Time Resolved Fluorescence & Anisotropy Data Processor Advanced

Version 1.4 for Windows 95/98/NT 4.0/ME/2000, Windows XP/Vista/7 32-bit
(works in WOW 32-bit emulator of Windows XP/Vista/7 64-bit)

Supported Methods and Models

Fluorescence decays analysis

Two deconvolution methods are supported for the analysis of fluorescence decays:

1. Deconvolution using Instrumental Response Function (IRF)
2. Deconvolution using single-exponential Reference Compound Function (REF)

In the first case (deconvolution with IRF) all models are based on the following common equation:

$$f^{th}(t) = \left(g(t+\delta) - b \right) \otimes I\left(t, \vec{A}\right) + \gamma B(t) + c + n\left(g(t+\delta) - b \right) \qquad (1)$$

where $g(t+\delta)$ denotes the instrument response function with a time shift of δ relative to the emission response; $B(t)$ is the measured intensity of background emission (Bg) and Rayleigh/Raman scatter contributing with a relative weight of γ, b and c are constant values for dark noise and time-uncorrelated background photons in g and f respectively; $I\left(t, \vec{A}\right)$ represents a model function with the vector of fit parameters $\vec{A}$ and n is the scattered light coefficient. Note: background is not subtracted from measured decay but added to theoretical decay accordingly to Eq. 1.

In the second case (Reference deconvolution) all models use the following general equation:

$$f^{th}(t) = \left(f_{ref}(t) - b \right) \otimes \left(\delta(t) I\left(0, \vec{A}\right) + \frac{I\left(t, \vec{A}\right)}{\tau_{ref}} + \frac{dI\left(t, \vec{A}\right)}{dt} \right) + \gamma B(t) + c \qquad (2)$$

where $f_{ref}(t)$ denotes the measured one-exponential reference compound decay with decay time τ_{ref}; $B(t)$ is the measured intensity of background emission contributing with a relative weight of γ; b and c are the levels of time-uncorrelated background in reference and sample decays respectively, $\delta(t)$ - Dirac delta-function.

The software provides the deconvolution of the multi-excitation decays [24, 25] (see figure bellow).
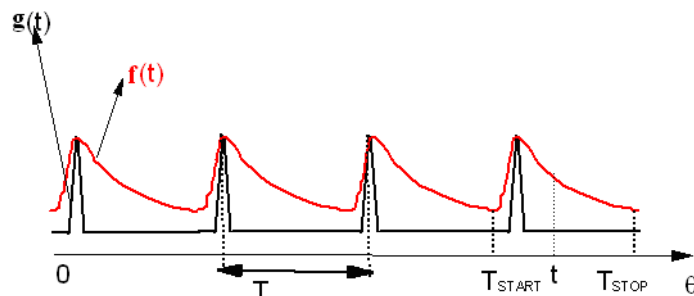
Usually in this type of experiments t changes from $-\infty$ to T_{STOP}. The formula for the convoluted sample decay takes the form

$$f(t) = g(t) \otimes I(t) = \int_{-\infty}^{t} I(t-\theta) g(\theta) d\theta. \quad (3)$$

If a finite lower limit (e.g. $t = 0$) is used then $f(t)$ is only more or less correct in the last interval, because the fluorescence excited by some of amount the previous pulses has to be taken into account.

Note: The Eqs. 1 and 2 can be applied for fitting both measured and simulated data. The simulated data can be obtained by the built-in simulator.

Since the calculations with equations described above require a set of measured data (fluorescence decays, IRF/REF, background) we introduce a term **Data Set** that will be further used as a reference to the complete set of analyzed data. Thus, **Data Set** contains all necessary information that describes the data, related to the one measurement (simulation). In the case if data come from polarization experiment **Data Set** may contain either both parallel and perpendicular components or separately one of them. Also **Data Set** can contain some values (External parameters) that define the conditions under which the data was measured (e.g. temperature, etc.).

Besides the model-specific parameters included in the vector $\vec{A}$ a number of common parameters (that participate in the Eqs. 1 and 2) can be estimated during the analysis. A set of available fit parameters in each case is defined by the configuration of each particular data set. Common fit parameters are:

Parameter name	Description
Shift	Non-integer channel-shift of IRF to the sample decay (δ in Eq. 1)
IRF/REF_Bg	Dark noise parameter (b in Eq. 1 and 2)
TCBg_factor	Time correlated background multiplication factor (γ in Eq. 1 and 2). Defines the relative weight of background emission $B(t)$. This parameter is available only in the case if model is attached to the data set that contains $B(t)$.
Const_Bg	Takes into account time-uncorrelated background photons in the sample decay (c in Eq. (1) and (2))
IRF_Contrib	Coefficient of scattered light contribution (n in Eq. 1)
tau_ref	A decay time of one-exponential Reference compound (τ_{ref} in Eq. 2). This parameter is available only if Reference deconvolution method is used.

The Dataset properties are listed below:

1. Time Step indicates the width of the time channel for measured (simulated) fluorescence decay. The value of this property is calculated as original time channel width that was set during measurement (simulation) multiplied on the value of **Binning** property.

2. ***Binning*** allows to join the values of measured (simulated) data taken from two or more neighboring channels. The value of this property must be integer and indicates the number of united channels.

3. ***Channels count*** indicates the total number of points in the measured (simulated) data. The value of this property is calculated as original number of points that was set during measurement (simulation) divided on the value of ***Binning*** property. This property is read only if ***Data Set*** contains measured data, otherwise it is changeable.

4. ***Start analysis at*** defines the initial point for analysis. The fluorescence decay contained in the ***Data Set*** is analyzed starting at this point.

5. ***End analysis at*** defines the end point for analysis.

6. ***Start criterion at*** specifies the initial point for the target fit criterion calculation (normally should be equal to ***Start analysis at*** but can differ for fine tuning).

7. ***Ref. life time*** keeps the value of life time of single exponential reference compound. This property is available only for data sets that contain reference compound data. The value of this property is used as initial guess for τ_{ref} fit parameter.

8. ***G-Factor*** keeps the value of G-Factor that is used for combining sample and reference compound parallel and perpendicular polarization components to the total decay (decay at magic angle). This property is available only for data sets that contain parallel and perpendicular polarization components.

9. ***Scatter G-Factor*** keeps the value of G-Factor that is used for combining Instrumental response function parallel and perpendicular polarization components to the total Instrumental response function (IRF at magic angle). This property is available only for polarized data.

10. ***Shift (Parallel Shift and Perpendicular Shift)*** keeps the value of shift between sample decay and Instrumental response function expressed in time channels. The value of this property is used as initial guess for δ fit parameter.

11. ***IRF/REF Bg (Par. IRF/REF Bg and Perp. IRF/REF Bg)*** keeps the level of time-independent background in Instrumental response function or Reference compound. The value of this property is used as initial guess for b fit parameter.

12. ***TC Bg factor (Par. TC Bg factor and Perp. TC Bg factor)*** keeps the multiplication factors for time dependent background. These parameters allow taking into account difference in measurement time between sample and background. For example, if sample decay was measured two times longer than background then ***TC Bg factor*** is 2. The value of this property is used as initial guess for γ fit parameter.

13. ***Const Bg (Par. const Bg and Perp. const Bg)*** keeps the level of time-independent background in sample total decay (sample parallel and perpendicular polarization components). The value of this property is used as initial guess for c fit parameter.

14. ***IRF Contrib. (Par. IRF Contrib. and Perp. IRF Contrib.)*** keeps the value of scattered light contribution in sample total decay (sample parallel and perpendicular polarization components). The value of this property is used as initial guess for n fit parameter.

Models:

All models that will be described below are used for calculation of δ-function response $I(t,\vec{A})$ within Eqs. 1 or 2 which are responsible for getting the final theoretical decay that is fitted to the measured data during the analysis. In result, if any new model is implemented, all instrumental parameters, mentioned above, are automatically provided for this model. Each model has some properties and set of fit parameters that form vector $\vec{A}$. The use of some models requires that corresponding data set has necessary external parameters (like Polarization angle, Temperature, Concentration, etc.). The external parameters are the values that are presented in the model formulas but do not appear as fit parameters.

All models have the following common properties:

Property name	Description
Constraints	This property displays the configuration dialog box that allows to add for current model the complex constraints (defined as special functions)
Fluor IG type	determines how the initial guesses for fit parameters are generated

Multi-exponential model

In a variety of applications, fluorescence decay of the sample and the sample anisotropy decay can be adequately approximated by a sum of exponentials [1-3]. The form of the equations that underlie the multi-exponential model depends on the model property FluorParametersType, which can be one of the following: "Amplitudes and decay times", "Contributions and decay times", "Ratio and decay times", "Paired ratio and Decay times":

Amplitudes and decay times. The model generates fluorescence decay accordingly to the formula:

$$I(t,\vec{A}) = \sum_{j=1}^{M} p_j e^{-t/\tau_j} \qquad (4)$$

where p_j and τ_j ($j=1,...,M$) are, respectively, the amplitudes and decay times of corresponding exponents; M is the number of exponents in the sum.

The following model-specific fit parameters are added:

Parameter name	Description
p	Amplitude of corresponding fluorescence component
tau	Decay time of corresponding fluorescence component

Contributions and decay times. Sometimes it is more convenient to use contributions of exponents instead of amplitudes. In this case pre-exponential factors are replaced by the normalization parameter N and contributions α_j and model formula takes the form:

$$I(t,\vec{A}) = N\sum_{j=1}^{M} \alpha_j e^{-t/\tau_j}, \quad \sum_{j=1}^{M} \alpha_j = 1. \qquad (5)$$

The sum of contributions is always equal to 1. Therefore such coefficients (being multiplied on 100) show the contribution of each exponent in percentage. Since in some cases the negative pre-exponential multipliers are required to describe the sample fluorescence decay (for example, in the case if δ-impulse response function of the sample $I\left(t,\vec{A}\right)$ rises at the beginning) the equations for $I\left(t,\vec{A}\right)$ can contain some exponents with pre-exponential factors less than 0. Switching between amplitudes and contributions is controlled by **Normalization** property of multi-exponential model and can be done independently for positive and negative pre-exponential factors. Therefore in the case if contributions are used, $I\left(t,\vec{A}\right)$ is calculated as follows:

1. The value of **Normalization** property is set to **Positive**:

$$I\left(t,\vec{A}\right)= N\sum_{j=1}^{M} \alpha_j e^{-t/\tau_j} + \sum_{j=0}^{M^n} p_j^n e^{-t/\tau_j^n} \qquad (6)$$

2. The value of **Normalization** property is set to **Negative**:

$$I\left(t,\vec{A}\right)= \sum_{j=1}^{M} p_j e^{-t/\tau_j} + N^n\sum_{j=0}^{M^n} \alpha_j^n e^{-t/\tau_j^n} \qquad (7)$$

3. The value of **Normalization** property is set to **Positive and Negative**:

$$I\left(t,\vec{A}\right)= N\left(\sum_{j=1}^{M} \alpha_j e^{-t/\tau_j} + r\sum_{j=0}^{M^n} \alpha_j^n e^{-t/\tau_j^n} \right) \qquad (8)$$

where p_j, α_j and τ_j ($j=1,...,\ M$) are respectively amplitudes, contributions and decay times of corresponding exponents with positive pre-exponential factors; N is normalization parameter for exponents with positive pre-exponential factors; p_j^n, α_j^n and τ_j^n ($j=0,...,\ M^n$) are respectively amplitudes, contributions and decay times of corresponding exponents with negative pre-exponential factors; N^n is normalization parameter for exponents with negative pre-exponential factors; M^n is a number of exponents with negative pre-exponential factors, r is the ratio of positive and negative normalization parameters.

In addition the normalization parameters N, N^n and r (we can call it Common Multipliers) can be either fitted or fixed to some value. If normalization parameters are fixed, the model is no longer holds the relations $\sum_{j=1}^{M}\alpha_j =1$ and $\sum_{j=1}^{M^n}\alpha_j^n =-1$ and preexponential factors fit the amplitude of corresponding fluorescence component.

Set of amplitude parameters can be easily recalculated to the pre-exponential factors and back. This is done by the software automatically while changing the set of parameters to use.

The list of additional fit parameters will be following:

Parameter name	Description
N	Normalization parameter for exponents with positive pre-exponential factors
N_n	Normalization parameter for exponents with negative pre-exponential factors

r	ratio of positive and negative normalization parameters
a	Contribution of corresponding positive fluorescence component
a_n	Contribution of corresponding negative exponent
tau	Decay time of corresponding fluorescence component
tau_n	Decay time of corresponding exponent with negative pre-exponential factor

Ratio and decay times. In this case pre-exponential factors are replaced by one normalization parameter N and ratio parameters r_{j-1}, where $j = 1...M$ and M is the number of exponents. Model takes the following form:

$$I\left(t, \vec{A}\right) = N\left(e^{-t/\tau_1} + \sum_{j=2}^{M} r_{j-1} e^{-t/\tau_j} \right) \qquad (9)$$

In this case model has the following additional fit parameters:

Parameter name	Description
N	Normalization parameter
r	Ratio of amplitude of corresponding fluorescence component to the amplitude of first fluorescence component
tau	Decay time of corresponding fluorescence component

Paired ratio and Decay times. In this case second pre-exponential factor from a pair is replaced by the ratio parameter $r_{2i,2i-1}$, where $i = 1...M/2$ and M is the number of exponents. Model takes the following form:

$$I\left(t, \vec{A}\right) = p_1(e^{-t/\tau_1} + r_{21} e^{-t/\tau_2}) + p_3(e^{-t/\tau_3} + r_{43} e^{-t/\tau_4}) + ... = \sum_{i=1}^{M/2} p_{2i-1}(e^{-t/\tau_{2i-1}} + r_{2i,2i-1} e^{-t/\tau_{2i}}) \qquad (10)$$

In this case model has the following additional fit parameters:

Parameter name	Description
p	Amplitude of first fluorescence component in each pair
r	Ratio of amplitude of second fluorescence component in each pair to the amplitude of the first fluorescence component from the same pair
tau	Decay time of corresponding fluorescence component

By default, the property ***FluorParametersType*** is set to "Amplitudes and decay times".

Multi-exponential model properties are:
1. ***FluorParametersType*** defines the form of equation (see Eqs. 4-10 above) that is used to generate the δ-impulse response function of the sample $I\left(t, \vec{A}\right)$;

2. ***FluorExponentsCount*** defines the number of exponents in Eq. 4-10 shown above. This property is available only in the case if ***FluorParametersType*** property is set either to ***Amplitudes and decay times*** or to ***Ratio and decay times***;

3. **PosFluorExponentsCount** defines the number of exponents with positive pre-exponential factors in equations shown above. This property is available only in the case if **FluorParametersType** property is set to **Contributions and decay times**;

4. **NegFluorExponentsCount** defines the number of exponents with negative pre-exponential factors in equations shown above. This property is available only in the case if **FluorParametersType** property is set to **Contributions and decay times**;

5. **Normalization** selects the type of positive and negative pre-exponential factors (amplitudes or contributions) that are used by multi-exponential model for calculating $I(t, \vec{A})$. This property is available only in the case if **FluorParametersType** property is set to **Contributions and decay times**.

6. **FitCommonMultiplier** selects the type of normalization of the preexponential factors. If this property is set to **True**, the normalization parameters N, N^n and r fits the common amplitude and sum of contributions is always equal to unity for positive exponents and to -1 for negative exponents. If the property is set to **False**, the model does not hold the relations $\sum_{j=1}^{M} \alpha_j = 1$ and $\sum_{j=1}^{M^n} \alpha_j^n = -1$. This property is available only in the case if **FluorParametersType** property is set to **Contributions and decay times**.

Gauss distribution of decay rates model

The gauss distribution of decay rates model [4, 5] generates the impulse response function of the sample $I(t)$ according to the following formula:

$$I(t, \vec{A}) = A\left\{ e^{-\frac{\sigma^2 t^2}{2} - \mu t - k_0 t} erfc\left(\frac{\sigma t}{\sqrt{2}} - \frac{\mu}{\sigma\sqrt{2}} \right) + \alpha e^{-\lambda_2 t} \right\} \qquad (11)$$

where A is the normalization parameter; σ is the standard deviation on decay rate; μ is average decay rate; k_0 denotes the minimum decay rate; *erfc* denotes the complementary error function; α defines the relative contribution of second fluorescence component; λ_2 is the inverted decay time of second fluorescence component.

Gauss distribution of decay rates model parameters are:

Parameter name	Description
A	is the normalization parameter;
sigma	is standard deviation on decay rate;
mu	is average decay rate;
k0	is minimum decay rate;
alpha	defines the relative contribution of second fluorescence component;
lambda2	is inverted decay time of second fluorescence component.

This model has no additional properties.

Poisson distribution of decay rates model

This model generates the fluorescence decay according to the following formula:

$$I\left(t,\vec{A}\right) = A\left\{e^{-(\lambda_1 + c\beta_1)t - c\mu\left(1 - e^{-\varepsilon t}\right)} + \alpha e^{-\lambda_2 t}\right\} \quad (12)$$

where A is the normalization parameter; λ_1 is the inverted decay time of first fluorescence component in absence of energy transfer or other quenching; c is concentration; β_1 is quenching efficiency of first fluorescence component; μ is average number of quenchers; ε is the rate of quenching for a probe interacting with one quencher; α defines the relative contribution of second fluorescence component; λ_2 is the inverted decay time of second fluorescence component in absence of energy transfer or other quenching.

This model has the following additional fit parameters:

Parameter name	Description
A	Amplitude parameter
lambda1	Inverted decay time of first fluorescence component in absence of energy transferor other quenching
beta1	Parameter for quenching efficiency of first fluorescence component
mu	Average number of quenchers seen by the probe
epsilon	Rate of quenching for a probe interacting with one quencher
alpha	Parameter that defines the relative contribution of second fluorescence component
lambda2	Inverted decay time of second fluorescence component in absence of energy transferor other quenching

Required external parameters:

External parameter name	Description
Concentration	This external parameter defines the concentration. The value of this external parameter is used as c in model formulas mentioned above.

This model has no additional properties.

Excited-state reactions

Compartmental models are used for analysis of *excited-state reactions*. Compartmental model in general form is defined by the following matrix equation:

$$I\left(t,\vec{A}\right) = DC\exp(tA)B \quad (13)$$

where: $I\left(t,\vec{A}\right)$ is a δ-impulse response function calculated according to the compartmental model; D is a proportionality coefficient; C is a $(1 \times N)$ vector: $C = \begin{bmatrix} C_1 & C_2 & \ldots & C_N \end{bmatrix}$ that contains the emission weighting factors of species $1, \ldots N$;

A is a $(N \times N)$ matrix: $A = \begin{bmatrix} A_{11} & A_{12} & \cdots & A_{1N} \\ A_{21} & A_{22} & \cdots & A_{2N} \\ \vdots & \vdots & \vdots & \vdots \\ A_{N1} & A_{N2} & \cdots & A_{NN} \end{bmatrix}$ that is defined by the rate constants of the

reactions between compartments in the system;

B is a $(N \times 1)$ vector: $B = \begin{bmatrix} B_1 \\ B_2 \\ \vdots \\ B_N \end{bmatrix}$ that represents the concentration of the species 1,…,N.

The current version of TRFA Data Processor Advanced software contains 2-compartmental and 3-compartmental models described bellow.

2-compartmental model

The 2-compartmental model [8, 9] is designed to describe the behavior of a dynamic, linear, time-invariant, intermolecular system consisting of two distinct types of ground-state species and two corresponding excited-state species. This system can be presented by the following scheme:
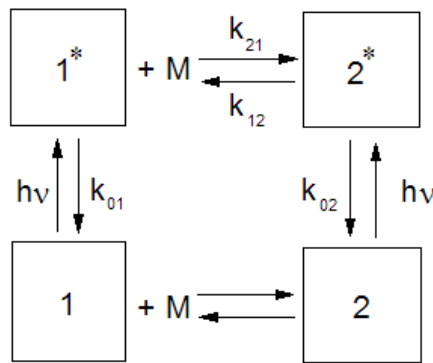
As it is shown on the scheme above ground-state species 1 can reversibly react with co-reactant M to form ground-state species 2. Excitation by light creates the excited-state species 1* and 2*, which can decay by fluorescence (F) and non-radiative (NR) processes. The composite rate constant for these processes is denoted by k_{0i} ($= k_{Fi} + k_{NRi}$) for species i* ($i = 1,2$). k_{21} represents the second-order rate constant for the association 1* + M → 2*, while k_{12} is the first-order rate constant for dissociation of 2* into 1* and M.

The impulse response function of the sample $I(t, \vec{A})$ for the system described above can be calculated according to the equation (10), where C is the 1 x 2 vector that contains the emission weighting factors of species 1* and 2* at emission wavelength; B is the 2 x 1 vector whose elements represent the concentration of species 1* and 2* at time zero. A is 2 x 2 matrix defined as follows:

$$A = \begin{bmatrix} -(k_{01} + k_{21}[M]) & k_{12} \\ k_{21}[M] & -(k_{02} + k_{12}) \end{bmatrix}$$

where k_{01}, k_{02}, k_{12}, k_{21} are the rate constants described above; $[M]$ is the co-reactant concentration.

2-compartmental model parameters are:

| Parameter name	Description
D	is the normalization parameter
C1	is the emission weighting factor of species 1*
C2	is the emission weighting factor of species 2*
K01	is the composite rate constant of species 1*
K12	is the first-order rate constant for dissociation of 2* into 1* and co-reactant M
K21	represents the second-order rate constant for the association 1* + M → 2*
K02	is the composite rate constant of species 2*
B1	is the concentration of species 1* at time zero
B2	is the concentration of species 2* at time zero

The 2-compartmental model contains no additional properties.

External parameters required by 2-compartmental model:
1. **[M]** is the co-reactant concentration.

3-compartmental model

The 3-compartmental model [10-12] is designed to describe the behavior of a causal, linear, time-invariant, intermolecular system consisting of three distinct types of ground-state species (1, 2, 3) and three corresponding excited-state species (1*, 2*, 3*). This system can be presented by the following scheme:
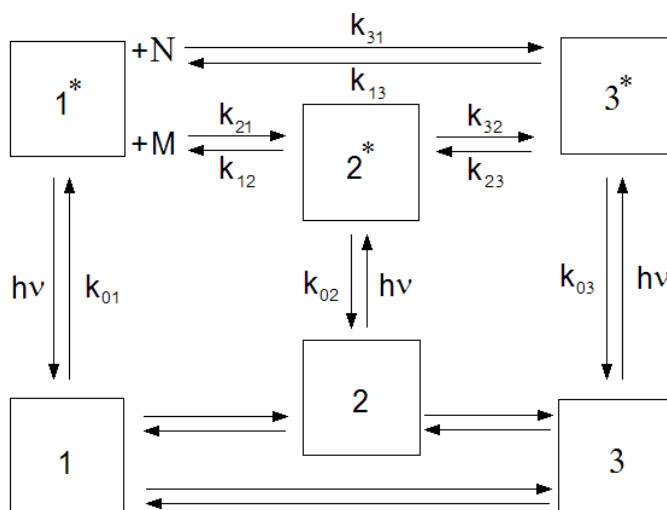
As it is shown on the scheme above species 1 (respectively 1*) can reversibly react with co-reactant M to form species 2 (respectively 2*). Also species 1 (respectively 1*) can reversibly react with co-reactant N to form species 3 (respectively 3*). Also the excited-state interconversion between states 2* and 3* exists. The excited-state species 1*, 2* and 3* created by light excitation can decay by fluorescence (F) and non-radiative (NR) processes. The composite rate constant for these processes is denoted by k_{0i} (= k_{Fi} + k_{NRi}) for species i* (i = 1, 2, 3). k_{21} and k_{31} represent respectively the second-order rate constants for the association 1* + M → 2* and 1* + N → 3*. The dissociations

$2^* \rightarrow 1^* + M$ and $3^* \rightarrow 1^* + N$ are respectively characterized by first-order rate constants k_{12} and k_{13}. The rate constants k_{23} and k_{32} describe the direct interconversion between species 2^* and 3^*.

The impulse response function of the sample $I(t, \vec{A})$ for the system described above can be calculated according to the equation (10), where C is the 1 x 3 vector that contains the emission weighting factors of species 1^*, 2^* and 3^* at emission wavelength; B is the 3 x 1 vector whose elements represent the concentration of species 1^*, 2^* and 3^* at time zero. A is 3 x 3 matrix defined as follows:

$$
A = \begin{bmatrix}
-(k_{01} + k_{21}[M] + k_{31}[N]) & k_{12} & k_{13} \\
k_{21}[M] & -(k_{02} + k_{12} + k_{32}) & k_{23} \\
k_{31}[N] & k_{32} & -(k_{03} + k_{13} + k_{23})
\end{bmatrix}
$$

where k_{01}, k_{12}, k_{13}, k_{21}, k_{02}, k_{23}, k_{31}, k_{32}, k_{03}, are the rate constants described above; $[M]$ and $[N]$ are concentrations for corresponding co-reactants.

3-compartmental model parameters:

Parameter name	Description
D	is the normalization parameter
C1	is the emission weighting factor of species 1^*
C2	is the emission weighting factor of species 2^*
C3	is the emission weighting factor of species 3^*
K01	is the composite rate constant of species 1^*
K12	is the first-order rate constant for dissociation of 2^* into 1^* and co-reactant M
K13	is the first-order rate constant for dissociation of 3^* into 1^* and co-reactant N
K21	represents the second-order rate constant for the association $1^* + M \rightarrow 2^*$
K02	is the composite rate constant of species 2^*
K23	is the interconversion rate constant from state 3^* to 2^*
K31	represents the second-order rate constant for the association $1^* + N \rightarrow 3^*$
K32	is the interconversion rate constant from state 2^* to 3^*
K03	is the composite rate constant of species 3^*
B1	is the concentration of species 1^* at time zero
B2	is the concentration of species 2^* at time zero
B3	is the concentration of species 2^* at time zero

The 3-compartmental model contains no additional properties.
External parameters required by 3-compartmental model:
1. $[M]$ is the co-reactant M concentration.
2. $[N]$ is the co-reactant N concentration.

Anisotropy analysis

The analysis of time-dependent anisotropy decays is very effective approach to study the structure of molecular systems, their behavior and processes that take place inside them. The shape of time-dependent anisotropy decays can be affected by the non-spherical form of the molecules under study, the segmental flexibility of the macromolecules, depolarization due to presence of resonance energy transfer between molecules of the same type, etc. Therefore the fit of the theoretical curve generated by the model to the measured anisotropy decay and estimation in this way the parameters that can affect the theoretical curve shape can deliver the information about the investigated molecular system.

The **_TRFA Data Processor Advanced_** software provides two approaches to perform the anisotropy analysis:

1. The _two stage anisotropy_ analysis based on sequential fit of measured sample total fluorescence decay and measured sample parallel and perpendicular polarization components.
2. The anisotropy analysis based on direct _global fit of sample parallel and perpendicular polarization components_. This approach also supports associative anisotropy analysis.

Two stage anisotropy analysis

This approach to the anisotropy analysis consists of two sequential stages and can be applied to the Datasets that contain both parallel and perpendicular polarization decay components.

On the first analysis stage the measured total fluorescence decay $f(t)$ is calculated from parallel $f_{//}(t)$ and perpendicular $f_\perp(t)$ decay components according to the equation:

$$f(t) = f_{\parallel}(t) + 2Gf_{\perp}(t), \qquad (14)$$

where G is a G-factor that can be calculated as the ratio of emission channel sensitivity for the vertically polarized light to the emission channel sensitivity for the horizontally polarized light at the sample wavelength. Further the measured total fluorescence decay is fitted to the model that generates the theoretical curve using the general equation that depends on the type of the Dataset.

In the case if measured data contain sample polarization decay components and Instrumental response function polarization components $g_{//}(t)$ and $g_\perp(t)$ the theoretical curve for total fluorescence decay is calculated by the following general equation:

$$f^{th}(t) = g(t) \otimes I(t, \vec{A}) + [\gamma_{\parallel}B_{\parallel}(t) + 2G\gamma_{\perp}B_{\perp}(t)] + [c_{\parallel} + 2Gc_{\perp}] + [n_{\parallel} + 2Gn_{\perp}]g(t), \qquad (15)$$

where

$$g(t) = g_{\parallel}(t + \delta_{\parallel}) - b_{\parallel} + 2G_{IRF}[g_{\perp}(t + \delta_{\perp}) - b_{\perp}] \qquad (16)$$

and $I(t, \vec{A})$ represents an δ-impulse response function of the total sample fluorescence decay that is different for each individual model; $B_{//}(t)$ and $B_\perp(t)$ are the measured parallel and perpendicular components of background emission intensity contributing with a relative weights of $\gamma_{//}$ and $\gamma_\perp$; $c_{//}$ and $c_\perp$ are values for time-uncorrelated background photons in the parallel and perpendicular sample decays; $n_{//}$ and $n_\perp$ are the scattered light coefficients; $\delta_{//}$ is the time shift in channels between

$g_{//}(t)$ and $f_{//}(t)$; $\delta_\perp$ is the time shift in channels between $g_\perp(t)$ and $f_\perp(t)$; $b_{//}$ and $b_\perp$ are the levels of dark noise in the parallel and perpendicular components of instrumental response function.

In the case if reference compound decay parallel and perpendicular polarization components have been obtained from the measurement instead of $g_{//}(t)$ and $g_\perp(t)$ the theoretical curve for total fluorescence decay is calculated by the following general equation:

$$f^{th}(t) = f_{ref}(t) \otimes \left(\delta(t) I(0,\vec{A}) + \frac{I(t,\vec{A})}{\tau_{ref}} + \frac{dI(t,\vec{A})}{dt} \right) + [\gamma_{\|} B_{\|}(t) + 2G\gamma_\perp B_\perp(t)] + [c_{\|} + 2Gc_\perp], \quad (17)$$

where

$$f_{ref}(t) = f_{ref\|}(t) - b_{\|} + 2G[f_{ref\perp}(t) - b_\perp], \quad (18)$$

$\delta(t)$ − Dirac delta-function; $f_{ref//}(t)$ and $f_{ref\perp}(t)$ denote the measured parallel and perpendicular components of one-exponential reference compound decay with decay time τ_{ref}. All other values in Eq. 17 and 18 are equal to corresponding values in Eq. 15 and 16.

The analysis of total fluorescence decay allows to obtain the estimations of model parameters that have an influence on the shape of the $I(t,\vec{A})$.

On the second stage of anisotropy analysis parameters obtained on the previous stage can be fixed and global analysis of parallel $f_{//}(t)$ and perpendicular $f_\perp(t)$ sample decay components is performed. Since both parallel and perpendicular sample decay components are stored in one data set the theoretical curves for both of them are generated by the same model object associated with given data set. Also theoretical curves for both polarization components depend on one set of fit parameters defined by the model.

In the case if measured data contain sample polarization decay components and instrumental response function polarization components $g_{//}(t)$ and $g_\perp(t)$ the theoretical curves for parallel and perpendicular sample decay components are calculated by the following general equations:

$$f_{\|}^{th}(t) = g(t) \otimes I_{\|}(t,\vec{A}) + \gamma_{\|} B_{\|}(t) + c_{\|} + n_{\|} g(t)$$
$$f_\perp^{th}(t) = g(t) \otimes I_\perp(t,\vec{A}) + \gamma_\perp B_\perp(t) + c_\perp + n_\perp g(t) \quad (19)$$

where

$$I_{\|}(t,\vec{A}) = \frac{1}{3} I(t,\vec{A})(1 + 2r(t,\vec{A})); \quad I_\perp(t,\vec{A}) = \frac{1}{3G} I(t,\vec{A})(1 - r(t,\vec{A}));$$

are respectively parallel and perpendicular components of impulse response function of the sample fluorescence decay and $r(t,\vec{A})$ is the model-dependent function that describes the anisotropy decay.

In the case if reference compound decay parallel and perpendicular polarization components have been obtained from the measurement instead of $g_{//}(t)$ and $g_\perp(t)$ the theoretical curves for parallel and perpendicular sample decay components are calculated by the following general equations:

$$f_{\parallel}^{th}(t) = f_{ref}(t) \otimes \left(\delta(t) I_{\parallel}(0,\vec{A}) + \frac{I_{\parallel}(t,\vec{A})}{\tau_{ref}} + \frac{dI_{\parallel}(t,\vec{A})}{dt} \right) + \gamma_{\parallel} B_{\parallel}(t) + c_{\parallel}$$

$$f_{\perp}^{th}(t) = f_{ref}(t) \otimes \left(\delta(t) I_{\perp}(0,\vec{A}) + \frac{I_{\perp}(t,\vec{A})}{\tau_{ref}} + \frac{dI_{\perp}(t,\vec{A})}{dt} \right) + \gamma_{\perp} B_{\perp}(t) + c_{\perp}$$

(20)

The global analysis of parallel and perpendicular sample decay components allows to obtain the estimations of model parameters that have an influence on the shape of the $I_{\parallel}(t,\vec{A})$ and $I_{\perp}(t,\vec{A})$ and thereby fit the anisotropy decay.

In order to switch between two analysis stages described above use the Experiment object property *Analysis type*. In the case if this property is set to *Anisotropy* the anisotropy decay and related dependencies can be viewed using Experiment window charts.
In the case if data set selected for the analysis contains polarization components, the model attached to such data set provides the following set of common fit parameters:

Parameter name	Description
TCBg_factor_par	Time correlated background multiplication factor ($\gamma_{\parallel}$ in Eqs. 15, 17, 19, 20) for parallel polarized decay. Defines the relative weight of parallel polarized background emission. This parameter is available only in the case if model is attached to the data set that contains background.
TCBg_factor_perp	Time correlated background multiplication factor ($\gamma_{\perp}$ in Eqs. 15, 17, 19, 20) for perpendicular polarized decay. Defines the relative weight of parallel polarized background emission. This parameter is available only in the case if model is attached to the data set that contains background.
Const_Bg_par	Accounts for time-uncorrelated background photons in the sample parallel decay component ($c_{\parallel}$ in Eqs. 15, 17, 19, 20)
Const_Bg_perp	Accounts for time-uncorrelated background photons in the sample perpendicular decay component ($c_{\perp}$ in Eqs. 15, 17, 19, 20)
IRF_Contrib_par	Coefficient of scattered light contribution in the sample parallel decay component ($n_{\parallel}$ in Eqs. 15, 19)
IRF_Contrib_perp	Coefficient of scattered light contribution in the sample perpendicular decay component ($n_{\perp}$ in Eqs. 15, 19)
tau_ref	A decay time of one-exponential Reference compound (τ_{ref} in Eq. 17, 20). This parameter is available only if Reference deconvolution method is used.

Anisotropy analysis based on the global fit

The ***TRFA Data Processor Advanced*** provides the ability to perform the anisotropy analysis as a global fit of fluorescence decays measured on the different polarization angles with respect to the excitation light.

To perform the anisotropy analysis in this way one should load the sample polarization decay components as separate Datasets. Each Dataset should contain the external parameter *Polarization angle* expressed in degrees. This external parameter is required by **anisotropy model** that was specially developed to perform anisotropy analysis via the global fit.

Anisotropy model should be associated with each Dataset (for each Dataset a separate model must be used, not the same one) and for all such models the parameters with the same name should be linked together. The only parameter of anisotropy model that must not be linked is the parameter *A* that is responsible for the fit of decay amplitude. Since the sample polarization decay components are considered as separate Datasets and fitted to separate models the general equations shown in the Analysis of single fluorescence decay section are used for generating theoretical curves by the anisotropy model.

The anisotropy model can be configured to perform *non-associative* and *associative* anisotropy analysis. The required type of the anisotropy analysis can be selected with *Associations property* of the model.

The anisotropy model is inherited from the multiexponential model (so, it inherits all properties and parameters of the multiexponential model) and generates the fluorescence decay according to the following formula:

$$I(t) = I_{fluor}(t) \times \left[1 + \left(3\cos^2\theta - 1 \right) \left(r_\infty + \sum_k T_{jk}\beta_k \exp(-t/\phi_k) \right) \right] \qquad (21)$$

where $I_{fluor}(t)$ is calculated by Eqs. 4-10 depending on the property FluorParametersType; Θ is the polarization angle, β_k and φ_k are, respectively, the amplitudes and rotational correlation times of corresponding anisotropy exponents and T_{jk} controls the associations: $T_{jk} = 1$, if fluorescence (from multiexponential model) and anisotropy components are associated, and $T_{jk} = 0$, if not.

By default the anisotropy model is configured for non-associative anisotropy analysis (all coefficients T_{jk} are set to 1). In the case of associative anisotropy analysis (only certain associations between fluorescence and anisotropy exponents exist) the coefficients T_{jk} that correspond to the unnecessary associations should be set to 0.

Required external parameters:

External parameter name	Description
Polarization angle	This external parameter defines the angle of polarizer orientation in degrees. The value of this external parameter is used as Θ in model formulas.

Additional properties:

Property name	Description
AnisExponentsCount	This property defines number of anisotropy exponents
Associations	This property provides access to the *"Associations" dialog box* that was designed for making associations between fluorescence and

	anisotropy exponents.

Additional fit parameters:

Parameter name	Description
r_inf	Residual polarization
beta	Amplitude of corresponding anisotropy component
phi	decay time of corresponding anisotropy component

Constraints

Specific constraints for the parameters of any model are implemented. Constraint can operate with any parameters according to its formula and therefore it is independent on a type of the model. The current version of TRFA Data Processor has just one constraint named "Ratio". It is defined by the following formulas (comparison operator can be either more or less):

$$\frac{\sum_i List1 - \sum_j List2}{\sum_n List3 - \sum_m List4} > Value \; , \; \frac{\sum_i List1 - \sum_j List2}{\sum_n List3 - \sum_m List4} < Value \qquad (22)$$

List1 – **List4** can contain any parameters. If no parameters have been included in any list, it means that this list is absent in the formula. 'Value' contains any number. Several examples of possible application of "Ratio" constraint for the multi-exponential model are shown bellow:

Value that is used as constraint	Implementation in "Ratio" constraint
Sum of pre-exponential factors	**List1** contains necessary parameters. Other Lists are empty. Value is any number
Difference of pre-exponential factors	**List1** and **List2** contain necessary parameters. Other Lists are empty. Value is any number
Ratio of sums of pre-exponential factors	**List1** and **List3** contain necessary parameters. Other Lists are empty. Value is any number
Ratio of differences of pre-exponential factors	All lists contain necessary parameters. Value is any number
Relations between sums or differences of pre-exponential factors	Similar to one of previous topics, but the 'Value'=0.

Methods

The parameters of the models can be estimated by a global fitting procedure. In a global analysis, several characteristics (fluorescence decays) are analyzed simultaneously. Certain fit parameters can be linked. The values of parameters linked together are kept equal to each other. Increasing number of fitted characteristics increases sensitivity and accuracy of the analysis while keeping the total number of parameters unchanged [23]

In TRFA Data Processor the global fitting procedure is based on the Marquardt-Levenberg non-linear method of Least Squares (Marquardt 1963) and Maximum-Likelihood Method. Since the non-linear method of least squares is a special case of Maximum-Likelihood method (when statistics of the noise in the measured data is Gaussian) the general fitting algorithm is developed where both analysis methods differ just in a target fit criterion: χ^2 criterion [13, 14] for the least squares method and Maximum-Likelihood Estimator for Maximum-Likelihood method.

Likelihood

The aim is to maximize the Likelihood given by:

$$L(\mathbf{a}) = P(x_1, x_2, ..., x_n, \mathbf{a}), \qquad (23)$$

where $\mathbf{a} = \{a_1, a_2, ..., a_m\}$ is the vector of m unknown parameters, x_i are the number of counts in the i-th channel ($i=1...,n$) and $P(x_1, x_2, ..., x_n, \mathbf{a})$ is the probability to obtain $x_1, x_2, ..., x_n$ counts in $1, 2, ..., n$–th channel, respectively. In other words, one has to find such a combination of parameters $a_1, a_2, ..., a_m$, which will ensure the maximum value for the Likelihood $L(\mathbf{a})$.

In case of independent channels, Eq. 23 can be rewritten as:

$$L(\mathbf{a}) = \prod_{i=1}^{n} P(x_i, \mathbf{a}). \qquad (24)$$

The maximization of Eq. 21 is equivalent to maximization of the logarithm of the Likelihood:

$$\ln L(\mathbf{a}) = \sum_{i=1}^{n} \ln P(x_i, \mathbf{a}), \qquad (25)$$

which is often used in practice.

Experimental statistics

Further practical treatment is strongly dependent on the used experimental statistics, i.e. on the shape of the functions $P(x_1, x_2, ..., x_n, \mathbf{a})$ or $P(x_i, \mathbf{a})$. In fluorescence studies there are three basic possibilities: Gaussian, Poissonian and multinomial. TRFA Data Processor supports two first statistics:

1. <u>Gaussian statistics.</u> It is assumed that the channels are independent and $P(x_i, \mathbf{a})$ is given by

$$P(x_i, \mathbf{a}) = \frac{\exp\left(-\dfrac{(x_i - y_i(\mathbf{a}))^2}{2w_i^2}\right)}{w_i \sqrt{2\pi}}, \qquad (26)$$

where $y_i(\mathbf{a})$ is the "theoretical" or "predicted" number of counts in the i-th channel. Substituting Eq. (26) into Eq. (25) yields

$$\ln L(\mathbf{a}) = \sum_{i=1}^{n} \ln P(x_i, \mathbf{a}) = -\sum_{i=1}^{n} \frac{\left(x_i - y_i(\mathbf{a})\right)^2}{2w_i^2} - \sum_{i=1}^{n} \ln w_i \sqrt{2\pi}. \quad (27)$$

Since the second term in the right-hand side of Eq. (27) is independent on $\mathbf{a}$, the maximization of $L(\mathbf{a})$ is equivalent to minimization of the following expression, taken from the first term in right-hand side of Eq (27),

$$\chi^2(\mathbf{a}) = 2\sum_{i=1}^{n} \frac{\left(x_i - y_i(\mathbf{a})\right)^2}{2w_i^2}, \quad (28)$$

which exactly corresponds to usual χ^2 criterion. In this case the method based on minimization of the χ^2 criterion (Least-Squares) is equivalent to the method based on the maximization of the Likelihood.

   2. <u>Poissonian statistics.</u> The channels are independent and $P(x_i, \mathbf{a})$ is given by

$$P(x_i, \mathbf{a}) = \frac{\exp\left(-y_i(\mathbf{a})\right)\left[y_i(\mathbf{a})\right]^{x_i}}{x_i!}, \quad (29)$$

Substituting Eq. (29) into Eq. (25) yields

$$\ln L(\mathbf{a}) = \sum_{i=1}^{n} \ln P(x_i, \mathbf{a}) = \sum_{i=1}^{n} -y_i(\mathbf{a}) + \sum_{i=1}^{n} x_i \ln y_i(\mathbf{a}) - \sum_{i=1}^{n} \ln x_i!, \quad (30)$$

It can be shown that the maximization of the Likelihood (Eq. (30)) is equivalent to the minimization of the following expression

$$\chi_p^2(\mathbf{a}) = 2\sum_{i=1}^{n} \left[y_i(\mathbf{a}) - x_i + x_i \ln\left(x_i / y_i(\mathbf{a})\right) \right]. \quad (31)$$

The quantity χ_p^2 from Eq. (31) is equivalent to "usual" χ^2 criterion with exactly the same statistical properties: $\chi_p^2/(n-m)$ should be around unity in a case of good fitting.

Minimization procedure

TRFA Data Processor Advanced uses gradient Marquardt-Levenberg optimization method [13, 16] in the minimization procedure.

Let us consider the case of Gaussian statistics (χ^2 criterion, given by Eq. (28)). The χ^2 can be represented in series around "true" values of the parameter vector $\mathbf{a}_0$:

$$\chi^2(\mathbf{a}) = \chi^2(\mathbf{a}_0) + \sum_{j=1}^{m} \left.\frac{\partial \chi^2(\mathbf{a})}{\partial a_j}\right|_{\mathbf{a}=\mathbf{a}_0} \Delta a_j + \sum_{k=1}^{m}\sum_{j=1}^{m} \left.\frac{\partial^2 \chi^2(\mathbf{a})}{\partial a_j \partial a_k}\right|_{\mathbf{a}=\mathbf{a}_0} \Delta a_j \Delta a_k + \ldots, \quad (32)$$

The condition for the minimum of χ^2:

$$\frac{\partial \chi^2(\mathbf{a})}{\partial \mathbf{a}} = 0, \quad (33)$$

yields a set of linear algebraic equations with respect to small increments $\Delta a_j, j=1\ldots m$

$$\left.\frac{\partial \chi^2(\mathbf{a})}{\partial a_j}\right|_{\mathbf{a}=\mathbf{a}_0} = -\sum_{k=1}^{m} \left.\frac{\partial^2 \chi^2(\mathbf{a})}{\partial a_j \partial a_k}\right|_{\mathbf{a}=\mathbf{a}_0} \Delta a_k + \ldots, \ j=1,\ldots,m. \quad (34)$$

The partial derivatives in Eq. (34) take the form:

$$\frac{\partial \chi^2(\mathbf{a})}{\partial a_j} = -2 \sum_{i=1}^{n} \frac{(x_i - y_i(\mathbf{a}))}{w_i^2} \frac{\partial y_i(\mathbf{a})}{\partial a_j}, \qquad (35)$$

and

$$\frac{\partial^2 \chi^2(\mathbf{a})}{\partial a_j \partial a_k} = 2 \sum_{i=1}^{n} \frac{1}{w_i^2} \frac{\partial y_i(\mathbf{a})}{\partial a_k} \frac{\partial y_i(\mathbf{a})}{\partial a_j} - 2 \sum_{i=1}^{n} \frac{(x_i - y_i(\mathbf{a}))}{w_i^2} \frac{\partial^2 y_i(\mathbf{a})}{\partial a_k \partial a_j}, \qquad (36)$$

It can be shown that with the increase of n the second term in right-hand side of Eq. (36) converges to zero much quicker than the first one. Therefore, in practical computations the second term in right-hand side of Eq. (36) is ignored and the matrix of the second derivatives can be written in the following way:

$$\frac{\partial^2 \chi^2(\mathbf{a})}{\partial a_j \partial a_k} \cong 2 \sum_{i=1}^{n} \frac{1}{w_i^2} \frac{\partial y_i(\mathbf{a})}{\partial a_k} \frac{\partial y_i(\mathbf{a})}{\partial a_j}, \qquad (37)$$

The logic of minimization of χ_p^2 is totally similar to the minimization of χ^2. It is clear that Eqs. (32), (33) and (34) are exactly the same for all statistics. The difference is only in the derivatives. Let us consider the derivatives given by Eq. (35) and (36) for the Poissonian statistics. The first and second derivatives can be found from Eq. (31) as:

$$\frac{\partial \chi_p^2(\mathbf{a})}{\partial a_j} = 2 \sum_{i=1}^{n} \frac{\partial y_i(\mathbf{a})}{\partial a_j} \left[1 - \frac{x_i}{y_i(\mathbf{a})} \right], \qquad (38)$$

$$\frac{\partial^2 \chi_p^2(\mathbf{a})}{\partial a_k \partial a_j} = 2 \sum_{i=1}^{n} \frac{\partial^2 y_i(\mathbf{a})}{\partial a_k \partial a_j} \left[1 - \frac{x_i}{y_i(\mathbf{a})} \right] + 2 \sum_{i=1}^{n} \frac{x_i}{[y_i(\mathbf{a})]^2} \frac{\partial y_i(\mathbf{a})}{\partial a_j} \frac{\partial y_i(\mathbf{a})}{\partial a_k}, \qquad (39)$$

The first term in right-hand side of Eq. (39) can be ignored due to the same reason as before and the matrix of second derivatives takes the form:

$$\frac{\partial^2 \chi_p^2(\mathbf{a})}{\partial a_k \partial a_j} = 2 \sum_{i=1}^{n} \frac{x_i}{[y_i(\mathbf{a})]^2} \frac{\partial y_i(\mathbf{a})}{\partial a_j} \frac{\partial y_i(\mathbf{a})}{\partial a_k}. \qquad (40)$$

Global target fit criterion

In the case of Gaussian statistics the global target fit criterion is calculated by the equation [23]:

$$\chi_{gl}^2 = \frac{1}{\left(N - m + \sum_{i=1}^{M} m_i^{lnk} - m^{gr} - 1\right)} \sum_{i=1}^{M} \sum_{j=1}^{n_i} \frac{\left(x_{ij} - y_{ij}(\mathbf{a})\right)^2}{w_{ij}^2}, \qquad (41)$$

where x_{ij} and ω_{ij} are the measured value and the standard deviation of the ith experimental data point in the jth measured curve and $y_{ij}(\mathbf{a})$ is the theoretical value for this point; M is the number of globally analyzed curves; n_i is the number of points in i^{th} curve ; m_i and m_i^{lnk} is the number of free and linked parameters in i^{th} model respectively, m^{gr} is a number of parameter groups (sets of linked parameters), $N = \sum_{i=1}^{M} n_i$, $m = \sum_{i=1}^{M} m_i$.

For a good fit the final value of target fit criterion obtained after the analysis should be close to 1. This is possible if weighting factors w_{ij} are calculated properly. Calculation of weighting factors depends on the analysis type (fluorescence decay or anisotropy analysis), the convolution method used in the analysis and performed using the general formula:

$$w_i = \frac{1}{(\omega_i + \gamma^2 bg_i)} \qquad (42)$$

where calculation of ω_i is calculated accordingly following formulas:

Fluorescence decay analysis:

IRF:

$$\omega = y_T^S;$$

REF:

$$w_T^{REF} = w_T^{IRF} + \left[f_T^S(0) \right]^2 d^{REF}$$

$$\omega = y_T^S + \left[f_T^S(0) \right]^2 y_T^{REF};$$

where y_T^S - sample total (measured at the magic angle) decay, y_T^{REF} - reference decay, f_T^S - calculated sample decay without the convolution;

Anisotropy analysis via parallel and perpendicular components:

IRF:

$$\omega_T = y_\parallel^S + 4G^2 y_\perp^S$$

$$\omega_\parallel = y_\parallel^S$$

$$\omega_\perp = y_\perp^S$$

$$\omega_r = \frac{1 + G + 3Gr - 3r^2 - 2(2G-1)r^3}{3y_T^S}$$

where $y_T^S = y_\parallel^S + 2G y_\perp^S$, $r = \dfrac{y_\parallel^S - G y_\perp^S}{y_\parallel^S + 2G y_\perp^S}$, G – G-factor;

REF:

$$\omega_T = y_T^S + \left[f_T^S(0) \right]^2 \left(y_\parallel^S + 4G^2 y_\perp^S \right)$$

$$\omega_\parallel = y_\parallel^S + \left[f_\parallel^S(0) \right]^2 \left(y_\parallel^S + 4G^2 y_\perp^S \right)$$

$$\omega_\perp = y_\perp^S + \left[f_\perp^S(0) \right]^2 \left(y_\parallel^S + 4G^2 y_\perp^S \right)$$

$$\omega_r = \frac{1 + G + 3Gr - 3r^2 - 2(2G-1)r^3}{3y_T^S}$$

For MLE with Poissonian statistics the global target fit criterion is calculated as following:

$$\chi_{P\,gl}^2 = \frac{2}{(N - m + \sum_{i=1}^{M} m_i^{lnk} - m^{gr} - 1)} \sum_{i=1}^{M} \sum_{j=1}^{n_i} \left[y_{ij}(\mathbf{a}) - x_{ij} + x_{ij} \ln\left(x_{ij} / y_{ij}(\mathbf{a}) \right) \right], \qquad (43)$$

For a good fit the final value of target fit criterion should also be close to 1.

Additional fit quality criteria

- Standard normal deviation $Z\chi^2$;

Standard normal deviation $Z\chi 2$ criterion is calculated by the following equation [17, 18]:

$$Z_{\chi^2} = \sqrt{\frac{k-m-1}{2}}(\chi^2 - 1), \qquad (44)$$

where k is the number of points in all decays that are used for the calculation of χ2; m is the number of fit parameters that should be estimated. The value Z_{χ^2} is normally distributed, so that the tables for normal distribution can be used to judge the quality of the fit at certain confidence level. Thus $|Z_{\chi^2}| < 1.96$ for testing at confidence level 95%; $|Z_{\chi^2}| < 2.58$ (99%) and $|Z_{\chi^2}| < 3.3$ (99,9%).

Note. Strictly speaking, Z_{χ^2} is derived and must be used only for χ2 criterion, but in TRFA Data Processor Advanced this and all following additional fit quality criteria is allowed to use also for MLE with Poissonian statistics.

- Durbin Watson parameter;

The Durbin-Watson parameter d is used to test for serial correlation between residuals and can be calculated by the following formula [18, 19]:

$$d = \sum_{i=2}^{K}(R_i - R_{i-1})^2 \Big/ \sum_{i=1}^{K} R_i^2 , \qquad (45)$$

where R_i is the value of the residual in the i-th point; k is the number of points where residuals are calculated. To make decision about the quality of the fit the calculated Durbin-Watson parameter value should be compared with the values dL and dU (lesser and upper values). These values can be taken from corresponding tables and depend on the number of points k (see equation above) and on the numbers of independent variables. If $d < dL$ the residuals are correlated. In the case if $d > dU$ the residuals are regarded as uncorrelated. When $dL < d < dU$ no conclusions about correlation of residuals can be made.

- Runs Test;

The ordinary runs test [20] is applied to check the randomness of the residuals signs versus channel number. In this test the actual number of ordinary runs R is calculated and compared with expected number R_{exp}. The term ordinary run denotes the consecutive sequence of residuals with the same sign. If the sequence of residuals is random the expected number of runs R_{exp} and number of runs variance are calculated as follows:

$$R_{exp} = 1 + \frac{2PN}{P+N}, \qquad \sigma_R^2 = \frac{2PN(2PN - P - N)}{(P+N)^2(P+N-1)} \qquad (46)$$

where P and N are respectively the number of residuals with positive and negative sign. The values defined above enable to calculate the runs test parameter Z that will be distributed approximately as a standard normal deviate:

$$Z = (R - R_{exp} + C)\big/\sigma_R , \qquad (47)$$

where C is the continuity correction which partially compensates for the approximation of a discrete distribution by a continuous distribution. The value of C depends on the number of tested runs R. In the case if R is low ($R < R_{exp}$) $C = 0.5$. Otherwise ($R > R_{exp}$) $C = -0.5$. The calculated value Z can be used to make decision about the randomness of the residuals. The value $|Z|$ should be compared at the certain confidence level with the value taken from tables for normal distribution. Thus $|Z| < 1.96$

for testing at confidence level 95%. The greater the value of |Z|, the greater the probability that some correlation exists in the residuals.

- ***Weighed residuals***

The weighted residuals provide visual estimation of conformity between measured (simulated) and theoretical decay. The equation that is used for calculation of weighted residuals depends on the type of target fit criterion that is applied for the analysis.

In the case if $\chi 2$ target fit criterion is used for the fit the weighted residuals in i-th channel R_i are calculated by the following equation:

$$R_i = (f_i - f_i^{th})/\sqrt{f},$$ (48)

where f_i and f_i^{th} are, respectively, the values of measured (simulated) and theoretical fluorescence decays in i-th channel.

In the case if MLE poissonian target fit criterion is used for the fit the weighted residuals in i-th channel R_i are calculated by the following equation:

$$R_i = sign(f_i - f_i^{th})\sqrt{2\left(f_i \ln(f_i/f_i^{th}) + f_i^{th} - f_i\right)},$$ (49)

where function $sign(x)$ is equal to 1 in the case if x is positive and -1 in the case if x is negative. For the successful fits the residuals should be randomly distributed around 0 and they should not exceed the range [-3; 3] for the normal statistics of the noise (the 3-sigma rule).

- ***Autocorrelation function of residuals***

The plot of residuals autocorrelation function [1, 17] against the time channel provides the useful visual check of the quality of the fit. The autocorrelation function shows the correlation between the residuals that are certain channels apart. The autocorrelation function of weighted residuals in the j-th channel C_j ($j = 1,...,m$) is calculated by the following equation:

$$C_i = \frac{1}{k - j + 1} \sum_{k=1}^{k-j+1} R_i R_{i+j-1} \bigg/ \frac{1}{k} \sum_{k=1}^{k} R_i^2,$$ (50)

where R_i is the value of weighted residuals calculated in i-th channel; k is the number of channels where weighted residuals are calculated. The plots of autocorrelation are usually made across half of the data channels ($m = k/2$) were residuals are calculated. This is done in order to ensure the sufficient number of terms in the sum located in the numerator of the equation shown above thus providing the proper averaging.

In some cases the visual inspection of autocorrelation function is more sensitive test for the goodness of the fit than the plot of weighted residuals. For successful fits the residuals autocorrelation is randomly distributed around zero. Bad fits provide low frequency periodicity in an autocorrelation plot that can be detected visually.

Note: As it is follows from the equation shown above, $C_1 = 1$. This means that each R_i value is fully correlated to itself. In order to make autocorrelation plots more convenient for visual inspection in **TRFA Data Processor Advanced** the value C_1 is replaced on the graphs by 0.

- *Heterosedasticity of the residuals;*

Heterosedasticity of the residuals shows the dependence of weighted residuals on the sorted ascending values of theoretical decay. For successful fits this plot should not demonstrate the broadening of one or the other side of the graph.

- *Normal probability function of the residuals;*

This graphical fit quality criterion allows to inspect visually if weighted residuals correspond to the standard normal distribution. For this purpose the theoretical probability density function for standard normal distribution is displayed together with inspected probability density function calculated from weighted residuals. For successful fits two curves described above should be in a good agreement to each other.

References

1. J. R. Lakowicz. **Principles of Fluorescence Spectroscopy.** Plenum Press, New York. 1983.
2. D. V. O'Connor, D. Phillips. **Time-Correlated Single Photon Counting.** Academic Press, London. 1984.
3. J. N. Demas. **Excited State Lifetime Measurements.** Academic Press, New York. 1983.
4. G.Verbeek, A. Vaes, M. Van der Auweraer, F.C. De Schryver, C. Geelen, D. Terrell, S. De Meuter. **Gaussian distributions of the decay times of the singlet excited state of aromatic amines dispersed in polymer films.** Macromolecules, 26, 1993, pp. 472-478.
5. F. Imans, L. Viaene, M. Van der Auweraer, F.C. De Schryver. **Local properties of adsorption sites probed by the emission of fluorophores with different size and shape.** Chem. Phys. Lett., 262, 1996, pp.583-588.
6. P. P. Infelta, M. Gratzel. **Dynamics of fluorescence quenching in micellar systems.** J. Chem. Phys. 78, 1983, p. 5280.
7. M. Tachiya. **On the kinetics of luminescence quenching in micellar systems.** J. Chem. Phys, 78, 1983, p. 5282.
8. V. Van den Bergh, A. Kowalczyk, N. Boens, F.C. De Schryver. **Experimental design in the Global Compartmental Analysis of Intermolecular Two-State Excited-State Processes.** J. Phys. Chem., 98, 1994, pp. 9503-9508.
9. N. Boens, J. Szubiakowski, E. Novikov. **Testing the identifiability of a model for reversible intermolecular two-state excited-state processes.** J. Chem. Phys. 112, 2000, p. 8260.
10. A. Kowalczyk, K. Meuwis, N. Boens, F.C. De Schryver. **Kinetics and Identifiability of an Intermolecular Two-State Excited-State Processes in the Presence of a Fluorescent Impurity.** J. Phys. Chem., 99, 1995, pp. 17349-17353.
11. N. Boens, A. Kowalczyk. **Identifiability of competitive intermolecular three-state excited-state processes.** Chem. Phys. Lett., 260, 1996, pp. 326-330.
12. J. van Stam et al. **Intermolecular excited-state processes of a Halato-Telechelic Polymer, Evaluated by Global Compartmental Analysis of the Fluorescence Decay Surface with the Use of Model Compounds.** Macromolecules, 30, 1997, pp. 5582-5590.
13. P.R. Bevington, D.K. Robinson. **Data Reduction and Error Analysis for the Physical Sciences.** McGraw-Hill, New Yourk, 2003.
14. A. Grinvald, I. Z. Steinberg. **On the analysis of fluorescence decay kinetics by the method of least squares.** Anal. Biochem, 59, 1974, pp. 583-598.

15. Z. Bajzer, T. M. Therneau, J. C. Sharp, F. G. Prendergast. **Maximum likelihood method for the analysis of time-resolved fluorescence decay curves.** Eur. Biophys. J., 20, 1991, pp. 247-262.

16. D.W. Marquardt. **An algorithm for least-squares estimation of non-linear parameters.** J. Soc. Ind. Appl. Math., 11, 1963, pp. 431-441.

17. D.F. Eaton. **Recommended methods for fluorescence decay analysis.** Pure &App/Chem., Vol. 62, No. 8, 1990, pp. 1631-1648.

18. N. Boens, M. Van Den Zegel, F.C. De Schryver. **Picosecond lifetime determination of the second exited singlet state of xanthione in solution.** Chem. Phys. Lett., 111, 1984, pp. 340-346.

19. J. Durbin, G.C. Watson. **Testing for serial correlation in least squares regression.** Biometrika, 37, 1950, pp. 409-428.

20. J. R. Lakowicz. **Topics in Fluorescence Spectroscopy.** Vol. 2, Kluwer Academic Publishers, New York. 2002.

21. V. V. Apanasovich, E. G. Novikov. **Deconvolution method for fluorescence decays.** Optics communications, v. 78, 1990, pp. 279-282.

22. E. G. Novikov. **Reference reconvolution analysis by phase plane method.** Rev. Sci. Instr., 69, 1998, pp. 2603-2610.

23. J. M. Beechem, E. Gratton, M. Ameloot, J. R. Knutson and L. Brand: Analysis of fluorescence intensity and anisotropy decay data: Second generation theory and programs. In: *Topics in Fluorescence Spectroscopy.* Ed. J. R. Lakowicz. Plenum Press, New York, Vol. 2; 241- 305 (1991)

24. J.C. Andre, M. Vincent, D.V.O' Connor, W.R. Ware, J Phys Chem., 84, 1974, 2285.

25 U.Wild, A. Holzwarth, H.P. Good, Rev. Sci. Instr., 48, 1977, 1627