EMS Microscopy Academy

Endless Possibilities ...

Kirsch notes

Providing Solutions: TEM Imaging Issues & Solutions

TEM Imaging Issues and Solutions

Low contrast of biological TEM thin sections is a constant challenge. The use of digital image acquisition has helped tremendously but it is still important to process the sample, stain the section and use the proper parameters on the microscope before resorting to digital corrections. Here are a few ways to ensure the best possible specimen makes a great image.

Low Contrast

Contrast, intensity difference between adjacent areas, in the TEM is generated through elastic interactions between the beam electron and the mass thickness (pt) of the sample. The larger angles of Rutherford scattering deflect the electron outside of the objective aperture's collection angle, eliminating them from the beam path. This scattering loss is referred to as Amplitude Contrast, a hole will not scatter any electrons allowing all the electrons down to the screen, = bright and a dense object will scatter a large portion of electrons which will be eliminated by the objective aperture = darker. In biological TEM the number and degrees of these interactions is small due to the low average atomic mass of the sample, or low Z. To increase this scattering effect there are sample preparations and instrument parameters which are used to obtain sufficient contrast for morphological and aesthetic evaluations.

Sample Preparation

Secondary fixation using osmium tetroxide (OsO_4) is the first step in processing which has an effect on contrast. Osmium is the densest element in the periodic table which, having been reduced in its interaction with unsaturated lipid groups, deposits metallic osmium within the tissue thus increasing the scattering cross section. The osmium also acts as a mordant for lead which is often used as a post stain on the sectioned tissue.

The use of uranyl acetate (UA) as a tertiary fixative also acted as an enbloc stain by depositing uranium, another high mass, dense element. Unfortunately, this compound is toxic and slightly radioactive, so its use as a fixative and even as a post stain is being phased out.

Potassium permanganate ($KMnO_4$) is a fixative which also results in the increase of the average atomic mass by depositing manganese in its reaction with tissue. This compound is usually used on botanical or bacterial specimens with coatings or membranes which are hard to penetrate. Potassium permanganate was an early fixative for EM providing excellent high contrast images of membranes but this was due to significant extraction of the cytoplasmic background so is only used in specific instances.

Post Staining Sections (Positive Staining)

Uranyl acetate is an excellent general stain for sectioned tissues dissolved in either an aqueous or alcoholic solvent. Its affinity for nucleic acids and proteins and, to a lesser extent, other cellular moieties makes it invaluable. Again, as above, UA is toxic and slightly radioactive so its use and availability is declining. UA is typically followed by a second stain, lead citrate, for optimum contrast.

Uranyless, a non-radioactive, non-toxic replacement for UA uses gadolinium and other lanthanides to add density to thin sections giving similar results as UA. Also followed by a second stain using lead citrate for optimum contrast.

Lead citrate (Reynolds) is the second post stain after UA or Uranyless. Preferentially binds to sites previously stained with osmium due to its mordant effect. An excellent stain but tends to react with carbon dioxide in the air forming insoluble lead carbonate which is deposited on the sections as lead "cannonballs" or a fine precipitate over the entire field of view. The use of NaOH pellets in the staining dish and a high native pH reduces this negative effect.

Sectioning

There is a trade-off when it comes to section thickness as it relates to contrast. The mass-thickness (pt) does increase with section thickness but due to the increase in chromatic aberration, Cc, there is a loss in resolution which must be weighed against the gain in contrast.

Instrument Parameters

Accelerating Voltage controls the beam electron's energy or momentum. The higher the electron's energy (100 kV) the smaller the angle of scattering allowing more electrons to be gathered by the collection angle of the objective aperture, decreasing contrast. Conversely a lower kV, 60, reduces the electron's speed creating larger angle scattering, increasing contrast.

Objective Aperture eliminates scattered electrons created by the beam/sample interactions. The smaller the objective (25 um) the smaller the collection angle, eliminating more scattered electrons, providing more contrast. Besides increasing contrast, a smaller aperture increases resolution due to decreased spherical aberration, Cs.

The beam intensity as controlled by focusing of the C2 lens also has an effect on contrast, although to much less degree than kV and objective aperture size. Contrast will increase as the C2 is defocused, reducing the intensity of the image. Back when film was used this had a more dramatic effect, increasing the exposure time and S/N of the recorded image. But even with digital imaging a dimmer, less intense beam will provide more contrast.

Spot size, C1, is another parameter which slightly affects contrast. The C1 lens effectively reduces the size of the electron source which increases the beams coherency. A smaller spot size or stronger C1 lens then provides increased contrast over a weaker, larger spot size. This reduction in spot size does come with a decrease in beam intensity causing the operator to work with a more focused C2 setting or increased gun brightness which shortens the filament life. Be aware, this increase in gun brightness requires a decrease in the gun bias setting which reduces both temporal and spatial coherence of the electron source. So typically, the spot size is set at an intermediate setting and is not often changed.

Digital Imaging

Histogram equalization and gamma adjustment are very effective ways of image contrast and intensity control. After image collection, having used all of the above parameters first, the "almost" white and black points of the histogram are set using the software sliders which spreads out the histogram incorporating a wider range of gray scales. With current 12 – 16 bit dynamic range, there is no combing or segmentation of gray scales, providing a continuous gray scale from a black grid bar, to an almost white hole.

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