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ATOMIC FORCE MICROSCOPY AS A TOOL FOR TESTING BIOMEDICAL SAMPLES AND ELIMINATION PROBE ARTIFACTS

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Abstract One of the most perspective available techniques for investigation of the composition, structure and properties of materials, is scanning probe microscopy (SPM), respectively its components scanning tunneling microscopy (STM) and atomic force microscopy (AFM). This technique is used in multidisciplinary research in the field of medicine, pharmacy, dentistry, material science, etc., for study of biological samples, chemical compounds, pharmaceutical products, artificial tissues, implantology materials, and all other materials that have nanotechnological impact on application in these scientific fields. This is because the probes have not perfect size and geometry, which leads to the appearance of artifacts. They are defined as characteristics that appear on the image and are not present on the sample. These effects caused by convolutions between the probe and sample can be corrected to a certain extent by mathematical manipulation of topographic data. The methodology used in this paper is based on algebra of sets, and basic tools of mathematical morphology. Mathematical algorithms for the „blind reconstruction" of the tip were used, and then in order to detect the parts of the sample surface which is not available in real-time scanning deconvolution was applied. The limit of the real probe tip is calculated from the image, using the morphological limitations inherent in the recording process. The result acuired as an image of the reconstructed surface out of the used images, with the reconstruction of the real tip. The presented results are clear proof of the usability of atomic force microscopy as a technique for imaging of biological materials on nano-level, and the applied algorithms increase the usability of the images in terms of a better conclusion based on precise numerical data taken from the processed images.

Keywords: Characterization, materials, SPM, AFM, probes, artefacts, convolution, post-processing, emulation.

1. INTRODUCTION

The beginnings of nanotechnology research have been traced back to the middle of the last century by presenting the human ability to design a set of precision tools for creation and management of another proportionally smaller set, moving vertically to the depths of the smallest dimensions [1]. In the coming decades, miniaturization of sensors and machines is perceived as the dominant trend in science and technology. At the end of the last and the beginning of this century, nanotechnology has become necessary in every segment of our society, including the widest areas of industry, biomedicine, as well as in all forms of production [2–4]. The application of nanotechnology in biomedicine has led to the development of medicines adapted to the individual, and exactly delivered to the affected area, new and better surgical techniques and tools have emerged, robotics have been used, the production of implants has been facilitated and improved.

The basic tools for nanotechnology research are Scanning Tunnelling Microscope (STM), the principle of which is based on the effect tunnelling of electrons, and Atomic Force Microscope (AFM), the principle of which is based on the interaction between atoms at close range and interatomic forces. Microscopic techniques STM/AFM and instrumentation are used to achieve extreme magnification, ranging from 10^3 to 10^9, in all three dimensions and with high image resolution, as well as for spectroscopy. The great advantage of these

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instruments is that they can be used in any environment, such as air and various gases [5], liquids [6], vacuum [7], at low temperatures (lower than 100K) [8] and high temperatures [9].

Both of these techniques are successfully applied in medical applications, for examining samples of biological origin, as well as materials used in medicine, dentistry and pharmacy. Images obtained with atomic force microscopy (AFM) originate from physical interactions that are completely different from those used for image forming in conventional light and electron microscopy. Furthermore, with the application of a wide range of software computing tools, the analysis of the obtained data at nanometre levels is performed.

One of the effects that inevitably accompany this process, being a direct consequence of the physical interaction of the probe/sample is the emergence of artefacts. An artefact on an image is defined as any characteristic that appears on the image that is not present on the original object. In reality, any AFM component (probe, piezoelectric ceramics, reverse electronic circuit, computer system for image generating and displaying, etc.) can be the source of artefacts.

In this paper is explained the way to solve the problem of the probe tip shape as a source of disorders (artefacts), which is why it is necessary to develop methods for determining the probe geometry and methods of postprocessing the obtained images in order to eliminate the disorders caused by the said artefacts.

2. METHODS

2.1. Principles of AFM

As one of the most important techniques for nanotechnology imaging, atomic force microscopy (AFM) is used for the examination of physical and chemical properties of materials. AFM was constructed in 1986 by Binnig, Quate and Gerber [10]. It is a multifunctional tool for examination of a multitude of information related to mechanical, electrical, magnetic and chemical properties of sample surfaces on a nanometre scale [11].

AFM relies on a scanning technique to produce high-resolution sample surface images. AFM measures the forces less than 1 nN present between the AFM probe and the sample surface. These small forces are detected by measuring the movement of a very flexible probe of extremely low mass.

The basis for the microscope functioning is the detection of force between the sample and the probe. There are two operation modes: static and dynamic. In the static mode, also called contact mode[10], a sharp tip at the end of the probe is brought into contact with the sample surface. Initially, atoms on the tip have a very weak reflective force due to electron orbital overlaps with atoms on the sample surface. A force acting on the tip causes deformation of the probe measured by tunnel, capacitive or optical detectors. A measurable deflection is up to 0.02 nm, so that a typical spring constant of 10 N/m, force of less than 0.2 nN can be detected. In the dynamic mode, also called non-contact mode, the probe tip is brought in close proximity (several nm) of the sample. The console-shaped probe oscillates in the amplitude modulation mode [12] or in the frequency modulation mode (FM) [12–15]. On the probe-sample interface, Van der Waals forces of attraction are present. In order to obtain topographic information, the interaction force is either directly read or used as a feedback control parameter that maintains a constant force value. Using of AFM in the contact mode, topographic images with vertical resolution of less than 0.1nm (even as low as 0.01nm) and lateral resolution of about 0.2nm [16–22] were obtained. With movement sensitivity of 0.01 nm, measurable forces are from 10 nN to 1 pN. These forces are comparable to the forces of chemical bonds, e.g. 0.1 μN for ionic bond and 10 pN for hydrogen bond [5].

The schematic view of AFM is given in Figure 1.

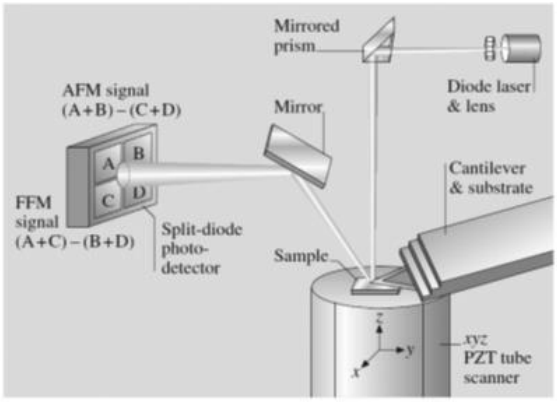


Figure 1. Schematic view of the AFM scanner

Attractive and repulsive forces depend on the sharpness of the sample surface and because of the change in the height of the shape, they bend the sensor. The control system (PC) keeps the sensor deflection constant but, due to the curvy surface of the sample, it constantly changes, which is registered with the laser beam reflected from the tip of the probe into the photo detector of the position, whose each point is equipped with a sensor-diode that turns laser reflected light into an electric signal. The position of the rejected beam, i.e. the position of the diode that

turns a light signal into an electrical one, allows constant monitoring of the vertical position – the sensor deflection.

The deflection information is transmitted to the correction element which, resulting from the deviation of the current value of the deflection from the equilibrium recorded at the start of the imaging, results in the error being corrected by moving the sample, using a piezoelectric actuator, in a vertical direction so that the nanoconsole deflection is continuously maintained at a constant value. All movements of the piezoelectric scanner are a consequence of changes in the shape of the sample surface, so that the image of the vertical movement of the sample (piezoelectric actuator) is actually on image of the appearance of the contour of the sample surface. Data for each line – cross section of the sample are recorded in the computer and eventually all lines connect, which gives 3D reconstruction of the sample surface topography.

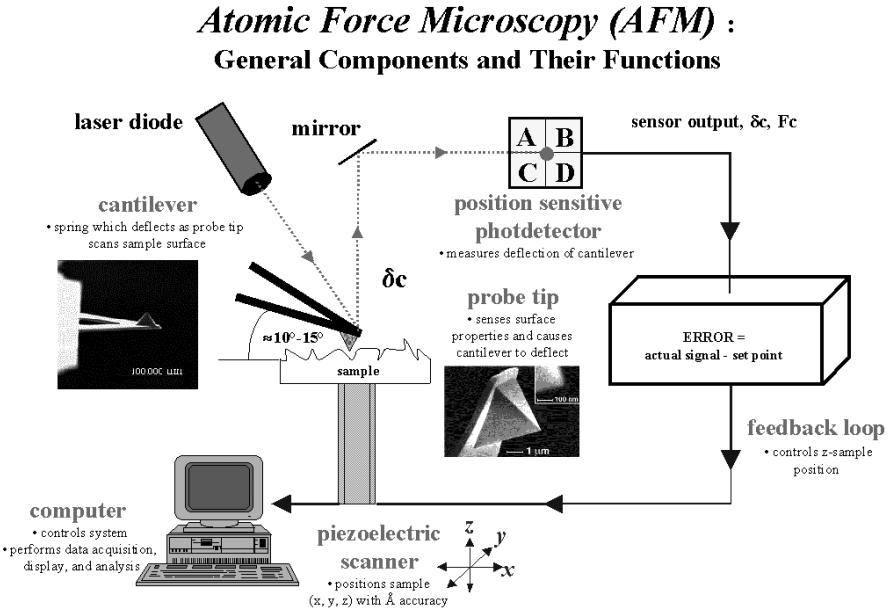


Figure 2. Schematic view of AFM device (JEOL SPM 5200 User Guide)

2.2. Nanotechnology device JSPM-5200

The JSPM-5200 device (JEOL, Japan) was used for sample imaging. It is a complex integrated device for the research and development of nanotechnologies and nanosystems where it is possible to implement several working modes: STM, AFM, MFM and others.

2.3. Probe selection methods

In the selection of probes used for AFM scanning, the material from which they are made should be taken into attention, in terms of module of elasticity, conductivity characteristics of the material and shape of the tip [23]. The dependence of these characteristics is directly related to the nature of the material and the scanning mode being performed.

An important factor in achieving atomic and molecular resolution is the sharpness of the probe tip. The probes of carbon nanotubes are unmatched by these characteristics. The choice of the probe according to the elasticity module depends on the object of observation. The low values of this module (<0.1 N/m) allow the scanning of biological materials.

2.4. Probe artefacts problem solving method

One of the methods to solve the problem of inaccuracy on images resulting from the probe artefacts is based on the concepts of mathematical morphology [24–28]. This approach is applied to any shape of the probe tip, i.e. any tip on a sample that can be presented as a set of points, and is most comprehensively described by Villarrubia [28,29]. Based on mathematical morphology, he proposed algorithms for the reconstruction of real surface shapes. The algorithms include:

(1) Simulation of the image of the given sample and probe tip

(2) Reconstruction of the sample of the probe tip surface and the resulting sample surface image

(3) Reconstruction of the shape of the tip from the sample image whose surface is known *a priori*

(4) Tip assessment from the sample image whose geometry is unknown *a priori.*

A new approach of the interaction between the sample and the tip of the probe is suggested. Theoretically, this reconstruction is based on algebra of sets. At the beginning, the sample and the probe tip are represented as sets A and B, and the scanning process is represented as an extension of the initial sets. The theory of sets applied here to describe the sample surface, the probe tip and the image formation, i.e. to reconstruct the surface and shape of the tip. The sample surface and the probe tip are considered functions of two variables. It's considered that the tip and the sample interact only over their surfaces. Having this approach of determining the geometry of the probe tip, it is not necessary to know its actual geometry. The probe tip border is calculated from the image using the morphological limitations inherent in the imaging process.

The "blind" probe tip assessment algorithm is a result of the well-known fact that on some surface structures, we can directly see the reflection of certain parts of the tip. The algorithm is repeated in all surface data, and at each point, it tries to simplify the tip in accordance with the steepest inclination in the direction between the concrete point of the probe and the tip.

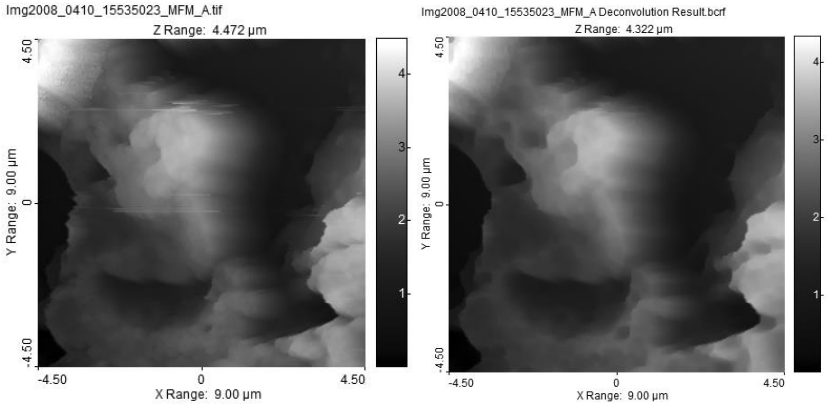
When we know the geometry of the probe tip, we use a convolution algorithm to simulate the data collection process. In addition to the visual overview of the elements on the image, this is particularly useful for working with data resulting from a numerical calculation or modelling, such as, for instance, calculation of roughness of the sample surface or a part of its surface from the image.

In the analysis of the processed images of the tested materials, roughness of the surfaces is calculated as variables and improvement of image quality: average roughness $S_{a,}$ maximum difference tip-tip S_z, maximum recess value S_v, and maximum tip height S_p. Average roughness S_a is defined by the standard DIN 4768 ASME B46.1 for 2D, and the standard ISO 25178-2ASME B46.1 for 3D. S_z dimension represents the maximum difference between the highest and lowest tips found on the image. It is defined by the standard ASME B46.1 for 2D, and the standard ISO 25178-2 ASME B46.1 for 3D. S_v dimension represents the value of the lowest point in the depth on the image. It is defined by the standard ASME B46.1 for 2D, and the standard ISO 25178-2 ASME B46.1 for 3D. S_p dimension represents the height value of the highest point on the image. It is defined by the standard ASME B46.1 for 2D, and the standard ISO 25178-2 ASME B46.1 for 3D.

3. RESULTS

Figure 3 shows an AFM image of a rat brain (a) as well as a processed image with a partially removed impact of artefacts (b). The tip of the scan probe is shown in the 2D and 3D projections in Figures (c) and (d), respectively, and its cross-sections and the radius of tip curvature in *xz* and *yz* planes in diagrams (e) and (f), respectively.

Table 1 shows numerical data of significance for the assessment of quality of sample images and elements.



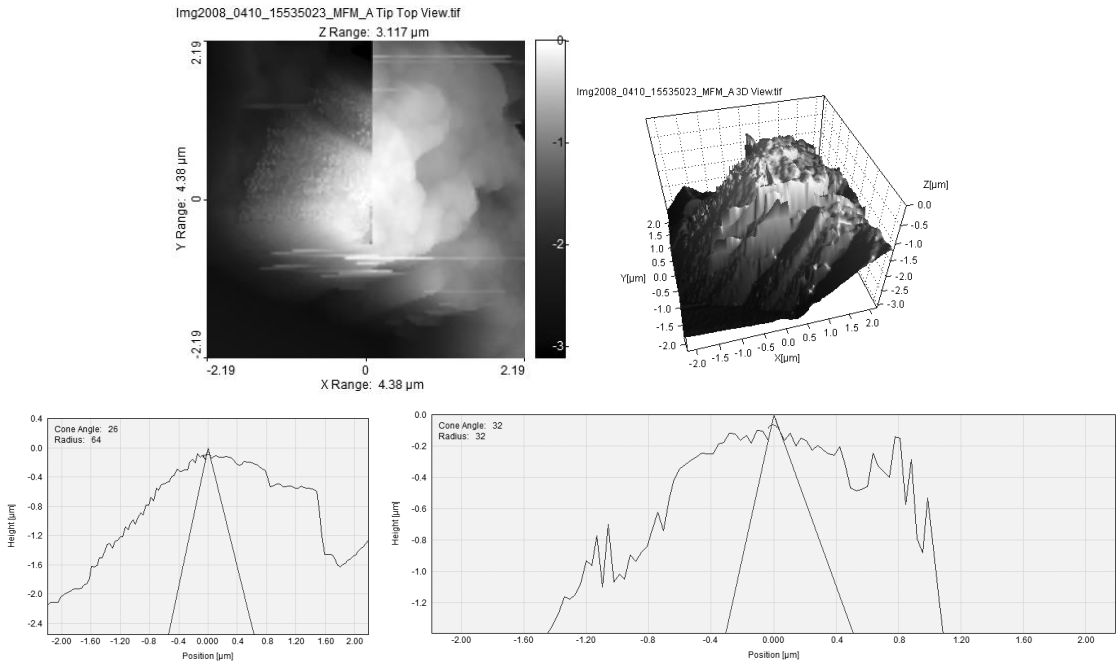


Figure 3. AFM rat brain image (a), a post-processed image obtained after application of algorithms for the elimination of probe artefact effect (b), 2D and 3D images of the real probe with which the imaging was done (c) and (d), and probe tip cross-sections in xz and yz planes (e) and (f)

Table 1. Numerical data on the image of the sample surface and the emulation after the application of algorithms

	Original	Emulation
Z	S_z [nm] 4472	S_z [nm] 3385.1
	S_v [nm] 2018.2	S_v [nm] 1607.5
	S_p [nm] 2453.8	S_p [nm] 1777.6
Roughness	S_a [nm] 689.58	S_a [nm] 550.84

4. DISCUSION

In this paper we present the results of imaging by atomic force microscopy of samples of biological tissues.

The presented sample is a rat brain. The image size is 9x9 µm and was obtained using a probe labelled NSC36/Co-Cr/AL BS. This probe is also used to test the magnetic properties of the sample in a contactless mode, and for this reason it is coated with magnetic material (Co-Cr). Due to the characteristic purpose of these probes and a large number of expected oscillations, due to the prevention of bending and increasing the reflection of the laser beam, the upper side of the probe is coated with a thin layer of aluminium. The typical tip radius specified by the manufacturer is 8 nm and the cone angle is 40°. The real tip of the probe with which the imaging was done has a radius of 64 nm and a cone angle of 26° in *xz* plane, i.e. 32 nm and 32 ° in *yz* plane. Under these conditions, the highest point values were obtained on a 2453.8 nm image with average roughness of 689.58 nm. After applying the algorithms for the elimination of probe artefacts, these values are reduced to 1777.6 nm for a maximum height point, and 550.84 nm for average roughness. From these values it is obvious that the algorithm allowed a better assessment of the sample surface, i.e. to reach, through several iterations, the shape of the probe tip, which is more sensitive to the crossings between the points on the sample surface. The indicated change is 27.5% for height and 20.1% for surface roughness. Based on this data, we obtained improved quality of image that corresponds better to real conditions of material surface.

The displayed results show that the real geometric properties of probes for imaging by atomic force microscopy have a great influence on the veracity of the obtained images, and consequently on the accuracy of the conclusions drawn from them, in terms of material characterisation.

5. CONCLUSION

During researching samples of biological tissues, presented in this paper, a known problem emerged – the appearance of artefacts of different origin, which affects the quality of the images and, therefore, the accuracy of the conclusions based on them. Consideration was given to the artefacts arising from the irregularities of the scan probe tips. These irregularities may be due to the imperfection of the production process or the worn tip of the probe. The long-term dealing with this problem in order to improve the quality of the already obtained images gave results in the form of mathematical algorithms, which simulate the imaging process.

The so-called "blind reconstruction" of the scan probe tip by means of atomic force microscopy does not imply previous knowledge of the probe data. They are obtained from the original image, upgraded with theory of sets and mathematical morphology.

Taking into account the real geometric characteristics of the probes, using mathematical algorithms, a simulated graphic interpretation of the original images was obtained. The imaging process generates a file, which, among other things, contains the values of all three coordinates of each point on the image. Applying statistical methods in the processing, the parameters of significance for the authenticity of the real sample surface are calculated. As a result, a more accurate image of the real state of the sample surface at the time of the imaging is obtained.

The purpose of the method is to obtain usable images, which would be discarded without further processing, since the irregularities that occurred during the imaging process were expressed to an extent that makes them unusable for the analysis of the real state of the tested material.

The presented results are clear proof of the usability of atomic force microscopy as a technique for imaging of biological materials on nano level, and the applied algorithms increase the usability of the images in terms of better conclusion based on precise numerical data taken from the processed images. In view of this conclusion, it is meaningful to continue the research, encompassing a wider range of biological and synthetic materials used in medicine.

6. REFERENCES

[1] R. P. Feynman, T*here's plenty of room at the bottom: An invitation to enter a new field of physics*, Eng Sci., Vol. 23 (1960) 22–35.

[2] P. Alivisatos, *The use of nanocrystals in biological detection*, Nat Biotechnol, Vol. 22–1 (2004) 47–52.

[3] C. Xu, Z. L. Wang, *Compact hybrid cell based on a convoluted nanowire structure for harvesting solar and mechanical energy*, Adv Mater., Vol. 23–7 (2011) 873–877.

[4] Y. G. Guo, J. S. Hu, L. J. Wan, *Nanostructured Materials for Electrochemical Energy Conversion and Storage Devices*. Adv Mater., Vol. 20–15 (2008) 2878–2887.

[5] G. Binnig, C. F. Quate, *Atomic Force Microscope*, Phys Rev Lett., Vol. 56–9 (1986) 930–933.

[6] T. Fukuma, K. Kobayashi, K. Matsushige, H. Yamada, *True atomic resolution in liquid by frequency-modulation atomic force microscopy*, Appl Phys Lett., Vol. 87–3 (2005) 034101.

[7] G. Binnig, H. Rohrer, C. Gerber, E. Weibel, *Surface Studies by Scanning Tunneling Microscopy*, Physical Review Letters, Vol. 49 (1982) 57–61.

[8] F. J. Giessibl, C. Gerber, G. Binnig, *A Low-Temperature Atomic Force Scanning Tunneling Microscope for Ultrahigh-Vacuum*, J Vac Sci Technol B., Vol. 9–2 (1991) 984–988.

[9] C. Basire, D. A. Ivanov, *Evolution of the lamellar structure during crystallization of a semicrystalline-amorphous polymer blend: Time-resolved hot-stage SPM study*, Phys Rev Lett., Vol. 85–26 (2000) 5587–5590.

[10] G. Binnig, C. Quate, C. Gerber, *Atomic Force Microscope*, Physical Review Letters, Vol. 56 (1986) 930–933.

[11] D. J. Müller, Y. F. Dufrêne, Atomic force microscopy as a multifunctional molecular toolbox in nanobiotechnology, Nat Nanotechnol., Vol. 3–5 (2008) 261–269.

[12] Y. Martin, C. C. Williams, H. K. Wickramasinghe, *Atomic force microscope-force mapping and profiling on a sub 100-Å scale*, J Appl Phys., Vol. 61–10 (1987) 4723–4729.

[13] D. Sarid, V. Elings, *Review of Scanning Force Microscopy*, J Vac Sci Technol B., Vol. 9–2 (1991) 431–437.

[14] F. J. Giessibl, *Atomic Resolution of the Silicon (111)-(7$\\times$7) Surface by Atomic Force Microscopy*, Science (80-), Vol. 267–5194 (1995) 68–71.

[15] B. Anczykowski, D. Krüger, K. L. Babcock, H. Fuchs, *Basic properties of dynamic force spectroscopy with the scanning force microscope in experiment and simulation*, Ultramicroscopy, Vol. 66–3/4 (1996) 251–259.

[16] G. Binnig, C. Gerber, E. Stoll, T. R. Albrecht, C. F. Quate, *Atomic resolution with atomic*

force microscope, Europhys Lett., Vol. 3–12 (1987) 1281–1286.

[17] O. Marti, B. Drake, P. K. Hansma, *Atomic force microscopy of liquid-covered surfaces: Atomic resolution images*, Appl Phys Lett., Vol. 51–7 (1987) 484–486.

[18] T. R. Albrecht, C. F. Quate, *Atomic resolution imaging of a nonconductor by atomic force microscopy*, J Appl Phys., Vol. 62–7 (1987) 2599–2602.

[19] S. Alexander, L. Hellemans, O. Marti, J. Schneir, V. Elings, P. K. Hansma, et al., *An atomic-resolution atomic-force microscope implemented using an optical lever*, J Appl Phys., Vol. 65–1 (1989) 164–167.

[20] G. Meyer, N. M. Amer, *Optical-beam-deflection atomic force microscopy: The NaCl (001) surface*, Appl Phys Lett., Vol. 56–21 (1990) 2100–2101.

[21] A. L. Weisenhorn, M. Egger, F. Ohnesorge, S. A. C. Gould, S. P. Heyn, H. G. Hansma, et al., *Molecular-Resolution Images of Langmuir-Blodgett Films and DNA by Atomic Force Microscopy*, Langmuir, Vol. 7–1 (1991) 8–12.

[22] J. A. Ruan, B. Bhushan, *Atomic-scale and microscale friction studies of graphite and diamond using friction force microscopy*, J Appl Phys., Vol. 76–9 (1994) 5022–5035.

[23] I. Hut, M. Marjanović, V. Miletić, Lj. Petrov, I.Djuričić, *Effects of brushing on surface roughness of microhybrid and nanohybrid composite resins*, Book of abstracts: V International Scientific Conference „Contemporary Materials 2012“, Banja Luka 2012, 102.

[24] D. J. Keller, F. S. Franke, *Envelope reconstruction of probe microscope images*, Surf Sci, Vol. 294–3 (1993) 409–419.

[25] N. Bonnet, S. Dongmo, P. Vautrot, M. C. N. Troyon, *A Mathematical Morphology Approach to Image-Formation and Image-Restoration in Scanning Tunneling and Atomic-Force Microscopies*, Microsc Microanal Microstruct., Vol. 5 (1994) 477–487.

[26] P. Markiewicz, M. C. Goh, *Atomic Force Microscopy Probe Tip Visualization and Improvement of Images Using a Simple Deconvolution Procedure*, Langmuir, Vol. 10–1 (1994) 5–7.

[27] D. L. Wilson, K. S. Kump, S. J. Eppell, R. E. Marchant, *Morphological Restoration of Atomic-Force Microscopy Images*, Langmuir, Vol. 11–1 (1995) 265–272.

[28] J. S. Villarrubia, Morphological estimation of tip geometry for scanned probe microscopy, Surf Sci., Vol. 321–3 (1994) 287–300.

[29] J. S. Villarrubia, *Algorithms for scanned probe microscope image simulation, surface reconstruction, and tip estimation*, J Res Natl Inst Stand Technol, Vol. 102–4 (1997) 425.

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МИКРОСКОПИЈА АТОМСКИМ СИЛАМА КАО АЛАТ ЗА ИСПИТИВАЊЕ БИОМЕДИЦИНСКИХ УЗОРАКА И ЕЛИМИНАЦИЈА АРТЕФАКАТА СОНДИ

Сажетак: Једна од најперспективнијих техника за испитивање састава, структуре и својстава материјала јесте микроскопија сондама за скенирање (SPM), односно њене компоненте микроскопија тунеловањем електрона (STM) и микроскопија атомским силама (AFM). Овим методама се рутински постиже нанометарска и атомска резолуција. Посебно истакнута предност методе је да не постоје ограничења у смислу порекла и састава узорака, те је могуће испитивање органских и неорганских материјала. Ова техника се примењује у савременим мултидисциплинарним истраживањима у области медицине, фармације, стоматологије, науке о материјалима, итд., и то за испитивање биолошких узорака, хемијских једињења, фармацеутских производа, вештачких ткива, материјала за имплантологију, и свих осталих материјала чија нанотехнолошка својства имају утицај на примену у наведеним научним областима. Међутим, снимци добијени помоћу AFM-а само су апроксимације површина узорака, јер сонде немају ни савршену величину ни геометрију, услед чега долази до појаве артефаката који се дефинишу као карактеристике које се појављују на снимку а које нису присутне на испитиваном узорку. Ови ефекти изазвани конволуцијом између сонде и узорка могу до извесне мере да буду кориговани математичком манипулацијом топографским подацима. Методологија која је у овом раду коришћена заснива се на алгебри скупова и основним алатима математичке морфологије. Искоришћени су математички алгоритми за “слепу реконструкцију” врхова сонди, а потом је извршена деконволуција, да би се открили делови површине узорка који у реалности нису били доступни. Граница реалног врха

сонде израчунава се из слике помоћу морфолошких ограничења која су инхерентна у процесу снимања. Резултат се добија у виду снимка реконструисане површине узорка из добијених снимака, уз помоћ реконструкције врха сонде којом је узорак сниман. Приказани резултати очигледан су доказ употребне вредности микроскопије атомским силама као технике за снимања биолошких материјала у нанодимензионалном свету, а примењени алгоритми повећавају употребну вредност снимака у смислу бољег закључивања на основу прецизнијих нумеричких података узетих са процесуираних снимака.

Кључне речи: карактеризација, материјали, SPM, AFM, сонде, артефакти, конволуција, постпроцесуирање, емулација.

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