

Solutions for Innovation

CryoNote

Cryo History

A wide range of Cryo-techniques

Freezing

Sectioning / Fracturing

Etching (Ice sublimation)

Cryo-TEM

Freeze Substitution

Freeze Replication

Cryo-SEM

Low-Vacuum SEM and Cooling with Peltier element

Cryo-FIB

Cooling CP (Cryo-CP)

CryoNote

Introduction

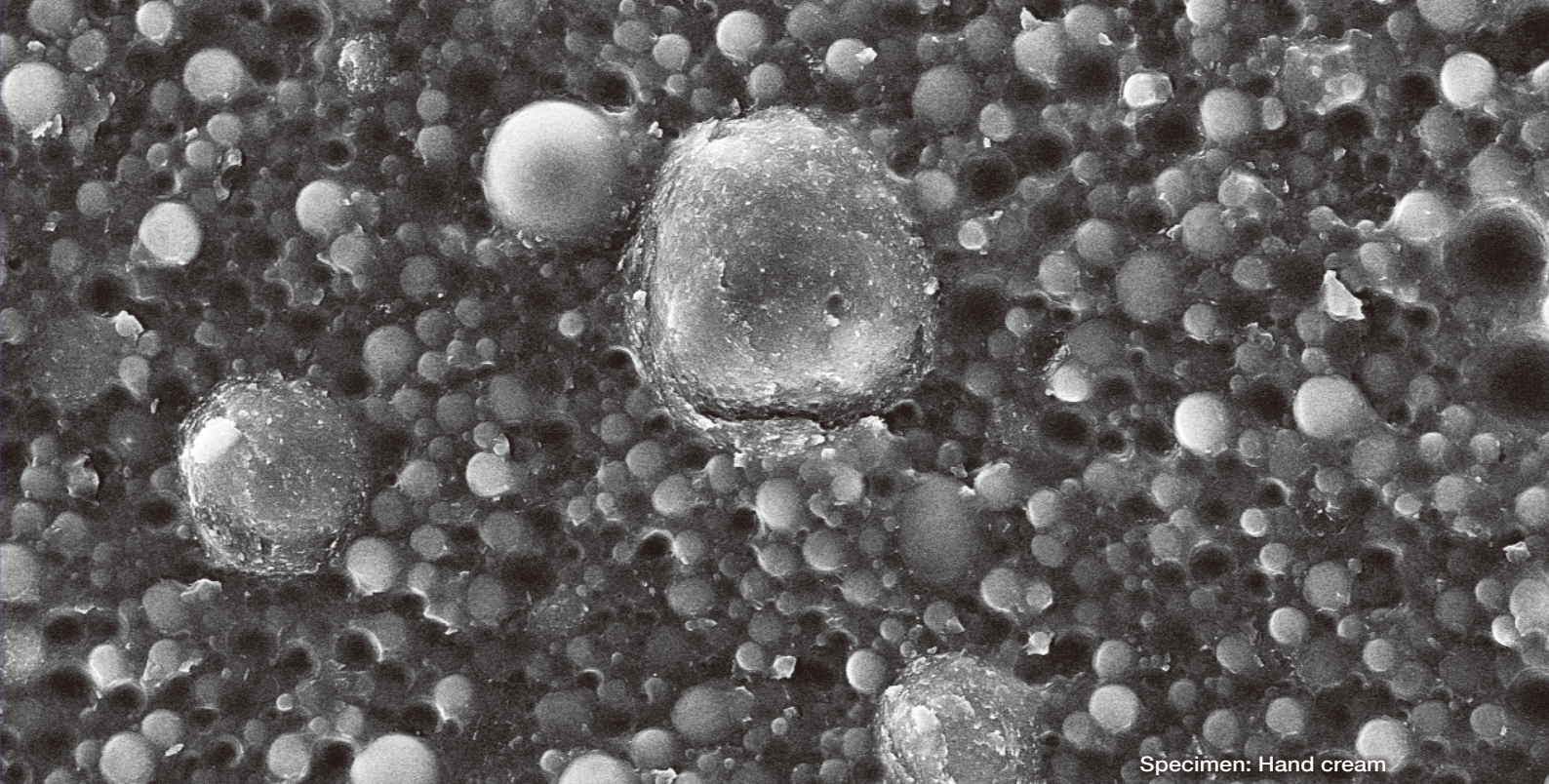
“Visualize the truth” is a hope of researchers who use various measuring equipments. Researchers who use electron microscopes as well have a desire to observe the real structure.

But actually, in experiments using electron microscopes, many problems arise: They include damage regions of the specimen when it is cut for the size suited to observation, artifacts due to the staining that is applied to enhance image contrast, deformation caused by substitution of water to resin for withstanding vacuum exposure, and thermal damage to the specimen with electron-beam irradiation. As a result, the visualization of the real structure in the microscope image becomes increasingly difficult.

One recommended solution is to cool the specimen, that is, “Cryo” techniques. This “Cryo Note” introduces some of the diversified cryo-techniques. We sincerely hope your challenge to observe the “real structure” will be solved by “Cryo” methods.

Cryo History

- 1932** First transmission electron microscope (TEM) designed and built by Ernst Ruska.
- 1939** First commercial TEM released by Siemens.
- 1949** Japan Electron Optics Laboratory (present JEOL) released its first commercial TEM, JEM-1.
- 1952** Freeze substitution technique developed by Fernández-Moán.
Rapid freezing technique (Plunge freezing) developed by Glick and Malmstrom.
- 1957** Freeze replication technique (Freeze-etching-replica) developed by Steere.
- 1963** With Freeze fracturing technique (Freeze etching), Moor and Mühletaler observed yeast.
- 1964** Metal mirror freezing (metal contact) developed by van Harrevelt and Crowell.
- 1965** First commercial SEM (named MK1 Stereoscan) released by Cambridge Instruments.
Freeze sectioning technique developed and released by Bernhard.
- 1966** JEOL released its first commercial SEM, JSM-1.
- 1968** High pressure freezing-preparation tool developed by Moor and Riehle.
- 1970** With Cryo-SEM, Echlin and coworkers observed frozen biological specimens.
- 1973** Tokuyasu method developed by Tokuyasu.
- 1974** Freeze-etching (Freeze-fracture deep-etch method) developed by Nei.
- 1975** Structure analysis of Bacteriorhodopsin by Henderson and Unwin.
- 1982** Spray freezing developed by Dubochet and Homo.
- 1983** JEOL started development of Cryo-TEM.
- 1984** Studies on vitrified water emerge from Dubochet and Lepault.
- 1985** Studies on viruses in vitrified samples emerge (Adrian, et al.).
- 1986** Nobel Prize in Physics awarded to Dr. E. Ruska for invention of Electron Microscopes.
JEOL developed an advanced Cryo-TEM, JEM-4000SF.
Balzers released a high-pressure freezing instrument, HPM10.
- 1998** Controlled-Environment Vitrification System (CEVS) was developed by Talmon and coworkers,
which combines rapid freezing with a temperature control chamber.
- 2004** Dubochet coined sectioning of frozen-hydrated sections “CEMOVIS” (Cryo-Electron Microscopy Of Vitreous Sections).
Direct electron detector developed by Xuong and coworkers (imaging test was done on a JEOL JEM-1200).
- 2017** Nobel Prize in Chemistry awarded to Dr. J. Dubochet, Dr. J. Frank and Dr. R. Henderson for their advanced development of Cryo-Electron Microscopy.
- 2020** With Cryo-FIB-SEM, Kuba and coworkers reported serial observation of rapidly-frozen Chlamydomonas cross sections.



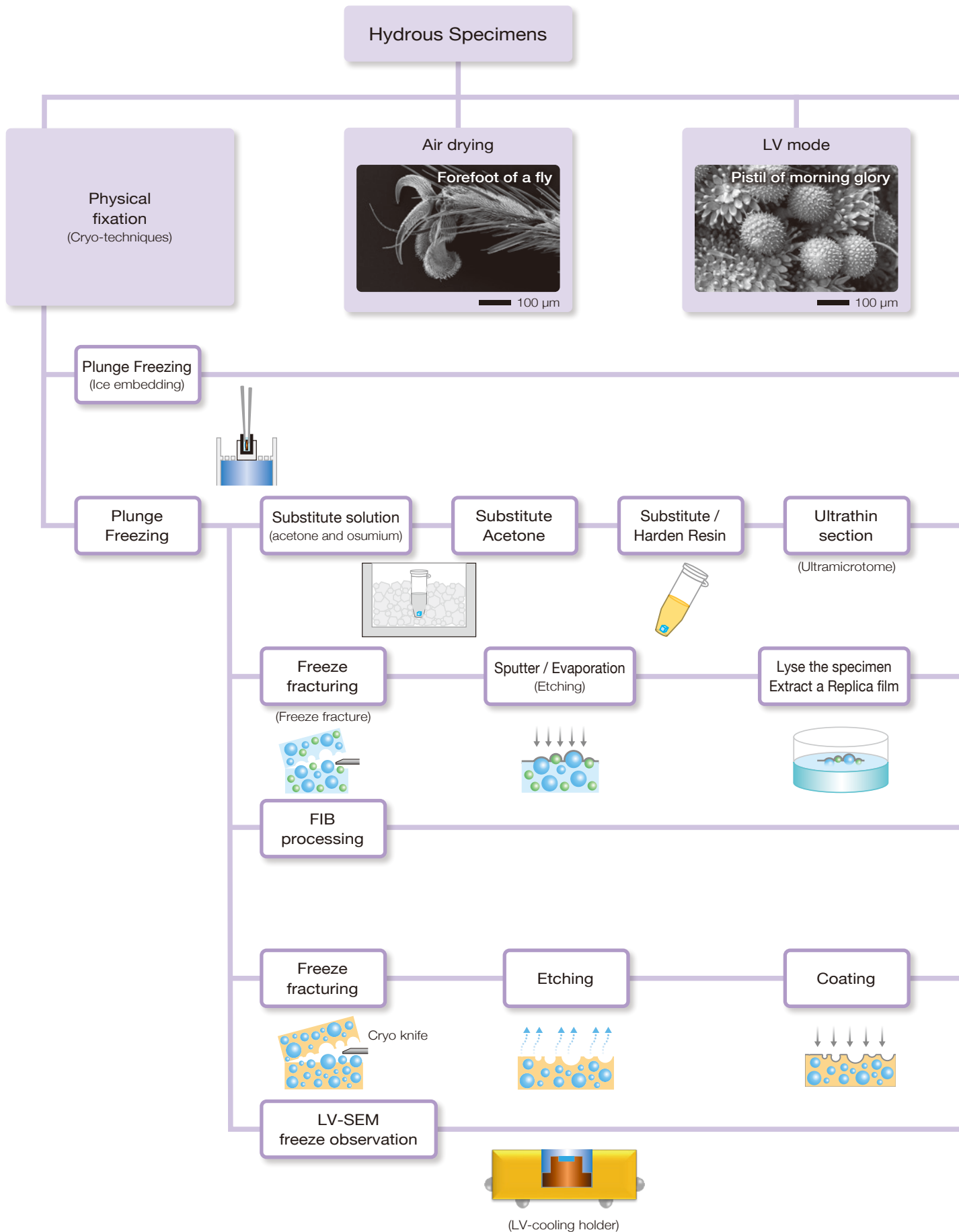
Specimen: Hand cream

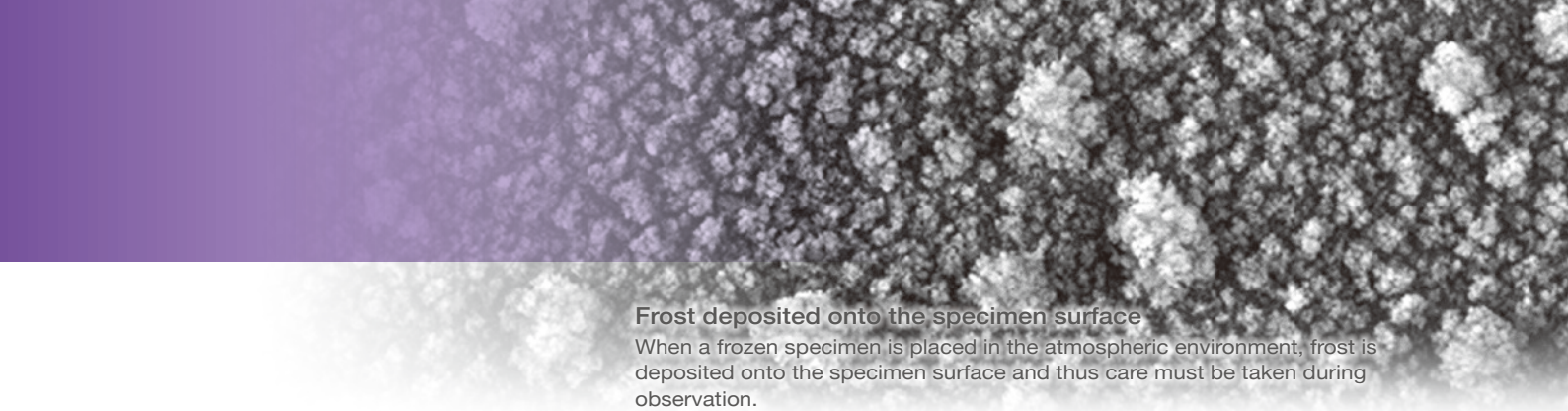
INDEX

Introduction	P1
Cryo History	P1
1 A wide range of Cryo-techniques	P3
2 Freezing	P5
2-1 Plunge Freezing (Rapid Freezing)	P5
2-2 Immersion	P5
2-3 Metal Mirror Freezing (slam freezing)	P6
2-4 High Pressure Freezing	P6
3 Sectioning / Fracturing	P7
3-1 Freeze Sectioning / Cryo-Microtomy	P7
3-2 Freeze Fracturing	P8
4 Etching (Ice sublimation)	P8
4-1 Etching	P8
5 Cryo-TEM	P9
5-1 Cryo-TEM	P9
5-2 TEM Applications using Cryo-transfer holder	P10
5-3 Cryo-TEM with Automated Specimen Transfer System	P13
5-4 Cryo-TEM Applications with Automated Specimen Transfer System	P14
6 Freeze Substitution	P15
6-1 Freeze Substitution	P15
7 Freeze Replication	P16
7-1 Freeze Replication	P16
Freeze fracturing	P16
Freeze etching (Deep etching)	P16
Cryo-extraction replication	P16
7-2 Freeze Replication: Applications	P17
8 Cryo-SEM	P19
8-1 Cryo-SEM	P19
8-2 Cryo-SEM Applications	P20
8-3 Simple Cryo-SEM	P24
9 Low-Vacuum SEM and Cooling with Peltier element	P25
9-1 Low-Vacuum SEM / Peltier Cooling Stage	P25
9-2 Low-Vacuum SEM / Peltier Cooling Stage / Wet Cover Method Application	P25
10 Cryo-FIB	P26
10-1 Cryo-FIB	P26
10-2 Cryo-TEM Observation with Cryo-FIB Milling	P27
11 Cooling CP (Cryo-CP)	P29
11-1 Cooling CP (Cryo-CP) Mechanism	P29
11-2 Cooling CP (Cryo-CP) Applications	P29

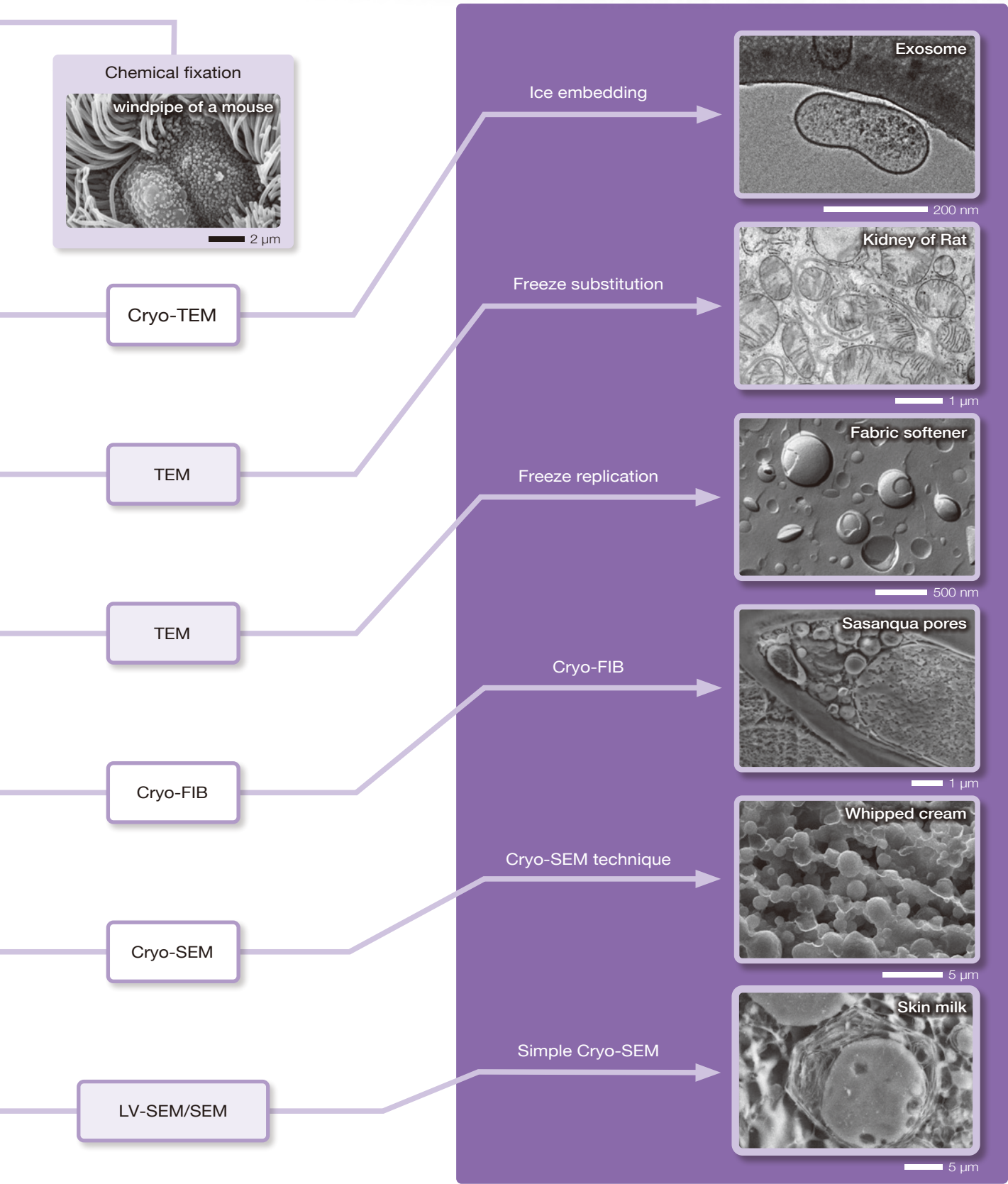
A wide range of Cryo-techniques

When you observe hydrous specimens with an electron microscope, you need to select the optimal specimen preparation technique suitable for the purpose of the observation. Cryo Note introduces to you a wide range of Physical fixations (Cryo-techniques).





Frost deposited onto the specimen surface
When a frozen specimen is placed in the atmospheric environment, frost is deposited onto the specimen surface and thus care must be taken during observation.

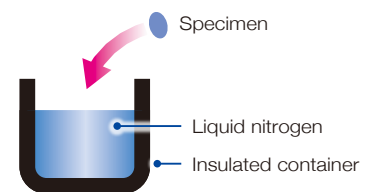


2 Freezing

2-1 Plunge Freezing

Plunge freezing is a method to rapidly freeze water-containing specimens, such as biomacromolecules, tissues and even living organisms, foods and paints, to keep them close to their native state for observation in a microscope and compatible with the vacuum required for imaging using electrons. If a specimen is slowly frozen in a refrigerator, or with dry ice, the specimen typically deforms due to water flow from the specimen, volume expansion of the ice, and ice-crystal formation. If the water specimen is rapidly frozen, these phenomena are suppressed and the native structure of the specimen is preserved. For this, a freezing rate of 10^4 °C/sec or better is required. Plunge freezing and the metal mirror freezing approaches typically achieve this.

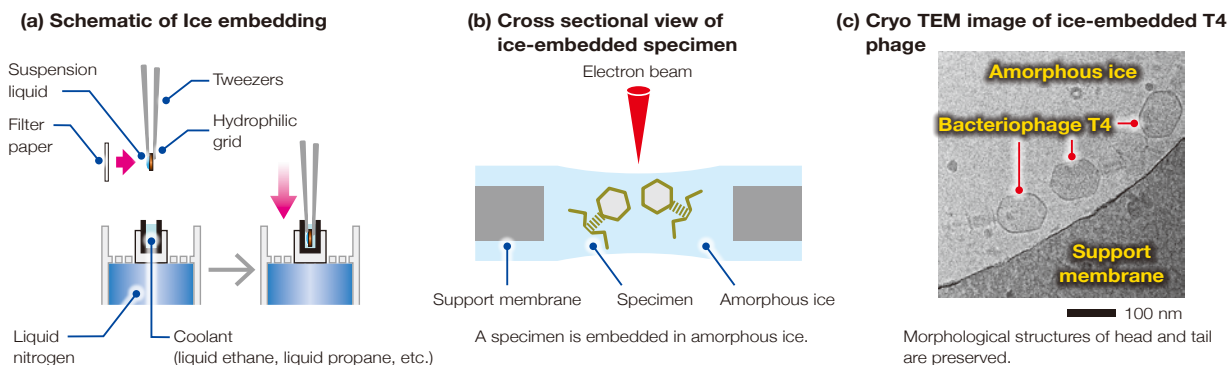
2-2 Immersion



Immersion is a method to introduce a specimen into a coolant that boils hardly. The coolants used include liquid ethane, liquid propane and slash nitrogen, any of which has a large difference between melting point and boiling point. In this case, the freezing depth which preserves the fine structure to be able to observe with an electron microscope, is 5 to 10 μm from the specimen surface.

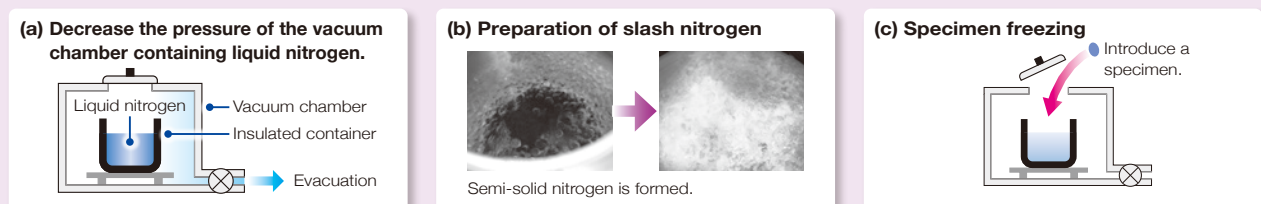
Ice Embedding with plunge freezing

For plunge freezing, a few μL of suspension liquid is placed onto a micro grid that has been subjected to hydrophilic treatment. Excess liquid is removed by blotting with filter paper, after which the specimen is rapidly frozen by plunging it into liquid ethane or propane (Fig. a). The ethane or propane is kept close to its freezing point using a secondary cryogen, liquid nitrogen. This technique embeds a suspended specimen of biological macromolecules (purified proteins, viruses) in an amorphous ice membrane with a thickness of several 10 nm to several 100 nm (Fig. b). It enables us to observe a molecular state of the specimen in a liquid solution (Fig. c).



Slash nitrogen

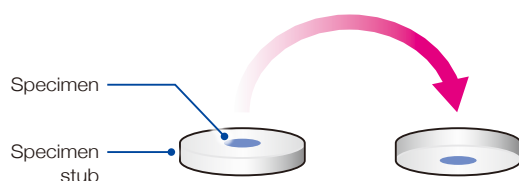
When a specimen is put in liquid nitrogen, instant boiling ensues with the resulting gas layer surrounding the specimen. The gas layer effectively insulates the specimen slowing down its cooling rate markedly. This is also known as the Leidenfrost effect. Thus, the specimen will slowly cool down, which results in crystalline ice growth. In contrast, slash nitrogen, a combination of liquid and solid nitrogen, will show far less boiling and thus more rapid cooling of the specimen is possible. Slash nitrogen is obtained by evacuating the gaseous nitrogen using a rotary pump (Fig. a). Vigorous boiling ensues as the pressure drops, which in turns draws heat from the liquid nitrogen until ultimately solid nitrogen is formed (Fig. b). Note that nitrogen boils at -195.7 °C and solidifies at -210 °C. Once normal pressure is restored samples can be introduced into the slash for freezing (Fig. c).



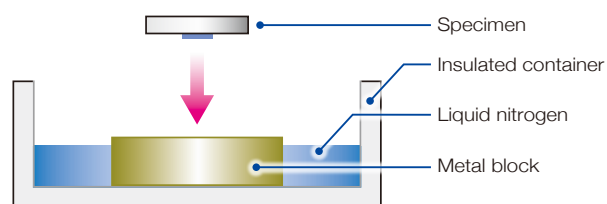
2-3 Metal Mirror Freezing (Slam freezing)

This method slams a specimen against a metal block cooled by liquid nitrogen or liquid helium. In many cases, high-thermal-conductivity, high-purity copper with gold plating is used for a metal block. To increase the temperature drop efficiency of the specimen, the surface of the metal block is prepared into a flat mirror-surface. This method enables freezing using a relatively inexpensive device, and the freezing depth suitable for electron-microscope observation, about 20 μm from the specimen surface, is achieved. Compared to immersion and high-pressure freezing, this method allows for wide-area freezing. After the freezing in TEM, the frozen specimen is subjected to one of the following three procedures for subsequent observation. (1) Freeze sectioning to the specimen, (2) Freeze substitution followed by resin embedding and ultrathin sectioning to the specimen at room temperature, and (3) Freeze fracturing followed by low-angle shadowing to form a surface replica of the specimen. In SEM, frost removal by etching is applied to the specimen for subsequent observation.

(a) Thinly paint a specimen onto a specimen stub and reverse the stub.



(b) Vertically punch a specimen against a cooled metal block.

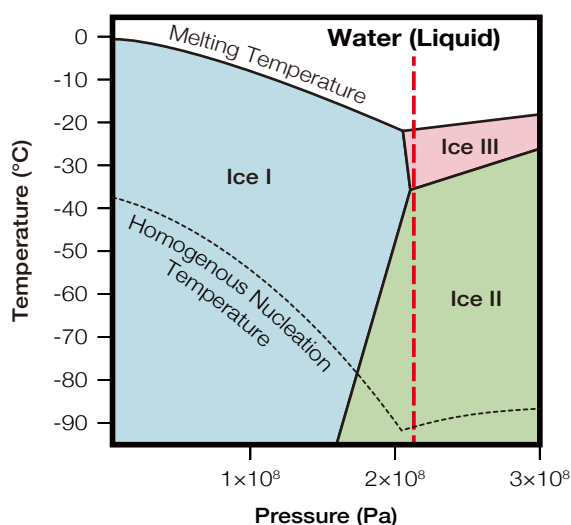


* The use of a dedicated device facilitates vertical punching.

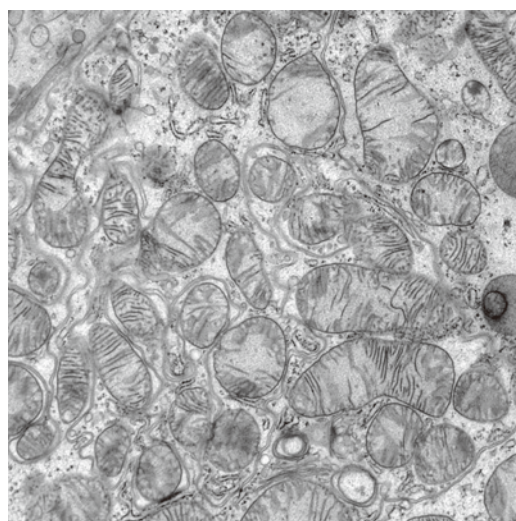
2-4 High Pressure Freezing

This method freezes a specimen with liquid nitrogen under a high pressure of approximately 2.1×10^8 Pa applied by an air compressor. The melting point of water decreases near this high pressure (red dotted line in the lower left figure) and the viscosity of the water increases. Thus, even when the time is spent for freezing, ice-crystal formation is suppressed, thus minimizing tissue destruction. This feature allows observation of a large specimen in the native state of the specimen up to depths of a few hundreds of micrometers.

Diagram of water state



Rat kidney



Specimen: Rat kidney
High pressure freezing, Freeze substitution
Staining with uranyl acetate and lead citrate
TEM observation

1 μm

3 Sectioning / Fracturing

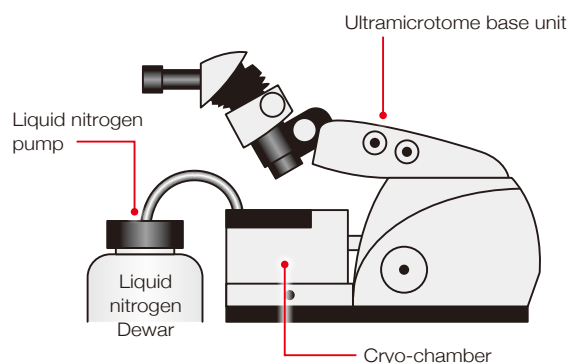
3-1 Freeze Sectioning / Cryo-Microtomy

An ultramicrotome is a specimen preparation tool for making ultrathin sections of vitrified specimens for TEM observation. Using this tool, a specimen block set out with a preset feed rate (several 10 nm to several 100 nm) is sliced into ultrathin sections by moving the block from top to bottom against the diamond knife.

Freeze sectioning is a method of sectioning by freezing a specimen which is soft and difficult in preparing an ultrathin section at room temperature, under liquid nitrogen temperature. Specimens prepared by this method include biological specimens without chemical fixation and high-polymers (rubber, etc.). A cryo-microtome equipped with a cryo-chamber and a liquid-nitrogen Dewar is used for freeze sectioning.

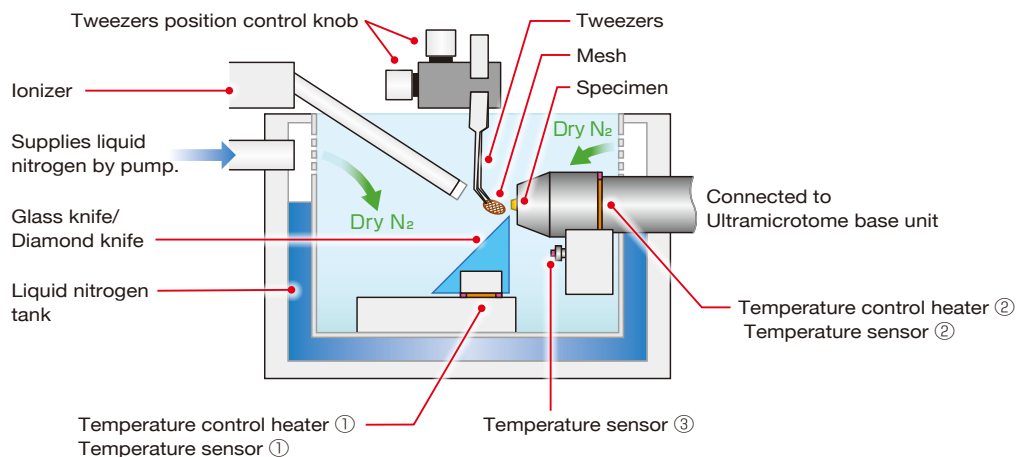
Preparation and collection of an ultrathin section is performed in the cryo-chamber filled with cold nitrogen gases. In the case of a biological specimen, the specimen does not suffer deformation caused by chemical fixation and dehydration

because the section is created from a frozen specimen prepared by rapid freeze fixation. Thus, this method allows for observation of tissues close to their living state. The use of a cryo-transfer holder enables ultrathin sections to be observed by TEM with the sections kept frozen.



Schematic of the cryo-microtome

Schematic of the Cryo-chamber



Liquid nitrogen tank: Liquid nitrogen is supplied automatically from pump.

Temperature control heater / Temperature sensor ① : Preserves the set temperature of Glass knife / Diamond knife.

Temperature control heater / Temperature sensor ② : Preserves the set temperature of the specimen.

Temperature sensor ③ : Detects the temperature in the chamber.

Specimen: Used to prepare ultrathin sections. A specimen block set out with a preset feed rate (several 10 nm to several 100 nm) is sliced into ultrathin sections by moving the block from top to bottom against the diamond knife.

Glass knife / Diamond knife: Knives to trim a specimen and prepare ultrathin sections.

Mesh: Picks up ultrathin sections.

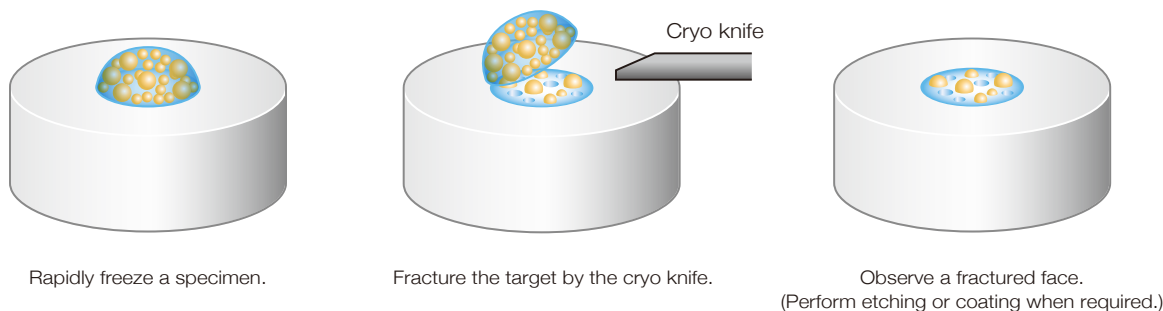
Tweezers: Preserves the mesh.

Tweezers position control knob: Adjusts the tweezers position so that the mesh is placed near Glass knife / Diamond knife.

Ionizer (static electricity control system): Assists collection of the sections using storage and discharge capabilities of static electricity.

3-2 Freeze Fracturing

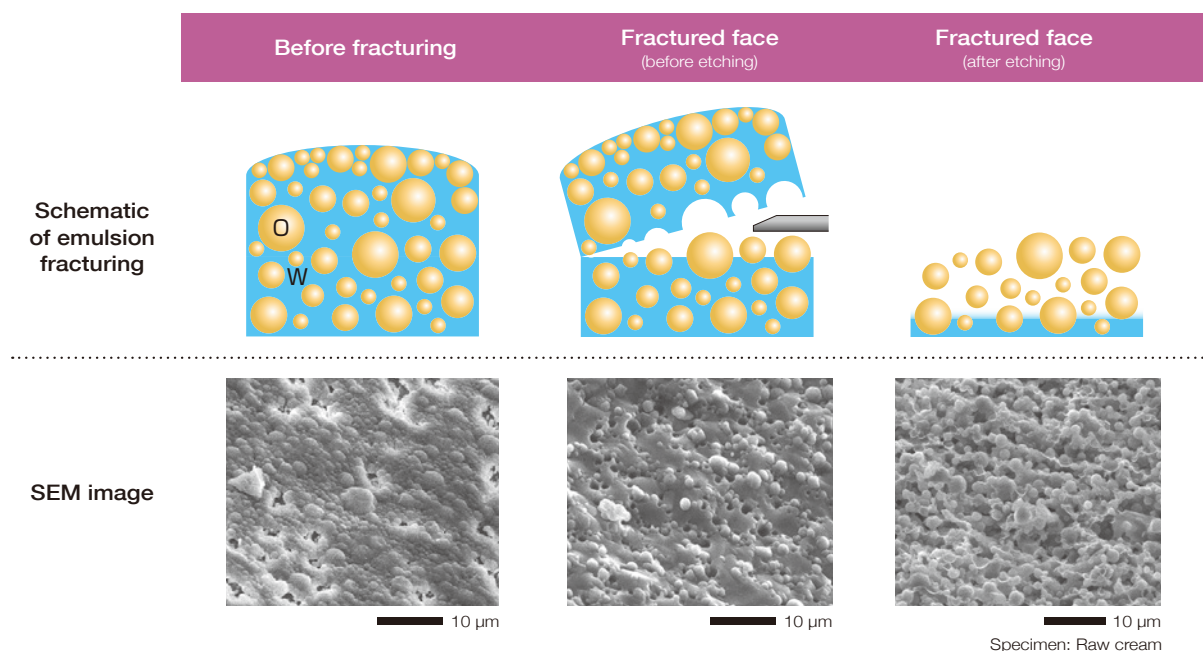
Freeze fracturing is a method to observe a fractured face of a frozen specimen. Depending on the specimen and research purpose, fracturing is performed by cutting protruding regions or pipes filled with the specimen, using a cryo knife. This method is effective for observation of the inner structure of the specimen and substances in liquid.



4 Etching (Ice sublimation)

4-1 Etching

Etching is a method which sublimates frost on the surface of a frozen specimen or ice inside the specimen, so as to expose and observe the inner structure of the specimen under the ice. The figures below show observation examples of oil-in-water emulsion; its surface, its fractured face, and its etched surface of the fractured face. Three images confirm the following phenomena. In the image before fracturing, fat balls collect on the surface. In the image of the fractured face (before etching), fat balls in water appear on the face. In the image of the etched surface (of the fractured face), distribution of fat balls in water is observed.



5

Cryo-TEM

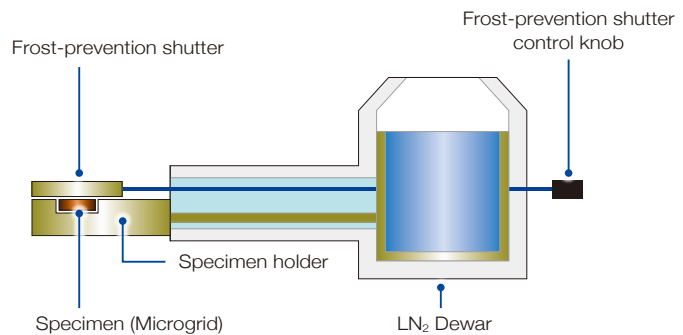
5-1

Cryo-TEM

“Cryo-electron microscopy” is a microscopy method used for the observation of biological specimens at the temperature of liquid nitrogen or liquid helium without staining. The specimen is subjected to a freezing technique, such as plunging or freeze fracturing, and the frozen specimen is introduced into the microscope column. Since biological specimens are mostly composed of light elements, scattering contrast is extremely weak to observe. Thus, the specimen is observed using phase contrast produced at defocus values ranging from less than 1 to several micrometers. Combining the omega filter (energy filter) with a phase plate leads to improvement of the image contrast. 3D structure analysis methods using cryo-electron microscopy include single particle analysis and tomography.

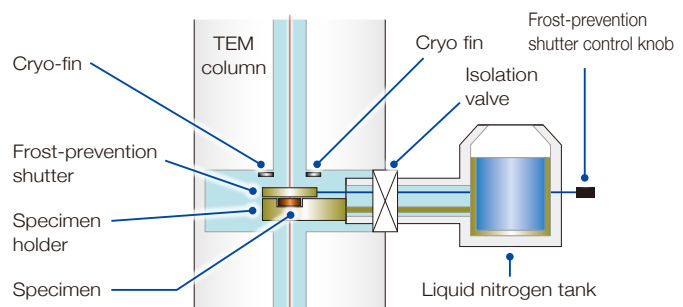
Cryo-Transfer holder

A Cryo-transfer holder is a holder that allows us to observe frozen specimens such as those prepared by plunging, at low temperature. The holder can be introduced into a TEM, SEM or FIB. The holder has a reservoir for LN₂ which maintains the tip of the holder close to liquid-nitrogen temperatures. When the holder is inserted into the electron-microscope column, the tip of the holder is exposed to air (though for a short time), thus the holder incorporates a shutter to prevent frost deposition onto the specimen.



Anti-contamination device

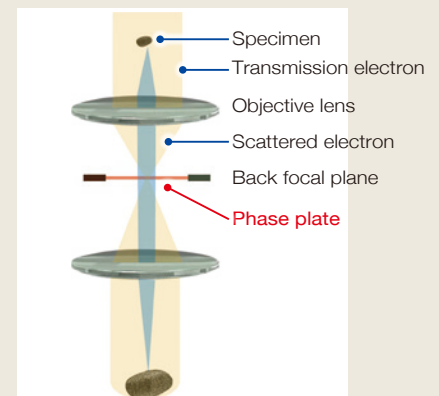
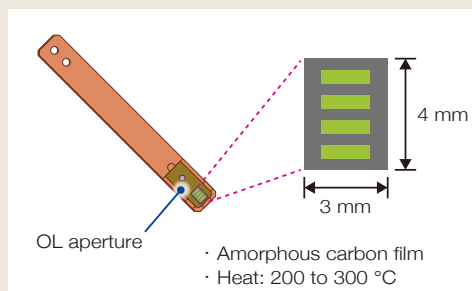
When a Cryo-transfer holder is mounted to the electron microscope, an Anti-contamination device or a cryo fin at lower temperature than the specimen is required. With this device, it helps the surface of specimen keep frost-free during observation.



Hole-free phase plate

A hole-free phase plate consists of a thin carbon film placed into the back focal plane of the objective lens. Unscattered electrons are focused onto this film, producing a charge spot that shifts the phase of the electron wave by 90°. Thus, low-contrast ice-embedded specimens can be imaged with near perfect contrast very close to Gaussian focus.

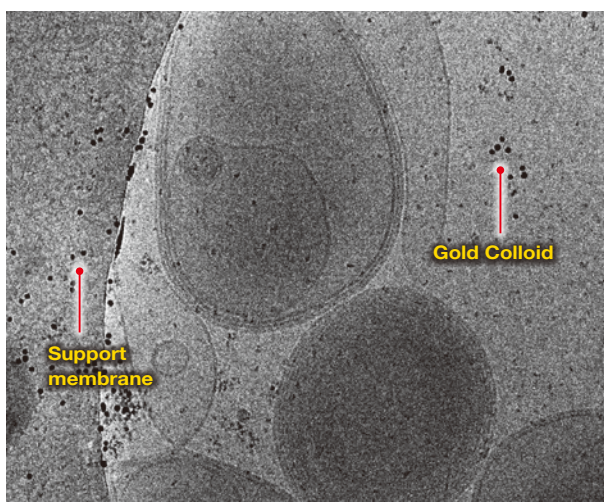
Hole-free phase plate



5-2 TEM Applications using Cryo-transfer holder

Liposome

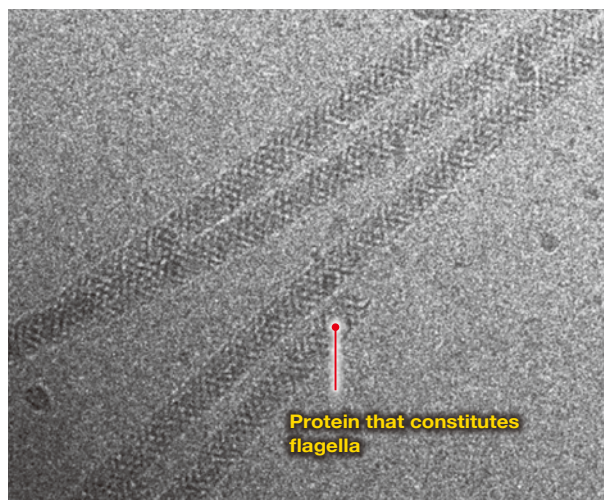
Liposomal particles composed of lipid bilayer membrane were observed at high-contrast.



Ice embedding Omega filter slit In 200 nm

Flagellar poly-hook

Long fibrous flagellum and protein, its composition element, protein, were observed at high-contrast.

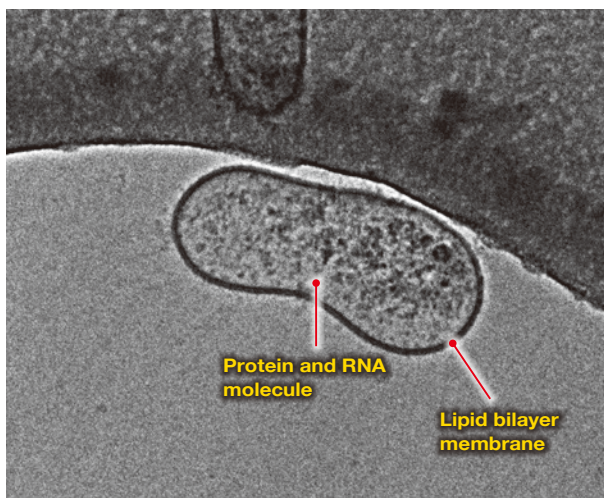


Ice embedding Omega filter slit In 100 nm

Fujii et al., Specific Arrangement of α -Helical Coiled Coils in the Core Domain of the Bacterial FlagellarHook for the Universal Joint Function. 2009, Structure.

Exosome

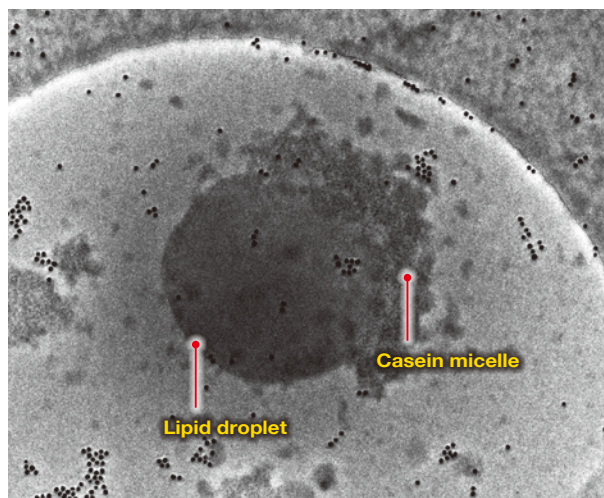
Protein and RNA molecule, which are encapsulated in a liposomal particle composed of a lipid bilayer membrane, are observed at high-contrast.



Ice embedding Phase Plate In 200 nm

Casein micelle

Casein micelle is observed at high-contrast, and that confirmed them existing around a bulb-shaped lipid drop.

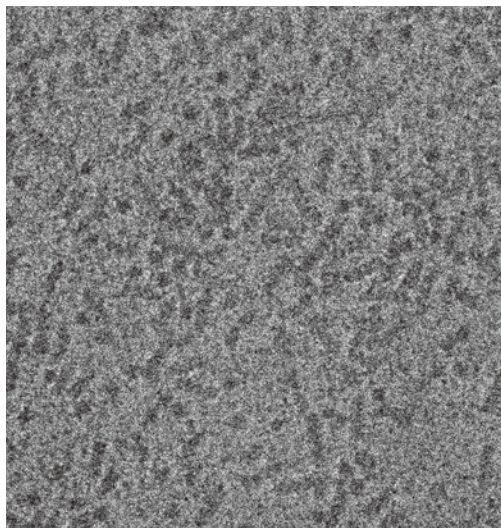


Ice embedding Omega filter slit In Phase Plate In 20 nm

Stathmine + Tubulins

Phase plate In

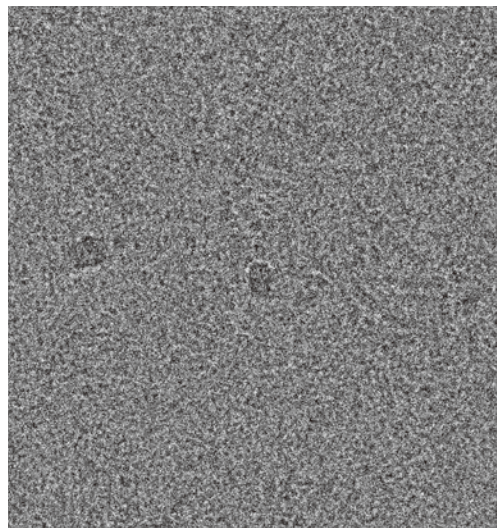
Stathmine and Tubulins complex is observed using the phase plate.



Detector: K2 Summit
Total dose: 30e⁻ / Å²

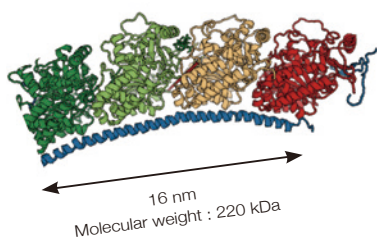
Phase plate Out

In normal observation, Stathmine and Tubulins complex does not appear as clearly as the case using the phase plate.



Using the phase plate, the particle images of the complex are cut out from the acquired images, for classifying these particle images into different groups based on external shape and density distribution. Then, the particle images of each group are added and averaged to create representative 2D-average images (three images to the right). In these images, structures that are similar to the known structures in the model image (left end) are seen.

Representative 2D class average



Specimen courtesy of Prof. Kikkawa (The Univ. of Tokyo)

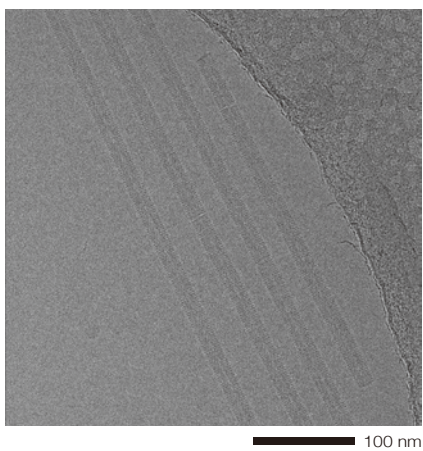
Three-dimensional (3D) reconstruction of protein and virus

In 3D reconstruction, first, the particle images projected from various directions are collected for classifying these particle images into different groups based on external shape and density distribution. Then, the particle images of each group are added and averaged to improve the signal-to-noise ratio of the images. Next, the angles of the projection orientations are estimated. Finally, the 3D structure of the protein is reconstructed by back projection of these particle images along the estimated angles.

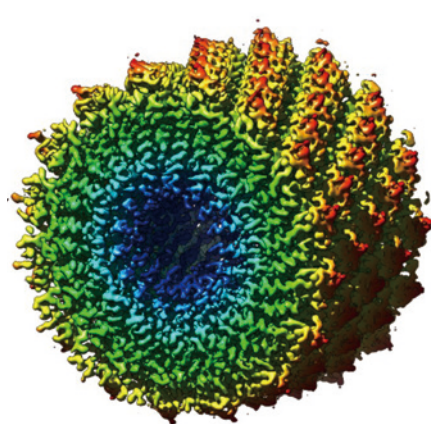
Tabacco mosaic virus

Tabacco mosaic virus at 3.8 Å resolution achieved by only 27 micrographs are shown in a cryo-electron micrographs (left) and 3D density map (right). It confirmed that the structure of virus is in spiral.

Cryo-electron micrograph



3D density map

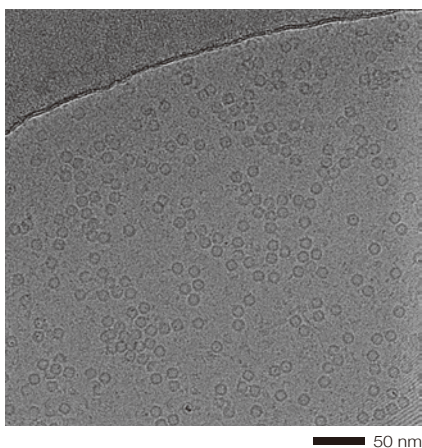


Specimen courtesy of Prof. Kikkawa
(The Univ. of Tokyo)

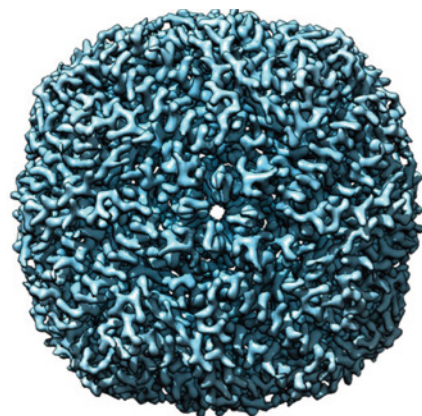
Apoferitin

Single particle analysis at 2.9 Å resolution for Apoferitin was confirmed as follows.

Cryo-electron micrograph



3D density map

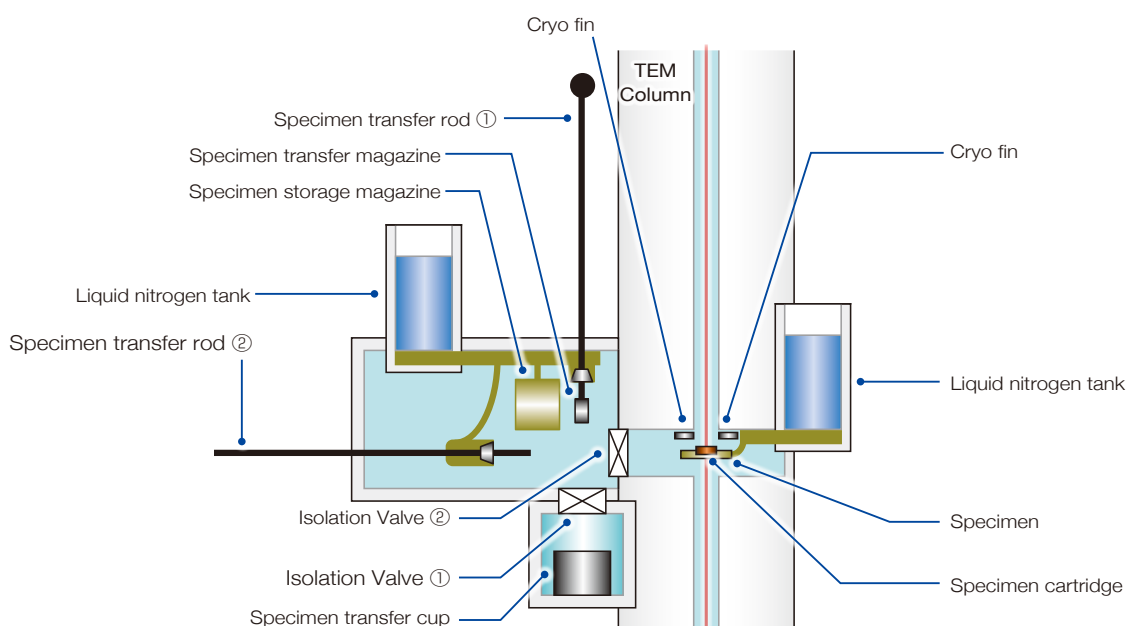


5-3

Cryo-TEM with Automated Specimen Transfer System

Frozen specimens are clipped in specimen cartridges and using a transfer magazine kept in a transfer cup filled with liquid nitrogen is introduced into the vacuum of the autoloader. The transfer cup is mounted into the instrument. After the chamber is filled with vaporized nitrogen, liquid nitrogen is vacuumed out from the cup. When the vacuum reaches a sufficient level, the isolation valve ① is opened, the specimen magazine is pulled out with the specimen transfer rod ① and the valve ① is closed. If the specimen cartridge is transferred to the specimen storage magazine from the transfer magazine, specimens can be stored there for about a few weeks. To observe a frozen-hydrated grid the appropriate cartridge needs to be pulled out from the storage magazine using the specimen transfer rod ② and put onto the TEM stage for observation.

Diagram of Cryo-TEM with Automated Specimen Transfer System



- **Specimen Cartridge**
- **Liquid nitrogen tank**

This cartridge holds a frozen-hydrated specimen.

This tank holds liquid nitrogen to cool down the specimen transfer rod, the specimen storage magazine, cryo-fin and the specimen cartridge.

- **Specimen transfer rod ①**
- **Specimen transfer rod ②**

This rod lifts the transfer magazine with cartridges from the specimen transfer cup.

This rod transfers the specimen cartridge to and from the specimen transfer magazine, the specimen storage magazine and the specimen chamber.

- **Specimen transfer cup**

This cup holds the transfer magazine with frozen specimens.

- **Isolation Valve ①**

Gate valve separating the autoloader from the specimen transfer cup.

- **Isolation Valve ②**

Gate valve separating the autoloader from the specimen chamber.

- **Specimen transfer magazine**

This magazine holds the frozen-hydrated specimens and transports them into the autoloader.

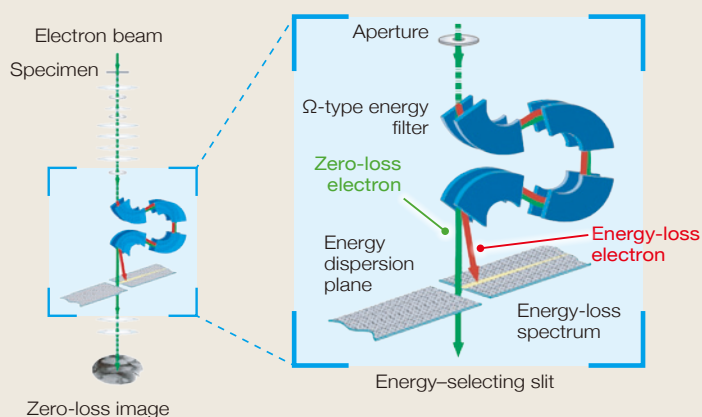
- **Specimen storage magazine**

This magazine holds the frozen-hydrated specimens for long-term storage.

Omega filter (energy filter)

Omega filter is an in-column energy filter installed in the TEM column. This filter enables us to acquire an energy-filtered image and an energy-loss spectrum.

Omega filter can also eliminate energy loss electrons and only pick up zero-loss electrons. It enables us to observe a high-contrast image with reduced chromatic aberration.



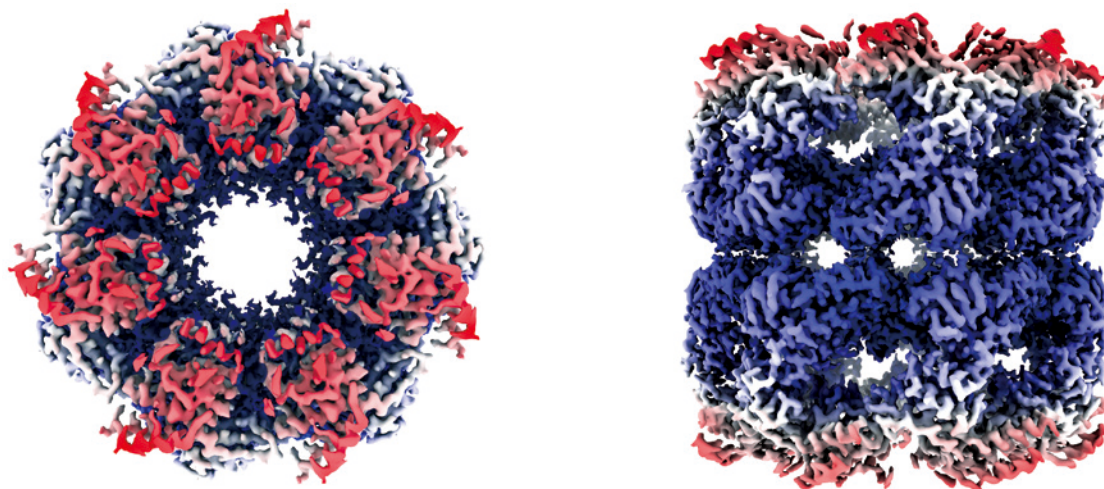
5-4

Cryo-TEM Applications with Automated Specimen Transfer System

GroEL

GroEL structure at 1.98 Å resolution achieved by only 504 micrographs, a dramatic improvement from 3.1 Å resolution from 1,883 micrographs in a previous study. (as of Oct. 26, 2020 at EMDB)

3D density map

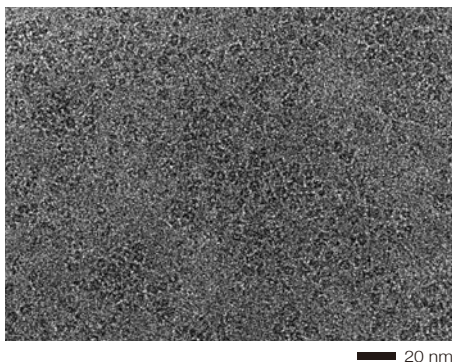


Data courtesy of Dr. Junso Fujita at Osaka University

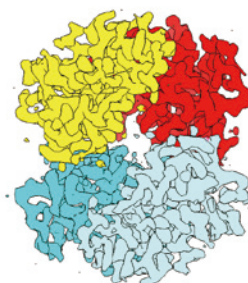
Hemoglobin

Cryo-electron micrograph (left), 3D density map (upper right) and fitted atomic model (lower right) of human hemoglobin obtained by high speed data collection, 850 movies per hour.

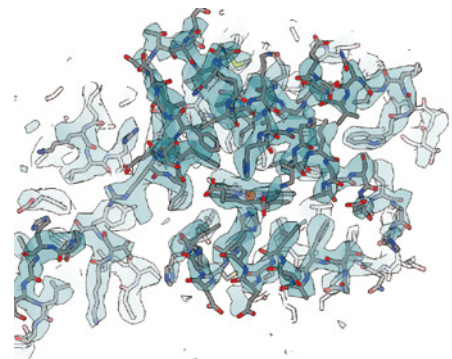
Cryo-electron micrograph



3D density map



Fitted atomic model onto 3D density map



Data courtesy of Dr. Miki Kinoshita at Osaka University

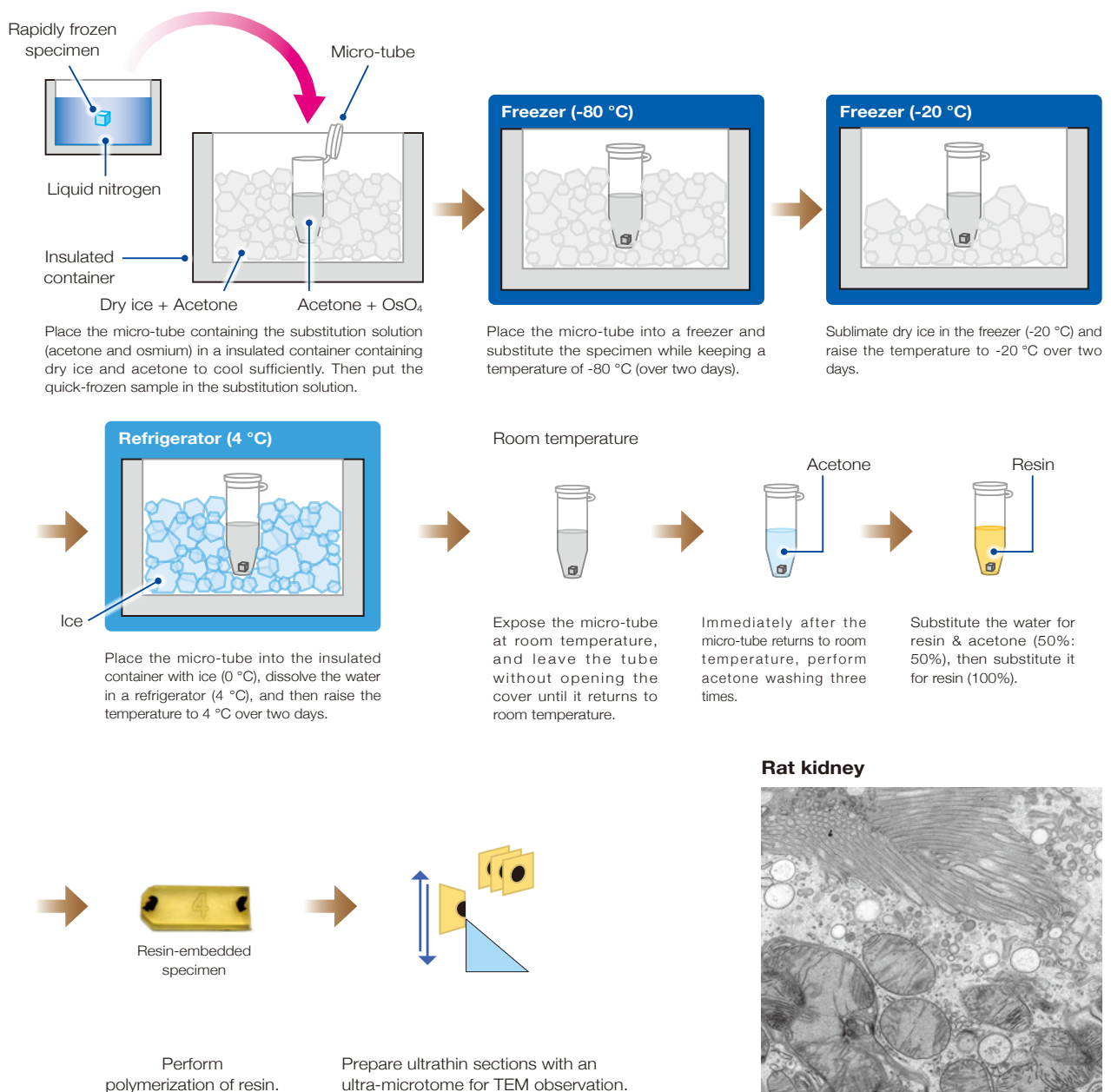
6

Freeze Substitution

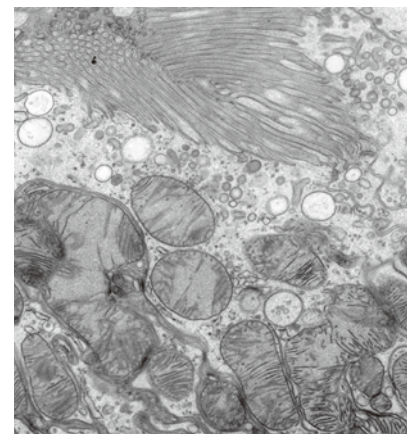
6-1

Freeze Substitution

Freeze substitution is a method to replace water with an organic solvent (acetone, etc.) in a biological specimen fixed by freeze fixation, where the specimen is subject to rapid freeze fixation (high pressure freezing or metal mirror freezing). In the course of freeze substitution, chemical fixation and electron staining can be simultaneously performed by adding osmium tetroxide and/or uranyl acetate. After the substitution, the specimen is gradually returned to room temperature, then it is subject to resin embedding and ultrathin sectioning for TEM observation. It is possible to replace water while preserving a morphological structure close to that of a living state. It is possible to replace water while preserving a morphological structure close to that of a living state.



Rat kidney



High pressure freezing
Freeze substitution
Staining with uranyl acetate and lead citrate
TEM observation

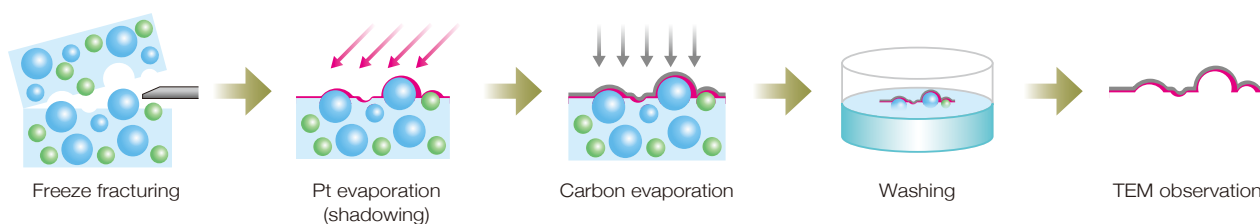
7 Freeze Replication

7-1 Freeze Replication

Freeze replication is a method of transferring the morphology of a frozen fractured face of a frozen (physically fixed) solution or soft material to a thin replica film and observing it with a TEM. Freeze replication is classified into three methods; Freeze fracturing, Freeze etching (deep etching), and Cryo-extraction replication. In the freeze fracture method and freeze etching method, a three-dimensional TEM image can be observed by the shadow effect obtained by depositing platinum on the sample. This contributes to the observation of a 3D TEM image. In Cryo-replication, particles dispersed in liquid are directly extracted to the replica film. The cryo-extraction replication is a method of directly extracting particles dispersed in a liquid onto a replica film. Since the sample extracted from the replica film can be directly observed, elemental analysis by EDS or EELS can be performed.

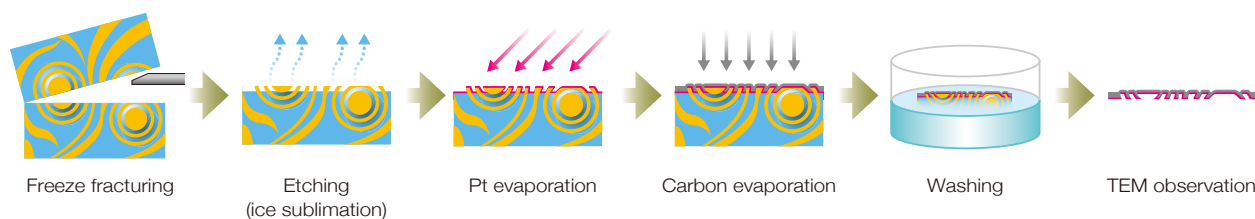
Freeze fracturing

When a frozen specimen is fractured, the resultant fracture goes through regions where resistance is low while the specimen splits further at the micro-level. In the actual experiment, fracturing occurs easily at physically weak sites under a cryo-environment. Typical examples include sites between a hydrophobic region and a hydrophilic region, and the interface between water and oil where physical properties are different. Therefore, this method is used to observe the interface between water and oil, the inner structure of a lipid bilayer membrane, and the structure of micelles or liquid crystal.



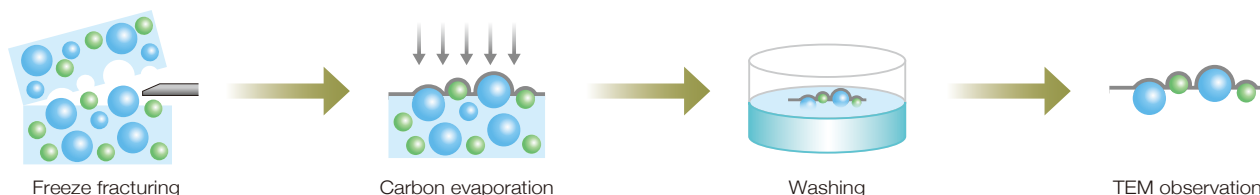
Freeze etching (Deep etching)

After the frozen specimen is fractured, ice on the freeze-fractured surface is etched (sublimated) to observe the exposed internal structure. This method is effective when you want to observe the internal structure of the cell. In addition, image contrast due to surface unevenness is obtained because the ice on the surface is removed by sublimation. The method is effective for visualizing the existence of water in the structure of oil-in-water emulsion, water-in-oil emulsion, or Bicontinuous structure.



Cryo-extraction replication

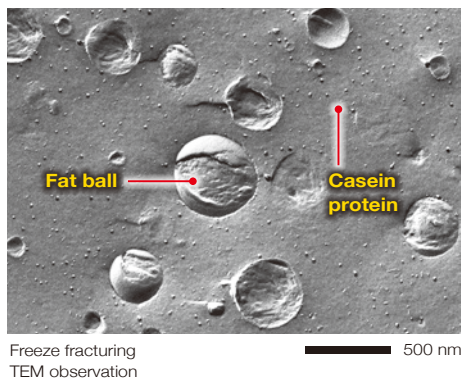
Cryo-extraction replication is a method to directly extract particles dispersed in liquid onto a replica film. Since the specimen can be observed directly, elemental analysis by EDS and EELS is possible. In addition, this method does not evaporate Pt, so it is possible to observe at a higher magnification.



7-2 Freeze Replication: Applications

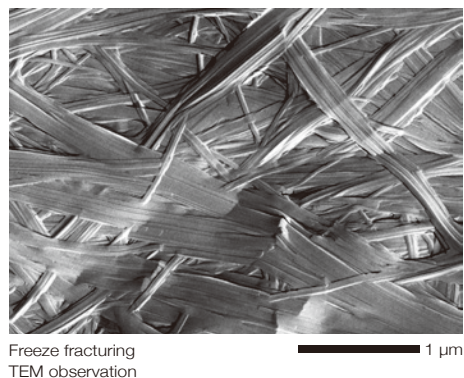
Coffee milk

Fat balls and casein proteins are observed.



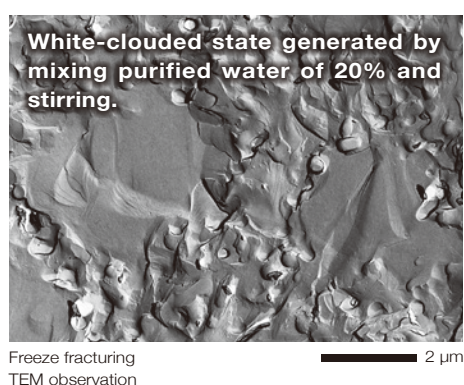
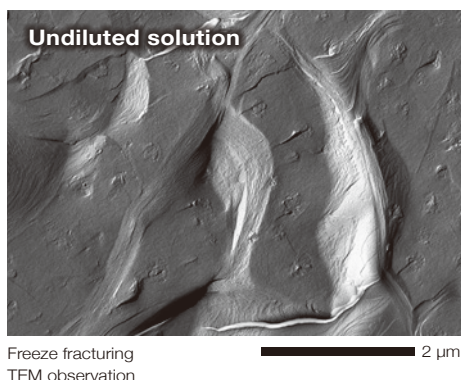
Soap

The morphological structure of a soap, which is susceptible to heat and electron-beam irradiation, can easily be observed.



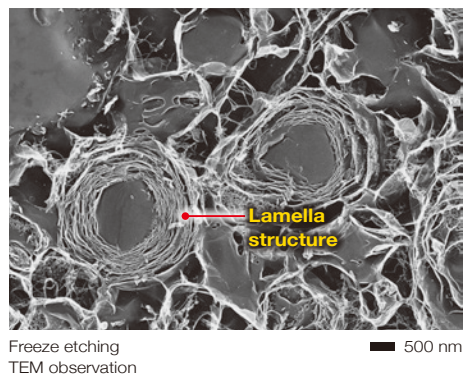
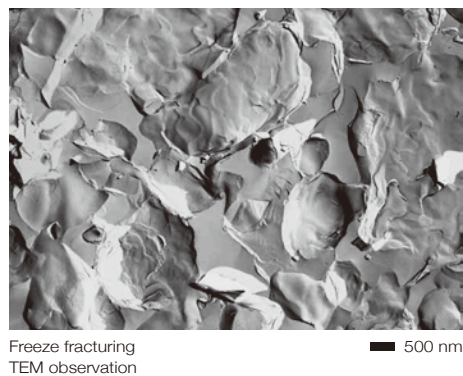
Cleansing oil

20% water is mixed to an undiluted solution of transparent cleansing oil. The morphological structure of this mixed liquid specimen is observed as a white-clouded state. The resultant image confirms that the fine morphology of the uniform layered structure is transformed to a ball-like structure (about 500 nm in size).



Emulsion (cream)

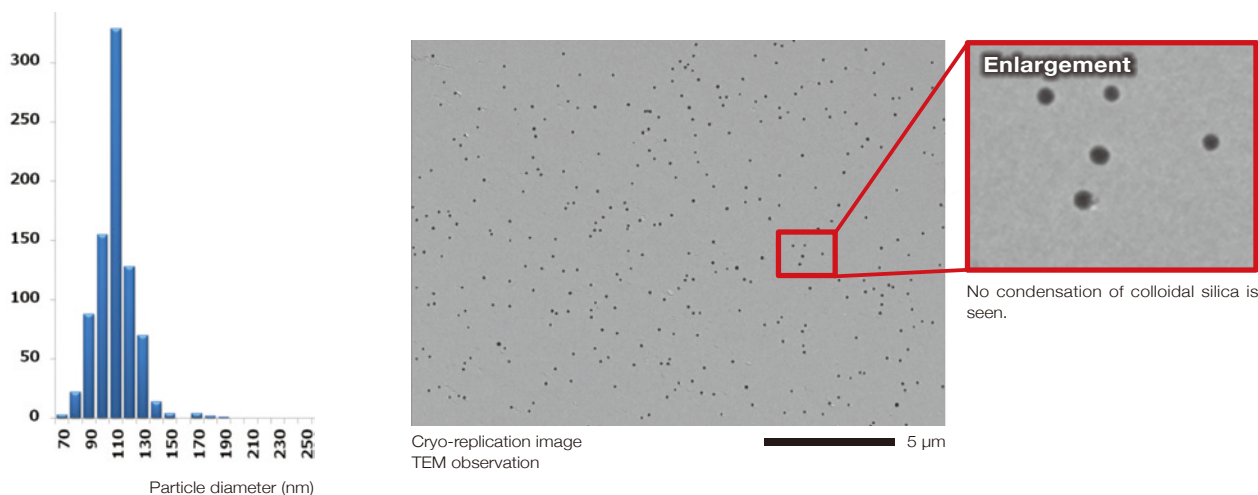
The fractured face of a freeze-fractured, water-containing specimen is imaged. The fractured structure, along a brittle oil-water interface is observed. In Freeze etching, etching is applied by cutting and preparing a smooth surface. As a result, the topographic shape and the inner structure of emulsion particles which are obtained by ice sublimation, can be observed.



Particle size distribution of colloidal silica

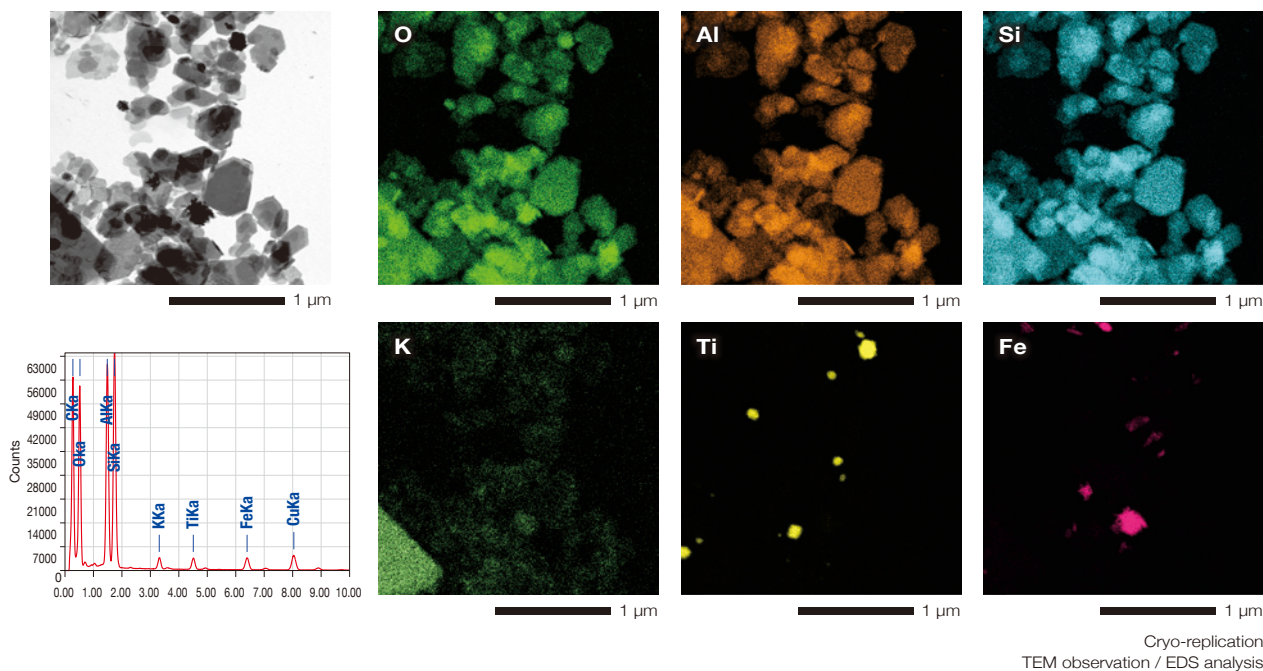
The following results show analysis of the particle size distribution of colloidal silica using Cryo-replication. Using the particle image of a large area (30 $\mu\text{m} \times 30 \mu\text{m}$ square) taken by montage, binarization of the image was conducted for detecting and extracting particles, then the particle diameters were calculated by circle approximation.

In Cryo-replication, no condensation of the particles is seen and thus, this method is effective for the accurate measurement of the particle diameter.



Elemental analysis of inclusions dispersed in emulsion (liquid foundation)

An inorganic dopant dispersed in emulsion was subject to EDS elemental mapping. Since the replica film is formed by high-purity, thin carbon, this method is suitable for high-resolution observation and analysis.

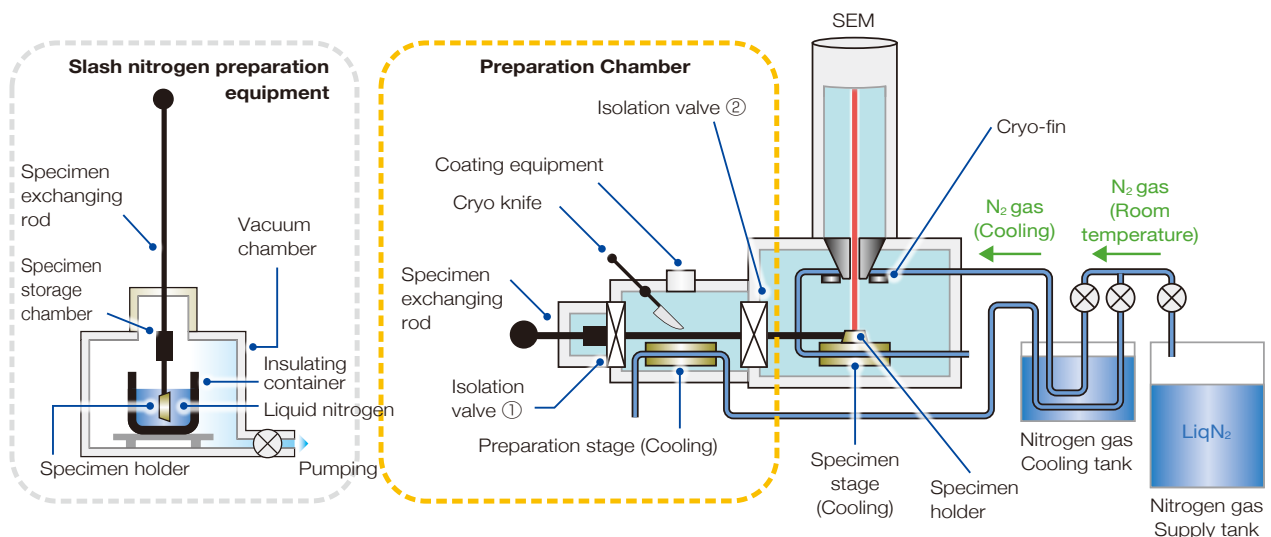


8-1

Cryo-SEM

Cryo-SEM is a method that makes it possible for hydrous specimens to be observed in their natural form by freezing. It is also used for protecting specimens like polymers, which are susceptible to heat damage from electron beam irradiation. After freezing the specimen, freeze fracturing or etching (sublimating the ice) is performed as appropriate to expose the target part of the specimen. It allows us to observe the specimen in a frozen state using a cooling stage.

Diagram of Cryo-SEM



[SEM]

Specimen stage (Cooling): This specimen stage is a cooling stage that allows a frozen specimen to be observed in this state.

Cryo fin: These cooling fins are used to prevent frozen specimens from getting ice-contaminated. The temperature is set lower than that of the specimen stage.

[Preparation Chamber]

Isolation valve ①: This valve is used for the specimen frozen outside of the instrument, to be placed into or taken out of the Preparation stage.

Isolation valve ②: This valve is used for the specimen to be placed into the SEM from the Preparation Chamber or taken out from the SEM to the Preparation Chamber.

Cryo knife: This knife is used to cut the specimen.

Preparation stage: This stage is a cooling stage to control the specimen temperature during the preparation. It is also used for etching.

Coating equipment: This equipment provides coating with gold, platinum and carbon etc., as appropriate.

[Accessory Equipment]

Nitrogen gas supply tank: This tank is a supply source of nitrogen gas to cool down the specimen stage, the preparation stage and the cooling fins. >99.999 vol%, H₂O free dry nitrogen gas can be used instead. It consumes a 7 m³ container of the gas a day.

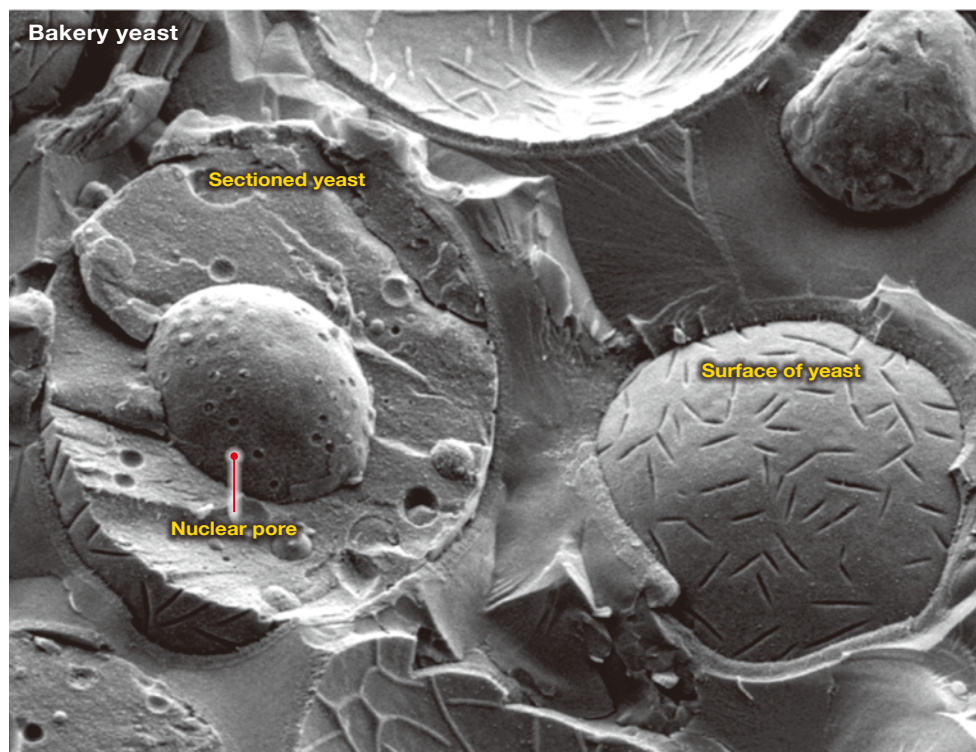
Nitrogen gas cooling tank: This tank is to cool down the nitrogen gas with liquid nitrogen that is supplied from the Nitrogen gas supply tank.

Slash nitrogen preparation equipment: This equipment produces Slash-nitrogen. A frozen specimen can be stored in the storage chamber which is mounted on the Specimen exchanging rod. It is able to carry the specimen into the Preparation Chamber without exposing it to air.

8-2 Cryo-SEM Applications

Microorganism

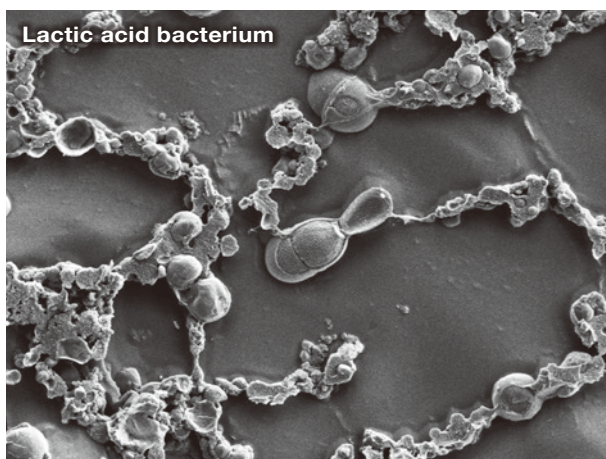
Bakery yeast was rapid frozen and fractured in a vacuum. This is a high resolution SEM image obtained by an FE-SEM equipped with Cryo-System. It enabled us to observe the surface of yeast and the nuclear pore.



Rapid freezing (Slash-nitrogen) Freeze fracturing
Pt Sputter Cryo-SEM Observation

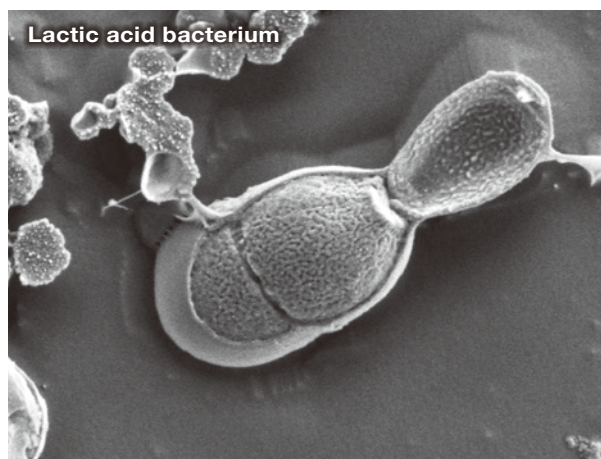
1 μm

These are SEM images of lactic acid bacterium that appeared after rapid frozen yogurt was fractured and subjected to etching.



Rapid freezing (Slash-nitrogen) Freeze fracturing Etching
Pt Sputter Cryo-SEM Observation

1 μm



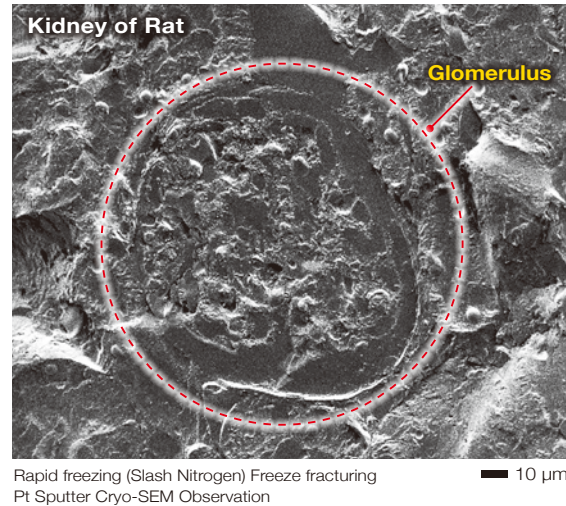
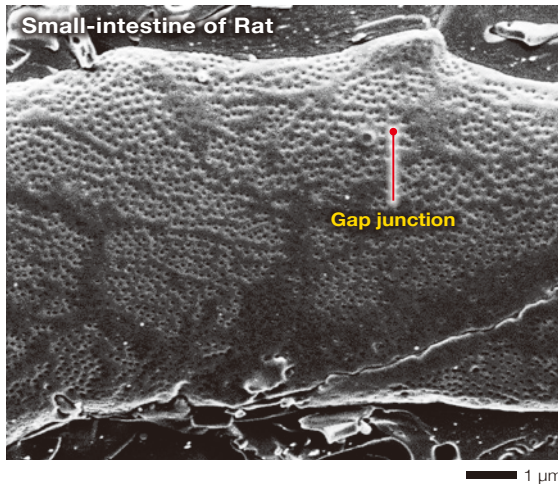
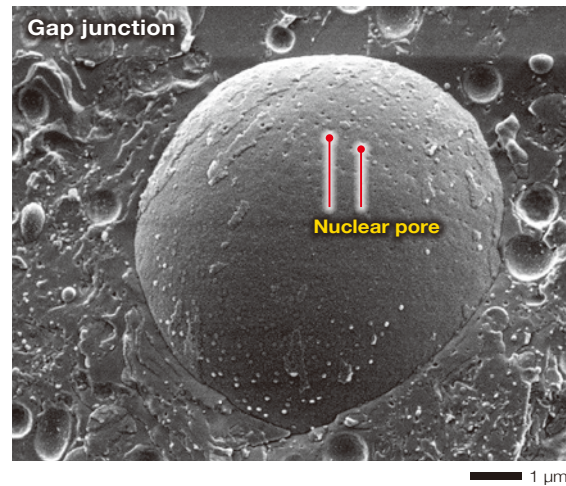
100 nm

Animal Tissue

Observation of rat tissue with Cryo-SEM revealed the nuclear pore and gap junction.

By freezing the specimen before SEM observation, it is not necessary to dry the specimen. Therefore, the observation can be performed without specimen shrinkage due to drying.

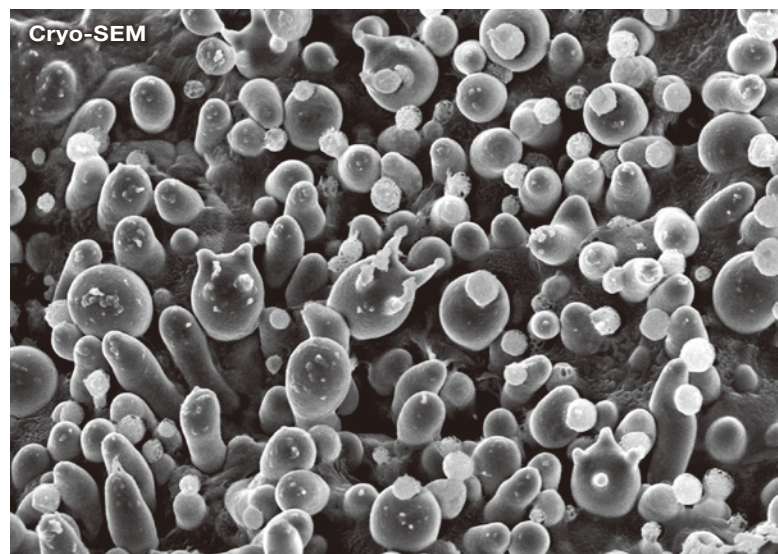
1. Remove a tissue after perfusion fixation
2. Wash after perfusion fixation
3. Substitute 20% of the specimen's solution by a sucrose solution
4. Fracture and observe it after rapid freezing



Rapid freezing (Slash Nitrogen) Freeze fracturing
Pt Sputter Cryo-SEM Observation

Basidiospore of Mushrooms

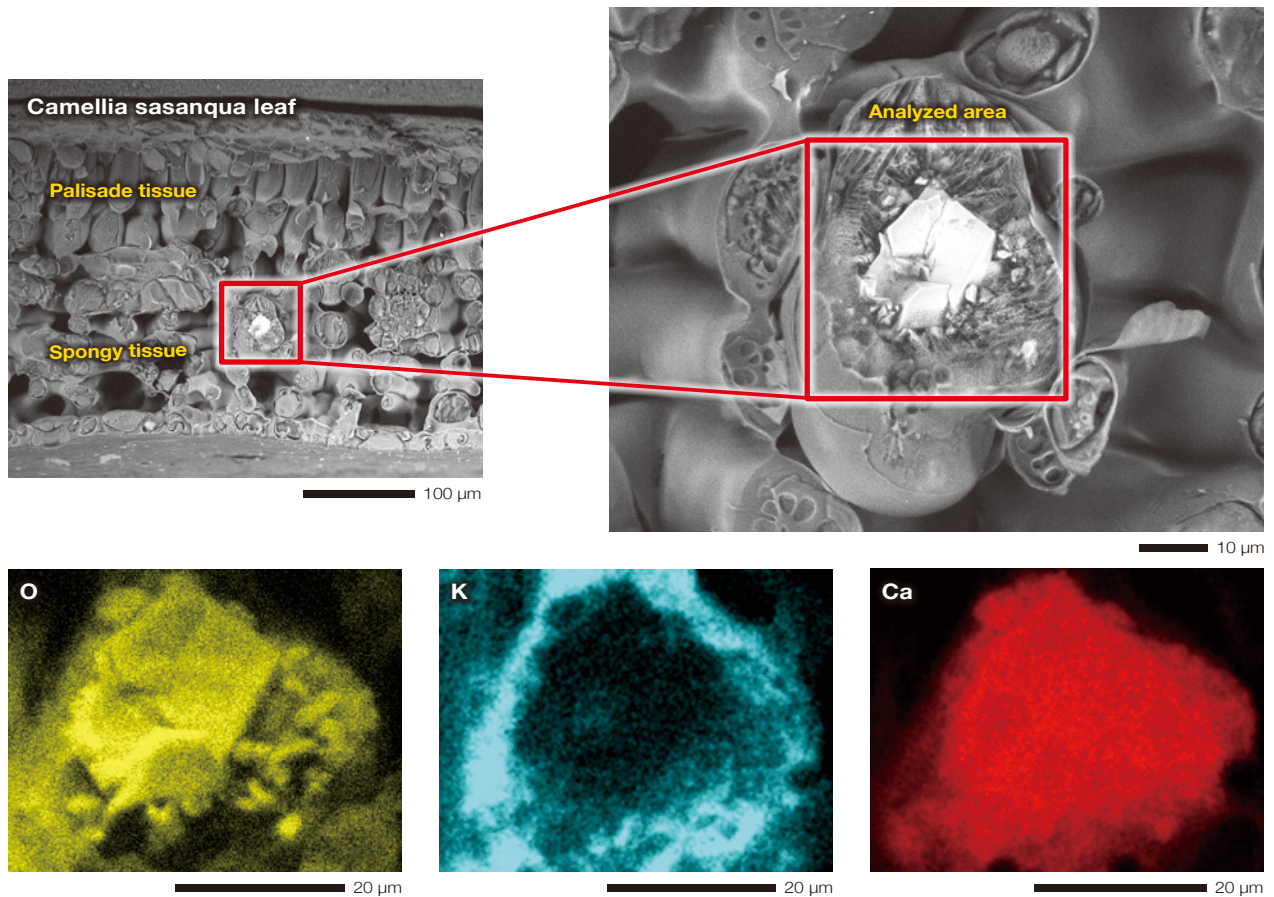
Basidiospore of Mushrooms are easy to deform and that makes it difficult to observe them. Once they are frozen, they retain their configuration and this allows us to observe them in detail.



Rapid freezing (Liquid Nitrogen)
Evaporated Au Cryo-SEM Observation

Plant

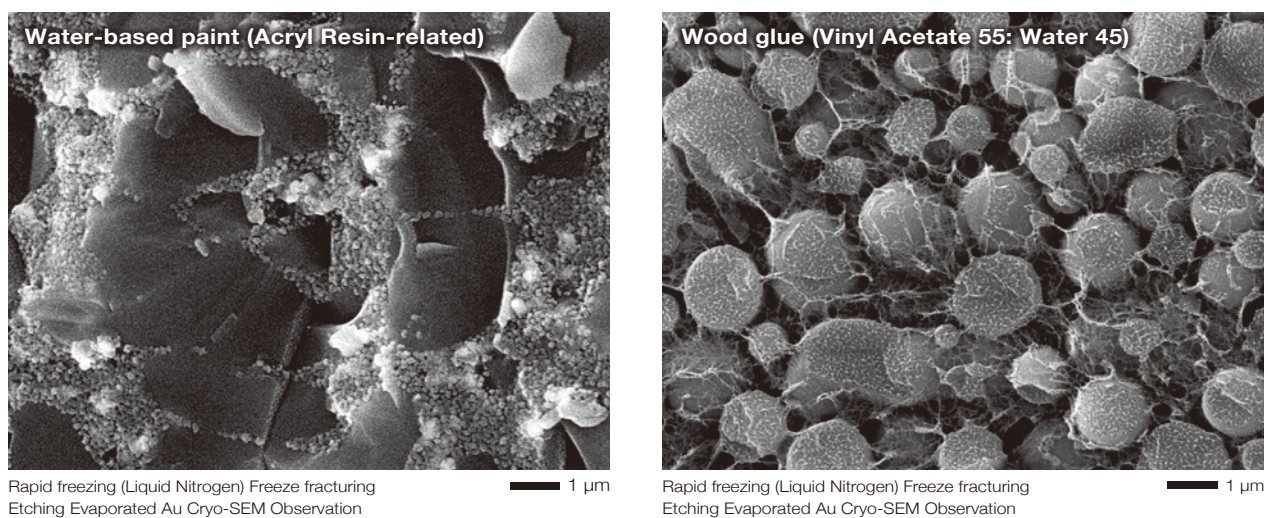
Cryo-SEM enables observation of a cross section of a frozen plant without draining its minerals. The left image below shows that a crystal of mineral was observed on the cross-section of a *Camellia sasanqua* leaf. EDS elemental analysis (right image below) found that this crystal has Ca and K distributed in its surrounding area.



Rapid freezing (Slash Nitrogen) Freeze fracturing
Etching Pt Sputter Cryo-SEM Observation / EDS Analysis

Emulsion

After an emulsion was frozen, etching was applied to the frozen emulsion and then its particle diameter and distribution was observed.

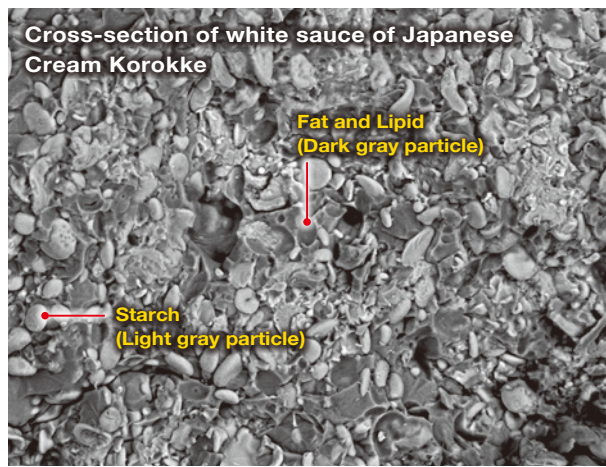


Rapid freezing (Liquid Nitrogen) Freeze fracturing
Etching Evaporated Au Cryo-SEM Observation

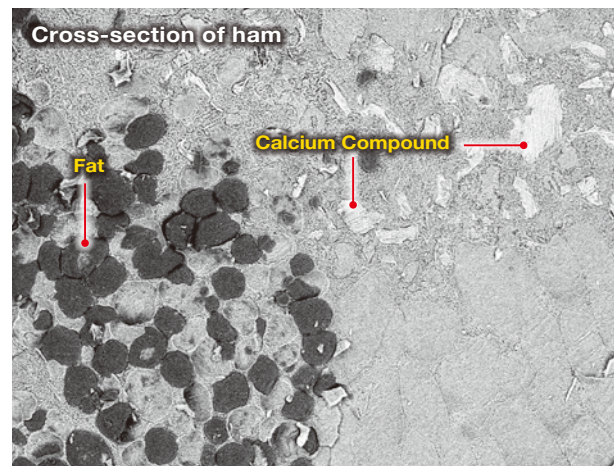
Rapid freezing (Liquid Nitrogen) Freeze fracturing
Etching Evaporated Au Cryo-SEM Observation

Food

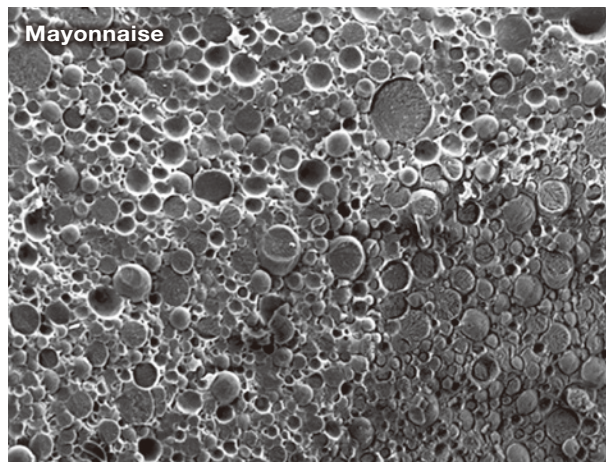
The morphology and distribution of fats, lipids or minerals contained in high moisture food can be observed.



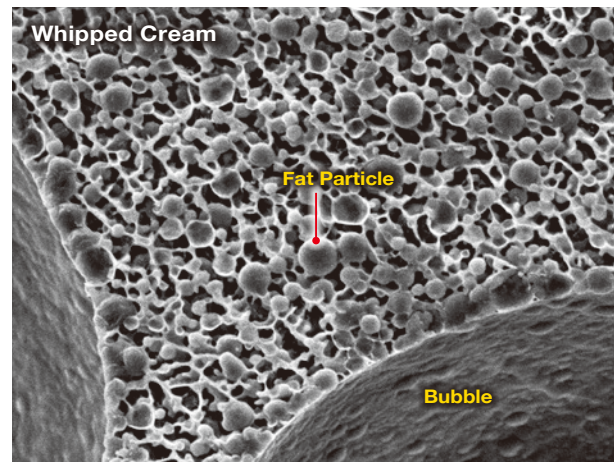
Rapid freezing (Liquid Nitrogen)
Cryo-SEM Observation (Low vacuum mode) 50 µm



Rapid freezing (Liquid Nitrogen)
Cryo-SEM Observation (Low vacuum mode) 100 µm



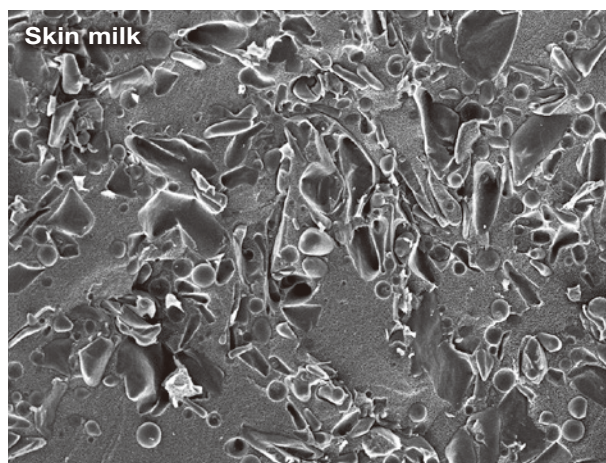
Rapid freezing (Liquid Nitrogen) Freeze fracturing
Etching Evaporated Au Cryo-SEM Observation 10 µm



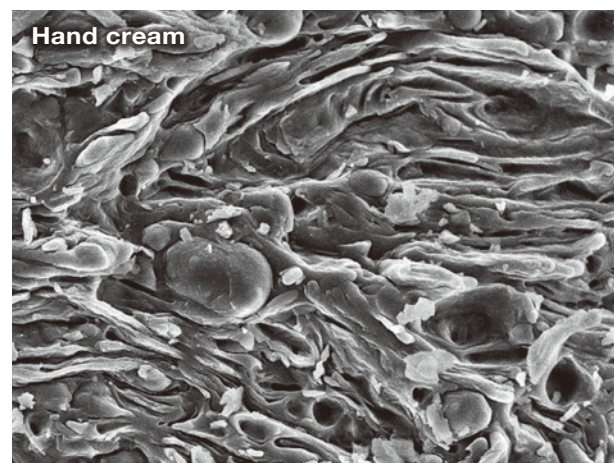
Rapid freezing (Liquid Nitrogen) Freeze fracturing
Etching Evaporated Au Cryo-SEM Observation 5 µm

Cosmetics

The structure of cream materials can be observed by freezing before SEM observation.



Rapid freezing (Liquid Nitrogen) Freeze fracturing
Etching Pt Sputter Cryo-SEM Observation 20 µm



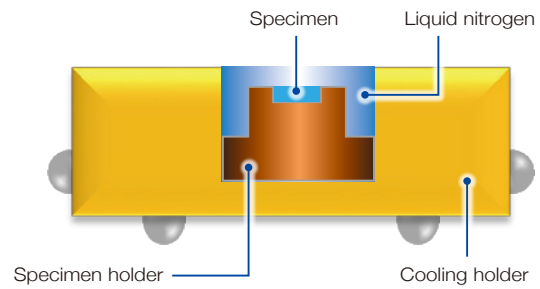
Rapid freezing (Liquid Nitrogen) Freeze fracturing
Etching Evaporated Au Cryo-SEM Observation 5 µm

8-3 Simple Cryo-SEM

Simple Cryo-SEM is a method to observe hydrous specimens or soft specimens using an LV Cooling holder with LV-SEM. The specimen is cooled by liquid nitrogen. This LV Cooling holder can be installed to almost all LV-SEMs, and allows us to perform simple Cryo-SEM observation or freeze-drying.

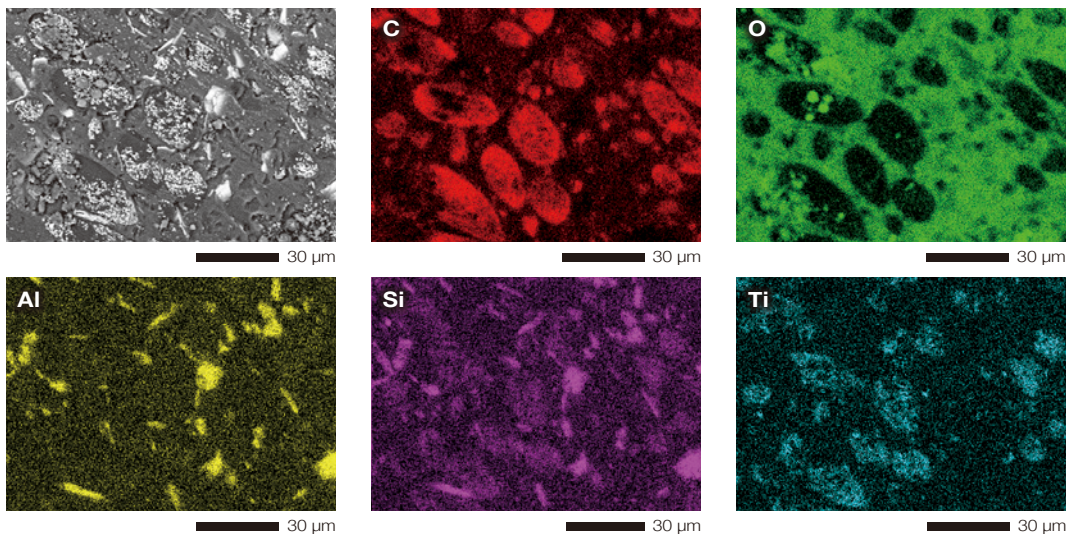
Using a Large capacity LV Cooling holder equipped with a frost-free function can provide full-scale Cryo-SEM observation.

Large capacity LV Cooling holder



Liquid foundation

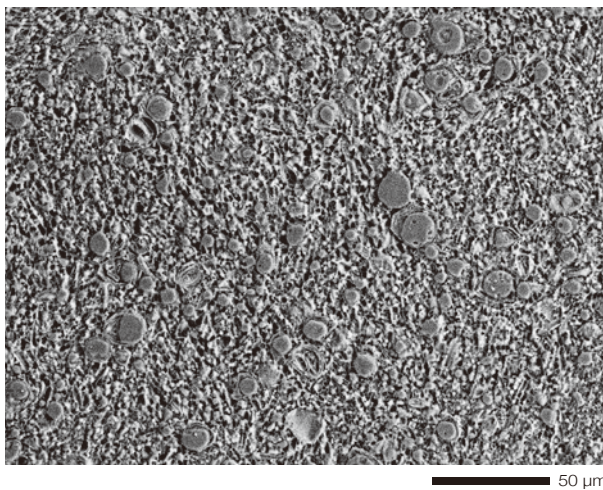
Elemental mapping for a frozen specimen allows us to observe not only inorganic materials but also the distribution of elements (C, O, etc.) at a stable liquid state.



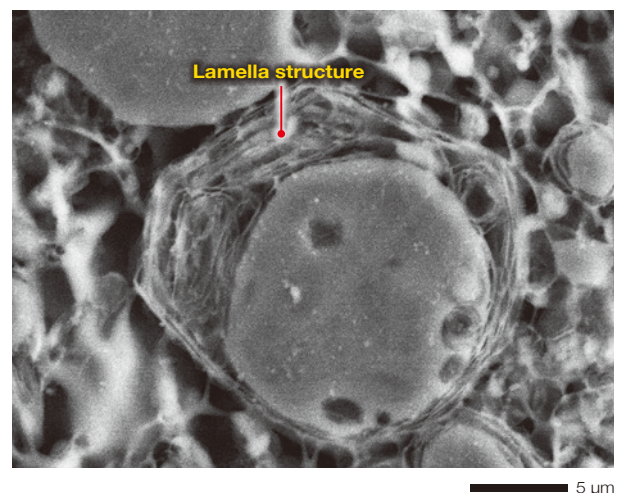
Rapid freezing (Liquid Nitrogen) Etching Using LV cooling holder SEM Observation / EDS Analysis (Low vacuum mode)

Skin milk

Lamella structure was easily observed by etching of the frozen cross-section that was prepared using Cryo-microtome to sublimate water (ice) inside of the specimen.



Rapid freezing (Liquid Nitrogen) Polished surface by Cryo-microtome Etching Using Colling holder SEM Observation (Low vacuum mode)

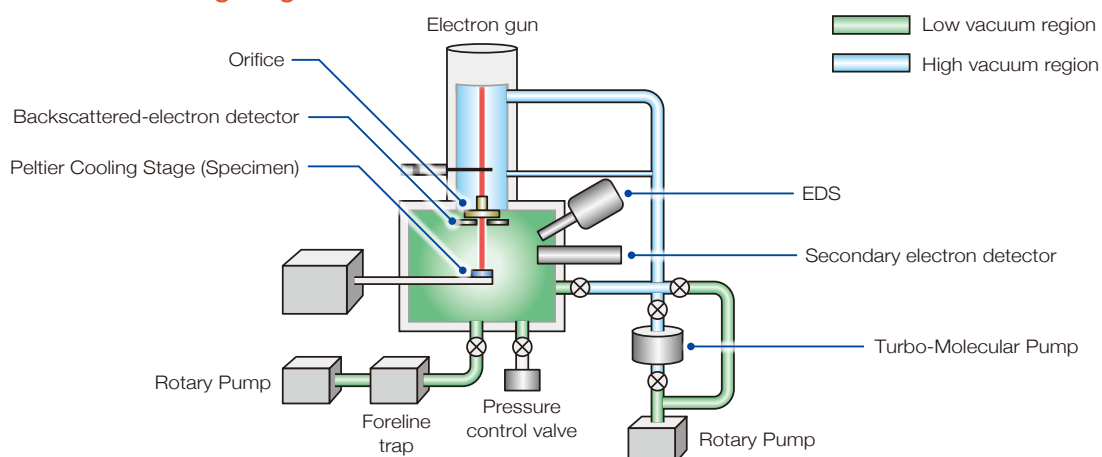


9-1

Low-Vacuum SEM / Peltier Cooling Stage

The Low vacuum SEM (LV-SEM) is designed to increase the pressure in the specimen chamber between a few Pa and a few 100 Pa (low vacuum range). In an LV-SEM, cations which are produced from the residual gas molecules in the specimen chamber colliding with the incident electrons or with the electrons emitted from the specimen, neutralize any electrically negative charging of the specimen surface. This phenomenon allows a non-conductive specimen to be observed without conductive coating while avoiding the influence of charging. A Peltier Cooling Stage can cool specimens down to -25 °C using a Peltier element. An LV-SEM equipped with a Peltier Cooling Stage can reduce a certain level of water evaporation from a hydrous specimen. When the partial pressure of water vapor and the specimen temperature are properly controlled using a special cover (wet cover), the specimen can be kept in a liquid state. This method (Wet Cover Method) enables us to observe it in a SEM image.

LV-SEM / Peltier Cooling Stage

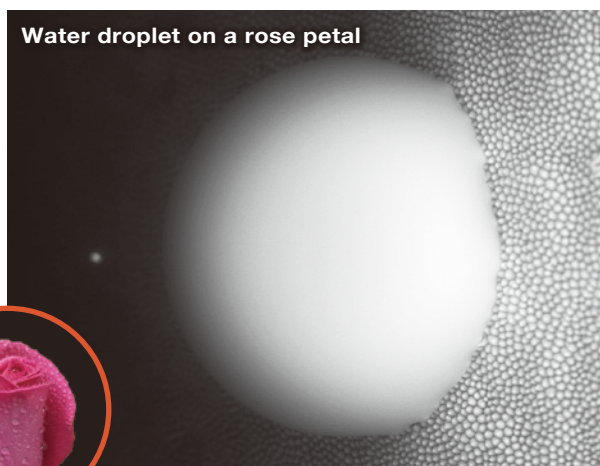


9-2

Low-Vacuum SEM / Peltier Cooling Stage / Wet Cover Method Application

Observation of a water droplet on the surface of leaf veins

This image shows a water droplet on a rose petal using the Wet Cover Method. This method enables us to observe an SEM image of a naturally spread water droplet on the petal without drying out. The observation confirmed how the water droplet repels the surface boundary.



Non-Preparation LV-SEM (Low-vacuum mode)
Wet Cover Method Peltier cooling: 0 °C

200 μm

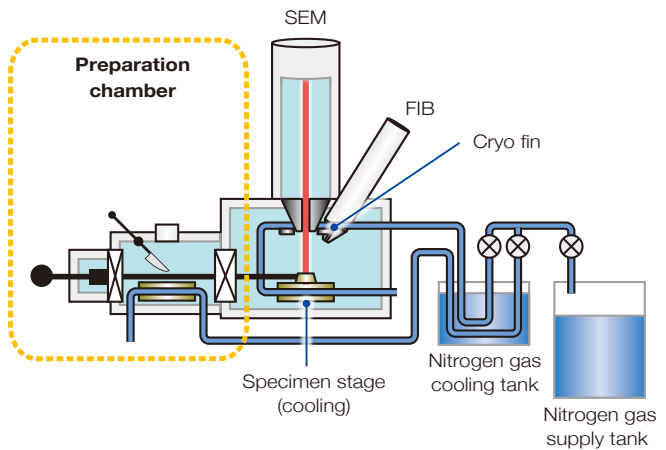
10 Cryo-FIB

10-1 Cryo-FIB

FIB is an instrument that irradiates a sample with a focused ion beam (gallium ion beam with an energy of a few keV to 30 keV), to mill and observe the milled specimen. In the Cryo-FIB-SEM, both SEM and FIB columns are placed in a specimen chamber and a Cryo- system is added to the equipment. This configuration enables a frozen hydrous specimen to be milled by FIB and imaged by SEM while the specimen morphology is preserved.

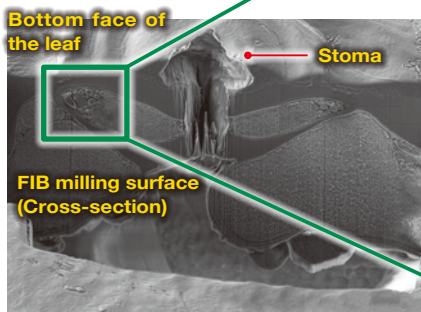
With the Cryo-FIB-SEM technique, the specimen, such as cell tissue or a cosmetic product, can be rapidly frozen and its specific site can be subject to cross-section milling. Thus, observation and analysis of its internal structure can be made.

Cryo-FIB-SEM

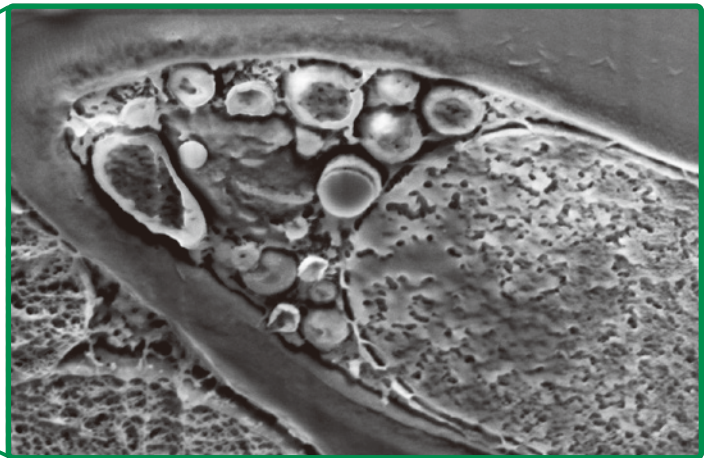


FIB column is mounted into Cryo-SEM, and that makes it possible to observe a cross-section of the specimen prepared by FIB milling.

Stoma of *Camellia Sasanqua*



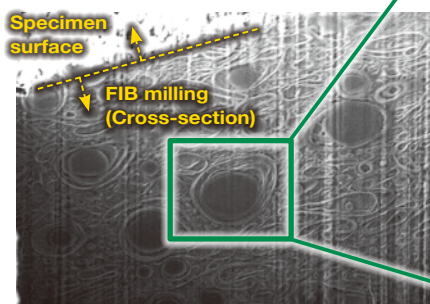
5 μ m



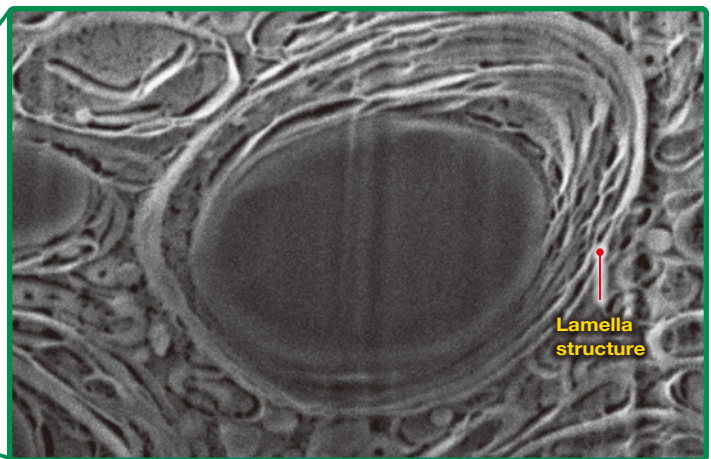
1 μ m

Rapid freezing (Liquid Nitrogen)
Cross-sectioned by Cryo-FIB Pt Sputter Cryo-SEM Observation

Skin-care cream



5 μ m



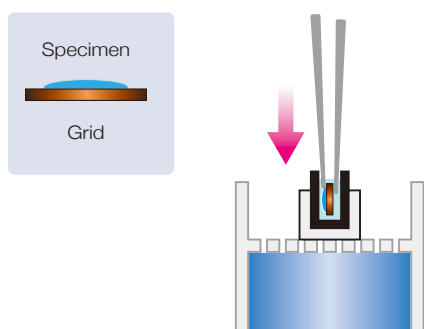
1 μ m

Rapid freezing (Liquid Nitrogen)
Cross-sectioned by Cryo-FIB Pt Sputter Cryo-SEM Observation

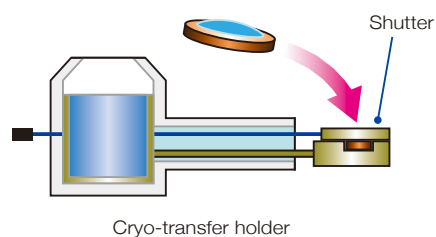
10-2 Cryo-TEM Observation with Cryo-FIB Milling

Single-beam FIB using Cryo-transfer holder ~ Observation with TEM

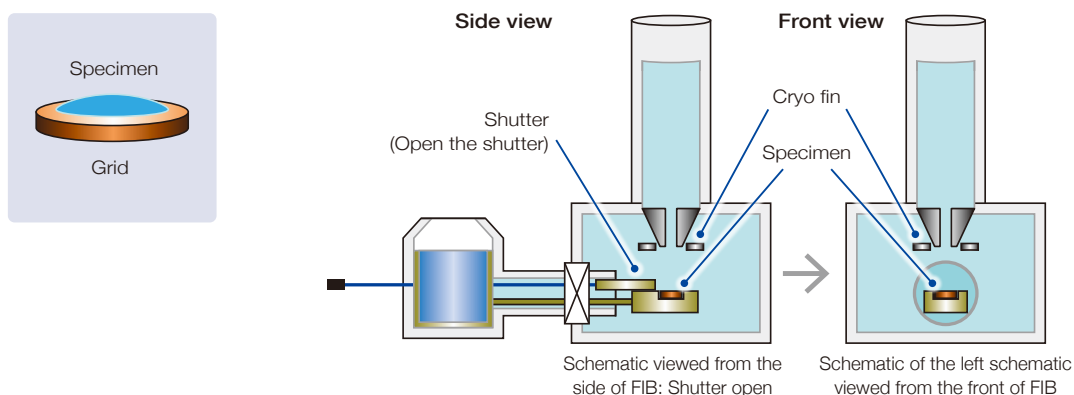
① Freeze a specimen (Ice embedding)



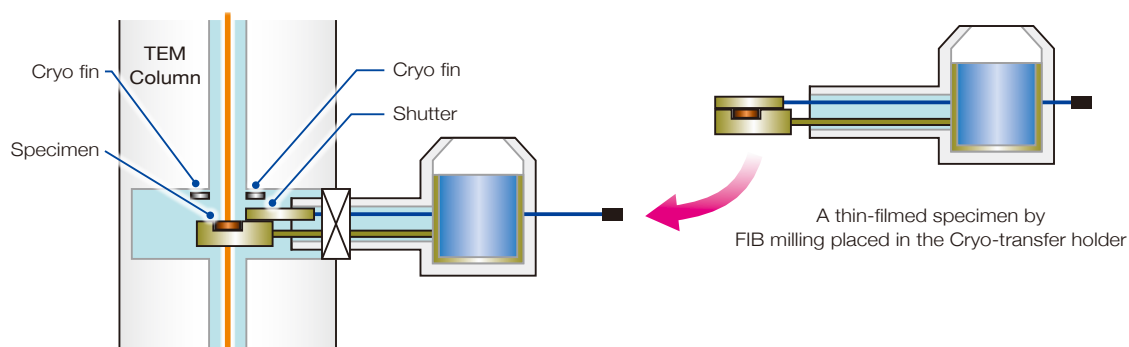
② Fix the grid into the Cryo-transfer holder and close the shutter



④ Open the shutter of the Cryo-transfer holder, observe the specimen with the FIB and decide the position for FIB milling

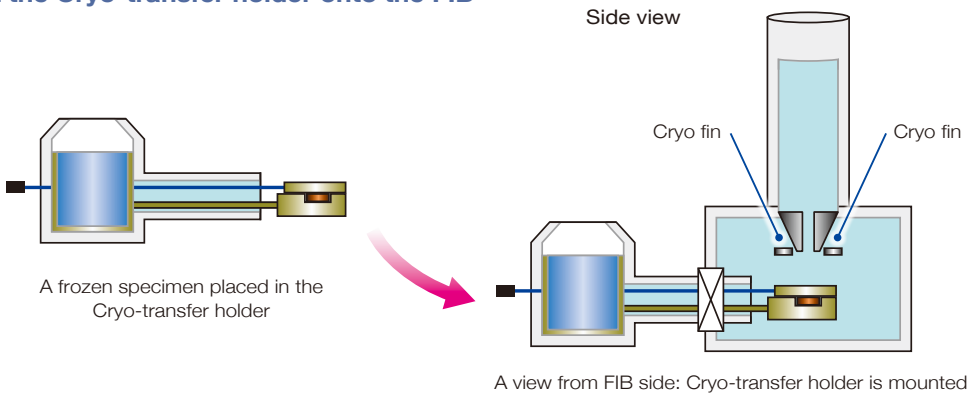


⑥ Close the shutter of the Cryo-transfer holder, convey the holder into TEM and open the shutter

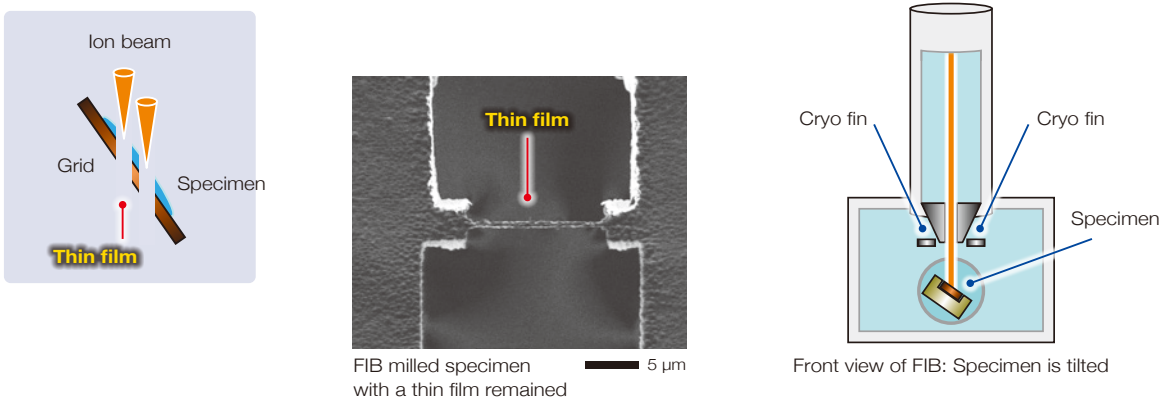


FIB has two types of configuration for cooling stages. One is used for bulk specimen called the bulk specimen motor stage and another is called the side-entry goniometer stage that allows direct insertion of the TEM tip-on holder. Since there is no need to remove the specimen from the specimen holder, it is possible to alternately perform FIB milling and TEM observation. When Cryo-transfer holder (*See P.10) is used with this goniometer stage, the operator can directly observe Cryo-TEM images of a thin section milled by FIB while cooling it.

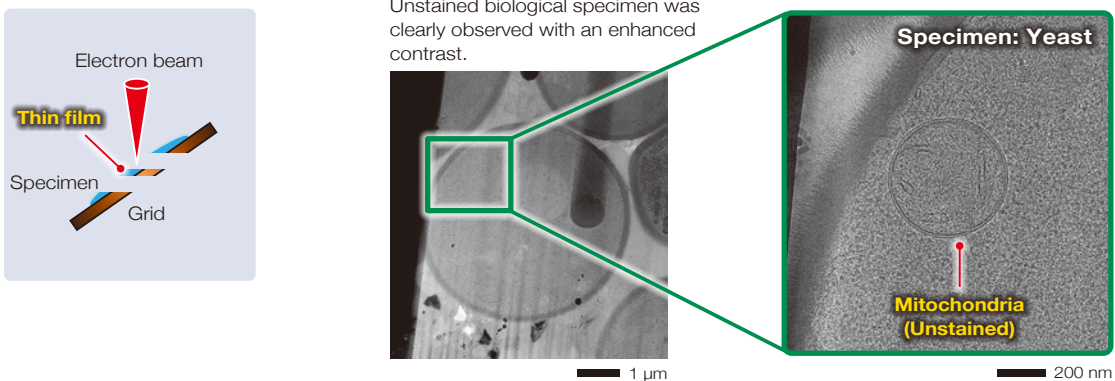
③ Mount the Cryo-transfer holder onto the FIB



⑤ Tilt the Cryo-transfer holder and perform FIB milling to the specimen from the oblique side



⑦ Tilt the Cryo-transfer holder in the opposite direction from the FIB milling side and observe the thin film with TEM.

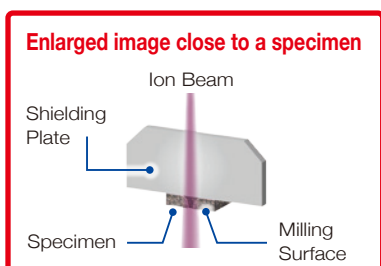


11

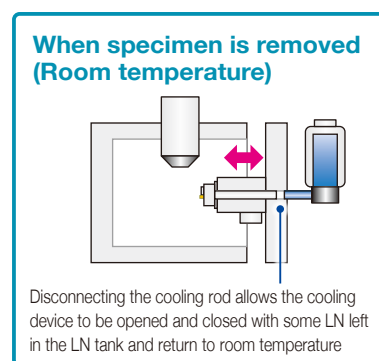
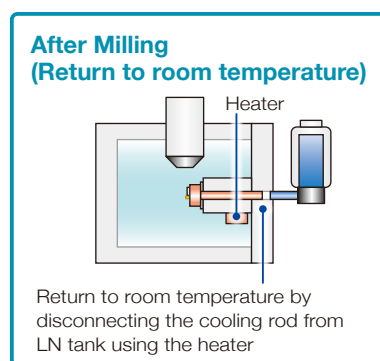
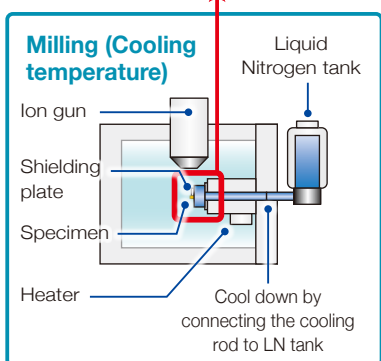
Cooling CP (Cryo-CP)

11-1

Cooling CP (Cryo-CP) Mechanism



CROSS SECTION POLISHER™ CP, is a milling device to prepare cross-section of a specimen using an Argon Broad Ion Beam, BIB. A slow-sputtering shielding plate is set directly above the specimen. Then, etching is performed by irradiating the Argon BIB from the top. A polished cross section is prepared along the edge face of the shielding plate. A cooling system is mounted to control temperature. While milling the specimen, the temperature can be lowered to the appropriate temperature. The heat damage to the specimen can be reduced in this manner. This device can automatically cool down the temperature of specimen, preparing a cross-section, return the specimen to room temperature using a heater after CP process.

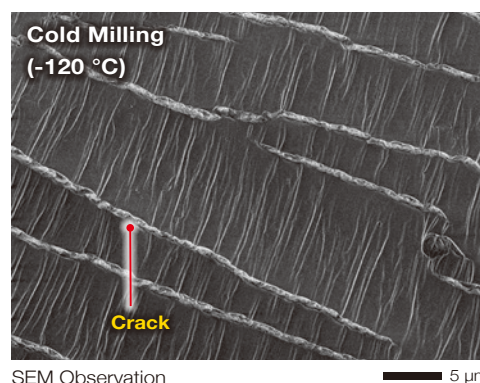
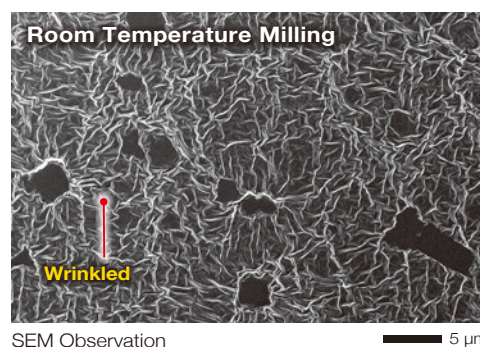
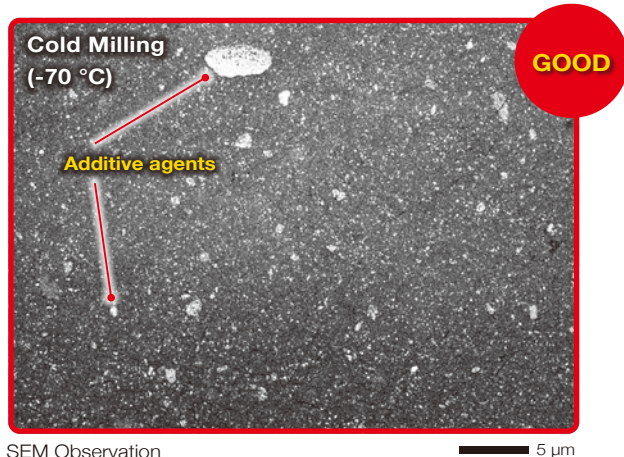


11-2

Cooling CP (Cryo-CP) Applications

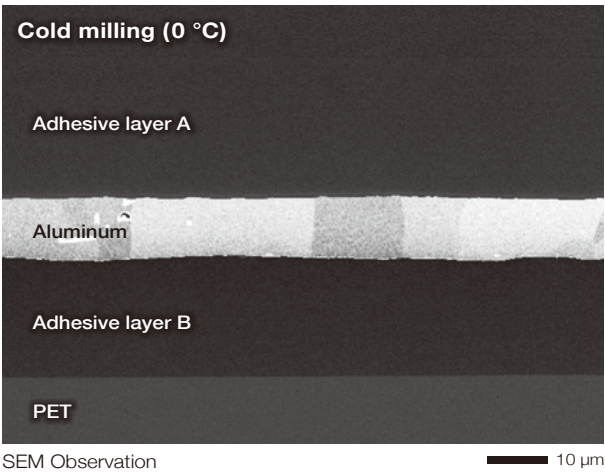
High-polymer material (Rubber band)

Rubber is sensitive to not only being heated but also being supercooled and that may change its shape. When Cooling CP was applied to a rubber band at room temperature, the material was wrinkled by the processing heat, and it cracked by excessive cooling at -120°C . When Cooling CP was applied to the material by controlling the temperature to -70°C , the cold milling enabled us to acquire a milling surface which appeared less damaged by heating and supercooling. This image allowed us to observe additive agent distributions.



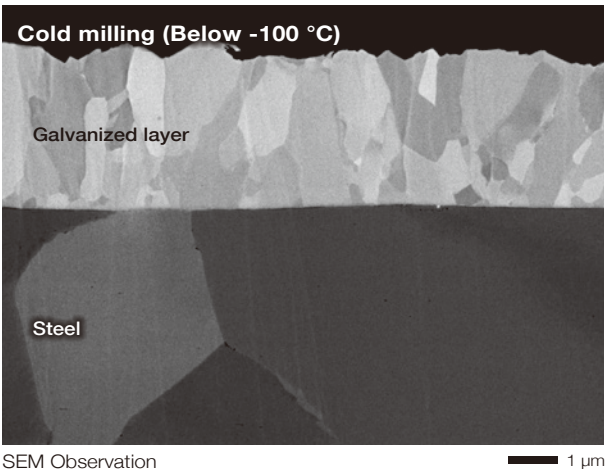
High-polymer material (Packing film)

This image shows a cold milling case applied to a multi-layer film coated material which is composed of soft adhesives and hard metal. In the cold milling application to this complex material, the layers may be delaminated by microtome fabrication, but a smoothed milling surface with less damage appeared with cooling CP.



Low-melting-point metal material (Galvanized steel)

This image shows a cold milling case applied to a low-melting-point metal. It resulted in reducing the damage of galvanized iron steel layer with a low melting point.



Index for specimens

Biological specimen (T4 Phage)	P5	Food (Whipped cream)	P8, 23
Biological specimen (Liposome).....	P10	Food (Coffee creamer / Casein micelle).....	P10, 17
Biological specimen (Flagellum)	P10	Food (White sauce in a frozen cream Korokke).....	P23
Biological specimen (Exosome).....	P10	Food (Ham)	P23
Biological specimen (Stathmine)	P11	Food (Mayonnaise)	P23
Biological specimen (Tublin).....	P11		
Biological specimen (Apoferitin)	P12	Cosmetics (Soap)	P17
Biological specimen (GroEL)	P14	Cosmetics (Cleansing oil).....	P17
Biological specimen (Hemoglobin)	P14	Cosmetics (Skin milk / Cream)	P17, 23, 24, 26
Biological specimen		Cosmetics (Foundation).....	P18, 24
(Liver / Small-intestine / Kidney of Rat)	P6, 15, 21		
		Material (Colloidal silica)	P18
Micro-organism (Virus).....	P12	Material (Paint).....	P22
Micro-organism (Lactic acid bacterium)	P20	Material (Glue).....	P22
Micro-organism (Yeast).....	P20, 28	Material (Packing film)	P30
Micro-organism (Fungus and Shiitake mushroom)	P21	Material (Rubber band)	P30
Plant (Camellia sasanqua leaf).....	P22, 26	Metal (Low-melting-point metal material).....	P30
Water droplet (A water droplet on a rose petal)	P25		

*Specifications are subject to change without notice.

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