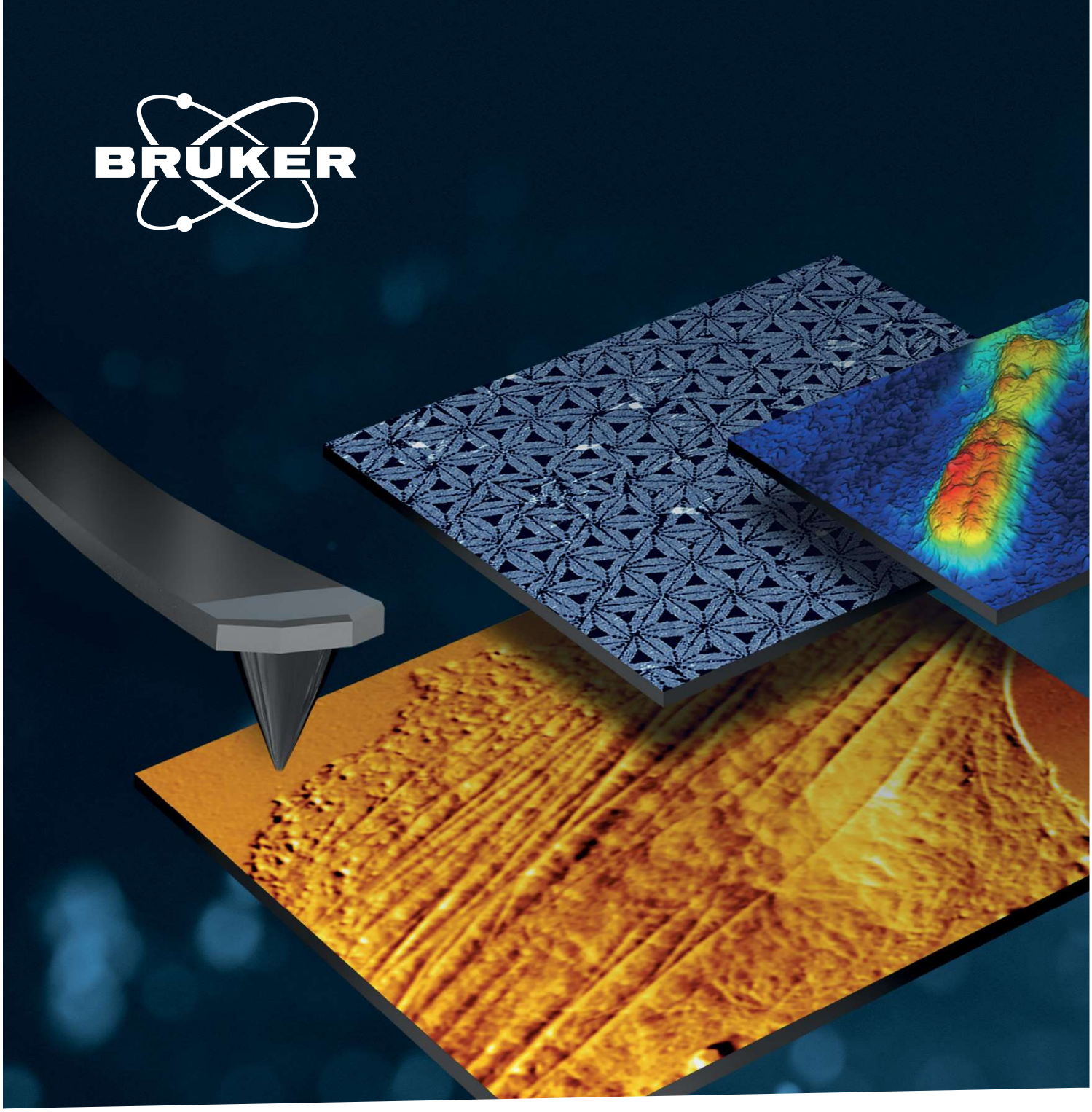

ATOMIC FORCE MICROSCOPY

AFM in the Life Sciences

An Overview

Table of Contents

Foreword

Over the last three decades atomic force microscopy (AFM), a high-resolution surface analysis technique, has become an invaluable research method in labs around the world.

Since its introduction in 1986, the use of AFM in the investigation of biological samples has grown steadily and the technique has gradually developed into a complete toolbox for imaging soft biological samples in liquid. The instruments developed around this technique, commonly referred to as BioAFMs, enable the unique investigation of samples ranging in size from single molecules, viruses, and proteins to living cells and tissues.

Unlike other conventional microscopic techniques, such as fluorescence and electron microscopy, AFM can easily be performed under near-physiological environmental conditions without the need for further sample processing or modification. It enables the real-time, high-resolution visualization of complex biological systems and dynamic processes, as well as the comprehensive characterization of their biomechanical properties.

Furthermore, the ability to easily combine AFM with advanced optical microscopy leverages the advantages of immunolabelling techniques to enable true correlative microscopy.

Introduction

Origins of Microscopy for Biological Sciences

Living cells and tissues are highly organized systems comprised of functional and structural subunits ranging from single molecules to macromolecular structures and organelles that are each vital for life. The fascination for investigating biological matter dates back to the first successful microscopic attempt to observe the cell walls of plant cork by Robert Hooke in 1665 [1]. The first microscopic observation of living cells (*Spirogyra* algae), however, is credited to Antonie van Leeuwenhoek in 1674 [2]. The optical microscope used by van Leeuwenhoek had an approximate two-hundredfold magnification [3]. Since then, various microscopy techniques have been developed that surpass the resolution limit of conventional optical microscopes, revealing continuously greater details in the samples under investigation.

A fundamental leap in microscopy resolution was the invention of the electron microscope in 1930 by Ernst Ruska and Max Knoll [4]. This electron microscope was used by Ladislaus Marton from 1932-1934 to image the first fixed biological specimen, sundew plant leaves [5]. Then, in 1934-35, Eberhard Driest and Heinz-Otto Müller imaged the first biospecimen, the wings and legs of a housefly [6]. Shortly after, Friedrich Krause imaged diatoms, epithelial cells, and bacteria [7, 8].

The principle of both optical and electron microscopy is based on the interaction of either photons or electrons with a sample surface, which, depending on the wavelength used, results in a different resolution. However, in general, neither of these imaging techniques deliver information on quantitative topography of the sample, making them a two-dimensional imaging techniques [9].

The first three-dimensional nanoscale images were acquired in 1982 by Gerd Binnig and Heinrich Rohrer with a technique called *scanning tunneling microscopy* (STM) [10]. In STM, a sharp metal tip

is brought very close to the surface of a sample while an electrical voltage is applied between the tip and the sample, causing electrons to flow between them. This so-called tunnel current is a quantum mechanical effect. By keeping the distance-dependent tunneling current constant while scanning the tip over an area of interest, it is possible to map the topography of the surface at the molecular or even atomic scale. However, to use STM, the sample must be conductive. This is a fundamental limitation for studying biological matter, as the sample needs to be coated with a conductive material in order to be imaged [11]. Nonetheless, STM was used for the first time in 1985 to image biological specimens by Baro et al. [12].

In 1986, the same year in which Binnig and Rohrer were awarded the Nobel Prize in Physics for inventing STM, Binnig and his colleagues replaced the original stiff STM tip with a flexible mechanical cantilever, also known as probe, which led to the invention of atomic force microscopy (AFM) [13]. AFM is based solely on the detection of the interaction forces between the sample surface and a sharp tip attached to the outer end of a flexible lever (mechanical spring), whereby the force-dependent deflection of the lever is measured and quantified (see Figure 1).

The invention of the atomic force microscope not only overcame the resolution limitations of optical microscopes, but also eliminated the need for samples to be conductive. The development of probes with nanometer-sized tip radii and precise XYZ-positioning systems advanced the capabilities of AFMs to enable routine high-resolution, 3D topographic imaging and nanomechanical characterization of biological samples.

Atomic force microscopy has come a long way since its introduction in 1986 [13]. Notable landmarks in the characterization of biological matter include the first image acquired in liquid in 1987 [14], the first observation of a biomolecular process in 1989 [15], the first scientific article on high-speed AFM in 1991 [16], the first high-resolution protein scan in liquid in 1994 [17], the first paper to describe the use of AFM in the study of protein unfolding in 1997 [18], and the measurement of cell-cell adhesion in 2000 [19].

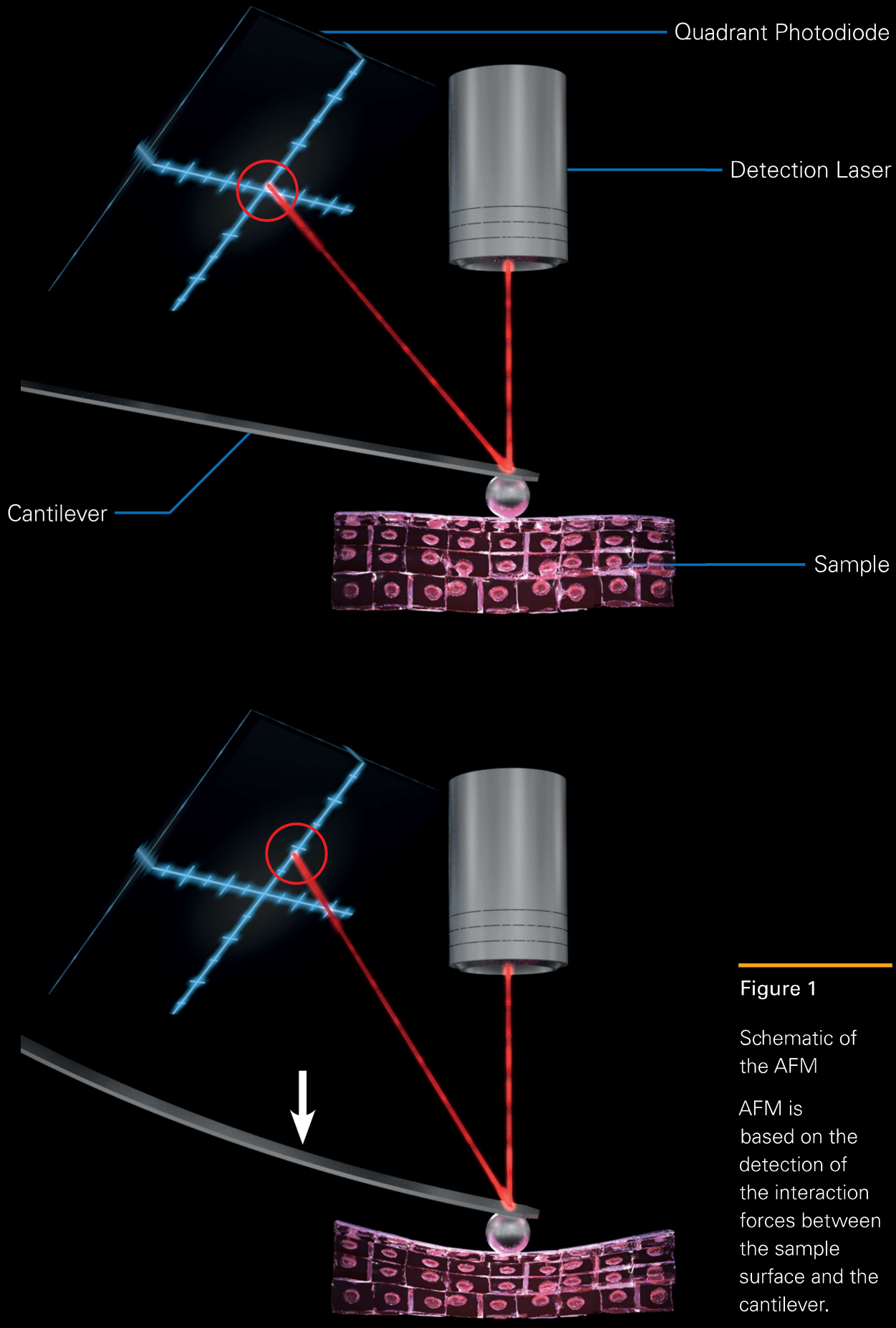

Figure 1

Schematic of the AFM

AFM is based on the detection of the interaction forces between the sample surface and the cantilever.

Comparison of Atomic Force Microscopy with Other Microscopy Techniques

The three most common microscopy methods used in the study of biological matter are optical microscopy, electron microscopy, and atomic force microscopy. The operating principles, imaging conditions, resolution, and sample preparation necessary for each technique differ considerably. A brief overview of the main characteristics and differences is outlined below.

Optical microscopy can be divided into multiple categories, the main ones being bright-field, dark-field, oblique-illumination, fluorescence, phase-contrast, confocal, deconvolution, differential interference-contrast, and dispersion-staining microscopy. Over the past ten years, super-resolution techniques, a sub-category of fluorescence microscopy, have gained particular interest as the resolution of super-resolution microscopy (around 10-20 nm) overcomes the diffraction limit of classical optical microscopy (around 200 nm) [20, 21]. A main advantage of fluorescence labelling is its specificity, which facilitates the highly sensitive identification of specific molecules of interest. However, this mandatory sample treatment, particularly in living systems, is often complex, time-consuming, and, in certain single-molecule scenarios, not feasible without disrupting the function of individual proteins.

Electron microscopy is generally divided into two types: transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Electron microscopy uses an electron beam to create an image, replacing traditional optical lenses with electromagnets. The electron beam interacts with the sample to produce an electron diffraction pattern that is converted into an image. The very short wavelength of the beam enables an approximate thousandfold increase in resolution over classical optical microscopy. In order to be 'visible' to the electron beam, biological samples must be stained or coated with a conductive metal, which results in static snapshots of individual proteins, cells, tissues, and molecular interactions. In addition, electron microscopy operates under vacuum to reduce non-sample related electron scattering, making it impossible to image biological samples under physiological conditions.

Although efforts have been made to develop environmental electron microscopy techniques capable of imaging unstained biological specimens in solution, sample degradation is unavoidable due to the strong electron dose required to achieve high contrast and spatial resolution [22].

While atomic force microscopy was originally invented to visualize atoms on solid interfaces [13], it has gradually evolved into a technique capable of measuring samples of almost any material in any environment. It differs from both optical and electron microscopy in that it does not "see" the sample, instead an AFM "feels" or "probes" the surface. The simplicity of this approach enables the 3D visualization of molecules and living cells in their near native environment.

The use of very sharp tips with optional chemical modifications ensures a resolution on the nanometer scale, high enough to image individual molecules, and comparable to high-resolution electron microscopy. In addition, the use of calibrated probes enables highly accurate force measurements and the comprehensive quantitative characterization of both molecular interactions and the nanomechanical properties of a sample.

AFM is an ideal tool for studying delicate and challenging, living biological samples under near-native conditions. Minimal sample preparation is required and the technique is minimally invasive, allowing direct access to the sample. High-speed imaging capabilities enable the investigation of dynamic cellular and molecular processes [23]. In addition, the ability to combine atomic force microscopy with other microscopy techniques (correlative microscopy) and collect data from the exact same area of the sample, harnesses the advantages of both techniques and delivers complementary data.