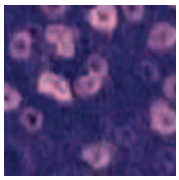
cutting edge developments



AFM Tomography

Looking inside the 3D matter

NTEGRA Tomo® features:

- Natural integration of AFM with ultramicrotome
- Tomography with ~ 10-20 nm resolution
- Large variety of imaging methods available

NT-MDT
Molecular Devices and Tools for NanoTechnology

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AFM principles

Atomic Force Microscopy (AFM) is a kind of Scanning Probe Microscopy allowing to get the surface images within microns-nanometers scale. In contrast to any other microscopy approaches AFM is based on registration of forces occuring between a very sharp probe and a sample surface.

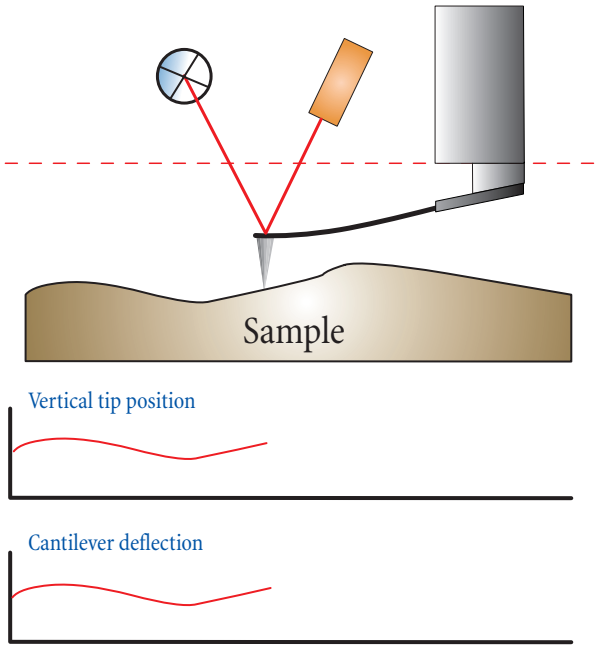

Fig.1 **Principle of the contact AFM mode. The probe attached to the long flexible cantilever is put in contact with the surface and then is moved along it. Following the surface relief the cantilever bends. This bending causes the laser beam deflection serving as an imaging signal.**

There is a good metaphor helping to understand how the image is built in the simplest AFM mode (the so called contact AFM). It is reminiscent to an old gramophone because in both systems sharp needle tip follow the surface relief and the signal is generated when the tip moves up or down (*Fig.1*). In AFM a very sharp tip is attached to a long, flat, and flexible cantilever. The back side of the cantilever is reflective. Laser beam falls on it so that even the slightest cantilever bending causes significant deflection of the reflected beam traceable by a photodiod detector. Thus in contact AFM techniques the image is made point-by-point and line-by-line based on the needle tip positioning information. This way the surface nano-scale topography can be drawn.

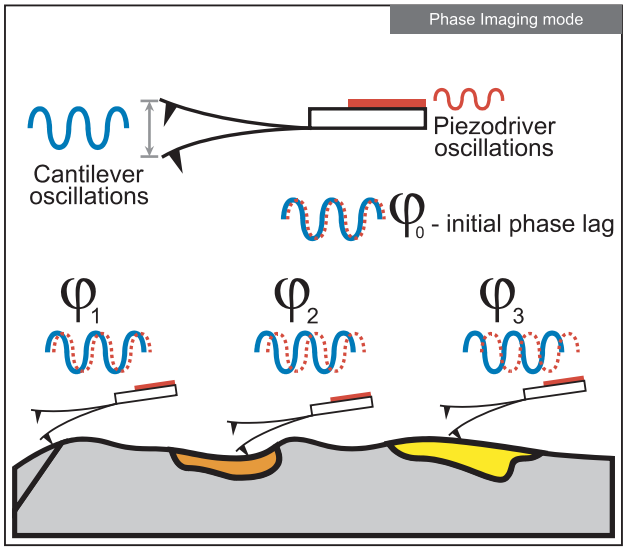

Fig.2 **In semi-contact and non-contact AFM modes the probe is forced to oscillate. Precise probe position all the time is still monitored by the laser beam deflection. When the tip strikes the surface oscillation amplitude is damped and the phase shifts. Depending on local material features oscillation parameters can differ in two neighboring points and this is the cause of an image contrast.**

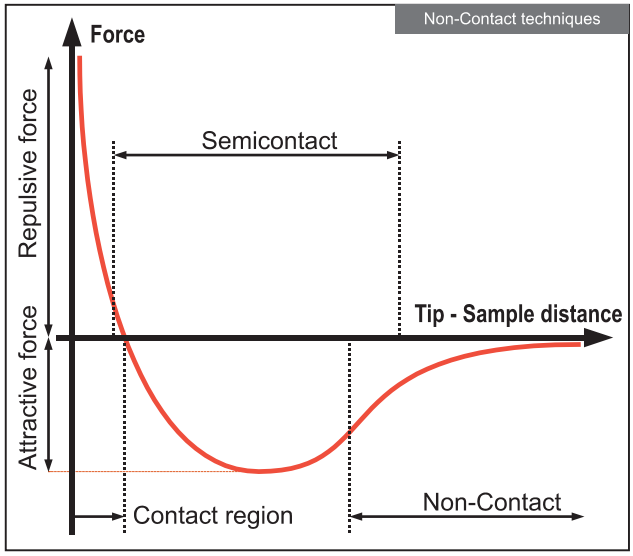

Fig.3 **Plot of forces acting between the probe and the sample in the proximity of the surface. When the probe is close enough to the surface attractive forces become detectable without direct tip-to-surface contact.**

In a most advanced set of AFM techniques (named the semi-contact and non-contact AFM) the needle goes repetitively downward the surface and upward again (usually with kilohertz frequencies). Instead of tip position itself the oscillation parameters are traced (like phase or amplitude) and point-by-point changes in it form the image contrast *(Fig. 2)*. This way even very smooth surfaces can be imaged with high contrast based on local differences in material characteristics.

Notice that the tip can "sense" the surface without direct touching (interacting through long-range attractive forces *Fig. 3)*.

AFM can reveal details of a polymers and biomaterials in the nanometre range

Nowadays the scanning probe instrument characteristics (probe geometry and planar interface, signal-to-noise ratio) allows one to access structural details on a sub-nanometer scale (*Fig. 4*). It means that AFM imaging can be done at the level of individual macromolecule (*Fig. 5, 6*).

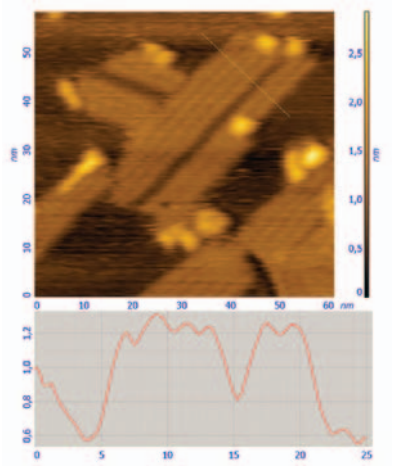

Fig. 4 **AFM image of two-dimensional dodecylamine crystals. Line profile shows the critical resolution of about 1 nm.**

Image courtesy of Dr. D.V. Klinov, Shemyakin & Ovchinikov Institute of Bioorganic Chemistry, RAS, Moscow, Russia.

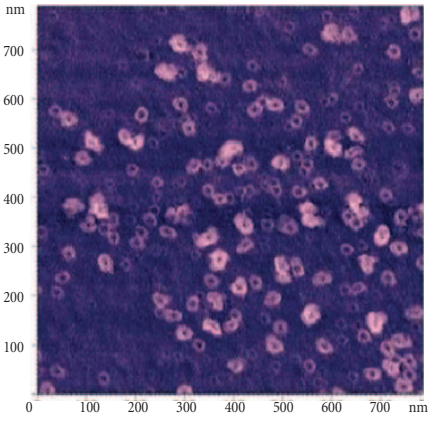

Fig. 5 **Ring-like molecules of poly-(phenylenevinylene) as seen in phase imaging (semi-contact) AFM mode. Ring diameter is about 20 nm.**

Sample courtesy of Dr. M.M.Koetse, DPI, Eindhoven, Netherlands.

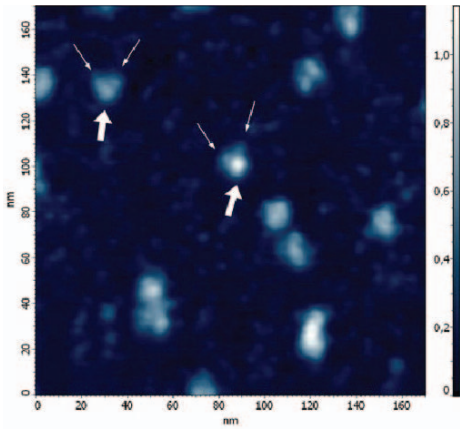

Fig. 6 **AFM image of ricin molecules (plant ribosome-inactivating protein type II). In some molecules the sub-molecular structure can be recognized: A-subunit domain (fat arrows) and two B-subunit domains (fine arrows).**

Sample courtesy of Prof. A.G. Tonevitsky and Dr. I. I. Agapov, Institute for transplantation and artificial organs, Moscow, Russia.

AFM image of an embedded biological object can be referred to the TEM one

The interpretation of new results in any subdivision of microscopy in life sciences is based on our knowledge about cellular ultrastructure that has been obtained by transmission electron microscopy of ultrathin section of biological material. In TEM, the ultrastructure is visualized by staining with heavy metals salts. Thus, only the structures, which react with the staining agents, and which can be reached by the staining agents are detected. Consequently, cell organelles, membranes, protein filaments, and nucleic acids are clearly observed, but many proteins in the cytoplasm of the cell are practically invisible. AFM, on the contrary, provides information about the cell constituents that are distributed on the surface of the section or block face. Therefore, due to the nature of the collected signal the macromolecular content (proteins, DNA, RNA, polysaccharides) in the cytoplasm can be detected by AFM (provided the cellular constituense are not damaged during the sample prepa-

ration). Using AFM, therefore, as a complementary to TEM microscopic technique gives us a chance to detect protein content of the epoxy fixed biosample and show new ultrastructural aspects in addition to TEM data (*Fig. 7*).

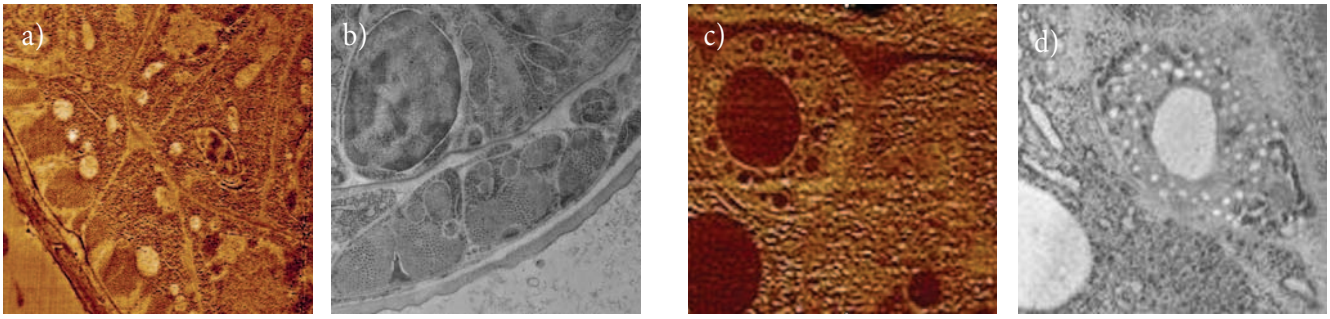

Fig. 7 **Two pairs of images of the similar object (high- pressure frozen and freeze- substituted nematode *C. elegans*, embedded in epoxy resin) obtained in AFM (left) and TEM (right). a) and b) — image size is 10x10 µm, c) and d) — image size is 2.8x3 µm. Sample and TEM images courtesy of Dr. M. Mueller and Dr. N. Matsko, ETH, Zurich, Switzerland**

AFM Tomography

The ability of modern high-resolution electron microscopes to produce images at near atomic resolution has led to tremendous progress in many fields of biological and material research. However, the vast majority of images are simply two-dimensional projections of a three-dimensional structure (Midgley & Weyland, 2003). This has enabled microscopists in many circumstances to regard the third dimension as constant and interpret the image accordingly. Therefore, the development of high-resolution tomography techniques is of major importance for many material and biological science disciplines.

At present, nanoscale resolution in three dimensions can be obtained with transmission microscopy (e.g. electron (TEM) or X-ray transmission(TXM)). However, these methods are only applicable to very thin samples (100nm in TEM), and this thickness restriction contradicts the requirement of larger volumes that have to be analyzed for the reconstruction of continuous network structures. Also should be noted that TEM or TXM can only be applied to very thin electron- and photon-transparent samples.

AFM tomography is based on a serial sectioning procedure, and allows one to reconstruct almost any polymer material including rather hard ones. 3D reconstruction in the **NTEGRA** Tomo can be obtained by automated block-face imaging combined with serial sectioning inside the chamber of an ultramicrotome (see specifications for **NTEGRA** Tomo later here) (*Fig. 8*). The spacing between the single sections is less than 20nm that allows one to reach the relatively similar resolution along all three orthogonal axes. In this way, the stack of acquired images can be directly transformed into a data volume.

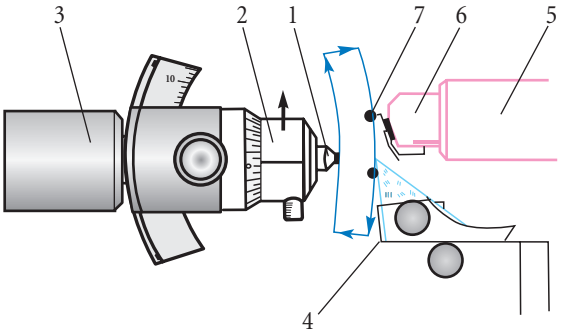

Fig. 8 **Principle scheme of the AFM tomography setup:**

1 – sample
2 – sample holder
3 – movable ultramicrotome arm
4 – ultramicrotome knife
5 – AFM scanner
6 – probe holder
7 – AFM probe

For example the 3D distribution of silica nanoparticles within the nano-composite polymer is shown on *Fig. 9*. Multicomponent polymer blends and polymer embedded biological samples also can be easily reconstructed (*Fig. 10 and 11* respectively).

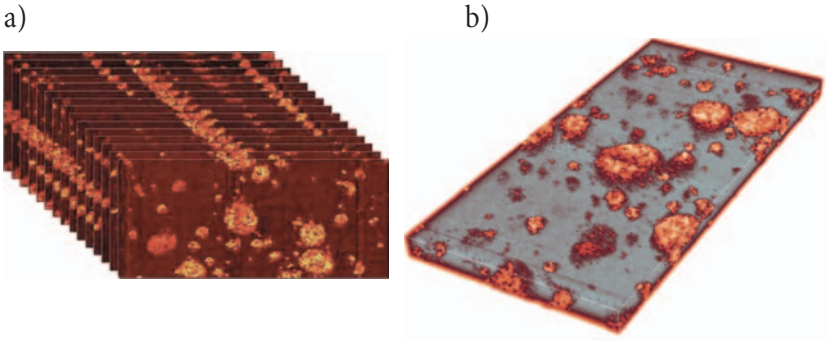

Fig. 9 **Sequential AFM images (a) and 3D reconstruction (b) of nanocomposite polymer material. Each individual image is 20x40 µm, spaces between them are 200 nm. Silica nanoparticles distribution within the polymer blend can be quantitatively evaluated on the resulting 3D model.**

Sample courtesy of Dr. Aliza Tzur, Technion, Israel.

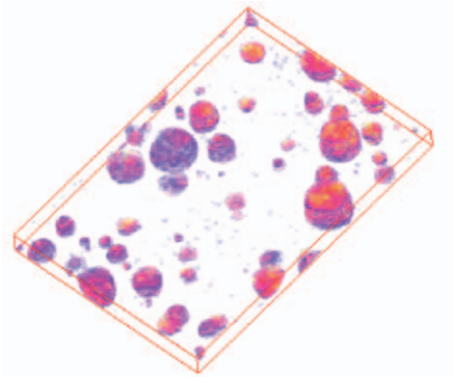

Fig. 10 **3D model of ABS/PA6 (Acrylonitrile-butadiene-styrene/polyamide 6) polymer blend structure (8.0x5.6x0.6 um, spaces between sections 40 nm).**

Sample courtesy of Christian Sailer, Institut f. Polymere, ETH-Honggerberg, Switzerland

Now commercially available AFM tomography system has been developed by the NT-MDT based on the most advanced LEICA Microsystems ultramicrotome UC6NT. **NTEGRA** Tomo uses convenient and highly automated working algorithm. The sectioning process controlled by a touch-sensitive display can be visually monitored by a stereomicroscope. The "clever" pre-programmed AFM needs only several mouse clicks to start and get high-quality images. 2-3 day training is enough even for non-experienced operator to become skilled and deal successfully with most of standard specimens.

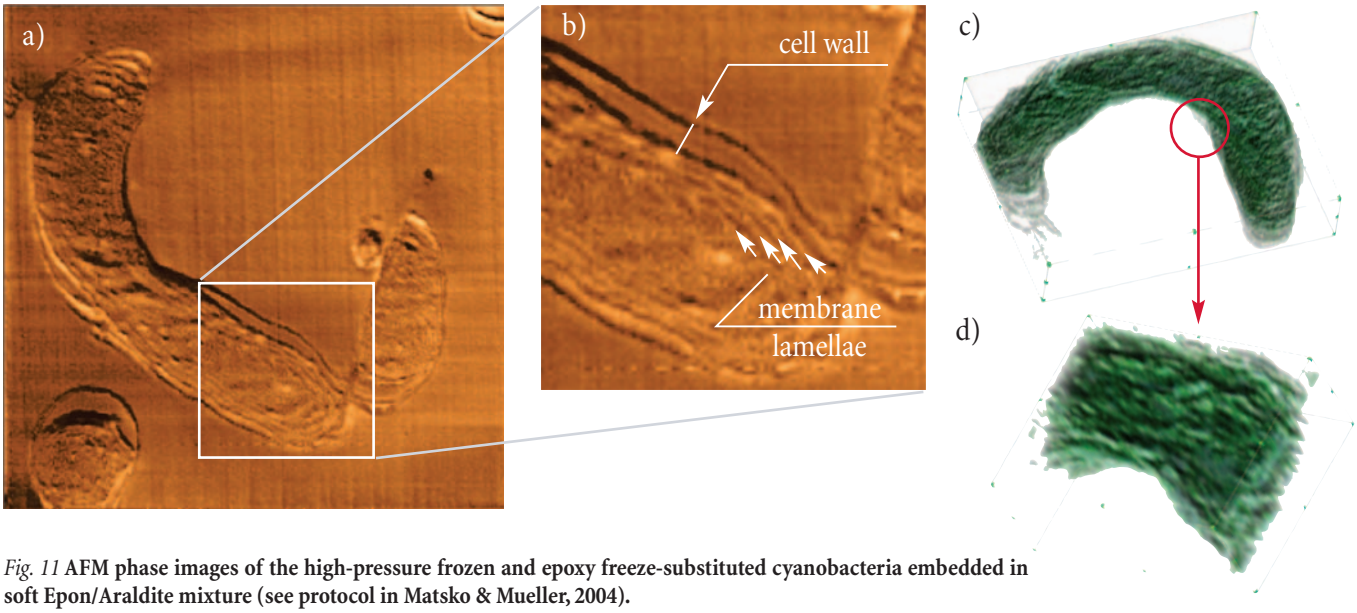

Fig. 11 **AFM phase images of the high-pressure frozen and epoxy freeze-substituted cyanobacteria embedded in soft Epon/Araldite mixture (see protocol in Matsko & Mueller, 2004).**
a) Typical 2D image (5x5µm, (phase imaging).
b) Enlarged fragment marked by frame on A. Note the internal membranes organized into photosynthetic lamellae.
c) 3D reconstruction made from a stack of 2D images of the whole bacterial cell (4.9x4.6x0.9 um, spaces between sections 50 nm).
d) Enlarged fragment of 3D volume reconstruction revealing the fine ulltrastructural details.
Sample courtesy of Dr. N. Matsko, ETH, Zurich, Switzerland.

Specifications

Scanning probe microscopy

in-situ: AFM (contact + semi-contact + non-contact) / Lateral Force Microscopy / Phase Imaging/Force Modulation/ Adhesion Force Imaging/ Magnetic Force Microscopy/ Electrostatic Force Microscopy / Scanning Capacitance Microscopy/ Kelvin Probe Microscopy/ Spreading Resistance Imaging/ Lithography: AFM (Force and Current)

Sample size		10x5x5 mm
Sample weight		Up to 10 g
Scan range		100x100x7 μm
Positioning resolution		5 μm
Non-linearity, XY		<0.15%
Noise level, Z (RMS in bandwidth 1000 Hz)		0.06 nm (typically), ⩽0.07 nm
Noise level, XY (RMS in bandwidth 200 Hz)		0.2 nm (typically), ⩽0.3 nm
Vibration isolation	Dynamic	Frequency range 0.7 – 1000Hz
	Passive	For frequencies above 1kHz

Ultratomy

Self locking		Yes
Graduation		±30° graduation
Clearance angle adjustment		-2° to 15° with 1°scale
Knife holder		For 6-12 mm knives
Coarse knife-movements	N-S	10 mm stepping motor
	E-W	25 mm stepping motor
Cutting window		0.2-15 mm adjustable
Cutting speed		0.05-100 mm/s wheel contr.
Section thickness		0-15000 nm wheel contr.
FEED / SPEED storage		5
Return speeds		10, 30, 50 mm/s
Step control		0.1-15 μm steps

Section counter		Yes
Feed totalizer		Yes
Count down		Yes
Rocking mode		Yes
E-W measurement		Yes
Auto trim		Yes
Specimen advance indicator		Yes
Working distance		110 mm
Universal specimen holder		2pcs.
Flat specimen holder		1p.
Instrument table	Dimensions	0.67 x 1.15 m
	Shock-absorbing elements	Yes

References:

- *" N. Matsko and M. Mueller. AFM of biological material embedded in epoxy resin. Journal of Structural Biology (2004)*
- *" P. Midgley, M. Weyland. 3D electron microscopy in the physical sciences: the development of Z-contrast and EFTEM tomography. Ultramicroscopy 96 (2003)*

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