Applications of Electron Microscopy in Medicine

The State of the Art

Applications of electron microscopy, stand-alone and in combination with other microscopy methods, in diverse fields of biological and medical research as well as in medical diagnosis will be one of the main topics at the Microscopy Conference (MC 2007), to be held from 2 to 7 September 2007 at the Saarland University in Saarbrücken (Germany). This is the 33rd Conference of the Deutsche Gesellschaft für Elektronenmikroskopie e. V. (DGE), which was initially conceived as a national meeting but has achieved in recent years a remarkable international character. Based on the experiences in past conferences, approximately 500 participants and over 20 exhibitors are expected in Saarbrücken. This conference is an important scientific highlight for the Saarland University, coinciding with the celebrations of the 60th anniversary of its foundation by the French administration in 1947. Information about the program, commercial exhibition, deadlines, etc. is available on the meeting homepage: http://www.uni-saarland.de/mc2007.

In the 1950s and 1960s transmission electron microscopy (TEM) was applied intensively in medicine and especially in pathology. The acceptance of this new technique was facilitated because light microscopy (LM) and electron microscopy (EM) are in principle closely related, the former using light radiations and the latter electron rays, both resulting in imaging by transmission through the object. The differences in resolution and enlargement between LM and TEM define very well the domains of these techniques. Whereas LM is appropriate for groups of cells and large areas, EM is highly effective in evaluating single cells or more subtle structures, such as organelles, cytoskeleton and other sub-cellular elements. For a long time both techniques were undoubtedly fundamental in histo-pathological diagnosis. However, with the advent of immuno-histochemical techniques the histo-pathological diagnosis was transformed considerably. For instance, in cases of tumors that are difficult to identify, an accurate diagnosis and typification can be achieved using a well-selected set of antibodies. Thus, in the current situation electron microscopy might appear as a redundant or even superfluous technique
Immuno-histochemical and immuno-cytochemical techniques are not the only factors responsible for this development. Other ancillary techniques, such as cytogenetics and molecular biological techniques like in-situ hybridization and gene rearrangement demonstration have also taken a place in the diagnostic protocols of pathologists, resulting in a reduced importance of EM in pathology. This development has been particularly obvious since the 1980s. An additional more logistical factor contributing to these trends is the eminent development of the instruments. The microscopes have become more operator-friendly than in the past, but at the same time they are complex and require competent service for maintenance, repair, etc. Other important factors are increased time requirements for staff training and specimen preparation. For many laboratories an infrastructure with these characteristics is not accessible, as the use of EM as a matter of routine is becoming very expensive in many respects. Under such conditions an expertise in EM diagnosis remains reserved for a few large and well-equipped centers, where diagnosis and also research, especially in the field of methodological development, can take place in addition to medical routine. These issues are on the agenda of the symposium LS 7.

The Value of Electron Microscopy in Diagnosis

Reports evaluating the role of EM in diagnosis are published regularly. These studies provide estimates of the usefulness of EM in certain fields (pathology of heart, kidney, skin, etc.), but are mostly valid for a short time only. Because the methods keep improving, it is difficult to predict the positive or negative consequences for EM. In order to give an idea on the situation, the EM contribution to the diagnosis of kidney biopsy will be briefly considered. The performance of EM is estimated in several hundred cases and the results of the technique divided into
three categories:

- cases in which EM was an essential technique for diagnosis,
- cases in which EM was a great aid for diagnosis,
- cases in which EM had no influence on diagnosis.

The case list consists of kidney biopsies from patients with kidney disease and also from patients with kidney transplants. The results do not differ very much between the different countries. Approximately 20% of the cases were classified as category 1, 50% as category 2 and 25% as category 3 [2]. On the basis of these estimates, many experts recommend the inclusion of EM in kidney biopsy protocols, or at least to take some biopsy tissue in reserve for additional EM studies, should they become necessary. This is surely one if not the standard paradigmatic case in which it can be said that EM is necessary as a routine method in biopsy diagnosis. In some countries, these circumstances alone have moved those responsible for education to include EM in the training for pathologists.

In other organs (heart, skeletal muscle, skin) and systems these estimates can differ considerably and must be studied individually.

**EM Methods Currently Used in Medical Diagnosis**

Generally it can be stated that EM is of high value in the investigation of clinical specimens related to renal diseases (see above), tumor processes (especially for questions concerning the grade of differentiation of tumor cells), storage disorders and the identification of infectious agents (see later). The majority of laboratories use the standard technique, i.e., chemical fixation (buffered aldehydes) followed by dehydration and embedding of objects in resins. With semi-thin and ultra-thin sections the experienced pathologist, following a strategy of correlative microscopy, is able to obtain valuable data for a considerable number of cases.

An example would be a group of patients with a clinical situation characterized by repeated infections of the upper and lower respiratory tracts. These patients moreover have a reduced mucociliary clearance that can be well measured with colored indicators at the nasal fossae. The reason for these deficits is the reduced or altered motility of the kinocilia (for instance, Kartagener syndrome). Both TEM and scanning electron microscopy (SEM) contribute significantly to the clarification of the structure of the kinocilia in biopsies obtained from the wall of the respiratory tract, which are of course indicated in these patients. Depending on the type of disorder, the external shape ("hockey-stick") or the exonemal microtubular apparatus of the kinocilia can be altered. EM is indicated in cases in which suspicion of a primary ciliary diskynesia (PCD) exists [3].
In the case of other diseases it is necessary to identify and locate specific molecules (markers), for which immuno-cytochemistry can be used (in pre- and post-embedding). For this purpose, numerous variations in the chemical composition of fixatives, temperature conditions during preparation and embedding (PLT, freeze-substitution) as well as the most useful type of resin are available. For instance, in studies on sperm fertility the criteria used to evaluate the state of the cells include both structural, immuno-cytochemical and microanalytical aspects. In such investigations cryo-techniques are used increasingly in TEM (cryo-fixation and cryo-ultramicrotomy to observe native vitrified sections).

In pathological processes like storage disorders and those related to occupational medicine, heavy metals and crystals can accumulate in cells and organs. In such cases microanalytical investigations (EDX, EELS) have an important place in the diagnostic strategy. The specimens can be prepared directly from fresh tissue, but material already embedded in paraffin for light microscopy can also be used for correlative LM/TEM studies. In this last case the preservation of ultrastructure is rather poor, which can mean a significant handicap for evaluation. Depending on which elements are to be micro-analyzed, appropriate fixation and embedding protocols are necessary. For easily diffusible elements cryo-preparation and cryosectioning are required (see Jonas et al. in this meeting).

In recent years the application of EM techniques in pathology has been aided by continuous progress in the technical development of specimen preparation. An important disadvantage of standard TEM protocols is the duration of processing, which can be as long as 3 to 5 or more days. Relevant modifications in fixation, dehydration and embedding management allow shortening processing times of small tissue blocs considerably. Today rapid processing for TEM can be performed in 2-3 hours, which is very attractive for diagnosis [4].

**Infectious Disease Emergencies**

Among the objects observed in very early times of EM [5] were the so-called submicroscopic pathogens, i.e., virus particles (tobacco virus) and bacteria. The magnification power of EM and the possibility to identify morphologically virus particles and the virus family was a decisive factor in the introduction of EM in virology.

Today the qualities and properties of EM in this field are still the same. As already mentioned, the preparation technique became very fast and was also improved. These circumstances in addition to a series of instrument developments make EM an essential method in the diagnosis of infectious diseases. Since 1994 a series of international Workshops has been taking place at the Robert Koch Institute in Berlin. The indication, specimen processing and interpretation of results are
regularly discussed topics during these workshops, which at the same time serve as a coordination vehicle for activities with identical quality criteria in a number of countries throughout the world. One of these workshops will be held in Saarbrücken.

**Interactions of Cells and Bacteria with Natural and Artificial Surfaces**

The term "bio-adhesion" applies primarily to interactions between proteins and surfaces. However, it also applies to the attachment of cells and bacteria to surfaces. A surface in this context is a natural surface, for example the tooth surface, as opposed to artificial surfaces such as cover slips, implants, stents or silicon-chips, on which cells are growing and which will be used for microphysiometry studies.

The anchorage of protein to a surface implies changes in the surface properties. This is known as "conditioning", a process that influences the cell behavior. In dentistry this is a very important field, because on the one side bioadhesion is involved in biofilm formation and on the other side it plays a role in bacterial invasion of the mouth. The same or similar mechanisms are involved in processes occurring on the surface of dental implants, in which cells and tissue of the implantation location are involved [6].

The formation of proteinaceous biofilms can be studied with TEM but also with SEM. A close analysis of the proteins forming the biofilm is performed with colloidal-gold immunolabelling.

In relation to the adhesion of bacteria to surfaces, the study of the ultrastructure of flagella is currently a much-discussed topic. The structure of flagella can be clarified down to the macromolecular level using EM methods such as negative staining and electron tomography [7].

The attachment of cells to a surface is a complex phenomenon in which the properties of the surface (topography, roughness) and the cells themselves are very important. During attachment and depending on the affinity to the surface they become flattened, displaying characteristic morphologies. During the cell cycle, the cell shape changes in correlation with variations in the cell adhesion. The density and distribution patterns of surface profiles (microvilli, kinocilia, small blebs) in adherent cells depend on the grade of attachment to the substrate. With SEM and also with ESEM (wet mode as well as low pressure and large field detector, LFD)
the changes of the cell surface associated with these processes can easily be investigated. But with this approach only the upper side of cells is accessible to observation.

One way to obtain information about the attachment side or bottom side of the cells is using an FIB SEM. The cell can be observed in the scanning electron microscope and a very precise selection of the point or area that should be cut is possible. For example in the case of neurons growing in vitro, with a cell body of 20-30µm diameter and a long thin axon it is possible to find relevant areas like growth cones, the axon itself or dendrites, etc. On the section surface both the cell and the adhesion points or areas of the membrane in contact with the substrate can be seen. It is important to compare the quality of the surface section produced by the FIB with the quality well known from conventional ultra-thin sections obtained from similar objects. In this context not only the structure but also the subcellular location of organelles and cytoskeleton elements is very important. FIB sections in different directions of the same object could give good results about the bottom side of cells attached to the substrate [8] (see also Bittermann et al. in this meeting). However, a view like that obtained with SEM of the upper side cannot be obtained in this way.

Meanwhile a technique to expose the bottom side of the cells, i.e., the side of attachment to the substrate, has been developed and promises to solve the problem of visualization of the membrane at this part of the surface. This method is based on the use of nitro-lacquer that is applied in a fluid state to cells. After the layer of lacquer has hardened, it can be stripped out together with the cells. The cells growing e.g. on the silicon-chip will be fixed briefly in a buffered aldehyde mixture, postfixed with osmium, bloc-stained with uranyl acetate and dehydrated in a series of solutions with increasing ethanol concentration. The last step is performed with 100% acetone and then the lacquer is poured. The specimen with the cells at the upper side is examined under a light microscope for areas of interest and then mounted on a stub and finally shadowed with carbon. For examination both the BSE and the LFD detector modes are used. Because the cells are thinly covered with lacquer on the bottom side, the very fine details of the cell surface might be unclear or imprecise in SE imaging. To overcome this problem some ultra-thin sections can be made, considerably improving the image quality in both SE and BSE detection. (Mestres et al., in preparation and in this meeting.)

In medical research the situation is completely different from the one in clinical laboratory medicine, and the newest EM methods can be applied, the only limitation being the financial support. The topic of neurosciences is a good example, represented at the MC 2007 with a plenary lecture and a workshop dedicated to the ultrastructure of the synapse and discussing the contribution of EM methods such as tomography to understand the crucial structure of the nervous tissue.
In conclusion, the right attitude at the moment seems to be to consider the efficiency of EM as a fundamental and very versatile technique and especially its correlation and integration in multi-modal microscopies in order to solve problems mostly in research but also in specific fields of medical diagnosis.

References:

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