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MICROSTRUCTURING SUBSTRATES FORMED BY ION IMPLANTATION FOR ANALYSIS OF SMALL BIOMATERIALS

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Abstract. The paper shows a new method of forming substrates (devices) with periodic micron surface grids for statistical analysis and visual characterization of ultra-small biological objects and microorganisms. When using implantation with Ar^+ of silicate glasses through surface masks in the form of wire meshes, surface periodic structures in the form of gratings with cell sizes of 25×25 μm and a depth of 100 nm were fabricated. Testing of new types of devices was carried out using scanning electron and atomic force microscopy, as well as applying EDX analysis using the example of substrates with deposited bacteria as *Bacillus subtilis*.

Keywords: *Mask-ion implantation, Silver nanoparticles, Counting biological grid.*

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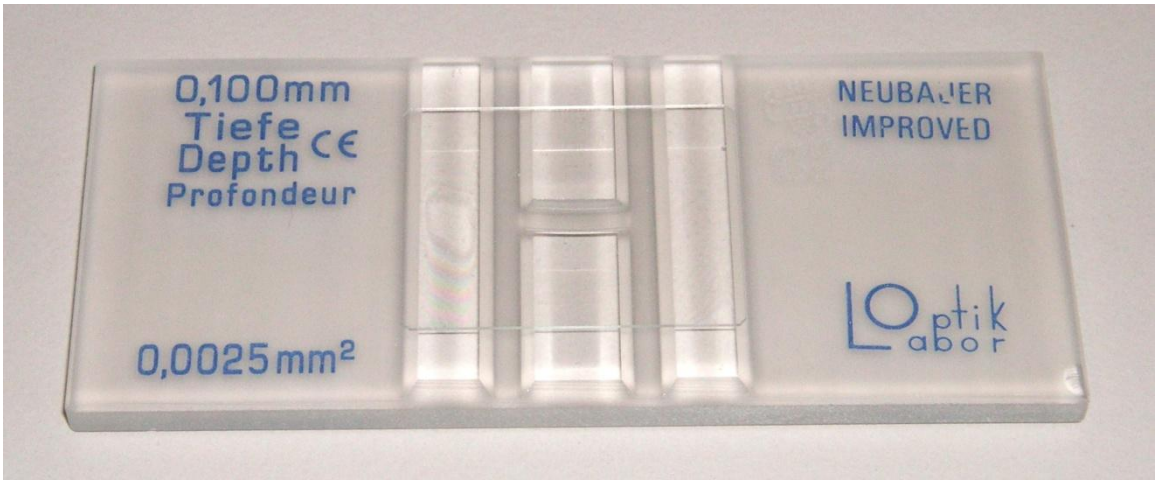
1. Introduction

The proposed work is associated with the implementation of the strategy of scientific and technological development in terms of the transition to personalized medicine. One of the potential solutions to the problems of identifying and subsequent localization of the spread, as well as the treatment of various types of infectious diseases is the timely and prompt diagnosis of various bioobjects – sources of diseases. For this purpose, new microstructured materials could be effectively used for carrying out standardized counting statistical analysis in work and in research in medicine during sequencing, separation, detection, identification, quantitative and structural analysis of biological molecules and micro-objects such as cell populations (blood, cell cultures), microorganisms, viruses, etc.

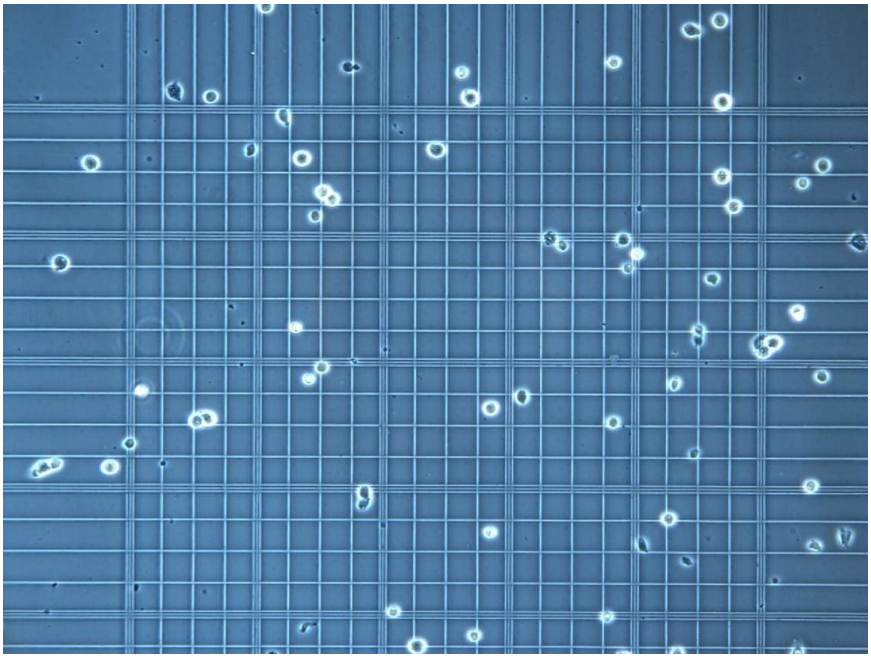
A commercial device is known (Fig. 1), called a counting chamber with Goryaev nets, which serves to count biological objects (Benumovich, 1999). This device was

proposed by a Russian doctor, a professor at Kazan University N.K. Goryaev (1875-1943). The essential features of the counting chamber with Goryaev's nets are the following: an optically transparent glass object, in which there is a groove with meshes engraved on its bottom. The Goryaev grid consists of large and small squares. The area of one large square is 1/25 mm^2, the area of one small square is 1/400 mm^2. Statistical analysis of biological objects is carried out according to establish the standardized counting procedures in medicine and biology, using fixed periodically grids on the bottom surface of the chamber and distribution of the counting biomaterial on them. A definite restriction for the use of this device is that the minimum mesh size is so large that it complicates or eliminates the possibility of counting ultra-small biological objects, several microns in size. Moreover, since the grid is made using the engraving method, the grid cells rise above their boundaries – the grooves of the engraving. In this case, mesh cells cannot play the role of sinks for microobjects – trap cells, which makes it difficult to fix microobjects in separate traps.

In recent years, in medicine and biology (Khalilov *et al.*, 2018; Samadishadlou *et al.*, 2018), various methods are increasingly being applied to analyze ultra-small biological objects using high-resolution electron and probe microscopy. The development of microscopic methods is due to the advent of new hardware and the use of modern advances in the development of various composite materials, derived from the study of their chemical and physical properties. The creation of fundamentally new technological control and analysis systems, as well as the development of new methods based on them, could open new perspectives for the diagnosis and characterization of ultra-small cell biological material (bacterial cells and viruses). Therefore, there are previously unrealized opportunities for their calculation and statistical processing, form analysis, etc. All this allows to significantly speed up the diagnosis and analysis of pathogenic and conditionally pathogenic biomaterial, which will lead closer to solving a number of problems in personalized medicine and improving healthcare.



(a)



(b)

Fig. 1. The Goryaev camera commercial device (a) and the image of blood elements against the background of the Goryaev camera grid obtained with an optical microscope (b).
Images are taken from https://ru.wikipedia.org/wiki/Kamera_Goryaeva

The objective of this work is the development, creation and testing of specific devices (substrates) with periodic micron surface grids with nanostructured cells in depth to ensure the counting of ultra-small biological micro-objects using high-resolution electron and probe microscopy. To form such biological devices, it is proposed to use the technology of ion implantation for modification and controlled structuring of the surface of dielectric materials (Stepanov, 2018). Ion implantation is currently one of the main techniques used in industrial semiconductor microelectronics for the formation of various types of nano and microdevices (Fink *et al.*, 2012). The possibility of creating periodic surface with optical diffraction structures by implanting various ions through masks on materials such as silicon (Azimi *et al.*, 2012), silica glass (Stepanov *et al.*, 2013; Wang *et al.*, 2017), polymethylmethacrylate (Nuzhdin *et al.*, 2018) and diamond (Stepanov *et al.*, 2018) was previously demonstrated. In the present study, it is proposed for the first time to use the implantation by inert gas ion with a biocompatible material – silicate glass through a metal mask to form surface microstructures suitable for use in the analysis of ultra-small biological objects.

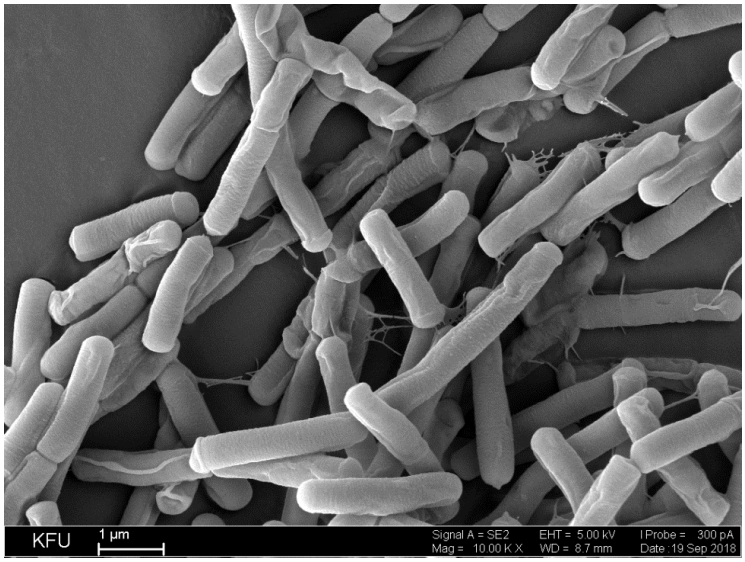
2. Experimental

The implantation by single-charged Ar^+ ions with an energy of E = 40 keV, an irradiation dose of $D = 3.1 \times 10^{17}$ ion/cm^2 and a current density of J = 20 μA cm^2 was carried out by an ILU-3 ion beam accelerator through a copper wire mesh mask with a size square cells 25 microns in cover silicate glasses (20 × 20 mm, thickness 170 microns). The choice of silicate glass is due to biocompatibility with various biological objects.

The morphology of the structured surface of an implanted glass with deposited microorganisms was studied using a Merlin (Carl Zeiss) high-resolution scanning electron microscope (SEM) at a low accelerating voltage of 5 keV in the secondary electron detection mode. Atomic force microscopy (AFM) was also used to characterize the morphology of local areas of the structured surface applying a Dimension Fast Scan microscope (Bruker) with Bruker ScanAssyst Air probes with a radius of curvature of ~5 nm and a hardness of 0.4 Nm). Elemental analysis was carried out using energy dispersive spectrometry (EDX) on an X-Max spectrometer (Oxford Instruments) combined with an SEM, at an accelerating voltage of 20 keV.

The bacterial *Bacillus subtilis* MG4 from the collection of microorganisms from the laboratory of biosynthesis and bioengineering of the Institute of Fundamental Medicine and Biology, Kazan Federal University was used as a biomaterial for deposition on the proposed substrates (Fig. 2). The cultivation of bacteria was carried out in a liquid nutrient medium LB (Luria-Bertani): trypton – 10 g/L, yeast extract – 5 g/L, sodium chloride – 5 g/L at a temperature of 37ºC and with stirring 200-250 rpm for 16-18 h. Subsequently, the suspension of microorganisms was precipitated by centrifugation (5000 rpm, Biosan centrifuge), the precipitate was resuspended in 1% glutaraldehyde (EM grade, Sigma-Aldrich) and kept for 18 hours to pass chemical fixation. After the chemical fixation procedure, microorganisms were precipitated by centrifugation and washed by resuspension in phosphate buffer, then dehydration was carried out in ethylalcohol (50-96%). From 96% alcohol, the suspension was deposited on a glass substrate nanostructured by ion implantation. To carry out SEM and EDX analysis, a gold/palladium conducting layer (Quorum Q-150T ES) 10-15 nm thick was applied onto the substrate surface with microorganisms.

(a)



(b)

Fig. 2. Petri cap with cultured bacteria culture of *Bacillus subtilis* (a) and their SEM image (b)

3. Results and discussions

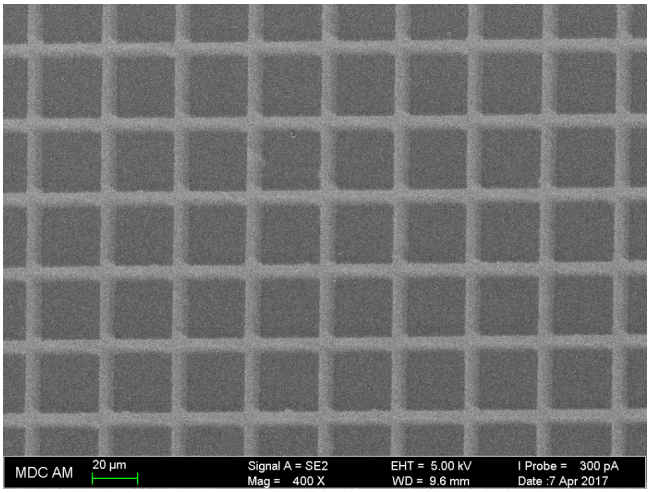
Ion implantation is the process of introducing accelerated ions into the irradiated matrix at a certain depth depending on energy. The process of ion collisions with the surface may be accompanied by partial sputtering or swelling. The nature of these processes depends on a number of factors such as acceleration energy, ion mass, density of the target substance, etc. Obviously, if material is implanted through a mask, areas of irradiated and non-irradiated matter form on such surface. As was shown in the case of the implantation of diamond single crystals with high doses of B^+ ions (Stepanov *et al.*, 2018), the irradiated areas turn into porous graphite, swell and rise above the initial level of the substrate. On the contrary, when silica glass was implanted with Cu^+ or/and Ag^+ ions, because of surface sputtering a decrease in the level of the irradiated area relative to the initial substrate were detected (Stepanov *et al.*, 2013; Wang *et al.*, 2017; Nuzhdin *et al.*, 2018).

In the present work, the observation of a change in the morphology of the surface of silicate glass subjected to high-dose implantation with Ar^+ ions through a mask was carried out by SEM and AFM (Fig. 3). SEM images allow visualization of a large sample area (Fig. 3a), on which a periodic grating pattern is formed with square implanted cells alternating with unirradiated silicate glass walls. The micron size of cells coincides with the parameters of the superimposed mask. AFM allows you to capture local surface areas with a higher scale, and at the same time to conduct quantitative measurements of the depth of the sputtered implanted areas (Fig. 3b). The thickness of the sputtered layer of glass (the depth of the lattice cells) for the selected radiation dose is near 100 nm, as these follow from the measured profile of the step (Fig. 3c), (shown by the segments in Fig. 3b).

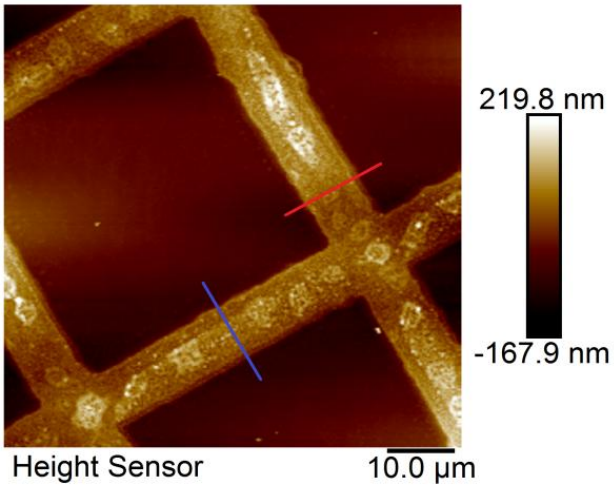
In Fig. 4 it is shown the AFM images of the surface grid on a glass substrate with deposited bacteria *Bacillus subtilis*. It is clearly seen that the dimensional parameters of the formed grid provide a convenient for work ratio with the size of small microorganisms, such as bacteria of medium (3-5 microns) sizes. As follows from the

figure, part of the bacteria is deposited on the edges of the grid, which could lead to some error in their statistical analysis. However, by optimizing the ion implantation parameters (Stepanov, 2018), it is possible to form grids of various depths up to hundreds of nanometers, and thus ensure a more efficient distribution of bacteria in the buried cells and minimize or eliminate the biomaterial deposition on the walls of the grid.

Fig. 5a shows an SEM image of *Bacillus subtilis* bacteria, deposited in a small concentration on a substrate with a periodic structure, and recorded against the background of a single cell. There is a relatively uniform distribution of bacteria over the entire surface of the implanted glass, and with some localization, mainly in the recesses of the cells. It can be seen that cell sizes are typical for representatives of the *Bacillus subtilis* species in a vegetative physiological state: the bacteria length is 3-5 μm, width is 500 nm.



(a)



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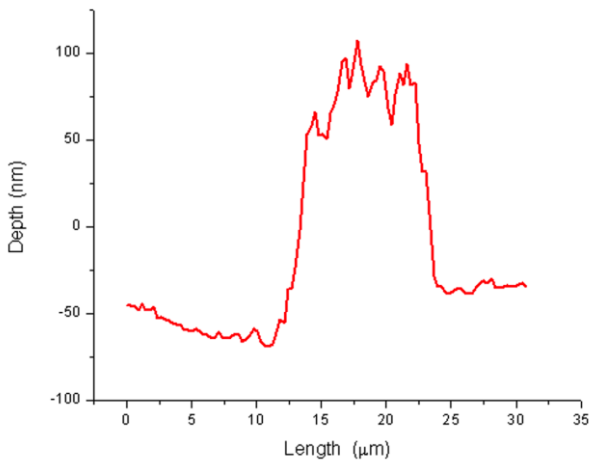
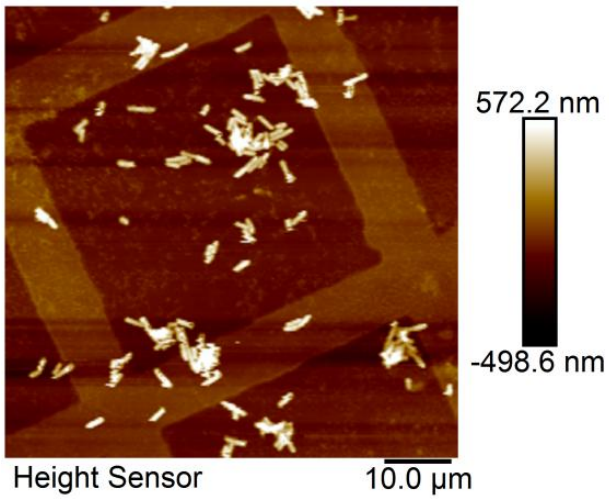
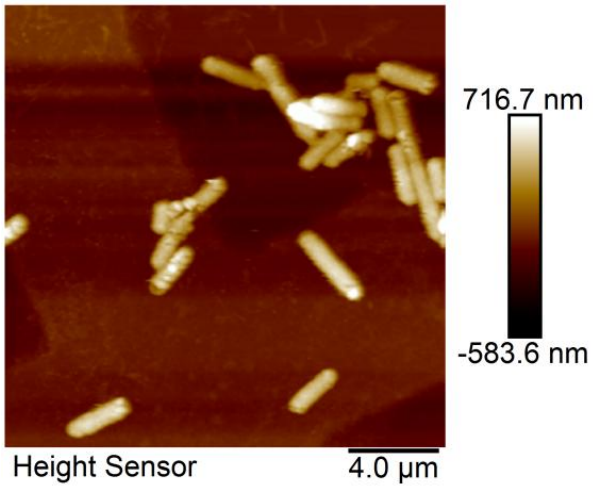


(c)

Fig. 3. SEM (a) and AFM images (b) of the surface grid formed by implantation with Ar ions, as well as the profile of the step (c), measured in the directions shown in (b)



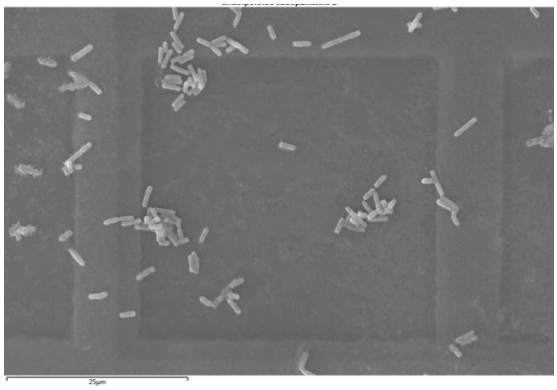
(a)



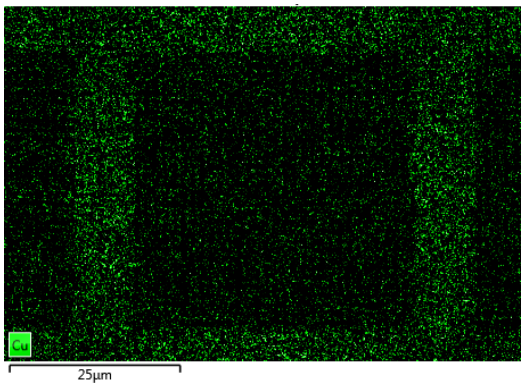
(b)

Fig. 4. AFM images at various scales of a single cell of the surface grid (small (a) and enlarged (b)), with bacteria *Bacillus subtilis* applied on it

By the method of EDX mapping (Fig. 5b-d) it was established that after implantation at high current densities in the ion beam and the heating caused by them, Cu is diffused from the mask into the volume of irradiated glass in those parts of the surface adjacent to the grid screen. In other words, controlled doping of the glass with Cu^{+} ions occurs over the mask pattern (Fig. 5b). When using EDX mapping in different chemical elements (carbon and Cu), one could observe the distribution of bacteria by carbon (Fig. 5c) over the surface relative to the Cu-doped parts of the grid (Fig. 5d). With this approach, it becomes possible to perform statistical analysis, distribution and packing density of bacterial material even in the absence of burial of the grid cells, as is done during AFM analysis (Fig. 4). This approach to EDX mapping could allow working not only with small concentrations of biological material, but also with their thick layers, since the electron beam penetrates through them easily.



(a)



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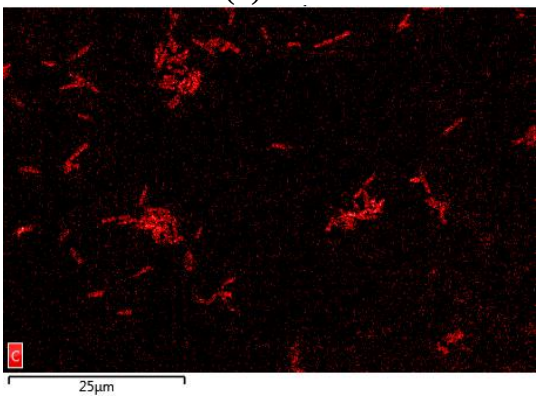

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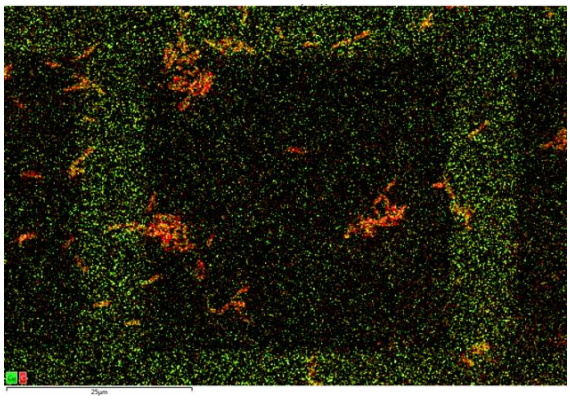

(d)

Fig. 5. SEM-image of a single cell of the surface grid (a) with *Bacillus subtilis* bacteria applied on it and the corresponding EDX maps for various chemical elements:
Cu (b), carbon (c) and Cu + carbon (d)

4. Conclusion

Thus, the paper demonstrated the possibility of creating new technological controlled analyzing devices (glass substrates with periodic micron surface grids with nanostructured cells in depth), and laid the basement for the development of new techniques for characterizing small cellular material using AFM, SEM and EDX analysis. The use of substrates with a cellular structure, formed by the method of ion implantation through a mask, allows for calculations of biological objects of ultra-small sizes. The large depth of penetration of electrons into organic media during EDX mapping makes it possible to obtain an image of the formed grid even from under a dense layer of biological material, which significantly expands the possibilities of analyzing microbial biofilms. In the future, the use of such devices with the involvement of AFM, SEM and EDX analysis will allow for the automatic processing of statistical information on biological objects.

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