Confocal and Atomic Force Microscopy

Confocal and Atomic Force Microscopy: A wide range of different forms of microscopy may be applied to the visualisation of biological specimens. Confocal microscopy can provide 3D information about fluorescently labelled cells with the added advantage that it excludes out of focus light. Atomic force microscopy (AFM) provides direct high resolution images of surface features of a sample. Here we look at the microscope configuration necessary for simultaneous confocal and AFM, and examine how the combination of the two techniques together with the 'Direct Overlay' function can be used to great advantage in cell research for correlating surface and internal features.

Configuring Confocal and AFM

Combined AFM and confocal microscopy requires a special microscope configuration. The JPK Nanowizard II (see fig. 1) designed for simultaneous confocal and AFM imaging can be installed on an inverted microscope. There is bottom-up access to the sample for light microscopy techniques (incl. confocal) and top-down access for AFM imaging. The inverted microscope must be fitted with a high stability stage. To further improve the configuration, JPK has designed special sample holders, such as the BioCell, that allow stable mounting of samples on a coverglass without compromising image quality. High magnification objectives (typically used in confocal microscopy) with high numerical apertures (requiring 0.17 mm coverslips) can now be combined with AFM without loss of stability.

System calibration is necessary for the precise overlay of confocal and AFM images. The JPK's alignment process is precise to 3Å in the x and y directions. However, because distortion may occur in the optical image, confocal and AFM images may not overlay accurately. This can be a problem particularly when users wish to correlate fluorescent confocal signals with small structures visualised by AFM (such as endocytic pits) on the cell surface.

As the AFM image is generated using very precise linearised piezos, it may be treated as "real-space". The cantilever of the AFM is raster-scanned over the surface to build an image but can also be moved precisely to fixed points.
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 determined automatically. A "transform" function is then calculated using both sets of 25 points, and this transformation algorithm (Direct Overlay function) is applied to the optical image as it is imported into the AFM acquisition and analysis software (fig. 2). In this way the optical image is calibrated and imported into the scanning probe microscopy environment automatically.

Once the image space of both microscopes has been cross correlated, the confocal image can be imported into AFM software to allow imaging of specific labelled areas, manipulation of specific regions of the cell, and/or precise offline overlays can be used to accurately map labelled components to their corresponding structures.

Applications

Combined Imaging of MDCK Cells

The surface of MDCK cells is covered by microvilli that can be imaged directly using AFM. Actin forming the structural base of the microvilli can be imaged using confocal microscopy (after staining with fluorescently labelled phalloidin). Previously, such images did not allow direct overlay of all actin signals at the protrusions of the cell surface due to slight shifts in both images (Poole et al, 2004). However, after calibration as described earlier, the overlay of confocal and AFM images is precise (fig. 3).

This precise overlay is not critical for cell structures such as microvilli at the apical surface of MDCK cells where they are the dominant feature. However, when cells with a very heterogeneous surface are imaged, it is extremely difficult to assign specific functions to various surface structures without specific labels. In such a case, if the overlay of the two types of image is not precise, mistakes in cross
correlation can be made.

**Combined Imaging of Clathrin Pits on Embryonic Fibroblasts**

To demonstrate Direct Overlay in a more complex system, cells were labelled with either anti-clathrin or anti-caveolin antibody, followed by a TRITC-labelled secondary antibody. Cells were then imaged using both confocal and AFM. After calibration of the confocal images, it is possible to determine which pits on the surface correspond to which specific fluorescently labelled structures (fig. 4 and fig. 5). This combined imaging allows the user to obtain an overview of how such structures relate to other structures at the cell surface.

**Conclusion**

Calibration of the confocal image with the absolute dimensions obtained by AFM, allows precise matching of surface structures acquired by AFM to specifically labelled proteins or bio-molecules imaged by confocal microscopy. Additionally, confocal imaging in combination with manipulation by AFM may allow imaging of biological processes, such as signal transduction. The combination of a Nikon confocal with the JPK AFM on a Nikon inverted microscope provides a highly flexible imaging system.

**Reference:**


**Author's background**

Kate Poole is product specialist for atomic force microscopy applications at JPK instruments in Berlin. Maarten Balzar is an application manager for Nikon Instruments Europe BV and is responsible for (biological) research applications such as TIRF, confocal, and advanced fluorescence microscopy.

Maarten joined Nikon in 1999 after completing his PhD at the Department of Pathology, Leiden University Medical Centre (LUMC), the Netherlands. His current activities are focussed on new product developments for the (confocal) microscopy product line.

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