Leica TCS SMD Series

Single Molecule Detection Platform
for Obtaining Meaningful, Reliable Results:
FCS, FCCS, FLIM, FRET, FLCS, Gated FCS

Living up to Life
• All in one – a single platform to study the dynamics of life
• Global control by full system integration
• Quantification with maximum content
• Dedicated application wizards for fast, reproducible work
• Immediate results by intelligent hardware and software presets
Molecular interactions, such as protein complex formation, protein-DNA or ligand-receptor binding are extremely significant for modern biology \(^1,^2\). The specific identification of interaction partners and quantification of binding parameters are crucial for understanding the biological, chemical, and physical processes in live cells. This information is necessary for goal-oriented development of agents that influence biochemical reactions on a molecular level, which is essential in pharmaceutical drug design and biomedical research.

**Leica TCS SMD Series**

*Quantify Life!*

Single Molecule Detection (SMD) and analysis is an elegant way to examine dynamics and interactions inside cellular systems. This includes the quantitative characterization of biochemical reaction kinetics and equilibrium on a molecular level. Besides giving deeper insight into complex systems, such data enables the development of predictive models.

Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Lifetime Imaging (FLIM) are well-established SMD technologies for investigating biological specimens. Upcoming methods such as gated FCS or Fluorescence Lifetime Correlation Spectroscopy (FLCS) combine these two approaches.

The Leica TCS SMD Series is a flexible platform for a variety of single molecule detection technologies, particularly FCS and FLIM. A single measurement allows the extraction of multiple biophysical parameters. New recording procedures complement the flexibility of the underlying data format, which enables the design of completely new experiments.

Straightforward application wizards guide the researcher through an SMD experiment to maximize reproducibility. Intelligent hardware and software presets allow the researcher to focus on what is most important – obtaining meaningful and reliable results.
FLIM – nano-environment

Protein activity is often affected by the local environment, in particular by pH, ion concentration, and polarity. Fluorescence Lifetime Imaging (FLIM) identifies environmental changes, both spatially and temporally resolved. Subcellular localization of GFP fusion protein gives rise to two protein populations, each exhibiting a different average lifetime as revealed by the lifetime histogram (shown here). Their relative contribution to the average lifetime changes with localization to different organelles (yellow and red lines).

FCCS – molecular interactions

Fluorescence Cross-Correlation Spectroscopy (FCCS) facilitates the analysis of molecular binding. Interactions are directly evident by the presence of a cross-correlation between two spectrally distinct channels. Furthermore, the ratio of free versus bound fraction in equilibrium reactions can be quantified. Caspase 6, a downstream effector of the TNF-dependent apoptosis pathway, dimerizes upon activation (shown here). Both a GFP-labeled and an mCherry-labeled construct were expressed in HeLa cells. 50% of the resulting double-labeled dimers (gray box) are detected in the cross-correlation signal. FCCS thus proves the existence of the active form of Caspase 6.
**FLIM-FRET – molecular distances**

Fluorescence Resonance Energy Transfer (FRET) probes the proximity of fluorescently labeled molecules. FRET unravels molecular interactions, both in vitro and in vivo. A tandem construct of GFP (donor) and mCherry (acceptor) exhibits FRET, which results in a 0.2 ns drop in the average fluorescence lifetime. This shift is observed by comparing a HeLa cell expressing only GFP (yellow) with a cell expressing the GFP-mCherry tandem construct (green).

**FLCS – molecular dynamics in difficult samples**

Many biomolecules are only active at a specific concentration and a precise quantification of physiological concentrations is mandatory in order to study their biological activity. Fluorescence Lifetime Correlation Spectroscopy (FLCS) reduces concentration measurement artifacts such as detector afterpulsing. The GFP fusion protein localizing to the endoplasmic reticulum (ER) was expressed in HeLa cells and excited with a pulsed laser at 470 nm. Influences of the detector after pulsing were successfully removed, which improved parameter accuracy.
High performance meets ease-of-use

SMD experiments involve complex specimen preparation, data acquisition, and analysis. To obtain reliable data these studies require excellent optical performance combined with a high degree of system stability. Leica Microsystems has developed an easy-to-use, comfortable platform specifically designed for single molecule detection: the Leica TCS SMD series.

All systems in the Leica TCS SMD series combine the full versatility of the Leica TCS SP5 confocal microscope with special equipment dedicated to single molecule detection and analysis. Fast switching to live cell imaging at the highest optical performance offers a wide range of application possibilities.

Highest flexibility for various methods

Depending on the scientific questions, a system can be configured that adapts perfectly to the user's individual requirements. With the Leica TCS SMD series, three products with variable configurations are available:

- Leica TCS SMD FCS (page 13)
- Leica TCS SMD FLIM (page 17)
- Leica TCS SMD FLCS (page 22)

“The Leica TCS SMD series is an excellent workhorse for monitoring dynamic biochemical events in the live cell at an advanced level.”

Dr. Matthias Weiss
BIOMS Group leader
Cellular Biophysics Group (B085)
German Cancer Research Center
Heidelberg, Germany

A Single Platform to Study Dynamics of Life
Quantification with maximum content

The universal raw data format of SMD data makes the Leica TCS SMD series highly flexible and versatile. This data format allows the researcher to analyze the same raw data file in different ways. The file format is based on Time-Correlated Single Photon Counting (TCSPC) data acquisition in the Time-Tagged Time Resolved (TTTR) measurement mode. It is compatible with any set of analysis tools, such as intensity time trace, FCS, FCCS, FLIM, and burst analysis. Time gating and photon filtering can be used for background suppression to improve reliability of results or for dye separation. The very same raw data file can be analyzed in different ways. This allows a researcher to extract the maximum amount of information inherent to the data.

Fast, reproducible results with dedicated application wizards

Leica Application Suite Advanced Fluorescence (LAS AF) includes dedicated application wizards guiding the user step-by-step to and through SMD experiments. An intuitive workflow ensures that all relevant steps for setting up an experiment are followed, while providing the flexibility to define complex recording scenarios. Reproducible results are obtained quickly and easily. Furthermore, optional application wizards, such as FRET and FRAP, complement the SMD application wizards.
Global control by full system integration

LAS AF confocal software and the SMD analysis software, SymPhoTime, run on separate PCs to maximize performance of each software package. Both packages are synchronized via a comprehensive ethernet based client-server connection. Via this connection LAS AF remotely controls the entire data acquisition process. All relevant experimental parameters are set up in the LAS AF wizard, which means that the user can concentrate on the LAS AF computer.

Data handling made easy

The transfer of meta data, including experiment file names or user comments from LAS AF to SymPhoTime, facilitates documentation and data management. All necessary information is bundled with the SMD raw data files for easy data handling.
Tight online control of data quality

The seamless software and hardware integration ensures tight online control of data quality: during running experiments users see a display of photon count rates in LAS AF as well as an online FCS curve or a FLIM image. Immediate interactive feedback helps to optimize experimental conditions, while full data analysis can be performed in a separate session. This saves valuable measurement time for data acquisition.

Intelligent presets – save time, minimize mistakes, increase efficiency

The user selects an acquisition method, appropriate excitation lasers, and emission detectors – the beam path of the confocal system is configured automatically. The configuration includes, for example, automated setting of optimal filters, beam splitters, and the exit port. Consequently, the confocal system's diverse hardware components are always set correctly.

Researchers can design customized experiments by defining and saving instrument parameter settings. Loading the previously defined settings helps to quick-start complex recording scenarios and increases experimental reproducibility. This is particularly important in multi-user environments.

“The Leica TCS SMD series integrates FLIM, FCS and spectral confocal in vivo imaging in an impressively comprehensive and unprecedented way.”

Dr. Malte Wachsmuth
EMBL
Cell Biology & Biophysics
Meyerhofstrasse 1
69117 Heidelberg
Germany
Precise analysis of particle transport and molecular binding

Over the last few decades, important advances in the development of fluorescence-based methods have given rise to many fluorescence staining methods in modern biology. In addition, GFP and spectrally related substances were discovered and refined in the last few years. This has opened the door to a rapidly growing number of in vitro and in vivo studies, both using FCS and FCCS and focusing on quantitative analysis of transport and binding processes.

The high specificity of the data obtained helps to explain basic processes in complex systems as well as to develop predictive models. The Leica TCS SMD FCS is a system that has been designed for reliable acquisition and analysis of such quantitative data, both in vitro and in vivo.

Specialized detectors with high quantum efficiency

A typical FCS experiment focuses on only a small number of molecules. The fluorescence signal is best registered by single photon counting. This is why very sensitive detectors with low background noise, like Avalanche Photodiodes (APDs), are particularly well-suited for FCS. Generally, all Leica TCS SMD FCS systems are equipped with 2 APDs for simultaneous detection in two channels, which is necessary for FCCS experiments.

Selecting the best detector: two different APD types are available for the Leica TCS SMD FCS system. APDs of the SPCM-AQRH type, by Perkin-Elmer, yield the highest quantum efficiency. These detectors give the best signal-to-noise ratio for FCS, especially at wavelengths above 600 nm. PDM APDs by Micro Photon Devices (MPD), on the other hand, offer the best trade-off between quantum efficiency and time resolution. These detectors allow future upgrade to a TCS SMD FLCS system.
The FCS and FCCS Method

FCS is a fluorescence-based measurement method. Fluorescent molecules passing through a strongly-focused laser beam are excited for fluorescence emission, and the emitted photons are registered using very sensitive detectors. Instead of using a cuvette with comparatively large volume, a diffraction-limited spot is used as the measurement volume while maintaining full confocality.

Moving particles give rise to intensity fluctuations. An autocorrelation of this reveals information on the molecular scale: the amplitude of the correlation curve reveals the particle number. The time at the inflection point contains information about particle mobility. Finally, the steepness and shape of the curve reflect the type of diffusion. Particle concentration, particle mass, viscosity, and bound fraction can be derived from these parameters. The high sensitivity of FCS permits the detection of nanomolar concentrations and dynamic processes in the range of microseconds to seconds – with confocal resolution.

An even higher sensitivity can be achieved in dual channel mode called FCCS (Fluorescence Cross-Correlation Spectroscopy): potential binding partners are marked with spectrally different fluorophores, and their emitted photons are detected in two separate channels. The diffusion of double-marked dimers creates synchronized (i.e., correlated) signal fluctuations, while single marked monomers cause independent fluctuations. An increasing amplitude of the cross-correlation curve reflects an enlargement of the bound fraction.

Principle of FCS data acquisition and analysis:

1. Laser illumination at a fixed point of interest (beam parking) excites fluorescent particles in the excitation volume. The particle movement in and out of the confocal volume causes intensity fluctuations.
2. Registration of intensity fluctuations using detectors in single photon counting mode.
3. Calculation of the correlation function.
4. Fitting of the corresponding biophysical model to the correlation curve. Obtain parameters of interest:
   - particle concentration
   - diffusion coefficient
   - viscosity
   - molecular mass
   - binding constant
   - photo-physical properties
Quantitative in vivo cell biology made easy

FCS is a point measurement method. The researcher obtains spatial information by defining FCS points in previously acquired images or image stacks. The sequence of image acquisition, positioning of FCS measurement spots, and performing the FCS measurement is best supported by the SMD FCS wizard. Within the wizard it is possible to conduct nested FCS experiments with up to four dimensions: 1. Multiple FCS measurement spots in image stacks. 2. Repetitions at each single spot. 3. Time series over all spots. 4. Multiple runs of the same FCS experiment. Additionally, the researcher can freely zoom in and out of the images, while the software retains the absolute position of the measurement spot.

Highly precise positioning on membranes and vesicles: for the first time, FCS measurement spots can be defined in both, previously acquired xyz-image stacks as well as in xzy-image stacks. This facilitates performing FCS measurements on thin and highly anisotropic structures, such as membranes, with highly precise positioning.

Evaluating data quality during a running experiment: for maximum control of data quality, LAS AF continuously displays the count rate of each APD and the counts per molecule. In SymPhoTime an on-line correlation allows users to judge the significance of the data as it is acquired.

Using FCS overview scan for maximum reproducibility: during a running FCS time series, control images can be acquired automatically using independent settings for imaging and FCS. Any change of the specimen during an FCS time series becomes visible. The specimen’s morphology as well as consistent positioning of the measurement spots can be documented and kept for reference. This ensures data reproducibility and consequently, high quality data. The FCS overview scan is particularly interesting for longer FCS time series in live cells or tissues.
Imaging faintly-stained specimens with high quality data: typically, cells or structures labeled for FCS measurements are only stained faintly. To obtain high quality images, fluorescence detection with very sensitive detectors and low background noise is mandatory. APDs fulfill this requirement for both, FCS measurements at faintly stained structures and their visualization.

Fast visualization of concentrations: the combination of FCS and APD imaging provides a fast and elegant way to map local concentrations in a cell or another structure. FCS measurements alone are point measurements; they deliver local information about single spots within the sample.

To obtain concentration information over a whole image, FCS and APD imaging can be combined using the same detector type. This enables scientists to calibrate the APD image intensity by the particle number obtained from the FCS measurement. Consequently, expression levels, which are displayed as an image intensity, can be quantified.

Concentration Mapping

Concentration of TIF1A - GFP

![Graph showing concentration of TIF1A - GFP with normalized autocorrelation vs. lag time.]
FLIM is a complementary way to extract quantitative information about cellular dynamics and interaction processes. It reveals information about the direct neighborhood of molecules on the nanometer scale. These local conditions can determine folding states that influence the reactivity of molecules. FLIM can thus explain or predict a certain behavior of biomolecules. Furthermore, FLIM is used to identify natural metabolites under physiological conditions.

**Versatility in pulsed excitation**

A variety of pulsed lasers ranging from UV via VIS to MP excitation is available for the Leica TCS SMD FLIM system.

MP excitation for thick samples: the use of an IR laser as a FLIM excitation source offers all the advantages of multiphoton excitation, such as deep tissue penetration and strongly reduced out-of-focus bleaching. The tuneable excitation wavelength facilitates the observation of a wide variety of dyes.

Pulsed diode laser excitation for dyes with longer decay times: the following wavelengths are available with the Leica TCS SMD FLIM: 405 nm, 470 nm, and 640 nm. The pulsed lasers can run at different pulse frequencies, which are selectable in the LAS AF software (5, 10, 20, and 40 MHz). Changing the pulse frequency allows researchers to optimally adapt to a variety of lifetimes to ensure the highest quality data.

**Highest time resolution by APDs from MPD**

Lifetime measurements require single photon counting detectors with high time resolution. PDM APDs yield the highest performance with respect to quantum efficiency, time resolution, and background level. Systems equipped with PDM APDs can upgrade to a TCS SMD FCS FLIM system in the future.
The FLIM Method

The fluorescence lifetime is a measure of how long a fluorophore remains on average in its excited state before returning to the ground state by emitting a fluorescence photon. The emission of a fluorescence photon from a fluorophore does not occur at a fixed time. Instead, a distribution of times is observed, which can be described by an exponential decay function. The characteristic time constant of this decay, the fluorescence lifetime, is in the range of a few picoseconds (10^{-12} s) to several tens of nanoseconds (10^{-9} s).

This lifetime is a characteristic parameter of each fluorescent dye that may change with its micro-surrounding or its conformational state. Lifetime information probes the molecular environment for its composition, such as ion concentration, pH, lipophilicity or the binding to other molecules.

FLIM combines lifetime measurements with imaging: lifetimes obtained at the pixel-level are color-coded to produce images. Thus, FLIM delivers information about the spatial distribution of a fluorescent molecule together with information about its nano-environment. This way an additional dimension of information is obtained.

Principle of FLIM data acquisition and analysis:

1. Repeated measurement of the time between laser pulse and fluorescence photon at each pixel
2. Calculation of a histogram of photon counts over arrival time after the laser pulse
3. Fit of an exponential decay to each histogram. The amplitude reflects the total number of photons, the time constant \( \tau \) is called the fluorescence lifetime
4. Display of lifetime image using a false-color look-up table
SP FLIM PMTs, which are integrated into the SP module of the scan head, ensure optimal adjustment to experimental conditions and removal of autofluorescence. Software-controlled mirror sliders in front of the detector select the wavelength range of interest. This gives the freedom and flexibility to choose the spectral detection range for FLIM.

**SMD FLIM wizard: complex experiments easily run**

Using the SMD FLIM wizard the user defines all relevant parameters for FLIM acquisition, such as scan speed and format or acquisition time of single FLIM images. These settings are transferred to and automatically used by SymPhoTime Software.

The SMD FLIM wizard offers a variety of FLIM scan modes to set up more complex sequences of FLIM data acquisition: FLIM volume stacks give information about the lifetime distribution in tissues or small organisms. With FLIM time series the researcher can follow dynamic changes of fluorescence lifetimes, especially in live cells or tissues. Volume stacks and time series can be easily combined.

A new dimension of knowledge – FLIM lambda stacks: SP FLIM detectors are used for automated acquisition of FLIM lambda stacks, i.e., FLIM image series at defined bands of the emission spectrum. Lifetime emission spectra are especially useful for characterization and identification of autofluorescence or new chromophores, for better separation of dyes with similar properties, and for identification of conformational states and aggregation of chromophores.

Automated brightness control provides maximum data reliability: this unique feature generates images with pre-defined brightness. It ensures that the photon statistics of all recorded images are consistent. Automated brightness control can be used for volume and lambda stacks. Intrinsically it compensates for a fluorescence intensity decrease caused by photo-bleaching or light absorbance in deeper sample sections.
Host – Pathogen Interaction – Identification of Invading Hyphae in Tomato Fruit

1. FLIM overview image

2. FLIM λ stack

3. Fluorescence intensity spectra

4. Spectra of average lifetimes

5. Spectral lifetime fingerprinting

Note: Data visualization using external software
FLIM-FRET: The Molecular Ruler

A typical application of FLIM is FLIM-FRET. FRET is a well-established technique to study molecular interactions. It scrutinizes protein binding and estimates intermolecular distances on an Angström scale as well.

In a FRET experiment the potential binding partners are labeled with spectrally distinct fluorophores in such a way that the emission spectrum of the donor molecule overlaps the excitation spectrum of the acceptor molecule. If both interaction partners are in close contact at a distance of only a few nanometers, the excited donor can transfer its energy to the acceptor. In turn, the acceptor emits a fluorescence photon and the fluorescence lifetime of the donor molecule decreases.

Intensity-based FRET methods are quite susceptible to variations in expression level or molecule diffusion inherent in the sample. This also applies to external influences such as sample movements and excitation fluctuation. Regarding this, FLIM-FRET is a great advantage as it is calibrated internally. It allows FRET measurements independent of such disturbances.
Contamination-free FRET using FLIM $\lambda$-stacks

1. Set up $\lambda$-stack using the FLIM wizard

2. Acquire FLIM data for donor-only, FRET and control samples

3. Identify optimal spectral detection range (blue area)

4. Compute FRET efficiency from optimal detection range

$$E = 1 - \frac{\tau_{FRET}}{\tau_{noFRET}} = 15\%$$

Note: Data visualization using external software
Towards multi-parametric biophysics

The Leica TCS SMD FLCS system conveniently combines both FCS and FLIM in one system. This ensures the highest flexibility in biophysics. Moreover, the use of common detectors and identical data format even allows completely new ways of data analysis, such as gated FCS and FLCS. The result: a variety of information can be extracted from a single measurement.

Maximum data reliability

FLCS and gated FCS increase the signal-to-noise ratio in correlation data. Time-dependent photon filtering removes scattered light or stray light, autofluorescence, detector after-pulsing, and other non-specific background signals. The in this way obtained parameters are more reliable even in difficult and weakly stained samples.

Using FLCS separates fluorescent species by their lifetimes before calculating auto- and cross-correlation curves. Correlation analysis using two spectrally identical dyes while avoiding any chromatic effect is now possible.

Applications of gated FCS and FLCS

- Differentiate micro-environments in one FCS measurement
- Low concentration measurements in sub-nanomolar range
- Quantitative analysis in samples with low signal and high background noise
- Binding studies of spectrally similar dyes using cross-correlation

One for all – sensitive fluorescence detection at highest time resolution

A detector appointed to FLIM and FCS measurements needs to have both high quantum efficiency and high time resolution. All Leica TCS SMD FLCS systems are equipped with 2 PDM APDs. These detectors offer full flexibility which results in high-quality data. Additionally, for spectral FLIM acquisition two SP FLIM PMTs can supplement the system.
The FLCS and Gated FCS Methods

Essentially, FCS can be performed with a continuous-wave laser, but using 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, selects for analysis of only those photons that can be clearly attributed to the fluorescence decay. Suppression of scattered light (Rayleigh, Raman) from the detected signal allows an accurate measurement of concentration. In FLCS each photon is statistically weighted as to how much it contributes to the FCS data analysis. It can be used to correct for detector artifacts or scattering contributions. It even allows simultaneous monitoring of two dyes with completely overlapping emission spectra and with very similar diffusion coefficients by using only a single detector.

Removal of detector artifacts by lifetime filtering (FLCS)

1. Measurement in vivo

2. FCS autocorrelation (no time filter)

3. Fitting lifetime decay and computation of time filter pattern

4. FLCS autocorrelation after time filtering
Top features
• True optical sectioning
• Adaptable spectral emission bands
• Prism-based spectral dispersion
• Programmable illumination
• Full transmission beam splitting
• High speed resonant scanning
• Triplet suppression
• Tandem Scanner
• Beam parking

Top application areas
• Neurobiology
• Embryology
• Cell Biology
• Genetics

The Ideal Base for SMD: Leica TCS SP5

Top-of-the-line technology

The Leica TCS SMD series is based on the highly approved Leica TCS SP5 confocal and multiphoton microscope system. The Leica TCS SP5 provides high photon efficiency and ingenious innovations in spectral fluorescence detection, which are the ideal preconditions for quantitative measurements.

Spectral multiband SP detector

The spectral detector module offers simultaneous detection of gapless variable emission bands. The SP detector resembles a multiband spectrophotometer, based on a prism and mirror sliders. Two of the five channels can be equipped with fast PMTs for FLIM measurements. This allows convenient optimizing of spectral separation and also for recording a comprehensive spectral sequence of tau-maps.

Acousto-Optical Beam Splitter (A0BS)

A critical element of incident light fluorescence microscopy is the beam splitter. Leica Microsystems has set the standard with the introduction of the A0BS. This optical device is a programmable deflection crystal, which very specifically directs narrow excitation lines onto the sample while passing the full emission onto the detection module. The efficiency is in the range of 95% transmission. As the excitation lines are computer-controlled, the system can switch the excitation regimes of various laser lines in a matter of a few microseconds. The high performance in reflection suppression allows high quality SMD measurements even in close proximity to reflective structures, as for example cover glass.
Tandem scanning system

The Leica Microsystems tandem scanning system comprises both variants within the very same system – conventional and resonant scanner – and addresses many different applications. Extra slow scanning and beam parking: the conventional scanner allows optimal user-defined adjustment of scan speed to the experimental requirements. For FLIM and single molecule imaging the ability to slow the scan down to one line per second is of special interest. Its controlled positioning capability is a prerequisite for FCS and FLCS multispot measurements.

High-speed imaging: recording of fast changing structures (such as cilia movement) or concentration values (such as calcium waves) requires high-speed data acquisition. The resonant scanning system, which operates at a line frequency of 16,000 Hz (bidirectional), provides this speed.

Specialized objectives for superior optical performance

SMD technologies require specialized objectives with high resolution and transmission, and high correction of chromatic and spherical aberration.

Reliable and reproducible FCS and FLCS data are only obtained by using objectives with the best possible aberration correction. Only then will the shape of the confocal volume closely resemble the mathematical model used in FCS analysis. Furthermore, it is crucial to match the refractive index (RI) of the immersion media with that of the sample under investigation. The 63x HCX Plan Apo 1.2 water immersion objective meets these requirements for live cells, tissues and other aqueous samples. A correction collar allows adjusting for deviations of cover glass thickness, temperature and RI. The high numerical aperture ensures a confocal volume small enough to observe single molecule fluctuations even in living cells. The objective with its excellent color correction is even suitable for FCCS experiments.

To maximize photon yield in FLIM measurements in fixed samples, the 63x HCX Plan Apo 1.3 glycerol immersion objective, with its ability to be adjusted for cover glass thickness, temperature, and RI, can be used together with a customized glycerol-water mixture to match the RI of the sample as closely as possible.
<table>
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<tr>
<th>TCS SMD FCS</th>
<th>TCS SMD FLIM</th>
<th>TCS SMD FLCS</th>
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<tr>
<td><strong>Choice of lasers:</strong></td>
<td><strong>Exclusive choice of pulsed laser combinations:</strong></td>
<td><strong>Choice of lasers for FCS:</strong></td>
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<tr>
<td>Use built-in cw lasers of TCS SP5 (laser lines available depending on TCS SP5 configuration: 405, 442, 458, 488, 514, 543, 561, 594, 633)</td>
<td>– 405</td>
<td>Use built-in cw lasers of TCS SP5 (laser lines available depending on TCS SP5 configuration: 405, 442, 458, 488, 514, 543, 561, 594, 633) and</td>
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<td>– 470 + 640 + Fiber Coupling Unit II – MP</td>
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<td>Appropriate laser drivers (PDL 800-B, PDL 800-D, PDL 808 “Sepia I”, or PDL 828 “Sepia II”) are included</td>
<td>– 405 + MP</td>
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<tr>
<td><strong>Exclusive choice of detectors:</strong></td>
<td><strong>Exclusive choice of detector combinations:</strong></td>
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<td>2 SPCM-AQRH APDs from PE, or 2 PDM APDs from MPD</td>
<td>– 2 PDM APDs from MPD, 2 PDM APDs from MPD + 2 SP FLIM PMTs, 1 or 2 SP FLIM PMTs, 1 external FLIM PMT</td>
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<td><strong>Components of data acquisition instrumentation:</strong></td>
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<td>TCSPC module PicoHarp 300, and Detector router PHR 800</td>
<td>TCSPC module PicoHarp 300, and at more than one FLIM detector: detector router PHR 800</td>
<td>1. TCSPC module PicoHarp 300, and 2. At more than one FLIM detector: detector router PHR 800</td>
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<td><strong>Software:</strong></td>
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<td>SymPhoTime SPT-1, and SMD FCS wizard in LAS AF</td>
<td>SymPhoTime SPT-2 SMD FLIM wizard in LAS AF</td>
<td>SymPhoTime SPT-1 SymPhoTime SPT-2 SMD FCS wizard in LAS AF SMD FLIM wizard in LAS AF</td>
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Leica TCS SP5 (AOBS) +

Trigger unit, pulse interface, 1 additional PC, 2 additional monitors, mounting rack, monitor array, APD imaging interface depending on configuration: X1 port adaptation, laser incoupling ports
SMD Specific Devices

1. Trigger Unit
2. 4 Channel Detector Router PHR 800
3. TCSPC PicoHarp 300
4. Dual SPAD Power Supply DSN 101
5. PDM APD Channel 1
6. PDM APD Channel 2
7. Fiber Coupling Unit FCU II Housing Picosecond Pulsed Diode Laser Heads
8. Pulse Interface
9. Multichannel Picosecond Diode Laser Driver PDL 828 “Sepia II”
10. Mounting Rack

visible and ultraviolet radiation:

LASER RADIATION
AVOID DIRECT EXPOSURE TO BEAM
VISIBLE AND/OR INVISIBLE LASER RADIATION
P=500mW / λ = 350-450 nm
CLASS 3B LASER PRODUCT

infrared radiation:

LASER RADIATION
AVOID EYE OR SKIN EXPOSURE TO DIRECT OR SCATTERED RADIATION
VISIBLE AND/OR INVISIBLE LASER RADIATION
P=4mW / λ = 355-1050 nm
CLASS 1 LASER PRODUCT
Acknowledgements:

We gratefully acknowledge the following scientists for providing images and data, and valuable support:

1. Antennae of scale insect. 3D rendering of FLIM z-Stack using autofluorescence. Color-coded lifetime images were segmented according to chitin lifetime for isosurface rendering (green) and volume rendering of surrounding structures (red). Clearly the antennae, body hairs, as well as other chitinous structures were reconstructed. Rendering was done using external software. Sample courtesy of Prof. Kees Jalink, NKI, Amsterdam.

2. Diffusion of Alexa 488-labeled BSA (bovine serum albumin) in polymer matrix. Investigation of subdiffusion threshold using different molecular weights of dextran as a model for visco-elastic matrix properties inside live cells. We thank Dr. Matthias Weiss, Dr. Jedrzej Szymanski, and Nina Malchus, DKFZ, Heidelberg.

3. CFP FLIM of a FRET construct in RBK cells. Courtesy of Prof. Gregory Harms, University of Würzburg, Germany. We acknowledge experimental support by Dr. Benedikt Krämer (Picoquant), Jan-Hendrik Spille, and Wiebke Buck.

4. Autofluorescence FLIM image of single cell in fungal hypha. Clearly the short lifetime of chitinous cell wall materials is visible (blue) as compared to cytoplasm and subcellular structures (red and yellow).

5. We thank Prof. Dr. Roland Eils and Dr. Joel Beaudouin (Bioquant, University of Heidelberg) for providing samples, conducting the experiment and advice on the interpretation of the results.

6. We thank Dr. Matthias Weiss, Dr. Jedrzej Szymanski and Nina Malchus, DKFZ, Heidelberg.

7. We thank Dr. Matthias Weiss, Dr. Jedrzej Szymanski and Nina Malchus, DKFZ, Heidelberg.

8. We thank Dr. Matthias Weiss, Dr. Jedrzej Szymanski and Nina Malchus, DKFZ, Heidelberg.

9. We thank Dr. Matthias Weiss, Dr. Jedrzej Szymanski and Nina Malchus, DKFZ, Heidelberg.

10. XZY section of fibroblast cell with labelled actin cytoskeleton. Positioning of measurement points in 3D data sets is possible both in the XY plane and the XZ plane. This facilitates measurements in different subcellular compartments such as cytoskeleton or membranes.

11. Concentration mapping of TIF1a-GFP in HeLa cells. Calibration of image taken with SPAD detector with FCS point measurements. In this example a spatial map of intracellular concentrations is obtained. ROIs for FCS measurements (red and blue graphs) shown as black crosses. We thank Dr. Matthias Weiss, Dr. Jedrzej Szymanski and Nina Malchus, DKFZ, Heidelberg.

12. Autofluorescence image of pathogenic plant fungi highlighting host pathogen interactions.

13. FLIM image of stained Paramecium spec. We thank Prof. Dr. Alberto Diaspro.

14. 3D reconstruction of autofluorescence FLIM z-stack of lily pollen grain acquired using the SMD FLIM wizard. At least two distinguishable lifetime components are rendered in green and blue respectively. Visualization was done using external software.

15. Lambda-Stacks of FLIM images of invading pathogenic hyphae in tomato fruit (1) using autofluorescence excited with 470 nm. Spectral scan of 50 nm steps from 440 to 740 nm (2). Intensity spectra (3) and lifetime spectra (4) show strongly overlapping, non-separable species. Spectral information with fluorescence lifetimes allows disentangling a complex mixture of (auto-)fluorescent species (i.e. “fingerprinting” of each species).

16. Contamination-free FRET: Optimal detection range for FRET (1). Lambda series of three samples, a donor-only control, a co-transfection as negative control and a GFP-mCherry tandem as FRET sample (2). Detection window free of autofluorescence or acceptor fluorescence ranges from 485–545 nm (constant average lifetime) (3). Artefacts above 545 nm. Spectral detectors hence help to maximize the usable wavelength range for FRET imaging.

17. FLCS in solution of Atto 425 excited at 405 nm. Detector artefacts and background noise removed by lifetime filtering. Improved signal-to-noise ratio evidenced by larger amplitude in FLCS mode (blue curve) compared to standard FCS (red curve). We thank Dr. Volker Buschmann, PicoQuant GmbH, Berlin for experimental support.

18. Principle of FLCS. GalNacT2-GFP expressed in HeLa cells analyzed with FLCS. Fluorescence image using SPAD detectors (yellow) with DIC overlay (1). Autocorrelation without lifetime filtering (2). Lifetime of GFP determined by curve fitting (3). Time-filtered curve (blue) and standard FCS curve (red) reveal removal of autofluorescence and noise (4). We thank Dr. Matthias Weiss, Dr. Jedrzej Szymanski and Nina Malchus, DKFZ, Heidelberg.

19. Perfect localization of differentially stained structures in xz: Hela cells stained with DAPI (nucleus), actin (Alexa 488), filaments (TRITC), and mitochondria (Mitotracker). Excitation with 405 nm, 498 nm, and 543 nm.
Abbreviations:

- APD: Avalanche Photo Diode
- FCCS: Fluorescence Cross-correlation Spectroscopy
- FCS: Fluorescence Correlation Spectroscopy
- FLCS: Fluorescence Lifetime Correlation Spectroscopy
- FLIM: Fluorescence Lifetime Imaging Microscopy
- LDM: Live Data Mode
- MFD: Micro Photon Devices
- PDM: Photo Diode Module
- PE: Perkin Elmer
- PMT: Photo Multiplier Tube
- PQ: PicoQuant GmbH
- RI: Refractive Index
- RS: Resonant Scanner
- SMD: Single Molecule Detection
- SP: Spectral
- SPCM: Single Photon Counting Module
- SPT: SymPhoTime Software
- TCS: True Confocal Scanner
- TCSPC: Time Correlated Single Photon Counting

References:


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