



## The Time-Resolved Fluorescence Techniques

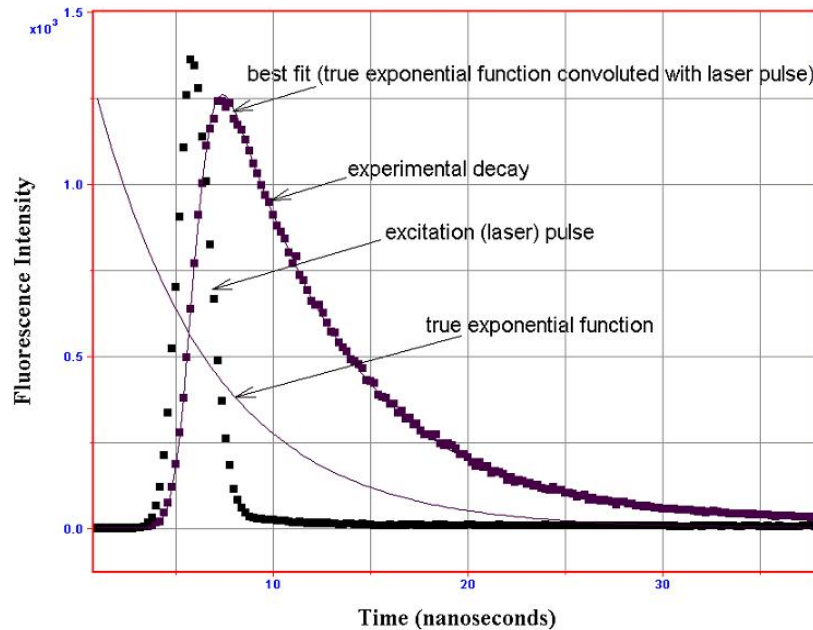
There are three common techniques used in time-resolved fluorescence spectroscopy: *the stroboscopic technique (strobe)*, *the time-correlated single photon counting technique (TCSPC)* and *the frequency modulation or phase shift technique (phase)*. The first two are *time-domain* techniques while the last one is a *frequency-domain* technique.

The *time-domain* techniques, i.e. the *strobe* and *TCSPC*, are very similar in what they measure and in the way data are analyzed. The differences are mainly in the way the data is collected and the hardware. They use different *detection electronics*. The light sources used are basically the same, although the *strobe* can use simpler light sources, which may not be very useful with *TCSPC*.

The time-domain techniques are *direct* techniques. They measure fluorescence decay curves (i.e. fluorescence intensity as a function of time) directly and the experimenter has full advantage of seeing the physical mechanism in the course of the experiment. Frequently, by examining raw decay data one can make a qualitative judgement about a particular mechanism and a proper fitting function can be thus selected.

In order to obtain the fluorescence lifetime, the profile of *instrument response function* (excitation pulse) has to be measured in addition to the fluorescence decay. This is because the lamp (laser) pulse has a finite temporal width, which distorts the intrinsic fluorescence response from the sample. This effect is called *convolution* (*for more information refer to our tech note on Convolution*).

In a typical experiment, two curves are measured: the *instrument response function (IRF)* using a scatterer solution and the *decay curve*. Analysis is then performed by convoluting the IRF with a *model function* (e.g. a single exponential decay or a double exponential decay or some other function) and then comparing the result with the experimental decay. This is done by an iterative numerical procedure until the best agreement with the experimental decay curve is achieved.



**Fig. 1** Experimental laser profile and decay (dotted functions) and the best numerical fit in a typical time-domain experiment. The true exponential function represents the model decay, i.e. a hypothetical decay if the laser pulse was infinitely narrow.

The *phase* technique is totally different, both in terms of what it measures and in the way the detection is performed. It utilizes a *sinusoidally modulated light* for the excitation of the fluorescence. The emitted light becomes also modulated at the same frequency, but it will show some shift (delay) relative to the excitation light. This shift is called a *phase shift* and it contains the information about the lifetime. Depending on the lifetime, the fluorescence will show a decreased depth of the sinusoidal modulation relative to the excitation light.

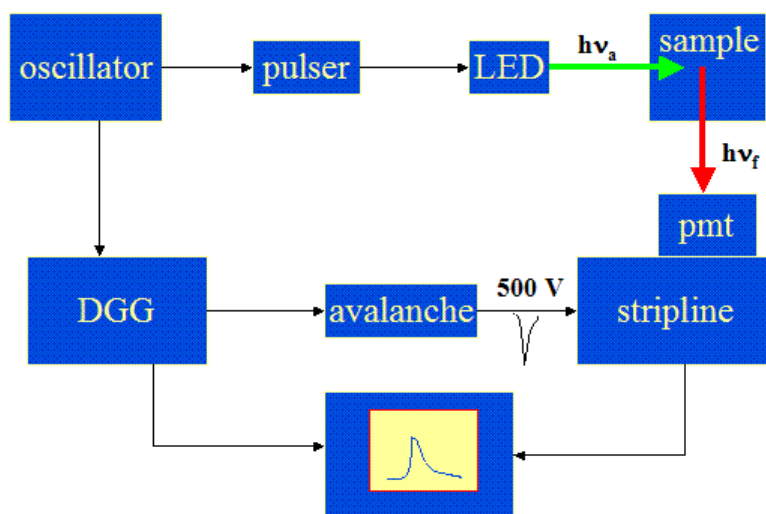
The *shift values* and the *degree of demodulation* of the emitted light can be expressed as complicated functions of fluorescence lifetime(s). The lifetimes and other parameters of interest are then obtained by numerical fits of assumed model functions to the phase shift and demodulation data measured at different modulation frequencies.

*The phase technique does not record a decay curve directly and it provides no intuitive insight into the physical process under study.*

### **The Stroboscopic Technique (Strobe)**

This is the most recent and electronically the simplest technique. While the technique is the newest, it is already more than 10 years old and well established and validated. It utilizes a *pulsed light source* (an LED, a laser diode or a nitrogen/dye laser) and measures fluorescence intensity at different time delays after the pulse. As a result, a

*fluorescence decay curve* is collected. The diagram below shows the basic elements of a strobe instrument that utilizes a *pulsed LED*.



**Fig. 2** Block diagram of an **LED-based EasyLife** stroboscopic system

A *master clock (oscillator)* generates pulses at a fixed 25 kHz frequency. The pulses are routed simultaneously to the *LED pulser* and a *digital delay gate generator (DGG)* unit.

The pulser triggers the LED; the LED flashes and excites the sample, which subsequently emits fluorescence. At the same time the pulse synchronized with the LED pulse triggers the *DGG*, which outputs a delayed TTL pulse.

The *DDG is under computer control* and the value of the TTL pulse delay is determined in the acquisition software. The delayed pulse triggers an *avalanche circuit*, which provides a *high voltage pulse* (ca. 500 V) for the detection circuitry. This pulse creates the *gain* and the *temporal discrimination gate* for the photomultiplier.

An important feature is that the strobe technique does not use a conventional voltage divider network for providing inter-dynode voltages in the photomultiplier (PMT). Instead, the PMT dynodes are interconnected by a *stripline circuit*. The pulse from the avalanche is injected in the stripline at the time delay specified by the DGG. *The pulse travels along the dynode chain amplifying the primary photoelectrons generated at the specific time delay*. This way *high amplification and time gating are simultaneously achieved in the PMT strobe circuit*. The measured analog signal is fed to a 12-bit A/D converter. Scanning the gate (time delay) across the fluorescence decay allows the acquisition of fluorescence intensity as a function of time.

One of the advantages of the stroboscopic technique is the ability to utilize relatively *inexpensive pulsed LEDs*.

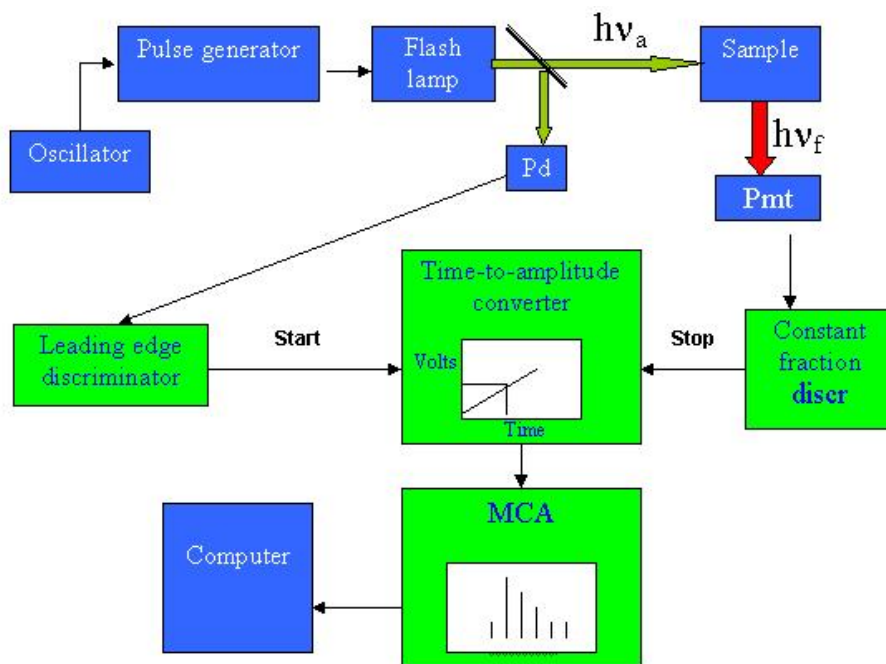
The strobe technique can also be *very fast*; this is because *it measures fluorescence intensity directly* and, unlike photon counting techniques, *is not limited by photon counting statistic* and can therefore take advantage of high intensity fluorescence.

A unique feature of the strobe is the ability to measure decays with the use of *non-linear timescale*. This is possible because the software controls the delayed output of the DGG. The stroboscopic instruments employ *arithmetic progression* and *logarithmic timescale acquisition protocols* in addition to the conventional linear timescale. These non-linear timescale protocols enhance the lifetime resolving power and allow for the acquisition of complex decays with underlying lifetimes differing by orders of magnitude using fewer data points than would be required with the linear timescale.

### Time-Correlated Single Photon Counting (TCSPC)

The *TCSPC* is also the *time-domain* technique. It is one of the oldest techniques dating back to 1970's. Like the strobe, *it utilizes pulsed light sources (flash lamps, lasers, LEDs)*. It also measures the same experimental functions as strobe, i.e. the *fluorescence decay* and the *IRF*; however, its detection is based on a different principle.

The block diagram shows the principle of TCSPC operation. *The clock triggers the lamp; the lamp fires and a photodiode (Pd) in front of the lamp triggers the electronics ("start" pulse).*



**Fig. 3** Block diagram of a TCSPC system

The *start pulse* is “cleaned” by the *leading edge (LE) discriminator* and it starts the *time-to-amplitude converter (TAC)*. The TAC is the key element of the technique. After being triggered, the *TAC creates a voltage ramp, which is linear in time* (e.g. it starts charging a capacitor).

In the mean time, the sample has been excited and emitted fluorescence photons. *When the PMT detects a photon, a short pulse is created at the output of the PMT. The pulse is “cleaned” by the constant fraction (CF) discriminator and enters the TAC as a “stop” pulse.* Once the stop pulse (i.e. the first arriving photon) has been detected, the voltage ramp is stopped and the voltage value (equivalent to the time difference between the start and stop pulses) is transmitted to the *multichannel analyzer*, which increments the counts in the channel corresponding to the detected voltage (time). This process is repeated with each flash and eventually, after many cycles, a histogram of counts vs. the channel number (time) is created. If certain conditions are met, the histogram represents the fluorescence intensity as a function of time, i.e. the fluorescence decay.

The main condition is that the *probability of the 2nd photon detection occurring after the start pulse should be negligible*. This implies that no more than one photon per about fifty flashes is detected, i.e. the *duty cycle of the detection is very low*. This is a limitation especially for strong emitting samples, as the technique cannot take advantage of the inherent high intensity in those samples.

As in the strobe technique, the analysis requires in most cases that the decay and the IRF be collected. The model parameters (e.g. lifetimes and pre-exponential factors) are recovered from the non-linear least squares fitting procedure that involves iterative re-convolution of the IRF and model function.

The low photon count relative to the lamp frequency requirement means that *only high-frequency light sources should be used. This eliminates some powerful and spectrally versatile sources like a nitrogen laser pumping a dye laser*. Typically, nanosecond flash lamps and mode-locked lasers are used, the latter being quite *expensive* and often *troublesome* to maintain. Recently, solid-state diode lasers and LEDs are becoming popular.

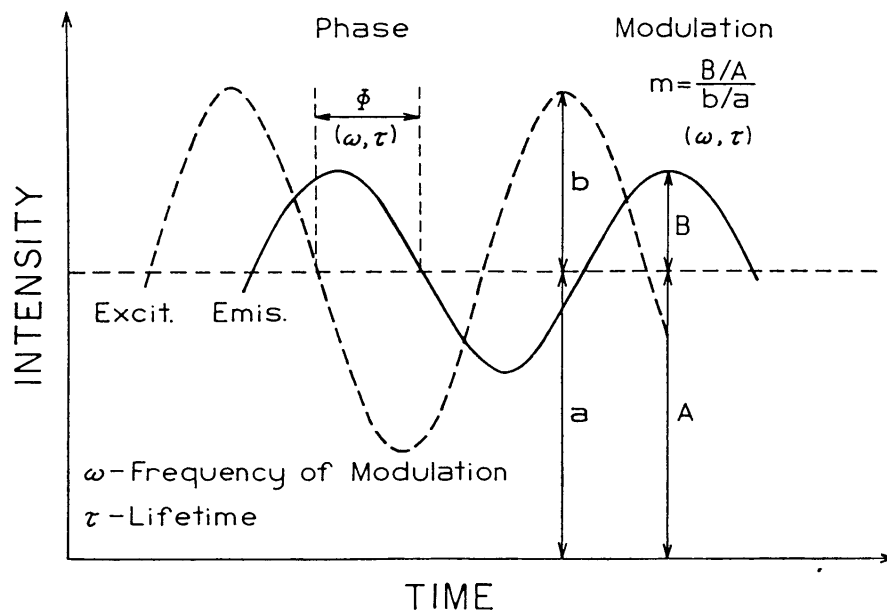
The *TAC is a linear device*, so the *technique can only work with equal time increments*. That means that a large number of channels (data points) are needed to adequately record fluorescence decays with very short and very long lifetimes present.

### **The Phase Shift Technique**

This technique too is of the 1970's vintage. Unlike the pulse methods, *the phase shift technique employs a continuous light source (e.g. xenon arc lamp) whose intensity is sinusoidally modulated at a frequency  $\omega$* . The fluorescence response can be determined by convoluting the excitation function with the intrinsic decay law (e.g. a single exponential function). The *resulting fluorescence response will also be modulated at the frequency  $\omega$ , but it will exhibit a phase shift  $\Phi$  and a reduced depth of modulation as*

**compared with the excitation light** (see diagram). If the underlying fluorescence decay is single exponential, the lifetime  $\tau$  can be easily and quickly determined from the phase shift  $\Phi$  and degree of modulation  $m$ :  $\tau = \tan(\Phi)/\omega$  and  $m = (1 + \omega^2 \tau^2)^{-1/2}$ , where  $m = (B/A)/(b/a)$ .

However, **with a single modulation frequency, only a single lifetime can be determined. In order to analyze complex fluorescence decays, the shift and the modulation depth should be measured with a large range of modulation frequencies.** The frequency response of the shift and modulation is then subjected to the non-linear least squares fitting procedure, from which the model parameters (lifetimes, pre-exponential factors) are recovered.



**Fig. 4** Illustration of the phase shift and change in the modulation depth of the emitted light relative to the excitation light

For typical lifetimes in the range of **hundreds of picoseconds to hundreds of nanoseconds megahertz frequencies are used.** These are usually obtained with **electro optical (EO) modulators** and the **upper limit is currently 350MHz** in commercial systems based on **arc lamps**. One problem is a **significant intensity loss** when conventional light sources (lamps) are used due to a **narrow range of acceptance angles for such modulators.**

EO modulators are special crystals that rotate polarized light when a certain voltage is applied. The modulator is placed between a pair of crossed polarizers, so no light is transmitted in the absence of voltage. In order to transmit light quite high voltages (1-7kV) should be applied. However, at MHz frequencies that are needed for the light modulation, only voltages of about 100V can be achieved. **This causes low depth of modulation and poor light transmittance.**

*Another major disadvantage of EO modulators is that they require collimated light. With arc lamps the light transmittance is less than 5% and this is before any monochromators or filters!*

The phase shift instrumentation becomes much more complex when the picosecond lifetime range is required. In order to obtain gigahertz frequencies, higher harmonics of *mode-locked picoseconds lasers* are used and also *faster multi-channel plates (MCP)* replace conventional photomultipliers. The cost of such systems grows enormously! The so-called cross-correlation technique has been developed to facilitate signal detection at high frequencies. In this technique the light source and the detector are modulated at somewhat different frequencies. This results in an additional signal at the much lower difference frequency, which contains the phase and modulation information of the original signal.

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