Cominbining Atomic Force Microscopy and Laser Scanning Confocal Microscopy

Introduction

The visualisation of sample elements using various forms of microscopy relies not only on magnification but also contrast. As such, differing forms of microscopy offer different information about a sample. Laser scanning confocal microscopy (LSCM) provides information about the 3D location of a particular, labelled component within a sample and has the additional advantage of excluding out of focus light. This can lead to sharper images of a given focal plane in a sample. Atomic force microscopy (AFM), on the other hand, provides direct structural information about the surface of a sample. The combination of these two forms of microscopy could be a very powerful tool in research as it is difficult to "stain" structures for AFM imaging, hence distinguishing specific elements on a structural basis alone is not always possible. The use of fluorescent labels and imaging the optical slice corresponding to the sample surface with LSCM means that, if the two techniques can be combined then labelling in the LCSM image can be cross correlated to structures imaged with AFM. This has the added benefit of also contextualizing the location of labelled proteins (identified with LSCM) with respect to specific structures.

To combine the two microscopy techniques the two separate instruments need to be built together, such that neither disturbs the function of the other. The combination of the Nikon C1 confocal microscope and the JPK Nanowizard® allows imaging of the same sample area with both techniques, as the JPK Nano Wizard® is designed to be installed on top of an inverted light microscope. Hence there is bottom-up access to the sample for the light microscopy techniques and top-down access for the AFM stylus. The JPK NanoWizard® only requires that the stage of the Nikon confocal is exchanged for one of highly stable construction. There is a light path through the AFM that means that transmission as well as reflection light microscopy can be conducted with the AFM in place. Hence, the basic hardware of the two imaging devices is compatible for simultaneous functioning.

For AFM imaging stability is extremely important (hence the requirement for the more stable stage). This has meant that the use of thin coverglass as sample supports for AFM imaging was for a long time incompatible with high quality AFM images. However, for superior optical images it is often better to image samples through thin coverglass, particularly when high magnification, immersion lenses are used. For such experiments, where the user wishes to combine high quality imaging from both systems, JPK has designed sample holders, such as the BioCell™, that allow the user to stably mount samples on coverglass, without compromising image quality.

Additionally, for true integration, there must be some way to calibrate the image space of the two techniques so that they can be precisely overlaid. This is necessary to compensate for the unavoidable, albeit small, spatial aberrations that arise from the use of optics in the light microscope. However, the piezos in each JPK instrument are linearized such that the AFM image is precise to 3Å in the x and y directions. As there is distortion in the optical image that is not replicated in the AFM, in most cases the images from the separate sources do not accurately overlay. This is particularly a problem when the user wishes to correlate fluorescent signals with small structures, such as endocytic pits, on the surface of a cell. Overlaying the images and then warping one by eye involves significant subjective input that is prone to error.

Accurate calibration of confocal optical images

As the AFM image is generated using very precise linearized piezos it can be treated as "real-space". The cantilever (imaging stylus) of the AFM is usually raster-scanned over the surface to build an image. However, this cantilever can also be moved precisely to fixed points. This means that the cantilever can be used to calibrate the optical image by empirically determining the cantilever position in a set of optical images, where the precise position in the AFM is already known. 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 applied to the optical image as it is imported into the SPM software (required for running the JPK NanoWizard AFMs). In such a way the optical image is calibrated and imported into the SPM environment, in an automated process.

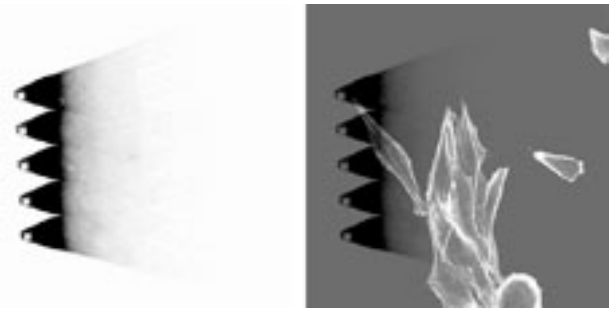

Fig. 1 Calibration of LSCM images using the Direct Overlay function. The AFM cantilever can be imaged in reflection mode using LSCM. Here, five individual cantilever images are superimposed (A), and then superimposed over a corresponding LSCM image of FITC-phalloidin labelled mouse embryonic fibroblasts (B).

In the case of combining AFM with LSCM the optical image of the AFM cantilever is generated in reflection mode, i.e. the emission filter is removed from in front of the detector and the reflection of the excitation laser from the cantilever is imaged (Figure 1). In such a way the cantilever position within the LSCM image range can be detected using essentially the same light path as for imaging the sample.

Figure 1 shows an overlay of five of the cantilever positions (A) with the corresponding LSCM image of FITC-phalloidin labelled mouse embryonic fibroblasts (B). As the precise position of the cantilever in the AFM space is known, calculation of the corresponding tip position in the LSCM images can be calculated and a transform function deduced to allow precise overlays of the two images.

Overlay of calibrated optical images and AFM images

Once the image space of both microscopes has been cross-correlated a number of interesting possibilities arise. The confocal image can be imported into the AFM software to allow imaging of specific, labelled areas, or manipulation of specific regions of the cell, and precise offline overlays can accurately map labelled components to their corresponding structures. For instance, the surface of MDCK cells is covered by actin-based microvilli that can be directly imaged using AFM and the actin that forms the structural basis of the microvilli can be imaged with LSCM after staining with fluorescently labelled phalloidin. Previously, comparison of such images did not lead to direct overlay of all of the actin signal with the protrusions at the surface the cell, due to the slight differences in both images. However, after calibration of the confocal image and transformation, overlay of the confocal and AFM images is precise (Figure 2).

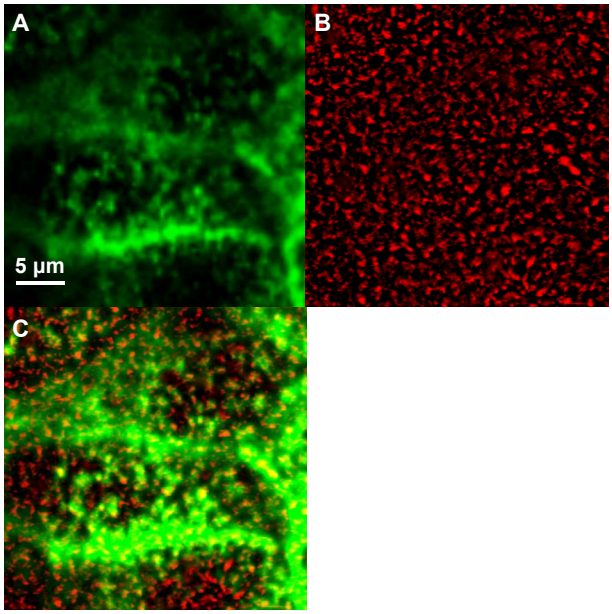

Fig. 2 Combined imaging of MDCK cells. Fixed MDCK cells, actin labelled with FITC-phalloidin. (A) Confocal microscopy image of the surface of the MDCK monolayer, showing surface associated actin-based microvilli and the cell junctions. The AFM image (B) of the same region has been processed to remove the curvature of the cell and just contain information about the surface protrusions (microvilli). In (C) the two images are overlaid.

The MDCK cells were fixed with paraformaldehyde (4% in PBS, 20 min), labelled with FITC-phalloidin and the top surface of the cells were imaged with AFM and LSCM. In the confocal image (A) the features correspond to the surface associated actin-based microvilli and the cell junctions, where filamentous actin is localised. In the AFM topographic image (B) the microvilli and cell junctions are also apparent. As the AFM image contains the structural information of the whole surface, not just specific components, the image has been processed to remove the curvature of the cells and just show the smaller surface protrusions (microvilli). In (C) the red-coloured AFM image has been overlaid with the green fluorescence.

Clathrin and caveolin on mouse embryonic fibroblasts

The microvilli at the apical surface of MDCK cells are the dominant structure on the surface in AFM images, so the correct overlay is clear. However, when cells that have a very heterogeneous surface are imaged it is extremely difficult to assign specific functions to various surface structures without some form of specific label. In such a case, if the overlay of the two types of images is not precise mistakes in cross correlation can be made. To demonstrate the potential of the use of the Direct Overlay for combining LSCM with AFM a more complex system was chosen. Mouse embryonic fibroblast cells were labelled with either anti-caveolin or anti-clathrin antibody, followed by a TRITC-labelled secondary antibody.

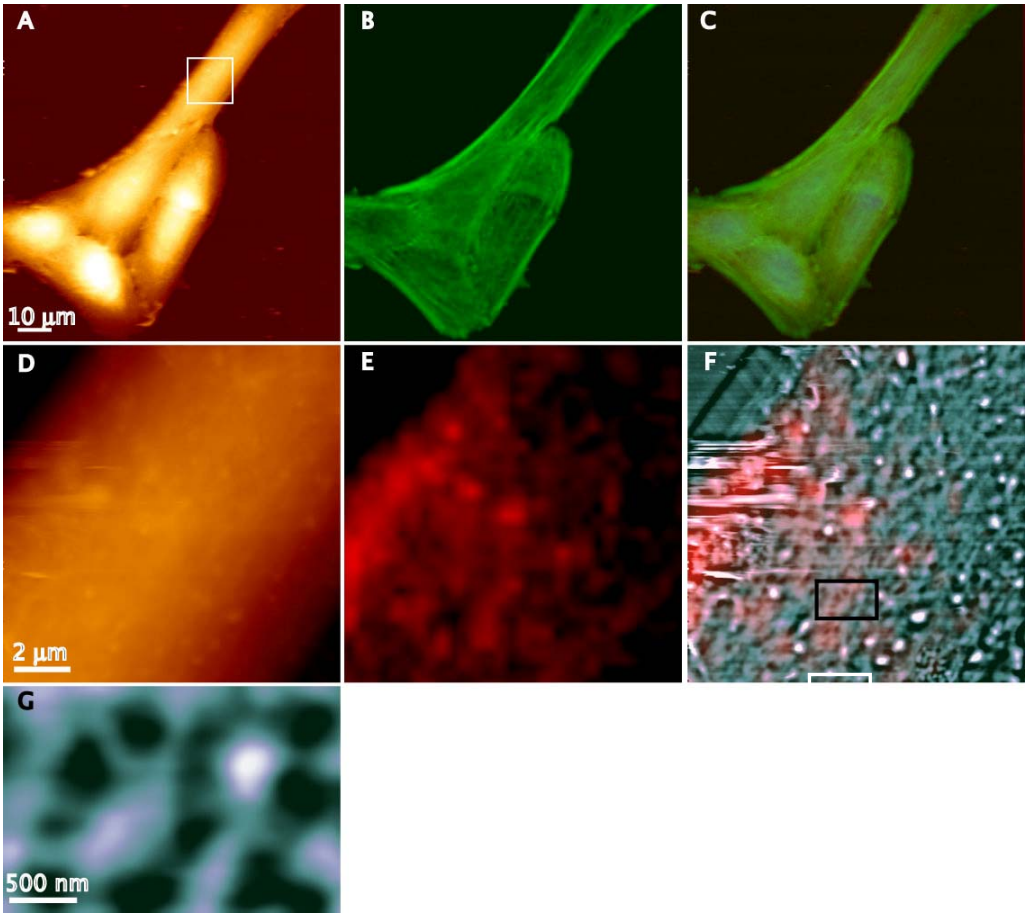

Figure 3: Combined imaging of clathrin coated pits at the surface of MEF cells.
Mouse embryonic fibroblasts were labelled with anti-clathrin heavy chain antibody. To visualise clathrin coated pits a TRITC-labelled secondary antibody was added, and filamentous actin was stained using FITC-phalloidin. An overview of the cells as AFM topography (A), labelled actin (B), and an overlay of the two (C) is provided. A higher resolution AFM topography (D) was acquired as was a LSCM image (E) of an area exhibiting labelled clathrin at the cell surface. An overlay of the two images (F) shows that fluorescent label corresponds to pits at the cell surface. An electronic zoom of the topography from one relevant area (marked in F) is presented in (G).

The cells were cooled to 4°C and labelled with anti-clathrin heavy chain antibody and then fixed with paraformaldehyde (4% in PBS, 20 min). To visualise clathrin coated pits a TRITC-labelled secondary antibody was added, and filamentous actin was stained using FITC-phalloidin. The DirectOverlay function was used to calibrate the LSCM image against the AFM image as described previously. After calibration of the confocal images, one can determine which pits on the surface correspond to the clathrin-labelled features in the fluorescence images (Figure 3).

To visualise surface caveolin, mouse embryonic fibroblasts were fixed with paraformaldehyde (4% in PBS, 20 min) and then labelled with anti-caveolin-1 antibody. This primary antibody was then stained using a TRITC-labelled secondary antibody. Again, the DirectOverlay function was used to calibrate the LSCM image against the AFM image. The images can then be compared to interpret the surface features seen by the AFM (Figure 4). As the cells only need to be fixed, not treated in any other way, this combined imaging allows the user to obtain an overview of how such structures relate to other structures at the surface of the cell.

Conclusions

The combination of AFM with LSCM can further extend the applications of both techniques. From the point of view of AFM imaging, combination with LSCM and calibration of the images allows precise determination of particular structures at the surface of cells, i.e. cavaeoli and clathrin-coated pits. Additionally, LSCM imaging in combination with manipulation by using the AFM allows imaging of processes downstream of the cell manipulation. The possibilities extend much further, with any combination of AFM manipulation and LSCM imaging now possible.

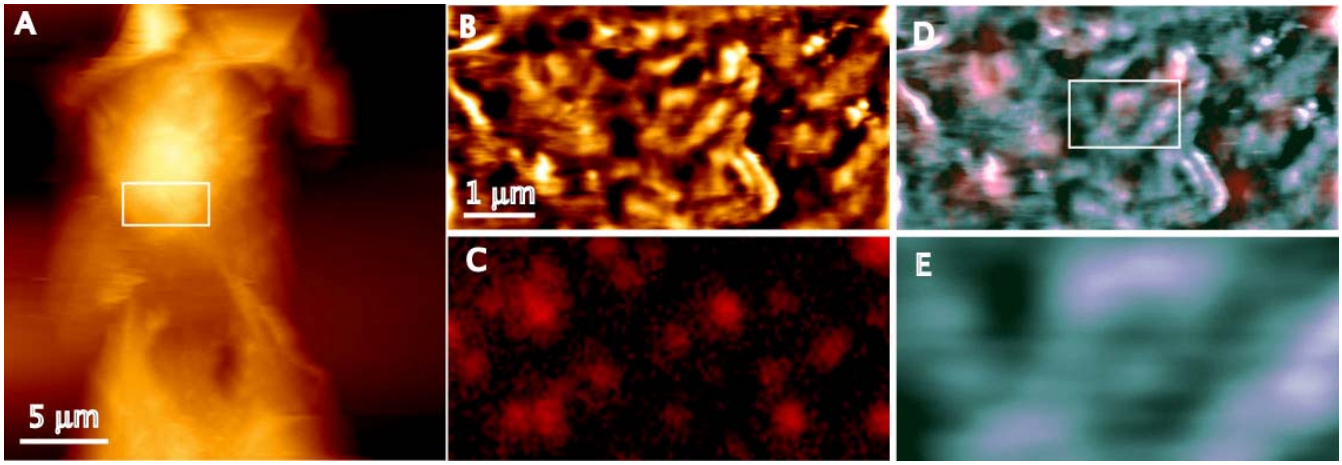

Figure 4: Combined imaging of caveolae at the surface of MEF cells.
Mouse embryonic fibroblasts were fixed with paraformaldehyde and then labelled with anti-caveolin-1 antibody. This primary antibody was then stained using a TRITC-labelled secondary antibody. An overview AFM topography image of the cell was acquired (A) and the LSCM image space calibrated to allow comparison of the AFM and LSCM. A higher resolution AFM image was acquired (B) and compared with the corresponding LSCM image of surface associated caveolae (C). An overlay is presented in (D), and a zoom into the marked area in (E).