PicoQuant

Combining the MicroTime 200 with the Bruker BioScope Catalyst AFM for Multiparameter Cell Imaging

Marcelle König[1], Marcus Sackrow[1], Andrea Bleckmann[1], Felix Koberling[1], Johannes Kindt[2], Alexander Berquand[2]

[1] PicoQuant GmbH, Rudower Chaussee 29, 12489 Berlin, Germany
[2] Bruker, Nano Surfaces Division, Dynamostrasse 19, 68165 Mannheim, Germany

Motivation

Characteristics of surfaces on a single molecular level can be examined using both confocal fluorescence microscopy and atomic force microscopy (AFM). The techniques analyze different aspects of the sample e.g. molecular dynamics are analyzed by time correlated fluorescence spectroscopy and surface topography is studied by AFM. Thus, a combination of both techniques into a single instrument enhances the methodology and opens up new investigation schemes, including the following topics:

1. Live cell imaging

Cell growth and differentiation are associated with changes in cell shape and content. By the synchronized acquisition of fluorescence and AFM images, protein concentrations and localizations during cell development can be monitored together with the mechanical properties of the cell surface. For instance, the topographical changes in cell shapes are induced by changes in the cell cytoskeleton. The reorganization of the involved proteins can be associated with changes in protein conformation, localization, or orientation. The variation in the environment and in the protein structure itself can influence the fluorophore behavior as well.

2. High-resolution molecular-scale imaging

AFM can reveal the packing and structure of both native and reconstituted membranes. Such membranes often consist of a heterogeneous distribution of lipid, sterol, and protein components. The simultaneous study of AFM with fluorescence techniques could reveal an association of such structures with protein compartmentalization and thereby reveal their function.

3. Force and mechanical studies

A well established AFM technique is single molecule force spectroscopy, wherein single molecules or pairs of molecules are mechanically interrogated to measure inter- and intra-molecular forces. With the parallel investigation of a distance dependent fluorescence parameter like fluorescence lifetime quenching and Förster Resonance Energy Transfer (FRET), it is possible to determine the inter- or intramolecular distances.

Fig. 1: Combined setup of the MicroTime 200 and the BioScope Catalyst. The AFM with its sample stage is mounted on the inverted microscope body of the MicroTime 200. The MicroTime 200 is configured for objective scanning.

This technical note describes the combination of two commercially available systems: the PicoQuant time-resolved confocal fluorescence microscope MicroTime 200 and the Bruker BioScope Catalyst AFM. Its versatility is demonstrated by the synchronized acquisition of FLIM and AFM images from fluorescent TetraSpeck beads as well as from fixed glioblastoma cells.

System description

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 from Olympus (IX71/81) and uses the method of Time-Correlated Single Photon Counting (TCSPC) for time-resolved data acquisition along with single photon sensitive detectors and picosecond pulsed diode lasers for excitation. For imaging measurements such as Fluorescence Lifetime Imaging (FLIM), the system is equipped with a 3D piezo scanning system with an effective scan range of 80 µm x 80 µm x 80 µm. In the standard configuration, the sample is placed on the scanner and moved across the stationary excitation beam ("sample scanning"). In an alternative configuration, the objective is mounted on the scanner and moved below the stationary sample ("objective scanning"). This special configuration allows free access to the sample from above and is therefore well suited for applications requiring special sample compartments or for integration with other techniques including patch-clamp, cryostats, or as demonstrated here, AFM (Fig. 1).

The BioScope Catalyst from Bruker is a high-performance AFM designed specifically for biological applications [2]. The AFM uses a flat sample scanner

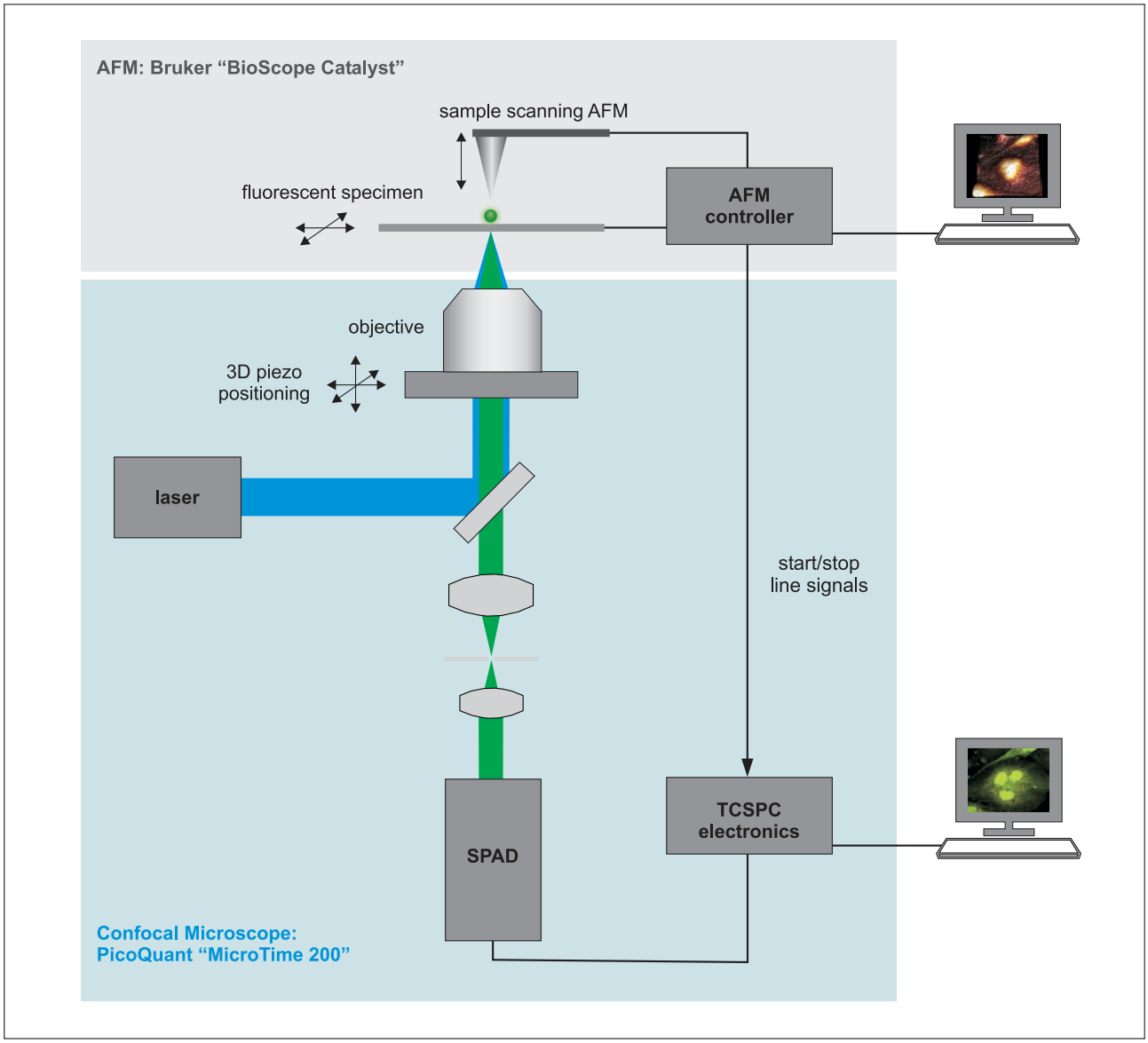

Fig. 2: Schematic view of the combined setup: The sample is placed onto the scanner of the AFM and the MicroTime 200 data acquisition is synchronized with the AFM scanner movement by incorporating corresponding electronic marker signals from the AFM controller into the collected photon data stream.

design that isolates the piezos from damage by fluids. The open design of the AFM head allows virtually unrestricted optical and physical access from above the sample. The scanning range is 150 µm x 150 µm in closed loop operation with extremely quiet sensors to ensure high resolution imaging and precise force measurements. The tip can be moved independently with a maximum range of more than 20 µm (in z-direction), which enables measurements of large samples such as living cells. The complete AFM also includes a sample stage with tip-sample alignment and tip-optics alignment, suited for the IX71/81 inverted microscope body from Olympus. The AFM control software is optimized for exceptional ease of use, including an exclusive AFM imaging mode called ScanAsyst that automatically optimizes AFM imaging parameters. It also includes microscope image registration and overlay (MIRO) software, which enables direct calibration and correlation of AFM images with light microscopy images. Using the new PeakForce QNM mode, one can additionally get nanomechanical information like sample modulus, tip-sample adhesion, and dissipation by measuring and analyzing force curves at every image point while simultaneously imaging sample topography at high resolution.

The Bruker BioScope Catalyst AFM is well suited for the combination with the MicroTime 200 as it fulfills all necessary requirements, which are:

1. The BioScope Catalyst is a sample scanning AFM.
2. The BioScope Catalyst controller provides suitable scan synchronization signals.
3. The BioScope Catalyst scan head fits mechanically onto the Olympus IX71/81 microscope body without interfering with normal light microscopy operation.
4. The BioScope Catalyst sample baseplate allows adjustment of the AFM tip in x, y and z relative to the confocal beam.

One of the key goals in the combination of the MicroTime 200 with the BioScope Catalyst is to enable synchronized recordings of AFM and optical images within the same sample region. It is therefore necessary to design a setup where the confocal volume of the MicroTime 200 can be precisely aligned with the AFM tip. This can be achieved by using the objective scanning configuration of the MicroTime 200. This allows for a precise localization of the AFM tip within the optical scan range. After the exact overlay of the confocal volume with the AFM tip, all scanning procedures are directly controlled by the AFM software. Therefore, the functionality of the AFM is not compromised in any way.

Synchronization of the two systems

The combination of the MicroTime 200 with the BioScope Catalyst allows the parallel acquisition of fluorescence (lifetime) and AFM images. The key for this procedure is the special time-tagged time-resolved (TTTR) mode of the PicoQuant TCSPC units, which allows the insertion of external synchronization signals (“marker signals”) into the continuously recorded data stream of photon arrival times. In case of the AFM scanning system, these marker signals are provided at the beginning and end of each scanning line. Basically, the acquired photon stream is divided into the scan lines and further allows the assignment of photons into line pixels [3]. The required “line start” and “line stop” synchronization signals are ready available from the BioScope Catalyst AFM controller.

Optical alignment

Before starting measurements, the AFM cantilever tip and the laser focus of the microscope must be aligned. The cantilever is observable through the eyepieces of the MicroTime 200. In one eyepiece crosshairs are visible which are aligned with the optical axis of the microscope (Fig. 3A). The first coarse alignment can be done by moving the AFM sample baseplate until the cantilever tip is placed directly in the center of the crosshairs (Fig. 3B).

The first coarse alignment is sufficient to position the cantilever tip within the scan area of the MicroTime 200. Now, the fine alignment of the laser focus with the cantilever tip in x and y can be done with the MicroTime 200 using backscattered light from the AFM cantilever. Starting well below the tip, 2D images from the backscattered light are taken in different focal planes. To avoid a sudden damage of the AFM probe, only small steps in z should be performed (~2 µm). The resulting images of a dry sample are shown in Fig. 4. Once the cantilever tip appears in the focal plane it becomes visible as a bright spot in the image (Fig. 4F). The laser focus is aligned with the AFM by defining a point measurement exactly at the cantilever tip. The fine alignment of the focal plane can be done using the photon count rate of the probe as an indicator. This alignment procedure has been shown to work with bare silicon and with silicon nitride AFM tips on glass surfaces.

Depending on the investigated sample, the tip apex might not show up as a sharp bright spot in the optical image. In this case one must use the shape of the tip to determine the position of the tip apex, as shown in Fig. 5. Again, the focal plane can be found using the photon count rate as an indicator. Since the laser power needed for this calibration procedure is very low, photobleaching during the alignment is not an issue.

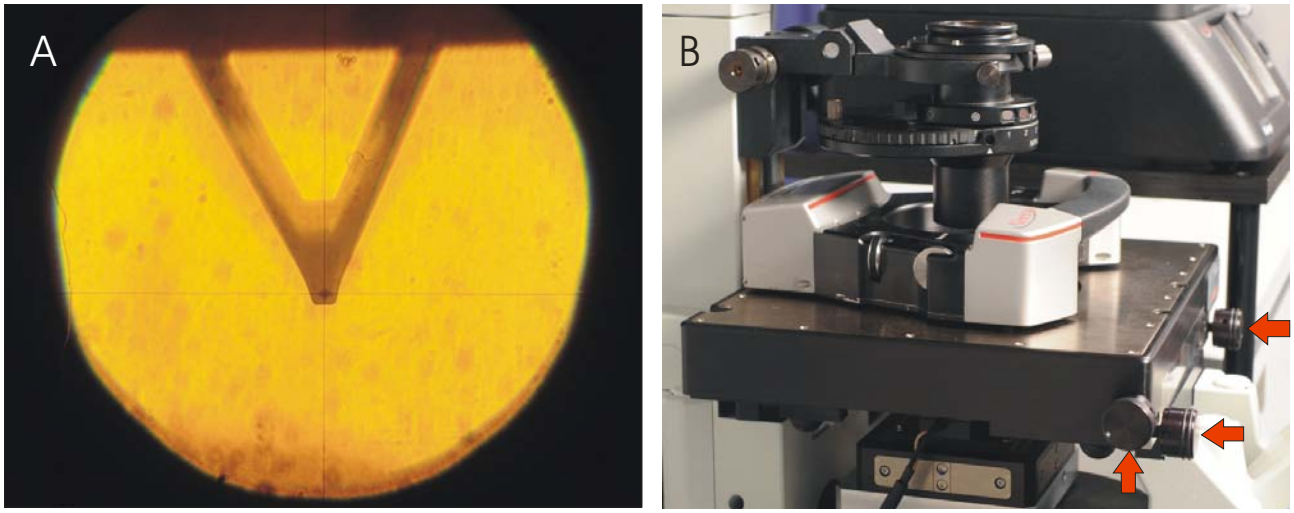

Fig. 3: (A) Cantilever with tip aligned to the crosshairs in the eyepiece of the MicroTime 200. (B) This coarse positioning can be done by using the 3 micrometer screws at the sample stage (red arrows).

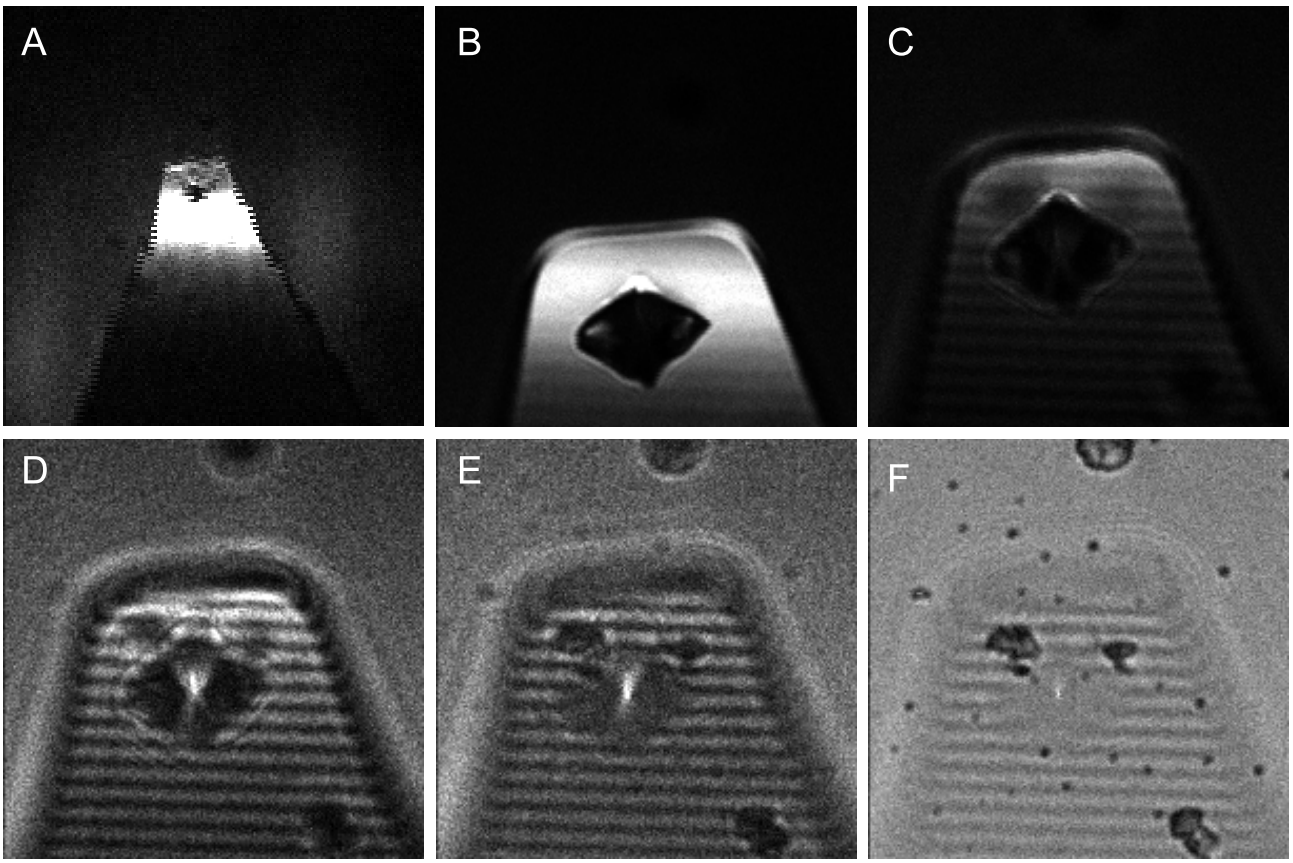

Fig. 4: Images from an AFM cantilever with SiN tip using backscattered light. The bright spot in the image center corresponds to the tip apex. After iteratively zooming in and scanning the AFM tip, the laser focus of the MicroTime 200 can be directly positioned at the tip apex. The sample stage is moved 2 µm in z-direction for every image B-F.

First results

Imaging of fluorescence beads

In a first experiment, fluorescence beads (TetraSpeck, 100 nm diameter, Molecular Probes) on glass were imaged with the combined setup of the MicroTime 200 and the BioScope Catalyst. The results are shown in fig. 6. The AFM was used in ScanAsyst mode. FLIM images were recorded with the resolution of the AFM scanner. The excitation wavelength was 470 nm with a LP510 emission longpass filter and SP750 short-pass in front of a single photon avalanche diode (SPAD) from MPD (10 µm x 10 µm, 128 x 128 pixels, Olympus 60X, 1.2 NA, water objective). The simultaneously taken images clearly show how both

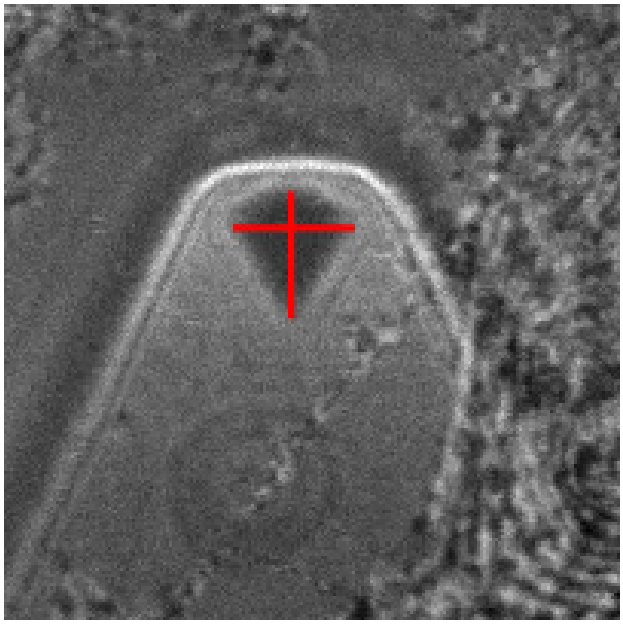

Fig. 5: Backscatter image of a probe tip with marked tip apex (red cross) which is determined by the shape of the tip base.

techniques allow to image the beads although not all beads visible in the AFM image also show fluorescence in the FLIM image. Fig. 6C shows a 3D representation of the merged FLIM and AFM height images.

To quantify the achieved overlay in the simultaneous recordings, a series of combined AFM and fluorescence images of one fluorescent bead was acquired. The fluorescence channel of the SPAD was introduced into an auxiliary input of the AFM controller so that the fluorescence intensity was acquired simultaneously with the AFM data by the BioScope Catalyst AFM software. The NanoScope xy drift analysis feature was used to measure drift rates for the AFM itself, as well as for the correlation between the AFM and the optical setup. The maximum drift rate observed for the AFM was 2.8 nm/min. The maximum drift rate observed for combined AFM and fluorescence imaging was 11.5 nm/min. This suggests that the alignment between AFM tip and a diffraction-limited spot at high NA could be held for at least a good fraction of an hour. BioScope Catalyst AFM controller.

Fixed cells

The Bruker BioScope Catalyst is optimized for biological samples, thus also fixed glioblastoma cells (brain cancer cells) stored in PBS buffer (pH 7.2) were investigated (Sample Courtesy of Celine Heu, FEMTO, Besancon, France). The transfected cells expressed a free diffusible Green Fluorescent Protein (GFP) visible both in the cytoplasm and nucleus. The combined setup of the MicroTime 200 and the BioScope Catalyst makes it possible to examine both cell topography and fluorescence lifetimes of the GFP. The excitation wavelength was 470 nm with a HC520/35 emission bandpass filter and SP750 shortpass in front of a single photon avalanche diode (SPAD) from MPD (100 µm x 100 µm, 512 x 512 pixels, Olympus 60X, 1.2 NA, water objective). Fig. 7A shows the intensity modulated GFP lifetimes in the cells taken by the MicroTime 200. The free GFP shows a homogeneous distribution in all parts of the cells. In Fig. 7B-H the information taken by the BioScope Catalyst in PeakForce QNM mode is displayed. By measuring and analyzing a force curve at every image point, one obtains topography, modulus, adhesion, deformation, and dissipation of the cell. The measurement was made quantitatively by prior spring constant calibration of the AFM cantilever, using the integrated thermal-noise calibration method. Fluorescence intensity (photon counts) was recorded pixel-synchronously with the AFM measurement, by distributing the MicroTime 200's digital APD signal into the Nanoscope-V AFM controller's pulse counting input. All images visualize the investigated cells whereas each image contains a distinct fluorescence or nanomechanical information. The overlay of the images from two instruments is possible by exporting the FLIM image and overlaying it in Bruker's MIRO software.

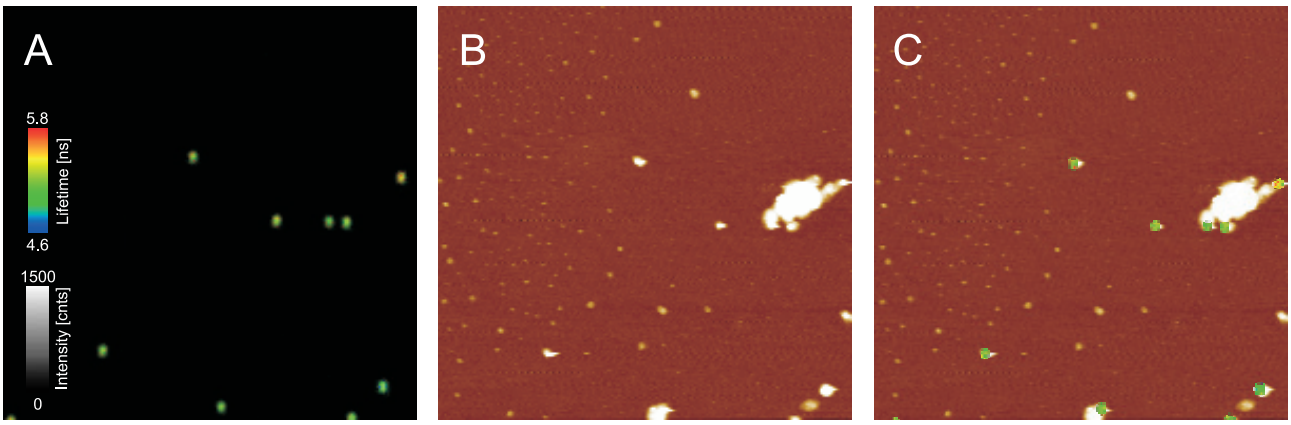

Fig. 6: Synchronized acquisition of fluorescence beads measuring both fluorescence lifetime and topography. (A) Intensity modulated FLIM image (B) AFM image and (C) 3D representation of the merged FLIM and AFM height image. Not all beads which are visible in the AFM picture show also fluorescence in the FLIM image. Scalebar 5 µm.

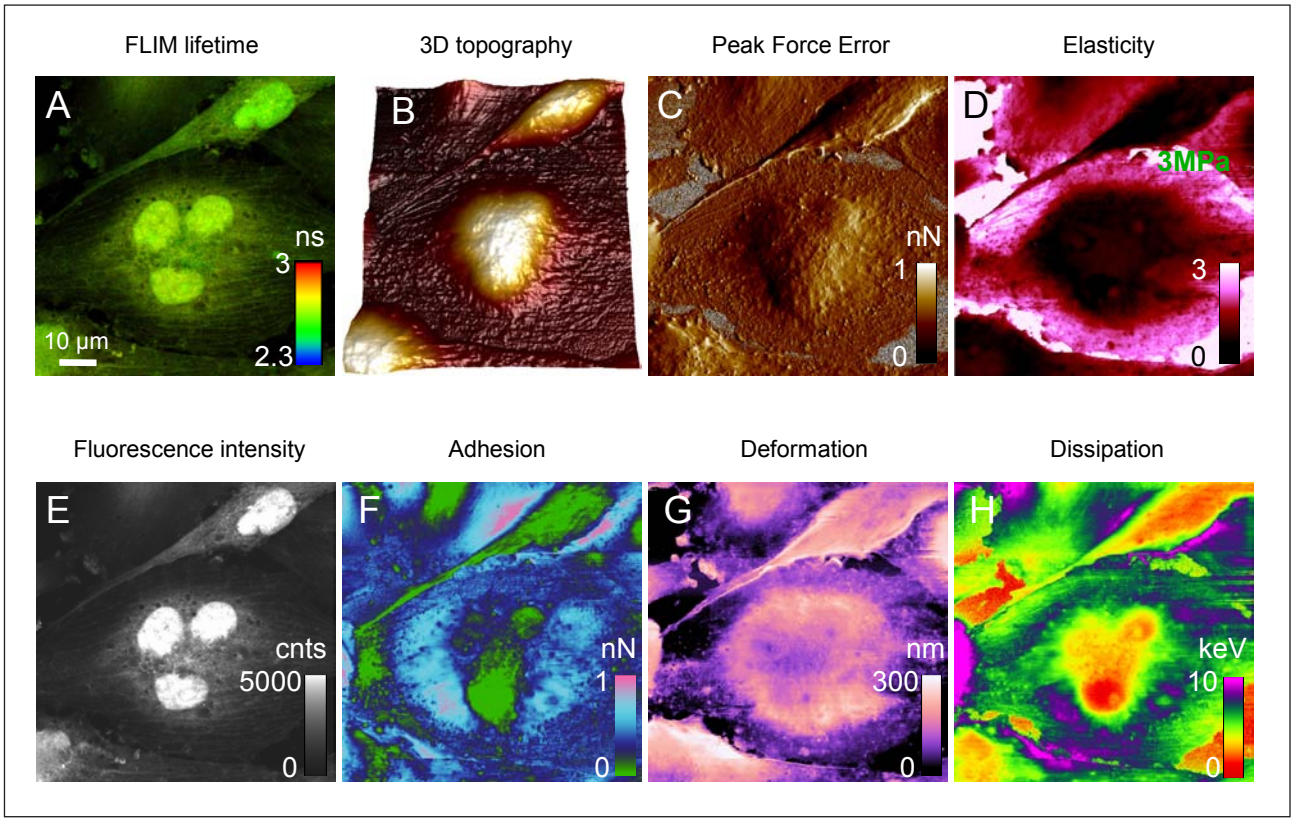

Fig. 7: Synchronized acquisition of fixed glioblastoma cells expressing GFP using the MicroTime 200 in combination with the BioScope Catalyst. (A) Fluorescence lifetime distribution in the cells. (100x100 µm, acquired by MicroTime 200, and aligned using MIRO Software.) (B-D, F-H) Data taken by the BioScope Catalyst AFM in PeakForce QNM mode, showing topography, and spatially resolved quantitative mechanical cell properties. (E) Photon counts from the MicroTime 200, synchronously recorded by Nanoscope AFM controller.

Both techniques and instruments, the MicroTime 200 time-resolved fluorescence microscope and Bioscope Catalyst AFM, are routinely used individually for live cell studies, therefore this proof of performance experiment can be fully extended towards combined FLIM and AFM live cell imaging. For long term studies (hours to days) of sensitive eucariotic cells without introducing environmental stresses, combined FLIM-AFM studies can be performed in sterile, CO2-buffered media at physiological temperatures, using the Catalyst Heater Stage and Catalyst Perfusing Stage Incubator (PSI) accessories.

ties of such a combined setup. Prospective applications are:

- Live cell imaging: Impact of protein changes on cell shape and structure
- High-resolution molecular-scale imaging: Merging of sub-nanometer AFM topographic imaging with optically encoded functionality
- Force and mechanical studies: Investigations of inter- and intramolecular distances using force spectroscopy, nanomanipulation on the single molecule level

Summary

The combination of AFM and time-resolved confocal microscopy opens up many new ways of investigation schemes in the characterisations of surfaces on a single molecular level. The presented combination of the PicoQuant MicroTime 200 and the Bruker BioScope Catalyst has been shown to be relatively straightforward without need of larger modifications to the systems. An alignment procedure has been developed and first results demonstrate the capabili-

References

[1] http://www.picoquant.com/products/microtime200/microtime200.htm

[2] http://www.bruker-axs.com/bioscope-catalyst-atomic-force-microscope.html

[3] http://www.picoquant.com/technotes/technote_lsm_upgrade_kit.pdf – section “Synchronization”

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