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INVESTIGATION OF THE MICRO- AND NANO-STRUCTURE OF LIVER CELLS CULTURED ON BIODEGRADABLE SILK FIBROIN-BASED SCAFFOLDS USING SCANNING PROBE OPTICAL NANOTOMOGRAPHY

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Objective: to analyze the 3D micro- and nano-structure and quantitative morphological parameters of liver cells cultured on biodegradable silk fibroin-based film scaffolds. **Materials and methods.** Samples of biodegradable silk fibroin-based scaffolds with cultured Wistar rat liver cells were obtained for the study. The 3D structure of liver cells cultivated on the scaffolds was studied by scanning probe optical nanotomography using an experimental setup combining an ultramicrotome and a scanning probe microscope in correlation with fluorescence microscopy. **Results.** Nanoscale images and 3D nanotomographic reconstructions of rat liver cells cultured on scaffold were obtained. The morphological parameters of liver cells (average roughness, specific effective area) were determined. The average surface roughness of the liver cells R_a was found to be 124.8 ± 8.2 nm, while the effective surface area σ was 1.13 ± 0.02. Analysis of the volume distribution of lipid droplets showed that they occupy 28% of the cell volume. **Conclusion.** Scanning probe optical nanotomography can successfully analyze the nanostructure and quantify the nanomorphology of liver cells cultured on biodegradable scaffolds.

Keywords: liver cells, biodegradable scaffolds, silk fibroin, scanning probe microscopy, fluorescence microscopy, nanotomography.

INTRODUCTION

One of the tasks in modern transplantation include the search for new materials for creating scaffolds with improved adhesive properties for the development of constructs for tissue engineering and regenerative medicine. When solving these problems, it is necessary to use new technological approaches to analyze the micro- and nanostructural features of constructs and cells in them, as well as the parameters of interaction between the cells and the surface of the constructs at the micro- and nanolevel.

A combination of various microscopy techniques (electron microscopy, scanning probe microscopy, high-resolution optical micro-spectroscopy) makes it possible to carry out correlative studies of micro- and nanostructures with high resolution [1]. The information obtained during the analysis using various microscopic technologies makes it possible to comprehensively characterize the structure of biological objects and obtain new knowledge about their properties [2].

One of the most in-demand areas in this field is the development of methods and approaches for analyzing the three-dimensional micro- and nanostructure of biomaterials, biological objects, and tissue-engineered constructs, which would provide correlative information about both nanomorphology and the biological structure of the objects under study.

The unique technique of scanning probe optical nanotomography (SPONT) allows combining the functional capabilities of a scanning probe microscope (SPM) and an ultramicrotome in one device in correlation with optical fluorescence microscopy methods [3, 4]. SPONT makes it possible to study the 3D nanostructure of biomaterials, cells, tissues, and tissue-engineered constructs, in correlation with the study of fluorescent marker localization in the volume of measured samples. Analysis of the obtained 3D reconstructions of the structure of biological objects and materials allows one to quantitatively determine such important parameters of their nanomorphology as micro- and nanoporosity [5], effective surface area and surface roughness [6], and the surface area to volume ratio [7].

Silk fibroin-based biodegradable film scaffolds with cultured rat liver cells were chosen as objects of this study.

Silk fibroin, derived from Bombyx mori cocoons, is one of the promising materials for tissue engineering. This biopolymer not only meets all the requirements for

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materials in regenerative medicine, but also has a number of advantages over other materials. Its biocompatibility, optimal mechanical properties, and biodegradability provide possible application in many areas of regenerative medicine, both as an independent material and as a component of various composites based on it [8]. All these properties make it possible to obtain from it two-dimensional and three-dimensional constructs for various purposes [9–11].

At present, many materials that can be effectively used to make scaffolds for culturing liver cells are being considered. There are known materials, whose coatings support hepatocyte adhesion, but formation of constructs from them is associated with difficulties. Such materials include, for example, chitosan, gelatin, collagen, etc. Silk fibroin has attracted the attention of researchers since it supports hepatocyte adhesion and proliferation, and also has suitable mechanical properties [12]. Fibroin can also be used in composite materials. For example, 3D porous scaffolds made of fibroin and chitosan that are mixed in different ratios support adhesion and proliferation of human hepatocellular carcinoma in vitro, and adhesion capacity of cells on scaffolds is much higher than on a control substrate [13]. Scaffolds of similar composition, made using electrospinning, were also used for culturing mouse hepatocytes [12].

In this work, the micro- and nanostructure of rat liver cells adhered to biodegradable silk fibroin-based film scaffolds were studied. A similar study for these cell types using SPONT has been performed for the first time.

MATERIALS AND METHODS

Production of film scaffolds for cells based on silkworm fibroin *Bombyx mori*

Fibroin for manufacturing film scaffolds for cells was isolated from silk filaments of the *Bombyx mori* silkworm. For this purpose, 1 g of silk filaments was weighed, and the filaments were cut into 0.5 cm fragments. The filaments were boiled in a water bath for 40 minutes in a solution with the addition of sodium bicarbonate to clean the filaments of sericin and other impurities. Then the filaments were washed with 3600 mL of distilled water. The filaments were boiled 3 times for 30 minutes each in double-distilled water, rinsing after each boiling with distilled water. After that, they were dried in a desiccator.

To obtain an aqueous fibroin solution, a sample of fibroin weighing 130 mg/ml was added to a solution prepared at the rate of 389 mg $CaCl_2$, 388 μl C_2H_5OH, and 544 μl H_2O per 1 ml of solution. The solution was heated in a water bath at 40 °C for 4 hours. Then the solution was dialyzed against double-distilled water. The optical density of the silk fibroin solution in the solution obtained after dialysis was measured spectrophotometrically at 280 nm wavelength, then the concentration was calculated using the formula D = ECL, where E is the extinction coefficient of silk fibroin equal to 1.07.

Next, an aqueous silk fibroin solution obtained by the technique described above was applied to the bottom of a Petri dish and dried at room temperature. The dried silk fibroin was dissolved in formic acid at 20 mg/mL by heating to 40 °C in a water bath for 30 minutes. The resulting solution was centrifuged for 5 minutes at 12,100 g.

Samples of biodegradable scaffolds in the form of silk fibroin films were obtained by irrigation. To make the films, 150 μL of a solution of a given composition was applied to the bottom wells of a 48-well culture plate and dried for two days at room temperature. The total protein concentration in the film was 20 mg/mL. After the fibroin solution had dried, 100 μL of 10 μg/mL fibronectin solution was applied to the wells of the plate and dried for two days at room temperature.

Before cell culturing, film scaffolds were sterilized: treated with 70% ethanol for 30 minutes, then irradiated with ultraviolet light for 30 minutes. After that, a sterile phosphate-salt buffer solution was introduced into the wells of the plate and incubated for 15 minutes, after which the phosphate-buffered saline was changed. This procedure was repeated three times. Then, 300 μL of incubation medium was added to the wells of the plate for 30 minutes.

Preparation of Wistar rat liver cell samples cultured on silk fibroin scaffolds for scanning probe optical nanotomography

Rat liver cells were isolated as follows. In the experiment, 1 year old male Wistar rats (weighing 250–350 g) were used. The rat was put to sleep using diethyl ether inhalation anesthesia. Then, the rat was placed belly-up on the operating table and the paws were straightened. Using tweezers, pulling aside the skin on the abdomen, a longitudinal skin incision was made with scissors in the midline of the abdominal side of the body from the genital opening to the sternum. The skin was turned away and secured. Then the abdominal cavity was opened, making a longitudinal incision along the midline; the muscle flaps were turned aside. The liver was harvested by cutting off the vena cava, portal vein, hepatic arteries, and veins. After sampling, the liver was placed in a sterile 0.9% sodium chloride solution and washed of blood. Next, the liver fragment was transferred into a second portion of sterile 0.9% sodium chloride solution and the liver was cut into 5×5 mm fragments using scissors. Then each liver fragment was washed with 0.9% sodium chloride solution using a syringe without a needle. After wa-

shing off blood, the liver fragment was transferred into a sterile dry Petri dish and injected with a collagenase type 2 solution with a 2 mg/mL concentration using an insulin syringe. Then the tissue was incubated for 20 minutes. Next, the liver was chopped with scissors and incubated for another 5 minutes, then transferred to ice. After that, 5 mL of 0.9% sodium chloride solution was added to the liver tissue. The resulting solution was transferred to a test tube, stirred, and centrifuged for 5 minutes at 1770 g and –5 °C. The supernatant was removed, the culture medium was added to the resulting precipitate, stirred and the cell suspension was transferred into previously prepared 48-well plates. The cells were incubated in a thermostat at 37 °C and 5% CO_2 for 2 days. Liver cells for further experiments using the SPONT method were visually localized using an optical microscope.

To examine cells on scaffolds by fluorescence SPONT, cells were pre-stained with fluorescent dyes FITC and DAPI. The wells were washed twice with sterile 0.9% sodium chloride solution, then a DAPI solution with 3 μg/mL concentration was added and incubated for 5 minutes at 37 °C. Two short washes were then performed with a sterile 0.9% sodium chloride solution, after which a 2.5% glutaraldehyde solution in phosphate-buffered saline (pH = 7.4) was added to the wells and incubated for 2 hours in the dark at +4 °C. Next, one short wash with a sterile 0.9% sodium chloride solution and one short wash with a carbonate-bicarbonate buffer solution (pH = 9.0) were performed. Then an FITC solution in dimethyl sulfoxide with a 1 mg/mL concentration was added to the wells and incubated for 40 minutes in the dark, followed by two short washes with a carbonate-bicarbonate buffer solution (pH = 9.0).

To prepare a preparation for SPONT, dehydration of scaffold samples with cultured liver cells was performed by conducting through alcohols with increasing concentration according to the following scheme:

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.

The samples were washed three times with 100% ethyl alcohol for 10 minutes each, and then incubated in a mixture of 100% ethyl alcohol and epoxy resin in a 1 : 1 ratio for 30 minutes, after which the samples were transferred into a mixture of 100% ethyl alcohol 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 12 hours, after which the incubation was continued for 72 hours at 60 °C. The scaffolds in epoxy resin were then separated from the bottom of the plates using a scalpel.

To embed the samples, we used an epoxy medium (Epoxy Embedding Medium) mixed with an equal weight of the embedding medium hardener (dodecenyl succinic anhydride DDSA) and 4% by weight of 2,4,6-Tris(dimethylaminomethyl)phenol (DMP-30).

Scanning probe optical nanotomography

An experimental unit Ntegra Tomo was used to study samples of rat liver cells by scanning probe nanotomography techniques. This unit allows for sequential SPM measurements of sample surface immediately after cutting into slices with an ultramicrotome. Consecutive 100 nm thick slices were made using a Diatome Ultra AFM 35 diamond knife (Diatome AG, Switzerland) with 2.0 mm cutting edge width.

SPM measurements were carried out in a semi-contact mode at 1.0 Hz scanning speed using NSG10 silicon cantilever probes (NT-MDT, Moscow) with 240 kHz resonance frequency and <10 nm tip curvature radius. Primary image processing was carried out using the Nova ImageAnalysis 1.0.26.1443 software (NT-MDT, Moscow); 3D tomographic reconstructions of the liver cell structure were obtained using the ImagePro Plus 6.0 software (Media Cybernetics, Inc, USA).

Correlative measurements of slices using optical fluorescence microscopy were performed using an AxioVert.A1 fluorescence inverted microscope (Carl Zeiss, Germany) equipped with an HBO 100 mercury lamp. Fluorescence images were obtained using oil-immersion lens Carl Zeiss EC Plan-Neofluar 100x/1.30 Oil Ph3. The Zen software package (Carl Zeiss, Germany) was used to analyze fluorescence images.

Analysis of surfaces reconstructed by the SPONT method using ImagePro Plus 6.0 software (Media Cybernetics, Inc, USA) makes it possible to determine and analyze the nanoscale parameters of these surfaces, such as average roughness Ra and effective surface area σ. The algorithms used for calculating these parameters are given in [6].

RESULTS AND DISCUSSION

Prepared samples of rat liver cells cultured on silk fibroin scaffolds were examined using SPONT technology. After separation of the scaffold samples embedded in epoxy medium from the plates, the cultured liver cells were localized in the scaffold plane using fluorescence microscopy. Based on analysis of the obtained images, scaffolds were selected for further SPONT analysis, then primary cutting of the sample into slices were performed with an ultramicrotome in a plane perpendicular to the scaffold plane. The distance from the plane of the primary slice to the liver cells being registered was measured using fluorescence microscopy. In accordance with the

measurements taken, subsequent slices of the scaffold with a controlled total thickness were made in order to obtain liver cell slices. Fig. 1 shows a fluorescence image of a sample of a silk fibroin-based film with liver cells adhered to it. The line marks the slice plane, the arrow indicates the cell localization area where SPONT analysis was performed.

Fig. 2 shows images obtained during analysis of liver cells adhered to silk fibroin-based film scaffolds. SPM analysis (Fig. 2a) revealed the presence of a nucleus, as well as inclusions in the form of a lipid droplet occupying a significant volume of the cell. Such inclusions are one of the characteristic features of liver cells. The findings were confirmed by correlative fluorescence images. The DAPI dye distribution confirms the presence of a nucleus in the cell (Fig. 2c); no dye fluorescence was observed in the lipid droplet area (Fig. 2b, c).

Further analysis of the structure of rat liver cells adhered to silk fibroin-based film scaffolds revealed the presence of a large number of inclusions. Analysis of the images presented in Fig. 3 revealed inclusions in the form of lipid droplets in the structure of liver cells, in the area of which no fluorescence was registered (Fig. 3b), and shaped granules, presumably glycogen (indicated by arrows in Fig. 3b).

Analysis of the structure of rat liver cells adhered to silk fibroin-based film scaffolds with high resolution revealed extensive areas of cytoplasm with a heterogeneous structure (Fig. 4), which may indicate a high level of cell metabolism due to developed endoplasmic reticulum system.

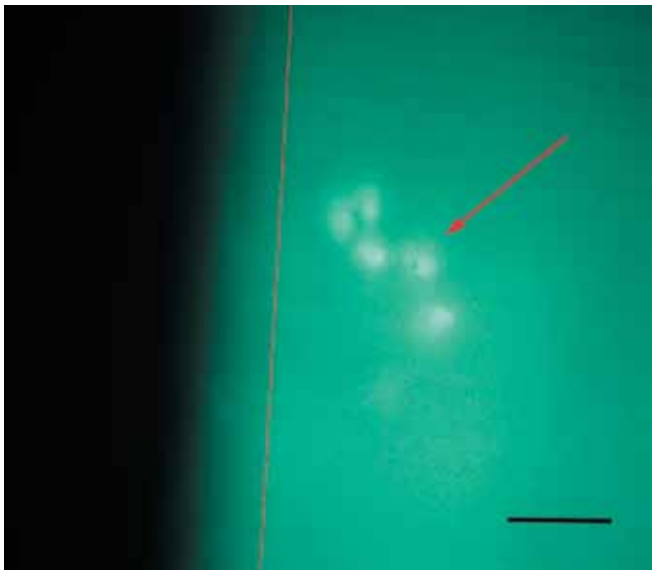

Fig. 1. Fluorescence image of a silk fibroin film sample with liver cells adhered to it; FITC stain, magnification 200×, 50 μm scale bar

To assess the 3D morphology of a liver cell adhered to the surface of a silk fibroin-based film scaffold, a 3D reconstruction of the cell fragment was performed using SPONT. For this purpose, 14 successive 200 nm thick slices of the sample were made, and 14 successive SPM images of the scaffold area measuring 32×16 μm

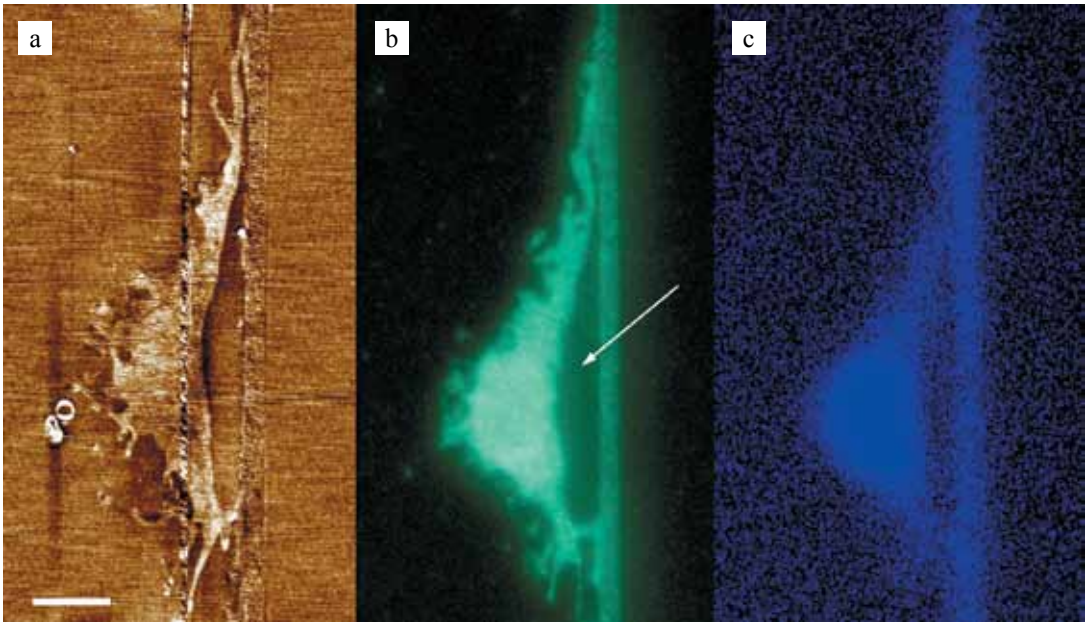

Fig. 2. Analysis of liver cell samples cultured on silk fibroin-based film scaffolds. a) SPM image of the surface topography of a cross-section of the silk fibroin-based film scaffold with adhered liver cell: scan size 25×12 μm, height variation range 11 nm, scale bar 3 μm. b) correlative fluorescence image of a cross-section of the silk fibroin-based film scaffolds with adhered liver cell, FITC stain, magnification 1000×, the arrow indicates a lipid droplet. c) correlative fluorescence image of a cross-section of the silk fibroin-based film scaffold with adhered liver cell, DAPI stain, magnification 1000×

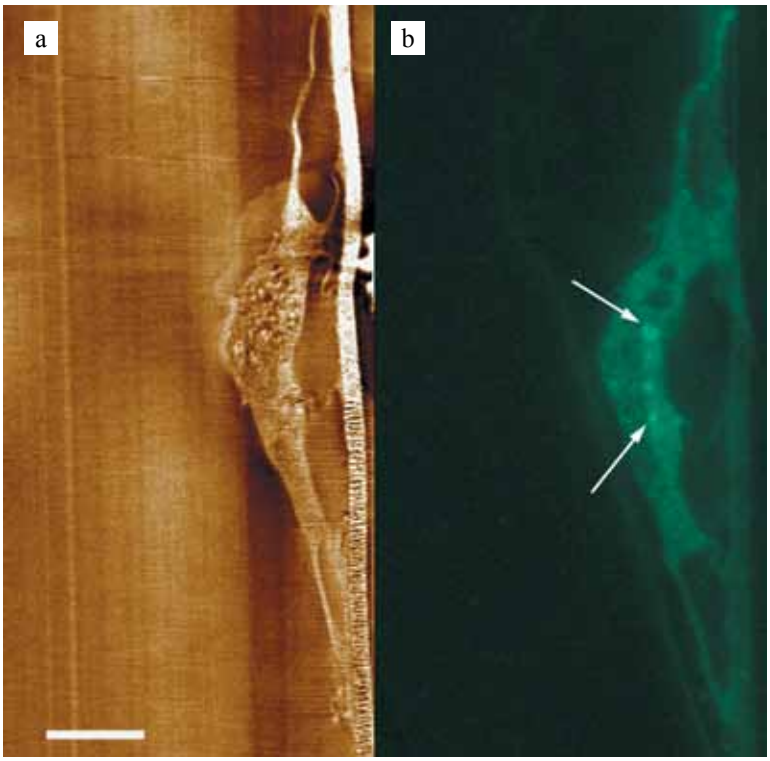

Fig. 3. Analysis of liver cell samples cultured on silk fibroin-based film scaffold. a) SPM image of the surface topography of a cross-section of the silk fibroin-based film scaffold with adhered liver cell: scan size 32×15 μm, height variation range 40 nm, scale bar 4 μm; b) correlative fluorescence image of a cross-section of the silk fibroin-based film scaffold with adhered liver cell, FITC stain, magnification 1000×, arrows indicate glycogen granules

Fig. 4. SPM image of the topography of a cross-section of the silk fibroin-based film scaffold with adhered liver cell: scan size 7.0×6.0 μm, height variation range 20 nm, scale bar 1 μm

in size were obtained. The resulting visualization of the 3D structure of the liver cell is shown in Fig. 5.

Analysis of the obtained 3D data made it possible to determine morphological parameters of the reconstructed 3D structure of the liver cell. So, the average cell surface roughness Ra is 124.8 ± 8.2 nm, while the effective surface area σ, calculated as the ratio of surface area to the area of its 2D projection onto the plane, is 1.13 ± 0.02. This dimensionless parameter determines the degree of surface development. Analysis of volumetric distribution of lipid droplets showed that they occupy 28% of the volume of the reconstructed cell fragment.

The technique developed by us for studying the nanoscale structures of rat liver cells that are adhered to biodegradable scaffolds using SPONT is applicable for studying the features of the 3D structure of cells of different types in micro- and nanoscale.

CONCLUSION

In this work, the nanoscale structures of rat liver cells adhered to the silk fibroin-based biodegradable film scaffolds were studied using the SPONT method. Three-dimensional reconstructions of a rat liver cell and its correlative fluorescence images were obtained; the parameters of three-dimensional cell nanomorphology were determined. The results obtained demonstrated that

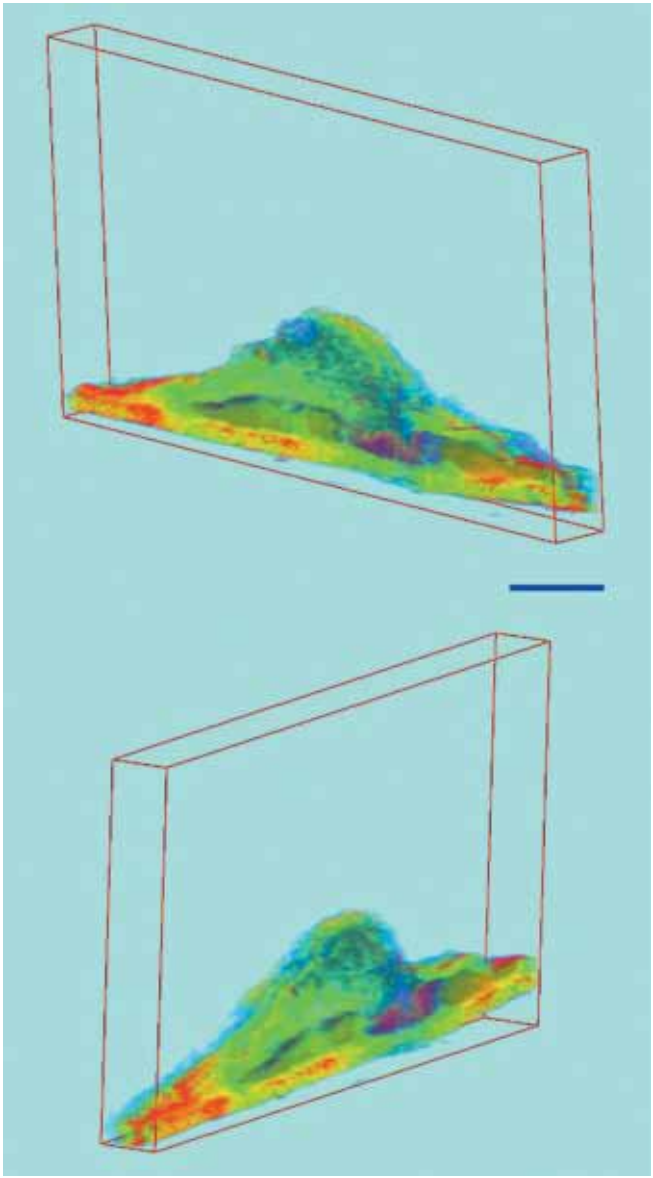

Fig. 5. SPONT 3D-reconstruction of the liver cell adhered to the silk fibroin-based film scaffold, 32.0×15.0×2.8 μm, slice thickness 200 nm, scale bar 5 μm

the developed method of fluorescence scanning probe optical nanotomography can be effectively used for correlation analysis of the structure of biodegradable silk fibroin-reinforced scaffolds with cells cultured on them.

The authors declare no conflict of interest.

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