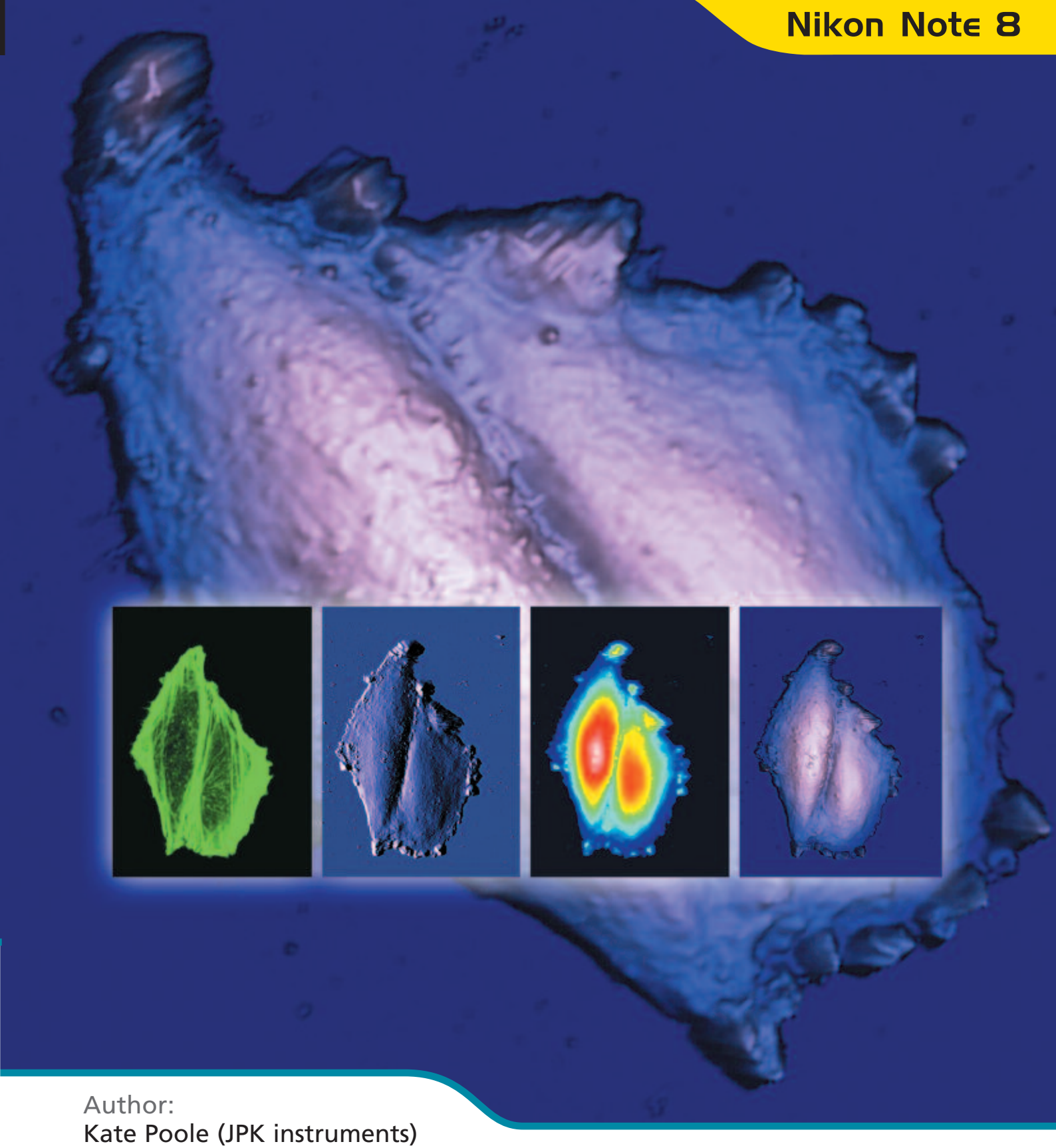

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Combining Confocal and Atomic Force Microscopy (AFM)

Abstract:

Different forms of microscopy may be applied for the visualisation of biological specimen. Confocal microscopy provides three dimensional information about the specimen by excluding out of focus light. Atomic force microscopy (AFM) 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; confocal microscopy for three dimensional imaging of e.g. fluorescent labelled cells and AFM for high resolution imaging of surface structures.

Introduction:

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

Figure 1: Configuration of the Nikon confocal and JPK AFM on an inverted microscope. Please note that the microscope stage is replaced by the AFM optimized stage for improved stability.

Technology:

Additionally, for complete integration, calibration of the system is required in order to correctly overlay images acquired with confocal and AFM. 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 may be distortion in the optical image, the images from the confocal and AFM do not accurately overlay. This is particularly a problem when the user wishes to correlate fluorescent signals (confocal) with small structures visualized by AFM (such as endocytic pits) on the cell surface. As the AFM image is generated using very precise linearized piezos, it may be treated as "real-space". The cantilever of the AFM is 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 detail, 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 AFM acquisition and analysis software. In this way the optical image is calibrated and imported into the SPM environment in an automated process.

Figure 2: Calibration of LSCM images using the Direct Overlay function.

The AFM cantilever can be imaged in reflection mode by confocal microscopy. Here, five individual images (the first line of a grid of 25 points) are superimposed Figure 2(A), and then superimposed over a corresponding confocal image of FITC-phalloidin labelled mouse embryonic fibroblasts (B). As the precise position of the cantilever in the AFM space is known, the corresponding tip position in the confocal images can be calculated and a transform function emplyed to allow precise overlays of the two 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 microvilli that can be directly imaged using AFM and the actin that forms the structural basis of the microvilli can be imaged with the confocal (after staining with fluorescently labelled phalloidin). Previously, comparison of such images did not lead to direct overlay of all of the actin signals at the protrusions of the cell surface, due to the slight shifts of both images (Poole et al, 2004). However, after calibration of the confocal image and a transformation process, the overlay of confocal and AFM images is precise (Figure 3).

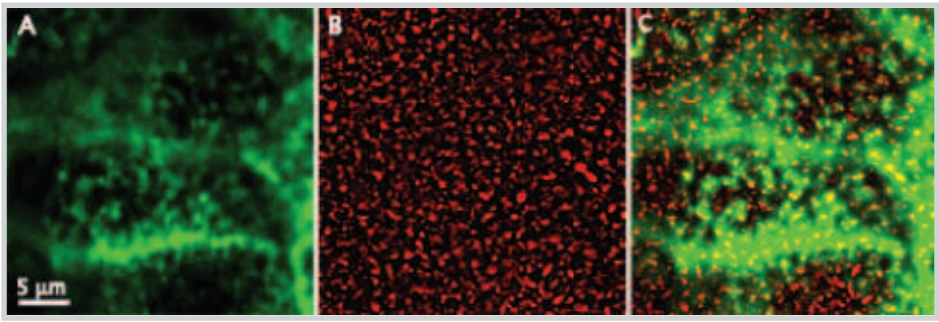


Figure 3: Combined imaging of MDCK cells.

Application:

MDCK cells, fixed with paraformaldehyde (4% in PBS, 20 min) were labelled with FITC-phalloidin and then imaged with AFM and confocal microscopy. The surface of the MDCK monolayer was imaged with confocal (A) and AFM (B). In the confocal image (A) the apparent 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, however, as the AFM image contains the structural information of the whole surface, not just specific components, the curvature of the cells is also apparent. In (C) the two images are overlayed, however in this case the AFM image has been processed to remove the curvature of the cell and just contain information about the surface protrusions (microvilli). In (C) the AFM image has been false-coloured red.

This precise overlay is not critical for cell structures such as the microvilli at the apical surface of MDCK cells, where the microvilli are the dominant structure on the cell surface. 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 demonstate the potential of applying Direct Overlay for combining confocal and AFM images, cells were labelled with either anti-caveolin or anti-clathrin antibody, followed by a TRITC-labelled secondary antibody. Cells were then imaged using both confocal and AFM. After calibration of the confocal images, one can determine which pits on the surface correspond to which specific (fluorescently labelled) structures (Figure 4 and Figure 5). This combined imaging allows the user to obtain an overview of how such structures relate to other structures at the cell surface.

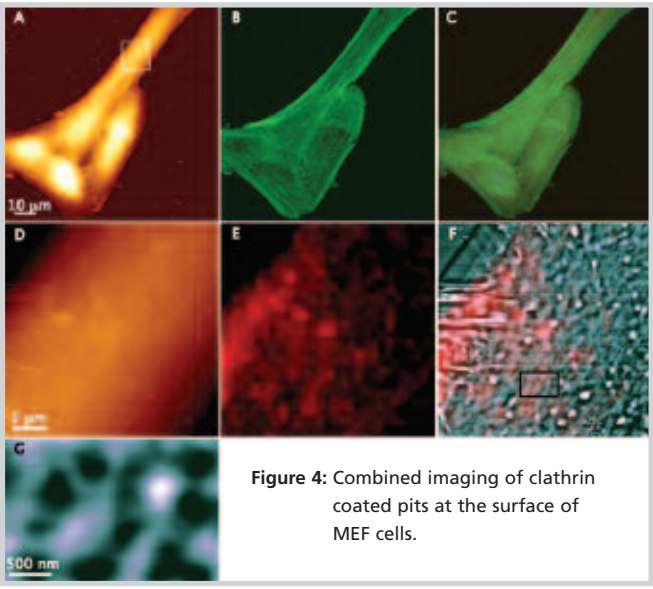


Figure 4: Combined imaging of clathrin coated pits at the surface of MEF cells.

Mouse embryonic fibroblasts 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 Direct Overlay function was used to calibrate the confocal image against the AFM image. An overview of the cells as AFM topography Figure 4(A), labelled actin (B), and an overlay of the two (C) is provided. A higher resolution AFM topography (D) was acquired in addition to a confocal 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 is presented in (G).

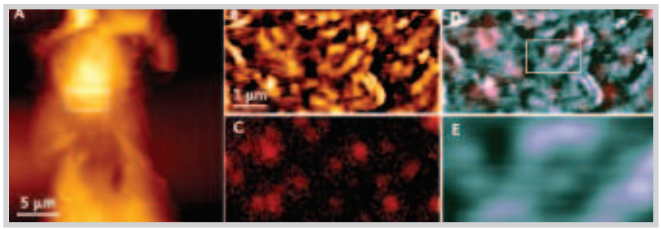


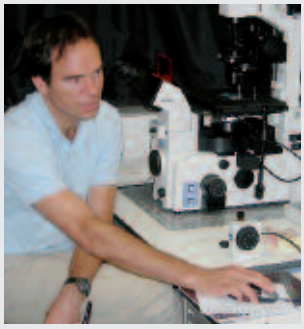
Figure 5: Combined imaging of caveolae at the surface of MEF cells.

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. An overview AFM topography image of the cell was acquired Figure 5(A) and the confocal image space calibrated to allow comparison of the AFM and confocal data. A higher resolution AFM image was acquired (B) and compared with the corresponding confocal image of surface associated caveolae (C). An overlay is presented in (D).

Conclusion:

The combination of confocal and AFM can further extend the applications of both techniques. Calibration of the confocal image with the absolute dimensions obtained by AFM, allows us to very precisely match surface structures (such as cavaeoli and clathrin-coated pits) acquired by AFM to specifically labelled proteins or bio-molecules imaged by confocal microscopy. Additionally, confocal imaging in combination with manipulation by using 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 an optimally flexible imaging system.

Kate Poole



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Authors' 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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References:

Poole K, Meder D, Simons K, Muller D. The effect of raft lipid depletion on microvilli formation in MDCK cells, visualized by atomic force microscopy. FEBS Lett. (2004) 565(1-3):53-8.

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- AFM complements confocal microscopy to provide greater visualisation of a sample
- AFM provides structural information about the surface of a sample
- Confocal microscopy provides 3D information of the sample by excluding out of focus light
- Calibration of the system is required to enable direct image overlay

For more information on Nikon confocal imaging systems go to:

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