Combining AFM with super-resolution STED (stimulated emission depletion) microscopy

Research in life sciences demands more and more on the analysis of biological samples with the highest resolution. This requires the development of new imaging techniques, both optical and non-optical. Non-invasive instrumentation such as the atomic force microscopy (AFM) provides high resolution topography without requiring labelling of the sample. The label-free topographical, electrical, magnetic and mechanical information gained from the AFM correlated with the colour-encoded molecular fluorescence map provides an important tool for deeper investigation of the sample properties. When the AFM is combined with a super-resolution microscopy system (such as stimulated emission depletion, STED) it generates a nanoscopic tool with very high versatility to answer sub-diffraction level morphology questions. This note shows how a system integration of AFM and STED works. The following data and results have been reported in a paper published by the group of Prof. Alberto Diaspro from IIT in Genova, Italy in the journal *Optical Nanoscopy* [1].

STED method

Fluorescence microscopy plays a key role in life science research, but diffraction limits the imaging of structures which are smaller than about half of the excitation wavelength. This limitation can be circumvented by the usage of super-resolution microscopy strategies like STED, PALM (photoactivated localization microscopy) and STORM (stochastic optical reconstruction microscopy). STED microscopy possesses a unique set of advantages, such as rapid image acquisition that enables the study of fast dynamics and the possibility of being adapted to other fluorescence based techniques [8]. This offers the possibility to analyse processes like vesicle movement in vivo, dendritic spine activity or actin dynamics within living brain cultures [9-11].

The focus of a single laser beam is limited by the wavelength of the light. The principle of STED is to combine two different (pulsed) lasers at the same focus position and control the fluorescence through the overlap of the point spread functions [8]. The first laser pulse is used to excite the fluorescent molecules. The second laser pulse (Fig. 2 STED beam) has a phase modulation to control the spatial intensity distribution, and a time delay which should be less than the spontaneous fluorescence emission time for the fluorophore. The second pulse depletes the excited state and transfers these molecules back to their ground state, suppressing spontaneous fluorescence emission from part of the first pulse focus. The spontaneous emission from the remaining excited molecules is collected as the STED fluorescence signal, so highly photo stable labels (dyes) are preferred.

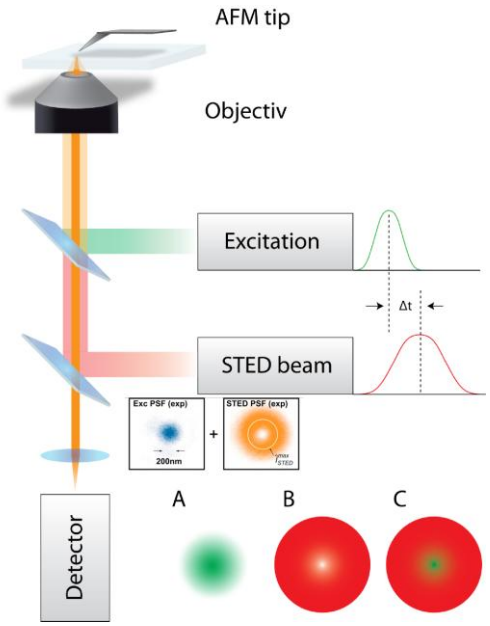

Fig. 1: *Scheme of an AFM and STED based system showing the major components. The sketch includes the time shifted pulses of the excitation source and the STED beam, the detector unit and the AFM. Schematic of the intensities of excitation laser (A), donut shaped STED laser (B) and the overlapping of both (C). A donut shaped STED beam is most common and it used to provide a lateral resolution enhancement, but it is not the only implementation.*

For imaging below the standard diffraction limit the STED-laser exhibits a donut-shaped pattern [12,13]. Thus a spatial resolution of tens of nanometers can be reached. The use of a spherical focus spot allows a 3D resolution of 40–45 nm inside a cell [13].

AFM method

Today AFM is a standard technique which measures the "nano-texture" of sample surfaces, thus enabling the reconstruction of the sample topography with sub-nanometer spatial resolution. Especially AFM under aqueous conditions has been of wide interest for modern research, particularly in biosciences. Studies on a large class of bio-samples, such as proteins, DNA, membranes, viruses and living cells have been performed successfully [2].

Besides the high resolution topography, JPK's NanoWizard® AFM technology (Fig. 1) provides information about the structural and for example mechanical properties of a sample. The AFM tip can be used to indent the sample surface in order to measure the local stiffness [3, 4], with a lateral resolution limited by the tip shape and the indentation depth. Recent developments like JPK's QI™ mode enable the measurement of mechanical properties with resolution and speed of standard AFM imaging.

In general, a label-free imaging technique like AFM works non-specifically, which can be a drawback for a variety of biological studies. An optical fluorescence microscope platform can give this specificity and is, therefore, often seen together with the AFM. The variety of implemented fluorescence strategies from confocal laser scanning microscopy to fluorescence correlation spectroscopy (FCS) [5], fluorescence recovery after photobleaching (FRAP) [6], fluorescence lifetime image (FLIM), Förster resonance energy transfer (FRET), total internal reflection fluorescence (TIRF) etc. [7] show the potential of this combination.

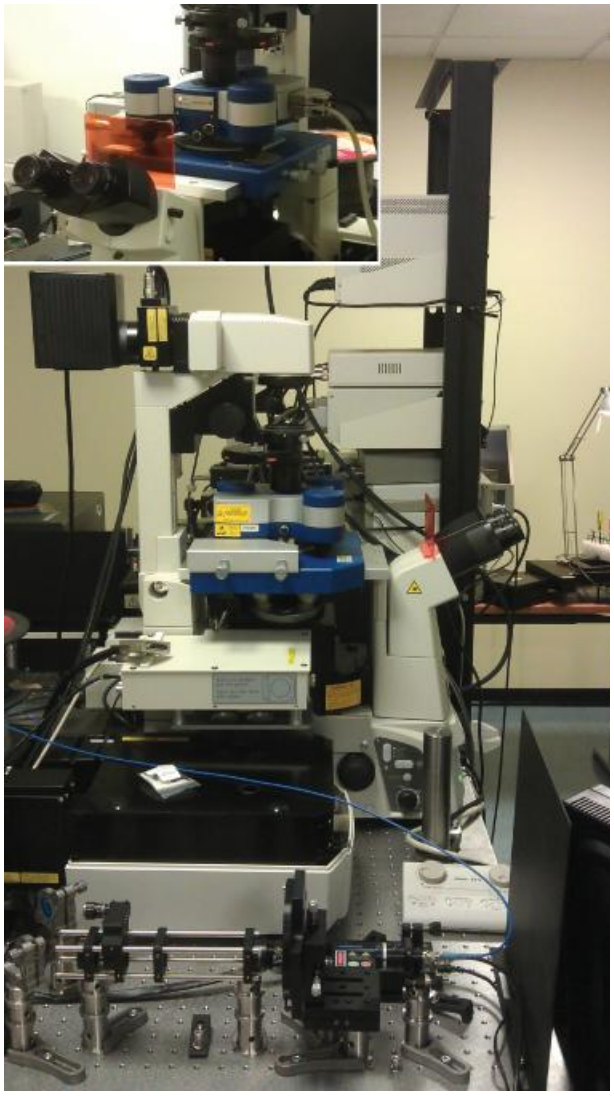

Fig. 2: *Combined NanoWizard® 3 AFM and Nikon based STED system in the laboratory of Prof. Alberto Diaspro from IIT in Genua, Italy.*

Integrated AFM + STED system

As a proof of principle and a test of performance it is recommended to use an easy sample in terms of its simplicity of preparation and its stable response to environmental perturbations. Fluorescent spheres have been employed successfully and can also serve as a routine test.

The results of such measurements are shown in Figure 3. The benefits of the AFM-STED combination in comparison to images acquired by a confocal microscope are clearly visible. The STED images (c) show a significantly better resolution than the confocal images (a). Every single 40 nm fluorescence sphere can be resolved by STED and AFM (b) whereas the confocal mode fails to resolve individual spheres within a group. This becomes obvious by comparing the enlarged regions (marked by dashed squares) and superimposing the AFM images with the corresponding confocal (d) and STED image (e) by the usage of the unique JPK DirectOverlay™ software feature. The 3D rendered view of the AFM topography in combination with the corresponding raw data of the optical images underscores this fact (f, g).

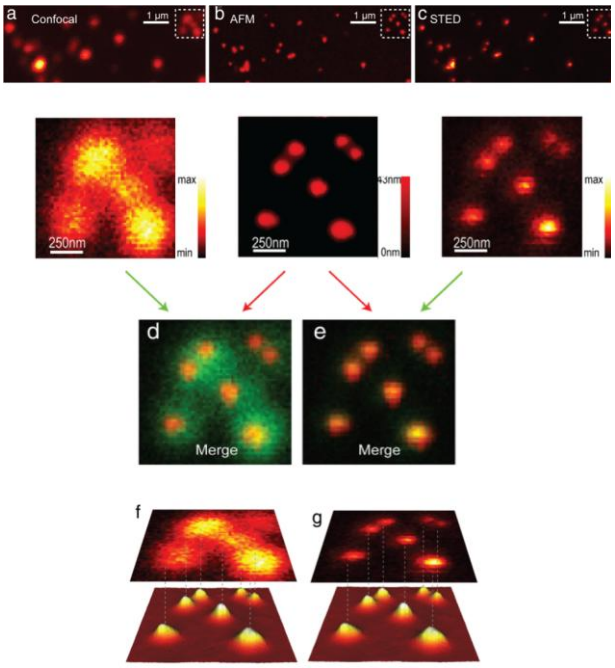

Fig. 3: *Raw data of confocal (a), AFM (b) and STED measurement (c) of 40nm fluorescent spheres. Zoomed areas of optical images and new AFM scan are highlighted. Overlay of AFM image with the confocal (d) and STED data (e). 3D AFM topography correlated with confocal (f) and STED data (g).*

Application example cellular complexes

After a performance test investigations of samples with higher complexity and scientific interest were performed. Recently, fascinating explorations by studying biological cell samples in aqueous conditions have been performed successfully [1]. Microtubules, an important component of the cytoskeleton, can be investigated within antibody labelled COS7 cells in PBS solution. In figure 4 images collected by a confocal (a) and a STED system (b) of the same area are shown.

The force mapping mode or the less time consuming and high resolution JPK QI™ mode can be applied to record simultaneous topographical and e.g. elasticity information of the sample. The advantages of these modes are the reduced lateral interactions between tip and sample and the prevention of possible stretching of the sample. The topographical information can be extracted from each single force curve. A convincing data set is shown as a 3D plot in figure 4c. The corresponding confocal (3d) and STED images (3e) reveal a meshwork with a junction of a tubule bundle and single filaments. Once more the insufficient resolution of confocal microscopy is shown. The confocal data are not able to confirm the AFM data whereas the STED data clearly displays single filaments within the meshwork.

Performing force mapping or JPK's further developed QI™ mode each single force curve can be investigated also in order to calculate the Young's modulus which gives information about the elasticity of the sample. In figure 4 the match of a collected STED image (4e), a rendered 3D AFM height image (4c) and a Young's modulus map (4f) of the same analysed area on a single cell is demonstrated. Microtubules which are visible in the STED image can be observed in the 3D height image and traced in the Young's modulus map.

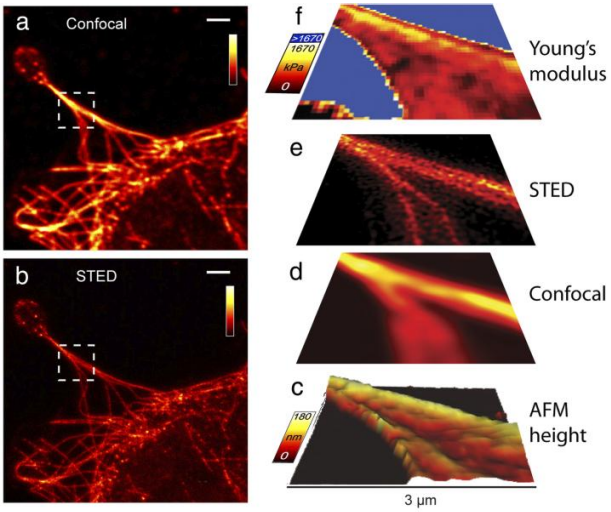

Fig. 4: *Confocal image (a) and STED image (b) (both raw data) of Atto 647 N labeled microtubules in COS7 cells in PBS. From AFM mapping extracted 3D height image (c) and calculated elasticity map (f) correlated with the STED (e) and confocal image (d) (both linear deconvolved) collected from same area. Scale bars in (a) and (b): 2 µm, axes bars in (c)-(f): 3 µm.*

Conclusion

The combination of JPK's state-of-the-art NanoWizard® AFM technology with a super-resolution STED system can be applied to various kinds of samples and applications where specificity and targeting can enhance the versatility of AFM. Both systems are commercially available which makes this type of nanoscopic tool very attractive for various research fields by their prompt availability. Many possible artefacts in each of both techniques can be excluded and structures of interest can be selected and brought into scientific focus.

The shown data have previously been published in **Optical Nanoscopy No. 1/2012**.For more information about the new QI™ mode please visit **http://www.jpk.com/qi-mode-overview.662.en.html**

Literature

[1] Harke, B, Chacko, JV, Haschke, H, Canale, C, & Diaspro, A (2012). A novel nanoscopic tool by combining AFM with STED microscopy. *Optical Nanoscopy*, *1*(1), 1–6.

[2] Dante S, Haufl T, Steitz R et al (2011) Nanoscale structural and mechanical effects of beta-amyloid (1–42) on polymer cushioned membranes: A combined study by neutron reflectometry and AFM Force Spectroscopy. Biochimica et Biophysica Acta (BBA)-Biomembranes 1808:2646–2655

[3] Le Grimellec C, Lesniewska E, Giocondi MC et al (1998) Imaging of the surface of living cells by low-force contact-mode atomic force microscopy. Biophys J 75:695–703

[4] Chiantia S, Ries J, Kahya N, and Schwille P (2006) Combined AFM and two-focus SFCS study of raft-exhibiting model membranes. Chem Phys Chem 7, 2409-2418

[5] Chiantia S., et al (2008) Role of ceramide in membrane protein organization investigated by combined AFM and FCS. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* 2008*, 1778*, 1356-1364

[6] Micic M, Hu D, Suh YD et al (2004) Correlated atomic force microscopy and fluorescence lifetime imaging of live bacterial cells. Colloids Surf B Biointerfaces 34:205–212. doi:10.1016/j.colsurfb.2003.10.020

[7] Vickery SA, Dunn RC (2001) Combining AFM and FRET for high resolution fluorescence microscopy. J Microsc 202:408–412. doi:10.1046/j.1365-2818.2001.00857.x

[8] Klar TA, Jakobs S, Dyba M, Egner A, and Hell SW (2000) Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission. PNAS 97(15), 8206–8210

[9] Westphal V, Rizzoli SO, Lauterbach MA et al (2008) Video-rate far-field optical nanoscopy dissects synaptic vesicle movement. Science 320:246

[10] Nägerl UV, Willig KI, Hein B et al (2008) Live-cell imaging of dendritic spines by STED microscopy. Proc Natl Acad Sci 105:18982

[11] Urban NT, Willig KI, Hell SW, Nägerl UV (2011) STED nanoscopy of actin dynamics in synapses deep inside living brain slices. Biophys J 101:1277–1284

[12] Harke B, Keller J, Ullal CK et al (2008) Resolution scaling in STED microscopy. Opt Express 16:4154–4162

Deckert-Gaudig, T, & Deckert, V (2010). Tip-enhanced Raman scattering (TERS) and high-resolution bio nano-analysis-a comparison. Physical Chemistry Chemical Physics, 12(38), 12040–12049. doi:10.1039/c003316b