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CSMS

Specimen preparation for SEM

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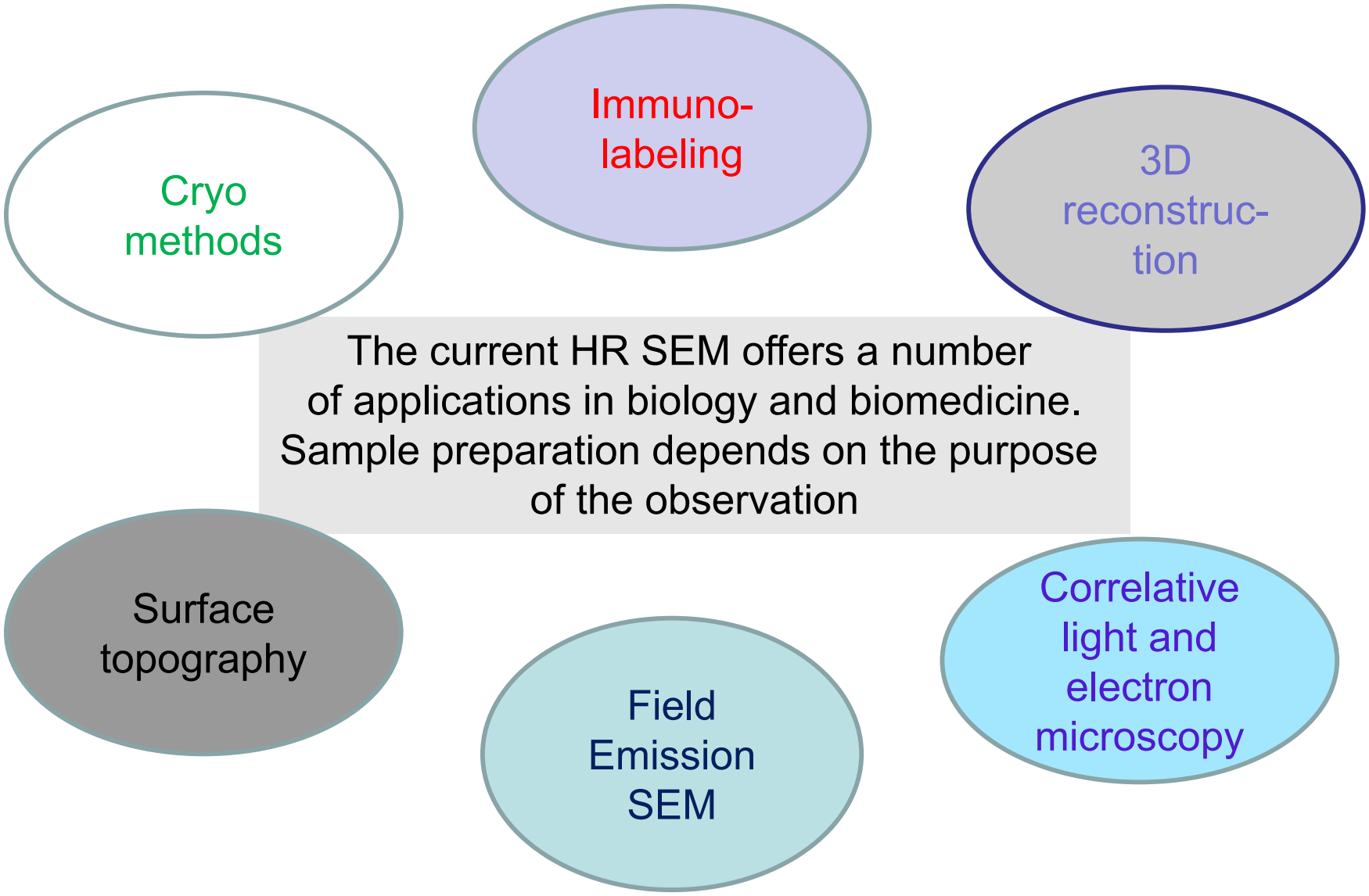
SEM Appplications



Surface topography

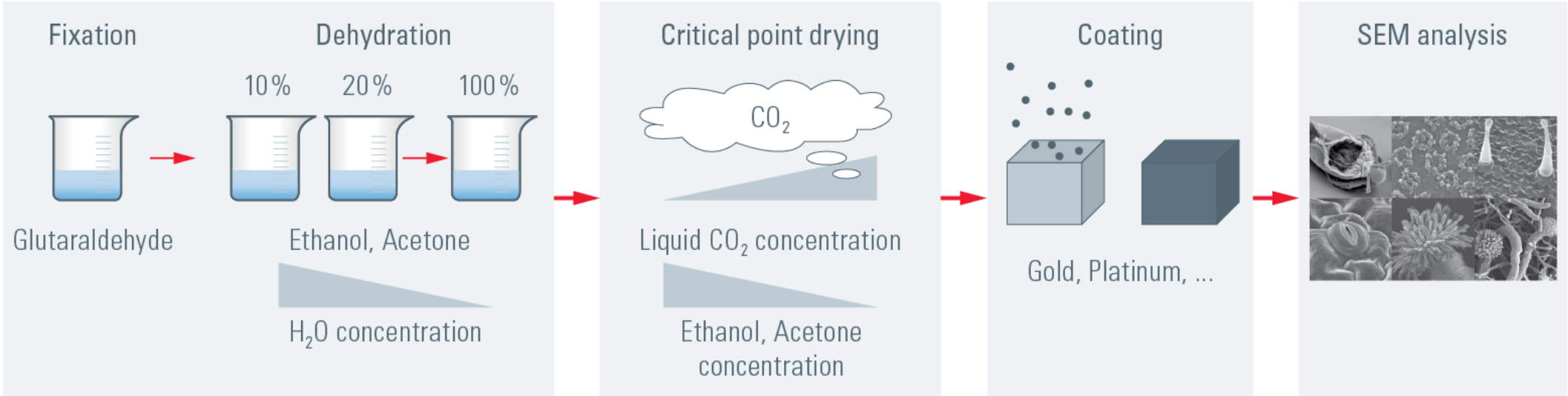
Specimen criteria for the EM examination

- Removing of water or other volatile components from the specimen:
  - totaly – HV SEM (dry specimen)
  - partly – LV SEM (70 %)
  - without – ESEM (wet)
- Ability to remain unchanged under high vacuum conditions
- Stability when exposed to electron beam
- Sufficient production of detected signals
- Stopping the changes associated with removing and processing of the sample
- Appropriate size for the SEM

Surface topography

Standard procedure of specimen preparation at RT

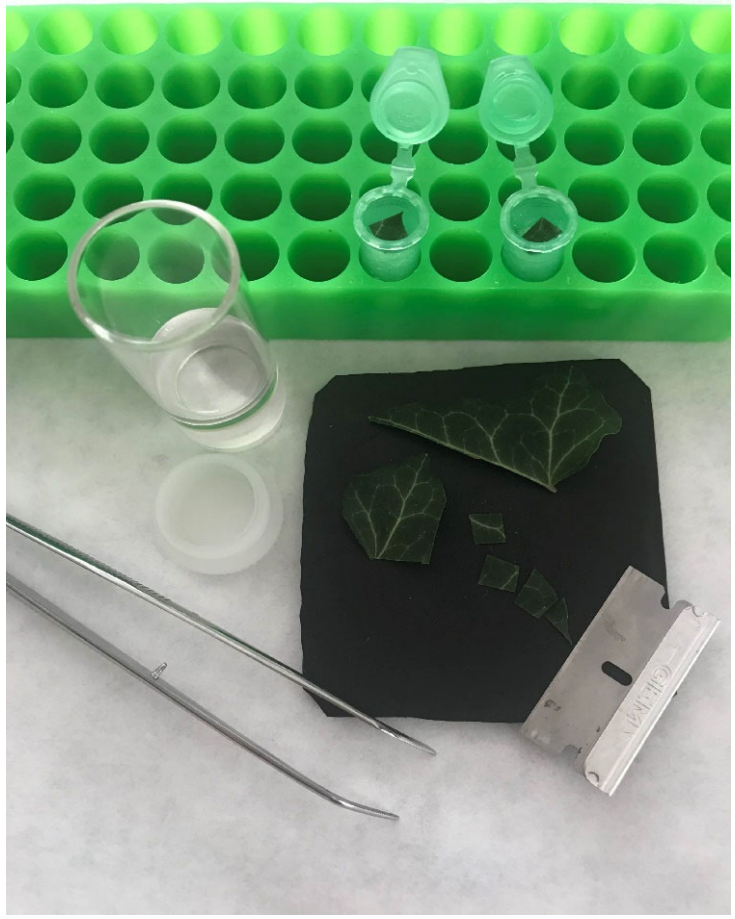
- Fixation
- Dehydration
- Drying
- Coating
- Examination in SEM



Surface topography

Chemical fixation

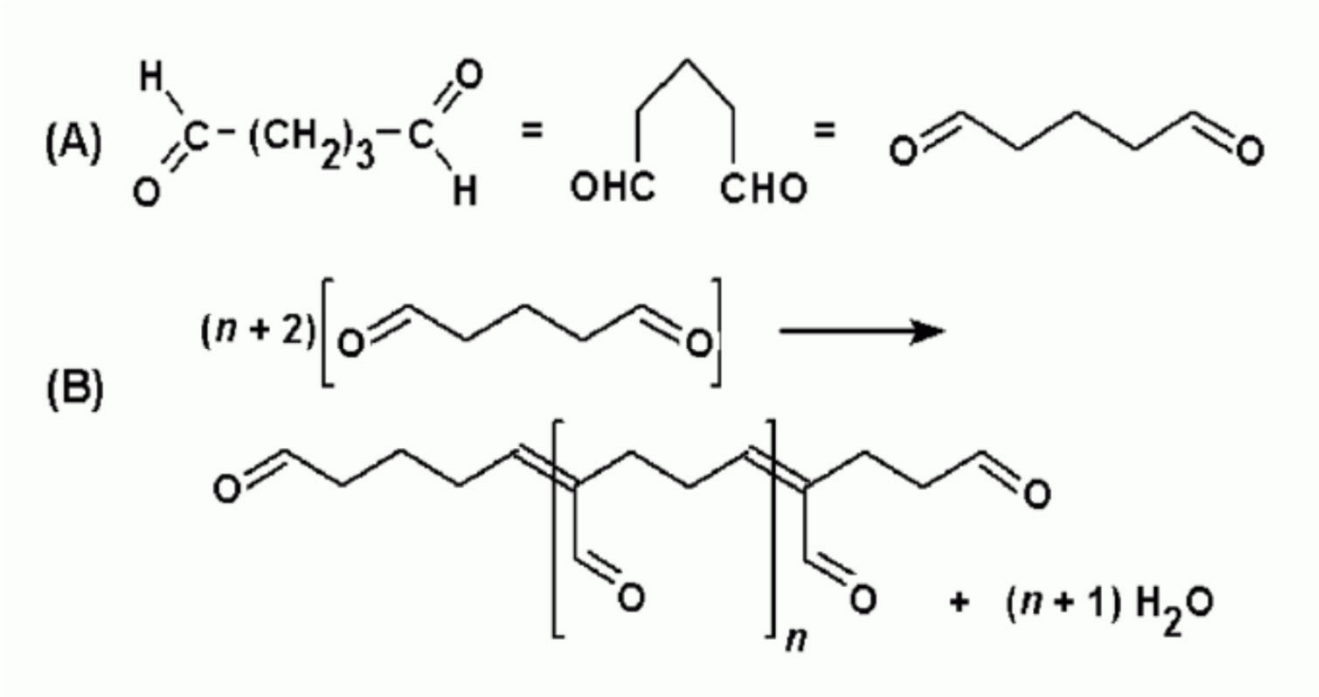
- To preserve cell and tissue organization as near as possible to the native state.
- To arrest living process in cells.
- To protect the sample against all damages in following preparation steps leading to minimal deterioration of fine structure.
- To arrest autolysis and bacterial decay.



Plant sample sizing

Glutaraldehyde $OHC-(CH_2)_3-CHO$

Introduced by Sabatini et al, 1962



- Aliphatic dialdehyde which forms colourless crystals that are highly soluble in water, ethanol and most organic solvents.
- In aqueous solutions, GA polymerizes into polymer chains with variable size.
- GA fixative solutions must contain monomer and low polymers with small molecules to penetrate cell membranes fast enough
- GA (EM grade,8-25 %) diluted in solutions (1-4%) are used for fixation, which are relatively stable with a pungent odour.

- GA is able to cross-link proteins rapidly, effectively and irreversibly and forms large, three-dimensional network throughout the cytoplasm in tenths of second to minutes

- GA in solution is uncharged and can thus rapidly cross all biological membranes.

Surface topography

Glutaraldehyde

- GA preserves mainly proteins, because it can react with several functional groups of proteins, such as amine, thiol, phenol and imidazol.
- GA cross linking preserves 3D structure of proteins
- It preserve also other mocromolecules associated with proteins (lipoproteins, histoproteins associated with DNA). GA cross-linking reactions are horribly complex leading to the formation of broad spectrum of conjugates.
- Glycogen may be preserved but the majority of carbohydrates will be extracted in the next preparation steps.
- Most lipids do not react with GA, with exception of phospholipids containing primary amines.
- Many enzymes remain active after aldehyde treatment. Molecules that are not immobilized may be relocated resulting in a false location or negative results in immunolabelling.

Surface topography

Glutaraldehyde

- The reaction is influenced by a ratio of GA to free amines (2:1), too high concentration of GA can inhibit the formation of the rapid cross-links
- The reaction with amines is accompained by a significant release of protons and ensuing drop in pH – buffered fixation solution
- The fixation process consumes oxygen - adding azide (inhibit respiration) or hydrogen peroxide hepl cross-linking.

Work with GA carefully! Avoid the contact with the skin and eyes,prolonged breathing. Reapeted exposure to GA may cause contact dermatitis. A well- ventilated hood, gloves and protective clothing are necessary!

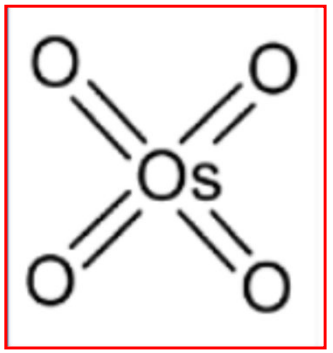
Surface topography

Formaldehyde H-CHO

- At room temperature FA is a colorless gas highly soluble in water. Both liquid and gas polymerize spontanously.
- FA is commercially available as a concentrated aqueous solution (Formalin) or in a polymerized state as a dehydrated powder – paraformaldehyde (PFA).
- FA molecule is smaller than GA, thus rapidly penetrates into sample (5 times faster than GA).
- Karnovsky‘s fixative – the combination of FA and GA (recommeded for larger size samples with a poor penetration).
- Substances such as carbohydrates, lipids and nucleic acids are trapped in a matrix of insolubilized and cross-linked protein molecules but they are not chemically changed by formaldehyde.
- FA links weakly proteins, therefore it is a prefered fixative for immunolabeling techniques.

Surface topography

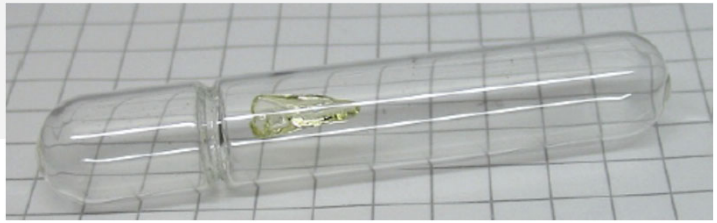
Osmium tetroxide



Introduced by Claude in 1948

- The molecule is symmetrical and contains four double bonded oxygen atoms.
- Osmium can exist in nine oxidative states, five of which are quite stable
- Osmium tetroxide is soluble in both polar (aqueous) and non–polar media. Thus can fix both hydrofobic (e.g. membrane phospolipids) and hydrofilic domains in cells.
- It is highly volatile, it can be used also as a vapor fixative.
- Os is electron opaque, works as a stain, as well as fixative.
- Osmium tetroxide also act as a mordant, it enhances lead staining.
- It causes rapid permeabilization of membranes with cessation of cytoplasmic movement within second to minutes.
- It is the most slowly penetrating fixative and has no cross-linking capabilities.
- It reacts with ethanol to form black precipitates:

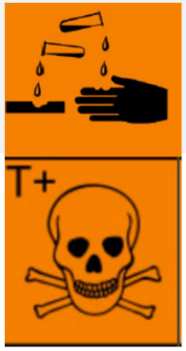
$$OsO_4 + C_2H_5OH = OsO_2 + 2\ CH_3CHO + 2\ H_2O$$



Surface topography

Osmium tetroxide

- Interact directly with unsaturated lipids oxidizing double bonds, leading to the formation of monoesters, diesters and dimeric monoesters.
- Specimens generally turn black after osmification.
- It causes hardening of tisues.
- Osmium tetroxide can also react with some proteins and lipoproteins complexes. Prolonged fixation results in the progressive denaturation of proteins.
- As a strong oxidant it damages the majority of antigents
- Limited penetration into tissue (200 μm)



Be extremely careful in handling OsO_4. It is dangerous to eyes, respiratory and alimentary membranes. Used or excess of OsO_4 must be stored in sealed glass containers. Avoid the direct contact by wearing plastic gloves and working in well-ventilated hood.

Surface topography

Fixation solution

- Glutaraldehyde, formaldehyde: 1-4%
- Osmium tetroxide: 1-4%

- Buffers - phosphate, cacodylate, HEPES…
- Other substances improving fixation like hydrogen peroxide, tannic acid etc.
- Isotonic or slightly hypertonic solution

Surface topography

Dehydration

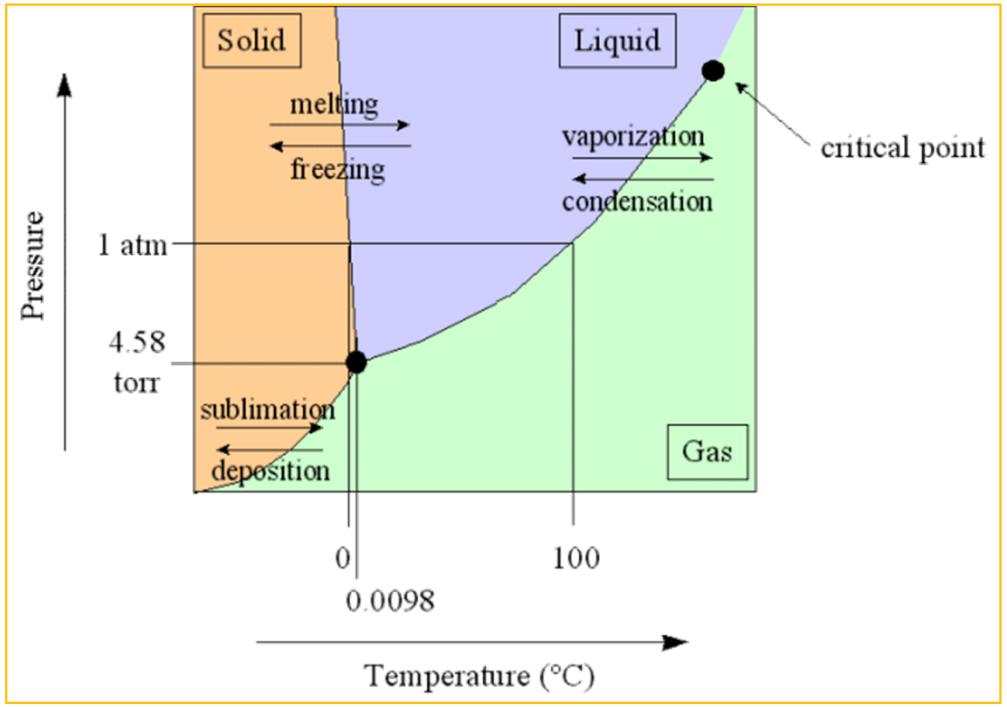
- To replace water from samples by organic solvents (ethanol, acetone, propylene oxide)
- Caused shrinkage, extraction of various celular components, changes in shape and size of sample
- Usually performed using a solution series with increasing concentration of organic solvent

Dehydrants are hygroscopic, they are able to absorb water from the air. It is important to keep dehydrants sealed!

Surface topography

Drying

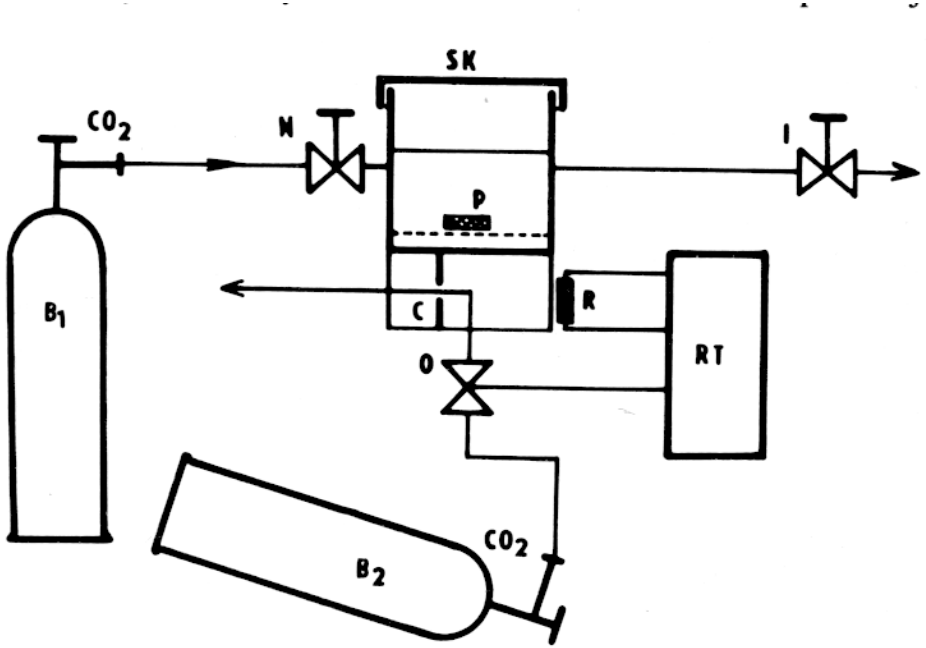
Critical point drying method - phase change from the liquid to the dry gas without the effects of surface tension on a specimen



CRITICAL CONSTANTS		
Substance	Temp. °C	P.S.I
HYDROGEN	-234.5	294
OXYGEN	-118	735
NITROGEN	146	485
CARBON DIOXIDE	+31.1	1072
CARBON MONOXIDE	+141.1	528
WATER	+374	3212

Surface topography

CPD method



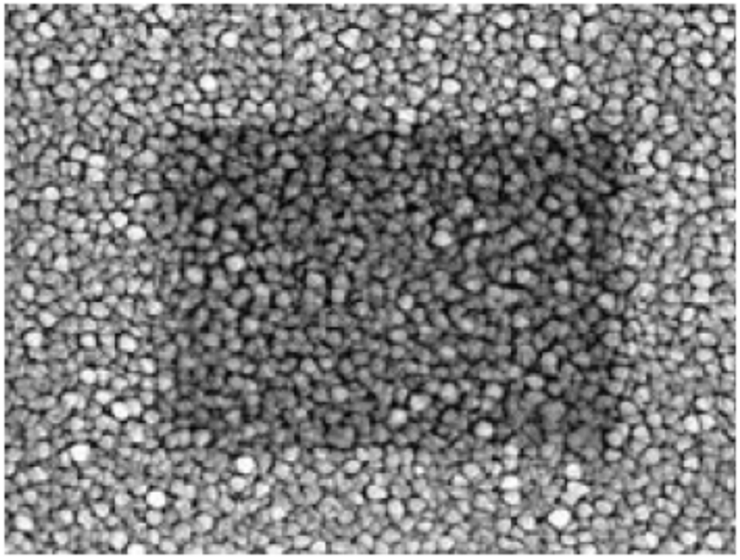
The ethanol/aceton is replaced by liquid carbon dioxide, then the CO_2 is brought to its critical point and converted to the gaseous phase without crossing the interfaces between liquid and gaseous avoiding the damaging effects.

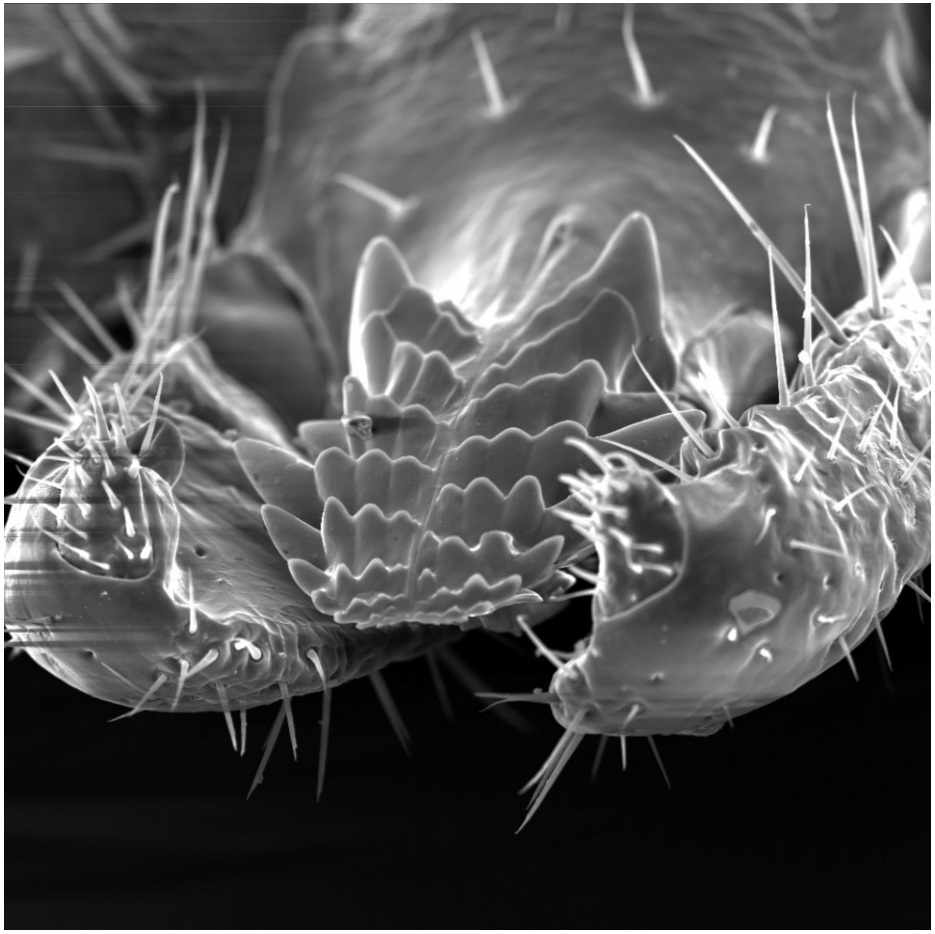
Surface topography

Specimen Coating

Charging – a material cannot conduct the beam energy imparted to it.

Contamination – the interaction of the electron beam with residual gases and hydrocarbons on the specimen surface





Surface topography

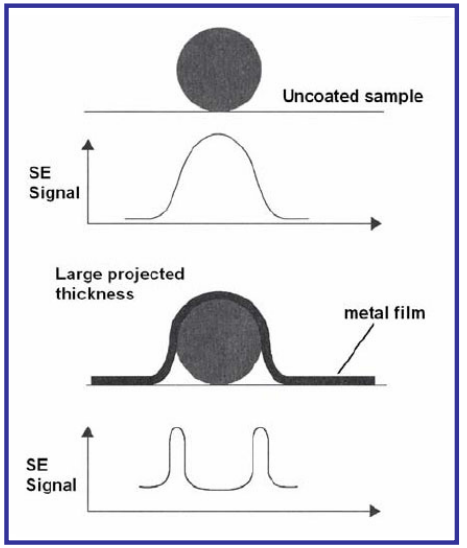
Specimen mounting

- The dry specimen is mounted on a metal stub using a sticky carbon/copper disc/tape which increases conductivity. Silver-containing glue can additionally be applied for even more conductivity.
- Dry biological samples are not conductive. To prevent charge build-up on specimen surface, it is coated with a conductive material. The metal is applied in a controlled manner in special devices. It is critical that the coating is thick enough to prevent charging (typically around 10 nm) but not thick enough to obscure specimen surface details

Surface topography

Specimen Coating

- increasing of the conductivity of dry specimen
- reduction of the charging efect
- reduction of thermal damage
- improvement of SE and BSE emission



Element	Z	Thermal conductivity at RT (W/cm/K)	Resistivity at RT (W .m)
Carbon	6	1.29	3.5x10-5
Aluminium	13	2.37	2.82x10-8
Palladium	46	0.72	1.1x10-7
Silver	47	4.29	1.60x10-8
Platinum	78	0.72	1x10-7
Gold	79	3.17	2.44x10-8

Surface topography

Specimen coating

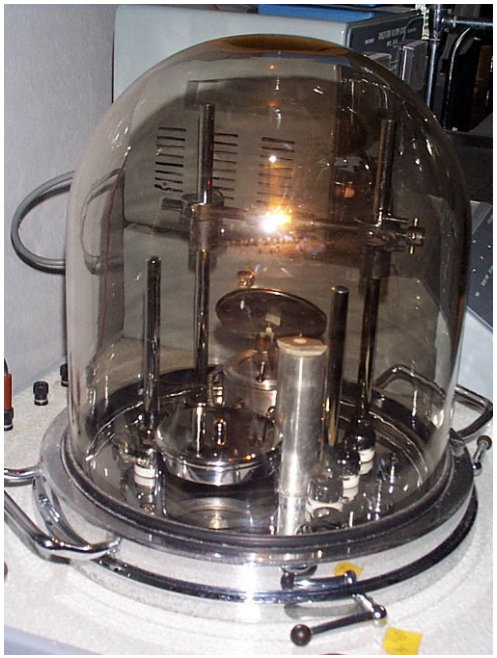
The most important parameters – film thickness, homogeneity and granularity
The film thickness depends – deposition rate, sputtered materiál, the distance between the metal target and sample position

The measurement of film thickness:
Quartz crystal thickness monitor
Contaminations:
The cleanness of vacuum chamber
The film granularity:
Selection of coating Metals.
Au, Pt, Pd for HR
W, Ir, Ch for ultra HR

Sputter coater

BalTec SCD050

Vacuum evaporator



JEOL JEE4C

Surface topography

Special methods

- Fixation in OsO_4 vapors:
- the sample is enclosed in a sealed container together with the crystal of OsO_4 and placed in the freezer for 2-3 weeks. Fixation with OsO_4 vapors strengthens the sample surface, which then gradually dries without any manipulation and deformation.
- Suitable for gentle samples

Surface topography

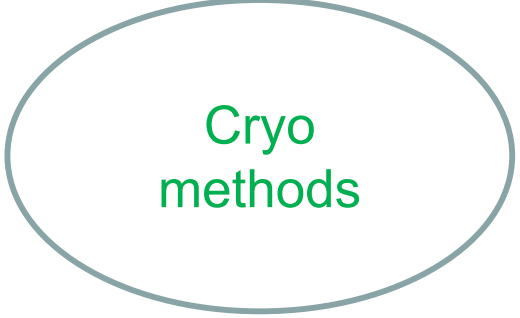
Special methods

- Dehydration with t-Butyl Alcohol:
- Ethanol is used as a dehydration agent. A sample in 100% ethanol is transferred to 100% t-BA at a temperature above 25°C.
- Then the sample is placed in the refrigerator (6 °C). Amorphous solid tBA is sublimated using a rotary vacuum pump.
- Suitable for gentle samples

Surface topography

Artifacts

- Sample compression or shape changes caused by dehydration and high pressure during CPD
- Insufficient or irregular thickness of conductive layers can cause charging
- Microwave irradiation can be used in each step of specimen preparation and improves the sample preservation



Cryo methods

Advantages:

- structure preservation close to native state
- no fixation, no dehydration, no coating
- low accelerating voltage (>5 kV)
- observation of time depending processes in cells

Disadvantages:

- expensive equipment (cryo-attachment)
- necessity to store the sample in liquid nitrogen

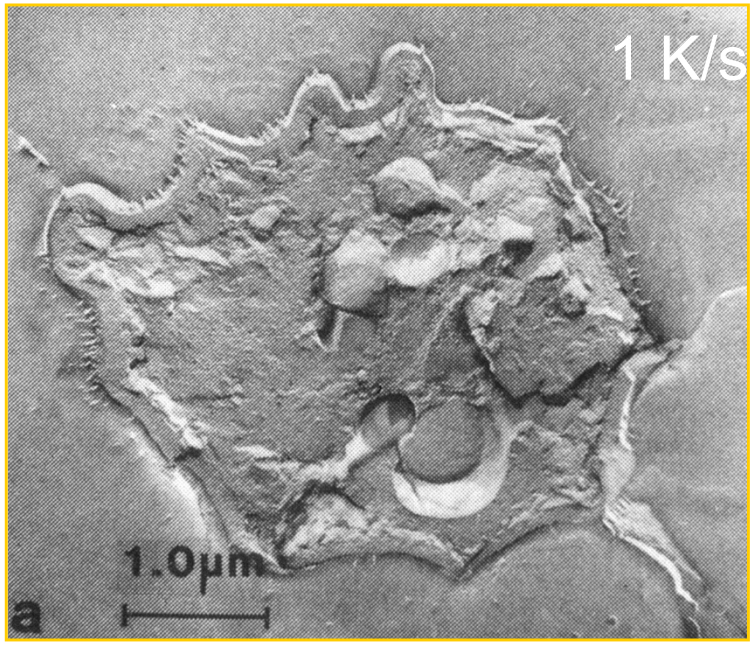
Cryo methods

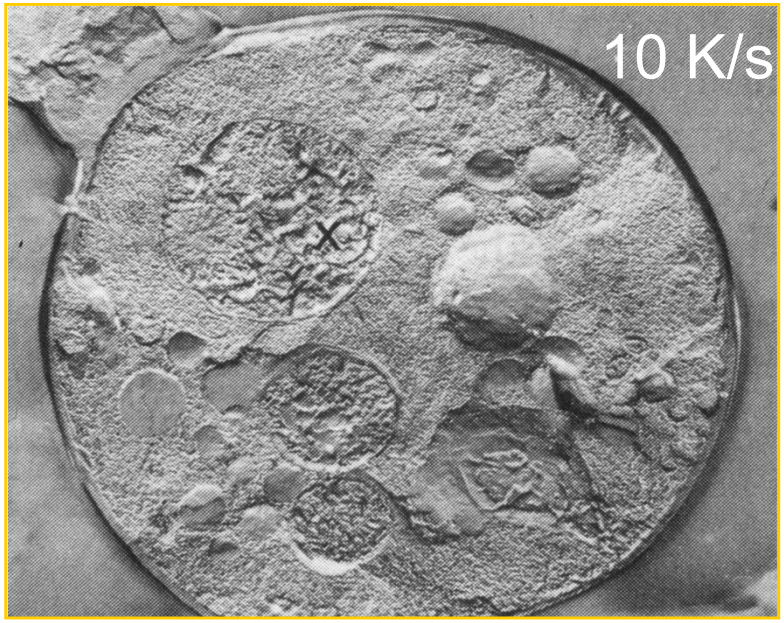
Cryo fixation

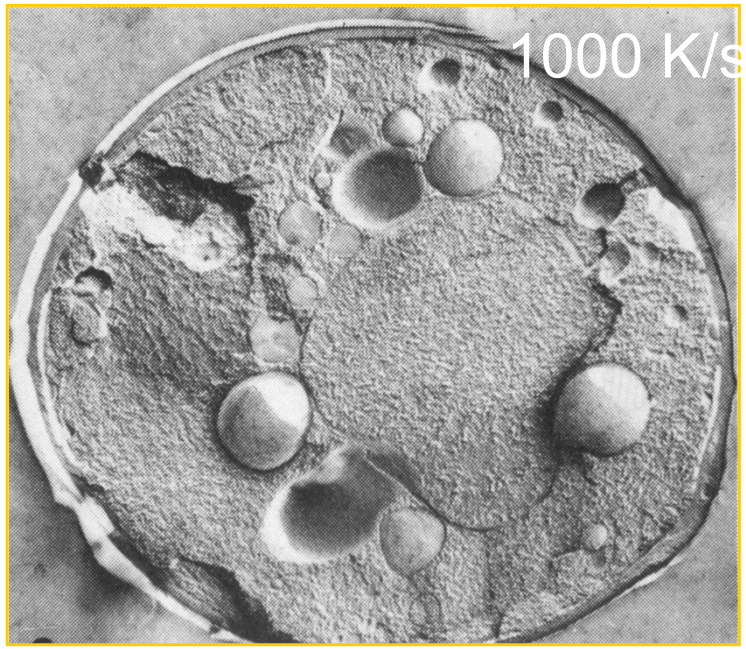
- Cryo-fixation - based on vitrification of samples without ice crystal formation during cooling (amorphous ice)
- High pressure freezing (HPF) – the best methods of vitrification of bulk samples containing water (biological samples, hydrogels etc.)
- *HPF - Moor H, Riehle U (1968) Snap-freezing under high pressure: a new fixation technique for freeze-etching. In: Bocciarelli DS (ed) Proceedings of the fourth European regional conference on electron microscopy vol 2. Rome, pp 33–34*

Cryo methods

Cryo - fixation





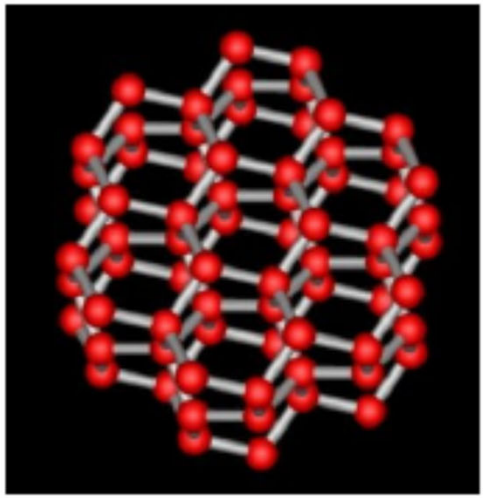


Cooling speed
> 10^5 K/s

Robards AW, Sleytr UB, Low Temperature Methods in Biological Electron Microscopy. In: *Practical Methods in Electron Microscopy,* Glauert AM (ed), vol 10, Elsevier, Amsterdam, 1985.

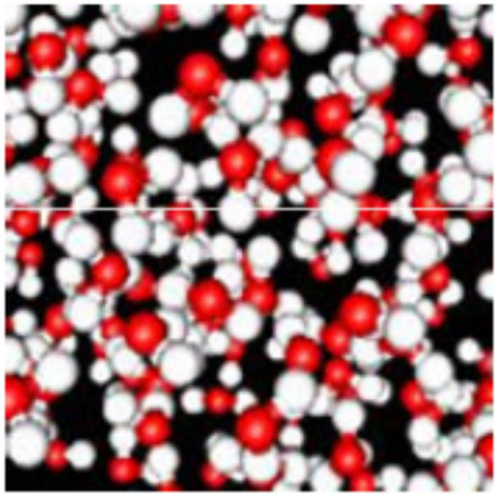
Freezing and vitrification

Vitrification *(from Latin vitreum, "glass" via French vitrifier) is the transformation of a substance into a glass.*



crystalline ice

-lower density than water in liquid state



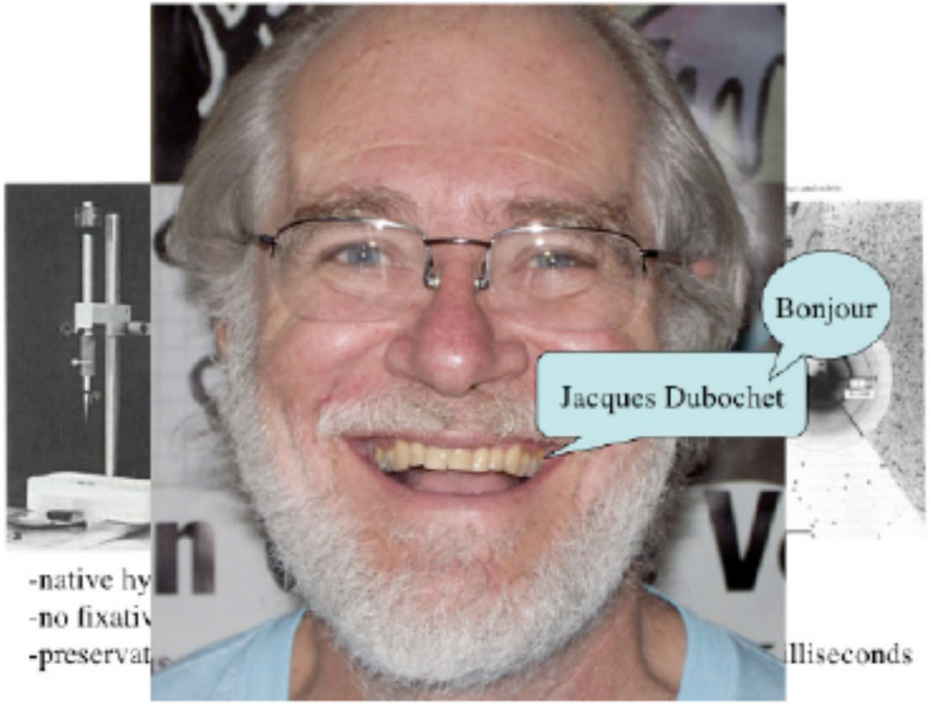
vitreous = amorphous = glassy ice

-density of liquid water and vitreous ice is about the same

- no segregation of solutes and solvents

Vitrification and amorphous ice

- **Brügeller P and Mayer E. 1980 –** vitrification of water
- **Dubochet and McDowel** introduced water in electron microscopy. Discovery of water vitrification and development of cryo-electron microscopy (1981) .



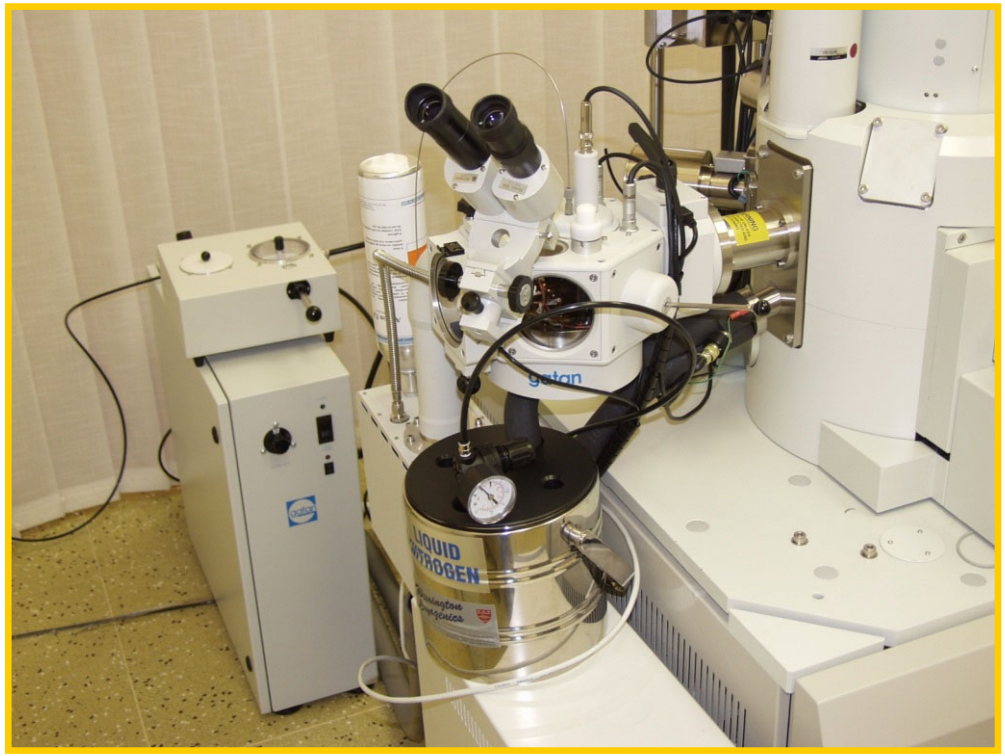
Water becomes solid while remaining in amorphous state.
Cooling rate must be high enough that the crystals do not have time to form

Cryo methods

Cryo - SEM

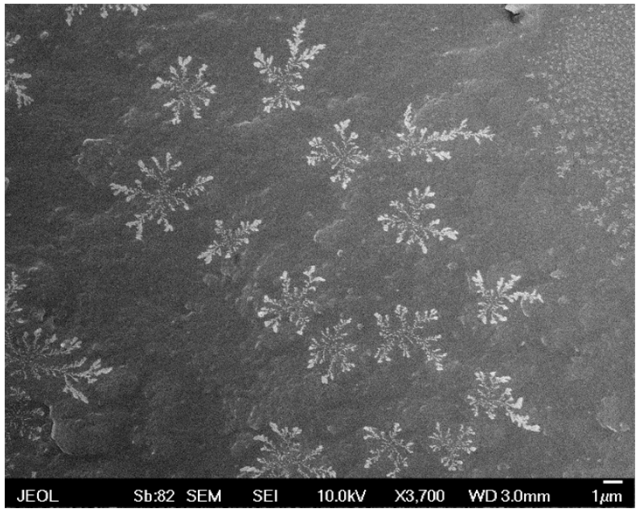
- Direct observation of frozen specimens in FESEM equipped with cryo-attachment

1. Cryofixation with slushy nitrogen
2. Transfer to preparation chamber under vacuum
3. Treatment of sample: Fracturing, etching, coating
4. Transfer to the cold stage of SEM and the examination



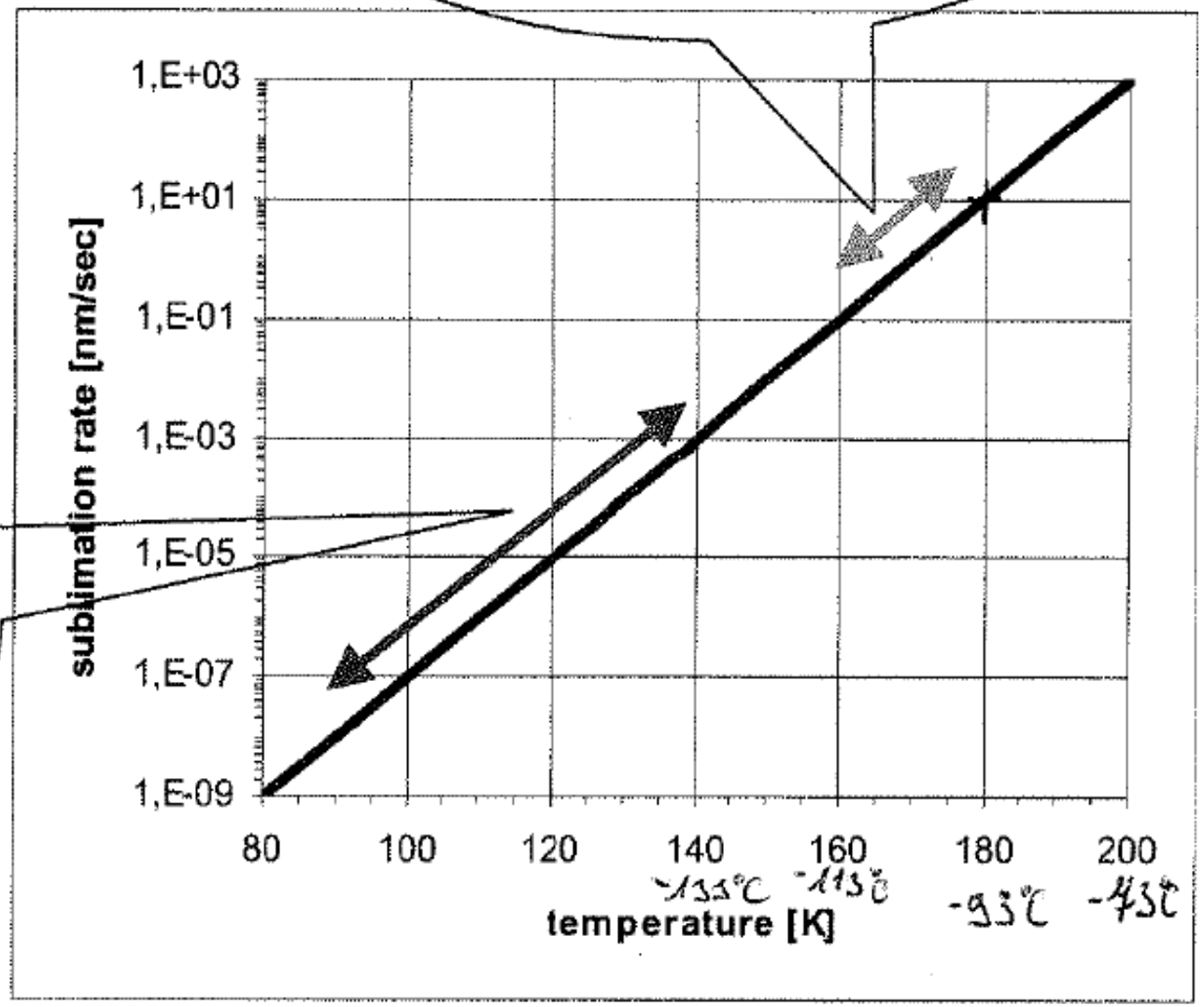
Cryo methods

Ice sublimation



Sublimation
(freeze etching)
T~160K to 180K
(-110°C to –90°C)

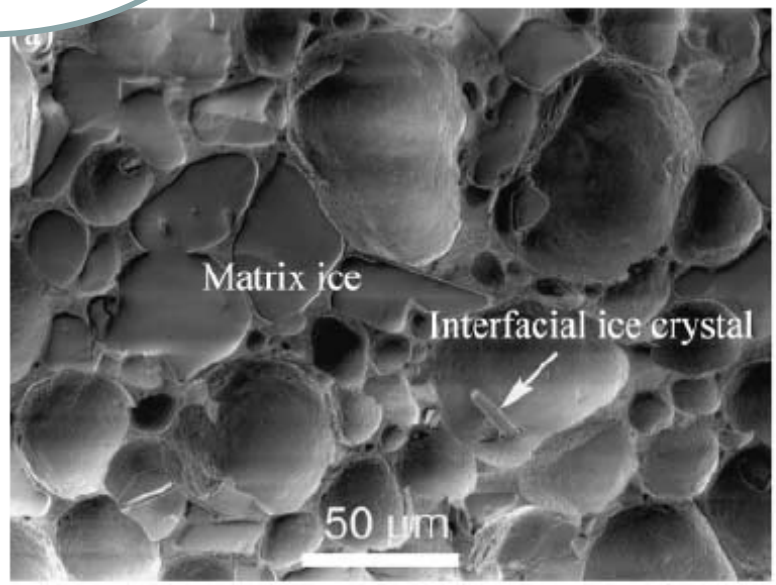
Examination:
T < 140K (-130°C)
Sublimation rate
<0,001nm/s
<4nm/h

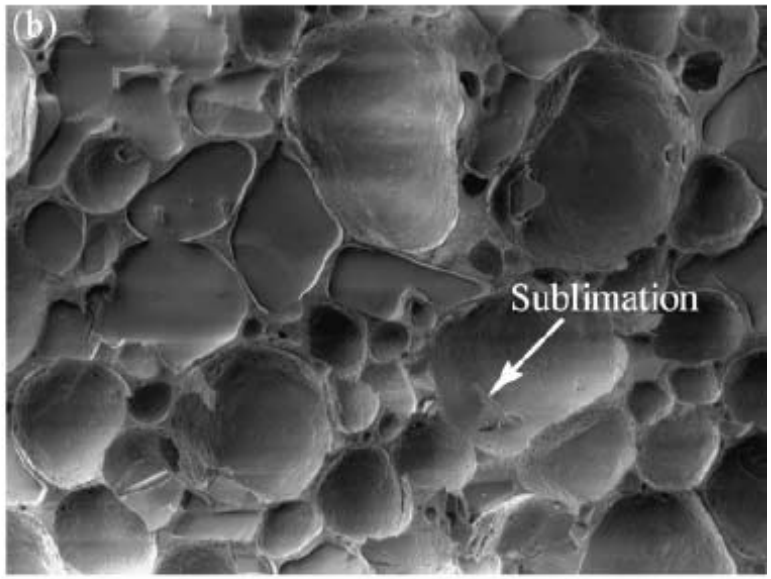


Cryo methods

Ice sublimation of ice cream

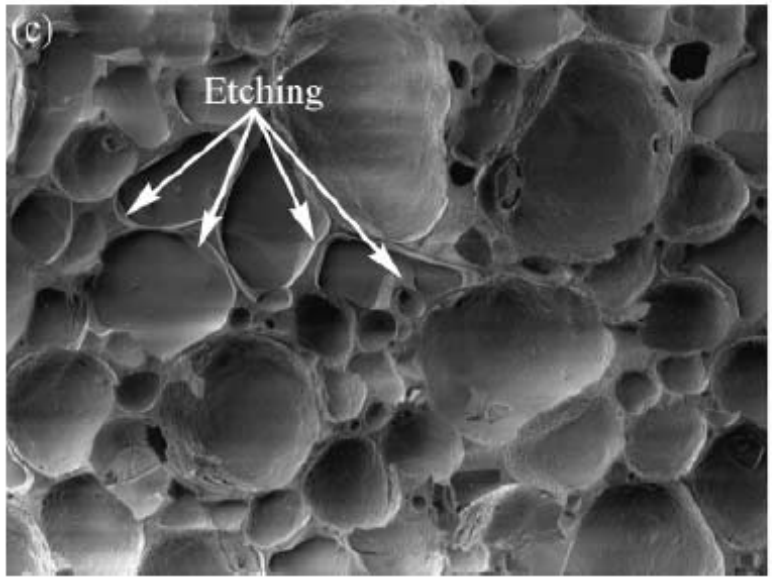
5min
-110°C

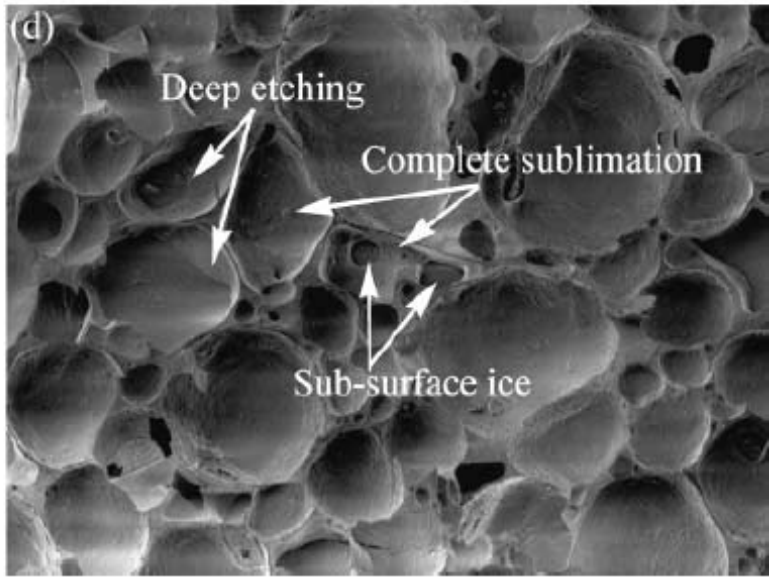




5min
-100°C

7min
-95°C



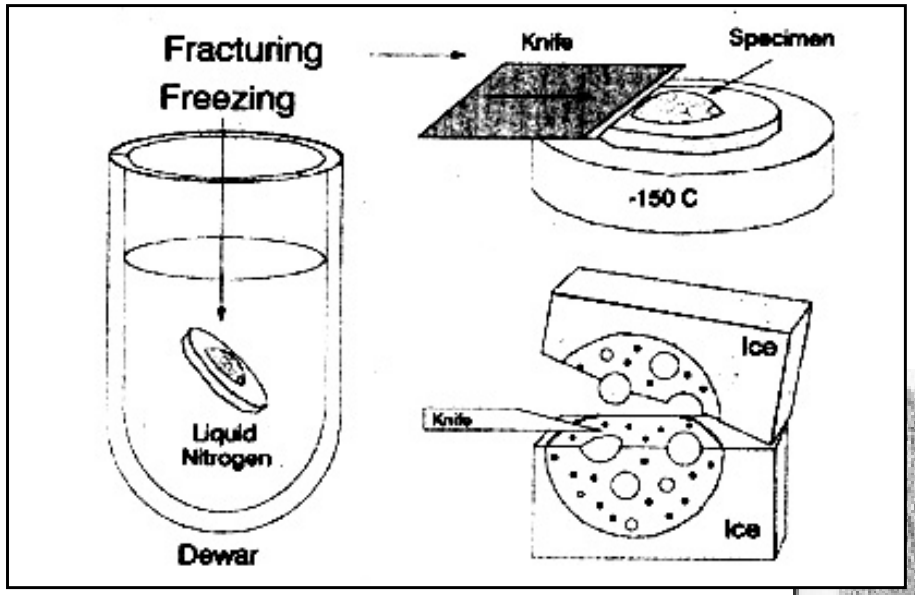


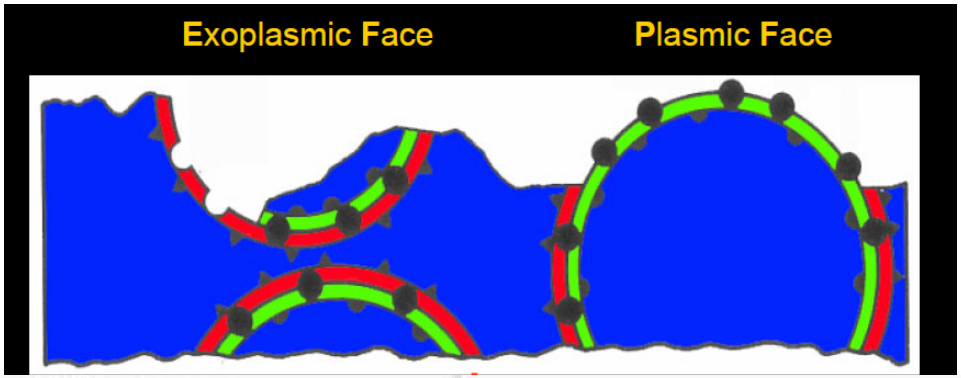
4min
-90°C

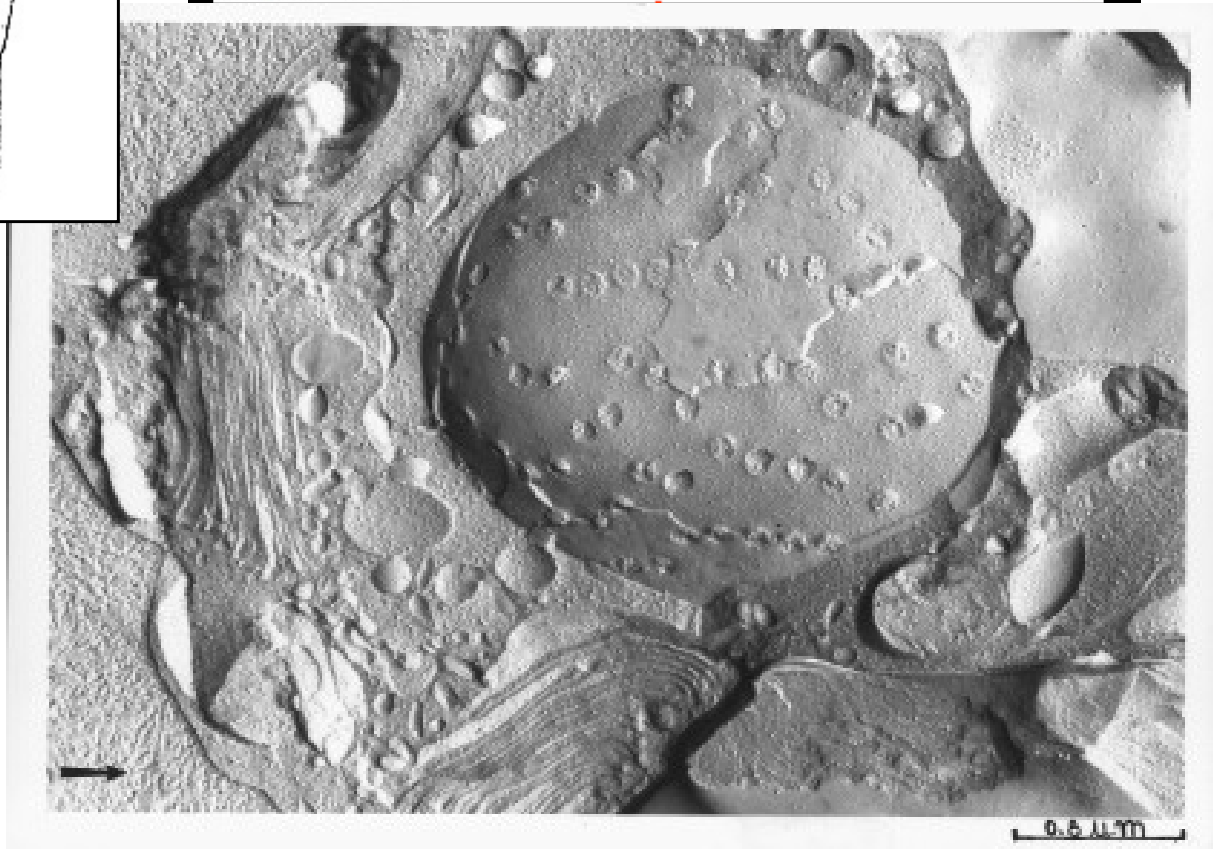
Stokes D.J. et *all,Journal of Microscopy*, 213, 2004,198-204

Cryo methods

Freeze fracturing







The technique used to look at inner ultrastructure of a frozen sample

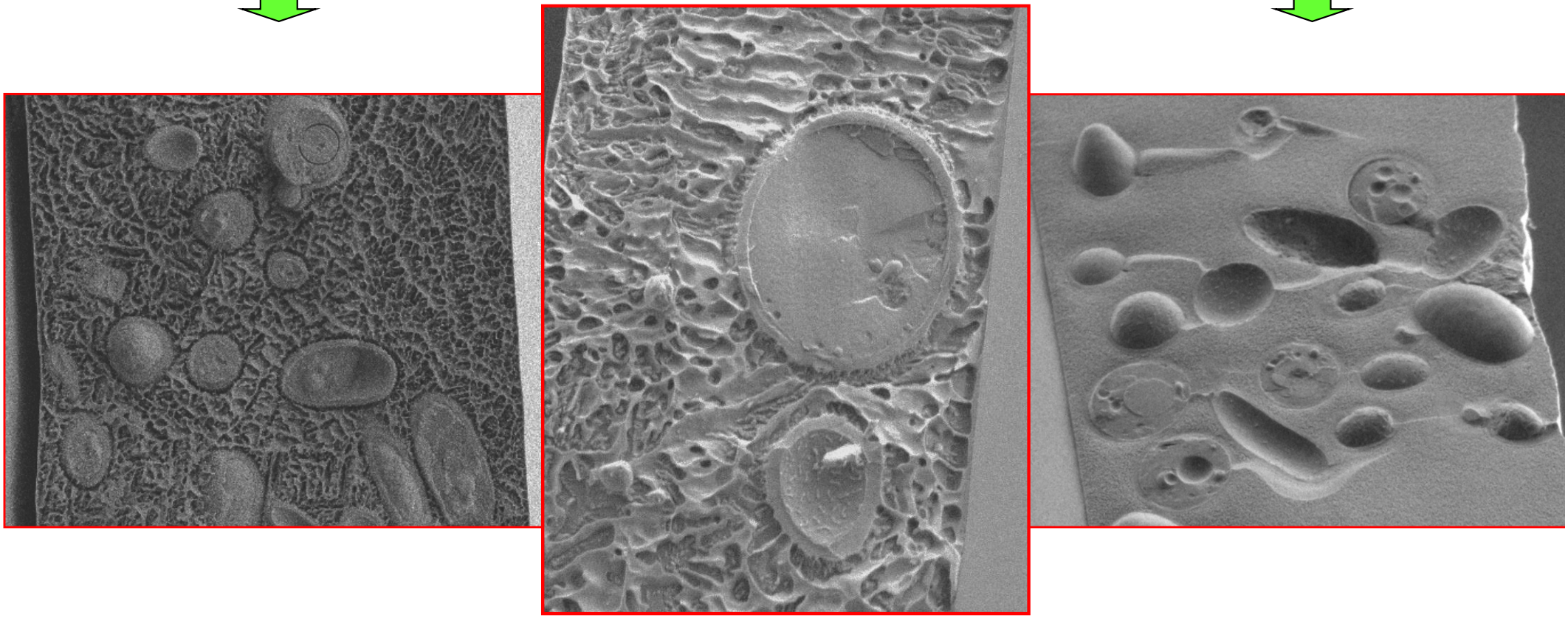
Cryo methods

Characterization of yeast biofilm

plunging into LN_2

plunging into liquid propane

high pressure freezing

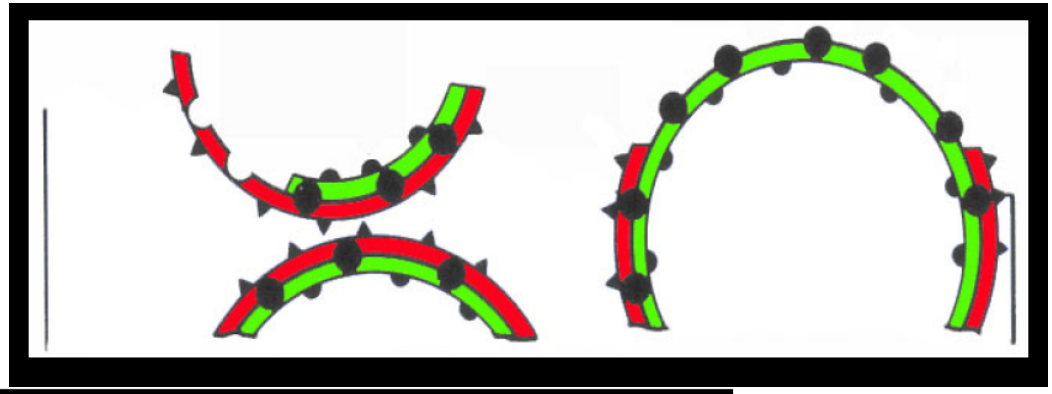


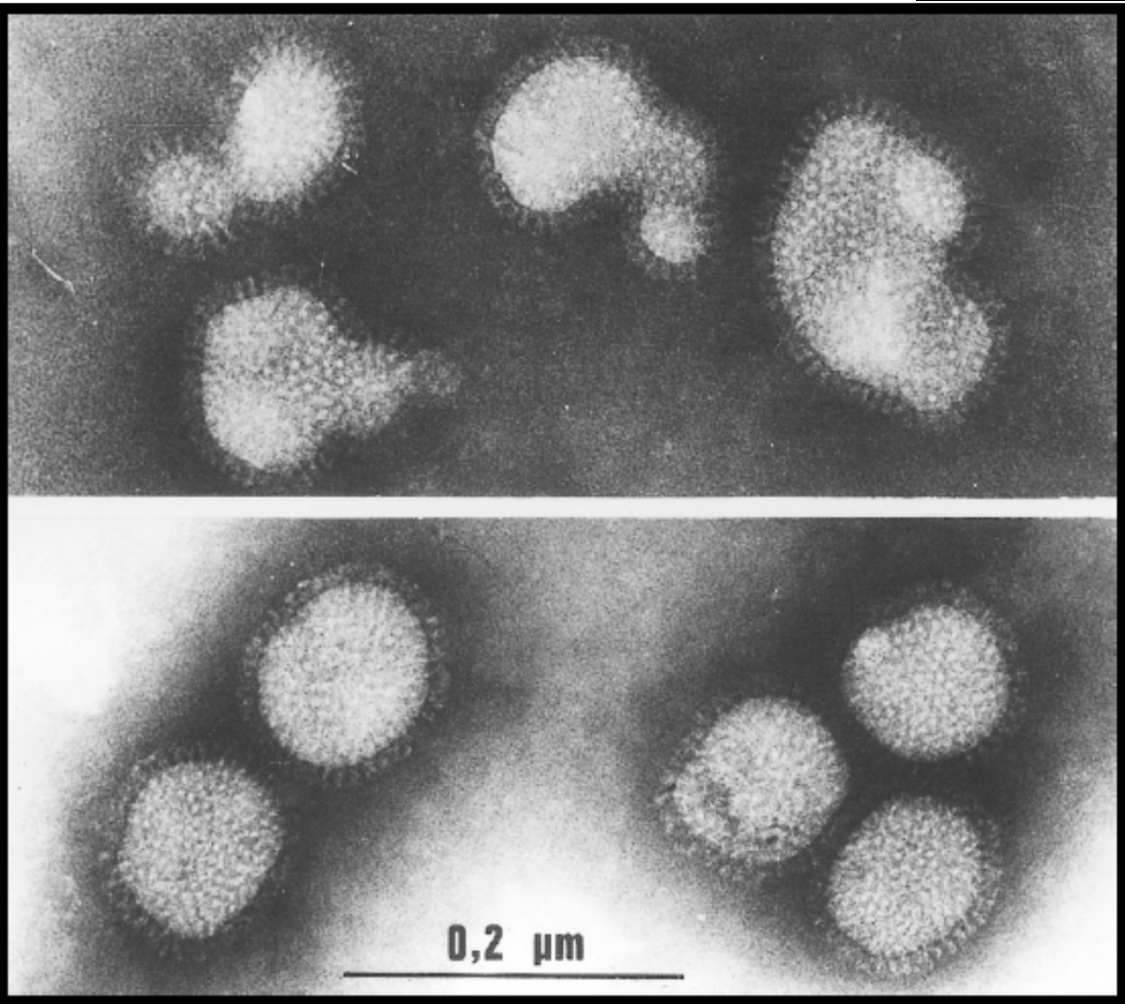
C. parapsilosis and C.albicans

K.Hrubanova et al.: Micron,110,2018

Cryo methods

Freeze drying





Air-dried

Freeze-dried

Nermut, M.V. and Frank, H. (1971) Fine structure of influenza A2 as revealed by negative staining, freze-drying and freze-etching. J. Gen. Virology 10, 37-51.

Cryo methods

Freeeze substitution

Living biological specimen

↓

Cryofixation (HPF) — HPF, plunge freezing

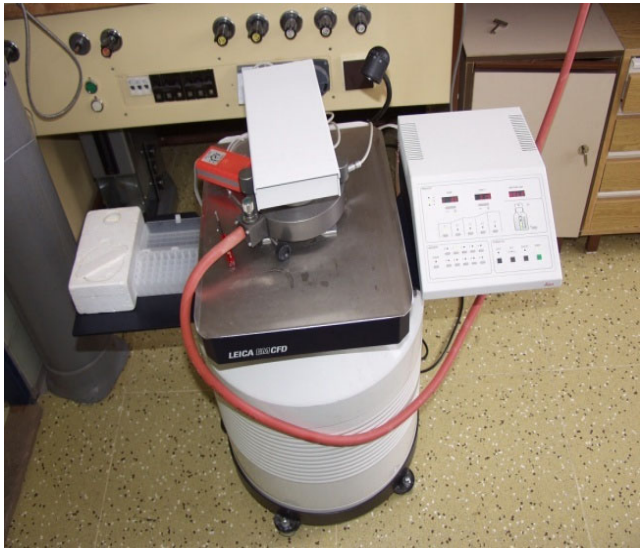
↓

Freeze substitution — Aceton with GA, OsO_4

↓

Drying (CPD)

- The combination of chemical methods with cryomethods
- The ice is replaced at low temperature by anhydrous solvent
- The organic solvent must be liquid at low temperature and dissolve additives such as osmium tetroxide or GA
- Reduced shrinkage and shape changes
- Resulting in dry samples

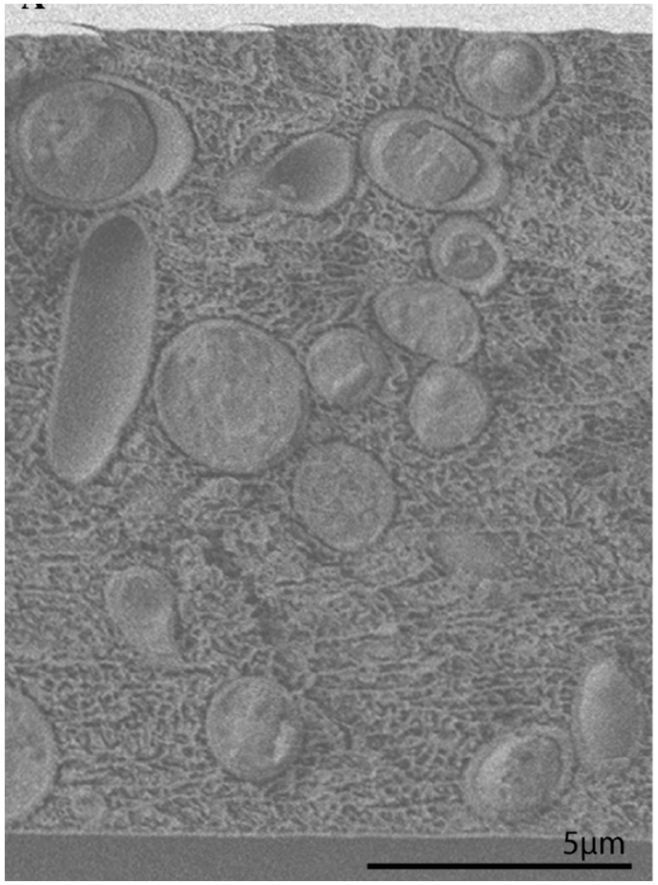


FS unit Leica
Temperature range:
-90°C - 20 °C

Cryo methods

Application: Characterization of yeast biofilm

Candida parapsilosis grown on the glass substrate and fixed by plunging in nitrogen slush

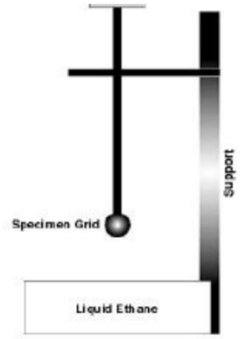


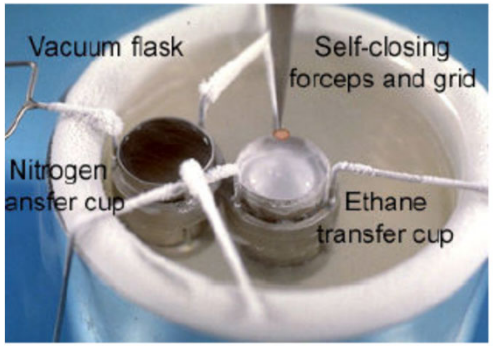
V.Krzyzanek, K.Hurbanová, J.Nebesářová, F.Růžička: Novel technique In cryo-SEM freeze fracturing demonstrated on microbial film. Submitted

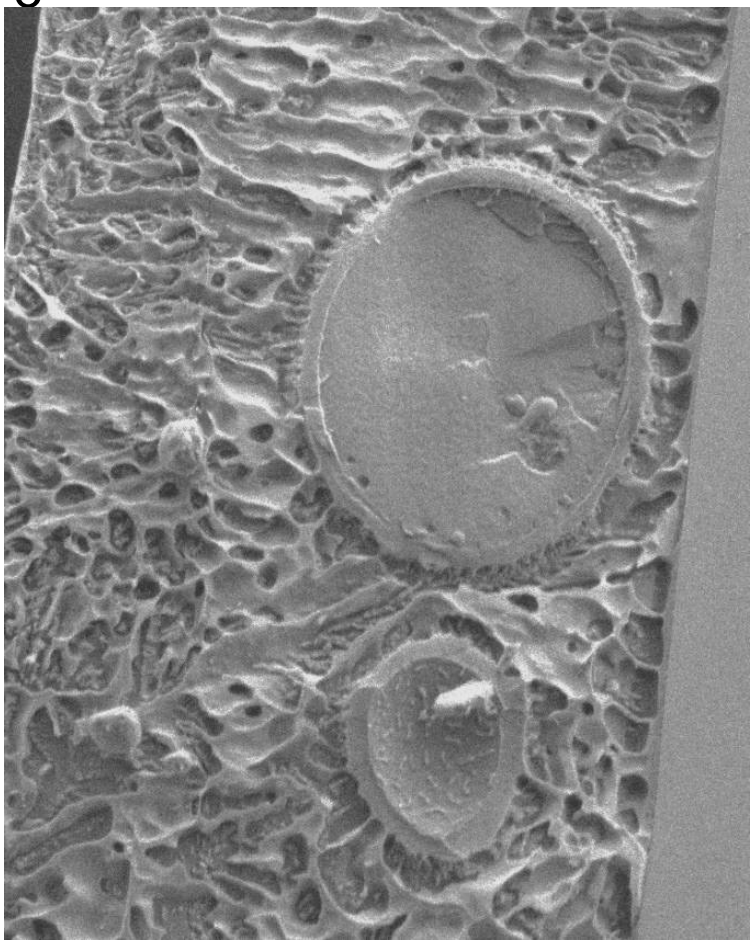
Cryo methods

Application:
Characterization of yeast biofilm

- Freeze fracture of *Candida parapsilosis* grown o
- n a saphire discs and
- fixed by plunging in
- liquid ethan/propan

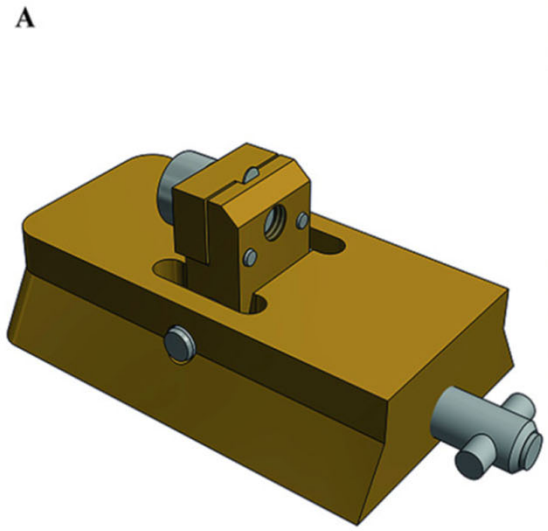


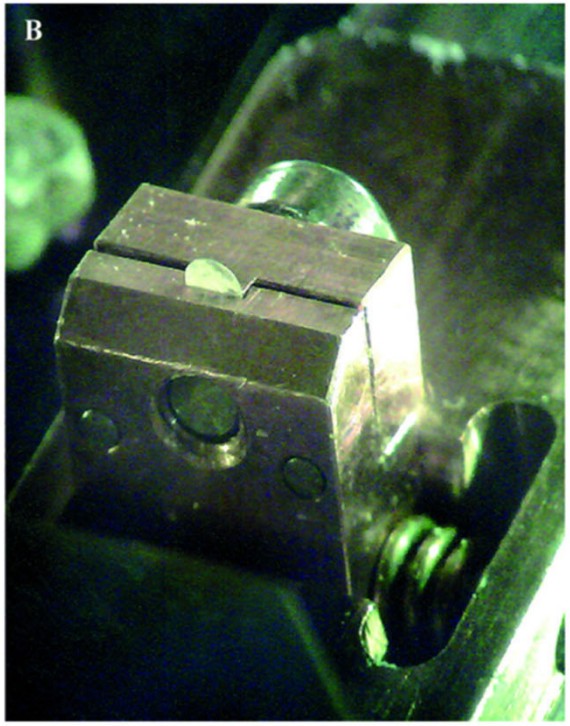




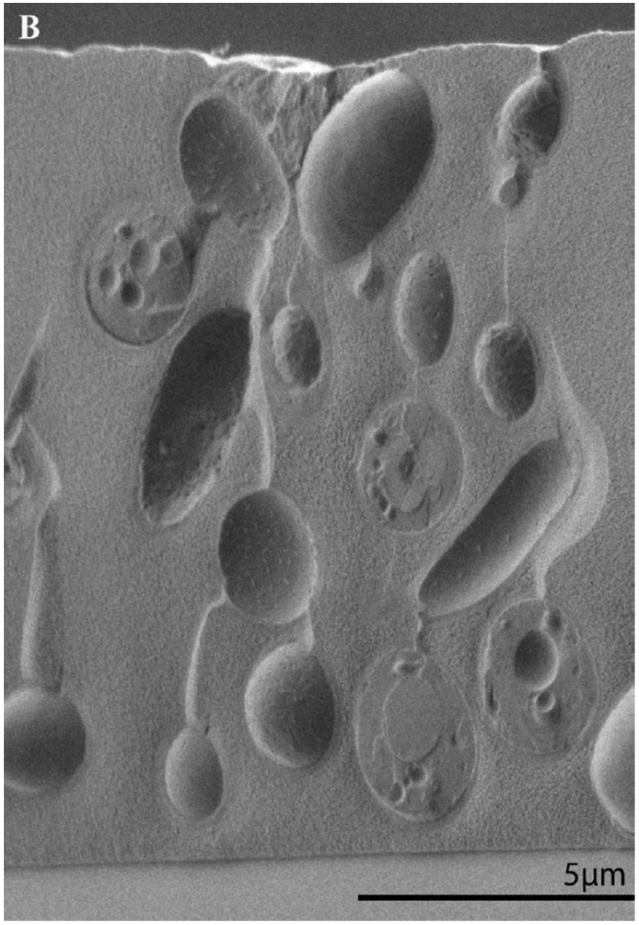
Cryo methods

Application:
Characterization of yeast biofilm



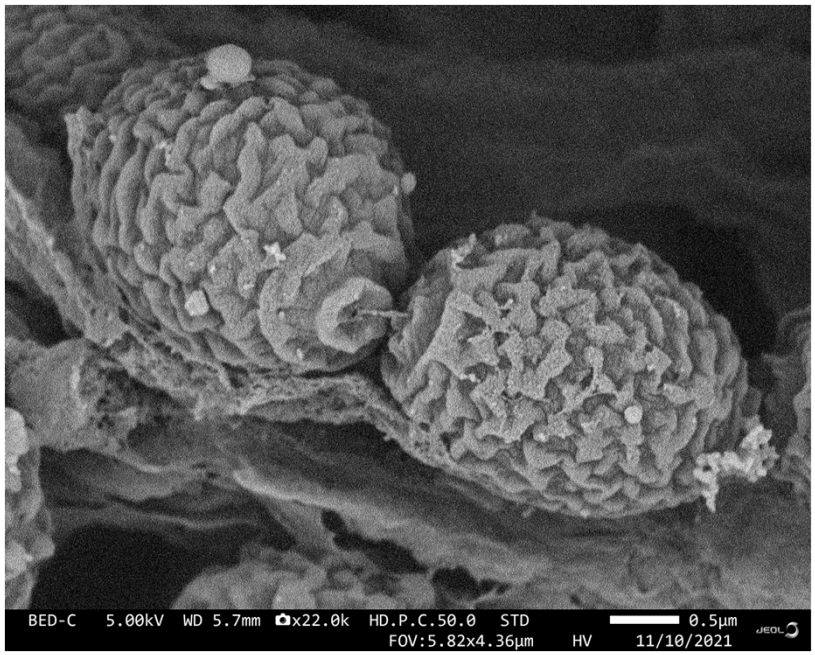


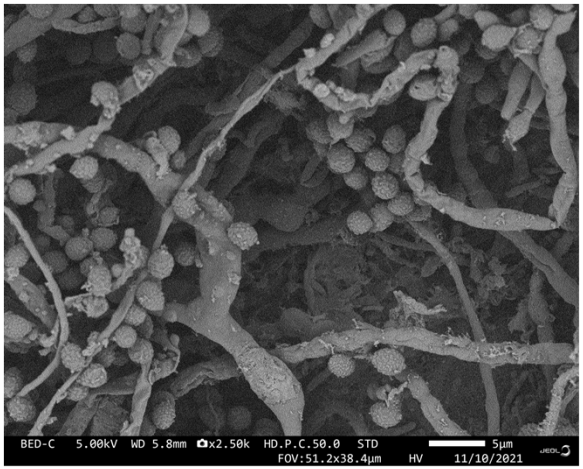
HPF Leica EM Pact II

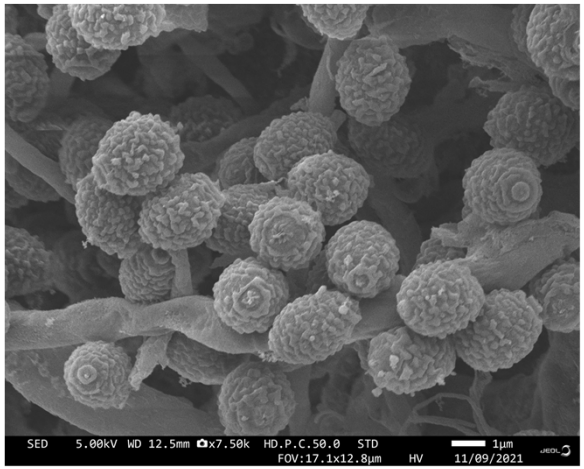


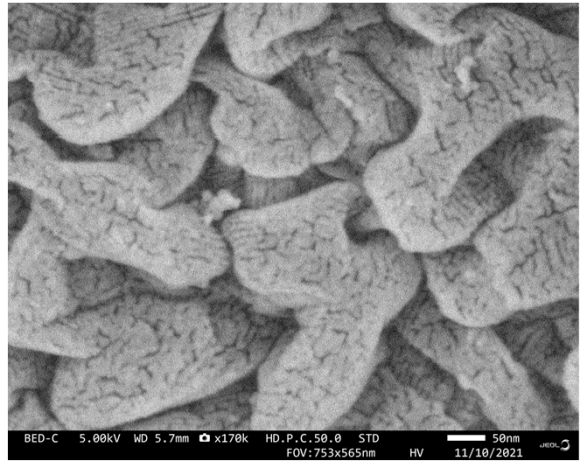
Penicillium

Fixation: 1% OsO_4
Dehydration: aceton
Drying: CPD
Coating: Au



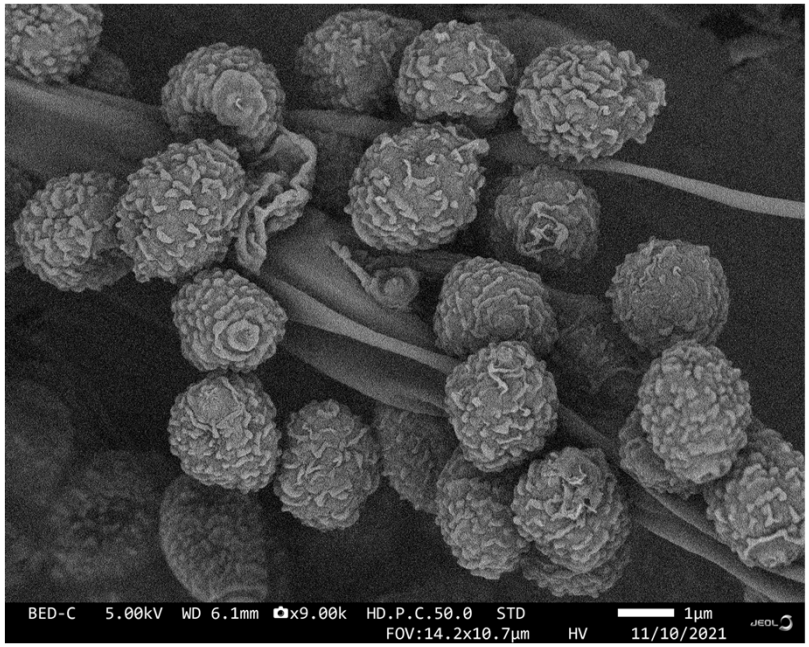


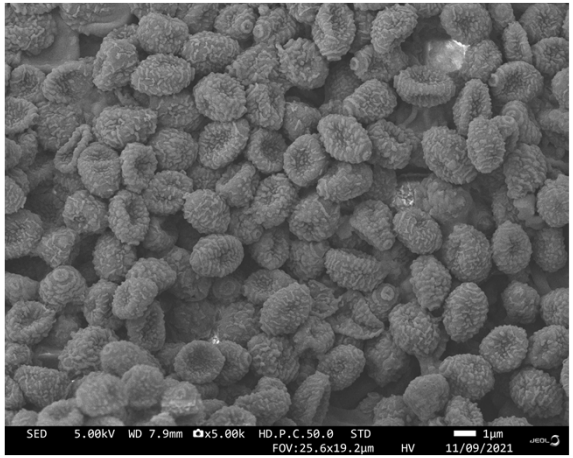


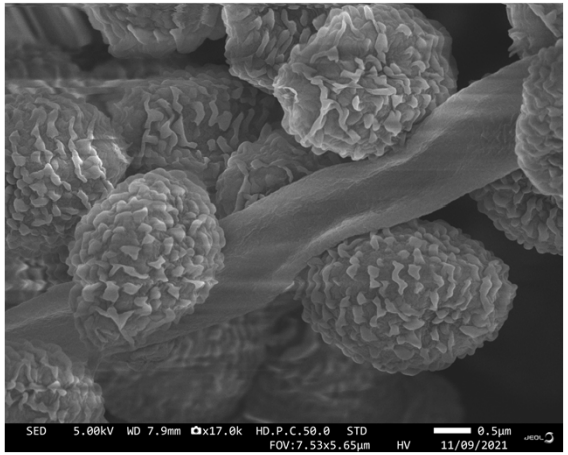


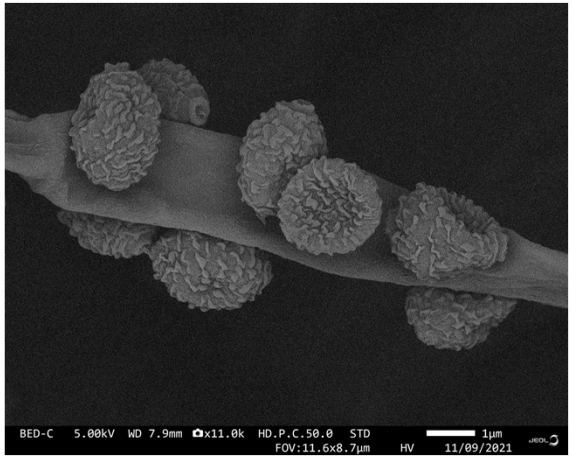
Penicillium

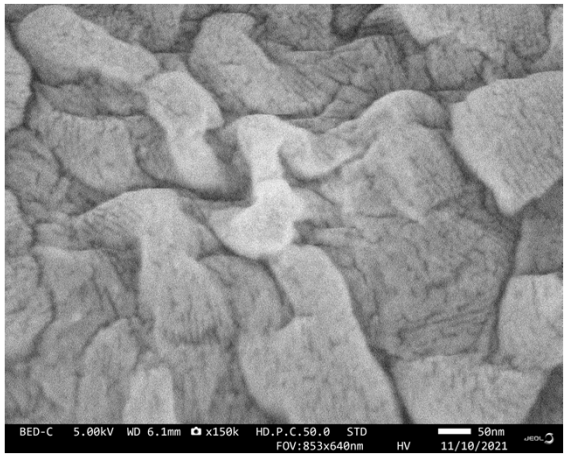
Fixation: OsO_4 vapors at freezer temperature
Drying: freeze drying
Coating: Au





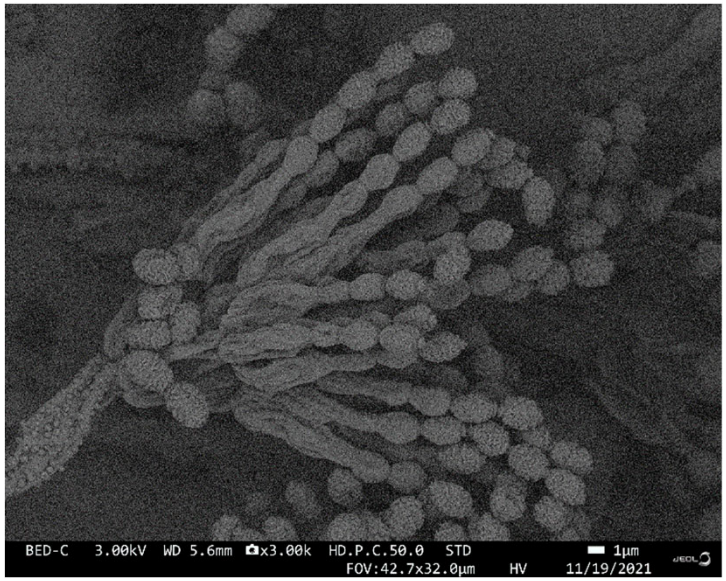


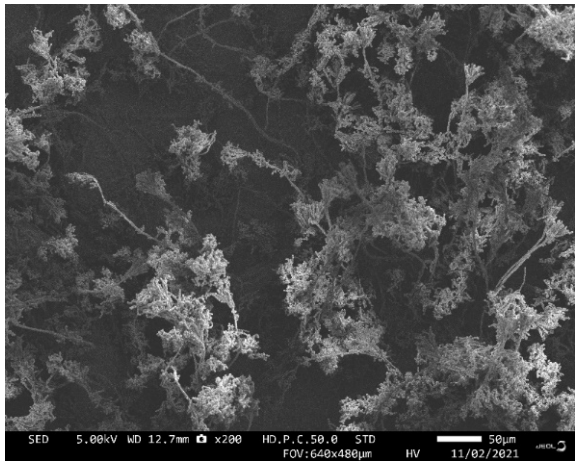


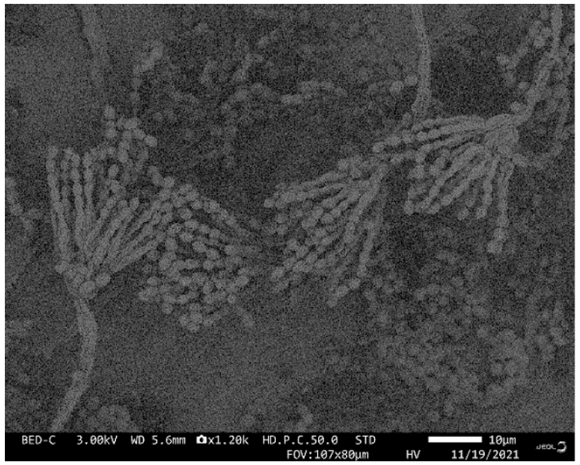


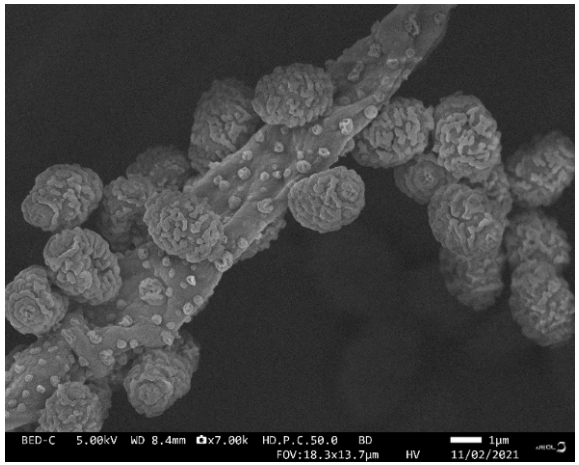
Penicillium

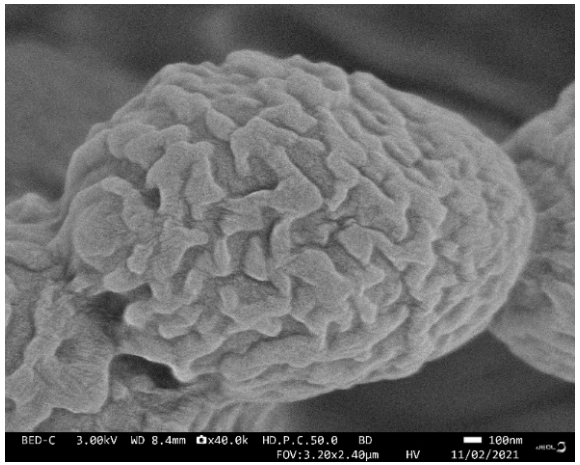
Fixation: Cryo-fixation
Freeze substitution: aceton with 1%OsO_4
Dehydration: aceton
Drying: CPD, Coating: Au





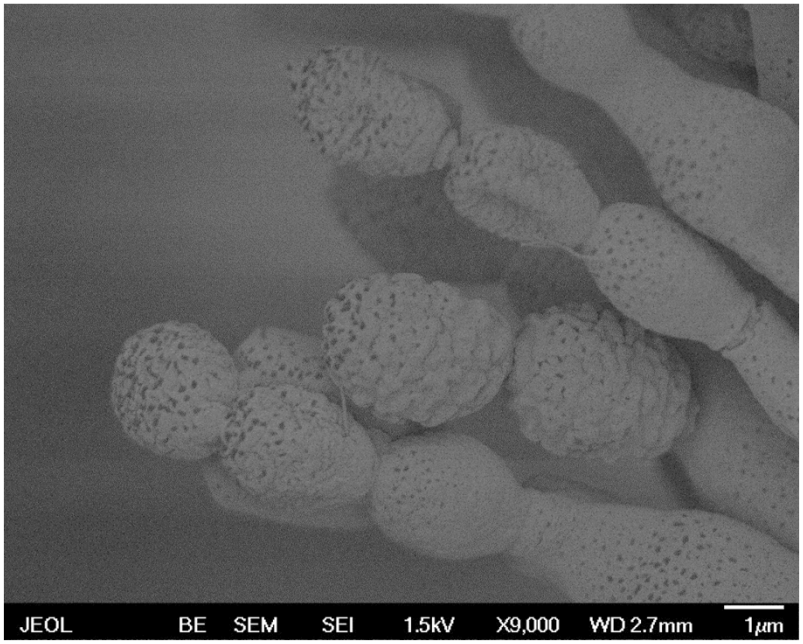


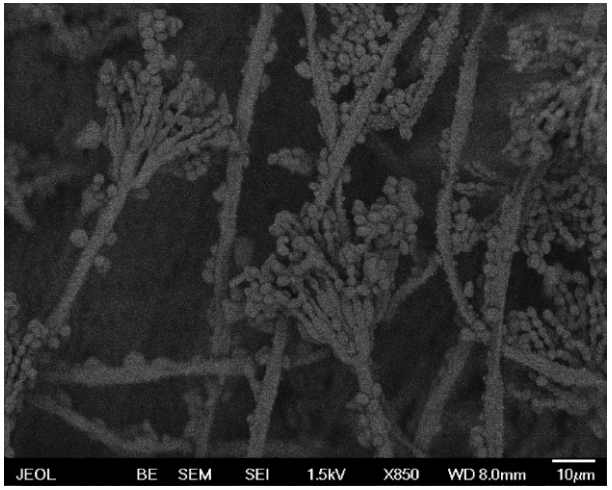


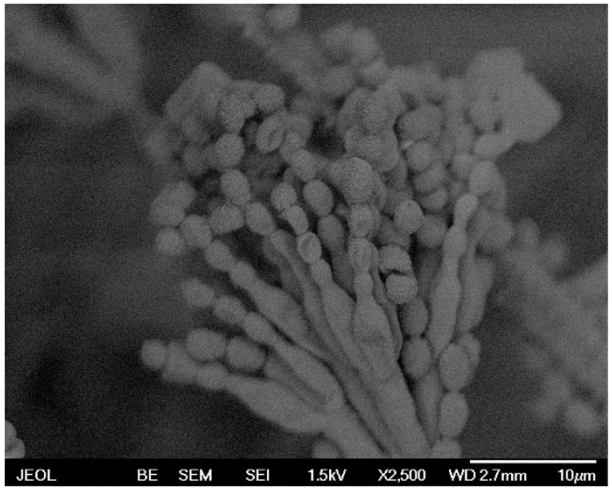


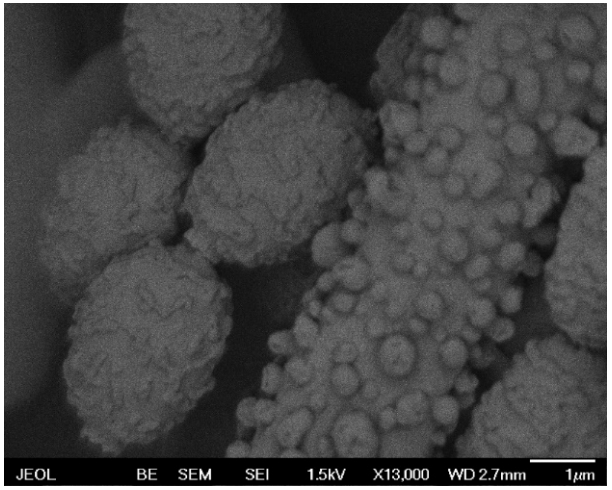
Penicillium

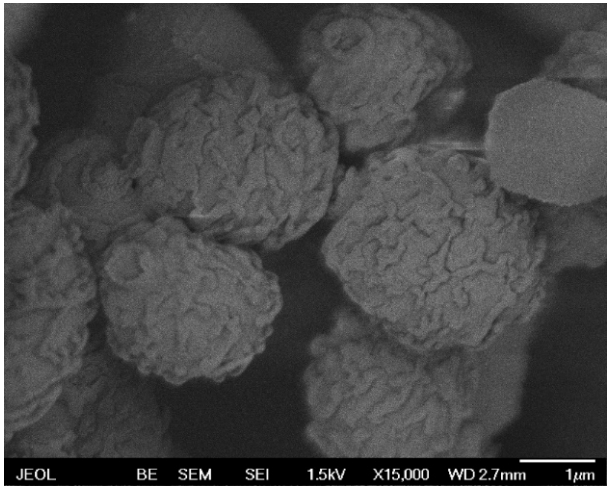
Cryo-SEM
Cryo-fixation
Sublimation at -95°C
Coating: Pt







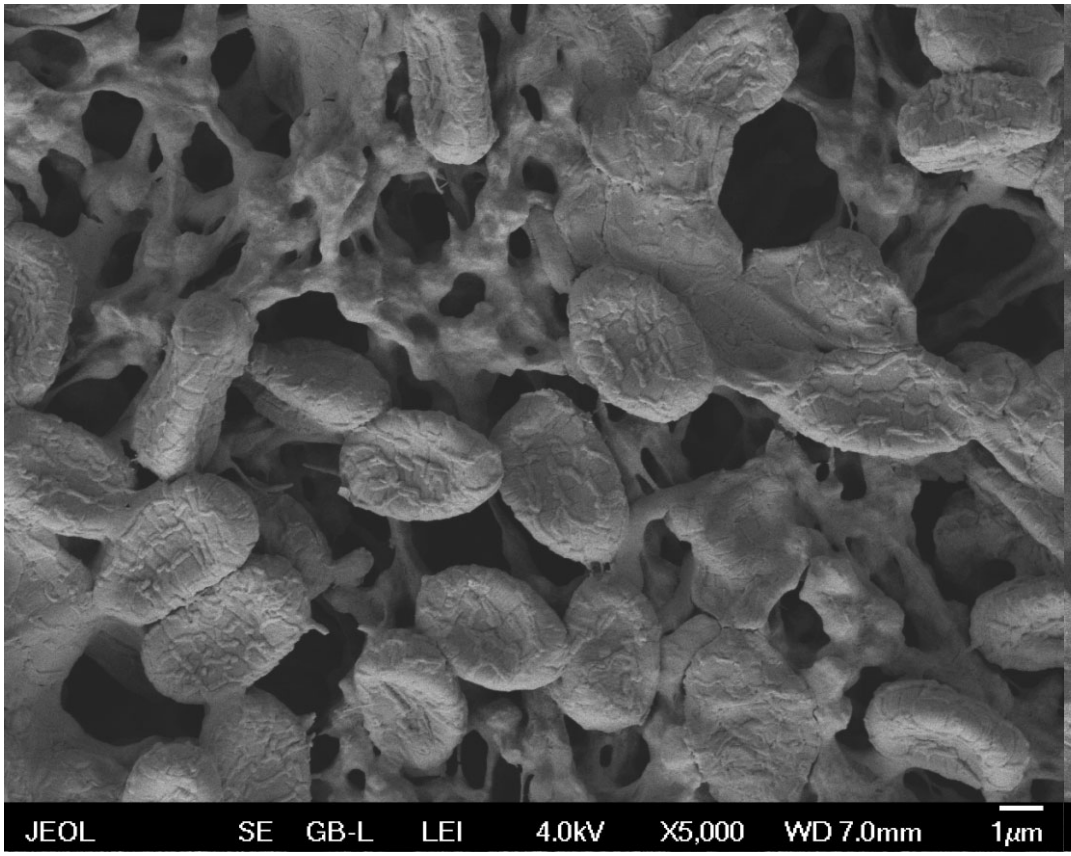




Cryo methods

Freeze drying and cryo SEM

- Suitable filter for water removing

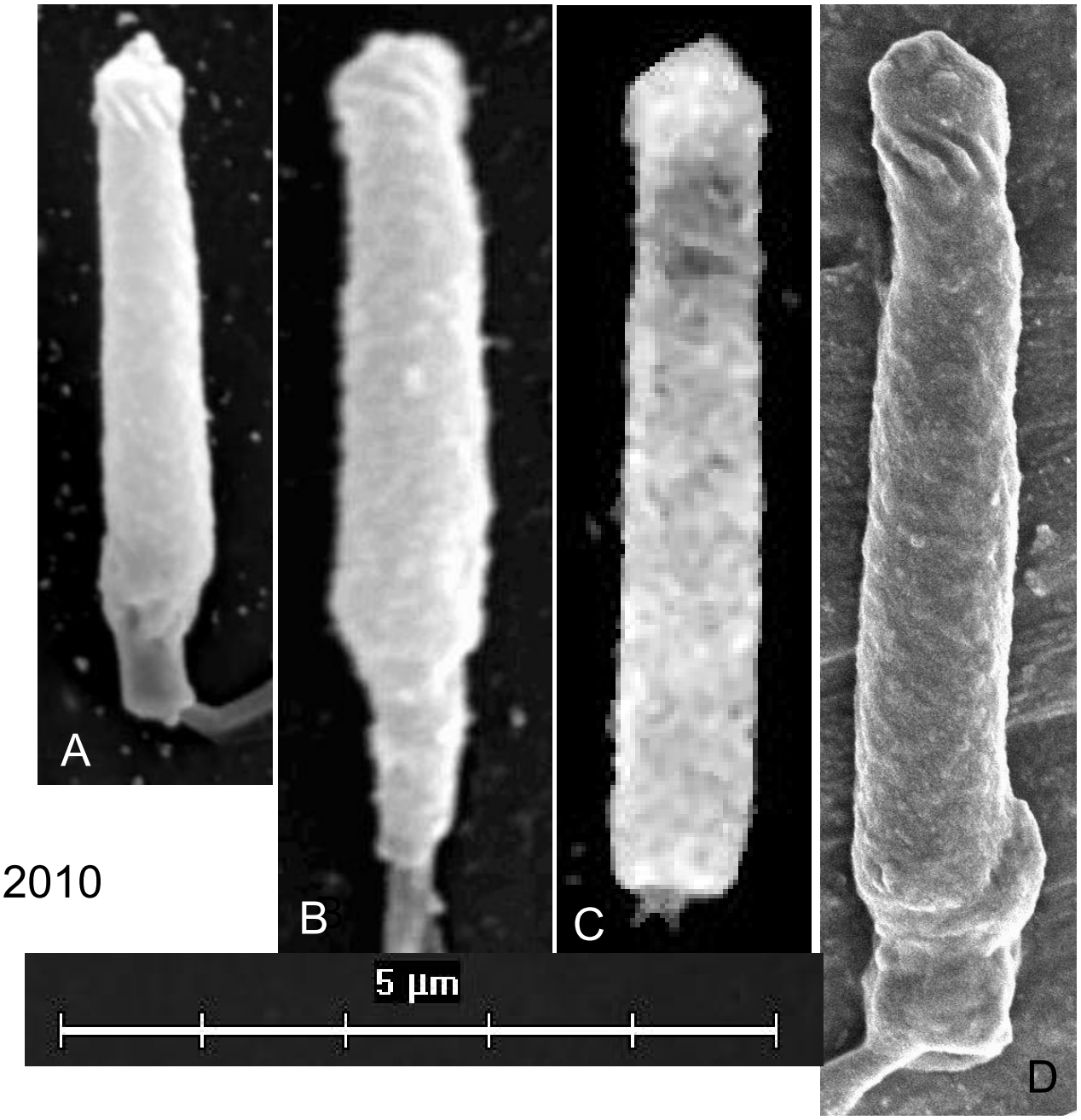


Undescribed species of Microsporidia from microcrustacea (Vavra J. 2013)

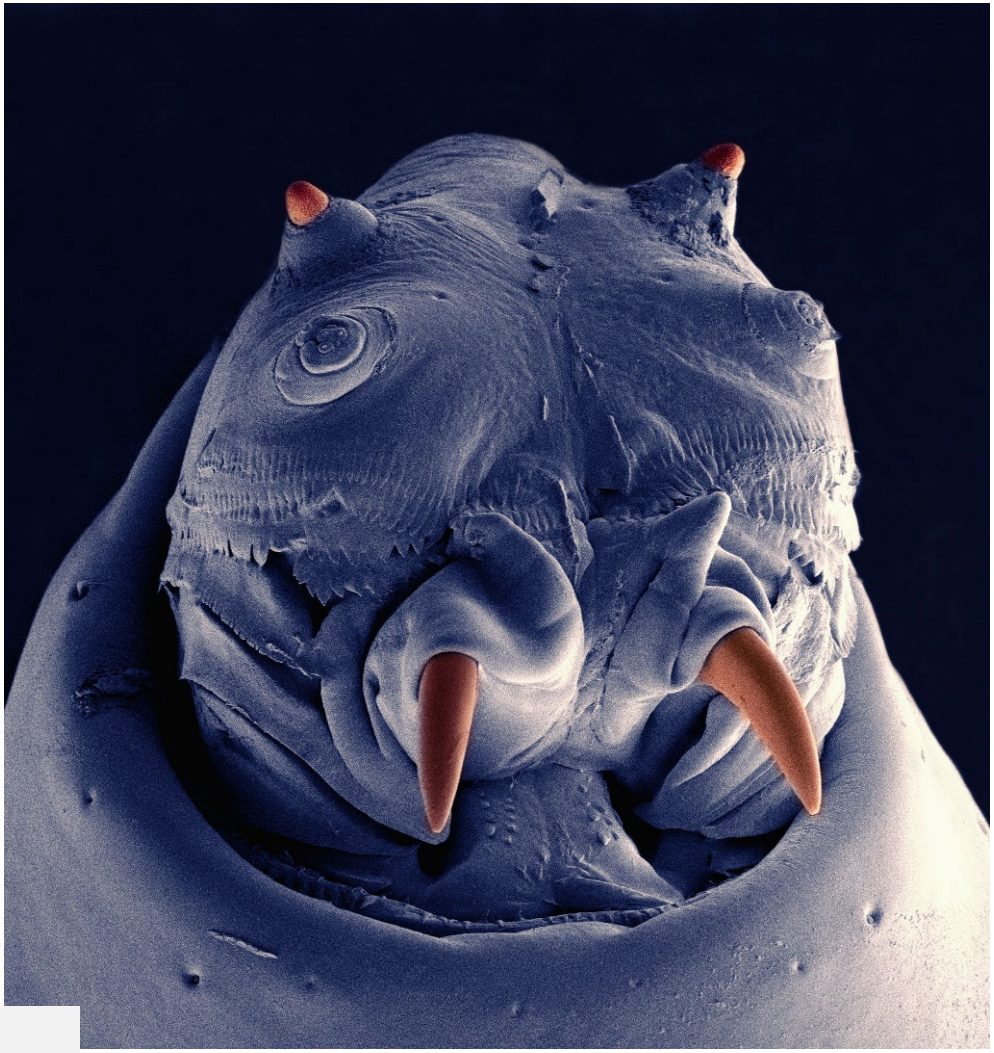
Comparison of sample size prepared by different methods

Size distribution of sturgeon Sperm depending on the method of prepartion:
A/ CPD drying
B/ t-butylalcohol
C/ ESEM
D/ cryo-SEM

Pšenička et.al.: Micron, 41(5), 2010



Thanks for your attention!



Cryo-SEM:
Larva of Chymomyza Costata (Drosophilidae)