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# reSOLUTION

## A User's Guide to STED-FCS

**St**imulated **E**mission **D**epletion-**F**luorescence **C**orrelation **S**pectroscopy

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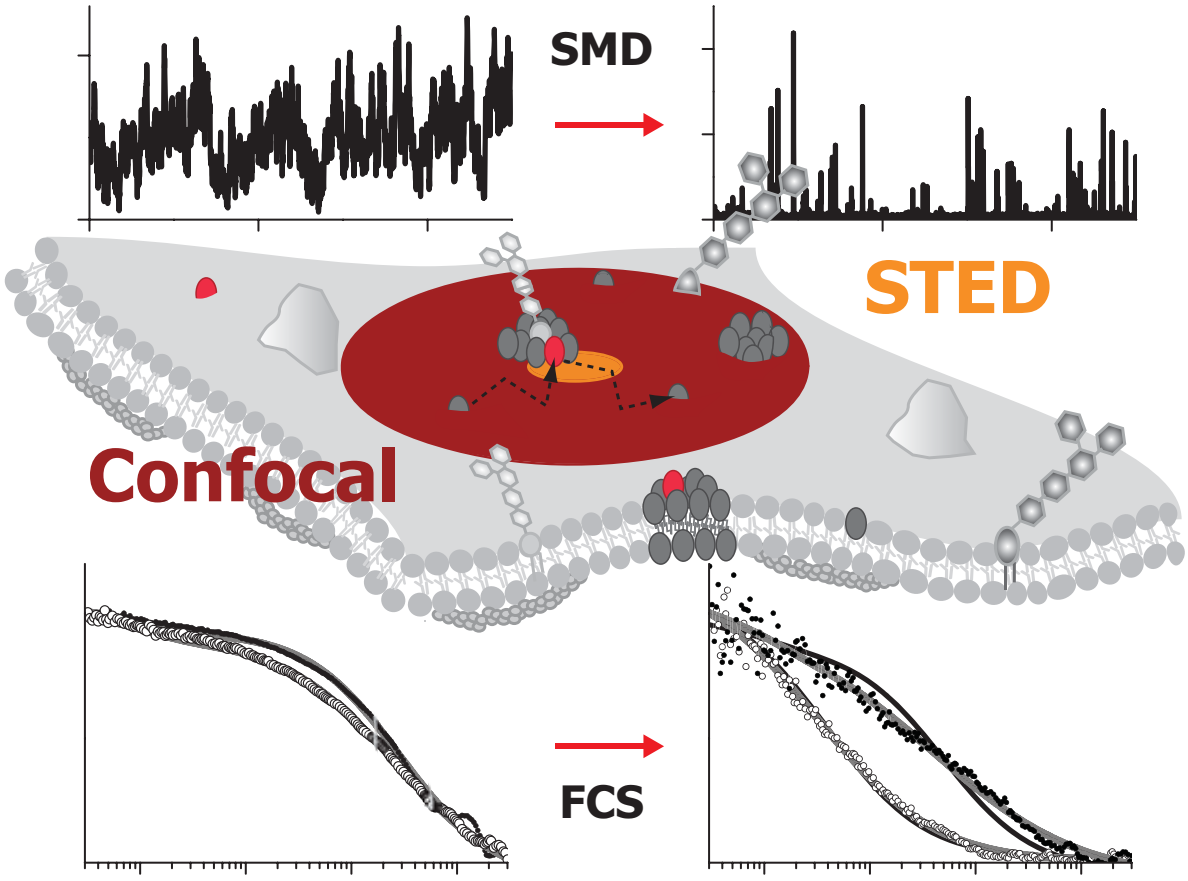


Leica
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# Contents

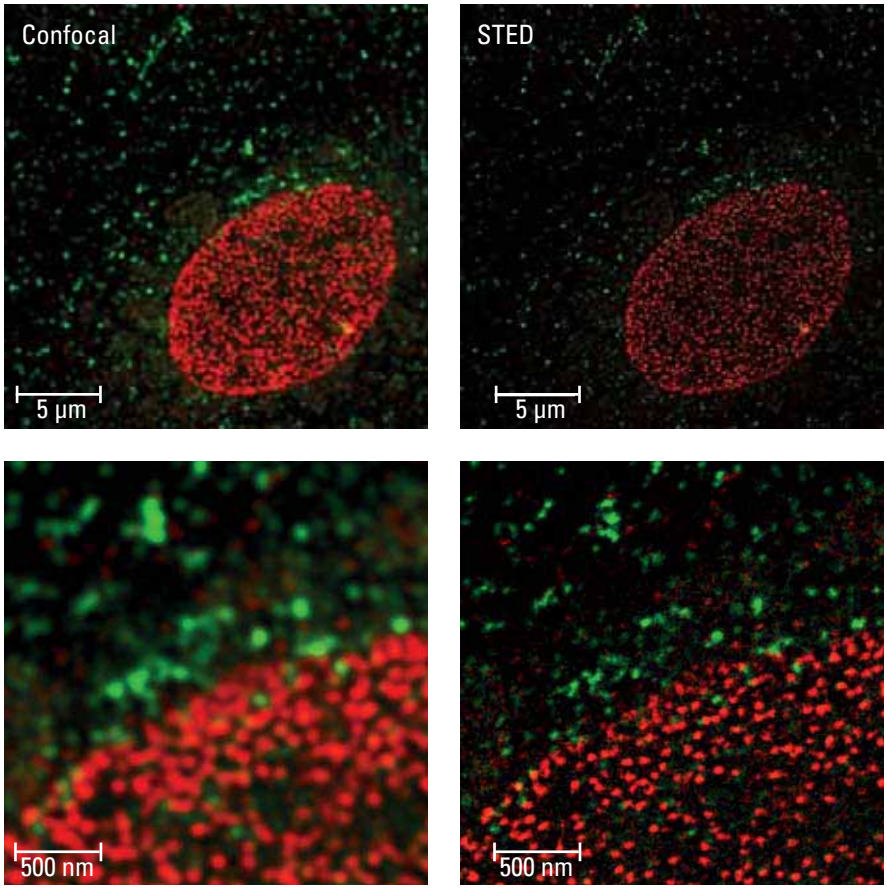


Cover:
Single molecule fluctuations (upper panel) and respective correlation curves (lower panel) of fluorescent lipid analogs diffusing in a supported bilayer. The data were recorded either from a confocal volume (left) or STED volume (right).

This application letter is a practical guideline on how to set up and analyze STED-FCS experiments. It consists of two basic parts:

The first part (chapters 1 to 4), written by Christian Eggeling, covers theoretical background and provides essential information on how to plan and set up a STED-FCS experiment. Further, it describes data analysis and discusses the example data.

The second part (chapter 5), by Leica Microsystems, describes data fitting using a Leica TCS STED CW with SMD FCS.




Nuclear pore complexes and clathrin coated vesicles of adherent cell stained with BD Horizon V500 (red) and Oregon Green 488 (green), respectively.

# 1. STED-FCS: Introduction

Fluorescence Correlation Spectroscopy (FCS) is a non-invasive and very sensitive analysis technique, allowing for the disclosure of complex dynamic processes such as diffusion and kinetics. FCS is usually combined with far-field (confocal) microscopy, which allows experiments using living cells. Often however, prominent, biological problems cannot be solved due to the limited resolution (> 200 nm) of conventional optical microscopy. For example, using a conventional optical microscope, FCS requires rather low concentrations of fluorescently-labeled molecules. Sometimes, this can preclude its otherwise very promising application to (endogenous) systems where molecular concentrations of several micro-mol per liter (µM) is required. Further, the limited resolution cannot fully characterize dynamic interactions on the nanoscale, since it cannot directly measure on the relevant spatial scales. Examples are lipid-lipid and lipid-protein interactions such as their transient integration into nanodomains ('rafts'), which are considered to play a functional part in a whole range of membrane-associated processes. The spatial dimension of these domains is supposed to be on molecular scales and the direct, non-invasive observation of them in living cells is thus impeded by the resolution limit.

Using the superior spatial resolution of STimulated Emission Depletion (STED) far-field microscopy with effective observation areas down to 20-30 nm in diameter in living cells [1], FCS can now be applied at rather large fluorophore concentrations (Fig. 1A) [2,3], or it can directly and non-invasively detect nanoscopic anomalous diffusion, such as of single lipid or protein molecules in the plasma membrane of living cells (Fig. 1B) [4]. Combining a (tunable) resolution of down to molecular scales with FCS, new details of molecular membrane dynamics can be obtained. Using STED-FCS it was, for example, shown that sphingolipids or 'raft'-associated proteins, unlike phosphoglycerolipids, were transiently (~ 10 ms) trapped on the nanoscale in cholesterol-mediated molecular complexes [4,5].

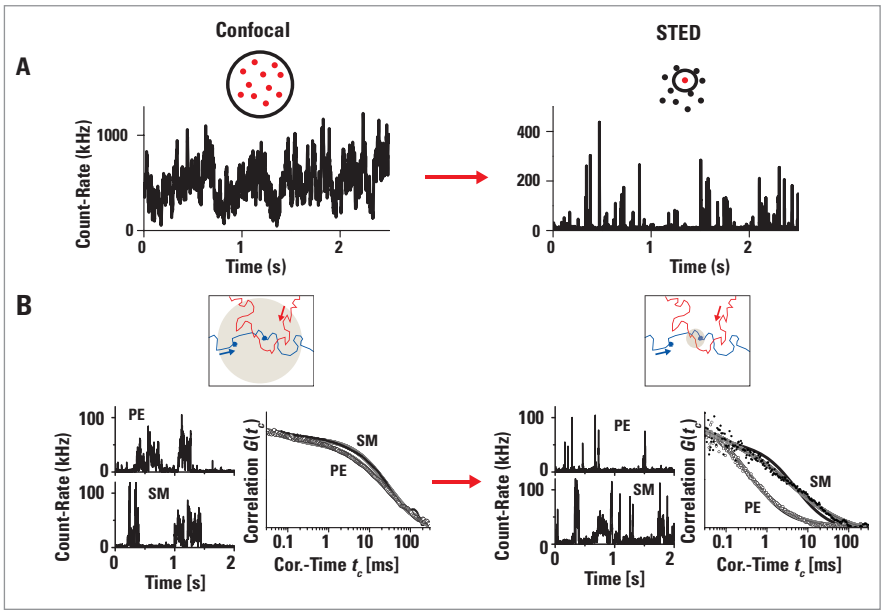



**Figure 1:** Small observation areas created by STED allow A) the recording of single molecule based fluctuations, i.e., FCS data at much higher concentrations than on a diffraction-limited confocal microscope, and B) the discrimination of free and anomalous diffusion due to interactions on the nanoscale.

A) Detected fluorescence count-rate over time of fluorescent lipid analogs diffusing in a supported membrane bilayer for confocal (left, diameter ~240 nm) and STED (right, diameter ~50 nm) recordings. At the same concentration of fluorescent lipids, the small observation area of the STED recordings reveals single molecule bursts while the large confocal spot averages over several molecules.

B) Molecular interactions result in an anomalous diffusion of molecules with trapping on the nanoscale (blue track). The small observation area of the STED recordings ensures a significantly reduced transient time of free diffusion and allows distinguishing between free (red track) and hindered diffusion (blue track), while confocal recordings average over these details. Shown are fluorescence count-rate time traces of single-molecule transits and FCS data of a non-interacting fluorescent phospholipid (PE) and an interacting fluorescent sphingolipid (SM) diffusing in the plasma membrane of living cells. Only STED reveals that single-molecule transits of SM are much more heterogeneous than those of PE, also disclosed by a large difference in decay times of the STED-FCS data. In contrast to the confocal FCS data, the STED-FCS data of SM cannot be described by normal free diffusion (black line, right panel), but only by anomalous diffusion (gray line). Confocal recordings would regard diffusion of SM to be slower than PE, but still normal.

# 2. STED-FCS: Fitting

**2.1. Models**

STED-FCS data may be analyzed according to common FCS theory, including diffusion and dark (triplet) state dynamics [2-4].

$$G_N(t_c) = DC + G_N(0) G_{Diff}(t_c) G_T(t_c)$$

Here, $G_N(t_c)$ denotes the normalized correlation function with correlation time $t_c$, DC the offset (usually DC = 1 for normalized correlation data), $G_N(0)$ the amplitude, $G_{Diff}(t_c)$ the diffusion term, and $G_T(t_c)$ the dark state term.

The amplitude $G_N(0) \sim 1/N$ of the FCS data usually scales inversely with the average number N of fluorescent molecules in the detection volume. At constant fluorophore concentration, N scales linearly with the size of the observation volume (or, for two-dimensional data, of the observation area).

Assuming a spatial three- (or two-) dimensional Gaussian profile of the detected fluorescence (observation volume), the diffusion term may be given by

$$G_{Diff}(t_c) = A_1 \left[ \left(1 + (t_c / t_{xy,1})^{\alpha_j}\right)^{-1} \left(1 + t_c / t_{z,1}\right)^{-1/2} \right] + \sum_{j=2} A_j \left[ \left(1 + (t_c / t_{xy,j})^{\alpha_j}\right)^{-1} \left(1 + t_c / t_{z,j}\right)^{-1/2} \right]$$

$$\text{with} \quad \sum_{j=1} A_j = 1 \quad \text{, i.e., } A_1 = 1 - \sum_{j=2} A_j$$

$G_{Diff}(t_c)$ may include several (j = 1, 2, …) differently diffusing components with relative amplitudes $A_j$, lateral $t_{xy,j} = FWHM^2/(8D_j \ln 2)$, and axial transit times $t_{z,j} = FWHM_z^2/(8D_j \ln 2)$, through the observation volume given by the diffusion coefficient Dj and the lateral and axial full-width-at-half-maximum (FWHM) diameters $FWHM_{xy}$ and $FWHM_z$ of the observation volume, respectively, and the anomalous coefficients $\alpha j$. These coefficients are = 1 for free Brownian diffusion and < 1 for anomalous diffusion, e.g., due to obstacles or transient interactions that hinder the diffusion pathway. The contribution of the axial (z) diffusion (i.e. of $t_{z,j}$) on the FCS data is usually much less than of the lateral (x,y) diffusion. Therefore, one can usually neglect anomalous coefficients for $t_{z,j}$. In case of two-dimensional diffusion, such as that of fluorescent lipids or proteins on membranes, the second terms including the axial diffusion $t_{z,j}$ can be neglected, $(1+t_c/t_{z,j}) = 1$.

The dark state term may be approximated by

$$G_T(t_c) = 1 + \sum_{i=1} T_i / (1 - T_i) \exp(-t_c / \tau_{Ti}) \quad \text{or} \quad G_T(t_c) = 1 - \sum_{i=1} T_i + \sum_{i=1} T_i \exp(-t_c / \tau_{Ti})$$

potentially including several dark states (i = 1, 2 …) with the average dark state populations $T_i$ and dark state correlation times $\tau_{Ti}$.

Usually STED light features a doughnut-shaped intensity distribution with a central zero along lateral x/y-directions (inset Fig. 2A). Increasing the power $P_{STED}$ of the STED light consequently reduces the observation volume along the lateral directions only, leaving the axial extension unchanged. As a result, the values of $t_{xy}$ decrease, and those of $t_z$ remain unchanged [3]. This user guide will concentrate on this special case.

Experiments have shown so far that STED light does not alter dark state populations [3,4].

N should scale with the observation volume (three-dimensional) or observation area (two-dimensional), and thus decrease with $P_{STED}$ in a similar way as $t_{xy}(P_{STED})$. This is not true in the case of increasing background contribution – as highlighted below for STED-FCS studies of three-dimensional diffusion.

**2.2. Calibration**

The calibration of a STED-FCS measurement is the dependence of the lateral diameter $FWHM(P_{STED})$ $(= FWHM_{xy}(P_{STED}))$ of the observation volume on the applied STED power $P_{STED}$ and may be determined from the dependency $t_{xy}(P_{STED})$ measured for an absolute freely diffusing sample (such as a pure dye in solution, a fluorescent lipid in a one-component model membrane, or a fluorescent lipid freely diffusing in the plasma membrane) [3,4].

1) Fit the confocal FCS data with one diffusion component (j = 1), no dark state, and $\alpha_1 = 1$ fixed. Float DC, $G_N(0)$, $t_{xy',1}$, and $t_{z',1}$ (the latter is not needed in the case of two-dimensional diffusion; DC may often be fixed). If the fit is bad, add as many dark states i as necessary to describe the data properly (with floating $T_i$ and $\tau_{Ti}$). In some cases it may be appropriate to fix $\tau_{Ti}$ to a value approximated from literature (for example the correlation time $\tau_{Ti}$ of the triplet state of a usual organic dye in aqueous environment is ≈ 5 µs).

2) Determine $t_{z',1}$, $T_i$, and $\tau_{Ti}$ for the confocal FCS data, and fix their values throughout further analysis ($t_{z',1}$ is not needed in the case of two-dimensional diffusion).

3) Fit the FCS data recorded for different $P_{STED}$ with free-floating parameters DC, $G_N(0)$, $t_{xy',1}$, and $\alpha_1$. $\alpha_1 = 1$ may also be fixed, since the calibration sample should be characterized by normal diffusion.

4) Plot the parameters $t_{xy',1}$ and $\alpha_1$ against $P_{STED}$. Values of $t_{xy',1}$ should decrease as indicated in Fig. 2. $\alpha_1$ should be close to 1 (otherwise check the adjustment of the microscope or the purity of the sample).

5) Calculate $FWHM(P_{STED})$:
   - $FWHM(P_{STED}) = \sqrt{(8D_0 \ln 2\, t_{xy',1}(P_{STED}))}$ if the diffusion coefficient $D_1$ is known;
   - $FWHM(P_{STED}) = FWHM(0)\, \sqrt{(t_{xy',1}(P_{STED})/t_{xy',1}(0))}$ if the confocal diameter FWHM(0) is known.

For a diffraction-limited spot, $FWHM(0) \approx 0.6\, \lambda/NA$ may be approximated from the applied wavelength and from the numerical aperture NA of the objective.

**A few general things to ensure for STED-FCS measurements:**

– Make sure that the calibration FWHM($P_{STED}$) is recorded for the same dye with approximately the same fluorescence lifetime as observed later. Molecular parameters such as fluorescence spectrum or lifetime determine the efficiency and thus, calibration of the STED nanoscope.

– Determine FCS parameters from the average (or median) of at least 5-10 measurements. In living cells, avoid measurement times of more than 10-15 seconds. The probability of detecting rare unrelated events such as diffusing aggregates, vesicles, etc., increases with increasing measurement time.

– The calibration (as done above with values of $t_{xy,1}(P_{STED})$) may similarly be performed using decreasing values of $N(P_{STED})$. Due to out-of-axis background (from areas where the STED light is not effective), the signal-to-background ratio of the FCS data of three-dimensional diffusion decreases with increasing $P_{STED}$, damping the amplitude $G_N(0)$, which results in too large values of N, and thus makes a calibration via $N(P_{STED})$ infeasible, as shown in Fig. 2A and as in detail discussed in [3].

This damping due to out-of-axis signal is usually not present for recordings on two-dimensional samples such as membranes (unless non-specific background signal from above/below the membrane is present) [3]. However, the signal-to-background ratio often decreases for very large $P_{STED}$, usually due to fluorescence contributions that are directly excited by the STED light. Therefore, it is recommended to calibrate the STED-FCS experiments with $t_{xy,1}(P_{STED})$ and not with $N(P_{STED})$.

– Ensure that the excitation power is low enough – an excitation power that is too high may induce too much photobleaching and thus shorten $t_{xy}$. This can, for example, be checked by plotting $t_{xy}$ determined from confocal FCS data for different excitation powers.

– Photobleaching by the STED light may result in the lowering of the number of fluorescing molecules in the effective observation volume (since molecules may be photobleached while traversing the STED-doughnut). However, photobleaching by STED has less influence on the transit time $t_{xy}$. The transit time through the effective observation volume and thus the probability of photobleaching during this transit is significantly shortened for the STED recordings [3,4].

– Usually FCS data recorded for large STED powers is rather noisy, especially for short correlation times $t_c$. Therefore, it does not make sense to start fitting before $t_c$ = 1-5 µs (model membranes, solution) or $t_c$ = 10 µs (plasma membrane living cell). However, one should use a starting point $t_c$ = 1 µs for adjustment of dark state parameters on the confocal data (for example, decay of the triplet is usually around 2-5 µs).

### 2.3. Sample: Fitting Steps

1) Fit the confocal data: Start with one diffusion component (j = 1), without a dark state, float DC, $G_N(0)$, $t_{xy,1}$, and $t_{z,1}$ (the latter is not needed in the case of two-dimensional diffusion, DC may often be fixed), and fix $\alpha_1 = 1$ (usually the confocal data is not too anomalous). If the fit is bad, add as many dark states i as necessary to properly describe the data (with floating $T_i$ and $\tau_{Ti}$). Fix the determined values of $t_{z,1}$, $T_i$, and $\tau_{Ti}$ throughout further analysis ($t_{z,1}$ is not needed in the case of two-dimensional diffusion).

2) Fit the FCS data recorded for different $P_{STED}$ with free-floating parameters DC, $G_N(0)$, $t_{xy,1}$, and $\alpha_1$ (DC may often be fixed).

3) Plot $t_{xy,1}$ and $\alpha_1$ against $FWHM^2$ (compare Fig. 2C). Normal diffusion is indicated by a linear dependency $t_{xy,1}(FWHM^2)$ and constant values of $\alpha_1$, anomalous diffusion by a change of $\alpha_1$ (specifically a decrease towards small FWHM), and a non-linear dependency of $t_{xy,1}$ ($FWHM^2$). See references [3-5] for interpreting the dependencies and for further analysis possibilities of the anomalous diffusion (for example, for extracting kinetic parameters of transient trapping).

4) One may also calculate the diffusion coefficient $D_1(P_{STED}) = FWHM^2(P_{STED}) / (8\ln2\, t_{xy,1}(P_{STED}))$ and plot it against FWHM. $D_1$ should stay constant for free diffusion, decrease toward small FWHM for trapping-like, and increase toward large FWHM for hopping-like diffusion constraints [5].

**Comment to 1):**
Additional terms may not only result from dark state kinetics, but also from additional diffusing species (such as unbound fluorophore). How to differentiate dark state kinetics from diffusion:

– Continue fitting data by adding dark state terms (floating parameters $T_i$ and $\tau_{Ti}$).

– Values $\tau_{Ti} < 10$ µs usually result from dark state transitions, since diffusion is slower; i.e., such terms can be assigned to dark state kinetics (with parameters $T_i$ and $\tau_{Ti}$).

– Record confocal FCS data for different excitation intensities – dark state kinetics often depend on excitation intensity, i.e., especially $T_i$ should increase with intensity. Therefore, a term definitely describes dark state kinetics if its amplitude $T_i$ strongly depends on the excitation intensity.

– Analyze FCS data with floating dark state parameters for a low STED power also, i.e., for a slightly reduced observation volume, maybe fix $T_i$ to a value known from the confocal fits. Diffusion depends on the size of the observation volume, while dark state kinetics do not, i.e., diffusion is present if $\tau_{Ti}$ decreases with STED power.

**In the case of positive checks for diffusion:**
– Add a diffusion term (j = 2) instead of a dark state term. Usually, one can assume this additional component to be diffusing normal (e.g., unbound fluorophore), i.e., fix $\alpha_2 = 1$. Fit the confocal FCS data with floating $t_{xy,1}(0)$, $A_2$, and $t_{xy,2}(0)$. Determine the values of the transit times $t_{xy,2}(P_{STED})$ of an additional component for different STED powers, either by

$$t_{xy,2}(P_{STED}) = t_{xy,2}(0)\, FWHM^2(P_{STED})/FWHM^2(0)$$

or by

$$t_{xy,2}(P_{STED}) = FWHM^2(P_{STED}) / (8D_2\ln2) \text{ with } D_2 = FWHM^2(0) / (8\ln2\, t_{xy,2}(0))$$

– Fit FCS data for each $P_{STED}$ with fixed $\alpha_2 = 1$, fixed $A_2$ (from confocal fit) and fixed, calculated $t_{xy,2}(P_{STED})$, and float DC, $G_N(0)$, $t_{xy,1}$, and $\alpha_1$ (DC may often be fixed).

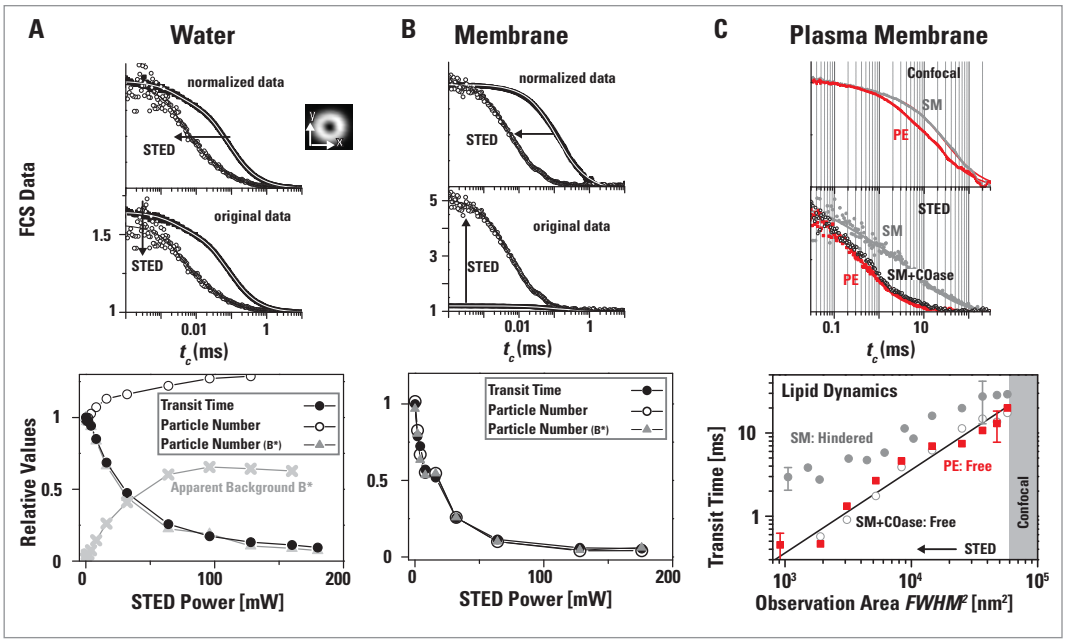



**Figure 2:** Performance of STED-FCS: Normalized and original STED-FCS data (upper panels) and dependence of transit time $t_{xy,1}$ and particle number N on STED power $P_{STED}$ or observation area FWHM² (lower panels).

**A)** Three-dimensional diffusion of an organic dye in water. While $t_{xy,1}$ (black circles) decreases with $P_{STED}$ as expected, N (open circles) increases due to an apparent out-of-axis background (cross). This bias, however, may be corrected (triangles) [3]. Inset: Doughnut-shaped intensity distribution of the STED focus.

**B)** Two-dimensional diffusion of a fluorescent lipid in a supported membrane. $t_{xy,1}$ (black circles) and N (open circles) decrease with $P_{STED}$ as expected, since no out-of-axis background is present [3].

**C)** Two-dimensional diffusion of fluorescent lipids (PE: phosphoglycerolipid, SM: sphingomyelin, SM+COase: SM after cholesterol depletion by COase treatment) in the plasma membrane of living cells. $t_{xy,1}$ depends linearly on FWHM² for PE or SM+COase (as expected for free diffusion), while this dependency is non-linear for SM due to hindered diffusion [4].

# 3. STED-FCS on the Leica Microscope

Fig. 2 shows STED-FCS data recorded with the same lasers as supplied by the pulsed Leica STED microscope (pulsed excitation at 635 nm and pulsed STED at 780 nm).

Fig. 3 shows STED-FCS data recorded on the continuous-wave (CW) Leica TCS STED microscope or with the same lasers as supplied by the Leica TCS STED CW microscope (CW or pulsed excitation at 488 nm and CW STED at 592 nm). Starting from diffraction-limited 180–190 nm, the achievable FWHM usually does not get below 80–100 nm due to the characteristics of CW STED-FCS [6], but it is improved by gated STED-FCS [7].

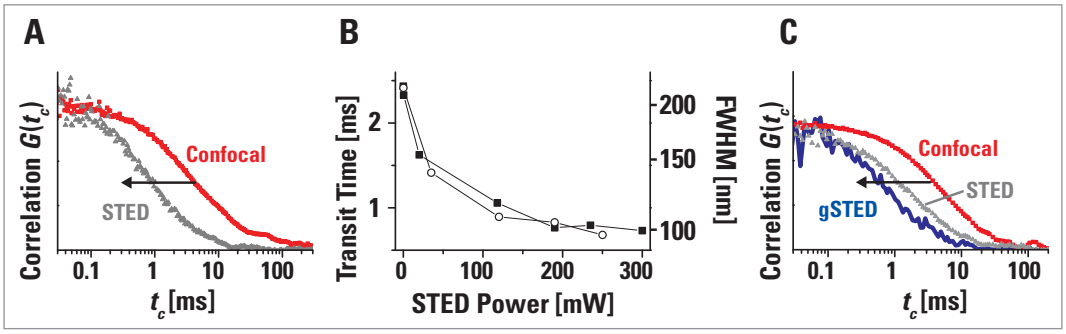



**Figure 3:** Leica STED-FCS of Bodipy-labeled lipids with CW STED lasers (488 nm excitation, 592 nm STED).
**A)** Normalized STED-FCS data (red: confocal, gray: STED) recorded on a Leica microscope.
**B)** Dependence of transit time $t_{xy,1}$ and calculated diameter FWHM of the observation area on CW STED power $P_{STED}$ for two different lipids.
**C)** Normalized STED-FCS data (red: confocal, gray: STED, blue: gSTED) showing that the resolution of FCS is further increased with gated STED (gSTED) (see [7]). Data in B and C were recorded on a home-built STED nanoscope.

**4. Literature**

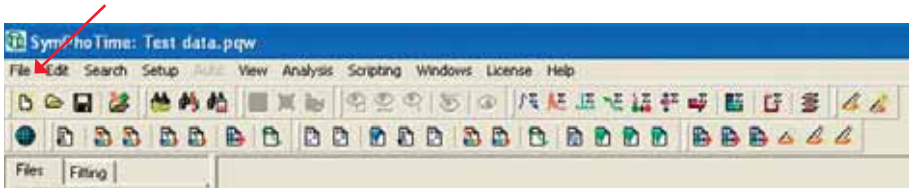
[1] S.W. Hell. "Far-Field Optical Nanoscopy", Science, 316, 1153, (2007).
[2] L. Kastrup et al. "Fluorescence fluctuation spectroscopy in subdiffraction focal volumes", Phys. Rev. Lett., 94, 178104, (2005).
[3] C. Ringemann et al. "Exploring single-molecule dynamics with fluorescence nanoscopy", New J. Physics, 11, 103054, (2009).
[4] C. Eggeling et al. "Direct observation of the nanoscale dynamics of membrane lipids in a living cell", Nature, 457, 1159, (2009).
[5] V. Mueller et al. "STED nanoscopy reveals molecular details of cholesterol- and cytoskeleton-modulated lipid interactions in living cells", Biophys. J., 101, 1651, (2011).
[6] M. Leutenegger et al. "Analytical description of STED microscopy performance", Opt. Expr., 18, 26417, (2010).
[7] G. Vicidomini et al. "Sharper low power STED nanoscopy by time gating", Nat. Meth., 8, 571 (2011).

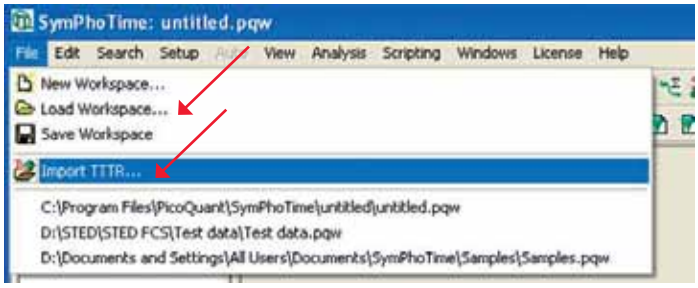
# 5. STED-FCS Curve Fitting Using PicoQuant's SymPhoTime Software

The Leica TCS STED CW system with integrated SMD FCS includes the data analysis software "SymPhoTime" (SPT) from PicoQuant GmbH. For two-dimensional experiments, e.g., for STED-FCS of membrane diffusion, built-in models can be used for data analysis (as described below). For three-dimensional STED-FCS measurements, e.g., in solutions, there is no built-in model in SPT. However, correlation curves can be exported as ASCII files and then analyzed using other programs.

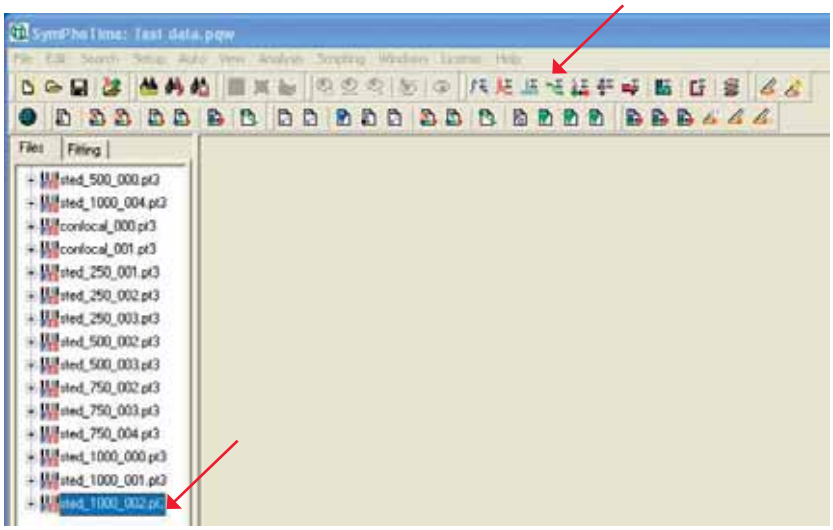
**How to fit two-dimensional STED-FCS data into the SPT software – step-by-step:**

**1.** Open the workspace containing the raw data or import the raw data into a new workspace.

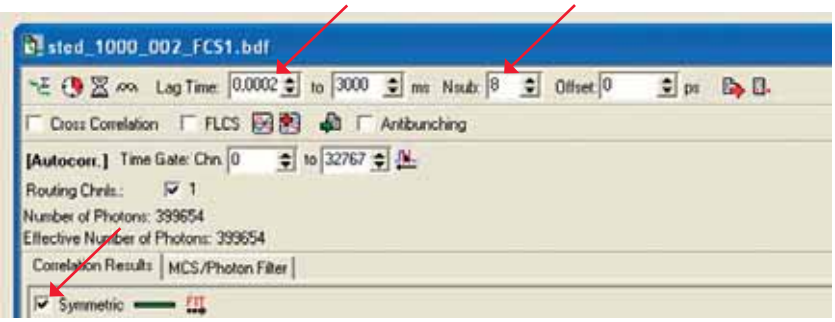


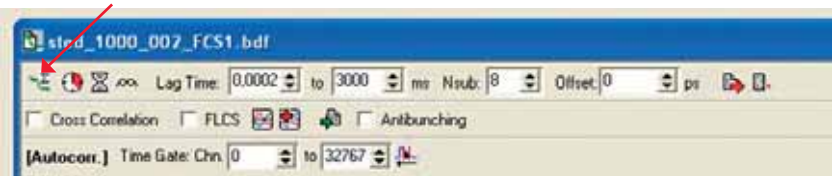

**2.** Highlight the data set to be processed and calculate its correlation curve using "FCS Trace".



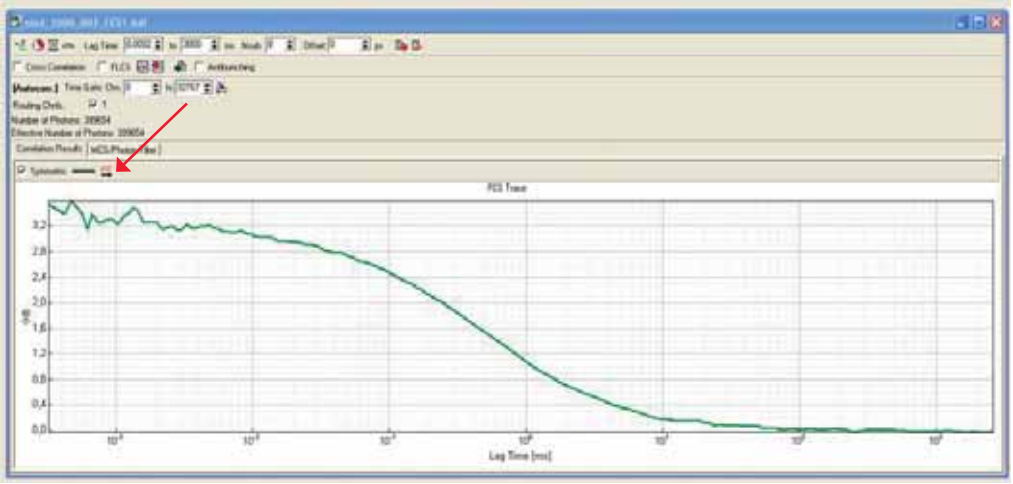
**3.** Recommended settings: Lag Time: 0.0002, Nsub: 8, Offset: 0, and Symmetric.



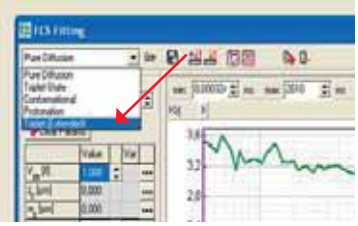

**4.** Press "Correlate". Now the correlation curve will be calculated and displayed.



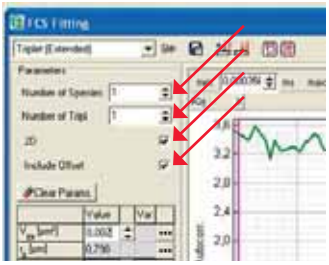

**5.** Press "Fit".



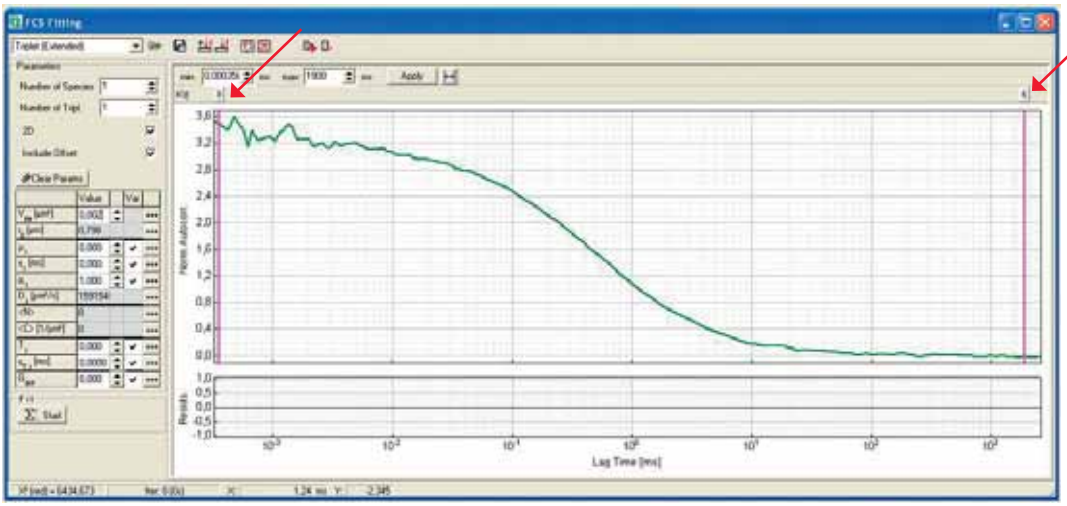
**6.** Within the fitting dialogue, select the model, "Triplet Extended".



**7.** Within the fitting dialogue, select the number of fluorescent species (1, 2, 3, or 4, j in Eq. 1), the number of triplet states (1 or 2, *i* in Eq. 2), check the box "2D". If the correlation curve does not approach the zero line, check the box "Include Offset" as well.



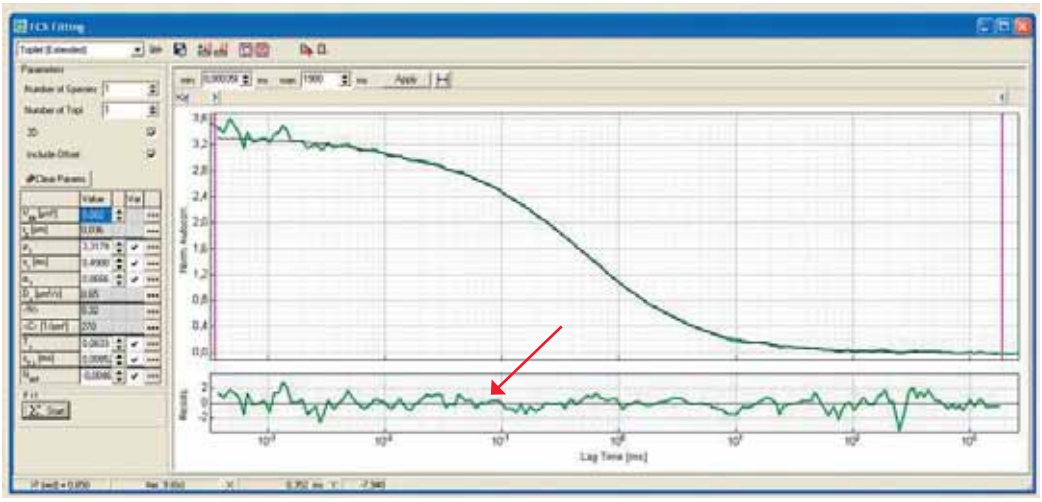
**8.** Select an appropriate fitting range. For example, start at 1 µs (for adjustment of dark state parameters on the confocal data), 1-5 µs (model membranes, solution) or 10 µs (plasma membrane of living cells) and end at 1000 ms – see comment above.



**9.** If known, specify the effective observation area of $V_{Eff2D}$. This value is not fitted, but serves only for the calculation of diffusion coefficients, etc.. r0 indicates the corresponding effective radius at $1/e^2$ intensity (FWHM = $r_0 \sqrt{(8 \ln 2)}$, Eq. 1).

**10.** To start the fitting process, press the "Start" button.

**11.** If the residual curve is scattering around zero without showing waves (as shown here), the selected fitting model well matches the data. Otherwise, add another dark state or another diffusion time as outlined before.



**12.** The following parameters can be obtained from the fit:

**a.** $\rho_1$, $\rho_2$, $\rho_3$, $\rho_4$ – contribution (amplitude of diffusion term) of the fluorescent species j = 1, 2, 3, and 4 ($A_j$ in Eq. 1)

**b.** $\tau_1$, $\tau_2$, $\tau_3$, $\tau_4$ – transit times of the fluorescent species j = 1, 2, 3, and 4 ($t_{xy,j}$ in Eq. 1)

**c.** $\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_4$ – anomaly coefficients of the fluorescent species *j* = 1, 2, 3, and 4 (equivalent to $\alpha_j$ in Eq. 1)

**d.** $D_1$, $D_2$, $D_3$, $D_4$ – diffusion coefficients of the fluorescent species *j* = 1, 2, 3, and 4. This value is calculated based on the fitted diffusion times $\tau_j$ and on the entered value of $V_{Eff2D}$:

$$D_j = \frac{V_{Eff\,2D}}{2\pi\tau_i}$$

This means it is only valid if the effective observation area is correctly entered. Otherwise, $D_j$ can be calculated according to the procedure outlined in chapter 1.3.

**e.** N – total average particle number of all fluorescent species within the observation area. The value is calculated from $G_N(0) = 1/N$ (Eq. 1).

**f.** C – total concentration of all fluorescent species within the observation area. This value is calculated based on the values N and $V_{Eff2D}$. It is correct only if the effective area, $V_{Eff2D}$, is correctly entered.

$$C = \frac{N}{V_{Eff\,2D}\,N_A}$$ with $N_A$ – Avogadro constant

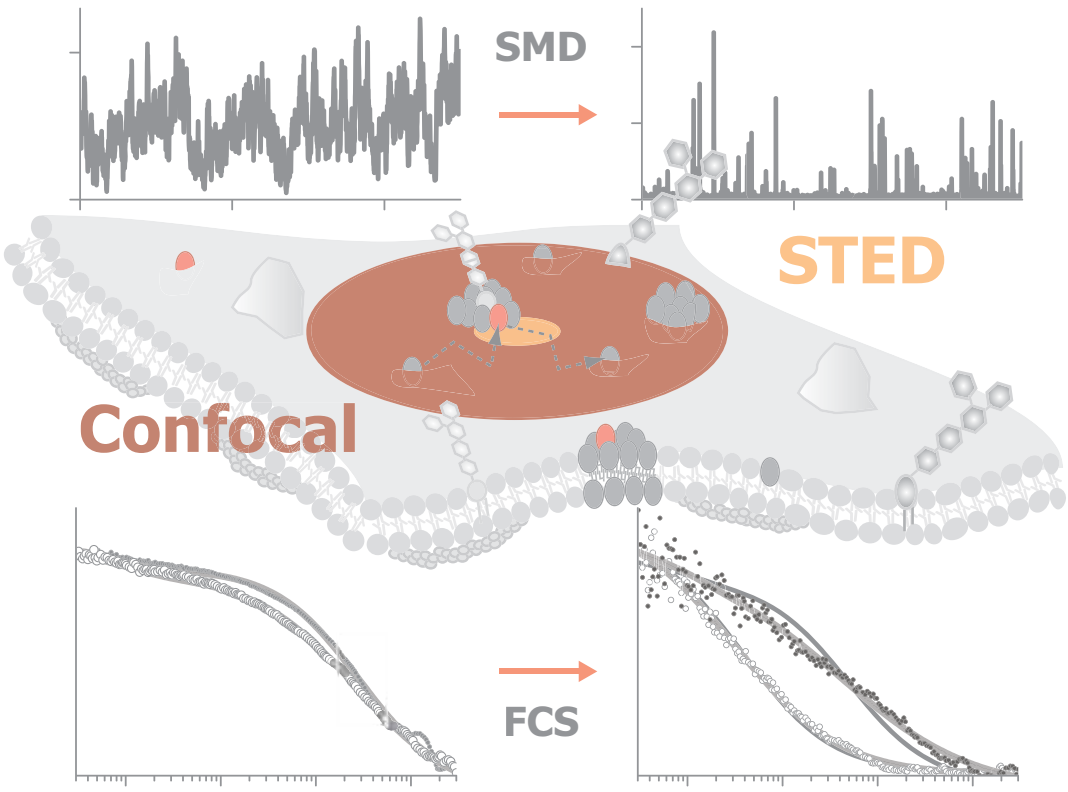
**g.** $T_1$, $T_2$ – fraction of molecules in dark (triplet) state i = 1, 2 ($T_i$ in Eq. 2).

**h.** $\tau_{T1}$, $\tau_{T2}$ – correlation time of the dark (triplet) states i = 1, 2 ($\tau_{Ti}$ in Eq. 2).

**i.** $G_{INF}$ – offset of the correlation curve (DC in Eq. 1).

The fitting procedure described in chapter 5 can be used for two-dimensional experiments for both the system calibration and the data analysis. System calibration requires, a freely diffusing sample in a membrane with a known diffusion coefficient. The diffusion time at different STED laser powers $P_{STED}$ is measured and the corresponding effective volume $V_{Eff2D}$ calculated (see chapter 2.2).

Now, the sample of interest can be analyzed. For each STED laser power $P_{STED}$ the effective volume $V_{Eff2D}$ can be typed into the software and the diffusion time, the anomaly coefficient, and the concentration are automatically derived. Chapter 2.3 gives a detailed description how to continue the analysis of data.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