Multidimensional microscopy on living cells

Introduction
An ongoing trend in scientific research is the development of techniques that can address multiple components of a system at a single time. DNA microarray analysis provides a snapshot of the set of genes expressed within a cell at a given time [1]. Multidimensional protein identification technology is being developed to determine the contents of heterogeneous samples of proteins [2]. Force spectroscopy analysis can address the complexity of cell adhesion by distinguishing between the contribution of individual elements to the binding process [3]. Advancements in microscopy techniques and sample preparations are extending this type of multi-component approach to the imaging of cells.

The information generated about cellular structure and function using various microscopy techniques will differ depending on how contrast is generated. By combining techniques, information about different properties of the one sample can be monitored simultaneously. The JPK Nanowizard® atomic force microscope is designed to be installed on an inverted light microscope, enabling simultaneous imaging of a sample by atomic force microscopy (AFM) and various optical microscopy techniques from phase contrast [4] to epi-fluorescence [5], total internal reflection fluorescence (TIRF) microscopy and laser scanning confocal microscopy (CLSM) [6] to name a few. This combination of AFM and other microscopy methods has, thus far, been mainly utilised for structure-function studies. However, the JPK Nanowizard® can be used to obtain superior images of living cells, simultaneously with additional microscopic techniques (figure 1). This makes the JPK Nanowizard® a powerful and versatile tool for multidimensional microscopy studies.

Multidimensional microscopy refers to the generation of multiple images of different properties of a sample over time. As an example, multidimensional microscopy has been used to great effect in the study of the composition and dynamics of focal adhesion structures [7].

Fig. 1 Successive error signal images of a living cell at progressively smaller scan sizes. All images taken in low force, constant contact mode.
In this case, multiple fluorescent channels were used to characterise the components of focal adhesions and their distribution in relation to each other and to actin within the cell. Advances in available fluorophores and sample preparation techniques mean that multiple channel fluorescence can be conducted on both living and fixed cells.

This simultaneous imaging of multiple fluorescent channels generates information about the localisation of multiple components in the cell, however such an approach could be further extended by combining AFM with fluorescence microscopy and phase contrast microscopy. In such a manner, not only can one investigate the location of particular proteins but also the dynamics of surface structures and subsurface cytoskeleton in addition to the overall cell morphology, simultaneously. Here we have imaged REF52 cells, expressing paxillin-GFP, with phase contrast, epi-fluorescence and AFM.

**Experimental setup**

In order to acquire multiple microscopy images on living cells, the cells were grown on coverslips, which were then mounted into the JPK Biocell™ for imaging. The Biocell™ (figure 2) is designed to optimise acquisition of simultaneous optical and AFM images, while maintaining an environment reflective of physiological conditions.

![Fig. 2 The JPK Biocell™.](image)

The cells were imaged at 37°C in media containing HEPES. The JPK Nanowizard® was installed on a Zeiss Axiovert 200M inverted light microscope. Cells were imaged in low force constant contact mode, with a flexible, unsharpened cantilever. At the beginning of each scan a phase contrast and fluorescence images were obtained.

**Living cell images**

As can be seen above, in figure 1, contact mode AFM imaging can be used to obtain overview images of a whole cell, or can be operated using small scan sizes to resolve surface structures beyond the resolution of conventional light microscopy. While the resolution and signal to noise ration of AFM are major advantages of this imaging technique the information generated in images of larger scan sizes can also be useful, and complementary to other imaging techniques. AFM imaging is a mechanical process, based on the interaction of the very flexible AFM probe with the surface. Consequently, the information generated is structural and mechanical. Additionally, AFM is a surface technique, so AFM images are "focussed" on the surface of the cell and the mechanical structures, such as the actin cytoskeleton, that underlie it.

The combination of imaging the surface using AFM and fluorescence imaging of focal adhesions allows the investigator to compare the dynamics of focal adhesion structures at the base of the cell with the actin structures at the surface of the cell. The additional phase contrast images give an overall impression cell morphology and can help to determine whether structures observed at the cell surface are due to vesicles that are clearly visualised in the phase contrast image. As such, images are generated of dynamics happening at the base, in the body and at the surface of the cell.

In figure 3 a series of images is presented – each row shows phase contrast, epifluorescence and contact mode error signal images taken at 15 minute intervals. The cells were dense on the coverslip, to inhibit cell motility, such that smaller scale movement could be investigated. Larger changes in cell structure can be seen in the phase contrast images (circled in A,D,G). Vesicles within the cell can be seen to have moved positions. A comparison of the three epi-fluorescent images of GFP-labelled paxillin show little change between each image.
Fig. 3 Multiple channel time-lapse images of REF52 fibroblast cells. Living fibroblast cells were imaged in phase contrast (A,D,G) epi-fluorescence (B,E,H) and contact mode with the atomic force microscope (C,F,I) at t = 0 (A,B,C), t = 15 (D,E,F) and t = 30 (G,H,I).
The white arrows in the fluorescent images (B,E,H) highlight focal adhesion structures that do not seem to change over the course of the scans. Interestingly, when compared to the actin structure in the AFM images, the focal adhesions do not seem to change, whereas there is a distinct alignment of the actin fibres along the body of the cell. In the original image a network is visible in the sub-membranous cytoskeleton. With each successive image the cytoskeletal fibres become more aligned (black arrows) and the cell body higher. In addition, there are many small, flexible, highly dynamic protrusions on the flat areas of the cell (circled in black). From these images the subsurface-cytoskeleton seems to be considerably more dynamic over this time scale than the focal adhesions at the interface between the cell and the support.

While here only one fluorophore is used, obviously the fluorescence microscopy could be extended to include multiple fluorescence channels. The laser in the JPK Nanowizard® is of a wavelength beyond the visible spectrum such that red channel fluorescence is not disrupted by the presence of the AFM laser.

At smaller scan sizes, the degree of dynamism at the cell surface can be more clearly seen. Two successive images of the cell surface are presented in figure 4 as three dimensional projections of topographic data. The two images were taken successively, 15 minutes apart. The large cytoskeletal structures at the top of both images are similar, however much of the rest of the structure has changed between images. A flexible ridge (black arrow) seen in the first image has disappeared in the second. Finer sub-surface structure can also be seen to have changes considerable between the two images.

**Conclusion**

The AFM can be used to image living cells at unprecedented resolution and on a time scale that is suitable for addressing a number of biological processes. The design of the JPK Nanowizard® enables multidimensional microscopy combining AFM with optical imaging techniques.

**Fig. 4** Successive topographic images of the same region of the cell surface. In both cases the scan size is 5 x 5 µm and the z range 0-200 nm.

Such a setup can be used to investigate, simultaneously, events occurring in different regions of the cell. Alternatively, information gleaned from the different contrast methods could be used to generate information on structure-function relationships [8]. While AFM can generate interesting and unique information about cells as a stand-alone imaging technique, the integration of the JPK Nanowizard® into a fully functional, inverted light microscope extends the potential for AFM for cell imaging.

**References:**


