Combining fluorescence and atomic force microscopy (AFM) to image individual microtubules in vitro

Atomic force microscopy (AFM) can be used in buffer, to image samples at a resolution superior to light-based microscopy techniques, and, unlike electron microscopy (EM), under physiological conditions [1]. However, the heterogeneity of most biological samples requires that, to distinguish specific components within a system, a label must be used. The lack of such labelling techniques for AFM makes it difficult to characterize structures in complex biological systems using AFM alone. An imaging setup capable of combining multiple imaging techniques, such as the JPK NanoWizard® AFM mounted on an inverted light microscope enables the correlation of images generated on a single sample by multiple microscopy techniques. While this set up is clearly essential for the characterization of cell surface structures [2,3], it is also useful for the study of individual cellular components used in in vitro assays.

Here, we show that the NanoWizard® can collect simultaneously both high quality fluorescence and AFM images of microtubules polymerized in vitro. The in vitro studies of microtubule dynamics have traditionally utilized fluorescent imaging, the use of AFM enables increased level of characterization of the microtubule structure, enabling the scope of such in vitro studies to be expanded.

Microtubules form dynamically within cells from a cytoplasmic pool of a/b tubulin dimers. Structurally, microtubules are linear polymers of the globular tubulin heterodimers, known as protofilaments (Figure 1). Protofilaments, consisting of alpha/beta tubulin heterodimers, laterally associate to form the microtubule. Polymerization and depolymerization can occur at both ends with both processes being faster at the plus end of the microtubule.

As a part of the cellular cytoskeleton, microtubules play a significant role in cell structure. Additionally, microtubules support the directed motion of chromosomes, vesicles and organelles. This second function is reliant on the polarity of the microtubules. While many studies have looked at microtubule polymerization in situ, microtubules can also be polymerized and studied in vitro, allowing investigation of the interaction of motor proteins responsible for this directed transport of organelles along the microtubule length.

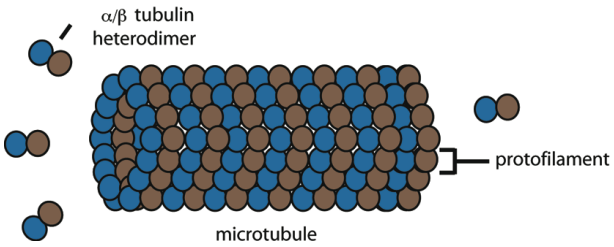

Fig. 1 Microtubule structure. Microtubules have a filamentous structure, 25 nm in diameter. Protofilaments, consisting of alpha/beta tubulin heterodimers, laterally associate to form the microtubule. Polymerization and depolymerization can occur at both ends with both processes being faster at the plus end of the microtubule.

For the following experiments tubulin, purified from pig brain, was polymerized in the presence of TRITC labeled tubulin in a 3:1 ratio. The microtubules were stabilized with a slowly hydrolysable GTP (GMPCPP) to inhibit depolymerization. The stabilized microtubules were then deposited on a silanized glass coverslip and imaged in the buffer most commonly used in microtubule assays (BRB80: 80 mM PIPES pH 6.8, 1 mM MgCl2, 1 mM EGTA) using the JPK coverslip holder. Fluorescent images were obtained using a 63 x 1.25 NA objective. To generate the AFM images microtubules were imaged using intermittent contact mode with a sharpened, triangular silicon nitride cantilever, spring constant 0.32 N/m.

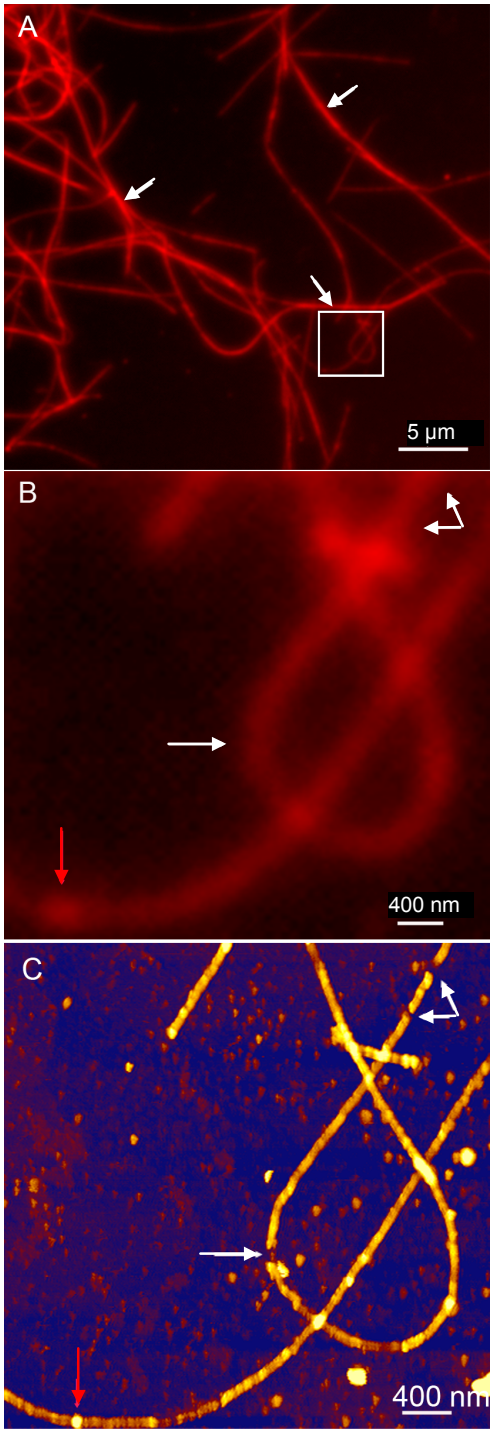

Fig. 2 Fluorescent (A,B) and AFM (C) images of microtubules deposited on silanized glass, imaged in buffer BRB80. (B) and (C) both show the region highlighted in (A).

Microtubules are clearly visible in the fluorescent image (Figure 2A), the brighter lengths highlight where the microtubules have started to bundle (white arrows). Some of the microtubules have a slightly speckled appearance, which results from using a mixture of labelled and non-labelled tubulin. A region was selected for imaging using AFM in which the microtubules did not appear bundled (square region marked in white). An electronic zoom of the relevant area of the light micrograph is presented in Figure 2B. The AFM image simultaneously acquired from the same sample area clearly shows more of the structural characteristics of the microtubules than the image obtained using epi-fluorescence. Breaks in the microtubule are clear in the AFM image that are not visible in the epi-fluorescent image (Figure 2B and C, white arrows). Additionally, in some case the brighter fluorescent spots correspond to large structures on the microtubules (Figure 2B and C, red arrows).

The use of fluorescence microscopy to image biological components cannot generate structural information at a resolution below 250 nm. As a consequence, the surface structure of such biological components cannot be characterized. While images can be obtained showing the interaction of fluorescently labelled components with the microtubules, the effect of the binding of other compounds such as salts to the surface is beyond the scope of light microscopy. Microtubules have a high affinity for salt ions, and are also sensitive to the salt composition of their environment. Taxol-stabilized microtubules were allowed to adhere to silanized glass and dried.

The surface of untreated microtubules appears smooth (Figure 3A). The surface of the microtubules changes after the addition of a salt-containing buffer. In Figure 3B, the microtubules have been washed with the buffer and then ultrapure water before imaging, resulting in globular appearance. Such a change in surface structure is exacerbated when microtubules are washed with salt-containing buffer and dried without the ultra-pure water washing step (Figure 3C).

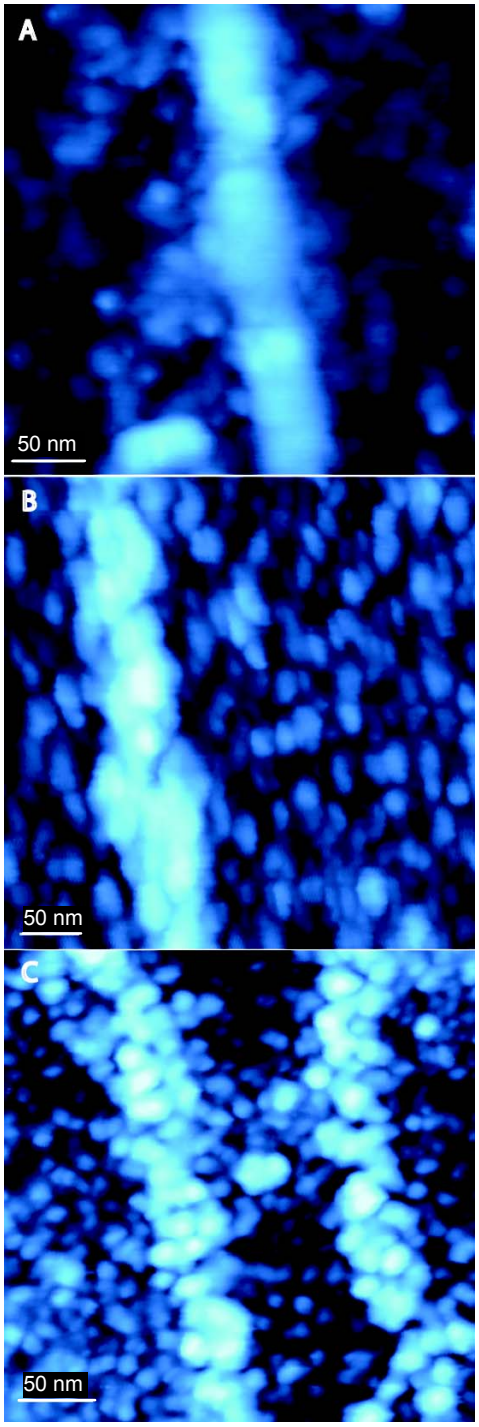

Fig. 3 AFM images of taxol-stabilized microtubules in air. Microtubules were untreated (A), treated with salt buffer (B), or treated with salt buffer and then rinsed (C).

As salt concentration also affects the activity of microtubule associated motor proteins, the local concentration of salts at the surface of the microtubule may also effect the in vitro analysis of motor protein activity.

A three dimensional representation of Figure 3A is presented in Figure 4. In this image, striations along the microtubule are visible. These structures likely represent the protofilaments within the microtubule.

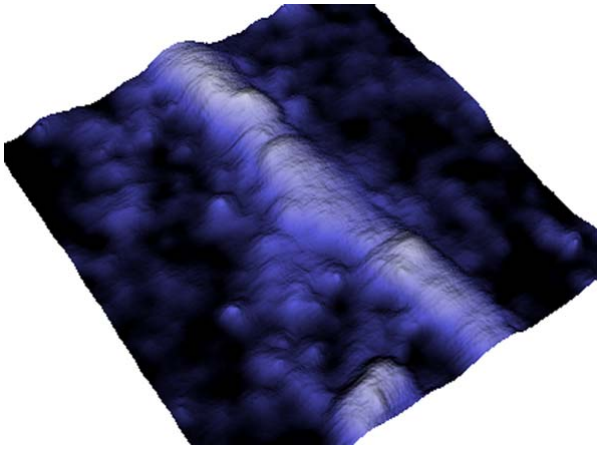

Fig. 4 Three-dimensional representation of the microtubule image shown in Figure 3A.

The polymerization of microtubules is characterized by dynamic instability, in that depolymerizing microtubules coexist side by side with polymerizing microtubules. In in vitro studies it has been found that the presence or absence of various microtubule associated proteins can alter the rate of either polymerization or depolymerization, suggesting a role for such proteins in the control of microtubule dynamics during various stages of the cell cycle (see reference [4] for a review). Microtubule dynamics and the directional movement of motor proteins along microtubules constitute a fascinating system that can be studied effectively in vitro.

Traditionally, light microscopy has been used to investigate the interaction and movement of various motor proteins along microtubules and the interaction of microtubule stabilizers and destabilizers. However, these experiments can produce variable results, particularly in the case of proteins that bind ends of microtubules, such as the microtubule destabilizer XKCM1. Similar samples will show protein binding along the microtubule, not just the ends. Here we have shown that there are breaks in the microtubules visible only when the sample is imaged using AFM. Additionally, the surface structure of the microtubules, as imaged with AFM, is dependent on salt concentration. The activity of certain microtubule associated motor proteins is influenced by ionic strength and salt composition of the buffering solution. It may be that variations in microtubule surface-associated ions are responsible for variability of results between experiments.

The combination of the high spatial resolution of AFM with light microscopy can be used to better investigate this in vitro system. Microtubule structure could be characterized using AFM, and subsequent microtubule associated protein dynamics with epi-fluorescence, confocal or total internal reflection fluorescence microscopy. Additionally, the ability to image a single sample using both epi-fluorescence and AFM would enable structural characterization with the AFM of microtubule-bound proteins located within the sample using epi-fluorescence. There is also the possibility that the breakdown-products after treatment with microtubule destabilizers could be imaged using AFM, providing additional information about the mode of action of the destabilizing protein.

The study of microtubules in vitro has hitherto relied on light microscopic techniques. While much information about the dynamics and function of microtubules has been elucidated by such experiments, high resolution structural information has not be obtained. The NanoWizard® AFM mounted on a inverted light microscope extends the possible scope of such in vitro studies without limitations of the optics. Techniques like CLSM, FRET, TIRF, IRM even with the use of the condenser techniques like DIC or phase contrast can be used with the AFM at the same time. This makes the NanoWizard® AFM to a real BIO-AFM.

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Literature

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