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3D ANALYSIS OF THE MICRO- AND NANOSTRUCTURE OF LUNG TISSUE BY SCANNING PROBE NANOTOMOGRAPHY

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Objective: to analyze the 3D micro- and nanostructure and quantitative morphological parameters of rat lung tissue. **Materials and methods.** Wistar rat lung tissue samples were obtained for the study. The 3D structure of the lung tissue was studied via scanning probe nanotomography using an experimental setup combining an ultramicrotome and a scanning probe microscope. **Results.** Nanoscale images and 3D nanotomographic reconstructions of the interalveolar septal sections of the rat lung were obtained. Morphological parameters (average roughness and specific effective area) of the interalveolar septal surface were determined. It was found that the average roughness of the reconstructed septal surface was 345.4 ± 24.5 nm, and the specific effective area was 2.7 ± 0.2 units. **Conclusions.** Results obtained demonstrate that scanning probe nanotomography allows to quantify lung morphology. The use of scanning probe nanotomography for 3D analysis of the structure and characteristics of lung tissue will increase the efficiency of future developments on creation of new criteria for diagnosing pathological conditions.

Keywords: lung, alveolus, interalveolar septum, scanning probe microscopy, nanotomography.

INTRODUCTION

The development of methods for microscopic studies of the three-dimensional nanostructure of cells and tissues is extremely important for understanding the structural and molecular mechanisms responsible for their functionality and the timely detection of pathological changes. Thus, modern methods of studying the three-dimensional micro- and nanostructure of biological objects are of great importance, in particular, such high-resolution microscopy methods as scanning probe microscopy (SPM) and scanning electron microscopy with a focused ion beam (SEM/FIB) [1, 2].

Studies using SEM/FIP made it possible to reconstruct the three-dimensional organization of alveoli in mouse lung tissue [3] and myofibrils, T-tubules, sarcoplasmic reticulum and mitochondria in myocytes [4, 5]. The recent discovery of interconnected networks of mitochondria in muscle cells [6] convincingly proves that nanotomography techniques can effectively investigate three-dimensional relationships between cell organelles, compartments, and systems. These structures are almost impossible to fully characterize using conventional microscopic techniques, which only provide two-dimensional images and projections of cellular structures. However, SEM/FIB cannot be called the optimal method for the analysis of biological objects, since the used electron and ion beams can cause undesirable damage to the surface structure, and the contrast and resolution on SEM images of samples after exposure to an ion beam can be reduced [1, 2, 7, 8].

The physical principles of SPM imaging are fundamentally different from the principles of both optical and electron microscopy [9–11]. In the case of SPM, raster images of the topography and distributions of the physical properties of the sample surface are constructed by analyzing the features of the physical interaction of the ultra-sharp probe (cantilever) with the scanned surface. The integration of the technique for obtaining ultrathin sample sections (ultramicrotomy) with SPM methods within the framework of a single instrument complex makes it possible to implement the scanning probe nanotomography (SPN) technology [2]. Computer processing of a series of sequential SPM images of the sample surface obtained immediately after ultrathin sections makes it possible, using specialized software, to perform three-dimensional reconstruction of the micro- and nanostructure of the samples under study, which makes it possible to apply any SPM techniques for the tasks of three-dimensional analysis. Thus, the analysis of three-dimensional structures of tissues of various organs by the SPN method makes it possible to obtain unique information about their nanoscale organization, which is inaccessible to other methods. It is also important that the analysis of three-dimensional SPN-reconstructions of biological objects and materials makes it possible to quantitatively evaluate such important parameters of their nanomorphology as micro- and nanoporosity [12], effective surface area and roughness, surface area to volume ratio [13].

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The progress of SPN technology as applied to the study of organs and tissues may allow the development of new methods and criteria for diagnosing the state of organs of donors and patients for the tasks of modern transplantology. This paper presents studies of three-dimensional structures of rat lung tissue using SPN methods.

MATERIALS AND METHODS

Preparation of rat lung tissue samples for scanning probe nanotomography

Male Wistar rats (250–350 g weight) were used in the experiments. The operation with the animals was carried out under inhalation ether anesthesia. The rat was placed belly up on the operating table and the legs were straightened. The skin on the abdomen was pulled with tweezers, and a longitudinal skin incision was made on the midline of the abdominal side of the body from the genital opening to the sternum with scissors. The skin was turned away and secured. Then the chest cavity was opened, the lungs were taken, cutting off the trachea and arteries. After sampling, the lungs were placed in a sodium phosphate buffer solution (pH = 7.4) and washed from blood.

For preparation for SPN, fragments of lung tissue 3 × 3 mm in size were excised with a scalpel. Then, to fix the obtained samples, the fragments were placed in a 2.5% solution of glutaraldehyde in sodium phosphate buffer (pH = 7.4) and incubated for 2 hours in the dark at + 4 °C. Then there were three washes of the samples in sodium phosphate buffer for 10 minutes. After that, the samples were dehydrated by wiring through alcohols with increasing concentration by the following pattern:

a) 30% ethanol solution – 10 min;
b) 50% ethanol solution – 10 min;
c) 70% ethanol solution – 10 min;
d) 80% ethanol solution – 10 min;
e) 96% ethanol solution – 10 min.

Next, the samples were washed three times in propylene oxide for 10 minutes each, and then incubated in a mixture of propylene oxide and epoxy resin in a 1:1 ratio for 30 minutes, after which the samples were transferred into a mixture of propylene oxide and epoxy resin in a ratio of 1:2 and incubated for 30 minutes. Then the samples were embedded in epoxy resin, incubated in a thermostat at 45 °C for 24 hours, after which the incubation was continued for 48 h at 60 °C.

To fill the samples, an epoxy medium (Epoxy Embedding Medium, Sigma-Aldrich, cat. No. 45345) was used, mixed with an equal weight of the embedding medium hardener (dodecenyl succinic anhydride, Sigma-Aldrich, USA, cat. No. 45346) and 4% by weight DMP-30 (Sigma-Aldrich, USA, cat # 45348).

Scanning probe nanotomography of rat lung tissue samples

Ntegra Tomo experimental device was used to study samples of rat lung tissue by SPN methods. This complex allows for sequential SPM measurements of the sample surface immediately after cutting with an ultramicrotome. Consecutive sections of a 60 nm thick sample were made using a Diatome Ultra AFM 35 diamond knife (Diatome AG, Switzerland) with a cutting-edge width of 2.0 mm.

SPM measurements were performed in the semicontact mode at a scanning speed of 1.0 Hz using NSG10 silicon cantilever probes (NT-MDT, Moscow) with a resonance frequency of 240 kHz and a tip curvature radius of <10 nm. Primary image processing was carried out using the Nova ImageAnalysis 1.0.26.1443 software (NT-MDT, Moscow), three-dimensional tomographic reconstructions of the lung structure were obtained using the ImagePro Plus 6.0 software (Media Cybernetics, Inc, USA).

Calculation of morphological parameters of 3D surfaces

Analysis of the surfaces reconstructed by the SPN method using the ImagePro Plus 6.0 software (Media Cybernetics, Inc, USA) allows one to determine and analyze the nanoscale parameters of these surfaces, such as the average roughness R_a and the effective surface area σ.

To calculate these parameters, the reconstructed surface is considered as a two-dimensional data array (values of heights Z) of size $N \times M$, where N and M are the number of columns and rows, respectively. Before calculations, a first-order surface (plane) is subtracted from the surface, which corresponds to the elimination of the slope.

Average surface roughness R_a is calculated as the average value of the modulus of the deviation of the height of the array points from the average height:

$$R_a = \frac{1}{N}\frac{1}{M}\sum_{i=1}^{N}\sum_{j=1}^{M}|Z_{i,j} - \langle Z\rangle|, \quad (1)$$

where $Z_{i,j}$ – the height value at a point in the array (i, j), $\langle Z\rangle$ – average height Z, averaged over the entire array:

$$\langle Z\rangle = \frac{1}{N}\frac{1}{M}\sum_{i=1}^{N}\sum_{j=1}^{M}Z_{i,j}. \quad (2)$$

Effective surface area σ is calculated as the ratio of the surface area to the area of its two-dimensional projection onto the plane. This parameter determines the degree of surface development [14]. Reconstructed surface area S is calculated using the triangulation method as a sum over an array of elementary areas $s_{i,j}$ of the surfaces of unit cells between 4 adjacent points of the array (i, j), $(i + 1, j)$, $(i, j + 1)$, $(i + 1, j + 1)$. When using the triangulation

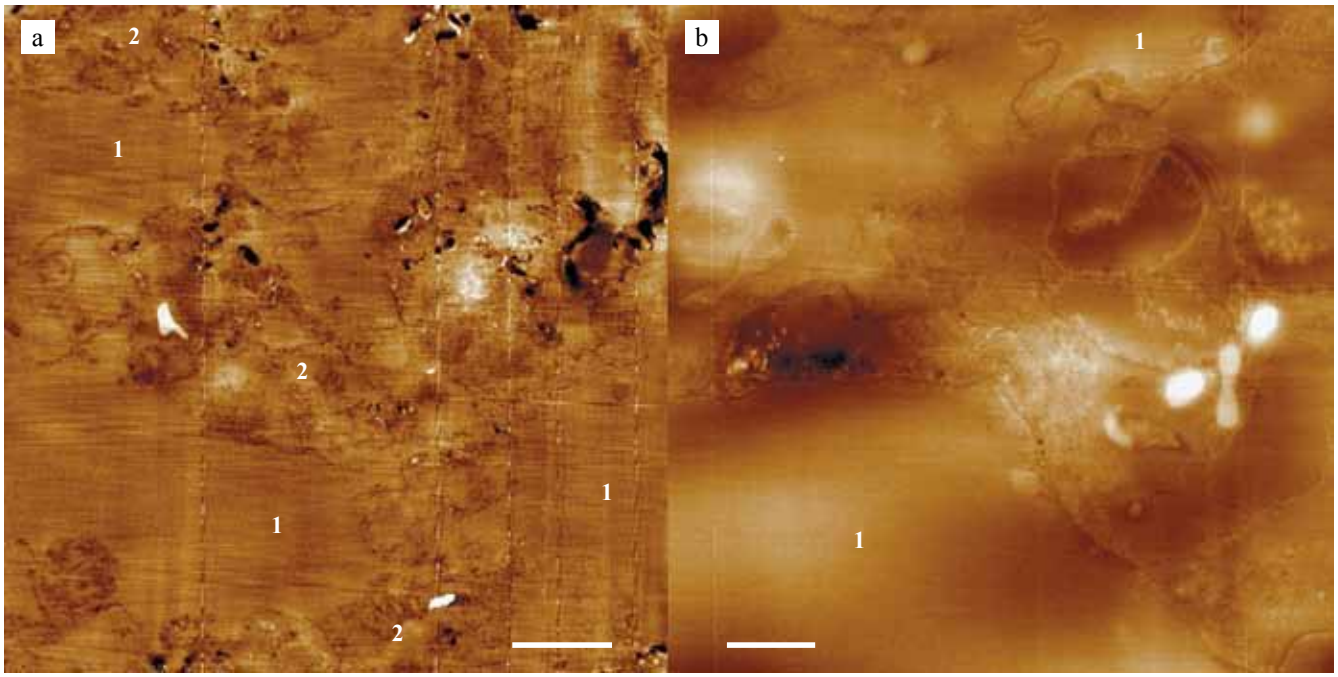

Fig. 1. SPM topography image of surface areas of rat lung tissue after ultramicrotome section: a – SPM image of region of respiratory lung area, scan size 65×65 μm, height variation 18 nm, scale bar 10 μm; б – SPM image of region of alveolar septum, scan size 15×15 μm, height variation 48 nm, scale bar 2 μm; 1 – areas of alveolus; 2 – areas of alveolar septum

method to calculate the elementary area, the midpoint is also entered with height $\langle Z_{i,j}\rangle = (Z_{i,j} + Z_{i+1,j} + Z_{i,j+1} + Z_{i+1,j+1})/4$ and effective coordinates $(i + 1/2, j + 1/2)$, and the elementary area is calculated as the area of four triangles, each of which is formed by adjacent unit cell vertices and a midpoint. So, for example, the area of a triangle formed by points (i, j), $(i + 1, j)$ and the midpoint is given by:

$$\frac{d_x d_y}{4}\sqrt{1+\left(\frac{Z_{i,j}-Z_{i,j+1}}{d_x}\right)^2+\left(\frac{2\langle Z_{i,j}\rangle - Z_{i,j}-Z_{i,j+1}}{d_y}\right)^2}, \quad (3)$$

where d_x и d_y – physical dimensions of pixels along the corresponding axes. Since we are using a surface reconstructed by the SPN method, in our case d_x will be determined by the pixel resolution of the SPM measurement, and d_y – by the slice thickness between successive SPM measurements. Accordingly, the effective surface area will be set as

$$\sigma = \frac{1}{Nd_x}\frac{1}{Md_y}\sum_{i=1}^{N}\sum_{j=1}^{M} s_{i,j}. \quad (4)$$

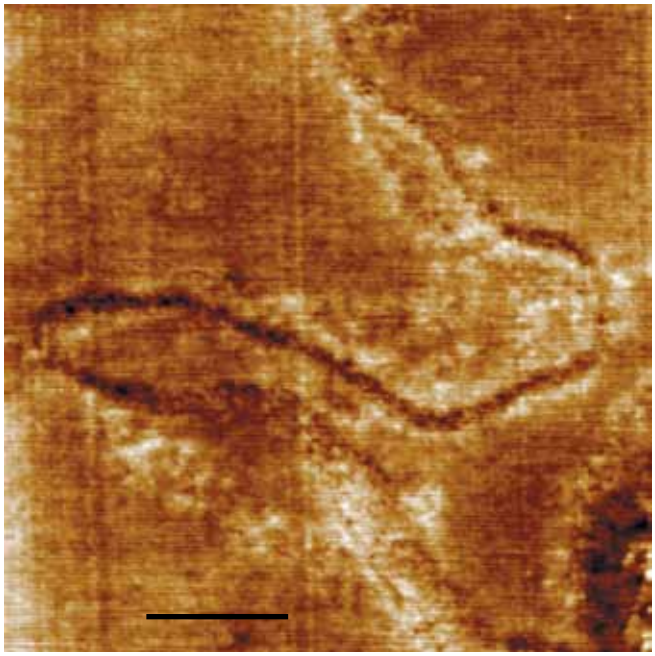

Fig. 2. SPM topography image of surface of alveolar septum surface folding; scan size 2.5×2.5 μm, height variation 13 nm, scale bar 500 nm

RESULTS AND DISCUSSION

Fig. 1, a, shows an example of the obtained SPM image (65 × 65 μm) of a section of the respiratory part of the lung. In this image, one can distinguish areas of bubbles with sizes of about 20–30 μm and nanostructured interalveolar septa with a width of 10–20 μm. In Fig. 2 shows an SPM image (15 × 15 μm) of a portion of the interalveolar septum between two regions of the bubbles. This image shows that the interalveolar septum has a complex nanoscale morphology and a pronounced nanostructure. The topology and morphological parameters of the septa are of great importance for the functionality of the alveoli and the efficiency of gas exchange processes [15, 16].

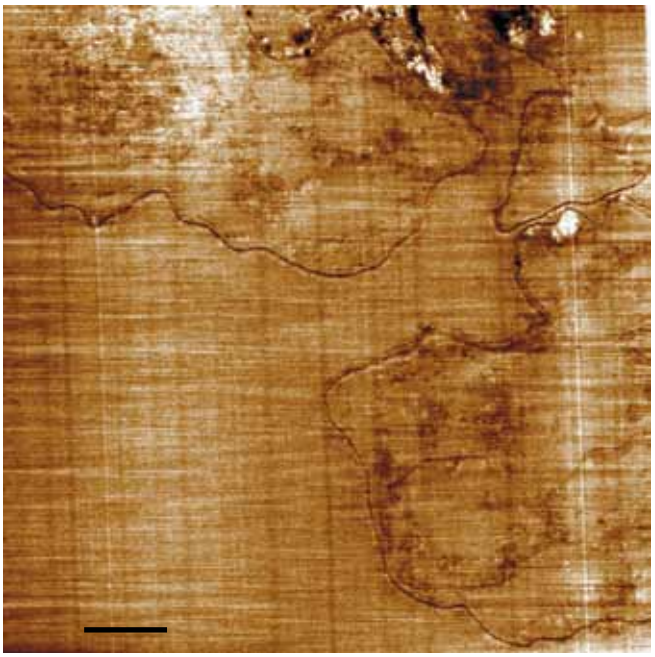

Fig. 3. SPM topography image of the surface of a region of alveolar septum border with a alveolus; scan size 16×16 μm, height variation 24 nm, scale bar 2 μm

The SPN technology used makes it possible to study the nanoscale features of the surface of the interalveolar septum with high resolution. In Fig. 2 shows an enlarged image (2.5 × 2.5 μm) of a section of the folds of the surface of the interalveolar septum, characterized by a complex shape and nanostructure and surrounded by a surfactant film.

Also, the most important advantage of the SPN method is the possibility of three-dimensional reconstruction of tissue structures, in particular, the surface of the septum. In Fig. 3 shows an SPM image of a fragment of the region of the interface of the interalveolar septum with a bubble (16 × 16 μm), which has a complex morphology. To assess the three-dimensional morphology of the interalveolar septum, a three-dimensional reconstruction of this area was performed using the SPN method. For this, 10 successive sections of a sample with a thickness of 120 nm were made, and 10 successive SPM images of a 16 × 16 μm surface area were obtained. The resulting visualization of the three-dimensional surface is shown in Fig. 4.

It should be noted that the complex morphology of the surface of the interalveolar septum is characterized by a significant increase in the effective area. Specialized software used for visualization of three-dimensional reconstructions makes it possible to determine both the surface roughness of the partition Ra and the specific effective area of its surface σ, calculated as the ratio of the area of the three-dimensional surface to the area of its projection onto the plane. For the three-dimensional reconstruction we have obtained, the value of the specific

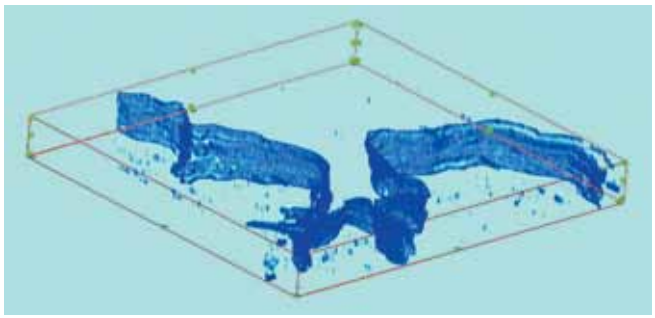

Fig. 4. SPNT three-dimensional reconstruction of a fragment of surface of the alveolar septum: reconstructed volume 16×16×1.2 μm, section thickness 120 nm

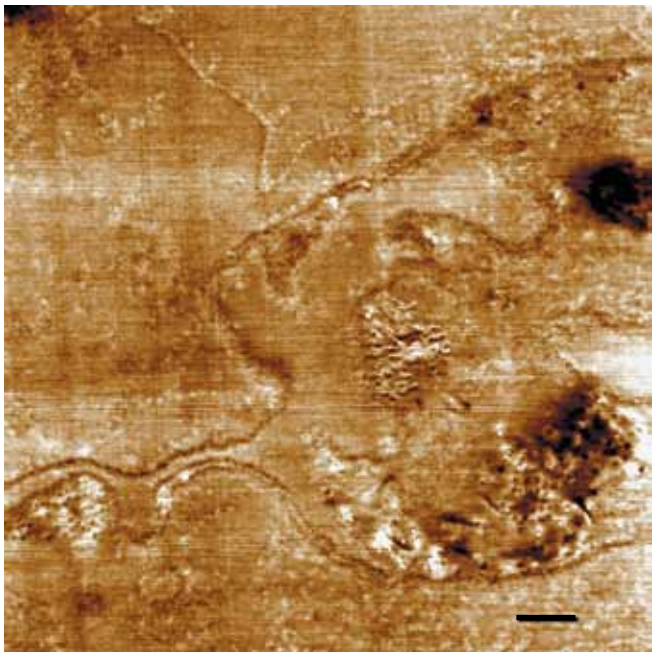

Fig. 5. SPM topography image of the surface area of an inner alveolus; scan size 5.5×5.5 μm, height variation 17 nm, scale bar 500 nm

effective area σ is 2.7 ± 0.2, which indicates a high degree of surface development. The nanoroughness of the reconstructed three-dimensional surface Ra is 345.4 ± 24.5 nm.

Interestingly, the surface of the interalveolar septum often forms bends and folds, resulting in the formation of internal vesicles or sacs several microns in size with a narrow entrance slit. An example of an image of such a structure is shown in Fig. 5. Note that the width of the air "neck" of this inner bubble is about 120 nm, and the surface of the partition in the inner region thickens and has a heterogeneous structure. These structures are associated with the processes of gas exchange in the alveoli.

The developed technique for studying the nanoscale structures of the alveoli with the scanning probe nanotomography is applicable to solving a number of problems in structural biology, for example, the urgent

problem of studying the three-dimensional organization of interalveolar septa and determining their quantitative morphological parameters at the nanoscale.

CONCLUSION

In the present work, studies of the nanostructural features of rat lung tissue were carried out using the SPN method. 3D reconstruction of the surface of the interalveolar septum in rat lung tissue was obtained. It is shown that the complex three-dimensional morphology of its surface is characterized by a significant increase in the effective surface area of the partition. The specific effective surface area σ of the reconstructed section of the interalveolar septum is 2.7 ± 0.2, which indicates a high degree of surface development.

SPN technology allows obtaining unique information about the relationship between nanoscale features of the structure and functional activity of cells and tissues.

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The authors declare no conflict of interest.

REFERENCES

1. *Zankel A, Wagner J, Poelt P.* Serial sectioning methods for 3D investigations in materials science. *Micron*. 2014; 62: 66–78. doi: 10.1016/*j*.micron.2014.03.002.
2. *Alekseev A, Efimov A, Loos J, Matsko N, Syurik J.* Three-dimensional imaging of polymer materials by Scanning Probe Tomography. *Eur Polym J.* 2014; 52: 154–165. doi: 10.1016/*j*.eurpolymj.2014.01.003.
3. *Kremer A, Lippens S, Bartunkova S, Asselbergh B, Blanpain C, Fendrych M et al*. Developing 3D SEM in a broad biological context. *J Microsc.* 2015; 259 (2): 80–96. doi: 10.1111/jmi.12211.
4. *Sulkin MS, Yang F, Holzem KM, Van Leer B, Bugge C, Laughner JI et al*. Nanoscale three-dimensional imaging of the human myocyte. *J Struct Biol*. 2014; 188 (1): 55–60. doi: 10.1016/*j*.jsb.2014.08.005.
5. *Dahl R, Larsen S, Dohlmann TL, Qvortrup K, Helge JW, Dela F, Prats C.* Three-dimensional reconstruction of the human skeletal muscle mitochondrial network as a tool to assess mitochondrial content and structural organization. *Acta Physiol*. 2015; 213: 145–155. doi: 10.1111/apha.12289.
6. *Glancy B, Hartnell LM, Malide D, Yu Z-X, Combs CA, Connelly PS et al*. Mitochondrial reticulum for cellular energy distribution in muscle. *Nature*. 2015; 523: 617–620. doi: 10.1038/nature14614.
7. *Bailey RJ, Geurts R, Stokes DJ, de Jong F, Barber AH.* Evaluating focused ion beam induced damage in soft materials. *Micron*. 2013; 50: 51–56. doi: 10.1016/*j*.micron.2013.04.005.
8. *Linkov P, Artemyev M, Efimov AE, Nabiev I.* Comparative advantages and limitations of the basic metrology methods applied to the characterization of nanomaterials *Nanoscale.* 2013; 5: 8781–8798. doi: 10.1039/c3nr02372a.
9. *Binnig G, Quate CF, Gerber C.* Atomic force microscope. *Phys Rev Lett.* 1986; 56 (9): 930–933. doi: 10.1103/PhysRevLett.56.930.
10. *Magonov SN, Reneker DH.* Characterization of polymer surfaces with atomic force microscopy. *Annu Rev Mater Sci.* 1997; 27 (1): 175–222. doi: 10.1146/annurev.matsci.27.1.175.
11. *Gallyamov MO.* Scanning Force Microscopy as Applied to Conformational Studies in Macromolecular Research. *Macromol Rapid Commun.* 2011; 32 (16): 1210–1246. doi: 10.1002/marc.201100150.
12. *Efimov AE, Moisenovich MM, Bogush VG, Agapov II.* 3D nanostructural analysis of silk fibroin and recombinant spidroin 1 scaffolds by scanning probe nanotomography. *RSC Adv.* 2014; 4: 60943–60947. doi: 10.1039/c4ra08341e.
13. *Efimov AE, Agapova OI, Safonova LA, Bobrova MM, Parfenov VA, Koudan EV et al*. 3D scanning probe nanotomography of tissue spheroid fibroblasts interacting with electrospun polyurethane scaffold. *Express Polymer Letters.* 2019; 13 (7): 632–641. doi: 10.3144/expresspolymlett.2019.53.
14. *Gómez-Pozos H, González-Vidal JL, Torres GA, de la Luz Olvera M, Castañeda L.* Physical Characterization and Effect of Effective Surface Area on the Sensing Properties of Tin Dioxide Thin Solid Films in a Propane Atmosphere. *Sensors.* 2014; 14: 403–415. doi: 10.3390/s140100403.
15. *Hsia CCW, Hyde DM, Ochs M, Weibel ER.* How to Measure Lung Structure – What For? On the "Standards for the Quantitative Assessment of Lung Structure". *Respir Physiol Neurobiol*. 2010; 171 (2): 72–74. doi: 10.1016/*j*.resp.2010.02.016.
16. *Yang L, Feuchtinger A, Möller W, Ding Y, Kutschke D, Möller G et al*. Three-Dimensional Quantitative Co-Mapping of Pulmonary Morphology and Nanoparticle Distribution with Cellular Resolution in Nondissected Murine Lungs. *ACS Nano.* 2019; 13 (2): 1029–1041. doi: 10.1021/acsnano.8b07524.

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