

Conference Paper

Correlative Microscopy: A Potent Tool for Biomedicine

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Abstract

The correlative microscopy method based on a combination of optical and electronic techniques that is increasingly widely used now, has a number of limitations. Here, an alternative approach is considered that uses scanning probe microscopy (SPM) technique to get high-resolution and ultra-high-resolution data. SPM greatly increases the possibilities of collecting new information (on topological, morphological, electrical, magnetic etc. properties). To obtain three-dimensional distributions of different parameters of the sample, ultramicrotomography is used, which allows to scan the sample in steps of up to 20 nm. The principal advantage of the approach is that spectral data are used which due to the combination in near field microscopy can be gained with high and ultrahigh resolution. All above mentioned features are implemented in a single instrument, which allows to have 3-D data and their distributions at the same instrumental platform. A special feature of the approach is the possibility to use all the power of micro(nano)spectral methods. Therefore, it would be more correct to name the proposed approach "Correlative microspectroscopy".

Keywords: correlative microscopy, correlative microspectroscopy, scanning near-field optical microscopy, ultramicrotomography, Raman, TERS

1. Introduction

The term "Correlative Microscopy" arose in the middle of the second half of the last century. In 1987, the book "Correlative Microscopy in Biology. Instrumentation and Methods" [1] was published. At that time, under the term "Correlative microscopy" we understood a combination of two methods: optical microscopy (OM) and electron microscopy (EM).

What is important in combining these two methods, is that their joint use allows to solve two problems: (1) to obtain an image of an object with a high and ultra-high resolution (units of nanometers), and, (2) to identify objects in the resulting image,

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including information about their composition. In some cases, optical spectroscopy yields information on structure–functional characteristics of a specific local area of the sample. Certain modifications make it possible to solve the third task: to reconstruct the 3-dimensional structure of the object.

Correlative microscopy declares the solving of three tasks: (1) to obtain an image with a high and ultra-high resolution (nanometers scale); (2) to identify objects presented in the image, and (3) to reconstruct a 3-D picture and to record 3-D maps of some parameters distribution.

The classical electron microscopy method to get images with a high and ultra-high resolution has some disadvantages. It becomes evident especially when studying biological objects. These are: the need for vacuum, the requirement of the sample surface conductivity, and, in most cases, the need for biological samples contrasting. For this, metal conductive layers are deposited on biological samples surfaces, which can distort the surface structure and hamper the subsequent steps of biological objects identification and obtaining physical and chemical data about the biological sample under study using optical microscopy.

An alternative method, which allows avoiding such drawbacks, is the technique of scanning probe microscopy (SPM), Fig.1. This method was developed much later than electron microscopy and, by now, it is just beginning to gain a decent position in medical diagnostic applications. The potential of the method is huge. Using cantilevers of different types, one can gain information not only about the topography of the surface, but also about its morphology, about distribution of electrical and magnetic parameters, and distribution of electronic and ionic conductivity. By the localization of the recognition molecule on the tip of cantilever with high affinity to the specific molecular structures of the sample, it is possible to obtain maps of the distribution of such targets on the sample surface. In addition, the SPM technique allows dealing with non-conductive samples. The method does not require vacuum. Now, microscopes are available which work not only in the air, but also in liquids including biological ones.

2. High-resolution data collection

A wide range of SPM capabilities became the main reason for our choice of this method to gain high-resolution data when creating our correlation tool.

The principal feature of SPM is that the method works with a very thin surface layer and cannot be used in transparency regimes. On the one hand, it permits to avoid the problem of overlapping of non-transparent elements, for example, in the case

of SEM studies of slides (Fig. 1A, B). On the other hand, it does not allow using 3-D reconstruction techniques, based on the analysis of sample projections recorded at different angles of irradiation.

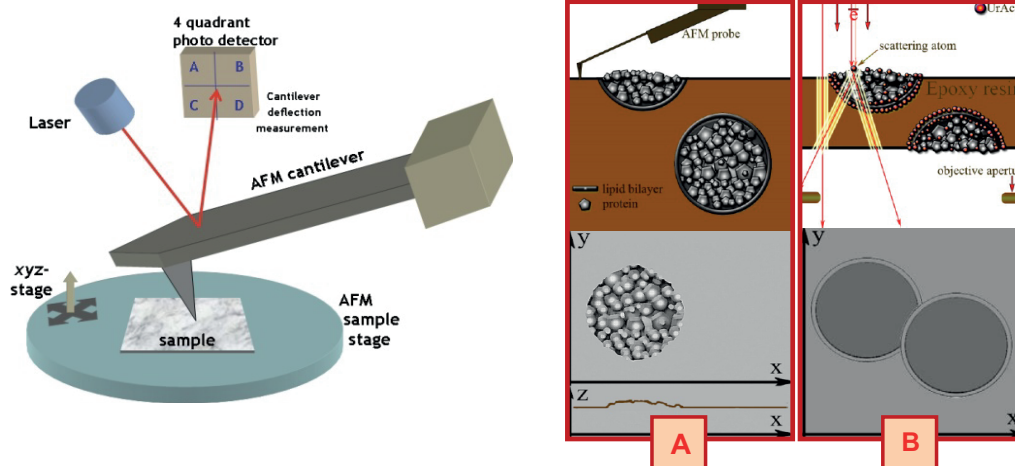


Figure 1: On the left – scheme of Scanning Probe Microscope (SPM); on the right (A) - subject to SPM study is the thin surface layer. Therefore the problems of 3D objects configurations reconstruction from its single 2D projections taken by TEM (B).

3. Three-dimensional data reconstruction

A specific feature of the SPM technique is that this method deals with a thin surfaces layer of samples. It defines that the most suitable method for taking 3-D information is the layer-by-layer removal of the sample material to open the next layer for scanning. Each 2-D scan is accumulated to reconstruct 3-D data. In our approach, the ultramicrotomography method was chosen, in which the removal of the layer is carried out with a diamond knife. The advantage of this method is that the thickness of the cut-off layer is always the same and does not depend on physical-chemical properties of the sample, which are different in different parts of the surface. The difference leads to a different rate of material removal during surface treatment. For example, in a discharge plasma or an ion beam the rate of etching and the thick of removal layer differs from point to point depending on the materials in each area. This leads to inhomogeneity of the material removing and is able to significantly distort the reconstructed 3D data. It can be noted that the approach does not need any alignment due to fixed sample position relative to orientation and direction of scanning cantilever moving.

The developed process to get 3-D information is presented in the Figure 2. After each overcut by diamond knife, the distribution maps of the characteristics of the sample are recorded by the SPM method, using broad capabilities of the selected probe depending

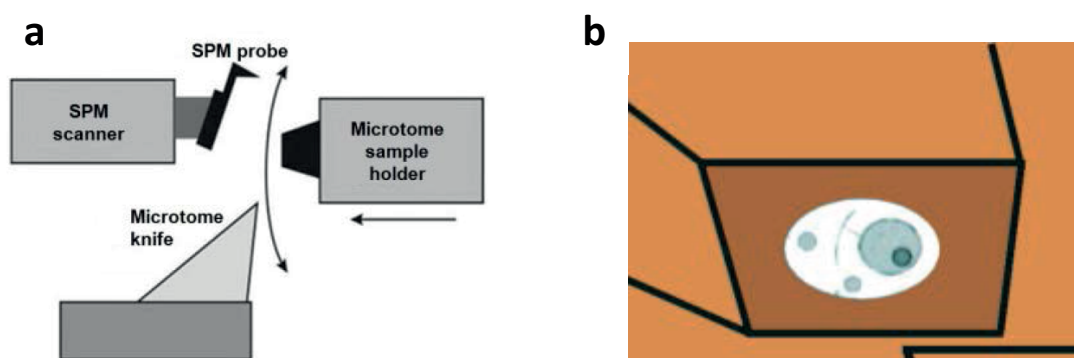


Figure 2: (a) - scheme of a step-by-step sample overcut followed by SPM picture recording at each step; (b) - flash-cut surface of the sample.

of its characteristic data (morphological parameters of the surface, electrical, magnetic, etc.). It should be emphasized that from cut to cut, the sample does not change its position and orientation. The recording of 2-D images is performed under identical conditions. Therefore, there is no need for complex procedures to combine individual 2-D images, as it is the case with EM. Examples of the reconstruction of the 3-D structure are given in [3, 4].

It should be noted that the method of mechanical removal of thin layers of a sample using microtomography is also used in EM. In particular, the equipment which can be embedded in the scanning electron microscope is offered by [5], where the strategy of recording 2-D information from the surface after the overcut is also used, instead of the slide resulted after cutting.

4. Optical microscopy/microspectroscopy data

To get information on the distribution of physical-chemical parameters and for small objects identification, methods of optical microscopy and microspectroscopy are usually used. Examples of the implementation of such a system are presented in [6-8]. In particular, a correlation was established in [7] between the 3-D distribution of nano-sized fluorescent nanocrystals (quantum dots) in the liquid crystal matrix and the local degree of fluorescence depolarization in the local nanoscale regions of the nanomaterial LC matrix. In [8], the "anatomy" of micro-dimensional microspheres possessing both fluorescent and magnetic properties, intended for medical diagnostic applications, has been determined. However, these tasks did not require a high resolution of the optical microscope, so the use of confocal scanning microscopy was sufficient.

However, to solve problems related to the study of the fine structure of biological objects (cells, tissues), resolution of conventional optical microscopes is not sufficient. The SPM technique allows getting information at the level of units and tens of nanometers. Unfortunately, for optics there is diffraction limit, in the optical range resolving power is not better than 200 nm. This applies not only to the lateral, but also to the axial resolution. In other words, the scanning of the sample by the optical method occurs up to depth of about 200 nm. Since the minimum thickness of the overcut is about 20 nm, the optical method becomes a limiting factor in the possibilities of such a correlation approach as a whole. Recent methods of ultra-high resolution optical microscopy do not solve the problem. They are all based on the use of fluorescence, and mainly require fluorophores with special properties. That is, not the object itself is registered, but the label associated with this object. This closes the possibility of direct application of spectral methods to gain information about the object.

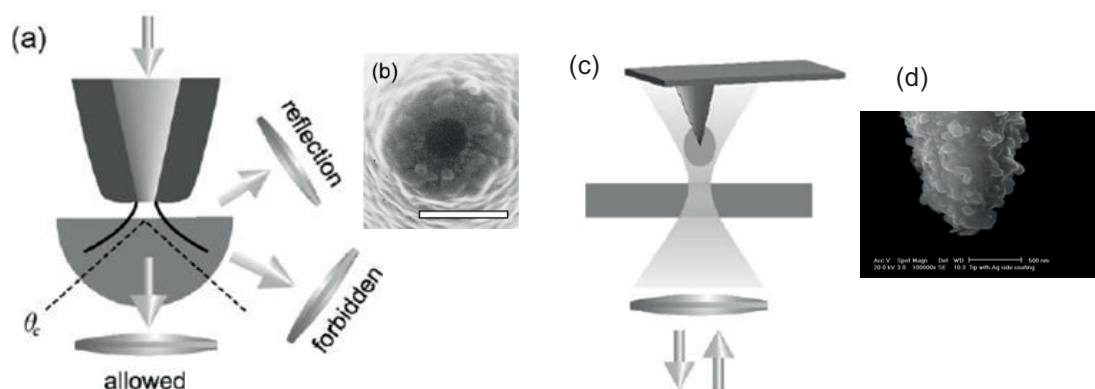


Figure 3: (a) - Scheme aperture SNOM; (b) - typical SNOM-probe, scale bar – 300 nm; (c) - scheme of apertureless SNOM; (d) - tip of TERS-active probe with adsorbed Ag nanoparticles (diameter near 20 nm). In this design, there are conditions for the narrow gaps (hot spot) formations.

Near-field microscopy principle was proposed in 1927 [9]. Since there were no technical means for its experimental implementation, the method was forgotten. It was re-invented in the 1980s and is practically implemented in scanning probe microscopes. As applicable to correlation microscopy, two ways are promising: illumination of the object through a small hole, and the use of the probe tip as an optical effect enhancer, which makes it possible to get information from a small local area near the probe. Both of them allow implementing a high-resolution system (Fig. 3). The resolution of such a system is determined by the size of the hole or the size of the tip (particles on the tip) of the probe. In the first case, this size is less 100 nm, in the second case, it can be about 10 nm. These values define both lateral and axial resolution. That is, the method makes it possible to get sufficiently close resolution values for SPM, UMT, and OM.

The approach presented makes it possible to overcome the limitations of ultra-high-resolution fluorescence-based methods. Since optical information is gained directly from the object, it allows us to use spectral methods to have information about the object. It is known that vibrational spectroscopy is most informative and Raman spectroscopy in principle would be most comparable with scanning near-field optical microscopy. The main problem is the huge loss of light energy on their way through the thin optical canal. When light passes through a small aperture, only a small fraction of the radiation reaches the sample. And since the non-resonance Raman scattering cross section is also extremely small, it is practically impossible to record the Raman signal from the local region of the biological sample in the SNOM mode.

This problem can be solved by amplifying the Raman signal by a specially prepared surface, effect of Surface Enhanced Raman Scattering (SERS) spectroscopy. The method was opened in the 1980's, demonstrated ultrahigh sensitivity (up to a single molecule detection), and successfully used to solve specific tasks related to distant dependents data or requiring high sensitivity. Their applications were limited due to poor reproducibility. However, in the last decade, mainly due to advances in the technique of preparing substrates for SERS, the interest in the applications of this method has increased dramatically. To date, different companies produce SERS-active substrates that allow getting not maximally enhanced, but reproducible results. Availability of reproducible SERS-active substrates opens the way to combine SERS with SNOM. This combination makes it possible to get spectral vibrational information from local sample volumes with a sufficiently high resolution.

Plasmonic effects that occur on the sharp tip of the SPM cantilever or near metal nanoparticles localized on the tip are able to enhanced electromagnetic field in local volume near their surface and enhance the Raman signal of the molecules localized in this area. Now, this so-called Tip Enhanced Raman Scattering (TERS) effect is used widely [10]. Commercial TERS-probes are available that enhance Raman scattering by several orders of magnitude. For this purposes, we developed special technique for preparation of stable TERS-probes by deposition of nanoparticles on the cantilever tip (Fig. 3d) [10].

Based on the described strategy of using methods combining the acquisition of structural information and data on the physical-chemical properties of local nanoscale sample regions with the possibility of reconstructing the three-dimensional distribution of these parameters by sample volume, we developed and manufactured an experimental tool (Fig. 4). This is the unique scientific setup "System for probe-optical 3D correlative microscopy" of the IBCh RAS (<http://ckp-rf.ru/usu/486825/>). Equipment

has mounted in the IBChH core facility (CKP IBCH, supported by Russian Ministry of Education and Science, grant RFMEFI62117X0018).

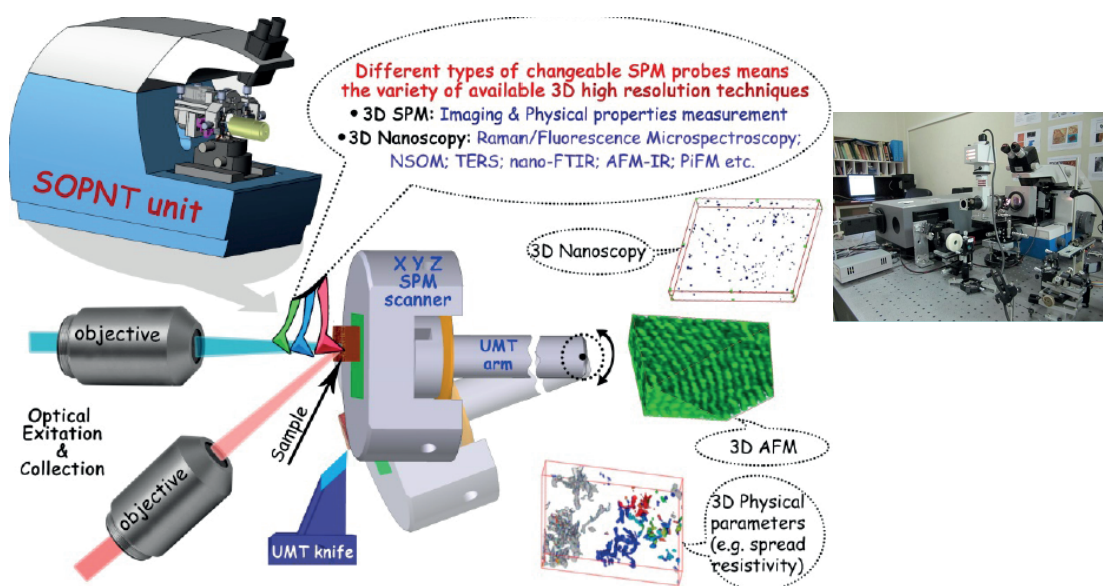


Figure 4: Scheme and photo of the tool combining scanning probe microscopy, confocal or/and scanning near-field optical microscopy and 3-D ultramicrotomography.

5. Summary

The approach developed and implemented as an experimental tool, is based on the use of the entire potential of scanning probe microscopy and microscopy/near-field microspectroscopy. In contrast to Correlative microscopy that uses as high resolution electron microscopy technique, the approach presented uses other principles. We propose another term that more accurately reflects the complex of basic methods and experimental possibilities, namely: "Correlative Microspectroscopy."

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