Cryo-Transmission electron microscopy: Sample preparation and material science applications

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Department of Applied Physics/
Nanomicroscopy center

PHYS-E0526 Microscopy of Nanomaterials, laboratory course P (5 cr)

Update for the schedule .. Due to covit-19 restrictions group sizes are smaller and number of exercises bit less ..

Course starting at week 16 (19.4 →)

Basic exercises – 2 Demos:

(4 persons per group):

1) High resolution TEM (Jeol 2200FS Cs-corrected TEM)
2) 3D tomography data collection (Jeol 3200FSC liquid helium cryo TEM or Jeol2800) + Tomography data processing (Computer room)

Small group exercises: (Select 2)

(2 persons per group)

1) basic-TEM imaging
2) SEM imaging
3) FIB -SEM processing/imaging
4) (AFM is currenty broken..)

Independent Small group exercises

(without supervision.. 2 person per group)

(1 exercises)

1) TEM imaging
2) SEM imaging
3) (AFM??)

Why CRYO?

- Electron microscope column is in vacuum
- Traditionally aqueous (water-containing) EM samples have been dehydrated
- Cryo-EM allows electron microscopy of aqueous specimen in their natural, hydrated state (also other solvents are possible for materials science applications: gels, polymeric vesicles and micels)
- Reducing beam damage for all materials..

Beam damage

I) Radiolysis: Inelastic scattering (mainly electron-electron interactions such as ionization) breaks the chemical bonds of certain materials such as polymers and alkali halides. Polymers: Chain scission (produce low molecular weight compounds) or Cross-linking.

II) Knock-on damage (direct displacement of atoms..) Knock-on damage or sputtering: Knock-on damage is the displacement of atoms from the crystal lattice and creates point defects. If atoms are ejected from the **specimen surface** we call it **sputtering.** These processes are ubiquitous if the beam energy (E_0) is high enough.

III) Heating: Phonons heat your specimen and heat is a major source of damage to polymers and biological tissue.

•If thermal conduction is very high, heating is negligible.

• If thermal conduction is poor, heating can be quite substantial

So, beam heating for metals is usually minimal but small ceramic particles may be heated by the beam to temperatures of 1700ºC.

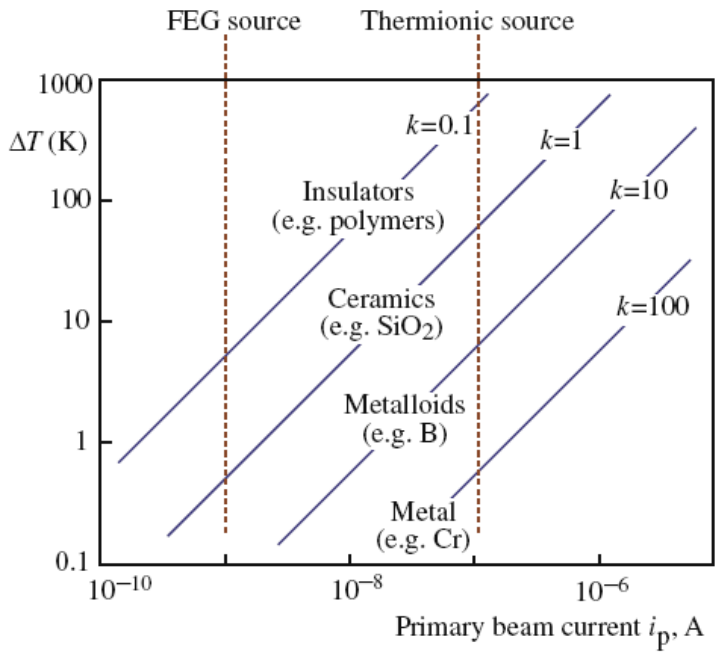


FIGURE 4.11. The increase in specimen temperature as a function of the beam current and the thermal conductivity of the specimen (k, in W/m K). Typical materials are noted, but should not be considered representative, since k varies substantially in any class of materials.

How to minimize…

1. Low dose TEM: - As small intensity as possible

Small spot size, small cond. aperture, spread the beam, **low magnification**

Focusing etc. in the adjacent area or the specimen - **Automatic low dose software**

2. High voltage TEM

+ Electron mean free path longer when *V* increased → less inelastic scattering → less damage

- knock-on damage is increased above 200 - 300kV

- Less contrast in high voltages

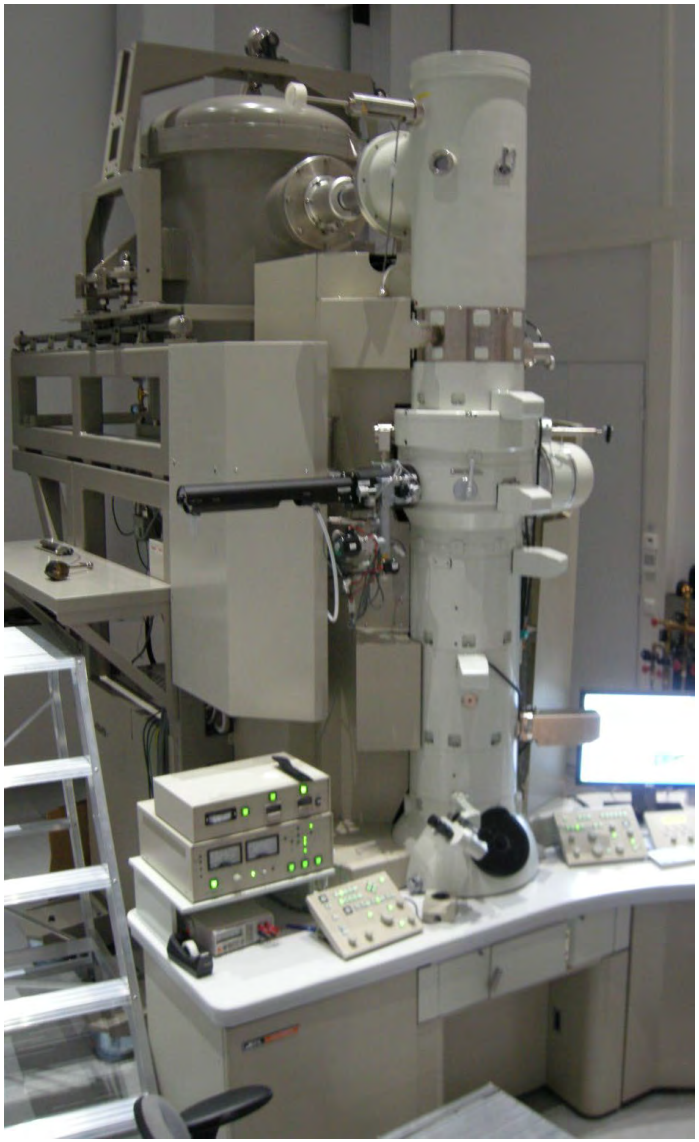
However carbon nanotubes quite low voltage 80kV is a better ..

3. Cryo microscopy

At low temperatures degradation slower (either **liquid nitrogen** or liquid Helium cooling) (Dose typically ~10 electrons/Å^2 or *some polymers even much less* – but then the signal to noise ratio is a problem…)

4. Sample coating (thin film of carbon film), Replica …

Nanomicroscopy center cryo-TEM's



One Dedicated cryo-TEM:
Liquid nitrogen or liquid helium

+ Other TEM's with cryo-Holders:

- Single tilt cryo Holder
- Multible specimen cryo holder
- Cryo-rotation holder

Nobel Prize for Chemistry 2017 for "developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution".

The Nobel Prize in Chemistry 2017

Vitrification of biological samples

Jacques Dubochet
Prize share: 1/3

Joachim Frank
Prize share: 1/3

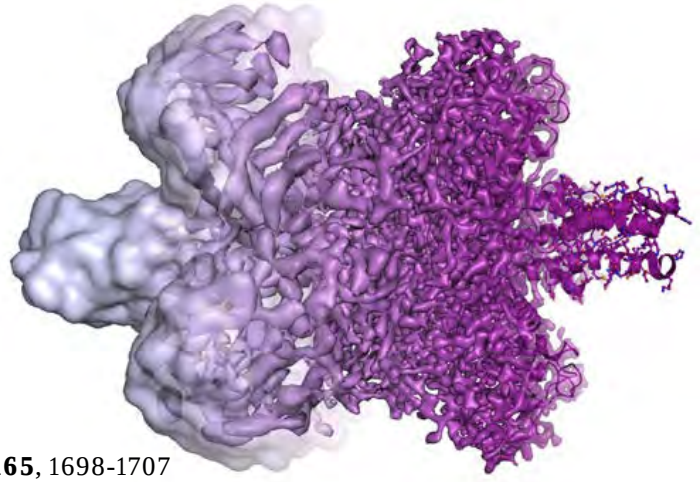
Richard Henderson
Prize share: 1/3

Used EM to solve structure of bacteriorhodopsin
Development of DEDs

DED = Direct Electron Detector Camera

The Nobel Prize in Chemistry 2017 was awarded jointly to Jacques Dubochet, Joachim Frank and Richard Henderson "for developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution."

Advancements in image processing and development of the image processing software, Spider



The resolution progression of cryo-EM, illustrated by a representation of glutamate dehydroge-nase with an increasing level of detail from left to right. For a protein of this size, 334 kDa, the 1.8 Å resolution to the right (38) could only be achieved after 2012/13.

38. Merk, A., et al. (2016) Breaking cryo-EM resolution barriers to facilitate drug discovery. *Cell* **165**, 1698-1707

Sample preparation for cryo-TEM?

Quarterly Review of Biophysics **21**, 2 (1988), pp. 129–228
Printed in Great Britain

Cryo-electron microscopy of vitrified specimens

JACQUES DUBOCHET[1], MARC ADRIAN[2], JIIN-JU CHANG[3], JEAN-CLAUDE HOMO[4], JEAN LEPAULT[5], ALASDAIR W. McDOWALL[6] AND PATRICK SCHULTZ[4]

European Molecular Biology Laboratory (EMBL), Postfach 10. 2209, D-6900 Heidelberg, FRG

Preparation pathways overview

RT specimen processing

Low temperature processing

WARM SPECIMEN

Plunge freezing

Propane jet freezing

High pressure freezing

FROZEN SPECIMEN

Chemical fixation

Dehydration

Embedding

Freeze-substitution

Freeze-fracturing/Freeze-drying/Coating

Cryo-Ultramicrotomy

Critical Point Drying

Low-temperature embedding
RT-embedding

Ultramicrotomy

thawing

Cryo thin section

Coating

Immunolabeling

Staining

Replica

Freeze-dried specimen

Freeze-fractured/etched specimen

RT-SEM

RT-TEM

RT-TEM

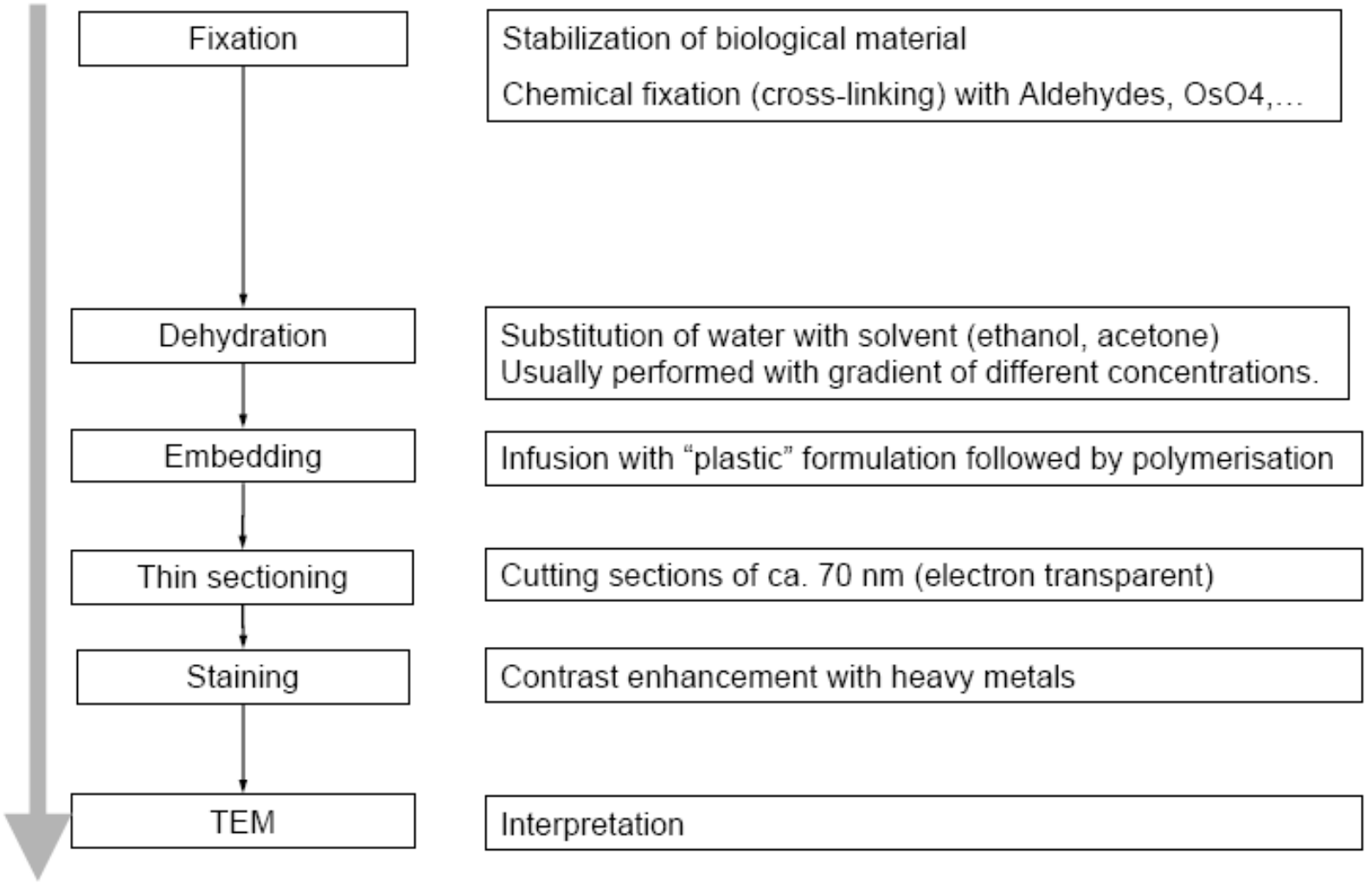
RT-SEM

Cryo-SEM

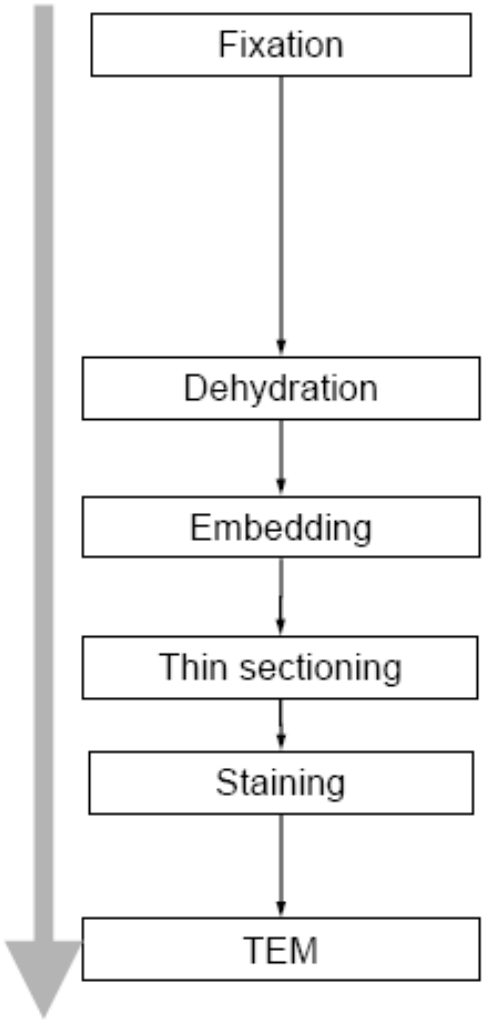
Cryo-TEM

(From Andres Kaech University of Zurich)

Room temperature processing for TEM

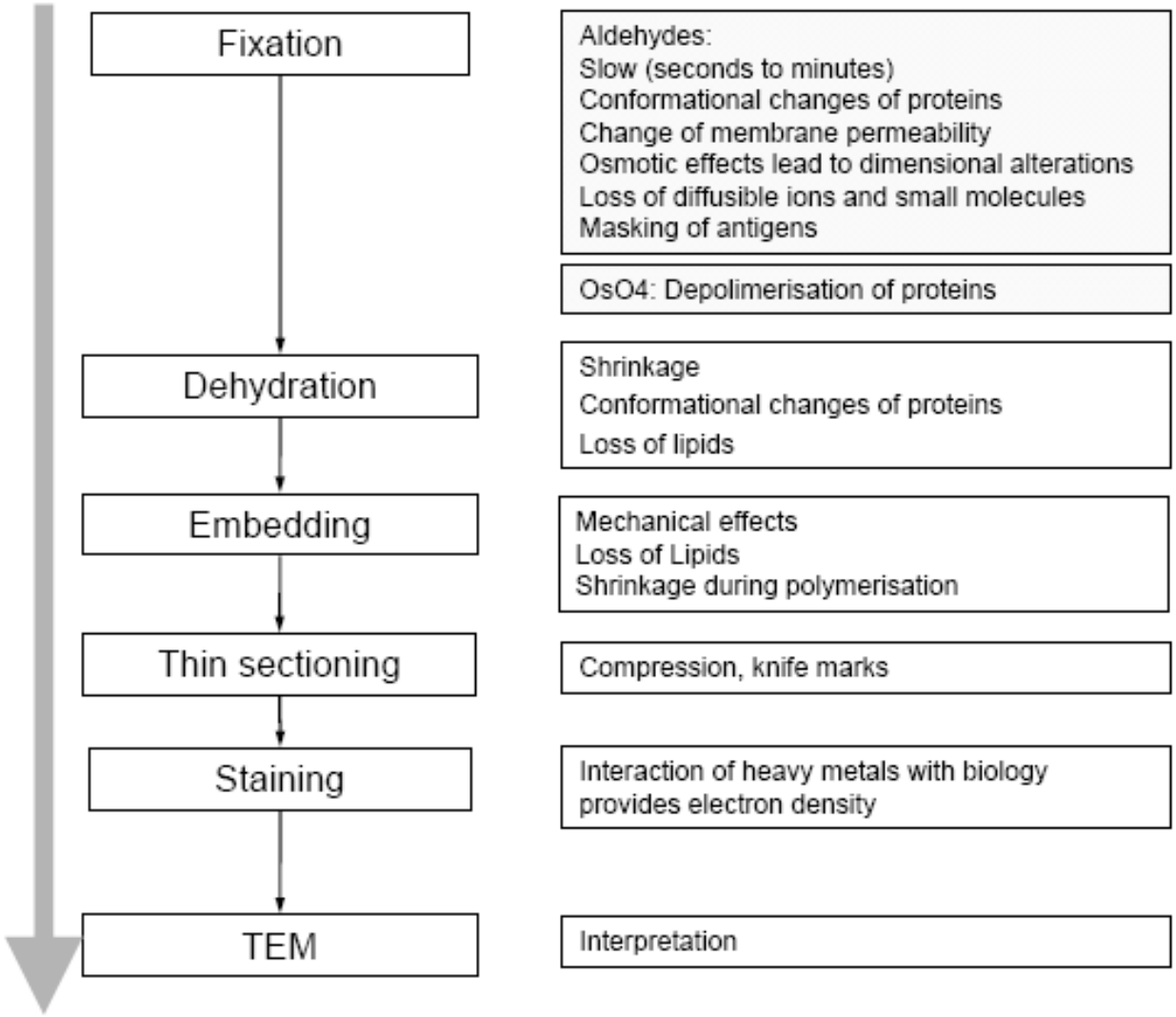


Room temperature processing for TEM

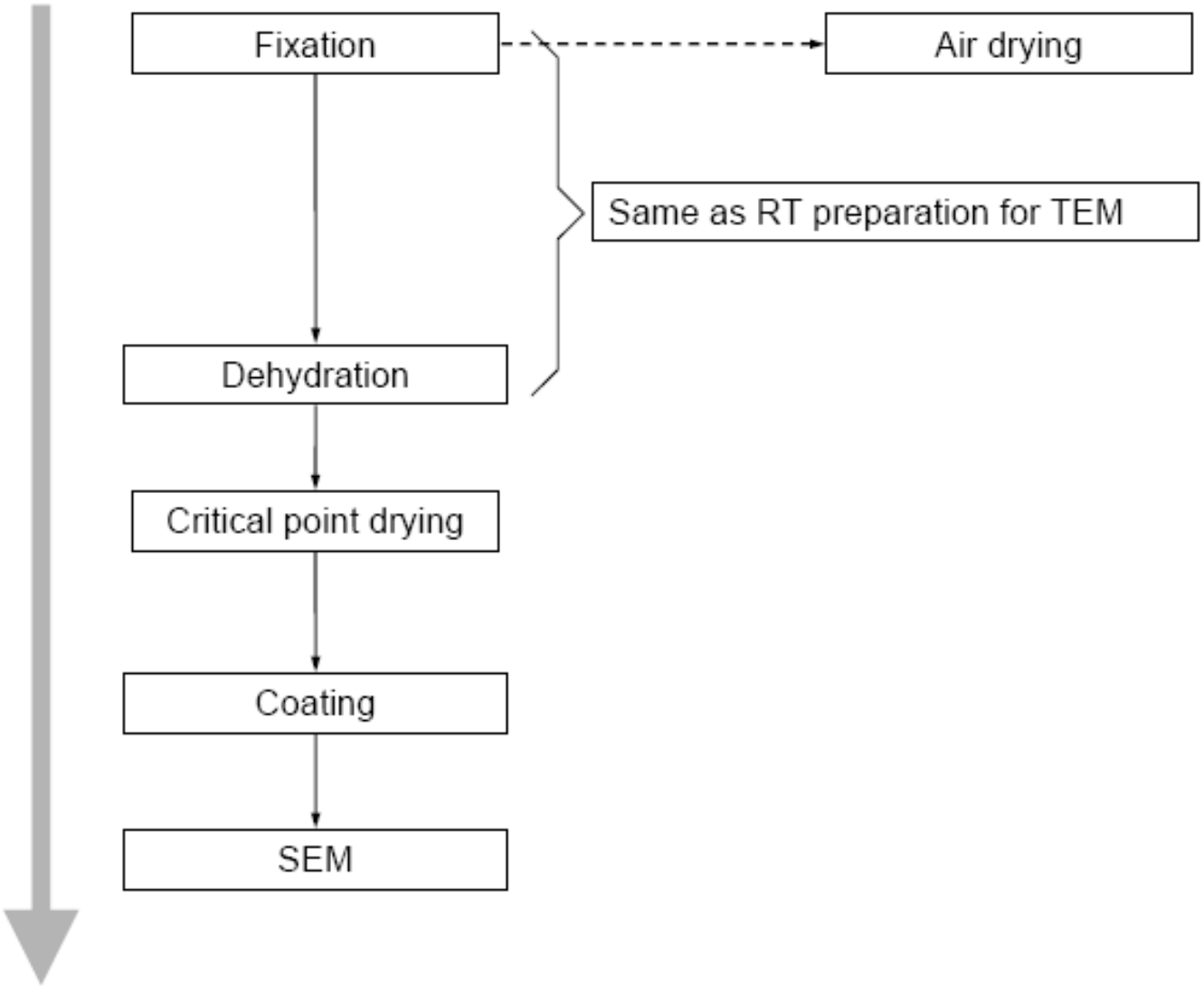


There is an automatic tissue processing devide available for all these steps.. (but not in NMC..)

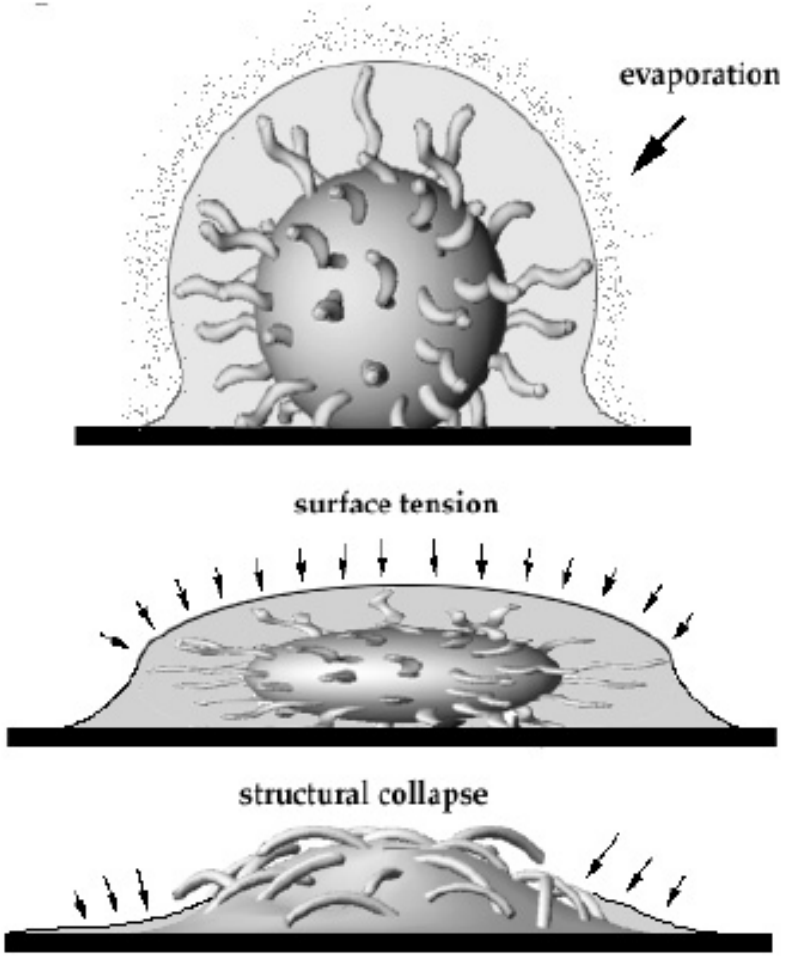
Room temperature processing for TEM - Problems and Artefacts



How about just drying the samples..



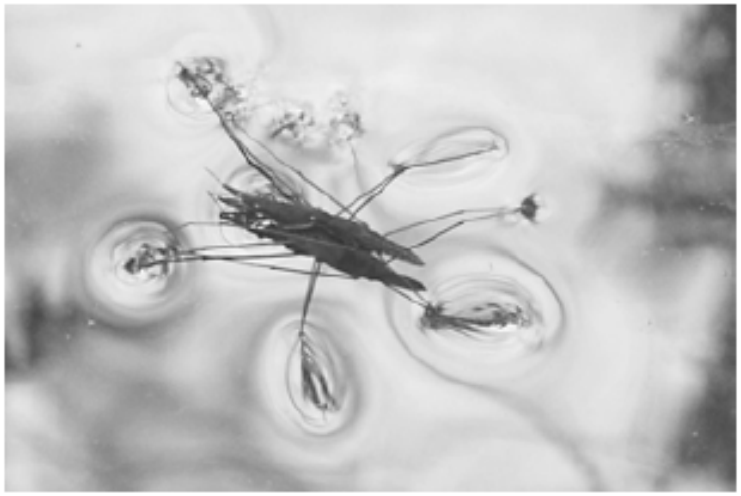
Air drying

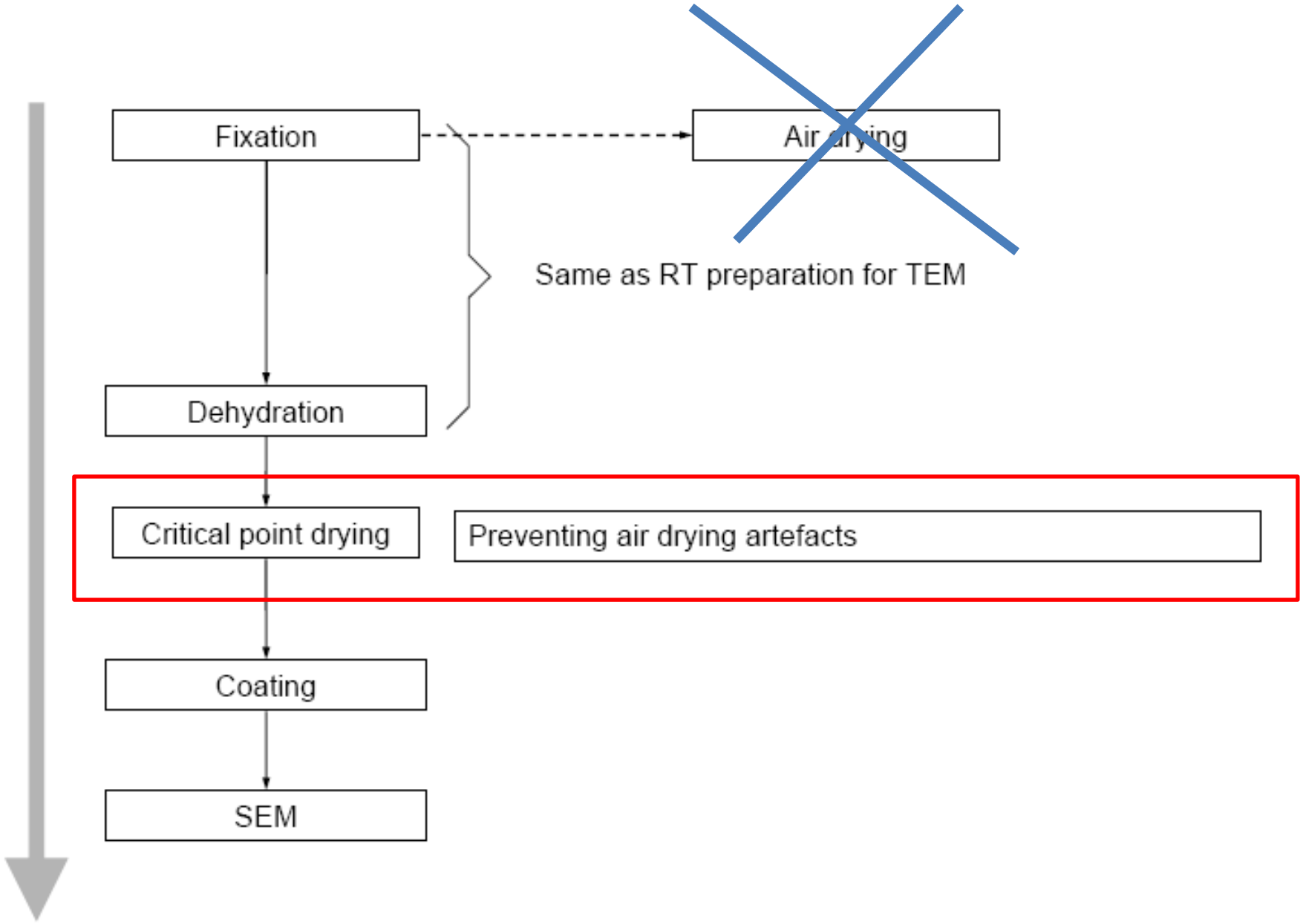


Problem of air drying:

High surface tension of water leads to a collaps of structures

Surface tension of water

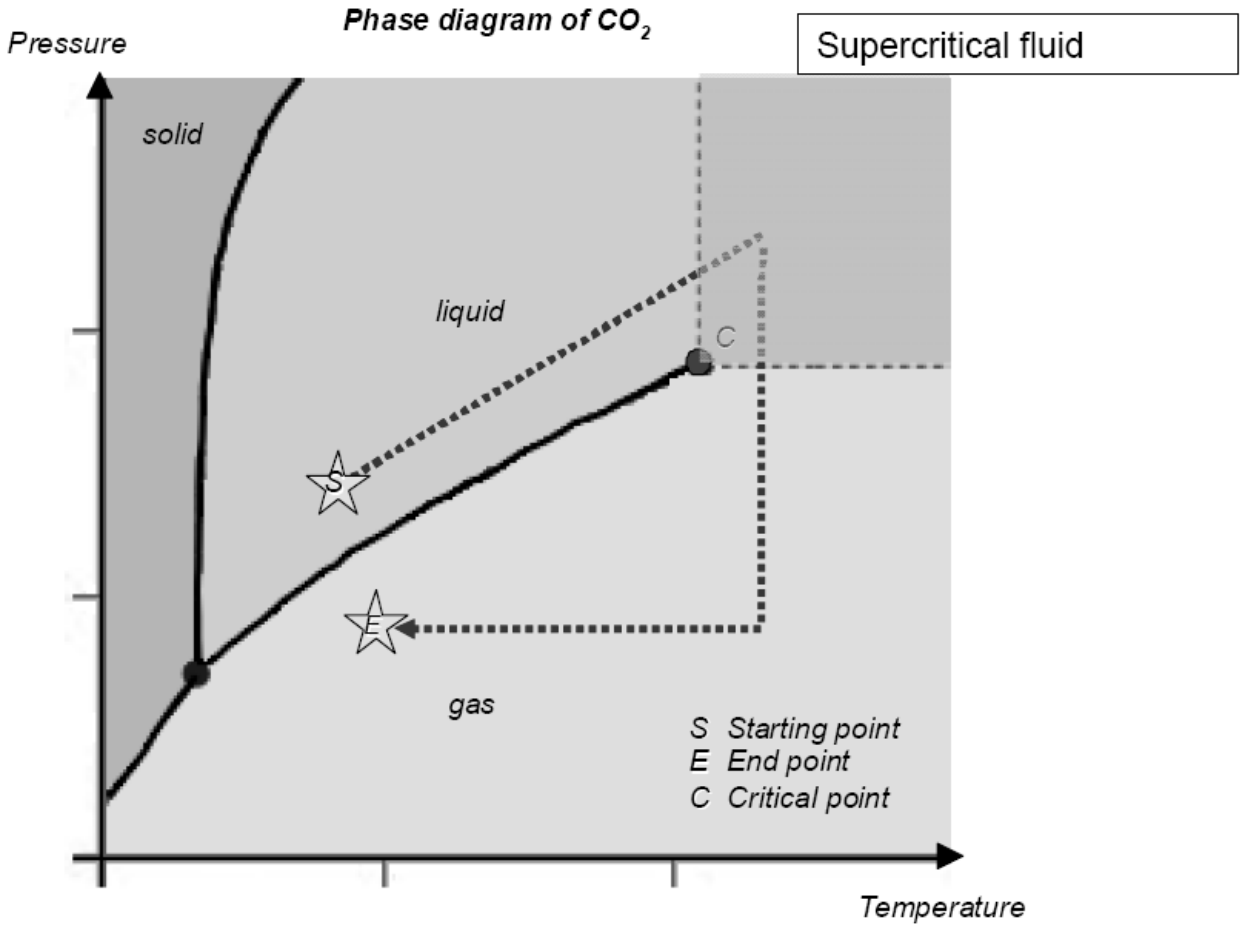



Fixation
Air drying
Same as RT preparation for TEM
Dehydration
Critical point drying
Preventing air drying artefacts
Coating
SEM

Critical point drying

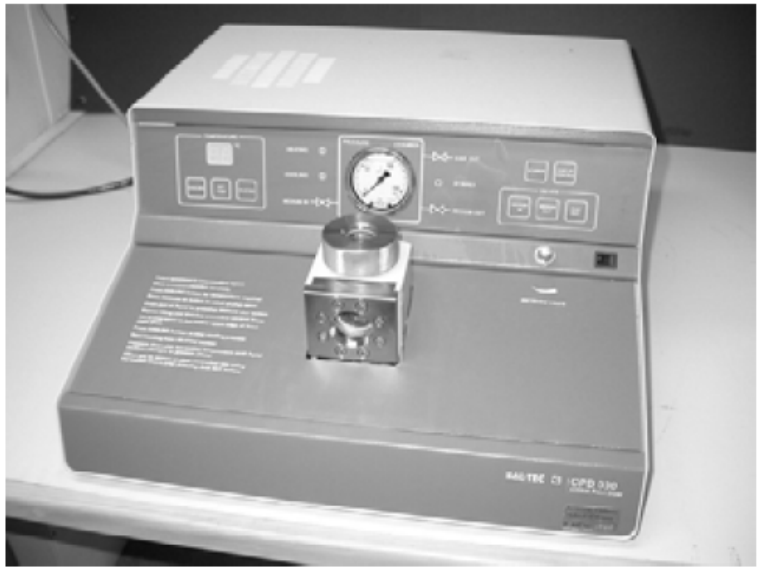
Critical point of CO_2: 31°C, 74 bar

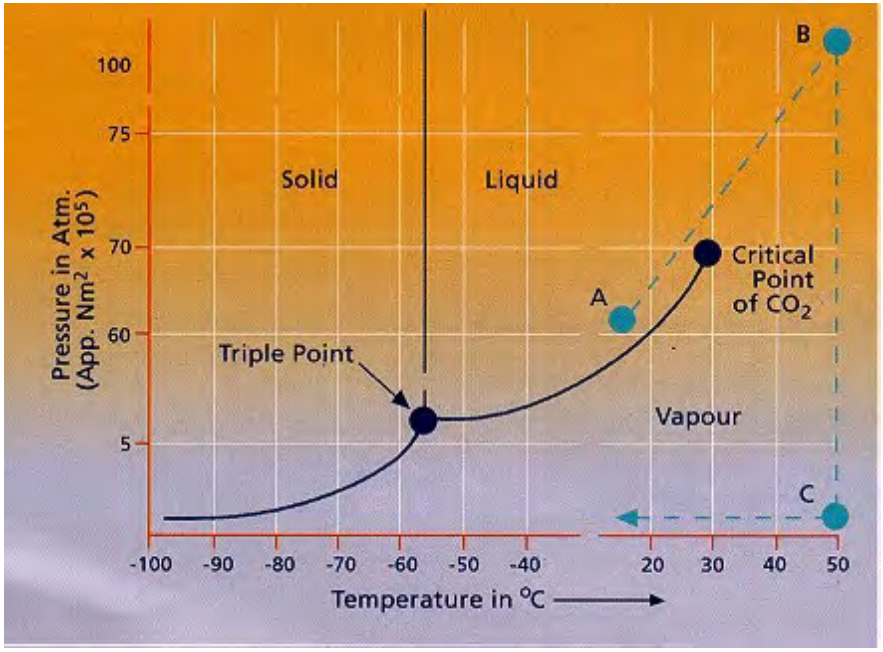
Critical point of H_2O: 374°C and 221 bar



Critical point drying

- Water is barely soluble in CO_2
- Sample must be transferred into solvent like ethanol, aceton (dehydration)
- Dehydration requires fixation of the specimen
- Solvent is exchanged with CO_2 in critical point dryer and processed thereafter

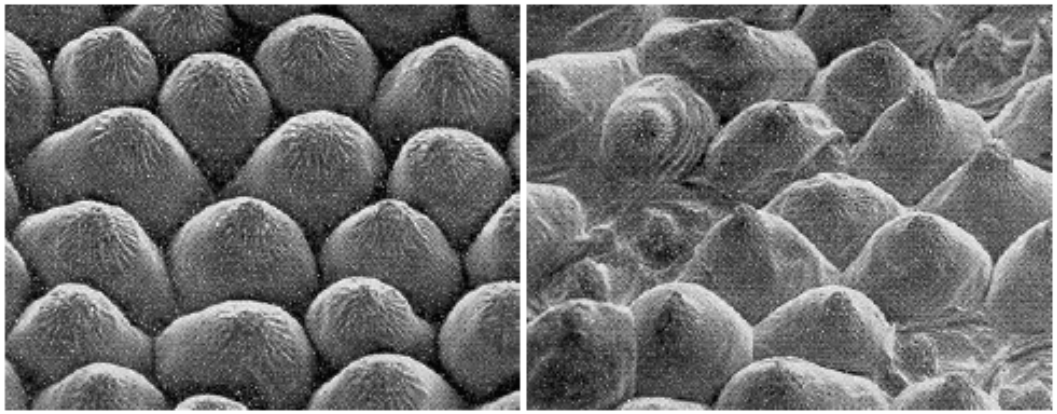




Critical point drying

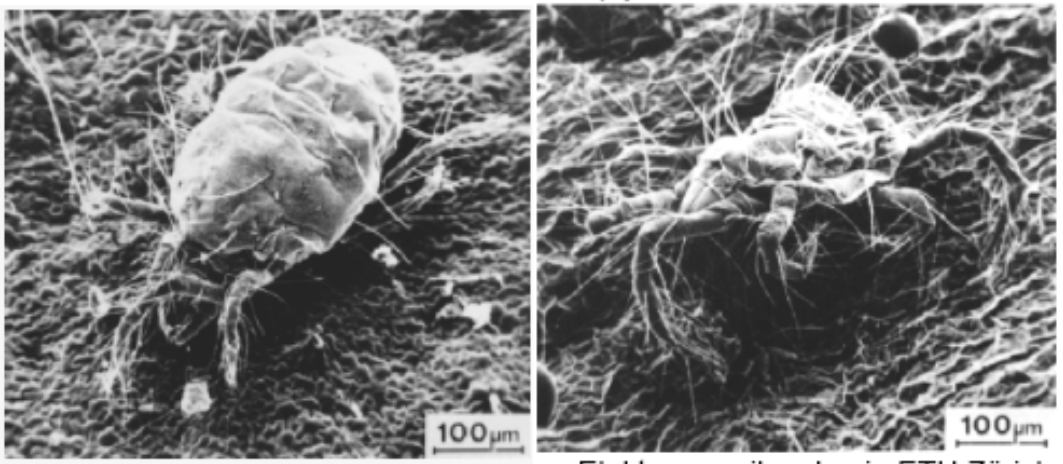
Critical point drying *Air drying*

Surface of rose blossom



SPI

Spider mite



Elektronenmikroskopie ETH Zürich

For TEM critical point drying is good for ”thin” samples like viruses etc.

Preparation pathways overview

RT specimen processing

Low temperature processing

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FROZEN SPECIMEN

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High pressure freezing

Dehydration

Embedding

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Coating

Immunolabeling

Freeze-dried specimen

Freeze-fractured/etched specimen

Staining

Replica

RT-SEM

RT-TEM

RT-TEM

RT-SEM

Cryo-SEM

Cryo-TEM

(From Andres Kaech University of Zurich)

Cryo sample preparation methods

- **1)** Bulk samples? (> 500 nm) (i.e too thick for TEM)

Cells, tissue, bulk materials, thick hydrogels,

❖Freeze and then prepare thin sample for TEM
(example: cryo-microtoming thin sections)

- **2)** Already ”thin” samples: e.g. small particles? (<500 nm, preferably < 200 nm)

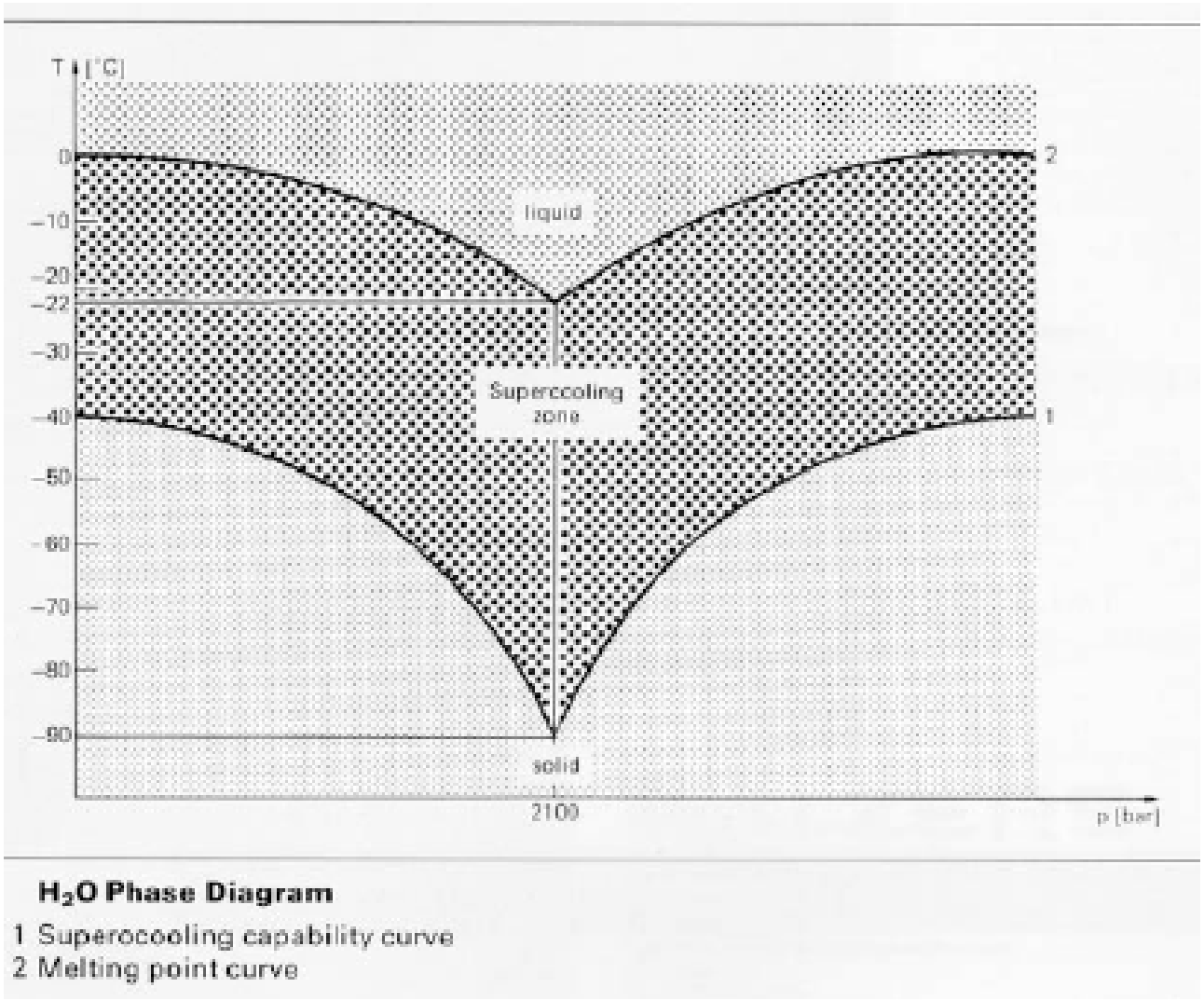
Viruses, liposomes, vesicles, micels, solutions, hydrogels

❖Freeze and then just transfer directly to the TEM

Handbook of Cryo-Preparation Methods for Electron Microscopy, 2008
Edited by Annie Cavalier, Daniele Spehner, Bruno M. Humbel

Bulk samples: High pressure freezing

- High pressure (2000 bars) delays ice crystal nucleation
- Sample thickness can be up to 200 micrometer



H_2O Phase Diagram

1 Supercooling capability curve
2 Melting point curve

High pressure freezing

Sample thickness can be up to 200 micrometers

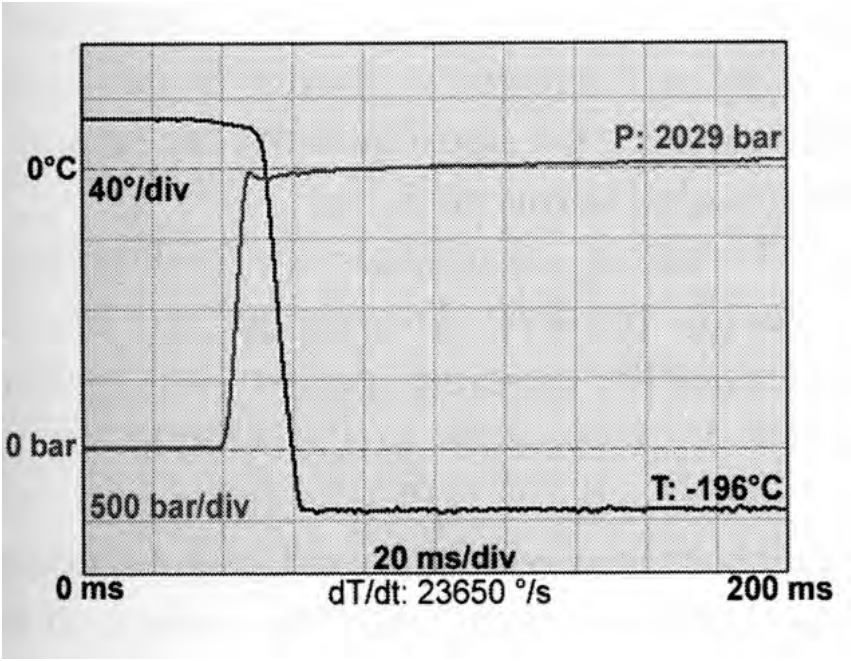
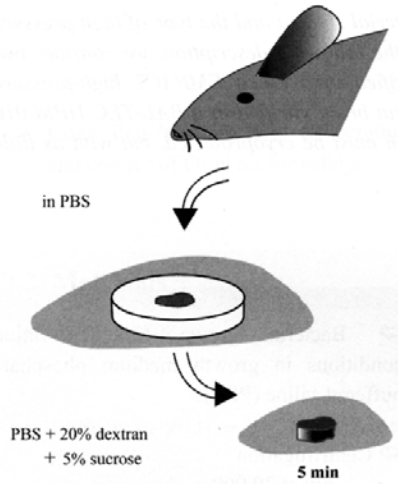


Figure 6.3 An optimal high-pressure cooling cycle: The pressure slope reaches 2045 bars in 20 msec. The pressure increase is nicely synchronized with the temperature decrease. The cooling rate is indicated as dT/dt: 23,650 K/sec.

High pressure freezing: Protocol (Book…

4.1.2. Tissues

Tissues from the central nervous system are especially difficult to vitrify, probably because they have a higher than average water content. Vitrification of the cells could only be achieved after a slight osmotic dehydration of the tissue by 5% sucrose (total osmolarity: 600 mOs).



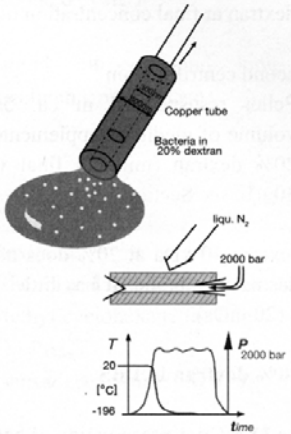
⇨ Organotypic slice of rat hypocampus in experimental culture medium; 5 × 2 mm, thickness: 200 µm.
Extraction of a 1 mm diameter slice

⇨ The small slice is placed for five minutes in the culture medium supplemented with 20% dextran and 5% sucrose. After this time, the tissue remains functional.

Figure 11.6 Extraction of a tissue sample for vitrification

4.2. Vitrification

4.2.1. Cell suspension vitrified in a Leica EM-PACT high-pressure freezer



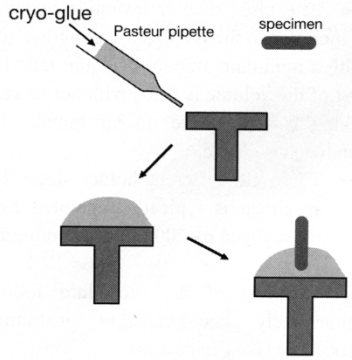
⇨ 30 µL of bacteria suspension on a Parafilm.
⇨ Suck the suspension into the copper tube with a piston until full. No air bubble must remain in the tube.

- Dimensions of the copper tube:
  - Length: 20 mm
  - External diameter: 600 µm
  - Internal diameter: 300 µm

The specimen is thus separated from the cryogen by 150 µm of copper.
⇨ High-pressure vitrification according to instrument procedure (*see* Chapter 6).
⇨ Pressure should not exceed 2000 bars; higher pressure favours formation of high-pressure ice.
⇨ After cooling, the tube can be stored at liquid nitrogen temperature or directly mounted in the cryo-ultramicrotome.

Figure 11.7 Vitrification in EM-PACT high-pressure freezer.

4.3.1. Mounting the specimen in the cryo-ultramicrotome

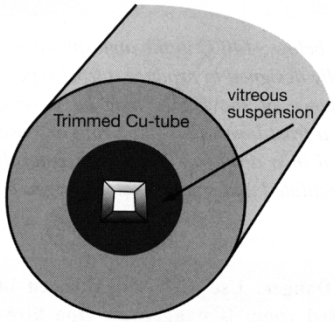


⇨ The copper tube from Leica EM PACT2 can be directly mounted in the specimen holder.
⇨ The fragment of material extracted from the sandwich of the BAL-TEC HPM 010 freezer is mounted with cryoglue.[18]

1. Cryoglue is a mixture of ethanol and 2-propanol in a 2:3 ratio.
2. A drop of cryoglue is applied on the specimen head at −140°C. The glue is slightly soft at this temperature.
3. The specimen fragment is forced into the glue and correctly oriented.
4. Temperature is reduced to −160°C. The glue becomes rigid enough for further operations.

Figure 11.9 Vitreous specimen mounted in a cryo-ultramicrotome

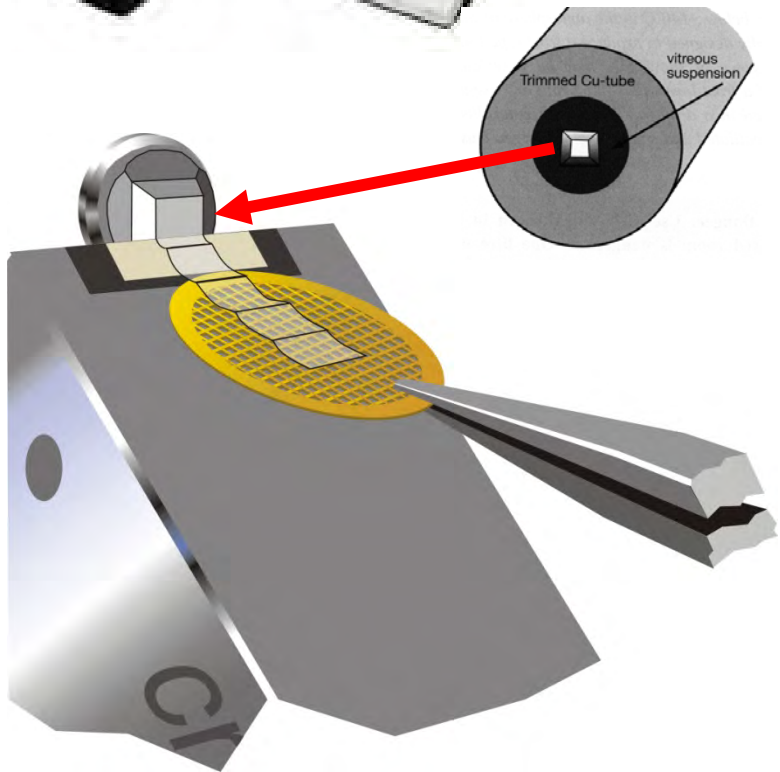
4.3.2. Trimming the specimen



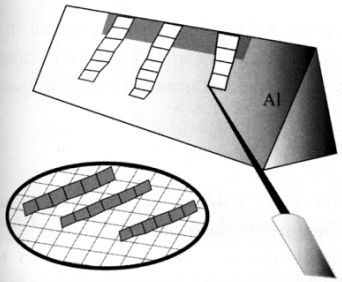
⇨ The specimen surface is prepared flat with a trimming knife. The whole section of the EM PACT2 copper tube is sectioned with ca. 0.05 µm (cryoglued and/or fragile specimens) to 1 µm strokes (clamped copper tubes). Typically, some 200 µm must be removed from the tube; less from a glued specimen.
⇨ The first evaluation of the state of the specimen can generally be made at this stage. A well vitrified specimen seen with top illumination is uniformly black in the copper tube. A non vitrified region or inhomogeneous material is shiny or milky. Many bad specimens can already be discarded at this stage.
⇨ A square-based pyramid is an excellent shape for final preparation of the specimen. A 45° trimming knife must be used. Suitable dimensions are 100 to 200 µm at the base and 10 to 100 µm high.
A rectangular parallelepiped trimmed with a 90° knife may offer advantages, but it can be dangerously prone to fracture.

Figure 11.10 Vitreous specimen trimmed in a cryo-ultramicrotome.

Cryo sectioning and section pick-up and direct mounting on grid



4.3.3. Cutting sections



➪ The conditions for obtaining good sections are a well-prepared, homogeneously vitrified specimen, careful trimming, and an excellent cutting knife. The latter is of utmost importance. Besides being sharp and clean, it must have good gliding properties. The manufacturing procedures are not known publicly. Research on this matter should be encouraged.

➪ In addition, the working room must be reasonably free of snowflake contamination and the forces induced by electric charges must be neutralised. This is best done with the ion shower incorporated into the cryo-ultramicrotome. Operating conditions must be adjusted according to the situation.

➪ Under good cutting conditions, the sections appear as regular ribbons, which neither stick to the surface of the knife nor fly away. Adjust the ion shower by changing the distance of the ion source to the knife edge and the power of the ionizer.

➪ The ribbons are manipulated with the eyelash. Under well-adjusted conditions, the ribbon just sticks to the eyelash that can be transferred directly onto the prepositioned grid. Two to 10 ribbons can be mounted on each grid.

➪ Some operators hold the growing ribbon with the eyelash and pull it gently. This longer ribbons can be obtained.
The incorporation of a micromanipulator into future instruments could help in democratising the method.

Figure 11.11 Ribbons of vitreous sections are transferred from knife to grid.

For the ”thick samples” also TEM Cryo-lamella preparation using Focused ion beam (FIB) is a good option (instead of microtoming)

- Either conventional FIB lamella preparation as shown in the FIB lecture (Lide) – but using a cryo stage, *cryo liftout* and cryo transfer..

- Or in-situ Cryo lamella milling (cryo stage and cryo transfer – but no need for cryo lift out..)

  Example: https://www.fei.com/aquilos/

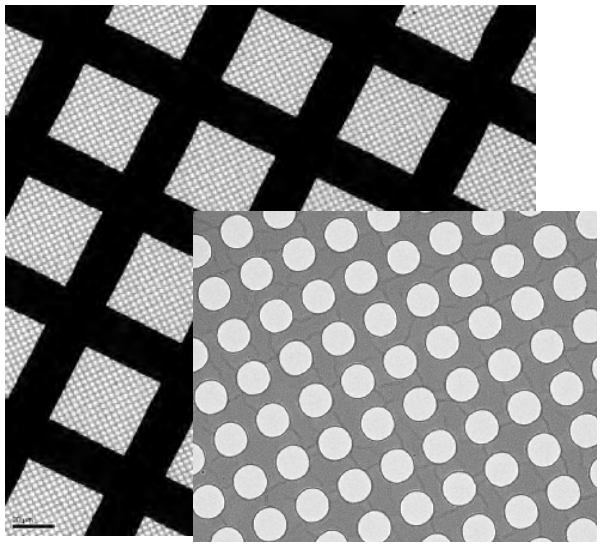
(NMC will get cryo stage and cryo transfer system to Jeol FIB next summer - July 2021)

2. Thin samples, small particles?

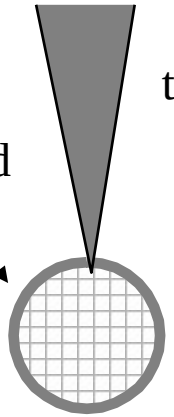
Viruses, liposomes, vesicles, micels, solutions, hydrogels

Vitrification of water by Rapid freezing of thin film suspensions

Sample preparation for cryo-EM: vitrification of water (solvent)

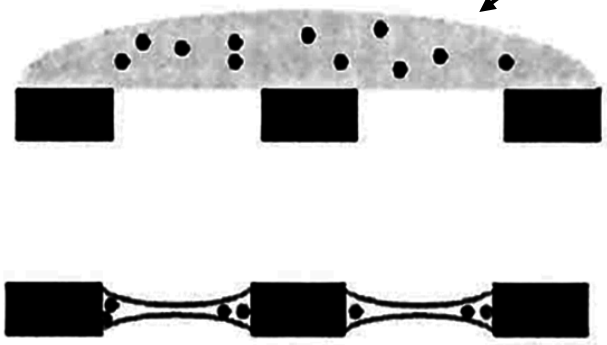


EM-grid



tweezers

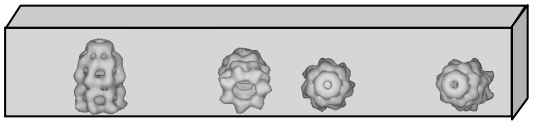
Pipette 3ul sample



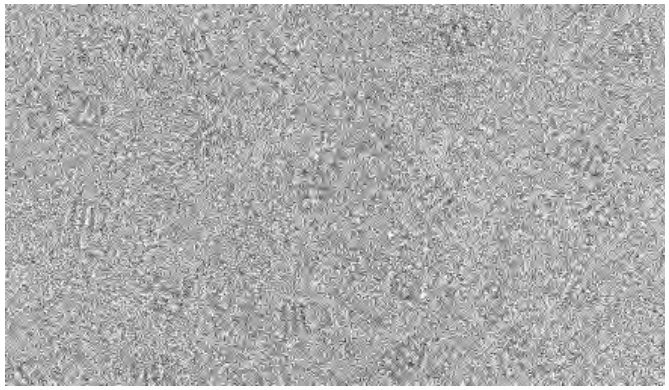
Plot excess liquid away using filter paper

Liquid ethane /propane (-175 to -188 °C)

→ Quench to the liquid ethane/propane



Native, aqueous sample is embedded in vitreous water and stored below -160°C

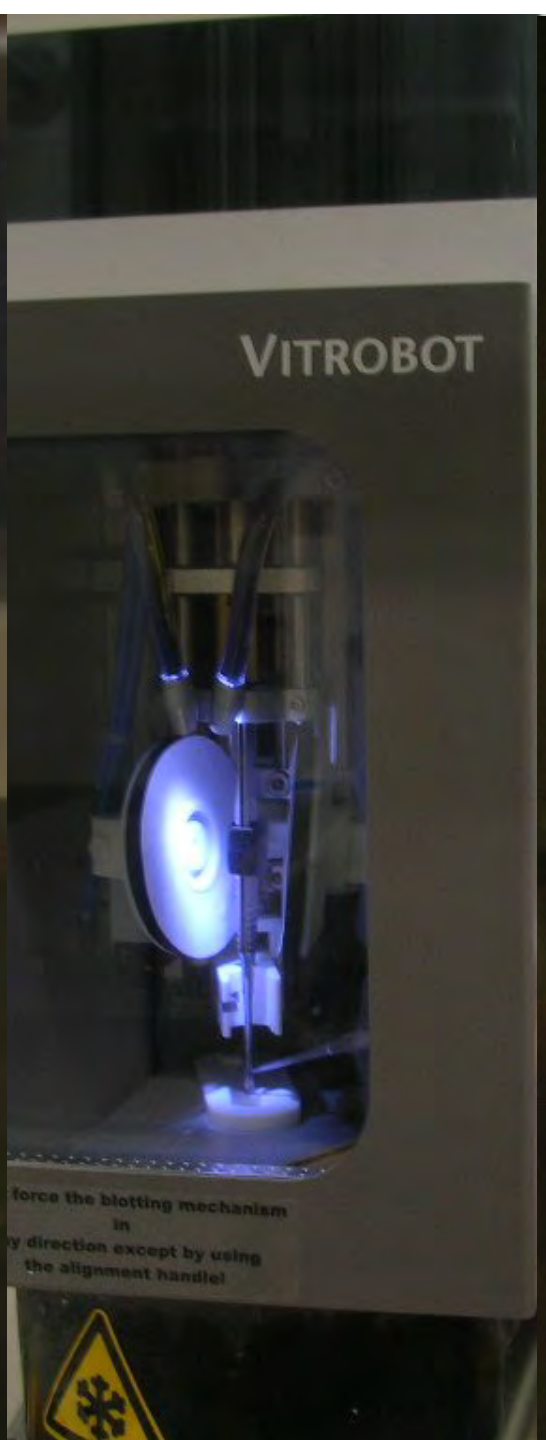


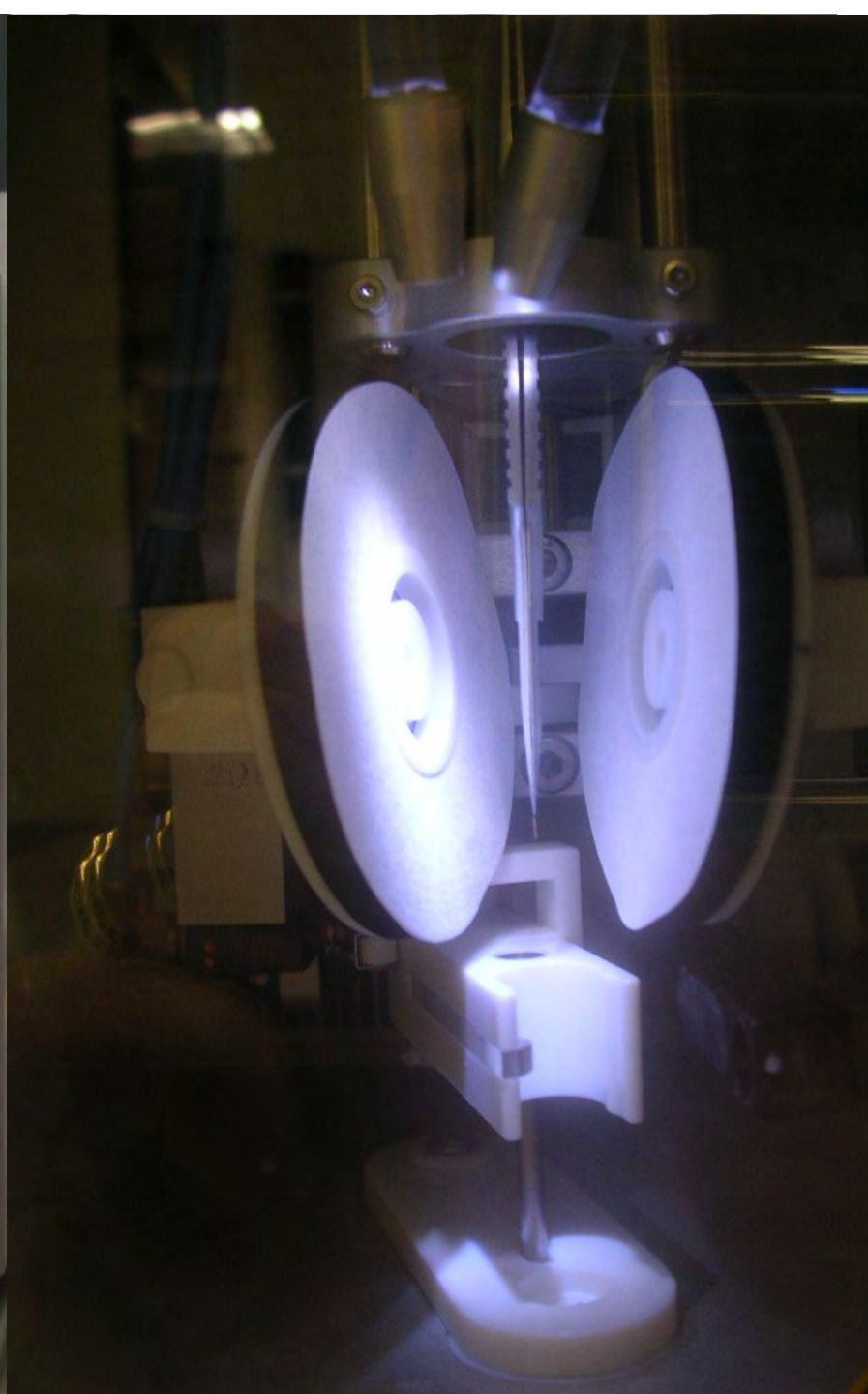
Micrograph(2D-projection)

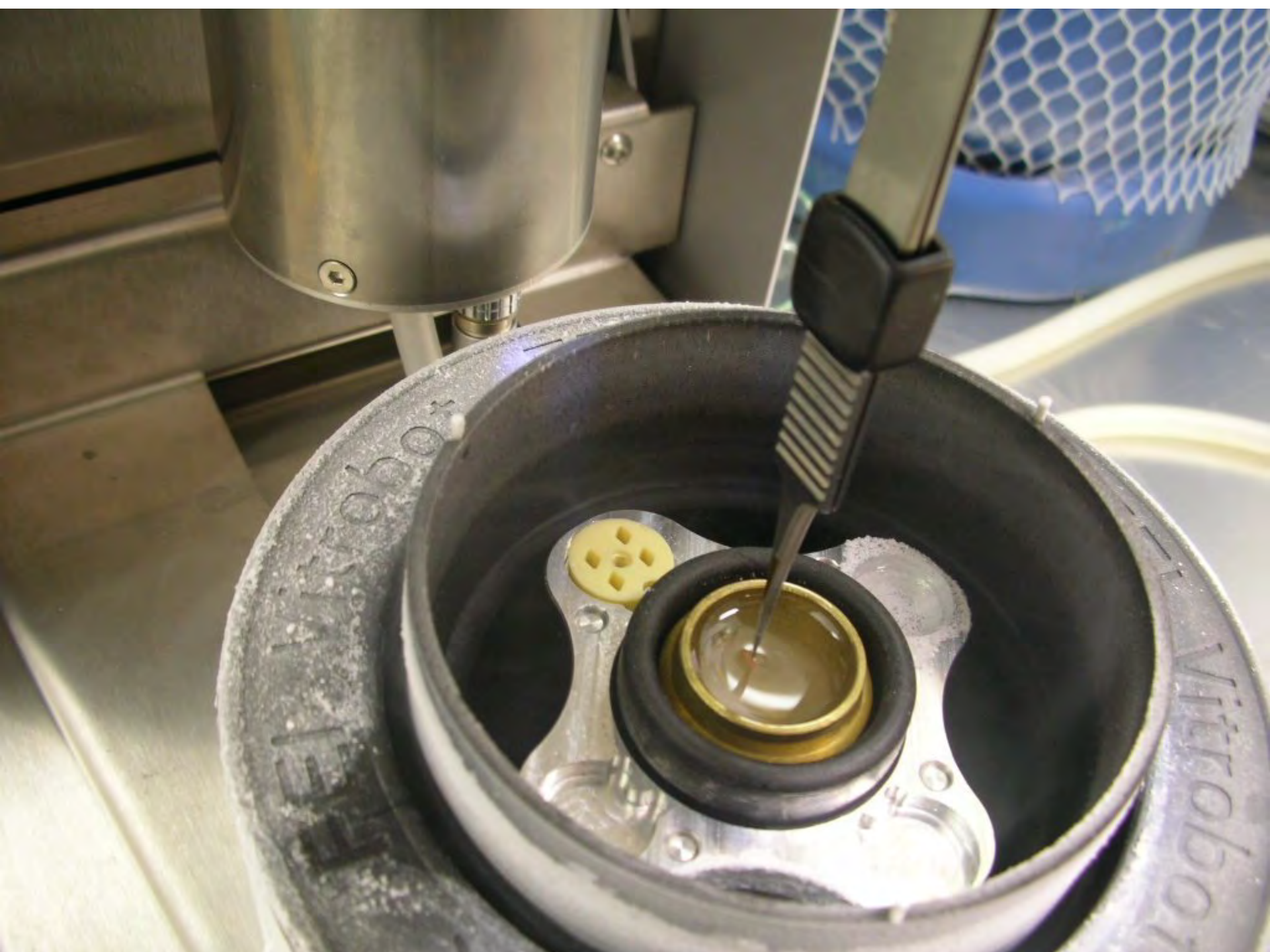
Sample Preparation: Automatic system FEI Vitrobot™

Automate the cryo-fixation process at constant and user-definable physical and mechanical conditions e.g. **temperature, relative humidity, blotting conditions (blot time, number of blots, blot pressure..)**

Because the freezing rate in liquid ethane is very fast (~1 x 10^4 °C/sec) and the amorphous ice layer produced is very thin (<100nm), ice crystals do not have time to form and thus the molecular integrity of the specimen is preserved.


VITROBOT
force the blotting mechanism
in
y direction except by using
the alignment handle!

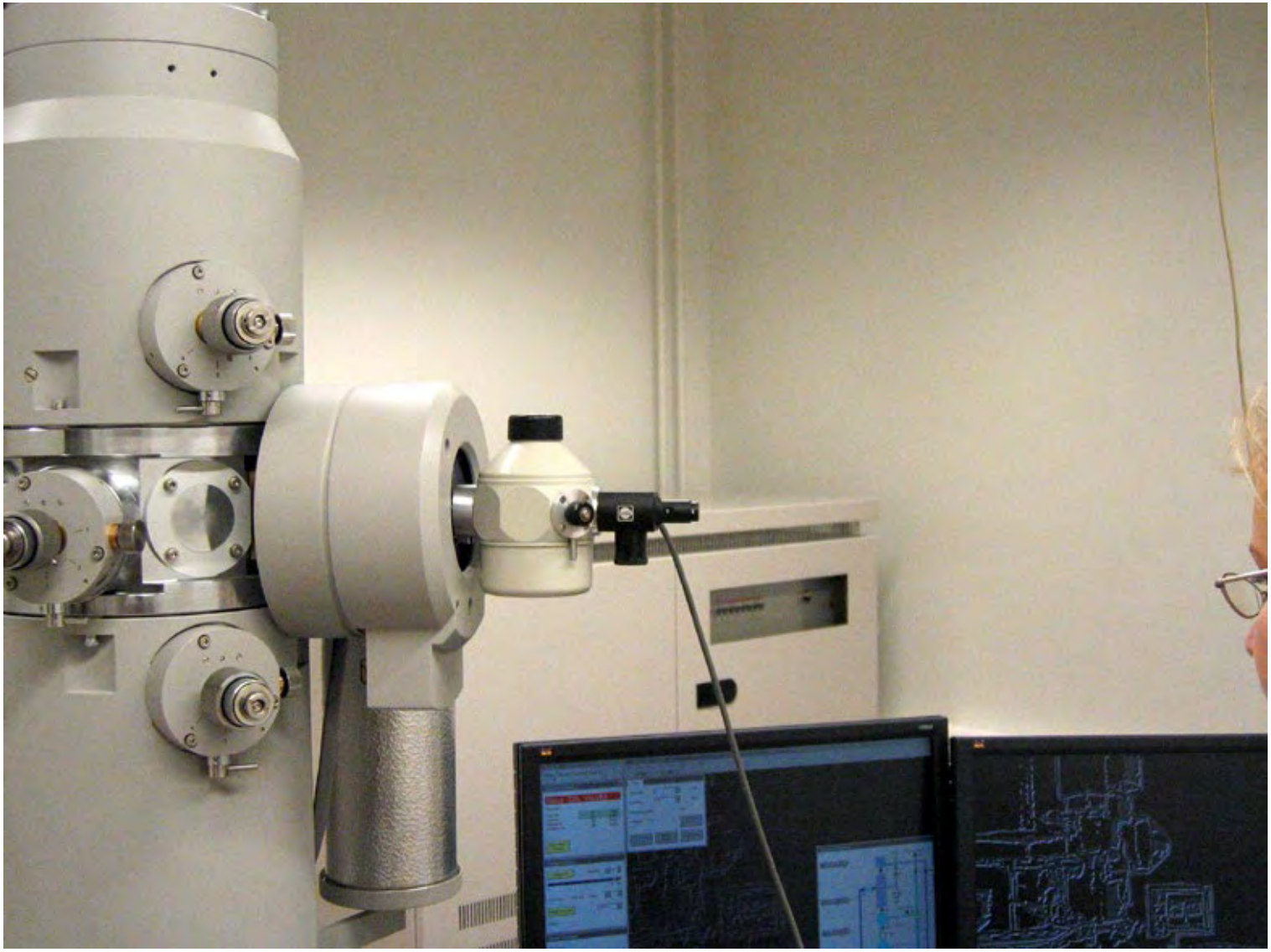



Vitroboost

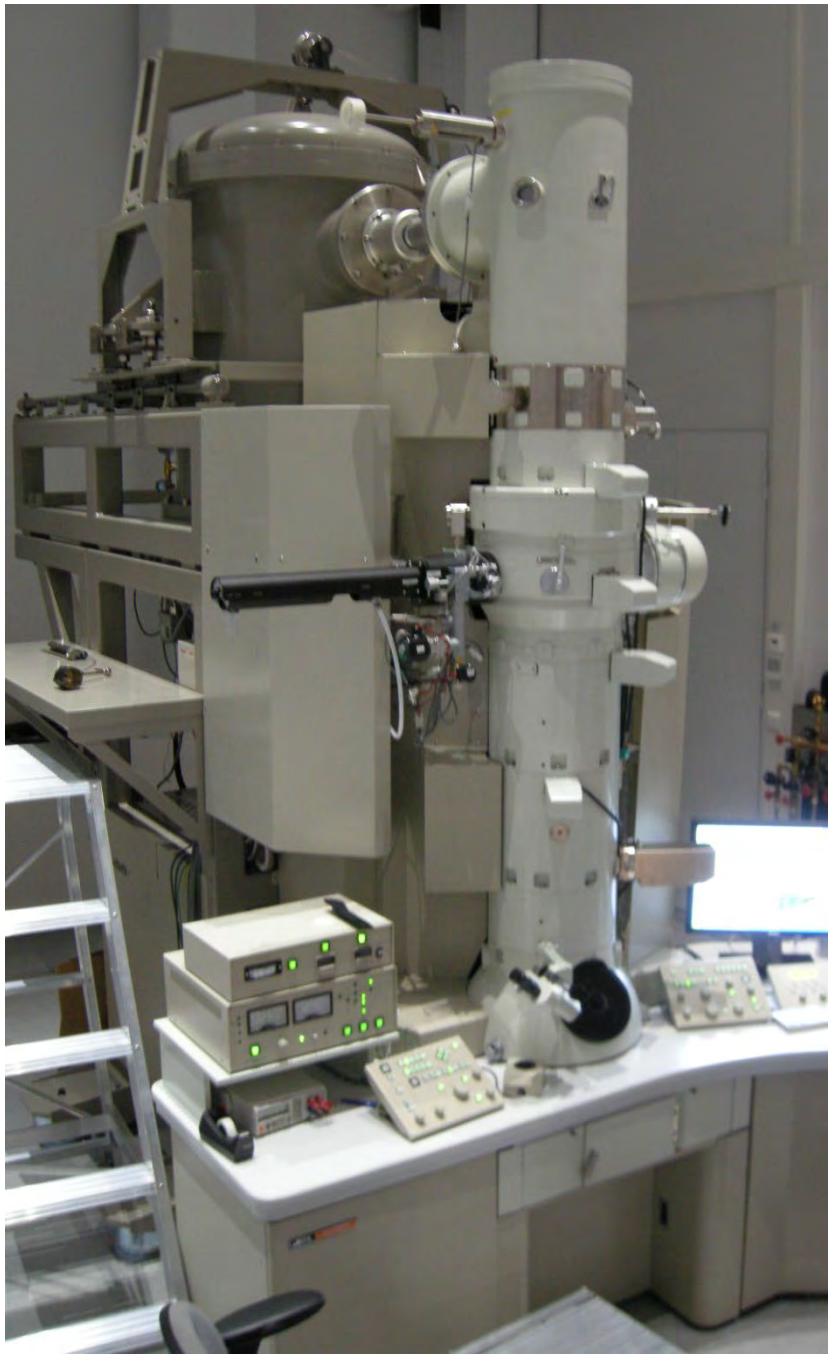
Cryo-transfer

Stantard cryo transfer holder...

we have two holders: multiple specimen (3 samples) holder & cryo-rotation holder



Resolution is limitted by sample holder due to thermal drift and vibrations - caused by external liquid nitrogen dewar.



Dedicated cryo-TEM

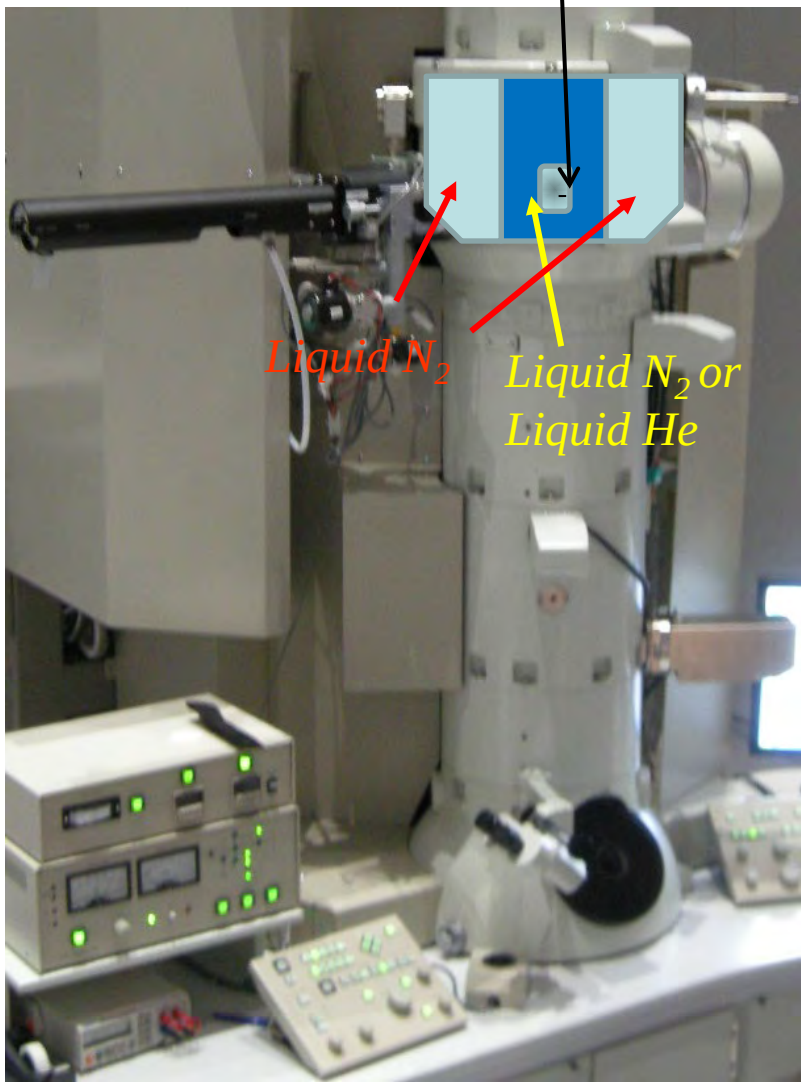
Liquid helium Jeol 3200FSC

Samples are surrounded by liquid helium/nitrogen cooling system inside the microscope - No external liquid nitrogen/helium dewar and samples are in small cartridges.

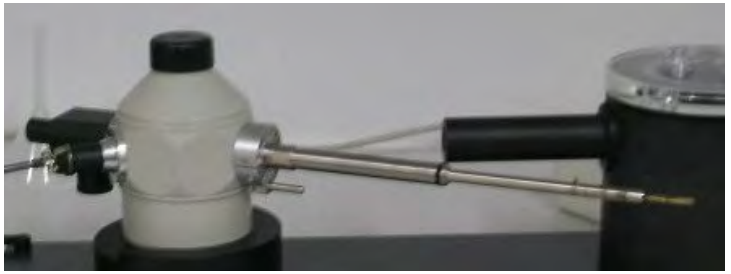
→ Fast temperature stabilization after the sample loading
→ Stable temperature – minimum thermal drift
→ No vibration due to external devar
→ True atomistic resolution (~2Å)

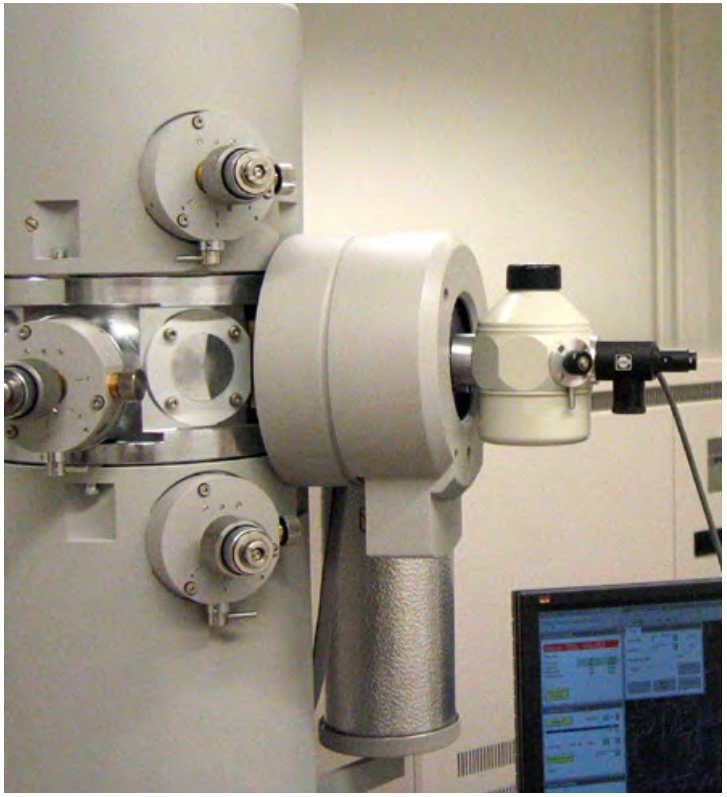
Eucentric tilt for tomography (±70 degrees)
Lattice resolution 0.204 nm at 18K and liquid nitrogen temperature

Cryo-Stage



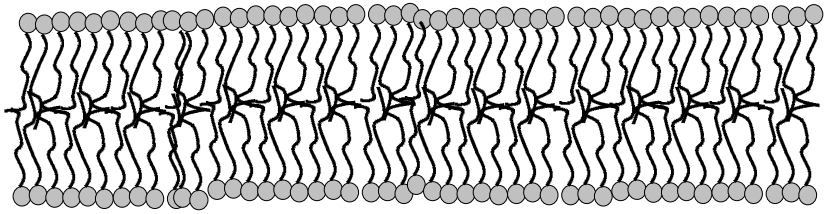
cryo transfer holder for ”normal” TEM



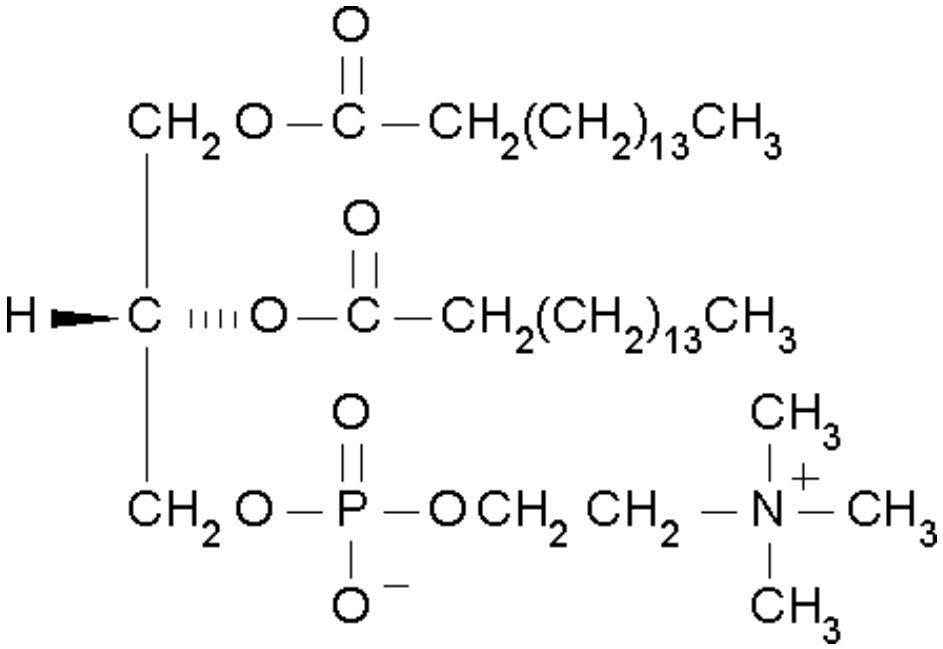


Material Science Cryo-TEM Applications

Example: vesicles in water.. Phosfolipid, polymeric etc.

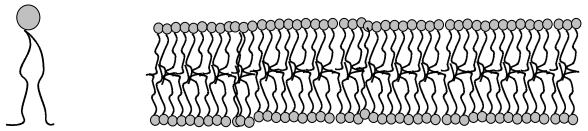


Lipid bi-layers

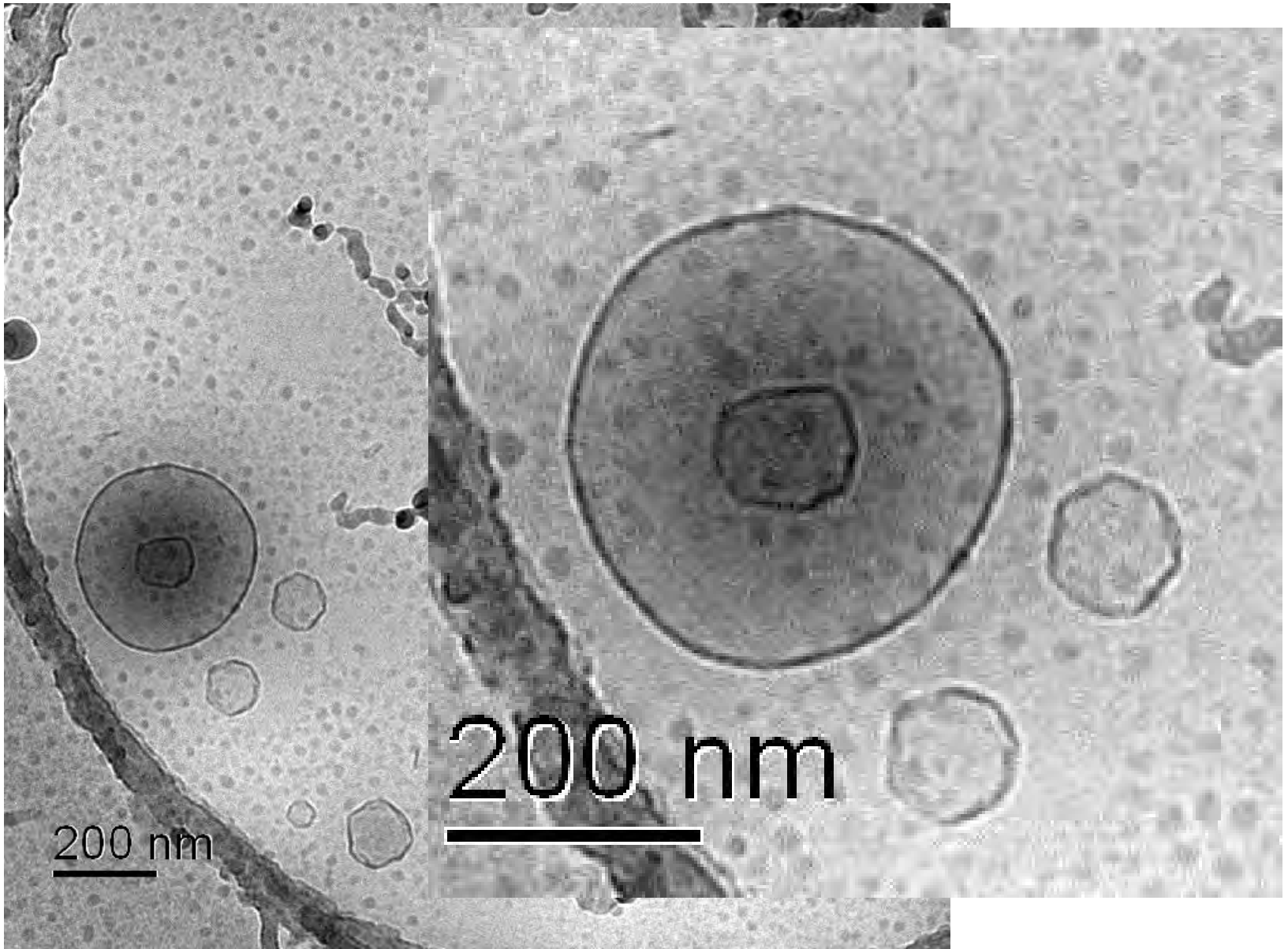


Phosfolipid

Example: Vesicles



Lipid bi-layers

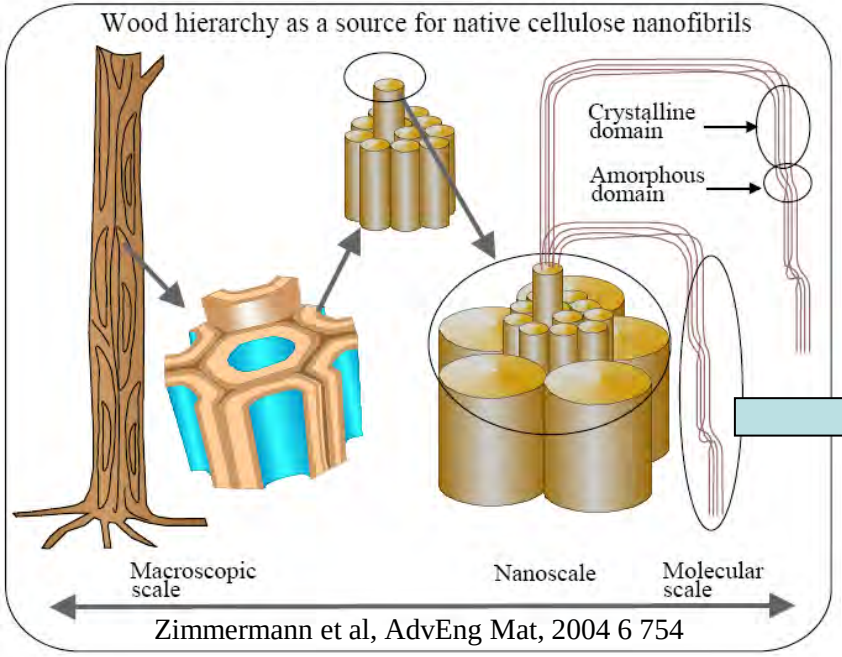


Example: Cellulose Nanofibers

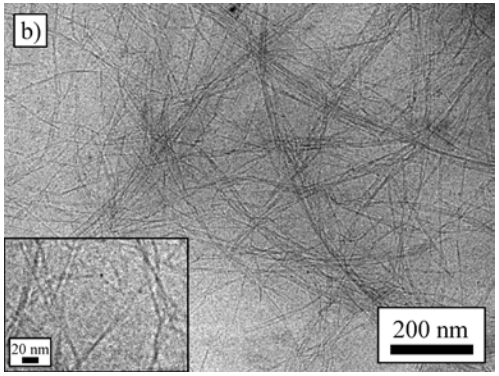
Biological hierarchical self-assemblied as templates for functionalities
Native (Cellulose I) 3-10 nm nanofibers

Exceptional properties
Sustainable

Large scale processes under developments (cf. silk)



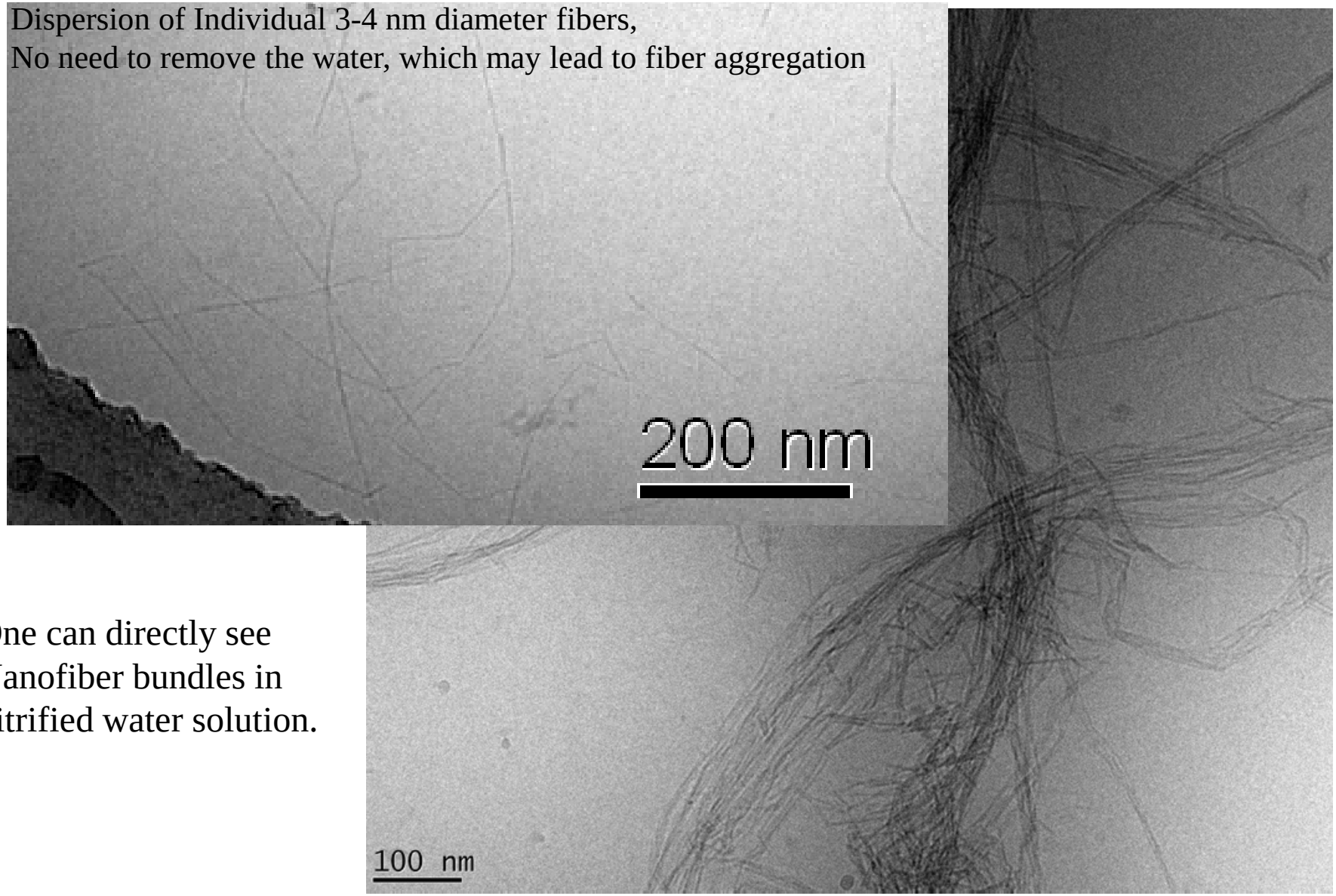
Zimmermann et al, AdvEng Mat, 2004 6 754



Pääkkö, Ankerfors, Kosonen, Nykänen, Ahola, Österberg, Ruokolainen, Laine, Larsson, Lindström, Ikkala, *Biomacromolecules* 2007 8, 1934

Cryo TEM applications:

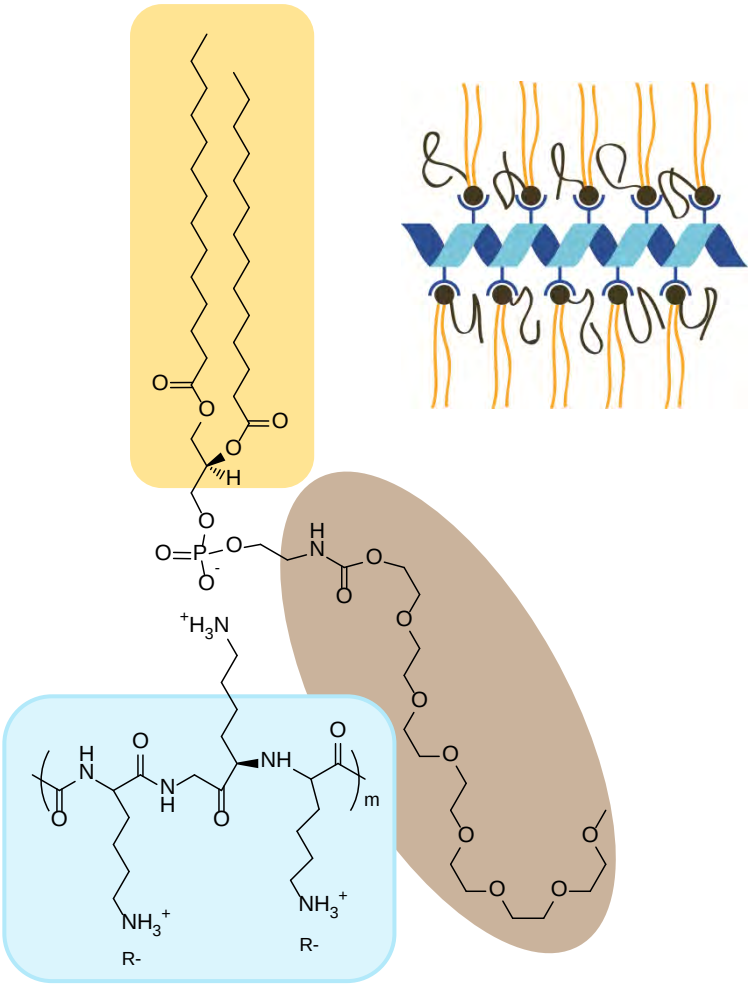
Example: Cellulose Nanofibers in water solution

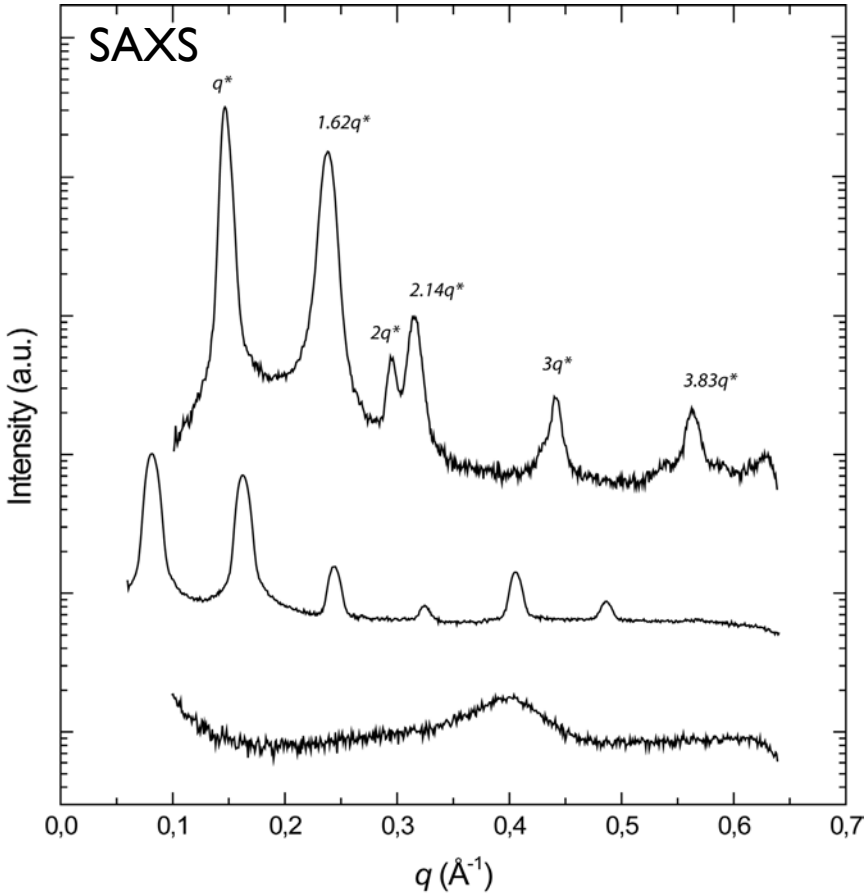


One can directly see Nanofiber bundles in vitrified water solution.

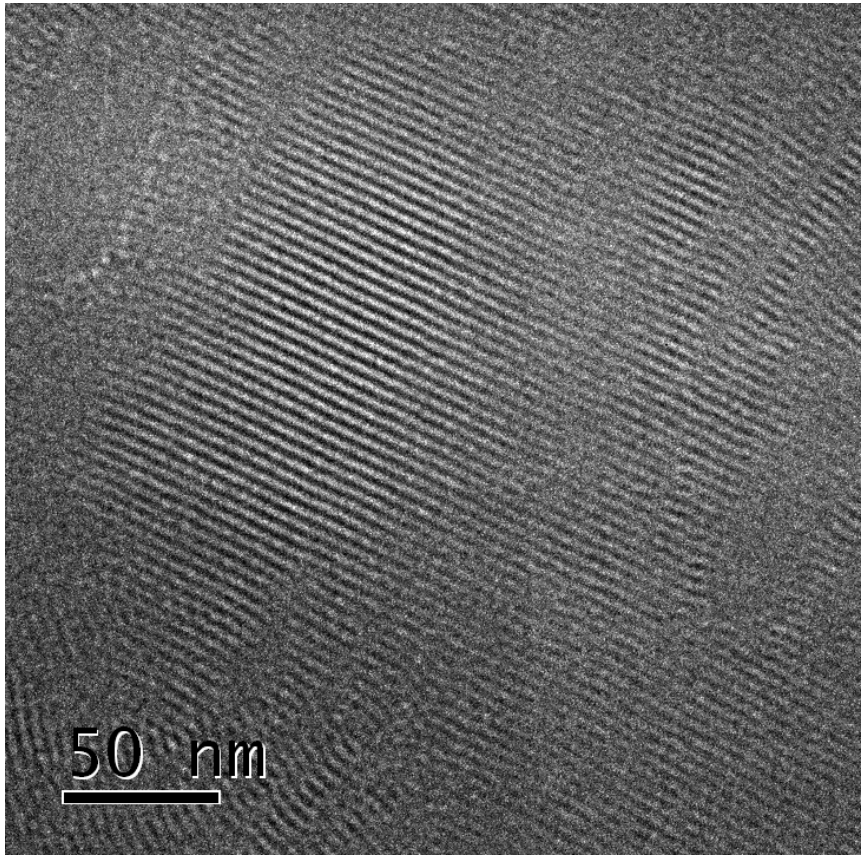
Liquid helium cryo TEM - Reducing the **Beam damage**

Polylysine-lipid(DMPE-EG_7) complex

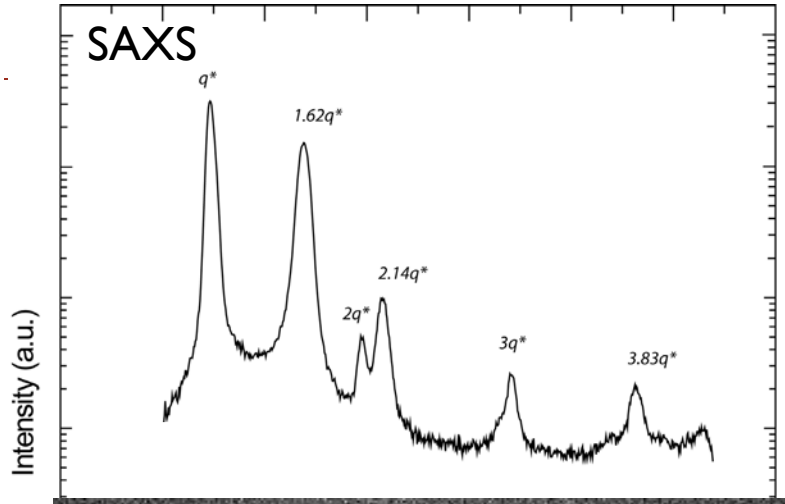


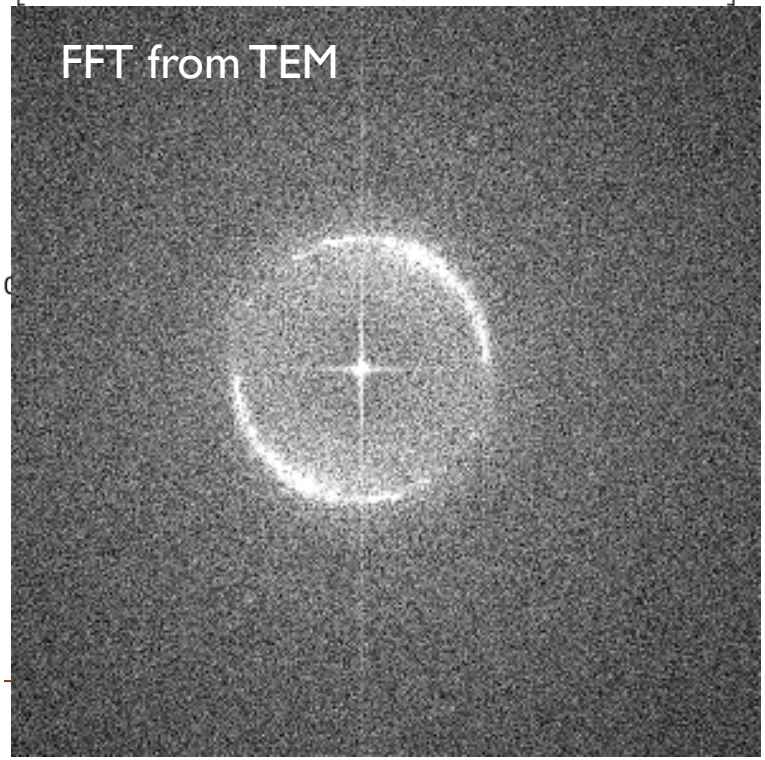


Cryo-TEM at 86 K (Liquid nitrogen cooling)



Low dose image taken at 86 K (- 187 °C)

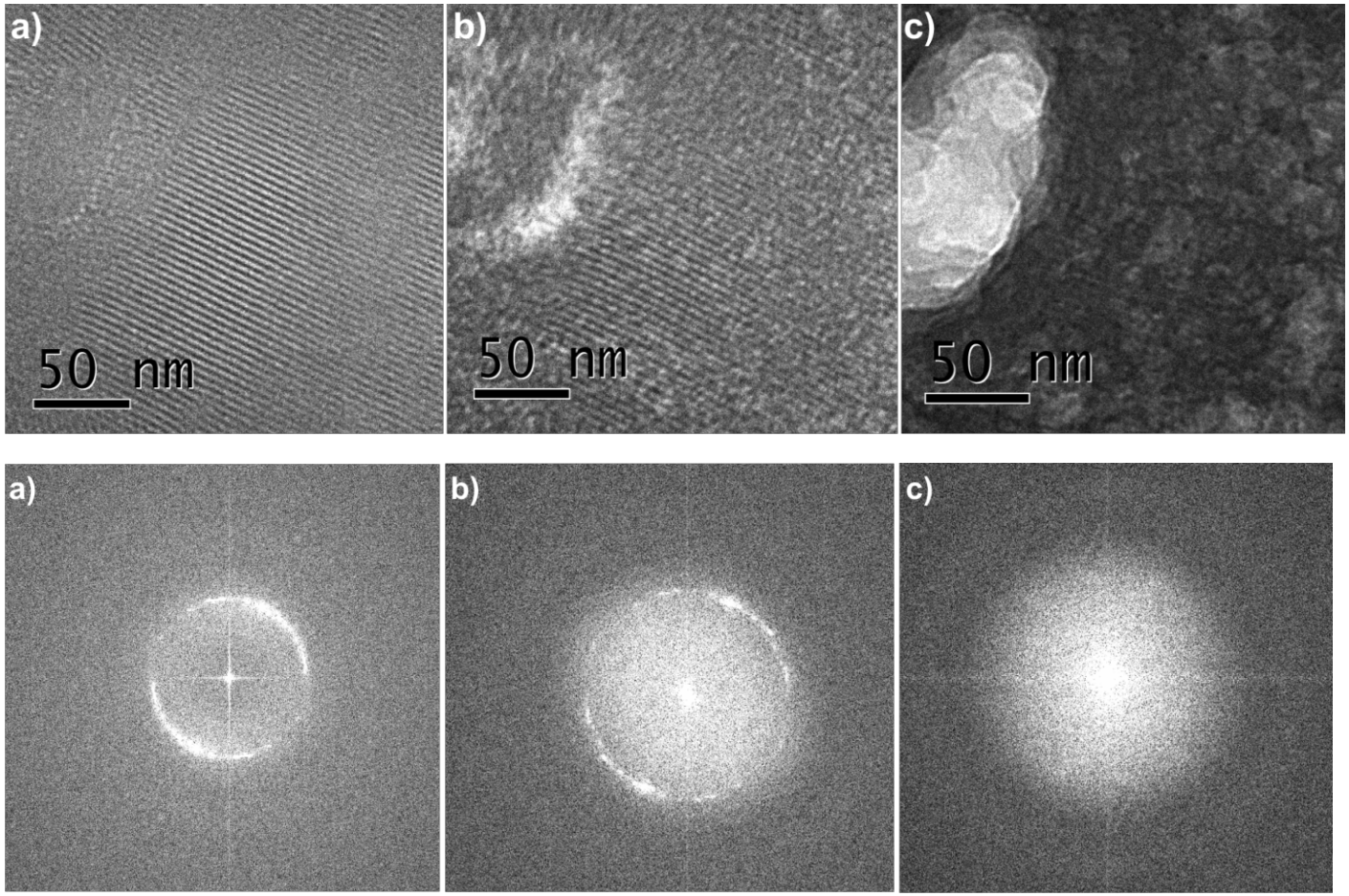




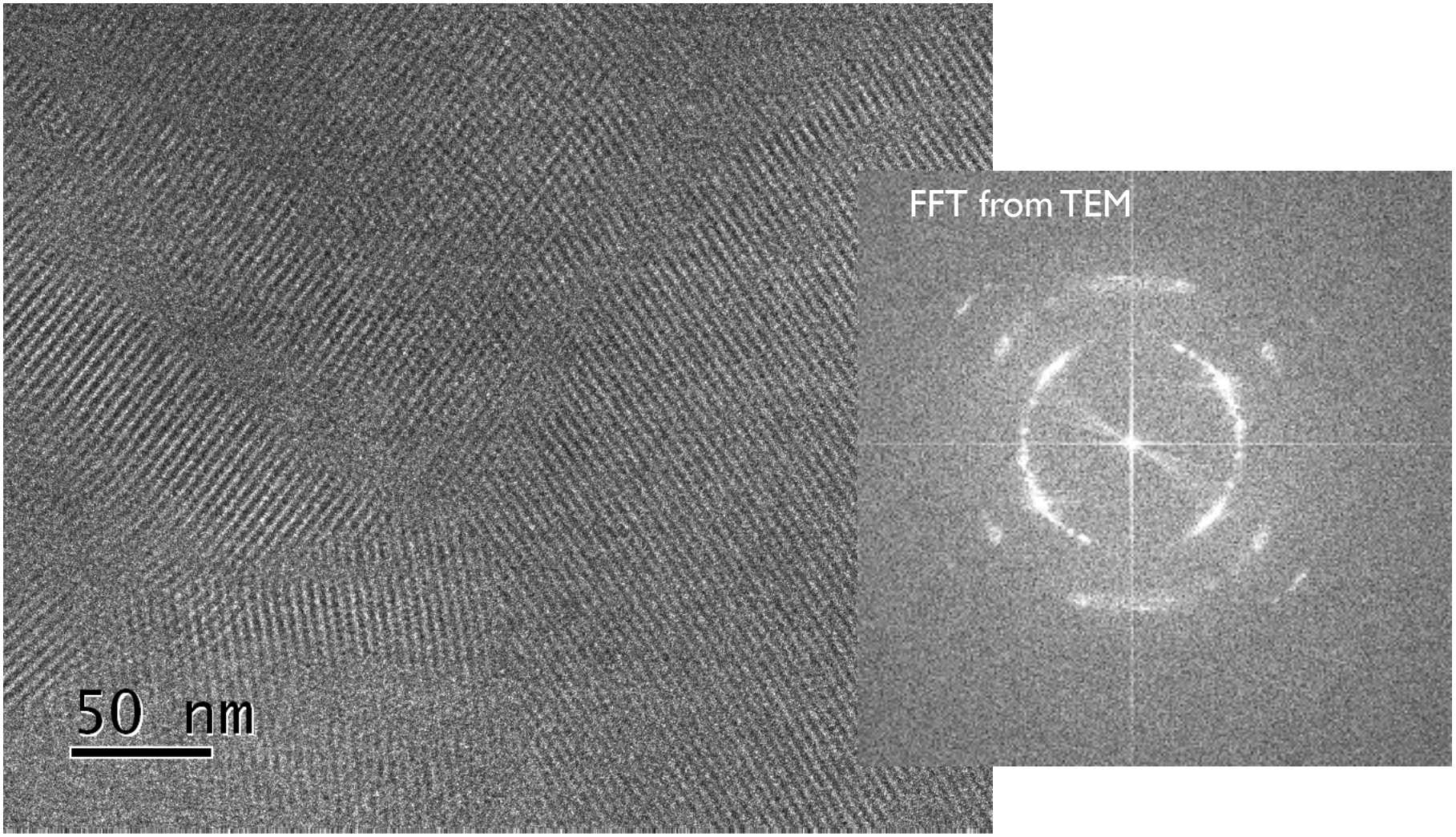
Cryo-TEM at 86 K (Liquid nitrogen cooling)

After one second

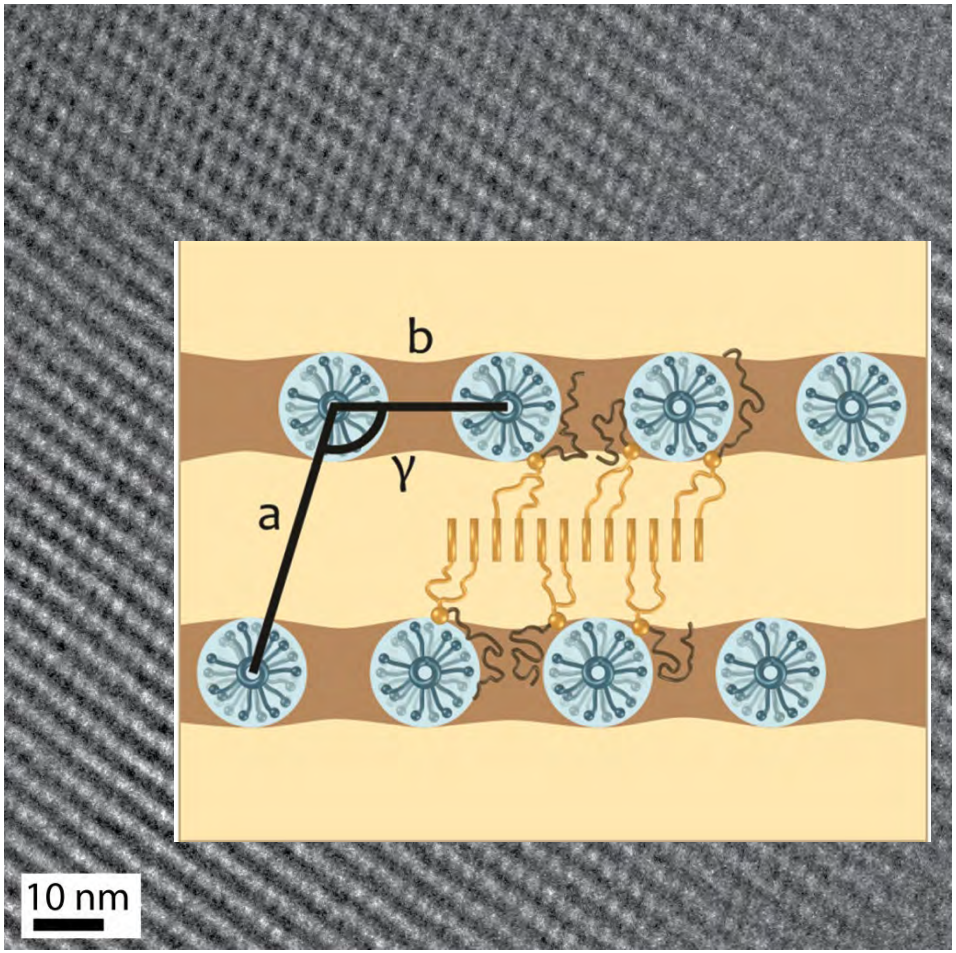
After few exposures ...

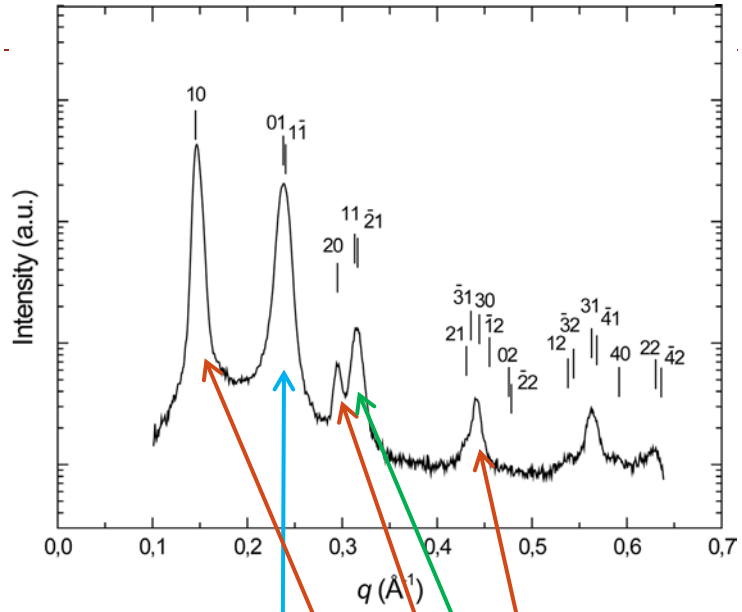


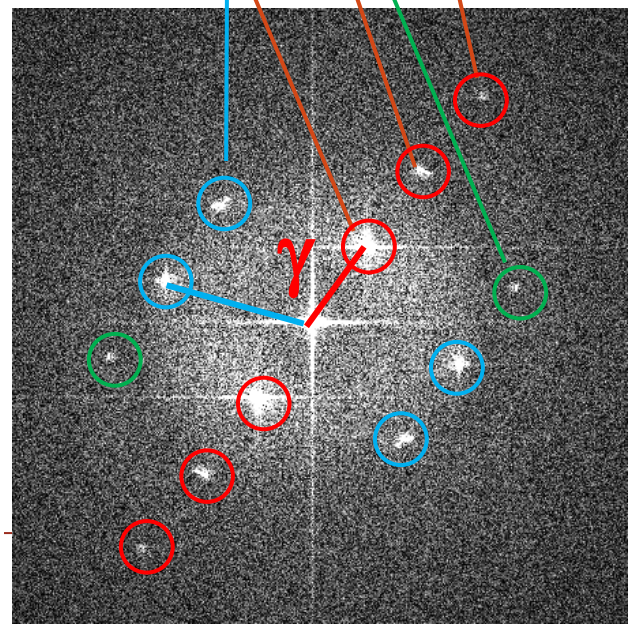
Cryo-TEM at 18 K (Liquid helium cooling)



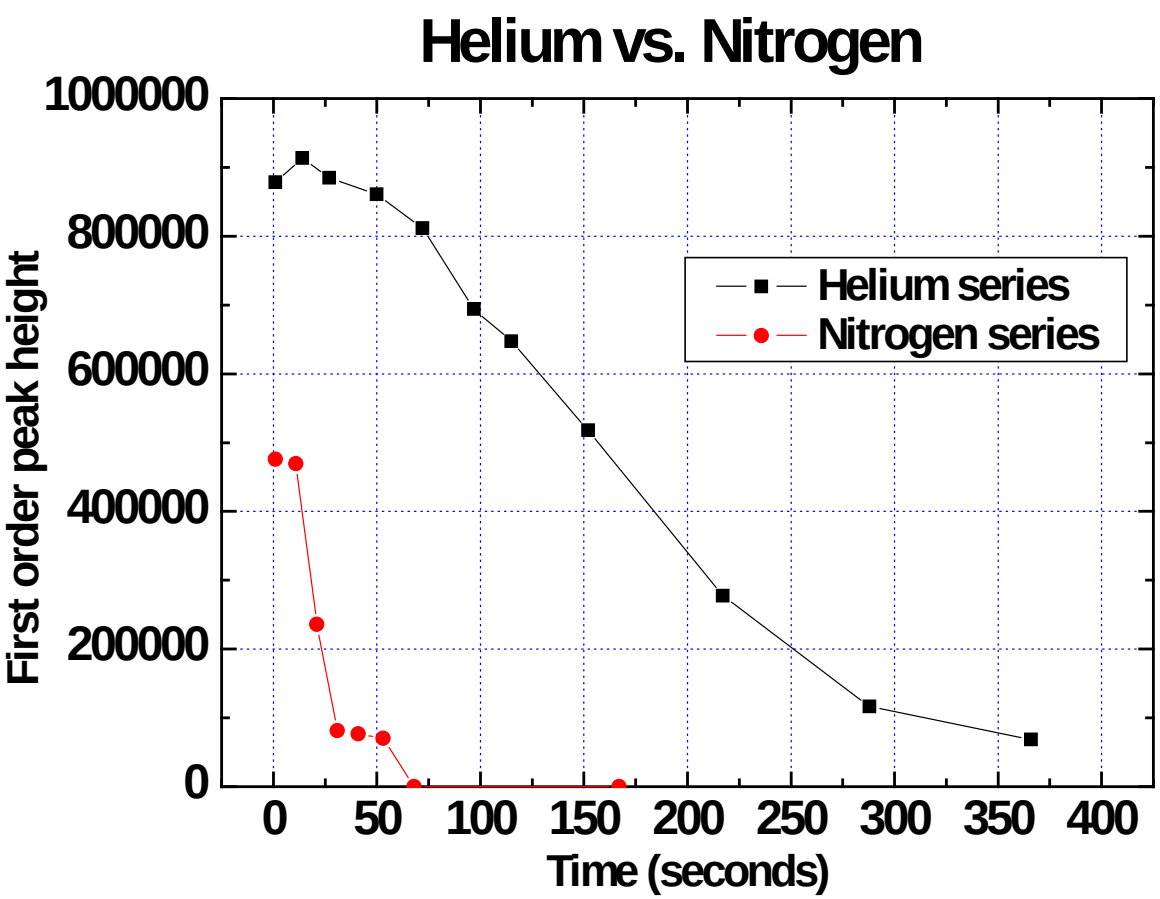
Cryo-TEM


b
γ
a
10 nm

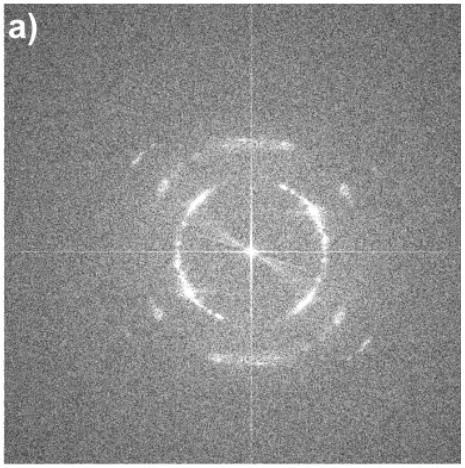

Intensity (a.u.)
10
01 1̄1
11 2̄1
20
3̄1 30
21 1̄2
02 2̄2
12 3̄2
31 4̄1
40
22 4̄2
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
q (Å⁻¹)


γ

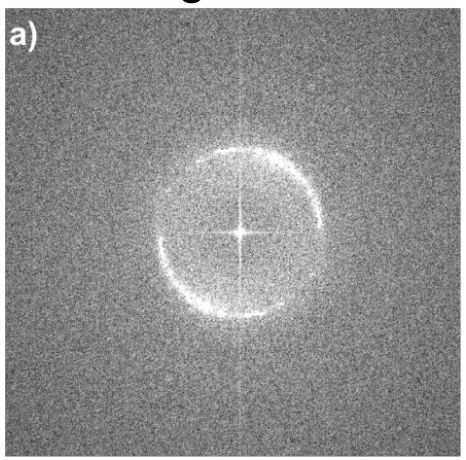
Summary: Cryo-TEM Helium vs. Nitrogen



Helium



Nitrogen

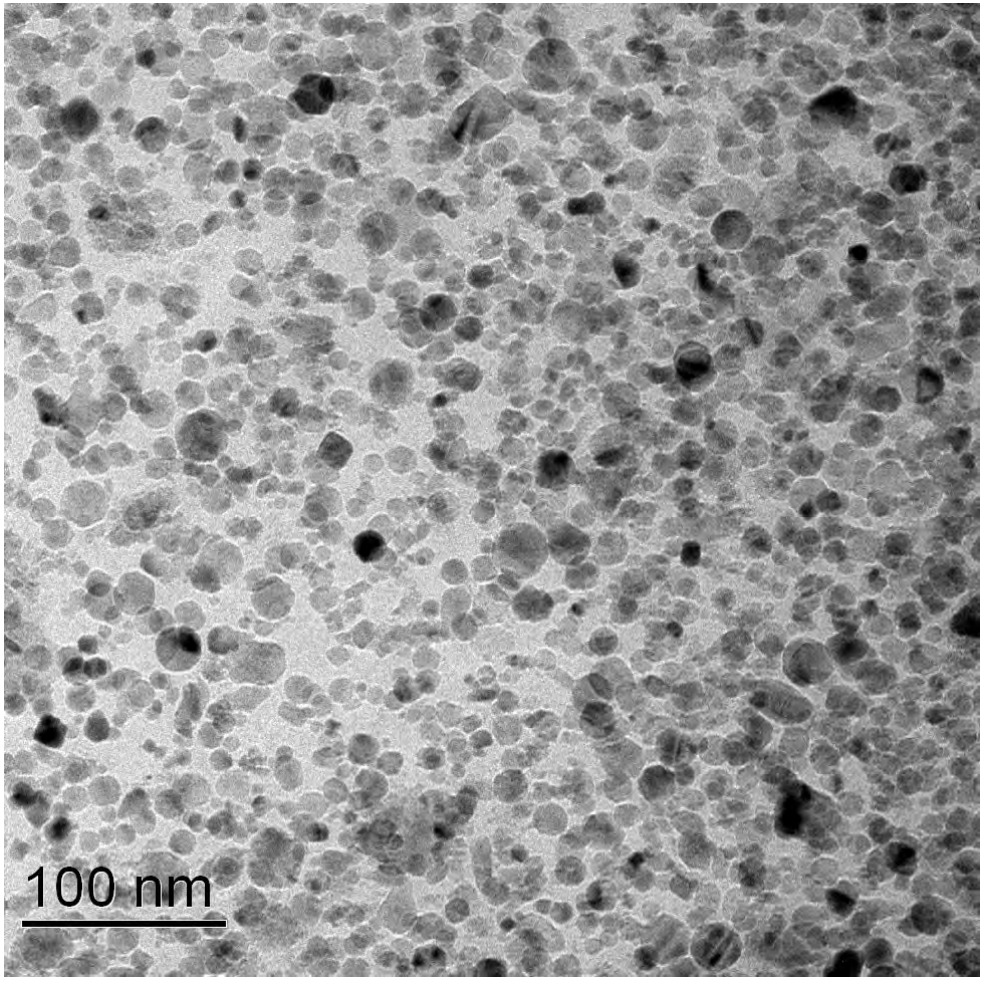


Cryo Vitrification: also other solvents possible

- Toluene
- Tetrahydrofuran (THF)
- THF/methanol mixture
- Alkohols (methanol, ethanol..)
- ..

Example: Nanoparticles in solution (organic solvent)

How stable is the solution – is there any aggregation?



•Cryo electron microscopy by direct vitrification of solutions –

Benefit: There is no need to evaporate solvent – which may induce aggregation – but solvent is just vitrified in amorphous state.

Cryo electron microscopy image after solution vitrification

Example: rod-coil block copolymer aggregation in THF/methanol mixtures