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Application Notes

Methodology of biological macromolecules study with AFM in a semicontact mode

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Sample preparation

An initial protein specimen is dissolved in a proper buffer solution or water to working concentration. Normally, to obtain a sample with separated molecules, the concentration should be about 1 mg per 1 ml. For dissolving a specimen and a buffer or for rinsing, only pure water (distilled twice or deionized) should be used. It is useful to control the quality of the water sample prior to measurements.

To study protein molecules, mica is usually employed as a substrate. Proteins are bound to it due to electrostatic attraction. Before a specimen deposition, the mica surface layer is to be cleaved (cleaned with a scotch tape; this procedure is described in detail for graphite in the microscope Operation Manual).

The protein solution must be deposited onto the mica just after the cleavage. The volume of deposited specimen depends on a substrate size. The mica is the hydrophilic material so the drop is spread over all the substrate. The amount of the specimen should be such that it covers the convenient for the study area and does not spill. If mica size is 1 cm^2, the solution volume can be 5 – 10 ml.

Next, the protein should be incubated – allowed some time for the molecules to bind to the mica substrate. The recommended incubation time is 1-2 min. This time is enough to bind sufficient number of molecules and to keep sample from drying. If longer incubation time is needed (10 min and more), the sample should be put into a wet camera to prevent the liquid evaporation. Upon incubation, not drying the drop, the sample should be flushed to remove the unbound molecules and salts dissolved in the buffer. For that, the sample can be just put into water. Two rinses of 5 minutes in small amount (20 ml) of water are enough.

After the sample has been rinsed it should be dried – the remaining water is blown away with a compressed clean and dry gas (air, nitrogen, argon). It is important to blow the water drops away but not to dry them. In the absence of such a gas one may use a compressed air can for office equipment cleaning.

Thus we have prepared the sample – a piece of mica with deposited molecules on one side. It makes sense to mark this side, e.g. with a soft-tip pen, because sample can fall down and turn over.

Now, one can, in principle, proceed to the measurements. However, sometimes on fresh samples there are observed topography artifacts,

probably, due to mica properties and adsorbed water
(see. http://www.ntmdt.ru/SPM-Techniques/SPM-Methodology/Methodology_of_biological_macromolecules_study/Examples/text281.html#ref_1).

Also, study of fresh samples may result in tips contamination.
Therefore, if it is enough time, do the following. Take image of the fresh sample to make sure there are enough molecules, and then dry it for some more time in order to obtain better results. Drying of the sample in a desiccator with RH 5-10% for some hours (through the night) is a good choice. Moreover, samples should be kept under low humidity conditions.

Equipment requirements

That protein molecules are very small makes this study specific. For example, the immunoglobulin G (IgG) molecule with molecular mass 150 kDa consists of three domains, each having diameter about 5 nm. This demands special requirements to an instrument and a tip.

Instrument requirements

Both noise and drift characteristics of the instrument are of great importance. However, it is difficult to present numerical values of noise and drift necessary to obtain a good quality image because transition between good and poor quality image is not sharp but relative. Nevertheless, a good condition of quality image acquiring is the instrument ability to reach an atomic resolution. Good quality image, by experience, can be obtained with Solver and NTEGRA instruments in scan-by-probe configuration with 3 and 12 um scanners. Instruments with 50 um scanner give images of worse quality. Scanning measuring heads with 50 or 100 um scanners produces poor quality images.
Regardless of the instrument type, it is necessary that it is properly installed and the noise level in the room is minimal.

Probe requirements

In order to just visualize molecules on the substrate one can use standard silicon probes for the simicontact mode (NSG10 or 01). If protein subunits should be imaged, it is necessary to use tips with curvature radius less then 7 nm. Standard probes NSG10 or 01 of «S» series meet this requirement only partly: number of useful for the high resolution probes in the batch is from 0 to 20%. The proved for this purpose probes are “Nanowisker” and “HR-wisker” having curvature radius down to 5 nm
(see http://www.ntmdt.ru/SPM-Techniques/SPM-Methodology/Methodology_of_biological_macromolecules_study/Examples/text281.html#ref_2).

Measurements

Microscope preparation

To avoid drifts due to the instrument parts heating and laser warming up, the microscope should be warmed up before measurements for at least one hour with the laser on.

Sample mounting

The instrument manual recommends using a scotch tape to mount a sample to the polycrystalline sapphire substrate. However, this way of mounting is inconvenient for two reasons: first, mica fixing is valid for only one occasion because sample can not be unstuck without its destruction; second, a scotch tape has its own vertical drift, so to avoid this drift, one should wait for some hours. From our experience it is not necessary to glue or otherwise fix a sample when operating in air but just enough to place it onto the substrate.
Handling the mica, one should remember that it is a dielectric and is readily charged with static electricity. The static charge in the sample leads to practical impossibility of an image acquiring when cantilever oscillation amplitude is set for measurements of biological macromolecules. In the process of measurements, the charge presence can be determined by examining the amplitude curve shape
(see http://www.ntmdt.ru/SPM-Techniques/SPM-Methodology/Methodology_of_biological_macromolecules_study/Examples/text281.html#ref_5).

Mica is charged as a result of cleavage but this charge is removed in process of sample incubation and rinsing. Later on, a sample can be charged from

an operator or via contact with dielectrics: polycrystalline sapphire substrates, plastic boxes, packs, etc. Because it takes a long time to remove this charge (several hours depending on ambient air humidity) the following care should be taken to avoid sample charging:

- prepare and temporarily keep a sample on conducting grounded surface;
- prevent charge transfer from an operator to a sample during manipulations: ground forceps, choose proper garment, footwear, furniture;
- keep samples in special antistatic boxes or packs.

All these requirements are especially important if RH in the room is less than 45 %. Therefore, one should use for measurements plates with a grounded contact.

Laser adjustment

Laser should be adjusted to obtain the maximal ***MAG*** signal but not the laser maximum signal.

Control block diagram settings

Choose a microscope configuration for operation in the semicontact mode.

- Choosing and setting the probe oscillation initial (free) amplitude

Choosing parameters, one should remember that protein molecules are soft enough objects which can be deformed by a tip. That is why the obtained resolution is much affected by the cantilever oscillation initial amplitude (see http://www.ntmdt.ru/SPM-Techniques/SPM-Methodology/Methodology_of_biological_macromolecules_study/Examples/text281.html#ref_3).

Oscillation amplitude depends on the given probe characteristics so it will differ for various probes at the same generator output voltage. The oscillation amplitude of each probe can be determined more or less accurately from an experimental MAG(z) amplitude curve prior to scanning; nevertheless, it is recommended to preset the following parameters (for air-operated Smena or P-47):

set generator voltage 0.05 - 0.5 V using the «**(v)**» button and attenuate it 10 times by switching the amplification/attenuation stage to «**0.1**». For the microscopes and NSG11 cantilevers utilized in this study, this voltage applied to the piezo produced oscillation amplitude 5-20 nm depending on the cantilever. For the universal Smena SPM the voltage should be 10 times more.

- Choosing the «MAG» signal value

It makes no sense to set large values of this signal because with increasing the lock-in amplifier gain the «**FBgain**» value, at which the generation starts, is lowered. On the other hand, setting too small signal is inconvenient since proper selection of the operating point in this case is not always available. Choosing a value of few nA seems to be the best. It is selected via the lock-in amplifier gain.

Approaching

In order to reduce the tip influence upon the sample during the approaching one should set possibly large value of the operating point. In our case this was 2/3 of the «**MAG**» signal initial value that corresponded to the maximal value of **SP** at which approaching is performed at one attempt. This operation should be executed in the «**fine**» regime at speed 20. When tip-sample separation becomes 15-40 micron, an operator may manually decrease the speed. This moment is easily detected by the start of the amplitude decrease. For time consuming one may approach the sample manually to the distance 0.3-0.5 mm.

Correcting the amplitude and operating point from spectroscopy data

Before starting the scanning it is recommended to obtain amplitude and phase curves - MAG(z) and phase(z) plots. From these curves it is possible to determine first, the tip-sample interaction regime (see http://www.ntmdt.ru/SPM-Techniques/SPM-Methodology/Methodology_of_biological_macromolecules_study/Examples/text281.html#ref_4), and second, the true value of the probe oscillation initial

amplitude. Moreover, abnormal shape of the amplitude curve can evidently reveal the sample charging or the tip sticking (http://www.ntmdt.ru/SPM-Techniques/SPM-Methodology/Methodology_of_biological_macromolecules_study/Examples/text281.html#ref_5). If everything is in order (no tip sticking and sample charging take place) one can proceed to scanning, correcting, if necessary, the initial amplitude of the probe oscillations and working amplitude (http://www.ntmdt.ru/SPM-Techniques/SPM-Methodology/Methodology_of_biological_macromolecules_study/Examples/text281.html#ref_6).

Scanning

To diminish the image distortions due to the scanner nonlinearity and creeps, one should choose the area of interest in the center of the maximum scanning area. Upon changing the area, it is necessary to pause scanning before the start for some time until the drift ends.

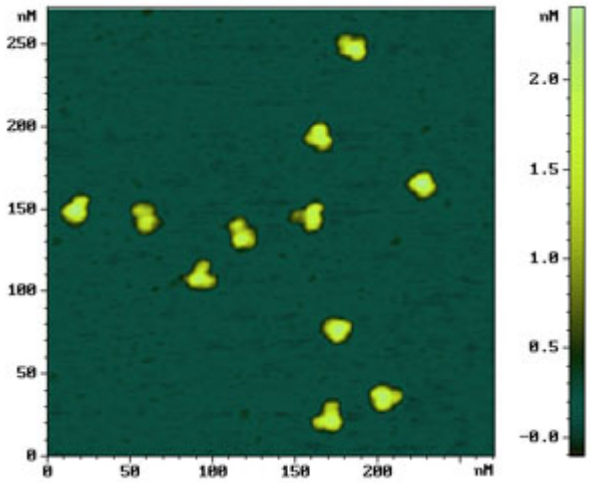
The approximate scan settings can be as follows:
- signal – height;
- scan size – 1 micron and less, in the center of the field, the “move” button released;
- number of data points – 512*512, if points number is less, the image quality is poor;
- velocity – 1-2 Hz;
- ADC gain – 10;
- substrate slope – for preliminary scans; the final scans are performed without any filters (for the DOS version of the program).

Image processing

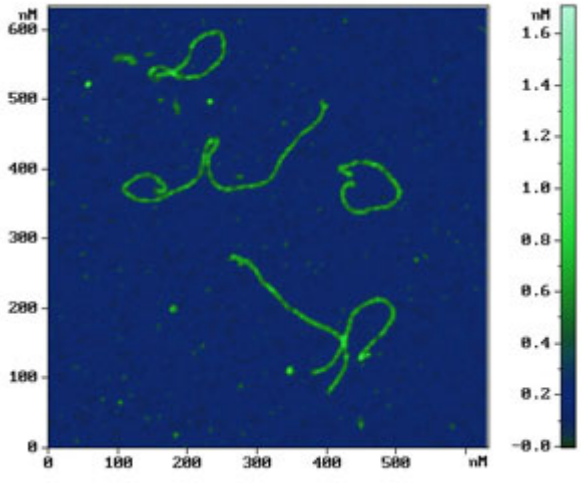
Typical operations of acquired images processing are:
- plane subtraction;
- slopes of different order subtraction;
- fit-lines over several areas with no molecules;
- filtering, e.g. median 3x3, 5x5 or 7x7 (for quite small scans).

Results

Results of plant toxins and immunoglobulin molecules study conducted in accordance with described procedure are given in [http://www.ntmdt.ru/SPM-Techniques/SPM-Methodology/Methodology_of_biological_macromolecules_study/Examples/text281.html#ref_1]. Moreover, in the scan-gallery of the NT-MDT Co. site are presented images of antibodies of isotype IgG and collagen molecules (Fig. 1).



a)



b)

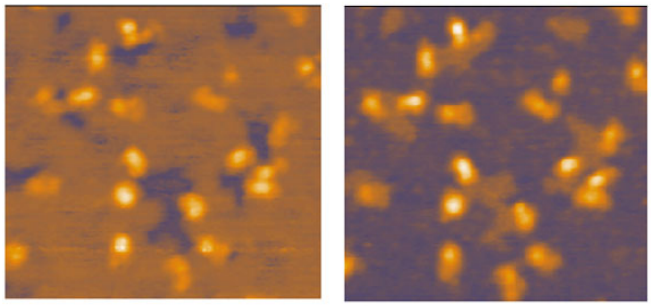
Fig 1.
a) Monoclonal antibodies 1RK2 against A-chain of ricin (IgG1)
b) Collagen molecules (native collagen of I-III type from rabbit skin)

Examples

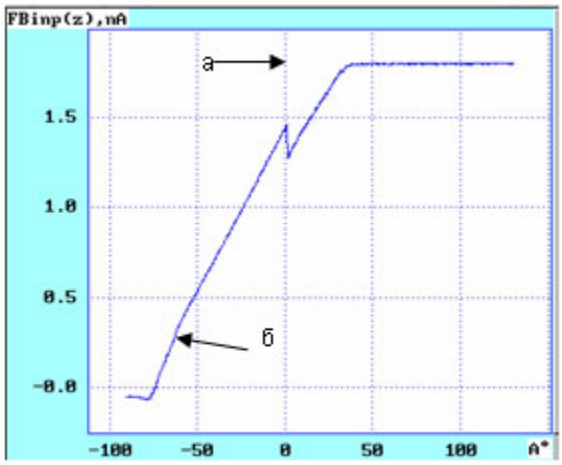
- Example 1

Fresh samples topography features

In the course of fresh samples study there often are imaged features that change the contrast depending on the measurement regime (Fig. 1): if the working amplitude is a slightly below than the initial one, they look as holes (Fig. 1a) but if the working amplitude is close to zero, they look as hills (Fig. 1b). Such «hill-valley» features make the image interpretation difficult and deteriorate resolution. As the sample is drying, the number of such objects, as well as their depth (height) and area are decreasing down to almost complete disappearing (dry sample images obtained under various measurement regimes are shown below in Fig. 9).



a) b)



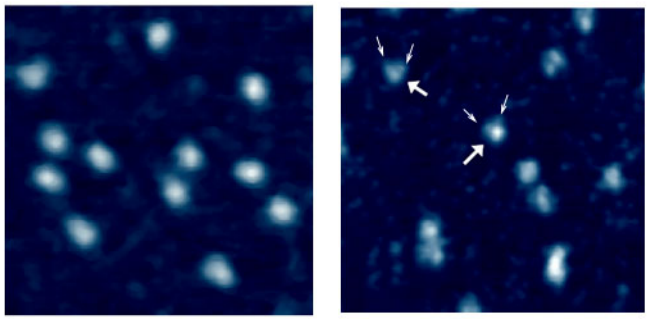
c)

Fig 1. Images of the same sample area, illustrating the effect of measurement conditions. Sample – ricinus agglutinin molecules on mica. a) working amplitude slightly below initial, b) working amplitude close to zero. Scan size 180x180 nm. c – amplitude-distance curve. Arrows mark working amplitudes for images a) and b)

- Example 2

Effect of tip sharpness

Images of objects whose size is about the tip curvature radius depend much on the employed tip sharpness (Fig. 2).



a) b)

Fig 2. Images of the plant toxin ricin molecules with molecular mass about 60 kDa, illustrating the effect of the probe sharpness. Scan size 170x170 nm, height – 2 nm. a) Image obtained using standard silicon probe of NSG11S series. Molecules in the image appear globular with no domain structure. b) Image obtained using "Nanowisker" probe. This probe allows to examine the structure of ricin molecules in detail. One can see that molecules on the substrate have different conformations. Some well situated molecules demonstrate their domain structure: A-subunit domain (thick arrow) and two B-subunit domains (thin arrows) are well resolved

- Example 3

Effect of the probe oscillation initial amplitude on the image quality

The image quality depends on the probe oscillation initial amplitude. The best quality images for standard cantilevers NSG10S with spring constant about 10 N/m can be obtained in the amplitude range 5-15 nm. The increasing in the initial amplitude results in resolution deterioration (Fig. 3b).

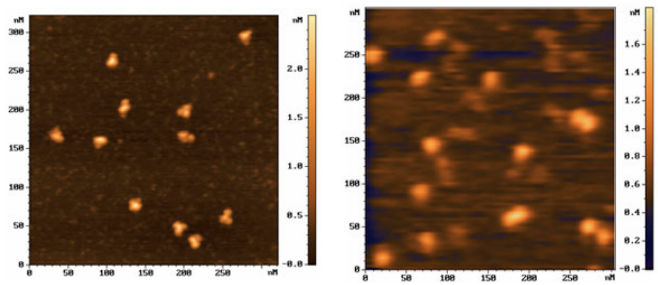


Fig 3. a) Image of immunoglobulin IgG molecules obtained at initial amplitude 10 nm. b) Same at initial amplitude 34 nm.

- Example 4

Tip-sample interaction regimes

A probe's oscillations near a sample surface are influenced by different forces which can be both attractive and repulsive. At large tip-sample separations the attractive Van der Waals forces dominate but when tip makes contact with the surface, the elastic repulsion and adhesion forces appear. When measurements are conducted in air, the capillary forces accrue because both tip and sample are covered with adsorbed water. Operation in the «semicontact» mode is based on the fact that a cantilever *effective* resonant frequency changes due to these forces which results in the cantilever oscillation amplitude change at constant drive frequency. In [4] it was shown that there are two major regimes of tip-sample interaction: *attractive regime* and *repulsive regime*. The *attractive regime* takes place when the resultant interaction force is the attraction force. If, vice versa, the resultant force is repulsive one, the *repulsive regime* takes place. According to this model the tip-sample interaction is as follows. As tip approaches the surface, the attractive regime dominates first because only attractive long-range Van der Waals forces act upon the vibrating probe decreasing the effective resonant frequency of the cantilever. Upon further approaching the surface, the tip starts to contact it part of period time and repulsive forces begin to contribute to the interaction. However, as long as attractive forces exceed the repulsive ones, the system remains in the attractive regime. When the tip is brought so close to the surface that averaged over period repulsive forces begin to exceed the attractive ones, the resonant frequency sharply increases and the system turns to the repulsive regime.

Notice that this model does not take into consideration the capillary forces. In practice, to determine which tip-sample interaction regime takes place, one should examine the amplitude and phase curves. In Fig. 4 numbers mark the curves parts corresponding to different interaction regimes:

- *No interaction*: amplitude and phase do not change because distance to the surface is large and there is no interaction.
- *Attractive regime*: oscillation amplitude decreases due to attraction forces. Phase exceeds 90 degrees.
- *Transition between regimes*: amplitude suddenly increases while phase suddenly decreases to less than 90°.
- *Repulsive regime*: oscillation amplitude decreases due to repulsive forces. Phase is below 90 degrees.

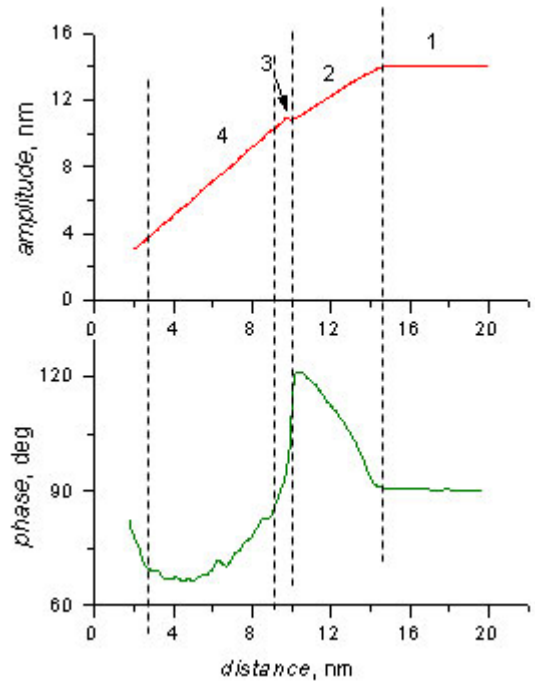


Fig 4. Probe oscillation amplitude (upper curve) and phase (bottom curve) vs. distance plots obtained at the same point of the sample. Numbers mark parts corresponding to different tip-sample interaction regimes: 1 - no interaction, 2 - attractive regime, 3 – transition between regimes, 4 – repulsive regime. Probe NSG10.

The working amplitude at which the system transition between regimes takes place depends on the cantilever stiffness and initial (free) amplitude of the probe oscillations (Fig. 5). At relatively large amplitudes (more than 25 nm for the probe NSG10 with resonant frequency about 250 kHz) this transition occurs at the working amplitude equal 0.8 of the initial one. In case of small amplitude (5.5 nm for the same probe) there can be no transition to the repulsive regime at all. For the case of intermediate amplitude (14 nm in Fig. 4) switching to another regime takes place in about the middle of the working amplitude range.

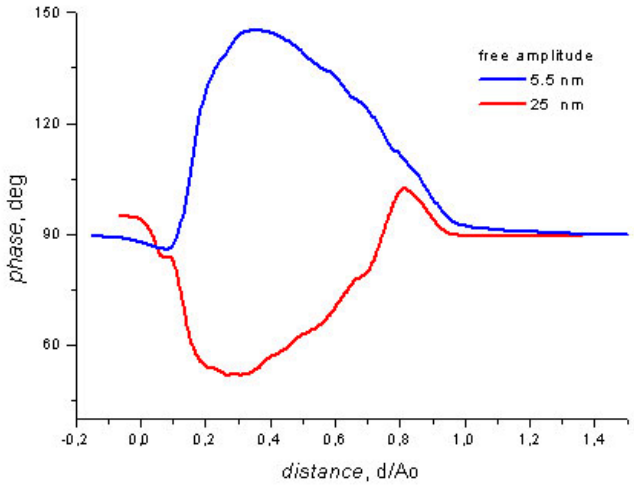


Fig 5. Probe oscillation phase vs. distance plots at different free amplitudes. If free amplitude is 25 nm, the transition to the repulsive regime occurs slightly below this amplitude. If free amplitude is 5.5 nm, there is no transition at all

- Example 5

Abnormal amplitude-distance curves

In the previous example the amplitude-distance curve dominated by Van der Waals, adhesion and elastic repulsive forces was examined in detail. Such dependence can be considered as the desired one for the «semicontact» mode. However, sometimes another amplitude curves are observed.

1. Charged sample

Examining the amplitude curve shape one can make sure that sample is charged (Fig. 6). In this case, as it is seen from the picture, the amplitude begins to decrease gradually at large distance from the sample surface. This occurs because the cantilever resonant frequency shifts due to the long-range electrostatic forces action. As a result, first, approaching is a problem since the amplitude decreases to the set working value far from the surface. Second, imaging is possible only at small working amplitudes (less than 0.5 nm in the given example) because at large amplitudes scanning will take place in air far from the sample.

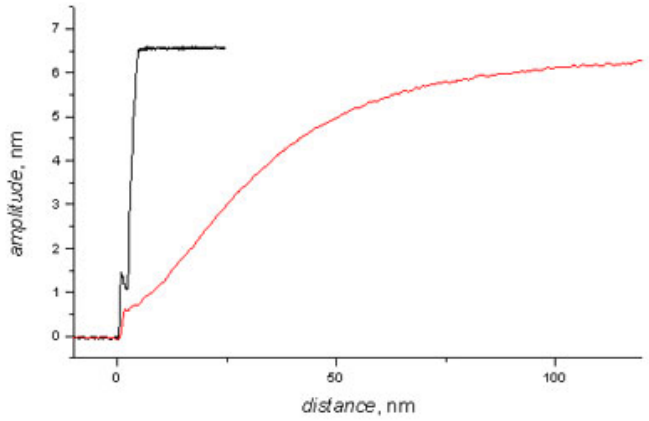
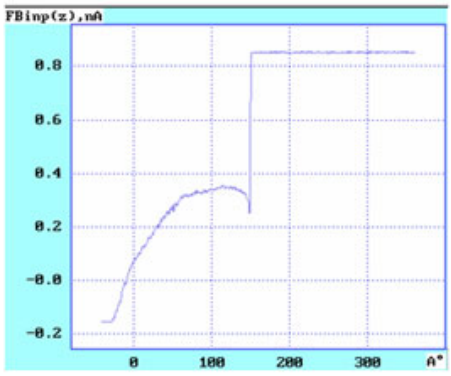


Fig 6. Amplitude curves for the non-charged (black) and charged (red) samples obtained at same settings

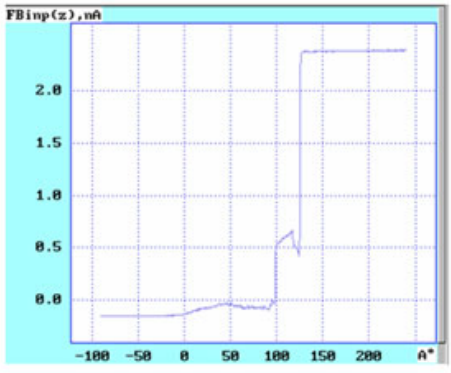
2. Tip sticking

While imaging biological specimens, tips readily become contaminated. This results in images with poor resolution and in the change of the amplitude curve shape – appearance of vertical lines (Fig. 7). Such a vertical line in the curve means that the tip sticks.

This line of working points is unusable; choosing the working point on the line leads to the generation onset. To avoid sticking one may try to increase the probe oscillation initial amplitude but it is better to change the probe.



a)



b)

Fig 7. Amplitude-distance curves with unusable vertical lines

- Example 6

Choosing parameters: initial amplitude and working amplitude (working point)

As mentioned in http://www.ntmdt.ru/SPM-Techniques/SPM-Methodology/Methodology_of_biological_macromolecules_study/Examples/text281.html#ref_4, three characteristic regions can be distinguished in the amplitude (or phase) curves: attractive region, transition region and repulsive region. To each of these regions corresponds a certain range of working amplitudes (working points).

The transition region is anyway unusable because of the instability. If the working amplitude is set in this region, the system will switch between regimes during scanning which will result in topography artifacts: streaks and inversion (Fig. 8, b). Evidently, one should choose only such working points where the system stays stable in a certain regime.

To study macromolecules, the attractive regime is the best as it provides minimum action upon the sample. At large amplitudes this regime is hardly realized because the region of attraction is rather narrow (see Fig. 8). Therefore, to operate in the attractive regime small initial amplitudes should be chosen. The working amplitude in this case is set slightly below the initial one (Fig. 8, c).

The repulsive regime is normally not used for the study of biological molecules.

In [http://www.ntmdt.ru/SPM-Techniques/SPM-Methodology/Methodology_of_biological_macromolecules_study/Examples/text281.html#ref_5] it was shown that scanning in the repulsive regime may result in irreversible deformation of an object. Nevertheless, images acquired at small (close to zero) working amplitudes can be of higher quality than those in the attractive regime (Fig. 4, a) [http://www.ntmdt.ru/SPM-Techniques/SPM-Methodology/Methodology_of_biological_macromolecules_study/Examples/text281.html#ref_4].

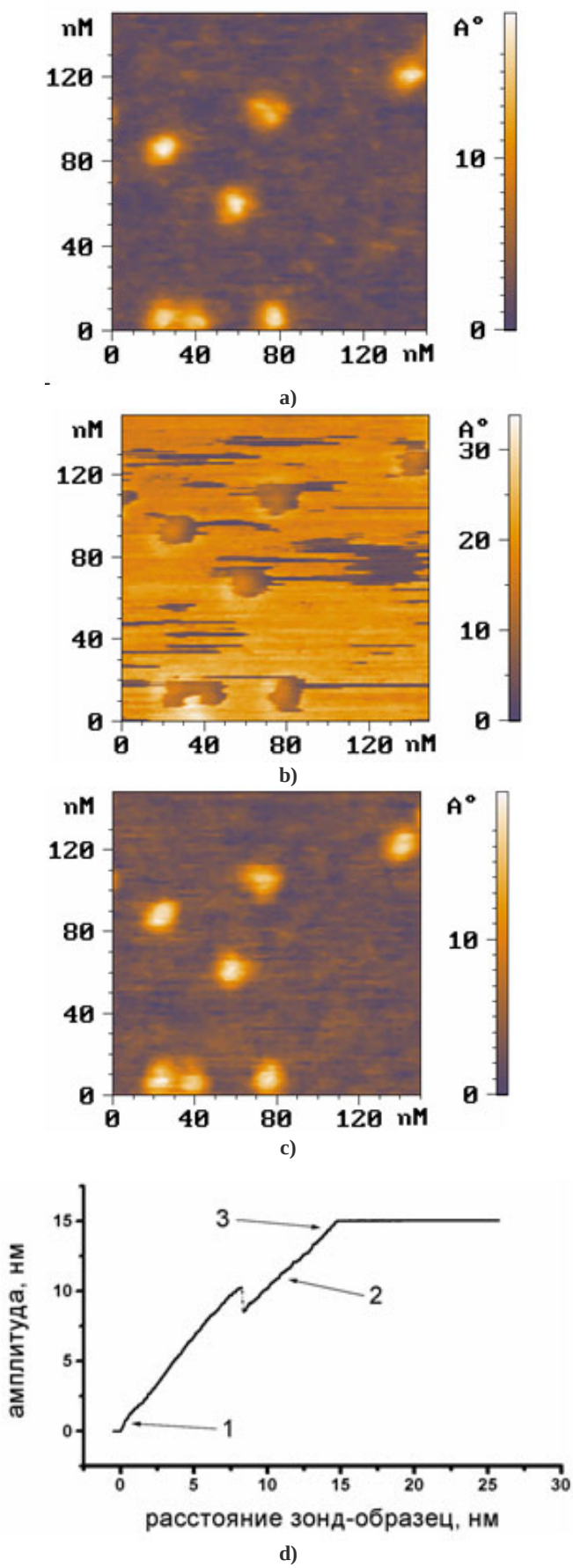


Fig 7. Images of IgG1 molecules are obtained at different working amplitudes corresponding to regimes marked with arrows on the amplitude curve. The amplitude-distance curve was obtained on the mica area free of the molecules.

References

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