Introduction

Already for more than 20 years, confocal laser scanning microscopy is a widely used tool in biochemistry, cell biology and other related sciences. However, the usually applied continuous-wave (cw) laser scanning microscopy, which measures fluorescence intensity, is strongly depending on the knowledge of system parameters for a quantitative analysis. A technique disregarding these drawbacks is therefore preferable. Fortunately, fluorophores can not only be investigated by their emission wavelength or overall intensity, but also by their fluorescence lifetime.

The use of time-resolved techniques in confocal laser scanning microscopy will grant the following advantages:

- Identification of fluorophores by their fluorescence lifetime
- Discrimination of fluorescence light from background scattering by temporal separation
- Reduction of needed detectors: One detector can determine different lifetimes simultaneously, however, only one spectral range.
- Fluorescence lifetime as a quantitative measure enhances the accuracy of analytical measurements
- Quantitative Förster Resonance Energy Transfer (FRET) efficiency through fluorescence lifetime analysis

Useful applications of lifetime as a parameter can be found in the investigation of environmental parameters. Typical examples are the mapping of cellular conditions such as pH [1], ion concentration or oxygen saturation by fluorescence quenching [2]. Förster Resonance Energy Transfer (FRET) [3,4] between different fluorophores allows to study the binding of reaction partners in living cells as well as measurements of distances in the range of only a few nanometres.

Alternatively, the temporal resolution can be used to monitor fluorescence intensity fluctuations, e.g. for Fluorescence (Lifetime) Correlation Spectroscopy (F(L)CS) [3,5]. The method can be applied to investigate diffusion processes and to measure the concentration of selected molecules.

With the ongoing technical development of time-resolved instrumentation, the combination of LSMs with time-resolved techniques was imminent. This technical note describes how common Laser Scanning Microscopes can be upgraded towards FLIM, FCS and other time-resolved methods using the dedicated products from PicoQuant.
**Technical aspects**

The upgrade kit is based on the method of Time-Correlated Single Photon Counting (TCSPC) for data acquisition and sensitive photon counting detectors. Along with a suited short pulsed laser at high repetition rates, the upgrade kit offers an excellent detection efficiency at high temporal resolution. It can be used with one photon and two photon excitation schemes using pulsed diode lasers or Titanium:Sapphire lasers.

The complete set up consists of the following components:

- **Commercial Laser Scanning Microscope such as**
  - FluoView FV 1000(MPE) or FluoView FV 300 from Olympus
  - SP5 from Leica
  - A1 or C1(s) from Nikon
  - LSM 510 / 710 from Zeiss
- **Excitation sources, which are either picosecond diode lasers from PicoQuant (LDH Series) along with a dedicated driver (PDL Series) or Titanium:Sapphire lasers**
- **Single Photon Avalanche Diodes (SPAD) or photon counting Photomultiplier Tubes (PMT)**
- **TCSPC data acquisition system (PicoHarp 300, HydraHarp 400)**
- **Data acquisition and analysis software (SymPhoTime)**
- **Set of necessary cables**
- **Computer**

The upgrade kit is added as an external extension to the LSM, as schematically shown in Fig. 2. To ensure an accurate system performance, several preconditions must be met:

1. It must be possible to guide the pulsed excitation light into the LSM. For single photon excitation schemes this is realised by one or more fibre input ports. Such ports are standard for each LSM allowing to connect the cw lasers from the LSM laser combiner to one or more of these ports. Titanium:Sapphire lasers for two-photon excitation are usually coupled via a free space entrance port.

2. It must be possible to guide the fluorescence light out of the microscope. This is crucial because the build-in detectors of the LSM are typically not suited for photon counting applications. The external detectors can be attached in two ways: either via a fibre exit port for descanned detection, which is the usual configuration, or directly to a side port for non-descanned detection (NDT, mainly used in combination with two-photon excitation).

3. It must be possible to synchronize the data acquisition with the movement of the laser scanner in order to actually generate the image. Typically synchronization signals at the beginning and end of each line and as well as at the beginning of each frame are used ("line start, line stop and frame start"). These synchronization signals are available or can be made available at the LSM controller.

For all above mentioned LSMs these technical issues are addressed by the upgrade kit from PicoQuant. It might, however, in some cases be necessary to modify the LSM itself, e.g. by adding an entrance or exit port or modifying the LSM controller firmware.
Special considerations for each supported LSM

Leica SP2

The external detectors are attached via a multimode fibre to the X1 exit port of the SP2.

Pulsed UV excitation at 405 nm is coupled to the SP2 via a corresponding UV fibre port. Due to the FC/0PC type of the fibre entrance port, back-reflections might occur that could disturb the TCSPC analysis. The usage of the internal cw 405 nm laser (if present) will be disabled. The coupling of other pulsed lasers at different wavelengths is not possible. Alternatively, a laser for two photon excitation is attached via a multi function port (MFP) that could be used for FLIM. There should be enough space inside the laser safety shielding to obtain a trigger signal for the TCSPC unit.

Leica SP5

The external detectors are attached via multimode fibres to the X1 exit port of the SP5.

Pulsed UV excitation at 405 nm is coupled to the SP5 via a corresponding UV fibre port. All other excitation wavelengths (407, 640 nm) are coupled to the SP5 via a multi function port (MFP), which acts alternatively for two photon excitation.

For FLIM, the SP5 must be equipped with a “Scanner Trigger Unit”, which is available from Leica. This box generates the necessary marker signals for the synchronization between data acquisition and movement of the scanner. Leica also provides a laser pulse unit which enables ROI scanning and allows to control the laser repetition frequency by the SP5 software.

PicoQuant and Leica have also introduced the TCS SMD series, which integrate hard- and software from PicoQuant with the confocal system TCS SP5. In the TCS SMD, the complete data acquisition is controlled by one single software. Straightforward application wizards guarantee a comfortable performance and allow for automated recording of FLIM volume stacks and FLIM lambda stacks for spectral and time resolved imaging. FCS time series performed at data points predefined in three dimensions enable automated measurements of diffusional parameters in living cells.

Nikon C1si

The Nikon C1si contains two fibre exit ports. The upgrade kit from PicoQuant is attached to the spectral exit port. In case the system is already equipped with the spectral detection box, it is necessary to swap fibres between spectral and FLIM / FCS operation.

The Nikon laser combiner must be equipped with a fibre switch with an open AFC connector to allow the incoupling of the PicoQuant pulsed lasers into the C1si. This switch is not standard and can be supplied by PicoQuant upon request. Due to this fibre switch pulsed and internal cw lasers can not be used simultaneously.

Nikon A1

The external detectors are attached via a multimode fibre to the AUX fibre exit port of the scan head, which needs to be modified by PicoQuant with a special APC fibre coupler. Nikon must
remove the focusing lens from the AUX port prior to installation.

Pulsed laser diodes ranging from 405 nm up to 640 nm can be coupled into the Nikon scan head via the VIS fibre input port. The Nikon laser combiner must be equipped with a fibre switch from PicoQuant with an open AFC connector to allow the incoupling of the PicoQuant pulsed lasers into the A1. Due to this fibre switch pulsed and internal cw lasers cannot be used simultaneously. For the implementation of pulsed laser diodes at 485 nm and 532 nm special main dichroics are necessary which must be built in by Nikon prior to the installation.

Olympus FluoView FV 300

The upgrade of the FluoView FV 300 requires a special fibre outcoupler, which is not offered by Olympus. PicoQuant is able to provide such a solution for newer versions of the FluoView FV 300. The fibre outcoupler is added to the top cover of the scan unit. In addition PicoQuant delivers a modified dichroic detection slider which guides the light to the exit port.

The pulsed lasers (405 nm - 640 nm) are coupled to the FluoView FV 300 using the UV/IR port, which must be available. A specially developed polarisation combiner is inserted in the FluoView FV 300 and allows the simultaneous usage of pulsed and cw lasers.

Olympus FluoView FV 1000(MPE)

The upgrade of the FluoView FV 1000 requires the integration of a fibre exit port to attach the external detectors. This port is provided by Olympus.

For the FluoView FV 1000MPE (mult-photon system) it is alternatively possible to use the Olympus NDD-detectors for FLIM measurements. Suitable units are:

- FV10MP-IXD2CH (inverse body, 2 Channels),
- FV10MP-BXD2CH (upright body, 2 Channels),
- FV10MP-IXD4CH (inverse body, 4 Channels)
- FV10MP-BXD4CH (upright body, 4 Channels)

All 4 PMTs can be used for FLIM. It is possible to easily switch between FLIM and FluoView NDD imaging, but simultaneous FluoView NDD-imaging and FLIM is not possible.

The pulsed lasers are coupled to the FluoView FV 1000 via the existing UV and IR ports. The UV Laser (375 nm) is directly connected to the UV port and the VIS wavelengths ranging from 405 nm to 640 nm are coupled into the FluoView FV 1000 via the IR port. The corresponding ports must therefore be present and unused. A specially developed polarisation combiner is inserted in the IR port and allows the simultaneous usage of (VIS) pulsed and cw lasers.

The serial number on the PSU (LSM controller) backplane must be 5H... 6H... and higher and the construction year of the system should be later than 2005.

Zeiss LSM 510

The upgrade requires a LSM 510 based on an inverted microscope body with the scan unit attached to the left side port. The system must further be equipped with a free space exit port along with a suited tubing. These parts are available from Zeiss upon request. If these preconditions are met, the fibre outcoupler is added externally by PicoQuant. Therefore the system must stand on an optical table with either metric or inch threads. Furthermore, the
breadboard must extend 20 cm to the left side over the LSM 510 unit.

If the system is equipped with a Confocor 3, an optical switch from Zeiss with a free space exit port must be implemented between the LSM 510 and the Confocor 3. The free space exit port must contain a tubing.

For UV lasers (375 - 440 nm) an additional UV port must be added by Zeiss. Two separate LCUs from PicoQuant are needed each for the UV wavelengths (375 nm - 440 nm) and the VIS wavelengths (470 nm - 640 nm). The lasers from the Zeiss laser combiner must be guided through the PicoQuant Laser Combining Unit (LCU) using the bypass option.

On the LSM 510 controller port B must be free. The data base has to be changed by Zeiss to allow the generation of marker signals for the line and frame synchronization at port B. Due to this alterations the 477 nm line of the Ar-Ion laser will not be available for the LSM any more. The controller must further have at least a hardware version of 3.5, available since 2004.

**Zeiss LSM 710 and 780**

The system must be equipped with a free space exit port along with a suited tubing. These parts are available from Zeiss upon request. If these preconditions are fulfilled, the fibre outcoupler is added externally by PicoQuant. Therefore the system must stand on an optical table with either metric or inch threads. The breadboard should extend 50 cm around the LSM 710 unit for easy access.

If the system is equipped with a Confocor 3, an optical switch from Zeiss with a free space exit port must be built in between the LSM 710 and the Confocor 3. The free space exit port must contain a tubing. Only pulsed lasers provided by Zeiss can be integrated into the set up.

**Detailed description of the involved components for the upgrade kit**

In the following pages, the individual components, that are part of the upgrade kit are described in more detail. These subsystems include the excitation unit, the detector configurations as well as the measurement device for Time-Correlated Single Photon Counting (TCSPC) along with the corresponding hardware and system software.

**Excitation**

![Fig. 3: Laser head of the LDH Series and single channel laser driver of the PDL Series](image)

As excitation source a short pulsed laser with a high repetition rate is the system of choice for time-resolved measurements using Time-Correlated Single Photon Counting (TCSPC). A short laser pulse excites the fluorophore and the subsequent fluorescence light decay can be measured. Typically, picosecond diode lasers are used, which are available as a combination of a laser driver unit (PDL Series) and a variety of laser diode heads (LDH Series) ranging from 375 nm to 800 nm (see Fig. 3). These laser heads work in pulsed mode at variable repetition rates from single shot up to 80 MHz or even in cw mode. They are controlled by a driver of the PDL Series, which are available as single channel or multi-channel versions with manual or computer control. All drivers allow to change the the laser output power – an essential feature to avoid photo-bleaching. Furthermore the drivers enable to adjust the repetition rate of the laser diodes to match the excitation pulse period to the fluorescence decay of the fluorophore. The multi-channel driver versions further permit to use several laser heads simultaneously for advanced

![Fig. 4: Laser Combining Unit (LCU) – in normal operation the LCU will be closed by a suited lid to ensure laser safety](image)
excitation schemes like Pulsed Interleaved Excitation (PIE). All drivers directly generate a synchronization signal for each pulse, suitable for TCSPC measurements.

The laser heads are typically included in a special Laser Combining Unit (LCU) that can couple up to five different laser heads into one polarisation maintaining single mode fibre (see Fig. 4). In addition the LCU allows to control the laser intensity via a ND-filter wheel as well as a scaffold.

If only a single laser diode should be implemented, it can be directly coupled via a fibre into the LSM (see Fig. 5). This compact coupler also includes a scaffolding for fine tuning the laser intensity. This configuration requires a free fibre coupling port at the LSM for the corresponding wavelength.

The laser safety of the additional laser sources is guaranteed since under normal operation conditions all laser light is shielded and guided through optical fibres.

As an alternative to pulsed diode lasers, especially for two photon excitation schemes, short pulse laser systems such as Titanium:Sapphire lasers can be integrated as well. In some cases an additional trigger diode is necessary to generate a stable trigger signal with optimal timing performance for TCSPC measurements.

For all excitation wavelengths it is mandatory that the LSM contains all needed dichroics or beam splitters (also for Leica LSMs with AOBS beamsplitter). Any necessary change of the LSM configuration must, however, be performed by the manufacturer of the LSM itself and can not be realised by PicoQuant.

Detection

The standard photomultiplier detectors present within the LSM are operated in analogue mode, where the current signal from the photon detector is used as a measure of fluorescence intensity. This allows an easy change of the detector sensitivity by variation of the supply voltage (HV) of the detector, but on the other hand prevents the usage of these detectors in time-resolved measurements. For these purposes specially designed photon counting detectors such as Single Photon Avalanche Diodes (SPADs) or photon counting Photomultiplier Tubes (PMTs) are used. These detectors are in most cases not integrated in the LSM, but must be added externally via an exit port and a multimode optical fibre. A suitable exit port is available as an option for all mentioned LSMs.

The upgrade kit can be configured for a single or dual channel detector version (Fig. 6 and 7). Typically SPAD detectors are used for the upgrade kit as these detectors feature a very high detection efficiency up to 50 % (wavelength dependent), which is essential for e.g. single molecule studies like Fluorescence Correlation Spectroscopy (FCS). The best currently available types are the PDM modules from MPD, which also feature an extremely high temporal resolution of 50 ps FWHM (at 640 nm). SPADs in general have a rather small active area in the micrometer range, which therefore requires an accurate opto-mechanical set up. Thus the detectors are mounted into a self-contained housing, which also includes filter holders for optical filters (bandpass, longpass, etc., Fig. 6). The applicable filters have a standard diameter of 25 mm (one inch) and can easily be changed to account for different excitation or detection schemes. The dual channel set up additionally contains suited mounts for beam splitting optics, which are included in a three-position tower, that can be operated from outside. Different beam configurations can thus be easily realized without greater efforts.

As an alternative to SPADs, the Photomultiplier Tubes of the PMA-M Series can be used. Compared to SPADs they have a lower detection efficiency and are thus not suitable for single molecule applications like FCS. However, for FLIM they are well-suited. All PMTs feature an excellent RF shielding as well as a built-in pre-amplifier. Since PMTs have a relatively large active area of several millimetres, they do not need sophisticated optics and opto-
mechanics like SPADs and are thus not included in a self-contained housing (Fig. 7).

Eventually, PMT detectors can further be attached to the microscope in a so called "non-descanned mode (NDD)". Here the detector is coupled via a dichroic mirror and appropriate filter set to a non-confocal exit port of the microscope. In this detection mode only two photon excitation can be used, where the confocal volume is no longer mainly defined by the detection pinhole, but rather by the extremely small volume where the necessary high excitation radiation is present. This option allows to upgrade a multi-photon excitation (MPE) LSM towards photon counting and fluorescence lifetime capability without the need of a fibre exit port. In case of the Olympus FV1000MPE it is actually possible to use the NDD detectors of that system for FLIM measurements as these can also be operated in a photon counting mode.

**Data acquisition and synchronization**

The data acquisition of the upgrade kit is based on the principle of Time-Correlated Single Photon Counting (TCSPC). TCSPC is the most powerful and sensitive method to measure fluorescence lifetimes. Briefly, the method is based on the precise measurement determination of the time difference between the moment of excitation and the arrival of the first fluorescence photon at the detector ("TCSPC time"). The measurement of this time difference is repeated several million times to account for the statistical nature of fluorescence emission and all TCSPC times are sorted into a histogram. Such a histogram of photon arrival times is generated for each image pixel, which does of course require to synchronize the data acquisition with the movement of the laser scanner. For FLIM, the histogram is analysed to extract the fluorescence lifetime, which is displayed in a false colour code.

![Scheme of the TTTR mode](image)

**Fig. 8: Scheme of the TTTR mode**
From a technical point of view the synchronization of the data acquisition with the movement of the laser scanner can be realized in several ways. The most flexible solution is a special measurement mode called Time-Tagged Time-Resolved mode (TTTR), whose basic ideas are (see Fig. 8):

- add an additional timing information (the “time tag”) to each TCSPC time, which re-presents the arrival time of each photon relative to the beginning of the experiment. This time tag is taken from a second, continuously running clock with nanosecond resolution.
- do not immediately form a histogram for each pixel during the measurement, but write the measured data (“TCSPC time” and corresponding “time tag”) into one data file.
- include external synchronization signals (“marker”) from the laser scanner at the beginning and end of each line as well as the beginning of each frame into the data file. A time tag needs to be added to each marker signal in order to sort the marker signal into the temporal order of the events.
- in case of a multi-channel set up add a channel identifier (“which detector?”) to each measured TCSPC time

One photon event in the TTTR data stream therefore consists of four individual pieces of information: the “TCSPC time”, the “time tag”, channel information and synchronization information. Sorting photons into their corresponding “image pixel” is now a simple mathematical procedure, which can be summarized for each detection channel as:

- split the measurement data into frames according to the marker signals for “frame start”
- split each frame into lines according to the marker signals “line start” and “line stop”
- calculate the difference of the time tags from

“line start” and “line stop” to get the “scanning time per line”
- divide the “scanning time per line” by the pixel number per line to get the “scanning time per pixel”
- sort each photon into the corresponding pixel using the time tag and the “scanning time per pixel”

This procedure does of course require the knowledge about the image size and that the scanner moves with a constant speed. The data analysis is already possible during the measurement by a parallel data processing and allows an online view of the lifetime image, FCS curves as well as the fluorescence intensity time trace. As great advantage of the TTTR approach the complete photon dynamics are preserved. The concept of TTTR allows the data storage without redundancy and without any loss of information, in contrast to e.g. on-board histogramming. Therefore, virtually all algorithms and methods for the analysis of fluorescence dynamics can be implemented and a very sophisticated offline data analysis is possible (see Fig. 9).

The data acquisition is done using the PicoHarp 300 USB 2.0 stand-alone module [6]. This unique device features a temporal resolution of 4 ps and allows to resolve fluorescence lifetimes well below 100 ps – even down to 60 ps in combination with a very fast detector of the PDM Series. A special routing device, PHR 800, allows to connect more than one detector to the TCSPC unit and thus enables multi-channel detection (Fig. 10 a and b).
Software

The SymPhoTime software is used for data acquisition and analysis. During FLIM measurements the software already calculates and displays a FLIM image ("online-FLIM"), which is calculated by the mean TCSPC arrival time of the fluorescence photons. It permits a quick assessment of the image quality and lifetime contrast. The advanced analysis functions of the SymPhoTime software for FLIM measurement include fitting the data in each image pixel to an exponential decay function to extract the fluorescence lifetime. The SymPhoTime software supports fitting of the measurement data to an exponential decay function up to the 4th order including numerical deconvolution algorithms. A special fitting routine can be employed to account for areas with low signal intensity. The fitting results (amplitudes, lifetimes or lifetime distributions) can be visualized individually or combined with freely adjustable colouring schemes. Besides the whole image, it is also possible to define several arbitrary areas (ROI) inside the image for a detailed analysis.

Also during FCS measurements an online correlation is performed and displayed for fast assessment of the measurement data. For detailed analysis, the SymPhoTime software provides an efficient correlator for FCS and supports autocorrelation and cross correlation between two channels. A user defined time gate can also be applied, which can be very useful to suppress scattered light contributions. As an advanced method, the software includes analysis routines for Fluorescence Lifetime Correlation Spectroscopy (FLCS) [5]. FLCS allows background and afterpulsing suppression without the need for a cross correlation. In addition, for dyes with different lifetimes but overlapping spectral properties, individual FCS curves can be calculated. Also cross correlation between these dyes is possible. Fitting routines for different established FCS models are already included and all results can be exported for further offline analysis.

The SymPhoTime software contains further analysis routines for many fluorescence related procedures. A special scripting language also permits to generate special user-defined analysis procedures.

Fig. 11: Screenshots from the software system SymPhoTime
**Experimental results**

1) FLIM-FRET measurements with cw acceptor bleaching

In a FLIM-FRET measurement only the fluorescence lifetime of the donor molecule is monitored. If donor and acceptor fluorophores are close enough to each other (in the range of several nanometers), the excitation energy can be transferred from the donor to the acceptor molecule. As a result of the energy transfer, the FRET process can be identified by a decrease of the fluorescence lifetime (quenching) of the donor in comparison to the lifetime of the individual molecule. Since the lifetime is an absolute measure, the FRET efficiency can be quantified from the ratio between the lifetime of molecules showing FRET and molecules that are not undergoing FRET in each image pixel. A direct approach to obtain the lifetime image of the FRET molecules not undergoing FRET is obtained by photo-bleaching the acceptor dye with a second laser. FRET analysis with the lifetime approach allows for quantitative results, no correction factors have to be applied.

The second method to obtain FRET efficiencies is using the ratio of the fluorescence intensity of donor and acceptor applying two detector channels. However, in order to quantify FRET efficiency by intensity based FRET measurements correction factors as for the quantum efficiencies of dyes and detectors have to be used which are difficult to obtain.

The example (Fig. 12) demonstrates FLIM-FRET measurements of fluorescent proteins inside living cells.

**Fig. 12:** FLIM-FRET measurements of EGFP/RFP with cw bleaching of the acceptor molecule. Data courtesy of Philippe Bastiaens, Max Planck Institute of Molecular Physiology, Dortmund, Germany

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**Fig. 13:** FLIM-FRET measurements of Cerulean/YFP using a dual channel setup. Data courtesy of Sandra Orthaus, former member of Fritz Lipmann Institute, Jena, Germany.
cells (12V HC Red cells), labelled with EGFP and RFP. The data are taken with an upgraded Fluoview 1000 from Olympus using an Apo 60x objective (1.4 N.A. Oil immersion) with fluorescence detection between 500 nm and 540 nm.

The left image in Fig. 12 shows the fluorescence lifetime image of EGFP in the presence of RFP molecules. The mean lifetime value is found to be 2.2 ns indicative of FRET. If the acceptor molecule is bleached by strong laser irradiation (i.e. cw excitation at 568 nm) no energy can be transferred from the donor to the acceptor any more. Thus the fluorescence lifetime of EGFP increases (see right image in Fig. 12). The mean lifetime value is now found to be 2.9 ns.

2) Protein interactions of human Centromere Proteins via FLIM-FRET

As a second example of FLIM-FRET measurements, protein interactions between human Centromere Proteins (CEPN) have been analysed. These proteins are part of the chromosome and ensure correct segregation of every chromosome during cell division. For this example, U2OS cells have been transfected with two centromere proteins, that were fused to Cerulean (CEPN-B-Cerulean) as the donor and EYFP (EYFP-CEPN-A) as the acceptor. The data were taken with an upgraded Fluoview FV 1000 from Olympus using a 1.35 N.A. oil immersion objective and a dual channel detection set up to measure the fluorescence from the donor and acceptor molecules simultaneously. The results in Fig. 13 clearly allow to distinguish different cells. In cell 1, the fluorescence lifetime in the donor as well as in the acceptor channel was found to be approx. 3 ns, which corresponds to the lifetime of “pure” CEPN-B-Cerulean as shown by an independent measurement. It can therefore be concluded that this cell is not transfected by EYFP-CEPN-A. The situation is different for the second cell. Here, the fluorescence lifetime was found to be 1.2 ns in the donor channel and 2.8 ns in the acceptor channel. A more detailed analysis revealed a rise of the fluorescence intensity at early times in the acceptor channel with a time constant of approx. 0.5 s. This rise is due to the time needed for the energy transfer from the donor to the acceptor fluorophore and is thus (along with the different lifetimes) a clear indicator that in this cell both CEPN-B-Cerulean and EYFP-CEPN-A were present and undergo FRET. The mean FRET efficiency E of cell 2 can be calculated to be E= 1 – (1.2 ns / 3 ns) = 0.6. The data thus reveal a centromere specific clustering between CEPN-A and CEPN-B centromere proteins.

3) Afterpulsing removal with Fluorescence Lifetime Correlation Spectroscopy (FLCS)

Fluorescence Lifetime Correlation Spectroscopy (FLCS) is basically a fusion of Time-Correlated Single Photon Counting and Fluorescence Correlation Spectroscopy. This method uses picosecond pulsed excitation in conjunction with time-resolved fluorescence detection for separating different FCS contributions. The core of the method is a statistical separation of different photon contributions due to their picosecond timing information, performed on a single photon level. One possible application of FLCS is the removal of detector afterpulsing artefacts common to SPAD detectors. These contributions are visible at short lag times and thus often prevent the correct determination of e.g. triplet lifetimes or dye concentration. One possible solution in order to circumvent these influences is to split the fluorescence signal equally onto two detectors and to perform a cross-correlation. However, with FLCS

![Fig. 14: Example of FLCS to remove the effect of detector afterpulsing. Classical autocorrelation-FCS (left) clearly shows the influence of afterpulsing, whereas FLCS (right) removes this artefact.](image-url)
the different temporal properties of the afterpulsing events in comparison to fluorescence photons allows to statistically filter the acquired data before the correlation calculation. The result of such a calculation is free from afterpulsing effects without the need for a second detection channel.

An example of a corresponding FLCS measurements of a 10 nM solution of ATTO 488 in water is shown in Fig. 14. The data is taken with an upgraded Nikon C1si and the measurement time was 60 s. The left image in Fig. 14 shows a classical autocorrelation function for FCS. Clearly the influence of detector afterpulsing is visible at early ag times and an evaluation of e.g. the concentration of the dye via the fitting of the data is problematic. If the same data is evaluated using FLCS, the influence of detector afterpulsing is removed as can be seen in the right graph of Fig. 14.

Further reading