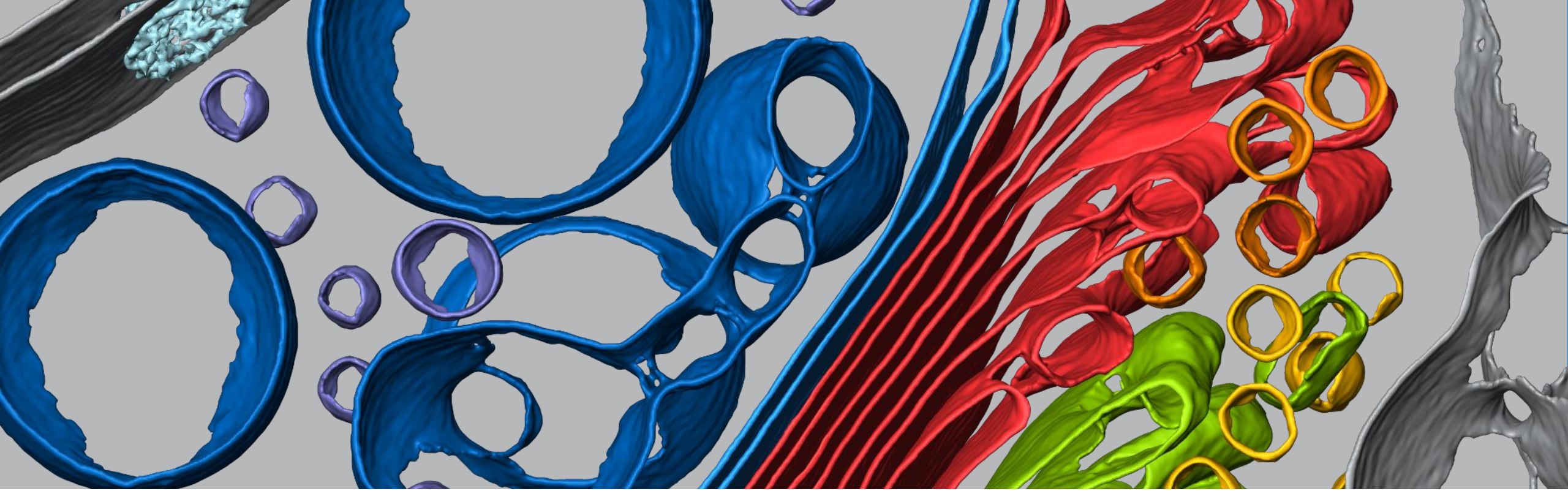
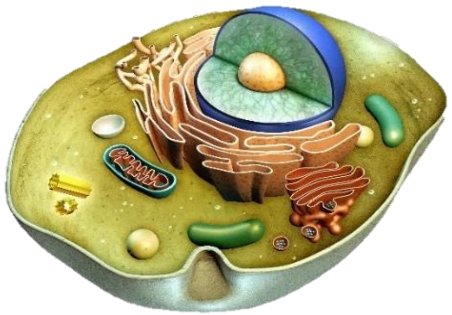


ThermoFisher SCIENTIFIC

MUNI

Cryo electron microscopy (for LS)

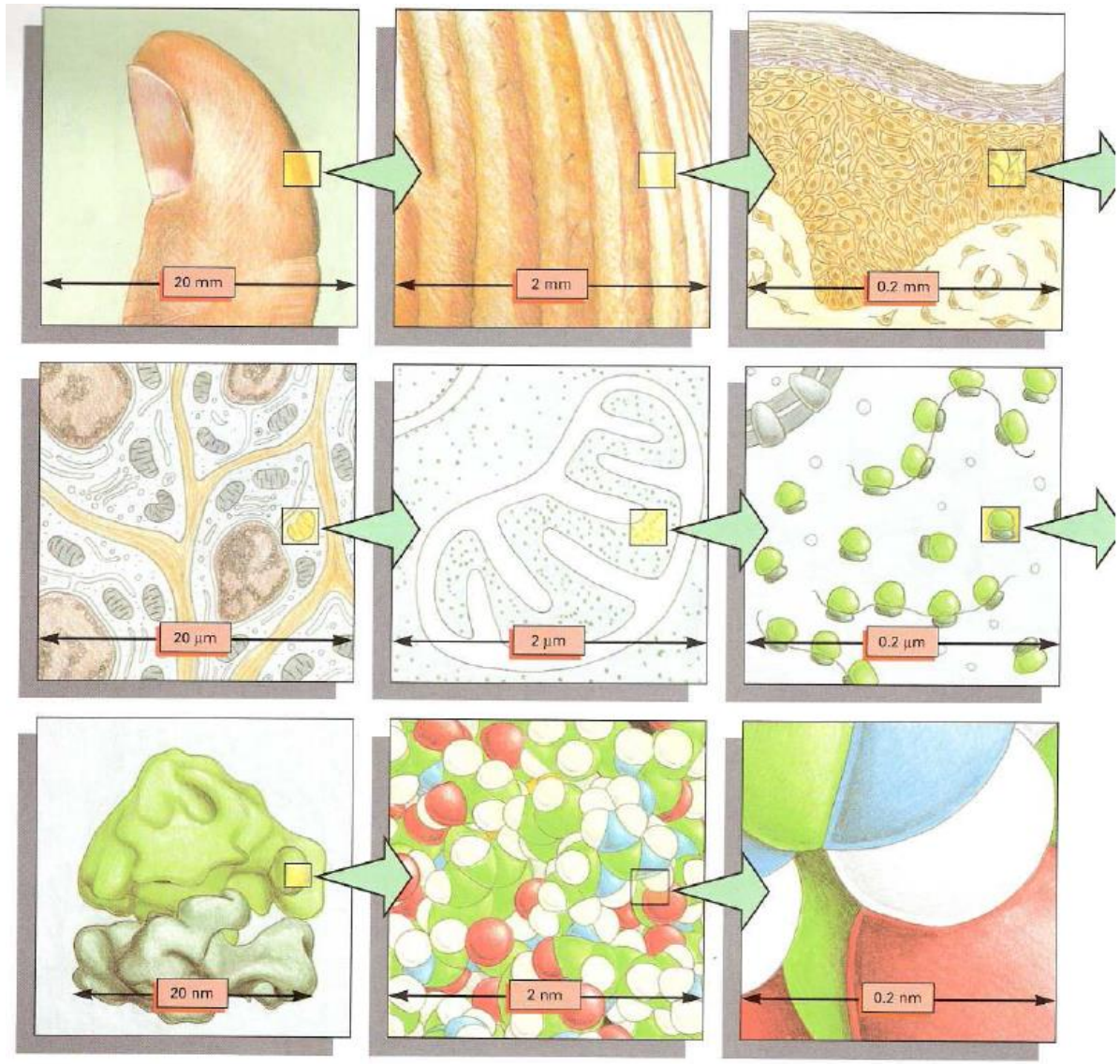
2020/05/28 (Vybrané kapitoly z elektronové mikroskopie, MUNI.)



Content

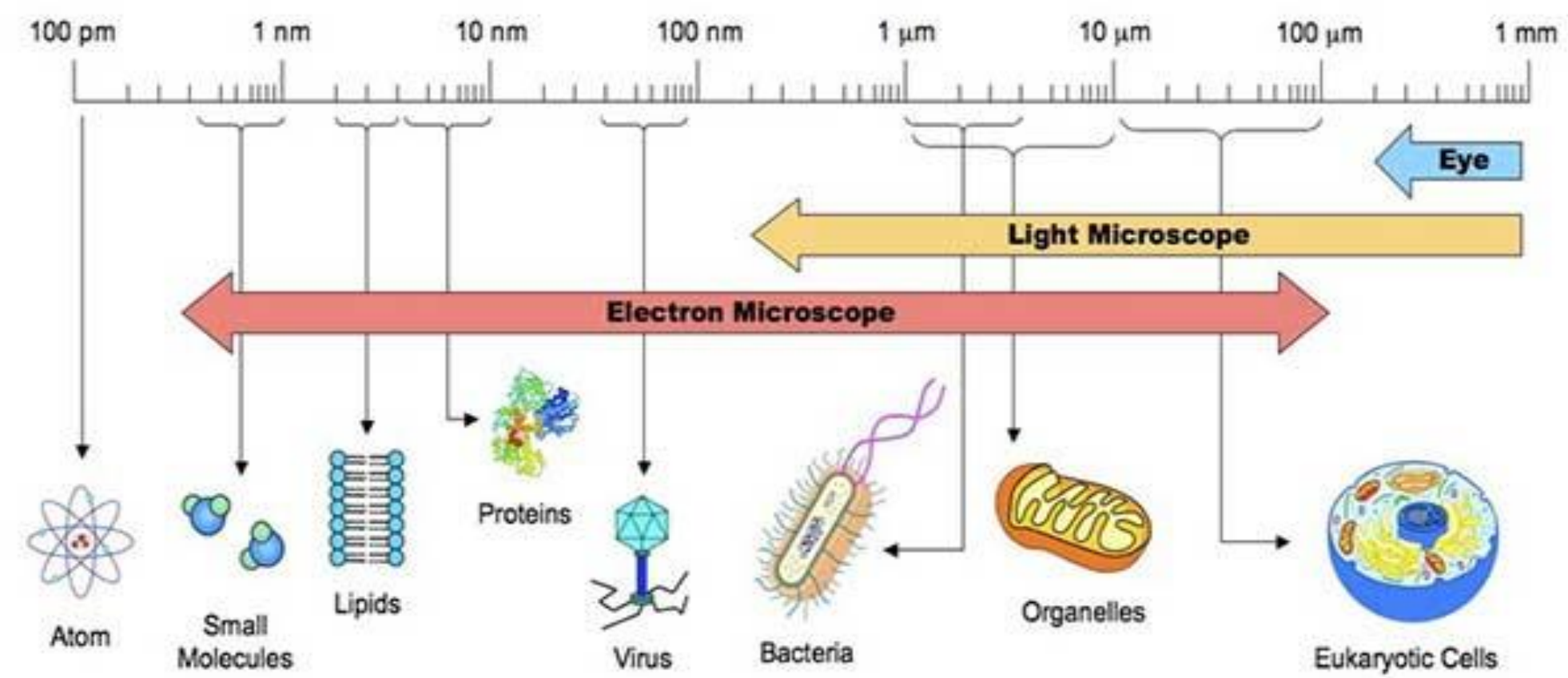
- **Introduction**: bio-samples, scale, methods, etc.
- **Sample preparation, navigation**
  - Fixation / vitrification,
  - Plunge freezing (PF),
  - High pressure freezing (HPF),
  - Correlative microscopy (CLEM),
  - Lamella preparation with ion beam (for SDB Tomo Workflow).
- **Methods**
  - Cryo-Electron Tomography (cryo-ET) + STA,
  - Single Particle Analysis (SPA),
  - MicroED.

Bio-Samples: Wide Scale Across the Space



Adapted from: Alberts, Základy buněčné biologie, 2004.

Different Methods See Different Details

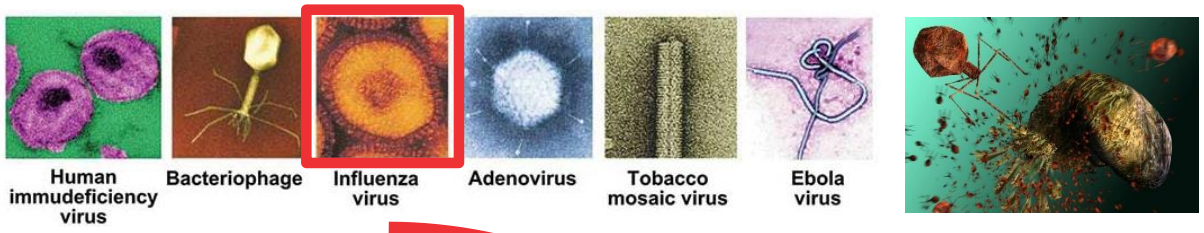


MUNI

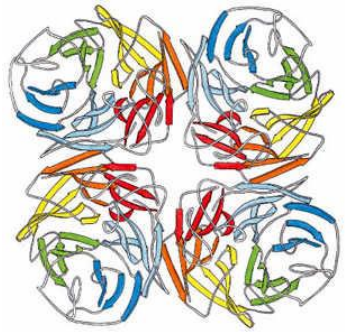
Adapted from: N/A; www.npt.co.uk/biotechnology/research/super-resolution-imaging.

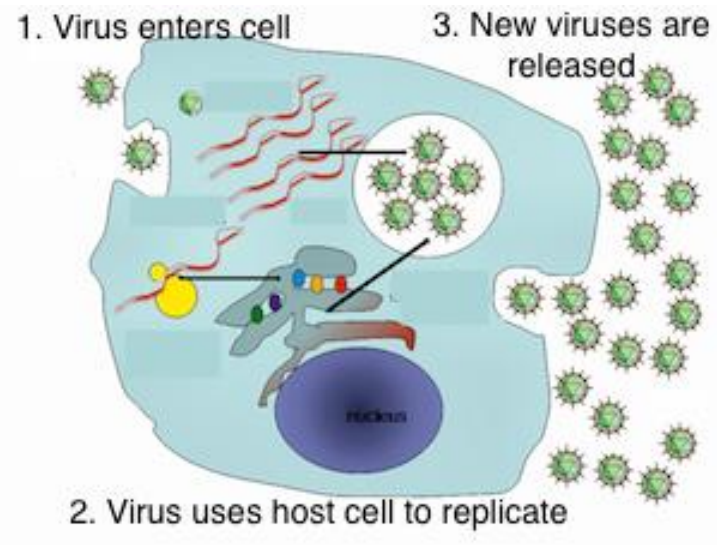
ThermoFisher SCIENTIFIC

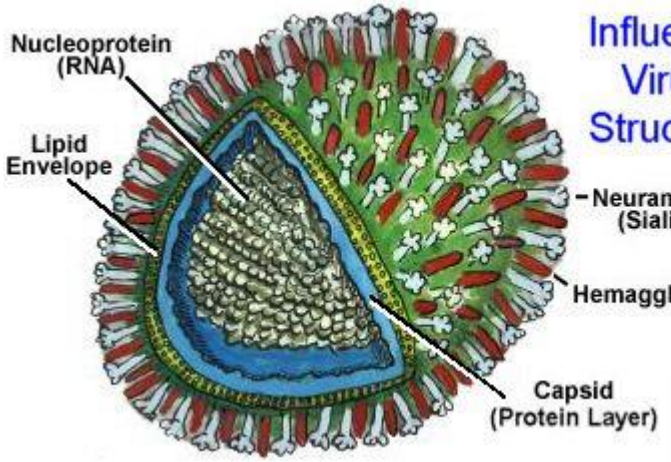
From Image to Function…To reveal a structure and understand its function.



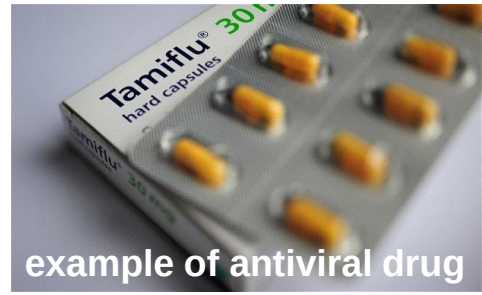
TEM image







Influenza Virus Structure



example of antiviral drug

MUNI

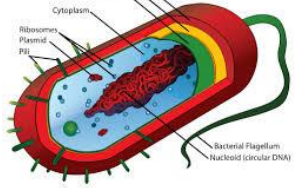
Adapted from Wiki, https://www.scientificpsychic.com/health/virus.html.

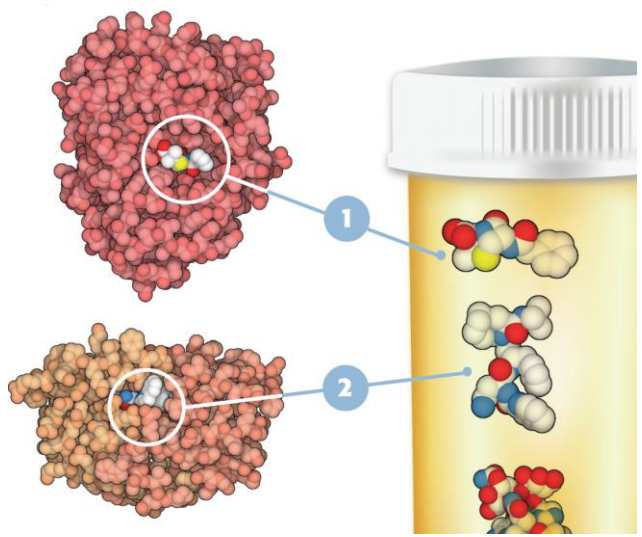
ThermoFisher SCIENTIFIC

From Image to Function…Drug Design

- **PROTEINS: “**tiny molecular machines. Perform most of the tasks needed to keep cells alive. Drugs can be used to turn proteins on or off, to affect their action.”

- **DRUGS: “**small molecules that bind to one specific protein and modify its action. Antibiotics or anticancer drugs are used to completely disable a critical molecular machine. These drugs can kill a bacterial or cancer cell. Other molecules, such as aspirin, gently block less-critical proteins for a few hours.”



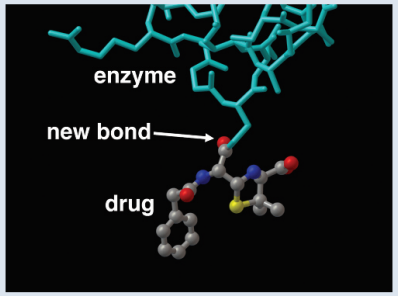


1 2 Antibiotics & Antivirals

Antibiotics and antiviral drugs are specific poisons. They need to kill pathogenic organisms like bacteria and viruses without poisoning the patient at the same time. Often, these drugs attack proteins that are only found in the targeted bacterium or virus and which are crucial for their survival or multiplication. For instance, **penicillin** attacks the enzyme that builds bacterial cell walls, and HIV protease inhibitors like **saquinavir** attack an enzyme that is needed for HIV maturation.

1. D-alanyl-D-alanine carboxypeptidase with penicillin (**1pwc**)
2. HIV protease with saquinavir (**1hxb**)

Suicide Inhibitors



Some drugs are particularly effective because they form a chemical bond to the protein target (shown in turquoise), totally disabling it in the process. Penicillin (shown at the bottom with atomic colors) reacts with a serine amino acid in the bacterial enzyme, forming a new covalent bond to the enzyme. This completely blocks the active site, so the enzyme is unable to perform its role in cell wall synthesis. Another suicide inhibitor, aspirin (shown in #7), attaches an acetyl group to its target which blocks an inflammation pathway.

Penicillin bound structure of D-alanyl-D-alanine carboxypeptidase (PDB entry **1pwc**)

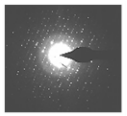
MUNI

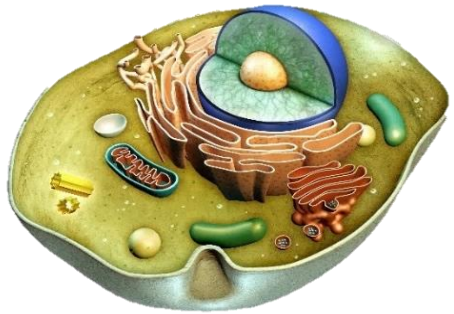
Adapted from https://cdn.rcsb.org/pdb101/learn/resources/flyers/how-do-drugs-work-flyer.pdf.

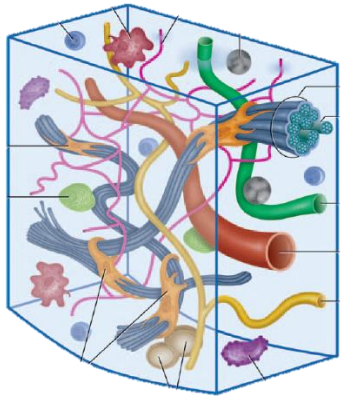
ThermoFisher SCIENTIFIC

Selected Life Science Workflows to Study Objects of Interest

SPA	Cryo-ET	LVA
Structure of folded Protein	***Function of Protein in Cells***	***Cellular Organisation in Tissue***







CryoEM: Single Particle Analysis	CryoEM: Tomography	Large Volume Analysis
Structure determination of proteins and protein complexes in their native state at “near-atomic” resolution.	High resolution reconstruction of large molecular complexes in their functional environment in cells and tissues.	Modelling of cell-cell interactions and organ function based on volume imaging at nm resolution.

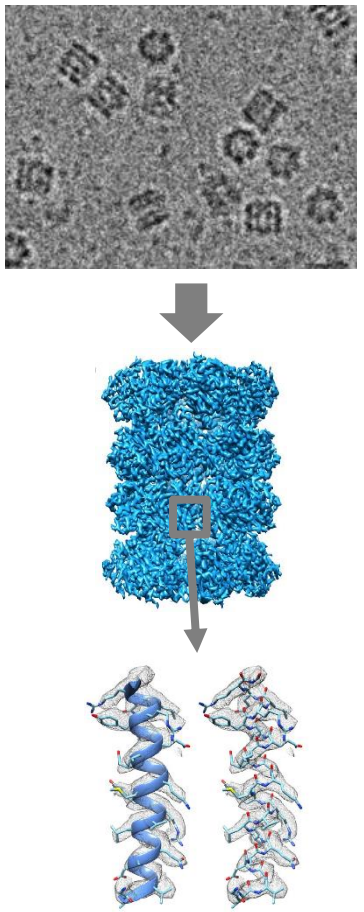
Illustration: Charis Tsevis (flickr)

Remark. Micro-ED: structure of protein crystals. Room Temperature Tomography: 3D imaging on RT TEM.

Advantages of Cryo-EM

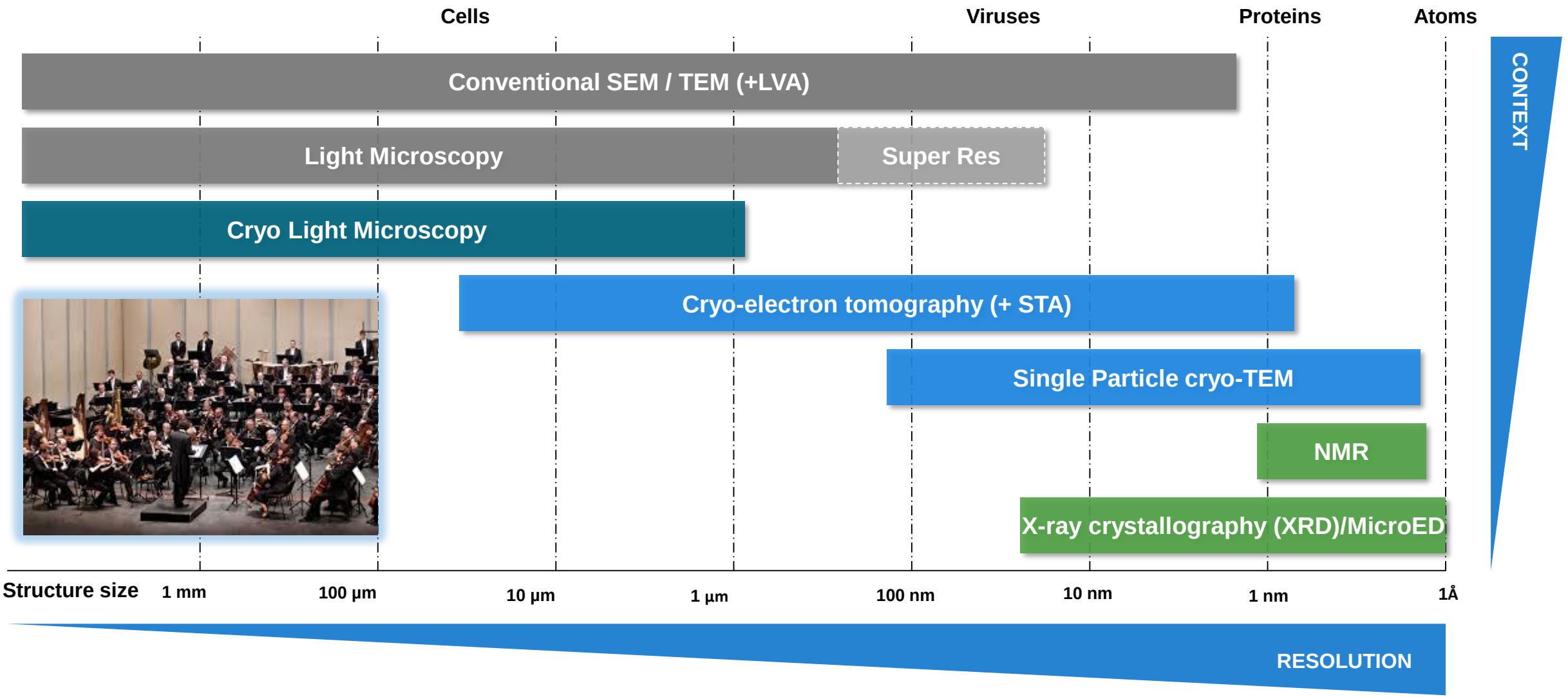
- Observation of biological systems in their native hydrated state.
- No artifacts that occur in chemical fixation and negative staining (direct visualization of biological macromolecules instead of their contours in background of negative stain).
- No need for the macromolecule crystallization.
- Suitable for larger protein complexes and pleomorphic structures.
- Possible 3D structure determination up to “near-atomic” resolution.
- Time-freezing of dynamic processes allows determination of molecules in multiple different functional conformations. Cryo-ET enables to study these conformations in the context of their cell environment.
- Cryo-ET bridges the gap between imaging of isolated macromolecules at near-atomic resolution and large volume analysis at the cell-cell/tissue level; “opens the window into the cell”.

20S proteasome



Remark. Images on the right: 20S Proteasome particles; near-atomic resolution model by Cryo-EM.

Structural Symphony: Role of Cryo-Electron Tomography / SPA within Imaging Technologies

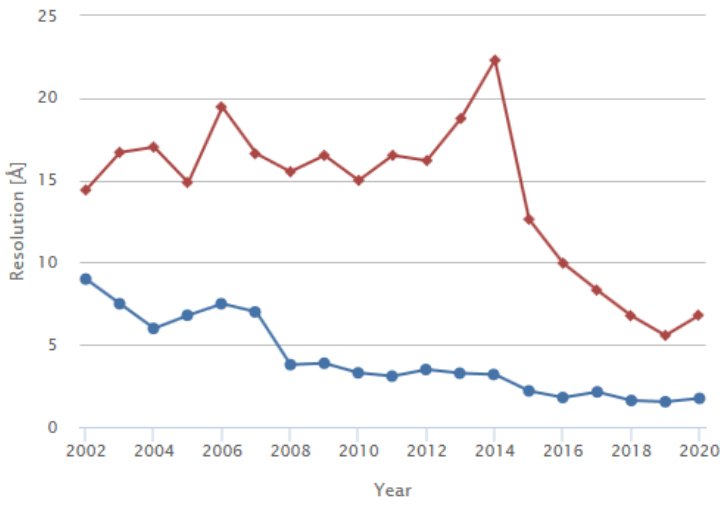


Brno Philharmonic Orchestra image: the-wagnerian.com.

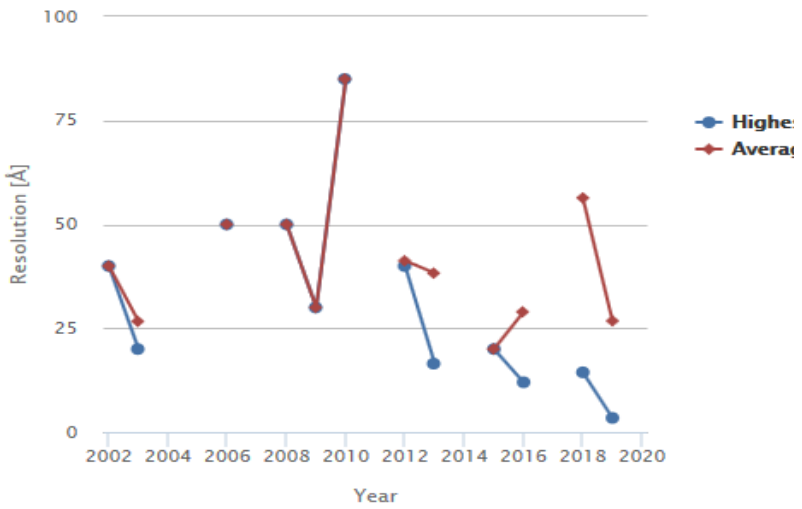
Deposited Structures in EMDB Solved by Cryo-EM

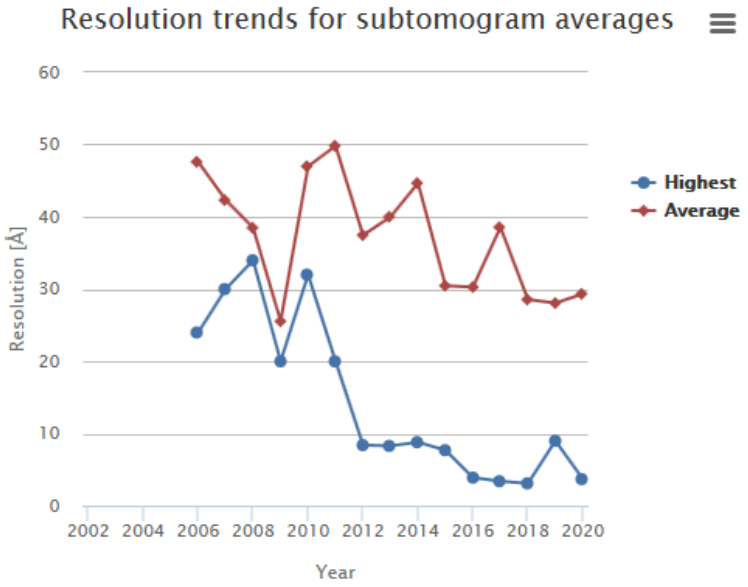
EMBL-EBI
Protein Data Bank in Europe
Bringing Structure to Biology

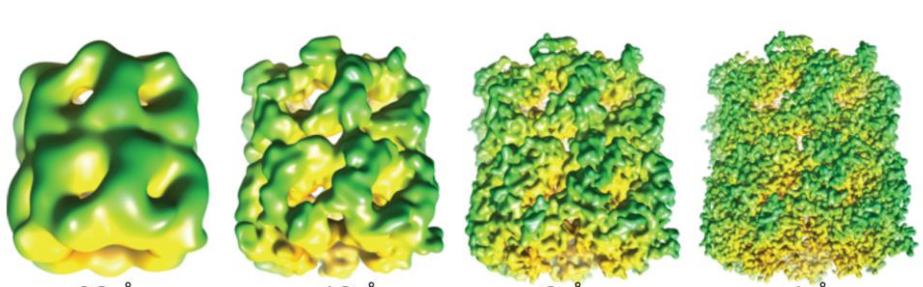
Single-particle released maps - resolution trends



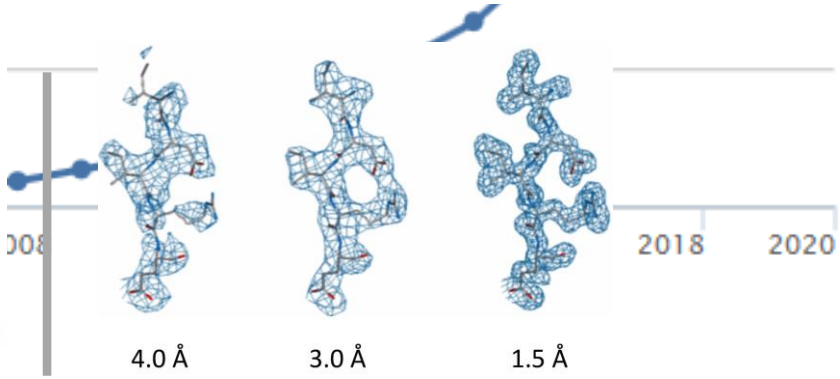
Tomography - resolution trends for released maps







Series of Resolutions for GroEL. From right to left, 4 Angstrom (Å), 8 Å, 16 Å, and 32 Å resolution. The details are smeared away as the resolution becomes lower.



MUNI

https://www.ebi.ac.uk/pdbe/emdb/index.html; Shashi Bushan: EMBO course: SPA presentation.

ThermoFisher SCIENTIFIC

Sample Preparation is the Key…Fixation

- **Bio-samples are full of water** (body water content around 60%, brain 73%, cell 70%) **=>** implications for the sample preparation and observation in EM. (Most/less abundant elements: H, C, N, O/Na, Mg, P, S.)

- **Fixation** = to stop the biological activity and to preserve the tissue structure for subsequent treatments.

- "The objective is to process tissues and cells without significant change in size, shape, positional relationship of the cellular components and to preserve as much of the biological activity and chemical nature of cellular components…"*

MUNI

*Dykstra M.J. et al., Biological EM microscopy (2003).

ThermoFisher SCIENTIFIC

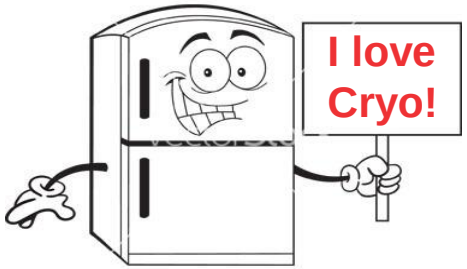
Sample Preparation is the Key…Fixation

Normal Strawberry

Heat Preservation

Freeze Dried

Chemical Fixation

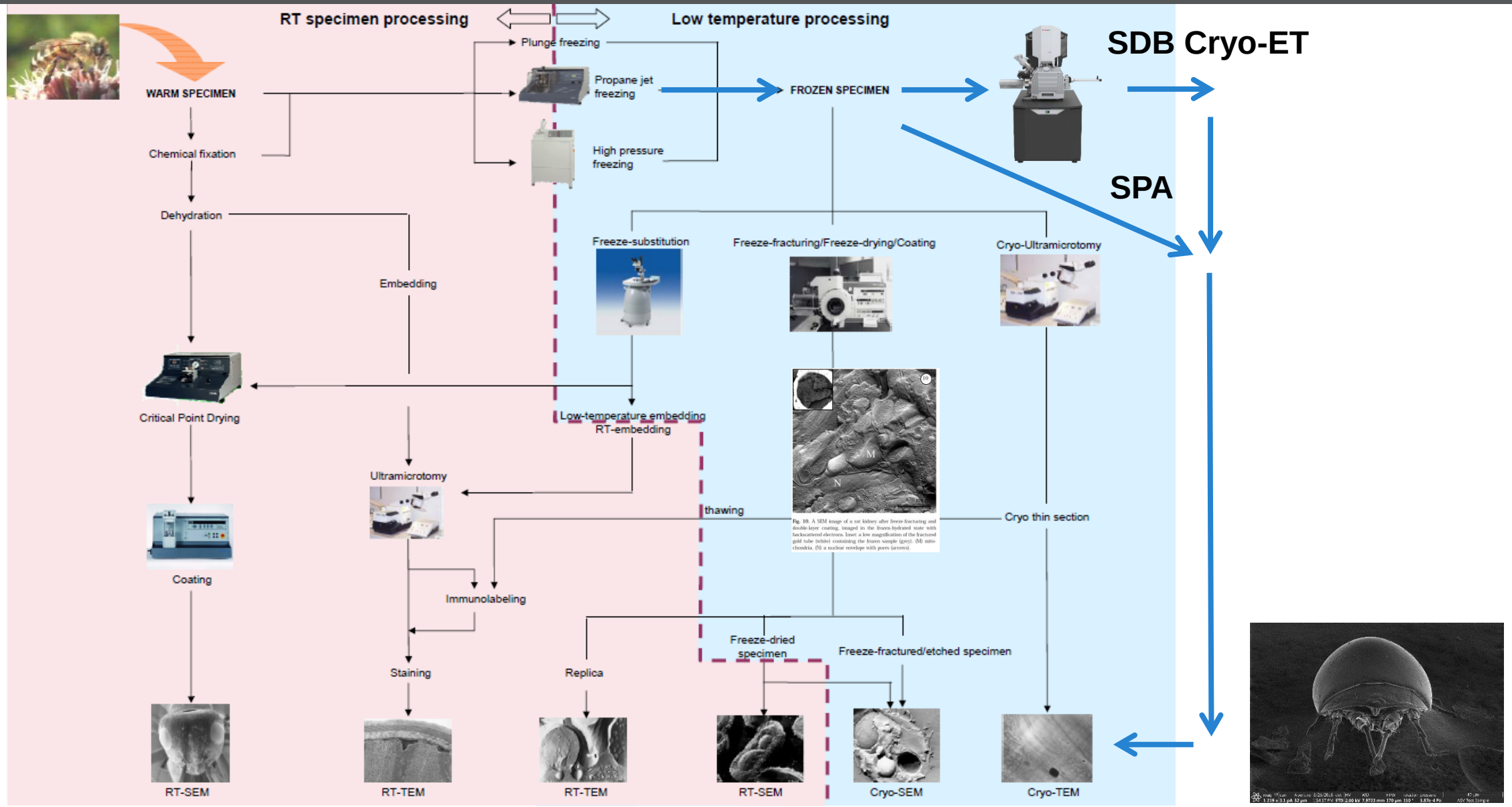


Cryo Preservation

MUNI

ThermoFisher SCIENTIFIC

Bio-Sample Preparation

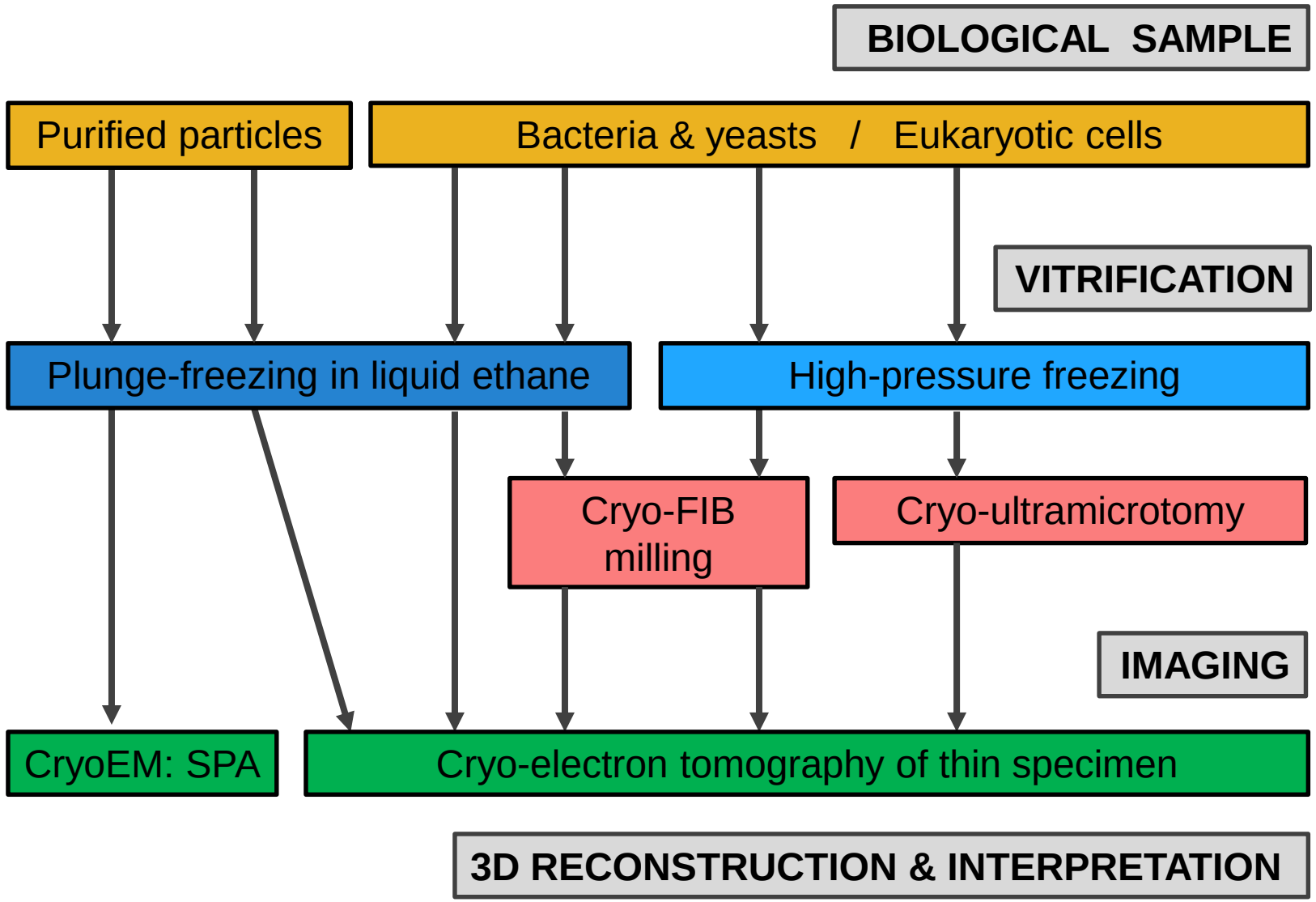


MUNI

Adapted image by Andreas Kaech, University of Zürich. See as well: Handbook of Cryo-preparation methods for EM, edited by Cavalier A., Spehner D., Humbel B.M. (2009).

ThermoFisher SCIENTIFIC

Preparation of Biological Samples for Cryo Electron Microscopy

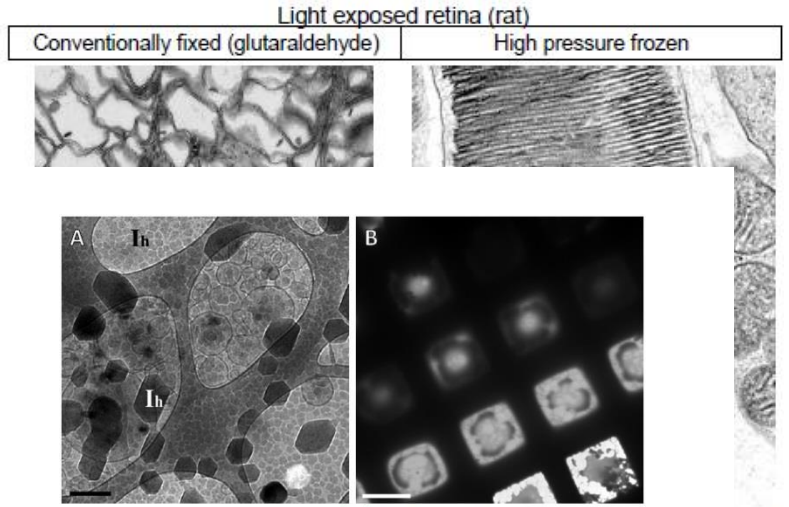


MUNI

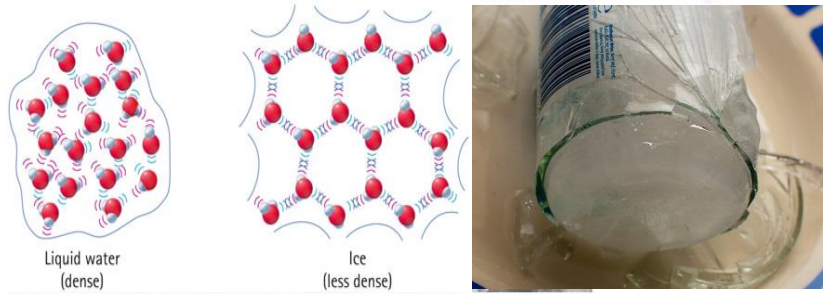
ThermoFisher SCIENTIFIC

Cryofixation (**cryo/cryos** means "icy cold" (from crystallos))

- **Why, advantages**
  - The best method of preservation. Rapid freezing in millise (minimal chemical and physical changes if done well).
  - Offers a SnapShot at a particular time, very important whe



- **Sample vitrification**
  - Cool the specimen so rapidly that there is not time for ICE what does the damage as it rips structures apart.
  - Increase cooling speed by the reducing size of the specim



- **Methods**
  - **High pressure freezing** (**HPF**, commonly up to ~200 µm), **plunge freezing (PF)**, slam (metal-mirror) freezing, double jet propane freezing, spray freezing (all up to units or tens of µm).

MUNI

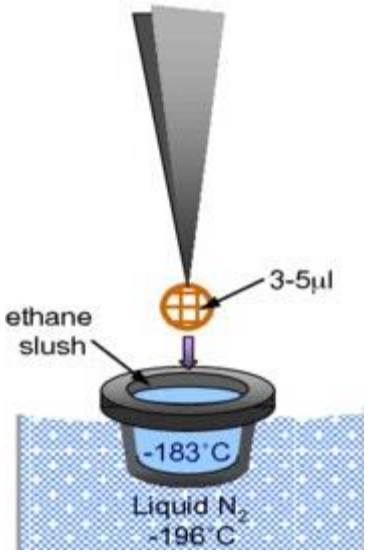
Adapted from Cheng et al., Current Micros. Contrib. to Advan. in Scien.and Tech. (2012); Szczesny et al.: doi.org/10.3109/02713689609017621.

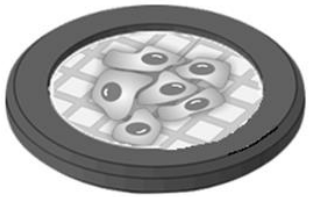
ThermoFisher SCIENTIFIC

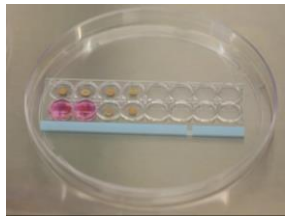
Plunge Freezing vs High Pressure Freezing (vitrification of native bio-specimens)

Plunge Freezing

- sample thickness: up to ~5-10 µm (cells, proteins, virus particles),
- 3 mm TEM grids,
- cryogen: liquid ethane/propane, atm pressure.

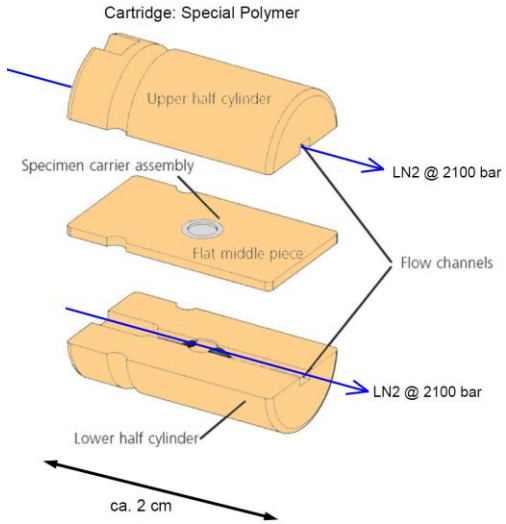






High Pressure Freezing

- sample thickness: commonly ~50-200 µm (small organisms, tissue, cell cultures),
- 3 – 6 mm carriers,
- cryogen: “open system”: pressurized LN2.



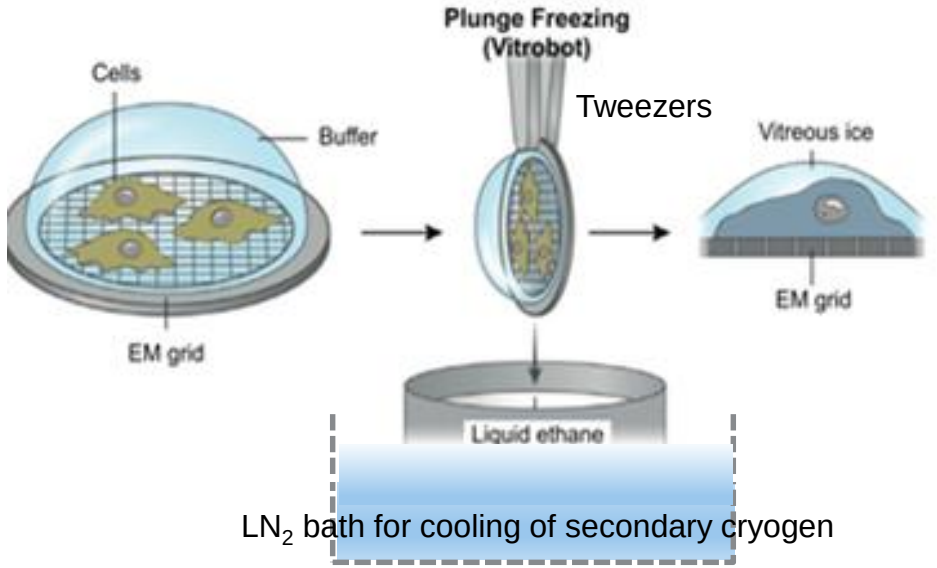
HPF: Images adapted from leica.com; Andres Kaech: High pressure freezing / presentation.

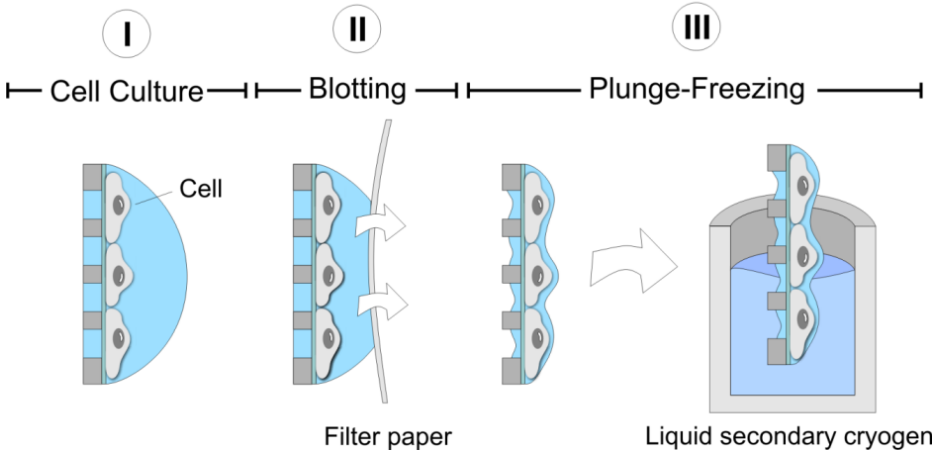
Plunge Freezing

- Needs high rate of cooling (~10^5 K/s) to temperatures below -140 °C.
- Fast plunge of blotted specimens (m/s).
- Liquid nitrogen cannot be used because of low heat capacity.
- Liquid ethane or propane have good properties and T_m close to T_b of LN_2.

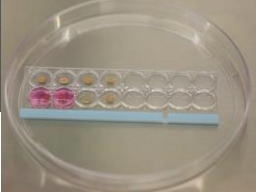
Cryogen	T_m (°C)	T_b (°C)	c_p (J/mol·K)	Rel. cooling effic.
Ethane	-183	-89	68.50	1.3
Propane	-189	-42	98.36	1.0
Nitrogen	-210	-196	4.08	0.1

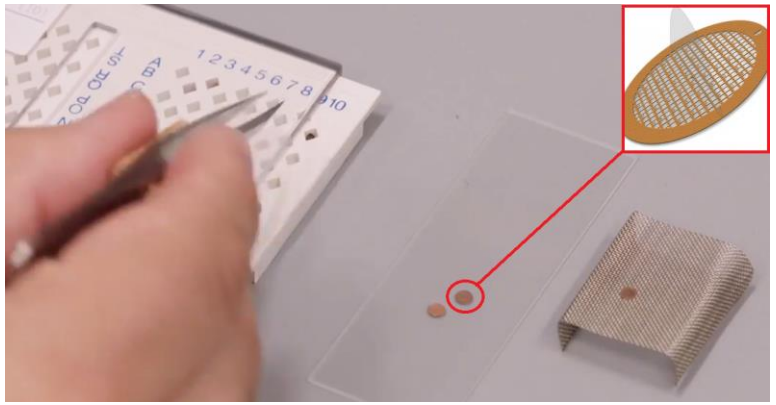
R. A. Steinbrecht, K. Zierold, eds. Berlin: Springer-Verlag. 1987; 88-113.

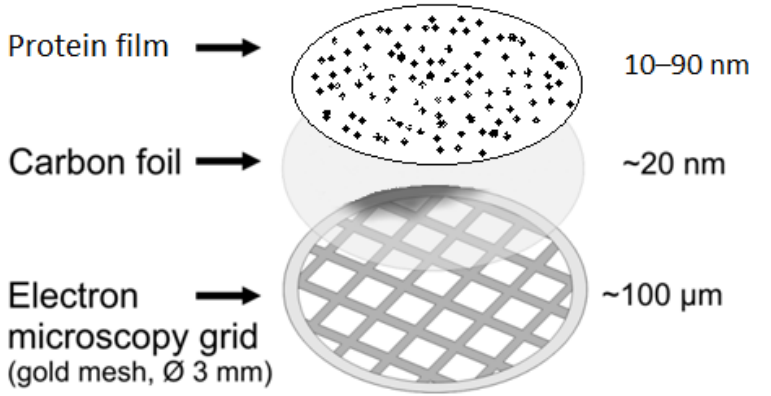


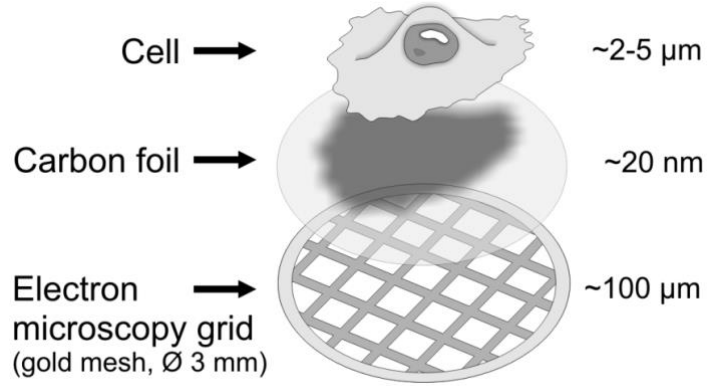


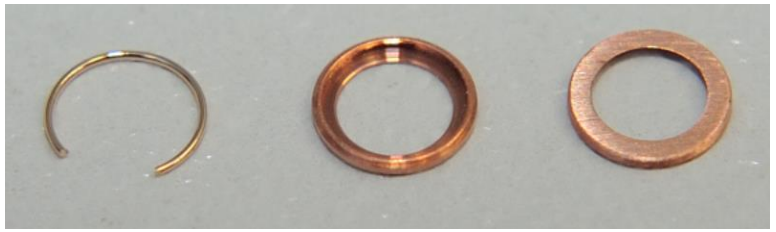
Sample and TEM Grid Support

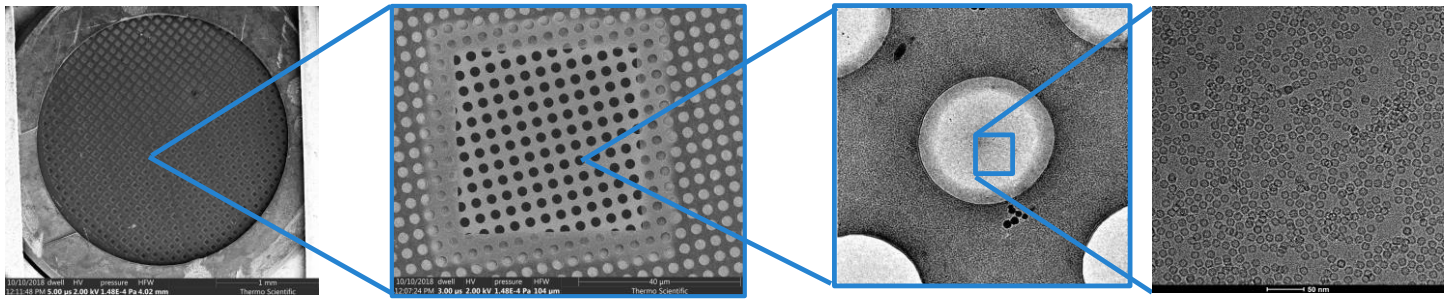










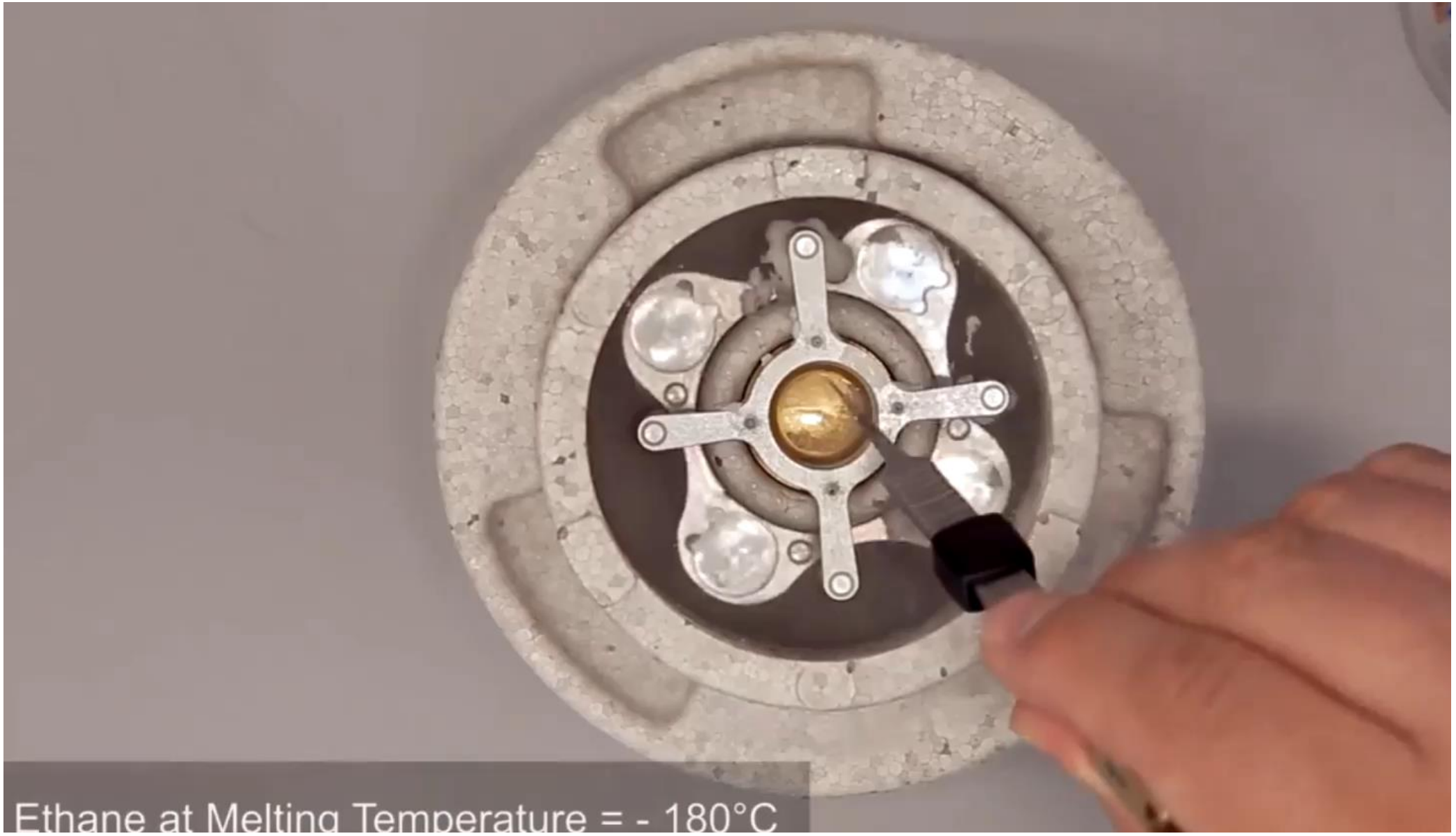


- Support to fragile TEM grids and possibility of robotic handling and automated TEM loading.

MUNI

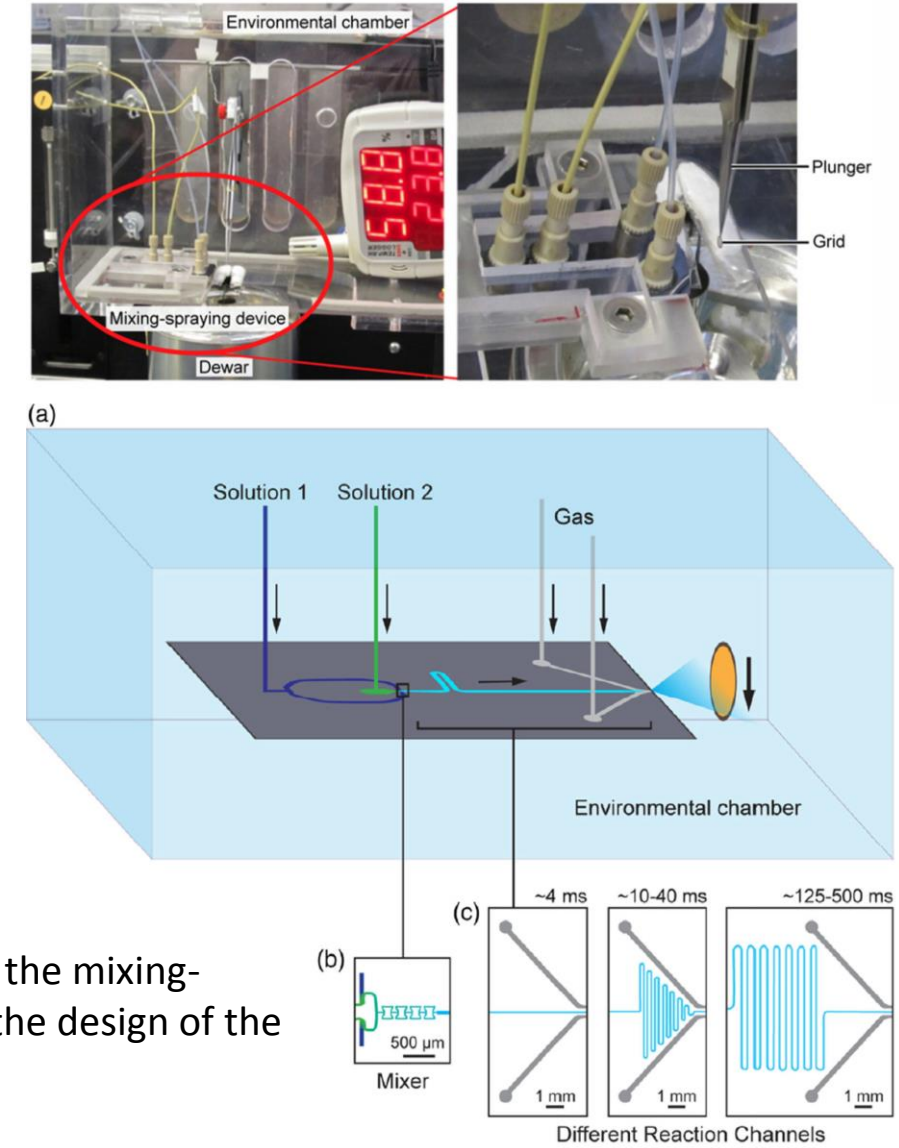
ThermoFisher SCIENTIFIC

Plunge Freezing Process

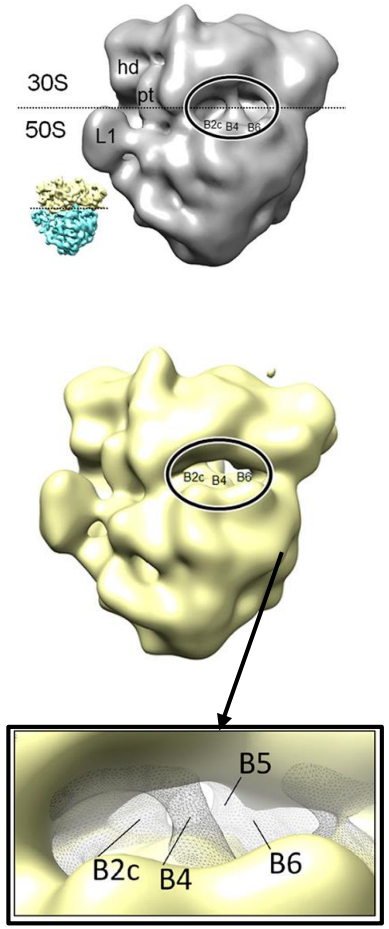


Time-Resolved Cryo-EM (TR Cryo-EM)

- *"*Combination of the structural study with kinetics*, by capturing kinetic intermediate states in a biological reaction."
- *"Movies of a biological complex functioning in real time."*
- "Fast reactions require a means of mixing, reacting and depositing the product on the grid in a fast, controlled way."
- **Different approaches**: *mixing-spraying, spraying-freezing, flash-photolysis, etc.*



Experimental setup of the mixing-spraying method and the design of the mixing-spraying chip.



Cryo-EM maps of the 70S ribosomes formed within 9.4 ms.

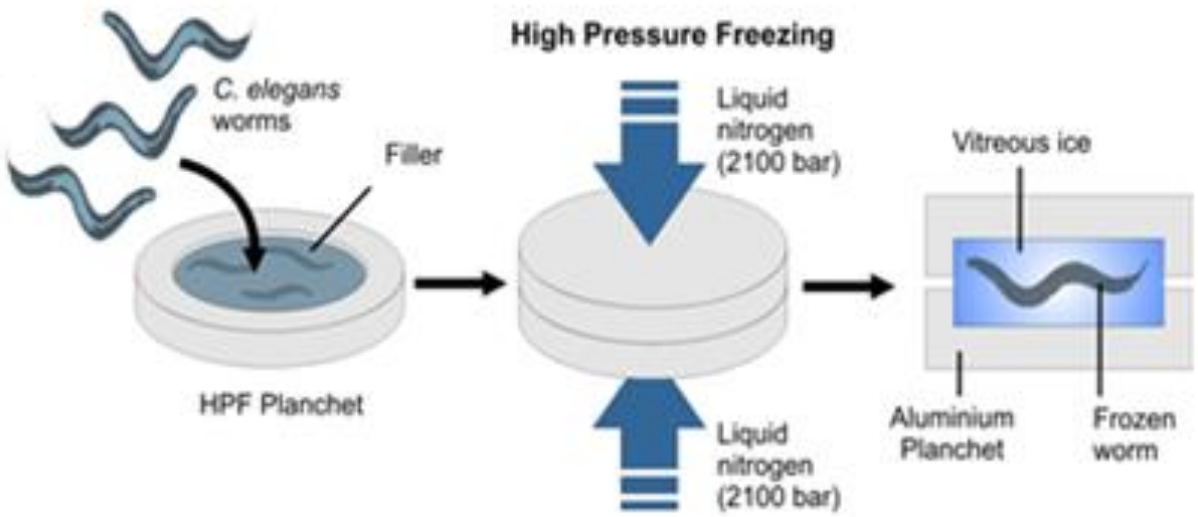
MUNI

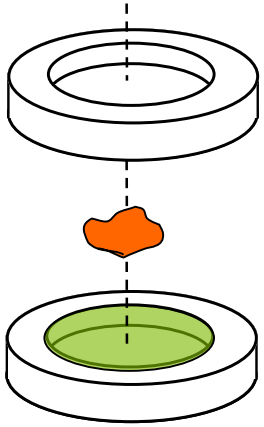
*Adapted from: *Frank J., J. Struct. Biol. 200(3) (2017); Shaikh T.R. et al., PNAS 111 (27) (2014).*

ThermoFisher SCIENTIFIC

High Pressure Freezing

- Simultaneous high rate of cooling (~10^4 K/s) to temperatures below -140 °C at high pressure (2100 bars).
- Pressurized LN_2 flows over the sample inside the carrier (“open system”).
- Rapid cooling at high pressure allows for the transition to amorphous (glassy-like) state even for “thicker” samples.





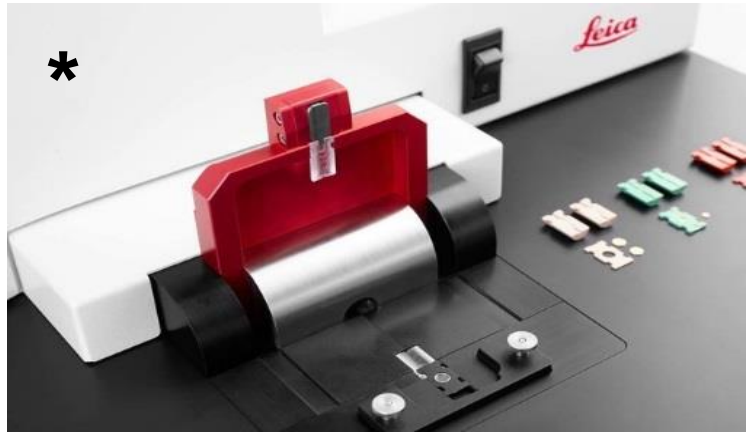
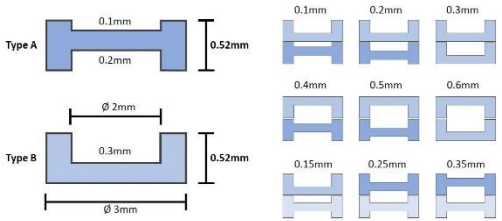
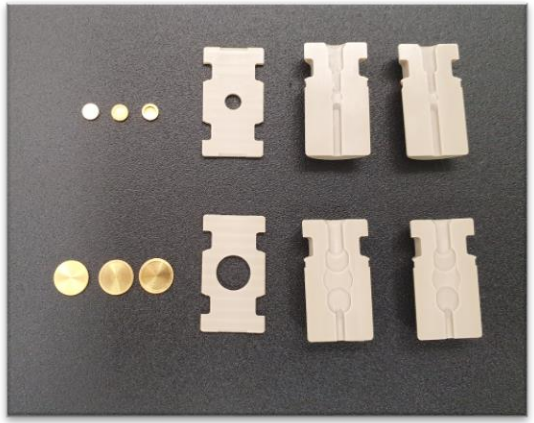
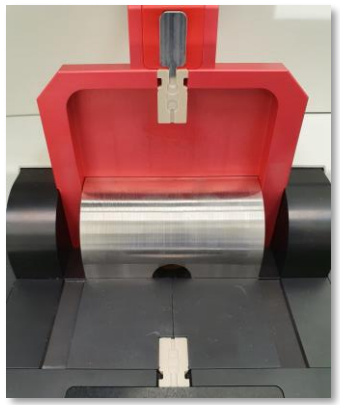


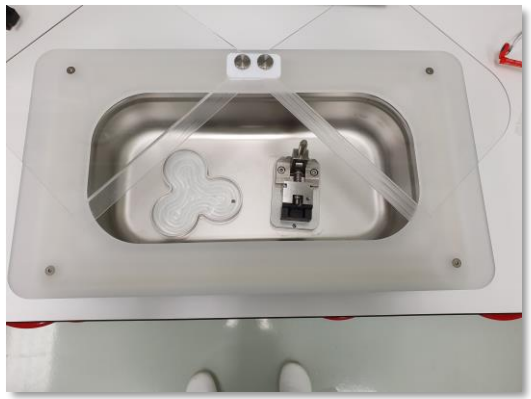
Image of HPF (*) adapted from leica.com.

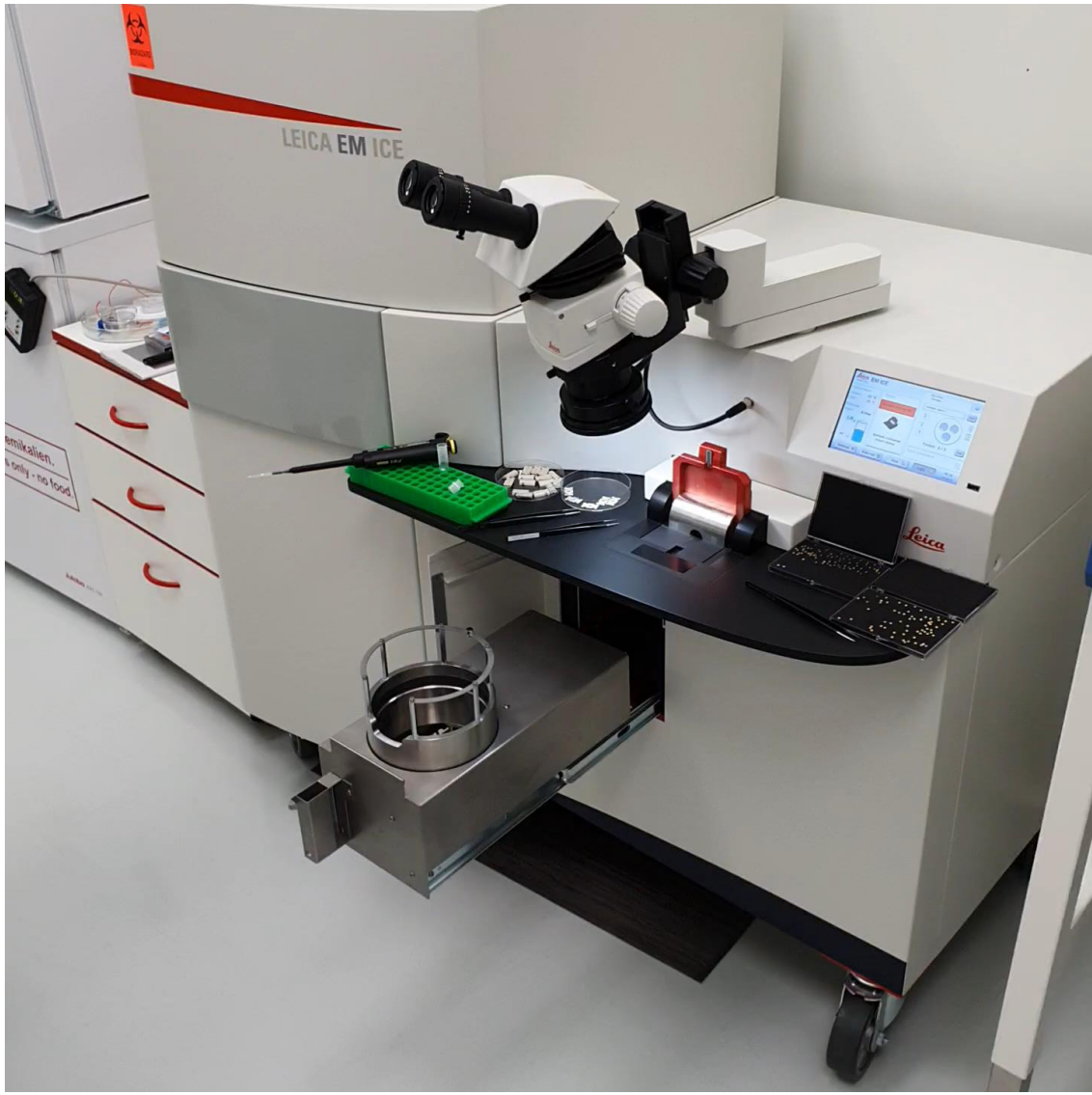
HPF Freezer











Depicted HPF Freezer: High Pressure Freezer Leica EM ICE.

The pressure-temperature dependent phase diagram of water

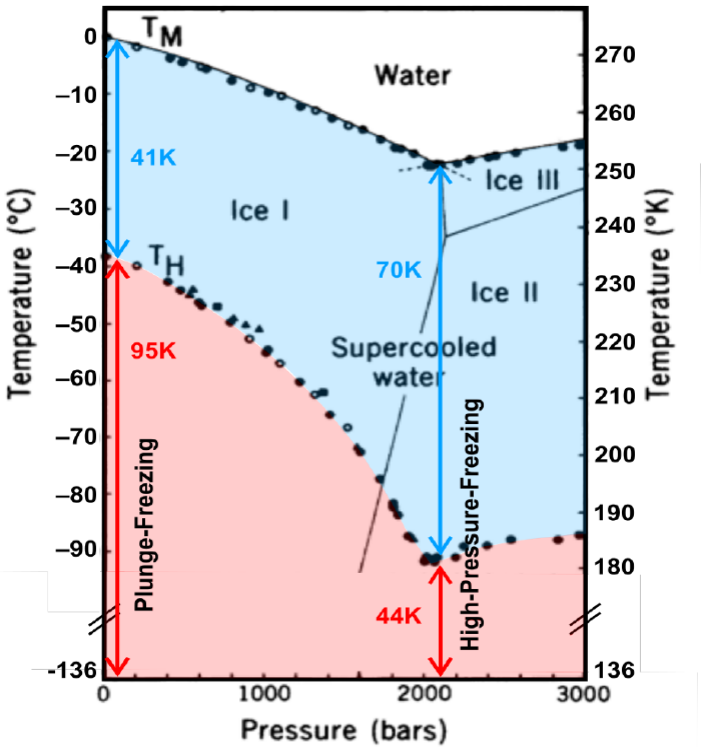


Fig. 1. Homogeneous nucleation (T_H) and equilibrium melting temperatures (T_M) for water in emulsion form as a function of pressure. Results obtained with different pressure cells and emulsion carrier fluids are distinguished as follows. Cell 1: △, heptane; ■, pentane; △, methylcyclohexane. Cell 2: ○, heptane; ●, methylcyclopentane + methylcyclohexane. Solid lines are the accepted equilibrium phase boundaries. The dashed, vertical line indicates the conditions under which ice II and ice III may be produced in high pressure frozen samples. (Adapted from Kanno et al., 1975.)

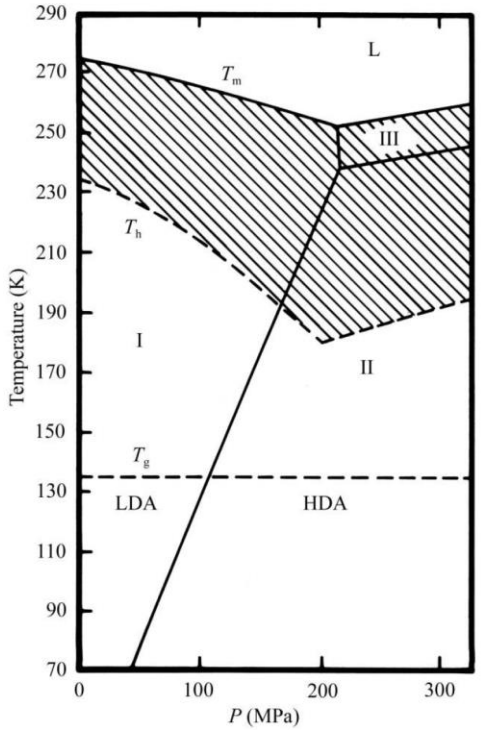
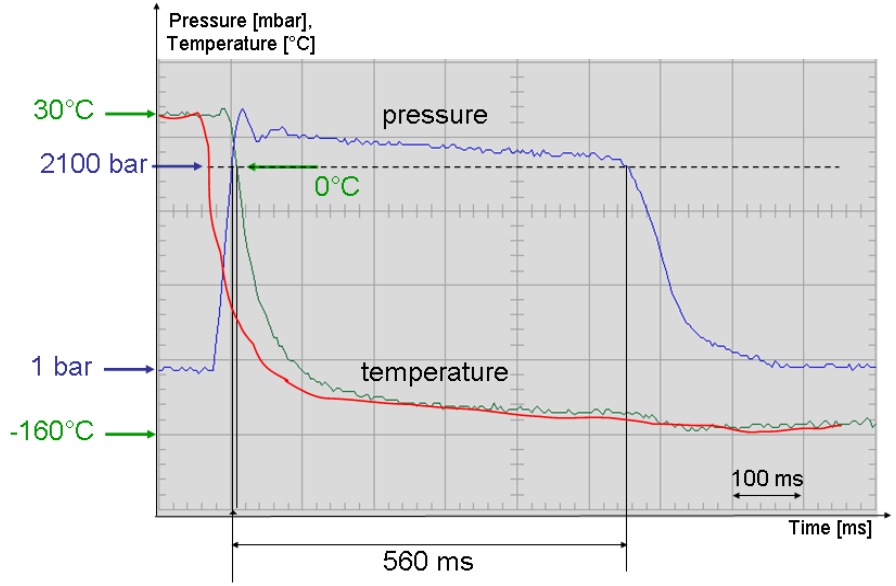


Figure 6
The pressure–temperature phase diagram of H_2O [adapted from Kanno *et al.* (1975), Franks (1985), Garman & Schneider (1997) and Mishima & Stanley (1998)]. Amorphous ices form when liquid water is rapidly cooled below the glass-transition temperature (T_g), preventing the nucleation and growth of crystalline ices. HDA ice may form upon cooling above ~100 MPa. LDA ice is formed below ~100 MPa. The hatched region is the allowed region for supercooled liquid water. The melting point (T_m) and the lowest temperature (T_h) for supercooled water both decrease with pressure up to 210 MPa. The region in which the amorphous phase can exist depends on the thermal/pressure history of the system. HDA ice is metastable at ambient pressure as long as the temperature is kept below 120 K. Note that the glass-transition line (T_g) is very hard to determine exactly experimentally and should be taken as an estimate.



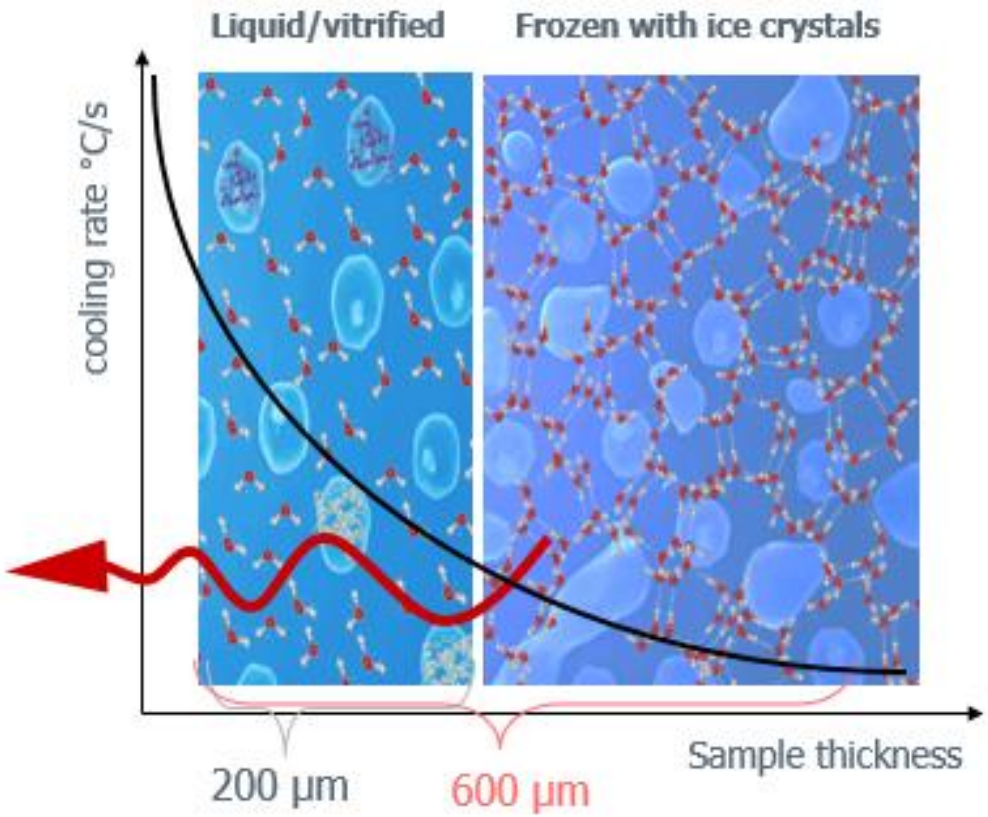
MUNI

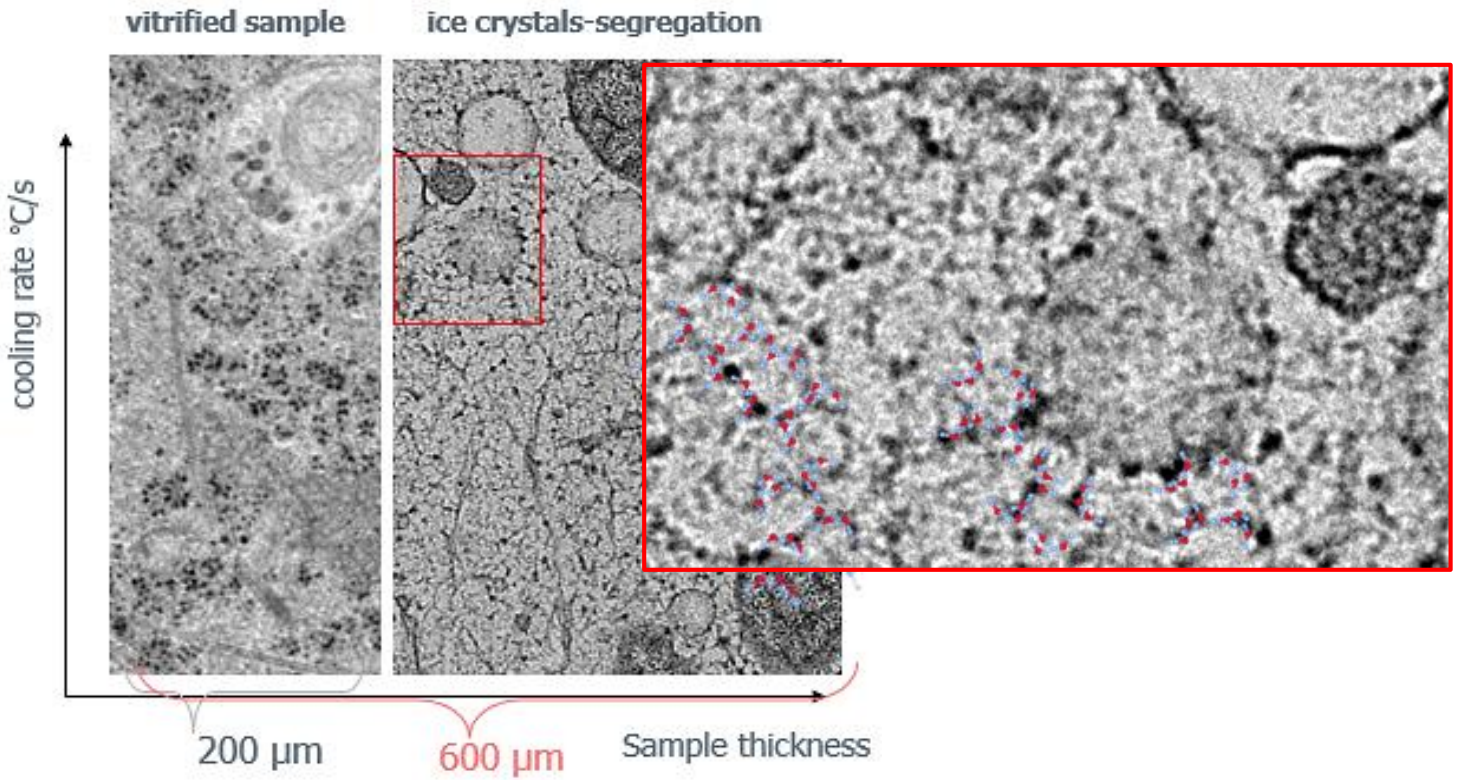
Images Fig.1, Fig. 6 adapted from: Dahl R. et al., J. of El. Micr. Tech. 13 (1989) + Bäuerlein F.J.B., PhD. thesis, 2018 (TUM/MPI); Kim Ch.U. et al., Acta Cryst. D61 (2005).

ThermoFisher SCIENTIFIC

HPF: Cooling Rates

- Water has a very poor heat conductivity.



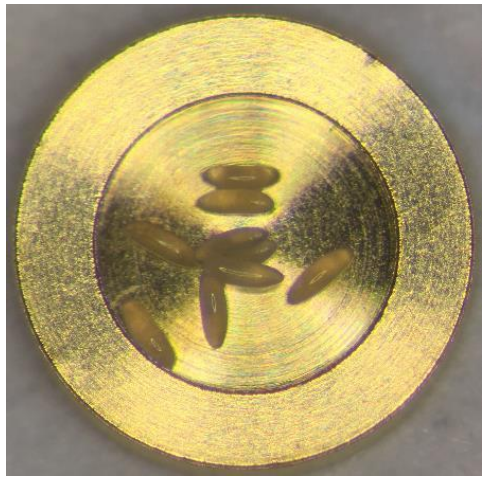


thicker specimen=lower cooling rate

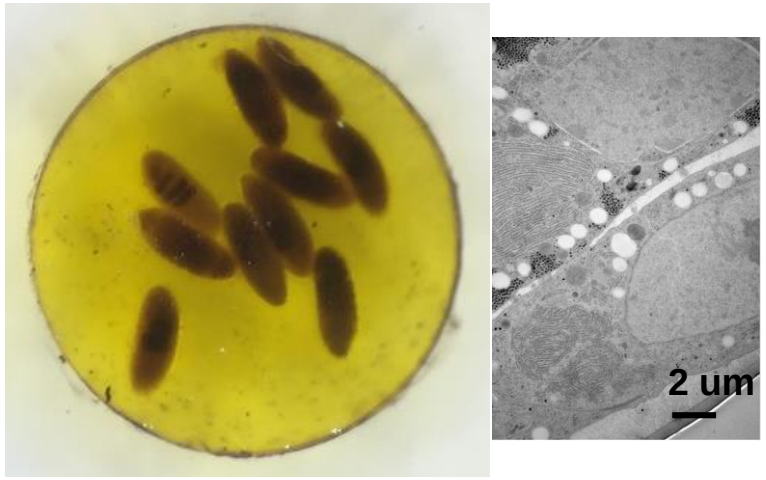
ice crystal formation=Segregation

HPF: FS and observation of resin embedded samples at RT

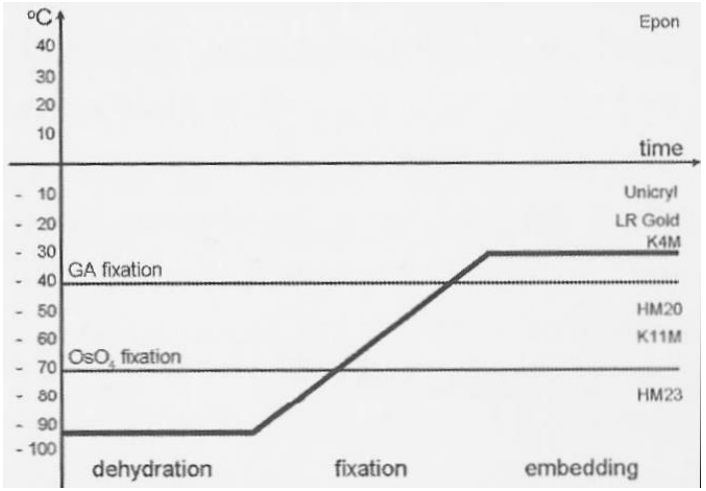
Drosophilla embryo



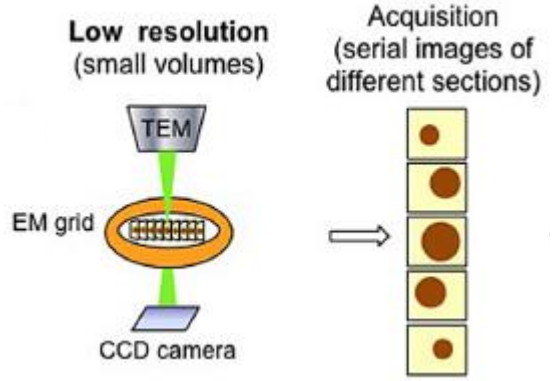
In 20% Dextran before freezing.

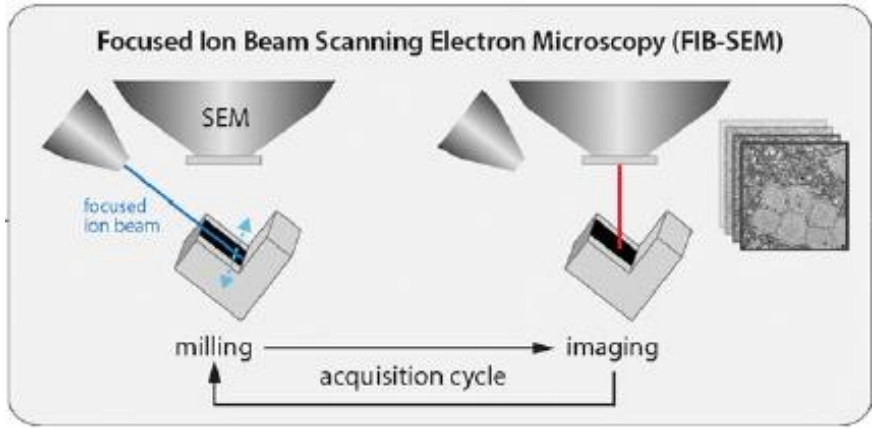


After Freeze Substitution (FS) in Epon resin.



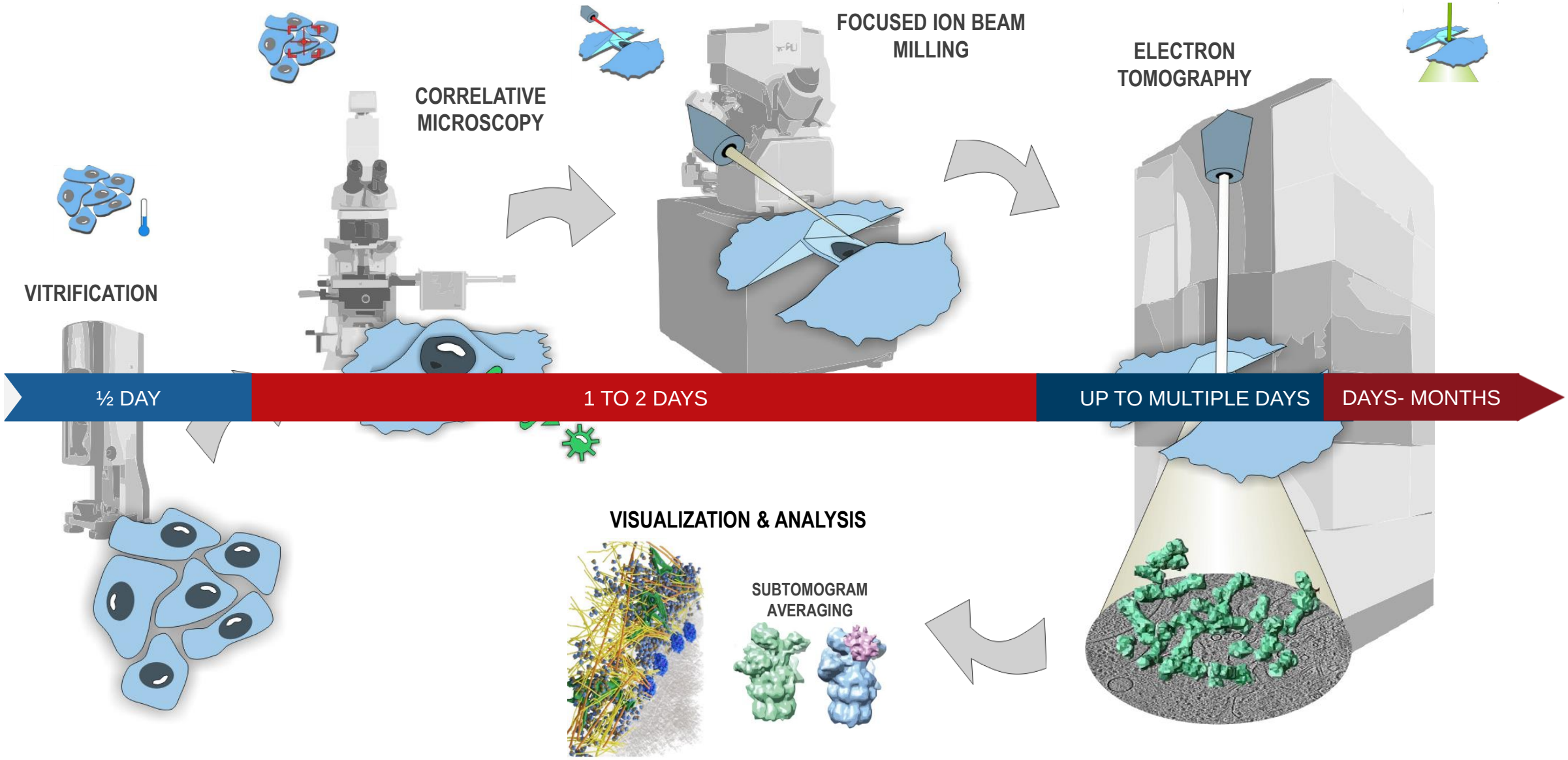
Schematic representation of FS process.





Images at the bottom adapted from Titze B. et al., Biol. Cell 108 (2016); Miranda K. et al., Molecular Rep. & Dev. 82 (2015).

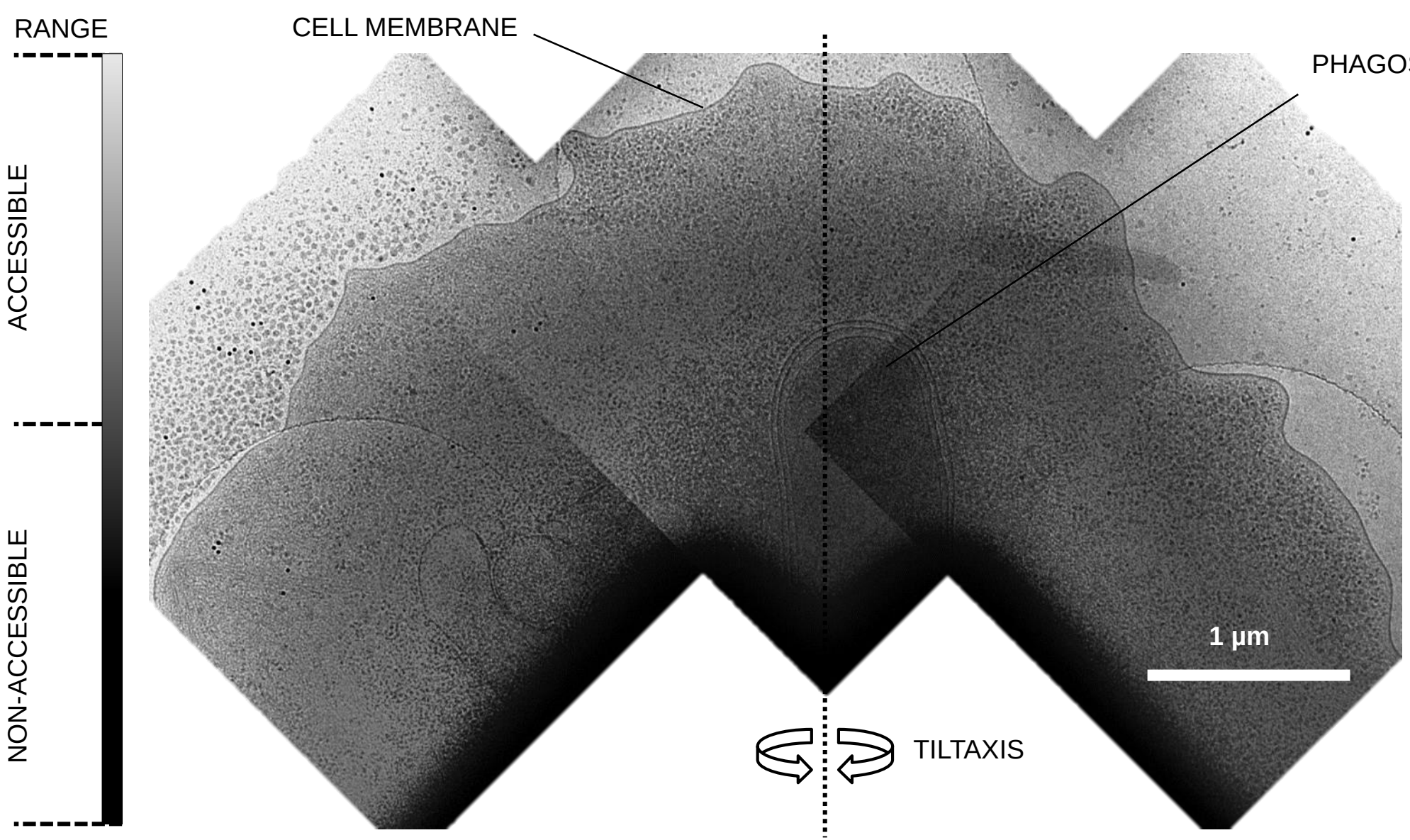
Doing Cryo Electron Tomography on Vitrified Cells Requires a Workflow

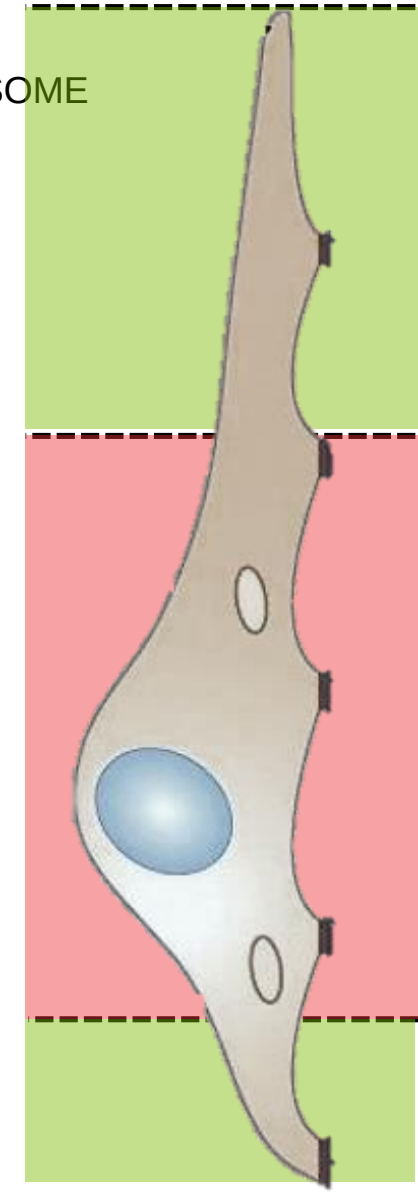


MUNI

ThermoFisher SCIENTIFIC

Many Cells are Too Thick for Electron Cryo-Tomography



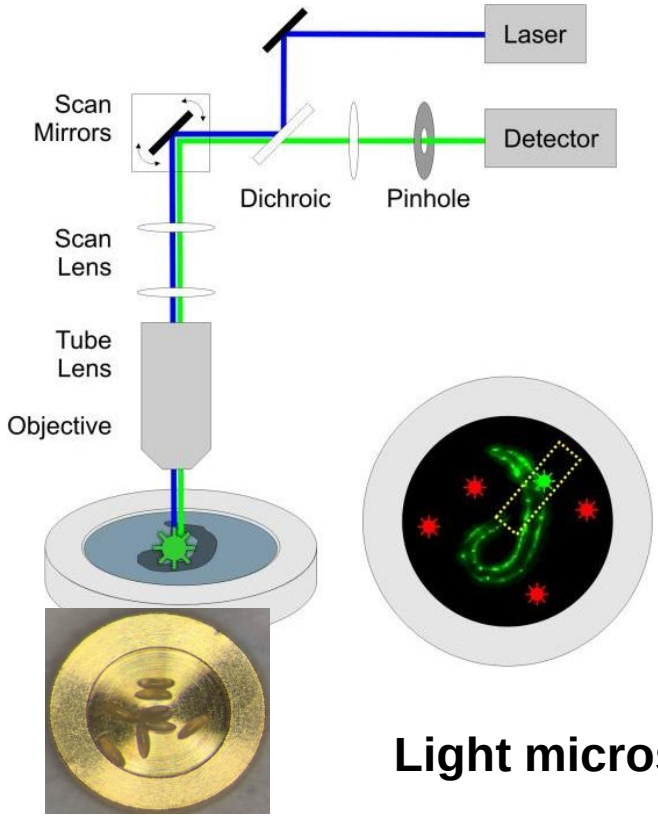


MUNI

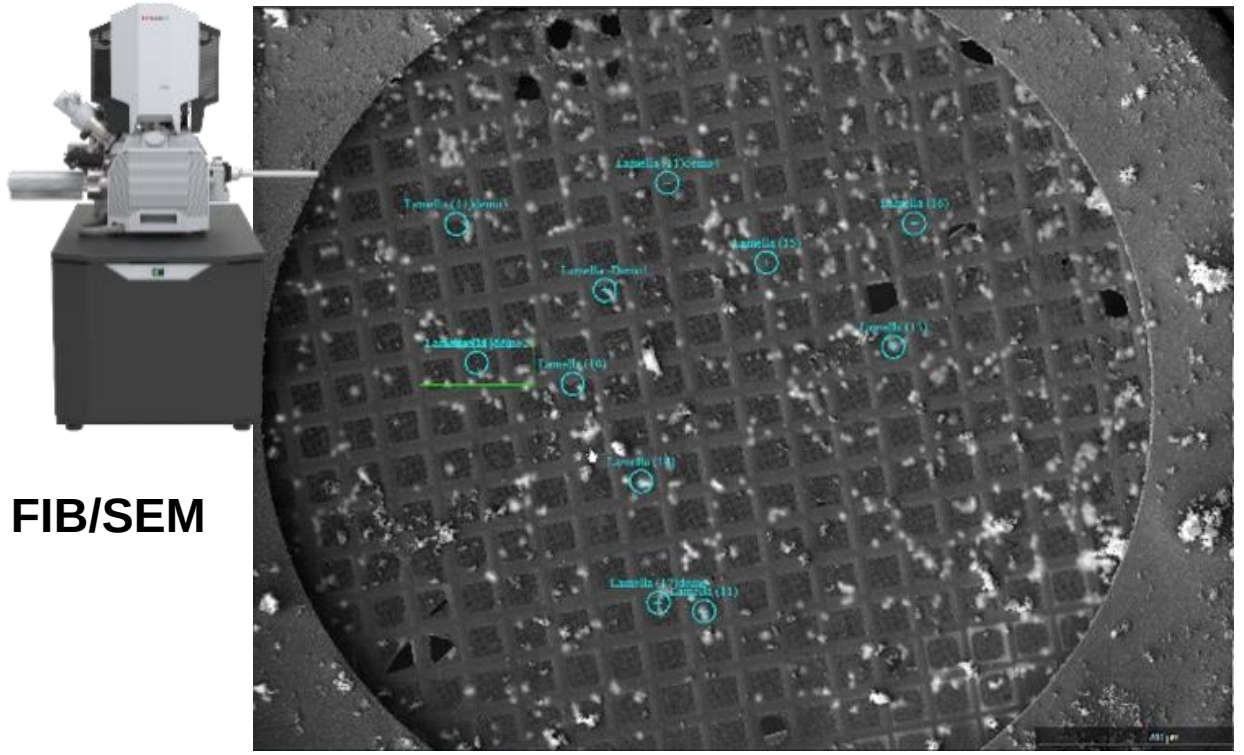
ThermoFisher SCIENTIFIC

A need for correlative microscopy / CLEM

Correlative microscopy: use of two or more microscopy techniques – preferably with different spatial and/or temporal resolutions – to characterize the same region of interest in a sample.



Light microscope

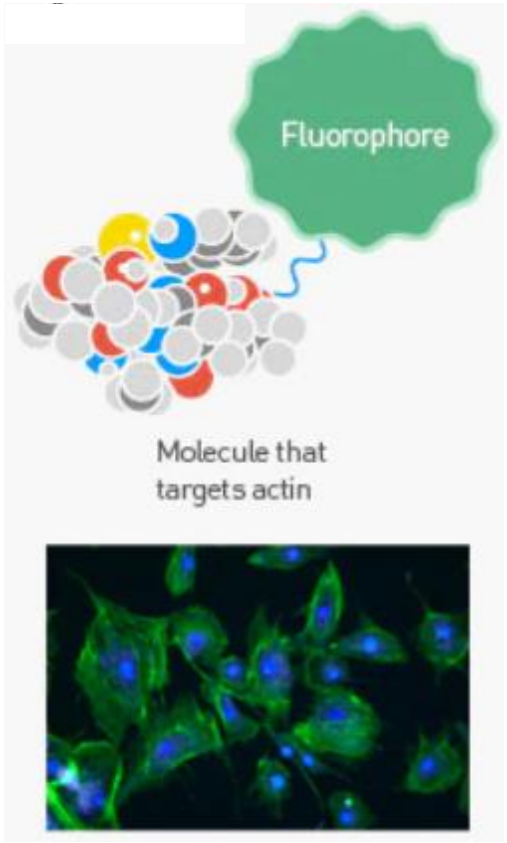


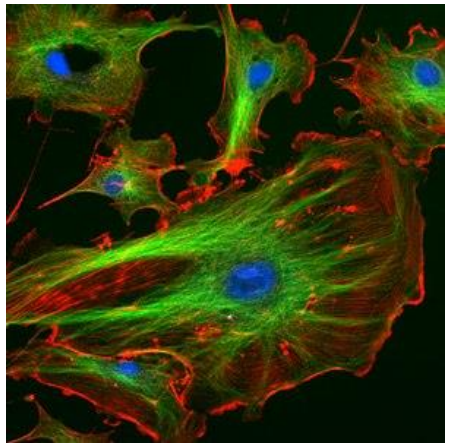
FIB/SEM

MUNI

ThermoFisher SCIENTIFIC

Cryo-Fluorescence Microscopy: Fluorescence Mediated Targeting

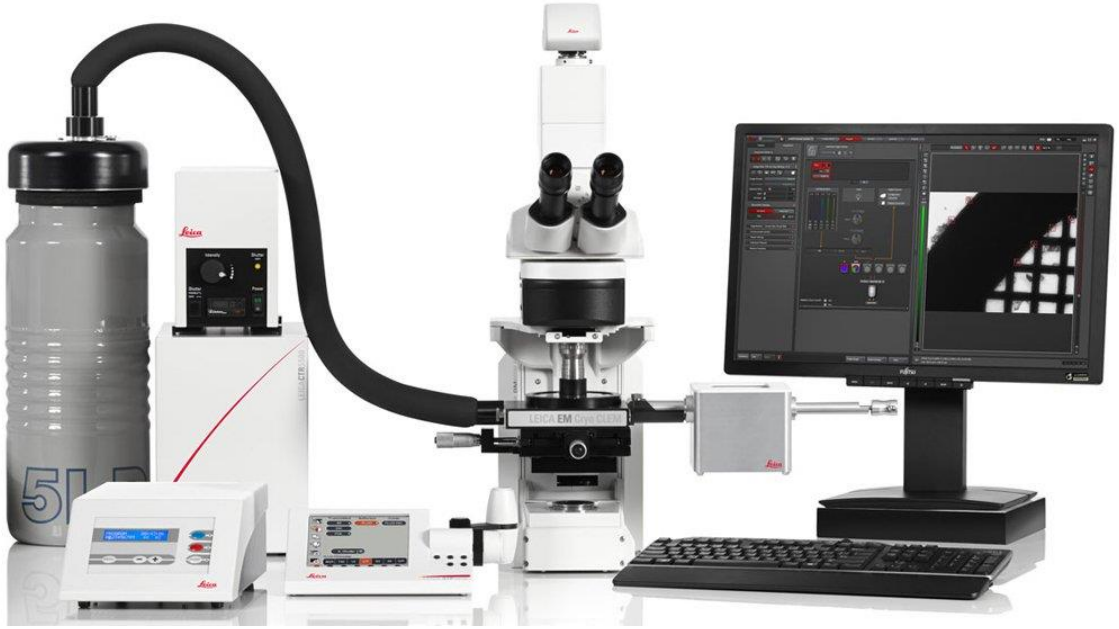


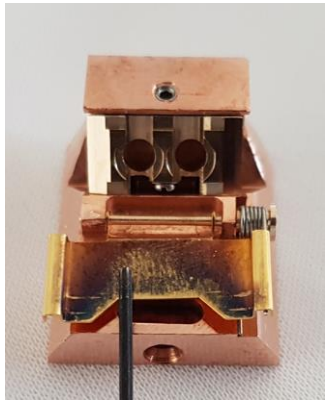


The eukaryotic cytoskeleton. Actin filaments are shown in red,and microtubules compo sed of beta tubulin are in green.

Blue: nucleus stained with DAPI; Green: Tubulin (microtubules); Red: F-Actin stained with Texas Red X-Phalloidin.

Cryo-Light Microscope (cryo-LM, Leica)





Catridge

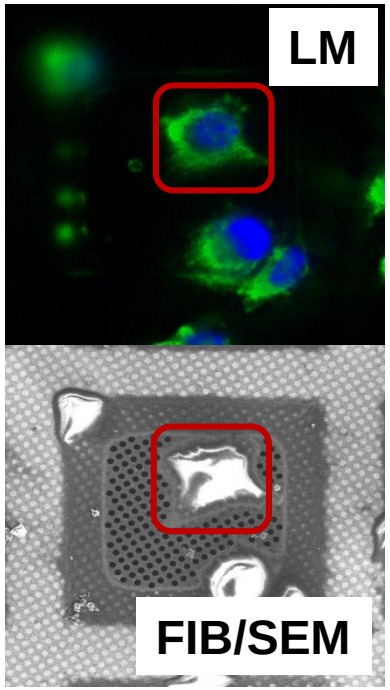
Transfer device

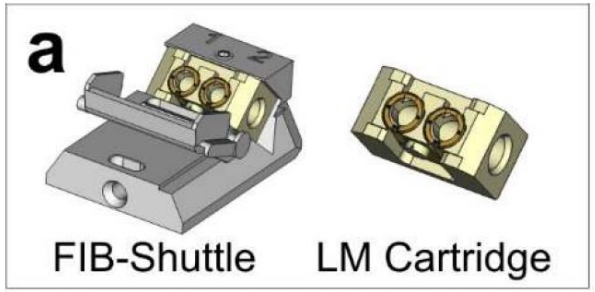
MUNI

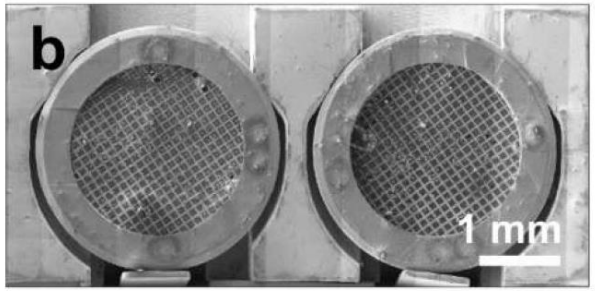
Images adapted from: thermofisher.com; http://rsb.info.nih.gov/ij/images/; leica.com.

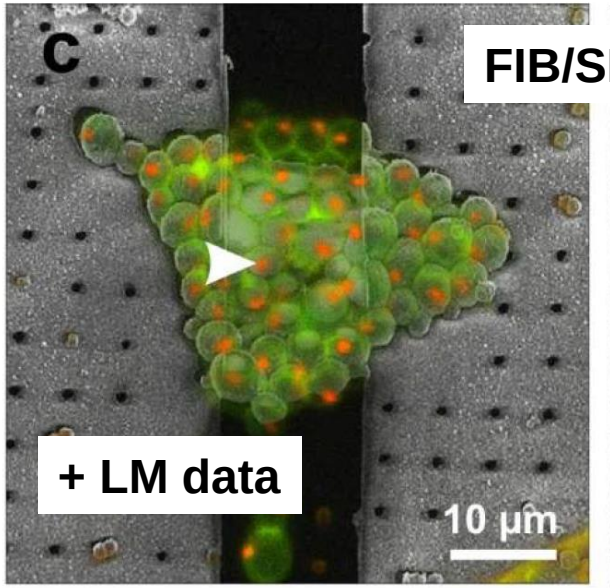
ThermoFisher SCIENTIFIC

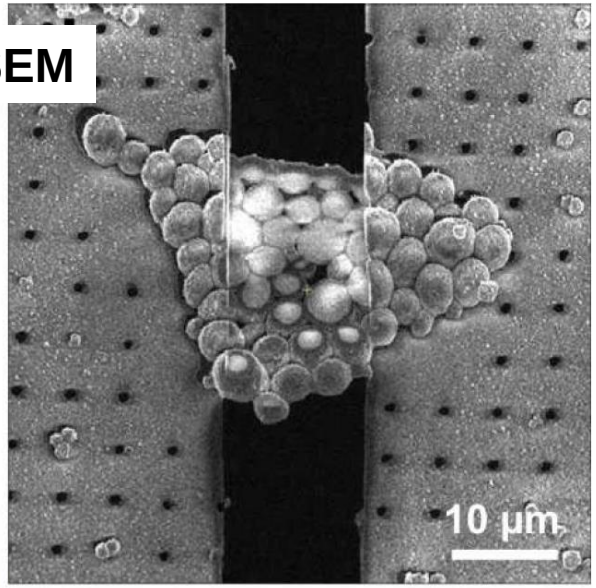
Cryo-CLEM: Relocation of Region of Interest in the Cryo-EM

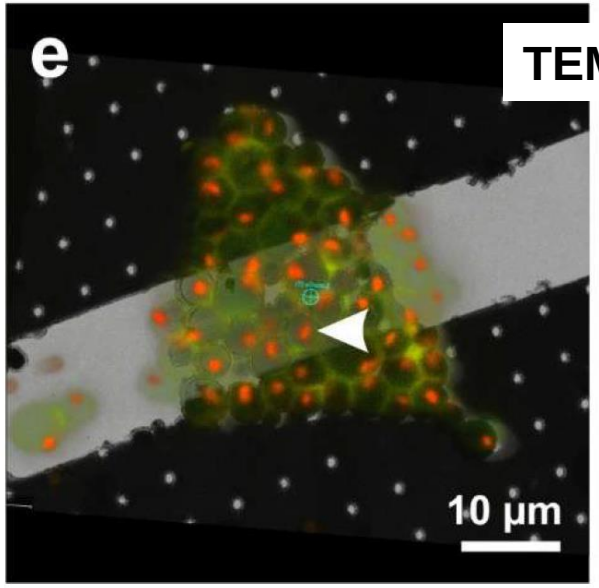


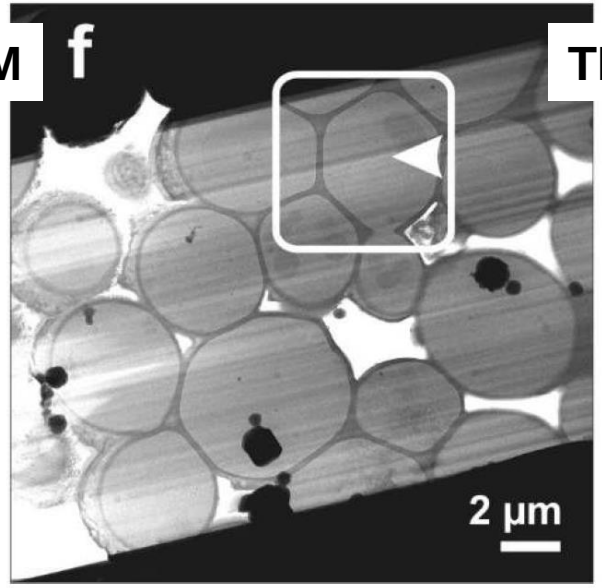


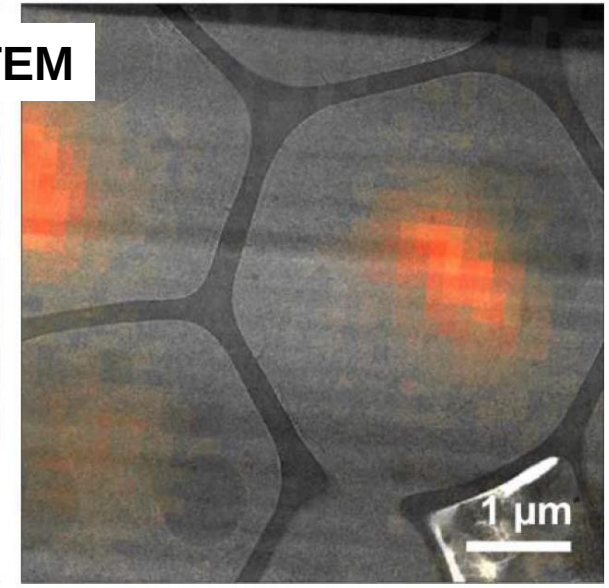












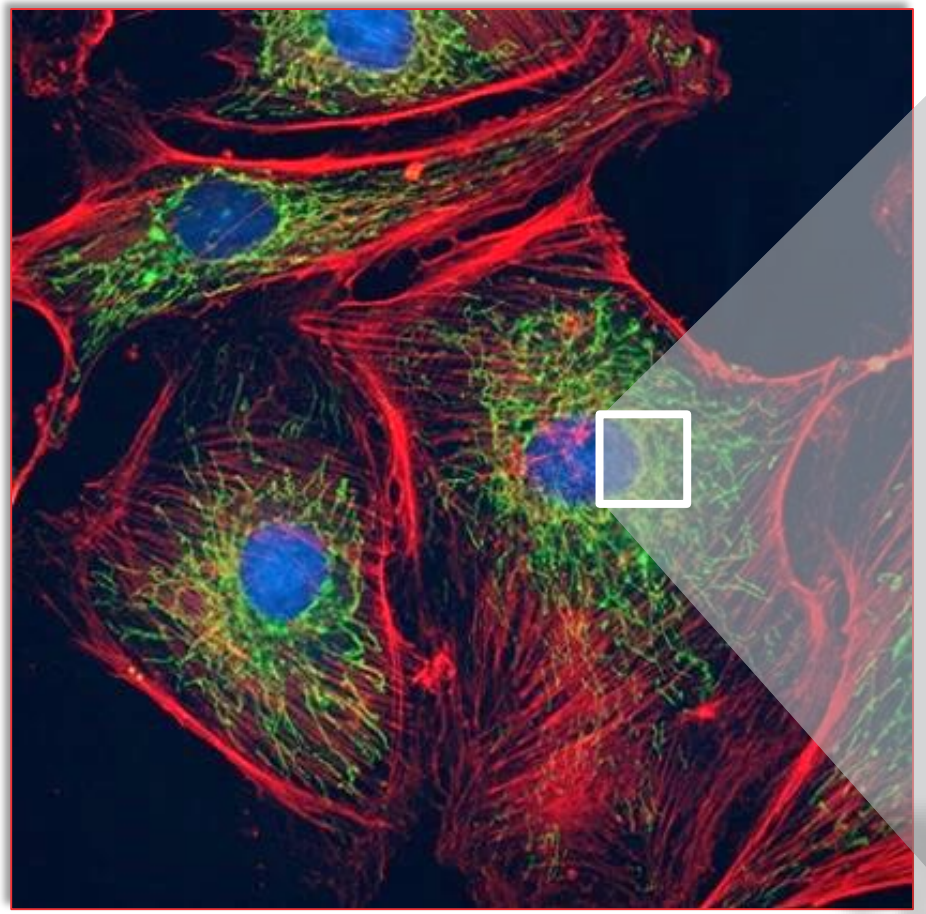
MUNI

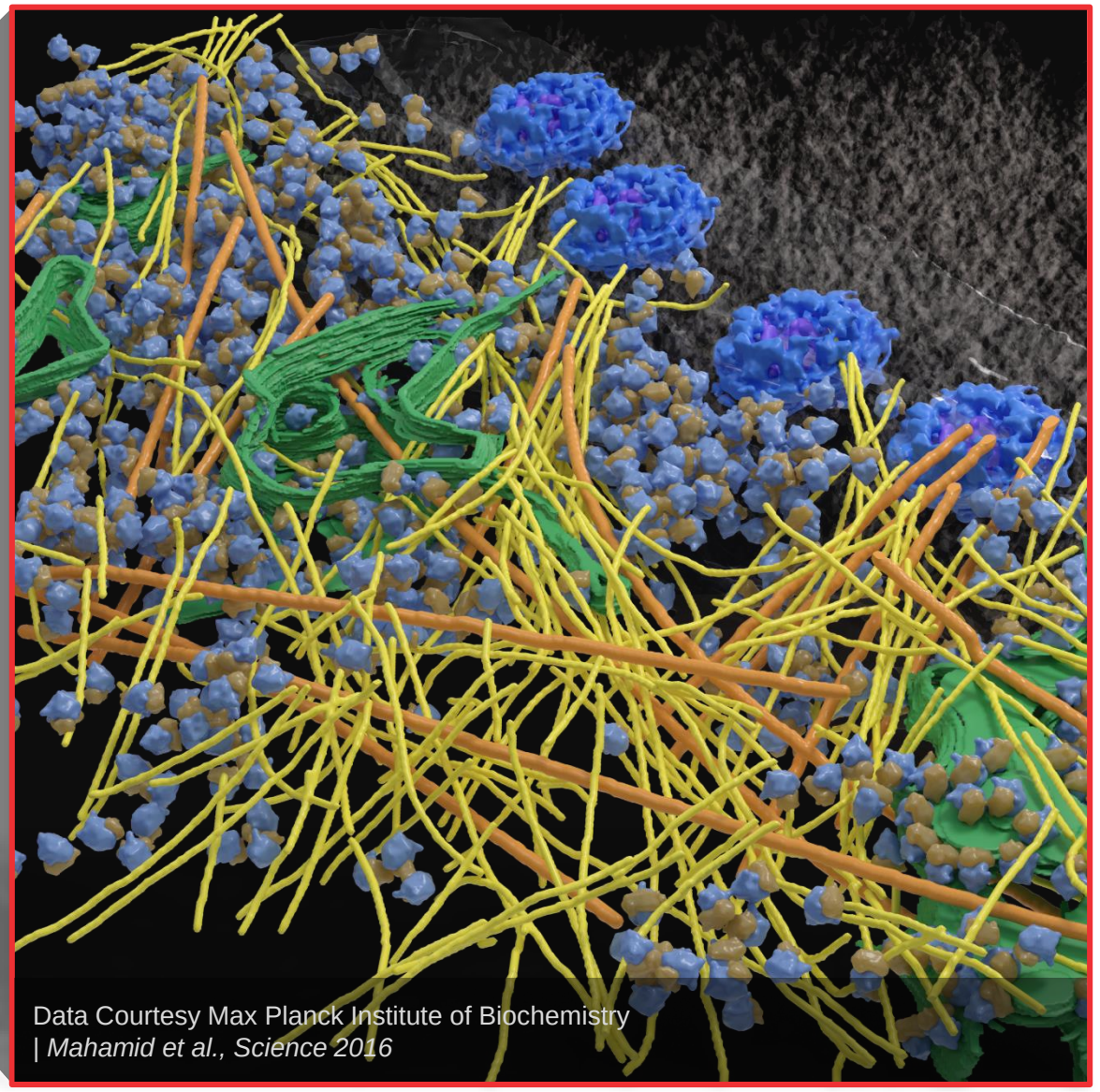
Right image adapted from: Kuba J. et al., Journal of Microscopy (in review) (2020).

ThermoFisher SCIENTIFIC

Cryo-Tomography: high resolution imaging of structure and context for cell biology

FLUORESCENCE IMAGING





Data Courtesy Max Planck Institute of Biochemistry
| *Mahamid et al., Science 2016*

⊕ Molecular Identification
Dynamics
Large Volumes

⊖ Only see what is tagged
No structure
No context

MUNI

ThermoFisher SCIENTIFIC

Cryo-FIB: Cryo-Sample Preparation…opening the window into the cell

GUIDED USER SOFTWARE

LN2 DEWAR

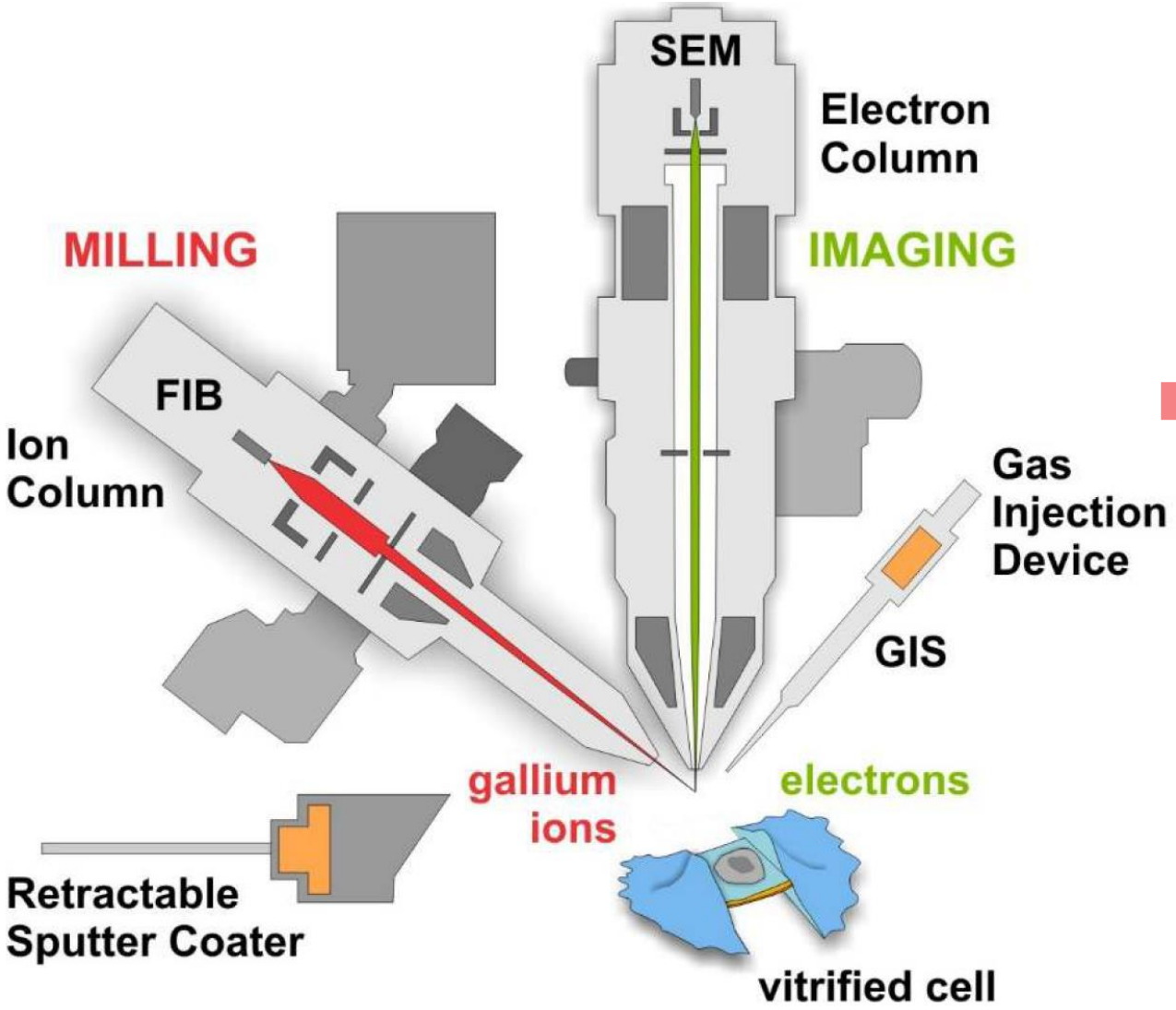
AQUILOS CRYO-FIB

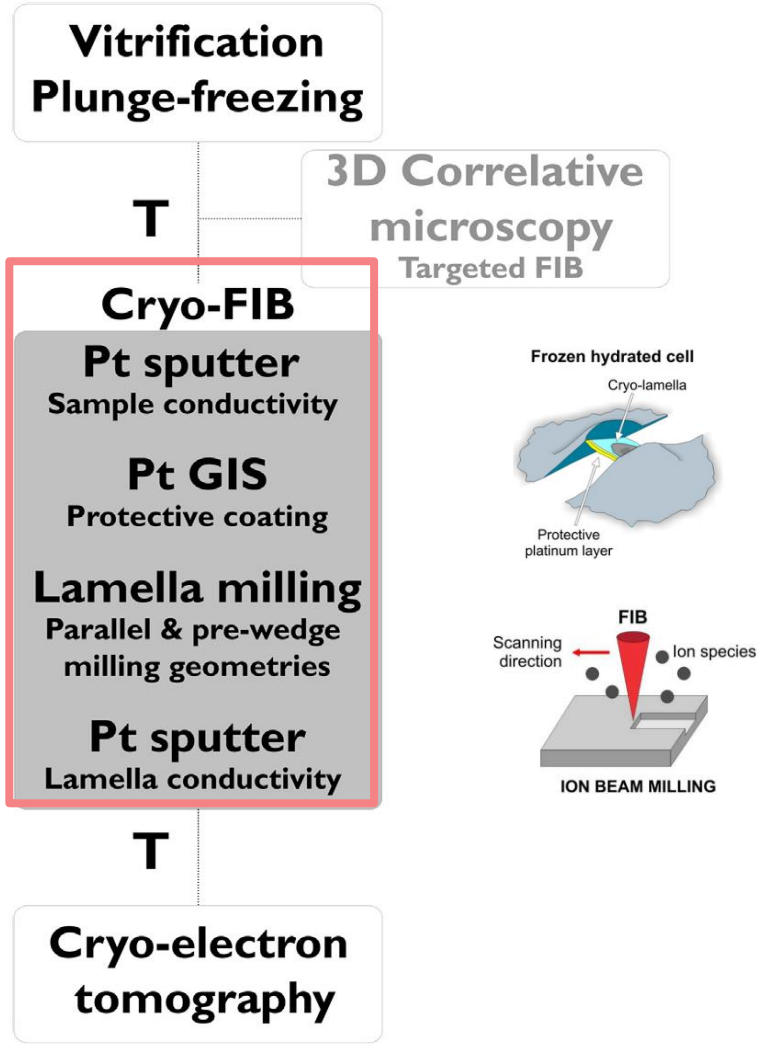
SAMPLE LOADING STATION

MUNI

ThermoFisher SCIENTIFIC

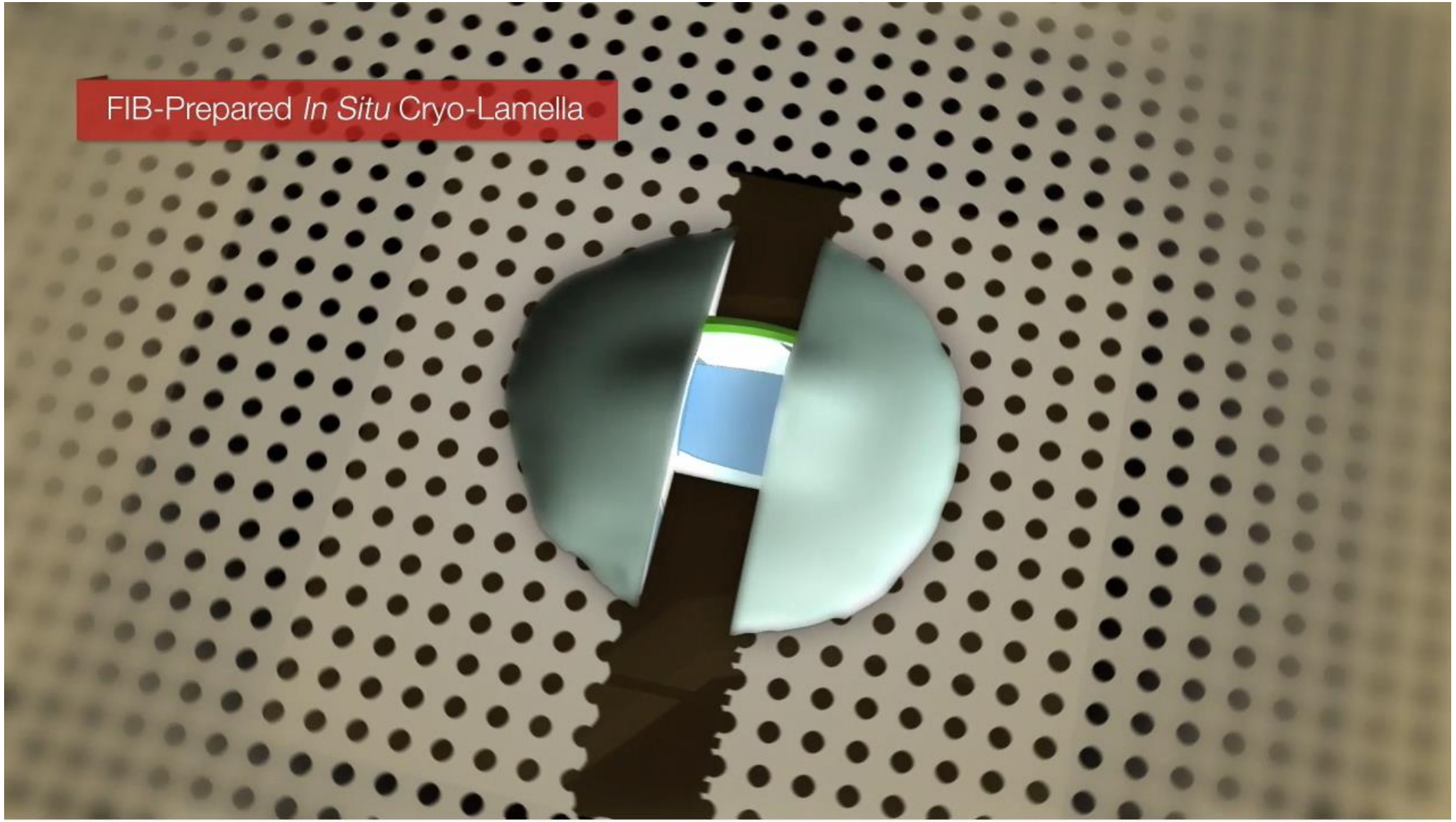
Operating Principle and Function of Cryo-FIB/SEM in SDB Cryo-Tomo Workflow





Left: adapted from Kuba J. et al., J. of Micr. 2020 (in review). Right: Shaffer M. et al., J. Struct. Biol., Vol. 197(2) (2017).

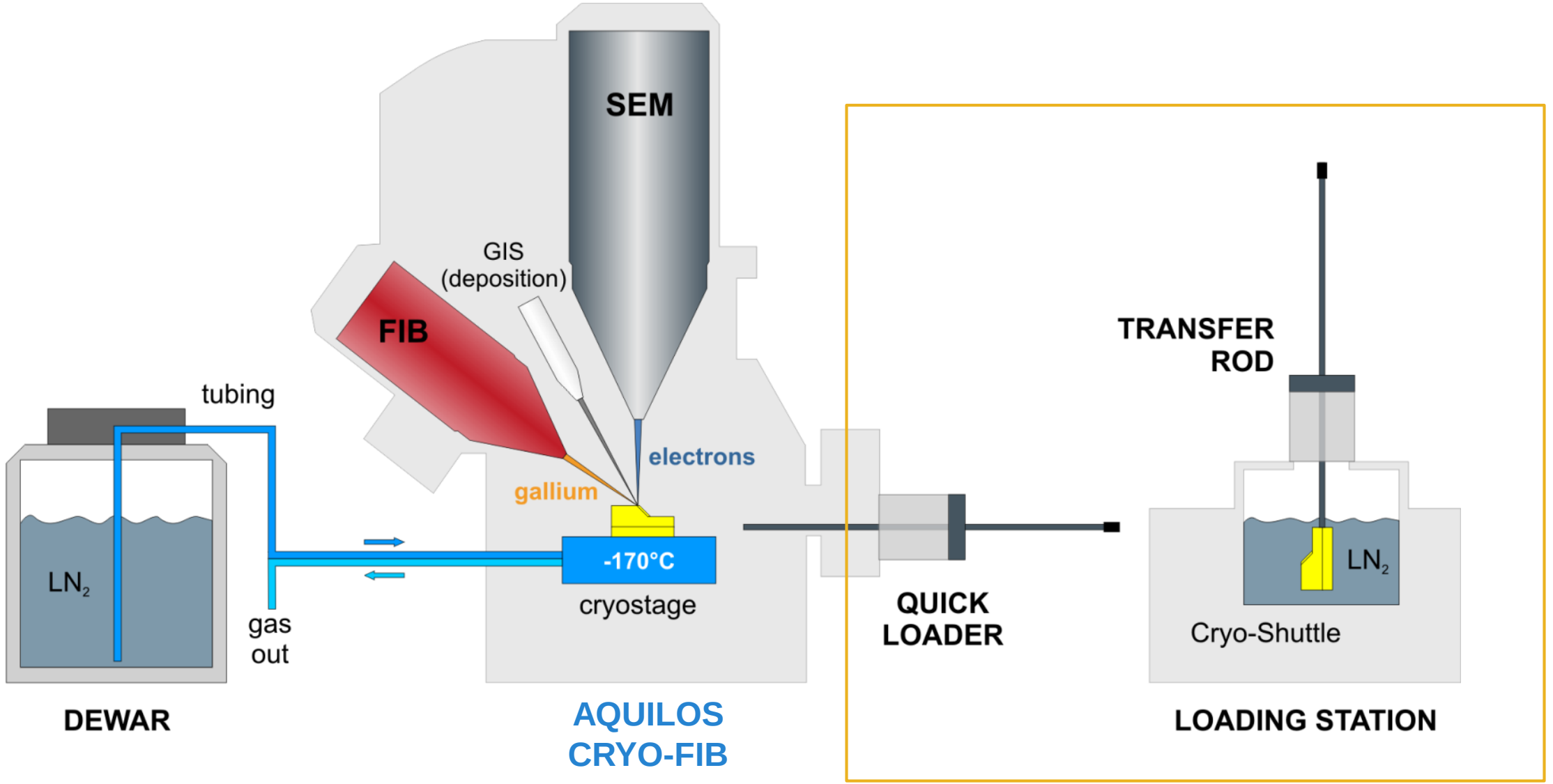
Cryo-FIB: Cryo-Sample Preparation Flow



MUNI

ThermoFisher SCIENTIFIC

Cryo-FIB: Cryo Infrastructure

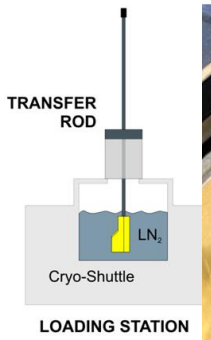


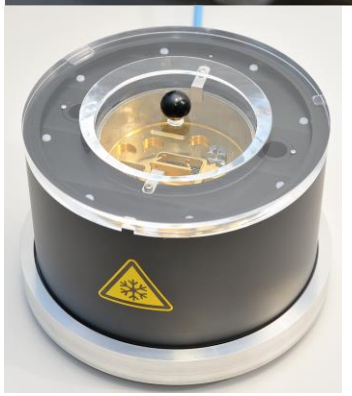
MUNI

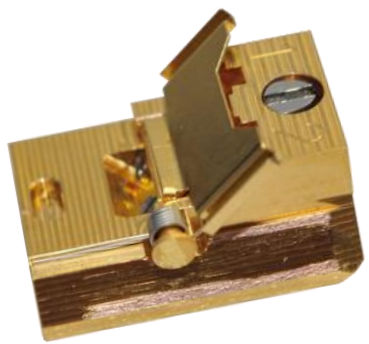
ThermoFisher SCIENTIFIC

Aquilos: Sample Loading, Transfer, Preparation Station

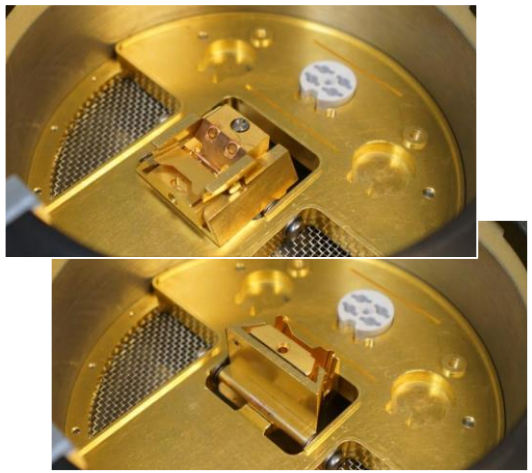
Transfer Pot
(+ docked Transfer Rod)



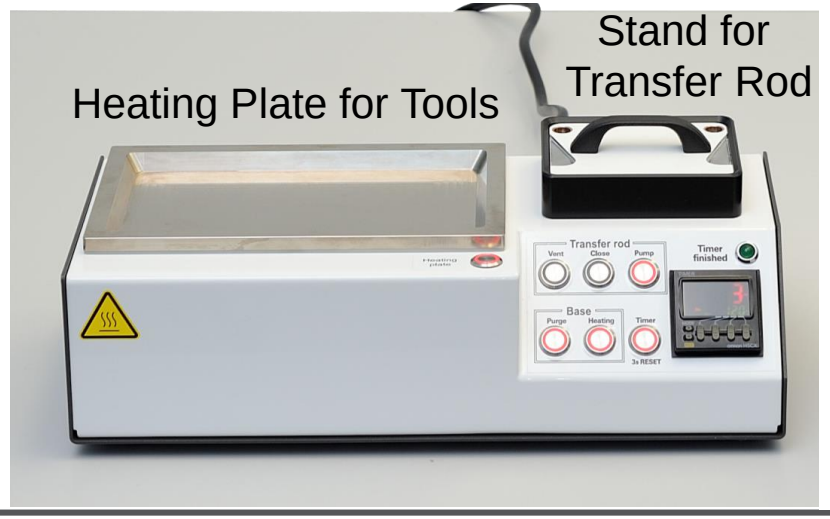




Cryo-FIB
Autogrid shuttle
(closed shield)



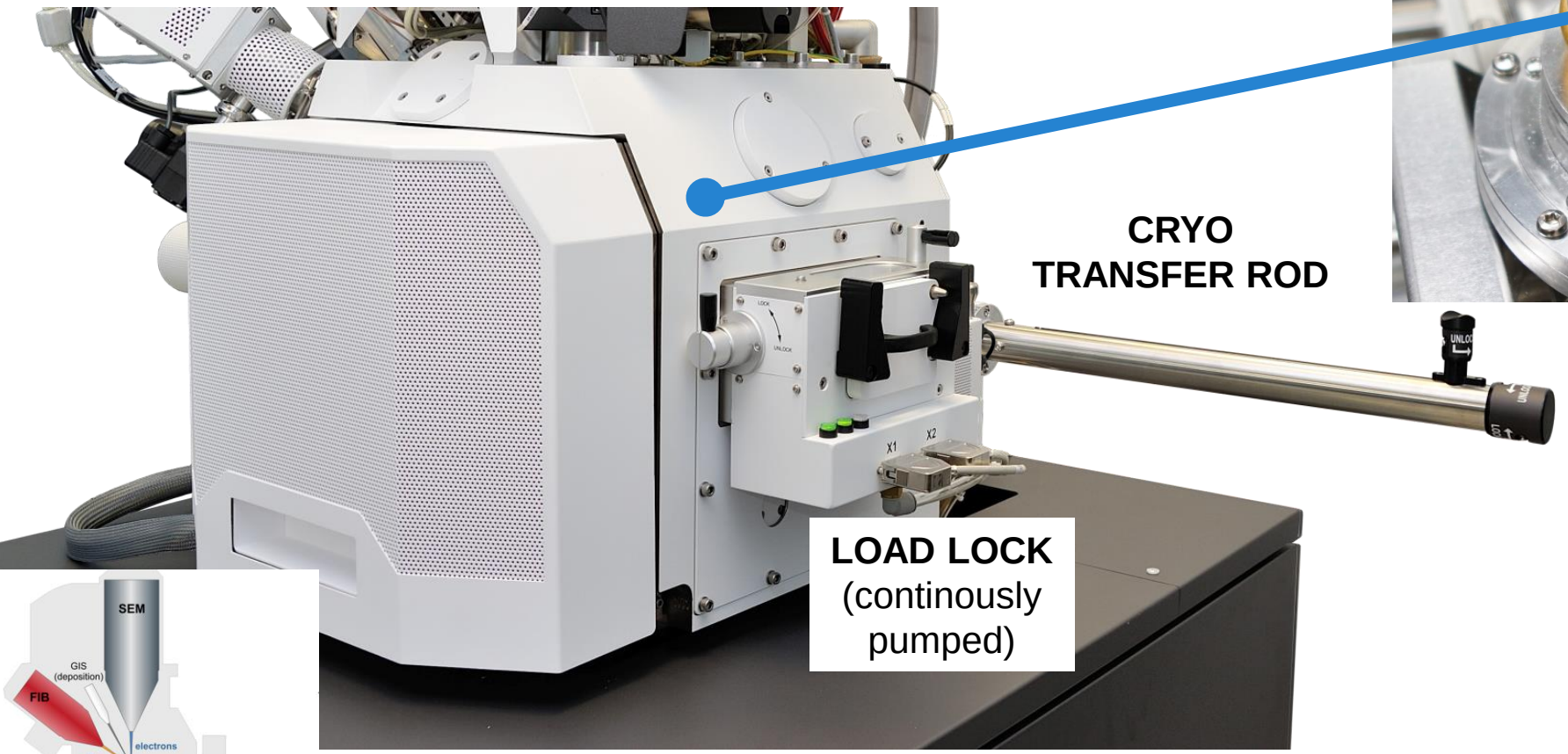
Prep Station Controller
(pumping, venting, heating)



MUNI

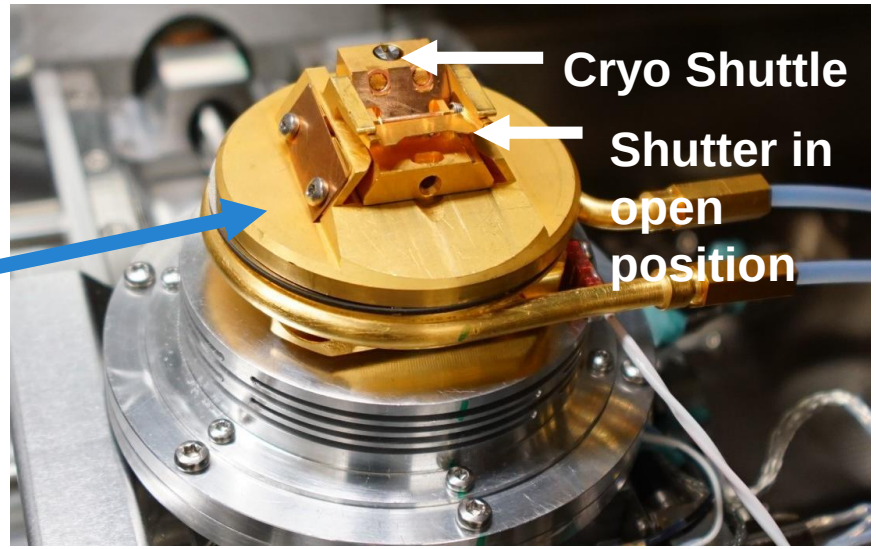
ThermoFisher SCIENTIFIC

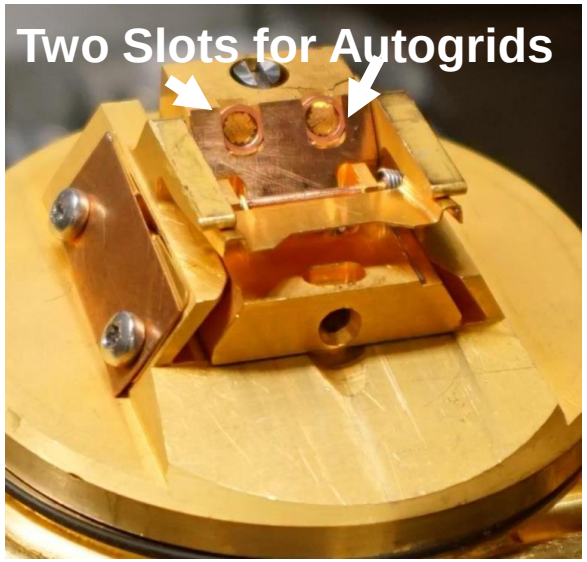
Aquilos: Load Lock, Cryo Transfer Rod, Stage



CRYO TRANSFER ROD

LOAD LOCK (continously pumped)

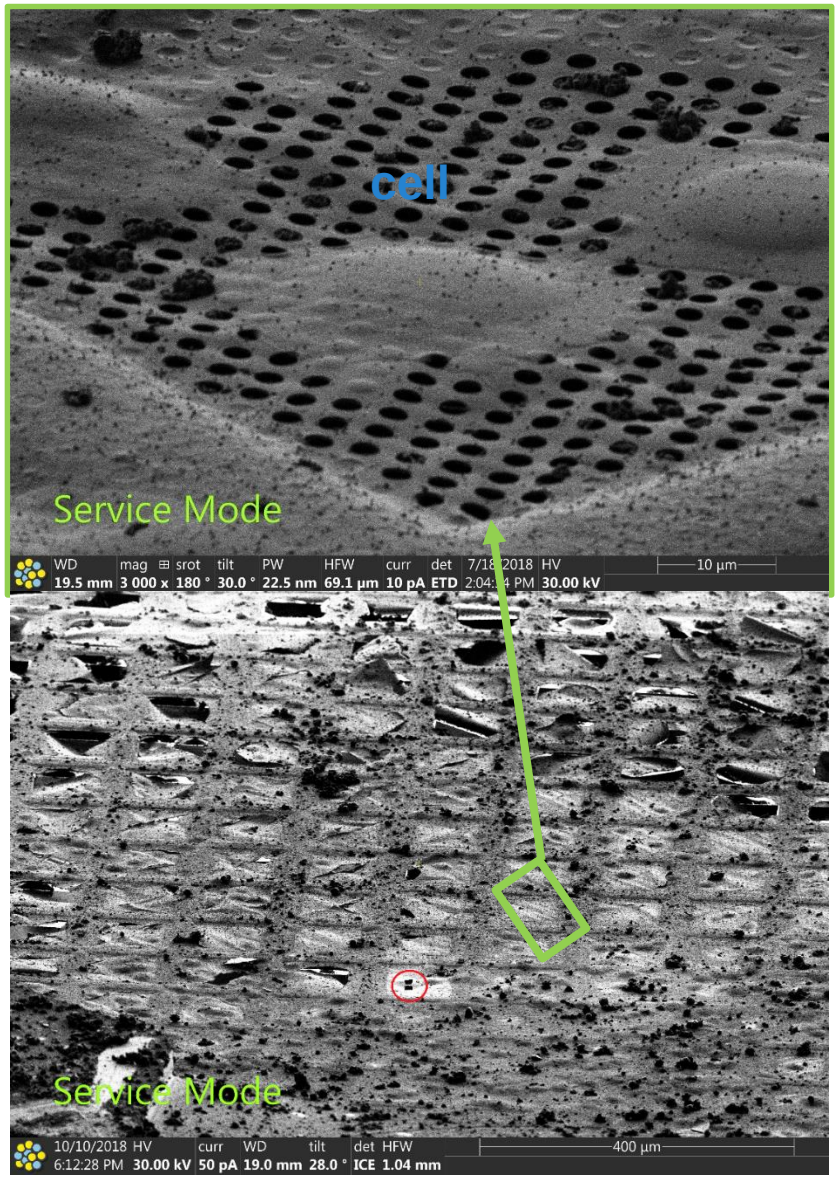




MUNI

ThermoFisher SCIENTIFIC

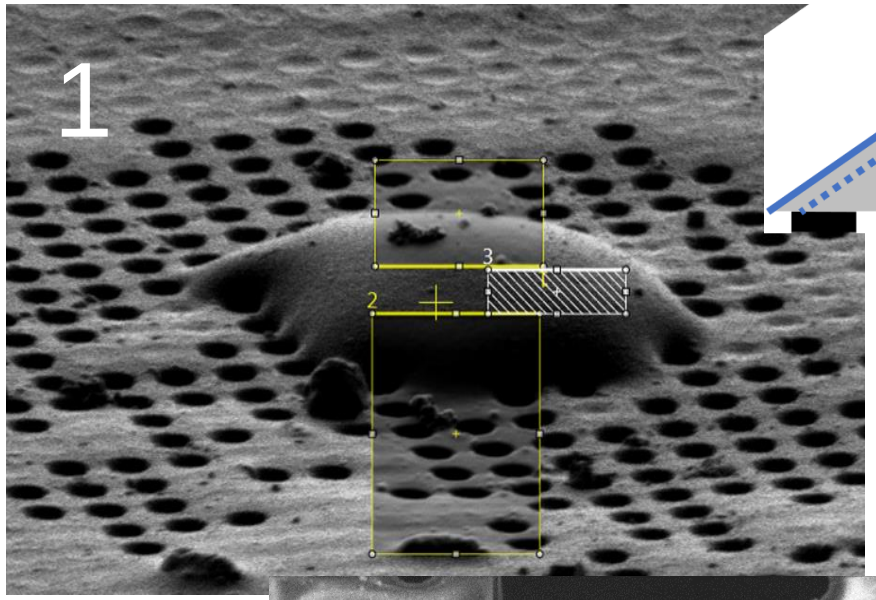
Contamination: specles, large particles, homogeneous layer; devitrification

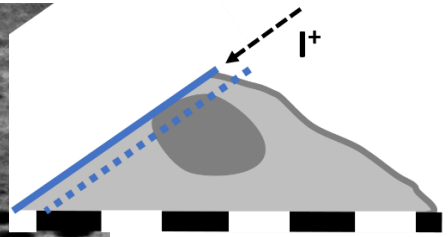


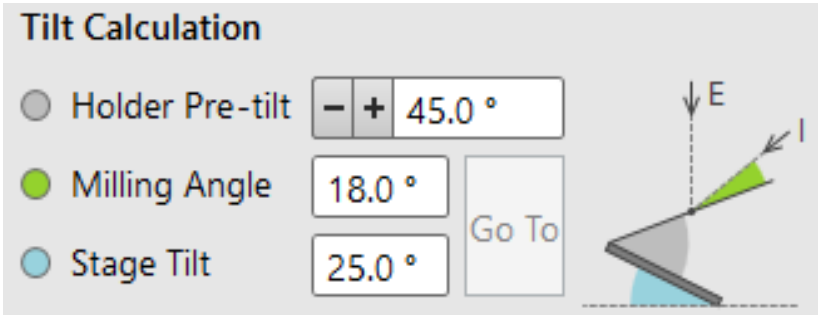
MUNI

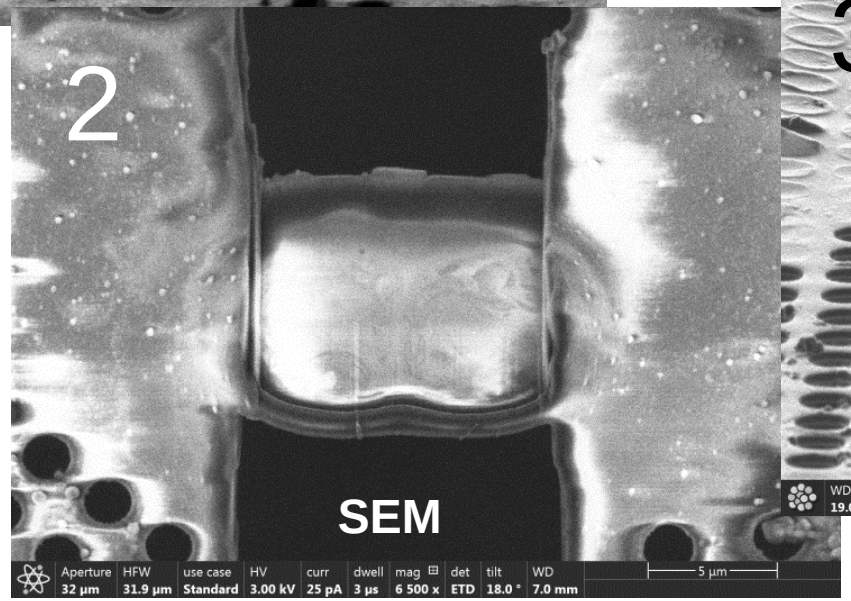
ThermoFisher SCIENTIFIC

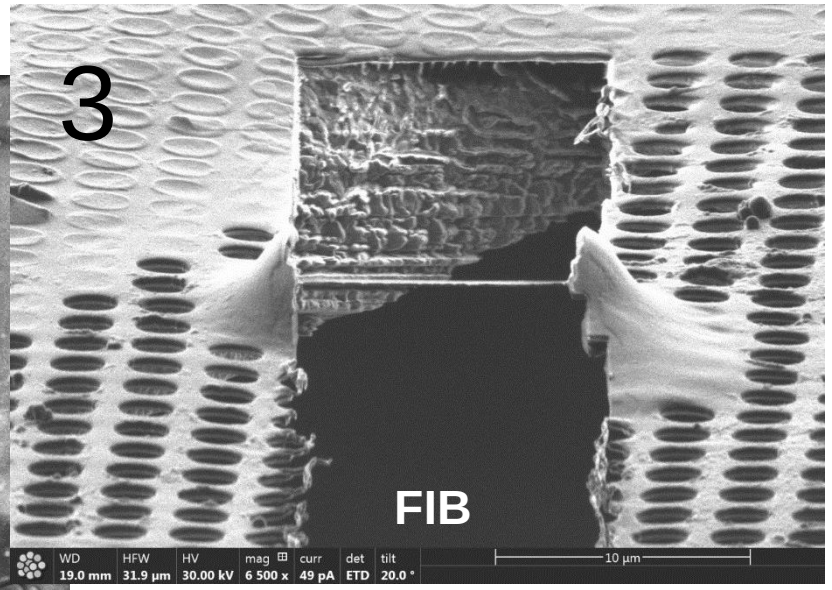
Lamella Preparation

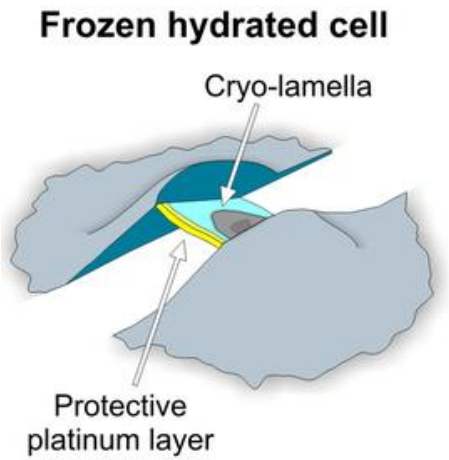








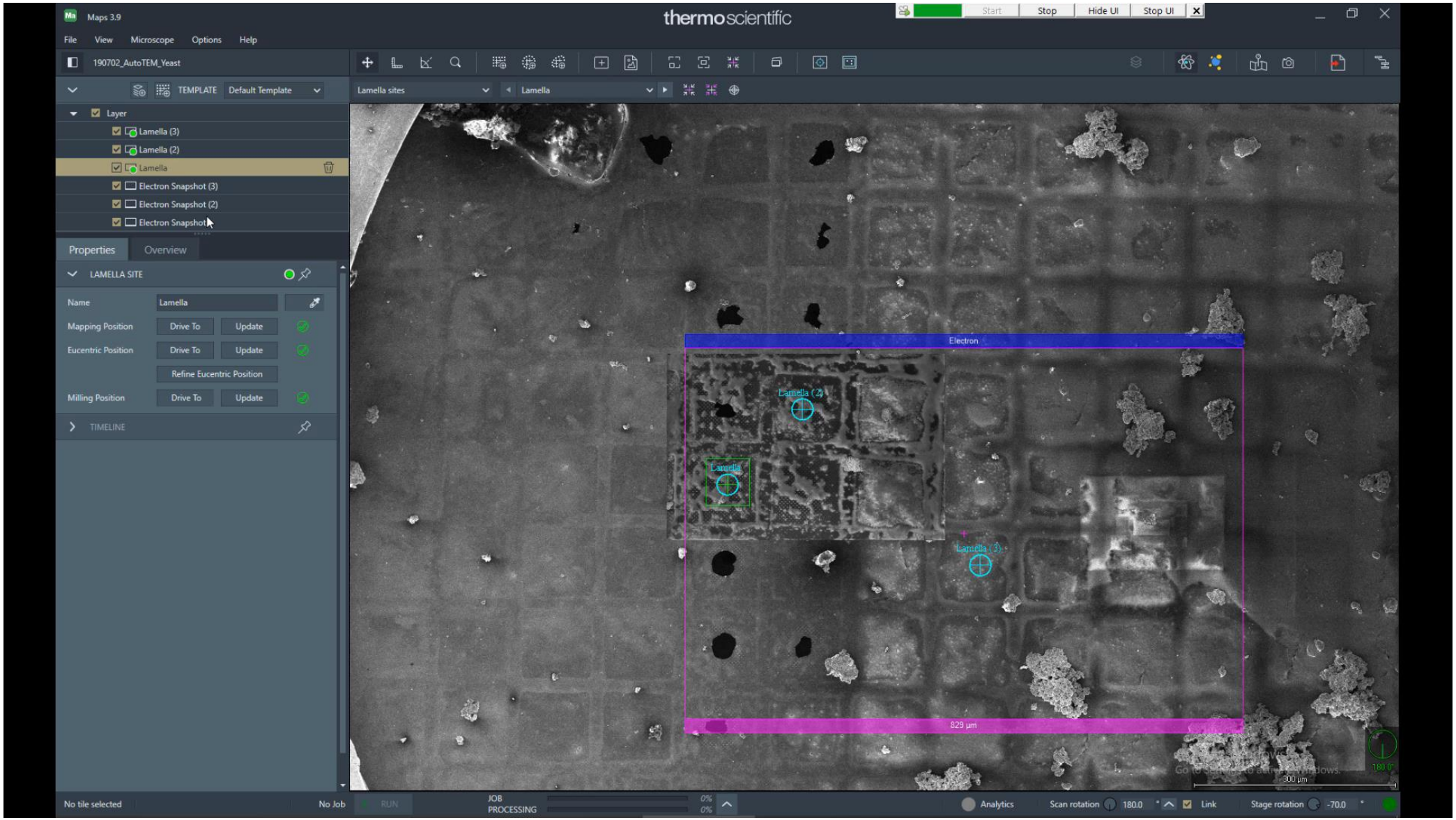




MUNI

ThermoFisher SCIENTIFIC

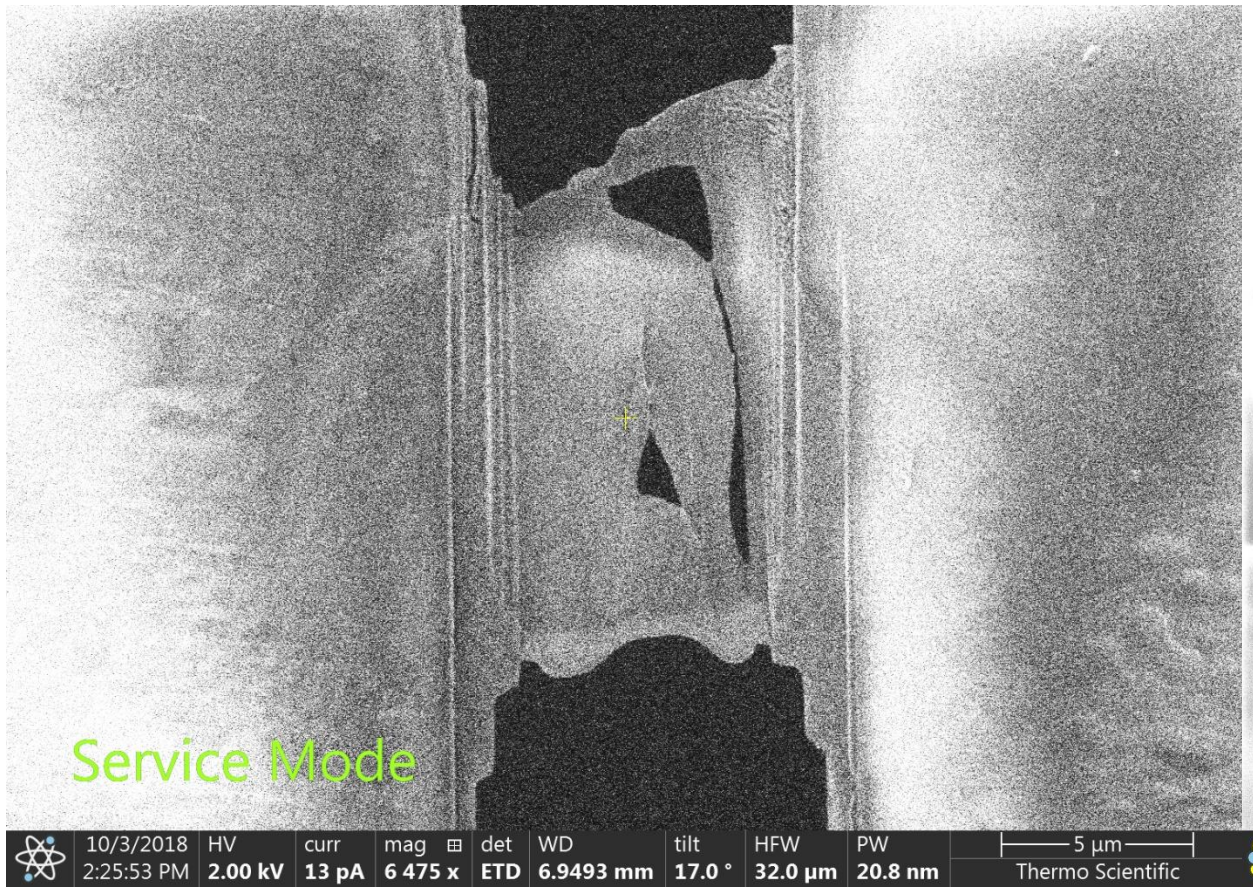
Lamella Preparation Automation: Cryo AutoTEM live

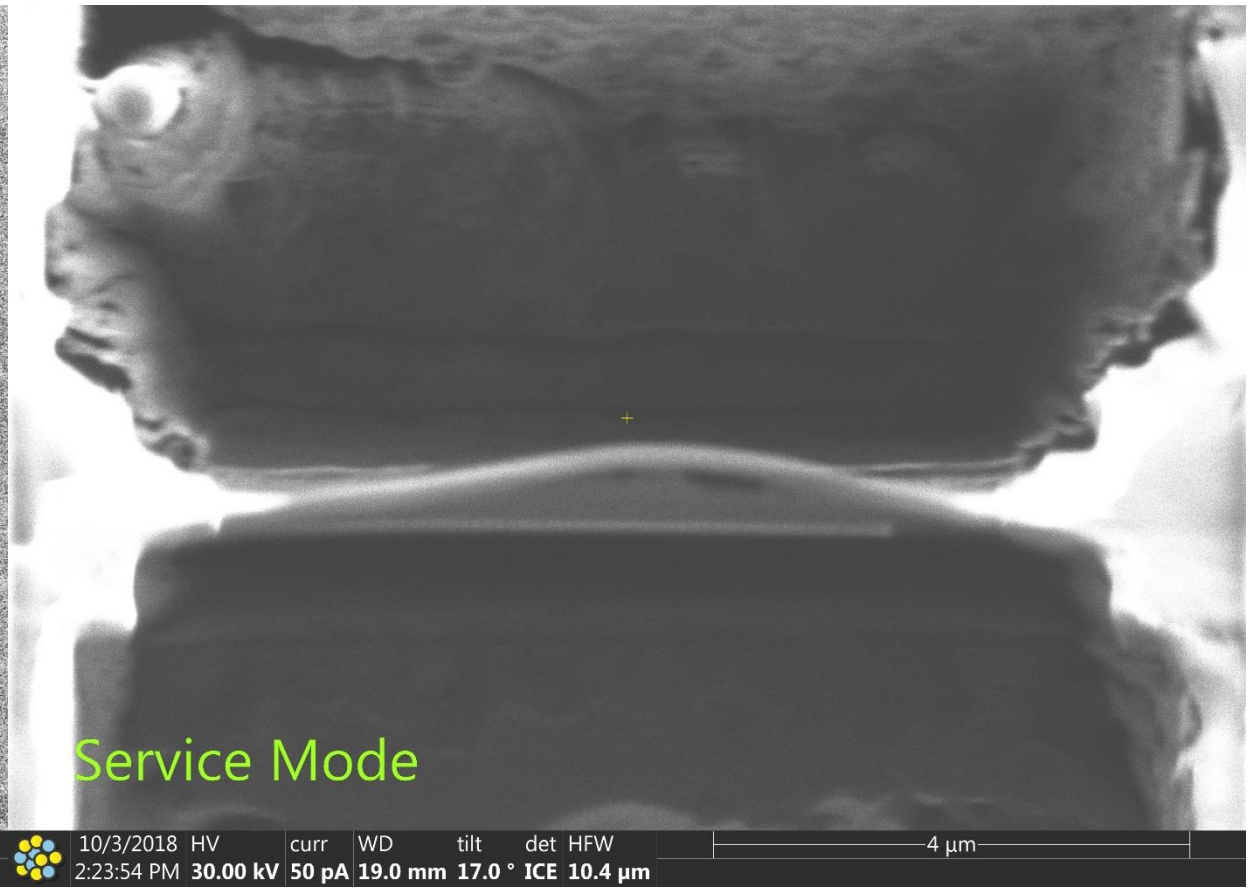


MUNI

ThermoFisher SCIENTIFIC

Lamella Milling Issues

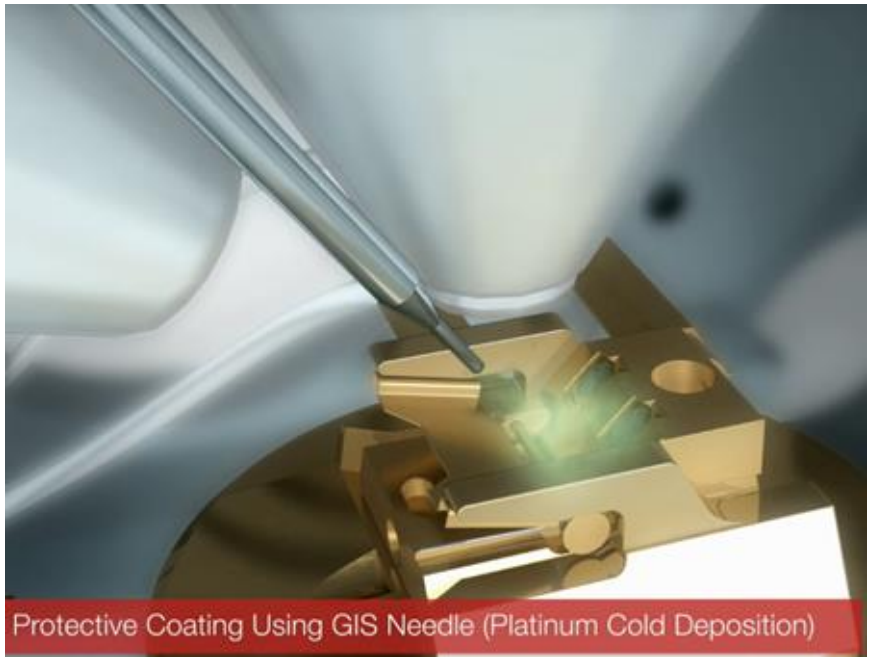


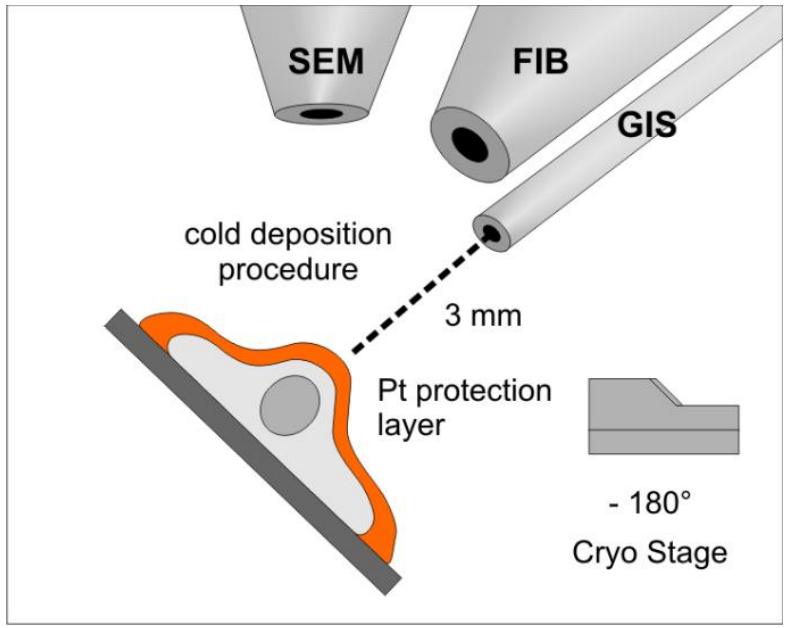


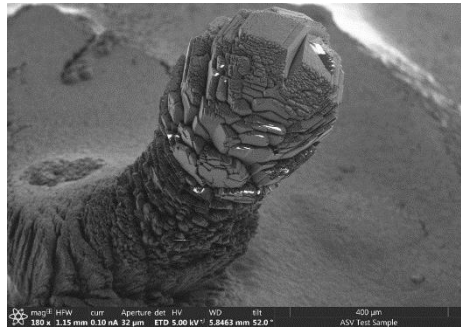
Electrons **Ions**

Protective Coating: Cold Deposition (using GIS = gas injection system)

Cryo

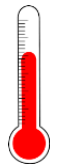


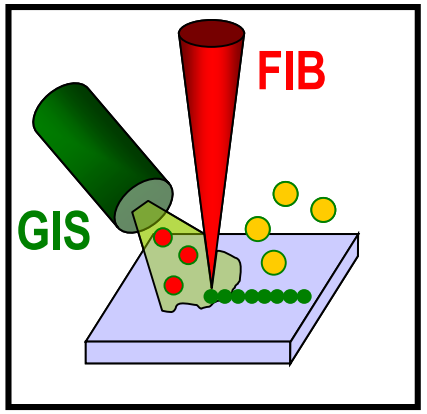




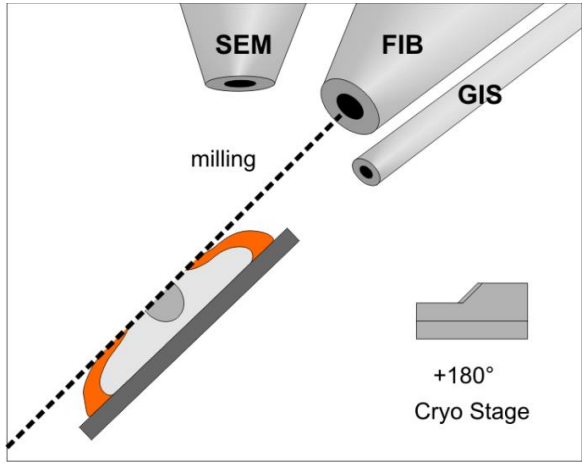
Do not forget to close the GIS valve…

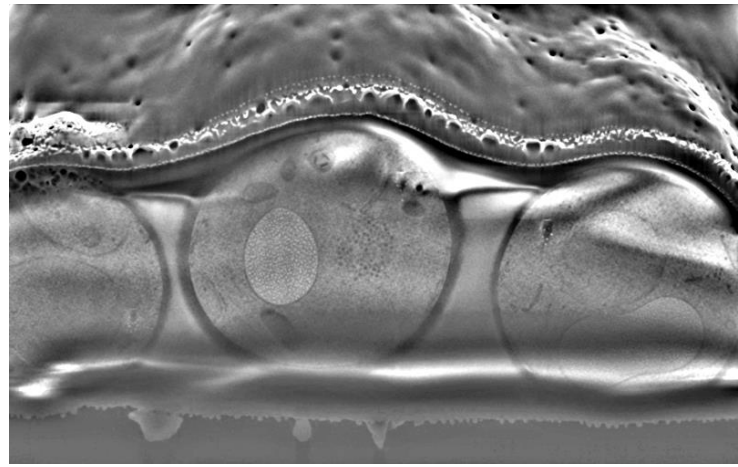
RT





- Gas molecules
- Deposited material
- Volatile products



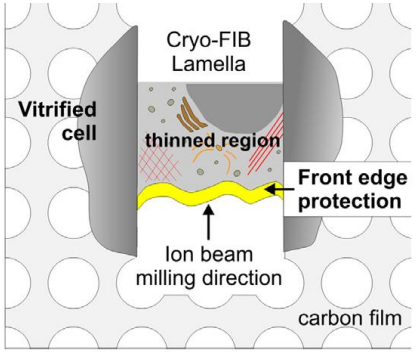


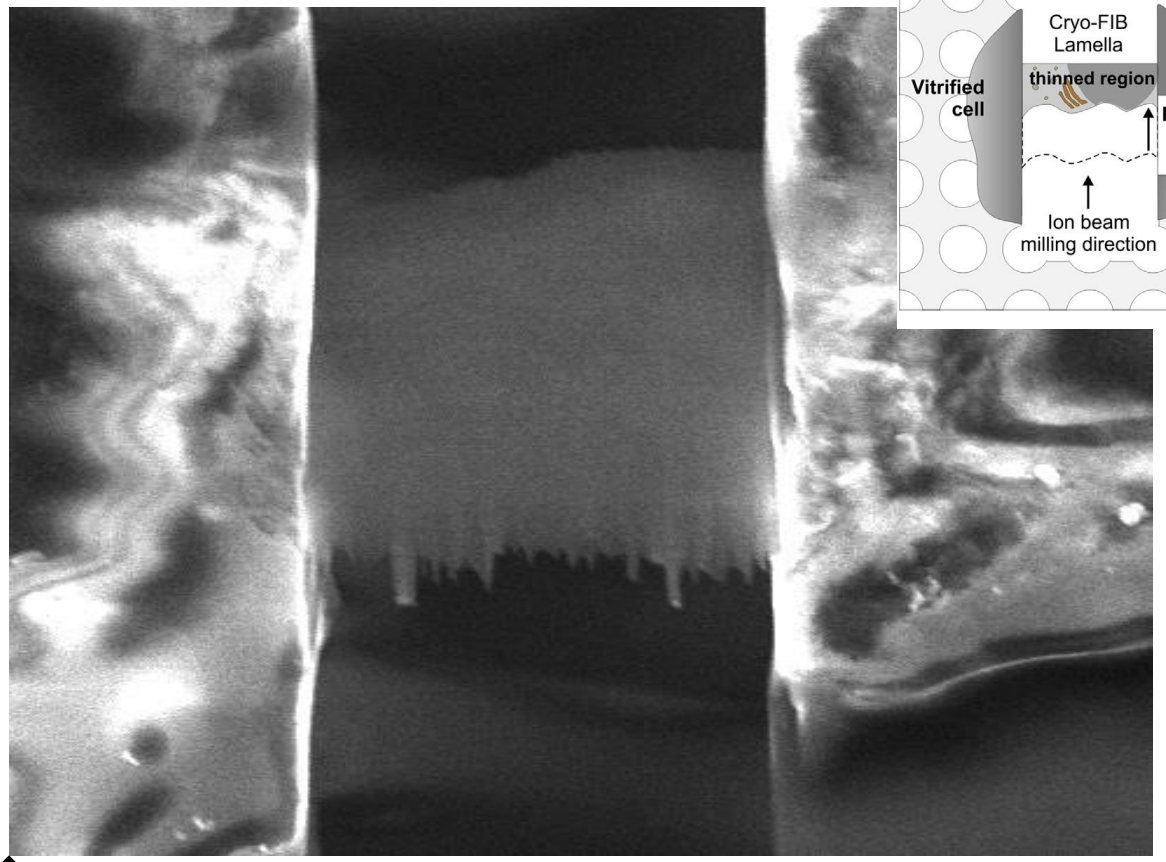
MUNI

GIS scheme on the right adapted from Rigort A. et al., Archives of Biochem. and Biophys. 581 (2015).
Remark. Hayles M. F. et al., A technique for improved FIB milling of cryo-prepared LS specimens, JOM 226 (Pt 3) (2007).

ThermoFisher SCIENTIFIC

Protective Coating: Preventing Beam Erosion





with Pt GIS coating

without Pt GIS coating

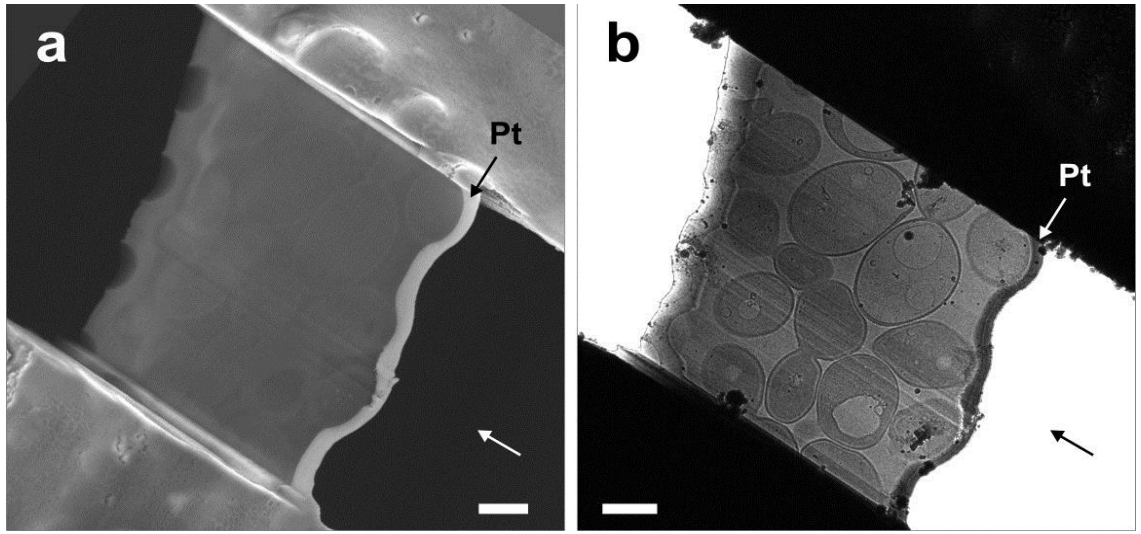
Milling direction

MUNI

Adapted from Shaffer M. et al., J. Struct. Biol., Vol. 197(2) (2017); Rigort A. et al., Archives of Biochem. and Biophys. 581 (2015).

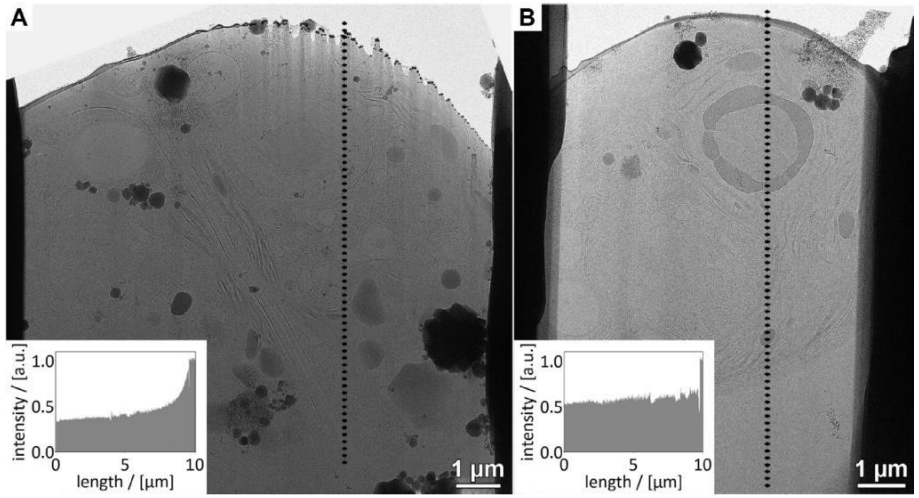
ThermoFisher SCIENTIFIC

Cryo-FIB Lamella: Milling Strategy

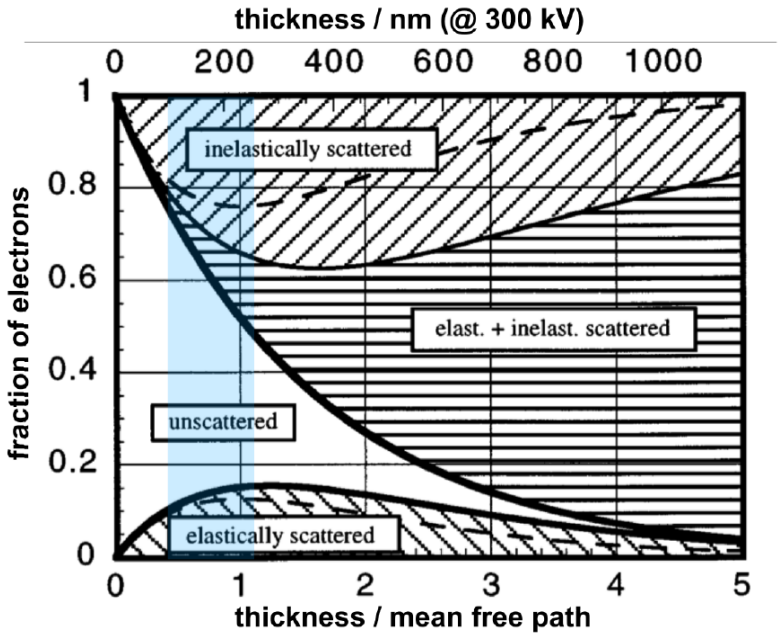


SEM (+ contrast difference)

TEM



Lamella thickness and uniformity, tilting during milling.



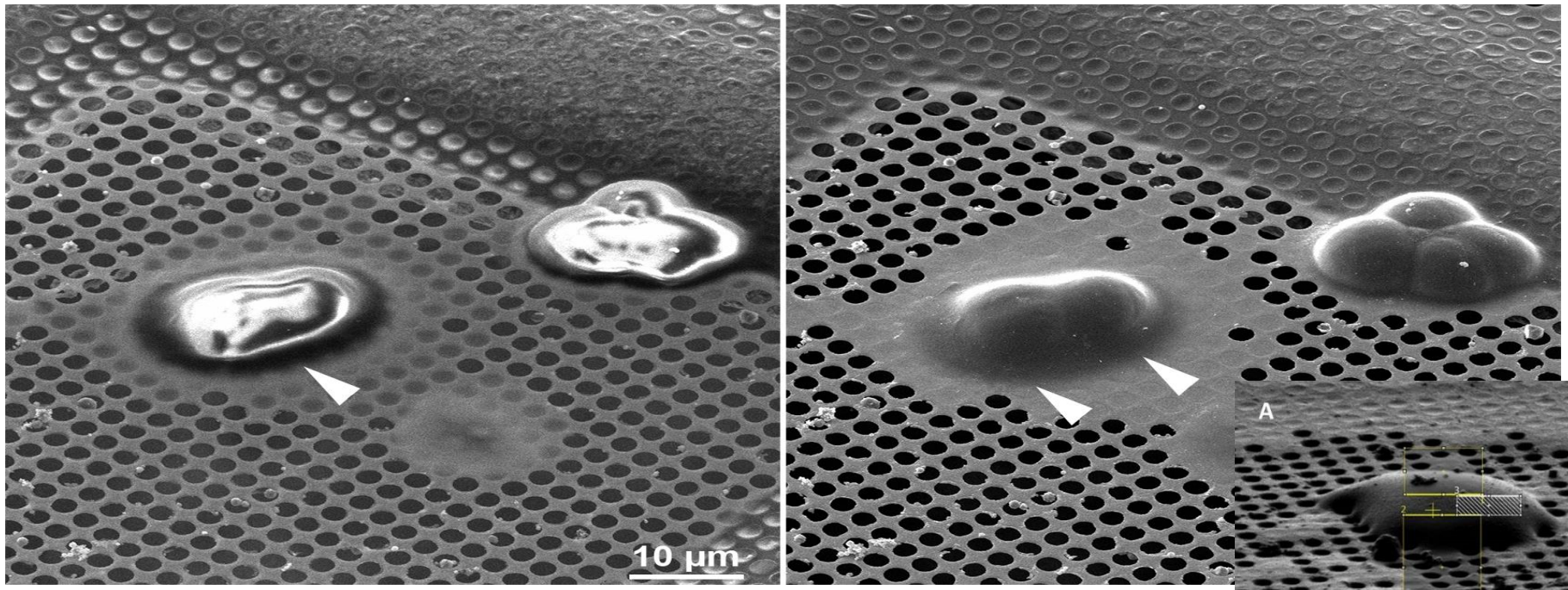
Distribution of scattered electrons for vitreous ice.

MUNI

Fig. a,b adapted from Rigort A. et al., Archives of Biochem. and Biophys. 581 (2015). Bottom image: Schaffer et al., Journal of Structural Biology 2016. Image on the right: Electron tomography (2010), edited by J. Frank.

ThermoFisher SCIENTIFIC

Conductive Coating: SEM, TEM

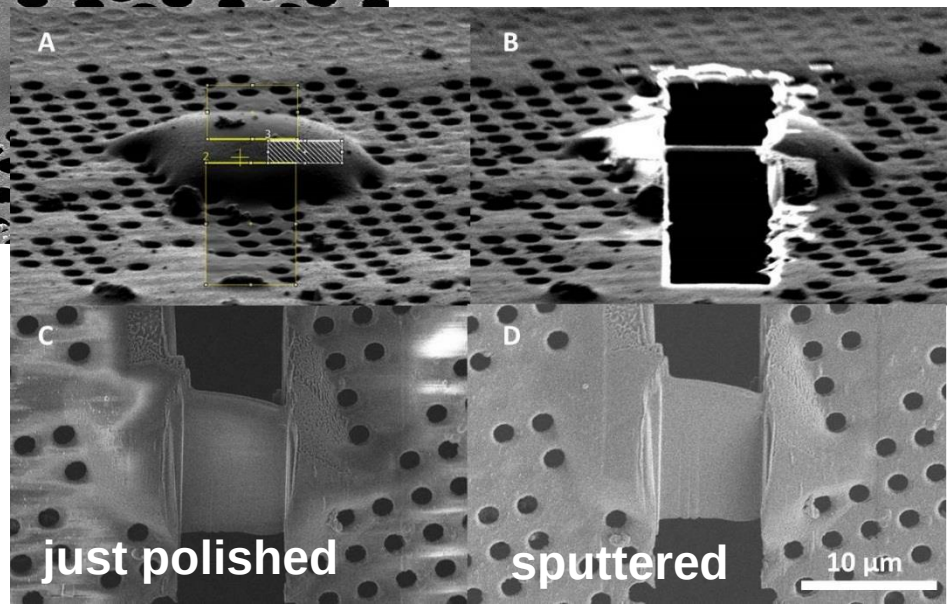


uncoated

Pt sputter coated

cells on the TEM grid

lamella

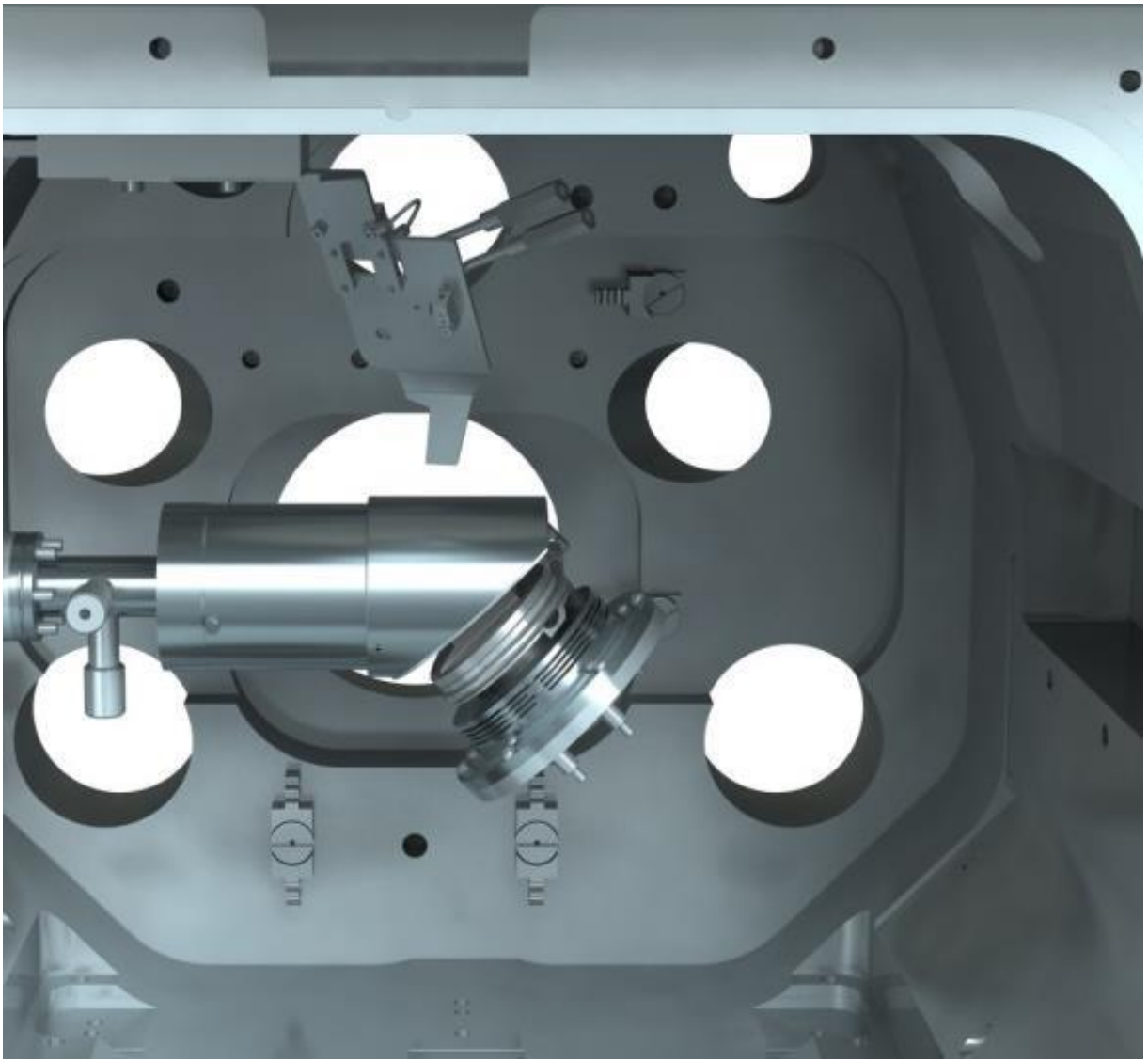


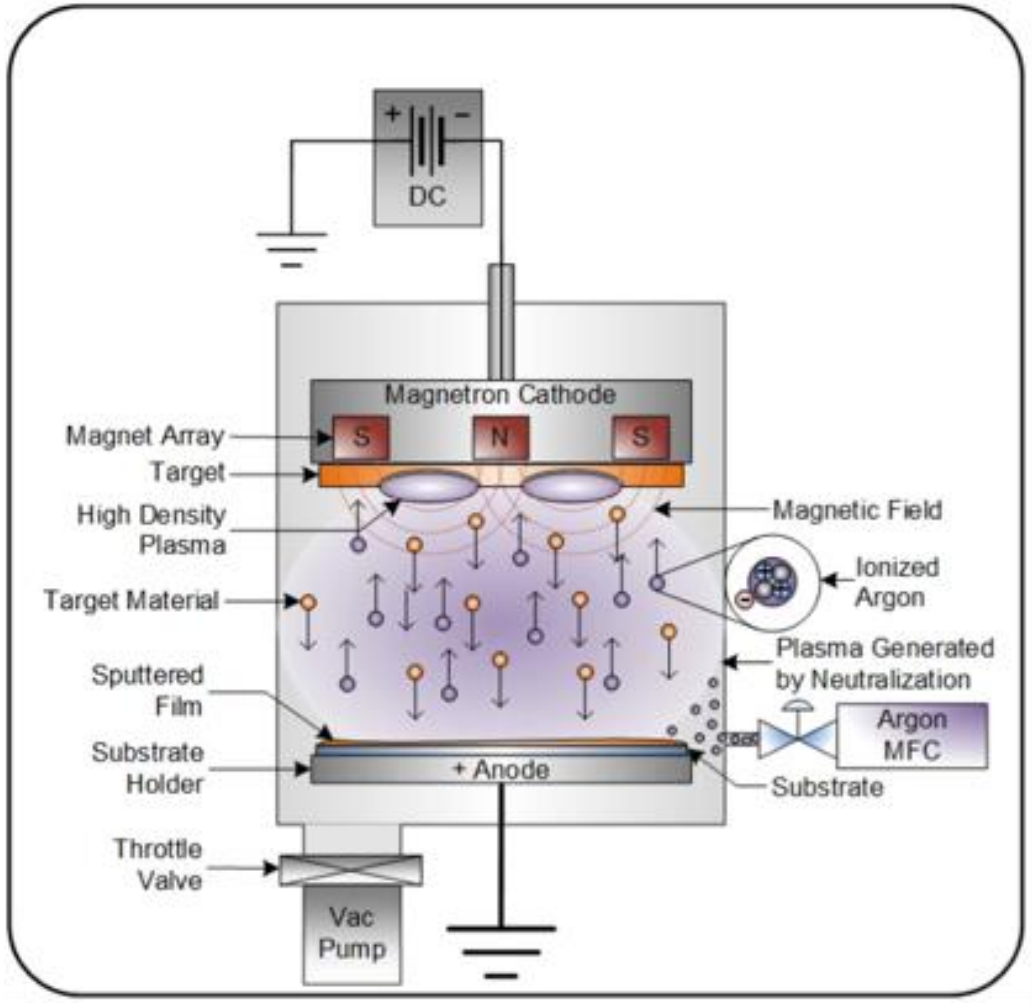
MUNI

Adapted from (image on the left) Schaffer et al., Journal of Structural Biology (2016), doi:10.1016/j.jsb.2016.07.010.

ThermoFisher SCIENTIFIC

Conductive Coating: Example of Retractable In-Chamber Magnetron Sputter





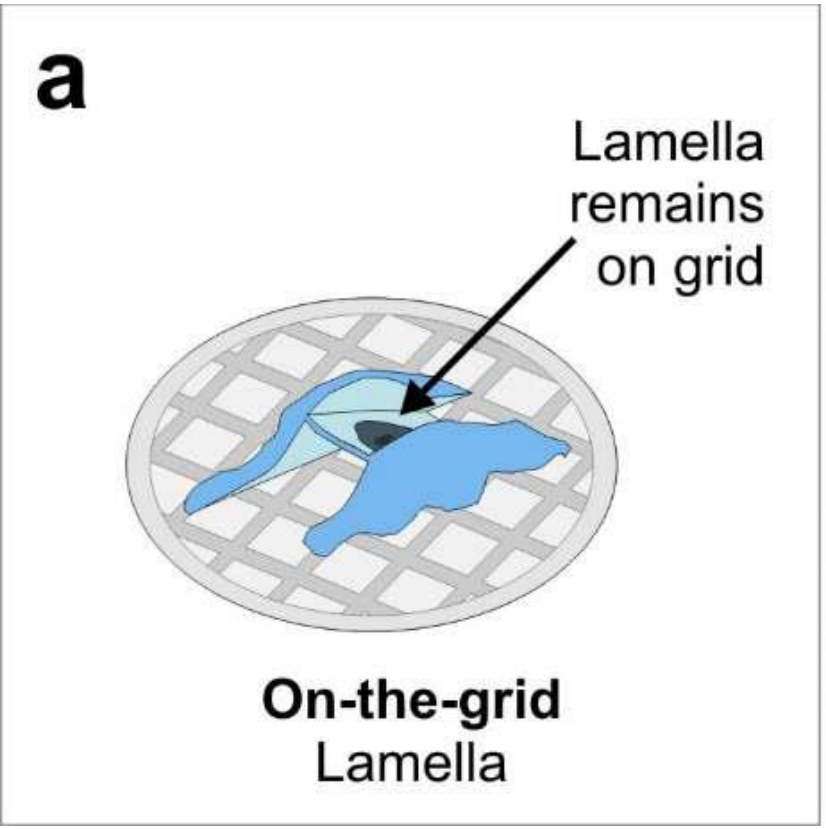
Principle of DC Magnetron Sputtering.

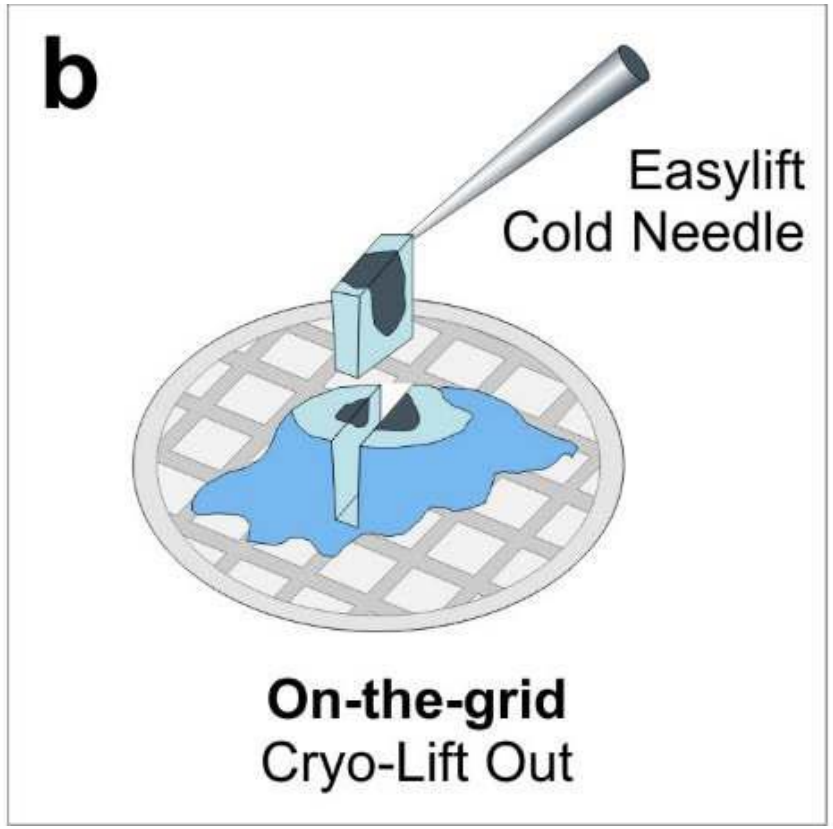
MUNI

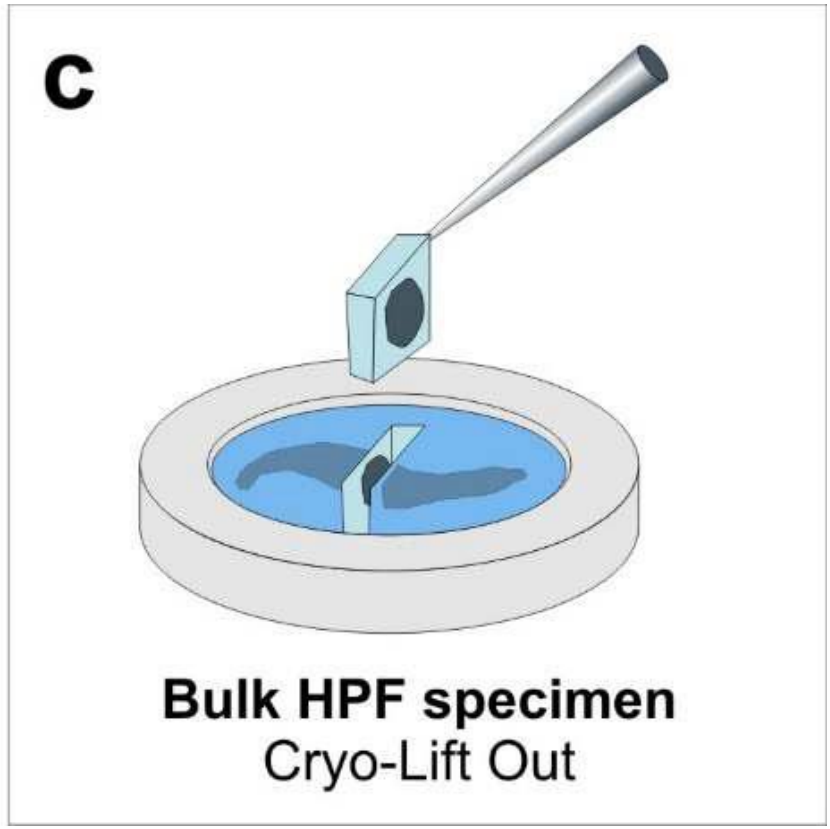
Image on the right adapted from: http://www.semicore.com/news/94-what-is-dc-sputtering.

ThermoFisher SCIENTIFIC

Lamella Preparation Approaches

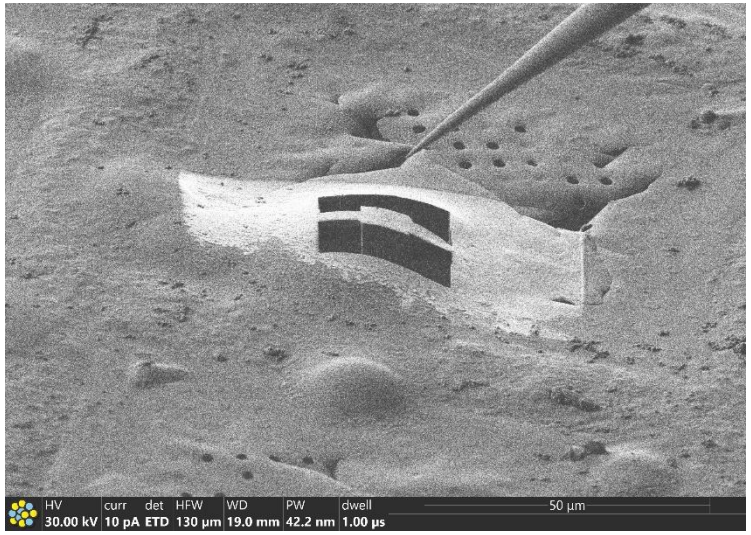


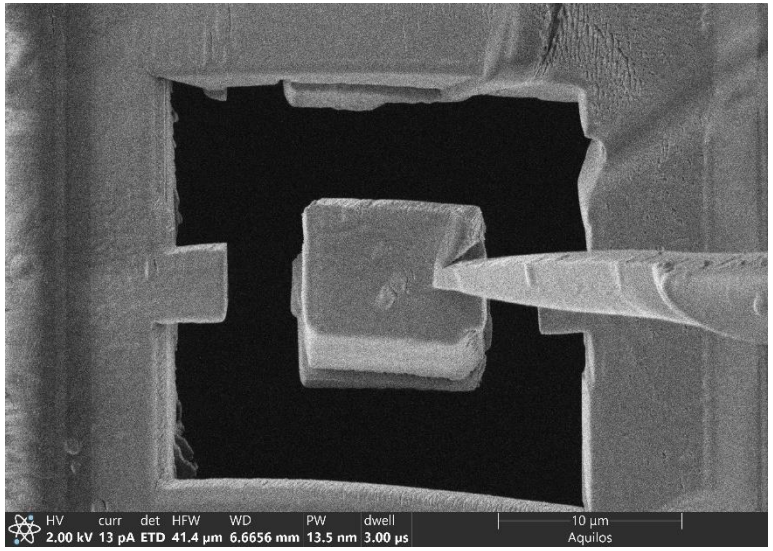


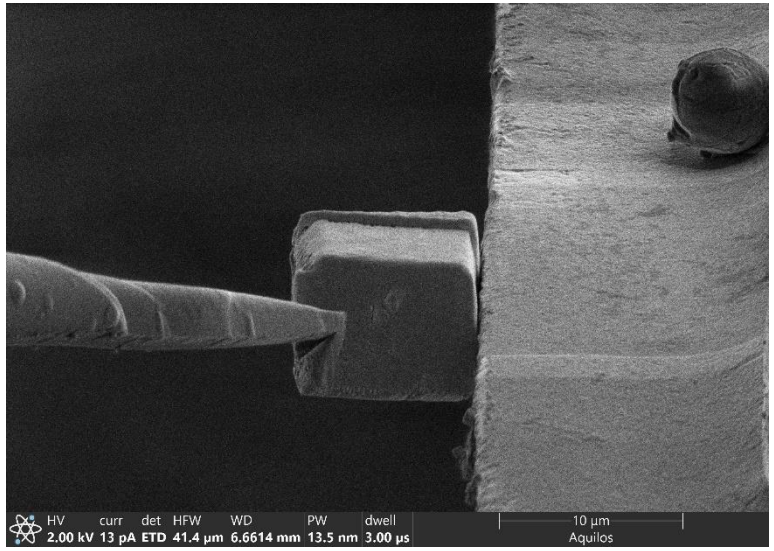


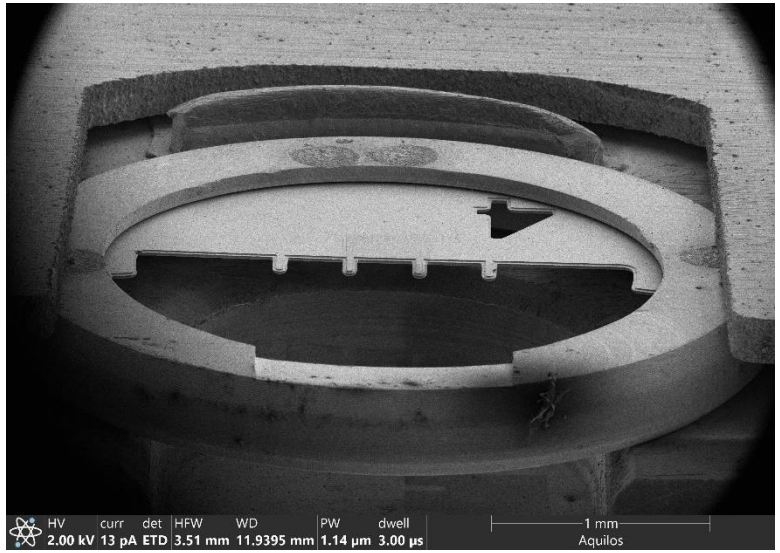
Adapted from Kuba J. et al., Journal of Microscopy 2020 (in review).

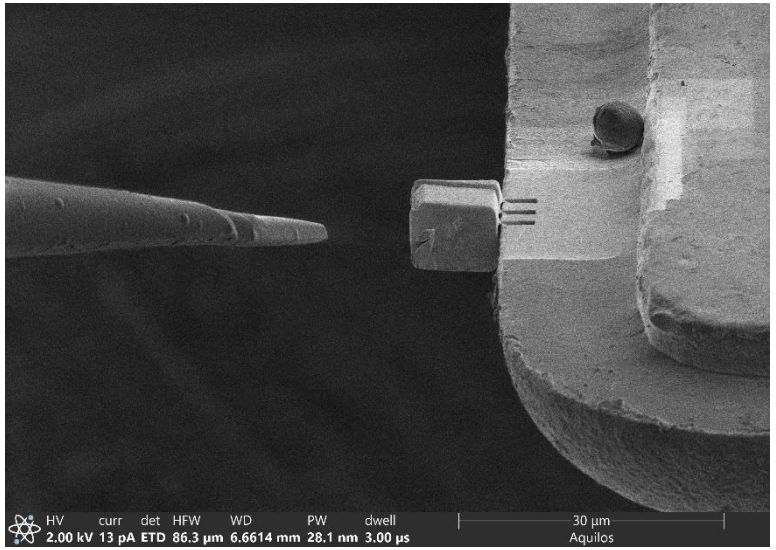
CLO Flow

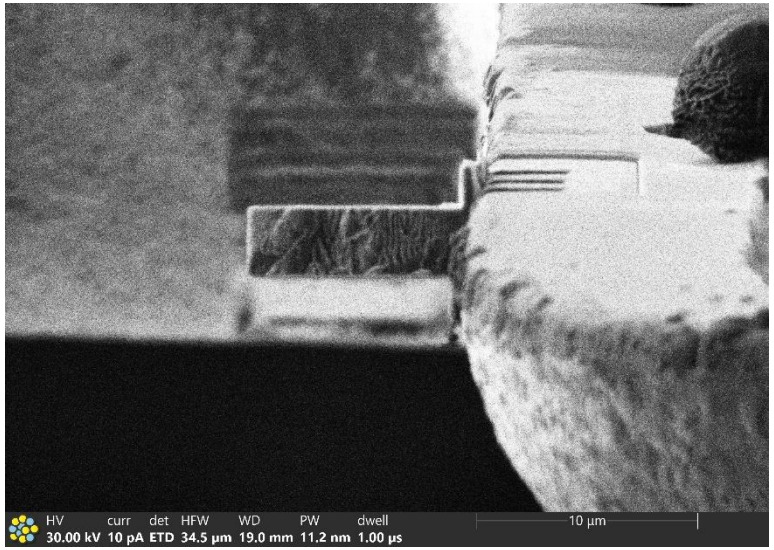








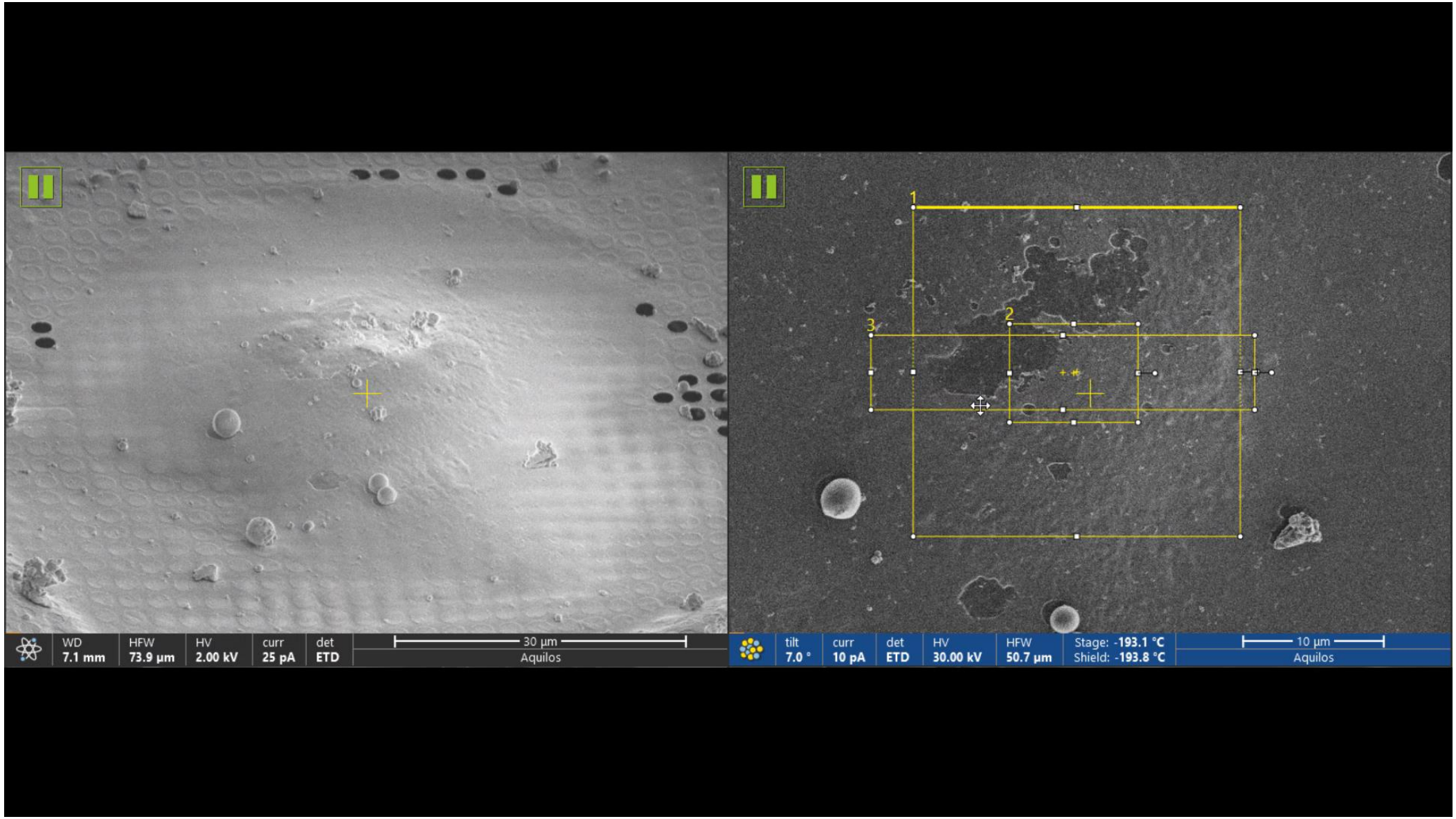




MUNI

ThermoFisher SCIENTIFIC

CLO live

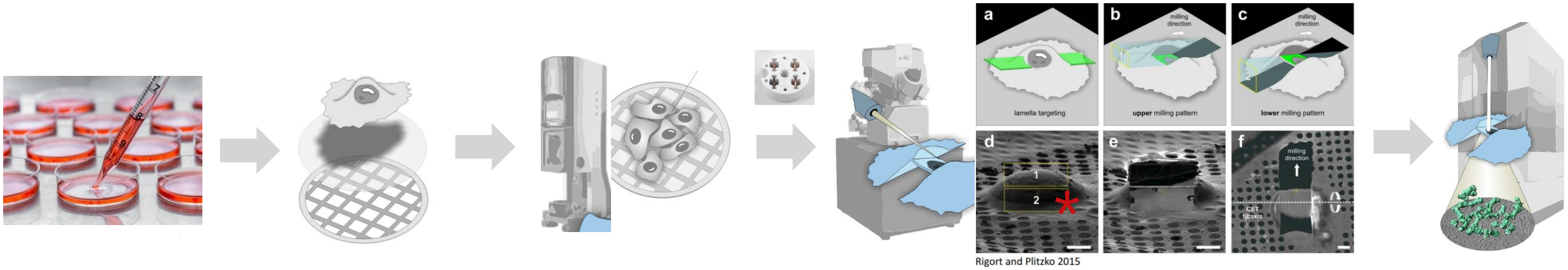


MUNI

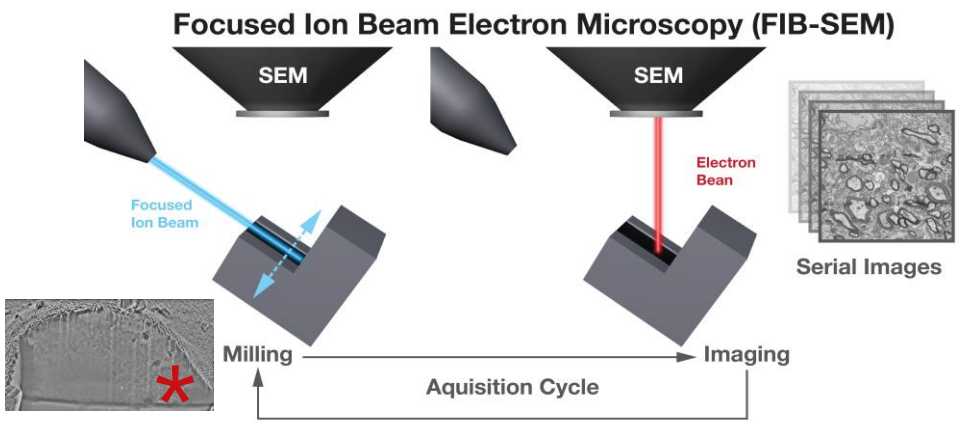
ThermoFisher SCIENTIFIC

SDB Cryo-Tomo Workflow: SEM Cryo-Imaging

SDB cryo-tomo workflow for structure determination of cells.

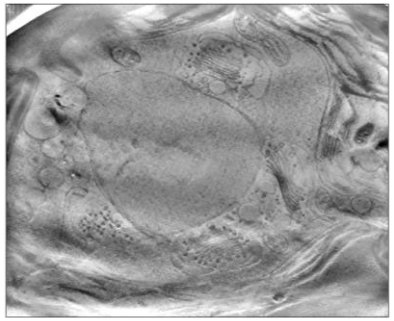


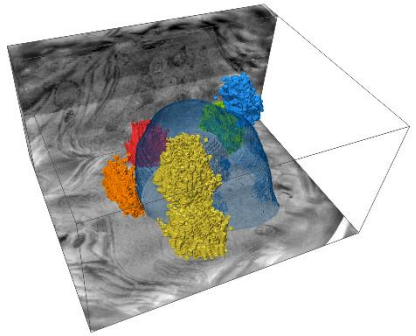
Rigort and Plitzko 2015



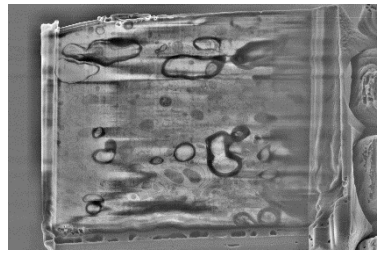
vitrified cell

Cryo Auto Slice and View or end-pointing during lamella preparation.





3D display, Chlamydomonas



cryo-lamella (mouse brain)

MUNI

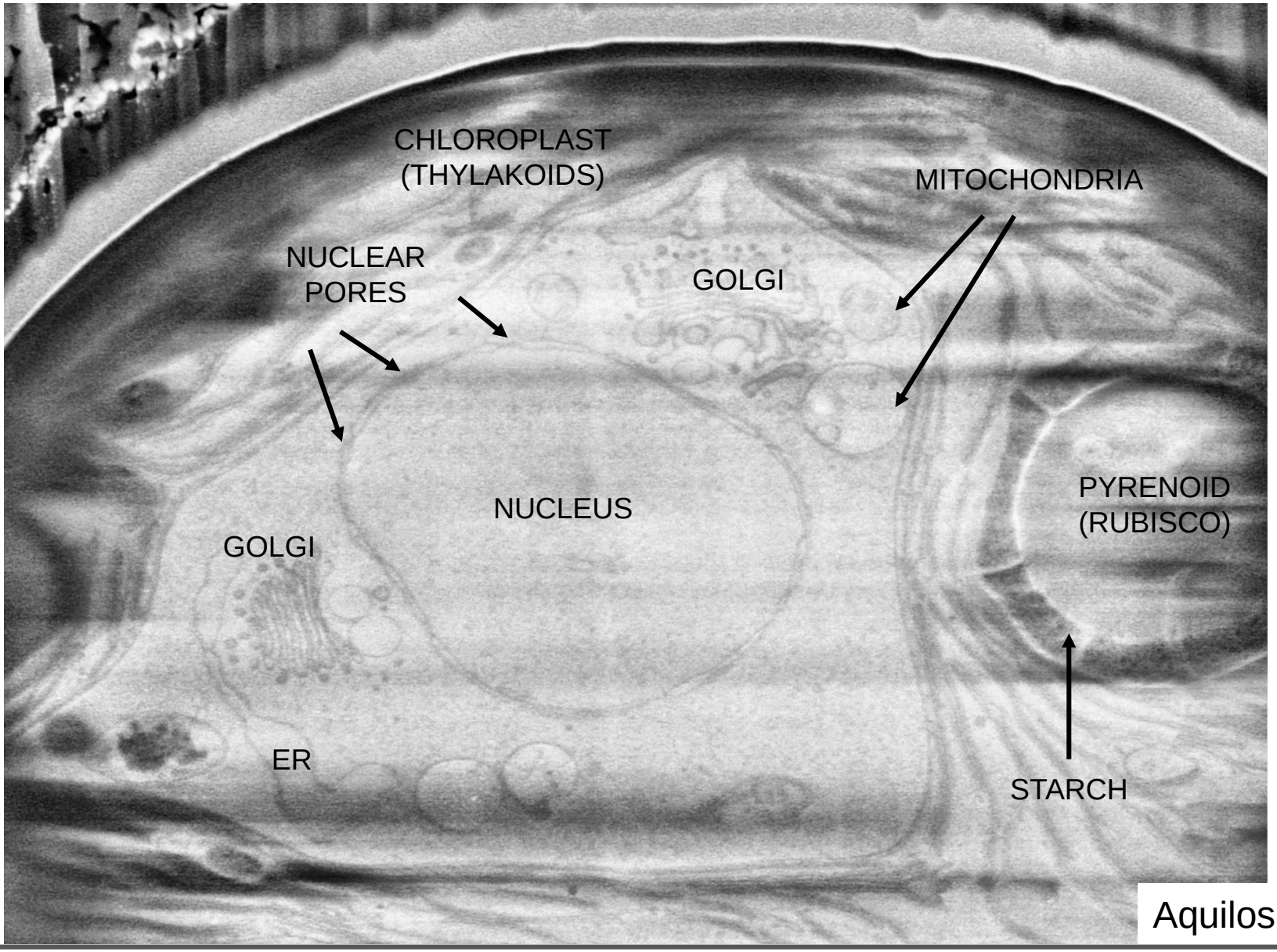
ThermoFisher SCIENTIFIC

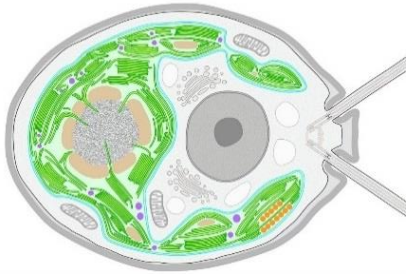
Cryo-ASV: Site Preparation

MUNI

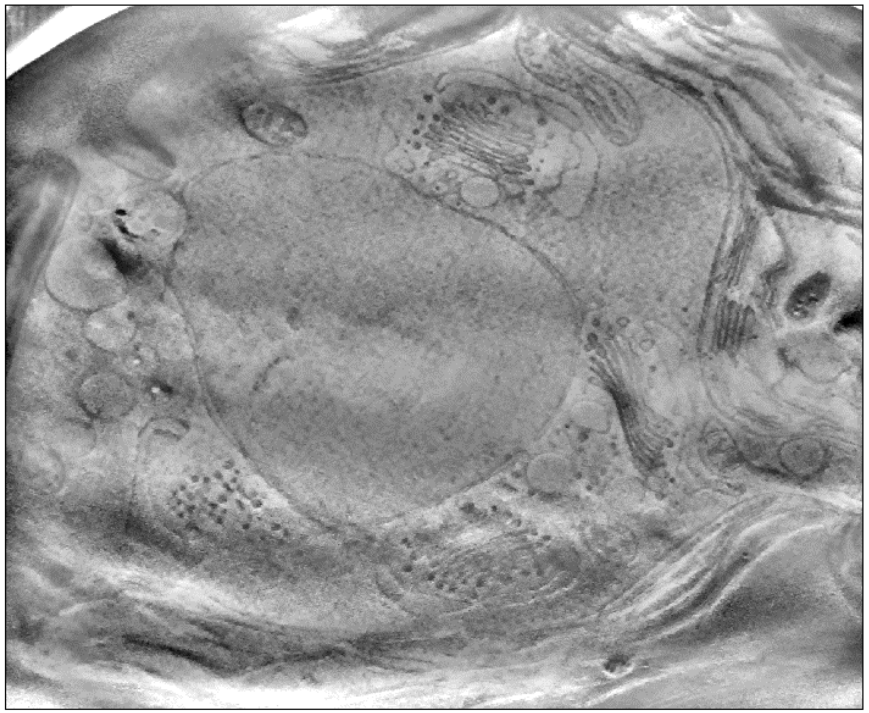
ThermoFisher SCIENTIFIC

SEM Cryo Imaging: Visualization of subcellular features.





Chlamydomonas Cell (schematic)



Aquilos Cryo-FIB: **Cryo ASV of** *Chlamydomonas*

MUNI

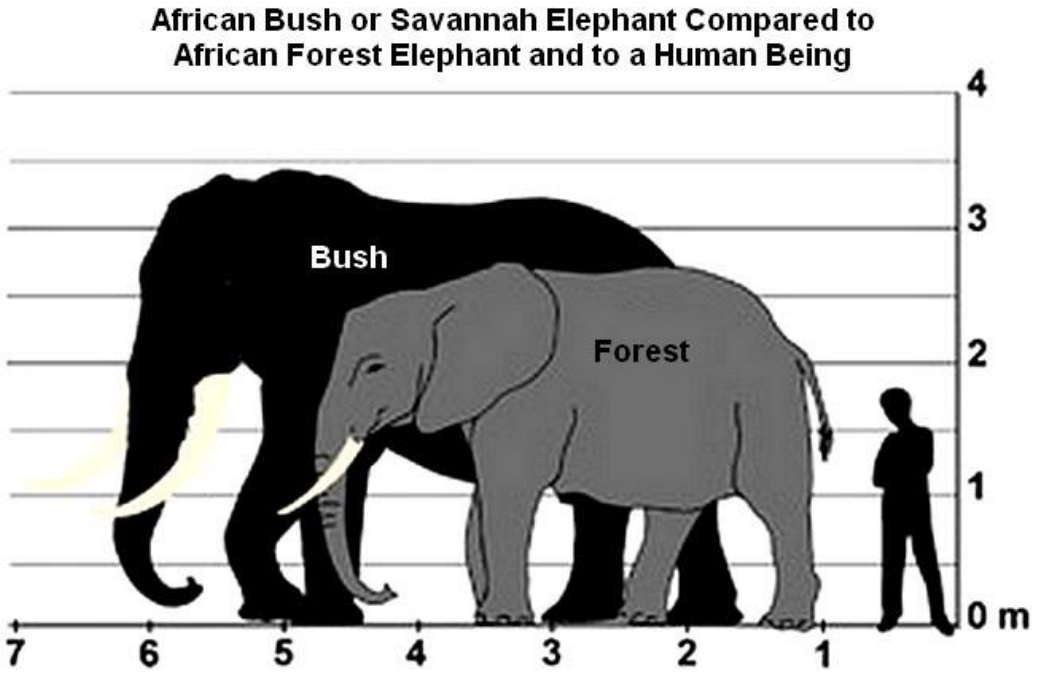
ThermoFisher SCIENTIFIC

Aquilos: Live Demo

MUNI

ThermoFisher SCIENTIFIC

Data collection: meet the big guy.



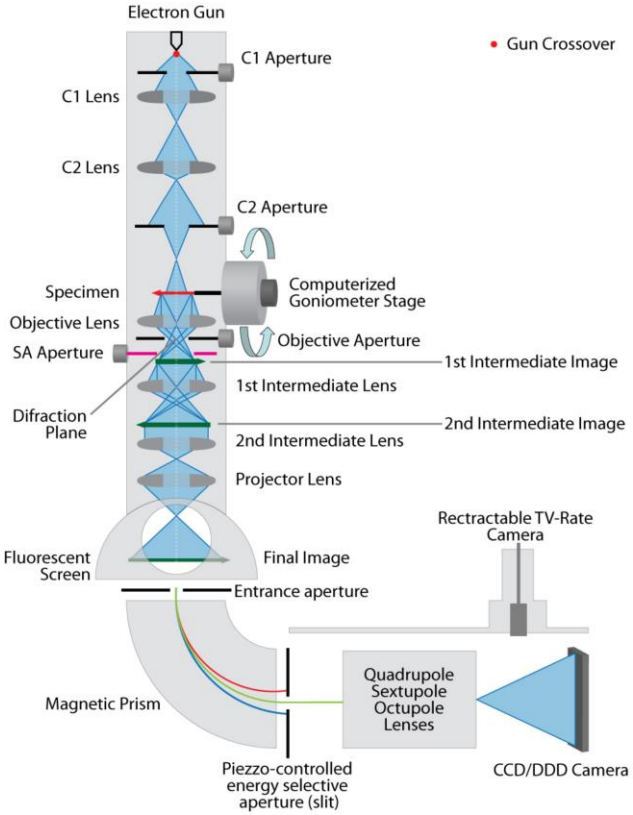
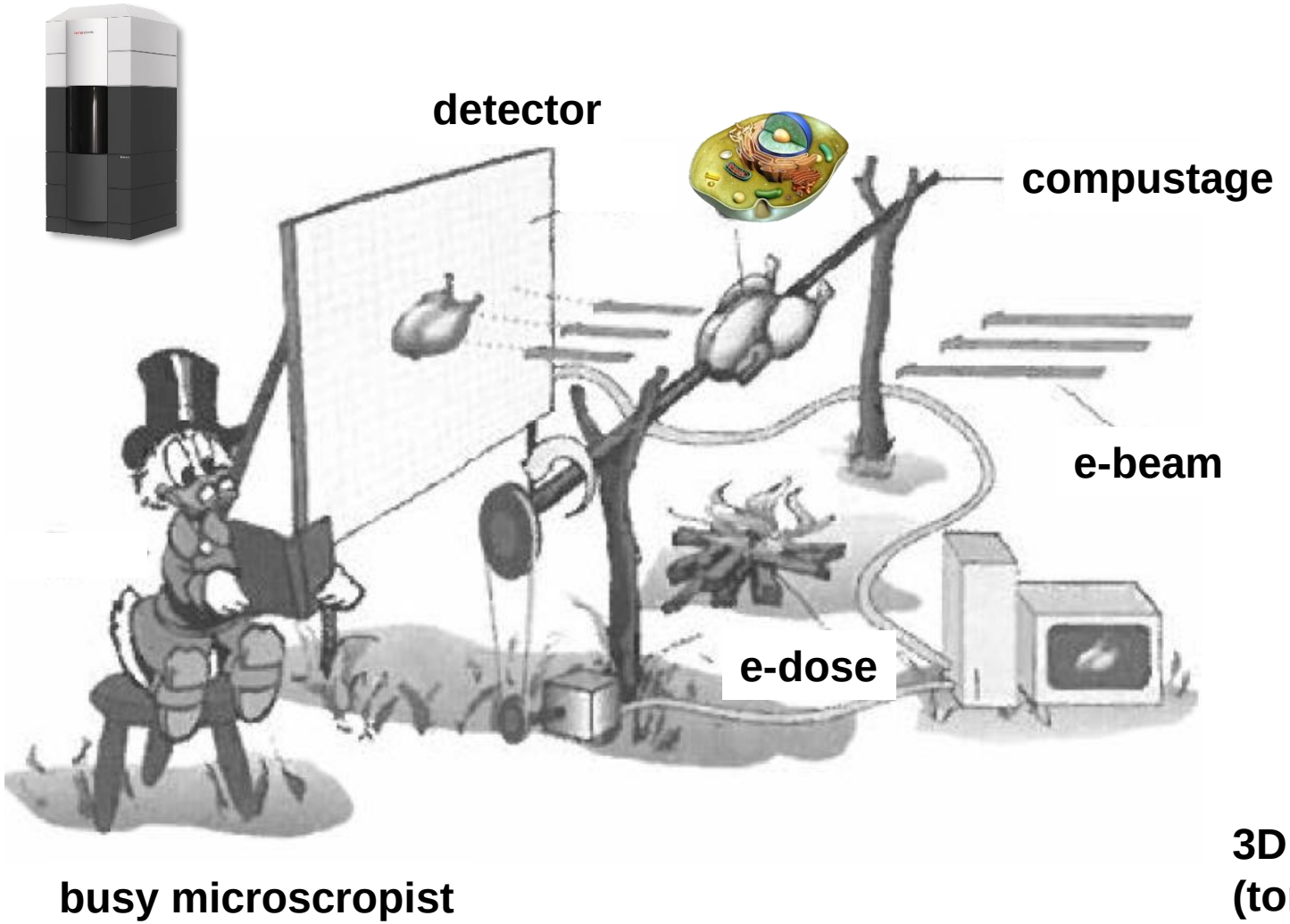
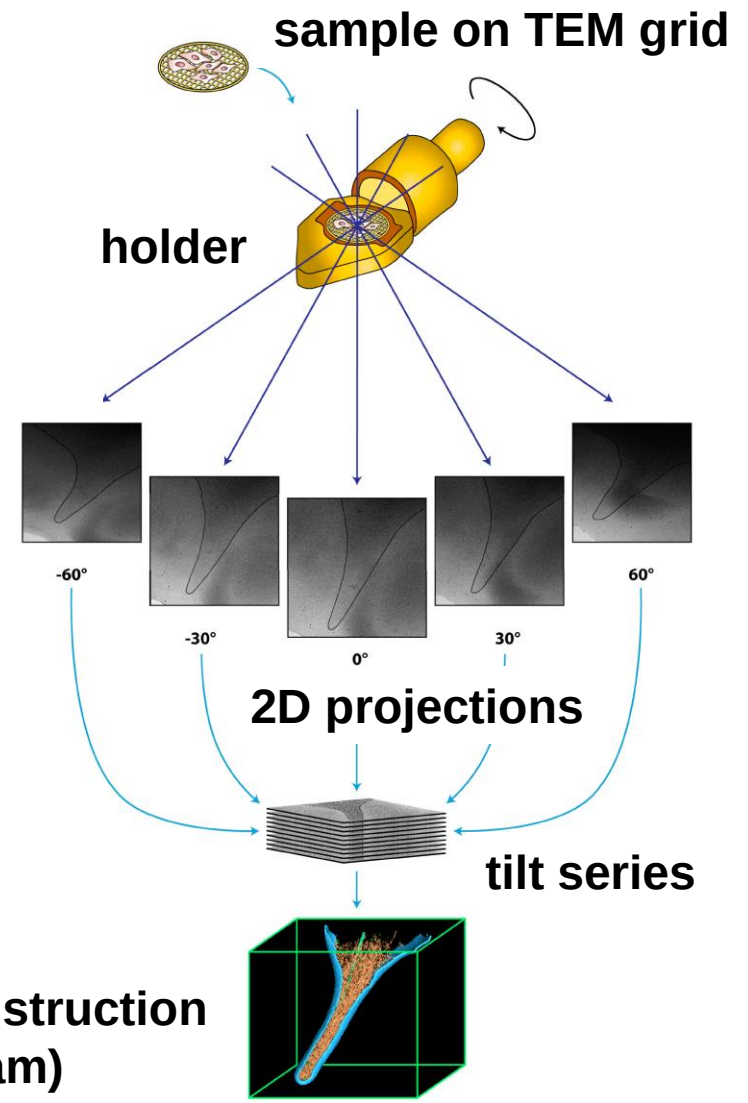


Image on the left: https://blogs.ubc.ca/corinnedy/history-2/. TEM scheme: Bäuerlein F.J.B., PhD. thesis, 2018 (TUM/MPI).

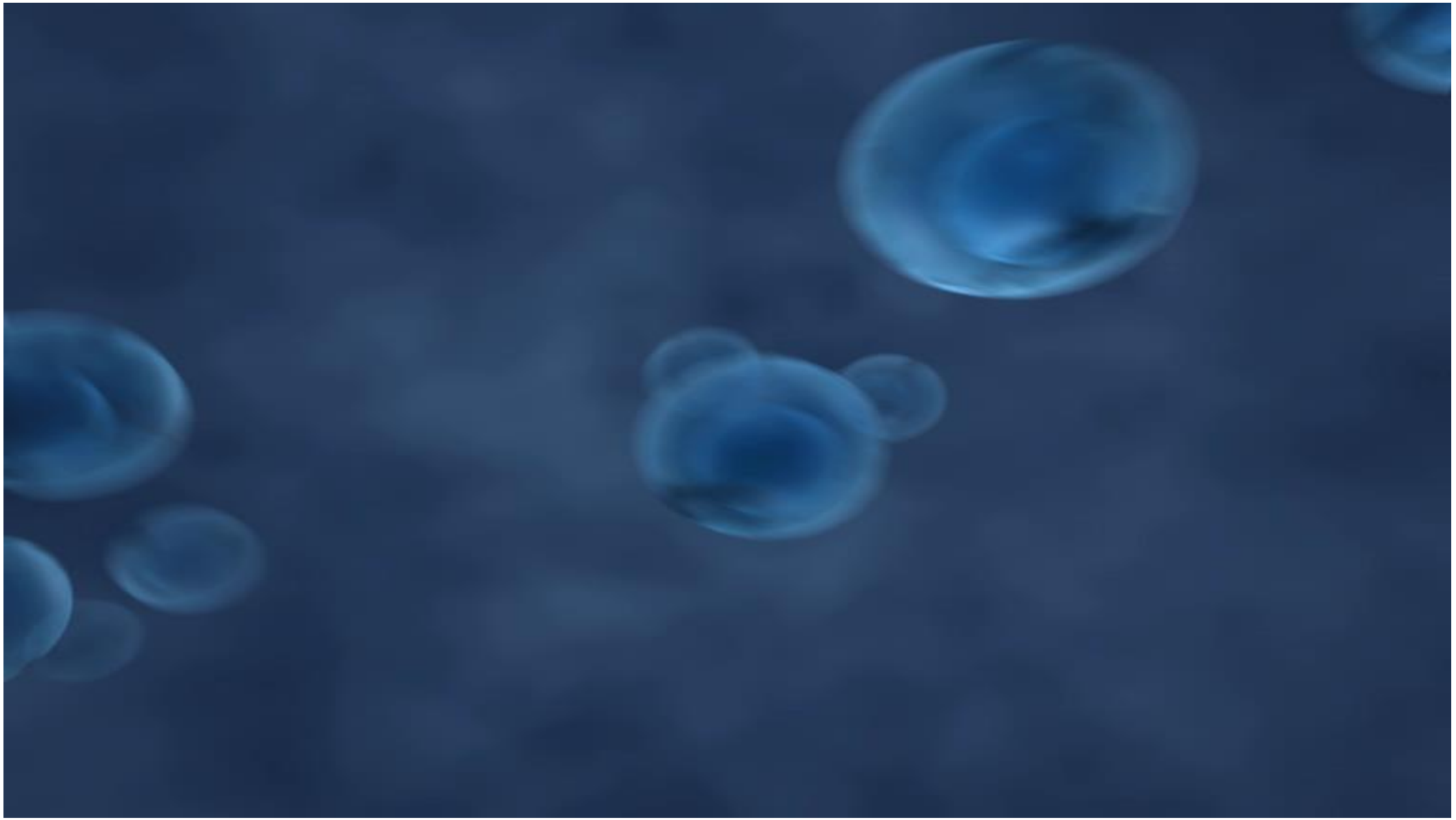
Cryo electron tomography (Cryo-ET) principle





Adapted from: left - Electron tomography (2010), edited by J. Frank; right: Weber M.S. et al., Cells 8 (57) (2019).

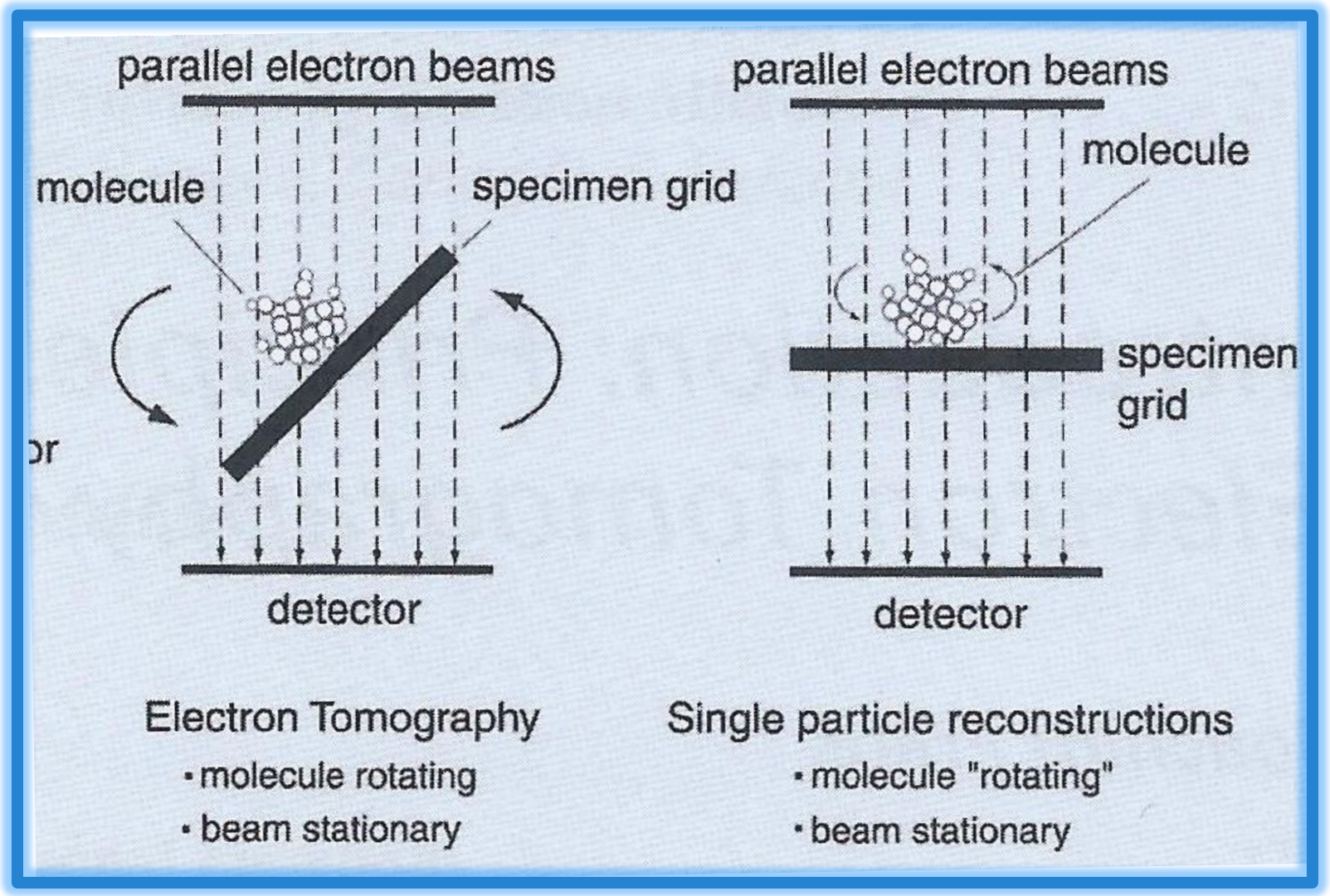
Cryo-ET principle



MUNI

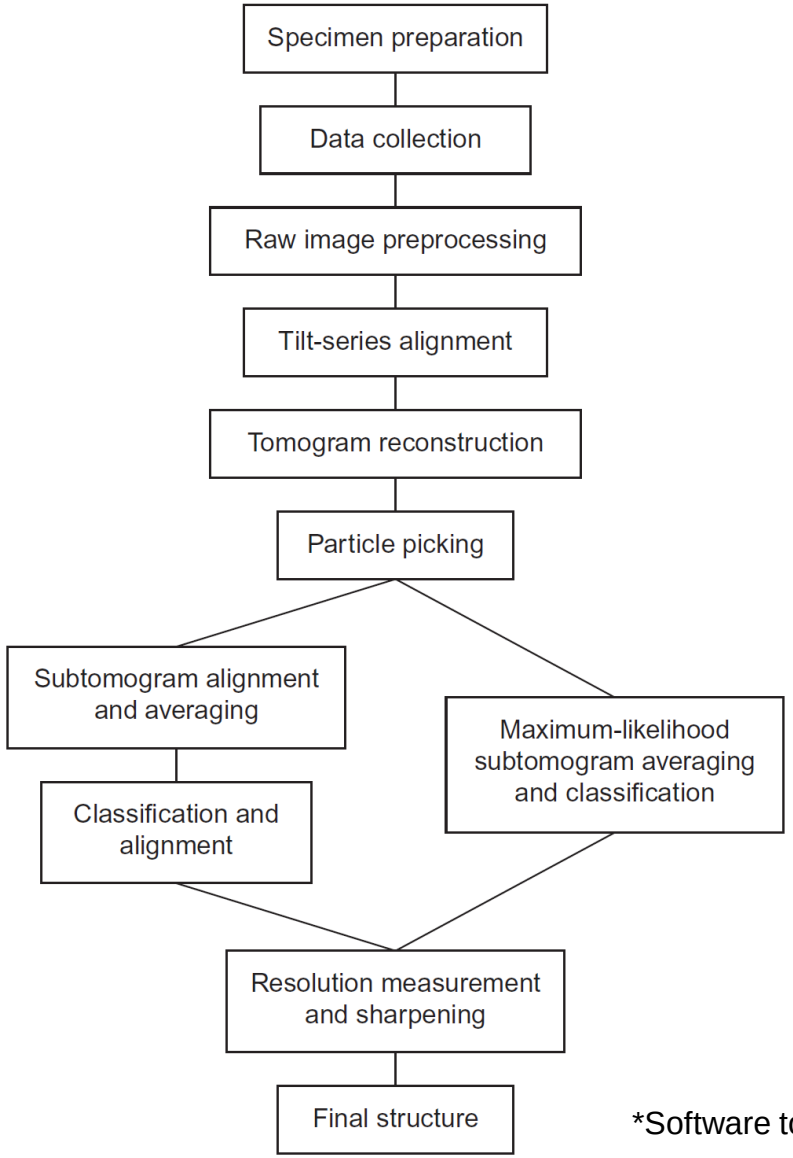
ThermoFisher SCIENTIFIC

Data collection geometry: ET vs SPA



Adapted from Electron tomography (2010), edited by J. Frank.

Cryo-ET (with STA) workflow*



- *Data collection* (automated by dedicated SW): collection geometry (single-, dual-tilt axis etc. strategy), tilt scheme; stage drift: ROI tracking, autofocus).
- *Image pre-processing*: frame alignment (DED); defocus determination (gradient: tilt, sample thickness) > CTF correction (3D).
- *Tilt series alignment*: correction for shifts, rotation, magnification changes; fiducial or feature/patch tracking based.
- *Tomogram Reconstruction* (dedicated SW packages: Eman, Spider, Scipion, …): different algorithms (WBP, ART, SIRT, DFM…) to process tilt series into tomogram.
- Particle picking for STA (template matching and/or manual; starting reference vs biased structure determination).
- STA = sub-tomogram averaging (3D particle averaging from reconstructed volume).
- Post-processing and visualization.

*Software tools for Molecular microscopy: https://en.wikibooks.org/wiki/Software_Tools_For_Molecular_Microscopy.

MUNI

Scheme adapted from Briggs J.A.G. et al.: Methods in Enzymology 579 (2016).

ThermoFisher SCIENTIFIC

Tilt scheme, dose distribution

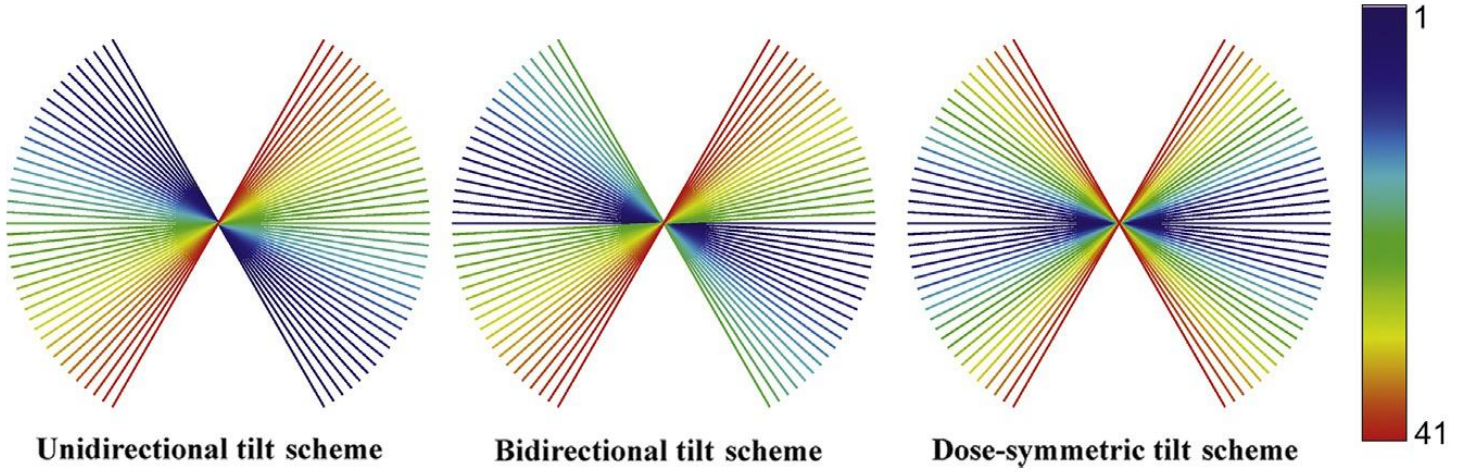
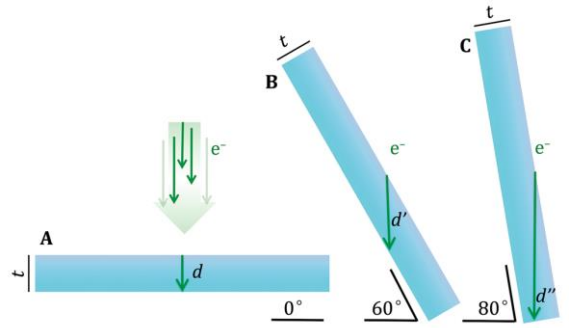
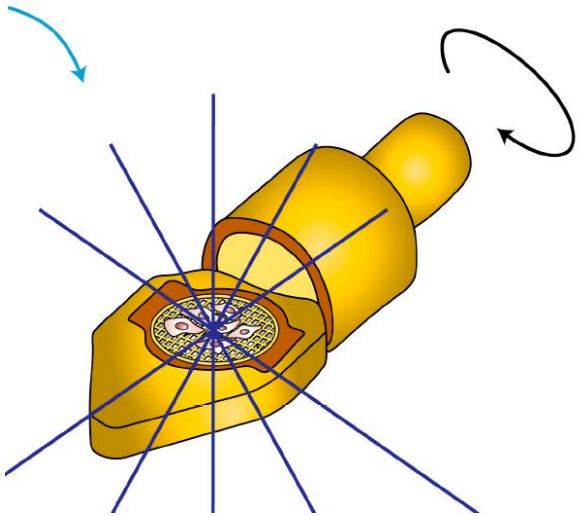


Fig. 4 Schematic showing the order in which tilts are collected in unidirectional, bidirectional, and "dual-walkup" tilt schemes. Tilts are shown from −60 to +60 degrees in 3 degree increments for a total of 41 tilts. *Gray values/colors* correspond to the collection order of each tilt according to the *color* map shown on the *right*. When tilts are collected with constant exposure times, tilt order is directly related to accumulated electron dose on each image. The unidirectional tilt scheme shows a linear sweep from one angular extreme to the other. The bidirectional tilt scheme shows the discontinuity when the tilt-increment direction is changed. The dual-walkup tilt scheme shows near-symmetric accumulated electron dose.

increase of the sample effective thickness





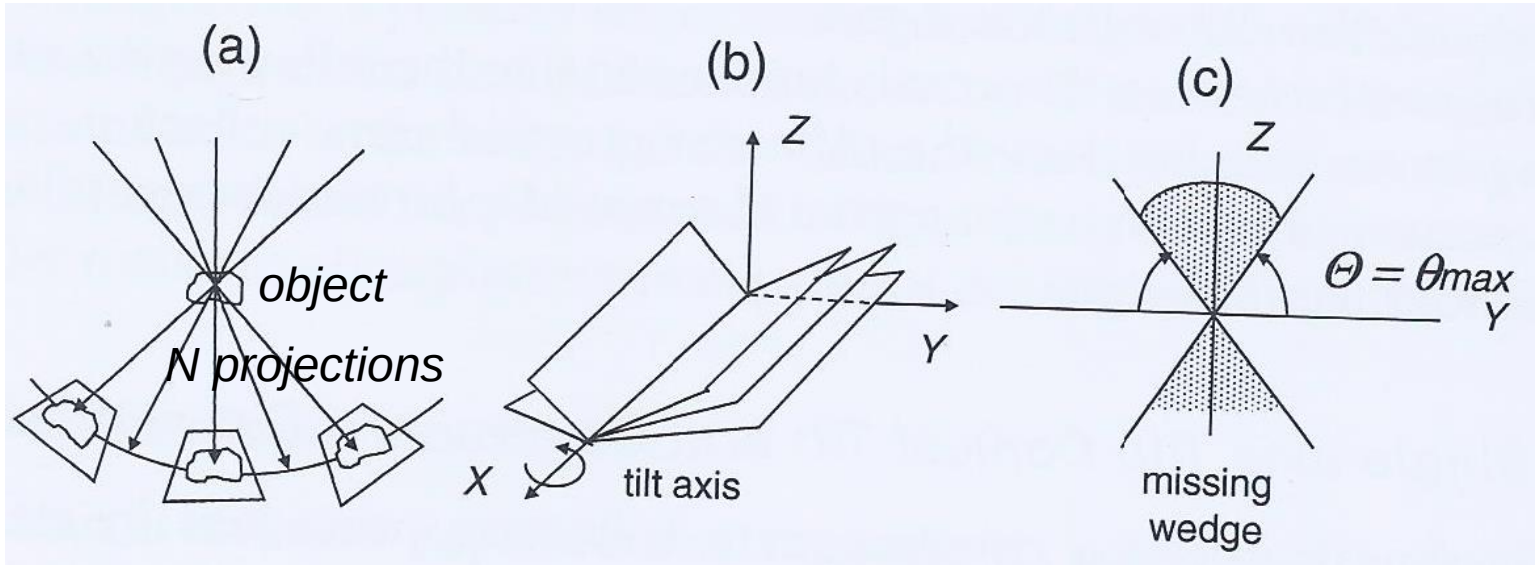
sample holder, single tilt

MUNI

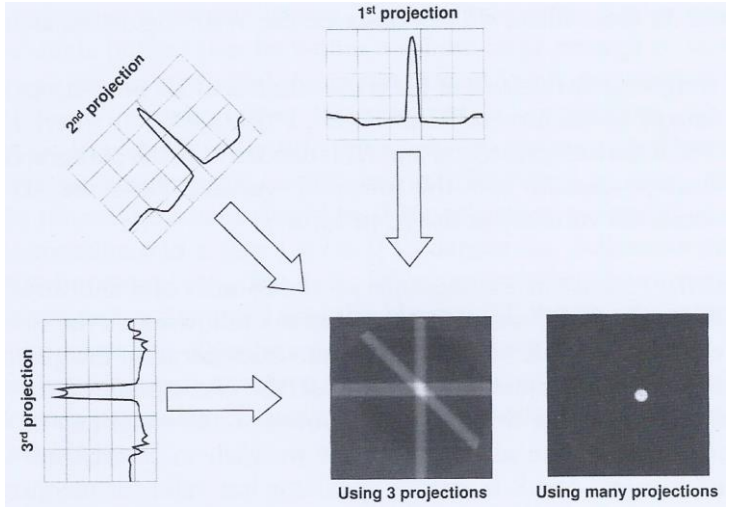
Adapted from: left - Briggs J.A.G. et al.: Methods in Enzymology 579 (2016); right top: Galaz-Montoya J.G. et al., Biophys. Rep. 3 (1-3) 2017.

ThermoFisher SCIENTIFIC

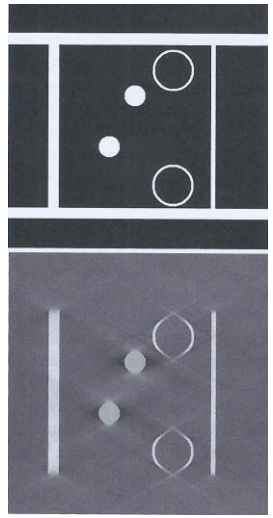
Projection theorem and missing wedge, WBP



real space *Fourier space*



WBP reconstruction



Effects of the missing wedge on a 2D image.

Projection theorem. "The 2D Fourier transform of a projection of the object is identical to a central section of the object's 3D Fourier transform."

N ~ D/d (Crowther; cylindrical object, fully mapped); D object dia, d resolution.

MUNI

Adapted from Electron tomography (2010), edited by J. Frank.

ThermoFisher SCIENTIFIC

Missing wedge

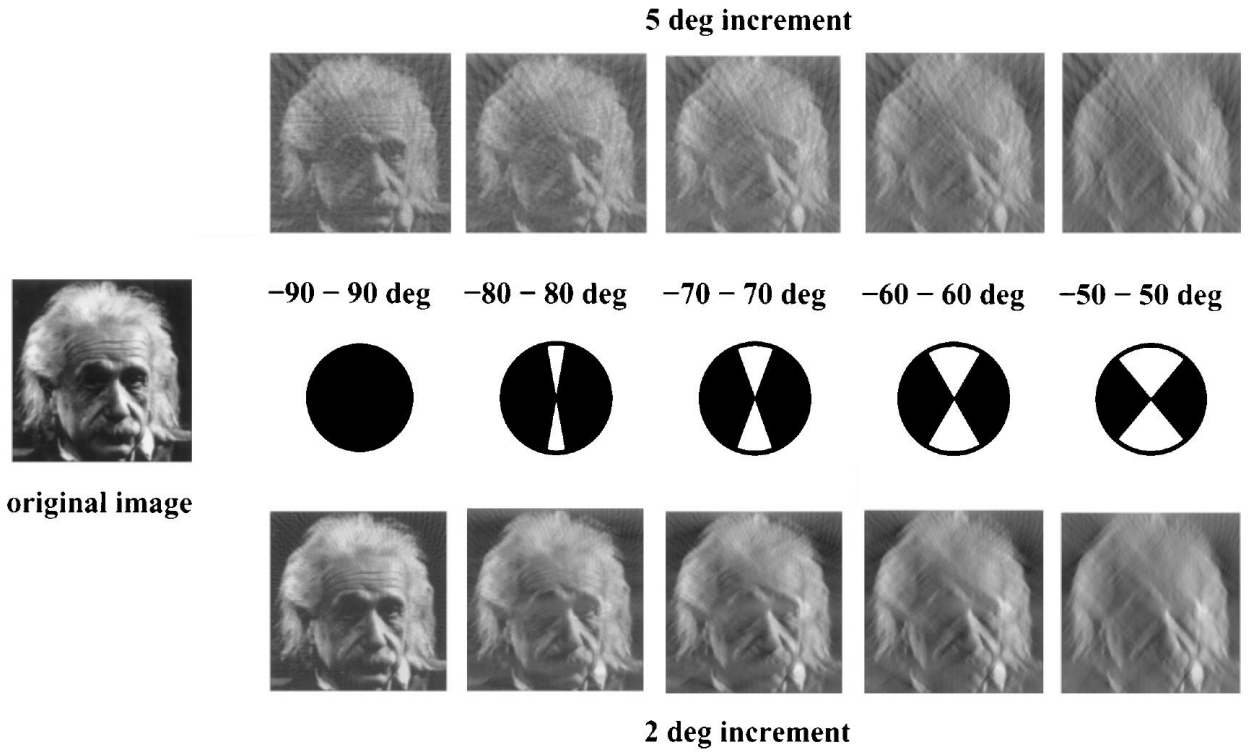


FIG. 2.2. Illustration of the missing wedge effect. The deteriorating influence of the missing gap depends on the tilt range and tilt increment. With a tilt range of ±90° and an increment of 2° the reconstruction is almost identical to the original image; with a tilt range of ±50° and an increment of 5° the similarity is very poor.

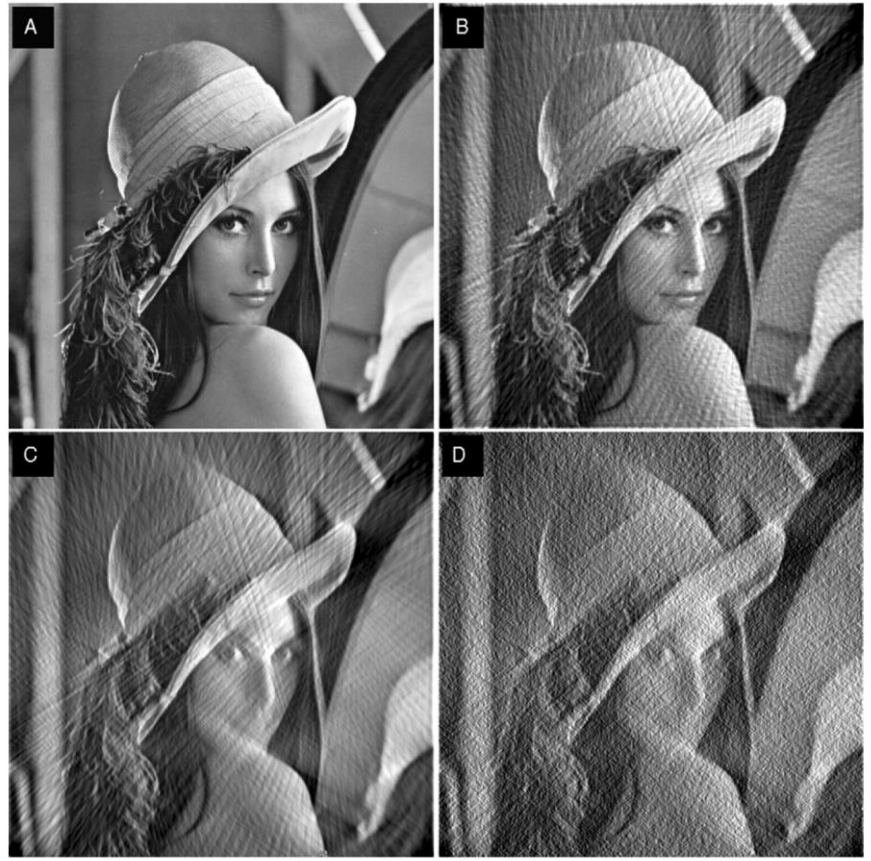


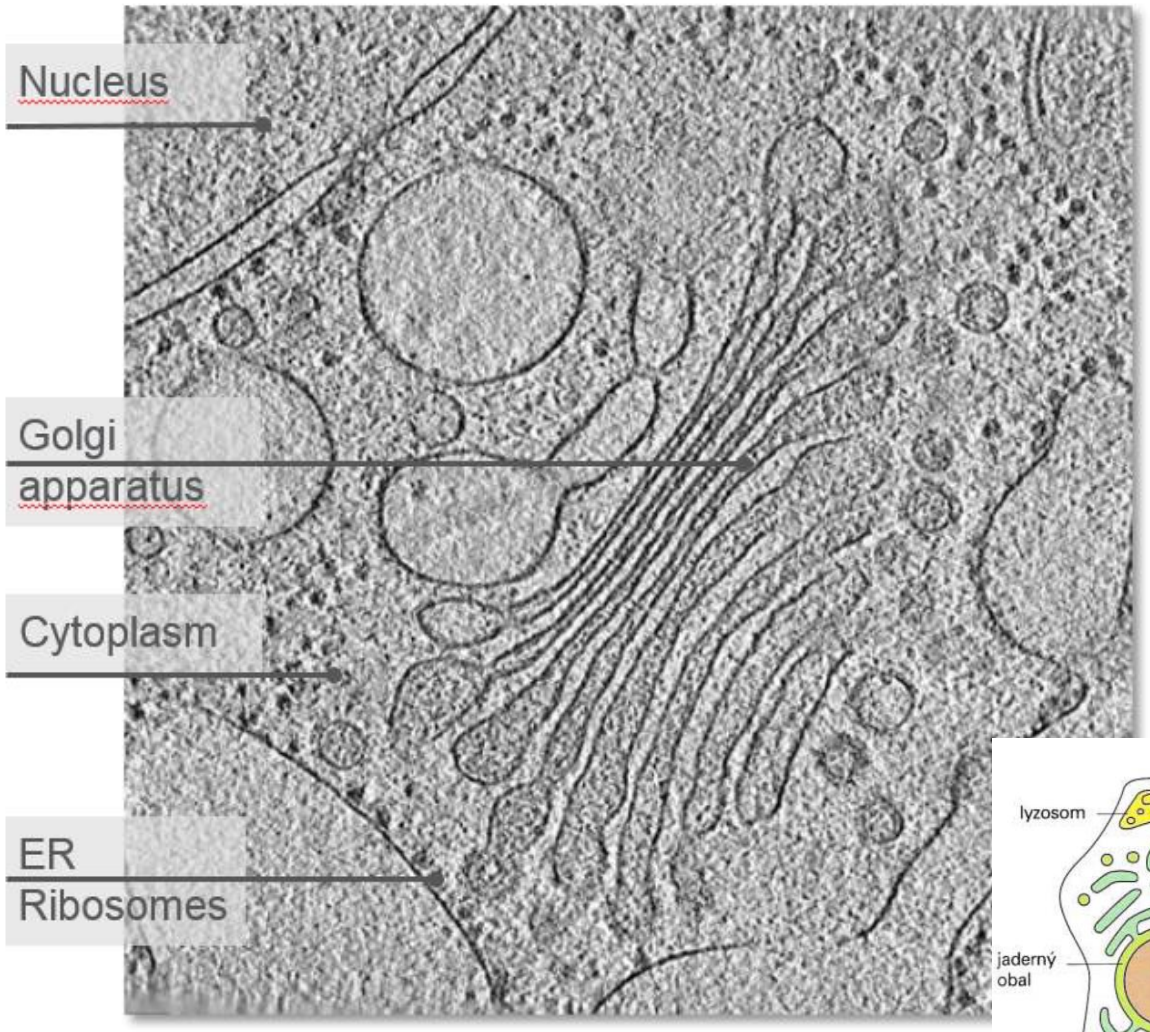
Fig. 8 Illustration of real-space artifacts arising from limited angular sampling and low SNR. (A) Original image of "Lena," a popular test image. (B) The 2D image reconstructed from 90 1D projections evenly distributed over a 180° tilt range. Note the numerous streaks that are produced by gaps between successive tilt images. (C) The 2D image reconstructed from 61 1D projections covering a 120° tilt range. Here, the missing wedge produces a smearing out of details in the vertical direction (e.g., the lips are nearly lost). (D) The 2D image reconstructed from 61 1D projections covering a 120° tilt range, with simulated shot noise added to each projection (SNR~2) before calculation of the reconstruction. Note that high-resolution features, such as the feathers in Lena's hat, are present in (B) and (C) but overlaid with streaks. Fewer high-resolution features are present in (D) than in (C) because of the lower SNR, but the streak artifacts are also less prominent.

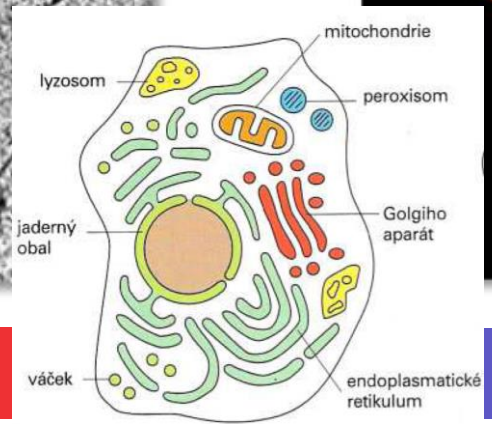
MUNI

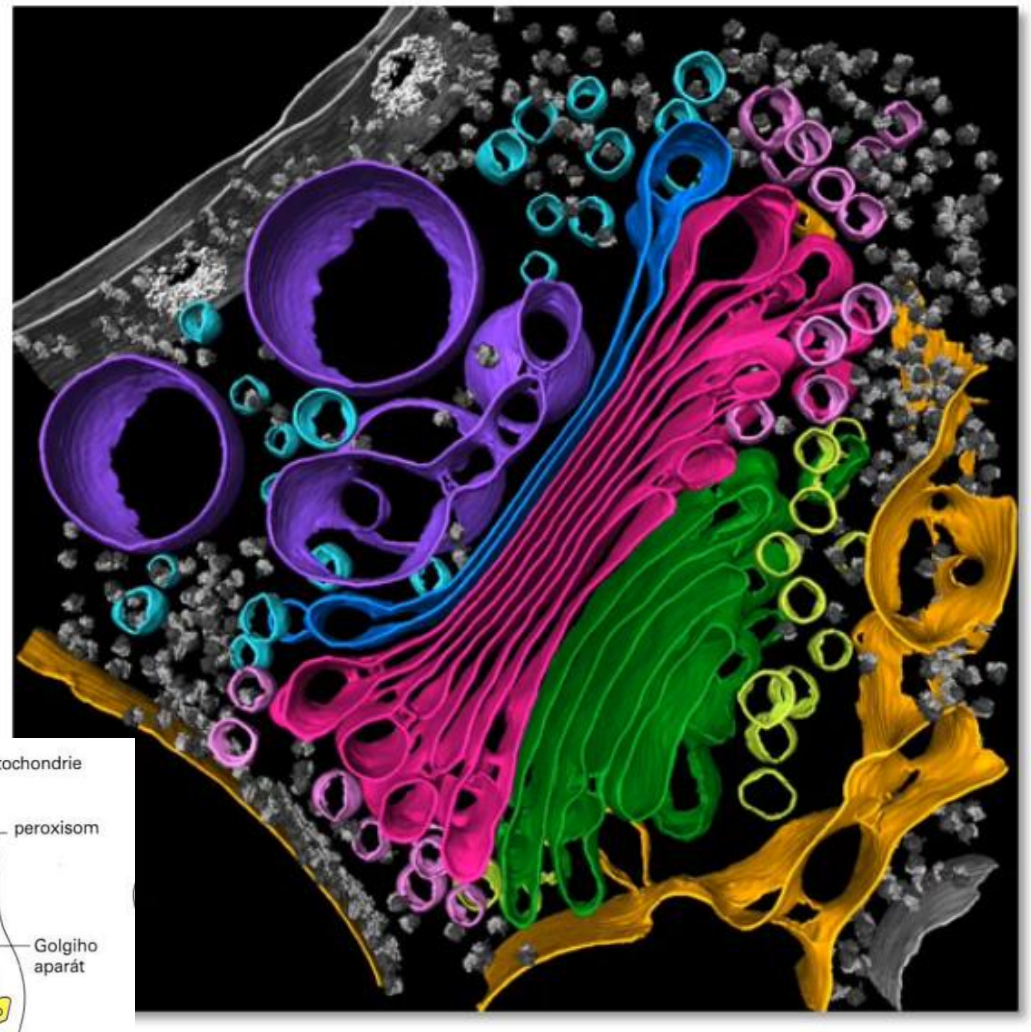
Adapted from: left – Koster A.J. et al., J. of Struct. Bio. **120** (1997); *right – McEwen B.F. et al., Methods in Cell Bio. 49 (ch. 6).*

ThermoFisher SCIENTIFIC

How does tomography data look like?







TOMOGRAM

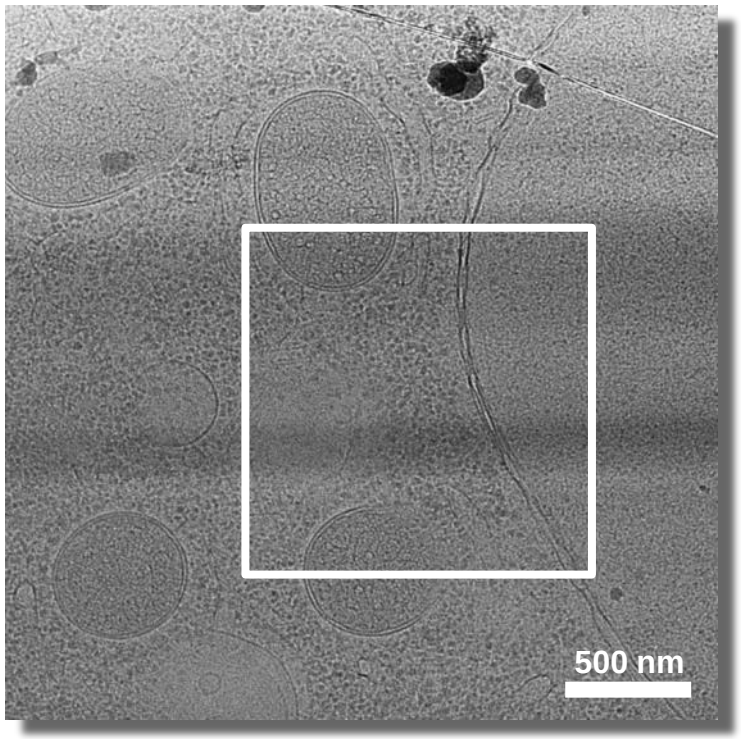
VISUALIZATION

MUNI

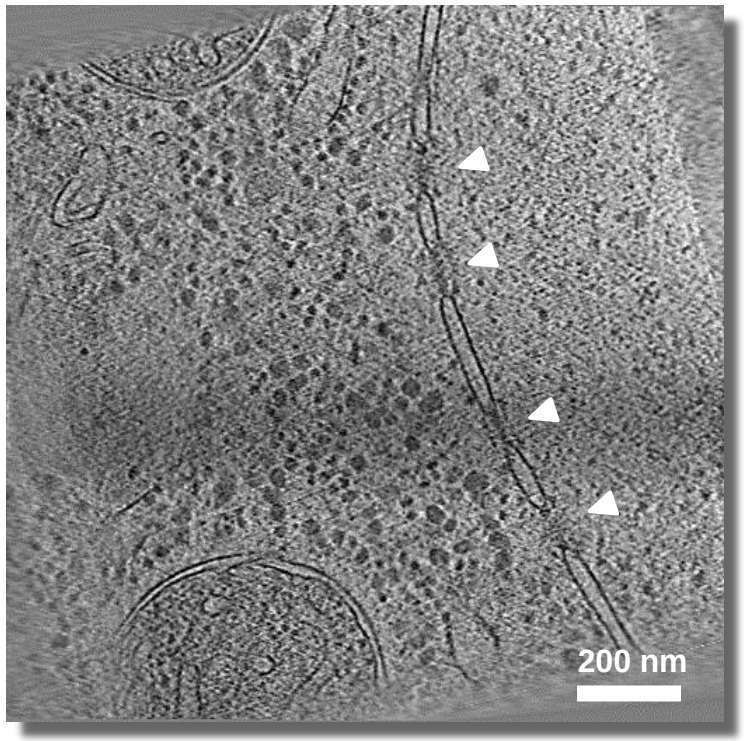
Data Courtesy Max Planck Institute of Biochemistry | Bykov et al., eLife 2017.

ThermoFisher SCIENTIFIC

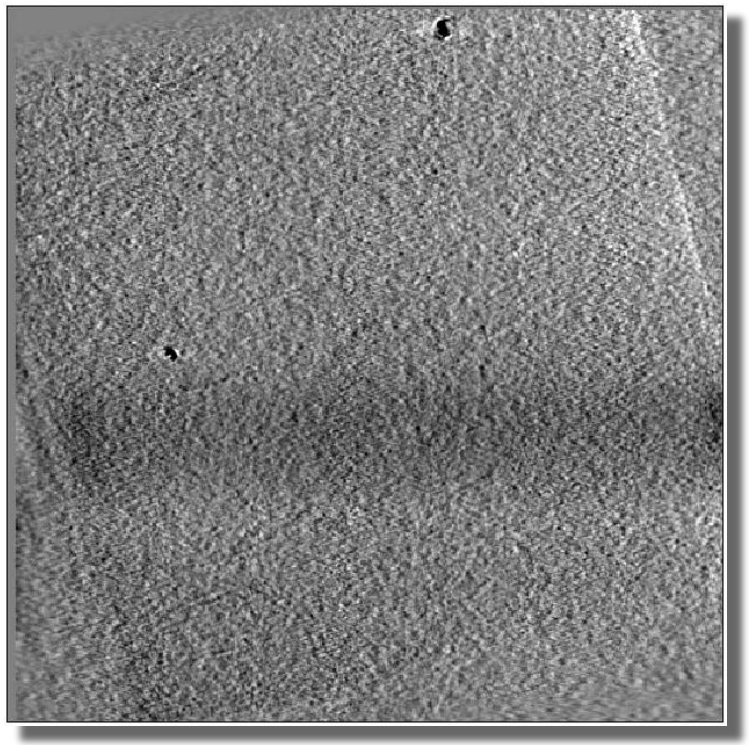
Cryo-ET at the Cytoplasm/Nucleoplasm Border



TEM projection



Slice from 3D reconstruction

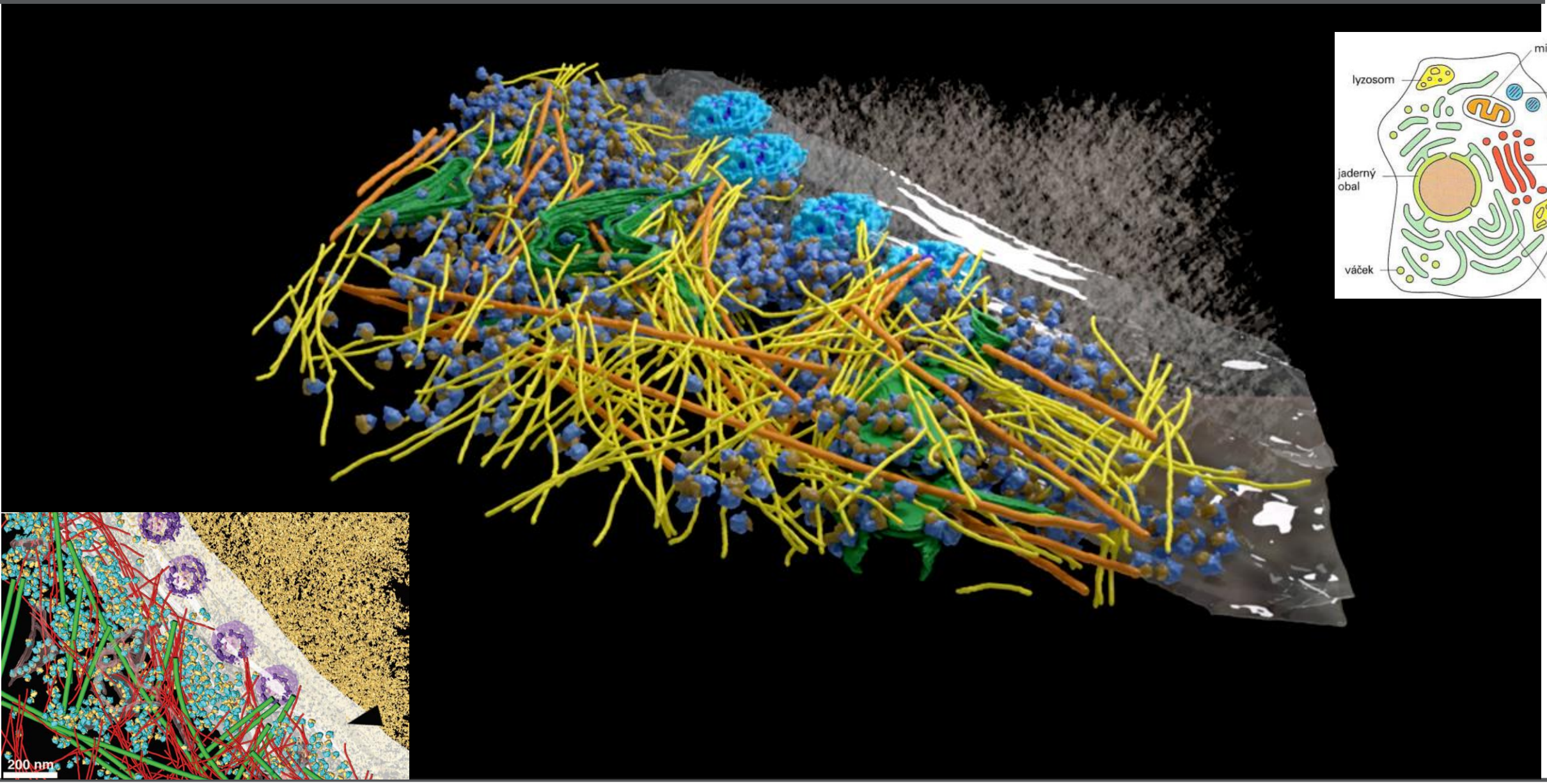


3D reconstruction (300 nm)

MUNI

ThermoFisher SCIENTIFIC

Cryo-ET: Visualizing the Molecular Sociology at the Hela Cell Nuclear Periphery

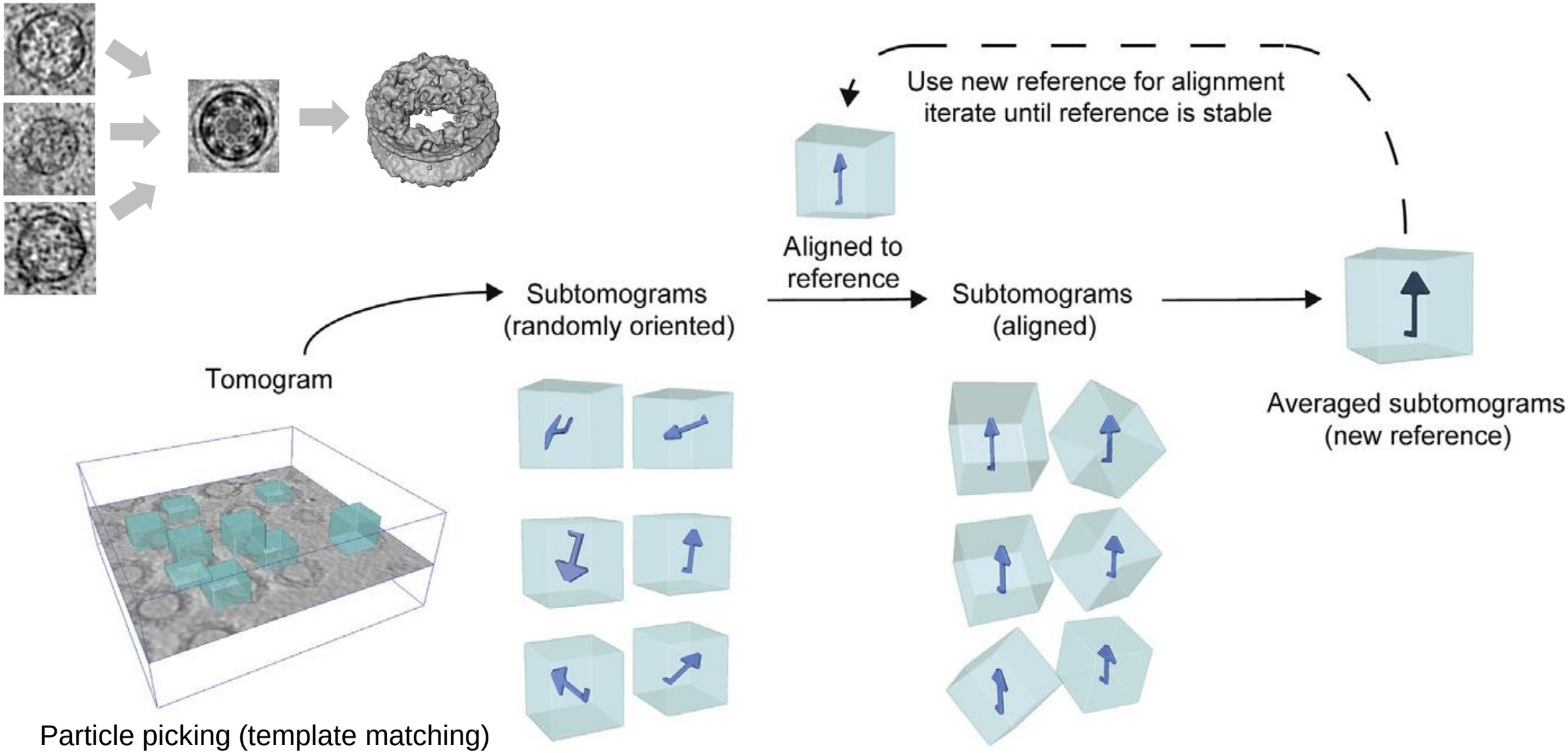


MUNI

Mahamid et al., Science, 351(6276) 2016; cell scheme on the right: Alberts, Základy buněčné biologie, 2004.

ThermoFisher SCIENTIFIC

Subtomogram averaging (STA/STP): structural biology in-situ

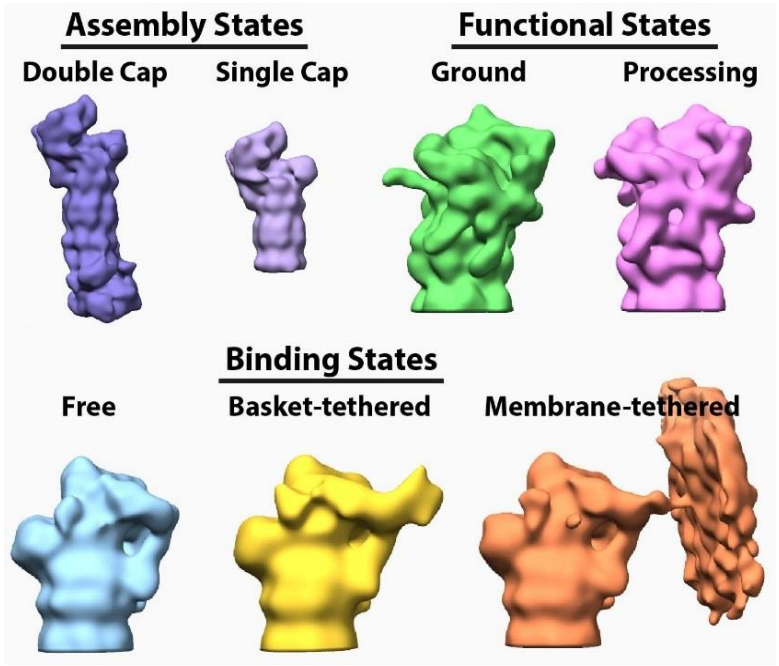


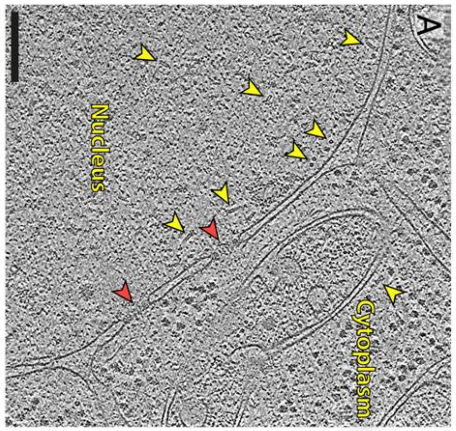
MUNI

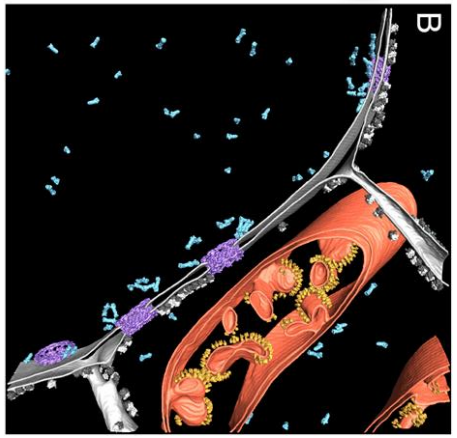
Adapted from Briggs Briggs J.A.G. et al.: Methods in Enzymology 579 (2016); Mahamid et al., Science. (2016).

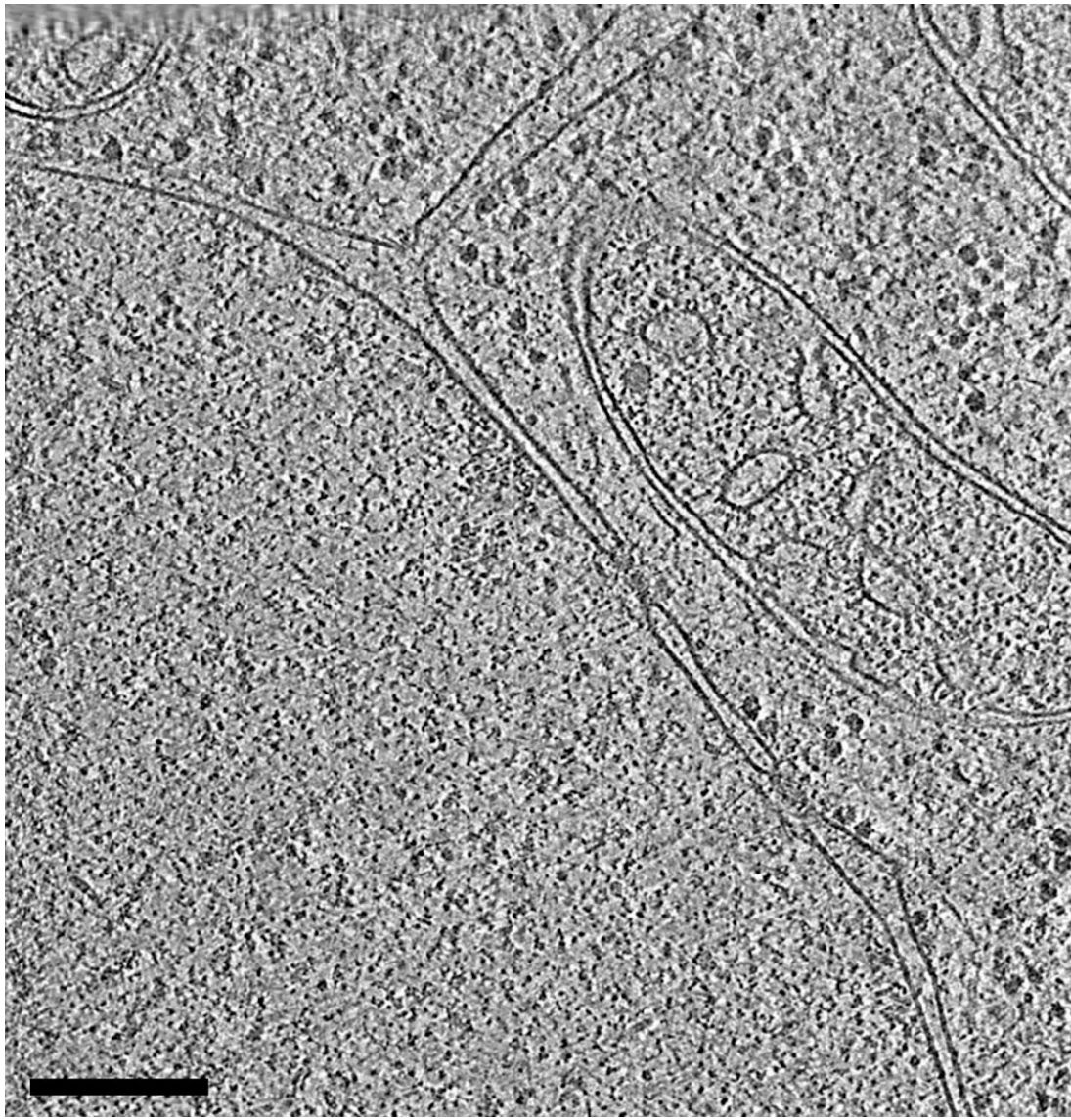
ThermoFisher SCIENTIFIC

STA allows to map different functional states of the same protein









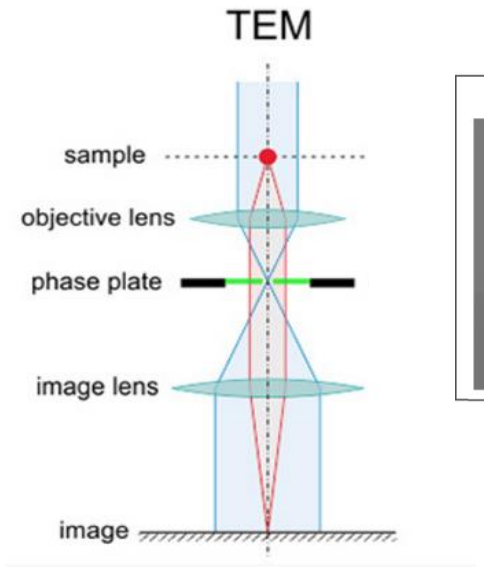
Subtomogram averages of 26S assembly states (res. ~ 21 Å).

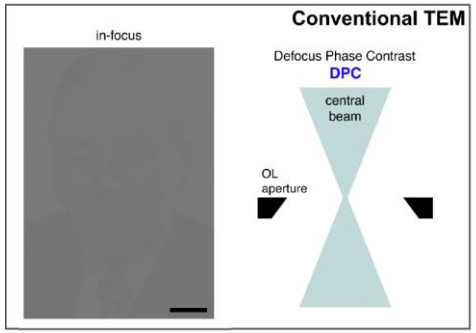
(Remark. Particle picking: SPA template from EMDB: low-pass filtered 20S core particle attached to one 19S regulatory particle.)

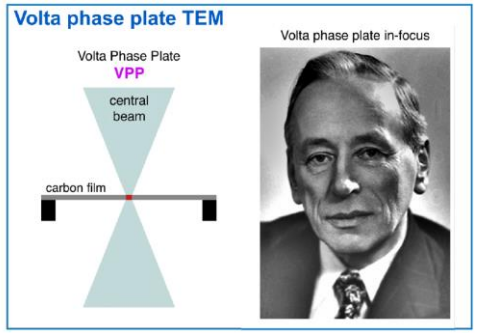
Proteasome localization and activity (functional state and interaction partners) by visualizing its macromolecular structure within the native cellular environment (*Chlamydomonas reinhardtii*). Scale bar: 200 nm. Tomo: lam. thick. < 200 nm, tilt incr. 2°, ±60°, px 3.4 Å, defocus form -4 to -5.5 µm, total dose < 100 e/Å.

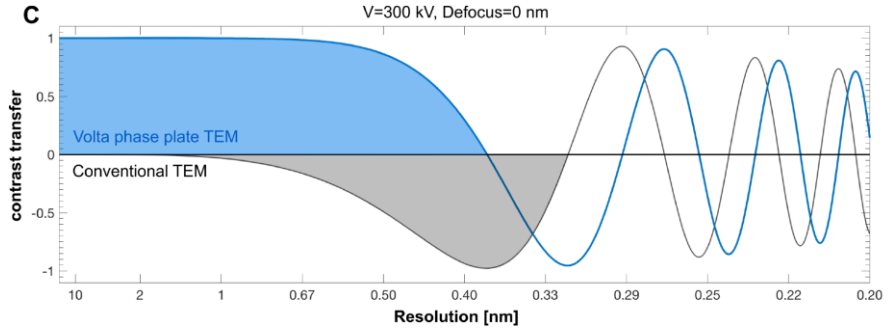
Adapted from Albert et al., PNAS 114 (2017).

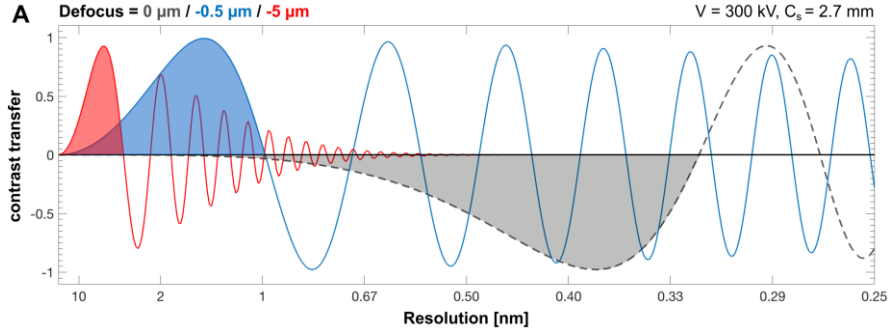
Volta Phase Plate (VPP)



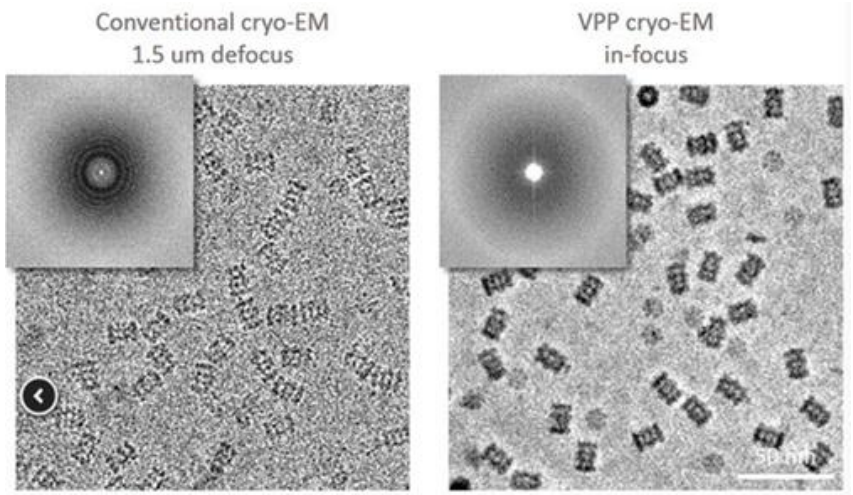




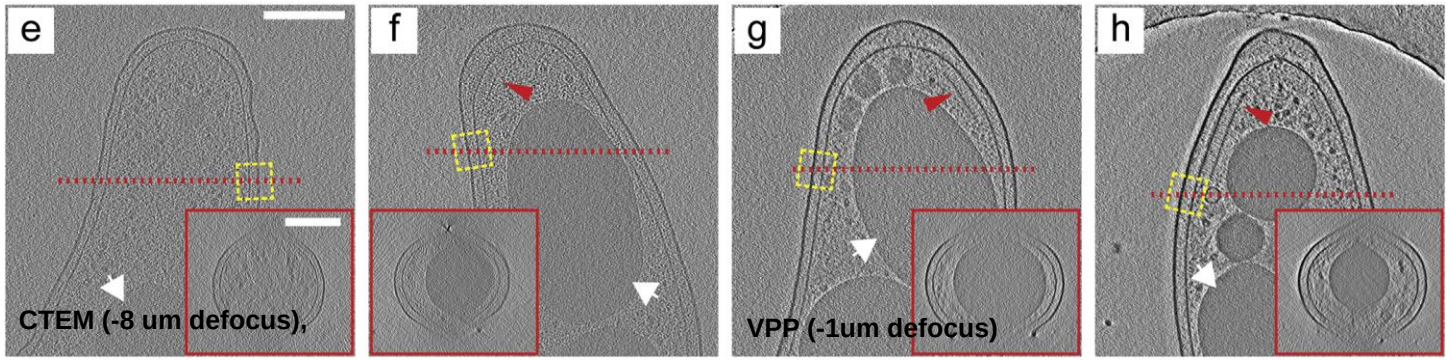




SPA



Cryo-ET

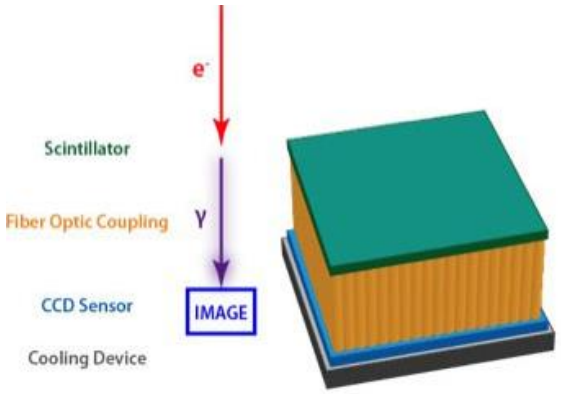


MUNI

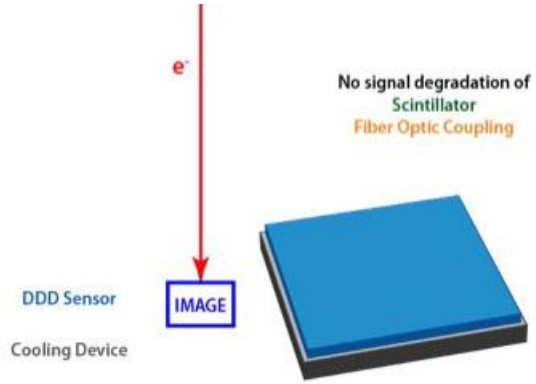
Adapted from: Danev R. et al., PNAS, 111 (2014); Bäuerlein F.J.B., PhD. thesis, 2018 (TUM/MPI); Fukuda Y. et al., Journal of Structural Biology 190 (2015).

ThermoFisher SCIENTIFIC

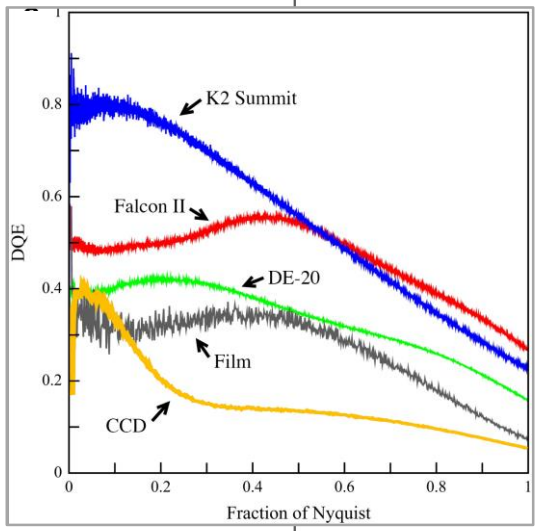
Direct Detectors & Dose Fractionation & Drift Correction



CCD Camera



Direct Detection Camera



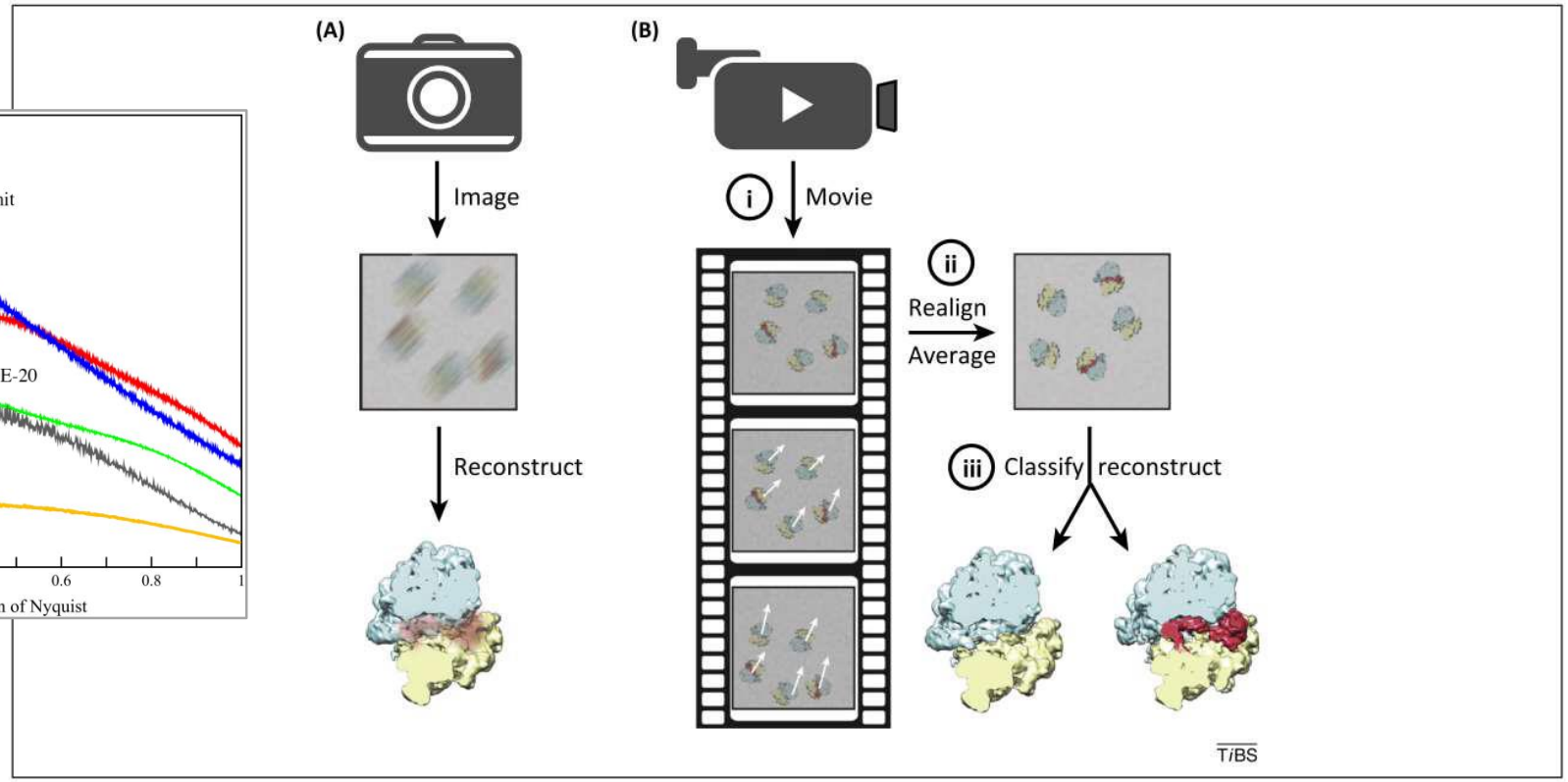


Figure 2. Recent technological advances. **(A)** Previously, noisier images were recorded on photographic film, beam-induced sample motion led to image blurring, and structurally different particles were often mixed in a single reconstruction. **(B)** Three recent advances yield better reconstructions: (i) digital direct-electron detectors yield data of unprecedented quality and allow recording movies during exposure; (ii) computer programs to realign the movie frames may correct for sample movements that are induced by the electron beam; and (iii) powerful classification methods lead to multiple structures from a sample mixture.

MUNI

Image on the left: http://www.directelectron.com/products/de-series; DQE graph: Bäuerlein F.J.B., PhD. thesis, 2018 (TUM/MPI).

ThermoFisher SCIENTIFIC

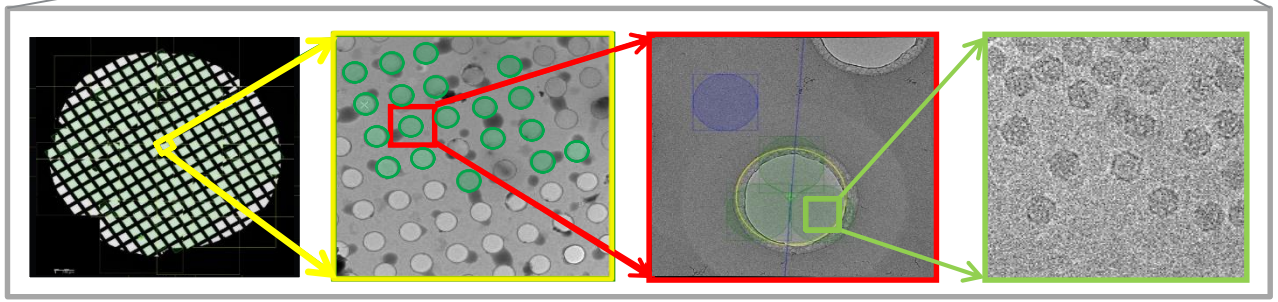
Single Particle Analysis (SPA) Workflow

Biochemistry
(protein purification)

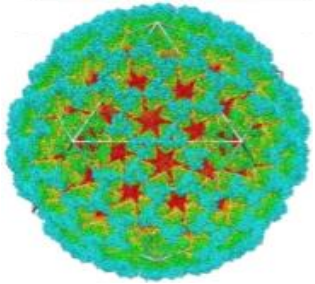
Sample preparation
(vitrification)

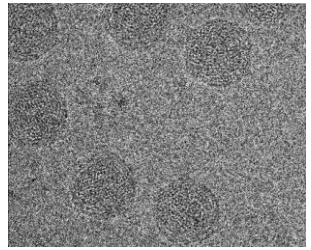
Data acquisition
(2D images of particles)

automated data collection: e.g. EPU sw



Reconstruction
(3D model)



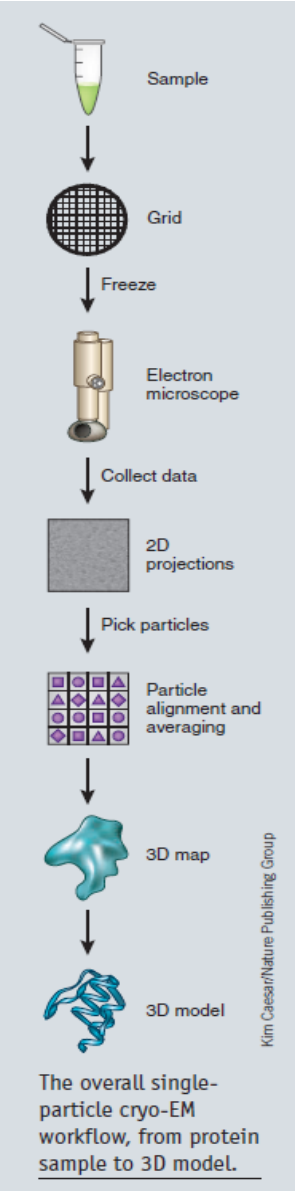


RELION
Frealign
EMAN
others…

MUNI

ThermoFisher SCIENTIFIC

SPA Workflow

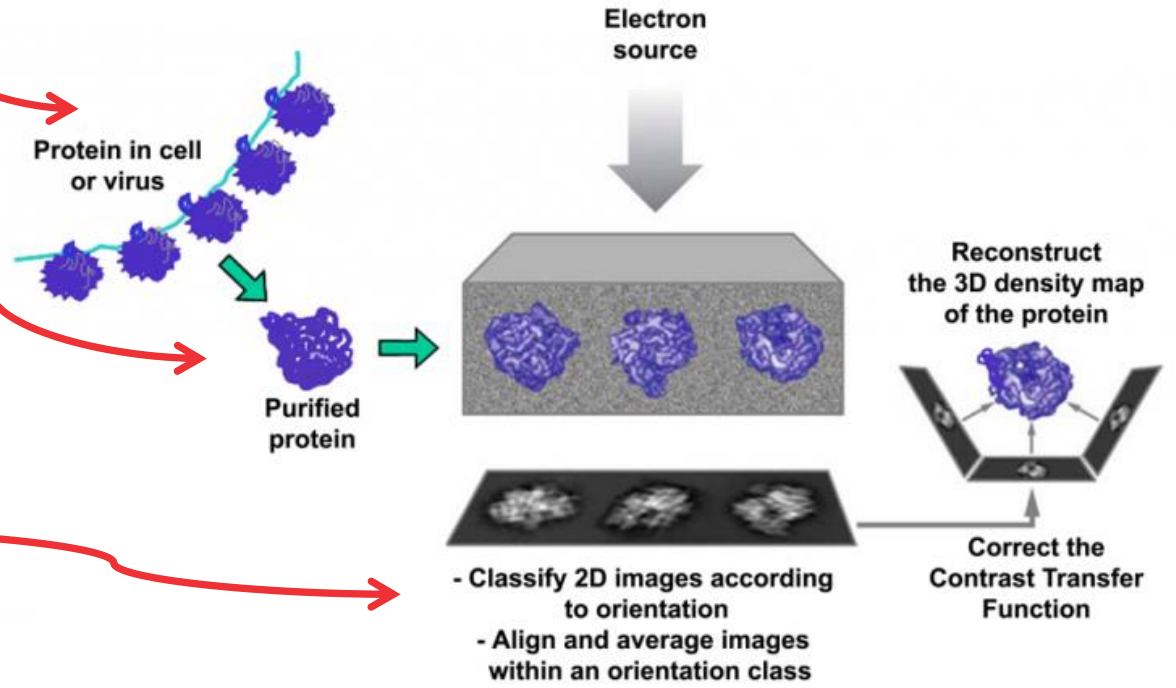


The overall single-particle cryo-EM workflow, from protein sample to 3D model.

Sample preparation (proteins, viral particles, …)

Data acquisition (no tilt series)

Data processing

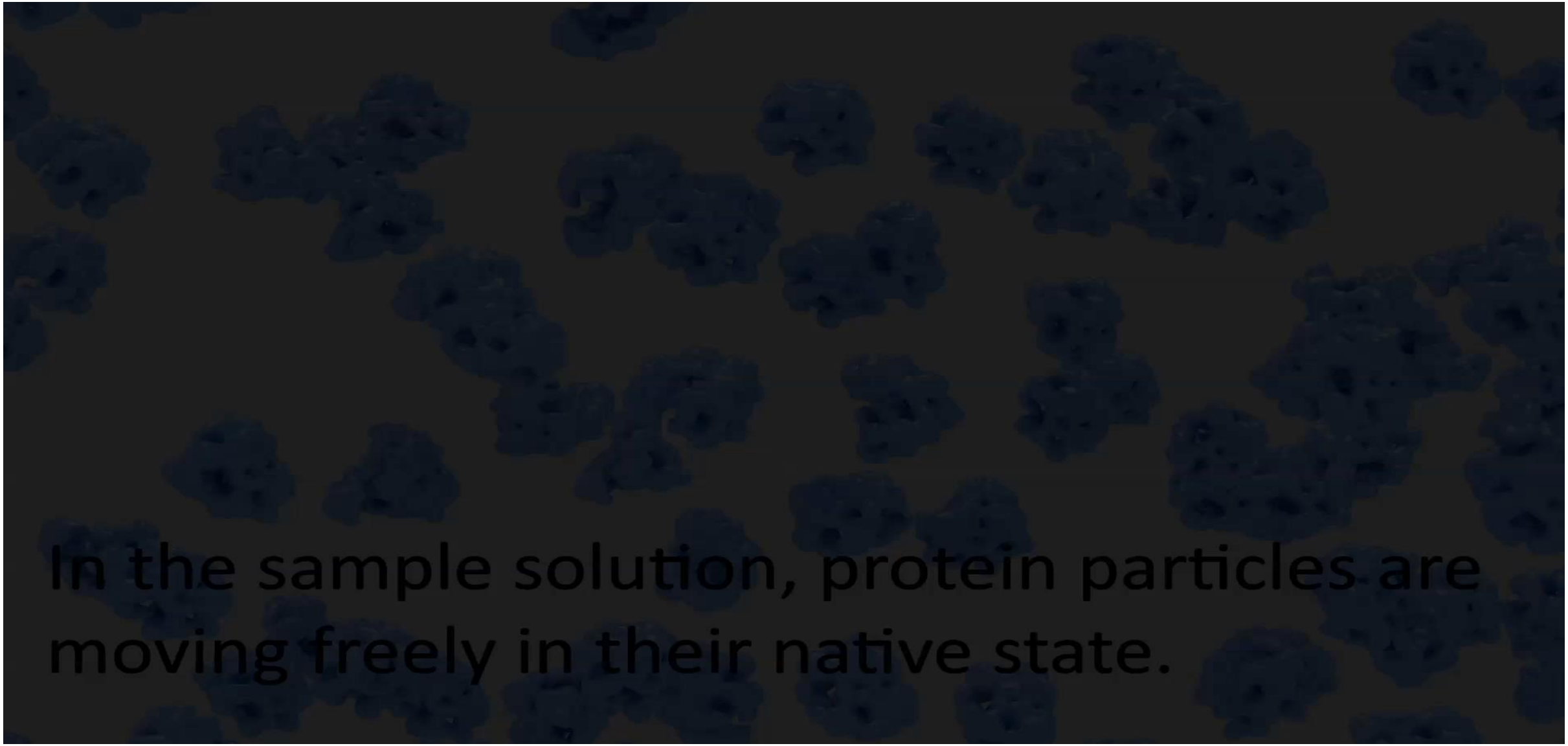


MUNI

Adapted from: image on the right - http://cns.fas.harvard.edu/CryoEM.

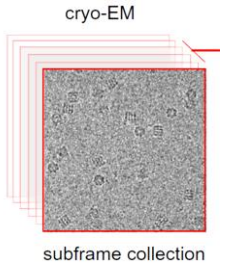
ThermoFisher SCIENTIFIC

SPA Principle - video

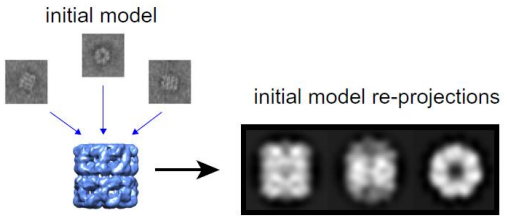


Dr. Wen-Ti Liu: http://www.novalix-pharma.com/.

SPA: Data Processing Chain



- known structure,
- tomography,
- de novo / common lines
- random conical tilt, …



initial model

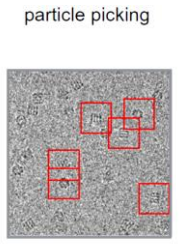
refined class average

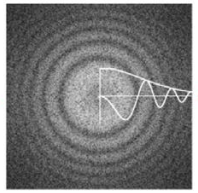
2D images / projections → class averages → 3D model → reprojection

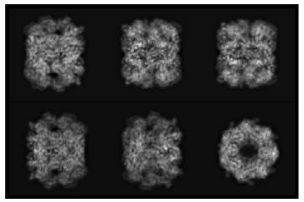
- frames: motion correction,
- particle picking,
- CTF correction,
- alignments and classification (particle shifts, rotation; classes)

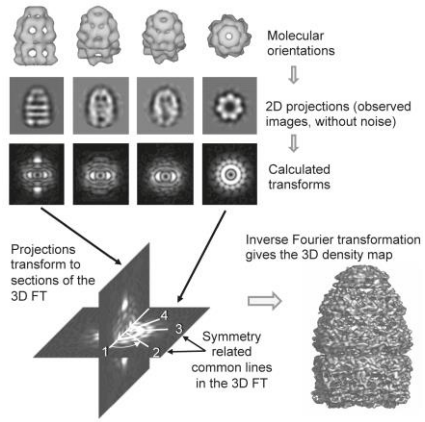
- relative orientation of the particles / classes in the space (euler angles),
- reconstruction algorithm (2D slices), e.g.: FT > filling of 3D FT space > FT^{-1}

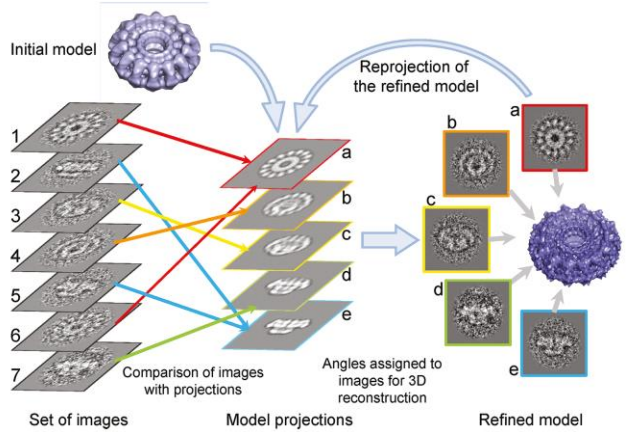
- reproject in all directions (angular step),
- realign, reclassify (filters),
- filling of 3D FT space > FT^{-1}




defocus determination and CTF correction


particle alignment and classification





MUNI

Adapted from: Jensen G.: Getting started in cryo-EM; Orlova E.V. et al., Chem. Rev. 111 (2011); Carroni M. et al., Methods 95 (2016).

ThermoFisher SCIENTIFIC

Example: Apoferritin sample reconstructed at 1.6 Å.

- *Data Acquisition* on Krios TEM using EPU sw & Falcon 3 EC camera.
- *Sample*: apoferritin ~3 mg/ml.

Camera	Falcon 3 EC
Pixel size(Å)	0.52
Dose rate (e/pix/sec)	0.5
Total dose (e/Å^2)	52
Dose fractions	100
Exposure time (sec)	29
Number of images	3740
Defocus values	-1.2, -1, -0.8, -0.6,-0.4

3740 Micrographs

Micrograph selection

2392 Micrographs

Particle picking

357,022 Particles

2D classification

265,955 Particles

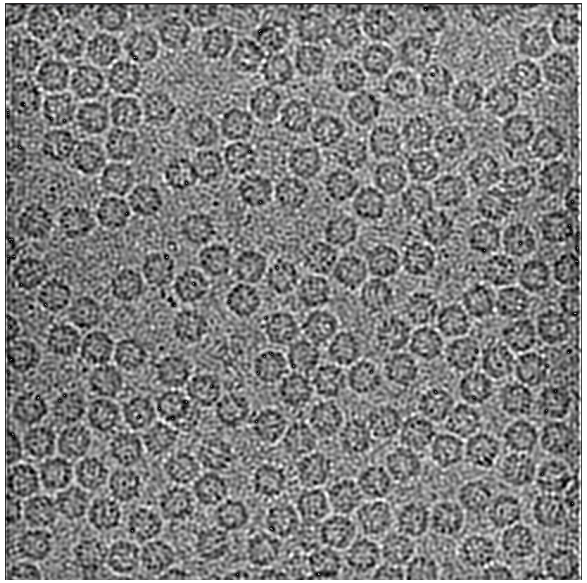
3D refinement + Postprocessing

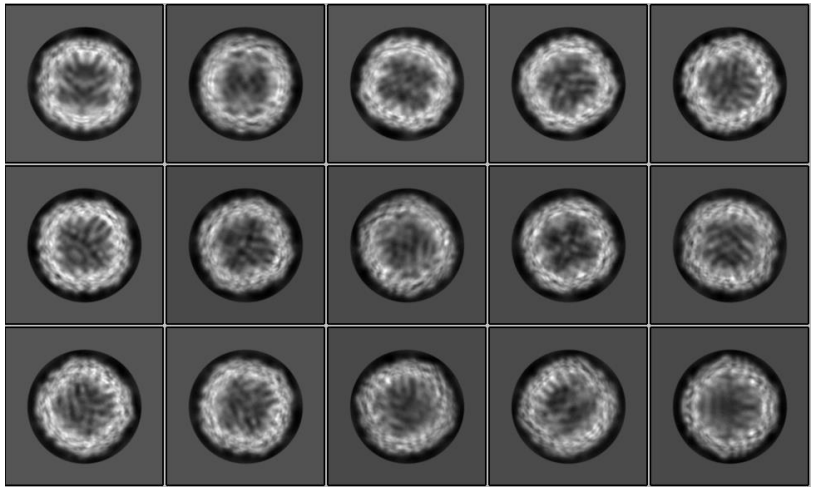
1.9 Å Reconstruction

Ctf Refinement + Particle Polishing

1.62 Å Reconstruction

Final 3D Refinement + Postprocessing



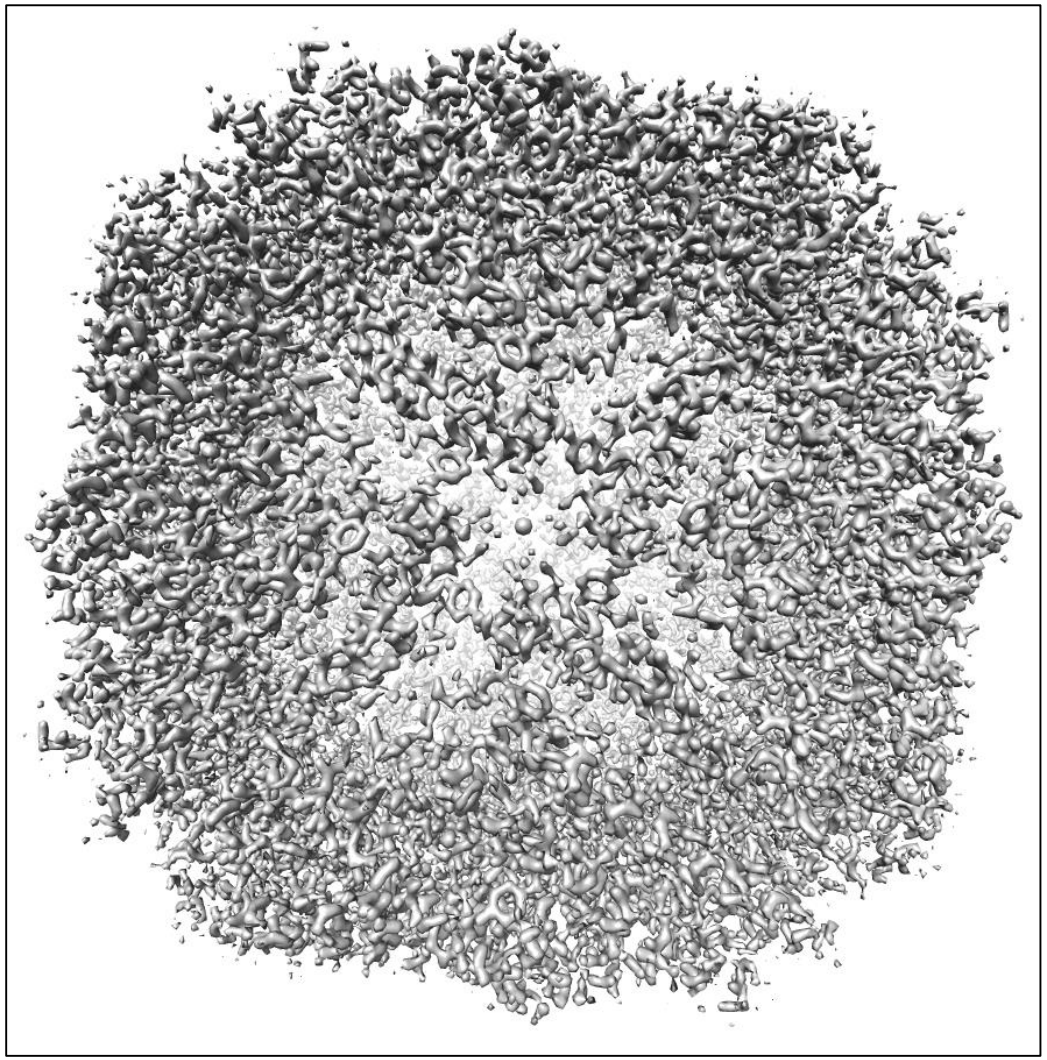


MUNI

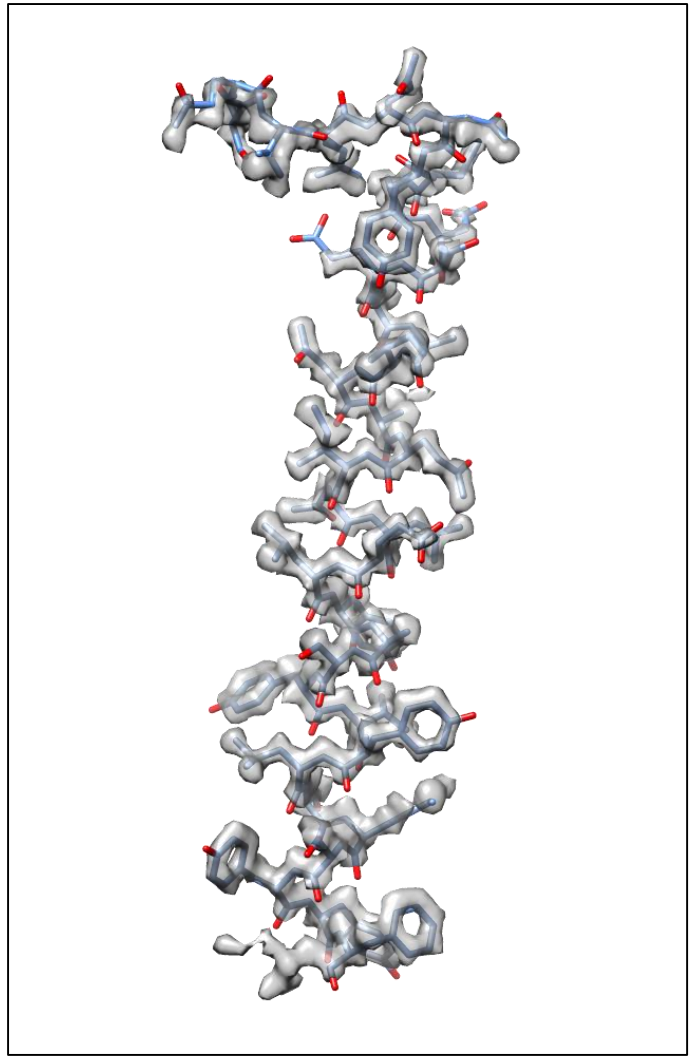
ThermoFisher SCIENTIFIC

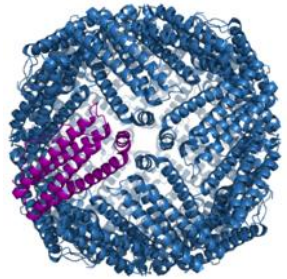
Example: Apoferritin reconstruction at 1.6 Å resolution.

3D reconstruction of Apoferritin



Atomic Structure Docking



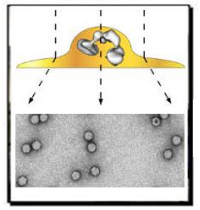


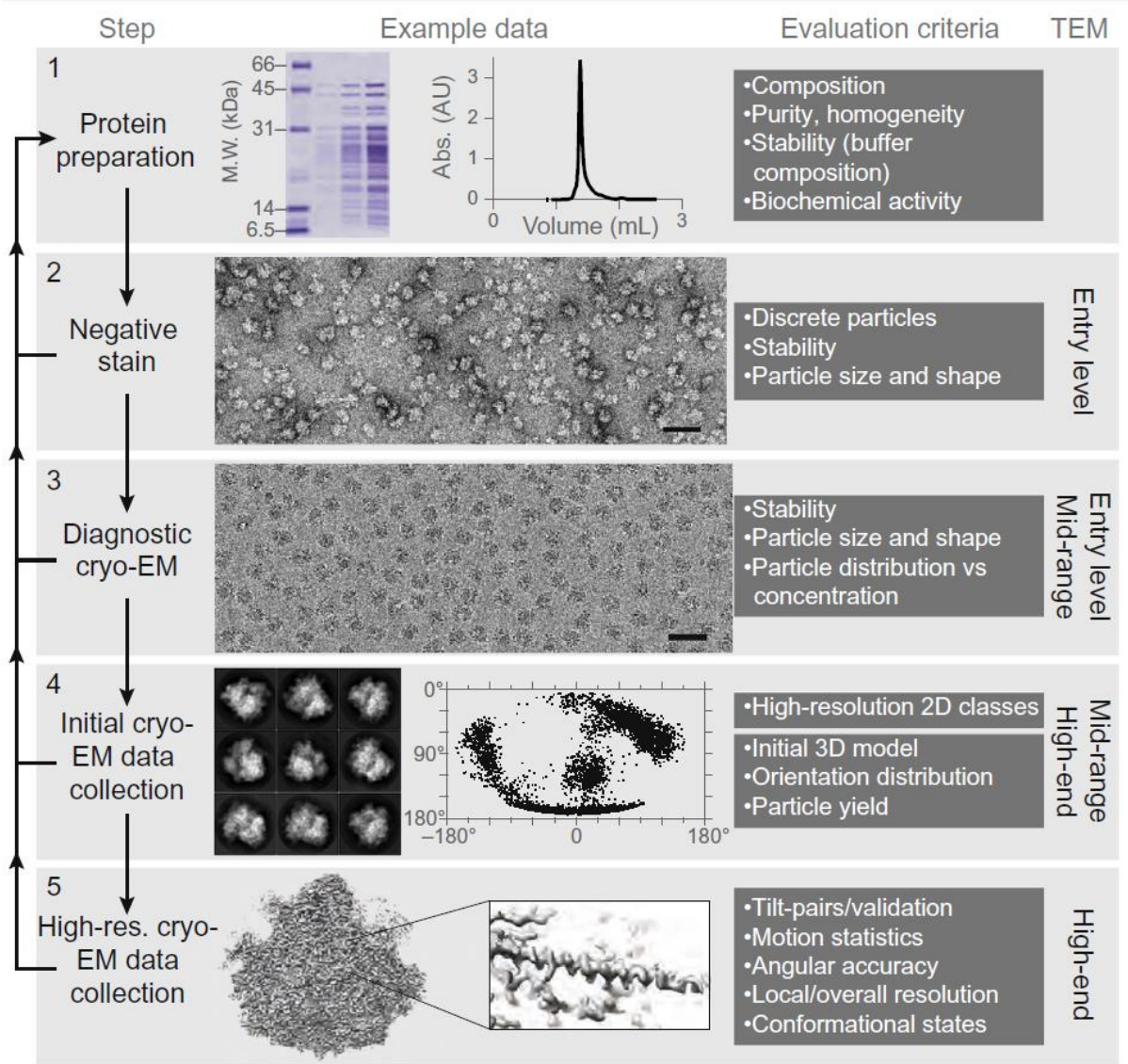
Ferritin

MUNI

ThermoFisher SCIENTIFIC

From Sample Screening to High Resolution Data Acquisition





Talos L120C

Glacios

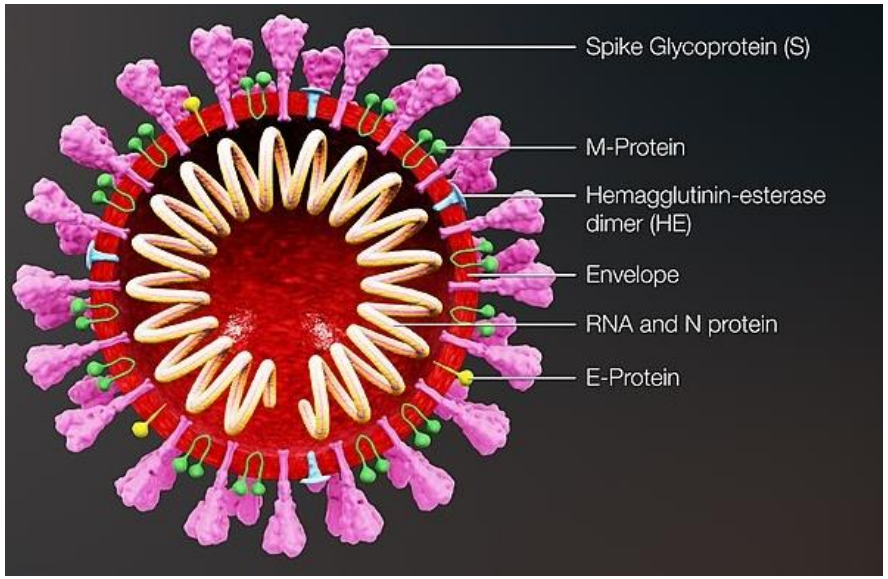
Titan Krios

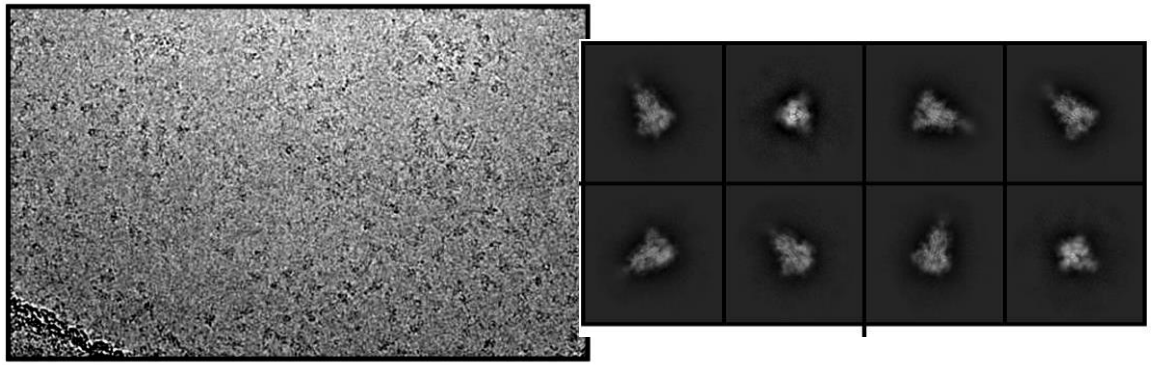
MUNI

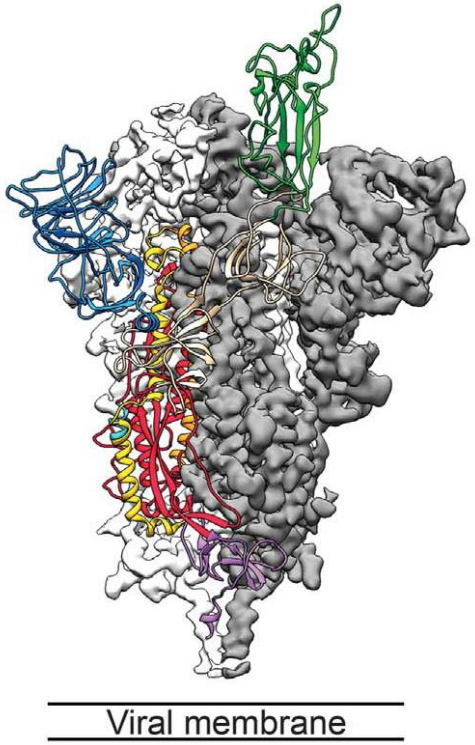
Image on the left adopted from Passmore & Russo, Methods Enzymol, 579 (2016).

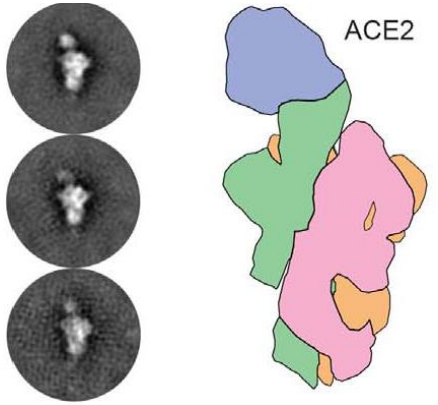
ThermoFisher SCIENTIFIC

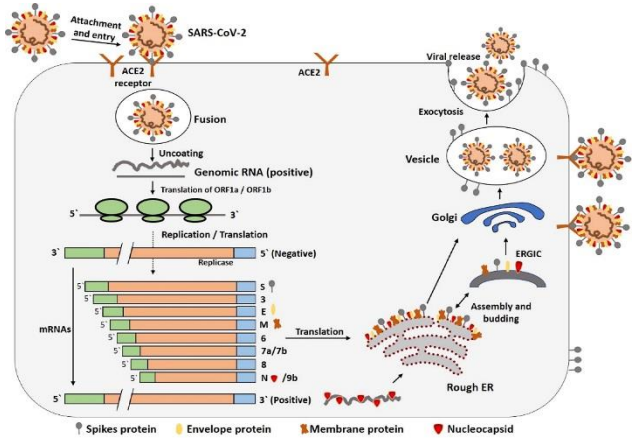
Example: Cryo-EM structure of the 2019-nCoV spike (S) glycoprotein.









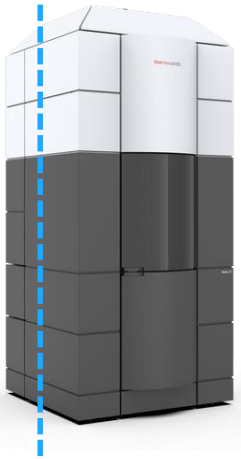


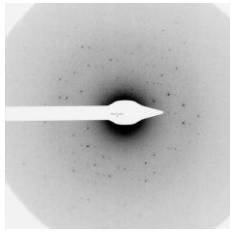
MUNI

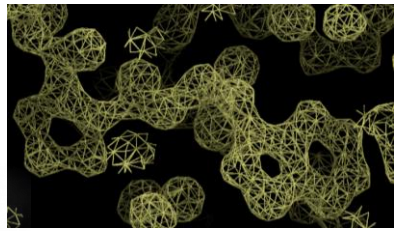
Adapted from: left - https://www.scientificanimations.com; right - Wrapp et al., Science 367 (2020) and Sheeren M.A. et al., Journal of Advanced Research 24 (2020). Remark. Build you own virus: ***CellPAINT-2D****.*

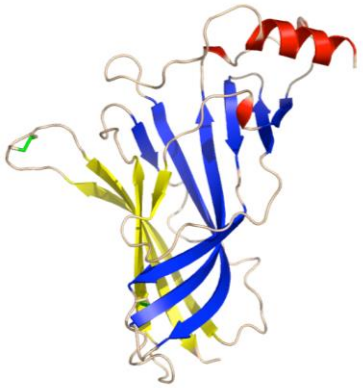
ThermoFisher SCIENTIFIC

MicroED: Crystal Protein Lamella Workflow



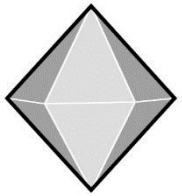




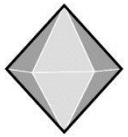


PRODUCTION + PURIFICATION → CRYSTALLIZATION → FREEZING → CRYO-FIB MILLING → DATA COLLECTION → DATA PROCESSING → REFINEMENT & VISUALIZATION

FEW DAYS | 1-2 DAYS | ½ DAY | FEW HOURS | MINUTES | MINUTES TO HOURS | FEW DAYS



X-ray crystallography (XRC)
>50 µm



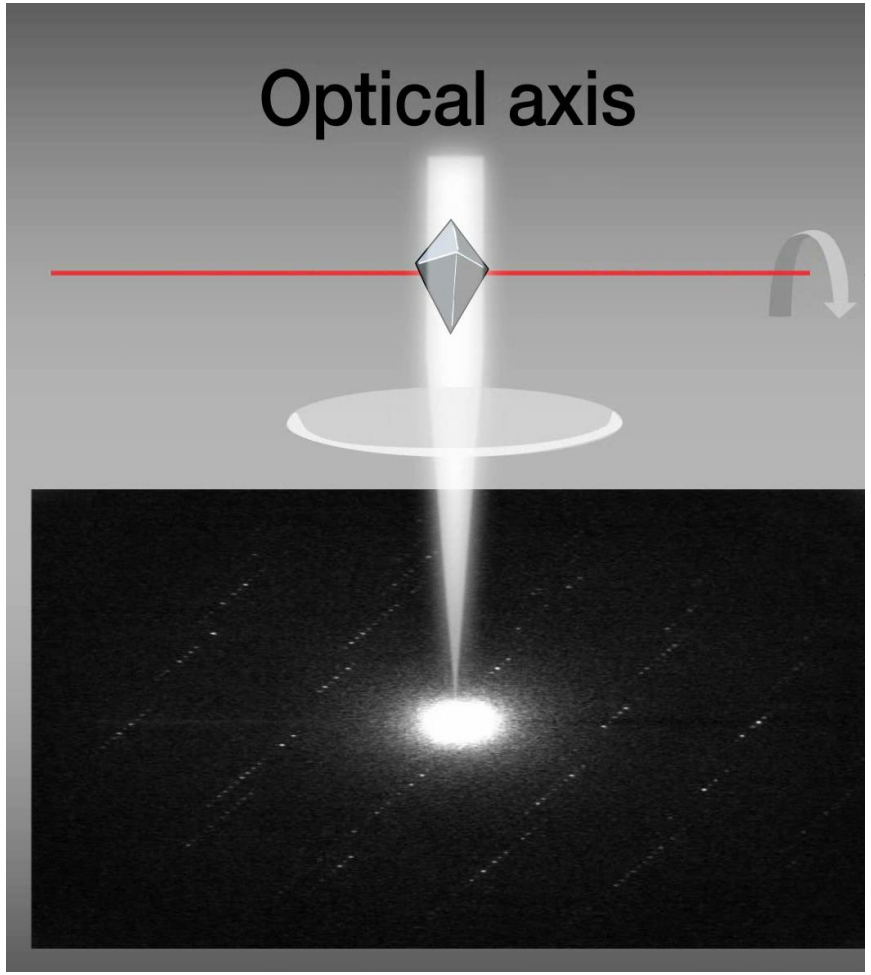
Too small for XRC
Too large for MED
0.5-50 µm

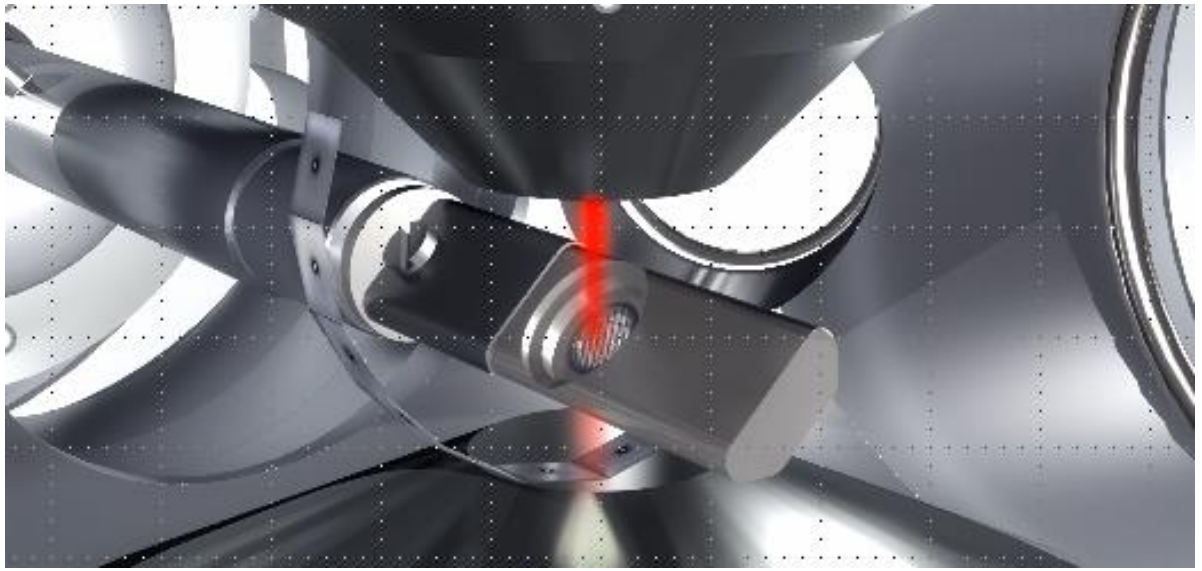
MicroED (MED)
<0.5 µm

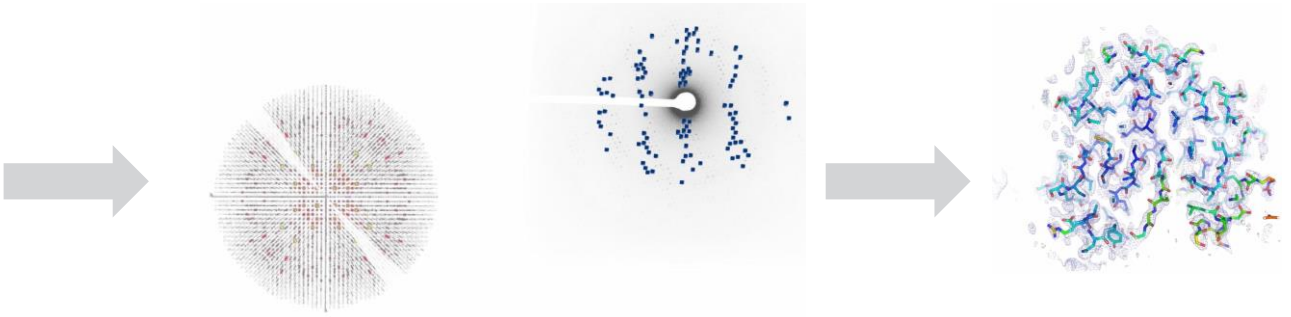
MUNI

ThermoFisher SCIENTIFIC

Principle of MicroED







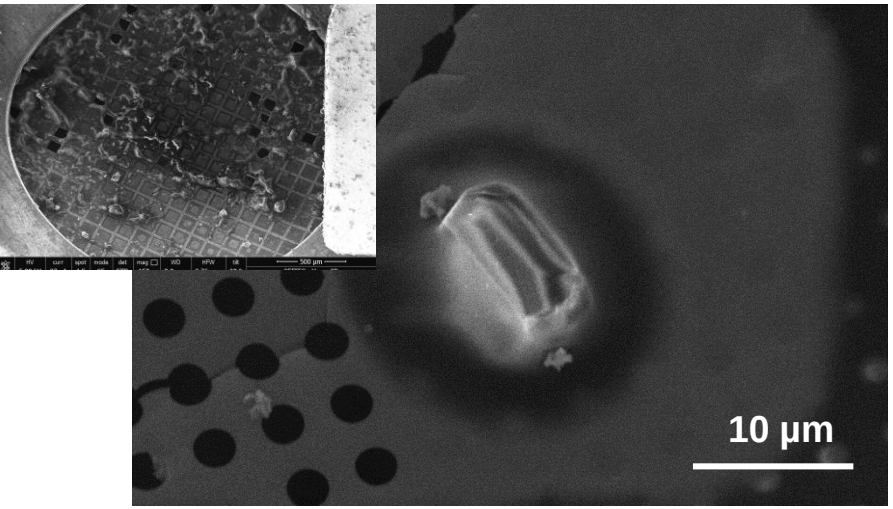
FT space

3D structure

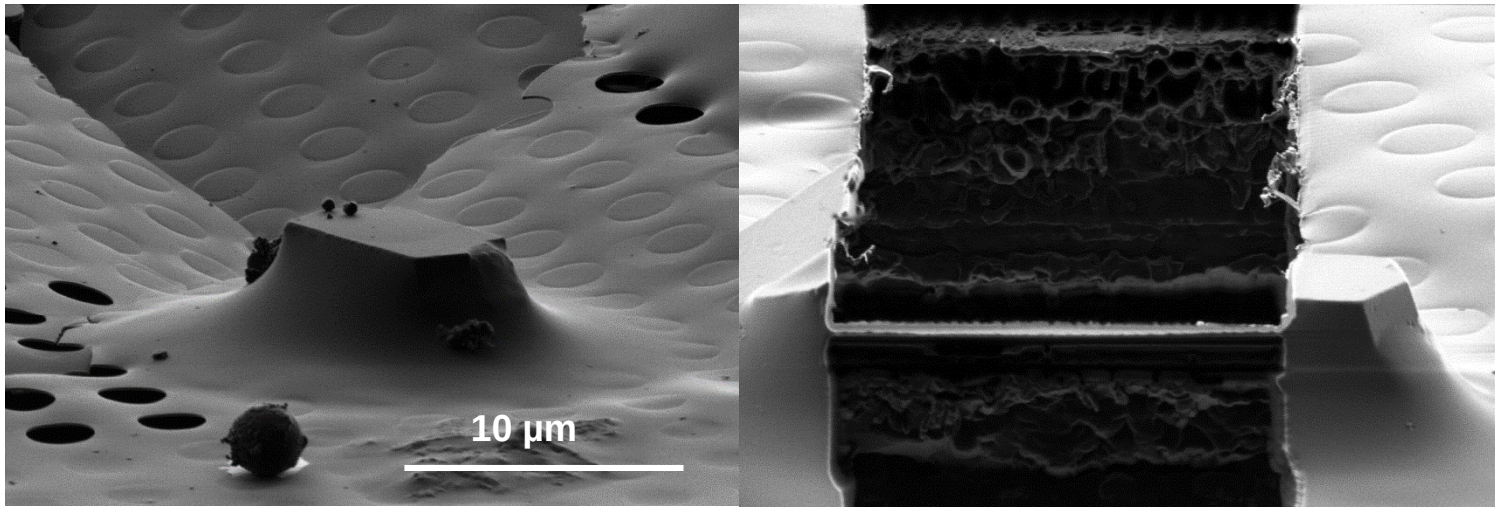
MUNI

ThermoFisher SCIENTIFIC

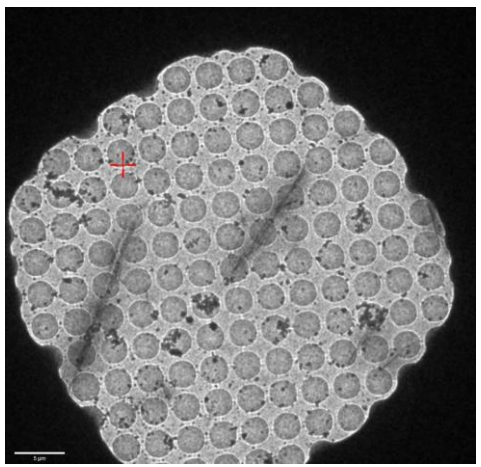
Micro-crystals of Lysozyme

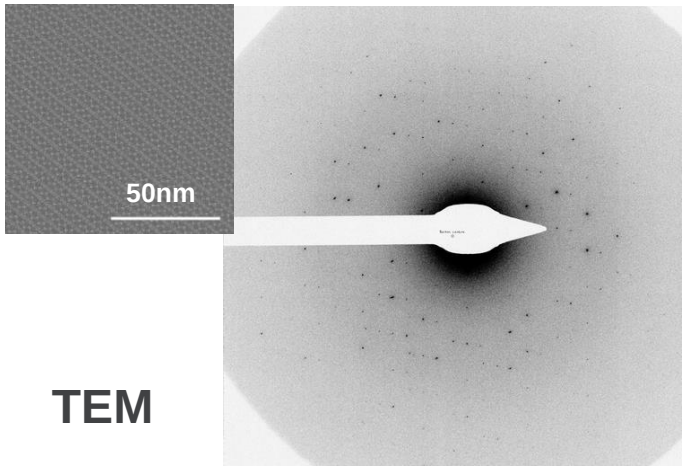


SDB: Electrons

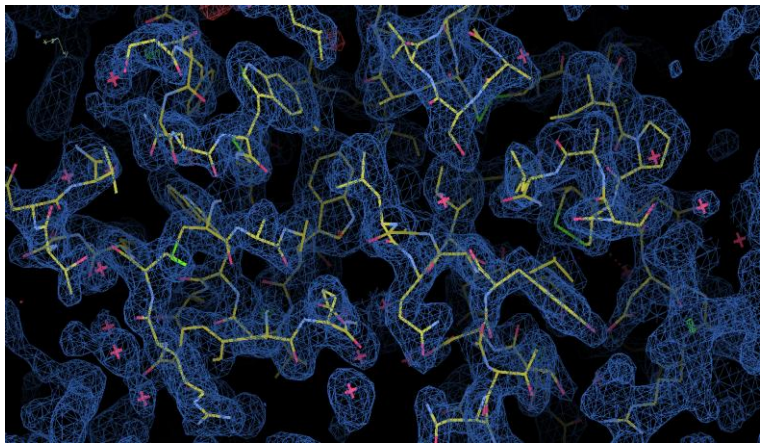


SDB: Ions





TEM

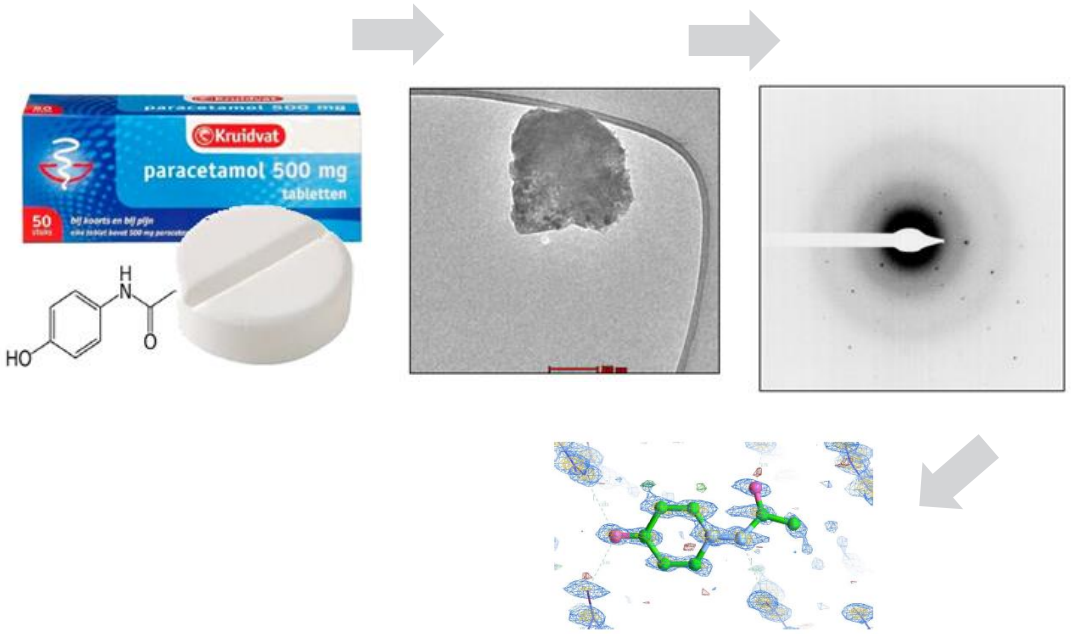


Electron Density Map

MUNI

ThermoFisher SCIENTIFIC

MicroED for Pharmaceutical Molecule Structure Workflow (RT)



- Drug (paracetamol, ibuprofen,…) is turned into to smooth powder.
- Powder is applied on carbon film on TEM grid and observed in TEM.
- No cryo temperature needed.

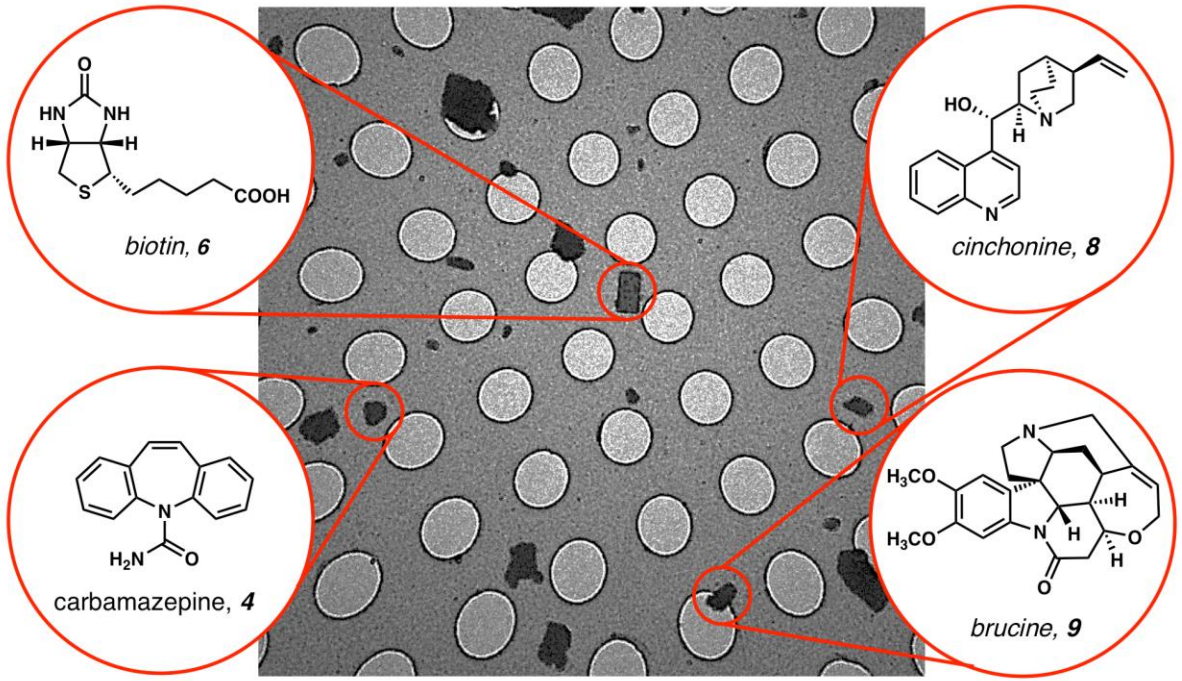


Fig. 3. Identification of compounds from heterogeneous mixtures. EM grid prepared as above with biotin, brucine, carbamazepine, and cinchonine powders mixed together. All four compounds identified by unit cell parameters using MicroED data from within the same grid square. All structures were solved to ~1Å resolution. Grid holes are 2μm in diameter.

Product quality control.

MUNI

Image on the right: Jones C.G. et al. ACS Cent Sci. 4(11) 2018.

ThermoFisher SCIENTIFIC