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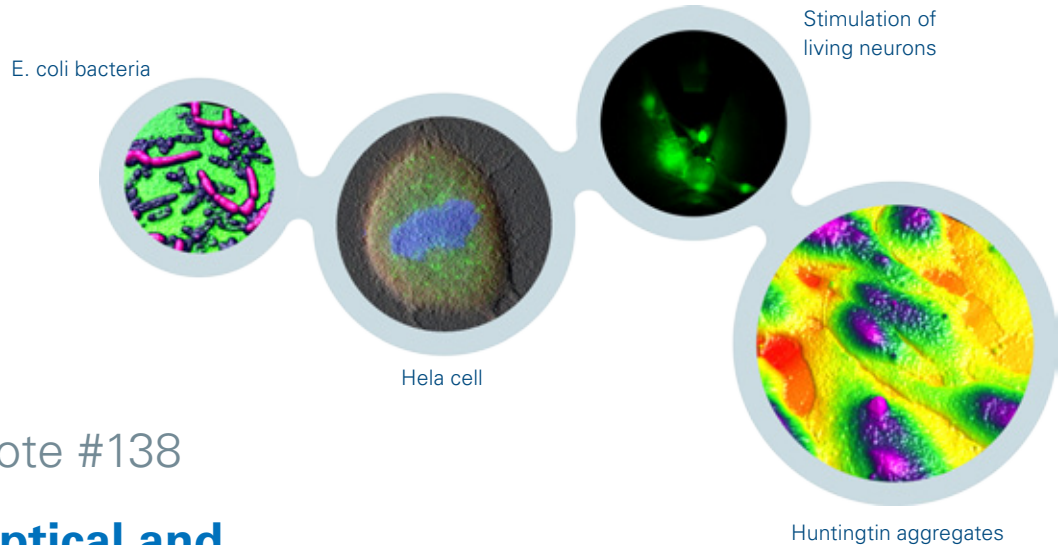
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Application Note #138

Combined Optical and Atomic Force Microscopy

Both Inverted Optical Microscopy (IOM) and Atomic Force Microscopy can be operated in a very wide range of specialty modes, and both have proven to be essential to the study of biological specimens in near-physiological environments. Where IOM, particularly fluorescence microscopy, is based on the staining and tracking of molecules inside the cells or tissues to allow direct observation of cell structure and dynamic processes, atomic force microscopy is primarily a surface investigation technique where the tip physically interacts with the sample to enable topographical, electrical, mechanical or chemical characterization. To monitor both types of information simultaneously, fully integrated optical and atomic force microscope (AFM) systems, such as the Bioscope Catalyst™, have been developed. This application note reviews a few representative applications that can be covered by this type of AFM/IOM approach.

Combining Atomic Force Microscopy and Optical Information in a Single Image

All atomic force microscopy techniques are based on the same principle: a sharp tip mounted on a reflective-backed cantilever is scanned over the sample surface. A laser

beam is focused onto the cantilever and each time the tip gets deflected by the local change in topography, the laser spot position on the photodiode is changed. This is how a height profile of the surface can be generated. Historically, contact mode atomic force microscopy was developed to get topographical information from the sample, as well as data concerning the friction forces between the tip and the surface.¹ In the early nineties, force spectroscopy was developed, which brings the tip into contact with the surface and retracts. This technique has been widely used to determine the specific interaction force between a molecule attached to the AFM tip and the complementary molecule present on the surface.² Around the same time, TappingMode™ was developed, where the AFM tip only interacts intermittently with the surface and thus negligible shear and friction forces are applied to the sample.³ Most recently, Peak Force Tapping® Mode has been developed, which is based both on the oscillation of the cantilever and the fact that a force curve is captured each time the probe contacts with the surface.⁴ It's the first technique that provides direct nanoquantification of the AFM signals at high resolution. This mode has been proven to provide very valuable results on a wide range of samples.⁵⁻⁷

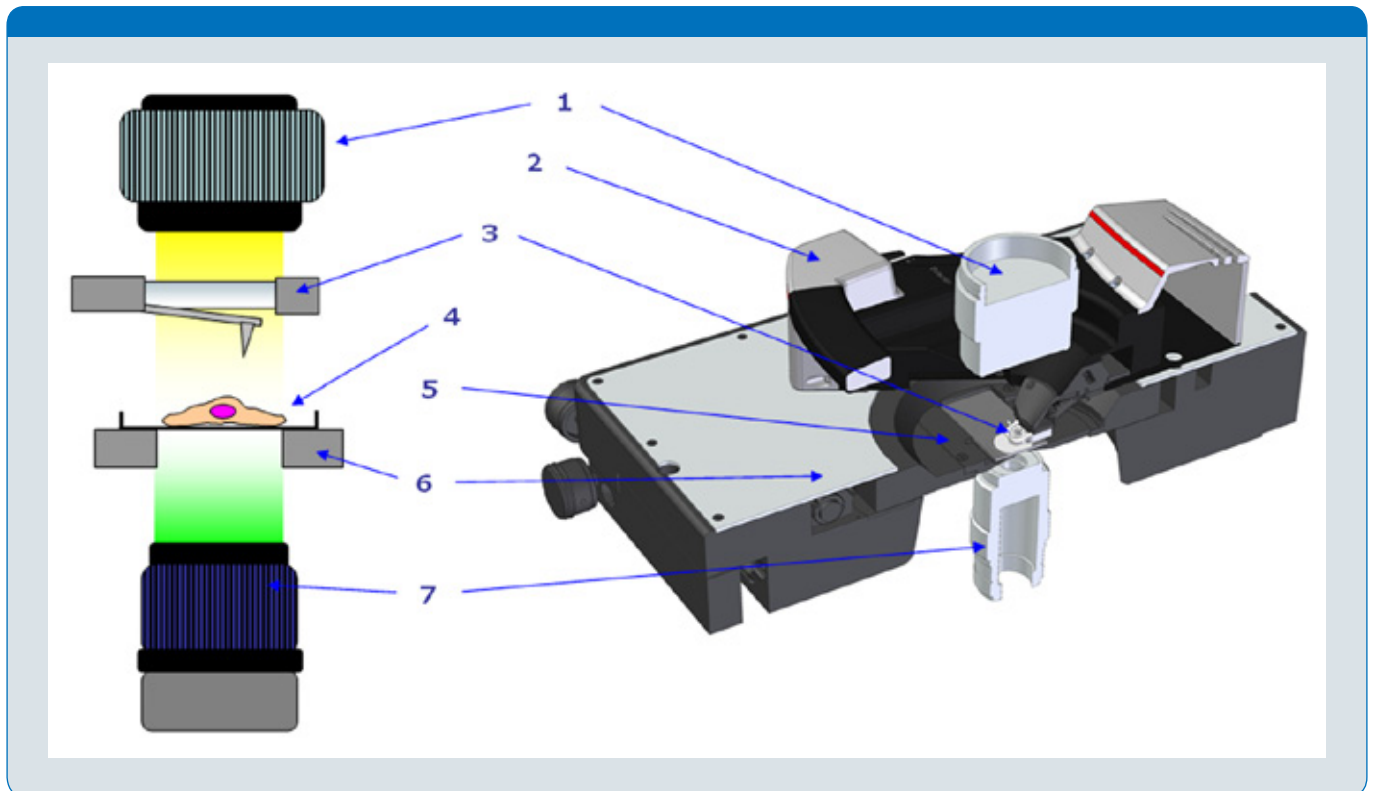


Figure 1: Required setup for IOM/AFM combination. Most of existing condensers (1) (0.5, 0.52, 0.55) should fit the AFM head (2). The cantilever holder (3) should contain a transparent part to let the incident light go through. The sample itself should lay on a substrate which enables both optical and AFM measurements (4), like glass-bottom Petri dishes. The sample insert (5) should be large enough to allow using high magnification objectives and have some heating capabilities to keep the specimens at 37°C. The x,y stage (6) should move the sample in x,y, have good feedback capabilities and allow navigating on a large optical window. The turret should be equipped with large range objectives for easier tip/sample positioning.

Any of the above-mentioned atomic force microscopy modes can be combined with most of the existing optical and fluorescence techniques, although the most commonly used setup is the combination of atomic force microscopy and epifluorescence.^{8,9} In fluorescence microscopy techniques, a sample is illuminated with a light of a specific wavelength that is absorbed by the fluorophores. As a consequence, those molecules emit a light of a longer wavelength, detected through a microscope objective. Unlike phase contrast or differential contrast microscopy techniques, which are based on the transmission or reflection of the white light, these techniques allow the labeling of any kind of organelle and other subcellular components, from nucleic acids to proteins or lipids. Figure 1 shows a schematic setup detailing how such a combination is possible. A key to success in this type of experiments is to have enough space around the sample to analyze so that the AFM head fits with any type of condenser and also allows compatibility with any type of objective. Keeping cells alive for an extended period of time is made possible by the use of features such as the

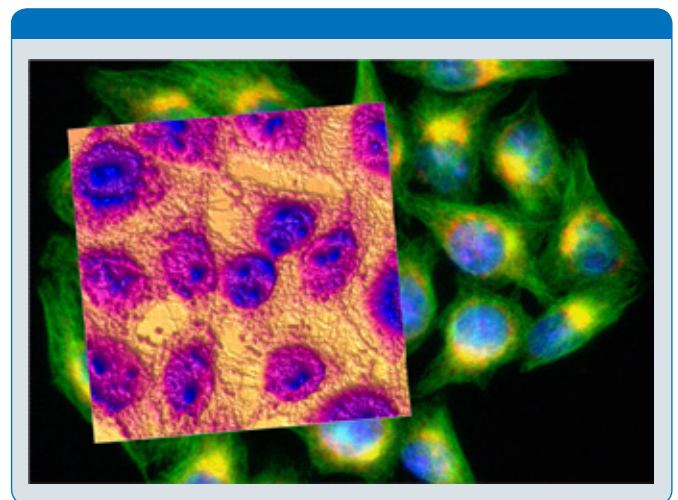


Figure 2: Atomic force microscopy/epifluorescence overlay of Lovo cells. The atomic force microscopy image (100x100µm) is a mix of topography and amplitude error channels. Automatic overlays can be achieved by making a quick calibration and registration. Then each objective will have a specific calibration file that can be loaded and re-used for further experiments. If the tip has to be changed, the user will only have to capture an image of the new tip to update the file.

Perfusing Stage Incubator, which maintains the sample in a fully closed environment and under constant gas and fluid flow.¹⁰

Overlaying atomic force microscopy and optical images is possible by using the Bruker's Microscopy Image Registration Overlay (MIRO[®]) option. An example achieved on Lovo cells is shown in figure 2. On the fluorescence part of the image, the cell nuclei have been labeled with DAPI and the actin cytoskeleton with α -phalloidin. The AFM image has been acquired in TappingMode and is a mix of topography and amplitude error channels. This type of software can be used to overlap atomic force microscopy and fluorescence images and also to target specific locations to perform force curves directly from the optical image.¹¹ This feature is of particular importance for point-and-shoot experiments carried out with functionalized tips.

Correlating Fluorescence Data and Measurement of Specific Unbinding Events

The combination of atomic force microscopy and spectroscopy can be used to detect specific interaction forces between two molecules with piconewton sensitivity.^{2,12,13} The experiment described in figure 3 illustrates how in situ fluorescence measurements can be associated to the presence of specific force measurements detectable by an AFM tip. In this instance, an AFM tip was functionalized with a pore-forming toxin called Pro-Aerolysin (PA), which has the ability to recognize any inositol group present at the surface of living cells.^{14,15} On the other hand, cells were transfected with a specific plasmid so that they over-express GlycosylPhosphatidylinositol (GPI)-anchored proteins at their surface. The plasmid also contains a GFP-coding sequence so that all cells over-expressing

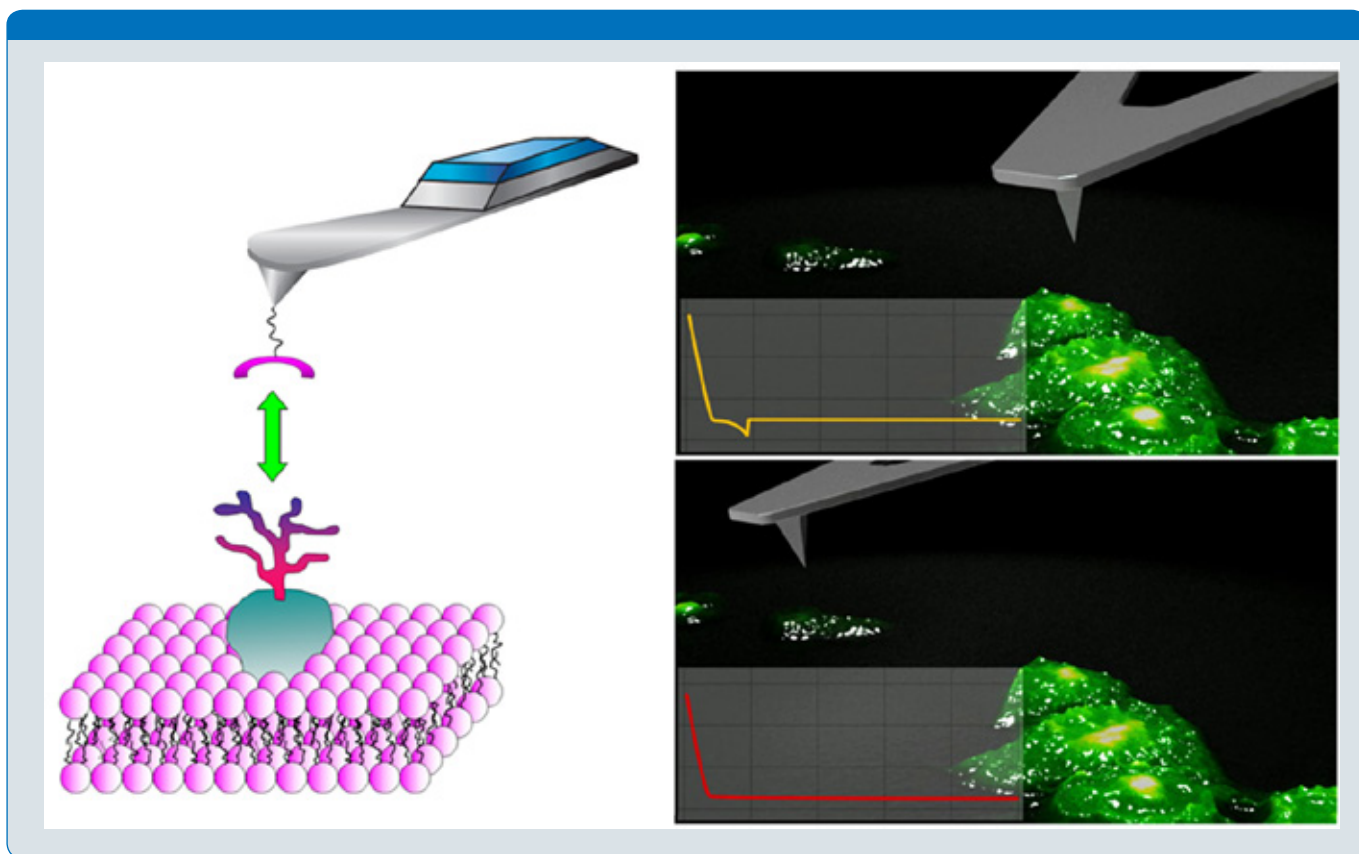


Figure 3: Correlation between fluorescence intensity and specific AFM force measurements on living Hela cells. A tip was functionalized with a toxin able to target any inositol-containing molecule. Fluorescent cells over-express GPI-anchored proteins and exhibit a high number of specific unbinding events (yellow retraction curve) whereas for most of the non-fluorescent cells, no event (red retraction curve), or a much lower number, can be detected.

the proteins are highly fluorescent. In highly fluorescent cells (top right of figure 3), specific PA-GPI events can be detected within a range of 100 pN (see representative retraction curve in yellow). In non-fluorescent cells, some GPI-anchored proteins are still present inside the plasma membrane, but as they are not over-expressed, the number of specific unbinding events is decreased by approximately a factor of five. This type of work highlights how straightforward it is to correlate the number of specific events and the fluorescence intensity of the targeted cells.

AFM Probe-Induced Mechanical Stimulation of Living Neurons and Simultaneous Fluorescence Monitoring of Transduction Signals

In vivo pain-evoking stimuli are detected by the primary sensory neurons through their endings. Signals are transmitted by a series of action potentials along the neurons until the spinal chord, and then to the brain where they are interpreted as pain. Those specific neurons that enable the transmission of painful noxious stimuli are called nociceptors.¹⁶ Although it's accepted that mechanically induced pain plays a clear role in trauma- and inflammation-

mediated death, the detailed process (particularly the signaling pathways following the stimulation) remains poorly understood. A first step to enhanced understanding is to develop a new device that allows both application of carefully controlled mechanical stimuli and the monitoring of the consequences at the biochemical level.

A tipless cantilever modified with a 10 μm polystyrene bead at its end can be used to apply a controlled nominal force on living dorsal root ganglions. Simultaneously, the intracellular calcium concentration can be measured by monitoring the intensity of the fluorescence signal on the optical image. The somas can easily be targeted by using 20x objectives and navigating the AFM probe on the (bright field or DIC) optical image, but the stimulation of the neurite endings requires a much more accurate localization and the use of high-magnification objectives. Single force stimulations were applied with the tip at different locations of the neurites, and each time a fluorescence change was systematically observed at the contact point or far from it (sometimes several hundreds of micrometers away) proving that the signals are transmitted along the cell and between

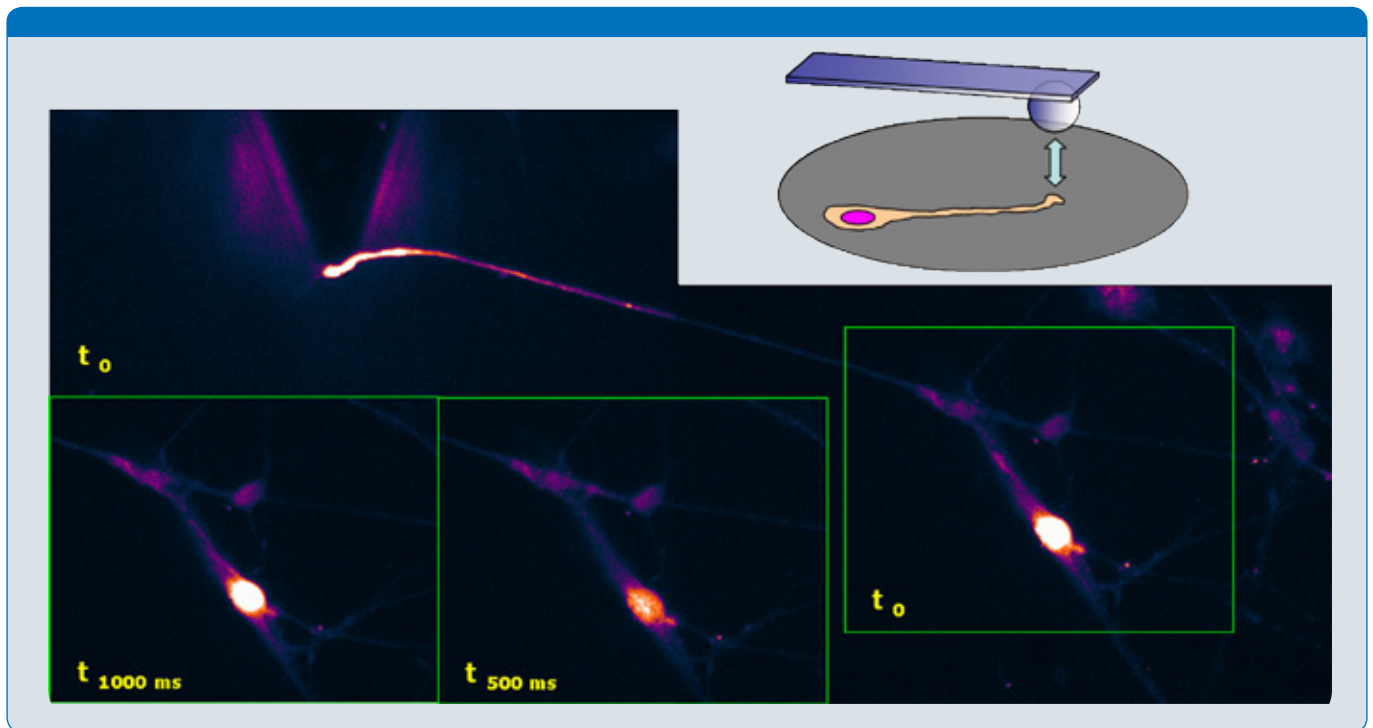


Figure 4: Mechanical stimulation of living dorsal root ganglions by a modified AFM probe. As soon as the tip touches the surface, some primary and secondary flows of action potentials can be observed, indicating a mechano-sensitive activation and transduction of the signal, through calcium and/or sodium receptors. In force volume mode, regular indentations can be applied onto the endings, which are perfectly synchronized with the fluorescence pulses seen on the cell core.

neighbor cells.¹⁷ The fluorescence intensity could be quantified and clearly distinguished from the basal activity, but one interesting unanswered question was whether or not the cells can be stimulated several times by the AFM tip. To address that question, the modified tip was engaged in force volume mode with a surface delay of 0 ms, a retraction delay of 500 ms and a scan size of 0 nm (see figure 4). The results indicate that the living neurites can be stimulated 4 or 5 times by the tip before being completely depolarized. These types of discoveries demonstrate the potential of such an approach to detect signal propagation and transduction in living neurons and to obtain better insight into the mechanisms of mechanotransduction in those cells.

Combining Confocal Laser Scanning Microscopy and AFM Data

Fluorescence microscopy techniques have been becoming more and more important in biology over the past decade or so, and they have enabled the development of more advanced applications, such as confocal¹⁸ or total internal reflection microscopies.

Voluminous samples, even when properly stained, are hard to image in standard fluorescence microscopy, and generally result in blurry images. To overcome this problem, confocal microscopy is often used since most of the light from the sample that is not from the microscope's focal plane is excluded. As a result, the corresponding image has much better contrast and represents a thin section of the sample at a certain height. By stacking several of those images taken at different heights, it is possible to reconstruct a three-dimensional image of the specimen.

Figure 5 shows confocal and atomic force microscopy images of Hela cells labeled with DAPI and α -phalloidin. A, B, C and D images were captured on a dense cluster of cells having more than 15 μm in thickness. A, B and C are three thin-sections of a z-stack movie recorded on a few cells protruding from the rest of the cluster, at different heights along the vertical axis. In A, the nuclei are clearly visible because they are very close to the bottom layer of underlying cells (whereas the actin cytoskeleton exhibits an average illumination density). In B, the actin fibers are even more visible, whereas the nuclei tend to disappear. In C, only the very top part of the sample is visible, here,

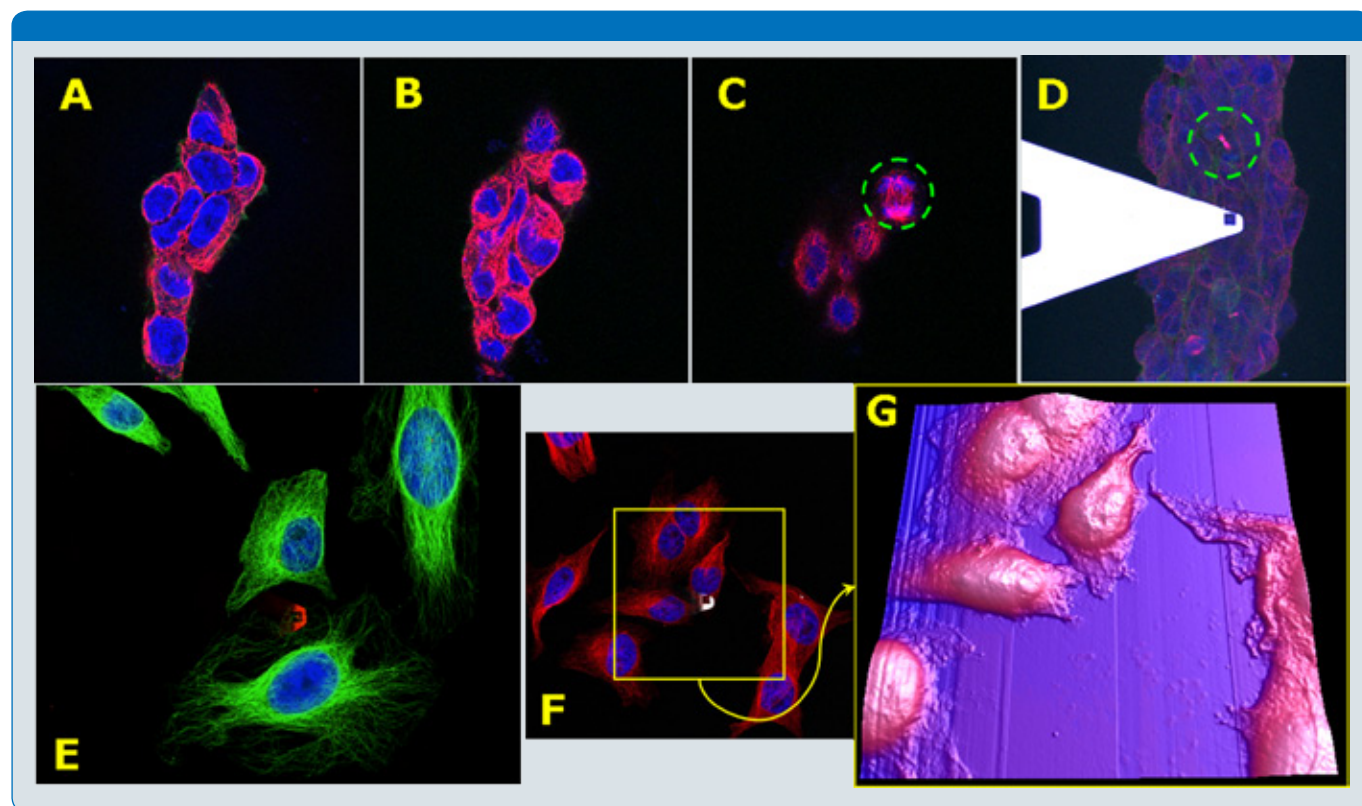


Figure 5: Full compatibility of AFM and confocal laser scanning microscopy. In confocal microscopy, several thin sections of the specimen can be captured along the z-axis (A, B, C and D) to reconstruct a 3D model. The green dashed circle spots cells in mitosis, both visible on the thin section (C) at high resolution and on a higher plane (D). A key requirement to overlay atomic force microscopy and fluorescence images is to make sure the AFM tip can easily be located on the optical images. Most of the commonly used AFM probes like MCLT (D), DNP (E and F), OBL, OTESPA (not shown here) were successfully tested. We also made sure that none of the confocal parameters (like the wavelength, the size of the pinhole or the rotation scanfield) impact the quality of the AFM operation and vice versa. An example of overlay with an atomic force microscopy height image is shown in G.

highlighting the two cells under mitosis. D is a zoom out of the same area showing the AFM cantilever tip in contact with the sample. As in epifluorescence, overlaying AFM and optical images is possible but requires precise localization of the AFM tip. Images D, E and F show different confocal images with different types of AFM tips clearly visible on each. The yellow square in F indicates where the topography AFM image (represented in 3D in G) has been performed. This example is focusing on the combination of fluorescence data with atomic force microscopy height information. However, with these two techniques it would also be possible to combine confocal images with local mechanical properties of the sample to provide even more relevant information.¹⁹

Combining TIRF and Atomic Force Microscopy Data

In regular epifluorescence, the intrinsic fluorescent signal of the fluorophores bound to the feature of interest is over-imposed with the background fluorescence of the unbound molecules, which causes the resulting image to exhibit a certain part of non-specific fluorescence and be blurry. TIRF was developed to selectively illuminate and excite fluorophores at the very neighborhood of the glass-water interface by using the evanescent wave that is generated only when the incident light is totally and internally reflected at this interface. The evanescent field decays very quickly from the interface and penetrates into the sample by a depth inferior to 200 nm. TIRF can hence be used to

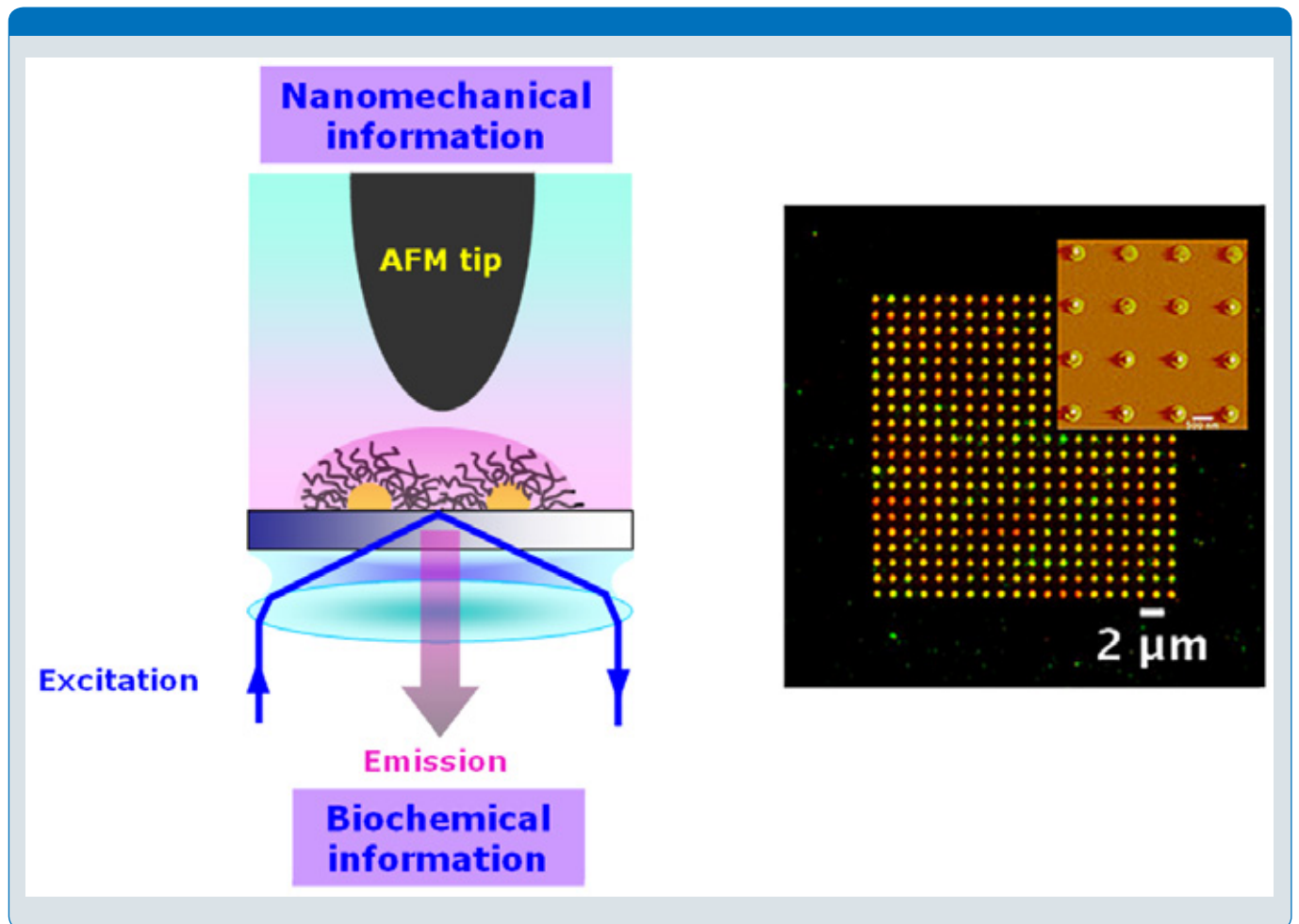


Figure 6: Combination of TIRF and atomic force volume measurements for biomolecular detection. The TIRF principle (left) is based on the evanescent wave. Unlike in confocal where thick features can be analyzed, in TIRF, only the first 100 nm close to the surface can be analyzed. The PEG-ylated nano-rings are mounted on a glass slide so that they are 1.3 μm apart from each other and have an inner diameter of less than 30 nm. As soon as different antibodies bind to the PEG layer, the TIRF signal is modified and can be monitored (right, background image), simultaneously with atomic force volume measurements (right; inset), which enable tracking the local changes in topography and mechanical properties.

visualize very thin structures like basal plasma membrane or small features like proteins or DNA, with a remarkable sharpness. The atomic force microscopy/TIRF combination has proven to be extremely powerful for investigating the properties of biological fibrils, such as myosin filaments.²⁰

The future of TIRF/atomic force microscopy combination might also dwell in biomimetic devices. Biomaterials capable of biological recognition with a high specificity are of considerable importance in the field of biosensors and several key medical applications.^{21,22} By taking advantage of the intrinsic affinity between Poly(ethylene-glycol) PEG and PEG-binding antibodies, some researchers²³ have recently proposed an original device (figure 6) based on a combination of TIRF and AFM that perfectly mimics the structure and function of biomolecular adaptors that transport molecules from a very complex biological environment to site-specific targets. PEG is known for its unique properties of biocompatibility and protein resistance.²⁴ In this setup, gold nano-rings are functionalized

with various types of thiol-based PEG derivatives in regular micro-patterns. Several types of anti-PEG antibodies are placed in the buffer and exhibit various affinity toward the PEG molecules. Such specific bindings result in a conformational change of the so-called “protein-like” structure of the PEG and can be monitored by using TIRF. On the other hand, force volume can be simultaneously used to probe the different changes in height and mechanical properties versus the different antibodies bound to the polymer brush. Figure 6 represents the TIRF/AFM setup (left) and the type of data that can be collected (right). As the antibodies bind to the PEG-coated ring, the TIRF signal can be detected (background image) correlated with any corresponding changes in height and mechanical properties via force volume measurements, and compared with AFM images (inset). This type of device is rather unique since it allows the detection of both nanomechanical and biochemical information, and opens the way to many promising outcomes in nano-biomimetism and biosensing.

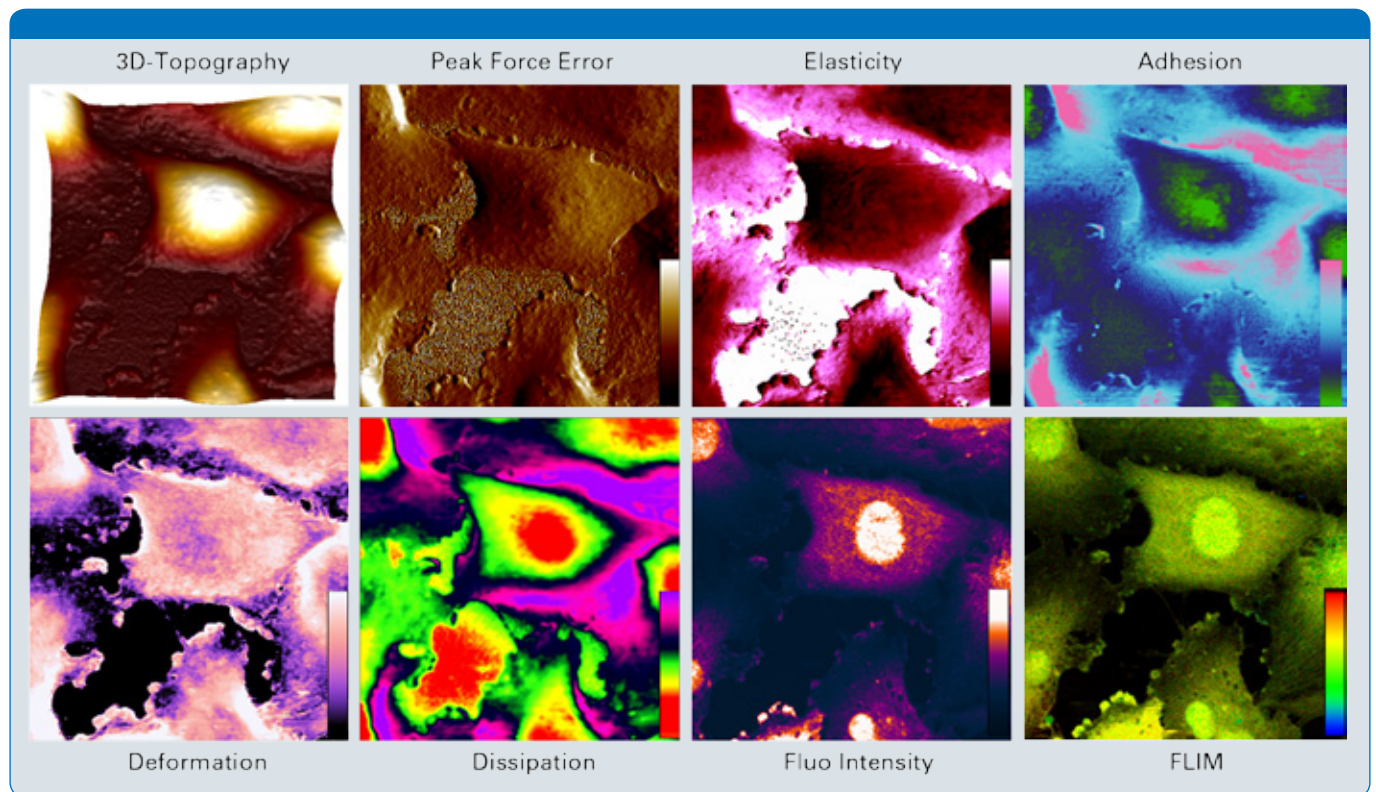


Figure 7: 100x100 μm scans of fixed glioblastoma cells recorded on a FLIM/AFM setup. The fluorescence intensity and FLIM signals are expressed in arbitrary units but all AFM channels are directly quantitative. The z-scales are (from bottom to top of the color bar): topography = 0–5 μm ; PeakForce error = 0–400 pN; Elasticity = 0–700 kPa; Adhesion = 0–150 pN = Deformation = 0–150 nm; and Dissipation = 0–3 keV.

Combining AFM with Fluorescence Lifetime Imaging Microscopy Measurements

Every fluorescent dye has a specific lifetime at the excited state. By monitoring differences in lifetime, one can distinguish between different molecular species, even if they have the same fluorescence color. The lifetime of a molecule is influenced by such factors as the intermolecular binding or the ionic strength, but is not dependent of the fluorophores concentration, the light intensity, or the photobleaching. One of the major benefits of using FLIM versus other fluorescence techniques is that it is possible to observe specific molecules while imaging an entire cell.

As mentioned above, atomic force microscopy is a surface investigation technique that can be combined with FLIM in one single tool. An ideal example of this is the BioScope Catalyst, which is a sample-scanning system with a powerful NanoScope® V controller that allows scan synchronization signals and very accurate positioning of the AFM tip in x,y,z relative to the confocal beam. With respect to the FLIM integration, the Catalyst can be combined with a MicroTime 200 (PicoQuant, Berlin), which is a high-performance time-resolved fluorescence microscope with single-molecule sensitivity.²⁵ The synchronization of the two techniques is possible via the so-called time-tagged time-resolved mode, which allows the branching of external “marker” synchronization signals within the continuously monitored flow of photon arrival times. For each AFM scan, the markers are given at the beginning and at the end of each scan line, where the recorded photon signals are ultimately converted into pixels. The “line start” and “line stop” synchronization signals are readily available from the NanoScope V controller. Figure 7 gives a representative example of simultaneously acquired FLIM, fluorescence intensity and PeakForce QNM® AFM signals on fixed glioblastoma cells. PeakForce QNM is the only AFM mode that provides directly quantitative mechanical information of the sample at a pixel resolution, making it the most informative AFM mode that can be combined with the FLIM technique. After data acquisition, the images can be post-processed in 3D so that atomic force microscopy and fluorescence data are overlaid, allowing a straightforward identification of specific features and immediate correlation with their intrinsic mechanical, chemical or fluorescence properties.

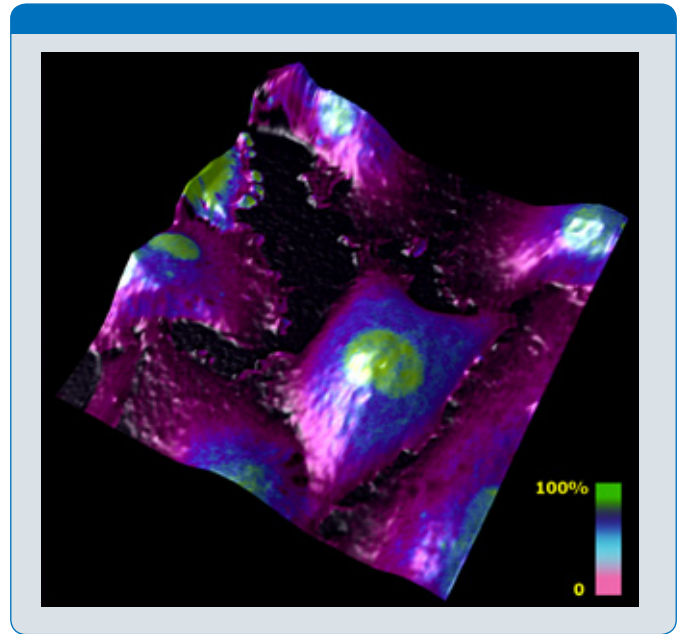


Figure 8: 3D representation of a 100x100 μm AFM height image of fixed glioblastoma cells with a fluorescence intensity skin. The center part of the cell (nucleus) is highly fluorescent. The intensity tends to decrease in the rest of the cell, to eventually be weak on the edges. Such a rendering allows a direct identification of the cell parts and immediate correlation with the fluorescence lifetime of fluorophores.

Conclusions

This application note provides a non-exhaustive list of combinations of atomic force microscopy and various fluorescence microscopy techniques, as well as the typical applications that can be addressed by using such setups. The fact that all of these combinations are made possible by the use of a single fully integrated tool makes the operator's task easy and user-friendly. The development of newly released AFM modes (such as PeakForce QNM) opens the way to even more promising applications.

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