

## Time-resolved fluorescence lifetime measurements

*The radiative emission of light from a molecule after excitation has a multiparameter nature. The objective of a measurement is therefore to gain information concerning as many parameters as possible. A steady state measurement of the fluorescence emission (intensity vs wavelength) gives an average and also relative representation. The fluorescence lifetime gives an absolute (independent of concentration) measure and allows a dynamic picture of the fluorescence to be obtained, factors that explain the appeal of this form of measurement.*

### The fluorescence decay

The decay of the excited state of a molecule to the ground state can be expressed as;

$$I(t) = I_0 \exp(-t / \tau)$$

where,  $I_0$  is the intensity at time zero (upon excitation) and  $\tau$  is the lifetime. This is defined as the time for the intensity to drop by 1/e or to ~37%. In terms of rate constants ( $k_r$  – radiative rate,  $k_{nr}$  – non radiative rate) the lifetime can be written as below, which can be compared to the fluorescence quantum yield ( $\phi$ )

$$\tau = \frac{1}{k_r + k_{nr}}, \quad \phi = \frac{k_r}{k_r + k_{nr}}$$

The fluorescence (FL) signal is multiparametric and can be considered as follows, along with the measurements that can elucidate them,

$$FL = f(I, \lambda_{exc}, \lambda_{em}, p, x, t)$$

where;

$I$  = intensity - measurement is quantum yield ( $\phi$ ),

$\lambda_{exc}$  = excitation wavelength

–measurement of absorption spectrum,

$\lambda_{em}$  = emission wavelength

–measurement of fluorescence spectrum,

$p$  = polarisation –measurement of anisotropy,

$x$  = position –measurement by fluorescence microscopy,

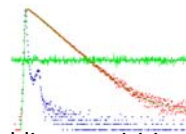
$t$  = time –measurement of fluorescence lifetime.

Although the decay law is based on first order kinetics, in practice, many fluorescence decays are more complex. Often populations of excited molecules are in an inhomogeneous environment and quenching processes and other environmental influences can lead to multi- or non exponential decay behaviour.

One of the major advantages of using the fluorescence lifetime is the fact that it is an absolute measurement, unlike the steady state intensity, which is relative. The fluorescence lifetime is an intrinsic molecular property and, within certain constraints, independent of concentration. This means that changes in concentration, whether caused by photobleaching or diluting/ concentrating the sample, would not affect the lifetime value. This is in contrast to a steady state measurement, where a change in intensity of the recorded emission would be observed. Furthermore using time-correlated single-photon counting (TCSPC), the lifetime is not influenced by fluctuations in excitation source intensity. The implication is that a measurement taken on one piece of equipment at a certain time and place should be capable of being repeated later elsewhere (provided the sample is stable and measurement conditions are preserved).

Fluorescence is an ideal nanoscale probe, as the fluorescence decay take places on the nanosecond timescale and it can be influenced by molecular processes occurring on the nanometre range. The emission of a fluorophore can be highly influenced by its environment or the presence of other interacting molecules, which can affect  $k_{nr}$ . Thus the fluorescence lifetime is useful in elucidating;

- changes in the nanoenvironment
  - viscosity, pH, polarity, solvation
- size and shape of molecules
- molecular interactions



- inter- and intramolecular distances
- kinetic and dynamic rates
- resolution of molecular mixtures

The extra specificity of the fluorescence lifetime allows easy discrimination against scattered excitation and background fluorescence. Determination of Förster resonance energy transfer (FRET) is much simpler using the fluorescence lifetime, as are quenching and fluorescence anisotropy measurements, allowing more parameters to be recovered.

However, it should be remembered that the use of steady state and time-resolved techniques are complementary in elucidating as much of the multiparametric fluorescence signature as possible.

### Time-correlated single-photon counting (TCSPC)

This is considered the most sensitive method for determining fluorescence lifetimes. It is a digital technique, with well defined (Poisson) statistics, which is not affected by changes in source intensity, unlike some analogue measurement techniques. TCSPC is based on the detection of the arrival times of individual photons after optical excitation of a sample. It makes use of a pulsed excitation source (typically laser or LED), as shown schematically in Fig. 1. Production of a light pulse “starts” the timing electronics. These are “stopped” by a signal from the detector, triggered when a fluorescence photon hits the detector. The difference in time between these two signals is then outputted to a histogram, consisting of time bins, with fixed time width ( $\Delta t$ ). So signals arriving within  $t + \Delta t$  go into one specific bin. The width of the bins will also influence the time resolution of the system.

The excitation - emission process is repeated many times (usually to 10,000 counts in the peak channel). This builds up a histogram of number of events (counts) versus time. However, for the histogram to be representative of the decay (i.e. the number of counts at a certain time after excitation to be equivalent to the intensity at that time) a certain condition needs to be observed. This is that only one photon can be counted at any one time, so it is required statistically to count only one photon and for it not to arrive during the dead time of the timing electronics. In practical terms the stop count rate needs to be limited to ca. 2% of the excitation rate. Failure to do so can lead to a biasing towards detection of photons arriving at shorter times, a phenomenon known as pulse pile up. This apparent inefficiency of the TCSPC technique is usually not considered a problem as the

excitation rate can be in the MHz range, enabling acquisitions under a second to be made. Historically using lower repetition rate sources led to longer data acquisition times.

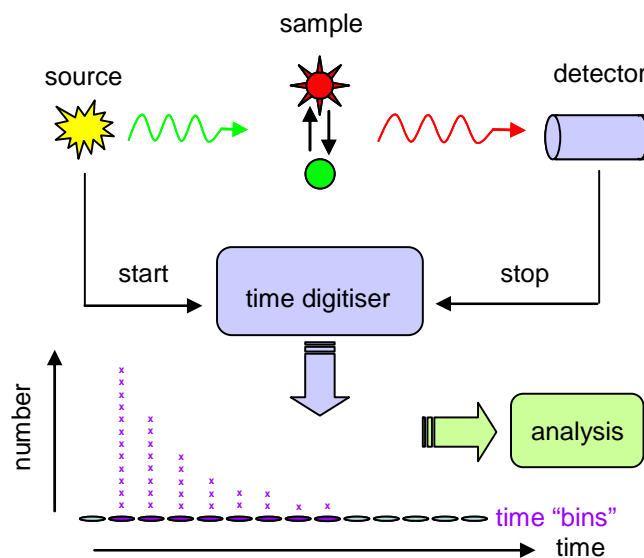
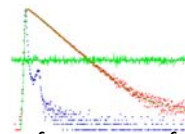


Fig. 1. Schematic for TCSPC

When measuring longer-lived decays, re-excitation of the sample before it has fully decayed should be avoided, thus the excitation rate should be set to less than  $1/(\text{time range})$ . Time ranges are normally selected to be 10 to 20 times that of the lifetime to be measured. This restriction on the maximum excitation rate and hence data collection rate, means that the measurement of longer-lived decays can be quite time consuming on time to amplitude converter (TAC) based systems. Normally a different approach is used when measuring decays on the phosphorescence ( $\geq \mu\text{s}$ ) timescale to overcome this restriction and reduce the data collection time.

### Typical components and TCSPC system response

The scheme shown in Fig. 1 is obviously very general. The pulsed source is usually a solid state LED or laser, although pulsed supercontinuum or femtosecond lasers can also be used. Since fluorescence occurs at longer wavelengths some form of wavelength discrimination, such as filters or monochromator is employed to select the desired fluorescence emission. The type of detector used is highly dependent on the required wavelength and time resolution. It often requires an additional amplifier, along with a discriminator. Sometimes all these components are available in one module. The timing device should have a low dead time to efficiently record all photon events, low timing jitter, a small time channel width and a reasonable number of channels in the histogram. Typically the number of histogram channels is  $\sim 4\text{k}$ . The timing electronics may be a card in a computer or a stand alone device. The latter is advantageous



as it avoids the “electronically noisy” environment of the computer and obsolescence of computer hardware.

Ideally the light exciting the sample should be a delta function (intensity with no temporal width) and the detector should instantaneously transform an incident photon into an electrical output. In reality each component has a response time. This means that the excitation source response, usually indicated by its temporal full width at half maximum intensity (FWHM), is a factor along with timing electronics jitter. Another advantage of TCSPC is that only the transit time spread (TTS) of the detector, rather than the overall response time, needs to be considered. Note that, the TTS is often much shorter than the detector response time. An estimate of the system response broadening caused by these factors is given by the following formula,

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

Where,  $\Delta t_m$  is the measured response, the subscripts *exc*, *det* and *elect* refer to the excitation source, detector and timing electronics respectively. Other factors (subscript *i*) can also be present, but generally uncertainty in the measured response is dominated by that of the source and detector.

Since the system response is not a delta function response, the measured decay can also be distorted by these instrumental factors and it may be thought that it would not be possible to recover lifetime values less than the measured response. However by making use of reconvolution analysis it is possible to overcome some of these problems. This requires an additional measurement of the instrumental response (commonly called the IRF or prompt), usually using a scattering solution and measured at the excitation wavelength. The reconvolution analysis takes into account the distortion caused by the instrumental response by assuming a series of delta functions and allows lifetimes down to approximately 1/10 of the instrumental FWHM to be recovered. Note that this is a generalisation and other sample and equipment factors need to be considered.

### Measuring longer-lived decays

The TCSPC technique may not be the most efficient one for measuring longer-lived decays (phosphorescence timescale) as the excitation source repetition rate may need to be lowered to avoid re-excitation of the sample before it has fully decayed. The disadvantage is the 2% restriction on the data collection rate and the reduced average excitation

power on the sample. Typically the minimum frequency of a TCSPC source is 10kHz (limits  $\tau$  to  $\sim 1\mu s$ ). To overcome this aspect, multichannel scaling (MCS) or similar can be used. This can require additional electronics to allow a complete measurement range. However, single timing modules are available that seamlessly change from one time range to another, allowing lifetimes from  $\sim 20ps$  to 1s to be measured.

### Measurement techniques

In this section, some of the more common forms of fluorescence measurements are briefly summarised with their defining equations and advantages. Further details on these can be found in separate Technical notes.

- Fluorescence anisotropy**

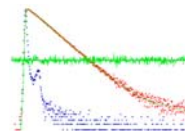
This basically is a measure of how ordered a molecular system is and follows its disorder via molecular (rotational) diffusion with time. It uses polarised light to excite a sample and the parallel and perpendicular polarisations are monitored on the emission. Information that can be obtained is given in the following formula,

$$r(t) = r_\infty + (r_0 - r_\infty) \exp(-t / \tau_r)$$

$$\tau_r = \frac{V\eta}{kT}$$

where  $r$  is the anisotropy,  $r_\infty$  is the anisotropy at infinite time,  $r_0$  is the initial anisotropy.  $\tau_r$  is the rotational correlation time, which can be determined and related to the effective volume of a molecule ( $V$ ) and the local viscosity ( $\eta$ ). Also  $T$  is absolute temperature and  $k$  is Boltzman’s constant. A time-resolved measurement permits the determination of  $r_0$ ,  $r_\infty$ ,  $\tau_r$  and  $\tau$ . This compares to the single value ( $\bar{r}$ ) obtained for steady state anisotropy.

It should also be noted in standard lifetime measurements, that when exciting a sample with a fast polarised laser pulse, polarisation effects can be present that can make the decay appear more complex. This relates to depolarisation effects. It is advisable to use a vertically orientated polarizer on the excitation and the emission polarizer at the magic angle ( $54.7^\circ$  to the vertical) to remove these depolarisation effects.



- **Förster resonance energy transfer**

This coulombic dipole-dipole interaction requires spectral overlap between the donor emission and an acceptor's absorption. It is manifest over the 10 to 100 Å range, which makes it well suited to follow molecular interactions and determine distances on the nanometre scale. There is a  $R^6$  distance dependence on the rate of energy transfer which effects the lifetime, allowing distance ( $R$ ) and efficiency ( $E$ ) of energy transfer to be determined. If only the lifetime of the unquenched donor ( $\tau_d$ ) is available this can be relative, or made absolute if the distance at which FRET is 50% efficient ( $R_0$  or Förster distance) is known for the system under study.

Rate of energy transfer  $k_{et} = \frac{1}{\tau_d} \left[ \frac{R_0}{R} \right]^6$

Efficiency  $E = 1 - \frac{\tau_d}{\tau_{da}}$

Distance  $R = R_0 \left[ \frac{1}{E - 1} \right]^{\frac{1}{6}}$

Where  $\tau_{da}$  is the donor lifetime in the presence of the acceptor.

- **Time-resolved emission spectra / decay associated spectra**

This technique is very useful if there are several emitting species present with overlapping emission spectra. Fluorescence lifetimes are measured at wavelength increments for the same data collection time. The resultant intensity-wavelength-time surface can then simply be "time-sliced" in the intensity-time axis to provide spectra at different times after excitation (Time-Resolved Emission Spectra). Alternatively the dataset can be analysed globally, linking common lifetimes. The pre-exponential factors then relate to the amount of each species present at a certain wavelength. Weighting these by their associated lifetime and plotting versus wavelength can provide spectra associated to that lifetime (Decay Associated Spectra). Summation of the individual spectra plotted in this manner should return the equivalent of the steady state spectrum.

**Further reading**

An increasing number of texts exist relating to fluorescence and TCSPC and notable ones are from O'Connor and Philips, Valeur and a series edited by Lakowicz.

