What is meant by the "lifetime" of a fluorophore???

Although we often speak of the properties of fluorophores as if they are studied in isolation, such is not usually the case.

Absorption and emission processes are almost always studied on *populations* of molecules and the properties of the supposed typical members of the population are deduced from the macroscopic properties of the process.

In general, the behavior of an excited population of fluorophores is described by a familiar rate equation:

$$\frac{dn^*}{dt} = -n^* \Gamma + f(t)$$

where n^* is the number of excited elements at time t, Γ is the rate constant of emission and f(t) is an arbitrary function of the time, describing the time course of the excitation . The dimensions of Γ are sec^{-1} (transitions per molecule per unit time).

If excitation occurs at t = 0, the last equation, takes the form:

$$\frac{dn^*}{dt} = -n^* \Gamma$$

and describes the decrease in excited molecules at all further times. Integration gives:

$$n^*(t) = n^*(0)\exp(-\Gamma t)$$

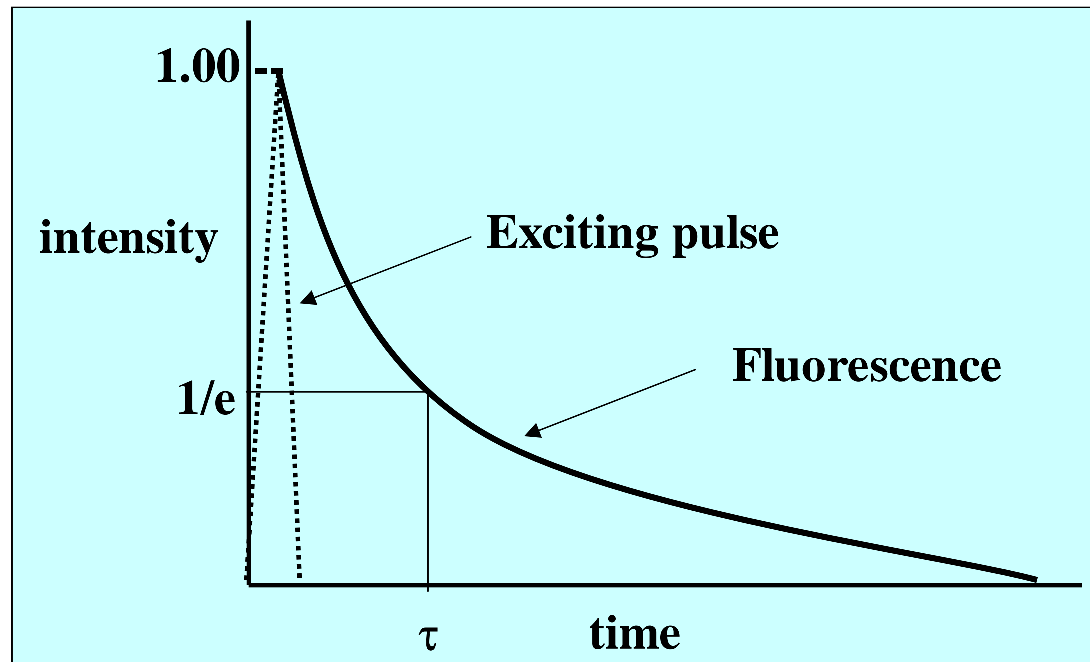
The lifetime, τ, is equal to Γ^{-1}

If a population of fluorophores are excited, the lifetime is the time it takes for the number of excited molecules to decay to 1/e or 36.8% of the original population according to:

$$\frac{n^*(t)}{n^*(0)} = e^{-t/\tau}$$

In pictorial form:

$$\frac{n^*(t)}{n^*(0)} = e^{-t/\tau}$$



Knowledge of a fluorophore's excited state lifetime is crucial for quantitative interpretations of numerous fluorescence measurements such as quenching, polarization and FRET.

In most cases of interest, it is virtually impossible to predict *a priori* the excited state lifetime of a fluorescent molecule. The true molecular lifetime, i.e., the lifetime one expects in the absence of any excited state deactivation processes – can be approximated by the Strickler-Berg equation (1962, J. Chem. Phys. 37:814).

$$\tau_m^{-1} = 2.88 \times 10^{-9} n^2 \langle \nu_f^{-3} \rangle \int_{\Delta\bar{\nu}_a} \varepsilon(\bar{\nu}) \, d\ln\bar{\nu}$$

where

$$\langle \overline{\nu_f^{-3}} \rangle = \frac{\int_{\Delta\bar{\nu}_e} F(\bar{\nu}) \, d\nu}{\int_{\Delta\bar{\nu}_a} F(\bar{\nu}) \overline{\nu^{-3}} \, d\nu}$$

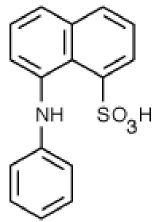
τ_m is the molecular lifetime, n is the refractive index of the solvent, $\Delta\nu_e$ and $\Delta\nu_a$ correspond to the experimental limits of the absorption and emission bands (S_0 - S_1 transitions), ε is the molar absorption and $F(\nu)$ describes the spectral distribution of the emission in photons per wavelength interval.

How well do these equations actually work?

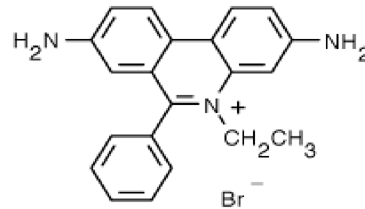
Not very well – usually off by factors of 2 – 5 fold.

The lifetime and quantum yield for a given fluorophore is often dramatically affected by its environment.

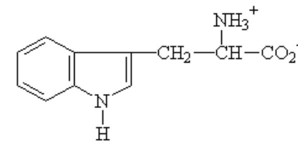
Examples of this fact would be NADH, which in water has a lifetime of ~0.4 ns but bound to dehydrogenases can be a long as 9 ns.



ANS in water is ~100 picoseconds but can be 8 – 10 ns bound to proteins



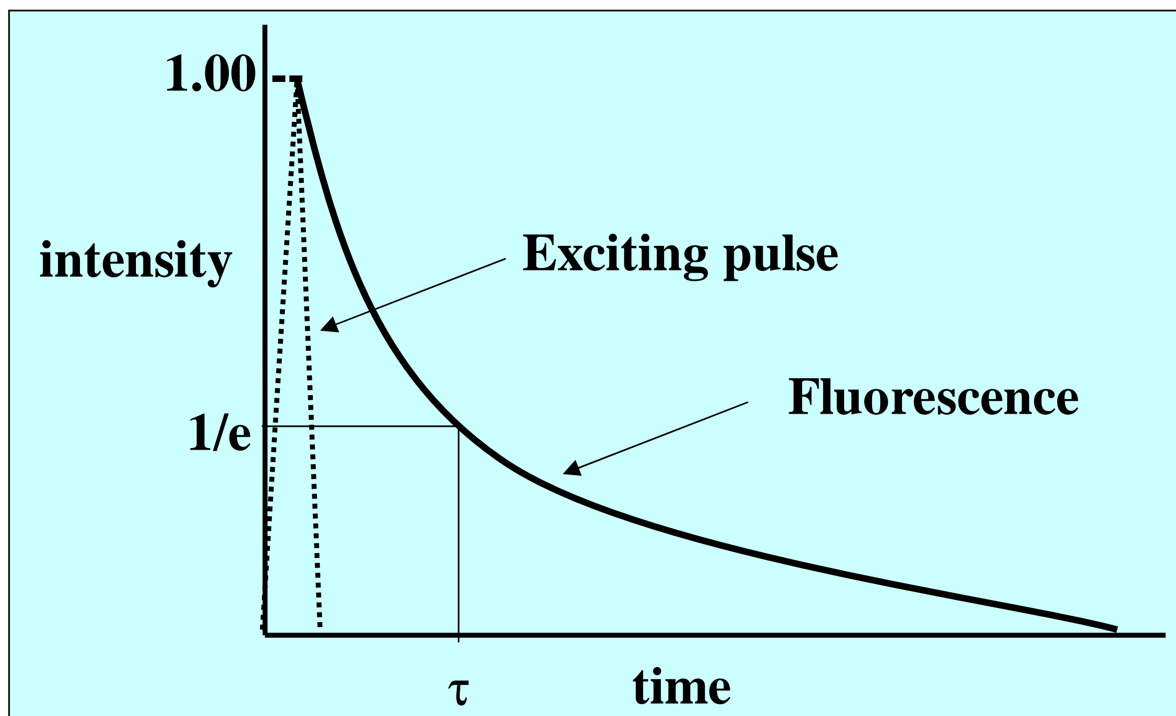
Ethidium bromide is 1.8 ns in water, 22 ns bound to DNA and 27ns bound to tRNA



The lifetime of tryptophan in proteins ranges from ~0.1 ns up to ~8 ns

Excited state lifetimes have traditionally been measured using either the *impulse* response or the *harmonic* response method. In principle both methods have the same information content. These methods are also referred to as either the "time domain" method or the "frequency domain" method.

In the *impulse (*or pulse) method, the sample is illuminated with a short pulse of light and the intensity of the emission versus time is recorded. Originally these short light pulses were generated using *flashlamps* which had widths on the order of several nanoseconds. Modern laser sources can now routinely generate pulses with widths on the order of picoseconds or shorter.

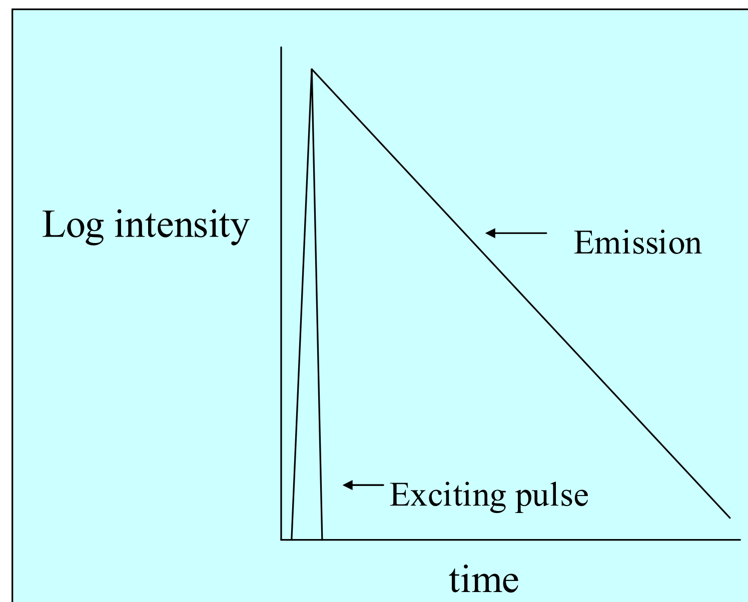


As shown in the intensity decay figure, the *fluorescence* lifetime, t , is the time at which the intensity has decayed to 1/e of the original value. The decay of the intensity with time is given by the relation:

$$I_t = \alpha e^{-t/\tau}$$

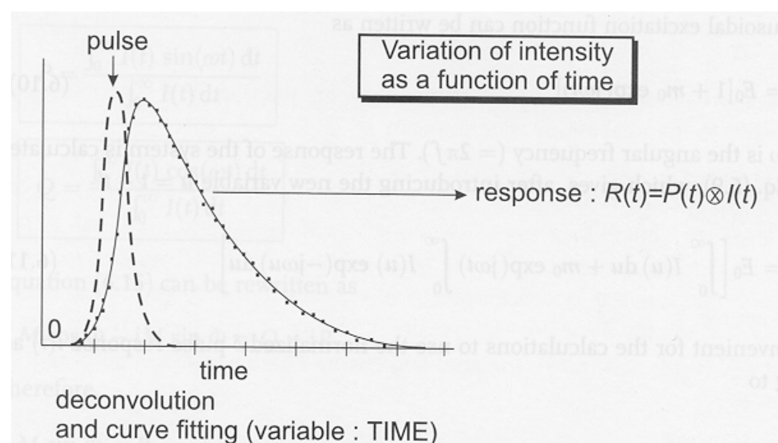
Where I_t is the intensity at time t, α is a normalization term (the pre-exponential factor) and τ is the lifetime.

It is more common to plot the fluorescence decay data using a logarithmic scale as shown here.



If the decay is a single exponential and if the lifetime is long compared to the exciting light then the lifetime can be determined directly from the slope of the curve.

If the lifetime and the excitation pulse width are comparable some type of *deconvolution* method must be used to extract the lifetime.



Great effort has been expended on developing mathematical methods to "deconvolve" the effect of the exciting pulse shape on the observed fluorescence decay.

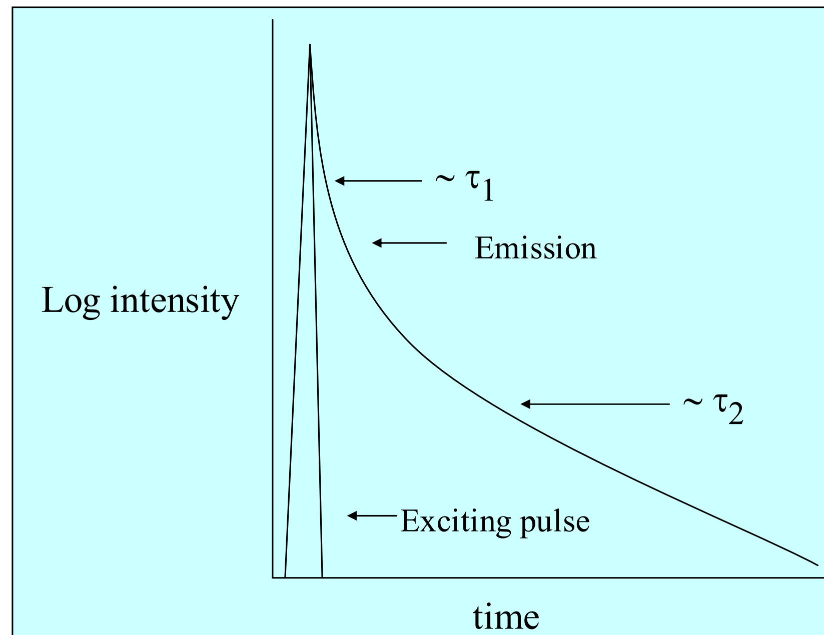
With the advent of very fast laser pulses these deconvolution procedures became less important for most lifetime determinations, although they are still required whenever the lifetime is of comparable duration to the light pulse.

If the decay is multiexponential, the relation between the intensity and time after excitation is given by:

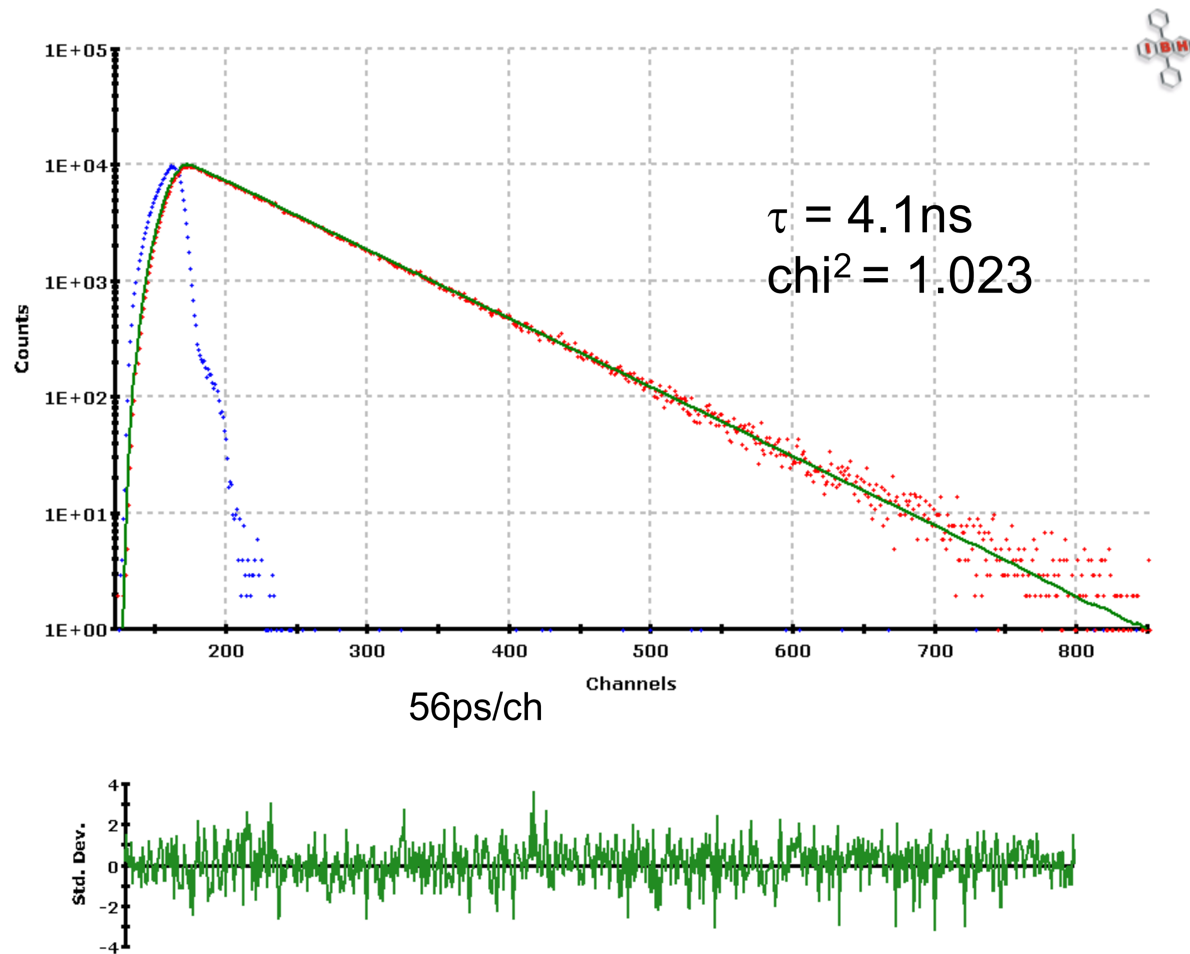
$$I(t) = \sum_i \alpha_i e^{-t/\tau_i}$$

One may then observe data such as those sketched below:

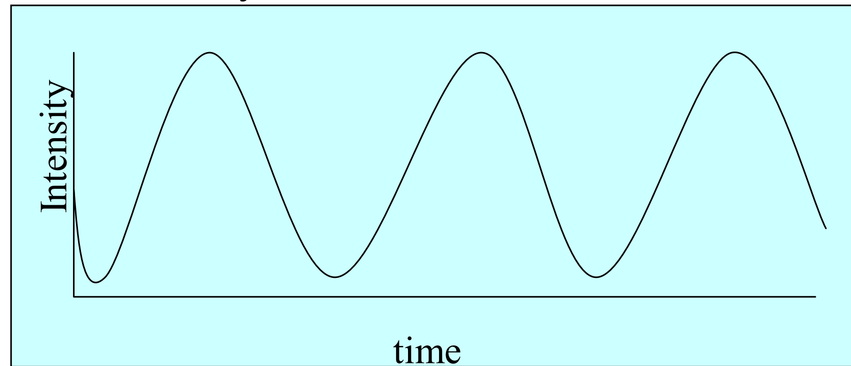
Here we can discern at least two lifetime components indicated as τ_1 and τ_2 . This presentation is oversimplified but illustrates the point.



Here are pulse decay data on anthracene in cyclohexane taken on an IBH 5000U Time-correlated single photon counting instrument equipped with an LED short pulse diode excitation source.



In the harmonic method (also known as the phase and modulation or frequency domain method) a continuous light source is utilized, such as a laser or xenon arc, and the intensity of this light source is modulated sinusoidally at high frequency as depicted below. Typically, an *electro-optic* device, such as a *Pockels cell* is used to modulate a continuous light source, such as a CW laser or a xenon arc lamp. Alternatively, LEDs or laser diodes can be directly modulated.



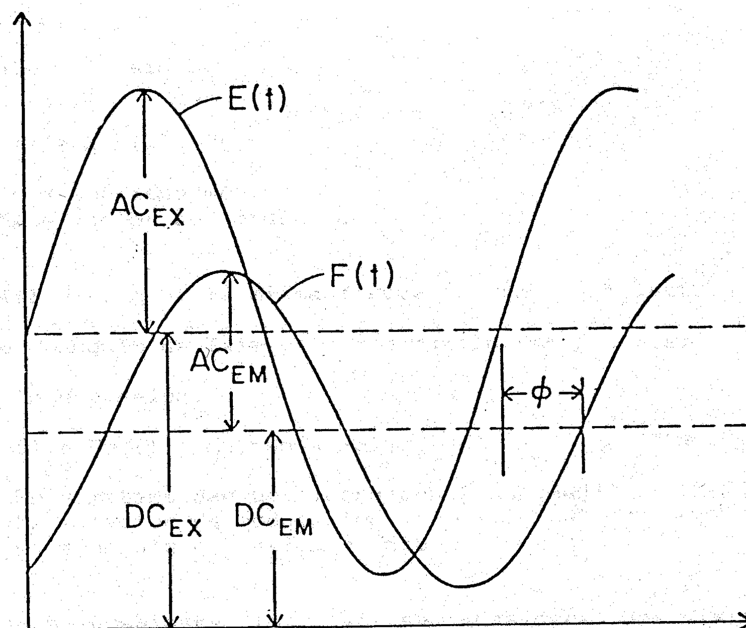
In such a case, the excitation frequency is described by:

$$E(t) = Eo\,[1 + M_E \sin \omega t]$$

E(t) and Eo are the intensities at time t and o, M_E is the modulation factor which is related to the ratio of the AC and DC parts of the signal and ω is the angular modulation frequency.

$\omega = 2\pi f$ where f is the linear modulation frequency

Due to the persistence of the excited state, fluorophores subjected to such an excitation will give rise to a modulated emission which is shifted in phase relative to the exciting light as depicted below.



This sketch illustrates the phase delay (ϕ) between the excitation, E(t), and the emission, F(t). Also shown are the AC and DC levels associated with the excitation and emission waveforms.

One can demonstrate that:

$$\mathbf{F(t) = Fo\ [1 + M_F \sin(\omega t + \phi)]}$$

This relationship signifies that measurement of the phase delay, ϕ, forms the basis of one measurement of the lifetime, τ. In particular one can demonstrate that:

$$\tan \phi = \omega\tau$$

The *modulations* of the excitation (M_E) and the emission (M_F) are given by:

$$M_E = \left(\frac{AC}{DC}\right)_E \quad \text{and} \quad M_F = \left(\frac{AC}{DC}\right)_F$$

The *relative modulation*, M, of the emission is then:

$$M = \frac{(AC/DC)_F}{(AC/DC)_E}$$

τ can also be determined from M according to the relation: $M = \frac{1}{\sqrt{1 + (\omega\tau)^2}}$

Using the *phase shift* and *relative modulation* one can thus determine a *phase lifetime* (τ_P) and a *modulation lifetime* (τ_M).

If the fluorescence decay is a single exponential, then τ_P and τ_M will be equal at all modulation frequencies.

If, however, the fluorescence decay is multiexponential then $\tau_P < \tau_M$ and, moreover, the values of both τ_P and τ_M will depend upon the modulation frequency, i.e.,

$$\tau_P(\omega_1) < \tau_P(\omega_2) \quad \text{if} \quad \omega_1 > \omega_2$$

To get a feeling for typical phase and modulation data, consider the following data set.

Frequency (MHz)	τ_P (ns)	τ_M (ns)
5	6.76	10.24
10	6.02	9.70
30	3.17	6.87
70	1.93	4.27

These differences between τ_P and τ_M and their frequency dependence form the basis of the methods used to analyze for lifetime heterogeneity, i.e., the component lifetimes and amplitudes.

In the case just shown, the actual system being measured was a mixture of two fluorophores with lifetimes of 12.08 ns and 1.38 ns, with relative contributions to the total intensity of 53% and 47% respectively.

Here must must be careful to distinguish the term *fractional contribution to the total intensity* (usually designated as f) from α, the pre-exponential term referred to earlier. The relation between these two terms is given by:

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j}$$

where j represents the sum of all components. In the case just given then, the ratio of the pre-exponential factors corresponding to the 12.08 ns and 1.38 ns components is approximately 1/3. In other words, there are three times as many molecules in solution with the 1.38 ns lifetime as there are molecules with the 12.08 ns lifetime.

Multifrequency phase and modulation data are usually presented as shown below:

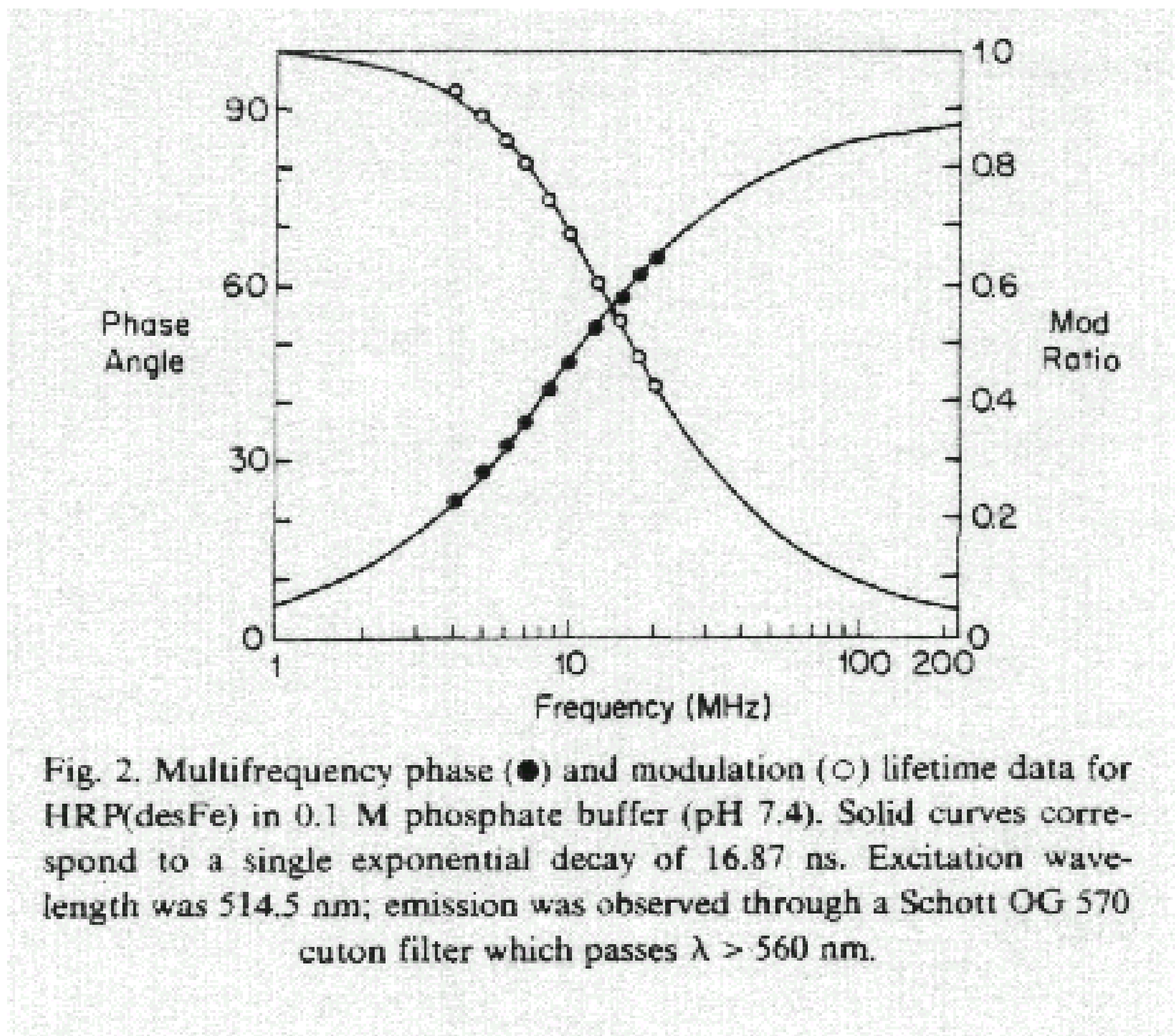
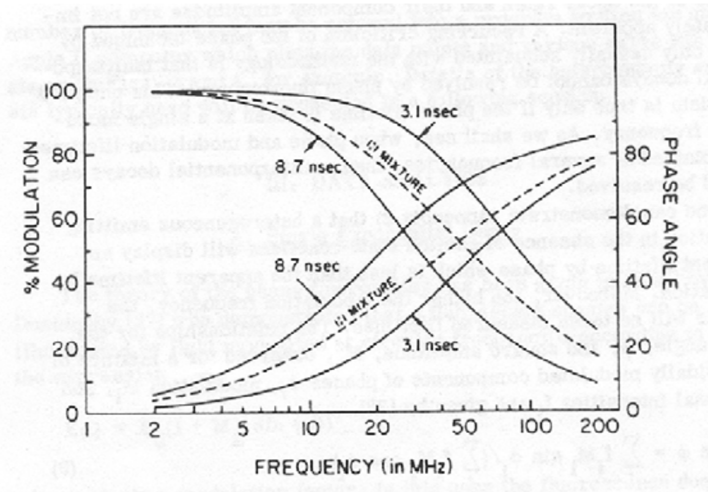


Fig. 2. Multifrequency phase (●) and modulation (○) lifetime data for HRP(desFe) in 0.1 M phosphate buffer (pH 7.4). Solid curves correspond to a single exponential decay of 16.87 ns. Excitation wavelength was 514.5 nm; emission was observed through a Schott OG 570 cuton filter which passes λ > 560 nm.

A case of multi-exponential decays is shown here for a system of two lifetime species of 8.7ns and 3.1ns and a 1 to 1 mixture (in terms of fractional intenisties)



Multifrequency phase and modulation data is usually analyzed using a non-linear least squares method in which the actual phase and modulation ratio data (not the lifetime values) are fit to different models such as single or double exponential decays.

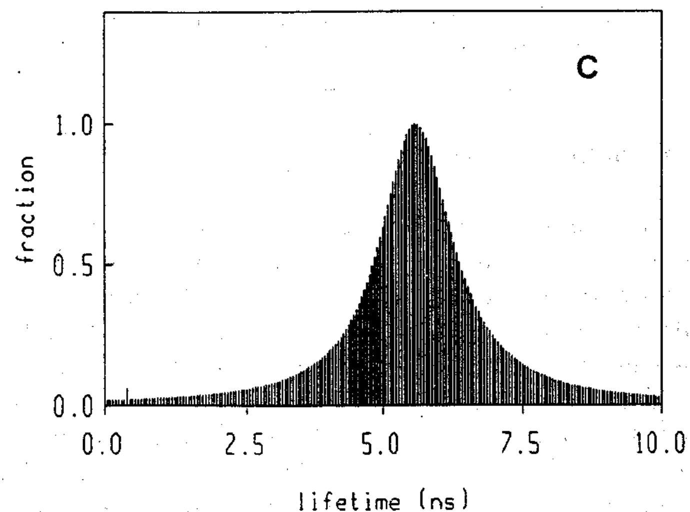
The quality of the fit is then judged by the *chi-square value* (χ^2) which is given by:

$$\chi^2 = \{[(Pc - Pm)/\sigma^P] + (Mc - Mm)/\sigma^M]\}/(2n - f - 1)$$

where P and M refer to phase and modulation data, respectively, c and m refer to calculated and measured values and σ^P and σ^M refer to the standard deviations of each phase and modulation measurement, respectively. n is the number of modulation frequencies and f is the number of free parameters.

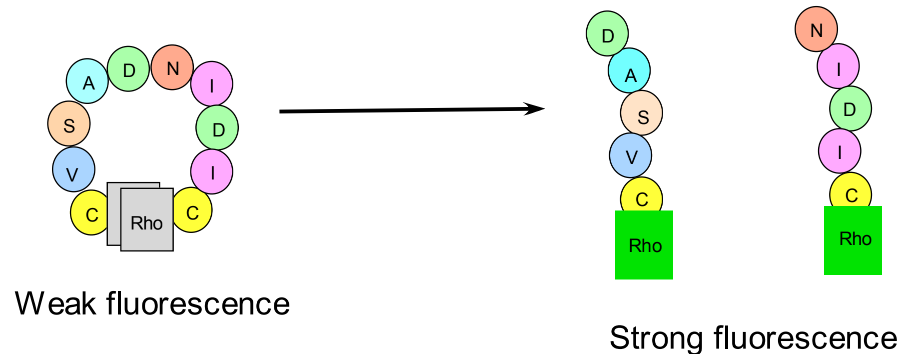
In addition to decay analysis using discrete exponential decay models, one may also choose to fit the data to *distribution* models. In this case, it is assumed that the excited state decay characteristics of the emitting species actually results in a large number of lifetime components. Shown below is a typical lifetime distribution plot for the case of single tryptophan containing protein – human serum albumin.

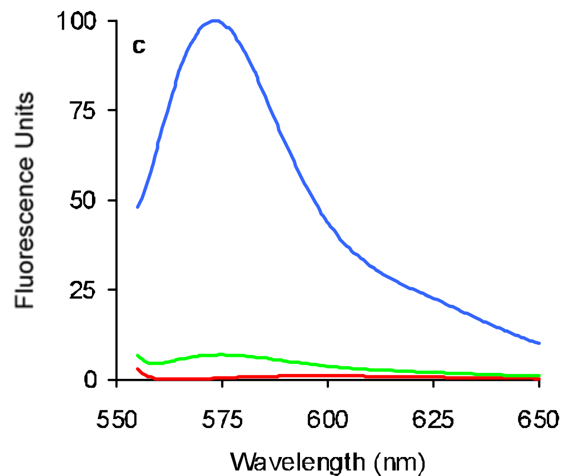
The distribution shown here is Lorentzian but depending on the system different types of distributions, e.g., Gaussian or asymmetric distributions, may be utilized. This approach to lifetime analysis is described in: Alcala, J. R., E. Gratton and F. G. Prendergast. Fluorescence lifetime distributions in proteins. *Biophys. J. 51*, 597-604 (1987).



Another popular lifetime analysis method is the *Maximum Entropy Method* (MEM). In this method no *a priori* intensity decay model is assumed.

An example of the use of lifetime data is given by a study of a rhodamine labeled peptide which can be cleaved by a protease (from Blackman et al. (2002) Biochemistry *41*:12244)





In the intact peptide the rhodamine molecules form a ground-state dimer with a low quantum yield (green curve). Upon cleavage and of the peptide the rhodamine dimer breaks apart and the fluorescence is greatly enhanced (blue curve).

Lifetime data allow us to better understand the photophysics of this system

Lifetime data for two rhodamine isomers (5’ and 6’) linked to the peptide

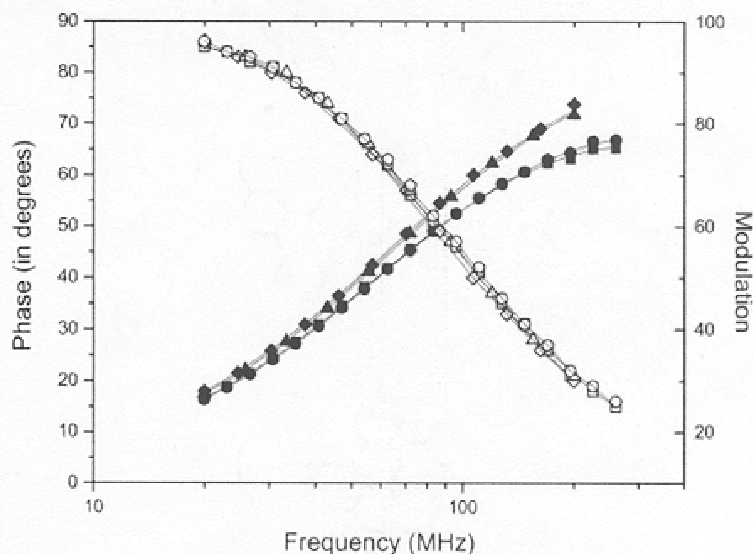


FIGURE 6: Typical phase (filled symbols) and modulation (open symbols) data for pepF1-5R before (circles) and after (triangles) Pronase treatment and for pepF1-6R before (squares) and after (diamonds) Pronase treatment.

Table 3: Fluorescence Lifetime Parameters of Intact and Cleaved Labeled Peptides[a]

sample	τ_1 (ns)	f_1	α_1	τ_2 (ns)	f_2	α_2
pepF1-5R	2.44	0.95	0.52	0.14	0.05	0.48
pepF1-5R + Pronase	2.43	1.00				
pepF1-6R	2.50	0.95	0.37	0.076	0.05	0.63
pepF1-6R + Pronase	2.50	1.00				

As the lifetime data indicate, before protease treatment the rhodamine lifetime was biexponential with 95% of the intensity due to a long component and 5% due to a short component. Assuming that the quantum yields and lifetimes are linked, however, one can calculate that molar ratios of the long and short components are nearly equal. Hence one can argue that the intact peptide exists in an equilibrium between open (unquenched) and closed (quenched) forms.

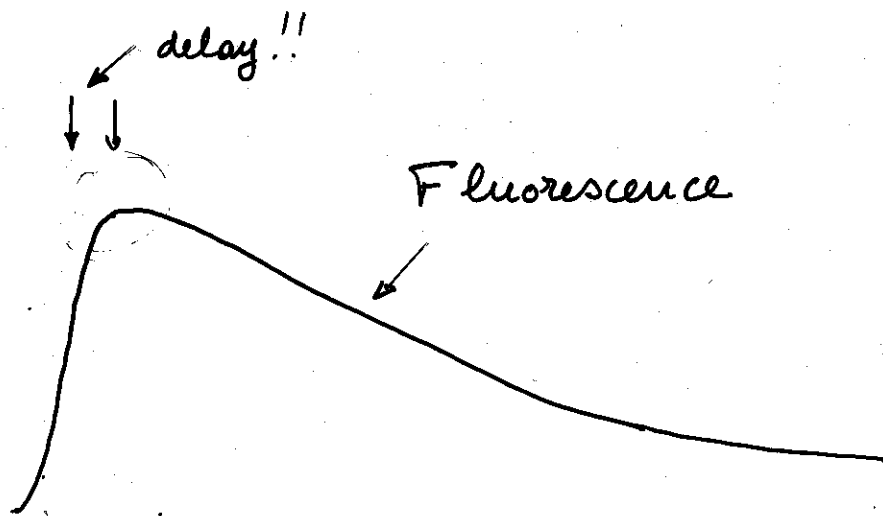
Time resolved fluorescence spectroscopy

Two different approaches

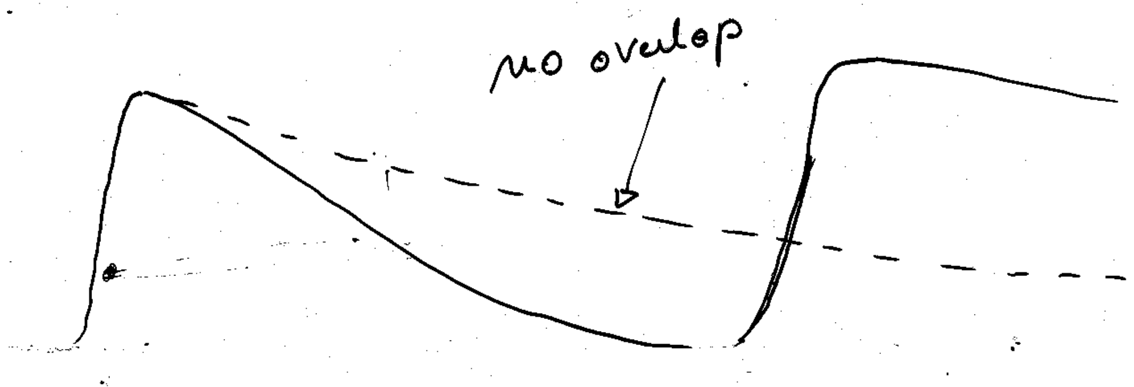
TIME DOMAIN
FREQUENCY DOMAIN

Time domain representation of the decay.

The excitation pulse must be short, much shorter than the decay to be measured.

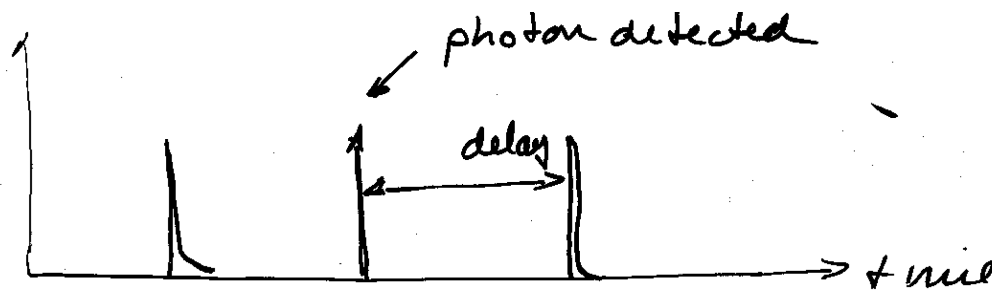


Only one photon is collected per pulse. The next pulse cannot arrive before the decay is completely finished. This method is called Time Correlated Single Photon Counting or TCSPC or simple photon counting.

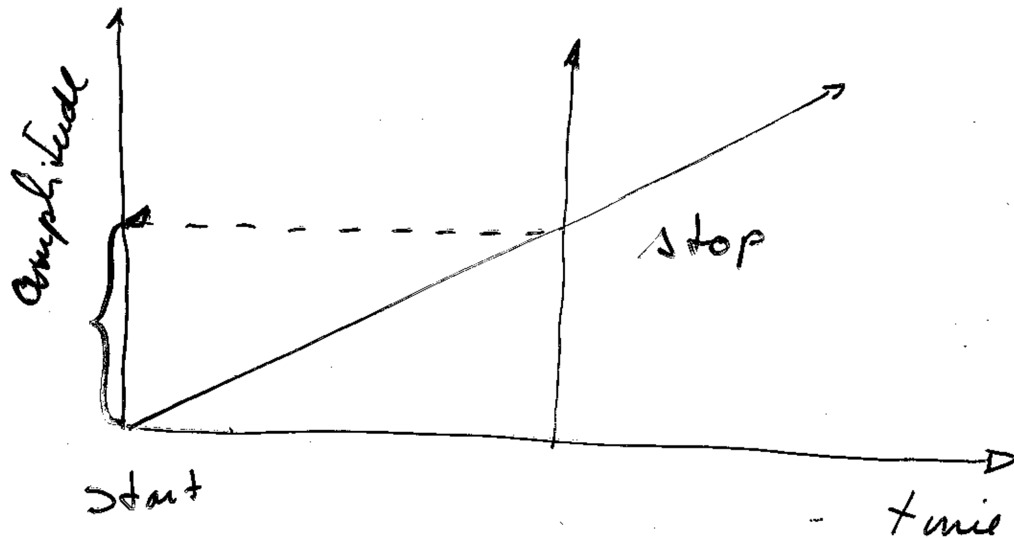


The decay is represented using a histogram of delays

The start-stop method. Start with the photon detected and stop with the next light pulse. Or start with the light pulse and then stop with the photon detected. The first method is normally used today because the recovery time of the electronics is very long.



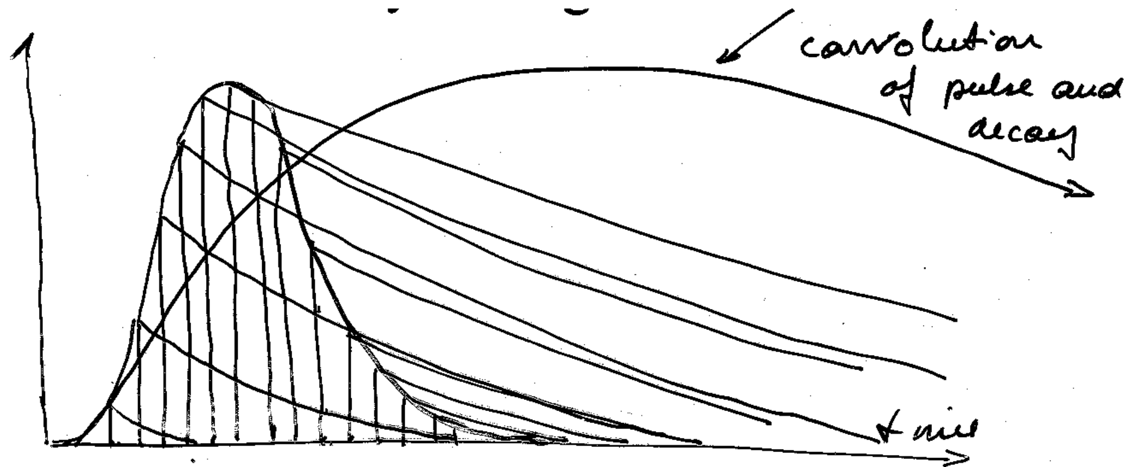
The time-to-amplitude converter (TAC) is the heart of the instrument. A discharge of a capacitor produces a linear voltage ramp. At the stop pulse the value of the ramp is sampled and digitized. The amplitude of the voltage recorded is proportional to time.



Problems of calibration and linearity of the TAC.

The duty cycle of the TAC.

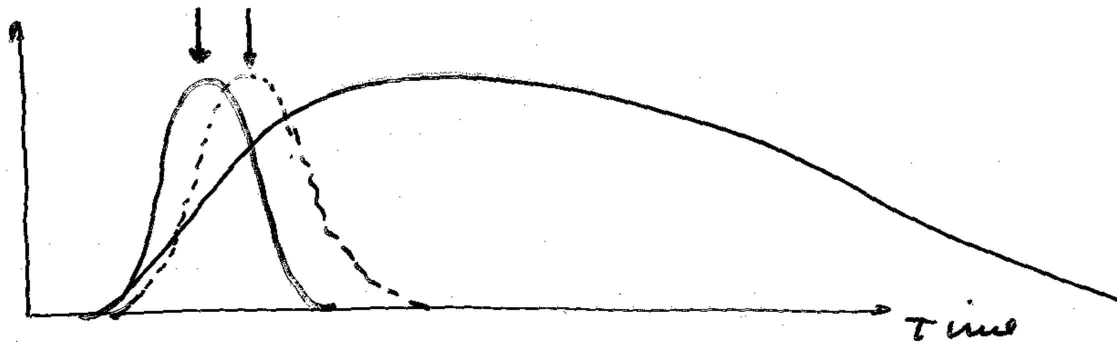
The instrument response function modifies the delays histogram.



A mathematical deconvolution technique must be applied to recover the correct decay. Iterative methods are generally used for deconvolution. Few years ago an expensive computer was needed. Today the deconvolution process is relatively inexpensive.

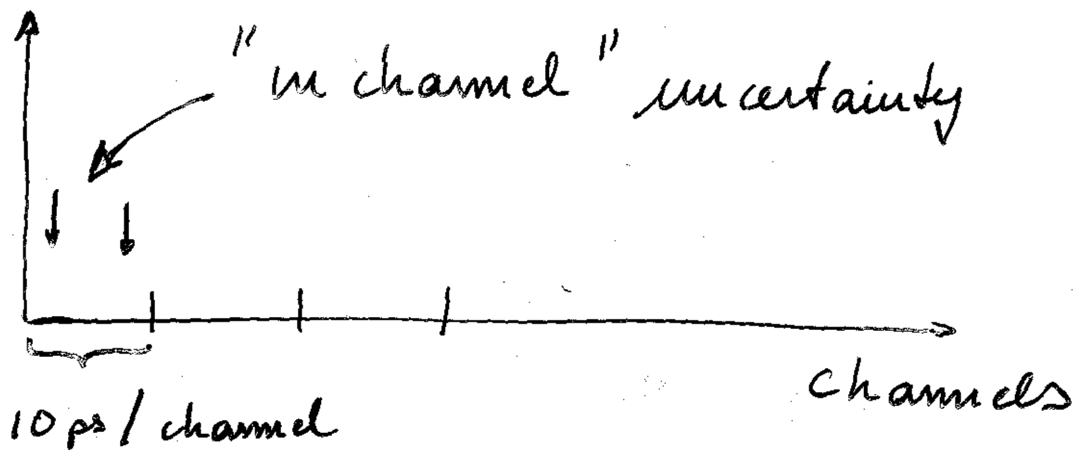
HOW accurately the instrument response function must be known? Of course, very accurately! It must be measured prior to each measurement and using the identical conditions later used

for the sample. The instrument response function, for obvious reasons, is often called the lamp curve. The lamp curve must be properly "Phased" to correctly perform the de-convolution.



How accurately can we assign a photon delay to a given bin? For modem instrumentation, this is not a big problem.

Bin widths as small as one picosecond can be obtained.



What range of lifetime values can we separate given this limitation?

Two lifetime components that differ by less than a factor of two are not resolvable!

2 ns from 4 ns
20 ns from 40 ns

200 ns from 400 ns

What is the statistic of each bin? Each bin follows a Poisson statistic, i.e. the uncertainty of the counts in each bin is given by the square-root of the number of counts in the bin.

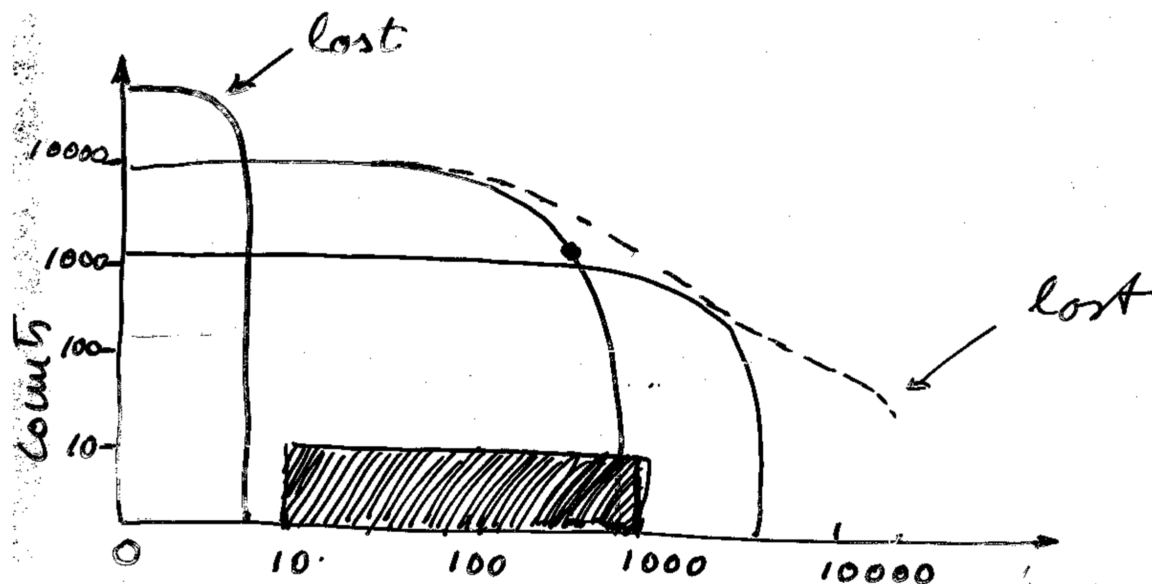
$N = N^* \pm \sqrt{N^*}$

How many delays do we have to measure per bin? To obtain an uncertainty of 1 % in a bin count, we must collect 10000 counts per bin!

The real limit is in the total number of bins, which determines the range of lifetime values that can be determined.
This is a very important limitation of the time domain methods. At most 1 000 to 10000 bins are recorded.
Due to uncertainties of the starting point, and due to noise at the

earlier bins, it is customary to disregard the 10-20 bins at the shorter times.

Logarithmically speaking, 2 to 2.5 decades in time are recorded at most.



Assume your light source gives 100 pulses per seconds (typical of a low repetition pulsed laser) and you want an uncertainty of 1 %

on the first bin, and that you collect at least 1 000 bins, how long a typical measurement will last?

time=10000x1000/100 seconds

This is almost a day!

Of course, if you use a high repetition mode-locked laser, (very expensive $200K) the time can be reduced. However, the maximum count rate cannot exceed 10,000-30,000, due to the TAC duty cycle. The best time domain system can collect decent data in about 10 to 30 minutes.

The alternative is

FREQUENCY DOMAIN

The sample is illuminated by a sinusoidal modulated light source.

The emission is also sinusoidal modulated at the same modulation frequency.

The emission is phase shifted and due to the finite persistence of the excited state.

Therefore, the phase shift and the demodulation carry the information about the lifetime of the excited state.

IMPORTANT: the light modulation frequency must match the characteristic decay rate.

$$\omega\tau=1$$

For example to match a decay time of 1 ns we must modulated the light at 160 MHz.

The basic relationships in frequency domain fluorometry are
Phase equation

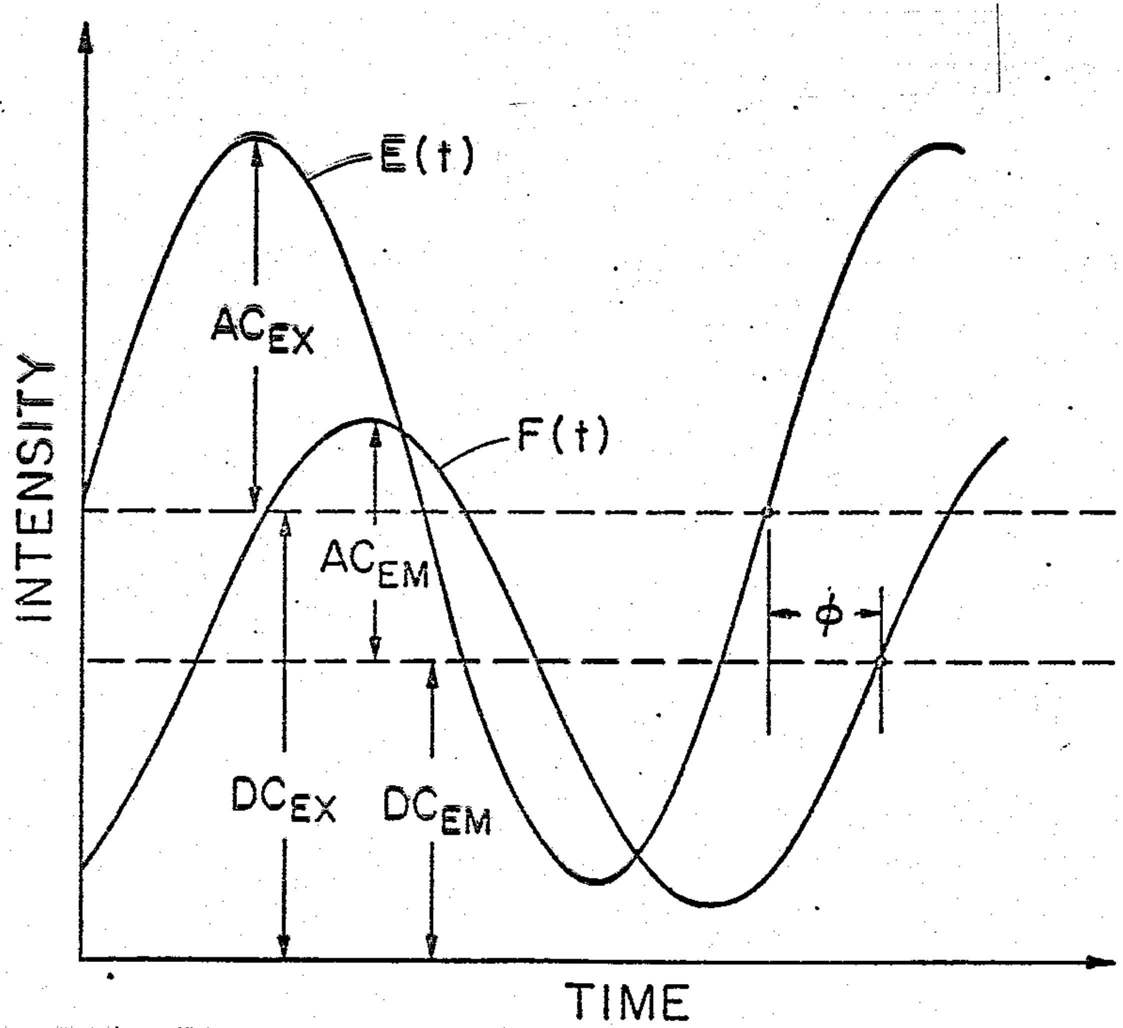
$$\Phi=\tan^{-1}\omega\tau$$

Modulation equation

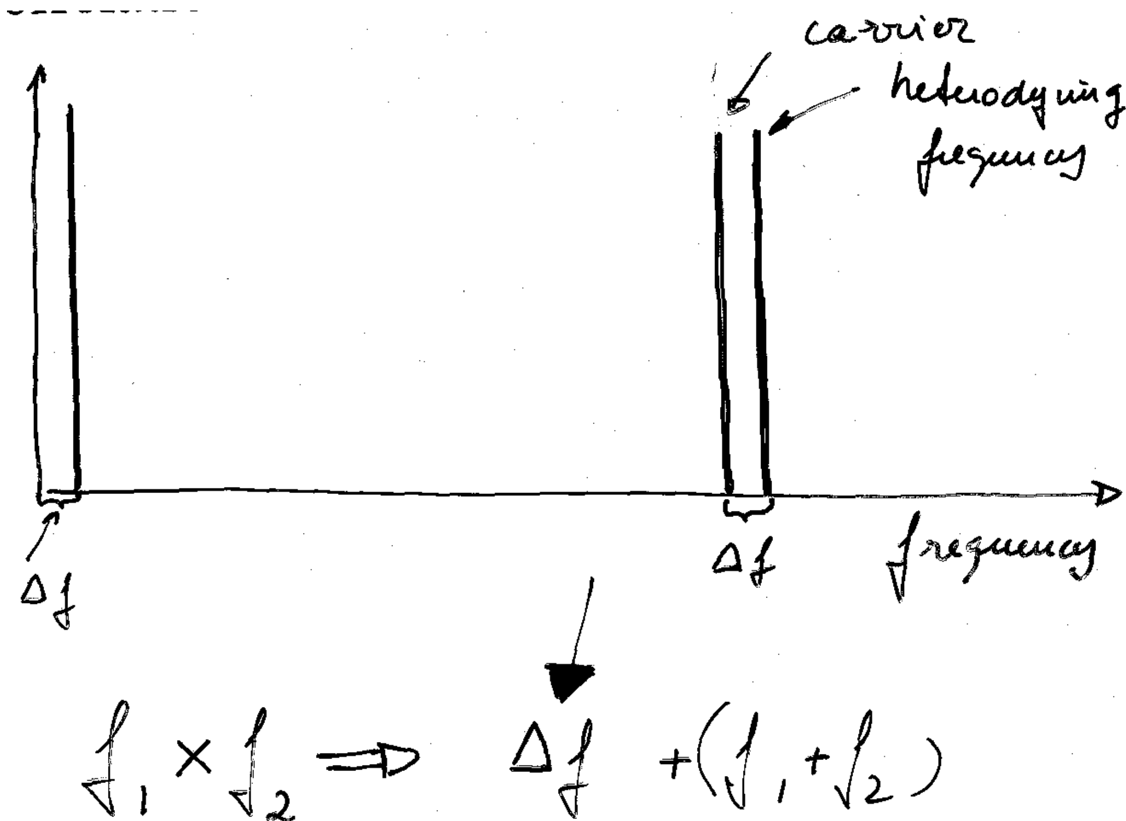
$$M=1/\sqrt{(1+\omega^2\tau^2)}$$

How to accurately measure the phase and the modulation at such a high frequency?

Use the cross-correlation principle (Spencer and Weber 1969).


E(t)
F(t)
INTENSITY
AC_{EX}
AC_{EM}
ϕ
DC_{EX}
DC_{EM}
TIME

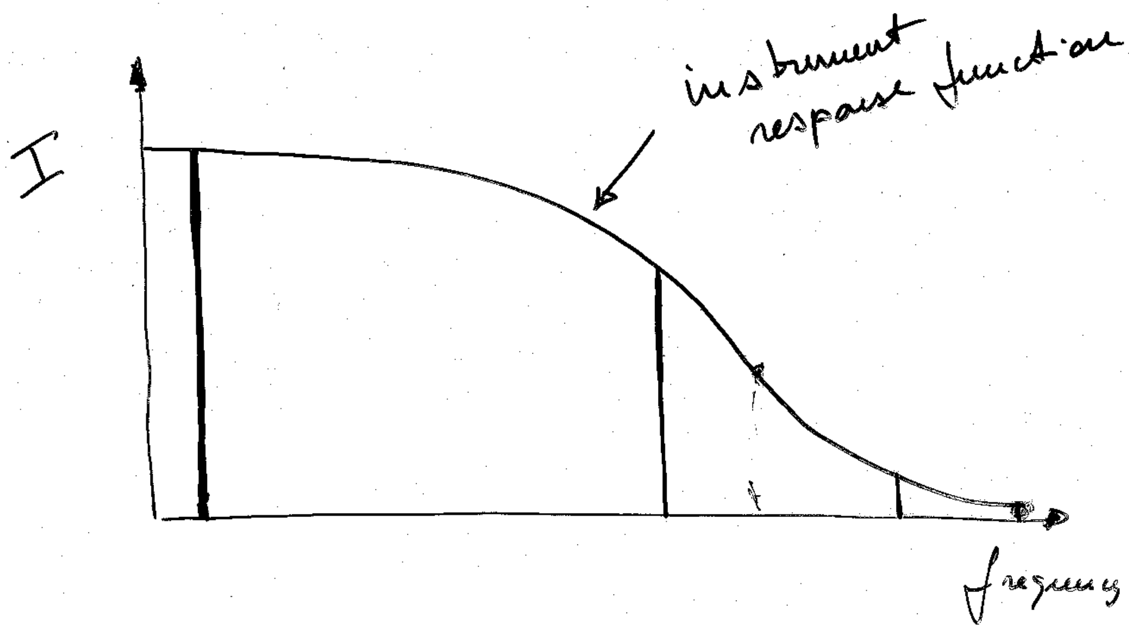
Cross-correlation and heterodyning are similar concepts. Cross-correlation, which requires phase coherence heterodyning is what is used in your radio to decode the amplitude or frequency modulated signals. Today, every good radio uses cross-correlation.



The mixing is performed in the detector itself. The output of the detector is a very low frequency signal, generally below 1000 Hz. At such a low frequency, powerful digital filtering and data processing can be applied.

The result is a very high accuracy in the phase and in the modulation determination is excellent. Generally 0.1% uncertainty is routinely obtained using few seconds integration. Compare this result with the time required to obtain the same uncertainty in the t e correlated single photon counting method.

What happens of the instrument response function? In the frequency domain the response of the instrument appear as a phase shift and as an additional demodulation. It is trivial to correct for the in instrument response. Actually, in the frequency domain, the phase is always measured relative to the phase of the excitation and the demodulation is measured with respect to the modulation of the source.



no attenuation at low frequency

large attenuation at high frequency.

Calibration is not necessary in the frequency domain: the modulation frequency is always known with very high precision. Phase measurements are measured in an absolute scale relative

to one period of the cross-correlation frequency. Modulation values are also measured with respect to the modulation of the excitation and they are independent from all instrument parameters such as photomultiplier response, monochromators light transmission and amplifiers gain.

Important: Frequency domain provides two independent determination of the fluorescence lifetime:

The phase determination

$$\phi = \tan^{-1} \omega\tau$$

$$\tau_\phi = \frac{1}{\omega} \tan\phi = \frac{1}{2\pi f} \tan\phi$$

The modulation determination

$$M = \frac{1}{\sqrt{1 + \omega^2 \tau^2}}$$

$$\tau_M = \frac{1}{\omega}\sqrt{\frac{1}{M^2} - 1} = \frac{1}{2\pi f}\sqrt{\frac{1}{M^2} - 1}$$

Evaluation of the minimum resolvable lifetime.

Assume that phase and modulation can be measured with an uncertainty of 0.1 % and that the maximum modulation frequency of a commercial frequency domain fluorometer is about 500 MHz, calculate the minimum lifetime that can be measured

$$\Delta \phi \cong 2\pi f \Delta \tau$$

$$\Delta \tau = \frac{\Delta \phi}{2\pi f} = 0.1 \times \frac{2\pi}{360} \cdot \frac{1}{2\pi f} = 0.5 \times 10^{-13}\ \text{s}$$

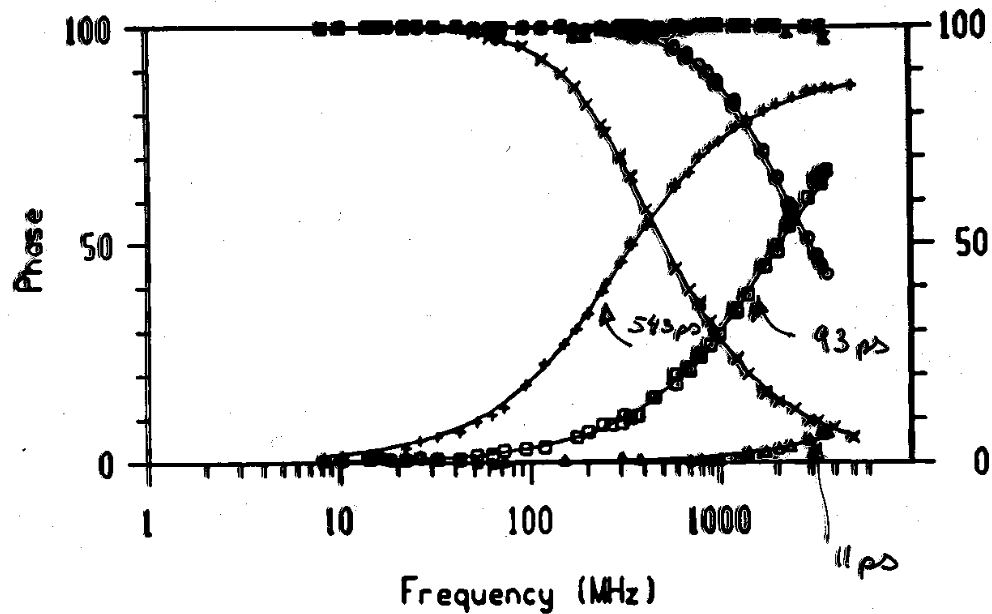
Picosecond resolution!!

What happens of the instrument response function? Apparently, the instrument response function is not a limiting factor!

Typical phase fluorometer layout

M
FREQ.
DOUBL.
M
REF
PMT
ADJ
CD
2 MHz
CD
DRIV
DL
S
S
PMT
A
D
J
MON
$\Omega + \Delta\Omega$
38 MHz
A 2
FREQ
SYNT
FREQ
SYNT
A 1
ML
DRIV
DIG. ACQ.
ELECTR.
COMP
ND-YAG

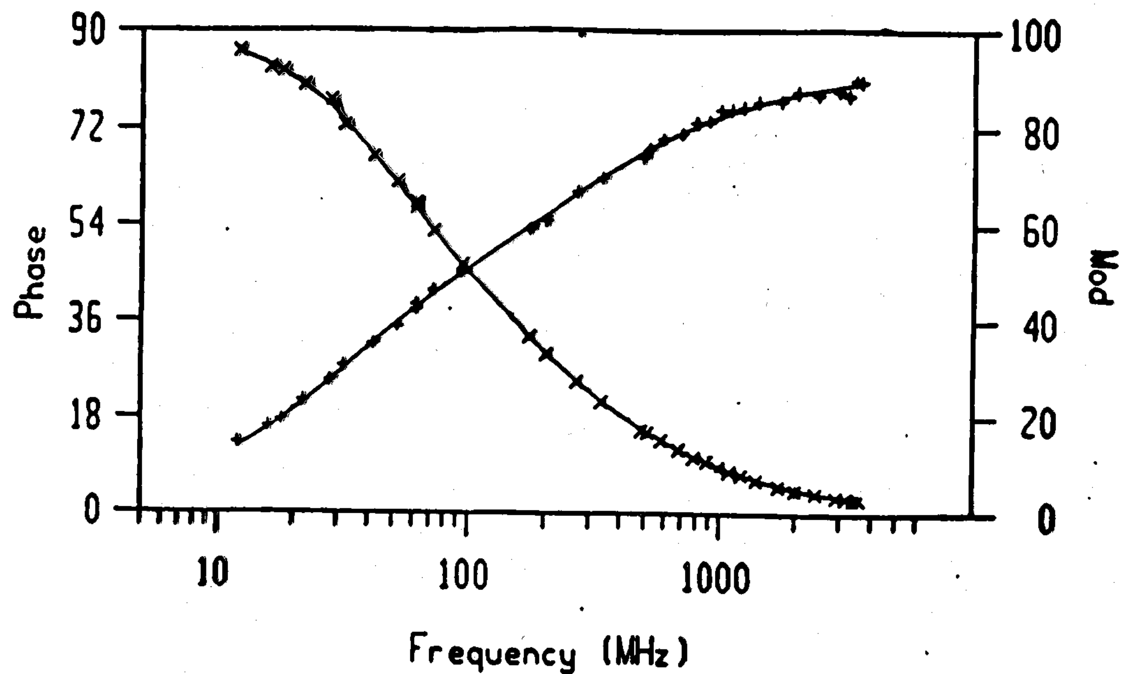
Frequency domain representation of the fluorescence decay



phase
- + Rose Bengale in alcohol
- □ Rose Bengal in water
- Δ Pinacyanol in ethanol

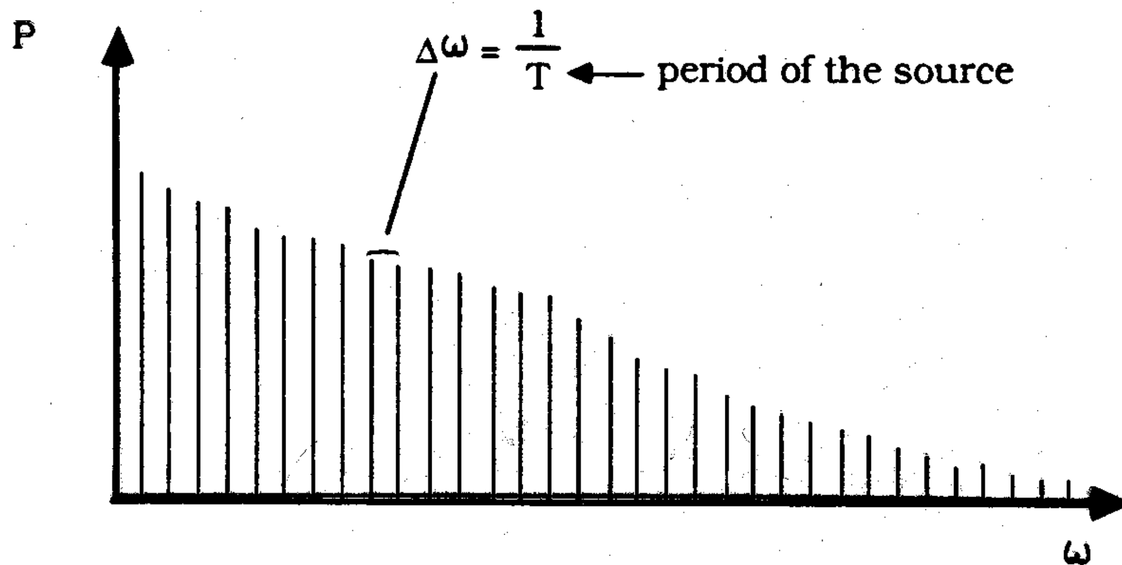
Each exponential decay has a characteristic curve, shifted in the frequency axis

Frequency domain representation of the decay of the PLA2 protein. The deviation from exponential is very evident.



USING PULSES OF LIGHT

a) The power spectrum is distributed from 0 to 1.0 GHz



What is the intensity of each harmonic?

All photons contribute to each harmonic!

b) How many photons do we need to measure an angle of 0.5 at 10 MHz (it is the same for all frequencies!)

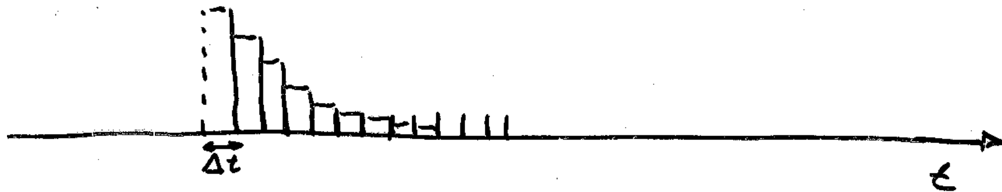
Very few - 100 per second!!

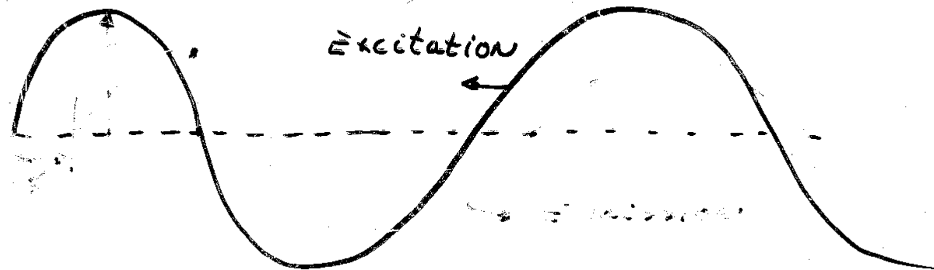
Comparison between time-domain and frequency-domain

Pulse fluorometry

Excitation

Emission





$$\phi = \tan^{-1} \omega \tau_\phi$$

$$\tau_\phi = \frac{1}{\omega} \tan \phi$$

Layout of a commercial frequency-domain fluorometer

DETAILED DESCRIPTION

P.C. Freq. Synth.

P.M. Freq. Synth.

Analog Electronics

Computer and Peripherals

He-Cd laser

BLOCK DIAGRAM

P.C. : Pockels cell
L : lens
F : filter holder
S : manual shutter
EL. S : electronic shutter
M1. M2 M3 mirrors
P Glan-Taylor polarizer
P.M. : photomultiplier
B.S : beam splitter
T.W.P. : two-way polarizer

Detail of the light modulator used in commercial frequency-domain fluorometers