True integration of optical and atomic force microscopy

Atomic force microscopy (AFM) and optical microscopy, in particular fluorescence microscopy, make a powerful combination in the study of biological samples. AFM is not subject to Abbe's resolution limit, and can generate images with a much higher resolution than light microscopy. However, as contrast is generated in response to the structural properties of the sample, it can be challenging to detect specific structures in a heterogeneous sample, such as a cell. By combining the two techniques, higher resolution structural information can be generated using AFM. Subsequent correlation with fluorescently labelled markers can provide information about the composition, and consequently the function, of the identified structures.

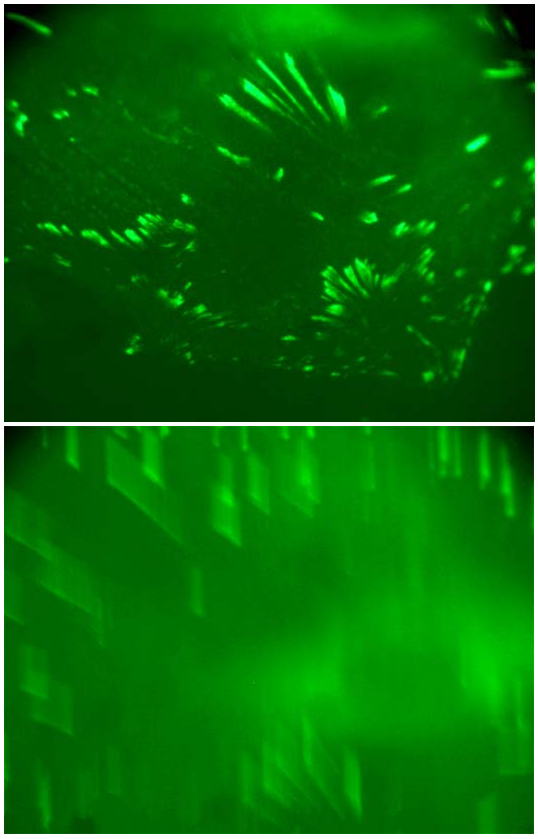

Fig. 1 Tip scanner vs sample scanner. The acquisition of epi-fluorescent images during AFM image acquisition with a tip scanner (A) and a sample scanner (B). As the sample is moving in (B) the fluorescent structures can no longer be imaged without smearing. The same cell is shown in both images.

The design of the Nanowizard® AFM from JPK is such that the atomic force microscope is integerated into an inverted light microscope, without affecting its functionality. This allows AFM imaging to be combined with optical contrast techniques including phase contrast, DIC, laser scanning confocal and TIRF microscopy. An important factor in the efficacy of this integration is that the JPK instruments are tip-scanners. That is, during AFM imaging the sample is held still, while the tip moves in raster fashion over the surface to build the image. In the case of sample-scanning instruments, the sample to be imaged is constantly moving in the optical microscopy picture while AFM scanning is in progress, meaning that true simultaneous imaging is not really possible (figure 1).

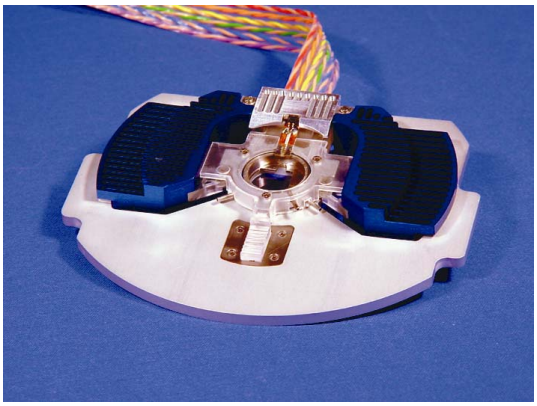

Fig. 2 The Biocell™ is designed to optimise image quality during experiments combining AFM and light microscopy while at the same time allowing temperature control. Peltier elements allow rapid adjustment of temperature.

While these hardware design features are the start of providing true integration there are also other factors that need to be addressed. The first is the sample holder used. As AFM and optical microscopy are fundamentally different techniques (one being based on physical interaction and the other on the diffraction of light) there are different requirements for effective sample supports. For the acquisition of high quality optical images, particularly with high magnification lenses (63x and 100x) it is best to use

very thin cover-glass with a thickness of around 170 µm. On the other hand, as AFM imaging is very sensitive to physical instability, the support for such imaging must be very stable. With these two fundamental requirements in mind, JPK designed the CoverslipHolder and the BioCell™ (figure 2). Both of these sample holders are designed to hold coverslips for uncompromised, combined AFM and light microscopy imaging. As such, with innovative sample holders and the fundamental design of the JPK instruments, simultaneous, high quality AFM and light microscopy images can be acquired. However, to be truly integrated we have now introduced the patented DirectOverlay™ feature, a software solution that involves calibration of the optical image and integration into the SPM software.

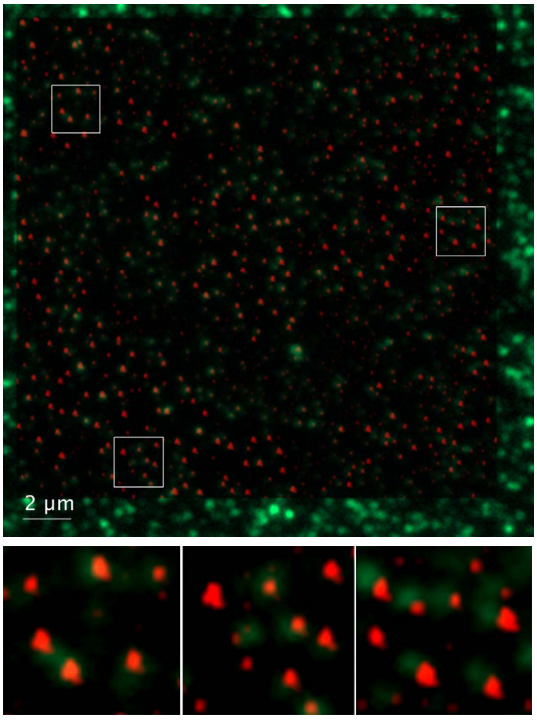

Fig 3. Overlay of topography (red) and untransformed fluorescence (green) images of 50nm beads. The lower panel is a set of digital zooms of the marked regions. While in the first two of the zoomed regions the overlay is good, in panel 3 there is a shear of the optical image.

As optical microscopy is based on the use of lenses, any aberrations in such lenses will lead to distortions in the final image. However, as the piezos in the JPK instruments

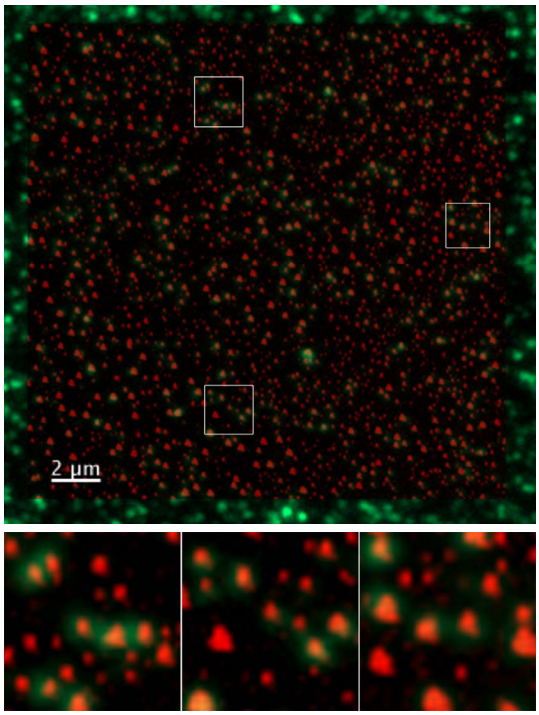

Fig 4. Overlay of topography (red) and untransformed fluorescence (green) images of 50nm beads. The same area that was imaged in Figure 3 is shown here. In all zoomed regions the overlay is now precise.

are linearized the AFM image is precise to 4Å in the x and y directions. As such, in most cases the AFM image and the light microscopy image do not accurately overlay, with shear or stretch in the optical image a common problem.

As the AFM image is generated using very precise linearized piezos it can be treated as “real-space”. Additionally, as the cantilever used for AFM imaging can be moved to fixed points, as well as raster-scanned over the surface, it can be used to calibrate the optical image. In short, the cantilever is moved to a set of 25 points in real-space, using the piezos. At each point an optical image is acquired and subsequently the tip location within the optical image is automatically determined. A transform function is then calculated using both sets of 25 points, and this transform is applied to the optical image as it is imported into the SPM software. In such a way the optical image is calibrated and imported into the SPM environment, in an automated process.

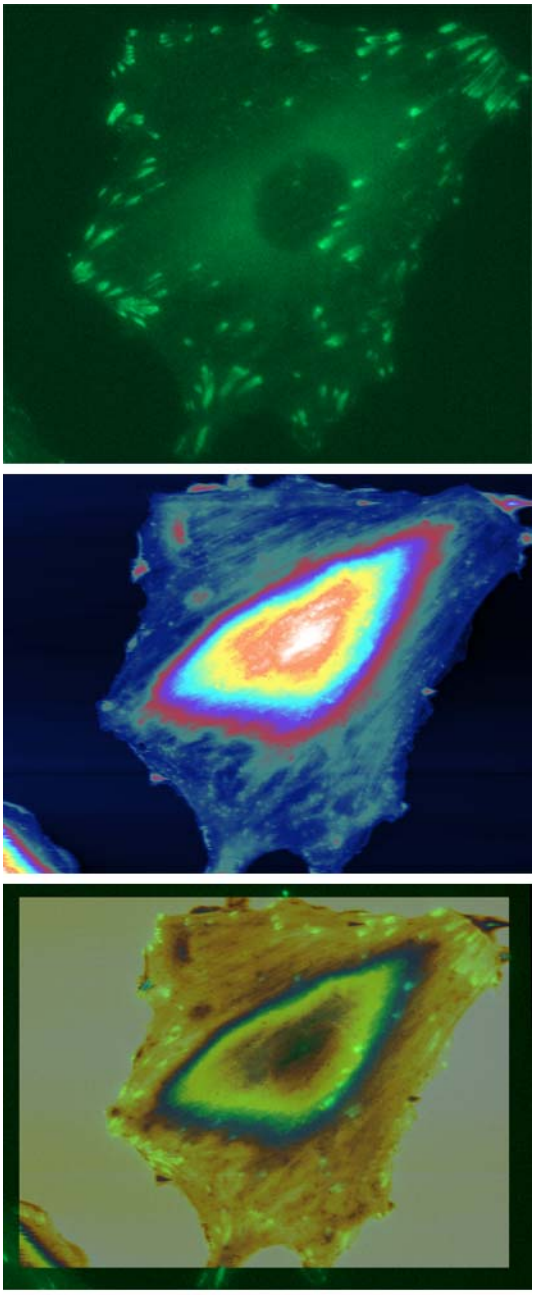

Fig. 5. Overlay of fluorescence and topography of REF52 fibroblast cells. In panel A is the transformed fluorescence image and B topography. In C an overlay of the two is displayed

In order to test the calibration of the optical image, 50 nm fluorescent beads were imaged using both AFM and epi-fluorescence and both the untransformed and the transformed optical images compared with the AFM. It can be seen that there is a shear in the right hand side of the untransformed optical image (figure 3). Once the calibration procedure has been applied, however, the overlay between the fluorescent and topographic signals is precise (figure 4).

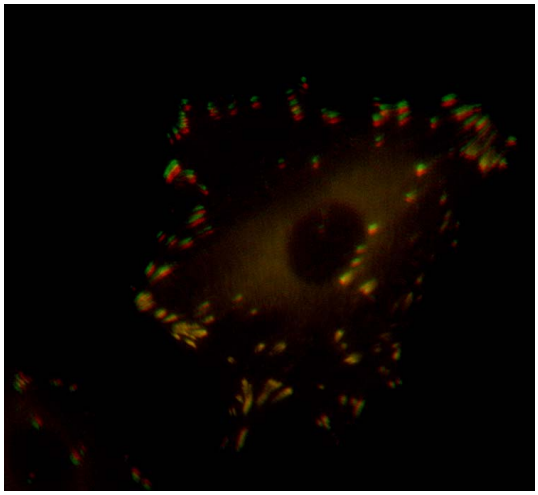

Fig. 6. Overlay of an image that has been calibrated (red) and the same image in its raw, uncalibrated form (green) is presented. It can clearly be seen that the uncalibrated image is stretched in one direction.

The benefits of such a software feature are extensive. For instance, in the comparison of fluorescence and AFM images there may not be easily identified fixed points within the two images to conduct such a transformation offline, nor even to overlay the edges. As seen in figure 5, the overlay of the AFM image of a REF52 fibroblast (stably transfected with YFP-paxillin) and the corresponding fluorescence image of the focal adhesions, there are no points in both images that can easily be identified for use in accurately mapping one image to the other. In this case the focal adhesions are at the basal side the cell and the AFM image generates a topograph of the apical side of the cell. Thus the use of the cantilever as a tool for calibrating the optical image is essential. In figure 6 the difference between the transformed and non-transformed optical image is displayed. In this case the predominant artefact is a stretch along one axis.

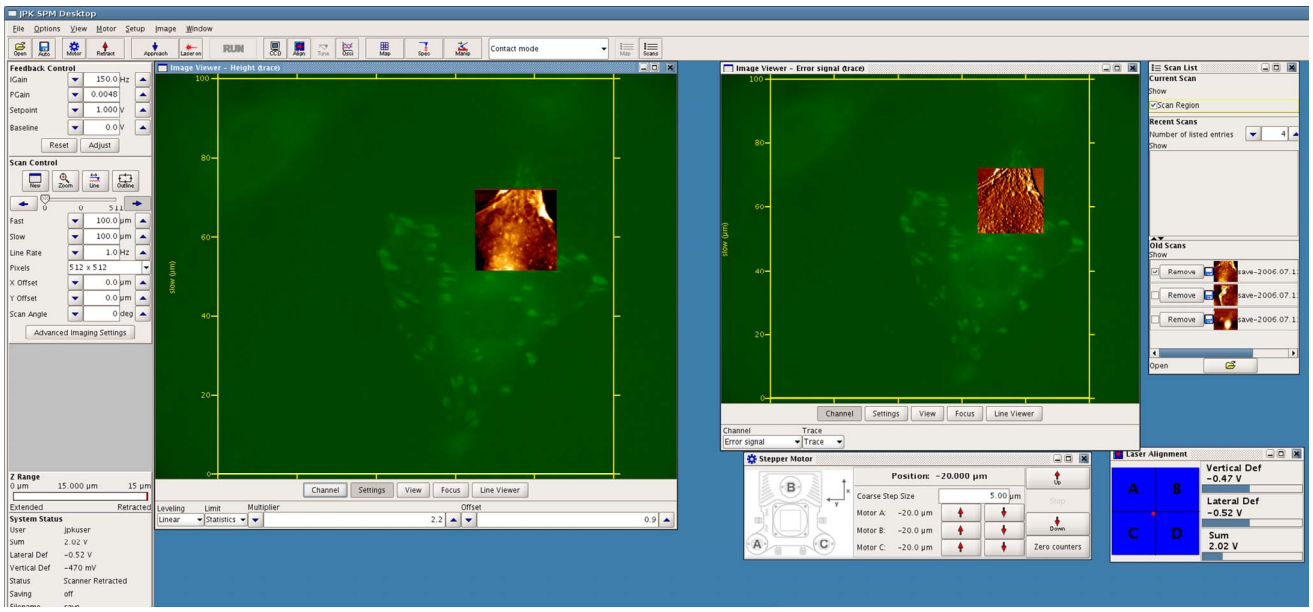

Fig. 7. Import of optical images into SPM software. A calibrated optical image can be imported into the SPM software and displayed in the background. This allows the selection of scan regions and force-spectroscopy points based on the optical image, removing the need for an overview AFM image.

Additionally, such a feature can save the user a considerable amount of time. While AFM has high spatial resolution, the temporal resolution of the technique is far lower than that of light microscopy, due to the longer acquisition times. With a calibrated optical image in the background of the SPM software (figure 7), the need to acquire an overview AFM image of the cell before focussing on a region of interest is removed. This can be particularly important for the scanning of regions of a living cell, where time may be important. Obviously, force-spectroscopy points can also be selected on the optical image, again removing the need to acquire an AFM image before starting force-spectroscopy experiments. When a functionalized tip is being used this could prove critical, as if a scan is taken before the force-spectroscopy data is obtained the tip may be passivated, leading to false negative results.

The alterations in atomic force microscope design that led to its installation on an inverted light microscope opened the possibility for the simultaneous acquisition of light and AFM images, crucial for the effective investigation of cells and biological systems in vitro. However for true optical integration with AFM, more is required than just the colocalisation of the two microscopes. A tip scanner is essential such that the sample is still during AFM imaging, so that optical images can be acquired. Sample holders must be optimised for both forms of microscopy, i.e. thin coverslips for light microscopy and stability for AFM imaging. Finally, a calibrated optical image must be available in the AFM imaging software, to enable accurate overlays, with artefacts from aberrations in the optical image removed. The NanoWizard AFM provides all of these features, allowing for the first time true integration of optics and AFM.