Correction of experimental distortions in analysis of fluorescence intensity decays by the phase plane method

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Abstract: *In this paper we advance the phase plane method for analysis of fluorescence intensity decays to take into account the presence of such instrumental artifacts as time-uncorrelated background radiation and time shift between instrumental response function and sample fluorescence intensity decay. The developed non-iterative algorithm does not require prior selection of initial guesses and ensures accurate estimates for parameters. The results of simulation experiments are presented to demonstrate the applicability of the developed method.*

Keywords**:** Fluorescence decay analysis, Phase plane method, Time shift, Background.

1. INTRODUCTION

Study of structure and dynamics of molecular systems in biology, chemistry and medicine requires application of informative and very sensitive experimental methods. Time-resolved fluorescence spectroscopy is one of the most widely used techniques employed for such investigations [1, 2, 3]. Among experimental fluorescence spectroscopy methods that are applied for getting information about the sample under study the best known is time-correlated single photon counting (TCSPC) [1]. This experimental approach is used to measure as single fluorescence decays [1] so fluorescence decay images in Fluorescence Lifetime Imaging Microscopy [4]. Sample fluorescence decay and instrumental response function (IRF) measured by this method are usually subjected to the influence of experimental distortions [2, 3] which can hardly be removed even in optimized experimental setups. Therefore, an important task is to develop appropriate analysis methods that can explicitly take into account the presence of instrumental distortions in the measured data.

The aim of the fluorescence decay analysis is to obtain the estimations of fit parameters that characterize the sample and belong to the selected model. For many molecular systems the fluorescence dynamics can be adequately approximated by the sum of exponentials [1]. The common approach that is frequently used to estimate unknown parameter values is based on least squares method. In this method the fit parameter values are searched to minimize the chi-square statistical criterion used to estimate the conformity between measured fluorescence decay and theoretical curve generated by the model [1]. The least squares method requires the application of iterative optimization routines [5, 6] for the models that are nonlinear with respect to the fit parameters (for example, multi-exponential model). The iterative algorithms are general enough to take into account instrumental distortions that present in measured data [2, 3]. However, the efficiency of these routines substantially depends on the initial guesses that should be selected for the fitted parameters. If the initial guesses are chosen far from their optimal values iterative fitting can be very time consuming. Moreover, if behavior of χ^2 is complex in the space of fit parameters the iterative procedure with inadequate initial guesses can be trapped in the local minimum thus delivering biased estimations for parameters.

Along with the iterative fit algorithms that can be applied in a similar way to fit fluorescence decays by different models, a number of non-iterative approaches have been developed for particular models. The common idea that underlies these methods consists in specific transformation of the mathematical model. Taking into account particularity of the model makes these algorithms fast and independent of the initial guesses. For the multi-exponential model, the most often used non-iterative methods are the Laplace transform method [7], method of moments [8], method of modulating functions [9] and phase plane method [10]. The influence of the instrumental distortions has to be explicitly accounted in these methods to ensure correct data processing. Such corrections so far have been made only for the Laplace transform method [7] and method of moments [8]. However, these methods require careful selection of the additional parameters (the parameter of transformation for the Laplace transform method and the cut-off ratio for the method of moments) that can greatly affect the fit as shown in [11, 12]. The phase plane method has been proven to be one of the most rigorous non-iterative algorithms, which does not require any extra information.

In this paper we revise the phase plane method for the analysis of fluorescence intensity decays obtained in the presence of time-uncorrelated background radiation and time shift between sample decay and instrumental response function.

2. METHOD

As the instrumental response function in TCSPC experiment differs from the delta function, the measured fluorescence intensity decay of the sample is represented by the convolution integral:

$$f(t) = g(t) \otimes I(t), \tag{1}$$

where $I(t)$ is an impulse response function of the sample;

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$g(t)$ is an IRF obtained in a separate measurement where the sample is replaced by the scatter [13]. The multi-exponential impulse response $I(t)$ takes the form:

$$I(t)=\sum_{k=1}^{n}a_k e^{-t/\tau_k}, \qquad (2)$$

where n is the number of exponentials, a_k and τ_k, $k=1,\dots,n$ are the amplitudes and decay times, respectively. The aim of the fit is to find estimations of a_k and τ_k.

According to the phase plane method [10], the convolution integral (1) with the multi-exponential representation (2) is equivalent to the integral equation:

$$\sum_{k=0}^{n}c_k F_k(t)=\sum_{k=1}^{n}r_k G_k(t), \qquad (3)$$

where

$$\begin{aligned}
&F_0(t)=f(t)\\
&F_k(t)=\int_o^t f(x)(t-x)^{k-1}/(k-1)!\,dx\,.\\
&G_k(t)=\int_o^t g(x)(t-x)^{k-1}/(k-1)!\,dx\\
&k=1,\dots,n
\end{aligned} \qquad (4)$$

Coefficients $c_k, k=0,\dots,n$ and $r_k, k=1,\dots,n$ in equation (3) should be fitted and may be further used to estimate τ_k as roots of the polynomial [10]:

$$\sum_{k=0}^{n}(-1)^k c_k \tau^{k-n}=0, \qquad (5)$$

and to estimate a_k as:

$$a_m=\frac{\sum_{k=0}^{n-1}(-1)^k r_{k-1}\tau_m{}^k}{\prod_{j=1,j\neq m}^{n}\left(1-\frac{\tau_m}{\tau_j}\right)},\ m=1\dots n\,. \qquad (6)$$

If instrumental distortions are present, the undistorted sample fluorescence intensity decay $f(t)$ and IRF $g(t)$ can be expressed through the corresponding distorted functions. Therefore we can modify equation (3) to take into account new representation of $f(t)$ and $g(t)$.

We consider two types of instrumental distortions in this paper. The first is the time shift between sample fluorescence decay and IRF. As IRF is measured on the excitation wavelength whereas sample fluorescence decay is measured on the emission wavelength, fluorescence decay is shifted to the red side of spectra (Stocks shift [1]). Photons with different wavelengths have different speeds in monochromator and different transition times in photomultiplier (transition time of photons with longer wavelength is longer). The second distortion is time-uncorrelated background added to the sample fluorescence decay and IRF. This can be due to, for example, dark noise of the detector.

If we combine these two types of distortions the undistorted IRF will be defined as:

$$g(t)=g^*(t-\Delta)-\beta\,, \qquad (7)$$

where Δ denotes a shift between sample and IRF curves, β is a time uncorrelated background in IRF and $g^*(t)$ is the distorted IRF.

By expanding function $g^*(t-\Delta)$ in Taylor series one obtains:

$$\begin{aligned}
g^*(t-\Delta)&=\sum_{k=0}^{\infty}\left(g^*(t)\right)^{(k)}(-\Delta)^k/k!\approx\\
&\approx g^*(t)-\Delta\left(g^*(t)\right)'
\end{aligned} \qquad (8)$$

As the expansion in equation (8) is truncated by the two terms of Taylor series, such approximation can be used when Δ is relatively small. Substituting equation (8) in eq. (7)**Ошибка! Источник ссылки не найден.** yields:

$$g(t)=g^*(t)-\Delta\left(g^*(t)\right)'-\beta\,, \qquad (9)$$

Undistorted sample fluorescence intensity decay is defined as follows:

$$f(t)=f^*(t)-\varepsilon\,, \qquad (10)$$

where ε is a time-uncorrelated background in sample fluorescence decay and $f^*(t)$ denotes distorted sample fluorescence intensity decay.

Now functions $F_k(t)$ and $G_k(t)$ in (4) can be expressed in terms of their distorted counterparts. Substituting (9) and (10) into (4) we get:

$$\begin{aligned}
G_k(t)&=\int_0^t\left(g^*(x)-\Delta\left(g^*(x)\right)'-\beta\right)\frac{(t-x)^{k-1}}{(k-1)!}dx=\\
&=G_k^*(t)-\Delta G_{k-1}^*(t)-\beta\frac{t^k}{k!}
\end{aligned}, \qquad (11)$$

$$F_k(t)=\int_0^t\left(f^*(x)-\varepsilon\right)\frac{(t-x)^{k-1}}{(k-1)!}dx=F_k^*(t)-\varepsilon\frac{t^k}{k!}\,.$$

where $F_k^*(t)$ and $G_k^*(t)$ are defined as

$$\begin{aligned}
&F_k^*(t)=\int_o^t f^*(x)(t-x)^{k-1}/(k-1)!\,dx\\
&G_k^*(t)=\int_o^t g^*(x)(t-x)^{k-1}/(k-1)!\,dx\,,\\
&k=1,\dots,n
\end{aligned} \qquad (12)$$

Substituting equations (11) into (3) yields:

$$\begin{aligned}
&\sum_{k=0}^{n}c_k F_k^*(t)+\sum_{k=0}^{n}(\beta r_k-\varepsilon c_k)\frac{t^k}{k!}=\\
&=\sum_{k=0}^{n}(r_k-\Delta r_{k+1})G_k^*(t)
\end{aligned}, \qquad (13)$$

where $r_0=r_{n+1}\equiv 0$. Equation (13) can be rewritten using new designations:

$$\sum_{k=0}^{n} X_k^F F_k^*(t) + \sum_{k=0}^{n} X_k^T T_k(t) - \sum_{k=0}^{n} X_k^G G_k^*(t) = 0, \qquad (14)$$

where $T_k(t) = t^k / k!$ and coefficients $X_k^F = c_k$, $X_k^T = \beta r_k - \varepsilon c_k$ and $X_k^G = r_k - \Delta r_{k+1}$ ($k = 0 \ldots n$) depend only on parameters of the multi-exponential model, a_k and τ_k ($k = 1 \ldots n$), and distortion parameters Δ, β and ε.

Equation (14) is a linear functional relationship with respect to the coefficients X_k^F, X_k^T and X_k^G ($k = 0 \ldots n$). Since $F_k^*(t)$ and $G_k^*(t)$ are functions of measured data that are distorted by statistical noise, the left-hand side of equation (14) will never be exactly equal to 0. Therefore the coefficients X_k^F, X_k^T and X_k^G can be found using the linear least-squares method [14, 15] by minimizing the quadratic form:

$$\chi^2 = \sum_{i=1}^{N} \left(\sum_{k=0}^{n} X_k^F F_{ki}^* + X_k^C T_{ki} - X_k^G G_{ki}^* \right)^2 = \min, \qquad (15)$$

where N is a number of points in measured data. In order to avoid trivial zero solution an additional restriction to the coefficients $X_0^F = c_0 = 1$ should be imposed.

Since coefficients X_k^F, X_k^T and X_k^G have been found, the parameters of multi-exponential model, a_k and τ_k ($k = 1 \ldots n$), and the distortion parameters Δ, β and ε can be calculated. The shift parameter Δ can be obtained as one of the roots of polynomial:

$$\sum_{k=0}^{n} \Delta^k X_k^G = 0, \qquad (16)$$

Taking into account that $r_0 \equiv 0$ and $c_0 = 1$, the time-uncorrelated background ε in the sample fluorescence intensity decay can be calculated using coefficient X_0^T:

$$\varepsilon = -X_0^T, \qquad (17)$$

Since the shift parameter Δ has been identified from equation (16) coefficients r_k ($k = 1 \ldots n$) can be restored as:

$$r_k = \begin{cases} X_n^G & k = n \\ \Delta r_{k+1} - X_k^G & k = 1 \ldots n-1 \end{cases}. \qquad (18)$$

The time-uncorrelated background, in IRF is estimated as:

$$\beta = \frac{X_n^T - X_0^T X_n^F}{X_n^G}, \qquad (19)$$

As soon as coefficients $c_k = X_k^F$ and r_k are known from (18) we can find the decay times τ_k and pre-exponential factors a_k from equations (5) and (6).

3. TESTING

Standard phase plane method (3) and phase plane method with corrections for instrumental distortions (13) have been implemented and tested on simulated data to prove the applicability of the proposed algorithms. Each simulated data set contains instrumental response function and sample fluorescence decay generated at 512 time channels. The width of time channel is 0.04. All time values are expressed in relative units. The IRF $g(t)$ is modeled as [3]:

$$g(t) = \exp(-0.6t) - \exp(-1.1t) \qquad (20)$$

Sample fluorescence decay $f(t)$ is calculated as a digital convolution of discrete representation of IRF $g(t)$ (eq. (20)) and sample impulse response function $I(t)$ (eq. (2)). In all simulations, we use bi-exponential (n = 2, $a_1 = 0.67$, $\tau_1 = 1$, $a_2 = 0.33$, $\tau_2 = 2$) function for $I(t)$. The instrumental distortions are implemented by shifting sample fluorescence decay to the right with respect to IRF and by adding time-uncorrelated background to both $g(t)$ and $f(t)$. Finally, statistical noise is added to $g(t)$ and $f(t)$ by rescaling them to the predefined maximum values (10^4 for IRF and $5*10^3$ for sample decay curve) and further replacing the exact values of both curves at all time points by realizations of Poisson random variable with the mean value equal to the corresponding exact value.

Each simulation experiment consists of analyzing 100 simulated data sets, each one with the different realization of statistical noise. After analysis of each data set the estimated parameters of multi-exponential model and distortion parameters are obtained and stored to calculate the mean values:

$$M(p) = \frac{1}{100} \sum_{i=1}^{100} p^i, \qquad (21)$$

and the variance:

$$\sigma^2(p_k) = \frac{1}{100} \sum_{i=1}^{100} \left(p^i - M(p)\right)^2, \qquad (22)$$

where p^i are the estimators obtained after the analysis of the i-th data set. p^i can be substituted either by one of multi-exponential model parameters (either a_k or τ_k, $k = 1 \ldots n$) or by one of distortion parameters (Δ, β or ε).

The results of simulation experiments are presented in table 1 for different combinations of the distortion parameter values (Δ, β and ε). The results show that the developed modification of the phase plane method is capable of correcting the instrumental distortions ensuring accurate estimates for the double-exponential model parameters as well as for the distortion parameters. The estimates obtained using the modified algorithm are more accurate than the corresponding values calculated with standard phase plane method at all tested time shifts and time-uncorrelated backgrounds. Moreover, at larger values of the time shift and time-uncorrelated background the standard phase plane method can not resolve two exponentials at all.

Table 1. Double-exponential fit by the standard phase plane method (PPM, eq. (3)) and phase plane method with corrections for instrumental distortions (PPMD, eq. (13)). Sample decay peak channel is $5*10^3$ counts. IRF peak channel is 10^4 counts. Number of time channels is 512. Width of a channel is 0.04. *M* is the mean value of the estimator (eq. (21)), σ is the standard deviation of the estimator (eq. (22)).

True values			Method	Estimations							
Δ	***β*, counts**	***ε*, counts**			**a_1 (0.67)**	**τ_1 (1.0)**	**a_2 (0.33)**	**τ_2 (2.0)**	**Δ**	***β*, counts**	***ε*, counts**
0	0	0	PPM	*M*	0.67	1.00	0.33	2.01	-	-	-
				σ	0.035	0.039	0.035	0.054	-	-	-
			PPMD	*M*	0.68	1.01	0.32	2.04	0.005	7.7	5.0
				σ	0.079	0.087	0.079	0.144	0.0086	8.98	6.52
0.020	50	25	PPM	*M*	0.54	1.01	0.46	1.79	-	-	-
				σ	0.065	0.065	0.065	0.055	-	-	-
			PPMD	*M*	0.69	1.02	0.31	2.06	0.020	44.1	20.1
				σ	0.078	0.087	0.078	0.170	0.0148	16.0	11.3
0.020	100	50	PPM	*M*	0.99	1.41	0.01	17.48	-	-	-
				σ	0.004	0.014	0.004	32.99	-	-	-
			PPMD	*M*	0.68	1.01	0.32	2.05	0.025	81.9	36.5
				σ	0.074	0.082	0.074	0.165	0.0131	14.7	10.3
0.040	50	25	PPM	*M*	0.84	1.29	0.16	2.17	-	-	-
				σ	0.056	0.040	0.056	0.146	-	-	-
			PPMD	*M*	0.70	1.02	0.30	2.07	0.039	52.2	26.1
				σ	0.075	0.083	0.075	0.152	0.0149	14.4	10.2
0.040	100	50	PPM	*M*	unresolved				-	-	-
				σ					-	-	-
			PPMD	*M*	0.70	1.03	0.30	2.08	0.038	103.4	51.9
				σ	0.073	0.076	0.073	0.146	0.0138	14.0	9.8

Another advantage of the modified algorithm, as compared to the standard one, is that it also gives reasonable estimates for the instrumental parameters (time shift and background), which can be considered as valuable supplementary information for the experimenter.

4. CONCLUSION

In this paper we describe the algorithm of phase plane method corrected for accounting the presence of such instrumental distortions as time-uncorrelated background and time shift between sample fluorescence intensity decay and instrumental response function.

The undertaken testing shows that in contrast to standard phase plane method proposed algorithm allows to obtain acceptable estimations for multi-exponential model and distortion parameters at different levels of instrumental distortions.

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