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Study on Lifetime Specific, Laser-induced Ultrafast Fluorescence Detection in the Time and Frequency Domain for In-situ Environmental Analysis of Organic Pollutants

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ABSTRACT

The capability and applicability of time resolved fluorescence detection techniques is investigated. Directly time resolved and phase modulation measuring techniques are able to resolve excited molecular state lifetimes on the picosecond time scale by application of modern laser and detection systems. The temporal resolution and its precision is discussed for highly developed phase-modulation instrumention and the older, phase resolved fluorescence emission detection method. A comment on the application of lifetime techniques for in situ environmental analysis is given.

Studie über lebensdauerspezifische, Laser-induzierte ultraschnelle Fluoreszenzdetektionsmethoden zur in-situ Analyse organischer Schadstoffe in der Umwelt

ZUSAMMENFASSUNG

Kapazität und Einsatzfähigkeit von spezifischen Meßtechniken in der zeitaufgelösten Fluoreszenzspektroskopie werden untersucht. Mit der direkt zeitaufgelösten Meßtechnik und der Phasen-Modulationstechnik ist es möglich, unter Einsatz moderner Laser- und Detektionssysteme Lebensdauern angeregter molekularer Zustände im Picosekundenbereich aufzulösen.
Zeitliches Auflösungsvermögen und Genauigkeit werden explizit sowohl für hoch entwickelte Phasen-Modulationsinstrumente als auch für ältere, rein phasenauflösende Fluorimeter diskutiert. Die Einsatzmöglichkeit von Lebensdauertechniken in der in-situ Umweltanalytik wird kommentiert.

CONTENTS

I. INTRODUCTION

Fluorescence spectroscopic methods have got increasing importance as useful, powerful techniques in physics, chemistry and biophysics-biochemistry in the last two decades. Modern lasertechnology coupled with fiberoptical methods opens new possibilities in remote-fluorosensing or -monitoring for fast detection in process and environmental control. Detection of surface oils /CCKOQR91/ or organic molecules /CCK85,NRK89/ in aqueous systems is already practised. The fluorescence emission fine structure can be used to identify polycyclic aromatic hydrocarbons in oil products e.g. in seawater /DBPS91,ITTL90/. Chudyk et al. /CCK85/ were able to detect e.g. phenols in the ppb concentration range. Recent results demonstrate the detection of organic compounds such as fluoranthene and pyrene in idealized aqueous systems by measuring the fluorescence emission and decay behavior /K91/. Fluorosensing of chlorophyll(a) concentrations in water could be achieved by exploiting the inelastic scattered light in water for correction of the fluorescence signal of chlorophyll(a), determining quality parameters of surface waters /BNBF81/.

Generally a pulsed laserystem with pulse widths of about 5-15ns consisting of, e.g. an excimer- or Nd:YAG-laser used separately or to pump a dyelaser, is used as exciting lightsource /CCKOQR90,NRK89,CCK85/. Fluorescence detection can be achieved either by a fast photomultiplier tube coupled with a boxcarintegator, a sampling oscilloscope or a gated optical multichannel detector system (OMA) /ITTL90/ in order to gain information about the fluorescence emission or the decay and hence the lifetime of excited molecular states of the pollutants. New developments present the detection of two-dimensional fluorescence excitation and emission spectra by charge-coupled-device-cameras (CCD's) /DBPS91/. Nevertheless, determination of the total chemical composition of e.g. complex conglomerates of different substances in seawater seems to be a difficult task.

Analytical detection of oil contaminants is mainly restricted to large concentrations as the fluorescent signal of very small concentrations is overlapped by the fluorescence of the naturally occuring organic matter; also, selective wavelength excitation does not yield any improvement /DBPS91/. New results /CP91/ refer to the practical limits of fiberoptic coupled fluorescence measurements in field screening.
In this work the capability and applicability of lifetime specific fluorescence detection techniques for enhancement of the resolution of multicomponent systems is investigated. The study is organized as follows: A summary about fluorometric techniques in the time domain (measurement of the impulse-response to a light pulse, delayed coincidence technique: "directly time-resolved") is presented followed by a review of phase-modulation-fluorometry, an experimental technique in the frequency domain (measurement of the harmonic response to sinusoidally modulated light or to a train of light pulses, a phase shifted technique). Both techniques reconstruct the time-resolved fluorescent decay. The theoretical background, experimental equipment and the properties and capability coupled with a variety of applications of phase-modulation-fluorometry are represented. Finally purely phase-resolved fluorometry at only very few modulation frequencies as first applied technique of phase-modulation fluorometry in analytical chemistry is described. In the last chapter some test measurements applying phase-modulation fluorometry are shown.

II. MEASUREMENTS OF EXCITED STATE DECAY TIMES, FLUORESCENCE LIFETIMES

Figure 1 demonstrates the well known Jablonski diagramm which schematically depicts different physical deactivation processes of a molecular system from an electronic ground state S_0 with paired electron spin (singlet state) into S_1, S_2 or higher excited electronic

states after excitation by light.

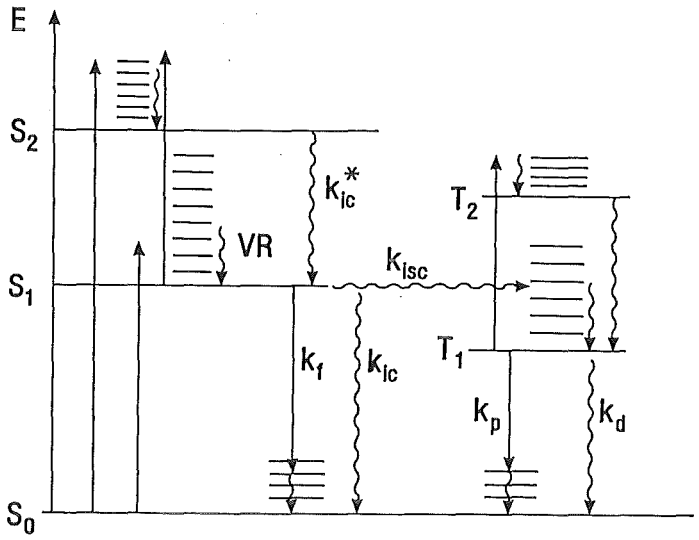


Fig. 1: Jablonski diagramm

Radiative and nonradiative transitions between different molecular states are governed by selection rules e.g the rule of (total) spin conservation resulting in singlet-singlet-transitions if spin and orbit momentum are only very weakly coupled. Population of triplet states as T_1 or T_2 occur at enhanced spin-orbit coupling which can be induced on organic molecules e.g. by the heavy atom effect /B70/.
Also vibrational relaxation (VR) occurs with electronic excitation (Franck-Condon principle), fast processes on the picosecond or less time scale.
Internal conversion (IC) may follow vibrational relaxation by conversion of the electronic energy to vibrational energy of a lower electronic state of the same multiplicity within a time range as vibrational relaxation and with rate constant k_{IC} of about $10^7 - 10^{11}$ s^{-1}. Kasha's rule tells that intersystem crossing is faster for higher excited electronic states as for the lowest excited singlet state. That's why processes as fluorescence with a rate constant k_f, intersystem crossing (k_{ISC}) and photochemical processes usually occur from the lowest excited vibrationally relaxed state. The radiative transition from the lowest excited electronic singlet state to the singlet ground state with

rate constant $k_f = 1/\tau_f$ is called fluorescence. Intersystem crossing is equivalent to the radiationless population of a triplet state from the corresponding singlet state, a usually spin forbidden process with rate constants k_{isc} of about $10^6 - 10^7\ s^{-1}$.
Typical fluorescence lifetimes are of a few nanoseconds. Excited state lifetimes of a few picoseconds to a few ten picoseconds can also occur whereas lifetimes of a few hundreds of nanoseconds as for only a few condensed aromatic hydrocarbons /BFG90/ are unusual.
Table 1 summarizes some characteristic lifetimes of chemical and physical processes.

process	lifetime/s
vibrational motion	$10^{-14}-10^{-12}$
vibrational relaxation	$10^{-13}-10^{-10}$
electron-hole-recombination	$10^{-13}-10^{-10}$
electronic relaxation	$10^{-12}-10^{-8}$
photoionisation, photodissociation	$10^{-14}-10^{-12}$
proton transfer	$10^{-12}-10^{-10}$
rotational and translational motion of small molecules	$10^{-10}-10^{-8}$
enery transfer	
in singlet states	$10^{-13}-10^{-10}$
in triplet states	$10^{-6}-10^{-2}$
electron transfer	
in singlet states	$10^{-12}-10^{-8}$
in triplet states	$10^{-6}-10^{-2}$

Table 1: Lifetimes of physical and chemical processes /BFG90/

II.1 PULSE FLUOROMETRIC MEASURING TECHNIQUE

Measuring the impulse response of a fluorescence light emitting system is equivalent to the detection of the luminescence emission in dependence of time I(t), the decay behavior, after an exciting light pulse which ideally should be described by a delta-function:

$$I(t) = I_0 \exp (-t/ \tau) \qquad [1]$$

The exciting light pulse can be created by flashlamps (arclamps), pulsed lasers and lasersystems /S77,K88/ or synchrotron radiation /LTM74/, see table 2a), 2b).

lifetimes/s	exciting light sources
10^{-3}	pulsed Xenonlamp pulsed laser (Excimer/dye)
10^{-6} - 10^{-7}	pulsed flashlamp (N_2, D_2), pulsed laser: pulse widths 10^{-8} - 10^{-7} ns
10^{-8} - 10^{-9}	compromise: flashlamps: pulse width 1-5ns pulsed laser: pulse width up to 10ns, e.g. N_2-laser, better: pulsed laser: pulse width << 1ns
10^{-12} - 10^{-10}	pulsed laser: pulse width of a few ps

table 2a): Exciting light sources and lifetimes

Table 2a) presents examples for exciting light sources for measuring specific lifetimes, table 2b) commercially available laser systems including the pulse widths.

lasersystem	pulse width
N_2-laser	5-8ns
Excimer-laser	about 10ns, > 10ns
Nd:YAG-laser (Q-switch)	5ns
cw-Nd:YAG-laser (modelocked)	100-850ps
coupled with a dye laser (cavity-dumped)	< 5ps
cw-Ar-ion-laser (modelocked)	> 100ps
coupled with a dye-laser (cavity dumped)	about 5ps

table 2b): commercial lasersystems

Lasersystems generating light pulses of ultrashort pulse widths on the picosecond time scale are nowadays widely applied for investigations of ultrafast interactions of solids, e.g. as semiconductor devices /RDBKA81,L88/, of biological processes /HJ88/, as the fluorescence decay of protein compounds as tryptophan /SWKA89/, of ultrafast chemical reactions as photoisomerization, charge-transfer reactions, electron-proton transfer /E88,DSET89/ and also for detection of the fluorescence of polynuclear aromatic hydrocarbons coupled with liquid chromatography /VHGBV89/.

If the decay is described by a single exponential law, equation [1], and the fluorescence lifetime is long compared to the pulse width of the exciting light pulse and the response function of the detection system the lifetime can directly be determined by the time-resolved decay.
Detection of the fluorescence decay curve can be realized by a time-resolving detector as a streak camera, a gated, intensified optical multichannel analyzer (OMA) /ITTL90/, yielding information about decay times on the nanosecond time scale within a few milliseconds measuring time, a fast photomultiplier coupled either with boxcar sampling technique or a sampling oscilloscope or used as a digital detector for single-photon-counting (SPC) /OP84/. Detection of 2-dimensional excitation-emission spectra can be performed by CCD-cameras /DBPS91/, a further innovative detection system. Also streakcameras /B74/, extremely fast detectors, coupled e.g. with an optical multichannel system (OMA) /FMR76/, can be established.
If the fluorescence decay to be measured is comparable to the instrumental temporal response, see fig. 2, deconvolution techniques have to be applied to separate the characteristic decay from the overall response F(t):

$$F(t) = \int_0^t E(t)\ I(t-t')\ dt \qquad [2]$$

E(t): pulse profile function of the exciting light pulse

I(t-t'): true fluorescence response

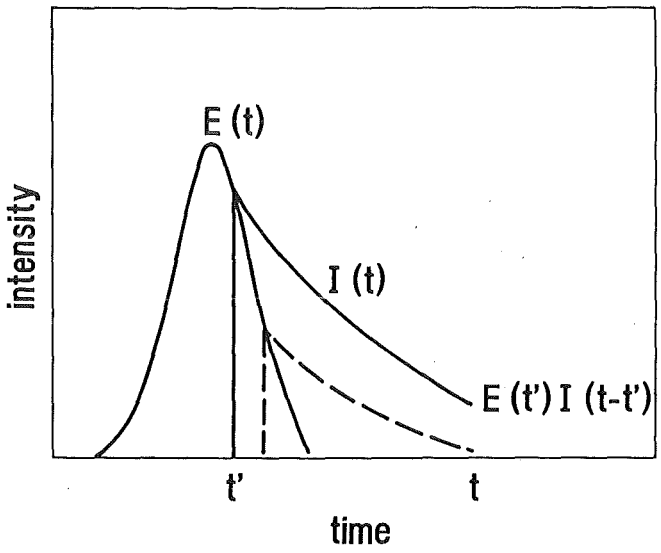


fig. 2: Convoluted decay curve in the time-domain, schematically

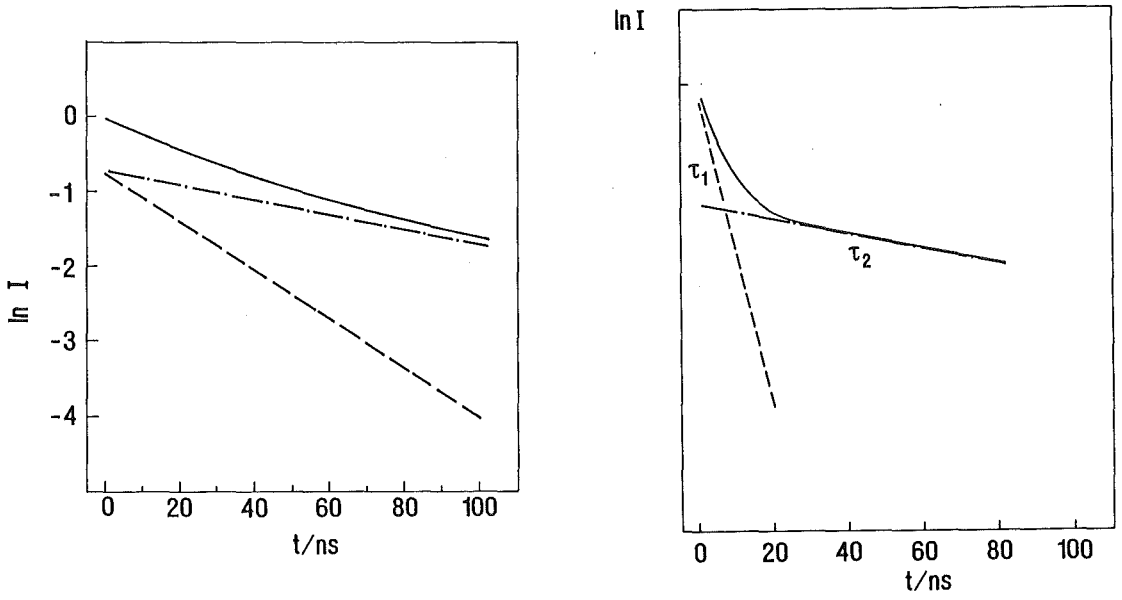
A lifetime, which is equivalent to the 1/2 FWHM (full width at half maximum) of the exciting light pulse, is thought to be still determinable in the time domain /L83/.
For a multicomponent decay the fluorescence intensity can be described by a sum of exponentials:

$$I(t) = \sum_{i=1}^{n} \alpha_i\ e^{-t/\tau_i} \qquad [3]$$

The intensity contribution of each fluorescent component to the steady state spectrum is given by:

$$f_i = \frac{\alpha_i \tau_i}{\sum_i \alpha_i \tau_i} \qquad [4]$$

Study of decays is difficult specially for small lifetime differences of the lifetimes of the individual components. Figur 3 a), b) describe the decay of a two component mixture with lifetimes τ_1 = 30ns and τ_2 = 100ns, fig. 3a), and τ_1 = 5ns and τ_2 = 100ns, fig. 3b), on a nanosecond time scale in the time domain if excitation is applied by a pulsed laser system of a nanosecond pulse width.



a) b)

fig. 3: Double-exponential decay with two decay times
a) τ_1 = 30ns and τ_2 = 100ns, b) τ_1 = 5ns and τ_2 = 100ns; preexponential parameters α_1 , α_2 = 0.5;

An excellent signal-to-noise ratio is required for separation of closely spaced lifetimes. In the time-domain the single-photon-counting method, SPC, /OP84,BUS85/ is the most frequently used and the most sensitive available technique. This method is based on the principle that the

probability distribution of the time of a photon arriving at the detector is directly proportional to the probability distribution of the photoemission of a photon of the fluorescent sample after the exciting light pulse at these times resulting in an intensity-time profile. This probability distribution can be determined by repetetive irradiation of the sample, e.g. with a flash lamp or a pulsed laser, and by succesively measuring the arrival time of the first fluorescence photon at the detector by a time-to-amplitude-converter (TAC) after each pulse, see fig. 4. The time detection is started by a reference channel and stopped by the fluorescence detection channel.

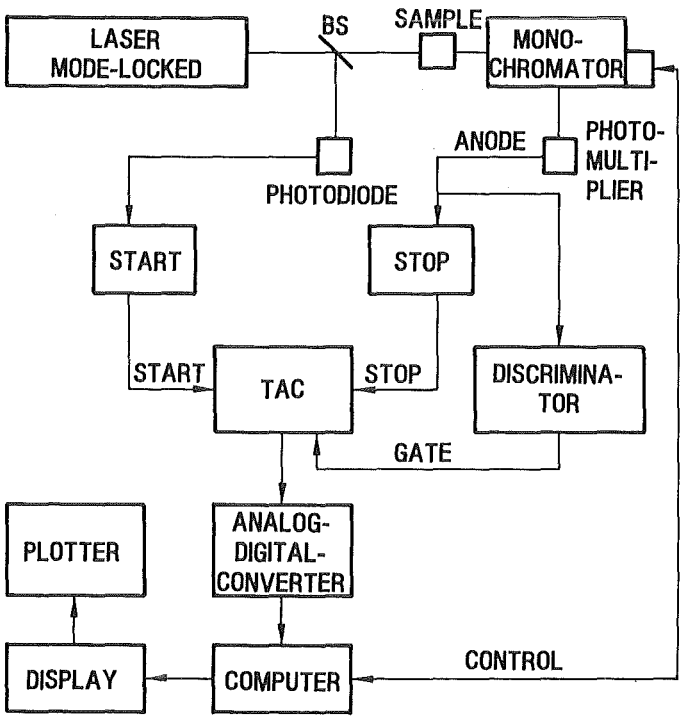


fig. 4: Fluorescence equipment with single-photon-counting detection technique, from /H82/;

As the instrumental temporal response of the detector system, (e.g. including the "transit-time-jitter" of a

photomultiplier, which can be about 100 - 200 picoseconds /H82/), is the most limiting factor, the best time resolution using ultrafast time-resolved measuring techniques, as SPC coupled with deconvolution, is about τ = 20 - 40 ps /H82,MF87,KWT91/. Table 3 presents a few examples of time resolution. The accuracy can be about $\Delta\tau$ = ± 5 ps /H82/.

exciting light source pulse width, FWHM	instr. response FWHM	time resolution	accuracy
0.5ps /RDBKA81/	streakcamera	39ps	±7ps
some ps /MF87/	110ps	30ps	
6-8ps /FMR76/	streakcamera	100ps	
10ps /WPB83/	1180ps	32ps	±3ps
15ps /SWKA89/	60ps	24ps	±1ps
ca.100 ps /MH90/	streakcamera + SPC	85ps	
1ns (pulsed lamp) /KGMI89/		3ns	

table 3: Time resolution in the time-domain depending on the pulse width of the exciting lightsource and the instrumental response; SPC: single-photon-counting;

In the time domain a streakcamera is a "real" picosecond pulse detector capable of resolving ultrafast times greater than τ = 50 ps without the necessity to deconvolute the instrumental response pulse from the decay curves /FMR76,H82/, see table 3.

Nowadays, femtosecond laser-systems are available which allow a further increase in ultrafast time resolution. It

should also be mentioned that fluorescence-up-conversion detection yields a subpicosecond time resolution /RTF90/.
In order to separate single components in a mixture deconvolution techniques have to be applied using time-resolved measuring techniques.
Twofold-exponential /LS83,WPB83,HSS89/ and also threefold-exponential decays /SLI84,LSSW83,SW89/ can be separated.
Some examples of multiple exponential decays up to four resolved decay times (τ) in the time-domain are listed in table 4.

exciting light source pulse width, FWHM	instr. response FWHM	τ_1	τ_2	τ_3	τ_4
10ps /WPB83/	1180ps	93ps	32 ps		
<10ps /HJTP89/	80ps	1430ps	790ps		
/ " /		3.78ns	650ps	30ps	
10ps /HSP88/	90ps	2.36ns	510ps	173ps	
30ps /CRVT89/	240ps	3.7ns	6.3ns		
a few ps /SW89/		205ns	119ns	25.3ns	3.3ns
ca. 100ps /MH90/	streakc. + SPC	1.36ns	290ps	80ps	
1ns (pulsed lamp) /KGMI89/		ca. 2.4ns	ca. 7ns		

table 4: Multiexponential analysis in the time domain

II.2 PHASE-MODULATION FLUOROMETRIC TECHNIQUES

a) BASIC PRINCIPLES, SOME THEORETICAL ASPECTS

Formerly, phase-resolved fluorescence detection was superior in subnanosecond time resolution which could more easily be

achieved than by the time-domain techniques requiring complex laser systems with small pulse widths. Also deconvolution techniques need not to be applied /HH75/ to reconstruct the time-resolved fluorescence spectra from phase and modulation data. The instrumental phase shift is cancelled caused by a relative measurement comparing the phase shift of the sample to the one of an ideal scattering solution or an ideal reference fluorophore.
Nowadays phase-modulation fluorometric techniques are e.g. widely used in basic research on chemistry, biochemistry and -physics to investigate e.g. rotational diffusion of dyes in micellar solutions /KH78,HKHH77/, energy transfer phenomena for determining static and dynamic conformational properties of macromolecules in solution /L83,LGWLPJ90/, anisotropy decays and collisional quenching of proteins /L83,LGSCJ91/. In the last years application of these techniques in analytical chemistry /M84,MB87,BL89/ has found interest for multicomponent analysis. Also, coupling of this technique with chromatographic methods was introduced /CM90/.
The phase-resolved technique is based on the harmonic modulation of light of a continuous radiating light source and the detection of the resulting demodulated and phase shifted emitted fluorescence light of the sample to determine decay times /G26,G27/, see fig. 5.
The measured phase shift, ϕ, and demodulation, m, carry information about the decay behavior of the excited system. They are correlated to the excited states' lifetimes.
The exciting light, E(t), can be sinusoidally modulated by modulation frequencies of a few Hertz up to the Gigahertz frequency range by exploiting e.g. the electro-optical effect (Pockels effect). E(t) can now be described by:

$$E(t) = E_0 + E_0 M_e \sin \omega t \qquad [5]$$

M_e is the modulation depth (ratio of the AC-amplitude (A) to the DC intensity (B); $M_e = A/B$).
The emitted light of a single sample, F(t), modulated by the same frequency is phase shifted by the phase angle ϕ, see

also fig. 5.

$$F(t) = F_0 + F_0 M_f \sin(\omega t - \phi) \qquad [6]$$

M_f : in analogy to M_e ; $M_f = C/D$.

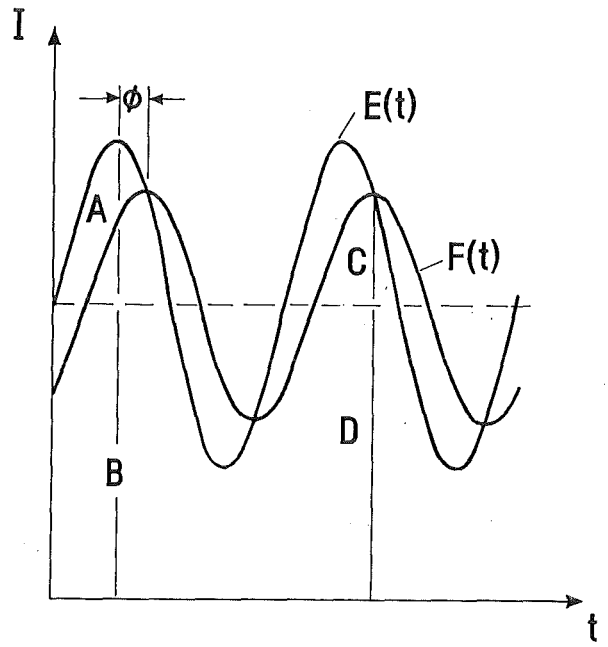


fig. 5: Sinusoidally modulated exciting cw-light, E(t), and emitted fluorescence light, F(t), schematically, for description see text;

The amplitude of the fluorescence signal can be reduced to the original one caused by processes competetive to the fluorescence process, e.g. non-radiative transitions.
The phase shift can be determined by detection of the emitted fluorescence light by a detector modulated by a modulation frequency, $f + \Delta f$, only slightly different from the original modulation frequency, f /SW69,JGH84/. The resulting photocurrent of the detector can now be described by a product function of the modulation function of the emitted light (F(t)) and an additional modulation function. The product signal contains all phase and modulation

information of the former high frequency signal in a low frequency term which can be isolated electronically. Higher harmonics of the low frequency fundamental can also be eliminated. For a more detailed description see /SW69,GL80,GJRW84,JGH84/. This cross-correlation detection technique (heterodyne method) is commonly applied in phase-modulation fluorometry nowadays /JGH84,LM85,BGL91/. The method is characterized by an improved signal-to-noise ratio and a high accuracy in determination of the phase delay and the modulation ratio /GJRW84/.

In the case of a single emitting fluorophore, a single exponential decay behavior, the fluorescence lifetime τ_p or τ_m can be calculated from the phase shift ϕ (τ_p) or from the 'demodulation factor' m (τ_m), the ratio of the modulation depths of the emitted to the excited light, M_f / M_e :

$$\tau_p = (1/\omega) \tan \phi \qquad [7]$$

$$\tau_m = (1/\omega) [(1/m^2) - 1]^{1/2} \qquad [8]$$

It is important to note that both, ϕ and m, the measured phase shift and demodulation factor, depend on the fluorescence lifetime and also on the applied modulation frequency.

For phase-modulation fluorometry it is useful to have a wide range of modulation frequencies which are high enough to investigate short lifetimes, see fig. 6 and also fig. 1.21 in /BFG90/ depicting the optimum modulation frequency for pico- and nanosecond lifetimes .

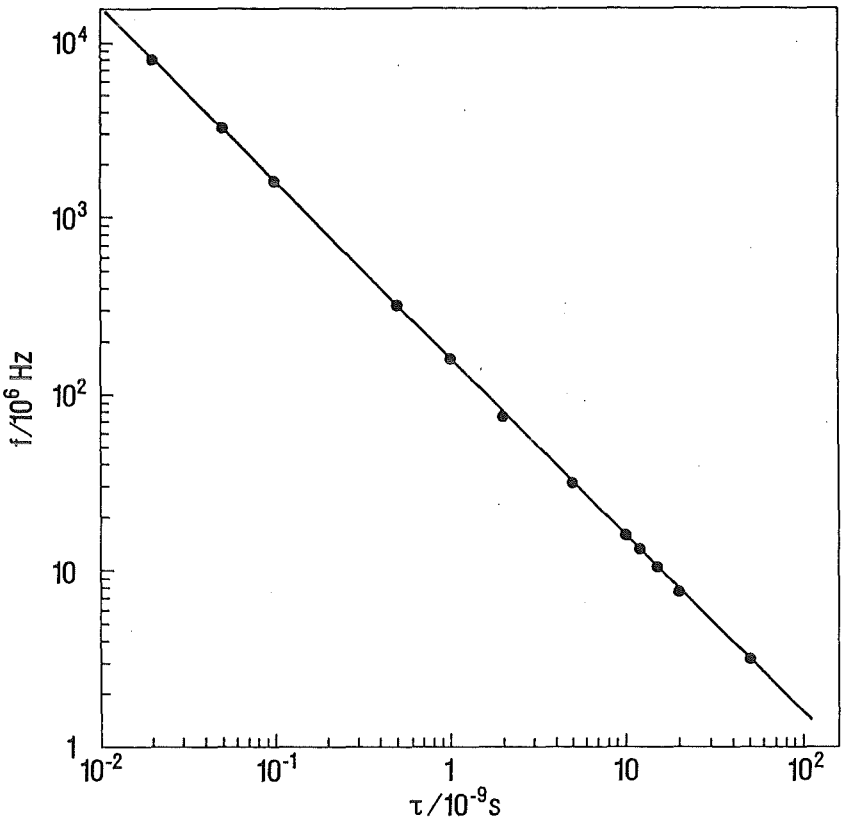


fig. 6: Optimum modulation frequencies f versus lifetime τ

Short-lived, s, and long-lived, l, fluorophores demonstrate a different response (s(t) and l(t)) to modulation frequencies resulting in different phase angles and also different modulation degrees, fig. 7 and fig. 8 a), b).

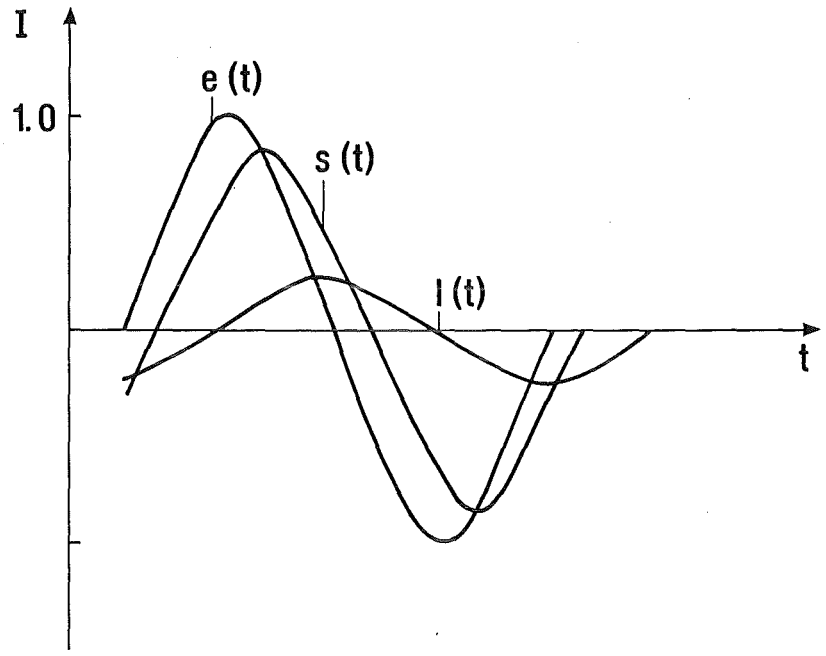
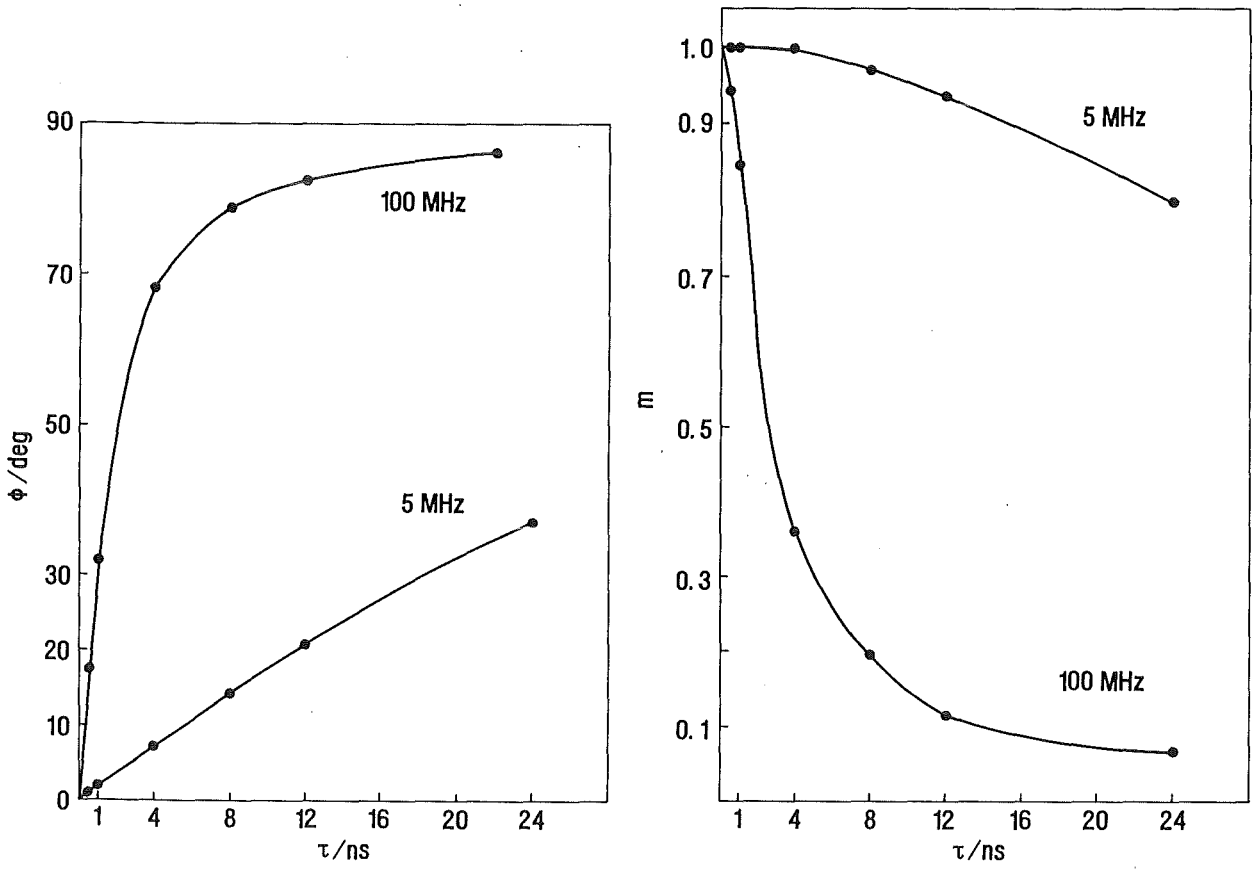


fig. 7: Phase shifted and demodulated response s(t) and l(t) to a single frequency modulated excitation e(t) schematically

Roughly spoken, the short-lived components 'react faster' to a high frequency than the long-lived ones resulting in a smaller phase shift and smaller modulation degree for the former, see also fig. 8 a), b).



a) b)

fig. 8: Phase shifts ϕ, a) and relative modulations m, b) for selected lifetimes at 100MHz and 5MHz modulation frequencies

Fig. 9 presents e.g. the response at variable modulation frequencies of two selected fluorophores in solution with lifetimes τ = 29.28ns and τ = 980ps, after /GHJ84/. Each individual decay corresponds to a single exponential decay law.

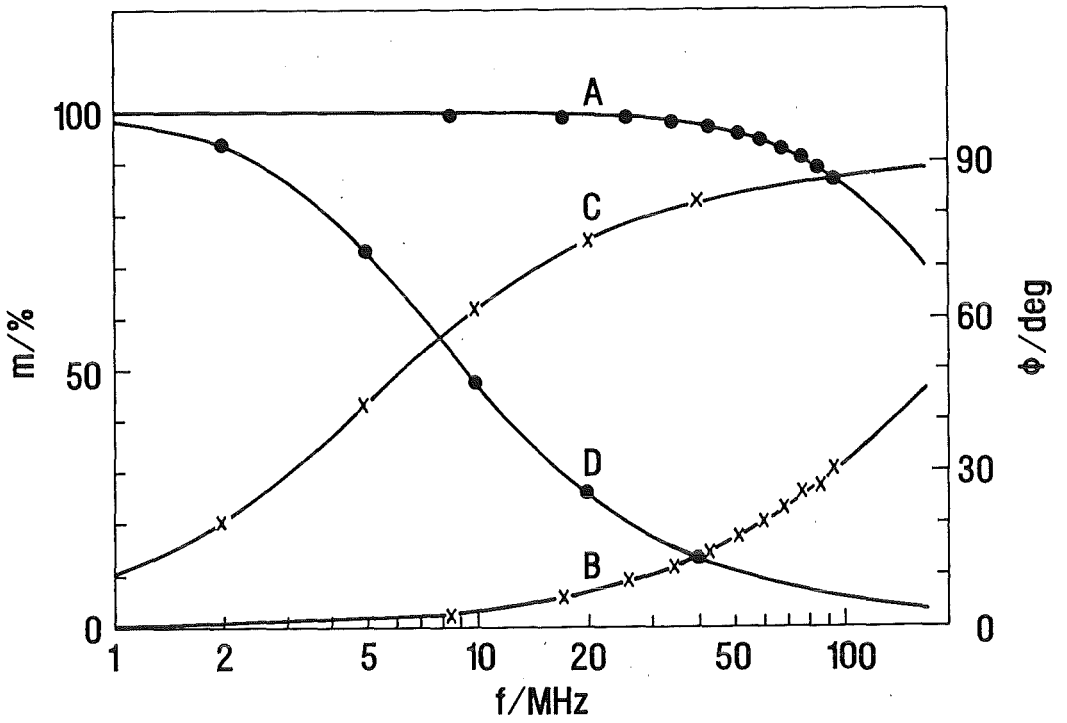


fig. 9: Phase (x) and modulation data (o) for DENS (2,5-diethyl-aminonaphthalene sulfonate) (C and D) in water , τ = 29.28ns, and p-terphenyl (A and B) in cyclohexane, τ = 980ps, from /GHJ84/, fig.3;

Directly time-resolved fluorescence detecting spectroscopy (pulse fluorometry) was often thought to be superior to the phase-resolved method in analyzing multiexponential decays /D88/.

In principle it seems to be possible to resolve the luminescence emission of different components in a multicomponent-system by phase angle sensitive measurements. An ensemble of fluorophores with different lifetime characteristics excited by harmonically modulated light emits light at different phase angles. The total fluorescence emission is a superposition of the harmonically modulated emission of each individual component in the mixture.

The use of variable modulation frequencies extends the phase-resolved technique which was shown by Weber who presents an analytical solution of the problem /W81/.
Also, Balter /B82/ discussing double exponential decay laws demonstrates that even for a single modulation frequency information about the complexity of a fluorescence decay can be gained by phase and modulation measurements.

As the impulse and the harmonic response to a pulsed excitation at a time $t = 0$ are identical and exchangeable within times $t = 0$ to $t = \infty$ the impulse response and hence the time-resolved fluorescence spectra and decay can be determined by phase-modulation data by Fourier or Laplace transformation.
The response function F(t) of a system to pulsed excitation with a time profile E(t) is already described by equation [2], see above.
Substitution of equations [5] and [6] into [2] yields the following equations, the cosine- and sine-Fouriertransforms of I(t), see equation [3] describing a multiexponential decay law, in the frequency domain:

$$m \cos \phi = \int_0^\infty I_f(t) \cos \omega t \, dt = C(\omega) \qquad [9]$$

$$m \sin \phi = -\int_0^\infty I_f(t) \sin \omega t \, dt = S(\omega) \qquad [10],$$

whereas I_f is the normalized decay function:

$$I_f(t) = I(t) \Big/ \int_0^\infty I(t) \, dt \quad \text{and} \quad \int_0^\infty I_f(t) \, dt = 1 \qquad [11]$$

The phase shift and the demodulation are hence described by:

$$\tan \phi = S / C \quad \text{and} \quad m = (S^2 + C^2)^{1/2} \qquad [12]$$

For more detailed description of these derivations see /J73,W81/.

Fig. 10 shows a double exponential decay in the frequency-domain /GLLMCL84/. The dashed curve represents the best one-component fit.

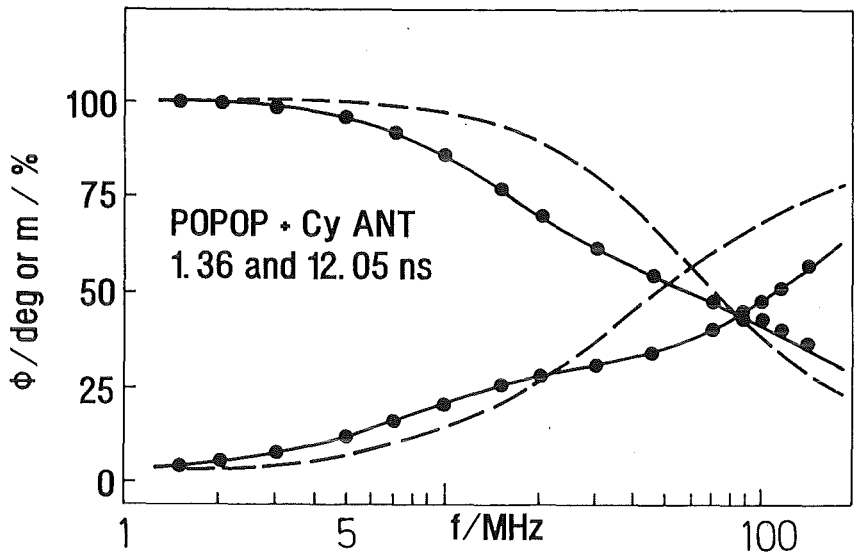


fig. 10: Phase and modulation data and best fit (o o o) according to a double exponential decay model of a mixture of POPOP (p-bis(2-(5-phenyloxazolyl)benzene) and 9-cyanoanthracene (Cy Ant), from /GLLMLC84/, fig.3;

The experimental data (phase and modulation data) of fluorescence decays can be compared with models of assumed decay behaviors (single - and multi-exponentials and also non-exponentials). The data are fitted by variation of the parameters (α_i and τ_i) to yield the best fit (agreement of experimental data and assumed model). Several statistical methods are integrated in these fitting methods as e.g. linear and non -linear least square analysis /D83,L83/ and algorithms as the Marquardt-algorithm /M63/ or Weber's algorithm /W81/. Knutson et al. /KBB83/ presented a global approach, 'Global analysis', to analyze multiple fluorescence decays simultaneously.

The value of the error-weighted sum of the squared deviations of the measured and calculated data, CHI_R^2 [13], is a measure of the probability for describing the experimental data set by the assumed model. The minimum value of CHI_R^2, which should be near unity, characterizes the goodness of the fit and the best possible matching of the model with the experiment. If CHI_R^2 is largely greater than unity the assumed decay model does not correlate with the real experiment.

$$CHI_R^2 = \sum_N \left[\left(\frac{\phi_N - \phi^c{}_N}{\sigma^P{}_N} \right)^2 + \left(\frac{m_N - m^c{}_N}{\sigma^m{}_N} \right)^2 \right], \quad /LLCGL84/, \quad [13]$$

$\sigma^P{}_N$ and σ_m are the estimated random errors in measured phase and modulation data, ϕ_N and m_N, for each modulation frequency N, $\phi^c{}_N$ and $m^c{}_N$ the calculated phase and modulation data of a specific model.
On the other hand the variation of CHI_R^2 is a measure for the extension of uncertainty of a parameter value. For closely spaced lifetimes CHI_R^2 can vary only slightly for widely different parameter values /LLCGL84/. For simplicity multiple emission wavelenght selectivity is not involved in the above equation.

b) INSTRUMENTATION AND EXPERIMENT

The main components of phase-modulation fluorometers, fig. 11, are: an exciting light source L, a light modulator M, a wavelenght selective unit as a monochromator MC or a bandpass filter and a modulated photodetector MD (measuring detector). The measured phase and modulation data are processed electronically (ph, m, C) to determine the phase shifts and fluorescence intensities. A second photodetector RD detecting the exciting light partially separated by a beam splitter BS is used as reference.

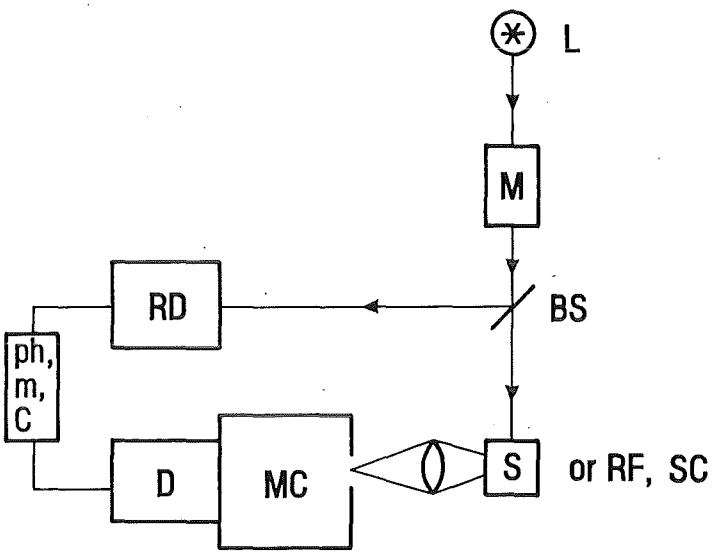


fig. 11: Experimental setup for phase-resolved fluorescence detection schematically; S: sample, RF: reference fluorophore, SC: scattering solution, for further description see text;

Early phase-modulation fluorescence spectrometers /G27,SW69,SLM Inc. (early commercial lifetime instrument)/ are indeed "purely" phase-resolved instruments as only 1 to 3 modulation frequencies were available. Gaviola used an arc lamp as exciting light source and introduced a Kerr-cell, based on the electro-optical effect, in two configurations coupled with polarizers as modulator with a modulation frequency of about f = 10MHz /G26,G27/. The lifetime dependent phase shift of a fluorophore was also measured via a Kerr-cell. Spencer and Weber used a phase fluorometer consisting of an ultrasonic unit, a Debey-Sears modulator, to modulate the exciting light source (Xe-arc lamp) at modulation frequencies of f = 14.2MHz and f = 28.4MHz in combination with cross-correlation detection techniques /SW69/.

The commerially available phase-resolved fluorometer SLM4800C /SLM Inc./ works at modulation frequencies of f = 6, 18 and 30MHz, also using a Debye-Sears modulator.

The availability of laser light sources promoted the

development of frequency-domain fluorometers with continuously variable wideband light modulation at modulation frequencies from f = 0.1MHz up to f = 200MHz /HH75,IEP83,GL83,LM85/ using standard electro-optical modulation by a Pockels cell, fig. 12.

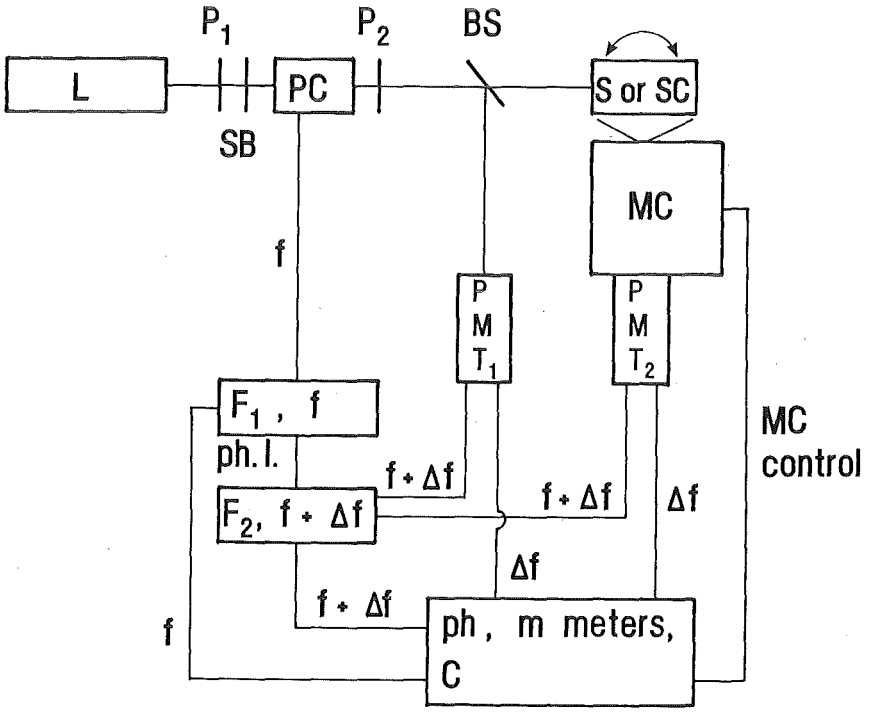


fig. 12: phase-modulation fluorometer with variable modulation frequencies of f = 1 - 200MHz and cross-correlation detection, schematically according to /LM85/; P_1, P_2: polarizers, SB: Soleil-Babinet polarizer, MC. monochromator, BS: beam splitter, PMT_1: reference photomultiplier, ph, m: phase and modulation;

The output of a cw-HeCd-laser L is sinusoidally modulated by a Pockels cell PC coupled with a frequency synthesizer F_1 yielding the modulation frequency f. A Pockels cell, in combination with polarizers, can be used as fast optical switch. The main component is an electro-optical crystal as

e.g. KDP (kalium hydrogen phosphate) which rotates the plane of light when an electric field is applied, see /DV78/ for detailed description. A second frequency synthesizer F_2, phase locked (ph.l.) with the first, generates the modulation frequency $f + \Delta f$, $\Delta f = 25Hz$, to modulate the gain of the fluorescence emission detecting photomultiplier PMT_2. Data storage and analysis is achieved by an interfaced computer C.

The electro-optical modulator is capable of providing modulation frequencies greater than f = 300MHz /KH78/. Optical frequency doubling of the modulation frequency enlarges the frequency range to about f = 500MHz cutoff frequency /HH78/ with a picosecond time-resolving power. Difficulties in gaining usable light modulation at frequencies greater as f = 200MHz due to the capacitance of the modulators are reported by Lakowicz et al. /LLG86/.
The limited bandwidth of commonly used fast photomultipliers (up to about f = 300-500MHz /GL80,CJEP89/) with rise times of one to a few nanoseconds usually restricts the temporal resolution. This can be extended by the use of modern microchannelplate photomultipliers (MCP-PMT, e.g. models of Hamamatsu) with pulse widths of about 30ps correlated to appropriate electronic circuits /LLG86/.
In addition to sinusoidal modulation of a cw-light source modulation frequencies can also be introduced by exploiting the harmonic content of pulsed light sources of synchrotron radiation sources /GJRW84/ or pulsed laser systems /BDP82,AGJ85,LLG86,BH87/ to extend the modulation frequency range.
In the Fourier space, an ideal impulse function described by a delta-function is equivalent to a frequency spectrum containing all frequencies /B86/. In the frequency domain a pulse train emitted by pulsed light sources can be described by a set of harmonic frequencies. Pulsed laser sources with pulse widths of a few picoseconds are characterized by a harmonic content over a wide frequency range up to several Gigahertz with hardly any loss in amplitude or intensity

/LLG86,GJRW84/. The Fourier transform of an intensity-time profile is schematically shown in fig. 13 assuming a Gaussian distribution for the impulse function.

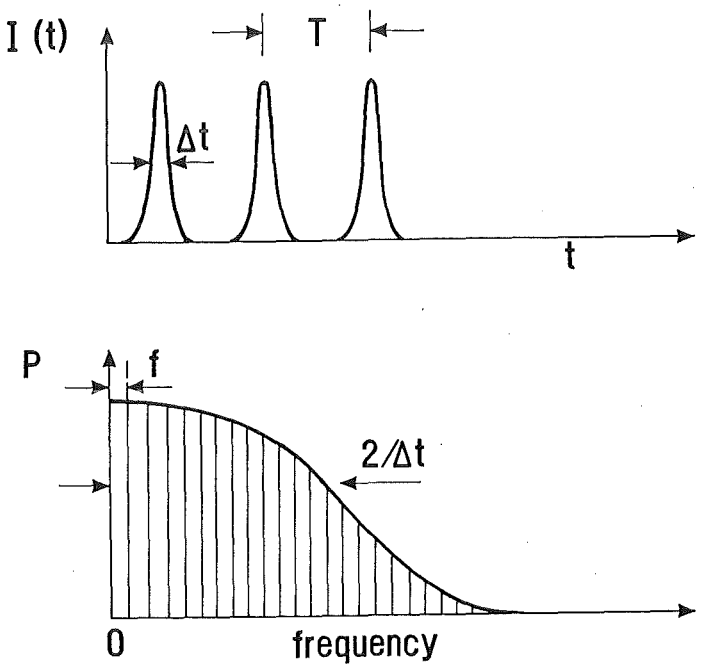


fig. 13: Intensity-time profile of a pulse train I(t) and its frequency spectrum P, schematically; for description see text;

The pulses of a finite pulse width, Δt, are separated in time, T. The pulse width, the full width of half maximum (FWHM), determines the bandwidth of the frequency spectrum ($2/\Delta t$).
In the past six years frequency-domain fluorometers with modulation frequencies greater than f = 500MHz up to f = 10GHz were developed /BDP82,AGJ85,LLG86,LGGWML90/ to resolve ultrafast decay times on the picosecond time scale, table 5 and fig. 14.

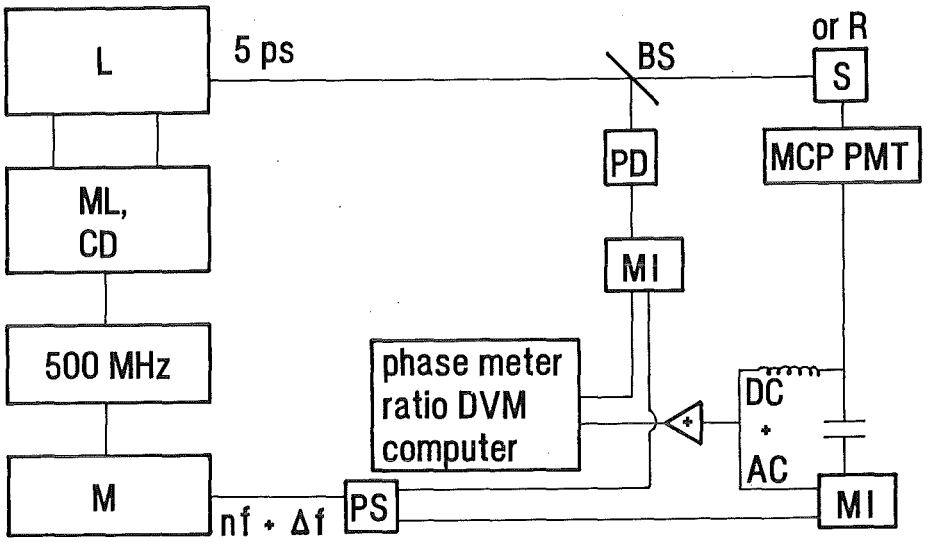


fig. 14: Phase-modulation fluorometer with modulation frequencies up to f= 2GHz and a MCP-PMT detector with an external cross-correlation circuit according to /LLG86/; L: lasersystem consisting of a mode-locked Ar^+-laser and cavity dumped dye laser (5ps pulse width), ML: mode-locker and CD: cavity dumper phaselocked to a 500MHz frequency syntheziser, fundamental frequency f = 3.79MHz, f: cross-correlation frequency, M: electronic multiplier, PS: power splitter, MI: mixer, PD: photodiode, DVM: digital voltage meter, BS: beam splitter, S: sample, R: reference fluorophore;

Insertion of harmonics between the harmonics of the laser system to increase the variability of available modulation frequencies from nearly DC to Gigahertz can be introduced by amplitude modulation by an external modulator (acousto- or electro-optical) /AGJ85/.

The capability of fluorescence lifetime determination by the phase-modulation method including laser application is demonstrated in table 5. The theoretically determinable longest decay times are estimated.

phase-modulation instr. and frequency range	time range
125 - 1000MHz (pulsed) /BDP82/	ca. 100ps - ca. 10ns
ca. 3.8 - 10GHz (") /LGGWML90/	2ps - ca. 50ns(**)
ca. 3.8 - 2GHz (") /LLG86,	7ps - ca. 50ns(**)
/LLGSW88/ a few Hz - 1GHz (") /AGJ85/	86ps - ms's
400kHz - 300MHz (") /CJEP89/	ca. 10ps(*) - 400ns(**)
5 - 250 MHz, SLM48000MHF /SLM Inc./	ca. 23ps - ca. 30ns(**)
5 - 500MHz /HH78/	25ps - ca. 30ns(**)
1 - 200MHz /LM85/	47ps - ca. 150ns(**)
0.1 - 200MHz /PMV89/	65ps - ca. 1.5µs(**)
1 - 160MHz	ca. 17ps(*) - ca. 150ns(**)
/GL83/ 0.1 - 50MHz /IEP83/	59ps(+) - ca. 1.5µs(**)

table 5: Modulation frequency range of selective frequency-domain fluorometric applications and decay time ranges; the lifetimes are cited from literature (without any asterisk), evaluated assuming 1° phase shift at the highest modulation frequency (*) and estimated considering the repetition rates of the (intrinsically pulsed) light sources and modulation frequencies (**); (+): only valid for phase shift data;

A commercially available "Fourier transform" phase-modulation instrument, model SLM 48000MHF /SLM Inc./, with pulsed excitation achieved by modulating a cw-HeCd-laser externally by a pulsed Pockels cell with modulation frequencies from f = 5 - 250MHz and successive heterodyne

detection is presented in fig. 15, see also table 5. For detailed descriptions see /SLM Inc./.

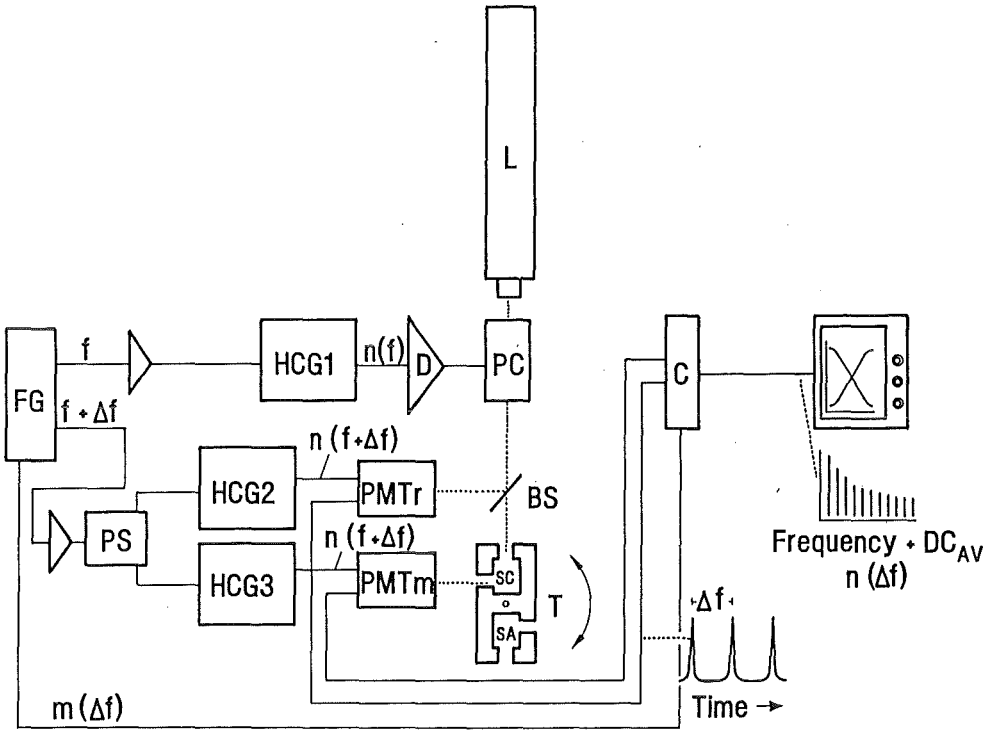


fig. 15: SLM48000 (MHF) Multi-Harmonic-Fourier-Transform fluorometer by SLM Instruments /SLM Inc./; L: laser, PC: Pockels cell, FG: frequency generator, HCG1, -2, -3: harmonic comb generators, PS: power splitter, D: triode driver, f: fundamental frequency, Δ f: repetition frequency, n(f + Δ f), n(f): integer harmonics, m(Δ f): binary multiple of Δ f, SC: scatterer, SA: sample, T: turret, PMTm, -r: measuring and reference photomultipliers, C: digitizer (A-D-converter),

Analysis of the phase sensitive fluorescence spectra requires exact knowledge about the relationship between the

phase angle arbitrarily found and the absolute phase angle of the modulated exciting light. This can practically be carried out by measuring the phase angle e.g. of a scattering solution. The absolute phase difference ϕ can be determined by:

$$\phi = (\phi_R - \phi_F) - (\phi_R - \phi_S) \quad /JGH84/ \qquad [14]$$

ϕ_R is the phase angle of the reference photomultiplier, ϕ_S the one of a scatterer and ϕ_F the one of the fluorescent sample.

It is important to mention that data based on phase matching by using a scattering solution are not absolutely reliable caused by systematic errors due to the temporal response of the detector, the so-called 'color effect'. The temporal detector response depends on the wavelength and the spatial distribution of the incoming light on the photocathode.

Instead of a scattering solution, a reference fluorophore with known decay behavior, a short decay time and equivalent spectral characteristics as the fluorescent sample can be used to reduce this error source /LC81/. It is critical whether any reference fulfills these idealized conditions. POPOP (p-bis(2-(5-phenyloxazolyl))benzene) and Me_2POPOP (1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene) are commonly used as reference fluorophores.

Pouget et al. /PMV89/ suggest a time-consuming measuring procedure for the case of lack of information about the reference decay time using a scatterer again. This can be performed in three steps: a) determination of the phase shift and relative modulation of the sample compared to the data of a scatterer as reference, b) determination of the temporal shift of a fluorophore of the same spectral characteristics as the sample without changing any experimental parameters by measuring the phase shift and demodulation, c) correction of the phase data relevant to the fluorescent sample.

Measurements of phase shifts and relative modulations over a wide frequency range are necessary to get reliable information about the overall decay behavior of complex systems as e.g. intrinsic decays in the case of heterogeneous emission of a fluorophore, lifetime distributions and multicomponent systems. Again I like to refer to fig. 6 presenting the optimum modulation frequency for specific lifetimes of excited states. Resolving individual decays and hence determining individual lifetimes requires the analysis of the overall decay.

c) ERROR SOURCES, LIFETIME RESOLUTION AND PRECISION, RESOLUTION CAPABILITY OF MULTICOMPONENT DECAYS

Random errors in phase and modulation data are critical factors influencing the lifetime resolution, the precision of the determined lifetime values and hence the resolvability of multicomponent systems.

Random errors can have different origins depending on the instrumental setup (e.g. frequency resolution of the frequency synthezisers, frequency response and stability of the electronics and the detector, intensity stability of the light source, temperature drifts (electronic and optical material parameters) etc.), on the measuring procedure and also on the samples to be investigated (standards, purity, preparation, time stability, matrix effects).
The principle of phase and modulation measurements can give rise to `intrinsic' random errors.
Lakowicz /L83/ roughly estimated errors and measurement accuracies assuming an uncertainty of $\Delta \phi = 2°$ for a measured phase shift greater than $\phi = 7.0°$ and a minor error of 1% in the demodulation factor m:
At 30MHz modulation frequency the imprecision in lifetime determination for a fluorescence lifetime of ca. $\tau = 5ns$ is ca. $\Delta \tau = 0.35ns$ (rel. error of 7%). Assuming a lifetime of ca. $\tau = 20ns$ the lifetime imprecision is ca. $\Delta \tau = 2.8ns$

(rel. error of 14%). The effect is more dramatic if one considers errors in the modulation degree. At 10MHz modulation frequency the demodulation factor is about m = 0.983 for a ca. 3ns lifetime. A deviation of 1% measuring the degree of modulation can cause errors greater than 50%.
The phase shift imprecision should be smaller than $\Delta \phi = 0.5°$ and the deviation in modulation data smaller than $\Delta m = 0.005$ to get reliable results /LLCGL84/.
Another important fact which should also be mentioned in error discussion is the use of standards (scatterers or reference fluorophores). The accuracy of lifetime determination of a fluorophore is correlated with the accuracy of the determined lifetime of the standard. The use of scatterers is critical because of the 'color effect' of the photodetectors. The 'color effect' can result in a time delay of $\Delta \tau$ = 100ps - 1ns for photomultiplier tubes /BDP83,BFG90/ and hence in an additional phase shift which has to be considered. This time delay can be reduced or eliminated by the above cited measuring procedure suggested by Pouget et. al /PMV89/. Modern research instruments with high speed detectors as silicon avalanche diodes /BDP83/ or microchannelplate-photomultipliers /LLG86/ suffer from 'color-delay error' of about $\Delta \tau$ = 20ps which enhances their temporal resolution in addition to a multifrequency application of high frequencies up to Gigahertz.

If reference fluorophores are used as standards with a fixed known lifetime it has to be taken into account that first their lifetime values do not agree better than ca. 5% in literature /TG88/, see also table 6, and second their emission characteristics do not totally overlapp with the total fluorescence emission range at wavelengths of ca. 250nm to 800nm.

POPOP	lifetimes:
1.35 ± 0.2 ns	/LCB81/
1.37 ns	/GL83/
1.32 ± 0.01 ns	/GLLMCL84/
1.34 ns	/MB85/
1.305 ns	/PMV89/

table 6: Different, experimentally determined lifetimes of POPOP (p-bis(2-(5-phenyloxazolyl))benzene) often used as reference fluorophore;

Errors in reference lifetimes have also to be taken into consideration during determination of the sample lifetime and its precision which can be reduced due to error propagation.
Thompson and Gratton /TG88/ suggested a differential method for accurately determining sample lifetimes without the use of a reference to eliminate the above error source by measuring the ratio of phase and modulation data of two compounds with similar emission characteristics. The results become uncertain if the two lifetimes of the compounds are too close (e.g. if the difference is close to the precision of the instrument, see the following). This method is limited to ca. 10% lifetime difference /TG88/.

Now I like to emphasize some more details on errors originated by using a modulation frequency which is not in coincidence with maximum lifetime selectivity requiring an optimum modulation frequency as worked out by Berndt /B85/, see also fig. 6 and /BFG90/. Relative errors in lifetime determination versus modulation frequencies are shown in fig. 16 for four selective lifetimes assuming a fixed random phase error of $\Delta \phi = 0.35°$.

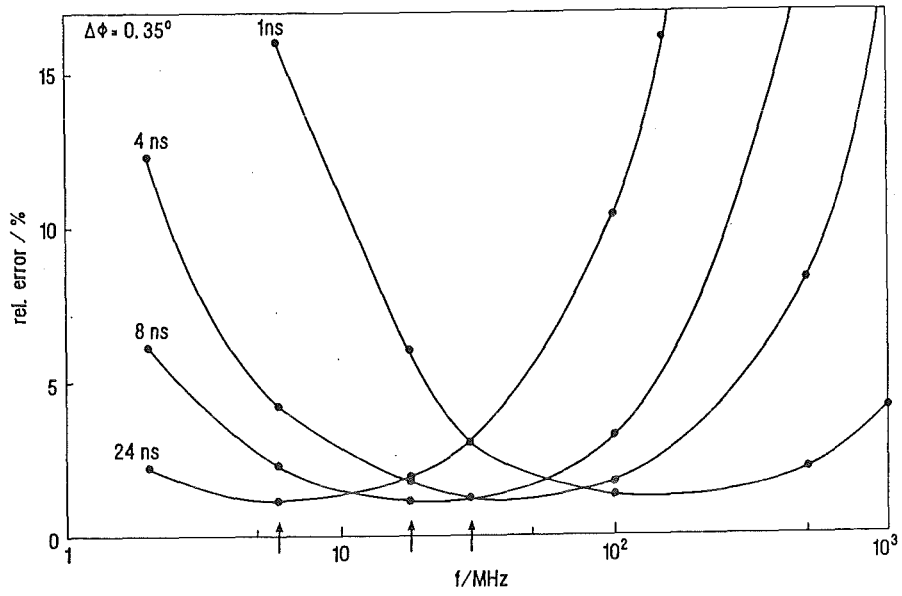


fig. 16: Estimated relative errors of lifetimes versus modulation frequency for τ = 1ns, 4ns, 8ns and 24ns lifetimes; random phase error: ϕ = 0.35°, see Spencer's and Weber's instrument /SW69/; the arrows indicate 3 available modulation frequencies of f = 6, 18 and 30MHz of the SLM 4800C instrument /SLM Inc./ with non-variable modulation frequencies;

In the case of the assumptions in fig. 16, an unevitable error of the determined lifetimes of about 1% has always to be taken into account due to the large random phase error. Let us consider a fluorophore with an excited state lifetime of ca. τ = 1ns modulated by a frequency of f = 6MHz. The relative error in lifetime determination by the phase shift will be greater than 15%, fig. 16. A random error in phase angle of $\Delta \phi$ = 0.1° reduces the error in lifetime determination by a factor of 3 (e.g. the relative error will be 4.6% for a lifetime of 1ns at 6MHz modulation frequency). Using the modulation data for determination of a lifetime of τ = 1ns at 6MHz modulation frequency assuming a measurement sensitivity of 10^{-3} (Δ m = 0.001) can lead to large errors in lifetime greater than 50%, fig. 17. Such errors can be reduced to smaller than 5% by application of the optimum modulation frequency or frequency range; (These estimations do not include additional error sources by error propagation, e.g. by errors in reference lifetimes).

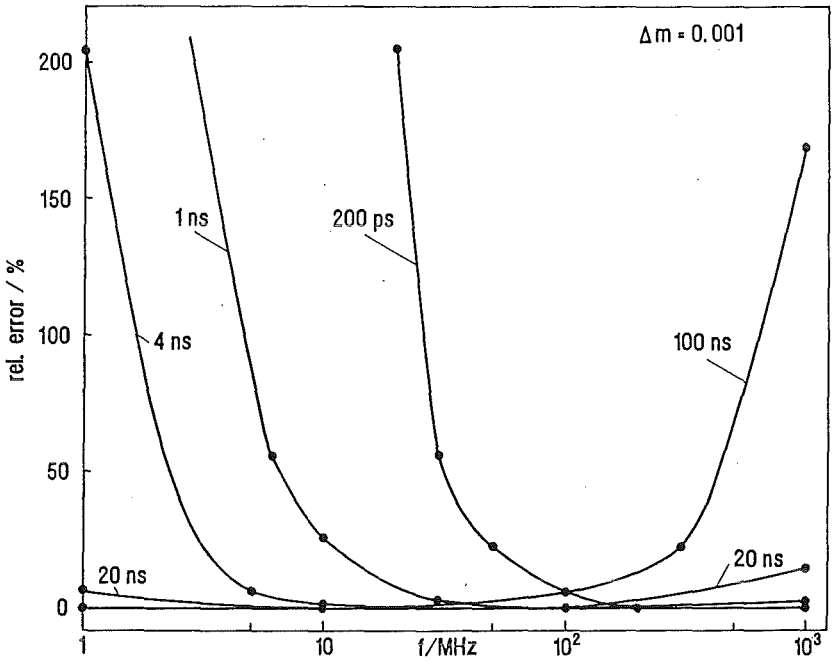


fig. 17: Estimated relative errors of lifetimes by lifetime determination by modulation data for selective lifetimes of τ = 200ps, 1ns, 4ns, 20ns and 100ns versus modulation frequencies from 1MHz to 1GHz; assumed fixed modulation deviation: Δ m = 0.001 at any modulation frequency;

These considerations show that the time resolution in phase-modulation fluorometry is a function which is very sensitive to the modulation frequency, modulation and dephasing angle coupled with the excited state lifetime to be investigated, an intrinsic characteristic of the method. Even with high precision in modulation data as presented in fig. 17, estimated in /GL80/ and also summerized in table 7 the lifetime resolution can be poor e.g. of about $\Delta\tau = \pm$ 0.55ns for a τ = 1ns fluorescence lifetime at 6MHz modulation frequency and about $\Delta\tau = \pm$ 23ns for a τ = 100ns lifetime at f= 300MHz.

Nevertheless, nowadays it is indeed possible to reduce the errors in lifetime determination of about 1% at optimum conditions, table 7, applying GHz frequencies as modulation

frequencies for lifetime resolutions of a few picoseconds to a few ten picoseconds. Highly developed phase-modulation instruments with available modulation frequencies up to ca. 2GHz for determination of hundred picoseconds to a few nanoseconds lifetimes can yield very high precision of the measured data. Table 7 summarizes precision and accuracy data for phase-modulation fluorometers in use.

phase-modulation instruments	phase and modulation uncertainties	lifetime accuracy	
up to 2GHz	0.3°, 0.005	±2ps	/LLG86, LLGSW88/
4GHz and 10GHz	2°	1 - 0.3ps	/LGGWML-90,BL90/
a few Hz - 1GHz,	±0.2°	1ps (400MHz)	/AGJ85/
5-500MHz	±0.1°	1ps	/HH78/
400kHz-300MHz	±0.1°	5-15ps	/CJEP89/
5-250MHz, SLM 48000MHF	<0.5°, <0.005	6ps	/SLM Inc./
1-200MHz	<0.2°, <0.002	3ps	/LM85/
0.1 - 200MHz	0.1°, 0.002	several ps	/PMV89/
1 - 160MHz	0.1°, 0.002	" "	/GL83/
0.1 - 50MHz	ca. 0.2°	±10ps	/IEP83/

table 7: Instrumental precision and lifetime accuracy in phase-modulation fluorometry (assuming a single exponential decay) either directly cited from literature or estimated by assuming a random phase error at maximum available modulation frequency at a phase shift of $\phi = 1°$.

The resolvability of the frequency-domain technique in simultaneous lifetime determination of individual fluorescence lifetimes of a multicomponent mixture depends on the instrumental time resolution (available modulation frequencies and time range), the precision of the measured data coupled with the lifetimes and the lifetime

differences to be investigated. The level of random error is the most critical factor.

The lifetime resolvability is intrinsically dependent on the intensity contribution of each individual component whose detectability is determined by the response of the individual component to the specific experimental conditions (e.g. applied modulation frequency).
In this framework another important fact, the components' concentration, has to be considered: e.g. the resolution of a low concentrated component with a high or low degree of demodulation at a specific modulation frequency in competition with a component of high concentration and nearly optimum response to the same modulation frequency can become difficult.
Again, knowledge of the overall frequency response of the system under investigation is necessary to get the most reliable results resolving mixtures.

A priori, individual lifetimes, e.g. in a two component system, can be distinguished if the lifetimes are well separated and if the measurement is performed over an adequate frequency range.

A lifetime resolvability plot of lifetimes between τ = 0.1ns and τ = 1000ns for a two component mixture and equal fractional contributions of the two components assuming a double exponential decay is presented by /GJH84/, see fig. 18.

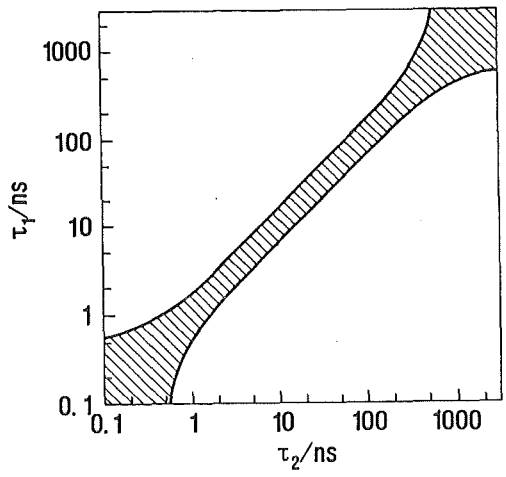


fig. 18: Double exponential resolvability plot for a set of eight modulation frequencies between f = 1MHz and f = 128MHz assuming a phase shift and modulation precision of $\Delta \phi > 0.2°$ and $\Delta m > 0.004$, from /GJH84/, fig. 5.

Within a lifetime range of τ = 1ns - 500ns two components of a lifetime ratio of ca. 1.6 can be distinguished under the presented conditions /GJH84/. Lifetimes in the shaded region of fig. 18 cannot be further resolved. If the number of frequencies used for analysis is increased errors in the parameters (lifetime and fractional contribution) can be decreased /GJH84/.

According to the picosecond lifetime resolution capability 2 to 3 lifetimes, closely spaced of a few ten to hundred picosecond, should be simultaneously resolvable with GHz-frequency-domain fluorometers.
Simultaneous resolution of 2 to 3 lifetimes with lifetime differences much greater than the instrumental resolution, e.g. of a few hundred picoseconds, should be possible performing phase-modulation experiments up to 200MHz with lifetime resolution of a few ten picoseconds.
Lakowicz et al. /LLCGL84/ demonstrate the distinction of double and triple exponential decays from single- or double

exponentials in phase-modulation fluorometry combined with appropriate software assuming random errors of 0.1% to 1% by simulations of phase-modulation data. For modulation frequencies of f = 1 - 200MHz a double exponential decay with lifetime differences of 40% of the individuals (e.g. lifetimes on the nanosecond scale as τ_1 = 20ns and $\tau_2 \leq$ 12ns) can be separated at a random error of 0.5% and assuming CHI_R^2 = 3 (see also equation [13]) as "cut-off" value to recognize the presence of an additional component /LLCGL84/. A triple exponential decay for lifetimes of τ_1 = 5ns, τ_2 = 10ns and τ_3 = 20ns could not be resolved with these conditions.

The lifetime resolvability can be enhanced by reduction of the random error and introducing variable modulation frequency and emission wavelength selectivity. Table 8 shows examples of the possible lifetime resolvability in dependence of the random error after computer simulations in /LLCGL84/. The random errors are referred to a maximum modulation degree of 1.0 and a maximum phase shift of 90°.

random errors	decays	$\Delta\tau$	fractional contrib.
	2-exponential at one wavelength:		
0.5%	20ns, <12ns	40%	$f_1 = f_2 = 0.5$
0.1%	20ns, <16ns	20%	"
<0.1%	4ns, 5ns	"	"
	at various wavelengths:		
0.5%	18ns, 20ns		f_1 from 0.2 to 0.8 f_2 from 0.8 to 0.2 (complementary to f_1)
0.1%	19.5ns, 20ns		"
1%	16ns, 20ns		"
	3-exponential at one wavelength:		
0.5%	20ns, <6ns, <1.8ns	70%	
0.1%	5ns, 10ns, 20ns	50%	$f_1 = f_2 = f_3 = 1/3$

table 8: Resolved lifetimes; data according to computer simulations in /LLCGL84/

The use of various emission wavelengths can enhance the resolving power (resolution of more closely spaced lifetimes), in the case of a double exponential decay with different emission spectra for each component. Introducing wavelength selectivity means that the fractional intensity contributions, f_i, of components with different emission characteristics are different at a selected emission wavelength. Nevertheless, the simultaneous identification and hence lifetime determination of three components is the more difficult case than to identify two components /LLCGL84/.

The accuracy of the resolved lifetime values of two- or threefold exponentials and hence the determined fractional intensities which can be correlated to the concentrations of the components has to be looked on very carefully to get reliable results. The dependence and sensitivity of CHI_R^2 (see [13]) on the chosen (simulations) or measured parameter values can be used as a measure for the precision of the determined data, presented in fig.5 in /LLCGL84/ for the case of a double exponential decay at selected random errors and also in table 9. Table 9 roughly presents lifetime accuracies according to the calculated CHI_R^2 response in /LLCGL84/.

random error:	lifetimes:	lifetime precision:
0.1%	20ns, 5ns	high
0.1%	20ns, 10ns	little uncertainty
0.1%	20ns, 15ns	less certain!
0.5%	20ns, 15ns	cannot be resolved

table 9: Lifetime precision for resolved lifetimes of double exponential decays; data of computer simulations in /LLCGL84/.

The confidence of lifetime values of components in the case of a small amplitude contribution to the phase resolved fluorescence spectrum, again based on the CHI_R^2 response, is shown in fig. 19.

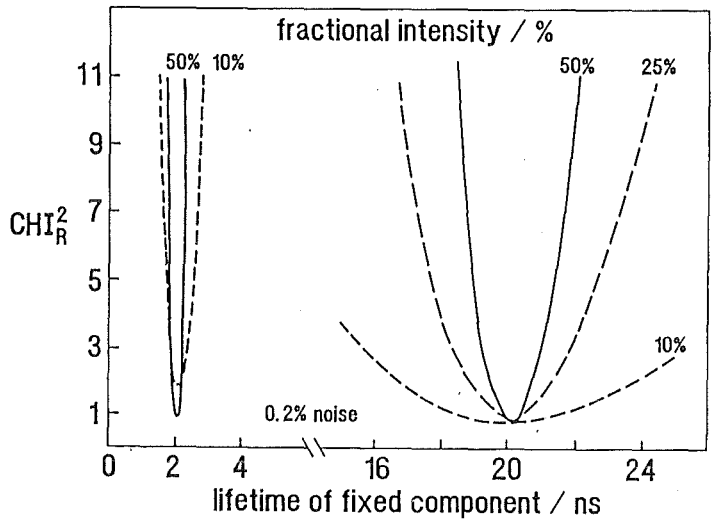


fig. 19: CHI_R^2 sensitivity versus lifetime for two-component decay of τ_1 = 2ns and τ_2 = 20ns and steady state fractional amplitudes of 10% and 50% of each component at assumed random error of 0.2%, after fig. 6 in /LLCGL84/;

A decrease in the amplitude of a component expressed by the fractional intensities reduces the change of CHI_R^2. CHI_R^2 becomes less sensitive to lifetime changes and the determined lifetime data uncertainty increases. This effect is more pronounced for the 20ns lifetime, fig. 19. In a two component system with a small amplitude of one component the components can be separated within a good precision if the individual lifetimes are well separated.
Again, wavelength selectivity enhances the lifetime resolution of more closely spaced lifetimes and the precision /LLCGL84/.
For a three component simulation CHI_R^2 reacts much more insensitive to a variation of the lifetimes for different amplitudes of the individual components at a single emission

bandpass without any knowledge about the lifetime of one component. The reliability of the determined lifetime values is increased if one of the three lifetimes is independently known and fixed /LLCGL84/.

phase-modulation instrument	lifetimes	fractional intensities	molecules, ref.
1 - 200MHz	τ_1 = 4.13ns τ_2 = 4.41ns, d.-factor: 1.07	$f_1 + f_2 = 1.0$, f_1 : 0.05, 0.24, 0.45, 0.86	A, 9-MA /LM85/
	τ_1 = 0.20ns τ_2 = 0.83ns d.-factor: 4.15	f_1 = 0.57 f_2 = 0.47	NADH /LM85/
0.1 - 200MHz	τ_1 = 11.71ns τ_2 = 5.95 d.-factor: 2	f_1 = 0.52 (f_2 = 0.48)	9-CA, 9,10-DPA
	τ_1 = 6.32ns τ_2 = 3.71ns d.-factor: 1.7	f_1 = 0.45 (f_2 = 0.55)	9,10-DPA Coum. 2 /PMV89/
400kHz - 300MHz,	τ_1 = 1.296ns, τ_2 = 4.6ns d.- factor: 3.54	$f_1 \approx 0.8$ ($f_2 \approx 0.2$)	POPOP, A, /CJEP89/
up to 2GHz	τ_1 = 51ps τ_2 = 756ps d.-factor: 15	$f_1 \approx f_2 = 0.5$	t-St p-Qu /LLGSW88/
	τ_1 = 17ps τ_2 = 73ps d.-factor: 4.3		indole, /LLGSW88/
4, 10GHz	τ_1 = 145ps τ_2 = 342ps d.-factor: 2.4	f_1 = 0.46 f_2 = 0.54	DFS, DBS, /LGGWML90/

table 10a): experimentally resolved two-fold exponential decays; abbreviations: d.-factor: lifetime difference factor; A: anthracene, DPA: diphenylanthracene, MA: methylanthracene, CA: cyanoanthracene, NADH: nicotineamid adenine dinucleotide, t-St: t-stilbene, p-Qu: p-Quaterphenyl, Coum.: Coumarine 2, DFS: 4-dimethylamino-4-fluorostilbene, DBS: 4-dimethylamino-4-bromostilbene, VA: vinylanthracene;

Table 10 a), b) present a few examples of experimentally determined lifetimes on the nanosecond time scale and fractional contributions of individual components in multicomponent mixtures for double- and triple- exponential decays cited in literature.

phase-modulation molecules, instrument	lifetimes	fractional intensities	ref.
1 - 200MHz	τ_1 = 1.24ns τ_2 = 4.13ns τ_3 = 7.7ns d.-factor: 3.3, 1.9	f_1 = 0.15 f_2 = 0.52 f_3 = 0.34	POPOP, A, 9-VA /LM85/
400kHz - 300MHz	τ_1 = 1.319ns τ_2 = 3.61ns-----(minor contrib.) τ_3 = 5.897ns d.-factor: 2.7, 1.6		POPOP, A, DPA /CJEP89/
up to 2GHz	τ_1 = 0.018ns τ_2 = 0.501ns τ_3 = 4.458ns d.-factor: 27, 9	f_1 = 0.6 f_2 = 0.32 f_3 = 0.08	Oxyhemo- globin /BMFGLL88/
4,10GHz	τ_1 = 0.002ns τ_2 = 0.028ns τ_3 = 0.820ns d.-factor: 14, 29	f_1 = 0.25 f_2 = 0.66 f_3 = 0.09	Deoxyhe- moglobin /LGGWML90/

table 10b): Experimentally resolved three-fold exponential decays; abbreviations, see table 10a);

Sometimes, the resolved lifetimes on the nanosecond time scale, table 10a), 10b), roughly differ by a factor of 1.7 (2-fold exponential decays), mostly by a factor greater 1.7 (2-fold- and 3-fold-exponentials). The lifetime differences are mostly greater than a factor of 2 for a double

exponential decay and much greater than a factor of 10 for a triple exponential decay for lifetime resolution on the picosecond time scale.
Further it should be mentioned that large deviations, relative errors, in experimentally determined and expected fractional contributions can occur in a multicomponent mixture, table 11. The smaller the fractional contribution of a component the poorer is the coincidence of the experimentally determined and the expected fractional intensity, the more uncertain is its value.

fractional contrib., experimental	fractional contrib., expected	rel. error
anthracene (4.13ns, f_1) and 9-methylanthracene (4.41ns) /LM85/:		
f_1 = 0.86	0.8	7%
f_1 = 0.45	0.6	ca. 33%
f_1 = 0.24	0.4	ca. 66%
f_1 = 0.05	0.2	ca. 75%
POPOP, anthracene , 9-vinylanthracene (1.24ns), (4.13ns), (7.7ns) /LM85/:		
f_1 = 0.15	0.2	25%
f_2 = 0.51	0.5	2%
f_3 = 0.34	0.3	ca. 13.3%
f_1 = 0.4	0.5	20%
f_2 = 0.44	0.3	ca. 46.7%
f_3 = 0.15	0.2	25%

table 11: Examples for experimentally measured and expected fractional contributions and relative errors of these contributions of a 2-fold- and 3-fold exponential decay.

I like to summerize:
If fluorescent, multicomponent systems are resolved by phase-modulation fluorometry simultaneously, the best results with good precision can be achieved resolving a mixture of two different components or one component with

heterogenous emission with nearly equal fractional intensity contributions of the individuals and sufficiently large lifetime differences at one emission wavelength. Such a fluorescent system can be treated by a double exponential decay law. Wavelength selectivity can enhance the lifetime resolution of closely spaced lifetimes. Triple exponential decay laws can also be resolved within the error limits. It has to be taken into account that the determined data, the lifetime and the fractional contribution of one component can be highly imprecise.
All these considerations already include modulation selectivity according to the time scale of the lifetimes to be determined and the required resolution capability.
Phase-modulation fluorometry can result in an excellent time resolution and precision specially for single exponential decays using highly developed instrumentation.

II.3 PURELY PHASE SENSITIVE FLUORESCENCE DETECTION, (PHASE-RESOLVED METHOD)

a) BASIC PRINCIPLES AND APPLICATION

Early phase-modulation fluorometers were restricted to phase sensitive fluorescence detection at non-variable, only one to three available modulation frequencies for resolving mixtures of two fluorescent components simultaneously in the error limits /SW69,VCS70,LCB81,LB82,L83,JGH84,MB87/. This technique was often used to measure and to determine the fluorescence emission coupled with lifetime selectivity.
The phase resolved detection of the fluorescence emission can easily be performed by measuring the resulting photocurrent with a phase-sensitive detector e.g. with lock-in technique for determining the phases /LCB81,B85/.
In a multicomponent system the total fluorescence emission is a superposition of the sinusoidally modulated emission of each individual fluorescent component of the system. As a result of the convolution of a periodic detector function

and this sinusoidally modulated fluorescence emission function the partial output signal is proportional to cos ($\phi_D - \phi_i$) and the phase-sensitive fluorescence spectrum of a multicomponent system can be described by :

$$P(x, \phi_D) = \Sigma I_i(x)\ \alpha_i\ m_i \cos(\phi_D - \phi_i) \qquad [14],\ /JGH84/$$

x: wavenumber or wavelength

ϕ_D : detector phase angle

ϕ_i : phase shift of the i-th component

The phase-sensitive spectrum depends on the detector phase angle ϕ_D, so that a defined phase shift between the detector and the waveform to be investigated can be adjusted.
By setting the detector phase angle $\phi_D = \phi_i \pm \pi/2$ the amplitudes of the detector and the i-th component are out of phase and hence no intensity contribution to the overall fluorescence intensity of this component can be observed. The resulting current presents the fluorescence intensity of the remaining components.
A two component mixture of components A, B is discussed in the following. For more detailed description see /L83/.
In the case of sufficiently different lifetimes τ_A, τ_B but overlapping spectral excitation and emission characteristics of A and B the intensity contribution to the overall intensity of component A can be suppressed by setting the detector phase angle to e.g $\phi_D = \phi_A + \pi/2$, see fig. 20. The phase-sensitive spectrum only consists of the contribution of component B:

$$P(x, \phi_D) = P_B(x)\ m_B \sin(\phi_B - \phi_A) \qquad [15]$$

(vice versa for the intensity contribution of component A suppressing B)

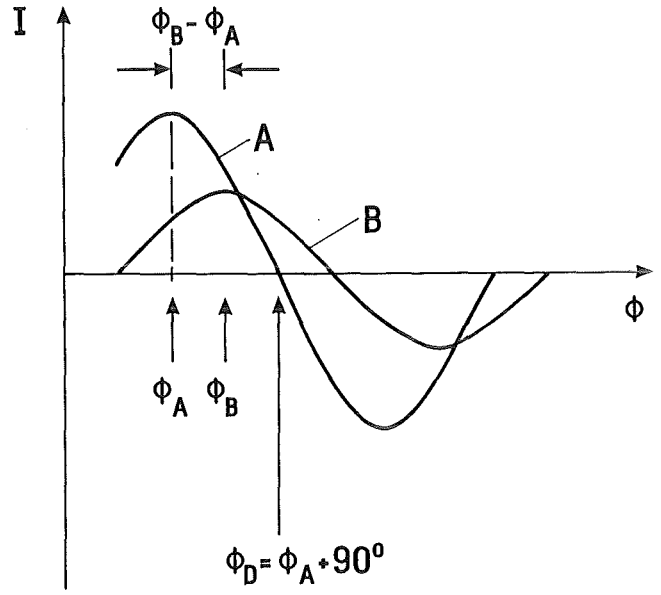


fig. 20: Suppression of the phase resolved intensity of component A by detector phase angle setting $\phi_D = \phi_A + \pi/2$, schematically, see text for description;

The resolution capability of phase-sensitive detection depends on the lifetime difference between the individual components. The observed phase resolved intensities of each component are attenuated as by a factor of sin ($\phi_B - \phi_A$) compared to the original modulated light intensity as by the demodulation factors, m_i, of the individual components. The responses of the amplitudes to modulation strongly depends on each component's lifetime. It also differs for long-lived and short-lived components, see fig. 7. Hence, the individual intensity contributions to the overall fluorescence intensity can differ strongly. These characteristics of phase resolved fluorescence detection can result in problems if only one modulation frequency is available ("response mismatch"). This has carefully to be taken into consideration if the phase-sensitive method is used for quantitative analysis, e.g. determining fractional contributions and hence concentrations of individual components.

Selecting the appropiate detector phase angle for one selective component to be suppressed requires knowledge about either the lifetime or the emission spectrum of the components.
Alternatively, the phase-sensitive fluorescence emission spectra of a two component mixture can be detected at different phase angles and be compared with the steady state emission spectrum of the individuals.
Two component systems can be resolved by phase sensitive detection and their lifetimes can be determined in the error limit caused by using one or two available, instrument dependent modulation frequencies only, which do not give an overall information about the investigated waveform, see also above and later.

Veselova et al. /VCS70/ were the first using this technique resolving the mixture of a two component system. They detected the monomer and aggregate emission spectrum of quick-frozen anthracene. Components with overlapping steady state emission spectra, e.g. in the case of 2-p-toluidinyl-6-naphthalenesulfonic acid (TNS) and 6-propionyl-2-(dimethylamino)naphthalene (PRODAN), anthracene and its exciplex with diethylaniline /LC81/ and naphthol and naphtholate /LB82/, could successfully be separated by phase-sensitive fluorescence detection. As an example, figure 21 demonstrates the phase resolved spectra of a two component mixture consisting of dibenzo(a,h)anthracene and dibenzo(c,g)carbazole by purely phase-resolved fluorescence emission spectroscopy at 10MHz modulation frequency in comparison with the steady state spectra of the individuals /LC81/.
The individual decay times could be determined within a precision of ± 0.1ns by knowledge about the steady state spectrum of the unsuppressed component.

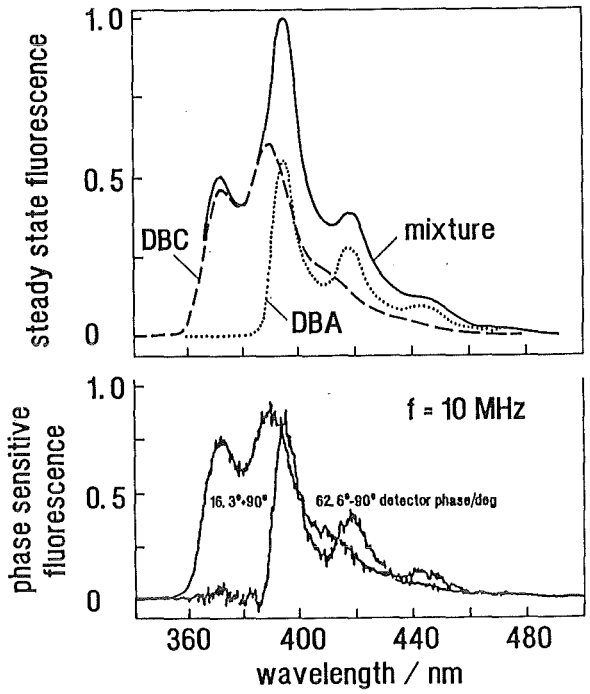


fig. 21: Steady state (top) and phase resolved (bottom) fluorescence emission spectra of a mixture of dibenzo(a,h)anthracene (DBA) and dibenzo(c,g)carbazole (DBC) from /LC81/, fig. 8;

Phase-resolved suppression of scattering background can also be performed to enhance the signal-to-noise ratio to 10^2 detecting "low intensity" fluorophores /DK85/.
McGown and Bright /M84,MB85,BM85,MB87/ applied phase sensitive fluorescence detection for quantitative chemical analysis on two to four component systems combined with emission wavelength selectivity. Using several detector phase angles, without suppressing one component explicitely, coupled with the solution of a linear equation system is an additional approach /M84/. All solutions to be analyzed are measured at n detector phase angles ($n \geq m$ (m: number of components in the mixture)) at one modulation frequency. The average relative error of the determined concentrations of a mixture of POPOP/DimethylPOPOP is reported to be 3.1% for POPOP and 5.3% for DimethylPOPOP for eight detector phase angle settings at 30MHz modulation frequency /M84/.

b) ERROR SOURCES, LIFETIME RESOLUTION AND ACCURACY, RESOLUTION CAPABILITY

The problems in phase-modulation fluorometry Lakowicz mentioned /L83/, see also above, have especially to be considered in purely phase sensitive fluorescence detection at limited available modulation frequencies.
The lifetime resolution and precision depends on the error in determining the phase angles which can be correlated with the applied modulation frequency if this is not in coincidence with the lifetime of the components, see fig. 6.

Phase angle imprecisions can be recognized by observations of differences in the fluorescence intensity of the phase-resolved and the steady state spectrum. Also a shift in the phase resolved emission maximum can occur indicating heterogenous emission of the sample /L83/.

An imprecision in measuring the phase angle of about $\Delta \phi = 0.43°$ (e.g. evaluated by lifetime resolution data of an SLM instrument model 4800 /SLM Inc./) can produce a difference in intensity of about $2.8*10^{-5}$ of the maximum intensity assuming a sinusoidal waveform (resulting in a deviation of about 4ps to 21ps if determining the lifetime by demodulation data) on the one hand. On the other hand an additional modulation frequency dependent time delay of ± 40ps to ± 0.2ns at 30MHz to 6MHz occurs, see fig. 22.

Assuming a larger imprecision of 1% in determining the maximum fluorescence intensity, e.g. by locating the detector phase angle for suppressing one component of a two component mixture, may result in an error of the phase shift of about 8.1°. Now, the determined lifetime can scarcely be evaluated to be accurate to ± 0.76ns at 30MHz, ± 1.26 ns at 18MHz and ± 3.78ns at 6MHz modulation frequency.

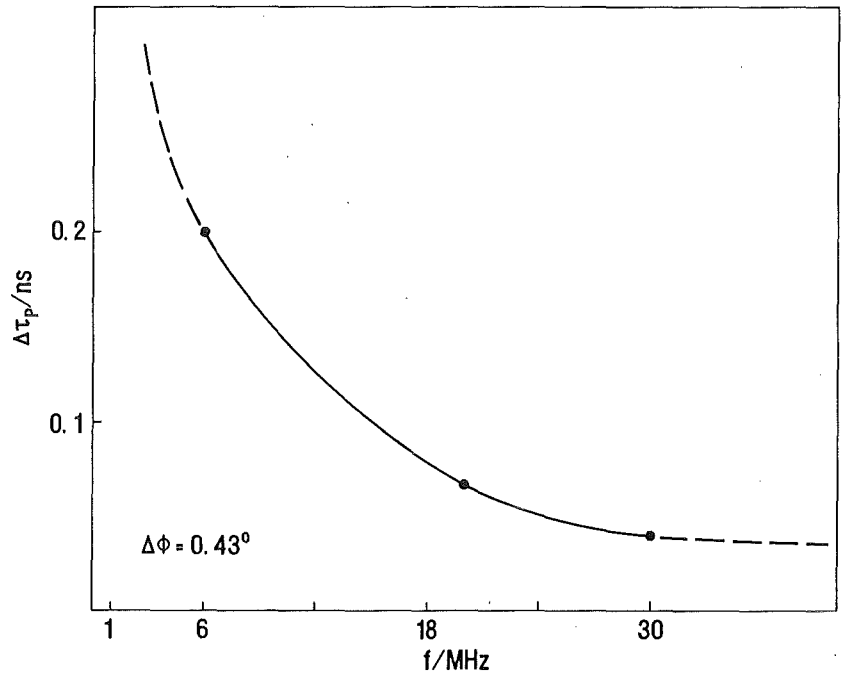


fig. 22: Deviation of lifetime τ_P, $\Delta\tau_P$, versus modulation frequency at a fixed phase angle error of $\Delta\phi = 0.43°$

As the resolution capability of a two component system can be limited by such imprecisions the lifetimes of the components should differ sufficiently.
Again, in analogy to fig. 16 and fig. 17, figures 23 and 24 selectively depict relative errors of determined lifetimes for phase and modulation data at three fixed, non variable modulation frequencies available in purely phase resolved fluorometers, see /SLM Inc./.

A broad minimum in the relative lifetime error can be distinguished at lifetimes of about τ = 5ns and τ = 7ns for 30MHz and 18MHz modulation frequency. This minimum shifts to shorter lifetimes with increasing modulation frequency. The error in lifetime determination for a fluorescence lifetime of τ = 1ns is greater than 15% at a modulation frequency of 6MHz which further increases strongly for lifetimes τ < 1ns.

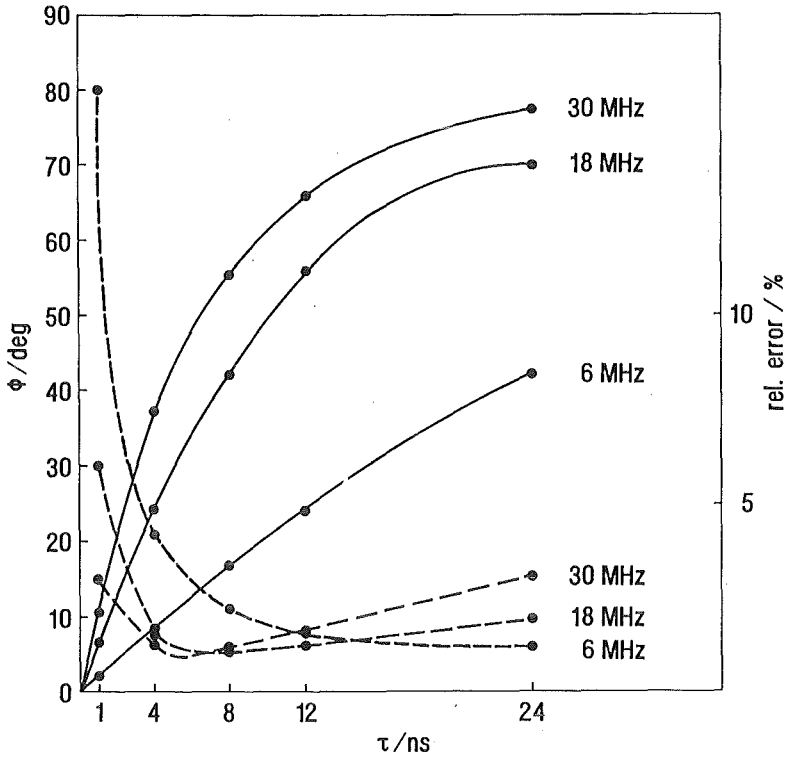


fig. 23: phase shifts (solid plots) and relative errors in determined lifetimes (dashed plots) at 30MHz, 18MHz and 6MHz modulation frequencies versus fluorescence lifetimes for a fixed phase angle error, $\Delta \phi = 0.35°$, (according to Spencer's and Weber's early instrument /SW69/).

These considerations are again in coincidence with a maximum lifetime selectivity obtainable at the optimum lifetime dependent modulation frequency /B85/.

The relative error of the lifetime of a component determined by demodulation can easily exceed 50% for e.g. fluorescence lifetimes $\tau < 1$ ns at 30MHz even for a minor deviation of 1%. (The relative error of the lifetime for long-lived components, e.g. with $\tau > 20$ns, can be underestimated in this plot caused by their poor response to high modulation frequencies. This results in an additional random error resolving their fluorescence intensities experimentally which may be large and is not included in these considerations).

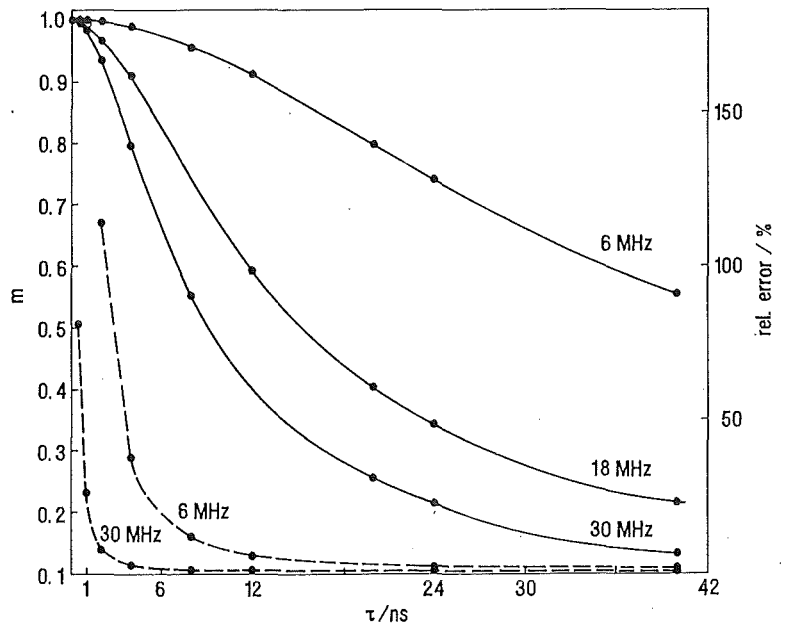


fig. 24: Demodulation data, m (solid plots), at 6MHz, 18MHz and 30MHz modulation frequency and relative errors of lifetime τ_M at a theoretically assumed imprecision of 1% for the demodulation factor at 6MHz and 30MHz (dashed plots).

Jameson and Weber /JW81/ investigated systematic and random errors and also propagated errors of resolved lifetimes at a fixed value of $\Delta\phi = 0.15°$ for phase angle imprecision and a variable error of demodulation data of $\Delta m = 0.0012$ to $\Delta m = 0.0037$ for 6MHz to 30MHz modulation frequency respectively. The authors report the lifetimes of a binary mixture to be determined within 5%-20% of their true values.

Table 12 summarizes some examples for lifetime resolution and precision data of phase resolved fluorescence detection at fixed, non variable modulation frequencies from literature.

lifetime precision/ modulation frequency		components/ mixtures and resolved lifetimes
± 0.03 ns	28.4MHZ	Fluorescein (10µg/ml in 0.1M NaOH/0.8M KJ solution), 0.39 ns, /SW69/
± 0.2 ns	10MHz	POPOP/DimethylPOPOP, 1.35ns/ 1.45ns /LCB81/
± 0.4-1.7 ns	6, 18, 30MHz	Tryptophan at various pH-values (binary mixture), 8.7ns anion, 3.1ns zwitterion /JW81/
± 0.8 ns	10 MHz	TNS/PRODAN, 11.5 ns/ 3.6 ns /LC81/
± 0.1 ns	30 MHz	evaluated /LC81/

table 12: Lifetime precision data for resolved lifetimes in multicomponent systems;

These data show that determination of individual fluorescence lifetimes $\tau < 1$ns is also possible by purely phase sensitive detection of fluorescence intensities. The resolution capability for resolving the lifetimes of two components with a small lifetime difference of about $\Delta\tau = 0.1$ns could also be demonstrated for a mixture of POPOP/DimethylPOPOP /LCB81/. Nevertheless, in this case a relative deviation of about 14% of the true individual lifetime values has to be taken into account.

For extended overall application of phase resolved fluorescence detection, including improvement of lifetime resolution even to the femtosecond range, improvement of the lifetime resolution capability of picosecond lifetimes of more than one component coupled with fast detection and analysis, "lifetime instruments" with variable modulation frequencies up to 10GHz were developed as described above.

III. TESTING PHASE-MODULATION FLUOROMETRY

A laser-induced fluorometric equipment, developed and used for uranium detection in reprocessing plants at the Karlsruhe Nuclear Research Center, consists of an excimer-laser pumped dye-laser as exciting light source and a fast photomultiplier coupled with a boxcarintegrator or a sampling oscilloscope as detection system, fig. 25.

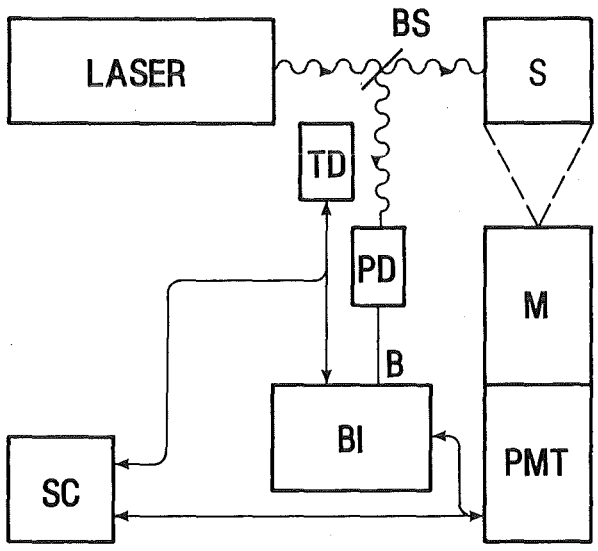


fig. 25: Time resolved, laser-induced fluorometric equipment; BS: beam splitter, S: sample, M: monochromator, PMT: photomultiplier tube, PD: photdiode, TD: trigger diode, BI: boxcar integrator, SC: sampling oscilloscope

Due to the pulse width of the lasersystem of about 15 ns, lifetime investigations of organic pollutants by a directly time-resolved measurement to enhance the selectivity using fluorometric methods as an analytical tool are restricted to a very few substances with "long" lifetimes as e.g. pyrene (τ ca. 100ns in water) or fluoranthene (τ ca. 37ns in water)

/K91/.
So phase-modulation fluorometry was taken into consideration as an alternative method.
Experiences with this method were gained during visits of several US laboratories (SLM-Aminco, Inc., distributor of commercial instruments, and US universities applying phase-modulation).
At the Pittsburgh Conference and Exhibition of Analytical Chemistry and Applied Spectroscopy in Chicago, March 1991, the fastness of data acquisition and analysis of the SLM48000MHF fluorometer applying Fourier transformation by SLM-Aminco, Inc., could be demonstrated. The lifetime of excited quininesulfate in sulfuric medium (1µg/ml) of τ = 18.4ns could be determined versus a scattering solution in the frequency range of 5MHz to 160MHz. A fitting procedure with a double exponential fit yielding a "good" CHI_R^2 = 1.1 was applied. An additional intensity contribution of < 1% and an additional lifetime of ca. 0.8ns could also be observed, probably due to scattered light. In a mixture of quininesulfate/Rhodamine 6G (unknown small quantity) fluorescence lifetimes of τ_1 = 18.9ns, τ_2 = 4.4ns and τ_3 = 0.72ns were resolved versus POPOP as reference fluorophore (triple exponential with CHI_R^2 = 1.3). The expected lifetimes of quinine and Rhodamine 6G in pure water are τ_1 = 18.5ns and τ_2 = 1-2ns /S91/. The lifetime of Rhodamine 6G of ca. 4.4ns does not agree with the one for the single component. It might have been changed. The additionally observed short living component of ca. 0.72ns cannot be identified exactly.
In order to test the SLM 48000 S phase-modulation fluorometer with sequential variable modulation frequencies from 5 - 200MHz and a HeCd-laser working at 325 nm emission wavelength as exciting light source anthracene in EtOH (< 1.3µg/ml) and a water/EtOH solution (90%/10%, < 0.13µg/ml) was used as sample, fig. 26 a), b), at the laboratory of SLM-Aminco, Inc..

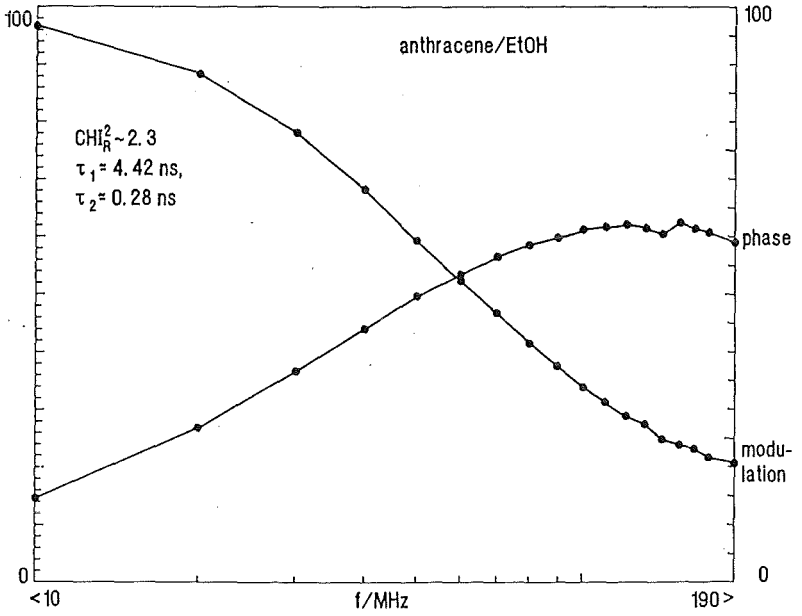


fig. 26: Phase and modulation data for anthracene in EtOH and a water/EtOH solution;
a) anthracene concentration: < 1.3µg/ml;

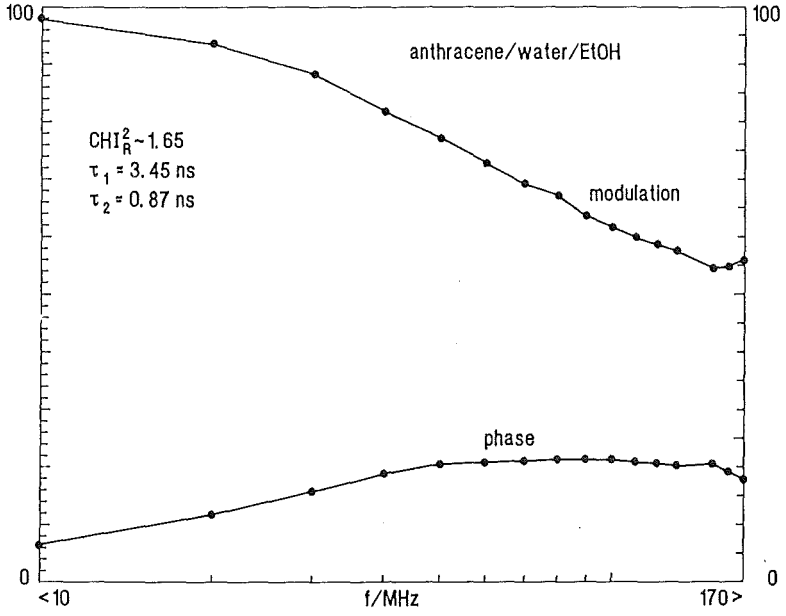


fig. 26 b): analog fig. 26 a); anthracene concentration: < 0.13µg/ml;

As anthracene did not seem to be totally dissolved in EtOH the real concentrations were estimated to be half the above cited values: probably ca. 0.6µg/ml and 0.06µg/ml.
A double exponential model yielded the best fits to the experimental data with CHI_R^2 = 2.3, fig. 26 a), and CHI_R^2 = 1.65, fig. 26 b) resolving anthracene fluorescence lifetimes of ca. 4.42ns and 3.45ns. The fractional contribution of a second component with a lifetime of ca. 0.28ns, fig. 26 a), and ca. 0.86ns, fig. 26 b), was also observed which could be assigned to scattered light originated either by the solvents themselves or by an instrumental artefact. A lifetime of τ = 1.61ns for POPOP /B71/ was used as reference lifetime. The scattering contribution increases by lowering the anthracene concentration.
Also at high modulation frequencies the noise increases which results in larger phase and modulation deviations, see fig. 27 as example.

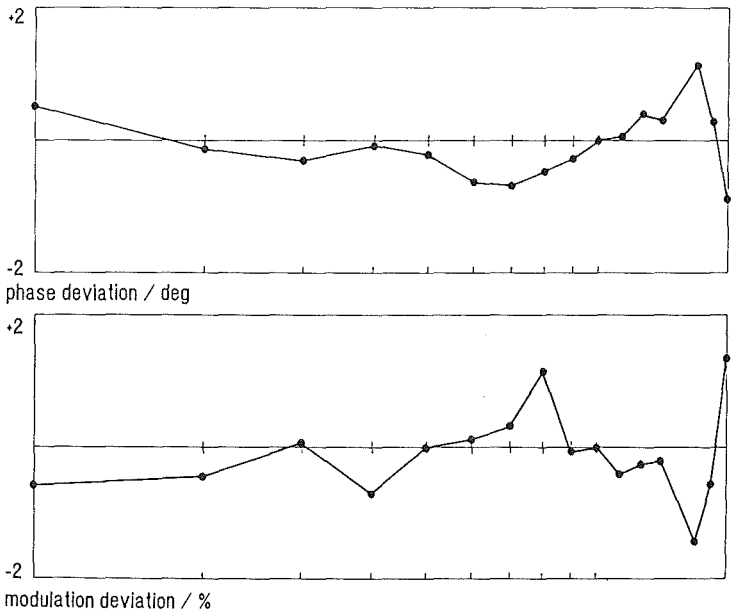


fig. 27: Phase and demodulation deviations of the experimental date in fig. 26b) versus modulation frequency

Instabilities of the Pockels cell, of the modulated UV-light of the HeCd-laser or the frequency response of the detector could be noise sources.

Fig. 28 presents the results of a second measurement at the same low anthracene concentration as in fig. 26 b) of anthracene dissolved in EtOH. The resolved lifetime τ is about 4.06ns. Although the CHI_R^2 value, $CHI_R^2 = 14.5$, is much greater than unity, which is unacceptable, the lifetime value of $\tau = 4.06$ns agrees with the ones in /LM85/, see also table 13.

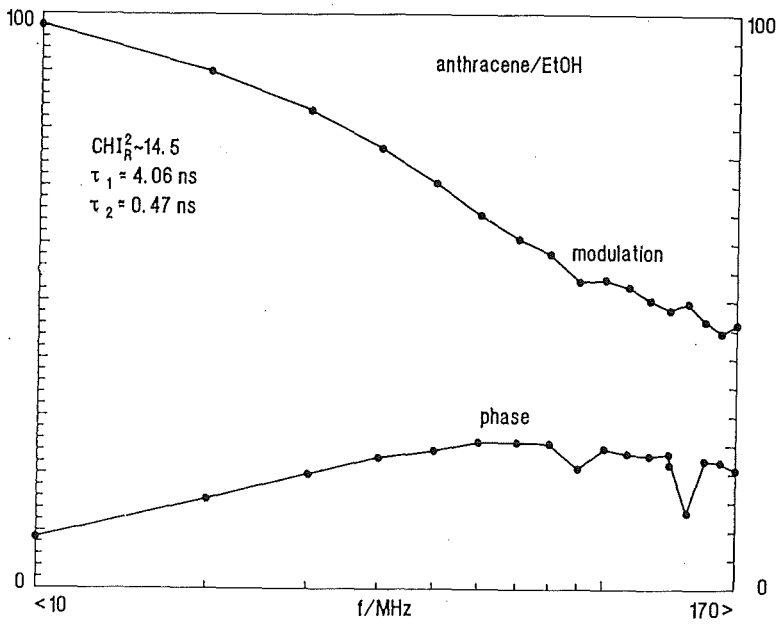


fig. 28: Phase and modulation data of anthracene in EtOH; anthracene concentration: < 0.13µg/ml;

measured anthracene decay times	solvent
4.9 ns	cyclohexane /B71/
4.13 ns	in a 2-component mixture, solvent not named /LM85/
4.6 ns	in a 2-component mixture, solvent not named /CJEP89/
3.61 ns	minor contribution in a 3-component mixture, solvent not named /CJEP89/

table 13: Determined lifetimes of anthracene from literature.

Table 13 and these test measurements show that the anthracene lifetime can be a concentration and solvent dependent parameter.
It should also be mentioned that at very low concentrations difficulties in the measurements occurred which were probably due to the low light intensity provided by the Liconix HeCd-laser in the UV.
Experiments with the SLM model 48000S fluorometer require long measuring times to gain reliable results, e.g. each test measurement took more than 15 minutes.
The SLM 48000MHF instrument working with fast Fourier transformation technique is superior, considering the duration of measuring times. For comparison, to gain thousand averages with the SLM 48000MHF at all available modulation frequencies takes ca. 15 minutes (measuring time, data storage and analysis), whereas with the sequential single frequency instrument, SLM 48000 S, it will take about two hours /B91/.
Nevertheless, at extremely low concentrations, long integration times, reference measurements and background subtractions, performed for the reference samples and the samples separately, also increase the time of a measuring

cycle needed for an overall, reliable analysis. This can also take about 2 hours including 4 measuring steps /B91/.

At Frank Bright's laboratory, State University of New York at Buffalo, indeed fluorescence phase shift and modulation data of lowly concentrated fluorophores could be measured with an extended SLM 48000MHF. This was due to the different exciting light source, an Ar^+-ion laser with an intensity (ca. 100mW at 351 nm), greater than one order of magnitude in the UV as the one of the HeCd-laser of the commercially available SLM-system, and the use of cooled photomultipliers (reference PMT and measuring PMT) improving the signal to noise ratio. A signal of anthracene in water (1.6ng/ml) which was 2.5 times larger than the background signal, could be detected, fig. 29.

Kannen/Bright

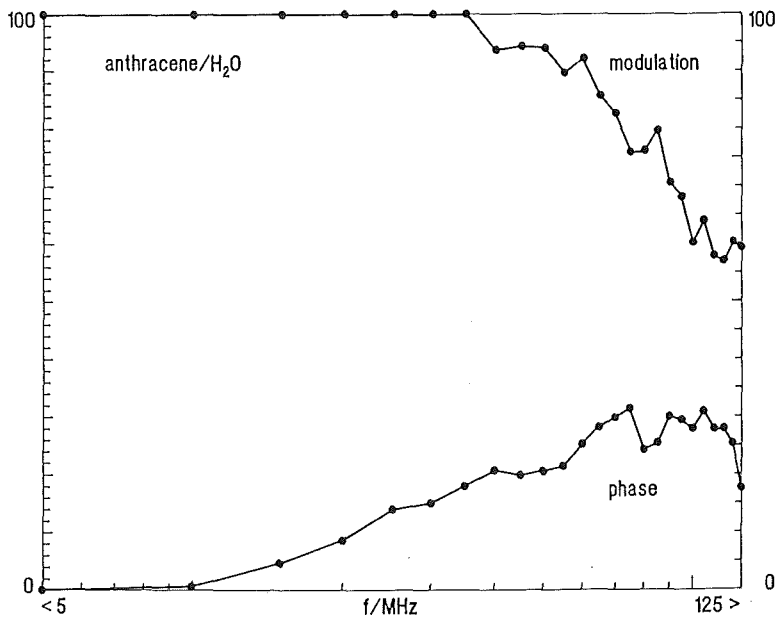


fig. 29: Phase and modulation data at modulation frequencies of 5-125MHz of anthracene (ca. 1.6 ng/ml) in twofold distilled water (with a minor amount of EtOH);

The noise of the spectra indicates a detection limit. Water (assuming τ = 0.0ns) was used as the reference sample. The detection limit in water is about 0.5 ng/ml for a single component /B91/. For getting better results background subtractions have to be applied, a time consuming procedure which can take ca. 2 hours for one sample /B91/.
It should be mentioned that for all measurements a long wavelength bandpass filter for λ > 400nm was used instead of a monochromator further reducing the intensity to be detected.
Phase and modulation data for a two- and a three-component mixture are presented in fig. 30 and fig. 31.

Kannen/Bright

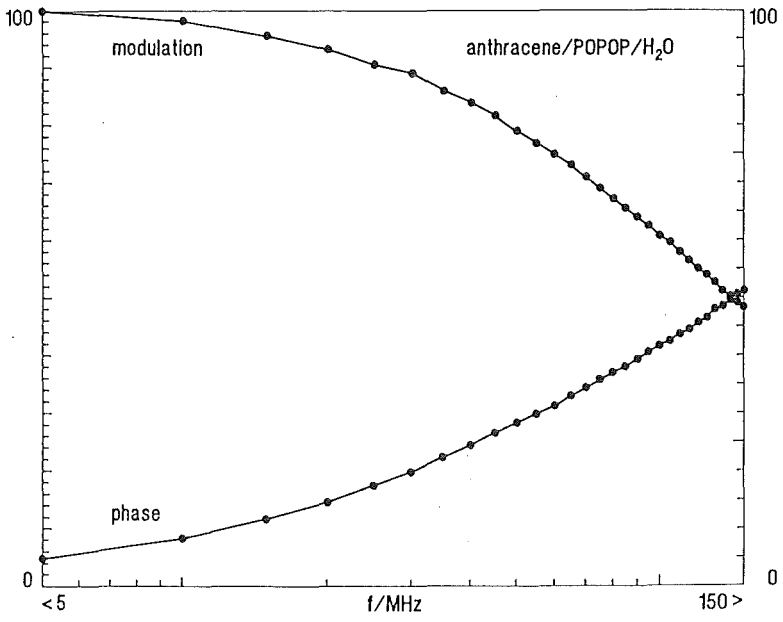


fig. 30: Phase and modulation data of a mixture of anthracene and POPOP in two-fold distilled water (EtOH) between 5MHz and 150MHz modulation frequency; concentrations: 16ng/ml (anthracence) and 20ng/ml (POPOP);

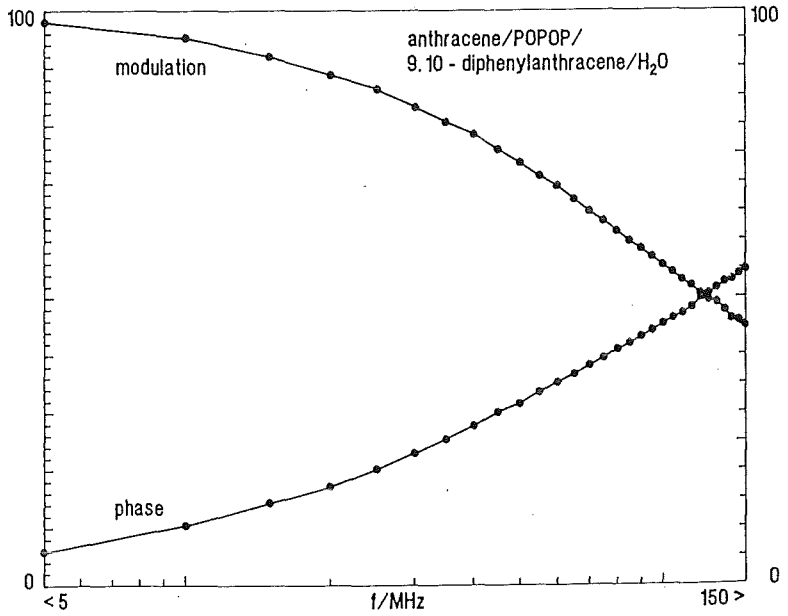


fig. 31: analog fig. 30; mixture of anthracene, 16 ng/ml, POPOP, 20 ng/ml, and 9,10-diphenylanthracene, ca. 10 ng/ml, in twofold distilled water (EtOH);

The brackets indicate an additional minor amount of EtOH as the molecules were first dissolved in EtOH and the solutions succesively diluted with water.
These data show that two- and three component mixtures could be resolved applying threefold exponential mathematical models additionally. Dimethyl-POPOP (τ = 1.35 ns in EtOH) was used as reference fluorophore. Results with a "good" CHI_R^2 value were gained. The determined lifetimes τ_i and fractional contributions f_i are listed for a:

— 2-component mixture of anthracence (ca. 16ng/ml)/ POPOP (ca. 20ng/ml) in water:
τ_1 = 5.21ns, f_1 = 0.247
τ_2 = 1.51ns, f_1 = 0.679
τ_3 = 7.93ps, f_1 = 0.074 (scattered light),
CHI_R^2: 0.72;

— 3-component mixture of anthracene (ca. 16 ng/ml) /POPOP (ca. 20 ng/ml) /9,10-dimethylanthracene (ca. 10 ng/ml) in water:

τ_1 = 13.37ns, f_1 = 0.1082

τ_2 = 3.23ns, f_1 = 0.452

τ_3 = 1.09ns, f_1 = 0.44

CHI_R^2: 0.22;

Deviations of the lifetime values of the components in the mixtures compared to the lifetime of the single component solutions occurred. Again, the anthracene lifetime seems to differ strongly in aqueous systems.
Extensive investigations of the single components, e.g. in different media, should precede in the case of analyzing mixtures.
Background subtractions would probably improve the results. Nevertheless, lifetime and fractional contribution determinations with high precision of organics at extremely low concentrations (ppb, subppb), as e.g. in above systems, seem to be critical.

IV. COMMENT ON THE APPLICATION OF LIFETIME TECHNIQUES

Determination of concentrations of organic molecules by fluorescence lifetime measurements requires exact knowledge of the lifetime of the excited state of a molecule to be investigated. Every effect influencing the excited state and hence changing the lifetime has to be taken into consideration and must be systematically investigated in order to get reliable results. I would like to mention some of those effects changing the fluorescence intensity: e.g. collisional quenching by oxygen, influences by H_2O_2 or heavy atoms (intramolecular), solvent effects, charge transfer reactions e.g. with NO, NO_2, NO_3^- (intermolecular) or complexations with molecules or ions, excited state reactions. The occurence of heterogeneous emission of a single fluorophore resulting in a non-single exponential decay law for that individual fluorophore in a mixture can

lead to an additional complication.
The situation becomes even more complex, if multiple component systems, environmental samples consist of, have to be resolved. Strong light scattering by small particles in real aqueous samples, e.g. from surface waters, can act as a limiting factor by overlapping the fluorescence intensity of fluorescent pollutants. Also incontrollable influences by oxygen or oxygen concentration gradients might exist. Without preceding preparations of environmental samples, e.g. solvent extraction and other separation methods, a fast simultaneous reliable overall analysis of extremely low (ppb and subppb) concentrated pollutants by fluorometric methods exclusively is indeed rather critical. Chromatographic methods as gas or liquid chromatography are more powerful analytical tools for the analysis and resolution of multicomponent systems of very low analyte concentrations /S87/, and see also examples in /KfK/TNO/ and /FQS88/.
Nevertheless, detection of the fluorescence emission of very few pollutants with different emission characteristics can be used for fast screening (see also the papers cited above).
Lifetime measurements require well defined conditions or extensive knowledge on lifetime changes.
Lifetime specific fluorescence detection methods as phase modulation fluorometry can be applied for fast remote control and sensing of well-known and well-defined systems e.g. in a chemical process separating two or three components without the need of additional selectivity parameters. This indeed is difficult considering real-time, in situ environmental analysis due to uncontrollable parameters, e.g. oxygen influences.
On the other hand, preparation and separation methods might cause important changes of the sample and can influence individual components to be analyzed. These changes have also to be known if such methods are applied for sample treatment.
In this context, it should be mentioned that e.g. HPLC (high performance liquid chromatography), a well known method for

separating polycyclic aromatic hydrocarbons in aqueous environments, can also be limited. HPLC is not able to separate polycyclic aromatic hydrocarbons as benzo(b)fluoranthene and benzo(k)fluoranthene /FQS88/.
Coupling of fluorometric and chromatographic methods is an approach for further enhancement of resolving and analyzing multicomponent systems.
A combination of phase resolved fluorometry and HPLC in flow systems is practised by Linda McGown /CM89/. A chromatographic peak at a selected retention time originated by two individual fluorophores is tried to be separated by lifetime measurements simultaneously at one modulation frequency. Fast Fourier transformed phase modulation fluorescence detection at all available modulation frequencies improves the method a) by higher accuracy and b) by fastness.
Also, in the case of application of preceding steps separating components from a mixture, analyzing real environmental samples by lifetime specific fluorometric methods would be difficult and critical, due to the above mentioned uncontrollable parameters changing the molecular lifetimes, e.g during separation, if these cannot be excluded.

V. SUMMARY AND CONCLUSION

In this work the capability of time resolved fluorescence detection methods, directly time resolved measuring techniques also correlated with single photon counting and phase-modulation techniques is investigated and discussed. Both techniques are able to resolve excited molecular states' lifetimes on the picosecond scale by application of modern laser and detection systems. The phase-modulation technique can be superior by further resolution on the subpicosecond time scale for lifetime determination of single component systems. Also, high resolution phase-modulation techniques do not need deconvolution techniques to determine and reconstruct the decay and hence the

fluorescence lifetimes. Measuring and data acquisition times can also be kept short for both methods. A progress in former time consuming single photon counting methods was gained by application of innovative pulsed lasers systems of high intensities and high repetition rates. Also, both methods are able to resolve two to three component systems simultaneously exclusively by lifetime measurements. They are indeed excellent and very sensitive techniques analyzing and investigating ultrafast phenomena under well-defined conditions.

Nevertheless, problems may arise especially during application of these lifetime specific methods for remote monitoring in environmental in situ analysis, due to a variety of possible uncontrollable effects changing the excited molecular states to be analyzed.
I would not recommend fluorometric lifetime techniques exclusively for an overall in situ detection of environmental pollutants without combination of additional methods yielding further information on the systems under study. Coupling of such fluorometric techniques with separation and preselection techniques offers new possibilities:
Well-defined measuring devices could be developed, each either consisting of one or two pollutants to be analyzed after a preceding separation process or acting as selective element for one or two pollutants, so that lifetime influencing parameters can be controlled or excluded on the one hand. On the other hand, lifetime specific fluorescence detection methods could also be used to gain additional analytical information on compounds influencing excited state lifetimes. In this sense, fluorosensing of organic pollutants by highly developed fluorescence lifetime techniques can become a powerfool analytical tool in environmental analysis.

However, we are just at the beginning of the use of innovative optical techniques in chemical analysis e.g. in

respect to laser application. At the end I want to indicate to the enormous potential included in innovative optical techniques. Fast optical techniques will become rather important in future as can already be observed in the development of optoelectronics, integrated optics and optical communication engineering.

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