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Quick Freeze Substitution Processing of Biological Samples for Serial Block-face Scanning Electron Microscopy.

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Until recently the only way to obtain three-dimensional electron microscopy data over a large area of a biological sample was via serial sectioning, a tedious and technically challenging technique. Serial block-face scanning electron microscopy (SBF-SEM) has recently been used as a technique to obtain similar results to serial sectioning transmission electron microscopy but in a simpler and semi-automated way. It involves working with a resin embedded sample similar to that used for transmission electron microscopy. However, the imaging is performed on the block-face rather than the section cut from it. Image collection is done using a backscattered electron detector in a scanning electron microscope. An image is taken of the block-face and then a thin section is cut from the block using a diamond knife. The newly exposed block-face is then imaged, another section cut from it, it is imaged again etc. This process of imaging and sectioning is performed over and over building up a three-dimensional data set. Two commercial instruments are available that will perform SBF-SEM: the Gatan 3View and the FEI Teneo Volumescope.

SBF-SEM suffers from a couple of major problems. As the sample is embedded in resin, a non-conductive material, they charge badly and this can have a significant impact on the imaging quality. As a result a lot of this work has been carried out operating the SEM in variable pressure mode. However, this also effects the image quality. Also standard processing protocols used for preparing samples for biological electron microscopy do not leave enough metal in the sample to produce a strong backscattered electron signal to give good imaging. As a result new protocols have been developed that deposit substantial amounts of metal into the sample in an attempt to overcome both these issues. Nguyen et al [1] and Tapia et al [2] have published protocols that use a combination of metals, including osmium-thiocarbohrdrazide-osmium, *en bloc* uranyl acetate and *en bloc* lead aspartate. Most groups are now following the "NCMIR Methods for 3D EM" [3] with small variations on it.

The problem with this protocol is that the samples are fixed chemically at room temperature. It has long been known that rapid freezing followed by freeze substitution preserves the morphology of biological samples in a form that is close the native state, avoiding most of the artefacts induced by processing chemically at room temperature. In 2011 McDonald and Webb [4] published a method for performing quick freeze substitution in which they could process samples in less than 3 hours, a process that up until that time was usually performed over several days.

The metals introduced into the sample by freeze substitution, usually osmium tetroxide and uranyl acetate, do not impart enough conductivity to make samples produced in this way useful for SBF-SEM. Unfortunately the metals used in the NCMIR methods paper [3] were all in aqueous form so are not useable in a freeze substitution processing protocol. New protocols that utilize the quick freeze substitution in combination with metals in an organic solvent such as acetone or methanol have been shown to produce samples that work well for SBF-SEM (Figs. 1 and 2). In fact, these samples can be readily viewed with the SEM operated in high vacuum mode. Chemicals such as lanthanum chloride or imidazole can be included into the freeze substitution media with the osmium tetroxide and these impart

a high electron density to the sample. A double osmium method can be performed by using tannic acid [5]. Once warmed to room temperature other metal containing solutions such as uranyl acetate, phosphotungstic acid [6] and lead acetate [7] are used to give the sample more electron density. By utilizing the quick freeze substitution and rapid embedding methods [8] the entire process can be completed in a day.

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References:

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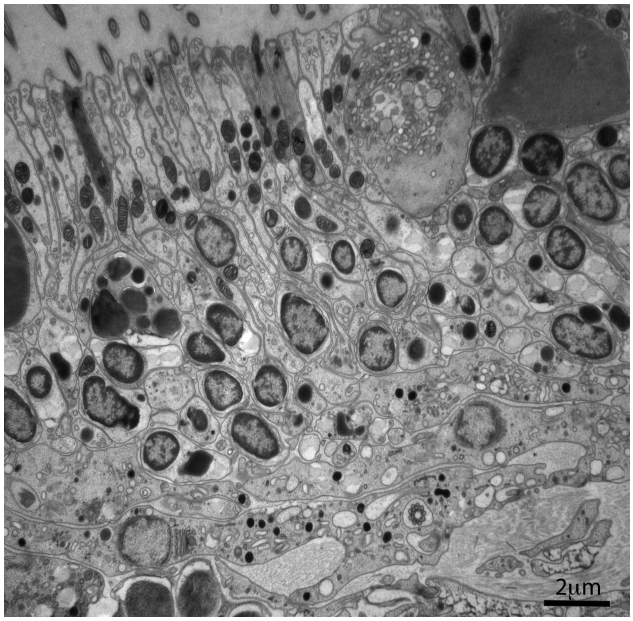

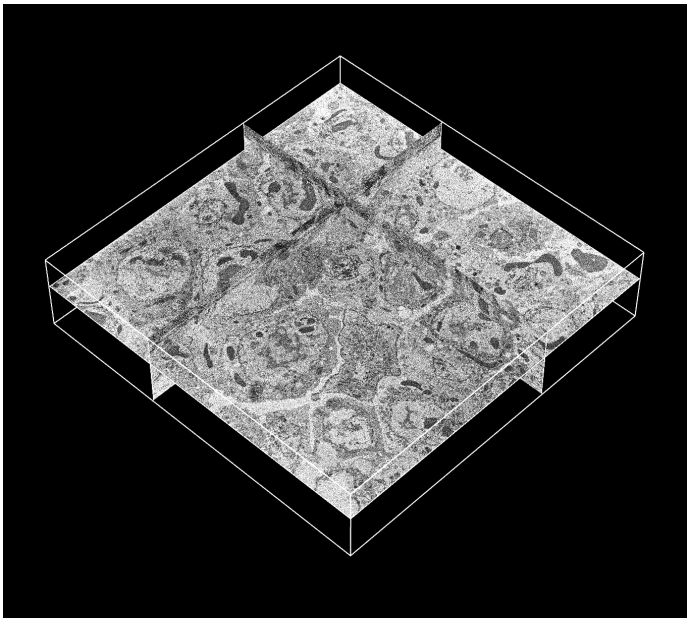

Figure 1. Single image from a 3D data set of a sponge larva taken using SBF-SEM. The sample was processed by quick freeze substitution in imidazole and osmium tetroxide in acetone, followed by tannic acid in acetone, another osmium tetroxide, *en bloc* uranyl acetate and *en bloc* lead acetate.
Figure 2. 3D data set of cultured insect cells infected with baculovirus, processed in a manner similar to the sample shown in Figure 1.