ORTEC®

AN50

APPLICATION NOTE (REVISION 2)

Instrumentation for Fluorescence Lifetime Spectrometry

Summary

This application note explains the basic principles of Fluorescence Lifetime Spectrometry and describes the electronic instrumentation needed to construct a system with picosecond time resolution. Guidelines are provided for adjusting the key operating parameters. A list of recommended instrumentation is included.

Function and Purpose

Fluorescence Lifetime Spectrometry is a popular technique used by biologists, chemists, biochemists, and biophysicists to study molecular structure and molecular interactions. It can also be used to identify the presence of specific molecular species.

In the simplest spectrometers, a pulse of light is directed at the sample to excite the molecules into a higher energy state. The excited molecules typically decay back to their lower energy state by emitting a photon. Since the probability of decay is an exponential function of time, the observed rate of fluoresced photon emission from the sample is given by:

$$R = (N_0/\tau)\, e^{-t/\tau} \quad (1)$$

where R is the rate in photons/s, N_0 is the number of molecules excited by the light pulse, t is the observed time of photon emission, and τ is the characteristic decay time constant of the excited molecule. The logarithm of the observed emission rate can be plotted versus time to obtain a straight line, whose slope is $-1/\tau$ [Fig. 1(a)]. This defines the lifetime, τ, of the excited state. For molecules of interest, τ typically falls in the range from 10 ps to 10 ns.

The value of τ is primarily determined by the structure of the molecule, but it is also affected by the environment surrounding the molecule. Thus, the measurement of the fluorescence lifetime can be used to study molecular structure and molecular interactions.

Instrumentation

The functional diagram for a typical fluorescence lifetime spectrometer is illustrated in Fig. 2. The beam splitter delivers a portion of the laser pulse to the sample while simultaneously feeding a large fraction of the light pulse to a fast photodiode. The function of the photodiode is to sense "time zero", the time at which the excitation pulse hits the sample.

A monochromator is usually inserted between the sample and the microchannel plate detector to select the desired wavelength of fluoresced photons from the sample. For reasons explained below, the light intensity is adjusted so

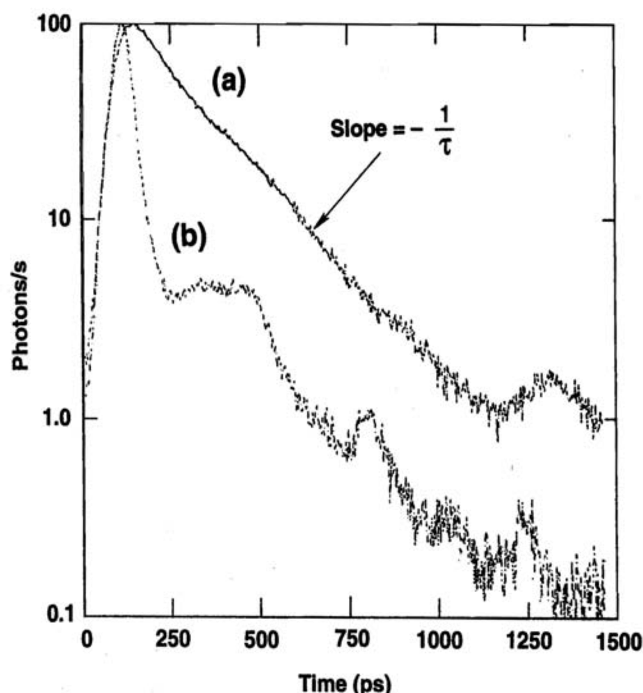

Fig. 1. (a) The Fluorescence Decay of Diphenylbutadiene in Butanol (τ = 128 ps). (b) The Instrument Response Function for τ = 0. (Courtesy of Dr. John Kauffman, University of Missouri-Columbia.)

that the probability of detecting a fluoresced photon after each laser pulse is somewhat less than 1%. Thus, the microchannel plate photomultiplier tube (PMT) is responding to the arrival of single photons. This means that the time interval between the laser excitation pulse and the detected fluoresced photon will randomly sample the shape of the fluorescence decay curve as the laser pulse is periodically repeated. The function of the remaining instrumentation in Fig. 2 is to record and sort these time intervals to form the spectrum in Fig. 1.

In the microchannel plate PMT, the fluoresced photon causes ejection of a single electron from the photocathode, and this photoelectron is accelerated by the voltage applied to the microchannel plate structure. As the electron hits the walls of the microchannel tube, it knocks out more electrons. This electron multiplication process is repeated numerous times as the group of electrons cascades along the tube towards the positive

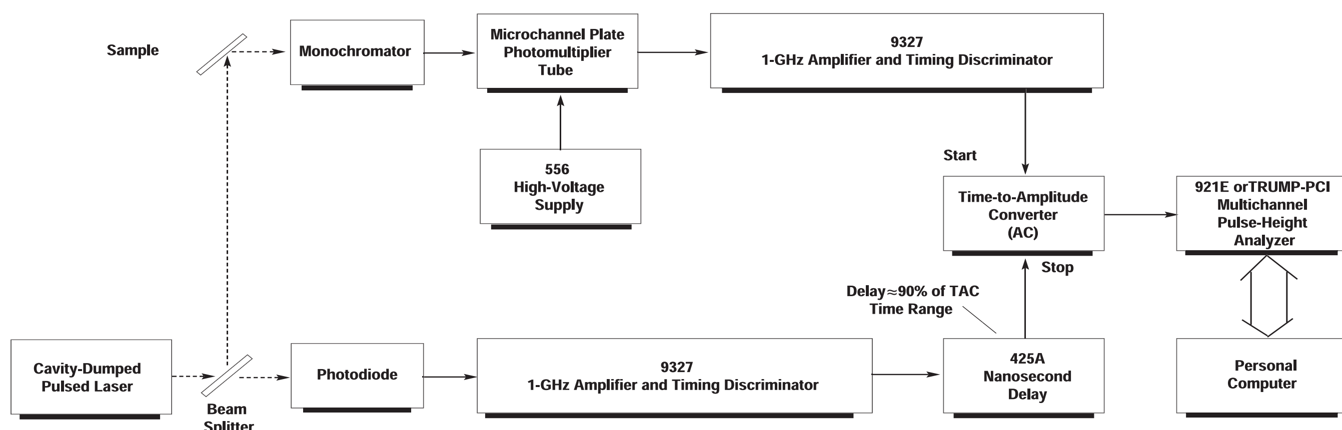

Fig. 2. Typical Block Diagram for a Fluorescence Lifetime Spectrometer.

anode. When the group of electrons arrives at the anode, it generates a negative polarity pulse on the 50-Ω load, with an amplitude on the order of 20 mV and a width of approximately 400 ps FWHM (Full Width at Half Maximum).

Further amplification of the microchannel plate PMT output is necessary before the pulse can be used to generate a precise timing signal. The amplifier section of the Model 9327 1-GHz Amplifier and Timing Discriminator provides that function, with a gain and rise time that are matched to the microchannel plate signal. At the output of the amplifier section the pulse is still extremely narrow (~600 ps FWHM). The amplitude also varies over a wide range, because of fluctuations in the electron multiplication yields in the microchannel plate PMT. The difficult task of deriving the timing information from these narrow pulses, in spite of their varying amplitudes, is handled by the timing discriminator in the 9327. A special technology employing ultra-fast circuits enables the timing discriminator to define the arrival time of these narrow pulses with picosecond precision, while exhibiting negligible sensitivity to the amplitude variations. The output of the timing discriminator is a fast logic pulse that tells the time-to-amplitude converter exactly when the fluoresced photon arrived.

Detecting the arrival of the laser pulse is somewhat easier, because the pulse consists of a large number of photons. Usually a fast photodiode provides adequate sensitivity to convert the detected laser pulse into a fast electronic pulse. Depending on the light pulse intensity, a small amount of gain may be needed before feeding the pulse to the timing discriminator. The amplifier section of the Model 9327 provides that gain, and produces a pulse that is quite narrow and suffers moderate pulse amplitude fluctuations. The timing discriminator section of the Model 9327 accurately defines the arrival time of these pulses with negligible sensitivity to the amplitude fluctuations. As a result, the fast logic output of the Model 9327 tells the time-to-amplitude converter precisely when the laser pulse occurred.

Time spectrometry in the range of 10 ps to 100 ns requires the use of a Time-to-Amplitude Converter (TAC)*. Receipt of a "start" signal initiates the linear charging of a capacitor in the TAC. When the "stop" signal arrives, the charging of the capacitor ceases, so that the voltage left on the capacitor is proportional to the time interval between the "start" and "stop" pulses. The final voltage on the capacitor is strobed to form an output pulse whose amplitude is proportional to the time interval.

Logically, one would expect the laser pulse to be used as the "start" signal and the fluoresced photon for the "stop" pulse. However, this would result in more than 99% of the conversions consisting of "start" pulses without "stop" pulses. That generates excessive dead time in the TAC and multichannel analyzer, due to a high rate of useless events. The excessive dead time can be eliminated by reversing the sources of "start" and "stop" pulses. The fluoresced photon pulse is used to start the TAC, while the laser pulse is delayed by about 45 ns and becomes the "stop" pulse. With this arrangement every useful "start" pulse is accompanied by a "stop" pulse, and the TAC operates at the much lower rate set by the fluoresced photons. As a consequence of the reversed start/stop scheme the time spectrum is reversed, with zero time at the right and longer times towards the left. That is easily corrected when plotting the data.

*See Application Note AN52 for solutions employing the Model 9308 picosecond TIME ANALYZER.

The multichannel analyzer (MCA) measures the amplitude of the analog pulse from the TAC, converts the measurement to a digital number, and adds the event to the memory location designated by the digital number. This process is repeated on a pulse-by-pulse basis to build a histogram that forms the spectrum illustrated in Fig. 1(a).

Accounting for the Instrument Response Function

The instrument response function (IRF) is the shape of the time spectrum when the fluorescence decay time is negligible [Fig. 1(b)]. The IRF can be recorded by reflecting a small portion of the laser pulse into the microchannel plate PMT from a sample that does not fluoresce. Instrument response functions with a full width at half maximum (FWHM) of 30 ps to 60 ps can be obtained with the apparatus in Fig. 2. The measured fluorescence decay curve [Fig. 1(a)] is the convolution of the IRF with the exponential decay of the molecule. If the decay time constant is long compared to the FWHM of the IRF, it is relatively easy to determine τ from the slope of the decay curve. When τ approaches the FWHM of the IRF, a fitting function must be generated by convolution of a measured IRF with Equation 1. The resulting function is fitted to the spectrum by a computer to determine the value of τ that yields the best fit to the measured data. With this technique, decay time constants as short as 10 ps can be measured.

Some systems of molecules exhibit multiple decay time constants. In such cases, the software adjusts multiple exponential decays to fit the measured data.

The Laser Repetition Rate and the Fluoresced Photon Rate

A high-resolution TAC measures the time interval from the first accepted "start" pulse to the next "stop" pulse. It ignores all subsequent "start" pulses and any additional "stop" pulses until it has finished converting the first pair of "start" and "stop" pulses. If the probability of detecting more than one fluoresced photon after each laser pulse is high, the TAC will give preference to the early photons, and will tend to exclude the photons from later decays. Such a bias towards shorter time intervals would distort the shape of the spectrum and lead to an erroneous value for τ. The distortion of the spectrum can be rendered negligible by adjusting the detected counting rate of the fluoresced photons so that the probability of detecting a single photon after a laser pulse is less than 0.01 (i.e., 1%). This implies that the probability of detecting two photons after a laser pulse is less than $(0.01)^2$ which is less than 1% of the single photon probability. This makes the spectrum distortion negligible. If the laser repetition rate is r_L pulses/s, then the optics must be restricted so that the fluoresced photon counting rate, r_f, satisfies Equation 2.

$$r_f < 0.01\ r_L \qquad (2)$$

The interval between successive excitation pulses from the laser must be greater than the longest time to be recorded in the spectrum (typically 50 ns). Usually the laser repetition rate is too high, and it must be reduced by employing a "pulse picker (pockels cell)" or a "cavity dump" to transmit to the sample only the pulses that are spaced far enough apart.

Further restriction of the laser repetition rate or the optics may be necessary to accommodate the total processing dead time T_d, of the TAC and multichannel analyzer. The total dead time is the sum of (a) the TAC time range, (b) the TAC output pulse width, and (c) the longer of i) the TAC reset time, or ii) the MCA processing time. To keep dead time losses less than 50%, Equation 3 must be satisfied.

$$r_f < 0.5\ /\ T_d \qquad (3)$$

For a total dead time of 9 µs, which is typical of the TRUMP-PCI-8k MCA coupled with the Model 566 TAC, the fluoresced counting rate must be kept below 55,555 photons/s. Faster MCAs, such as the Model 921E, can reduce the dead time to about 2.5 µs for a factor of 3.6 improvement. The condition in Equation 3 can be met either by further restriction of the optics to lower r_f, or by reducing the laser repetition rate.

Alternate Excitation Sources and Detectors

Flashlamps are often used in place of the laser because the cost and complexity of a flashlamp system is substantially lower. On the other hand, the pulse width of a flashlamp (0.8 ns FWHM) is much greater than available laser pulse widths (<10 ps). Consequently, minimum measurable lifetimes with a flashlamp are about 200 ps, compared to 10 ps with a pulsed laser. Flashlamps also have an intrinsically lower repetition rate (<100 kHz) than lasers (~100 MHz).

When flashlamps are used, the expensive micro-channel plate PMT can be replaced with a more economical, 12-stage photomultiplier tube. Some flashlamps provide an electronic pulse suitable for operating the "stop" input of the TAC. If such a signal is not available, the photodiode in Fig. 2 can be replaced with a conventional PMT. Avalanche photodiodes can also be used to detect the excitation light pulse with either flashlamps or lasers.

Adapting to Phosphorescence Decay Times

It is possible to measure phosphorescence decay times up to 100 µs with a time-to-amplitude converter, but the limitations from Equations 2 and 3 result in unproductively low data acquisition rates for the longer time scales. A better solution for measuring such long lifetimes is to replace the TAC and MCA in Fig. 2 with the multiple stop capability of a multichannel scaler (MCS). The laser pulse starts the MCS scan, and the fluoresced photons are counted at the event input. The resulting spectrum is similar to Fig. 1, but with a longer time scale. This method eliminates the restrictions represented by Equations 2 and 3, resulting in significantly higher data acquisition rates. A third alternative is to use the ORTEC Model 9308 picosecond TIME ANALYZER in place of the TAC and MCA. Because of its multistop capability and its picosecond resolution, the Model 9308 can accommodate both picosecond fluorescence decay times and microsecond phosphorescence decay times at exceptionally high rates. See Application Note AN52.

EQUIPMENT LIST

Item Number	Quantity	Model Number	Description
1	1	556	High-Voltage Power Supply (3 kV)
2	1	C-36-12	SHV High-Voltage Cable (12-ft. length)
3	2	9327	1-GHz Amplifier and Timing Discriminator
4	2	SMA58-0.15	50-Ω Cable with SMA Connectors (0.15-m length)
5	1	BNC/SMA	BNC to SMA Adapter (with male BNC, female SMA) (for photodiode to Model 9327 connection)
6	1	425A	Nanosecond Delay
7	1	4006	Minibin and Power Supply
8	2	C-25-1	50-Ω Cable with BNC plugs (1-ft. length)
9	2	C-25-4	50-Ω Cable with BNC plugs (4-ft. length)
10	1	C-24-4	93-Ω Cable with BNC plugs (4-ft. length) (connects TAC to MCA)
(a) For Counting Rates <50,000 Counts/s:			
11a	1	566	Time-to-Amplitude Converter
12a	1	TRUMP-PCI-8k	TRUMP-pci MCA for Windows (8-µs conversion time, 8k channels) (includes MAESTRO™ -32)
(b) For Counting rates >50,000 Counts/s:			
11b	1	566	Time-to-Amplitude Converter
12b	1	921E	High-Rate MCA (1.5-µs conversion time, 16k channels) (requires ethernet port on PC)
13b	1	A65-B32	MAESTRO™-32 MCA Emulator Software
(c) For Fluorescence and Phosphorescence at High Counting Rates			
11c	1	9308	picosecond TIME ANALYZER
12c	1	PCBCBL1	Interface to IBM-compatible PC
13c	1	425A	Nanosecond Delay (if the reversed start/stop mode is used)

Other Suppliers:

Analytical Software: Edinburgh Instruments, Globals Unlimited (Physics Dept., University of Illinois), IBH Consultants Ltd.

Avalanche Photodiodes: PerkinElmer Optoelectronics

Flashlamps: Edinburgh Instruments, IBH Consultants Ltd.

Microchannel Plate PMTs: Hamamatsu, ITT

Photodiodes: Hamamatsu, Hewlett-Packard, Spectra-Physics, Antel Optronics Inc. (Model AR-S1)

Photomultiplier Tubes: Burle, Hamamatsu, Philips, Thorn EMI

Pulsed Lasers: A-B Lasers Inc., Antel Optronics Inc., Coherent Inc., Lambda Physik, Laser Diode Inc., Opto-Electronics Inc., Quantel International, Spectra-Physics

Specifications subject to change
041805

ORTEC®
www.ortec-online.com
Tel. (865) 482-4411 • Fax (865) 483-0396 • info@ortec-online.com
801 South Illinois Ave., Oak Ridge, TN 37831-0895 U.S.A.
For International Office Locations, Visit Our Website
AMETEK® ADVANCED MEASUREMENT TECHNOLOGY