PicoQuant

Resolve Time and Spectral Information Simultaneously: Spectrograph Add-on for the MicroTime 200

Marcelle König, Sebastian Tannert

Introduction

This technical note describes the combination of the PicoQuant MicroTime 200 with the spectrograph SR-163 equipped with a Newton 970 EMCCD camera from Andor Technology. This combination allows to simultaneously record intensity fluctuations, fluorescence decay properties and spectral data of the sample and opens up new prospects in the investigations of nano-structures and single molecules [3, 4, 5].

The time-resolved confocal microscope MicroTime 200 is a powerful instrument for the investigation of fluorescent samples. It is built around an inverted microscope body and uses the method of Time-Correlated

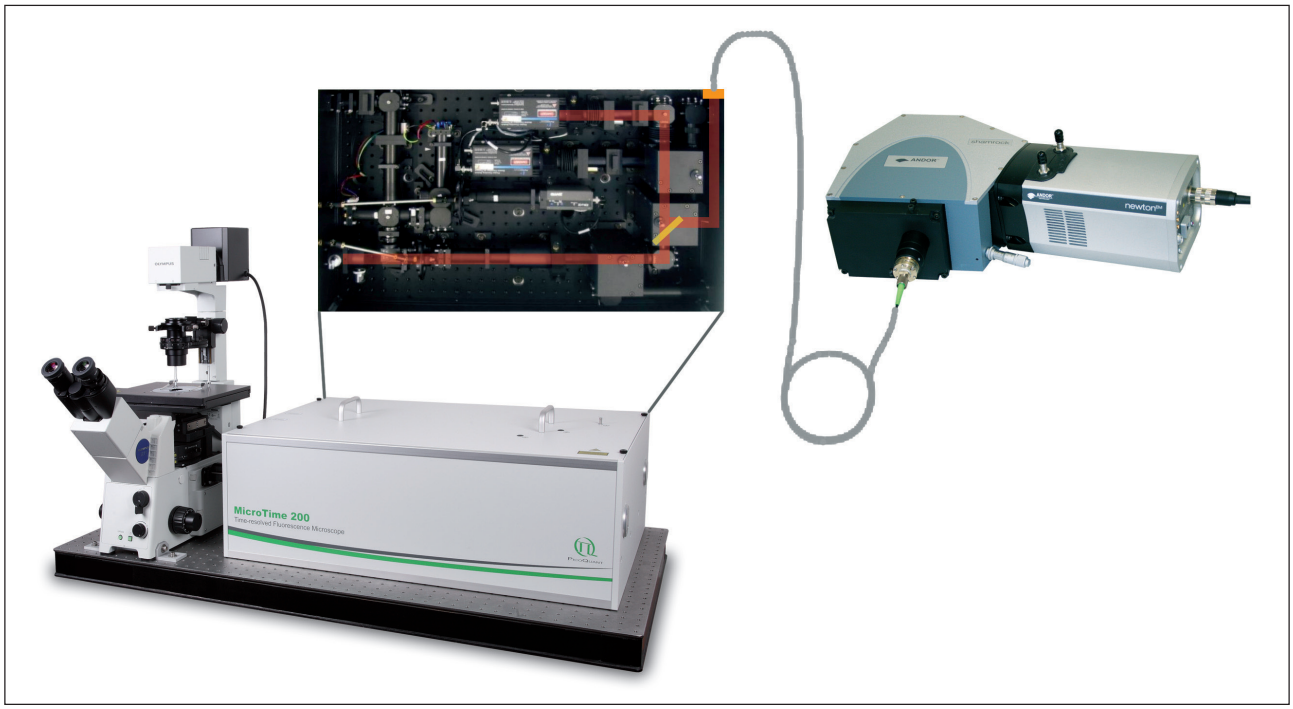

Fig. 1: Graphical presentation of the MicroTime 200 combined with a spectrograph. One example for the fluorescence beampath inside the main optical unit of the MicroTime 200 is shown in red. A beamsplitter (displayed in yellow) defines the part of the fluorescence that is directed towards the exit port (displayed in orange). The fluorescence is coupled into a multimode fiber and directed into the spectrograph SR-163 equipped with a Newton 970 EMCCD (Andor Technology).

Single Photon Counting (TCSPC) for time-resolved data acquisition along with single photon sensitive detectors and picosecond pulsed diode lasers for excitation [1]. Various techniques like Fluorescence Lifetime Imaging (FLIM), Fluorescence Correlation Spectroscopy (FCS) and Förster Resonance Energy Transfer (FRET) are supported and enable studies of microscopic samples down to the single molecule level. Due to the modular conception of the MicroTime 200, various instrumental add-ons can be included that enable further investigations of the obtained fluorescence [2].

System Description

The spectrograph is attached to an exit port of the main optical unit of the MicroTime 200 as shown in Fig. 1. Inside the main optical unit, a suitable optical element is used to direct a defined part of the emitted light towards the exit port. An easy exchange of the optical element (100% mirror, 50/50 beamsplitter, ...) allows for the customization of the experimental conditions. At the exit port, the fluorescence is efficiently coupled into a multimode fiber (50 µm diameter) and directed into the spectrograph. In order to remove Raman and Rayleigh scattered light from fluorescence, a bandpass filter might be used prior to the detection.

The Andor SR-163 [6] is a compact research-grade Czerny-Turner spectrograph with 163 mm focal length. For the combination with the MicroTime 200, a grating with 600 l/mm, blazed at 500 nm, enables data acquisition covering a window of 260 nm within the range of 300 nm to 1050 nm. The corresponding optical resolution is < 2 nm. The grating is manually exchangeable, it can be replaced by various different gratings from Andor [7] in order to alter wavelength coverage and resolution. The fluorescence is detected by the Newton 970 EMCCD, which features 1600 x 200 pixel (16 µm pixel size) with thermoelectric cooling down to -100°C [8]. The very high detection efficiency of up to 95% in the wavelength range between 400 nm and 750 nm makes the camera especially suited for single molecule studies.

In order to ensure the highest possible sensitivity, the coupling of the fluorescence into the spectrograph needs to be specially adapted. The modified xy-adjustable fiber coupler includes an FC/APC fiber connector with an adjustable f-matching lens. No slit is used in order to maximize the light collection efficiency. The alignment is easily verified utilizing the 2D array readout of the spectroscopic detector in the imaging mode.

The SR-163 with the Newton 970 EMCCD camera is used with its original software “Solis” from Andor. As the spectrograph is fibercoupled, it is easily unmounted and thus available for other applications, while maintaining the alignment of the exit port at the MicroTime 200.

Experimental Results

The results of a proof of principle experiment using immobilized Atto655 fluorophores (ATTO-TEC) on glass are shown in Fig. 2. Fluorescence Lifetime Imaging (FLIM) was first performed in order to identify the single molecules on the surface. They appear as diffraction limited spots with fluorescence lifetimes of roughly 4 ns. Some of the spots show black lines caused by the typical blinking behaviour of single fluorophores. By positioning one molecule into the laser focus, the fluorescence can be monitored as a function of time. The fluorescence intensity time trace (bin width 1 ms) again shows the blinking behavior of the single molecule on a millisecond time scale (middle panel). From the same data set, the TCSPC histogram can be calculated which can be analyzed to determine the exact fluorescence lifetime(s) of the single molecule (upper panel). Other fluorescence parameters like anisotropy or FRET efficiency are in principle also accessible, depending on the configuration of the experimental setup.

With the spectrograph attached, the fluorescence is split in order to simultaneously obtain fluorescence properties and spectral data. In the shown example, the fraction of fluorescence directed towards the spectrograph has been defined by a 20/80 fluorescence beamsplitter with 80% of the fluorescence being used for the spectra. As the detected photons are spread over many spectral channels of the EMCCD camera, the necessary integration time for a spectrum with an acceptable signal-to-noise ratio is in the order of seconds. Spectra of the selected single molecule using an integration time of 3 s are shown in the lower part of Fig. 2.

In the FLIM image of Fig. 2, a second, much dimmer molecule is visible on the lower left. A direct comparison of the spectra of the two fluorophores is shown in Fig. 3. Here, the data of the successively taken spectra are summed up and normalized. Evidently, even minor differences in the spectra of two different molecules can be directly observed.

Summary

Adding the spectrograph SR-163 equipped with the EMCCD Newton 970 to the time-resolved confocal microscope MicroTime 200 opens up new prospects in the investigations of single molecules. The presented results show the high sensitivity offered by

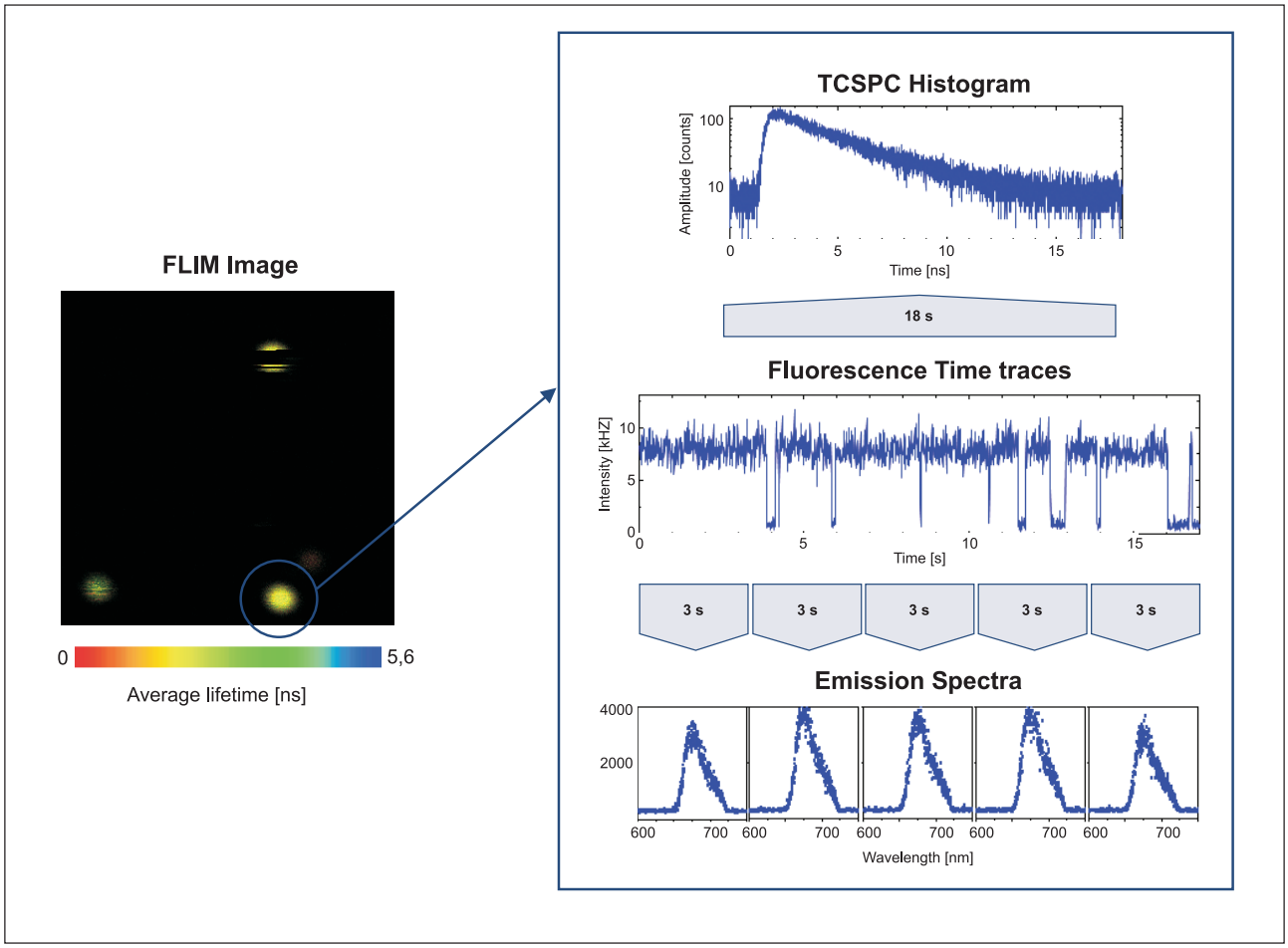

Fig. 2: Fluorescence data from single Atto655 molecules immobilized on a glass surface (image size: 4.3 x 4.3 µm). Left: The encircled molecule was placed into the excitation focus and the intensity fluctuations, fluorescence decay properties and spectral data were collected simultaneously. Right: The fluorescence time trace was recorded over 18 s (displayed binning 1 ms). The TCSPC histogram is calculated for the whole time trace. The simultaneously taken emission spectra are integrated over 3 seconds each. Excitation 635 nm, 40 Mhz, 100x/1,4 oil objective, optical filters: BS470/640, BP685/70, BS 20/80

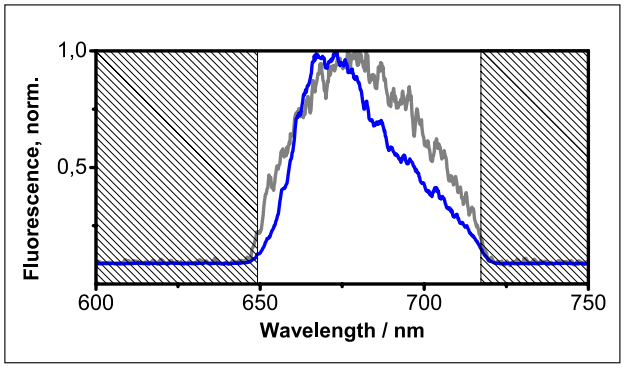

Fig. 3: Direct comparison of fluorescence spectra from single Atto655 molecules immobilized on a glass surface.

Excitation 635 nm, 40 MHz, 100x/NA 1,4 oil immersion objective, optical filters: BS470/640, BP685/70, BS 20/80. The black stripe pattern indicates the spectral range rejected by the utilized spectral bandpass filter.

this specific combination. Depending on the context of the experiment, a defined part of the fluorescence is utilized to acquire the spectral information. Intensity fluctuations, fluorescence decay properties and spectral data of a single molecule can be obtained simultaneously. Both, the observation of spectral changes as a function of time, and the comparison of spectra from different molecules are possible with this combination.

Supplemental Information

If single molecule sensitivity is not required, a cost-saving version for low-light level applications (QE65000 spectrograph from Ocean Optics) is available for the combination with the MicroTime 200. The QE65000 is a Czerny-Turner spectrograph with 101.6 mm focal length. For the combination with the MicroTime 200, an HC1 grating and a longpass filter OFLV-QE are recommend. This permits data acquisition within a spectral range from 200 nm to 950 nm. The corresponding optical resolution is < 3 nm. No slit is applied in order to achieve maximum light collection efficiency. The QE65000 uses a Hamamatsu FFT-CCD detector, which serves as spectroscopic

detector. It features 1600 x 200 pixel (24.6 µm² pixel size) with thermoelectric cooling down to -15°C [9].

Comparable to the SR-163, the QE65000 is connected to the exit port of the main optical unit of the MicroTime 200 using a multimode fiber (50 µm diameter, input with FC-APC connector, output with SMA connector). The QE65000 is supplied with its original software “SpectraSuite” from Ocean Optics. It is easily unmounted and thus available for other applications, while maintaining the alignment of the exit port at the MicroTime 200.

References

[1] Brochure of the time-resolved confocal microscope MicroTime 200. Available at http://www.picoquant.com/datasheets/systems/MicroTime200_Brochure.pdf

[2] “Combining Atomic Force Microscopy with Confocal Microscopy”, Technical Note, PicoQuant GmbH, available at http://www.picoquant.com/technotes/technote_mt200_afm.pdf

[3] Th. Christ, F. Kulzer, P. Bordat, Th. Basché: Watching the Photooxidation of a Single Molecule, Ang. Chem. 113 (2001) 4323-4326, Angewandte Chemie Int. Ed. 40 (2001) 4192-4195

[4] F. Stracke, C. Blum, S. Becker, K. Müllen, A.J. Meixner: Two and multilevel spectral switching of single molecules in polystyrene at room temperature, Chem. Phys. 300 (2004) 153-164.

[5] C. Blum, A. J. Meixner, V. Subramaniam: Transitions between different photophysical forms of Aequorea GFP variants revealed by spectrally resolved single molecule spectroscopy, Biophysical Journal 87 (2004) 4172-1479.

[6] Brochure of the Andor spectrograph SR-163. Available at http://www.andor.com/spectrographs/czerny-turner_spectrographs/shamrock_163/

[7] http://www.andor.com/spectrographs/czerny-turner_spectrographs/shamrock_163/gratings/Default.aspx

[8] Brochure of the Andor EMCCD camera Newton 970. Available at http://www.andor.com/pdfs/specifications/Andor_Newton_EMCCD_Specifications.pdf

[9] Brochure of the Ocean Optics spectrograph QE65000. Available at http://www.oceanoptics.com/productsheets/qe65000_product_sheet.pdf

PicoQuant

PicoQuant GmbH
Rudower Chaussee 29 (IGZ)
12489 Berlin
Germany

Phone +49-(0)30-6392-6929
Fax +49-(0)30-6392-6561
Email info@picoquant.com
WWW http://www.picoquant.com