

# Combining Atomic Force Microscopy with Confocal Microscopy



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## Motivation

Confocal fluorescence microscopy and atomic force microscopy (AFM) are two established techniques that can be used to study single molecules. Both techniques have their individual capabilities that permit the study different aspects such as topography using AFMs, or dynamics using fluorescence spectroscopy. Thus, a combination of both techniques into a single instrument enhances the methodology and opens up new investigation schemes, including the following topics:

1. Investigation, prediction and control of photophysical processes on the nm-scale

Variations in the immediate vicinity of a fluorophore can have an influence on its fluorescence properties. Hence, depending on the design of the experiment, the detected fluorescence intensity of a sample can be decreased (quenched) or increased (enhanced) by the presence of the AFM tip. Quenching, i.e. decrease of fluorescence intensity, is due to a radiationless transfer of energy from the fluorophore to the quenching agent (like a metal particle or the AFM tip). Enhancement, i.e. the increase of fluorescence intensity, is due to an amplification of the electric field by confinement of the energy in the vicinity of the tip ("nanophotonic effects"), thus resulting in a stronger excitation of the fluorophore. However, the exact influence of an AFM tip onto single fluorophores has not been extensively studied so far.

2. High-resolution imaging: Merging of sub-nanometer topography mapping with optically encoded functionality

Quenching or enhancement of fluorescence can effectively be used to improve the resolution of the fluorescence image by correlating the fluorescence data with the position of the AFM tip. Depending on the distance between AFM tip and fluorophore, quenching or enhancement of the fluorescence will appear as a dark or bright center in the otherwise larger, diffraction-limited fluorescence image. Using time-resolved data acquisition for e.g. fluorescence lifetime imaging (FLIM), even more information about the system can be gathered and an optically encoded functionality can be mapped with nm-precision.

3. Investigation of inter- and intramolecular distances using force spectroscopy

A very established AFM technique is the pulling of molecules bound to the surface and thereby the examination of inter- and intramolecular forces. However, by the parallel observance of fluorescence parameters, it is e.g. possible to study FRET processes induced by the movement in order to determine inter- or intramolecular distances.

In addition, quenching and enhancement induced by the tip can provide information about the distance between fluorescent parts of the molecule and the tip.

4. Nanomanipulation: Assembly and fabrication of nanomaterials

The AFM tip can be used to move fluorescent or metallic nanoparticles on the sample surface. Even fluorescent proteins can be moved using the AFM tip. Making use of this technique, two-dimensional structures of different fluorophores or fluorescence modification agents can be created to probe nanophotonic effects.

This technical note describes the first realization of the combination between two commercially available systems: the AFM MFP-3D BIO from Asylum Research and the time-resolved confocal fluorescence microscope MicroTime 200 from PicoQuant.

## 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 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  $\mu\text{m}$  x 80  $\mu\text{m}$  x 80  $\mu\text{m}$ . 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 therefore permits to place the sample in bulky compartments or realize setups using patch-clamps or cryostats. It is also the required configuration to combine the MicroTime 200 with the AFM.



Fig. 1: The MicroTime 200 is used in objective scanning mode, in which the objective is mounted directly on the scanner and moved below the stationary sample.

The MFP-3D BIO from Asylum Research [2] is a high-performance Atomic Force Microscope designed specifically for biological applications. It is

imaging and pN force-based measurements on an inverted optical microscope. The AFM uses a flat sample scanner design that isolates the piezos from damage by fluids. The scanning range is 90µm x 90µm with closed loop operation with extremely quiet sensors to ensure precise motion for force measurements. The tip can be moved independently with a maximum range of up to 28 µm (in z-direction). The complete AFM also includes a sample stage with coarse alignment features of the tip relative to the sample, suited for the IX71/81 inverted microscope body from Olympus. The Top View module of the AFM uses a Plan APO 10x/NA 0.28 objective integrated in the scan head and enables viewing of opaque samples as well as easy alignment of the cantilever relative to the internal IR laser beam.

The MFP-3D BIO is especially suited for the combination with the MicroTime 200 as it fulfils all necessary requirements, which are:

1. The MFP-3D BIO is a sample scanning AFM
2. The controller of the MFP-3D BIO provides suited scanning synchronization signals (“line start” and “line stop”).
3. The scan head of the MFP-3D BIO fits mechanically onto the Olympus IX71/81 microscope body.
4. The sample table of the MFP-3D BIO allows coarse adjustment of the AFM tip in x, y and z

## Setup, synchronization and alignment

### Combined setup

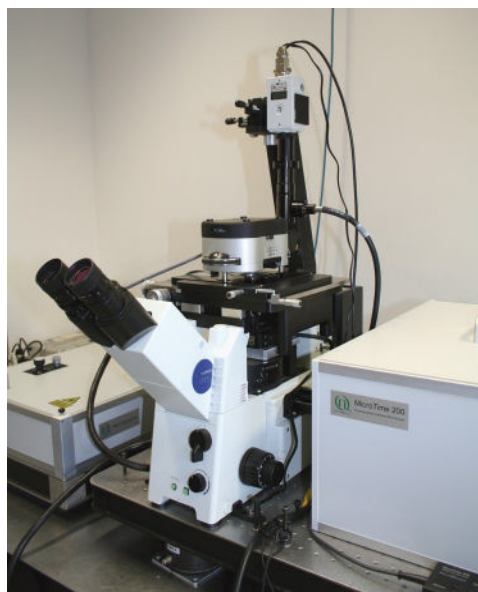
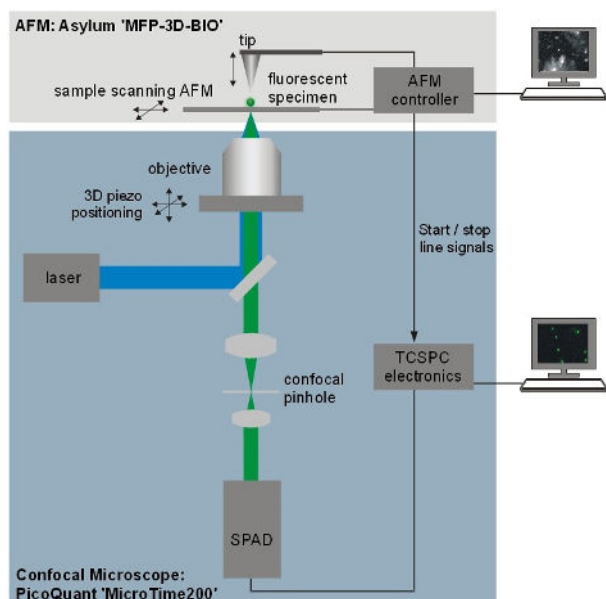


Fig. 2: Schematic sketch and picture of the combined setup: The AFM along with its sample stage is mounted on the inverse microscope body of the MicroTime 200, which is configured for objective scanning. The sample is placed on the scanner of the AFM and the data acquisition of the MicroTime 200 is synchronized with the scanner movement by including corresponding electronic marker signal into the collected photon data stream.

a versatile AFM that combines molecular resolution

relative to the confocal beam.

One of the key goals in the combination of the MicroTime 200 with the MFP-3D BIO, is to enable simultaneous recording of AFM and optical images of the same sample region. It is therefore necessary to design a setup where the confocal volume of the MicroTime 200 can be precisely overlaid with the AFM tip. This can be achieved by using the objective scanning mode of the MicroTime 200 and by placing the complete AFM onto the sample table. The sample table itself and thus the complete AFM can be moved in x and y direction, which is useful for the alignment of the cantilever tip relative to the optical axis of the MicroTime 200. The sample is then placed onto the scanning table of the AFM (see Fig. 2). All scanning procedures are directly controlled by the AFM and the AFM software. The functionality of the AFM is thus not influenced at all.

### Synchronization of the two systems

The combination of the MicroTime 200 with the MFP-3D BIO allows for 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 to insert external synchronization signals (“marker”) in the continuously recorded data stream of photon arrival times. In case of the AFM scanning systems, these markers are signals at the start and end of each scanning line, which basically divide the acquired photon stream into the scanned lines and further allow to sort the photons into image pixels [3]. The required “line start” and “line stop” synchronization signals are readily available at the DAC output of the AFM controller.

### Optical alignment

For coarse alignment, the AFM cantilever and the laser beam of the MicroTime 200 are monitored through the AFM Top View module (see Fig. 3) by looking at backscattered light on the illumination pillar. The complete AFM is then moved using the alignment features of the sample table until the confocal laser beam is positioned close to the AFM tip.

For the fine alignment, the AFM cantilever with its tip is then imaged with the MicroTime 200 configured for the detection of scattered/reflected light. With the AFM tip being already roughly aligned with respect to the laser focus of the MicroTime 200 and being in contact with the surface (contact or AC mode), it is possible to acquire an image indicating the position of the tip (see Fig. 4). By iteratively zooming in and scanning the AFM tip apex with the MicroTime 200 objective scanner, the objective is finally placed nicely centred below the AFM tip apex.

Since the laser power needed for this alignment procedure is very low, photobleaching during the alignment is not an issue. Also, since no AFM scanning is needed for the alignment, the AFM tip stays sharp and clean.

This alignment procedure has been shown to work with bare silicon and with gold coated AFM tips on glass and on mica surfaces.

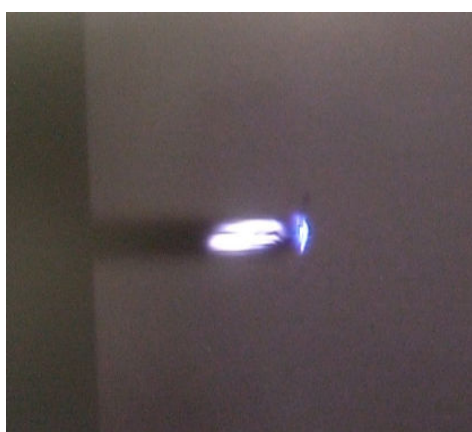


Fig. 3: AFM topview image (left) showing the cantilever with the AFM laser (blue/white) and the laser spot from the MicroTime 200 (blue) close to the cantilever. The cantilever can be moved using the dedicated alignment screws of the AFM sample table (right image)

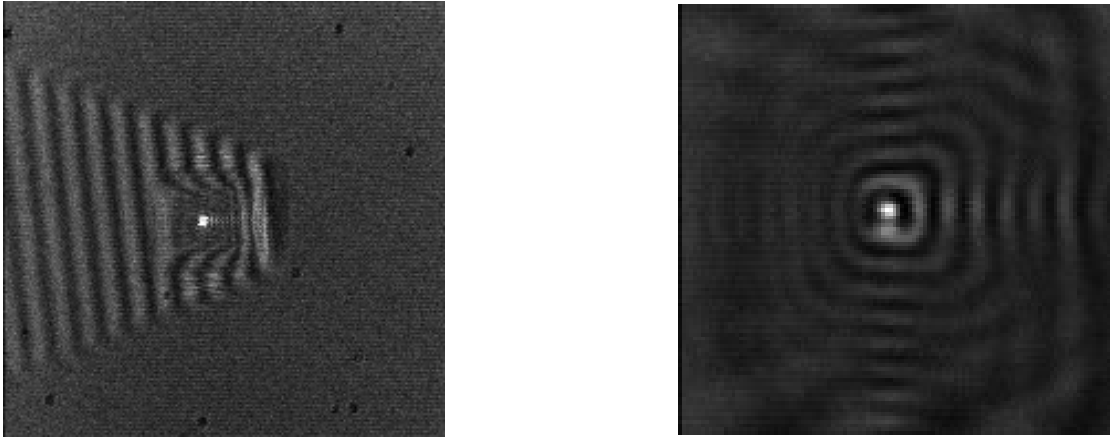


Fig. 4: 27  $\mu\text{m}$  x 27  $\mu\text{m}$  backscatter image of a gold coated SiN tip (left). The bright spot in the image center is the tip apex. After iteratively zooming in and scanning the AFM tip, the objective of the MicroTime 200 can be directly positioned below the tip apex (right).

## 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 MFP-3D BIO. The results are shown in fig.5. The AFM was used in tapping mode (Olympus TR800 SiN Cantilever). The excitation wavelength was 470 nm (9  $\mu\text{m}$  x 9  $\mu\text{m}$ , 480 x 480 pixels, Olympus 100 x 1.4 oil objective).

The images clearly show how both techniques allow to image the beads, but they also show the differences. While the individual beads are visible in both images, the needle-shaped salt crystals in the lower left are only visible in the AFM image (left image). This becomes even more visible by merging both images into one (see fig. 6)

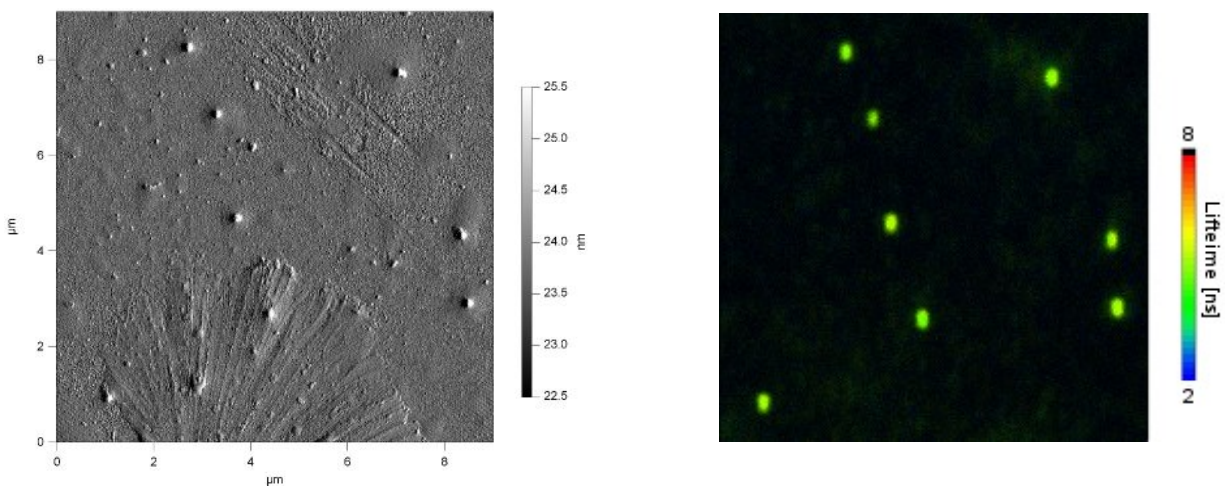
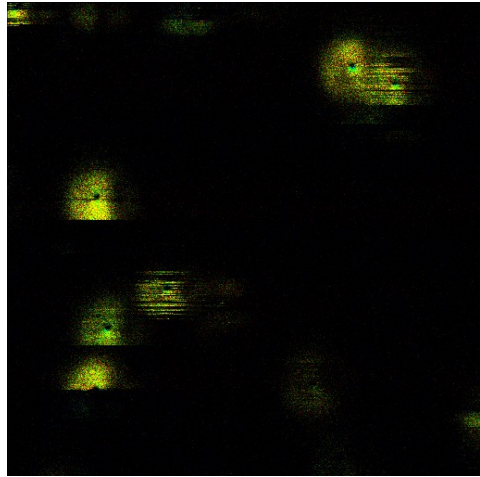


Fig 5: AFM image (left, display as Z sensor data (ZST)) and FLIM image (right) of fluorescent beads (100 nm diameter, Molecular Probes) together with non-fluorescent needle-shaped salt crystals



*Fig. 7: Image of single Atto 647N molecules on glass shows "holes inside" the larger spots, proving that the fluorescence was quenched by the AFM tip. The image was taken using contact mode in ambient conditions. Image dimension: 5  $\mu\text{m}$  x 5  $\mu\text{m}$*

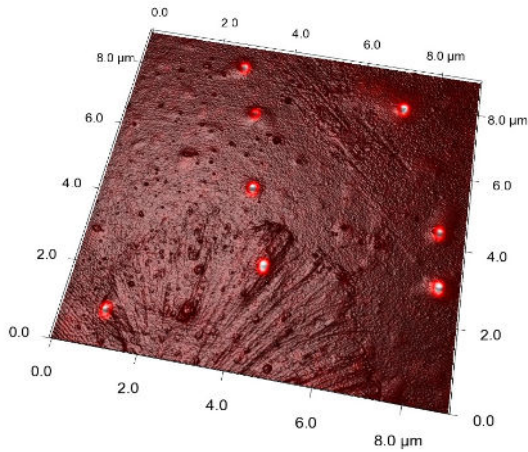


Fig. 6: The overlay of AFM and FLIM image nicely shows the location of the fluorescing beads in the AFM image. Image generated with 3<sup>rd</sup> party software.

## Synchronised AFM and Fluorescence Scanning of Single Nanodiamonds

Nitrogen-vacancy (NV) diamond color centers recently have been intensely investigated. Owing to their strong optical transition, high fluorescence quantum yield and exceptional photostability, they are ideal non-toxic markers for bioimaging applications. In order to study their photophysical properties in more detail, combined AFM and FLIM measurements have been performed by the group of Jörg Wachtrup at the University of Stuttgart [4]. Fig. 8 shows some results of these studies that for example indicated that 35 % of the produced nanocrystals contained a fluorescent defect center with fluorescence decay times between 9ns and 25 ns. The studies also showed that there is no correlation of nanocrystal size and lifetime.

## Fluorescence Quenching by AFM tip

First experiments of Robert Ros using single Atto647N molecules on glass substrates nicely show the quenching effect of the silicon AFM tip (see Fig. 7). The sample was scanned in a combined scan with AFM contact mode in ambient conditions. The image shows the fluorescence intensity/lifetime image. The dark areas in the center of the diffraction limited spots are attributed to quenching due to the AFM tip. Also, the change in fluorescence lifetime near these “holes”, shows that the AFM tip has an influence on the radiative and non-radiative decay rates of the fluorescent molecule.

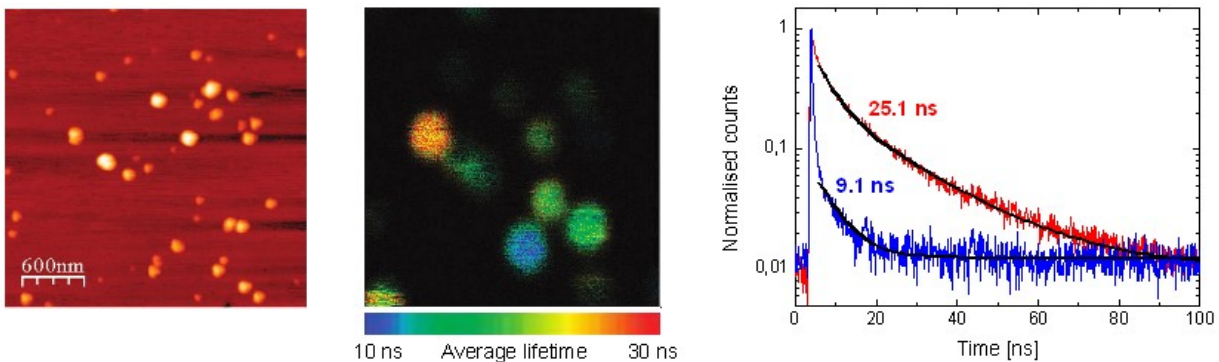


Fig 8: AFM topography (left) and FLIM image (middle) of NV diamond color centers. From the AFM image one can determine a mean particle size of 9 nm and by comparison with the FLIM image it can be calculated that 35 % of the nanocrystals contain a fluorescent nitrogen-vacant defect center with fluorescence lifetimes between 9 and 25 ns (right). Data courtesy of Prof. Joerg Wrachtrup, University of Stuttgart

## Summary

The combination of AFM and time-resolved confocal microscopy opens up many new ways of investigating single molecules. The presented combination of the MicroTime 200 from PicoQuant and the MFP-3D BIO from Asylum Research has been shown to be relatively straightforward without the need of larger modifications of the systems. An alignment procedure has been developed and first results demonstrate the capabilities of such a combined setup. Prospective applications are

- the investigation, prediction and control of photophysical processes on the nm-scale
- high-resolution imaging by merging of sub nanometer topography mapping with optically encoded functionality
- the investigation of inter- and intramolecular distances using force spectroscopy
- nanomanipulation on the single molecule level

## References:

- [1] Visit the product page of the MicroTime 200 on [picoquant.com](http://picoquant.com)
- [2] <http://www.asylumresearch.com/Products/MFP-3D-BIO/MFP-3D-BIO.shtml>
- [3] Read technical note about LSM Upgrade Kits
- [4] Tisler J., et.al., "Fluorescence and spin properties of defects in single digit nanodiamonds", ACS Nano, Vol.03, p.1959-1965 (2009)

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