Time-resolved (Time correlated single photon counting-TCSPC) fluorescence:

Measurement of fluorescence life time:

As we already know, the life time of a fluorophore is an average value of time spent by the molecules in the excited state prior to return to the ground state.

Let consider, you have a large collection of molecules and excite them by an instantaneous flash (time taken to excite the molecules is infinitely small) so that all the molecules are now in the excited state. Next, these excited molecules will come back to the ground state. It is unlikely that all the molecules will come back to the ground state all of a sudden. As fluorescence emission is a random process, some molecules will come back to the ground state at early time; some molecule will come back later and so on. Initially (at time zero), fluorescence intensity will be maximum as all the molecules are in the excited state. With time passes, the fluorescence intensity will decay as described in Fig.1.

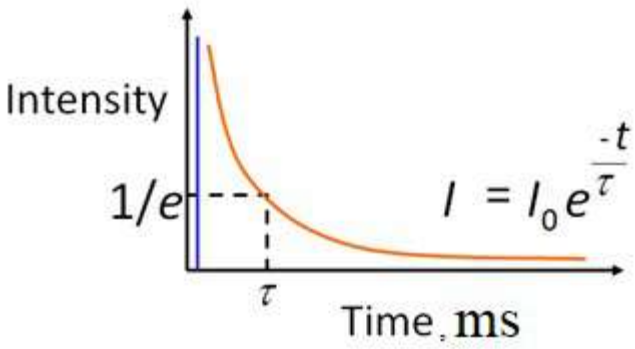

Fig 1 Fluorescence intensity vs. time plot.

For a single exponential decay 63% of the molecules have decayed prior to $t = \tau$ and 37% decay at $t > \tau$. An example of two similar molecules with different lifetimes and quantum yields is shown in Figure 2. The differences in lifetime and quantum yield for eosin and erythrosine B are due to differences in non-radiative decay rates. Eosin and erythrosin B have essentially the same

extinction coefficient and the same radiative decay rate. Heavy atoms such as iodine typically result in shorter lifetimes and lower quantum yields.

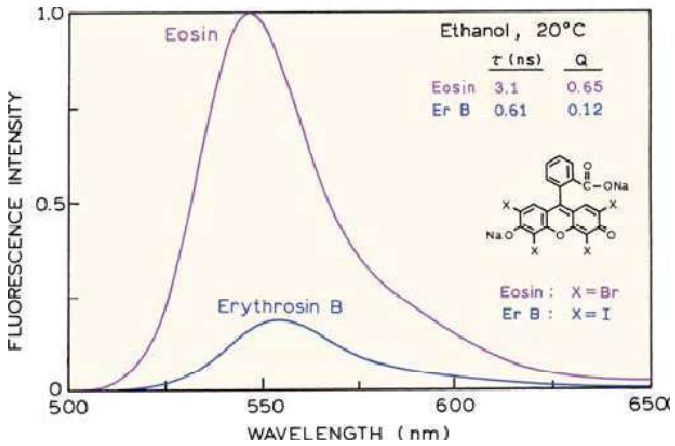

Figure 2. Emission spectra of eosin and erythrosin B (ErB).

Now, how we can measure the value of τ, or construct intensity vs. time plot (as shown in Fig.1) for a fluorophore?

If we can construct intensity (photon count) vs. time plot, we can easily calculate the value of τ, just by simply seeing the time when the fluorescence intensity decreased ~ 67% of its initial value.

If the time scale in figure 1 is in the order of hour, we can easily construct the plot by simply measuring the fluorescence intensity at different time (like after 2 second, 20 second and so on). But in reality the life time of maximum fluorophres are in the order of nano second. So you need to practically measure the fluorescence at picosecond (ps) range (after 10ps, 100ps, 200ps …..) as shown in figure **1. A simple stopwatch which can measure time in the ps (10^{-12} s) is not available easily. So we need special instrumental arrangements or technique to measure the fluorescence in such short time range.**

The basic principle of life time measurement is as follow. First, you have to excite the molecules using an instantaneous pulse and all the molecules will go to the excited state (which is not always true, but for this case we assume 100% excitation). Now the molecules will come down to the excited state through a random process and the emission will decay exponentially. You will get an exponential curve in intensity vs. time plot (as in fig.1). The curve may be single exponential or multi -exponential (distorted curve). From that curve we will get the value of τ.

Life time can be measured in two different ways (i) time domain method (ii) frequency domain method.

Time domain lifetime measurement: In time domain method, molecules are excited with instantaneous pulse (Fig. 3). The time of instantaneous pulse will be very short compared to the time of decay and this pulse is called excitation pulse which will excite all molecules at once. Then the decay of emission intensity with time is monitored to get the plot described in figure 3. From the plot one can get the value of life time.

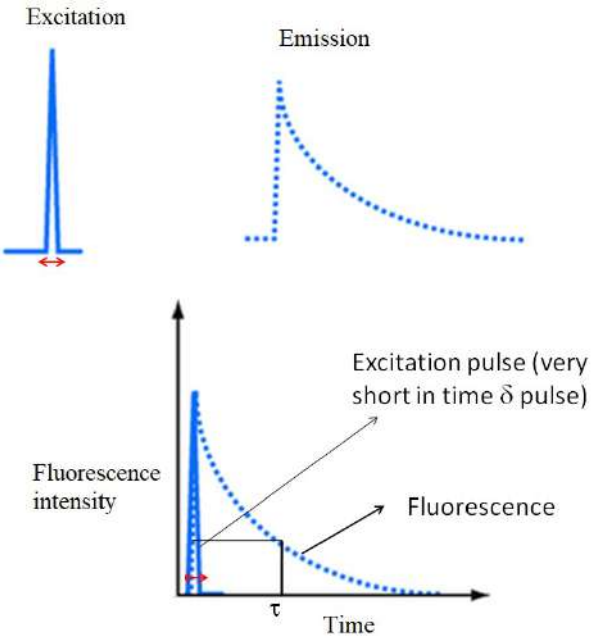

Fig. 3 describing the basic principle of time domain measurement.

In time domain method there are three different way of measuring the life time which are

(i) Time correlated single photon counting (TCSPC) (Time resolution ~ 50 -1500 ps)

(ii) Streak camera (time resolution ~ 2-20 ps)

(iii) Fluorescence up-conversion (time resolution ~ 0.2-0.4 ps, time window is small)

Fluorescence up-conversion method is more superior in terms time resolution. But in case of TCSPC, the time window is very large.

Time correlated single photon counting:

At present most of the time-domain measurements are performed using time-correlated single-photon counting, but other methods can be used when rapid measurements are needed. These instruments use **high repetition rate mode-locked picosecond (ps) or femtosecond (fs) laser light sources,** and **high-speed microchannel plate (MCP) photomultiplier tubes (PMTs).** For many applications, these expensive systems are being rapidly replaced by systems using pulsed-laser diodes (LDs), light-emitting diodes (LEDs), and small, fast PMTs.

Laser has unique property that it can create ultra short light pulse. So, for TCSPC, laser is used as light source to excite the molecules.

For a light bulb if you turn on and off very quickly, you can generate the shortest light pulse of 10^{-1} sec, i.e.; light will be on at least 10^{-1} second. But we need a light pulse of 10^{-12} sec. **We need special kind of light source which can generate such short light pulse and that can be done by laser.**

Let consider a fluorophore has life time of 10 ns. From the equation

$I = I_0 e^{-t/\tau}$,

$\tau = 10$ ns,

When $I = 0.01I_0$, we can assume that the fluorescence intensity is almost close to zero.

$\ln (0.01I_0 / I_0) = -t/\tau$

or $\ln (0.01) = -t/\tau$,

or $-4.6 = -t/\tau$,

$t = 46$ ns

From the above calculation it is clear that when life time of a fluorophore is 10 second, the total time window require to observe the complete decay will be 46 ns (Figure 4b). Now to get a complete exponential plot, you need several points (as shown as black dot in fig. 4b) within 46 ns time window. So you need to measure the fluorescence at different time as possible in nanosecond time domain. It cannot be measured by standard fluorometer. For fluorescence measurement in every nanosecond or picoseconds time, we need a special instrumental set up that allows measurement in pico second or nanosecond time domain.

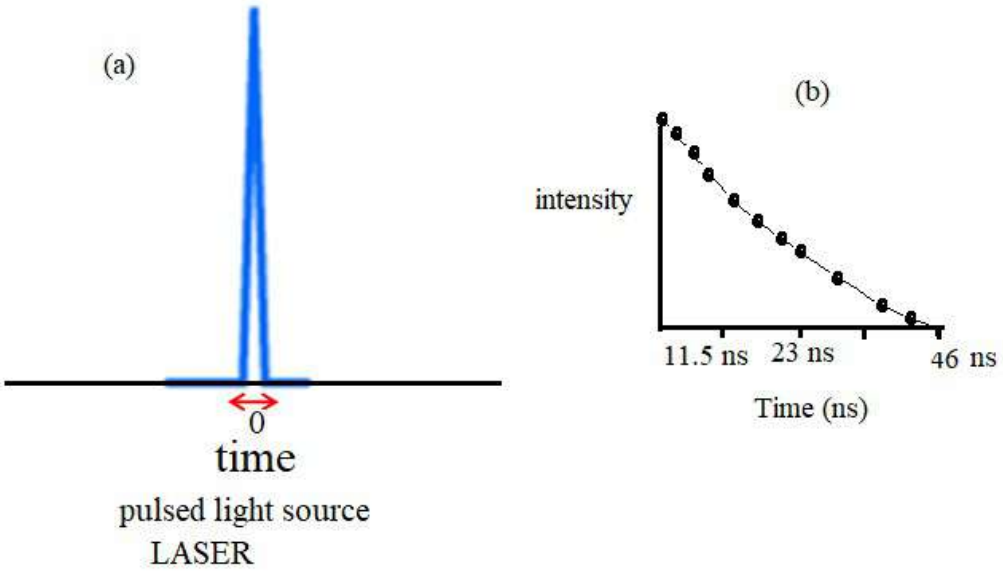

Fig. 4 Schematic of TCSPC measurement

In TCSPC set up laser is used to generate ultra short light pulse. The duration of the light pulse must be much shorter than the time window of the fluorescence decay. For example if your fluorescence decay window is of 46 ns, and duration of the light pulse is 80ns, you will not be able to measure the life time of that fluorophore using that light pulse. If fluorophore life time is 10 ns, you should use light pulse of 1ns or less.

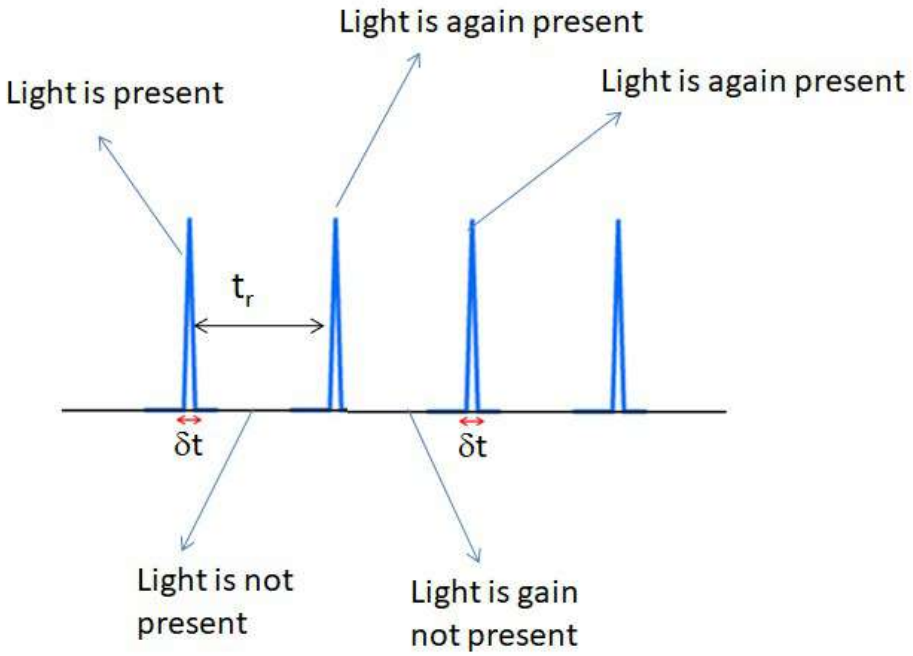

Fig. 5 Schematic of ultrafast light pulse using laser.

Pulse light mean light will successively on and off for some time (Fig. 5). In this way, a series of light pulses are generated and used for TCSPC. These series of light pulses are characterized by repetition time (t_r) (time between two successive pulse) and pulse width (δt) (Fig.5) and wavelength. The excitation pulse will then excite the sample in the sample chamber and fluorescence will be generated. This fluorescence light is not light of single wavelength. So it is

to passed through a monochromator to select particular λ_{em} what you want to study. After the monochromator, it will come to detector where its intensity is measured.

Basic principle of TCSPC: The detailed schematic diagram describing basic instrumentation of TCSPC is shown in Figure 6. In TCSPC, the experiments start with the excitation pulse that excites the samples and at the same time a signal is sent to a device called TAC (time-to-amplitude converter). The signal is passed through a constant function discriminator (CFD), which accurately measures the arrival time of the pulse. The TAC can be considered as one kind of stopwatch; once signal is sent to TAC it will start count the time. The special characteristic of this stop watch (TAC) is that it can measure the time in the order of pico second. Once laser send signal to TAC means sample is excited and TAC will start counting the time immediately. As sample is already excited which means several molecules are in the excited state. The number of molecules in excited state obviously will depend on parameters like concentration of molecules and number of photon in each pulse. Now, all the molecules will not come down to the ground state at once, some will go to the ground state early, some will go late and some at the time of fluorescence life time and so on. The emission from the sample will be detected at the PMT (photomultiplier tube). **But in case of TCSPC, the condition is that when a single excited photon from the sample will come to the PMT, the PMT will convert the photon to the electron and an electron pulse/current pulse will be generated.** This current pulse will reach to TAC and it will stop the stop watch and stop watch (TAC) will count the time. The stop watch will count the time difference between start and the stop. The time difference or time delay is the difference between excitation and emission and the time required to complete the path and to reach TAC. For each light pulse, one time is measured (which is difference between stop) and against this time we will get one count of photon emission.

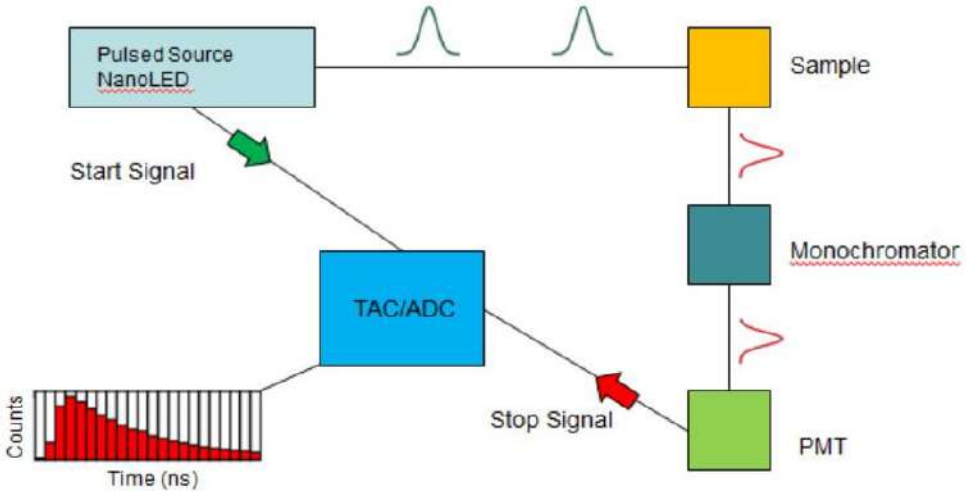

Figure 6 schematic diagram describing basic instrumentation of TCSPC.

Now imagine that you have excited a large number of molecules, some of them will emit early time and some of them emit later. **Here one need to understand that, the first photon what is detected in PMT is not necessarily the first emitted photon from the sample.** In the decay profile (Figure 7a) let assume the marked point corresponds to the emission of the first photon as the point arise at very early time (the first photon mean photon emitted from the excited molecule which first come down to the ground state). Now as we know that fluorescence

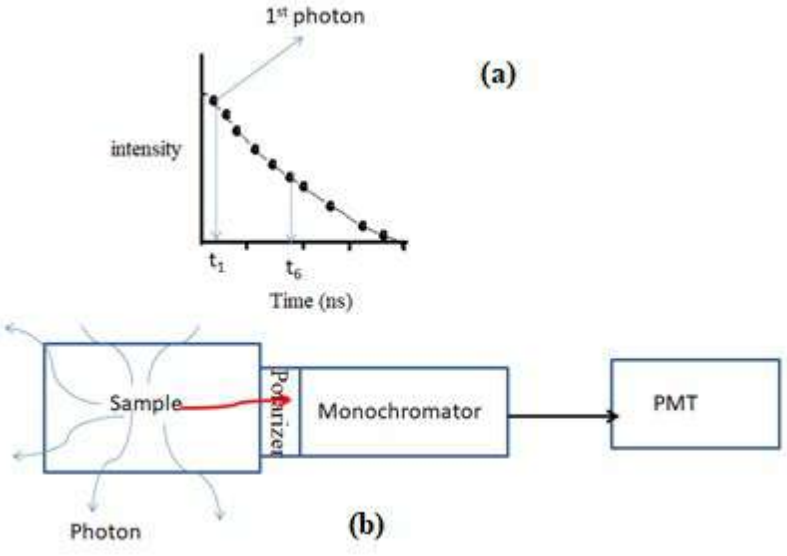

Fig. 7 Schematic of TCSPC

is a spontaneous emission and the emission is directionless (Figure 7b). It is not necessary that the first emitted photon will be in the direction of PMT (red arrow). If it is directed to PMT it will be counted by the photo detector. But as emitted photons are directionless, it is highly probable that 1st emitted photon from the sample will be in the other direction and not will be detected by PMT. **In TCSPC, once a photon is detected, the stop watch will stop and experiment will be finished.** Next it will wait for the second excitation pulse to come and excite the sample. **So for one turn, one light pulse arrives, excite the sample and one photon is counted.** The photon which comes first to the detector for this light pulse may be at time t_1 or t_2 or any arbitrary time t_6 within the time window. Let consider, for this case the first photon comes to the detector at time t_6 (in the figure 7a). But, the excited molecules are large in number, some may emit photon at time t_1, some may be at time t_2 or some may at different time. But for this excitation pulse, we will only consider the single emitted photon at time t_6 as the first photon comes for this excitation pulse comes to PMT at time t_6. Next, we will wait until all the molecules to come in the ground state and the case will be like before excitation. Then another excitation pulse will be used and it will excite all the molecules from ground to the excited state. But in this case may be a photon at different time (t_2) will be in the direction of PMT and will be detected in PMT.

For the first cycle (pulse), we count a photon at time t_6

For the 2nd cycle (pulse), we count a photon at time t_2

For the first cycle (pulse), let say, we count a photon at time t_{17}

…….. and so on.

Now if the cycles are repeated for millions of time, so you will get a large number of time associated with large number of photon count. We will finally get a histogram type of plot that is

shown in the figure 8c. As shown in the for the first cycle (Fig. 8b) no photon is detected, for 2nd cycle one photon is detected at early time,

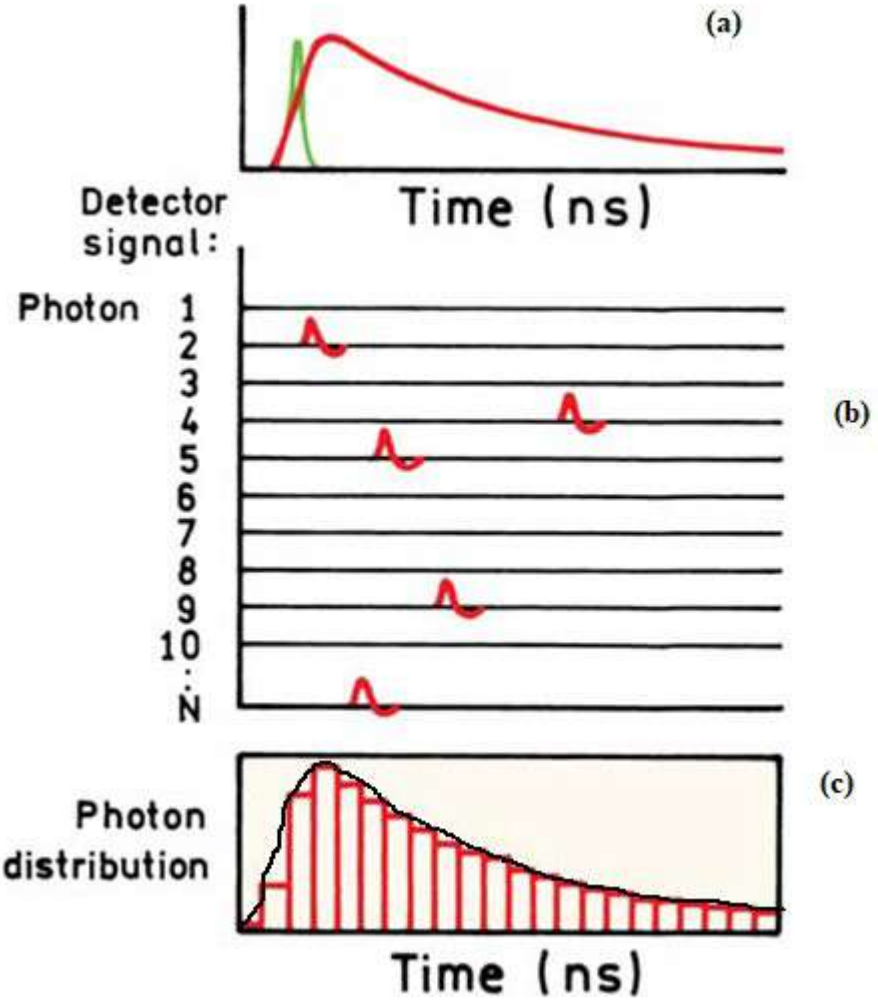

Figure 8 Principle of TCSPC. The pulses in the middle panel represent the output from a constant fraction discriminator

for 3rd cycle no photon detected and so on. So if we sum up all these photons at different time for millions of cycle, we will get a histogram time plot. The x-axis is the time difference and the y-

axis the number of photons detected for this time difference. And if we fit the histogram (black line) and by using the equation $I = I_0 e^{-t/\tau}$, we can get the value of τ, the fluorescence life time.

Basic electronics: In case of TCSPC only single photon (the first photon which will come to PMT) can be detected for each pulse. This is because the electronics are not fast enough to measure multiple photons per pulse when the lifetimes are in the nanosecond range. Multiple photons per pulse can be measured for decay times near a microsecond or longer. Specialized electronics are used for measuring the time delay between the excitation and emission (Figure 9). The experiment starts with the excitation pulse that excites the samples and sends a signal to the electronics. This signal is passed through a constant function discriminator (CFD), which accurately measures the arrival time of the pulse. This signal is passed to a time-to-amplitude converter (TAC), which generates a voltage ramp that is a voltage that increases linearly with time on the nanosecond

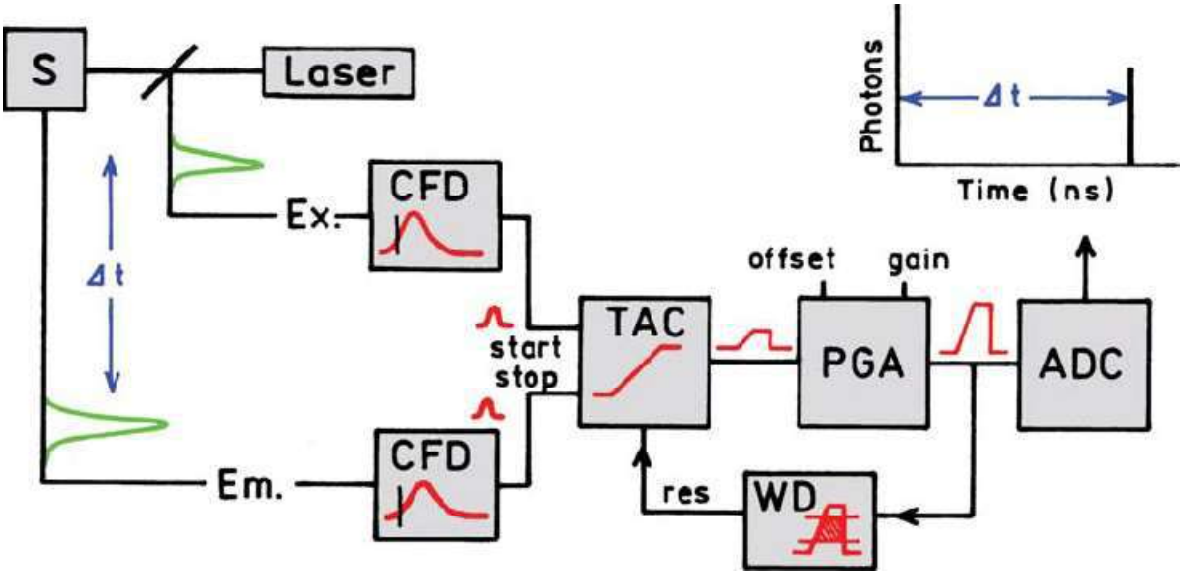

Figure 9 Electronic schematic for TCSPC.

timescale. A second channel detects the pulse from the single detected photon. The arrival time of the signal is accurately determined using a CFD, which sends a signal to stop the voltage ramp. The TAC now contains a voltage proportional to the time delay (Δt) between the excitation and emission signals. As needed the voltage is amplified by a programmable gain amplifier (PGA) and converted to a numerical value by the analog- to-digital converter (ADC). To minimize false readings the signal is restricted to given range of voltages. If the signal is not within this range the event is suppressed by a window discriminator (WD). The voltage is converted to a digital value that is stored as a single event with the measured time delay. A histogram of the decay is measured by repeating this process numerous times with a pulsed-light source.

Example of TCSPC Data:

Intensity decay for 2,5-diphenyl-1,3,4-oxadiazole (PPD) is shown in Figure 10. These data were taken using a cavity-dumped R6G dye laser that was cavity dumped at 1 MHz and frequency-doubled to 300 nm. The detector was an MCP PMT. The intensity decay plot is associated with three curves, measured data $N(t_k)$, the instrument response function $L(t_k)$, and the calculated decay $N_c(t_k)$. These functions are plotted against discrete times (t_k) because the counted photons are collected into channels each with a known time (t_k) and width (Δt). The instrument response function (IRF) is the response of the instrument to a zero lifetime sample. This curve is typically collected using a dilute scattering solution such as colloidal silica (Ludox) and no emission filter. This decay represents the shortest time profile that can be measured by the instrument. The width of the IRF is due to both the characteristics of the detector and the timing electronics. IRF is very important parameter for a TCSPC system which summarizes its overall timing precision. The basic idea is that if the system is ideal, i.e., has an infinitely sharp excitation pulse and infinitely

accurate detectors and electronics; it should have an infinitely narrow IRF. Any deviation from these ideal results in a broadening of the IRF. The IRF value is measured as the full width of the half maximum intensity (FWHM) and for this case the IRF value is quite narrow, about 60 ps. The plots are also drawn in logarithm scale as the intensity scale will more exaggerate the low intensity region of the profile.

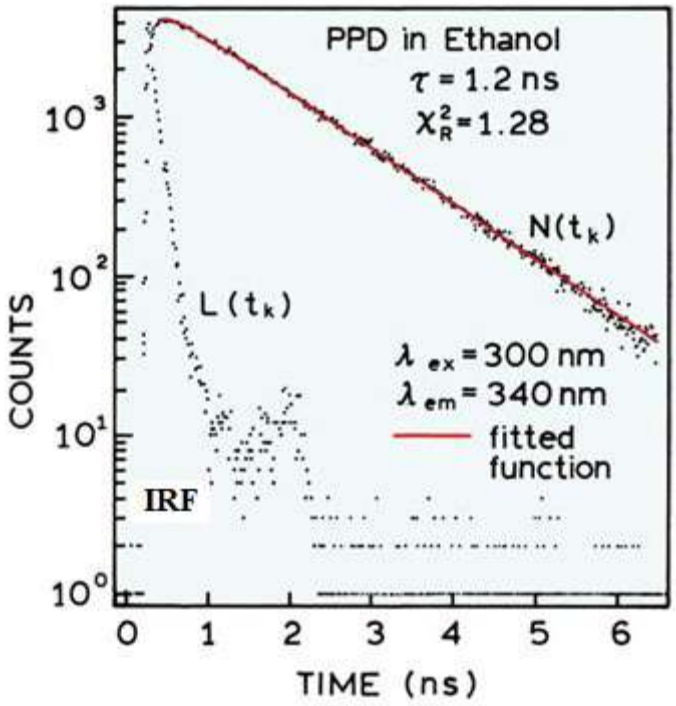

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Figure 10 TCSPC data for 2,5-diphenyl-1,3,4-oxadiazole (PPD) in ethanol. The light source was an R6G dye laser, cavity dumped at 1 MHz. The detector was an R2809 MCP PMT