



Live Cell Imaging with the AFM

Application Note

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Introduction

Atomic Force Microscopy (AFM) is a powerful, non-destructive technique that can be applied to the study of a variety of materials of biological significance and/or biological origin. It provides a means to study the structural elements of very delicate structures, such as healthy or diseased living cells and offers unique opportunities to image, identify and study biological features on the surface of cells and even certain structural elements inside of cells. Using AFM to image living cells, important information on the architecture of membranes, organelles, and cytoskeletal structures can directly be gathered, without the use of potentially interfering fluorescent labels or probes. Cellular studies that have been aided by AFM have provided information about living cell dynamics, intercellular communication and responses to stimulus, drugs, or toxic substances; all in real-time. Non-destructive AFM images of cellular structures can quickly and easily be obtained with the Agilent 5500 AFM. Whether this AFM is used as a stand alone system or it is combined with an inverted optical microscope (ILM), images of living cells and cellular structures, far below the limits of optical resolution, can be quickly and relatively easily obtained.

There are three key operating principles of the AFM; 1) an AFM probe which has a sharp tip attached

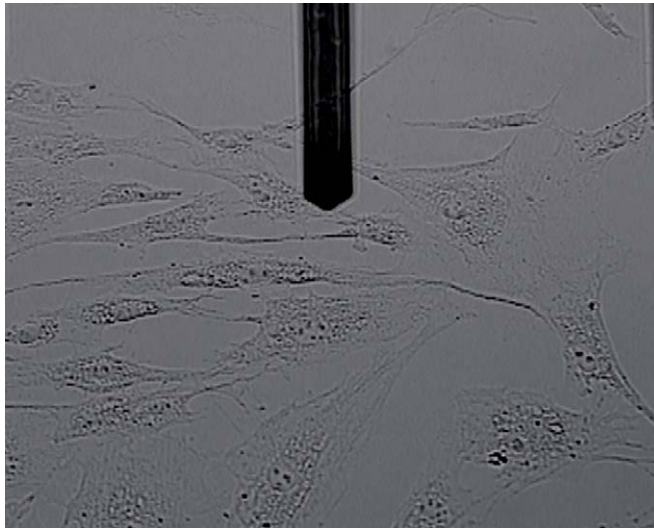


The Agilent 5500 AFM combined with an inverted optical microscope (ILM).

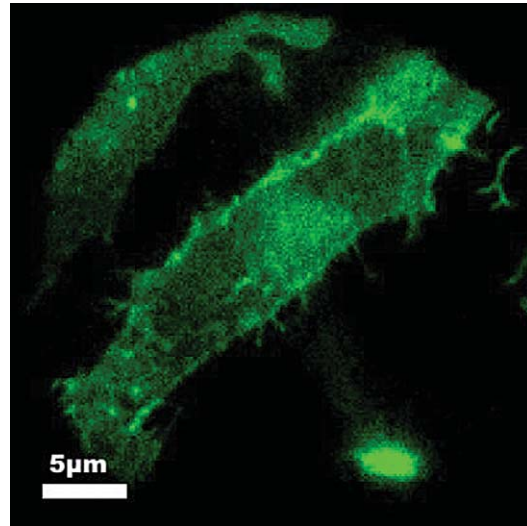
near the end of a reflective, flexible cantilever which is gently scanned across the surface of a sample in a raster-like manner, 2) a feedback loop that maintains constant deflection of the cantilever in the vertical (z) plane with sub-Angstrom accuracy and 3) a piezo-actuated scanner or stage that moves the cantilever in the horizontal (x-y) plane. The probe tip interacts with the sample surface as it is raster scanned in the x-y plane which changes the location of the reflected laser spot. The feedback circuit, which is connected to a photosensitive cantilever deflection sensor, keeps the distance between the tip and the sample at a fixed value. The feedback signal is combined with the x-y information to form an image which represents a 3D reconstruction of the sample's topographic features.



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Bovine capillary endothelial cells. Optical 10X.



CHO fluorescence.

AFM Imaging of Biological Samples

As described, AFM is a scanning probe technique in which a mechanical probe can be used to image a variety of surfaces at the molecular level with sub-nanometer scale resolution, even under physiological conditions. AFM images generally can be obtained without fixation or staining methods, and without the use of extraneous labels or probes. AFM images can be obtained in buffer or even in filtered growth media, which makes it a very useful technique for studies of delicate samples, such as living cells. AFM has been used to image a variety of samples, including proteins, DNA, cell membranes, lipids, viruses, fixed or living cells and other materials of biological significance or origin [Allison 2002, Kada 2008, Kienberger 2006, Sun 2006]. Since it is capable of resolving these materials under physiological conditions; it is a powerful imaging tool in the life sciences.

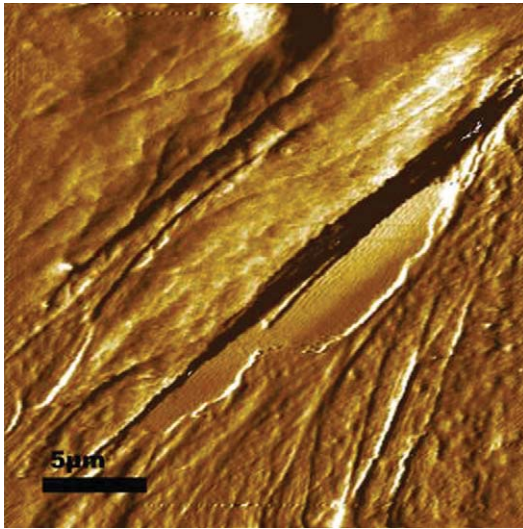
AFM was originally developed to overcome some of the limitations of its immediate predecessor, the scanning tunneling microscope (STM), which can only be applied to conducting samples [Binnig 1982]. Soon after its invention in 1986 [Binnig 1986],

AFM attracted the attention of the researchers in the biological and biophysical scientific communities and it was quickly used to achieve resolution of biological samples far beyond that which was obtainable with optical microscopy [Engel 1991, Lindsay 1994, Marti 1988, Radmacher 1992]. The range of biological samples studied by AFM vary from the smallest biomolecules, such as DNA, RNA, phospholipids, and single protein molecules, to sub-cellular structures, such as membranes and membrane components and even relatively larger samples, such as living cells and tissue samples. AFM is particularly useful for studies that involve living cells, at least in part because of its ability to yield high resolution images under physiological conditions. In studies of microbial or mammalian cells, AFM has been used to obtain high resolution images of the soft outer membrane and even of cytoskeletal structures underneath the cell membrane. In addition to imaging, the properties of various samples can be probed and measured with the AFM. Consequently, AFM can also be used to measure key structural parameters of biologically significant materials, including their mechanical, chemical and functional properties. For example, AFM has

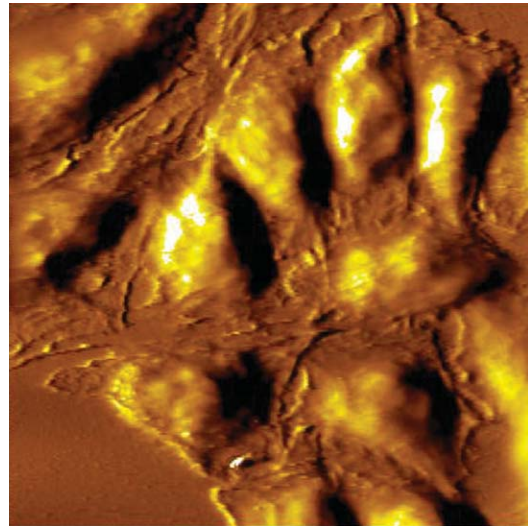
been effectively utilized to measure the compliance of biological materials and living cells, and even the minute forces which occur between biological entities; including, receptor-ligand interactions [Yang 2007], antibody-antigen interactions [Kienberger 2005, Stroh 2004], DNA-DNA and DNA-protein interactions. It has even been used to map a variety of other molecular interactions on mammalian cells [Chitchevlova 2007, Lee 2007].

Advantages of AFM over other Imaging Methods

AFM has several distinct advantages over some other, more common, imaging techniques. Standard optical imaging generally gives information about the exterior of the cell, such as the cell membrane, mainly because the depth of focus of an optical microscope is on the order of a few hundred nanometers. Contrast enhancing techniques, such as Phase Contrast and DIC optical microscopy, can be used to help provide a view of some internal cell structures or organelles. And fluorescence microscopy can give a detailed picture of the distribution of selectively labeled cell components. Confocal microscopy is able to more accurately focus on



CHO AFM.



CHO cells deflection. Scan size: 82 μm.

narrow plans or sections inside the cell and, therefore, give additional information about organelles and even physiological processes. However, unlike optical microscopy, AFM provides nanometer-scale resolution without the use of extraneous labels.

Electron microscopy (EM) has also played a fundamental role in characterizing microbial organisms and mammalian cells and tissues. However, even though it is a relatively high-resolution technique, there are inherent requirements for fixation and staining of samples for image contrast. These are not applicable to the study of living organisms. Unlike SEM (scanning electron microscopy) and TEM (transmission electron microscopy), AFM is a high resolution imaging technique that can be operated under more biocompatible conditions. In contrast, AFM avoids complex sample preparation procedures and the artifacts that are inherent to these measures. This significant and appealing advantage of AFM for studies in the biological sciences permits measurements on native biological samples under relatively mild conditions, even in liquids. Another unique capability of AFM is its ability to detect and quantify molecular interactions

between single receptor-ligand complexes and antibody-antigen interactions [Stroh 2004]. Furthermore, unlike other microscopy techniques such as EM or conventional optical microscopy, which generally only give information about cell morphology and composition, AFM can be used to measure the mechanical properties of cell membranes.

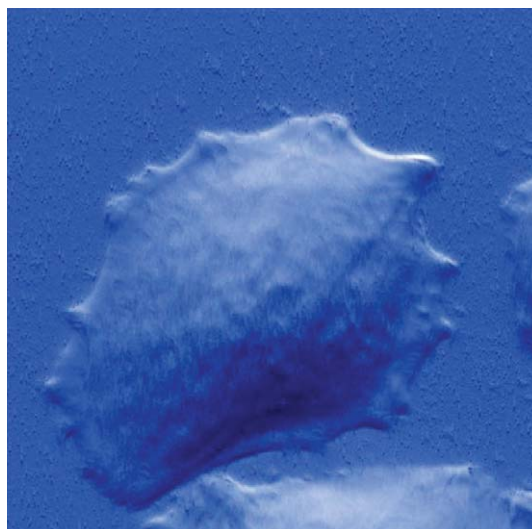
Combining AFM with Optical Microscopy (AFM/ILM)

While optical microscopy has proven itself to be an extremely useful imaging technique, the resolution of a standard optical microscope is limited by the wavelength of light. In contrast, image resolution with an AFM is limited only by the radius of the tip the AFM probe. Consequently, high resolution images of living cells can be obtained in their native, hydrated states, so the AFM can often provide data that is complementary to the information obtained using more conventional microscopy techniques. A feature of Agilent's 5500 AFM is that it can be interfaced to a number of conventional, commercially available ILMs through the use of Agilent's Quickslide adaptor which permits the 5500 AFM to be quickly and easily converted from a stand-alone AFM to

an AFM/ILM combination. Quickslide allows for accurate positioning of samples under the ILM because the sample holder can be moved relative to the optical axis of the objective, and also relative to the AFM cantilever. This unique, convertible ILM/AFM configuration can be used to gather important, complementary information about cell morphology, composition and mechanical properties, along with information regarding the architecture of membranes, cellular processes, organelles, and cytoskeletal structures; further enhancing the power and utility of the 5500 AFM and the optical microscope beyond that which might be attainable by either technique alone [Madl 2006].

Combining AFM with Fluorescence Microscopy

Fluorescence microscopy can be used to visualize labeled molecules specifically and with high resolution; even down to the single-molecule level. It is a powerful tool in its own right which can be used to follow cellular processes and to monitor the dynamics of living cell components and to observe diffusion or intracellular trafficking processes in living cells. By combining the Agilent



Human lung cancer cell. Scan size: 45 μm .

5500 AFM with an ILM outfitted with fluorescence microscopy capabilities, the advantages and benefits of both methods can exceed the capabilities of either method alone. Such a system allows a more comprehensive and detailed characterization of a sample's cellular structures and internal and external biochemical processes; the information from which can give insight into many physiological processes. There are other advantages to a combined AFM fluorescence microscope system, including assisting the study of fluorescence-labeled membrane receptors via atomic force spectroscopy with antibody-functionalized AFM tips [Madl 2006].

Imaging Live Mammalian Cells with the AFM

The study of living cells by atomic force microscopy is a relatively new discipline and a consequence of the convergence of two independent technologies; cell biology, which has a relatively long history, and scanning probe microscopy, which has a history spanning less than two decades. Fixation refers to the process by which proteins in the cell membrane and other cellular components are

cross-linked with various reagents [Rhode 2004]. Cells that have been fixed in such a manner are often used for EM and AFM imaging because the fixation process strengthens the cell membrane dramatically and freezes it in a particular shape or morphology. Therefore, fixed cells can be imaged with the AFM more easily than live cells. Unfortunately, the fixation process also kills the cell and changes its mechanical properties, rendering the outer membrane much stiffer, and making it less likely that structures such as cytoskeletal elements underneath the cell membrane can be detected. Fixation also stops most or all biochemical processes within the cell, so these events can not be studied with fixed cells. Consequently, when it is technically feasible, it is almost always advantageous to perform AFM studies on living cells.

The membranes of living mammalian cells are very compliant but mechanically inhomogeneous. This lack of homogeneity arises from organelles and some other sub-cellular structures but primarily from the cytoskeleton which is a structure underneath the cell membrane that lends it support. The cytoskeleton is the primary

determinant to the overall shape or morphology of the cell. A contact-mode AFM image of a mammalian cell, obtained under physiological conditions can yield several different images for the purpose of resolving the soft outer membrane of a cell from the cytoskeleton underneath. But we will concern ourselves with only two of these, the height or raw topography image, and the corresponding 'error' or deflection signal image. The force between the tip of the AFM probe and the cell membrane indents the membrane slightly, and if the force is increased beyond the compliance of the cell membrane, it can be pressed into cytosol underneath. In this manner relatively stiff, large fiber structures, which are the actin filaments that make up the cell's cytoskeleton can be resolved from the cell's soft outer membrane.

Bundles of actin filaments can quickly polymerize or depolymerize, rapidly adding or removing the components of the cytoskeleton to different locations within the cell. It is by this mechanism that cells change shape and the means by which cell motility occurs. The polymerization and depolymerization of actin filaments are, therefore, important in many normal and disease processes, including metastasis, which is the spread of malignant tumor cells from one organ or tissue to another location. Since the cytoskeleton is responsible for many mechanical properties of the cell, including shape, activity and mobility, real-time monitoring of living cell dynamics, intercellular communication and response to stimulus, drugs, or toxic substances is another powerful application of cellular studies with AFM.

One interesting study of mammalian cells with the AFM utilized an Agilent 5500 AFM combined with a fluorescence microscope to investigate Chinese hamster ovary (CHO) cells [Rhode 2004, Madl 2006]. These cells are widely used as expression system for a number

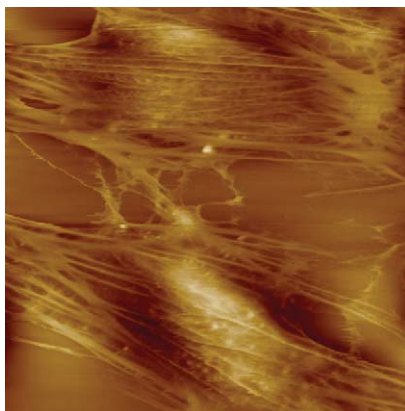
of proteins and are relatively easily grown in defined media and they are adherent to various solid matrices; often without the need for additional immobilization chemistries. The CHO cells that were utilized expressed high levels of recombinant scavenger receptor class B type 1 (SRB1) proteins which were fused to green fluorescent protein (GFP), which can be excited with 488 nm and light detected at 508 nm. The information obtained from fluorescence microscopy was used to accurately position an antibody-modified tip above specific antigens on CHO cell membranes so that AFM force spectroscopy experiments could be performed.

Imaging Microbial Cells with the AFM

The AFM can also be used to study living microbial cells under physiological conditions on native surfaces. This reveals minute details under conditions that are not possible using electron microscopy [Doktycz 2003, Sullivan 2007]. Significant morphological differences, which are dependent on the conditions of bacterial growth, can be observed with an Agilent 5500 AFM. For example, the AFM has been used to distinguish bacteria grown in liquid media, which can be rod shaped, from bacteria grown in the air showed, which can have irregular folding patterns on the surface.

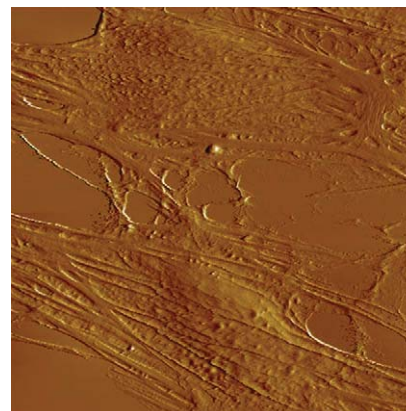
Selecting AFM Probes for Live Cell Imaging

Since the tip of the AFM probe is in contact or near contact with the sample surface, it is a critical component of the AFM's overall performance. Most AFM probes are manufactured using silicon micromachining technology [Alessandrini 2005]. Depending on the imaging mode and the sample's properties, different types of probes with different cantilever and tip characteristics may be chosen. When the AFM is operated in contact mode, which is the primary imaging mode

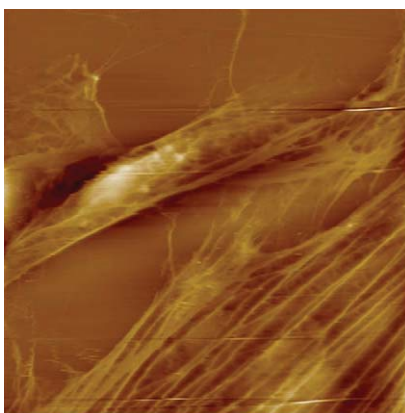


Scan size: 105 μm .

Topograph

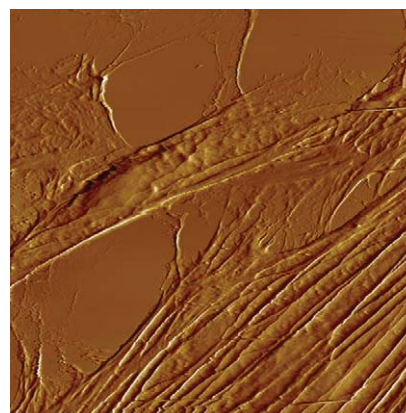


Deflection

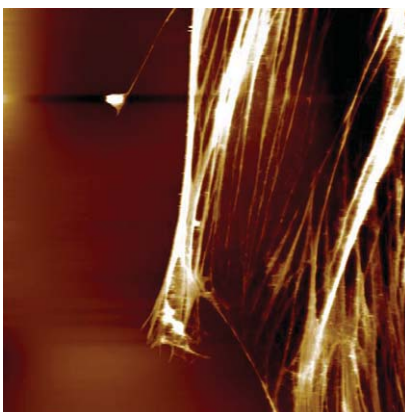


Scan size: 56 μm .

Topograph



Deflection



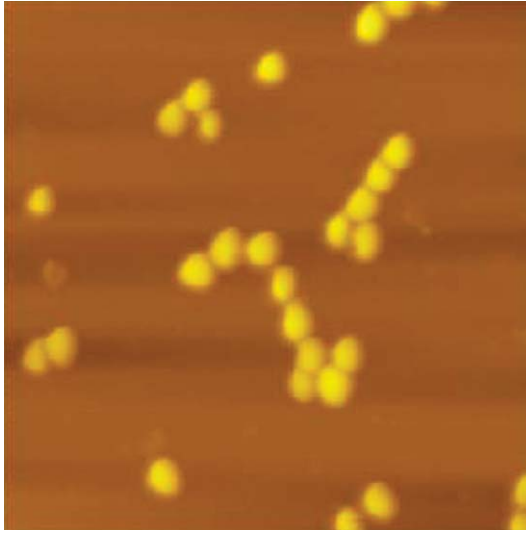
Scan size: 87 μm .

Topograph

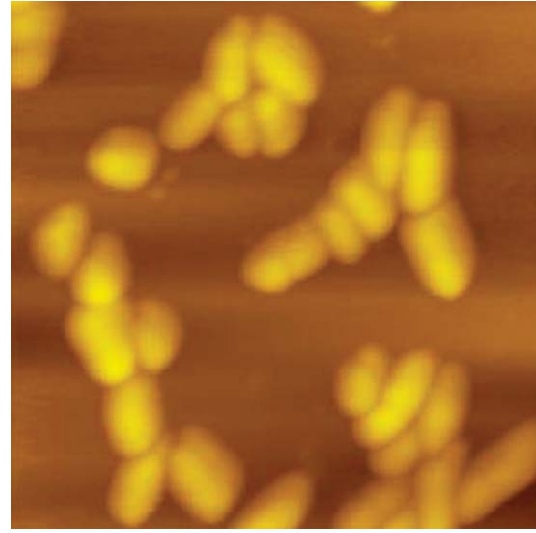


Deflection

Bovine capillary endothelial cells.



Staphylococcus aureus. Scan size: 15 μm .



Escherichia coli. Scan size: 15 μm .

for live cells, the cantilever generally should be as flexible as possible; for example, softer than the outer membranes of the cells being imaged. Typically, the spring constants (k) for AFM probe cantilevers range from 0.01 N/m to 100 N/m, with softer cantilevers being used for live cell imaging applications. In many AFM imaging applications it is advantageous to utilize the sharpest probe tips available in order to optimize imaging resolution. However, in the case of live cells, extremely sharp tips such as these can tear or damage the surface of the cell's soft outer membrane, so sharp tips generally offer little advantage in live cell imaging.

Immobilizing Mammalian Cells for AFM Imaging

Most eukaryotic cells are relatively large, so unlike smaller samples, one needn't necessarily be over concerned about the flatness of the surface upon which the cells are to be imaged. This is in contrast to protein or DNA imaging, both of which require atomically flat substrates, such as mica. Glass or plastic substrates will often suffice for cell imaging. However, many cells do adhere tightly to bare

glass or plastics. Furthermore, most cells do not naturally reside on glass or plastic in their native environments, so these materials can cause changes in cell morphology and alter normal cell processes. Fibronectin, gelatin or collagen coated glass or plastic substrates may more closely mimic the surfaces of biological substrates and surfaces. These substrates are quickly and easily made from glass cover slips that are commercially available [Doktycz 2003, Sullivan 2007]. Another important factor regarding coated matrices is the fact that many cells adhere more tightly to fibronectin, gelatin, or collagen coated surfaces, which further enhances most AFM imaging applications.

Immobilizing Microbial Cells for AFM Imaging

Poly-L-lysine coated mica surfaces are often suitable for immobilizing both gram positive bacteria and gram negative bacteria in order that they can be imaged in air or liquid environments. However, it has been demonstrated that gelatin coated surfaces may be preferable to poly-L-lysine coated surfaces, especially for *Rhodospseudomonas palustris* in liquid media [Doktycz 2003, Sullivan 2007].

Using PicoTREC to Probe Molecular Interactions on Cell Surfaces

AFM Topography and Recognition (TREC) imaging is the simultaneous resolution of topographical information while mapping the locations of specific molecular recognition events on heterogeneous surfaces. TREC imaging is made possible with PicoTREC; an option that is only available on Agilent AFMs. PicoTREC enables the determination of the precise locations of specific molecular recognition events; including the identification of specific receptor binding sites on cell membranes. TREC imaging represents a novel method to quickly resolve the local distribution of receptors on cellular surfaces with unprecedented lateral resolution (approximately 5 nm).

In an example of TREC imaging applied to mammalian cells, Chtcheglova et al., used PicoTREC to identify binding sites of vascular endothelial (VE)-cadherin on mouse myocardium endothelial (MyEnd) cells. VE-cadherin plays a crucial role in calcium-dependent, homophilic cell-to-cell adhesion. The TREC images were acquired using MAC Mode probes that were functionalized with recombinant

VE-cadherin-Fc cis-dimers. The recognition images revealed single molecular binding sites ranging from 10 to 100 nm, which were correlated to specific binding interactions between active VE-cadherin cis-dimers. Clusters of VE-cadherin binding sites were subsequently assigned to topographic features by overlaying the recognition images with the MAC Mode topography images [Chtcheglova 2007].

In another interesting example of TREC imaging applied to mammalian cells, Lee et al. used PicoTREC, molecular force spectroscopy, AFM tip-bound monoclonal antibodies, and living human microvascular endothelial (HUVEC) cells to map the locations of individual vascular endothelial growth factor (VEGF) receptors. Since interactions between extracellular ligands and cell surface receptors are considered to be critical to many intracellular and intercellular processes, locating and measuring these interactions on living cells, is important to understanding this and similar processes. They were able to determine the binding kinetics, calculate the distribution, and the association/dissociation rate constants of the molecular interactions on the cells. By simultaneously imaging the surface of the cells and identifying the distribution of the VEGF receptors, then quantifying their binding kinetics with respect to their underlying cytoskeletal structural elements, a map of the VEGF receptor-mediated cell behavior was obtained [Lee 2007].

Other Agilent AFM Options that Assist Live Cell Imaging Applications

Due to the relatively large size of most cells (often tens of microns or more), an Agilent large multipurpose is generally recommended for live cell scanning applications. If precise temperature control is of concern, live cell imaging can be performed with Agilent's heating or cooling stages. Continuous renewal of the cell culture medium using Agilent's unique flow-through liquid cell and a peristaltic pump can be a distinct advantage in live cell imaging because it prevents changes in the ionic strength of the imaging medium due to evaporation, and provides fresh culture medium to cells on a continuous basis, both of which are basic requirements for relatively long-term studies of living cells. Using Agilent's environmental control chamber, the atmosphere around living cells can be tightly controlled. Closely maintaining optimal concentrations of, for example, CO₂ or O₂, enables continuous, long-term imaging studies, or imaging studies under aberrant conditions.

Conclusion

AFM has a wide variety of imaging applications in the life sciences; including imaging DNA, protein, lipids, isolated cell membranes, viruses, as well as living microbial or mammalian cells. It has been proven to be an indispensable tool for establishing structure-function relationships among the various components of living cells. In that light, the Agilent 5500 is an extraordinary AFM with an impressive record of rigorous studies involving the morphology and mechanical properties of living cells under physiological conditions. By combining the 5500 AFM with an ILM through the use of a Quickslide adaptor, its capabilities can quickly and easily be extended even further; seamlessly and simultaneously combining information about the cell's topography and mechanical properties with experimental data regarding cellular events and processes. Moreover, along with Agilent's unique topography and recognition imaging option, PicoTREC, the 5500 AFM becomes a powerful tool to map out the locations of molecular binding interactions on cell surfaces. Whether it is used as a stand alone system, with PicoTREC and/or combined with an ILM, the unsurpassed resolution of the Agilent 5500 AFM, combined with its flexibility, and ability to generate high resolution, real time images suggest that it will continue to contribute to the explosive growth of novel research and applications at the frontier of nanobiology, where biology, chemistry and physics are merged into one discipline.

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