Combining fluorescence and atomic force microscopy

Integration of optical and atomic force microscopy (AFM) provides a powerful tool to obtain comprehensive information on a variety of samples. Especially combining fluorescence microscopy and the AFM technique provides complementary imformation: the fluorescence about the location of labelled molecules not detectable by transmission light microscopy, the AFM finally on the topology of the sample.

The design of the NanoWizard®II AFM (JPK Instruments, Berlin) allows its integration into inverted optical devices providing different optical techniques like epifluorescence. The use of the DirectOverlay™ feature available for the JPK SPM software enables real optical integration, not only by detecting the position of the cantilever within the otpical image but also by correcting optical distortions caused by the use of lenses. DirectOverlay™ can be performed using a wide range of cameras or even with confocal techniques, but the most convenient way is to use cameras that can be controlled by the SPM software. For basic applications like phase contrast or DIC the DFK 31AF03 camera from Imaging Source that is usually provided by JPK is a reasonable solution. For advanced applications as sensitive fluorescence techniques JPK offers software integration of Jenoptik cameras as the ProgRes® MF^{cool} and ProgRes® CF^{cool}.

In this report the combining of sensitive fluorescence detection and AFM, and the way how these techniques can complement each other are described by different applications.

Optical integration

Using the ProgRes® camera instead of the ImagingSource makes no difference concerning camera mounting or the optical overlay procedure. Figure 1 shows the setup used for this report, a Nikon Eclipse TE2000 hosting the ProgRes® camera and the JPK NanoWizard®II AFM. The camera is mounted to one of the side ports of the microscope and connected to the JPK NanoWizard®II controller via fire wire. The camera software can then be opened via the camera button within the JPK SPM software. Crucial parameters as exposure time and gain can be adjusted and the fan can be controlled manually, turned off when performing AFM measurements. In case of very sensitive dyes that suffer fast bleaching there is the possibility to operate a shutter using the input/output trigger option.

Fig. 1: Fluorescence setup (Nikon Eclipse TE2000) with the ProgRes® MF^{cool} camera and the JPK NanoWizard®II.

To perform the DirectOverlay™ procedure, the cantilever is moved to 25 defined piezo positions and an optical image is taken at each point. The tip locations of these images are then automatically assigned to the corresponding piezo positions. Thus the software can correlate the piezo or rather tip

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position to the appropriate pixel within the optical image. After this procedure the field of view recorded by the camera is calibrated to the JPK SPM software. At the end of the calibration procedure a snapshot of the sample can be taken or another image taken by the same camera with the same resolution, can be imported. If examining very photosensitive samples it is recommended to take the fluorescence image before the calibration images. Finally interesting regions of the sample can be chosen within the optical image and scanned by AFM.

Fluorescence detection and high resolution AFM imaging of nano structures

Imaging structures in the nanometer range such as beads or quantum dots is a classical example for the use of AFM technique. Sensitive fluorescence microscopy emerges as a useful tool to get a first impression of the composition of the sample and to localize the particles or rather to find the optimal sample position.

In the first example 40 nm red fluorescent spheres were deposited on a mica substrate. The spheres were focused and an optical image taken using the ProgRes® MF^{cool} (exposure time 1 sec). After the optical system was calibrated to the SPM software using the DirectOverlay™ feature an interesting scan region was specified. AFM scanning of this region revealed clusters of beads, appearing as high fluorescent spots within the optical image, but also single beads showing only weak fluorescence (figure 2).

In the next example sensitive fluorescence detection was used to localise quantum dots deposited on mica in a low concentration to finally resolve their topology and composition by AFM imaging (figure 3). In low concentrations quantum dots are almost impossible to be visualised by eye. Then it helps to use sensitive fluorescence detection as shown here using the monochrome camera ProgRes® MF^{cool}.

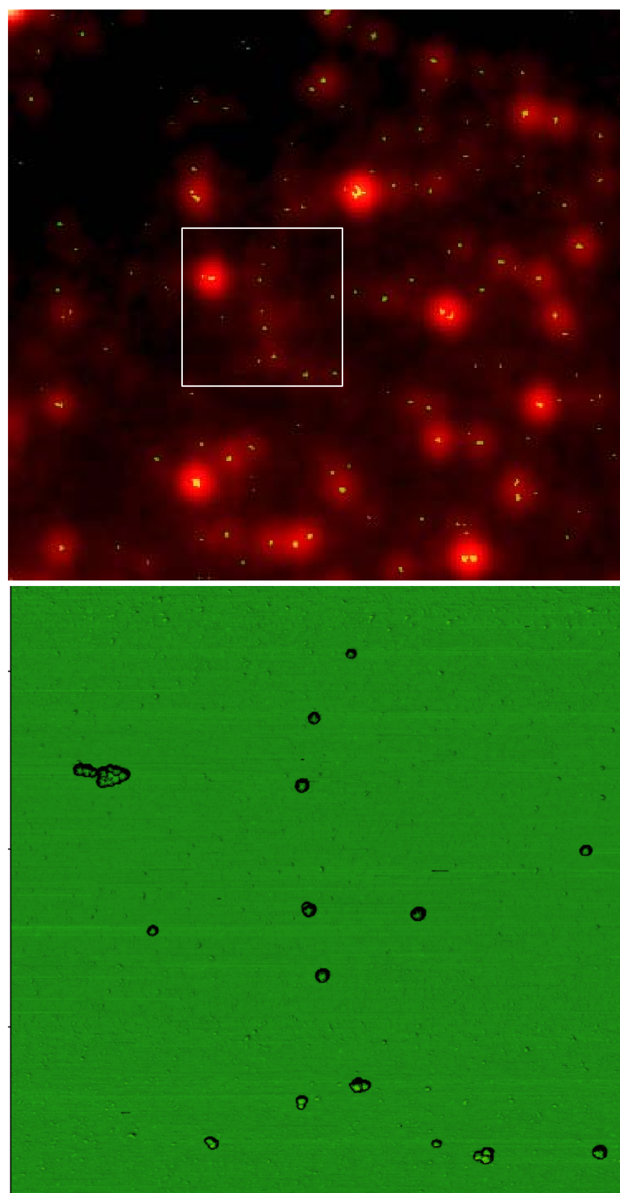

Fig. 2. Red fluoerescent 40 nm beads on mica. Overlay of the optical and AFM height image (top, scan region 15 µm) and phase image of the zoomed region (bottom). AFM images were taken with the JPK NanoWizard®II using IC mode, the optical image using the ProgRes® MF^{cool} camera. The optical image was colourized with the GIMP software.

Since relatively long exposure times (around 7 sec) are necessary to take high quality images the use of the binning option is very helpful. The light information of several pixels is summed and the exposure time automatically decreased to keep the

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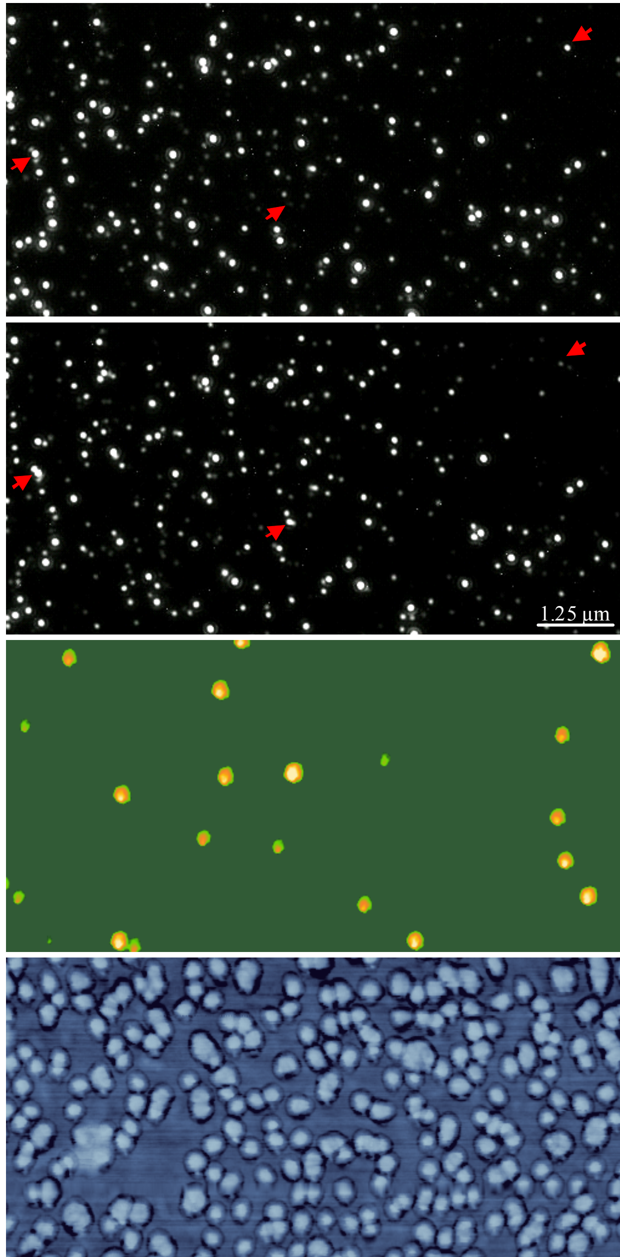

Fig. 3: Red fluorescent quantum dots on mica. The two optical images on top show the same sample region recorded consecutively with the MF[ccd] camera . The lower images show a height image of a region with low dot concentration (1.5 µm scan range, max. height around 8 nm) and a phase image with high dot concentration (1 µm scan range).

chosen brightness of the raw image constant. If for instance 3-fold binning is chosen the resolution decreases by summing squares of 3x3 pixels. But the exposure time also decreases to the same extent, allowing quicker focussing on the right plane. When the optimal focus and sample region is found the maximum resolution (no binning, low gain, high exposure time) can be used to get high quality images. To take high resolution and quality images it is recommended not to use binning for such small structures like quantum dots, where the size of the structure is similar to the pixel size. When using the binning option anyway, it is essential to use the same binning for the calibration images, since the overlay feature is based on the correlation of the pixel position of the cantilever tip to the pixels within the snapshot of the sample.

The blinking nature of quantum dots makes it difficult to localize all dots in one region at the same time (compare areas indicated by the red arrowheads of the two optical images in figure 3). However, using optics it is possible to distinguish between areas of high and low quantum dot concentration and the optical image helps to orientate and choose an adequate scan region.

Imaging cellular structures and extracellular matrix proteins

To localize cellular and extracellular structures down to the molecular level fluorescence labeling is an indispensable technique. Topographic information of the corresponding structures or the cellular compartments hosting the structures is another refinement that can be provided by AFM imaging.

Here L929 fibroblasts (figure 4) and MC3T3 osteoblasts (figure 5) were stained for f-actin using AlexaFluor546- and FITC-phalloidin respectively. L929 cells were grown and fixed on glass slides, the MC3T3 cells on coverslips for the use with the CoverslipHolder™. To prevent photo bleaching, focus and adequate sample position were adjusted using fluorescence detection in binning mode. High quality images for the overlay were taken before calibration images. Applying phalloidin staining the actin stress fibers could be visualized using

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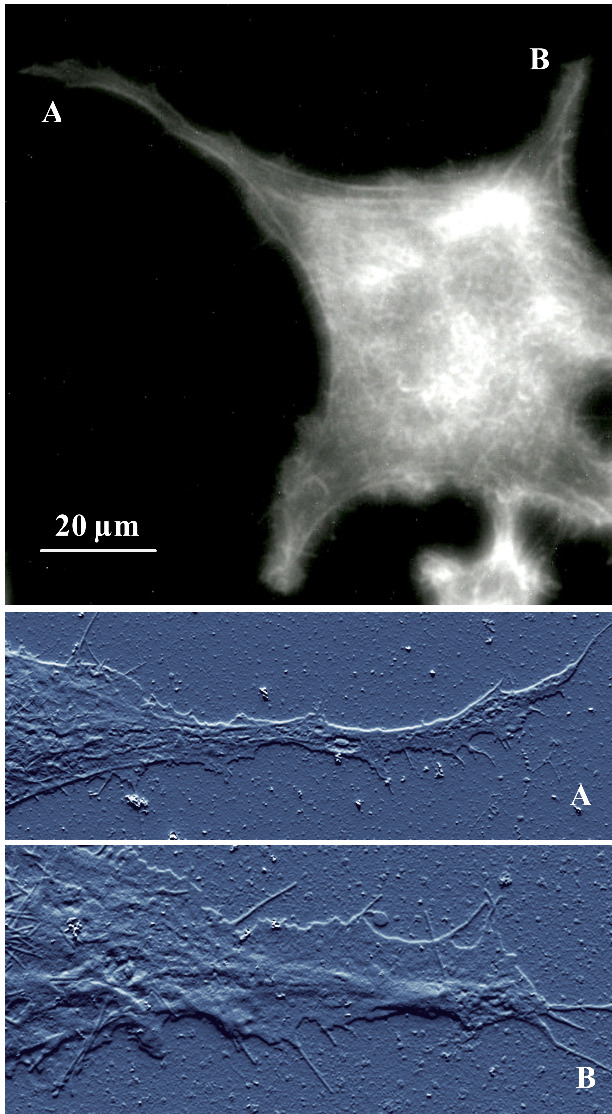

Fig. 4: Fluorescence image of a mouse fibroblast (L929) stained for filamentous actin (top) and deflection images of two cellular extensions (below). Cells were stained with AlexaFluore546-phalloidin and acquired with the MF^{cool}. Extensions were imaged using contact mode in air.

fluorescence microscopy. Additional topographical information could be derived by AFM imaging, also revealing the fine structure of the filaments and thus complementing the optical image.

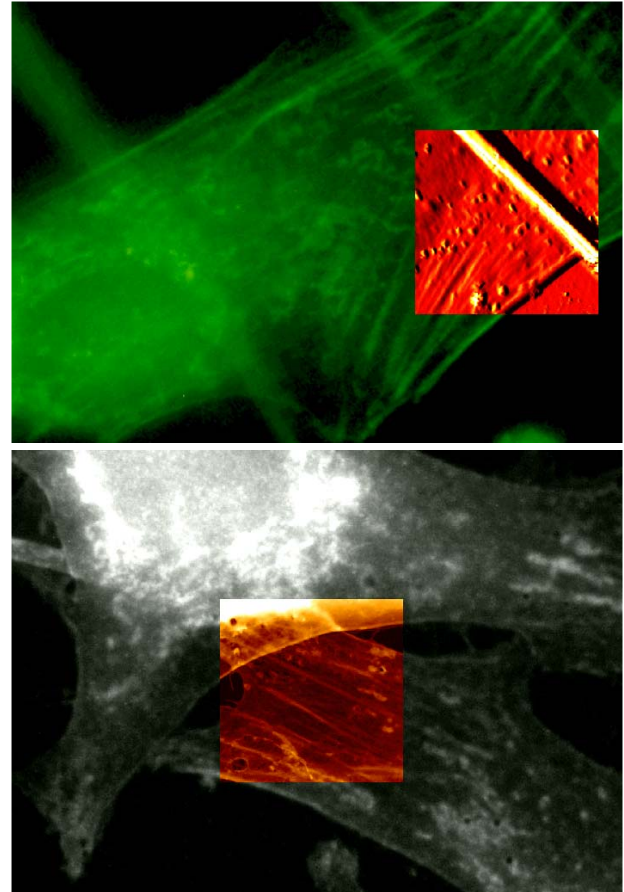

Fig. 5: Fluorescence images of MC3T3 fibroblasts stained for filamentous actin with inserted deflection (top) or height image (bottom) of the stress fibres. Cells were stained with FITC-phalloidin and acquired with the CF^{cool} and the MF^{cool}. AFM images were taken using contact mode in liquid (top) and air (bottom). Both scan regions 15 µm, height range around 300 nm.

Another example where AFM imaging reveals substructures invisible by fluorescence imaging is D-periodic collagen. Collagen type 1 forms thick (~30 nm), D-banded collagen fibers if polymerized on glass with buffer of a potassium ion concentration of at least 5 mM (*Cisneros et al. 2006, J Struct Biol 154:232-245*). The collagen fibers can be stained with flourescein and thus be detected by fluorescence microscopy. The D-banding pattern can finally be resolved using AFM imaging (figures 6 and 7).

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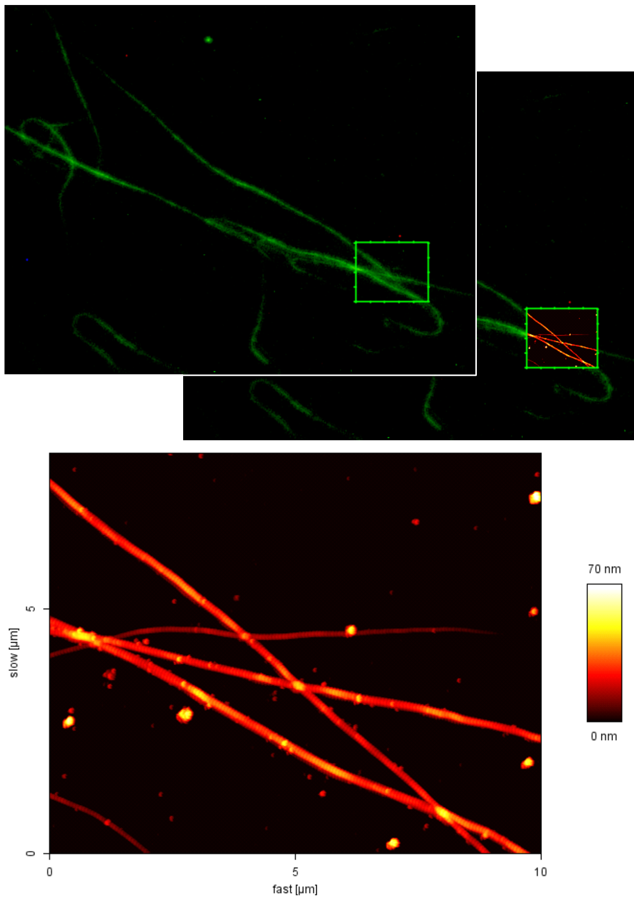

Fig. 6: Fluorescence image of fluorescently labelled collagen type 1 on glass and AFM image of the marked region (see optical overlay). Acquisition occurred with the CF^{cool}. AFM imaging in intermittent contact mode in air.

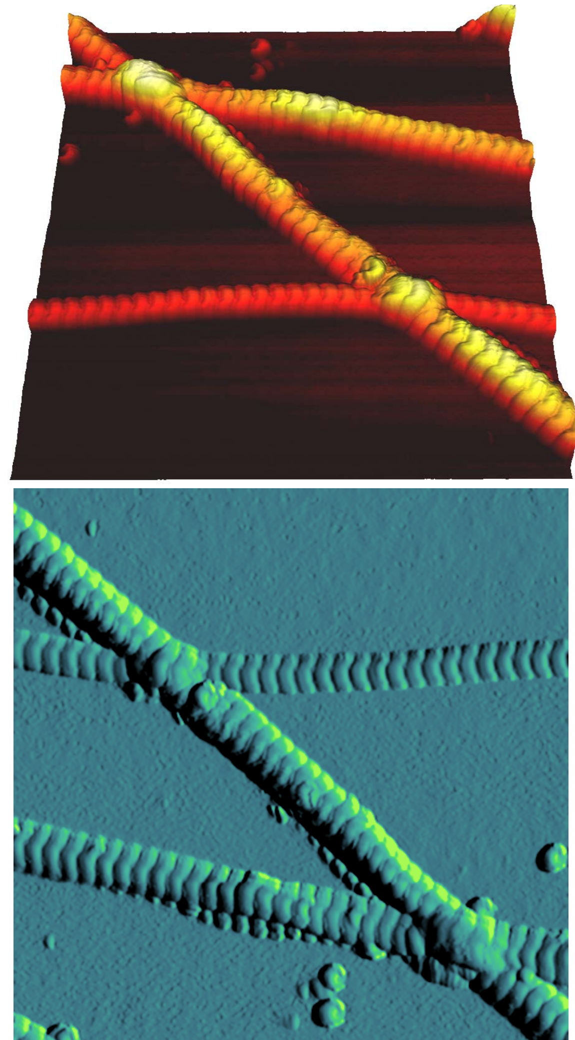

Fig. 7: Height and deflection image of the collagen fibers in figure 6. Both scan regions 2 µm, height range around 50 nm. Intermittent cantact mode in air.

Conclusion

As shown in this report, combining sensitive fluorescence and atomic force microscopy can be provided by integrating the JPK NanoWizard®II AFM and the ProgRes® CF^{cool} or MF^{cool} into the fluorescence setup. The DirectOverlay™ feature allows for real optical integration and provides optical and topographic information within one region. Fluorescence labeled cellular components like the cytoskeleton or trafficking molecules can be optically tagged and the morphology of the corresponding region characterised by AFM imaging. Investigating structures in the nanometer range that cannot be optically resolved, fluorescence can help to roughly detect the structures and thus serve as an orientation tool for the search for interesting regions to be finally resolved by AFM imaging.

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