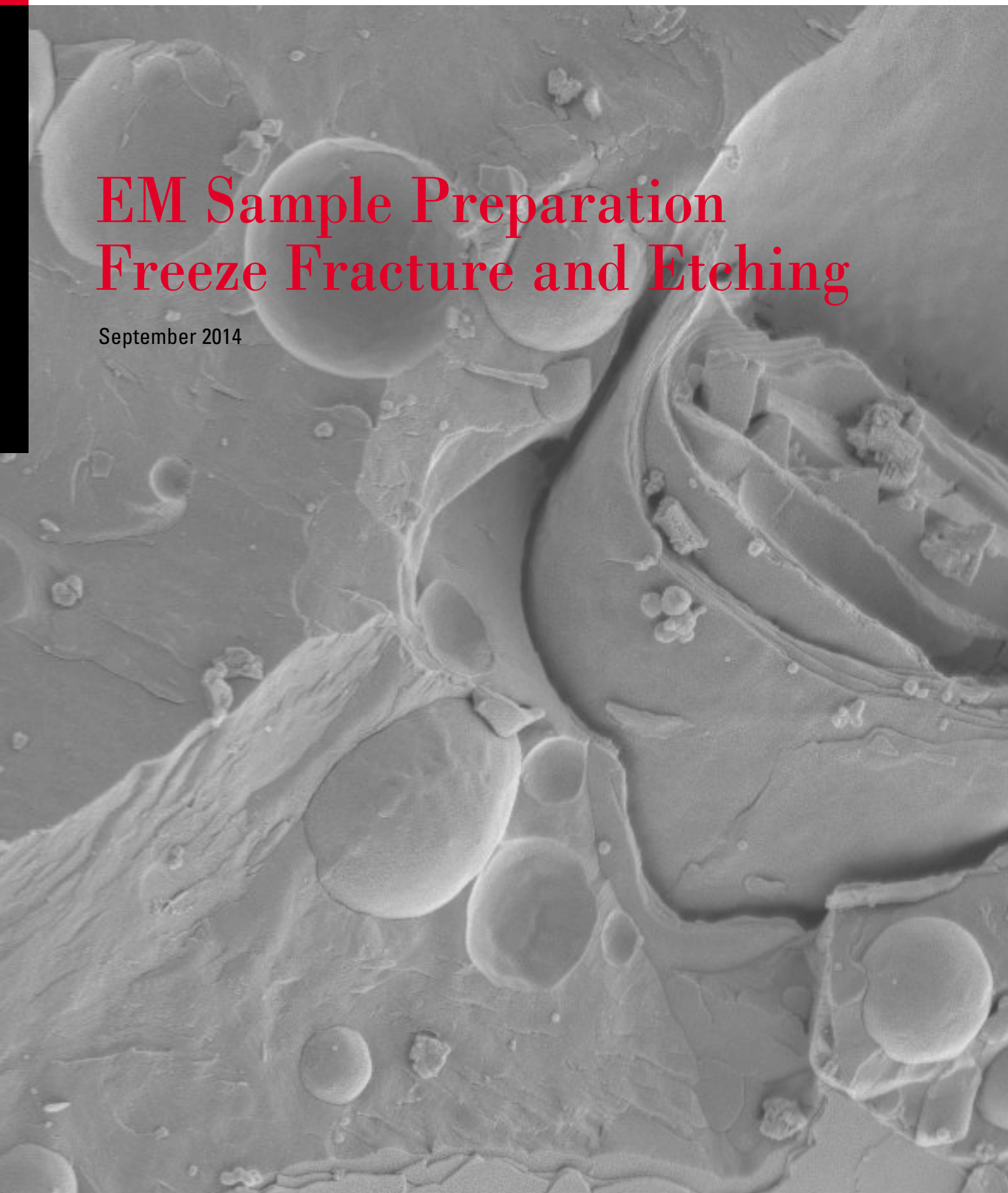
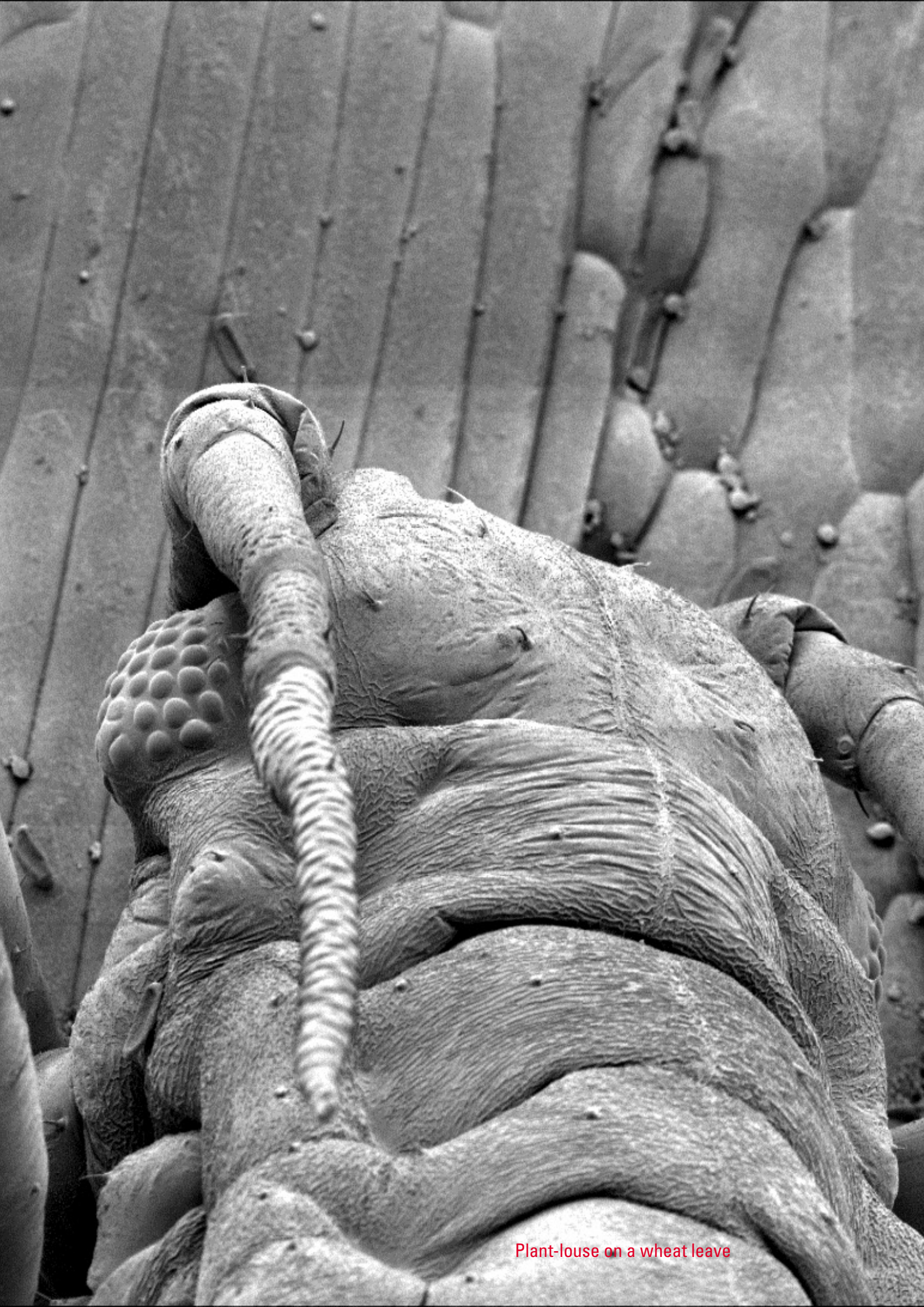
Living up to Life

Leica
MICROSYSTEMS

EM Sample Preparation
Freeze Fracture and Etching

September 2014





Plant-louse on a wheat leave

Freeze Fracture and Etching - a brief introduction

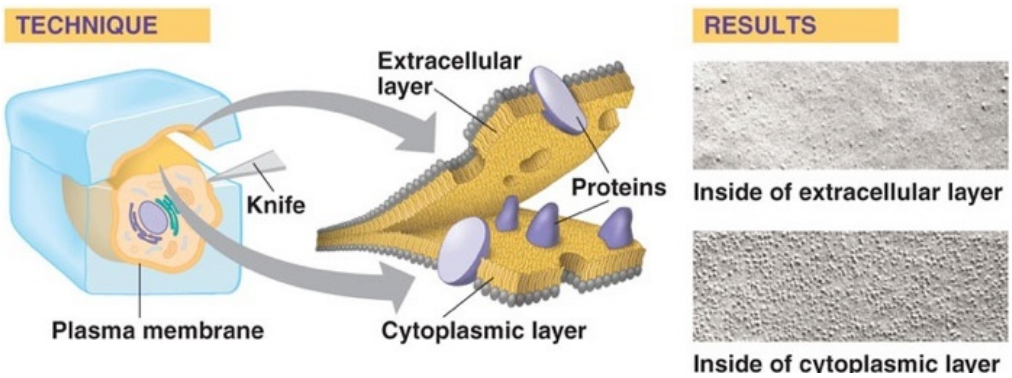
Freeze fracture describes the technique of breaking a frozen specimen to reveal internal structures. Freeze etching is the sublimation of surface ice under vacuum to reveal details of the fractured face that were originally hidden. A metal/carbon mix enables the sample to be imaged in a SEM (block-face) or TEM (replica). It is used to investigate for instance cell organelles, membranes, layers and emulsions. The technique is traditionally used for biological applications but started to develop significance in physics and material science. Recently, freeze fracture electron microscopy, particularly freeze replica immunolabelling (FRIL), has provided new insights into the roles of membrane proteins in dynamic cellular processes.

Fit for the environment of an electron microscope

The chamber of an electron microscope is evacuated to a very low pressure. The structure of a living cell placed into this environment cannot be preserved due to the extremely quick evaporation of the water which makes most part of the cell.

There are a number of preparation possibilities for biological samples. The material could be preserved (fixed) so the subsequent dehydration produces minimal damage to the in vivo structure, an environmental SEM could be used or the water could be frozen. High pressure freezing is the only way to observe hydrated structures in their natural state. The ice built by high pressure freezing is not hexagonal ice, which shows an increase of volume from water to ice but amorphous ice, where the volume stays constant. Therefore structures which are sensitive to osmotic and temperature change are preserved (see the article "Brief introduction to High Pressure Freezing").

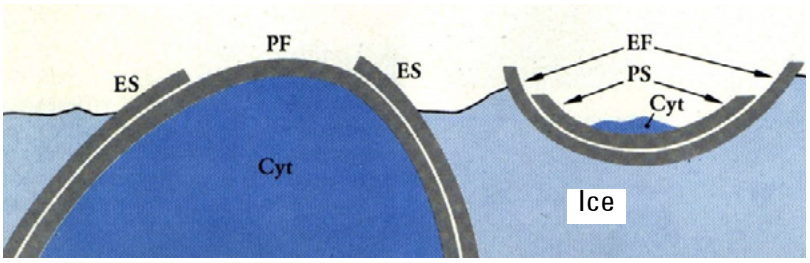
To observe structures such as cell organelles, membranes, emulsions or surface interfaces of liquids, freeze fracture is the only way to do that. The frozen sample is broken by force of a knife (or similar) or a released spring load and breaks along the lines of least resistance.



Source: http://en.wikibooks.org/wiki/Structural_Biochemistry/Lipids/Membrane_Fluidity

Sublimation and condensation of water - Freeze etching and contamination

To reveal details of the fractured surface , ice has to be removed. This needs to be done by sublimating the ice to preserve the structure of the specimen. Ice is directly transformed into water vapour without going through the liquid state which would lead to a change in volume and structural damage.

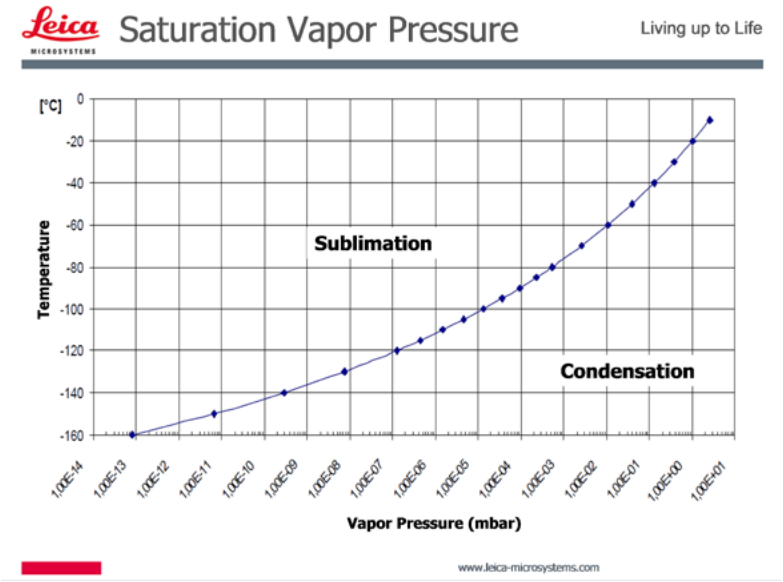


ES...Exoplasmic surface
PF...Plasmatic fracture face
EF...Exoplasmatic fracture face
PS...Plasmatic fracture face
Cyt...Cytoplasma

The sublimation/condensation process of water depends on the saturation pressure at a particular temperature and the effective partial water pressure of water or ice in the chamber. Note: a good vacuum reduces the partial water pressure.

For example: Ice or frozen specimens with a temperature of -120°C have a saturation pressure of about 10-7 mbar. If this pressure is established in the chamber, condensation and evaporation are in equilibrium. The amount of evaporated molecules is equal to the amount of condensed molecules. At a higher pressure the condensation rate is higher than the sublimation rate – ice crystals grow on the specimen's surface. This has to be avoided by all means. A colder (than the specimen) plate above the specimen reduces the local pressure and works as a condensation trap. Water molecules driven up from the specimen preferentially attach to the colder surface. At a lower pressure than the saturation pressure more molecules sublimate than condensate and freeze etching takes place.

Performing freeze etching until the sample is completely ice free, is called freeze drying. This process only works for small samples to be performed in a reasonable time. It is done in several steps by heating up from around -120°C to -60°C maintaining the temperature of each step for a certain time. This can take up to days.



Umrath 1982

At specimen temperatures below -120°C the etching rate is very low, etching times increase to impractical durations. If the pressure of the vacuum chamber is fixed, it is possible to increase the etching rate by raising the specimen temperature. Careful with temperatures higher than -90°C for biological samples. Etching rates increase tremendously. Additionally hexagonal ice forms from the vitrified ice and causes dehydration artefacts.

The theoretical sublimation rates of pure water are reduced because:

- Water from the depth of a specimen sublimates slower than water from the surface.
- Solvents of salt and macromolecules reduce the sublimation speed.
- The bound water which is a considerable part of biological samples have a lower sublimation rate.

Freeze fracture to generate images

Freeze-fracture and freeze etching techniques require ultrathin heavy metal and carbon films deposited under vacuum on the fractured surface.

Freeze fractured samples are coated under an angle with metal followed by a carbon backing up film (Leica EM ACE600 freeze fracture or Leica EM BAF060 with Leica EM VCT100) to produce either a replica to be imaged in a TEM or a block face for the SEM.

For both methods the fractured surface is coated after a certain amount of etching time the same way. First a thin (2-7nm) heavy metal coating under an angle to produce topographic contrast (shadowing). Second a thick carbon layer (15-20nm) coated under 90° to stabilize the ultra-thin metal film. At this point the etching process is stopped. To image very small structures the heavy metal is applied in a very low angle (2-8°) and the sample is rotated during coating. This adds contrast to filamentous and small structures. Such technique is called low angle rotary shadowing.

E-beam evaporation should be used for the heavy metal film. This is the coating technique giving a very fine and directional deposition. The supporting layer of carbon stabilizes the structures which are uncovered by metal. Those structures would change their contour during the increase of temperature, the sample would not be completely conductive and a replica would not stick together.

Replica for TEM

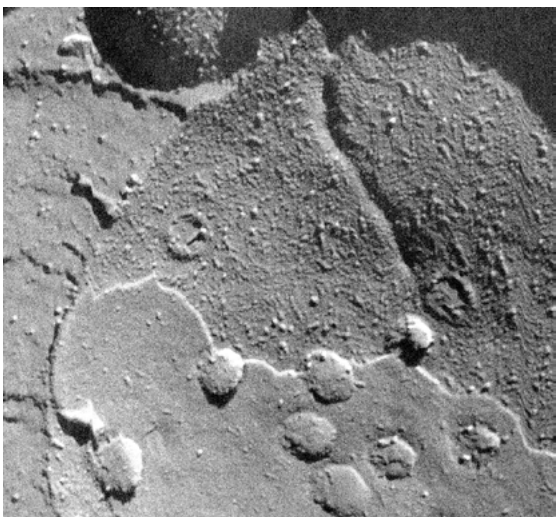
After the coating process the biological structure is washed away with acids and cleaned in distilled water. Only the replica made of metal and carbon is left. It is placed on a TEM grid and transferred into the microscope.

Block-face for SEM

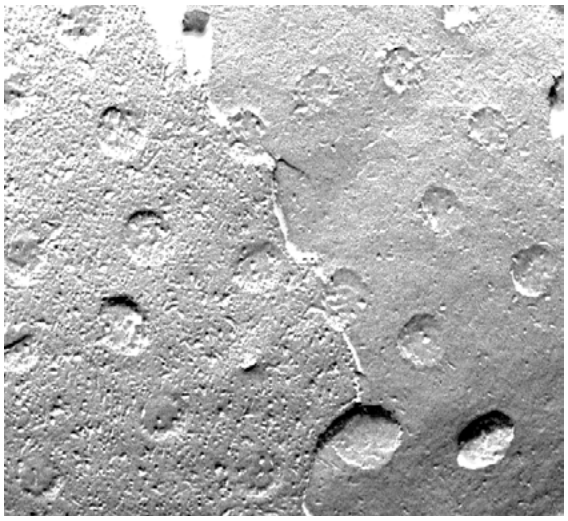
After the coating process the sample is kept under cryogenic conditions and transferred into a cryo- SEM. It is very important to keep the sample under constant temperature otherwise artefacts can be introduced in this last step. The Leica EM VCT100 is ideal to transfer the frozen specimen from the coater into the SEM via the transfer shuttle.

Unidirectional shadowing of freeze fractured yeast

Cryo SEM, BSE (back scattered electron) image



Replica, TEM image



Electronmicroscopy ETH Zürich

Applications

Image 1:
HPF, EM VCT100 transfer to the EM BAF060 for freeze etching and cryo coating using the electron beam gun and rotating specimen holder. EM VCT100 transfer to the cryo SEM.
Deo formula on an oil/water base, approx. 3 minutes by -100°C (sublimation) exposing lipid layers (rough texture wafer + emulsifier).
Courtesy of: Dr. Stefan Wiesner, Beiersdorf, Hamburg

Image 2:
HPF, EM VCT100 transfer to the EM BAF060 for freeze-fracture/freeze-etching and cryo-coating using the electron beam gun and rotating specimen holder. EM VCT100 transfer to the cryo SEM. Pennate diatom from a mixed culture of the protist Euplotes.
Courtesy of: Dr. Roland Fleck, NIBSC, Potters Bar, Uk.

Image 3:
HPF, freeze fractured, freeze etched and cryo caoted with the EM BAF060/EM VCT100 transfert to the cryo SEM.
Oil/water emulsion fractured exposing onion-like composition of lamellae forming a droplet.
Courtesy of: Dr. Stefan Wiesner, Beiersdorf, Hamburg

Image 4:
Yeast cell replica in TEM
High pressure frozen in gold specimen carriers and freeze-fractured in EM BAF060.
Courtesy of: Elektronenmikroskopie ETH Zürich

Image 5:
Funghus on Barley Leafs
Mounted on the BAF060 specimen table and frozen via the cooled specimen table in liquid nitrogen. EM BAF060 partially freeze dried (freeze drying at higher specimens temperatures). Coated with tungsten. EM VCT100 transfer to the cryo FESEM 5keV.

Related Instruments: Leica EM BAF060 /Leica EM VCT100

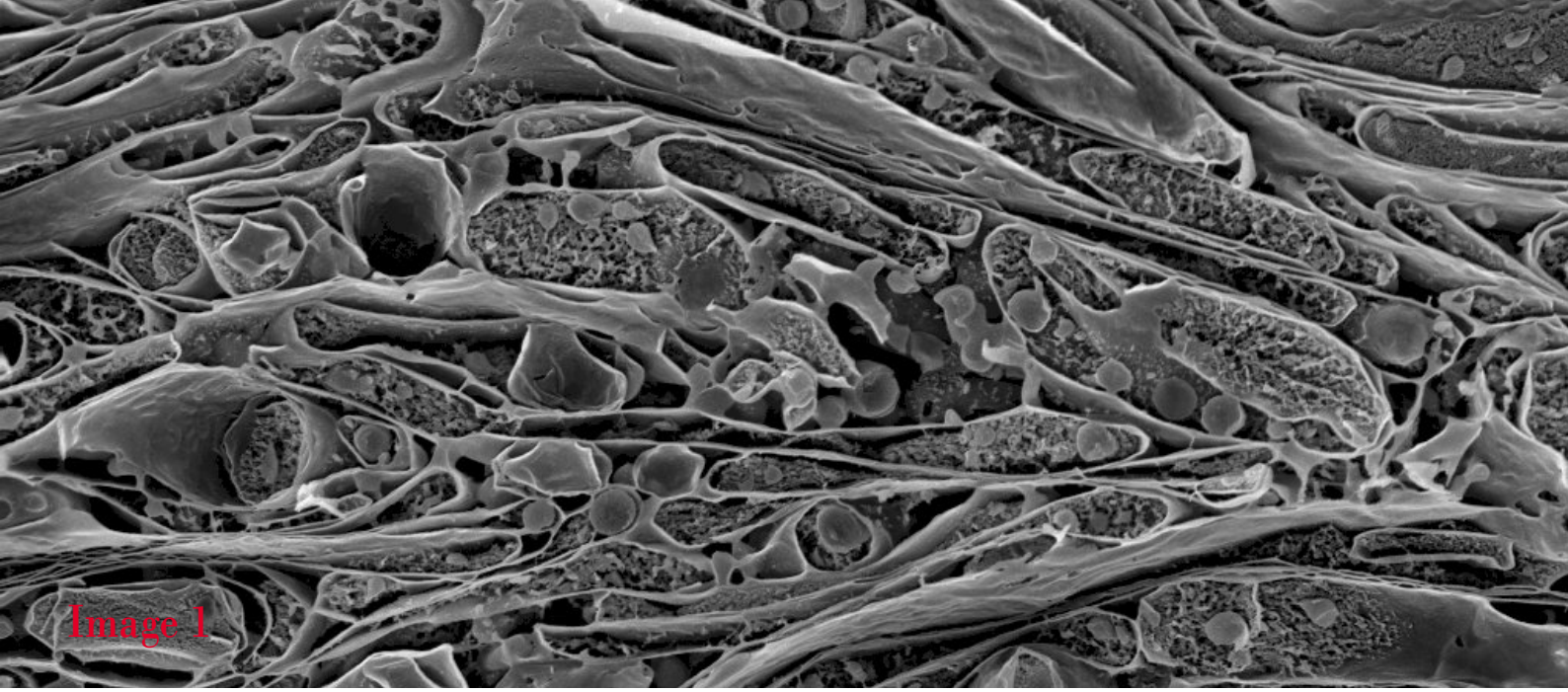


Image 1

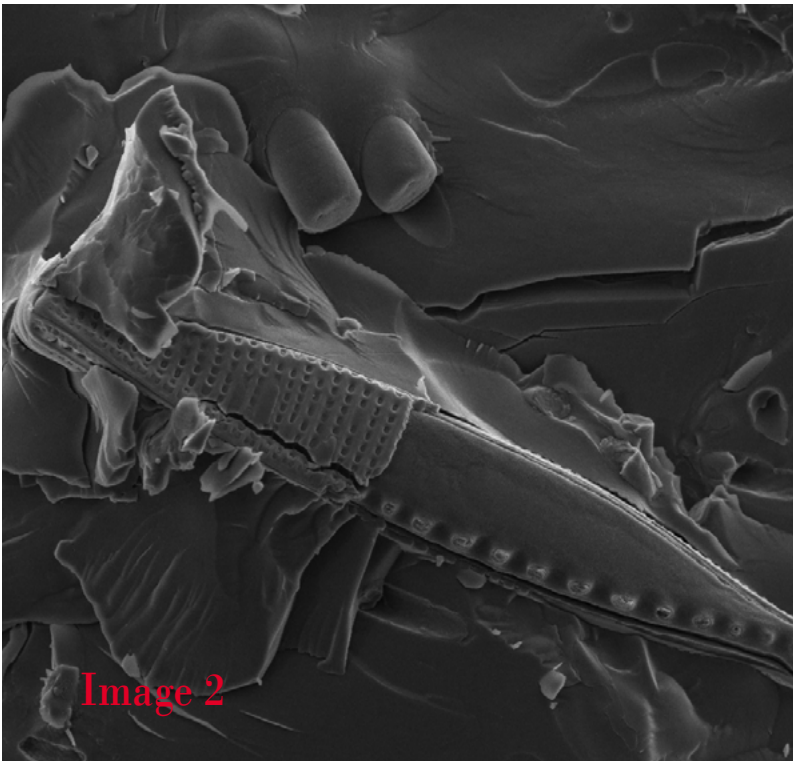


Image 2

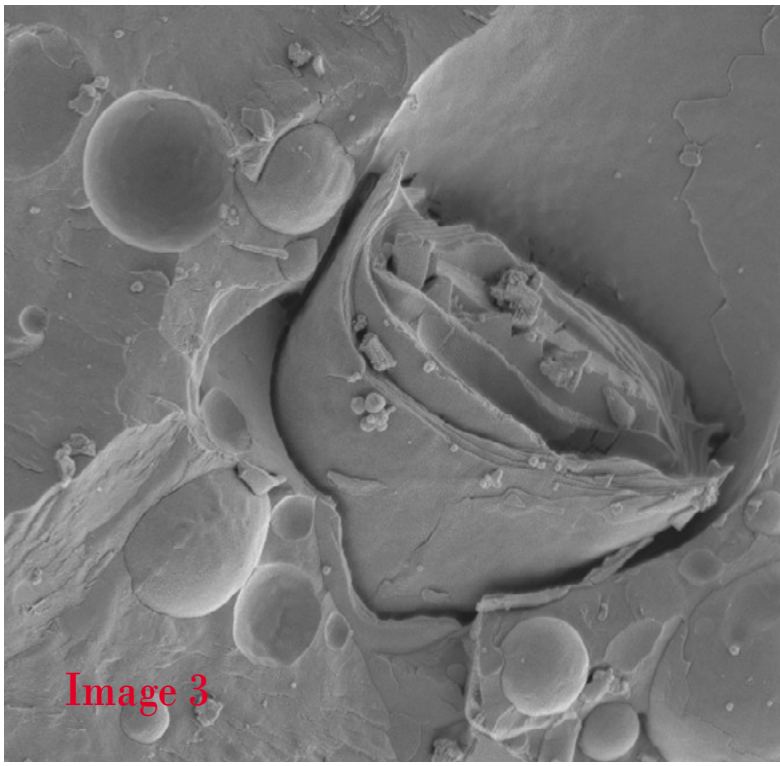


Image 3

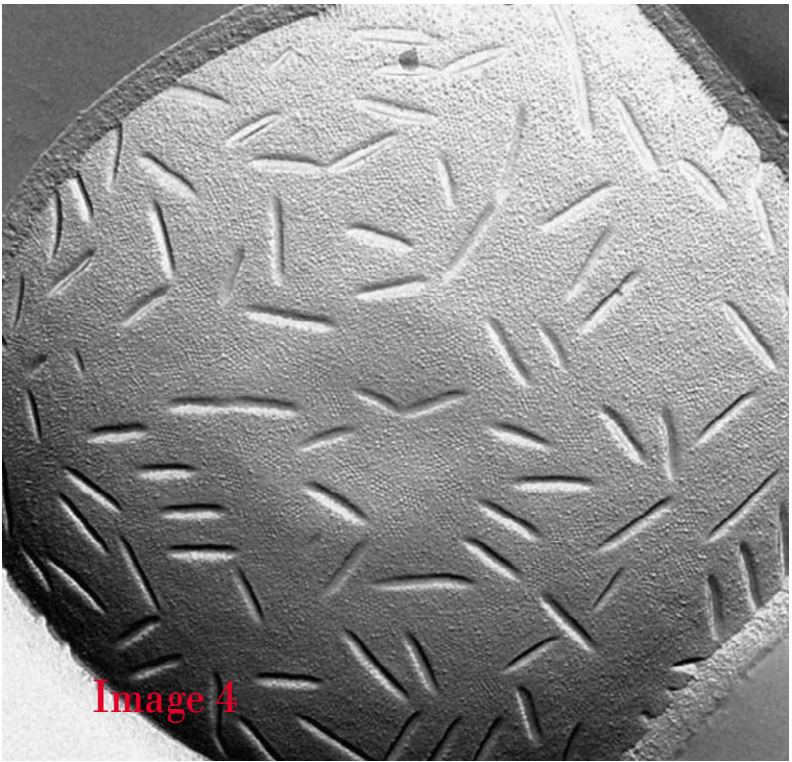


Image 4

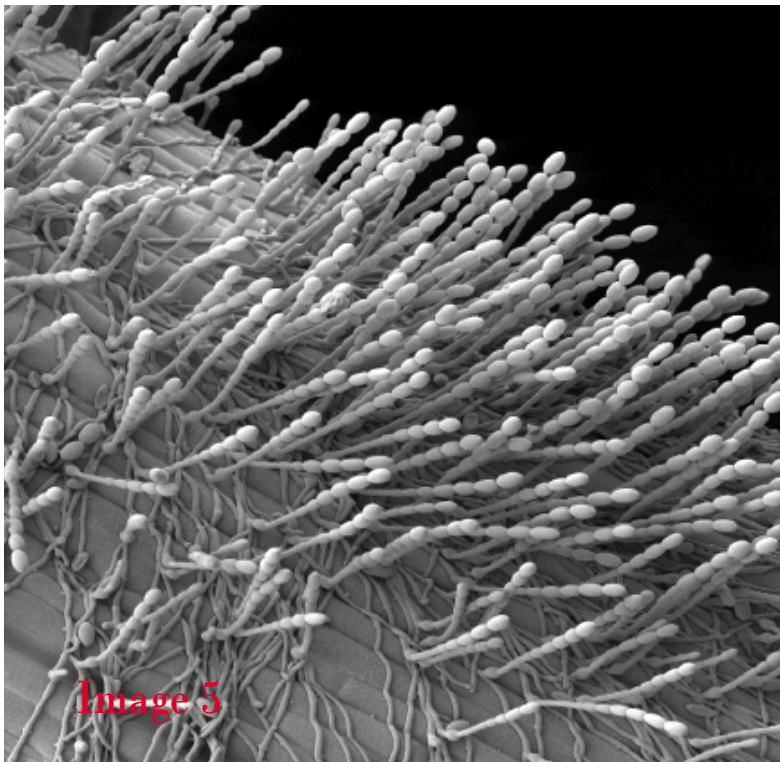


Image 5

Leica EM BAF060

The Leica EM BAF060 is a fully automatic high-end preparation unit for:

- freeze fracturing
- freeze etching
- freeze drying
- double replica (mirror fracturing)
- high resolution carbon/metal mix coatings for TEM/SEM analysis
- specimen replication by electron beam evaporation
- double layer coating of specimens for cryo SEM analysis
- cryo coating for cryo SEM using the EM VCT100 vacuum cryo transfer system

Visit the Website: Leica EM BAF060

Publications

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Leica EM VCT100

Setup Advantages and Key Features

- Contamination-free transfer between preparation and analysis unit
- Unique shuttle system for the vacuum cryo transfer of specimens
- Special shuttle and load-lock design maintains resolution of the SEM, vibration free
- Suitable for either room or cryo transfer
- Versatile protective gas transfer such as argon to prevent oxidation
- Preparation and analysis can be performed at different locations
- Space saving design offers minimal interference with the analysis system
- Sample preparation and analysis can be performed independently without interruption of either process
- Possible to repeat preparation with the same specimen
- Adaptable to more than one SEM
- Preparation units can be linked to several Leica EM VCT100 adapted analysis units such as the EM ACE200, EM ACE600 or the EM BAF060

Visit the Website: Leica EM VCT100

Publications

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www.leica-microsystems.com

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Leica EM BAF060

Leica EM VCT100

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