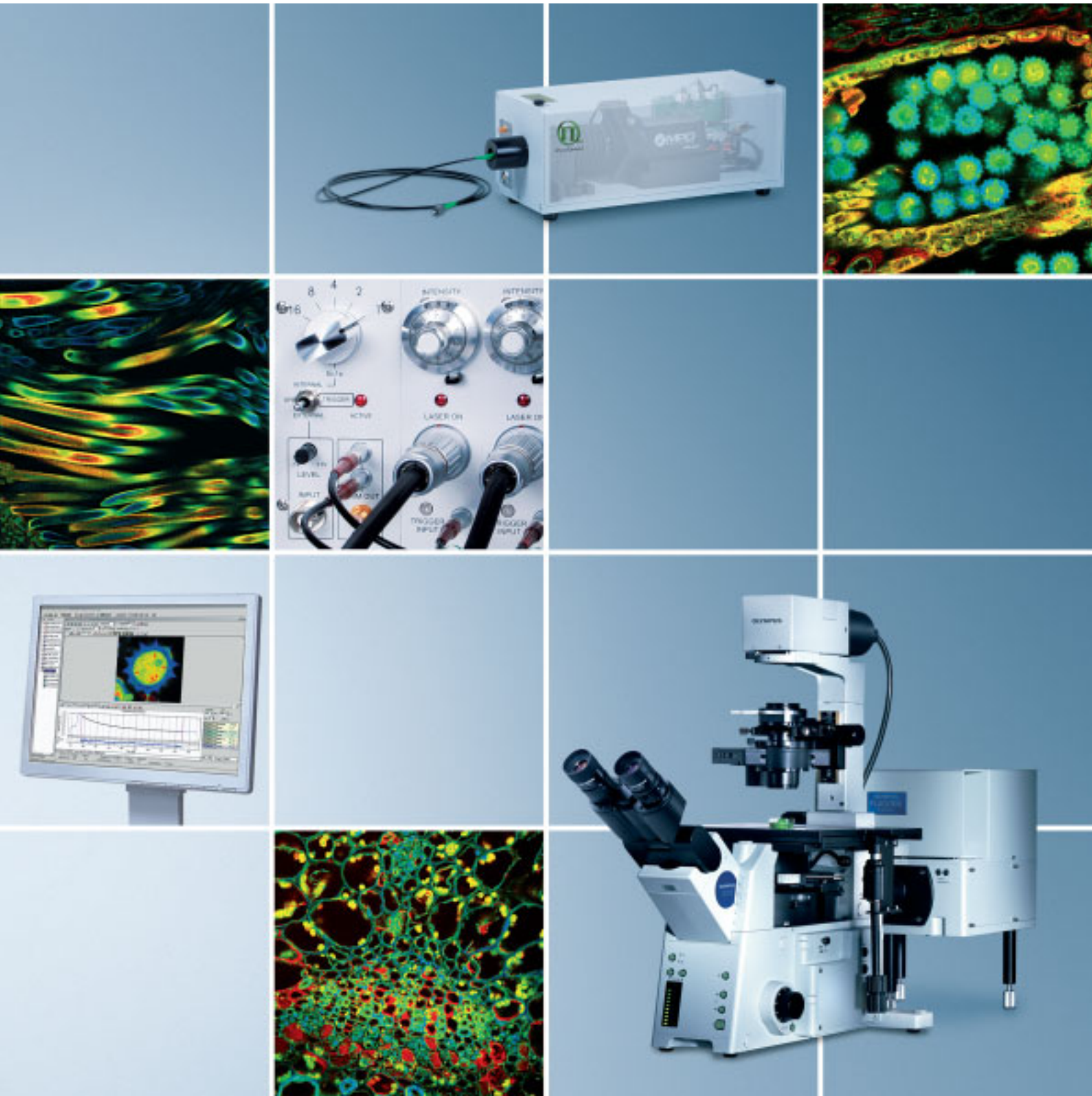


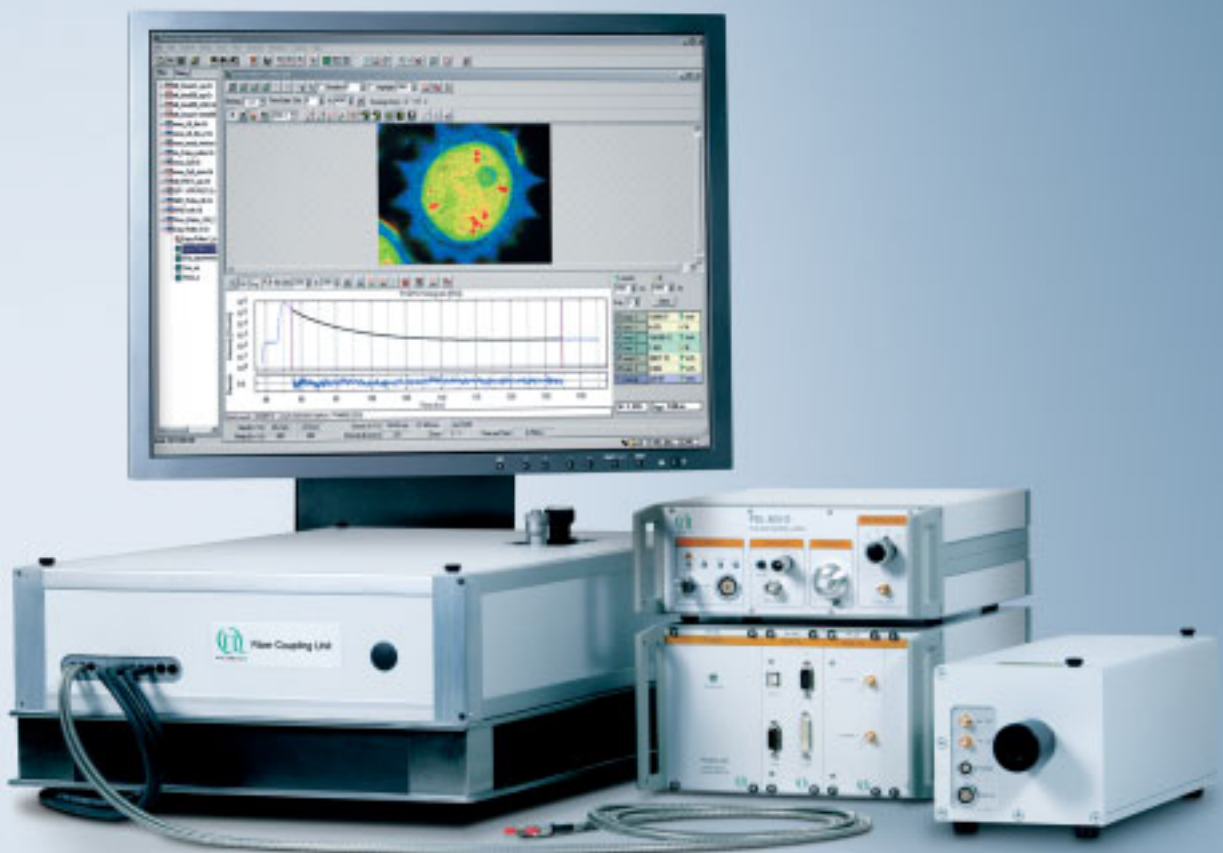
Add time as another dimension –  
FLIM & FCS Upgrade Kit from PicoQuant for the FluoView FV1000



# FLUORESCENCE MICROSCOPY

## A fundamental measurement procedure

Fluorescence microscopy is the most fundamental measurement procedure of a laser scanning microscope. In fluorescence microscopy, the intensity of the detected fluorescence emission is used to monitor chemical reactions or to localise the fluorophore in imaging experiments. The fluorescence intensity is, however, not the only parameter that can be monitored. Even more information is available by looking at the temporal characteristics of the fluorescence emission with time-resolved techniques such as fluorescence lifetime imaging (FLIM) or fluorescence correlation spectroscopy (FCS).



# FLUORESCENCE LIFETIME

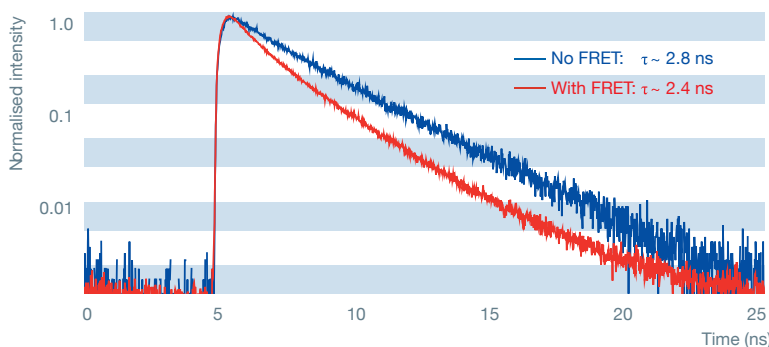
The fluorescence lifetime reflects the temporal evolution of the fluorescence emission and is characteristic for each fluorophore.

## Fluorescence emission is not “instantaneous”

Time-resolved fluorescence microscopy enables the extraction even more information about a fluorophore or its chemical environment. The key is to monitor not only the fluorescence intensity, but also the temporal characteristics of the fluorescence emission. Contrary to scattering phenomena, like Rayleigh or Raman scattering, the fluorescence emission is not “instantaneous”. Instead, it takes a certain amount of time for the fluorophore to return from the excited state to the ground state. Due to the quantum nature of this process, the emission of a fluorescence photon from each fluorophore does not occur at a fixed time, but a distribution of times is observed. This distribution can be described by an exponential decay function. The characteristic time constant of this decay, the “fluorescence lifetime”, is in the range of several picoseconds (ps,  $10^{-12}$  s) to several tens of nanoseconds (ns,  $10^{-9}$  s).

## An indicator for environmental conditions

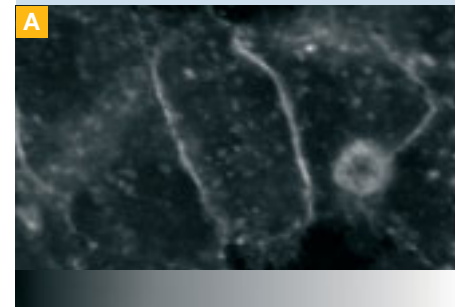
**A** The fluorescence lifetime is characteristic for each fluorophore. It is, however, not always constant as it can be influenced by the chemical composition of the environment. The reason for this is that the excited fluorophore does not necessarily emit the excitation energy as fluorescence. Instead, the energy can also be transferred to other molecules. This process (“quenching”) has an influence on the fluorescence lifetime and can therefore be used to gain information about the chemical environment. The energy can also be transferred between fluorophores – a process known as fluorescence resonance energy transfer (FRET). Again, the fluorescence lifetime is influenced, and hence FRET analysis can also be performed in the time domain.



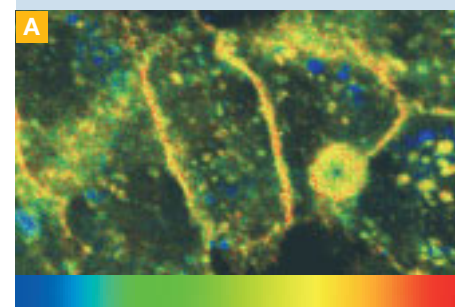
Typical example of the fluorescence decay of GFP (blue) and GFP in the presence of RFP (red). The measured fluorescence lifetimes ( $\tau$ ) of 2.8 ns for GFP is reduced to 2.4 ns in the presence of RFP due to the FRET process.

\* The lipid-bound NBD shows a broad lifetime distribution from approx. 6 to 12 ns, indicating the different molecular environments of the fluorescing molecules. Long lifetimes (red) are found in the membrane of the cells while the cytoplasm exhibits lower lifetimes (green), indicating a less ordered environment. The lowest lifetime values (blue) are found in vacuoles, presumably due to destruction of the fluorescent lipid and cleavage of the NBD moiety.

Data courtesy of Andreas Herrmann, Humboldt University Berlin, Germany



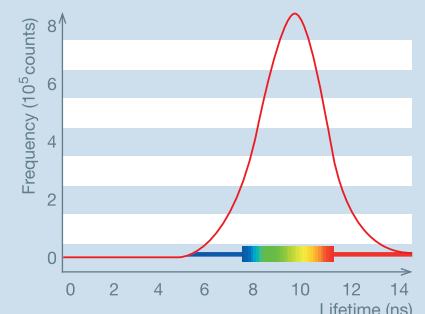
0 cps 400 cps

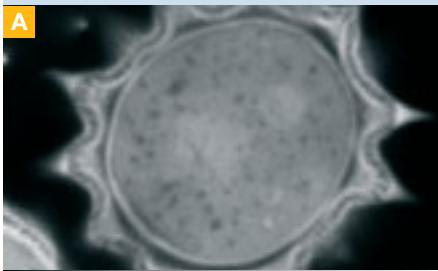


7.5 ns 11 ns

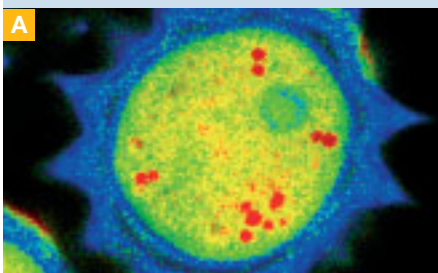
Images of hepatocytes of liver cells stained with lipid-bound nitrobenzoxadiazole (NBD), whose fluorescence lifetime is dependent on the water concentration in the environment. The fluorescence intensity image (upper image) does not allow differentiation between various environments, whereas the FLIM image (lower image) clearly shows different lifetimes, which in turn indicates different water concentrations in the various cell compartments.

## A Lifetime distribution\*



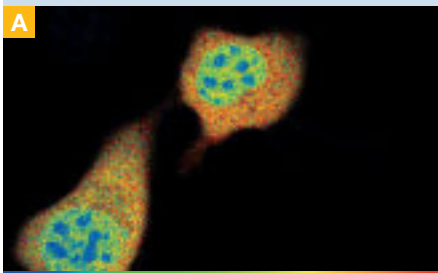


0 cps 3,000 cps



1 ns 1.8 ns

Fluorescence intensity (above) and fluorescence lifetime image (below) of the autofluorescence of a daisy pollen. Regions that look very uniform in the intensity image clearly show pronounced differences in the FLIM image.



2.2 ns 2.7 ns

FLIM-FRET measurements of protein partners in their natural environment inside living cells. The technique was used to characterise intranuclear dimer formation for the transcription factor C/EBP $\alpha$  in living pituitary GHFT1-5 cells of mice. Dimerisation of CFP-YFP-C/EBP $\Delta$ 154 protein molecules in the cell nucleus could be detected with FRET.

Sample courtesy of Ye Chen and Ammasi Periasamy, W.M. Keck Center for Cellular Imaging, University of Virginia, USA

## FLUORESCENCE LIFETIME IMAGING

Fluorescence lifetime imaging (FLIM) adds another dimension to conventional intensity-based imaging methods.

### Creating a fluorescence lifetime image

**A** In fluorescence microscopy, the measured fluorescence intensity in every image pixel is often displayed in a false colour scale – the same principle is applied in FLIM, except that the fluorescence lifetime is used as the display parameter. However, as the fluorescence lifetime is not directly accessible from the measurement, an analysis is needed prior to the image forming. Briefly, the distribution of photon arrival times is measured for each image pixel, which is then analysed to extract the fluorescence lifetime. The different fluorescence lifetimes are finally assigned to a false colour scale, which is of course arbitrary and can be freely changed to generate increased contrast, for example. The necessary analysis procedure is actually fast enough to display the FLIM image during the measurement itself.

### Time-domain analogue to multi-colour imaging

Fluorescence lifetime imaging can be viewed as the time-domain analogue to multi-colour imaging. It can be performed with just one detector and is not affected by fluctuations in the fluorescence intensity. The influence of different fluorophore concentrations or sample thickness is therefore meaningless in FLIM, and further structural insight can often be gained. FLIM also enables discrimination between fluorophores with similar emission spectra (like GFP and YFP) and from autofluorescence. Hence, in combination with intensity-based fluorescence microscopy, a broad range of fluorophore combinations inside the sample becomes possible.

In combination with other techniques, FLIM can for example be used to determine:

- Oxygen, water or Ca<sup>2+</sup> concentration
- pH value
- Distances on a nanometre scale
- Intracellular signal transduction
- Molecular structure and dynamics
- ...

### Complementing FRET with FLIM

**B** FLIM measurements can also be combined with FRET analysis. Using the fluorescence lifetime for FRET analysis avoids all problems associated with intensity fluctuations of the fluorescence emission. A FLIM-FRET measurement images the FRET efficiency and therefore directly visualises the proximity of the donor and the acceptor fluorophore on a nanometre scale. In a FLIM-FRET measurement, only the fluorescence lifetime of the donor fluorophore is used as a probe. 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. FLIM-FRET even allows differentiation between fluorophores showing FRET and fluorophores that do not undergo FRET in each image pixel, which is impossible with intensity-based FRET measurements. In those cases, the measured fluorescence decay is a superposition of two decays, corresponding to the fluorophore with and without FRET. A closer analysis of the measured decay also yields the ratio between these two types.

# FLUORESCENCE CORRELATION SPECTROSCOPY

Fluorescence correlation spectroscopy (FCS) can be used to measure molecular properties, diffusion and concentrations in solution at the single-molecule level.

## A highly precise and versatile method

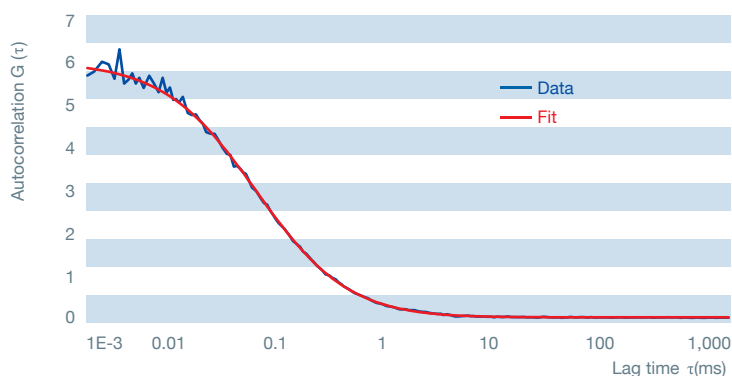
Fluorescence correlation spectroscopy (FCS) is a highly precise and versatile method which has demonstrated its great potential for many different applications. The method records the temporal changes in the fluorescence emission intensity caused by single fluorophores passing through the excitation volume. These intensity changes can be quantified in their strength and duration by temporally autocorrelating the recorded intensity signal.

Typical applications for FCS include:

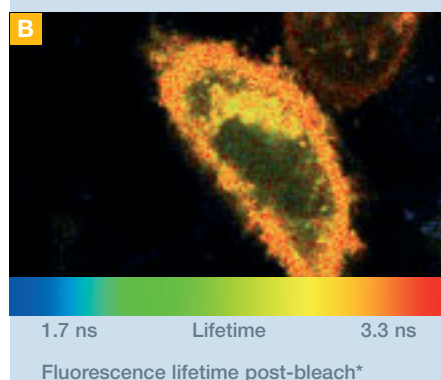
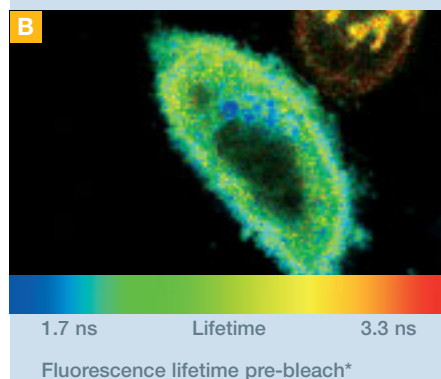
- Molecular association and dissociation
- Measurement of sample concentration in femtolitre volume
- Investigation of lateral and rotational diffusion of fluorophores
- Conformational dynamics
- Measurement of kinetic rate constants
- Enzyme dynamics and intramolecular dynamics in vitro, but also in the living cell
- ...

## Time-gated FCS and FLCS

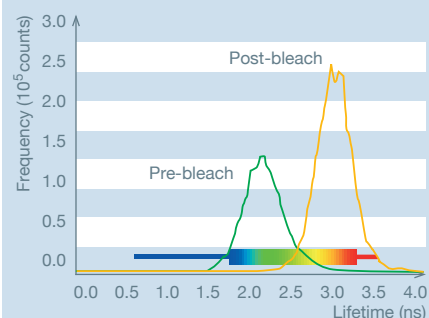
FCS can essentially be performed with a continuous-wave laser, but the usage of pulsed lasers allows even more sophisticated analysis possibilities such as time-gated FCS or fluorescence lifetime FCS (FLCS). Both methods make use of the additional information obtained by the simultaneous measurement of the fluorescence lifetime. A time gate, for example, can be used to suppress scattered light contributions (Rayleigh, Raman) from the detected signal. Only those photons that can clearly be attributed to the fluorescence decay can be selected for the analysis, which allows a more accurate concentration measurement, for example. In FLCS, the measurement data is used for a statistical evaluation of the fluorescence emission. FLCS can be used, for example, to correct for detector artifacts (e.g. afterpulsing) or scattering contributions. It even allows simultaneous monitoring of the concentration and diffusion speed of two dyes with completely overlapping emission spectra, but with differing lifetimes, by using only a single detector.



FCS measurements of freely diffusing Atto655 molecules in water (shown in black). Using a standard FCS model for diffusing species (shown in red), a fluorophore concentration of 0.6 nM with a diffusion coefficient of  $4 \cdot 10^{-6} \text{ cm}^2/\text{s}$  can be calculated.



## B Lifetime distribution\*



\* Interactions of fluorescent proteins inside living cells (12 V HC red cells) labelled with GFP and RFP and attached to each other. The very heterogeneous environment in these samples complicates the analysis of the FRET process between GFP and RFP, as it can cause a quenching of the donor and therefore affect its lifetime. In order to distinguish between GFP-RFP FRET and environmental effects, the acceptor was bleached with intense CW light. The corresponding lifetime histogram after the bleaching shows a global increase in the donor lifetime, indicating that the FRET processes were actually the main reason for the decreased lifetime.

Sample courtesy of Philippe Bastiaens, Max Planck Institute of Molecular Physiology, Dortmund, Germany

**A Pulsed diode laser**

Compact pulsed diode lasers with picosecond pulses are used for excitation.

**B Fibre coupling unit**

The fibre coupling unit makes it possible to couple up to four pulsed lasers into one optical fibre and makes wavelength changes or simultaneous excitation schemes very easy.

**C PicoHarp 300**

An outstanding time-correlated single photon counting system with picosecond resolution.

**D Single-channel detection unit**

Single-channel SPAD detection unit, suitable for FLIM and FCS measurements. A dual-channel version is also available.



## COMPONENTS OF THE UPGRADE KIT

The key components of this upgrade kit are pulsed lasers along with electronics for time-resolved measurements, special detectors and software to measure and analyse the fluorescence lifetime.

### Fluorescence lifetimes below 100 picoseconds

The upgrade for the FluoView FV1000 is manufactured by PicoQuant GmbH and is based on the method of time-correlated single photon counting (TCSPC) using time-tagged time-resolved (TTTR) data handling, which is considered to be the most precise technique with the highest temporal resolution. Along with a pulsed laser with picosecond or femtosecond pulses at high repetition rates and a detector able to detect single fluorescence photons, fluorescence lifetimes well below 100 picoseconds can be resolved.

Briefly, the method is based on the repetitive measurement of the time difference between the emission of the laser pulse and the first detected fluorescence photon. These time differences are sorted into a histogram for each image pixel from which the lifetime(s) can be extracted and false colour-coded. This procedure is actually possible during the data acquisition itself ("online FLIM") and allows a quick assessment of the data quality.

### Excitation subsystem

**A** The excitation subsystem consists of compact turnkey picosecond pulsed diode lasers, which are available in a wavelength range covering 375 nm to 900 nm and emit pulses as short as 50 ps. The lasers are variable in output power and repetition rate, which is controlled by specialised drivers that are available in single and multi-channel versions. With the multichannel versions, special excitation schemes, such as pulsed interleaved excitation (PIE), become possible. Instead of pulsed diode lasers, the upgrade kit can also work with other suitable external pulsed light sources such as Titanium:Sapphire lasers used in the FV1000MPE for multi-photon excitation.

**B** All laser heads are included in a unique fibre coupling unit (FCU), that makes it possible to couple up to four pulsed lasers into a polarisation-maintaining single-mode fibre. The fibre is attached to the FluoView FV1000 via the modified IR port and therefore ensures an independent and, if necessary, also simultaneous operation of pulsed and continuous-wave lasers. A single laser head can also be directly coupled to the FluoView FV1000 for an even more compact design.

### TCSPC data acquisition

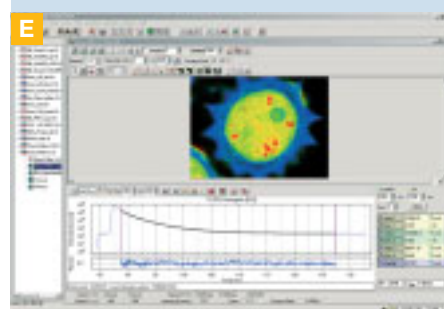
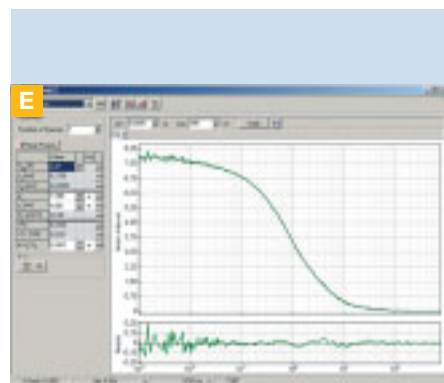
**C** The unique time-correlated single photon counting (TCSPC) data acquisition unit PicoHarp 300 is used for the upgrade kit. This stand-alone unit has a very high temporal resolution of 4 ps and is attached to the system computer via a high-speed USB connection. The data acquisition is done in the unique time-tagged time-resolved (TTTR) mode in which the temporal information of each detected photon is preserved, which allows a very sophisticated offline analysis.

## Detectors

**D** The upgrade kit can work with one or two special photon-counting detectors of different types. The detectors are incorporated in a self-contained housing and are typically attached to the fibre exit port of the FluoView FV1000. The available detectors are either single-photon avalanche diodes (SPADs) or photomultiplier tubes (PMTs). The PMTs have a lower detection efficiency than SPADs and are therefore not suited for fluorescence correlation spectroscopy, but are highly suited for fluorescence lifetime imaging. For measurements that require high detection efficiencies, two types of SPADs are available for the upgrade kit (PDM Series or SPCM-AQR Series), which differ in their spectral and temporal responses.

## System software – SymPhoTime

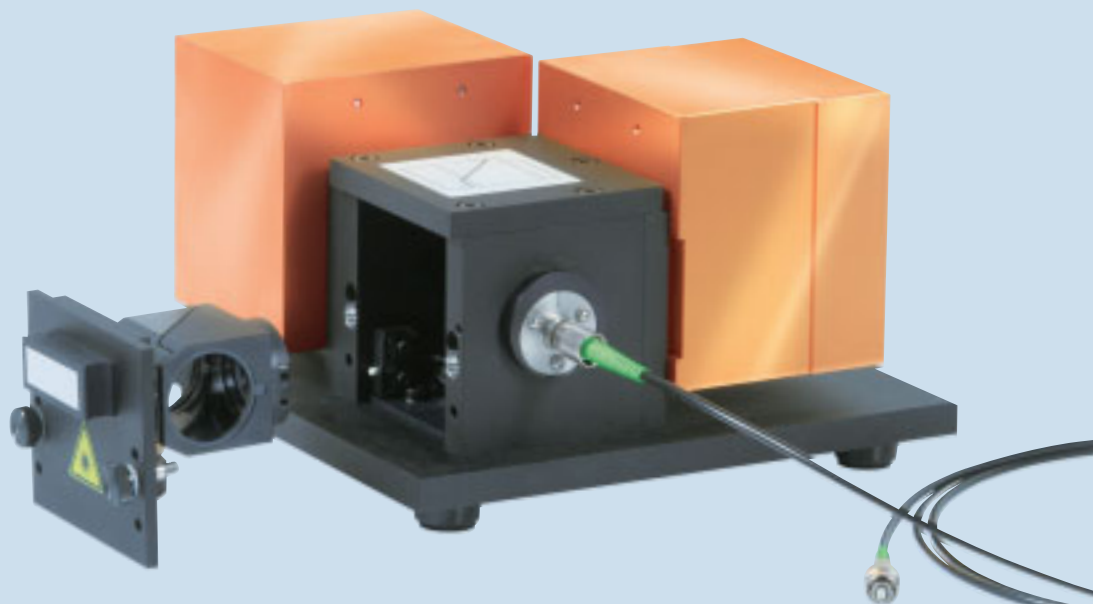
**E** The data acquisition and analysis software “SymPhoTime” is based on powerful but generic TTR data collection. FCS correlation curves and FLIM images are already displayed during the data acquisition to permit a quick assessment of the data quality respectively lifetime contrast in the sample. The software allows to perform an unlimited number of analysis steps without losing track of the interdependence and origin of their measurement and analysis data. All derived data is maintained in the hierarchic workspace, including a log file, keeping track of all measurement and analysis steps. The analysis possibilities include fluorescence correlation spectroscopy (FCS), fluorescence lifetime correlation spectroscopy (FLCS), fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging (FLIM), to name only a few. All data can be exported to standard formats. A specially developed scripting language even enables the generation of user-defined analysis routines.



Screenshots from the operation and analysis software suite “SymPhoTime” showing FCS (above) and FLIM analysis (below).

### **D** Dual-channel detection unit

Dual-channel PMT detection unit, suitable for FLIM measurements. A single-channel version is also available.



## Specifications

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### Excitation system

- Picosecond pulsed diode lasers with adjustable output power and repetition rates up to 80 MHz inside a fibre coupling unit for up to four laser heads
  - Wavelengths between 375 and 900 nm, pulsed and optional continuous-wave operation
  - Single or multi-channel laser driver
  - Optional: external laser (e.g. Titanium:Sapphire laser) for 2-photon excitation
- 

### Detectors

- Single-photon avalanche diodes or photomultiplier tubes
  - Integrated in compact housing with all necessary optical elements (beamsplitters, filters, etc.)
  - One or two detector channel setups
- 

### Data acquisition

- Based on the method of time-correlated single photon counting (TCSPC) in the unique time-tagged time resolved (TTTR) mode
  - Fluorescence lifetime resolution less than 100 ps
- 

### 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
  - 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, photon-counting histogram, lifetime histogram
  - Imaging measurement analysis: fluorescence intensity images, fluorescence lifetime images, time-gated analysis, TCSPC histogram for region of interest
  - Scripting language for user-defined data analysis routines
- 



The manufacturer reserves the right to make technical changes without prior notice.

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