Roma, 16 settembre 2020

Nano Innovation 2020
Rome, 15-18 September
Conference & Exhibition

Cryoelectron microscopy: a dream for the microscopist and a primer for the non-microscopist

Luciana Dini

SAPIENZA UNIVERSITA' DI ROMA

Dipartimento di Biologia e Biotecnologie Charles Darwin

CNR-Nanotec LECCE

SAPIENZA UNIVERSITÀ DI ROMA

CNR NANOTEC INSTITUTE OF NANOTECHNOLOGY

Sample Preparation Pathways

Specimen

Chemical fixation

Dehydration

SEM

Critical point drying

Metal Coating

Carbon Coating

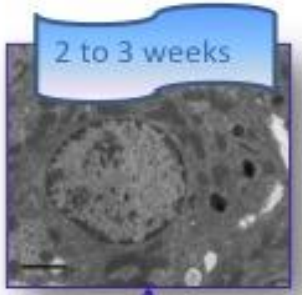
Immunolabeling

Spread onto support grid

TEM

Embedding

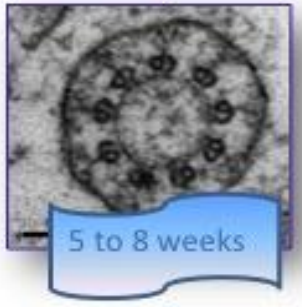
Ultramicrotomy



Immunolabeling

specific area (i.e. cilia in trachea or in eyes, islets in pancreas): thick sections stained with toluidine blue first

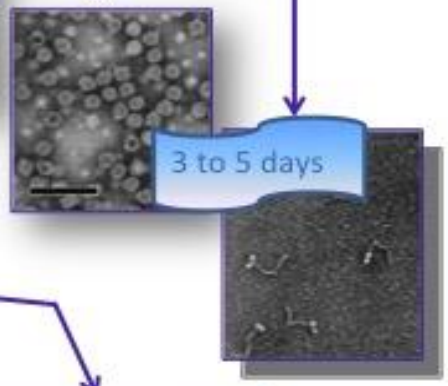
Contrasting



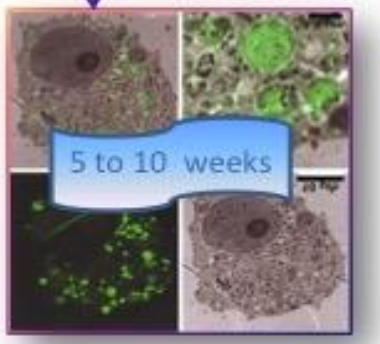
Negative Stain

or

Metal shadow



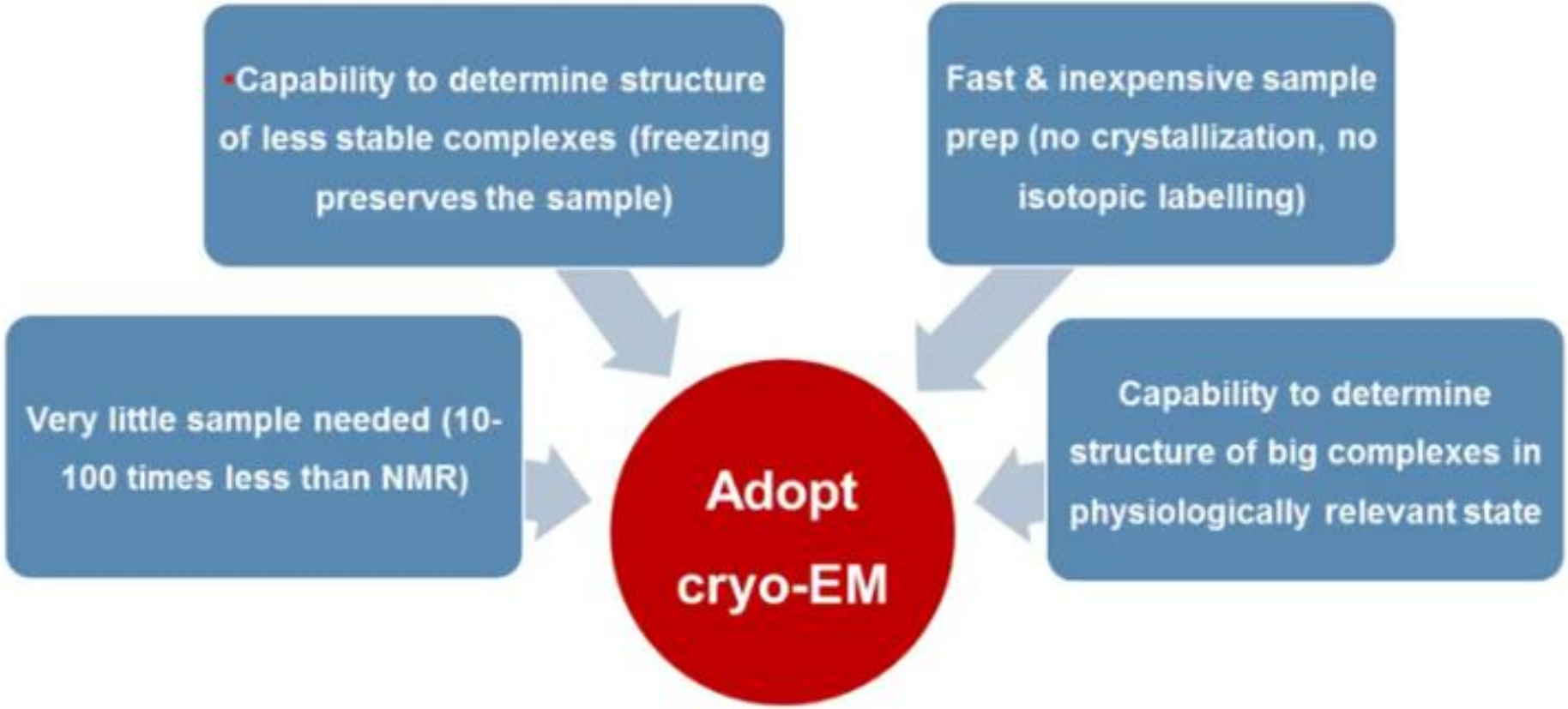
Correlative LM & TEM



The examination of frozen-hydrated specimens by ***cryo-electron microscopy*** is a rapidly developing technique, which has much potential for the analysis of low atomic number samples.

Although there are several technical difficulties, cryo-electron microscopy represents a powerful analytical tool.

Why Use Cryo-EM?

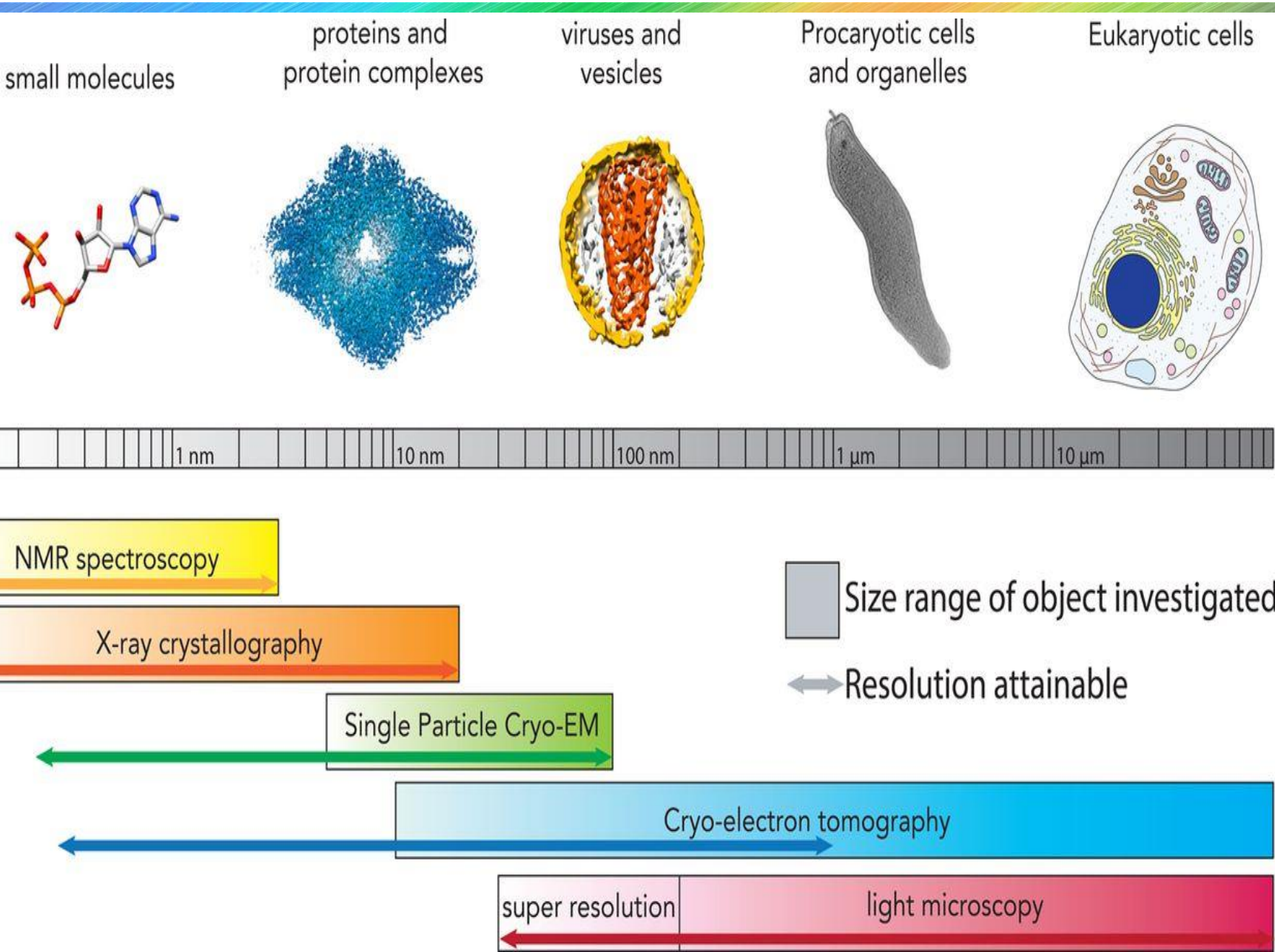


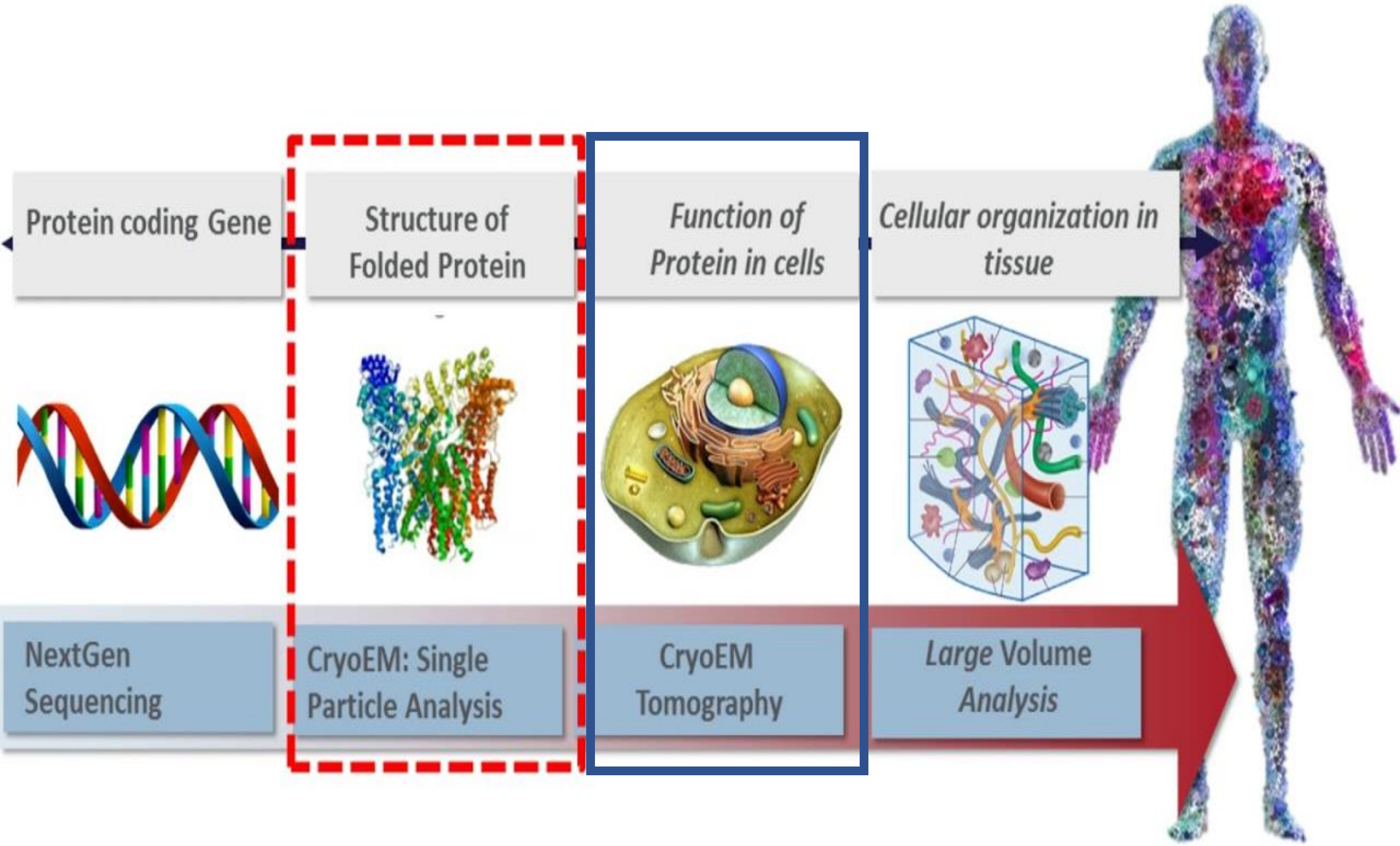
CRYO –TEM APPLICATION

Thin films like transmembrane proteins or helical arrays of proteins, can provide structural information about 2-D ordered arrays of protein.

Thin film of vitrified aqueous suspension allow to observe particles in their native state (colloids like nanoparticles, liposomes or micelles)

Vitreous sections of larger objects such as cells and tissues that cannot generally observed directly.


small molecules
proteins and protein complexes
viruses and vesicles
Procaryotic cells and organelles
Eukaryotic cells
1 Å
1 nm
10 nm
100 nm
1 µm
10 µm
NMR spectroscopy
X-ray crystallography
Single Particle Cryo-EM
Cryo-electron tomography
super resolution
light microscopy
Size range of object investigated
Resolution attainable


Protein coding Gene
Structure of Folded Protein
Function of Protein in cells
Cellular organization in tissue
NextGen Sequencing
CryoEM: Single Particle Analysis
CryoEM Tomography
Large Volume Analysis

Transmission Electron Microscopy studies

Limited by four general factors:

1. Electrons need **high vacuum** to form a beam able to go throw the sample.
2. Electrons **heat** the sample.
3. **Low penetration power** of electrons.
4. Images are **2D projections** of the specimen.

Samples that have low Z or containing water pose serious limitations

Conventional TEM preparation:

- ***Chemical fixation***: glutaraldehyde (GA) and osmium tetraoxide (OsO_4)
- ***Dehydration***
- ***Resin embedding***
- ***Sectioning***
- ***Staining*** with heavy metals: uranyl acetate (UA) and lead citrate (LC)

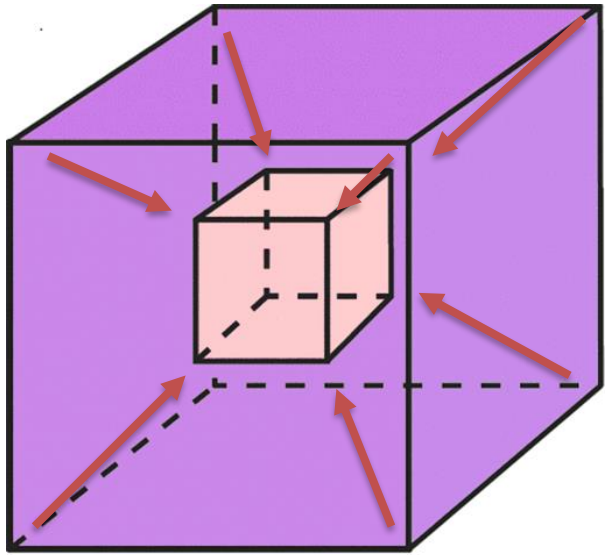
SOURCE OF ARTIFACTS

PRESERVATION LIMITED

Sample fixation

10 μm fixation

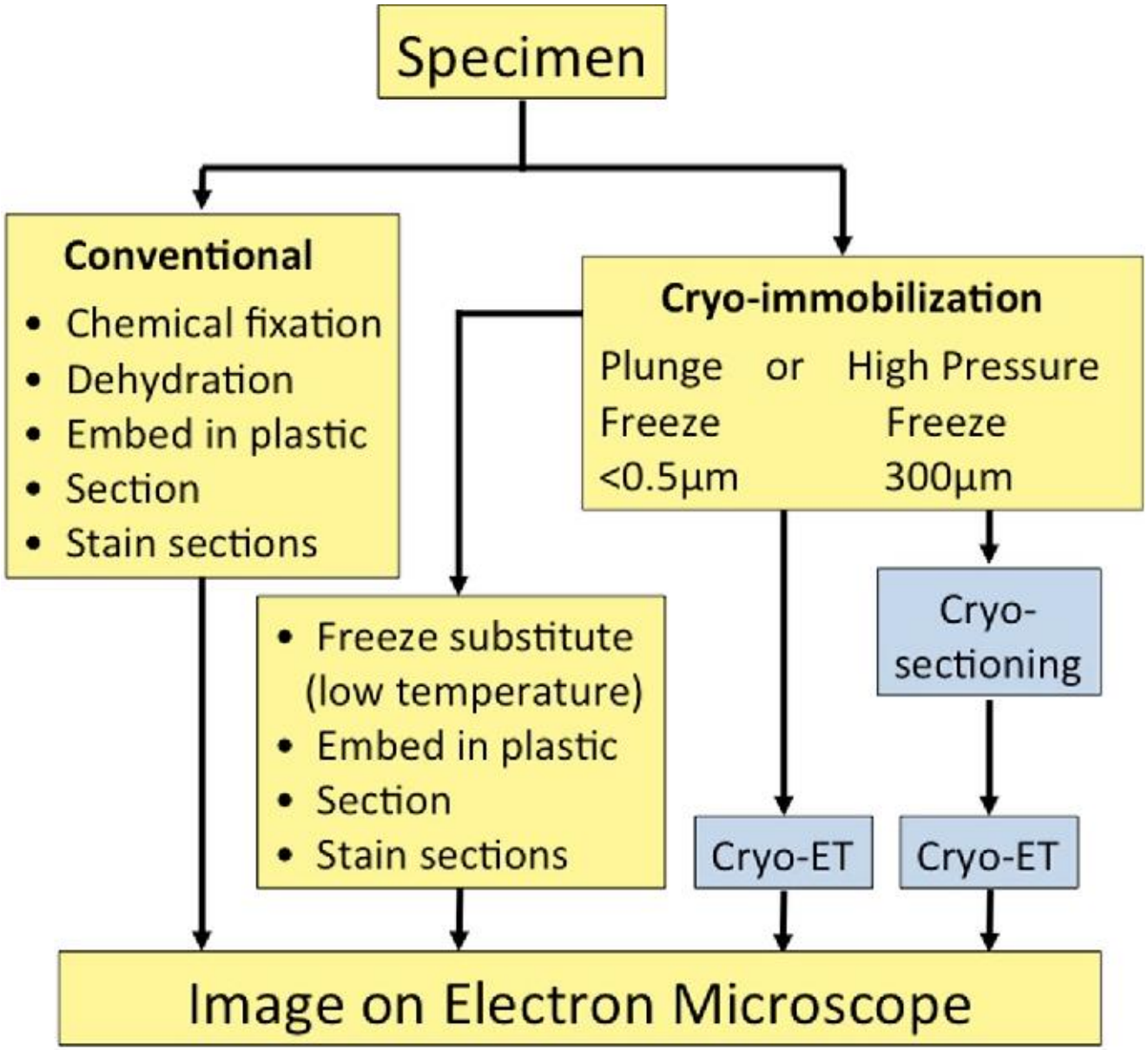
Chemical Fixation
- 5-6 seconds



Cryo-fixation
- 0.5 milliseconds

Physiological events: 1-5 milliseconds!

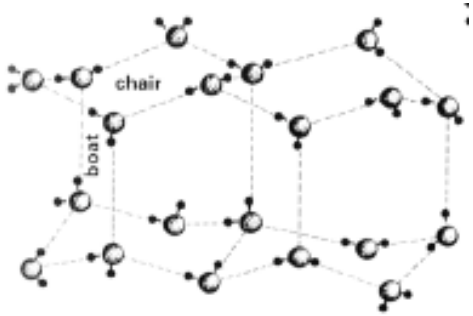
Electrolyte diffusion: 2 micrometers per second!


Specimen
Conventional
• Chemical fixation
• Dehydration
• Embed in plastic
• Section
• Stain sections
Cryo-immobilization
Plunge Freeze <0.5µm
or
High Pressure Freeze 300µm
• Freeze substitute (low temperature)
• Embed in plastic
• Section
• Stain sections
Cryo-sectioning
Cryo-ET
Cryo-ET
Image on Electron Microscope

Cryo-immobilization or Cryo-fixation

Can also add artifacts to the sample! → **WATER CRYSTALLIZATION**

Water



Ice

→ **Solutes segregation**

→ **Distortion of structure**

Ice crystals formation depends on:

- Cooling rate
- Pressure
- Water content of the sample
- Solute concentration in the aqueous phase

Cryo-immobilization or Cryo-fixation

Types of ice:

- Crystalline
  - Hexagonal ice
  - Cubic ice

→ STRUCTURAL DAMAGES!

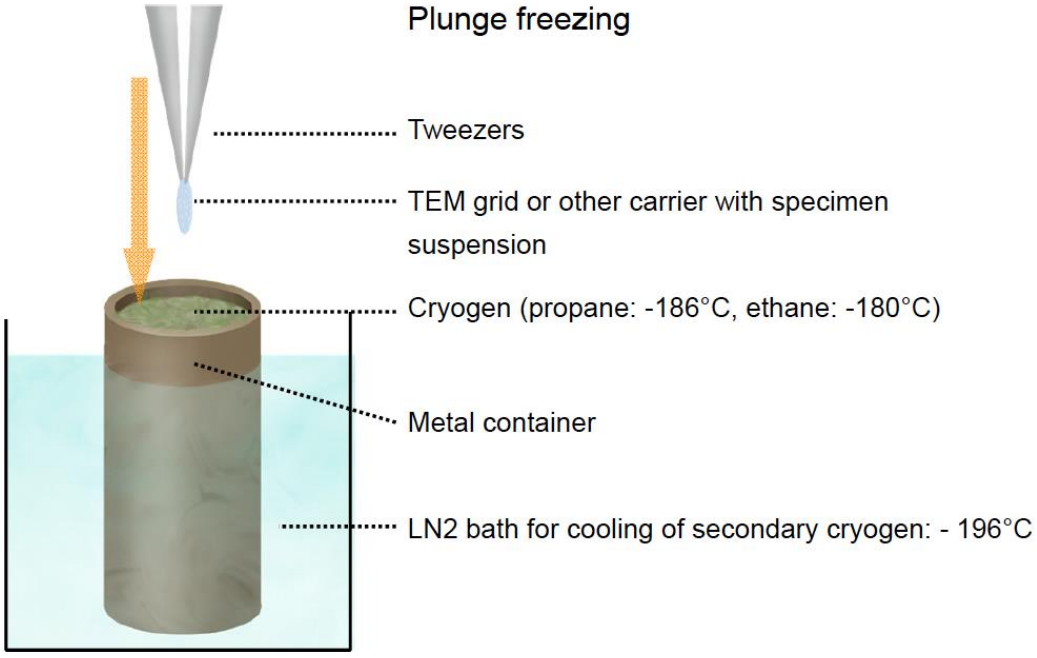
- Non crystalline
  - Vitreous/amorphous ice

→ **STRUCTURE REMAINS IN A CLOSE-TO-NATIVE STATE**

Should mean ***vitrification***: transformation of intrinsic water of the sample to vitreous ice directly, avoiding crystallization.

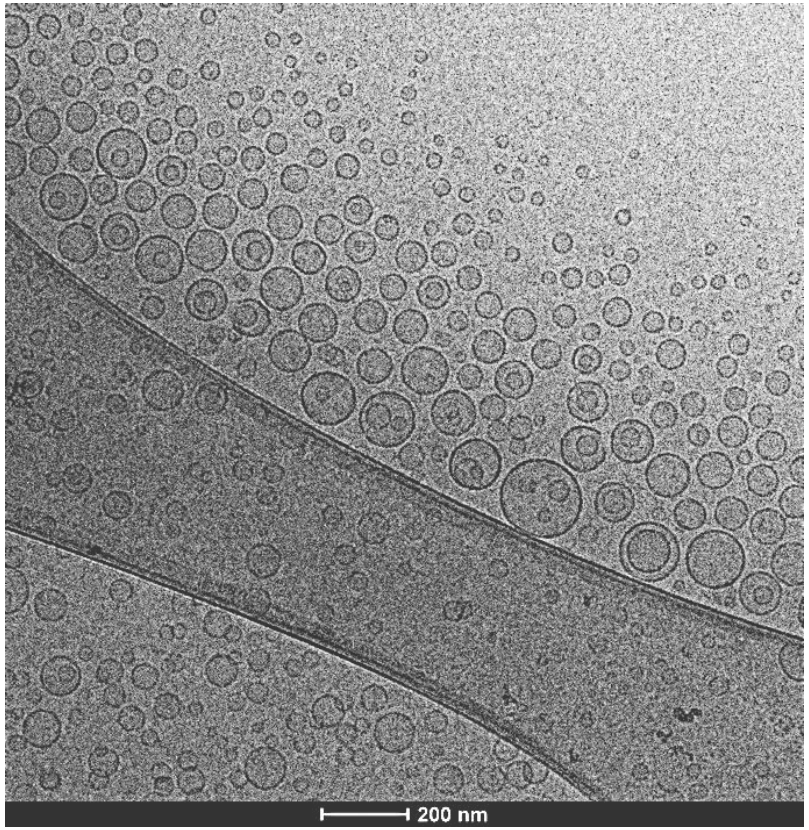
Cryo-immobilization by plunge freezing (PF)

- Freezing by immersion in a liquefied gas (primary cryogen: ethane, propane).
- Small pieces of tissue. Manual plunge freezing (chemical fixation, cryo-protection).
- Suspensions and cells grown on a grid. Automated plunge freezing. Direct observation of the prepared grids (samples < 500 nm).
- Cooling rate 10,000-15,000ºC/s.

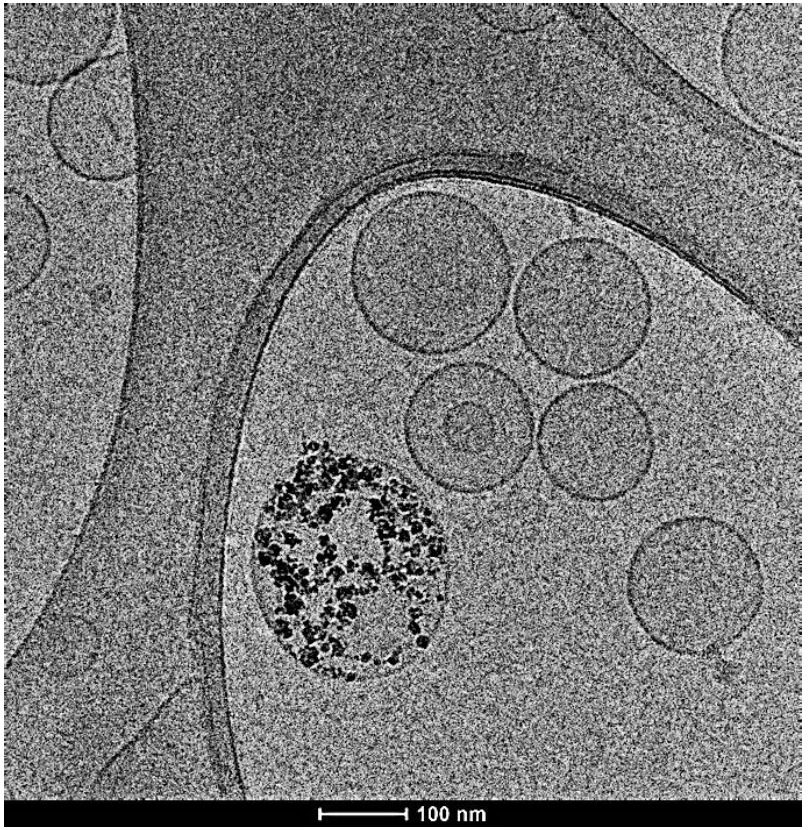


Cryo-TEM observation: plunge frozen samples

Liposomes

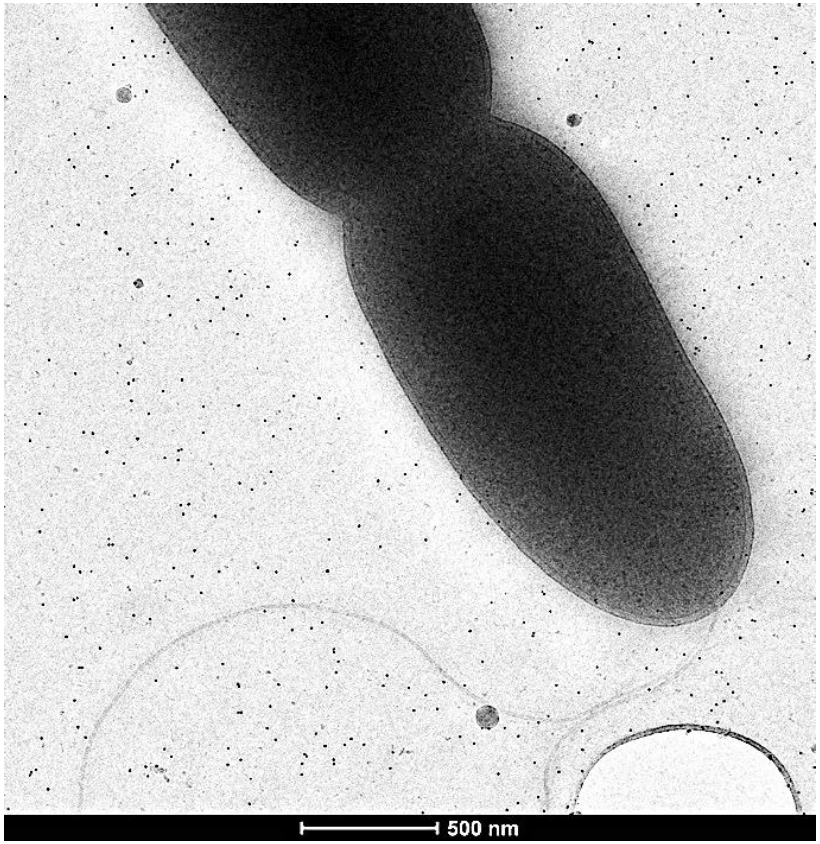


Liposomes containing iron particles

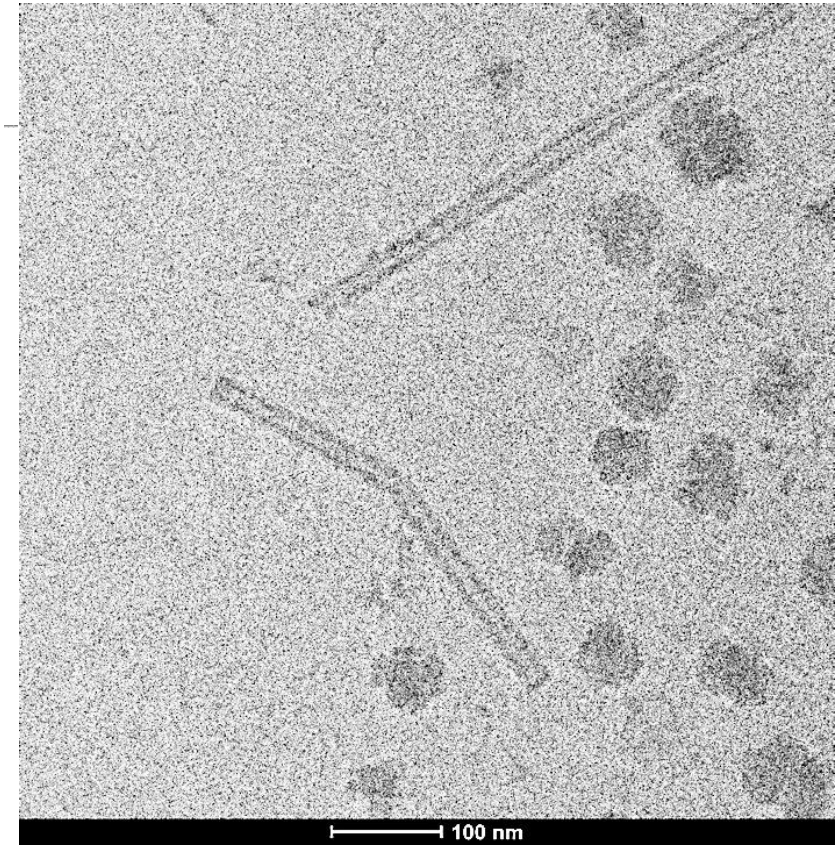


Cryo-TEM observation: plunge frozen samples

Pseudomonas deceptionensis M1^{T}

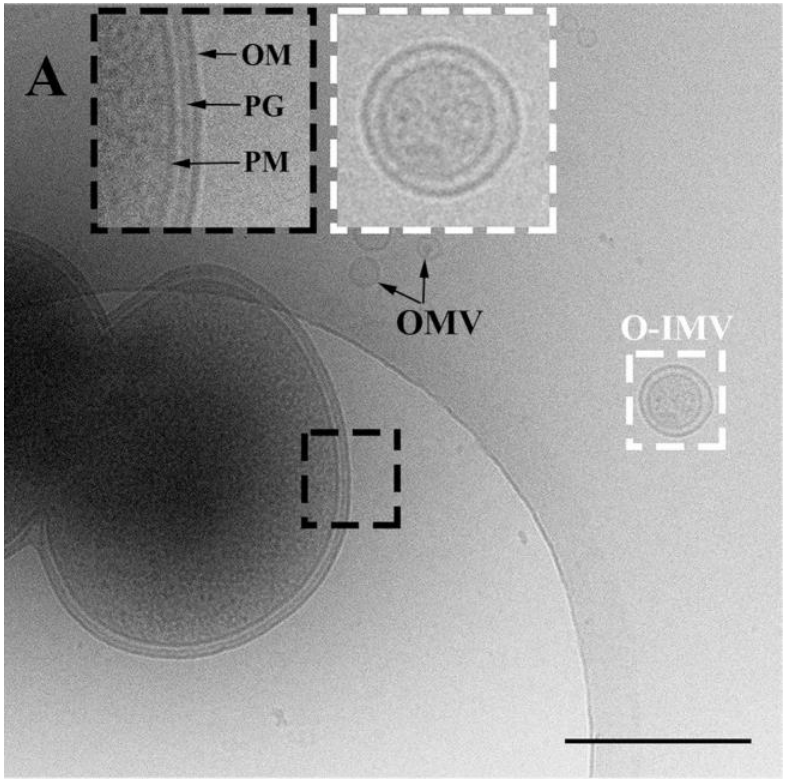


Lateral flagella isolated from *Aeromonas hydrophila*

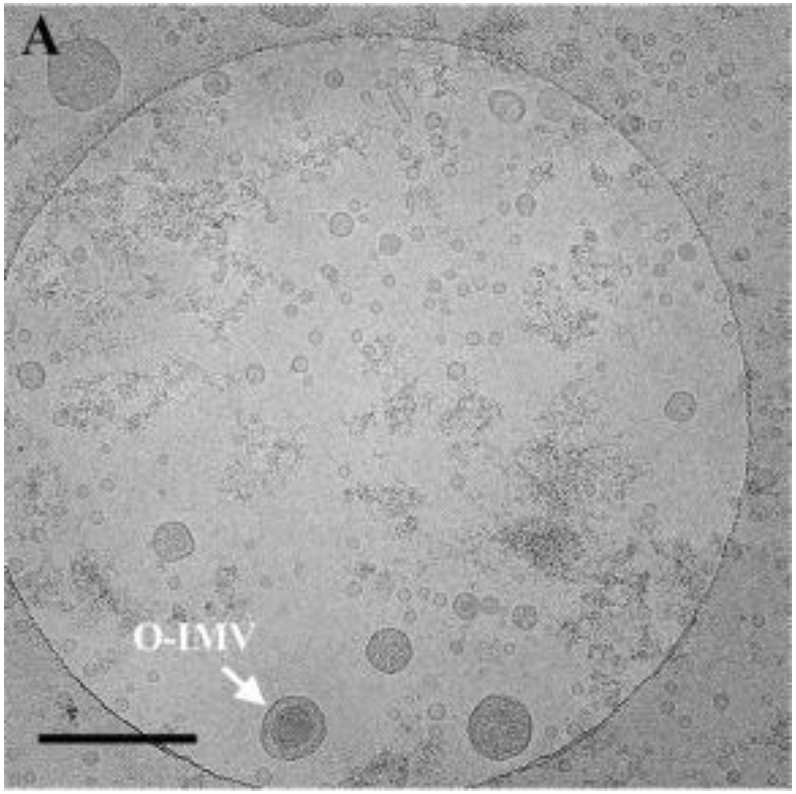


Cryo-TEM observation: plunge frozen samples

Membrane vesicles
Neisseria gonorrhoeae



Membrane vesicles
Acinetobacter baumannii



Perez C *et al* 2015, Plos ONE

Cryo-immobilization: High-pressure freezing (HPF)

- Freezing under high pressure: 2,100 bar
- Freezing rate: 10,000-30,000ºC/s
- Pressure built-up must be as quick as possible
- Samples < 200 µm

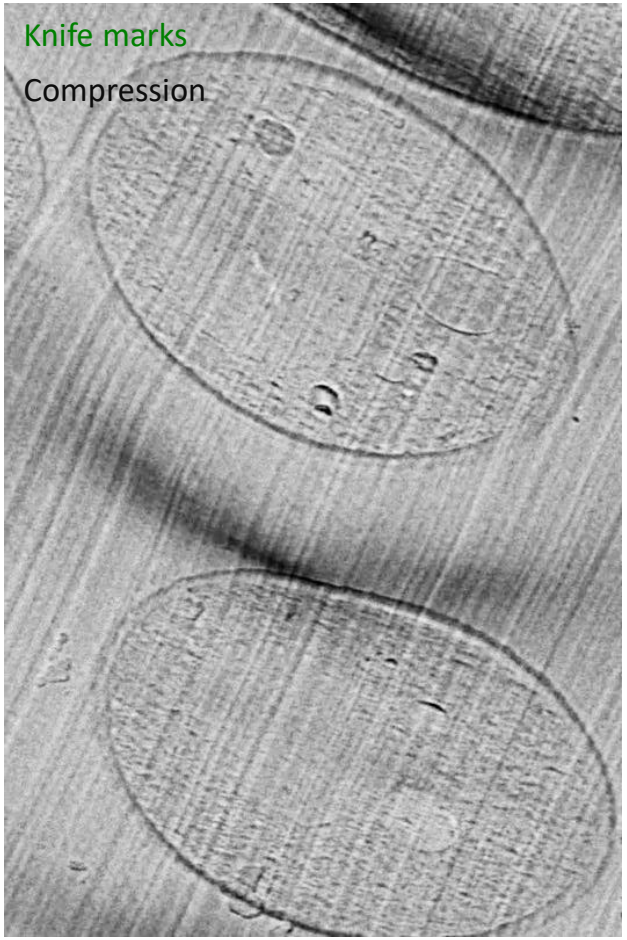
Flat carrier system
(3 mm and 6 mm)

Tube system

Cryo-immobilization: High-pressure freezing (HPF)

Cryo-Electron Microscopy of Vitreous Sections (CEMOVIS)

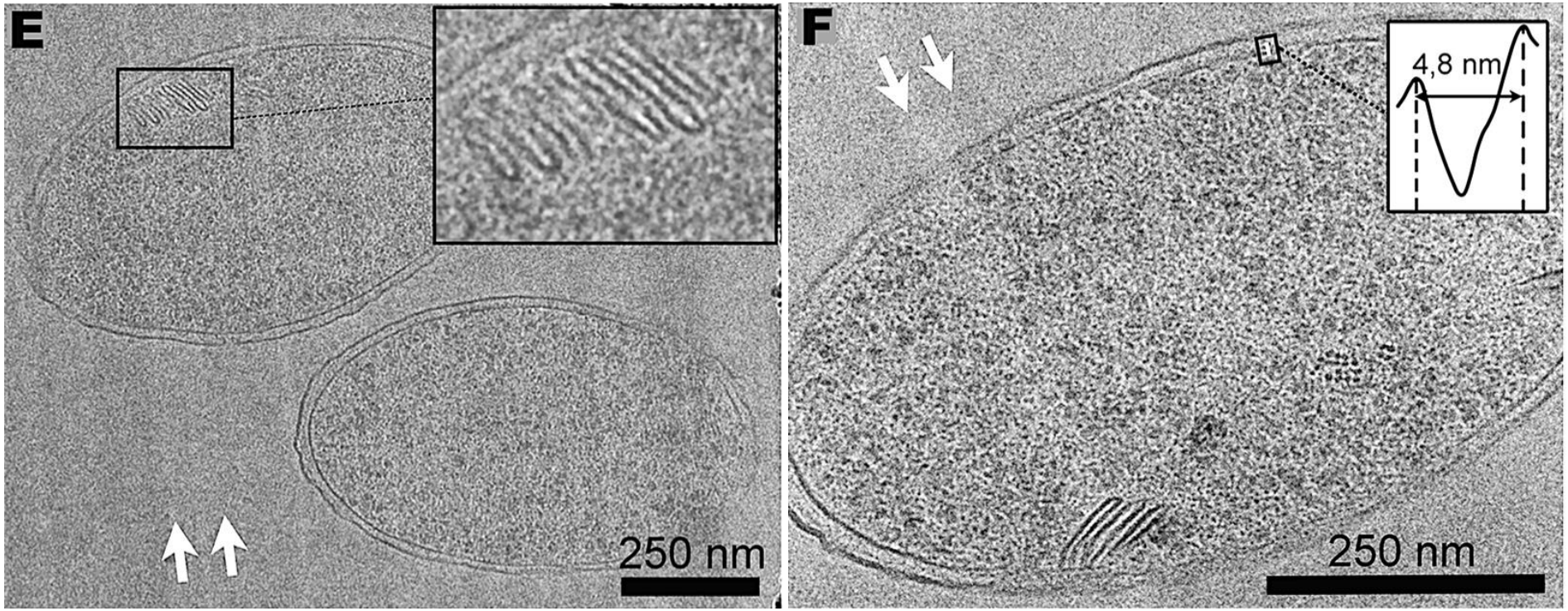
Dealing with mechanic artifacts…



Cryo-TEM observation: high-pressure frozen samples

Cryo-Electron Microscopy of Vitreous Sections (CEMOVIS)

Pseudomonas deceptionensis M1^T



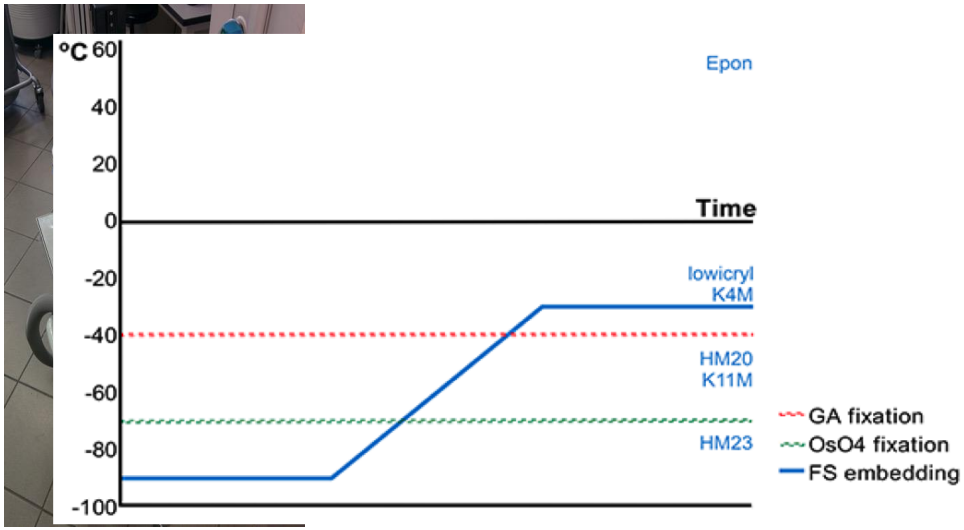
Delgado L *et al* 2015, J Struct Biol

Freeze substitution (FS)

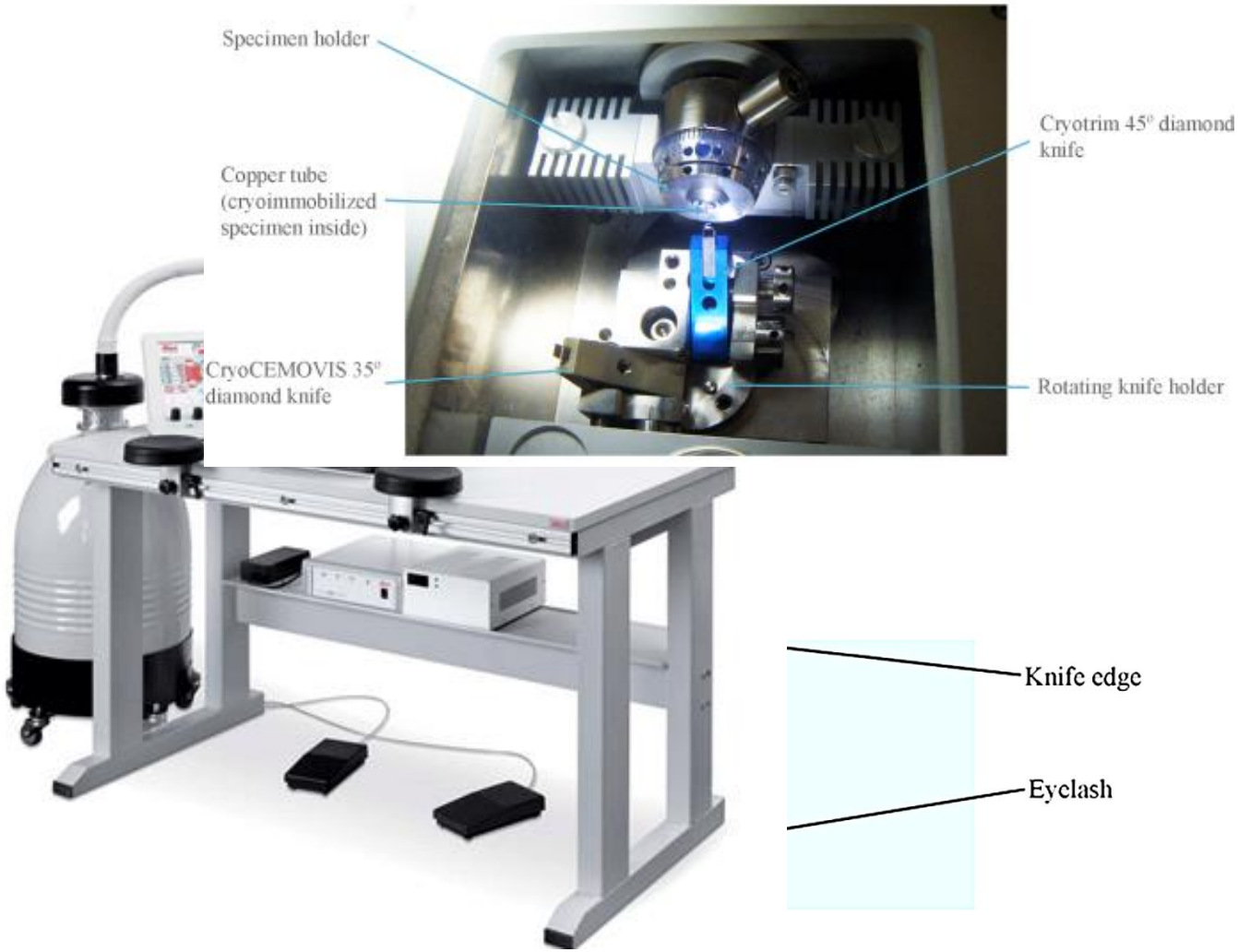
- Combines cryo-immobilization and resin embedding, allowing observation at room temperature.

- Provides complementary information that is not provided by only cryo-electron microscopy methods: tomography of thick sections (200-300 nm), immunolabeling.

Replacing the ice of the cryo-immobilized sample (HPF or PF) by an organic solvent, at -90°C and in the presence of chemical fixatives (OsO4, GA, UA).

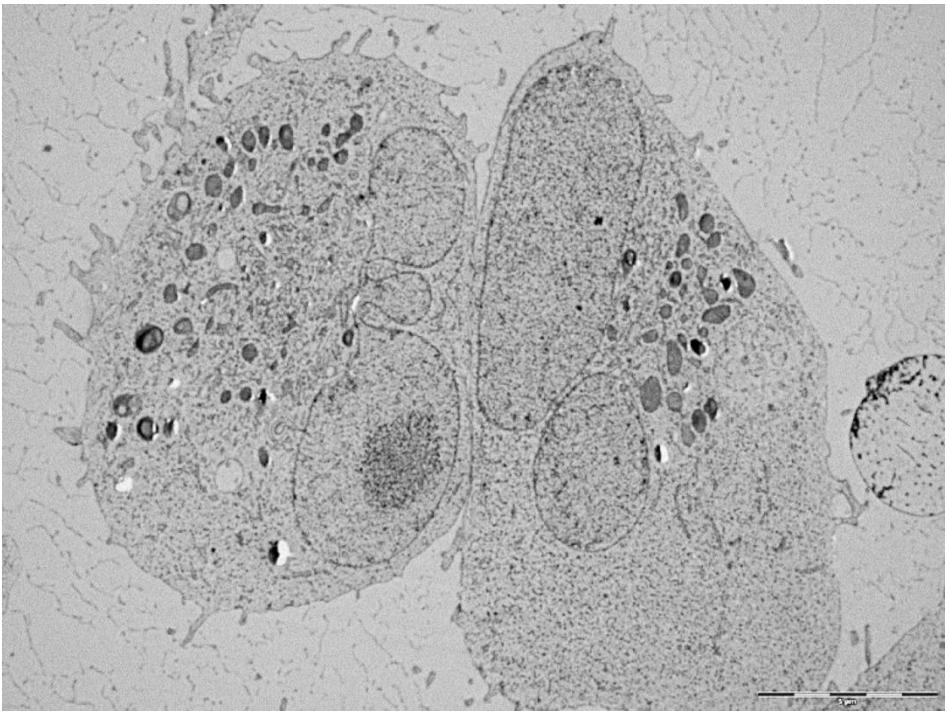


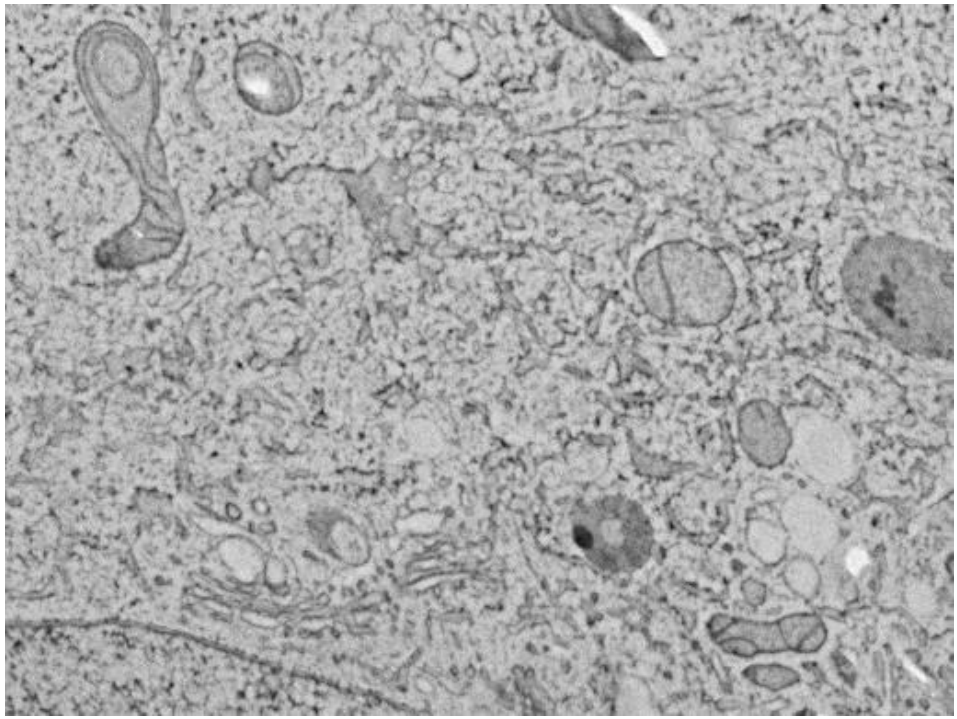
•Cryo-ultramicrotomy of vitreous sections



Room temperature TEM observation: PF+ FS

Tumoral cell line. On grid growth.

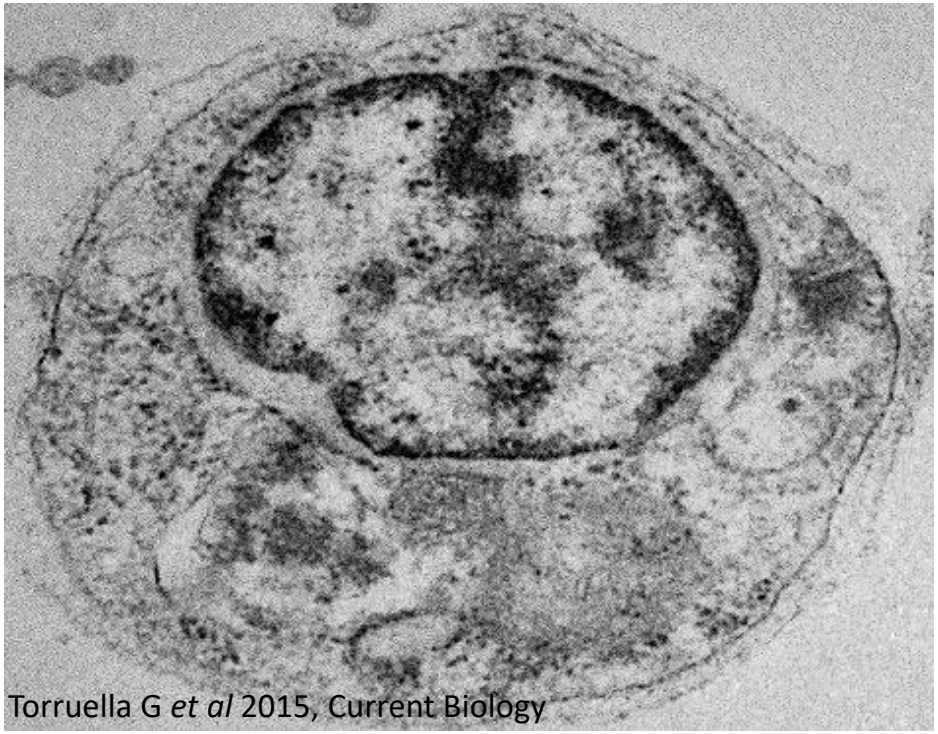




Room temperature TEM observation:

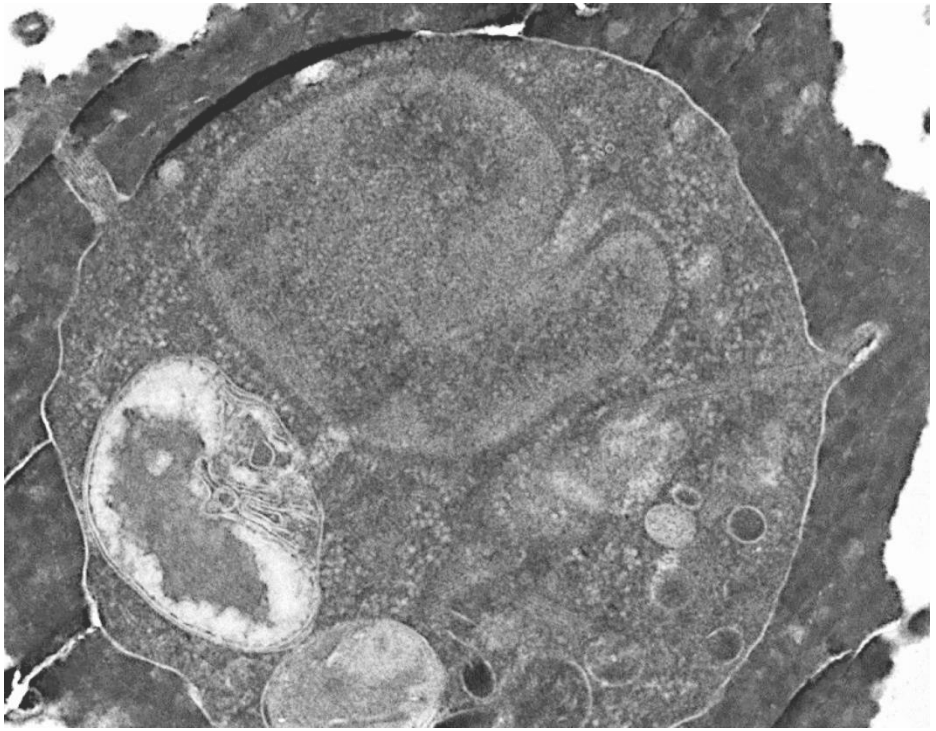
manual plunge freezing + FS vs HPF + FS

Ministeria sp. **Manual plunge freezing**.
Chemically fixed: GA
Cryo-protectants: glycerol



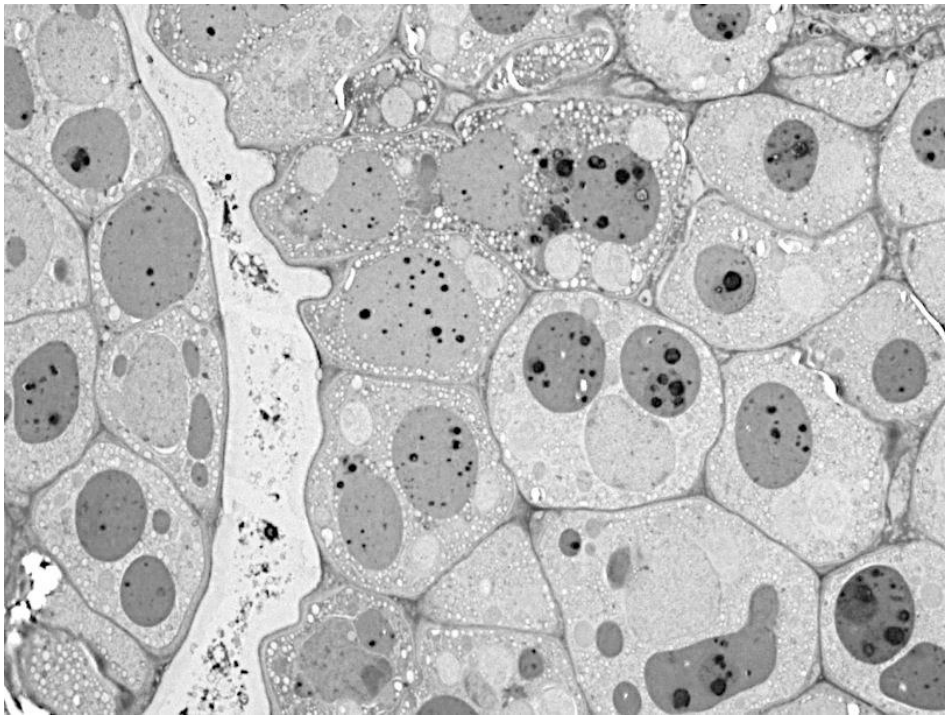
Torruella G *et al* 2015, Current Biology

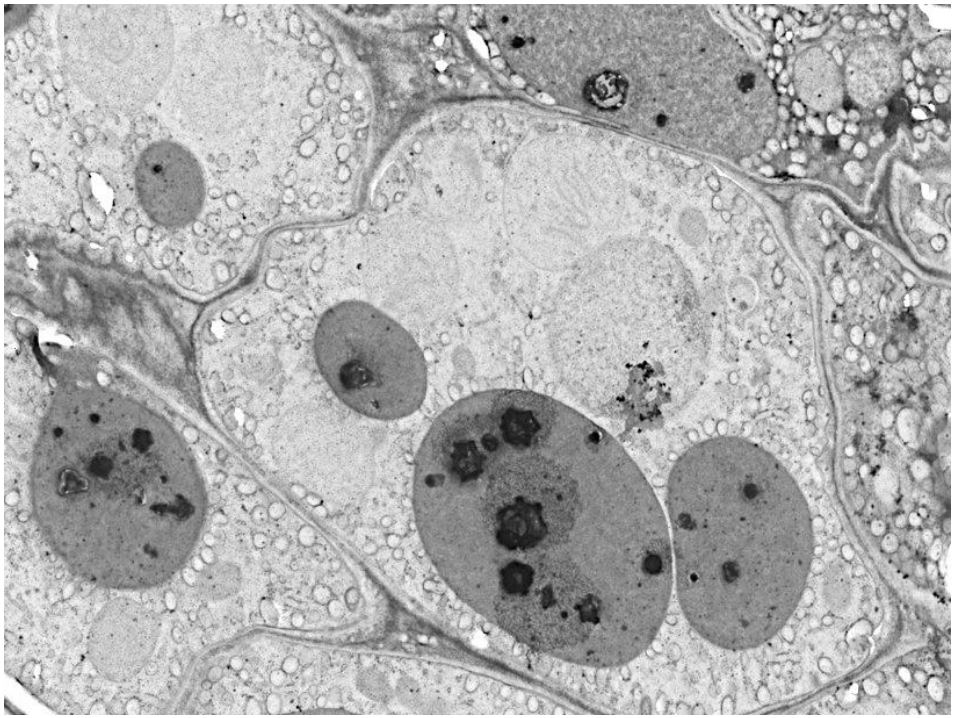
Ministeria sp. **High-pressure freezing**.
Cryo-protectants: no added



Room temperature TEM observation: HPF + FS

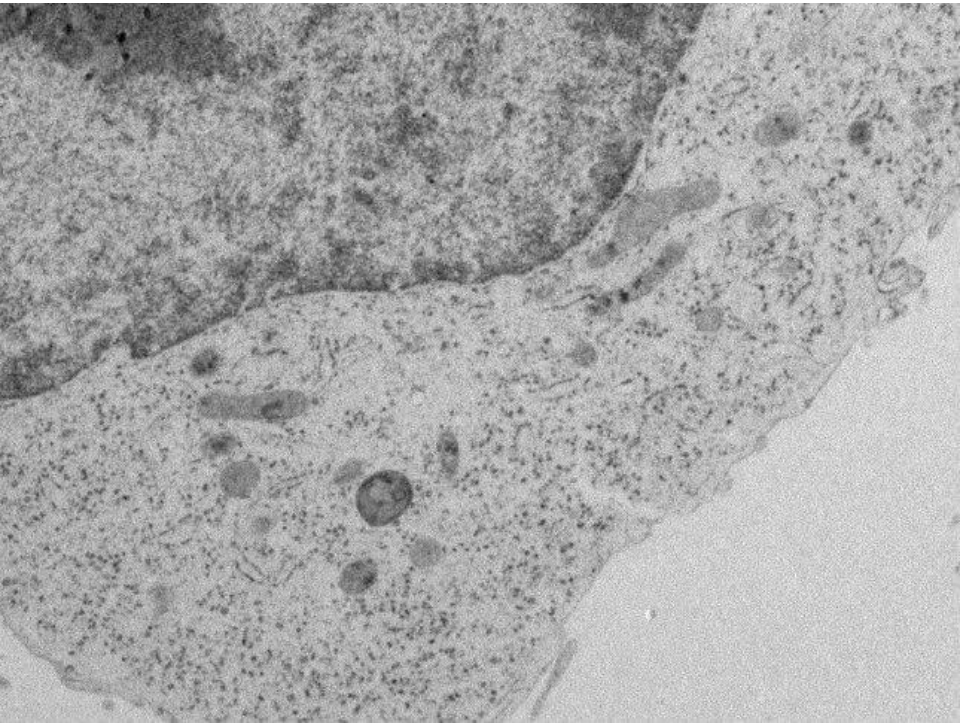
Hydrated seed
Filler/Cryo-protectant: yeast paste



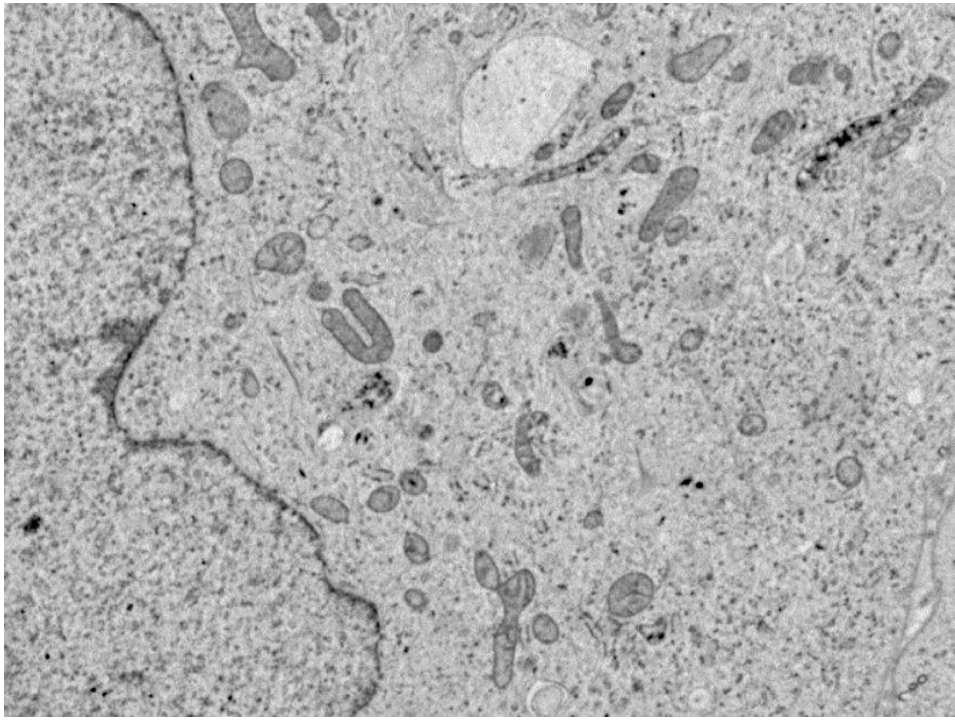


Room temperature TEM observation: HPF + FS

Cell line
Cryo-protectant: no added



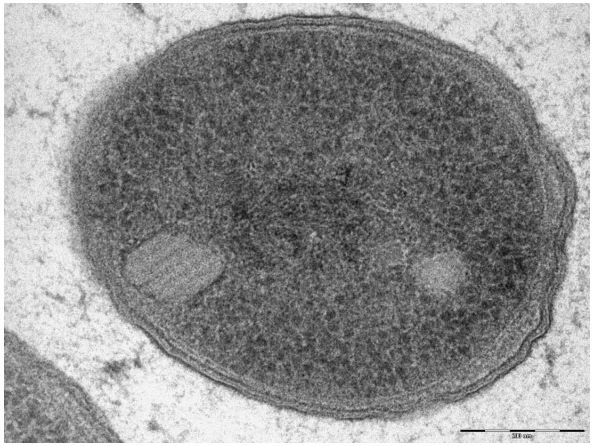
Cell line
Cryoprotectant: 20% dextran PBS



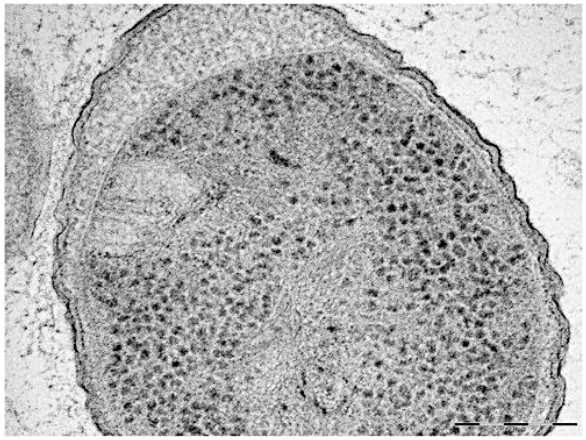
Room temperature TEM observation: HPF + FS

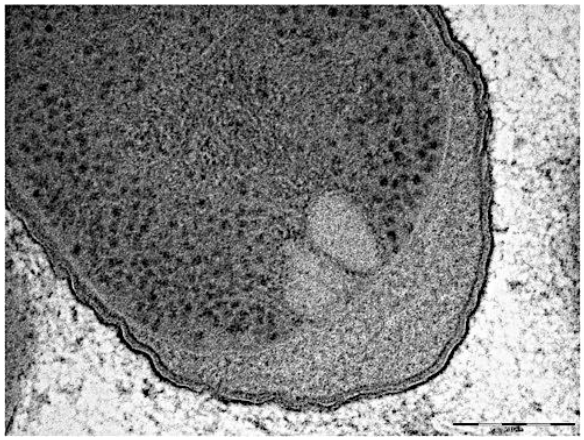
Pseudomonas deceptionensis M1^{T}. Freeze-substitution cocktails.

Structure

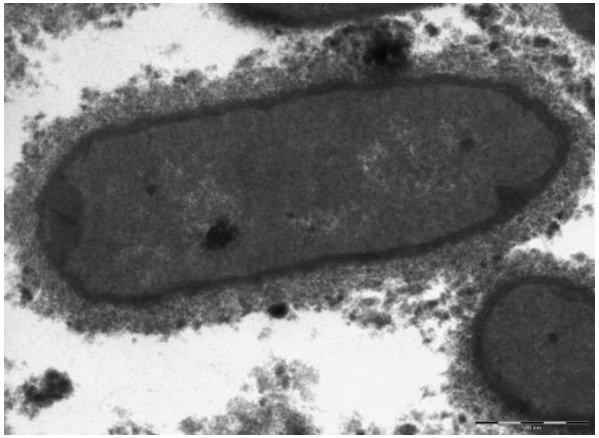


Membranes



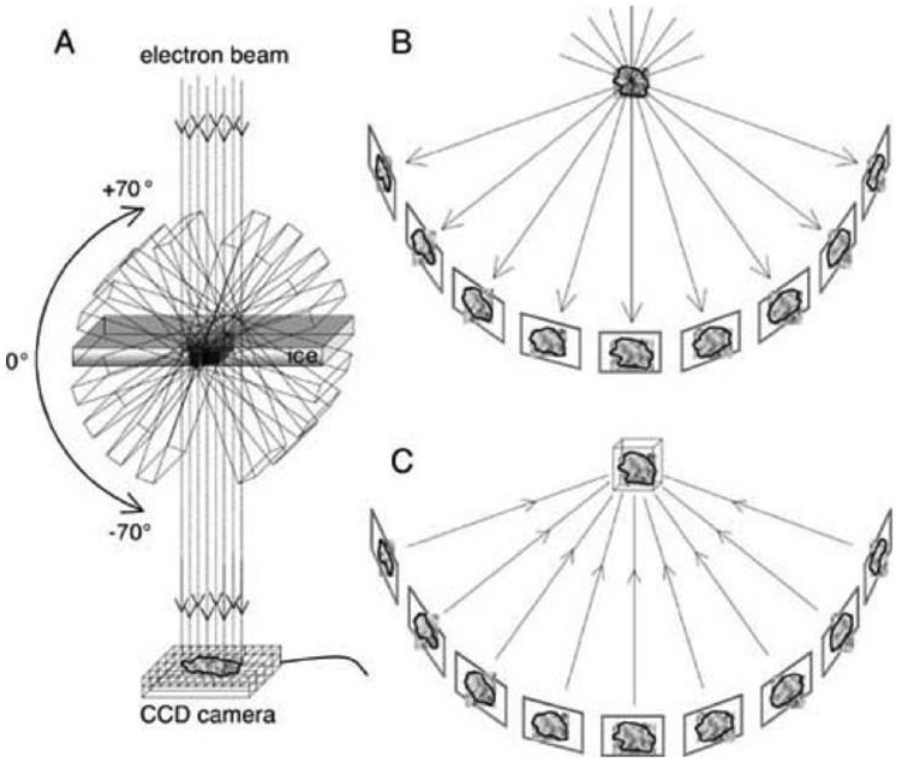


Fatty acid



Visualizing samples in 3D: Electron tomography

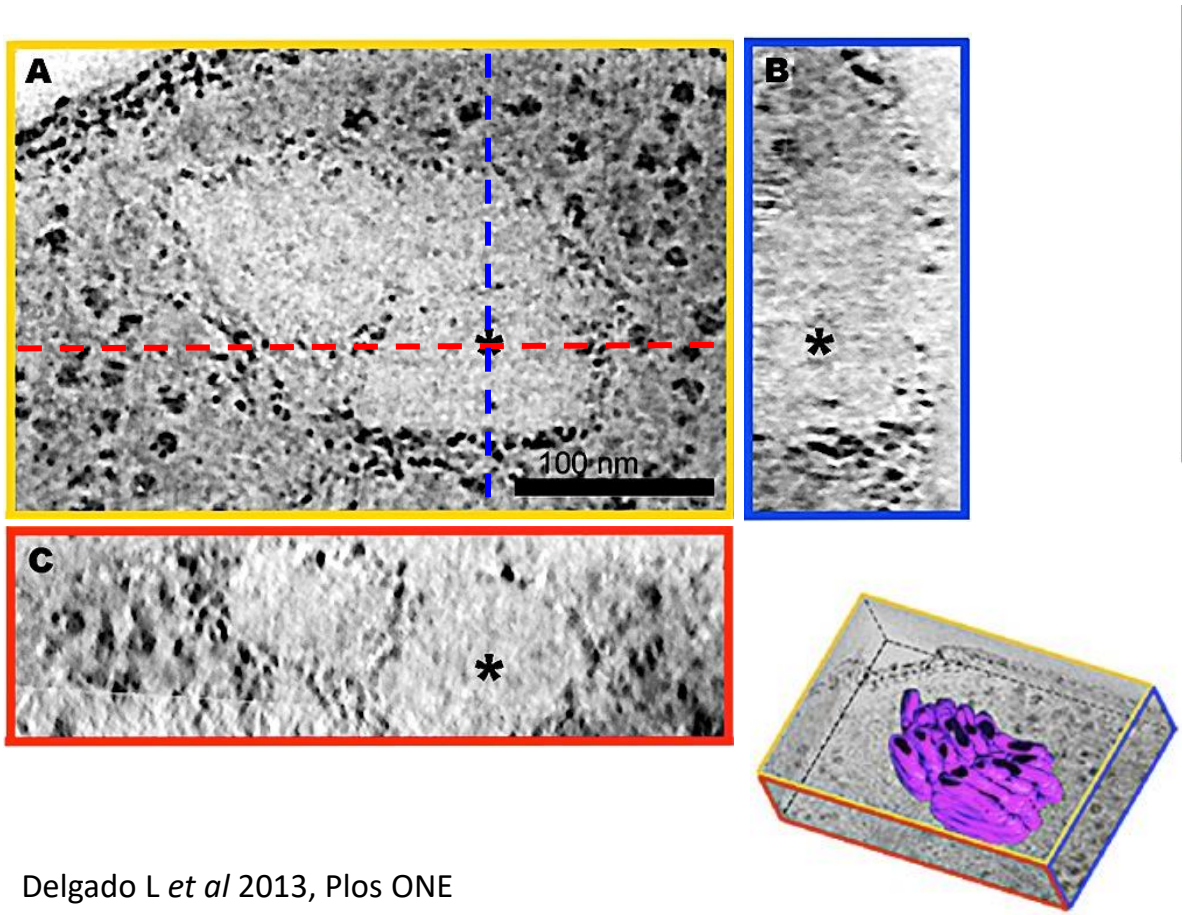
- Several projections of the same structure at different orientations (tilting from -70º to +70º).
- Pictures are processed in a 3D map.



Steven and Belnap, 2005

Visualizing samples in 3D: Electron tomography

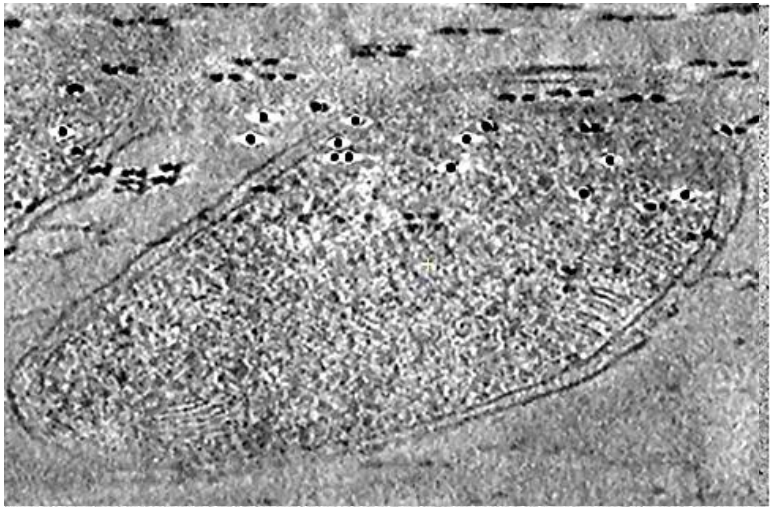
250-nm sections of *Pseudomonas deceptionensis* M1^T processed by HPF-FS

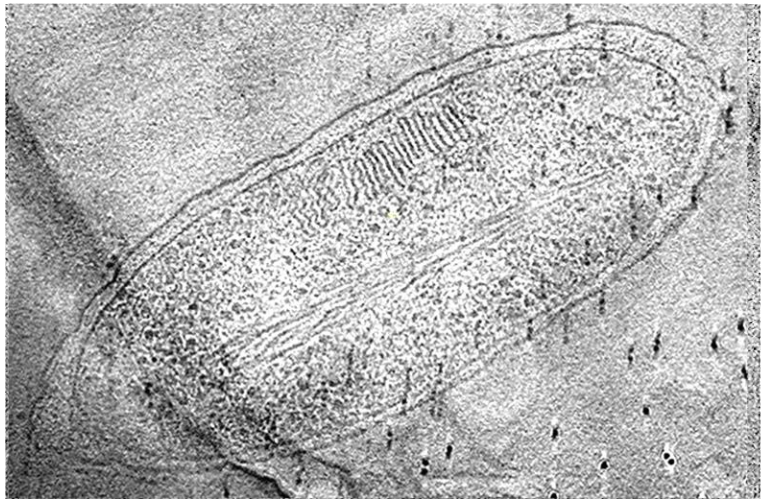


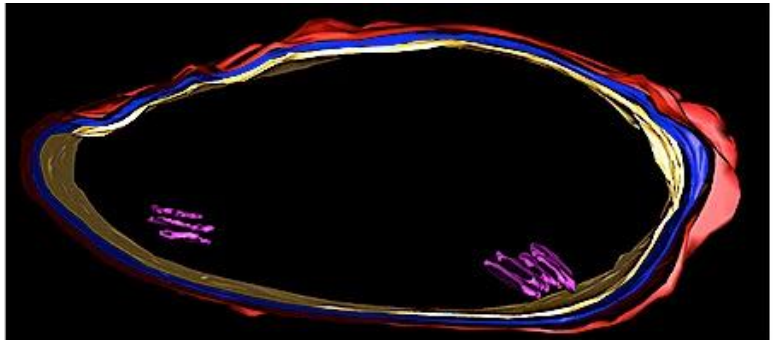
Delgado L *et al* 2013, Plos ONE

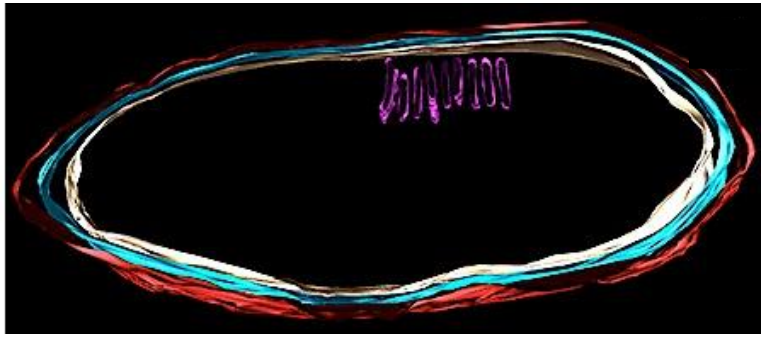
Visualizing samples in 3D: Electron tomography

Vitreous sections of *Pseudomonas deceptionensis* M1^{T}. HPF.





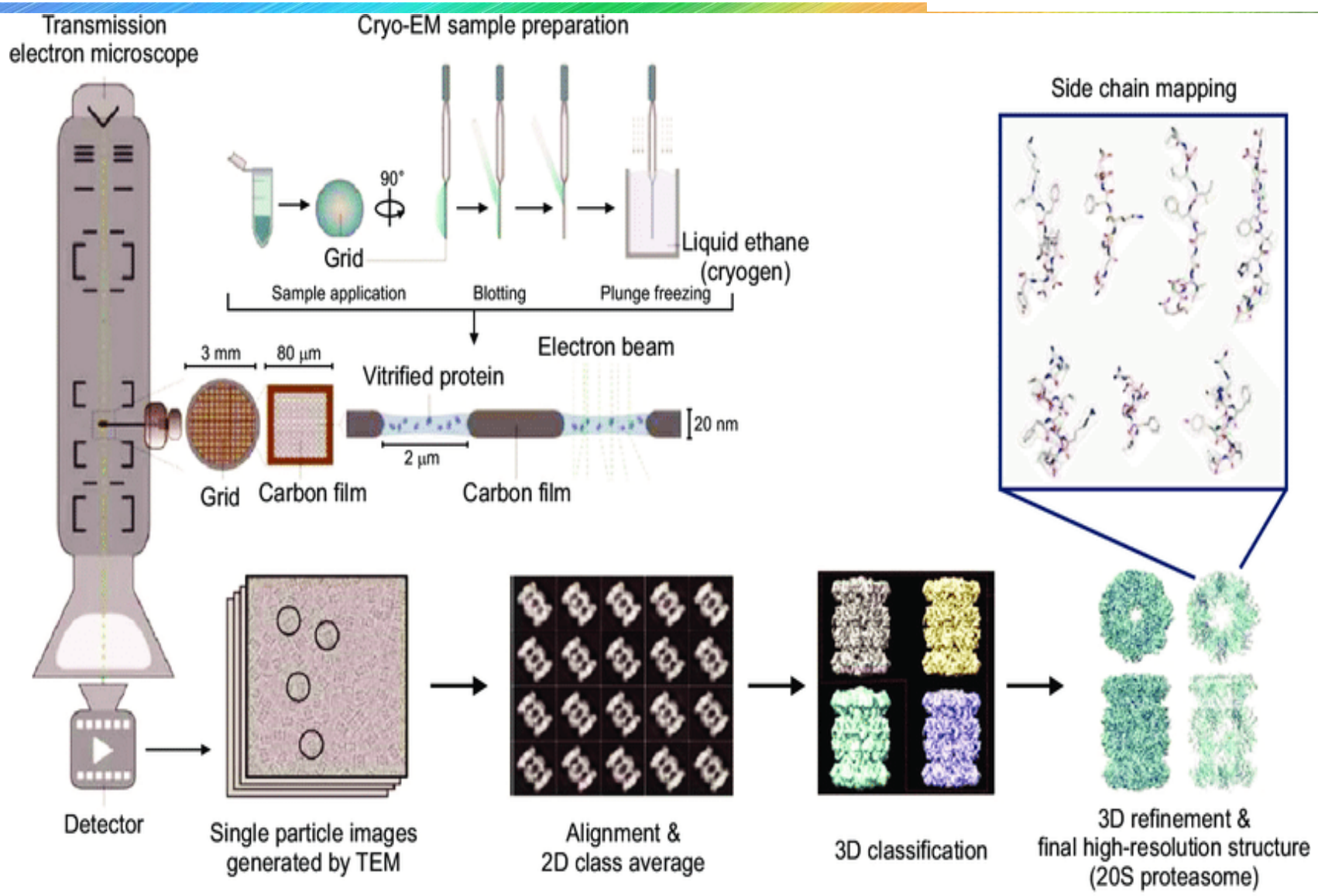




Delgado L *et al* 2015, J Struct Biol

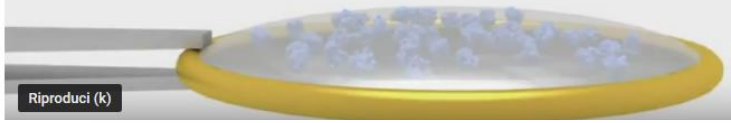
The quality of cryo-EM images now rivals that of X-ray crystallography, long the dominant technique for solving protein structures.

The technique has also succeeded where crystallography has struggled: showing, for instance, how temperature-sensitive ion channels work, characterizing pathological proteins in neurodegenerative disease and detailing how viruses can interact with antibodies

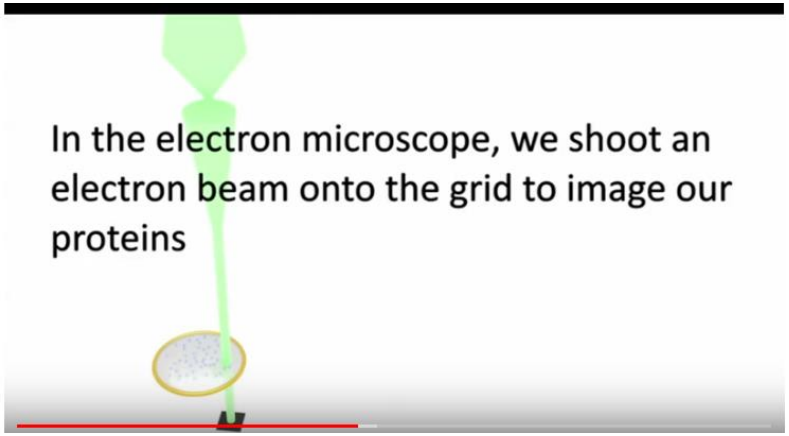


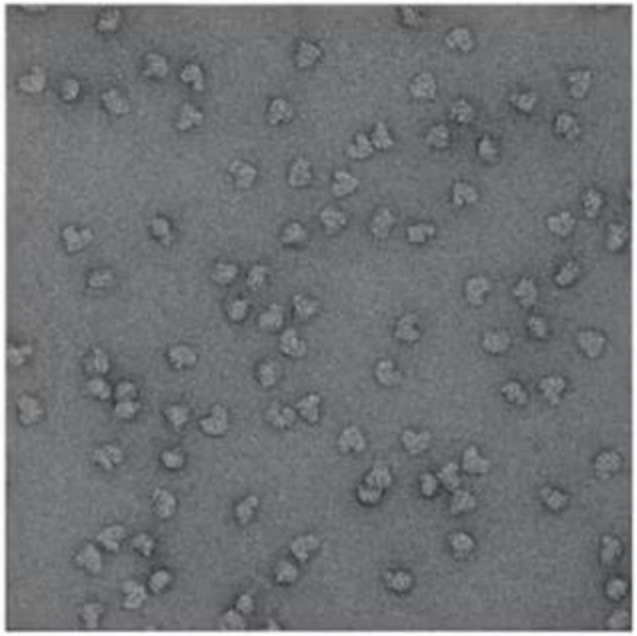
Determination of high-resolution structure through image processing

This is how our sample grid like: our protein particles are embedded in ice, with their native form preserved.



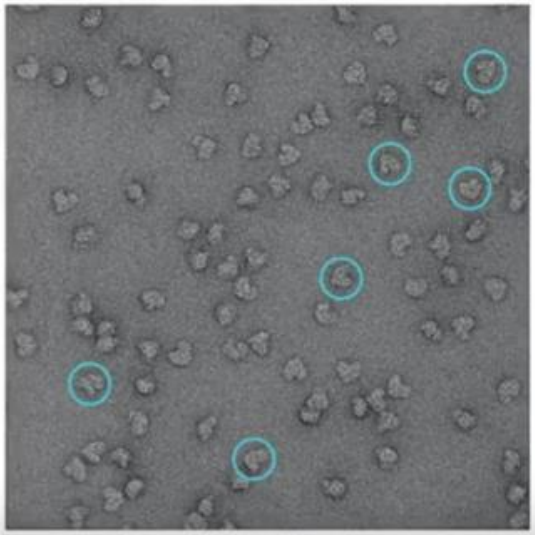
In the electron microscope, we shoot an electron beam onto the grid to image our proteins

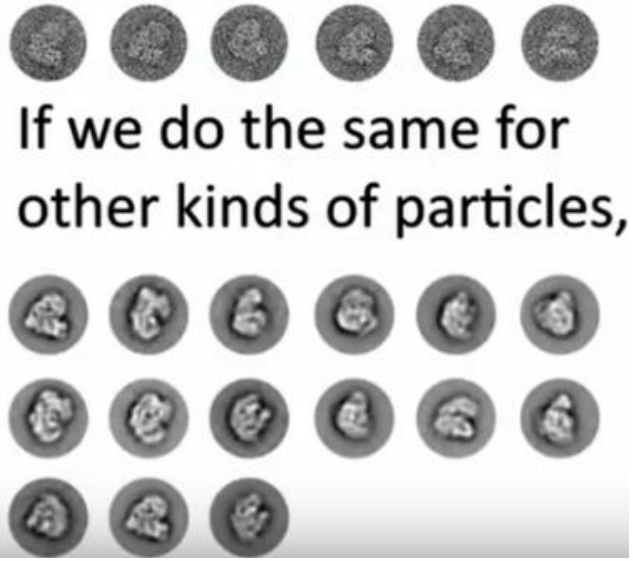




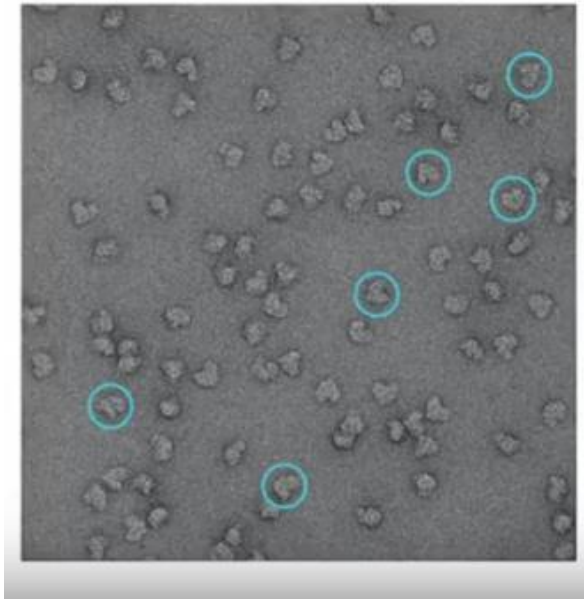
A typical EM image looks like this.

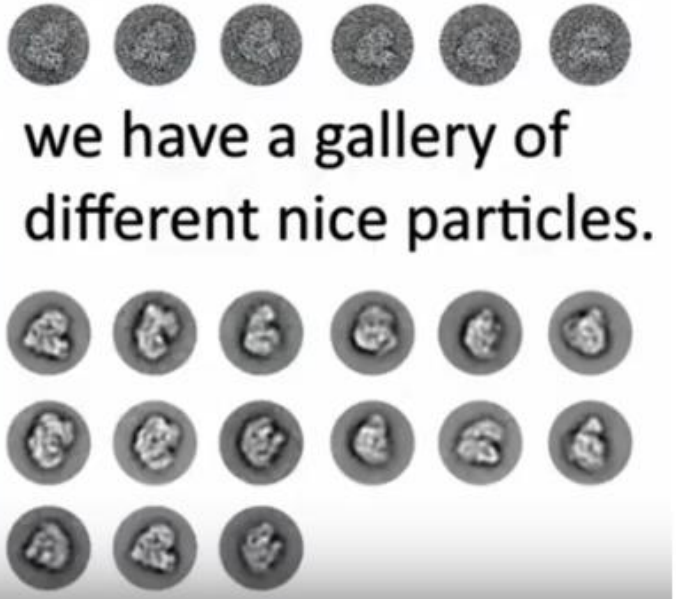
It looks pretty noisy, so we try to make it look clearer.



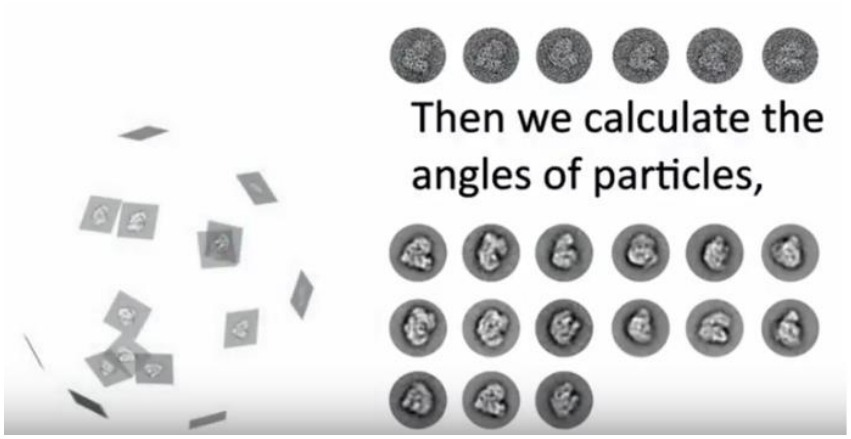


If we do the same for other kinds of particles,

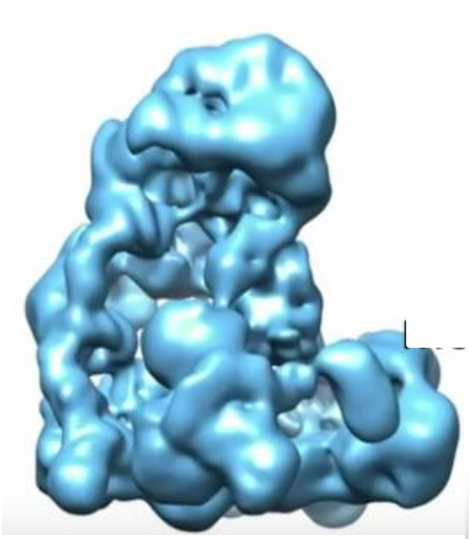


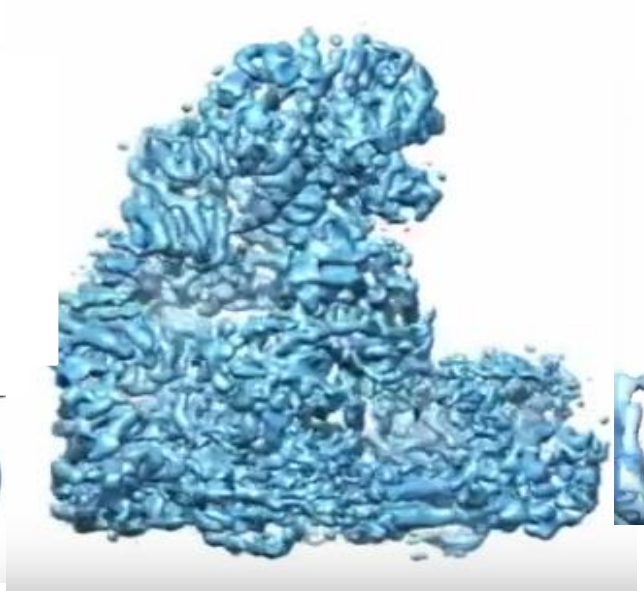


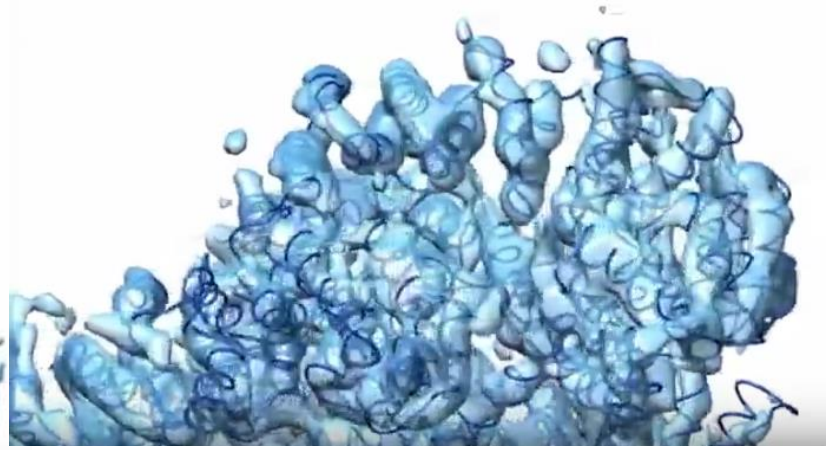
we have a gallery of different nice particles.


Then we calculate the
angles of particles,


and get our first 3D model!







Thank you