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A guide to the interpretation of the electron micrographs contained within the "Ken Clarke Collection"

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Electron microscope specimen preparation techniques:

General. Electrons are easily absorbed by all materials, even by gases. The electron microscope, to be effective, uses electro-magnetic lenses to focus the electron beam and runs under a high vacuum. Specimens for transmission electron microscopy must also be extremely thin to enable examination of the subject's finest ultrastructural detail. A number of techniques have been developed to accomplish this and the basic methods for many of the collection's investigations are given below. Contact the FBA for a more detailed procedure.

Fixation. Interaction by electrons with living material can cause detrimental changes to the specimen under examination. This means that in most cases biological material examined by electron microscopy must be dead but preserved as in a living state. To prevent any changes occurring in the material due to its treatment during any electron microscope examination, live material must first be chemically or physically 'fixed'. In this process the metabolic processes of the biological cells under examination are 'locked-down' so that biological deterioration is prevented.

a) In **chemical fixation**, a protocol is adopted that takes the biological sample through a series of diluted chemicals such as osmium tetroxide and glutaraldehyde. Experimentally, time, temperature, molarity, and pH are established for each sample in each solution for each step of the procedure. Molecules of the chemicals used form a stable cross-linked matrix with the biological molecules of the sample material (proteins and lipids) so preserving the biological ultrastructure as in living material.
b) In **physical fixation**, the biological material is plunged into a slurry of liquid/solid nitrogen gas at below -200 degrees Centigrade. This chills the material so fast its metabolic processes again are locked-down.

Freshwater Biological Association

Shadowed direct preparation. Chemically fixed material is washed in distilled water and a suspension placed on a plastic-coated (polyvinyl formal, or 'Formvar') electron microscope specimen grid. The grid is then transferred to a vacuum evaporator where an incandescent source coats a noble metal such as gold or platinum onto the grid's surface at a known angle between 20 to 30 degrees to the horizontal. The metal creates a fine cross-lighting effect when the grid with its specimen is examined in the electron microscope, thereby enhancing detail and aiding height measurements. An electron micrograph resulting from this procedure will show a silhouette image in areas of higher density but exhibit surface detail in low density areas. A negative or a positive shadow-cast will accompany image detail.

Sectioned material. Chemically fixed material is dehydrated with ethanol and embedded in a liquid plastic resin. When the specimen material is completely permeated by the resin, the resin is polymerised by heating in an oven. A small block is trimmed from the embedded material and placed in an ultra-microtome. Here, thin successive cuts each of a few nanometres in thickness are cut with a diamond knife and caught on a water surface and then dried onto an electron microscope specimen grid. When dry, the ribbon of successive sections are stained with an electron dense series of stains and examined in the transmission electron microscope. Specimens treated this way show the internal organisation of the biological sample, and can also show food ingested by the sample organism, and infection by bacteria or viruses.

Replicated material. Chemically fixed material is washed in distilled water and coated onto glass microscope slides or onto mica sheets. When dried the slides are transferred to a vacuum evaporator and a metal shadow is cast, as above. The shadowed sample is then coated with a layer of carbon from another incandescent source and then coated with liquid polyvinal formal dissolved in chloroform. When dry, the area of interest on the slide is scribed with a scalpel, lifted and floated onto an enzyme solution or a dilute solution of sulphuric or hydrochloric acid. This dissolves away the biological material from under the replicating carbon layer. After washing, the area of interest is transferred to an electron microscope grid and the polyvinyl formal washed away using chloroform applied with a micro-pipette. When dry the material is now ready for electron microscope examination. The use of carbon evaporation replicates the surface of a cell or other material and, after the removal of the biological content from beneath this carbon, enables the cell surface and its features to be examined in fine detail.

Negative staining. Chemically fixed material for examination is washed in distilled water and a drop placed onto a polyvinyl coated electron microscope specimen grid. When dried, a droplet of electron dense stain (eg, uranyl acetate or sodium dodeca-phosphotungstic acid) is added to the surface, allowed to remain for around 30 seconds and then removed to leave a small remaining film. When dry, this can be examined in the electron microscope. The method builds a dense stain **around** the structure being examined, not staining the structure itself. This is a method for examining extremely fine detail and will resolve detail on virus particles showing protein assembly structures.

Freshwater Biological Association

Positive staining. As ‘negative staining’ above, but using a stain such as lead citrate to enhance the fine structure by staining the specimen itself.

Freeze-fracture. A method used for scanning as well as transmission electron microscopy.

a) **Freeze-fracture for scanning electron microscopy**. Biological cells are physically fixed in a slurry of liquid/solid nitrogen. Droplets of cells are then crushed and freeze-dried under vacuum, so removing all frozen water. The dried material is then transferred to a scanning electron microscope specimen stub and then placed in a gold or platinum sputter-coater where an all-covering layer of metal is sputtered onto the cell material. It is then ready for examination by scanning electron microscopy. It will be found that the crushed and dehydrated cells under examination are randomly scattered and should show themselves fractured through most planes through the cell. Internal organisation can be examined as well as food vacuoles, infection etc. The three-dimensional aspect of this technique can prove invaluable.
b) **Freeze-fracture for transmission electron microscopy**. Chemically fixed material is washed in distilled water and placed in a fine metal tube. The tube is then plunged into a slurry of liquid and solid nitrogen at below -200 degrees Centigrade. The tube is then fractured and in so doing fractures the frozen material inside it. The two pieces of the tube pieces are transferred to a frozen block within a vacuum evaporator, presenting their fractured surfaces toward the evaporants. A shadowed and carbon replica is made (see above for replica procedures)and the biological material removed by digestion in enzyme or dissolved in acid solution. In the transmission electron microscope it will be found that the cleaved cells of the sample material will show respective halves of lipid bi-layers of membrane as well as other internal ultrastructure.

Scanning electron microscopy. General methodology. Chemically fixed cells are washed in distilled water and dehydrated through ethanol. The biological material is then transferred to a critical-point dryer where the ethanol is replaced with liquid carbon dioxide. Critical-point is reached by heating the dryer with hot water. The carbon dioxide sublimates to gas without passing through a liquid- meniscus phase and in so doing preserves the morphology of the sample specimen. The dried material is then transferred to scanning electron microscope specimen stubs and sputter coated (as above). The material is then ready for examination. In an ideally prepared sample, whole cells should be preserved and their appendages displayed. However, there can be a little overall shrinkage in materials prepared by this method.

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