PicoQuant

MicroTime 200

A versatile platform for time-resolved confocal microscopy

Foreword

Dear Researcher,

PicoQuant has a long and successful history in instrumentation for time-resolved spectroscopy and single molecule detection. Our picosecond pulsed diode lasers and photon counting electronics can be found in more than a thousand systems around the world. Several years ago, we have used all our experience in time-resolved spectroscopy and single molecule analysis to engineer an extraordinary single molecule sensitive turn-key microscope system, the MicroTime 200. After more than 10 years on the market, the system is used in leading research institutes and industry laboratories around the world. It is under constant development and always adapted to match the requirements of today's cutting-edge research.

If you are interested in this outstanding instrument, please contact us – we are always happy to discuss your individual requirements in detail. Your needs drive our development.

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Vision

An unmatched time-resolved confocal microscope

The MicroTime 200 is the only commercial instrument that provides single molecule sensitivity and extremely high temporal resolution combined with outstanding capabilities and ease-of-use. It allows numerous measurement and analysis modes and is established as a versatile tool for current research and analysis topics.

Today's research puts high demands on the capabilities of analytical instruments. They must be powerful, flexible and extendible, yet easy to use. This philosophy is one of the underlying principles in the sophisticated design of the MicroTime 200 – all systems are individually configured to meet the needs of the user and can always be modified and extended. The major components are proven turn-key devices that provide the capability of simultaneous excitation over a broad wavelength range, multi-channel time-resolved photon detection as well as 2D and 3D high resolution imaging.

High resolution scanning

2D and 3D objective or sample scanning with nominal 1 nm positioning accuracy enabling single molecule studies

Turn-key excitation sources

Picosecond pulsed diode lasers covering the complete spectral range from 375 nm to 900 nm as well as alternative excitation sources (e.g. multi-photon laser)

Specially designed right side port

Port for confocal optics designed by PicoQuant

Comprehensive system software

Intuitive user-interface with extensive analysis and export features for various applications

Open, modular, and flexible design

Easy access to all optical elements and exit ports, e.g., for external devices like spectrographs

Picosecond time resolution

Temporal resolution for lifetime and correlation measurements down to 1 ps

Fully functional microscope

All remaining ports of the microscope are still accessible for, e.g., widefield illumination or TIRF experiments

Up to four parallel detection channels

Confocal set-up using different detector types with variable beam splitting possibilities

Phosphorescence option

Lifetime measurements from picosecond up to microseconds possible

Accessible Main Optical Unit

Containing all optical elements to guide the excitation laser towards the objective and the fluorescence light towards the detectors. Layout of the optics is very streamline and the design allows for easy realignment and monitoring of the laser beam.

Methods and Applications

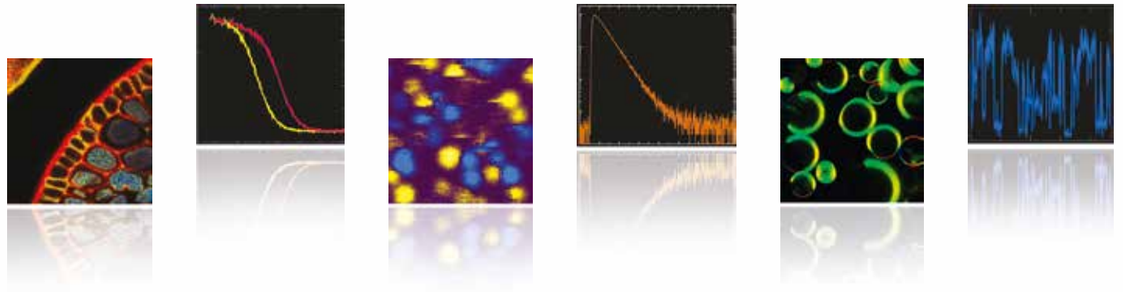
A system with ultimate flexibility for high-end research

The MicroTime 200 is designed as an extremely versatile platform for many different applications. Its unique measurement mode allows to record virtually all aspects of the fluorescence dynamics users may require.

The proven concept of the MicroTime 200 allows its regular usage in a broad range of applications and research topics. On the one hand, its open design allows researchers to adapt the system to their highly specialized experimental needs, while on the other hand it is also perfectly suited for regular analytical tasks owing to its unprecedented ease-of-use. The whole instrument hardware is controlled by the powerful system software SymPhoTime 64. The philosophy of PicoQuant, “making a complex technique easy and accessible for beginners and to provide experts all needed tools”, has been nicely fused. Consequently, the MicroTime 200 can be found in laboratories throughout the world, spanning broad research topics from biological, medical and chemical to physical sciences, such as

- **Single Molecule Studies**
- **Materials Research**
- **Molecular Sensing**
- **Interaction and Conformation Studies**
- **Quantitative Microscopy**
- **Cell Biology**
- **Protein Folding**

Due to its extremely flexible measurement principle, numerous acquisition and analysis methods are available to support this diverse range of applications. The most prominent examples are:



Fluorescence Correlation Spectroscopy (FCS)

- to determine the mobility of molecules
- to observe molecular movement, aggregation, association, dissociation, and conformational changes
- to measure low molecular concentrations
- to study intracellular dynamics (diffusion, active transport, etc.)
- to determine the stoichiometry of complexes
- to quantify concentrations and interaction strengths (e.g., in living cells) Förster Resonance Energy Transfer (FRET)
- to measure inter- and intramolecular distances
- to detect molecular interactions
- to monitor conformational changes of molecules
- to determine environmental parameters (e.g., pH) via FRET sensors

Fluorescence Lifetime Imaging (FLIM)

- to discriminate between spectrally similar fluorophores
- to determine local environment parameters (e.g., pH)
- to analyze oxygen, water, or ion concentrations
- to differentiate structures deep inside tissue
- to eliminate background fluorescence

Fluorescence Lifetime Measurements

- to discriminate fluorophore emission from autofluorescence
- to determine the number of fluorescent compounds (species)
- to study photophysics on the single molecule level

Fluorescence Anisotropy

- to study molecular movement, mobility and symmetry
- to follow conformational changes of molecules
- to analyze surface topology

Fluorescence Intensity Time Traces

- to observe blinking from single particles
- to identify and analyze individual photon bursts
- to assess molecular brightness and stability
- to identify fluorescence photobleaching

Simultaneous FLIM and AFM

- to monitor changes in fluorescence lifetime depending on AFM-tip proximity
- for super-resolution imaging via tip-fluorophore interactions
- to identify individual nano-particles
- to correlate mechanical properties and fluorescence of a cell

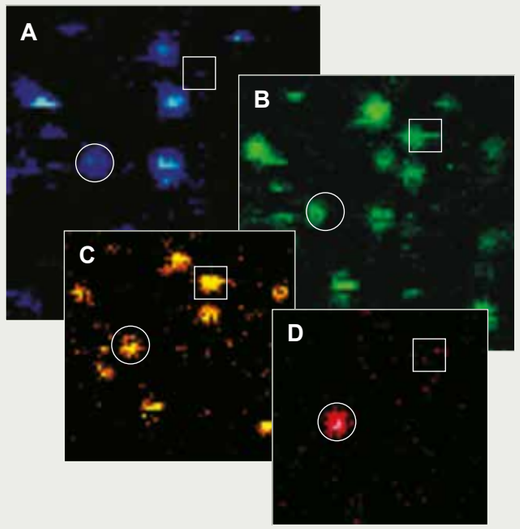
Imaging Measurements

The MicroTime 200 is designed for 2-dimensional and 3-dimensional imaging experiments as well as high resolution line scans. Conventional intensity-based imaging and Fluorescence Lifetime Imaging (FLIM) is supported at a sensitivity that even permits imaging of single molecules.

The MicroTime 200 is equipped with a high resolution piezo scanner for imaging measurements. The standard scanning table has a scan range of 80 µm x 80 µm with a nominal positioning accuracy of 1 nm. A special learning algorithm corrects for positioning uncertainties at different scanning speeds and ensures a highly reproducible and distortion free scanning process. Mono- as well as bi-directional scanning is supported. An optional wide range scanning table can be supplied for imaging of areas in the centimeter range with nanometer positioning accuracy.

A special feature of the MicroTime 200 is the objective scanning mode in which the objective is mounted on the scanning stage and the sample remains stationary. This offers access to larger sample compartments like cryostats and provides a stage ready to add an AFM for simultaneous FLIM/AFM studies.
Sample scanning and objective scanning can be easily exchan-ged. In contrast to galvometric scanners, the confocal volume
(< 1 femtoliter) is independent from the position of the scanner and is undistorted by the scanning process for both scanning modes.

Example



Multichannel imaging of the FRET process inside immobilized photonic wires. The photonic wires are labeled with five different dyes, which fluoresce in different spectral regions. One dye is excited at 470 nm and the energy is transferred inside the wire step by step via FRET to all other dyes. The images show the result of a simultaneous measurement of the fluorescence in four different spectral regions, corresponding to the different dyes in the wire. Differences in the FRET process can clearly be seen: the wire marked with
the circle is visible in all images, while the wire inside the rectangle is only visible in two images. This difference can be either related to different efficiencies of the FRET process or to incomplete labeling of the wires.

Data courtesy of Markus Sauer, University of Würzburg

High precision 3D scanning

The MicroTime 200 can also be equipped with a z-piezo element (PIFOC) which moves the objective up and down to allow 3-dimensional imaging. Combined with the scanning table it allows all kinds of 2-dimensional imaging (X/Y, X/Z, Y/Z) as well as 3-dimensional imaging (z-stacks). With this feature, precise focussing of single molecules becomes easy and thicker structures like cells can be imaged at defined confocal planes.

Optimized light throughput

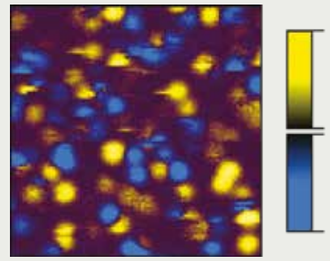
The design of the MicroTime 200 allows to acquire images with up to four parallel operating detection channels in the spectral range from the ultraviolet to the near infrared. The detected light is split either spectrally or according to its polarization or even both. Owing to the selection criterion of highest throughput for the optical elements inside the optical path, the needed intensity of the excitation source is minimal. This permits to work with low laser power and diminishes all problems usually encountered with high laser intensity like backscattering and photobleaching.

The open and modular design philosophy of the MicroTime 200 allows for additional measurement modes. It is, for example, possible to use Total Internal Reflection (TIRF) excitation and the confocal set-up for detection. Options for widefield imaging as well as the combination with Atomic Force Microscopes (AFM) are also available.

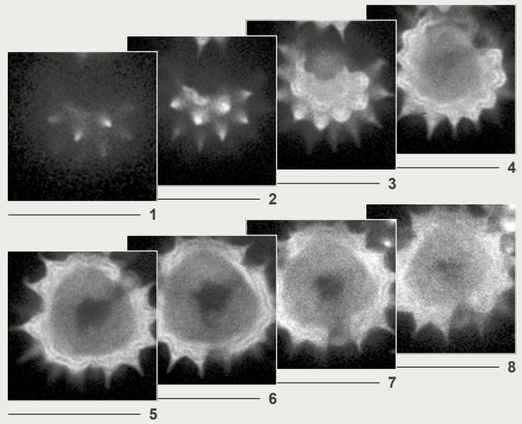
"The MicroTime 200 is an ideal tool for FRET microscopy."

Zygmunt & Ignacy Gryczynski,
University of North Texas Health Science Center

Examples



Polarization-resolved fluorescence of isolated, single Cy5 molecules on top of a standard glass cover slip (image dimension 6 µm x 6 µm). The collected fluorescence light was divided with a polarizing beam splitter cube and simultaneously detected with two SPAD detectors. The image contains all molecules which exhibit either a predominant parallel (yellow) or perpendicular (blue) polarized emission.

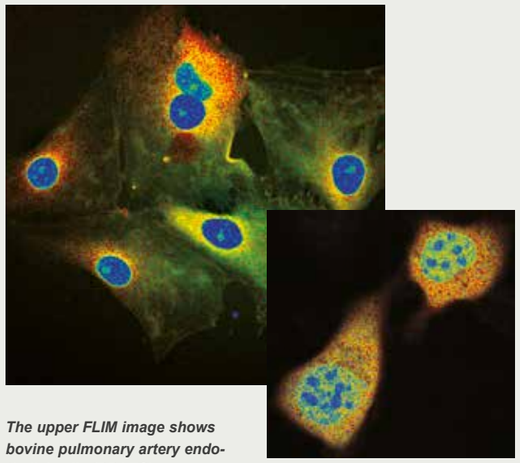


Optical sectioning: the images show the recording of a z-stack of autofluorescence from a daisy pollen. The image size is 20 µm x 20 µm and the images are separated by 2 µm in z-direction.

FLIM

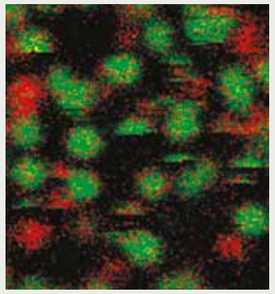
Fluorescence Lifetime Imaging (FLIM) adds another dimension to conventional intensity based imaging methods. FLIM is not affected by fluctuations in the fluorescence intensity and permits to discriminate between fluorophores with similar emission spectra (like Atto655 and Cy5) and from autofluorescence. It can be used to probe local environmental conditions (e.g., pH value), to determine ion concentrations, to study intracellular signal transduction or to distinguish between different tissue components.

Examples



The upper FLIM image shows bovine pulmonary artery endothelial cells (BPAEC).
The mitochondria, the F-actin and the nuclei are labeled with different dyes that have different lifetimes and can therefore easily be distinguished (blue: 3 ns; red: 6 ns). The right image shows the interactions of protein partners in their natural environment inside living cells, studied by FLIM-FRET (blue: 2.2 ns; red: 2.7 ns). The technique was used to characterize intra-nuclear dimer formation for the transcription factor C/EBP a in living pituitary GHFT1-5 cells of mice.

Sample courtesy of Ammasi Periasamy, Keck Center for Cellular Imaging, University of Virginia



FLIM measurements of single Cy5 molecules adsorbed on a glass cover slip. Two types of emitters with different lifetimes due to different molecular orientation, conformations, or interactions with the glass surface can be identified (green: 1.9 ns; red: 0.9 ns).
Visible dark lines are due to blinking of Cy5 molecules.

Fluorescence Lifetime Imaging can be viewed as the time-domain analogue to multi-color imaging without having its inherent problems. Essentially, FLIM can already be performed with only one detector and avoids all problems associated with different detection efficiencies at different wavelengths, bleed through or chromatic aberrations of the optical elements thus eliminating the need of spectral mixing and/or unmixing procedures. Consequently, also the total number of available fluorophores to label molecules is increased, as lifetime and spectral separation of their emission can be combined. The fluorescence lifetime can furthermore be used to extract information about the local environment of the fluorophore, which enables to probe, e.g., pH or ion concentration inside living cells.

Complementing FRET with FLIM

FLIM measurements significantly improve FRET studies. Using the fluorescence lifetime for FRET analysis avoids all problems associated with intensity fluctuations of the fluorescence emission.

In a FLIM-FRET measurement, the fluorescence lifetime of the donor molecule is used as a probe. The energy transfer to the acceptor molecule results in a decrease of the fluorescence lifetime (“quenching”) of the donor molecule in comparison to the lifetime of the individual molecule. In contrast to intensity-based FRET measurements, FLIM-FRET allows to distinguish between molecules that do or do not show FRET in each image pixel. In those cases, the measured fluorescence decay is a super-position of two decays, corresponding to the molecule fractions with and without FRET. A closer analysis of the measured decay even yields the ratio between these two populations.

FLIM with single molecule sensitivity

The superior detection sensitivity and scanning accuracy of the MicroTime 200 enables FLIM down to the fluorescence stemming from a single emitting molecule. Again, the lifetime allows to unravel local changes in the environment based on the smallest possible probe – a single molecule. Differences in individual lifetime and brightness can be traced back to environmental inhomogeneities and competing nonradiative decay channels. This approach also allows to, e.g., monitor conformational changes in molecules or to follow protein folding processes.

“The MicroTime 200 is simply a superb instrument with regard to its optical design, electronics and software.”

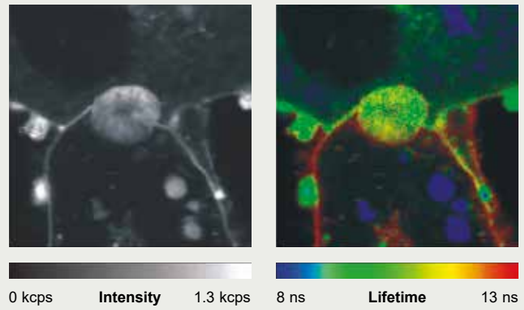
Joseph R. Lakowicz, Center for Fluorescence Spectroscopy, University of Maryland

FLIM images already during data acquisition

An online preview based on the average arrival time of the fluorescence photon is already displayed during the scanning process (“fast FLIM”) and permits a quick assessment of the image quality and lifetime contrast. For a detailed FLIM analysis, the SymPhoTime 64 software can fit a multi-exponential decay function to the fluorescence decay in each image pixel. This analysis is available for the entire image or in arbitrarily shaped regions of interest inside the image.

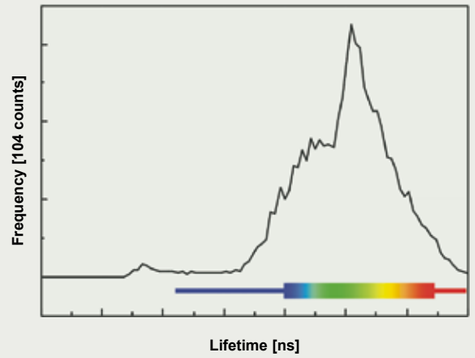
Examples

Fluorescence intensity **Fluorescence lifetime**



Images of hepatocytes of liver cells stained with lipid bound Nitrobenzoxadiazole whose fluorescence lifetime is quenched by water. The intensity image (left) does not allow to distinguish between distinct cellular environments, whereas the FLIM image (right) shows clear variations in fluorescence lifetime indicative of different lipid compositions inside the cell compartments.

Lifetime distribution



Distribution of the measured lifetimes in the upper right fluorescence lifetime image. The lipid bound NBD shows a broad lifetime distribution from approx. 8 ns to 12 ns, indicating different molecular environments of the fluorescing molecules. Long lifetimes (red) are found in the membrane of the cells while the cytoplasm exhibits shorter lifetimes (green) indicating less ordered environment. The shortest 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

FRET

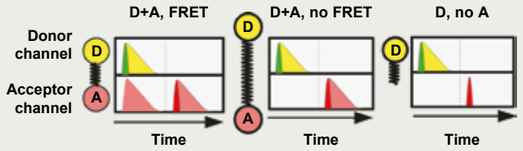
Förster Resonance Energy Transfer (FRET) is a nonradiative process in which energy from an excited molecule (donor) is transferred to a second molecule (acceptor), which leads to changes in the fluorescence intensity and the fluorescence decay kinetics of the two chromophores. The rate of energy transfer is sensitive to the distance between these two molecules. Hence, this technique is used to measure inter- and intramolecular distances on a nanometer scale and has found a broad range of applications: FRET can be used to study in vivo molecular interactions, protein folding kinetics, protein subunit exchange, or enzyme activity. The method is suited to examine the structure of DNA fragments, translocation of genes between two chromosomes, detection of nucleic acid hybridization, and automated DNA sequencing.

The design of the MicroTime 200 allows to efficiently study FRET processes in detail. The emission from both, the acceptor and the donor molecules can be measured simultaneously and enables analysis based on fluorescence intensity as well as lifetime. This permits quantitative study of FRET between donors and acceptors exhibiting strong spectral crosstalk, as well as with non-fluorescing acceptors (e.g., quenchers).

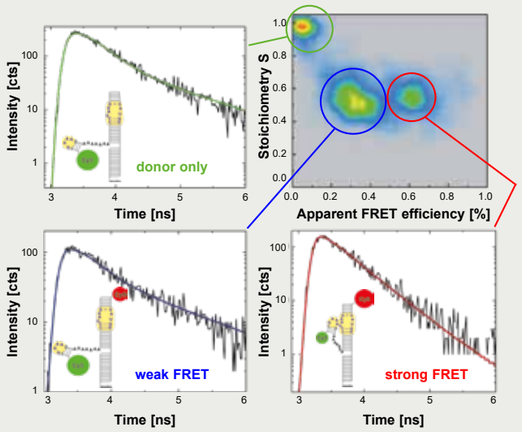
PIE for accurate FRET measurements

One special feature of the MicroTime 200 is the ability to perform and analyze Pulsed-Interleaved Excitation (PIE)-FRET measurements. In this special measurement mode, a second laser pulse excites the acceptor molecule directly, independent of the FRET process, in order to prove its existence. This technique allows to distinguish a FRET pair with very low FRET efficiency from a donor molecule with an absent acceptor and avoids in that way the occurrence of an additional apparent low efficiency peak in the FRET histogram. The additional information gained with the PIE technique allows to determine molecular distances with very high accuracy. The SymPhoTime 64 software readily supports the analysis of PIE-FRET measurements as well as the calculation of FRET efficiency diagrams and the histogram of the donor-acceptor distances in units of the Förster distance R_0.

Examples



In Pulsed-Interleaved Excitation two laser pulses are used sequentially to excite the donor and the acceptor molecule independently. The resulting fluorescence emission patterns can be used to discriminate between molecules showing FRET, molecules that do not show FRET and molecules without the acceptor molecule.



FRET analysis of freely diffusing RNA. The experiment aimed at localizing interactions between specific elements of RNA secondary structure, in this case a GAAA tetraloop motif (Cy3 label, green) and its corresponding receptor region (Cy5 label, red). Using PIE it was possible to calculate a 2D plot of FRET efficiency versus stoichiometry, that enables easy identification of subpopulations for further FRET evaluation.

Data courtesy of Julie Fiore and David Nesbitt, University of Boulder, USA

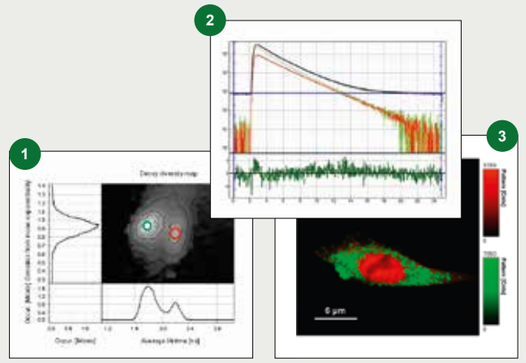
Fluorescence Pattern Matching

Fluorescence pattern matching refers to a special imaging analysis mode that uses the typical fluorescence decay of a fluorescent species as a fingerprint for separation of different lifetime contributions within a FLIM image. Pattern matching overcomes problems with samples that do not exhibit a mono-exponential decay behavior and allows to determine the fluorescence lifetime analytically without the need of time-consuming fitting.

In various applications it is necessary to separate fluorescent species in biological samples. Examples are multi-labeling without crosstalk, differentiating cell types based on their endo-genous protein fluorescence for cancer diagnosis, investigating environmental changes, removal of signal background or autofluorescence as well as separating locations in the sample with and without FRET. As a prerequisite for applying quantitative FLIM techniques, the different constituents must exhibit a mono-exponential decay behavior. Such mono-exponential decays are, however, not observed for most dyes investigated inside biological specimens. The separation of fluorescent species is further complicated by the fact that numerous components like background or autofluorescence are often included in the measured fluorescence decay.

In order to overcome these problems, the fluorescence pattern matching analysis can be applied. In fluorescence pattern matching, the decay “fingerprint” of each fluorescent species within the sample is taken as a pattern and then used to decompose a recorded image on a pixel-by-pixel basis into the different user-defined patterns. Patterns are either imported from control measurements or can even be defined within the image using the so-called “Decay Diversity Map (DDM)”. The DDM consists of a two-dimensional histogram displaying on the x-axis the average fluorescence lifetime, while the y-axis is a measure for the deviation from a mono-exponential decay. Patterns can actually be defined without knowledge about the exact lifetime behavior of the fluorophores involved. Once the patterns are defined, a simple fitting routine is used to assign the patterns to each pixel of the image. The residuals of the fit indicate whether the selected patterns describe the fluorescent decays inherent to the image completely.
During the analysis, for each pixel in the FLIM image the intensity of each user-defined pattern is determined and the distribution of all patterns is displayed. Since a low number of fit parameters is involved, the resulting images for each pattern show high quality, low noise and allow an unambiguous separation of the species under investigation. All patterns can of course be further analyzed in detail by multi-exponential lifetime fitting.

Examples



Binding between N-WASP (labeled with GFP) and TOCA-1 (labeled with mRFP) inside vesicles of CHO cells. The DDM (1) shows two distinct maxima representing a partial FRET pattern (green) as well as the non-FRET pattern (red). The partial FRET population exhibits a shorter lifetime and a slightly bigger deviations from a mono-exponential decay resulting from the fact, that the fluorescence decay contains a mixture of functional FRET pairs and donor only or non-FRET molecules. The lifetime decays are used as patterns for the two species (2). On a pixel per pixel basis the Pattern Matching Analysis then determines the distribution of the partial FRET and non-FRET contributions. The resulting image (3) demonstrates, that FRET takes place inside vesicles while inside the nucleus only free donor molecules (N-WASP linked to GFP) were found. This indicates specific binding of nWASP to TOCA-1 inside of vesicles but no other cellular regions.

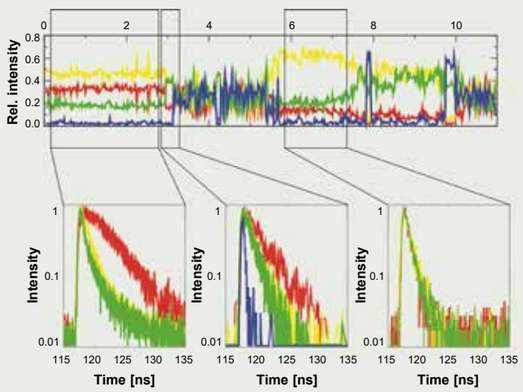
Data courtesy of Sohail Ahmed and Thankiah Sudhaharan, Institute of Medical Biology, Singapore

Point Measurements

Point measurements refer to measurement conditions with a fixed sample volume and a corresponding stationary sample scanner during the data acquisition. Different points of interest in the sample can be identified and studied in close detail. Typical application examples are Fluorescence Correlation Spectroscopy (FCS), Förster Resonance Energy Transfer (FRET), and fluorescence intensity time traces.

Point measurements with the MicroTime 200 benefit from the diffraction limited confocal volume. The size and shape of the confocal volume (< 1 femtoliter) is absolutely independent from the position of the objective scanner – a feature that is especially important for quantitative analysis approaches. The carefully selected optical elements in the beam path guarantee a very good overlap of the confocal volume for different excitation wavelengths, which is of crucial importance, e.g., for Pulsed-Interleaved Excitation (PIE) experiments. The excellent overlap is highly reproducible and can be easily monitored with the 3-dimensional scanning device of the MicroTime 200. The high sensitivity permits time-resolved single molecule experiments with up to four detection channels for, e.g., spectrally separated data acquisition, anisotropy measurements or cross-correlation spectroscopy.

Example



Fluorescence time traces of the FRET process inside an immobilized photonic wire. The photonic wire is labeled with five different dyes, which fluoresce in different spectral regions. One dye is excited at 470 nm and the energy is transferred inside the wire step by step via FRET to all other dyes. The fluorescence from a single wire is monitored with four detectors in four different wavelength ranges with suited beam-splitting optics. The time traces and the corresponding lifetime histograms show the dynamic behavior of the energy transfer process and can be used to study the FRET processes in detail.

Data courtesy of Markus Sauer, University of Würzburg, Germany

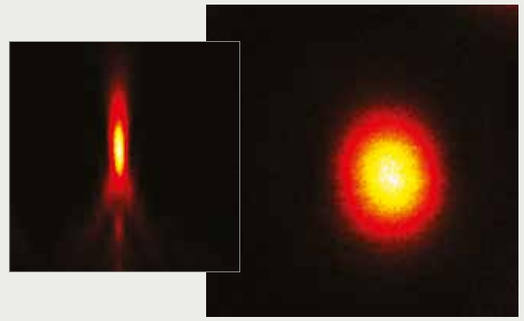
Superior positioning accuracy

The MicroTime 200 scanning stage is characterized by a very high repositioning accuracy with nominal 1 nm resolution. The stage position stays extremely stable even during long time measurements. Due to the principle of objective scanning, the excitation and detection beam path is undistorted and does not depend on the position of the scanning stage. This allows, for example, to acquire fluorescence time traces even for single molecules over long time periods and analyze them for intensity, spectral and lifetime fluctuations. In addition, the focus diagnostic tool helps to easily identify characteristic surfaces in the sample (typically the glass coverslip surface) based on the backscattered excitation light. This facilitates to find thin samples on surfaces independent from their fluorescence or to position the confocal investigation volume in a well-defined distance to the surface, which is necessary for quantitative FCS measurements.

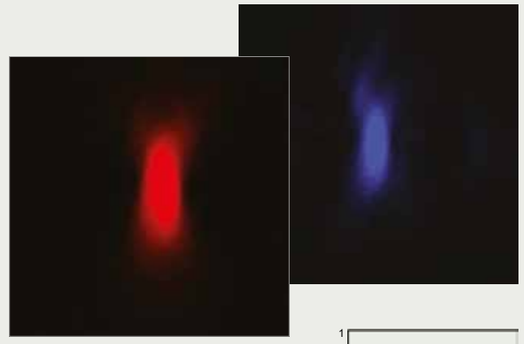
"By far the best instrument of its type on the market."

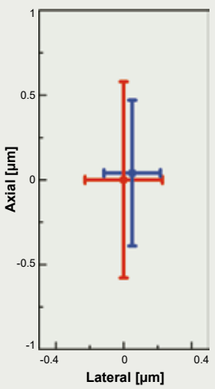
Benjamin Schuler, University of Zürich

Examples



The 3-dimensional scanning stage of the MicroTime 200 can be used to image and quantify the size of the confocal volume (Point Spread Function – PSF) using nm-sized fluorescent beads, which is, e.g., important for analyzing FCS measurements. The displayed images were measured with a 100x/NA1.4 objective. The left image shows an x-z scan (image size: 7.5 µm x 7.5 µm) and the right image an x-y scan (image size: 1.7 µm x 1.7 µm) of a 100 nm diameter bead.



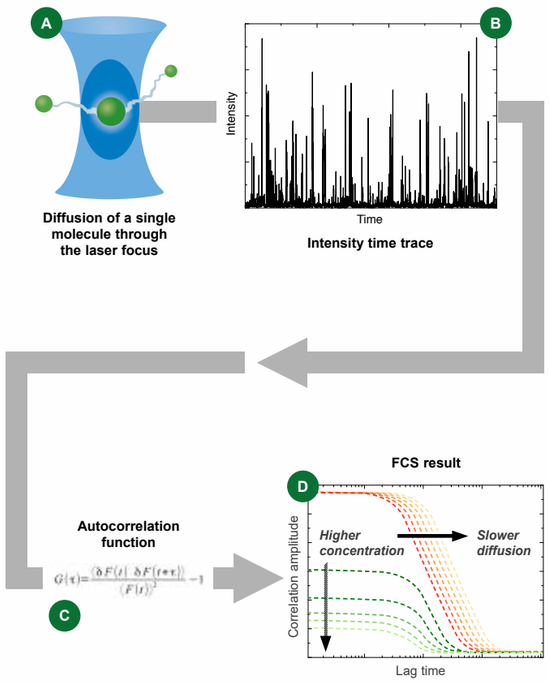


Measurement of the PSF of a 60x/NA1.2 water immersion objective with different excitation wavelengths. The confocal volume was imaged by an x-z scan using 100 nm beads dried on a cover glass, excited at 635 nm (left image) and 470 nm (right image). The displayed image sizes are 3.8 µm x 3.8 µm. The bars in the right graph indicate the full width at half maximum (FWHM) values of the intensity distributions. Due to the shorter wavelength the FWHM values are smaller for blue excitation. The graph clearly demonstrates the very good overlap of the confocal volumes for both wavelengths.

FCS

Fluorescence Correlation Spectroscopy (FCS) is a high precision and versatile method which has demonstrated its great potential for many different applications. It can be employed to measure concentrations, to investigate lateral and rotational diffusion of molecules, conformational dynamics, the stoichiometry of complex molecules, as well as molecular association and dissociation, enzyme dynamics and intramolecular dynamics in vitro, but also in the living cell.

In a classical FCS experiment, continuous excitation light is focussed onto a point in a sample that contains diffusing fluorophores. The observation volume is designed to be very small (sub-femtoliter range) so that in average, fluorescence emission from only a single molecule can be detected (Fig. A). FCS then monitors the emission intensity fluctuations caused by the single molecules passing through the excitation volume. The primary result of such a measurement is an intensity time trace reflecting the fluorescence intensity fluctuations over time (Fig. B).



Autocorrelation to visualize dynamics

The first analysis step for FCS is to apply an autocorrelation function on the acquired intensity time trace (Fig. C). In essence, the autocorrelation calculates the similarity of the time trace with a temporally shifted copy of itself. The resulting sigmoidal curve of correlation over time interval (lag time) shows high autocorrelation for small lag times, dropping relative to the velocity of the diffusing sample. It thus reflects the probability that fluorescence signals between two time points have the same molecular origin. From the autocorrelation curve, two values can be derived: the turning point of the curve corresponds to the diffusion time of the fluorophore through the optical volume, whereas the y-axis intercept is inversely proportional to the number of particles in the observation (Fig. D).

The second analysis step is to fit a suitable biophysical model function to the obtained autocorrelation function characterizing the source of the fluctuations. This enables to calculate the number of fluorophores in the optical volume and the diffusion time through it. By determining the optical volume size, the concentration and the diffusion coefficient of the molecules under investigation can finally be derived from the fit.

Cross-correlation for binding studies

Fluorescence Cross-Correlation Spectroscopy (FCCS), a widely used daughter technique of FCS, measures the simultaneous occurrence of signal fluctuations originating from two different fluorophores with different emission wavelengths. By correlating time traces from two individually labeled molecules that bind to each other, their fluorescence fluctuations are largely identical and their cross-correlation amplitude is high. This principle of dual color FCCS is typically applied for interaction studies allowing to quantify the degree of binding and to access binding kinetics at low molecular concentrations in solution as well as living cells. FCS and FCCS thus provide a highly quantitative tool for measurements of concentration, molecular diffusion, and studies of binding and rotational diffusion.

Lifetime correlation yields artifact free results

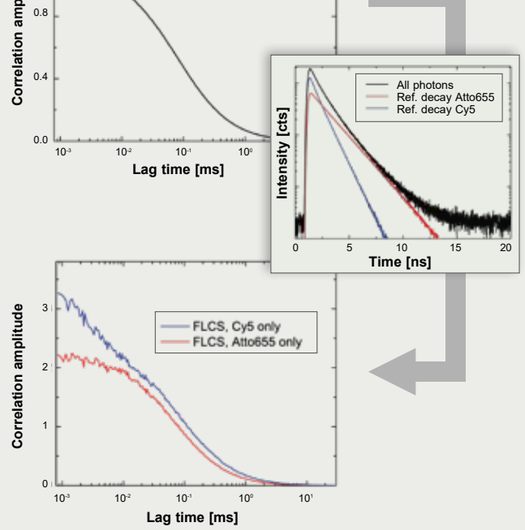
One challenge in FCS has been the influence of experimental factors on the recorded fluorescence signal, such as background fluorescence, spectral bleed-through, light scattering or detector afterpulsing. These challenges can be circumvented with an advanced FCS method possible with the MicroTime 200: Fluorescence Lifetime Correlation Spectroscopy (FLCS). Due to the unique data acquisition concept of the MicroTime 200, the complete photon dynamics during the measurement are available: the absolute photon arrival time relative to the measurement start as well as the arrival time relative to a laser pulse for lifetime information. For a conventional FCS analysis, only the absolute arrival time is used. In FLCS, the fluorescence lifetime information contained in the measurement data is also included into the analysis procedure.

The algorithm behind FLCS is based on a statistical separation of different intensity contributions based on their fluorescence lifetime. This allows, e.g., to remove influence from scattered or background light or to separate the FCS contributions of two dyes measured from a mixture, based on their different fluorescence lifetimes.

Dual-focus FCS (2fFCS)

The MicroTime 200 offers an even more advanced method called dual-focus FCS (2fFCS). In contrast to conventional FCS, it allows to measure absolute diffusion coefficients of dyes without referencing against a sample with a known diffusion coefficient. This is achieved by generating not only one, but two laterally shifted, slightly overlapping laser foci at a fixed distance. Instead of the optical volume of the instrument, 2fFCS uses the known distance between two confocal volumina as a reference. Consequently, this method is independent of the confocal volume's size and shape. It is robust against common measurement artifacts like refractive index mismatch of the sample solution and the objective's immersion medium, coverslide thickness variations and especially the dependence on optical saturation of the fluorescent dye.

Examples



FLCS allows to separate the diffusion characteristics of two dyes measured from a mixture based on their different fluorescence lifetimes. The example shows the measured FCS curve of a solution containing Atto655 and Cy5 molecules in aqueous buffer. By a statistical evaluation which takes the different decay characteristics of the dyes into account, the individual autocorrelation curves of Atto655 and Cy5 can be reconstructed. As expected, the increase of the correlation amplitude at µs lag times is caused by Cy5 only (cis-trans isomerization blinking).

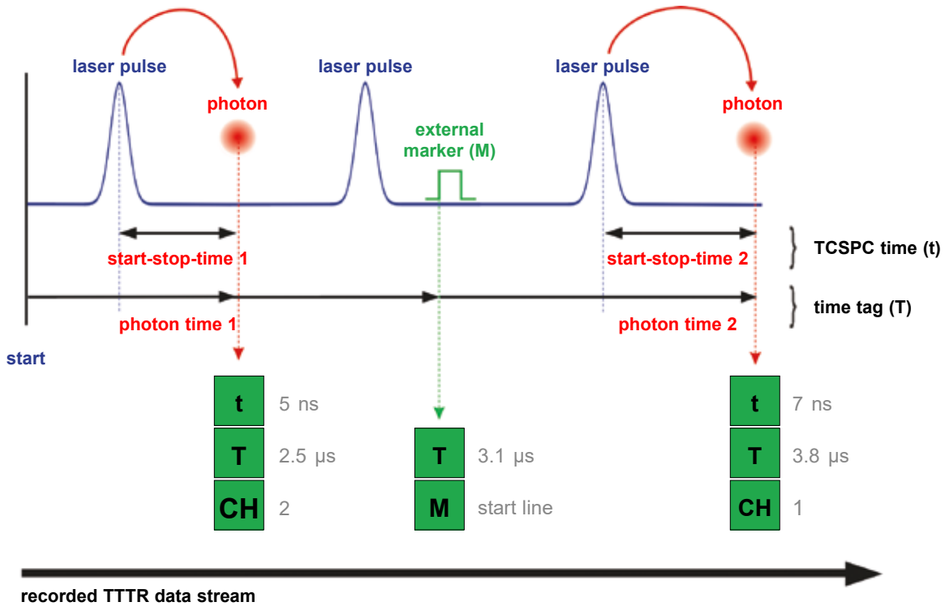
Data Acquisition Principle

Time-Correlated Single Photon Counting (TCSPC)

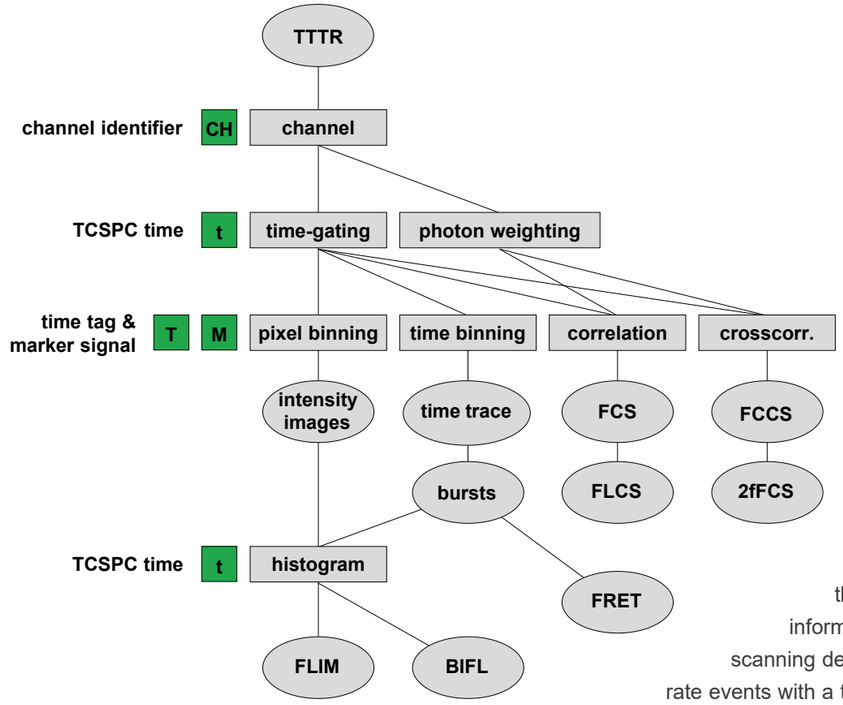
Time-Correlated Single Photon Counting (TCSPC) is the most powerful and sensitive method to measure fluorescence lifetimes. TCSPC can be used to study the emission properties of single molecules and allows to resolve fluorescence lifetimes down to some picoseconds. The method is based on the precise measurement 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 the time difference is repeated for several million photons to account for the statistical nature of fluorescence emission and all measured time differences are sorted into a histogram. This histogram of photon arrival times can then be analyzed to extract the fluorescence lifetime and signal amplitude. PicoQuant's TCSPC electronics PicoHarp 300, HydraHarp 400, and TimeHarp 260 step beyond this simple histogramming and use the Time-Tagged Time-Resolved (TTTR) data acquisition mode since classical TCSPC is not capable of, e.g., measuring the fluctuations in the fluorescence intensity (used for FCS) or to synchronize the data acquisition with a scanning device for FLIM.

"The MicroTime 200 is a powerful and easy to use system with a user-friendly system software."

Andong Xia, Chinese Academy of Science, Beijing"



Measurement scheme of the Time-Tagged Time Resolved (TTTR) mode. Like in classical TCSPC the time difference between the moment of excitation and the arrival of the first fluorescence photon at the detector is measured. This TCSPC time (t) is stored into the TTTR data stream along with a global tag (T) and information about the detection channel (CH). Additional external synchronization signals (markers, M) can be included in the data stream for imaging.



The TTTR data file is the basis for all analysis methods. Depending on how the different information in the data stream (TCSPC time, time tag, channel identifier, and marker signal) are combined and evaluated many different analysis procedures are possible. Only the most common methods are mentioned in this chart.

Time-Tagged Time-Resolved mode (TTTR)

The basic idea of the Time-Tagged Time-Resolved measurement mode (TTTR) is to add a second timing information to each measured TCSPC time. This "time tag" is measured on an absolute scale and represents the arrival time of each photon relative to the beginning of the experiment. In multi-detector set-ups, channel information, i.e., in which detector the photon was registered, is also included and used, e.g., for FCCS and spectral FLIM. The TTTR data stream can be extended even more to contain up to four external synchronization signals (markers) derived from an imaging device like the piezo scanner. This enables to reconstruct the 2D or 3D image from the stream of TTTR records, since the relevant XYZ position of the scanner can always be determined.

Thus, the TTTR data stream consists of photon events with three individual pieces of information: the TCSPC time, the time tag, and the channel information. The markers from a scanning device are incorporated as separate events with a time tag.

Consequently, in the TTTR measurement mode the complete photon dynamics are conserved and no information is lost. The data generated is free of redundancy and can be transferred in real-time.

The Time-Tagged Time-Resolved (TTTR) acquisition mode allows to perform vastly different measurement tasks based on one single data format. This in turn allows to handle all measurement data in a standardized, yet flexible, way. Therefore, virtually all algorithms and methods for the analysis of fluorescence dynamics can be implemented.

The starting point for all data analysis procedures is the TTTR data file. Depending on how the individual information in the data stream are combined, different analysis procedures are possible, ranging from intensity time trace analysis, burst analysis, lifetime histogramming, Burst Integrated Fluorescence Lifetime (BIFL), Fluorescence Correlation Spectroscopy (FCS) or Fluorescence Lifetime Correlation Spectroscopy (FLCS), to Fluorescence Lifetime Imaging (FLIM), Förster Resonance Energy Transfer (FRET), and anisotropy to name only a few.

System Software

SymPhoTime 64 – A Symphony of Photons in Time

The SymPhoTime 64 software package is an integrated solution for data acquisition and analysis for the MicroTime 200. The clearly structured layout and powerful analysis routines allow the user to focus on the data and results rather than on data processing.

Extended hardware control

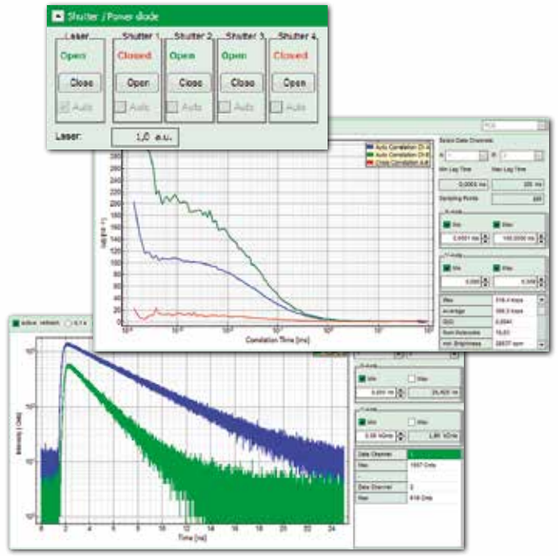
The SymPhoTime 64 software controls all relevant hardware of the MicroTime 200, such as the available piezo scanners, video camera, power photodiode and all installed shutters. It supports all available PicoQuant TCSPC units: PicoHarp 300, HydraHarp 400, and TimeHarp 260. Relevant settings, such as the temporal bin width or the discriminator settings of the input channels can be adjusted within the software. Laser power and repetition rates of the connected lasers can also be controlled directly from SymPhoTime 64.

Automated measurements

SymPhoTime 64 also supports special measurement modes like Time Scan, in which the measurement is repeated at defined time intervals with identical data acquisition parameters. A second acquisition mode is z-stack, in which the z-position of the objective is varied in order to acquire 3-dimensional images.

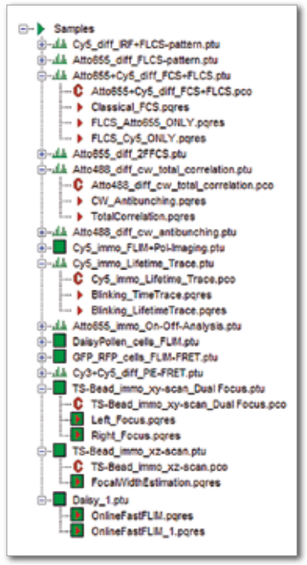
Extensive online visualization

SymPhoTime 64 features online display of up to four indepen-dent preview windows during data acquisition. Data that can be calculated in real time include fast FLIM, intensity time trace, lifetime histograms or auto- or cross-correlation for FCS. This special feature allows for a quick judgment of data quality or changes of the sample properties already during the measurement. It also permits to adjust signal intensity and to optimize the alignment. All calculated previews are saved along with the raw data file in the workspace for later analysis.



Intuitive data acquisition

The interface for the data acquisition is characterized by a clear and comprehensive design. SymPhoTime 64 features a dedicated pre-measurement mode (“oscilloscope mode” or “test mode”), which permits to fine tune system parameters and data acquisition settings without actually creating any measurement data. The interface also shows the image from the video camera that monitors the focal plane. The camera image can be used to position the sample as well as to assess the focus quality.



Intelligent data storage

Another very helpful feature of the SymPhoTime 64 software is its data storage architecture. All measurement data files and all related analysis results are stored in a clearly arranged workspace, with the familiar tree structure of a hard drive directory. Data dependencies are visible at first glance and a log-file keeps track of all measurements and analysis steps performed. Each measurement or analysis can also be commented so that each step is retraceable at any time later. All data is automatically saved, preventing loss of valuable information. It can additionally be exported to standard formats and analyzed in custom-made software for novel approaches.

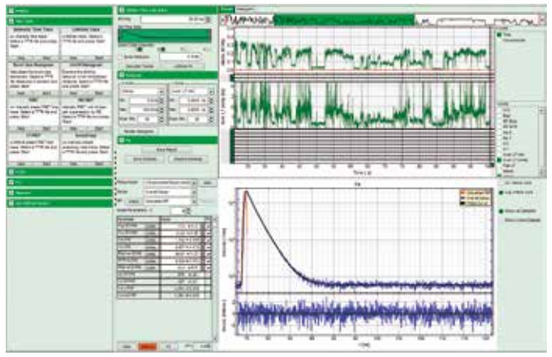
Comprehensive analysis tool

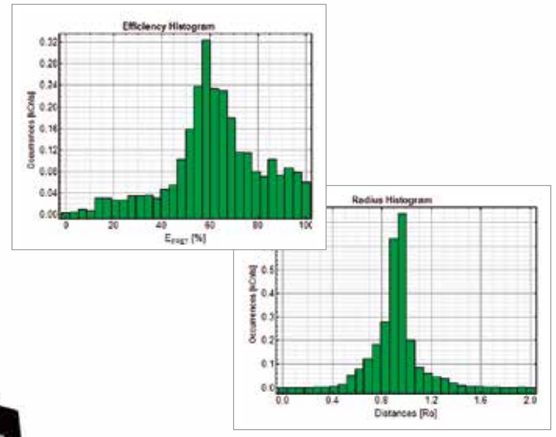
The SymPhoTime 64 is designed to guide the user through all necessary steps for an individual analysis or measurement process. This is achieved by a clearly structured graphical user interface (GUI) and specially adapted analysis procedures for analysis of Fluorescence Intensity Time Traces, FCS, (PIE-) FRET, Anisotropy, and TCSPC Histogram – as well as for imaging measurements like FLIM with up to four detection channels. The SymPhoTime 64 can be further customized using the integrated scripting language “STUPSLANG” that enables the user to add, e.g., additional fit models, GUI components or analysis procedures. Detailed tutorials of data analysis are constantly updated and free to use.

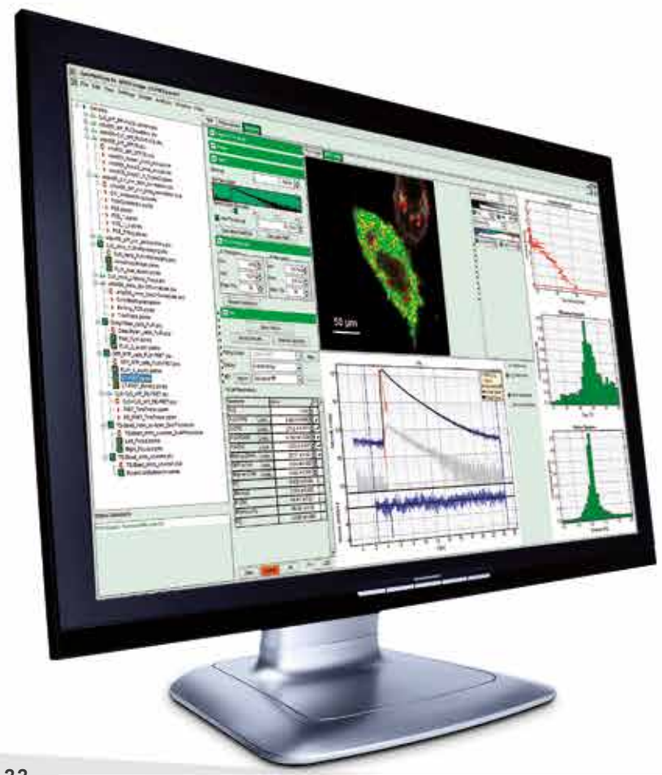
System Software

Fluorescence intensity time traces

The analysis of fluorescence intensity time traces is a core feature of SymPhoTime 64. Fluorescence intensity time traces display the measured fluorescence dynamics and, due to PicoQuant's TTTR-mode, can be analyzed in a variety of ways. SymPhoTime 64 features eight specially adapted user interfaces for the most common time-trace analysis procedures such as intensity time traces, lifetime traces, FRET, Pulsed-Interleaved Excitation FRET (PIE-FRET), or anisotropy. The individual interfaces include all parameters and tools required for a specific data analysis. As an example, the analysis interface for lifetime fluctuations includes an efficient decay fitting algorithm, and provides the determination of up to five different fluorescence lifetimes. The software supports tail as well as reconvolution fitting using either measured or calculated Instrument Response Functions (IRF). All fits are automatically followed by a sophisticated error analysis based on the bootstrap method.







Förster Resonance Energy Transfer (FRET)

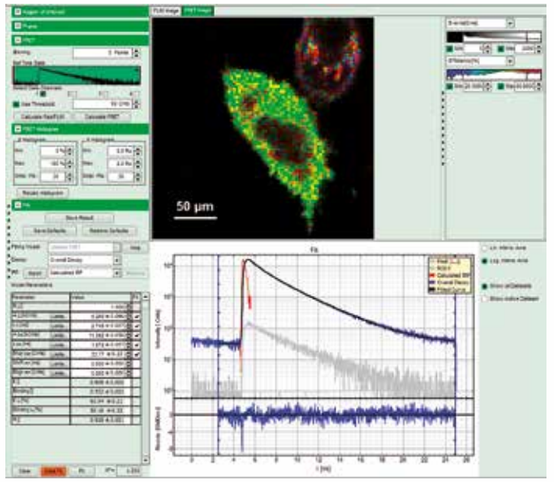
The SymPhoTime 64 offers the analysis of FRET measurements and calculates the FRET efficiency histogram and the histogram of the donor-acceptor distances in units of the Förster distance. Bleed-through, direct excitation, and the differences in the detection efficienc can be taken into account. The special measurement mode of Pulsed-Interleaved Excitation (PIE) that allows to identify incomplete FRET pairs is also supported.

"The MicroTime 200 has made complex technology usable for every lab. We have been able to publish our first paper within a few months."

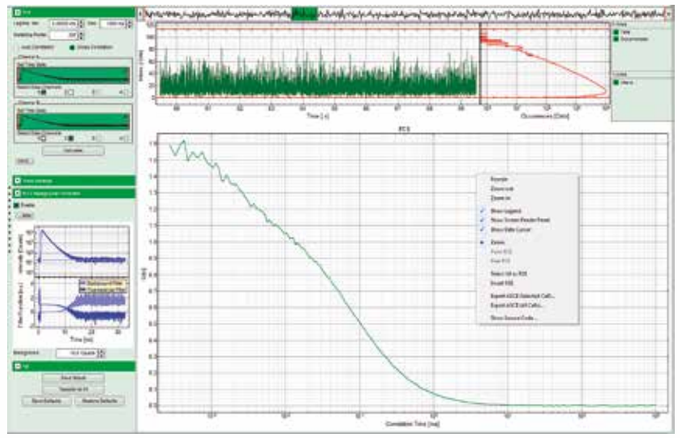
Kankan Bhattacharyya, Indian Association for the Cultivation of Science

Image analysis

With SymPhoTime 64, analysis of (time-resolved) imaging measurements are easier and faster than ever before. The software includes specially adapted, straight forward interfaces for many standard analysis procedures ranging from Fluorescence Lifetime Imaging (FLIM) to Förster Resonance Energy Transfer (FRET) and anisotropy. In FLIM analysis, the SymPhoTime 64 offers a special "fast FLIM" procedure that yields quick



preview results for assessment of the image quality or the selection of regions-of-interest (ROIs) for more detailed analysis. A detailed FLIM analysis is then based on fitting an exponential decay function to the acquired fluorescence decay in each image pixel.



Fluorescence Correlation Spectroscopy (FCS)

SymPhoTime 64 includes one of the fastest software correlators. It supports autocorrelation, cross-correlation, grouped FCS analysis for larger data volumes, Fluorescence Lifetime Correlation Spectroscopy (FLCS), as well as total correlation. A basic analysis routine for 2fFCS measurements is also included. The software features a complete analysis tool to fit different already included established FCS models to the correlation curves. At the same time SymPhoTime 64 allows adding of new fitting functions as well as export of all results for further off-line analysis.

System Set-up and Components

A modular and flexible system

The MicroTime 200 gains its exceptional sensitivity and flexibility in combination with unprecedented ease-of-use from a unique fusion of miniaturized and sophisticated state-of-the-art technologies. The underlying key technologies are PicoQuant's well-established picosecond diode lasers and the Time-Correlated Single Photon Counting (TCSPC) electronics. The system, including the confocal optics, is built around the latest inverse microscope from Olympus and is complemented by piezo-scanning technology and accessories from industry leaders.

Microscope body

The MicroTime 200 is built around the IX 73 microscope body from Olympus. For applications that require a high degree of automation, the fully motorized IX 83 is available. The microscopes are equipped with a transmission illumination unit and a set of different high-end objectives. Compatible accessories are available for integration.

The MicroTime 200 is built around the latest inverse microscope from Olympus. Free access ports on the microscope allow the enhancement of the system and adaption to further measurements tasks.

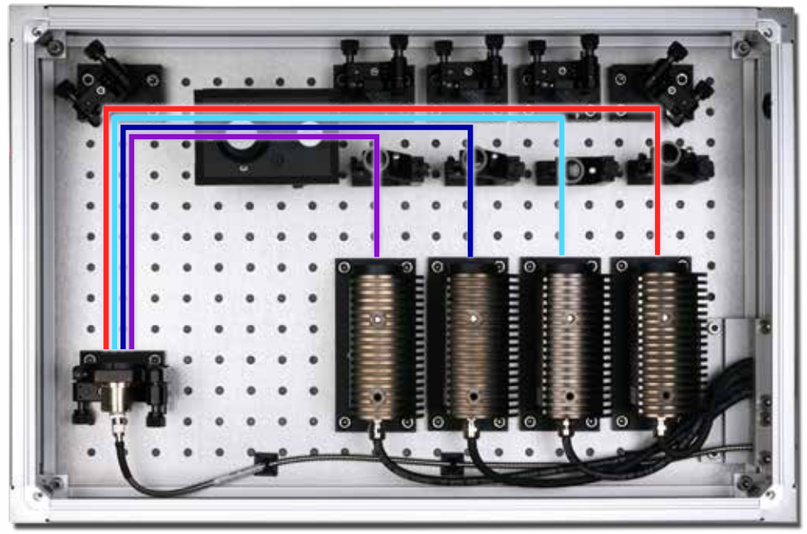
The confocal unit of the MicroTime 200, the Main Optical Unit (MOU), is attached to the right side port. The port is specially designed by PicoQuant and includes a movable 100 % mirror to guide the emission and excitation light into and out of the microscope. Two other ports of the microscope are still accessible for, e.g., widefield detection using a CCD camera at the left side port. As light from the back port is not influenced by the mirror position, it enables to use Total Internal Reflection (TIRF) for excitation and the confocal unit for detection.

Excitation system

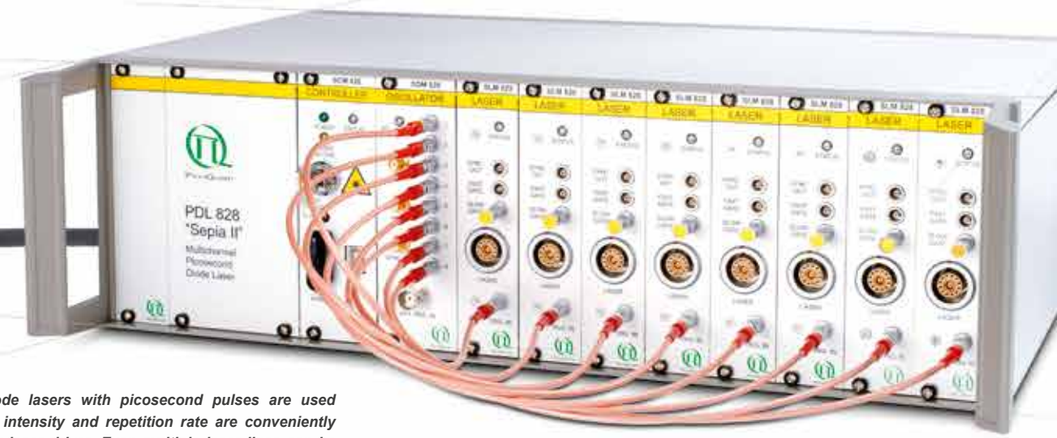
The excitation system consists of compact turn-key picosecond diode lasers, which are available in a wavelength range covering 375 nm to 900 nm and emit pulses as short as 50 ps. Output power and repetition rate can be adapted to the experimental needs, and are thus ideal excitation sources for fluorescence lifetime measurements. The lasers are controlled by a specialized driver, available as a single- and a multi-channel version to allow multi-color experiments as well as Pulsed-Interleaved Excitation (PIE) schemes.

All laser heads are included in a unique Laser Combining Unit (LCU).

This unit couples the emission of up to five lasers into a polarization maintaining single mode fiber, which is connected to the main optical unit of the MicroTime 200. The LCU offers the possibility to adjust the excitation intensity and allows easy and flexible exchange of lasers.
In addition to using pulsed diode lasers for excitation, the MicroTime 200 can also work with other suitable external pulsed light sources like Titanium:Sapphire lasers, mode-locked solid state lasers, and optical parametric oscillators. These lasers are connected to the MicroTime 200 via a dedicated, free-space excitation port. Easy integration and beam adjustment is achieved via a specially designed coupling unit.



The Laser Combining Unit (LCU) allows to couple up to five lasers into one polarization maintaining single mode fiber and makes wavelength changes or simultaneous excitation schemes very easy.

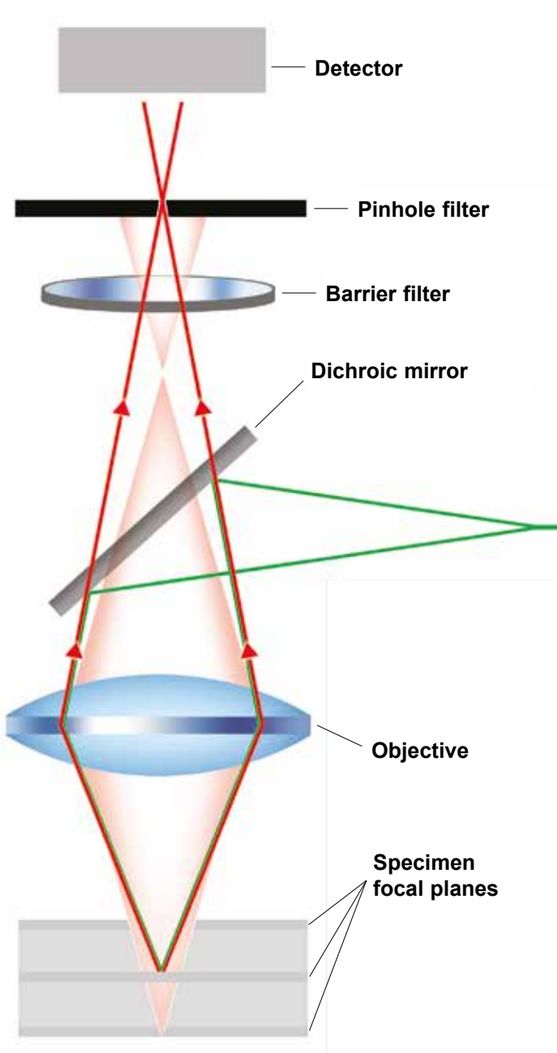


Compact pulsed diode lasers with picosecond pulses are used for excitation. Laser intensity and repetition rate are conveniently changed through our laser driver. Even multiple laser lines can be operated at the same time for multi-wavelength applications.

System Set-up and Components

Confocal detection system

The MicroTime 200 uses the principle of confocal microscopy for data acquisition. In a confocal microscope, a pinhole is used to reject out-of-focus light which leads to a detection volume below 1 femtoliter. The pinhole and the excitation focus are in conjugated image planes of the microscope system – thus the name confocal. By using the confocal pinholes of different sizes, the optical section thickness can be optimized for the chosen objective and wavelength of light. Of course, such a set-up only allows “point” measurements – in order to acquire images, the sample is scanned across a stationary excitation beam.



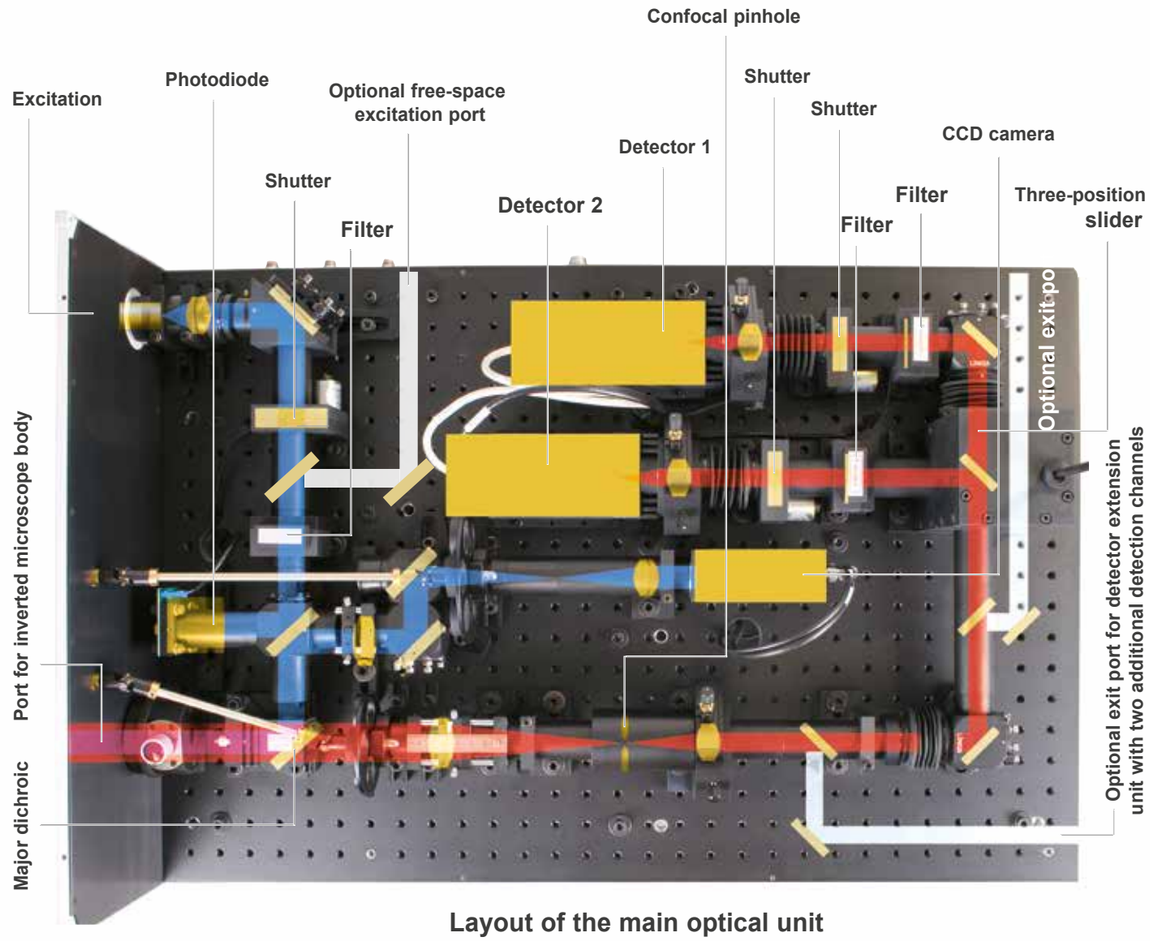
Principle of confocal imaging: A pinhole in the intermediate image plane ensures that only the in-focus portion of the light reaches the detector, which permits imaging with near-diffraction limited resolution.

The confocal detection system in the MicroTime 200 is contained in the Main Optical Unit (MOU). The layout of the MOU permits an easy exchange and adjustment of all optical elements by the user if required. All optical elements inside the MOU are carefully selected for maximum sensitivity and their number is kept to the absolutely necessary minimum to ensure single molecule sensitivity.

In the MOU, the laser light is coupled out of the delivery fiber from the LCU or through the free-space coupling port in case of external excitation sources such as Titanium:Sapphire lasers for two-photon excitation schemes. In the beam path towards the sample, a part of the laser light is deflected onto a photodiode by a beam splitter allowing for online excitation power control. The same beam splitter also leads light that is scattered and reflected at the sample surface onto a CCD camera, which is used for easy focussing and monitoring of the beam quality.

Variable beam splitting

A 3 mm thick major dichroic, mounted in an exchangeable holder inside the MOU separates the fluorescence from scattered excitation light. These thick dichroics have been chosen for excellent beam quality and robustness and allow an easy change of the configuration without cumbersome realignment procedures. After the dichroic, the fluorescence light is focussed onto an exchangeable pinhole and then guided to the detectors. The detection path after the pinhole contains a beam-splitting element with a three-position slider, which is used to split the light onto different detectors, depending on polarization or wavelength. Two more optional beam splitters can be introduced to guide the light to a second detector unit for simultaneous four-channel measurements, or to an exit port in order to attach, e.g., a spectrometer. The sliders can be accessed from outside the MOU and their excellent positioning repeatability makes realignment of the beam splitters by the user unnecessary. The beam splitters and also emission filters before the detectors have standard sizes and can be easily exchanged.



Layout of the main optical unit

Electro-mechanical shutters are installed in the excitation beam path as well as in front of each detector to ensure safe operation of the system – the shutters can be controlled from the system software or manually via the shutter remote control module.

System Set-up and Components

High resolution scanning

The MicroTime 200 is equipped with a specially developed positioning stage with 25 mm range that allows the coarse adjustment of the sample position. For standard image acquisition, the MicroTime 200 uses the principle of objective scanning. Here, the objective is moved across a stationary sample using a piezo scanner with very high repositioning accuracy. This is crucial and the prerequisite for single molecule measurements at surfaces. This scanner configuration also enables for experiments requiring free access to the sample, e.g., patch-clamp investigations, applications using a cryostat or combined FLIM and AFM measurements. The 2-dimensional piezo scanner from Physik Instrumente is mounted on top of the objective holder and features a scan range of 80 µm x 80 µm with a nominal positioning accuracy of 1 nm. Since the objective is maximally moved 40 micrometers to either side, the symmetry of the laser beam entering the objective is practically not affected. An optional z-piezo element moves the objective up and down (PIFOC) and permits 3-dimensional scanning with a scanning range of 80 µm (nominal positioning accuracy: 1 nm). PIFOC and objective scanner are completely controlled by the SymPhoTime 64 software. Alternatively, a sample scanner moving the sample across a stationary excitation beam can be used instead of objective scanning. If a larger scanning range is necessary, a wide range scanner covering a range up to several centimeters with nanometer positioning accuracy can be provided.

TCSPC electronics for data acquisition

Three outstanding Time-Correlated Single Photon Counting (TCSPC) data acquisition systems are available for the MicroTime 200: the HydraHarp 400, the MultiHarp 150, and the TimeHarp 260. All PicoQuant TCSPC electronics work in the special Time-Tagged Time Resolved (TTTR) measurement mode enabling the numerous analysis methods available with the MicroTime 200.

The HydraHarp 400 is a stand-alone unit with high-speed USB 3.0 connection to the system computer. It features a temporal resolution of 1 ps and up to eight independent detection channels. This permits real parallel measurements with multiple detectors leading to increased data throughput and consequently shorter measurement times.

Like the HydraHarp 400, the MultiHarp 150 4P is a standalone unit that connects to the system computer via a high-speed USB connection. It features a temporal resolution of 10 ps and 4 independent but identical detection channels, which is ideal for multi-channel set-ups.

The TimeHarp 260 is a PCI-express board with two independent detection channels. It has a temporal resolution of 25 ps and an ultra short dead time for high data throughput. The board can cover the time range up to seconds for phosphorescence applications.

The variable beam-splitting unit can hold two different dichroics or beam splitters and is integrated into the main optical unit. It is operated from outside and permits the adaption of the system to the current measurement task.

"Just days after the installation we were up and running and making state-of-the-art single molecule measurements."

Thomas Huser, Department of Physics, Universitiy of Bielefeld

The TimeHarp 260 is a PCIe card type TCSPC electronics component with ultra short dead time. It is used for fluorescence as well as phosphorescence studies due to its extended range up to seconds.

HydraHarp 400 - an outstanding Time-Correlated Single Photon Counting system with picosecond resolution.

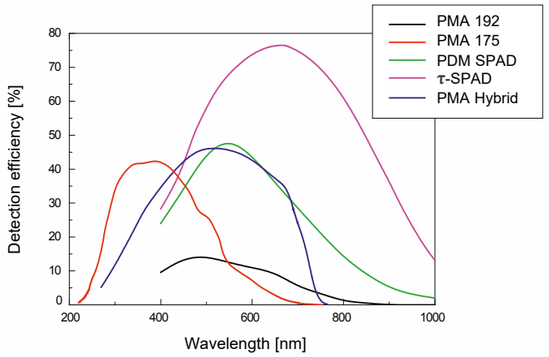
System Components

Single photon sensitive detectors

The MicroTime 200 can be equipped with up to four detectors of various types. Each detection channel has its dedicated filter holder and a electro-mechanical shutter, which is operated from the system software or manually via a remote control module. The available detectors are Single Photon Avalanche Diodes (SPADs), Photomultiplier Tubes (PMTs), or Hybrid-Photomultiplier Tubes (Hybrid-PMTs).

The PMTs (PMA Series) are an economic variant for lifetime based applications such as Fluorescence Lifetime Imaging. They are also the detector of choice for detection in the ultraviolet. However, due to their lower detection efficiency in the visible spectral range compared to SPADs or Hybrid-PMTs, they are not ideal detectors for single molecule studies (e.g., Fluorescence Correlation Spectroscopy). For applications that require very high detection efficiencies, but modest temporal resolution such as Fluorescence Correlation Spectroscopy, the τ-SPADs are an ideal choice as they feature a detection efficiency of up to 70 % in the red spectral region. Their temporal resolution is, however, limited which restrict their usage in high-resolution lifetime based applications. For applications requiring very high temporal resolutions, either the SPADs of the PDM Series or the Hybrid PMT should be used. Both detectors feature a high detection efficiency in the visible spectral region as well as a very high temporal resolution. Depending on the experimental conditions and the type of detector used, the resolvable lifetime can be as fast as 10 ps.

As each detector type carries a special strength, the user has the full choice of selecting the detector that fits the experimental requirements best, be it FLIM, FCS, or single molecule studies. Naturally, different detector types can be mixed in one system to allow for best flexibility.



Detection efficiency as a function of wavelength of some of the available detector types for the MicroTime 200.

Various detector types are available for the MicroTime 200, including PMTs of the PMA Series, SPADs of the PDM Series, τ-SPADs, and Hybrid-PMT detectors.

MicroTime 200 extended

The MicroTime 200 can be extended by integrating instruments that complement its capabilities. We are constantly working on upgrades and new features for the MicroTime 200 to stay at the leading edge of science. Feedback from researchers is always welcome and woven into new extensions.

UV exitation for label-free experiments

Many molecules, ranging from small aromatic compounds to large biomolecules, can be detected by their intrinsic fluorescence in the deep ultraviolet (UV) as they contain appropriate chromophoric groups. Proteins are, e.g., accessible in their native form as they include the fluorophores tryptophan, tyrosine, and phenylalanine.

These three amino acids are, however, relatively rare in proteins and contribute to their UV fluorescence to different degrees. Tryptophan is the main contributor that also shows an emission that is highly sensitive to its local environment. It is therefore commonly used to study protein structure, folding and function. To detect and employ this fluorescence which offers the immense advantage of label-free experiments, a uniquely sensitive UV-option has been added to the MicroTime 200.

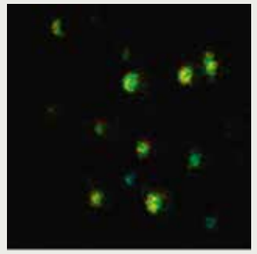
Parallel UV and VIS detection

The UV-option includes a modification of the MicroTime 200 along the optical path to enable fluorescence excitation down to 266 nm and detection in the UV spectral region. For fluorescence excitation, a suitable ultraviolet emitting pulsed laser is incorporated into the system along with suitable optics, and electronics allowing to record photon timing in the UV with picosecond temporal resolution.

The MOU is further equipped with quartz optics and a high-end glycerin immersion quartz objective to enable parallel VIS and UV detection. The fluorescence light is detected by a ultrabialkali photon counting photomultiplier tube. This detector offers a detection efficiency of up to 42 % at 350 nm matching the fluorescence maximum of, e.g., tryptophan.

The MicroTime 200 is particulary suited to implement deep UV experimentation because it uses a high-resolution piezo scanning system, which shows the same performance independent from the excitation wavelength. This is contrary to galvo scanners, that usually do not reflect UV excitation light.

Examples

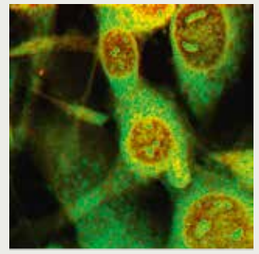


0 ns **Lifetime** 7 ns

Measurements in the ultraviolet allow label-free FLIM with biological cells. The example shows a FLIM image of fixed 3T3-L1 cells that contain tryptophans. Nucleus, nucleoli and the cytoplasm can clearly be distinguished. The sample was excited at 266 nm through a dedicated quartz objective and the fluorescence of the sample was detected by a ultrabialkali PMT.

FLIM image of streptavidin-coated beads (500 nm diameter, 1 streptavidin contains 24 tryptophans), immobilized on a quartz coverslip. Image size is 13 µm x 13 µm. The sample was excited at 266 nm through a dedicated quartz objective and the fluorescence of the sample was detected by a ultrabialkali PMT.

Sample courtesy of Astrid Tannert, University of Leipzig, Germany



2.3 ns **Lifetime** 3.5 ns

MicroTime 200 extended

MicroTime 200 combined with an AFM

The MicroTime 200 can also be combined with an Atomic Force Microscope (AFM). This is a unique combination of two complementing methods in one experiment, one detecting molecular dynamics at diffraction limited resolution and the other detecting topography at sub-nm resolution. Besides gaining additional information about the sample surface, the AFM-tip can be used to manipulate the sample on a nanometer scale down to the single molecule level. The combined MicroTime 200 AFM system then enables to study sample reaction monitored by FLIM in response to manipulation with the AFM-tip. The interaction of the tip with the sample fluorophores can also be investigated and used for high resolution imaging. This unique AFM/FLIM combination allows to address questions that cannot be answered with either technique alone.

Several AFM model supported

The combination of the MicroTime 200 with three different commercially available AFMs has so far been tested:
- MFP-3D BIO from Asylum Research
- BioScope Catalyst from Bruker AXS
- NanoWizard3 from JPK Instruments

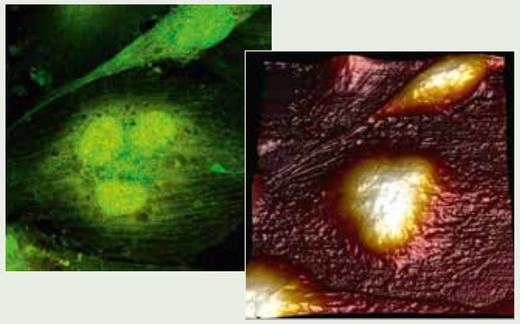
Synchronized recordings of AFM and optical images within the sample region are enabled by convenient alignment of the AFM-tip position with the confocal volume and integration of position markers from the AFM scanning system into the TTTR data stream for digital image assembly.

The objective scanning configuration makes the MicroTime 200 particularly suitable for the extension with a AFM, because the objective is mounted on the scanner and moved below the stationary sample. The AFM sample holder, including the AFM scanner and AFM-tip can therefore be mounted on the freely accessible Olympus IX 73 microscope stand.

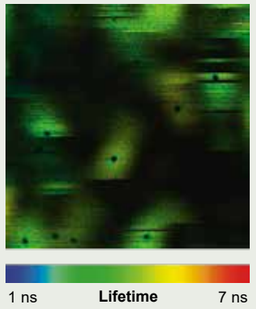
Investigation of cells and single molecules

The applications of the combination of AFM and time-resolved fluorescence microscopy range from investigation of cells and tissues to studies of single molecules. The information which is gained from measurements on cells include topography and the mechanical response of a cell to the AFM-tip (stiffness). Other applications include, for example, to look at the response of fluorescence labeled proteins to mechanical influence from the AFM-tip. Measurements on single molecules reveal the (time-resolved) nanophotonic interaction between the AFM-tip and the fluorophore which can, e.g., be used for tip enhanced Raman spectroscopy and super-resolution microscopy.

Example



Synchronized acquisition of fluorescence lifetime and topography of fixed glioblastoma cells expressing GFP using the MicroTime 200 in combination with the BioScope Catalyst (Bruker, Madison, WI, USA).



AFM-tip with single fluorophores can be studied and used for super-resolution fluorescence imaging along with topography. The FLIM image shows Atto655 molecules scanned with an AFM-tip in the center of the excitation volume. The round dark spots stem from energy transfer to the AFM-tip.

Sample courtesy of Robert Ros, Arizona State University, Tempe, USA

Specifications

Excitation system
- Picosecond diode lasers (375 nm – 900 nm) with repetition rates up to 80 MHz inside a compact Laser Combining Unit
- Single- or multichannel laser driver
- Optional: excitation down to 266 nm
- Optional: external laser (e.g., Titanium:Sapphire laser)

Microscope
- Inverted microscope IX 73 or IX 83 from Olympus
- Specially designed right side port for confocal microscopy
- Left side port and back port still accessible (for e.g., widefield imaging or TIRF)
- Transmission illumination unit included
- Special manual sample positioning stage with 25 mm range
- Standard sample holder for 20 mm x 20 mm cover slips
- Optional: epifluorescence illumination, optional: cryostat for low temperature measurements
- Optional: combination with Atomic Force Microscope (AFM)

Objectives
- Air objectives with 20x and 40x magnification (standard)
- Various high-end objectives available (Oil/Water immersion, air spaced, IR/UV-enhanced, TIRF, or long working distance objectives)

Scanning
- Computer controlled 2-dimensional piezo objective scanning with 80 µm x 80 µm scan range at nominal 1 nm positioning accuracy
- PIFOC for 3-dimensional imaging, 80 µm range at nominal 1 nm positioning accuracy
- Optional: sample scanning
- Optional: large area scanning table with centimeter range

Main optical unit
- Confocal detection set-up in a compact housing with up to four parallel detection channels
- Specialized high-end major dichroics with enhanced stability
- All optical elements easily accessible, adjustable and exchangeable
- CCD camera for beam diagnostics and photodiode for relative power measurements
- Variable beam-splitting units and exit ports to connect external devices

Detectors
- Single Photon Avalanche Diodes (PDM Series, τ-SPAD)
- Photomultiplier Tubes (PMA Series), Hybrid-Photomultiplier Tubes (PMA Hybrid Series)

Data acquisition
- Based on the method of Time-Correlated Single Photon Counting (TCSPC) in the unique Time-Tagged Time Resolved (TTTR) mode
- Simultaneous data acquisition of up to four detection channels

System software
- Easy to use and comprehensive Windows system and analysis software
- 1-, 2-, and 3-dimensional data acquisition based on the versatile TTTR file format
- Data archiving in workspace, time-gating for all methods, data export features, binning, TCSPC Fitting (Multi-Exponential Decay (1 to 5 Exponentials), Least-Squares Fitting, MLE Fitting, IRF Reconvolution, Tailfit, Bootstrap error analysis)
- Point measurement analysis: FCS, FCCS, FLCS, PIE-FCS, FCS Fitting (models: diffusion constants, triplet state, conformational, protonation, gaussian PSF, bootstrap error analysis), FCS calibration, antibunching/coincidence correlation, total correlation, blinking (on/off histogramming), count rate histogram (PCH), intensity-gated TCSPC, fluorescence lifetime and intensity traces, lifetime histogram, BIFL (Burst Integrated Analysis)
- Imaging measurement analysis: FLIM, FLIM-FRET, intensity FRET, anisotropy imaging, (time-gated) fluorescence intensity imaging, pattern matching, region-of-interest (ROI)
- Scripting language (“STUPSLANG”) for user-defined analysis procedures, fitting functions, and GUI components

Not all options can be combined with each other.

PicoQuant for Scientists

The annual workshop on Single Molecule Spectroscopy brings together the top researchers in the field.

Application Lab

PicoQuant always welcomes scientists to visit our application labs, to see the MicroTime 200 working, and to perform test measurements with their own samples. We guarantee a quick and qualified investigation of your experimental needs and to provide a solution supporting you in your future research work.
Of course, all of our other products including cuvette based fluorescence lifetime systems for sample pre-testing are also available.

Workshop on “Single Molecule Spectroscopy”

Since 1995, the scientists from PicoQuant organize the annual workshop on “Single Molecule Spectroscopy and Ultrasensitive Analysis in the Life Sciences”, which brings together the top researchers in the field. With this event, we continue to encourage the exchange of knowledge and new ideas between the experts in single molecule spectroscopy, interested scientists from other fields and potential users from Life Science Industry.

Courses on “Time-Resolved Fluorescence”

To improve the understanding and usefulness of time-resolved fluorescence spectroscopy and microscopy, PicoQuant established the “European Short Course on Principles and Applications of Time-resolved Fuorescence Spectroscopy” as an annual event since 2003. In 2009, an additional event was introduced focusing on time-resolved microscopy and its applications. Both courses are intended for individuals wishing an in-depth introduction to the principles of fluorescence spectroscopy and microscopy and their applications to the Life Sciences. They consist of lectures as well as comprehensive instrumentation and software hands-on training.

PicoQuant

PicoQuant was founded in 1996 with the goal to develop robust, compact, and easy to use time-resolved instrumentation. Since April 2008 sales and support in North America is handled by PicoQuant Photonics North America Inc.
Today, PicoQuant is known as a leading company in the field of pulsed diode lasers, time-resolved data acquisition, single photon counting, and fluorescence instrumentation.
Our instruments are present all over the world. They are used in the laboratories of Nobel Laureates as well as for carrying out routine quality control in production processes of global industry players. Starting from traditional time-resolved fluorescence detection, the range of covered applications continuously grew to include semiconductor quality control, diffuse optical imaging, materials research, quantum information processing, optical detector testing, and telecommunications. Due to the ease of use of our products, researchers can focus on their scientific questions in biology, medicine, environmental science, quantum optics, or chemistry without needing a large background in physics, electronics, or optics.

We offer state-of-the-art technology

Our goal is to offer state-of-the-art technology that has been co-developed and tested by renowned researchers, at an affordable price for both scientists and price conscious industry.

We have successfully teamed up with major confocal microscopy companies to develop dedicated equipment that permits carrying out time-resolved fluorescence studies on their laser scanning microscopes. Following this philosophy, we are always looking for new challenges. PicoQuant especially encourages OEM inquiries for its products, notably for applications where implementing time-resolved techniques were considered too expensive or cumbersome.

More than 20 years of R & D work

The combination of more than 20 years of R & D work, several thousand units sold, and cooperation with international experts forms the basis for new outstanding developments which are always driven by our customers’ needs and inspirations. Visit our website or contact our product and application specialists directly to discuss your needs. Of course, you are always welcome to visit our application labs during your travels to Germany.

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