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Dual-color Fluorescence Cross-Correlation Spectroscopy to study Protein-Protein Interaction and Protein Dynamics in Live Cells

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TITLE:

Dual-Color Fluorescence Cross-Correlation Spectroscopy to Study Protein-Protein Interaction and Protein Dynamics in Live Cells

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SUMMARY

We present an experimental protocol and data analysis workflow to perform live cell dual-color fluorescence cross correlation spectroscopy (FCCS) combined with Förster Resonance Energy transfer (FRET) to study membrane receptor dynamics in live cells using modern fluorescence labeling techniques.

ABSTRACT:

We present a protocol and workflow to perform live cell dual-color fluorescence cross-correlation spectroscopy (FCCS) combined with Förster Resonance Energy transfer (FRET) to study membrane receptor dynamics in live cells using modern fluorescence labeling techniques. In dual-color FCCS, where the fluctuations in fluorescence intensity represent the dynamic "fingerprint" of the respective fluorescent biomolecule, we can probe co-diffusion or binding of the receptors. FRET, with its high sensitivity to molecular distances, serves as a well-known "nanoruler" to monitor intramolecular changes. Taken together, conformational changes and key parameters such as local receptor concentrations and mobility constants become accessible in cellular settings.

Quantitative fluorescence approaches are challenging in cells due to high noise levels and the vulnerability of the sample. Here we show how to perform this experiment, including the calibration steps using dual-color labeled β2-adrenergic receptor (β_2AR) labeled with eGFP and SNAPtag-TAMRA. A step-by-step data analysis procedure is provided using open-source software and templates that are easy to customize.

Our guideline enables researchers to unravel molecular interactions of biomolecules in live cells *in situ* with high reliability despite the limited signal-to-noise levels in live cell experiments. The operational window of FRET and particularly FCCS at low concentrations allows quantitative analysis at near-physiological conditions.

INTRODUCTION:

Fluorescence spectroscopy is one of the main methods to quantify protein dynamics and protein-protein interactions with minimal perturbation in a cellular context. Confocal fluorescence correlation spectroscopy (FCS) is one of the powerful methods to analyze molecular dynamics as it is single-molecule sensitive, highly selective, and live-cell compatible [1]. Compared with other dynamics-orientated approaches, FCS has a broader measurable time range spanning from ~ ns to ~ s, most importantly covering the fast time scales that are often inaccessible by imaging-based methods. Moreover, it also provides spatial selectivity so that membrane, cytoplasmic, and nucleus molecular dynamics can be easily distinguished [2]. Thus, molecular blinking, the average local concentration, and the diffusion coefficient can be quantitatively analyzed with FCS. Intermolecular dynamics such as binding become easily accessible when probing co-diffusion of two molecular species in fluorescence cross-correlation spectroscopy (FCCS) analysis [3-5] in a dual-color approach.

The main underlying principle in correlation spectroscopy is the statistical analysis of intensity fluctuations emitted by fluorescently labeled biomolecules diffusing in and out of a laser focus (**Figure 1A**). The resulting auto- or cross-correlation functions then can be further analyzed by curve fitting to eventually derive the rate constants of interest. In other words, the statistical methods FCS and FCCS do not provide single molecule traces like in single particle tracking, but a dynamic pattern or "fingerprint" of a probed specimen with high temporal resolution. When combined with Förster resonance energy transfer (FRET), intramolecular dynamics such as conformational changes can be monitored at the same time in a common confocal setup [5,6]. FRET probes the distance of two fluorophores and is often referred to as a molecular "nanoruler". Energy transfer takes place only when the molecules are in close vicinity (3-10 nm), the emission spectrum of the donor significantly overlaps with the absorption spectrum of the acceptor molecule, and the dipole orientation of the donor and acceptor is (almost) parallel. Thus, the combination of FRET and FCCS provides a technique with very high spatio-temporal resolution. When spatial selectivity, sensitivity as well as live-cell compatibility is required, FRET-FCCS has obvious advantageous over other methods such as Isothermal Titration Calorimetry (ITC)[7], Surface Plasmon Resonance (SPR) [8], or Nuclear Magnetic Resonance (NMR) [9,10] for measuring protein dynamics and interactions.

Despite the capabilities and promise of dual-color fluorescence cross-correlation spectroscopy (dc-FCCS), performing dc-FCCS in live cells is technically challenging due to the spectral bleed-through or crosstalk between the channels[3,4], the difference in the confocal volumes due to the spectrally distinct laser lines[3,4,11], background signal, and noise or limited photostability of the samples[12-15]. The introduction of pulse interleaved excitation (PIE) to FCCS was an important tweak to temporally decouple the different laser excitations for reducing the spectral crosstalk between the channels [16]. Other correction methods to counter spectral bleed-through[17-19] and

background corrections have also been well-accepted[17-19]. For details and basics on FCS, PIE or FRET the reader is referred to the following references[2,4,6,16,20-24].

Here all necessary calibration experiments and analysis along with the experimental results of a prototypical G-protein coupled receptor, β_2-adrenergic receptor (β_2AR), for three different scenarios are presented: (1) single-labeled molecules carrying either a "green" (eGFP) or a "red" (SNAPtag-based labeling) [25] fluorophore; (2) a double-labeled construct, which carries an N-terminal SNAPtag and intracellular eGFP (NT-SNAP) [in this case, both labels are at the same protein. Thus 100% co-diffusion was expected];, and (3) a double-labeled sample, where both fluorophores are on the same side of the cell membrane (CT-SNAP). It carries a C-terminal SNAPtag and an intracellular eGFP. Here, again both labels are at the same protein with again 100% co-diffusion expected. As both labels are very close to each other, on the same side of the cell membrane, it shows the potential to observe FRET and anticorrelated behavior. All constructs were transfected in Chinese Hamster Ovary (CHO) cells and later labeled with a red fluorescent substrate which is membrane-impermeable for the NT-SNAP construct and a membrane-permeable substrate for the CT-SNAP construct. Finally, simulated data exemplifies the influence of experimental parameters on the FRET-induced anticorrelation, and the effect of protein-protein interactions on the co-diffusion amplitude.

Thus, this protocol provides a complete guide to performing the combined FRET-FCCS in living cells to understand protein dynamics and protein-protein interactions while making aware of technical/physical artifacts, challenges, and possible solutions.

PROTOCOL:

1. Experimental Protocol

1.1. Sample preparation

NOTE: Perform cell seeding and transfection under sterile conditions.

1.1.1. Place a cleaned coverslip per well onto a 6-well culture plate and wash three times with sterile phosphate-buffered saline (PBS).

NOTE: The coverslip cleaning protocol is detailed in **Supplementary Note 1.**

1.1.2. To each well, add 2 mL of the complete cell culture medium containing phenol red (supplemented with 10% fetal bovine serum (FBS), 100 µg/mL penicillin and 100 µg/mL streptomycin) and keep the plate aside.

1.1.3. Culture the CHO cells in the same medium containing phenol red at 37 °C and 5% CO_2. Wash the cells with 5 mL of PBS to remove the dead cells.

1.1.4. Add 2 mL of trypsin and incubate for 2 min at room temperature (RT).

1.1.5. Dilute the detached cells with 8 mL of medium containing phenol red and mix carefully by pipetting.

1.1.6. Count the cells in a Neubauer chamber and seed at a density of 1.5 x 10^5 cells/well in the 6-well cell culture plate containing the coverslips (prepared in step 1.1.1-1.1.2).

1.1.7. Let the cells grow in an incubator (37 °C, 5% CO_2) for 24 h in order to achieve approximately 80% confluency.

1.1.8. Dilute 2 µg of the desired vector DNA (e.g., CT-SNAP or NT-SNAP) and 6 µL of the transfection reagent in two separate tubes, each containing 500 µL of the reduced-serum medium for each well and incubate for 5 min at RT.

1.1.9. Mix the two solutions together to obtain the transfection mixture and incubate it for 20 min at RT.

1.1.10. In the meantime, wash the seeded CHO cells once with sterile PBS.

1.1.11. Replace the PBS with 1 mL/well of phenol red-free medium supplemented with 10% FBS without any antibiotics.

1.1.12. Add the entire 1 mL of transfection mixture dropwise to each well and incubate the cells overnight at 37 °C, in 5% CO_2.

1.1.13. For labeling of the SNAP construct, dilute the appropriate SNAP substrate stock solution in 1 mL of the medium supplemented with 10% FBS to obtain a final concentration of 1 µM.

1.1.14. Wash the transfected cells once with PBS and add 1 mL per well of 1 µM SNAP substrate solution. Incubate the cells for 20 min at 37 °C in 5% CO_2.

1.1.15. Wash the cells thrice with phenol red-free medium and add 2 mL per well phenol red-free medium. Incubate the cells for 30 min at 37 °C in 5% CO_2.

1.1.16. Transfer the coverslips of all samples subsequently into the imaging chamber and wash with 500 µL imaging buffer. Add 500 µL imaging buffer before moving to the FRET-FCS setup.

1.2. Calibration Measurements

NOTE: The FRET-FCS setup is equipped with a confocal microscope water objective, two laser lines, a Time-Correlated Single Photon Counting (TCSPC) system, two hybrid photomultiplier tubes (PMT) and two avalanche photodiodes (APD) for photon collection and the data collection software. It is very important to align the setup every time before live cell measurements. The detailed setup description can be found in **Supplementary Note 2.** Both lasers and all detectors

(two PMTs and two APDs) are always ON during the measurements, as all measurements need to be conducted under identical conditions. For the calibration measurements, use a coverslip from the same lot on which the cells were seeded, this decreases the variation in collar ring correction.

1.2.1. For adjusting the focus, pinhole and collar ring position place 2 nM green calibration solution on a glass coverslip and switch on the 485 nm and 560 nm laser. Operate laser in Pulsed Interleaved Excitation (PIE) mode[16].

1.2.2. Focus on the solution and adjust the pinhole and collar ring position such that the highest count rate and smallest confocal volume are obtained to get the maximum molecular brightness.

1.2.3. Repeat this process for the red channels with 10 nM red calibration solution and a mixture of both, the green and red calibration solution.

1.2.4. Place the 10 nM DNA solution on a glass coverslip and adjust the focus, pinhole, and collar ring position such that the cross-correlation between the green and red detection channels is highest, i.e., shows the highest amplitude.

NOTE: Steps 1.2.1 and 1.2.4 might have to be repeated back and forth to find the optimal alignment. Take 3-5 measurements from each calibration solution for 30 s – 120 s after the focus, pinhole, and collar ring position have been aligned optimally for the green and red detection channels and the confocal overlap volume.

1.2.5. Measure a drop of ddH_2O, the imaging medium, and a non-transfected cell 3-5 times each for 30 s – 120 s to determine the background count rates.

1.2.6. Collect the instrument response function with 3-5 measurements for 30 s – 120 s. This is optional but highly recommended.

1.3. Live cell measurements

1.3.1. Find a suitable cell by illuminating with the mercury lamp and observing through the ocular.

NOTE: Suitable cells are alive showing the typical morphology of the respective adherent cell line. The fluorescence of the protein of interest, here a surface receptor, is visible all over the surface. Less bright cells are more suitable than brighter ones due to the better contrast in FCS when a low number of molecules are in focus.

1.3.2. Switch on both lasers in PIE mode and focus on the membrane by looking at the maximum counts per second.

NOTE: The laser power might need to be reduced for the cell samples (less than 5 µW at

objective). This depends upon the used fluorophores and the setup.

1.3.3. Observe the auto- and cross-correlation curves of the β_2AR bound to eGFP and/or SNAP-tagged probes in the online preview of the data collection software and collect several short measurements (~2 -10) with an acquisition time between 60 -180 s.

NOTE: Do not excite the cells for a long time continuously as the fluorophores may bleach. However, it will depend on the brightness of each cell, how long the measurements can be, and how many measurements in total can be performed.

2. Data analysis

2.1. Data Export

2.1.1. Export the correlation curves, G(t_c) and count rates, CR, from all measurements.

2.1.2. Take care to correctly define the "prompt" and "delay" time windows and use the "microtime gating" option in the data correlation software.

NOTE: In total, three different correlations are required: (1) autocorrelation of the green channel in the prompt time window (ACF_{gp}) (2) autocorrelation of the red channels in the delay time window (ACF_{rd}), and finally (3) the cross-correlation of the green channel signal in the prompt time window with the red channel signal in the delay time window (CCF_{PIE}). The data export is shown step-by-step for different software in **Supplementary Note 3**.

2.2. Calibration measurements

2.2.1. Use the autocorrelation functions of the green (ACF_{gp}) and red (ACF_{rd}) fluorophore solutions, and fit them to a 3D diffusion model with an additional triplet term if required (eq. 1) to calibrate the shape and size of the confocal detection volume for the two used color channels:

$$G_{ACF}(t_c) = b + \frac{1}{N} * \cdot \left(\left(\frac{1}{1+t_c/t_D} \right) \left(\frac{1}{\sqrt{1+s^2(t_c/t_D)}} \right) \right) \left[1 - a_R + a_R \cdot \exp\left(-\frac{t_c}{t_R} \right) \right] \quad \text{eq. 1}$$

Here, b is the baseline of the curve, N the number of molecules in focus, t_D the diffusion time (in ms), and $s = z_0/\omega_0$ the shape factor of the confocal volume element. The triplet blinking or other photophysics is described by its amplitude a_R and relaxation time t_R

NOTE: All variables and symbols used within the protocol are listed in Table 1.

2.2.2. Use the known diffusion coefficients D for the green [26] and red calibration standard [27] and the obtained shape factors s_{green} and s_{red} to determine the dimensions (width ω_0 and height z_0) and volume V_{eff} of the confocal volume element (eq. 2a-c).

$$w_0^2 = 4 t_D D \quad \text{eq.2a}$$

$$z_0 = s\, w_0 \quad \text{eq.2b}$$

$$V_{eff} = \pi^{3/2} z_0 w_0^2 \quad \text{eq.2c}$$

NOTE: Templates for calculation of the calibration parameter are provided as supplementary files (S7).

2.2.3. Calculate the spectral crosstalk α of the green fluorescence signal (collected in channels 0 and 2) into the red detection channels (channel number 1 and 3) as a ratio of the background-corrected (BG) signals (eq. 3).

$$\alpha = \frac{((CR_{1,green} - CR_{1,BG}) + (CR_{3,green} - CR_{3,BG}))}{((CR_{0,green} - CR_{0,BG}) + (CR_{2,green} - CR_{2,BG}))} * 100\% \quad \text{eq. 3}$$

2.2.4. Determine the direct excitation of the acceptor fluorophore δ by the donor excitation wavelength by the ratio of the background-corrected count rate of the red calibration measurements in the "prompt" time window (excitation by green laser) to the background-corrected count rate in the "delay" time window (excitation by red laser) (eq. 4).

$$\delta = \frac{((CR_{1,prompt,red} - CR_{1,prompt,BG}) + (CR_{3,prompt,red} - CR_{3,prompt,BG}))}{((CR_{1,delay,red} - CR_{1,delay,BG}) + (CR_{3,delay,red} - CR_{3,delay,BG}))} * 100\% \quad \text{eq. 4}$$

2.2.5. Calculate the molecular brightness *B* of both the green and red fluorophores (eq. 5a-b) based on the background-corrected count rates and the obtained number of molecules in focus, *N*, from the 3D diffusion fit (eq. 1):

$$B_{green} = \frac{CR_{corr,green,0} + CR_{corr,green,2}}{N_{green}} \quad \text{eq. 5a}$$

$$B_{red} = \frac{CR_{corr,red,1} + CR_{corr,red,3}}{N_{red}} \quad \text{eq. 5b}$$

2.2.6. Fit both ACF_{gp} and ACF_{rd} as well as $CCF_{gp\text{-}rd}$ of the double-labeled DNA to the 3D diffusion model (eq. 1). Keep the obtained shape factors, s_{green} and s_{red}, constant for ACF_{gp} and ACF_{rd}, respectively. The shape factor for the $CCF_{gp\text{-}rd}$, $s_{PIE,}$ is usually in between these two values.

NOTE: In an ideal setup, both $V_{eff,\ green}$ and $V_{eff,\ red}$ would have the same size and overlap perfectly.

2.2.7. Determine the amplitude at zero correlation time, $G_0(t_c)$, based on the found values of the apparent number of molecules in focus (N_{green}, N_{red} and N_{PIE}).

2.2.8. Calculate the amplitude ratio r_{GR} and r_{RG} for a sample with 100% co-diffusion of green and red fluorophores (eq. 6). Be aware that N_{PIE} does not reflect the number of double-labeled molecules in focus but reflects only the $1/G_0(t_c)$.

$$r_{GR} = \frac{G_{0,CCF}(t_c)}{G_{0,ACFgp}(t_c)} \text{ and } r_{RG} = \frac{G_{0,CCF}(t_c)}{G_{0,ACFrd}(t_c)} \quad \text{eq. 6}$$

2.3. Live cell experiments

2.3.1. For single-labeled constructs, fit the cell samples to an appropriate model. For the shown membrane receptor, diffusion occurs in a bimodal fashion with a short and a long diffusion time. Additionally, the photophysics and blinking of the fluorophores have to be considered:

$$G_{ACF,2D}(t_c) = b + \frac{1}{N}\left[\frac{a_1}{1+{}^{t_c}/_{t_{d1}}} + \frac{1-a_1}{1+{}^{t_c}/_{t_{d2}}}\right]\left[1 - a_R + a_R \cdot \exp\left(-\frac{t_c}{t_R}\right)\right] \quad \text{eq. 7}$$

Here, t_{d1} and t_{d2} are the two required diffusion times, and a_1 is the fraction of the first diffusion time.

NOTE: In contrast to the calibration measurements, in which the free dyes and DNA strands freely diffuse in all directions, the membrane receptor shows only 2D diffusion along the cell membranes. This difference between 3D and 2D diffusion is reflected by the modified diffusion term (compare eq. 1), where t_D in the 2D case does not depend upon the shape factor s of the confocal volume element.

2.3.2. Calculate the concentration c of green or red labeled proteins from the respective N and V_{eff} using basic math (eq. 8):

$c = \frac{N}{V_{eff} N_A}$ where N_A = Avogadro's number eq. 8

2.3.3. For N-terminal SNAP label and intracellular eGFP, fit the two autocorrelations (ACF_{gp} and ACF_{rd}) of the double-labeled sample using the same model as for the single-labeled constructs for the ACFs (eq. 7) and the CCF_{PIE} using a bimodal diffusion model (eq. 9):

$$G_{CCF,2D}(t_c) = b + \frac{1}{N}\left[\frac{a_1}{1+{}^{t_c}/_{t_{d1}}} + \frac{1-a_1}{1+{}^{t_c}/_{t_{d2}}}\right] \quad \text{eq. 9}$$

NOTE: For a global description of the system, all three curves have to be fit jointly: The diffusion term is identical for all three curves and the only difference is the relaxation term for the CCF_{PIE}. As photophysics of two fluorophores is usually unrelated, no correlation term is required. This absence of relaxation terms results in a flat CCF_{PIE} at short correlation times. However, crosstalk and direct excitation of the acceptor due to the donor fluorophore might show false-positive amplitudes and should be carefully checked for using the calibration measurements.

2.3.4. Calculate the concentration c of green or red labeled proteins from the respective N and V_{eff} using equation 8.

2.3.5. Estimate the fraction or concentration, c_{GR} or c_{RG}, of interacting green and red labeled proteins from the cell samples using the correction factors obtained from the DNA

samples, the amplitude ratios r_{GR} and r_{RG} of the cell sample and their respective obtained concentrations (eq. 10).

$$c_{GR} = r_{GR} \cdot c_{green} \text{ and } c_{RG} = r_{RG} \cdot c_{red} \quad \text{eq. 10}$$

2.3.6. For C-terminal SNAP label and intracellular eGFP, fit the two autocorrelations (ACF_{gp} and ACF_{rd}) of the FRET sample as the single-labeled samples (equation 7) and the CCF_{FRET} to a bimodal diffusion model containing an anti-correlation term (equation 11)

$$G_{CCF,2D}(t_c) = b + \frac{1}{N}\left[\frac{a_1}{1+{}^{t_c}/_{t_{d1}}} + \frac{1-a_1}{1+{}^{t_c}/_{t_{d2}}}\right]\left[1 - a_f\right]\left[1 - \sum_i a_{Ri} \cdot \exp\left(-\frac{t_c}{t_{Ri}}\right)\right] \quad \text{eq. 11}$$

where a_f reflects the amplitude of the total anti-correlation and a_R and t_R the respective amplitude and relaxation time.

NOTE: In case of anti-correlated fluorescence changes due to FRET, one or several anti-correlation terms might be required (eq. 11), resulting in a "dip" of CCF_{FRET} at low correlation times coinciding with a rise in the two autocorrelations (ACF_{gp} and ACF_{rd}). However, be aware that photophysics such as triplet blinking might mask the anti-correlation term by dampening the FRET-induced anti-correlation. A joint analysis supplemented with filtered FCS methods might help to unmask the anti-correlation term. Additionally, technical artifacts stemming from dead times in the counting electronics in the nanoseconds range should be excluded [16]. A more detailed step-by-step procedure on how to perform the analysis in ChiSurf [28] and templates for the calculation of confocal volume or molecular brightness are provided on the Github repository (https://github.com/HeinzeLab/JOVE-FCS) and as supplementary files (**Supplementary Note 4** and **Supplementary Note 6**). Additionally, the python-scripts for batch export of data acquired with the Symphotime software in .ptu format can be found there.

REPRESENTATIVE RESULTS:

Exemplary results of the calibration and live-cell measurements are discussed below. Additionally, the effect of FRET on the cross-correlation curves is demonstrated based on simulated data next to the effect of protein-protein-interaction increasing the CCF_{PIE} amplitude.

PIE-based FCS data export

In PIE experiments, data are collected in the time-tag time-resolved mode (TTTR) [29,30]. **Figure 1B** shows the photon arrival time histograms of a PIE measurement of a double-labeled DNA strand on the described setup (**Supplementary Note 1**). The setup has four detection channels. The fluorescence emission is first split by polarization in "S" and "P" directions (referring to the perpendicular and parallel plane in which the electric field of a light wave is oscillating). Secondly, each polarization direction is then split in two color channels (green, red) before detection, resulting in four channels (S-green, S-red, P-green, P-red). In the "prompt" time window, the green fluorophore gets excited, and the signal is detected in both the green and red channels due to FRET. In the delay time window, only the red fluorophore (in the red channel) is visible. Based

on the detection channels and "prompt" versus "delayed" time windows, at least five different correlation curves (3 autocorrelation curves (ACFs) and 2 cross-correlation curves (CCFs)) can be obtained (**Figure 1C-D**): (1) green signal in the prompt time window (ACF_{gp}) (2) red signal in the prompt time window (in case of FRET, ACF_{rp}) and (3) red signal in the delay time window (ACF_{rd}). These ACFs report on the protein mobility, photophysics (e.g., triplet blinking) and other time-correlated brightness changes in the fluorophores (e.g., due to FRET). (4) The PIE-based cross-correlation CCF_{PIE} of the green signal in the prompt time window with the red signal in the delay time window allows determining the fraction of co-diffusion of the green and red fluorophore[16]. (5) The FRET-based cross-correlation CCF_{FRET} of the green with the red signal in the prompt time window is related to FRET-induced, anticorrelated brightness changes in the green and red signals [31-33].

[Place **Figure 1** here]

Calibration

Figure 2A-B shows a calibration measurement of the singly diffusing green and red fluorophores, respectively. Based on a fit with eq. 1 and the known diffusion coefficient D_{green} [26] and D_{red} [27] the shape (z_0 and ω_0) and size (V_{eff}) of the detection volume are calculated using eq. 2a-c. The fit results from the ACF_{gp} from the green fluorophore and ACF_{rd} from the red fluorophore are summarized in **Figure 2C**. Both fluorophores show an additional relaxation time constant of 8.6 µs (18%) and 36 µs (15%), respectively. The molecular brightness (eq. 5a-b) of the green and red fluorophore amounts to 12.5 kHz per molecule and 2.7 kHz per molecule, respectively.

For a reliable estimation of the confocal volume size and shape as well as the molecular brightness, it is recommended to perform 3-5 measurements per calibration experiments and a joint (or global) fit of all repeats.

The crosstalk α (**Figure 2D**, eq. 3) and the direct excitation of acceptor by the green laser δ (**Figure 2E**, eq. 4) for this fluorophore pair lie at ~15% and ~ 38%, respectively.

[Place **Figure 2** here]

To determine and calibrate the overlap of the green and red excitation volume, a double-labeled DNA double strand is used (**Figure 3A**) as described above. Here, the fluorophores are spaced 40 bp apart such that no FRET can occur between the green and red fluorophores attached to the ends of the DNA double strands. **Figure 3B** shows the autocorrelations from both fluorophores in green (ACF_{gp}) and magenta (ACF_{rd}) and the PIE-cross correlation, CCF_{PIE}, in cyan. Please note that for CCF_{PIE}, the signal in the green channels in the prompt time window is correlated with the signal in the red channels in the delay time window [16].

Here, an average diffusion coefficient for the DNA strand of D_{DNA} = 77 µm²/s is obtained. More details on the calculation can be found in the step-by-step protocol, **Supplementary Note 4**. This value is obtained by inserting the calibrated green and red detection volumes size (**Figure 2**) and the respective diffusion times of ACF_{gp} and ACF_{rd} of the DNA strand (**Figure 3C**) into equation 2a.

Next, using the obtained correction values r_{GR} and r_{RG} and using eq. 6 later on, the amount of co-diffusion, i.e., double-labeled molecules (or protein complexes in case of co-transfection of two different proteins) can be determined from the cell samples.

[Place **Figure 3** here]

Live-cell experiments

In the following section, the analysis of live-cell experiments for different β_2AR constructs is presented. As β_2AR is a membrane protein, its diffusion is largely limited to a two-dimensional diffusion (**Figure 4A**) along the cell membrane (except for transport or recycling processes to or from the membrane) [2]. With the restriction to the 2D diffusion the shape factor $s = z_0/\omega_0$ in eq. 1 becomes obsolete resulting in a simplified diffusion model (eq. 9).

Single-labeled constructs: β_2AR-IL3-eGFP and NT-SNAP-β_2AR

Figure 4 shows exemplary measurements of the single-label construct β_2AR-IL3-eGFP (**Figure 4B**), where eGFP is inserted into the intracellular loop 3, and the construct NT-SNAP-β_2AR (**Figure 4C**), where the SNAP tag is conjugated to the N-terminus of β_2AR. The SNAP tag is labeled with a membrane-impermeable SNAP surface substrate. The representative curves show the average of 4-6 repeated measurements with acquisition times of 120 - 200 s each. The respective autocorrelations ACF_{gp} and ACF_{rd} of the eGFP and SNAP signal are fitted to a bimodal, two-dimensional diffusion model (eq. 9). In terms of fast dynamics, eGFP shows only the expected triplet blinking at t_{R1} ~ 9 µs while the SNAP signal requires two relaxation times, one at the typical triplet blinking time of t_{R1} ~ 5 µs and a second one at t_{R2} ~ 180 µs.

The molecular brightness of the substrates in living cells is 0.8 KHz (GFP) and 1.7 kHz (SNAP) per molecule under the given excitation conditions (eqs. 5a-b). The concentration of the labeled β_2AR constructs incorporated in the cell membrane should be in the nano-molar range and can be determined by the average number of molecules (eq. 9, **Figure 4C**) and the size of the respective confocal volume for the green and red channel (**Figure 2**) using eq. 8.

[Place **Figure 4** here]

Double-labeled construct: NT-SNAP-β_2AR-IL3-eGFP

In the double-labeled construct NT-SNAP-β_2AR-IL3-eGFP (short NT-SNAP), eGFP is inserted into the intracellular loop 3, and, the SNAP tag conjugated to the N-terminus of β_2AR (**Figure 5A**). In this configuration, the GFP is on the inner side of the membrane and the SNAP on the outer side with too large distances for FRET. In an ideal case, this construct would show 100% co-diffusion of the green and red fluorophore, and no FRET signal. **Figure 5B-D** shows two measurements of the NT-SNAP in two cells on two different measurement days. Fitting the ACF_{gp} and ACF_{rd} of the "better" measurement shown in **Figure 5B** with eq. 7 and the CCF_{PIE} with eq. 9, reveals 50- 60 molecules in focus for the ACF_{gp} and ACF_{rd}, whereas N_{app}, thus $1/G_0(t_c)$ ~ 114 for the CCF_{PIE} (**Figure 5C**). The concentration of labeled receptors lies in the ~100 nM range as determined with eq. 8. To determine the average concentration of double-labeled molecules, first, the ratio of $G_0(t_c)$ (represented by $1/N_{(app)}$) of the CCF_{PIE} to ACF_{gp} and ACF_{rd}, respectively, is calculated (eq. 6). Next,

these values, r_{GRcell} = 0.43 and r_{RGcell} = 0.53 are compared to the values obtained from the DNA measurement ($r_{GR,DNA}$ = 0.51 and $r_{RG,DNA}$ = 0.79 on this measurement day). Using the rule of proportions, a r_{GRcell} = 0.43 from the ACF_{gp} of the eGFP signal reflects to a fraction of co-diffusion ($r_{GRcell}/r_{GR,DNA}$) of 0.84, where for the other case of ACF_{rd} of the SNAP substrate signal, this value amounts to 0.67. The average concentration of the double-labeled NT-SNAP construct can finally be calculated based on eq. 10. In contrast, in the measurement shown in **Figure 5D** from a different day, the concentration of receptors is quite low and the data very noisy such that the fit range is limited up to ~ 10 µs. In addition, only a low amount of co-diffusion is observed (15 – 26%).

[Place **Figure 5** here]

Double-labeled construct undergoing FRET: β_2AR-IL3-eGFP-CT-SNAP

In the double-labeled construct β_2AR-IL3-eGFP-CT-SNAP (**Figure 6A**), the eGFP is inserted into the intracellular loop 3 identical to the NT-SNAP-β_2AR-IL3-eGFP construct with the SNAP tag attached to the C-terminus. Here, both labels are on the same side of the cells' plasma membrane, so that the fluorophores are in close vicinity so that FRET occurs as indicated by the quenched eGFP lifetime (**Supplementary Note 5**). Considering the flexibility of relatively unstructured protein regions like the C-terminus [34] and at least two different protein conformations of GPCRs [35], "high FRET" (HF) or "low FRET" (LF), dynamic changes in the FRET efficiency due to eGFP-SNAP distance changes could be observed and identified by an anticorrelation term in the CCF_{FRET} (orange curve in **Figure 6B**). FRET fluctuations have been shown to be anticorrelated as the receptor can only be in one state at a time, either HF or LF. Joint (or global) fit of all five correlation curves (Figure 6B) reveals ~70% of slowly diffusing molecules at ~100 ms while the rest diffuses with ~ 1 ms. All autocorrelations and CCF_{FRET} show relaxation terms at 37 µs and 3 µs; those correlations dominated by red signal (ACF_{rp}, ACF_{rd} and CCF_{FRET}) show an additional slow component ~ 50 ms (**Figure 6C**).

FRET-induced changes on the CCF_{FRET} under different conditions (**Figure 6D**) are demonstrated by a series of simulations of a two-state system with a fluctuation time of 70 µs between LF and HF states. Upon switching from the LF to the HF state, changes in the anticorrelated signal are observed in the prompt time window: The green signal decreases and the red signal increases (vice versa for the HF -> LF switching). If HF-LF switching occurs on timescales faster than the diffusion time, in other words during the residence time of the molecule in the focus, the rate can be derived from the anticorrelation in the CCF_{FRET} [6,31,36]. Please note that dynamic processes slower than the diffusion time cannot be observed in FCS.

In this demonstration, two different FRET scenarios were assumed, showing either a moderate or maximal change in FRET efficiency between the two states. The simulations were performed using Burbulator[37] and consider absence or presence of triplet blinking and increasing amount of donor crosstalk into the red channels. The diffusion term was modeled as a bimodal distribution with 30% of fast diffusing molecules at t_{D1} = 1 ms and the rest of the molecules diffusing slowly with t_{D2} = 100 ms. In total, 10^7 photons were simulated in a 3D Gaussian-shaped volume with w_0 = 0.5 µm and z_0 = 1.5 µm, a box size of 20, and N_{FCS} = 0.01.

Figure 6E-F shows the simulation results for the FRET-induced cross-correlation CCF_{FRET} for moderate (**Figure 6E**) and maximal FRET contrast (**Figure 6F**) in the absence (solid lines) and presence of triplet blinking (dashed lines). The FRET-induced anti-correlation can easily be seen in **Figure 6F**. The "dampening" effect upon adding an additional triplet state reduces the correlation amplitude (**Figure 6E-F**) [38,39].

[Place **Figure 6** here]

However, in the simulation most similar to the experimental conditions (α = 10%, 15% triplet blinking and moderate FRET contrast, dashed yellow line in Figure 6E), the anticorrelation term is nearly diminished. **Figure 7** shows the result of analyzing this simulated data using the information encoded in the photon arrival time histograms (i.e., the fluorescence lifetime) by means of Fluorescence Lifetime Correlation Spectroscopy (FLCS) [17,19] or species-filtered FCS (fFCS) [18]. Here, the fluorescence lifetime of the known HF and LF species (**Figure 7A**) is used to generate weights or "filters" (**Figure 7B**) which are applied during the correlation procedure. In the obtained species-auto- and cross-correlation curves (**Figure 7C-D**) the anticorrelation can be clearly observed.

[Place **Figure 7** here]

CCF_{PIE} amplitude to study Protein-Protein Interaction (PPI)

Finally, a common use case for PIE-based FCS in live cells is to study the interaction between two different proteins. Here, the read-out parameter is the amplitude of the CCF_{PIE}, or more precisely the ratio of the autocorrelation amplitudes ACF_{gp} and ACF_{rd} to the amplitude of CCF_{PIE}. To show the effect of increasing co-diffusion on CCF_{PIE}, simulations have been performed based on the two single-labeled constructs, β_2AR-IL3-eGFP and NT-SNAP-β_2AR (**Figure 8A**). **Figure 8B** shows how the amplitude of CCF_{PIE} increases when the fraction of co-diffusing molecules changes from 0% to 100%. Please note that a 1% crosstalk of green signal into the red channels in the delay time window was added with the diffusion components otherwise modeled as shown above.

[Place **Figure 8** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Pulsed-interleaved excitation (PIE) based fluorescence (cross) correlation spectroscopy (F(C)CS). (**A**) In FCS fluorescently labeled molecules diffuse freely in and out of a (diffraction-limited) focal volume shaped by a focused laser that induces fluorescence within this tiny volume. The resulting intensity fluctuations of molecules entering and leaving the volume are correlated and provide information on the mobility of the molecules. (**B**) In PIE, two different laser lines ("prompt" and "delay") are used to excite the sample labeled with two different fluorophores ("green" and "red"). The time difference between both excitation pulses is adapted to the fluorescence lifetimes of the respective fluorophores so that one has decayed before the other is excited. In the double-labeled sample shown, both fluorophores are sufficiently close to

undergo Förster Resonance Energy Transfer (FRET) from the "green" donor fluorophore to the "red" acceptor fluorophore. Thus, red fluorescence emission can be detected in the "prompt" time-window upon excitation of the green donor. In the used setup (**Supplementary Note 2**), two detectors are used for each color, one oriented parallel to the excitation beam orientation (denoted "p") and the second perpendicular (denoted "s"). (C) Three different autocorrelation functions can be determined in a PIE experiment: Correlation of the i) green channel signals in the prompt time window (ACF_{gp}), ii) red channel signals in the prompt time window (ACF_{rp}) and iii) red channel signals in the delay time window (ACF_{rd}). (D) Two different cross-correlation functions can be determined: iv) The "PIE" cross-correlation (CCF_{PIE}) with green channel signals in the prompt time window correlated with the red channel signals in the delay window, where the amplitude of this curve is related to the co-diffusion of fluorophores; and v) the "FRET" cross-correlation (CCF_{FRET}) with the green channel signals in the prompt time window correlated with the red channel signals in the same prompt window; here the shape of this curve at times faster than diffusion is related to the FRET-induced intensity changes.

Figure 2: Calibration measurements of freely diffusing green and red calibration standard. (**A-B**) Representative 60 s measurement of a 2 nM green (**A**) and a 10 nM red (**B**) calibration standard measurement fitted to the 3D Diffusion model including an additional relaxation time (**eq. 1**). The table in panel (**C**) shows the fit results and the derived parameter based on **eq. 2a-c** and **eq. 5a-b**. *Diffusion coefficients were taken from literature [26,27]. (**D**) Determination of the crosstalk α of the green signal into the red channels (**eq. 3**). The excitation spectrum of the green standard is shown in cyan, the emission spectrum in green. The excitation laser lines at 485 nm (blue) and 561 nm (orange) are shown as dashed lines. Transparent green and magenta boxes show the collected emission range (**Supplementary Note 2**). (**E**) Determination of the direct excitation δ of the red fluorophore by the 485 nm laser (eq. 4). Color code is identical to (**D**), light and dark orange show the excitation and emission spectrum of the red standard, respectively.

Figure 3: Calibration of the green-red overlap volume using a DNA sample. (**A**) The DNA strand used for calibration carries a green and a red calibration fluorophore, with a distance of 40 bp in between. The interdye distance must be sufficiently large to exclude FRET between the fluorophores. (**B**) Representative 60 s measurement of a 10 nM DNA solution. Autocorrelations from both fluorophores in green (ACF_{gp}, green standard) and magenta (ACF_{rd}, red standard) and the PIE-crosscorrelation, CCF_{PIE}, in blue. The table in panel (**C**) shows the fit results based on the 3D Diffusion model including an additional relaxation term (**eq. 1**) and the derived parameter diffusion coefficient of DNA, D_{DNA} (**eq. 2a**), the size and shape of the overlap volume (**eq. 2a-c**) and the correction ratios r_{GR} and r_{RG} (**eq. 6**). Please note the values for the green and red detection volume (labeled with *) were taken from the fit of the individual fluorophores shown in **Figure 2**.

Figure 4: Representative measurement of single-label constructs. (**A**) In this study, the membrane receptor β_2AR was used as an example. In contrast to the fluorophores and DNA strand used for calibration, which could freely float through the detection volume, membrane proteins diffuse mainly laterally along the membrane, described as 2-dimensional diffusion. (**B**, D) ACF_{gp} and ACF_{rd} of the single-label constructs β_2AR-IL3-eGFP (**B**) and NT-SNAP-β_2AR (**D**). Shown is the average of 4-6 measurements each collected for 120 – 200 s. The table in panel (**C**) shows

the fit results of the data to the bimodal two-dimensional diffusion model including additional relaxation terms (**eq. 7**).

Figure 5: Double-labeled NT-SNAP-β_2AR-IL3-eGFP construct. (**A**) In the double labeled construct, the eGFP is inserted into the intracellular loop 3 and the SNAP tag attached to the N-terminus of β_2AR (NT-SNAP). (**B, D**) ACF_{gp}, ACF_{rd} and CCF_{PIE} of two measurements of the double-labeled construct. The data is fit to a bimodal two-dimensional diffusion model (**eq. 9**, CCF_{PIE}) and including additional relaxation terms (**eq. 7**, ACF_{gp} and ACF_{rd}). The table in panel (**C**) shows the fit results and the derived parameter concentration (**eq. 8**), the ratio of the correlation amplitude at zero correlation time ($G_0(t_c)$) and the fraction of co-diffusing molecules (**eq. 10**). Please note that the measurements were acquired on different days, thus slightly different factor for the amplitude correction were used (B: $r_{GR,DNA}$ = 0.51 and $r_{RG,DNA}$ = 0.79; D: $r_{GR,DNA}$ = 0.51 and $r_{RG,DNA}$ = 0.56).

Figure 6: Simulation of double-labeled sample showing dynamic FRET. (**A**) Double-labeled β_2AR with an eGFP inserted into the intracellular loop 3 and a C-terminal SNAP tag. Both fluorophores are close enough to undergo FRET and show changes in the FRET efficiency if the receptor undergoes protein dynamics. (**B**) Autocorrelation (ACF_{gp}, ACF_{rp} and ACF_{rd}, fit with **eq. 7**) and cross-correlation curves (CCF_{FRET} (**eq. 7**) and CCF_{PIE} (**eq. 9**)) of an example measurement. Table in panel (**C**) shows the fit results. (**D-F**) To show the influence of experimental parameter on the expected, FRET-induced anticorrelation term, 12 simulations were performed, in which the change in the FRET efficiency (small or large), different amount of donor crosstalk into the acceptor channels (0%, 1% or 10%) and the absence and presence of triplet blinking were modeled. The equilibrium fraction of both FRET-states was assumed to 50:50 and their exchange rates adjusted such that the obtained relaxation time t_R = 70 µs. More details on the simulations see in the text. (**E**) CCF_{FRET} of the simulation results with a moderate FRET contrast and in the absence of crosstalk (dark orange), 1% crosstalk (orange) and 10% crosstalk (light orange). Solid lines show results in the absence of triplet, dashed lines in the presence of triplet. (**F**) CCF_{FRET} of the simulation results with maximal FRET contrast. The color code is identical to (**E**).

Figure 7: Lifetime-filtered FCS can help to uncover the protein dynamics based fluctuations in FRET efficiency in samples with high crosstalk, significant triplet blinking or other photophysical or experimental properties masking the FRET-induced anticorrelation in the CCF_{FRET}. Here, the approach is shown exemplary for the data shown in Figure 6E for the simulation containing 10% crosstalk and 5% triplet blinking. (**A**) Normalized fluorescence intensity decay patterns for the two FRET-species (light and dark green for high and low FRET, respectively) and the IRF (grey). The pattern for the parallel detection channel is shown in solid lines, dashed lines for the perpendicular detection channel. (**B**) The weighting function or "filter" were generated based on the patterns shown in (**A**), color code is identical to (**A**). Please note that only the signal in the green detection channels, and thus the FRET-induced donor quenching, is considered here. (**C**) Four different species-selective correlations are obtained: species-autocorrelations of the low FRET state ($sACF_{LF\text{-}LF}$, dark green) and the high FRET state ($sACF_{HF\text{-}HF}$, light green), and the two species-crosscorrelations between the low FRET to the high FRET state ($sCCF_{LF\text{-}HF}$, dark orange) and vice versa ($sCCF_{HF\text{-}LF}$, orange). The sCCF clearly shows the anticorrelation in the µs-range.

Dashed black lines show the fits. sACF were fit with **eq. 9** and sCCF with **eq. 11**. Table in panel (**D**) shows the fit results.

Figure 8: The CCF_{PIE} can be used to study the interaction of two proteins. (**A**) Here, a co-transfection study of β_2AR-IL3-eGFP with NT-SNAP-β_2AR (carrying a "red" SNAP-label) was simulated. (**B**) For an increasing amount of co-diffusing molecules (0% (dark blue) -> 100% (light blue)) the amplitude G(t_c) increases. The diffusion term was again modeled as a bimodal distribution with 30% of fast diffusing molecules at t_{D1} = 1 ms and the rest of the molecules diffusing slowly with t_{D2} = 100 ms. Additionally, 1% crosstalk of green signal into the red delay time window was added.

Table 1: List of variables and abbreviations. For the use of symbols and definition in fluorescence and FRET experiments, the guidelines of the FRET community [40] are recommended.

SUPPLEMENTARY FILES:

SuppNote1_Coverslip cleaning.docx

SuppNote2_Confocal Setup.docx

SuppNote3_Data export.docx

SuppNote4_FCCS calibration analysis using ChiSurf.docx

SuppNote5_Fluorescence lifetime histograms.docx

S6_Scripts.zip

S7_Excel_templates.zip

DISCUSSION:

FCS techniques in GPCRs allow the mobility and interactions of receptors inside live cells to be assessed [41]. The advantage of the FRET-FCS technique is that, along with mobility, the conformational dynamics of GPCRs can be investigated. However, performing FRET-FCS in live cells is challenging and requires cells which show low (or maximal moderate) expression of the fluorescently labeled protein of interest, a well-calibrated setup and a good pipeline to analyze data. Here, first the critical points in sample preparation and experimental procedure are discussed concerning the biological, spectroscopic, and technical points of view.

Critical experimental steps include minimizing the background and autofluorescence (by using extensively cleaned coverslips and phenol-red free media), the optimization of transfection conditions (e.g., amount of plasmid DNA and time after transfection) to achieve low expression levels and efficient labeling. Of course, it is also vital to ensure that the function of the labeled protein is not hampered. Thus, in live-cell experiments, the decision for the labeling strategy and label position is often made in favor of fluorescent proteins or SNAP/CLIP tag attached to the

flexible N- or C-terminus [42,43]. Alternative labeling strategies like inserting an unnatural amino acid with a reactive side chain for labeling with an organic fluorophore have been emerging in the last years [44].

For dual-color PIE-FCS, where only the interaction of two molecules of interest is to be investigated, the fluorophores can be selected from a large variety of established fluorescent proteins or SNAP/CLIP substrates. Here, spectroscopy-wise the goal should be to select a pair such that little crosstalk or direct acceptor excitation occur. Additionally, the selected fluorophores should be photostable and show little or no bleaching under the chosen experimental conditions. It is recommended to select fluorophores in the red spectral range as (1) the autofluorescence background from the cell is reduced and (2) the excitation light is of longer wavelength, thus less phototoxic[14]. Photobleaching can be minimized by conducting a so-called "power series" first, in which the laser power is increased stepwise, and the molecular brightness is observed. The optimal excitation intensity range lies in the linear range of the results[45].

If the two labels are supposed to report also on protein conformational dynamics through FRET then the choice of available fluorophore is more restricted. Here, the possible minimal/maximal distance between the two fluorophores should be estimated beforehand e.g., based on available structures or molecular size, and a fluorophore pair selected with a reasonable Förster radius R_0 such that FRET can actually occur [20].

Here, eGFP and a SNAP tag were chosen for labeling, and the SNAP tag was labeled with either an intracellular or a membrane-impermeable surface substrate. The spectra are similar to the ones shown in Figure 2C-D. This combination of fluorophores shows high crosstalk of the eGFP into the red detection channels and direct acceptor excitation of the SNAP substrate by the green excitation in the prompt time window and results in a significant "false" signal in the red channels in the prompt time window. Ideally, both values, cross talk and direct acceptor excitation, should not exceed 5% [5,6,38]. However, with a Förster radius of 57 Å, it is ideally suited to probe the distance between the labels in the β_2AR-IL3-eGFP-CT-SNAP construct as can be evaluated from the quenched eGFP lifetime (Supplementary Note 5).

Technically, as for any fluorescence spectroscopy experiment, the device should be well aligned and should possess suitable excitation sources, emission filter, and sensitive detectors. To avoid artifacts from detector after pulsing on the µs timescale, at least two detectors of each color should be present, which can be cross-correlated. In modern time-correlated single photon counting electronics, the dead time of the detection card in the ns time range hardly plays a role due to the independent routing channels, however, it might be checked as proposed by Müller *et al* [16] provided the time range of interest lies in the sub-µs/ns time range. Additionally, for even higher time resolutions in the ps range, each detection channel should be doubled, i.e., four detector per color should be used, to also bypass detector dead times [2,15,29,46]. While the average fluorescence lifetime can be estimated using non-polarized fluorescence detection, for analysis of the distance (~distribution) between the fluorophores the emission has to be collected polarization-dependent. This is due to the fact that the efficiency of the energy transfer in FRET

relies on the orientation of the two fluorophores. More detailed information can be found here [20,28,47]. Finally, in PIE experiments, the distance between the prompt and delay pulse is critical and should be chosen such that the fluorescence intensity of the fluorophores has been largely decayed (Figure 1B). A common rule is to place the two pulses 5x the fluorescence lifetime apart, i.e. for eGFP with a fluorescence lifetime of 2.5 ns the distance should be 12.5 ns at minimum [22].

After having detailed all considerations for the experimental procedure, the data and its analysis is discussed in more detail. As mentioned in the protocol section, the alignment of the setup must be checked daily, including the analysis of the calibration measurements. The data shown in **Figure 2A-C** e.g., shows an additional relaxation component in 8-40 µs. Typical triplet blinking of the green calibration fluorophore is known to occur in the 2-10 µs range [13,15,48]. The slow relaxation component required in all curves of the DNA sample (**Figure 3C**), too slow for actual triplet blinking, might stem from interactions of the DNA with the fluorophores [39]. However, this component would not be expected in CCF_{PIE}, and most likely stems from residual crosstalk. Thus, it is highly advisable to perform the analysis of the calibration samples directly prior to proceeding to the cell experiments to judge the quality of the day's alignment.

The proper calibration of the confocal overlap volume requires a sample with 100% co-diffusion of the green and red label. Here, fluorescently labeled double stranded DNA is used. Both DNA strands can be tailored to have the desired fluorophore at the required distance from each other. The designed strands can be annealed with high yield. However, Good Laboratory Practice advises checking the integrity and labeling degree of the DNA strands by agarose gel electrophoresis and measuring the absorption spectrum. Also, the yield of the double-stranded assembly should be checked as this calibration measurement critically relies on the assumption that there is a 100% co-diffusion of the green with the red label. In case the assumption is not valid, correction factors might have to be applied [16,22]. In the calibration measurements shown in **Figure 2** and **Figure 3**, a detection volume of 1.4 fL and 1.9 fL in the green and red channel were obtained, respectively. This size difference is expected for a setup with nearly diffraction-limited excitation volumes (**Supplementary Note 2**). Under this condition, the size of the excitation volume scales with the excitation wavelength. This in turn explains the different correlation amplitudes observed in **Figure 3B**. The derived correction factors r_{GR} = 0.56 and r_{RG} = 0.72 correct for this size discrepancy and potential non-perfect overlap of the two excitation volumes [3,4].

Figure 4-7 showcase the workflow of a PIE-F(C)CS based study aimed toward understanding conformational protein dynamics. First, the two single-labeled constructs β_2AR-IL3-eGFP and NT-SNAP-β_2AR serve as controls to characterize the fluorophore properties in cells in the absence of the respective other fluorophore (**Figure 4**). Next, the double-labeled construct NT-SNAP-β_2AR-IL3-eGFP carrying a SNAPtag facing the cell exterior and an eGFP on the cytoplasmic side serves as a "100% co-diffusion" control (**Figure 5**). The last construct, β_2AR-IL3-eGFP-CT-SNAP, carries both fluorophores on the cytoplasmic side and close enough together to undergo FRET. Here, again a 100% co-diffusion would be expected in tandem with anti-correlated intensity fluctuations in the green and red channels signal in the prompt time window, i.e., after donor excitation, due to protein dynamics influencing the FRET efficiency [31-33]. This dynamics might show up as anti-correlation in the CCF_{FRET} (**Figure 6-7**).

All GPCR β_2AR constructs show bimodal diffusion on the cell membrane (**Figure 4A**). Whereas the β_2AR-IL3-eGFP shows only the expected triplet blinking (Figure 5B) [13,15], NT-SNAP-β_2AR shows an additional slow relaxation time (**Figure 5C-D**). It is likely that t_{R2} might stem from unbound SNAP substrate. This could be elucidated by further experiments, e.g., by also measuring the diffusion and photophysical properties of the used SNAP substrate in an aqueous solution. Of note, a straightforward experiment to differentiate between diffusion and relaxation times is to change the pinhole of the confocal setup, i.e., increasing the effective volume: While the diffusion times increase with increasing effective volumes, relaxation terms are unaltered [13]. When determining the concentration of fluorescent protein (FP) based on the fit results, be aware that FPs in general undergo a maturation process, in which finally the chromophore is formed [12]. This maturation time may differ from FP to FP in addition to photophysics that depends on the local chemical environment [13,15]. Thus, the actual protein concentration present in the sample reported by FCS is usually underestimated, which can be corrected if the fraction of non-fluorescent FPs can be determined in the experiment. Finally, it is advisable to check the fluorophore spectra in live cells to correct the values for α and δ, if required, as most fluorophores react sensitive to their environment [13,15,48]. The background to subtract is determined by the signal collected in non-transfected cells. Additionally, the autocorrelation of the respective other color channel and the CCF_{PIE} should be checked to be able to identify false signals (**Supplementary Note 4 – Figure 30**).

The two measurements from the NT-SNAP-β_2AR-IL3-eGFP (**Figure 5D**), where the fluorophores are located on different sides of the membrane, were acquired on different days and shows the importance of statistics in time-resolved single molecule fluorescence. Here, the different results may be due to the different degree of labeling: In one cell the higher degree of labeling and averaging of measurements resulted in relatively low noise (**Figure 5B**), while from the other cell, only two measurements could be collected (**Figure 5A**). Beyond collecting a sufficient amount of data, it is critical to evaluate the results timely, and maybe optimize the labeling strategy. When designing the experiments, it is important to remember that FRET is sensitive, but limited to distances up to 10 nm and "blind" otherwise. In our case, this "blindness" is indicated by the unaltered eGFP fluorescence lifetime (**Supplementary Note 5**). In the β_2AR-IL3-eGFP-CT-SNAP construct (**Figure 6A**), FRET can be pinpointed from the quenched eGFP lifetime (**Supplementary Note 5**). However, no anticorrelation term is observed (**Figure 6B**), which means that FRET is either not fluctuating or at a time scale slower than the diffusion time. Up to three additional relaxation term are required in ACF_{gp}, ACF_{rp}, ACF_{rd} and CCF_{FRET} (**Figure 6C**). The slow component in ACF_{rp}, ACF_{rd} and CCF_{FRET} might be due to acceptor bleaching and, of course, influences the obtained value of the slow diffusion found in these curves (~350 ms compared to 117 ms in ACF_{gp}). t_D in the red channel is supposed to be slightly larger than in the green channel due to the differently sized confocal volumes (**Figure 2**) – but only by a factor comparable to the size difference. The very fast relaxation time of 3 µs reflects the triplet blinking of the fluorophores [13,15,48], whereas the slower relaxation time of 37 µs might be due to FRET: Similarly, as FRET induces an anticorrelation in the CCF_{FRET}, positive correlations are expected in the autocorrelations [31-33]. The presence of this term as "positive" in CCF_{FRET} and its presence in the ACF_{rd} might be explained with the high crosstalk and should be further elucidated. Note that the CCF_{PIE} is flat at short correlation times as expected.

On the other hand, it should be noted that the occurrence of FRET in a system of interest leads to non-linear effects on the correlation curves [6]. The molecular brightness e.g., of a molecule scales into the correlation amplitude squared and each FRET-state (and the always present molecules without an active receptor) shows different molecular brightness. Indeed, FRET decreases the apparent concentration of green molecules detected (i.e., increases ACF_{gp} amplitude) and the number of red molecules (determined from red-prompt) is overestimated [5]. Both effects influence the amount of interaction derived from both CCF_{FRET} and CCF_{PIE}. However, global analysis as shown e.g. for the intramolecular dynamics of Calmodulin [31,32] or Syntaxin [33] can reveal the protein dynamics. When carefully calibrated, the average FRET efficiency may be extracted from the relative CCF_{PIE} and ACF amplitudes [22], whereas the limiting states might be determined from the analysis of the donor fluorescence lifetime distribution [33].

Considering the fact that in live cell experiments with large fluorophores like eGFP the FRET contrast is likely to be even lower than assumed for the simulations shown in Figure 6 and that the direct excitation of the acceptor was not added in the simulation, might explain why the identification of the anticorrelation in live cell experiments is very challenging. A promising analysis alternative relies on harvesting the information encoded in the photon arrival time histograms (**Figure 1B**) accessible due to the time-correlated single photon counting data collection [29,30]. If the fluorescence lifetime (~patterns) of the two (or more) (FRET) species inside the sample are known (**Figure 7A**), "filter" or weights can be chosen which are applied during the correlation process (**Figure 7B**) [17-19]. The correlation curves thus obtained, no longer represent the correlation of detection channels but rather the auto- or cross-correlations between two different (FRET) species, thus renamed to species-ACF (sACF) or species-CCF (sCCF). Applying this approach to the simulated data with moderate FRET contrast, high crosstalk and triplet blinking recovers the anticorrelation term (**Figure 7C-D**). However, it should be noted that relaxation times can be obtained but the relationship to amplitude is lost [18]. This approach has been applied previously in live cell experiment e.g. to study the interaction of EGFR with its antagonist [49] or to separate the fluorescence from proteins attached to eGFP variants with exceptionally short and long fluorescence lifetimes [50].

While PIE-based FRET measurements in purified proteins are largely used to study protein dynamics [36 22], in live cells it focuses on understanding protein-protein interactions. This approach has been applied to study the regulation of MAP kinase activity in yeast [51] or to resolve the interaction of membrane proteins with their cytosolic binding partner as summarized in this recent article [52]. Here, complications may arise when significant crosstalk of green fluorophores is still present in the delay time window of the red channels or red signal in the green channels in the prompt time window. The former might be caused by an insufficient delay of the red pulse with respect to the green pulse while both effects stem from too strongly overlapping excitation and emission spectra of the chosen fluorophores. It is recommended to check the respective single labeled constructs carefully and correct for false-positive CCF_{PIE} amplitudes, especially in cells where autofluorescence with very short fluorescence lifetime might be another complicating factor [22].

To conclude, the FRET-FCS approach described here has great potential to understand protein-protein interactions and protein dynamics in live cells at near physiological concentrations. In this protocol, the focus was laid on the required calibration measurements and the necessary quantitative analysis to be performed during live cell measurements. To this end, different live cell measurements were shown complemented with simulations. The simulations provide the general understanding here as parameter could be varied systematically with tailored fit models that describe the specific mobility and photophysical properties of the respective data. The analysis was performed with open-source software tools with an extensive step-by-step protocol and easy-to-adapt templates. Finally, the technical advancements, and thus the availability of ready-to-buy stable PIE-FCS systems together with the spread of open-source software for data analysis will make this technique more and more accessible for a larger research community to eventually unravel protein interaction and dynamics in live cells with highest sensitivity.

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DISCLOSURES:

The authors have no conflicts to declare.

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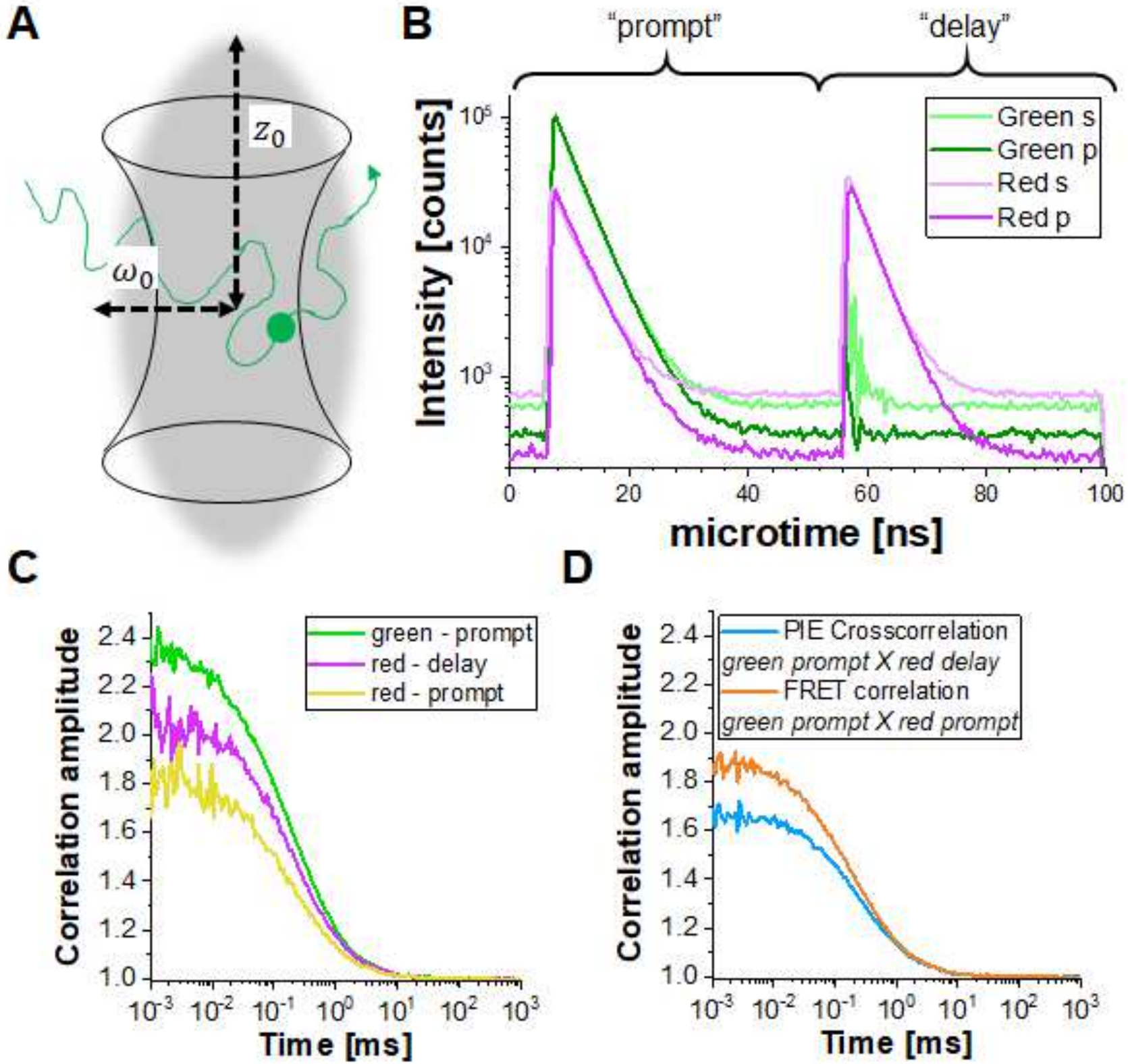
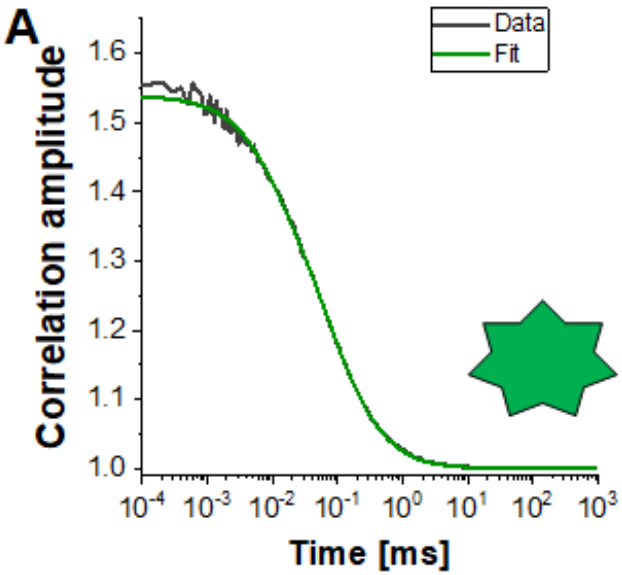
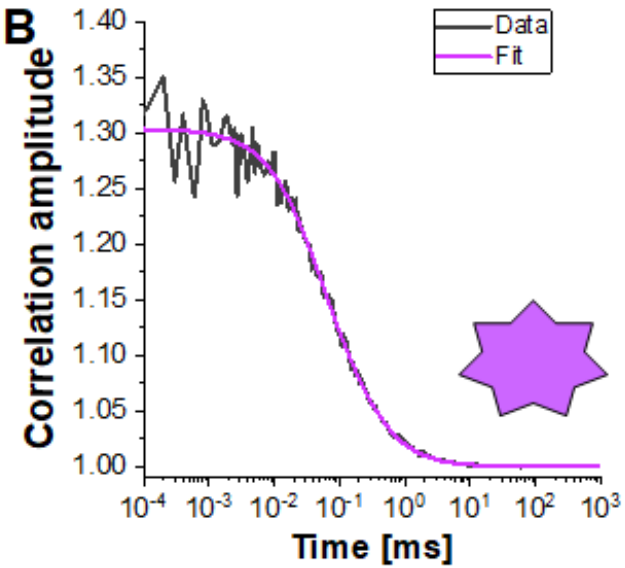
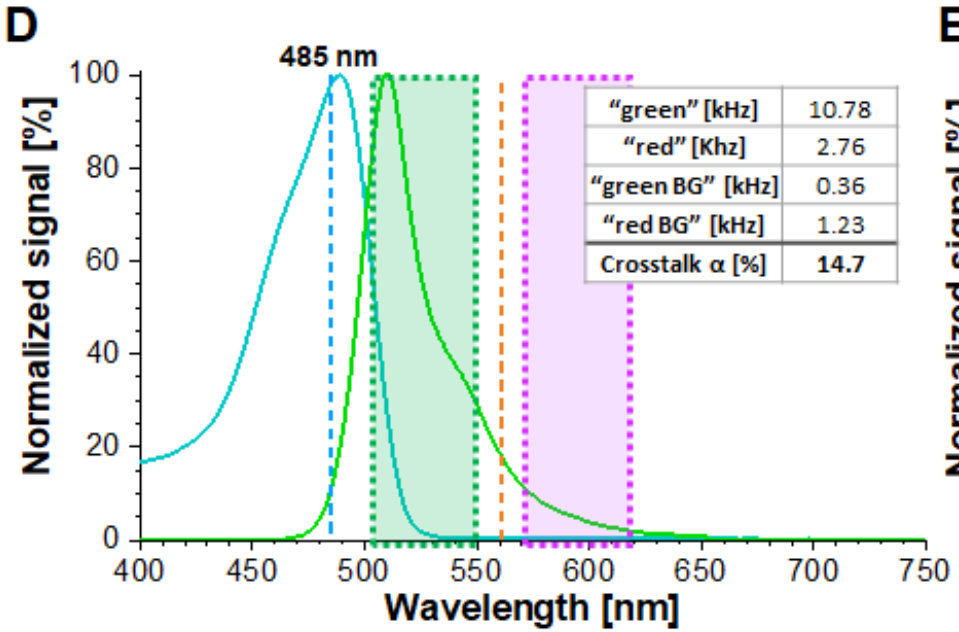


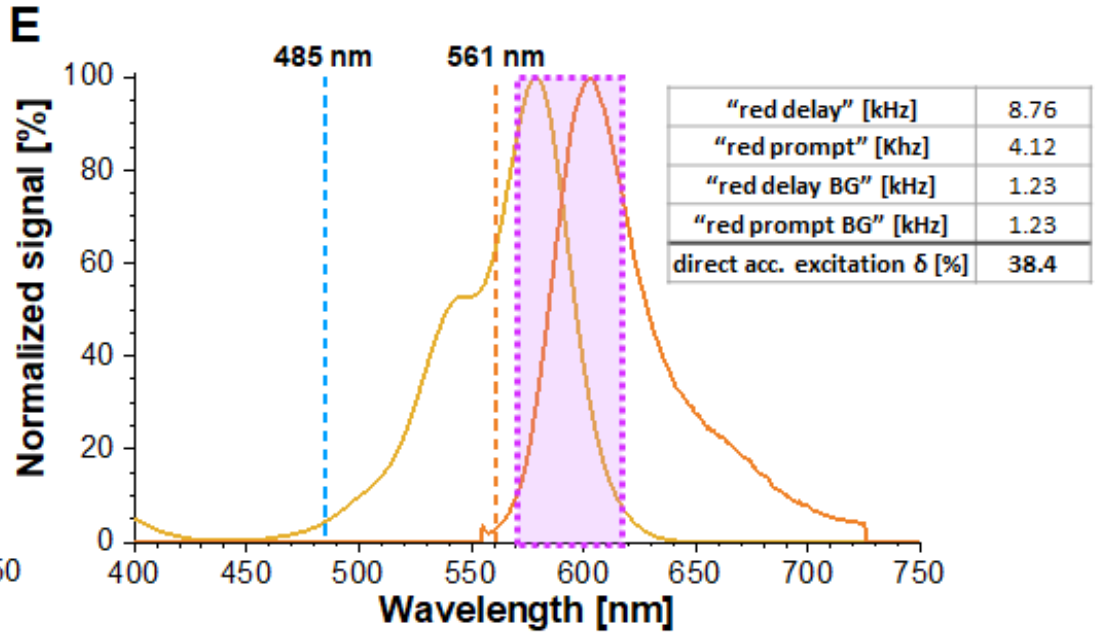




C

Parameter	Green	Red
D [µm²/s]*	414	363
s	6.49	6.78
t_D [µs]	69.0	99.5
N	1.85	3.44
t_R [µs]	8.59	36.4
a_R	0.18	0.15
Derived parameter		
w_0 [µm]	0.338	0.371
z_0 [µm]	2.19	2.51
V_{eff} [fL]	1.40	1.92
B [kHz/molecule]	12.5	2.68





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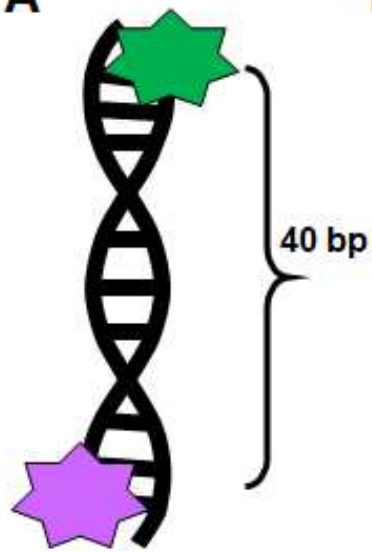
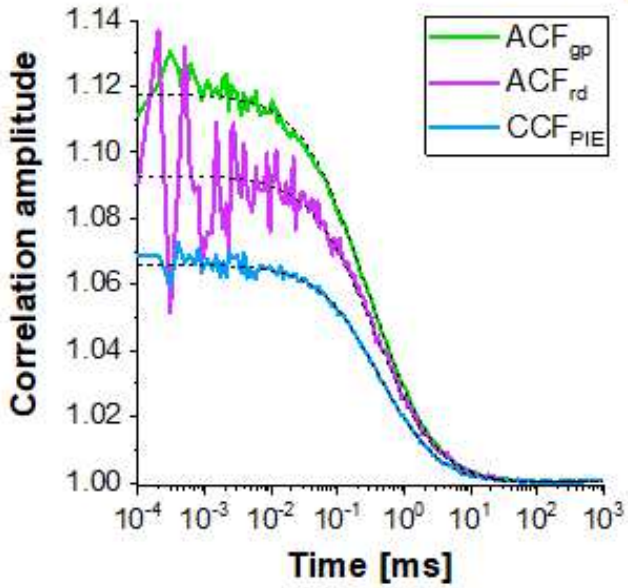


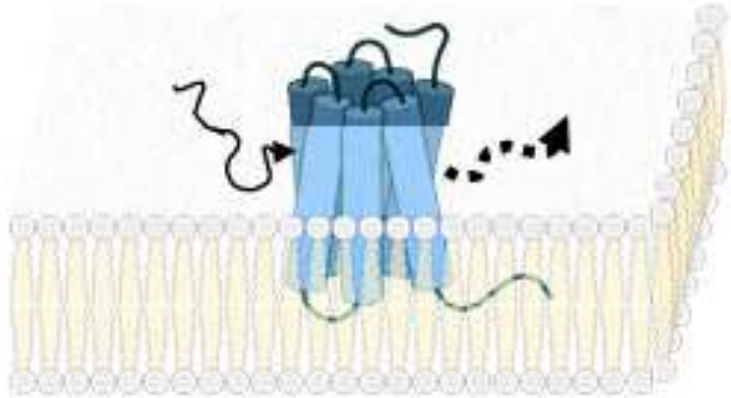
B



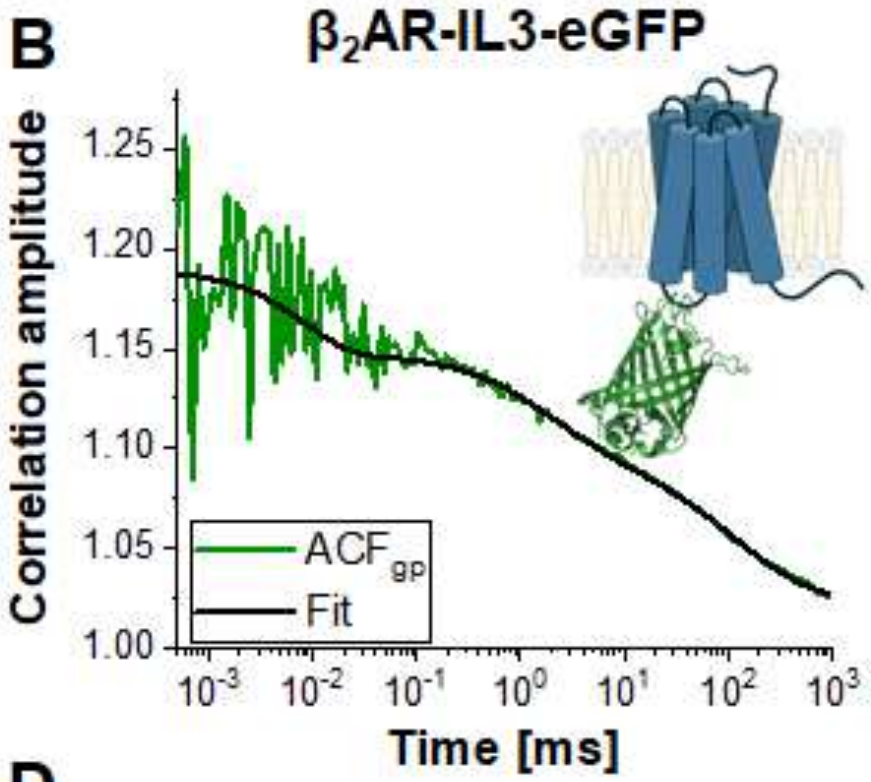
C

Parameter	ACF_{gp}	ACF_{rd}	CCF_{PIE}	
s	6.49*	6.78*	5.65	
t_D [µs]	462	437	470	
N	8.32	10.8	1/G(tc)	15.0
t_R [µs]		67.6		
a_R	0.223	0.088	0.066	
Derived Parameter				
D_{DNA} [µm²/s]		77.1		
w_0 [µm]	0.338*	0.371*	0.381	
z_0 [µm]	2.19*	2.51*	2.15	
V_{eff} [fL]	1.40*	1.92*	1.74	
r_{GR}			0.56	
r_{RG}			0.72	

A



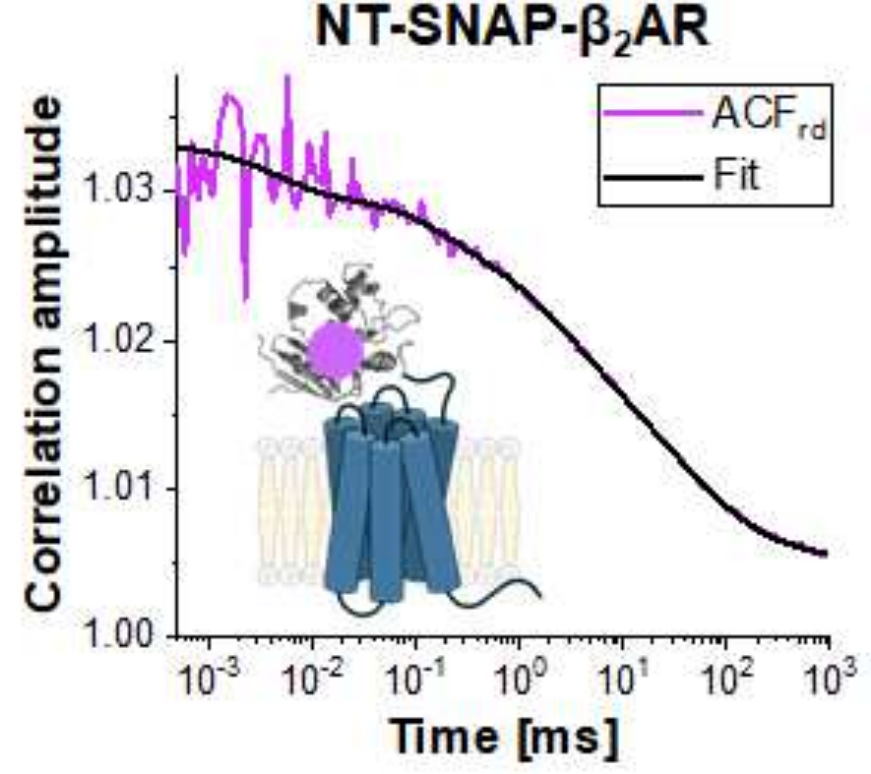
B



C

Parameter	ACF_{gp} (eGFP)	ACF_{rd} (SNAP)
t_{D1} [ms]	118	48.9
a_1	0.542	0.489
t_{D2} [ms]	1.88	2.72
N	5.86	35.3
t_{R1} [µs]	8.7	4.7
a_{R1}	0.252	0.120
t_{R2} [µs]		181
a_{R2}		0.110
Derived Parameter		
B [kHz/molecule]	0.80	1.72
c [nM]	4.30	67.5

D



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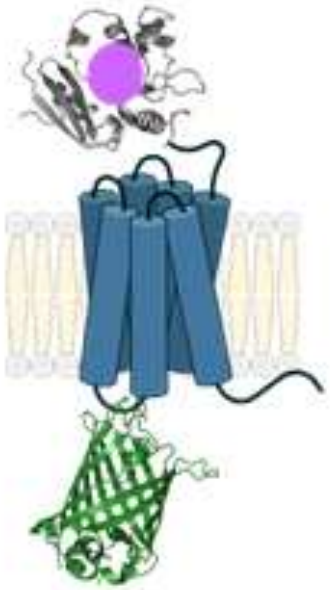
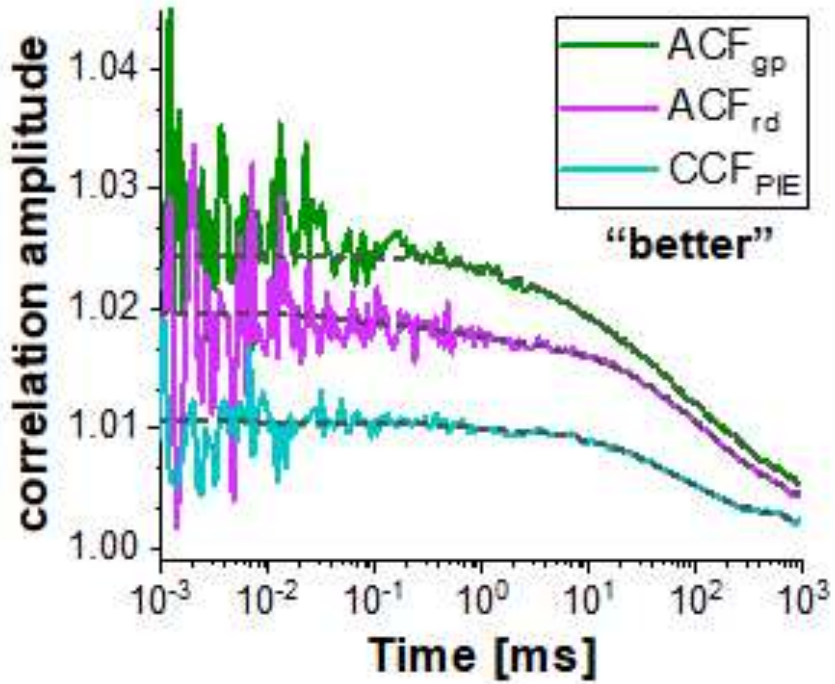


C

	"better"			"bad"		
Parameter	ACF_{gp}	ACF_{rd}	CCF_{PIE}	ACF_{gp}	ACF_{rd}	CCF_{PIE}
τ_{D1} [ms]	145	119	71.6	63.8	99.4	98.4
a_1	0.637	0.879	0.926	0.628	0.700	0.996
τ_{D2} [ms]	7.41	1.22	0.658	1.07	0.726	0.958
N	48.7	60.0	$1/G_0(t_c)$ 114	7.41	12.1	$1/G_0(t_c)$ 91.0
τ_R [µs]	---	86.0	---	4.3	---	---
a_R	---	0.063	---	0.150	---	---
Derived parameter						
c [nM]	182	118	115	5.44	23.2	3.41
ratio $G_0(t_c)$	0.43	0.53		0.08	0.13	
fraction co-diffusion	0.84	0.67		0.15	0.26	

B



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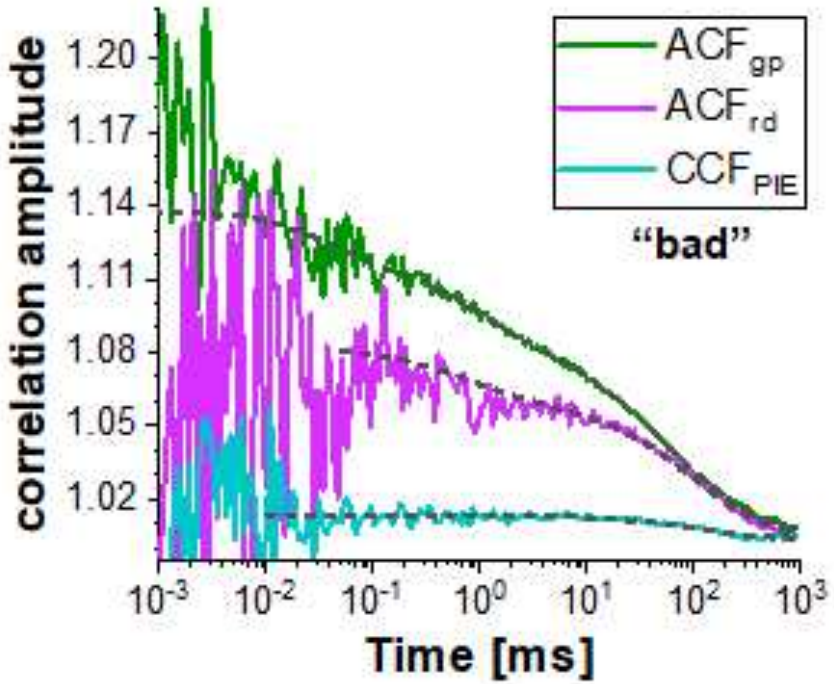

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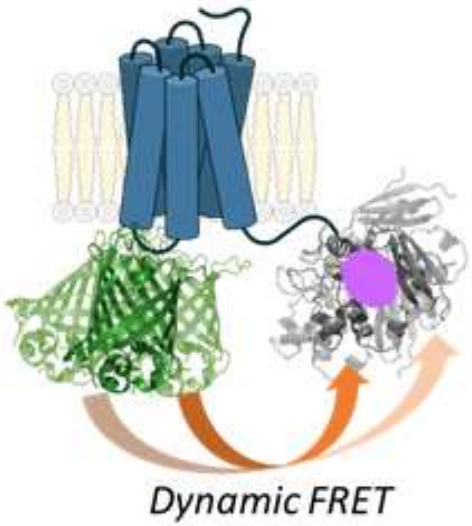
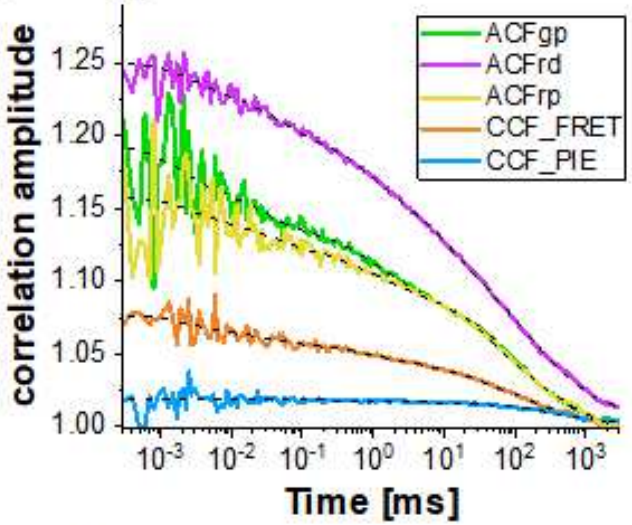


B β_2AR-IL3-eGFP-CT-SNAP

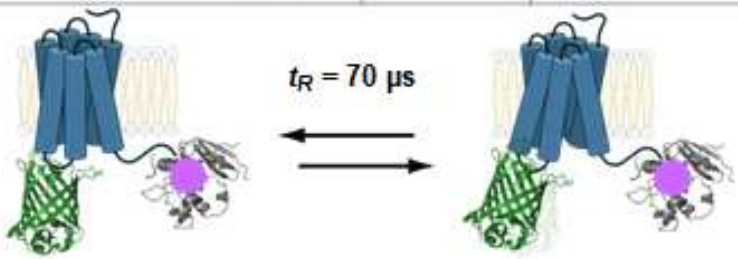


C

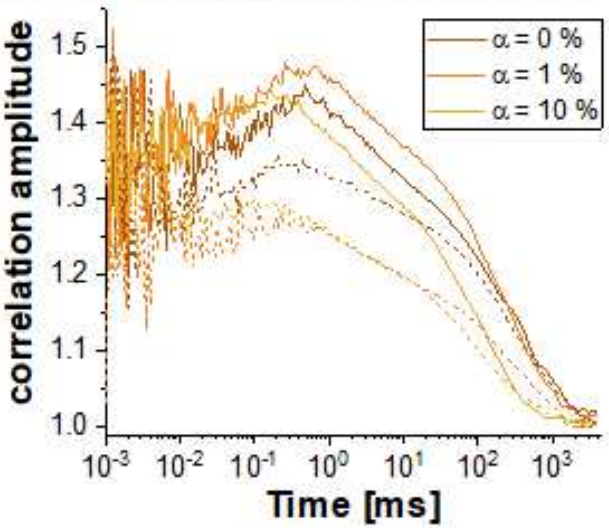
Parameter	ACF_{gp}	ACF_{rp}	ACF_{rd}	CCF_{FRET}	CCF_{PIE}
t_{D1} [ms]	117	329	372	310	341
a_1	0.608	0.727	0.637	0.731	0.896
t_{D2} [ms]	1.23	0.897	1.39	1.20	1.43
$N_{(app)}$	5.20	6.04	4.04	12.5	61.8
t_{R1} [ms]	---	51.6			---
a_{R1}	---	0.302	0.313	0.229	---
t_{R2} [µs]	37.1				---
a_{R2}	0.129	0.107	0.123	0.118	---
t_{R3} [µs]	3.06				---
a_{R3}	0.159	0.102	0.071	0.137	---

D Simulation of dynamic FRET

Parameter / FRET change	Crosstalk α [%]	Triplet
"Low FRET" (E = 0.2) <-> "High FRET" (E = 0.7)	0 – 1 –10	+/- 16 % at 5.5 µs
"Donly" (E = 0) <-> "Very High FRET" (E=0.9)	0 – 1 – 10	+/- 16 % at 5.5 µs



E moderate FRET contrast



F maximal FRET contrast

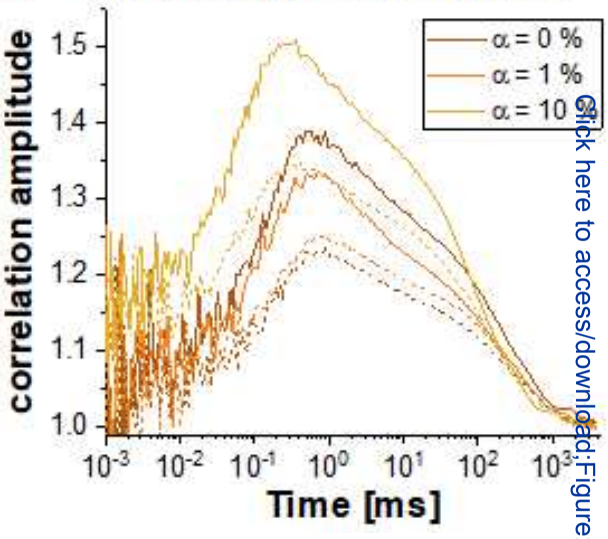
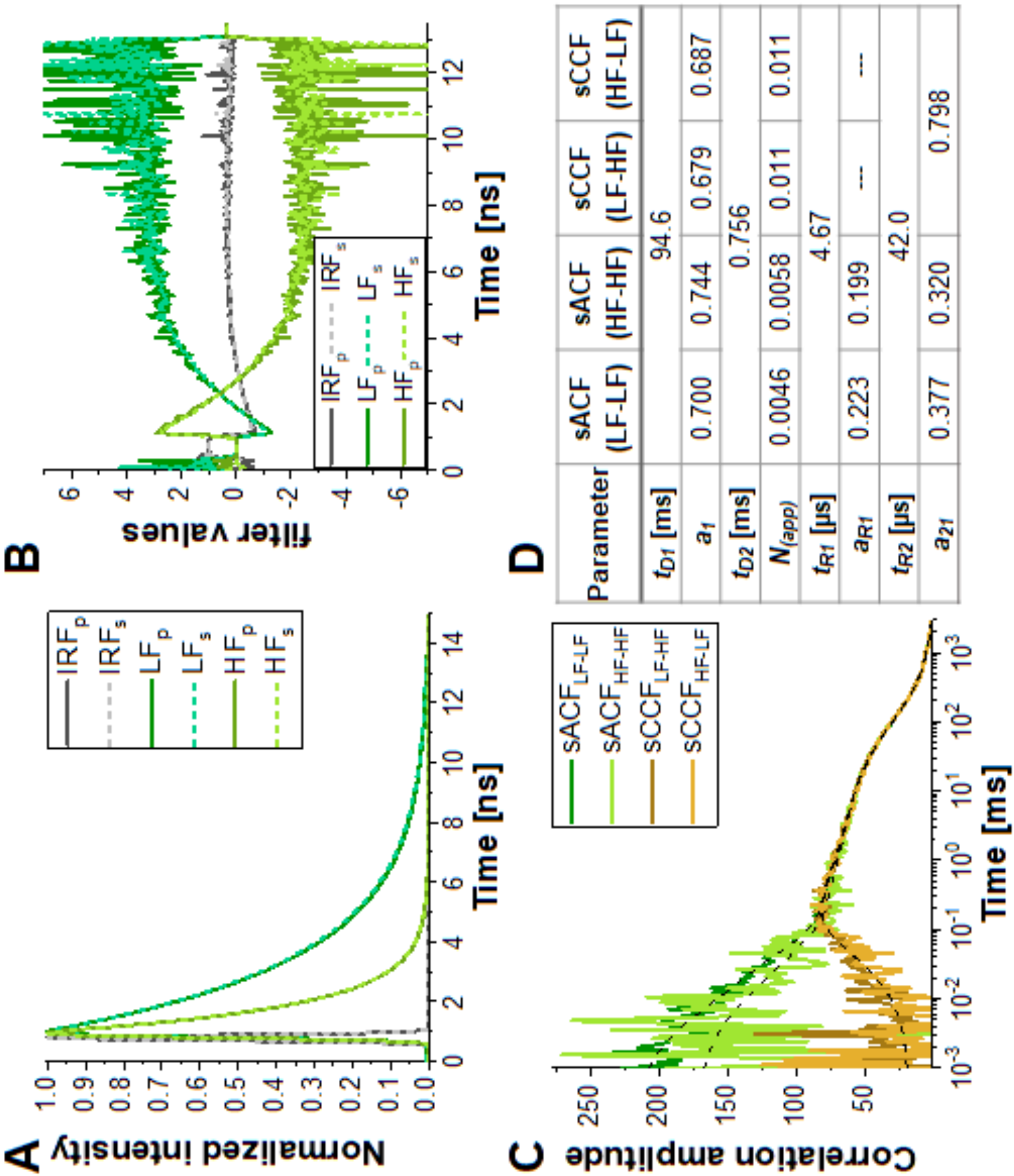
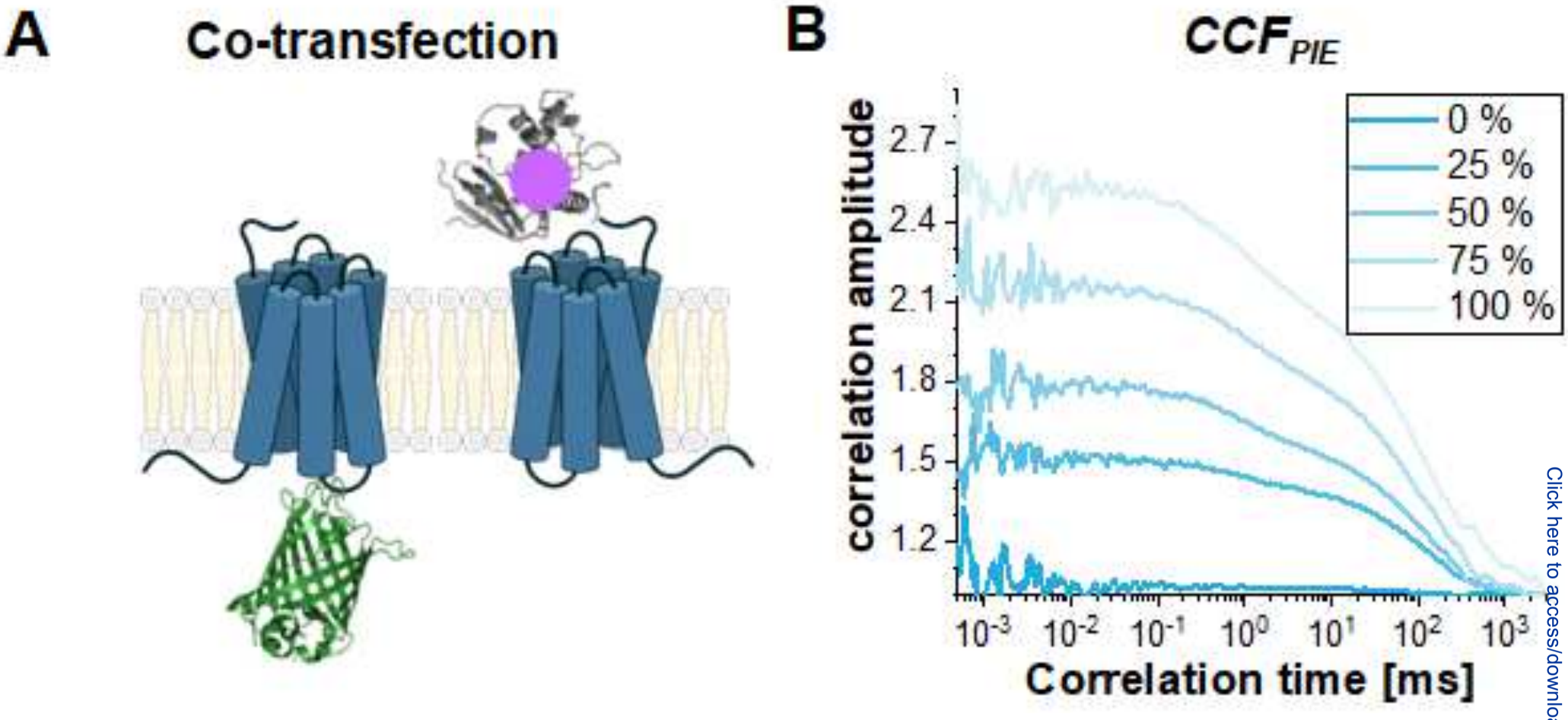


Figure 7

Click here to access/download;Figure;Figure 7.eps



Parameter	sACF (LF-LF)	sACF (HF-HF)	sCCF (LF-HF)	sCCF (HF-LF)
t_{D1} [ms]	94.6			
a_1	0.700	0.744	0.679	0.687
t_{D2} [ms]	0.756			
$N_{(app)}$	0.0046	0.0058	0.011	0.011
t_{R1} [µs]	4.67			
a_{R1}	0.223	0.199	---	---
t_{R2} [µs]	42.0			
a_{21}	0.377	0.320	0.798	



Table

Symbol
α
a_1
a_f
a_R
A488
A568
b
B
BG
c
CR
δ
D
$G(t_c)$
N
N_A
r_{GR}, r_{RG}
s
t_c
t_D
t_R
t_T
ω_0
z_0

Meaning (common unit)

crosstalk of the green fluorophore after green excitation into the red detection channels (%)

fraction of first diffusion component in bimodal diffusion model of membrane receptors

total amplitude of the anticorrelation term

amplitude of photophysics /triplet blinking

Alexa Flour 488, green calibration fluorophore

Alexa Flour 568, red calibration fluorophore

baseline / offset of a correlation curve

molecular brightness of a fluorophore ((kilo-)counts per molecule and second)

background (e.g. from an appropriate reference sample: ddH2O, buffer, untransfected cell etc.)

concentration

count rate (KHz or (kilo-) counts per second)

direct excitation of the red fluorophore after green excitation (%)

diffusion coefficient ($\mu m^2/s$)

correlation function

number of molecules in focus

Avogadro's number ($6.022*10^{23}$ Mol^{-1})

amplitude ratio of green or red autocorrelation function to the PIE-based cross-correlation function

shape factor of confocal volume element

correlation time (usually in millisecond)

diffusion time (usually in millisecond or microsecond)

relaxation time of photophysics (usually in microsecond)

relaxation time of triplet blinking (usually in microsecond)

half-width of confocal volume element (μm)

half-height of confocal volume element (μm)

Replies are written in blue.

Editorial and production comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have carefully checked the manuscript.

2. Please expand the Introduction include all of the following with citation:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

3. Please include citations in the introduction section.

We have extended the introduction section as requested and added citations.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Done.

5. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Done.

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

Done.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

8. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

We have reformatted the protocol section of the manuscript as requested.

9. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Hydraharp 400, ChiSurf , Excel, etc.

We have removed the commercial language and replaced e.g. by “data analysis software”.

10. Some of the analysis steps can be moved to a supplementary file.

We have moved few steps of protocol section to supplementary notes, e.g. the coverslip cleaning.

11. Please ensure the results are described in the context of the presented technique e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included. Please do not make points in the representative result section and use paragraph style instead.

We have extended the Representative Results section, also in line with the suggestions from the Reviewers, which points in the data analysis need to be clarified or making readers aware on possible (technical) artifacts.

12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

13. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

12-13: We have not taken any figures from any previous publication; all data are original and figures were created exclusively for this manuscript.

14. Please do not abbreviate the journal titles in the references section.

We have corrected the same in the revised manuscript. We have downloaded und used the Endnote-style provided on the JOVE website (filehttps://www.jove.com › files › JoVE).

15. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table in alphabetical order.

We have removed the symbols from the table and sorted in alphabetical order.

Changes to be made by the Author(s) regarding the video:

1. Please, increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.

We have added more figures in the written manuscript so that all figures shown in the video also appear in the results section of the written manuscript. However, in the results section more details are shown which are required for a full understanding and the requested discussion / classification of the results. In the video, we focus on the essence the figures as they will be shown for few seconds only.

2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol.

We have revised the protocol section of the written manuscript as requested above in the sections 4-8 and added the new voice-over to the video. However, according to the Comment 4 above at some points in the protocol we added some notes, which are crucial to follow the Protocol. In addition, one optional step, step 1.2.3, is not shown in the video but only present in the written protocol.

3. Please, ensure that the protocol subheadings are the same in the text and the video. Please also make a separate representative result section, which will contain all the figures associated with the result. Please make a separate conclusion section title card as well to be placed before the last interview.

We have split the content of the video in an experimental part, a data analysis and the results sections. Additionally, we have added section title cards.

4. Please, capitalize the first letter of every important word in the title of the manuscript.

We have changed the title accordingly:

Dual-color Fluorescence Cross-Correlation Spectroscopy to study Protein-Protein Interaction and Protein Dynamics in Live Cells

5. • Export the next version as 1920x1080, 4K is too high of a resolution

We have exported the video now with the required resolution of 1920x1080.

6. • At 2:00, 2:26, 2:30, 3:31, 8:54, 9:50 there are a few frames of black. They might be gaps in between the footage or an issue in the export of the video. Please either fill the gaps or double check the next exported version.

Done.

7. • Remove the windows task bar at the bottom of the screen capture footage.

Done.

8. • At 5:46 the on screen text graphic transitions out, then pops back in a second later. Please keep it on screen the whole time it's needed.

Done.

9. • At 7:34 there are two black lines on the bottom of the frame, please remove.

Done.

10. • At 10:19 there is a render issue where the lower right part of the screen "glitches" when transitioning from the computer monitor footage to the graphs/charts. Keep an eye on that for the next render.

Done.

11. • At 7:57 the voice over audio starts to sound compressed and echo-y. Please remove the effects on that audio.

Done.

12. • Remove the lab logo at the end of the video

Done.

Once done please ensure that the video is no more than 15 mins in length. Please upload the revised submission at https://www.dropbox.com/request/AzJMSp1stjeYnAdCSNAX?oref=e

Done.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The Jove article by Hemmen et al. is a protocol describing the use of dual-color fluorescence cross-correlation spectroscopy (DC-FCCS) to measure dynamics and interactions in cells. The article describes the theory and practical aspects of DC-FCCS and provides a software for the data evaluation. The article is clearly written and very useful for users of DC-FCCS but needs some clarifications.

We thank the reviewer for these positive comments. We agree that our article needs additional information / explanation for the readers. We would like to thank the reviewer for pinpointing where exactly additional content would make most sense. We have carefullly incorporated all the suggestions and answers to the queries of the learned reviewer. Wherever space was limiting in this short protocol, we have referred the readers to other specific sources if higher depth of information is required.

Please find below all the detailed answers and actions.

Major Concerns:

line 153: Please provide some more detail on the exporting option for Symphotime as either the full count rate trace as well as just the correlation functions can be exported. The Data format for ChiSurf seems to indicate a particular text format.

We have added a supplementary note (**Supplementary Note 3**) on how to export the required data using either Symphotime, Kristine or our self-written scripts. The data format for ChiSurf is explained on bottom of the 1st page of the supporting information concerning the step-by-step fitting with ChiSurf (now **Supplementary Note 4**).

line 176: If the systems are operated in PIE mode aren't the photons sorted according to excitation and emission? And I assume the correlation functions are corrected for background and after-pulsing by FLCS as the system is equipped with a TCSPC module? This could be explained here.

Yes, the system is operated in PIE-mode and like in any modern counting electronics with software-based correlation post-data acquisition, the TCSPC information is available and could be exploited. We are not doing this here for mainly two reasons:

1) Time: In our first draft of the manuscript / video we had included fitting of the fluorescence decay histograms and a detailed example on how to perform (at least) scatter-filtered FCS by exploiting the TCSPC-information. However, we had to realize that the maximum 15 min of video-material did not allow us to show this much of data analysis and that we had to cut ourselves short to the most important steps.

2) Readership: We would like this video to be helpful also for beginners in the field. The shown strategy in this video seemed us the easiest one, albeit encompassing all absolute essential steps to obtain reliable results.

We have now expanded the results and discussion section, in which we provide the readers with references to the more advanced techniques in FCS.

line 200: Can you actually guarantee a sample with 100% cross-correlation? This is a major assumption that should somehow be verified and problems if this is not true should be pointed out.

Yes, thanks for raising this important points. For synthesized DNA strands, we can assume nearly 100 % double labeling after annealing. This would translate into 100 % co-diffusion in our calibration experiment. However, in a dual color experiments this results usually in less than 100 % cross-correlation amplitude due to different sizes of focal volumes thus the incomplete overlap. In our setup, ~50 % (green ACF) - 70 % (red ACF) cross-correlation would reflect 100 % co-diffusion. This is a typical value. This means that if we observe 50 - 70 % cross-correlation in cells, the binding can be assumed to be “complete” (for 1:1 stoichiometry). We now mention this in the Discussion section of the calibration measurements.

line 223: eGPF is estimated to be only ~80% fluorescent, here also the assumption of 100% co-diffusion does not seem to be realistic. Corrections could be explained.

The reviewer is right that fluorescent proteins (FP) are usually not 100 % fluorescent due to their maturation process.

We now suggest the following correction in the Discussion section in the revised manuscript: If the value would be known exactly, the most straightforward correction would be to multiply *N* with (100 % / 80 % =) 1.25 and to use this value to determine $G_0(t_c)$.

However, even if this information is not available, this should not be overcritical here as the potentially reduced brightness or concentration of fluorescent eGFP would not change during the time course of the experiment, so that the maximal amplitude is not affected. Thus, we consider this sample as our "100 % co-diffusion" standard representing the maximal amplitude, which we can find in this described live cell system. Another source of bias is the high variability within live cell systems, where the fraction of fluorescent FPs might slightly differ from cell to cell. This would be challenging to decouple from general biological variances, which are the larger source of data uncertainty.

Line 236: The explanation of parameter a1 is missing. Perhaps also explain difference between 2D and 3D model? Readers might not be familiar with all FCS concepts.

Thanks for spotting the missing explanation of parameter a1. It describes the fraction of the first diffusion time of the bimodal diffusion model. We have added the explanation now and additionally added a note concerning the key difference between 2D and 3D diffusion models in the protocol, which we also explain in detail in the Representative Results section.

Line 258: The explanation why FRET should lead to negative cross-correlations is not clear. In principle, FRET leads to changed brightness of the fluorophores and thus to changed amplitudes overall (see Müller et al. Biophys J 2005). Could this rather be an issue of the detection card (see the same publication)?

We agree with the reviewer that the description / explanation of the source of the anticorrelated behavior needs adjustment, but also needs to be kept short to not cut any other essential information within the 15 min video time limit. I hope you agree that this section is improved in the revised version. To get an idea of the potential of correlation analysis and FRET we just briefly showed simulated data to give the reader a chance for general understanding and further reading if desired.

It is important to state, that there is conceptually no artefact to be expected as the biophysics of a fluctuating FRET state itself forces this anticorrelation as other groups have even experimentally verified. If a molecule switches between the low and the high-FRET (LF, HF) state at a timescale faster than its residence time in the focal volume, the photons coming from LF versus HF are anticorrelated as the molecule can only be in HF or LF, but never be in both at the same time. This is a classic case of anticorrelation. If there is no such thing as a FRET fluctuation between different FRET states like in DNA, meaning "stationary FRET", the anticorrelation vanishes and the curve is flat or "plateauing" at fast times (identical to the absence of FRET). Nevertheless, we have expanded the section about FRET-induced changes, complications (general brightness changes, triplet blinking, crosstalk, direct acceptor excitation. etc) and possible artifacts in the Discussion. We have added a CT-SNAP measurement now showing the absence of anticorrelation - although expected - in the CCF_{FRET}. We supplement these experimental results with simulations of different amounts of crosstalk, in the presence and absence of triplet blinking, and either small or large change in FRET efficiency to show the reader the influence of these parameters and the challenge in obtaining the anticorrelation (**Figure 6**). The new **Figure 7** shows how to "rescue" the anticorrelation term if the individual species analyze by species-filtered FCS.

Additionally, we make the reader now aware that at least two detectors of each color should be used to avoid after pulsing artifacts, while four detectors (two detectors of each polarization direction) would be required to also eliminate the effect of detector dead times.

Line 274: The triplet times are quite long, are these numbers reported correctly? See also Fig. 1 where at least the correlation function for A488 seems not to well fit the start of the function at short times.

The reviewer is right, Alexa488 triplet blinking is rather expected in the 2-10 µs range (e.g. Widengren 1995 JPhysChem).

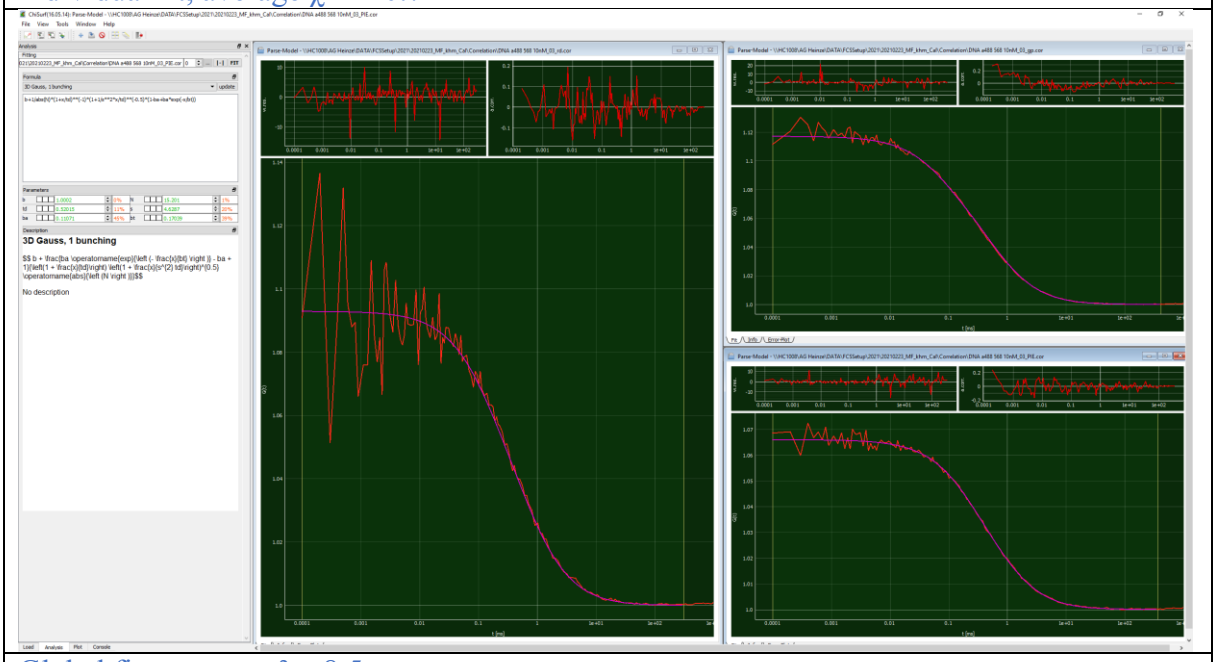
We have carefully checked our data again and have to admit that this it is a simple typo that we have corrected in the revised manuscript and recalculated respective results.

Fig. 2: Why is there a triplet time as long as 170 us in the CCF? The CCF should not contain a triplet time except there is cross-talk? But then the triplet time is independent of the focal volume so why is the CCF triplet twice longer than for the ACFs? Wouldn't that rather indicate that the times denoted as triplet times are rather another diffusive fraction? Note that I interpret tT as triplet relaxation time. This is not defined though in the list of symbols. Only tR is defined regarding photophysics.

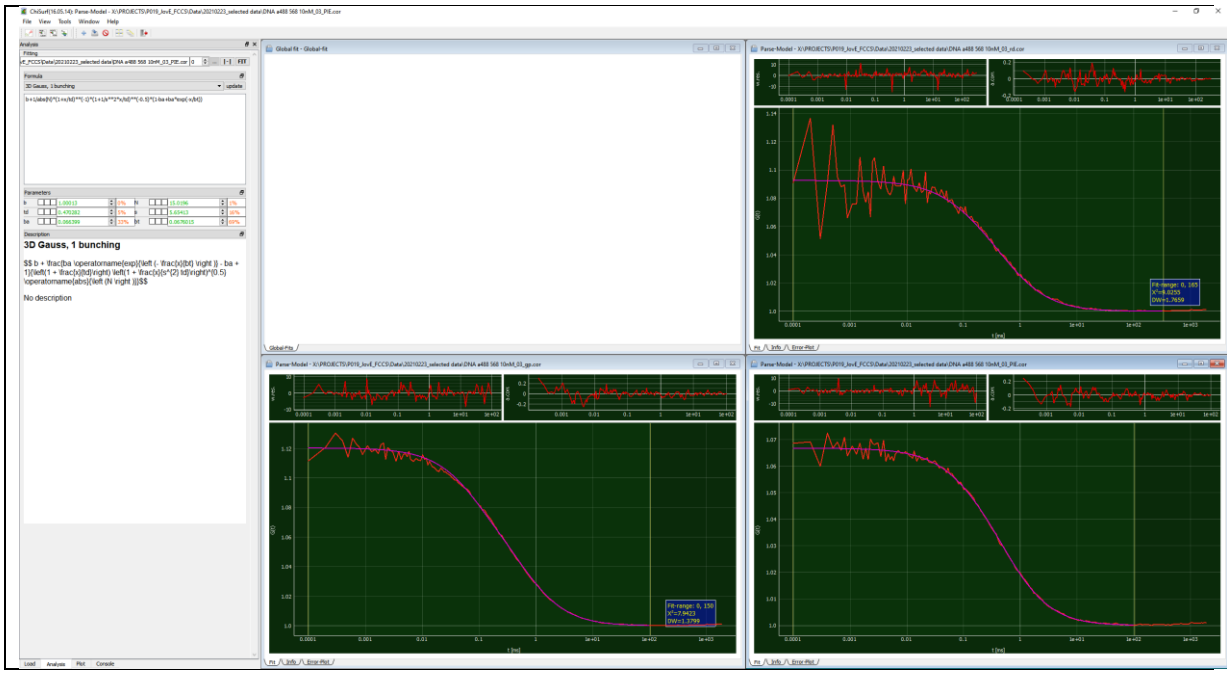
Thanks you for spotting that tR is missing so far in our list of symbols. Yes, by tT we mean the triplet relaxation time. We have changed here the tT to tR to not confuse the readers and make them aware of the possible sources.

We re-evaluated the fits for the relaxation time shown in the first version of the manuscript and tested the reviewers hypothesis that this constant should be independent of the focal volume if related to crosstalk, and thus identical for ACFs and CCF. The results are shown in the provided figure below.

Individual fit, average $\chi^2 = 10.7$



Global fit, average $\chi^2 = 8.5$



We doubt that the DNA strand shows multiple diffusion times as we excluded the presence of broken strands by agarose gel electrophoresis.

Consequently, we have repeated the data fitting routine with a global, linked tR and now obtained tR ~ 68 µs describing the data equally well when considering the reduced degrees of freedom. Thus, we follow the reviewer's thoughts and eventually decided to replace the fit result as shown consistent with the most likely scenario.

Line 280: By joint fit, do the authors mean global fit?

Yes, joint fit means global fit here. Both "joint" and "global" are been used frequently in this aspect so that we changed it along your recommendation but link the other term as equivalent to avoid readers confusion.

Line 322: Again the 100% co-diffusion is not a given as the authors already detect a possible free component in the red channel?

As we state also in the respective line "in an ideal case", we would like to make the reader first aware what would be expected and / or what the construct was designed for. In the next step, we then point to the "real case", where we have to consider the non-fluorescent fraction of eGFP (see your comment above), freely diffusing SNAP substrate and / or a low degree of SNAP labeling. We have extended and clarified this section in the Representative Results section.

Line 328: If the cell is suboptimal, do they have a better example as well?

Yes, we also have a better example; however, as JOVE guidelines encourage to also show "bad" results we decided to use this example as a showcase for a bad example along with a good example for comparison.

Reviewer #2:

Manuscript Summary:

The authors demonstrate and describe how to perform dual color FCCS in living cells, either by itself or in combination with FRET. Measurements are first performed on dye molecules in solution and then on a double labeled, double stranded DNA oligo in solution, followed by measurements of the transmembrane protein Beta 2 adrenergic receptor, B2AR, in living cells. B2AR is either fused to EGFP on the cytoplasmic side, or SNAP-tag labeled with alexa 568 on the extracellular side, or labeled with both of those two, or fused to EGFP on the cytoplasmic side as well as SNAP-tag labeled by alexa 568 also on the cytoplasmic side. When EGFP as well as alexa 568 are on the cytoplasmic side, dynamic FRET will occurr, which in principle can be analyzed by FRET-FCCS but instead the authors simulate such measurements and analyze the results. The manuscript is well written and easy to follow.

We would like to thank the reviewer for kind appreciation of our work.

Major Concerns:

I may have missed it, but I cannot recall that the authors comment on why they do not analyze any experimental results from FRET-fluctuations of the CT-SNAP and GFP-labeled protein. I assume that these data were too noisy or unsatisfactory in some other way. I realize that the authors themselves would have preferred if such data could have been analyzed, but nonetheless a short clarification regarding why that was not possible would be helpful for the reader.

We agree with the learned reviewer. We considered showing a promising example also for the CT-SNAP.

As the other reviewer requested as well, we have added an experimental example now for the CT-SNAP (**Figure 6B**) and show how to "rescue" the anticorrelation term with species-filtered FCS if the individual species can be characterized (**Figure 7**). Additionally, we have expanded the section about FRET-induced changes, complications (general brightness changes, crosstalk, direct acceptor excitation, triplet blinking etc) and possible artifacts in the Discussion making these measurements and explicitly their analysis challenging.

We hope that the Review Editor of JOVE allows us to add this additional information in written manuscript, as we can clearly not show all of this in the video.

Another general comment, which I am sure that the authors already have thought of, is that it would have been nice if cross-correlation had been measured of two interacting proteins on the plasma membrane, instead of detecting cross-correlation of a single, double-labeled protein. I know that FCCS analysis of protein-protein interactions in cells is not trivial, but again, just a short comment on why that was not done would be helpful for the reader.

Again, the reviewer is right, this would be great to show; however, as above this section was again omitted due to the strict length limit of the video given by the publisher. On the other hand, as we do not study Protein-Protein Interactions, thus, we do not have any plasmids which we could transfect and show this effect. We have extended the Representative Results section with the results with a simulation of two molecules with increasing co-diffusion fraction to show the reader what to look for in their data, which complications may arise and refer the readers to previous studies on Protein-Protein Interactions accessed with FCS.

We hope the reviewer find this extension sufficient.

Minor Concerns:

Row

54-55 This sounds like FCS can only study GPCR interactions.

We have extended the introduction section of the written manuscript as also requested by the Review Editor.

124 Incomplete sentence.

The complete protocol section has been rewritten now in imperative tense following the JOVE protocol template as requested by the Review Editor.

160 S should be 1/S

We would like to thank the reviewer to spot that typo and for checking the equations carefully. We have corrected it in revised manuscript. In the data analysis software, this was correct.

Figure 2 State the number of bp in the oligo in the figure.

The fluorophores are 40 bp apart. We have added the sequence in the materials list and added the information to the figure.

Figure 3b Write NT-SNAP in the figure.

We have added the construct names to the figure.

266 "exemplary the results"?

We have rewritten the sentence in less convoluted way.

275 The triplet decay time of 21 us seems too long, and in addition, the fit seems unsatisfactory in the range of a few us where the triplet time of alexa 488 usually is. This indicates that the component of 21 us is due to something other than the triplet process.

The reviewer is right, Alexa488 triplet blinking is rather expected in the 2-10 µs range (e.g. Widengren 1995 JPhysChem).

We have carefully checked our data again and have to admit that this it is a simple typo that we have corrected in the revised manuscript and recalculated respective results.

301 Which figure?

"Figure 3 shows exemplary measurements of the single-label constructs β_2AR-IL3-eGFP (Figure 3A), where the eGFP is inserted into the intracellular loop 3, and NT-SNAP-β_2AR (Figure 3B)".

We apologize but for whatever technical reason the "3" has disappeared from the JOVE-generated pdf-file. We spotted many mistakes, mainly missing references to figures, in the first version of the pdf. We thought we found all of them but apparently missed this one.

334-336 0.08/0.56=0.14, and 0.12/0.71=0.17. How did you get 0.13 and 0.26?

We apologize, and thank the reviewer for carefully checking all equations including the provided numbers. This number got wrong due to two reasons:

(A) "Copy-and-paste" mistake, on this measurement day $r_{RG,DNA}$ is in fact 0.51, and not 0.71.

(B) Wrong reference in the excel template: For both calculations of the fraction of co-diffusion the value of $r_{RG,DNA}$ was used instead of using the $r_{GR,DNA}$ for the calculation of the green molecules co-diffusing with the red molecules.

Single-labeled constructs

Sample	Average countrate [kHz]		Number of molecules	Mol. Brightness [kHz/molecule]	Approx. Concentration [nM]
	s	p			
eGFP	1.87	2.81	5.86	0.80	4.30
SNAP	21.1	39.6	35.28	1.72	67.47

Double-labeled construct: NT-SNAP & IL3-eGFP

Sample	Average countrate [kHz]		App. number of molecules	Approx. Concentration [nM]	ratio G_0(tc)	fraction co-diffusion
	s	p				
Green-prompt	3.16	4.85	7.41	3.56		
Red-delay	0.53	1.45	12.13	2.78		
PIE			91.0	0.52	0.08	0.15
				0.72	0.13	0.26

Example calculation:						
DNA -> 100 % co-diffusion						
$G_{0ACF,green}$ =	1		$r_{green,ideal}$=	0.5		
$G_{0ACF,red}$ =	0.8		$r_{red,ideal}$=	0.625		
G_{0CCF} =	0.5					
Cell sample shows					fraction co-diffusion:	
$G_{0ACF,green}$ =	0.12		$r_{green,cell}$=	0.167	33.3	%
$G_{0ACF,red}$ =	0.08		$r_{red,cell}$=	0.25	40.0	%
G_{0CCF} =	0.02					

Thus, we are obtaining a value of 0.15 for the fraction of eGFP-labeled molecules co-diffusing with the SNAP-labeled molecules and on the other hand, a fraction of 0.26 SNAP-labeled molecules co-diffusing with an eGFP-labeled molecule. We have corrected the mistakes.

There were a few more errors similar to the one on row 266, so reading thru the manuscript carefully is a good idea.

We carefully checked the manuscript to avoid any orthographic mistakes.

1 Coverslip cleaning for live cell FCS experiments

1.1 Goal

Cleaning of glass coverslips to minimize background signal

1.2 Comments

- Cleaning procedure must be performed under the hood
- Use safety gloves when handling chloroform
- Reuse chloroform and NaOH solution
- Prepare 5 M NaOH solution on ice
- Materials: ultrasonic bath, 2 glass containers, metal rack, small beaker, funnel, 25 ml glass pipet, glass petri dish
- Discard used chemicals according to the local regulations.

1.3 Cleaning Protocol

- Arrange single coverslips in metal rack

- Place glass container in ultrasonic bath, fill bath with deionized water
- Hang the rack into the glass container and fix with the loops

- **Fill the glass container with chloroform and sonicate for 1 hour**
- **Place the rack in an empty glass container and let it dry**
- Put on **safety gloves**!
- Pour the chloroform back into the bottle via a funnel by first scooping it with a beaker, then pipette the rest with a 25 ml glass pipette. Do **NOT** pour it directly out of the glass container! It will spill badly!
- Reinstall the rack in the glass container in the ultrasonic bath.
- **Fill the glass container with 5 M NaOH solution and sonicate for 1 hour**
- **Wash three times in ddH_2O in a second glass container**
- **Dry glass coverslips**
- **Store in 100 % ethanol in a glass petri dish**

- Pour the NaOH back into the bottle as described before. Clean pipette by pipetting some ddH_2O.

Confocal FCCS setup

A custom-built confocal setup based on an Olympus IX 71 stand (Olympus, Hamburg, Germany) equipped with a Time-Correlated Single Photon Counting (TCSPC) system (Hydraharp 400, Picoquant, Berlin, Germany) are used to perform FCS and fluorescence lifetime measurements. Two lasers (488 nm and 560 nm, PicoQuant, Berlin, Germany) are fiber coupled through a single mode fiber (PicoQuant, Berlin, Germany) and expanded via a telescope to fill the back aperture of the objective (60X, water immersion, NA 1.20. Olympus, Hamburg, Germany). A quad band beamsplitter (zt405/473-488/561/640 rpc phase r uf1, AHF, Tübingen, Germany) in the excitation path allows both 488 and 560 nm lasers through the objective to the sample. In the detection path a 50 µm pinhole (PNH-50, Newport, Darmstadt, Germany) rejects out of focus light before being projected on photon counting detectors (2x PMA Hybrid-40, Picoquant, Berlin, Germany and 2x APDs Perkin Elmer SPCM-AQR-14) by a telescope in a 4f configuration (focal length of lenses: 60 mm, G063126000, Qioptiq, Rhyl, UK). The beam was split via a polarizing beamsplitter (10FC16PB.3, Newport, Darmstadt, Germany) into parallel (detector 0 and 1) and perpendicular emission (detector 2 and 3) after the first lens of the telescope. A dichroic beam splitter (HC BS F38-573 Beamsplitter, AHF, Tübingen, Germany) in both parallel and perpendicular path allows the higher wavelength into the APDs (Red detection channels, detector 1 and 3) and reflects the lower wavelength emission into PMTs (green detection channels, detector 0 and 2). Band pass filters Brightline HC 525/50 and Brightline HC 600/52 (AHF, Tübingen, Germany) are used to reject unspecific light in each detection path.

Data export for PIE-based data analysis

Prerequisite for data analysis

In pulsed-interleaved excitation (ref PIE) experiments, the sample is excited by two subsequent laser pulses by two different laser. Commonly the first laser is lower wavelength ("prompt") and excites the green or donor fluorophore, while the second laser pulse ("delay") has a longer wavelength and excites the red or acceptor fluorophore. The timing between the two laser pulses is chosen such that the fluorescence lifetime of the fluorophore decays within their specific excitation window.

For a PIE-based data analysis, it is important to know the (time) distance between the two laser pulses and the time resolution, which with the data is saved, as in all data analysis software you will have to specify, which time range should be used in the correlation.

Up to five different correlation can be constructed:

- ACF_{gp}: Autocorrelation of the green channels in the "prompt" time window
- ACF_{rp}: Autocorrelation of the red channels in the "prompt" time window
- ACF_{rd}: Autocorrelation of the red channels in the "delay" time window
- CCF_{FRET}: Crosscorrelation of the green channels with the red channels in the "prompt" time window
- CCF_{PIE}: Crosscorrelation of the green channels in the "prompt" window with the red channels in the "delay" time window

Not all correlations are required for each sample.

Short list of available software

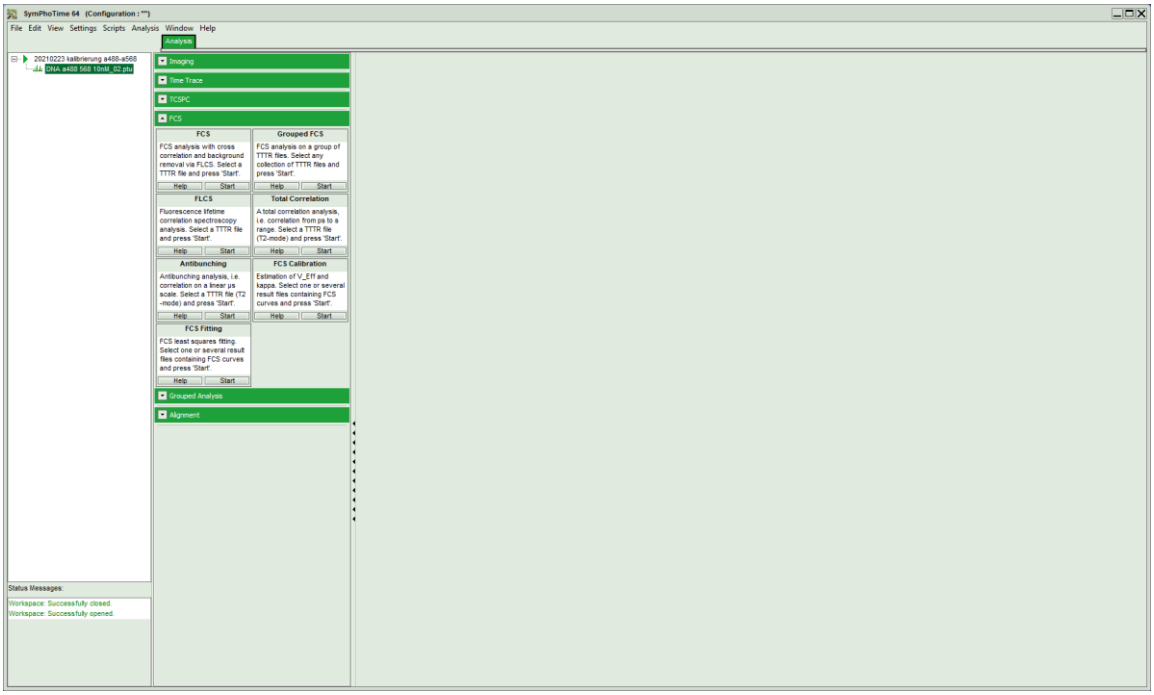
This list is not complete and only reflects the software we know of.

- Kristine (https://www.mpc.hhu.de/software/3-software-package-for-mfd-fcs-and-mfis)
- PAM (https://gitlab.com/PAM-PIE/PAM)
- Globals for FCS (https://www.lfd.uci.edu/globals/)
- Our self-written scripts for batch export (https://github.com/HeinzeLab/JOVE-FCS)
- Proprietary software from the manufacturer of the microscope, e.g. Symphotime (Picoquant).

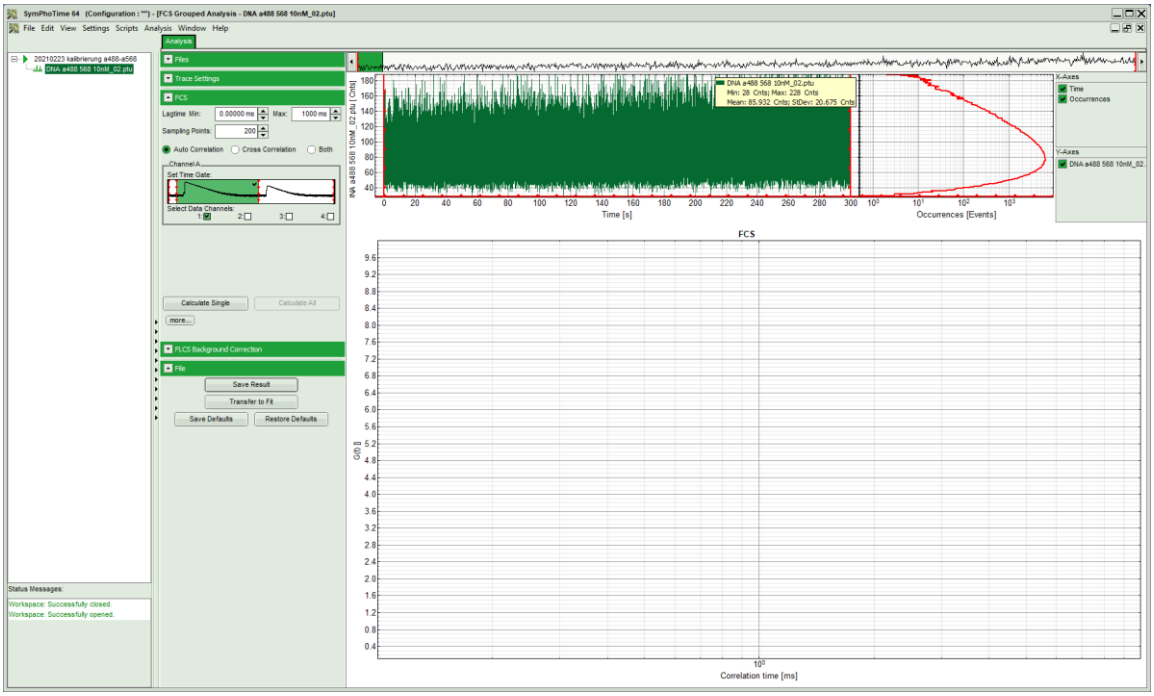
Example data export workflow

Symphotime

- Open the project file in Symphotime
    - Caution: The folder needs to contain the ending ".sptw" and must contain a file named "WSLogfile.sptl" to recognized as a project file.



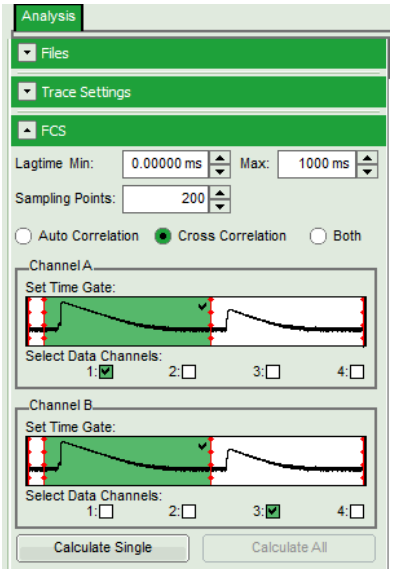
- Start a new "FCS´" and wait until it has opened the data:



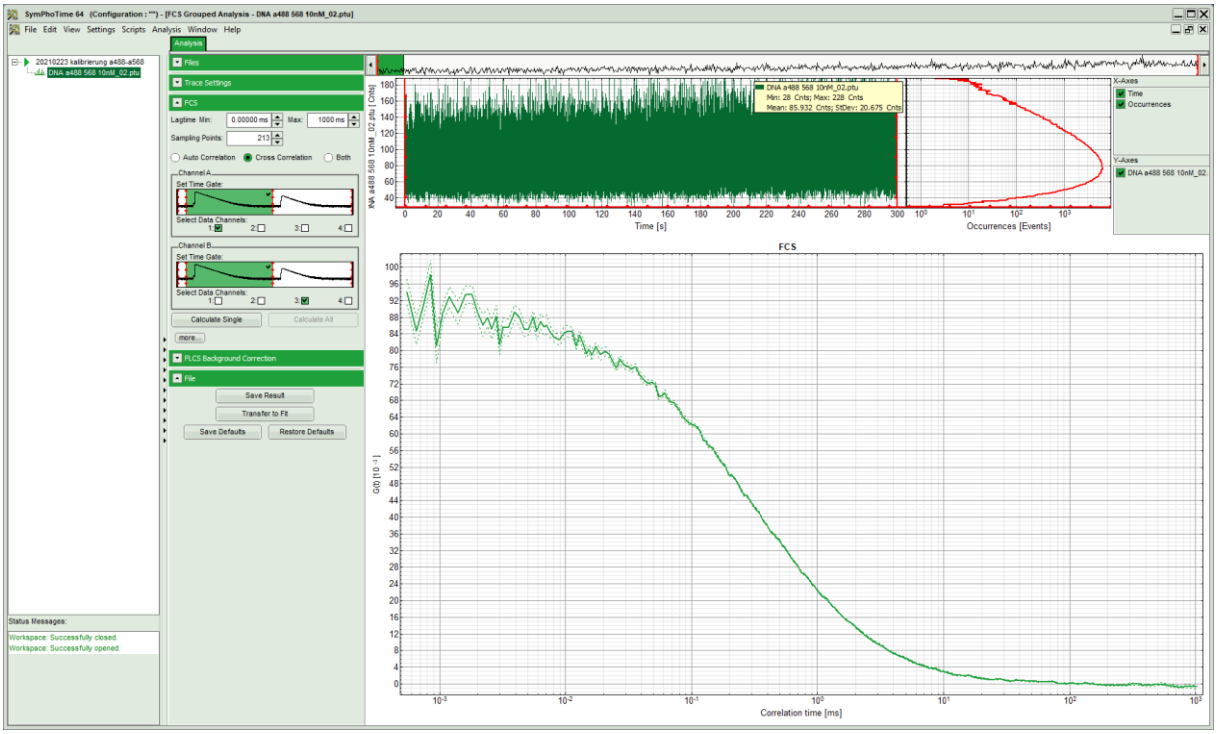
- In the top left corner the correlation setting can be determined:
    - Lagtime "min" and "max": a minimum of zero means it starts from earliest time point possible, i.e. the distance between two laser pulses.
    - Sampling points: the number of correlation point to be determined
    - Time window and channels selection:
        - Caution! Symphotime start counting the channels with 1 instead of 0!

- The green channels are 1 & 3 here and the red channels are 2 & 4.
- For the time windows, Symphotime determines them "automatically" and tends to remove some of the beginning, either do not move the red lines or take care to always move them to the same values/positions for all your data sets.

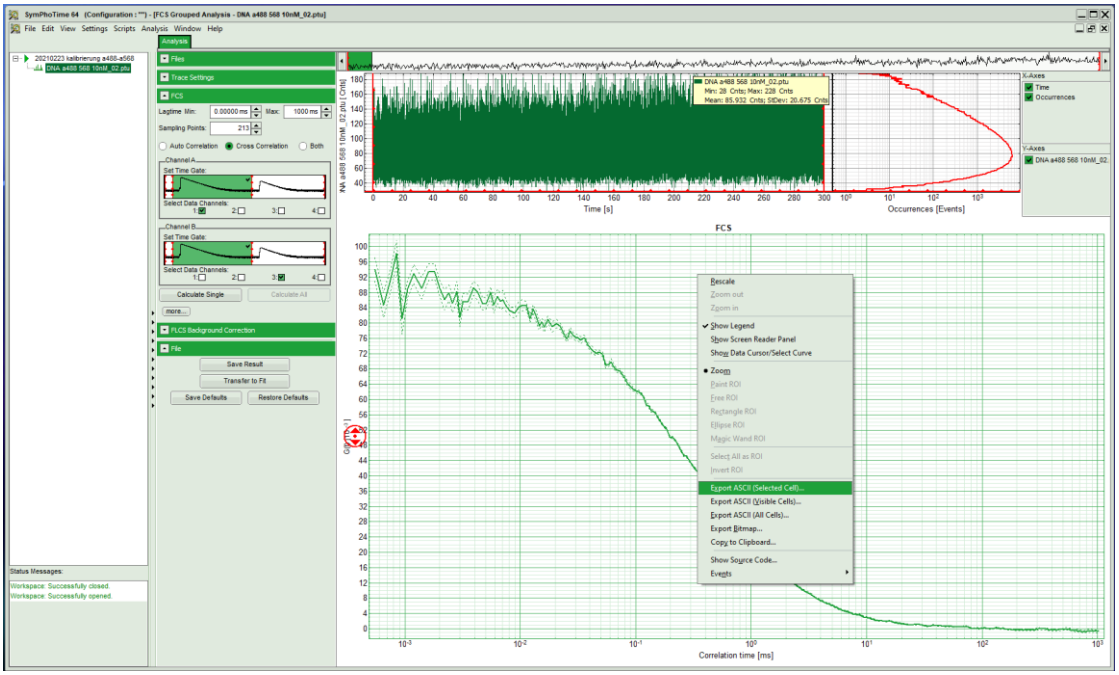
- For the autocorrelation of the signal in the green channels in the prompt time window, select "crosscorrelation" and tick the boxes of channel 1 and 3:



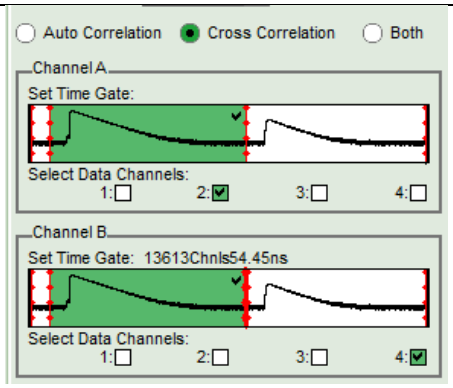
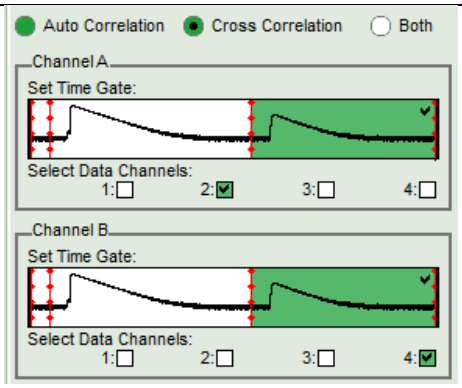
- Press "calculate single" and wait until the result is shown:



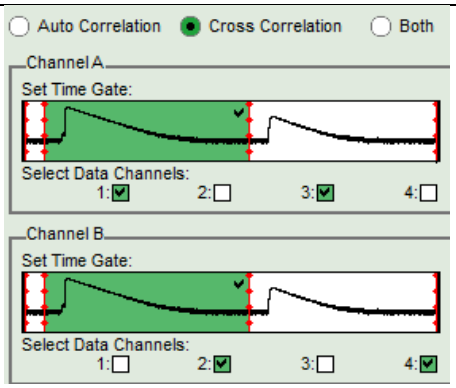
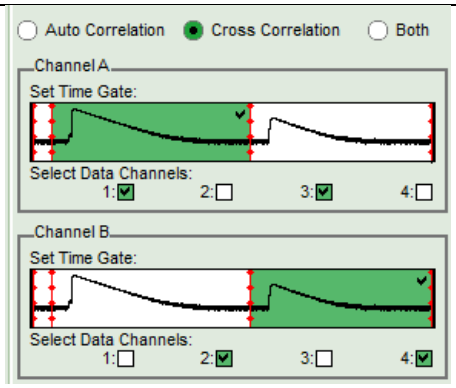
- Caution! Check the labeling on the y-axis, the correlation amplitude. Symphotime tends to report the data scaled by 10^3. You will need to divide the data manually by 10^{-3} to properly analyse the data in any other data analysis software than Symphotime.
- Export the data by right-click on the curve and select either of the ASCII export option (selected, visible, all cells)



- Proceed to calculate the other two autocorrelation functions:

Red signal in the prompt time window	red signal in the delay time window
	

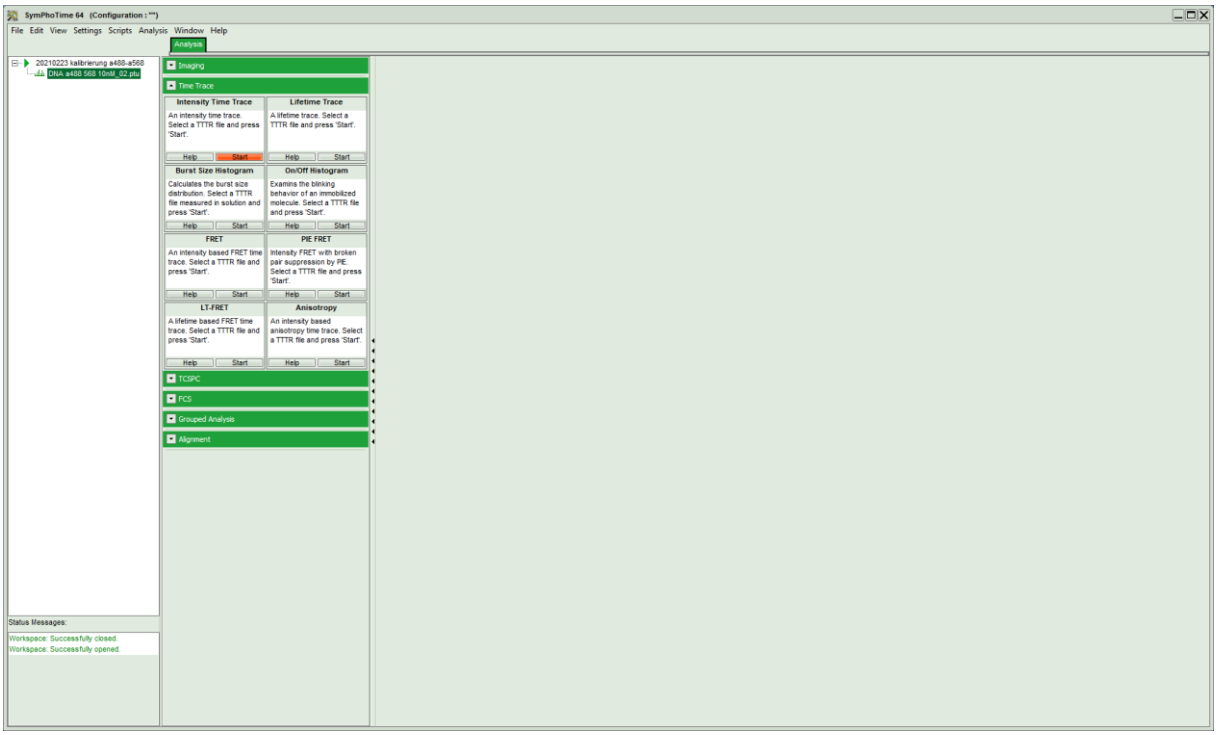
- Next, determine the two required crosscorrelations:

Green signal in prompt time window with red signal in prompt time window	Green signal in prompt time window with red signal in delay time window
	

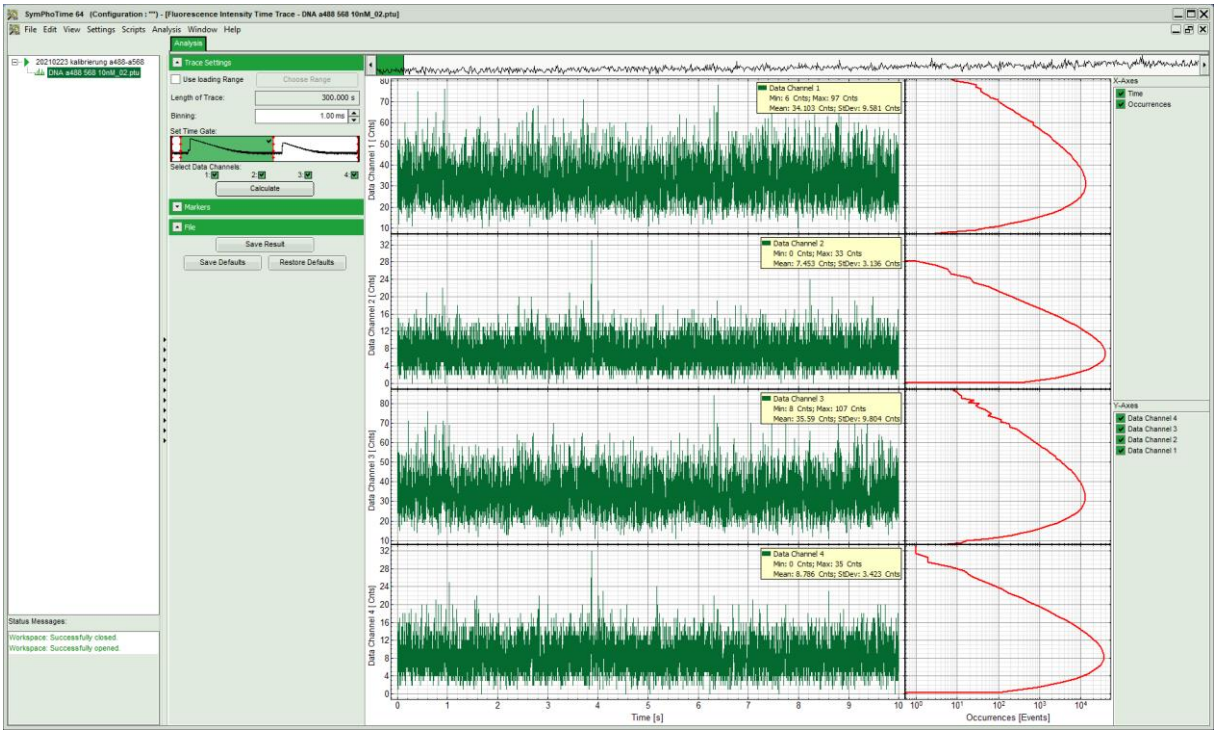
- Export all generated curves
    - For the data export, consider which software you would like to use for the fitting. Some software (Kristine, ChiSurf) might require a single
- Finally, determine the count rates for the different channels and time windows

- The "Header" file of your data set displays the average count rate for each channel, however, not separation between "prompt" and delay time window is made.

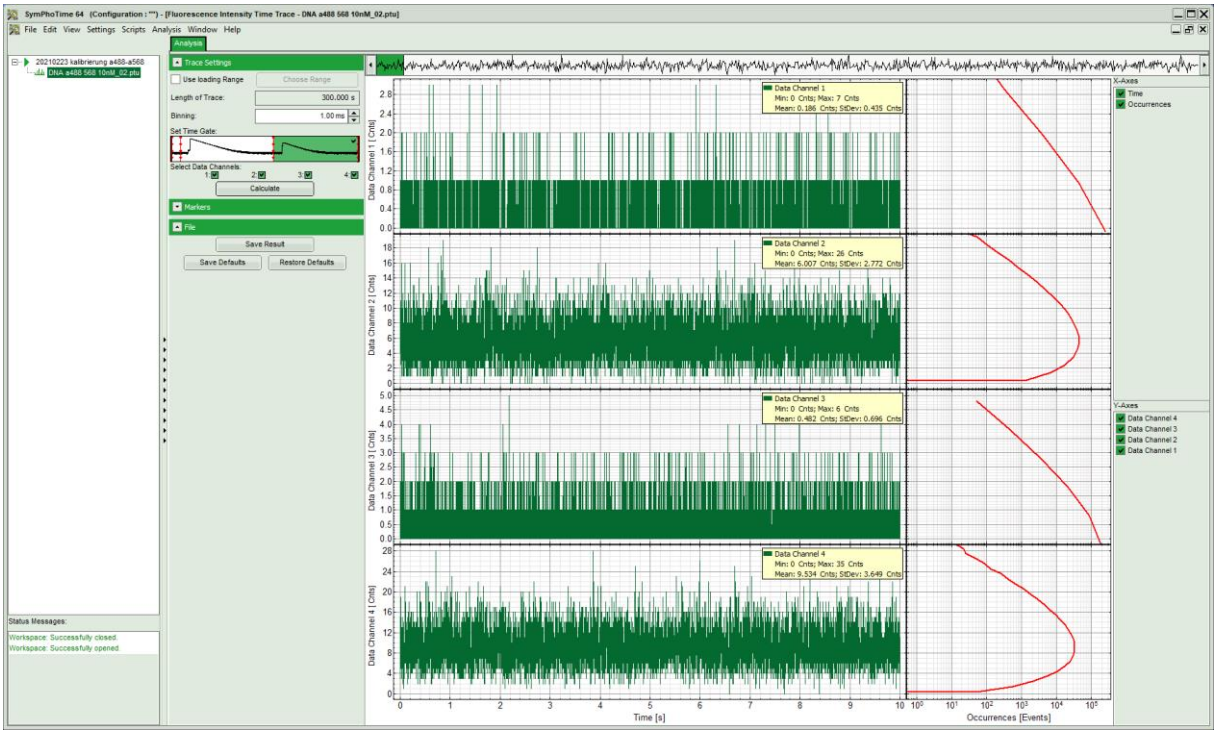
- Select "Intensity Time Trace" and press start.



- Select the "prompt" time window and leave all for channels selected and press calculate:



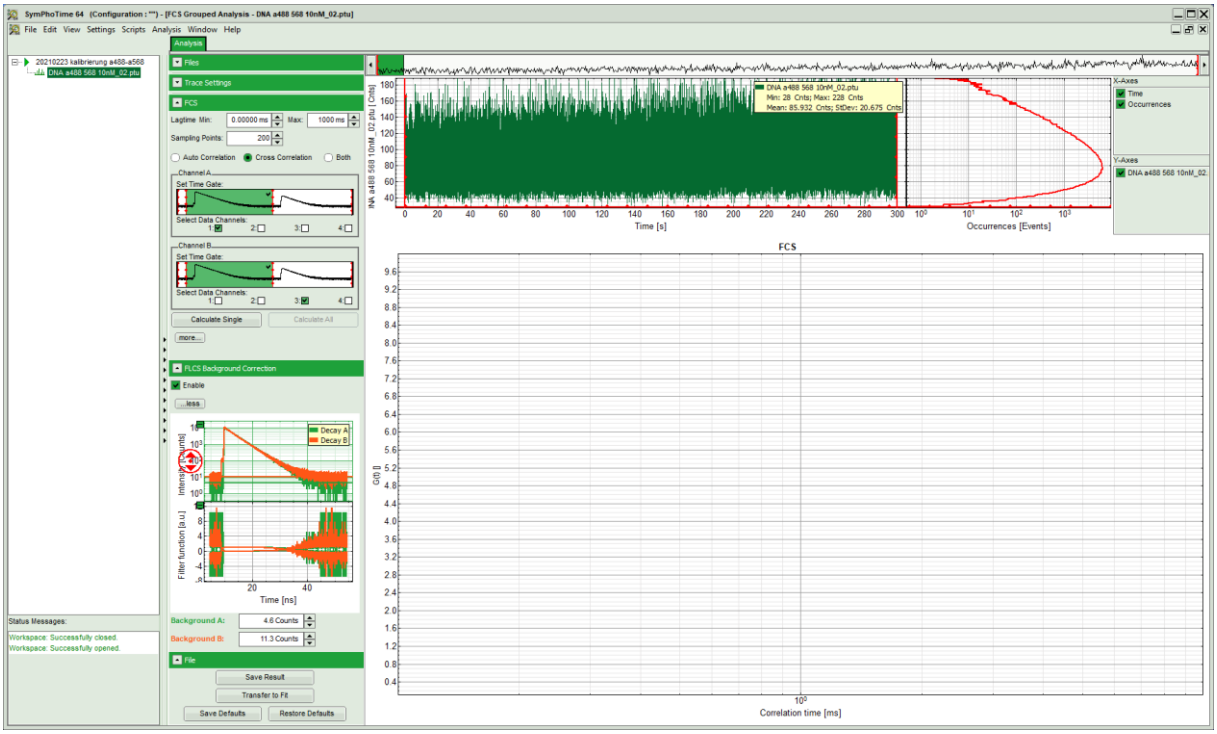
- Note down the mean values (and standard deviations, if required) in your protocol.
- Next, switch to the "delay" time window and repeat the calculation:



Advanced analysis: correcting for background

In cell measurements quite high background count rates might be reached compared to the relatively dim fluorescence of the fluorescent proteins. The Symphotime software offers an option to remove this background from your correlation.

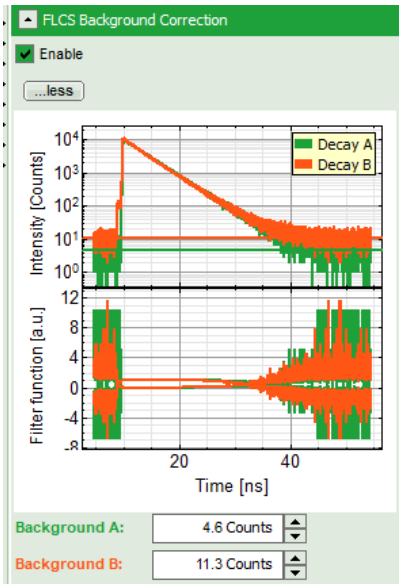
- Switch back to the “FCS” window and unfold the “FLCS background correction” option:



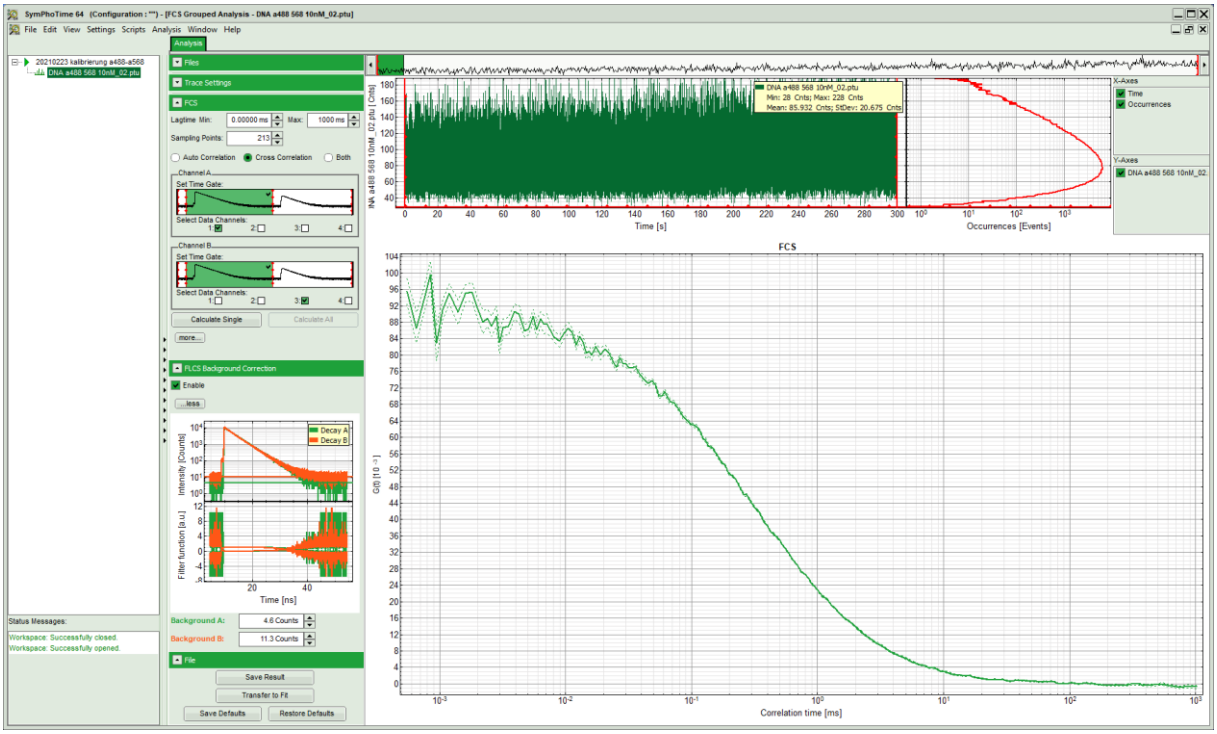
- In the bottom right, now two plots appear:
  - Top one shows the fluorescence intensity histogram on the nanosecond time scale

- Bottom one shows the weighting factor calculated based on these fluorescence intensity histograms. These weighting factors will be applied in the next step during the correlation.

- Adjust the background or offset of the fluorescence intensity histograms such that the horizontal green and orange lines run through the offset of the histogram:

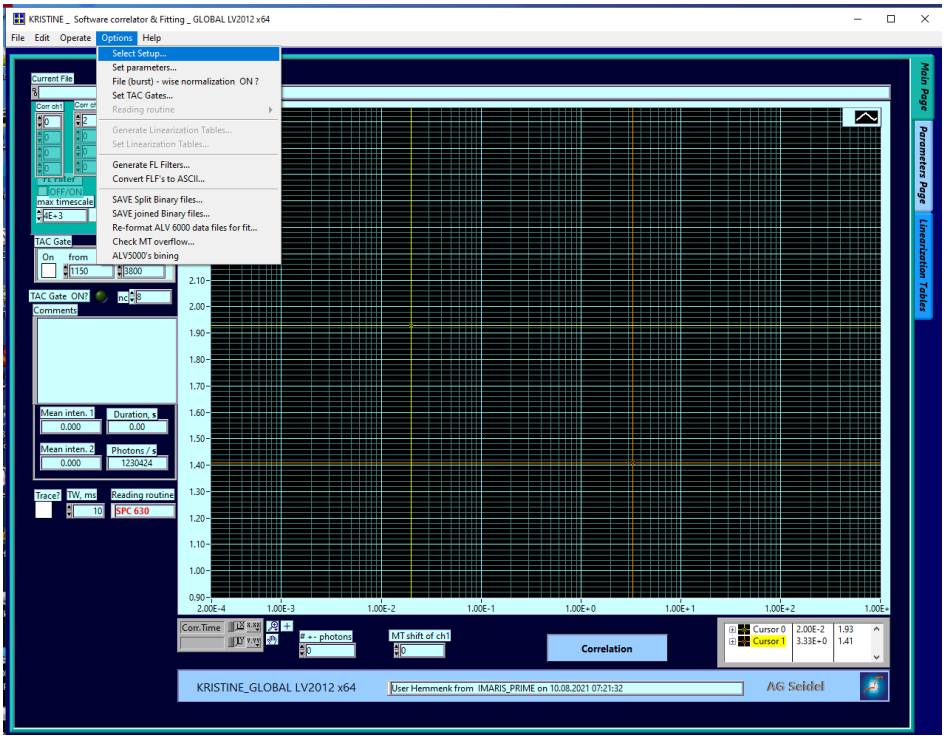


- Run the calculation by pressing “calculate single” and compare the results. For the shown DNA measurement, no big difference is visible. However, in cells with high background this analyse might improve the result.

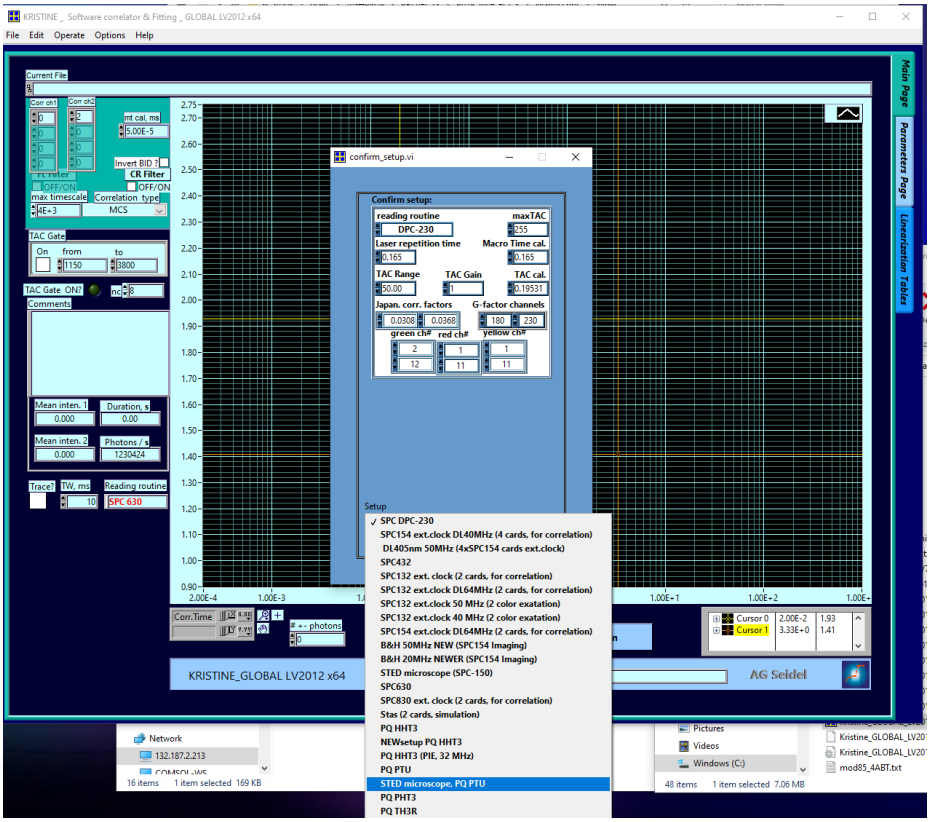


Kristine

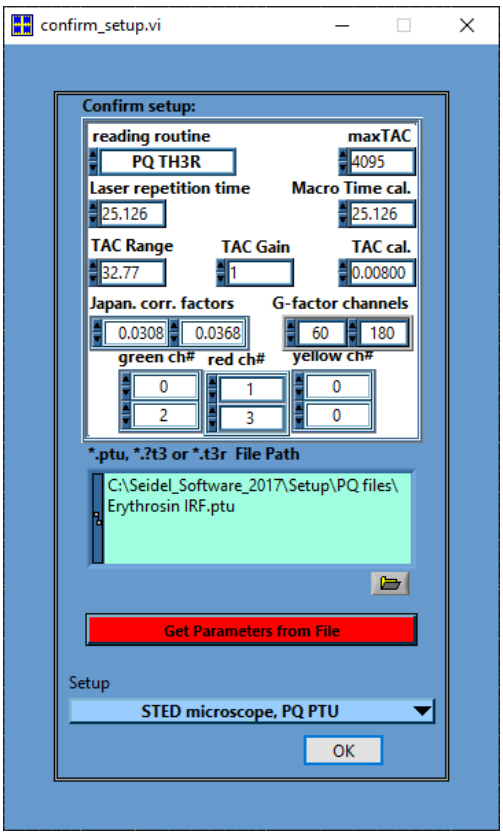
- Open Kristine_GLOBAL LV2012 x64_sf and select your data format via “Options” -> “Select Setup”

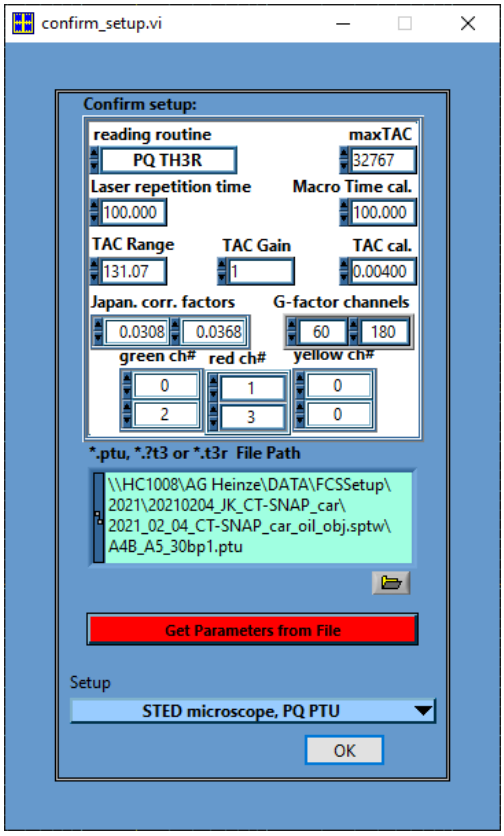


- In the newly opened window, select your data format from the drop-down list at the bottom

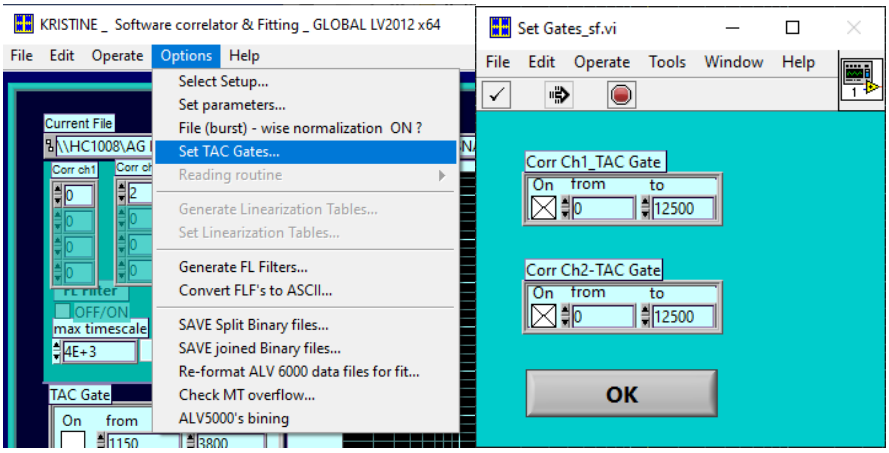


- Here, we select the “STED microscope, PQ PTU”. The name is not important, what matters is the reading routine: “PQ PTU” stands for Picoquant ptu- format.



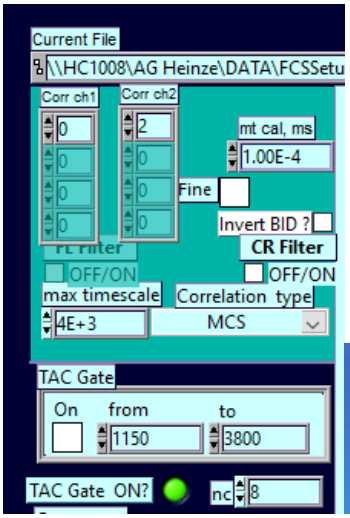


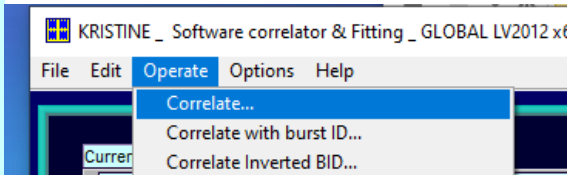
- Next, load an example file from your measurement day. This file s used to collect the header information about the measurement settings. Which channels were used? What was the repetition rate? What was the time resolution?
- Press “Get Parameters from file” and observe how the values change.
- Adjust the “Correction factors” and “G-factor channels”, if your plan to use the anisotropy information, usually not required in correlation analysis
- Adjust the channels numbers: Top row are the perpendicular channel numbers in green, red (and yellow) and bottom row are the parallel channels of the green, red (and yellow) detectors.
- Here, the numbers are correct.
- Press “OK” to save the settings and to return to the main window.
- Set your time gates for PIE correlation:



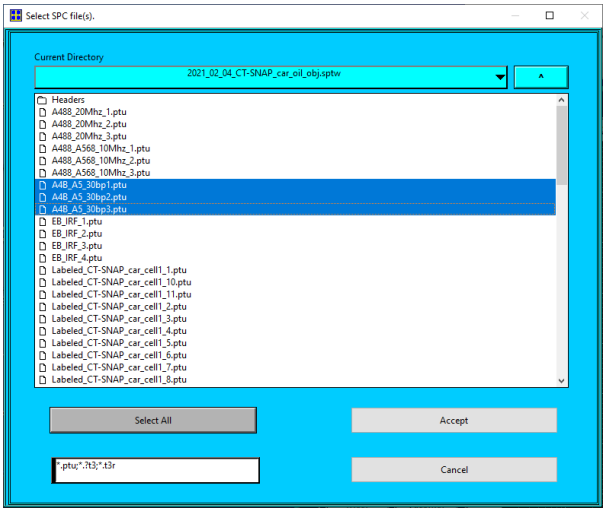
- In the shown example, the time window (TAC window) between two laser pulses of the same color is 100 ns, the base resolution is 4 ps, thus the data is distributed into in total 100 ns / 4 ps = 25’000 time channels. The first 12’500 channel represent the “prompt” time window, the second half the “delay” time window.

- Set the TAC gates for both correlation channels to 0 -12500, select the tick box and press ok.
- In the main window, set your correlation channels – we leave them at 0 and 2 for now, this will give us the autocorrelation of the green channels in the prompt time window.
- Additionally, you could increase the maximum correlation time, now 4000 ms, e.g. for slow diffusion molecules.
- Note: The bright green spot next to "TAC gate ON?" indicates that you will be using TAC gating during your correlation.

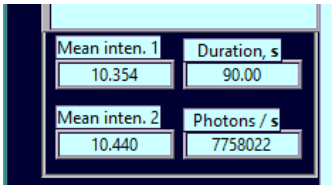


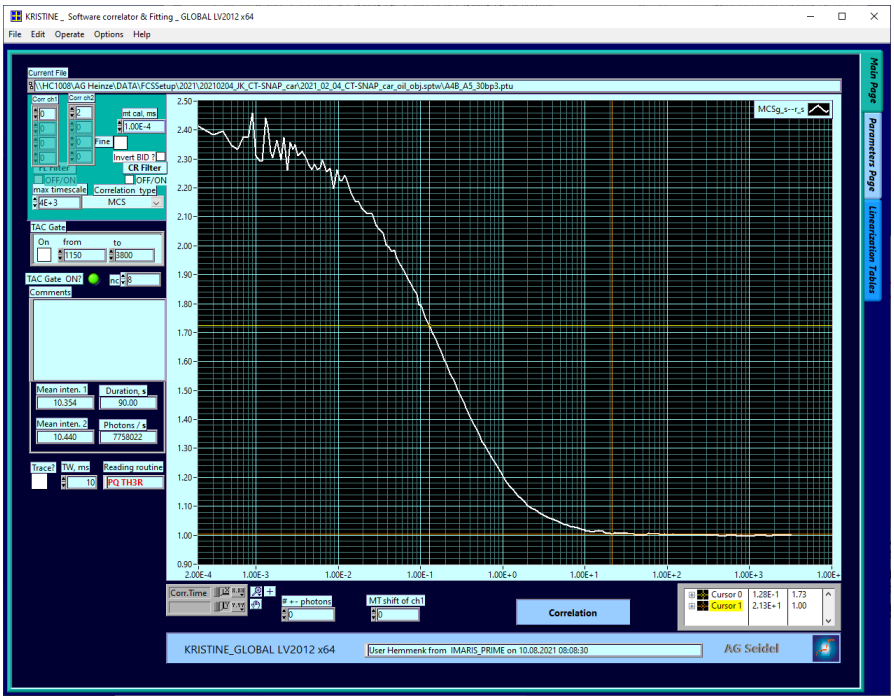


- Run the correlation via "Operate -> Correlate"
- Select your data to correlate in the file selection window:

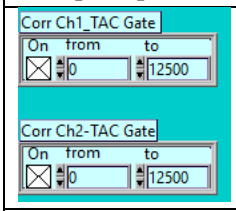
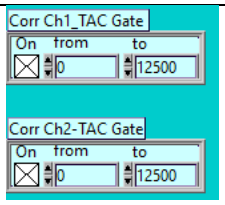
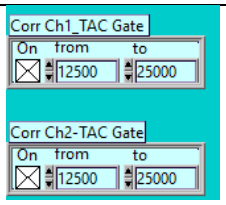
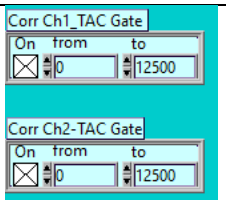
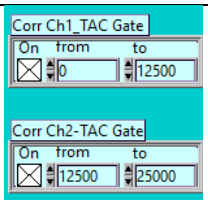
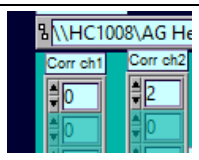
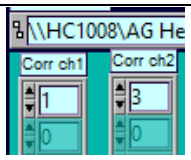
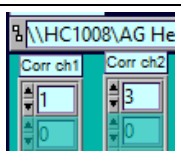
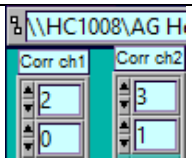
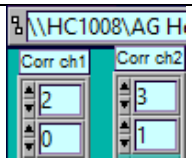


- You can select one or multiple files
    - Caution: multiple files will be correlated subsequently and averaged, thus they must belong to the sample.
    - For cell samples, it is recommended to first correlate each data set separately, then decide which correlations are "good ones", repeat the process with the selected measurements and obtain the average values.
- The average count rate per channel, the total measurement duration and read photons per second are summarized on the left-mid:

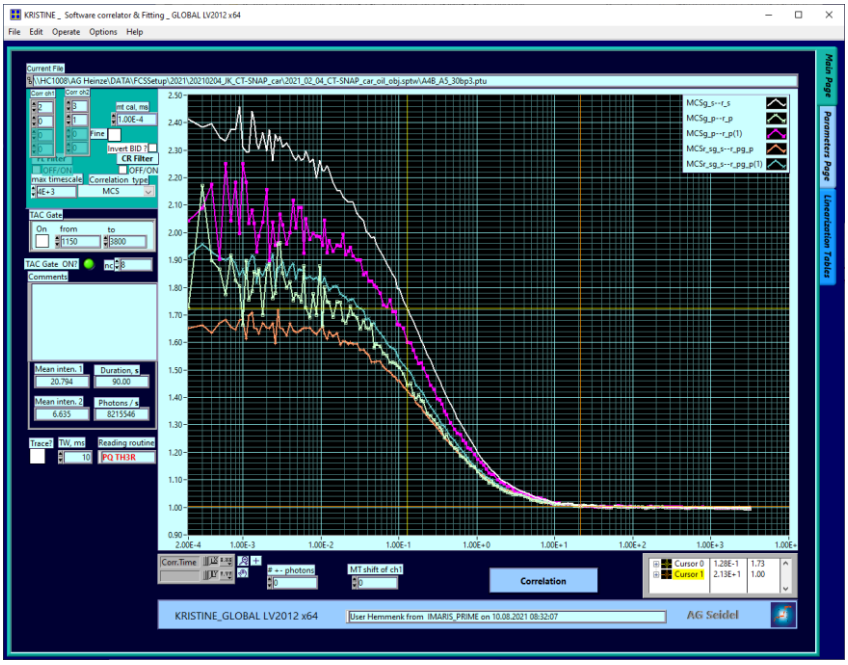




- Save the data by pressing “control + S” or via “File” -> “Save plots for fit”
    - Note: Kristine supports batch saving, thus it is most convenient to perform all correlations of sample first and finally save the data.
- In total, up to five correlations are required, the top row show the TAC gating setting, the bottom row the selected correlation channels.

ACF “green-prompt”	ACF “red-prompt”	ACF “red-delay”	CCF “FRET”	CCF “PIE”
				
				

- All five required correlations of a FRET sample:



- Save all curves and rename them if required.
- The exported .cor-files and .inf-files can be opened with common text editors, e.g. Notepad or Notepad++.
- The files contain four columns: (1) Time in milliseconds, (2) correlation value, (3) measurement time and average count rate and (4) standard deviation, if available, else this column is filled with zeros.
    - Note: In the absence of a standard deviation, the time and average count rate is used to estimate the weighting factors during the fitting

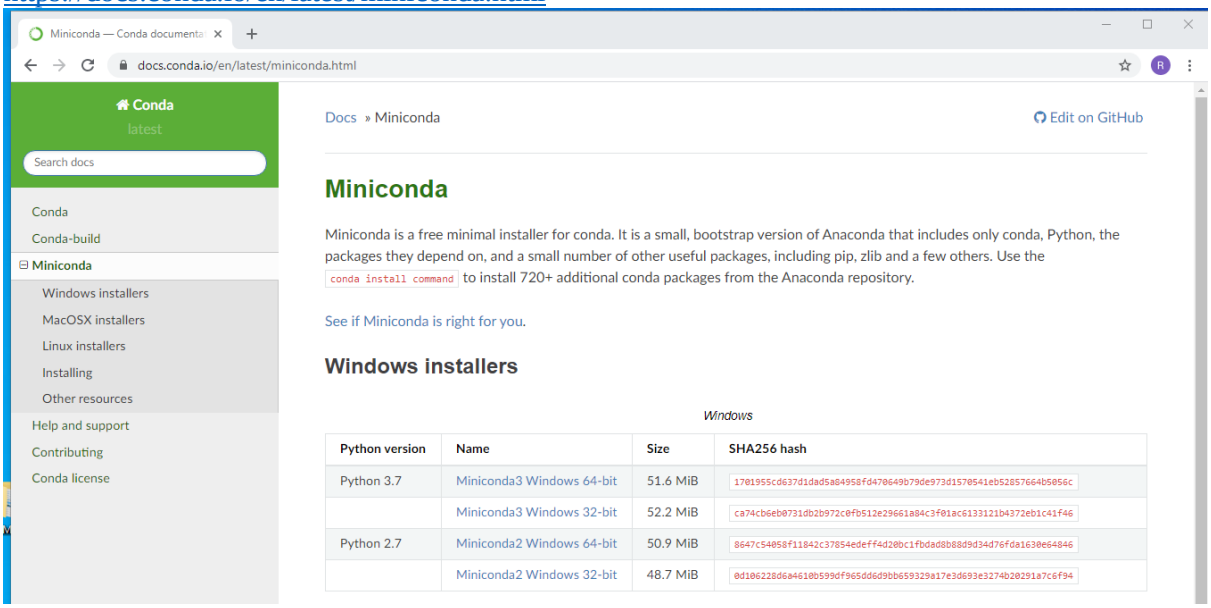
CCF_PIE.cor | ACF_rd.cor

```
1  0.000100000400→2.165642742129→89.999551096764→0.000000000000CRLF
2  0.000200000800→2.043236857090→3.461583932403→0.000000000000CRLF
3  0.000300001200→2.090316056636→0.000000000000→0.000000000000CRLF
4  0.000400001600→2.175058609139→0.000000000000→0.000000000000CRLF
5  0.000500002000→1.901999311684→0.000000000000→0.000000000000CRLF
6  0.000600002400→2.250385334222→0.000000000000→0.000000000000CRLF
7  0.000700002800→2.043236902496→0.000000000000→0.000000000000CRLF
8  0.000800003200→2.184474486110→0.000000000000→0.000000000000CRLF
9  0.000900003600→1.996157728938→0.000000000000→0.000000000000CRLF
```

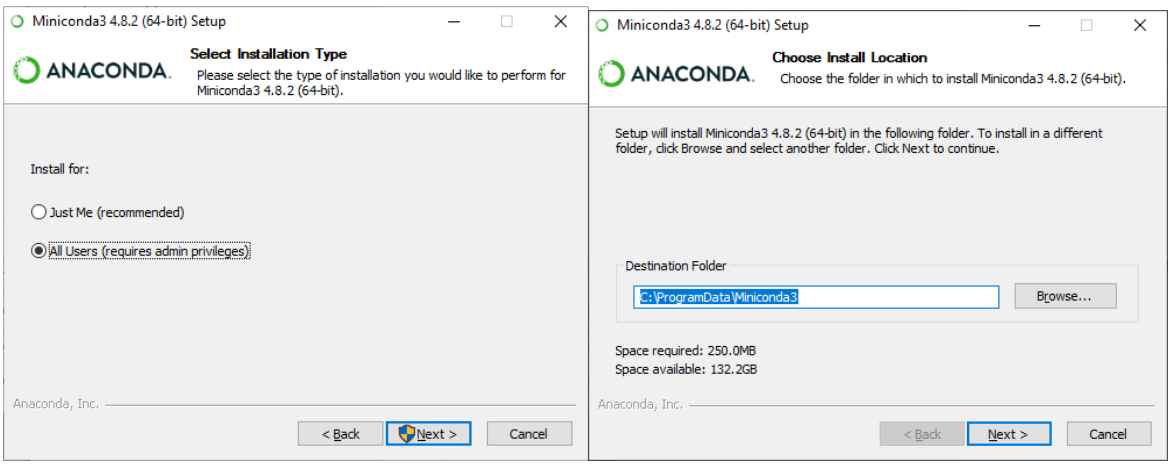
Python-scripts

Install Miniconda (Python3.7, usually 64-bit)

If you haven't done so, please install Miniconda first.
https://docs.conda.io/en/latest/miniconda.html

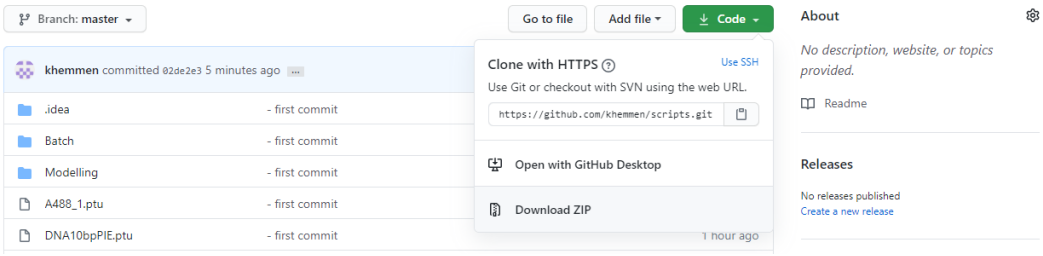


Install for all users:



Getting "scripts" from Github

Go to the following website and download the newest version:
https://github.com/HeinzeLab/JOVE-FCS



Best is to place the folder on "C:\Users\username"

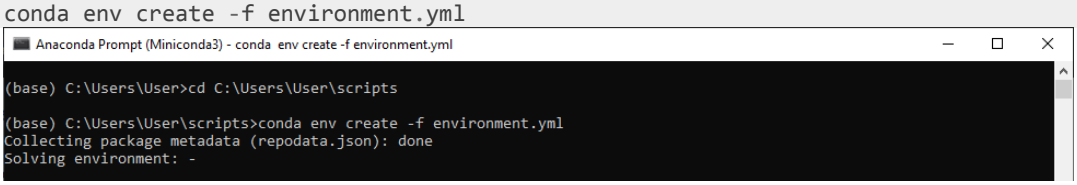
Generating the "scripts" environment

https://docs.conda.io/projects/conda/en/latest/user-guide/tasks/manage-environments.html
Option: "Creating an environment from an environment.yml file"
In case you closed the command window, open it again by typing into your windows search bar "miniconda".
Navigate to your folder by typing into the console:

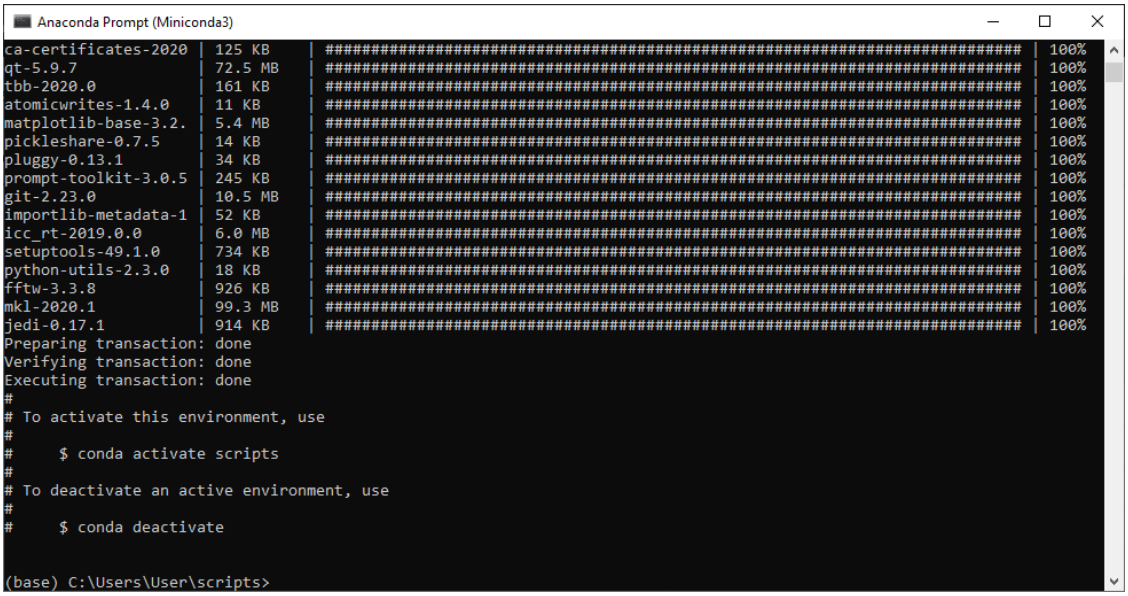
```
cd C:\Users\username\scripts
```

Generate the new environment named „scripts":

```
conda env create -f environment.yml
```



It will download quite some packages and dependencies.
When it is done, it will show the following:



Starting "scripts" environment

Activate the environment by typing:

```
conda activate scripts
```

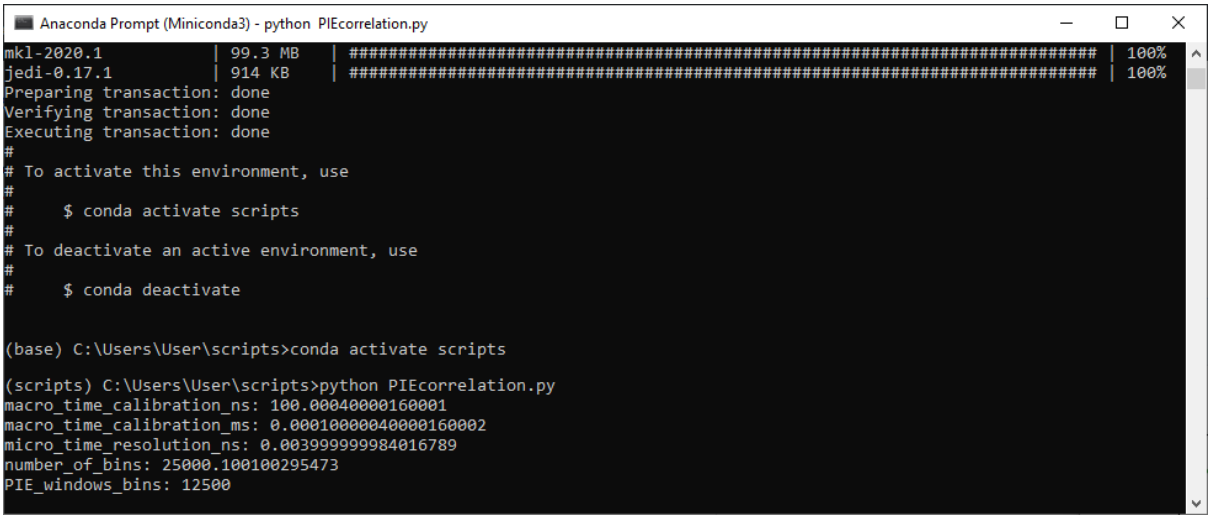
When you want to use "scripts" next time, you can directly start from section 4 using the environment activation.
Please make sure you navigate to the scripts-folder before trying to run any command.

```
(base) C:\Users\User > conda activate scripts
(scripts) C:\Users\User > cd C:\Users\username\scripts
(scripts) C:\Users\username\scripts >
```

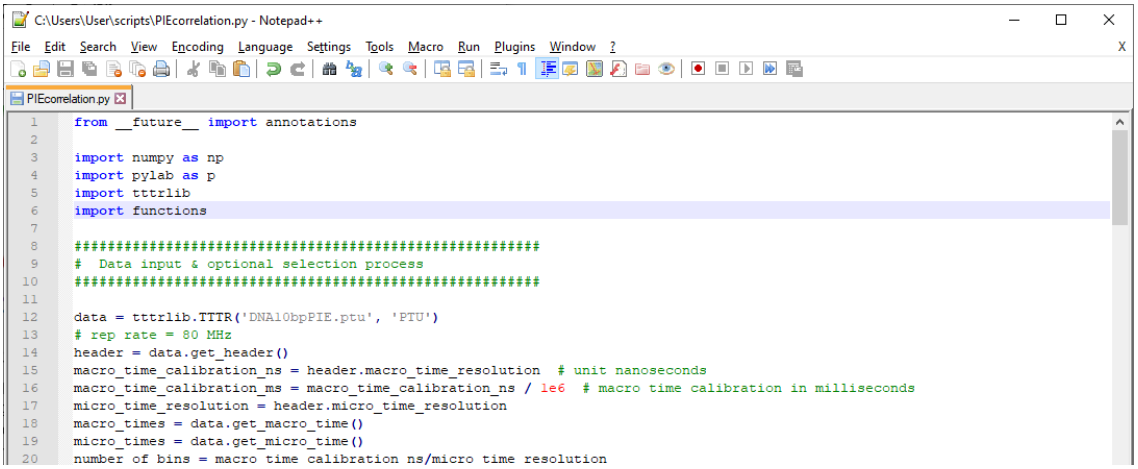
Running your first command

e.g. correlate a single data file, which had been obtained by a PIE measurement:
→ 2 channels from two colours each ("donor", green, and "acceptor", red), the two channels of a colour are arranged in two different polarizations "s" and "p", horizontally and vertically to the excitation laser beam.
The example data is called: DNA10bpPIE.ptu
Start the correlation by typing "python PIEcorelation.py"

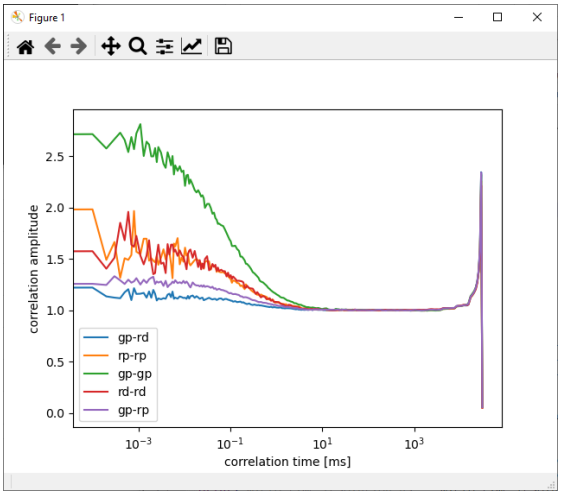
```
(scripts) C:\Users\user\scripts > python PIEcorrelation.py
```



You might want to check the script in Notepad++ (or any editor) to check it and to modify your filename (line 12):

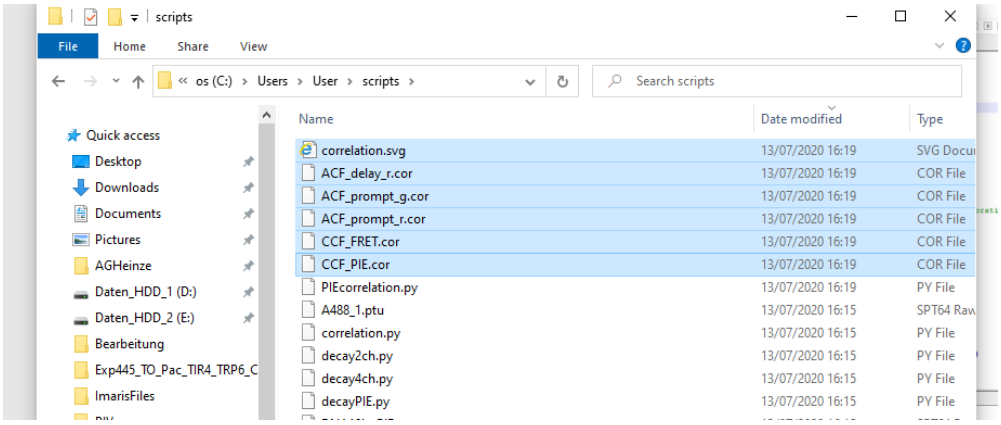


After the correlation is done, a new window containing the data plot is opened:



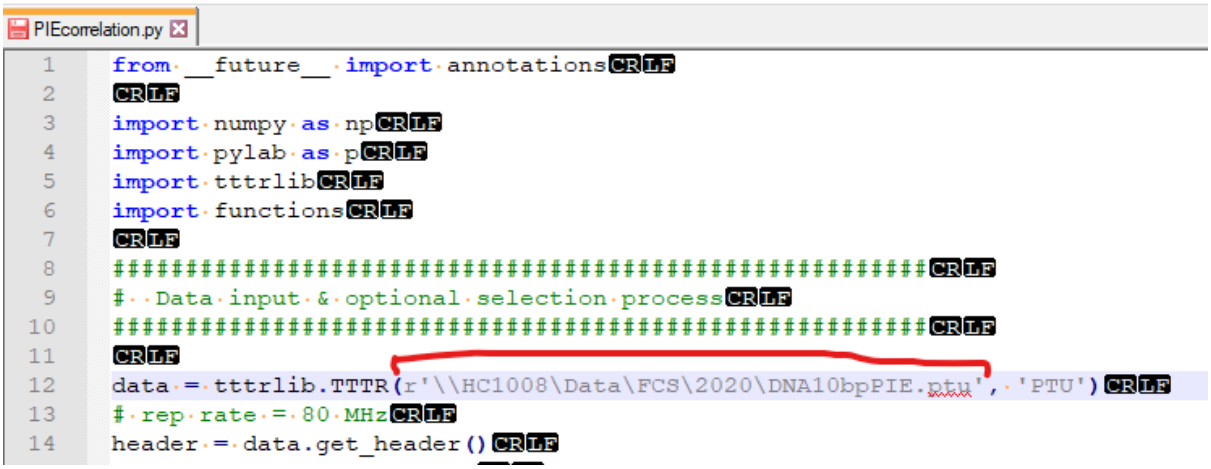
CLOSE this window to be able to proceed!

Additionally, the data is saved to your input folder:



To process a different data set change the filename in line 12 of the scripts.

As it is unpractical to copy all your data to your "C:\Users\User\scripts" folder, you can replace the filename by a data path:



Batch correlation of whole folders

If you have many data files, which you all would to process the same way, you can use the scripts in the "batch" folder. These are made for batch processing.
Navigate to the "batch folder" in the console:

```
(scripts) C:\Users\user\scripts > cd C:\Users\user\scripts\batch
(scripts) C:\Users\user\scripts\batch
```

This scripts consist of two parts:

- your python script, which does the job and
- your "settings" file, which is a so-called YAML-file. This can also be edited in Notepad.

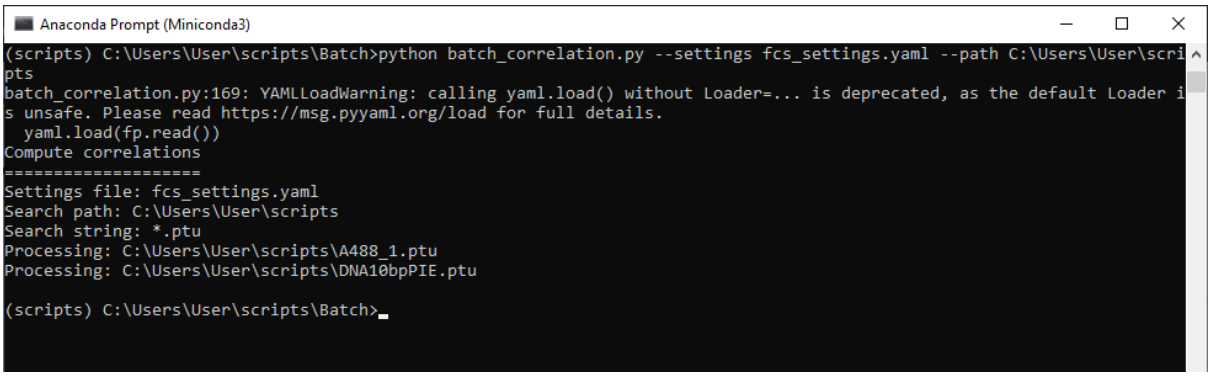
For correlating a folder of PIE data sets open the "PIEcorrelation_settings.yaml" file in Notepad and adjust your settings as required:
i.e.
- make sure the channel definitions are correct
- adjust the naming suffix for autosaving
Don't forget to press save after made your changes!

PIEcorrelation.py | PIEcorrelation_settings.yaml

```
search_string: '*.ptu'
filetype: 'PTU'
PIE_suffix: '_PIE.cor'
FRET_suffix: '_FRET.cor'
green_prompt_suffix: '_gp.cor'
red_prompt_suffix: '_rp.cor'
red_delay_suffix: '_rd.cor'
PIE_number_ch1: [0,2]
PIE_number_ch2: [1,3]
green_channel_ch1: [0]
green_channel_ch2: [2]
red_channel_ch1: [1]
red_channel_ch2: [3]
make_plots: true
display_plot: false
```

Now switch back to the console and type:

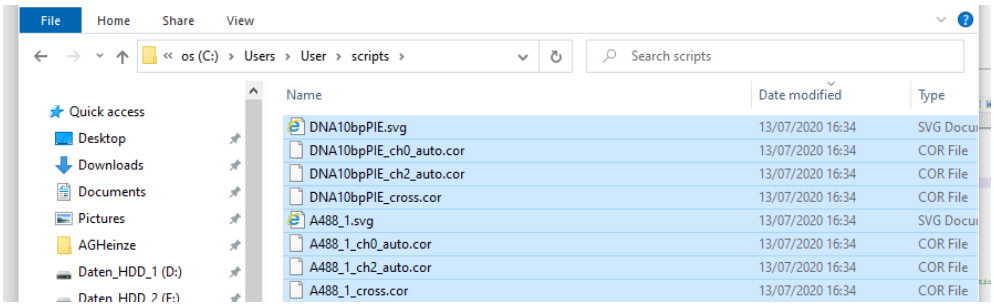
```
(scripts) C:\Users\user\scripts\batch > python batch_correlation.py --settings
PIEcorrelation_settings.yaml --path "\\HC1008\Data\FCS\2020"
```



This scripts needs two input parameter:
--settings: your modified settings file
--path: the path to your data, please enclose the path in " "
CAUTION!!!

- **No dots (.sptw!),**
- **no special characters (µm!),**
- **to change drive add path in "E:\path-to-data"**

Here, all folders within your specified path will be search recursive for the key phrase ".ptu" and all ptu-files will be correlated (ch0 and ch2), the auto- and crosscorrelation and a figure for visual inspection is saved:



In case you want to correlate e.g. only your cell data, you can change the search phrase to 'cell*.ptu', if you data is saved as cell1.ptu, cell2.ptu etc. Calibration measurements named e.g. A488.ptu will not be correlated.

Analysis of FCCS experiments using ChiSurf

Analysis of the Fluorescence Correlation curves

In this guide, we will first work solely with the Fluorescence Correlation (FCS) curves exported from your calibration data and use the different measurement to characterize the daily performance of your system. It is assumed that you have exported your data already using (i) the proprietary software of your setup (e.g. Symphotime from Picoquant), or (ii) other, maybe self-written software. In case you are still searching for a software, which fits your needs best, you might try

- Kristine (https://www.mpc.hhu.de/software/3-software-package-for-mfd-fcs-and-mfis)
- PAM (https://gitlab.com/PAM-PIE/PAM)
- Globals for FCS (https://www.lfd.uci.edu/globals/)
- Our self-written scripts for batch export (https://github.com/HeinzeLab/JOVE-FCS)

Once you have your workflow and templates established the calibration data is easily analyzed.

In the second section of this guide, we will show you how to (i) add your own fit models, and (ii) analyze live cell experiments.

All data analysis are performed using ChiSurf V2016 [1], which can be downloaded here:

https://github.com/Fluorescence-Tools/chisurf/releases/tag/16.05.14

Of course, all analysis can also be performed with other software, which supports the implementation of the described fit models, using weights in the fit routine and global analysis.

Data format

For analysis with ChiSurf, your correlation data has to be exported in text format with either three or four columns:

- Column 1: correlation time
- Column 2: correlation amplitude
- Column 3: first value reflects the measurement time, second value the average count rate, the rest of this column is filled with zeros OR: the whole column is filled with zeros.
- Column 4: standard deviation of the correlation amplitude

The measurement time and the average count rate in column 3 are used to estimate the uncertainties in your correlation amplitudes if the standard deviation is not available, i.e. single measurement was performed or the hardware / software used for correlation does not provide these values.

Analysis of Calibration measurements

Provided test data - calibration

Sample	Purpose	Measured
ddH2O / buffer	background / dark counts	2x
IRF (EB quenched with KI)	Instrument response function	2x
A488	green calibration dye	5x
A568	red calibration dye	5x
DA-labeled DNA	green-red overlap calibration	5x

Settings:

- 2-color excitation (PIE)
- Excitation power: 485 nm: 6 µW, 568 nm: 1.4 µW
    - measured at objective
- 10 MHz rep rate for each laser line (485 nm prompt, 568 nm delay)
- TAC window: 100 ns (50 ns delay between pulses)
    - TCSPC bin size: 4 ps
- Calibration samples were measured as drop on objective slides

Provided test data – live cell experiments

Sample	Purpose	Description
eGFP	Donor-only sample (DOnly)	eGFP inserted into the intracellular loop 3 of the β_2 adrenergic receptor (β_2AR)
SNAP	Acceptor-only sample (AOnly)	SNAP-tag attached to the C-terminus of the β_2AR, labeled with SNAP Surface DY-549
NT-SNAP	Double-labeled sample (DA) which does not show FRET	β_2AR construct with both eGFP inserted into the intracellular loop 3 and SNAP-tag attached to the N-terminus, labeled with SNAP Surface DY-549

Settings:

- Experimental settings are identical to the calibration measurements.

Provided test data – simulation

Sample	Purpose	Description
CT-SNAP	Double-labeled sample (DA) which shows FRET	β_2AR construct with both eGFP inserted into the intracellular loop 3 and SNAP-tag attached to the C-terminus, labeled with SNAP Cell TMR

Settings / Assumptions:

- Bimodal diffusion of molecules with 30 % of a fast diffusing species (t_{D1} = 1 ms) and the rest of the time diffusing slowly with t_{D2} ~ 100 µs
- Dynamic exchange between two equally populated FRET states, high FRET (HF; FRET efficiency E = 0.7) and low FRET (LF, with E = 0.2), with a time constant of t_R = 70 µs
- Additionally, 16 % of triplet blinking at 5.5 µs was added.

Determination of background / dark count rate

Firstly, the average count rate from ddH2O or appropriate buffer (untransfected cells in case of cells) is obtained – under the same excitation condition as for the sample measurements!

Channel number	Channel name	Count rate [kHz] (prompt)	Count rate [kHz] (delay)
0	g-p (green parallel)	0.13	
1	r-p (red-parallel)	0.445	0.375
2	g-s (green perpendicular)	0.23	
3	r-p (red perpendicular)	0.79	0.39

The average countrate can be determined from your data in different ways:

- If you measured with the Picoquant software, then the average countrate is written in the header section and can be easily obtained by opening the dataset in Symphotime.
- Else you can get the number of photons detected during measurement time from an export of a photon arrival time histogram and divide this number by your measurement time
- Additionally, you can use the "determine_countrates.py" script to export this information for your whole folder of image (see Help file for using scripts).

Calibration of detection volume – green excitation

In a first step, we will use the data from the individual diffusing red and green fluorophores and determine (i) the shape factor of the confocal detection volume, and next, based on this result and on the known diffusion coefficients, (ii) the confocal detection volume in femto liter. Additionally, the (iii) molecular brightness of your fluorophores and finally, (iv) the concentration of the samples can be calculated based on the fit results.

Fit of a FCS curve

➔ Open Chisurf2016
  - It might take a while to open – be patient!

➔ It has got two panels (Figure 1):
  - Left / white: Here, the data is loaded and the analysis method / model is selected. Additionally, you can use the integrated Python-console to run little scripts.
  - Right / grey: Here, each data set opens in its own window.

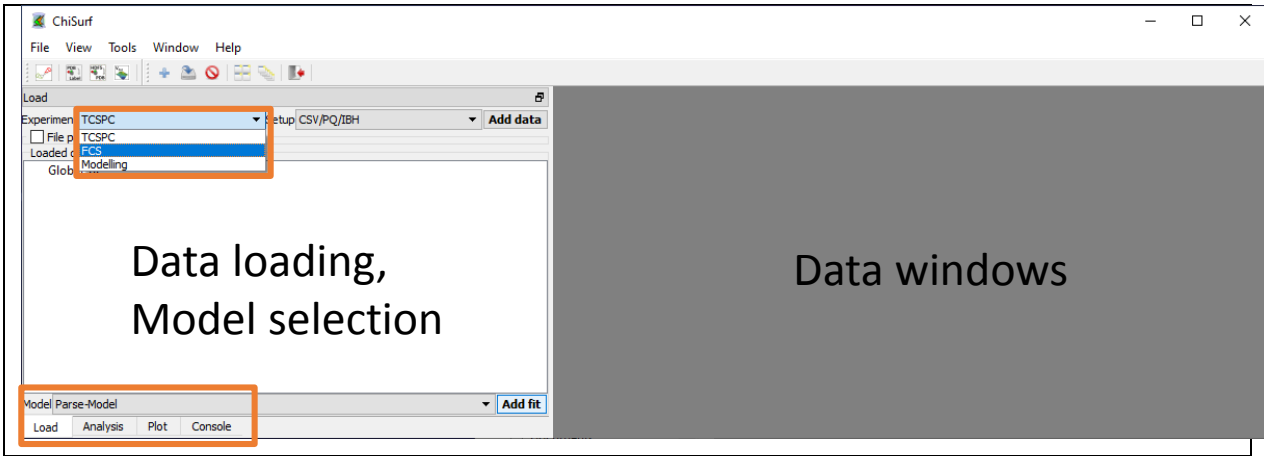


Figure 1 Start window of ChiSurf

➔ Change to the "FCS" mode und load your all "***A488_ACF_prompt.cor***" (auto correlation curves of green calibration fluorophore correlated within the prompt time window) dataset(s) by "File"-> "Add dataset" or simply by "drag'n'drop" into the white area (Figure 2).

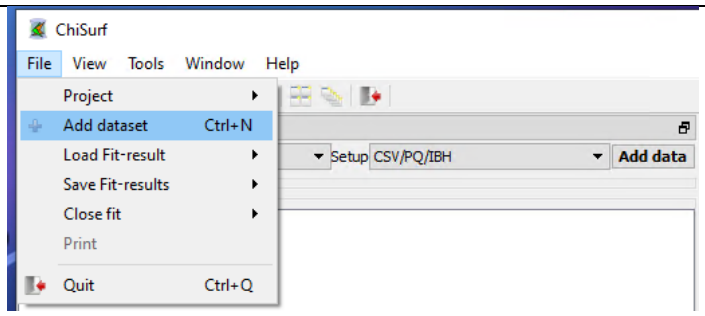


Figure 2 Adding new data sets

➔ Select your loaded dataset(s) and click on "Add fit" (Figure 3):

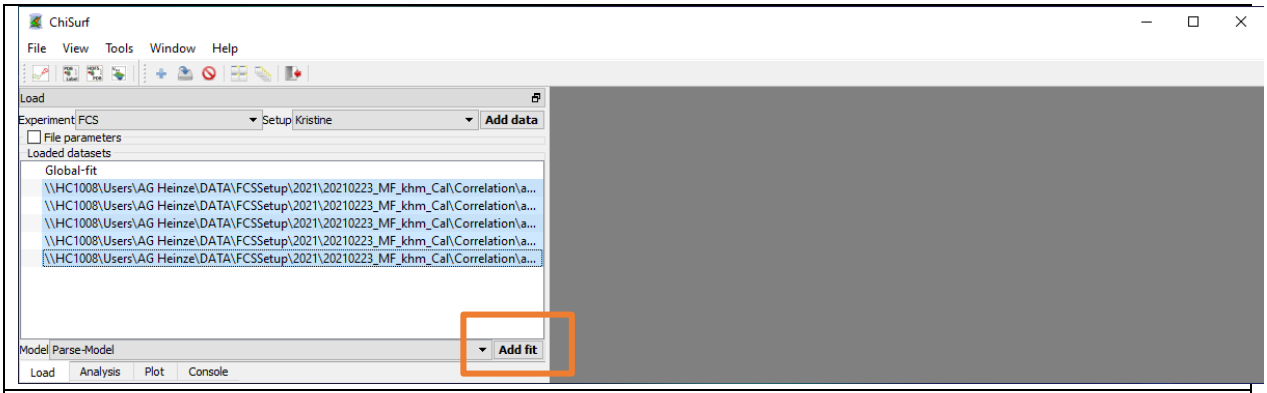


Figure 3 Adding new fits

➔ Here, five new windows will open in the grey working panel (Figure 4):

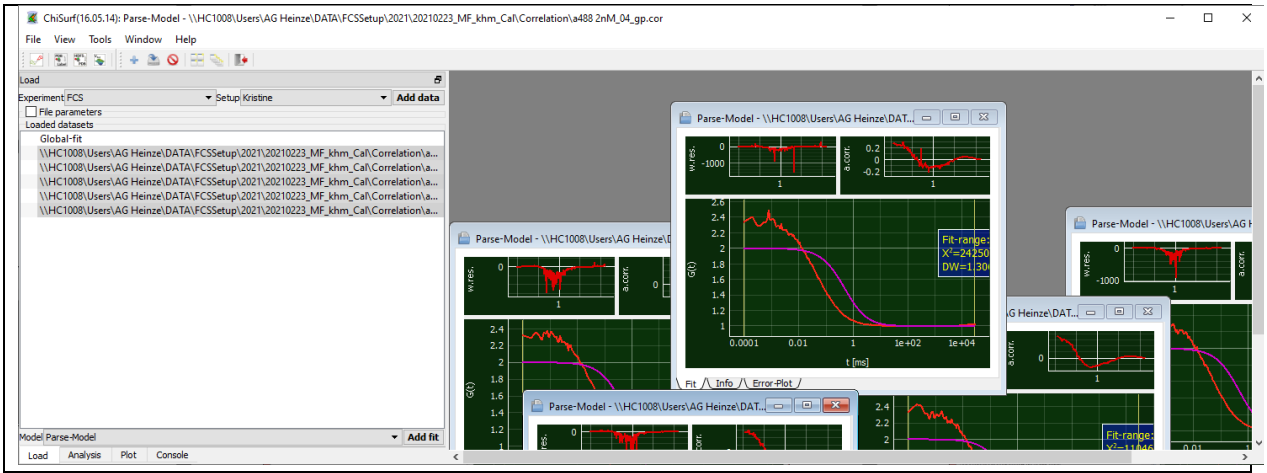


Figure 4 New fit windows open in the grey panel on the right side.

- ➔ Caution! The individual windows may lie on top of each other!
- ➔ Move the top-visible window aside and arrange the windows as you find it comfortable:
  - You can maximize the windows to see only one curve at a time. Minimize the window to see the other data again.
  - Alternatively, you can display each curve in full-size but in individual tabs (Figure 5). Then you can switch between the data by clicking on the respective tab and / or use the little arrows on the side to change between the tabs.

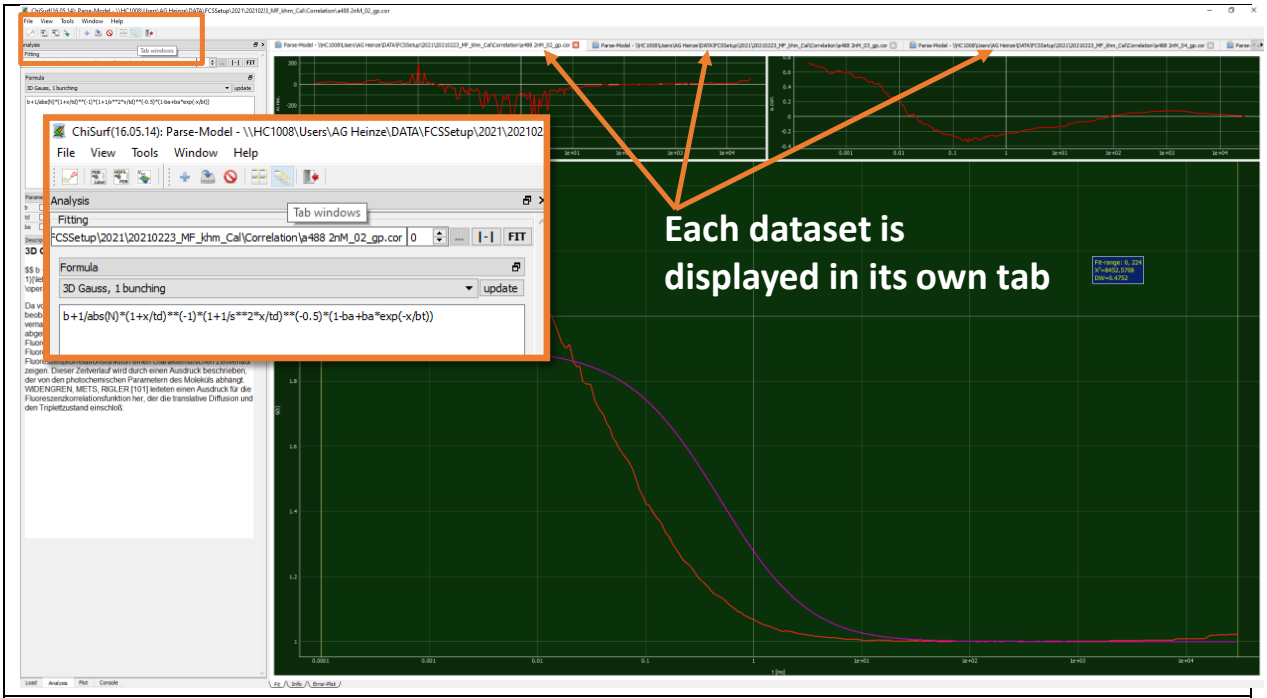


Figure 5 Fit windows of data sets arranged in individual tabs.

- Or you can distribute all windows automatically to evenly cover the full working panel (Figure 6):

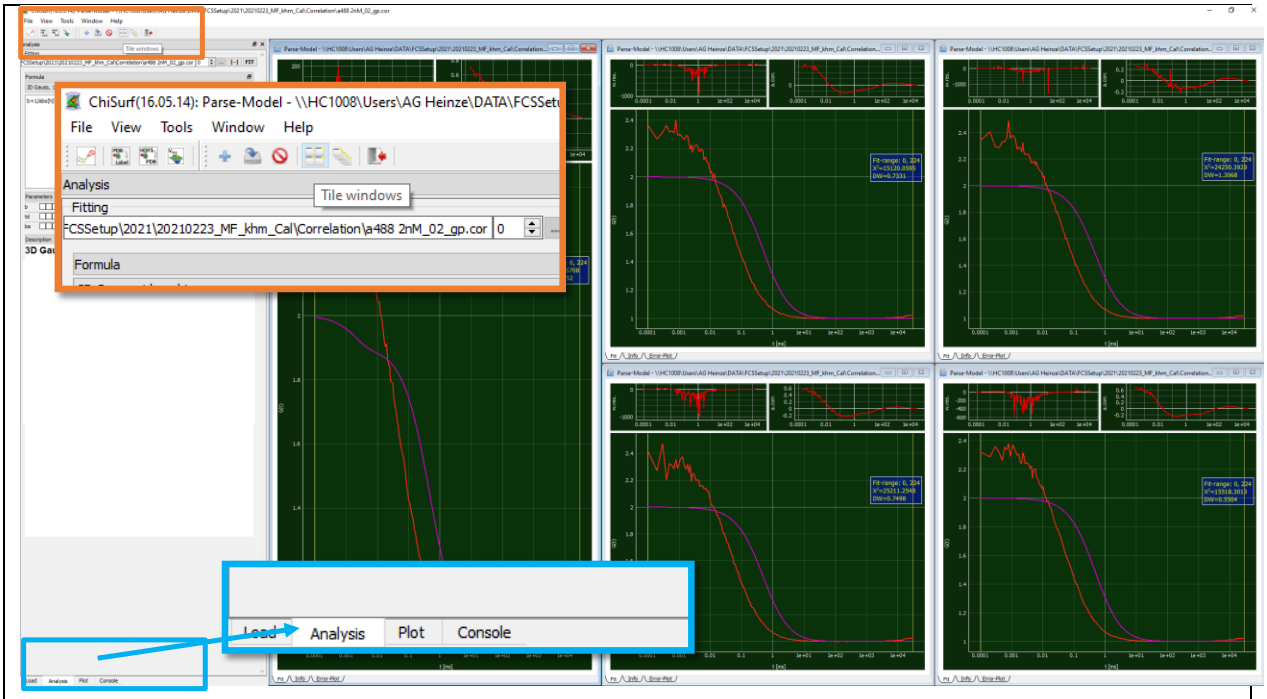


Figure 6 Fit windows of data sets distributed evenly over the data window pane.

➔ Now change to the analysis tab in the data panel and select the "3D Gauss 1 bunching" model from the drop-down menu for your first curve (Figure 7):

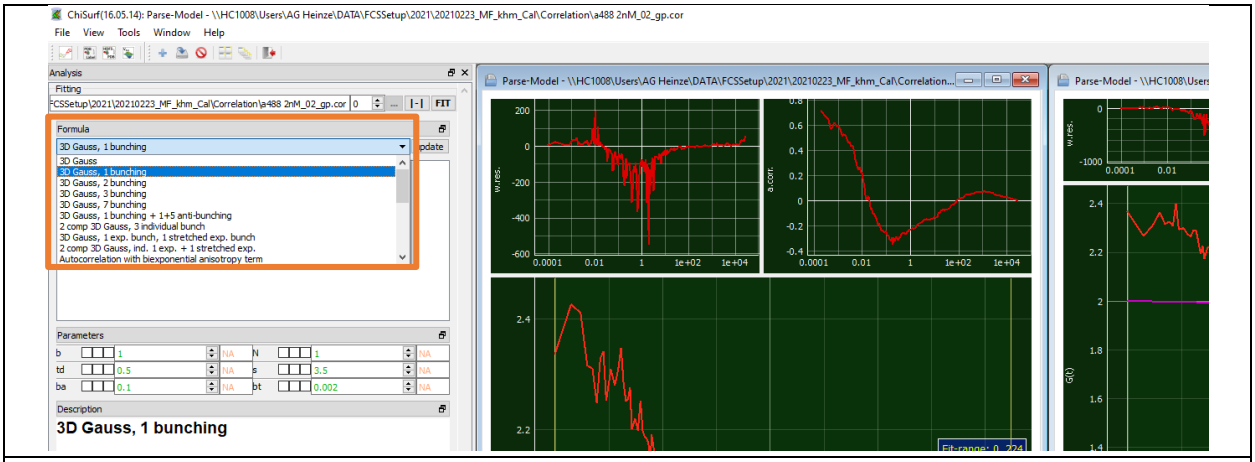


Figure 7 Selection of a fit model.

➔ This model has six parameters (Figure 8):

➔ The "1 Bunching" term as described by "ba" and "bt" is used to model the typical photophysical triplet blinking of many fluorophores in the µs time range.

- Note: ChiSurf comes upon installation with a selection of pre-defined fit models, you can modify these fit models or add your own models easily.
- This will be shown later.

b	offset of the curve (either 1 or 0)
N	number of molecules in focus
td	diffusion time of the molecule [ms]
s	shape factor of the confocal volume: $s = z_0/w_0$ (ratio height / width)
ba	amplitude of relaxation time
bt	time constant of relaxation time [ms]

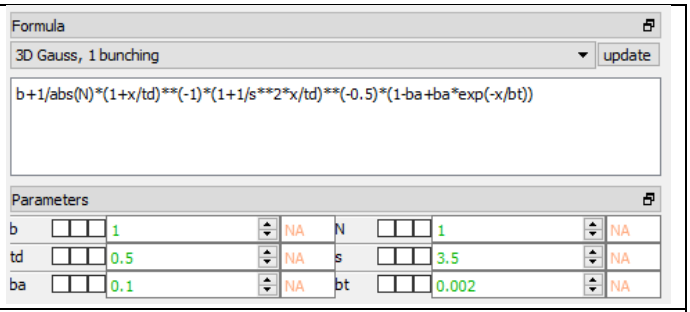


Figure 8 Fit parameter of the 3D diffusion model containing an additional relaxation term.

➔ Press "Fit" for fitting:

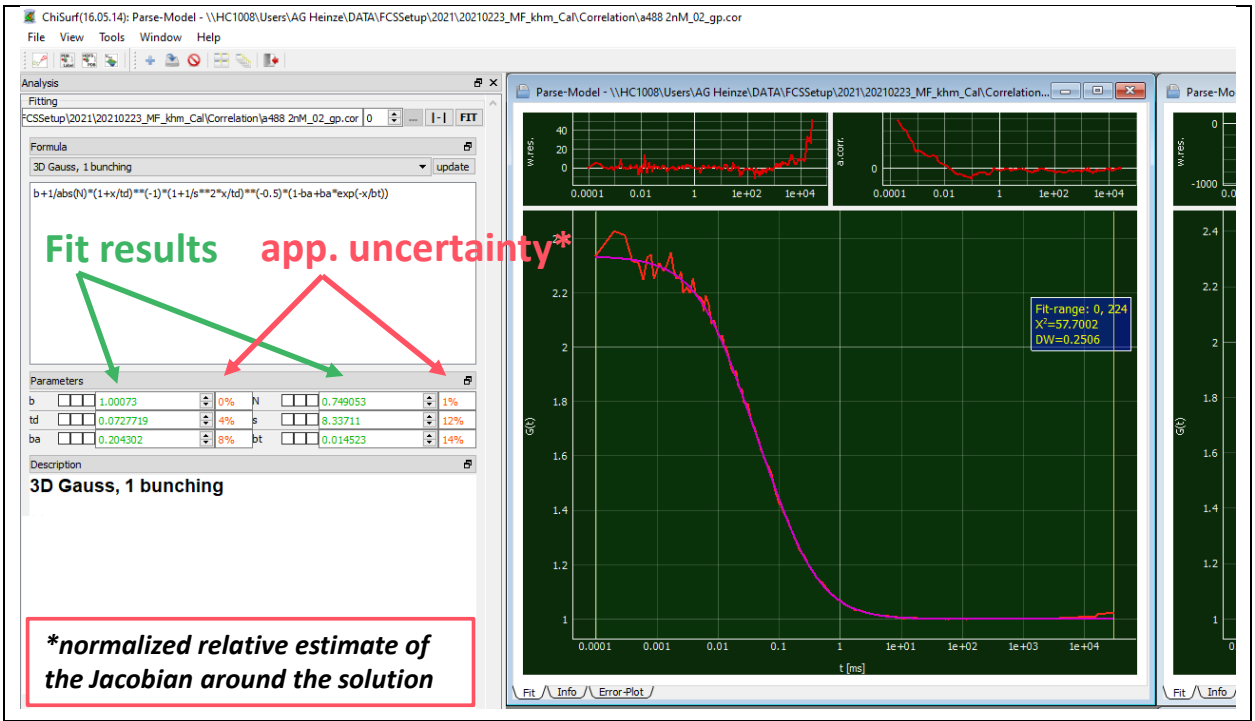


Figure 9 After the fitting, the obtained values for the parameter are shown and the apparent uncertainty is displayed. The small panel on top of the fit show the weighted residuals (left) and the autocorrelation of the weighted residuals (right)..

➔ We obtain a number of molecules in the focus N = 0.75, a diffusion time td = 0.073 ms (i.e. 73 µs) and triplet blinking time constant of 14.5 µs with an amplitude of 0.20. However, from the weighted residuals and the autocorrelation of the residuals, we can see a mismatch at long correlation times (Figure 9): This is because the absolute measurement time in this measurement was too short to reliable obtain these values.

➔ Next to the fit results, also the *normalized relative of the Jacobian Matrix around the solution* can be seen. This is **NOT** reflecting the **uncertainty** of the fit result, but the values might give a first hint whether the uncertainty is rather large or small. For more details on this topic, please check out the information provided on the following web page and the references cited herein:
https://root.cern.ch/doc/master/Minuit2Page.html

➔ For a more reliable estimate on the uncertainty of the fit parameter you have two options within ChiSurf to (i) sample the χ^2-surface or (ii) to run a Markov-Chain Monte-Carlo simulation, which also allows you to obtain the mutual dependencies between the fit parameter. However, this uncertainty analysis is beyond the scope of this analysis of the calibration samples and will be shown in a different tutorial.

➔ Here, we take advantage of multiple measurements of the same sample and take these as additional restraints.

➔ Shorten the fit range to ~ 100 ms by grabbing the right yellow line and move it to the left (Figure 10):

Figure 10 Modifying the fit range.

➔ *Of note*: To reliably fit your diffusion time, the **baseline** (0 or 1, depends on correlation algorithm) **MUST be reached** in your correlation curve (or in the fit range, respectively)

➔ Press "fit" and observe the changes:

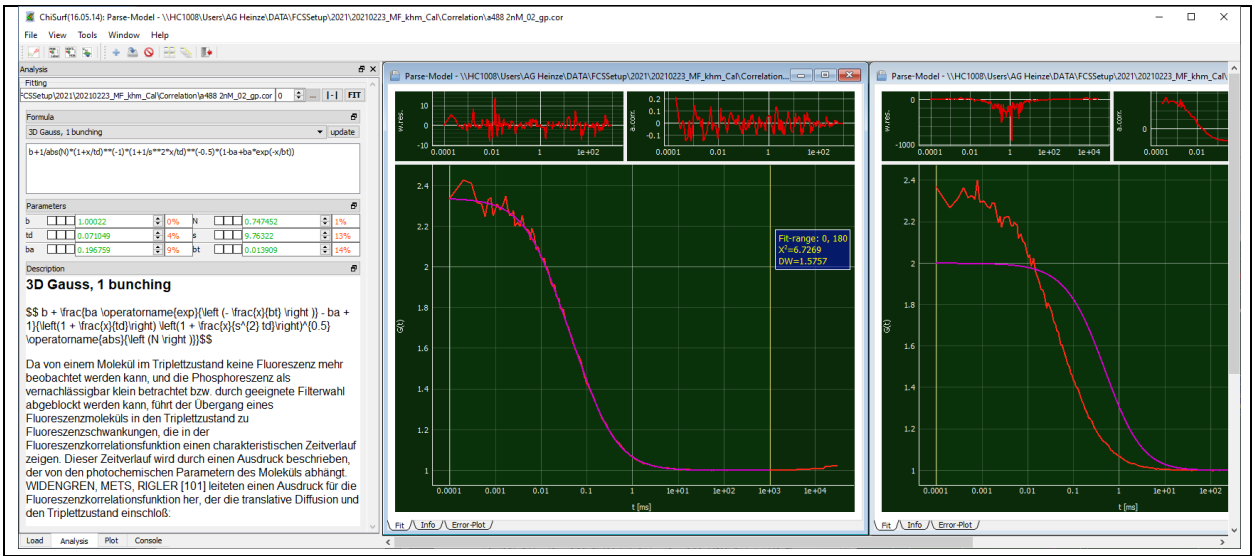


Figure 11 Individual fit of a single data set with the 3D diffusion model including one relaxation term with optimized fit range..

- ➔ Now the shape has increased to 9.76, which is very huge and would indicate a misalignment of your system. In ideal case, the shape factor should lie between 3 - 7. The other values have changed only slightly (Figure 11).
- ➔ Now let's add the other measurements into the play and see whether a global fit of all measurements stabilizes this value.
- ➔ Go to the other fit windows, change the fit to "3D Gauss, 1 bunching", adjust the fit range and fit them as done for the first curve (Figure 12):

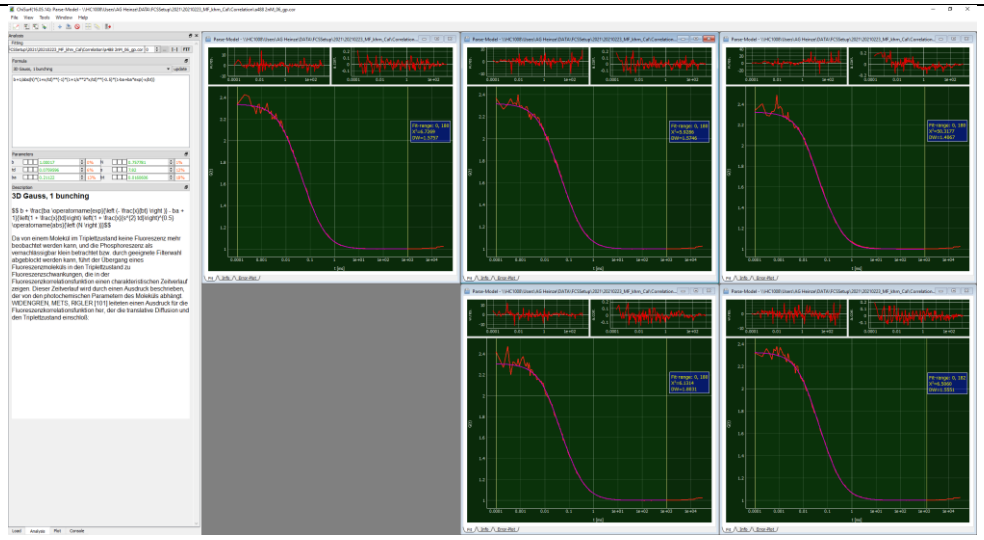


Figure 12 All five data sets of the A488 measurement were fitted individually with the 3D diffusion model including an additional relaxation term..

- ➔ Next, we will link the fits together such that the fit parameter are jointly minimized
- ➔ For this, first decide for one "parent" dataset, to which all other datasets are pointing. Here, I will simply take curve #1.

➔ Now, switch to the first of your "child" or "dependent" dataset. We will now work with the three checkboxes located between each variable name and variable value:

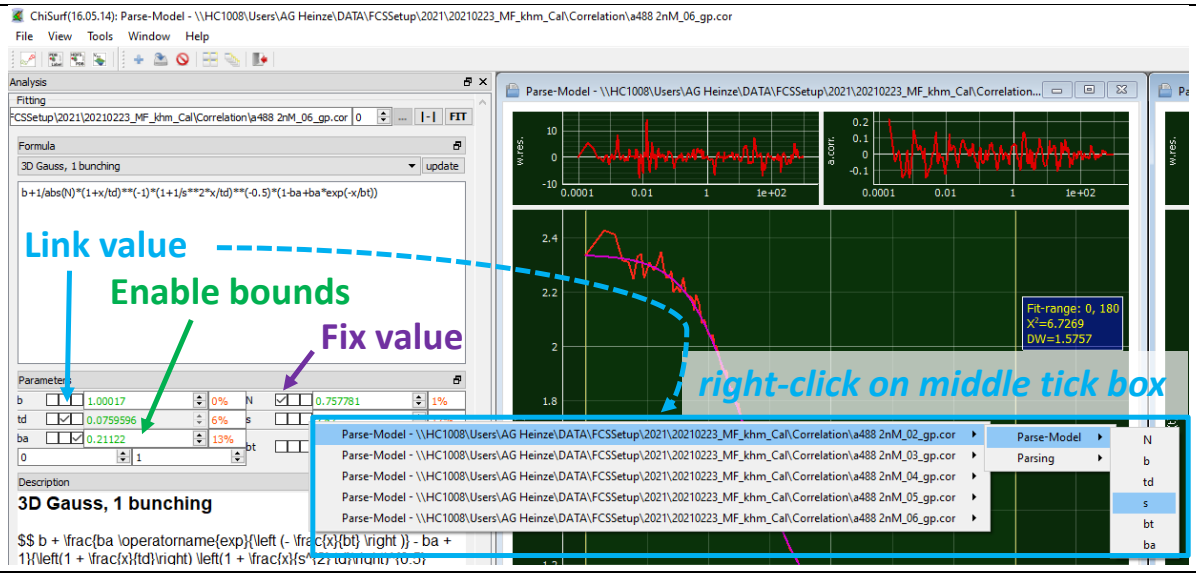


Figure 13 Linking the fit parameter in preparation for a global, joint fit.

➔ Each tick box has a different function (Figure 13):

- Left: fixes the value of the parameter to its current value
- Right: Two new parameter fields open, in these ones the lower (left) and upper (right) boundaries for this parameter during the fitting can be defined. An example is to define the allowed fit range for a correlation amplitude to be positive and lie between 0 -1.
- Middle: By right-clicking into this tick box a list of all opened fit windows opens. Move your mouse towards the right, as soon as you approach the little arrow, new options appear, move further right on the height of "Parse Model" until the list of fit parameter appears.

➔ From this list of fit parameter select the appropriate one.

➔ For our global fit, we will now link (i) the diffusion time td, (ii) the shape parameter s and (iii) the triplet time constant bt to the first data set (Figure 14):

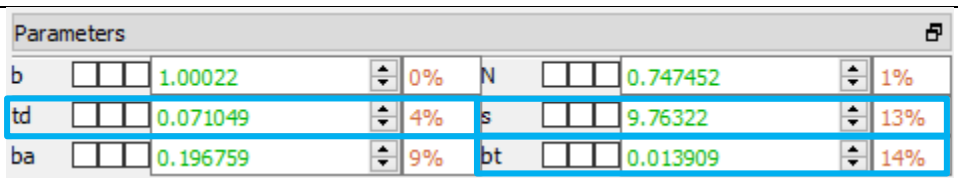


Figure 14 Global fit parameter: td, s and bt.

➔ Linked parameter will appear greyed out.

➔ After you are done with the linking, add a new global fit in the "load" tab:

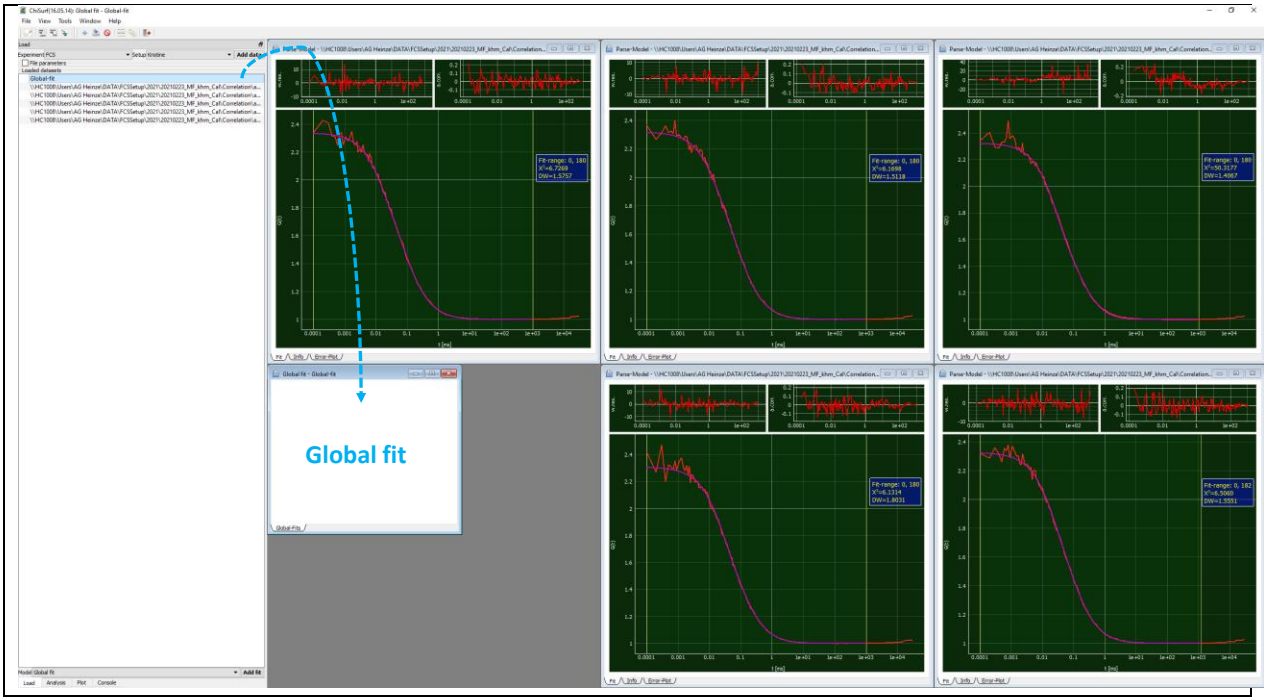


Figure 15 The fit window for the global fit parameter opens as a new, white window.

- ➔ It opens as empty / white window (Figure 15).
- ➔ Switch back to the analysis tab and add dataset which are to be jointly fitted by clicking firstly on "update" and then on "add".

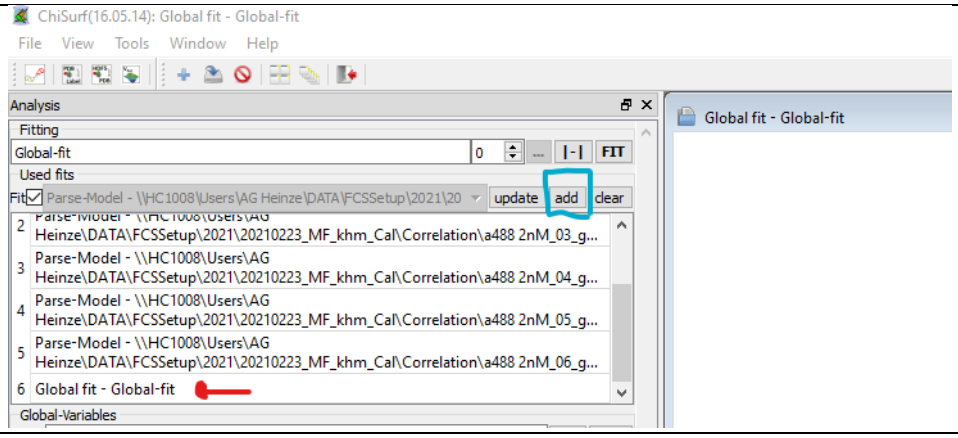


Figure 16 In a first step all open datasets are added to the global fit, next the global fit is removed from the list to avoid "circular reference".

- ➔ Now a list of all available fit windows appears (Figure 16).
- ➔ If you want to select which datasets to add, remove the tick behind "fits". Then you can choose from a drop-drown list which datasets are to be included in the global fit.
- ➔ We need to remove the "global fit" from list ("circular reference"). You do so by simply double-clicking on the item in the list.

➔ Now, press fit and observe what happens.

➔ If you fit many and / or complicated models, the program might take a few moments and display “not responding”. This is nothing to worry about and wait until it responds.

➔ Now all datasets have fitted jointly and we obtain the following values (Figure 17):

- td = 85.7 µs
- s = 5.84
- bt = 21.0 µs
- ba varies between 0.255 – 0.270
- N varies between 0.748 - 0.766

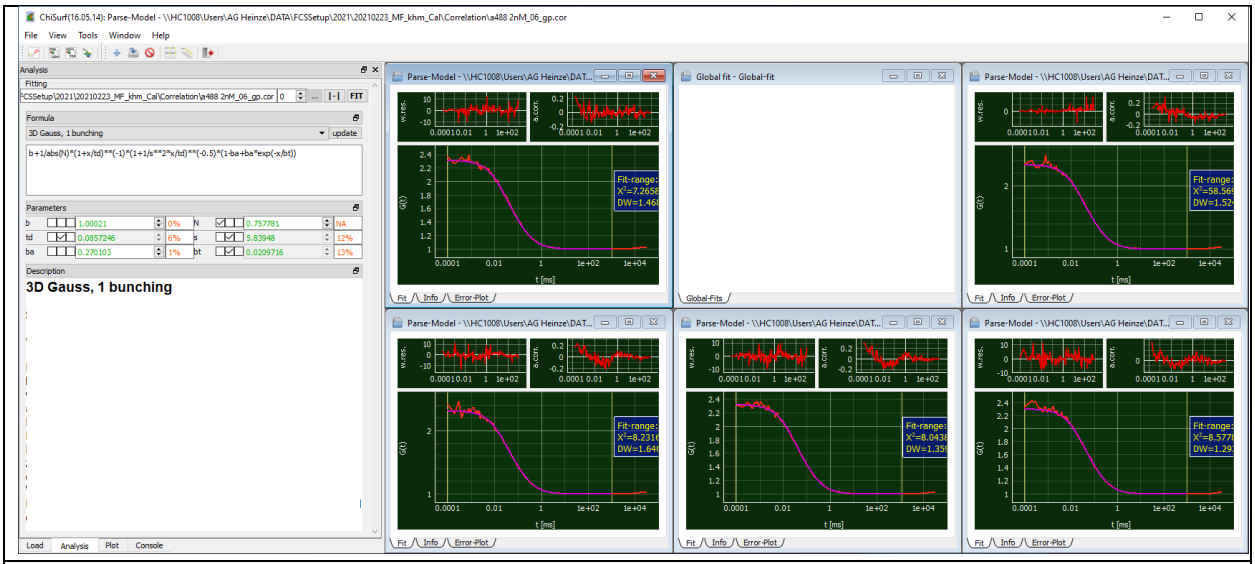


Figure 17 Result of the global fit.

➔ Finally, let’s save the fits by either selecting “File” -> “Save Fit-results” -> “current fit” or by pressing “Ctrl + S” (Figure 18).

➔ Caution! Saving all fits may not work, if the filenames are (a) similar and (b) the whole file path is too long. (AutoSaving uses complete path as automatic save name currently).

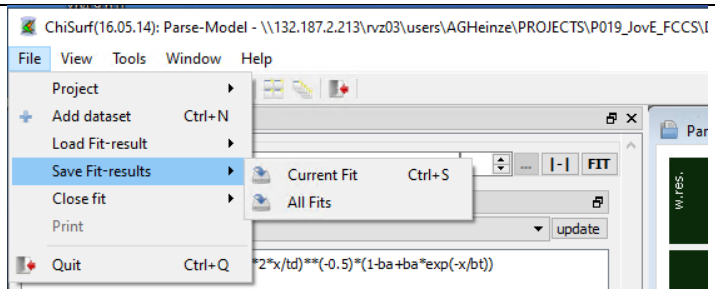


Figure 18 Saving of the fit results.

➔ Save the results of all measurements, we will need the fit results in the next step.

Calculation of confocal volume

Note:

- Use the provided excel sheet "*FCCS_calibration.xlsx*" to retrieve the results semi-automatically (Figure 19).
- Don't forget to update each time your fit results from the respective measurement day!
- Please note the different units (µm, m etc.) and unit conversions!

➔ To determine the confocal volume, we need three different values:

- Diffusion coefficient of the freely diffusing standard dye, here A488,
  - These values can be found in the literature for most common fluorophores.
  - Caution! They are temperature and solvent dependent, i.e. if you measure at low temperature or in a more viscous environment, you have to correct for the viscosity of the solution.
  - **D_{A488} = 414 µm²/s** (@25°C in ddH2O [2]
- Diffusion time t_D from our fit: **85.7 µs**
- Shape factor ***s*** from our fit: **5.84**

➔ Diffusion time and diffusion coefficient are related by the following relationship:

$$D = \frac{w_0^2}{4t_D} \quad \rightarrow \quad w_0^2 = 4t_D D$$

➔ Inserting our fit results and *D* into this equation, we obtain **w_0 = 0.377 µm**

➔ Based on the value from w_0, we can determine the height z_0 of the confocal volume:

$$s = \frac{z_0}{w_0} \quad \rightarrow \quad z_0 = s\, w_0$$

➔ For our example, **z_0 = 2.2 µm**

➔ Finally, the confocal volume is assumed to have in good approximation an elliptical shape from which the volume V_{eff} can be obtained using the following formula:

$$V = \pi^{3/2} z_0 w_0^2$$

➔ Thus, our confocal volume in the green excitation range has a size of **$V_{eff,green}$ = 1.74 fL**.

Estimation of the fluorophore concentration

➔ Using the above determined confocal volume, the concentration of the fluorophore in your measurement solution can be estimated.

➔ The following parameters are required:

- Confocal volume **$V_{eff,green}$ = 1.74 fL**
- Number of molecules in focus, ***N***, from our fit: **0.76** (in average)

- Avogadro's number: N_A = **6.022*10^{23} mol^{-1}**

➔ The concentration of the fluorophore is determined from the following relationship:

$$c = \frac{N}{V_{eff,green} N_A}$$

➔ The approximated concentration of green calibration dye **c_{A488} ~ 0.72 nM.**

Confocal volume			
D	414	µm²/s	*Literature value*
	4.14E-10	m²/s	
td	85.7	µs	*Fit result - please add!*
	0.0000857	s	
s	5.84		*Fit result - please add!*
w02	1.41919E-13		
w0	3.76722E-07	m	
	0.377	µm	
z0	2.20005E-06	m	
	2.200	µm	
pi^(3/2)	5.568327997		
V	1.7386E-18	m³	
	1.739	fl	1 fl = 1e-15 l

Concentration estimation			
Alexa488			
N	0.76	number	*Fit result - please add!*
c	4.34833E+14	number/liter	
c	7.22E-10	Mol/l	
	7.22E-07	mM	
	7.22E-04	µM	
	0.72	nM	

Figure 19 Calculation of the size of the green confocal volume and the concentration of the Alexa488 solution.

Calibration of detection volume – red excitation

➔ For the red detection volume, we proceed as described for the green detection volume.

➔ Only the fit results will be provided for a guidance (Figure 20).

➔ Important: Use the correlated signal of the directly excited red fluorophore within the delay time window of your measurement, e. g. "***A568_ACF_delay.cor***"

Fit of FCS curve

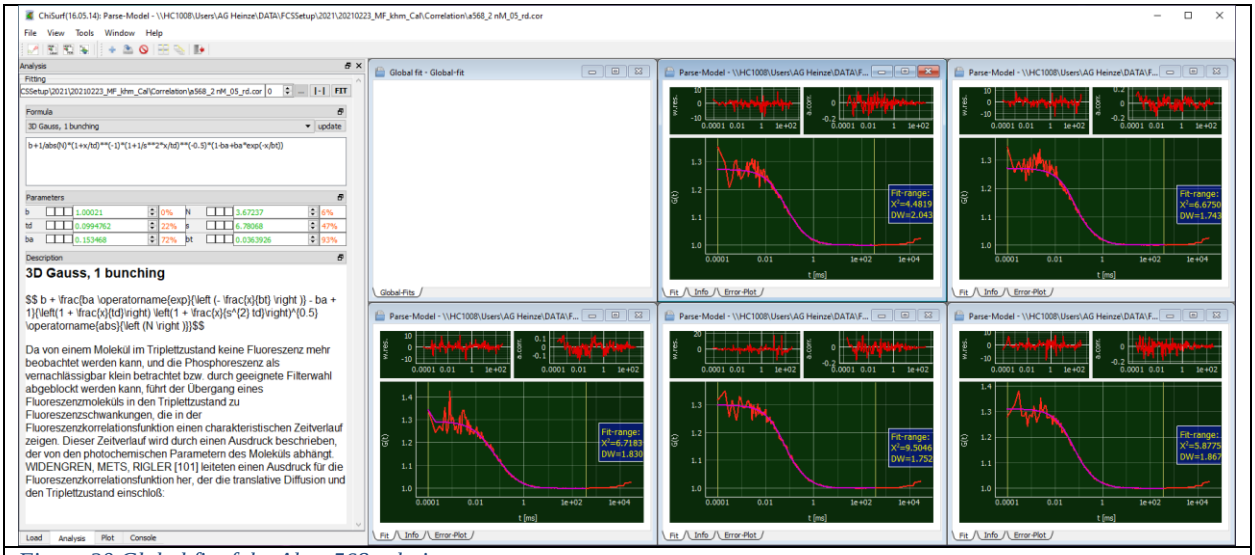


Figure 20 Global fit of the Alexa568 solution.

➔ Here, also the "3D Gauss, 1 Bunching" model will be sufficient.
➔ We obtain:
  - td = 99.5 µs
  - s = 6.78
  - N varies between 3.16 – 3.67
➔ Save the fit results and proceed with the calculations as described for the green detection volume.

Calculations

➔ As A568 is larger than A488, its diffusion coefficient is reduced compared to A488.
➔ There are different studies reporting a D_{A568} in the range of 330 -360 µm²/s.
➔ Here we use a value of **D_{A568} = 363 µm²/s** [3].

Confocal volume			
D	345	µm²/s	*Literature value*
	3.45E-10	m²/s	
td	99.5	µs	*Fit result - please add!*
	0.0000995	s	
s	6.78		*Fit result - please add!*
w02	1.3731E-13		
w0	3.70554E-07	m	
	0.371	µm	
z0	2.51235E-06	m	
	2.512	µm	
pi^(3/2)	5.568327997		
V	1.92091E-18	m³	
	1.921	fl	1 fl = 1e-15 l

Concentration estimation			
Alexa488			
N	3.44	number	*Fit result - please add!*
c	1.7929E+15	number/liter	
c	2.98E-09	Mol/l	
	2.98E-06	mM	
	2.98E-03	µM	
	2.98	nM	

Figure 21 Calculation of the size of the red confocal volume and the concentration of the Alexa488 solution.

➔ As expected for a measurement at red-shifted, thus longer wavelengths of excitation and emission, the confocal detection $V_{eff,red}$ volume is increased compared to $V_{eff,green}$ (Figure 21).

Correction factors for spectral crosstalk & direct acceptor excitation

➔ Absorption and emission spectra of fluorophores are usually quite broad and extent over quite a wavelength range, and not seldom the spectra from green and red fluorophores used in the measurement overlap (Figure 22).
➔ By introducing optical (bandpass) filters into the emission path of your setup, it is possible to minimize these effects.

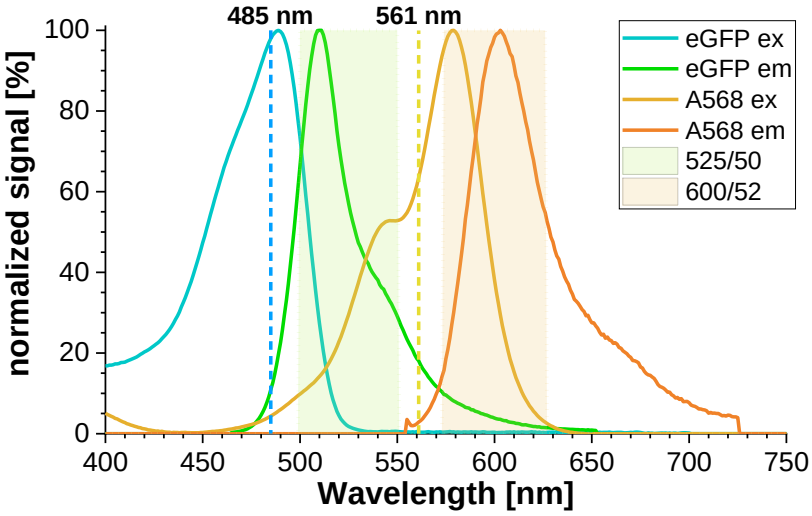


Figure 22 Normalized absorption and emission spectra for the eGFP and Alexa568 fluorophores used in the cell experiments. Excitation laser lines are shown as dashed lines and transparent boxes denote the bandpass filter through which the emitted fluorescence is collected.

- ➔ However, even if these contributions to your signal are small, they might be become critical when you have a low count rate / low signal in one channel compared to the other channels.
- ➔ For more detailed information on required corrections and the nomenclature of correction factors, we refer to the recently published multi-laboratory benchmark study of Hellenkamp *et al.* [4].

Spectral crosstalk of green fluorescence into red detection channel

- ➔ Required is the measurement from the freely diffusing green fluorophore.
- ➔ Collect the count rates in all four detection channels for the ddH2O /buffer measurement and the green dye (A488) measurement:

Channel number	Count rate [kHz] BG	Count rate [kHz] A488	Corrected count rate [Hz]
0	0.13	5.30	5.18
1	0.36	1.23	0.87
2	0.23	5.38	5.15
3	0.69	1.53	0.85

(Note: average values of repeated measurements are reported)

- ➔ The background count rate needs to be subtracted from value and then the ratio of count rates in the red channels to the green channel is determined:

$$\alpha = \frac{\left(\left(CR_{1,A488} - CR_{1,BG}\right) + \left(CR_{3,A488} - CR_{3,BG}\right)\right)}{\left(\left(CR_{0,A488} - CR_{0,BG}\right) + \left(CR_{2,A488} - CR_{2,BG}\right)\right)} * 100\%$$

- ➔ In our example the spectral crosstalk ***α* = 16.6 %.**

Determination of direct excitation of red fluorophore by green excitation

- ➔ Required is the measurement of the freely diffusing red fluorophore.
- ➔ Collect the count rates in the two red detection channels for the ddH2O / buffer measurement and the red dye measurement A568, take care to separate the count rate into the "prompt" and "delay" time window:

Channel number	Count rate [kHz] BG	Count rate [kHz] A568	Corrected count rate [Hz]
1 delay	0.36	4.24	3.88
1 prompt	0.36	1.90	1.54
3 delay	0.69	4.81	4.12
3 prompt	0.69	2.37	1.68

(Note: average values of repeated measurements are reported)

- ➔ In the "prompt" time window the green excitation pulse might excite a bit of the red fluorophores.
- ➔ In the "delay" time window the red fluorophores are directly excited.
- ➔ Subtract the background counts from each channel and the respective time window.
- ➔ Calculate the ratio of the "prompt" time window to the "delay" time window:

$$\delta = \frac{\left(\left(CR_{1,prompt,A568} - CR_{1,prompt,BG}\right) + \left(CR_{3,prompt,A568} - CR_{3,prompt,BG}\right)\right)}{\left(\left(CR_{1,delay,A568} - CR_{1,delay,BG}\right) + \left(CR_{3,delay,A568} - CR_{3,delay,BG}\right)\right)} * 100\%$$

- ➔ Caution! If you measure in a mixture of green and red fluorophores, you need to also subtract the green crosstalk into the red channels (α determined above!)
- ➔ For the shown example, the direct excitation of acceptor by green laser is **δ = 40.3 %.**

Determination of molecular brightness

- ➔ After having correcting the count rates, we can now determine the molecular brightness of our fluorophores (Figure 23).
- ➔ Note: It is advisable to monitor this molecular brightness of your calibration standards carefully as they can give you early hints about a possible misalignment and / or reduction of laser output power.
- ➔ Required parameter:
  - Number of molecules in focus as determined from the fits above
    - N_{green}: 0.76
    - N_{red}: 3.44
  - Corrected count rates:
    - $CR_{corr,green,0}$ = 5.18 kHz
    - $CR_{corr,green,2}$ = 5.15 kHz
    - $CR_{corr,red,1}$ = 3.88 kHz
    - $CR_{corr,red,3}$ = 4.12 kHz

➔ The molecular brightness is calculated as count per molecule and second:

$$B_{green} = \frac{CR_{corr,green,0} + CR_{corr,green,2}}{N_{green}}$$

$$B_{red} = \frac{CR_{corr,red,1} + CR_{corr,red,3}}{N_{red}}$$

➔ For the data shown here, the following molecular brightness is obtained:

Countrates A488

	ch 0 / s [kHz]	ch 2 / p [kHz]	sum [kHz]	N	brightness [kHz/molecule]
2	5.27	5.38	10.65	0.766	12.28
3	5.26	5.34	10.60	0.753	12.44
4	5.30	5.36	10.66	0.757	12.50
5	5.30	5.39	10.69	0.748	12.66
6	5.35	5.43	10.78	0.756	12.63
Mean	5.30	5.38	10.68	0.76	12.50
stdev	0.03	0.03	0.06	0.01	0.14

Countrate background / **Countrate red channels**

	ch 0 / s [kHz] ddH2O	ch 2 / p [kHz] ddH2O	ch 1 / s [kHz] A488	ch 3 / p [kHz] A488
1	0.13	0.23	1.25	1.57
2	0.13	0.23	1.23	1.54
3			1.20	1.52
4			1.22	1.52
5			1.23	1.53
Mean	0.13	0.23	1.23	1.53
stdev	0.00	0.00	0.02	0.02

Green crosstalk into red channel

	CR [kHz] A488	CR [kHz] BG	CR_{A488}-CR_{BG} [kHz]	
0 prompt	5.30	0.13	5.18	*spectral crosstalk [%] α*
1 prompt	1.23	0.36	0.87	16.61
2 prompt	5.38	0.23	5.15	
3 prompt	1.53	0.69	0.85	

Countrates A568

	ch 1 / s [kHz] delay	ch 3 / p [kHz] delay	sum [kHz]	N	brightness [kHz/molecule]
1	4.55	5.11	9.66	3.16	3.06
2	4.43	5.00	9.43	3.30	2.86
3	4.25	4.83	9.08	3.50	2.60
4	4.18	4.74	8.92	3.59	2.48
5	4.10	4.66	8.76	3.67	2.39
Mean	4.24	4.81	9.17	3.44	2.68
stdev	0.12	0.13	0.33	0.19	0.25

Countrate background

	ch 1 / s [kHz] ddH2O	ch 3 / p [kHz] ddH2O	ch 1 / s [kHz] prompt	ch 3 / p [kHz] prompt
1	0.390	0.69	2.01	2.46
2	0.36	0.69	1.95	2.41
3			1.88	2.35
4			1.85	2.33
5			1.83	2.29
Mean	0.36	0.69	1.90	2.37
stdev	0.00	0.00	0.07	0.06

Direct excitation of A568 by 488-laser line

	CR [kHz] A568	CR [kHz] BG	CR_{A568}-CR_{BG} [kHz]	
1 Delay	4.24	0.36	3.88	*direct excitation [%] δ*
1 Prompt	1.90	0.36	1.54	40.31
1 Delay	4.81	0.69	4.12	
1 Prompt	2.37	0.69	1.68	

Figure 23 Calculation of the molecular brightness of both calibration standards..

Determination of overlap of green & red detection volume

➔ For calibration of the overlap of the green and red excitation volume, DNA strands are used, which carry both the green and red calibration fluorophore. However, the fluorophores are placed usually so far apart that no energy transfer due to FRET can occur.

➔ For single-molecule measurements, a mixture of DOnly-labeled DNA strands and high- and low-FRET showing DNA strands is additionally used to calibrate the green-to-red detection efficiency ratio.

➔ For our determination of the confocal volume overlap, we will use the amplitude of the green (ACF_{green}) and red autocorrelation function (ACF_{red}) as well as from the "Green-prompt"-"Red-delay" cross-correlation function (CCF_{PIE}).

Important note!

The detection volume increases with the excitation and emission wavelength of the fluorophores. This effect is more prominent in diffraction-limited setup as used here and commonly for live cell FCS.

In setups for single-molecule experiments, with freely diffusing molecules, with larger detection volumes this effect can often be ignored and the diffusion terms in the green and red channels can be fit jointly.

Global fit of FCS curves

- ➔ Switch back to ChiSurf2016 and load the following correlation curves from your DNA-sample:
  - *DNA_gp.cor*: Autocrrelation of green channels in prompt time window
  - *DNA_rd.cor*: Autocorrelation of red channels in delay time window
  - *DNA_PIE.cor*: Crosscorrelation of green signal in the prompt time window with red signal in the delay time window
- ➔ Add a "3D Gauss, 1 bunching" model to your correlation functions
  - Of note: theoretically no bunching term should be required as the photophysics from the two fluorophores is independent from each other, and is thus not resulting in a correlating signal.
  - However, in practice, in case of significant photophysics / triplet blinking and considerable crosstalk as is the case here, we often observed an apparent "photophysics" term, which we model by the relaxation term.
- ➔ Fix the shape factor s_{green} and s_{red} to your determined values from the free dye measurements above.
- ➔ Remember to adjust your fit range at long lag times if required.
- ➔ Note: If you have multiple DNA measurements, fit the respective correlation curve from the same correlation channels jointly, i.e. link ***td***, and ***bt*** and – for the CCF_{PIE} also s_{PIE}
- ➔ Observe s_{PIE} in your CCF_{PIE}, it should get a "reasonable" number, usually between s_{green} and s_{red} (Figure 24).
- ➔ Save all fit results, we will need them for our calculations below.
- ➔ Here, we obtain the following fit results for our DNA samples:

Parameter	DNA_gp	DNA_rd	DNA_PIE	
s*	5.7*	6.78*	4.63	
***td* [µs]**	438	474	520	
N	11.6 – 7.54	14.4 – 8.73	1/G(tc)	20.4 – 13.4
***bt* [µs]**	88.6	101	170	
ba	5 - 22 %	1.3 – 24.5 %	0 – 15.7	

**s_{green} and s_{red} are fixed from the calibration measurements above.*

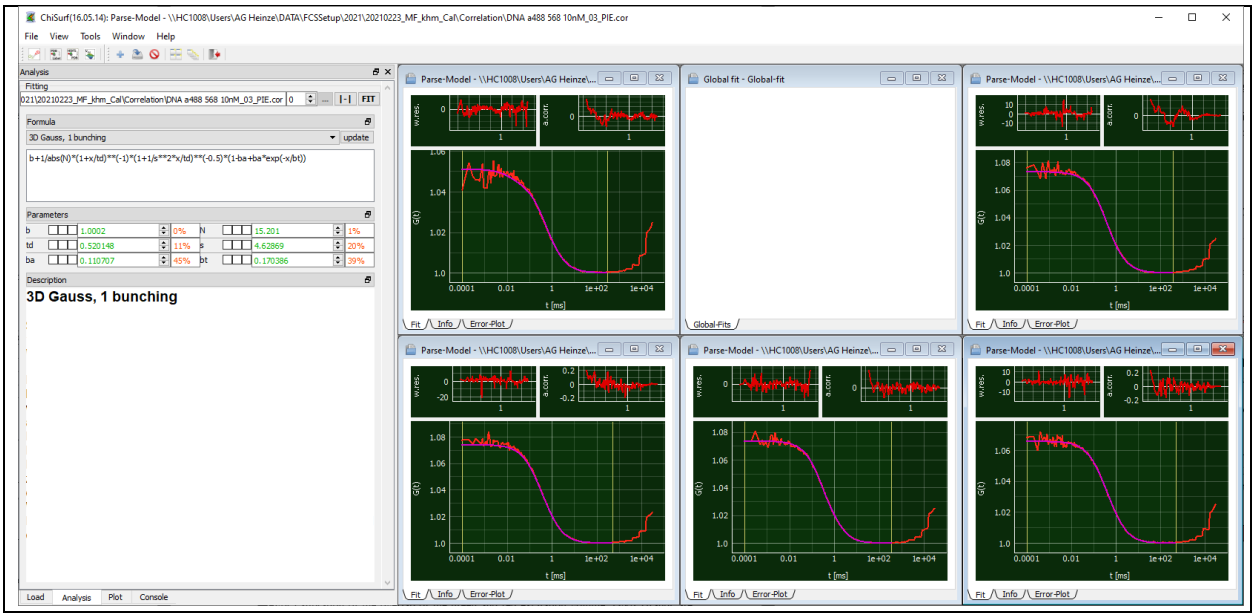


Figure 24 Global fit of the CCF_{PIE} correlations from the DNA sample..

Calculations

From our double-labeled DNA measurements, we need to derive two important parameter:

- The size of the overlapping confocal detection volume $V_{eff,PIE}$
- The Cross-correlation amplitude, which reflects 100 % co-diffusion

The size of the overlapping confocal detection volume can be determined using the already provided equations used in the sections above for the calculations of the confocal detection volume of the green and red channel.

In a first step, we use the obtained diffusion times from our *DNA_gp* and *DNA_rd* fits and determine the translational diffusion coefficient of our DNA sample:

$$D_{DNA,green} = \frac{w_0^2}{4t_{D,green}} \quad \text{and} \quad D_{DNA,red} = \frac{w_0^2}{4t_{D,red}}$$

Here, we obtain a value of $\mathbf{D_{DNA,green} = 81.8\ \mu m^2/s}$ and $\mathbf{D_{DNA,red} = 72.4\ \mu m^2/s}$ using the value of w_0 from A488 and A568 dye, respectively. Thus, in average $\mathbf{D_{DNA} = 77.1\ \mu m^2/s}$.

Based on this value, we can obtain $w_{0,PIE}$ and $z_{0,PIE}$:

$$w_0^2 = 4t_D D \quad \text{and} \quad z_0 = s\, w_0$$

Here, $\mathbf{w_{0,PIE} = 400\ nm}$ and $\mathbf{z_{0,PIE} = 1.85\ \mu m}$. This results in a $\mathbf{V_{eff,PIE}}$ of **1.66 fL**:

$$V = \pi^{3/2} z_0 w_0^2$$

Next, we observe the amplitudes of the auto- and cross correlation functions: In an ideal system the amplitudes of the three curves, *DNA_gp*, *DNA_rd* and *DNA_PIE* should be identical. However, as the detection volumes differ with the excitation and emission wavelength, this is rarely the case. In the next-optimal setting, the amplitude of *DNA_PIE* would be identical to the amplitude of the autocorrelation curve with the lower amplitude.
In common experimental settings, the overlap of the green and red confocal detection volumes is suboptimal and the apparent amplitude of a 100 % co-diffusion sample is required for calibration. The concentration, and thus, later the fraction of co-diffusing particles in your sample, of double-labeled particles can be calculated based on the ratio of the correlation amplitudes:

$$c_{RG} = \frac{G_{0,CCF}}{G_{0,ACFgreen}} \cdot c_{green} \qquad \text{and} \qquad c_{GR} = \frac{G_{0,CCF}}{G_{0,ACFred}} \cdot c_{red}$$

where the amplitudes $G_{0,ACFgreen}$ and $G_{0ACF,red}$ are the inverse of the respective number of particles, N_{green} and N_{red}, in focus.
Here, we obtain amplitude ratios for 100 % co-diffusion of ***ratio*$_{GR}$ = 0.57** for the green and of ***ratio*$_{RG}$ = 0.68** for the red autocorrelation curves (Figure 25).

Effective overlapping confocal volume			
D	77.09	µm²/s	based on green /red
	7.7086E-11	m²/s	
td	520	µs	Fit result - please add!
	0.00052	s	
s	4.63		Fit result - please add!
w02	1.6034E-13		
w0	4.0042E-07	m	
	0.400	µm	
z0	1.854E-06	m	
	1.854	µm	
pi^(3/2)	5.568328		
V	1.6552E-18	m³	
	1.655	fl	1 fl = 1e-15 l
			1000 l = 1 m³

	Green				Red			
	N	c [nM]	td [µs]*	D [µm²/s]	N	c [nM]	td [µs]*	D [µm²/s]
2	11.6	11.08	434	81.75	14.7	12.71	474	72.42
3	8.53	8.15	434	81.75	10.8	9.34	474	72.42
4	7.79	7.44	434	81.75	8.73	7.55	474	72.42
5	7.54	7.20	434	81.75	9.01	7.79	474	72.42
6	7.61	7.27	434	81.75	8.94	7.73	474	72.42
Mean	7.87	7.52	434.00	81.75	10.44	9.02	474.00	72.42
stdev	0.39	0.38	0.00	0.00	2.26	1.95	0.00	0.00

*global fitting — Average diffusion coefficient D [µm²/s]: 77.09

Green - Red Crosscorrelation						
	Napp	G(t,CC)	cRG [nM] (g)	$G_{0,CCF}/G_{0ACFgreen}$	cRG [nM] (r)	$G_{0,CCF}/G_{0ACFred}$	AVG cRG [nM]
2	20.4	0.049	6.30	0.57	9.16	0.72	7.73
3	15.2	0.066	4.57	0.56	6.64	0.71	5.60
4	13.6	0.074	4.26	0.57	4.85	0.64	4.55
5	13.5	0.074	4.02	0.56	5.20	0.67	4.61
6	13.4	0.075	4.13	0.57	5.16	0.67	4.64

$c_{RG} = \frac{G_{0,CCF}}{G_{0,ACF,green}} * c_{green}$ $c_{RG} = \frac{G_{0,CCF}}{G_{0,ACFred}} * c_{red}$

Figure 25 Calculation of the effective overlap volume and the correction factor for the co-diffusion amplitude.

Now we are ready to switch to our real samples measured in live cells.

Analysis of live cell experiments

Again, also here, we assume that you have already correlated your data using the software of your system or any of the software mentioned in the beginning.

We will show you first how to add your own fit models to ChiSurf and next analyze first the measurements of the singly labeled constructs (β_2AR-eGFP-IL3 and NT-SNAP-β_2AR) before switching to the double-labeled NT-SNAP-β_2AR-eGFP-IL3 sample.

Adding the membrane-diffusion models

Upon installation, ChiSurf comes with a bunch of FCS fit models; however, your required fit model might not be among them. The fit models are defined in a JSON-file ("fcs.model.json") which can be found in the installation folder of ChiSurf (Figure 26):

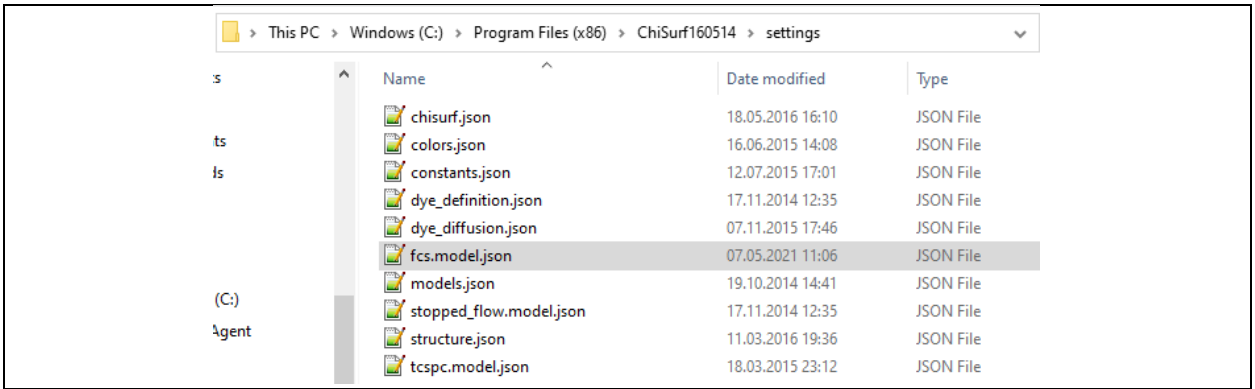


Figure 26 The settings file containing the fit models can be found directly in the installation folder.

Before modifying the file, (i) create a copy on a different place as a backup and (ii) make a second copy to work on as modifying / saving directly in the programs installation folder is usually not allowed.

Open the JSON-file using a text editor, e.g. Notepad++.

Each fit model consists of four sections (Figure 27):

- Model name
- Model equation
- Model parameter definition by initial values
- Model description

It is vital to keep this notation and take care of proper punctuation and indentation!

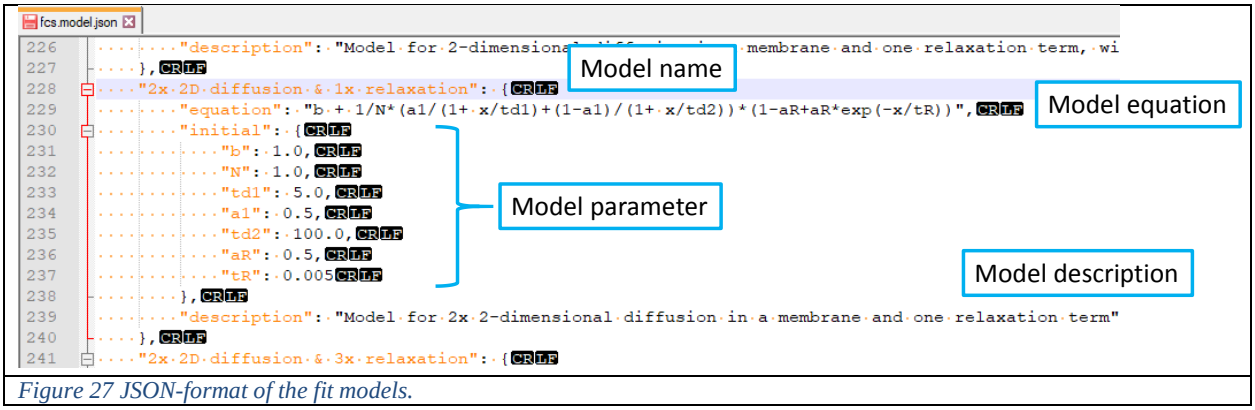


Figure 27 JSON-format of the fit models.

Add the two following fit models for bimodal membrane diffusion with or without and additional relaxation / triplet term to your JSON-file:

- For analysis of autocorrelation curves:

$$G_{ACF,2D}(t_c) = b + \frac{1}{N}\left[\frac{a_1}{1 + {t_c}/{t_{D1}}} + \frac{1 - a_1}{1 + {t_c}/{t_{D2}}}\right]\left[1 - a_R + a_R \cdot \exp\left(-\frac{t_c}{t_R}\right)\right]$$

where t_{D1} and t_{D2} are the two diffusion time and a_1 is the fraction of t_{D1}. a_R and t_R describe the triplet blinking / photophysics.

- For analysis of cross-correlation curves:

$$G_{CCF,2D}(t_c) = b + \frac{1}{N}\left[\frac{a_1}{1 + {t_c}/{t_{d1}}} + \frac{1 - a_1}{1 + {t_c}/{t_{d2}}}\right]$$

In cross-correlation curves, usually no triplet blinking can be seen.

Don't forget to define reasonable initial values for each of the model parameter.
Replace the original JSON-file in your programs folder with your modified version and restart ChiSurf.
Note: For changes to the JSON-file to take effect, ChiSurf must always be restarted!

Individual transfected β2AR-eGFP-IL3 and NT-SNAP-β2AR

Fit of autocorrelation curves

Open the autocorrelation of the green channels from the prompt time window from the β2AR-eGFP-IL3 measurements and the autocorrelation of the red channels in the delay time window from the NT-SNAP-β2AR measurements in ChiSurf.
Select the bimodal membrane diffusion model for autocorrelation curves, which we have just added to ChiSurf and fit the data (Figure 28):

For average of the β2AR-eGFP-IL3 measurements, a slow diffusion time t_{D1} = 118 ms and a fraction of 54 % is obtained. The faster diffusion lies at t_{D2} = 1.9 ms. Additionally, 25 % triplet blinking at t_R ~ 9 µs is observed. However, at this short correlation times the data is already quite noisy and care should be taken in the interpretation. The number of molecules in focus is 5.

For the average of the NT-SNAP-β2AR measurements, two additional relaxation terms seem to be required (modify your JSON-model file accordingly!) with relaxation times (and fractions) of t_{R1} ~ 5 µs (12 %) and t_{R2} ~ 180 µs (11%). The two diffusion components show times of t_{D1} = 49 ms (49 %) and t_{D2} = 2.7 ms. The number of molecules in focus lies at 35 and this is much higher compared to β2AR-eGFP-IL3. One could speculate whether t_{R2} ~ 180 µs might not be a photophysics-related term but rather unreacted SNAP substrate diffusing through the confocal volume.

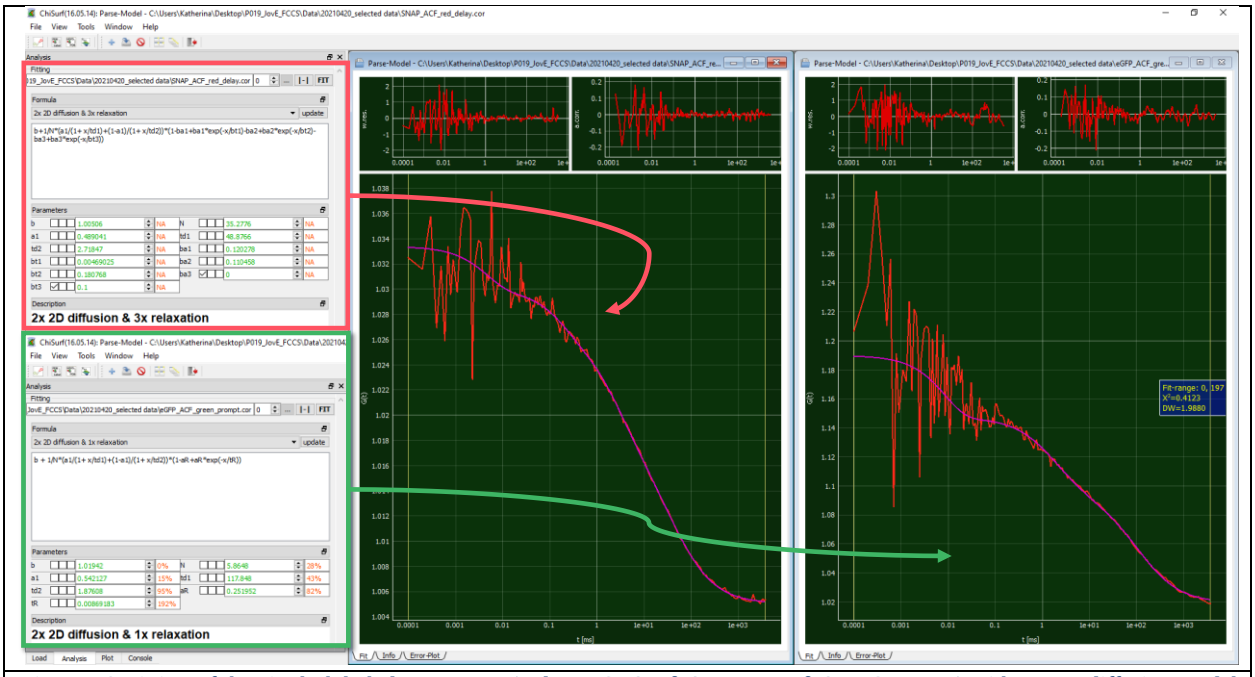


Figure 28 Fitting of the single-labeled constructs (red: NT-SNAP-β2AR, green: β2AR-eGFP-IL3) with 2x 2D diffusion model and additional relaxation times..

Based on the obtained number of molecules in focus and the known average count rates, we can also determine the molecular brightness of our fluorophores in the live cell settings and estimate the concentration of molecules using the equations explained in the calibration section.
Take care to subtract the background signal e.g. measured on non-transfected cells from the average count rate of your fluorescence samples (Figure 29).

Single-labeled constructs

Sample	Average countrate [kHz]		Number of molecules	Mol. Brightness [kHz/molecule]	Approx. Concentration [nM]
	s	p			
eGFP	1.87	2.81	5.86	0.80	4.30
SNAP	21.1	39.6	35.28	1.72	67.47

Figure 29 Characterization of the single-labeled constructs

Crosstalk-induced correlations

Based on these single-color experiments also one can test how much artificial / unwanted cross-correlation into the respective other color channel is present.
For this, we export and plot the following correlations:

- From the β2AR-eGFP-IL3 measurements: red channels in the delay time window and PIE cross-correlation (green channel prompt time window with red channels in the delay time window)

- From the NT-SNAP-β_2AR measurements: green channels in the prompt time window and PIE-cross-correlation (green channel prompt time window with red channels in the delay time window)

In ideal case, all of these combinations show flat curves or better-said noise distributed around 1. Here, this is the case only for the PIE-cross-correlations (Figure 30). The respective "false-color" autocorrelation functions, red-delay from β_2AR-eGFP-IL3 and green-prompt from CT-SNAP-β_2AR, reflect the crosstalk into the red channels and the direct excitation of red fluorophore by the green excitation wavelength, respectively.

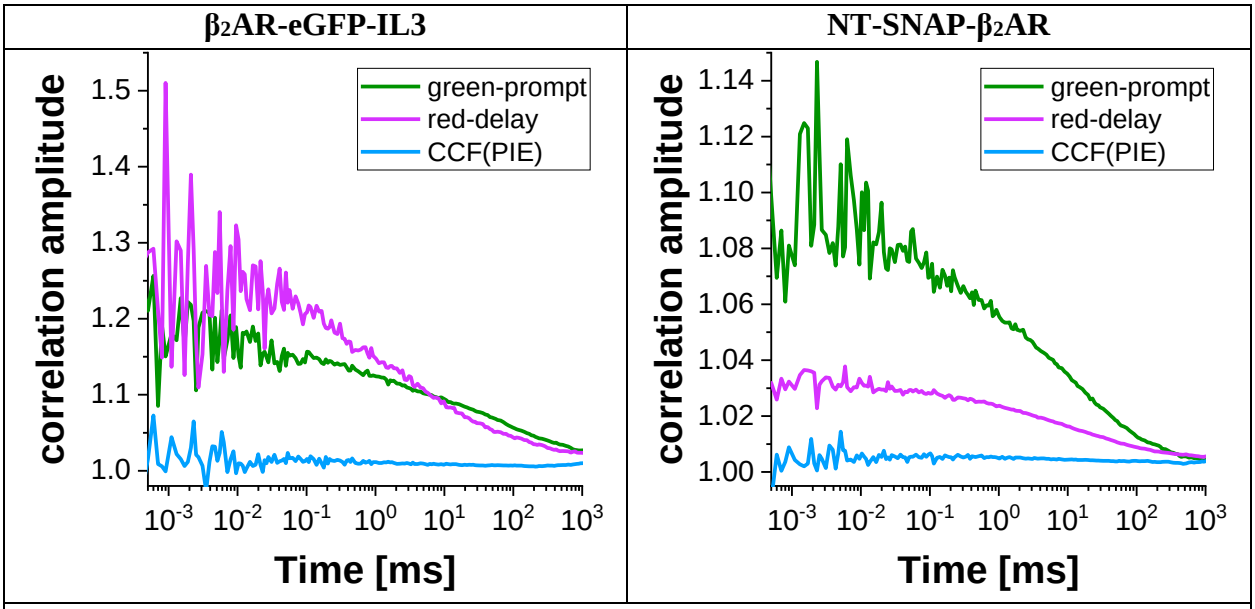


Figure 30 Autocorrelations and the PIE-based crosscorrelation of the single-labeled constructs.

Double-labeled sample: NT-SNAP-β_2AR-eGFP-IL3

Fit of auto- and cross-correlation curves

Open all three average curves, the green-prompt autocorrelation, red-delay autocorrelation and PIE-cross-correlation curve in ChiSurf. For both autocorrelation curves, we add the fit model with bimodal membrane diffusion and an additional relaxation term. For the PIE-cross-correlation curve, a bimodal membrane diffusion model is sufficient (Figure 31).

For the red autocorrelation and especially the PIE-cross-correlation, the fit ranges have to be adjusted as particularly for short correlation curves the noise is very high and no reliable fit could be acquired here.

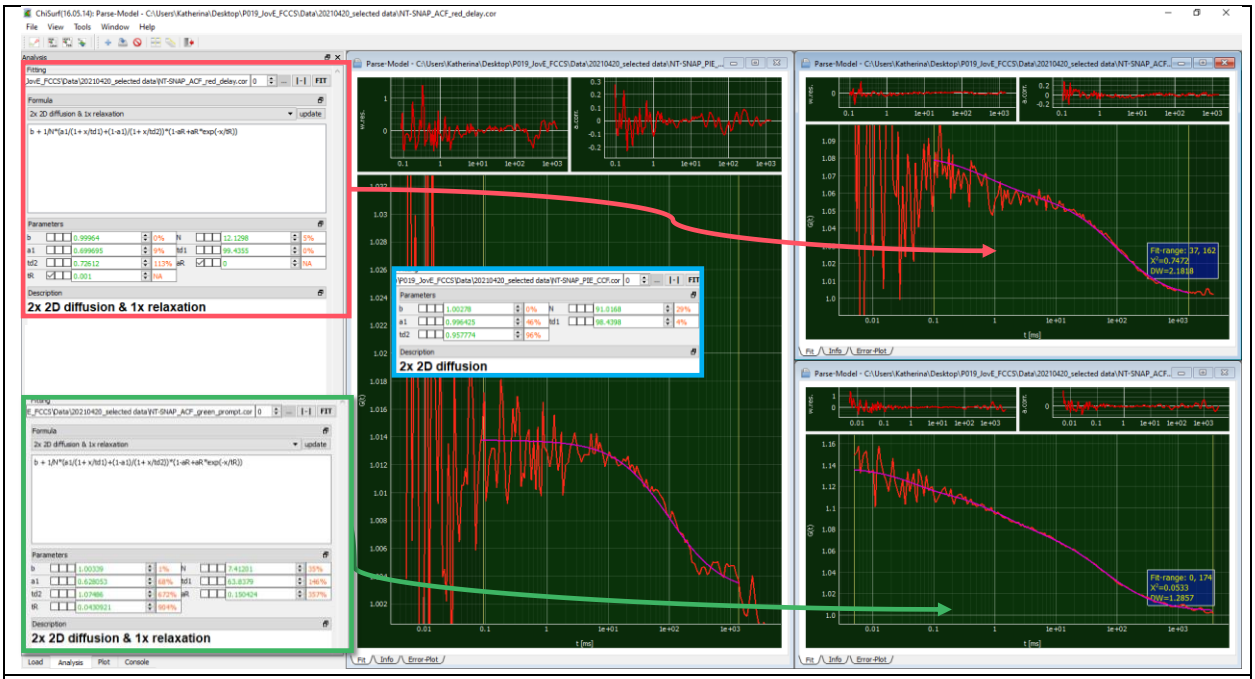


Figure 31 Global fit of both autocorrelation curves (green: eGFP, red: SNAP-substrate) and PIE-based crosscorrelation curve (blue, large window).

Calculation of co-diffusing molecules

Based on the (apparent) number of molecules in focus and our determined correction factors for the confocal overlap volume from the DNA measurements, the fraction of double-labeled molecules can be calculated (Figure 32).

Double-labeled construct: NT-SNAP & IL3-eGFP						
Sample	Average countrate [kHz]		App. number of molecules	Approx. Concentration [nM]	ratio $G_0(tc)$	fraction co-diffusion
	s	p				
Green-prompt	3.16	4.85	7.41	3.56		
Red-delay	0.53	1.45	12.13	2.78		
PIE			91.0	0.52	0.08	0.15
				0.72	0.13	0.26

Example calculation:						
DNA -> 100 % co-diffusion						
$G_{0ACF,green}$ =	1		$r_{green,ideal}$=	0.5		
$G_{0ACF,red}$ =	0.8		$r_{red,ideal}$=	0.625		
G_{0CCF} =	0.5					
Cell sample shows					fraction co-diffusion:	
$G_{0ACF,green}$ =	0.12		$r_{green,cell}$=	0.167	33.3	%
$G_{0ACF,red}$ =	0.08		$r_{red,cell}$=	0.25	40.0	%
G_{0CCF} =	0.02					

Figure 32 Calculation of the fraction of co-diffusing molecules.

Here, N_{eGFP} = 7.4, N_{SNAP} = 12.1 and $N_{app,PIE}$ = 91. Thus, the amplitudes are zero correlation time G(t_c=0) have the following values: $G_{eGFP}(0)$ = 0.135, $G_{SNAP}(0)$ = 0.082, and $G_{PIE}(0)$ = 0.011.
Using the correction factors from the DNA measurement, here only between 15 -26 % molecules show both labels. However, (i) the data is quite noisy and (ii) the correlation amplitudes are very low. Both factors lead to large errors.
Conclusion: Search for cells with low expression level, i.e. low fluorescence and take your time to collect a decent amount of photons to correlate!

Analysis of simulated data

Simulation details

The simulations of the β_2AR-eGFP-IL3-CT-SNAP measurements (short: CTSNAP) were performed using Burbulator [5] (part of the MFD software package, https://www.mpc.hhu.de/software/3-software-package-for-mfd-fcs-and-mfis).
The NTSNAP construct has both fluorophore on the inner side in a membrane and we assume (i) the fluorophore to be close enough to each other to undergo FRET and (ii) the membrane receptor β_2AR to show dynamics such that the fluorophores exchange between two different levels of FRET.
Burbulator uses the Becker&Hickl spc-file format with 4096 TAC channel with a width of 4.07 ps and a laser period of 13.596 ns. The green-to-red detection efficiency ratio and the g-factor was set 1 and the fundamental anisotropy to 0.38. The fluorescence lifetime of both eGFP and SNAP was set to 3 ns with a molecular brightness of 10 kHz/molecule, fluorescence quantum yield of 0.8 and a rotational correlation time of 100 ns. Additionally the background in the green channel was set to 1 kHz and in the red channel to 0.5 kHz. The green crosstalk into the red channels was set to 0.1. All listed values for the fluorophores were adopted based on our measurements from the NTSNAP construct and the setup-describing values were set to reasonable values or 1.
The mean lifetime of the low FRET (LF) and high FRET (HF) states for dynamic exchange was set to 2.4 ns (E = 0.2) and 0.9 ns (E = 0.7). The equilibrium fractions of LF and HF were set to 0.5 each with a relaxation rate of 71 µs and – in case of triplet – with 16 % triplet blinking at 5.5 µs (Caution: Burbulator adds triplet blinking to both donor and acceptor molecules at the same time!).
The diffusion term was modeled as a bimodal distribution with 30 % of fast diffusing molecules at t_{D1} = 1 ms and the rest of the molecules diffusing slowly with t_{D2} = 100 ms.
In total, 10^7 photons were simulated in a 3D Gaussian shaped volume with w_0 = 0.5 µm and z_0 = 1.5 µm, a box size of 20, and N_{FCS} = 0.01.
Of note: As the slower modeled diffusion time is quite long, the number of photons and the box size might have to be increased further to allow good fitting at large correlation times t_c. Here, for the sake of time / simplicity, the fitting was stopped at t_c = 1 sec.

Global fit of auto- and cross-correlation curves

Before starting ChiSurf, add the fit model for the cross-correlation curve to your JSON-file as described above:

$$G_{CCF,2D}(t_c) = b + \frac{1}{N}\left[\frac{a_1}{1 + {t_c}/{t_{d1}}} + \frac{1 - a_1}{1 + {t_c}/{t_{d2}}}\right]\left[1 - a_R \cdot \exp\left(-\frac{t_c}{t_R}\right)\right]$$

where a_R and t_R describe the amplitude and relaxation time of the anticorrelation.

A more general equation – in case of more than one relaxation term – would have the following form:

$$G_{CCF,2D}(t_c) = b + \frac{1}{N}\left[\frac{a_1}{1 + {t_c}/{t_{d1}}} + \frac{1 - a_1}{1 + {t_c}/{t_{d2}}}\right]\left[1 - a_f\right]\left[1 - \sum_i a_{Ri} \cdot \exp\left(-\frac{t_c}{t_{Ri}}\right)\right]$$

where a_f describes the total amplitude of the anticorrelation (identical to a_R in the single anticorrelation term model above) and a_{Ri} and t_{Ri} the respective relaxation times and amplitudes. Load in total five different correlation curves into ChiSurf:

- Green-prompt (autocorrelation of green signal in prompt time window)
- Red-prompt (autocorrelation of the FRET-induced red signal in the prompt time window)
- Red-delay (autocorrelation of the red signal (direct excitation) in the delay time window)
- FRET-CCF (cross-correlation of green prompt and red-prompt signal)
- PIE-CCF (cross-correlation of green-prompt with red-delay)

The two new curves (red-delay and FRET-CCF), which we have not used to far, both stem from the FRET-induced red signal now present in our data (Figure 33).

Figure 33 Global fit of all correlation curves of simulated data of a CT-SNAP construct. No triplet blinking was added.

Due to the FRET-induced anticorrelated behavior of green and red signal in the prompt time window. The FRET-CCF shows a "dip" at short correlation time, coinciding with a rise in both autocorrelation curves from the prompt time window.

All five loaded curves are fit jointly with linked t_{D1}, t_{D2} and t_R. The fit results are summarized in the table below. Please note that here the diffusion times can be fit jointly as the simulation software does not support the modelling of differently sized confocal detection volumes. For experimental results, this joint fitting of t_D might not be possible, however t_R should be linked.

Parameter	green-prompt	red-prompt	red-delay	FRET-CCF	PIE-CCF
N(app)	3.90	1.09	0.455	2.04	1.23
t_{D1} **[ms]**	86.0				
a_1	0.710	0.687	0.694	0.677	0.687
t_{D2} **[ms]**	0.928				
a_R	0.203	0.144	0	0.213	0
t_R **[µs]**	76.9				

Influence of FRET efficiency

The extent of the anticorrelation can be used also as a marker for the extent of change in FRET efficiency E: If we change the LF state to $E = 0$ and the HF state to $E = 0.95$, the induced dip in the FRET-CCF is much more pronounced than for the first example (Figure 34).

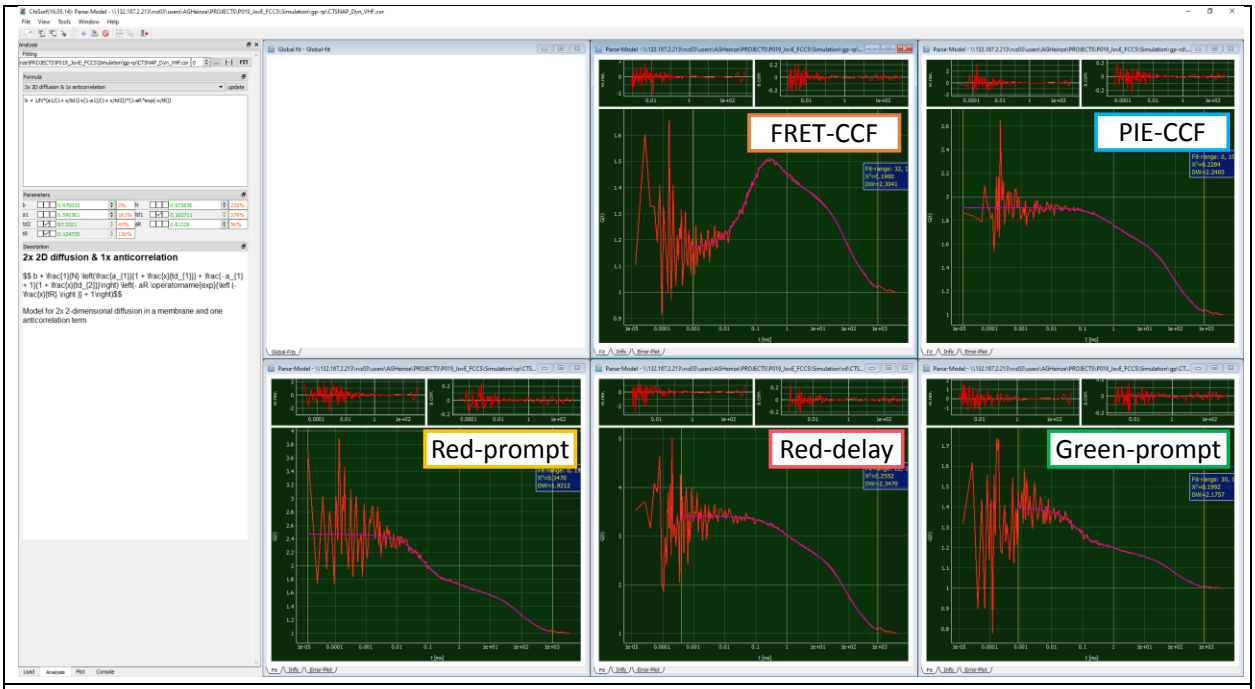


Figure 34 Global fit of all correlation curves of simulated data of a CT-SNAP construct with very high FRET contrast. No triplet blinking was added.

The amplitude of the anticorrelation has increased to a_R = 0.66 compared to an a_R = 0.21 from the previous case.

Parameter	green-prompt	red-prompt	red-delay	FRET-CCF	PIE-CCF
N(app)	2.45	0.664	0.397	0.973	1.05
t_{D1} **[ms]**	91.8				
a_1	0.722	0.704	0.728	0.713	0.77
t_{D2} **[ms]**	0.886				
a_R	0.39	0.392	0	0.662	0
t_R **[µs]**	70.5				

Influence of triplet blinking

Be aware that significant amount of triplet blinking in the µs time range of the donor and / or acceptor fluorophore might mask the anticorrelation induced due to FRET in the FRET-CCF.
In the example shown below (Figure 35), 16 % of additional triplet blinking at 5.5 µs was added to the example of LF(E = 0.2) <-> HF(E = 0.7).

Here, the FRET-CCF model has to be extended for a triplet-induced correlation term:

$$G_{CCF,2D}(t_c) = b + \frac{1}{N}\left[\frac{a_1}{1+{t_c}/{t_{d1}}} + \frac{1-a_1}{1+{t_c}/{t_{d2}}}\right]\left[1 - a_R \cdot \exp\left(-\frac{t_c}{t_R}\right)\right]\left[1 - a_T + a_T \cdot \exp\left(-\frac{t_c}{t_T}\right)\right]$$

where a_T and t_T describe the amplitude and relaxation time of the triplet component.

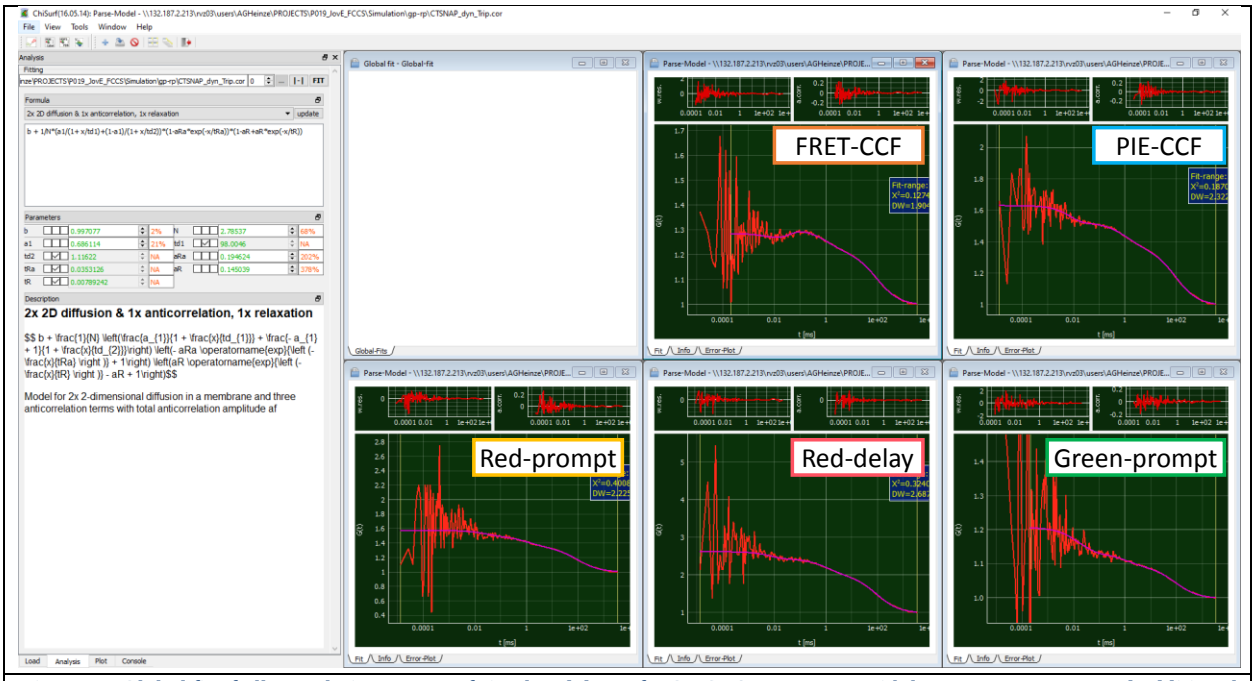


Figure 35 Global fit of all correlation curves of simulated data of a CT-SNAP construct with low FRET contrast and additional triplet blinking.

One can see quite nicely in the FRET-CCF how the "dip" in the curve due to the anticorrelation term is counteracted by the triplet component.

Parameter	green-prompt	red-prompt	red-delay	FRET-CCF	PIE-CCF
N(app)	4.79	1.73	0.611	1.73	1.57
t_{D1} **[ms]**			98.0		
a_1	0.678	0.670	0.677	0.670	0.674
t_{D2} **[ms]**			1.12		
a_R	0.210	0.115	0	0.115	0
t_R **[µs]**			35.3		
a_T	0.146	0.015	0.113	0.015	0.171
t_T **[µs]**			7.89		

Species-filtered FCS to recover dynamics

A method to recover the triplet-masked anticorrelations in the FRET-CCF is to make use of the microtimes (i.e. the fluorescence decay histograms) encoded in the data. Here, instead of direct photon traces, an additional weighting function is introduced based on the fluorescence decay shape of the (i) IRF, (ii) the LF state, and (iii) the HF state. More details on how to generate these weighting functions can be found e.g. in the following literature [6,7].
In this species-specific or filtered FCS approach, four different correlation pattern are generated:

- Species-autocorrelation of the LF state ($sACF_{LF\text{-}LF}$)
- Species-autocorrelation of the HF state ($sACF_{HF\text{-}HF}$)

- Species-cross-correlation of the LF state to the HF state ($sCCF_{LF\text{-}HF}$)
- Species-cross-correlation of the HF state to the LF state ($sCCF_{HF\text{-}LF}$)

Below, exemplary the work flow and input for a filteredFCS analysis of the LF(E = 0.2) <-> HF(E = 0.7) example with additional triplet is shown (Figure 36).

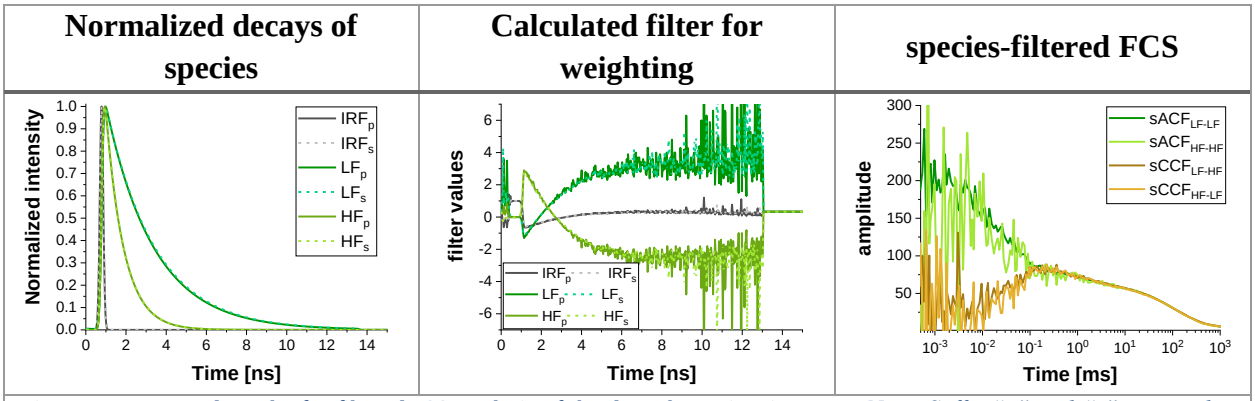


Figure 36 Input and results for filteredFCS analysis of the data shown in Figure 35. Note: Suffix "p" and "s" are used to discriminate between the parallel (p) and perpendicular (s) channel here.

For generation of the species-filtered FCS curves, the weights determined for the LF and HF species based on the normalized intensity decays are used during the correlation.

The resulting curves are fit to standard equations with bimodal membrane diffusion and relaxation and anticorrelation terms, respectively (Figure 37).

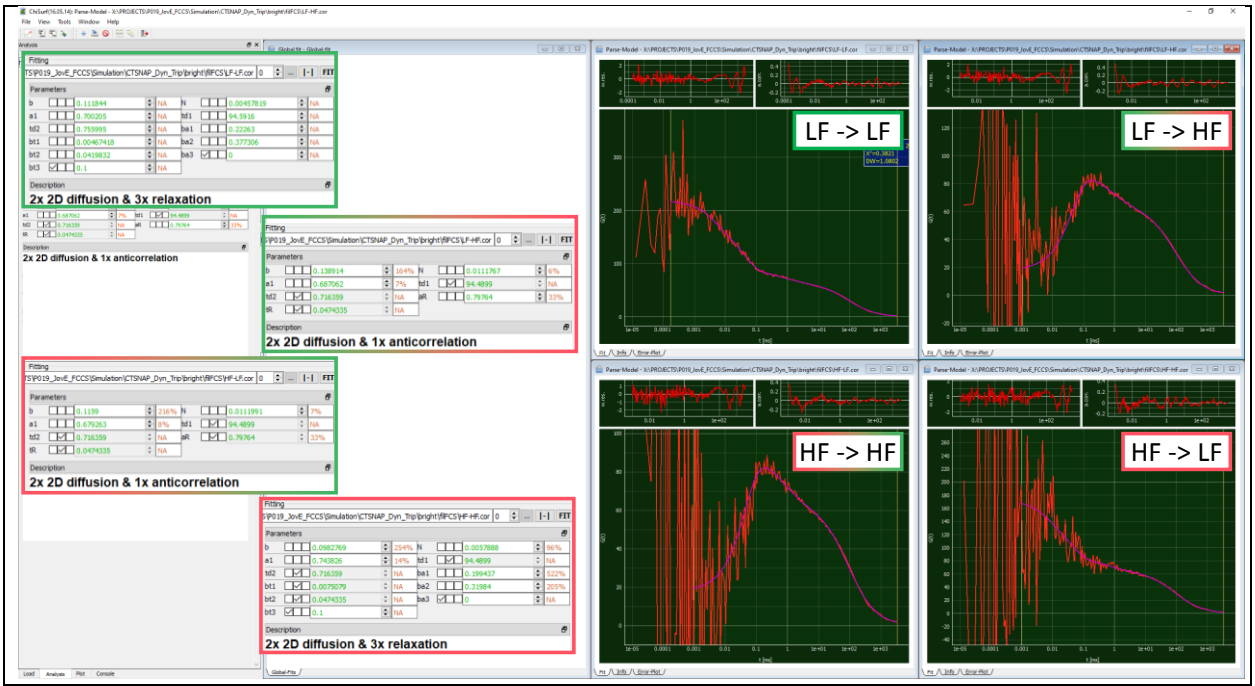


Figure 37 Global analysis of the generated filteredFCS curves

During the fit, both diffusion times t_{D1} and t_{D2} as well as the relaxation times are fit jointly and the FRET-induced relaxation of the $sACF_{LF\text{-}LF}$ and $sACF_{HF\text{-}HF}$ is linked to the anticorrelation term of the $sCCF_{LF\text{-}HF}$ and $sCCF_{HF\text{-}LF}$.
Be aware that the number of molecules in focus, N, is only an apparent number and does no longer relate to the concentration of molecules in the experiment / simulations!

Parameter	LF->LF	HF->HF	LF->HF	HF->LF
N(app)	0.0046	0.0058	0.011	0.011
t_{D1} **[ms]**	94.6			
a_1	0.700	0.687	0.687	0.679
t_{D2} **[ms]**	0.756			
a_R	0.378	0.320	0.798	
t_R **[µs]**	42.0			
a_T	0.223	0.199	0	0
t_T **[µs]**	4.67			

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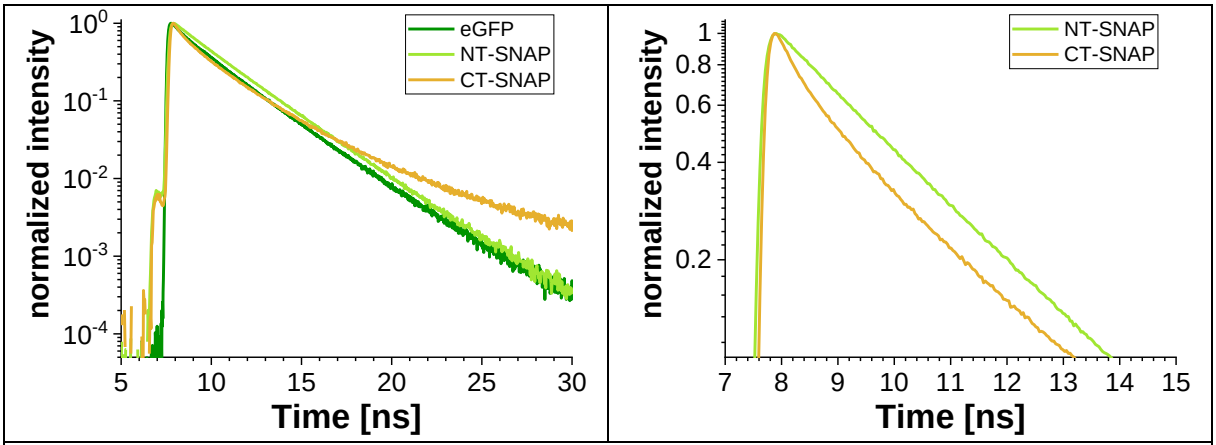
Fluorescence intensity of β_2AR-IL3-eGFP, NT-SNAP-β_2AR-IL3-eGFP and β_2AR-IL3-eGFP-CT-SNAP



Normalized photon arrival time histograms of β_2AR-IL3-eGFP (green), NT-SNAP-β_2AR-IL3-eGFP (light green) and β_2AR-IL3-eGFP-CT-SNAP (orange). The Zoom-in on the right side shows clearly the "curved" course of the fluorescence intensity decay in the CT-SNAP induced by FRET while in contrast the NT-SNAP sample shows a nearly linear decay.

Supplemental File (Figures, Permissions, etc.)

Click here to access/download;Supplemental File (Figures, Permissions, etc.);S7_FCCS Calibration.xlsx

Filename
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\\HC1008\Users\AG Heinze\DATA\FCSSetup\2021\20210223_MF_khm_Cal\a488 2nM_05.ptu
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\\HC1008\Users\AG Heinze\DATA\FCSSetup\2021\20210223_MF_khm_Cal\EB_02.ptu

Duration [s]	CR gs [kHz]	CR gp [kHz]	CR rs(prompt) [kHz]	CR rp (prompt) [kHz]
300.00	5.27	5.38	1.25	1.57
300.00	5.26	5.34	1.23	1.54
300.00	5.30	5.36	1.20	1.52
300.00	5.30	5.39	1.22	1.52
300.00	5.35	5.43	1.23	1.53
300.00	0.33	0.45	2.01	2.46
300.00	0.32	0.45	1.95	2.41
300.00	0.31	0.41	1.88	2.35
300.00	0.31	0.40	1.85	2.33
300.00	0.31	0.40	1.83	2.29
300.00	0.13	0.23	0.47	0.79
300.00	0.13	0.23	0.44	0.79
300.00	34.10	35.59	7.45	8.79
300.00	41.58	43.28	8.98	10.52
300.00	47.13	48.97	10.12	11.78
300.00	51.00	52.98	10.91	12.67
300.00	50.65	52.60	10.85	12.60
60.00	14.51	22.55	12.48	19.77
60.00	14.56	22.57	12.53	19.81

CR rs(delay) [kHz]	CR rp (delay) [kHz]
0.41	0.72
0.41	0.72
0.38	0.71
0.40	0.71
0.41	0.71
4.55	5.11
4.43	5.00
4.25	4.83
4.18	4.74
4.10	4.66
0.39	0.69
0.36	0.69
6.05	9.60
7.41	11.64
8.43	13.13
9.15	14.23
9.16	14.19
0.58	1.10
0.58	1.09

Confocal volume

D	414	µm²/s	***Literature value***
	4.14E-10	m²/s	
td	69	µs	***Fit result - please add!***
	0.000069	s	
s	6.49		***Fit result - please add!***

w0²	1.14264E-13	
w0	3.3803E-07	m
	0.338	**µm**
z0	2.19381E-06	m
	2.194	**µm**

pi^(3/2)	5.568327997		
V	1.39583E-18	m³	
	1.396	**fl**	1 fl = 1e-15 l
			1000 l = 1 m³

1 Mol 6.02E+23

Concentration estimation

Alexa488

N	0.76	number	***Fit result - please add!***
c	5.41612E+14	number/liter	
c	8.99E-10	Mol/l	
	8.99E-07	mM	
	8.99E-04	µM	
	0.90	**nM**	

Countrates A488

	ch 0 / s [kHz]	ch 2 / p [kHz]	sum [kHz]	N
2	5.27	5.38	10.65	0.766
3	5.26	5.34	10.60	0.753
4	5.30	5.36	10.66	0.757
5	5.30	5.39	10.69	0.748
6	5.35	5.43	10.78	0.756

Mean	5.30	5.38	10.68	0.76
stdev	0.03	0.03	0.06	0.01

Countrate background

Countrate red channels

	ch 0 / s [kHz] ddH2O	ch 2 / p [kHz] ddH2O	ch 1 / s [kHz] A488	ch 3 / p [kHz] A488
1	0.13	0.23	1.25	1.57
2	0.13	0.23	1.23	1.54
3			1.20	1.52
4			1.22	1.52
5			1.23	1.53

Mean	0.13	0.23	1.23	1.53
stdev	0.00	0.00	0.02	0.02

Green crosstalk into red channel

	CR [kHz] A488	CR [kHz] BG	CR_{A488}-CR_{BG} [kHz]
0 prompt	5.30	0.13	5.18
1 prompt	1.23	0.36	0.87
2 prompt	5.38	0.23	5.15
3 prompt	1.53	0.69	0.85

brightness [kHz/molecule]
12.28
12.44
12.50
12.66
12.63

12.50
0.14

spectral crosstalk [%] **α**
16.61

Confocal volume

D	414	µm²/s	***Literature value***
	4.14E-10	m²/s	
td	85.7	µs	***Fit result - please add!***
	0.0000857	s	
s	5.84		***Fit result - please add!***

w0²	1.41919E-13		
w0	3.76722E-07	m	
	0.377	**µm**	
z0	2.20005E-06	m	
	2.200	**µm**	

pi^(3/2)	5.568327997		
V	1.7386E-18	m³	
	1.739	**fl**	1 fl = 1e-15 l
			1000 l = 1 m³

1 Mol 6.02E+23

Concentration estimation

Alexa488

N	0.76	number	***Fit result - please add!***
c	4.34833E+14	number/liter	
c	7.22E-10	Mol/l	
	7.22E-07	mM	
	7.22E-04	µM	
	0.72	**nM**	

Countrates A488

	ch 0 / s [kHz]	ch 2 / p [kHz]	sum [kHz]	N
2	5.27	5.38	10.65	0.766
3	5.26	5.34	10.60	0.753
4	5.30	5.36	10.66	0.757
5	5.30	5.39	10.69	0.748
6	5.35	5.43	10.78	0.756

Mean	5.30	5.38	10.68	0.76
stdev	0.03	0.03	0.06	0.01

Countrate background

Countrate red channels

	ch 0 / s [kHz] ddH2O	ch 2 / p [kHz] ddH2O	ch 1 / s [kHz] A488	ch 3 / p [kHz] A488
1	0.13	0.23	1.25	1.57
2	0.13	0.23	1.23	1.54
3			1.20	1.52
4			1.22	1.52
5			1.23	1.53

Mean	0.13	0.23	1.23	1.53
stdev	0.00	0.00	0.02	0.02

Green crosstalk into red channel

	CR [kHz] A488	CR [kHz] BG	CR_{A488}-CR_{BG} [kHz]
0 prompt	5.30	0.13	5.18
1 prompt	1.23	0.36	0.87
2 prompt	5.38	0.23	5.15
3 prompt	1.53	0.69	0.85

brightness [kHz/molecule]
12.28
12.44
12.50
12.66
12.63

12.50
0.14

spectral crosstalk [%] **α**
16.61

Confocal volume

D	345	µm²/s	***Literature value***
	3.45E-10	m²/s	
td	99.5	µs	***Fit result - please add!***
	0.0000995	s	
s	6.78		***Fit result - please add!***
$w0^2$	1.3731E-13		
w0	3.70554E-07	m	
	0.371	**µm**	
z0	2.51235E-06	m	
	2.512	**µm**	
pi^(3/2)	5.568327997		
V	1.92091E-18	m³	
	1.921	**fl**	1 fl = 1e-15 l
			1000 l = 1 m³
1 Mol	6.02E+23		

Concentration estimation

Alexa488			
N	3.44	number	***Fit result - please add!***
c	1.7929E+15	number/liter	
c	2.98E-09	Mol/l	
	2.98E-06	mM	
	2.98E-03	µM	
	2.98	**nM**	

Countrates A568

	ch 1 / s [kHz] delay	ch 3 / p [kHz] delay	sum [kHz]	N
1	4.55	5.11	9.66	3.16
2	4.43	5.00	9.43	3.30
3	4.25	4.83	9.08	3.50
4	4.18	4.74	8.92	3.59
5	4.10	4.66	8.76	3.67

Mean	**4.24**	**4.81**	**9.17**	**3.44**
stdev	**0.12**	**0.13**	**0.33**	**0.19**

Countrate background

	ch 1 / s [kHz] ddH2O	ch 3 / p [kHz] ddH2O	ch 1 / s [kHz] prompt	ch 3 / p [kHz] prompt
1	0.390	0.69	2.01	2.46
2	0.36	0.69	1.95	2.41
3			1.88	2.35
4			1.85	2.33
5			1.83	2.29

Mean	**0.36**	**0.69**	**1.90**	**2.37**
stdev	**0.00**	**0.00**	**0.07**	**0.06**

Direct excitation of A568 by 488-laser line

	CR [kHz] A568	CR [kHz] BG	CR_{A568}-CR_{BG} [kHz]
1 Delay	4.24	0.36	3.88
1 Prompt	1.90	0.36	1.54
1 Delay	4.81	0.69	4.12
1 Prompt	2.37	0.69	1.68

brightness [kHz/molecule]
3.06
2.86
2.60
2.48
2.39

2.68
0.25

direct excitation [%] γ
40.31

Effective overlapping confocal volume

D	77.09	µm²/s	***based on green /red***
	7.7086E-11	m²/s	
td	520	µs	***Fit result - please add!***
	0.00052	s	
s	4.63		***Fit result - please add!***

w0²	1.6034E-13	
w0	4.0042E-07	m
	0.400	**µm**
z0	1.854E-06	m
	1.854	**µm**

pi^(3/2)	5.568328	
V	1.6552E-18	m³
	1.655	**fl**

1 fl = 1e-15 l

1000 l = 1 m³

	Green				
	N	c [nM]	td [µs]*	D [µm²/s]	N
2	11.6	11.08	434	81.75	14.7
3	8.53	8.15	434	81.75	10.8
4	7.79	7.44	434	81.75	8.73
5	7.54	7.20	434	81.75	9.01
6	7.61	7.27	434	81.75	8.94

Mean	**7.87**	**7.52**	**434.00**	**81.75**	**10.44**
stdev	**0.39**	**0.38**	**0.00**	**0.00**	**2.26**

*global fitting

Average diffusion coefficient D [µm²/s]:	**77.09**

			Green - Red Crosscorrelation		
	Napp	G(t,CC)	**cRG [nM] (g)**	**$G_{0,CCF}/G_{0ACFgreen}$**	**cRG [nM] (r)**
2	20.4	0.049	6.30	0.57	9.16
3	15.2	0.066	4.57	0.56	6.64
4	13.6	0.074	4.26	0.57	4.85
5	13.5	0.074	4.02	0.56	5.20
6	13.4	0.075	4.13	0.57	5.16

$$c_{RG} = \frac{G_{0,CCF}}{G_{0,ACF,green}} * c_{green}$$

$$c_{RG} = \frac{G_{0,C}}{G_{0,AC}}$$

Red		
c [nM]	td [µs]*	D [µm²/s]
12.71	474	72.42
9.34	474	72.42
7.55	474	72.42
7.79	474	72.42
7.73	474	72.42

9.02	**474.00**	**72.42**
1.95	**0.00**	**0.00**

$G_{0,CCF}/G_{0ACFred}$	AVG cRG [nM]
0.72	7.73
0.71	5.60
0.64	4.55
0.67	4.61
0.67	4.64

$\frac{CCF}{Fred} * c_{red}$

Supplemental File (Figures, Permissions, etc.)

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Filename	Duration [s]	CR gs [kHz]	CR gp [kHz]	CR rs(prompt) [kHz]
a488 2nM_06.ptu	300.00	5.35	5.43	1.23
a568_2 nM_05.ptu	300.00	0.31	0.40	1.83
DD_H2O_02.ptu	300.00	0.13	0.23	0.44
DNA a488 568 10nM_03.ptu	300.00	41.58	43.28	8.98
EB_01.ptu	60.00	14.51	22.55	12.48

CR rp (prompt) [kHz]	CR rs(delay) [kHz]	CR rp (delay) [kHz]
1.53	0.41	0.71
2.29	4.10	4.66
0.79	0.36	0.69
10.52	7.41	11.64
19.77	0.58	1.10

Confocal volume

D	414	µm²/s	*Literature value*	D	350	
	4.14E-10	m²/s			3.5E-10	
t_d	76	µs	*Fit result - please add!*	t_d	117	
	0.000076	s			0.000117	
s	7.75		*Fit result - please add!*	s	3.63	
w_0^2	1.26E-13			w_0^2	1.64E-13	
w_0	3.55E-07	m		w_0	4.05E-07	
	0.355	µm			0.405	
z_0	2.75E-06	m		z_0	1.47E-06	
	2.749	µm			1.469	
pi^(3/2)	5.568328			pi^(3/2)	5.568328	
V	1.93E-18	m³		V	1.34E-18	
	1.927	fl	1 fl = 1e-15 l		1.340	
			1000 l = 1 m³			

Concentration estimation

Co

Alexa488				Alexa488		
N	0.76	number	*Fit result - please add!*	N	3.58	
c	3.94E+14	number/liter		c	2.67E+15	
c	6.55E-10	Mol/l		c	4.44E-09	
	6.55E-07	mM			4.44E-06	
	6.55E-04	µM			4.44E-03	
	0.65	nM			4.44	

Effective overlapping confocal volume

D	79.62	µm²/s	*based on green /red*
	7.96E-11	m²/s	
t_d	502	µs	*Fit result - please add!*
	0.000502	s	
s	4.45		*Fit result - please add!*
w_0^2	1.6E-13		
w_0	4E-07	m	
	0.400	µm	
z_0	1.78E-06	m	
	1.779	µm	
pi^(3/2)	5.568328		
V	1.58E-18	m³	

	1.584	fl

Confocal volume

µm²/s	*Literature value*
m²/s	
µs	*Fit result - please add!*
s	
	Fit result - please add!

m	
µm	
m	
µm	

m³	
fl	1 fl = 1e-15 l
	1000 l = 1 m³

ncentration estimation

number	*Fit result - please add!*
number/liter	
Mol/l	
mM	
µM	
nM	

ch 0 / s [kHz]
5.35

Countrate backgr

ch 0 / s [kHz] ddH2O
0.13

	CR [kHz] A488
0 prompt	5.35
1 prompt	1.23
2 prompt	5.43
3 prompt	1.53

ch 1 / s [kHz] delay
4.10

ch 1 / s [kHz] ddH2O
0.440

	CR [kHz] A568
1 Delay	4.10
1 Prompt	1.83
3 Delay	4.66
3 Prompt	2.29

Green					
N	c [nM]	td [µs]	D [µm²/s]	N	c [nM]
8.27	7.13	433	72.67	10.9	13.51

Average diffusion coefficient D [µm²/s]:	79.62

Green - Red Crosscorrelation

Napp	G(t,CC)	cRG [nM] (g)	$G_{0,CCF}/G_{0ACFgreen}$	cRG [nM] (r)	$G_{0,CCF}/G_{0ACFred}$
15.1	0.066	3.90	0.55	9.75	0.72

$$c_{RG} = \frac{G_{0,CCF}}{G_{0,ACF,green}} * c_{green}$$

$$c_{RG} = \frac{G_{0,CCF}}{G_{0,ACFred}} * c_{red}$$

Countrates A488			
ch 2 / p [kHz]	sum [kHz]	N	brightness [kHz/molecule]
5.43	10.78	0.760	12.57

ound	Countrate red channels	
ch 2 / p [kHz] ddH2O	ch 1 / s [kHz] A488	ch 3 / p [kHz] A488
0.23	1.23	1.53

Green crosstalk into red channel	
CR [kHz] BG	CR_{A488}-CR_{BG} [kHz]
0.13	5.22
0.44	0.79
0.23	5.20
0.79	0.74

spectral crosstalk [%] α

14.65

Countrates A568			
ch 3 / p [kHz] delay	sum [kHz]	N	brightness [kHz/molecule]
4.66	8.76	3.58	2.45

Countrate background		
ch 3 / p [kHz] ddH2O	ch 1 / s [kHz] prompt	ch 3 / p [kHz] prompt
0.79	1.83	2.29

Direct excitation of A568 by 488-laser line	
CR [kHz] BG	CR_{A568}-CR_{BG} [kHz]
0.44	3.66
0.44	1.39
0.79	3.87
0.79	1.50

direct excitation [%] γ

38.40

Red	
td [µs]	D [µm²/s]
473	86.58

AVG cRG [nM]
6.83

Supplemental File (Figures, Permissions, etc.)

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# Filename	Duration [s]	CR gs [kHz]	CR gp [kHz]	CR rs(prompt) [kHz]
\\HC1008\Users\AG Heinze\DA	387.92	0.26	0.43	0.54
\\HC1008\Users\AG Heinze\DA	340.96	0.23	0.38	0.53
\\HC1008\Users\AG Heinze\DA	253.26	0.24	0.39	0.53
\\HC1008\Users\AG Heinze\DA	27.73	14.67	30.27	35.78
\\HC1008\Users\AG Heinze\DA	59.32	14.47	29.80	35.82
\\HC1008\Users\AG Heinze\DA	21.17	14.37	29.56	35.52
\\HC1008\Users\AG Heinze\DA	19.49	76.41	118.82	107.81
\\HC1008\Users\AG Heinze\DA	9.81	76.76	119.07	106.02
\\HC1008\Users\AG Heinze\DA	16.84	77.96	121.07	108.06
\\HC1008\Users\AG Heinze\DA	60.00	31.22	31.74	5.53
\\HC1008\Users\AG Heinze\DA	60.00	33.66	34.11	5.86
\\HC1008\Users\AG Heinze\DA	60.00	34.68	35.19	6.03
\\HC1008\Users\AG Heinze\DA	60.00	0.96	1.14	5.41
\\HC1008\Users\AG Heinze\DA	60.00	0.93	1.09	5.40
\\HC1008\Users\AG Heinze\DA	60.00	0.92	1.09	5.37
\\HC1008\Users\AG Heinze\DA	60.00	24.69	24.62	8.71
\\HC1008\Users\AG Heinze\DA	60.00	24.72	24.60	8.70
\\HC1008\Users\AG Heinze\DA	60.00	24.90	24.78	8.72
\\HC1008\Users\AG Heinze\DA	60.00	40.98	42.46	10.74
\\HC1008\Users\AG Heinze\DA	60.00	40.97	42.51	10.76
\\HC1008\Users\AG Heinze\DA	60.00	40.92	42.42	10.87
\\HC1008\Users\AG Heinze\DA	120.00	3.41	5.23	1.00
\\HC1008\Users\AG Heinze\DA	120.00	2.70	4.13	1.01
\\HC1008\Users\AG Heinze\DA	120.00	2.63	3.89	1.02
\\HC1008\Users\AG Heinze\DA	120.00	1.52	2.30	1.00
\\HC1008\Users\AG Heinze\DA	120.00	1.28	1.95	0.94
\\HC1008\Users\AG Heinze\DA	256.86	1.16	1.78	0.89
\\HC1008\Users\AG Heinze\DA	180.42	2.51	4.73	9.32
\\HC1008\Users\AG Heinze\DA	186.66	2.78	5.24	10.50
\\HC1008\Users\AG Heinze\DA	192.62	3.63	6.69	13.57
\\HC1008\Users\AG Heinze\DA	110.73	4.42	7.97	16.60
\\HC1008\Users\AG Heinze\DA	240.25	3.32	5.27	1.21
\\HC1008\Users\AG Heinze\DA	78.69	3.49	5.23	1.40

Average countrates

Sample	Duration [s]	CR gs [kHz]	CR gp [kHz]	CR rs(prompt) [kHz]
Untransfected cell	982.14	0.24	0.40	0.53
A488	180.00	33.19	33.68	5.81
A568	180.00	0.94	1.11	5.40
A488-A568	180.00	24.77	24.67	8.71
40bp DNA	180.00	40.96	42.46	10.79
eGFP cell 1	856.86	2.12	3.21	0.97
SNAP only cell 2	670.42	3.34	6.16	12.50
NTSNAP cell 5	318.95	3.40	5.25	1.31

CR rp (prompt) [kHz]	CR rs(delay) [kHz]	CR rp (delay) [kHz]	corrected cou
0.62	0.39	0.38	
0.58	0.39	0.38	
0.57	0.39	0.38	
69.45	3.98	7.56	
69.61	4.02	7.65	
69.12	4.01	7.66	
149.52	6.05	8.69	
147.12	5.89	8.42	
149.74	5.97	8.50	
5.06	0.43	0.57	
5.30	0.44	0.57	
5.48	0.47	0.61	
5.39	17.82	17.44	
5.40	17.81	17.49	
5.36	17.76	17.46	
8.10	18.26	18.01	
8.13	18.31	18.00	
8.11	18.21	17.98	
10.70	25.54	30.68	
10.68	25.58	30.68	
10.66	25.86	30.68	
1.55	0.72	1.07	3.17
1.43	0.78	1.06	2.46
1.30	0.79	0.91	2.38
1.23	0.85	1.03	1.27
1.15	0.88	1.10	1.04
1.02	0.84	0.97	0.92
17.82	14.32	27.10	2.27
19.89	17.58	33.07	2.54
25.35	24.10	44.59	3.39
30.80	29.88	55.26	4.18
2.34	0.84	1.72	3.08
2.51	1.01	1.95	3.24

CR rp (prompt) [kHz]	CR rs(delay) [kHz]	CR rp (delay) [kHz]	
0.59	0.39	0.38	
5.28	0.45	0.58	
5.38	17.80	17.46	
8.12	18.26	18.00	**Countrates**
10.68	25.66	30.68	
1.28	0.81	1.03	1.87
23.46	21.47	40.01	
2.42	0.93	1.83	3.16

nts

4.84			0.32	0.69	
3.73			0.39	0.68	
3.49			0.40	0.53	Donly
1.90			0.45	0.65	
1.55			0.49	0.72	
1.38			0.44	0.59	
4.33	4.41	8.95	13.93	26.72	
4.84	4.59	9.19	17.19	32.68	Aonly
6.29	5.67	11.13	23.70	44.21	
7.58	6.94	13.32	29.49	54.88	
4.87	-0.11	0.37	0.45	1.33	NTSNAP
4.83	0.00	0.48	0.62	1.57	

s corrected for background of untransfected cells, crosstalk & direct excitation

2.81				
			21.08	39.62
4.85			0.53	1.45

Confocal volume

D	414	µm²/s	***Literature value***
	4.14E-10	m²/s	
t_d	73	µs	***Fit result - please add!***
	0.000073	s	
s	9.67		***Fit result - please add!***

w_0^2	1.20888E-13	
w_0	3.4769E-07	m
	0.348	**µm**
z_0	3.36216E-06	m
	3.362	**µm**

pi^(3/2)	5.568327997		
V	2.26322E-18	m³	
	2.263	**fl**	1 fl = 1e-15 l
			1000 l = 1 m³

1 Mol 6.02E+23

Concentration estimation

Alexa488

N	1.67	number	***Fit result - please add!***
c	7.37888E+14	number/liter	
c	1.23E-09	Mol/l	
	1.23E-06	mM	
	1.23E-03	µM	
	1.23	**nM**	

Countrates A488

	ch 0 / s [kHz]	ch 2 / p [kHz]	sum [kHz]	N
1	31.22	31.74	62.97	1.67
2	33.66	34.11	67.77	1.67
3	34.68	35.19	69.87	1.67
4			0.00	
5			0.00	

Mean	34.17	34.65	40.12	1.67
stdev	0.51	0.54	32.84	0.00

Countrate background

Countrate red channels

	ch 0 / s [kHz] ddH2O	ch 2 / p [kHz] ddH2O	ch 1 / s [kHz] A488	ch 3 / p [kHz] A488
1	0.00	0.00	5.53	5.06
2	0.00	0.00	5.86	5.30
3	0.00	0.00	6.03	5.48
4				
5				

Mean	0.00	0.00	5.81	5.28
stdev	0.00	0.00	0.21	0.17

Green crosstalk into red channel

	CR [kHz] A488	CR [kHz] BG	CR_{A488}-CR_{BG} [kHz]
0 prompt	34.17	0.00	34.17
1 prompt	5.81	0.00	5.81
2 prompt	34.65	0.00	34.65
3 prompt	5.28	0.00	5.28

brightness [kHz/molecule]
34.40
37.07
38.23

36.56
1.61

spectral crosstalk [%] α
16.11

Confocal volume

D	350	µm²/s	***Literature value***
	3.5E-10	m²/s	
t_d	61	µs	***Fit result - please add!***
	0.000061	s	
s	6.25		***Fit result - please add!***
w_0^2	8.54E-14		
w_0	2.92233E-07	m	
	0.292	**µm**	
z_0	1.82645E-06	m	
	1.826	**µm**	
pi^(3/2)	5.568327997		
V	8.68544E-19	m³	
	0.869	**fl**	1 fl = 1e-15 l
			1000 l = 1 m³
1 Mol	6.02E+23		

Concentration estimation

Alexa488			
N	1.77	number	***Fit result - please add!***
c	2.03789E+15	number/liter	
c	3.38E-09	Mol/l	
	3.38E-06	mM	
	3.38E-03	µM	
	3.38	**nM**	

Countrates A568

	ch 1 / s [kHz] delay	ch 3 / p [kHz] delay	sum [kHz]	N
1	17.82	17.44	35.26	1.77
2	17.81	17.49	35.30	1.77
3	17.76	17.46	35.21	1.77
4			0.00	
5			0.00	

Mean	17.78	17.47	21.16	1.77
stdev	0.03	0.02	17.27	0.00

Countrate background

	ch 1 / s [kHz] ddH2O	ch 3 / p [kHz] ddH2O	ch 1 / s [kHz] prompt	ch 3 / p [kHz] prompt
1	0.000	0.00	5.41	5.39
2	0.00	0.00	5.40	5.40
3	0.000	0.00	5.37	5.36
4				
5				

Mean	0.00	0.00	5.40	5.38
stdev	0.00	0.00	0.02	0.02

Direct excitation of A568 by 488-laser line

	CR [kHz] A568	CR [kHz] BG	CR_{A568}-CR_{BG} [kHz]
1 Delay	17.78	0.00	17.78
1 Prompt	5.40	0.00	5.40
1 Delay	17.47	0.00	17.47
1 Prompt	5.38	0.00	5.38

brightness [kHz/molecule]
19.92
19.95
19.89

19.92
0.02

direct excitation [%] γ
30.56

Effective overlapping confocal volume

D	62.34	µm²/s	*based on green /red*
	6.2338E-11	m²/s	
t_d	527	µs	*Fit result - please add!*
	0.000527	s	
s	5.29		*Fit result - please add!*

w_0^2	1.3141E-13	
w_0	3.625E-07	m
	0.363	**µm**
z_0	1.9176E-06	m
	1.918	**µm**

pi^(3/2)	5.568328		
V	1.4032E-18	m³	
	1.403	**fl**	1 fl = 1e-15 l
			1000 l = 1 m³

	Green				Re	
	N	c [nM]	td [µs]*	D [µm²/s]	N	c [nM]
2	4.41	3.24	412	73.35	4.05	7.75
3		0.00				0.00
4	8.32	6.11			10.8	20.66
5		0.00				0.00
6		0.00				0.00

Mean	**8.32**	**1.53**	**#DIV/0!**	**73.35**	**7.43**	**5.68**
stdev	**0.00**	**2.64**	**#DIV/0!**	**0.00**	**3.38**	**8.07**

*global fitting

Average diffusion coefficient D [µm²/s]:	**62.34**

	Green - Red Crosscorrelation					
	N	G(0,CCF)	**cRG [nM] (g)**	**$G_{0,CCF}/G_{0ACFgree}$**	**cRG [nM] (r)**	**$G_{0,CCF}/G_{0ACFred}$**
2	7.9	0.127	1.81	0.56	3.97	0.51
3						
4	15	0.067	3.39	0.55	14.87	0.72
5						
6						

$$C_{RG} = \frac{G_{0,CCF}}{G_{0,ACF,green}} * c_{green}$$

$$C_{RG} = \frac{G_{0,CCF}}{G_{0,ACFred}} * c_{red}$$

ed

td [µs]*	D [µm²/s]
416	51.32

416.00	51.32
0.00	0.00

AVG cRG [nM]
2.89
9.13

Single-labeled constructs

Sample	Average countrate [kHz]		Number of molecules	Mol. Brightness [kHz/molecule]
	s	p		
eGFP	1.87	2.81	5.86	0.80
SNAP	21.1	39.6	35.28	1.72

Double-labeled construct: NT-SNAP & IL3-eGFP

Sample	Average countrate [kHz]		App. number of molecules	Approx. Concentration [nM]
	s	p		
Green-prompt	3.16	4.85	7.41	3.56
Red-delay	0.53	1.45	12.13	2.78
PIE			91.0	0.52
				0.72

Example calculation:				
DNA -> 100 % co-diffusion				
$G_{0ACF,green}$ =	1		$r_{green,ideal}$=	0.5
$G_{0ACF,red}$ =	0.8		$r_{red,ideal}$=	0.625
G_{0CCF} =	0.5			
Cell sample shows				
$G_{0ACF,green}$ =	0.12		$r_{green,cell}$=	0.167
$G_{0ACF,red}$ =	0.08		$r_{red,cell}$=	0.25
G_{0CCF} =	0.02			

[redacted]

Approx. Concentration [nM]
4.30
67.47

[redacted]

ratio $G_0(tc)$	fraction co-diffusion
0.08	**0.15**
0.13	**0.26**

fraction co-diffusion:	
33.3	%
40.0	%

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Supplemental Coding Files

S6_Scripts.zip