Quantitative and fast AFM co-localized with STED microscopy in living cell experiments

Atomic force microscopy (AFM) has become an essential tool for investigating a huge variety of samples with nanometer resolution under physiological conditions. Recently, in addition to topographic measurements, information about the interaction and mechanical properties like adhesion and elasticity has been extracted [1]. Furthermore, novel developments focus on fast AFM and high-speed AFM to increase the temporal resolution [2]. The integration of AFM with optical microscopy has increased the number of applications, in particular, when specificity is required [3, 4].

In the last decade, similar to AFM, super-resolution light microscopy has attracted a lot of attention in the scientific community, leading to the development of several far-field microscopy techniques, which have enabled researchers to resolve objects below Abbe's diffraction limit. In 1994, Hell and Wichmann proposed the concept of stimulated emission depletion (STED) as a method to break the diffraction limit [5].

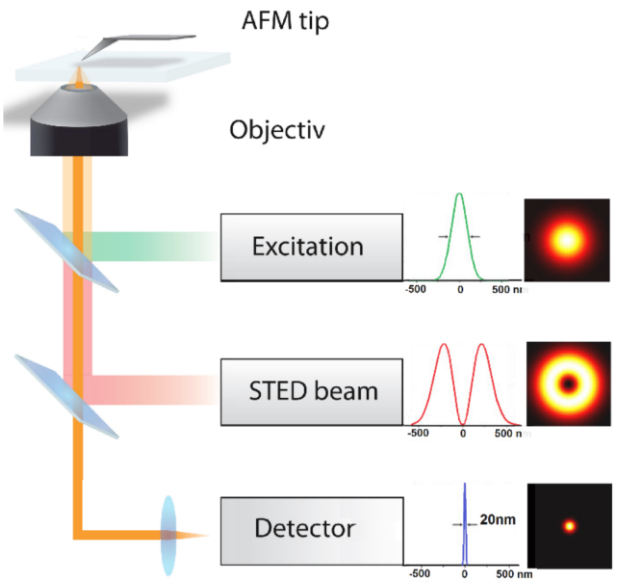

Fig. 1: *Scheme of an AFM and STED based system showing the major components. The sketch includes the excitation source, the STED beam, the detection unit and the AFM.*

The basic concept of STED microscopy is to confine the region of effective fluorescence by selectively switching off fluorophores at the rim of the focal spot. Thus, an excitation focus and a donut-shaped STED focus are scanned over the sample. The STED light is used to switch off all fluorophores except those within a nanometer-sized central region. Spontaneous fluorescence can only occur within this region. Since the experimental viability of the concept was first demonstration in 1999, STED microscopy has continuously been improved and has now become a standard technique in optical microscopy [6-9]. The importance of Stefan Hell's invention and the technical realization of STED microscopy were underlined by the Nobel Prize in Chemistry in 2014.

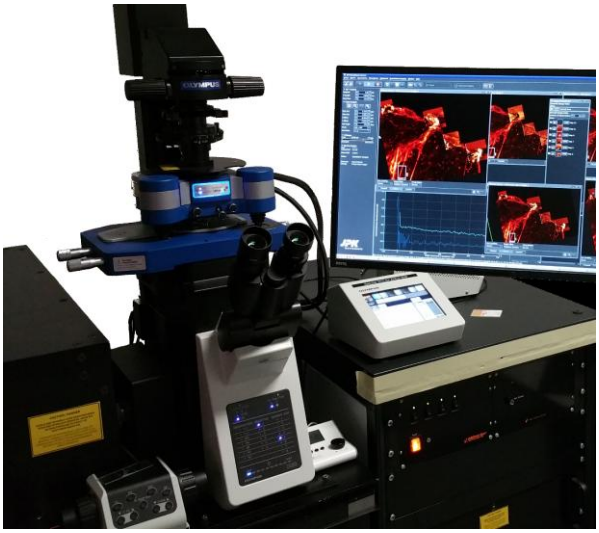

Fig. 2: *JPK NanoWizard® AFM integrated into an Abberior Instruments Expert Line STED microscope (as an example the NanoWizard® ULTRA Speed is shown).*

In recent years, there has been a trend towards combining microscopy techniques, such as AFM and super-resolution. This trend is driven by the ability to combine readouts of both microscopy techniques in single experiments and create datasets with much higher information content. In addition, better accessibility and

simplified usage concepts of AFM and STED microscopes have facilitated their combination.

In AFM, new modes like QI™ mode or easy-to-use features allow even beginners to create high quality data of topography, adhesion and mechanical information.
In STED microscopy, new lasers and concepts like easySTED have allowed the development of very robust super-resolution microscopes and have laid the path towards their use in routine applications. The combination of different microscopy techniques opens up new ways for studying biological samples under physiological conditions with versatile information output.

Correlative Nanoscopy: AFM-STED

Atomic force microscopy and STED microscopy address a similar nanometer resolution in biological samples. However, both techniques have individual limitations and suffer from drawbacks. A combination of both techniques is, therefore, favourable for creating a more complete set of data and gaining a deeper insight into the specimen under investigation.

AFM is a surface technique. With nanometer scale resolution, AFM can also be used as a force sensor for measuring interaction forces or mechanical properties. Furthermore, AFM can be used as a nanomanipulator with Piezo-controlled, sub-nanometer movement capability [10]. Nevertheless, AFM is a surface technique and is always limited to the investigation of the top layer of a sample. Another limitation of AFM is a lack of chemical specificity. There are techniques like AFM recognition (using functionalized cantilevers for, e.g., antigen-antibody interaction) or tip-enhanced Raman spectroscopy which provide optimal chemical specificity, but these techniques are limited due to their time-consuming analysis. A combination of AFM and optical/ fluorescence microscopy offers the best way to gain access to chemical recognition and to enlarge AFM capabilites [11].

Since the beginning, optical integration has been a major goal of JPK Instruments AG. The JPK NanoWizard® AFM is perfectly designed to combine with optical microscopes, due to its tip-scanning design. The sample remains fixed while the AFM is operating. The AFM head is designed to avoid disturbances of the optical path. Filters within the AFM head avoid crosstalk between the AFM laser and the optical signal. The two techniques can, therefore, work simultaneously.
Although a combination of AFM and conventional (diffraction-limited) light microscopes is widely used, it suffers from a mismatch of resolution of both techniques. The resolution of conventional light microscopes is limited by diffraction to about 220 nm in the imaging plane (XY). This mismatch can be overcome by the greatly improved resolution of some 10 nanometers in STED microscopy. The optical concept of the Abberior Instruments microscope product lines enables its use on existing microscope bodies and experimental setups. This creates a perfect platform for correlative nanoscopy.

Combination of Abberior STED and JPK NanoWizard® AFM: proof-of-principle

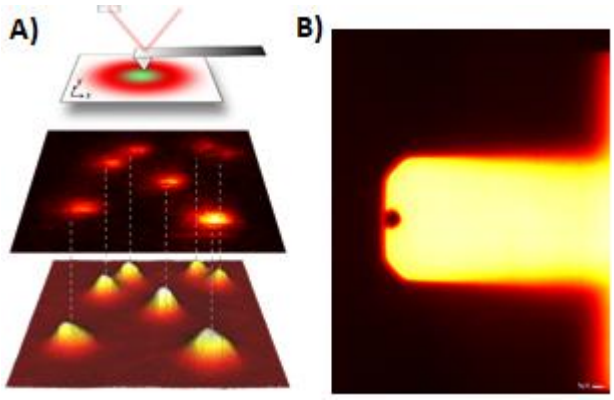

Fig. 3: *A) STED + AFM principle. STED and AFM images of fluorescence beads as well as correlation of both images are shown [12]. B) Reflection image of Nanoworld USC cantilever.*

Since 2011, JPK Instruments AG has continuously worked on the combination of AFM and super-resolution techniques [12]. In order to create perfect optical integration, the flexibility of JPK's patented DirectOverlay™ feature has been increased. The calibration procedure is done automatically and uses

known positions and offsets of the cantilever to calibrate the optical image into the AFM coordinates. The calibrated optical image is transferred into the JPK SPM software, so that scan regions can be selected within the optical image.

In 2016 JPK Instruments started a close collaboration with Abberior Instruments, a Spin-off from Stefan Hell´s group in Göttingen, to speed up the development of a correlative nanoscope. The open electronic and software architectures of both the JPK and Abberior systems allow easy and unique integration. DirectOverlay™ runs automatically as a result of TTL triggering and network assistants.

Abberior Instruments offer two microscope product lines, the Expert Line and the Compact Line:

The Expert Line STED / RESOLFT is a flexible, high-end platform and provides all cutting-edge STED options. It is highly research orientated and enables the user to continuously expand and upgrade the STED microscope (see figure 2).

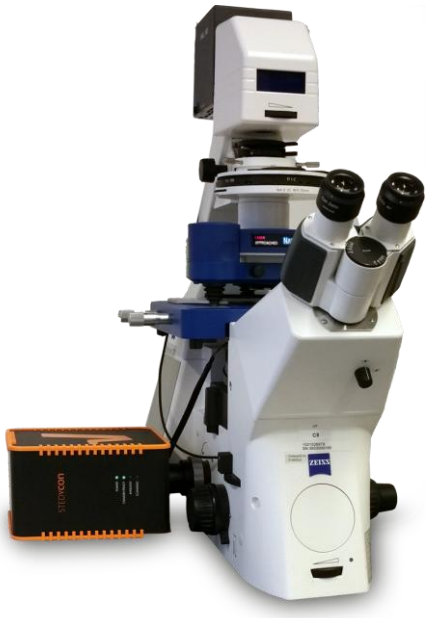

Fig. 4: *JPK NanoWizard® AFM integrated into Abberior Compact line – STEDYCON (as an example the NanoWizard® 4 BioScience is shown).*

The Compact Line STED microscope, STEDYCON, is a STED module which can be used as an add-on to any conventional epifluorescence microscope (see figure 4). It is a very compact STED system, aimed at providing a wide range of users simplified access to super-resolution light microscopy. The combination of AFM and STED has been intensively tested with both Abberior product lines.

In a first proof-of-principle experiment, a sample of two different fluorescent bead species, differing in color, were measured using a JPK NanoWizard® and an Abberior Expert Line STED microscope with two excitation lasers (561/ 640 nm) and a STED laser with a wavelength of 775 nm. The beads have a specified diameter of 40 nm and can be clearly identified by two-color STED microscopy. Crimson beads are shown in red (640 nm excitation laser) and red fluorescent beads (RFB) in green (561 nm excitation laser).

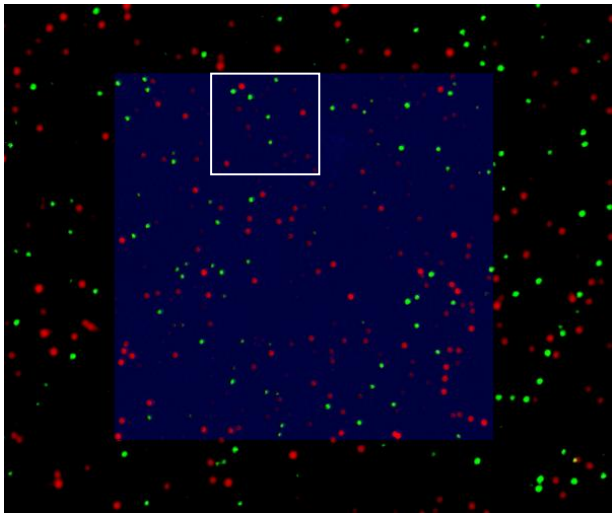

Fig. 5: *Overlay of AFM and STED image. Scan size: 5 µm x 5 µm*

For perfect integration of STED and AFM images, DirectOverlay™ was tested and the overlay accuracy within a 20 x 20 µm scan range of better than 15 nm was measured. In the scan area shown, the STED signal measured corresponds well to the AFM image (figure 5). A smaller scan area was chosen to check if all of the beads measured in AFM can be visualized in the STED image (figure 6). As described above, the overlay of both

measurements fits very well, however, at some positions, a bead visible in the AFM measurement does not appear to be present in the STED measurement (marked in figure 6A). A 3D topography analysis of the bead diameter shows that the beads vary in size, some even smaller than 25nm in diameter. In addition, some beads do not contain a fluorescent label and, therefore, can only be observed in the AFM images.

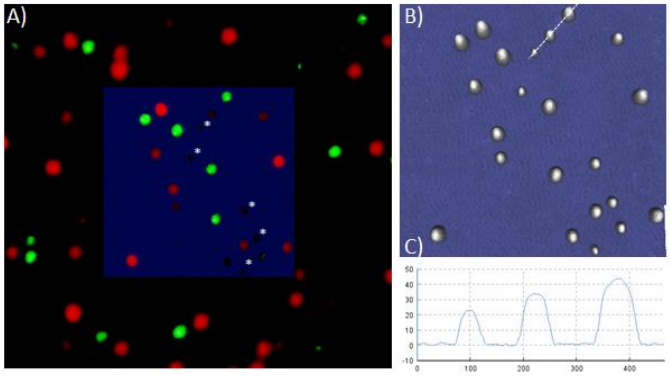

Fig. 6: *A) Overlay of AFM and STED image. Scan size: 1 µm x 1 µm. Beads measured in AFM that do not correspond to a STED signal are marked with a white star. B) AFM topography image in 3D clearly shows the divergent distribution of bead diameter C) Cross section of three beads.*

Although the experiment is relatively simple, a combination of both techniques provides further information about the sample quality. A correlative approach provides supplementary information and improves data from parallel techniques.

Simultaneous AFM and STED of Living fibroblasts – Nanomanipulation

In this experiment the NanoWizard® AFM was used in combination with an Abberior Instruments Expert Line STED microscope. For these measurements, living human skin fibroblasts were labelled with SiR Tubulin (Silicone containing Rhodamine with docetaxel as a tubulin binding moiety) and imaged in cell culture medium. To prove the capability of true simultaneous measurement, the labeled microtubules were manipulated by the AFM tip while the same area was being imaged in STED mode.

The bending, manoeuvring and rupturing of the microtubules by the AFM tip can clearly be monitored by the STED microscope in real-time. As a result of the DirectOverlay™ feature, microtubules can be targeted and manipulated on the nanometer scale. The pressure exerted by the AFM tip can be monitored and controlled. Figure 7 shows a time series of the manipulation and demonstrates a rupture of selected microtubules within the cell. It is also possible to adapt the manipulation force to stretch specific microtubules. This can provide more information about the elastic behaviour of microtubules.

As well as scratching or moving the sample, it is also possible to indent or apply a local stimulus to a cell. For a stimulus experiment, actin filaments were labelled with SiR Actin (Silicone containing Rhodamine with jasplakinolide as actin binding moiety).

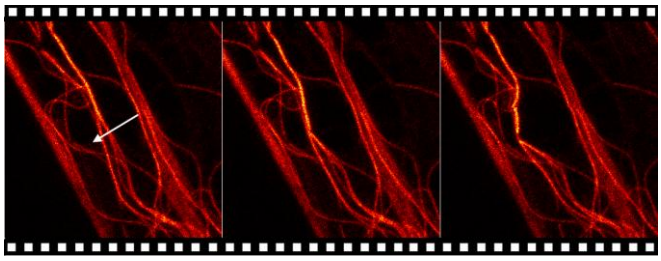

Fig. 7: *Time series of AFM manipulation-STED measurement. Microtubules labelled with SiR Tubulin and imaged with an Abberior Instruments Expert Line STED microscope (excitation, 640 nm; STED, 775 nm). The AFM manipulation path is marked with an arrow. The manipulation force was set to 5 nN with a velocity of 1 µm/s.*

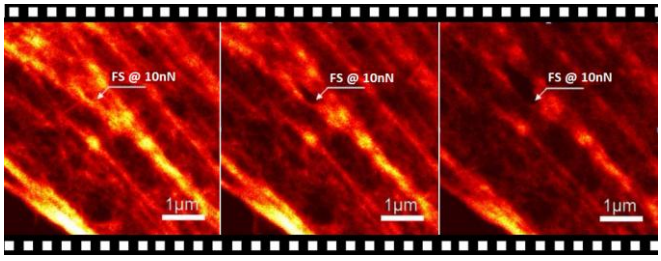

Fig. 8: *Time series of AFM stimulation-STED measurements. The actin cytoskeleton was labelled with SiR Actin and imaged with an Abberior Instruments Expert Line STED microscope (excitation, 640 nm; STED, 775 nm). The AFM stimulation position is marked with an arrow.*

To avoid bleaching in live cell STED measurements, Abberior Instruments' proprietary RESCue STED imaging mode was used. In this experiment, a force of 10 nN was selectively applied to the fibroblast cell. The JPK Force RampDesigner™ was used to define the stimulus parameter. The cell was continuously stimulated for 3 s and relaxed (no force applied) for 1 s. The change of the actin filament structure was monitored via STED.

It is known that local mechanical stimulation introduces a rearrangement of the cytoskeleton and the focal adhesion [14, 15], but to date, it has been impossible to follow the actin restructuring, polymerization and de-polymerization in correlation with nanometer-precise mechanical stimulus in such high optical resolution. Figure 8 shows the effect of the AFM stimulus to the actin fibre formation. It can be seen, that some actin filament structures are altered by the application of a 10 nN force which allows the user study the dynamics and recovery of the actin filament structure.

Living fibroblasts – Nanomechanical investigation

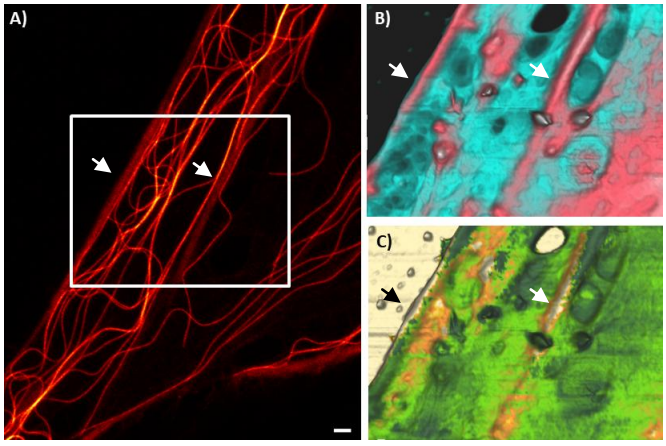

Fig. 9: *Living human skin fibroblast A) Microtubules labelled with SiR Tubulin and imaged with Abberior Instruments Expert Line STED B) 3D topography AFM image, 12.5 µm x 9 µm; range: 1 µm C) 3D topography image overlaid with Young's modulus range: 60 kPa.*

Furthermore, the mechanical information of living fibroblast cells was measured using JPKs QI™ mode. AFM can provide a high resolution surface topography image and enables the determination of elasticity, adhesion and other mechanical characteristics. In STED microscopy, it is possible to record a 3D stack in order to understand the underlying intracellular functionality. The combination and overlay of AFM and STED image makes it possible to correlate different information and to understand how the morphological features emerge from the underlying cytoskeleton fibres. In figure 9A) microtubule distribution can be seen with high resolution in the STED image. For AFM, a characteristic part of the cell was selected to correlate the surface topography and the elasticity image with the microtubules distribution.

The STED image shows two regions with several microtubule bundles arranged in parallel and close together (marked with an arrow). These regions can also be identified in the AFM images. In the topography image, the two parallel regions appear higher and in the Young´s modulus image stiffer. Additionally, different vesicles/ particle-like structures can be recognized on the cell surface.

While a few structures seem to be softer than the surrounding cell membrane, some other particle-like structures are stiffer. Living cells are constantly in contact with their surroundings. The cell membrane is highly dynamic and re-modulation, endocytosis and exocytosis take place. To get further information about these processes, it is possible to follow the cell dynamics and cytoskeleton reorganization with AFM high-speed measurements via the NanoWizard® ULTRA Speed or ULTRA Speed A.

Nanomechanical information was also correlated with actin labeled cells. The results are shown in figure 10. Thicker actin filament bundles can be identified in the Young´s modulus image as stiffer regions. Different actin filaments can also be seen in the height image. The combined approach of STED and AFM enables the correlation of information on chemical recognition, intracellular features and topography/nanomechanical

information with nanometer resolution. Therefore, it is a very powerful tool for investigating cells and biological specimen.

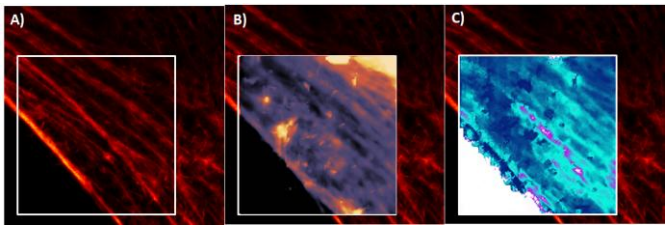

Fig. 10: *Living human skin fibroblast A) Actin labelled with SiR Actin and imaged with Abberior Instruments Expert Line STED B) Topography AFM image, 15 µm x 15 µm; range: 0.7 µm C) 3D topography image overlaid with Young's modulus range: 90 kPa.*

Conclusion

The performance, reliability and benefits of a combined AFM and STED show that correlative nanoscopy is a powerful tool for biological applications. As a result of the ability to perform measurements simultaneously, correlative microscopy provides supplementary information and improves data. Super-resolution light microscopy provides molecular specificity and intracellular information to the AFM data, while AFM delivers true surface visualization in 3D and nanomechanical information. The possibility to perform full simultaneous measurements was demonstrated in nanomanipulation experiments. Depicted measurements on living human skin fibroblasts are the first examples of an almost unlimited array of applications for the new and innovative correlative technique.

Sample courtesy

The proof-of-principle experiment with two different sorts of beads, the nanomanipulation experiment and the measurements on living human skin fibroblast were done in collaboration with Abberior Instruments GmbH. The samples were provided by Abberior Instruments GmbH.

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For further information on the Abberior STED & JPK AFM combination please see also the free webinar recording "JPK NanoWizard® AFMs in conjunction with Super-Resolution Abberior STED microscopes – towards live cell experiments".

For more details about the NanoWizard® AFM systems and their applications please contact us or visit the JPK AFM webpage.

For more details about the STED microscope product lines please visit the Abberior Instruments webpage.