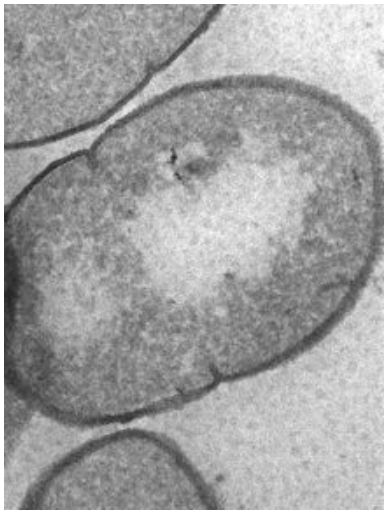
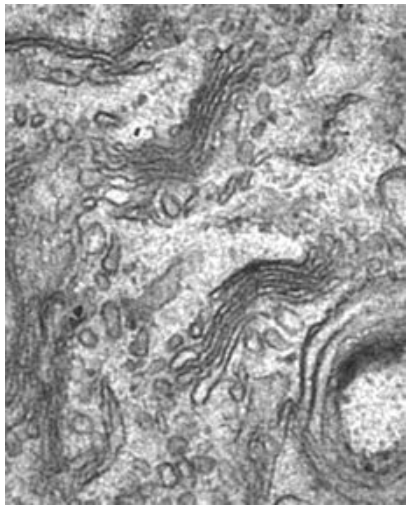
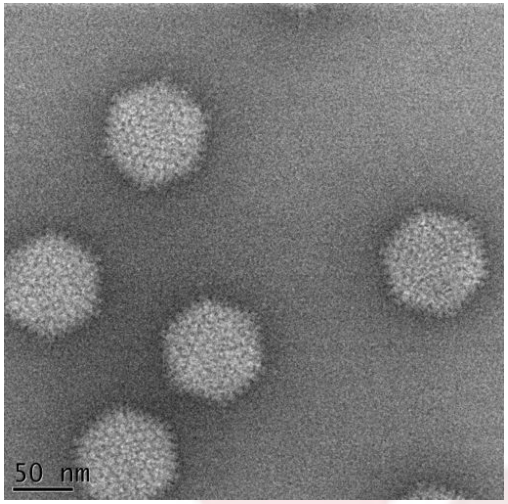
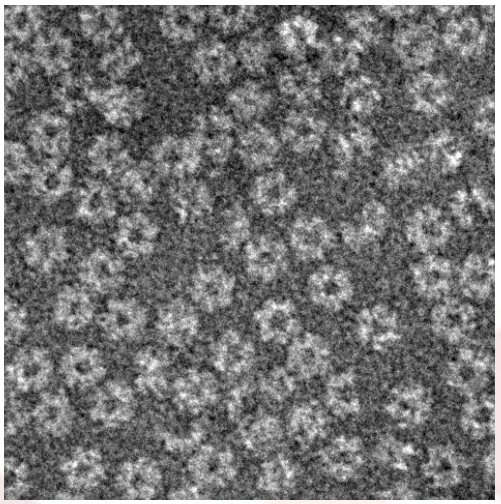
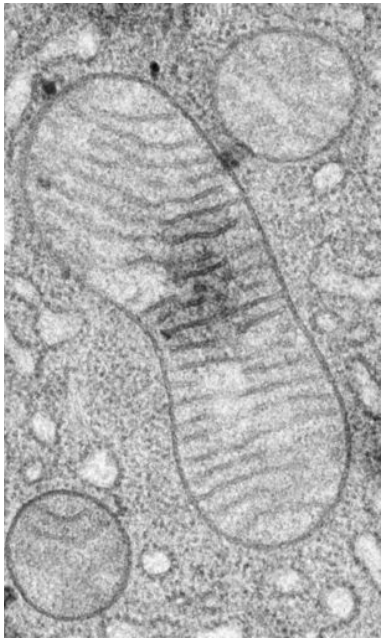
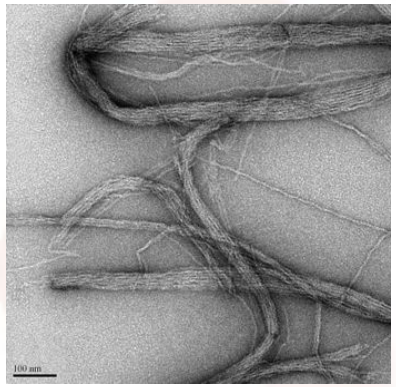
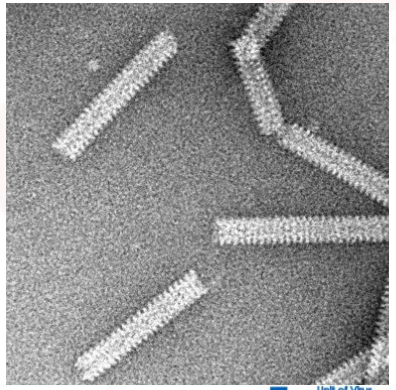




Sample preparation for Structural studies by EM

RéNaFoBiS Réseau National de Formation en Biologie Structurale Intégrative

Guy Schoehn IBS/UVHCI/ISBG
Guy.schoehn@ibs.fr

ibs Institut de Biologie Structurale

EM platform Partnership for Structural Biology

Oléron 2015

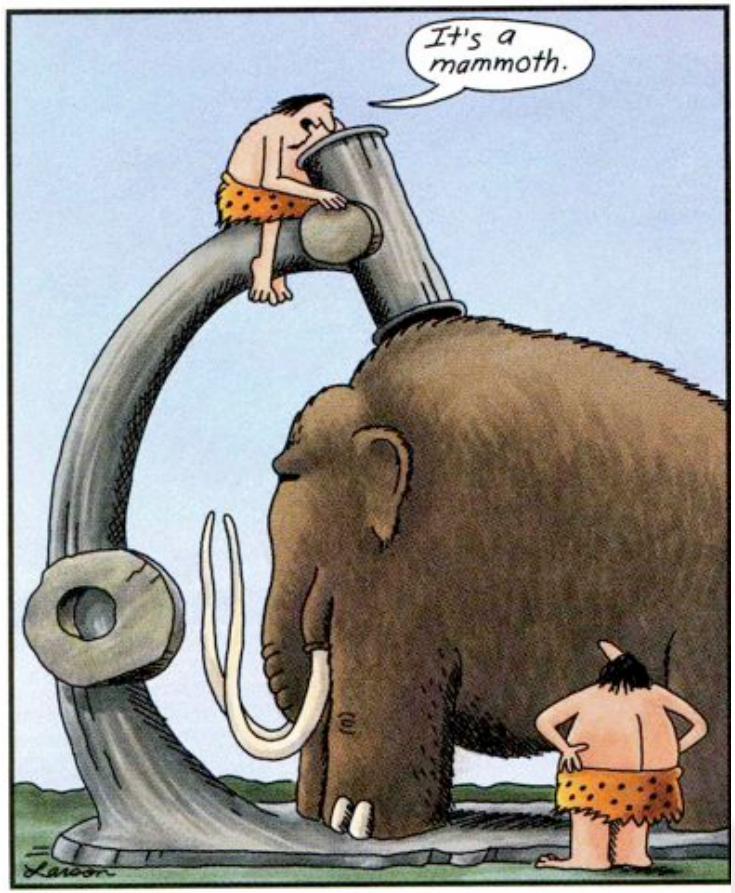
uvhci Unit of Virus Host Cell Interaction

Preparation of biological samples for electron microscopy

They have to:
- resist to the vacuum and to the electrons
- stay in their native form

Preparation of biological samples for electron microscopy

They have to: - being thin enough



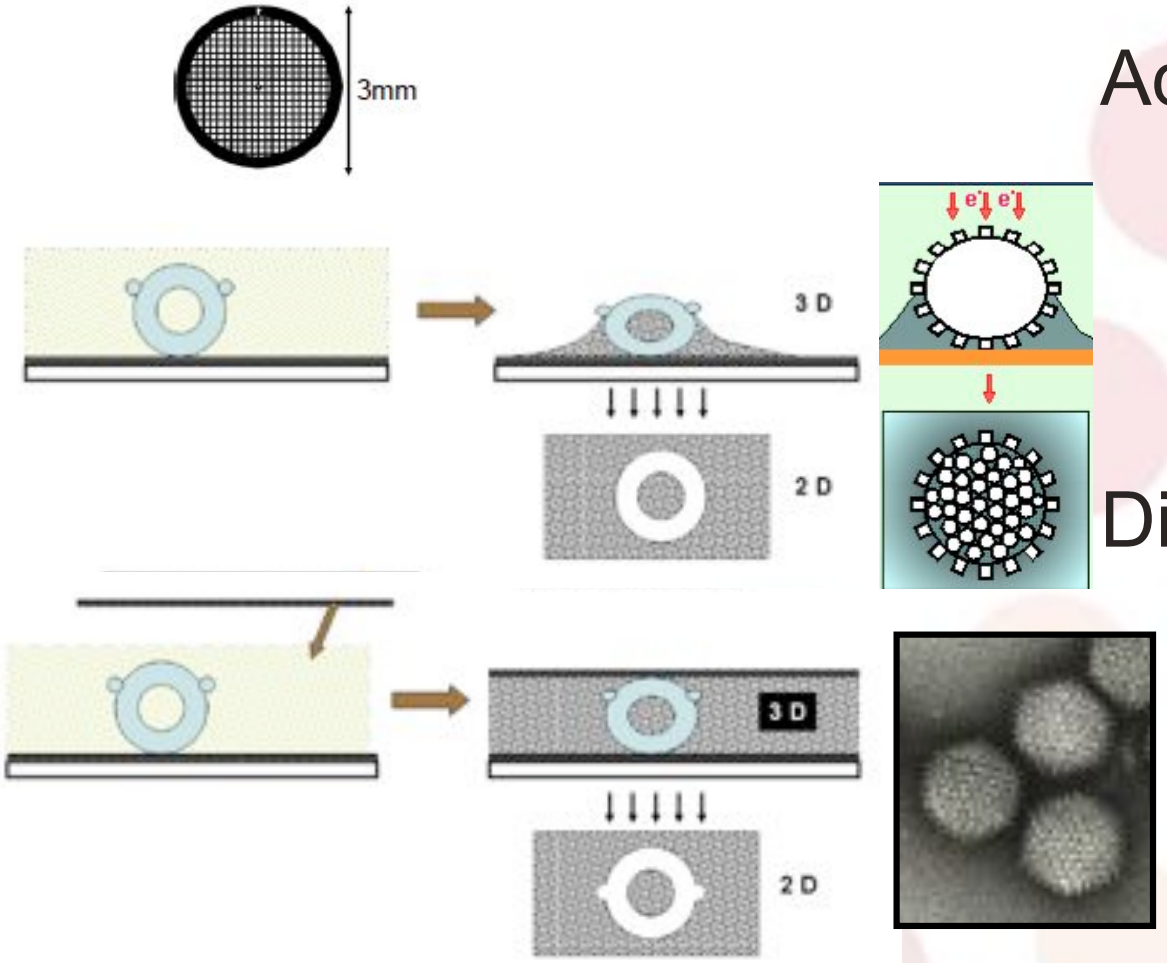
Early microscope

Thin sample

- Negative staining
- Cryo-electron microscopy
- Metal shadowing

The two mains techniques

Negative staining



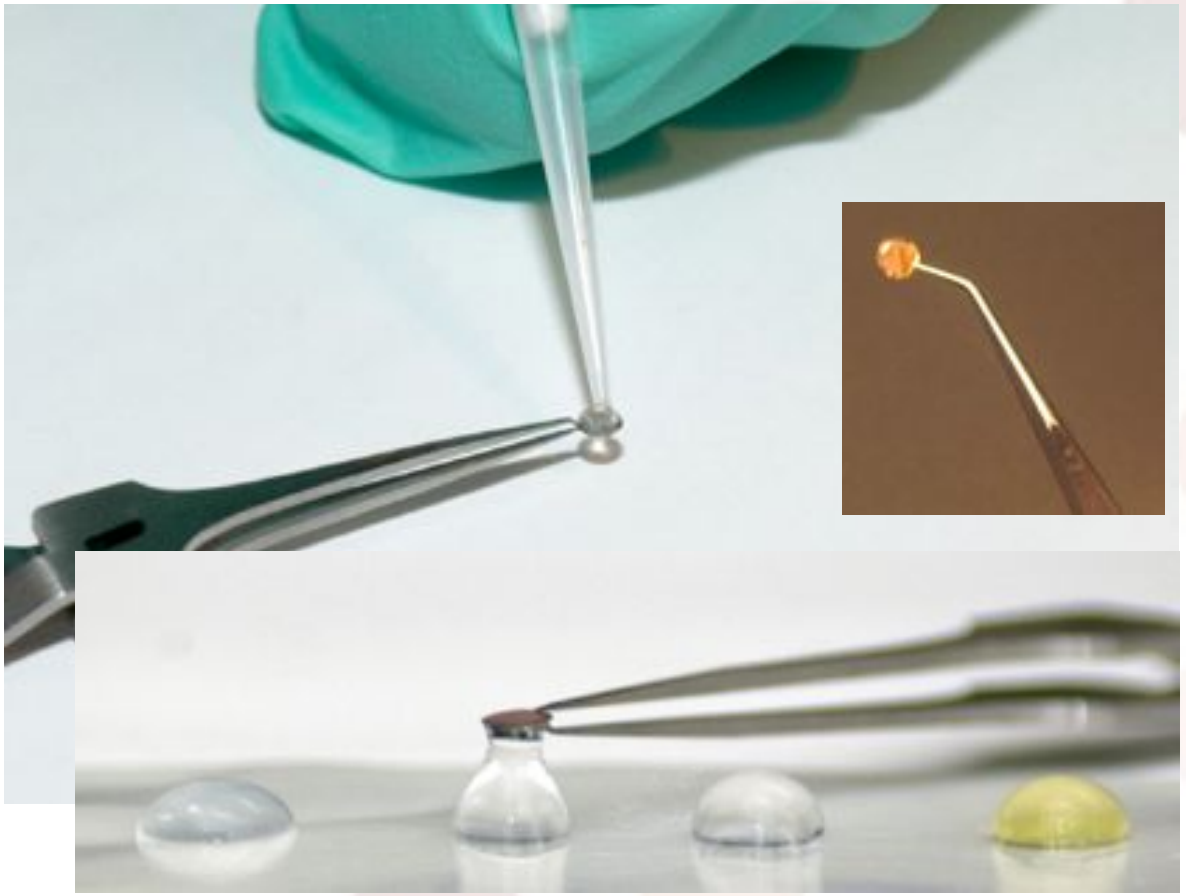
Advantages :

- Fast
- Small amount of protein (concentration 0.01-0.1mg/ml, few μl)
- Small proteins are visible
- High contrast

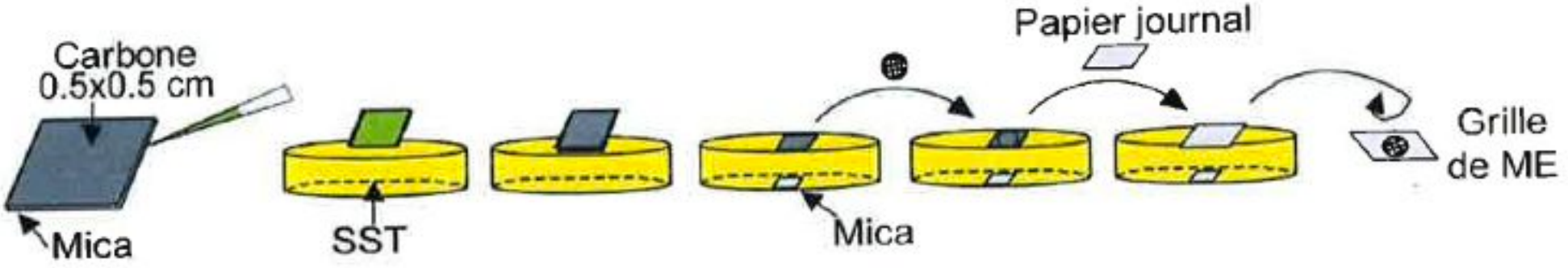
Disadvantages :

- Artefacts due to the stain
- Flattening and drying
- Footprint of the sample in the heavy atom
- Low resolution (15 Å)

Preparation method



Preparation method



The different stains

Stains:
Uranyl formate (finer grain, but unstable)
Uranyl acetate (coarser grain but stable)

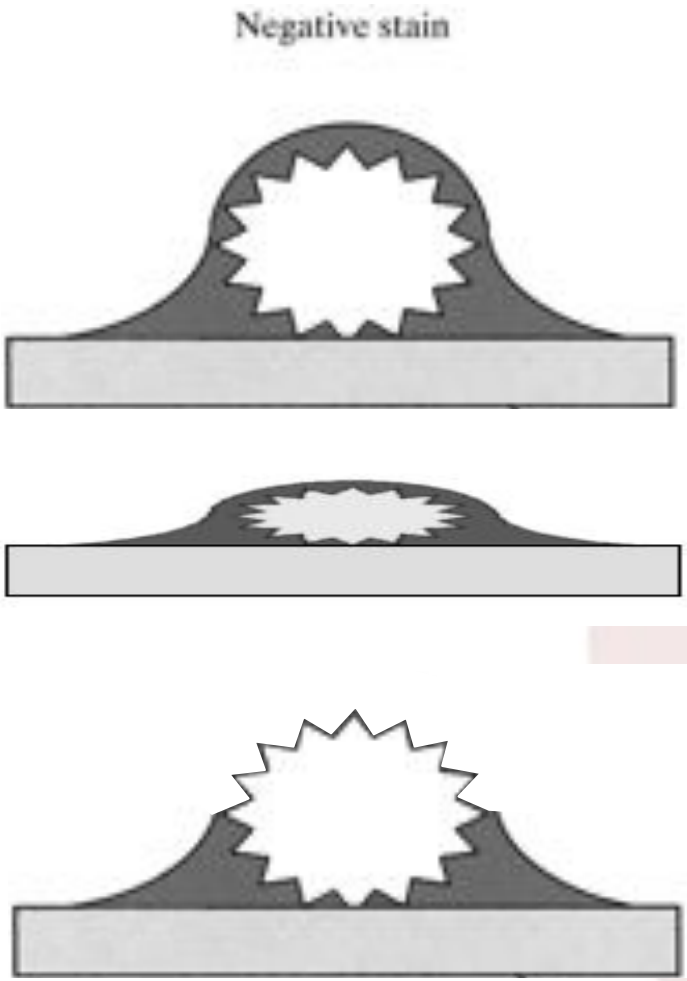
- Higher contrast and radiation resistant
- but acidic (pH 4)
- It also function as fixative !

Sodium silicotungstate
Sodium phosphotungstate (thin layer of stain)
Ammonium molybdate (thick layer of stain)
Aurothioglucose

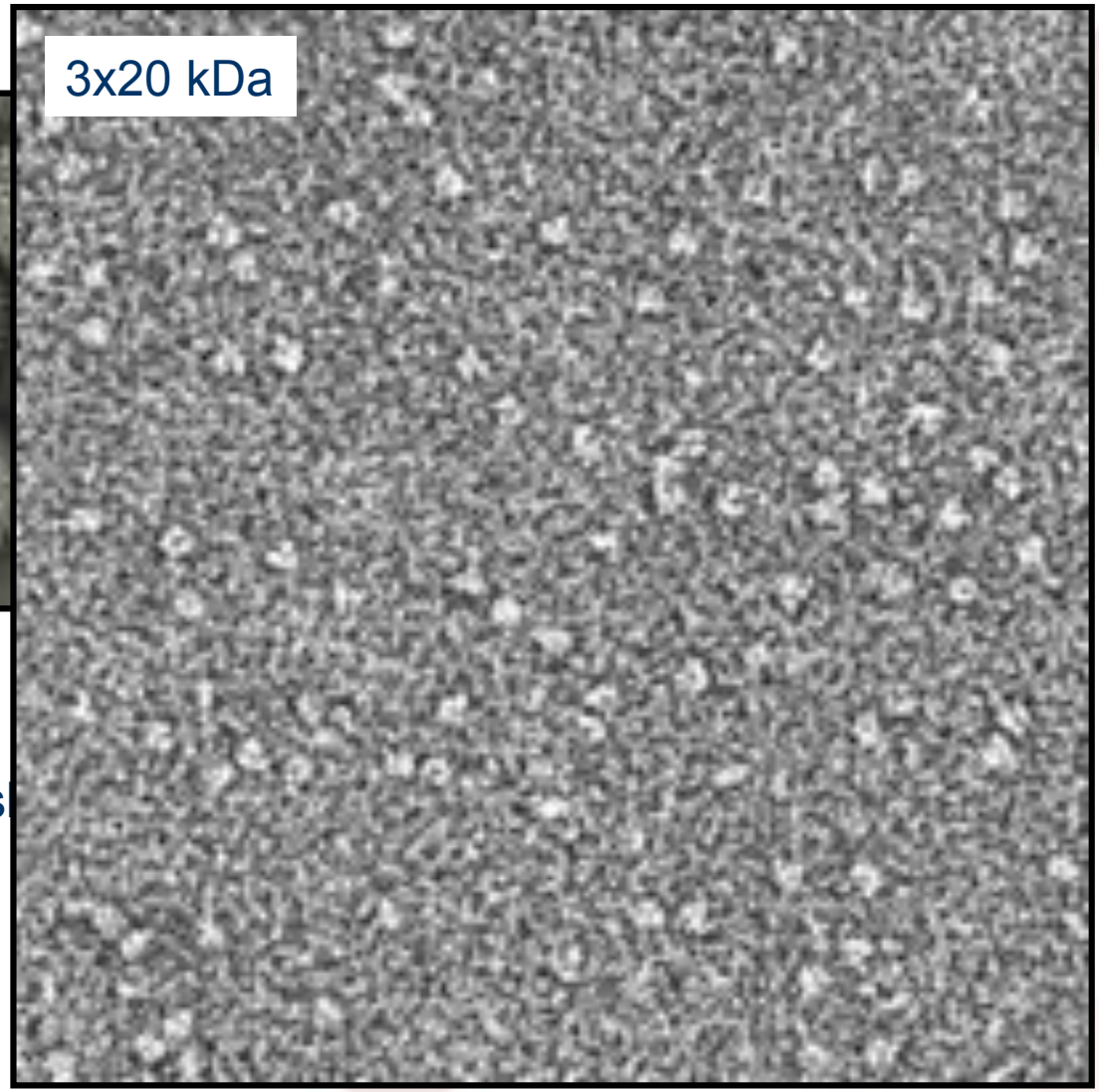
- - neutral pH
- - but lower contrast and less radiation-resistant

Ohi et al., 2004 Biol. Proced. Online 6: 23-34

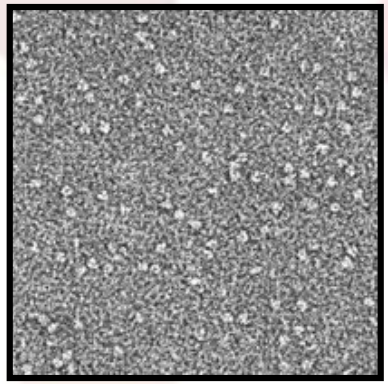
What we obtain in NS



Examples of negatively stained samples

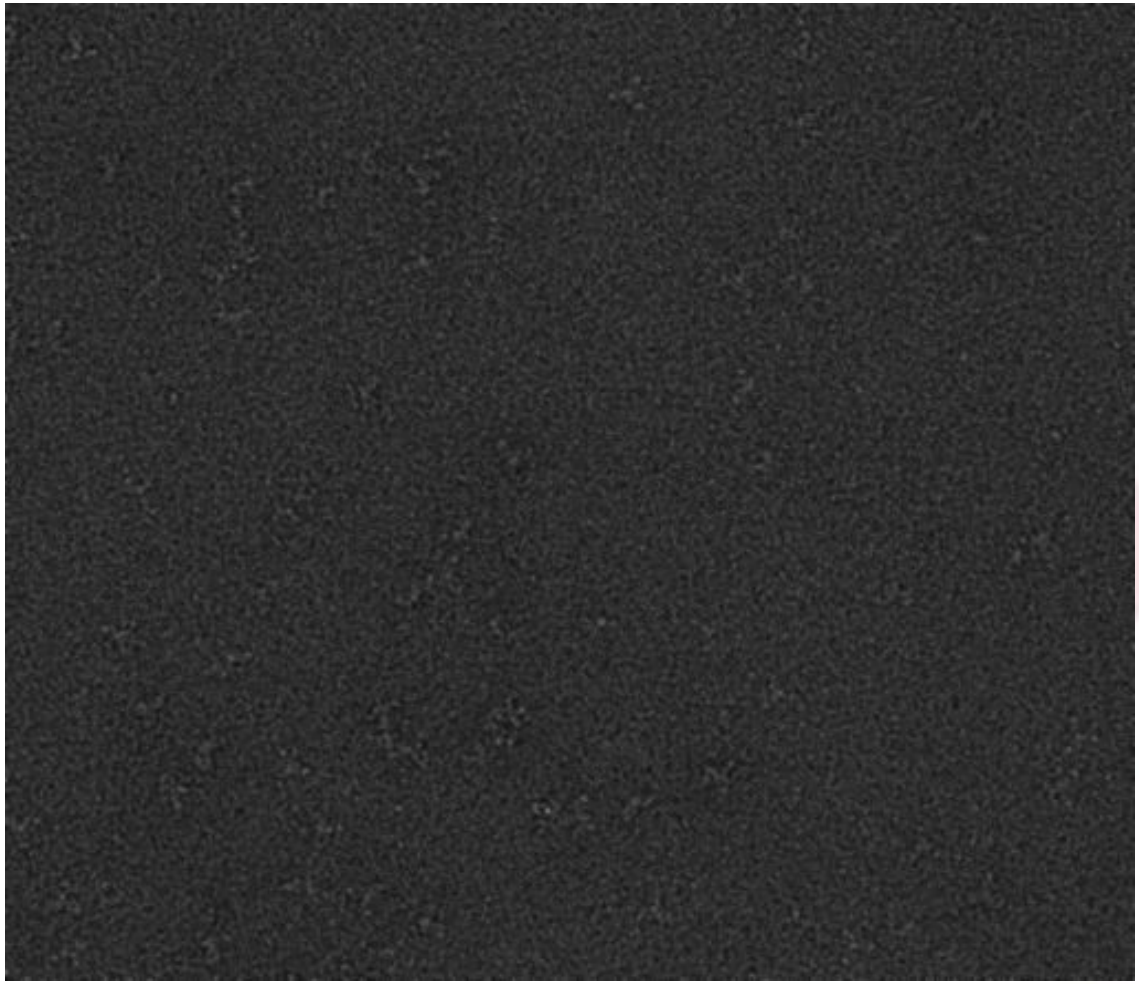


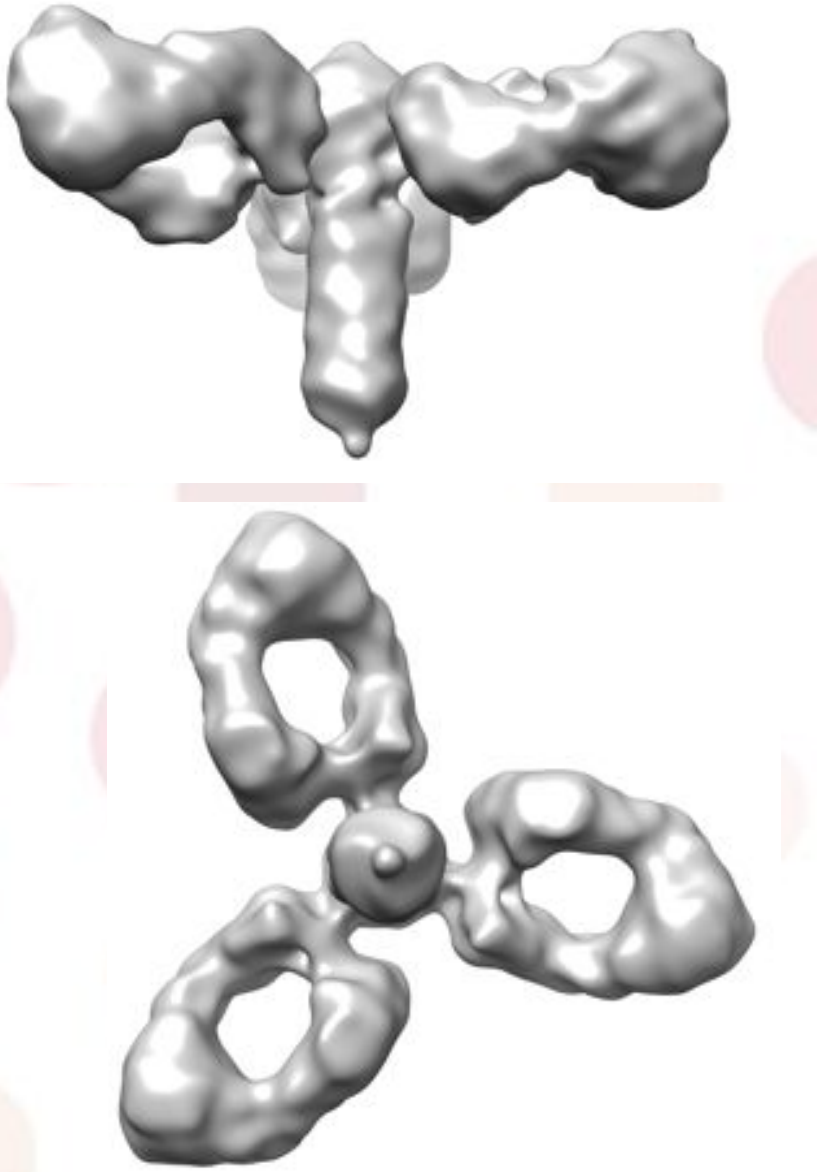
Measl
Pitch



Tetrahedral
aminopeptidase
480 kDa

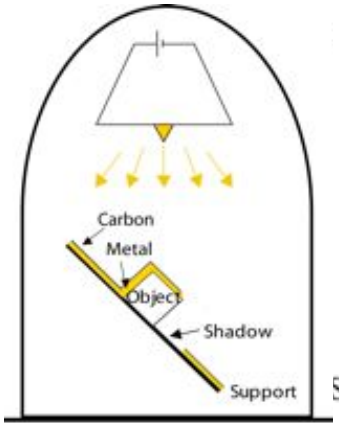
Negative staining

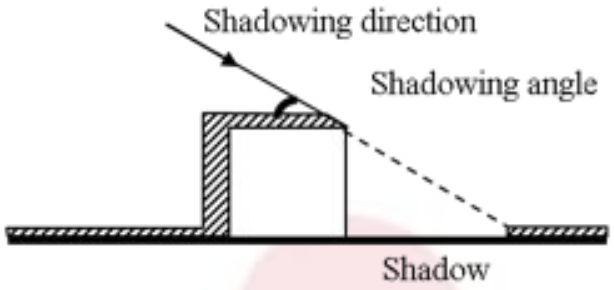


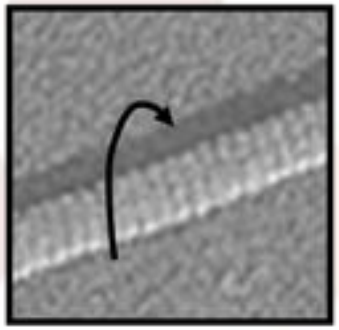


Still very usefull prior to cryo EM in most of the cases

Shadowing

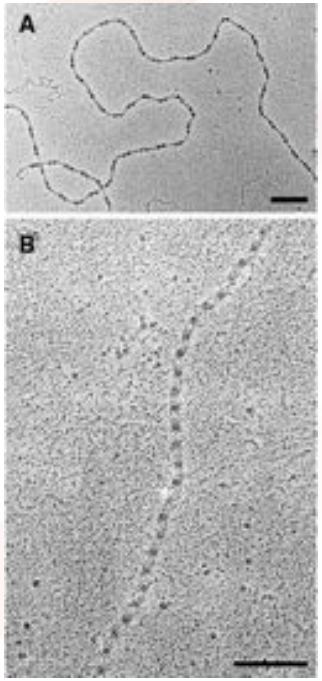






- Aim :
  - Visualize the surface of the particles
  - Handiness determination

- Technique :
  - Heavy atom evaporation
  - The sample is tilted in the evaporator
  - The tilted sample can also be rotated



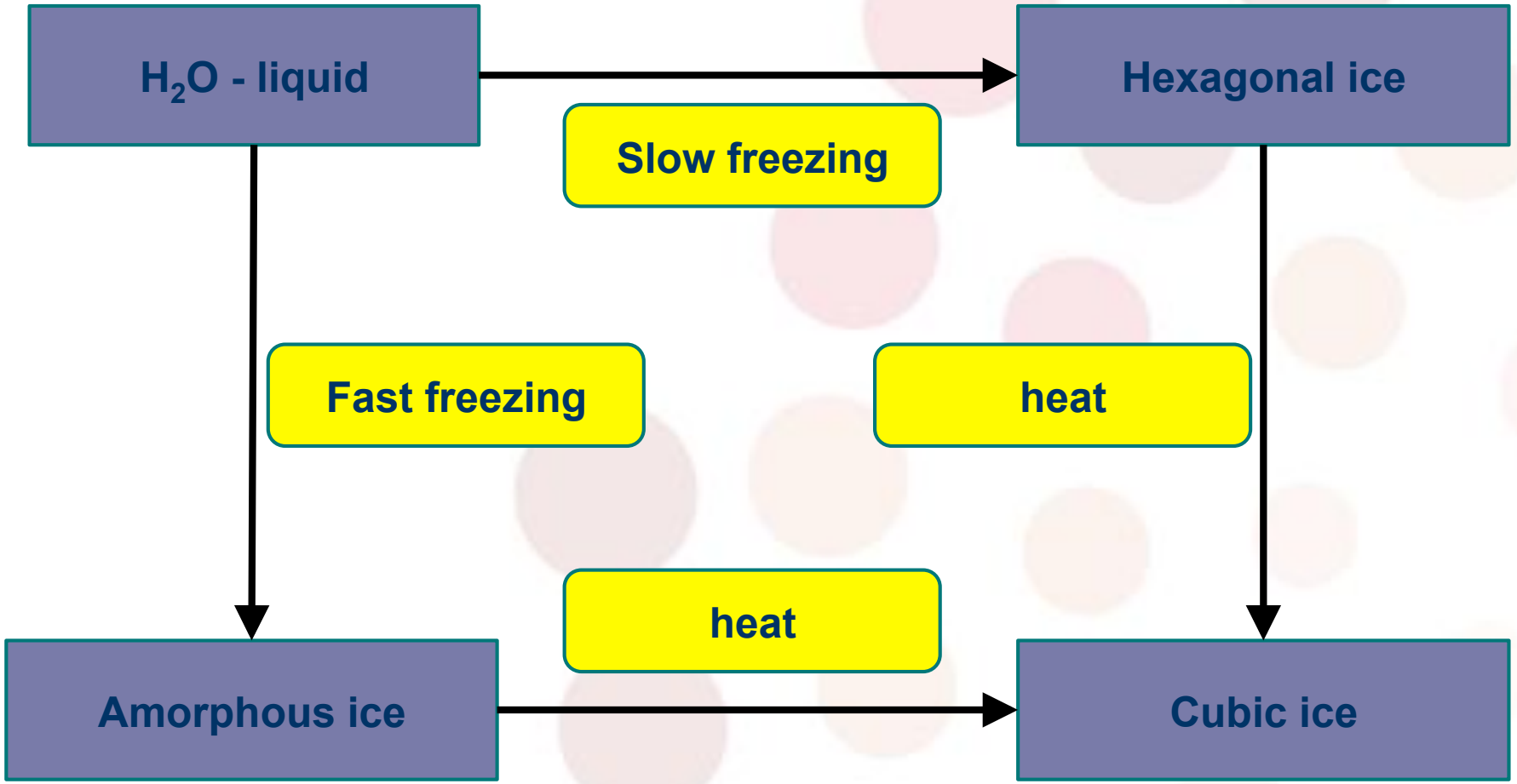
Negative staining and shadowing

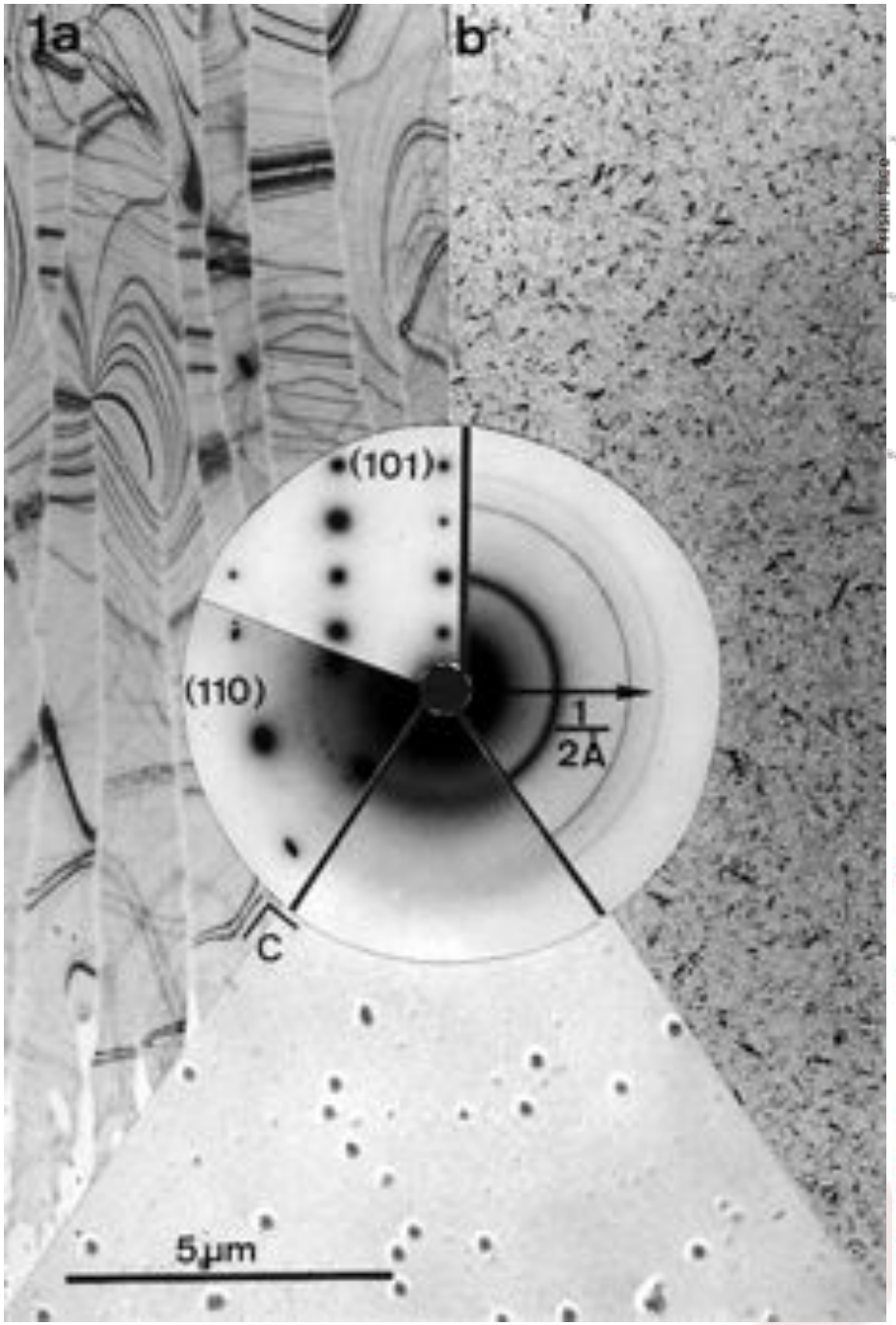
What we obtain

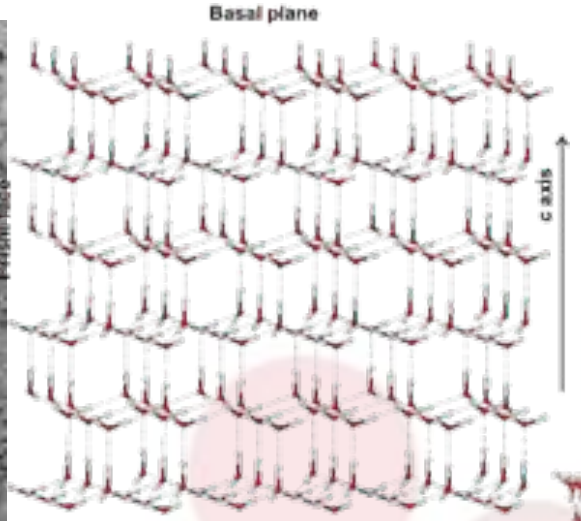
What we would like to have

For real structural work one need something else

Vitrification





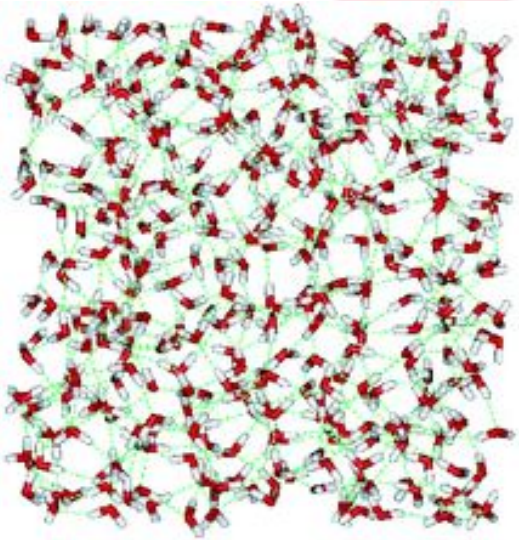


Dubochet et al., (1982)

a)

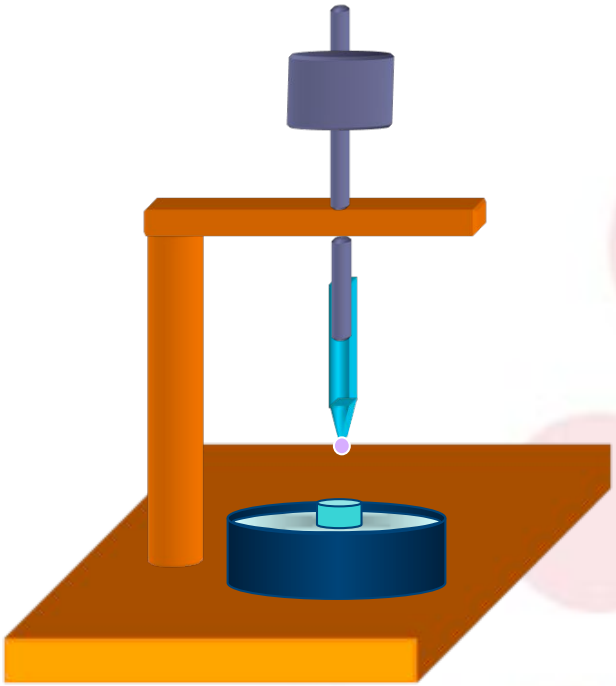
b)

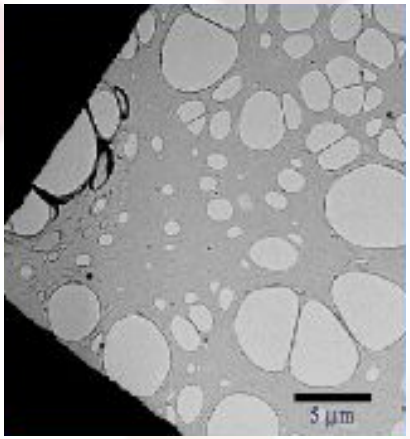
c)

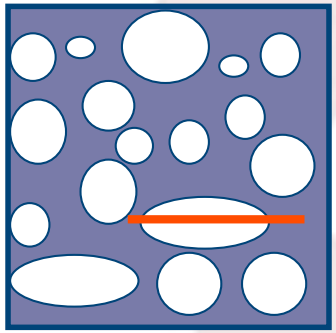


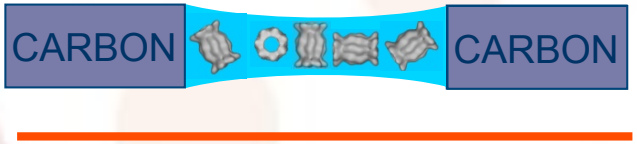
a) hexagonal b) cubic c) amorphous

Cryo Electron Microscopy

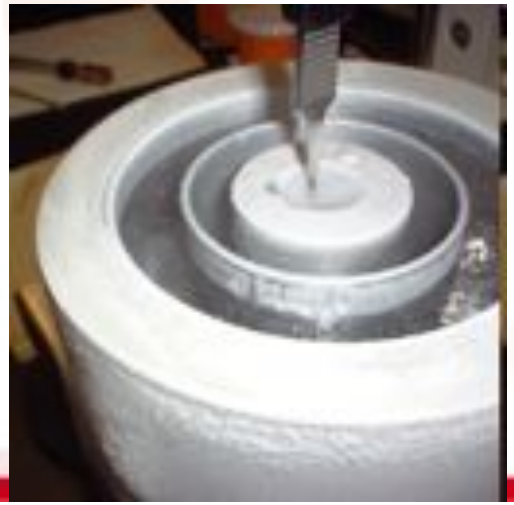




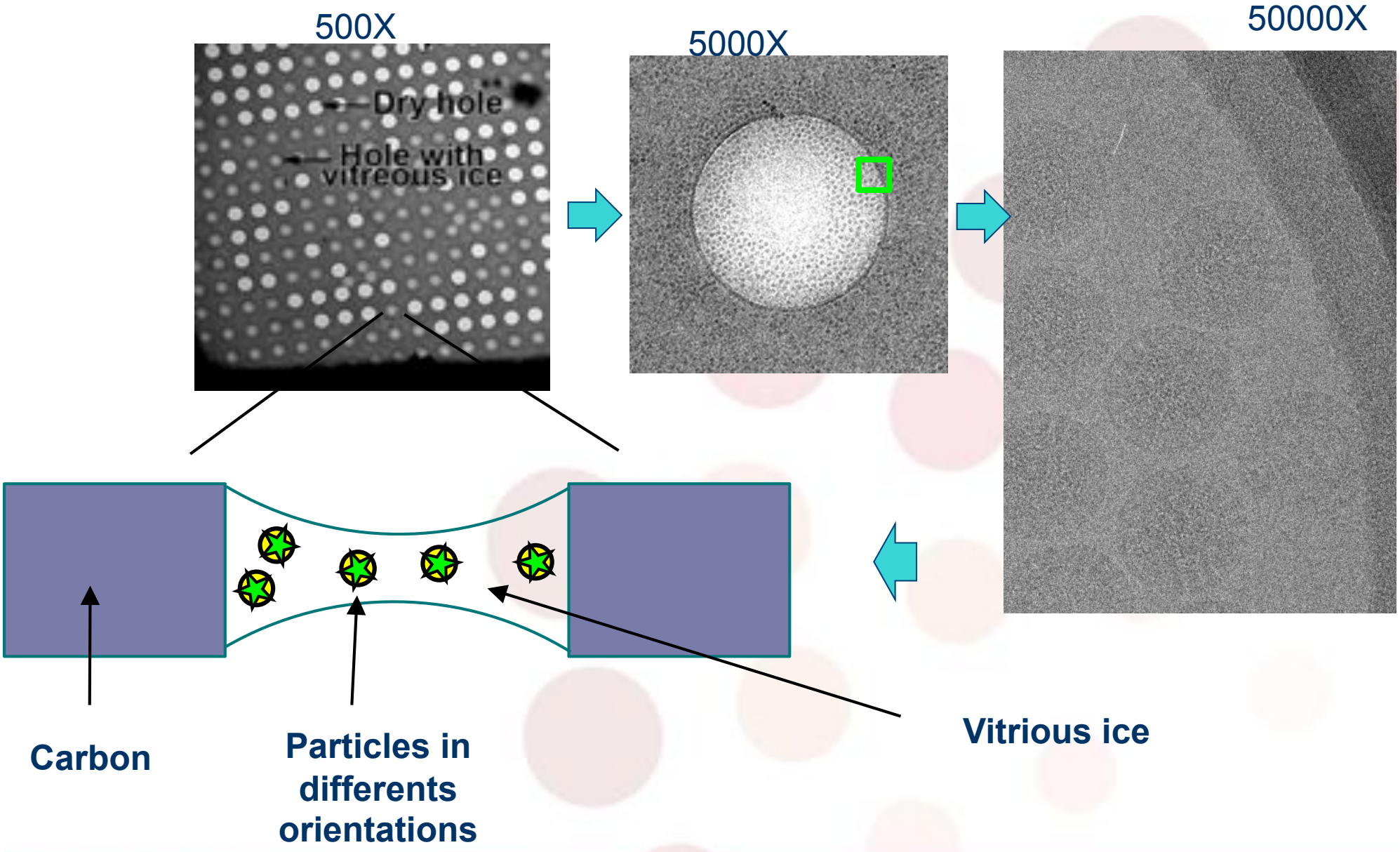




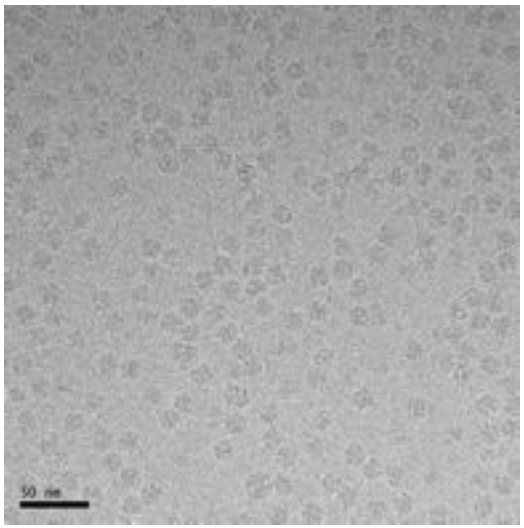
The freezing instrument

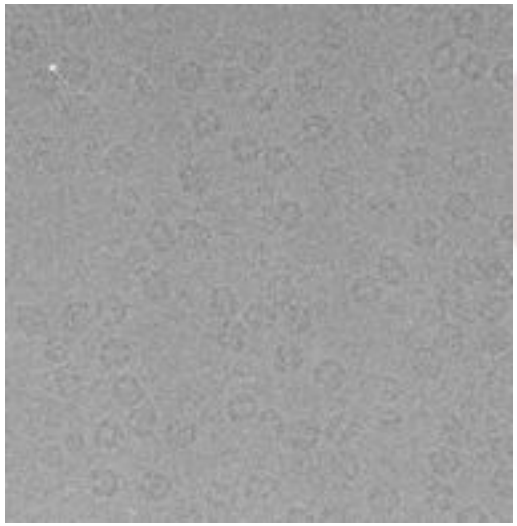


How does a frozen grid look like?



Cryo: advantages / disadvantages



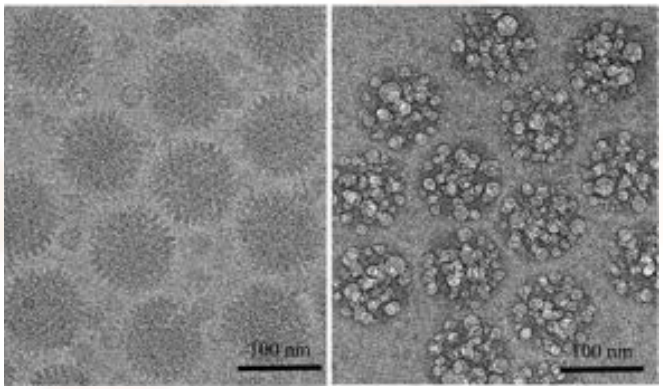


Advantages :

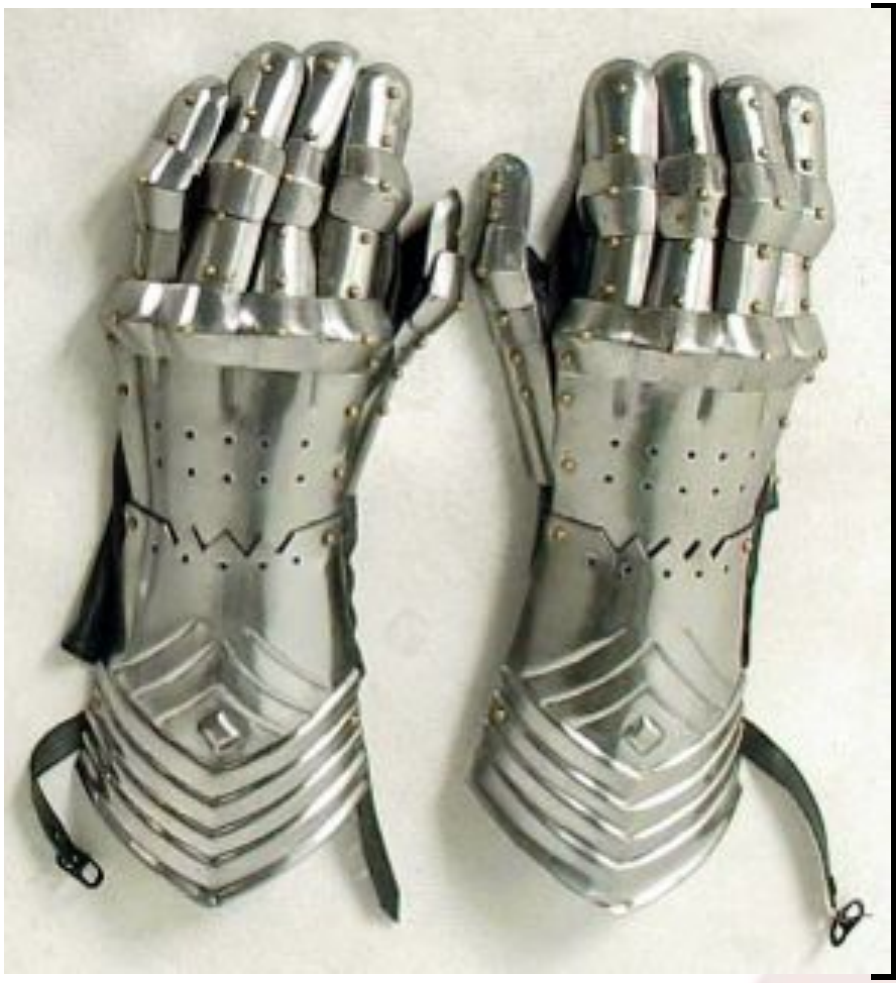
- Native state of the sample
- High resolution
- Small amount of sample

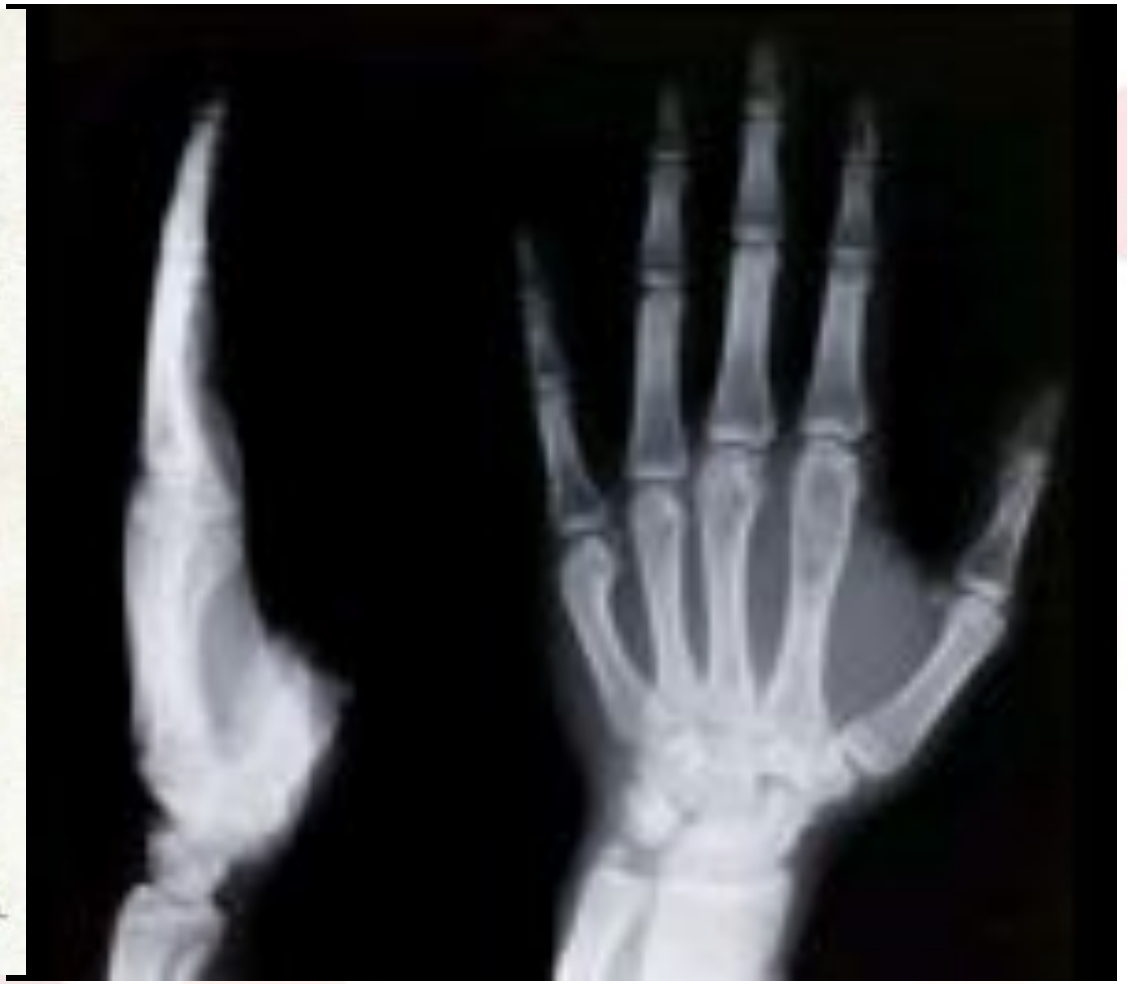
Disadvantages :

- Low contrast
- High sensitivity against electrons
- Size of the sample
- More expensive

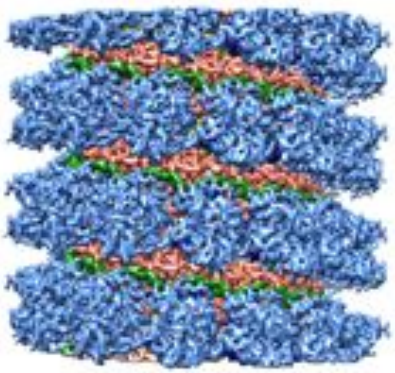


Negative staining vs. cryo

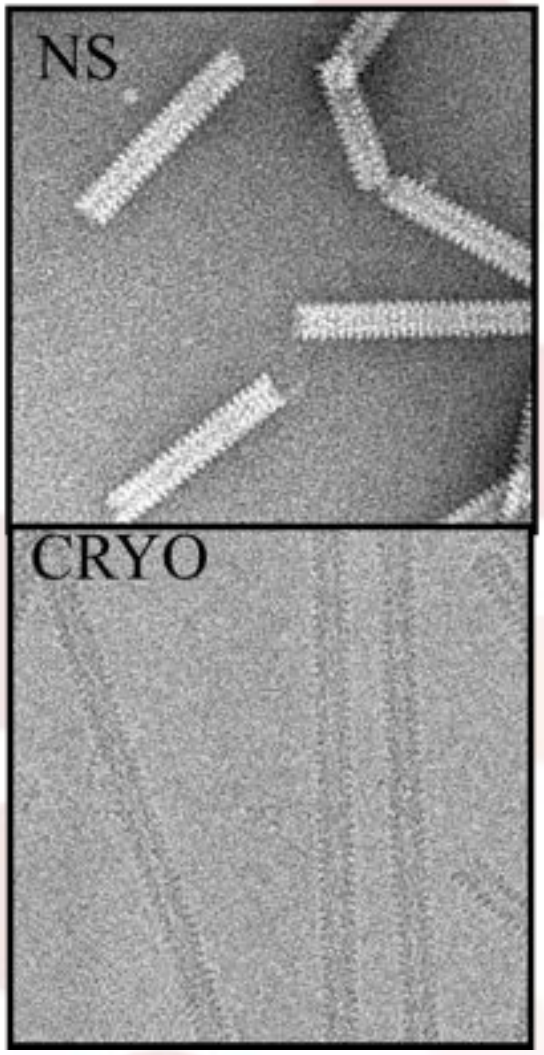


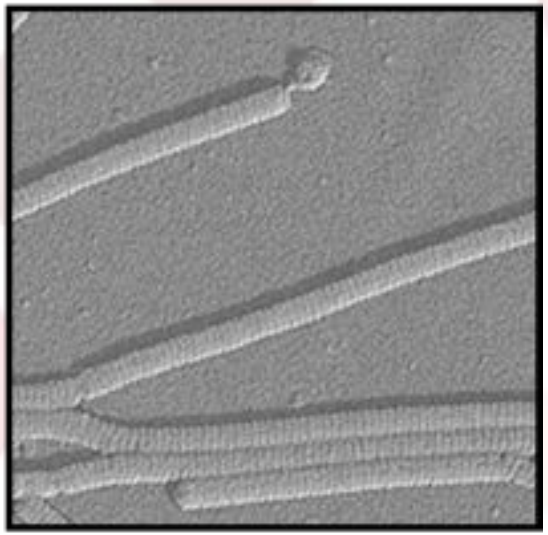


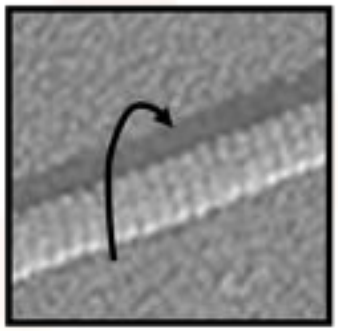
Comparison between negative staining, cryo and shadowing



4.3 A, Gutsche et al.,Science 2015







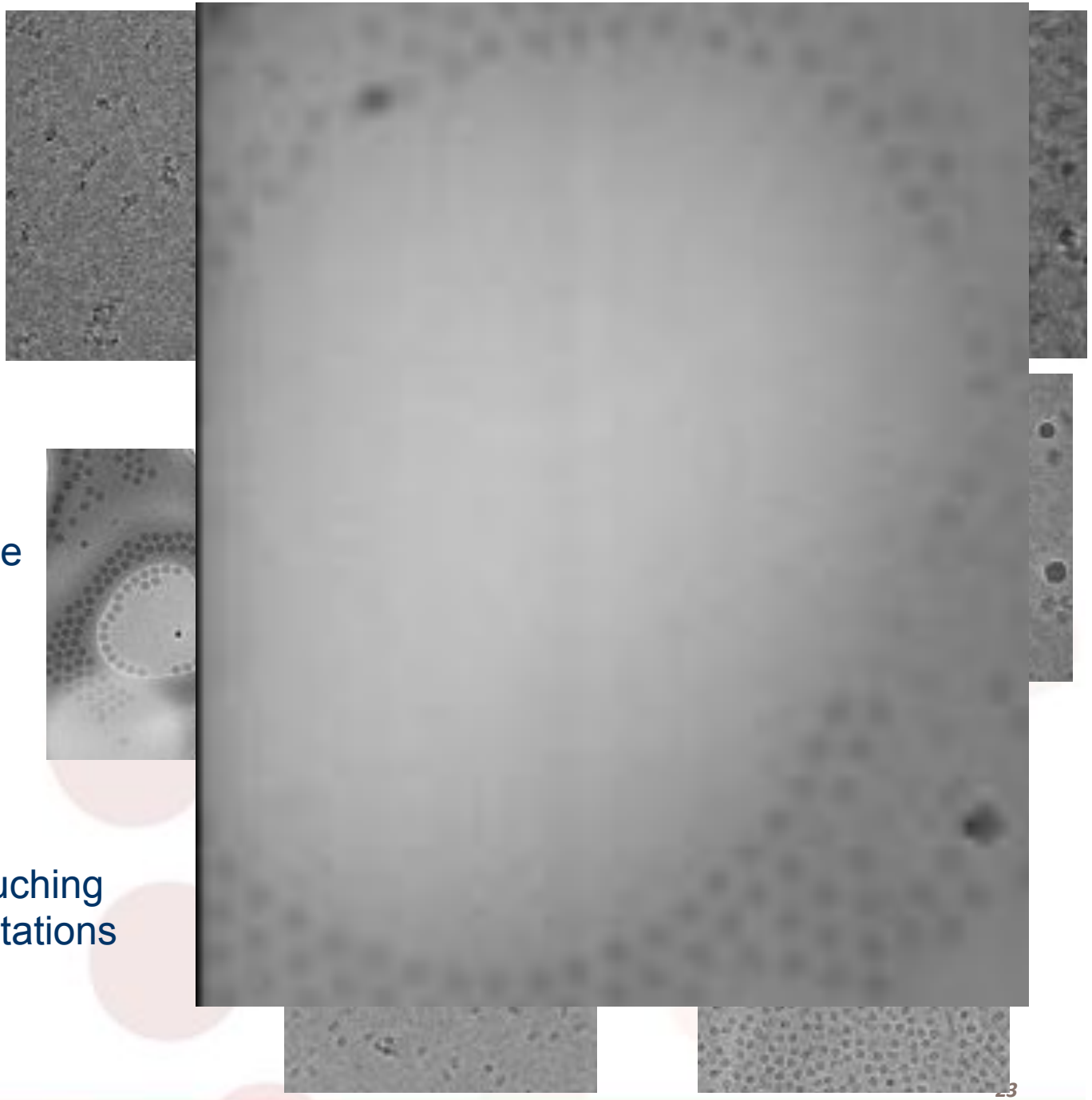
What is a good cryo grid?

Good amorphous ice
-not crystalline ice
-no « leopard skin »pattern
-no contamination

Appropriate ice thickness
-typically as thin as possible

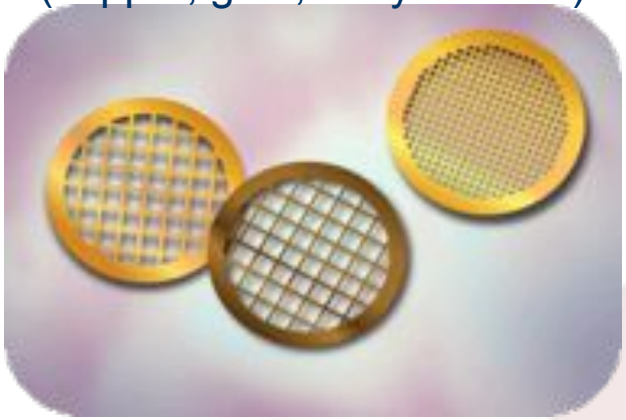
Clearly visible particles

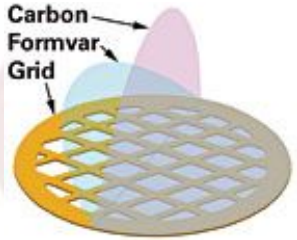
Good particle distribution
-in holes
-dense but particles not touching
-randomly distributed orientations



Variables in grid preparation

EM grid
(copper, gold, molybdenum)



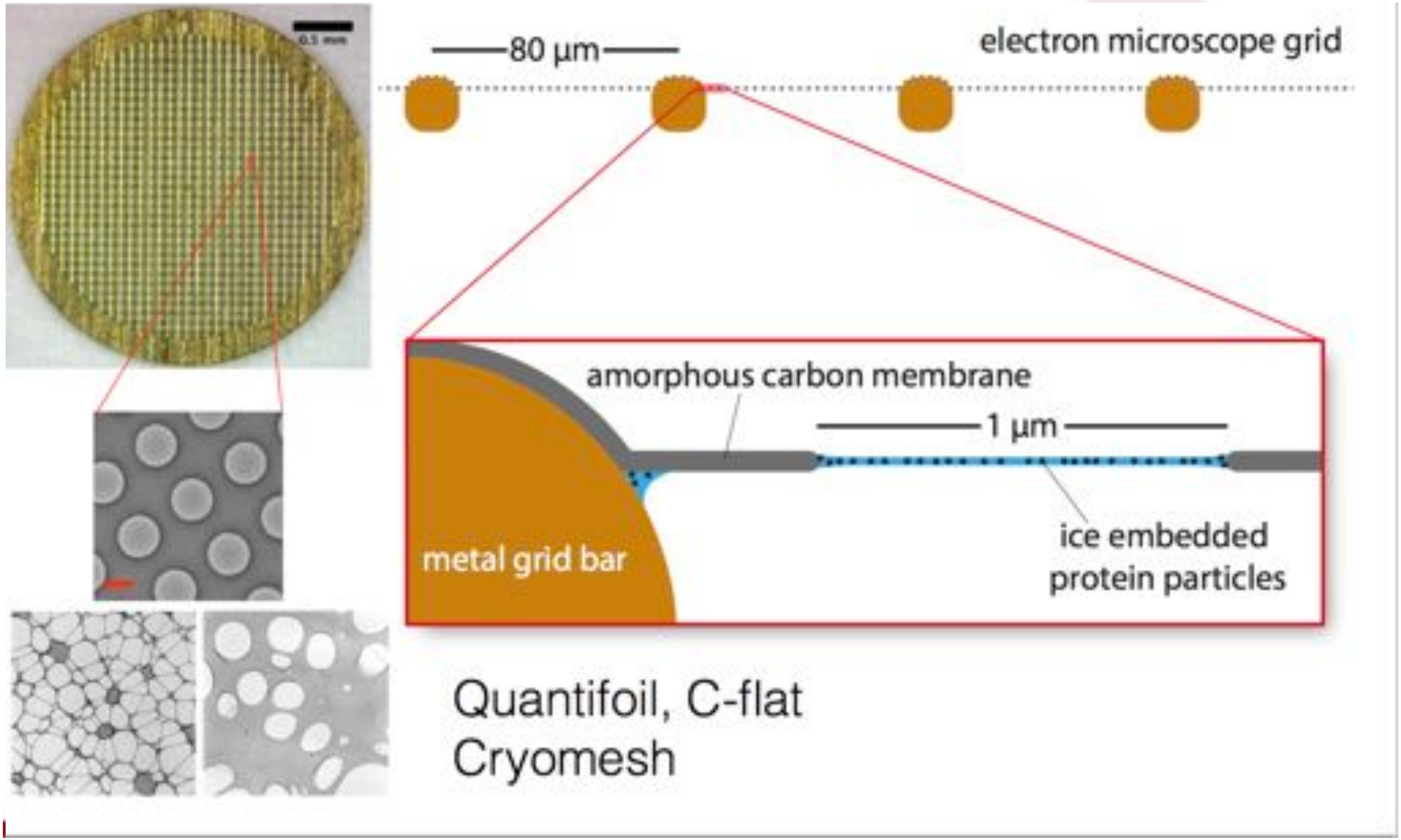


Grid
-type of grid and substrat
-batch and age of grid
-glow discharging

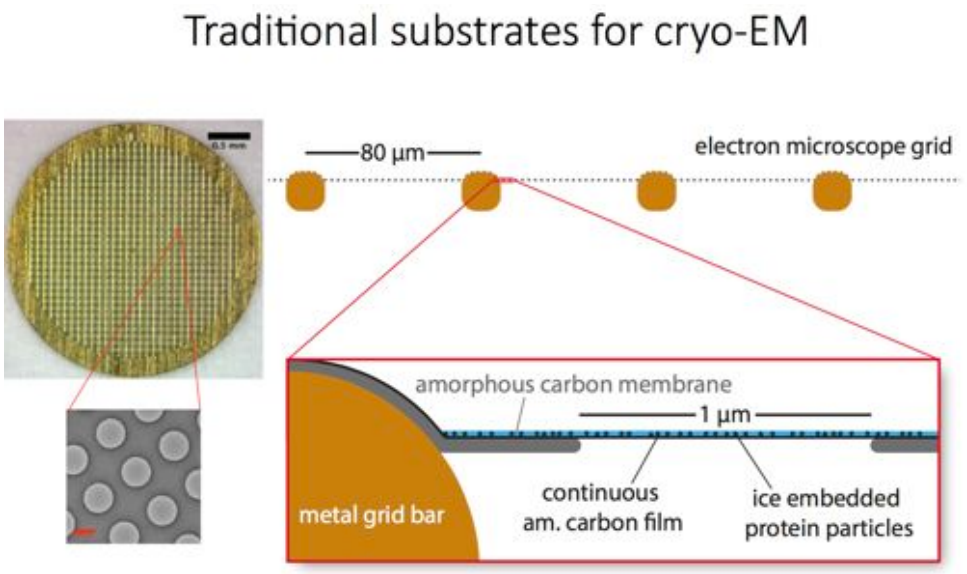
Sample
-concentration
-buffer composition
-detergent

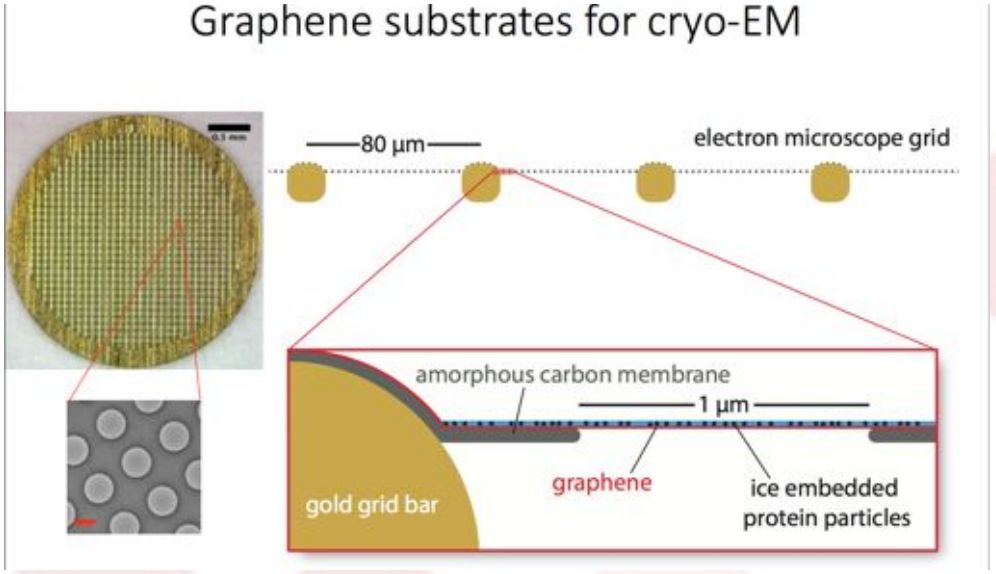
Freezing
-blotting time
-single or double side blotting
-waiting time
-multiple sample application

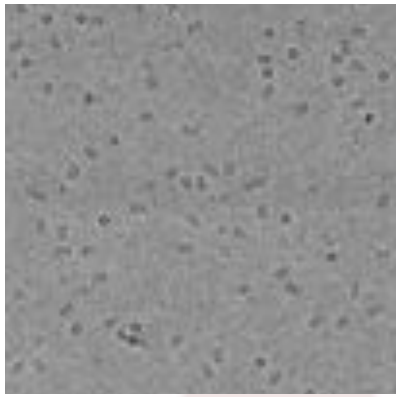
The different substrats on grid

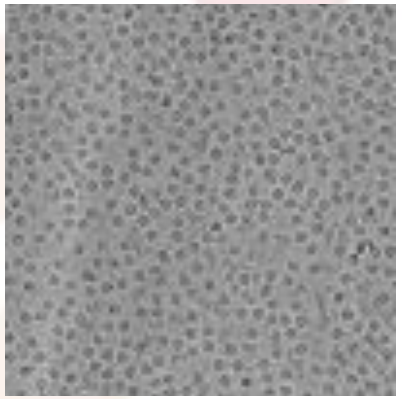


The different substrats on grid

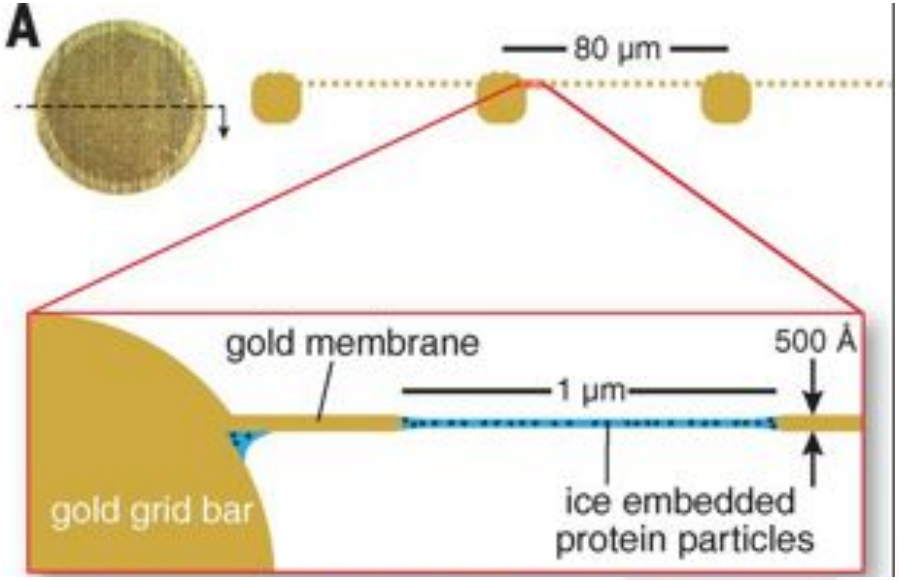


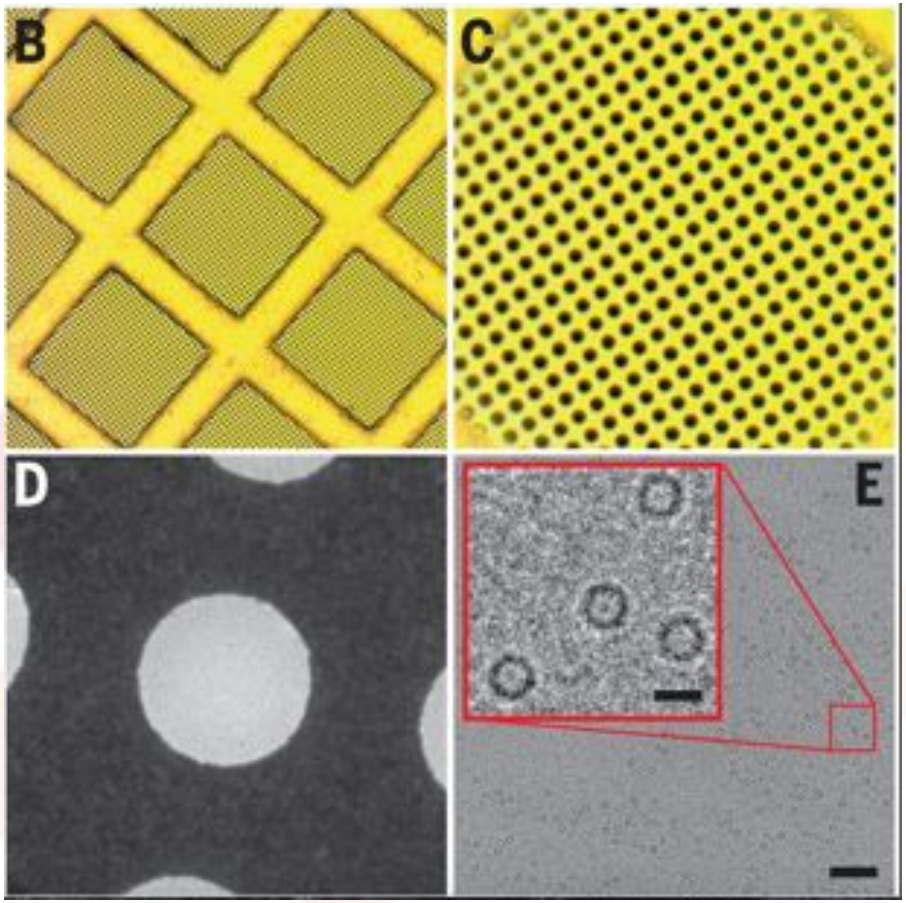






The different substrats on grid



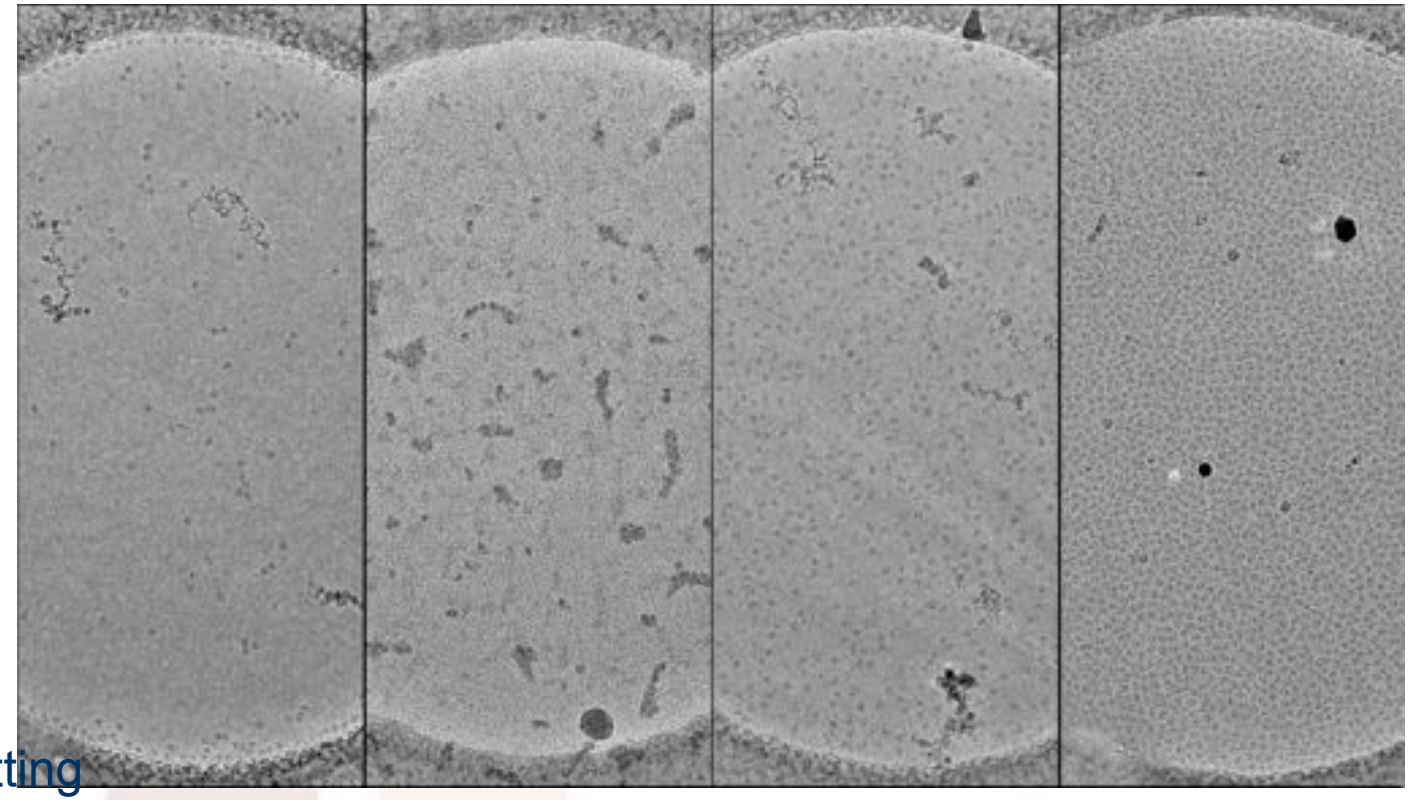


Glow discharge

Grid
-type of grid and substrat
-batch and age of grid
-glow discharging

Sample
-concentration
-buffer composition
-detergent

Freezing
-blotting time
-single or double side blotting
-waiting time
-multiple sample application



Buffer composition

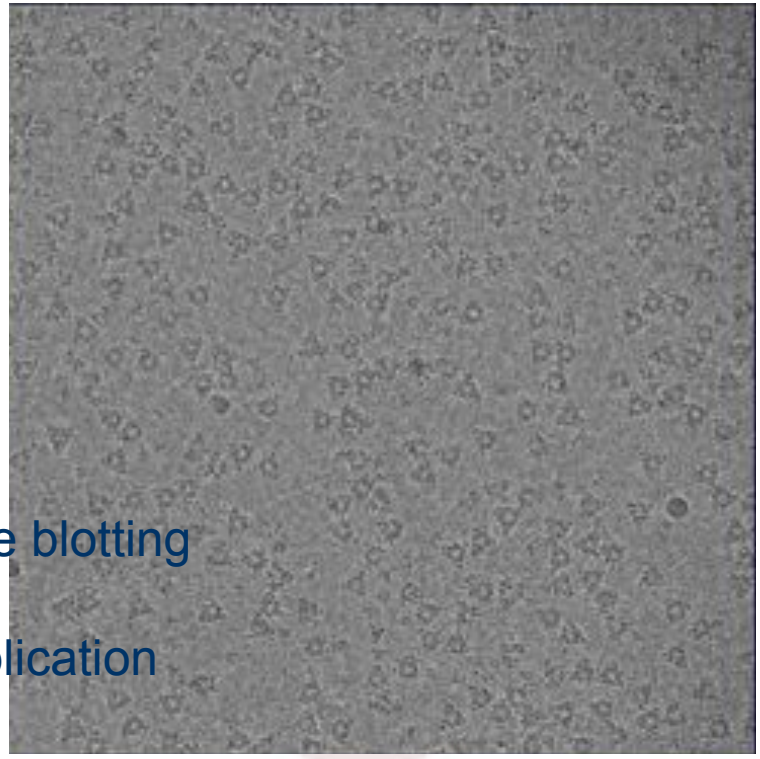
Buffer composition
Density of protein : 1.36 g/cm3
Density of water : 1 g /cm3
Density of glycerol : 1.26 g/cm3

Beware of high concentrations of
-glycerol
-sugars
-salts
-detergents

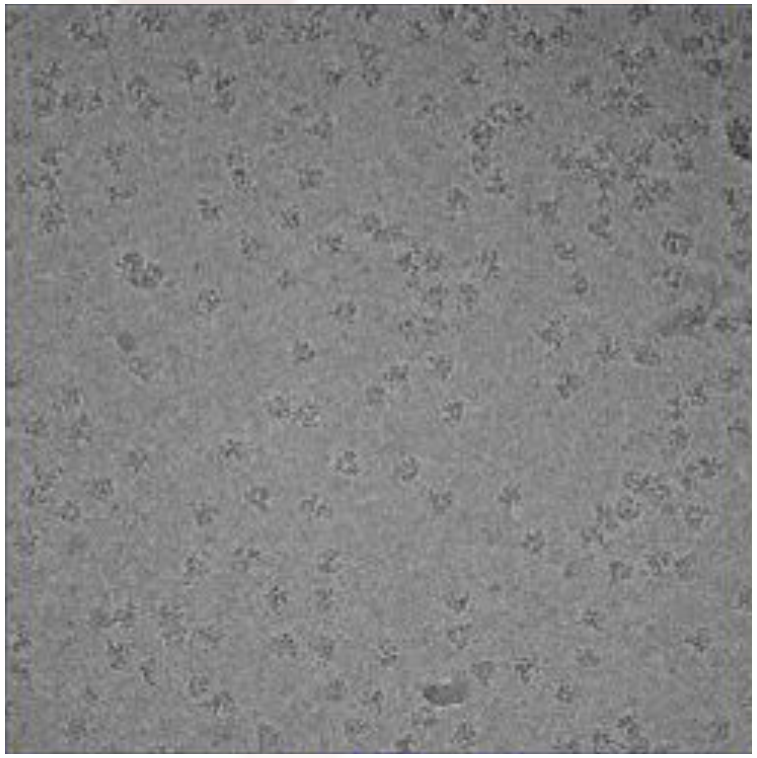
Grid
-type of grid and substrat
-batch and age of grid
-glow discharging

Sample
-concentration
-buffer composition
-detergent

Freezing
-blotting time
-single or double side blotting
-waiting time
-multiple sample application



No detergent



Detergent

Freezing device

Grid
-type of grid and substrat
-batch and age of grid
-glow discharging

Sample
-concentration
-buffer composition
-detergent

Freezing
-blotting time
-single or double side blotting
-waiting time
-multiple sample application

Home made	Gatan	FEI
		
€	€€	€€€

What is good for cryo EM?

Size
The bigger the better
200 kDa currently for near-atomic resolution

Symmetry
The higher the better
Pseudo symmetry can be a problem

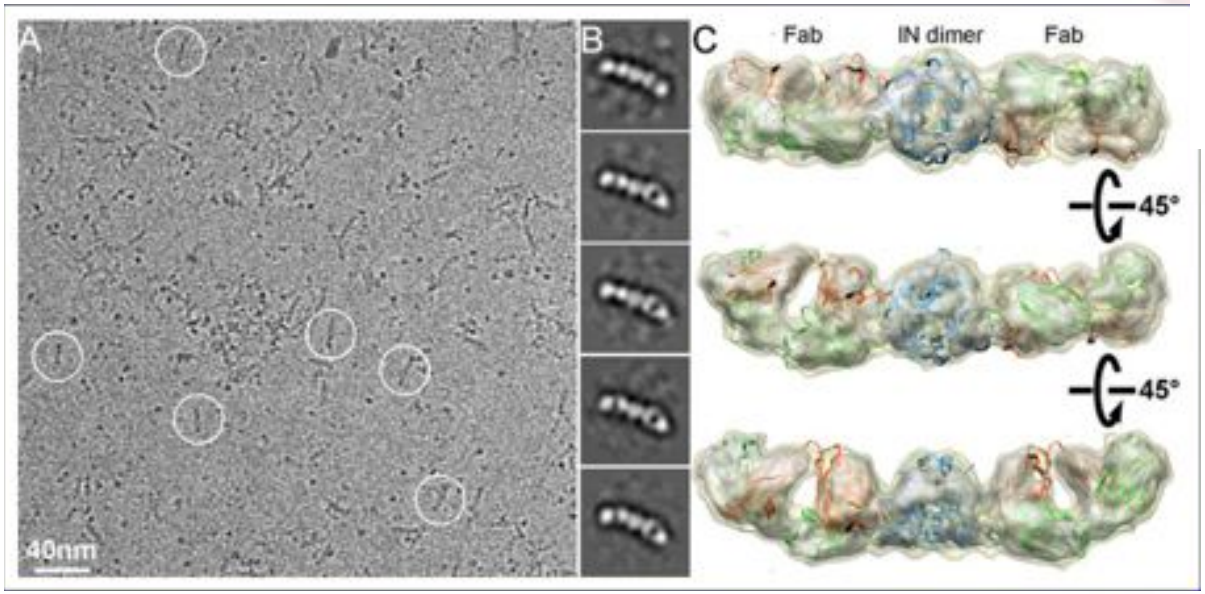
Shape
Globular better then extended
« extra features » highly beneficial

Homogeinity
The more homogenous the better but heterogeneity now more manageable due to
- Better image quality
- New software development

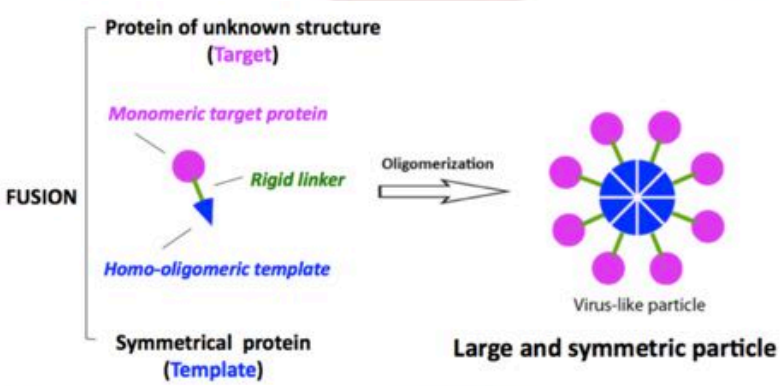
What is good for cryo EM?

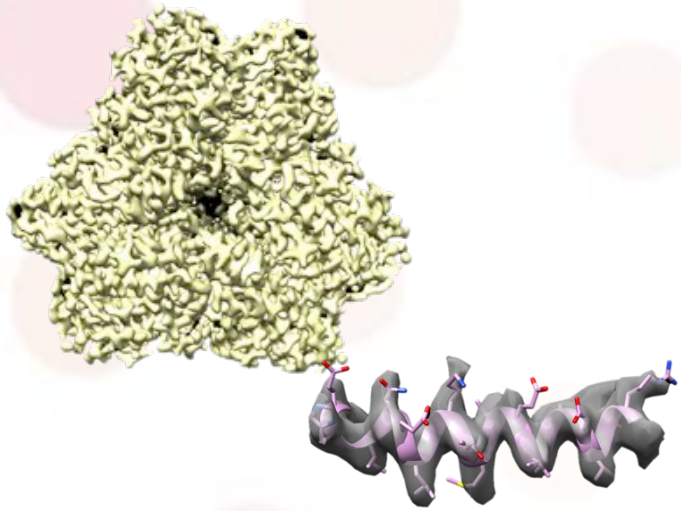
Size
The bigger the better
200 kDa currently for near-atomic resolution

Symmetry
The higher the better



HIV-1 integrase dimer (65 kDa) in complex with two Fabs (total of 165 kDa).
10 A resolution. Wu et al., 2012 Structure 20: 582-592.



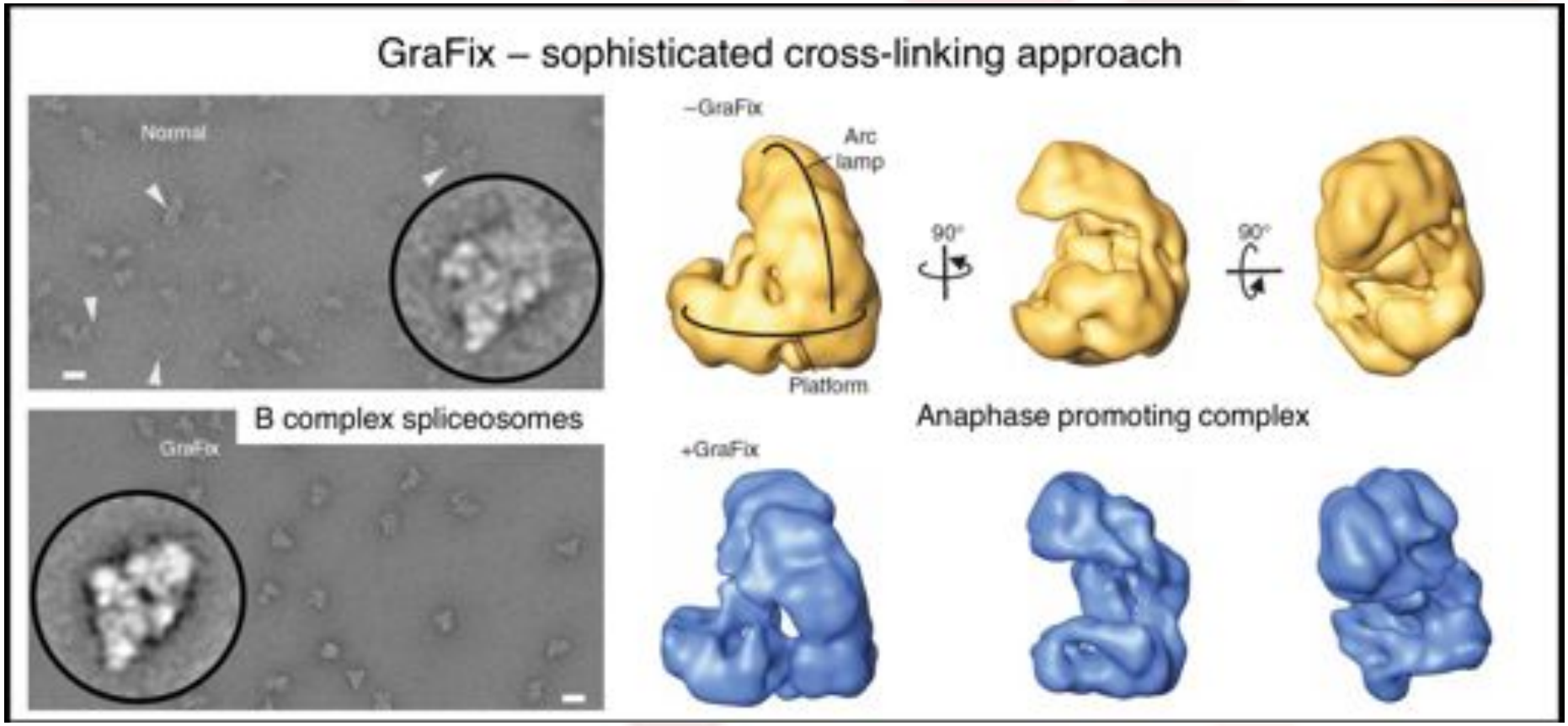


Tetrahedral
Amminopeptidase
480 kDa

What is good for cryo EM?

Shape
Globular better then extended
« extra features » highly beneficial

Homogeneity
The more homogenous the better but heterogeneity now more manageable due to
- Better image quality
- New software development



Kastner et al., (2008) Nat. Methods 5:53-55.

Thin sample

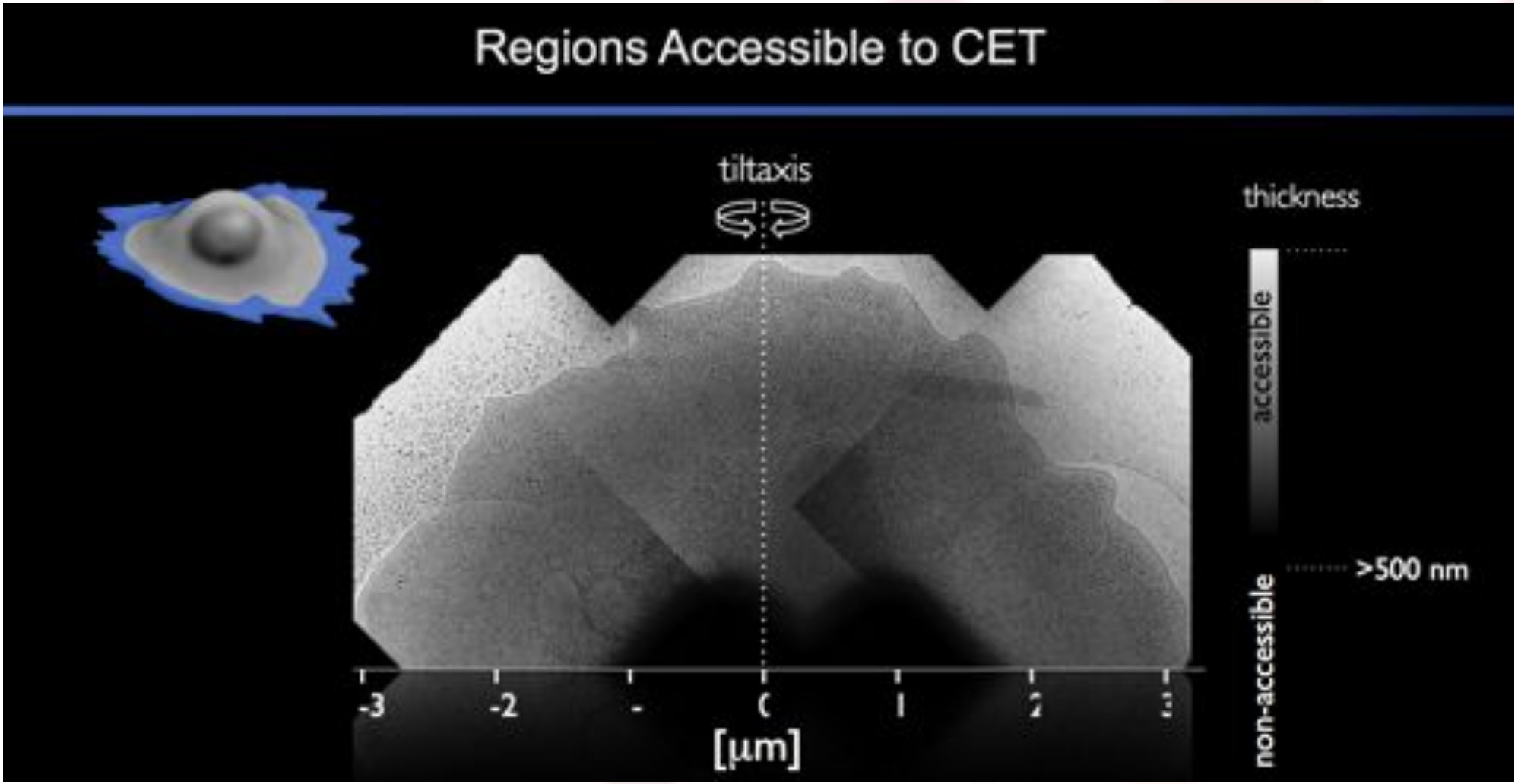
A lot of parameter can be changed

But start with the easier setting and if it doesn’t work then try the more sophisticated one

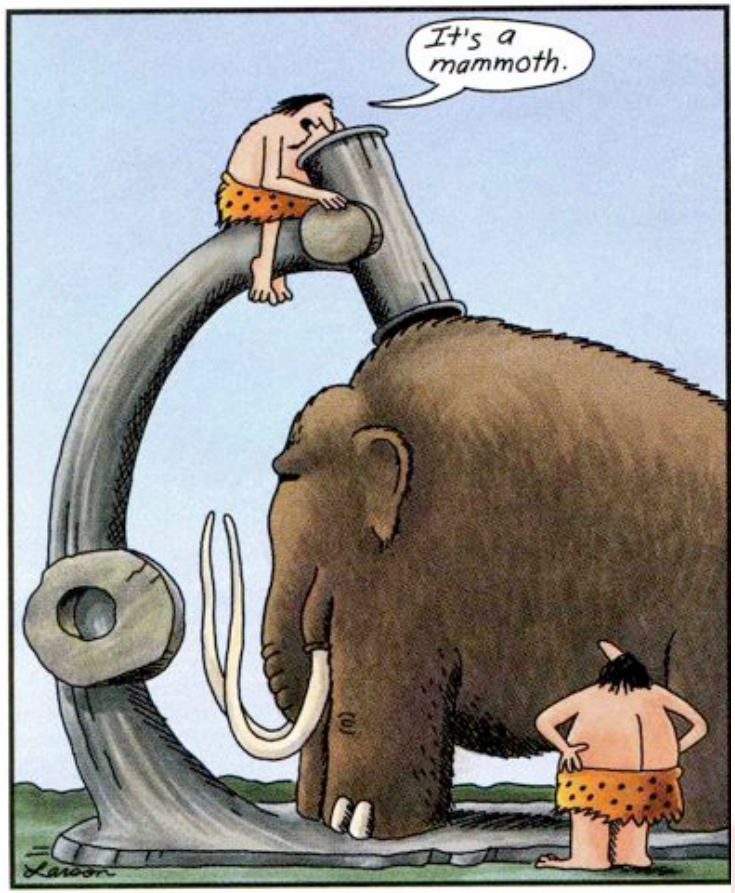
What can we freeze on the grid?

Bacterial Cells	Small Cells	Mammalian Cells
~0.5 um	~2 um	~5-10 um

« Thick samples »

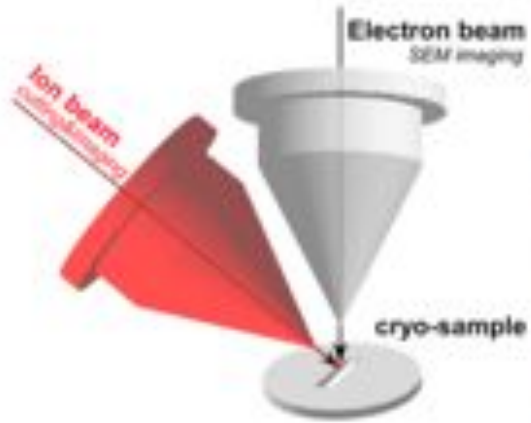


« Thick samples »



Early microscope

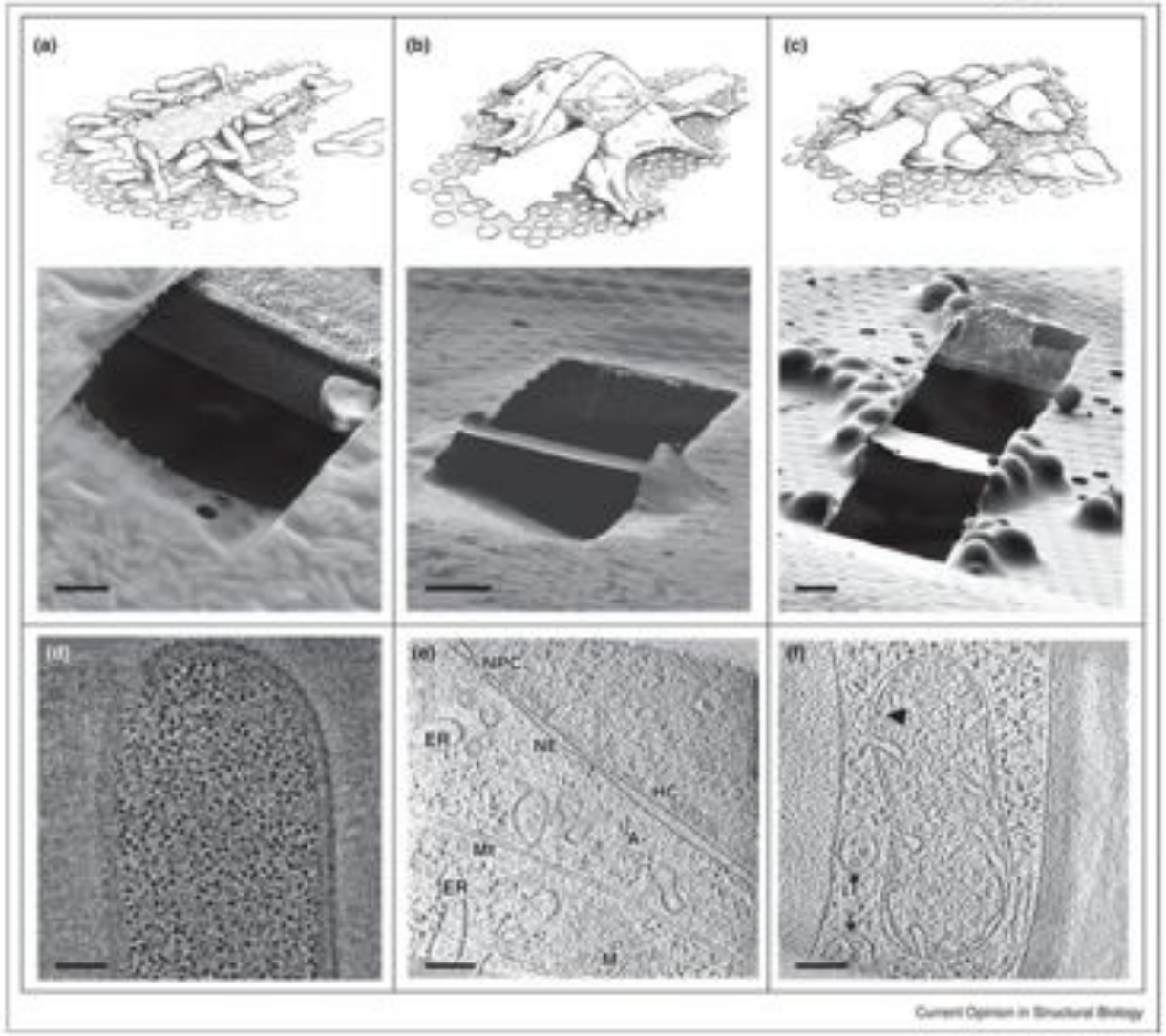
Focused Ion Beam – FIB



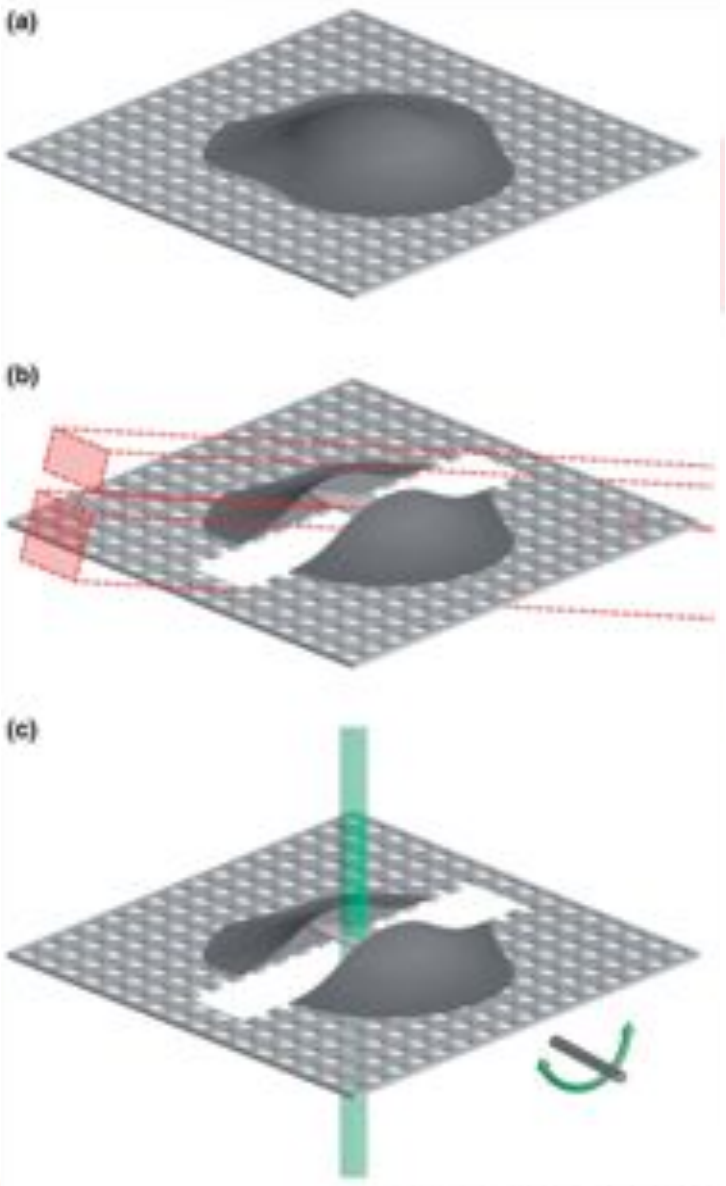
Bundesministerium
für Bildung
und Forschung

FEI Quanta 3D FEG dual beam FIB/SEM instrument as installed at the MPIB

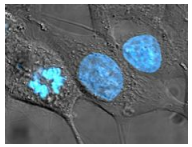
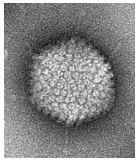
Focused ion beam milling

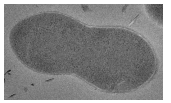


a et al. 2013



EM on Cellular sample

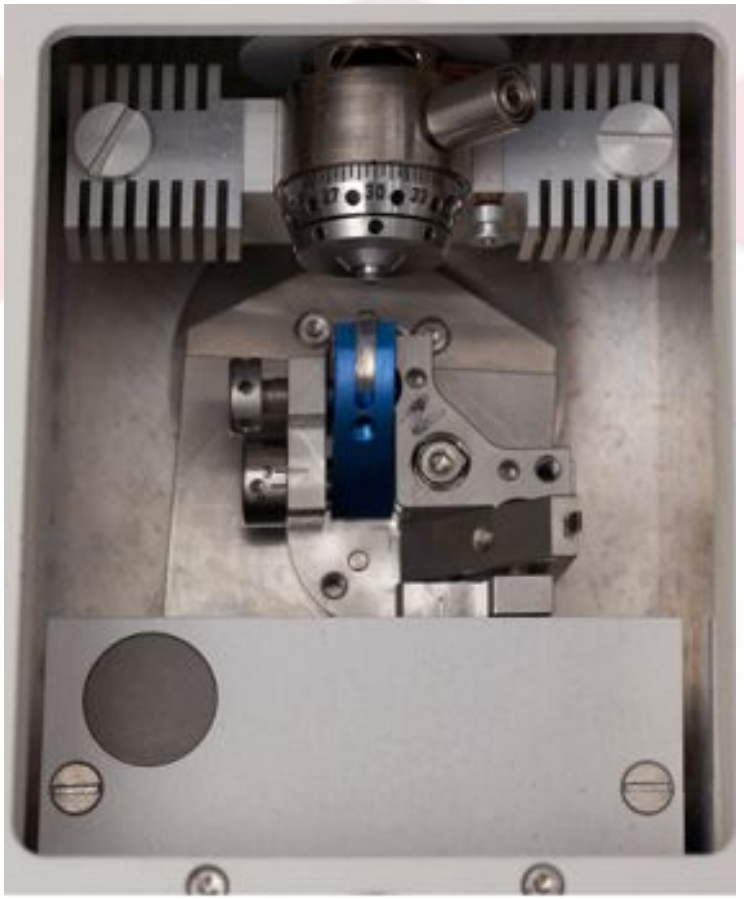


- Fixation (to stop all cellular process)
- « Rigidification »
- Sectionning
- -Visualization

	Cryo fixation *HPM100* 		**Chemical fixation** 		
CEMOVIS  -FC7	**Freeze substitution** 	**Tokuyasu** **RT gelatine embedding**	**RT resin embedding**	**PLT** 	
	RT sectionning 	**Cryosections Immunogold** UC7-FC7 	**RT sectionning** 	**RT sectionning** 	
Cryo-TEM	TEM	TEM	TEM	TEM	

Ultrastructure	+++++	+++	+/++	++	++
Immunolabelling	-	++	+++	+/-	++
Difficulty	+++	++	++(+)	+	+

The ultra microtome



The high pressure freezing device

Cellular sample

Chemical fixation

RT resin embedding

- Dehydration
- Impregnation
- Polymerization

} 4 days

RT sectionning

UC7

- Sectionning } 1 day

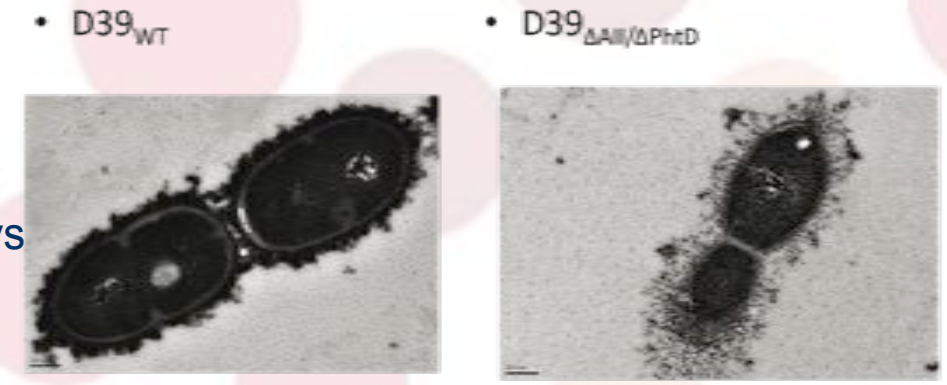
TEM

- Imaging } 1(2) day(s)

Ultrastructure	++
Immunolabelling	+/-
Difficulty	+

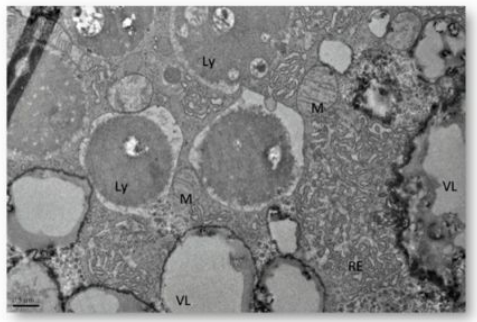
Classical embedding

- No specific instrument needed
- Easy
- Good contrast



S. pneumoniae, RR + EPON, T

Mitochondria in HeLa cell,



M : Mitochondrie / RE : Reticulum Endoplasmique / VL : Vesicule Lipidique
Ly : Lysosomes / AP : Autophagosomes

Drosophila larvae, fat body

Cellular sample

Chemical fixation

Tokuyasu

RT gelatine embedding

- Gelatine
- Cryo-protection
- Freezing

1 day

Cryosections Immunogold

UC7-FC7 IGL

- Sectionning

1 day

TEM

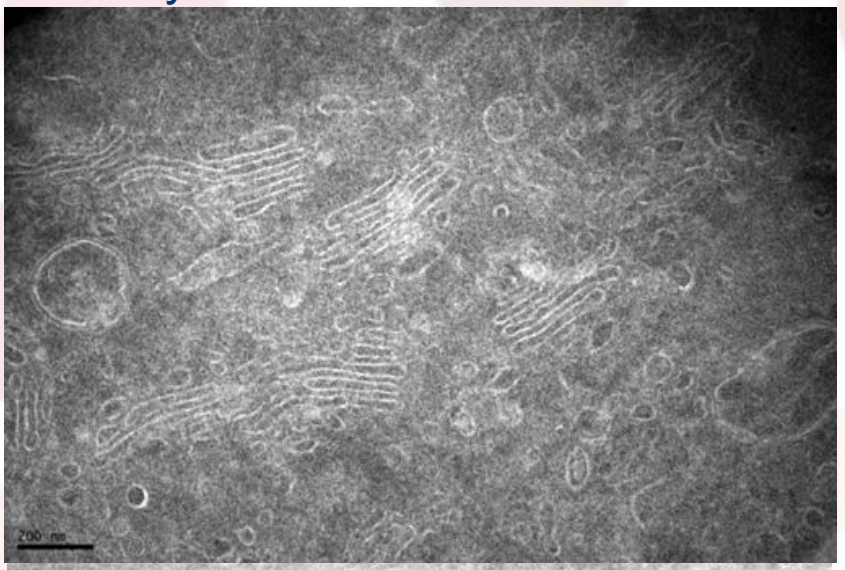
- Imaging

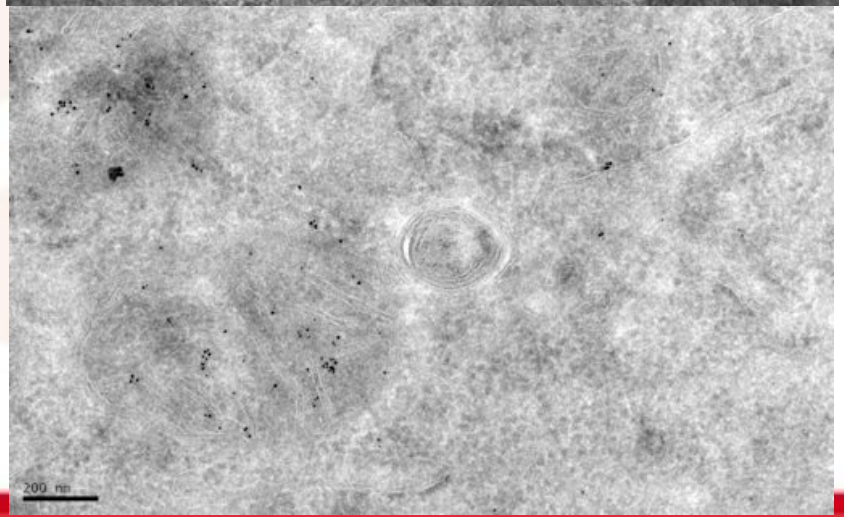
1 (2) days

Ultrastructure	+/++
Immunolabelling	+++
Difficulty	++(+)

Tokuyasu for immunogold

- Fast
- Good structure preservation
- Best for immunogold
- Cryosections





HPF & freeze substitution

Cellular sample

Cryo fixation

- HPF (HPM100) & FS (AFS2)
- Automated process
- RT sections

- Immuno-compatible (HM20)
- Good structure preservation

Freeze substitution

- Substitution
- Impregnation
- Polymerization

} 5-10 days

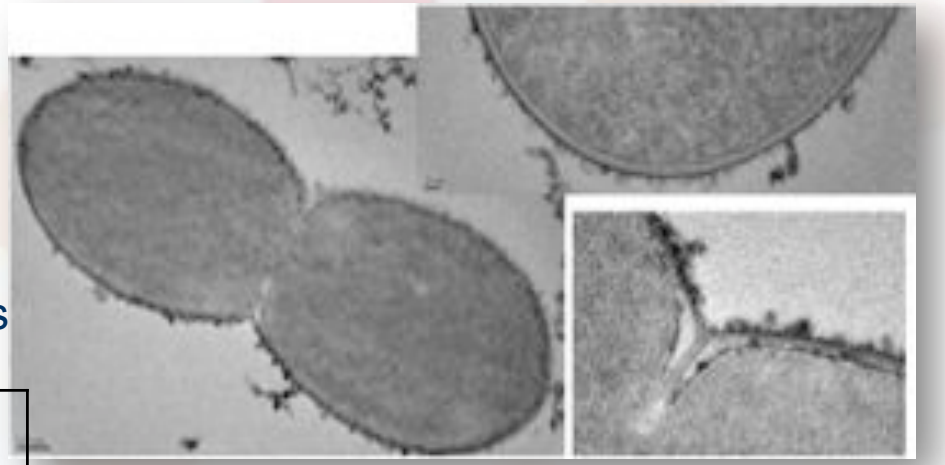
RT sectionning

- Sectionning } 1 day

TEM

- Imaging } 1(2) days

Ultrastructure	+++
Immunolabelling	++
Difficulty	++



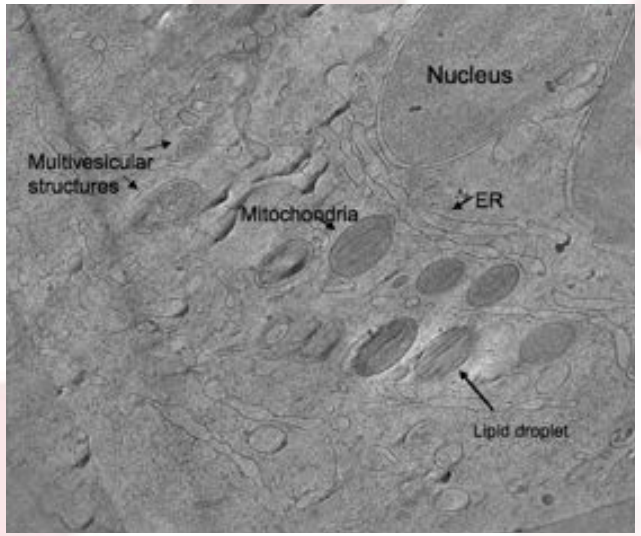
S. pneumoniae, R6

Cellular sample

Cryo fixation

HPM100

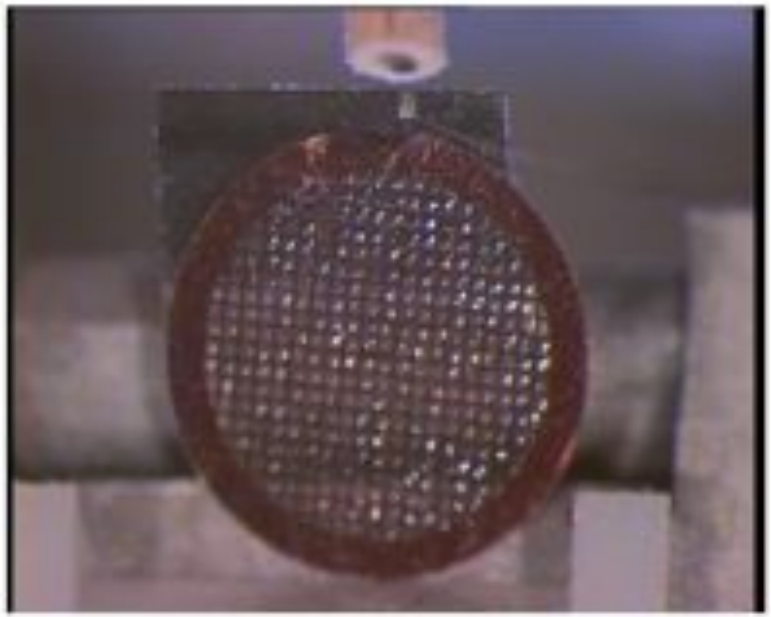
HPF device



CEMOVIS

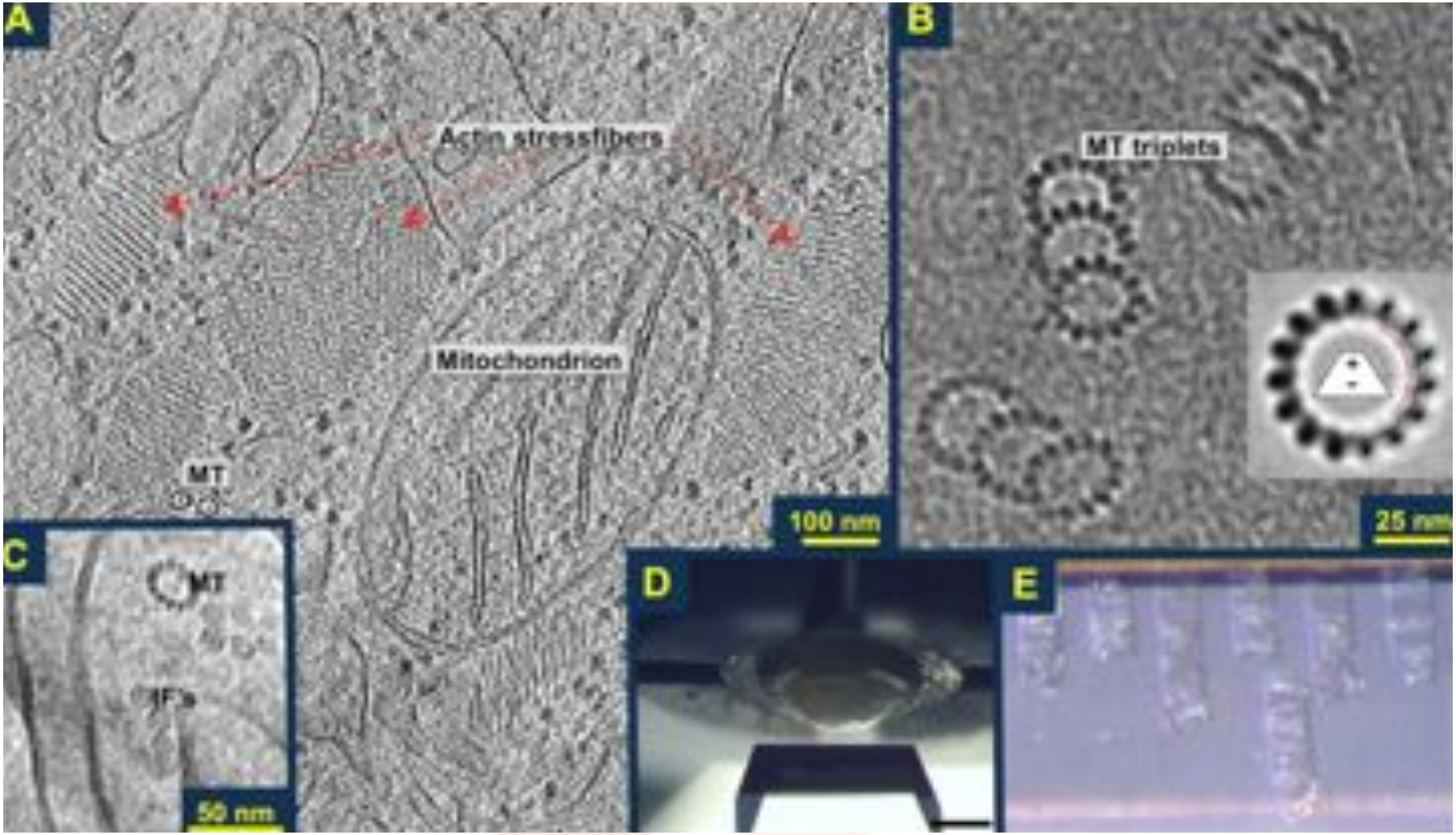
UC7-FC7

Cryo-TEM



picture by Peter Peters

Example of Cemovis

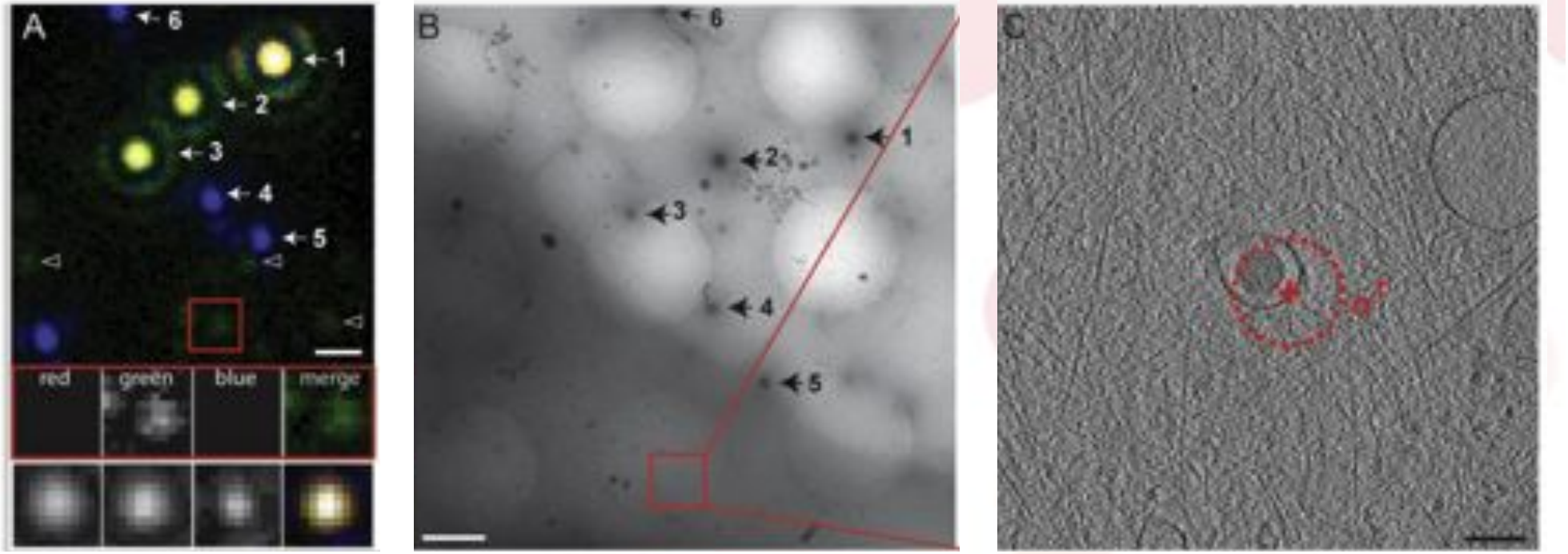


Very difficult

Correlative light electron microscopy

Locate an event by light microscopy and then find the same event in the EM to image it at higher mag

Use of probe that are electron dense and fluorescent



Schellenberger et al, ultramicroscopy 2014

The sample preparation step is crucial (as well as the purification and data collection steps)

Some other techniques exist for sample preparation:

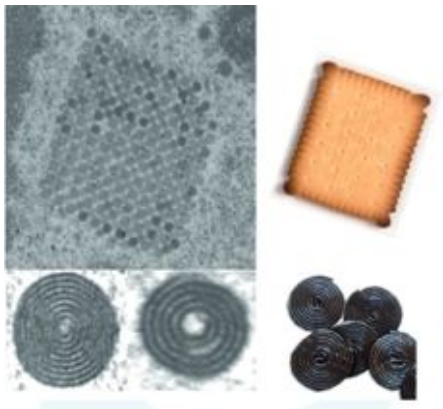
Time resolved EM

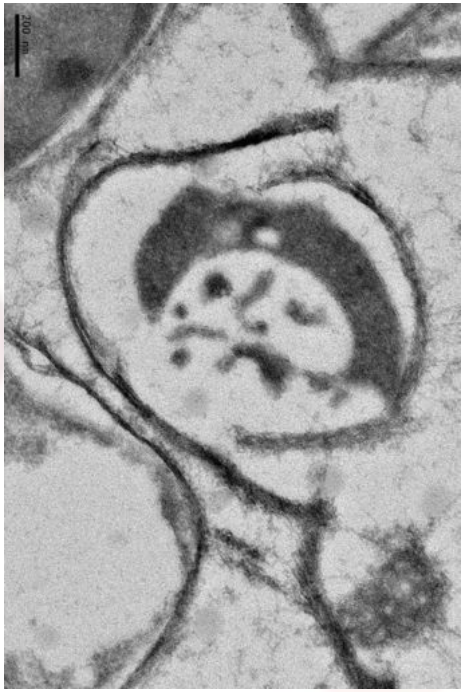
Cryo-negative staining

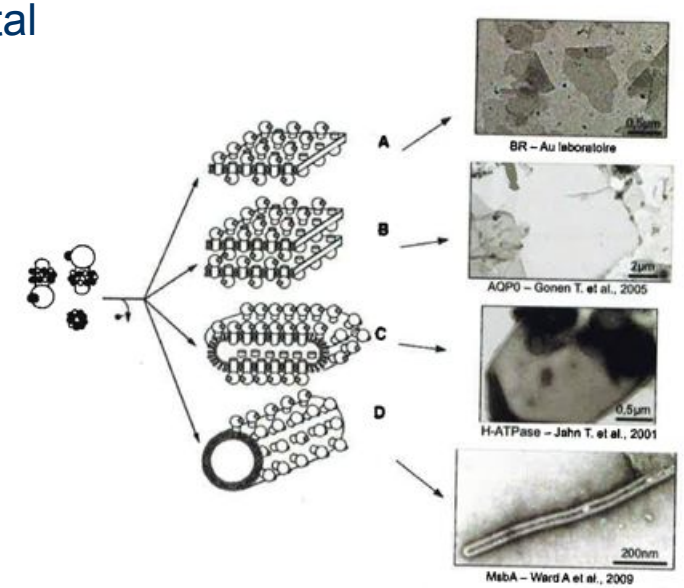
Specificity for tomography

For 2D crystal

Etc…







Questions????