Combined fluorescence and AFM imaging of cells

**Introduction**

Combining optical and AFM imaging of cells opens up many possibilities for correlating structural information about the cell surface with functional labelling of certain components [1-3]. In this report, examples are shown of AFM combined with optical microscopy for phase contrast, DIC and epi-fluorescence using a JPK NanoWizard® AFM mounted on a Zeiss Axiovert 200 inverted optical microscope.

As a particular application, cells were treated with hydrogen peroxide to induce apoptotic blebbing. The combination of optical and AFM imaging allows better interpretation of the links between the changes in the membrane surface as vesicles form, and the structure of the actin cytoskeleton beneath them.

**Mouse melanoma cells labelled with YFP**

In the first example, a mouse melanoma secondary cell line (B16) was used. In this cell line, the actin was labelled with yellow-fluorescent protein (YFP). The cells were a kind gift from Dr Clemens Franz (Cellular Machines Group, Biotechnologisches Zentrum, TU Dresden).

The cells in this cell line are particularly mobile and do not adhere well as they grow, so they were fixed before imaging. The cells were treated with 2% glutaraldehyde for only 45 seconds, to minimise the fluorescence contribution from the glutaraldehyde, and the cells were then fixed for 20 minutes in 4% paraformaldehyde.

Optical images of a B16 cell are shown in Figure 1. The upper panel is a phase contrast image obtained using a 20x water immersion objective. The lower panel shows the YFP fluorescence of a region near the cell edge and was obtained using a 63x oil immersion lens (NA 1.2). The YFP labels the actin monomers, so there is some contribution to the fluorescent signal from the cytoplasm as well as from actin filaments.

AFM imaging of the cells was carried out in Dulbecco’s PBS solution. For AFM imaging, intermittent contact mode was used, with triangular silicon nitride cantilevers that had a spring constant of around 0.3 N/m. In intermittent contact mode, the AFM sensor oscillates over the surface of the sample, and the amplitude of the oscillation is used to control the scanner. This mode gives both topography images of the cell surface and images from the amplitude signal, which highlight the finer details of the cell surface.
Figure 2 shows a comparison of optical and AFM images of the B16 mouse melanoma cell edge. The upper panel is a YFP fluorescence image, as in Figure 1, and the lower panel is a montage of two AFM amplitude signal images of the same region. Arrowheads – examples of actin filaments seen in both panels. Ring – example of rounded features on the cell surface, seen only in the AFM images. Scale bar 2 μm in both panels.

Figure 2 reveals that the AFM images show details of the cell surface, however, that do not appear in the fluorescence image, such as the group of rounded features marked with a white ring in Figure 2. The left hand side of the AFM panel particularly shows surface structure that is quite different from the pattern of the underlying actin cytoskeleton.

**Fibroblasts**

Mouse dermal fibroblasts were grown on glass coverslips. The cells were fixed with 2% glutaraldehyde for 45 seconds and then 4% paraformaldehyde for 20 minutes, as for the mouse melanoma cells. The actin filaments were fluorescently labelled by incubating the cells overnight at 4°C with phalloidin-FITC, as per manufacturers instructions. The cells were imaged in Dulbecco’s PBS solution. Fluorescence images were collected as described above, using a 63x oil immersion lens and FITC filter set.

Figure 3 shows an optical image of an isolated fibroblast. The image shows the fluorescence from the labelled actin filaments. The actin is more strongly labelled than for the YFP in Figure 1, and the cytoskeleton of the fibroblast is more developed than for the melanoma cell, so the actin cables stand out clearly in the fluorescence image.
The images in Figure 4 all show the region of the cell edge that is marked with a box in Figure 3. The optical fluorescence image (A) has been rotated to facilitate comparison with the AFM images (B, montage of two amplitude signal images and C, montage of two topography images) of the same area.

The fine protrusions at the cell boundary (marked with white arrowheads) are faintly visible in the fluorescence image (A), but can be clearly resolved in the AFM images (B,C). Other isolated features at the cell surface are only visible in the AFM images (for example, the large feature marked with a black arrowhead).

The AFM and optical images here give complementary information about both the surface of the cell and submembranous structures. The AFM images give both real 3-D information about the cell shape (from the topography signal) and an impression of the fine structures at the cell surface (from the amplitude signal). The combination of optical and structural information allows the identification of the cellular components underlying particular cell membrane structures.

**Treated fibroblasts**

To demonstrate an application for the different information that is available from this combination of microscopy techniques, a brief study of the apoptotic effects of hydrogen peroxide (H₂O₂) on the fibroblast cells is presented here.

The mouse dermal fibroblasts were treated with low concentrations of hydrogen peroxide to initiate a cell death cascade [4]. The treated cells were incubated with 100 μM hydrogen peroxide for either 30 minutes or one hour, before being fixed and the filamentous actin fluorescently labelled as described above for the standard imaging.

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Fig. 4 Comparison of optical and AFM images of a fibroblast using JPK NanoWizard®. A: fluorescently labelled actin (phalloidin-FITC). B: montage of two AFM amplitude signal images. C: montage of two AFM topography images. Scale bar 2 μm in all, height range of AFM image is 1.8 μm.
**Fig. 5** Combination of optical and AFM images of fibroblasts treated with hydrogen peroxide. Image series A-C shows control cells (no H$_2$O$_2$ treatment), D-F shows cells that have been treated with 100 μM H$_2$O$_2$ for 30 minutes before fixation, and G-I shows cells that have been treated for one hour before fixation. Phase contrast images (A,D,G) and epi-fluorescence from FITC-phalloidin labelled actin filaments (B,E,H) show the same scan area for each case. AFM topography images (C,F,I) are 20 μm x 20 μm scans in each case, for the region marked with a box in the preceding optical images. The total height scale for C and F is 2.3 μm in both cases, and the total height scale in I is 9 μm. AFM imaging was carried out in contact mode using unsharpened DNP cantilevers with spring constant 0.06 N/m.
An overview of the results is given in Figure 5 for control cells (no treatment, image series A-C) and cells treated with hydrogen peroxide for 15 minutes (image series D-F) or 30 minutes (G-I). The images along each row show different imaging techniques for the same cell sample. The phase contrast (A, D, G) and epi-fluorescence (B, E, H) images show the same sample area. The region for the AFM topography images (C, F, I) is marked in the optical images with a box. The scan area for the AFM images was 20 μm x 20 μm in each case.

Prominent actin cables can be seen in all three images (A-C) for the untreated control cells. In addition, vesicles can be seen in the phase contrast image, which correlate with protrusions on the cell surface in the AFM topography images (for example, the one circled in C).

After treatment for 30 minutes with the 100 μM H₂O₂, the actin filaments have started to retract from the edges of the cells. The fluorescence image in E shows faint staining at the periphery of the cell, but the details can be more clearly seen in the corresponding AFM image (F). A region of relatively flat membrane is left at the periphery (marked with an arrow in F) that has a fine mesh structure supporting it, but the prominent actin fibers no longer extend to the edge of the cell.

The cells treated with H₂O₂ for one hour show a dramatic change in cell morphology, and a complete rearrangement of the actin cytoskeleton. The cells have retracted and become rounder, the actin cables are reduced and blebs have started to form on the surface.

The blebs form large three-dimensional structures – the total height scale for the AFM images in both C and F is 2.3 μm, while the height range for the last image (I) is 9 μm. The AFM measurements allow a quantitative measure of the change in cell height as it rounds up, and the surface blebs could be analysed for size, shape, number or volume. Comparison with the fluorescence image in H shows the actin cytoskeleton underlying the bleb structures.

**Summary**

This report has introduced some of the possibilities of combining different optical imaging techniques with AFM for studying cell morphology and responses. Combining techniques widens the range of information that can be gathered about cell structure and function. Specific molecules on the cell surface or in the underlying parts of the cell can be labelled and imaged to explore their relationship to the morphology of the cell surface and particular structures. These investigations can be done under physiological conditions, or even on living cells, and present new opportunities to analyse the relationship between physical structures and cellular function.

**Literature**


