2 Basic Principles of the Single Photon Counting Lifetime Measurement

2.1 The Standard Experiment

A schematic diagram of a conventional single photon counting instrument is shown in Fig. 2.1. The single photon counting measurement relies on the concept that the probability distribution for emission of a single photon after

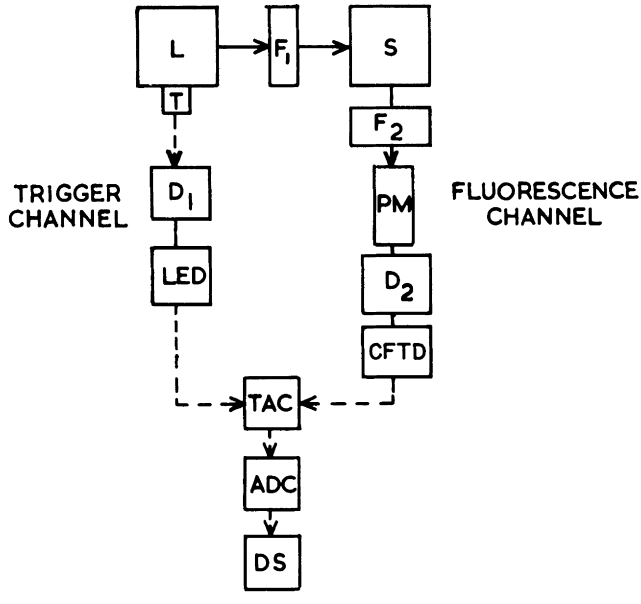

Figure 2.1 Block diagram of a conventional single photon counting apparatus. —— optical signal; ------ electronic signal; L, excitation source; T, trigger [Antenna, (fibre optic) and photomultiplier tube, etc.]; S, sample holder; F_1,F_2, filter or monochromator; PM, fast photomultiplier tube; D_1,D_2, delay lines; LED, leading edge timing discriminator; CFTD, constant fraction timing discriminator; TAC, time-to-amplitude converter; ADC, analogue-to-digital converter; DS, data store (multi-channel analyser or computer).

an excitation event yields the actual intensity against time distribution of all the photons emitted as a result of the excitation. By sampling the single photon emission following a large number of excitation flashes, the experiment constructs this probability distribution.

With reference to this diagram, the experiment is carried out as follows: the trigger T, which could be a photomultiplier, an antenna pick-up, or a logical sync. pulse from the electronics pulsing the excitation source, generates an electrical pulse at a time exactly correlated with the time of generation of the optical pulse. The trigger pulse is routed, perhaps through a discriminator, to the start input of the time-to-amplitude converter, (TAC) which initiates charging of a capacitor. In the meantime the optical pulse excites the sample, which subsequently fluoresces. An aperture has been adjusted so that at most one photon is "detected" for each exciting event. The signal resulting from this photon stops the charging ramp in the TAC, which puts out a pulse, the amplitude of which is proportional to the charge in the capacitor, and hence to the time difference between START and STOP pulses. The TAC output pulse is given a numerical value in the analogue-to-digital converter and a count is stored in the data storage device in an address corresponding to that number. Excitation and data storage are repeated in this way until the histogram of number of counts against address number in the storage device represents, to some required precision, the decay curve of the sample. If deconvolution is necessary, the time profile of the excitation pulse is collected in the same way by replacing the sample by a light scatterer.

2.2 Details

2.2.1 Statistics

In an ideal time-correlation experiment each photon emitted by the sample as a result of a given excitation flash would be timed and recorded. In fact the response time of the detection equipment and the mode of operation of the TAC render it necessary to time only the first photon in a given time interval after the occurrence of the flash. The statistics of general photon counting have been discussed by Morton (1968); a simpler discussion is given here following Pfeffer *et al.* (1962) and Wahl (1975).

During one excitation cycle an average number of photons, z_i, impinge on the cathode of the fluorescence photomultiplier during the time interval $t_{i-1/2}$ to $t_{i+1/2}$, corresponding to the time width of address i (usually a channel in a multichannel analyser) in the data store. These photons eject an average number w_i of photoelectrons from the photocathode, thus

$$w_i = qz_i, \tag{2.1}$$

where q is the quantum efficiency of the photocathode. The probability of emission of l photoelectrons in the ith time interval is given by the Poisson distribution (Mandel and Wolf, 1965):

$$p_l(i) = \frac{(w_i)^l}{l!} e^{-w_i} \tag{2.2}$$

with

$$\sum_{l=0}^{\infty} p_l = 1 \tag{2.3}$$

Therefore (Koechlin, 1961):

$$p_0(i) = e^{-w_i} \tag{2.4}$$

$$p_1(i) = w_i e^{-w_i} \tag{2.5}$$

$$\begin{aligned} p_{l>1}(i) &= 1 - p_0(i) - p_1(i) \\ &= 1 - e^{-w_i} - w_i e^{-w_i} \\ &= 1 - (1 + w_i) e^{-w_i}. \end{aligned} \tag{2.6}$$

After a large number of excitation cycles (N_E), the number of anode pulses in the ith time interval, N_A, is

$$N_A = N_E[p_1(i) + p_{l>1}(i)]. \tag{2.7}$$

If $w_i \ll 1$, then

$$p_1(i) = w_i \tag{2.8}$$

and

$$p_{l>1}(i) = w_i^2 \ll w_i.$$

Therefore the number of anode pulses, N_A, is proportional to the intensity of the fluorescence at time t_i since

$$\begin{aligned} N_A &\approx N_E(w_i + w_i^2) \\ &\approx N_E w_i \\ &= N_E q z_i. \end{aligned} \tag{2.9}$$

Because the TAC detects only the first photon in a given time interval for a given excitation cycle, N_A is not the number of counts in the ith channel. This number, N_i, is related to N_A by the equation (Coates, 1968):

$$N_A = \frac{N_i}{1 - \frac{1}{N_E} \sum_{j=1}^{i-1} N_j} \tag{2.10}$$

Since

$$\sum_j N_j \leqslant N_D,$$

the number of *detected* anode pulses, if $N_D \ll N_E$ it follows that $N_i = N_A$. Consequently the count in channel i is a measure of the fluorescence intensity at time t_i.

Generally, N_D is measured at the output of the TAC and N_D/N_E kept below a certain limit. If N_D is not very much less than N_E, data can be corrected using Equation 2.10 provided that $w_i \ll 1$ (see Section 6.4). Collection at high N_D/N_E ratios need not lead to distorted curves if pile-up inspection is performed (see Section 5.2.5(b)). However, it is simpler and probably just as efficient, when data transfer and analysis are taken into account, to keep the ratio N_D/N_E below a certain value.

It is clear that choice of a value for $N_D/N_E(=F_D)$ will depend on the level of distortion that can be accepted. Yguerabide (1972) has discussed this point; using numerical calculations with Equation 2.10 he concluded that an F_D as high as 0.05 was tolerable since at this count rate distortions in the early channels are negligible, while they amount to only a few per cent in the late channels. However, empirical tests in our laboratory indicate that significant distortions are still present when F_D is as high as 0.05. In fact most workers keep F_D lower than 0.01 or 0.02. As far as pile-up distortions are concerned, the lower the value of F_D the better; therefore, if collection times are short (as for instance in instruments with a cavity dumped laser as excitation source) an F_D of 0.005 or even 0.002 is more usual. It should be pointed out that excitation sources in SPC instruments are comparatively weak in intensity and in many experiments the low level of detected photons, N_D, renders discussion of tolerable F_D values somewhat academic. In conclusion, therefore, when pulse pile-up distortions are to be avoided by limiting the number of detected photons a value of F_D in the region of 0.001 or lower is to be preferred.

2.2.2 Sensitivity, precision and time resolution

When the fluorescence photomultiplier (the PM) is operated in the single photon mode, many of the distortions normally accompanying photomultiplier detection and associated recording circuitry are avoided, since the PM is used merely to time the arrival of a photon at the photocathode. In addition, amplitude jitter in the exciting light pulse is irrelevant provided that the pulse shape remains constant. The SPC technique therefore has all the advantages of digital signal processing and in addition, at least for decay time measurements (see Chapter 7 for spectroscopic measurements), is concerned only with the times of occurrence of events and not with intensities.

The effect of PM noise is greatly reduced in many experiments by the mode of operation of the TAC. When the TAC has received the START pulse it remains "dead" for some fixed time before the voltage sweep (see Section 5.2.5) is initiated. During the voltage sweep, which can continue for a time set by the operator (called the TAC range), it can accept at most one signal resulting from either noise or fluorescence. At the end of the TAC range, or after receiving the STOP signal, it remains "dead" until the next START signal arrives. Since the TAC range is usually set to span the maximum in the photon distribution function, data collection occurs at times of enhanced signal-to-noise ratio. Therefore, a PM noise count rate of say 1000 Hz after the CFTD discriminator may be reduced by a factor of 100 after the TAC when the trigger pulse is used to start the voltage sweep. Otherwise (see Section 2.2.6) noise reduction will be less. Generally background noise offers no serious difficulties since the PM is chosen with low noise characteristics and correction for background is quite straightforward (Section 2.2.5).

Sensitivity in SPC instruments has sometimes been reported in terms of the reciprocal of the product (optical density in 1 cm path length × quantum yield) for the sample, the decay time of which has been measured. Figures as high as 10^6 and 10^7 have been reported for flash lamp (Ware, 1971; Yguerabide, 1972) and laser-based systems (Harris *et al.*, 1977). Unless the degree of light attenuation in monochromators and filters is specified, this figure is virtually meaningless. Another approach is suggested by Knight and Selinger (1973) who used a concrete example. They detected (TAC conversions) 10 photons per second on a TAC range of 80 ns when the vapour over crystalline anthracene at 20°C ($P = 10^{-4}$ Torr) was excited at 337.1 nm. Slit widths and mode of filtering, however, were not specified. Haugen and Lytle (1981) have evaluated the sensitivity of their instrument as (counts actually measured)/(counts that should be measured), the latter calculated from known quantities such as number of excitation photons per second, optical density of sample, quantum yield, f-number of optics, reflections and air glass interfaces, spatial apertures, quantum efficiency of PM photocathode and transmission of filters at the appropriate wavelengths. (They achieved 2.0×10^{13} counts $\text{M}^{-1}\,\text{s}^{-1}$ out of a possible 2.8×10^{13}.) This approach, while rigorous, is difficult to follow in some experiments. We suggest, therefore, that if sensitivity is to be specified it should be done in terms of optical density and quantum yield with *full* details of slit widths and filters also specified.

In a fluorescence decay curve collected by the SPC technique there are two distinct types of noise. One, resulting from photomultiplier dark counts, is a constant background and is easily treated (see Section 2.2.5). The other, a counting error, varies from channel to channel and is of more fundamental importance. In the data store resulting from an SPC experiment there is, in a

given time interval (channel), a finite non-zero chance of observing any integral number of counts. The probability of observing any specific number of counts is given by the Poisson probability function (Bevington, 1969), with a mean μ and variance $\sigma^2 = \mu$. Every count in every channel represents an estimate of the mean of a Poisson distribution of counts for that channel. Hence the variance σ^2 equals the number of counts in that channel, N_i, and the uncertainty in the number of counts σ_i is given by

$$\sigma_i = \sqrt{N_i}. \tag{2.11}$$

The implications of this counting error in connection with data analysis are discussed in Chapter 6. Here we can make use of this uncertainty to estimate the number of detected counts that are necessary to record a decay curve with a given precision (Yguerabide, 1972).

If we wish to have a precision of 5% in the number of counts, N_i, in channel i, where the curve has decayed to 1% of its maximum value, then $0.05 = 1/\sigma_i = 1/\sqrt{N_i}$ and $N_i = 400$. Consequently the number of counts in the channel of maximum counts should be 40 000. This figure represents a very rough estimate. Experimentally it will probably be sufficient to accumulate 1 or 2 $\times 10^4$ counts in the maximum for a single exponential decay. The requirements for multiple exponential decays are more severe and depend on the lifetime values and the intensities of each component. For such decays a peak count of at least 3×10^4 should probably be accumulated, but frequently the choice of the acceptable number of counts will be a compromise between high precision and avoidance of distortions resulting from long-term instability in the excitation source. For measurements of the decay of fluorescence anisotropy, peak counts of the order of 10^5 or 10^6 may be advisable because the decay times extracted depend on the subtraction of two decay curves (see Chapter 8).

Many factors contribute to the uncertainties in the lifetimes that are derived from an SPC measurement. These include excitation pulse instability, spread in the photomultiplier transit time, jitter in the electronics and uncertainty in the channel calibration. It is important to realize that the width of the pump pulse profile is irrelevant if the shape of the profile remains absolutely constant. However, no excitation source is perfectly stable and instabilities are usually proportional to the pulse width. Hence it is desirable to have as narrow a pulse width as possible. Similarly, transit time spread will in general be smaller in a PM tube with a shorter transit time; hence the search for faster and faster detection devices. It must also be realized that the SPC experiment is, essentially, an averaging technique with the consequence that the transit time spread (typically 1 to 2 ns for modern tubes) does not lead to the same spread in the measured lifetime. They are proportional but the latter can be made as small as required to a certain limit, given perfect

stability in the other experimental conditions, by increasing the number of collected counts. The determination of this limit is basically empirical.

Some authors set it on the basis of the shortest lifetimes they have measured with confidence. Thus Ware (1971) reported a limit of 0.8 ns while Yguerabide (1972) reported 0.5 ns. Others prefer to estimate the time resolution as some fraction of the full width at half maximum (FWHM) of the instrument response function. This function is the curve that results when the pump pulse profile is measured in the way described in Section 2.1 and is a convolution of the true pump pulse profile with the response function of the detector. West (1979) puts this fraction as one tenth for single exponentials and one fifth for double exponentials, whereas one fifteenth is recommended by Grinwald (1976) and Cramer and Spears (1978). On pulsed laser based SPC equipment, instrument response functions can be as low as 75 ps (Murao *et al.*, 1982); lower limits of about 5 ps on measurable lifetimes are therefore set by the one fifteenth criterion. We believe that this figure is unrealistic and that errors of at least 100% should probably be quoted for lifetimes up to 60 ps if the result of a single measurement is given.

The figures for time resolution given in the preceding paragraph are rules of thumb based on authors' confidence in the lifetimes they have measured. If a more exact estimate is preferred, the following experiment (Zimmerman and Cutler, 1975) may be performed. Two pump pulse profiles are measured, a length of time corresponding to the time needed to collect an ordinary decay curve being allowed to elapse between the two measurements. Deconvolution of one pump pulse profile with the other will yield a "decay" time, which is a measure of the time resolution of the instrument. This experiment has yielded values of 18 ps (Zimmerman and Cutler, 1975) for instruments with flash lamp excitation. Koester and Dowben (1978), using a method based on the optical properties of the cavity-dumper in their dye laser excitation source, determined a timing resolution of 25 ps whereas for a similar excitation source using the method proposed by Zimmerman we estimate the resolution at 50 ps.

In spite of these low values, quoted in recent publications, the prospective user should not expect to be able to measure lifetimes less than 500 ps until each component of his instrument has been properly optimized. Even then, constant checks on excitation source stability and electronic jitter must be performed if decay times less than 100 ps are to be believed. Short decay times in two-component decay curves present even more difficulties. These decays should be measured a number of times in order to compensate for random errors. The detection of systematic errors can best be achieved by comparison of the results of SPC analysis with results of independent experiments. We strongly caution against the adoption of models based on extracted lifetimes from two-component decays of less than 100 ps unless the model is justified on other grounds.

2.2.3 Convolution

If the flash of light that excites the sample were infinitely narrow, and if the response of the detection system were infinitely fast, the observed decay curve would represent the true decay, or δ-pulse response, of the sample. We shall refer to this function as $G(t)$.

The form of the observed decay, $I(t)$, when the excitation function, $E(t)$, is not a δ-function can be deduced from the theory of impulse functions and leads to the convolution concept. Convolution, or folding together, occurs because molecules excited by photons at early times are decaying while others are being excited by photons in the tail of the excitation pulse. A simple deduction of the convolution equation is based on the diagram in Fig. 2.2. The pump pulse is assumed to be a sum of δ-pulses of amplitude $E(t')$ at any time t'. Since the number of sample molecules excited at time t' is proportional to $E(t')$ the number at any later time $x-t'$ is proportional to $E(t')G(x-t')$. The total number of excited state molecules at time x, written $[A*](x)$ is then a sum over all times t' preceding time x or, for an infinite sum,

$$[A*](x) \propto \int_0^x E(t')G(x-t')\,dt'. \tag{2.12}$$

We have so far neglected distortions introduced by the detection system.

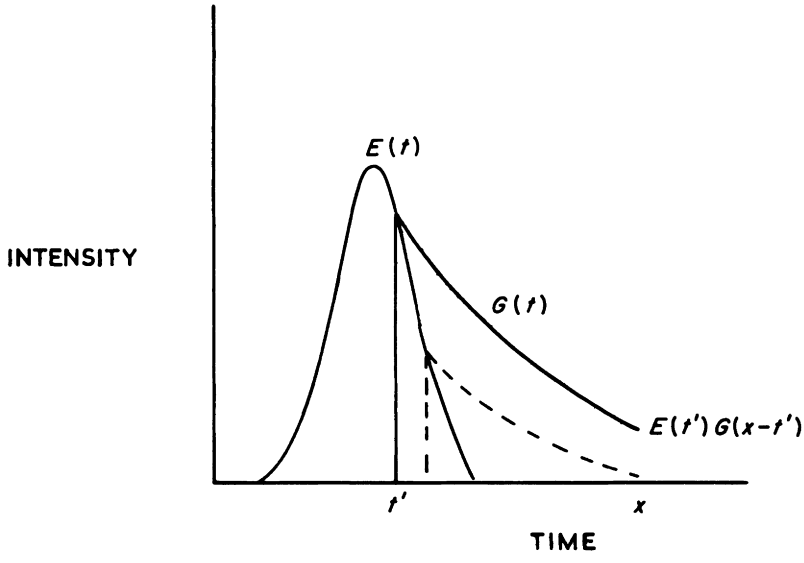

Figure 2.2 Schematic representation of the effect of convolution. $E(t)$ idealized pump pulse profile; $G(t)$ decay law (assumed single exponential) of sample.

Their effect can be evaluated through the use of Laplace transforms. Suppose that $H(t)$ is the δ-pulse response of the detection system, and $P(t)$ the measured time profile of the pump pulse, i.e., the instrument response function. $P(t)$ is a convolution of $E(t)$ and $H(t)$, which we shall write as

$$P(t) = E(t) \otimes H(t). \tag{2.13}$$

Writing the Laplace transform of a function $X(t)$ as $x(s)$ with

$$x(s) = L[X(t)] = \int_0^\infty e^{-st} X(t)\, dt,$$

it follows that

$$p(s) = e(s).h(s). \tag{2.14}$$

Similarly

$$a(s) = e(s).g(s) \tag{2.15}$$

and

$$i(s) = a(s).h(s). \tag{2.16}$$

Therefore

$$\begin{aligned} i(s) &= e(s).g(s).h(s) \\ &= p(s).g(s) \end{aligned} \tag{2.17}$$

and

$$\begin{aligned} I(t) &= P(t) \otimes G(t) \\ &= \int_0^t P(t')G(t-t')\, dt', \end{aligned} \tag{2.18}$$

where $I(t)$ represents the δ-function response of the sample distorted by convolution with both the pump pulse and the detector response, i.e., the measured decay curve.

Equation 2.18, the convolution integral, can be solved for $G(t)$ if $I(t)$ and $P(t)$, measured under the same conditions of instrumental distortion, are known. Methods for solving the convolution integral, or variants thereof, are discussed in Chapter 6.

Convolution will obviously become less significant as $P(t)$ becomes narrower with respect to $G(t)$. Errors attendant upon failure to deconvolve, in the case of instrument response functions of varying FWHM, have been estimated (Shaver and Cline Love, 1975), but only for single exponential decays.

Before a decision is reached that the assumption $I(t) = G(t)$ is valid it must

be borne in mind that $P(t)$ represents the instrument response function and not simply the excitation pulse. Many instrument response functions, while having quite a narrow FWHM (as short as 75 ps in some instruments), have an extended tail, usually with a secondary peak resulting from photomultiplier effects (Lewis *et al.*, 1973). If this tail cannot be eliminated it is neaningless to consider only the FWHM of $P(t)$ in deciding whether deconvolution is necessary or not. In addition, the sample decay function may contain a short-lived component which may be overlooked if convolution is not taken into account. For instance, Lopez-Delgado *et al.* (1974) state that with an instrument response function of FWHM 1.4 ns, lifetimes greater than 4 ns can be derived directly from the measured $I(t)$ without deconvolution. As an example they report the decay time of quinine bisulphate in 0.1 N H_2SO_4 as 18.8 ns; significantly, there is now strong evidence that a single exponential function is an inappropriate model for the decay of this fluorescence (Harris and Selinger, 1979; Meech *et al.*, 1982). We therefore suggest that all decay curves should be deconvolved, irrespective of the lifetime values.

When both $P(t)$ and $I(t)$ are employed in data analysis it is usually assumed that the counting errors in $P(t)$ are negligible. Experiments in our laboratory have confirmed the validity of this assumption, instrument response functions collected with varying numbers of counts yielding identical results. Provision for including the errors in $P(t)$ in the uncertainty in $I(t)$ is therefore unnecessary but can be made (see Chapter 6).

2.2.4 Measurement of the instrument response function

The validity of the convolution integral requires that the functions $I(t)$ and $P(t)$ should be measured under the same conditions of distortion. In practice one tries to scatter from the same volume as that from which fluorescence is viewed, thereby equalizing the illuminated areas of the PM tube photocathode, upon which the response function, $H(t)$ depends. Colloidal suspensions such as Ludox*, glycogen (Weber and Teale, 1957), barium sulphate (Bailey and Rollefson, 1953) or simply milk (Millar *et al.*, 1980) can be substituted for the sample and, at the appropriate concentration, will closely, but not exactly, mimic the emission geometry (Demas and Crosby, 1971). Some of these suspensions, however, fluoresce under short wavelength excitation (300 nm) (Eastman, 1967) and are therefore to be avoided at these wavelengths. Aluminium or silver foil or, better, roughened quartz, may be used as a diffuse reflector. A screen coated with magnesium oxide can also

*Ludox is a Trade name for an aqueous suspension of silica manufactured by E. I. du Pont de Nemour Co., Wilmington, Delaware, USA.

serve as a scatterer. A monochromator between the sample and PM tube renders the choice of scatterer or reflector less important, but if the PM tube photocathode views the fluorescing or scattering area directly the correct method should be determined empirically using a standard sample (Section 2.4) and deconvolution.

As might be expected, the photomultiplier response, $H(t)$, is dependent on the energy of the incident radiation. Since fluorescence is normally Stokes shifted from excitation it may not be possible to measure $I(t)$ and $P(t)$ at the same wavelength. Pulses from hydrogen and deuterium flash lamps and from storage ring radiation are thought to be wavelength invariant, so that the instrument response function for such sources can be measured at the wavelength of the fluorescence. Measurement of $P(t)$ for other excitation sources is discussed in Section 2.3.

Long-term drift in the time profile of the excitation source can be treated either experimentally or in the data analysis. The former is probably preferable. A slight elaboration of the simple experiment entails the collection of one instrument response function, then of the decay curve, and then of a second instrument response function (these counts can be simply added to the counts in the first instrument response function in the data store). An instrument that automatically switches data collection between instrument response function and decay curve after a certain number of excitation cycles has been described by Hazan *et al.* (1974). A special cell holder carries the scattering solution and the sample one above the other. The holder is moved up and down by a pneumatic piston and the entire operation of decay curve and instrument response function collection, and background subtraction is controlled electronically and repeated until the required precision has been reached. For long collection times this procedure has much to recommend it and should lead to an improvement in measured decay times. More elegant devices than a pneumatic piston for alternating between scattering solution and sample have been described (Easter *et al.*, 1976; Rayner *et al.*, 1976). Short-term drift or jitter in the pump pulse profile is less easily corrected for and should be eliminated by optimization of the operating conditions in the excitation source.

2.2.5 Correction for constant background

In a good SPC instrument there is usually a very low noise level arising from leaked room light and thermionic noise in the photomultiplier tube. The reason is that the fast PM tubes chosen for single photon counting have very good dark noise characteristics. Most u.v.-sensitive tubes do not need to be cooled but it is advisable to operate red sensitive tubes at a low temperature. All measured curves must be corrected for dark noise since long low-intensity

lifetimes are sometimes difficult to distinguish from the background. There are three commonly used methods for background correction.

Simplest and most widely used is the displacement of the decay curve in the data storage channels so that the first ten or twenty channels are used to accumulate only background counts. This background level is then estimated from a recount of the data and subtracted from the curve channel by channel. A justification for this subtraction has been given by Knight and Selinger (1973) who showed that the observed decay function $I(t)$ is given by

$$I(t) = C_1 I_0(t) + C_2, \tag{2.19}$$

where C_2 is the constant background component.

An automated version of background subtraction has been described by Hazan *et al.* (1974). In this version the analyser is run in subtract mode with an excitation shutter closed, for exactly the same number of excitation cycles as are used with the analyser in add mode and the shutter open. This procedure is also justified on statistical grounds but involves rather precise timing and is perhaps most suitable for a fully automated system such as that described by the authors.

More questionable is the inclusion of the constant background as a variable parameter in least-squares fitting procedure (Robbins *et al.*, 1980). There appears to be no advantage in increasing the number of fitting parameters in this way, especially in multi-exponential decays when a variable shift parameter is also included.

Subtraction of the background before deconvolution is therefore quite satisfactory. Care should be taken, if background is estimated from channels corresponding to a non-linear region of the TAC, that sufficient channels to give proper averaging are used. In addition the timescale should be such that in the channels before the curve the probability of collecting signal photons resulting from previous excitation events is negligible.

2.2.6 Operation at high excitation repetition rate

When the repetition rate of the excitation source is so high that the TAC has not time to reset between the occurrence of successive excitation pulses, distortions will be present in the decay curve if the data collection is performed in the conventional fashion. The present generation of TACs can operate at 1 or 2×10^5 Hz START rate (Haugen *et al.*, 1979) and therefore can be operated in conventional mode with most flash lamp excitation sources and with laser sources that employ a Pockels cell. Mode locked ion lasers, cavity dumped dye lasers and storage rings usually operate optimally at repetition rates in the MHz region. Consequently, for these excitation sources, the standard mode of data collection is not suitable.

In one variant of the conventional technique (Wild *et al.*, 1977) an inhibit function is used in such a way that data collection is blocked during periods of TAC recovery. Full advantage of the high repetition rate of excitation is, therefore, not taken. A more common variant, and one that also offers some advantages for low repetition rate excitation sources, is operation of the TAC in "reverse" mode (Swords, 1977; Lopez-Delgado *et al.*, 1974). Fluorescence signals are routed to the START input of the TAC and the trigger signals to the STOP input. It is preferable to arrange the delays (D_1 and D_2 in Fig. 2.1) so that the TAC sweep is stopped by a trigger signal corresponding to the excitation cycle in which the START signal occurred. If the delays are not so arranged the inter-pulse separation in the excitation source must be very stable.

The advantage of this technique is that, for high repetition rate sources, many more of the fluorescence signals (after suitable reduction to avoid pulse pile-up) are processed by the TAC. In a similar way, however, a correspondingly larger number of the dark noise signals are processed, and the need for a low noise fluorescence PM tube is therefore greater. In all other respects it is similar to the conventional mode except that the curves are collected in the data store with time increasing from higher to lower channel numbers. An example of a pump pulse and a decay curve collected with the TAC in "reverse" mode is shown in Fig. 2.3. It is usual to adjust data such as these to the more conventional arrangement before data analysis.

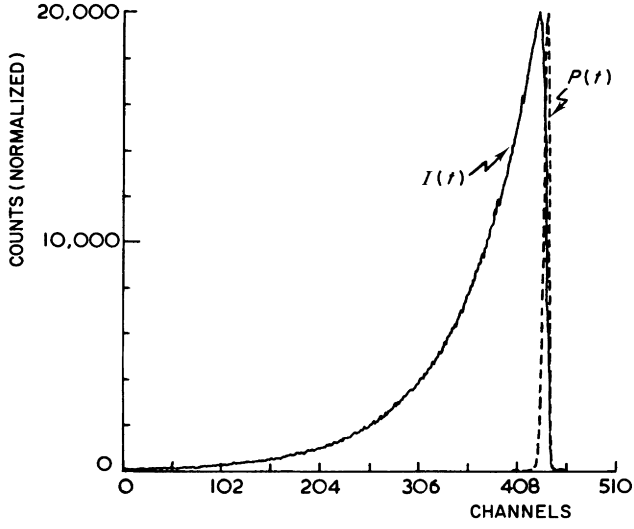

Figure 2.3 Decay curve of 1-methylindole in methylcyclohexane measured with the TAC operated in "reverse" mode. 1 channel = 0.082 ns.

2.3 Experiments to Correct for Wavelength-dependent PM Distortion

In the derivation of the convolution integral, Equation 2.18, wavelength-dependent effects on pump pulse shape and detector response were neglected. When they are taken into account we should write for the measured instrument response function $P(\lambda_E,t)$ or $P(\lambda_e,t)$

$$P(\lambda_E,t) = E(\lambda_E,t) \otimes H(\lambda_E,t) \tag{2.20}$$

$$P(\lambda_e,t) = E(\lambda_e,t) \otimes H(\lambda_e,t), \tag{2.21}$$

where λ_E signifies the wavelength of excitation and λ_e the wavelength at which the emission is observed. The measured decay curve should be written

$$I(\lambda_e,t) = E(\lambda_E,t) \otimes G(\lambda_e,t) \otimes H(\lambda_e,t). \tag{2.22}$$

If the pump pulse shape is wavelength independent, $E(\lambda_E,t) = E(\lambda_e,t)$ and when the instrument response function is measured at the wavelength of emission observation Equation 2.22 is soluble since

$$\begin{aligned} I(\lambda_e,t) &= E(\lambda_e,t) \otimes G(\lambda_e,t) \otimes H(\lambda_e,t) \\ &= P(\lambda_e,t) \otimes G(\lambda_e,t). \end{aligned} \tag{2.23}$$

The wavelength dependence of pump pulse profiles is discussed in Chapter 3. Here it may be noted that only hydrogen and deuterium flash lamps and storage ring radiation have pulse shapes that are believed to be constant over a wide wavelength range. For other sources, the pump pulse shape must be measured at the excitation wavelength and some sort of correction applied.

It has been suggested (Ricka, 1981) that in certain experiments in which two or more decays are measured at the same emission wavelength, having been excited at the same excitation wavelength, there is no need to measure an instrument response function since, if the decay functions are related (e.g., in a fluorescence depolarization measurement), the decay curves can be manipulated and the desired information extracted. While it is indeed possible to analyse some kinds of decay data in this way, we would strongly suggest that all decay curves should be deconvolved in order to ascertain whether the data are distortion free. Mathematical treatment of this wavelength-dependent distortion is discussed in Chapter 6. In general, it is preferable to eliminate experimental distortions by instrumental techniques rather than correct for them in data analysis. Hence, some effort has been made by workers in the SPC field to develop experiments by which the effect of a wavelength-dependent PM response may be counteracted.

The first such experiment appears to be that of Wahl *et al.* (1974), who proposed the use of a sample with a well-known single exponential decay

time. In essence their method rests on the solution of Equation 2.22 for the reference compound. The convolution product $(E(\lambda_E,t)\otimes H(\lambda_e,t)$ is then calculated, and may be substituted in Equation 2.22 when $I(t)$ is measured for an unknown decay. The disadvantages of the experiment are that reference compounds must be found for each emission wavelength and that the errors involved in the two deconvolutions as well as the small number of compounds with a definitely known single exponential decay must lead to large errors in the recovered $G(t)$.

Britten and Lockwood (1976) modified the method just described so that a knowledge of the exact lifetime of the reference compound was not required. Instead, two reference compounds, the decays of which are known to be single exponential, but the lifetimes of which need not be known accurately, are used to determine the function $E(\lambda_E,t)\otimes H(\lambda_e,t)$. Since this method entails preparation of two highly pure samples at each emission wavelength of interest its practicability is highly questionable. Wong and Halpern (1976) found that, by adjusting the measured function $P(\lambda_E,t)$ so that all counts at times longer than 3.6 ns after the function maximum were reduced by 20%, a function was obtained that yielded successful results when used in the deconvolution of decay curves of certain samples. Such an entirely empirical technique is not recommended since it could seriously mask real non-exponentiality in the decay curves of interest.

Probably the least objectionable of the techniques involving reference compounds was proposed by Szabo and co-workers (Rayner *et al.*, 1976, 1977). Consider a compound, R, which absorbs at a wavelength λ_a, shorter than the wavelength of excitation of the sample, and that emits both at this excitation wavelength and at the wavelength at which the sample's emission is observed. In addition, assume that the decay of this reference compound is wavelength independent [i.e., $G^R(\lambda_E,t)=G^R(\lambda_e,t)$]. One measures four functions: $P(\lambda_E,t)$ (Equation 2.20); $I(\lambda_e,t)$ (Equation 2.22); $I^R(\lambda_E,t)$ (Equation 2.24); and $I^R(\lambda_e,t)$ (Equation 2.25).

$$\begin{aligned} I^R(\lambda_E,t) &= E(\lambda_a,t)\otimes G^R(\lambda_E,t)\otimes H(\lambda_E,t) \\ &= E(\lambda_a,t)\otimes G^R(\lambda_e,t)\otimes H(\lambda_E,t) \end{aligned} \tag{2.24}$$

$$I^R(\lambda_e,t) = E(\lambda_a,t)\otimes G^R(\lambda_e,t)\otimes H(\lambda_e,t) \tag{2.25}$$

Convolve $I(\lambda_e,t)$ with $I^R(\lambda_E,t)$ to get

$$I(\lambda_e,t)\otimes I^R(\lambda_E,t) = G(\lambda_e,t)\otimes I^R(\lambda_e,t)\otimes P(\lambda_E,t). \tag{2.26}$$

Since $I^R(\lambda_e,t)$ and $P(\lambda_E,t)$ have been measured, Equation 2.26 is deconvolved to yield the decay of interest, $G(\lambda_e,t)$. Although the proposers of this technique have achieved excellent results, some drawbacks are obvious. For instance the reference compound, R, must have a broad fluorescence

wavelength range suitable for each sample of interest. It is therefore likely that a number of reference compounds, all in a high state of purity, will be required. In addition, the decay of the reference fluorescence must be wavelength independent; this property of decay times has been investigated only to a very limited extent. Finally, propagated errors are likely to be very large since four functions rather than two are involved in deconvolution. Nevertheless, the ratio correction technique is at present the best alternative to the zero-time shift outlined in Chapter 6 in experiments affected by a wavelength-dependent PM response.

2.4 Standards

In the following four chapters we describe the individual components of an SPC fluorimeter and the mathematical methods with which SPC data are analysed. During the course of this description we shall emphasize that a large number of instrumental and mathematical factors, some of them quite subtle, may interfere with the success of a fluorescence lifetime measurement. For example, excessive discrimination of the fluorescence PM pulses can bias the data collection towards multiphoton events in such a way that, while measured lifetimes will be in error, simple criteria by which data analysis is judged may indicate success. Consequently it is of the utmost importance to have available a range of standard compounds with definitely established single exponential decay times, which can be used to test the performance of the instrument.

Recently we have, together with our colleagues, reviewed the literature for standard compounds and prepared a list of lifetimes that we have measured ourselves (Lampert *et al.*, 1983). In general, the agreement between lifetimes measured in different laboratories is good, but there are some startling discrepancies. These discrepancies may arise from different states of purity of solvent or solute, from strong temperature dependence in the lifetime of interest or from maladjusted instruments or inadequate mathematical analysis techniques. For one compound, quinine bisulphate in sulphuric acid, the reported lifetimes show a wide variation. We believe that this compound has a double exponential fluorescence decay (Meech *et al.*, 1982) and we strongly caution against its use as a fluorescence lifetime standard. Perhaps the most popular standard is a dilute solution of anthracene in cyclohexane, the decay time of which at 25°C is independent of excitation and emission wavelength. Before use both the anthracene and cyclohexane must be rigorously purified as residual impurities have in the past led some workers to assume a rather low value for the decay time. It should also be noted that the decay time is temperature, viscosity and concentration dependent (Blatt *et al.*, 1982). In

Table 2.1

Compound	Solvent	Wavelength of observation of emission/nm	Lifetime, τ_0/ns
PPO[a]	Cyclohexane	440	1.42
PPO[a]	Cyclohexane (undegassed)	440	1.28
Anthracene	Cyclohexane	405	5.23
Anthracene	Cyclohexane (undegassed)	405	4.10
1-Cyanonaphthalene	Hexane	345	18.23
1-Cyanonaphthalene	Vapour[b]	345	23.77
1-Methylindole	Cyclohexane	330	6.24
3-Methylindole	Cyclohexane	330	4.36
3-Methylindole	Ethanol	330	8.17
1,2-Dimethylindole	Ethanol	330	5.71
DMNA[c]	CH_2Cl_2	375	2.40

[a] PPO is the symbol for 2,5-diphenyloxazole.
[b] At 0.3 Torr vibrationally relaxed with 1 atmosphere cyclohexane; $t = 185°C$.
[c] DMNA is the symbol for *N*,*N*-dimethyl-1-naphthylamine.

Table 2.1 we list a number of compounds that can be purified by standard techniques and that have single exponential decay times. The lifetimes have been measured at least twice on our SPC instrument, which employs a synchronously pumped dye laser as an excitation source. The method of data analysis was reiterative least-squares fitting (see Chapter 6). Further lists of standard compounds are given by Birks (1970) and Berlman (1965) but many of the results in these publications were obtained with inaccurate, or at least, unsophisticated techniques and it is now advisable to rely on more recent data.

We have not treated the transfer of data to a computer, nor do we consider the type of computer to be used since there will be enormous variation from laboratory to laboratory.

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