Strategies of Biological Sample Preparation for Scanning Electron Microscopy

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IMG

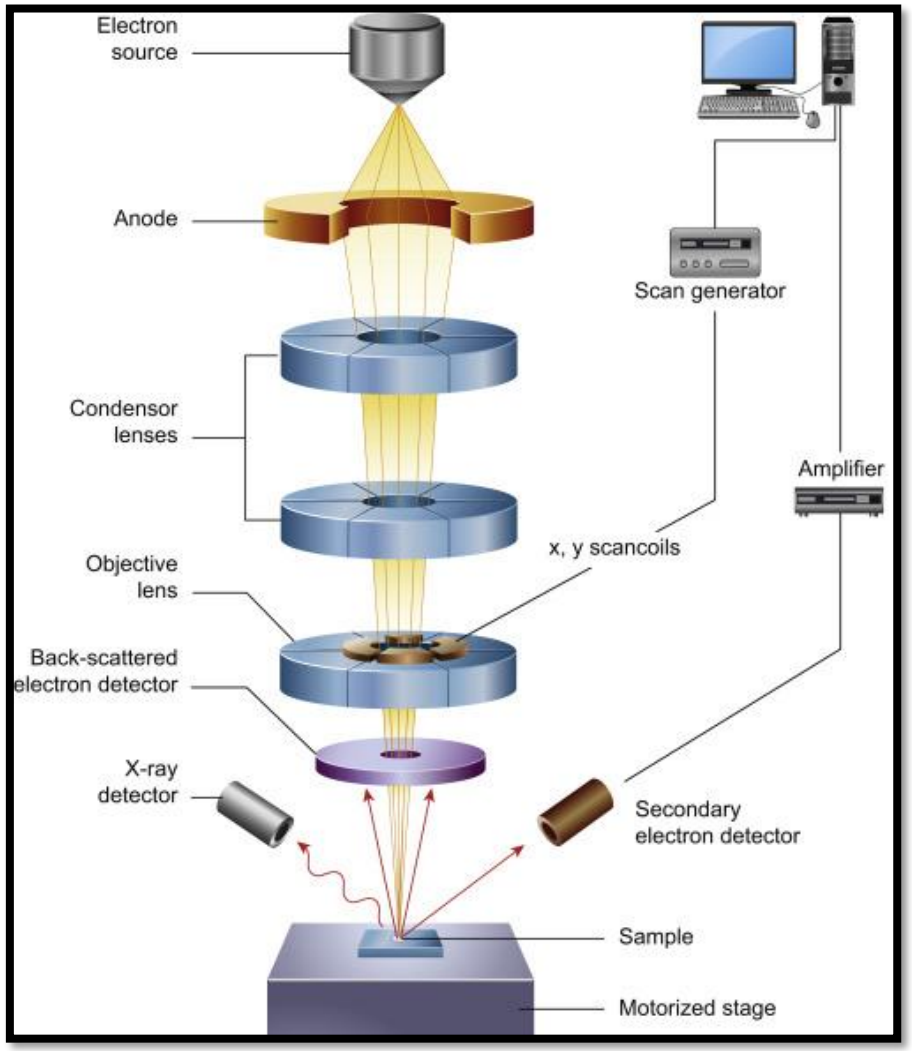
EURO BIOIMAGING
Image life, discover the future

CZECH BIOIMAGING
Imaging principles of life

Specimen criteria for the SEM examination

- Removing water or other volatile components from the specimen:
  totally 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
- Sufficient conductivity of the specimen surface
- Appropriate size for the SEM



https://doi.org/10.1016/B978-0-08-100040-3.00002-X

Sample preparation at RT

2D SEM imaging

1) Fixation – a) chemical with aldehydes

2) Postfixation and Contrasting with $0s0_4$ - not necessary (depands on sample)

↓

3) Dehydration with alcohol series

acetone or ethanol

↓

4) Drying

on air/with HMDS/by Critical Point Dryer (CPD) with CO_2

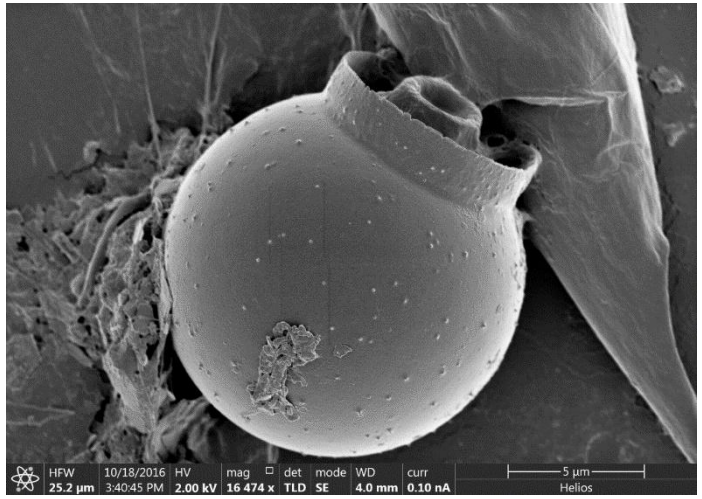
↓

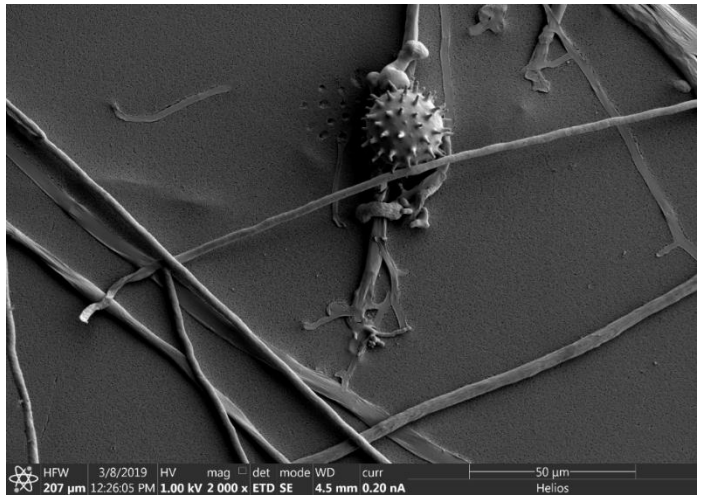
5) Sputter coating

Platinum or gold, the layer depands on sample (charging!) usually 2-10 nm

↓

6) SEM at RT

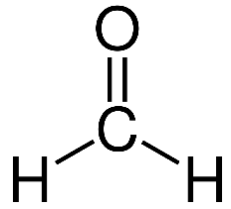


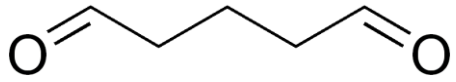


Steps during the sample processing

FIXATION

Chemically with aldehydes – Glutaraldehyde or Formaldehyde (or combination) to proteins cross-link = stabilisation of the ultrastructure before further processing



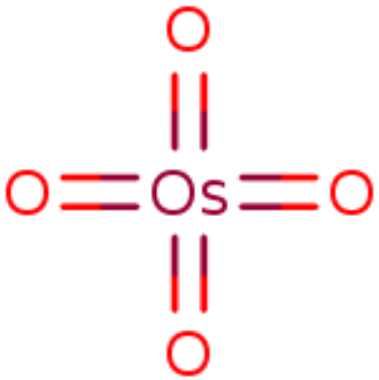


	formaldehyde	**glutaraldehyde**
Crosslinking Efficiency	low	high
Diffusion Speed	high	low

Steps during the sample processing

Postfixation and Contrasting with 0s04

- Bilipid membranes are fixed to prevent their extraction by solvents during dehydration
- The black osmium precipitate which is formed during this process increases sample conductivity and minimizes image distortions resulting from charging

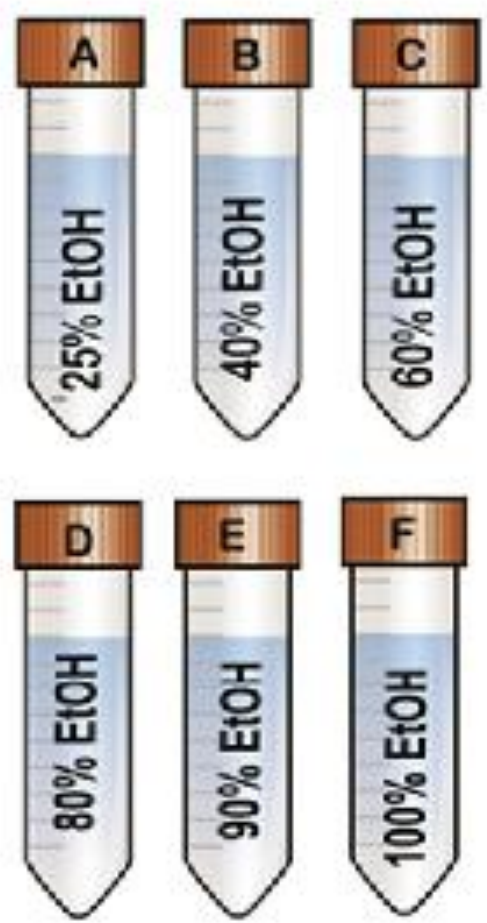


Steps during the sample processing

Dehydration

- A fixed specimen is dehydrated by incubation in a series of ethanol or acetone solutions

- Solvent concentration is increased gradually so that water is removed gently, without causing specimen shrinkage

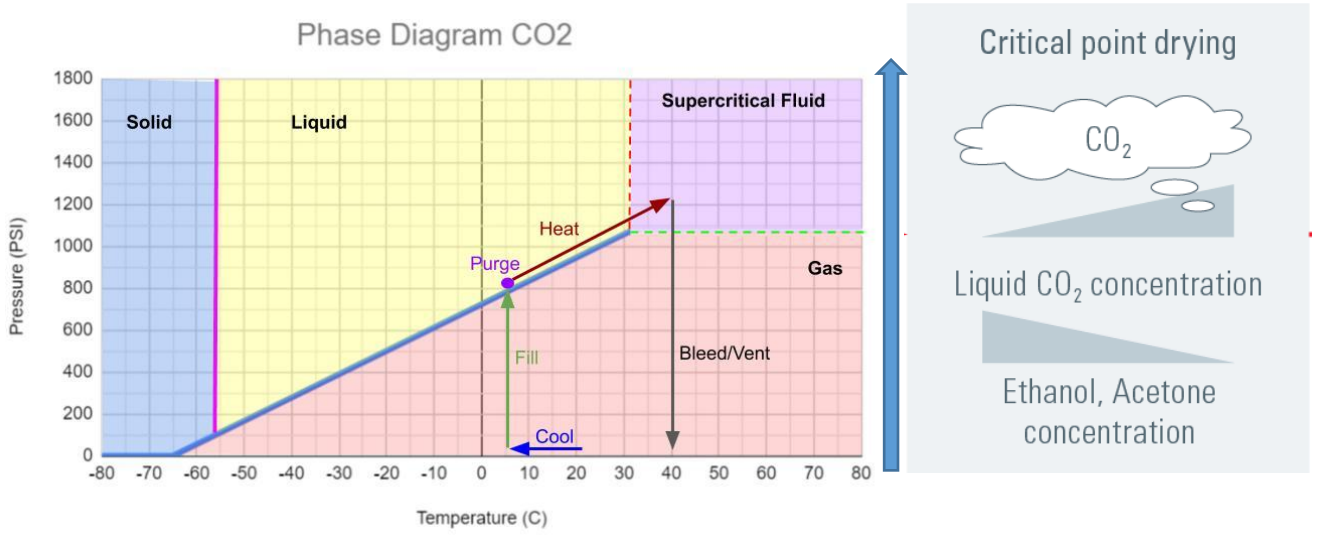


Steps during the sample processing

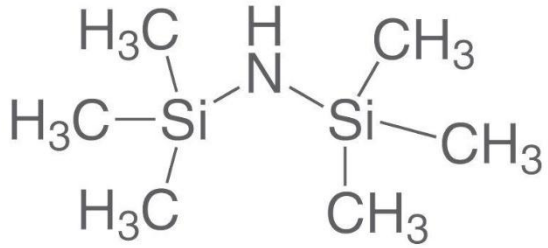
Drying

Allowing acetone or ethanol to simply evaporate from sample surface would create artefacts as these solvents have relatively high surface tension and would create micro-ripping of the surface upon leaving.

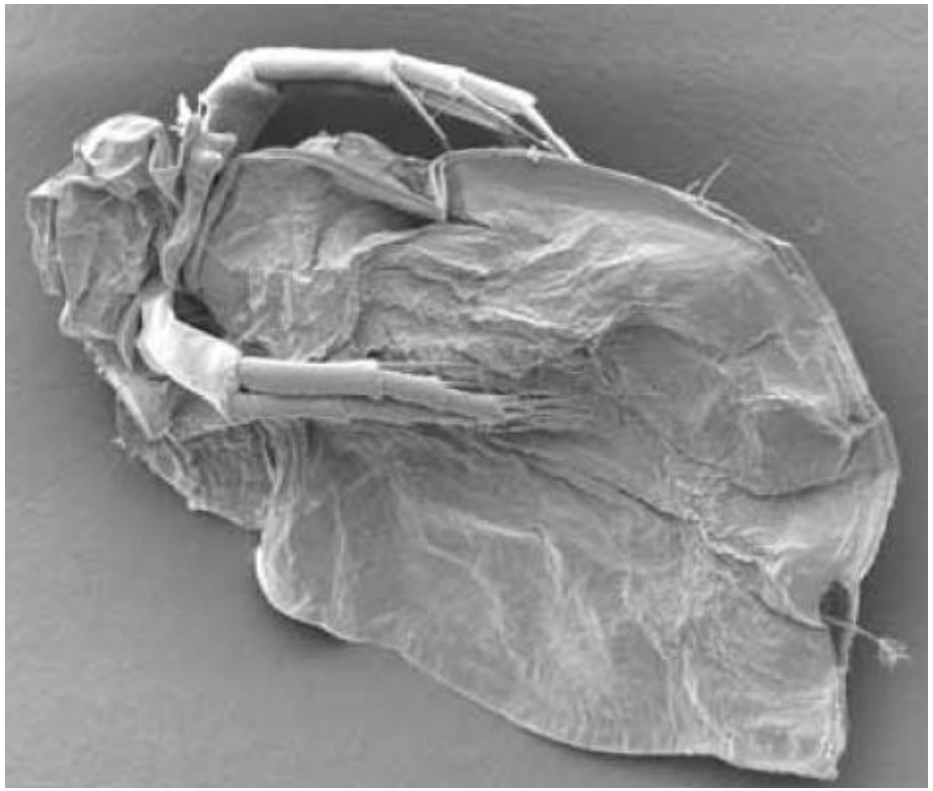
CPD (Critical Point Drying) – make use of liquid CO_2 under high pressure and lower temperatures to remove any water or liquid from specimen



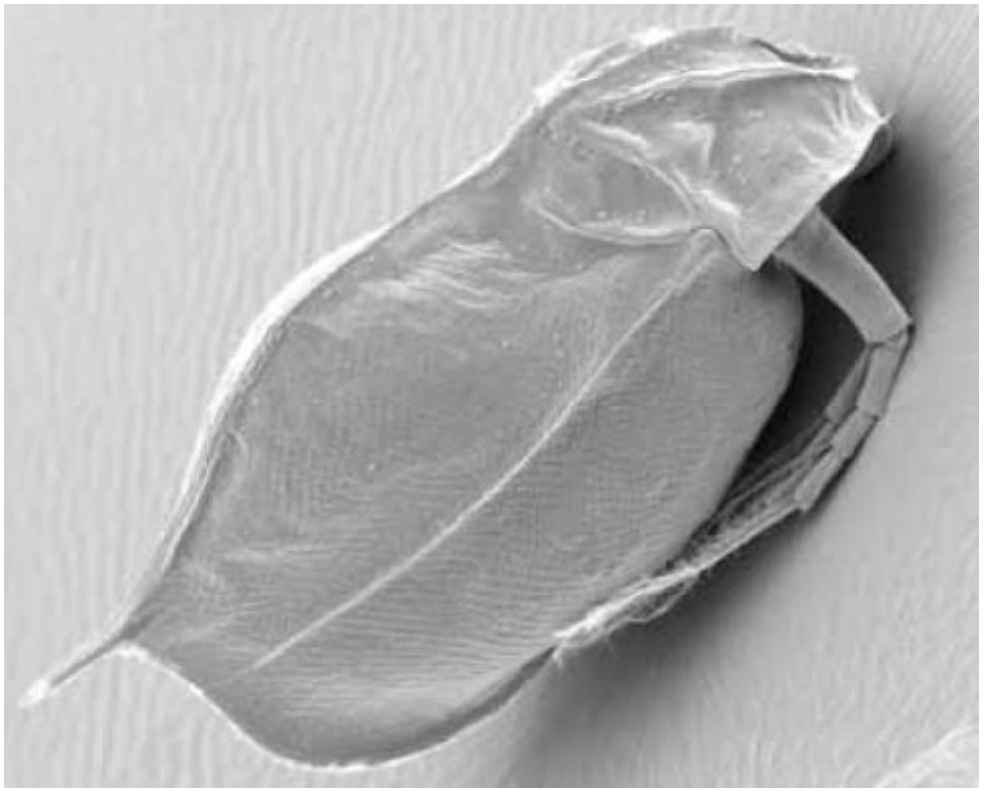
HMDS (Hexamethyldisilazane) – can be used in cell preparations and after a short (3 minute) incubation it is removed and excess is left to evaporate. A chemical with low surface tension = good alternative approach to CPD.



CPD method



Air dried sample (Water flea)



Critical point dried sample (Water flea)

Source: https://www.leica-microsystems.com

Steps during the sample processing

Mounting and sputter coating

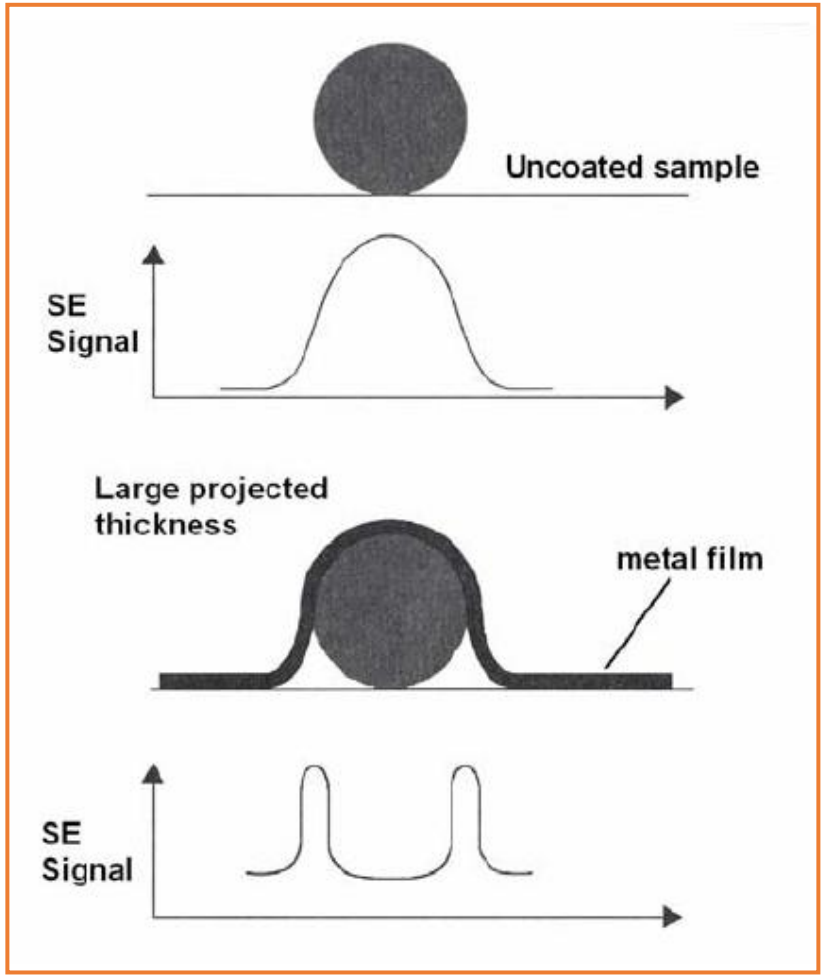
- The specimen is mounted on a metal stub using a sticky carbon disc which increases conductivity
- Silver-containing glue can additionally be applied for even more conductivity

- To prevent charge buildup on specimen surface, it is coated with a conductive material (Pt, Au)
- The metal is applied in a controlled manner in a sputter coater
- It is critical that the coating is thick enough to prevent charging but not thick enough to obscure specimen surface details

Specimen Coating

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

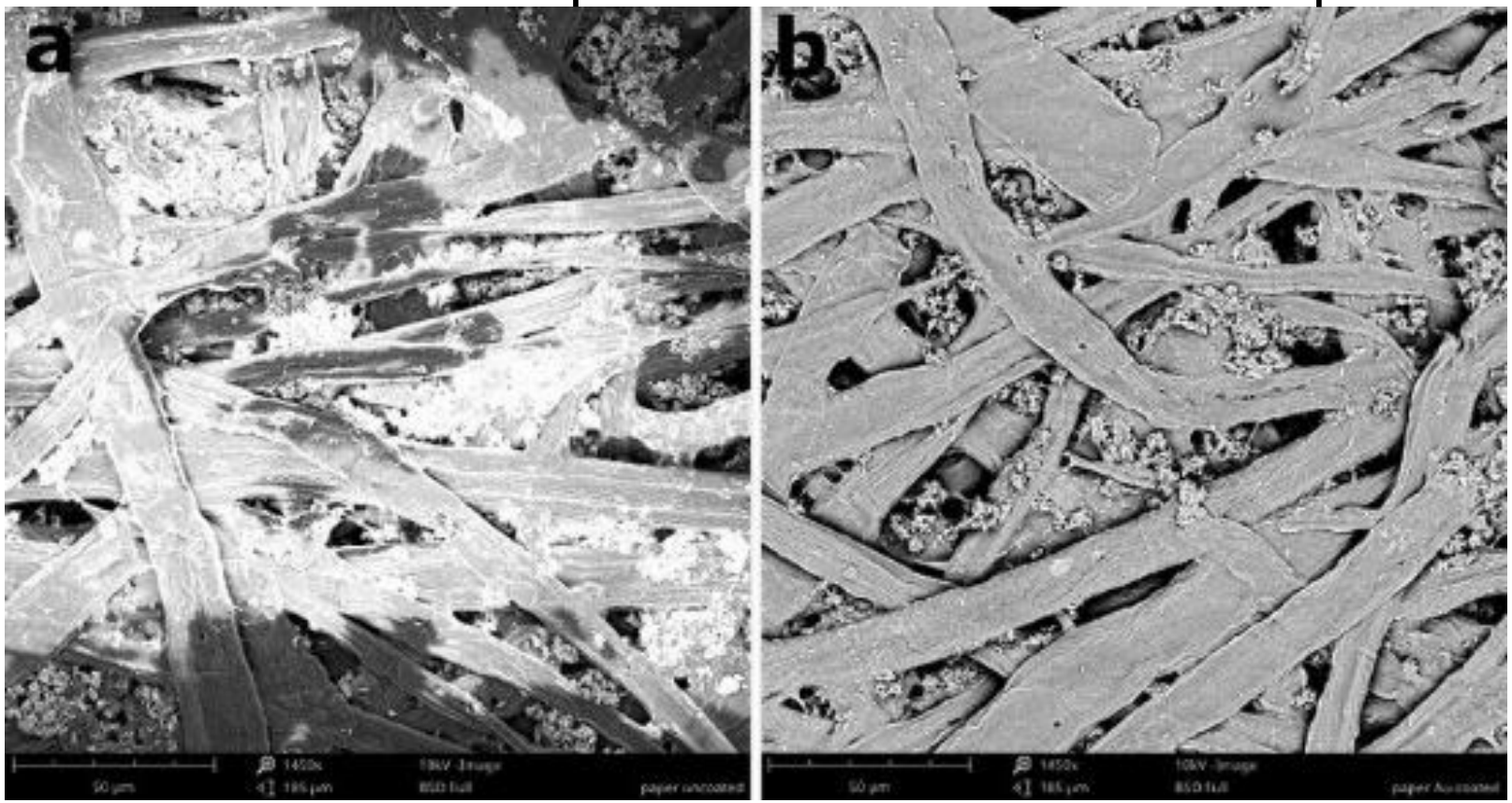
Element	Z	Thermal conductivity at RT (W/cm/K)
Carbon	6	1.29
Aluminium	13	2.37
Palladium	46	0.72
Silver	47	4.29
Platinum	78	0.72
Gold	79	3.17



Specimen Coating

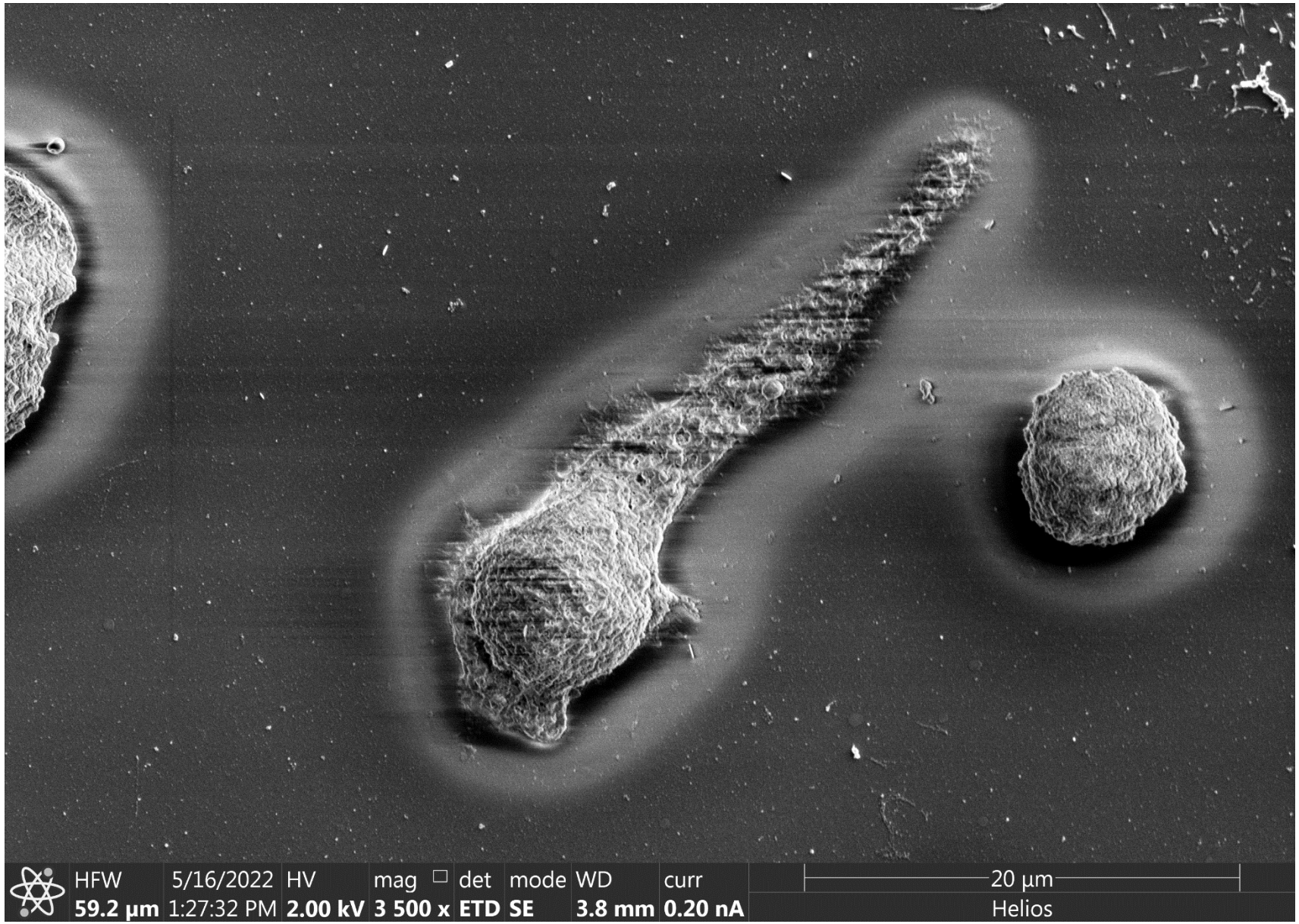
Non-coated sample

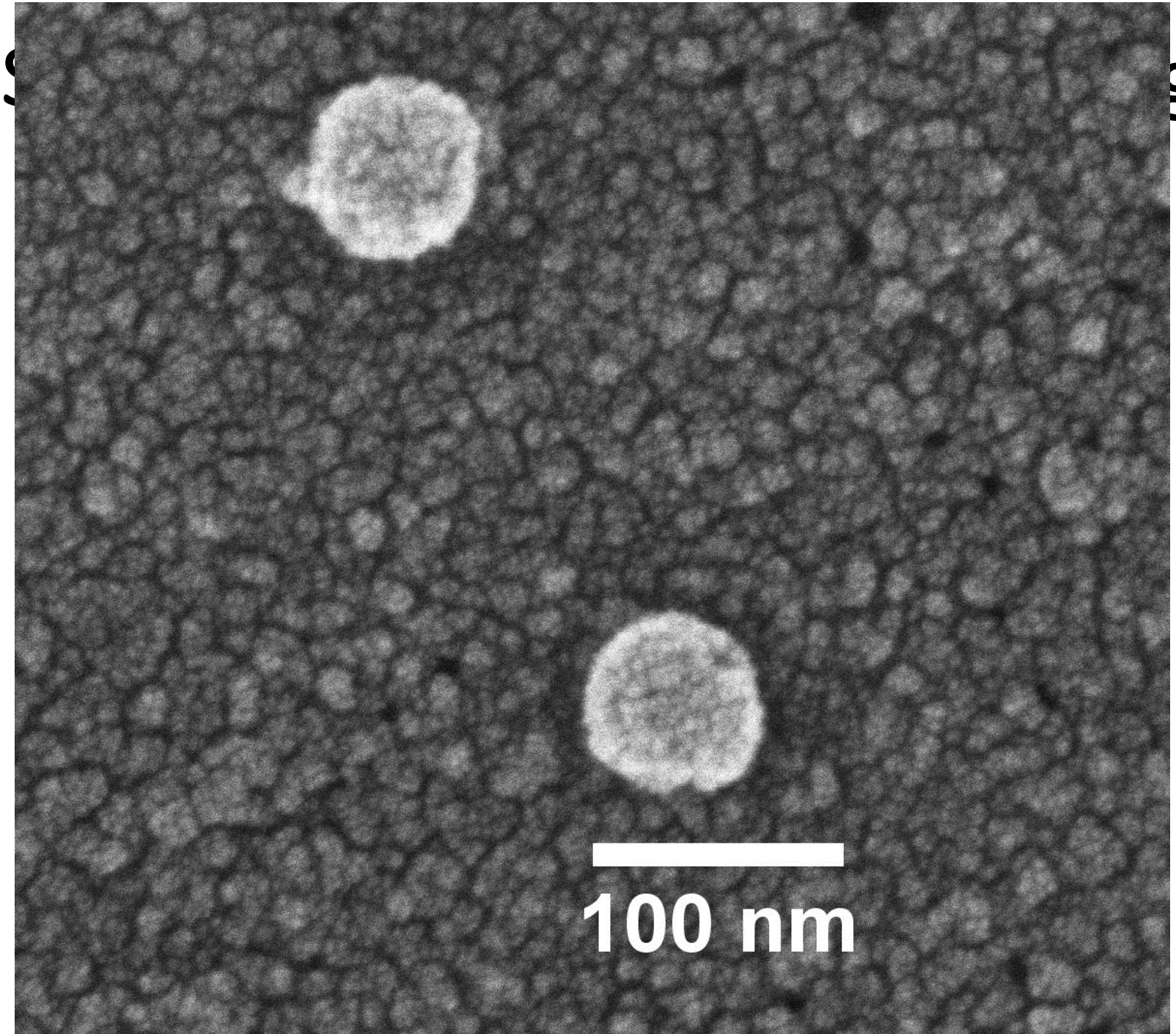
Gold-coated sample



https://www.thermofisher.com/cz/en/home/global/forms/industrial/sputter-coating-sem.html

Charging effect

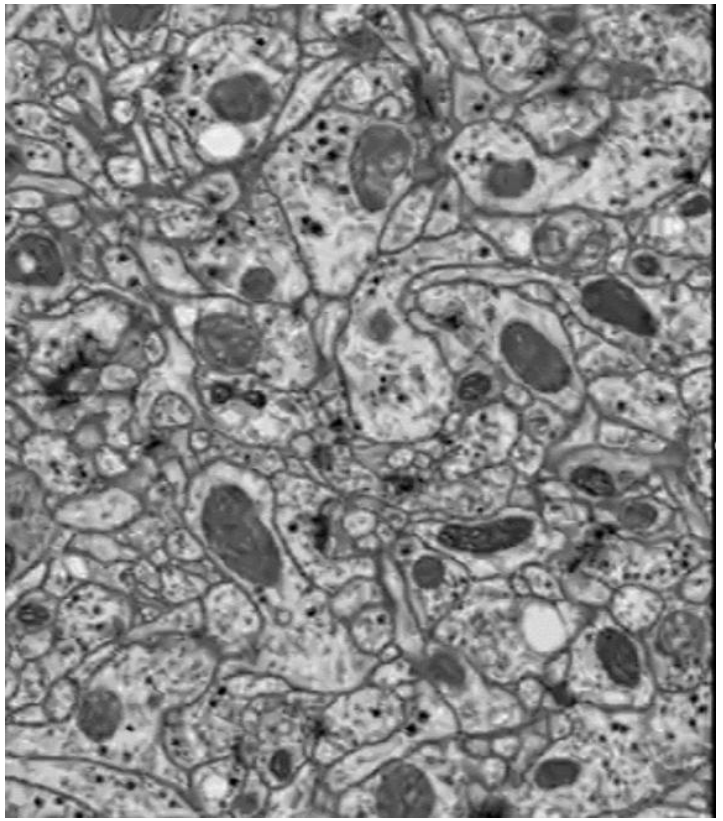



100 nm

Surface or ultrastructure in SEM

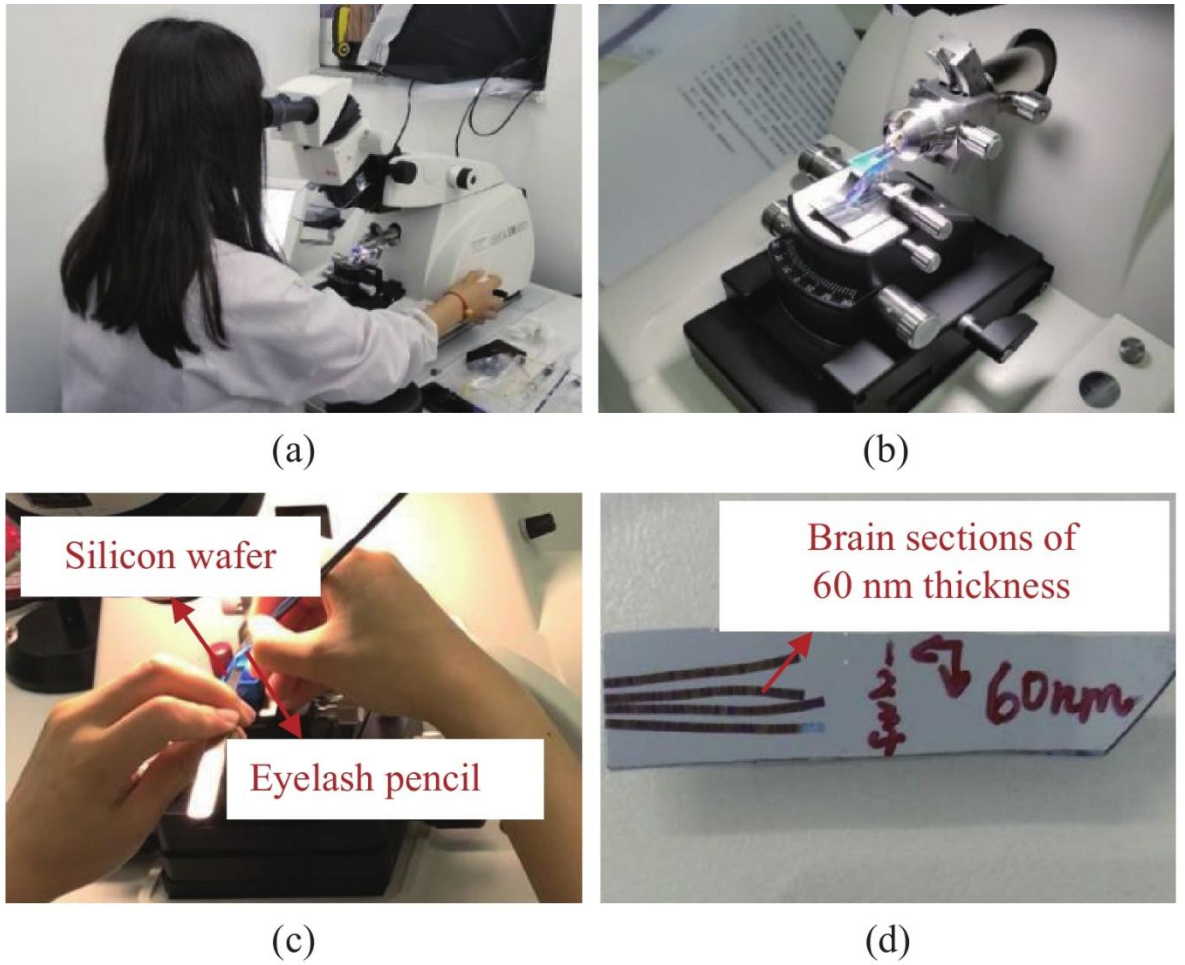
Whole-mount specimen:
imaging of surface

Sectioned specimen:
imaging of ultrastructure



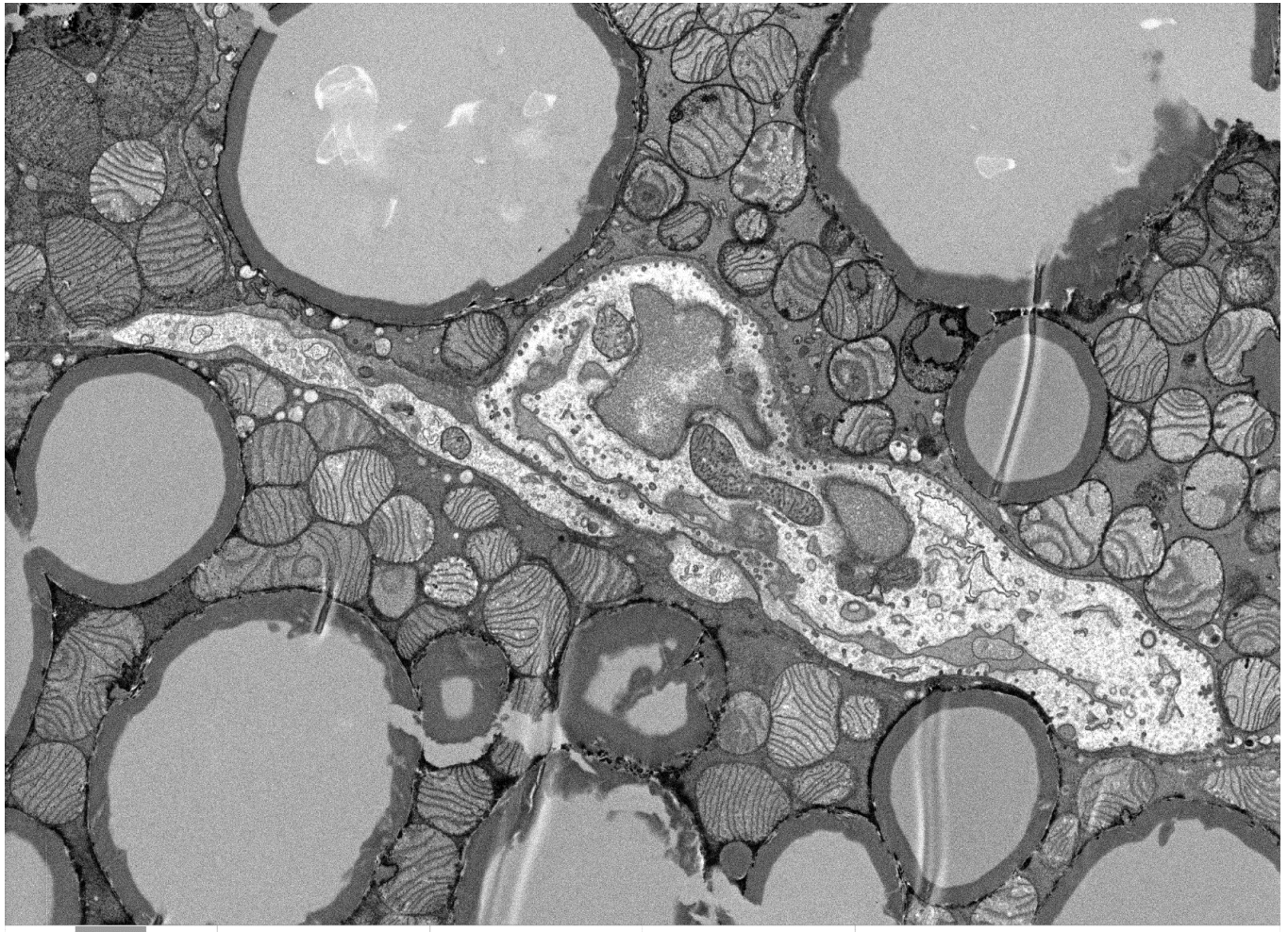
Specimen preparation for imaging of sections in SEM

- The sample must be fixed, stained and embedded as for TEM.
- Since the specimen block is viewed using backscatter detector, the increased staining is necessary for contrast (OTO)



doi: 10.1109/JAS.2021.1003829

Specimen preparation for imaging of sections



Mouse adrenal gland

D. Pinkas, IMG EM CF
Sample: A. Neuwirth, IMG

Cryo-methods for SEM

Cryo-fixation

Ice sublimation

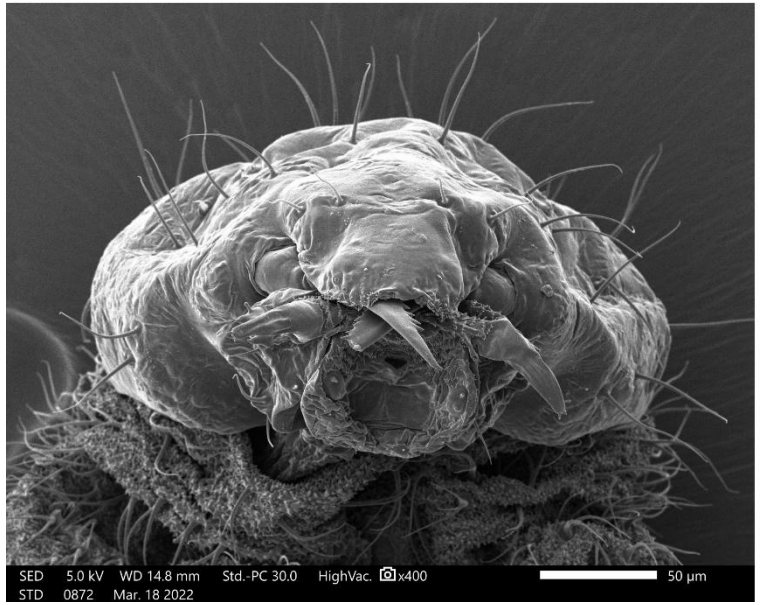
Freeze fracturing

Freeze drying

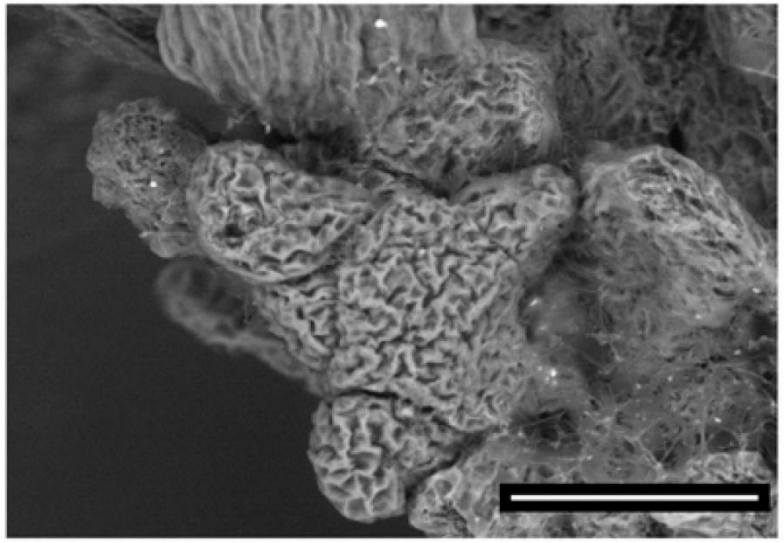
WHY CRYO-SEM?

SEM

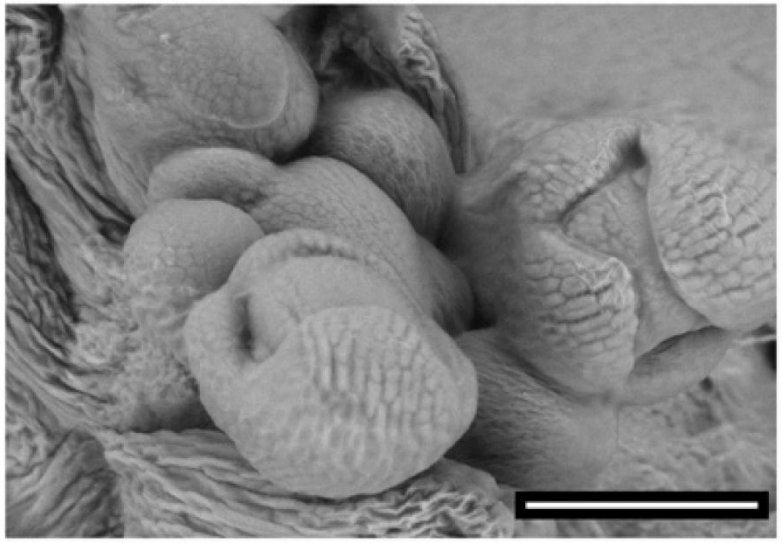
Cryo-SEM



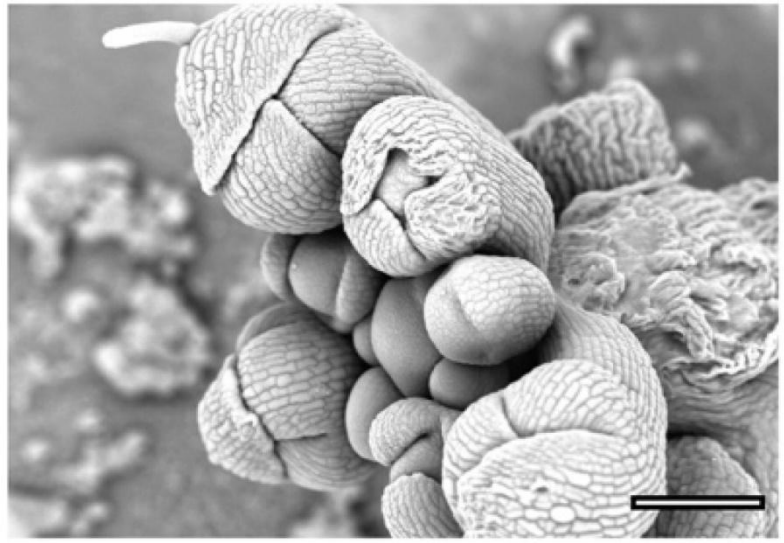
Amalia Pasolli, PhD (@APasolli) / Twitter



VP-SEM (30 Pa)



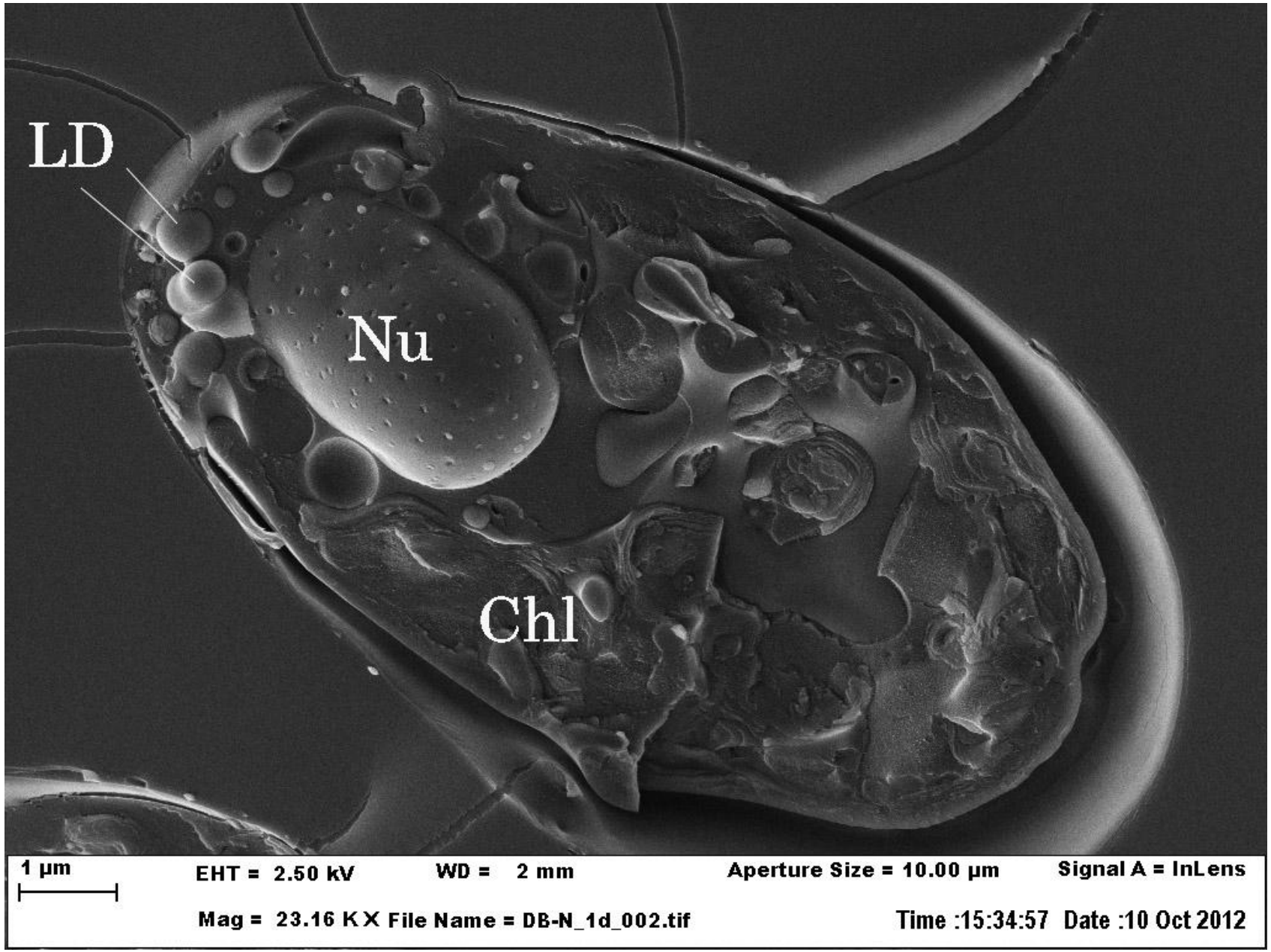
EP-SEM (507 Pa)



Cryo-SEM (high vacuum)

https://doi.org/10.3390/plants11091113

WHY CRYO-SEM?



http://www.weizmann.ac.il/Chemical_Research_Support/EM_Unit/Eyal/research-activities/cryo-sem

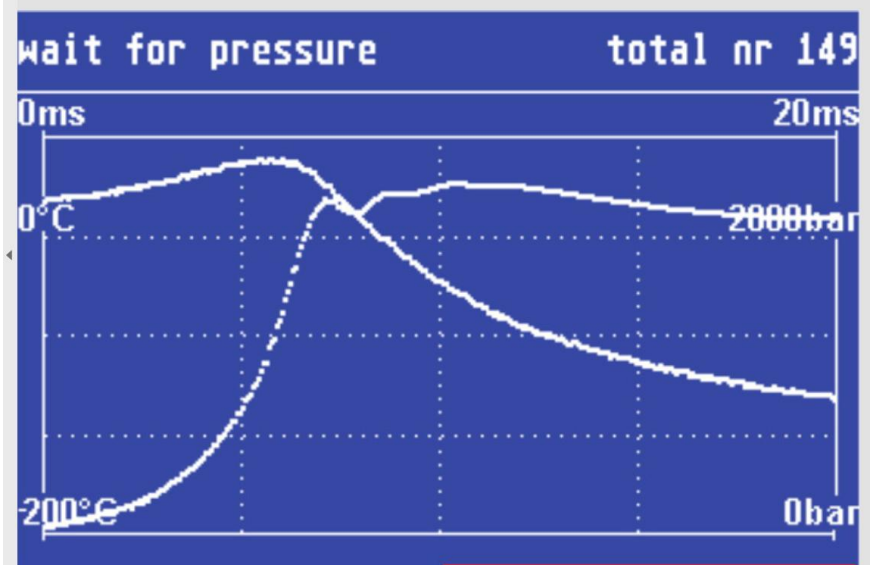
Steps during the sample processing

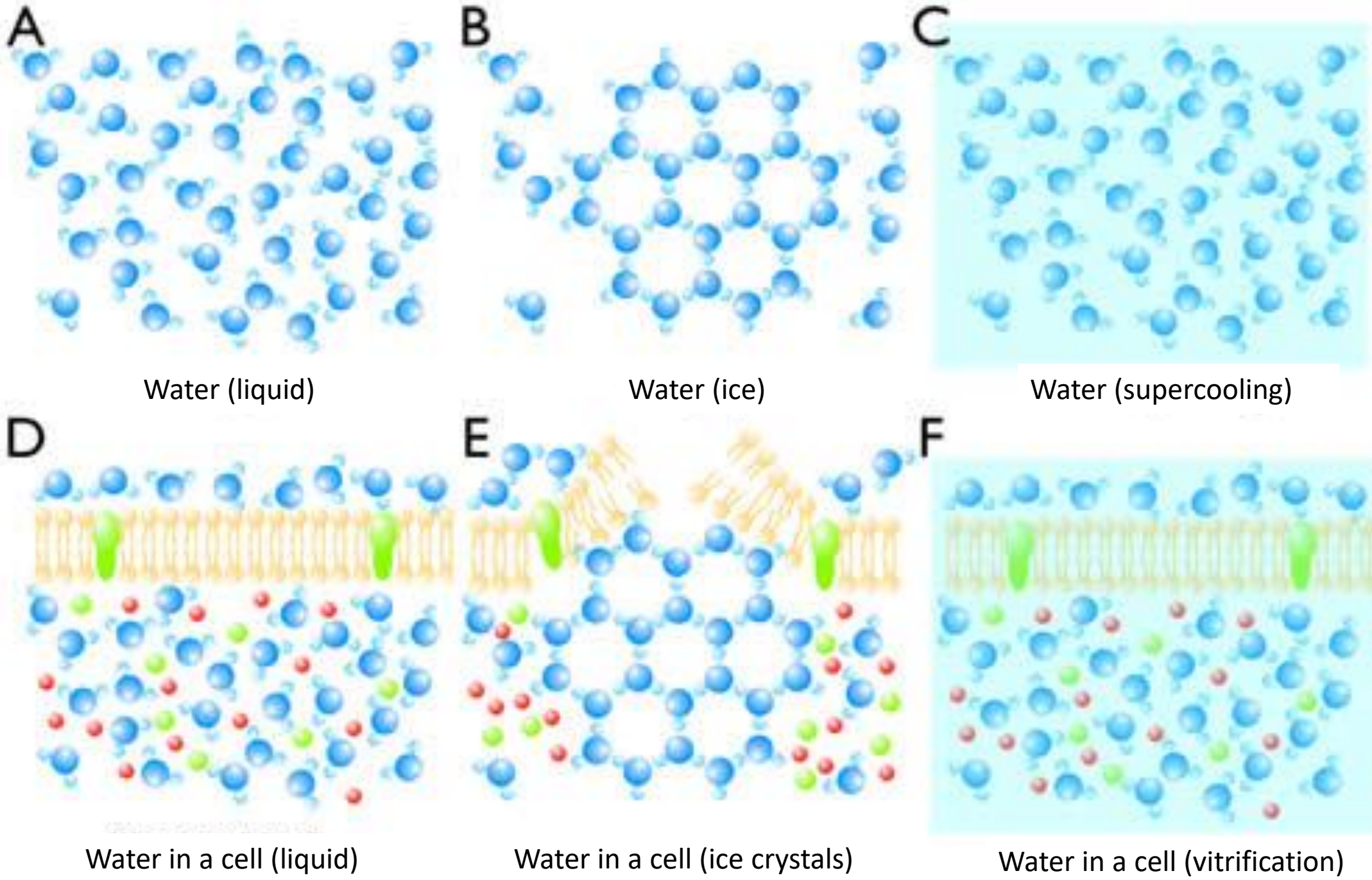
FIXATION

Chem **lehydes –** C or
Fo binati
link e
furthe

	ehyd	
Crossli Efficiency	low	
Diffusion Spe	high	w

B) Cryofixation - specimen is frozen quickly enough to cool water from its normal liquid state to its solid state (vitrification) without an intermediary ice-crystal phase




A
B
C
Water (liquid)
Water (ice)
Water (supercooling)
D
E
F
Water in a cell (liquid)
Water in a cell (ice crystals)
Water in a cell (vitrification)

Cryo-SEM

- For fresh frozen samples

1) Mounting of the hydrated sample on the SEM stub
with double-sided tape

↓

2) Place the stub with the specimens in the liquid nitrogen bath

↓

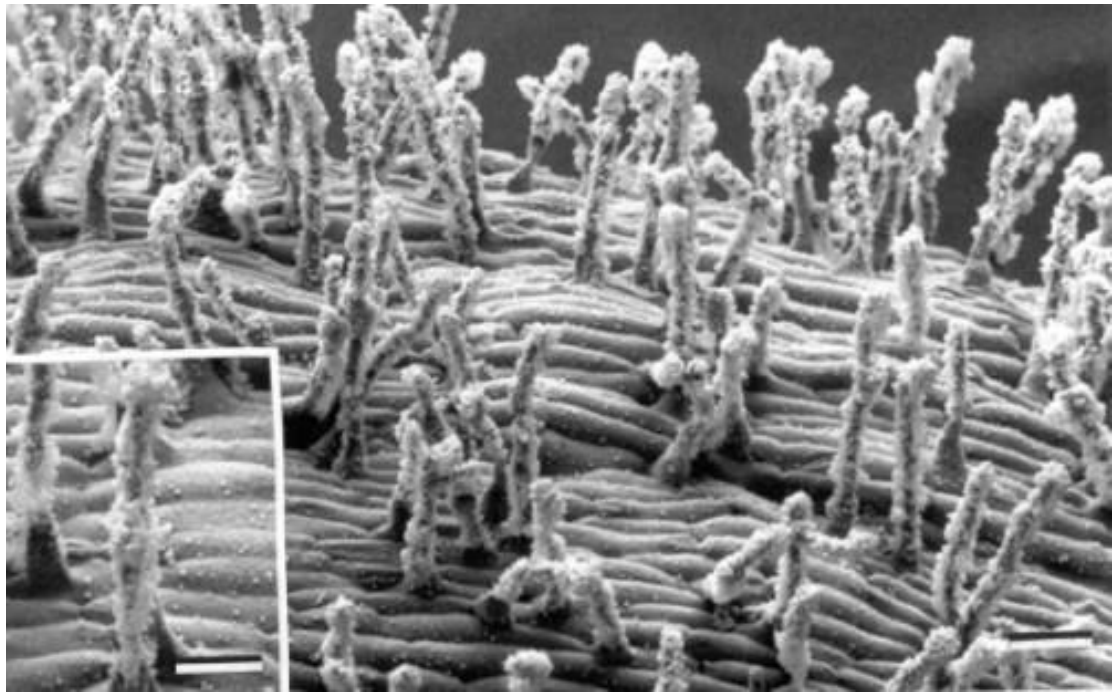
3) Freeze etching
the sublimation of surface ice under vacuum to reveal details of the fractured face that were originally hidden

↓

3) Sputter coating
Platinum in cryo condition

↓

4) Transfer the cryogenic stub and sample into the SEM chamber



Frozen hydrated root hairs
https://www.quorumtech.com/cryo-sem-images/

Cryo - SEM

- Direct observation of frozen specimens in SEM 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

Freeze Fracture and cryo-SEM

1) Physical fixation with high pressure freezer (HPF)

2) Freeze fracture

technique of breaking a frozen specimen to reveal internal structures

3) Freeze etching

the sublimation of surface ice under vacuum to reveal details of the fractured face that were originally hidden

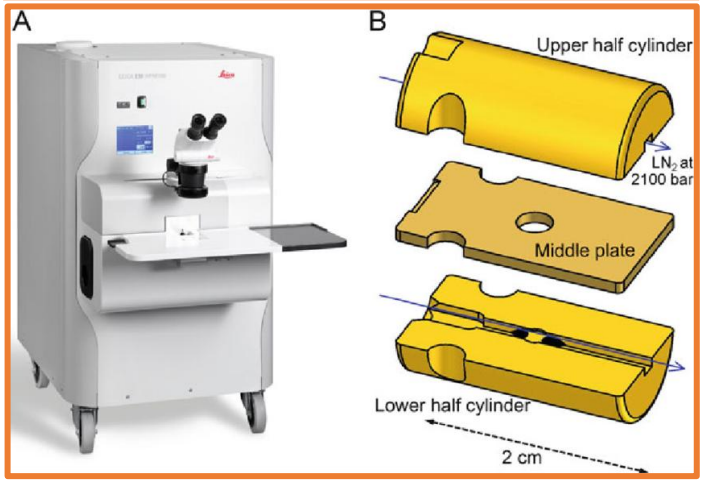
4) Sputter coating

Platinum in cryo condition

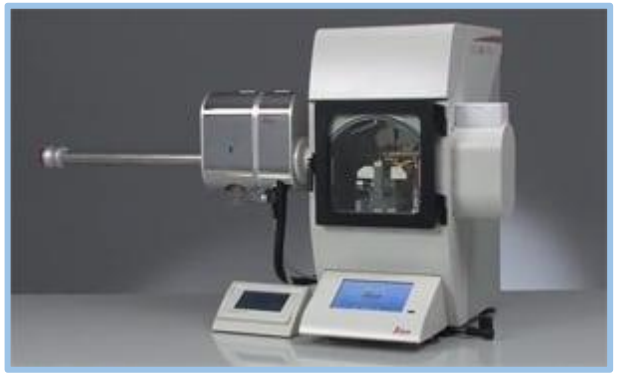
5) SEM at cryo condition

Temperature on stage -130°C

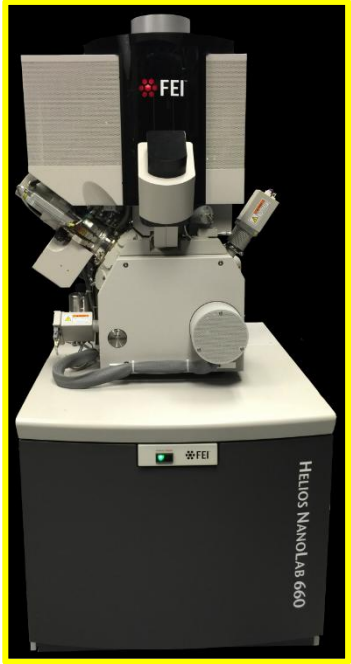
Leica HPM100



Leica ACE600 e-beam

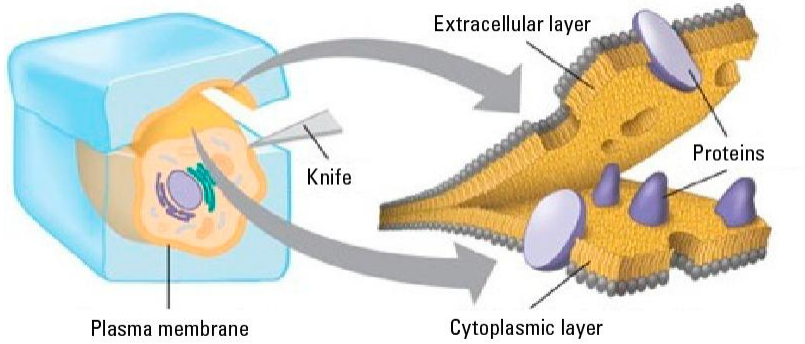


FEI Helios NanoLab 660 G3 UC



Freeze Fracture and cryo-SEM

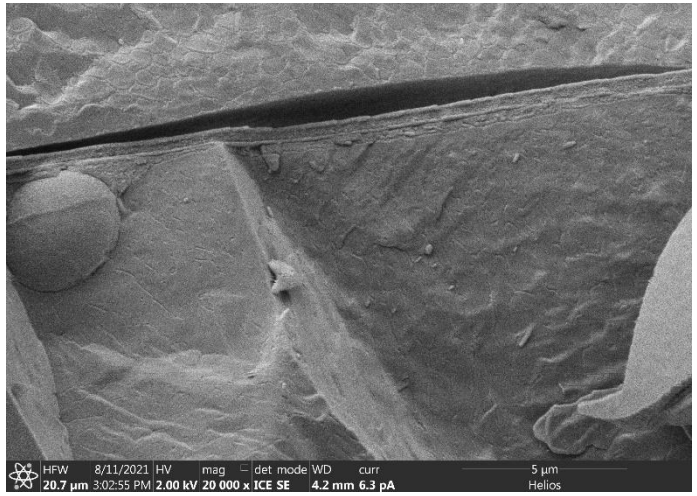
TECHNIQUE

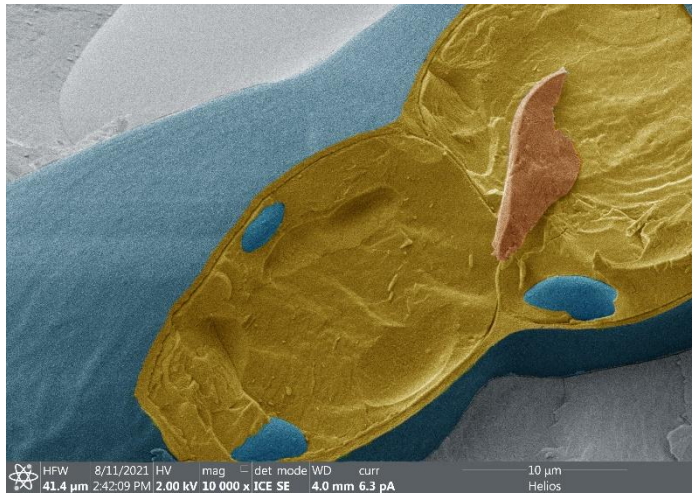


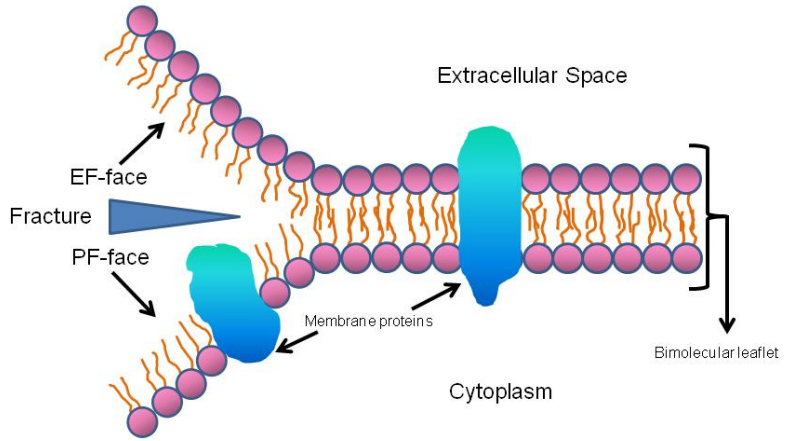
RESULTS

Inside of extracellular layer

Inside of cytoplasmic layer







https://www.jove.com/t/51694/fundamental-technical-elements-freeze-fracturefreeze-etch-biological

Freeze Fracture and cryo-SEM

- To observe structures such as cell organelles, membranes, emulsions or surface interfaces of liquids
- Preservation of membrane structures during sample preparation is the key to understand their true 3D nature
- Oraginzations of the proteins embedded in the membrane
- For the study of functions and structure - combined freeze-fracture replica immunogold labeling (FRIL)

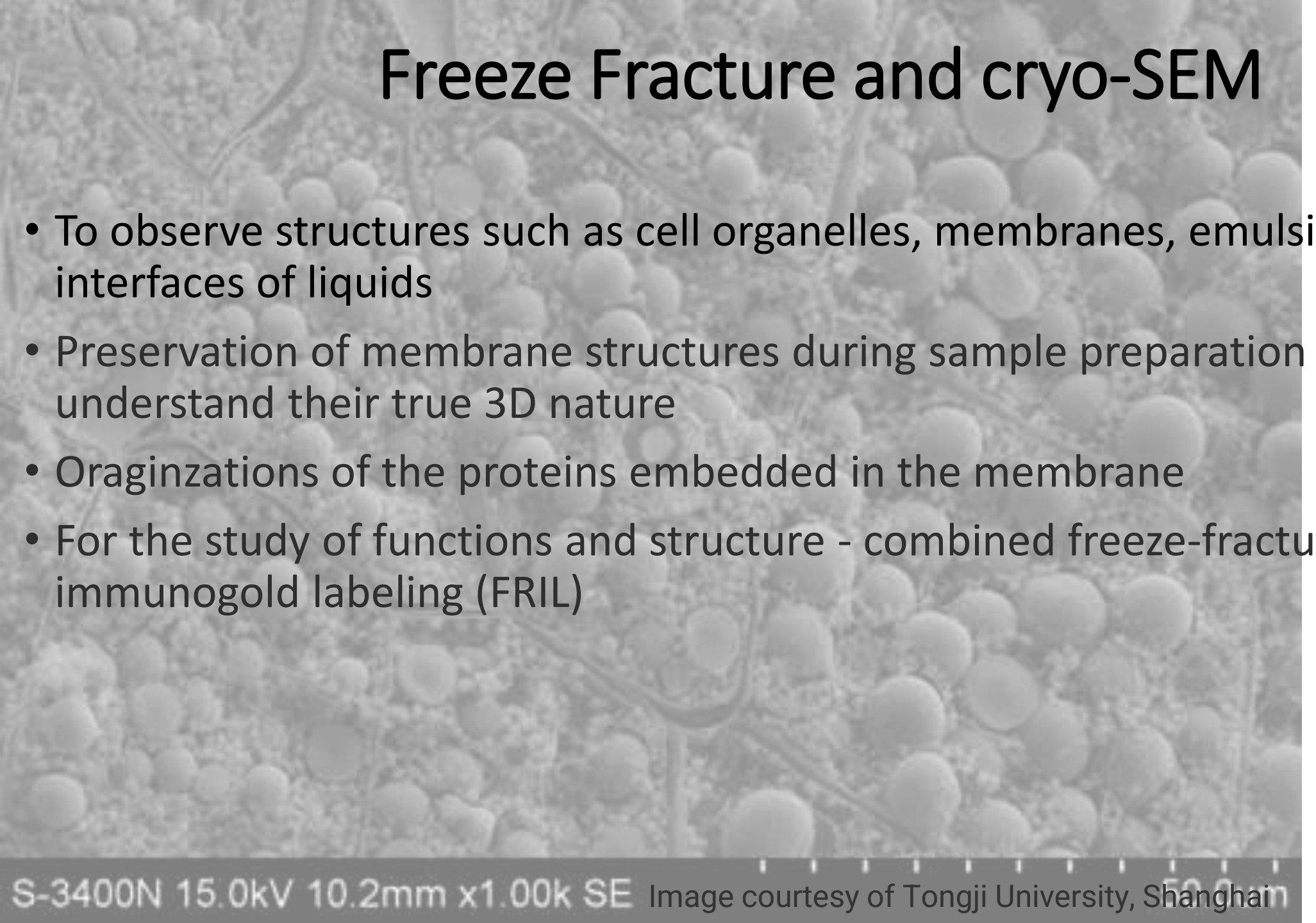
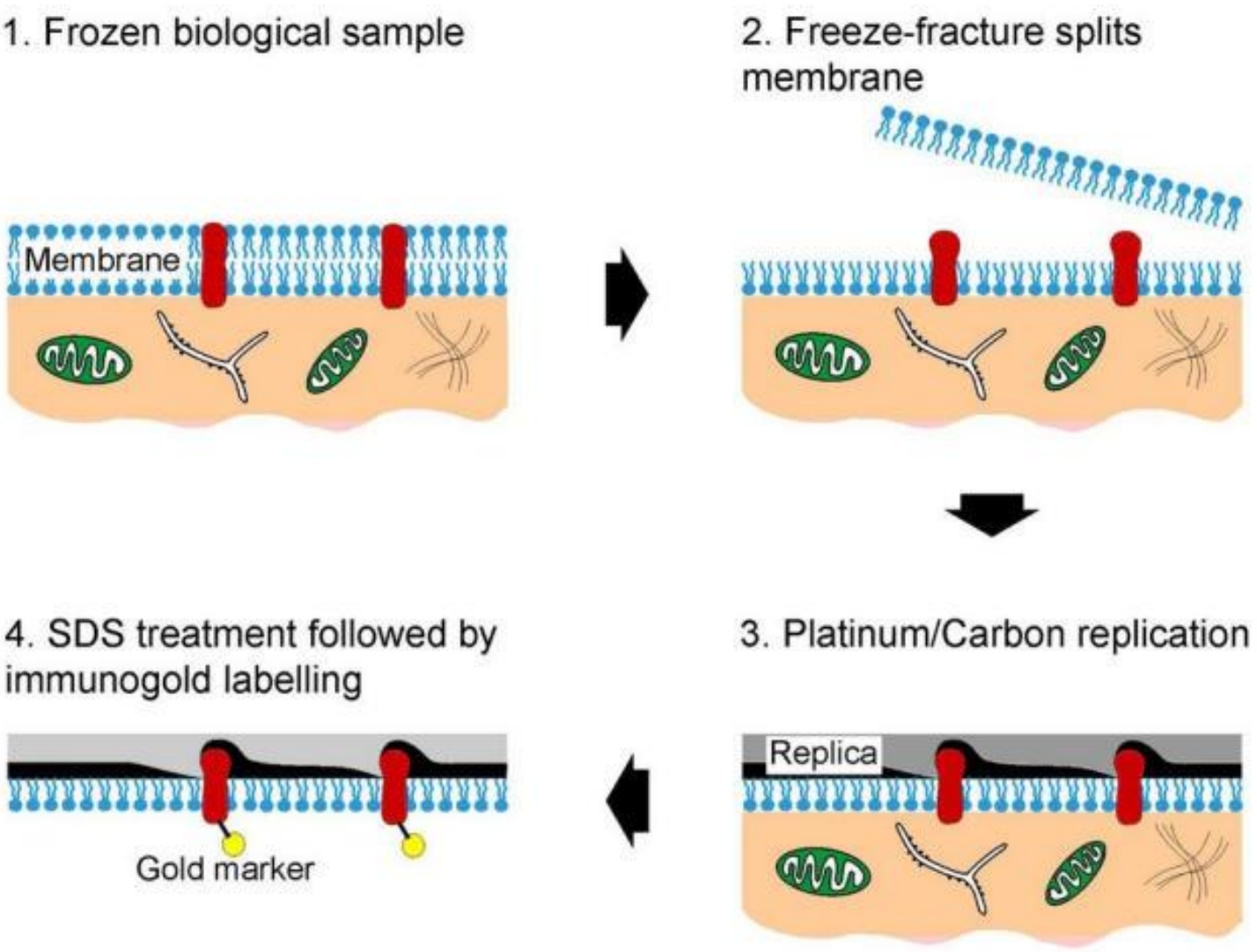
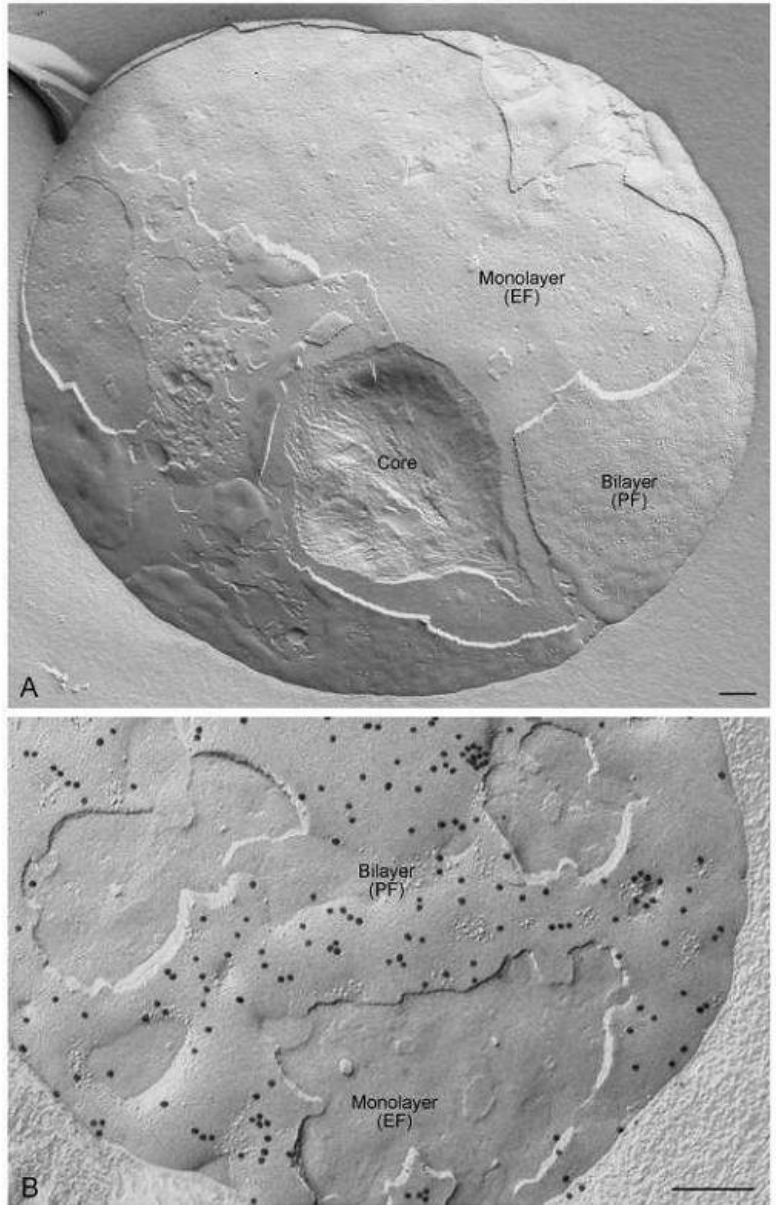


Image courtesy of Tongji University, Shanghai

Freeze Fracture Replica



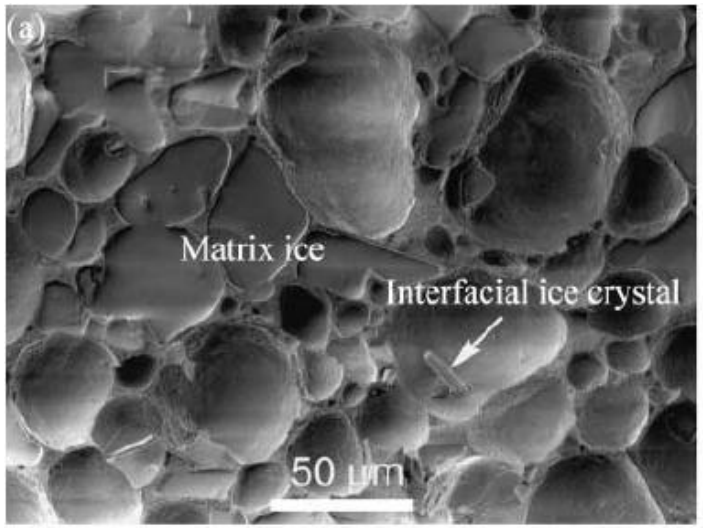


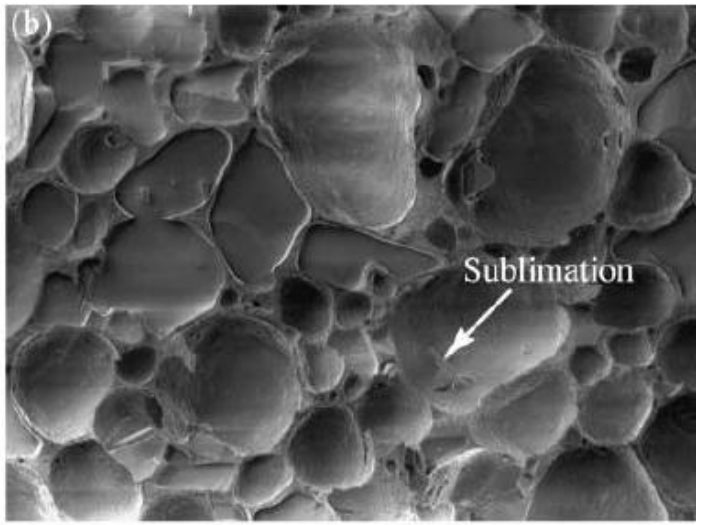
ROBENEK, Horst a Nicholas J. SEVERS. Recent advances in freeze-fracture electron microscop: the replica immunolabeling technique. *Biological Procedures Online* [online]. 2008, **10**(1), 9-19 [cit. 2023-04-18]. ISSN 1480-9222. Dostupné z: doi:10.1251/bpo138

Ice sublimation of ice cream

uncoated specimen
high vacuum
V0 = 1 kV

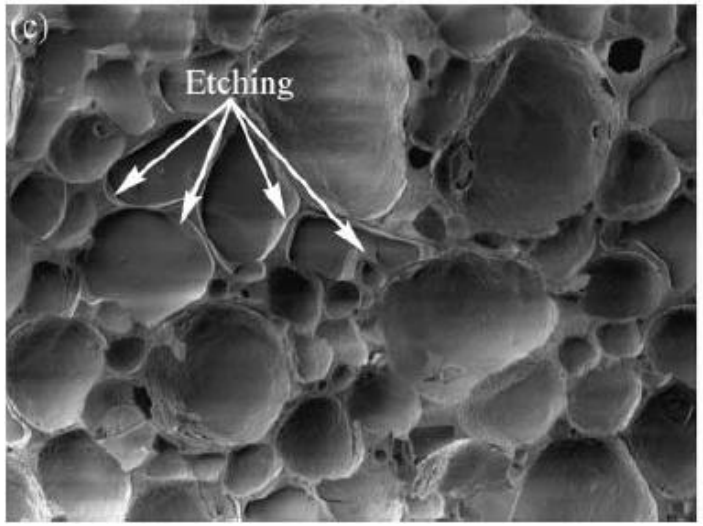
5min
-110°C

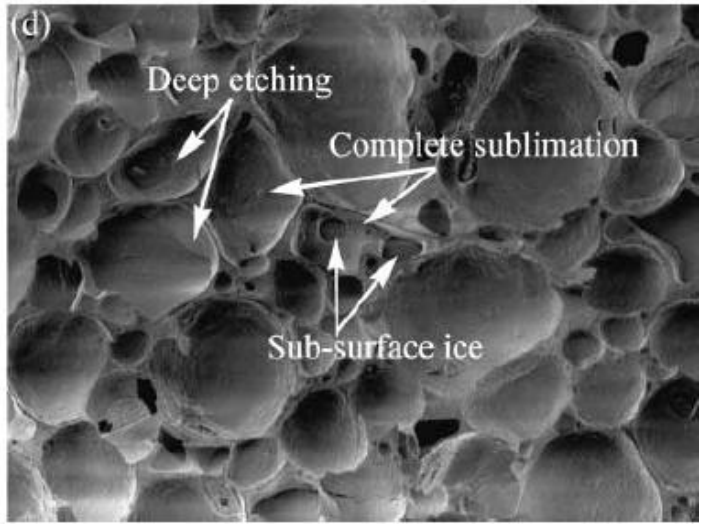




5min
-100°C

7min
-95°C

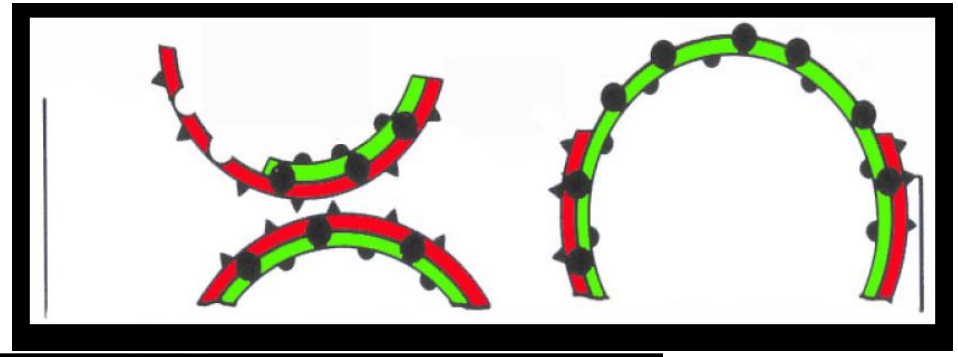


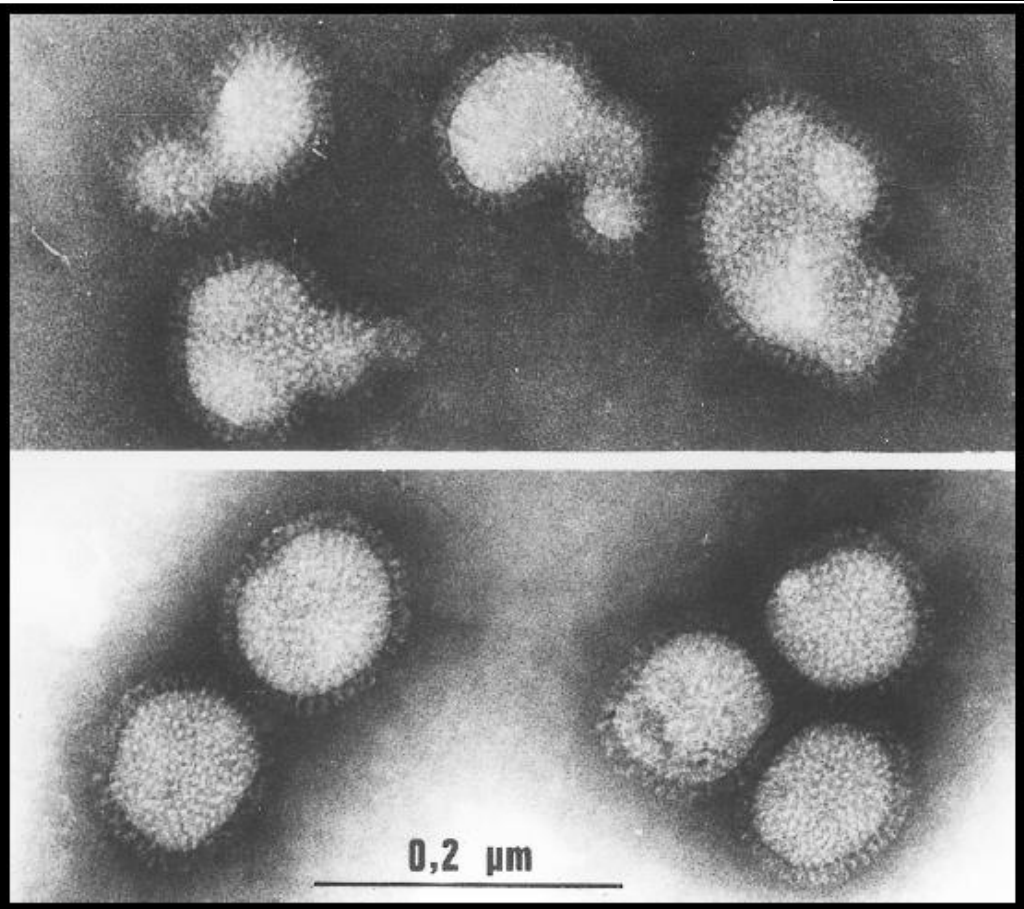


4min
-90°C

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

Freeze drying





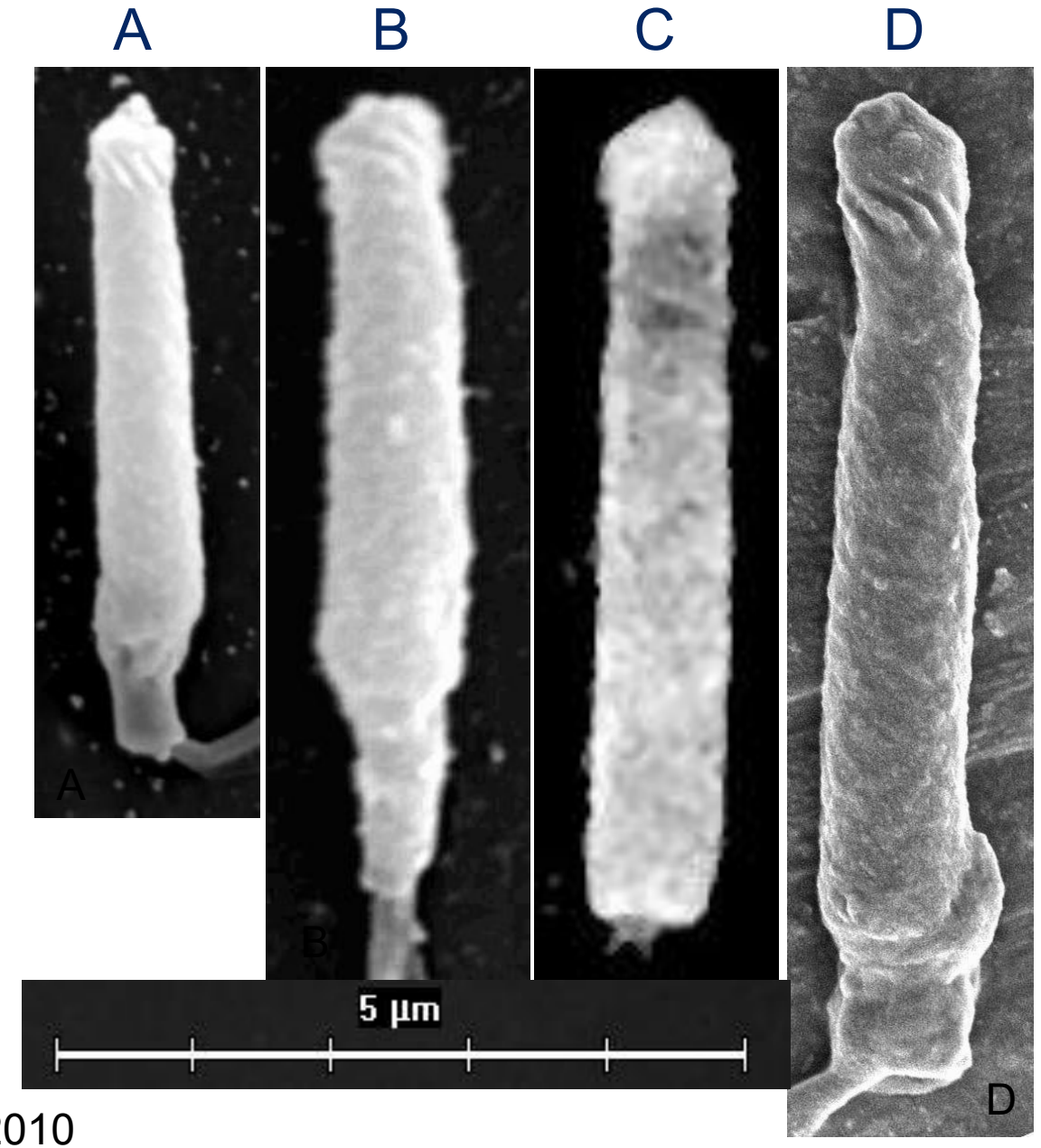
Air-dried

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.

Comparison

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

CLEM

- **C**orrelative **L**ight and **E**lectron **M**icroscopy
- combination of optical (mainly fluorescence) microscopy with electron microscopy

Benefits of fluorescence microscopy

- localizing specific cellular and subcellular targets
- Identification of specific molecules and study their biological roles
- Live cell imaging

Benefits of electron microscopy

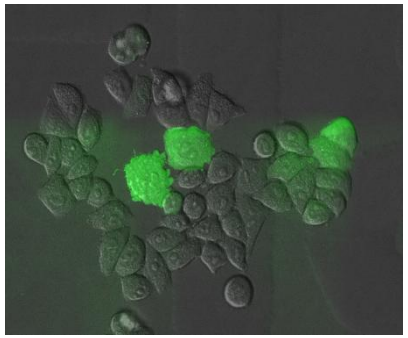
- imaging of ultrastructural details of cellular architectures
- ultrastructure detail beyond the limit of optical resolution

Bridging of two microscopic modalities

Combination the strengths of the two modalities and enables the analysis of cellular (or subcellular) events in their cellular context.

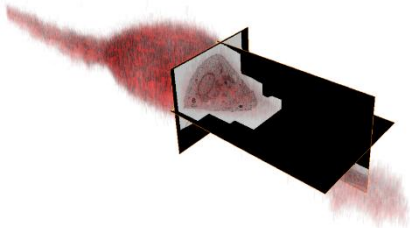
CLEM for select the cell

- Images from optical microscope are used only for the targeting one specific cell with the specific signal in an electron microscope



CLEM for registration of datasets

- datasets from optical and electron microscope are overlay for studying the complex relation between form and function in biology



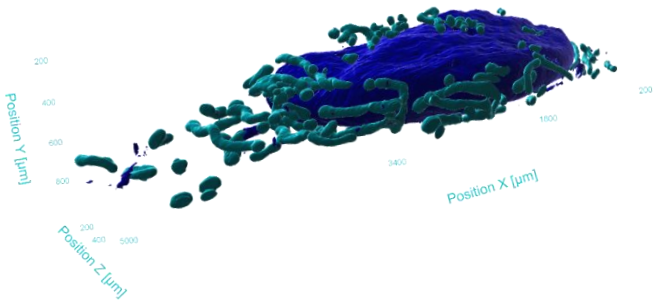
There are exist a lot of correlative approaches and many alternatives how to avoid the main sample prep cross-problem in CLEM but:

The most important questions for choosing the best method are:

✓What is the biological question = what do you need to see?

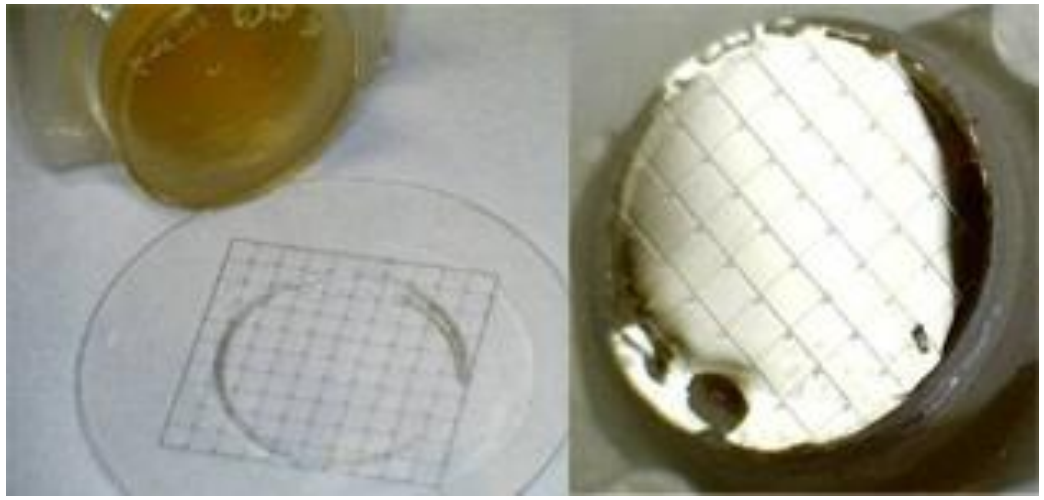
✓ What type of specimen do you have?

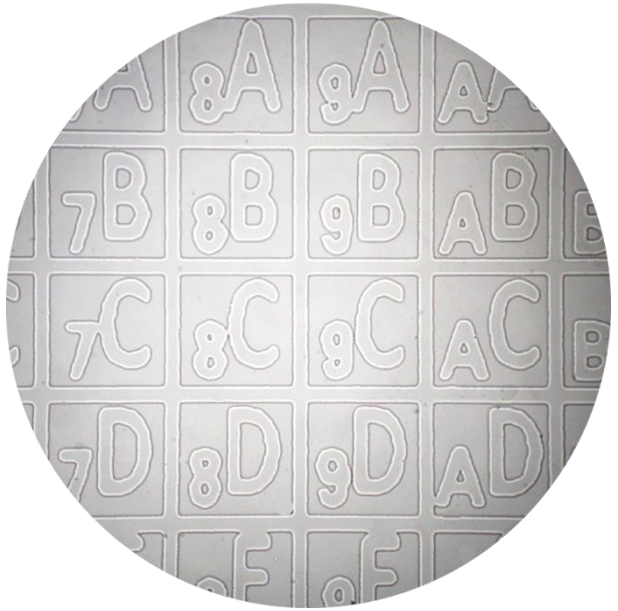
✓ What resolution, volume and number of samples do you require?



What is needed for finding the same area?

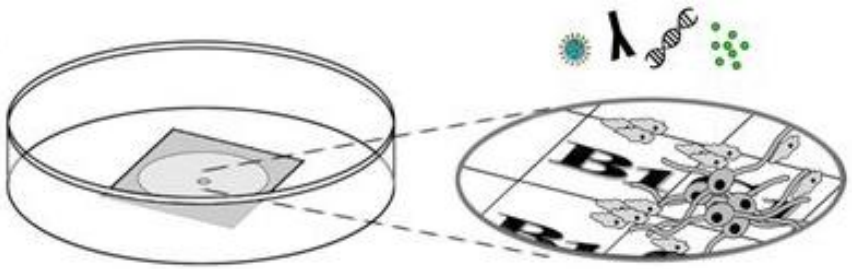
- Grid or pattern with good optical properties in brightfield/fluorescence and ability to imprented grid into resin or visibility on EM



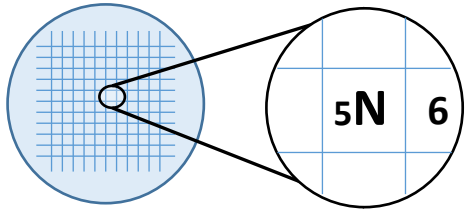


The footprint of the grid allows location of the cell position

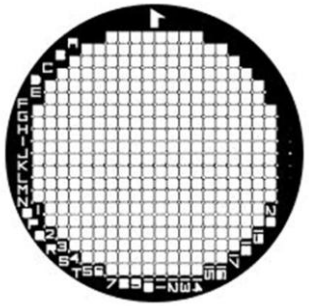
What is needed for finding the same area?



Mattek dish - disposable plastic petri dishes with the optical quality of glass in the middle with a grid

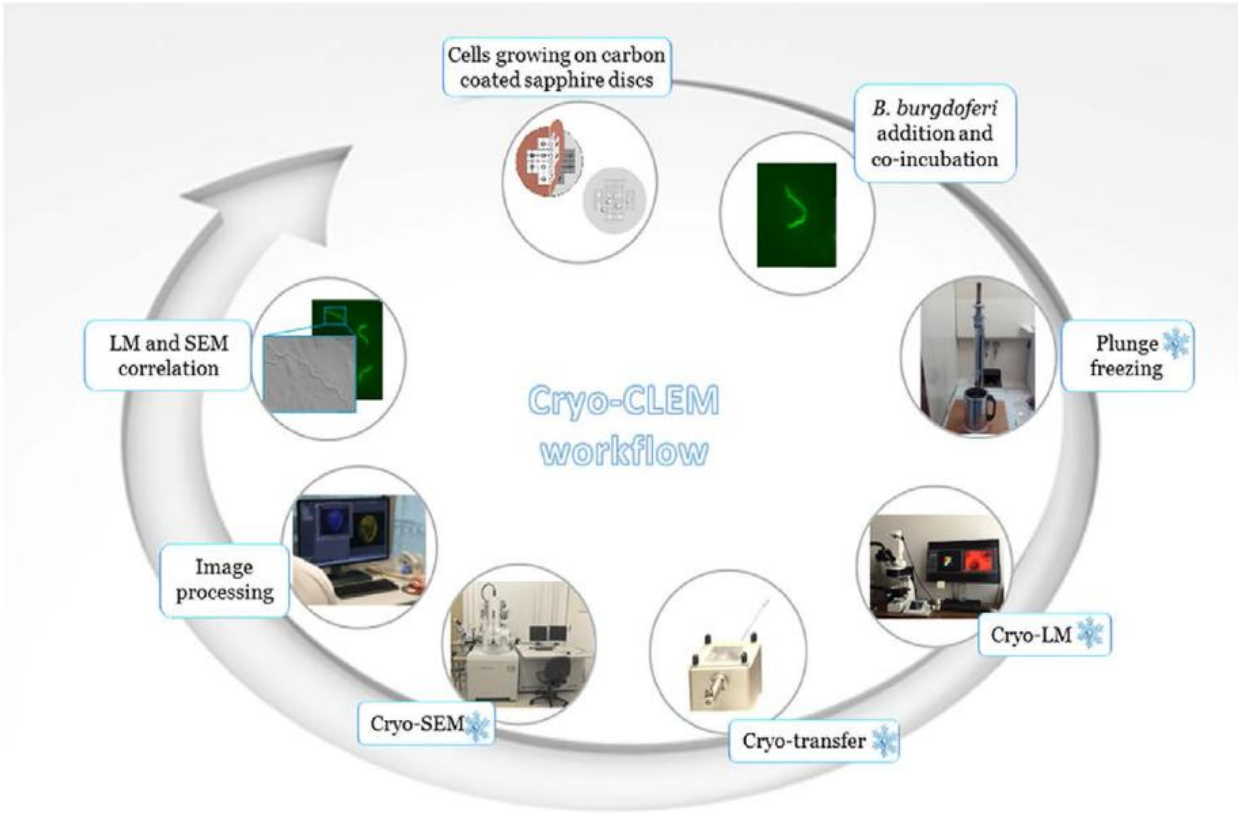


Sapphire disk with imprinted grid – cell monolayer cultivation



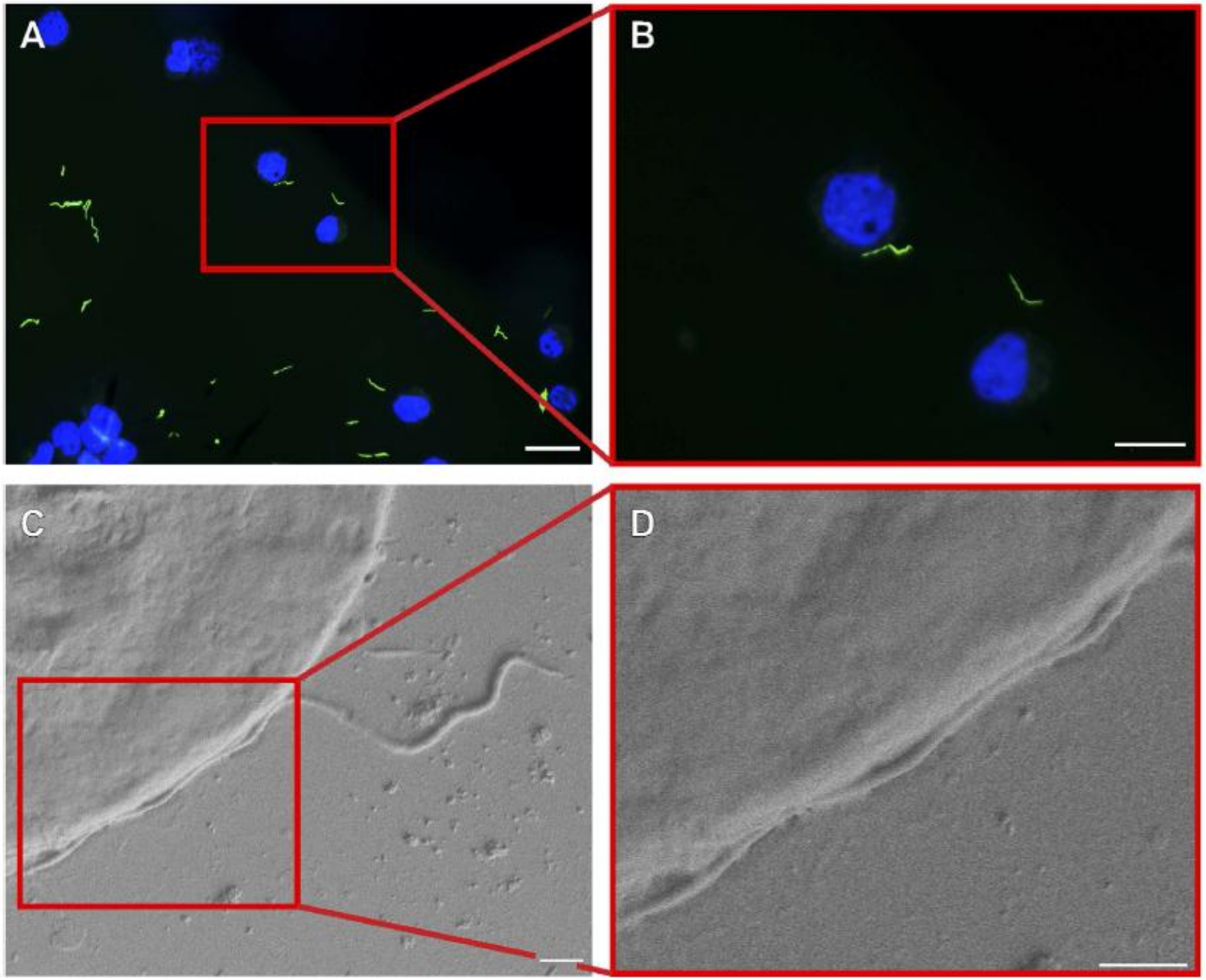
Finder grids with marks

Correlative microscopy: cryo FM a cryo 2D-SEM



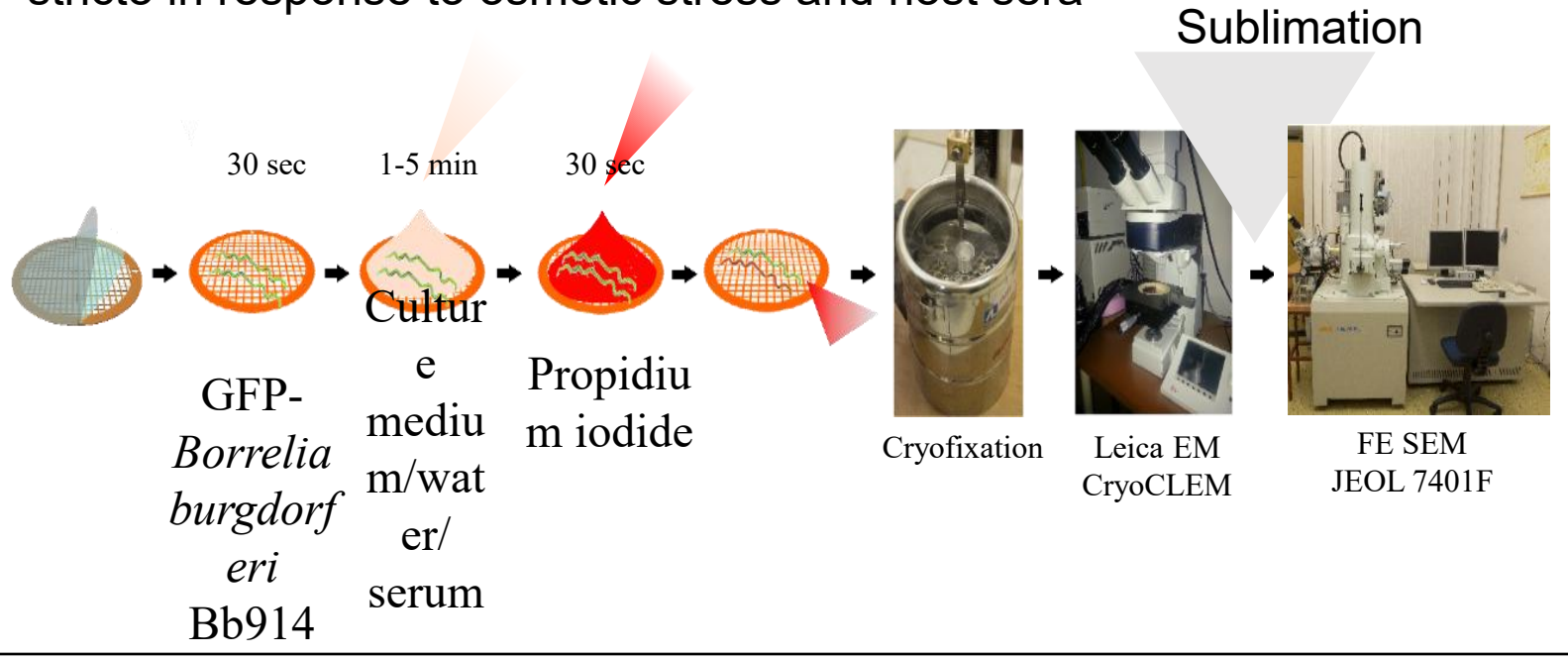
The interaction of bacterium *Borrelia burgdorferi* with human neuroblastoma cells

Strnad M. et al., Scientific Reports, 5, 2015, 18029

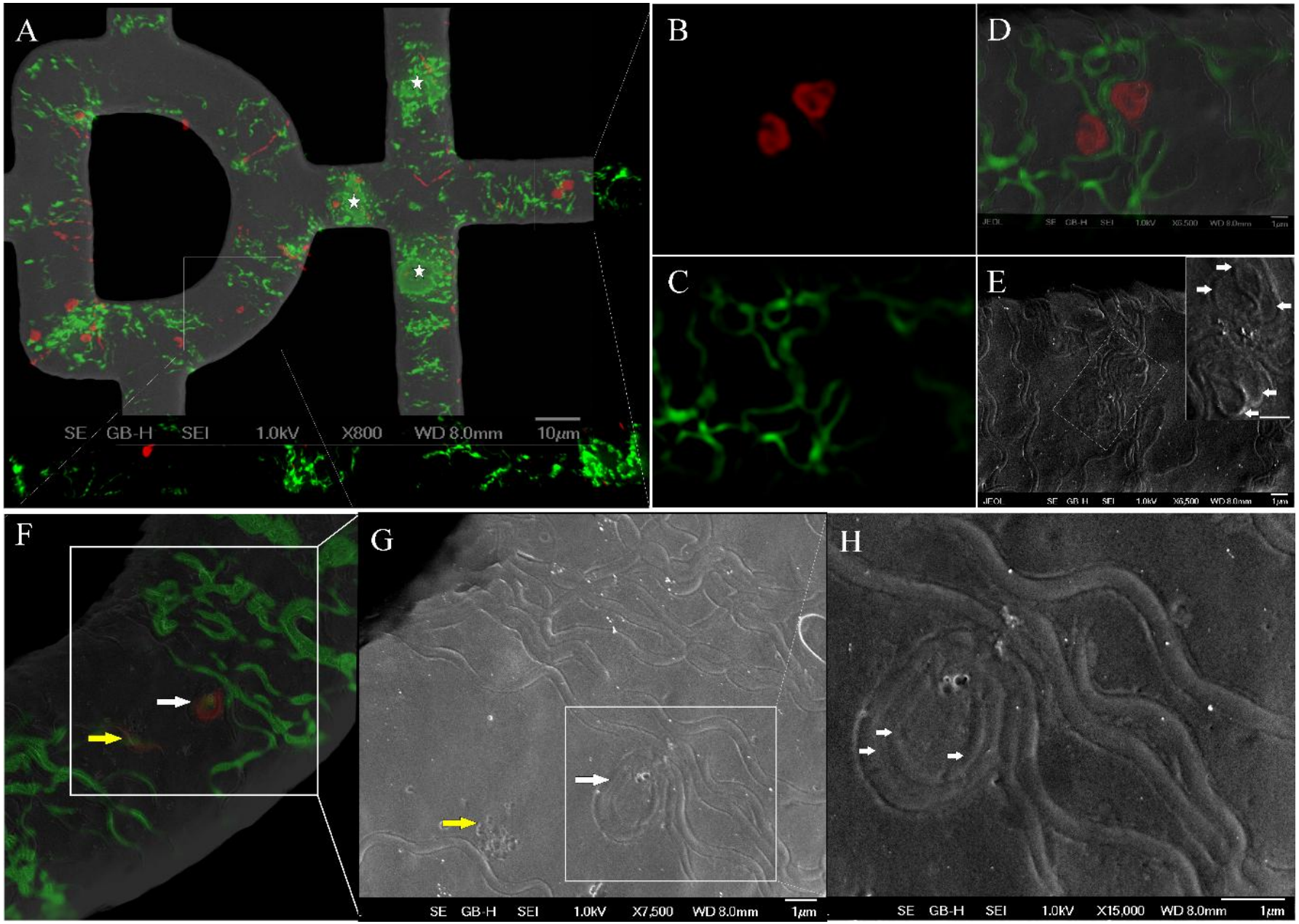

A
B
C
D

Correlative microscopy: cryo FM a cryo 2D-SEM

Morphological changes of GFP-expressing *Borrelia burgdorferi* sensu stricto in response to osmotic stress and host sera



Vancova M. et al., Scientific Reports, 2018



Thank you for attention

Thank to Jana Nebesářová (**Faculty of Science, Charles University, Prague, nebesaro@natur.cuni.cz Biology Centre v.v.i., Academy of Sciences of the CR, České Budějovice)** for some slides in this presentation.