# Structure determination by CryoEM, from macromolecules to cells

IMpaCT
IMAGING LIFE FROM MOLECULES TO CELLS

7th EUROPEAN CRYSTALLOGRAPHIC SCHOOL
LISBON | 2022
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ECS7

14th July 2022

7th European Crystallography School
Lisbon | 2022

*Célia V. Romão*

cmromao@itqb.unl.pt

CRYO EM PT

UNIVERSIDADE NOVA DE LISBOA
itqb nova
UNIVERSITY OF HELSINKI
CSIC
מכון ויצמן למדע
WEIZMANN INSTITUTE OF SCIENCE

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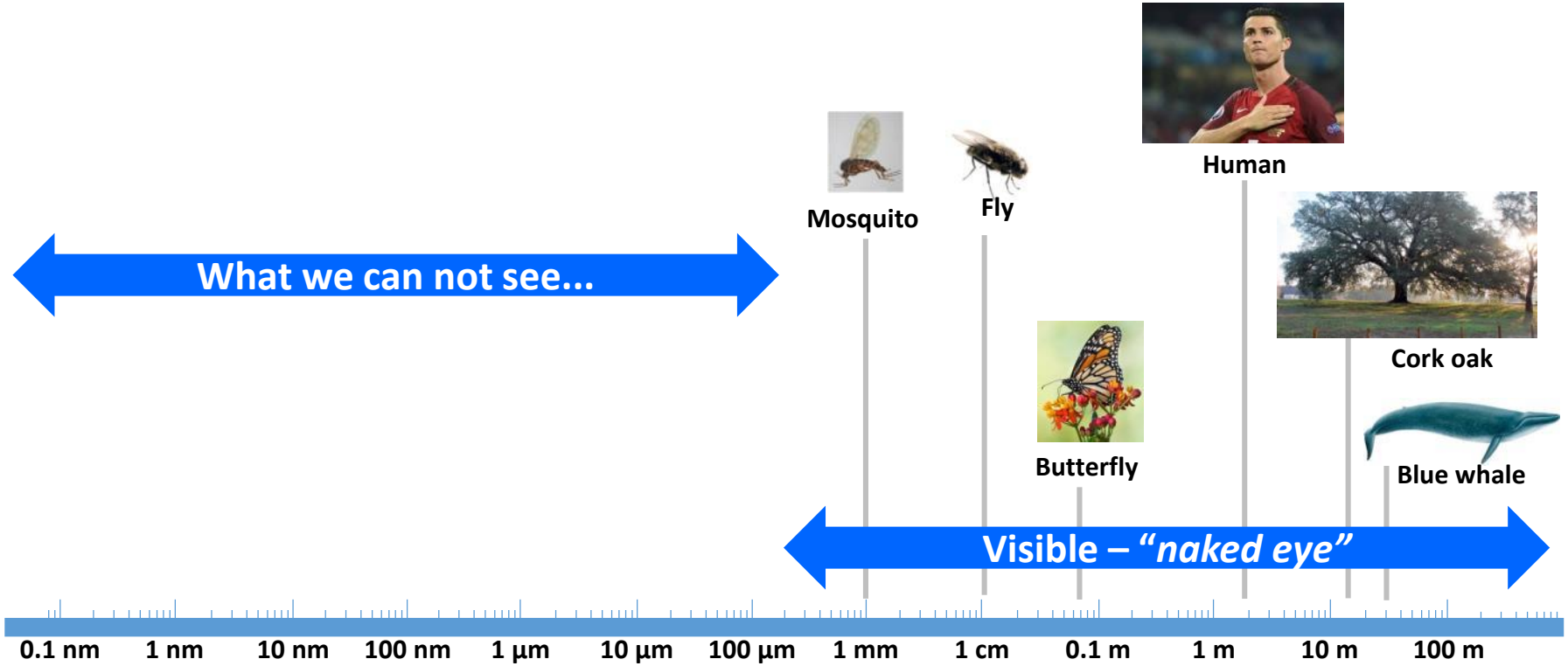
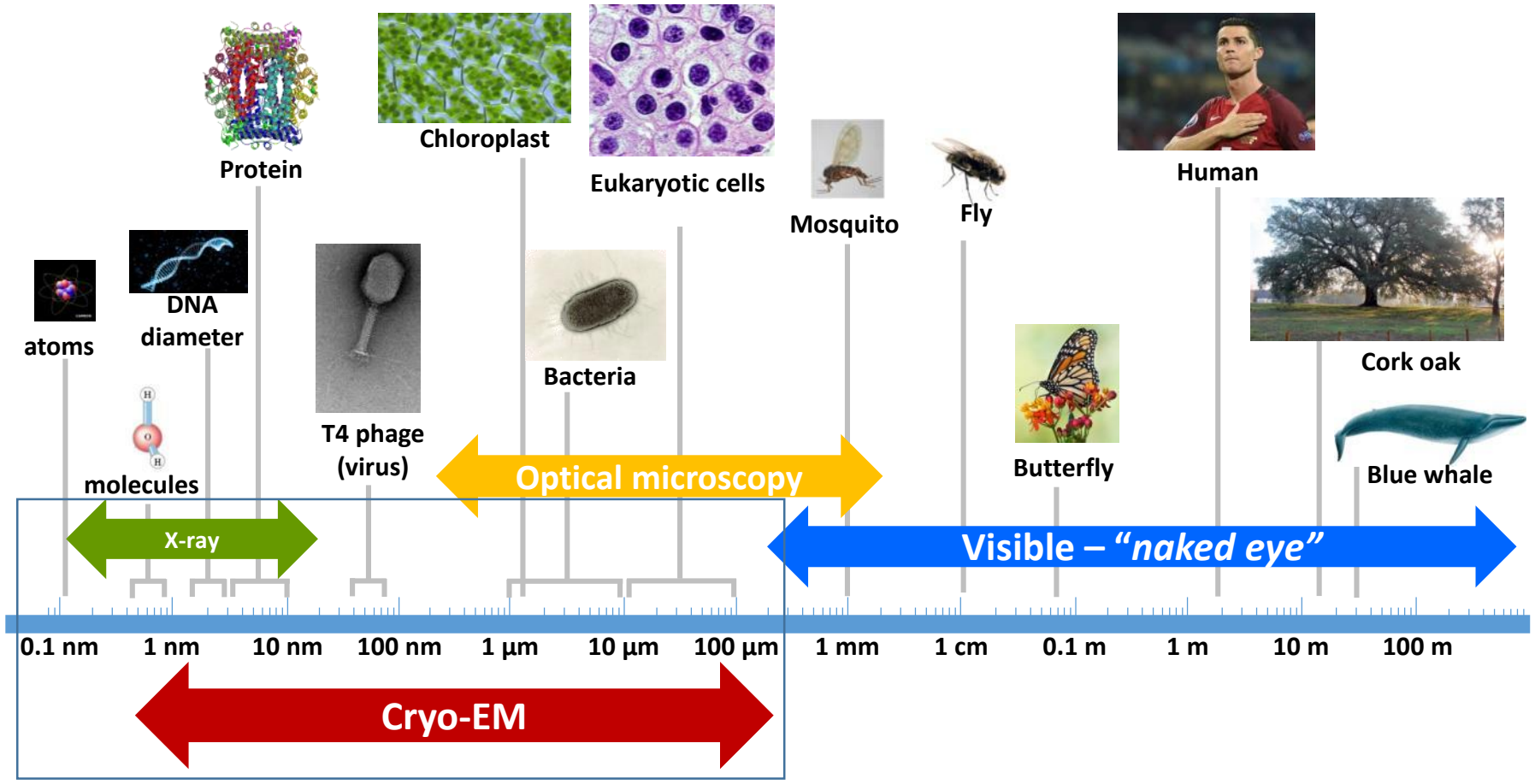
# *Life as we see*

# Length scales

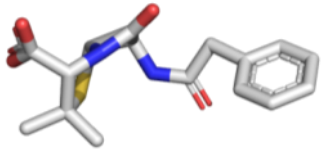


# Length scales and structural biology approaches

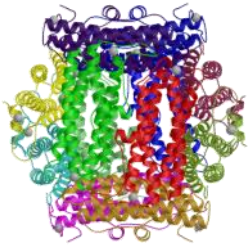


# Structural biology approaches

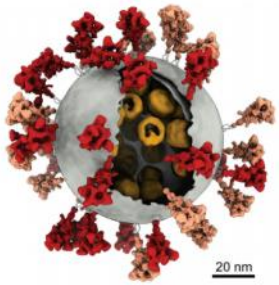
small molecules



Proteins and protein complexes

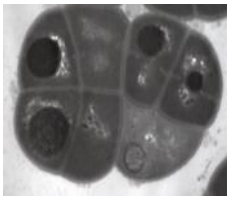


Viruses and vesicles

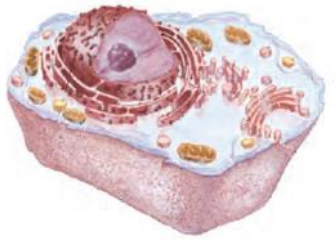


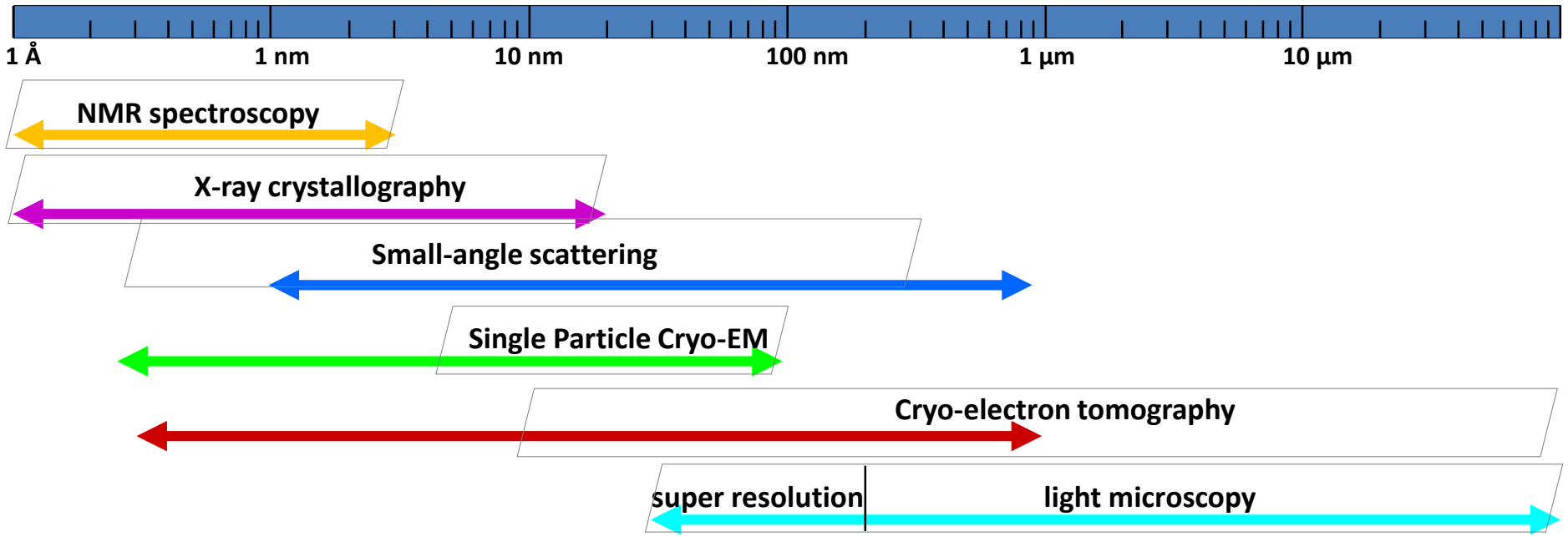

10.1016/j.cell.2020.09.018

Prokaryotic cells and organelles



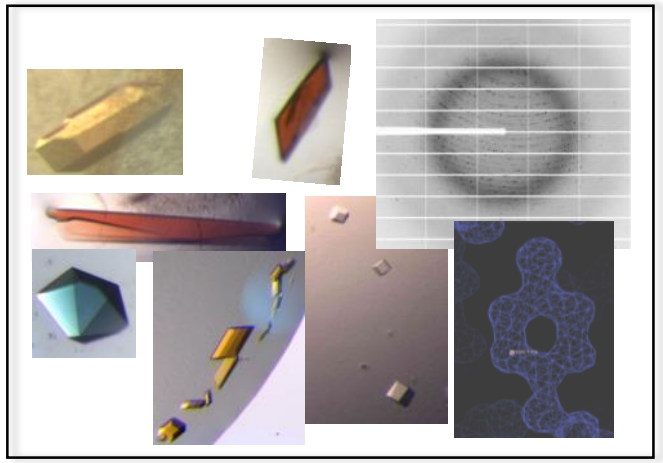
Eukaryotic cells



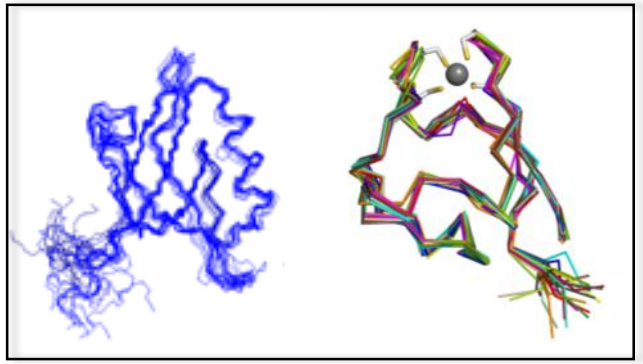


# Structural biology approaches

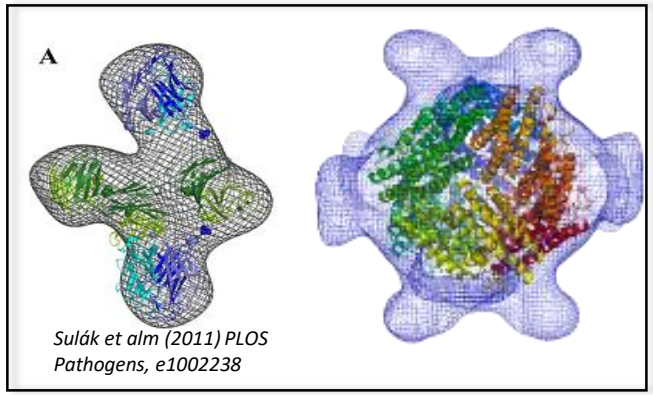
***Protein crystallography***



***NMR***

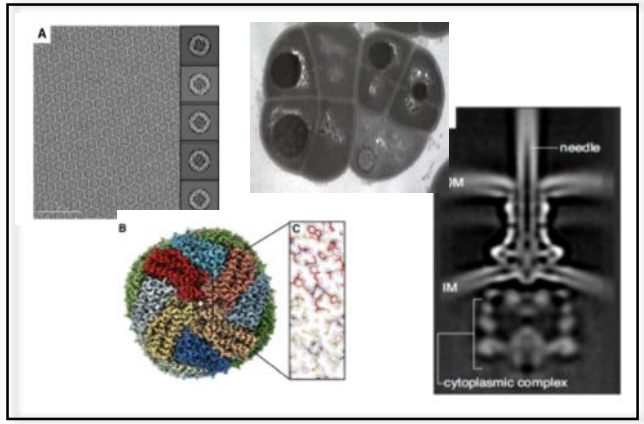


**INTEGRATIVE STRUCTURAL BIOLOGY**




*Sulák et alm (2011) PLOS Pathogens, e1002238*

***Small Angle X-ray Scattering***




***CryoEM (SPA, CET, microED)***

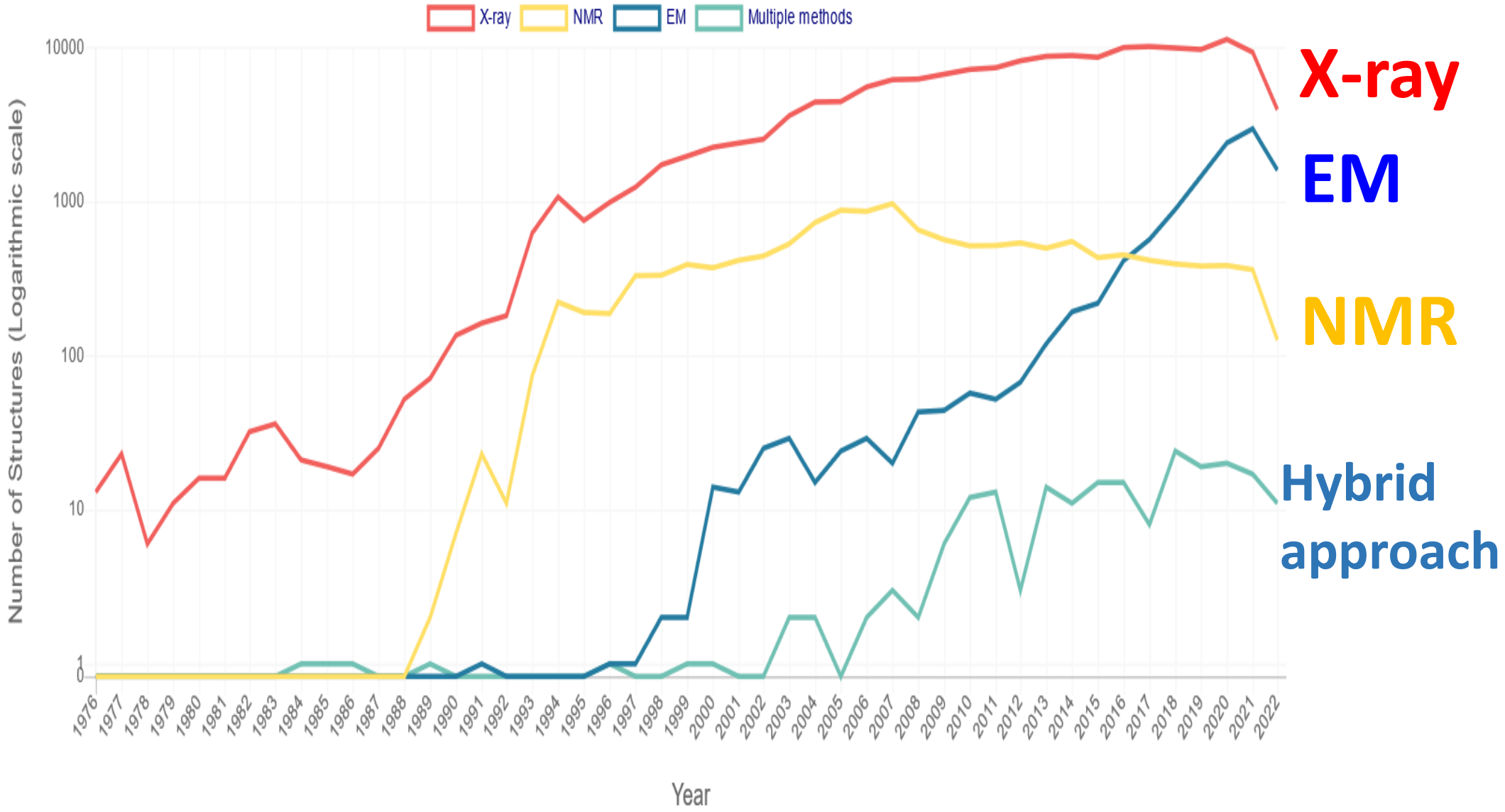
**Biophysical methods**

**Spectroscopies**

***Hybrid methodologies***

**Fluorescence Microscopies…**

# Structures deposit@ Protein Data Bank (PDB database)



# CryoEM, the revolution era

***simplify and improve the visualization of macromolecules.***

The Nobel Prize in Chemistry 2017


© Nobel Media AB. Photo: A.Mahmoud

**Jacques Dubochet**
University of Lausanne
Switzerland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**Joachim Frank**
Columbia University
New York


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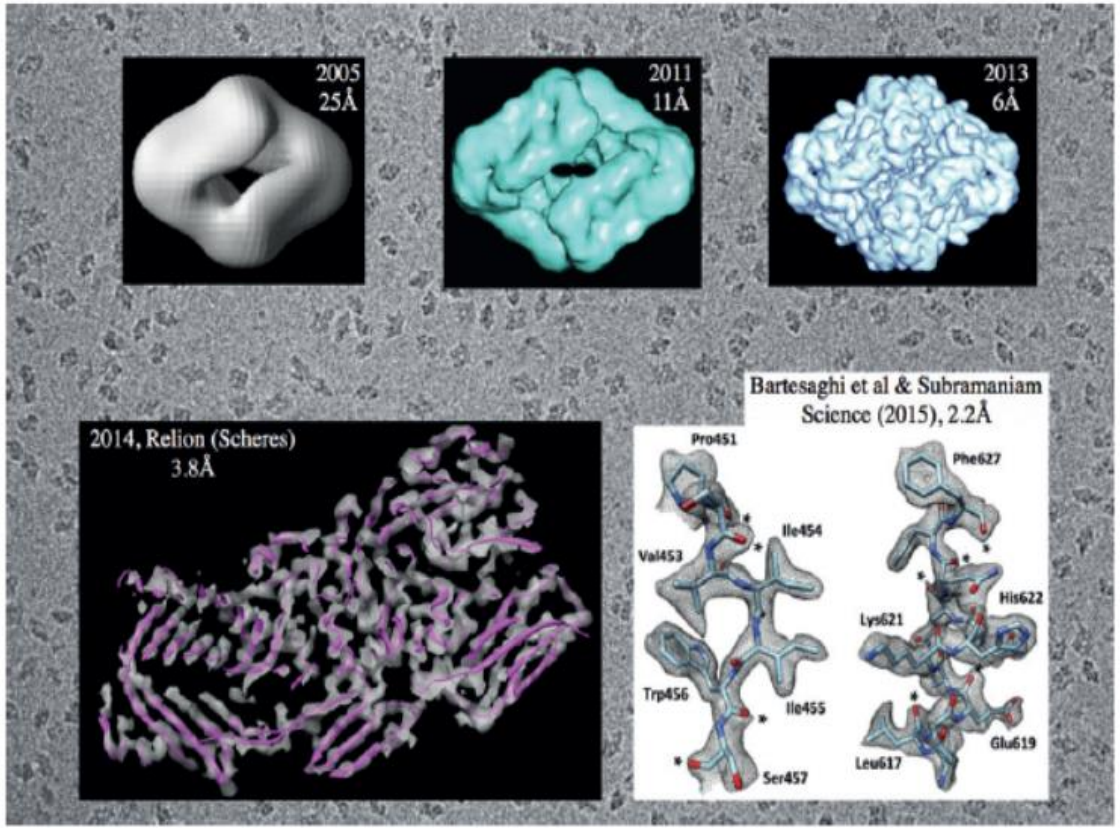
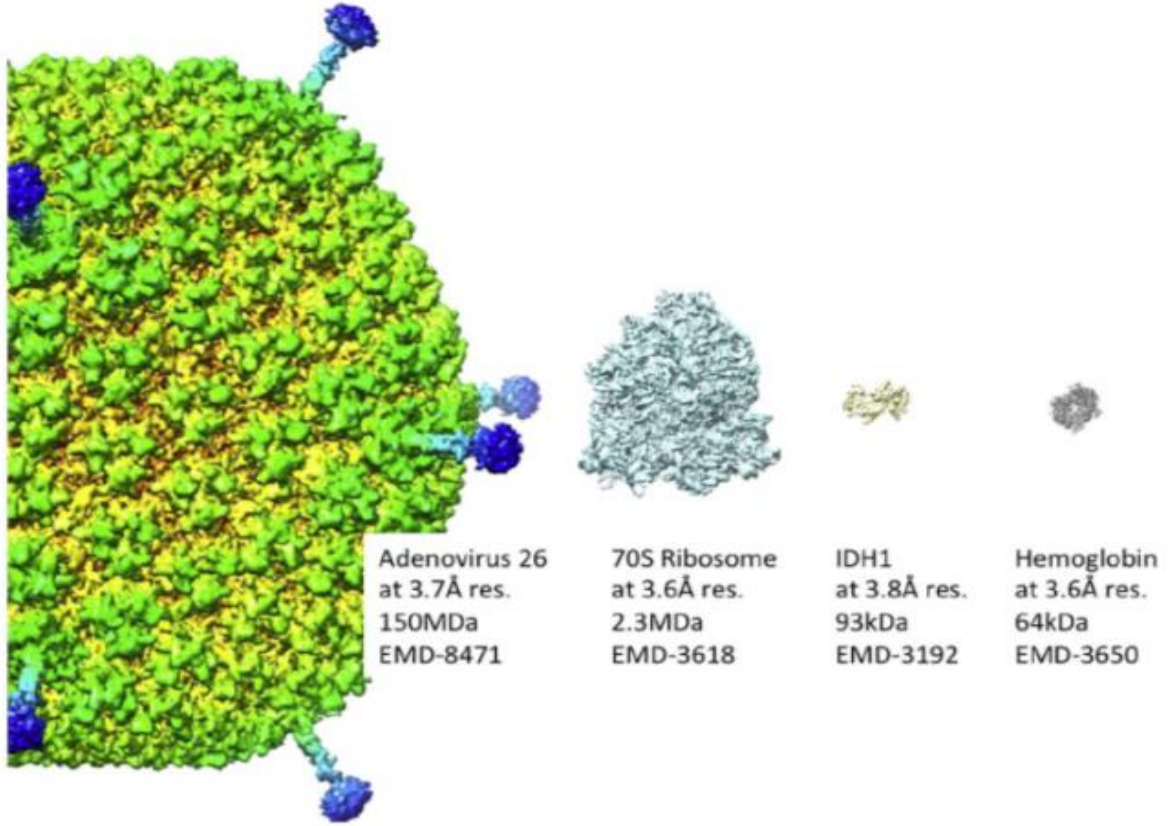
**Richard Henderson**
MRC Laboratory of Molecular Biology
Cambridge, U.K.

# Resolution and size

## Atomic detail - Resolution




## Size of Macromolecules




Murata and Wolf (2018) BBA 1862, 324-334

# Before the revolution era

***Working mode:*** **electron source – system in vacuum**

***Sample preparation***

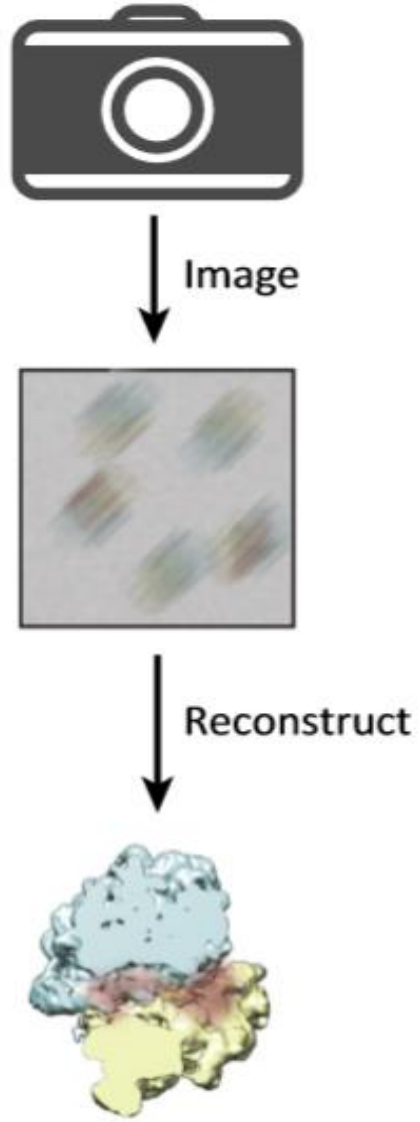
Macromolecules  collapse under vacuum conditions

Macromolecules are destroyed due to the radiation effects

Noiser images with low resolution- recorded on photographic film

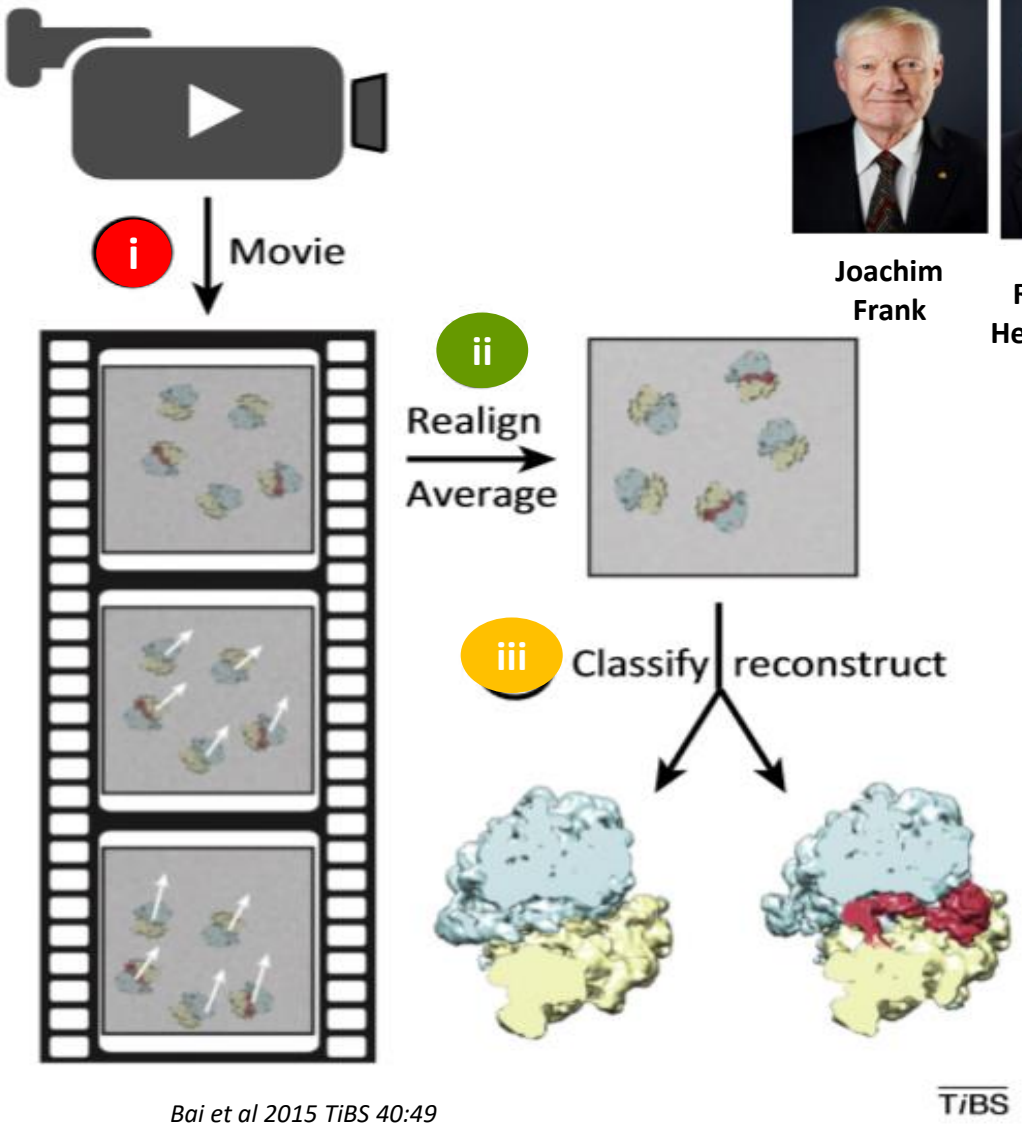
Image blurring due to beam-induced sample motion

Structurally different particles were often mixed in a single reconstruction.




*Bai et al 2015 TiBS 40:49*

# CryoEM, the revolution era




*Bai et al 2015 TiBS 40:49*



**Joachim Frank**

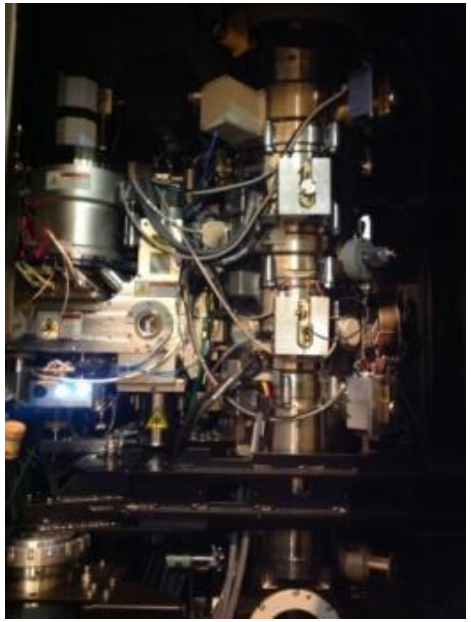


**Richard Henderson**

i **Digital direct-electron detectors :**
recording movies during exposure; data with high quality.

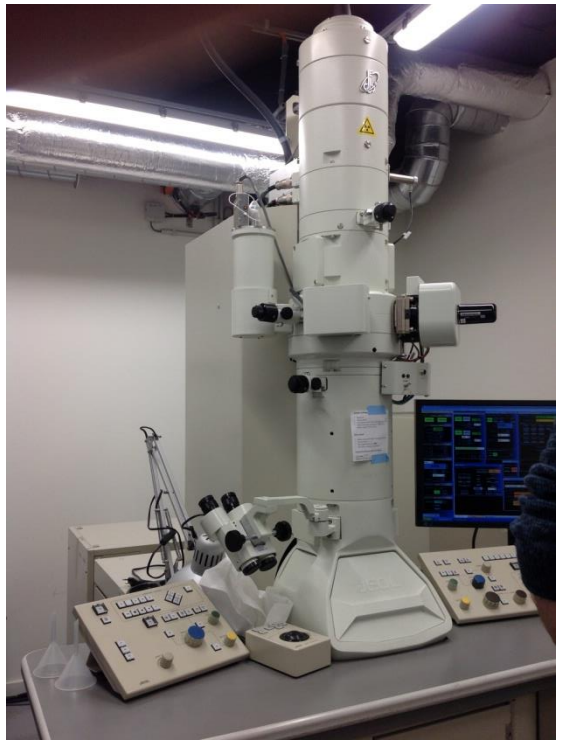
ii **Computer programs:**
realign the movie frames may correct for sample movements induced by the electron beam.

iii **Powerful classification methods:**
Identification of multiple structures from a sample mixture

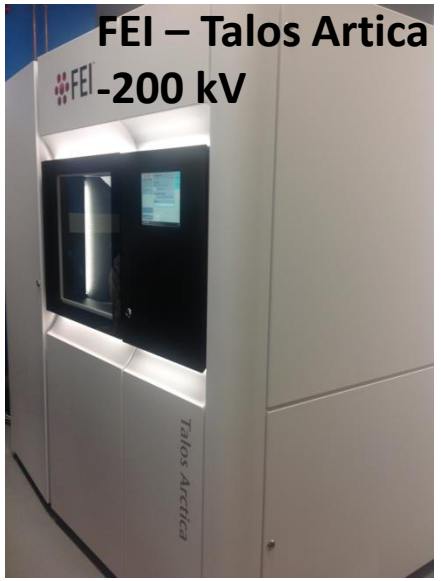
# Transmission electron microscopes

## Standard TEM
## 100 kV



## Cryo EM
## 200 kV
## 300 kV

Fisher – Titan Krios-300 kV




FEI – Talos Artica -200 kV

JEOL –300 kV

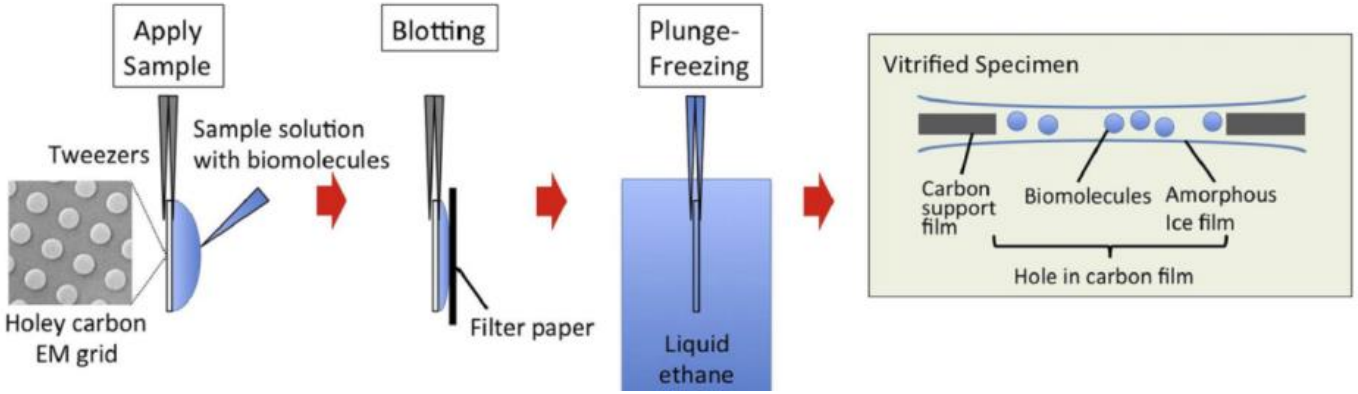
# CryoEM, the revolution era

Jacques Dubochet

## Sample preparation

***Vitrification – Plunge freezer***

Rapid cooling at low temperatures (in liquid ethane -188ºC) of the individual molecules in a very thin layer of vitrified water.




***Vitrification advantages:***

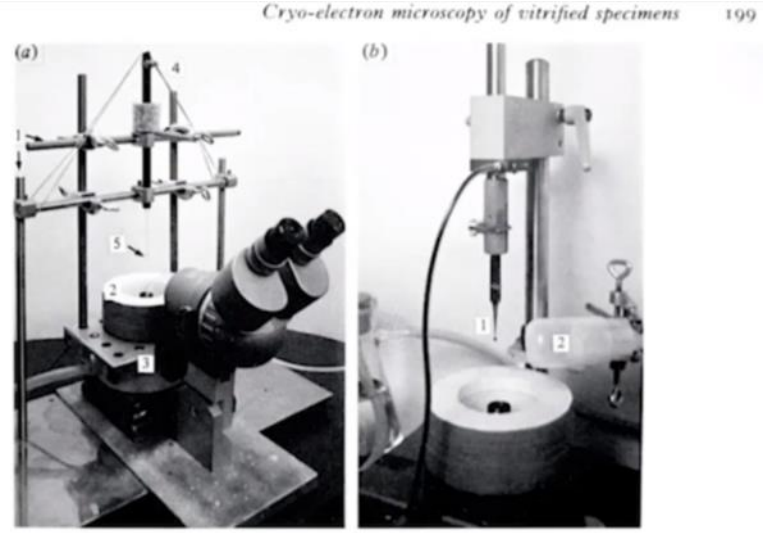
- Macromolecules are kept in a close-to-native environment, even under vacuum conditions

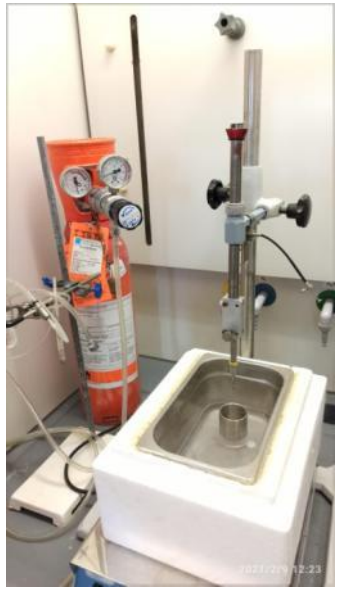
***Data collection at low temperature (liquid nitrogen):***

- Protects the macromolecules from the dehydration by imaging
- Cooling at low temperature helps to reduce the damage caused by the electron beam

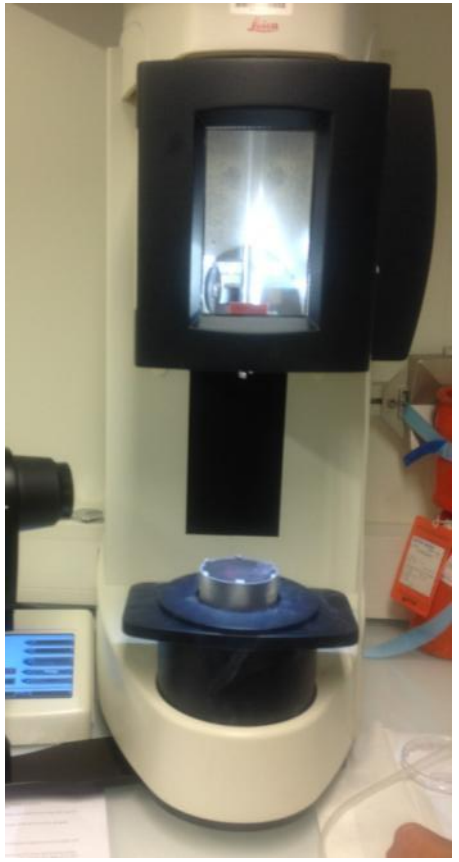
*Murata & Wolf 2018 BBA 1862:324*

# Freeze plunger

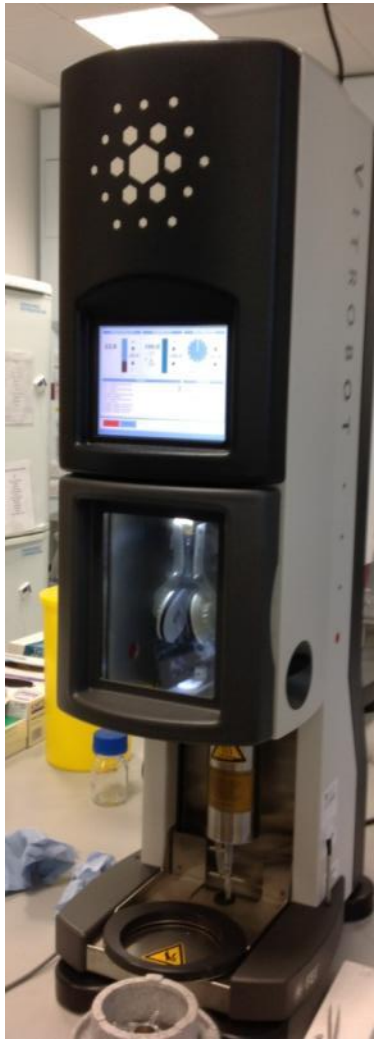


Gatan



Leica

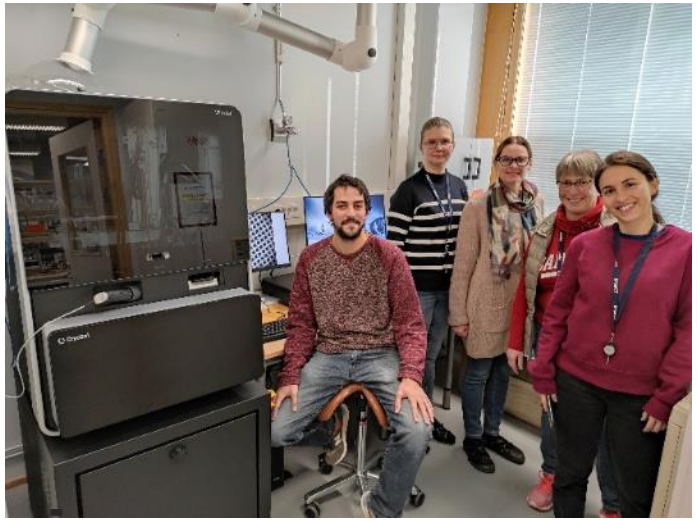


Fisher

# CryoEM, the revolution era

CryoSol

## VitroJet™

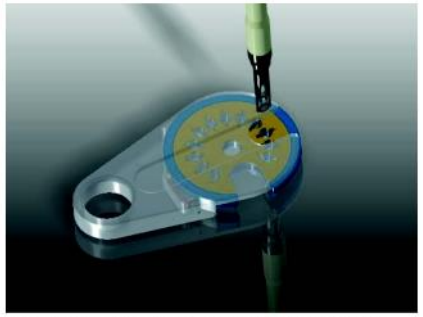


Sarah Butcher's Lab
@University Helsinki

**VitroJet™ :**

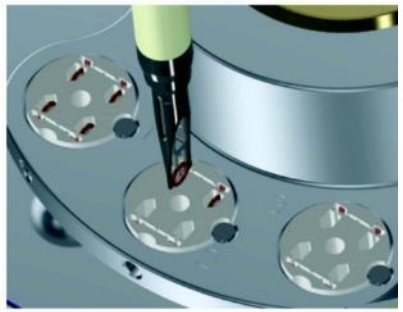
- brings control and automation to grid preparation.
- Method for better control and reproducibility in sample preparation, maximizing throughput, and minimizing the waste of valuable high-grade protein.
- pin printing technology
- *uses less overall sample for deposition by depositing the sample onto the grid with a pin and eliminates blotting - a method known for producing inconsistent ice quality.*
  - ✓ Eliminate blotting: apply only the amount of sample you want
  - ✓ Customize ice thickness: adjust parameters of sample size and vitrification based on user programmable specifications
  - ✓ Reproducible: consistent ice thickness, vitreous status, and overall quality

PRE-CLIPPED AUTOGRIDS



*The VitroJet™ freezes grids much faster than conventional plunge freezing techniques.*

AUTOMATION

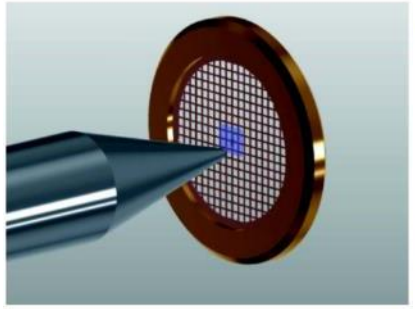


*Avoids grid damage. All the vital steps for preparation are done automatically without user intervention.*

PLASMA TREATMENT

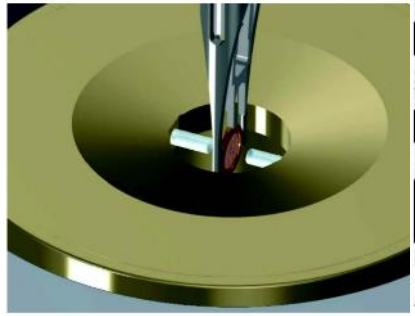
*The integrated plasma treatment provides a tunable and consistent method enhancing the wettability of the grid. Surface energy is controllable, reproducible, and designed for flexibility; so users can incorporate different to achieve results in wettability.*

PIN PRINTING



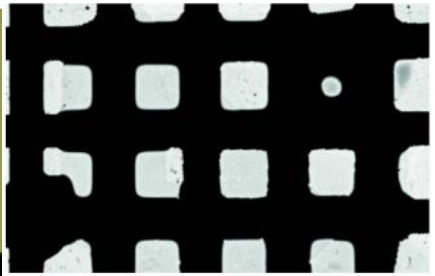
*The system uses less overall sample for deposition by depositing the sample onto the grid with a pin and eliminates blotting; a method known for producing inconsistent ice quality.*

ETHANE JETTING TECHNOLOGY



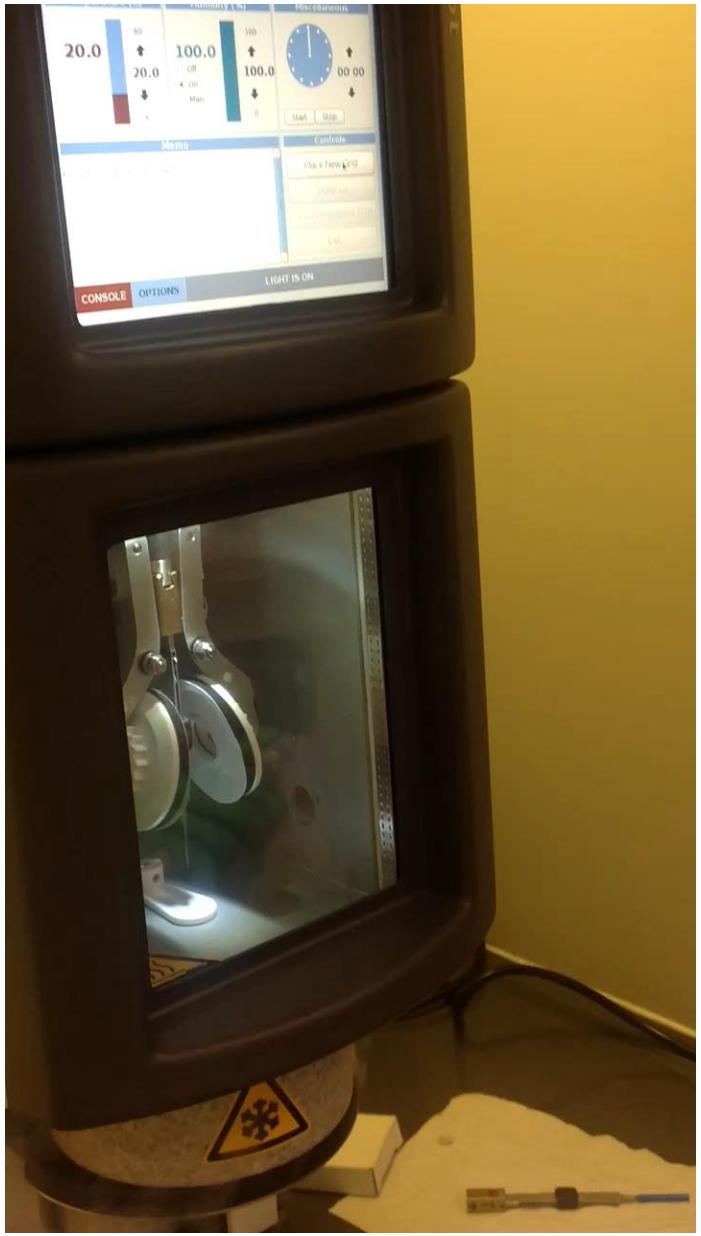
*Ensures vitrification of pre-clipped autogrids.*

VISUAL FEEDBACK



*Visualize the entire sample deposition process with a camera to monitor the success of deposition.*

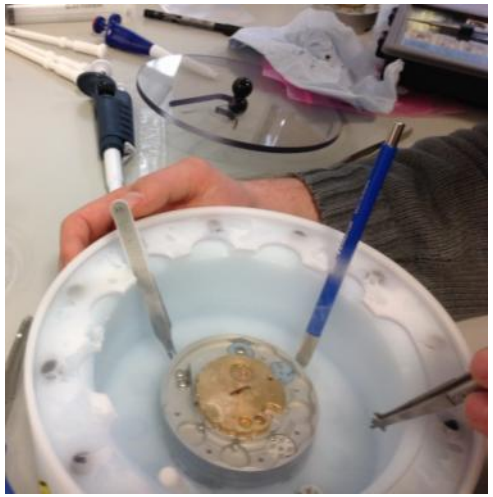
- ▸ Assess deposition quality prior to microscope screening
- ▸ Log videos, images, and settings for future export for lab journals

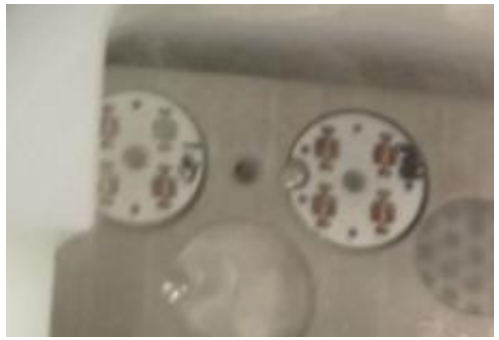

20.0
20.0
100.0
100.0
00 00
Start
Stop
Memo
Controls
CONSOLE
OPTIONS
LIGHT IS ON

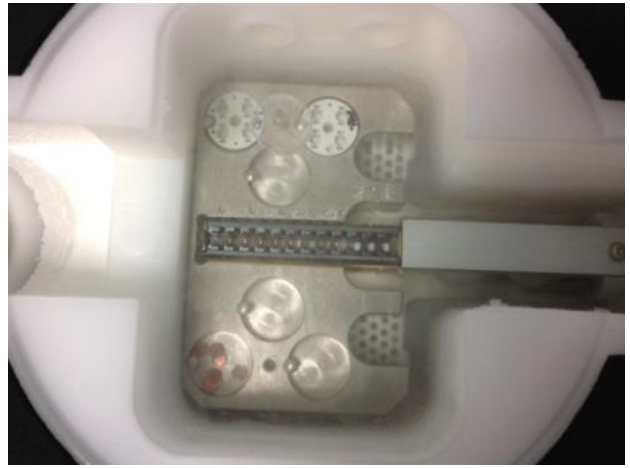
IMpaCT

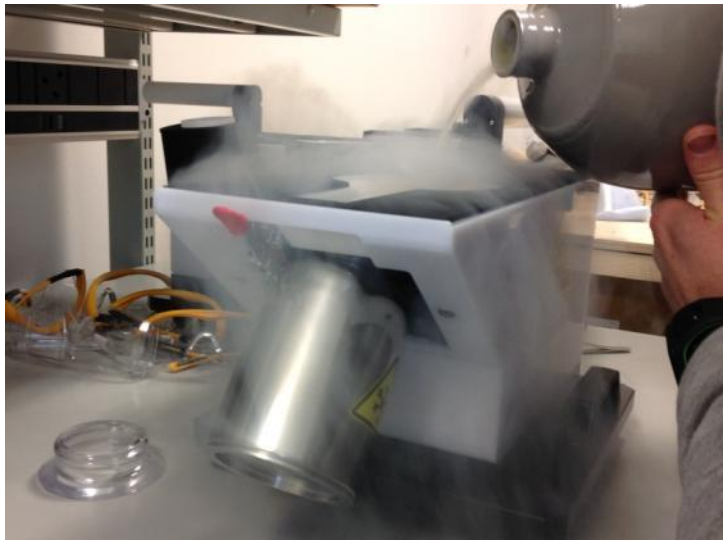
# Sample

***Sample – vitrified in liquid ethane, after is kept under liquid nitrogen temperature, ca. -180ºC***









# CryoEM, the revolution era

## Combination of two major communities

### Traditional electron microscopy

***Standard TEM***
***100 kV***

### Macromolecular X-Ray Crystallography

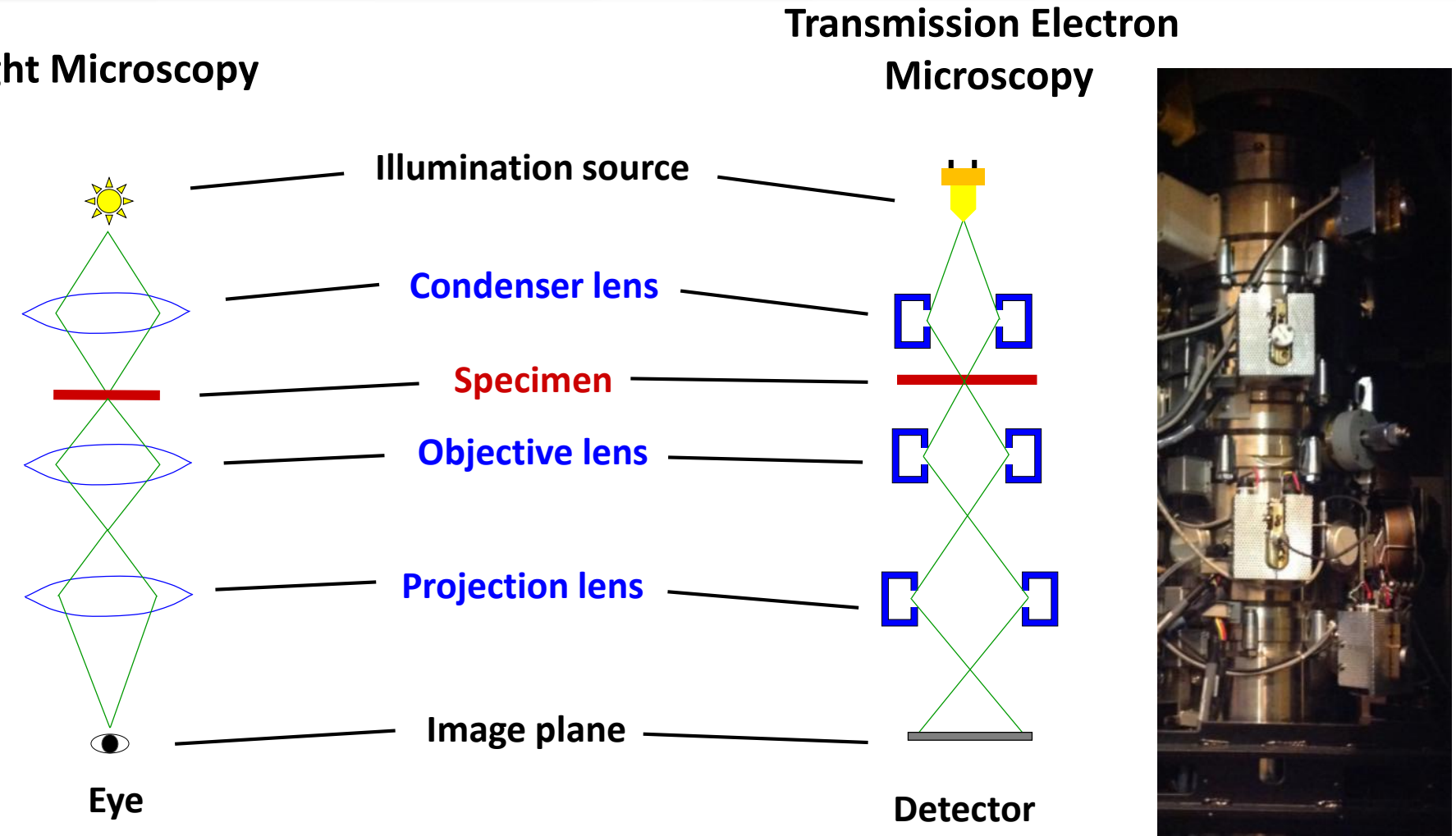
**Running CryoEM as "beamlines"**

**AUTOMATION**

**AUTOMATION**

*Automatic sample changer*
*Automatic data collection*
*Automatic data processing….*

# Light *versus* TElectron Microscopy






***The electrons travel in the vacuum tube, and the magnetic lenses are used to manipulate the path of the electrons.***

# *Transmission electron microscopy*

- Resolution of :

  ***light microscope*** is fundamentally limited by the wavelength of ***photons***.

  ***electron microscopes*** use electrons for imaging the sample.

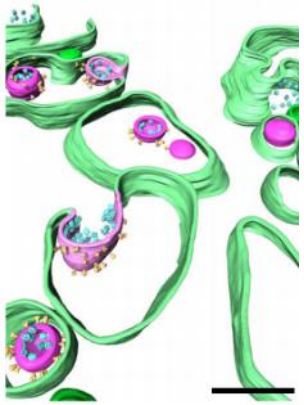
- ***Photons-Electrons:***

  high energy electrons have a wavelength that is shorter by several order of magnitude allowing higher resolution (than photons)

***Using electrons for imaging allows to directly view and further analyse the structures of viruses and even individual proteins.***

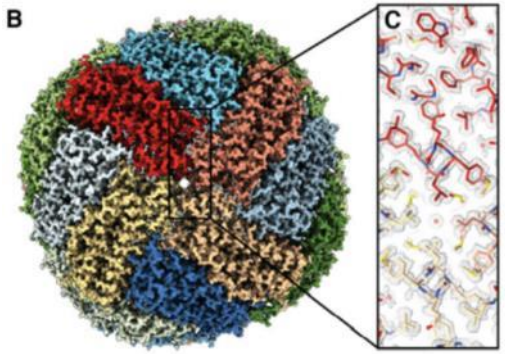
# CryoEM, the different approaches

## Electron Tomography



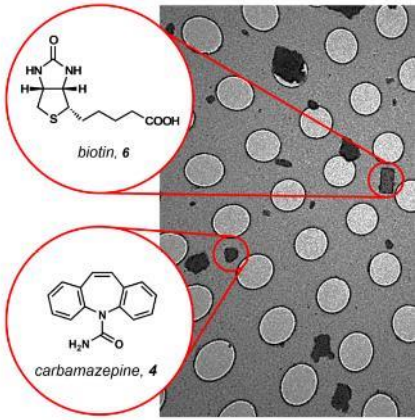
*Whole cells and organelles*

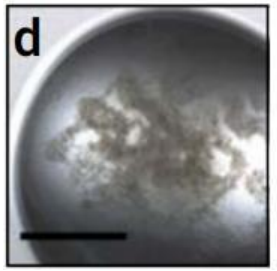
## Single-particle reconstruction

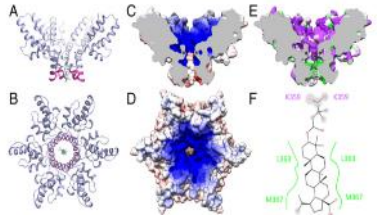



*Isolated single particles*

## Microcrystal electron diffraction (MicroED)




*3D micro-crystals*

**Imaging**

**Crystal based, diffraction**

# Cryo-EM workflow – *imaging*

Sample (aqueous solution)

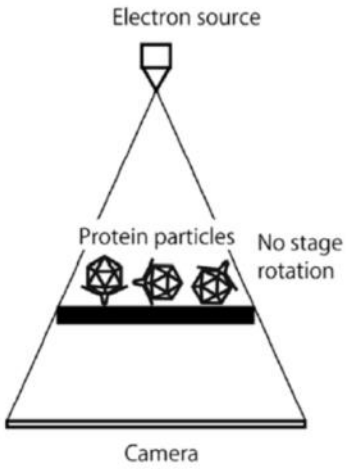
Vitrification

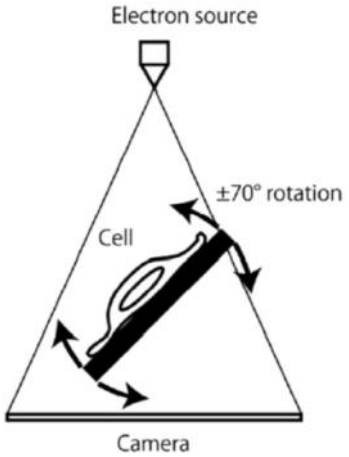
Recording images (low dose)

Single Particle Analysis (SPA)

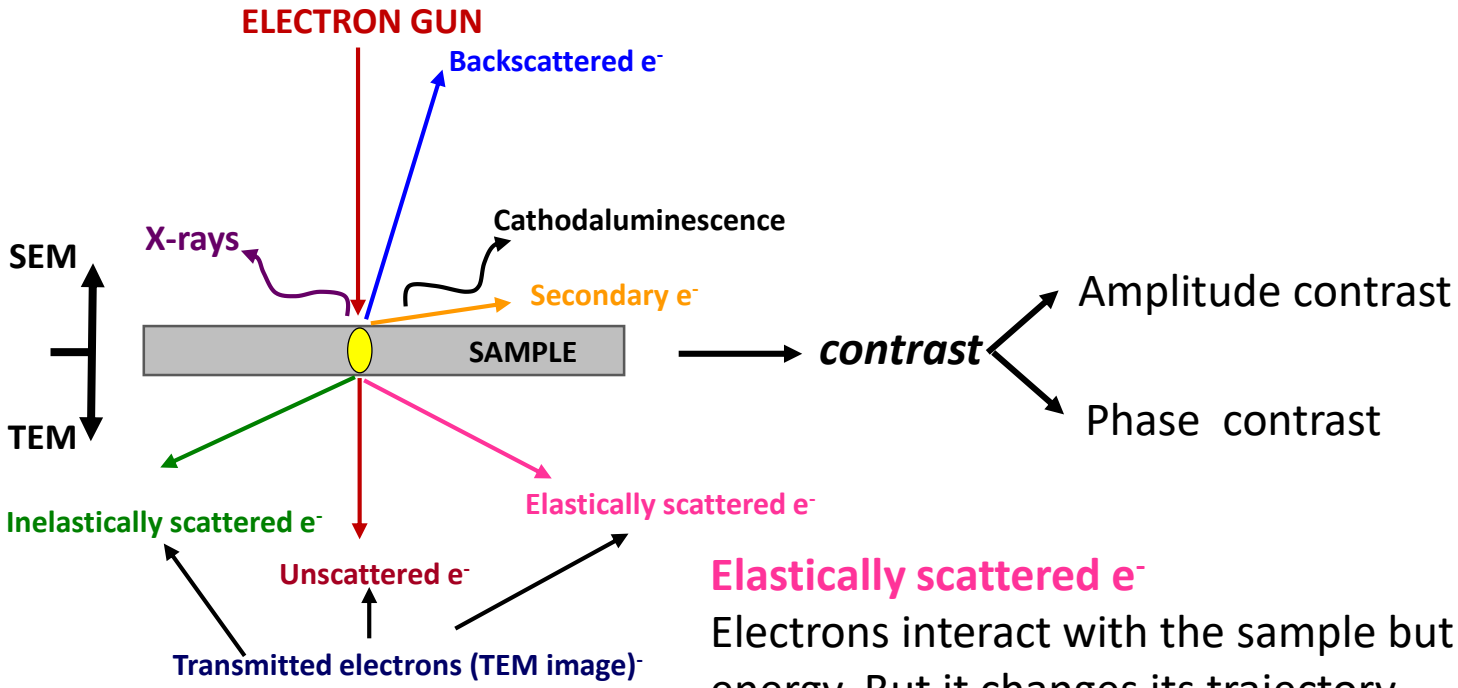
Cryo tomography (CTE)

Reconstructed electron potential map; Validation



# Electron interaction with the sample




**Inelastically scattered e-**

Electron interacts the sample, giving part of its energy.
This interaction usually leads to ionization of the sample.
Radiation damage, affecting the sample and a decrease in the quality of the final image.
***Contribute to the noise of the sample bad scattering***

**Unscattered e-**

Electron that pass the sample

**Elastically scattered e-**

Electrons interact with the sample but keep its full energy. But it changes its trajectory.
This kind of dispersion is responsible by the image formation.
It is more likely to occur to atoms with a high atomic number (Z).
Electron don't change energy
Good scattering
Contrast TEM

# Contrast

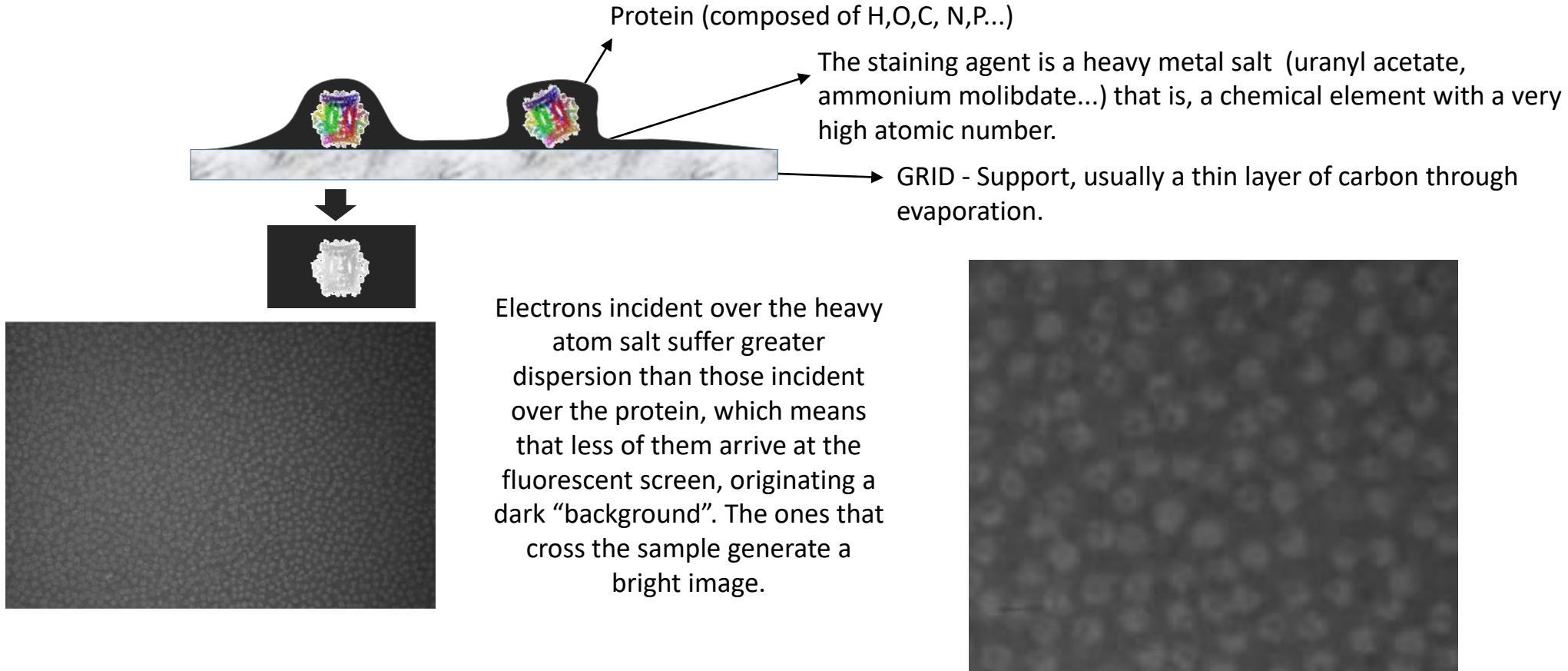
**Amplitude contrast –** in negative stain TEM the sample is stained with heavy metal salt, dehydrated and imaged. The dark areas contain electron-scattering heavy atoms from the stain.

**Phase contrast –** for high resolution structure determination, it is prefered to image unstained biological samples in aqueous solution (cryo-EM). With light elements embedded in a matrix of light elements, the amplitude contrast is very weak. It is require to introduce phase contrast in order to see the sample.

Phase contrast in TEM can be modulated by changing the defocus. In cryoEM it is intentionally collect underfocused data. This imaging strategy results in artefacts that have to be corrected (deconvoluted) during image processing.

# Negative staining

- Most projects start with negative staining analysis
- Initial analysis of sample homogeneity and protein concentration on grid.
- Soaking the sample with a “dye” that increases constrast




Electrons incident over the heavy atom salt suffer greater dispersion than those incident over the protein, which means that less of them arrive at the fluorescent screen, originating a dark “background”. The ones that cross the sample generate a bright image.

# Negative staining *vs.* Cryo-EM

## Negative staining EM

***Positive***

- Simple procedure and fast to prepare.
- High rate of success of the preparation process.
- High contrast. Easy posterior computational processing.
- Easy to analyse many samples.
- Samples can be analysed multiple times after fixation.

***Negative***

- Sample is dehydrated.
- Possible distortion and flattening of the structure.
- Possible partial staining.
- Resolution is limited by the stain's own nature, and no internal structure can be observed.

***For these reasons, it is only used for initial analysis of sample homogeneity and concentration on grid.***

## Cryogenic EM

***Negative***

- Sample preparation much more complex and tedious.
- Lower rate of success in sample preparation.
- Very low contrast. Complex posterior computational processing.
- Hard to visualise due to low contrast. Usually you cannot observe samples on the screen, and you need to draw upon negative staining micrographies.
- Sample storage is more difficult.

***Positive***

- Native, hydrated state.
- Physiological conditions – 3D structure is preserved.
- Fast freezing can preserve short lifetime conformational states.

***For these reasons, this is the technique used to acquire high resolution data and structure determination.***

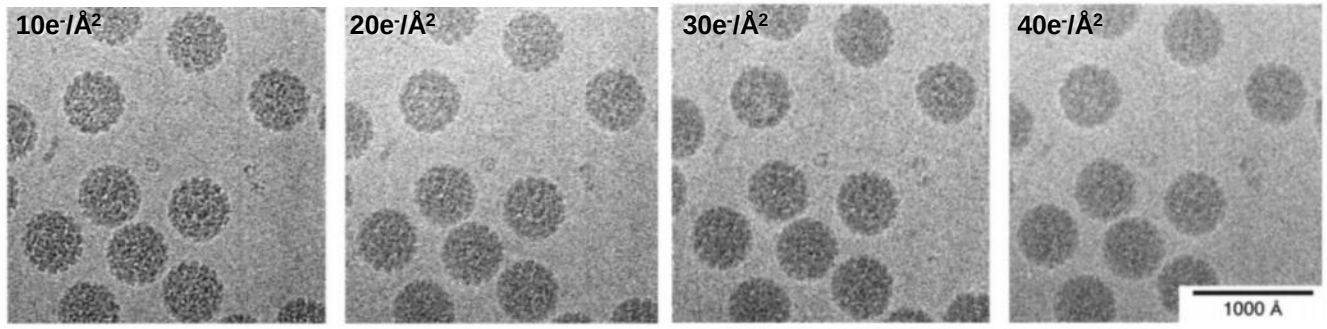
# Beam damage

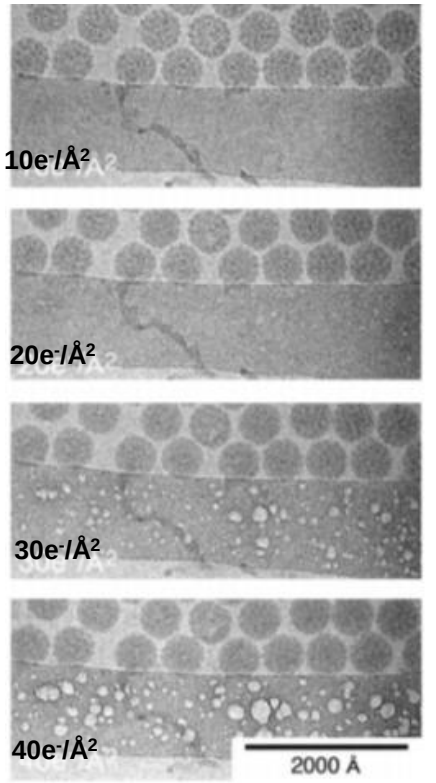
- High energy electron are a form of ionising radiation

- The high-energy electrons interact with the sample they break atomic bonds within the sample molecules and create free radicals that cause secondary alterations

- These beam damage effects accumulate rapidly and destroy the features of interest.

- ***Imaging strategy – manage beam damage – low dose strategy, minimal dose***

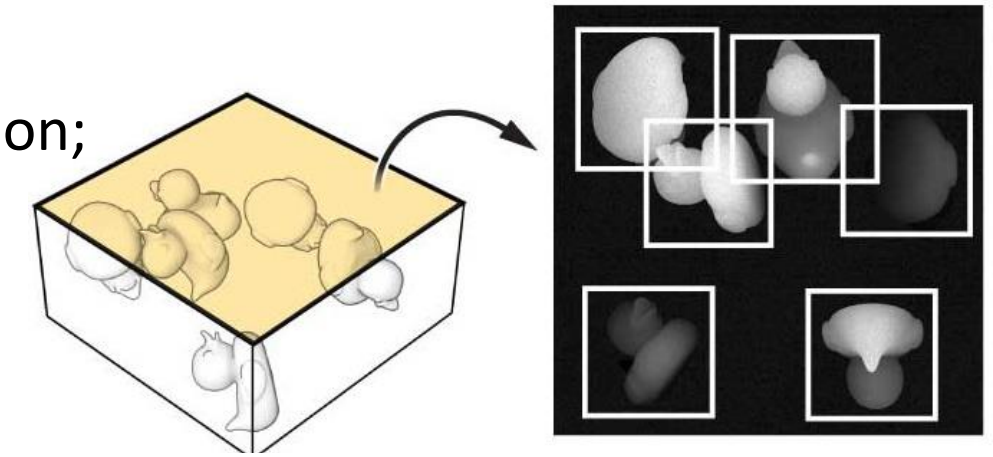


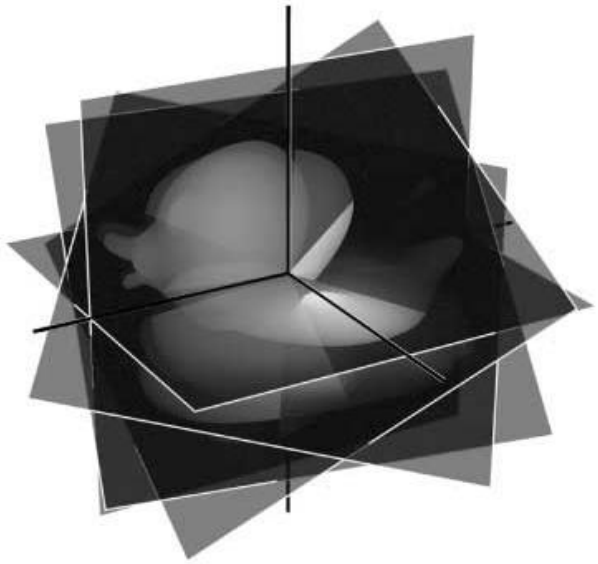

Baker TS, Olson NH, Fuller SD. Adding the third dimension to virus life cycles: three-dimensional reconstruction of icosahedral viruses from cryo-electron micrographs. Microbiol Mol Biol Rev. 1999 Dec;63(4):862-922, table of contents. doi: 10.1128/MMBR.63.4.862-922.1999

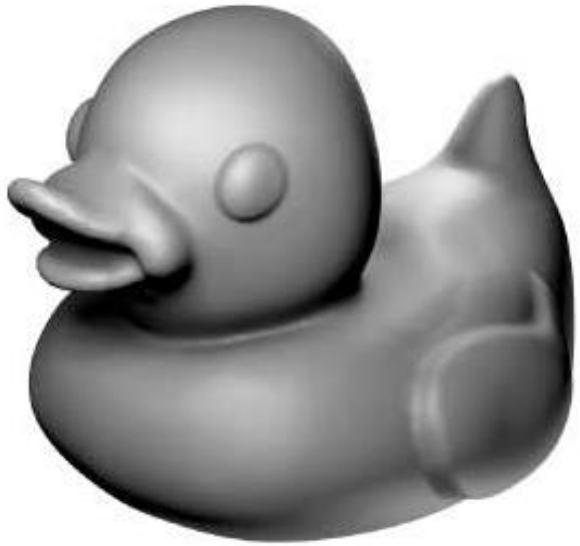
# Building a 3D picture

- individual proteins freeze in random orientations;
- the microscope generates 2D images of each orientation;



- a computer identifies the 2D projections
- and uses them to calculate the 3D structure



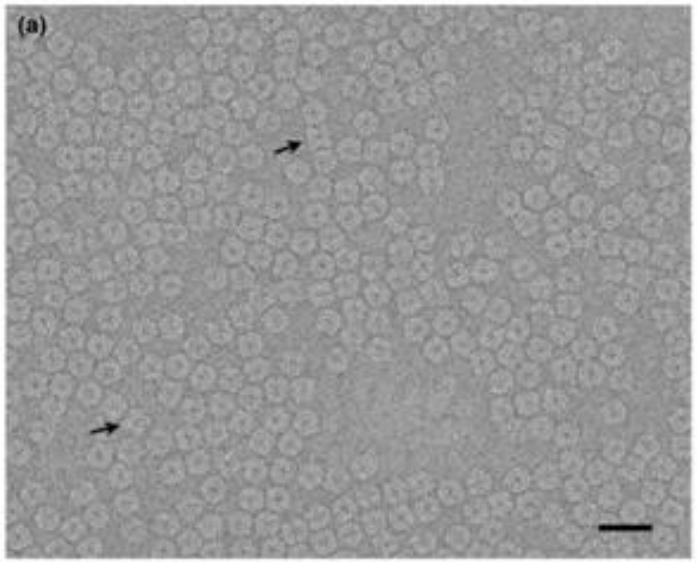


# SPA example: *alcohol oxidase*

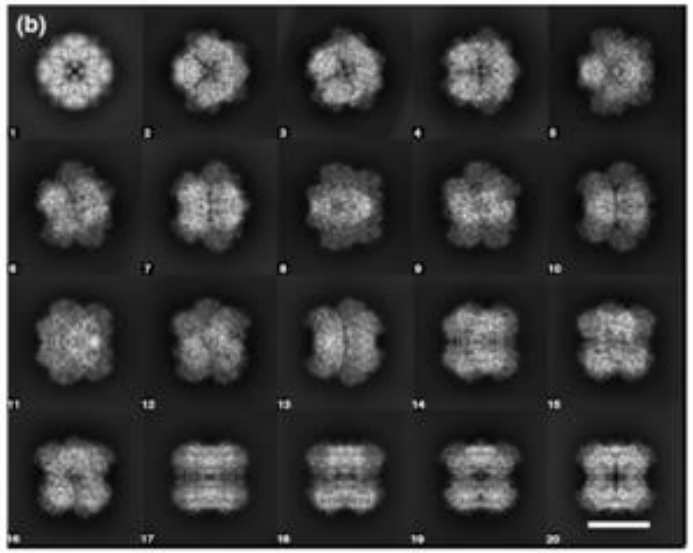
**Alcohol oxidase from *Pichia pastoris***

**– AOX – 600 kDa, 8 subunit, 3.4 Å resolution**

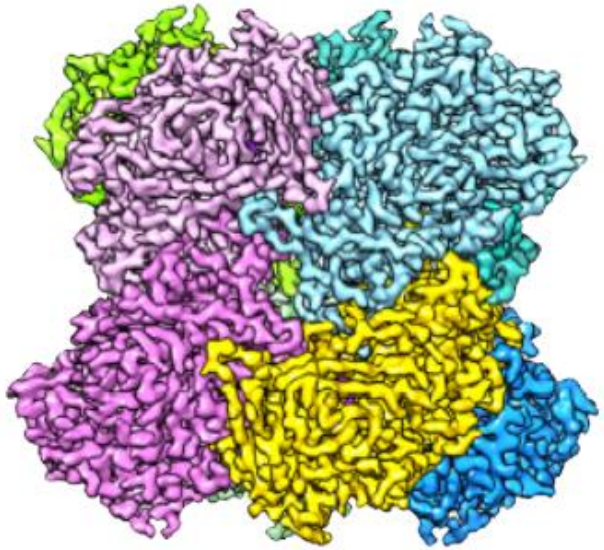
**Electron micrograph**




**Particle classification**




**3D Structural model**

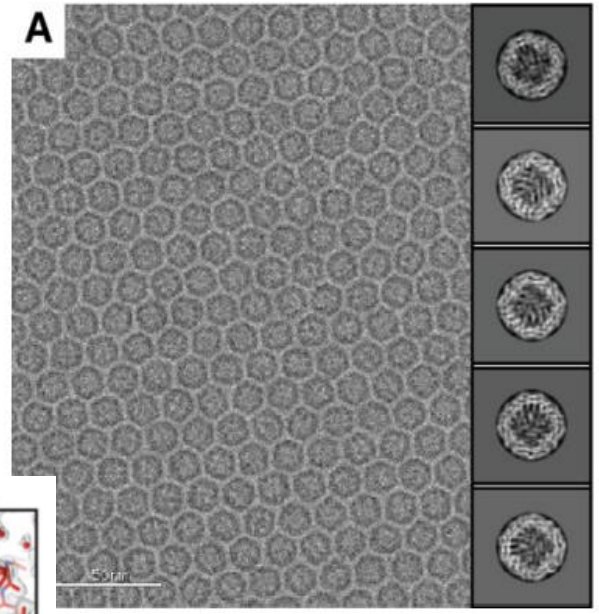


*Vonck et al 2017 Curr Opin Struct Biol 46:48*

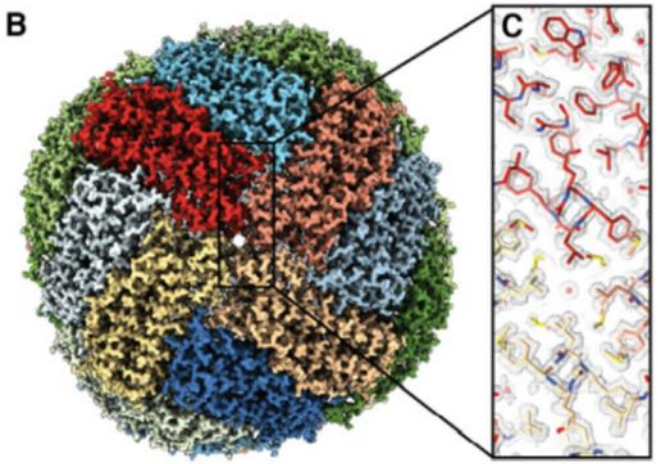
# SPA example: *ferritin*

## *Apoferritin - 1.75 Å resolution (mouse heavy chain apoferritin)*

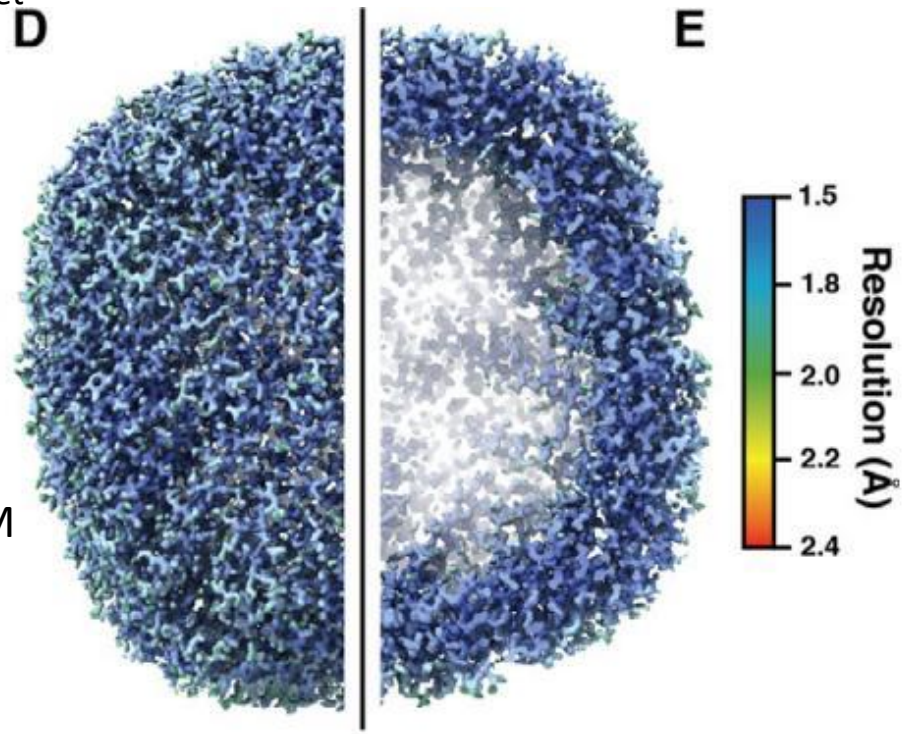
Representative aligned and dose-weighted micrograph (imaged at ~1.2 µm underfocus) of apoferritin in vitreous ice.




Representative reference-free 2D class averages are shown in the right-side inset



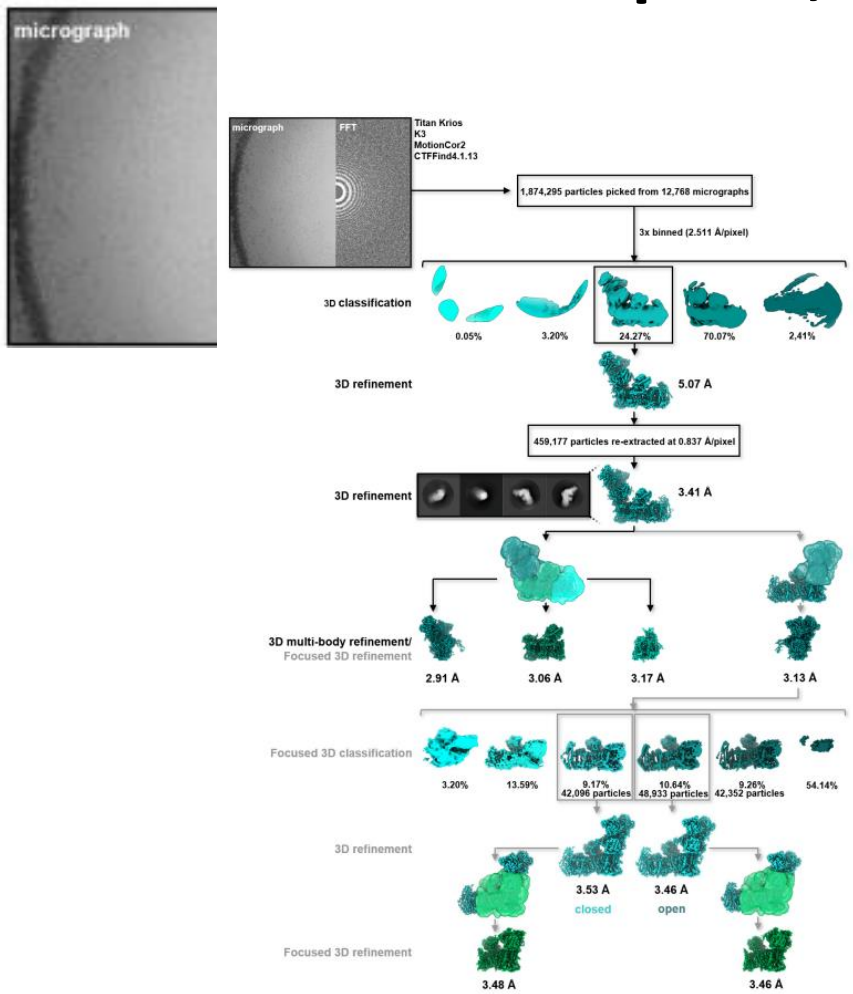

Final apoferritin EM density colored by local resolution

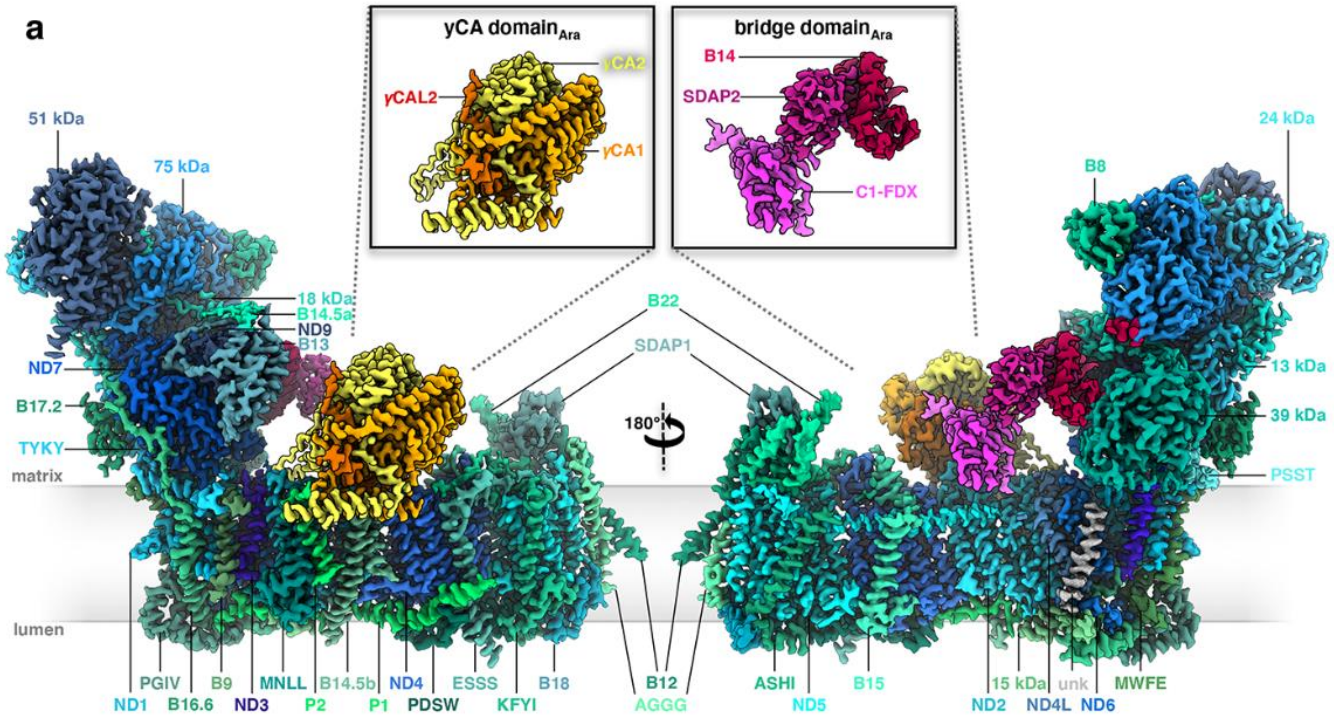



Mengyu Wu, Gabriel C. Lander, Mark A. Herzik, (2020) Sub-2 Angstrom resolution structure determination using single-particle cryo-EM at 200 keV,
Journal of Structural Biology: X, 4, https://doi.org/10.1016/j.yjsbx.2020.100020.

# SPA example: *ferritin*

## Mitochondrial complex I, *Arabidopsis thaliana – 2.9 Å resolution*





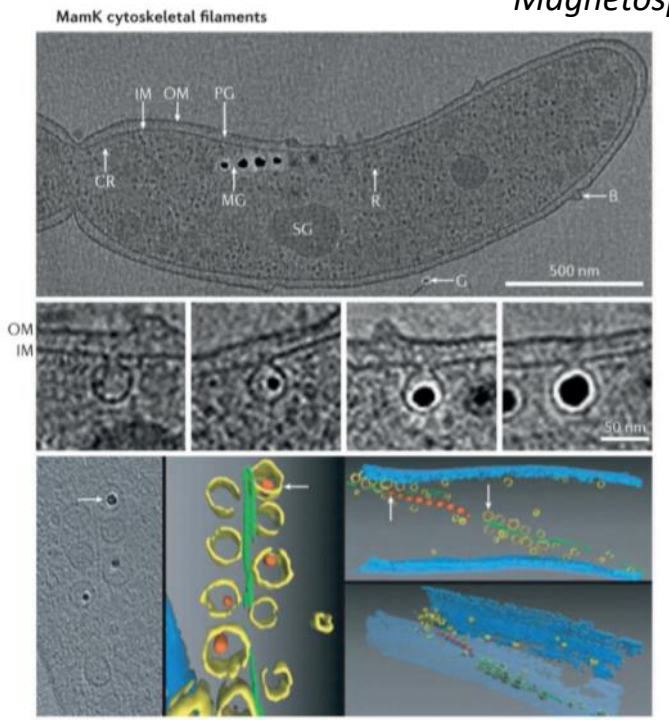
Klusch N et al. (2021) A ferredoxin bridge connects the two arms of plant mitochondrial complex I. The Plant Cell 33: 2072–2091

Werner Kuhlbrandt (2022) Forty years in cryoEM of membrane proteins Microscopy, 2022, 71(S1), i30–i50
DOI: https://doi.org/10.1093/jmicro/dfab041

# Cryo-electron tomography (CTE)

***CTE allow 3D structure determination in situ. Visualization of molecular structures to atomic detail in their biological environment, highly contributing to the understanding of molecular mechanisms. Macromolecular complexes, cell substructures, cellular envelope***

*Magnetospirillum spp.*




*Macromolecular complexes that can not be purified*:

the function is lost and may disassemble when are extracted from its natural environment

*Oikonomou et al., 2016 Nat Rev Micriolog 14:205*

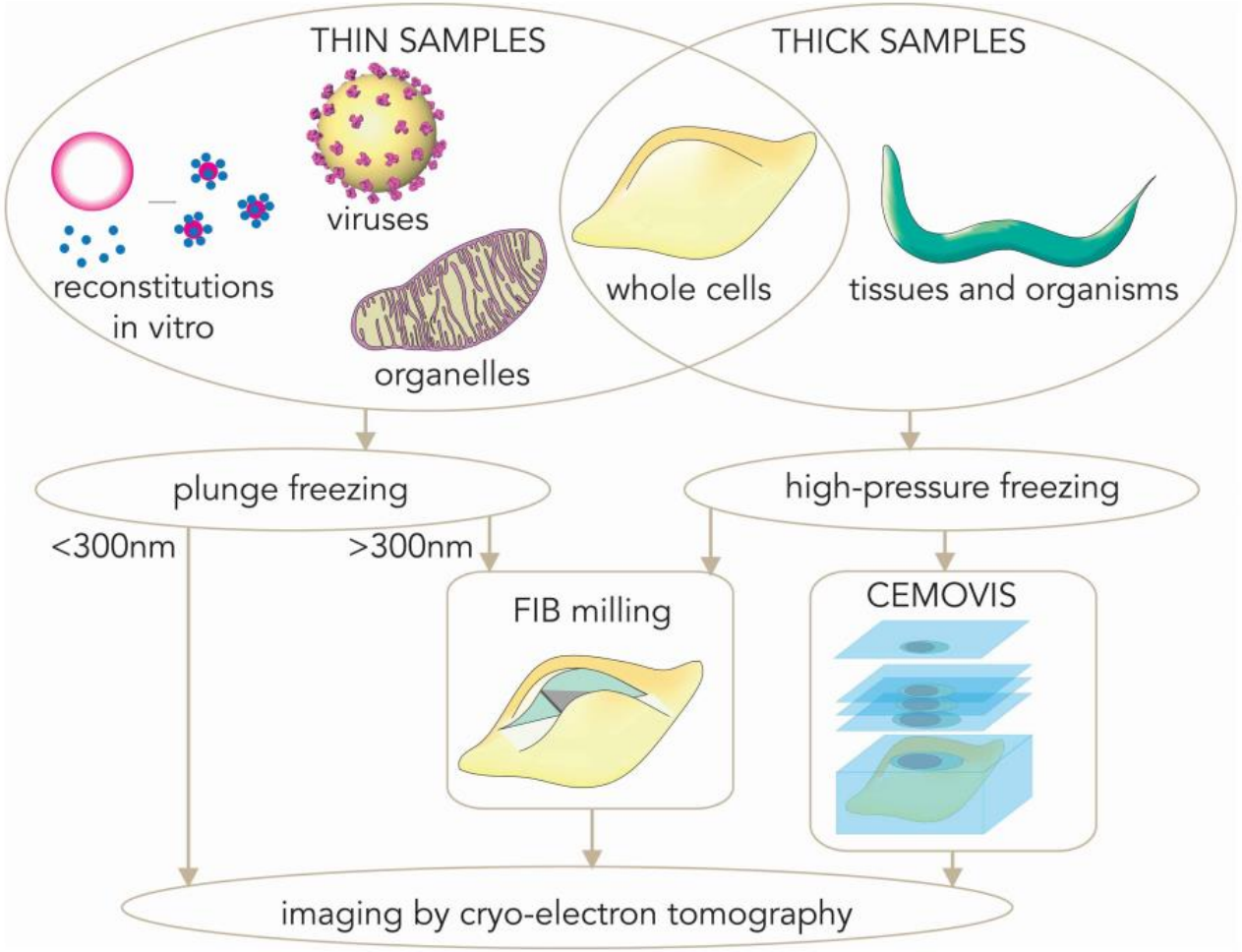
# CTE- *Sample preparation*

***Samples:***

viruses, isolated organelles, some bacterial cells, and peripheral regions of eukaryotic cells.

***Thin samples:*** plunge-freezing into liquid ethane.

***Thicker samples:***
high pressure freezing.



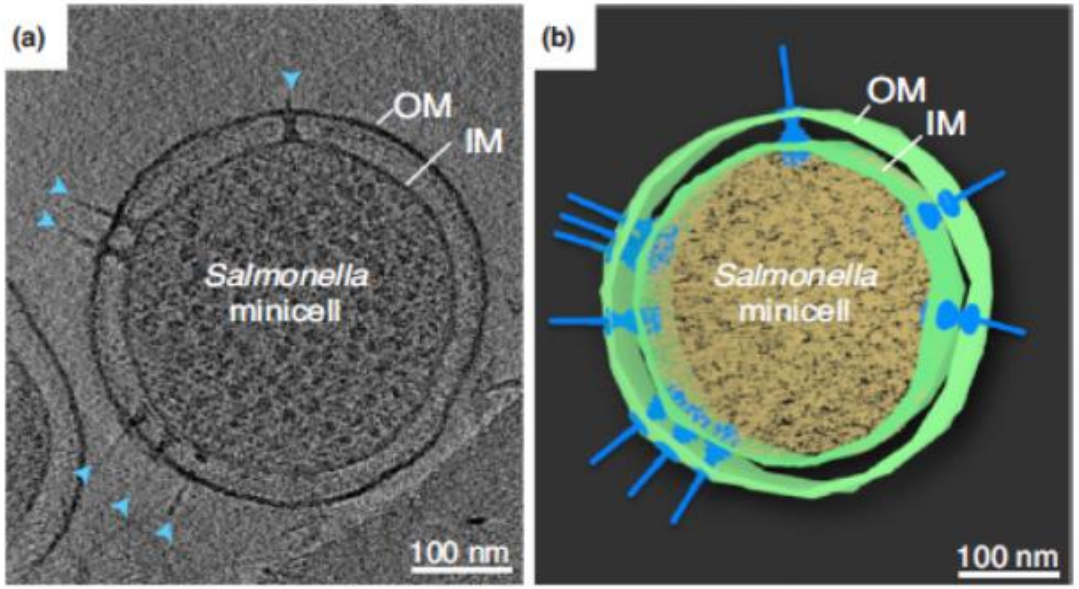

Hutchings J and Zanetti G (2018) Biochemical Society Transactions. https://doi.org/10.1042/BST20170351

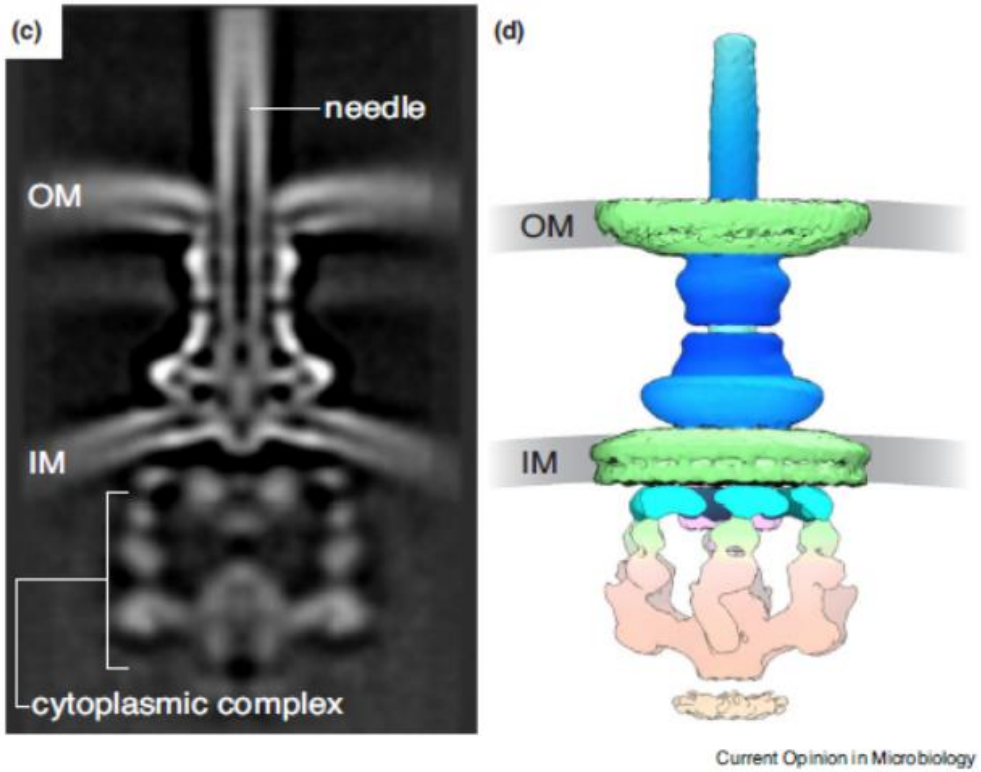
# CTE- *type III secretion system*

## *Secretion systems from bacteria*

Bacteria need to communicate with other cells (prokaryotic or eukaryotic cells).

The secretion systems are macromolecular membrane complexes, involved in energy production and contain a channel to secrete several substrates. They secrete numerous macromolecules such as DNA, polysaccharides, proteins, enzymes, toxins...



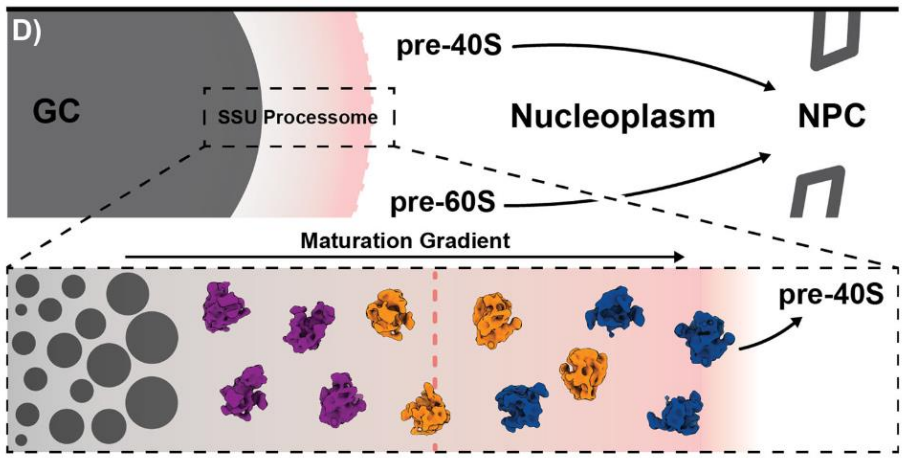



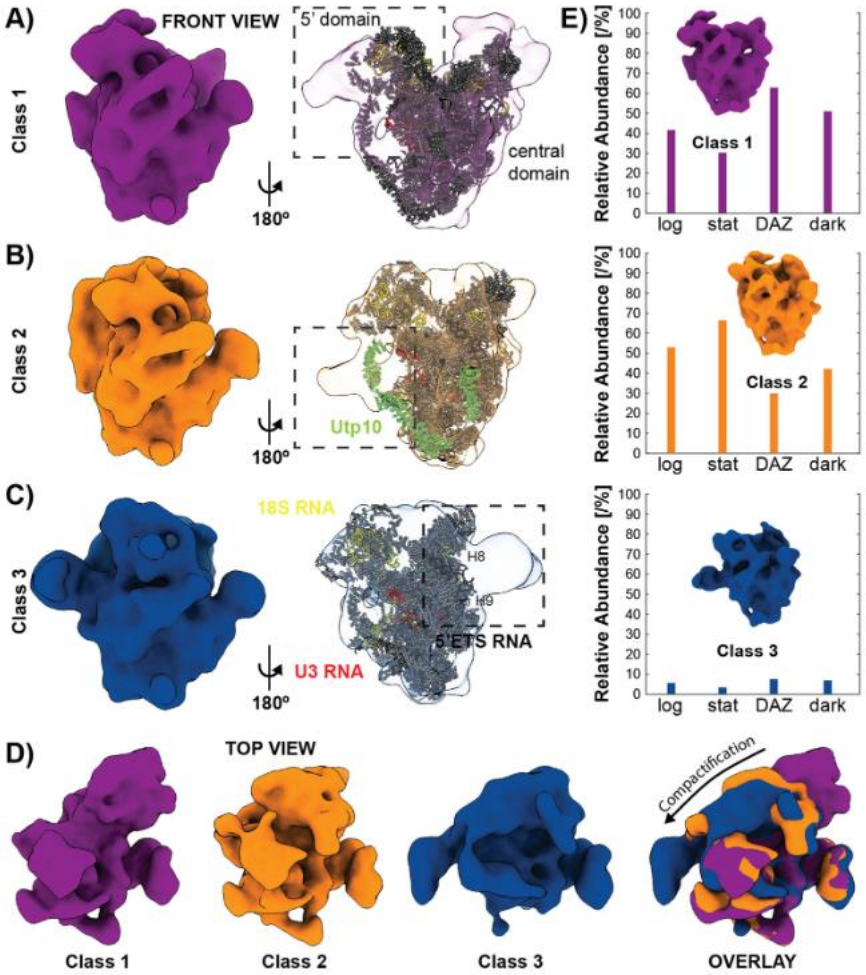

*Earl et al 2018 Cur Op Micr, 43:199*

# *in situ* Cryo-ET

## investigate the native organization of the nucleolus and its role in ribosome biogenesis

green alga *Chlamydomonas reinhardtii*




Erdmann, P.S., Hou, Z., Klumpe, S. *et al.* In situ cryo-electron tomography reveals gradient organization of ribosome biogenesis in intact nucleoli. *Nat Commun* **12,** 5364 (2021). https://doi.org/10.1038/s41467-021-25413-w

# *MicroED*

**MicroED -** collection of high-resolution electron diffraction data from extremely small protein microcrystals using an electron cryo-microscope – ***Diffraction mode***

**Samples** - depositing microcrystals in solution on a carbon-coated EM grid → solution removal by blotting → vitrification by plunge-freezing in liquid ethane or liquid nitrogen.

**Data collection** – ***Diffraction mode***

data collected on a complementary metal-oxide-semiconductor (CMOS)–based detector in a cryo-EM, eg. CMOS-based TVIPS F416

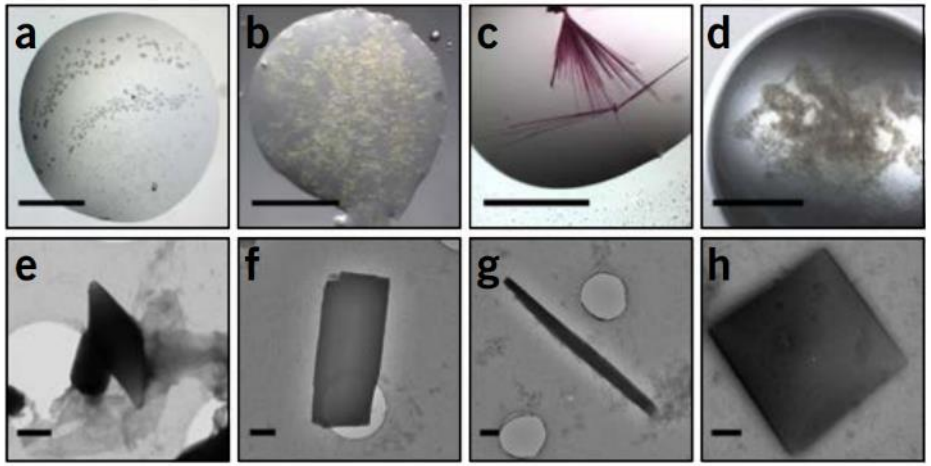
analogous to rotation method in X-ray; low-dose mode (low number of electrons/Å$^2$ per second) - limit the sample's exposure to damaging radiation.

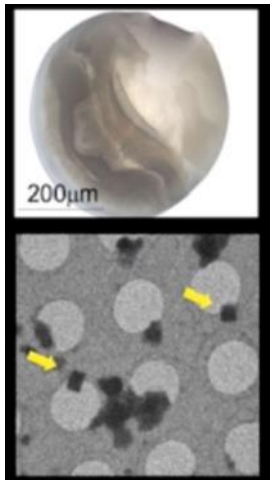
Low dose – 100 x less than SPA

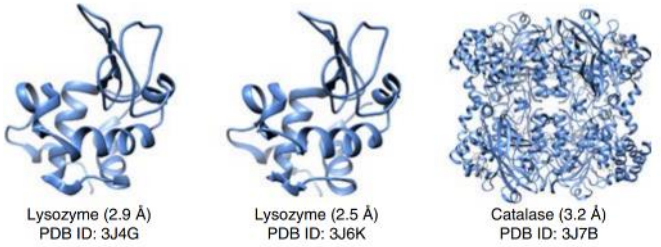
**Data processing** – using X-ray software

# MicroED

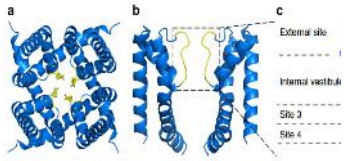
## Macromolecular Crystallography



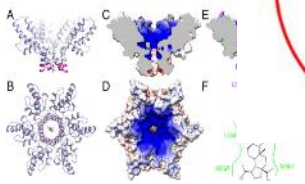



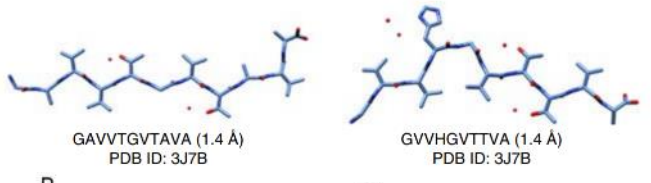

**NaK channel, 2.5 Å**

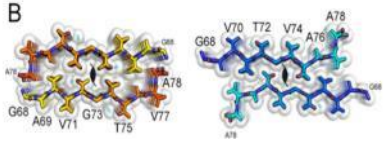


**HIV-1 maturation Inhibitor Bevirimat**



Purdy MD, et al 2018. *PNAS,* 115, 13258-13263. doi:10.1073/pnas.1806806115



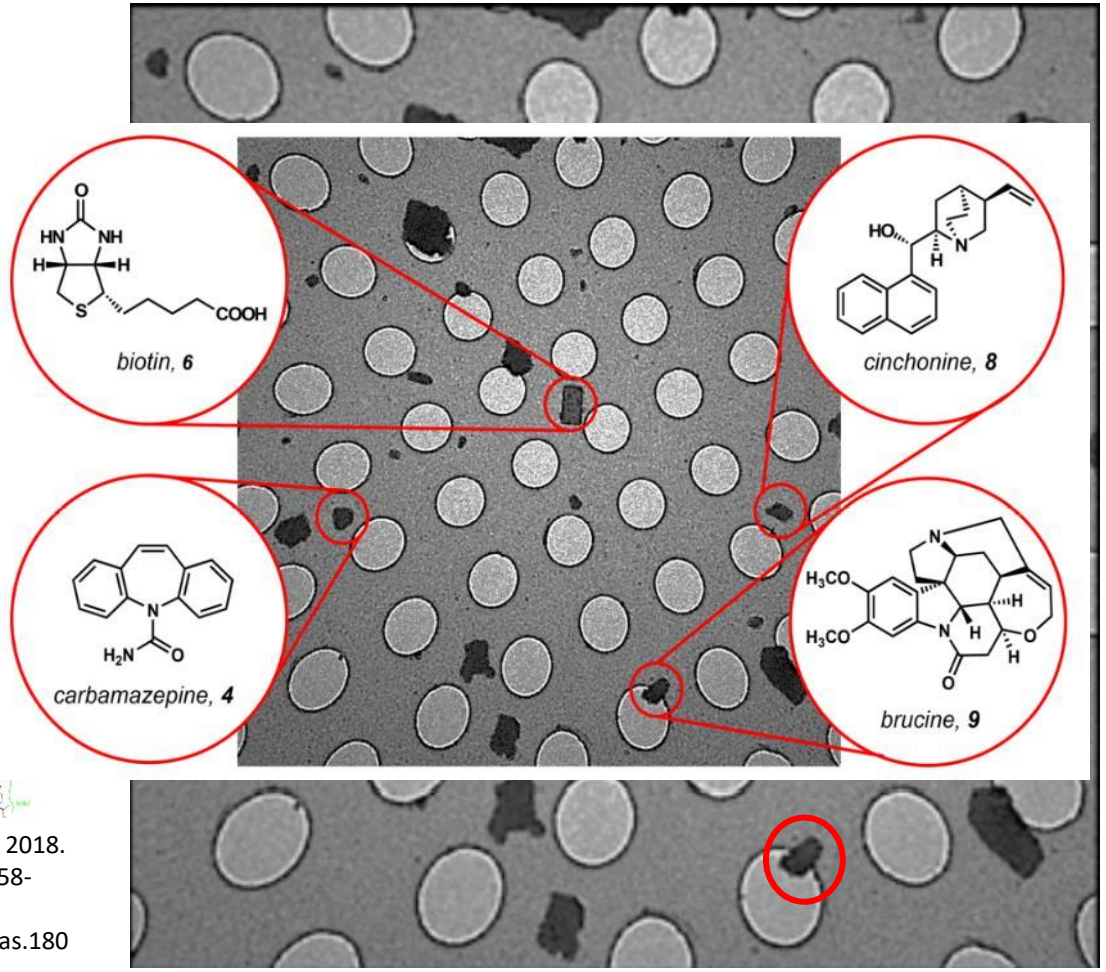






α-synuclein NACcore

Jones CG et al 2018 ACS Cent Sci. 2018 Nov 28;4(11):1587-1592. doi: 10.1021/acscentsci.8b00760.

## Small-molecules Crystallography

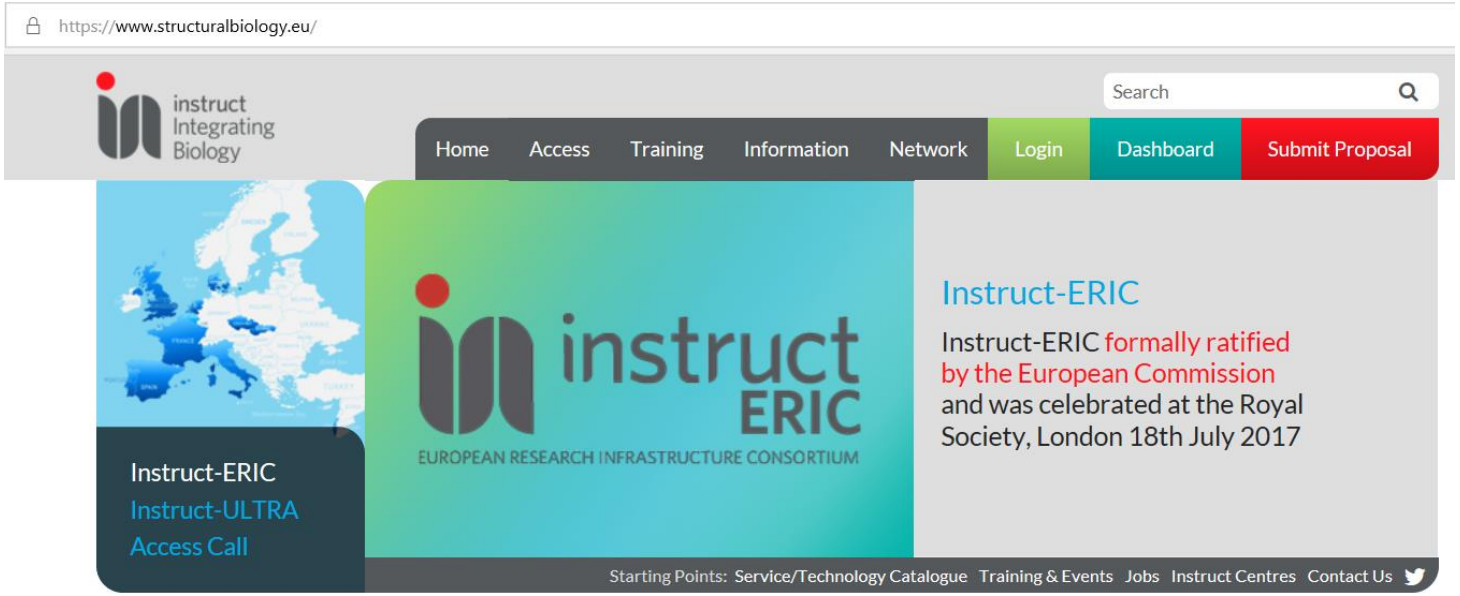



Liu, S., Gonen, T. *Commun Biol* **1,** 38 (2018). https://doi.org/10.1038/s42003-018-0040-8

Shi, D., Nannenga, B., de la Cruz, M. *et al.* The collection of MicroED data for macromolecular crystallography. *Nat Protoc* **11,** 895–904 (2016). https://doi.org/10.1038/nprot.2016.046

# Cryo-EM European centers

- **Access to cryo-EM european centers by portuguese scientists**
  - *High resolution centers – they require preliminary data of Cryo-EM of low/medium solution. Highly competetive.*
  - *Low/medium resolution – sample transport to the centers; depends on the available time of the collaborators*




IMpaCT

IMAGING LIFE FROM MOLECULES TO CELLS

www.itqb.unl.pt/impact

# Building knowledge on Cryo-electron microscopy methodologies at ITQB NOVA

*Coordination:* **Pedro Matias & Célia Romão**

*Project Manager:* **Ana Gomes**

**September 2019 – February 2023**

***Mission - bring Cryo-EM knowledge to ITQB NOVA, relying on a unique and highly experienced Cryo-EM network of partners that aims to develop ITQB NOVA scientific projects needing Cryo-EM methodologies.***

Main activities:

- Workshops with hands-on experience
- Exchange visits by ITQB Early Career Researchers to the partner labs
- Seminars by world-leading experts in the field
- Visits to ITQB by experts from the partner labs
- Softs Skills development courses for Early Career Researchers
- Outreach activities to the general public

**This project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 857203.**

# The project and our network

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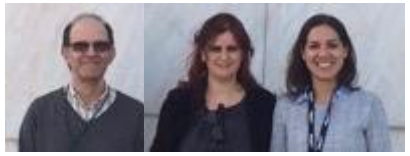
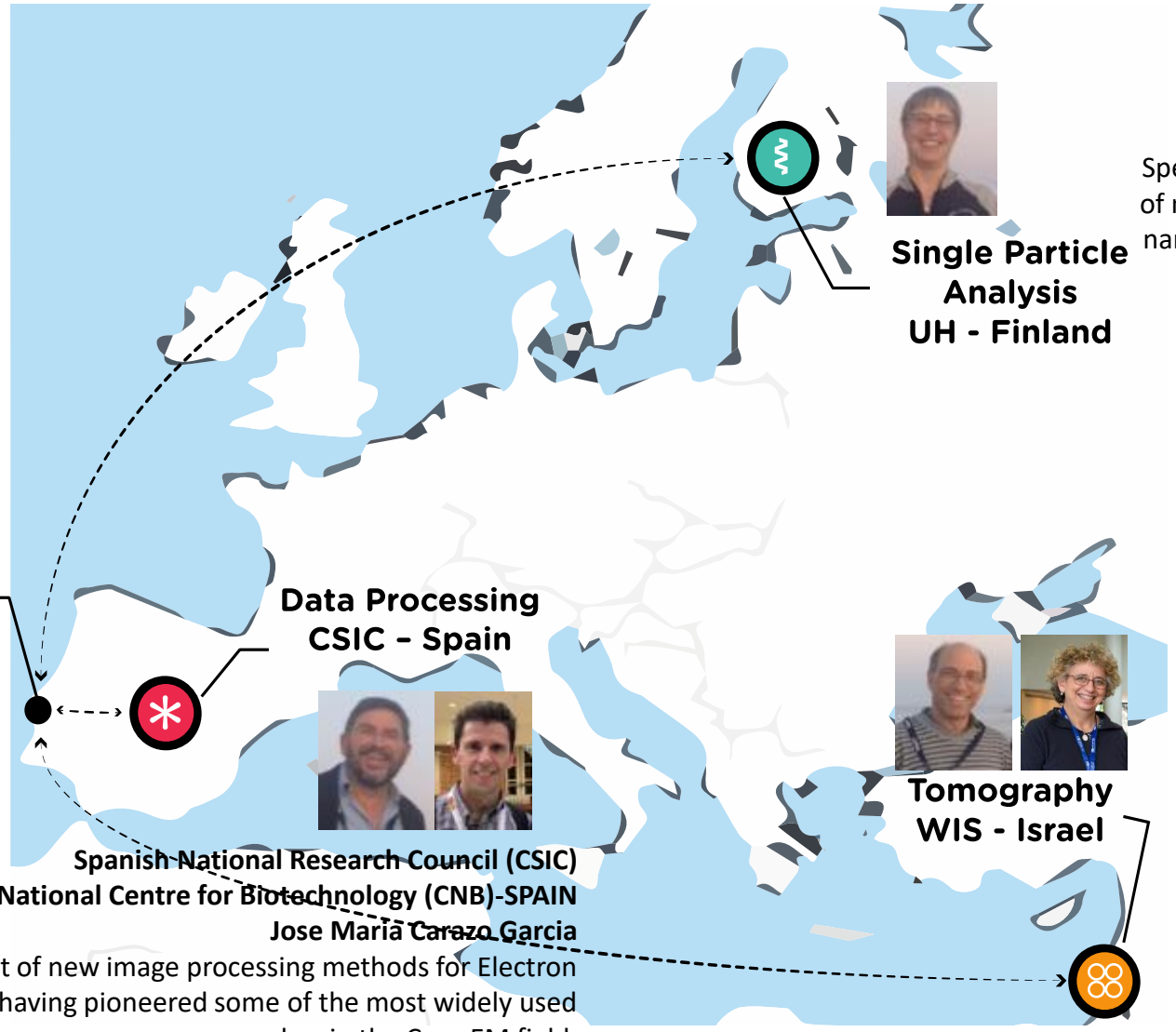
IMAGING LIFE FROM MOLECULES TO CELLS



ITQB NOVA
Portugal










**University of Helsinki (UH) – FINLAND**
**Sarah Butcher**
Specialized in the characterisation of macromolecular complexes and nanoparticles using single particle analysis Cryo-EM.

**Spanish National Research Council (CSIC)**
**National Centre for Biotechnology (CNB)-SPAIN**
**Jose Maria Carazo Garcia**
Development of new image processing methods for Electron Microscopy, having pioneered some of the most widely used approaches in the Cryo-EM field.

**Weizmann Institute of Science (WIS) – ISRAEL**
**Michael Elbaum, Sharon Wolf**
Are considered a reference for cryo-electron tomography (CET), a method used for probing the 3D structure of vitrified, intact cells.

IMpaCT

IMAGING LIFE FROM MOLECULES TO CELLS


*Kickoffmeeting, Sintra, Sept 2019*


*Tomography Workshop, Rehovot, Mar 2022*

**2019-2023**

***#cryoem_impact***

**www.itqb.unl.pt/impact**

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IMAGING LIFE FROM MOLECULES TO CELLS

instruct Integrating Biology

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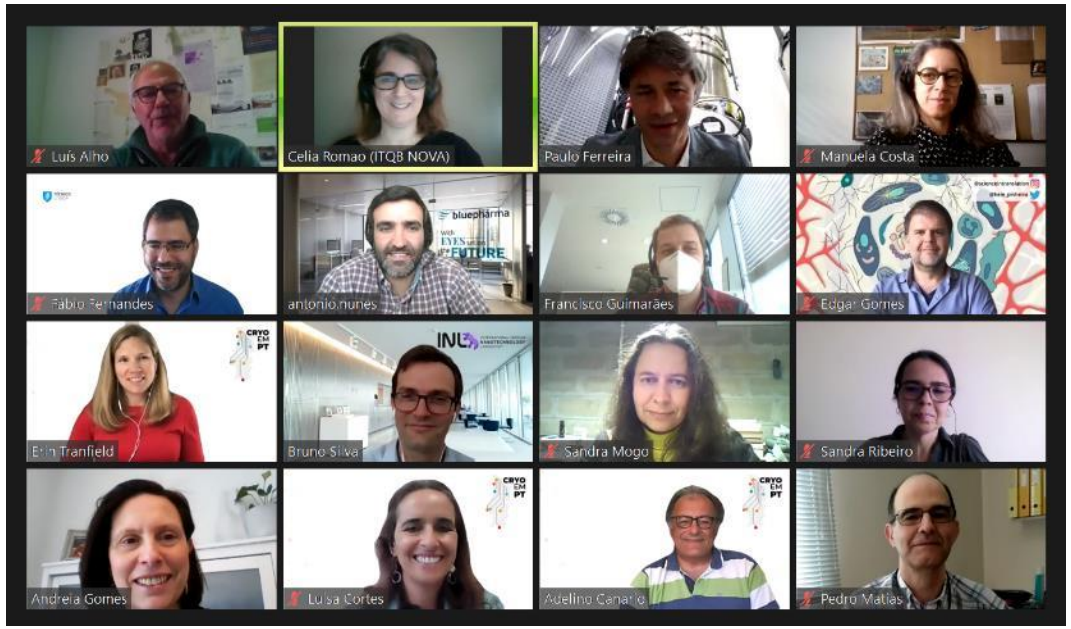
מכון ויצמן למדע

WEIZMANN INSTITUTE OF SCIENCE

**This project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 857203.**

# CryoEM at national level




***National Advanced Microscopy Network for Health and Life Sciences***

CRYO EM PT


**October 2022, Start the instalation**

**INL- Laboratório Ibérico Internacional de Nanotecnologia**

# Acknowledgments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**André Gouveia***
@ITQB NOVA
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**Ausra Domanska**
@University Helsinki
**Marcos Gragera**
@CSIC
**Eaazhisai Kandiah**
@ESRF

## *Electron dense granules*

**Sara Silva ***
**André Gouveia ***
@ITQB NOVA
**Sharon Wolf**
**Michael Elbaum**
@Weizmann Institute

## *Class F FDP*

**Bruno Salgueiro ***
**Filipe Folgosa**
**Miguel Teixeira**
@ITQB NOVA
**Alessandro Grinzato**
@ESRF
**Marcos Gragera**
**Roberto Melero**
@CSIC



**** Romão's Team@ ITQB NOVA***


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LS3131

PID: 12696, 19879, 20983

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