From Single Molecules to Tissues: Application of Correlative BioAFM in Microscopy Core Facilities

Introduction

Over the last three decades, atomic force microscopy (AFM) has been an indispensable tool for the analysis of a wide range of samples, from single molecules, proteins, and nucleic acids to complex biological systems such as cells, and tissues.[1] Unlike other conventional microscopic techniques, such as fluorescence and electron microscopy, AFM can be performed in a near physiological sample environment without further need for sample processing or modifications. It also provides 3D image-based information at the nanoscale level with enhanced resolution over other available techniques. This has led to an increase in its usage in life sciences and the subsequent development of biological AFM (bioAFM) for the analysis of biomolecules and cells at nanometre resolution, close to their native state.[2,3] BioAFM allows real-time, high-resolution monitoring of complex biological systems and dynamic processes in their physiological aqueous environment, augmented by information about their mechanical properties.

Biological systems, e.g., the surface of living cells, are heterogenous and dynamic. The presence of many different biomacromolecules, such as receptors, transporters, channels, and assemblies can make cell surface identification challenging.[4] The integration of AFM with advanced optical techniques is a powerful multi-dimensional correlative microscopy tool that could circumvent these challenges.[3] Correlative AFM is used in life science microscopy core facilities to understand the structural, functional, chemical, mechanical, and dynamic complexities of biological systems.

Advanced Optical Integration

Recent advancements in imaging techniques and software have reduced the gap in resolution between AFM and optical microscopy, by allowing users to overlay and compare two different data sets.[5] DirectOverlay™ is a patented calibration method that achieves perfect optical integration, precisely correlating AFM and optical data from various optical microscopes.[6] It can rectify lens imperfections associated with optical images by converting the optical signal into the linearized AFM length coordinates.[7] Currently, advanced optical microscope data can be perfectly overlaid in the same window using the DirectOverlay calibration method. It can also be utilized with a wide range of advanced microscopes, including, confocal microscopy and stimulated emission depletion (STED).[5] Figure 1 depicts the real-time correlation of nano stiffness and cytoskeletal organization of human dermal fibroblasts. There are many other examples of correlative AFM integrated with different optical microscopic techniques for application in life sciences (Table 1).

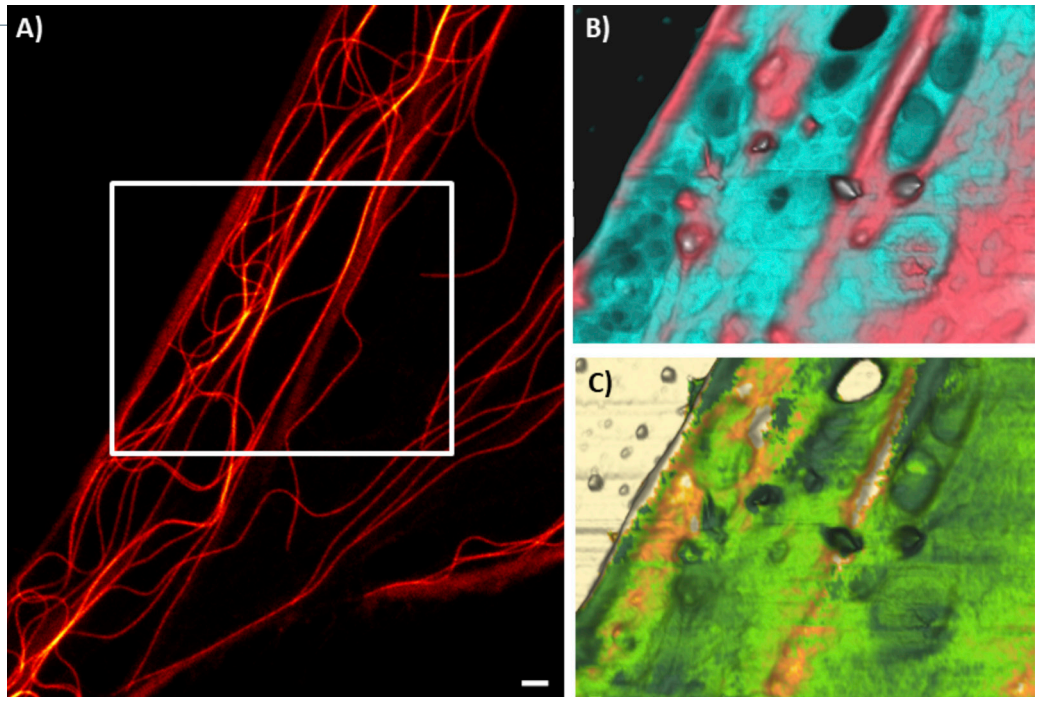

Figure 1: Correlative AFM-STED showing (A) microtubules of human skin fibroblasts labeled with silicon containing rhodamine (SiR), (B) 3D topography AFM image (12.5 µm x 9 µm; z-range: 1 µm), and (C) 3D topography image overlaid with a stiffness map (z-range: 60 kPa). Imaged in HDMEM cell culture medium with QI mode, sample courtesy of Abberior Instruments GmbH.

Table 1: Examples of correlative AFM integrated with different optical microscopic techniques.

Types of techniques	Applications
AFM and STED microscopy	Correlation of cytoskeletal information with the cell membrane physical properties of living astrocytes[8]
AFM and single molecule localization microscopy (SMLM)	Localization of specific labeled proteins in cell biology[9]
AFM and direct stochastic optical reconstruction microscopy (dSTORM)	Direct correlation and density quantification of F-actin cytoskeletal filaments,[10] and nanoscale organization of tetraspanins during HIV-1 budding[11]
AFM and photo activated light microscopy (PALM)	Generation of correlative images of live bacterial and mammalian cells[12]
AFM and super-resolved structured illumination microscopy (SR-SIM)	Simultaneous co-localization of nanomechanical data and cellular dynamics visualization using human bone osteosarcoma epithelial cells[13]
AFM and fluorescence microscopy	Real-time analysis of single-cell bio-physical properties in mixed human cell populations by co-localizing fluorescence imaging data with nanomechanical mapping[14]

Mechanobiology: Mechanical Characterization of Biological Samples

Mechanobiology provides insights about how cells, tissues, organs, and proteins respond to the mechanical cues and contribute towards dynamic molecular processes.[15] There are a few major challenges within mechanobiology, including the quantification of how exactly living systems sense, transduce, apply, and respond towards mechanical signals. To address these challenges, various nanotechnological and microtechnological approaches, such as bioAFM have been employed to quantify the mechanical properties of the biological systems. An advanced AFM mode, quantitative imaging (QI™) was specifically developed to produce quantitative images of soft or loosely bound biological samples with high speed and resolution. Compared to classical AFM modes, QI is a high-speed multiparametric mode that provides detailed structural and mechanical properties while mapping delicate biological samples at nanoscale resolution in physiological conditions.[16] Figure 2 (top panel) shows an example of QI mode-based AFM measurement resulting in a surface topography and a stiffness (Young's modulus) map of a living Vero cell. Young's modulus describes the composite mechanical properties of biological systems, while quantifying the elastic deformation (stiffness) of their components.

Due to their complex composition, biological systems, such as cells and tissues, are considered to be viscoelastic, behaving as both an elastic solid and a viscous fluid.[17] The cytoplasmic rheology of cells is described as a porous elastic solid meshwork of cytoskeleton, different organelles, and macromolecules submerged in interstitial fluid (cytosol).[18] The intracellular water redistribution affects the cellular properties, such as

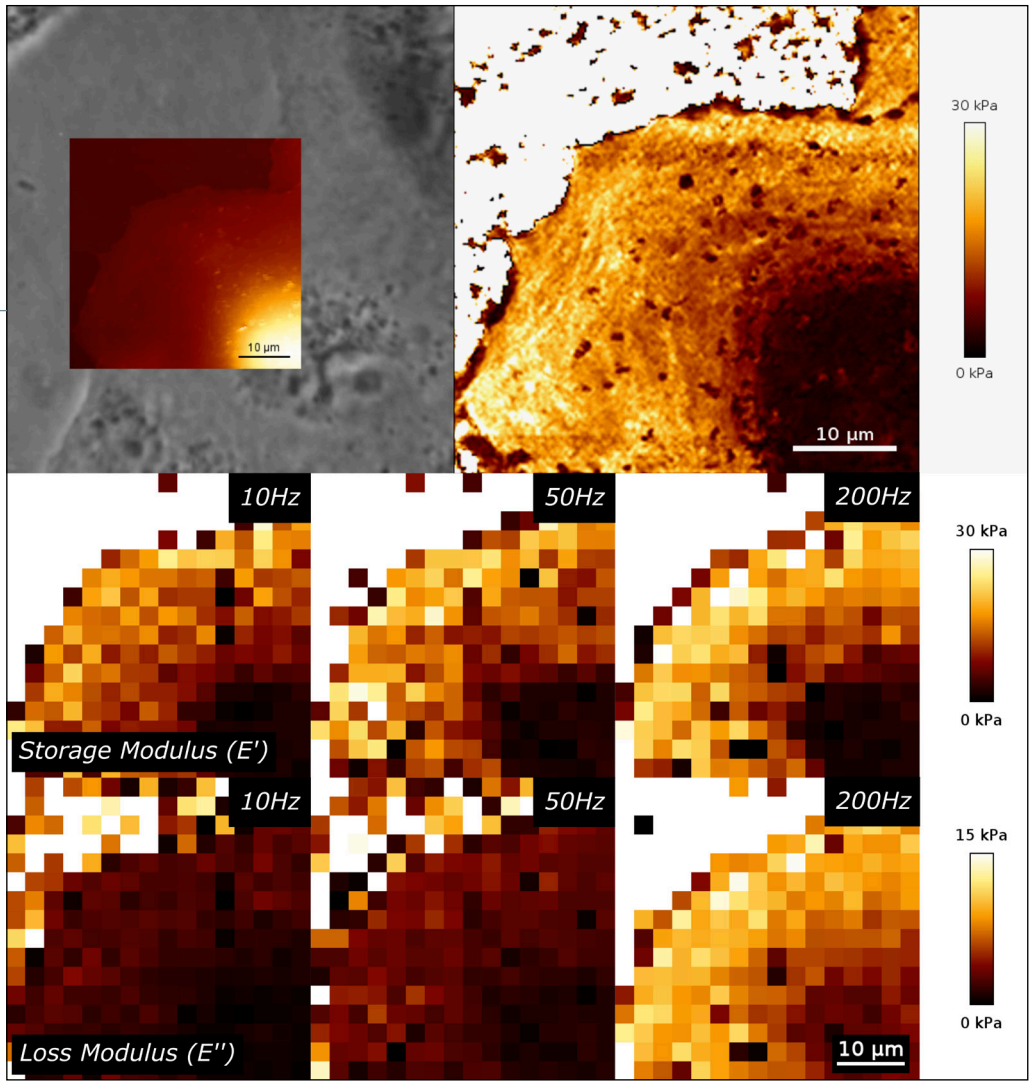

Figure 2: Overlay of living Vero cells with topography and static Young's modulus (stiffness) maps (upper left panel) recorded with QI Advanced Imaging. The top right panel shows a static Young's modulus (stiffness) map determined by a Hertz model fit, typically applied to linearly elastic samples. The same area subjected to rheological analysis, by means of sine modulation via the CellMech package, reveals differences in the dynamic modulus composition of the cells. These measurements derive frequency-dependent rheological properties, such as the elastic storage (middle) and the viscous loss modulus (bottom panel). Samples courtesy of Prof. Andreas Herrmann, Humboldt University Berlin (Germany).

cell division, cell migration, embryogenesis, metastasis, or the epithelial-mesenchymal transition. The microrheological characterization of such systems is therefore pertinent to undertanding how they sense and respond to mechanical cues. The CellMech™ package[19] is specifically designed to perform a frequency-dependent rheological analysis of such samples and can be directly applied for the determination of the individual elastic storage and viscous loss modulus in living cells (Figure 2, middle and bottom panels). Viscoelastic differences, particularly in cancerous versus normal cells, are considered important biomarkers of disease state and progression,[20,21] which often results in adaptive mechanosensing in certain cell types and the corresponding cytoskeleton tuning.[22] The measurement of difference in elasticity between cancer cells and normal cells might lead to the development of diagnostic tools that can identify malignant phenotypes.

Dynamics in Living Cells: Interactions and Manipulation

The direct visualization of living cells provides intricate details about cellular dynamic processes as well as pivotal information regarding the different biomolecules present in living cells.[23] In addition, investigation of cell-cell and cell-microenvironment interactions are important to understand dynamic cellular processes.

The development of high-speed AFM for dynamic biological process imaging enables faster image acquisition (~3 fold in magnitude). Some examples of the dynamic molecular and cellular processes that are investigated using this approach include conformational changes of bacteriorhodopsin protein,[24] nucleosome dynamics,[25] and rotary-catalysis of F1-ATPase.[26]

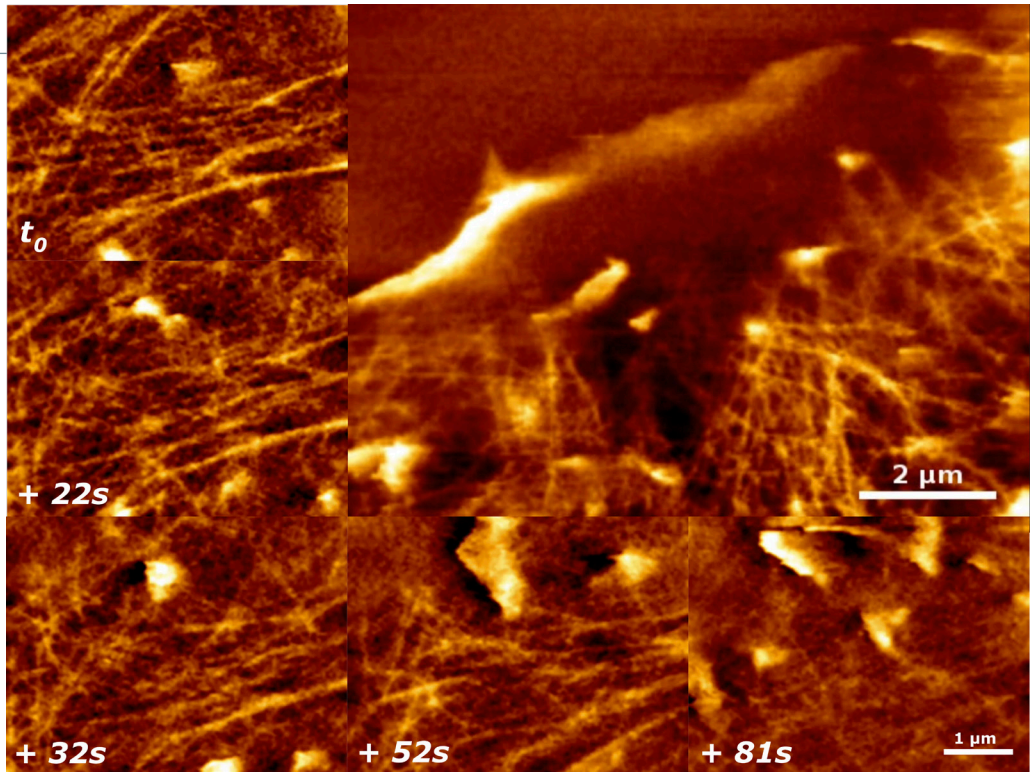

Figure 3: An overview (top right) of fast scanning of living Chinese hamster ovary (CHO) cells imaged in medium at 37 °C. Individual AFM time-lapse snapshots of an unassisted video acquisition (left and bottom panels), consisting of 600 consecutive AFM images taken with a line-rate of 48 lines/sec depicts remarkably diverse cell surface dynamics, involving cytoskeleton reorganization, as well as a highlighted membrane ruffle formation. Samples courtesy of Prof. Andreas Herrmann, Humboldt University Berlin (Germany).

This allows live cell surface imaging and non-invasive visualization of processes, such as filopodia morphogenesis, endo-/exocytosis, cytoskeletal reorganization and cell spreading with high spatiotemporal resolution. Figure 3 is a high-speed image of living Chinese hamster ovary (CHO) cells, indicating the cytoskeleton reorganization that occurs under the dynamic cell membrane at physiological conditions, in real-time. Long-term cell surface imaging with high spatial resolution using high-speed AFM is still restricted, due to the difference between the height of the cells and the typically

shorter z-range of the high-speed AFM scanners – often resulting in severe cellular damages. To circumvent these issues, a dual scanner technology (NestedScanner) has been developed which enables high-speed imaging of highly corrugated surfaces up to 16.5 µm.

Introduction of high-speed AFM in a correlative microscopy setup, is an example of how new biophysical imaging modalities are required for direct visualization of cellular behaviour and processes ("cellulomics"). A correlative microscope combining high-speed AFM, QI mode, and laser scanning confocal microscopy features mechanical stimulation and nano-manipulation, while simultaneously applying minimal force for real-time imaging of living bacteria, yeast, and mammalian cells for the study of cell mechanics and surface biochemistry.[17] This can be further extended by the application of single-cell fluidic force microscopy – a correlative integration with the fluidFM technology – recently employed to study cell-cell and cell-microenvironment interactions by measuring adhesion forces and stress dependent molecular interactions in living bacteria and yeast cells.[27, 28]

High-resolution Molecular Imaging

The transition from qualitative to quantitative data acquisition is enabling life scientists

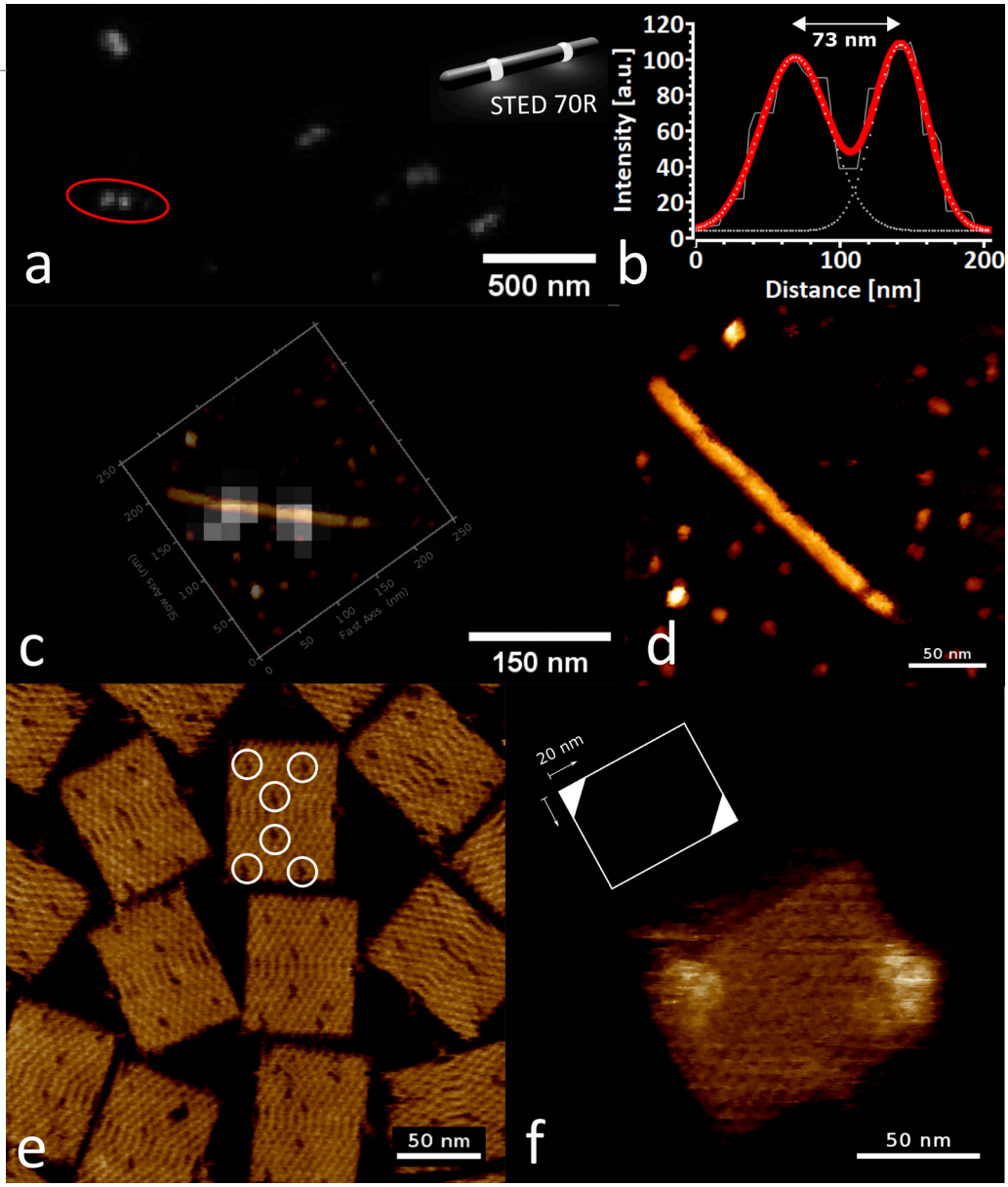

Figure 4: Correlative microscopy of DNA nanostructures, consisting of STED measurements of STED 70R nanorulers (a) and topographic AFM images (d). Inset in (a) is a sketch of GATTA quant nanoruler with a 70 nm mark-to-mark distance between two positions labeled with Atto 647N (reproduced with permission from Gattaquant DNA Nanotechnologies GmbH. (b) shows a bi-sigmoidal Gaussian fit of the intensity signal along the signified cross-section in (a). The optical correlation of the consecutively acquired STED and AFM images of the DNA nanorulers is shown in (c). Fast AFM images of two different DNA origami lattices, without a fluorophore (e), and carrying Atto 647N labels (f) have been recorded at 10 lines/s. The six visible gaps in (e), labeled with circles, are introduced via shortened staple strands, and serve as designated binding sites for other molecules. The inset (f) signifies the locations along the lattice, which are supposedly carrying the fluorescent tags.

to measure biomolecules at the molecular level. Using this data, researchers can explore various metabolic and developmental processes, namely understanding various diseases, disease progression, disease probability, self-assembly processes, and drug delivery.[29] The development of super-resolution microscopy surpassed the Abbe diffraction limit, which for STED microscopy led to a near 10-fold increase in optical resolution.[30]

With the recent advances in DNA nanotechnology, the ability to create DNA nanoscale structures has made possible new design strategies for research in synthetic biology and molecular machines. These strategies increase the development of diverse complex DNA-based structures/assemblies (DNA origami, DNA nanopillers, DNA nanochambers) for application in various fields, namely drug design and development, controlled and targeted drug delivery, and self-assembled electronics.[29, 31] DNA origami refers to the programmed folding of small molecules to create 2D and 3D shapes with nanometer precision. It is a flexible and versatile new tool that can be used in emerging super-resolution microscopes.[32] DNA origami nanorulers are commercially available as calibration standards for distance measurement in super-resolution systems, such as STED microscopy. Figure 4 shows correlative microscopy images of DNA nanostructures, such as images of nanorulers by AFM and AFM/STED microscopy. There is ongoing development in DNA origami and correlative AFM for non-invasive, high-resolution, and high-speed characterization of molecular structures in milliseconds with enhanced sensititvity.[33]

Tissue Mapping in Health and Disease

Diseases can cause various structural and compositional changes in tissues and organs at the cellular level. These changes can be studied to investigate disease initiation and progression, while tissue mapping serves as a tool for the identification of such markers. The softer nature of biological tissues and limited resolution are challenges associated with conventional imaging tools for mapping of diseased or healthy tissues. To circumvent these shortcomings, high-resolution correlative AFM can be used for tissue mapping at nanoscale resolution to identify structural or compositional changes in tissue samples, for example cancer and osteoarthritis pathological conditions.[34, 35]

Osteoarthritis is a degenerative disease that is characterized by articular cartilage

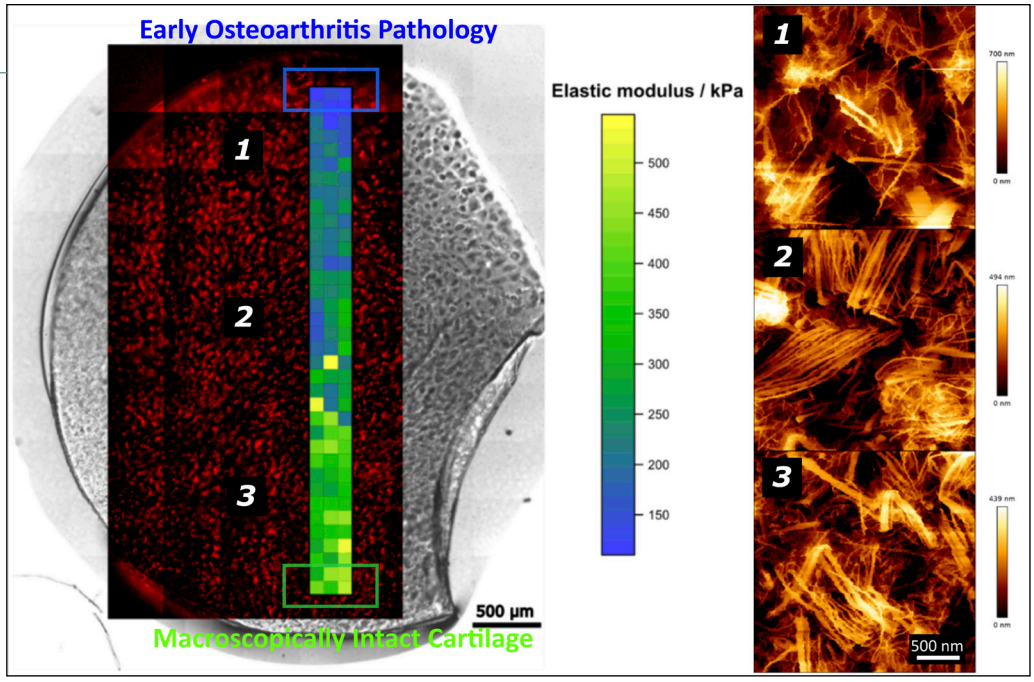

Figure 5: Large-scale force mapping and AFM imaging of a human osteoarthritic cartilage sample. Following a brightfield view of the 4 mm wide sample slab, with 50 fluorescence images mapped over its surface (left panel), an area was selected for automated analysis via 108 force maps, allowing determination of the mechanical properties of the sample. Correlating mechanics and topography in three arbitrary AFM regions (right panel) reveals two different superficial chondrocyte spatial organizations, namely early osteoarthritis pathology (top) and macroscopically intact cartilage (bottom). The higher resolution height images (right) reveal a clear loss of fibre alignment in the osteoarthritic zones, associated with cartilage remineralization. Sample and analysis in collaboration with the group of Thomas Hugel, University of Freiburg, Germany.

degeneration and bone sclerosis, however the current understanding of the structural and mechanical characteristics of healthy and diseased cartilage at the nanometre scale or molecular level is limited.[36] AFM is a minimally invasive nanoindentation tool, used for the real-time monitoring of age-related structural and biomechanical changes to help diagnose the onset of osteoarthritis.[37] Correlative AFM combined with quantitative AI-supported optical biopsy can also help the detection of early osteoarthritis pathology by measuring and correlating the nanostiffness and local tissue architecture.[38] Figure 5 shows a map of a human osteoarthritic cartilage sample and the correlation of nanomechanics and topography of two different superficial chondrocyte spatial organizations to better understand early osteoarthritis pathology. The hybrid fluorescence-AFM setup comprised of a HybridStage™ and an advanced optical tiling tool (DirectOverlay 2) was utilized to map local changes in the organization and stiffness in samples from patients with early osteoarthritis conditions with macro to nanoscale precision.[39] The HybridStage tool enables the AFM to conduct multi-parametric characterization of samples in high resolution. This piezo-based modular sample scanner avoids the constraints of the AFM piezo range and permits automatic, motorized sample movement in the XY directions by executing repetitive patterns. The corresponding optical images are tiled and displayed to enlarge the microscopic optical view.[40] The optical tiling procedure helps the user to select the AFM positions for mechanical maps in the integrated optical fluorescence image.

What Does Bruker Offer?

Bruker has developed sophisticated technology for correlative AFM microscopy that enables scientists to image living systems at the nanoscale with great ease. Figure 6 shows a correlative bioAFM setup integrated with confocal laser scanning microscope that is ideal for microscopy core facilities.

Figure 6: A NanoWizard AFM combined with a Leica TCS SP8 confocal LSM at the Institut für Physiologie II, Münster, Germany. Image courtesy of Prof. Herman Schillers.

The NanoWizard® 4 XP BioScience AFM

The NanoWizard 4 XP BioScience AFM delivers atomic resolution and a large scan range of 100 µm in a single system, enabling fast scanning (up to 150 lines/sec) and seamless integration with advanced optical techniques. The previously discussed NestedScanner technology enables high-speed imaging of corrugated surfaces up to 16.5 µm with outstanding resolution and stability. The optional HybridStage is a modular, piezo-based sample scanner stage combined with motorized XY sample movement, giving direct access to anything you can see. It is a versatile tool for the

Figure 7: NanoWizard 4 XP BioScience AFM mounted on an inverted optical microscope from Zeiss and a 300x300x300 µm³ scan range HybridStage.

advanced, multiparametric AFM characterization of mm-to-nm samples that alleviates the constraints of the AFM piezo range. A variety of samples can be studied, including biomaterials, cells and microaggregates, embryos and tissues, model organisms in developmental biology and implants.

Its wide range of modes and accessories for environmental control, as well as its capabilities for the mapping of nanomechanical, electrical, magnetic, or thermal properties, makes NanoWizard 4 the most flexible system available on the market today.

The NanoRacer® High-Speed AFM

Figure 8: NanoRacer – a high-speed AFM for real-time video rate measurements of molecular dynamics at 50 frames per second.

The NanonRacer stand-alone AFM marks a quantum leap in true high-speed imaging capabilities. It has never been easier to visualize dynamic biological processes in real-time with nanometre resolution. The NanoRacer opens a world of new and exciting possibilities for life science applications, enabling researchers to gain an in-depth understanding of complex biological systems and molecular mechanisms in a way that was not possible until now. Developed with the user in mind, NanoRacer features a robust, reliable design and a host of new features that make it extremely easy to use, even for AFM newcomers. All components are designed for convenient handling, from sample preparation through to fully motorized and automatic optical alignment. The streamlined handling enables easy data collection and fast results. A short time-to-data is vital for achieving dynamic results on active single molecule samples.The modularity and flexibility of the system ensures that the NanoRacer and NanoWizard 4 XP can be separately operated with a single controller, thus enabling the largest possible spectrum of measurements at the same core facility.

Outlook for High-speed Correlative Microscopy

High-speed correlative microscopy (integrated with high-end optical microscopy) has nanometre scale resolution and the ability to image living cells or tissues in liquid without damaging the biological samples. It is extensively utilized for measuring and correlating structural and functional properties of biological samples with high sensitivity, helping study dynamic cellular and molecular events, healthy and diseased tissue mapping, and mechanobiology. Correlative AFM offers possibilities for remarkable characterization of a wide range of biological systems and addresses outstanding questions in biology, promising further advancements in complementary microscopic techniques in the coming decades.

Learn more about how Bruker's BioAFM products, accessories, and applications can help advance your correlative life science research at www.bruker.com/bioAFM.

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