

Simultaneous Atomic Force and Fluorescence Microscopy Using the MFP-3D™ AFM

Combining Atomic Force Microscopy (AFM) and fluorescence microscopy (FM) has long been of interest for biologists. These two types of microscopies individually provide unique information about samples.

Fluorescence microscopy is typically used to detect specific molecules. However, this microscopy technique frequently has a number of drawbacks including lower spatial resolution and complex sample preparation. Another limitation of this microscopy is that the molecule must be bound to the fluorophore, which in turn, can alter the state of the molecule.

On the other hand, AFM is a measurement made with a cantilever that is scanned across the surface. It provides three dimensional molecular resolution of biological molecules with no additional contrasting agent (such as fluorophores, dyes, etc.) or compromise to the sample. In addition, AFM is also a higher resolution measurement that is non-fleeting.

Using these microscopy techniques simultaneously provides the user with high resolution imaging on specific molecules and also opens the door to a wide variety of research experiments. In this application note, we will describe the instrumentation and setup, sample preparation, applications, and give experimental examples of the technique.

Instrumentation Requirements

Successful simultaneous AFM and FM measurements require specific configurations of both instruments. First and foremost, the AFM must not interfere with FM measurement.

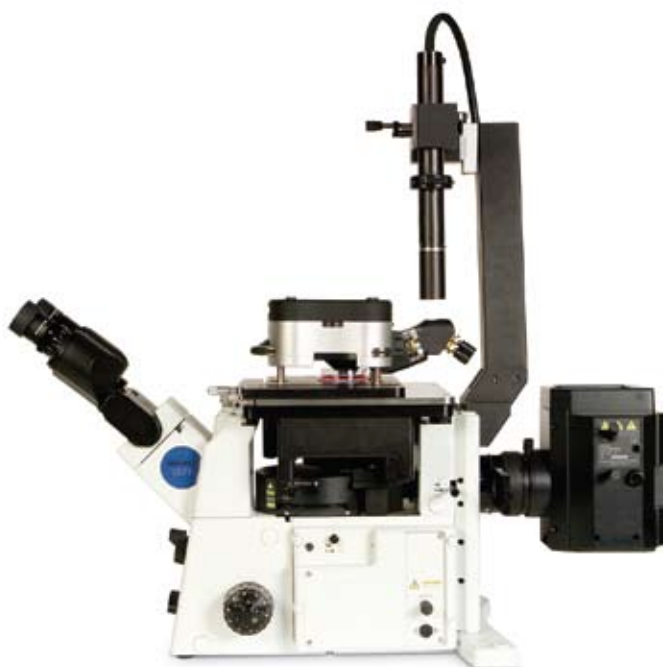


Figure 1: The MFP-3D-BIO allows simultaneous optical and AFM measurements on an inverted optical microscope, ideal for bioscience applications.

Because most commercial AFMs use red laser diodes to illuminate and detect the deflection of the cantilever, the fluorescent wavelengths cannot be observed. However, the Asylum Research MFP-3D uses an infrared superluminescent diode with reduced emission at most fluorescent wavelengths. This allows viewing of both the fluorescence and AFM image (Figure 2).

In addition, both measurements have potentially conflicting spatial constraints. The FM microscope requires that the sample be close to the inverted microscope objectives. For the AFM, the cantilever must also reside close to the sample for imaging.

The MFP-3D is designed specifically so that the sample can be accessed both by the inverted optical microscope and the scanning stage of the AFM. The MFP-3D scanner rests on the top of an inverted microscope and provides almost unlimited optical access to the sample from below, making it easy to combine with fluorescence microscopes, including epifluorescence, confocal, TIRF and FRET.

Description and Setup

The MFP-3D-BIO™ AFM is the ideal instrument to perform the simultaneous measurements. This model has a base plate and an X-Y scanner that rest on top of an inverted optical microscope (manufactured by Olympus, Zeiss or Nikon). This stage and scanner arrangement allows both simple alignment of the AFM tip with the optical axis and sub-micron positioning of the sample in two dimensions. It is also compatible with any inverted optical objectives including high numerical aperture 100X and TIRF objectives. Figure 1 shows a setup of the MFP-3D-BIO mounted on an Olympus IX71 equipped for fluorescence.

During operation, the MFP-3D AFM infrared SLD emits between 840 and 880nm. Because most fluorophores have excitation and emission wavelengths below these values, there is no interference (Figure 2).

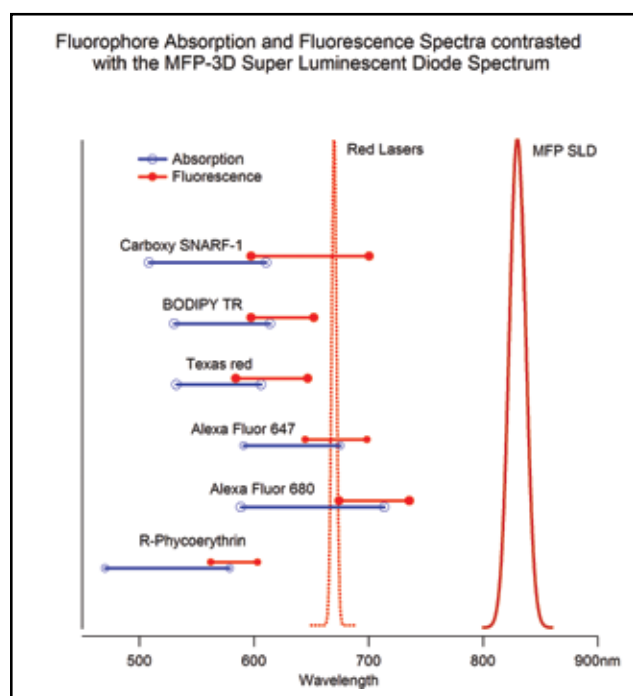


Figure 2: Fluorochrome Excitation (blue)/Emission (red) and MFP-3D SLD light wavelength.

Sample Preparation

The sample preparation for simultaneous measurements is similar to that used for AFM imaging. However, in fluorescence microscopy, light signals can be faint. Some common methods of increasing the efficiency of fluorescent emissions cannot be used in the MFP-3D because it would prevent the AFM probe from coming into contact with the sample – for example, using a hydrophobic medium and replacing water with glycerol, or adding antioxidants, to increase the efficiency of the fluorescence. These methods typically require that a coverslip be placed on top of the sample to protect and preserve it. Since such a coverslip prevents the AFM probe from interacting with the sample, this step in sample preparation must be omitted. Instead, preparation must be tailored to each sample and the amount of fluorescent dye has to be adapted to the new imaging conditions. For example, it might be necessary to increase the amount of fluorochrome that is going to bind to the specimen to obtain a larger signal for the FM measurement. Note, however, that since this may result in a loss of binding specificity of the dye, and a compromise may need to be found.

Image Examples

Figure 3 shows localization of actin filaments relative to the surface topography of single cells using a combined TIRFM/AFM. HeLa cells were grown on glass microscope coverslips, formaline-fixed, permeabilized with Triton X-100, labeled with TRITC-phalloidin, washed with acetone and dried. Imaging was carried out using a custom-built combination of the MFP-3D AFM and a TIRF microscope constructed on an Olympus IX81 microscope. Topographic and fluorescence data were collected simultaneously. Topographic data were acquired in non-contact mode using an SiN cantilever (Olympus AC160). The image shows the TIRF data channel overlaid on the rendered AFM topography.

Figure 4 shows an example of human lung fibroblasts. These images were taken with a long working distance 40X objective. The first image on the left is an optical phase contrast image. The second image in the sequence is the fluorescence image: the nucleus and actin filaments have been stained with DAPI and Alexa Fluor 488 phalloidin, respectively. The third image is the AFM deflection image of the same cells taken in contact mode. The last image

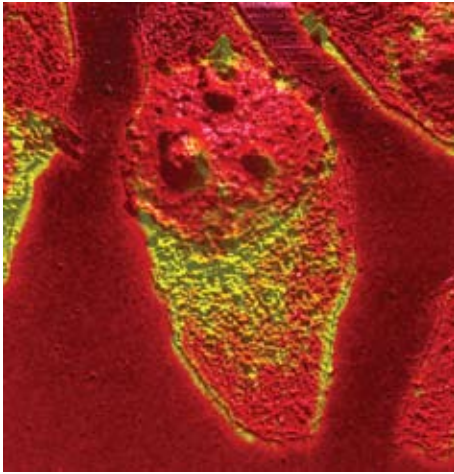


Figure 3: TIRF data overlaid on rendered AFM topography of HeLa Cells. Image courtesy Miklós S.Z. Kellermayer, Nanobiotechnology and Single-molecule Biophysics Group, University of Pécs, Hungary.

is an overlay of the AFM image with the fluorescence image. It is interesting to see fine details of the lamellipodia in the AFM image which are not visible in the fluorescence image. The MFP-3D software allows users to combine not only two different channels of the AFM image but AFM data with optical images.

Figure 5 is an optical image of bacteria (*Pseudomonas aeruginosa*) showing a Green Fluorescent Protein (GFP) and corresponding force curves. The image was taken with a 10X objective and used a Nikon FITC-HYQ cube fluorescent filter. White light from a fiber source was also used to illuminate the cantilever (visible on the left side of the image). The bacteria was first grown in a medium suspension. The suspension was then flushed over a glass slide for a few hours in order for the bacteria to grow a biofilm on the glass. The glass slide was then rinsed with the cell culture medium and imaged in the same medium. The cantilever

used in this measurement is a 60 μ m long Olympus Bio-Lever (visible as a dark shadow in the brightfield image).

Applications

There are many application examples of AFM and epifluorescence – Near Field Scanning Optical Microscope (NSOM), confocal, Total Internal Reflectance Fluorescence (TIRF), and Fluorescence Resonance Energy Transfer (FRET) methods. Researchers may be concerned with the risk of modifying the conformation of the specimen with the fluorophore, thus slightly compromising the AFM as well. However, there are labeling techniques that minimize this problem. One technique is to insert a modified and fluorescent amino acid in a protein (such as inserting methylglyoxal in collagen). Many experiments do not present this issue at all, particularly when the AFM is used to mechanically trigger a specimen and not to image it.

Epifluorescence and AFM

- Labeling nucleus, proteins or other components within a cell. Epifluorescence is used to locate the membrane optically and AFM is used to obtain a high molecular resolution image (as shown in Figure 4) or measure mechanical properties of the samples (Figure 5).
- Labeling certain lipids (like DPPC) to study the fluidity of cell membrane by correlating FM and AFM images.
- Studying the composition and distribution of lipids in vesicles through epifluorescence and using simultaneous AFM to obtain data about the mechanical and viscoelastic properties of the same vesicles.
- Studying the structure of chromophores, like carotenoids and chlorophyll, in relation to their optical behavior.

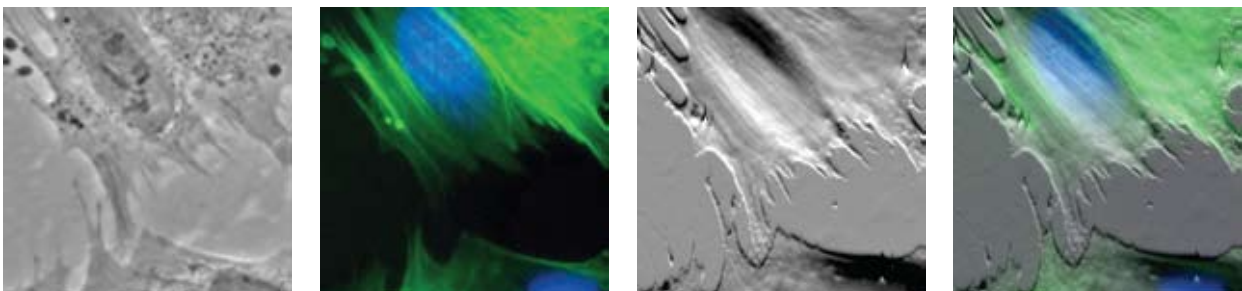


Figure 4: Left to right, optical phase contrast, fluorescence, AFM, and combined AFM/fluorescence of fixed human lung fibroblasts, 60 μ m scan.

- Mechanically excite a cell or even some receptors at the surface of a cell membrane with the AFM and using the FM to optically follow a triggered cascade of biochemical reactions, like Ca^{2+} flow inside the cell.

FRET and AFM

FRET is used for measuring proximity relations in proteins, nucleic acids, and membranes. Using a FRET method, it is possible to study the dynamic of changes in conformation like DNA, or the molecular interaction between two entities. AFM can be used simultaneously to visualize these events.

TIRF and AFM

This technique can be used to study the response of cells to localized forces applied via the cantilever on the top of the cells. More generally, it is used to manipulate cells on the nanometer level with the AFM probe used to follow the cell reaction with fluorescence (Figure 3).

Conclusion

The MFP-3D-BIO is the instrument of choice for simultaneous AFM and fluorescence measurements. Its unique design allows these measurements to be made easily and precisely. By simply altering the normal AFM sample preparation, researchers can perform these measurements and broaden their experiments to a limitless number of bioscience applications.

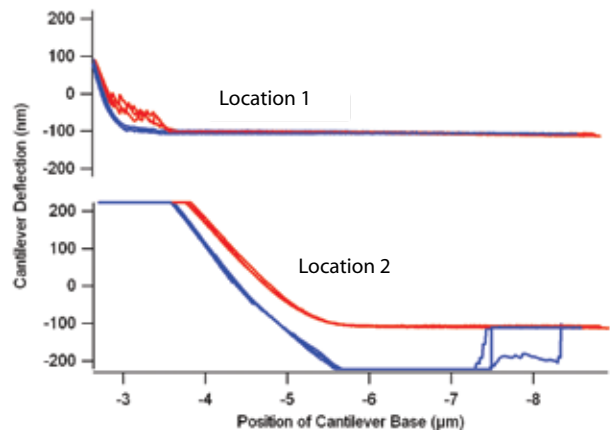
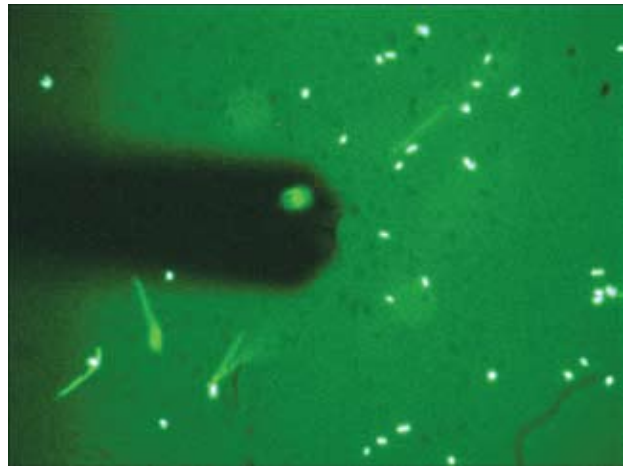


Figure 5: (Top) The optical image was used to locate the bacteria and position the cantilever. Force measurements were then made on the bacteria to assess the hardness of the cell (bottom image). The sample is *Pseudomonas aeruginosa* showing GFP.

In the force curves, the blue curve corresponds to the deflection of the cantilever when it is being withdrawn from the surface, and the red curve corresponds to the deflection as it is approaching the surface. Compare the force curve taken on the bacteria to that on a glass substrate. The most striking feature is the large adhesion (visible as a negative deflection on the blue retraction curve). Sample courtesy of Terry Beveridge, University of Guelph, Canada.