Strategies of Biological Sample Preparation for Electron Microscopy

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IMG

EURO BIOIMAGING
Image life, discover the future

CSMS

CZECH BIOIMAGING
Imaging principles of life

Why is it necessary to process biological samples?

Samples are too:

- Big
- Soft
- Wet
- Perishable

Why is it necessary to process biological samples?

- Stopping of postmortem decay
- Low penetration ability of primary electrons = the maximum thickness of specimen in low 100s nm for TEM:

Specimens with sizes **under** the penetration limit of primary electrons

Isolated cell organels
viruses, macromolecular complexes...

Specimens with sizes **above** the penetration limit of primary electrons

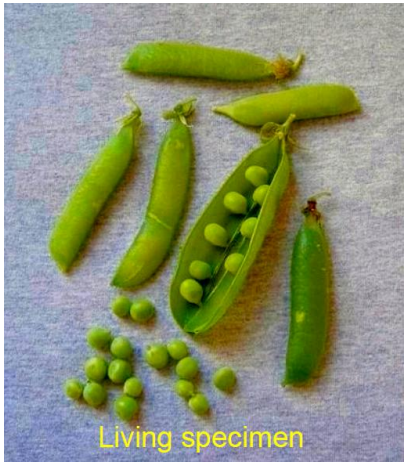
Cutting of ultrathin sections

- Vacuum inside electron microscopes = **no liquid water** in the specimen

How to remove liquid water?

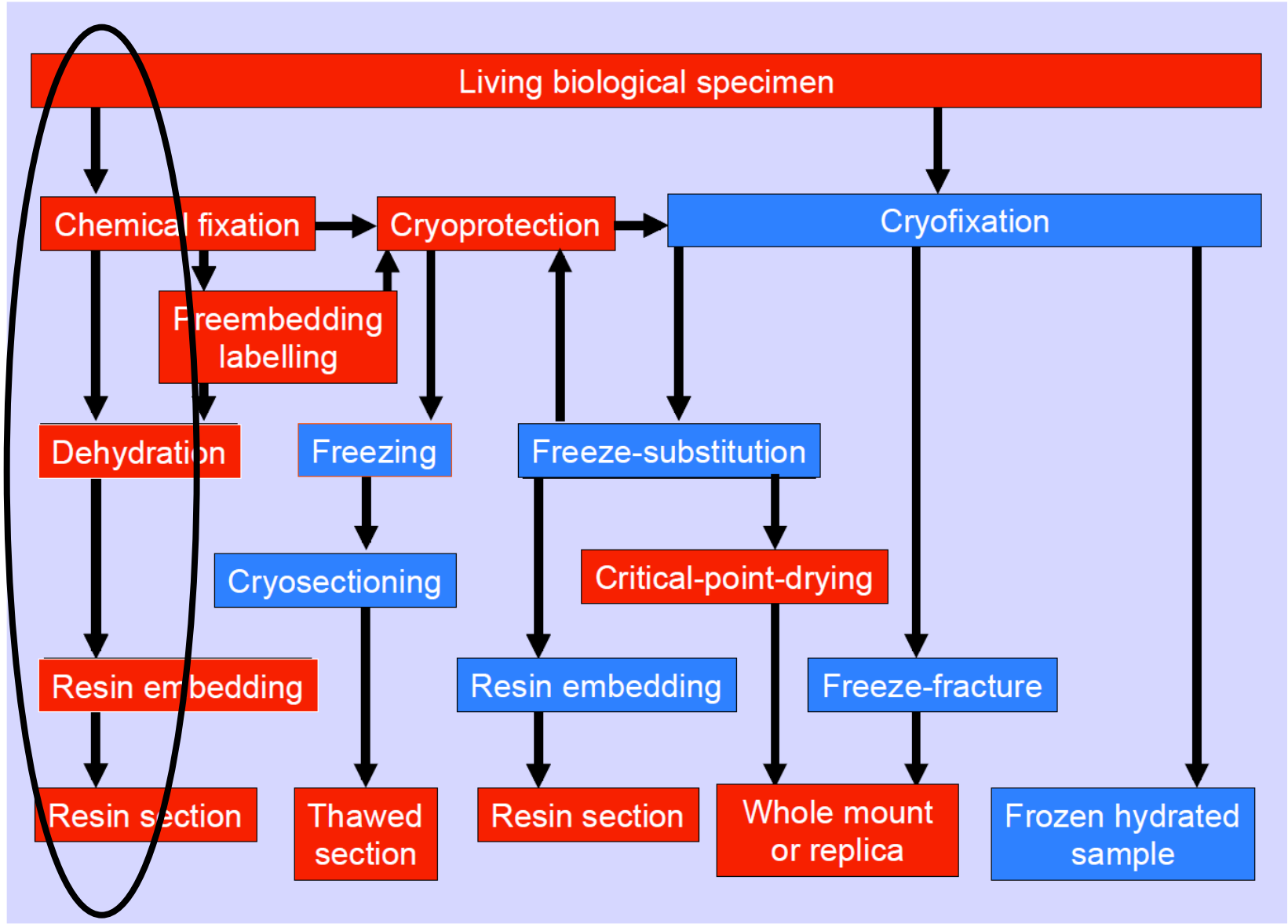
Drying

Freezing

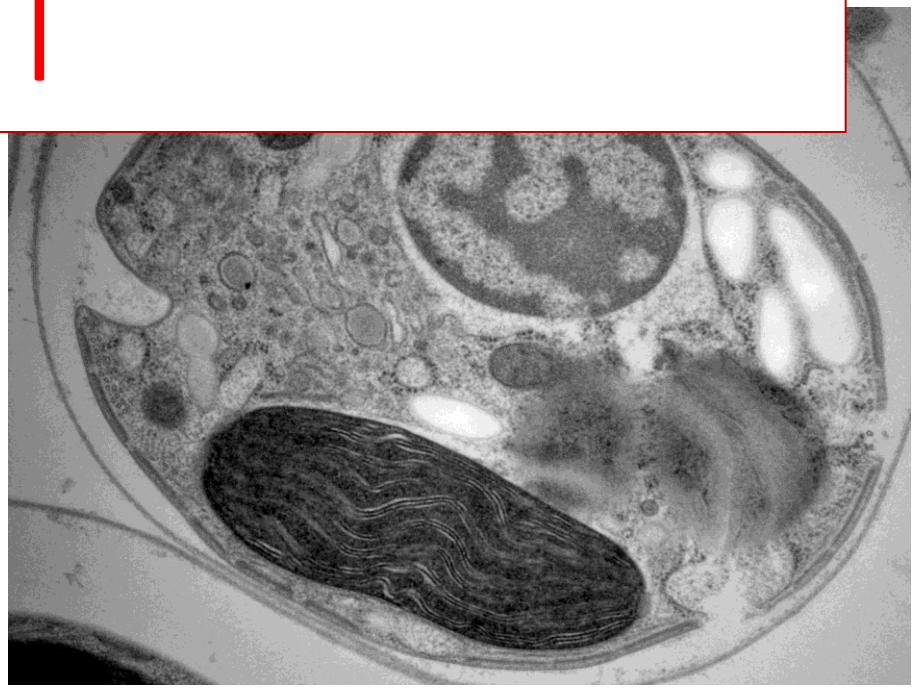


Kent McDonald, UC Berkeley

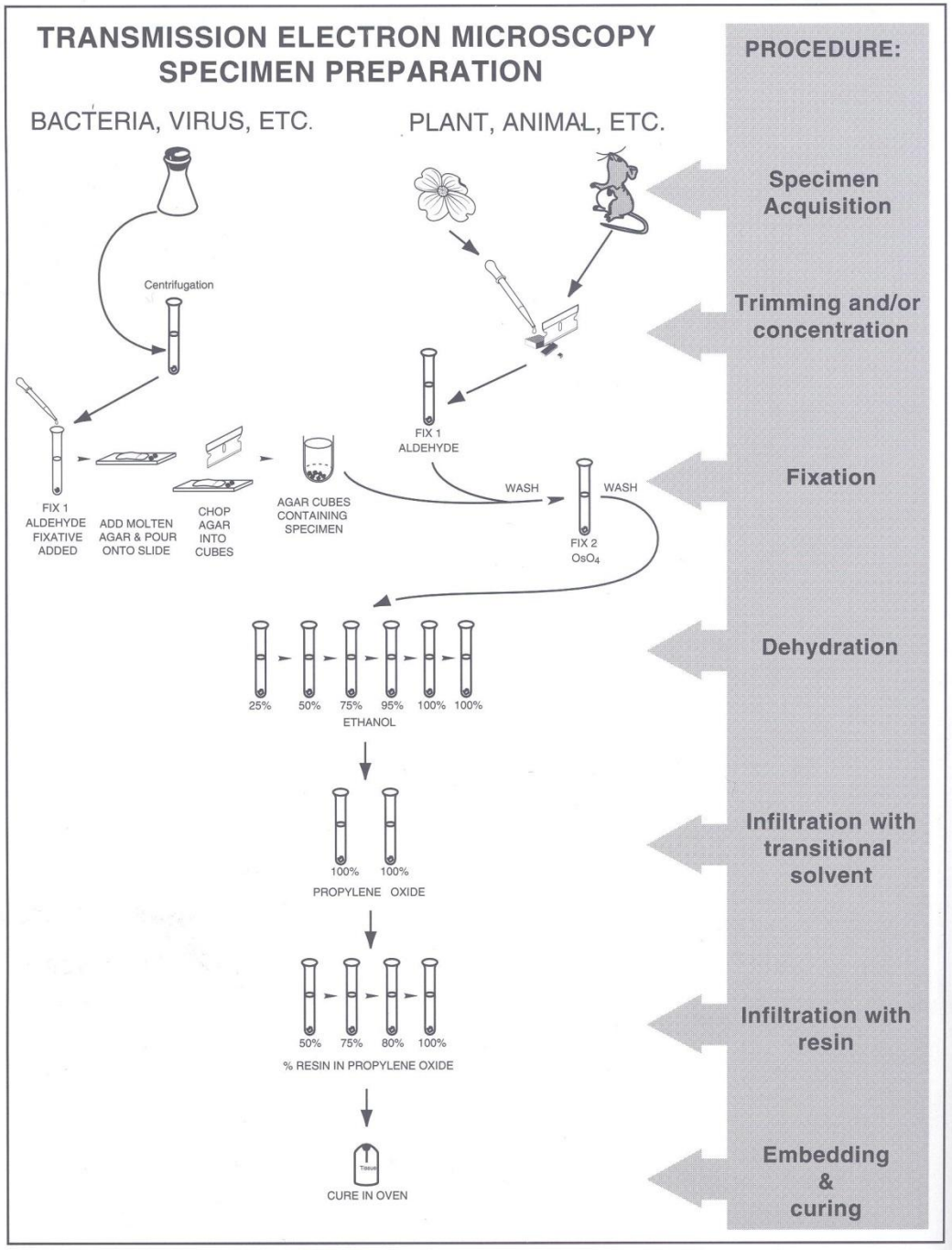
Strategies of biological specimen preparation



Specimen preparation for TEM at RT



Solving „perishable“



Chemical methods

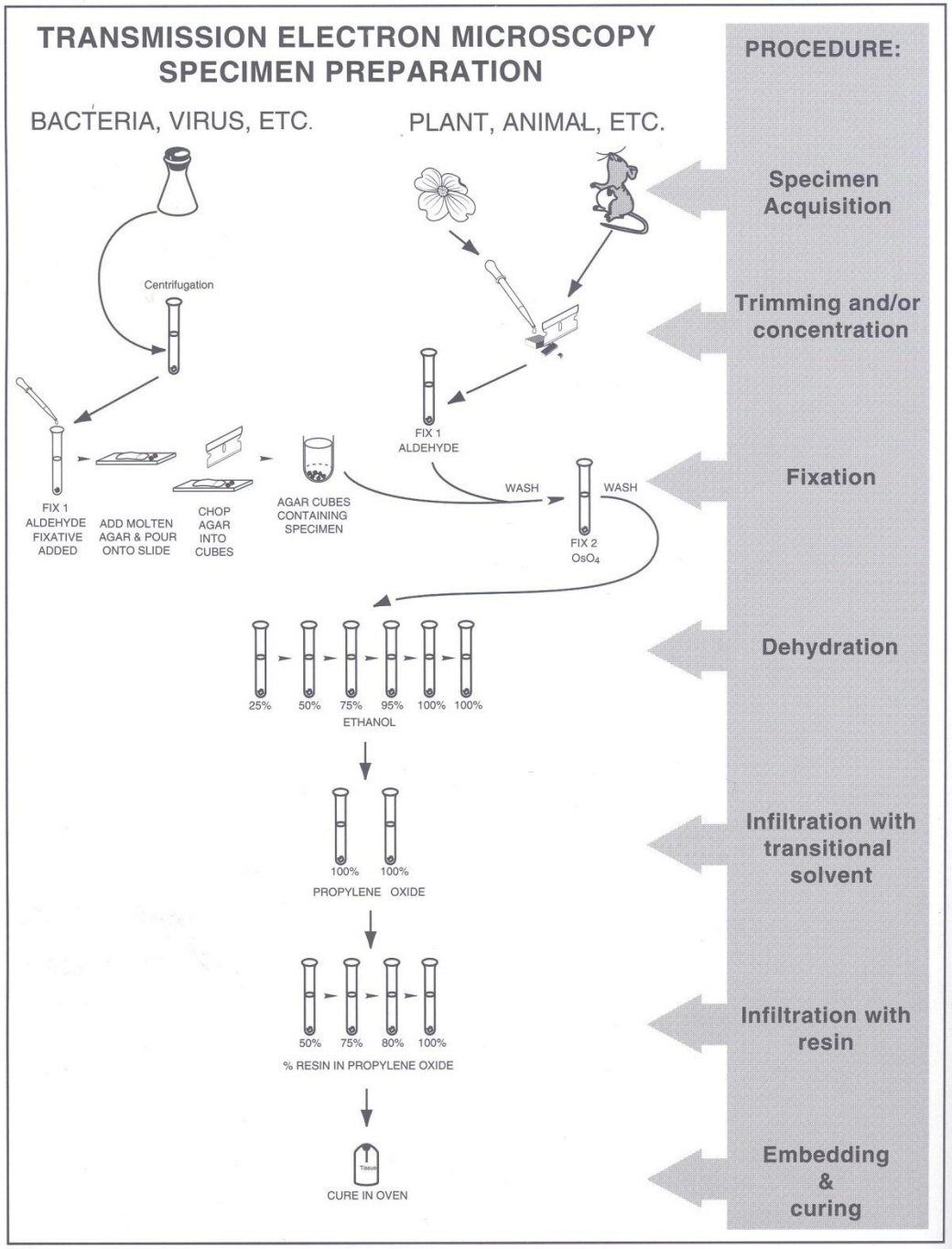
Main preparation pathway

Advantage:

no expensive equipment

Disadvantage:

production of artifacts



Fixation

- Stabilization and immobilization of specimen ultrastructure as close as possible to its native state
- Stopping post-mortem decay

- Aldehydes

glutaraldehyde and *formaldehyde*

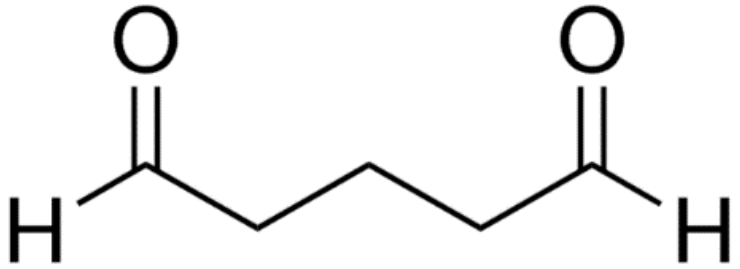
- Oxidation agents

osmium tetroxide

potasium permanganate

Glutaraldehyde $OHC\text{-}(CH_2)_3\text{-}CHO$

- Aliphatic dialdehyde which forms colorless crystals that are highly soluble in water, ethanol, and most organic solvents
- Aqueous solution (1-4%), which is relatively stable with a pungent odor
- GA can cross-link proteins rapidly, effectively, and irreversibly; forms a large, three-dimensional network throughout the cytoplasm in tenths of second to minutes
- Dissolved GA uncharged => can rapidly cross biological membranes.



Glutaraldehyde

- GA reacts predominantly with amines to form numerous products
- 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

Work with GA carefully!
Avoid:

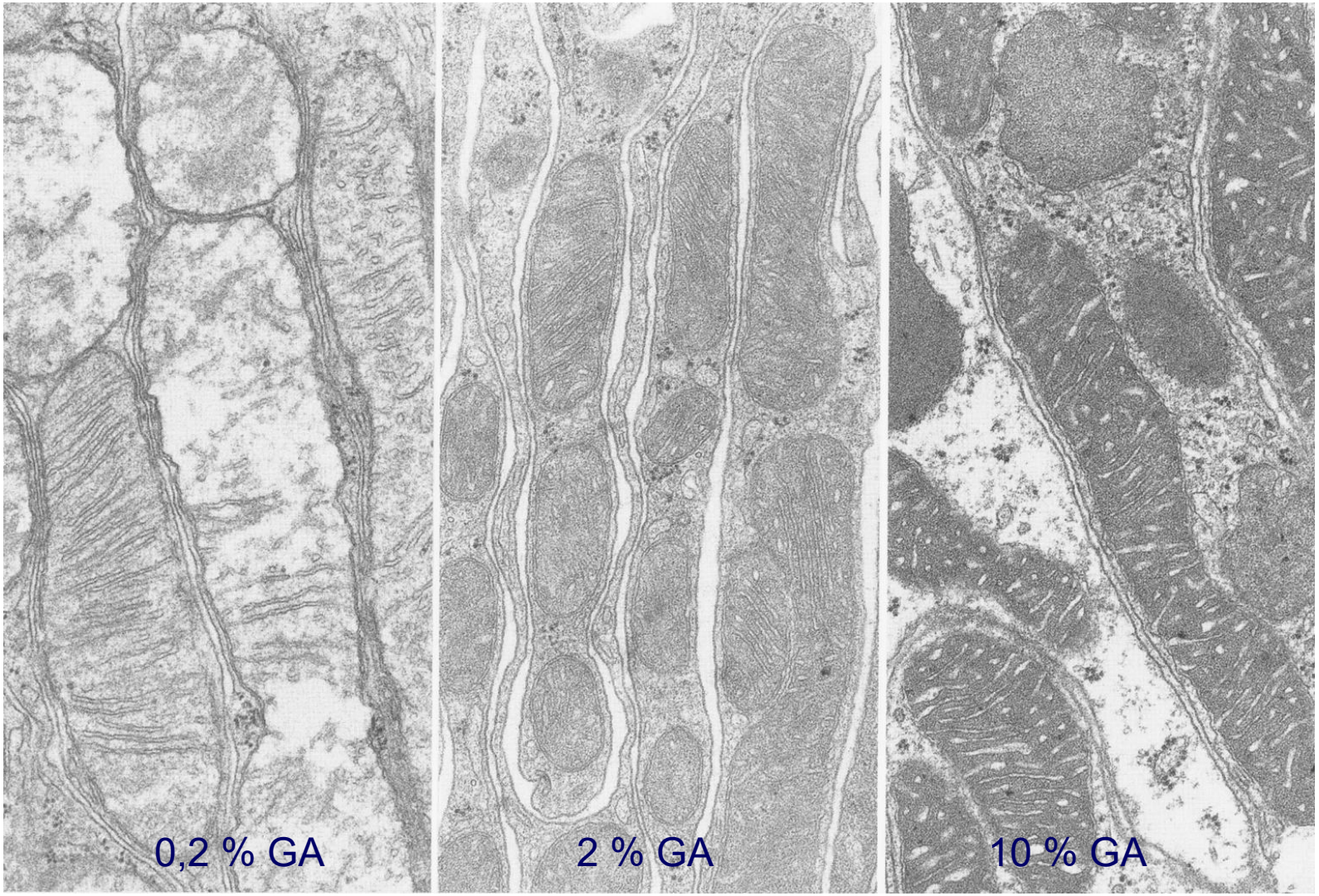
- contact with the skin and eyes
- prolonged inhalation

Reapeted exposure may cause contact dermatitis.

A well-ventilated hood is necessary!

POISON

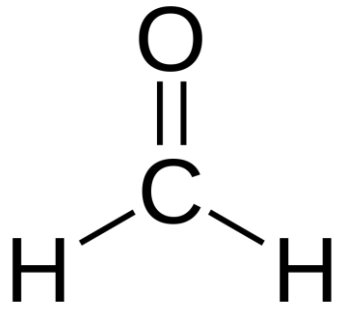
Concentration matters



Tubul cells of rat kidney, postfixation by OsO_4, Epon, stained

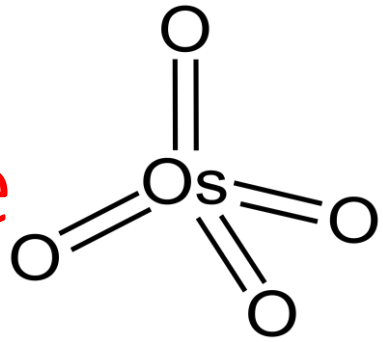
Recommended GA concentration : 1 - 4 %

Formaldehyde H-CHO



- At room temperature FA is a colorless gas highly soluble in water. Both liquid and gas polymerize spontanously.
- Commercially available as a concentrated aqueous solution or in a polymerized state as a dehydrated powder
- FA molecule is smaller than GA, penetrates into the sample 5 times faster than GA
- Karnovsky‘s fixative – the combination of FA and GA (recommeded for larger size samples with a poor penetration)
- Weakly links proteins =>prefered fixative for immunolabelling techniques

Osmium tetroxide



- The molecule is symmetrical and contains four double bonded oxygen atoms.
- Osmium tetroxide has a number of stable oxidation state and is soluble in polar and non –polar solvents. Thus can fix both hydrofobic and hydrofilic domains in cells.
- It is highly volatile, it can be used also as a vapor fixative.
- It causes rapid permeabilization of membranes with cessation of cytoplasmic movement within second to minutes.
- It reacts with ethanol to form black precipitates

  $OsO4 + C2H5OH = OsO2 + 2\ CH3CHO + 2\ H2O$

- It is the most slowly penetrating fixative and has no cross-linking capabilities
- Prolonged fixation results in the progressive denaturation of proteins
- As a strong oxidant it damages the majority of antigents

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

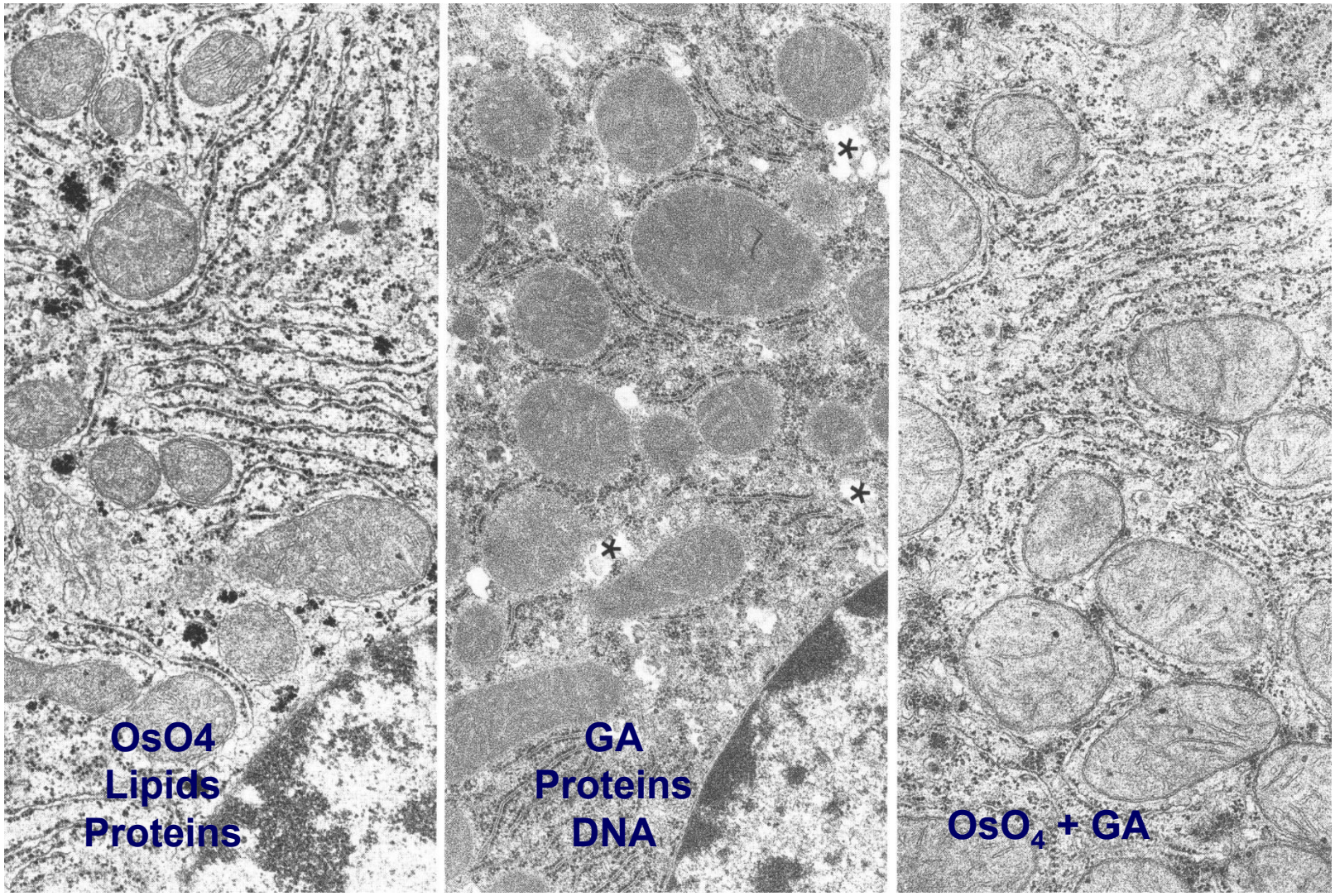
Be extremely careful in handling OsO_4!

It is highly toxic, volatile and always should be used under a fume hood!

- Dangerous to eyes, respiratory and alimentary membranes.
- Used or excess OsO_4 must be stored in sealed glass containers.
- Avoid the direct contact by wearing plastic gloves and working in well-ventilated hood.

POISON

Choice of fixative agents



Rat liver, Epon, stained

Fixative solution

- Fixative agent
- Buffer – phosphate, ~~cacodylate~~, HEPES…
- Distilled water
- Other substances improving fixation like hydrogen peroxide, tannic acid, dimethylsufoxid, uranylacetate

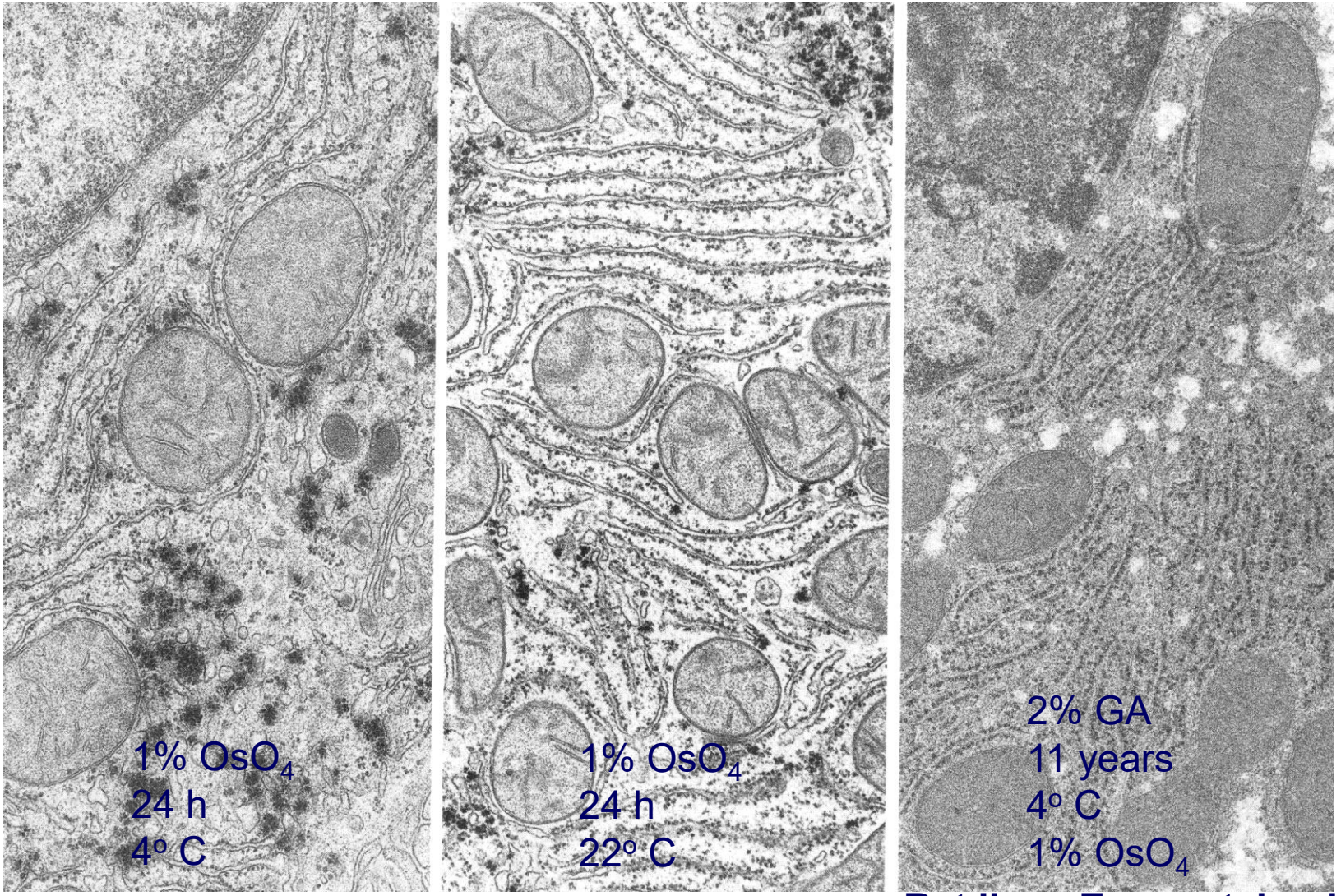
Isotonic or slightly hypertonic solution

pH 6,7-7,1

Factors affecting quality of fixation

- Concentration and lenght of fixation
- Temperature of fixation
- pH and buffer vehicle
- Osmolarity of fixative solution
- Purity of aldehyde fixative
- **Tissue type**
- Methods of fixation
- Rate of fixative penetration

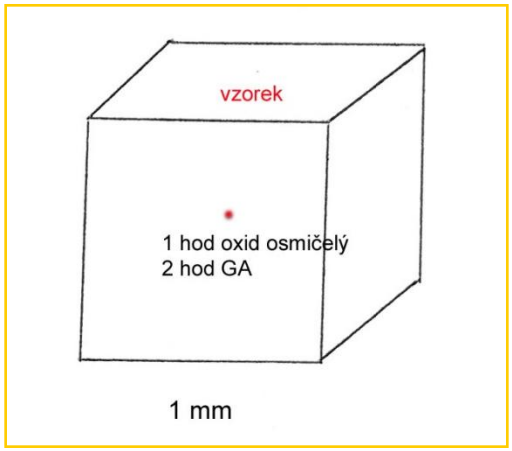
Time and temperature of fixation



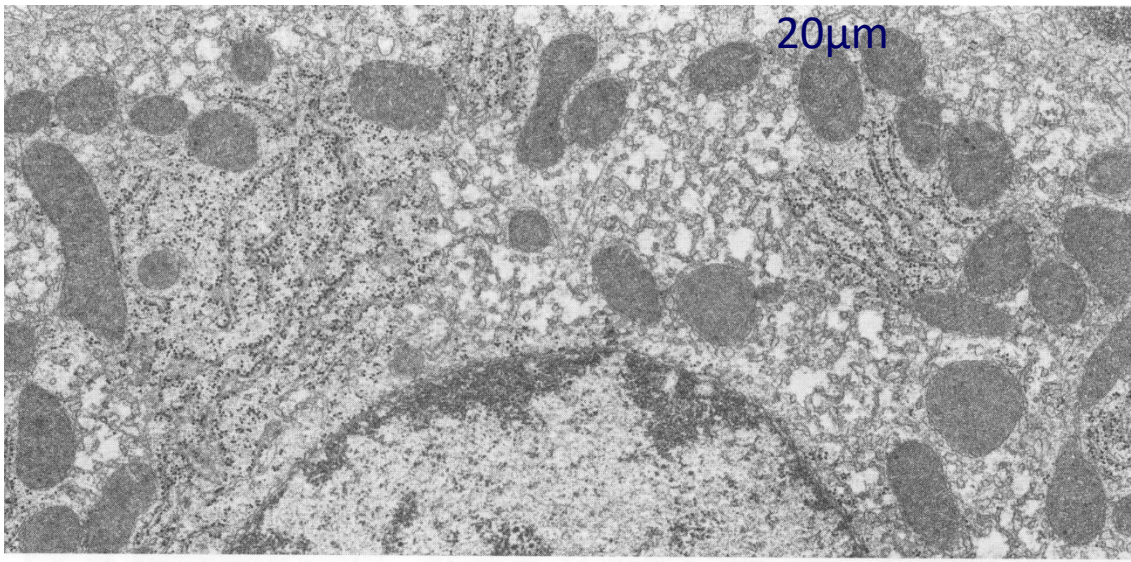
Rat liver, Epon, stained

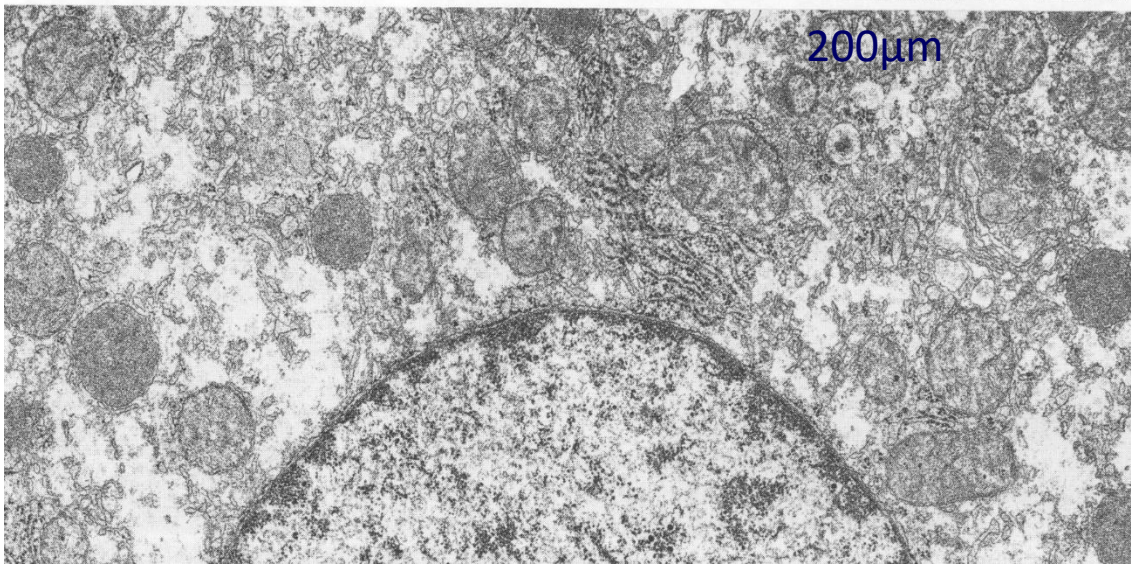
Size (depth) matters

Immersion fixation

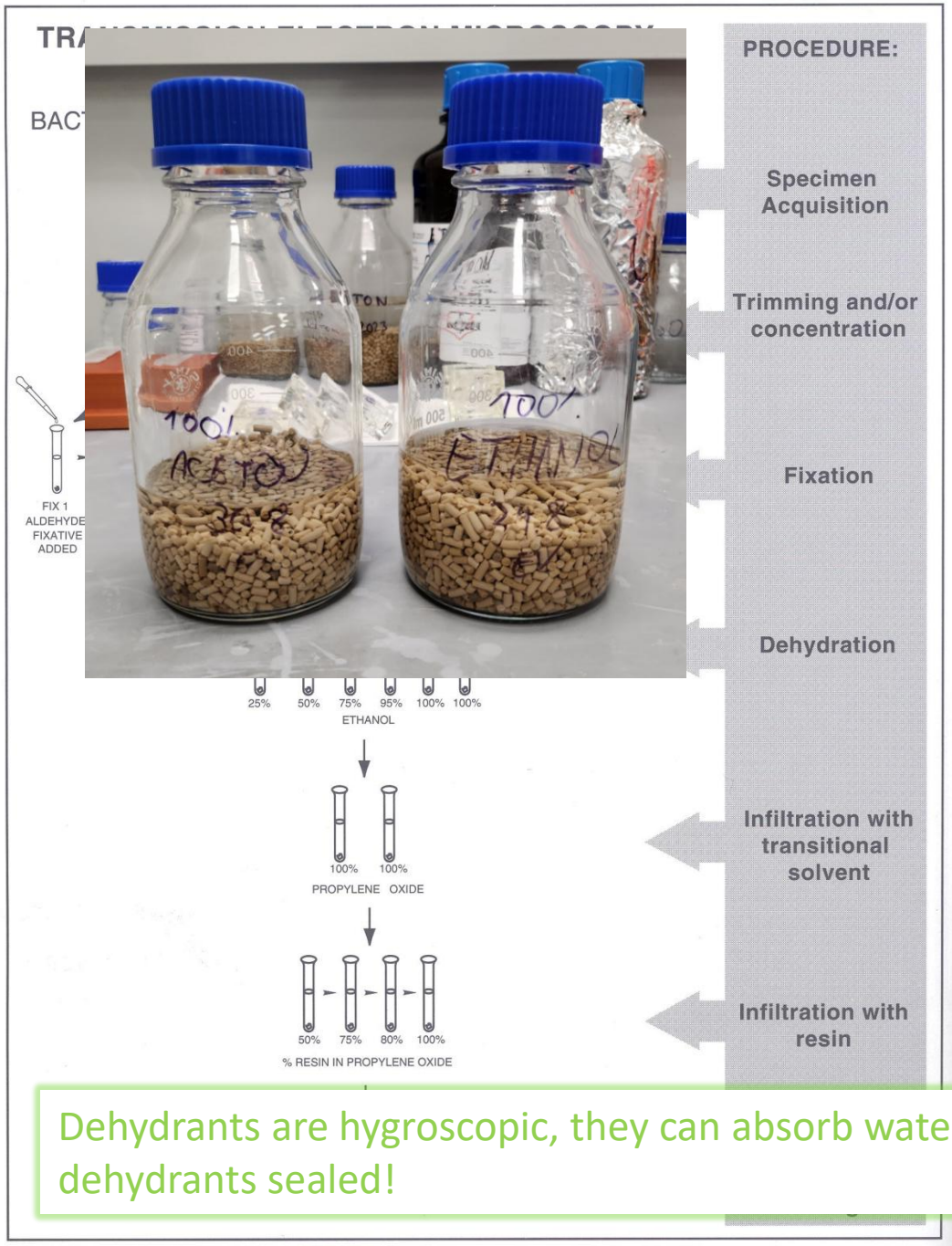


Rat liver, Epon,
1% OsO4
0,1 M cacodylate buffer
Epon
stained





Solving „wet“



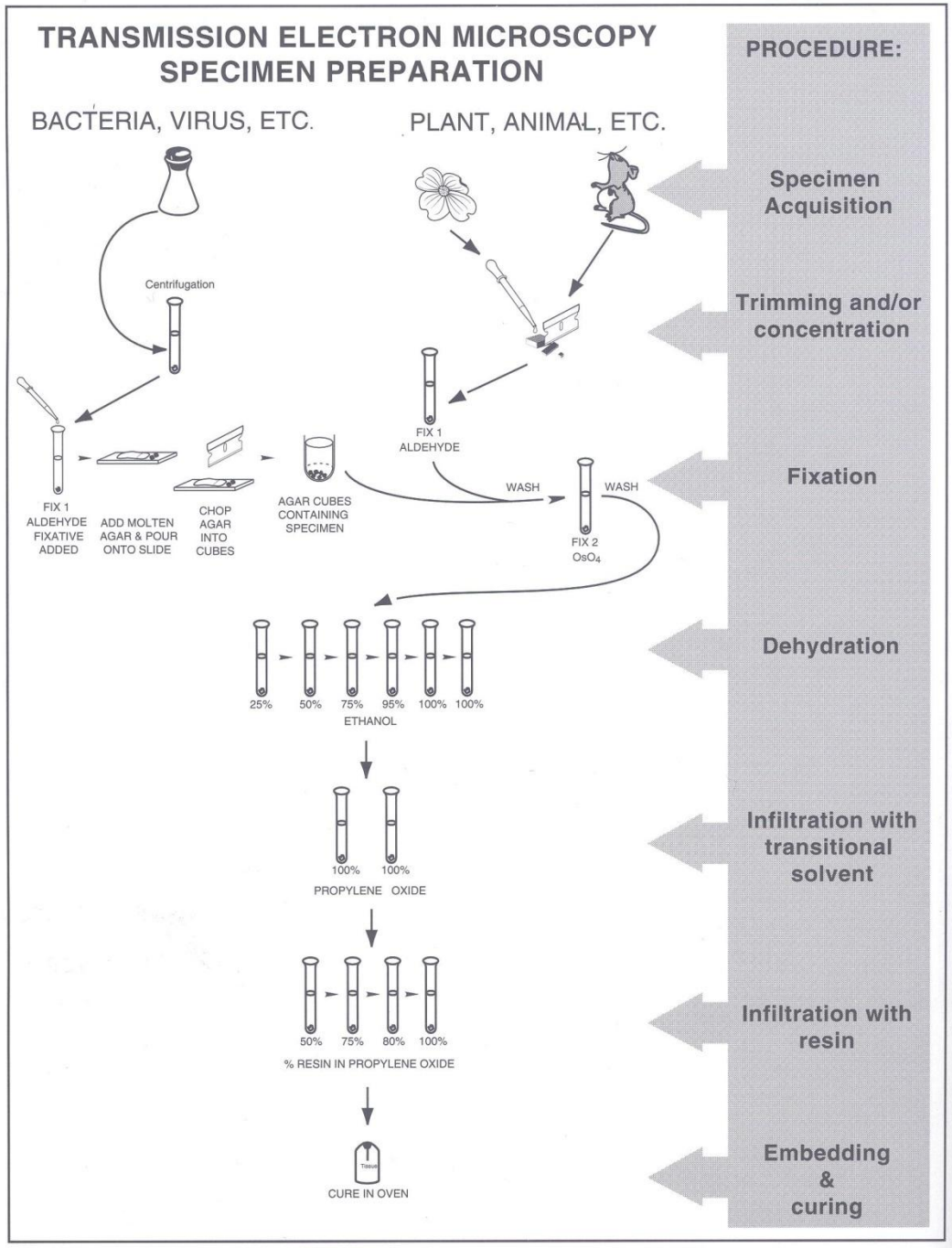
Dehydration

To replace the water in samples with organic solvent (ethanol, acetone, propylene oxide)

Caused shrinkage, extraction of various cellular components, changes in shape and size of the sample

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

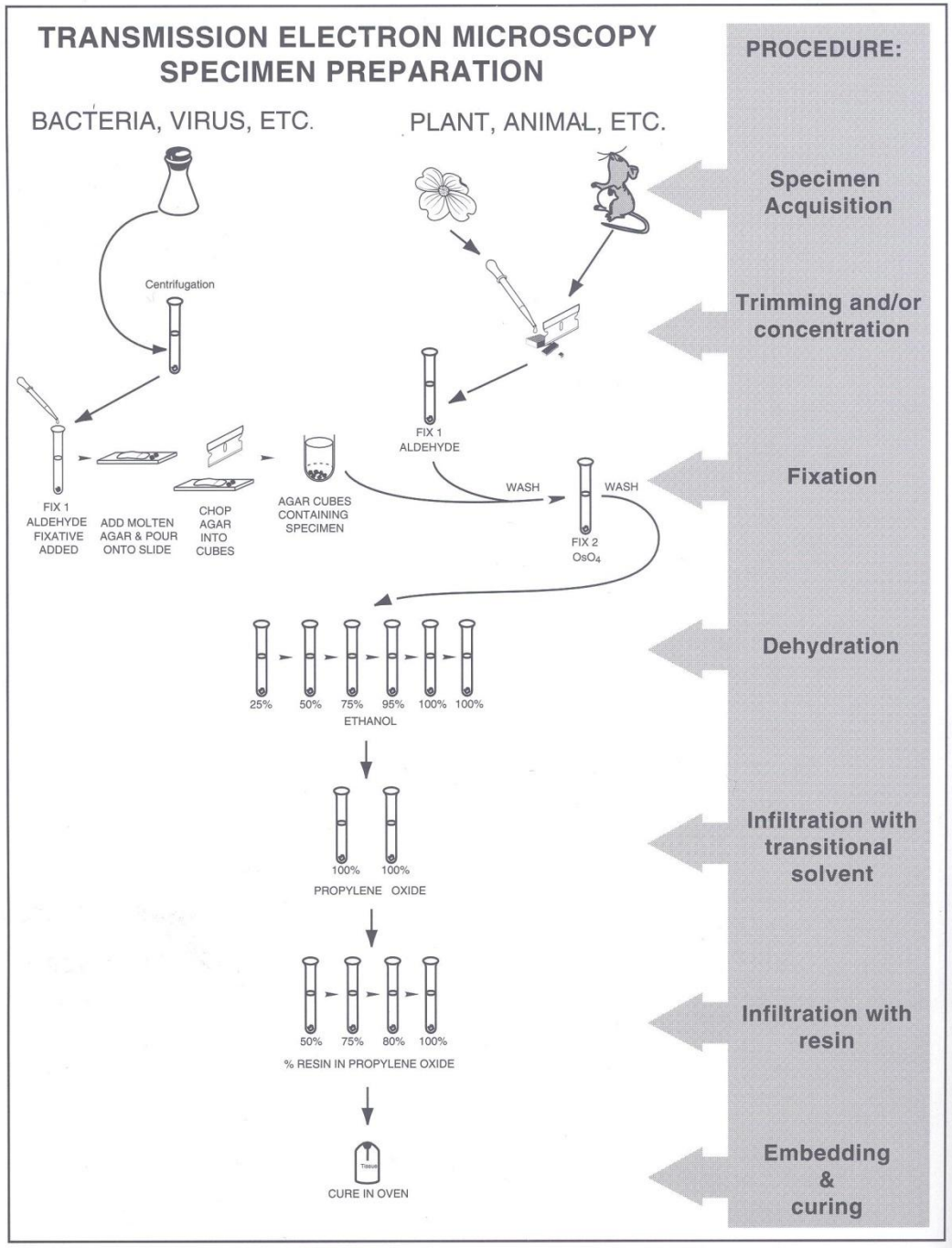
Solving „soft“



Infiltration

The sample is permeated with a liquid embedding media – resin monomers:

- Low viscosity
- Sufficient and uniform hardness
- Small volume changes during the polymerization
- Resistant to the irradiation by electron beam
- Without own ultrastructure

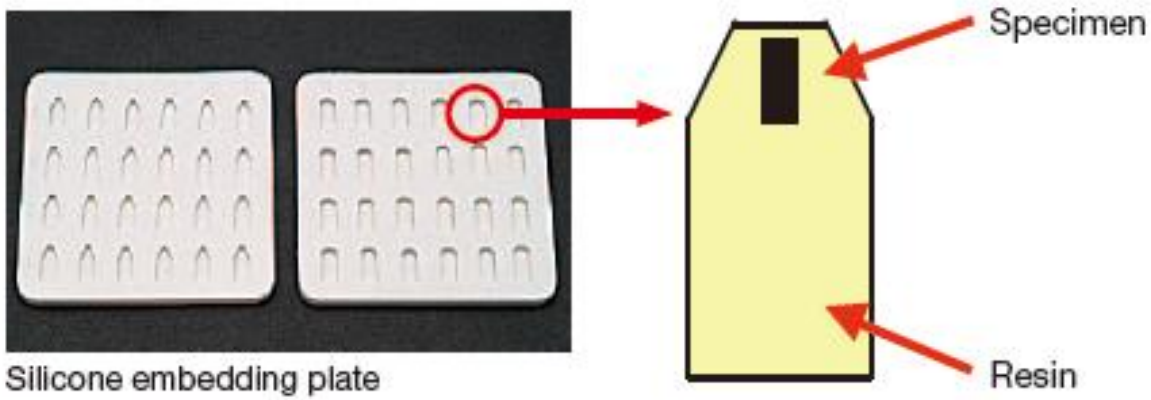


Embedding

The sample in a pure resin is placed in a suitable mold:

- Epoxide resins – ultrastructural studies (Epon, Spurr, Araldit)
- Acrylic resins – immunolocalization (LR White, LR Gold, Lowicryls)
- Polyester resins – Westopal
- Water-miscible media – Nanoplast, Durcupan

Embedding and polymerization



Silicone embedding plate

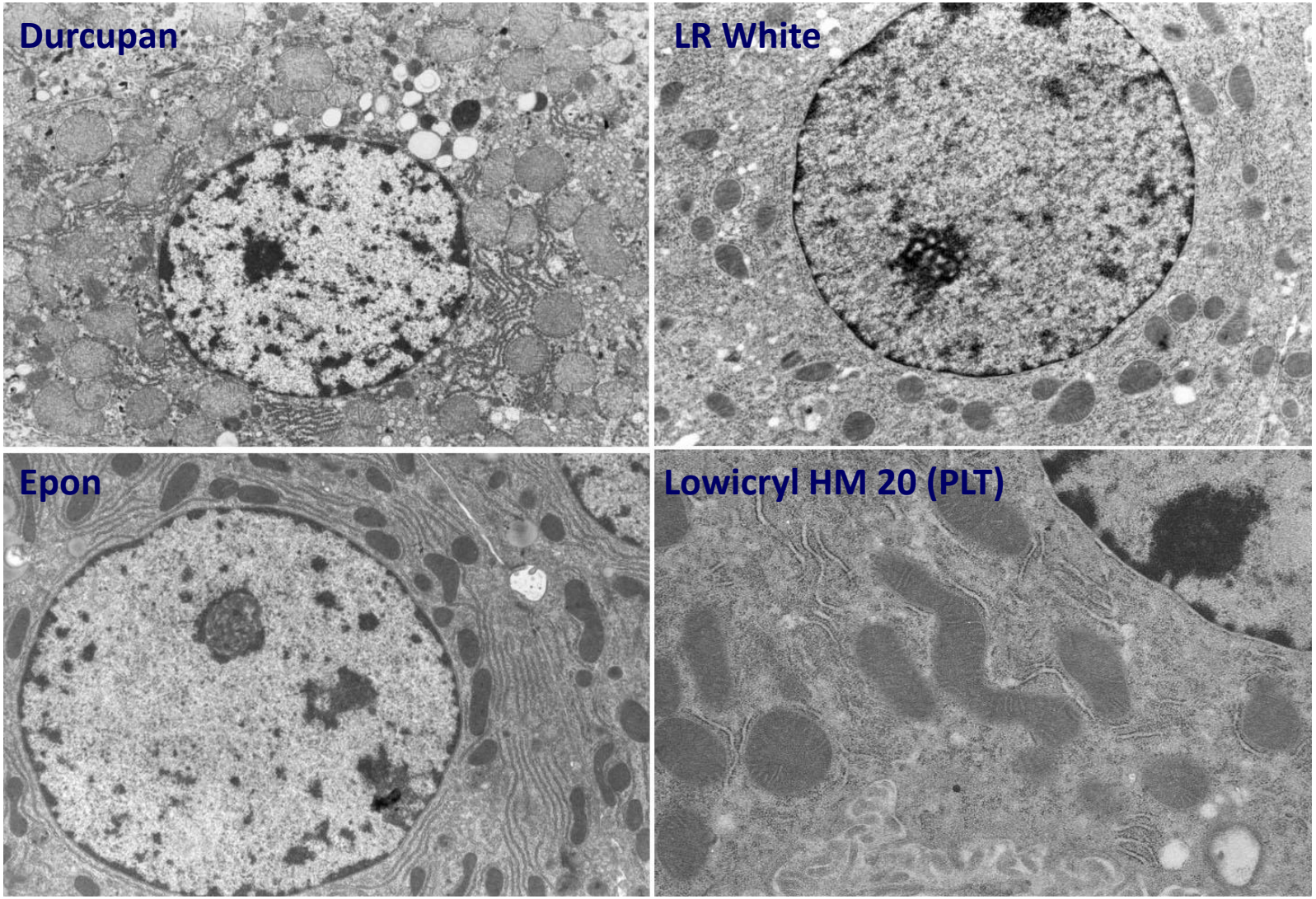
Polymerization:

- by heat (12-48 hours)
- by UV light (at RT or below)

Molds: flat capsules


Oven for polymerization (1 stage type)

Type of resin matters



Epon – Durcupan embedding – brown adipose tissue (var. IIE):

Day 1:

- fixation with 2% PFA + 2,5% GA/SB (prepare always just before fixation from stock 20% PFA, 25% GA to prevent polymerization) –overnight, (0.5 hr RT) 4°C – cut into really small pieces after 1.5 hr of fixation

Day 2:

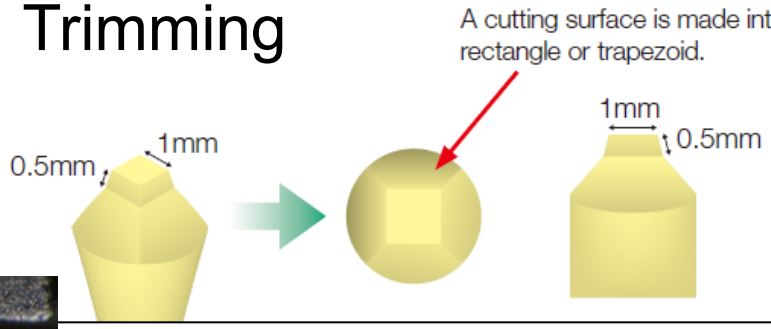
- wash with SB, 10 min, RT
- quench aldehydes with 0,02M glycine in SB, 30 min, RT
- wash with SB, 2x10 min, RT
- 1 % OsO4 in SB at RT (in the dark) – 1,5 hours
- Wash with SB - 3 x 10 min
- Rinse with water 3 x 10 min
- 30 % ethanol in water - 15 min, 4°C
- 50 % ethanol in water - 15 min, 4°C
- 70 % ethanol in water - 15 min, 4°C.
- 90 % ethanol in water – 2x15 min, RT
- 95 % ethanol – 2x15 min, RT
- 100 % ethanol water free - 20 min, RT
- 100 % ethanol water free - 15 min, RT
- propylene oxide 2x15 min, RT
- propylene oxide 1 : 1 Epon+C, 1 hr, RT
- propylene oxide 1 : 3 Epon+C, overnight, 4°C **Day 3:**
- 100 % Epon+C – 2 hrs, RT (open to allow evaporation)
- 100 % Epon+C – 2 hrs, RT (open to allow evaporation)
- Embedding
- Polymerization in oven at 60°C – 72 hrs

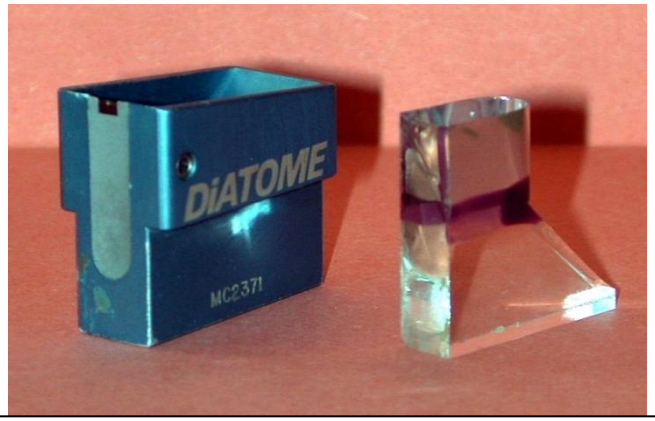
On shaker where possible!

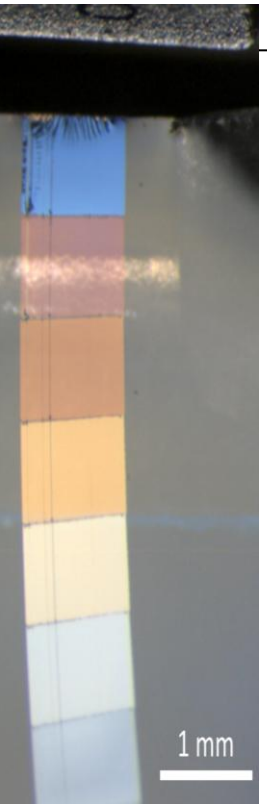
Solving „big“

Ultramicrotomy
cutting ultrathin sections

Trimming





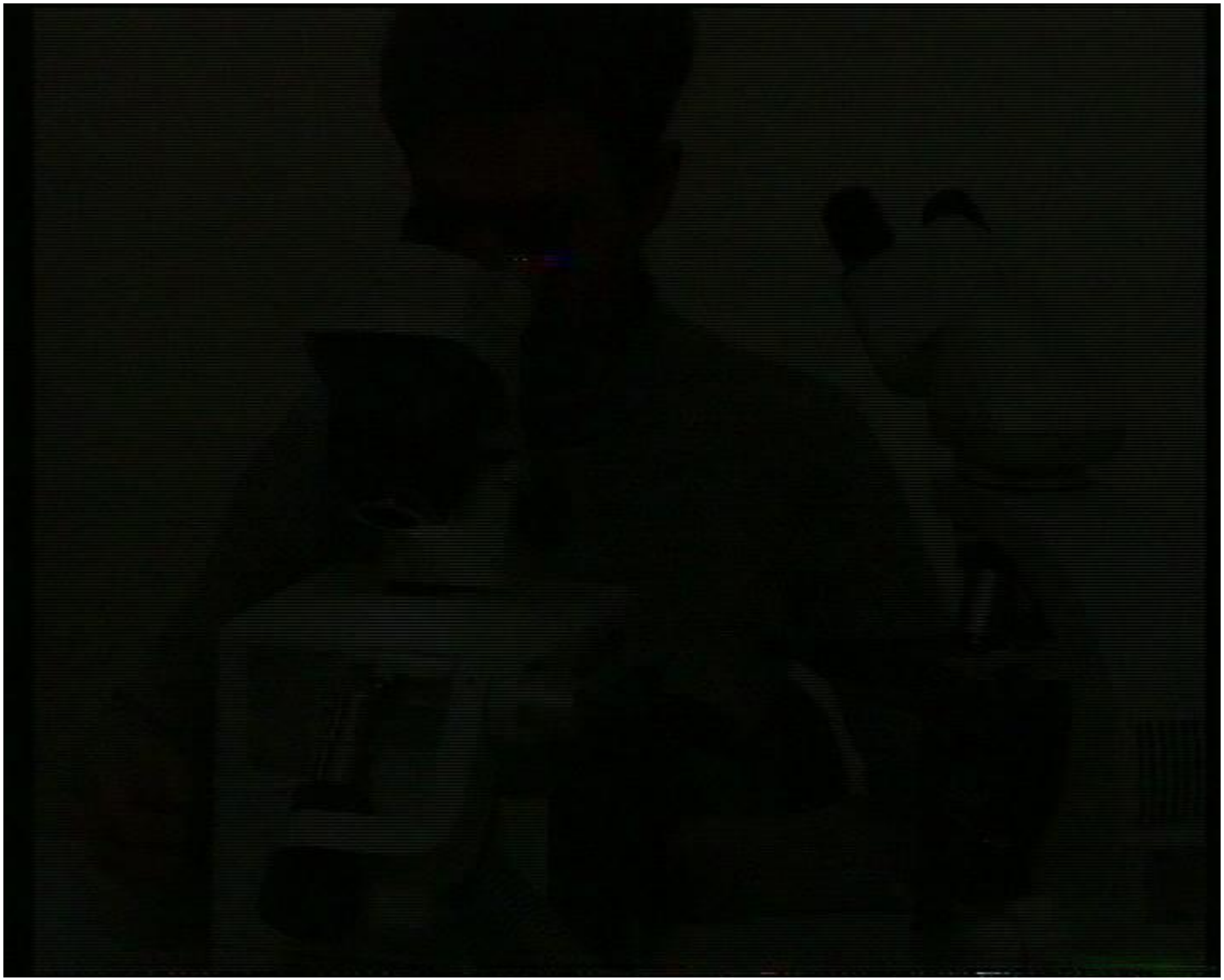


The interference color and thickness of an ultrathin section

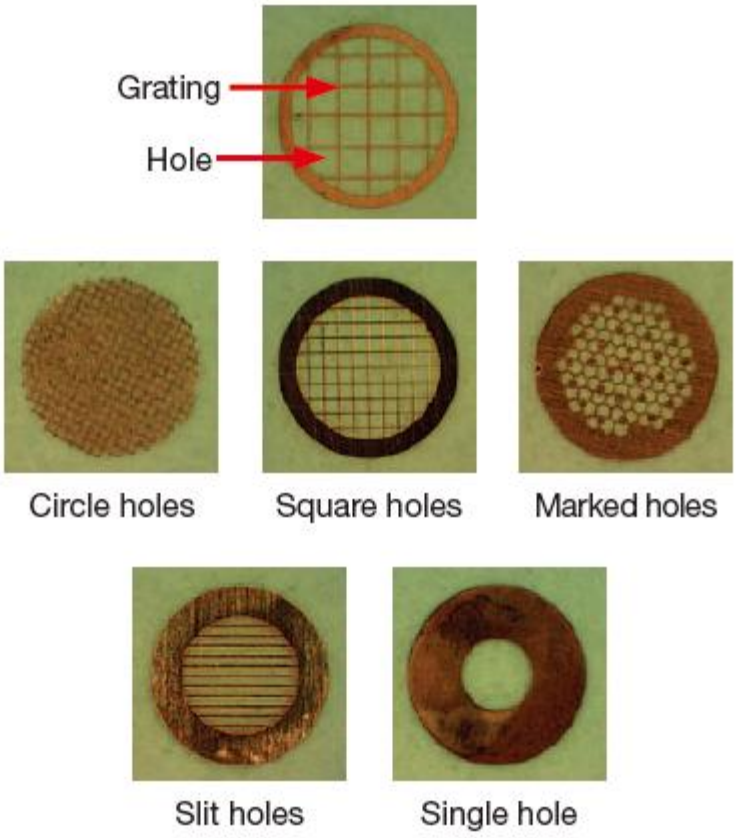
The optimal thickness	Thickness
Gray	< 60 nm
Silver	60 ~ 90 nm
Gold	90 ~ 150 nm
Purple	150 ~ 190 nm
Blue	190 ~ 240 nm
Green	240 ~ 280 nm
Yellow	280 ~ 320 nm

The optimal thickness for observation in a 120 kV TEM.

Ultramicrotomy
cutting ultrathin sections

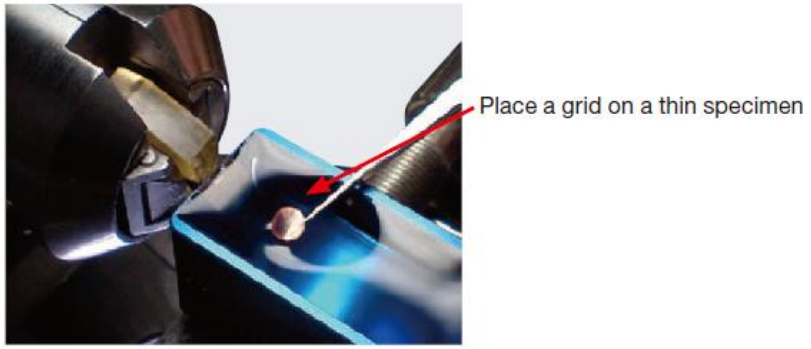


Picking up sections



A) Press Method

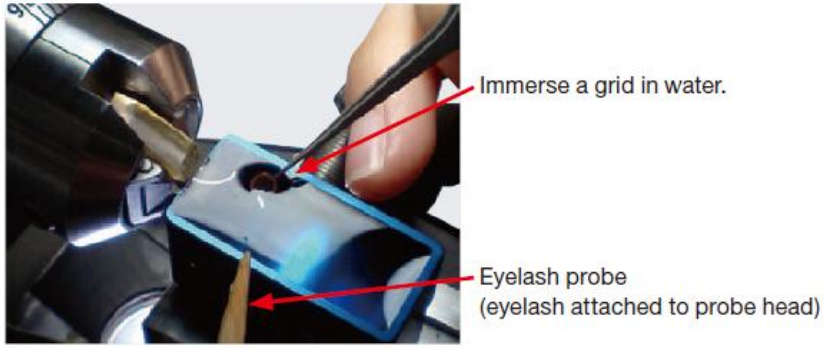
Place a grid on a thin specimen with a support film side down.



Advantage : Simple and easy
Disadvantage : Thin specimen may be wrinkled
Thin specimens may be overlaid

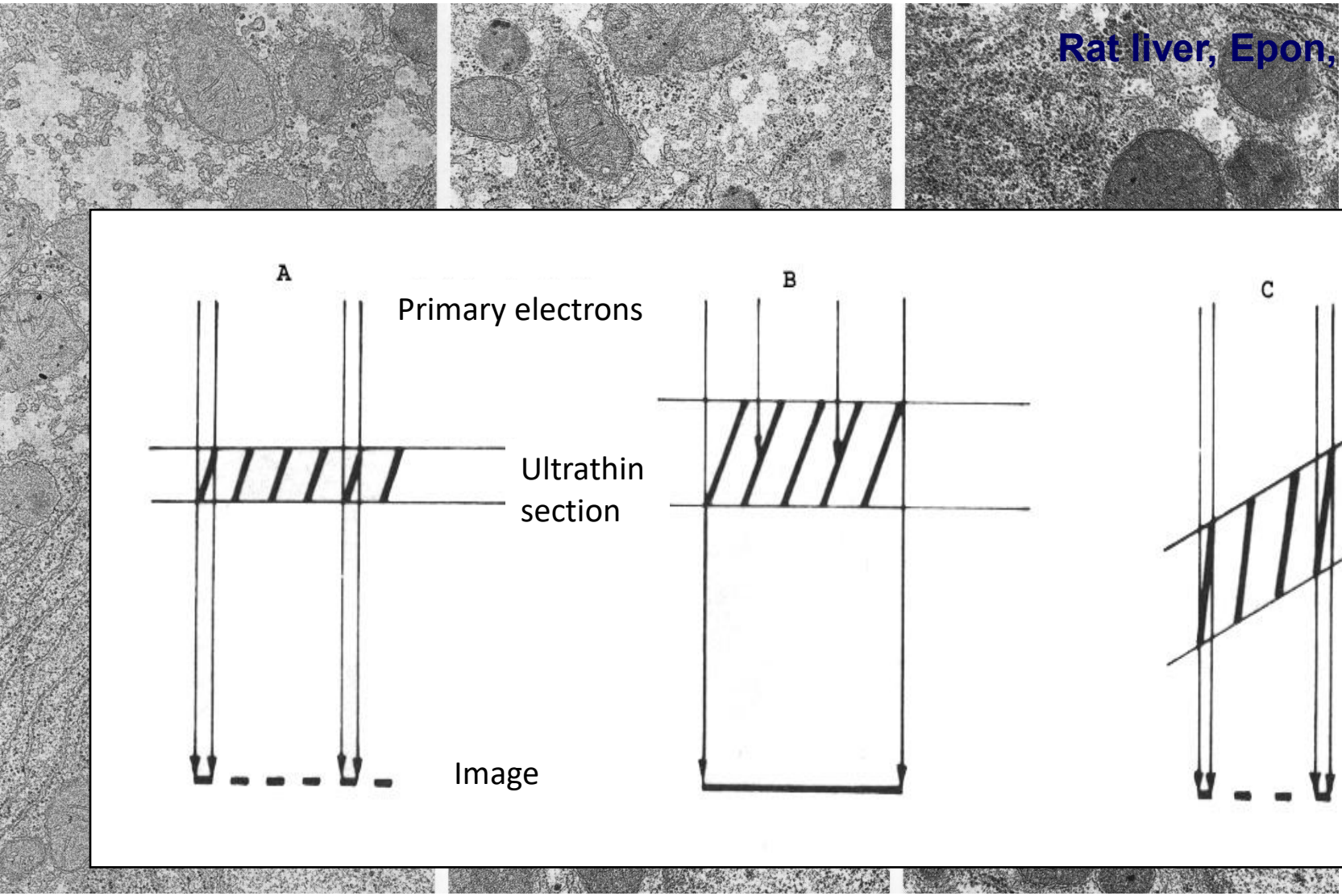
B) Pull up Method

Place a TEM grid under thin specimens with a support film on the top.
Bring thin specimens on the grid by using an eyelash probe.



Advantage : No wrinkles on specimens
You can place specimens as you desire
Disadvantage : Skill needed

Thickness matters

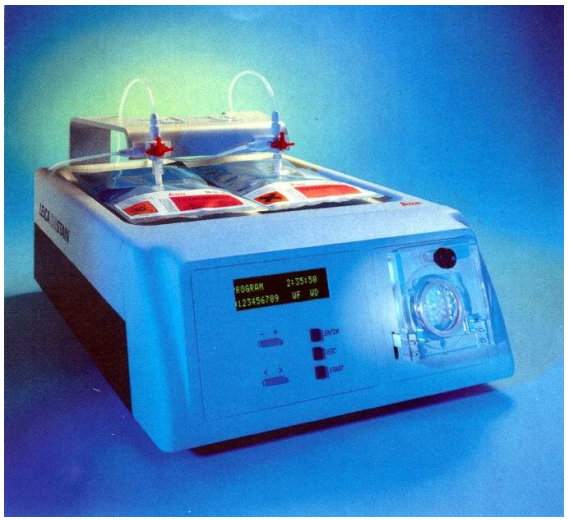


A side note for contrast

- Biological samples tend to have light atoms
- Resins have similar density

=> LOW CONTRAST
=> need to get some additional heavy nuclei there

→Stain sections with solutions of heavy atoms (uranyl acetate, lead citrate…) that will bind to structures of interest



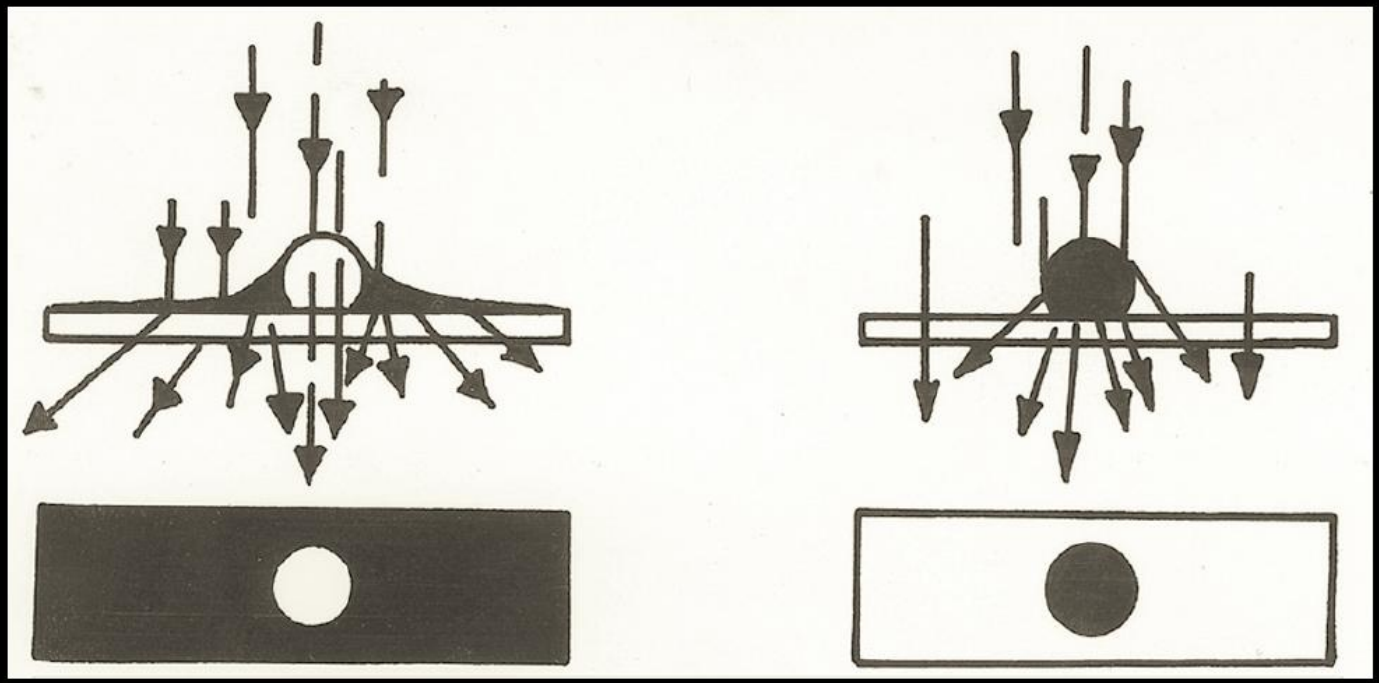

POISON

„BIG“ sample
(needs sectioning)

„small“ sample
(transparent)

Negative staining

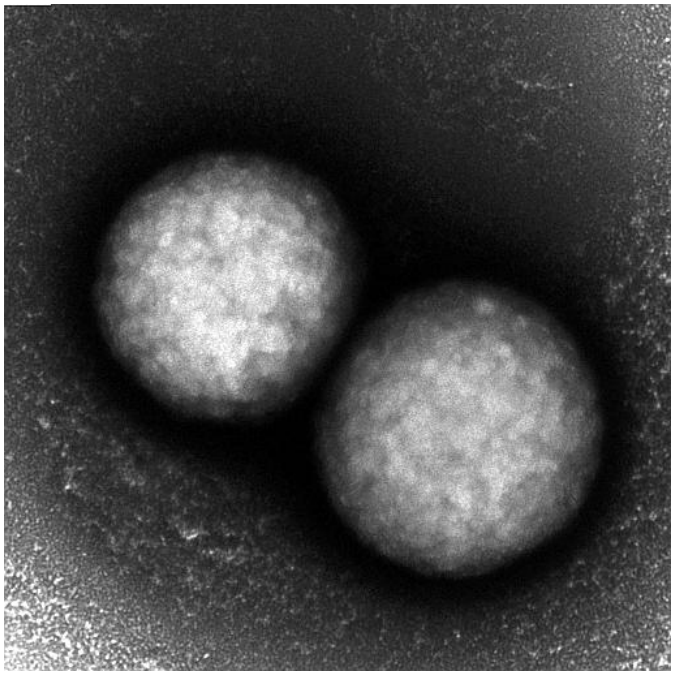
Staining with heavy metal salt solution for TEM



Negative Staining:
Sample is embedded in stain

Positive Staining:
Sample binds stain

Method of negative contrast



Supporting foils:

Plastic (Formvar…); amorphous carbon (~few nm)

Negative stains:

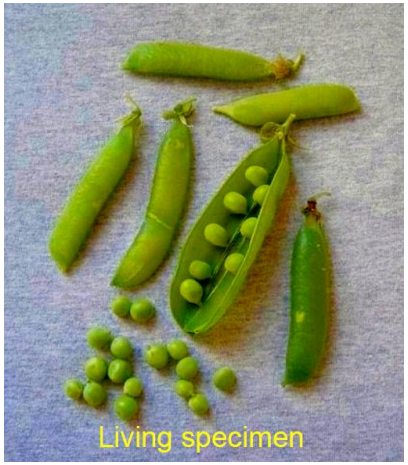
- Uranyl acetate – 0.5-2% aqueous
- Phosphotungstic acid - 0.5-2% aqueous (at pH 5.5 – 8.0)
- Amonium molybdate - 0.5-3% aqueous
- Sputtered metals
- New stains being developed (people don’t like the sound of “uranium”)


POISON

How to remove liquid water?

Drying

Freezing



Kent McDonald, UC Berkeley

Neatly solves:
„soft“
„wet“
„perishable“

Cryo methods in EM

Vitrification

Plunge freezing

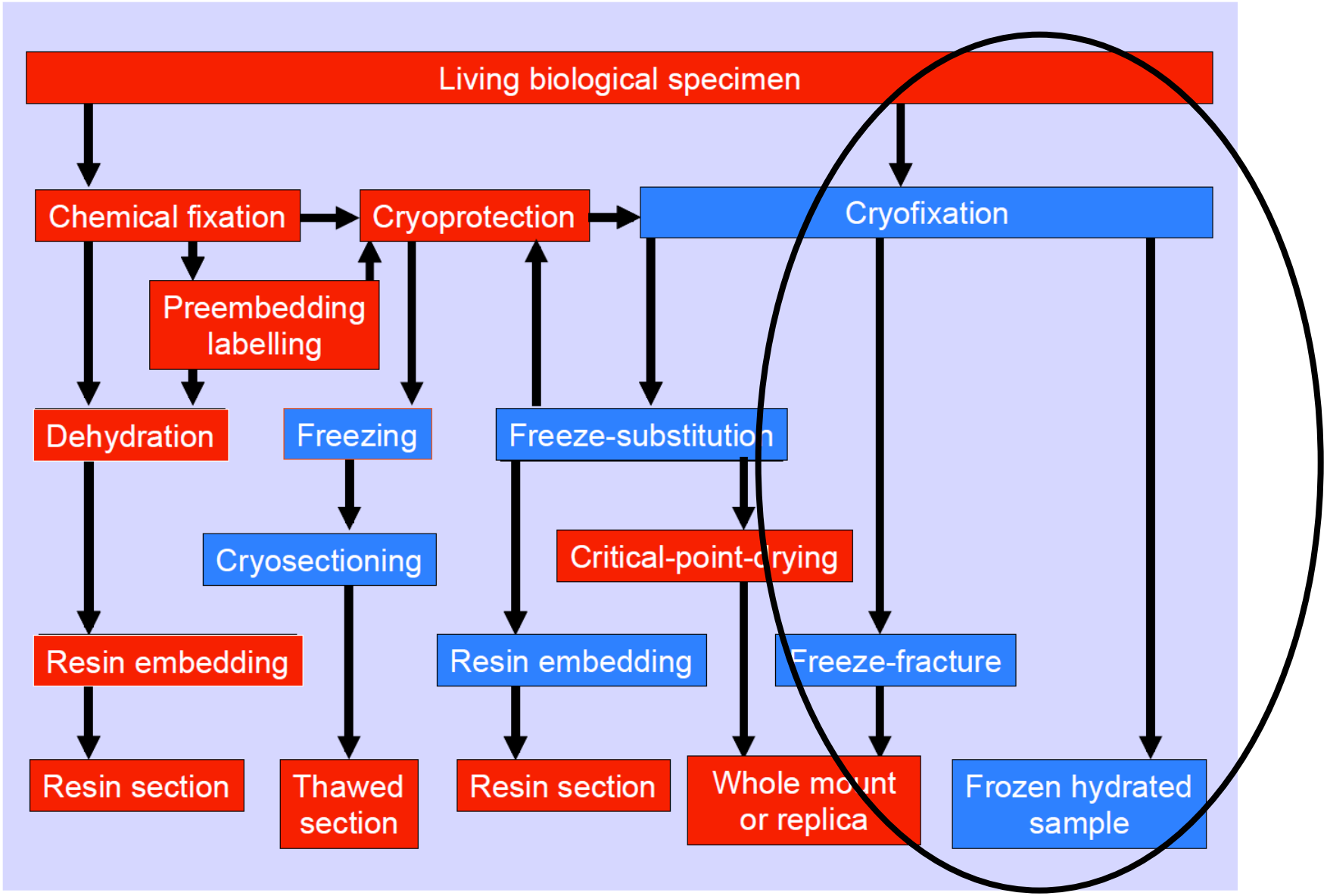
High pressure freezing

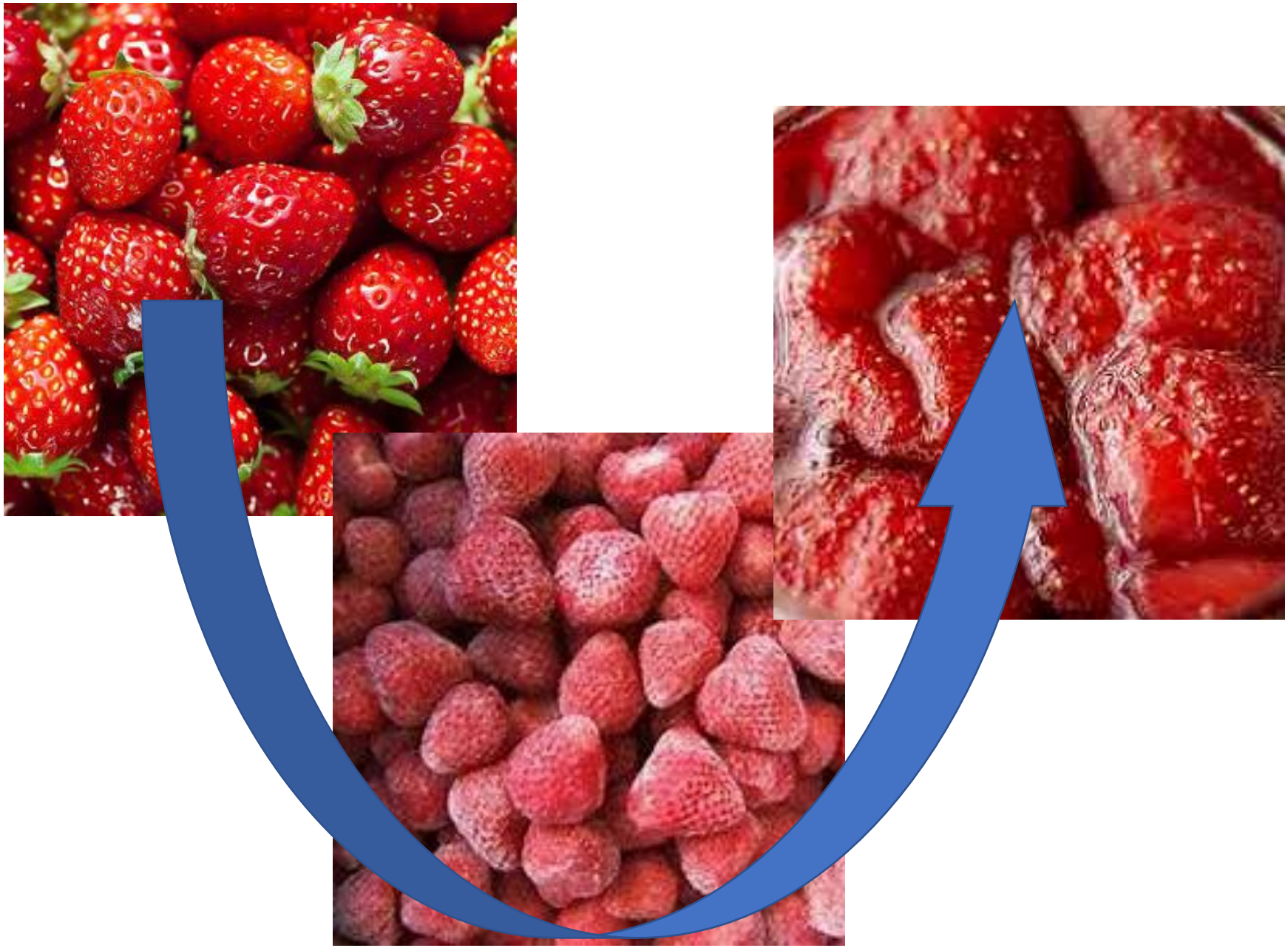
Freeze substitution

CEMOVIS

Cryo lift-out

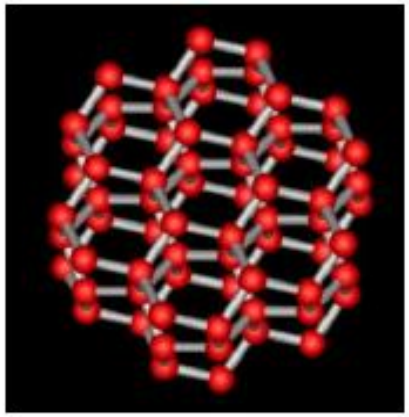
Strategies of biological specimen preparation

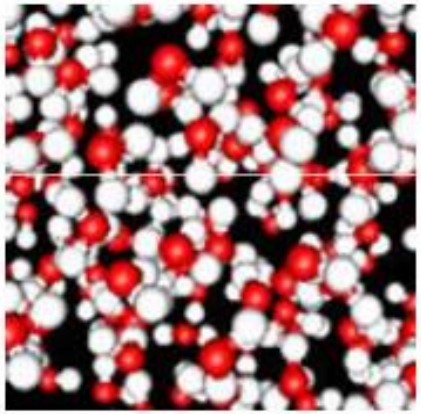




Freezing and vitrification

Vitrification (from Latin *„vitreum“* – „glass“) means turning something into glass)





Crystaline ice:

- Lower density than liquid water
- Purifies water, pushes dissolved stuff aside

Vitreous (glassy, amorphous) ice:

- Same density as liquid state
- No sequestration of solutes

Discovery of vitrification

Brüggeller & Mayer. Complete vitrification in pure liquid water and dilute aqueous solutions. Nature. 1980;288:569–571.

Dubochet & McDowall. Vitrification of pure water for electron microscopy. J. Microscopy 1981; 124, pp. KP3-RP4

only the reflexes of I_h are visible. Our experimental conditions inevitably lead to contamination of the original jet-frozen sample by ice I_h due to condensation of water vapour during the preparation and transfer of the sample to the X-ray camera, therefore, the resulting X-ray diffraction pattern at 95 K will contain an unknown amount of ice I_h.

Journal of Microscopy, Vol. 124, *Pt* 3, *December* 1981, *pp. RP3–RP4.*
Rapid Publication accepted 9 *November* 1981

VITRIFICATION OF PURE WATER FOR ELECTRON MICROSCOPY

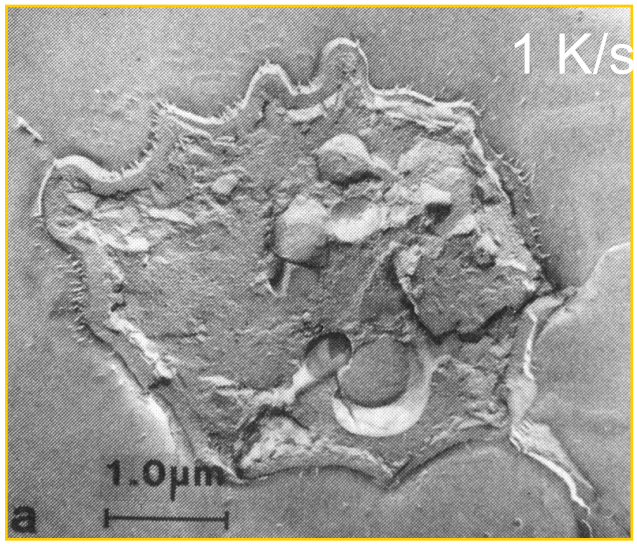
J. Dubochet and A.W. McDowall
European Molecular Biology Laboratory (EMBL)
Postfach 10.2209, D-6900 Heidelberg, F.R.G.

Vitrified ice (Iv), may be obtained by slow deposition of vapour on a cold substrate or rapid freezing of concentrated solution of cryoprotectant. It was recently claimed that if the cooling rate is high enough, the vitreous state can be obtained from dilute solutions or even pure water (Brügeller & Mayer, 1980). We have devised a method for preparing vitrified ice or any frozen aqueous solution for direct observation in the electron microscope.

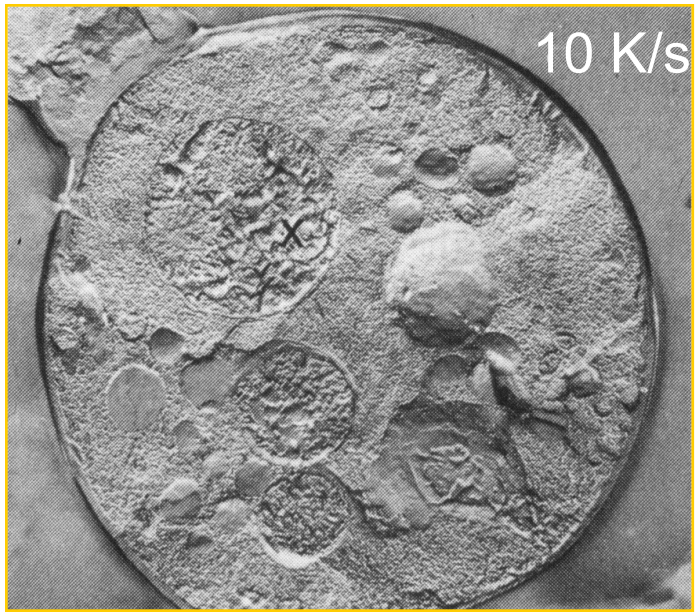
Slide shamelesly stolen from Mikhail Eltsov

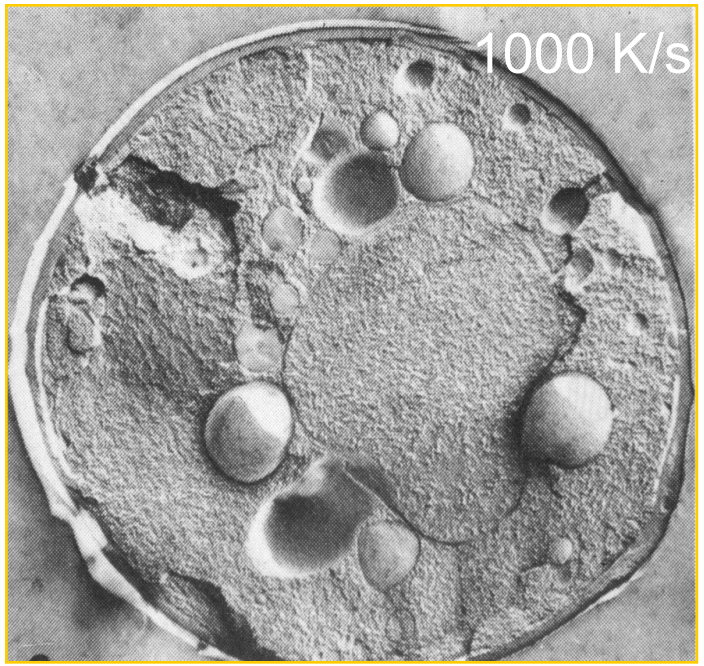
Cryo- fixation

Speed is key



Freezing 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.

How to practically achieve vitrification?

1. Speed – cooling rates in excess of 5*10e5 °C/s

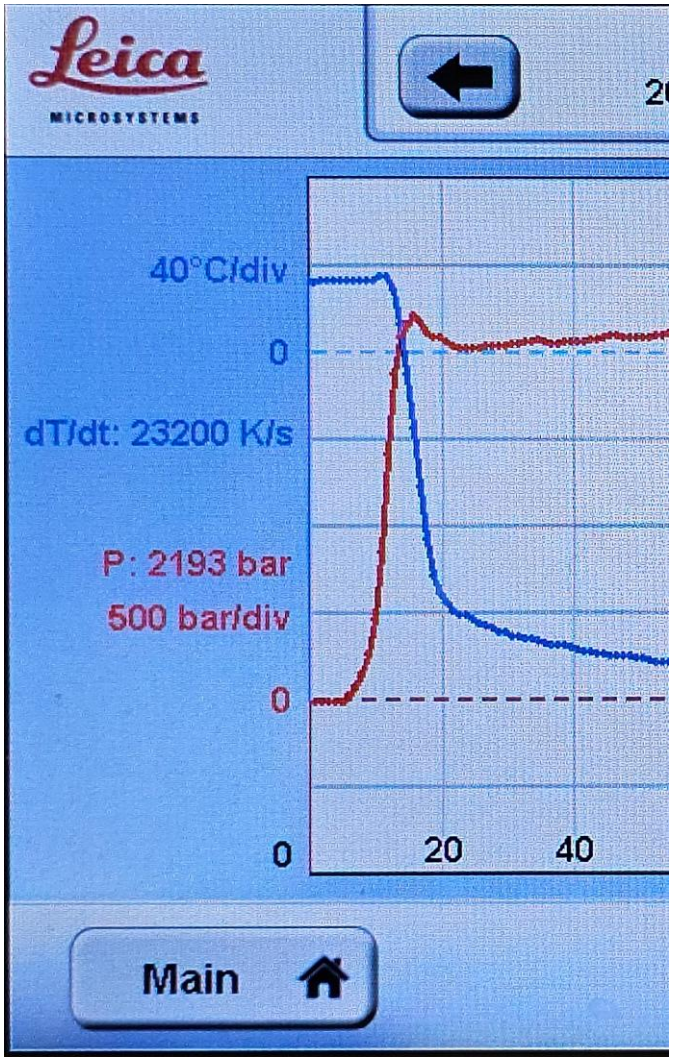
→Plunge freezing

2. High pressure (and speed)

→High presure freezing

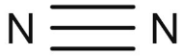
3. Cryoprotectants

→“Tokuyasu“



Cryogens

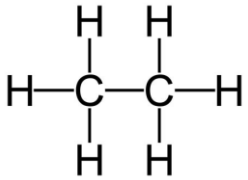
Liquid Nitrogen



Boiling point: -196 °C
Melting point: -210 °C
Low heat capacity

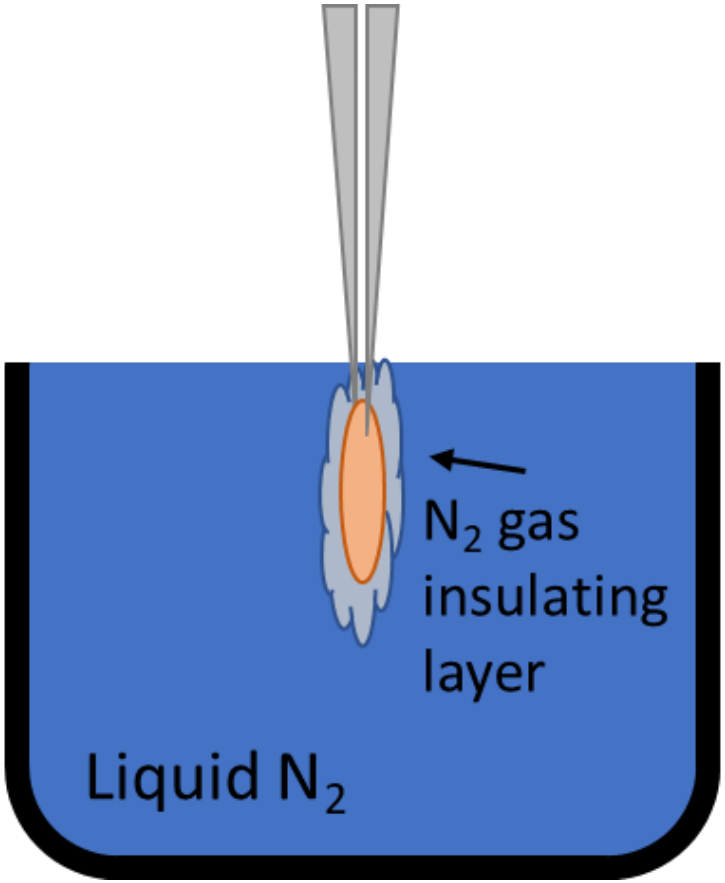
➔**BAD CRYOGEN**

Liquid Ethane



Boiling point: -88 °C
Melting point: -182 °C
High heat capacity

➔**GOOD CRYOGEN**



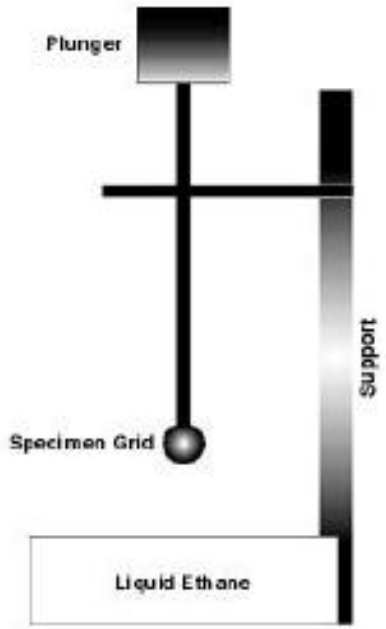
Leidenfrost effect:

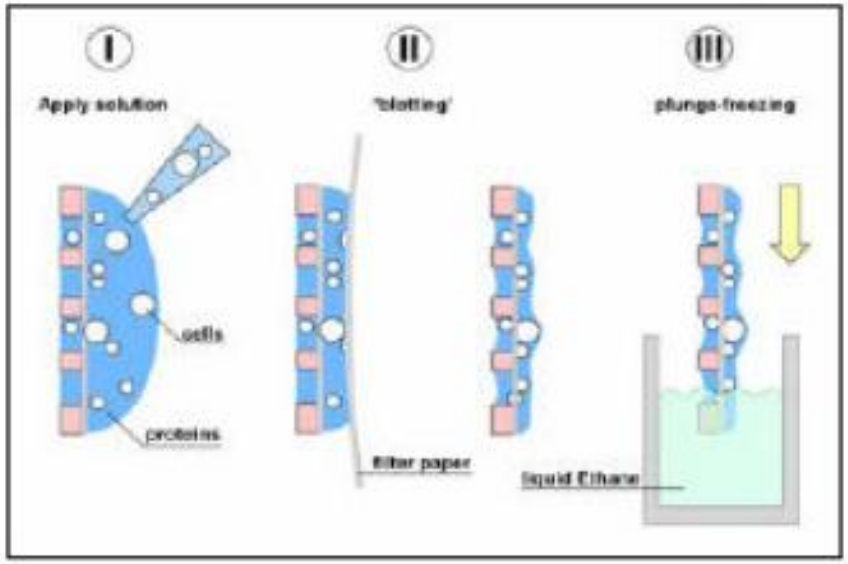
„Physical phenomenon in which a liquid close to a **very hot surface** produces an insulating vapor layer keeping the liquid from boiling rapidly“ (Wikipedia…)

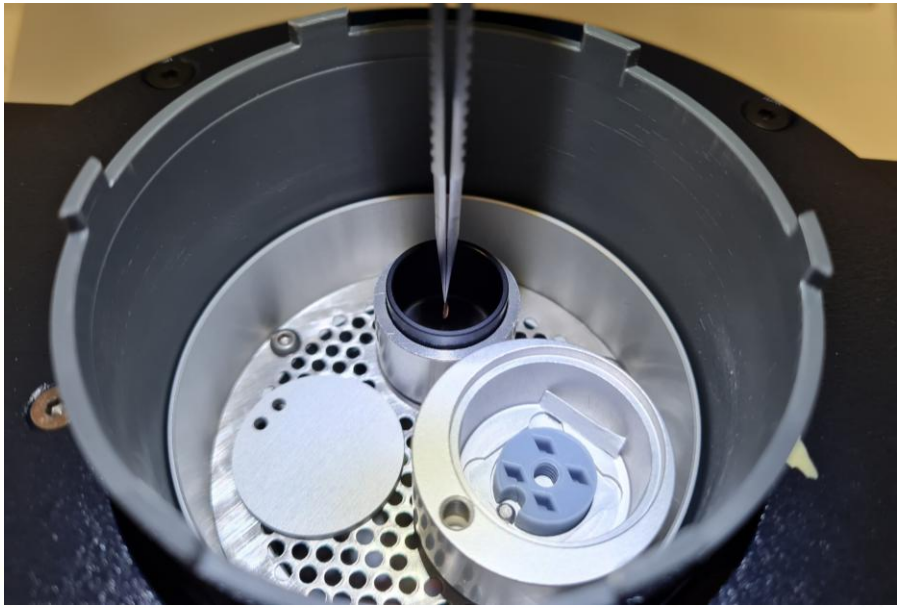
Plunge freezing

- Vitrification of thin film of sample suspension by plunging it into liquid ethane at ~ -180 °C

- Max thickness ~ 5-10 µm



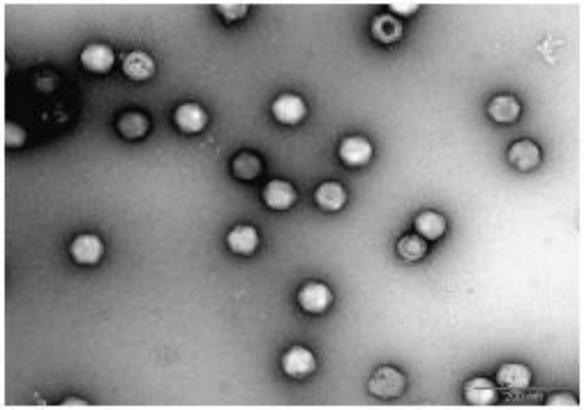


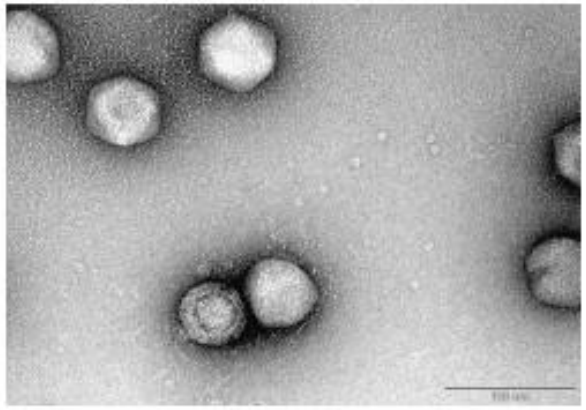


Single particles (examples)

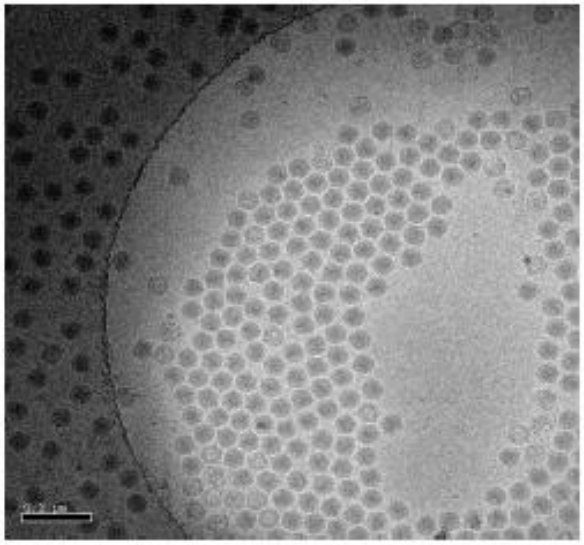
- Viruses, protein complexes

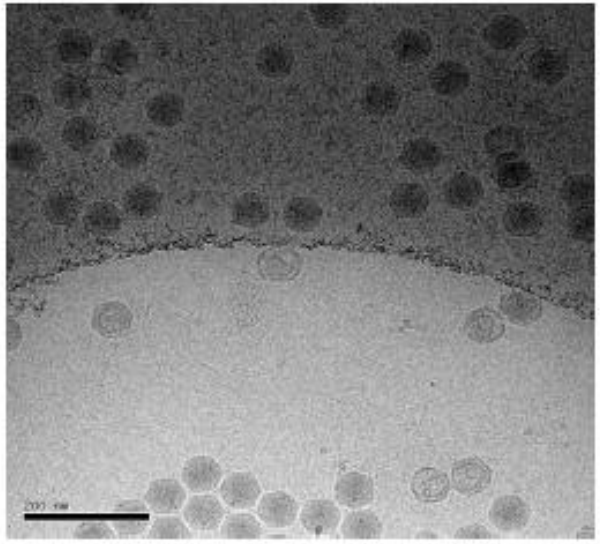
Negative staining





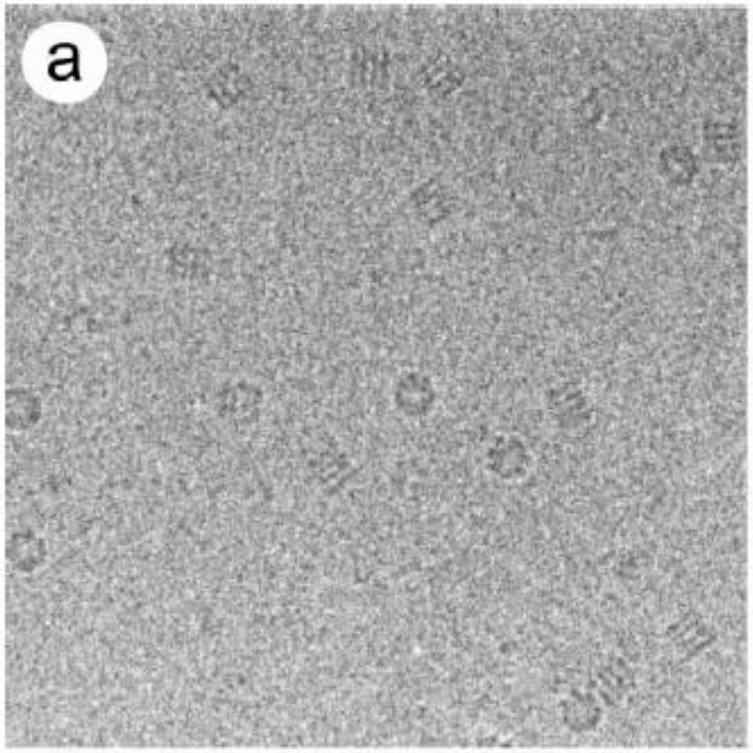
cryo



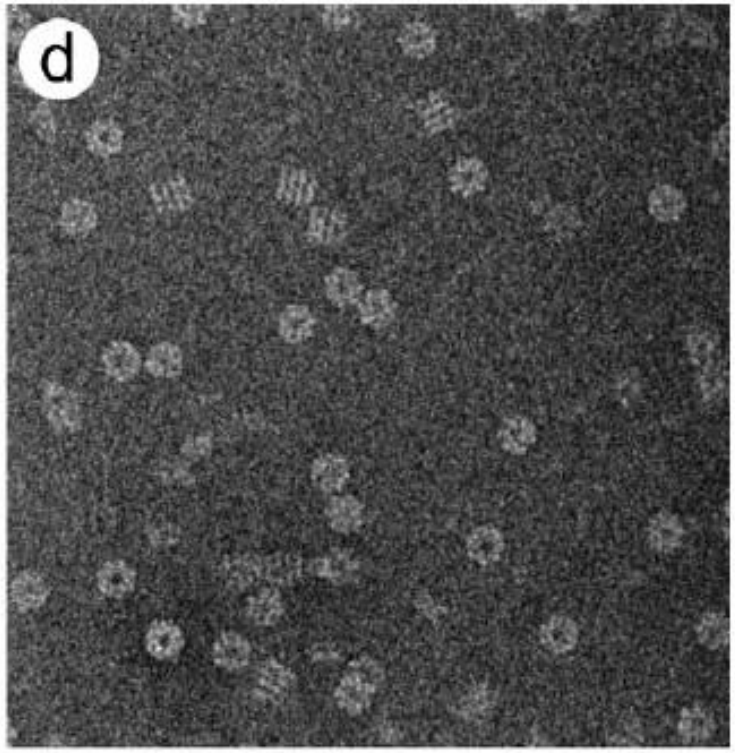


PRD1 bacteriophage,
(Simone Prinz)

Cryo-negative staining



No staining



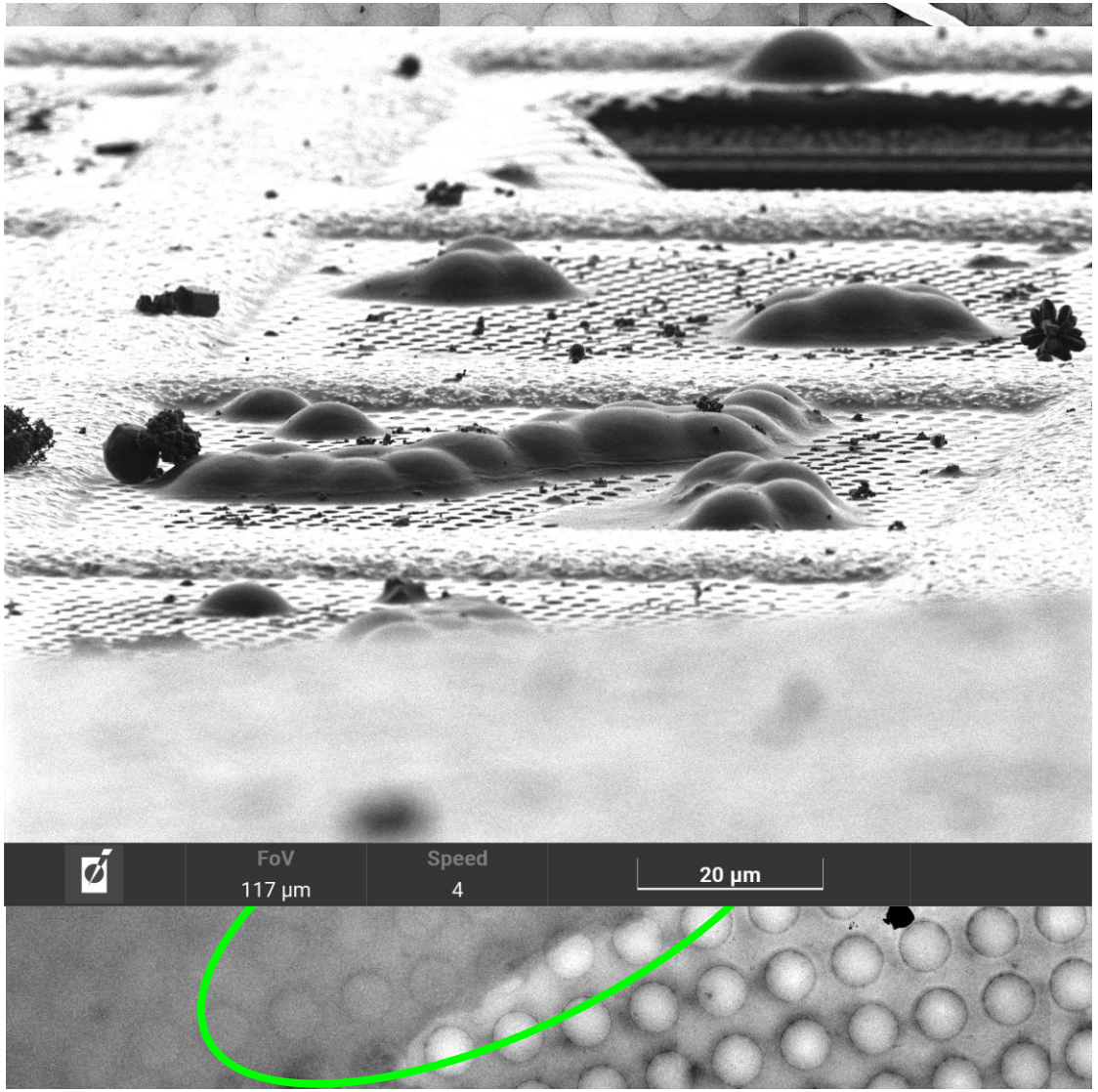
16% Amonium molybdate

S. De Carlo et al., 2002

GroEL

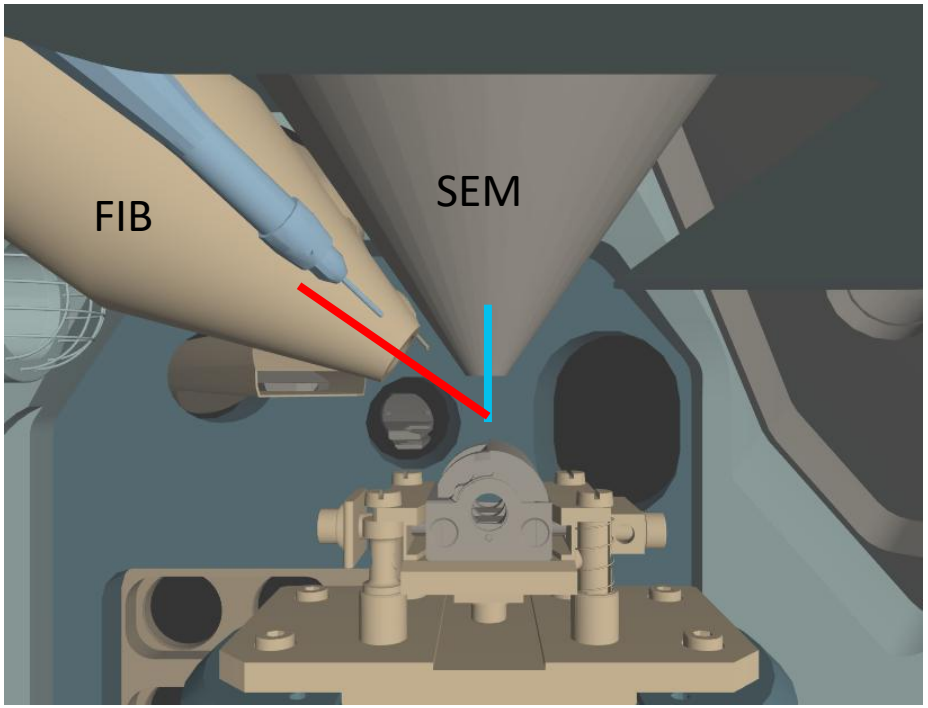
Cell suspensions/Cell monolayers

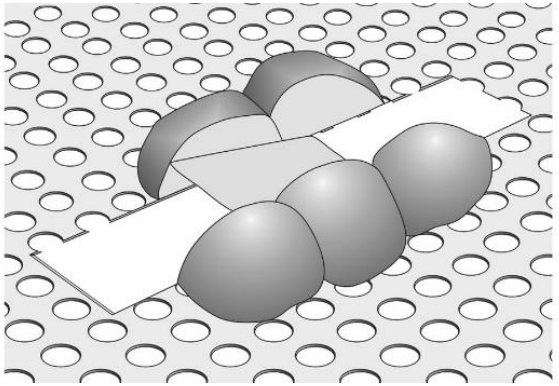
- Cell periphery directly observable (~<300nm)
- Anything else needs thinning…



Ion milling – lamella fabrication

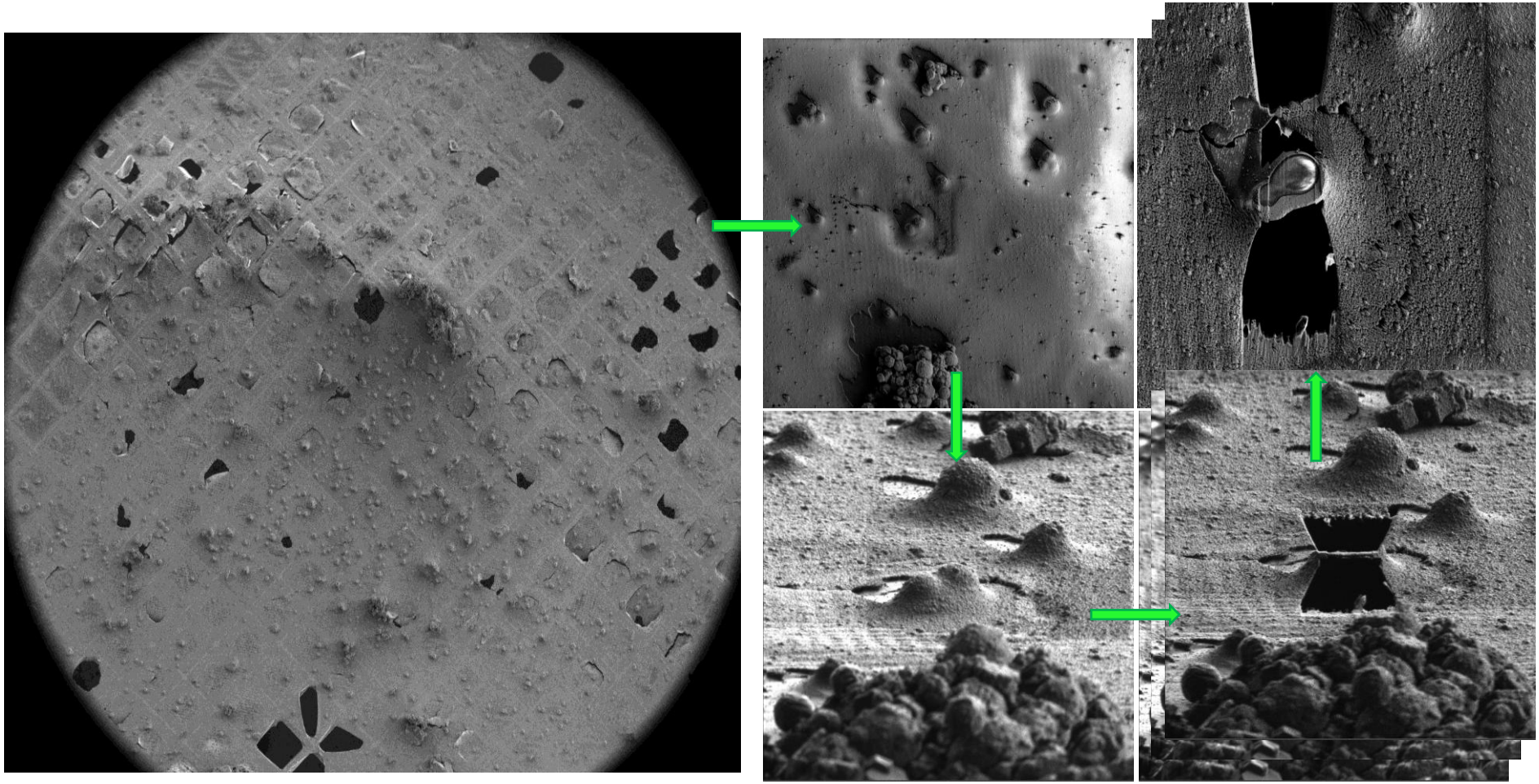
- Free of artifacts introduced by cryo-ultramicrotomy
- Site-specific sample preparation





Engel et al. 2015

Blasting actual cells with an Ion Gun



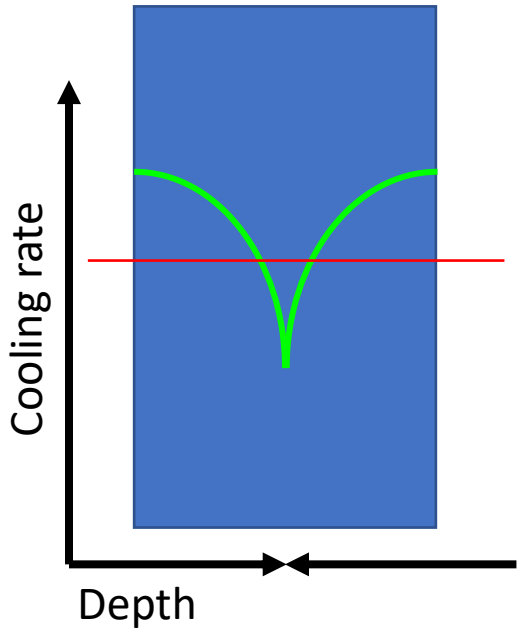
Chlorella culture courtesy of Dr. Forczek IEB CAS

Vitrification depth limit for **Plunge freezing** is ~ **5-10 µm**

- Limiting factor – heat transfer through sample

➢ Cooling rate in depth drops below the threshold for ambient pressure vitrification

Solution:
Don’t freeze at ambient pressure…

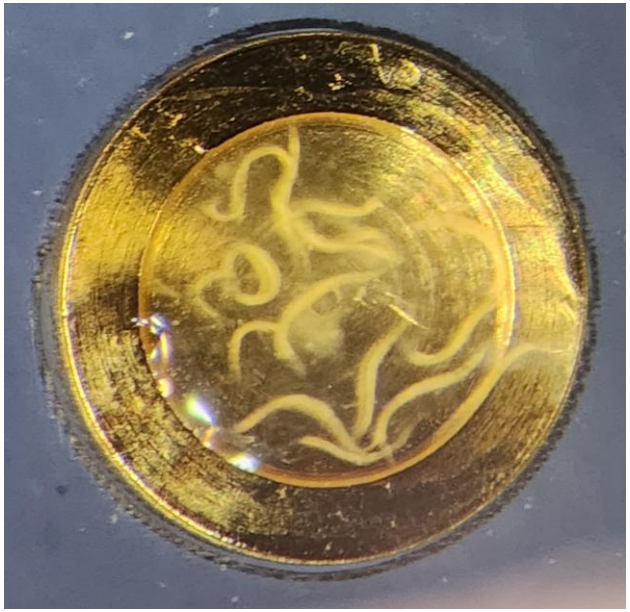


Freezing at ambient vs. high pressure

	0.1 MPa (atmospheric pressure)	210 MPa
Melting point	0°C (273 K)	-22°C (251 K)
Nucleation temperature (supercooling water limit)	-42°C (231 K)	-92°C (181 K)
Cooling rate necessary for vitrification (cell)	$>1x10^5$ K/s	$1x10^3$ K/s
Max. sample thickness which can be vitrified	> 5 µm	> 200 µm

High Pressure Freezer Leica EM ICE

HPF => Bulk sample enclosed in metal carrier



Freeze substitution

CEMOVIS

Cryo lift-out

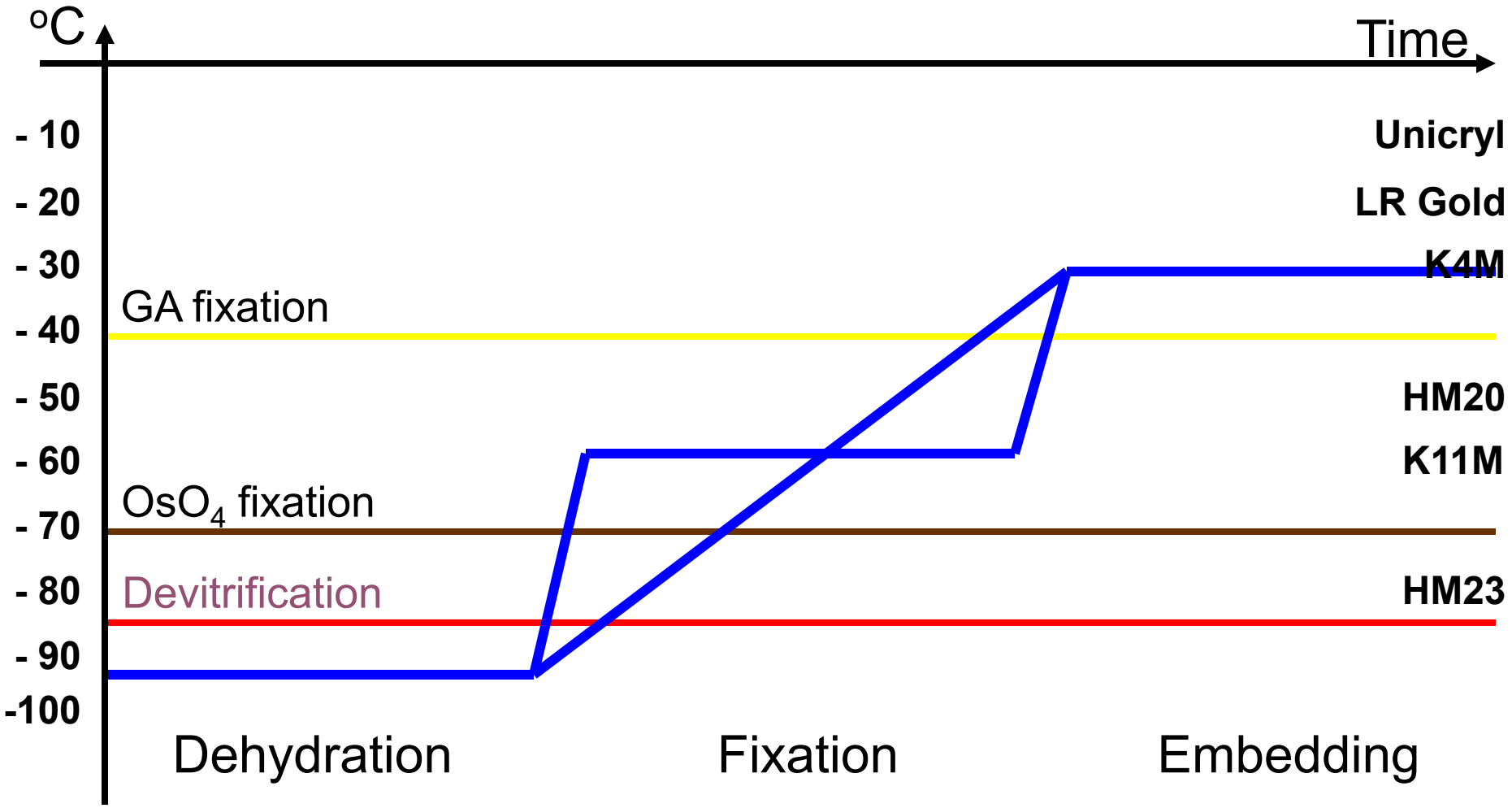
Waaaaay too thick to be directly usefull!!!

Freeze substitution

- The combination of chemical methods with cryomethods
- The ice in a frozen specimen is replaced at low temperature by anhydrous organic solvent followed by infiltration of resins
- The organic solvent must be liquid below recrystallization point and dissolve addittives such as osmium tetroxide, or GA
- Polymerization by UV

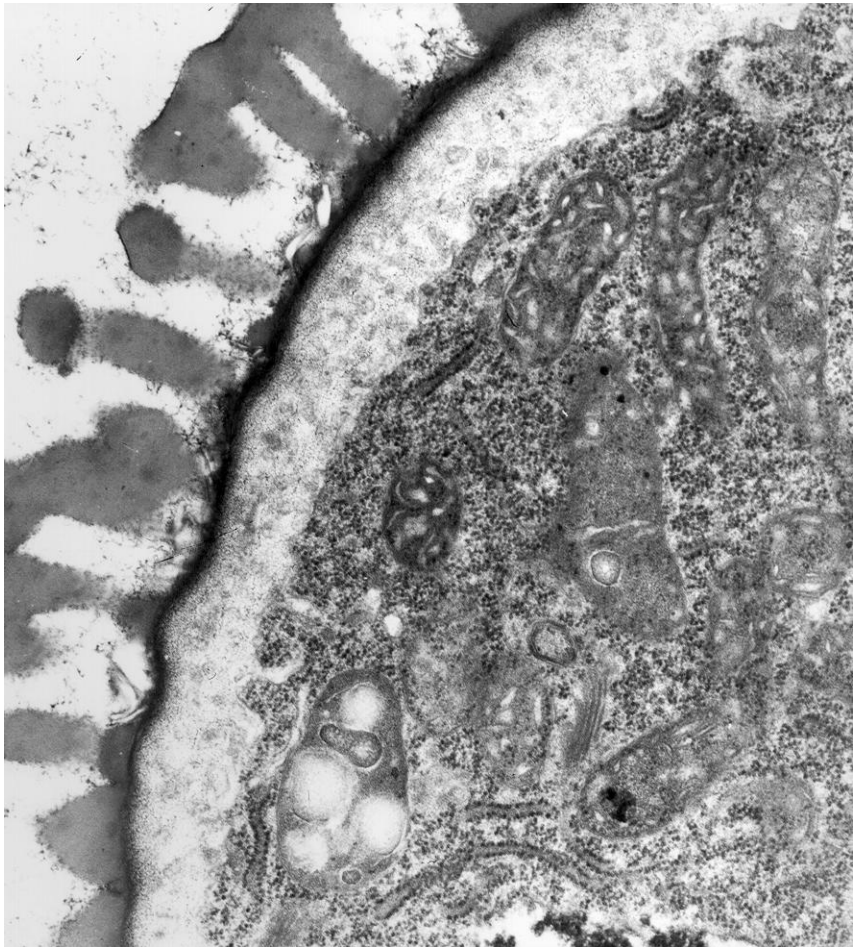
➢**Normal ultramicrotomy**

Freeze-Substitution

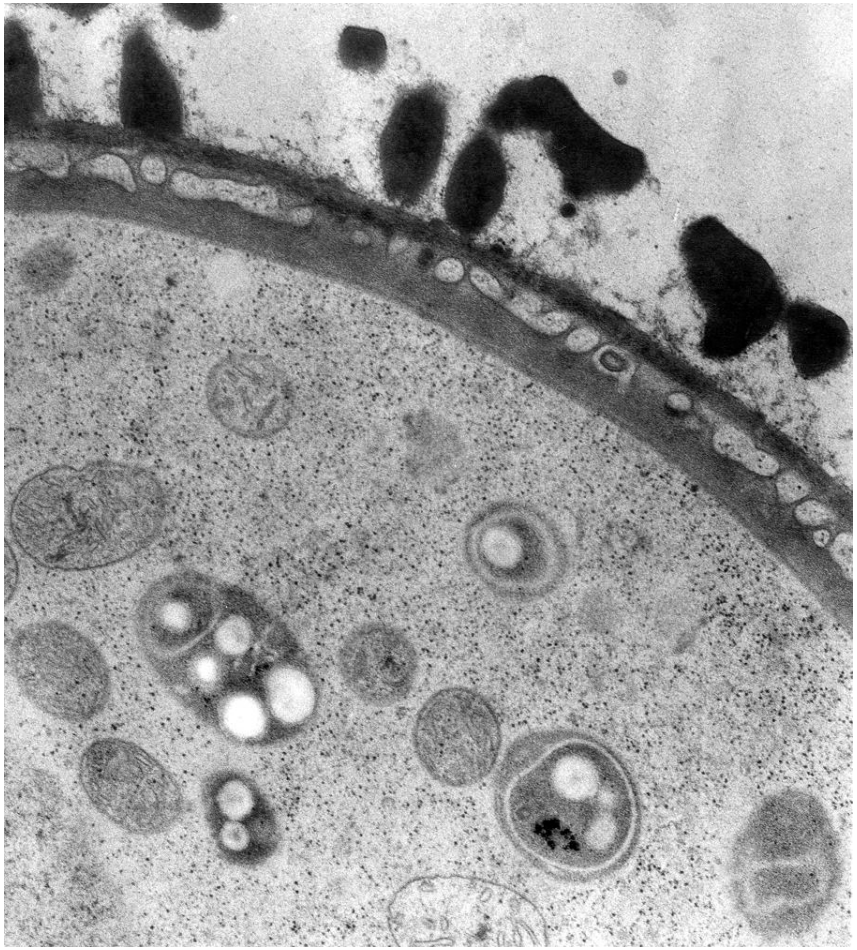


Arabidopsis thaliana

Conventional



Freeze-substitution



A. Omedilla, University of Granada, Granada

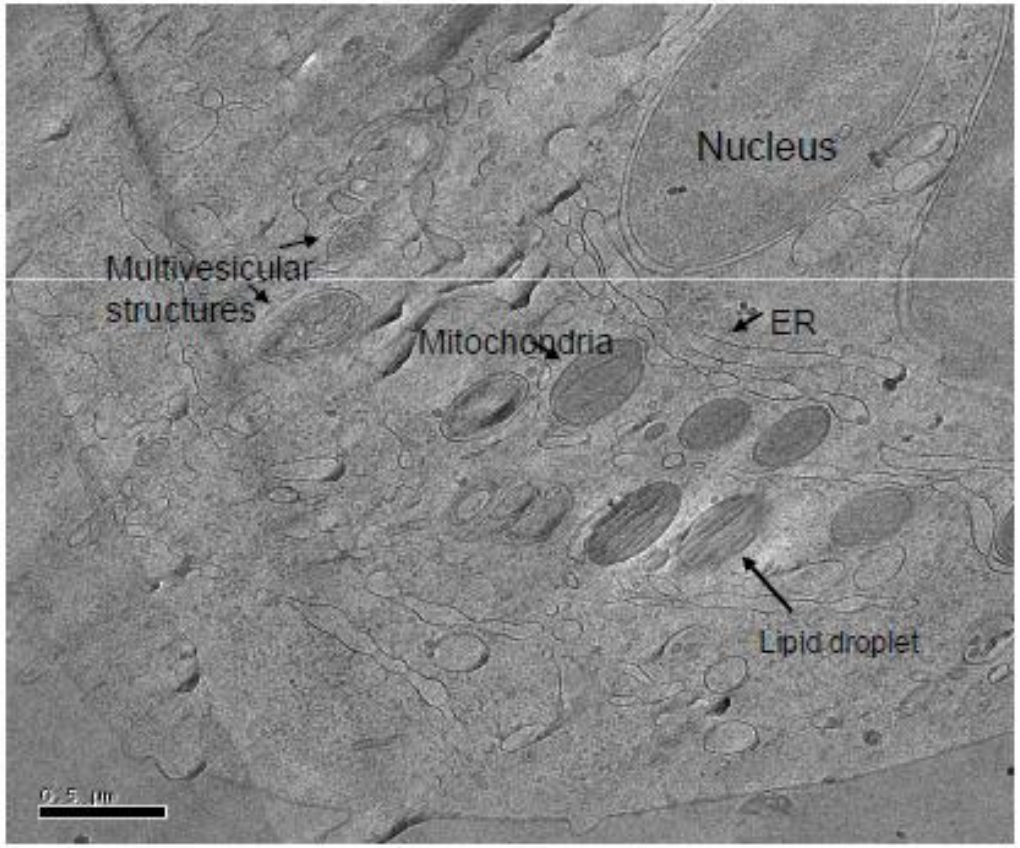
Cryo ultramicrotomy

Sectioning frozen samples

Cryo electron microscopy of vitrified sections (CEMOVIS)

HPF → cryo sectioning (T<135°C) → cryo TEM

Leica, Ultramicrotome, FC6

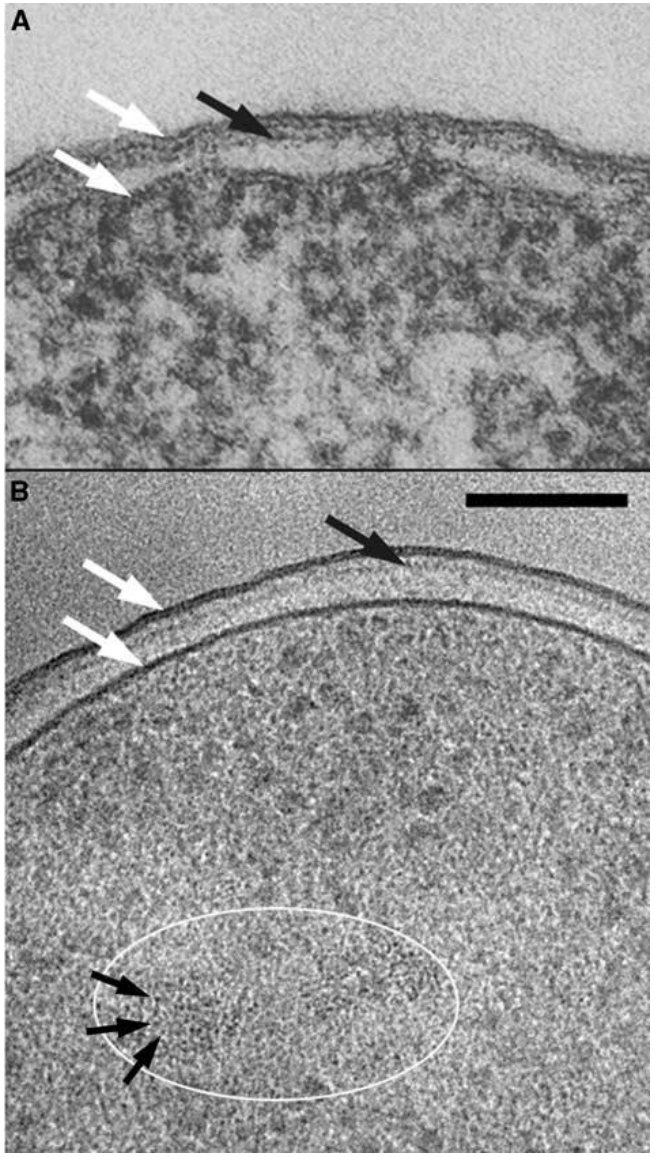


CEMOVIS

The ultrastructure of outer membrane of gram negative bacteria:

A. Escherichia coli
B. Pseudomonas aeruginosa

A. Image of staining agent distribution
B. Contrast of unaltered native specimen

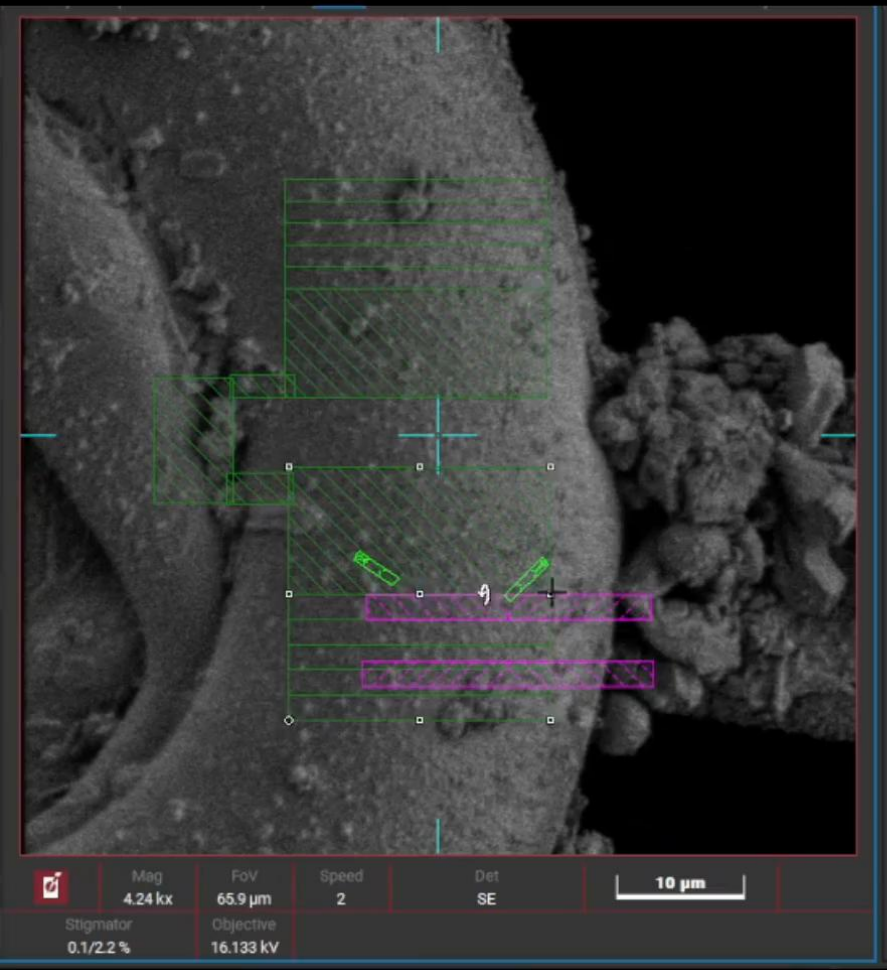


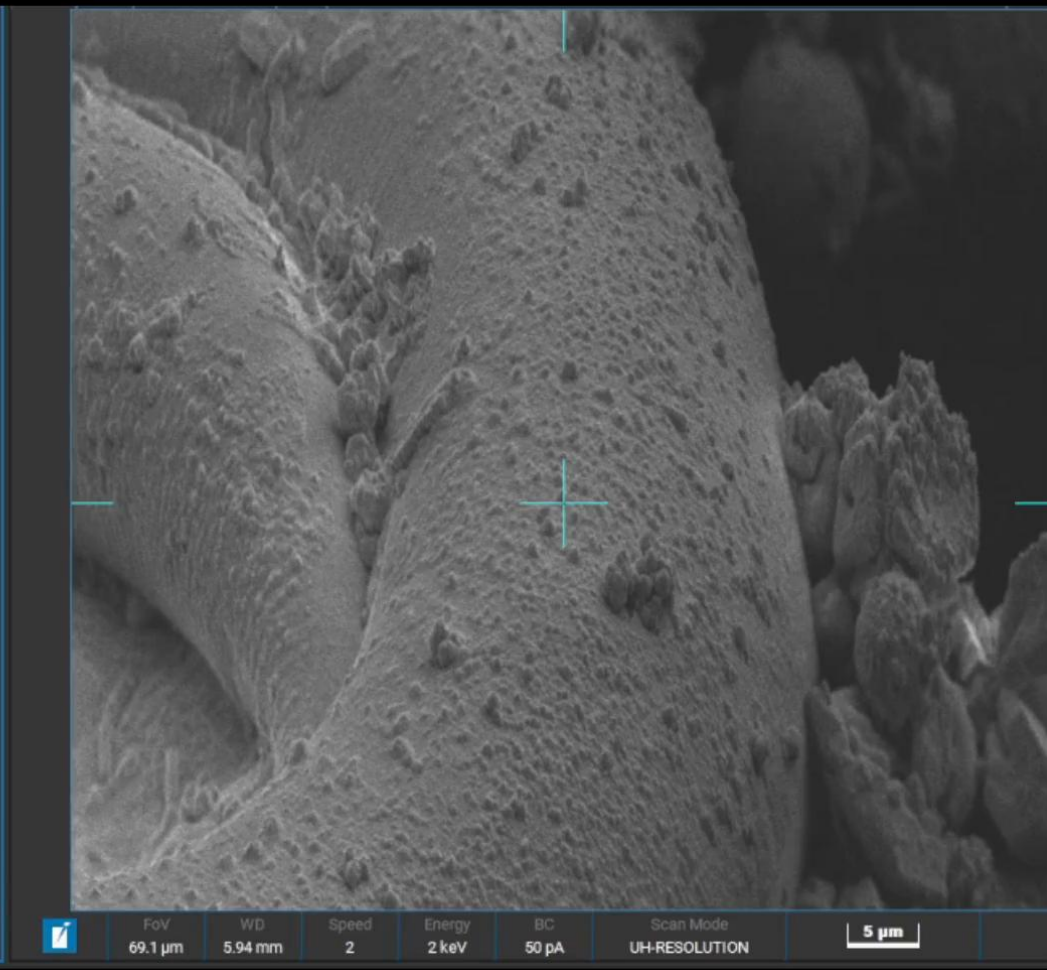
A.Al-Amoudi et al.: Cryo-electron microscopy of vitreous sections EMBO Journal (2004) 3583-3588

Cryo lift-out

Switching 1000€ knife for a 1 000 000€ FIB-SEM

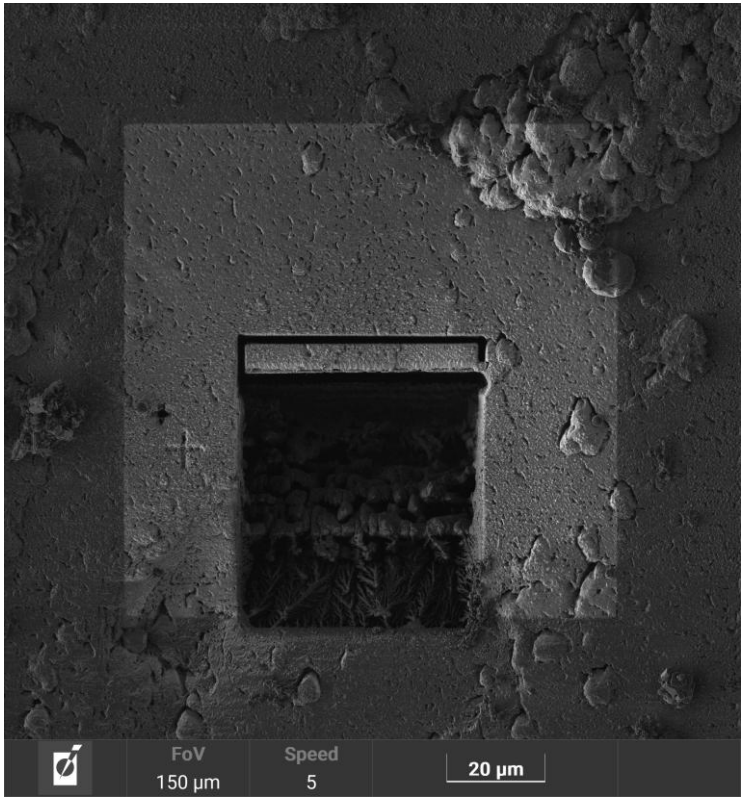
Cryo lift-out

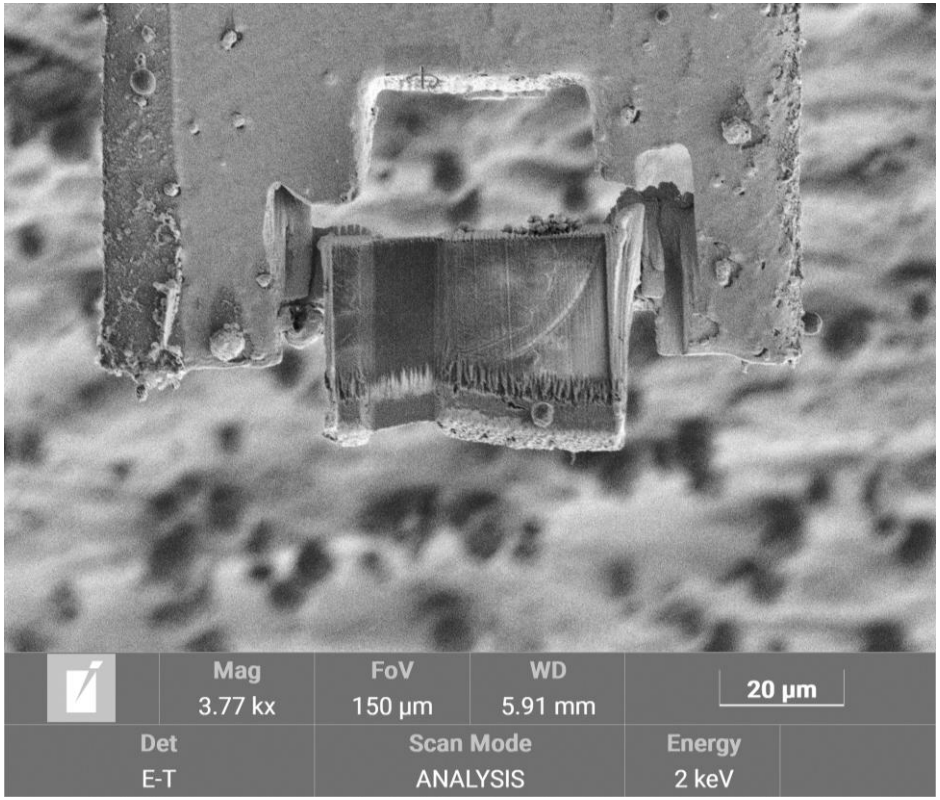




Cryo lift-out

Cryo lift-out





Thank you for attention…