This document gives an overview over the wide range of data analysis features offered by the SymPhoTime software. All analysis schemes are based on PicoQuant’s unique TTTR (time tagged time-resolved) data format offering e.g. spatial, spectral, polarisation and timing information for every detected single photon.

• All displayed results have been generated with SymPhoTime 4.7.2 (released April 2008).

• All shown analysis results and the corresponding raw data files are available in a demo workspace („samples“, 0.5 GByte, released June 2008). The demo workspace also includes more detailed information about the used specimen and the measurement parameters.

• In order to reproduce the displayed results starting from the raw data files please refer to the SymPhoTime manual (4.7. released April 2008) and the online help, which include several step by step protocols that guide you through the analysis.

• Chapter 11 and chapter 12 include results, which are generated with user configurable scripts („STUPSLANG“), that allow to program individual analysis schemes.

Please contact PicoQuant if you want to get the latest SymPhoTime release or additional information like the actual demo workspace and the SymPhoTime manual. Trial versions of the SymPhoTime software are also available for a limited period of time.

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1 IRF reconstruction (Cy5_diff_IRF+FLCS-pattern.pt3)

This feature can be used to get a reasonable IRF approximation in cases where experimental determination of the IRF is problematic. Typical examples are two photon excitation or measurements where the detection wavelength is far away from the excitation wavelength, so that the color effect of the detector cannot be neglected.

Sample

Freely diffusing Cy5 molecules (5 nM) in aqueous buffer solution at room temperature deposited on the top of a standard glass cover slip.

Acquisition Parameters

MicroTime 200 using pulsed excitation at 635 nm with 20 MHz repetition rate. The fluorescence light was split by a 50/50 beamsplitter cube onto two SPAD detectors.

Data Analysis

The IRF Reconstruction dialog shows the overall TCSPC histogram of the measurement and automatically calculates an approximation of the Instrument Response Function (IRF). Such IRFs can be calculated separately for each detection channel.

The calculation of a fluorescence intensity time trace (MCS trace) from the measurement data and subsequent TCSPC fitting of the selected area of interest makes it possible to extract the fluorescence lifetime(s). Without an IRF, however, only simple tail fitting is possible. This fitting technique has many inherent limitations. It can, for example, not provide the correct relative amplitudes of the lifetime components due to the undefined (arbitrary) beginning of the fitted region.

By including the previously recovered IRF approximation, it is possible to perform a full deconvolution fit. The fit quality is not perfect due to the approximations involved in the IRF reconstruction process. However, the lifetime values are now corrected for the finite IRF duration and the relative exponential amplitudes are correct.
2 FCS (Atto655_diff_FLCS-pattern.pt3)

This example shows the analysis of a Fluorescence Correlation Spectroscopy (FCS) measurement, which can be used to characterise the mobility and concentration of (typically) diffusing species. It is based on analysing the characteristic fluorescence intensity fluctuations of the particles when diffusing randomly through the confocal observation volume.

Sample
Freely diffusing Atto655 molecules (5 nM) in aqueous buffer solution at room temperature deposited on the top of a standard glass cover slip.

Acquisition Parameters
MicroTime 200 using pulsed excitation at 635 nm with 20 MHz repetition rate. The fluorescence light was split by a 50/50 beamsplitter cube onto two SPAD detectors.

Data Analysis

Autocorrelation FCS can be calculated for any selected routing channel or for a summed signal of a group of routing channels. Cross-correlation is also possible by defining two arbitrary data channels (A and B). These are usually associated with detector routing channels, but can also be two different time gates for the same detector signal as in case of PIE (or ALEX).

Advanced fitting of standard FCS models is supported. Weighted residuals and the optimized parameters are displayed immediately. It is possible to perform Support Plane and Bootstrap error analyses, as well as to fix or manually alter the model parameters.

FCS calibration data are stored in the workspace. New data can be added anytime, calibration results can be recalled, altered and deleted when necessary.

The fitting formulas and the definition of the model parameters are well documented.

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3 FLCS (Atto655+Cy5_diff_FCS+FLCS.pt3)

This example shows the advantage of Fluorescence Lifetime aided Correlation Spectroscopy (FLCS), which also takes the nanosecond timing information after pulsed excitation into account in order to separate the FCS curves for species with different lifetime characteristics.

Sample
Equimolar solution of Atto655 and Cy5 in aqueous buffer at room temperature (0.5nM concentration for each dye).

Acquisition Parameters
MicroTime 200 using pulsed excitation at 635 nm with 20 MHz repetition rate. The fluorescence light was split by a 50/50 beamsplitter cube onto two SPAD detectors.

Data Analysis

The two detector cross-correlation function (XCF), which corresponds to the autocorrelation function (ACF) of the mixture is free of afterpulsing artefacts. However, it is still complicated due to the photophysics of Cy5 (light driven transition between fluorescent and non-fluorescent state) and the presence of two independently diffusing components. As the diffusion coefficients of these dyes are similar, it is impossible to resolve their contributions by FCS model fitting. Indeed, already a model for a single diffusing particle with triplet term describes the XCF.

FLCS is much more powerful. Using the experimentally obtained decay patterns of the two dyes it is possible to decompose the complex TCSPC decay curve of the mixture into its components. Filter functions (right hand side) for the identified FCS components are then calculated. By selecting the corresponding filter function for correlation calculation the separated ACFs of the components are obtained.

Separated ACF of Atto655 and the fit of the single particle diffusion model. Note that there is no triplet term in the model. The recovered diffusion constant agrees well with that obtained for the pure Atto655 solution. The concentration calculated from the fitted parameters does also very well agree with the expected value of 0.5 nM.

Separated ACF of Cy5 and the fit of the single particle diffusion model with an additional triplet term to account for the Cy5 photophysics. The recovered diffusion constant agrees well with that obtained for the pure Cy5 solution. Again, the concentration calculated from the fitted parameters agrees with the expected value of 0.5 nM.
4  2FFCS (Atto655_diff_2FFCS.t3r)

This example shows the first steps in the analysis of a 2 Focus FCS (2FFCS) measurement. This method, as introduced by Jörg Enderlein, is based on a dual foci excitation geometry with a known distance between the two foci and allows a very precise determination of the diffusion coefficient.

Sample
Freely diffusing Atto655 (1nM) molecules in aqueous buffer solution.

Acquisition Parameters
MicroTime200 system using two interleaved pulsing 640 nm diode lasers at 10 MHz repetition rate with perpendicular polarisation and a Nomarski type DIC prism before the objective to create a double excitation focus. For the lateral excitation profile see (see chapter 13). The fluorescence light was collected through a common 150 µm pinhole and split by a 50/50 beamsplitter cube onto two SPAD detectors.

Data Analysis

To address the two different foci pulsed interleaved excitation (PIE) is used. Setting a suitable timegate in the shown TCSPC histogram (displayed is TG1: TCSPC channel 3121 – 3360) allows to identify e.g. just the photons stemming from excitation in the horizontally polarised excitation focus.

FCS analysis of the diffusion through the horizontally polarised excitation focus. The respective photons were identified by setting a suitable timegate (TG1: TCSPC channel 3121 - 3360). Cross-correlation was used to suppress detector afterpulsing. For the final 2FFCS analysis this curve is used to account for the size and shape of this focus volume.

FCS analysis of the diffusion through the perpendicular polarised excitation focus. The respective photons were identified by setting a suitable timegate (TG2: TCSPC channel 3554 - 3887). Cross-correlation was used to suppress detector afterpulsing. For the final 2FFCS analysis this curve is used to account for the size and shape of this focus volume.

Fluorescence Cross-correlation analysis to study the diffusion from the horizontal to the perpendicular polarised focus. The cross-correlation is carried out between photons in TG1 and photons in TG2. Please note the longer diffusion times given by the inter focus diffusion in comparison to the intra focus diffusion in the two files above. The final algorithm to determine the diffusion coefficient is not yet implemented, but the SymPhoTime allows an easy ASCII-export of all three necessary FCS curves. The final calculation can then be carried out with, e.g. MATLAB (c) routines, as developed by Jörg Enderlein.
5 Full Correlation and Antibunching
(Atto488_diff_cw(pulsed)_total_correlation.pt2)

This example illustrates the calculation of a full (FCS) correlation from picoseconds up to seconds as well as antibunching with either cw or pulsed excitation. The measurement data needs to be acquired in the T2 mode of the TCSPC device PicoHarp 300. In this special measurement mode, the absolute photon arrival time is stored with a resolution of 4 ps, measured from the start of the experiment.

Sample
Freely diffusing Atto488 molecules in water.

Acquisition Parameters
MicroTime 200 system with an adapted cabling for a Hanbury-Brown-Twiss (HBT) and the PicoHarp 300 TCSPC unit being operated in T2 mode. The excitation was carried out with a dual mode 470 nm laser diode which was operated either in cw or pulsed mode. Signals from two SPAD detectors are directly fed into the two PicoHarp 300 input channels to allow for cross correlation in order to suppress detector afterpulsing.

Data Analysis

Total correlation: FCS analysis with the standard logarithmic lag time scale down to the ps regime shows already the characteristic decrease of the correlation at early lag times due to photon antibunching. This experiment was carried out with cw excitation.

Analysis of the inter photon lag times after cw excitation around lag time zero displayed with a linear time scale. To visualize also „negative“ lag times, a delay of about 21 ns was introduced between the two channels. The local minimum clearly indicates photon antibunching, a typical effect in the single molecule regime representing a reduced (or for a single molecule zero) probability to emit two photons at the same time.

Analysis of the inter photon lag times after pulsed excitation (20 MHz repetition rate) around lag time zero displayed with a linear time scale. To visualize also „negative“ lag times a delay of about 170 ns was introduced between the two channels. Instead of an almost continuous lag time distribution as after cw excitation (see above), pulsed excitation leads to equidistant peaks, separated by the inverse of the repetition rate (here 50 ns). The reduced intensity of the central peak (at lag time zero) again indicates photon antibunching.
6 Antibunching for Immobilised Molecules
(Atto655_immo_Antibunching.t3r)

This example shows the feasibility of antibunching measurements with a single immobilised molecule under pulsed excitation.

Sample
Immobilised Atto 655 molecule hosted in a thin PVA film on top of a glass coverslip

Acquisition Parameters
MicroTime 200 system with an adapted cabling for a Hanbury-Brown-Twiss (HBT) setup using the TimeHarp 200 and pulsed excitation at 635 nm with 20 MHz repetition rate. The fluorescence light was split by a 50/50 beamsplitter cube onto two SPAD detectors.

Data Analysis

The fluorescence intensity time trace (MCS trace) of a single Atto 655 molecule shows strong blinking and finally photo-bleaching after about 2.1 s.

The TCSPC histogram calculated from the intensity time trace does not show the fluorescence decay but the expected structure of equidistant peaks, separated by the inverse of the repetition rate (here 50 ns). The missing central peak (between the red lines) indicates photon antibunching. Due to experimental reasons, time zero is shifted to 4.42 µs.
7 Immobilised Single Molecules (Cy5_immo_FLIM+Pol-Imaging.t3r)

This example shows the parallel analysis of properties of immobilised single molecules like fluorescence blinking and photobleaching, the fluorescence lifetime and the orientation of the emitting dipole.

Sample
Immobilised Cy5 molecules adsorbed on a glass coverslip without any matrix.

Acquisition Parameters
MicroTime 200 using pulsed excitation at 635 nm with 20 MHz repetition rate. The fluorescence light was split by a polarizing beamsplitter cube onto two SPAD detectors. The scan area corresponds to 6 µm x 6 µm (200 x 200 pixel) and the time per pixel was set to 9 ms.

Data Analysis

Fluorescence intensity (left, intensity displayed in grayscale) and lifetime image (right). The lifetime was analysed with a double exponential fit model and false color coded. The two resolved fluorescence lifetimes (red: 1.1 ns, green: 2.0 ns) indicate two dominant states.

Polarisation resolved imaging, analysed with a polarisation beamsplitter cube. The left subset shows the horizontal polarised part and the right subset the perpendicular polarised part.

The Image Calculator Tool allows to do basic arithmetics with different images, which can be used e.g. to find specific differences. In this case the perpendicular polarised image was subtracted from the horizontal polarised image.

Every image can be exported as ASCII array or Bitmap. In the latter case also custom colour schemes can be applied. In the example on the left predominantly horizontally polarised emitting molecules are coloured in yellow, perpendicular emitters are coloured in blue.
8 Lifetime Fluctuations of Immobilised Molecules
(Cy5_immo_Lifetime_Trace.t3r)

This example illustrates the fluorescence lifetime analysis of a blinking, immobilised single molecule and the correlation between intensity and lifetime fluctuations over time.

Sample
Single, immobilised Cy5 molecules adsorbed on a glass coverslip without any matrix.

Acquisition Parameters
MicroTime 200 using pulsed excitation at 635 nm with 20 MHz repetition rate without scanning and a single channel SPAD setup.

Data Analysis

The fluorescence intensity time trace (MCS trace) shows the blinking of a single immobilised Cy5 molecule over time under constant, pulsed illumination. Intensity thresholds (green and red line) allow to select regions from the measurement data within a certain count rate regime. Based on the photons from these regions the fluorescence decay can be calculated and fitted.

Intensity thresholds and ROI markers (vertical red lines) are used to select a certain region of the measurement data with a lower count rate. The lifetime deduced from this region is significantly shorter, indicating that the molecule emission is perhaps partly quenched temporarily by competition with a non radiative decay channel.

A configurable 2D histogram shows a clear dependence between lifetime and count rate. The histogram contains data from a 160 s time trace.

Fluorescence lifetime analysis showing the lifetime amplitudes time trace. In this case a double exponential model (e.g. unaffected and quenched state) was used to analyse the decay.
9 Fluorescence Blinking (Atto655_immo_On-Off-Analysis.pt3)

This examples illustrates the fluorescence intensity analysis of a blinking, immobilised single molecule.

Sample

Immobilised Atto 655 molecule hosted in a thin PVA film on top of a glass coverslip

Acquisition Parameters

MicroTime 200 using pulsed excitation at 635 nm with 20 MHz repetition rate without scanning and a single channel SPAD setup.

Data Analysis

The fluorescence intensity time trace (MCS trace) shows the blinking of a single immobilised Atto655 molecule over time under constant, pulsed illumination. The molecule was photobleached after 4.95 s. The count rate histogram on the right is used to identify a reasonable threshold (red line) to define „on“ and „off“ periods for the emitted fluorescence.

The „Off“ time histogram (logarithmic y-axis scaling) follows in this case almost a single exponential decay (red line).

The „On“ time histogram (logarithmic y-axis scaling) can also be described with single exponential decay (red line).

Intensity fluctuations like these can also be used for an FCS analysis to characterise the intensity fluctuations. The steep rising on the left side is due to detector afterpulsing and can be removed in this single detector channel experiment via FLCS (see chapter 3).
10 FLIM (DaisyPollen_cells_FLIM.t3r)

This example shows how Fluorescence Lifetime Imaging (FLIM) is used to map local variations in the fluorescence decay characteristics, which can e.g. be used to analyse material composition.

Sample
Daisy pollen (showing autofluorescence) stained with dye labels.

Acquisition Parameters
MicroTime 200 using pulsed excitation at 470 nm with 20 MHz repetition rate and a dual channel SPAD detector configuration. The scan area corresponds to 23.2 µm x 23.2 µm (200 x 200 pixel) and the time per pixel was set to 4 ms.

Data Analysis

**Fluorescence intensity image**, showing the number of detected photons per pixel in a grayscale.

**Fast FLIM analysis**: The average TCSPC channel is used as an indicator for the average lifetime. The colour of each pixel denotes the lifetime, while the intensity of each pixel is proportional to the overall fluorescence intensity.

*A single exponential fit* reproduces the overall shape of the fast FLIM analysis. The slight shift of the Fast FLIM to longer decay times is mainly due to the uncertainty of time zero estimation for the fluorescence decay.

*A double exponential fit* with two fixed lifetimes at the peak lifetimes in the lifetime histogram of the preceding example. The data is visualised in R(G)B mode (with the green channel unused).

*A triple exponential analysis* with three lifetimes fixed while fitting the image. In addition, a binning of 2x2 pixels was applied to provide enough photons per analysed pixel to account for the complex model used for fitting.
11 FLIM FRET in cells (GFP_RFP_cells_FLIM-FRET.pt3)

This example shows how FLIM can also be used to analyse FRET in e.g. living cells. In this case only the detected FRET donor emission is used for the analysis, because the donor lifetime is significantly reduced ("quenched") by the FRET process.

Sample

Living 12V HC Red cells, expressing a protein that should carry GFP (FRET donor) and RFP (FRET acceptor), connected via a fixed length linker. Sample courtesy of Philippe Bastiaens (MPI Dortmund, Germany).

Acquisition Parameters

Laser Scanning Confocal Microscope FluoView FV1000 from Olympus, equipped with the PicoQuant LSM Upgrade Kit for FLIM and FCS. The sample was excited using pulsed excitation at 470 nm with 20 MHz repetition and a single channel SPAD setup. A fluorescence filter (500-540 nm) limits the detection to the donor (GFP) fluorescence only. The image size is 256 × 256 pixels.

Data Analysis

**FLIM analysis using a single exponential tailfit.** This qualitative approach already indicates a cell with donor (GFP) only molecules, coloured in red in the upper right corner, and a cell in the center, expressing the complete FRET pair (GFP-RFP) leading to significantly shorter donor lifetimes.

The corresponding **lifetime histogram** for the previous image shows the lifetime distribution and allows to set a **false colour scheme** to visualise the lifetime variations in the image above.

**FLIM analysis with a double exponential decay model.** The short lifetime (1.0 ns, blue) visualises FRET quenched donor molecules and the longer lifetime (2.6 ns, red) visualises unaffected donor only molecules. The **RGB display** allows the separate visualisation of both components and their spatial distribution.

**Lifetime Histogram** illustrating the distribution for the fast (blue) and the slow (red) decay component. The grey line represents the weighted average lifetime distribution.

**Scripting based FLIM-FRET analysis** with a double exponential decay model. Left: 2D FRET efficiency plot based on the short lifetime component, Right: 2D plot of the amplitude ratio between short (FRET quenched) and long (donor only) lifetime component. Apparent FRET changes are in reality mainly due to different ratios between donor only molecules and FRET pairs with almost the same FRET efficiency.
12 FRET in Diffusion (Cy3+Cy5_diff_PIE-FRET.pt3)

This example shows an intensity and a lifetime based FRET analysis scheme for freely diffusing FRET pairs. Both schemes use the additional intensity information based on pulsed interleaved excitation (PIE) to identify and remove artefacts like donor only molecules.

Sample

RNA strands with a flexible tetraloop (labeled with Cy3) and the corresponding tetraloop receptor (Cy5) freely diffusing in aqueous buffer solution. Sample courtesy of Julie Fiore and David Nesbitt (JILA, Univ. of Colorado, Boulder, USA).

Acquisition Parameters

MicroTime 200 using pulsed interleaved excitation at 532 nm and 640 nm with 20 MHz repetition rate and a dual channel SPAD detector configuration. The fluorescence was spectrally separated on the two detectors.

Data Analysis

Freely diffusing FRET pairs are identified with an intensity threshold criterion and then analysed burst per burst. The intensity based FRET analysis allows to build up histograms for the FRET efficiency and the deduced FRET radius.

Using a pulsed interleaved excitation (PIE) scheme it is in addition possible to identify donor only molecules and to remove these artefacts from the final FRET histogram.

Scripting based FRET Analysis. This predefined analysis script allows to classify bursts based on the FRET efficiency and in addition on the stoichiometry (when using PIE, analysis introduced by Shimon Weiss / UCLA). In the resulting 2D burst plot (FRET Eff. vs. stoichiometry Seff) one can precisely identify specific FRET subpopulations and use the photon data of a selected region of interest to perform a lifetime analysis for this burst subpopulation (see below).

The displayed decay contains only photon information from the lower left bright spot in the 2D plot which represents RNA molecules in the open state.
13 Confocal Volume Geometry (TS-Bead_immo_xy-scan_Dual Focus.t3r)

This example shows the lateral and axial profile of the confocal volume, visualised by imaging a sub-diffraction size small fluorescent bead. In this special case a dual focus excitation geometry used for 2 Focus FCS measurements was imaged (see chapter 4).

Sample

Single TetraSpeck Bead (100 nm diameter, stained with 4 different fluorophores) on top of a glass coverslip.

Acquisition Parameters

MicroTime200 system using two interleaved pulsing 640 nm diode lasers at 10 MHz repetition rate with perpendicular polarisation and a Nomarski type DIC prism before the objective to create a double excitation focus. The fluorescence light was collected through a common 150 μm pinhole.

Data Analysis

Selectable ns timegates allow to limit the data visualisation and analysis to certain time windows relativ to the laser pulse. Here, the first decay is the fluorescence response to a vertical polarised laser, the second decay stems from 50 ns delayed horizontally polarised laser pulses.

x,y-Intensity Image of a single bead, raster scanned through the confocal volume (about 1x1 mm). The left image is based on the time gate for horizontally pulsed excitation (see above). The right image uses no time gate and visualises both polarised lasers.

A y-Line section through the left focus shows the lateral intensity profile, which can be nicely fitted to a gaussian distribution in order to extract the exact size (FWHM: 417 nm).

x,z-Intensity image (about 1.5 x 3 mm) of a single bead raster scanned through a single excitation focus and z-Line section through the single focus shows the axial intensity profile that can also be nicely fitted to a gaussian distribution (FWHM: 1062 nm).