

Combining Atomic Force Microscopy with Fluorescence Lifetime Imaging

J. Barner (JPK Instruments AG), M. Koenig and M. Sackrow (PicoQuant GmbH)

Confocal fluorescence microscopy provides fluorescence information from a sample volume close to the refraction limit with a spatial resolution down to approx. 300 nm whereas the resolution of atomic force microscopy (AFM) can be three orders of magnitude higher (e.g. fraction of a nanometer). Both of these established techniques can provide different types of information such as local fluorescence intensity and lifetime using time resolved fluorescence microscopy or high resolution spatial topography and mechanical properties using AFM. A combination of both techniques in a single instrument opens up new experiments for measuring and manipulating down to the molecular scale.



Fig. 1: Combined setup of MicroTime 200 and JPK NanoWizard[®]3 AFM. The AFM sample stage (TAO[™] module, 3-axis) is mounted to the IX71 body of the MicroTime 200. The MicroTime 200 is configured for 2D objective scanning.

Resolution combined with functionality: By correlating the diffraction-limited fluorescence data with the higher resolution AFM topography image, optically encoded functionality can be mapped with nanometer precision. Using time-resolved data acquisition for fluorescence lifetime imaging (FLIM), more information about the local environment of the fluorophores can be gathered.

Control of photophysical processes on the nm-scale: The AFM tip can also be used to deliberately influence the environment of a fluorophore. Tip-enhanced fluorescence increases the fluorescence intensity by locally amplifying the electric field in the vicinity of the tip. The converse effect, quenching, is seen when the fluorescence intensity is reduced in the vicinity of the tip. This is due to a radiationless transfer of energy from the fluorophore to the tip. Both these near-field effects occur over distances below the optical wavelength, so quenching or enhancement will be seen as a dark or bright center within the diffraction-limited fluorescence image.

Probing inter- or intramolecular distances: AFM force spectroscopy is a well-established technique to measure the force response of objects bound between the AFM tip and the supporting surface as they are stretched or compressed. This can give structural information e.g. about a single molecule being unfolded or a binding pair of molecules being separated. Simultaneously measuring fluorescence quenching or enhancement can provide information about the distance between fluorescent parts of the molecule and the tip. Using multiple fluorescent labels, e.g. for FRET measurements (nonradiative resonant energy transfer), during the force spectroscopy

movement, can determine inter- or intramolecular distances between defined parts of the molecules.

Assembly and testing of optical nanostructures: The AFM tip can apply defined forces to the sample, and can be used as a tool to move optically active particles e.g. fluorescent or metallic nanoparticles or even single macromolecules across the sample surface. Optically active structures can be made, for instance, to test plasmon resonance effects depending on the separation and orientation of the components.

This technical note describes the combination of the confocal time-resolved fluorescence microscope MicroTime 200 (PicoQuant) and the JPK NanoWizard® 3 AFM system for simultaneous AFM and FLIM measurements (see Fig. 1). Exemplary synchronized AFM and FLIM images are taken on fluorescent beads to demonstrate the basic operation of such a rather complex setup.

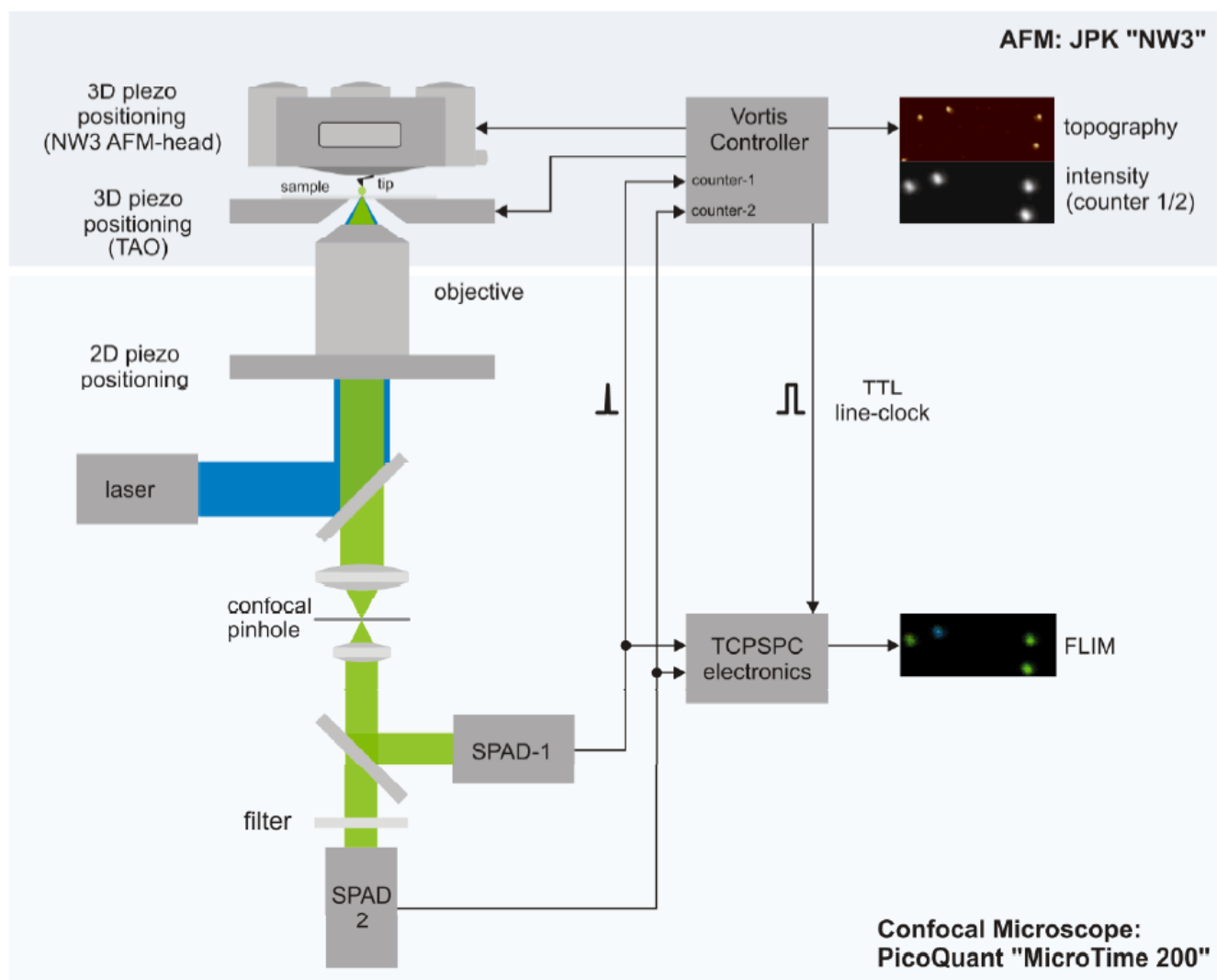


Fig. 2: Schematic view of the combined setup: The tip-scanning NW3 AFM head (3-axis tip scanner) in combination with the JPK TAO™ module (stage with integrated 3-axis sample scanner) is mounted onto the MicroTime 200 equipped with two SPAD detectors. Additionally, the SPAD signals are fed into the two counter modules of the JPK Vortis controller. The data acquisition is synchronized using a native, fast TTL line-clock of the AFM controller as an electronic marker for the collected photon stream.

System description

An integrated setup of optical fluorescence lifetime imaging and AFM used for synchronized imaging with both techniques has to fulfil several needs: The AFM tip position has to be locked to the excitation laser focus (confocal volume) position of the fluorescence microscope for the time scale of the experiment. For data acquisition the sample has to be scanned relative to the fixed AFM tip and laser focus, while the data acquisition of both systems has to be synchronized to correlate a pixel in the AFM image to the continuously recorded data stream of photon arrival times and, therefore, be able to reconstruct a spatially matched fluorescence intensity and lifetime image.

The MicroTime 200 [1] from PicoQuant is a confocal time-resolved fluorescence microscope with single molecule sensitivity. It is built around an inverted microscope body (Olympus, IX71) and uses Time-Correlated Single Photon Counting (TCSPC) for time-resolved data acquisition along with single photon sensitive detectors (single photon avalanche diode, SPAD) and picosecond pulsed diode lasers for excitation. For measurements such as Fluorescence Lifetime Imaging (FLIM), the system is equipped with a 3D piezo scanning system for the sample (2D PI scanning table for XY-axes, PI PIFOC for Z-axis) with an effective scan range of 80 μ m x 80 μ m x 80 μ m. In this configuration, the sample is moved across the stationary excitation beam ("sample scanning"). In the alternative configuration used here, the objective is mounted directly onto a 2D scanner ("objective scanning") and the PIFOC unit for z-axis focussing is removed for stability reasons. Immersion objectives are mechanically coupled to the coverslip sample through the immersion medium, so additional position noise from the PIFOC would be present in the AFM data.

The MicroTime 200 was equipped with two SPAD detectors. SPAD-1 was used to collect the backscattered

light from the AFM-tip used for optical alignment of the tip relative to the excitation laser focus. SPAD-2, equipped with an emission long-pass filter, was used for detecting the sample fluorescence. Both detectors were connected in parallel to the PicoQuant TCSPC electronics and to the two counter modules of the JPK Vortis™ Advanced AFM controller to allow synchronized recording of images of backscattered light (SPAD-1) as well as fluorescence intensity images (SPAD-2) alongside the standard AFM image channels such as topography. With freely configurable signal thresholds, polarity and termination impedance and a maximum count rate of 10MHz the JPK Vortis™ counters are perfectly suited to handle different detector pulse shapes standards (NIMM, TTL, LVTTTL etc.).

The JPK NW3 AFM head [2], a high resolution tip-scanning system (100 μ m x 100 μ m x 15 μ m) designed for operation on an inverted optical microscope, integrates seamlessly with the MicroTime 200 in combination with the JPK TAO™ module (Tip Assisted Optics) [3] without any modification of the two systems. The TAO™ module is a sample stage with integrated three-axis sample scanner (100 μ m x 100 μ m x 10 μ m) which can be used in parallel with the three axes of the AFM head tip scanner for maximum flexibility. There are six axes for closed-loop positioning:

- 1) Fine focussing of the MicroTime 200 to the sample (Z-axis of TAO™ stage)
- 2) Precisely locking the AFM-tip to the confocal volume of the MicroTime 200 (XY-axes of the AFM head)
- 3) Sample scanning (with fixed AFM tip and optical focus) for synchronized data acquisition (XY-axes of TAO™ stage)

All motions of the AFM tip and sample used for the experimental procedure, as well as the fine focussing of the sample to the confocal volume of the MicroTime 200, can be directly controlled by the AFM software for maximum ease of operation, keeping the spatial position of the confocal volume of the MicroTime 200 fixed.

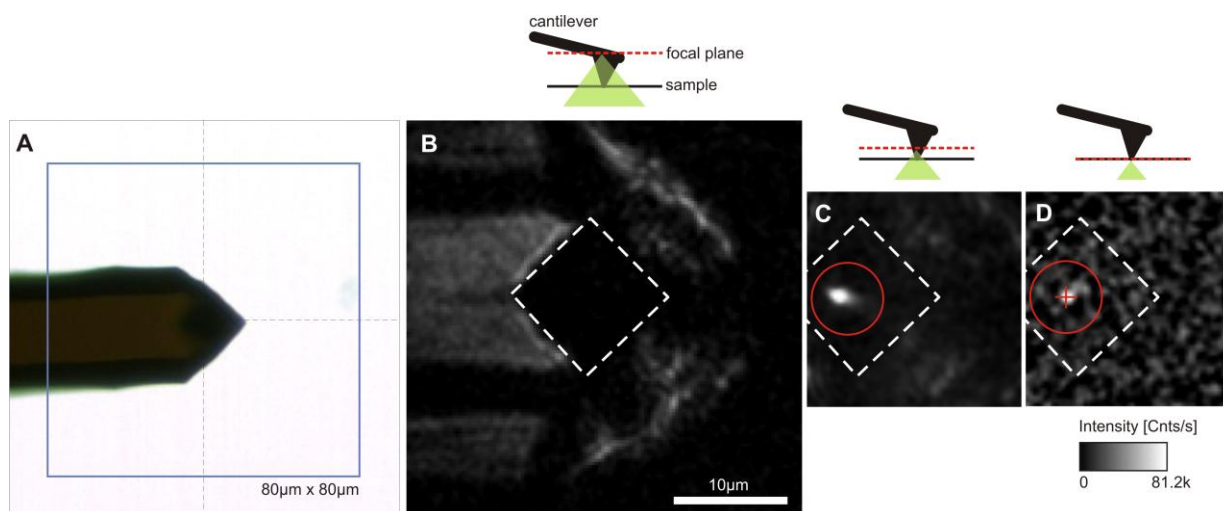


Fig. 3: Optical alignment: (A) The cantilever with tip is aligned to the crosshairs of the eyepieces of the MicroTime200 while the excitation laser is parked in the center of the $80 \times 80\mu\text{m}$ scan range (blue square) of the MicroTime 200 using the micrometer screws of the AFM stage. (B, C, D) Series of images of the backscattered light acquired by scanning the AFM tip relative to the steady laser focus. The focal plane is moved sequentially towards the AFM tip apex to indicate the position of the laser spot confocal volume

The synchronized data acquisition of the two instruments is achieved by using the special time-tagged time-resolved mode of the PicoQuant TCSPC units which allows the insertion of external synchronization signals (“markers”) into the continuously recorded data stream of photon arrival times. The marker signal (TTL) is generated by the JPK Vortis™ Advanced controller (“line-clock”), synchronized with the AFM scanning motion where the signal changes from low to high at the line start and from high to low at the line end. Based on this marker, the recorded photon stream is divided into scan lines and the photons of each line are binned into equidistant pixels. The scan lines were grouped finally into an image of fluorescence intensity and lifetime where the spatial dimensions of the image matches the scaling of the acquired AFM image.

Optical alignment

Before starting the measurement, the AFM tip has to be aligned with the laser focus of the MicroTime 200 to allow synchronized FLIM and AFM data recording. The AFM cantilever with integrated tip is observable through the eyepieces of the MicroTime 200 while the laser is parked in the center position of the optical scan range, which is

aligned with the crosshair of one of the microscope eyepieces. The first coarse alignment can be done by shifting the AFM-head and so the cantilever tip into the center of the crosshair by using the sample stage micrometer screws (Fig. 3A). For this procedure the AFM-tip was set to the center of the $100\mu\text{m} \times 100\mu\text{m}$ scan range of the tip-scanner so that the MicroTime 200 laser focus is now inside the tip-scanner scan range for the following fine alignment using the backscattered light from the cantilever.

Fig. 3B shows an image of the backscattered light using the SPAD-1 detector of the MicroTime 200 fed directly into counter channel (1) of the JPK Vortis™ Advanced AFM controller while scanning the cantilever relative to the steady laser focus. First the focal plane is set to the cantilever arm, well above the tip, to find the cantilever contour and therefore the position of the base of the pyramidal tip (dashed line). Then the focal plane was moved sequentially down towards the tip apex, as shown in Fig. 3C, 3D. If the focal plane is close to the end of the tip, the apex appears as a single spot in the image of the backscattered light and therefore the exact laser focus position is known precisely. Finally the tip is moved into the focus position using the tip-scanner and the photon count-rate of the backscattered light was optimized to its

maximum by shifting the tip position in plane at the nanometer scale. The optical alignment is established and any further scanning motion was done using the TAO™ sample scanner. The procedure needs only a low laser power and the sample area of interest can also be moved away from the focus of the MicroTime 200, so photo-bleaching is not an issue during the alignment procedure.

First results

To test the combined setup, images of fluorescent beads (TetraSpeck, diameter 100nm) prepared on a coverslip (thickness 170µm) were recorded. The AFM was operated using a standard silicon cantilever (Nanoworld, NCH, spring constant 42 N/m). The MicroTime 200 was set to an excitation wavelength of 470 nm with a LP510 emission longpass filter and a SP750 shortpass filter in front of SPAD-2. A 60x Olympus water immersion objective (1.2 NA) was used for detection.

Fig. 4 shows a set of images synchronously acquired with the MicroTime 200 and JPK NanoWizard® 3 AFM. In the AFM topography (A) individual beads are observed which are associated with spots in the fluorescence intensity image (B), recorded from detector SPAD-2 fed into the second counter channel of the JPK Vortis™ Advanced controller. The AFM data are recorded and displayed line by line, so the online comparison of topography and fluorescence intensity data during acquisition gives direct feedback on the system alignment and optical activity of the sample. After the scan finished, the data stream of photon arrival times and the AFM line-clock marker recorded with the MicroTime 200 were processed into a FLIM image (C), where the fluorescence intensity is encoded as brightness and the lifetime as color. Again, all individual beads observed in the AFM topography are visible in the lifetime image. An overlay of the FLIM image with the AFM topography (D) confirms a precise overlap of the bead positions in both image channels.

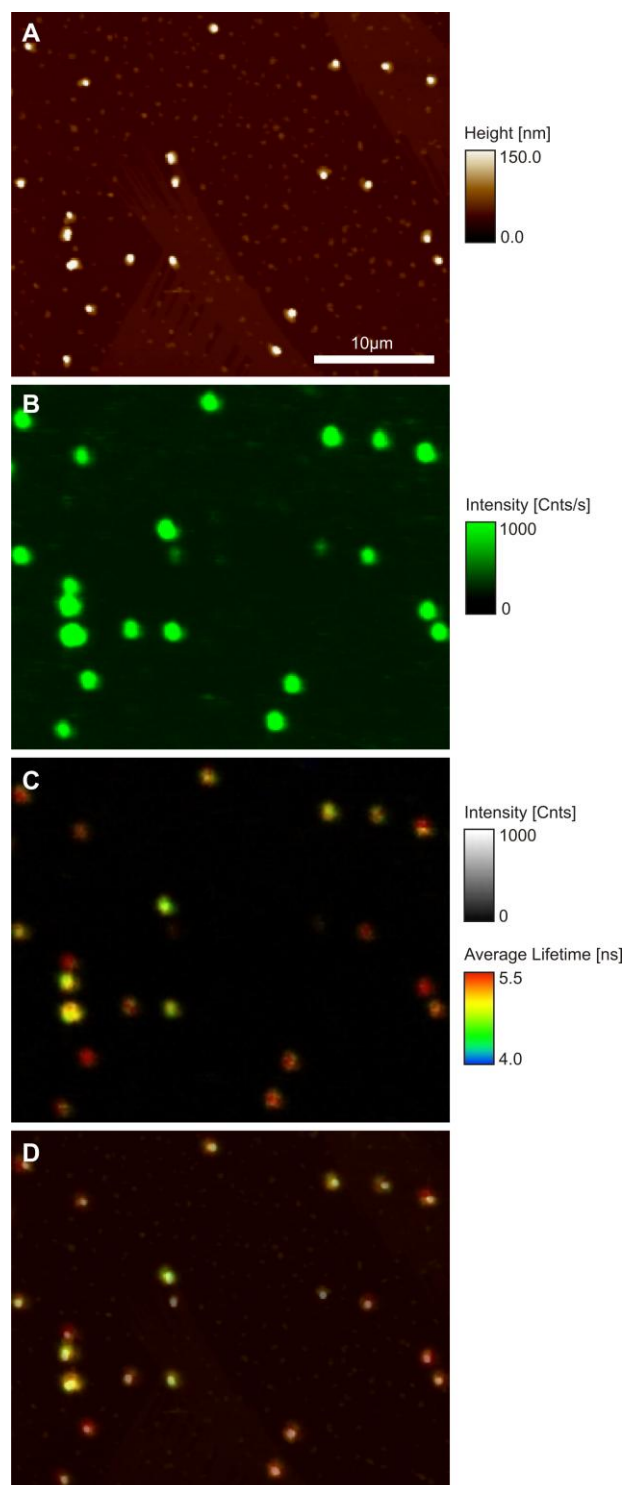


Fig 4: Synchronized recording of AFM topography (A), fluorescence intensity (B) and intensity-modulated FLIM (C) of fluorescence beads (TertaSpeck, $d = 100\text{nm}$). Topography and fluorescence lifetime are merged into one image (D) to verify the overlay accuracy.

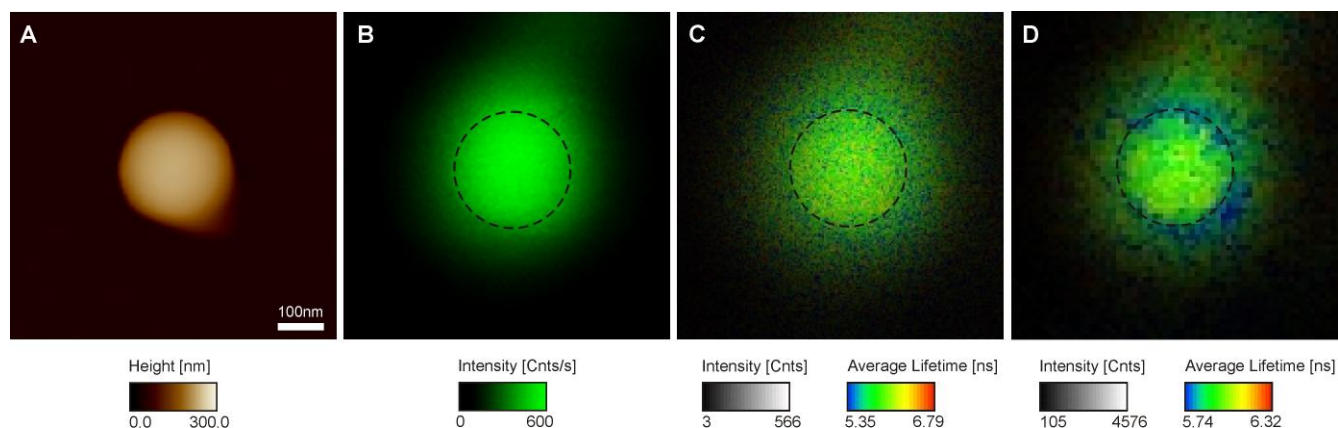


Fig. 5: AFM topography (A), fluorescence intensity (B) and intensity-modulated FLIM (C) of a single fluoresce bead (Molecular Probes, $d = 200\text{nm}$). The beads position, taken from the AFM topography, is marked in the corresponding fluorescence images (dashed line). (D) Reconstructed FLIM image using a lower pixel resolution to achieve a better signal to noise ratio. The fluorescence lifetime is significantly shortened at the bead edge.

During the whole experiment the backscattered light from the AFM tip was monitored using detector SPAD-1 to ensure that the AFM tip was perfectly locked to the position of confocal volume of the MicroTime 200 since any thermal drift would break the alignment. The NanoWizard[®] 3 AFM-head with its integrated tip scanner allows a comfortable, fast and precise realignment of the setup without the help of the MicroTime 200 objective scanner.

To confirm the precise position congruence between the two systems, in a second experiment AFM topography and FLIM images were recorded on a different type of fluorescence beads (fluoresceine labelled, Molecular Probes, diameter 200nm) cast onto a plasma-cleaned coverslip. Here, a Cr/Pt coated cantilever (Mikromasch, Multi75E-G, spring constant 2 N/m) was used. Fig. 5 shows the complete set of images. In the AFM topography (A) a single bead is visible in the center of the scan-frame. The fluorescence intensity image (B) and intensity-modulated FLIM image (C) show the optical signature at exactly the same XY-position, with the contour of the bead taken from the AFM topography superimposed as a dashed line.

A FLIM image with lower pixel resolution (D) was also generated from the data set of photon arrival times to

improve the signal-to-noise ratio, since more photons are binned to a pixel for the fluorescence lifetime analysis. The fluorescence lifetime is significantly shorter at the edge of the bead (ring of blue pixels) compared to the center. The thickness of the ring is clearly smaller than the diffraction limit of the detection objective ($\sim 300\text{nm}$), so the optical super-resolution observed here has to be the consequence of the contact between bead and AFM tip. Since the effective contact area between tip and bead is maximized at the bead edge, a pronounced shortening of the fluorescence lifetime can be caused by the close proximity of the fluorophores to the metal-coated tip.

Conclusions

The combination of time-resolved confocal microscopy and high-resolution AFM opens up many new ways to characterize of surfaces on a molecular level. The straightforward integration of the PicoQuant MicroTime 200 and JPK NanoWizard[®] 3 can be done without any modifications of the two systems. The JPK NanoWizard[®] 3 AFM head, combined with the JPK TAO[™] sample stage to give a total of six axes or degrees of freedom, allows easy and reliable alignment of the rather complex setup without permanent switching between the two systems.

The high spatial precision of the AFM- and FLIM-data overlay was demonstrated using one possible investigation scheme where the AFM tip stays fixed in the confocal volume of the MicroTime 200 to achieve a synchronized recording of topographical and fluorescence data using sample scanning.

References

- [1] PicoQuant MicroTime 200 specifications.
www.picoquant.com/products/microtime200/microtime200.htm
www.picoquant.com/technote_lsm_upgrade_kit.pdf
- [2] JPK NanoWizard[®] 3 specifications.
www.jpk.com/atomic-force-microscopy.27.en.html
- [3] JPK Tip Assisted Optics (TAO[™]) specifications.
<http://www.jpk.com/jpk-productnote-tao-module.download.f08389779209fb8487c70ab70dbf01a3>

Authors addresses:

J. Barner
JPK Instruments AG
Colditzstr. 34-36, 12099 Berlin, Germany

M. Koenig, M. Sackrow
PicoQuant GmbH
Rudower Chaussee 29, 12489 Berlin, Germany