A practical guide to time-resolved luminescence lifetime determination using dedicated Time-Correlated Single-Photon Counting systems.

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Contents

Overview

The purpose of this document is to give a brief introduction to fluorescence and the technique of time-correlated single-photon counting, commenting on the advantages of its use in the determination of fluorescence lifetimes. Also three dedicated time-resolved fluorescence measurement systems that make use of this technique will be highlighted and their capabilities and specifications stressed. They are;

TemPro Fluorescence Lifetime System

Compact TCSPC filter based system capable of measuring lifetimes from 100ps to s

FluoroCube

A time-resolved spectrofluorometer for determination of short fluorescence lifetimes from ps to s

DynaMyc

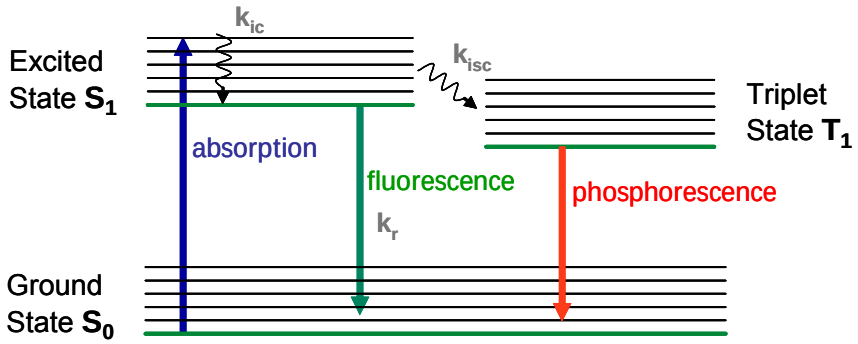
Microscope system with confocal and fluorescence lifetime mapping ability

Also common components, such as the **NanoLED** and **PicoBrite** excitation source, the **TBX** detector range, plus the **FluoroHub** photon counting electronics will be briefly described.

It should be noted that TCSPC upgrades are also available for the **FluoroLog** and **FluoroMax** steady state spectrophotometers. The **FluoroLog** is also capable of measuring lifetimes in the frequency domain. If further information is required please consult the **MF^2** application notes.

Luminescence

The term luminescence originates from the latin (*lumen* for light) and was first used as *luminesenz* by Wiedemann in 1888 for "*all those phenomena of light which are not solely conditioned by the rise in temperature*"[1]. It is now generally used to encompass the processes of *fluorescence* and *phosphorescence*, with these two phenomena simplistically distinguished by timescale. *Fluorescence* is associated with the absorption and re-emission of light on the pico- to nanosecond range, while the timescale *for phosphorescence* is normally associated with the microsecond to second range. The spectral range for these phenomena spans the ultra violet to near infra red regions. Both processes relate to the absorption of energy in the form of light, which causes a change in electron distribution, moving the molecule to an excited electronic state, which then relaxes back to the ground state via emission of light. In *fluorescence* this occurs between singlet energy levels, but with phosphorescence intersystem crossing occurs and the electronic triplet state becomes populated; emission to the ground state then occurs. As molecules tend to dissipate energy in the excited state this leads to the energy of the re-emitted light to be less than that of the excitation light, i.e. *fluorescence* and *phosphorescence* occur at longer wavelengths compared to the excitation. This is demonstrated in the simplified energy level diagram given below (known as a Jablonski diagram). Where k_{ic}, k_{isc} and k_r are the internal conversion, intersystem crossing and radiative rate constants respectively.



The timescale for the absorption process is typically femtoseconds (10^{-15}s) and the excited molecular rapidly goes to the lowest vibrational level of the first excited state on the order of picoseconds (10^{-12}s). From here the molecule can return to the ground state non-radiatively or by the emission of a photon. If the origin of the photon is the single state, this is *fluorescence* and usually occurs on the pico to nanosecond (10^{-9}s) timescale. If the molecule goes to the classically forbidden triplet state (with the associated change in electron spin) then the emission can be on the microsecond to second timescale and is termed *phosphorescence*.

[1] B. Valeur in New Trends in Fluorescence Spectroscopy: Applications to Chemical and Life Sciences. B. Valeur and J.-C. Brochon (eds), Springer Verlag (2001).

The main parameters of interest include the fluorescence quantum yield, Φ, which gives and indication of the efficiency of the fluorescence process. This can be determined experimentally from steady state spectroscopy and can be expressed by the following equation.

$$\Phi = \frac{number\ of\ photons\ emitted}{number\ of\ photons\ absorbed} = \frac{k_{nr}}{k_{nr} + k_r}$$

Where k_{nr} is the overall non radiative rate and k_r is the radiative rate. This means that a maximum value of 1 is achieved if for every photon absorbed a photon is emitted. Also of interest is the Stokes' shift, which relates in the difference in energy (wavelength) between the absorption and emission spectra, caused by excited state interactions of the molecule, principally with the environment. Finally and most importantly in relation to the equipment and techniques stressed here is the fluorescence lifetime. This is a measure of how long on average a molecule spends in the excited state, or more exactly how long it takes to decay to 1/e of its original intensity (I_0). A simple fluorescence decay takes the form of an exponential (given in the equation below), where τ is the fluorescence lifetime and can be related to the radiative and non radiative rate constants.

$$I = I_0 e^{-t/\tau} \qquad , \qquad \tau = \frac{1}{k_{nr} + k_r}$$

An important fact to keep in mind is that the fluorescence lifetime is an absolute measurement, whilst the intensity, usually taken from the emission spectrum, is only a relative measure, as depends on the number (ie concentration) of molecules present.

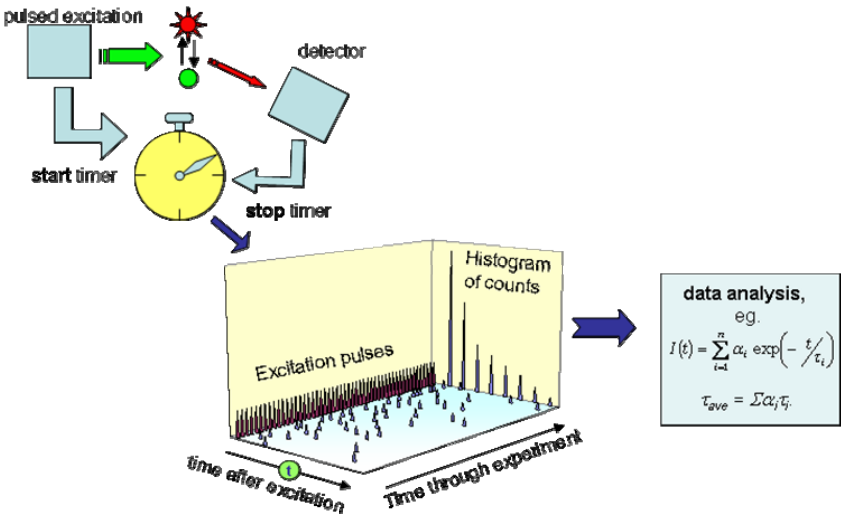
Many substances fluoresce (ranging from biological tissues to inorganic crystals) and this can be put to good use, since a molecule's fluorescent properties are highly dependent on its local environment. In some respects a fluorescent molecule can be considered a nanoscale reporter and a well characterised probe molecule can help elucidate information concerning local pH, viscosity, dielectric constant and molecular interactions. There are numerous books relating to this field and some worthy of note are those by Lakowicz[2] and Valeur[3].

[2] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Springer and the "Topics in Fluorescence Spectroscopy" series of books (J.R. Lakowicz (ed)), Springer.

[3] B. Valeur, Molecular Fluorescence: Principles and Applications, Valeur, B., Wiley-VCH, New York, Chapters 1-3, pages 3-71 (2002).

Why Time-Correlated Single-Photon Counting?

Time-correlated singe-photon counting is the most popular method of determining picosecond to microsecond fluorescence lifetimes. It is based on the fact that the probability of detection of a single photon at a certain time after an excitation pulse is proportional to the fluorescence intensity at that time. It is a pulsed technique that builds up a histogram of fluorescence photon arrival times from successive excitation-collection cycles, depicted schematically below.



Main aspects of this technique can be summarised below and details found in several texts[2,4,5].

- The histograms obtained give an immediate, qualitative, indication of the lifetime and can be indicative of the presence of more than one decay pathway.
- Very high sensitivity
- Large dynamic range (3-4 decades)
- Well defined statistics (Poisson)
- Digital technique (not susceptible to changes in excitation source intensity)

As with all techniques TCSPC has its advantages and drawbacks. On the plus side it offers the extremely high sensitivity of side photon counting, which is only possible in the time domain. The histograms obtained give an immediate, qualitative, indication of the lifetime and can be indicative of the presence of more than one decay pathway. As it involves timing the arrival of photons, rather than light intensities, it is not vulnerable to any fluctuation in excitation source intensity. However, in order to be certain of acquiring histograms representative of the decay process there should not be more than one photon detected per excitation-collection cycle. This restriction mainly relates to the instrumental dead time and is to avoid the possibility of a photon arriving while the equipment is not able to time it. In practice this has meant limiting the rate of detection to less than 2% that of the

[4] D.J.S. Birch and R.E. Imhof. *Time-domain fluorescence spectroscopy using time-correlated single photon counting* in Topics in Fluorescence Spectroscopy vol 1: Techniques" (J.R. Lakowicz (ed)), Plenum Press, New York pp 1-95 (1991).
[5] D.V O'Connor and D. Phillips. Time-correleated single photon counting. Academic Press, London (1984).

excitation frequency, a value that means distortion linked to undetected fluorescence photons will be less than other experimental errors. However, the absolute amount of distortion that can be tolerated depends on the individual measurement type. For example in a fluorescence lifetime imaging (FLIM) acquisition, where for example, contrast or trends require elucidation higher rates (> 5 %) can be acceptable.

Historically with the use of low repetition rate excitation sources, this has implied that to obtain statistically meaningful numbers of fluorescence photons long measurement times were necessary. This factor has previously led to the use other fluorescence lifetime measurements techniques, such as those in the frequency domain, when short measurement times were vital. However, advances in excitation source technology, especially the advent of diode lasers, have improved matters significantly, enabling much faster acquisition rates (many kHz compared to 100s of Hz). This has meant that data collection times have reduced significantly and depending on the requirements data acquisition in milliseconds is now possible.

Although the basis of the technique has not noticeably changed since its conception in the early 1960's successive improvements in instrumentation have combined to produce an extremely powerful and popular method for the determination of the excited state molecular kinetics.

Advantages over other time domain techniques

The fact that the TCSPC technique involves timing the difference between two defined events, rather than the direct measurement of intensity, means that it is a digital technique. This gives TCSPC great sensitivity and advantages over other time domain methods, eg time-gated (strobe) methods which measure intensities. These include;

- measurements not affected by fluctuations in excitation source intensity
- greater stability during a measurement allowing longer data collection times if required
- no need for intense excitation source (with associated risk of photobleaching sample)
- well defined statistics (Poisson) and error determination
- not limited to collecting on part of the temporal decay per measurement cycle (ie better efficiency)

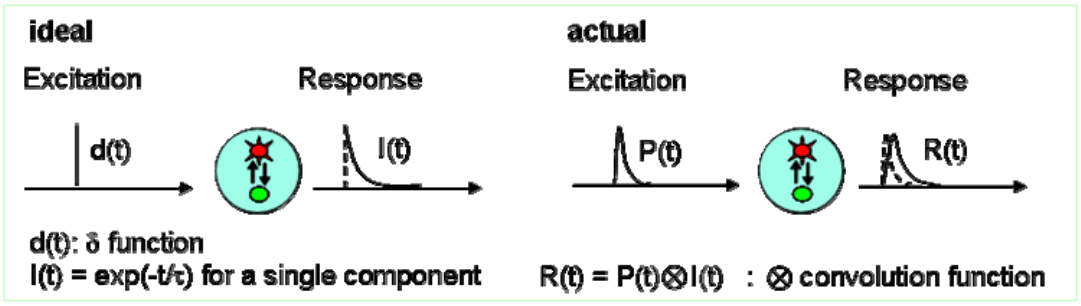
Fluorescence lifetimes can also be determined by the use of streak cameras, which can measure intensity time and wavelength (or position). However these are specialised devices that are not trivial to use and their usage is principally applied to weakly emitting ultrafast decays.

Instrumentation for time-resolved measurements

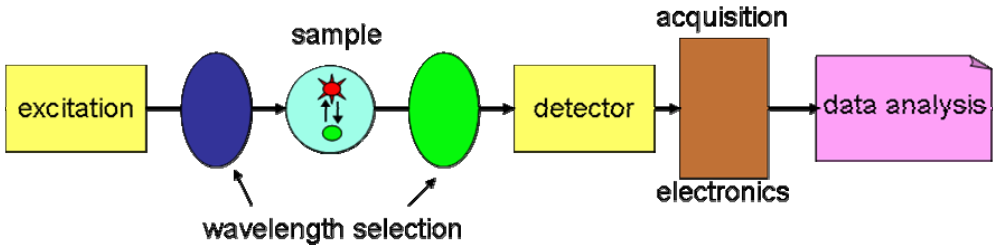
TCSPC, as any pulse technique, starts from the premise that the excitation pulse has the form of a δ function, i.e. all intensity and no temporal width (see below). This means that the measured fluorescence response is that coming directly from the fluorophore. However, in reality this will not be the case. The light pulse from the excitation source does not form and extinguish instantaneously, it takes time. Also the detector does not respond immediately to light hitting it and other factors, such as the electronics have their own response times. Simply put this can be approximated to the full width at half maximum of

$$(\text{instrumental response})^2 = (\text{optical excitation pulse})^2 + (\text{detector transit time spread})^2 + (\text{electronic jitter})^2 + (\text{optical dispersion})^2$$

This is rewritten more mathematically on the following page. Combining these aspects means that, on timescales where the lifetime to comparable to the instrumental FWHM, the observed decay contains distortion caused by this instrumental response (see below). However, there is a way to mitigate this and by measurement of both the fluorescence decay and the instrumental (or prompt) response it is possible during the analysis process to “remove” the instrumental distortion via reconvolution.



The main components of a time-correlated single-photon counting system are shown schematically in the following figure. From the user’s point of view, the important aspects are probably the resolution of the equipment, plus the time to make a measurement. Obviously the capability to measure a large range of decay times is also advantageous. Here we will briefly consider the component parts of the instrumentation that have the biggest influence of the performance.



In general the instrumentation can be divided into

1) light sources
2) detectors
3) electronics
4) optical components

The major factors that have been found to effect equipment performance are 1 and 2. The measured response of a time domain lifetime system depends on the components as indicated by following equation, although the electronic resolution (time per channel) also needs to be considered.

$$\Delta t_m \sim \left[\Delta t_{exc}^2 + \Delta t_{\text{det}}^2 + \sum_i \Delta t_i^2 \right]^{1/2}$$

Where Δt_m is the measured full width at half maximum (FWHM), Δt_{exc} is the FWHM of the excitation, Δt_{det} is that associated with the detector and Δt_i is related to the response of the i^{th} component in the system. The excitation source and detector, with associated electronics, are by far the greatest influences on the measured response and Δt_m can be approximated as just being dependent on these two components. In TCSPC an assumption is made of delta function excitation, which because of the time-response of the electrical components is clearly not the case. This leads to the need to measure the instrumental response (normally made with a scattering solution) as well as the decay and the use of reconvolution analysis[6] to recover the decay parameters. Generally speaking the shortest lifetime that can be determined with this approach is approximately a tenth of the instrumental full width at half maximum.

[6] D.J.S. Birch and R.E. Imhof. Anal. Instrum. 14, 293-329 (1985).

Light sources

The capabilities of the light sources have improved dramatically since the conception of the technique. The initial three choices of excitation sources, flashlamps, mode-locked laser and synchrotron radiation, have been augmented and superseded as (mainly) semiconductor technology has advanced. Today favoured excitation sources for TCSPC include femtosecond lasers, semiconductor laser diodes and LEDs and more recently supercontinuum lasers. Main aspects are summarised below.

fs	ps	ns	Phos
Ti:Sapphire, eg, Chameleon Mai Tai 2 photon exc High rep rate ~80MHz (fixed) Tunable Expensive bulky	**Diode lasers**, eg, NanoLED PicoBrite Small Interchangeable Variable rep rate (10kHz-1MHz NL) (10kHz-80MHz PB) Fixed wavelength maintenance free	**LEDs**, eg, NanoLED **Flashlamps**	**LEDs**, eg, SpectraLED **Flashlamps** **lasers**

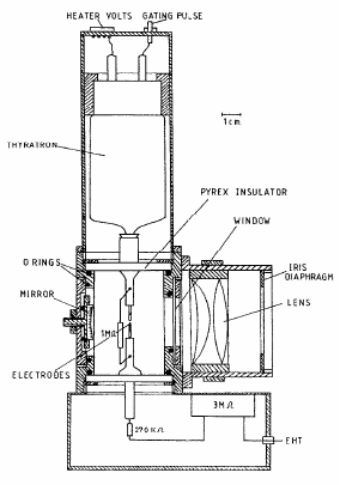
Supercontinuum (white light) lasers
Broad wavelength range (400-2000nm)
Fixed rep rate (MHz), short pulses

Flashlamps

Historically, the mainstay of fluorescence lifetime systems, they usually consist of a gas filled enclosure. A voltage applied to electrodes situated inside this produces a spark and the spectral output is determined by the gas filling the volume. Gating the voltage controls the repetition rate (typically 10's to 100 kHz), although some designs were "free running". The measured instrumental response of this type of source ranges from just under subnanosecond to a couple of nanoseconds depending on filler gas. The gases of choice tend to be hydrogen (continuum emission uv to *ca*. 500nm), nitrogen (intense lines at 316 nm, 337 nm and 358 nm) and a argon-hydrogen mixture, which exhibits spectral lines up to almost 800 nm[7]. The main drawbacks of this type of source is the fact they require cleaning, have a relatively low repetition rate (leads to slow data acquisition. An example is the **FluoroSource CF** nanosecond coaxial flashlamp illustrated on the following page. This latest generation flashlamp is derived from a coaxial design described in the early 1980's[8] and is still in production today. The coaxial design along with gold plating minimises electrical reflections and radio

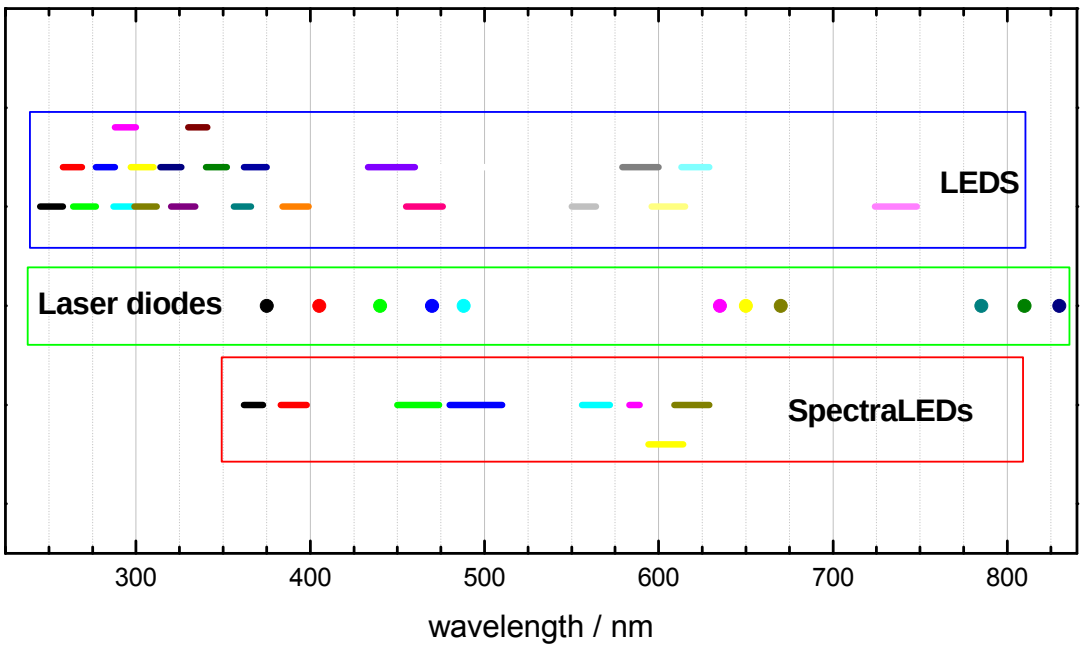
[7] D.J.S. Birch, G. Hungerford and R.E. Imhof. Rev. Sci. Instrum. 62, 2405-2408 (1991)
[8] D.J.S. Birch and R.E. Imhof. Rev. Sci. Instrum. 52, 1206-1212 (1981)

frequency interference produced by the electrical discharge. This was a common complaint of other types of design. For measurements of longer lived (phosphorescence) samples there is the slower pulsed **FluoroSource XF** microsecond xenon flashlamp.



Laser diodes and LEDs

Advances in semiconductor technology have enabled this class of excitation source to become the cost effective choice for routine time-resolved measurements. Although lacking the tuning range of the flashlamp, the combination of the different wavelength emitting sources covers an extensive wavelength range, from the uv (250 nm) up to the NIR (1310 nm). This is exemplified in the wavelength selection chart for the **PicoBrite**, **NanoLED** and **SpectraLED** ranges of sources below.



As well as the good wavelength coverage, these sources can pulse up to 80 MHz and the LEDs exhibit pulse widths from *ca*. 500 ps (for the uv variants) to ~1.5 ns (vis / NIR). Laser diodes typically have pulse widths < 100 ps and their cost, plus the fact that they rarely require maintenance has made them idea choices for routine fluorescence lifetime determination.

The **SpectraLED** range are designed for lifetime measurements on the phosphorescence timescale and have a variable optical pulse width from 100 ns to dc (giving a power up to 0.1mW) and are mechanically interchangeable with the **NanoLED** range.

Custom sources

It is possible to use certain third party excitation sources with our timing equipment (**FluoroHub**) as long as either a TTL or NIM signal for the START/STOP is available. High resolution spectroscopy is sometimes performed using femtoseond laser sources. As their name suggests their optical response is around 100 to 150 fs and they typically run close to 80 MHz with the output is usually routed via a pulse picker or cavity dumper (in pulse picking mode) to reduce this frequency (to 4 MHz for example). This is required in TCSPC to avoid re-excitation of the samples before their fluorescence has completely decayed. Most fs lasers are broadly tunable (~700 to 1100 nm), with more wavelengths available via second and third harmonic generation.

Recently the use of supercontinuum or white-light lasers to TCSPC has gained interest. These sources have narrow (fs) pulses with repetition rates of several MHz and emission from 400 nm to 2100 nm and have been touted to be the next state of the art source. Other excitation sources of note are mode-locked picosecond dye lasers. Of course the time-resolution and complexity of the sources mentioned here comes at a cost and for a majority of fluorescence applications laser diodes or LEDs more than suffice.

Detectors

The ideal choice of detector for TCSPC should exhibit most of the following[9];

- large wavelength coverage
- low transit time jitter across the device's detecting area (ideally < 20 ps mm^{-1})
- high gain (typically in excess of five million)
- negligible wavelength dependency of the temporal response (ideally < 0.5 ps nm^{-1})
- low noise (dominated by dark count and ideally < 100 cps)
- fast time response (typically < 1.5 ns rise time)
- few detector generated artefacts, e.g. secondary pulses
- sensitive area compatible with other optical components (eg ~10mm).

However, some of these ideals are conflicting, such as decrease in noise performance with increasing gain. A major factor when choosing a detector is its wavelength range. The lower limit is, in large,

[9] G. Hungerford and D.J.S. Birch. Meas. Sci. Technol. 7, 121-135 (1996).

determined by any window material, as well as the composition of the material responsible for converting the incoming light into an electrical signal. The latter also determines the longer wavelength response. As longer wavelengths correspond to lower energies this material is required to have a lower work function (ie less energy required to liberate a photoelectron). This fact is also problematic as heat energy, even at normal ambient conditions, can be sufficient to cause the liberation of these electrons and hence the subsequent secondary electrons and a signal output. As this does not correspond to an incident light pulse, it is commonly referred to as noise. Thus, detectors that are more sensitive to the red end of the spectrum tend to have higher noise counts. This can, in part, be counteracted by cooling them to reduce thermal influences. However, even with cooling, photomultiplier tubes sensitive over 1000 nm for example, may exhibit large a number (several tens of thousands) of noise counts. Another important factor to take into account is how much the choice of detector is by how much the incoming signal gets spread during its passage through the detector. A measure of this is called the transit time spread (TTS) and it is a good measure of temporal resolution. The following figure, gives a summary of representative detectors.

faster response light → electrical signal

Microchannel plate (MCP) PMT

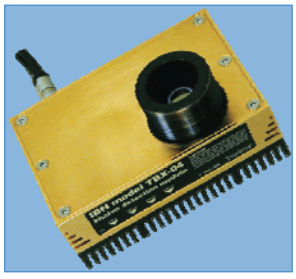
recommended for fs lasers
sensitive
count rate limited by damage threshold
expensive
FHWM count rate dependent

Avalanche photodiodes

sensitive
small active area
hard to use with monochromators
response wavelength dependent
robust

TBX detection module

sensitive
robust
well suited for use with picosecond laser diodes



side on and linear PMTs

nanosecond response
linear amplification
ideal for combined steady state / lifetime systems

detector	R928P	TBX	SPAD	MCP (R3809U)
TTS (ps)	1200	≤ 200	< 50	≤ 25

R928P - side on PMT, TBX – picosecond detection module,
SPAD – avalanche photodiode, MCP – microchannel plate PMT

TBX Picosecond detection module

For a vast majority of fluorescence life applications the **TBX** range of detectors will suffice. As can be seen from the summary figure above, they exhibit a good transit time spread in relation to a typical single-photon counting photomultiplier (R928P). Two main versions of this detector are available, one for the wavelength range 185 nm to 650 nm and another sensitive up to 850 nm (either cooled or uncooled). The variant with the higher blue sensitivity typically exhibits a noise count ~ 20 cps (specified to be < 80 cps). Because of the lower work function of the photocathode the red sensitive version has a higher noise count (couple of hundred cps), but this can be reduced by cooling (typically < 20 cps). These detectors are well suited for use with picosecond light sources and exhibit excellent performance per cost considerations.

Microchannel plate (MCP) detector

This type of detector has remained for a long time the main choice for measurements requiring the ultimate in resolution, especially for use with femtosecond light sources. The version supplied as standard with the **FluoroCube Ultrafast 01** is the Hamamatsu R3809-50. The wavelength response is determined by the photocathode material and some other examples are tabulated below for comparison (see manufacturers website for full details).

spectral range	peak λ	photocathode material	tube type
160 to 850nm	**430nm**	**multialkali**	**R3809-50**
160 to 910nm	600nm	extended red multialkali	R3809-51
160 to 650nm	400nm	bialkali	R3809-52

The MCP is typically provided with a cooled housing (eg. Hamamatsu C10373) facilitating the reduction in thermally generated photoelectrons, thus decreasing the noise counts. One drawback of the MCP is the count rate that it can endure, this is recommended to be < 20,000 cps by Hamamatsu in typical operation to avoid damage to the device. This type of detector is not suitable for steady state or phosphorescence measurements. MCPs are commonly used in conjunction with an amplifier-discriminator. In the **FluoroCube Ultrafast** this is close coupled to the MCP to minimise noise pick up and maximise timing properties.

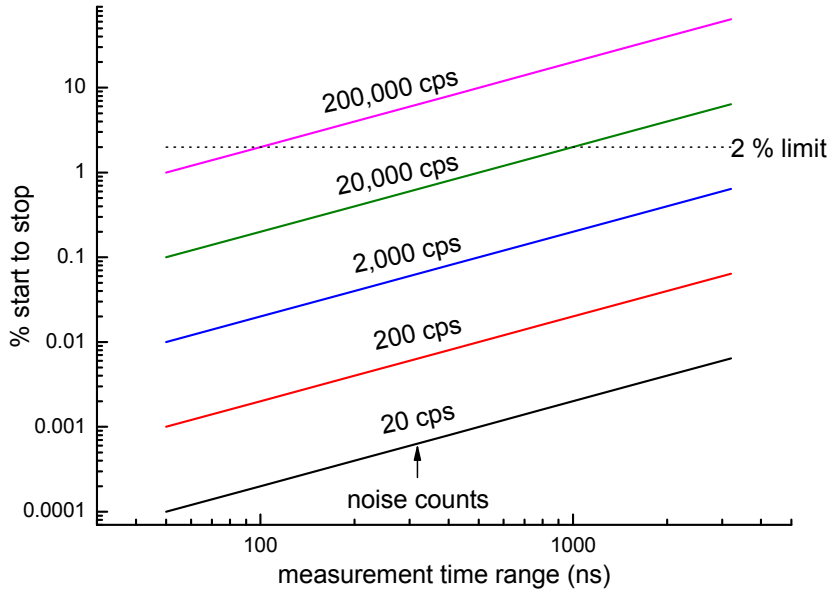
Single-photon counting avalanche photodiodes (SPADs) are also “fast” detectors, however their general usage is limited principally because of their small active area (~100 μm^2) and a wavelength dependent temporal response (can make reconvolution problematic). Their usage in recent times has been linked to that of fluorescence microspectroscopy, where focussing onto the active area is better defined and reconvolution is not always employed.

NIR sensitive photomultiplier tubes

The photomultipliers mentioned above are capable of detecting fluorescence up to 850 nm, which covers most applications. However, when there is a need to measure luminescence decays at longer wavelengths other detectors are required. The ones that are commonly recommended for use are the Hamamatsu H10330 and R5509 series (see manufacturer's website for details). The wavelength range and noise counts for these models, taken from manufacturer's literature, are given tabulated below.

detector		wavelength range (nm)	noise counts (cps)
H10330	-45	950 - 1400	20,000
	-75	950 - 1700	200,000
R5509	-42	300 - 1400	20,000
	-43	300 - 1400	16,000
	-72	300 - 1700	200,000
	-73	300 - 1700	160,000

Because of the sensitivity to longer wavelengths the work functions of the tubes photocathodes are relatively low, hence the higher noise values even when cooled (which is a requirement for these detectors). This fact is problematic when it comes to fluorescence lifetime determination using TCSPC, a good signal to noise ratio is usually a strength, but these detectors are extreme. In order to have single-photon statistics the fluorescence collection rate should be < 2 % that of the excitation rate. The noise counts will contribute to the fluorescence collection rate, thus reducing the amount of fluorescence that can be measured (ie reduced signal to noise ratio). This is also influenced by the time range selected for the measurement (this is usually set to be at least ten times the expected lifetime).

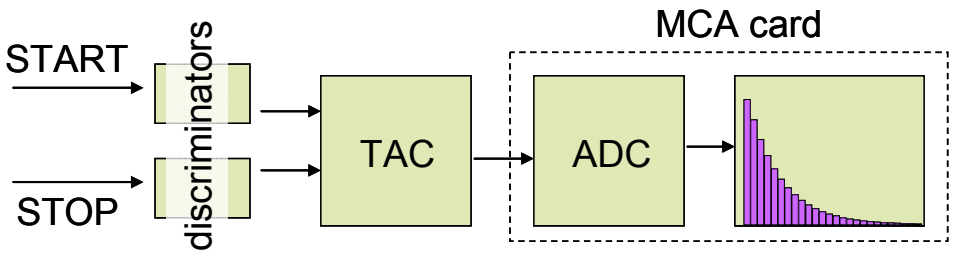


The effect of noise of the collection rate for different timescales is illustrated in the preceding figure and demonstrates that there is a narrow TCSPC operating range for detectors exhibiting high noise levels. It is clear from the graph that for detectors with high noise counts the 2 % limit is reached and exceeded by just considering the detector noise alone! Thus some time ranges are “off limits” to certain detectors and for example a detector with 200,000 cps noise can only be used to determine lifetimes to ~ 5 ns using TCSPC.

Hence determining lifetimes in the NIR using TCSPC is not a trivial matter and a good knowledge of the systems to be studied alone with the capabilities of the equipment is required.

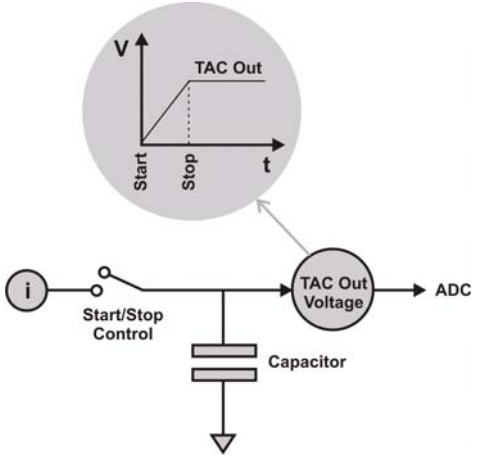
Electronics for data acquisition and hardware control electronics

In the main, because of historical reason the electronics used has been based on Nuclear Instrumentation Modules (NIM), which was developed as the name suggests for nuclear physics area. Until recently these modules have been employed with little customisation to fluorescence lifetime determination. The start and stop timing pulses are usually “cleaned up” using a discriminator, commonly a constant fraction discriminator (CFD) as it counteracts fluctuating pulse heights in the signals by producing a common timing point with low timing jitter.



Scheme of conventional TCSPC timing electronics. TAC- time to amplitude converter, ADC – analogue to digital converter, MCA multichannel analyser

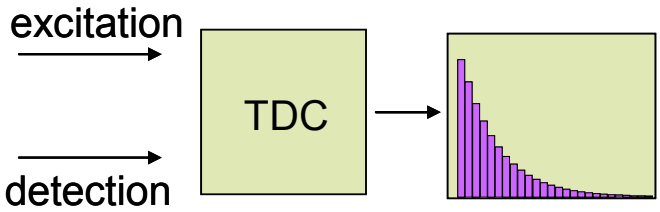
The “start” and “stop” signals are typically fed to a timing device, the TAC (time to amplitude converter), which when triggered ramps up the voltage on a capacitor, demonstrated in the figure below.



The arrival of a “stop” pulse then halts this increase and the voltage output is fed to an analogue to digital converter (ADC), which outputs the time to a histogram bin. Histogram sizes can vary depending on application. Typically they can be up to 8192 channels for high resolution measurements or only 256 channels, if data is to be acquired quickly at the expense of resolution, for example in fluorescence lifetime microscopy, with the width of the bins as little as 700 fs. The time width of the histogram bins is dependent on the time range selected and the number of points in the histogram. Typically the time range should be at least ten times that of the lifetime to be measured. The number of histogram points is normally a trade off between resolution and data collection time, as doubling the number points for example, will double the time to measure the data. Typically at least 1024 channels in the histogram are advisable, although this is highly application dependent.

The above scheme implies that for every pulse of excitation light a trigger pulse should start the TAC. However, as the “stop” rate is generally limited to 2% of the “start” rate, this means that the electronics are being triggered many times without any output expected. When using high repetition rate excitation sources this means that the equipment dead time is significant. One method used to overcome this is to run the equipment in “reverse mode”, with the TAC triggered using the signal from the detector and the voltage ramp halted by the correlated signal from the excitation source. As the TAC is started fewer times a reduction in equipment dead time is achieved. This has the small drawback that the resultant data appears noisier than when running in “forward mode” as noise counts from the detector are more likely to trigger the TAC.

The latest trend is to move away from MCA cards incorporated within computers and NIM orientated hardware. Computers can be “noisy” environments in which to process the small signals originating from the detectors and the very advances in computer technology that led this integration have made some older MCA cards obsolete, as they will not physically couple within newer computers. These two factors have lead to the return of more stand alone hardware with control and connection achieved *via* a USB connection to a computer. Combining the functionality of TACs and ADCs in newer devices called time to digital converters (TDC) has also occurred and a newer representation of the electronics is given below. A practical example is the HORIBA Scientific **FluoroHub-B**, where all electronics are in a stand alone module in communication *via* a USB link to a computer running control and analysis software.



Scheme of TCSPC timing electronics incorporating a time to digital converter (TDC)

The following figure summarises the two types of **FluoroHubs** available

FluoroHub-A

- Excellent time base linearity for high quality data / precision measurements
- TCSPC and MCS acquisition modes in one package
- Max. histrogram size 8k
- TCSPC resolution 7ps / channel standard and 0.7ps channel with FluoroHub-TE option
- MCS resolution from 500ns / point (optional)
- Integral cable delays for low temporal drift

FluoroHub-B

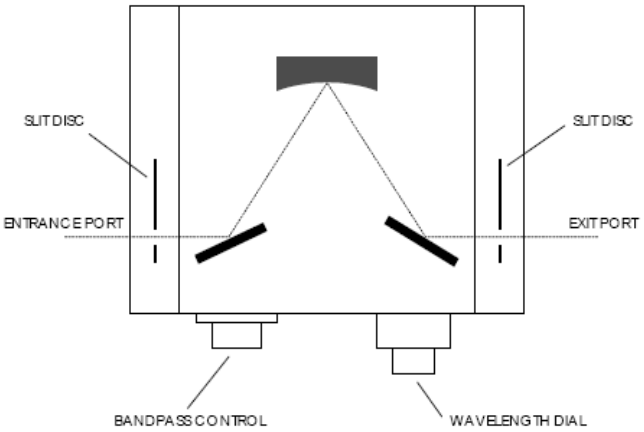
- Lifetime range from 100ps to 1s with same electronics
- Resolution 50ps / channel nominal
- Max. histrogram size 4k
- Single USB 2.0 connection
- NanoLED driver (FluoroHub-NL) cards (optional)
- All electronics included into the FluoroHub-B, no need for board integration in computer

The main distinction is that of time-resolution. For use with fast MCP-PMT detectors and picosecond laser sources the **FluoroHub-A** is the module of choice, while for general fluorescence lifetime measurements, plus ease to transform to the determination of phosphorescence lifetimes the **FluoroHub-B** is recommended.

Optical components

The main optical component that will be discussed here is the monochromator, which is used for wavelength selection. Because of the sensitivity of the TCSPC technique there is a requirement to be able to detect low light levels. To facilitate this any monochromator needs to have a good throughput. Unlike with steady state instruments where spectral resolution is highly important, this aspect is not ranked as high as the ability to transfer light efficiently.

As most sources, such as laser diodes, LEDs and lasers have a restricted spectral spread there is no specific need for a monochromator on the excitation side of the TCSPC fluorometer and it is now only usually situated on the emission side. The HORIBA Scientific **5000M** series, shown schematically below contains an aberration corrected holographic grating mounted in a Seya-Namioka arrangement, masked to give a f/3 aperture and it exhibits a temporal spread of 0.13 ps nm^{-1}.

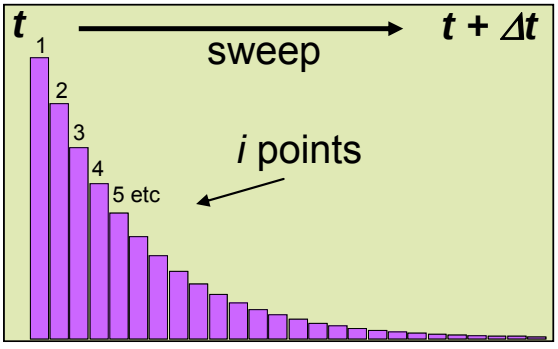


This particular monochromator has a focal length of 100 mm and spectral range is from 200 nm to 800 nm with a precision ± 1 nm. The optics are vertically dispersive and the monochromator includes an integrated safety shutter. Uniquely the internally mounted entrance and exit slits are synchronised for convenience when changing the bandpass.

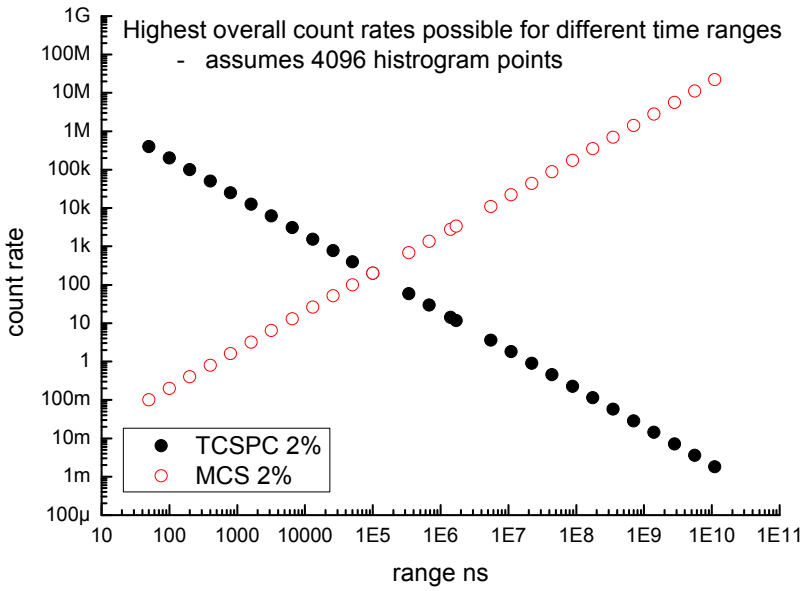
Longer lifetime measurements

Up until this point we have been mainly concerned with the fluorescence timescale and the use of the TCSPC technique. Much of the instrumentation can also be applied to the determination of lifetimes on the phosphorescence timescale. More traditionally rather than using TAC based systems multichannel scaling (MCS) has been employed. The **FluoroHub-A** contains a MCS card and the **FluoroHub-B** can be selected seamlessly to measure different lifetime ranges.

Multichannel scaling works by counting all events occurring within a specified time interval t to $t + \Delta t$ (the dwell time or bin width). The time t is divided into i histogram points (number of bins) and t is quantised by the relation $t = i\Delta t$. When the measurement starts it inputs counting events into the first channel, at the end of the dwell time selected counting is commenced in the next channel and so on, see following schematic. This continues until all (i) channels have been utilised (called a sweep). This process can then be repeated for a prerequisite number of sweeps, specified time or peak number of counts.



This type of operation means that the device is acting in a multiple stop mode as it collects all the counts that are occurring within a specified time interval. This means that the luminescence collection rate relative to the excitation rate can be higher than that of a TCSPC based system which only allows for one stop. However, this is not true for all time ranges as the electronic dead time effects dominate at shorter timescales. The graph below indicates the point at which MCS becomes more efficient (assuming for the 2 % limit for both techniques) and this helps explain why the two methods are preferentially for different timescales. In order to obtain the maximum count in any channel divide by 4096.



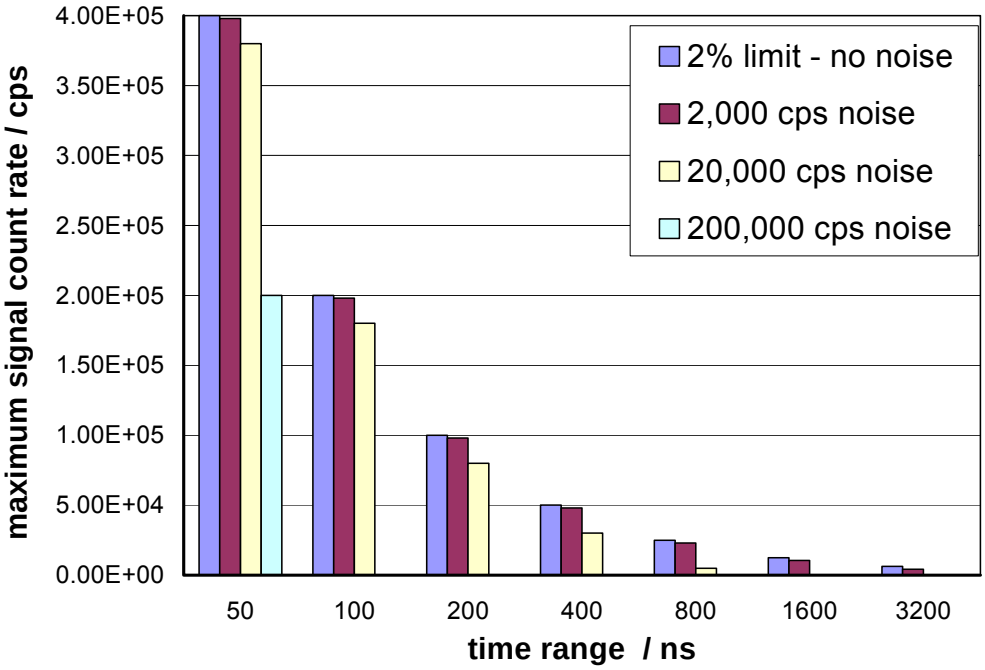
This form of operation is also advantageous when it comes to treatment of the detector noise, allowing even the noisiest of the previously mentioned NIR tubes to be used. This means in terms of NIR decay time determination that there is a inaccessible lifetime range, too long for TSCPC to be used and too short for MCS to be efficient.

Although the **FluoroHubs** can operate in MCS mode the **NanoLED** range of excitation sources are not designed for low repetition rate usage as their pulse energy is too low for this type of operation. Instead the meachanically interchangeable **SpectraLED** sources can be used. If a wavelength not included in the **SpectraLED** range is required there is the possibility of using the output from the **FluoroSource XF** microsecond xenon flashlamp. Of course custom sources may also be used provided they can provide the appropriate electronic signal (NIM or TTL).

Near Infrared decay measurements

The care required in undertaking these measurements, as has been mentioned in the previous sections (NIR detectors and longer lifetime measurements) prior knowledge about the samples (in terms of lifetimes and emission wavelengths) is required. This can then be matched to the

equipment capabilities in terms of which detector – simply choosing a detector sensitive to 1400 nm as opposed to 1700 nm reduces its noise counts tenfold and opens up the possibility to measure longer lived fluorescence. If the sample is know to be long lived then MCS can be employed, allowing detectors further into the red to be used. Often not all this information is available and this may lead to problem in obtaining meaningful data. The following chart shows the maximum count rate possible (assumed repetition rate = 1/time range) for each range (blue bar, 2% - no noise). This can be compared with the maximum signal possible for tubes with different noise levels. The difference between these rates is the noise contribution for the particular time range.



This indicates that that it is really only advisable to use a PMT with 200,000 cps on the 50 ns fluorescence time range (ie maximum lifetime that can be determined 5 ns), where the 2 % stop rate will consist of equal quantities of noise and signal. Thereafter this limit is reached by noise alone. This implies that a PMT exhibiting this level of noise should not be used with a **FluoroHub-B** to determine short (ns) lifetimes, as the minimum range is 200 ns and a **FluoroHub-A** should be considered. If the PMT is ten times less noisy then a time range of 800 ns (lifetime 80 ns) is just about accessible.

If considering MCS operation, which has different constraints in relation to noise then the **FluoroHub-A** contains a MCS card with a maximum resolution of 500 ns per channel. The **FluoroHub-B** can start this type of operation with a resolution of 83 ns per channel. Hence there is a "gap" in the time ranges (determined by detector noise) that is inaccessible between the TCSPC and MCS modes. The minimum lifetime possible then relates to the number of channels to be included within the analysis. For example if considering a minimum of 5 channels this means that a **FluoroHub-A** in MCS mode (500ns per point) could determine a lifetime of 2.5 μs and **a FluoroHub-B** 415 ns. Although it should be noted, that a faster MCS card (2 ns per point) is available, allowing for 10 ns lifetimes to be determined using the above criteria. Therefore a careful choice of both **FluoroHub** and detector is required and some prior knowledge of both the wavelength range and possible lifetime is highly advisable.

Time range selection

The time range required for a particular measurement depends on the lifetime to be determined. Generally speaking the time range should be ten times that of the longest lifetime to be determined. Obviously this is also influenced by the user's desired resolution. It is also desirable that the decay should cover several hundred channels within the selected time range. This maybe adjusted by changing the number of histogram points. Reducing the number of histogram points will also reduce the resolution, although there is a trade off between this and data collection time, the fewer the number of points the faster the collection time.

Problems can arise if the sample is multiexponential, with lifetimes that are very different. In which case, it is advisable to determine the longest-lived first, by using a longer time range. Then by selecting a shorter time range the measurement should be repeated, although in this case determination of the longer-lived decay will be more prone to error. This can be overcome by using the value obtained from the first measurement and fixing the lifetime value in the analysis. This will allow a more accurate determination of the shorter-lived emission.

The choice of time range also influences the repetition rate of the excitation source, which to avoid the presence of more than one pulse within the time window should be set to less than 1 / time range.

We have also seen that detector generated noise (*Detector* and *NIR decay measurement* sections) has a different influence depending on the time range.

Therefore when selecting the time range we should consider,

- the required resolution / data collection time
- range ideally ten times the longest lifetime to be determined (if high precision required)
- source repetition rate = 1 / time range
- take into account detector noise

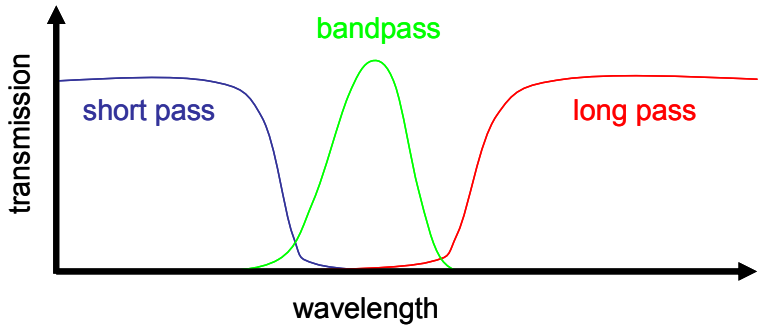
Count rates

It is generally recommended that the data collection rate should not be more than 2 % that of the repetition rate of the excitation source to avoid data distortion. Although this is dependent on what the researcher wants to obtain from the measurement and its precision. For example higher rates may be deemed acceptable if a low number of peak counts is collected or if accurate lifetime determination is not the objective. Such an application can be FLIM, where only contrast is required. This distortion is commonly known as pulse pile up and has the effect of decreasing the measured lifetime and, at times, the apparent presence of an extra decay component. This originates from the fact that that at "high" count rates only the photons arriving first will be counted and any arriving at a longer time will not be seen because of the electronic dead time. It is also important to factor in the detector noise as if it is high it will contribute significantly to the data collection rate (see *detector* and *NIR decay*

measurement sections). At this point, it should be pointed out that TAC based systems should be operated in "reverse mode" (detector provides start and source the stop) if the repetition rate of the excitation source is high (more than several hundred kHz).

Wavelength selection

The phenomenon of fluorescence occurs at longer wavelengths (lower energies) than the molecules absorption. To avoid scattered excitation reaching the detector, as well as choosing specific spectral regions of the emission a form of wavelength selection is required. For **flashlamp** excitation sources, which have a broad spectral emission a means to select the appropriate excitation wavelength is also required. Wavelength selection can be achieved by use of a monochromator (see *instrumentation* section) or filters. The latter can either be short pass, long pass or band pass and are illustrated below. Typically band or long pass filters are used to select the fluorescence.



When choosing a filter it should be kept in mind that the wavelength specified for long or short pass filters are usually those for 50% (highest) transmission. As the curves are typically "*S* shaped" a wavelength separation of about 50 nm is recommended between the excitation wavelength and the filter wavelength. Band pass filters are usually specified in terms of their central wavelength and how many nanometres can be passed, again there is a small tail either side so a small intensity out with these values maybe passed. Band pass filters are also occasionally used in conjunction with laser diodes to reduce any broad band LED emission, which can affect reconvolution analysis. Then they are referred to as "*clean up*" filters. A short pass (UG 1) or band pass (370 ± 5 nm) filter is also recommended for use with the **NanoLED N-370**, as this particular LED also exhibits a small amount of longer wavelength emission.

When using a filter on the emission side (after the sample) it needs to be removed when measuring the instrumental response. If a monochromator is employed the slit width (determining the spectral band pass) should be kept the same for both the decay and instrumental response measurements, with only the wavelength changed from that to collect the fluorescence to the excitation wavelength. Any required adjustment to the count rate should be made using neutral density filters.

Data analysis

Histograms obtained from the TCSPC technique provide intuitive lifetime information, allowing a simple qualitative assessment of the lifetime to be made without prior knowledge. However, detailed quantitive interpretation of the decay data is very application dependent and some knowledge about the photophysics of the molecules under study is highly advisable. This is not only useful in ascertaining if the results are sensible, but can be a diagnostic aid in relation to equipment performance. If there is any doubt about a lifetime value then it may be fruitful to use a "standard" to provide confidence in the measurement and analysis process. There are useful publications listing substances that can be used in both the fluorescence[10] and luminescence ranges[11].

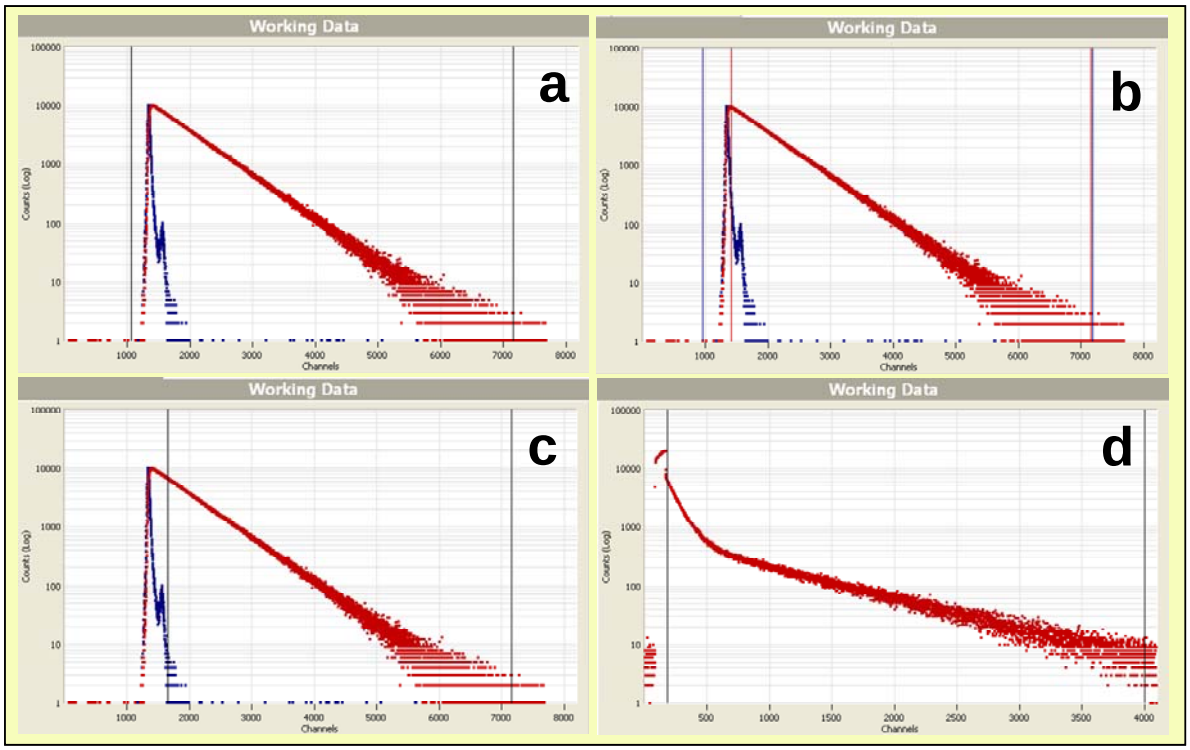
Another major consideration is if reconvolution analysis is to be used or not. If so, as well as the acquisition of the fluorescence decay, the instrumental (or prompt) response of the equipment also needs to be measured. This should be done to a similar precision (number of counts in the peak channel) as the decay. This is usually recommended to have 10,000 counts, although this maybe influenced by data acquisition time and other factors (eg photobleaching etc). If the lifetime is much longer than the instrumental FWHM then the data can be "tail fitted" without the need for reconvolution.

A fuller account is available in the **DAS6** and **DataStation** user guides and attention is draw to these. Here only a brief methodology will be given and it is assumed that the measurement has been made and data stored and opened using the **DAS6** analysis software. Here some basic practical issues will be addressed.

Selecting the analysis range

This is important as it has a bearing on the goodness of fit parameter (chi-squared, for example) as an incorrect choice may lead to fitting "nothing" to "nothing" which has the effect of improving the good of fit value. Thus, the range should be kept close to that containing the data of interest. The following examples demonstrate the fitting ranges that can be used when performing analysis both with and without reconvolution of the instrumental response.

[10] N. Boens *et al*. Anal. Chem. 79, 2137-2149 (2007).
[11] K.J. Morris *et al*. Anal. Chem. 79, 9310-9314 (2007).



a) This shows the suggested range when fitting the whole of the decay using reconvolution analysis, making use of the same analysis range for both decay and instrumental response.

b) Here separate ranges are used for the instrumental response (blue lines) and decay (red lines), but the program will still reconvolute the prompt from the decay. Sometimes fitting to the rising edge of the decay can be problematic (occasionally linked to experimental conditions) and hence two ranges are used, the extended range for the instrumental response allows the calculation of the noise background etc., while the decay range avoids the rising edge and just begins from the peak of the decay. When starting the decay from this position it is advisable to fix the shift parameter at 0 channels. This will not be necessary if the decay range begins before the peak channel.

c) Although the figure shows the position of the prompt, it need not be present and this is the range that could be employed in a "*classical tail fit*". The range is selected as to begin at a time where the influence of the instrumental response is negligible and hence is not necessary to be reconvoluted. If an instrumental response is recorded it can either be omitted in the analysis or the extended range (similar to blue lines in **b**)) used with the shift parameter fixed as 0.

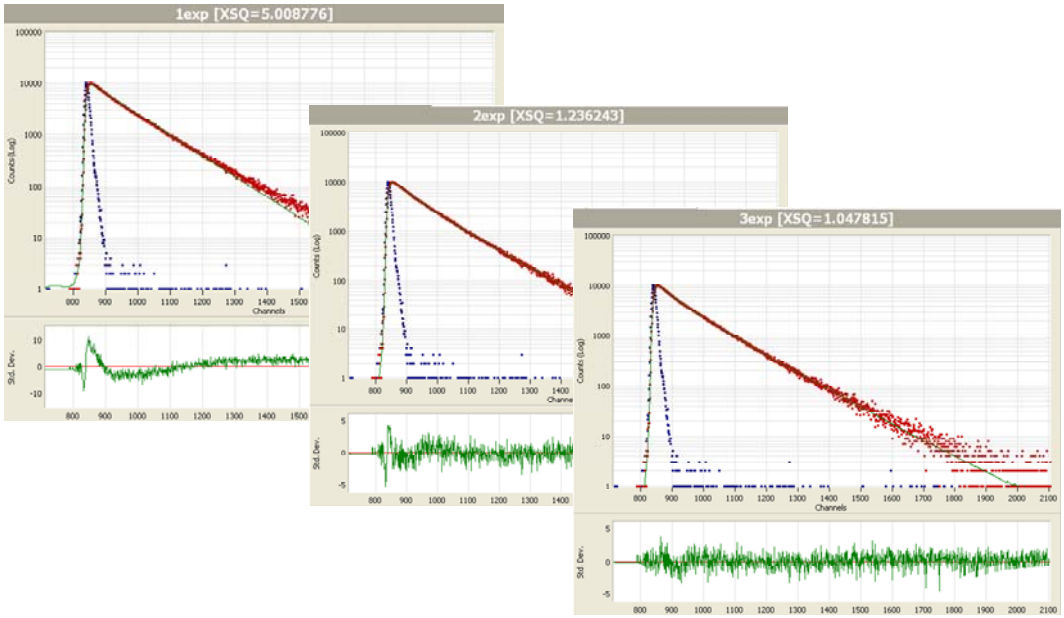
d) This figure is similar to **c**), but uses a phosphorescence decay as an example. Here the fit range is chosen to begin after the distortion caused by the excitation pulse and any short lived fluorescence. Again no reconvolution is necessary.

How do I know if I have a good fit?

The usual criteria to assess whether a fit is satisfactory are to use the reduced chi-squared value and to see if there are any trends in the weighted residuals. Also the fitting of an additional exponential decay component should not produce any significant improvement. The returned values should also be sensible. As a rough guide, reconvolution allows lifetimes ~ 10 times less than the instrumental full width at half maximum to be determined, although this also instrumentation dependent. The following should be considered in assessing the fit data, along with any prior knowledge of the system studied.

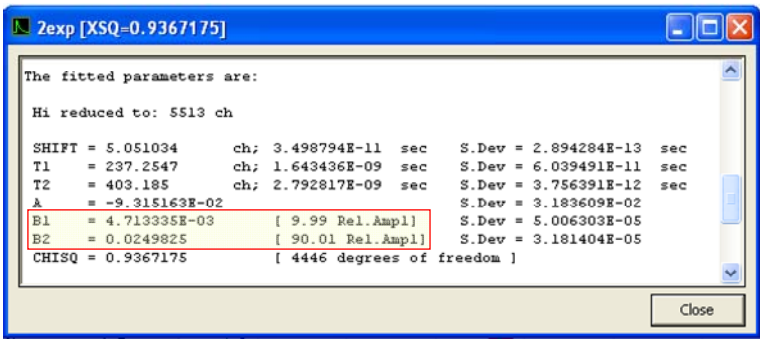
- Chi-squared value below 1.2, not meaningfully improved by adding an extra decay component.
- Randomly distributed weighted residuals.
- Do the lifetime(s) correspond to previous reports (measurements or literature)?
- Lifetime values of 10^{-11} seconds (0.01 ns) or equivalent to one data channel should be treated with caution and may relate to scattered excitation light reaching the detector.
- Large (e.g. more than ~3 channels) or negative shifts may indicate a problem.
- Excessive errors (s. dev) on the data. Note errors best taken as 3 std. dev.
- Negative pre-exponential components (B values) are not necessarily a sign of a bad fit; they can indicate the presence of an excited state process and are referred to as “rise times”.

The following figure demonstrates the effect on chi-squared value and weighted residuals of adding extra exponential components to a fit. The decay is in fact the sum of three exponentials.



Amount of fluorescing components (pre-exponentials and relative amplitudes)

When interpreting the recovered decay parameters, if the fit is multiexponential as shown below, it is useful to know how much of each fluorescing species (lifetime) there is. There are two ways which we shall concentrate on.



Relative Amplitude

This value is readily availble in the DAS6 analysis (see Rel. Ampl. values in box above). These are calculated from the pre-exponential values ("B" values in the box) weighted by the lifetime and given as a percentage. This is therefore more or less equivalent to a steady state value and can be compared to the steady state spectrum, where upon perturbing the system changes can be followed and related. It is sometimes also referred to as a fractional.

Normalised pre-exponential values

These are sometimes denoted α (see equation below) and are useful if the actual concentration of each fluorescing component is required.They are easily deduced from the pre-exponential "B" values or provided in the analysis. For the above data (B_1 ~ 0.00471, B_2 ~ 0.025)
For a decay of form

$$I(t) = \sum_i^n \alpha_i \exp(-t/\tau_i) \qquad \alpha_1 = B_1 / (B_1 + B_2) = 0.16$$

$$\alpha_2 = B_2 / (B_1 + B_2) = 0.84$$

Average lifetime

Sometimes these are useful in following trends in data and to realted to the overall (steady state) emission. There are two ways in which the average lifetime should be calculated and these are compared in a publication[12]. These values should be calculated from the multiexponetial analysis of the data, rather than just attempting a single exponential fit. In most cases it recommned to use the following equation,

$$\tau_{ave} = \sum_{i=1}^{n} \alpha_i \tau_i$$

In the case of Stern-Volmer type quenching then the follwing average lifetime is advised (see ref. 12 for details)

$$\tau_{ave} = \sum_{i=1}^{n} \alpha_i \tau_i^2 \Big/ \sum_{i=1}^{n} \alpha_i \tau_i$$

[12] A. Sillen and Y. Engelborghs. Photochem. 67, 475-486 (1998).

Systems

HORIBA Scientific produces three principal types of dedicated time-resolved TCSPC systems;

TemPro Fluorescence Lifetime System

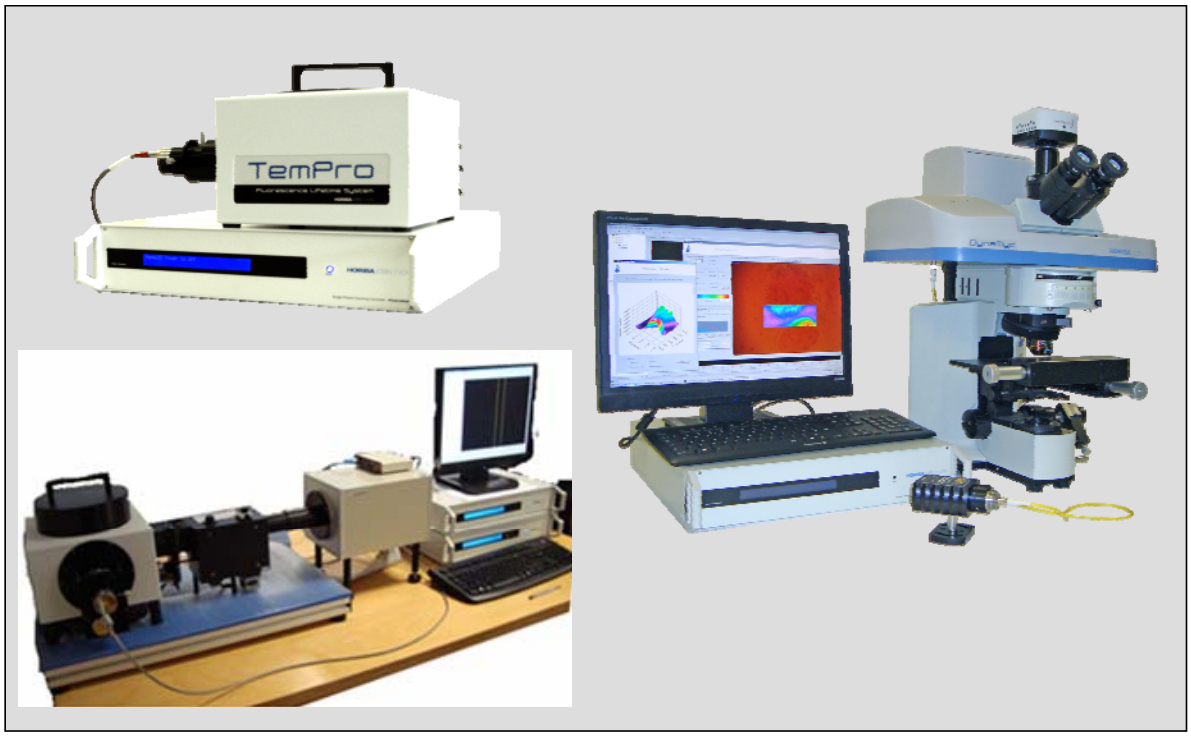
Compact TCSPC filter based system capable of measuring lifetimes from 100ps to s. The **FluoroHub-B** is at the heart of this system and it makes use of **NanoLED** and **SpectraLED** excitation sources, with a **TBX** detector.

FluoroCube

Time-resolved spectrofluorometer for determination of short fluorescence lifetimes from ps to s, with timing made using a **FluoroHub-A**. Can be used with the complete range of excitation sources (option of input for Ti : sapphire laser) and has the option of **MCP** detection.

DynaMyc

Microscope system with confocal and fluorescence lifetime mapping ability, making use of **PicoBrite** excitation sources.

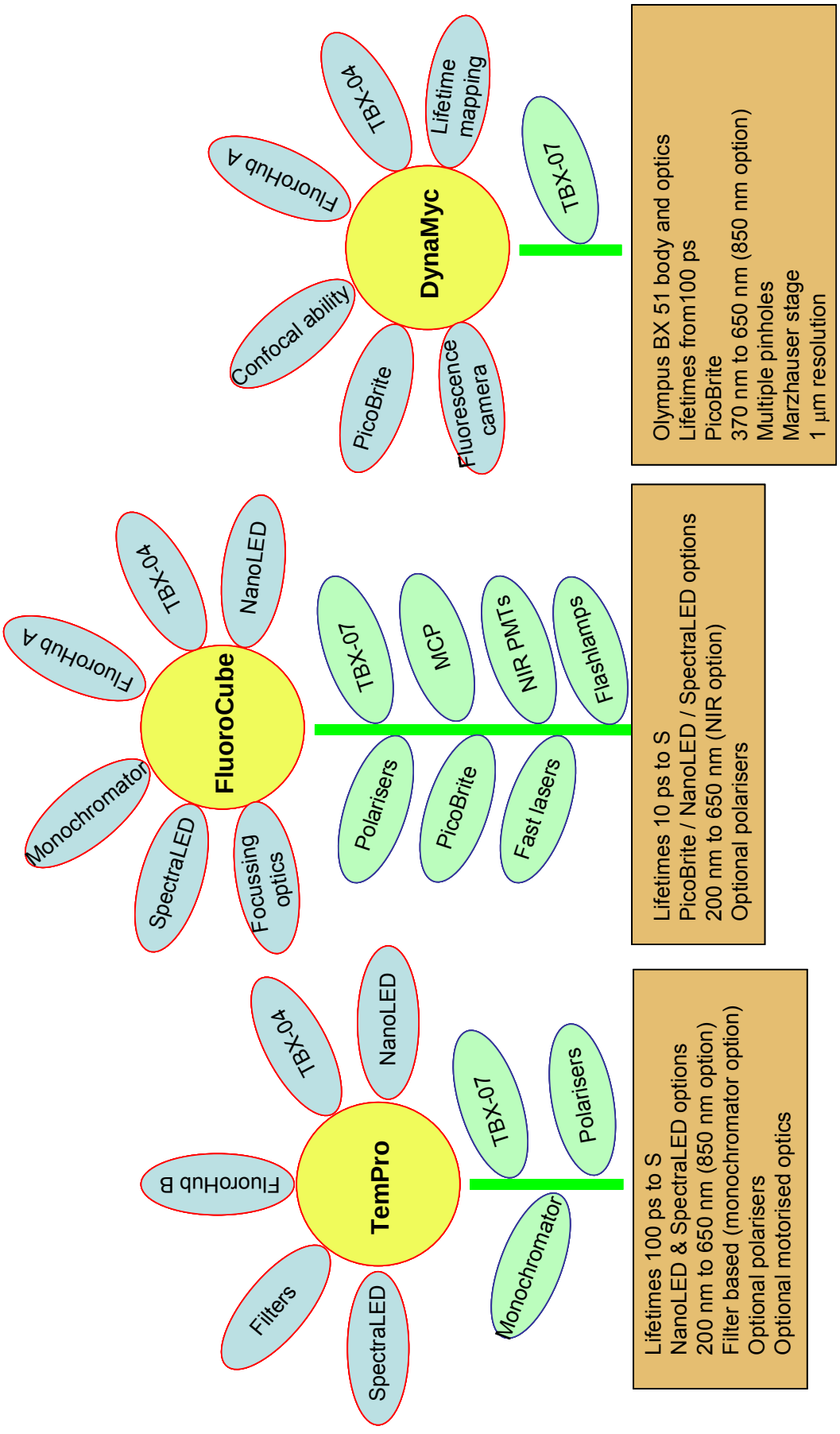


The above picture shows the **TemPro** (top left), a **FluoroCube** Ultra fast with MCP detector (bottom left) and a **DynaMyc** with **PicoBrite** excitation source coupled via an optical fibre (right).

A large range of accessories and upgrades exist for these systems, which allow them to be employed to measure a great range of lifetimes over large spectral range.

Additionally TCSPC upgrades are available for the **FluoroLog** and **FluoroMax** ranges of steady state spectrophotometers.

Overview – system components



Component specifications

This section is intended to highlight and summarise some of the specifications / capabilities of selected system components and accessories. However, it should be noted that an overall system performance is not simply obtained from that of the components as their interplay can be complex and connecting parts need to be considered.

Note that the values given below are open to change and dependent on the use of the component with the appropriate light source / detector / timing electronics. For an idea (for use as a rough guide only) a table is provided to give an indication of the shortest lifetimes possible with different combinations of components in a TCSPC system.

Light sources

PicoBrite

– LED for measurement of lifetimes 10% of nominal pulse duration. Repetition rate up to 80 MHz.

– Laser diode for lifetimes 10% of nominal pulse duration. Repetition rate up to 80 MHz.

NanoLED

– LED for measurement of lifetimes 10% of nominal pulse duration. Repetition rate up to 1 MHz.

– Laser diode for lifetimes 10% of nominal pulse duration. Repetition rate up to 1 MHz.

FluoroSource CF

– Gas filled coaxial flashlamp. Wavelength range, FWHM and repetition rate filler gas dependent

SpectraLED

– LED source for measurement of lifetimes 0.5 μs to seconds.

Phos Lamp

– Xe filled source for measurement of lifetimes 10 μs to 1 s. Below 50 μs requires the use of reconvolution analysis.

Custom sources

– In order to calculate the lifetime range, both the repetition rate and pulse width (obtained with measurement system) need to be known. The former to avoid re-excitation of the sample before it has decayed (to give longest measureable lifetime) and the latter to determine the shorter lifetime (one tenth of the FWHM with reconvolution).

– It is recommended that Ti : sapphire and supercontinuum lasers are used in conjunction with a **FluoroCube** with time expander and fast detector to give optimum time resolution. To use the Ti : Sapphire laser a pulse picker or cavity dumper working in pulse picking mode is required to reduce the repetition rate.

Note – the maximum usable repetition rate will depend on the sample lifetime and other factors.

Detectors

TBX Picosecond detection module

– TBX-04. Sensitive from 185 nm to 650 nm

– TBX-07. Uncooled detector, sensitive from 250 nm to 850 nm

– TBX-07C. Cooled detector, sensitive from 250 nm to 850 nm

Side on PMT

– R928P. Sensitive from 200 nm to 850 nm

MCP detectors

– R3809-50. Sensitive from 160 nm to 850 nm

NIR PMTs

– H10330-45. Sensitive from 950 nm to 1400 nm

– H10330-75. Sensitive from 950 nm to 1700 nm

– R5509-43. Sensitive from 300 nm to 1400 nm

– R5509-73. Sensitive from 300 nm to 1700 nm

NOTE – these are not trivial measurements because of the high noise levels of the detectors and shorter decays are best performed using a **FluoroHub-A** and longer lifetime measurements with a **FluoroHub-B**. Fast MCS options should also be considered, which also affects the measureable lifetime range.

Detector – source combinations for TCSPC (FluoroHub-A)

Detector		excitation source			
		flashlamp	LED	LD	fs laser
R928P	side on PMT	**100 ps**	**100 ps**	**100 ps**	**100 ps**
TBX-04	ps detection module	**100 ps**	**100 ps**	**50 ps**	**20 ps**
R3809-50	MCP	**100 ps**	**100 ps**	**10 ps**	**5 ps**
R5509	NIR PMT	**300 ps**	**300 ps**	**300 ps**	**-**
H100330	NIR detector	**200 ps**	**200 ps**	**200 ps**	**-**

IMPORTANT NOTES

FluoroHub-B the minimum lifetime is 100 ps, ie electronics limited.

NIR PMTs / detectors there is a limit to maximum lifetime using TCSPC see NIR section for details and shortest lifetime when using MCS mode