Basics of CryoET

From cells to tomograms

07 June 2024

Conny Leistner

Why cryoET?

Size of macromolecule of interest

Small | Big

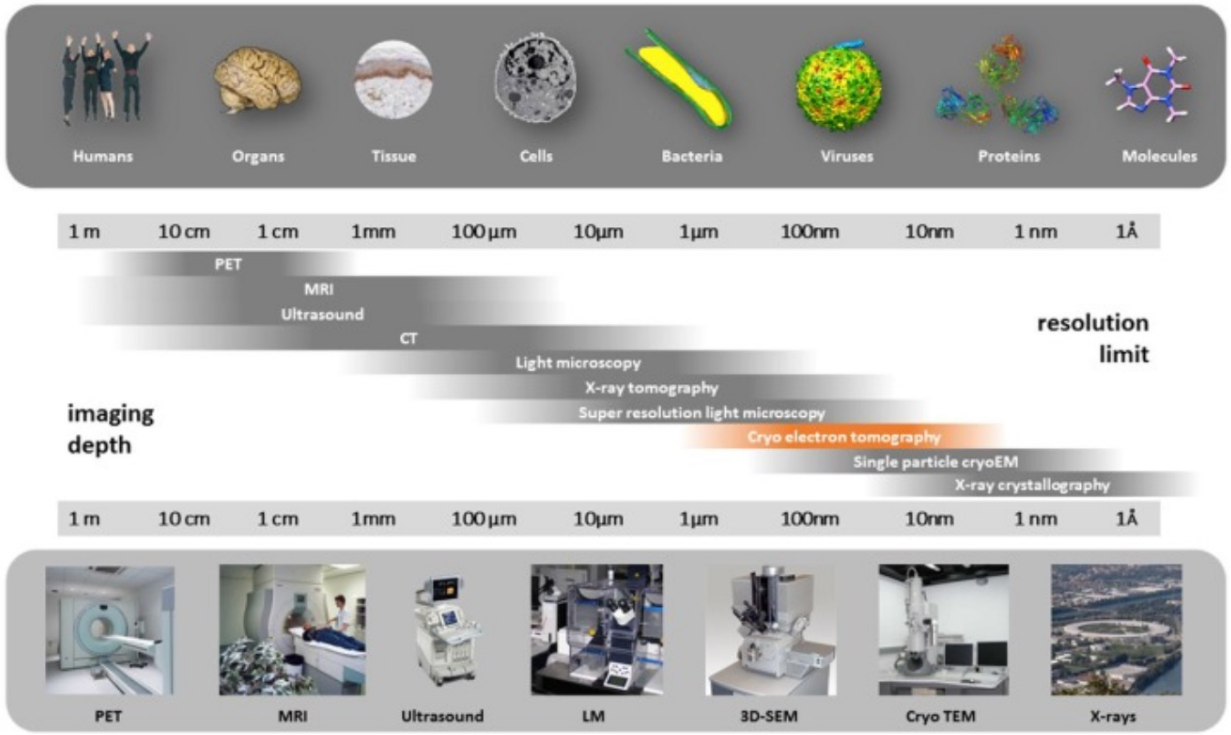
Abundance in cells

Low | High

Can you purify your macromolecule of interest?

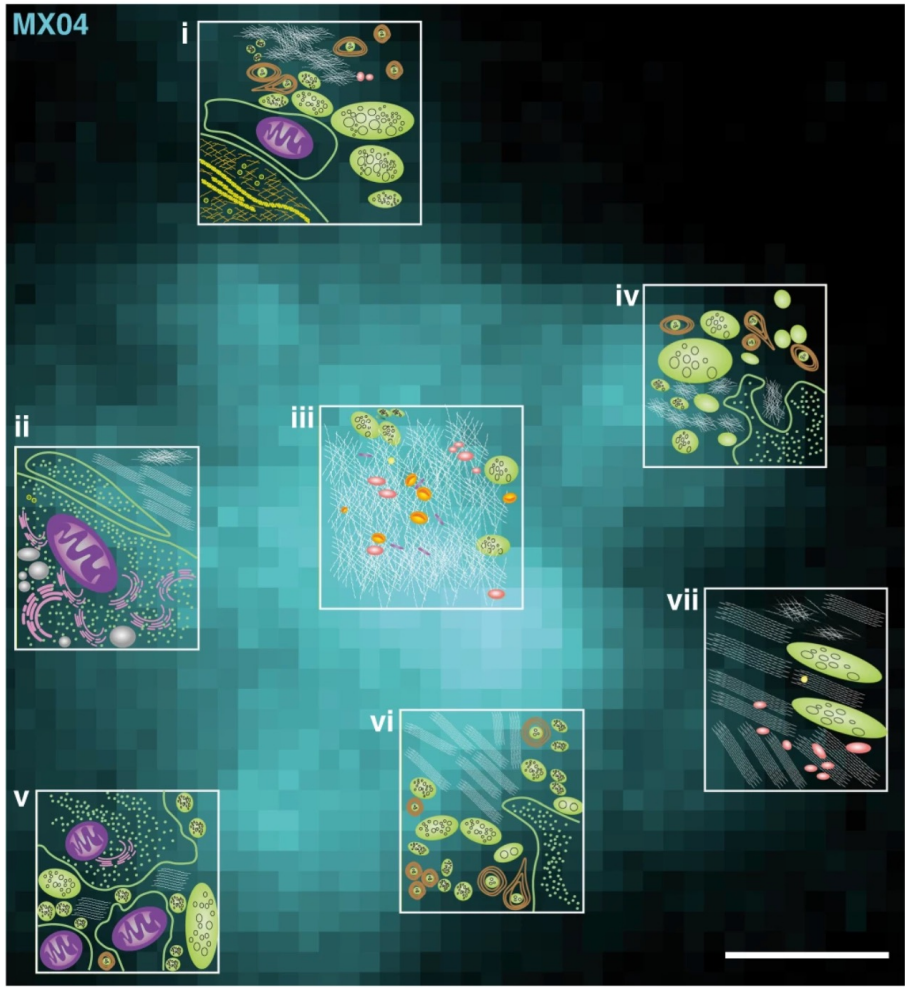
Yes → SPA

No → cryoET

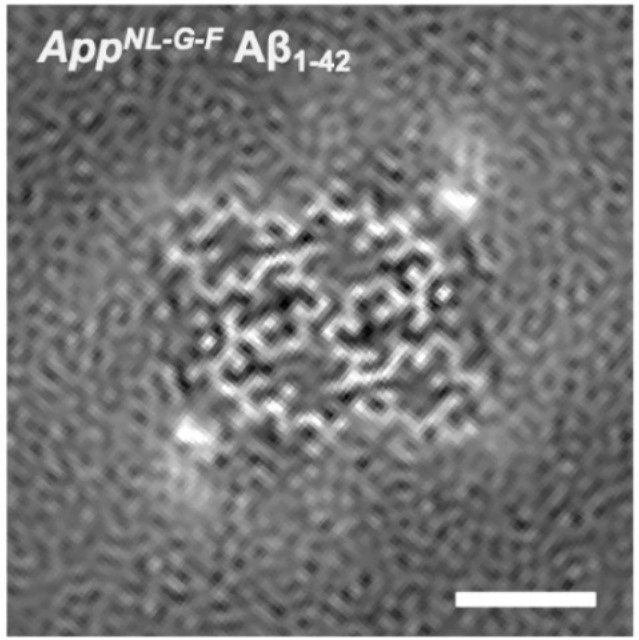


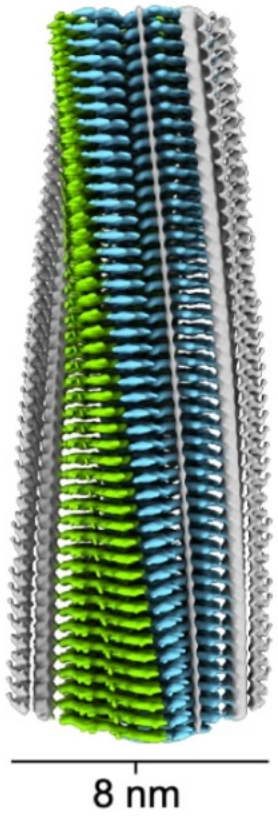
Combining SPA and cryoET

Architecture of Amyloid beta plaque from mouse brain tissue



SPA of purified amyloid beta fibrils

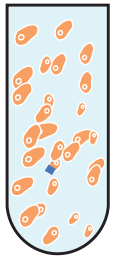




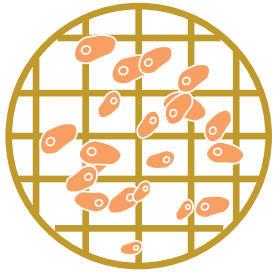
Leistner, C., Wilkinson, M., Burgess, A. et al. The in-tissue molecular architecture of β-amyloid pathology in the mammalian brain. Nat Commun 14, 2833 (2023). https://doi.org/10.1038/s41467-023-38495-5

Overview CryoET Workflow

Cells in suspension



Cells grown on grids



Plunge freezing

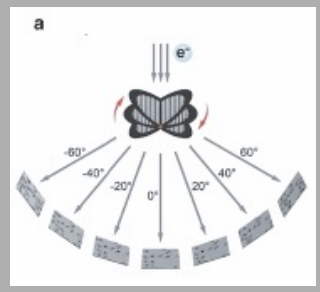
https://ppms.us/harvard-hms/req/?pf=8&training=true&form=27

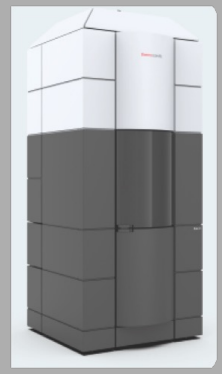
Screening and FIB milling:
Aquilos2

Lamella milling
Waffle method
Cryo-liftout
Volumetric SEM

https://ppms.us/harvard-hms/req/?pf=8&training=true&form=115

cryoET:
Titan Krios

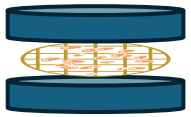




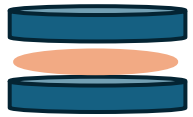
https://ppms.us/harvard-hms/req/?pf=8&training=true&form=123

High pressure freezing

cells on grids

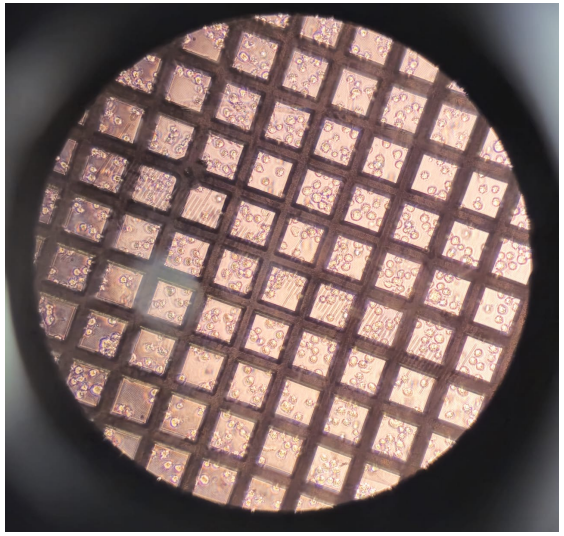


tissue biopsy



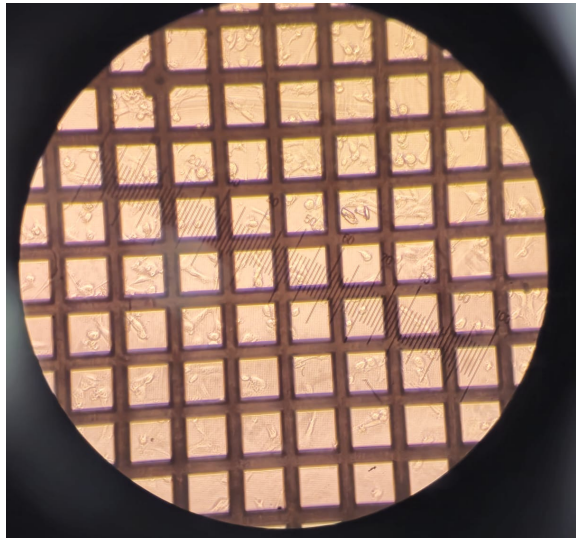
Growing cells on grids

- Find a protocol for your cell type
- 200 mesh Gold grids (copper is toxic for cells)
- Glow discharge grid
- Sterilise grids (70% Ethanol)
- Wash well with water and media
- Coat grids with poly-D-lysine or fibronectin
- Plate cells (avoid trypsin) 4h – over night



Cells on grid

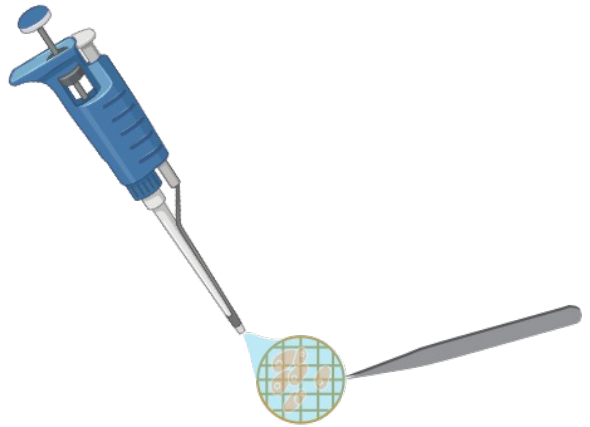
Over night



Plunge freezing

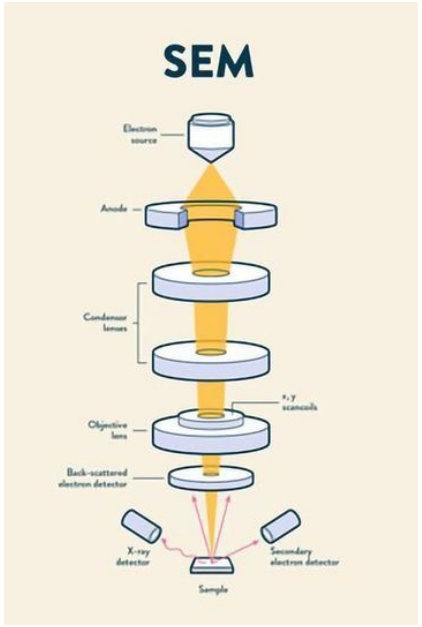
- 200 mesh grids
- Back blottingwith parafilm
- How thick are your cells/organism?

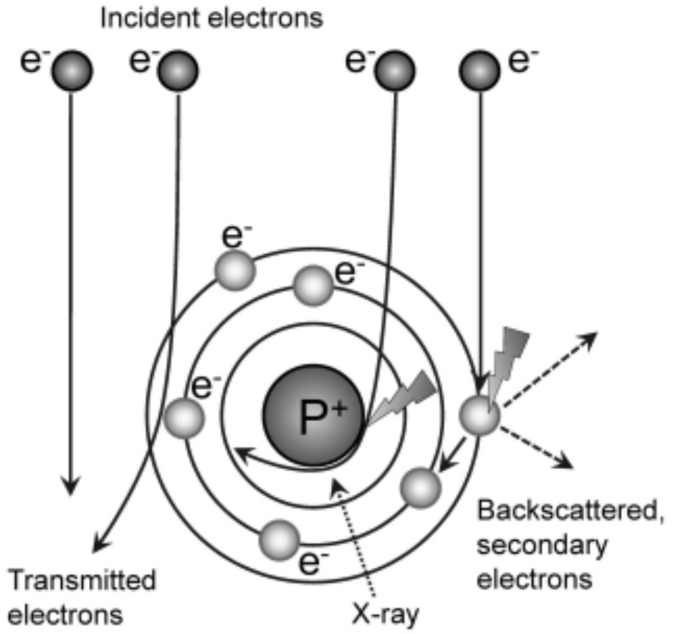
→Cryoprotectant/HPF?



SEM

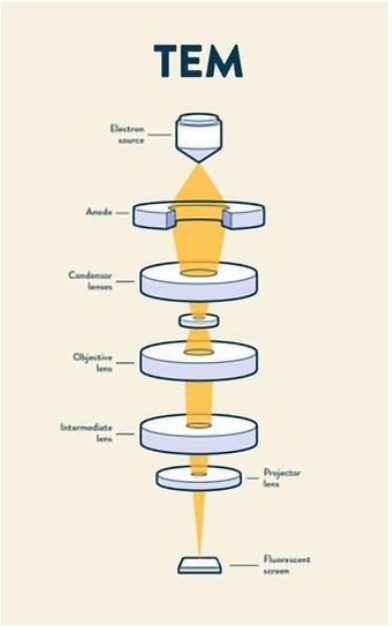
- Scanning SEM = images surface of samples
- Images back scattered and secondary electrons
- Faster





TEM

- Transmission EM = electrons are transmitted through the sample
- Higher resolution



Aquilos2: lamella milling

Tile scan

↓

Optional: fluorescence

↓

Coating the grid

↓

Lamella milling

- SEM
- FIB
- iFLM

Workflow for Aquilos2: lamella milling

Tile scan:SEM

↓

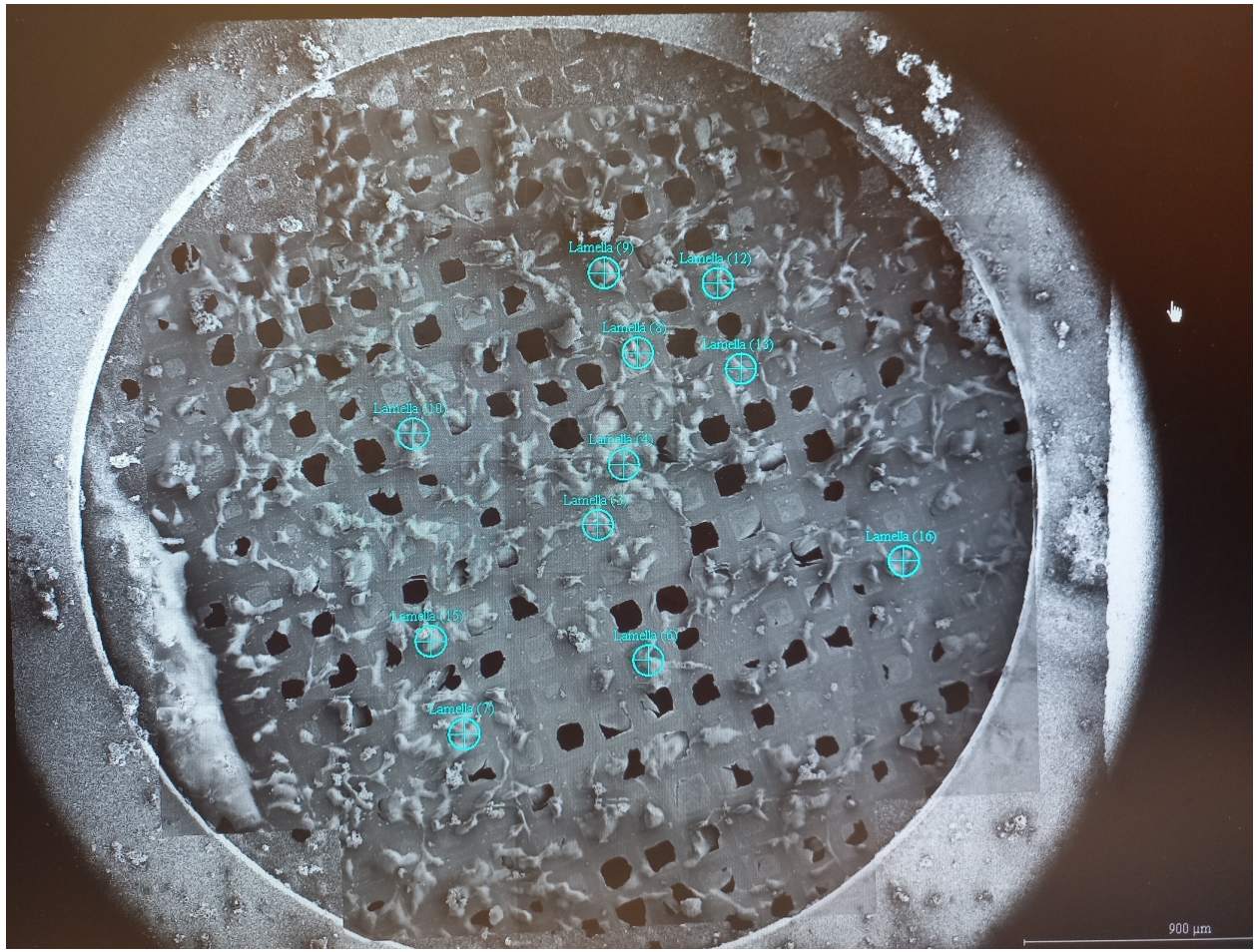
Optional: fluorescence

↓

Coating the grid

↓

Lamella milling



Workflow for Aquilos2: lamella milling

Tile scan

↓

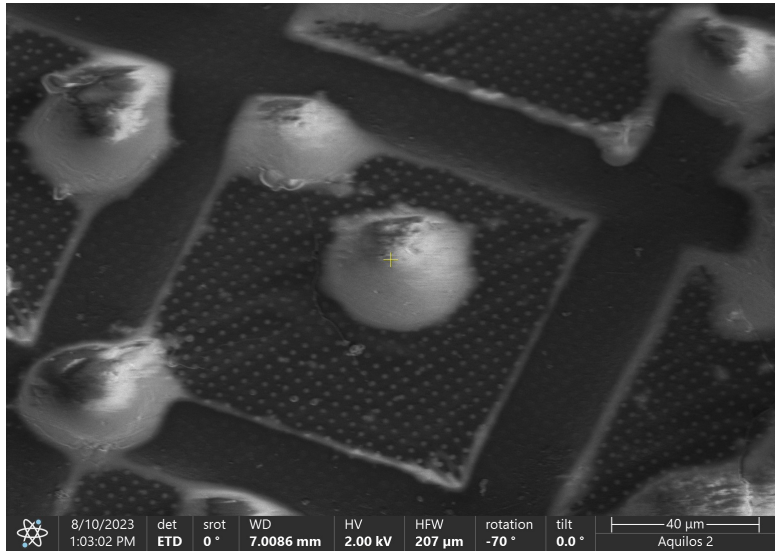
Optional: fluorescence

↓

Coating the grid

↓

Lamella milling



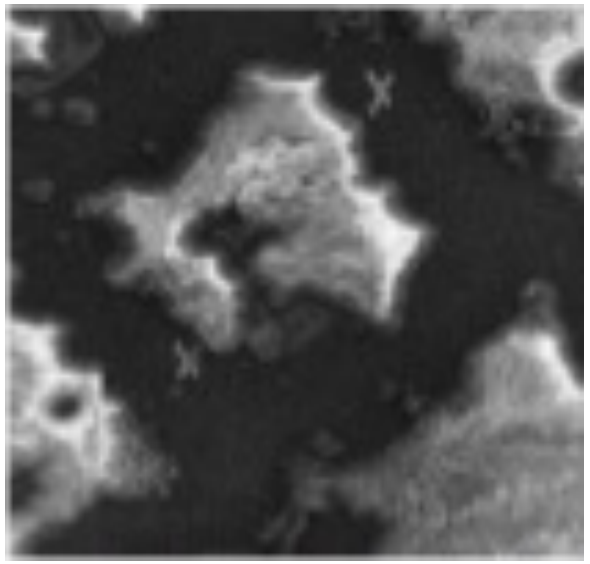
Aquilos2: lamella milling

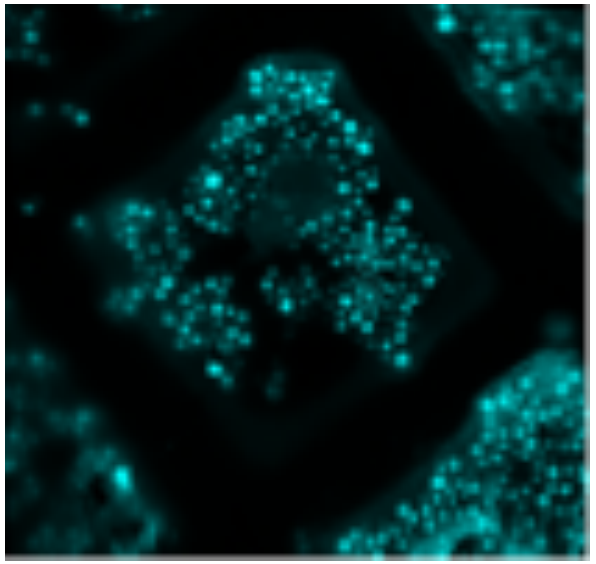
Tile scan

Optional: fluorescence

Coating the grid

Lamella milling: patterns





Aquilos2: lamella milling

Tile scan

↓

Optional: fluorescence

↓

Coating the grid

↓

Lamella milling: patterns

Sputter coating and GIS (gas injection system) deposition

- Protective platinum coating of sample
- Reduced charging
- Better conductivity

Aquilos2: lamella milling

Tile scan

↓

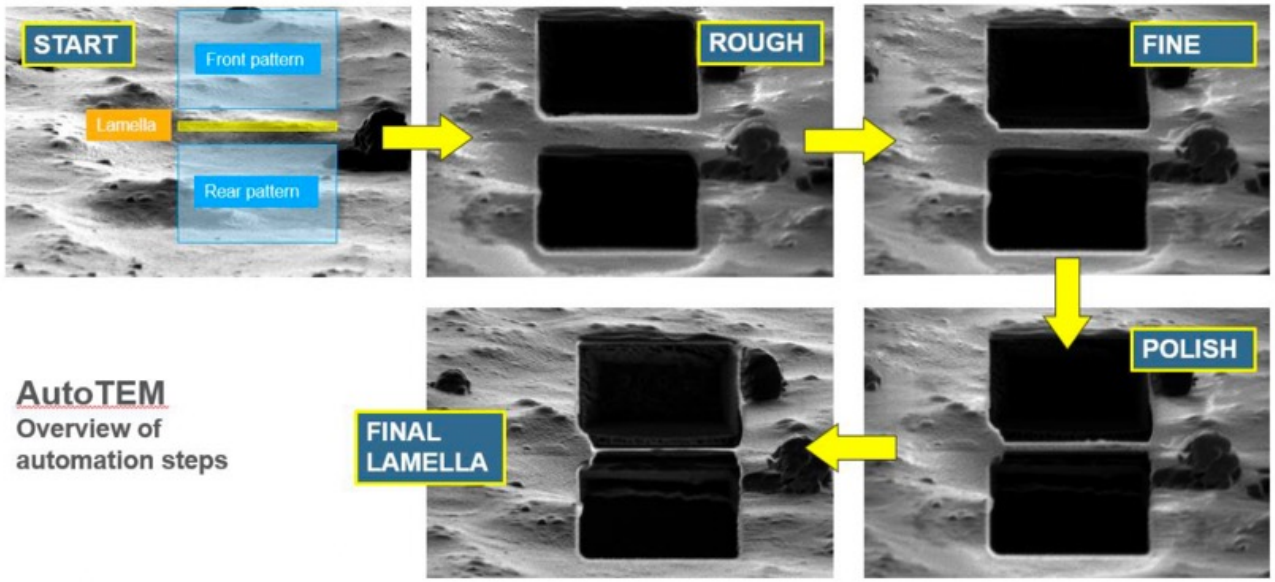
Optional: fluorescence

↓

Coating the grid

↓

Lamella milling



Aquilos2: lamella milling

Tile scan

↓

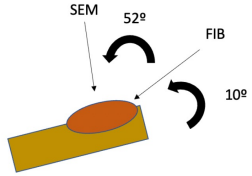
Optional: fluorescence

↓

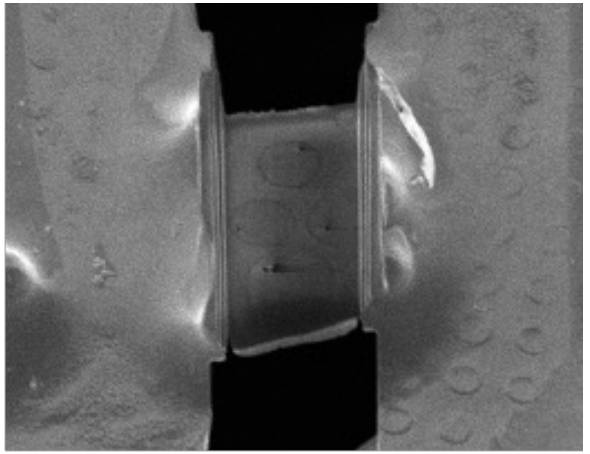
Coating the grid

↓

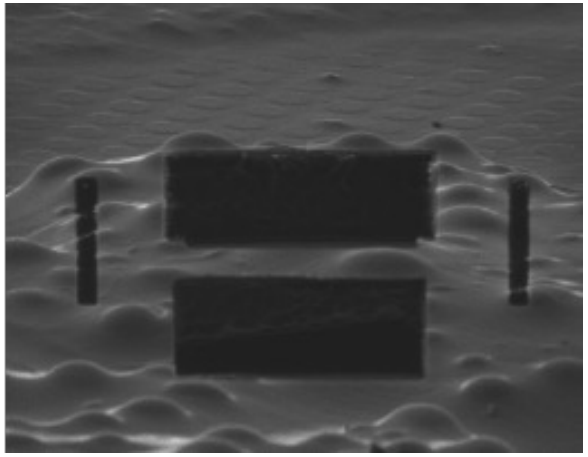
Lamella milling



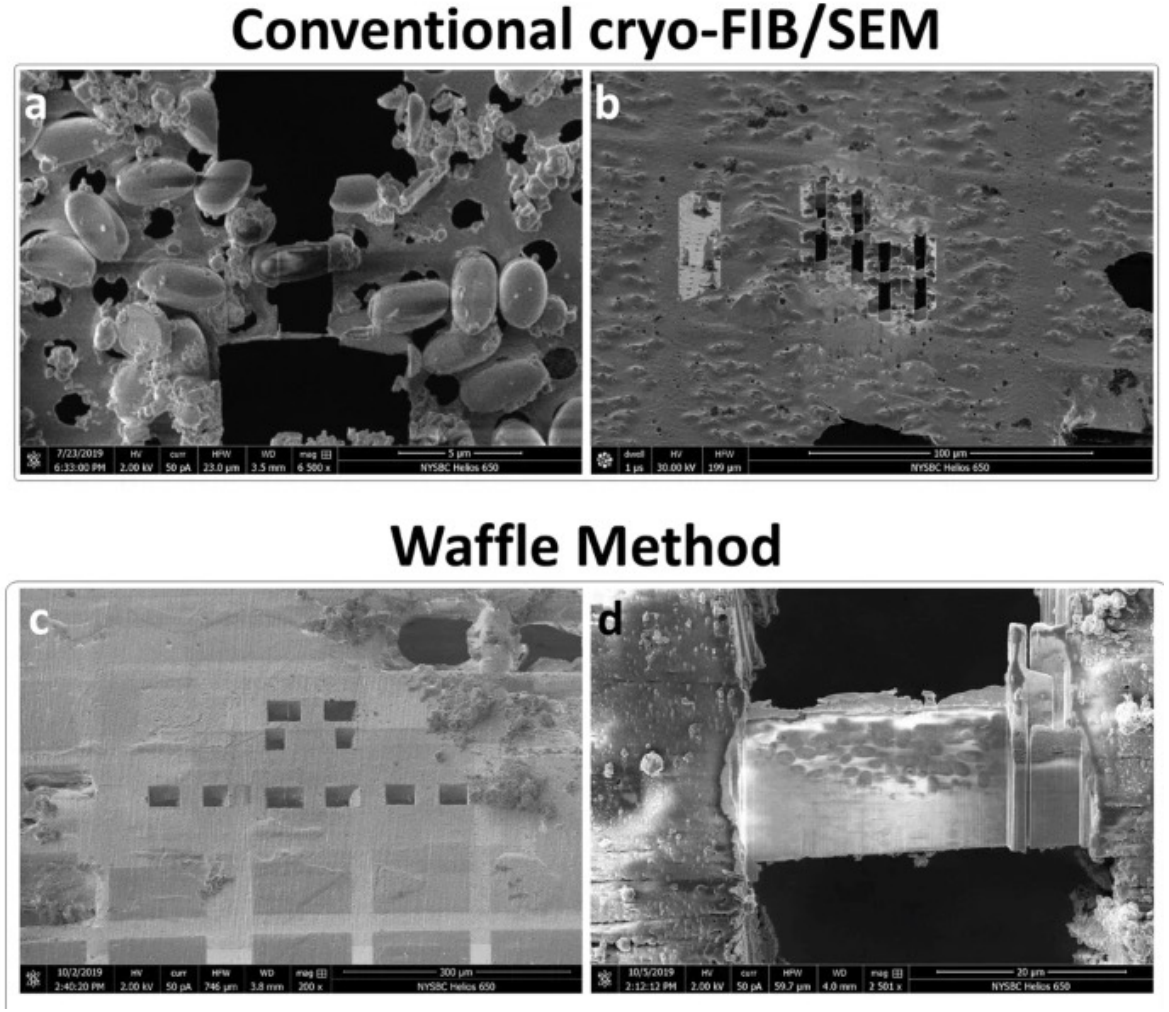
SEM



Ion Beam



Waffle Method

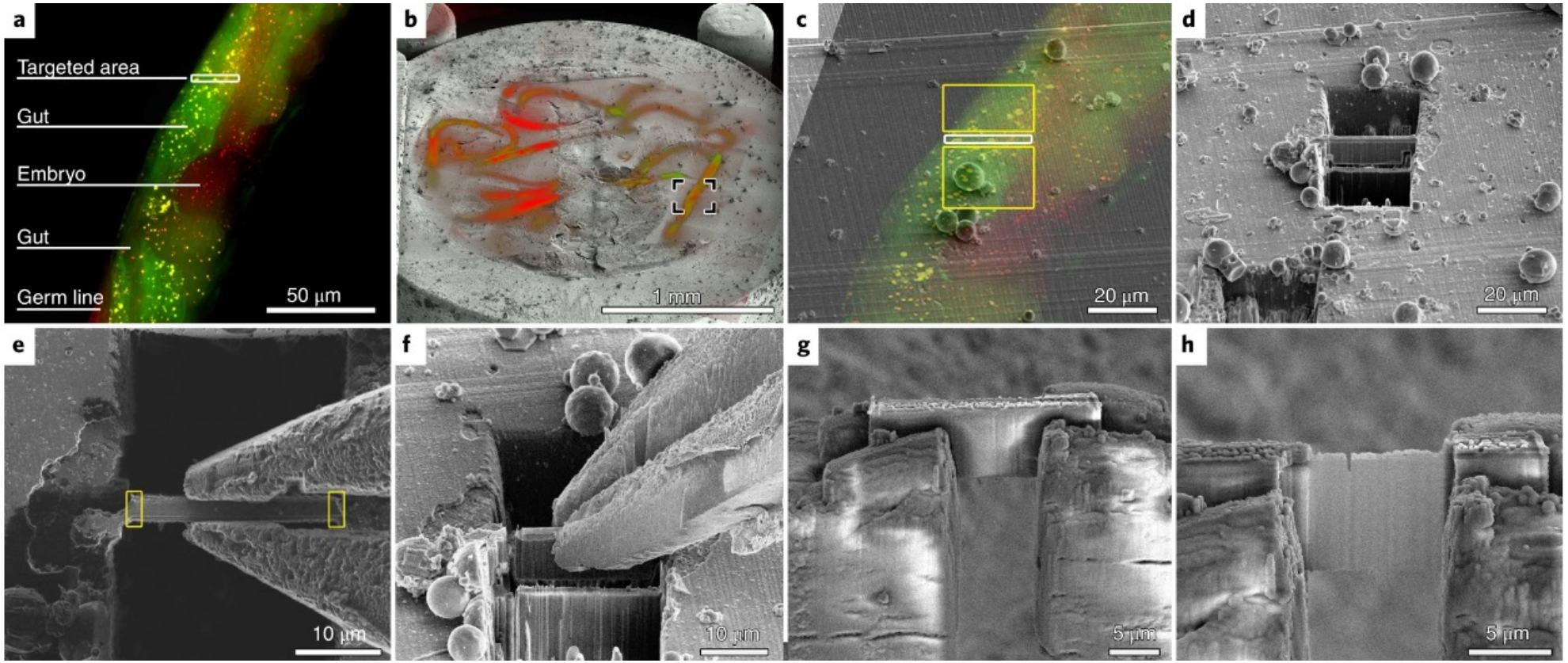


- High pressure freezing of samples into grids
- Vitrification of thick samples
- Cell-to-cell interaction
- High throughput for low concentrations
  → more sample on grid
- Not restricted to orientation of cells on the grid
- Similar to cryoliftout, higher throughput

Kelley, K., Raczkowski, A.M., Klykov, O. et al. Waffle Method: A general and flexible approach for improving throughput in FIB-milling. Nat Commun 13, 1857 (2022). https://doi.org/10.1038/s41467-022-29501-3

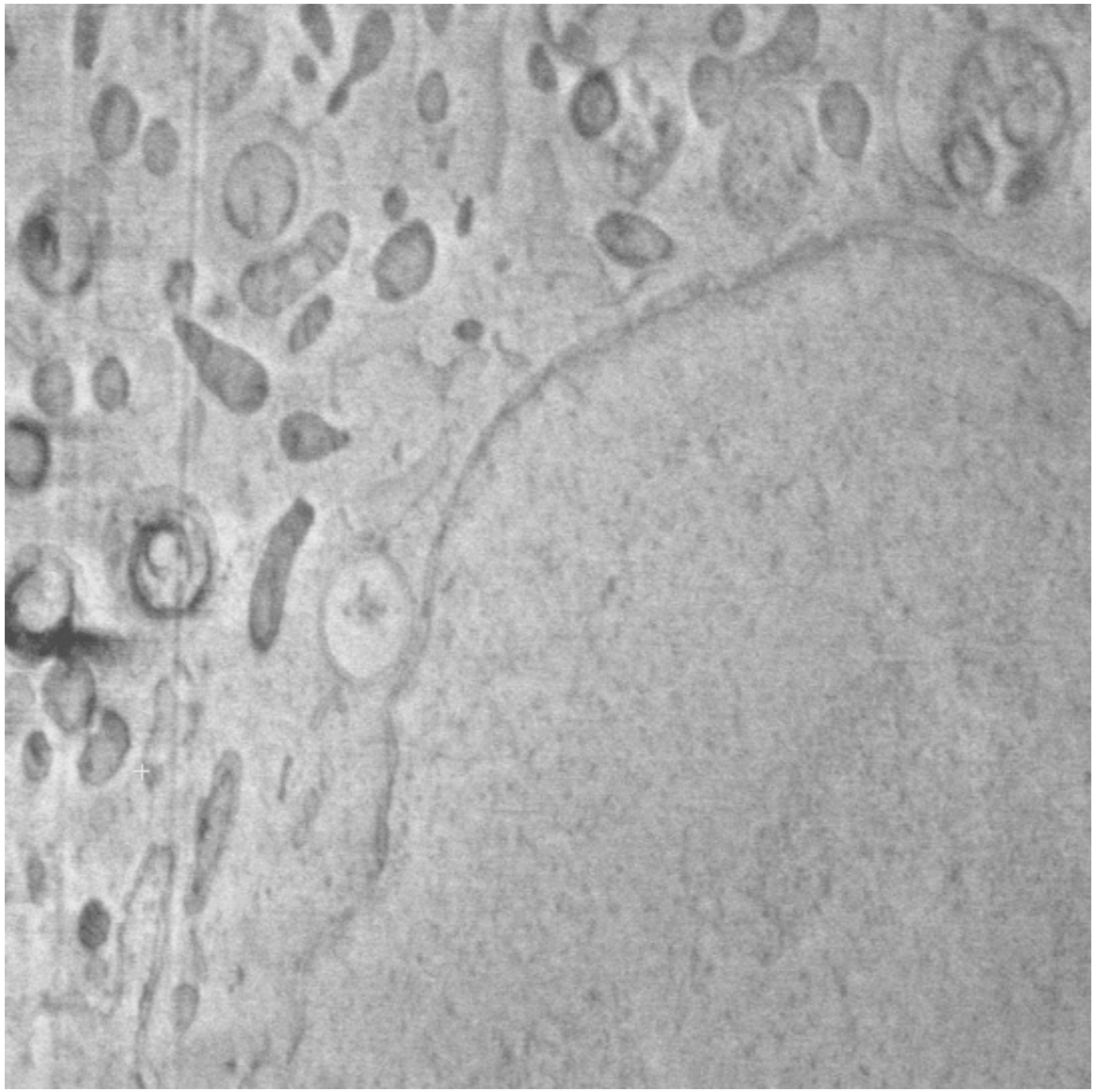
Cryoliftout

Cryoliftout of C.elegans



Schaffer, M., Pfeffer, S., Mahamid, J. et al. A cryo-FIB lift-out technique enables molecular-resolution cryo-ET within native Caenorhabditis elegans tissue. Nat Methods 16, 757–762 (2019). https://doi-org.ezp-prod1.hul.harvard.edu/10.1038/s41592-019-0497-5

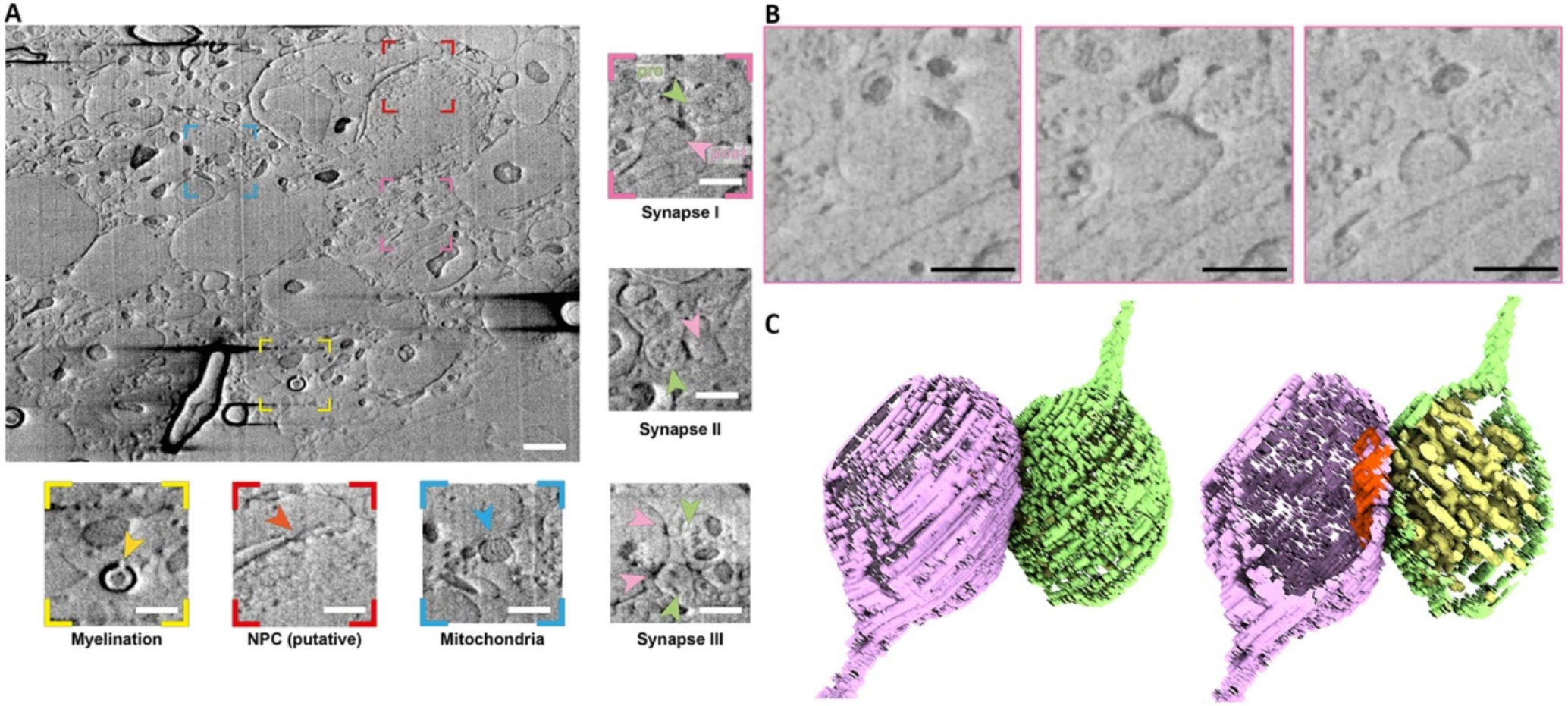
Volumetric SEM



Marilina deSautu, Conny Leistner

- 3D Volume of cells or tissue from different angles
- FIB milling combined with SEM imaging to reconstruct 3D volume
- It is possible to target cells and mill lamella

Volumetric SEM

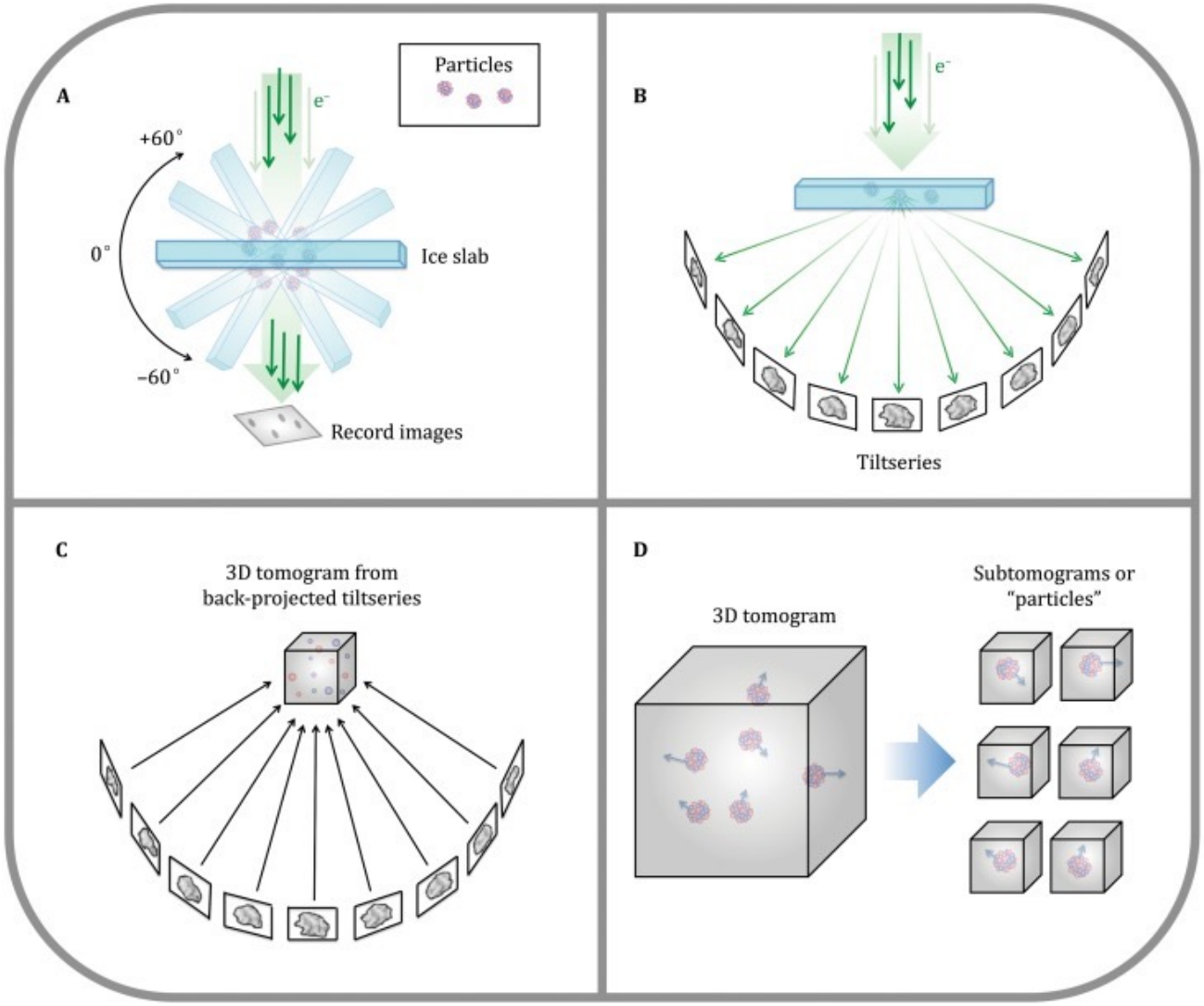


Maud Dumoux, Thomas Glen, Jake LR Smith, Elaine ML Ho, Luis MA Perdigão, Avery Pennington, Sven Klumpe, Neville BY Yee, David Andrew Farmer, Pui YA Lai, William Bowles, Ron Kelley, Jürgen M Plitzko, Liang Wu, Mark Basham, Daniel K Clare, C Alistair Siebert, Michele C Darrow, James H Naismith, Michael Grange (2023) Cryo-plasma FIB/SEM volume imaging of biological specimens eLife 12:e83623 https://doi.org/10.7554/eLife.83623

Cryo-ET: data collection

Cryo-ET

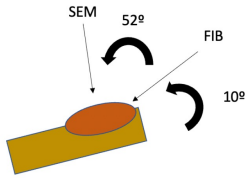
- 3D structures of biological molecules can be obtained in large, irregular macromolecular assemblies or *in situ,* without the need for purification.
- biological specimen is tilted to record 2D images
- Slower Data collection compared to SPA
  - ~50-100 tomograms per day vs. 10,000 SPA images per night on Krios



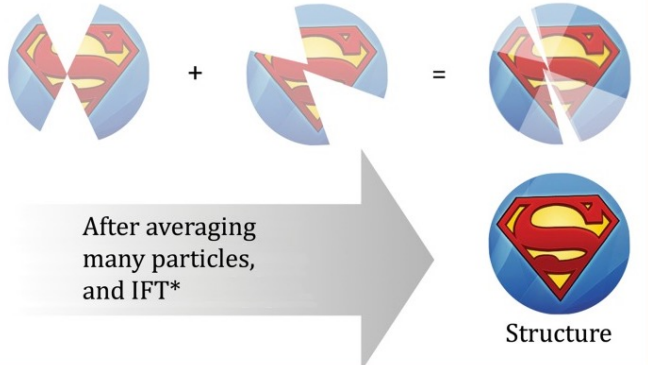
DOI: 10.1016/j.tibs.2021.10.006

Data collection

- Tomo5 or pace (serialEM)
- Min/Max tilt: +60º - -60º; +45º - -45º
- tilt increments: 2º, 3º
- Pretilt of lamella → start tilt angle

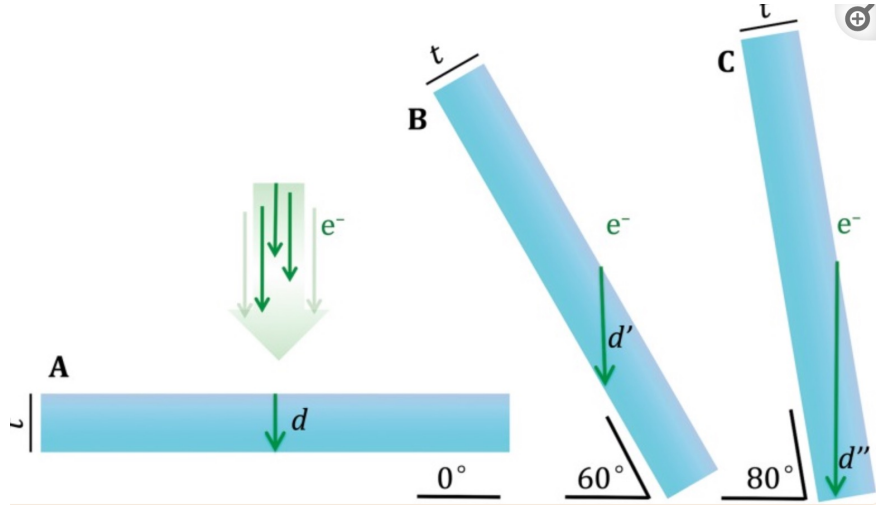


Missing wedge



- Total dose: depending on sample (100 – 150 e/A^2)
- Tilt scheme: dose-symmetric scheme* (best high resolution information at low tilts Ice thickness increases with higher tilt angle → lower contrast

- Processing: Imod, Aretomo, Relion5



Galaz-Montoya JG, Ludtke SJ. The advent of structural biology in situ by single particle cryo-electron tomography. Biophys Rep. 2017;3(1):17-35. doi: 10.1007/s41048-017-0040-0. Epub 2017 May 29. PMID: 28781998; PMCID: PMC5516000.

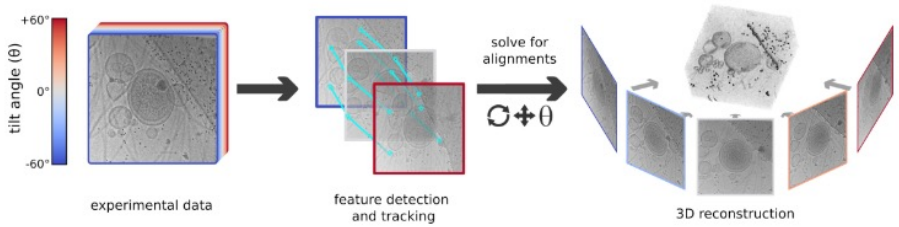
*Hagen WJH, Wan W, Briggs JAG. Implementation of a cryo-electron tomography tilt-scheme optimized for high resolution subtomogram averaging. J Struct Biol. 2017 Feb;197(2):191-198. doi: 10.1016/j.jsb.2016.06.007. Epub 2016 Jun 14. PMID: 27313000; PMCID: PMC5287356.

Data processing

- Motion correction: aligns 2D frames
- CTF correction: distortions of CTF (like lens aberrations and image defocus are estimated and corrected)

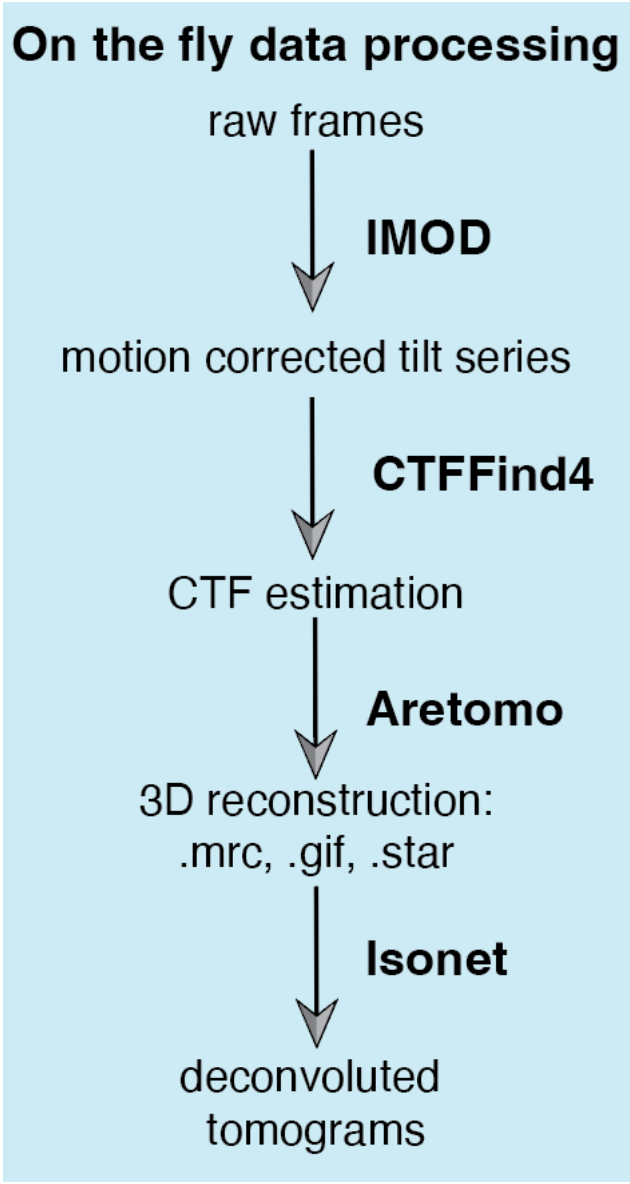
- Tilt Series Alignment (gold fiducials, local patch tracking, global alignment)
- Tomogram reconstruction

- Postprocessing: denoising filters, deconvolution, segmentation, subtomogram averaging



Data processing

- Motion correction: aligns 2D frames
- CTF correction: distortions of CTF (like lens aberrations and image defocus are estimated and corrected)
- Tilt Series Alignment (gold fiducials, local patch tracking, global alignment)
- Reconstruction: 2D images are back projected onto 3D volume
- Postprocessing: denoising filters, deconvolution, segmentation, subtomogram averaging



Alexis Rohou, Nikolaus Grigorieff, CTFFIND4: Fast and accurate defocus estimation from electron micrographs,
Journal of Structural Biology, Volume 192, Issue 2, 2015,Pages 216-221,ISSN 1047-8477, https://doi.org/10.1016/j.jsb.2015.08.008.
Mastronarde DN, Held SR. Automated tilt series alignment and tomographic reconstruction in IMOD. J Struct Biol. 2017 Feb;197(2):102-113. doi: 10.1016/j.jsb.2016.07.011. Epub 2016 Jul 19. PMID: 27444392; PMCID: PMC5247408.
Shawn Zheng, Georg Wolff, Garrett Greenan, Zhen Chen, Frank G.A. Faas, Montserrat Bárcena, Abraham J. Koster, Yifan Cheng, David A. Agard, AreTomo: An integrated software package for automated marker-free, motion-corrected cryo-electron tomographic alignment and reconstruction, Journal of Structural Biology: X, Volume 6,
2022,100068, ISSN 2590-1524, https://doi.org/10.1016/j.yjsbx.2022.100068.