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## Combined Fluorescence and Atomic Force Microscopy for Cytoskeleton Morphology

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### INTRODUCTION

Fluorescence microscopy has become an indispensable tool in cell biology because it allows specific proteins to be visualized. Atomic force microscopy (AFM) is also becoming extensively used in the life sciences, but its development has largely followed an independent path and is used for somewhat different, but often complementary, purposes. Both methods can be applied to hydrated biological samples. Fluorescence microscopy is routinely used to image and track specifically labeled biological molecules *in situ*, illuminating a wide variety of cellular processes. In contrast, AFM is used for high spatial resolution imaging but also introduces the possibility of mechanically probing and manipulating samples without the need of complicated sample preparation. In this note, we briefly

describe the application of an AFM mounted on an inverted optical microscope to the characterization of the morphology of axons from cultured chick neurons using specific fluorescent immunostaining and topographic imaging with AFM.

All of the measurements were carried out using a Veeco Bioscope II mounted on a Nikon TE 200 inverted optical microscope (see Figure 1 for experimental geometry). AFM images were taken in TappingMode™ in fluid. Fluorescence images were recorded using total internal reflection fluorescence (TIRF) microscopy excited with a 532 nm B&W TEK laser and collected with a Photometrics Cascade 512b camera through an Olympus 60x 1.45 NA oil immersion TIRF objective.

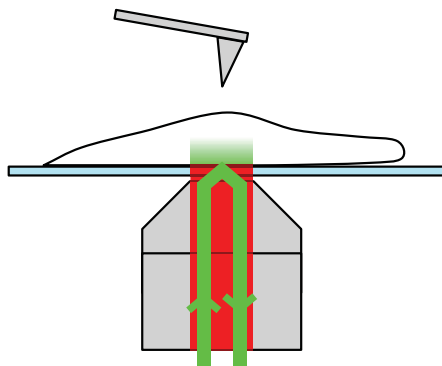


Figure 1: Experimental geometry. The microscope objective images sample fluorescence from the bottom while the AFM tip scans the top-surface topography. The illumination of the sample is restricted to within a few hundred nanometers of the cover slip surface because of the exponentially decaying intensity of the totally internally reflected laser light (shown in green). The sample fluorescence (red) is collected using the same objective that focuses the TIRF laser light onto the sample

### SAMPLE PREPARATION

Chick dorsal root ganglion (DRG) neurons were isolated and cultured on glass cover slips and lightly fixed and perforated using a buffer containing 0.15% glutaraldehyde and 0.1% Triton X. Following a thorough rinsing with PBS and quenching of the autofluorescence using sodium borohydride, the cells' microtubules were stained with Cy3-labeled monoclonal anti-beta-tubulin antibodies (Sigma). The cell-plated cover slips were then placed on the microscope sample stage and imaged in PBS without further preparation. Further details of the sample preparation can be found in reference 1.

### TIRF/AFM OF AN AXON

Figure 2a shows a TIRF image of an axon with fluorescently labeled microtubules. It is apparent from the TIRF image that microtubule bundles fill the body of the axon and splay into the growth cone, but the AFM height image of Figure 2b more clearly highlights the extent of spreading. This is because the cell extremities contain little or no tubulin and so are not prominent in fluorescence. The instrument used for these measurements is sensitive enough for single molecule fluorescence<sup>2</sup>, so it would be possible to determine conclusively whether there is labeled tubulin even in the cell extremities but the settings

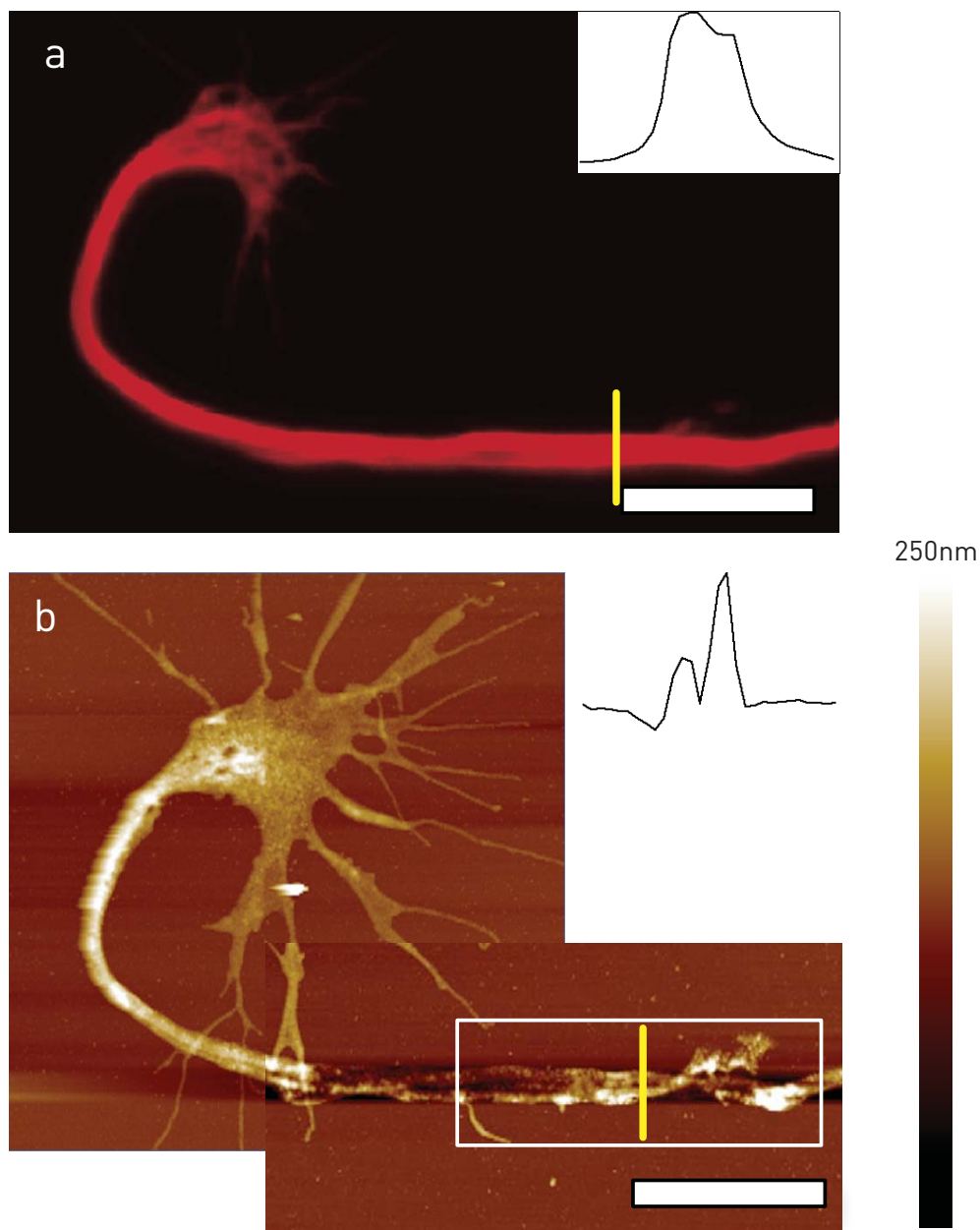


Figure 2: TIRF/AFM of an axon growth cone. a) TIRF image of the axon growth cone with fluorescently labeled microtubules. b) Composite Tapping Mode height image of the same region. The insets show the intensity and height profiles along the yellow lines in the TIRF and AFM images respectively. This clearly demonstrates the superior resolution obtained with AFM. Scale bar represents 10  $\mu\text{m}$ .

used in this case were optimized to visualize the densely labeled microtubules in the cell body instead.

The AFM height image also contains information about the microtubule arrangement in the axon that is not available from the TIRF image alone. For example, two microtubule bundles are visible running through the axon in the TIRF image, but in the region of the AFM image highlighted with a white box, their relative position becomes clear: the lower bundle is actually broken and frayed, passing over its neighbor and terminating above the main part of the axon. Without the AFM image one might falsely conclude that the bundles simply merged and continued unobstructed through the rest of the axon. The picture that emerges from the combined data set is thus more accurate and complete than that from either data set considered in isolation.

### SIMULTANEOUS IMAGING AND MICROMANIPULATION

In addition to high-resolution imaging, a hybrid TIRF/AFM allows new kinds of experiments that are impossible by either microscopy alone. Because the AFM tip can interact directly with the sample, micromechanical manipulations can be performed and simultaneously visualized using fluorescence. This approach has recently been demonstrated on fibrin fibers<sup>3</sup>, but could also be applied to cells.

As a simple demonstration, Figure 3 shows four consecutive TIRF images of an axon as the stage is scanned parallel to the sample plane (in the direction indicated by the arrow) with the AFM tip held at the position shown by the white dot. When the axon comes into contact with the tip, it begins to deform and eventually stretches to over twice its relaxed length. Repeating this kind of experiment on living cells while monitoring the lateral deflection of the cantilever would allow both the cell's mechanics and its response to different mechanical conditions to be determined—both properties that are increasingly recognized to play important roles in the function and regulation of diverse cell types<sup>4</sup>.

### CONCLUSION

The development of combined optical microscopy and AFM instrumentation, as offered by Veeco's BioScope II, has not only addressed some of the key limitations of both individual techniques, it has now opened the door for researchers to apply this type of integrated instrumentation to study fundamental challenges in cell biology and biophysics. From the direct correlation of complementary data in optical and AFM images to the *in vivo* measurement of biomolecular nanomechanical properties, these types of studies can now provide a new level of insight into a wide variety of biological systems.

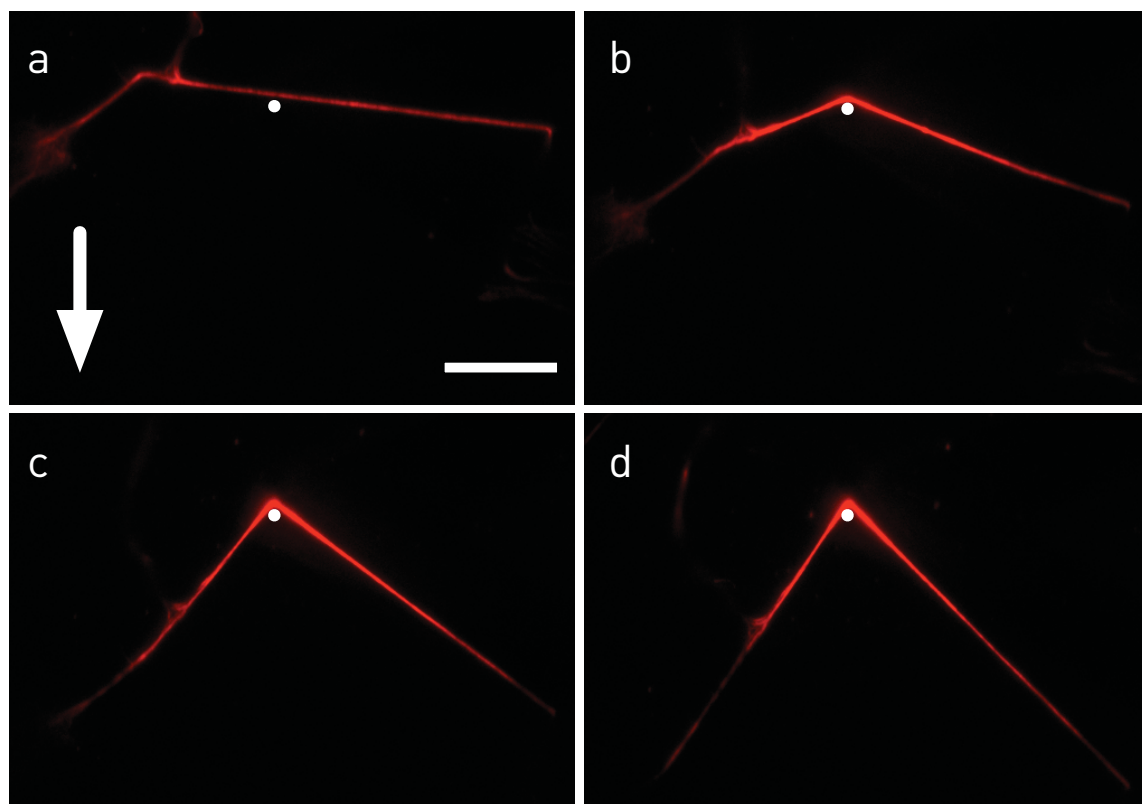


Figure 3: Manipulation of an axon with the AFM cantilever tip imaged with TIRF microscopy. The sample stage is moved in the direction of the arrow while the AFM tip is held at the position shown by the white dot. Images a–d show the progressive stretching of the axon as it is pulled against the unmoving tip. The scale bar represents 20  $\mu\text{m}$ .

## REFERENCES

1. He Y et al., 2002. Microtubule reconfiguration during axonal retraction induced by nitric oxide. *Journal of Neuroscience*. 22(14): 5982-91.
2. Rosenberg SA et al., 2005. Rotational motions of macro-molecules by single-molecule fluorescence microscopy. *Acc Chem Res*.38(7): 583-93.
3. W. Liu et al., 2006. Fibrin Fibers Have Extraordinary Extensibility and Elasticity. *Science*. 313:634 .
4. Dennis E. Discher, Paul Janmey, Yu-li Wang, 2005. Tissue Cells Feel and Respond to the Stiffness of Their Substrate. *Science*. 310:1139-1143.



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Printed in U.S.A. AN98, Rev. A0 3/07