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What do we know about stain distribution in cells and tissue? Using EDS to determine the quantity and distribution of common EM stains.

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Heavy metal stains are commonly used when preparing biological electron microscopy samples to contrast structures of interest. There are a huge variety of stains that have been used and many publications have gone into detail about how to apply the stains and their binding affinities in different sample types [1]. Research into chemical fixation protocols and the applications of stains fell out of favour with the rise of cryofixation and cryo-imaging techniques. However, in recent years volume electron microscopy has seen a resurgence in the need for general and specific stains [2, 3] to improve visualisation of cell structure and improve the ability of researchers and computer algorithms to segment and reconstruct those structures in 3D.

Much of our current understanding of stains and how they work is based on publications dating back several decades. The localisation and amount of stain within the tissue has been primarily based on the amount of contrast generated. However, there is increasing interest in determining accurate localisation of specific stains and measuring how effective the staining protocols are.

We used energy dispersive x-ray spectrometry (EDS) to measure the relative quantity and map the distribution of several common electron microscopy stains, including zinc, osmium, lead citrate, uranyl acetate and bismuth in both cells (cultured erythroblasts) and tissues (mouse liver tissue, and roots and/leaves from *Arabidopsis thaliana*, *Zea mays* and *Dionaea muscipula*). Samples were all embedded in epoxy resin and were imaged either as a diamond-knife trimmed resin block or in sections on copper grids. Resin blocks were coated with approximately 10nm of carbon and sections were left uncoated. All samples were analysed using an Ultim Extreme detector (Oxford Instruments, UK) in a FEGSEM with optimised working distance (5-10mm), accelerating voltages (5-6kV) and beam current or aperture settings depending on the make of microscope and the sample. Grids were placed into a STEM holder for analysis. EDS maps and spectra from specific points were collected for all the samples. Multiple points were measured across several cells for each of the regions examined. TruMap was applied to the map data for deconvolution of signals.

EDS revealed that common stains used for contrast in biological samples exhibit differential distribution at the cellular level in all samples examined. The ZIO stain applied to *Dionaea muscipula* had up to 10 distinct combinations of osmium and zinc (figure 1), significantly different from the 4:3 ratio commonly cited [1]. This was also observed in *Zea mays* root cells with variability in zinc, osmium and lead distributions, some areas showing distinct colocalization and specific organelles showing stain segregation (figure 2). Erythroblast cells also showed variability in the uptake of stains to different regions of the cell. Bismuth appeared to target the nucleolus and was not present in significant levels in either heterochromatin or the cytoplasm of the same cells. The bismuth results correlated with findings in other studies [4]. Osmium was very slightly higher in the nucleolus (1.4 x higher) compared to the heterochromatin and cell cytoplasm. Lead (from lead citrate) and uranium (from uranyl acetate) were 2-2.5x higher in both the heterochromatin and nucleolus compared to the cytoplasm, indicating that they probably account for the large degree of contrast for these areas.

Our results show that there is still a lot of information about stain distributions and quantities that have not yet been determined. EDS offers a quantitative measurement tool that is available to electron microscopists to further determine the effects of different sample preparation protocols on the results that we observe.

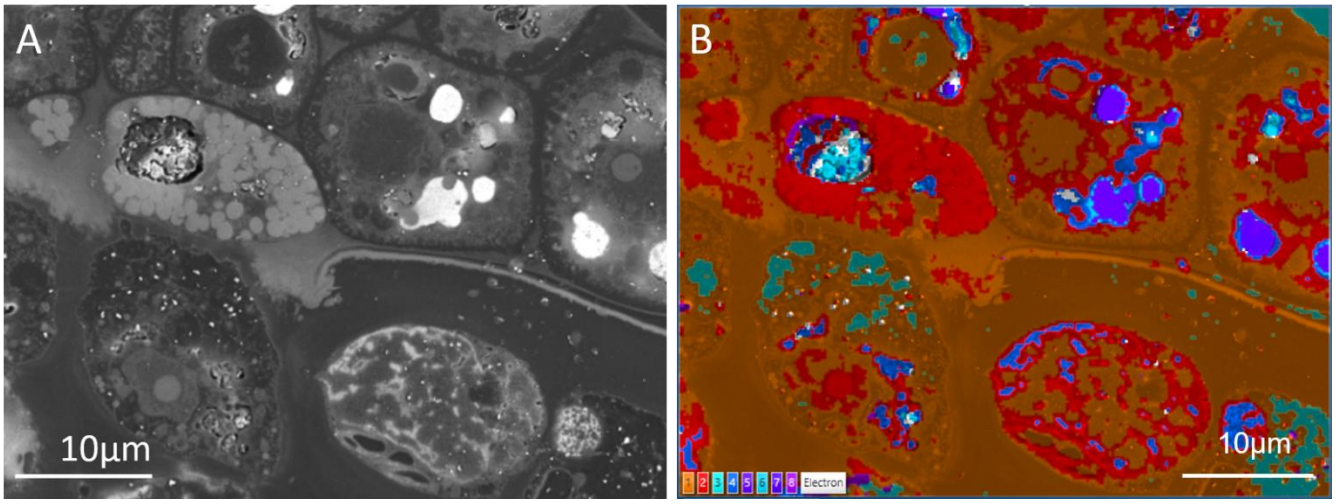

Figure 1. Figure 1. BSE image of Dionaea muscipula leaf showing the edge of a digestive gland (A) and the corresponding phase map produced from EDS analysis (B). A total of 10 phases were produced and 8 are shown superimposed on the BSE image. The colours are selected automatically and the closer the colours are in shade, the closer the chemistry detected in those regions, larger chemical differences are indicated by larger differences in the shade displayed.

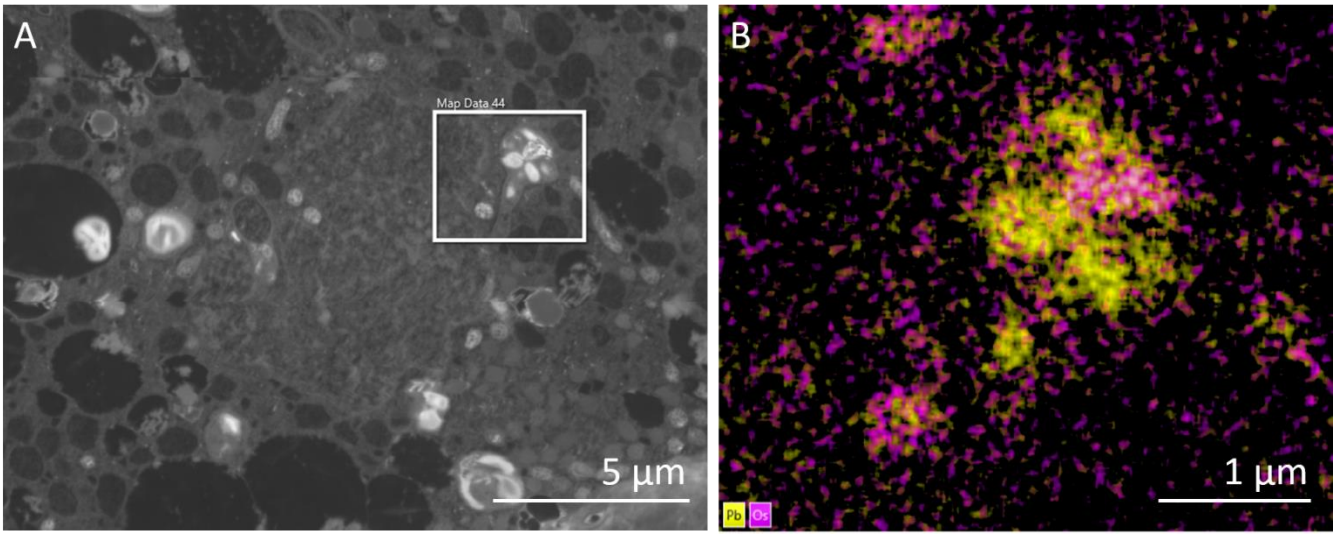

Figure 2. Figure 2. BSE image of a Zea mays root (A) with acquisition area showing the location of the EDS maps (B). The structure in the acquisition area is a proplastid with high levels of contrast. The EDS maps show a differential distribution of osmium (pink) and lead (yellow) from the stains that have been applied to the sample.

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