



Time adds another dimension

FLIM & FCS Upgrade Kit from PicoQuant for Nikon A1 LSM

Time-Resolved Fluorescence Microscopy

Time adds another dimension

About Nikon and PicoQuant

Nikon, as a leading supplier of top quality microscope imaging systems, selects its supply partners with care. The aim is to provide users with the best possible imaging performance and to provide a single point of contact for all essential imaging tools. Here you will find information on PicoQuant's FLIM and FCS upgrade kit. This dedicated upgrade kit for Laser Scanning Microscopes enhances the capabilities of Nikon's A1 confocal system by adding time-resolved data acquisition capabilities to monitor both fluorescence lifetime and fluorescence intensity fluctuations as new measurement parameters. This enables the visualisation of molecular and cellular structure and dynamics using Fluorescence Lifetime Imaging (FLIM): lateral and rotational diffusion of fluorophores using Fluorescence Correlation Spectroscopy (FCS) or the measurement of intra- and intermolecular distances on a nanometer scale using Förster Resonance Energy Transfer (FRET). All these capabilities are available in one easy-to-use, maintenance-free and reasonably priced system.

About PicoQuant

PicoQuant GmbH was founded in 1996 to develop robust, compact and easy-to-use time-resolved electro-optical instrumentation and systems. Today PicoQuant GmbH is known as a leading company in the field of systems and components for time-resolved fluorescence measurements. The company is based in Berlin, Germany.



Fluorescence lifetime

The fluorescence lifetime is a measure of "how fast" a fluorescence photon is emitted after excitation and is characteristic for each fluorophore.

In fluorescence microscopy, the fluorophore is transferred from the ground state to an excited state by absorption of light. The excited molecule then returns to the ground state and releases energy in the form of light emission, i.e. fluorescence. The excitation wavelength and the emission wavelength range is characteristic for each fluorophore. It can be used to identify the fluorophore, to follow chemical reactions or to localise the fluorophore in imaging experiments. However, the fluorescence emission is not "instantaneous", but decays with a certain temporal structure. This structure can be described by an exponential decay function. The characteristic time constant of this decay, the "fluorescence lifetime" is in the range of some picoseconds (10^{-12} s) to several tens of nanoseconds (10^{-9} s).

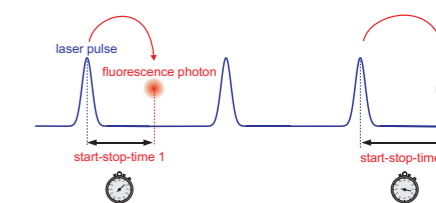
A probe for the molecular environment

The fluorescence lifetime is characteristic for each fluorophore and may be influenced by the chemical composition of its environment due to energy transfer to other molecules. This process ("quenching") usually influences the fluorescence lifetime. Changes in the lifetime can be used to gain information about the chemical environment such as pH or ion concentration e.g. at different intracellular locations. Energy can also be transferred between fluorophores – a process known as Förster Resonance Energy Transfer (FRET). Again, the fluorescence lifetime is affected and can be measured exactly in the time domain to detect and quantify the amount of FRET.

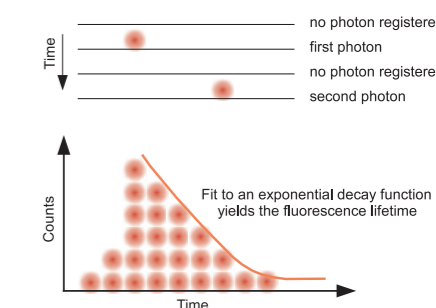
Compact upgrade solution for time-resolved measurements

The Nikon A1 can be upgraded to measure the fluorescence lifetime. The upgrade is based on the Time-Correlated Single Photon Counting (TCSPC) method, considered to be the most precise technique with the highest temporal resolution and sensitivity. Along with a pulsed laser with picosecond or femtosecond (10^{-15} s) pulses at high repetition rates, dedicated TCSPC electronics and a single photon sensitive detector, fluorescence lifetimes well below 100 ps up to several tens of nanoseconds can be resolved, covering the complete lifetime spectrum of commonly used fluorophores.

Time-Correlated Single Photon Counting (TCSPC)

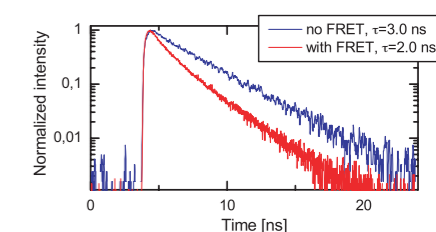


The fluorescence lifetime is defined as the average time that a molecule remains in the excited state before it returns to the ground state by emitting a photon. It can in principle be measured with a very fast and precise "stop watch", that is started with a very short laser pulse to excite the fluorophore and stopped by the first photon arriving at the detector.



The time difference between excitation and emission of a photon is measured several thousand times to build up a histogram of photon arrival times. This histogram represents the fluorescence decay and is analysed to obtain the fluorescence lifetime.

Fluorescence lifetime histogram



Typical example of the fluorescence lifetime decay change for FLIM-FRET analysis. The measured fluorescence lifetime (τ) of 3.0 ns for the donor EGFP (blue) is reduced to 2.0 ns in the presence of the acceptor mRFP (red) due to the FRET process.

Fluorescence Lifetime Imaging

Fluorescence Lifetime Imaging (FLIM) adds another parameter to conventional intensity based imaging methods.

Confocal fluorescence images are generated by measuring the fluorescence intensity in every image pixel and displayed by mapping the intensity e.g. a grey or an arbitrary color scale. Essentially the same principle is applied in FLIM by using the fluorescence lifetime as display parameter. However, as the fluorescence lifetime is not directly accessible from the measurement, the complete fluorescence decay is measured in each image pixel and then analysed to extract and display the fluorescence lifetime. The analysis is so fast that a preliminary FLIM image can be displayed during the measurement ("online-FLIM").

Fluorescence Lifetime Imaging allows fluorophores with similar emission spectra (like ATTO 655 and CY5) or autofluorescence to be separated. It can be performed with a single detector and is not affected by fluctuations in the fluorescence intensity. FLIM is used, for example, to determine

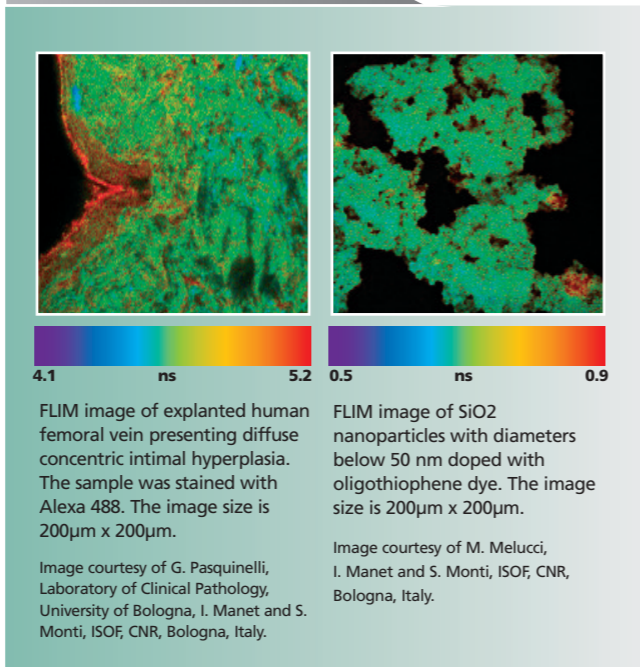
- Oxygen, water or ion concentration locally in cells
- pH value e.g. in different organelles
- Intra- or intermolecular distances on a nanometer scale
- Protein interaction *in vitro* and *in vivo*
- Intracellular signal transduction
- Molecular structure and conformational changes

VISUALISING MOLECULAR DISTANCES AND LOCALISATION DEPENDENT INTERACTION WITH FLIM-FRET

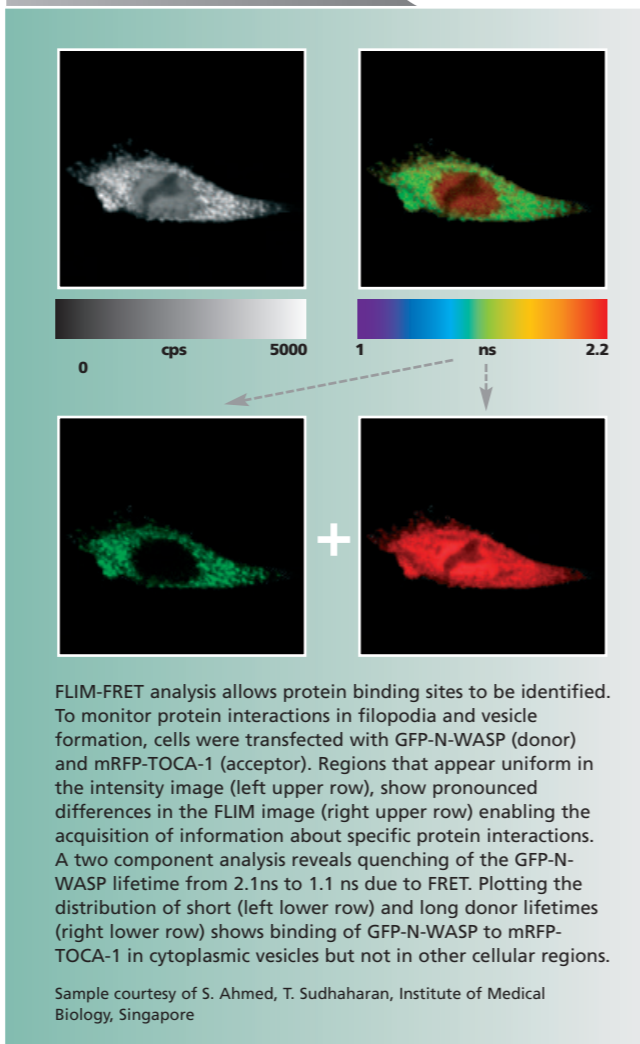
FRET is a widely used method to detect, for example, protein interactions. However, the quantification of intensity-based FRET measurements is often problematic as the monitored intensity changes depend strongly on the fluorophore concentration and thus the accuracy of the calibration. These limitations can be overcome by measuring the fluorescence lifetime of the FRET donor molecule using FLIM. As a result of the energy transfer to the acceptor fluorophore, 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 fluorophore. Comprehensive calibrations are no longer necessary as the fluorescence lifetime (over a broad range) is independent of the molecular concentration.

FLIM-FRET even allows discrimination between fluorophores showing FRET and fluorophores that are not undergoing FRET in each image pixel, which is impossible by intensity based FRET measurements. In these cases the measured fluorescence decay is a superposition of two decays, corresponding to the donor with and without FRET. A closer analysis of the measured donor lifetimes also yields the ratio between these two types and allows determination of, for example, the fraction of donor molecules bound in a complex with the acceptor.

FLIM EXAMPLE



FLIM-FRET EXAMPLE



FLUORESCENCE LIFETIME IMAGING

PICOQUANT

Ion concentration pH value Protein interactions Signal transduction Molecular structure

Fluorescence Correlation Spectroscopy

Fluorescence Correlation Spectroscopy (FCS) measures molecular properties, mobility and concentrations in solution as well as in cells at the single molecule level.

Fluorescence Correlation Spectroscopy (FCS) is a high precision and versatile method, which evaluates the temporal changes in the fluorescence emission intensity caused by single fluorophores passing through the excitation volume. These intensity changes are quantified in strength and duration by temporally autocorrelating the recorded intensity signal. FCS can be performed with a single detector, however, a dual-channel setup permits even more analysis methods by cross-correlating the signals received by both detectors (Fluorescence Cross Correlation Spectroscopy – FCCS).

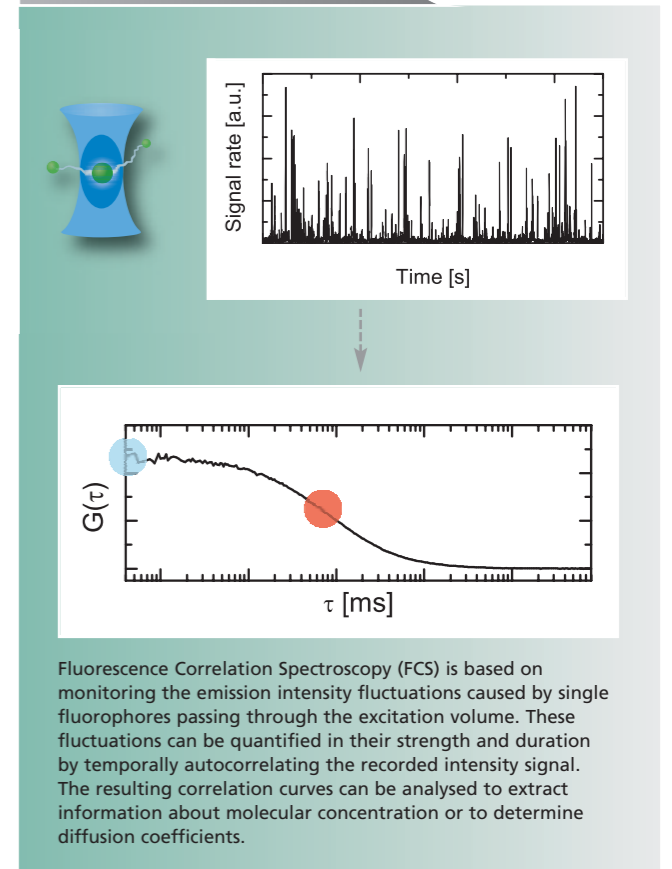
Typical applications for FCS include measurements of:

- Molecular movement e.g. protein mobility at different cellular locations and lipid dynamics in membranes
- Molecular concentrations in solutions and living cells, e.g. determination of expression levels
- Molecular interactions e.g. intracellular protein aggregation, association, dissociation and complex formation
- Lateral and rotational diffusion of fluorophores e.g. cis-trans isomerisation
- Conformational dynamics e.g. protein folding
- Enzyme dynamics and kinetic rate constants of reactions
- Intracellular dynamics, e.g. diffusion and active transport

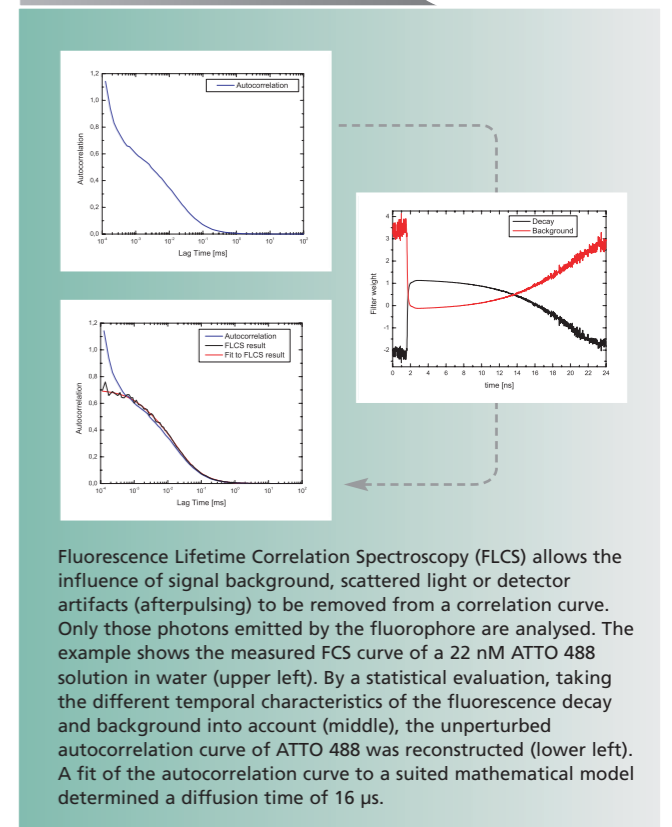
FLUORESCENCE LIFETIME CORRELATION SPECTROSCOPY (FLCS)

FCS and FCCS can be performed with the continuous-wave lasers of the A1, but the use of pulsed lasers allows even more sophisticated analytical possibilities such as Fluorescence Lifetime Correlation Spectroscopy (FLCS). This method makes use of both the simultaneously measured fluorescence lifetime and intensity fluctuations in the unique PicoQuant data format. The combined analysis of both parameters allows clear identification of photons resulting from fluorescence and discriminates them from detector artefacts such as afterpulsing without the need for cross correlation. FLCS also enables identification and removal of the influences of scattered or background light. As a consequence, FLCS provides more realistic fluorophore concentrations at higher dilutions. FCS curves of different dyes measured in a mixture can be separated based on their different fluorescence lifetimes. This allows cross-correlation measurements of two fluorophores using the same excitation wavelength and only one detector. Finally FLCS is superior for the separation of the fluorescence response after dual colour excitation and especially for identifying and overcoming spectral crosstalk.

FCS EXAMPLE



FLCS



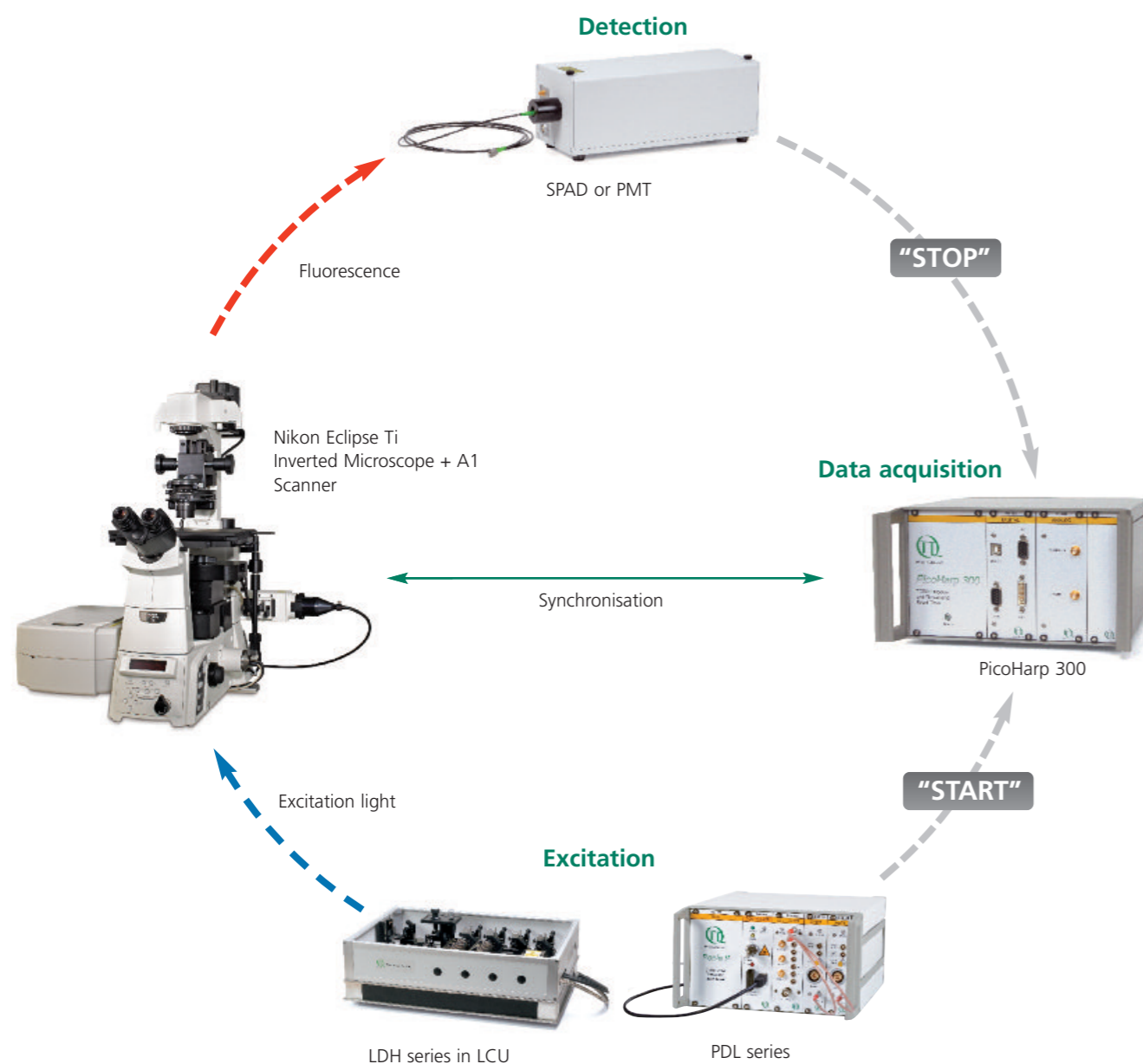
FLUORESCENCE CORRELATION SPECTROSCOPY

Molecular movement Concentration Diffusion Enzyme dynamics Intracellular dynamics

Components of the Upgrade Kit

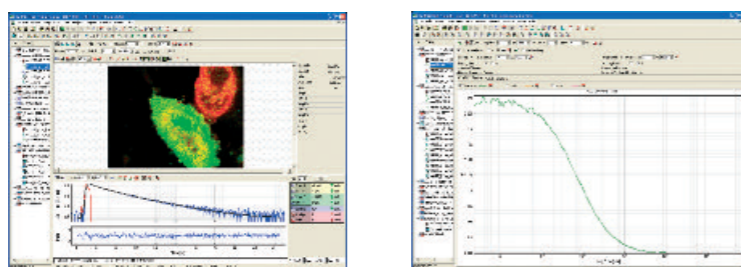
The FLIM and FCS upgrade kit is a compact, turn-key system offering ease-of-use, versatility and comfortable operation. The set-up is an external addition to the LSM and consists of four core components: TCSPC electronics for time-resolved data acquisition (PicoHarp 300), picosecond pulsed diode lasers (LDH series) including a dedicated laser driver (PDL series) along with a laser coupling unit (LCU) for up to five lasers, photon counting detectors (SPAD or PMT) and specialised data acquisition and analysis software

(SymPhoTime). For the reconstruction of 2D and 3D images the spatial origin of each photon is recovered via synchronisation signals from the LSM scanner. Typically scanner signals at the beginning and end of each line and frame are used (line and frame clock) which are provided by the LSM controller. The upgrade kit includes a dedicated system computer for optimum performance. The integration of the PicoQuant set-up into the Nikon A1 allows remote control of TCSPC data acquisition from the LSM desktop.



System software

The powerful SymPhoTime™ software is used for time-resolved data acquisition and analysis.



UPGRADE KIT SPECIFICATIONS

Excitation system

- Picosecond pulsed diode lasers with adjustable output power and variable repetition rates up to 40 MHz (adaptable to each fluorescence lifetime to be measured)
- Single or multi-channel laser driver for single or multi-colour excitation
- Wavelengths: 405, 440, 450, 470, 480, 530 and 635 nm, pulsed and optional continuous-wave operation
- Up to five laser heads inside a laser coupling unit provide full excitation flexibility
- Easy adjustment of laser power to minimise sample bleaching
- Low sample background due to narrow excitation spectra (<10 nm)
- Combination with continuous-wave lasers of LSM via a fibre switch
- Optional: external laser (e.g. Titanium:Sapphire laser) for 2-photon excitation, simultaneous incoupling possible

Detectors

- Single-Photon Avalanche Diodes (SPAD) for FLIM and FCS
- Photomultiplier tubes (PMT) for FLIM
- Integration in compact housing with all necessary optical elements (beamsplitters, filters, etc.)
- One or two detector channels

Data acquisition

- Based on Time-Correlated Single Photon Counting (TCSPC) ideal for counting single photons and analysing samples with low fluorescence signals
- Working in the unique Time-Tagged Time Resolved (TTTR) data acquisition mode that allows FLIM and FCS in one system
- Very high temporal resolution down to 4 ps allowing measurement of fluorescence lifetimes <100 ps and up to several ten nanoseconds
- Very high count rates and low dead time for fast data acquisition

Software

- Easy-to-use and comprehensive Windows™ system and analysis software
- 1- and 2-dimensional data acquisition based on the versatile TTTR file format
- Data archiving in workspace, time-gating for all methods, separation of up to four detector signals, data export features
- Application-oriented software including multiple, intuitive analysis tools
- Point measurement analysis: MCS trace, FCS, FCCS, FLCS calculation and fitting, TCSPC histogram and fitting, on/off-state histogram, burst size analysis, FRET histogram incl. Pulsed Interleaved Excitation (PIE), photon-counting histogram, lifetime histogram
- Imaging measurement analysis: fluorescence intensity images, fluorescence lifetime images, fluorescence lifetime histogram, TCSPC histogram for arbitrary region of interest, automated FRET analysis calculating FRET efficiency, FRET radius, Amplitude ratio (to determine the fraction of donor molecules undergoing FRET) etc. in each image pixel
- Multi-exponential decay analysis up to the fourth order which is essential for FLIM analysis in the heterogeneous cellular environment
- Online display of FLIM image, FCS curve and TCSPC histogram for quick assessment of data quality
- Scripting language for user-defined data analysis routines
- FLIM images up to 512x512 pixels

NIKON MICROSCOPE SETUP

Ideal for the Eclipse Ti inverted microscope with A1 confocal scanner. Contact your Nikon representative for advice on the ideal PicoQuant FLIM and FCS upgrade kit for your application.

For further information

For specific details on these products please contact

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