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Abstracting reliable parameters from time-correlated single photon counting experiments I. A consideration of how many photons need to be collected and a simple pileup error correction for poly-exponential decay profiles without convolution of the lamp profile☆

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Abstract

An investigation of how many photons need to be collected, when using the time-correlated single photon counting method, in order to reproduce, within a reasonable accuracy, the generating function of a poly-exponential decay, indicates that this number is quite often considerably greater than that usually published. This poses a series of potential problems if considerably longer counting times are used. It is proposed that the solution is to collect data with substantial pileup. An analytical expression is given for correcting the errors due to pileup in poly-exponential decay profiles. The method, which requires only a knowledge of the STOP/START ratio and the experimental decay profiles, generates results basically identical to those obtained when pileup is intentionally limited, even for high pileup distortion, thus greatly reducing the time necessary to make measurements.

Keywords: TCSPC; Poly-exponential decay; Correction of pileup error; Number of photons necessary

1. Introduction

The time-correlated single photon counting (TCSPC) method is the dominant method used for determining fluorescence decay times in the pico- and nanosecond time realms. These in turn are necessary in order to calculate excited singlet state kinetic rate constants. However, in the case of systems where more than one species is present in the excited state, even when the rate constants are not of primary interest, determining the time-resolved spectra is often necessary in order to understand [1] what chemical processes are operative. The basic idea of TCSPC is to split an exciting light pulse into two, the first of which passes through a photomultiplier tube (PMT), which in turn starts a voltage ramp (START signal). The second excites the sample. The first photon emitted from the sample and detected by the emission PMT then halts the voltage ramp (STOP signal) and the voltage built up by the ramp is proportional to the time between the excitation and emission processes. One count is then stored in the channel of a multi-channel analyzer (MCA), which corresponds to that time value. This procedure is repeated many times to

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have a sampling of times that faithfully represent the decay profile of the compound(s) being studied.

Because fluorescence normally occurs on the nanosecond (or faster) time scale and photomultiplier tubes have recovery times in this region, only the first photon (STOP) to arrive at the PMT after each excitation (START) pulse can be taken into account. The neglect of the later pulses causes a warping of the measured profile toward shorter decay times. This effect is called the pileup error and is minimized by running the experiment at very low "STOP/START" ratios, typically around 1–2%. This guarantees that rarely does a second photon arrive at the detector ($\leq$0.02%, in the case given above) and effectively eliminates pileup errors.

As long as the fluorescence decay profile is mono-exponential, using low "STOP/START" ratios poses no great problem. Standard wisdom for many years was that decay profiles needed to cover at least three decades, in order to guarantee that that there were no additional exponential terms in the fitted function. This implies at least 10^3 pulses in the maximum channel. More recently, it was shown [2] that, under optimum conditions, the lifetime of a mono-exponential decay could be determined, within an accuracy of 10%, with only 185 detected photons.

However, this result does not permit direct extrapolation to the cases of decay profiles whose order is not known a priori, much less those cases known to be poly-exponential, which are far more interesting. (In general, systems which fall into this category are those which undergo chemical reactions which occur on the same time scale as decay, as well as micro heterogeneous and almost all biological systems.) In qualitative terms, it is fairly obvious that the number of pulses necessary to guarantee recovery, within a given accuracy, of the $2n$ generating parameters (Eq. (1))

$$I(t) = A_1 \exp\left(\frac{-t}{\tau_1}\right) + \cdots + A_n \exp\left(\frac{-t}{\tau_n}\right) \quad (1)$$

will depend on the number of exponential terms, the accuracy desired, the relative values of the τ_i's and A_i's, as well as the optimization of the time scale of the MCA. In fact, only $2n-1$ (all the τ_i and the A_i/A_1 ratios) parameters need to be optimized. The absolute pre-exponential values are unnecessary for calculating excited state rate constants because the calculated function can be normalized to the same area as the experimental decay profile. But, having gotten this far, still does not permit one to estimate the number of pulses that it is necessary to collect in the MCA for a given set of parameters. As will be shown below, this number is often considerably larger than what is usually presented in the literature. The apparent implication is that, using low "STOP"/"START" ratios, much longer counting times should be used. This implies the possibility of introducing other errors into the measurement, due to instability of the sample (often a critical consideration when studying biological samples) and/or the equipment. In addition, longer determination times imply additional financial costs. This problem has been treated previously [3–9] however almost always limiting consideration to the case of mono-exponential decay. It has been pointed out that pileup errors can be minimized experimentally by, in addition to the usual technique of using low "STOP/START" ratios, discarding [6] all START pulses which produce two (or more) STOP pulses and multiplexing [8] which introduces more than one detector. In addition, corrections for pileup error [6,8,9] can be made in the mathematical treatment of the decay data.

Expansion of this problem to explicitly include any consideration of poly-exponential decay profiles [10] is considerably rarer. However, in this case the objective was limited to showing that it is possible to correct for pileup error in the case of a single bi-exponential decay profile.

What follows below is, to the best of our knowledge, the first general consideration of how many photons need to be collected in TCSPC experiments in order to obtain reliable parameter fits for poly-exponential decay profiles. Considering that this number is found to be significantly greater than what is normally collected, it is suggested that it is almost inevitable to run multi-exponential decay profiles under conditions of high "STOP"/"START" ratios. A simple method for correcting pileup errors, independent of the number of exponentials, is given.

2. Mathematical treatment

In order to scrutinize the proposed pileup correction algorithm, pileup corrupted data based on Monte Carlo experiments, which accurately mirror real data, were generated numerically. These data are analyzed subsequently and the results are compared with the original analytical generating functions. The quality of the recuperation of the generating parameters is then measured by an arbitrarily scaled function.

2.1. Generation of the pseudo-experimental decay profile, with pileup error

Starting with the initial τ_i (in units of channel number) and A_i-values, the analytical decay profile $I(t)$ is calculated from eq (1), for a convenient number (L) of channels, usually 500. The vector $I(t)$ is then normalized to have a sum of 1.0, i.e., $\sum[I_{\text{norm}}(t)] = 1.0$. The individual amplitudes of $I_{\text{norm}}(t)$ now represent the probability of a photon, which arrives within the time range of the MCA, falling into that channel (t). The MCA is then "reconstructed" into channels whose time widths are the values of I_{norm} at the corresponding t-values. A predetermined number of random numbers, whose value varies uniformly between 0 and 1.0, when assigned to the appropriate channel according to their value, should then be capable of generating a decay profile which would be the integer equivalent of that which could be generated by the analytical function; however, with the addition of correctly distributed random noise. But this decay profile would not contain pileup error.

In order to generate a simulated decay profile which also includes pileup error, using this Monte Carlo method, it was necessary to consider the following. The probability f_j of lamp pulses that result in j photons reaching the detector is given by the Poisson distribution. Experimentally, we know the "STOP"/"START" ratio $f \equiv \sum f_j (j \geq 1)$ and thus $f_0 = 1 - f$. The distribution can then be calculated as:

$$f_j = f_0 \frac{\left[\ln(1/f_0)\right]^j}{j!} \tag{2}$$

The f_j's, when multiplied by the number of lamp pulses (M) and converted into integer form, define the absolute number of events (N_j) in which j photons arrive at the detector. Only those q terms are retained that result in numbers $N_j \geq 1$. The Monte Carlo pseudo-experimental profiles $I_{MC}(t)$ were then constructed, as follows: for each value of $N_j \geq 1$, j random numbers are generated, *only the lowest being retained* and assigned to the appropriate channel in the MCA according to its value, as determined by I_{norm}. It should be noted that as f approaches its maximum value of 1.0, the N_j-values, other than N_0 and N_1 become increasingly important, or even dominant.

2.2. Analysis of the previously generated Monte Carlo decay profile

It is assumed that the value of f is known and constant. Commercial TCSPC instruments monitor and allow the user to read out both the number of lamp flashes per second ("START") and number of photons detected per second ("STOP"). These tend to be fairly constant, especially the former, once the instrument stabilizes. The Poisson distribution of events with j photons reaching the detector is calculated by eq. (2).

In general practice, the order of the poly-exponential is not known and various values are tried. For simplification, it is assumed here that this has been done and the correct order arrived at. Initial guessed parameters (τ_i, A_i) are fed in and a "true" analytical vector $I(t)$ generated from these parameters, eq (1). This distribution is correct for those N_1 events where exactly one photon is reaching the detector. Those events with more than one photon reaching the PMT result in a detected decay profile that is distorted towards shorter lifetimes. Considering first the two-photon events, the probability, $p(r)$, that a particular photon at time $t = r$ is detected is equal to the probability that the other photon reaches the detector at a time $t > r$. This probability is given by the ratio of the integrals over the "true" signal from r to ∞, divided by the integral from 0 to ∞. As the data are discrete, the integrals are replaced by the respective sums. Also, because in practice only a finite number of channels are used, the sum is taken from 1 until L.

$$p(r) = \frac{\sum_{t=r}^{L} I(t)}{\sum_{1}^{L} I(t)} \tag{3}$$

For three photons, the probability for observation is equal to the probability that both of the other photons are arriving after r, etc. Thus, the observed distributions, $I_j(t)$, for the j-photon events are:

$$I_j(t) = I(t)p(t)^{j-1} \tag{4}$$

where the multiplication is done element-by-element. Each distribution, $I_j(t)$, is normalized and multiplied by the number of events N_j. The calculated distribution, $I_{calc}(t)$, is the sum over all q terms of weighted $I_j(t)$:

$$I_{calc}(t) = \frac{\sum_{j=1}^{q} N_j I_j(t)}{\sum_{1}^{L} I_j(t)} \tag{5}$$

The sum of the squared weighted differences of χ $\{\chi(t) = [I_{MC}(t) - I_{calc}(t)]/\mathrm{sqrt}[I_{calc}(t)]\}$ is minimized, using the Newton–Gauss–Marquardt [11] algorithm, by varying the initial parameters. Convergence is considered to be attained when, in two consecutive iterations, the sums of the squares of the differences in the two profiles does not differ by more than 10^{-4}.

It can be noted that compared to previously published methods [4,10] the task of pileup correction is approached differently. Here, the theoretically calculated profiles are pileup-corrupted and these curves are compared with the actual measurements. In earlier approaches, the original data are "de-corrupted" and the derived profiles are compared with the theoretical ones. Pileup de-corruption of real and, thus, noisy data is inferior because error propagation is difficult to ascertain, particularly with high f-values. The corruption computation of theoretical data, however, is correct (within numerical precision) and thus comparison with the native, noisy data is statistically sounder. The additional computation time due to pileup corruption of the theoretical data during the fitting is not significant, as the formulae are explicit.

Accuracy in recovering the parameters which generated the Monte Carlo profile was judged using the Derringer D-function [12]. This function is defined as the product of the individual D_i-values, which measure the accuracy in recovery of each initial parameter. Arbitrarily, for any recovered parameter which was within 5% of the original generating value, $D_i = 1.0$. For any recovered parameter which was outside the limit of 50% from the original generating value, $D_i = 0$. Intermediate cases are calculated from eq. (6).

$$D_i = \frac{0.50 - \Delta}{0.45} \tag{6}$$

where Δ is the difference in fractional terms. It is reasonable to expect that more stringent demands for accuracy, as reflected in the defining Derringer parameters, should merely indicate the necessity for collecting a greater number of pulses.

All calculations were done using several different versions of MATLAB [13]. Copies of the subroutines are available from any of the authors.

3. Results and discussion

The quality of parameter recovery, as indicated by the Derringer Function, as a function of the number of pulses collected in the maximum channel was tested and the results of a few arbitrarily chosen combinations of parameters (τ's and ratio of the pre-exponential terms) are shown in Figs. 1 (bi-exponential decays) and 2 (tri-exponential decays), both include cases of rise times. All values refer to $f = 0.80$, unless indicated otherwise. The parameter values recovered reflect the average of five runs in all cases. These figures indicate how the variations in the parameters can have a large effect on the ease of recuperating them and that even the most difficult cases seem to converge if enough pulses are counted.

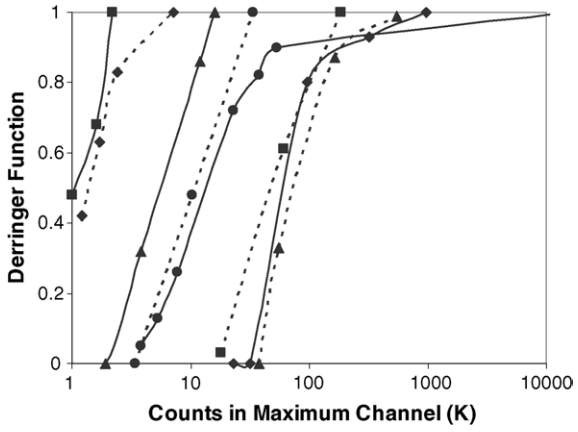

Fig. 1. Derringer function value as function of counts in maximum channel (in thousands) for different initial parameters of biexponentials. (A) (dotted line/circles) $\tau_1 = 5$, $\tau_2 = 50$, $A_2/A_1 = 10$; (B) (dotted line/squares) $\tau_1 = 10$, $\tau_2 = 30$, $A_2/A_1 = 3$; (C) (solid line/diamonds) $\tau_1 = 5$, $\tau_2 = 50$, $A_2/A_1 = 30$; (D) (dotted line/triangles) $\tau_1 = 10$, $\tau_2 = 30$, $A_2/A_1 = 9$; (E) (solid line/triangles) $\tau_1 = 5$, $\tau_2 = 50$, $A_2/A_1 = 3.33$; (F) (solid line/circles) $\tau_1 = 10$, $\tau_2 = 30$, $A_2/A_1 = 1$; (G) (dotted line/diamonds) and (H) (solid line/squares) $\tau_1 = 10$, $\tau_2 = 30$, $A_2/A_1 = -1$ (i.e., rise-time). $f = 0.8$ (A–G), $f = 0.9$ (H).

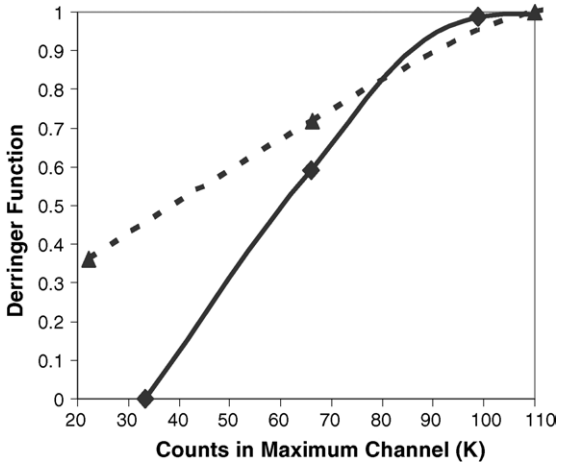

Fig. 2. Derringer function value as function of counts in maximum channel (in thousands) for different initial parameters of triexponentials. (A) (solid line) and (B) (dotted line) $\tau_1 = 5$, $\tau_2 = 20$, $\tau_3 = 60$, $A_3/A_1 = 12$, $f = 0.8$. $A_2/A_1 = 4$ (A) and $A_2/A_1 = -4$ (i.e., rise-time, B).

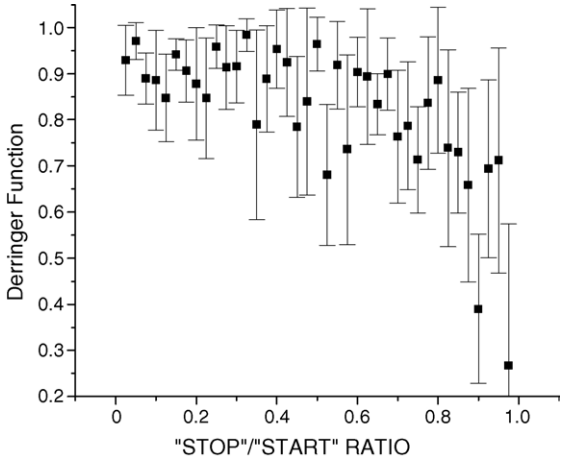

Fig. 3. Effect of varying the "STOP"/"START" ratio on model parameter recuperation.

From inspection of the results it is obvious that, to attain a reasonable recuperation of the generating values, accumulating a much larger number of pulses in the MCA than what is normally practiced is sometimes necessary.

Fig. 3 shows the variation of parameter recovery quality as f is varied. The data from this figure were generated by the tri-exponential

$$I(t) = \exp\left(\frac{-t}{10}\right) + 2\,\exp\left(\frac{-t}{50}\right) + 5\,\exp\left(\frac{-t}{100}\right) \qquad (7)$$

f was varied at intervals of 0.025, from 0.025 through 0.975. In order to have a reasonable basis of comparison, the number of pulses counted in each profile was the same, M being $10^6/f$. Twenty-five profiles were determined for each f-value, being five groups of five. In each group of five, the average was taken and the error flags represent the standard deviation of the five averages from the overall average. It appears reasonable to conclude that this variation is purely statistical and that no tendency for deterioration of the recovered parameters with increasing f-values is observed, at least as long as $f \leq 0.8$. It is important to point out that the measuring time at $f = 0.025$ is more than 30 times longer than at $f = 0.8$, with essentially identical parameter recovery. This can be a very valuable gain in terms of avoiding photo-degradation as well as problems related to instrument stability. The overall results are considered to be strong evidence that the pileup error correction procedure introduced here is correct. An incorrect procedure would be expected to either under-correct (more likely) or over-correct for pileup error, which would "tilt" the results shown in Fig. 3.

That the decrease in parameter recovery quality at very high f-values would be expected experimentally can be illustrated by the following consideration. Consider a TCSPC experiment in which the f-value is continually increased for successive decay profiles, for example, by opening the shutters. In principle, this can be done until $f = 1$, the point at which every lamp pulse causes a photon to be counted at the PMT. Upon opening the shutters even further, on the average,

even more photons per lamp pulse will reach the PMT, however the instrument will continue registering $f = 1$. Thus, at the upper limit, the photon distribution, and therefore the pileup correction which need be applied, is indeterminable. What would be expected at f-values approximating 1? Within experimental error, various distinct photon distributions would be capable of generating essentially the same f-value. Also, the slow tail of any distribution is increasingly reduced with increasing f-value and the recorded decay is increasingly compressed into early channels, rendering differences in lifetimes undetectable. Thus, it would not be advisable to carry out experiments at extremely high values and it seems reasonable to assume that the correction system presented here is valid for the entire range of f-values in which one would want to work.

Both Figs. 1 and 2 show that recovery of the generating parameters, for the cases of one rise time also converge to $D = 1$ as one increases the number of pulses collected, and, compared to the results for the same τ-values, but both being decay, this convergence is attained with even fewer collected photons when one of the terms refers to a rise time. The recovery is considerably worse (in fact, monotonic convergence was not observed) when $f = 0.90$, as compared to $f = 0.80$, consistent with the conclusion drawn from the results in Fig. 3.

4. Conclusions

It has been shown that using the TCSPC method, reliable recovery of generating parameters for poly-exponential decay profiles is possible, if one collects enough photon pulses. The number of pulses required is often considerably greater than that usually collected. In order to be able to do so within a reasonable period of time counting should be done with “STOP”/“START” ratios in the order of 0.80, i.e. approximately two orders of magnitude greater than that usually used. A method for correcting the resulting pileup error is given.

When real data is treated it is often necessary to convolute the trial fitting function with an experimentally determined (by scattering) lamp profile, unless the τ-values are considerably greater than the pulse width of the lamp. This procedure can potentially introduce additional uncertainties in the fit, especially because the lamp profile used for the convolution step is not the same as that which generated the decay profile. These additional uncertainties should be subject to minimization by increasing the number of pulses collected. This question will be taken up in the next paper of this series.

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