FIB/SEM/EDS complementary analysis for proper forensic interpretation

M. Milani¹, R. Gottardi², C. Savoia³ and C. Cattaneo⁴

¹ Dipartimento di Scienza dei Materiali - Università degli Studi di Milano-Bicocca, Via Cozzi 53, 20125 Milano, Italy
² Dipartimento di Biotecnologie e Bioscienze, Corso di laurea triennale in Scienze Biologiche - Università degli Studi di Milano-Bicocca, Piazza della Scienza 2, 20125 Milano, Italy
³ST Microelectronics, Via C. Olivetti 2, Agrate Brianza, MB 20864, Italy
⁴ Dipartimento di Medicina Legale – Università degli Studi di Milano, Via Mangiagalli 37, 20133 Milano. Italy

Scanning Electron Microscopy and Microanalysis have an established role in forensic biology. Up to now SEM has contributed mainly to areas such as ballistics, fibre analysis and a few others. However unexplored yet crucial areas of possible application are forensic pathology, taphonomy and anthropology, i.e. the realm of decomposed material contaminated by the environment, more difficult to read and interpret from a morphological point of view; hence the need to study electron microscopy images and signals of non prepared biological samples such as blood or other tissue cells, mixed with environmental contaminants, for example entomological and botanical residues. Such issues will be approached thoroughly and examples will be provided of how a FIB/SEM complementary analysis can be crucial for proper forensic interpretation of human remains by surface and subsurface imaging with sections created in situ during sample observation by ion milling in selected places and along selected and changeable lines. Furthermore attention will also be devoted to low-voltage electron microscopy and to sample preparation compliant with forensic evidence.

Keywords forensic pathology; taphonomy; erythrocytes, Scanning Electron Microscopy (SEM); Focused Ion Beam (FIB)

1. Introduction

Scanning Electron Microscope, Focused Ion Beam and Forensic

Scanning Electron Microscopy (SEM) and Microanalysis have an established role in forensic medicine; they contribute to the chain of evidence due to SEM ability to examine details on a wide range of materials in an easily interpreted manner, from high to low magnification and with an exceptional depth of focus. Anyway SEM is limited to few areas such as balistics, merceology, examination of paint and fibres, handwriting and print examination/forgery.

Forensic science involves analyzing and comparing known and unknown materials. There are also some issues that could affect the credibility of the results: it is imperative to preserve the integrity and evidentiary value of the sample and that the results stem from equipment that is in good working condition and properly calibrated.

Some modern SEMs offer additional advantages for forensic purposes because of new methods of biological sample analysis that do not corrupt the specimen, a major drawback with conventional SEMs. In conventional electron microscopy, biological samples have to be dehydrated and then coated with a material that conducts electricity, such as a thin layer of gold or carbon. Modern SEMs allow the adjustment of the internal pressure in the chamber to dissipate the electric charge that would otherwise charge the sample, so that coating and dehydration are no more necessary. Examples of non-conductive materials that require special preparation in conventional SEMs are paper, paint, textiles, bone, hair, and glass.

Moreover forensic evidence has to be presented in a simplified language so jurors can understand what scholars are talking about. This simplification often results in distorting its meaning; as a result, the defense may be able to appeal a valid conviction based on the improper presentation of scientific evidence.

Recently forensic pathology and anthropology interest for human rests moved to taphonomy and in general towards decomposition processes. This new perspective anyway unveil the difficulties of read out and interpretation of samples of human body that are ill preserved or heavily contaminated by environment.

The major point is to evaluate the morphology of structures that are relevant to the forensic medicine diagnostics (e.g. erythrocytes, spermatozoa, etc.) both in well preserved samples (i.e. that can be prepared according to standard protocols for electron microscopy) as well in the decomposed samples: so a fundamental topics is the interpretation of electron microscopy images (and not only images but the whole set of signals) of non prepared biological samples that are with an unknown history.

Examples will be provided of how a FIB/SEM complementary analysis can decide uncertain sample evaluations by surface and subsurface imaging. FIB could further create sections in situ during sample observation by ion milling in selected places and along selected and changeable lines.

Attention will also be devoted to low-voltage electron microscopy and to sample preparation compliant with forensic evidence.
Instrumentation in micron/submicron microscopy

The focused ion beam (FIB)/scanning electron microscope (SEM) is a scanning microscope with an electron column and an ion column embedded in the same specimen chamber. Both beams are aiming the same point on the specimen surface. The FIB, generated by a Ga Liquid Metal Ion Source (LMIS), impacts the sample normal to the surface and can be focused to a spot as small as about twenty nanometres. The FIB can be rastered in a user defined pattern over larger areas of the sample to mill acting as a „nano-microtome“.

Up to now, the FIB/SEM was applied on a variety of biological samples [1-5], however there are still many questions left opened which have to be answered before FIB/SEM is widely applied in life sciences.

The standard preparation of biological specimens for SEM / TEM (Transmission Electron Microscopy) is complex and time consuming while the use of the FIB/SEM can reduce the weight of sample preparation.

The application of FIB/SEM on yeast cells and an epithelial tissue is presented as a basis for the investigations on samples of forensic interest and the advantages of FIB/SEM over conventional SEM or TEM are discussed.

2. Sample preparation- Results in non forensic investigations

2a -The FIB/SEM application on unprepared cells [6].

The investigation on yeast cells is presented to demonstrate the potentiality of the FIB/SEM technique. Saccharomyces cerevisiae cells were fast hydrated in deionized water and glucose at room temperature.

Fig.1 Unprepared sample (air dried): electronic image after a series of operations at FIB/SEM (FEI Strata 235)

They were deposited in the form of a drop onto a silicon slice, dried at room temperature, inserted into the working chamber and brought to high vacuum. Yeast cells sustain very high vacuum without visible damage, [ see also ref. 7 ] they sustain ion beam milling and imaging and moreover ion milling revealed many internal features already of cells (Fig.1). This is a proof that the FIB/SEM operating in high vacuum environment can be applied also on air dried biological material, that is not prepared following standard procedure.

2b- Sample navigation : linking gross morphology to cell ultrastructure

It can be shown that a FIB/SEM system can be applied for simultaneous research of the digestive gland epithelium gross morphology and the cell ultrastructure [8,9]. Digestive gland tubes of Porcellio scaber were isolated and „standard“ fixed and prepared. They were mounted into the specimen chamber for FIB / SEM operation (FEI Strata DB 235 M). It is observed a connection between digestive gland gross morphological characteristics (extruding and non-extruding phase of a cell) and cell ultrastructure. A link can be established between larger scale tissue morphology and cellular and subcellular structures. The ability to reveal specific intracellular structural details and to link them to the gross morphology of the tissue or organ is at the moment among most promising and beneficial applications of FIB/SEM in life sciences. This is of particular interest when cells or cellular inclusions have a dynamic nature due to normal, stress or pathologic conditions.
2c – FIB/SEM versus TEM analysis [10]

Samples can be manipulated by FIB/SEM and results compared with TEM observation. Moreover resulting sections can be EDX analyzed. We have examined the potential of the focused ion beam/scanning electron microscope system for the investigation of biological tissues of the model organism Porcellio scaber (Crustacea: Isopoda). Tissue from digestive glands was prepared as for conventional SEM or for transmission electron microscopy (TEM). The samples were transferred into FIB/SEM for FIB milling and an imaging operation. FIB-milled regions were secondary electron imaged, back-scattered electron imaged, or energy dispersive X-ray (EDX) analyzed. Results demonstrated that FIB/SEM enables simultaneous investigation of sample gross morphology, cell surface characteristics, and subsurface structures. The same FIB-exposed regions were analyzed by EDX to provide basic compositional data. When samples were prepared as for TEM, the information obtained with FIB/SEM is comparable, though at limited magnification, to that obtained from TEM. A combination of imaging, micromanipulation, and compositional analysis appears of particular interest in the investigation of epithelial tissues, which are subjected to various endogenous and exogenous conditions affecting their structure and function. The FIB/SEM is a promising tool for an overall examination of epithelial tissue under normal, stressed, or pathological conditions.

2d – Cell morphology and individual characteristics[11]

The structure of the digestive gland epithelium of a terrestrial isopod Porcellio scaber has been investigated by conventional scanning electron microscopy (SEM), focused ion beam–scanning electron microscopy (FIB/SEM), and light microscopy in order to provide evidence on morphology of the gland epithelial surface in animals from a stock culture. Object of the analysis were the shape of cells, extrusion of lipid droplets, shape and distribution of microvilli, and the presence of bacteria on the cell surface in a total of 22 animals. Variability was documented in the appearance of the gland epithelial surface. Seventeen animals had dome-shaped digestive gland “normal” epithelial cells, which were densely and homogeneously covered by microvilli and varying proportions of which extruded lipid droplets. On the surface of microvilli we routinely observed sparsely distributed bacteria of different shapes. Five of the 22 animals had “abnormal” epithelial cells with a significantly altered shape. In three of these animals, the cells were much smaller, partly or completely flat or sometimes pyramid-like. A thick layer of bacteria was detected on the microvillous border, and in places, the shape and size of microvilli were altered. In two animals, hypertrophic cells containing large vacuoles were observed indicating a characteristic intracellular infection.

2e- Extracting information from the sample: biological sample preparation for backscattered (BSE) imaging [12]

Secondary electron (SE) imaging is the main method to understand structural characteristics of specimens; the backscattered (BSE) image contains more than just morphological information. As the atomic number increases, the yield of both secondary and backscattered electrons increase.

A main hindrance of BSE imaging of biological samples is that the specimen should be flat in order to reduce interfering topographic contrast. Flat surface is not (or very rarely) the case in biological samples. The sample preparation was similar to the standard one.

FIB/SEM can be used to make a flat surface for both SE and BSE imaging is provided. The cells investigated in our study store metals in metal granules, which can easily be identified by BSE. The BSE imaging can be used when investigating those biological samples where not only low atomic numbered elements are present, but also high atomic numbered elements are expected (metal storing tissue, skeleton etc.).

2f – Tests for different preparation procedures [13]

When a new approach in microscopy is introduced, broad interest is attracted only when the sample preparation procedure is elaborated and the results compared with the outcome of the existing methods. In [13] we tested different preparation procedures for focused ion beam (FIB) milling and scanning electron microscopy (SEM) of biological samples. The digestive gland epithelium of a terrestrial crustacean was prepared in a parallel for FIB/SEM and transmission electron microscope (TEM). All samples were aldehyde-fixed but followed by different further preparation steps. The results demonstrate that the FIB/SEM samples prepared for conventional scanning electron microscopy (dried) is suited for characterization of those intracellular morphological features, which have membranous/lamellar appearance and structures with composition of different density as the rest of the cell. The FIB/SEM of dried samples did not allow unambiguous recognition of cellular organelles. However, cellular organelles can be recognized by FIB/SEM when samples are embedded in plastic as for TEM and imaged by backscattered electrons. We provide evidence that FIB/SEM enables both, detailed recognition of cell ultrastructure, when samples are plastic embedded as for TEM or investigation of sample surface morphology and subcellular composition, when samples are dried as for conventional SEM.
3. Results

In the following images from blood drops of different origins are presented that underwent no preparation at all (Figs.2,3,4). A major point to which attention was devoted is the support (materials, shapes, deposition procedures, etc.) on which blood drops are deposited, since in the forensic practice usually analysis must be performed on samples whose constituents and shapes are not the best for electron microscopy investigations.

A second point towards the delineation of a satisfactory protocol is the acceleration of the electron beam in order to best preserve the sample and to highlight the cellular blood components from the messy background; furthermore the more invasive ion beam imaging provides further elements for identification and characterization of erythrocytes (Figs.5).

Finally attention is devoted to peculiar elements that can emerge during imaging processes that provides hints for the samples history that can be traced back to the blood drop deposition onto the support and to the environment in which the whole sample was immersed (Figs.6).

Fig.2a) Unprepared sample: blood drop over standard paper filter. The sample shows a thick layer of lipids, polymers and sugar that cover all the structures shielding any detecting/imaging abilities; drying the layer cracks a lot of sectors that show a wide difference in brightness and contrast due to different charging levels.

b) Unprepared sample: blood drop over standard paper filter. The thick border of the sectors shows embedded particles (erythrocytes?)

In figs. 3 the blood sample is deposited onto a mixed metal plastic surface for analytical purposes. The solid matrix is again present and in cracks erythrocytes are visible and recognizable as well as roleaux from erythrosedimentation. (fig.3a). Blood cells are clearly distinguishable and measurable, so they allow statistical analysis. Fig.3b is a magnification of the previous one.

Fig.3 a, b. The blood sample is deposited onto a mixed metal plastic surface for analytical purposes.
In figs. 4 the sample is deposited onto a conducting metal wire. After drying in air the wire appears covered by a plaque that cracks along lines driven by gravity hydrodynamic forces showing the layered structure of the plaque.

In the plaque structures are embedded and we proceeded to investigate the possible association of these structures with blood constituents by using different acceleration of the electron beam (Figs.5a, 5b), also imaging the samples by primary ion beam and secondary electron collection and investigating the role of tilting the samples with respect to the primary particle beam (Figs.6a, 6b).

The primary ion beam can image the sample surface (ions penetrate less than electrons at the same acceleration thanks to their larger mass).
Fig. 6. Role of tilt angle and of different primary beams: a) primary ion beam and 0° tilt angle; b) primary electron beam and 45° tilt angle

In figs. 7a, b again unprepared sample: blood drop over standard paper filter. Other elements are visible that can be associated to crystals possibly deriving from substances present in blood when the liquid part is absorbed by paper. Such crystals could be important in evidence building for forensic purposes.

Figs. 7a, b Blood drop over standard paper filter

Conclusion and future perspectives of FIB/SEM in forensic

The preliminary results presented about unprepared (air dried) blood samples on heterogeneous supports show that interesting information for the forensic applications can be obtained and pave the way towards a possible protocol of analysis. Of course this is only the beginning and deeper investigations can be planned on other biological samples of forensic interests, where the interplay of the use of electrons and ions as primary beam particles may be of help in resolving controversial object identification. There is a general belief that scanning electron microscopes are not suited for subsurface investigations and researchers should switch from using SEM to TEM. This, however may result in losing important information. Perhaps the upgraded SEM that allows in situ controlled and precise sample manipulation is probably the optimal choice for many imaging needs in forensic.
References