TCSPC Research

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Feburary 2024

1 Non-linear least squares fit assumptions

The primary objective of least squares is to evaluate whether a given mathematical model is consistent with the data and to ascertain the parameter values that have the highest likelihood of being accurate. However, this estimation process hinges on several critical assumptions [1]:

1. All experimental uncertainty resides solely in the dependent variable (y-axis).

2. The uncertainties in the dependent variable (measured values) follow a Gaussian distribution centred on the correct value.

3. No systematic errors affect either the dependent (y-axis) or independent (x-axis) variables.

4. The assumed fitting function accurately describes the system mathematically. Incorrect models yield incorrect parameters.

5. Each data point represents an independent observation.

6. There is a sufficient number of datapoints so that the parameters are overdetermined.

These assumptions generally hold true for Time-Correlated Single Photon Counting (TCSPC), making least squares a suitable method for analysis in such cases. However, in scenarios where these assumptions are not met – such as when variables are transformed to achieve linear plots, errors deviate from a Gaussian distribution, or errors affect the x-axis – least squares may not be the most appropriate analysis method. This is particularly evident in situations with a small number of photon counts, such as TCSPC measurements on single molecules. Nonetheless, TCSPC data typically align well with the assumptions of least squares analysis, facilitating its effective application in this context.

2 Sum of exponential fits

In the analytical framework of Time-Correlated Single Photon Counting (TCSPC), a sophisticated method deployed for the quantification of fluorescence decay profile often exhibits complexity that cannot be adequately modelled by a singular exponential function. This complexity arises from the presence of multiple fluorescence decay characteristics. To address this complexity, the decay curve is conventionally represented as a composite of multiple exponential decay components, with each component embodying a distinct fluorescent entity or lifetime within the sample. The mathematical representation of the fluorescence decay profile is articulated as follows:

$$I(t) = \sum_{i=1}^{n} a_i e^{-\frac{t}{\tau}_i} + b \tag{1}$$

Where *I(t)* denotes the fluorescence intensity at time, a_i represents the pre-exponential factors that signify the amplitude or contribution of each component to the overall fluorescence, τ are the lifetimes associated with the fluorescent components, b is a constant encapsulating any background signal, and n is the number of exponential components incorporated within the model[2].

The process of fitting the sum of exponential functions to the TSCPC data entails the determination of the parameters a_i and τ_i that optimally correspond to the observed fluorescence decay.

3 Problems with high correlation of parameters

An issue that can occur with exponential fitting for decay is that the amplitudes and decay lifetimes can be highly correlated. This causes the fit to be sensitive to initial guess parameters(ill-conditioning) and could lead to bias in the parameters. Furthermore, it makes the fit hard to interpret as, due to the variance increasing, 1 variable influences another variable and both variables affect the result. Another possible consequence is over-fitting. To fix the issues different approaches can be taken. If the fit was optimized using a computer algorithm a constraint on how large or small the parameters are can be placed. Introducing a new parameter that stabilizes the size of parameters can also be possible. For example, the original equation would be

$$x(t) = \sum_{i=1}^{M} A_i e^{-\frac{t}{\tau}_i} \tag{2}$$

But with new parameters it would be

$$x(t) = \sum_{i=1}^{M} A_i B_i e^{-\frac{t}{\omega_i \tau}_i} \tag{3}$$

where B and omega are to stabilize the fit. However, this can potentially lead to over-fitting and the interpretation of all the fit parameters becomes harder to understand.

4 Effects of noise

TCSPC stands as a pinnacle technique for fluorescence lifetime measurements, lauded for its sensitivity, precision, dynamic range, and data accuracy. This method is distinguished by its ability to disentangle complex multicomponent decays through deconvolution or tail fitting, enabling the identification of multiple lifetime components within a sample. A core strength of TCSPC is its governance by will-defined Poisson noise, which, combined with high timing accuracy achieved through constant fraction discrimination, elevates its capability to reach the quantum limits of sensitivity by recording single-photon event[3].

TCSPC Fluorescence Lifetime Imaging Microscopy (FLIM) further exemplifies the technique's prowess, delivering near-ideal photon efficiency and exceptional time resolution, independent of scanner speed. The signal-to-noise ratio in TCSPC FLIM is solely dependent on the number of recorded photons, thus directly tying the quality of data to the total acquisition time and available photon rate from the sample. The technique's time resolution is determined by the laser pulse width and the detector's transit-time spread, with fast hybrid detectors achieving an Instrument Response Function (IRF) width of less than 20 ps (FWHM). This high resolution allows for the resolution of decay functions into several components, revealing even those decay components typically obscured within the IRF[4].

One often-misunderstood aspect of TCSPC is the Pile-Up Effect, where the detection of a second photon within the same signal period as a previous one could potentially distort the recorded optical waveform. Contrary to the commonly cited pile-up limit of 0.1% of the excitation rate, the correct threshold is, in fact, ten times the excitation rate, or 10% of it. This correction, addressing a long-standing error propagated from a typo in TCSPC literature from the late 1970s, significantly relaxes constraints on the maximum count rate, allowing for a much higher throughput of photon detection than previously believed without inducing photo-induced changes in the molecular structure of the sample[5].

5 Resolution of closely spaced lifetime

To resolve closely spaced lifetimes, such as those exhibited by anthranilic acid (AA) and 2-amino purine (2-AP)[6] in this scenario, a multi-faceted approach is essential. Initially, employing multi-decay-time models rather than single exponential models facilitates capturing the intricate decay behaviour observed in mixtures of fluorophores. It's crucial to evaluate residuals, which represent the discrepancies between the fitted model and actual data, to gauge the goodness of fit, particularly noting systematic deviations indicative of multiple decay times. Chi-squared analysis provides a quantitative means to compare different fitting models, with a significant decrease in chi-square favouring models with multiple decay times. Additionally, confidence interval analysis aids in estimating uncertainties associated with recovered lifetimes, considering correlations between parameters, especially as lifetimes become closer.

Conducting measurements at additional wavelengths and employing global analysis is a practical strategy for improving the resolution of closely spaced lifetimes. Global analysis operates on the assumption that decay times remain consistent across all wavelengths while allowing for variations in amplitudes. Decay times are considered global parameters since they remain uniform across datasets, whereas amplitudes are non-global, being subject to differences among datasets. By applying global analysis to multi-wavelength data, chi-square surfaces become steeper [6], indicating an increased likelihood of accurately recovering lifetimes. The certainty associated with determining lifetimes is enhanced through global analysis due to the sharper chi-square surfaces and lower Fx values resulting from greater degrees of freedom provided by additional data. This methodology ensures a more precise characterization of the system's behaviour, with amplitudes obtained from global analysis better aligning with individual emission spectra compared to those derived from single-wavelength data. Thus, leveraging multiple wavelengths and global parameterization offers an effective approach for resolving closely spaced lifetimes.

6 Research on COBYLA method

COBYLA stands for Constrained Optimization By Linear Approximations. It is an iterative method for derivative-free constrained optimization. The method maintains and updates linear approximations to both the objective function and to each constraint. The approximations are based on objective and constraint values computed at the vertices of a well-formed simplex. Each iteration solves a linear programming problem inside a trust region whose radius decreases as the method progresses toward a constrained optimum.

Note that COBYLA treats simple bounds as constraints, which can cause bound violations.Suited for noisy functions but comes at a computational cost.Due to the fact that linear approximations tend to be inefficient at a higher number of variables, this algorithm is suited mostly for small dimension numbers of n less than 9.

The COBYLA method is a variation of Powell's nonlinear derivative-free constrained optimization that uses a linear approximation approach. Mathematically it wants to minimize a function f(x)

$$min_{x\in\Omega} f(x) \tag{4}$$

where f : $\Re^{\mathbf{n}} \rightarrow$ R is the objective function and $\mathbf{\Omega}\subseteq\Re^{\mathbf{n}}$ represents the feasible region(regions where the equation can be minimized). The equation to be solved in the

feasible region is given by

$$\Omega^{def} = [x \in \Re^n : c_i(x) \geq 0, i = 1, ..., m] \quad (5)$$

where $_{\mathbf{i}} : \Re^n \rightarrow R$ denotes the ith constraint function for each $\mathbf{i} \in [\mathbf{1}, ..., \mathbf{m}]$. All of the constraints are assumed to be obtained only through function values.

7 Hessian matrix to get covariance

The covariance matrix algebraically equals the negative inverse of the Hessian matrix, as long as the Hessian matrix is positive definite. The diagonal entries of the covariance matrix are the square of each variance and the other entries are the covariance between two independent variables.

8 How does number of photons improve the fit?

The number of photon counts significantly affects the fit of multi-exponential decay models, particularly in determining the resolution of parameter values. While a single-exponential decay can be accurately determined even with a small number of observed photons, for multi-exponential decays, it is crucial to measure as many photons as possible to achieve higher resolution in parameter values. In an illustrative example with a two-component mixture of p-terphenyl and indole [6], when the number of counts in the peak channel was tenfold less compared to a higher count scenario, the correct values for the two decay times were still recovered, but the relative decrease in the reduced chi-square for the two decay time models was much smaller. Increasing the number of counts improves the fit by providing more data points, resulting in a higher resolution of parameter values. With more photon counts, the chi-square surfaces rise more slowly as the lifetimes are varied, indicating that the lifetimes are determined with less precision when there are fewer counts. Therefore, increasing the number of photon counts leads to a smoother and more precise determination of decay lifetimes in multi-exponential decay models.

9 How does changing the range improve the fit?

The range of data points can drastically influence the fitting quality. At shorter ranges, the amplification of noise levels renders the data unsuitable for conducting meaningful analysis.[7] With data points too close to each other, it may exhibit high correlation, which is not suitable for multi-exponential decay fitting.

Besides, incorporating an excessively broad spectrum of data points is prone to artefacts of overfitting, wherein the model picks up on noise instead of the fundamental decay pattern. It is also worth mentioning that the duration for acquisition is constrained by both pile-up effects and the processing time of the time-measurement circuitry[8], so it is not pragmatic to process a big pile of data for fitting. By appropriately adjusting the range, it is easier to mitigate overfitting and promote the model's ability to generalise effectively to fresh data.

References

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