Supported methods

FCS [1-8, 17, 18, 19], FCCS [19], TIR-FCS [38], PCH [25, 23, 24, 41], PCMH [28], FIDA [21], FIMDA [22], FCA [15, 42], TIFCA [20, 42], coincidence bursts counting and coincidence analysis [16] are supported in FFS Data Processor (FFS DP).

Analyzed characteristics

Following statistical characteristics of the recorded photon stream can be analyzed globally [12, 26] in FFS DP:

1. Autocorrelation function (ACF)
2. Crosscorrelation function (CCF)
3. Photon Counting Distribution (PCD)
4. Fluorescence factorial cumulants (FFC)

In addition FFS DP can analyze two special characteristics:

5. Coincidence values histogram (CVH)
6. Fit parameters dependence (FPD)

FPD is a secondary characteristic that can be built from a number of fit parameter values of the same type obtained in the analysis of a number of datasets, e.g. diffusion time versus dataset number, molecular brightness versus binning time (at which a particular PCD was calculated), molecular fraction versus dilution factor, etc.
In global analysis the statistical characteristics of different type can be combined (e.g. global analysis of ACF and PCD). This allows increasing accuracy and robustness of analysis.

Models

FFS technique is aimed to the investigations of the intensity fluctuations of fluorescent molecules excited by a tightly focused laser beam. These fluctuations may arise from translational and rotational diffusion, chemical reactions, deexcitation of the triplet-state, conformational and structural changes, etc [1-5, 15, 16, 26].
To take into account any phenomenon mentioned above while analyzing the FFS data the appropriate mathematical model should be chosen. In the FFS Data Processor, the mathematical models are represented by the corresponding **Model Objects**.
Model Object can belong either to the Data Set or to the Simulation Data Source. In the first case **Model** is used to generate the theoretical characteristic (theoretical curve). In the second case it is used to generate noise-free source characteristic. Following models are available in **FFS Data Processor**:

Characteristics	**Models**	**Details**
Correlation function **(FCS, FCCS)**	Pure-Diffusion Triplet-State Conformational Protonation FCS flow Custom	(2D/3D free diffusion, 2D/3D anomalous diffusion, 2D/3D confined diffusion, TIR-FCS are supported) in all these models
Coincidence values histogram **(Coincidence analysis)**	Gaussian Custom	
Cumulants **(FCA, TIFCA)**	FFC Custom	
Photon counting distribution **(PCH, PCMH, FIDA, FIMDA)**	PCH Gaussian Poissonian Custom	FIDA can be done using PCH with the polynomial profile and normalization on two first PSF moments. FIMDA can be performed as a global analysis of a number of PCDs by PCH model with activated motion and process corrections and linking of all corresponding parameters . PCMH is a two-step analysis. First, a number of PCDs are analysed sequentially; second, the model Concentration vs. time or Brightness vs. time is used for fitting the obtained concentration (or brightness) versus binning time curve.
Fit parameters dependence **(PCMH, custom analysis)**	Concentration vs. time Brightness vs. time Gaussian Custom	

Identifiability

All models mentioned above (except Custom model and PCH with "FIDA-like" polynomial brightness profile approximation) are totally identifiable, i.e. all parameters of the models can be uniquely determined on the basis of only one measured characteristic. PCH with "FIDA-like" polynomial brightness profile approximation at fixed background can have up to three equivalent sets of parameters. Each of them describes experimental data equally [27].

Correlation function models

The fluorescence emitted by the molecules in the focal spot is recorded photon by photon. Assuming constant excitation power, the fluctuations of the fluorescence signal are defined as the deviations from the temporal average of the signal:

$$\delta F(t) = F(t) - \langle F(t) \rangle$$

$$\langle F(t) \rangle = \frac{1}{T} \int_0^T F(t)\, dt$$

The normalized fluorescence fluctuation autocorrelation function $G(\tau)$ for the fluctuation of the signal $\delta F(t)$ from the average fluorescence intensity is defined as:

$$G(\tau) = 1 + \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$

If diffusion is assumed to be the only process governing the number of fluorescent molecules in a 3D-Gaussian shaped observation volume ($B(\mathrm{r}) = \exp\left[-\frac{2(x^2+y^2)}{\omega_{xy}^2} - \frac{-2z^2}{\omega_z^2} \right]$, where ω_{xy} and ω_z are the lateral and axial radii, respectively), autocorrelation function is represented as [31]

$$G(\tau) = 1 + \frac{1}{N} \sum_j \frac{F_j}{\left(1 + \frac{\tau}{\tau_{dif\ j}}\right) \sqrt{1 + \left(\frac{\omega_{xy}}{\omega_z}\right)^2 \frac{\tau}{\tau_{dif\ j}}}}$$

where j=1,2,3… and $\sum_j F_j = 1$, N is the average number of fluorescent molecules in the effective volume $V_{eff} = \chi_1^2 / \chi_2$, $\chi_k = \int_V PSF^k(\mathbf{r}) d\mathbf{r}$, $V_{eff} = \pi^{3/2} . \omega_{xy}^2 . \omega_z$, F_j and $\tau_{dif\ j}$ are, respectively, the contribution and translational diffusion relaxation time of molecules of the j-th fluorescent component.

The lateral diffusion time τ_{dif} describes the residence time of a particle in the observation volume, which is related to the diffusion coefficient D_{tran} by:

$$\tau_{dif} = \frac{\omega_{xy}^2}{4 D_{tran}}$$

The amplitude of the correlation function, $G(0)$, represents the average number of molecules N found in the observation volume:

$$G(0) = 1 + 1/N$$

It was assumed that the fluorescent brightness of a molecule is not changed during binning time interval and additional contribution to the recorded signal due to background is negligible. Besides free diffusion there are some additional processes (intercombination conversion, conformational changes, etc.) that can cause fluctuations of the fluorescence intensity. There are also different kinds of molecular motion: flow, two dimensional, anomalous and constrained diffusion. With some modification of the instrument TIR-FCS is also possible.

The correlation function models that are included in FFSDP are based on the following general formula [31, 17, 18, 19]:

$$G(\tau) = G_{\inf} + \frac{1}{N} X_{BG} X_{kinetics}(\tau) G_{motion}(\tau)$$

where:

G_{inf} is the level of autocorrelation function at $\tau \to \infty$ (by default $G_{\inf} = 1$);

N denotes the average number of fluorescent particles in the effective volume V_{eff};

$X_{BG} = (1 - R_{bg})^2$ denotes the background correction multiplier ($R_{bg} = I_{bg} / (I_S + I_{bg})$ denotes background ratio, where I_s is the sample signal and I_{bg} is the uncorrelated background signal). Correction to background is performed if property **Background correction** is set on;

$X_{kinetics}(\tau)$ denotes kinetic process;

$G_{motion}(\tau)$ describes motion type of the particles.

Brightness correction is necessary if molecules with different weight have different quantum yield (it is performed if property **Brightness correction** is set on). It has two options: either correction to the absolute brightness or to its ratio. The last option allows to perform the global analysis of ACFs and PCDs if the brightness ratio option is selected in the PCH model. In the case of brightness correction for the absolute brightness the following replacement in the motion term of a model equation is made:

$$\frac{F_i}{N} = \frac{q_i^2 N_i}{\left(\sum_j q_j N_j\right)^2},$$

where q_i and N_i are, respectively, brightness and number of particles for *i*-th component, $N = \sum N_j$ is the average number of fluorescent particles, $F_i = N_i / N$ is the contribution of molecules of the *i*-th fluorescent component.
N, F_i (in the case if brightness correction is set off) and N_i, q_i (in the case if brightness correction is set on) are fit parameters, where *i=1,2,...*, *n* (*n* - number of fluorescent components).

The models are classified by kinetic term $X_{kinetics}(\tau)$. For each model with particular $X_{kinetics}(\tau)$ several types of motion are available trough the **Motion type property**. The following motion types of the particles are implemented:

- Free 2D Diffusion
- Free 3D Diffusion
- Anomalous 2D Diffusion
- Anomalous 3D Diffusion
- Confined 2D Diffusion
- Confined3D Diffusion
- TIRR-FCS

Description of the motion types is given below.

Free 2D Diffusion term

$$G_{motion}(\tau) = \sum_i \frac{F_i}{1 + \frac{\tau}{T_i^{diff}}}, \qquad \sum_j F_j = 1,$$

where F_i and T_i^{diff} are, respectively, fraction and translational diffusion relaxation time of molecules of the *i*-th fluorescent component.

Fit parameters added to the general model by Free 2D Diffusion term:

1. F_i is the contribution of molecules of the *i*-th fluorescent component. In the case if brightness of the particles should be taken into account **F_i** is replaced by **N_i** (the number of particles for *i*-th component). For more information see brightness correction topic.
2. T_i is the translational diffusion relaxation time of molecules of the *i*-th fluorescent component.

Free 3D Diffusion term

$$G_{motion}(\tau) = \sum_i \frac{F_i}{\left(1 + \frac{\tau}{T_i^{diff}}\right)\sqrt{1 + \frac{\tau}{a^2 T_i^{diff}}}}, \qquad \sum_j F_j = 1,$$

where F_i and T_i^{diff} are, respectively, fraction and translational diffusion relaxation time of molecules of the *i*-th fluorescent component, *a* is the structural parameter.

Fit parameters added to the general model by Free 3D Diffusion term:

1. F_i is the contribution of molecules of the *i*-th fluorescent component. In the case if brightness of the particles should be taken into account **F_i** is replaced by **N_i** (the number of particles for *i*-th component). For more information see brightness correction.
2. T_i is the translational diffusion relaxation time of molecules of the *i*-th fluorescent component.
3. *a* is the structural parameter.

Anomalous 2D Diffusion term

$$G_{motion}(\tau) = \sum_i \frac{F_i}{1 + \left(\frac{\tau}{T_i^{diff}}\right)^{\alpha}}, \qquad \sum_j F_j = 1,$$

where F_i and T_i^{diff} are, respectively, fraction and translational diffusion relaxation time of molecules of the *i*-th fluorescent component, α denotes the anomality factor.

Fit parameters added to the general model by Anomalous 2D Diffusion term:

1. F_i is the contribution of molecules of the *i*-th fluorescent component. In the case if brightness of the particles should be taken into account **F_i** is replaced by **N_i** (the number of particles for *i*-th component). For more information see brightness correction.
2. T_i is the translational diffusion relaxation time of molecules of the *i*-th fluorescent component.
3. *alpha* is the anomality factor α.

Anomalous 3D Diffusion term

$$G_{motion}(\tau)=\sum_i \frac{F_i}{\left(1+\left(\frac{\tau}{T_i^{diff}}\right)^{\alpha}\right)\sqrt{1+\frac{1}{a^2}\left(\frac{\tau}{T_i^{diff}}\right)^{\alpha}}}, \qquad \sum_j F_j=1,$$

where F_i and T_i^{diff} are, respectively, fraction and translational diffusion relaxation time of molecules of the *i*-th fluorescent component, *a* is the structural parameter, α denotes the anomality factor.

Fit parameters added to the general model by Anomalous 3D Diffusion term:

1. F_i is the contribution of molecules of the *i*-th fluorescent component. In the case if brightness of the particles should be taken into account **F_i** is replaced by **N_i** (the number of particles for *i*-th component). For more information see brightness correction.
2. T_i is the translational diffusion relaxation time of molecules of the *i*-th fluorescent component.
3. *alpha* is the anomality factor α.
4. *a* is the structural parameter.

Confined 2D Diffusion term [37]

$$G_{motion}(\tau)=\sum_i F_i g_x^i(\tau) g_y^i(\tau), \qquad \sum_j F_j=1, \text{ where:}$$

$$g_x^i(\tau)=\frac{1}{\sqrt{1+\frac{\tau}{T_i^{diff}}}}, \qquad g_y^i(\tau)=\frac{\sqrt{\pi}}{Y}\left[1+\left(\frac{Y}{\sqrt{\pi}}\cdot\frac{erf(Y)}{erf^2\left(Y/\sqrt{2}\right)}-1\right)\cdot\left(\frac{\exp\left(-K(Y)(\pi/Y)^2\frac{\tau}{T_i^{diff}}\right)}{\sqrt{1+\frac{\tau}{T_i^{diff}}}}\right)\right],$$

$K(i)=0.689+0.34e^{-0.37(i-0.5)^2}$ with *i*=number of fluorescent species $Y=d_y/r_{xy}$, F_i and T_i^{diff} are, respectively, fraction and translational diffusion relaxation time of molecules of the i-th fluorescent component, r_{xy} denotes the distance lateral direction at which the intensity of the exciting laser beam is dropped by e^{-2}, d_y is the confined volume diameter (f.e. diameter of celorganelle)

Fit parameters added to the general model by Confined 2D Diffusion term:

1. F_i is the contribution of molecules of the *i*-th fluorescent component. In the case if brightness of the particles should be taken into account **F_i** is replaced by **N_i** (the number of particles for *i*-th component). For more information see brightness correction.
2. T_i is the translational diffusion relaxation time of molecules of the *i*-th fluorescent component.
3. *Y* is d_y/r_{xy}.

Confined 3D Diffusion term [37]

$$G_{motion}(\tau)=\sum_i F_i g_x^i(\tau) g_y^i(\tau) g_z^i(\tau), \qquad \sum_j F_j=1,$$

where:

$$g_x^i(\tau)=\frac{1}{\sqrt{1+\frac{\tau}{T_i^{diff}}}}, \qquad g_y^i(\tau)=\frac{\sqrt{\pi}}{Y}\left[1+\left(\frac{Y}{\sqrt{\pi}}\cdot\frac{erf(Y)}{erf^2\left(Y/\sqrt{2}\right)}-1\right)\cdot\left(\frac{\exp\left(-K(Y)\left(\frac{\pi}{Y}\right)^2\frac{\tau}{T_i^{diff}}\right)}{\sqrt{1+\frac{\tau}{T_i^{diff}}}}\right)\right],$$

$$g_z^i(\tau)=\frac{\sqrt{\pi}}{Z}\left[1+\left(\frac{Z}{\sqrt{\pi}}\cdot\frac{erf(Z)}{erf^2\left(Z/\sqrt{2}\right)}-1\right)\cdot\left(\frac{\exp\left(-K(Z)\left(\frac{\pi}{a\cdot Z}\right)^2\frac{\tau}{T_i^{diff}}\right)}{\sqrt{1+\frac{\tau}{a^2T_i^{diff}}}}\right)\right],$$

$K(i)=0.689+0.34e^{-0.37(i-0.5)^2}$ with *i*=number of fluorescent species, $Y=d_y/r_{xy}$, F_i and T_i^{diff} are, respectively, fraction and translational diffusion relaxation time of molecules of the i -th fluorescent component, $a=\frac{r_z}{r_{xy}}$ is the structure parameter, r_z and r_{xy} distances in axial and lateral direction at which the intensity of the exciting laser beam is dropped by e^{-2}, d_z and d_y are the diameters of the small sample within the detection volume.

Fit parameters added to the general model by Confined 3D Diffusion term:

1. F_i is the contribution of molecules of the *i*-th fluorescent component. In the case if brightness of the particles should be taken into account **F_i** is replaced by **N_i** (the number of particles for *i*-th component). For more information see brightness correction.
2. T_i is the translational diffusion relaxation time of molecules of the *i*-th fluorescent component.
3. *Y* is d_y/r_{xy}.
4. *Z* is d_z/r_z.
5. *a* is the structural parameter.

TIR-FCS term [38]

$$G_{motion}(\tau)=\sum_i\frac{F_i}{2}\left(1+a^2\frac{t}{T_i^{diff}}\right)^{-1}\left[\left(1-\frac{t}{2T_i^{diff}}\right)erfcx\left(\sqrt{\frac{t}{4T_i^{diff}}}\right)+\sqrt{\frac{t}{\pi T_i^{diff}}}\right],\qquad\sum_j F_j=1,$$

where F_i and T_i^{diff} are, respectively, fraction and axial diffusion relaxation time of molecules of the *i*-th fluorescent component, *a* is the structural parameter $a=z_0/\omega_{xy}$, $erfcx(x)=\exp(x^2)\,erfc(x)$ is the scaled complementary error function.

Fit parameters added to the general model by TIR-FCS term:

1. F_i is the contribution of molecules of the *i*-th fluorescent component. In the case if brightness of the particles should be taken into account **F_i** is replaced by **N_i** (the number of particles for *i*-th component). For more information see brightness correction.
2. T_i is the axial diffusion relaxation time of molecules of the *i*-th fluorescent component.
3. *a* is the structural parameter.

According to the described kinetic types the following fitting models are available for the analysis of the experimental correlation functions:

- Pure diffusion model
- Triplet-State model
- Conformational model
- Protonation model
- Flow model
- Custom model

Description of the model types is given below:

Pure-Diffusion model

For Pure-Diffusion model [1, 2, 6, 7] kinetic term $X_{kinetics}(\tau)$ in general formula is defined by the following equation:

$$X_{kinetics}(\tau)\equiv 1.$$

Pure-Diffusion model properties:

1. IG type determines how the initial guesses of all model parameters are generated. See list of IG type values for details.
2. ComponentsCount defines number of fluorescent components.
3. Background correction determines if background correction is taken into account. If this property is true the Data Set associated with the model should contain the external parameter **BG ratio**.

4. Brightness correction determines if different quantum efficiency of each component is taken into account. See brightness correction topic for more details.
5. Motion type specifies the motion term $G_{motion}(\tau)$ in the model equation (see general formula).

Pure-Diffusion model parameters:

1. *Ginf* is the level of autocorrelation function when $\tau \to \infty$.
2. *N* is the average number of fluorescent molecules in the detection volume (exists only if brightness correction is not performed).
3. Fit parameters of selected motion term $G_{motion}(\tau)$.

Triplet-State model

For Triplet-State model [8] kinetic term $X_{kinetics}(\tau)$ in general formula is defined by the following equation:

$$X_{kinetics}(\tau) = 1 + \frac{F_{trip}}{1 - F_{trip}} e^{-\tau / \tau_{trip}},$$

where F_{trip} and T_{trip} are, respectively, the fractional population and relaxation time of the triplet state.

Triplet-State model properties:

1. IG type determines how the initial guesses of all model parameters are generated. See list of IG type values for details.
2. ComponentsCount defines number of fluorescent components.
3. Background correction determines if background correction is taken into account. If this property is true the Data Set associated with the model should contain the external parameter **BG ratio**.
4. Brightness correction determines if different quantum efficiency of each component is taken into account. See brightness correction topic for more details.
5. Motion type specifies the motion term $G_{motion}(\tau)$ in the model equation (see general formula).

Triplet-State model parameters:

1. *Ginf* is the level of autocorrelation function when $\tau \to \infty$.
2. *N* is the average number of fluorescent molecules in the detection volume (exists only if brightness correction is not performed).
3. F_{trip} is the fractional population of the triplet state.
4. T_{trip} is the relaxation time of the triplet state.
5. Fit parameters of selected motion term $G_{motion}(\tau)$.

Conformational model

For Conformational model [3, 4] kinetic term $X_{kinetics}(\tau)$ in general formula is defined by the following equation:

$$X_{kinetics}(\tau) = 1 + Ae^{-\left(\tau / \tau_{conf}\right)^{\beta}},$$

where A is the pre-exponential factor, β is the "stretch" parameter, τ_{conf} is the characteristic time of conformational relaxation.

Conformational model properties:

1. IG type determines how the initial guesses of all model parameters are generated. See list of IG type values for details.
2. ComponentsCount defines number of fluorescent components.
3. Background correction determines if background correction is taken into account. If this property is true the Data Set associated with the model should contain the external parameter **BG ratio**.
4. Brightness correction determines if different quantum efficiency of each component is taken into account. See brightness correction topic for more details.
5. Motion type specifies the motion term $G_{motion}(\tau)$ in the model equation (see general formula).

Conformational model parameters:

1. *Ginf* is the level of autocorrelation function when $\tau \to \infty$.
2. *N* is the average number of fluorescent molecules in the detection volume(exists only if brightness correction is not performed).
3. *A* is the pre-exponential factor.
4. *beta* is the "stretch" parameter.
5. *tau* is the characteristic time of conformational relaxation.

6. Fit parameters of selected motion term $G_{motion}(\tau)$.

Protonation model

For Protonation model [5] kinetic term $X_{kinetics}(\tau)$ in general formula is defined by the following equation:

$$X_{kinetics}(\tau) = 1 + P_1 e^{-\tau/\tau_1} + P_2 e^{-\tau/\tau_2},$$

where P_1 and P_2 are the pre-exponential factors, τ_1 and τ_2 are the decay constants, associated, respectively, with the external and internal protonation processes.

Protonation model properties:

1. IG type determines how the initial guesses of all model parameters are generated. See list of IG type values for details.
2. ComponentsCount defines number of fluorescent components.
3. Background correction determines if background correction is taken into account. If this property is true the Data Set associated with the model should contain the external parameter **BG ratio**.
4. Brightness correction determines if different quantum efficiency of each component is taken into account. See brightness correction topic for more details.
5. Motion type specifies the motion term $G_{motion}(\tau)$ in the model equation (see general formula).

Protonation model parameters:

1. *Ginf* is the level of autocorrelation function when $\tau \to \infty$.
2. *N* is the average number of fluorescent molecules in the detection volume(exists only if brightness correction is not performed).
3. *P1* is the pre-exponential factor, associated with the external protonation.
4. *P2* is the pre-exponential factor, associated with the internal protonation.
5. *tau1* is the decay constant, associated with the external protonation.
6. *tau2* is the decay constant, associated with the internal protonation.
7. Fit parameters of selected motion term $G_{motion}(\tau)$.

FCS flow model

For FCS flow model [19] kinetic term $X_{kinetics}(\tau)$ in general formula is defined by the following equation:

$$X_{kinetics}(\tau) = e^{-G_{motion}(\tau)\left(\tau/\tau_{fl}\right)^2},$$

where $G_{motion}(\tau)$ is the motion term in general formula, τ_{fl} is the average flow time of the fluorescent particles through the detection volume.

Flow model properties:

1. IG type determines how the initial guesses of all model parameters are generated. See list of IG type values for details.
2. ComponentsCount defines number of fluorescent components.
3. Background correction determines if background correction is taken into account. If this property is true the Data Set associated with the model should contain the external parameter **BG ratio**.
4. Motion type specifies the motion term $G_{motion}(\tau)$ in the model equation (see general formula).

Flow model parameters:

1. *Ginf* is the level of autocorrelation function when $\tau \to \infty$.
2. *N* is the average number of fluorescent molecules in the detection volume.
3. *Tfl* is the average flow time of the fluorescent particles through the detection volume.
4. Fit parameters of selected motion term $G_{motion}(\tau)$.

Weight factors for FCS analysis are calculated by the software in two ways: 1) accordingly to the algorithm proposed in [32] (third method) if auto(cross)correlation function is calculated from the raw data; 2) by standard procedure of standard deviations calculation from a number of independent repetitions of the experiment (i.e. from a number of auto(cross)correlation curves).

PCH model

The PCH model is used to analyze Photon Counting Distribution (PCD). PCD here refers to the data to be analyzed, whereas PCH is a commonly used term to specify the method of analysis. The total PCD from a number of molecules is calculated by successive convolutions of a single molecule PCD [23, 24, 25]:

$$p^{(1)}(n,Q,q) = \frac{1}{QV_{ref}} \int_{-\infty}^{\infty} Poi(n, qT\,PSF(\mathbf{r}))d\mathbf{r}, \quad n = 1,2,\mathrm{K}\ ,$$

where $Poi(n, \xi)$ denotes the Poisson distribution with the mean value ξ, T is the counting time interval, V_{ref} is the reference volume and Q is taken so that the product QV_{ref} is large enough to completely include the illuminated volume. The total distribution $P(n)$ is the weighted average of $p^{(1)}(n,Q,q)$ convolved M times [25]

$$P(n) = \sum_{M=0}^{\infty} p^{(M)}(n,Q,q)\, Poi(M, QN)$$

$$p^{(1)}(0,Q,q) = 1 - \sum\nolimits_{k=1}^{\infty} p^{(1)}(k,Q,q),$$

$$p^{(0)}(n) = \begin{cases} 1, n = 0 \\ 0, n \neq 0, \end{cases}$$

$$p^{(M)}(n) = \underbrace{p^{(1)} \otimes p^{(1)} \otimes \ldots \otimes p^{(1)}}_{M\ times}(n)$$

We use normalization either to the effective volume $V_{ref} = V_{eff} = \chi_1^2 / \chi_2$, $\chi_k = \int_V PSF^k(\mathbf{r})d\mathbf{r}$, or on two first moments of PSF [40] in order to relate N_i obtained by PCH and FCS. An additional convolution to the background term $P_{bg}(n) = Poi(n, \lambda T)$ can be taken in order to account for the background photons $P(n,\lambda) = P(n) \otimes P_{bg}(n)$. The total PCD of a number of independent species is given by a convolution of PCD of each species $P(n) = P(n, N_1, q_1) \otimes \ldots \otimes P(n, N_n, q_n)$.

Correction for the brightness profile nonideality can be done either by the use of polynomial approximation or by introducing additional fitting parameters F_k defined as the relative difference between the integral χ_k of the k^{th} power of the actual brightness profile function (normalized to unity) and that of its 2D/3D Gaussian (or Gaussian-Lorenzian) approximation χ_{Gk}.

The most practical way to account for diffusion and other time dependent processes like triplet-state relaxation is to correct the brightness and number of molecules such that the first and second factorial cumulants of PCD are exact. According to this theory one has to calculate the so-called binning correction factor $B_2(T) = \frac{2}{T^2}\int_0^T (T-t)g(t)dt$ where $g(t)$ is a time dependent term of autocorrelation function in FCS and to correct the brightness and the number of molecules in the following form:

$$q_0 = q(T)/B_2(T),$$

$$N_0 = N(T)B_2(T),$$

where $q(T)$ and $N(T)$ are apparent parameters of the model dependent on bin time T and q_0 and N_0 are absolute values of brightness and concentration that are independent on T. In general, the binning correction factor can be calculated assuming two or even more diffusing components (such correction can be applied to a mixture of species with approximately equal brightness values but quite different hydrodynamic radii). For a model with multiple brightness components this correction has to be applied independently to each component. Triplet and diffusion characteristics can be either different or the same for each brightness component.

PCH model properties:

1. IG type determines how the initial guesses of all model parameters are generated. See list of IG type values for details.

2. Afterpulses correction performs correction for afterpulses (accordingly to Palo et al., 2006 [30]).

3. Dead time correction performs dead time correction (accordingly to Palo et al., 2006 [30]).

4. Process correction determines if process correction is taken into account. The following correction types are available:

- None,
- Triplet,
- Conformation,
- Protonation.

5. Motion correction determines if motion correction is taken into account. The following correction types are available:

- None,
- Free 2D diffusion,

- Free 3D diffusion,
- Anomalous 2D diffusion,
- Anomalous 3D diffusion.

6. Profile type specifies the type of brightness profile *PSF(x,y,z)*. The following profile types are available:
- 2D Gaussian,
- 3D Gaussian,
- Gaussian-Lorenzian, squared (to fit two-photon excitation data), not normalized to unity, see notes below,
- Polynomial (accordingly to Palo et al., 2000 [22]).

7. Profile correction type specifies the type of brightness profile correction. The following profile correction types are available:
- None,
- First order (accordingly to Huang et al., 2005 [24]). It is not applicable for the polynomial brightness profile approximation.
- Second order (accordingly to Huang et al., 2005 [24]). It is not applicable for the polynomial brightness profile approximation.

8. Normalization type specifies the type of normalization (scaling). The following normalization types are available:
- Effective volume, by representation of a number of molecules in the effective volume $N_{eff} = CV_{eff}$, i.e. by introducing the unit measurement volume $V_{eff} = 1$,
- 1st and 2nd PSF moments, i.e. by setting $\int_V PSF(\mathbf{r})d\mathbf{r} = 1, \quad \int_V PSF^2(\mathbf{r})d\mathbf{r} = 1$ [22, 40].

7. Profile defaults allows to specify default values of brightness profile parameters. Initial guesses for two and more component data are calculated assuming these settings.
8. Parameters Type specifies the type of possible combinations of fit parameters. The following parameter types are available:
- Absolute *N* and *q* values,
- *N* ratio,
- *q* ratio,
- *N* and *q* ratio.

9. Components Count defines a number of molecular components with different brightness.

Common PCH model parameters:

1. *Bg* is the mean background count rate of detector λ. It defines the sample independent background.
2. *Ni* is the mean number of molecules of *i*-th brightness component. Note, it is the number of molecules in the effective volume. For conversion of this value to other types of normalizations, see chapter below.
3. q_i is the mean number of photons detected in a time interval (brightness, cpsm) of *i*-th brightness component. Note. This is an apparent, not true, brightness, which is depend on a type of normalization and profile correction. For conversion of this value to other types of normalizations, see chapter below.
4. T_i is the translational diffusion relaxation time of molecules of the *i*-th diffusion component (exists only if **Motion correction property** is not set to "None).
5. *a* is the structural parameter (exists only if **Motion correction property** is not set to "None).
6. F_{trip} is the fractional population of the triplet state (exists only if **Process correction property** is "Triplet State").
7. T_{trip} is the relaxation time of the triplet state (exists only if **Process correction property** is "Triplet State").
8. T_{dt} is the dead time (exists only if **Dead time correction property** is "true").
9. P_{ap} is the afterpulsing probability (exists only if **Afterpulses correction** property is "true").
10. N2/N1 is the concentration ratio (exists only if **Parameters Type** property is set to either "*N* ratio" or "*N* and *q* ratio").
11. q2/q1 is the brightness ratio (exists only if **Parameters Type** property is set to either "*N* ratio" or "*N* and *q* ratio").
12. Fit parameters of selected profile.

Supported approximations of PSF:

2D Gaussian profile

$$PSF(\mathbf{r}) = PSF(x, y) = \exp\left(-2\frac{(x^2 + y^2)}{\omega_0^2}\right)$$

3D Gaussian profile

$$PSF(\mathbf{r}) = PSF(x, y, z) = \exp\left(-2\frac{(x^2 + y^2)}{\omega_0^2} - 2\frac{z^2}{z_0^2}\right)$$

Gaussian-Lorenzian squared profile

$$PSF^2(\mathbf{r}) = PSF^2(x,y,z) = \frac{4}{\pi^2\left(1+\rho z^2\right)^2}\exp\left(-4\frac{(x^2+y^2)}{\omega_0^2(1+\rho z^2)}\right)$$

Note, we use here the not normalized to unity PSF, i.e. with a factor $B_0 = 4/\pi^2$. It leads to different brightness value because B_0 becomes confounded with q. It is not a problem if one is not interested in the absolute values of q. If the normalized to unity GL profile is used: $PSF^2(\mathbf{r}) = \frac{1}{\left(1+\rho z^2\right)^2}\exp\left(-4\frac{(x^2+y^2)}{\omega_0^2(1+\rho z^2)}\right)$ and one is interested in direct comparison of the estimated values returned by methods which use different PSF, one can easily convert the value of q using the relation $q_{norm} = 4q_{not\ norm}/\pi^2$. See chapter below how to calculate the true brightness.

Polynomial profile

$PSF(\mathbf{r})$ is approximated by the exponential function of one argument with polynomial transformation of the unit of volume (further simply FIDA-like polynomial approximation) [21, 22, 40]

$$PSF(\mathbf{r}) = PSF_0\, e^{-x},\ d\mathbf{r}/dx = A_0\ (x + a_1x^2 + a_2x^3),$$

where a_1, a_2 are adjusted instrumental parameters and PSF_0 is the value of $PSF(\mathbf{r})$ at $\mathbf{r}$ equal to 0. PSF_0 and A_0 are calculated from the system of normalization equations:

$$\int_V PSF(\mathbf{r})d\mathbf{r} = 1,$$
$$\int_V PSF^2(\mathbf{r})d\mathbf{r} = 1.$$

Solution of this system yields $PSF_0 = 8u/v$, $A_0 = v/8u^2$, where $u = 2a_1 + 6a_2 + 1$ and $v = 2a_1 + 3a_2 + 2$.

Application of this normalization changes the definition of number of molecules and brightness respectively:

$$N_i = c_i\,\chi_1^2/\chi_2 = c_iV_{eff},\ q_i = q_{true\,i}\chi_2/\chi_1 = q_{true\,i}\gamma_2.$$

Single molecular PCD takes the form: $p^{(1)}(n,q) = \frac{A_0}{Q}\int_0^\infty Poi(n, qPSF_0Te^{-x})(x + a_1x^2 + a_2x^3)dx$. In this definition, results of PCH analysis with the polynomial profile are equivalent to results of FIDA.

Specific PCH model parameters:

1. *a1* is the brightness profile parameter.
2. *a2* is the brightness profile parameter.

Out-of-focus correction of 2D/3D Gaussian and Gaussian-Lorenzian profiles

Out-of-focus correction is performed by introduction of additional fitting parameters F_k defined as [23, 24]:

$$F_k = (\chi_k - \chi_{G\,k})/\chi_{G\,k},\quad k > 0.$$

In the most cases only the first order correction (all F_k equal to zero except F_1) is sufficient to get the best fit to the experimental data. F_1 can be treated as an out-of-focus emission ratio. The second order correction (F_1 and F_2 are different from zero) can be also applied.
Single molecule PCD with out-of-focus correction [24] takes the form (written for the normalization to the effective volume):

$$p^{(1)}(n,Q,q) = \frac{1+F_2}{(1+F_1)^2}\left[p^{(1)}(n,Q,q) + \frac{1}{n!Q}\sum_{k=n}^{\infty}\frac{(-1)^{k-n}(qT)^kF_k}{(k-n)!}\gamma_2\gamma_k\right],$$

whrere $p^{(1)}(n,Q,q)$ is the single molecular PCD for pure Gaussian (or squared Gaussian-Lorenzian) approximation and $\gamma_k = 1/k^{d/2}$ (d is the dimensionality) for the case of 2D/3D Gaussian approximation and $\gamma_k = \frac{1}{k}\frac{C_{4k-4}^{2k-2}}{(2\pi)^{2k-2}}$ for the case of not normalized to unity Gaussian-Lorenzian approximation. Note, factor γ_2 appears here (in comparison with the original version [24]) due to normalization to the effective volume.
The first-order correction accounts for the emission from additional (out of-focus) molecules similarly to the background uncorrelated emission that is also independent from the observation profile shape. It means that the first-order out-of-focus correction works similarly to correction for background photons. Consequently, the larger value of the out-of-focus correction parameter F_1 can compensate for the background (e.g. after fixing λ to zero) and, conversely, the larger value of λ can completely compensate for the out-of-focus signal (after fixing F_1 to zero). In spite of the possibility to omit the first-order correction term we prefer to keep it. It standardizes the theory and enables an easier comparison of fit parameters between different analysis methods. In addition, there is a clear difference

between two sources of 'background' photons. The first source is the sample-independent background, e.g. noise of detector, etc, and the second source is the sample-dependent (depends on both concentration and brightness of the sample, see equation above) out-of-focus signal.

Specific PCH model parameters:

1. ***Fc1*** is first order correction parameter.
2. ***Fc2*** is second order correction parameter.

Afterpulses and dead time correction is performed accordingly to the algorithm described in [30]. Correction for afterpulses is done by the following formula

$$P_{APcorr}(n) = \sum_{j=0}^{n} P_0(n-j) P_{binomial}(j; n-j, p_{ap}) ,$$

where p_{ap} is the afterpulsing probability and $P_0(n)$ is the ideal PCD (i.e. without correction).
Correction for dead time is done by the following formula

$$P_{DTcorr}(n) = \sum_{j=0}^{\infty} P_0(n+j) P_{binomial}(j; n+j, \frac{(n+j)\tau_{dt}}{T+(n+j)\tau_{dt}}) ,$$

where τ_{dt} is the detector dead time and $P_0(n)$ is the ideal PCD (i.e. without correction).
Actually, PCH model is calculated using several algorithms. One realization of PCH model (is used for a case of relatively low product of q T) is based on the efficient algorithm that uses generation function (GF) approach and Taylor expansion of the exponent under the integral in the single-molecular PCD [29]. Description of the model given bellow is presented for the case of Gaussian brightness profile. The generation function of probability to detect n photons $P(n)$ can be written as [29]

$$G(\xi) = \exp\left\{ \lambda T(\xi - 1) + \sum_i c_i \sum_{k=1}^{\infty} \frac{(\xi-1)^k q_i^k T^k (1+F_k) \chi_{Gk}}{k!} \right\},$$

where i is an index of molecular species, c_i is the mean number of molecules in an unit volume (concentration), q_i is the specific brightness of molecules (in counts per second per molecule), $\chi_{Gk} = \int_V PSF_G^k(\mathbf{r}) d\mathbf{r}$, G denotes Gaussian approximation and λ is the mean background count rate of the detector. It is assumed that the contribution of each single molecule to the recorded photon trace is independent and the emission intensity is constant during the counting time interval T.
$P(n)$ is obtained by the Fast Fourier Transform (FFT) of the characteristic function, which can be obtained from GF by substituting ξ by the complex exponent $e^{i\varphi}$

$$P(n) = FFT^{-1}(G(e^{i\varphi})) ,\ n = 0,1,\text{K} ,m-1 ,\ \varphi = 2\pi n/m .$$

All χ_{Gk} can be calculated as $\chi_{Gk} = \int_V PSF_G^k(\mathbf{r}) d\mathbf{r} = k^{-3/2} (\pi/2)^{3/2} w_0^2 z_0$.

After normalization to the effective volume $V_{eff} = \chi_1^2 / \chi_2$ conventionally used in FCS we arrive at

$$G(\xi) = \exp\left\{ \lambda T(\xi - 1) + \frac{1+F_2}{(1+F_1)^2} \sum_i N_i \sum_{k=1}^{\infty} \frac{(\xi-1)^k q_i^k T^k (1+F_k)}{(2k)^{3/2} k!} \right\},$$

where N_i is a mean number of molecules in the effective volume and we used following relations

$$c_i = N_i / V_{eff} ,\ V_{eff} = (1+F_1)^2 V_{eff\,G} / (1+F_2) ,\ V_{eff\,G} = \pi^{3/2} \omega_0^2 z_0 .$$

After introducing brightness ratio $r_{qi} = q_i / q_1$ and concentration ratio $r_{Ni} = N_i / N_1$ one arrives at

$$G(\xi) = \exp\left\{ \lambda T(\xi - 1) + \frac{1+F_2}{(1+F_1)^2} \sum_{k=1}^{\infty} \frac{(\xi-1)^k (1+F_k)}{(2k)^{3/2} k!} N_1 q_1^k T^k \left[1 + \sum_{i=2}^{M} r_{Ni} r_{qi}^k \right] \right\}$$

where M is a number of components. Last equation allows fitting brightness and concentration ratios instead of brightness and concentration of each species. It also opens a possibility to set reasonable constraints on brightness and concentration ratios thus making analysis more robust. Any combinations of absolute fit parameter values and their ratios are possible in FFS DP. Since our realization of this algorithm works well only for product q and T up to 20 (often problems can already arise when this product is less than 20, depending on the brightness profile approximation and correction), the algorithm of PCH, described before, is used in this case.

Weighting factors are calculated as standard deviations of Binomial distribution given by $\sigma_i = \sqrt{MP^*(i)(1-P^*(i))}$, where M is the total number of bins and $P^*(i)$ is the measured PCD. Because M is usually large, Binomial distribution is approximated well by normal distribution and therefore the application of reduced χ^2 criterion is justified.

FFC model

The FFC model is used to analyze a set of Fluorescence Factorial Cumulants [15, 20]. Factorial cumulants K_k are calculated from the experimental data by formulas:

$$K_k = F_k - \sum_{i=1}^{k-1}\left(\frac{k-1}{i}\right)K_{k-i}F_i \text{ , where } \left(\frac{k-1}{i}\right) = C_{k-1}^{i} = \frac{(k-1)!}{i!(k-i-1)!},$$

$$F_k = < n(n-1)\text{K } (n-k+1) > = \sum_{n=k}^{N-1} n(n-1)\text{K } (n-k+1)P^*(n),$$

where $P^*(n)$ is a measured photon counting distribution i.e. probability to detect n photons within a counting time interval T, the angular brackets indicate averaging with the set of probabilities $P^*(n)$.
In general FFC model is defined by the following formula:

$$K_1 = \lambda T + \chi_1 \sum_i c_i q_i T$$

$$K_k = \chi_k \sum_i c_i q_i^k \Gamma_k(T, \tau_{diff\,i})$$

where q_i is the mean number of photons (expressed in counts per second per molecule) detected in a time interval T, c_i is the concentration of molecules of the *i-th* component, λ is the mean background count rate of the detector, $\chi_k = \int_V PSF^k(\mathbf{r})d\mathbf{r}$ and

$$\Gamma_k(T) = k! \int\limits_{0\le\tau_2\le\ldots\le\tau_k\le T} \ldots \int G(\tau_2,\ldots\tau_k)(T-\tau_2-\ldots-\tau_k)d\tau_2\ldots d\tau_k$$

is the diffusion correction term. $G(\tau_2,\text{K},\tau_k)$ is the normalized correlation function of *k*th order [20]. For the limit of short bin times it takes the form

$$\Gamma_k(T, \tau_{diff\,i}) = T^k .$$

We use normalization to the effective volume $V_{eff} = \chi_1^2 / \chi_2$ in order to relate N_i obtained by both FFC and FCS. After normalization one gets

$$K_1 = \lambda T + \gamma_2 \sum_i N_i q_i T$$

$$K_k = \gamma_2\gamma_k \sum_i N_i q_i^k \Gamma_k(T, \tau_{diff\,i}).$$

Factor γ_2 that appears in Eq. 1 is due to this type of normalization. γ-factors are defined as $\gamma_k = \chi_k / \chi_1$.
To correct for deviations of the actual observation profile from its ideal approximation three different approaches can be used in FFC (see **Profile type** property of the model):
1) γ-factors (γ_3, γ_4, ...) can be fitted during the analysis (applicable only in the global analysis with linking all fitted γ-factors) [20];
2) FIDA-like polynomial profile [21, 22] can be used for the approximation of the actual PSF [39];
3) out-of focus corrected 2D/3D Gaussian [23, 24] or squared (not normalized to unity) Gauss-Lorenzian profiles can be used for the approximation of the actual PSF [39].
In the case of out-of-focus correction general FFC formula becomes

$$K_1 = \lambda T + \gamma_2 \frac{(1+F_2)}{(1+F_1)} \sum_i N_i q_i T$$

$$K_k = \gamma_2\gamma_k \frac{(1+F_2)(1+F_k)}{(1+F_1)^2} \sum_i N_i q_i^k \Gamma_k(T, \tau_{diff\,i}), \quad k = 2,3,\text{K},$$

where q becomes different from the true brightness is the correction is applied, see chapter below, F_k are correction parameters defined as relative difference between integral of the *k-th* power of the actual observation profile χ_k and that of its approximation $F_k = (\chi_k - \chi_{G\,k})/\chi_{G\,k}, k > 0$. In the most cases just first order correction is needed (all $F_k = 0$ except F_1), and sometimes second order correction (all $F_k = 0$ except F_1 and F_2) is necessary to get best fit to the experimental data.
The first-order correction accounts for the emission from additional (out of-focus) molecules similarly to the background uncorrelated photons. It means that the first-order out-of-focus correction works similarly to correction for background photons. Consequently, the larger value of the out-of-focus correction parameter F_1 can compensate for the background (e.g. after fixing λ to zero) and, conversely, the larger value of λ can completely compensate for the out-of-focus signal (after fixing F_1 to zero). The first source is the sample-independent background, e.g. noise of detector, etc, and the second source is the sample-dependent (depends on both concentration and brightness of the sample, see equation above) out-of-focus signal.
For the polynomial profile the general FFC model takes the form

$$K_1 = \lambda T + \sum_i N_i q_i T$$

$$K_k = \chi_k \sum_i N_i q_i^k \Gamma_k(T, \tau_{diff\,i})$$

where $\chi_k = A_0 PSF_0^k (2a_1 k + 6a_2 + k^2)/k^4$, $PSF_0 = 8u/v$, $A_0 = v/8u^2$ and $u = 2a_1 + 6a_2 + 1$, $v = 2a_1 + 3a_2 + 2$. A_0 and PSF_0 are chosen so that the normalization conditions $\chi_1 = \chi_2 = 1$ are satisfied. Application of different normalization conditions change the values of q and N. For a conversion of q and N between different types of normalization see chapter below.

If a number of factorial cumulants used for analysis is equal to a number of fitted parameters, the exact solution is available that leads to zero (or very close to zero) χ^2 value. The reduced χ^2 that is the measure of adequacy of the fit model to the experimental data can be calculated only if number of factorial cumulants used for analysis is more than number of fitted parameters plus one.

Weight factors of the cumulants are calculated by the software in two ways: 1) the first five weighting factors are calculated according to formulas given in [15] (moments of moments technique) if cumulants are calculated from the raw data (weighting factors of higher order cumulants are set to zero; 2) all weighting factors are calculated by standard procedure of standard deviations calculation from a number of independent repetitions of the experiment.

Note: if standard deviations are calculated from the raw data, only first five cumulants are actually analyzed. Higher order cumulants do not participate in the analysis because their standard deviations equal to zero. To analyze higher order cumulants one either has to choose the second way of the standard deviations calculation or switch off the calculation of standard deviations while calculating cumulants.

FFC model parameters:

1. *Bg* is the mean background count rate of detector λ. It defines the sample independent background.

2. *Nj* is the mean number of molecules of *j*-th brightness component. Note, it is the number of molecules in the effective volume. For conversion of this value to other types of normalizations, see chapter below.

3. q_j is the mean number of photons detected in a time interval (brightness, cpsm) of *j*-th brightness component. Note. This is an apparent, not true, brightness, which is depend on a type of normalization and profile correction. For conversion of this value to other types of normalizations, see chapter below.

4. T_i is the translational diffusion relaxation time of molecules of the *i*-th diffusion component (exists only if **Diffusion correction property** is "true").

5. *a* is the structural parameter (exists only if **Diffusion correction property** is "true" and **Profile type property** is "3D Gaussian").

6. Fit parameters of selected profile.

FFC model properties:

1. IG type determines how the initial guesses of all model parameters are generated. See list of IG type values for details.

2. ComponentsCount defines a number of molecular components with different brightness.

3. Motion correction determines if motion correction (due to diffusion) is taken into account.

4. Profile type specifies the type of *PSF(x,y,z)*. The following profile types are available:

- 2D Gaussian,
- 2D Gaussian 1-st corr.,
- 2D Gaussian 2-nd corr.,
- 3D Gaussian,
- 3D Gaussian 1-st corr.,
- 3D Gaussian 2-nd corr.,
- 3D Gaussian fitted,
- Squared Gaussian-Lorenzian,
- Squared Gaussian-Lorenzian 1-st corr.,
- Squared Gaussian-Lorenzian 2-nd corr.,
- Polynomial,

2D Gaussian profile

$$PSF(\mathbf{r}) = PSF(x, y) = \exp\left(-2\frac{(x^2 + y^2)}{\omega_0^2}\right)$$

γ-factors are calculated by $\gamma_k = 1/k^{d/2}$, where *d* is the dimensionality (*d*=2).

3D Gaussian profile

$$PSF(\mathbf{r}) = PSF(x, y, z) = \exp\left(-2\frac{(x^2 + y^2)}{\omega_0^2} - 2\frac{z^2}{z_0^2}\right)$$

γ-factors are calculated by $\gamma_k = 1/k^{d/2}$, where *d* is the dimensionality (*d*=3).

3D Gaussian fitted profile

$$PSF(\mathbf{r}) = PSF(x,y,z) = \exp\left(-2\frac{(x^2+y^2)}{\omega_0^2} - 2\frac{z^2}{z_0^2}\right)$$

γ-factors for two first cumulants are calculated as $\gamma_1\gamma_2 = 1/2\sqrt{2}$, $\gamma_2\gamma_2 = 1/8$. All other γ-factors (actually products $\gamma_2\gamma_3$, $\gamma_2\gamma_4$, …) are fit parameters.

Gaussian-Lorenzian squared profile

$$PSF^2(\mathbf{r}) = PSF^2(x,y,z) = \frac{4}{\pi^2\left(1+\rho z^2\right)^2}\exp\left(-4\frac{(x^2+y^2)}{\omega_0^2(1+\rho z^2)}\right)$$

Note, we use here the not normalized to unity PSF, i.e. with a factor $B_0 = 4/\pi^2$. It leads to different brightness value because B_0 becomes confounded with q. It is not a problem if one is not interested in the absolute values of q. If the normalized to unity GL profile is used: $PSF^2(\mathbf{r}) = \frac{1}{\left(1+\rho z^2\right)^2}\exp\left(-4\frac{(x^2+y^2)}{\omega_0^2(1+\rho z^2)}\right)$ and one is interested in direct comparison of the estimated values returned by methods which use different PSF, one can easily convert the value of q using the relation $q_{norm} = 4q_{not\ norm}/\pi^2$. For calculation of true brightness see chapter below.

γ-factors are calculated by

$$\gamma_k = \frac{1}{k}\frac{C_{4n-4}^{2n-2}}{(2\pi)^{2n-2}},\ \gamma_2 = \frac{3}{4\pi^2}.$$

For comparison, one gets $\gamma_k = \frac{1}{k}\frac{C_{4n-4}^{2n-2}}{4^{2n-2}},\ \gamma_2 = \frac{3}{16}$ for the case on normalized to unity PSF.

First order correction (2D Gaussian 1-st corr.; 3D Gaussian 1-st corr.; Squared Gaussian-Lorenzian 1-st corr.)

all $F_k = 0$ except F_1

Second order correction (2D Gaussian 2-nd corr.; 3D Gaussian 2-nd corr.; Squared Gaussian-Lorenzian 2-nd corr.)

all $F_k = 0$ except F_1 and F_2

Specific FFC model parameters:

1. *Fc1* is first order correction factor.
2. *Fc2* is second order correction factor.

Polynomial profile

$PSF(r)$ is approximated by an exponential function of one argument with polynomial transformation of unit of volume [21, 22]

$$d\mathbf{r}/dx = A_0\ (x + a_1x^2 + a_2x^3),\ x = \ln\left[PSF_0/PSF(\mathbf{r})\right],$$

where a_1, a_2 are adjusted instrumental parameters and PSF_0 is the value of $PSF(\mathbf{r})$ at $\mathbf{r}$ equal to 0. PSF_0 and A_0 are calculated from the system of normalization equations:

$$\int_V PSF(\mathbf{r})d\mathbf{r} = 1,$$
$$\int_V PSF^2(\mathbf{r})d\mathbf{r} = 1.$$

Solution of the system yields $PSF_0 = 8u/v$, $A_0 = v/8u^2$, where $u = 2a_1 + 6a_2 + 1$ and $v = 2a_1 + 3a_2 + 2$. All geometric factors can be calculated as follows $\chi_k = A_0PSF_0^k(2a_1k + 6a_2 + k^2)/k^4$.

Specific FFC model parameters:

1. *a1* is the brightness profile parameter.
2. *a2* is the brightness profile parameter.

Monomer-N-mer FFC model

The Monomer-N-mer FFC is a modification of FFC model designed to analyze two component sample with known brightness ratio. For Gaussian profile with out-of-focus correction it is defined by the following formula ($F_1 = F_2 = 0$ if out-of-focus correction is not applied):

$$K_1 = Bg\,T + \gamma_2 \frac{(1+F_2)}{(1+F_1)}(N_1 qT + N_2 r qT)$$

$$K_k = \gamma_2\gamma_k \frac{(1+F_2)(1+F_k)}{(1+F_1)^2}(N_1 q^k \Gamma_k(T,\tau_{diff1}) + N_2 (rq)^k \Gamma_k(T,\tau_{diff2})), \quad k = 2,3,\mathrm{K}\ ,$$

where *r* is the brightness ratio. For polynomial profile it takes the form

$$K_1 = \lambda\,T + N_1 qT + N_2 kqT$$

$$K_k = \frac{A_0 PSF_0^k (2ak + 6b + k^2)}{k^4}(N_1 q^k \Gamma_k(T,\tau_{diff1}) + N_2 (kq)^k \Gamma_k(T,\tau_{diff2})), \quad k = 2,3,\mathrm{K}\ .$$

Monomer N-mer FFC model properties:

Properties of the model are similar to FFC model except of additional property **Brightness ratio**. Brightness ratio property is expressed in real numbers, not only integers.

Monomer N-mer FFC model parameters:

1. *Bg* is the mean background count rate of detector λ.
2. *N_monomer* is the mean number of monomer molecules in the effective volume.
3. *N_n-mer* is the mean number of N-mer molecules in the effective volume.
3. *q* is the mean number of photons detected in a time interval (brightness, cpsm) of monomer. Becomes a function of F_i, see chapter below.
4. T_i is the translational diffusion relaxation time of molecules of the *i*-th diffusion component (exists only if **Diffusion correction property** is "true").
5. *a* is the structural parameter (exists only if **Diffusion correction property** is "true" and **Profile type property** is "3D Gaussian").
6. Fit parameters of selected profile.

Conversion formulas for N and q [42]

Note that, FIDA, PCH and FCA are mathematically equivalent and, for the noise-free data, must result in exactly the same estimates of the model parameters. However, the application of different brightness profile approximations and normalizations in PCH and FIDA leads to different measurement units of the concentration and brightness. Brightness estimated using different methods may differ more than four times(!). It does not introduce any errors in the analysis when scientists are interested in relative changes of concentration and brightness in measurements performed at same experimental conditions. However, in PCH with out-of-focus correction a linear dependence of the brightness on the out-of-focus correction parameters is observed. Change of the measurement conditions may result in significant change of the out-of-focus correction parameters and consequently, estimated brightness.
The application of the different types of normalizations and brightness profile approximations leads to different values of brightness and number of molecules in the observation volume. Fortunately, the correction for an out-of-focus emission does not affect the value of the number of molecules in the effective volume. However, the estimators of brightness are different, depending on the correction order. Therefore, when a correction is applied (F_1, $F_2 \neq 0$), the estimated brightness represents, so called, apparent brightness *q*, which must be recalculated into the "true" one

$$q_{true} = q(1+F_2)/(1+F_1)\,.$$

While normalization to the observation volume $V_{PSF} = \int_V \bar{B}(\mathbf{r})dV$ both $N_{PSF} = cV_{PSF}$ and *q* depend on the correction parameters and hence they both vary from one type of correction to another

$$N = (1+F_1)N_{PSF}/((1+F_2)\gamma_2)\,.$$

While normalization on two first moments of brightness profile (FIDA-type normalization) the normalization conditions can be realized by a variable substitution $N_i = c_i\,\chi_1^2/\chi_2 = c_i V_{eff}$, $q_i = q_{truei}\chi_2/\chi_1 = q_{truei}\gamma_2$. Therefore, we still estimate the number of molecules in the effective volume but

$$q_{true} = q/\gamma_2\ .$$

In contrast to PCH and FCA with out-of-focus correction, FIDA-type normalization does not exhibit dependence of *N* and *q* on correction parameters, because of the chosen normalization. Two normalization conditions allow to simultaneously determine two unknown parameters: the size of the observation volume and the value of the brightness profile in the focus.
Relations between brightness at normalization on the effective volume and normalization on two first moments of brightness profile are given below (we added subscripts *FIDA* and *eff* to distinguish both estimates)

$$q_{FIDA} = \gamma_2 q_{eff}\ (1+F_2)/(1+F_1)\,.$$

Since at normalization on the effective volume number of molecules are the same for all methods (FCS, PCH, FIDA, FCA, …), used in FFS Data Processor and it does not depend on the profile correction parameters, we prefer to use

namely the normalization on the effective volume instead of the normalization to the observation volume V_{PSF}. It allows the global analysis of all these methods with direct linking of N.

Not also, that we use here the not normalized to unity squared Gaussian-Lorenzian profile (with $PSF_{GL}{}^2(0) = 4/\pi^2$). It leads to different brightness value because $PSF_{GL}{}^2(0)$ becomes confounded with q. If one is interested in direct comparison of the estimated brightness returned by their own realization of the method which uses the normalized to unity PSF, one can easily convert the value of q using the relation $q_{GL^2norm} = 4\,q_{GL^2not\ norm} \big/ \pi^2$. If the normalization on two first moments of brightness profile is applied, the true brightness should be calculated using the correct gamma factor $q_{true} = q \big/ \gamma_{GL^2 2}$, $\gamma_{GL^2 2} = \frac{3}{4\pi^2}$, not $\gamma_{GL^2 2} = \frac{3}{16}$ derived for the case on normalized to unity squared Gaussian-Lorenzian PSF.

Gaussian model

Gaussian model is used to analyse coincidence values histogram [16] and photon counting distribution. The model is based on the Gaussian distribution function

$$f(x) = \frac{1}{\sqrt{2\pi}\,\sigma} e^{-\frac{(x-m)^2}{2\sigma^2}}$$

where m is the mean, σ is the standard deviation.

Gaussian model properties:

1. IG type determines how the initial guesses of all model parameters are generated. See list of IG type values for details.

Gaussian model parameters:

1. *M* is the mean of the Gaussian distribution.
2. *Sigma* is the standard deviation of the Gaussian distribution.

Poissonian model

Poissonian model can used to analyse photon counting distributions

$$f(x) = \frac{\lambda^x}{x!} e^{-\lambda}$$

where λ is the mean of Poisson distribution and x must be integer.

Poissonian model properties:

1. IG type determines how the initial guesses of all model parameters are generated. See list of IG type values for details.

Poissonian model parameters:

1. *lambda* is the mean of Poisson distribution.

Brightness vs. time model

The Brightness vs. time model is used to analyze brightness versus bin time dependence that can be made from a number of fitted photon counting distributions calculated at different bin times. This model allows to accomplish PCMH analysis. It is defined by the following formula:

$$q(T) = q\,X_{BG} \frac{2}{T^2} \int_0^T (T-t) X_{kinetics}(t) G_{motion}(t)\,dt\ ,$$

where q is the true brightness, X_{bg}, $X_{kinetic}(t)$ and $G_{motion}(t)$ are background correction term, process and motion terms of FCS model.

Brightness vs. time model properties:

1. Background correction determines if background correction is taken into account. If this property is true the Data Set associated with the model should contain the external parameter **BG ratio**.
2. Process type specifies the process term $X_{kinetic}(t)$.
3. Components Count defines a number of molecular components with equal brightness.

Brightness vs. time model parameters:

1. F_i is the contribution of molecules of the *i*-th fluorescent component.

2. T_i is the translational diffusion relaxation time of molecules of the *i*-th fluorescent component.

3. *a* is the structural parameter.

4. *q* is the mean number of photons detected in a time interval (brightness, cpsm).

Concentration vs. time model

The Concentration vs. time model is used to analyze concentration versus bin time dependence that can be made from a number of fitted photon counting distributions calculated at different bin times. This model allows to accomplish PCMH analysis. It is defined by the following formula:

$$N(T) = X_{BG}\frac{2N}{T^2}\int_0^T (T-t)X_{kinetics}(t)G_{motion}(t)dt\,,$$

where N is the true number of molecules in the effective volume, X_{bg}, $X_{kinetic}(t)$ and $G_{motion}(t)$ are background correction term, process and motion terms of FCS model.

Concentration vs. time model properties:

1. Background correction determines if background correction is taken into account. If this property is true the Data Set associated with the model should contain the external parameter **BG ratio**.

2. Process type specifies the process term $X_{kinetic}(t)$.

3. ComponentsCount defines a number of molecular components with equal brightness.

Concentration vs. time model parameters:

1. F_i is the contribution of molecules of the *i*-th fluorescent component.

2. T_i is the translational diffusion relaxation time of molecules of the *i*-th fluorescent component.

3. *a* is the structural parameter.

4. *N* is the mean number of molecules.

Custom model

Custom model is designed to perform the data analysis with user defined mathematical description. The special Script programming language was developed for writing the user-defined models. Before constructing new model some fit parameters have to be created. The names assigned to the parameters can then be used in the model script.

Custom model properties:

1. IG type determines how the initial guesses of all model parameters are generated. See list of IG type values for details.

2. Configuration provides access to Custom model configuration dialog box. This dialog box can be accessed by pressing button located on the right side of the property value box.

3. Subtype provides quick selection of the needed custom model from the database.

Custom model parameters:

The parameters are defined by user. To learn more about custom models see **"Custom model"** section bellow.

Custom model

Script programming language

The following topics provide a formal definition of **Script programming language**, which is used for creating custom model scripts:

- Lexical elements
- Whitespace
- Tokens
- Variables
- Expressions
- Built in functions

Whitespace

Whitespace is the collective name given to spaces (blanks), horizontal and vertical tabs and newline characters. Whitespace can serve to indicate where tokens start and end.
For example, the following two sequences are lexically equivalent:

```
var: a, x, y;
a = x+y;
```

and

```
var:
   a, x, y;
a  =
  x+y;
```

Tokens

Tokens are word-like units recognized by a language. Script programming language recognizes five classes of tokens. Here is the formal definition of a token:

- keyword
- identifier
- operator
- built in function
- punctuator (also known as separator)

As the source code is scanned, tokens are extracted in such a way that the longest possible token from the character sequence is selected. For example, **external** would be parsed as a single identifier, rather than as the keyword **extern** followed by the identifier **al**.

Keywords

Keywords are words reserved for special purposes and must not be used as normal identifier names. Keyword ***var:*** is used to declare new variables.
Example:

```
var: x, y, z;
```

In this example three variables (x, y, and z) are declared.

Variable identifiers

Identifiers are arbitrary names of any length given to variables.
Naming and length restrictions
Identifiers can contain the letters **a** to **z** and **A** to **Z**, and the digits **0** to **9**. There is only one restriction: the first character must be a letter.
Case sensitivity
Script programming language identifiers are case sensitive, so that **Sum**, **sum** and **suM** are distinct identifiers.
Uniqueness
Although identifier names are arbitrary (within the rules stated), errors are generated if the same name is used for more than one identifier within the script.

Variables

Variable is a named storage location that can contain data that can be modified during script execution. Each variable has a name that uniquely identifies it within script. The variable names are sometimes referred to as identifiers .Variables can be of three types:

- external variables - fit parameters, time and external parameters.
- internal variables - can be defined by user in the script.

- result variable - contains script execution result.

Internal variables declaration

Internal variables declaration is a list of variable identifiers. The declaration begins with keyword **var:**. The identifiers are separated by commas and the list is terminated by a semicolon.

Example:

var: var1, var2, var3, ...;

where var1, var2, var3, ... are any sequence of distinct identifiers .

Operators

Operators are tokens that trigger some computation when applied to variables and other objects in an expression. The following operators are available in the Script programming language:

* Operator
/ Operator
+ Operator
- Operator
= Operator

= Operator

This operator assigns the value of an expression to an internal variable.

Syntax

varname = ***expression***

The **= *operator*** syntax has following parts:

Part	Description
varname	Required; any internal variable.
expression	Required; any numeric expression .

Example:

```
var: Var, Var1, Var2;
Var1 = 5;
Var2 = Var1+10;
Var = Var1 + Var2;
```

+ Operator

This operator is used to sum two numbers.

Syntax

result = ***expression1***+***expression2***

The **+ *operator*** syntax has following parts:

Part	Description
result	Required; any internal variable .
expression1	Required; any numeric expression .
expression2	Required; any numeric expression.

Example:

```
var: MyNumber, Var1, Var2;
MyNumber = 2 + 2;          ' Returns 4.
MyNumber = 4257.04 + 98112;     ' Returns 102369.04.

Var1 = 34; Var2 = 6;        ' Initialize variables.
MyNumber = Var1 + Var2;          ' Returns 40.
```

- Operator

This operator is used to find the difference between two numbers or to indicate the negative value of a numeric expression .

Syntax 1

result = ***expression1-expression2***

Syntax 2

-expression

The **- *operator*** syntax has following parts:

Part	Description
result	Required; any internal variable .
expression1	Required; any numeric expression .
expression2	Required; any numeric expression.
expression	Required; any numeric expression.

Remarks
In Syntax 1, the ***- operator*** is the arithmetic subtraction operator used to find the difference between two numbers.
In Syntax 2, the ***- operator*** is used as the unary negation operator to indicate the negative value of an expression.
Example:

```
var: MyResult, MyVar;
MyResult = 4 - 2;           ' Returns 2.
MyResult = 459.35 - 334.90;         ' Returns 124.45.

MyVar = 2;
MyResult = -MyVar;        ' Returns -2;
```

**** Operator***

This operator is used to multiply two numbers.
Syntax
result = ***expression1*expression2***
The **** operator*** syntax has following parts:

Part	Description
result	Required; any internal variable .
expression1	Required; any numeric expression .
expression2	Required; any numeric expression.

Example:

```
var: MyValue;
MyValue = 2 * 2;           ' Returns 4.
MyValue = 459.35 * 334.90;         ' Returns 153836.315.
```

/ Operator

This operator is used to divide two numbers.
Syntax
result = ***expression1/expression2***
The ***/ operator*** syntax has following parts:

Part	Description
result	Required; any internal variable .
expression1	Required; any numeric expression .
expression2	Required; any numeric expression.

Note: ***expression2*** must be nonzero ***expression2*** = 0 results in a runtime error. (You can't divide by zero.)

Example:

```
var: MyValue;
MyValue = 10 / 4;          ' Returns 2.5.
MyValue = 10 / 3;          ' Returns 3.333333.
```

Built in functions

Built in functions are used to perform mathematical calculations.
The following functions are available in the Script programming language:

abs	acos	asin	atan	cos	erf	erfc	exp
fact	Gamma	log	log10	logGamma		pow	
sin	sqrt	tan					

abs

Syntax

```
abs(x);
```

Description
Returns the absolute value of a number.
Return Value
abs returns the absolute value of **x**.

acos

Syntax

```
acos(x);
```

Description
Calculates the arc cosine.

Arguments to ***acos*** must be in the range -1 to 1. Otherwise a runtime error will occur.
Return Value
acos of an argument between -1 and +1 returns a value in the range 0 to pi.

asin

Syntax
asin(x);
Description
Calculates the arc sine.
Arguments to ***asin*** must be in the range -1 to 1. Otherwise a runtime error will occur.
Return Value
asin of an argument between -1 and +1 returns a value in the range -pi/2 to pi/2.

atan

Syntax
atan(x);
Description
Calculates the arc tangent.
Return Value
atan of **x** returns a value in the range -pi/2 to pi/2.

cos

Syntax
cos(x);
Description
Calculates the cosine of a number.
The angle is specified in radians.
Return Value
cos returns a value in the range -1 to 1.

exp

Syntax
exp(x);
Description
Calculates the exponential **e** to the **x**.
Return Value
exp returns **e** to the **x**.
The constant **e** is approximately 2.718282. If the value of **x** exceeds 709.782712893, a runtime error occurs.

Note: The **exp** function complements the action of the log function and is sometimes referred to as the antilogarithm.

log

Syntax
log(x);
Description
Calculates the natural logarithm of x.
If the argument x passed to this function is 0 or less than 0, runtime error occurs.
Return Value
On success, log returns the value calculated ln(x).

log10

Syntax
log10(x);
Description
Calculates the base ten logarithm of **x**.
If the argument **x** passed to this function is 0 or less than 0, runtime error occurs.
Return Value
On success, ***log10*** returns the calculated value log base ten of **x**.

pow

Syntax
pow(x, y);
Description

Calculates x to the power of y.
If the result of this function is more than $1.79 \cdot 10^{308}$, the overflow runtime error will occur. If the argument x passed to pow is real and less than 0, and y is not a whole number, or you call *pow(*0,0*)*, runtime error will occur.
Return Value
On success, pow returns the value calculated of x to the power of y.

sin

Syntax
sin(x);
Description
Calculates the sine of a value.
The angle is specified in radians.
Return Value
sin returns a value in the range -1 to 1.

sqrt

Syntax
sqrt(x);
Description
Calculates the positive square root.
If **x** is positive, the result is positive. If **x** is negative, runtime error will occur.
Return Value
On success, ***sqrt*** returns the square root of **x**.

tan

Syntax
tan(x);
Description
Calculates the tangent.
Angles are specified in radians.
Return Value
tan returns the tangent of x, sin(x)/cos(x) .

fact

Syntax
fact(x);
Description
Calculates the factorial of **x** rounded to the nearest lesser integer.
If **x** is negative, runtime error will occur.
If the result of this function is more than $1.79 \cdot 10^{308}$, the overflow runtime error will occur.
Return Value
On success, ***fact*** returns the factorial of **x**.

erf

Syntax
erf(x);
Description
Calculates the error function of **x**

$$erf(x) = \frac{2}{\sqrt{\pi}} \int_0^x e^{-t^2} dt$$

Return Value
erf returns the error function of **x**.

erfc

Syntax
erfc(x);
Description

Calculates the complementary error function of **x,** $erfc(x) = \frac{2}{\sqrt{\pi}} \int_x^\infty e^{-t^2} dt$

Return Value
erfc returns the complementary error function of **x**.

Gamma

Syntax
Gamma(x);
Description
Calculates the gamma function of **x**

$$Gamma(x) = \int_0^\infty t^{x-1} e^{-t} dt$$

Return Value
Gamma returns the gamma function of **x**.

logGamma

Syntax
logGamma(x);
Description
Calculates the natural logarithm of the gamma function of **x**.
Return Value
logGamma returns the natural logarithm of the gamma function of **x**.

Punctuators

The Script programming language punctuators (also known as separators) are:
( )
,
;

Parentheses

There are two cases when open and close parentheses ( ) are used:
• to indicate function calls and function parameters:
Example:
func(); /* function call, no arguments */
• to group expressions and change operator precedence:
Example:
d = c * (a + b); /* override normal precedence */

Comma

The comma (,) punctuator is used for:
• separation of the elements of a function argument list:
Example:
func(i, j); /* call function with two arguments */
• separation of the different variables while making variable declaration:
Example:
var: x, y, z;

Semicolon

The semicolon (;) is a statement terminator. Any legal Script programming language expression is followed by a semicolon.

Numeric expression

Any expression that can be evaluated is a number. Elements of an expression can include any combination of variables, built in functions and operators that result in a number.

Expression

An expression is a sequence of operators, operands, and punctuators that specifies a computation.
Syntax
variable part = numeric expression ;
Where ***variable part*** is any defined internal variable.

Note: The script must contain at least one expression with the ***result*** variable.

Methods

Global fit

The parameters of the models can be estimated by a global fitting procedure, based on the Marquardt-Levenberg non-linear method of least squares [9, 10]. In the global analysis, several measured and/or simulated characteristics are combined and simultaneously fitted. Certain parameters can be linked. The values of parameters linked together are kept equal to each other. Each parameter can be fixed into the predefined value. For each parameter the range of admissible values can be set by defining the constraints. The global χ^2 criterion is used as a target criterion. Local χ^2 criterion values of each analyzed characteristic can be inspected by correspondent property of the Dataset (Local fit criterion). Characteristics of different types (i.e. autocorrelations, photon counting distributions and fluorescence cumulants) can by analyzed together globally if there is a possibility to link some parameters of the corresponding models (f. e. number of molecules *N* in FCS, PCH and FFC models). To make it possible we apply normalization to the effective volume for all these models.

Sequential fit

The parameters of the models can be estimated by a sequential fitting procedure, based on the Marquardt-Levenberg non-linear method of least squares [9, 10]. In the sequential analysis, several measured and/or simulated characteristics are fitted one by one. Each parameter can be fixed into the predefined value. For each parameter the range of admissible values can be set by defining the constraints. Local χ^2 criterion values of each analyzed characteristic can be inspected by corresponding property of the Dataset (Local fit criterion).

Quality of fit and optimization

The quality of fit is judged by χ^2 criterion and visual inspection of the residuals between experimental and fitted curves. χ^2 criterion, which is a sum of squared weighted differences of an experimental $D^E(i)$ and theoretical $D^T(i,a)$ data, $i = 1,\ldots,N$ is defined by the formula:

$$\chi^2(\boldsymbol{a}) = \frac{1}{N-m-1}\sum_{i=1}^{N} w(i)\left(D^E(i) - D^T(i,\boldsymbol{a})\right)^2, \qquad (1)$$

where $\boldsymbol{a}$ is a vector of the unknown model parameters, N is the number of data points, m is a number of fitted parameters and $w(i)$ is the weighting factor (inverse value of the data point variance).

Global analysis of experimental data obeying different functional forms may result in overestimation (or underestimation) of some model parameters, if appropriate weighting factors are not applied to each data point. This is especially important when the number of data points in these functions is quite different, for instance the ACF usually has 175 experimental data points versus only 10-20 data points in PCD. Such difference in number of data points leads to a significant difference in the number of degrees of freedom corresponding to each analyzed curve. Thus, the standard global $\chi 2$ criterion (1) becomes relatively insensitive to small deviations between the measured and model-generated curves that have lower number of data points. To avoid this problem it is necessary to take into account the specific weight of each individual curve that participates in the global analysis. It can be done if χ^2 is modified in the following way

$$\chi^2_{\mathrm{mod}}(a) = \frac{N-m-M}{M(N-m+M^{gr}-1)}\sum_{i=1}^{M}\left(\frac{1}{n_i-m_i-1}\sum_{j=1}^{n_i} w_{ij}\left(D^E_{ij} - D^T_{ij}(\boldsymbol{a})\right)^2\right), \qquad (2)$$

where $M^{gr} = \sum_{i=1}^{M} m_i^{lnk} - m^{gr}$; M is the number of globally analyzed curves; n_i is the number of points in ith curve ; m_i and m_i^{lnk} is the number of free and linked parameters in ith model respectively, m^{gr} is a number of parameter groups (sets of linked parameters), $N = \sum_{i=1}^{M} n_i$, $m = \sum_{i=1}^{M} m_i$. As follows from the equation the contribution of each analyzed curve to the value of the global χ^2 is made equal by dividing the sum in brackets by the corresponding number of degrees of freedom (thus obtaining the local χ^2) and finally multiplying the total sum of the local χ^2 by the average number of degrees of freedom $\left(N-m-M\right)/M = \sum_{i=1}^{M}\left(n_i-m_i-1\right)/M$.

FFS DP supports both versions of the target criteria.

There are many various optimization algorithms, developed for the minimization of the criterion function χ^2 [33, 34]. These algorithms are usually based on the iterative searching, when, starting from a priory chosen initial guesses, a new set of parameters is generated after the comparison of the criterion on the current and previous iterations. The search stops when either the value of criterion or the values of parameters do not change more than a priory chosen threshold, or number of iterations exceeds some critical value. The implementations of the algorithms differ in a way of the generation of a new set of parameters. There are algorithms that optimize only one solution as well as some solution-population based methods that optimize a number of possible solutions simultaneously.

One of the most wide-spread and rigorous iterative algorithms is Marquardt non-linear least-squares algorithm [9, 35, 36]. The idea consists of the linearization of the model in a truncated Taylor series in order to make use of linear least-squares analysis, and attain the desired minimum value of χ^2 criterion by an iterative sequence of calculations.
The nonlinear theoretical model function $G^T(i,\mathbf{a})$ is linearized by the expansion in a truncated Taylor series near the vector of initial guesses $\boldsymbol{a^0}$:

$$G^T(i,\mathbf{a}) = G^T(i,\mathbf{a^0}) + \sum_{j=1}^{m}\left(\frac{\partial G^T(i,\mathbf{a^0})}{\partial a_j}\right)\delta a_j \tag{2}$$

where unknown coefficients $\delta a_1,\mathrm{K}\,,\delta a_m$ are corrections to the parameters $a_1^0,\mathrm{K}\,,a_m^0$, and are assumed to be small enough to expand $G^T(i,\mathbf{a})$ in a Taylor series and to truncate it after the first-order terms. Eq. 2 is the equation of linear regression with respect to the coefficients $\delta a_1,\mathrm{K}\,,\delta a_m$. These coefficients can be found by the linear least squares method, applied directly to Eq. 2, as the solution of a set of linear algebraic equations

$$\left(\frac{\partial \chi^2}{\partial \delta a_j}\right) = 0,\ j = 1,...,m. \tag{3}$$

After substituting Eq. 1 into Eq. 3, one obtains:

$$\sum_{i=1}^{n} w(t_i)\left[G^E(i) - G^T(i,\mathbf{a^0}) - \sum_{j=1}^{m}\left(\frac{\partial G^T(i,\mathbf{a^0})}{\partial a_j}\right)\delta a_j\right]\left(\frac{\partial G^T(i,\mathbf{a^0})}{\partial a_j}\right) = 0, \quad j = 1,\mathrm{K}\,,m. \tag{4}$$

Introducing weighted residuals $E(i) = w(i)\left(G^E(i) - G^T(i,\mathbf{a^0})\right)$, set 4 can be rewritten as:

$$\sum_{k=1}^{m}\sum_{i=1}^{n} w(i)\left(\frac{\partial G^T(i,\mathbf{a^0})}{\partial a_k}\right)\left(\frac{\partial G^T(i,\mathbf{a^0})}{\partial a_j}\right)\delta a_k = \sum_{i=1}^{n} w(i)E(i)\left(\frac{\partial G^T(i,\mathbf{a^0})}{\partial a_j}\right), \quad j = 1,\mathrm{K}\,,m. \tag{5}$$

Once the vector of coefficients $\delta a_1,\mathrm{K}\,,\delta a_m$ is obtained from the set 5, a new realization of the vector $\boldsymbol{a}$ can be calculated:

$$a_j = a_j^0 + \delta a_j,\ j = 1,...,m \tag{6}$$

The improved estimate of a_j replaces a_j^0 in Eq. 2 and iteration starts again.
Marquardt [36] had developed a method that exhibited a gradient like search direction when far from the minimum and then moved smoothly into the analytical method near the minimum. The method improves the conditioning of the matrix of partial derivatives

$$\mathbf{B} = \sum_{i=1}^{n} w(i)\left(\frac{\partial G^T(i,\mathbf{a^0})}{\partial a_k}\right)\left(\frac{\partial G^T(i,\mathbf{a^0})}{\partial a_j}\right), \quad j,k = 1,\mathrm{K}\,,m. \tag{7}$$

The off diagonal elements of Eq. 7 are left unchanged but the diagonal elements are redefined as follows:

$$b_{ii} = (1+\lambda)b_{ii}, \quad i = 1,\mathrm{K}\,,m \tag{8}$$

If λ = 0, the analytical solution is provided by Eq. 7. If λ is large, the off diagonal elements $b_{ij}(i \neq j)$ become insignificant compared to the diagonal elements. The search direction is then along the path of steepest descent or the gradient method.
The Marquardt method adjusts λ to ensure that after each iteration χ^2 decreases; λ is reduced at each iteration as long as χ^2 decreases. If the solution causes χ^2 to increase, however, λ is increased. In this manner, failure of the analytical-like solution causes λ to increase, which makes the solution more steepest-descent-like until χ^2 is reduced. As the minimum is approached, however, the analytical solution usually becomes more accurate and λ approaches zero.
The covariance matrix *C* of the fit parameters is given by

$$\mathbf{C}=\mathbf{B}^{-1} \tag{9}$$

when $\lambda=0$.

Initial guesses

In general, iterative methods, where a new set of parameters is generated on the basis of available initial guesses (IG), are used for fitting of a theoretical model to the experimental data [13]. If IG are in close proximity to the unknown parameters, they can significantly increase the efficiency and correctness of the fit. Moreover, if the target criterion

surface has a complex shape with many local minima, the possibility to reach global minima directly depends on the quality of the IG. Even with obviously reasonable, physically admissible, but randomly chosen IG, the iterative procedure may converge to situations where the fitting model becomes distorted or cannot be even numerically evaluated. It is also important that reliable algorithm for IG generation reduces user participation and renders the whole procedure more standardized. For correlation functions the initial guesses for parameters are generated by the phase plane method. For PCH and FFC method of moments is primarily used.
For example, for PCH model, user can select following types of IG:

IG type	Description
None	Generation of the initial guesses is not performed in any cases.
Method of moments	Generation of the initial guesses is performed by method of moments.
User-defined	The values defined by user will be used as initial guesses. In the case of Custom model these values can be set via Custom model configuration dialog box.
Predefined values	The predefined values will be used as initial guesses. These values are specific for each type of the model and do not depend on the data of characteristic associated with the model.

Confidence intervals calculation

Exhaustive search

Error estimation of the recovered parameters can be performed by the exhaustive search method [12] (other name is support plane). In this method the examined parameter is fixed at a number of particular values in a predetermined range, while other parameters are allowed to adjust to the minimum of χ^2. Thus, the dependence of the χ^2 values on the particular parameter is observed. Analysis stops when the calculated value of χ^2 becomes higher than the χ^2 level obtained from the statistical F-test (for that particular confidential probability and number of degrees of freedom) [9]. The value of the examined parameter obtained by the procedure described above is taken as the border of the confidential interval.
To set confidential probability use property CI Probability of the Experiment Object.

Asymptotic standard errors

Error estimation of the recovered parameters can be performed by calculating asymptotic standard errors (ASE) [14]. Confidential interval for the parameter p_j, $j = 0, 1,\ldots$ is calculated according to the following equation:

$$p_j - t_{\alpha/2,\nu}\sqrt{\chi^2_{\min} C_{ij}} \le p_j \le p_j + t_{\alpha/2,\nu}\sqrt{\chi^2_{\min} C_{ij}}$$

In this equation:
1. p_j - the value of estimated parameter j;
2. C_{jj} - the jj element of the inverted Marquardt matrix obtained after the analysis (Eq. 9);
3. $\alpha = 1 - \beta$ (β is confidential probability ($0 < \beta < 1$));
4. $t_{\alpha/2,\nu}$ - the upper percentage point of the t - distribution (Student's distribution) with $\nu=n-m-1$ degrees of freedom (n is the number of experimental points and m is the number of estimated parameters). It can be calculated from the definitions of percentage point of Student's distribution and its distribution function.
5. $\chi^2_{\min}$ - the value of χ^2 obtained after the analysis.

To set confidential probability use property CI Probability of the Experiment Object.

Fit parameters dependence (FPD) analysis

A special analyzable characteristic can be constructed in FFS Data Processor. It is a sequence of a certain fit parameter values versus either model number or any available external parameter like Time Step, Repeat Number, Duration, etc. So if one performs a number of experiments and the dependence of some model parameter versus parameter of the experiment (external parameters) is known, the construction and analysis of such parameters dependence curve is possible in FFS Data Processor. The typical application of this possibility is PCMH [28] where a brightness (or concentration) vs. bin time dependence is analyzed in order to get true brightness (or concentration) estimates. There are two special models: brightness vs. time and concentration vs. time, which are primarily designed to perform PCMH analysis. But one can also design his own custom models like exponential and fit necessary dependences. For example, dissociation constant K_D can be derived from a fit of the titration curve obtained as a dependence of the parameter F_2 of FCS model versus dilution coefficient (the latter can be stored as an external parameter or can by typed in any numerical field of the Files table of the Measurements database, e.g. in the field Duration) to the equation

$$Y = \frac{K_D + [B]_o + [A]_o - \sqrt{(K_D + [B]_o + [A]_o)^2 - 4[B]_o \cdot [A]_o}}{2[A]_o}$$

which can be easily programmed in the custom model script.

Coincidence analysis

Coincidence value $K(n)$ is calculated from experimental data by formula:

$$K(n) = n\frac{\sum_m N_1(m)N_2(m)}{\sum_m N_1(m)\sum_m N_2(m)}$$

$K(n)$ represents the coincidence value as a measure of the relative frequency of coincident events in two detection channels. $N_1(m)$ and $N_2(m)$ are the absolute photon count numbers of the emission signals (blue and red channels) in consecutive time channels m, and n is the total number of time channels in the trace.

The experimental data time trace can be subdivided into a number of sections. In that case coincidence values are calculated on a base of each section and then their histogram is calculated. The coincidence histogram can be fitted by Gaussian distribution revealing average coincidence value and its standard deviation.

Coincidence separation value is calculated according to the following equation:

$$Q_{sep} = \frac{2\left|< K_1 > - < K_2 >\right|}{\sigma_{K1} + \sigma_{K2}}$$

where σ_{K1} and σ_{K2} are the standard deviations, $<K_1>$ and $<K_2>$ are mean values of the coincidence values $K_1(n)$ and $K_2(n)$ for two samples.

Coincidence bursts counting

Coincidence bursts can be automatically counted in the FFS Data Processor. Following things are possible:

- Automatic selection and counting of bursts in both channels separately by setting min and max threshold on intensity graph;
- Lee filtration of intensity graph;
- Counting and selection of coincidence bursts by analyzing of start/end positions of each burst in both channels. Only bursts with given time overlap and photon counts ratio (implemented for the accounting the leaking of the fluorescence from blue to red channel) are counted;
- Highlighting of the leaked bursts (in the red channel chart) by blue color;
- Calculating of percentage of coincidence bursts from a number of bursts in selected channel.

Simulator

Simulation tools are aimed to investigate the performance of the fitting procedures with respect to the particular type of characteristic. Simulation consists of the numeric generation of the corresponding characteristic distorted by statistical noise.

The characteristics of statistical noise are strongly dependent on the experimental methods and apparatus tools. In the case of FCS measurements, for example, expressions, describing statistics of the obtained correlation function, are known only for a limited number of particular cases. That is why we assume that value in each sampling interval or channel is the random value with Gaussian probability function with mean value equal to the true value of correlation function and empirically derived standard deviation:

$$\sigma(t) = \alpha t^{\gamma} + \beta$$

where α, γ and β are the adjusted parameters (parameters of simulator).

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