Correlative AFM and TEM of Soft Material
About the Synergetic Use of Two Analytical Techniques

We describe a comprehensive characterization of biological and polymer samples, when a particular organelle or macromolecule cluster is cut into two parts: One part is used for AFM and the other for TEM. Information about the microstructure of the sample (from TEM) in combination with the data on distribution, morphology and mechanical properties of macromolecular/chain content (from AFM) reveals new structural aspects going beyond the possibilities offered by AFM or TEM alone.

What is more valid: information obtained by eyes or by hand?

The answer to this question is quite obvious: In order to obtain the comprehensive impression about surrounding objects, one needs both of these sensation systems. Importantly, the information obtained visually and tactiley is not the same since different detection mechanisms are applied. This example provides a perception of diversity and similarity between AFM and TEM data obtained from the same object due to the existing analogy between TEM and AFM detection mechanisms and human visual and tactile sensation systems. Figure 2 shows the basic principles of AFM (fig. 2(A, C)) and TEM (fig. 2(B, D)) image contrast formation. In TEM, the image contrast is formed by sufficiently scattering structures that are present in the 20 to 90 nm thick volume of the section. Most of biomacromolecules (proteins, polysaccharides, nucleic acids) as well as polymer/copolymer chains mainly consist of light elements (C, H, O, N), which scatter the incident electrons rather weakly and consequently can be detected on TEM micrographs only as a grayish background. Staining of the sample can considerably improve the image contrast, but the heavy metal salts, which are used for such purpose, have a certain size and can penetrate deep into sample only when macromolecule/chain matrix is relatively disengaged. Therefore, in TEM image even after staining one recognizes not the whole ultrastructure, but only those cellular/polymer components which can be reached by the staining agents and which can react with the latter [1].

On the block face [2] surface investigated by AFM, macromolecules/polymer chains create a pronounced topographical contrast due to the relaxation of the tension
inside the polymer block during or immediately after the sectioning process.

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Such tension most probably results from an electrostatic repulsion between side groups of (bio-)polymer chains, which are not cross-linked during polymerization. AFM also detects phase shifts, which in case of biological samples can be attributed to the different density of the pure embedding resin and the copolymerized cell components, surrounded by the resin [3]. For artificially synthesized polymers, phase contrast usually shows chain and crystalline order, structure and distribution of the highly oriented molecules in fibers and films etc. [4]. Therefore, the usage of AFM as a complementary microscopic technique to TEM allows the determination of the macromolecule content of biosamples embedded in epoxy resin as well as the morphology, distribution and the mechanical properties of polymer chains.

Only the investigation of exactly the same place of the specimen provides the most adequate correlative AFM/TEM analysis.

Technically such data can be obtained when the block-face of the embedded sample is used for AFM imaging, while the last ultrathin section is collected, heavy metal stained (if necessary) and then used for TEM. Here, the important point is that a particular organelle or macromolecule cluster is cut into two parts: one part for AFM and the other for TEM. Only such complementary pairs of images are indispensable for a serious interpretation of AFM and TEM data as well as for the discovery of new ultrastructural aspects of macromolecular/polymer chain morphology and their mechanical properties. Figure 3 clearly shows that the interpretation of the macromolecular content of the inner membrane space of mitochondrial cristae cannot be performed, when AFM and TEM images were collected from different places of the same sample (fig. 3(A, B)). From the AFM image, it remains uncertain, whether the tightly packed lines inside the mitochondria correspond to the structure of cristae or they belong to the matrix of
the organelle. Only (!) from the complementary pair of images (fig. 3(C, D)) it can be seen that the most tightly packed lines in mitochondria (AFM image) correspond to the cristae structure (TEM micrographs).

**The complementary pair of AFM and TEM images can be used for the investigation of dynamical processes within the cell or during polymer formation/modification.**

At present, the description of biological ultrastructure more closely related to the living state is important as a complementary study of dynamic events in living cells by fluorescent light microscopy. In polymer science as well, many important questions concerning the structural changes accompanying melting, crystallization or glass transition are very relevant [5]. In some cases, TEM of ultrathin sections can solve the problem, although the low electron microscopy contrast of biological/polymer samples, the necessity to use a two-dimensional projection of the sample volume and the issue of beam damage of sample strictly limit the number of topics which could be assigned to this method. The nondestructive character of AFM and the possibility to obtain information about location, architecture and mechanical properties of macromolecules/polymer chains in a nanometer range make this technique extraordinary useful for the investigation of local changes within the sample that take place during dynamic processes. However, the identification of the area where local changes occur still requires the complementary TEM image obtained from the same area of the specimen. Figure 4 shows a cross-section of high pressure frozen (HPF) and then epoxy freeze substituted (FS) C.elegans. Cryo-immobilization preserves biological structures in a state close to native, because of its microsecond time resolution for dynamic physiological processes in contrast to conventional chemical fixation that takes seconds to hours [6]. In the TEM image (fig. 4(B)), some areas of the nuclear membrane look slightly dissolved (see arrows). It cannot be clearly distinguished between three possibilities:

- Such areas correspond to nuclear pores involved in the transport of macromolecule across the nuclear envelope [7];

- The low contrast of these areas is a staining artifact;

- The membrane orientation was not parallel to the electron beam.

The AFM image (fig. 4(A)) shows that these areas correspond to almost solid bridges between cytoplasm and nucleoplasm, of most probably macromolecular nature [8]. It should be noted that the membrane transport is a highly dynamic
process [9] and its proper investigation requires additional biophysical methods, as well as an accumulation of a big statistical database. Hereby the direct imaging opens up new horizons for the investigation of dynamic membrane processes at the level of individual macromolecular components.

For polymer systems with a limited number of phases the correlative AFM/TEM analysis can be strengthened by the TEM analytical techniques.

Figure 5 represents a complementary pair of AFM/TEM images of the cross-section of a Teonex foil with deposited ZrO$_2$ clusters. The surface of the foil has been substantially modified during the deposition process (most probably due to local heating). In this particular case, the usage of each of the applied methods (AFM, TEM, elemental mapping by energy filtering TEM (EFTEM)) was necessary in order to give a comprehensive description of the sample. TEM provided information about the general organization of defects at the sample surface, ZrO$_2$ clusters have been localized by EFTEM elemental mapping [10]. The thickness and the morphology of the damaged layer near the surface as well as the size and the distribution of ZrO$_2$ clusters were characterized by AFM. In summary, the full potential of each of high resolution microscopic technique can be expressed only through the combination with other techniques, since very often the main limitation is not the technique, but the proper interpretation of the obtained results.

Acknowledgments
We are very grateful to Anna Rozhkova, Anja Haase, Harald Plank, Alexander Bluemel and especially Martin Mueller for the support and fruitful discussions. Also we would like to thank Helmut Gnaegi and Diatome Co of the generous supply with an AFM microtome knife. Financial support by ETH and the "Austrian NANO Initiative" is kindly acknowledged (project DevAna 0706 within the „Isotec" research cluster).

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