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A REVIEW ON TIME CORRELATED SINGLE PHOTON COUNTING

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ABSTRACT

The phenomenon of molecular fluorescence emission contains both spectral and time information it is former which has traditionally been more usually associated with routine analytical applications of fluorescence spectroscopy. In contrast, since the early seventies, the most important strides in fluorescence spectroscopy have been by and large concerned with the development and application of new time resolved techniques. Applications of these now span not only in the traditional areas of photochemistry but also research in polymers, membranes, liquid crystals, low-dimensional structures, solar energy, and semiconductors, to name but a few. Such diverse areas are linked by their common instrumentation needs, which have greatly benefited over the past decade from quite radical progress in optoelectronics and on-line data analysis. There is now widespread recognition of the advantages of studying and using fluorescence in the time domain. Quite simply, fluorescence measurements in the time domain (fluorometry) posses a much greater information content about the rates and hence kinetics of intra and intermolecular processes than is afforded by the wavelength spectroscopy of the energy domain (fluorimetry). A simple analogy to the greater detail shown by time domain as compared to steady state fluorescence measurements is that of motion pictures in comparision to still photographs. The rising importance of fluorescence measurements in the time domain has been reflected during the past decade in several texts devoted exclusively to this topic. Nevertheless, the field has not stood still, and, if anything, the rate of innovation has probably increased over the past few years.

Keywords: Time correlated single photon counting, Fluorescence lifetime, Photon signals, Luminescence.

INTRODUCTION

Time-correlated single photon counting (TCSPC) is a remarkable technique for recording low-level light signals with extremely high precision and picoseconds time resolution. TCSPC has developed from an intrinsically time-consuming and onedimensional technique into a fast, multi-dimensional technique to record light signals.

This method generates a histogram that represents the fluorescence lifetime.

This is an efficient process because it counts photons and records arrival time in picoseconds, wh ich directly represents the fluorescent decay. Additional information such as wavelength and spatial position can also berecordedto multiplex different fluorescent targets an d generate image.

The idea is that less than one photon per laser puls e period needs to be detected in a given signalperiod, with photon arrival time distribution built up over many pulses. Because today'slaser's offer high repetition, if time measurements can be performed with in milliseconds to seconds as compared to earlier generations. However, if the area being imaged has many pixels (e.g. 50 0x 500 pixels), then this may take significantly long (for a high number of photons (10,000) in the primary channel, one pixel will take approximately 12.5ms with account rate of 0.8MH. If there are 250,000pixels, then that will take almost one hour to collect data). There is a delicate balance between number of photons needed for life time accuracy and time for an experiment [1-3].

Why we use TCSPC for time resolved experiments?

- Variations of the excitation beam's intensity are irrelevant.

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- Detects individual photons.
- No pulse-smearing from boxcar gates.
- High speed data-acquisitions.
- Digital precision, and no analogue offset required for fitting results.
- The entire decay curve is examined at once.
- Noise reduction, time limiting.

THEORY INVOLVED

Time-Correlated Single Photon Counting is based on the detection of single photons of a periodical light signal, the measurement of the detection times of the individual photons and the reconstruction of the waveform from the individual time measurements. The method makes use of the fact that for low level, high repetition rate signals the light intensity is usually so low that the probability of detecting one photon in one signal period is much less than one. Therefore, the detection of several photons can be neglected and the principle shown in the figure below can be used.

The detector signal consists of a train of randomly distributed pulses due to the detection of the individual photons. There are many signal periods without photons; other signal periods contain one photon pulse. Periods with more than one photons are very rare. When a photon is detected, the time of the corresponding detector pulse is measured. The events are collected in memory by adding a '1' in a memory location with an address proportional to the detection time. After many photons the histogram of the detection times, i.e. the waveform of the optical pulse, builds up in the memory [4]. Although this principle looks complicated at the first glimpse, it is very efficient and accurate for the following reasons:

- The time resolution of TCSPC is limited by the transit time spread, not by the width of the output pulse of the detector
- TCSPC has a near-perfect counting efficiency and therefore achieves optimum signal-to-noise ratio for a given number of detected photons
- TCSPC is able to record the signals from several detectors simultaneously
- TCSPC can be combined with a fast scanning technique and therefore be used as a high resolution high efficiency lifetime imaging (FLIM) technique in confocal and two-photon laser scanning microscopes
- TCSPC is able to acquire fluorescence lifetime and fluorescence correlation data simultaneously
- State-of-the-art TCSPC devices achieve count rates in the MHz range and acquisition times down to a few milliseconds [5].

The histogram is collected in a block of memory, where one memory cell holds the photon counts for one corresponding time bin. These time bins are often referred to as time channels. The typical result is a histogram with an exponential drop of counts towards later times.

Count rates and single photon statistics

It was mentioned that it is necessary to maintain a low probability of registering more than one photon per cycle. This is to guarantee that the histogram of photon arrivals represents the time decay one would have obtained from a single shot time-resolved analogue recording. The reason for this is briefly the following: Detector and electronics have a "dead" time for at least some nanoseconds after a photon event. Because of these dead times TCSPC systems are usually designed to register only one photon per excitation cycle. If now the number of photons occurring in one excitation cycle were typically >1, the system would very often register the first photon but miss the following ones. This would lead to an over-representation of early photons in the histogram, an effect called 'pile-up'. It is therefore crucial to keep the probability of cycles with more than one photon low [6].

To quantify this demand one has to set acceptable error limits for the lifetime measurement and apply some statistics. For practical purposes one may use the following rule of thumb: In order to maintain single photon statistics, on average only one in 20...100 excitation pulses should generate a count at the detector. In other words: the average count rate at the detector should be at most 1...5% of the excitation rate. E.g. with the diode laser PDL 800-B, pulsed at 80 MHz repetition rate, the average detector count rate should not exceed 4 MHz This leads to another issue: the count rate the system (of both detector and electronics) can handle. In- deed 4 MHz are stretching the limits of many detectors and certainly are way beyond the capabilities of most conventional NIM based TCSPC systems. On the other hand, one wants high count rates, in order to acquire fluorescence decay histograms quickly. This may be of particular importance where dynamic lifetime changes or fast molecule- le transitions are to be studied or where large numbers of lifetime samples must be collected (e.g. in 2D scanning configurations). PMTs (dependent on the design) can handle count rates of up to 1...10 Millions of counts per second (cps), standard (pas-sively quenched) SPADs saturate at a few hundred kcps. Old fashioned NIM based TCSPC electronics can handle a maximum of 50,000 to 500,000 cps [7].

With modern integrated TCSPC designs count rates over 10 Mcps can be achieved. It is also worth noting that the actual count arrival times of course are random so that there can be bursts of high count rate and periods of low count rates. Bursts of photons may still exceed the average rate. This should be kept in mind when an experiment is planned. Even if an instrument can accommodate the average rate, it may drop photons in bursts. This is why the length of the dead-time is of interest too. This quantity describes the time the system cannot register photons while it is processing a previous photon event. The term is applicable to both detectors and electronics. Dead time or insufficient throughput of the electronics is usually not of detrimental effect on the decay

histogram or, more precisely, the lifetime to be extracted from the latter. However, the photon losses prolong the acquisition time or deteriorate the SNR if the acquisition time remains fixed [8].

Fluorescence lifetime

Fluorescence lifetime or decay time of a molecule can be defined in the domain in terms of the rate of depolarisation of the first excited singlet state following δ-function(i.e., impulse) optical excitation from the ground state.

The primary focus of this technique is fluorescence lifetime, which is an intrinsic property of a fluorophore and therefore does not depend on the method of measurement. Fluorescence lifetime can be considered as a state function because it also does not depend on initial perturbation conditions such as wavelength of excitation, duration of light exposure, one or multiphoton excitation, method of measurement and not affected by photo bleaching. In addition, fluorescence lifetime is a parameter largely independent of the fluorescence intensity and fluorophore concentration. Since this process is affiliated with an energetically unstable state, fluorescence lifetime can be sensitive to a great variety of internal factors defined by the fluorophore structure and external factors that include temperature, polarity, and the presence of fluorescence quenchers.

A combination of environmental sensitivity and parametric independence mentioned above renders fluorescence lifetime as a separate yet complementary method to traditional fluorescence intensity measurements. Although initial research activities have focused on determining the fluorescence lifetime chemical and biological analytes, this technique has found its way into the burgeoning field of molecular imaging.

Fluorescence lifetime imaging can be performed either directly, by measuring the fluorescence lifetime for each pixel and generating a lifetime map of the object, or via time-gated experiments, where the fluorescence intensity for each pixel is determined after a short time interval and an intensity map is produced [9].

Fluorescence Lifetime describes the average amount of time a fluorophore will remain in its exicted state before dropping down to a ground energy level. In addition to internal relaxation, fluorophores will emit photons after absorption. Fluorescence lifetimes occur on the order of picoseconds to nanoseconds while phosphorescence is on the order of milliseconds or longer.

In general one makes a lifetime measurement by exciting the fluorophore and then measure the amount of time it takes to decay. This paper will focus on the time domain method: TCSPC using a microscope for imaging.

Luminescence is the emission of light from any substance, and occurs from electronically excited states. Luminescence is formally divided into two categories—fluorescence and phosphorescence—depending on the nature of the excited state. In excited singlet states, the electron in the excited orbital is paired (by opposite spin) to the second electron in the ground-state orbital. Consequently, return to the ground state is spin allowed and occurs rapidly by emission of a photon. The emission rates of fluorescence are typically 108s–1, so that a typical fluorescence lifetime is near 10 ns (10 x 10–9s). The lifetime (τ) of a fluorophore is the average time between its excitation and return to the ground state. It is valuable to consider a 1-ns lifetime within the context of the speed of light. Light travels 30 cm, or about one foot, in one nanosecond. Many fluorophores display sub nanosecond\ lifetimes.

Because of the short timescale of fluorescence, measurement of the time-resolved emission requires sophisticated optics and electronics. In spite of the added complexity, time-resolved fluorescence is widely used because of the increased information available from the data, as compared with stationary or steady-state measurements. Additionally, advances in technology have made time-resolved measurements easier, even when using microscopes. Phosphorescence is emission of light from triplet excited states, in which the electron in the excited orbital has the same spin orientation as the ground-state electron. Transitions to the ground state are forbidden and the emission rates are slow (103to 100s–1), so that phosphorescence life-times are typically milliseconds to seconds. Even longer lifetimes are possible, as is seen from "glow-in-the-dark" toys. Following exposure to light, the phosphorescence sub-stances glow for several minutes while the excited phosphors slowly return to the ground state. Phosphorescence is usually not seen in fluid solutions at room temperature. This is because there exist many deactivation processes that compete with emission, such as non-radiative decay and quenching processes. It should be noted that the distinction between fluorescence and phosphorescence is not always clear. Transition metal ligand complexes (MLCs), which contain a metal and one or more organic ligands, display mixed singlet–triplet states. These MLCs display intermediate lifetimes of hundreds of nanoseconds to several microseconds. In this book we will concentrate mainly on the more rapid phenomenon of fluorescence. Fluorescence typically occurs from aromatic molecules. One widely encountered fluorophore is quinine, which is present in tonic water. If one observes a glass of tonic water that is exposed to sunlight, a faint blue glow is frequently visible at the surface. This glow is most apparent when the glass is observed at a right [10].

Vibrational levels are represented by three lines per electro nic energy level

Kasha's Rule: Emission occurs from the lowest energy exc ited state

Return to ground state rate is emission (Γ) + non-radiative decay (k)
Energy absorbed = hva; Energy emitted as photons during fluorescence = hvf
Energy emitted per photon during intersystem crossing pho sphorescence = hvp
Lifetime: Average time the molecule spends in the excited state prior return
to the ground state (emission is random). Note that fluores cence emission is a random process and $t \neq \tau$.
Forexponential decay of excited molecules; e(t/τ): 63% of molecules decayed before ($t<\tau$) while 37% after ($t> \tau$).

$$\tau = \frac{1}{\Gamma + k}$$

INSTRUMENTATION

Heart of this technique is "single photon counting", as the instruments usually have a dead time in the order of microsecond. By this technique only the first arriving photon is counted. So by doing the same process many times will eventually give a decay profile of a single shot measurement. To make sure that the decay is not biased to early arriving photon the photon count rate is kept law (usually less than 1% of excitation rate) [12].

- The Antares Pump Laser
- The Dye Lasers
- The Detection System
- The TCSPC Measurement
- The CFD
- The MCP-PMT
- Software Data Analysis

SOURCES

A Coherent Antares A76s Neodymium: YLF cw-modelocked laser acts as the pump for the lasers.

DYE LASERS

KTP (potassium tytanil phosphate) crystal to give a 527nm 76MHz rep-rate pulse train which is used to synchronously pump the dye lasers. The pulse width is on the order of 100ps. One of two Coherent 700 series dye lasers are employed. Dyes used in the lasers are Rhodamine 6G and Pyridine-1 which give wavelengths in the ranges 565-620nm and 680-720nm respectively. A Coherent 7220 cavity dumper is used to reduce the repetition rate and decrease the pulse width to about 6ps. Two BBO (beta barium borate) crystals cut at different angles are used to frequency double the dye laser outputs (290-310nm and 340-360nm respectively) and also to allow sum frequency generation of 413-428nm by adding the 680-720nm output to the 1053nm Antares fundamental [13].

DETECTORS

When afluorescence photon is detected by a Micro channel Plate Photomultiplier Tube (MCP or PMT) the stopwatch is stopped and the "time" measured is collected. Faster detectors (PMT, photodiodes, and streak cameras) have made it possible to record pico seconds lifetimes .SPAD (Single Photon Avalanche Diode) best suited for photon counting/timing applications.

TCPSC Measurements

Constant fraction discriminator

A constant fraction discriminator (CFD) is an electronic signal processing device, designed to mimic the mathematical operation of finding a maximum of a pulse by finding the zero of its slope. Some signals do not have a sharp maximum, but short rise times. Typical input signals for CFDs are pulses from plastic scintillation counters, such as those used for lifetime measurement in positron annihilation experiments. The scintillator pulses have identical rise times that are much longer than the desired temporal resolution. This forbids simple threshold triggering, which causes a dependence of the trigger time on the signal's peak height, an effect called time walk (see diagram). Identical rise times and peak shapes permit triggering not on a fixed threshold but on a constant fraction of the total peak height, yielding trigger times independent from peak heights.

Micro channel Plate Photomultiplier Tube

A micro channel plate is a secondary electron multiplier consisting of an array of millions of glass capillaries with an internal diameter from around 10 to 20 microns, fused into the form of a thin disk less than 1mm thick. The wall of each channel is secondary electron emissive; each end of the walls is covered with a metal thin film which acts as an electrode and a voltage VD is applied between both ends of the capillary. An electric field is thus created in the direction of the channel axis. After a wall-electron collision, secondary electrons are accelerated by this field and travel along the parabolic trajectories determined by their initial ejection velocity. As a result of repeated secondary electron emissions the electron current increases exponentially as the output end of the channel is approached. In the manufacturing process of micro channel plates, bundles of many capillaries are sliced into thin disks.

A slice angle is selected to prevent electrons passing through the channels without colliding with the walls. This bias angle is chosen to be 5°, 8° or 13° typically, depending on the application. Onto both input and output surfaces of an MCP, Fe-Cr is evaporated to form electrodes. The thickness of the electrodes is adjusted to make the surface resistance of 50 to 100Ohms between the MCP edges. The Gain, (g), of an MCP is a function of the length to diameter ratio of the channel as well as the electric field strength. Where G is a gain factor related to the secondary electron emission characteristics of the channel wall material and the electric field intensity inside

the channel. The useful gain of a single micro channel plate is limited to about 10000 [14].

MCP-PMTs are used because they are fast. That is, the time that it takes between the generation of the primary emissive electron at the cathode and the arrival of the corresponding electron bunch at the anode is very small. This TRANSIT TIME is a limiting factor in the TCSPC technique. Strictly speaking it is the transit time spread of photoelectrons or, better still, the statistical dispersion associated with this spread that affects the time resolution. As the transit time and corresponding spread become shorter the dispersion decreases and time resolution increases.

The width of the instrument response function in the single photon counting experiment is due also to timing jitter in the electronic components and problems encountered with frequency matching of the signal output of the MCP. The Instrument Response FWHM of the Centre TCSPC system is 80ps.

Software data analysis

Computers now have highertimeresolutionand data collection rates. Computers record the values and analysed by using software. These analysed results are stored in the form of histograms, diagrams and graphs.

WORKING

Pulsed laser or LED is used as an excitation light which is split into two, one passes through the sample other though the electronics part to act as a "time start". The emitted light passesthough a mono-chromator and amplified by a photomultiplier tube (PMT). The emitted light as well as reference light is processed through a constant fraction discriminator (CFD) (to eliminate timing jitter). After passing through CFD reference pulse activates a time-to-amplitude converter (TAC) circuit. This circuit begins to build a charge on a capacitor which will only be discharged once the PMT sends another electrical pulse to the circuit. This data is then further processed by analog to digital converter (ADC) and multichannel analyzer (MCA) to get a data output [15].

This electrical pulse comes after the second laser pulse excites the molecule to a higher energy state, and a photon is eventually emitted from a single molecule upon returning to its original state. Thus, the longer a molecule takes to emit a photon, the higher the voltage of the resulting pulse. The central concept of this technique is that only a single photon is needed to discharge the capacitor. Thus, this experiment must be repeated many times to gather the full range of delays between excitation and emission of a photon. After each trial, a pre-calibrated computer converts the voltage sent out by the TAC into a time and records the event in a histogram of time since excitation. Since the probability that no molecule will have relaxed decreases with time, a decay curve emerges that can then be analyzed to find out the decay rate of the event.

APPLICATIONS

- Fluorescence Lifetime Imaging.
- Lifetime imaging of Local Environment Parameters.
- FLIM-FRET: Single-exponential Model.
- FLIM-FRET by Double-exponential FLIM.
- Protein binding interactions.
- Auto fluorescence of tissue - the FLIM approach.
- Fast sequential measurements.
- Chlorophyll transients.
- Diffuse Optical Tomography.
- DOT Setup for Static Brain Imaging.
- DOT Setup for Dynamic Brain Imaging.
- Single Molecule Spectroscopy.
- Simultaneous Recording of Fluorescence Decay.
- Ultra-Fast Recording of Optical Waveforms.
- DNA Sequencing.

Figure 1. TCSPC Measurement Principle

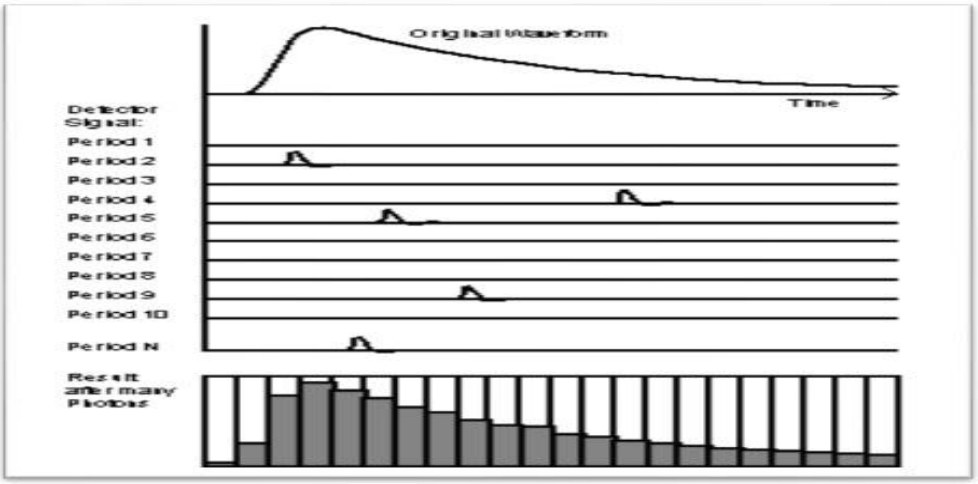


Figure 2. Recording of photon count

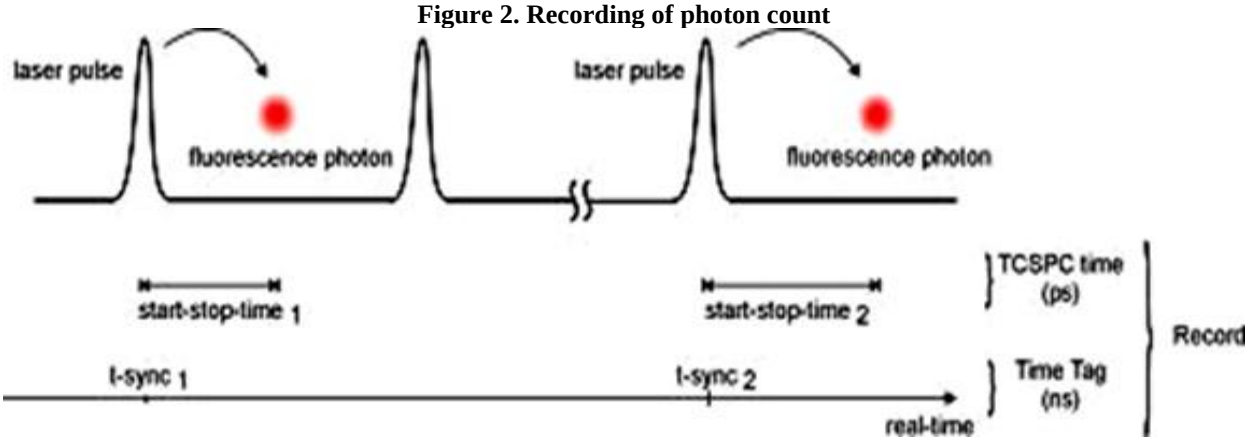


Figure 3. Histogram of photons recording

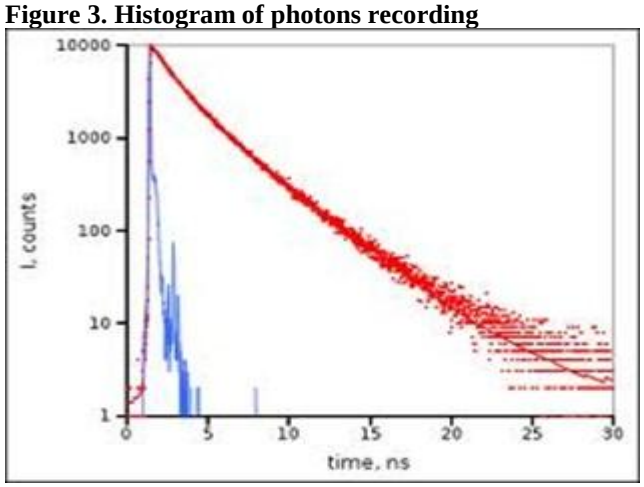


Figure 4. Jablonski Energy Diagram

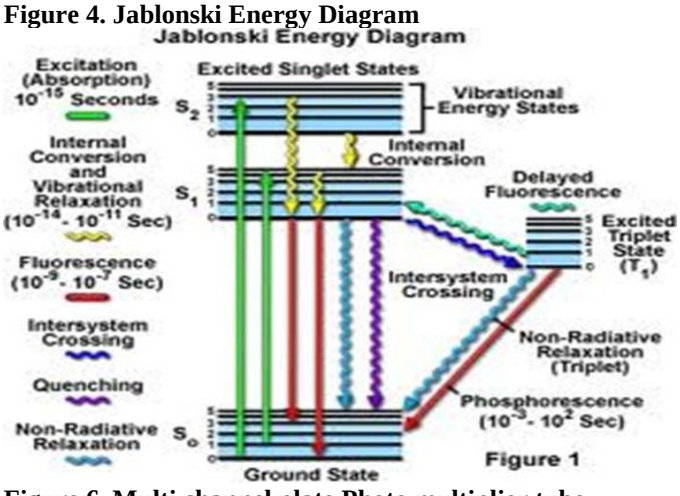


Figure 5. Graphs representing time walk and constant fraction respectively

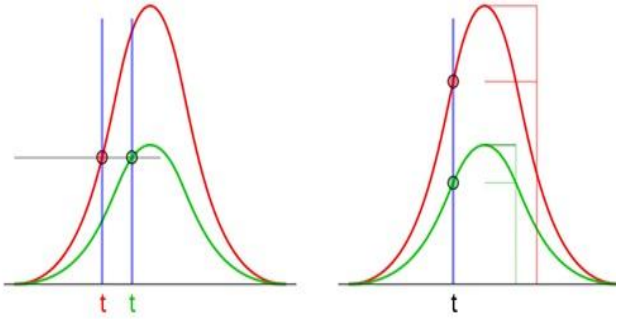


Figure 6. Multi channel plate Photo multiplier tube

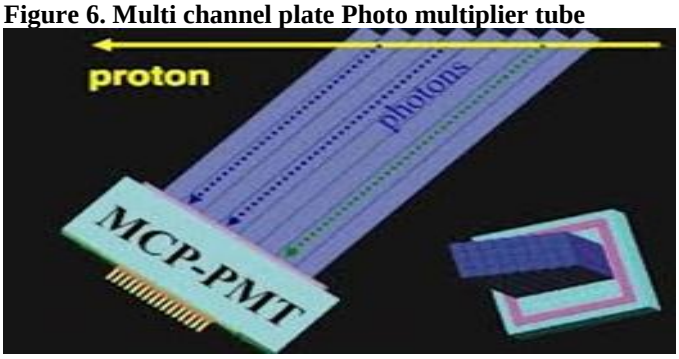


Figure 8. Photo multiplier tube

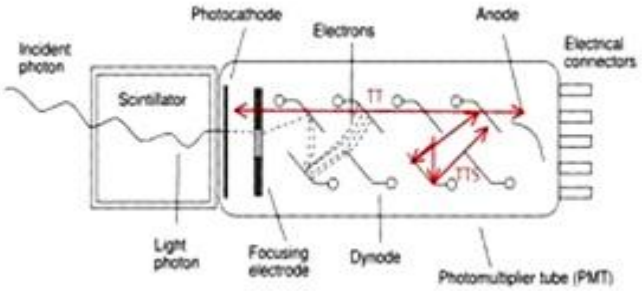
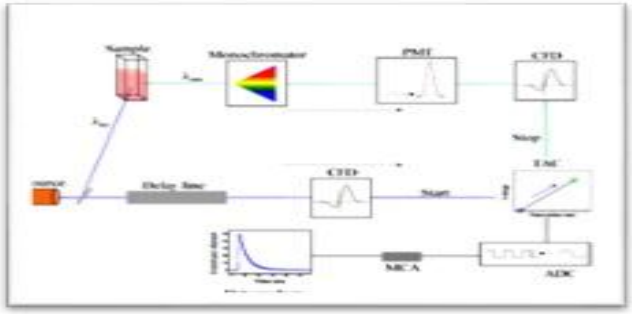


Figure 8. TCSPC Technique



CONCLUSION

Time Correlated Single Photon Counting (TCSPC) has been one of the best ways of measuring fluorescence decay times since the decay must be thought of as having a certain rate rather than occurring at a specific time after excitation. By observing how long individual molecules take to emit their photons, and then combining all these data points, an intensity vs. time graph can be generated that displays the exponential decay curve typical to these processes.

A major complicating factor is that many decay processes involve multiple energy states, and thus multiple rate constants. Though non-linear least squared analysis can usually detect the different rate constants, determining the processes involved is often very difficult and requires the combination of multiple ultra-fast techniques. Even more complicating is the presence of inter-system crossing and other non-radiative processes in a molecule. A limiting factor of this technique is that it is limited to studying energy states that result in fluorescent decay.

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