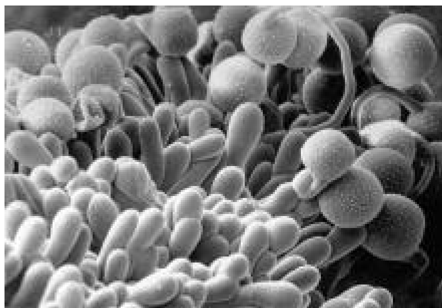
Cryo-SEM - the advantages.

The Scanning Electron Microscopist is faced with the inescapable fact that liquid is a fundamental part of practically all lifesciences – and many materials – specimens. Since water occupies up to 98% of some animal and plant tissues it represents a most formidable specimen problem to most Scanning Electron Microscopists.



Cryo-SEM is a quick, reliable and effective way to over come these not inconsiderable SEM preparation problems. Additionally the technique is widely used for observing 'difficult' samples, such as those with greater beam sensitivity and of an unstable nature. An important application, often overlooked, is the ability to use cryo-SEM to study dynamic processes (industrial or otherwise) by using a series of time resolved samples.

Naturally the advent of various "higher pressure" modes, such as VP, LV and ESEM has allowed such samples examined in SEM without resorting to freezing or drying methods. However, cryo-SEM is still by far the most effective method of preventing sample water loss, which will in fact occur at any vacuum level –even with Peltier stages fitted to the SEM and the careful addition of water vapour in the SEM chamber.
Cryo-SEM also a number of a additional advantages, including the ability to fracture and selectively remove surface water (ice) by controlled specimen sublimation.

The limitations of conventional SEM processing:

- Shrinkage and distortion
- Extraction of soluble materials
- Relocation of highly diffusable elements
- Mechanical damage. Fragile samples can be damaged during conventional samples processing
- Slow (24 hours plus….)
- Toxic reagents

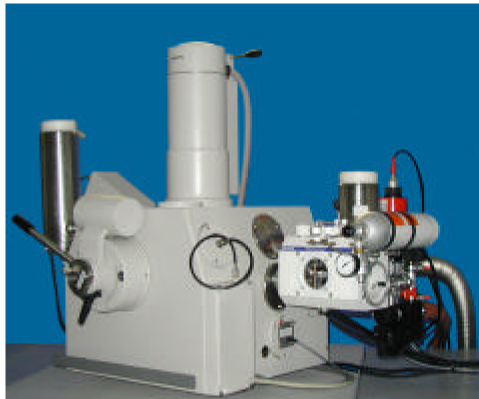
By contrast cryo-SEM has the following significant advantages:

- Soluble materials are retained
- Less relocation of highly diffusable elements
- Little or no mechanical damage.
- Usually no exposure to toxic reagents
- The only SEM method for dynamic time resolved experiments (freezing at timed intervals - in the lab or outside)
- Rapid process (from "fresh" material to viewing a frozen, fractured and sputter coated sample can take as little as five minutes.

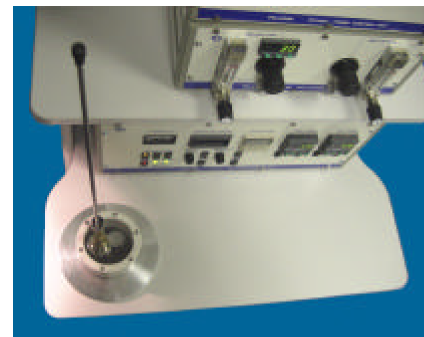
- High resolution capability (compared to low vacuum techniques)
- Extra information obtained by low temperature fracturing (compared with conventional and low vacuum methods)
- Good for liquid, semi-liquids and beam sensitive samples
- Ability to selectively etch (sublimate to reveal information)
- Ability to "rework" sample (e.g. re-fracture and coat the same sample)

The cryo-SEM method

The specimen is first mounted on an appropriate holder – a wide variety are available to suit most sample types – which is itself mounted on to a cleverly designed freezing / vacuum transfer rod. The sample is plunge frozen, usually in slushy nitrogen and then



transferred under vacuum onto the cool stage of the cryo-SEM preparation chamber of the Polaron PP2000, which is mounted directly onto a convenient 'port' of the SEM. The preparation chamber is pumped either by a rotary pump or by a specially designed turbomolecular pumping system (recommended for FESEM). The preparation chamber has facilities to manipulate, fracture, etch (sublimate) and sputter or carbon coater the specimen. Finally the simple-to-use gate valve between the preparation chamber and the SEM can be raised and the specimen transferred onto the cooled stage of the SEM.



Typical applications:
Biological sciences including botany, mycology, zoology, biotechnology and biomedical – plus economically import agricultural sciences.

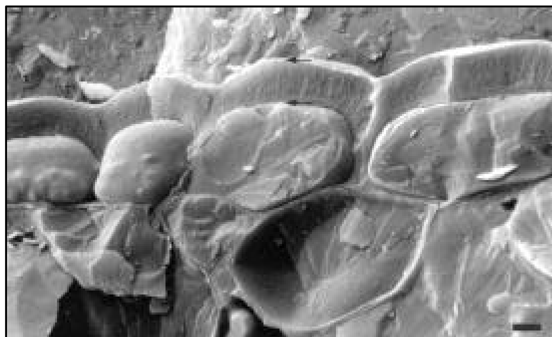
More recently cryo-SEM is becoming an essential tool for pharmaceutical, cosmetics and healthcare industries, where it is used in basic applied research and for routine QA of many products, such as creams, cosmetics and drug delivery systems.

Cryo-SEM has long been a standard preparation method in the food industry. Of interest are multi-phase products, such as ice cream, confectionery and dairy products.

Botanical
Cryo-SEM is the perfect method for highly hydrated botanical material.

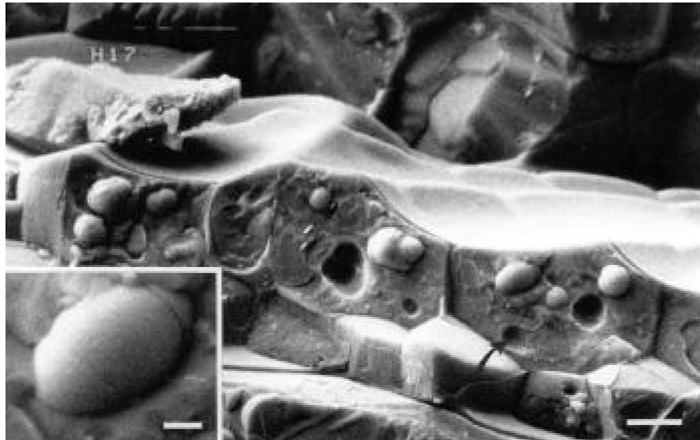
Figure 10. Epicuticular waxes on the adaxial surface of the 'wax plant' Hoya carnosa

These waxes are very delicate and damage can easily be damaged during conventional processing for SEM.



Hoya has a cutinised / suberised cuticle some 10 –15um thick (between arrows) with a delicate array of waxes on the outer surface. These are easily demonstrated using low temperature SEM, but would be lost during the critical point drying processing. Note also that cold fracturing allows sample to be broken to expose the many different faces – e.g. in this case between membranes, through cells and around cell organelles. The user can then selectively etch (sublimate) ice to reveal inner structure. This is a major advantage of cryo-SEM compared to conventional processing and variable pressure / environmental methods. This is illustrated in figure 11 (below).

Figure 11. Organelles in cells from the Apical Meristem



Cells of the Apical Meristem are non-vacuolated with a cytoplasm rich in organelles. There are no intercellular spaces between them. In many meristematic cells the fracture has passed through nuclei (arrows) and in places larger organelles (plastids and large vesicles) are obvious.

Animal tissue

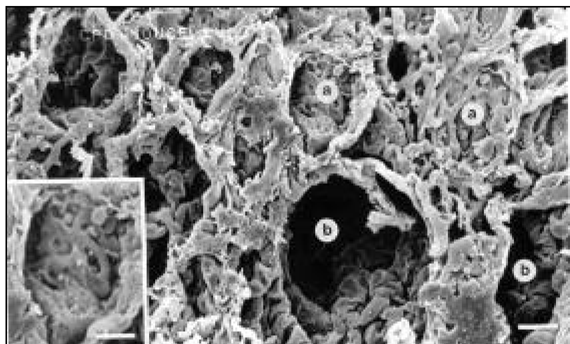


Figure 12a bar -5um, inset 10um

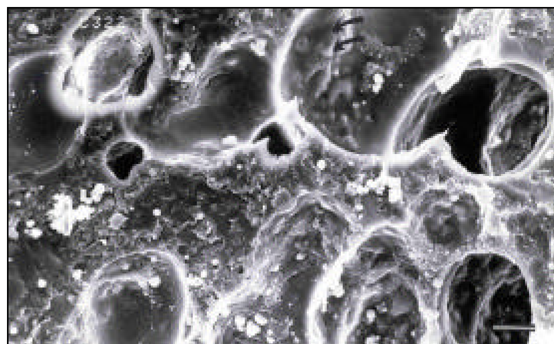


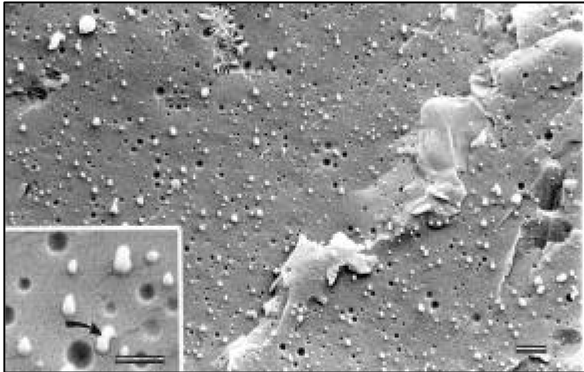
Figure 12b bar = 5um.

Figure 12a: Mouse Lung - Critical-Point Dried.
The lung consists of a mass of spongy alveoli where gaseous exchange occurs between blood and air. These are connected to the trachea via small airways called bronchioles. Figure 3a illustrates a mass of alveoli (a) associated with alveolar ducts into which the bronchioles open (b). A mass of capillaries are associated with the alveoli. The walls of the alveoli (septa) are composed of epithelial cells, connective tissue and elastin. The walls are generally one cell thick and highly hydrated. Consequently conventional preparation methods such as dehydration followed by critical point drying cause collapse and shrinkage of this very delicate tissue.

Figure 12b: Mouse Lung - Frozen Hydrated.
Low temperature SEM retains all of the cellular and extra-cellular fluids in the lung tissue. Consequently, the micrograph clearly illustrates that the bronchioles are covered with a thin layer of mucus (arrows). None of the alveoli show any collapse.

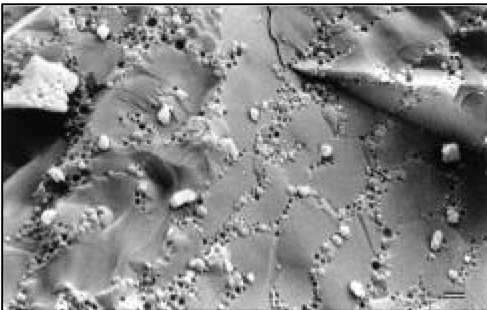
Materials

Figure 13: uncured epoxy resin



Many epoxy resins use “crack-stoppers” (shown here as white particles). Note that during the cold fracture some of these “crack-stoppers” are removed, while others remain. Some are partially removed (see insert arrow).

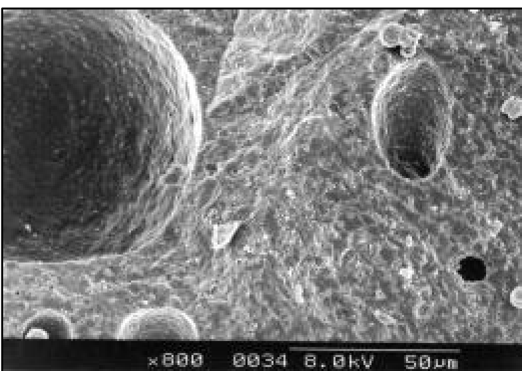
Figure 14: Rubber Particles in Natural Latex After Freezing



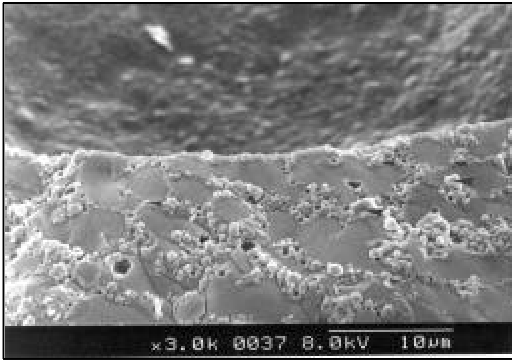
Natural latex consists of a suspension of rubber particles of approximately 0.1 - 1.0um in diameter, suspended in a buffered aqueous medium. One method for the manufacture of rubber - the Talalay process - includes a stage in which the latex is cooled to a temperature of -30°C. As a result of the crystallisation of water, the rubber particles are forced into a boundary around the ice, where partial coalescence occurs. Low temperature techniques such as LTSEM are ideal for monitoring this process.

Food stuffs

Figure 15a & 15b. Ice Cream



Despite starting as a liquid, ice cream is a solidified foamed emulsion containing air as the primary disperse phase. The air content of ice cream varies between products but is generally about 50%, with a total fat content of 10 -12%.
At low magnification air bubbles of a variety of diameters are visible; dispersed amongst a frozen emulsion. The ice crystal diameter is between about 5 and 10um.



At higher magnifications fat globules (small arrows) of a variety of sizes (between 200nm and 2um) are visible both at the gas/ice surface and also in the eutectic boundary between ice crystals. In addition, large angular lactose crystals are visible (larger arrows).
The texture and rheological properties of ice cream are directly related to structure, and in particular; the percentage and dispersion of fat, the ice crystal size and the size of air bubbles.

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6. Plunge freezing
7. Specimen transfer
8. View through window of PP2000T preparation chamber – showing cold stage, fracturing knife and cooled probe.
9. SEM cold stage and “anti-contaminator”