High Resolution Cryo Scanning Electron Microscopy of Macromolecular Complexes

Introduction

The beauty of Scanning Electron Microscopy (SEM) is its power to describe and integrate structural details, mainly surface related details, within the context of a complex system. Its unique ability compared to other Electron Microscopy techniques (STEM & TEM) is that handling and imaging of bulk samples is in principal possible and hence sectioning, thinning or replicating of the specimen is not essential when surfaces structures are to be investigated. With the introduction of Field Emission SEM (FESEM), especially in combination with improvements to the signal detection efficiency ("in-lens" detection and new type of detectors), high resolution SEM (HRSEM) has become a powerful approach to describe structural details even down to macromolecular dimensions (1-2nm) for structural studies in Biology and soft-material science.

Natural surfaces of living systems are usually not directly accessible. As a consequence and due to the fact that HRSEM is only possible under high vacuum conditions, the specimens have to be very carefully processed to obtain close to native surfaces at nanometer level for HRSEM investigations. The information density and the accuracy of structural information are strongly influenced by the preparation steps and imaging conditions.

The preparation workflow for any SEM investigation can be briefly given as follows:
1) Isolation of the specimen; 2) Immobilization; which contains adsorption or embedding as well as chemical or physical fixation; 3) Surface exposure; which contains chemical or physical dehydration and detail of interest exposure (e.g. fracturing) techniques; 4) Signal enhancement; consistent of contrasting techniques and imaging techniques; 5) Transfer to the microscope without structural changes 6) Imaging with minimal structural damage e.g. imaging mode (exposure time), beam current, specimen temperature etc.

In this short overview we try to outline the current knowledge of the different steps for HRSEM of macromolecular structures and describe technical approaches to preserve and enhance structural details at the nanometer level.
The challenge for the future will be to use the acquired knowledge to move to a level of higher structural complexity while preserving the macromolecular resolution achievable by HRSEM nowadays e.g. macromolecular resolution in tissues or soft-matter samples as will be outlined in a follow-up Book edited by Roland Fleck and Bruno Humble on “Biological Field Emission Scanning Electron Microscopy” Wiley.

Prerequisites for High Resolution SEM (HRSEM)

Macromolecular Structure Preservation

The goal of structural studies is to preserve the specimen in its native shape and conformation to provide structural data finally for structure-function correlation. Therefore, any specimen manipulation step has to be carefully investigated as a possible source of introducing structural artifacts to the specimen structure. The inherent problem is that the native state of living systems is a highly dynamic and typically aqueous environment, which has to be transferred to a “solid state”, to withstand the vacuum conditions of the microscope and the bombardment of electrons.

It is therefore essential to meticulously evaluate each preparation step concerning its influence on the structural integrity and to optimize the preparation protocol for every single specimen. For some rigid macromolecular complexes such as F-actin filaments, HPI-layer, T-phages a washing step in water does not change the protein complex arrangement, whereas in most cases distortion or disassembly may take place as shown in figure 1. Essentially for a good preservation of these fragile samples e.g. CCV, is the physical arresting in their natural media by vitrification and the subsequent exposure of their surface by freeze-fracturing and partial freeze-drying (b). The sample needs to be kept under cryogenic conditions until and during imaging since sublimating all water would lead to a total collapse of the structure (fig. 1 b inset).
Controlling the Freeze-Drying Process: Partial Freeze-Drying

As mentioned in the previous section, the gentlest approach for a partial or total dehydration of the sample material is the freeze-drying process. The process of freeze-drying can be easily explained by looking at the phase diagram of water as shown in figure 2. This diagram is calculated for pure water and can be used as a reference graph for any cryo-EM work and finally also for controlling freeze-drying processes [1, 2].

Sublimation of water (freeze-drying) – is the process of removal of water molecules from a frozen surface and it only takes place if the energy (temperature) of the samples matches a given local partial pressure of water in the surrounding environment (vacuum). The graph shows the saturation curve for water under vacuum conditions from 100 - 10-14 mbar and over a temperature range of 20°C to -160°C. The blue line follows the “steady-state” conditions where the total number of sublimating and condensing molecules equals zero. Above this curve (dark blue area) condensation dominates and these are the conditions, which need to be avoided in cryo-EM applications and where special anti-contamination strategies come into play. The area below (rainbow color) marks the conditions where water molecules can move directly from the solid phase into the gas phase. The amount of water molecules leaving the frozen bulk of material can be controlled by the amount of energy delivered to the sample in simply changing the temperature under a given vacuum condition.

E.g. at a pressure of 10-7 mbar at a temperature below -120°C no sublimation takes place, if one raises the temperature to -100°C the sublimation amounts to 2nm/sec; at -80°C it increases to about 100nm/sec and at -60°C the rate is approximately 1500 nm/sec. This simple relation allows us to carefully control the condition of drying and hence exposure of fine structures of our samples and stop it at any time by just lowering the temperature.

Knowing the physical background, one can now design gentle (partial) freeze-drying routines to preserve the 3D structure of a frozen macromolecular complex depending on the quality one would like to achieve. An often forgotten issue is the removal of structural water [3, 4], for most protein complexes this strongly bound water is removed above -40°C which leads to a further structure collapse (fig. 1b inset).

Mass Loss During Imaging

Having conserved a close to native state during sample preparation does not inevitably mean that this state is also imaged. Besides the artifacts related to sample preparation, also the electron impact might induce some structural re-arrangements. One is a mass loss of the specimen itself during exposure to the e-beam and the other is the opposite to avoid contamination built up during
observation and imaging in a SEM.
An exact determination of the dose-dependent mass loss can be performed by mass
determination via dark-field STEM [5, 6, 7, 8]. It has been shown that the
degradation of the structure can be prolonged by simply lowering the specimen
temperature to -140°C giving just 1-2 images at low dose (about 300e-/nm²)
conditions with minimal amount of mass loss [9].

Avoid Blurring and Noisy Images
Direct imaging of nm-surface structures depends very much on avoiding these structures to be buried or taking
images with a high level of noise. In the following we will discuss the best ways to avoid blurring and reduce
noise in HR-SEM imaging of macromolecular structures.

Contamination in HR-SEM
The interaction of electrons with the specimen surface produces various numbers of break down products (hydrocarbons, water etc.) which themselves can be attracted by the charges deposited in the imaged area. Such effects become even more pronounced as the amount of electrons per area increases, which is the case when working at high magnification.
In figure 3 freeze-dried and coated Semliki Forest Virus were imaged at -90°C (a) or at RT (b). The inset in B shows the amount of contamination built up during the first slow scan on a specimen area at a primary magnification of 150’000 (B) and with a beam current of 200 pA at RT in a XL-30FEG in a freshly pumped down microscope chamber. The first slow scan at a specimen temperature of -90°C (A) under identical imaging conditions shows no blurring or covering of fine specimen details by contaminants. Therefore, we can conclude that the amount of contamination caused by the electron bombardment, can be dramatically reduced in the presence of cold-traps at liquid nitrogen temperature and imaging at cryogenic specimen temperatures. Our experience is that at specimen temperature below -80°C several slow scans can be acquired before a significant contamination layer obscures fine specimen details (data not shown). However, one has to bear in mind that beam damaging causes shrinkage of the investigated specimen and hence more than one contamination free slow scan is ideally not needed. In addition the amount of contamination is proportional to the total beam current (electron dose) and can be minimized by using the minimal possible beam current in a given SEM-system which still delivers a sufficient signal to noise ratio for imaging.

A Versatile High-Vacuum Cryo-Transfer System (VCT)
To avoid contamination and alteration of samples during transfer from a cryo-
preparation chamber to any imaging system, a versatile high-vacuum cryo-transfer shuttle was developed. This VCT system had the potential to establish connectivity between different instruments and microscope types. Over the years it was adapted
not only to cryo-SEMs, but also to ESEMs for inert gas transfer, to cryo-FIB/SEMs, XPS, Tof-SIMS, APT and finally to in-lens SEM systems. Figure 4 shows historical versions of the VCT and the latest version for in-lens systems. Moreover, this figure illustrates the desired concept of connectivity, which allows finally a protected workflow to be established by transferring sensitive samples in either an inert-gas or high vacuum environment at room or cryogenic temperatures.

**Signal Enhancement and Signal Detection System**

**Contrasting Techniques for HR-SEM**

Several contrasting methods have been used in SEM to enhance the SE or BSE yield of biological specimen, such as negative staining, positive staining (OTOTO methods) and metal coating by different coating techniques [10, 11, 12, 13, 14]. These contrasting techniques are summarized in an experimental finding on F-actin filaments in figure 5.

Direct contrasting methods reveal a "volume contrast" related to the mass thickness of the specimen, if the penetration depth of the primary beam electrons is larger than or equals the specimen thickness. This "volume contrast" can be slightly enhanced if the macromolecules were positively stained with Uranylacetate (Uac) and/or Phosphortungstenacid (PTA) salts prior to freezing and freeze-drying (fig. 5B) compared to the uncoated freeze-dried F-actin filaments in the shadow area in figure 5A. Note that with both "volume-contrast" methods only a rod like filament with some density fluctuations became visible in SE images. Neither structural detail from the actin monomers nor from the topographic features of the F-actin filament is visible. Periodically every 37 nm along an uncoated filament (fig. 5A) the SE-signal reaches a maximum, which matches exactly with the location of the largest mass thickness at the cross-overs of the two monomer filament stands (fig. 5A). No structural details can be found in the noisy SE-image of positively stained filaments where the filament signal is weakly enhanced compared to the background stain pattern (fig. 5B). Whereas in both indirect staining methods structural details down to 2 nm are revealed (fig. 5A coated area & C). The contrast of the "heavy metal stain", in metal coated specimens (fig. 5A) reveals even a topographic contrast of the specimen surface by enhancing and localizing the SE signal within the replicating metal film grains. Surprisingly, also here the strongest SE-signal is found at the cross overs of the two actin strands. In comparison with the uncoated naked structure in the shadow area of a uni-directionally coated F-actin sample (fig. 5 A top left), from which no BSE-signal could be detected (not shown here), the single subunits and their orientation within the filament are clearly resolved. Negative staining, on the other hand, reveals a stain excluding pattern as shown in figure 5 C&D. Here, the main signal emerges from the metal, which is surrounding the protein mass (fig. 5D), revealing a similar topographic
view in the SE-mode in reverse contrast (fig. 5C) as on coated F-actin filaments (5A). While in the BSE-mode (fig. 5D) a projection view of the surrounding metal is obtained, similar to the contrast in TEM image of negatively stained F-actin filaments. At a closer look it becomes obvious that the stain penetrates further into the filament structure resulting in a more teetered projection view of the F-actin filament in the SE-image compared to the topographic appearance of coated F-actin filaments (fig. 5A). Therefore, the mini "arrow-head" of the F-actin filament is better resolved by negative staining than by metal coating.

**Coating Techniques**

As already stated in the previous section, soft-matter specimen, e.g. macromolecular complexes, consist of light elements, mainly carbon. Consequently, the secondary electron (SE) yield from such specimen is typically low and hence the signal to noise ratio (S/N) is inappropriate for HR-imaging. In order to increase the signal and to further enhance the localization of the SE signal, the sample material is typically coated with metal forming nanometer small metal grains. Since there are different methods available for lab usage here we only discuss the best way to perform HR-SEM imaging of macromolecular complexes. The two most spread metal coating techniques in electron microscopy belong to the so called physical vapor deposition (PVD) technologies. These devices are classified into two major categories: either the devices utilize “sputtering” or “evaporation” as technical concept for releasing metal from the solid phase into a vapor phase. Sputtering is based on the bombardment of a metal target by ions and evaporation by inducing heat and melting herewith the metal. Both techniques are broadly used in material science and semiconductor industry and have many more variations. Compared to sputtering where the metal atoms are randomly scattered and deposited from any angle and direction onto the sample surface, e-beam evaporation (EBE) allows shadowing to be controlled and adapted by the operator. To successfully deposit films of only 1-2 nm one uses a thin film microbalance (quartz crystal monitor) for all high resolution coating experiments.

**Example: Tungsten (W) PMS vs. EBE Coating**

A comparison of PMS and EBE coating on freeze-dried HPI Layer is shown in figure 6. The left column shows the SE, BSE and TEM images from samples coated by PMS at -80°C with 1 nm W and the right column shows the corresponding images from samples which were rotary shadowed from 45° at -80°C with 1 nm W averaged film thickness. The grains after planar magnetron sputtering (PMS) are significantly larger in all imaging modalities giving at a first glance a crisper image but by carefully comparing with the EBE images show less fine structural details in the raw images. The averaged images of about 250 unit cells are shown in the right upper corner for each imaging mode. By comparing the fine structures from EBE
coating it becomes clear that the PMS coated samples reveal a more “blurred” structure with less details and only in the TEM averaged image the handiness of the HPI outer layer becomes extractable. Whereas the EBE averaged structure all show the handiness of the HPI unit cell as well finer structures on the arm-like connections. Resolution assessments (FRC, S-Image and SSNR) of PMS and EBE coated HPI layers revealed 2-2.2 nm for W-PMS and 1.5-1.8 nm for W-EBE coating. The measured metal grain sizes are for PMS between 1.5-3 nm and for EBE between 1.2-2.2 nm.

For most biological applications it is interesting to know the maximal metal film thickness for an "optimal visibility" of fine structural details without the necessity to use averaging techniques. For “direct visibility” one would preferentially use a film thickness between 2 and 3 nm that will not mask too much of thin surface features. Any thinner film 1-2 nm makes sense only if averaging techniques can be used as shown in figure 5&6 insets. In general if averaging is not possible and direct visibility is requested, a lower elevation angle, a thicker metal film or, for sputter coating, a higher process gas pressure will help to enhance “visibility” of structure but blur fine structural details (low pass filtering effect). There is no general rule and depending on the object size, corrugation and dimensions, the coating may start from film thicknesses above 2 nm and needs a few iterations to allow highest possible resolution extraction while avoiding “covering” of fine details and still maintain charge balance during imaging.

**Which Metal?**

There remains one big question after investigating the different parameter in metal film deposition technology – which metal is the best to be used for HR-SEM? This depends very much on the final application, since some of the metals tend to oxidize (W, Cr) or produce a high backscattered signal (W, Pt, Au) or are not inert for replica production (W). There are hundreds of publications on coating films mainly from the 1960-1990s, mainly done to show that a certain product is the best and most of them are unfortunately not comparable nor run under best possible cryo-HR-SEM conditions in these days. But let’s try to summarize a few experiences from more than 20 years testing metals and metal-oxides from room temperature down to 15K sample temperature [15]. After comparison on HPI; T4 Polyhead and other structures, a general empirical rule emerged – “the higher the melting temperature the finer the metal gains at specimen temperature between 193-15 K are. Mainly W, Ta, Ta/W and Re revealed the finest metal grains if not exposed to oxygen or atmospheric conditions. An alternative way to reduce the grain size is to deposit simultaneously C or SiO2 – as eg. Pt/C; Pt/Ir/C [4, 16] have established. In addition, for HR-SEM imaging of insulating samples the “conductivity” of thin films becomes one of the major selection criteria. As has been reported [18] the electrical resistance of metal films depend on the metal type, the deposition
conditions, the sample temperature and the average film thickness. To summarize also these findings we focus just on the electrical resistance in Ω/mm² (sheet resistance); 1 nm W was found to be in the range of 104 Ω/mm²; for Ir about 105 Ω/mm², for Cr about 106 Ω/mm² and for Pt about 106 Ω/mm² - making W, Cr and Ir the top candidate to prevent charge instability. This has also been reported empirically e.g. by Peters et al. [19], Herman et al. [14, 20] and many more in their various HR-SEM publications.

Figure 7 tries to “high-light” the benefit of very thin W-films in a simple experiment. For testing the electrical conductivity (mainly charge redistribution) of sub-nm films 1 µm latex beads were adsorbed to 8 nm C-film (a) or to a freshly cleaved mica surfaces (b - an insulator). These latex beads were coated from different orientation at an elevation angle of 45° with various amount of tungsten. Leading to areas, which are not coated to films of 0,2 nm, 0,5 nm, 0,7 nm, 0,8 nm and 1 nm total average film thickness (as shown in figure 7. A) shows the experiment, performed on a 8 nm carbon supporting film and imaged with 1000e-/nm² in an in-lens FE-SEM. A 0,2 nm W film already enhances the SE-yield compared to the pure carbon film and allows also stable imaging of such a coated area on mica. Charge built-up and electron reflection (generally known as charging) on the insulator is prevented from expanding towards these sub-nm W metal film areas several nm before the edges of these coated areas. An average film thickness of 0,2 nm would be less than 2 atoms. A closer look at the film shows that even in these sub-nm thin films one can still find distinct W-grains of 0,8-1,5 nm in size. These grains act as distinct SE signal emitting entities - “lantern like” - and hence amplifies the signal yield and prevent SE to move as far as their mean free pathway (about 3-4 nm) would allow to. In other words these small W-grains improve not only the signal enhancement by their higher atomic number but also the localization accuracy of the emitted signal by preventing the signal to travel within a “continues” metal film. The individual grains are separated by vacuum on the support film and once an SE-signal is emitted at a metal grain surface the electron is attracted immediately by the collecting field or potential gradient towards the SE-detector. The distance between the grains is sufficiently small that charge can laterally flow avoiding charging arte-facts.

This means that metal films from 1 nm thickness are absolutely sufficient to locally prevent charging for HR-SEM imaging (see mica support area in fig. 7). The only remaining risks are that such films are “discontinuous” (shadowed areas; cracks etc) across the whole sample surface or badly grounded to the sample stage.

**Final Resolution Obtainable from Metal Coating for HR-SEM Work**

A resolution of 3-5 nm on freeze-dried thin biological specimens, coated with Cr by planar-magnetron sputtering (PMS) or double-axis rotary shadowing (DARS) at low
temperature (193 K) has been reported also by Hermann and Müller [14] and 2 nm resolution by high angle rotary shadowing by Wepf [4]. High resolution SE images were also shown on heavy metal coated samples e.g. on Pt, Cr or W sputter coated, frozen-hydrated yeast cells by Walther et al. [11, 21, 22] and Hermann et al. [23] and on coated freeze-dried macromolecular structures [4, 12, 24, 25].

To finally determine the resolution limit of metal coating of biological samples for HR-SEM, we compare coated and non-coated areas of freeze-dried HPI-layers and determined the resolution power by averaging techniques, using standard methods used in TEM 2D protein-crystal structure research.

Figure 8 shows a latex bead adsorbed on-top of a HPI-layer after freeze-drying and metal shadowed from one direction with 1nm W from an elevation angle of 45° resulting in a coated and an uncoated area on the same HPI-layer. The area in the shadow shows no BSE-signal (A) and a strong SE-signal (B), which allows to extract the protein crystal lattice and some fine structure without a surface contrast enhancement. Averaging of coated and uncoated HPI areas from SE-images reveals the unit cells of coated (C) and uncoated (D) HPI-layer. The uncoated area reveals an averaged core structure with a strong 6-fold core structure and very weakly resolved connecting arm-like structures, even they can be from time to time directly seen in the raw image. Using standard TEM frequency based resolution criteria (SSNR, FRC, S-image) a resolution of 3-5 nm was found on such areas. Whereas the coated area reveals a much more detailed unit cell with clear arm-like connections to the core-proteins and resolves a right handiness of the core structure. The resolution criteria show structural details down to 1.5 nm. Hence one has to follow that the single W-metal grain also here helps to keep the signal laterally and more importantly localized to the surface area where the metal was deposited. In other words, uncoated areas SE-images resemble more a “mass-density” representation as long as the sample is not much thicker than the mean free pathway of electrons. Metal coating on the other hand allows with its defined and discrete grains to replicate a surface area more accurately than a continuous metal film could. After averaging of 30-100 unit cells from SE images and 100-200 unit cells from BSE images these statistical grain locations are lost revealing a continuous signal, which virtually assembles a continuous “film” on a given surface topography with a replication potential of 1.5 nm.

Finally to summarize the findings – best structural preservation can only be achieved by cryo-fixation and partial freeze drying and best signal to noise ratio is obtained by coating the surface with 1-3 nm thin metal films, not continuous but with single grains enhancing SE and BSE signal generation at its site of deposition replicating slope and position more accurately than a continuous metal film could. Which coating techniques may be used is summarized in figure 9 were diode sputter coating (DS) can help to visualize sample details in the µm range, planar
magnetron sputtering down to the 2-3 nm range and only electron beam evaporation is capable to resolve details down to 1nm in SE or BSE images.

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References


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