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Maximum entropy analysis of data simulations and practical aspects of time-resolved fluorescence measurements in the study of molecular interactions ☆

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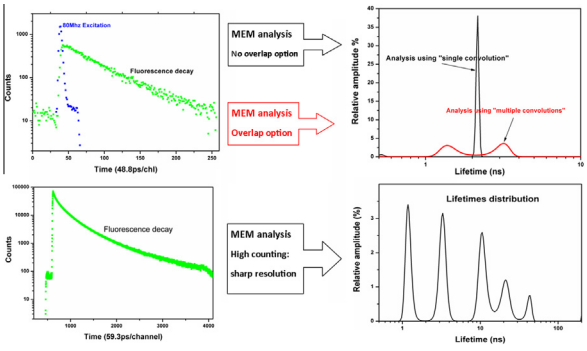
HIGHLIGHTS

- Advantage of prior knowledge option in the quantified MEM software of data analysis.
- Decays overlap should be taken into account in FLIM data analysis.
- Usefullness of simulated data with non-random noise for accuracy limit estimation.
- Increasing counting yields more accurate rotational correlation time determination.
- Too fast acquisition of excitation profile causes time shift and distortion.

GRAPHICAL ABSTRACT



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ABSTRACT

Time-resolved fluorescence spectroscopy and microscopy is increasingly used to probe molecular interactions and dynamics events in vitro and in vivo. We point out some pitfalls to avoid in the data acquisition procedure using time correlated single photon counting. A good accuracy in fluorescence decay measurements is not only linked to the counts in the peak channel but also to the statistics at the end of the curve. A too narrow time interval between successive excitation leads to an overlap of decays, and that should be taken into account in data analysis. The counting rate in the peak channel of the excitation profile should be close to the one of the fluorescence decay.

Many distributions of lifetimes can fit an incomplete or noisy data set to satisfying precisions corresponding to close values of χ^2. The maximum entropy principle is appropriate to distinguish among these in a consistent way. It is also shown that encoding a prior knowledge about the system study dramatically improves the quality of the recovered distribution, particularly in case of a set of discrete components. Based on simulated noisy quantify maximum entropy method (MEM) data analysis, we propose a simple strategy for estimating the quality of information and inferences we can draw from experimental results.

1. Introduction

Time-resolved fluorescence spectroscopy (TRF) and microscopy provide information on the temporal and spatial dynamics of molecules in solution, and in the cellular context give information about molecular interactions, lateral and rotational diffusion, and conformational fluctuations; these are particularly important parameters in characterizing biological systems.

Abbreviations: CPC, Count Peak Channel; FLIM, Fluorescence lifetime imaging; FRET, Förster resonance energy transfer; MCA, Multi-Channel Analyzer; TRF, Time-resolved fluorescence; TCSPC, Time correlated single photon counting.

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Fluorescence spectroscopy has two dimensions. First, the energy dimension is basically associated with the wavelengths of excitation and emission processes; second, the temporal dimension is related to the kinetics of the various processes at work until to return to ground state. In extending fluorescence spectroscopy to the time domain, a new field of information is available but more sophisticated technologies are needed than for steady-state fluorimetry, especially in the case of a short pulse source of excitation and fast sensitive detectors. Since complex kinetics can be determined from time-resolved fluorescence measurements, the three key items of information are lifetimes, the rotational correlation time, and the short range distance between molecules, from which inference can be made about molecular interactions.

This paper deals only with pulsed time-resolved fluorescence using time correlated single photon counting (TCSPC) technology, which is now the most common because of its high sensitivity, notable dynamic range and well-defined data statistics, and practically enabling visual examination and quick comparison of decays. In addition, present portable computer speed enables quick data analysis. In contrast to steady state fluorimetry, the time-resolved approach had been limited by technical difficulties and equipment cost. Today, advances in pulsed tunable lasers, detectors, time measurement electronics and computer performance allows the technique to be extended to new fields of research. These include biological application using 3D microscopy imaging and fluorescence lifetime imaging (FLIM) with intense femtosecond laser for two-photon excitation [1–4]. In the time dimension, the rotational and translational Brownian motion of molecules in solution can be monitored [5–7] even at the single molecule level [8–10]. Photoselection of an ensemble of orientated molecules by a fast linearly polarized pulse of excitation make it possible to monitor the speed of return to random orientation during the time interval in which fluorescence emission takes place. This is fluorescence anisotropy (polarization) decay. Analysis of fluorescence anisotropy decay provides information about the hydrated volume, geometry and internal flexibility of molecule alone or in interaction.

"One of the key aims of science is to gain increasing knowledge from experimental activities. The results of measurements must be compared to prior information and prediction, and the appropriate conclusions are then drawn about the model(s) under test, among other things, the number and quality of the measurements that can be taken are restricted by practical constraints on available time and apparatus capabilities" [11,12]. The amount and accuracy of information that can be extracted from data depends on the amplitude of the errors and also how the data analysis accounts for non-random errors. The TCSPC technique yields high quality data based on technological progress, but TRF experiments must be performed and analyzed with care.

The various pitfalls in sample preparation and optical artifacts have been already reviewed [13]. The present work is intended to provide insight into some limitations in applying the TCSPC technique, and to suggest precautions as well as a procedure to estimate, from simulations, the limits of such experiments.

2. Data acquisition

Many factors are involved in the accuracy of time-resolved fluorescence data. There are three main types of experimental uncertainty/error. First is random error arising from the single photon counting process itself; second are systematic errors due to limitations of the detection device and electronics, mainly timing and counting measurement errors; third are external perturbations such as radio frequency interference with electronics and laser instability in the intensity and pulse shape. For a detailed description of TCSPC technology, see the book by Becker [14].

3. Count in Peak Channel (CPC)

The accuracy in the lifetime determined from the data depends on the number of counts collected in the decay and also on parameters such as the time window Tw while the fluorescence decay lasts, the channel width δt, the number of channels k, and the amplitude of the background.

The time window should be long enough to capture the exponential decay over five decades. If the measured decay lasts five times the longest lifetime then the signal damping is $\exp(-Tw/\tau) = \exp(-5) = 0.67\%$ assuming a Dirac excitation profile, and the Count in the Peak Channel (CPC) reaches 10,000,then the count in the last data channel falls to 67 with an error of 8 (if we approximate the Poisson noise by a Gaussian distribution). For a CPC value of 100,000 we extend the time window to 6 times the lifetime value.

For good resolution, the width of the storage channel δt must be well below the shortest lifetime. Modern electronics offers flexibility in the channel width and in the number of channels so as to ensure a timescale covering the entire decay. Too many too narrow channels means a lower accuracy in each channel, and a longer time of measurement due to the dead time needed by the multichannel analyzer to store counts [14].

For multi-exponential decay it is necessary to consider how many counts must be collected in the entire decay and how many in the peak channel for good resolution of each individual component, or whether there is evidence for a distribution of lifetimes. Theoretical analysis can be used to estimate the minimum number of counts needed to determine the lifetime with a given uncertainty [15], with and without background. As an example, a few hundred counts in 256 channels are sufficient for a lifetime of 2 ns within 10% accuracy. However, 400,000 counts would be needed for two exponentials (2 and 4 ns) without background [15]. This number increases substantially when several exponentials with various amplitudes are to be separated. In practice, the user should run several simulations to estimate the total number of counts in the decay and the count in the peak channel which provide accurate results with the relevant analysis software [11,15–17].

As an example, the complex fluorescence decay of CdTe quantum dots in water [18] is shown in Fig. 1, with the result of data analysis in Fig. 1b. For good accuracy of short lifetimes the channel width δt is rather small (59.3 ps) and the requirement for following the decay as long as possible is satisfied with 4096 channels corresponding to a time window of 243 ns; the laser pulse frequency is 2 MHz.

4. Pileup

In the TCSPC technique the true probability distribution of the fluorescence decay is obtained correctly if the recorded photon is the only one reaching the detector prior to the next excitation. Owing to the strong intensity of excitation using a solid state laser the counting rate may be high and multiple events may occur at a single excitation. The time information comes from the first photoelectron generated by the photocathode which is then amplified as a pulse in the detector. Subsequent photoelectrons are ignored until the multichannel analyzer has stored the event and this is liable to distort the histogram of emission times. Assuming that the emitted photons falling on the photocathode of a detector conform to Poisson statistics and the emission of a photoelectron by the photocathode obeys a binomial law (1 or 0), the distribution of photoelectrons follows Poisson statistics.

If the quantum yield of the photocathode is q and N is the average number of photons received at the photocathode, the probability of detection of n photoelectrons is:

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