SCANNING PROBE MICROSCOPY AS A TOOL FOR INVESTIGATION OF BIOMATERIALS

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Abstract. *Super-microscopic techniques like scanning tunnelling microscopy, atomic force microscopy or scanning near-field optical microscopy allows investigate micro- and/or nano-scale surfaces and structures. In this paper, both Environmental scanning electron microscope (ESEM) and Scanning near field optical microscope (SNOM) have been applied to more closely study of biomaterials. The results of visualization of human osteo-sarcoma cell line (U2OS) are compared. SNOM and ESEM yield different, however, comparable and complementary information on studied biological samples.*

Keywords

Biological cell, ESEM, SNOM, U2OS cell line, visualization.

1. Introduction

Exploration of cell surface at nanoscopic level is an important issue of cell biology, since the morphology of cell membrane and its molecules distribution may provide important clues to cell physiology and pathology. Traditionally, conventional electron microscopy techniques, like scanning electron microscopy (SEM), are currently considered the best tools to investigate cell surfaces. The major limit of this approach is, however, due to invasive protocols like strong dehydration methods and metal coating of biological specimens.

Light based techniques are becoming an increasingly popular method of probing heavily scattering media such as body tissue [1]. At present, different types of commercial or scientific UV-visible-IR light microscopes still a plays significant role on the field of bio-medical and science. A current development in optical imaging technologies enables the study of biological samples with a resolution that is capable of detecting macromolecular complexes and signalling domains [2].

Several advanced non-conventional microscopy techniques like scanning near-field optical microscopy (SNOM) and atomic force microscopy (AFM) has been recently developed and might be used to explore the surface of biological membranes at high resolution. These novel instruments should supply important details of cell topography [3]. SNOM in particular is highly attractive because it combines scanning probe techniques (SPM) with optical microscopy, providing a lateral spatial resolution beyond the traditional diffraction limit [4], [5] using a point light source coming from a subwavelength aperture. SNOM offer a unique technology to simultaneously map cell surface topography and investigate the local optical properties (near-field) of the inner cell structures [6], [7], [8], [9].

On the other hand, the environmental scanning electron microscopy (ESEM) is a modern solution for the observation of any type of nonconductive objects, without the necessity of coating with a metallic or carbon layer. In addition, wet samples of animate or inanimate origin can be preserved and observed close to their natural status [10].

Both ESEM and SNOM can usually operate at similar room conditions - temperature, pressure, humidity, and allow observation of wet samples.

2. Materials and Methods

2.1. Sample Preparation

The sample preparation method is based on the same procedure like for visible-light microscope. It consists of several stages – sampling, fixation, embedding, slicing, staining and mounting of specimens [11]. Nevertheless, the SNOM samples preparation needs some experience.

Preparation and fixation was realized in cooperation with the Faculty of Medicine of Masaryk University, Brno.

For the experiment, the cell culture U2OS of human bone cancer-cells has been used. Specimen cells were cultivated on cover glasses inside cultivation medium (DMEM with 4,5 g/l Glucose, L-Glutamine by PAN Biotech (cat. no. P03-0710 with the $NaHCO_3$, penicillin or streptavidin and 10 % FBS) for 40 hours in CO_2 atmosphere at 37 °C.

Then the specimen were removed from medium and dried. For the specimen staining, the rapid staining set *Diff-Quick* (by Medion Diagnostics) was used, yielding results comparable to the *Pappenheim technique (Giemsa-May-Grünwald)*. Slides were gradually dipped in Fixative Solution and in Stain Solution (I and II), in each for 5 seconds. Thereafter specimen were shortly washed in distilled water and dried by air. Fixative Solution contains Fast Green (0,002 g/l) in methanol.

Stain Solution I contains Eosin Y (1,22 g/l) in phosphate buffer (pH 6,6) and Sodium azide (< 0,1 %) as preservative. Stain Solution II contains Thiazine Dye (1,1 g/l) in the same buffer like the previous one.

Some slides were embedded in one-component epoxy resin Epoxylite 6001-M, polymerized at 100 °C for 3 hours and at 180 °C for 1 hour.

3. Experimental Set-Up

To avoid a quite complicated preparation of samples for SEM microscope (metallization of sample, high vacuum conditions), the ESEM and SNOM measurements seem to be suitable for biological tissue measurements. Prepared samples were analysed by ESEM microscope ESEM SEM AQUASEM-II (Institute of Scientific Instruments Brno, Academy of Sciences of the Czech Republic) and SNOM microscope NT-MDT-Spectra.

3.1. Environmental Scanning Electron Microscope

An ESEM employs a scanned electron beam and electromagnetic lenses to focus and direct the beam on the specimen surface in an identical way as a conventional SEM. A very small focused electron spot (probe) is scanned in a raster form over a small specimen area. The beam electrons interact with the specimen surface layer and produce various signals (information) that are collected with appropriate detectors: secondary electron (SE) ionization detector, the back-scattered electron (BSE) scintillation detector [12]. The output of these detectors modulates, via appropriate electronics, the screen of a monitor to form an image that corresponds to the small raster and information, pixel by pixel, emanating from the specimen surface.

Environmental scanning electron microscope AQUASEM-II (which is actually the SEM VEGA from Tescan) allows visualization of samples in a gaseous environment. All measurements were carried out under constant operating conditions of experimental environmental at the high relative humidity of 85 %, high pressure of gas or water vapor (693–786 Pa) and temperature of 6 °C.

3.2. Scanning Near-Field Optical Microscope

SNOM experiments were carried out on a combined AFM/SNOM microscope based on NT-MDT–Spectra microscope working in reflection mode. A x-y-z piezo scanner with 50 μm lateral scan step serves for sample scanning. During the scan, the tip-sample distance was kept constant at (5±1) nm using an optical shear force feedback control.

The continuous red laser diode light was used for excitation (650 nm, 20 mW, linear polarization) transmitted through a single mode fibre with a sharp tip probe aperture. A very small area of the sample surface (approx. 120 or 200 nm in diameter) was locally illuminated (Fig. 1) [13].

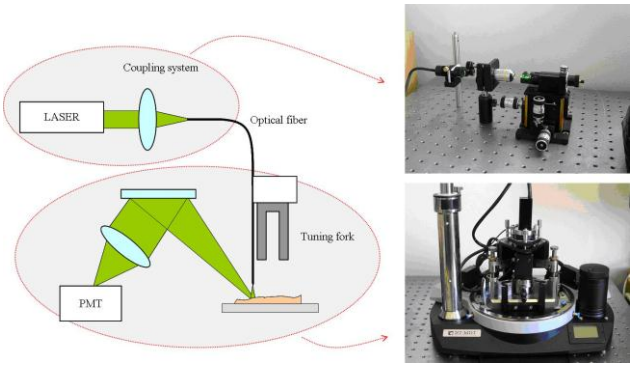

Fig. 1: SNOM experimental set-up for the measurement of effective reflectivity, and topography of cell culture sample.

The excitation light was amplitude-modulated by the light chopper at a frequency of 300 Hz. The input laser power coupled into the optical fibre was 3 mW, output power from the fibre sharpened probe varied

between 10 nW and 100 nW, when detected by remote avalanche photodiode detector (type R74000, Hamamatsu Photonics). Images were recorded in reflection mode at a scan rate of 0,25 Hz and a resolution of 512×512 for large-scale images and 256×256 for 5×5 μm^2 images. We were able to acquire optical signal and topography in a single experiment. The reflected light intensity across the specimen was detected as a function of the probe tip position above the sample surface mounted on an x-y-z piezo-stage.

SNOM probes were prepared from high GeO_2 doped single mode fibres with a core diameter of 3 µm using a two-step chemical etching method followed by aluminium deposition and focused ion beam milling to produce a flat circular aperture. Al-coated sharpened fibre probe with aperture diameters of ~60 and ~150 nm were used in the present work (estimated from SEM images) [14]. The line scan provides the topography and the reflectivity signal. The amplitude of the SNOM tip oscillation was reduced to match the oscillation amplitude of the engaged tip (approximately 10 nm). 2-D images were performed using modified NT-MDT software. The 3-D images can display a combination of the topographic data (height) and reflectivity (color-coded). Thanks to this set-up, the 2-D maps (100×100 μm^2) of topography and surface reflectivity have been obtained (Fig. 4, 5).

4. Results and Discussion

The ESEM images (Fig. 2, 3) show cell structure that was grown up on cover glasses.

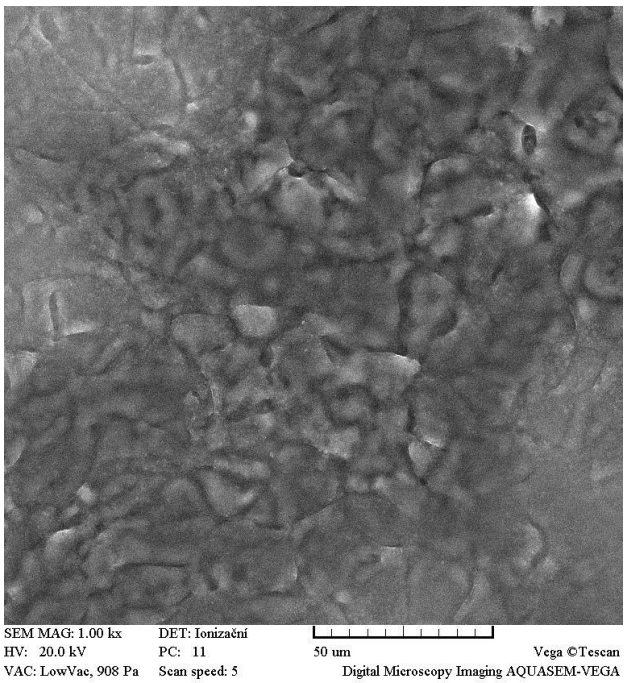

Fig. 2: U2OS in ESEM at 20 kV (SE ionization detector).

Cells are disorganized and the osteosarcoma-tissue has chaotic architecture. Thus, the observed cells are pleomorphic: slight differences in shape and the size of the cells are evident. The nuclei are irregular and hyperchromatic, and are large relative to the cytoplasm. That is caused because by the cancer-cells, which in general grow quickly and haphazardly. The tumour cells, which are derived from osteoblasts, are poorly differentiated. They do not respond to usual regular mechanisms, therefore have increased mitotic activity. By staining, it is possible to distinguish cytoplasm from the cell nucleus and also inner nuclear compartments of cells. Measured average size of the nucleus is about 17 µm.

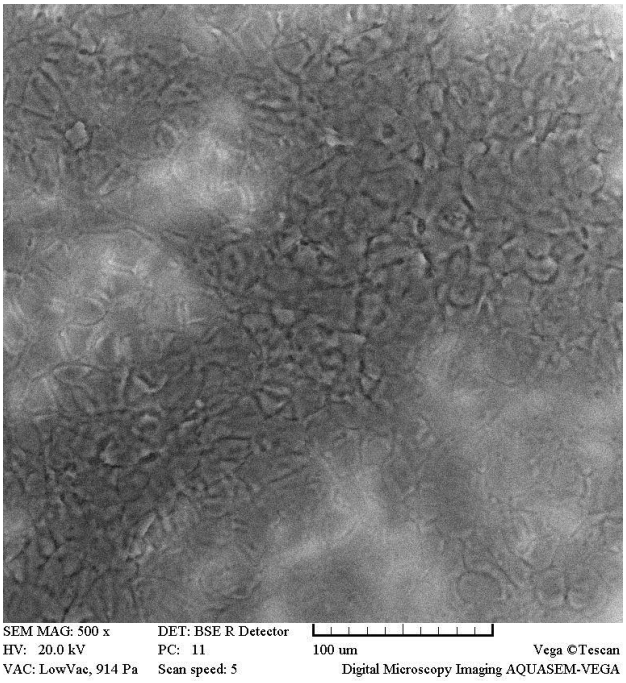

Fig. 3: U2OS in ESEM at 20 kV (BSE detector).

SNOM images (Fig. 4, 5) show the same detail of two cells originated from the central part of Fig. 2. Also, here a measured average size of the nucleus is about 17 µm.

Various SNOM operation modes (optical reflection and topography) make possible to observe the cell surface, alongside with intracellular space. In the displayed images the nucleus of each cell can be easily observed. However, the topography image (Fig. 4) is brighter than reflection one (Fig. 5). Bright areas in Fig. 4 correspond to the location of nuclei, which is caused due to the higher density and mass, comparing to surrounding cytoplasm. Higher density of cytoplasm is relates to increased synthesis in hypertrophic endoplasmic reticulum.

In both images, there are a low number of intercellular bindings. Cell membrane, which bounds the intracellular structure, can be observed as well. It can be presumed, that this method can be useful for instance,

while investigating membrane defects [12].

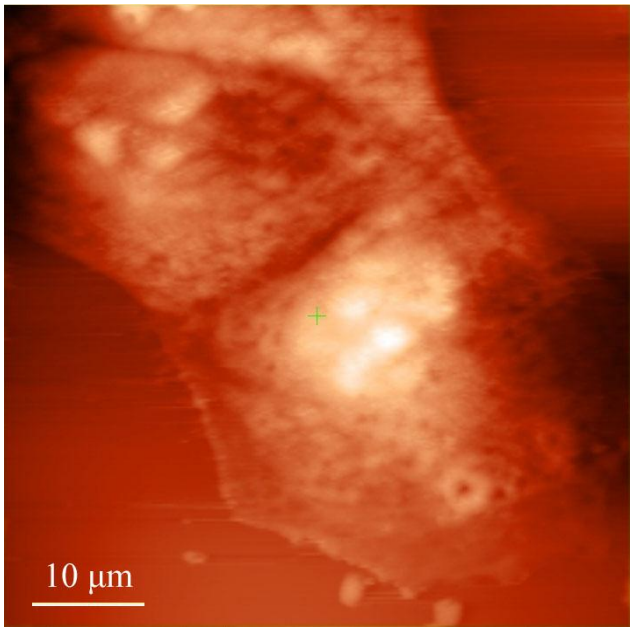

Fig. 4: U2OS SNOM topography.

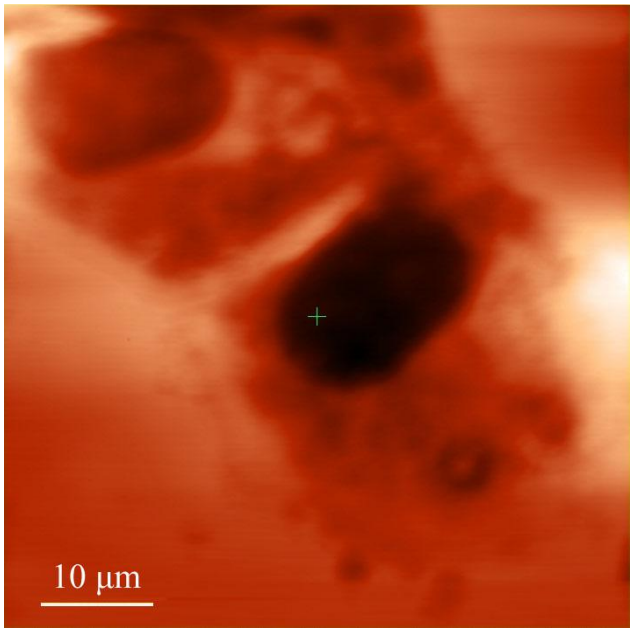

Fig. 5: U2OS SNOM optical reflection.

5. Conclusions

Our study shows that ESEM and SNOM yield different, however, comparable and complementary information of the cell sample. The different acquisition signals of ESEM (3-D topography by SE ionization and BSE detection] and SNOM (like 2-D topography and reflection) have been compared to obtain original observations of cell surface topography and sub-membrane structures with resolution below the conventional diffraction limit.

Although currently visible light microscopes and ESEM still play a significant role on the field of medical and biological science, SNOM incorporates additional mode, which allows recognise intracellular compartments (at the optical reflection mode) as well as the cell surface (at the topography mode).

The contrast of ESEM images decreases accordingly as the electron probe losses current with travel distance and increase of pressure. Nevertheless, increasing the incident-beam current, which is accompanied by an increase of the spot size, can compensate this loss. So ESEM practical resolution depends on the original specimen contrast of a given feature, on the design of the instrument that should provide minimal beam and signal losses and on the operator selecting the correct parameters for each application.

We have imaged stained cells to assess the contribution of topography-induced and reflectivity signals, as a result from SNOM images, shapes of the particular cells can be distinguished, as well as the intracellular membrane structures while using only the simple staining.

Our work illustrates the potential of SNOM for studying membrane domains on a length scale, which could exceed that available with classical optical microscopy. The results show, that SNOM is characterized by higher resolution and possibility to measure local optical properties as against ESEM.

Future development of scanning probe microscopy in biomedical applications will need some improvements in preparation techniques of bio-specimens. Moreover, an extended sensitivity of scanning will be necessary in order to obtain higher resolution and increase the signal-noise ratio of the acquired images.

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References

[1] TUCHIN, Valery V. *Tissue Optics: Light scattering methods and instruments for medical diagnosis*. Bellingham: SPIE - the international society for optics and photonics Press, 2007. ISBN 978-0-8194-6433-0.

[2] TSIEN, R. Y. Imagining imaging's future. *Nature Reviews Molecular Cell Biology*. 2003, vol. 4, iss. 16-21, pp. SS16-SS21. ISSN 1471-0072. DOI: 10.1038/nrm1196.

[3] De LANGE, F., A., CAMBI, R. HUIJBENS, B. De BAKKER, W. RENSEN, M. GARCIA-PARAJO, N. van HULST and C. G. FIGDOR. Cell biology beyond the diffraction limit: near-field scanning optical microscopy. *Journal of Cell Science*. 2001, vol. 114, no. 23, pp. 4153-4160. ISSN 1477-9137.

[4] POHL, D. W., W. DENK and M. LANZ. Optical stethoscopy: image recording with a resolution of λ/20. *Applied Physics Letters*. 1984, vol. 44, iss. 7, pp. 651-653. ISSN 0003-6951. DOI: 10.1063/1.94865.

[5] LEWIS, A., M. ISAACSON and A. LEWIS. Development of a 500 angstrom spatial resolution light microscope. *Ultramicroscopy*. 1984, vol. 13, iss. 3, pp. 227-232. ISSN 0304-3991.

[6] KELLER, T. H., T. RAYMENT, D. KLENERMAN and R. J. STEPHENSON. Scanning near-field optical microscopy in reflection mode in liquid. *Review of Scientific Instruments*. 1997, vol. 68, iss. 3, pp. 1448–1455. ISSN 0034-6748. DOI: 10.1063/1.1147937.

[7] SUBRAMANIAM, S., A. K. KIRSCH and T. M. JOVIN. Cell biological applications of scanning near-field optical microscopy (SNOM). *Cellular and Molecular Biology*. 1998, vol. 44, iss. 5, pp. 689-700. ISSN 1165-158X. DOI: 10.1007/978-1-59259-993-6_14.

[8] ZWEYER, M., B., B. TROIAN, V. SPREAFICO and S. PRATO. SNOM on cell thin sections: Observation of Jurkat and MDAMB453 cells. *Journal of Microscopy*. 2008, vol. 229, iss. 3, pp. 440-446. ISSN 1365-2818. DOI: 10.1111/j.1365-2818.2008.01925.x.

[9] KAUPP, G. Scanning near-field optical microscopy on rough surfaces: Applications in chemistry, biology, and medicine. *International Journal of Photoenergy*. 2006, vol. 2006, paper ID 69878-22, pp. 1-22. ISSN 1110-662X. DOI: 10.1155/IJP/2006/69878.

[10] NEDELA, V., P. JANSKY, B. LENCOVA and J. ZLAMAL. Experimental and simulated signal amplification in variable pressure SEM. In: *EMC 2008 14th EUROPEAN MICROSCOPY CONGRESS*. Berlin: Springer-Verlag, 2008, pp. 587-588. ISBN 978-3-540-85156-1. DOI: 10.1007/978-3-540-85156-1_294.

[11] ECHLIN, Patrick. *Handbook of sample preparation for scanning electron microscopy and X-ray microanalysis*. 2nd. New York: Springer Science+Business Media, 2009. ISBN 978-0387857305.

[12] JIRAK, J., V. NEDELA, P. CERNOCH, P. CUDEK and J. RUNSTUK. Scintillation SE detector for variable pressure scanning electron microscopes. *Journal of Microscopy*. 2010, vol. 3, iss. 239, pp. 233-238. ISSN 0022-2720. DOI: 10.1111/j.1365-2818.2010.03377.x.

[13] SKARVADA, P., P. TOMANEK, L. GRMELA and S. J. SMITH. Microscale localization of low light emitting spots in reverse-biesed silicon solar cells. *Solar Energy Materials and Solar Cells*. 2010, vol. 94, iss.12, pp. 2358-2361. ISSN 0927-0248. DOI: 10.1016/j.solmat.2010.08.014.

[14] TOMANEK, P. Fiber tips for reflection scanning near-field optical microscopy. In: *Near Field Optics NATO ASI series*. Dordrecht: Kluwer Academic Publishers, 1993, pp. 295-302. ISBN 0-7923-2394-7.

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