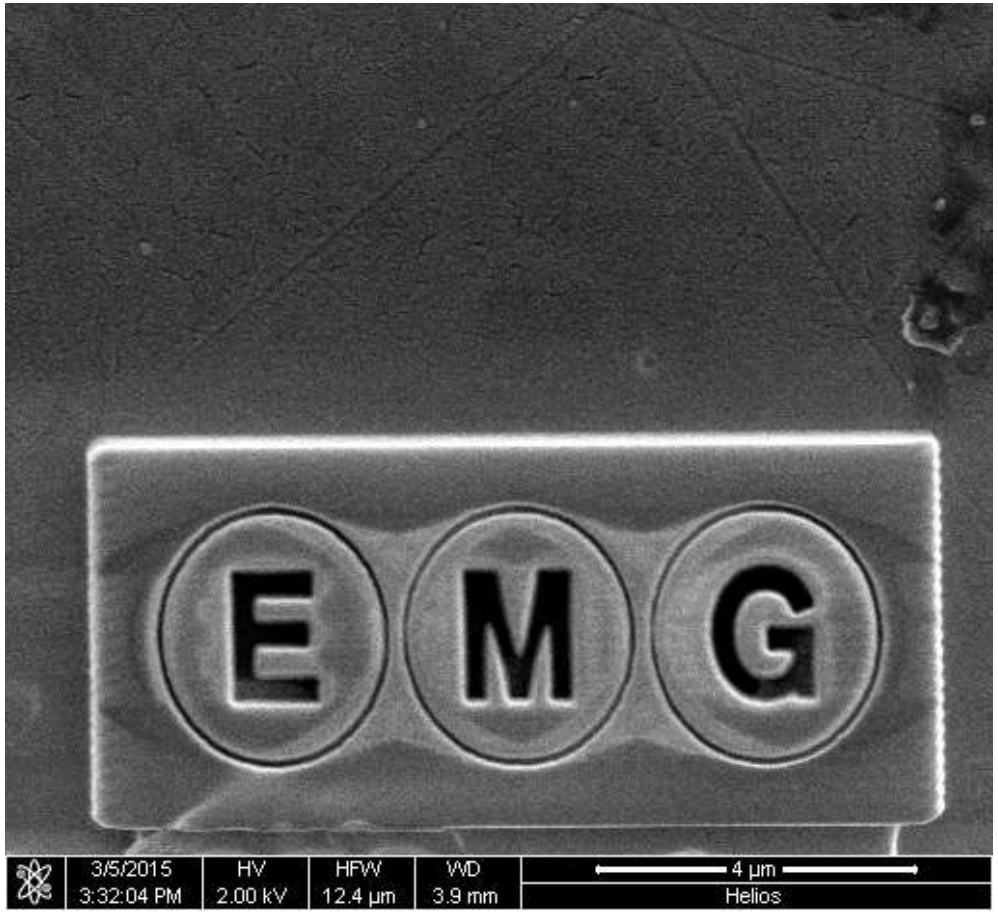
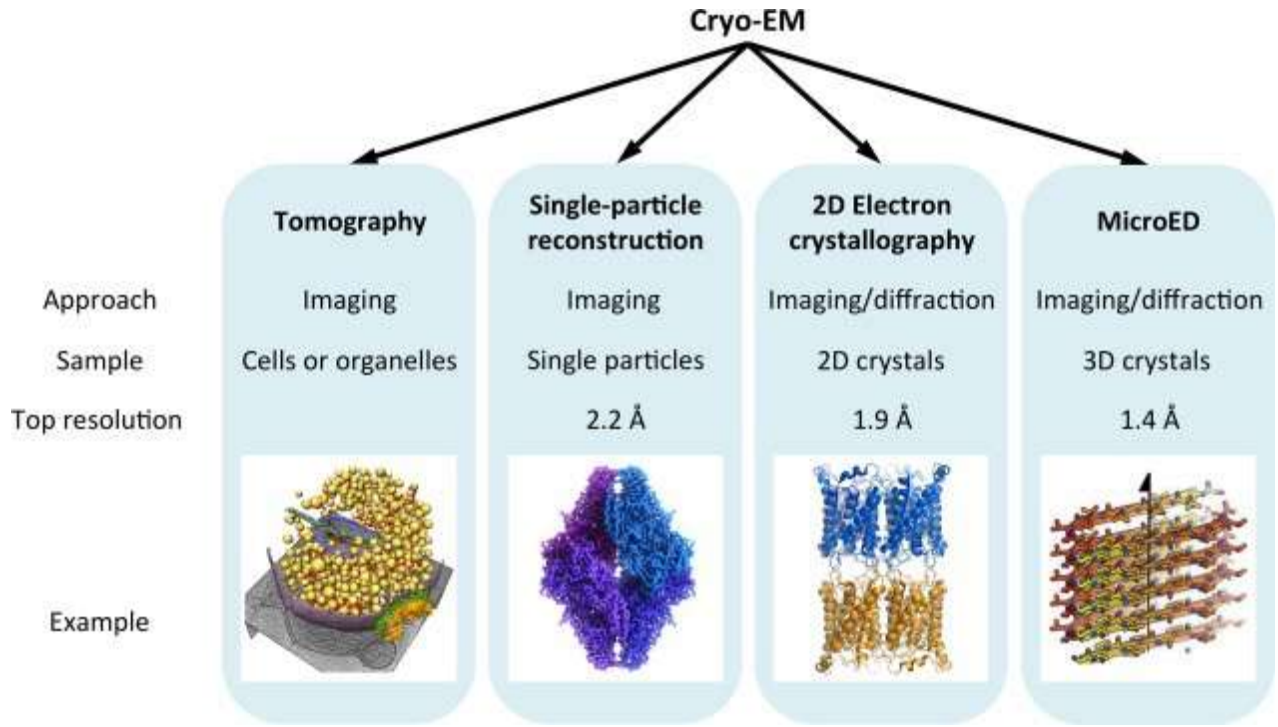
FIB SEM

April 7, 2025

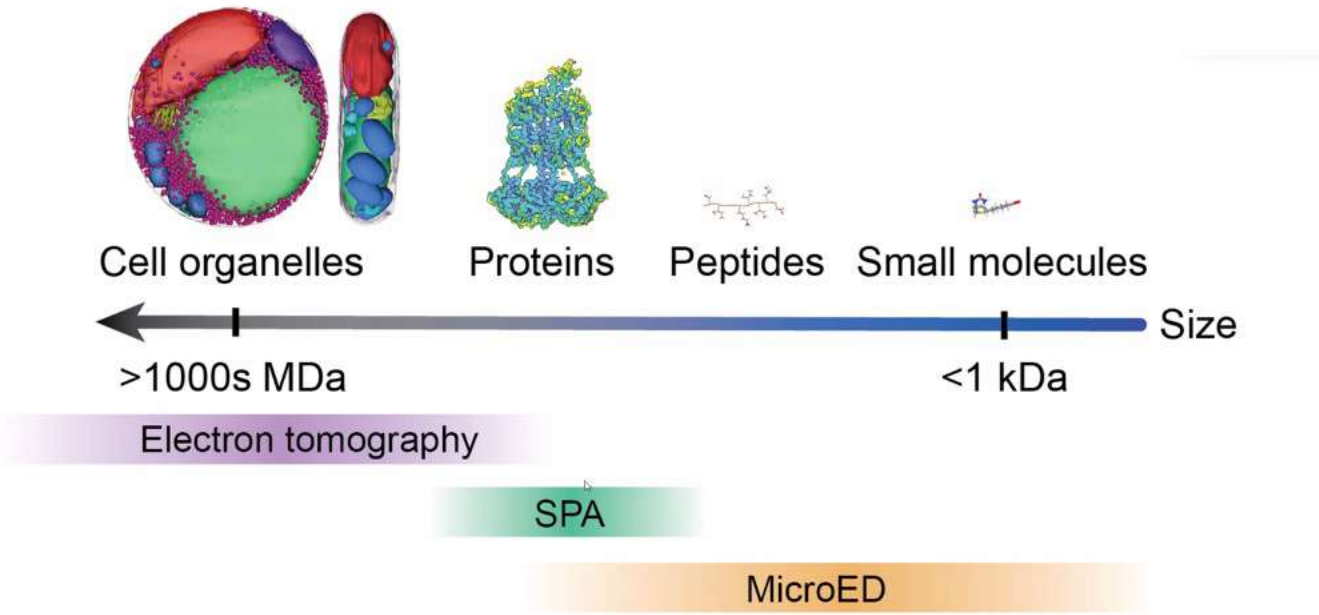
William Rice, NYU School of Medicine



Cryo-electron Imaging Modalities



Target Sizes

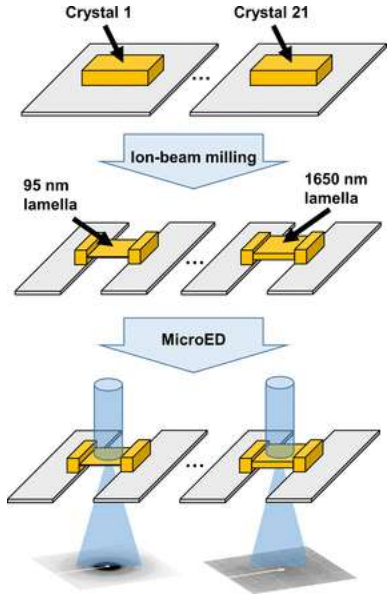


Sample thickness for TEM

- Lysozyme: crystals thicker then 500 nm unusable
- Martynowycz MW, Clabbers MTB, Unge J, Hattne J, Gonen T. Benchmarking the ideal sample thickness in cryo-EM. Proc Natl Acad Sci U S A. 2021 Dec 7;118(49):e2108884118. doi: 10.1073/pnas.2108884118. PMID: 34873060; PMCID: PMC8670461.
- Maximum usable thickness ~ 2X mean free path of electrons
  - 120 kev: 430 nm
  - 200 kev: 540 nm
  - 300 keV: 640 nm

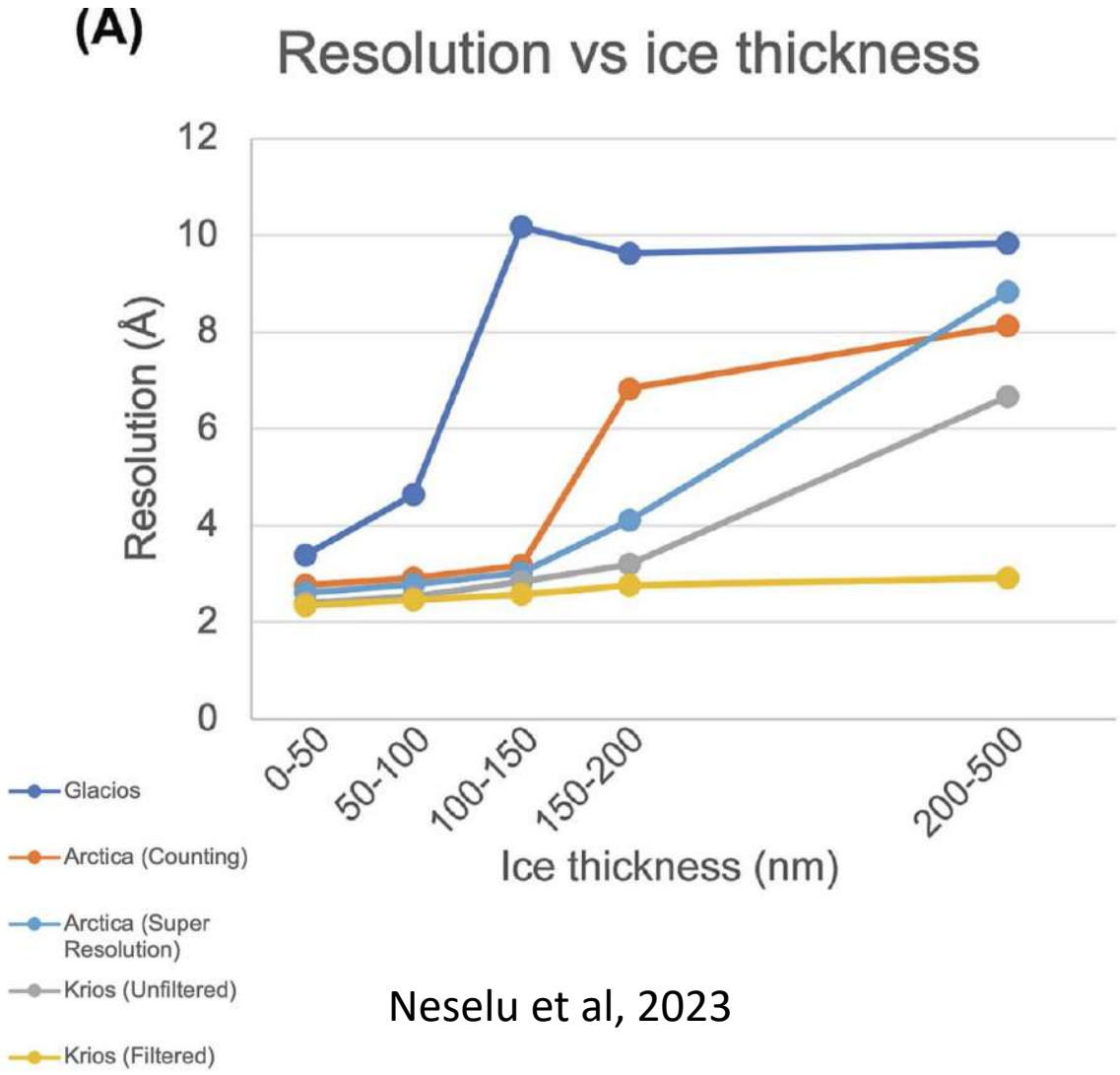
Thickness increases by a factor of 1/cos(θ)

60° tilt: twice as thick as nominal untilted



Typical sample thickness

- Single particle samples: 10nm – 200 nm
- Bacterial cell: 1-2 µm
- Typical eukaryotic cell: 5 µm
- Tissue samples: up to 200 µm



Neselu et al, 2023

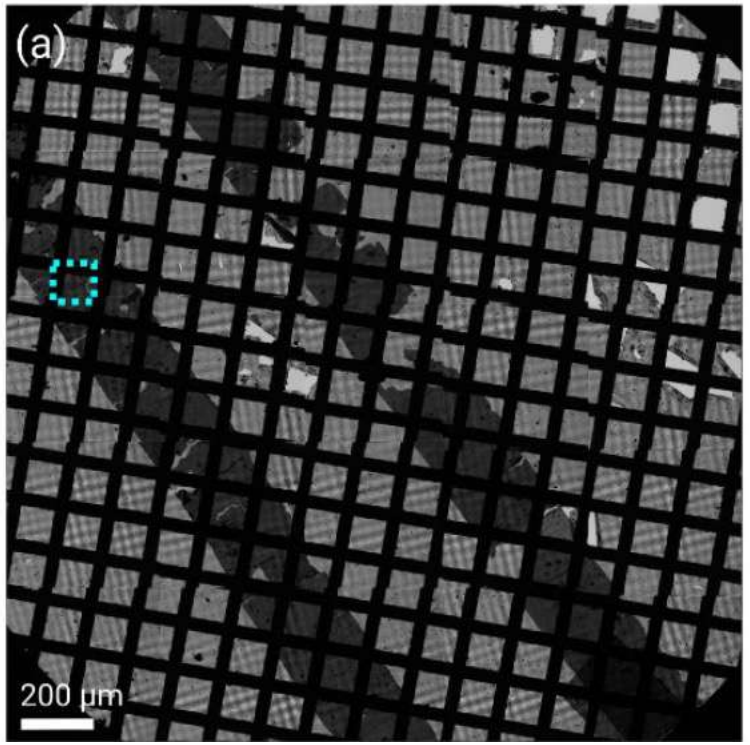
Megavolt electron microscopes

- Megavolt electron microscopes
  - Not commercially available
  - Space requirements: 2+ stories
  - X-ray safety
  - Detectors
  - Not made for cryo work

https://doi.org/10.1016/bs.aiep.2021.08.008.
(https://www.sciencedirect.com/science/article/pii/S1076567021000811)

Microtomy (CEMOVIS)

- Microtomy
  - Cryomicrotomy (CEMOVIS) is difficult
  - Sections hard to pick up
  - May be difficult to place on grids
  - Compression and knife artifacts
  - However, large areas are possible
  - Serial sectioning is possible, leading to greater volume sampling



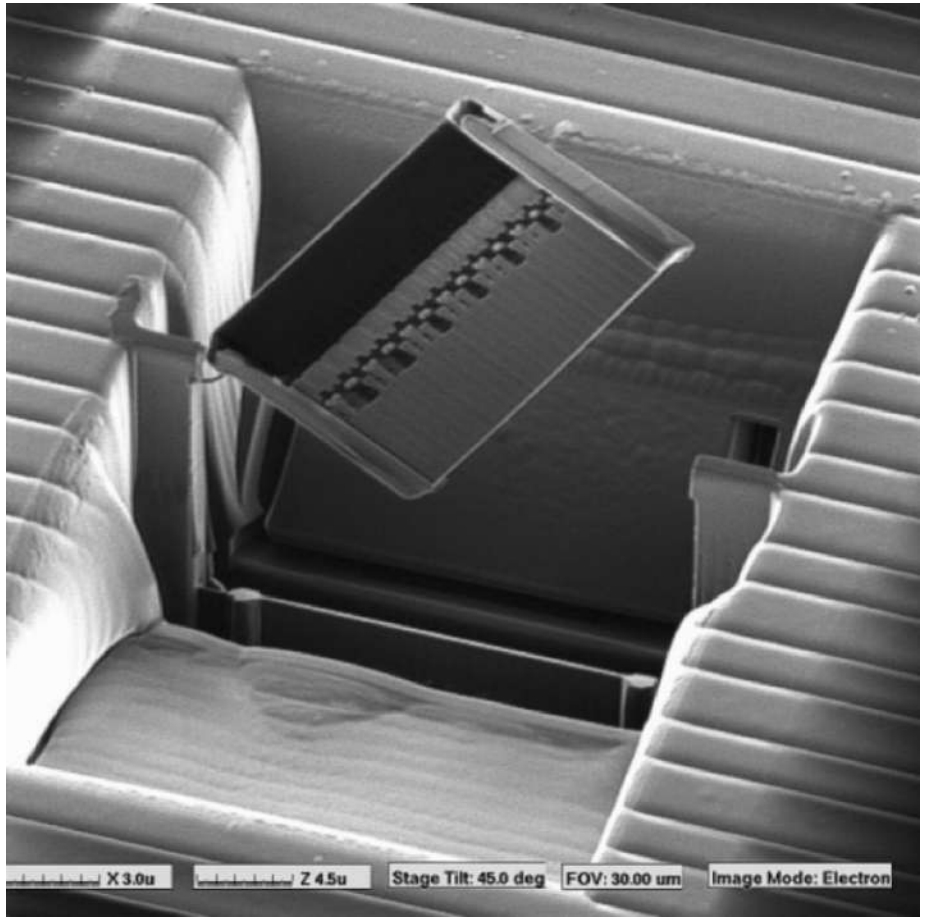
In-Situ High-Resolution Cryo-EM Reconstructions from CEMOVIS

Johannes Elferich, Marek Kaminek, Lingli Kong, Adolfo Odriozola, Wanda Kukulski, Benoît Zuber, Nikolaus Grigorieff

doi: https://doi.org/10.1101/2025.03.29.646093

FIB SEM

- Use a focused ion beam to thin the sample
- Cut out a thin (electron transparent) piece then place on a standard EM grid for TEM imaging



Solutions

- Use a focused ion beam to carve out a thin lamella from a frozen sample
- Transfer to cryo-TEM

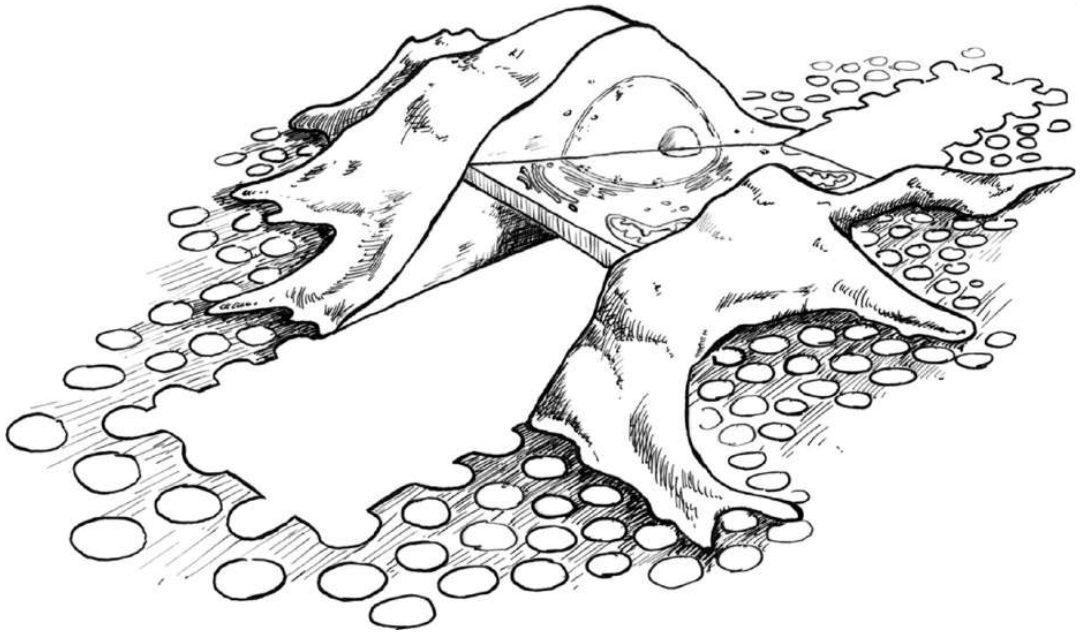


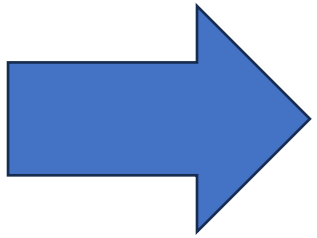
Image: Villa *et al*, 2013

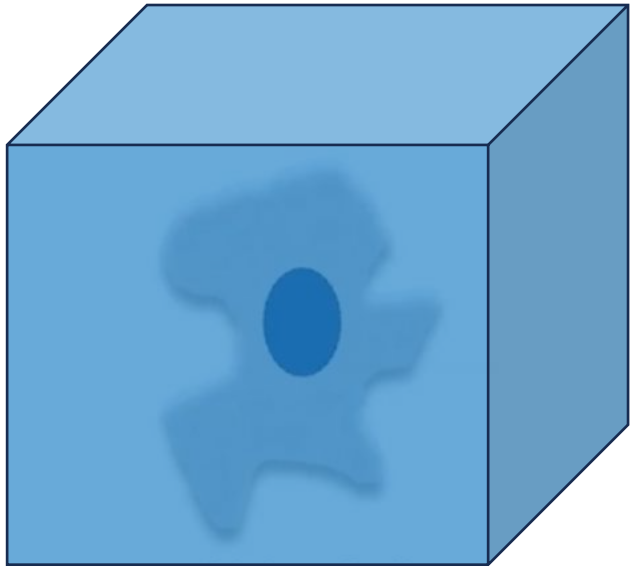
Freezing thick (> 5-10 µm) Samples

Frozen Samples

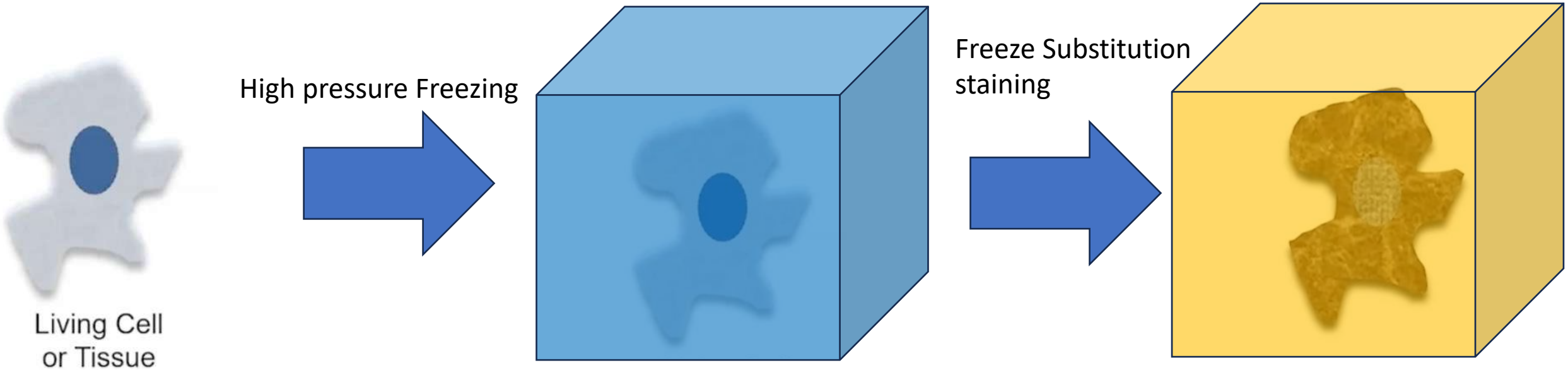
Living Cell
or Tissue

High pressure Freezing





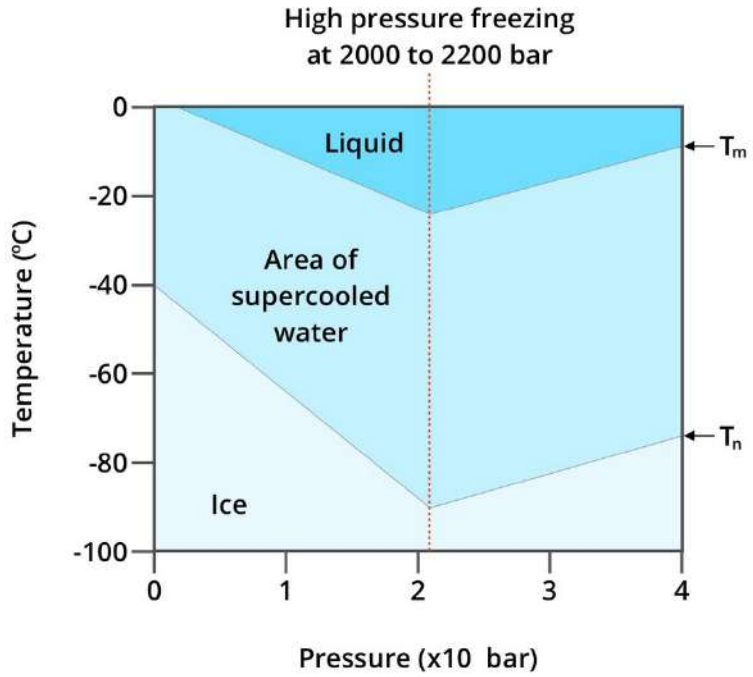
Freeze Substituted Samples



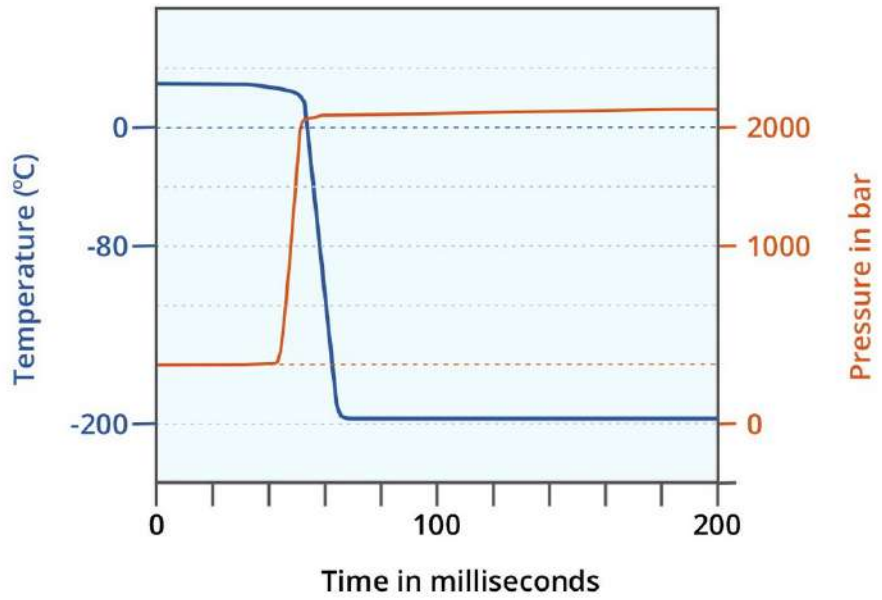
Size limit: ~200 µm thick

Ultrastructural details only

High Pressure Freezing



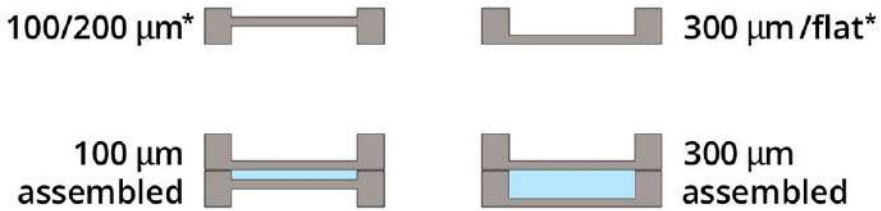
Phase diagram for water showing the minimal melting temperature T_m and minimal nucleation temperature T_n that delineate the region of supercooling. At 2045 bar both the minimal melting temperature and the minimal nucleation temperature are at their lowest limits showing this pressure is ideal for freezing of water.



Graph of pressures and temperatures of the sample during high pressure freezing. In less than 10 milliseconds from the start of the pressure being applied it has reached 2100bar. Cooling starts fractionally before this so that the cooling rate is 20,000°C/s when the sample reaches this pressure. Under these conditions freezing takes only a few milliseconds.

https://myscope.training/CRYO_High_pressure_freezing

High Pressure Freezing: Planchettes



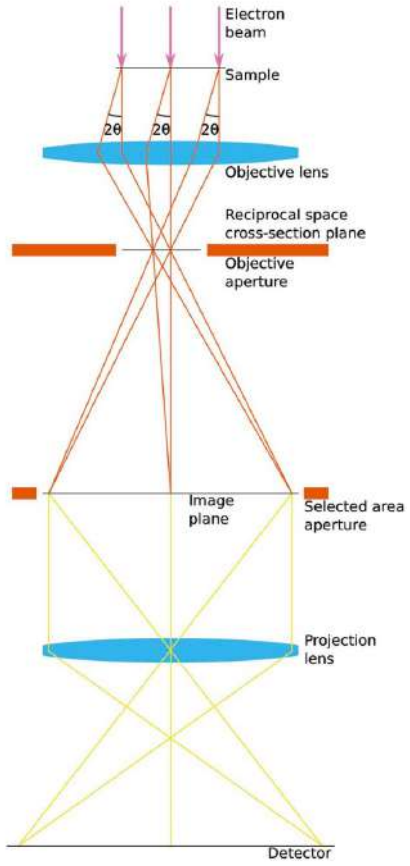
https://myscope.training/CRYO_High_pressure_freezing

FIB SEM:

Start with SEM Basics

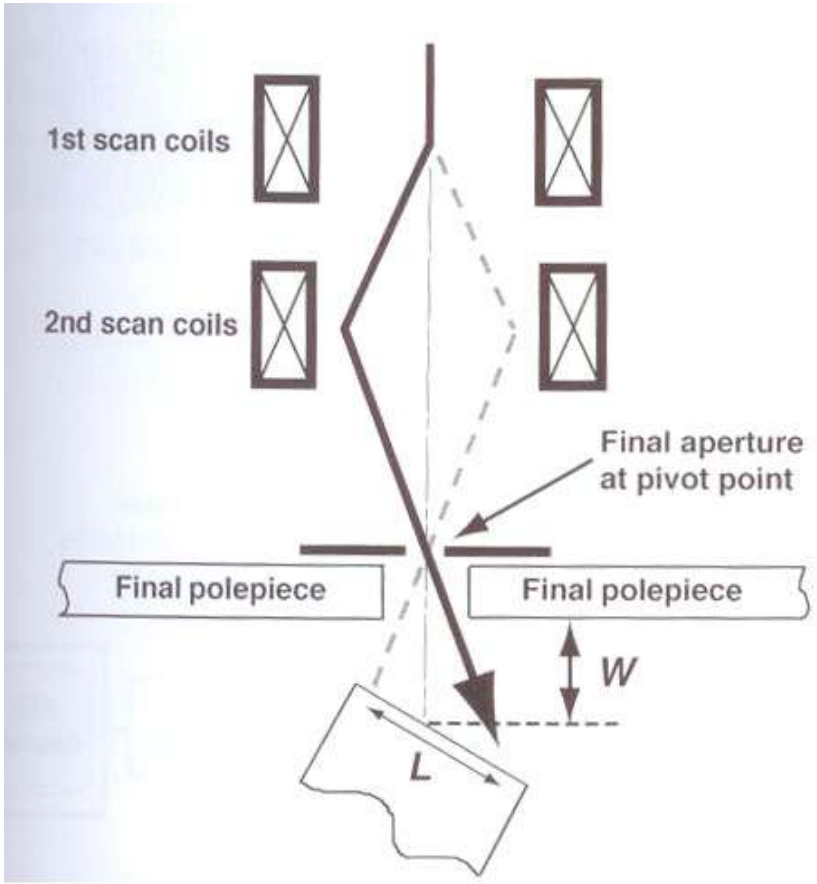
SEM versus TEM

TEM



Projection through sample

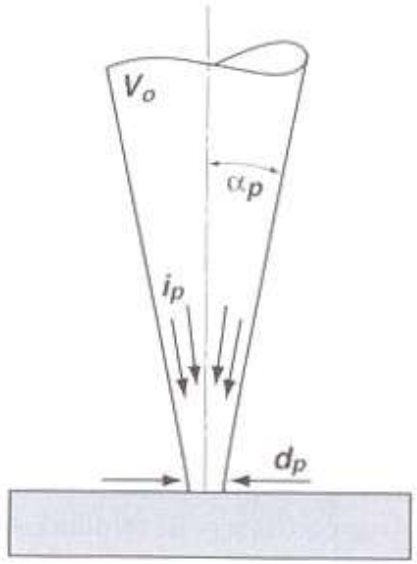
SEM



Surface imaging

SEM Beam: probe size

- Ideally want as small a probe as possible, relative to pixel size
- Probe size is determined by voltage, current, divergence angle
- Lens distortions
  - Spherical aberration (focus different at center and edge of lens) – proportional to focal length (working distance)
  - Aperture diffraction
  - Astigmatism (user correctable)
  - Chromatic aberration – voltage dependent (higher at low voltage)



Goldstein et al, 2003

Signal: Back Scattered Electrons (BSE's) and Secondary Electrons (SE's)

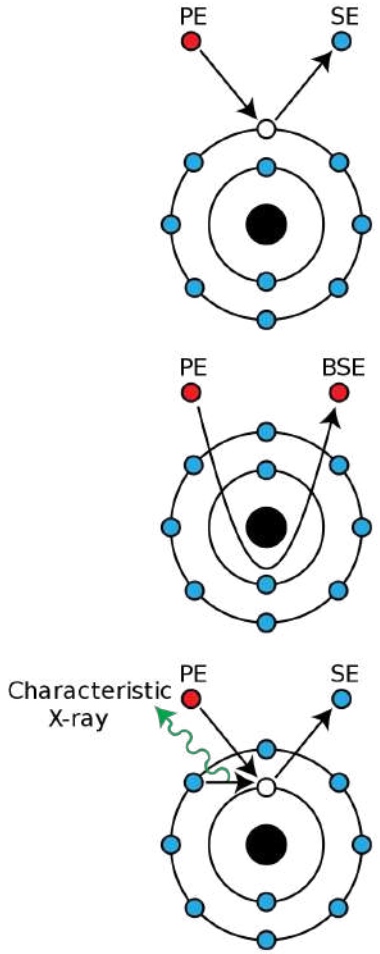
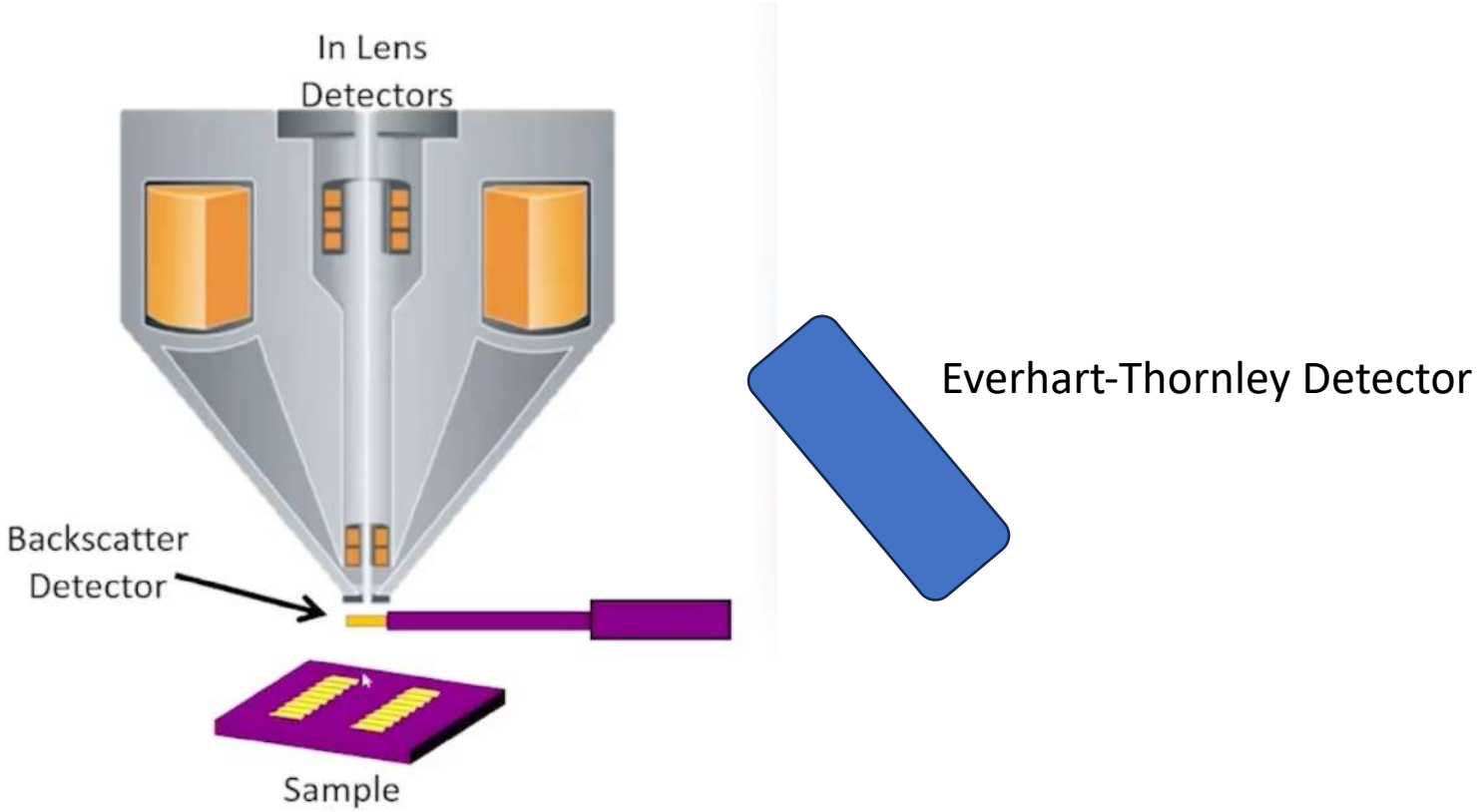


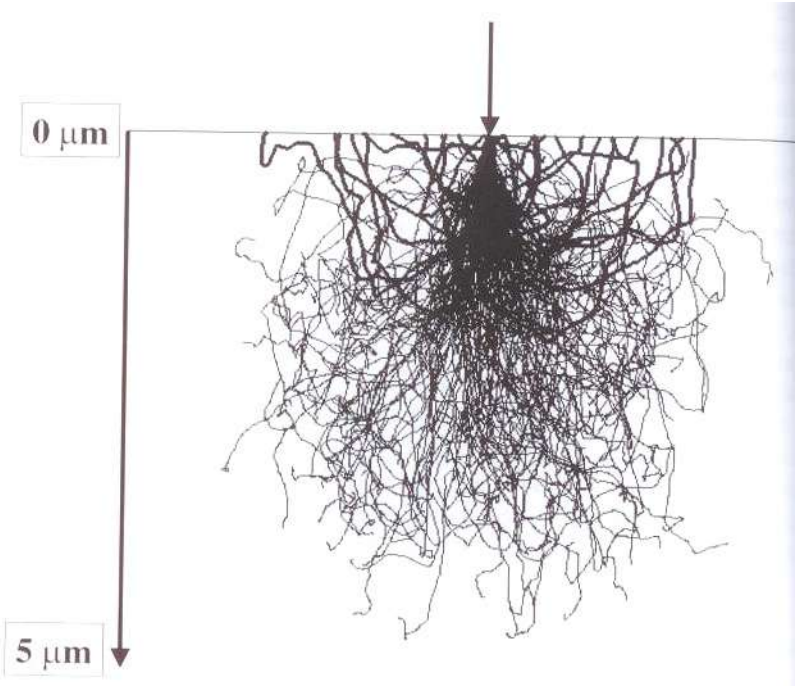
Image Source: Rob Hurt - Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=50931451

Detector Setup for SEM



Beam-Specimen Interaction

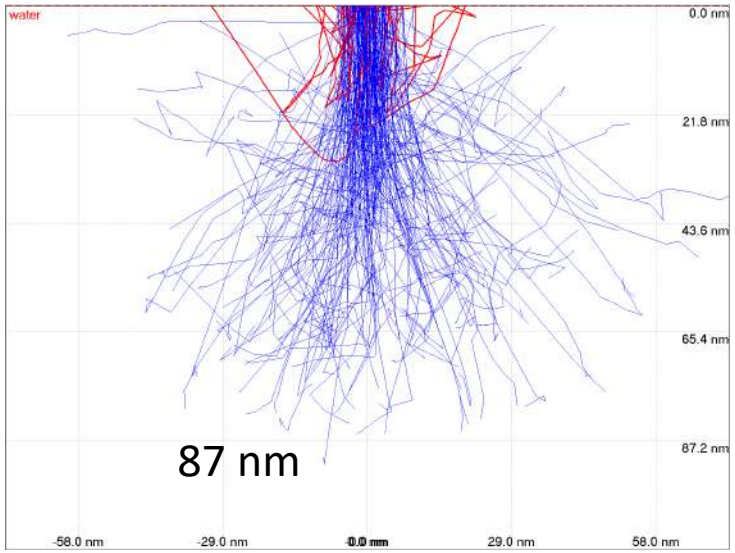
- Monte Carlo simulation of a 20 keV beam in Si
  - Dark traces: electrons which left the sample (BSE's)
- Electrons may be scattered elastically or inelastically
- Probability of elastic scattering ~ Z^2
- Inelastic scattering:
  - Secondary electrons
  - X-rays



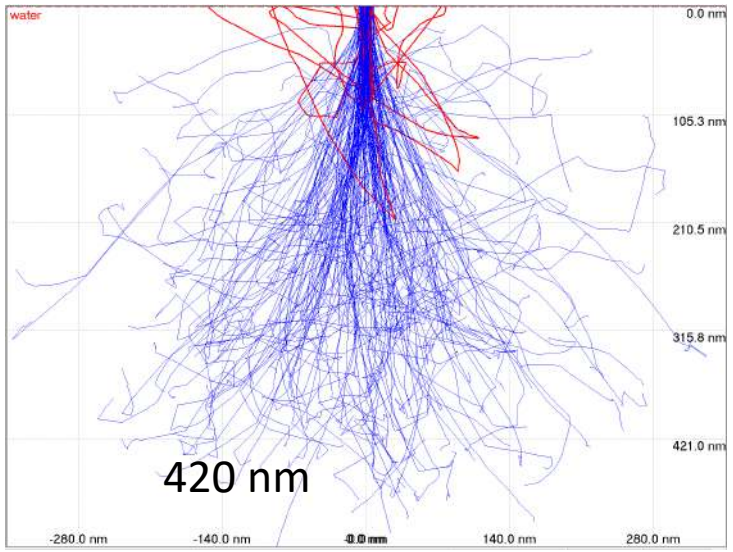
Goldstein et al, 2003

Monte Carlo simulation: water

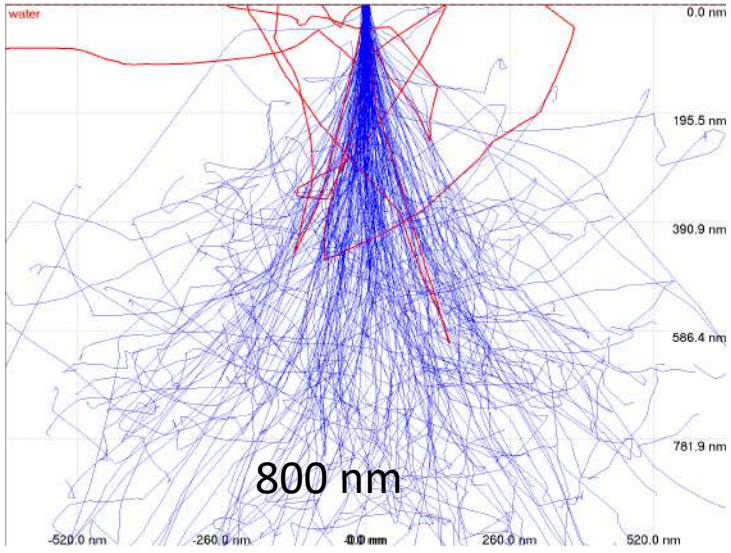
1 KEV



3 KEV

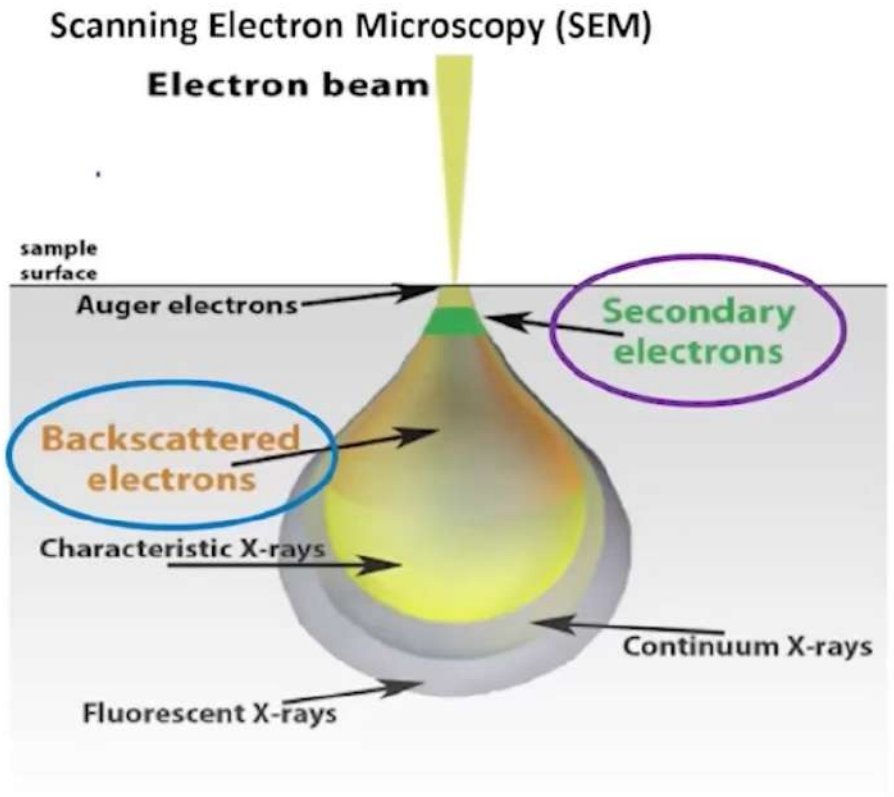


5 KEV



https://www.gel.usherbrooke.ca/casino

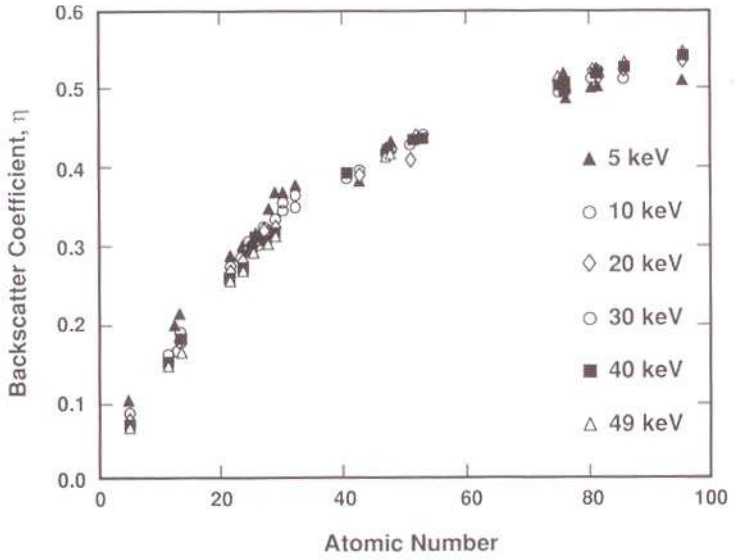
Interaction Volume



Schematic of electron beam interaction

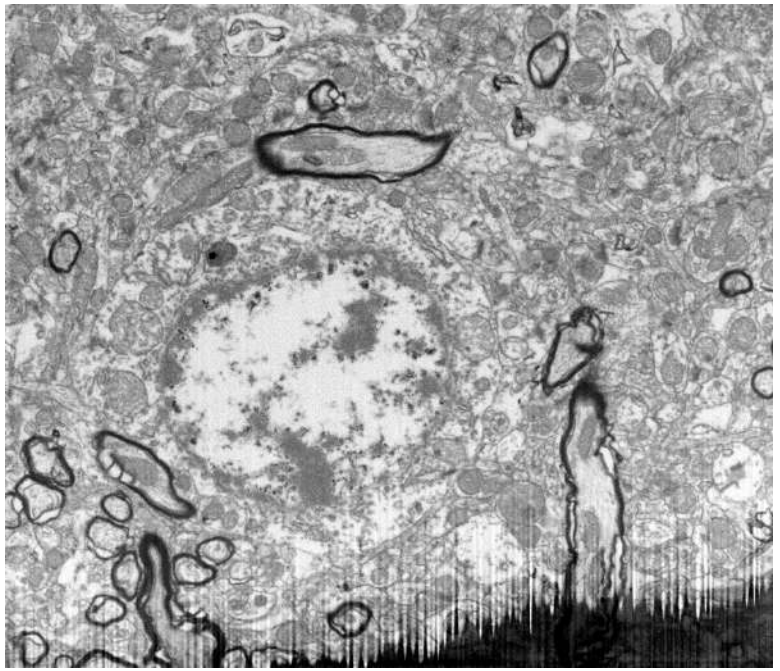
BSE efficiency is material dependent, voltage independent

Fraction of
electrons that backscatter



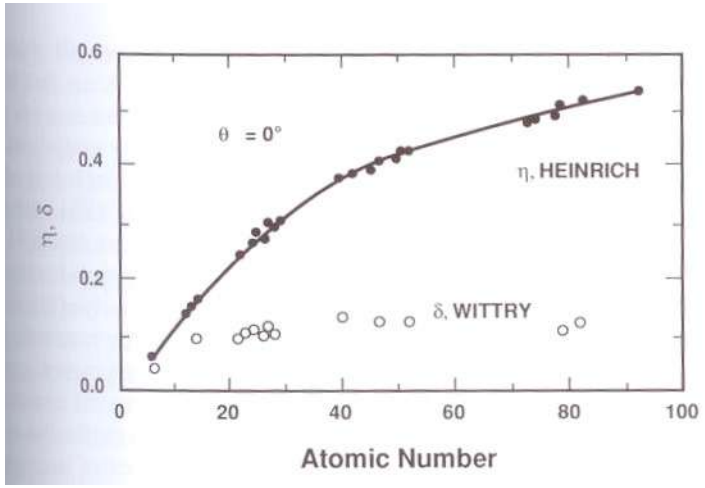
Goldstein et al, 2003

BSE's give contrast between
light and heavy elements

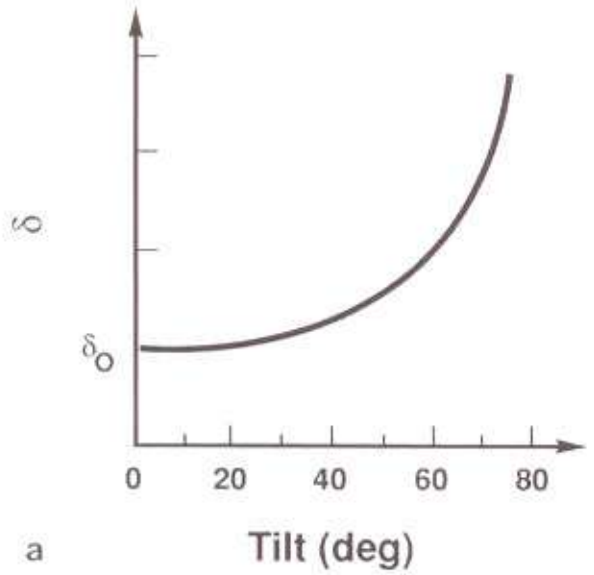


Osmium stained, resin-embedded tissue

Secondary Electrons are much less sensitive to element difference, more sensitive to topographic information



30 keV

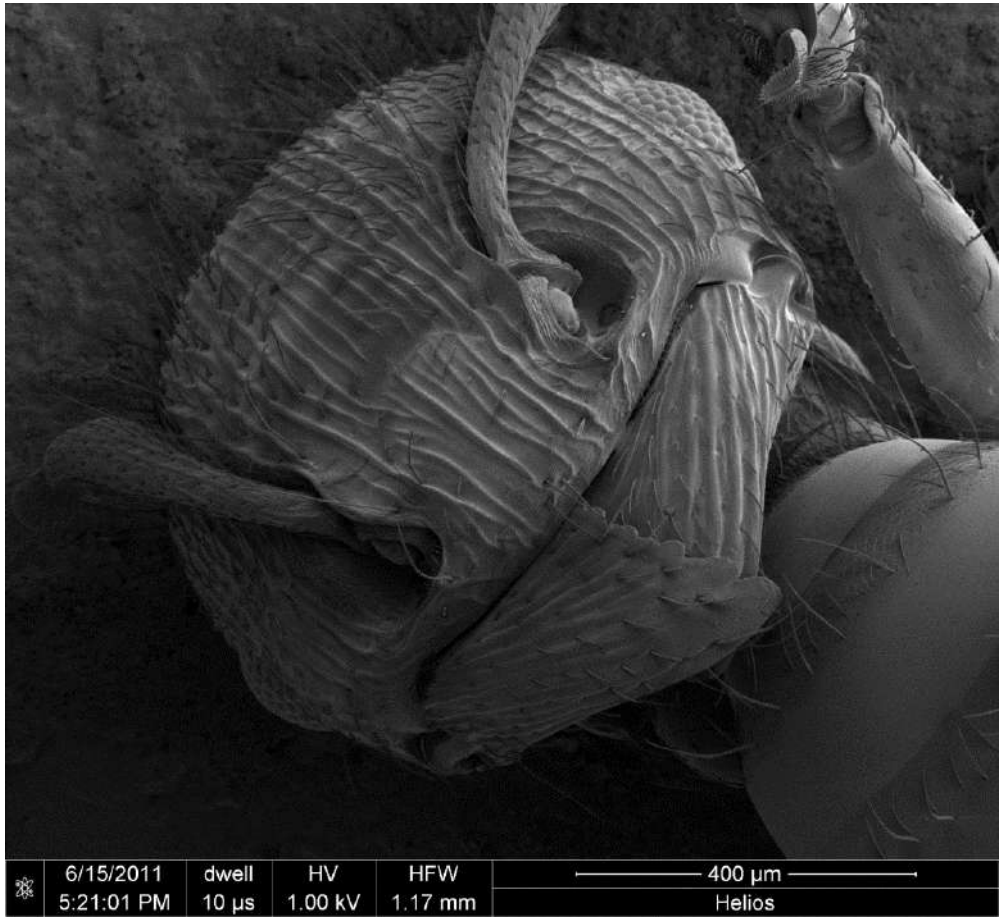


SE's are less sensitive to atomic number than BSE's (may be more sensitive at lower beam energies)

Signal is strongly dependent on viewing direction

Goldstein et al, 2003

SE’s give excellent topographic information



Non-conductive samples

- Imaging with electrons on non-conductive samples is difficult due to charging artifacts
  - Resin-embedded samples, biological specimens, frozen samples
- Generally make them conductive beforehand by sputter-coating with metal (Pt, Au)
- Image using low voltage (5 keV or less) and low current
  - Current too low requires longer scan/integration times
- Ideally, the SEM includes a pre-loading chamber for sputter coating

SEM versus TEM

SEM

- Large chamber
  - Harder to reach highest vacuum
  - Many ports for add-ons
- Voltage: < 1 keV to 30 keV
  - Commonly <5 keV for non-conductive specimens
- Large samples of varying shape
- Signal from surface or just beneath surface
- Non-coherent imaging, no phase information

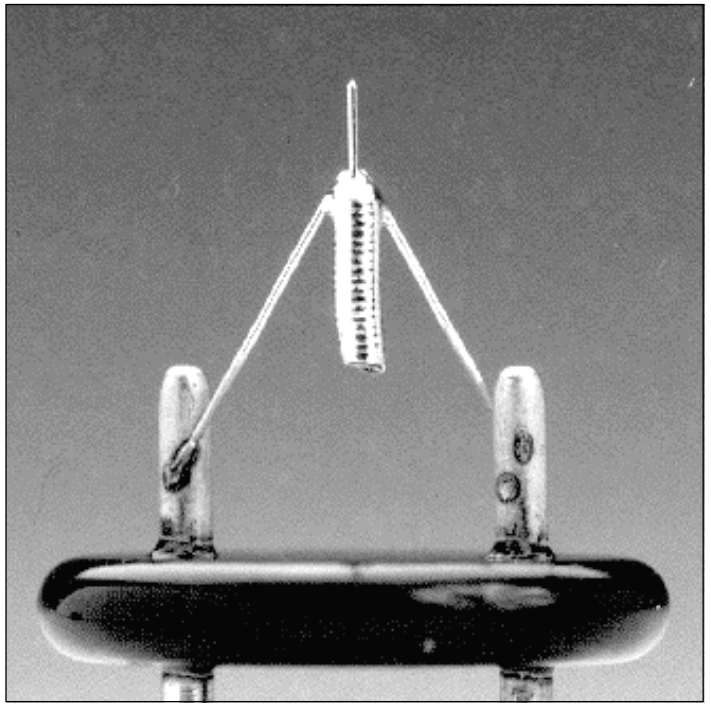
TEM

- Small Chamber
  - Easier to reach very high vacuum
  - Few ports for add-ons
- Voltage: 80-300 keV
  - 300 keV for highest resolution
  - Lower voltage DED now being released
- Thin samples (<500 nm) on TEM grid
- Projection images through sample
- Coherent beam imaging: phase preserved

FIB Operation

Gallium is the Most Popular LMIS

- A liquid metal
- Room temperature operation
- Long lived (500-1500 hr sources)
- High vacuum compatible
- Large ion for sputtering
- Other options
  - He, Ne, Xe
  - Mostly for materials sciences

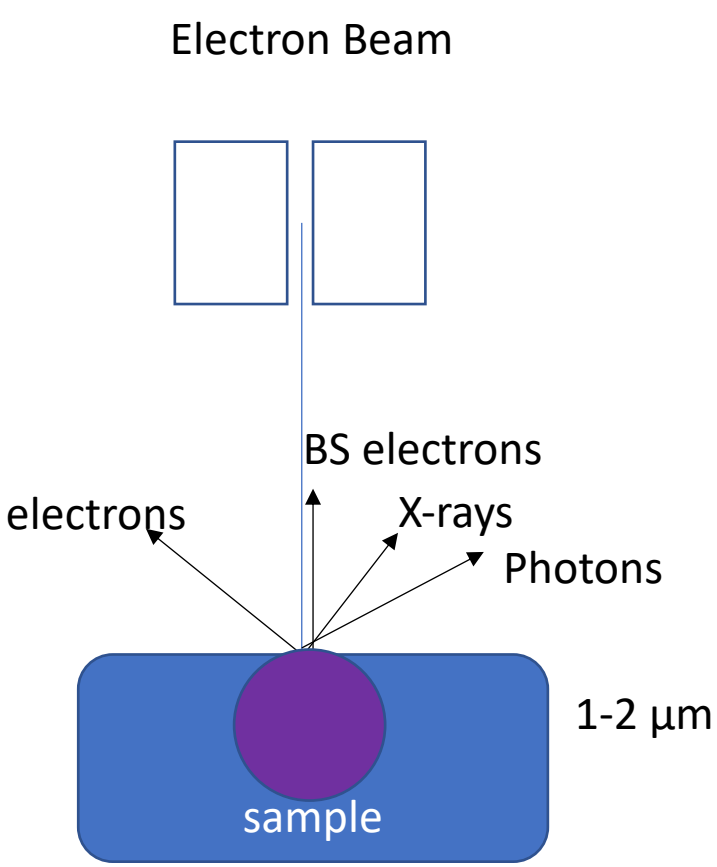


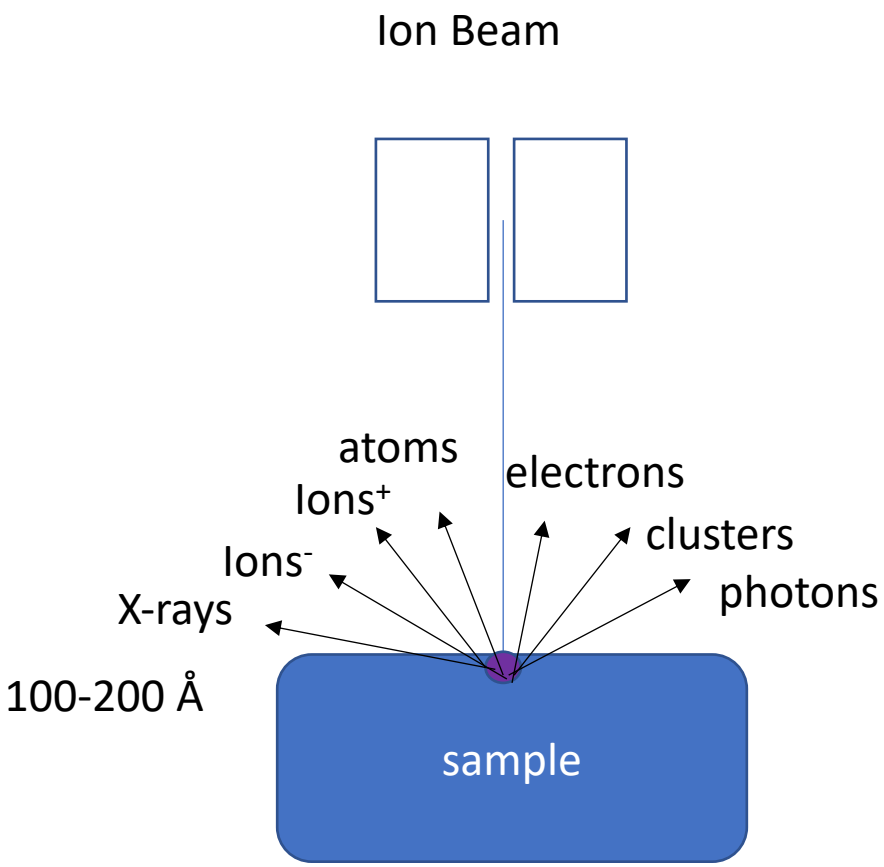
FEI™

Ion Column

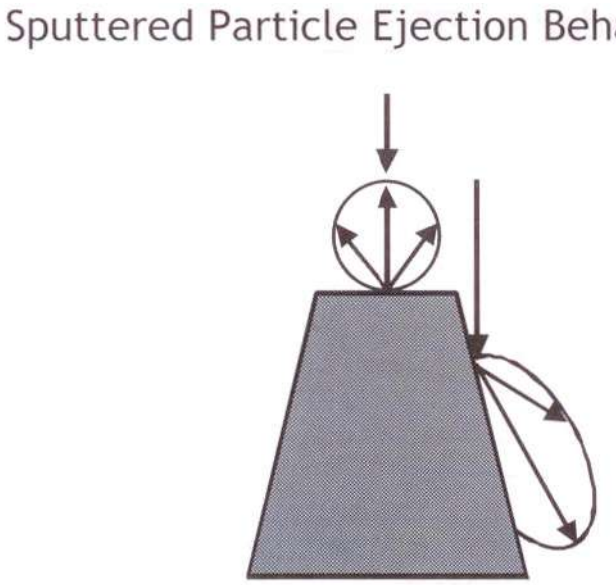
- ◆Source - LMIS at top
- ◆Focusing Optics
  - Use Electrostatic lenses since ions are heavier than electrons.
- ◆Deflection Electronics/Pattern Board
- ◆High-speed Blanking
  - Need to prevent milling while blanking
- Current is controlled by apertures
  - Apertures wear out over time and must be replaced!
- You can get images with FIB beam. Beam is much more damaging than electron beam so you need to image at as low current as possible
- Generally used at 30 keV, though voltage can be changed

Beam Interactions with Specimens

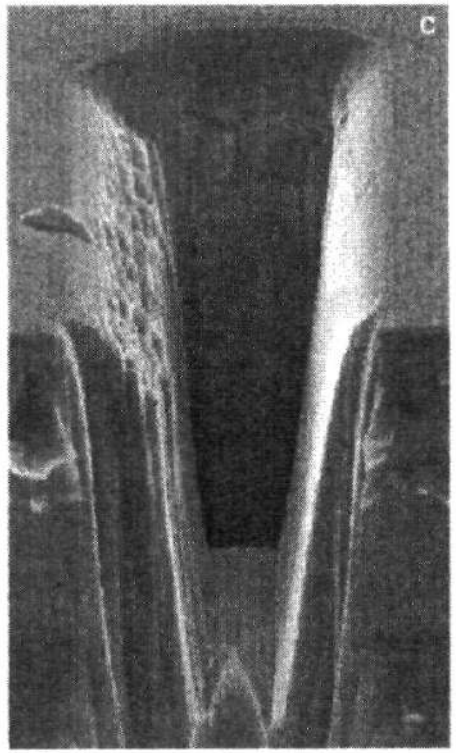




Common Use: Sputtering particles from substrate



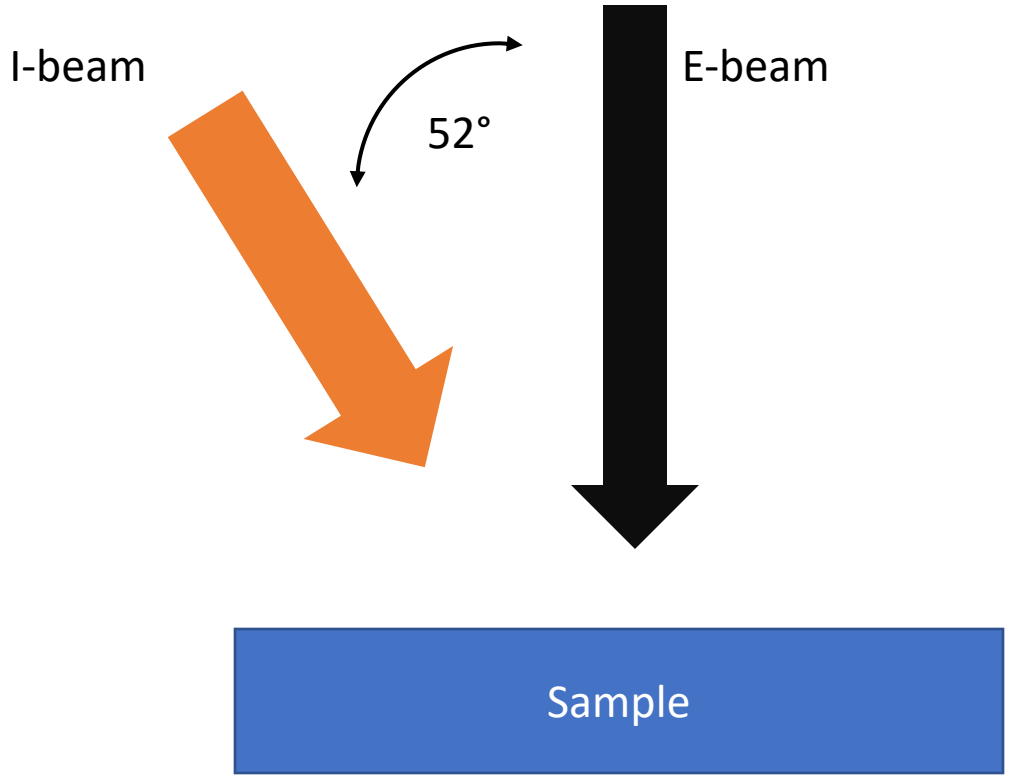
More efficient milling at edge than in bulk



Redeposition limits trench depth

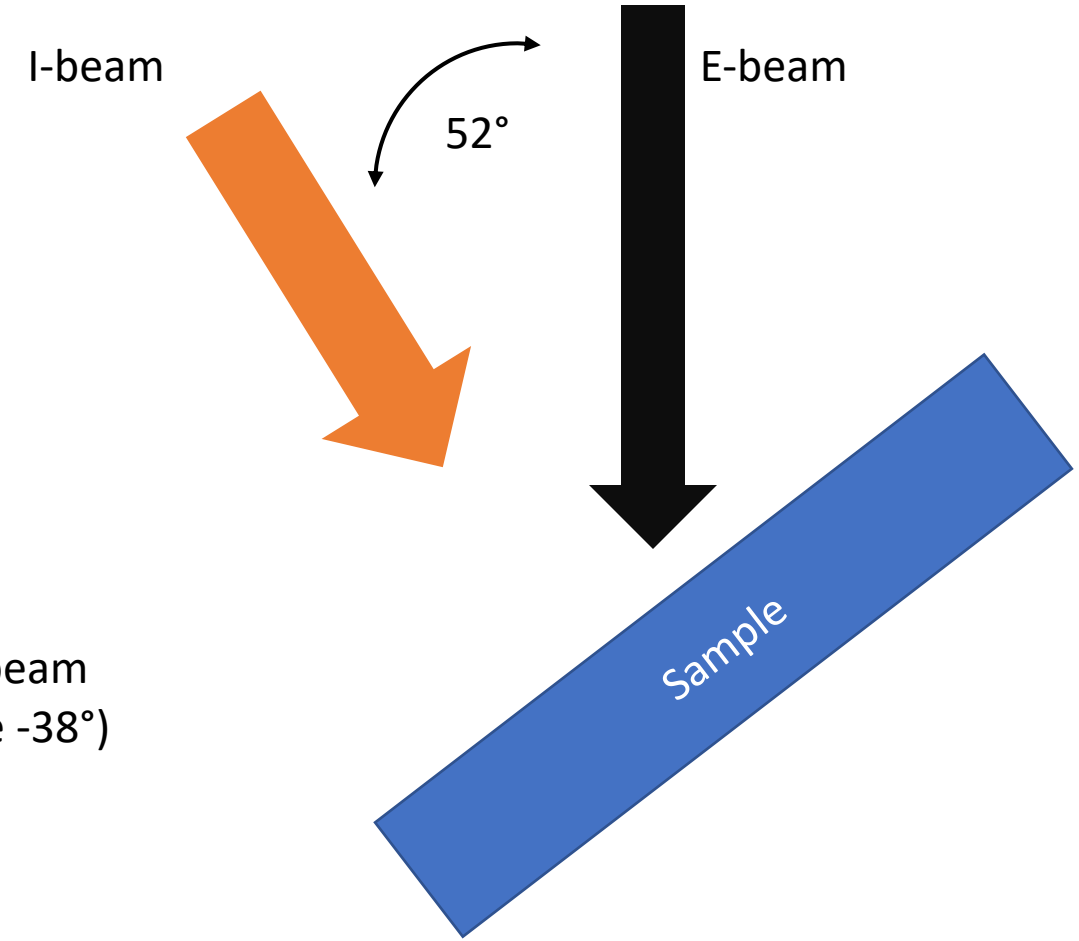
FEI™

Geometry



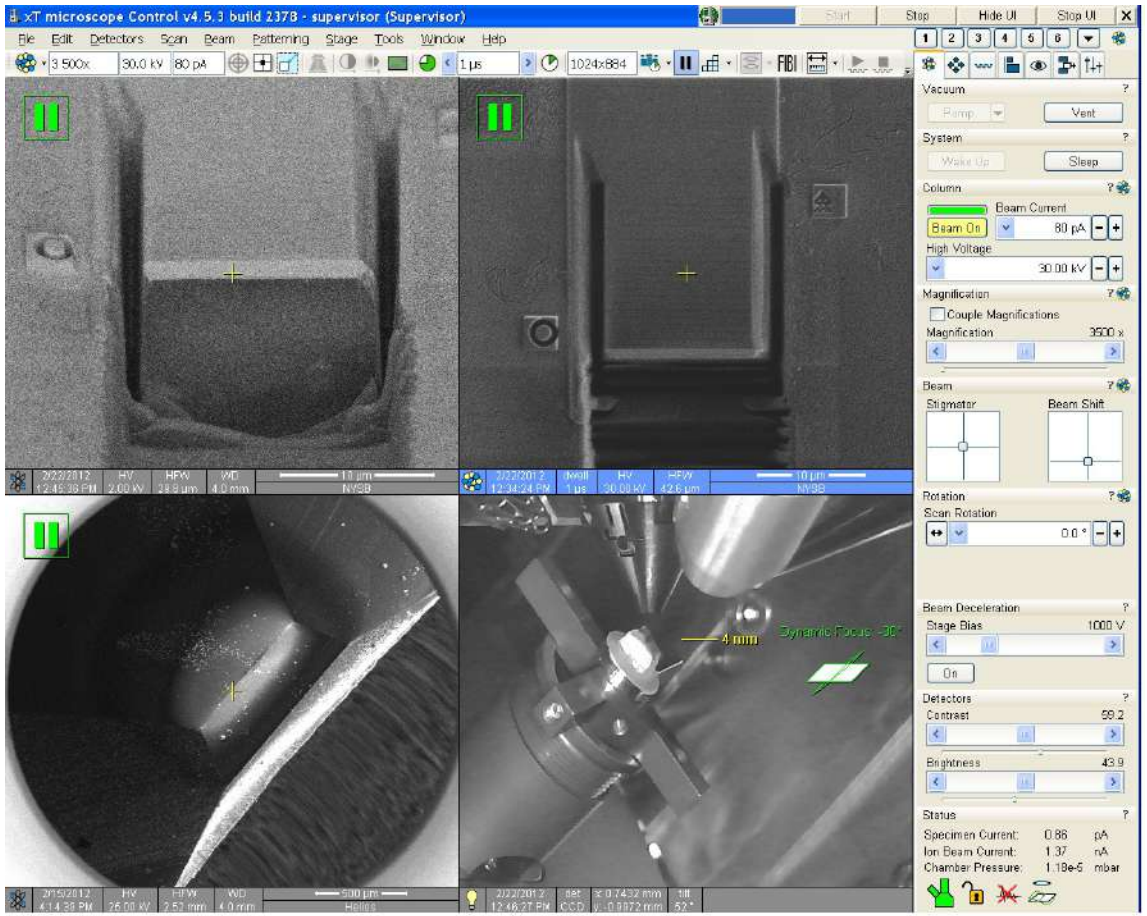
Sample:
Tilt from -10° to +70°

Geometry



Sample:
Tilt from -10° to +70°
Tilt to 52° for orthogonal i-beam
(cross-section viewing angle -38°)

Geometry


xT microscope Control v4.5.3 build 2378 - supervisor (Supervisor)
File Edit Detectors Scan Beam Patterning Stage Tools Window Help
Vacuum
Vent
System
Sleep
Column
Beam Current
Beam On
80 pA
High Voltage
30.00 kV
Magnification
Couple Magnifications
Magnification
3500 x
Beam
Stigmator
Beam Shift
Rotation
Scan Rotation
0.0 °
Beam Deceleration
Stage Bias
1000 V
On
Detectors
Contrast
99.2
Brightness
43.9
Status
Specimen Current: 0.86 pA
Ion Beam Current: 1.37 nA
Chamber Pressure: 1.18e-5 mbar
4 mm
Dynamic Focus

Metal Deposition for surface protection (GIS)

- (Methylcyclopentadienyl) trimethyl platinum
- Warm to gas, spray over sample with needle
- I-beam or e-beam interactions break it apart, deposit metal onto sample
  - Protection
  - Hard surface for mill
  - Prevents “curtaining”

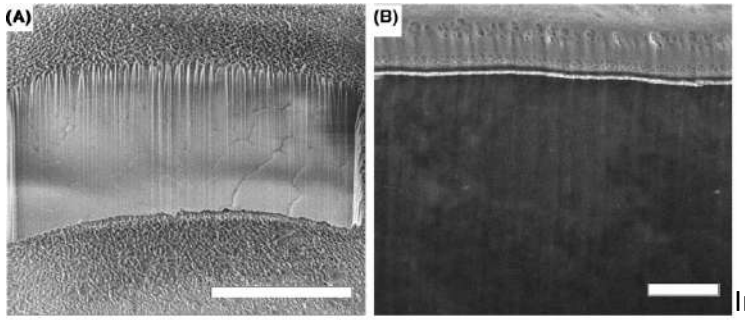
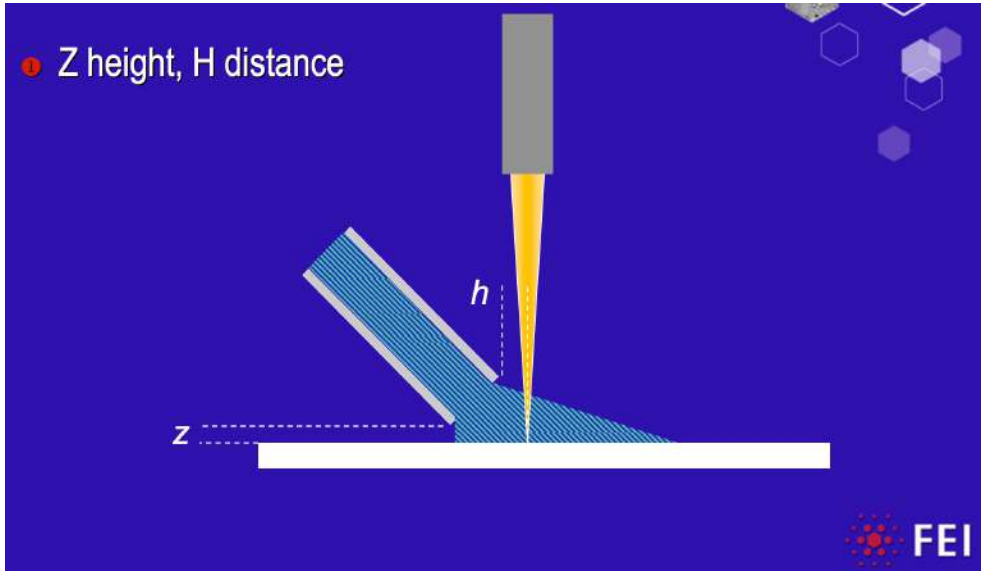


Image: Hayles and Winter, 2021



Cryo FIB/SEM for tomographic sample preparation

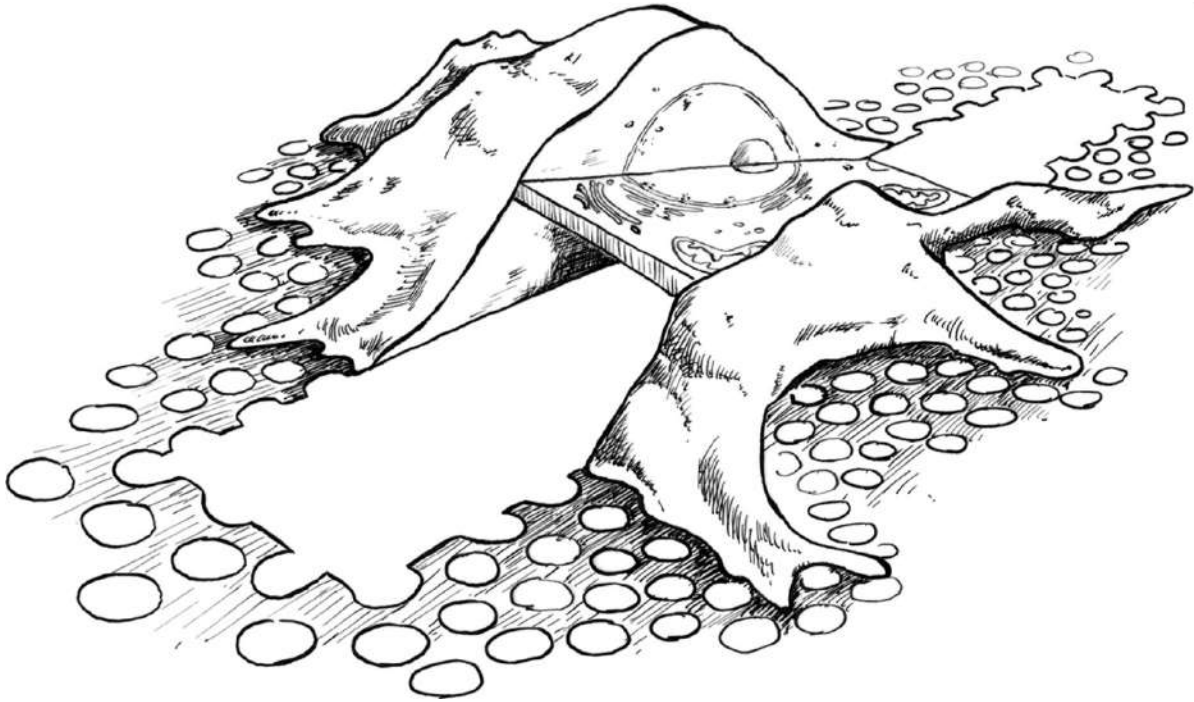


Image: Villa *et al*, 2013

2005: Shown that FIB milled cryo specimens remain vitreous

Journal of Microscopy, Vol. 222, Pt 1 April 2006, pp. 42–47
Received 8 July 2005; accepted 21 December 2005

Focused ion beam milling of vitreous water: prospects for an alternative to cryo-ultramicrotomy of frozen-hydrated biological samples

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Resource for Visualization of Biological Complexity, †Howard Hughes Medical Institute, Wadsworth Center, Empire State Plaza, Albany, NY 12201, U.S.A.
**Center for Nanoscale Systems, Harvard University, 17 Oxford St., Cambridge, MA 02138, U.S.A.*

Key words. Cryo-EM, devitrification, electron tomography, FIB, frozen-hydrated specimens, vitreous ice.

Place cells on Grids

- Need gold grids, not copper, for growing cells on grids
- Cells on carbon-facing side of grid
- If cells < 10 µm thick, plunge freezing should work
  - Back-blot to freeze grid
- For thicker specimens, a high pressure freezer is needed to vitrify

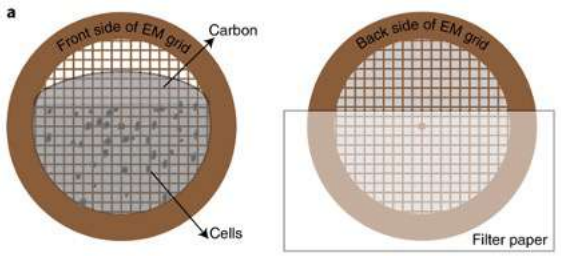


Image: Wagner *et al*, 2020

Grid Geometry

- After freezing, grids need to be clipped
  - Protection
  - Krios/Arctica
- Important to mark the autogrid!
- Autogrids with milling slot are commercially available
  - Milling slot allows lower angle of approach from ion beam

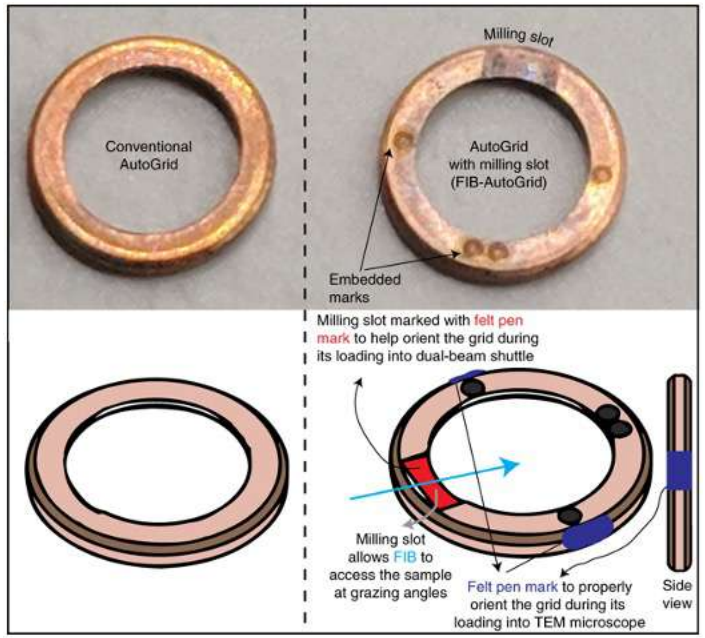


Image: Wagner *et al*, 2020

Grid Geometry

- Only the center of the grid is suitable for milling
- Cells are on flat-side of cartridge

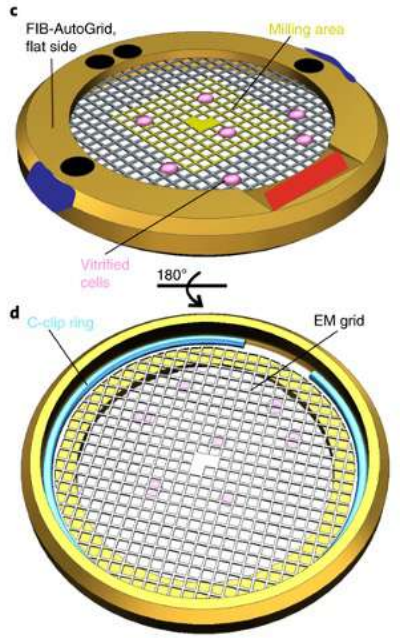
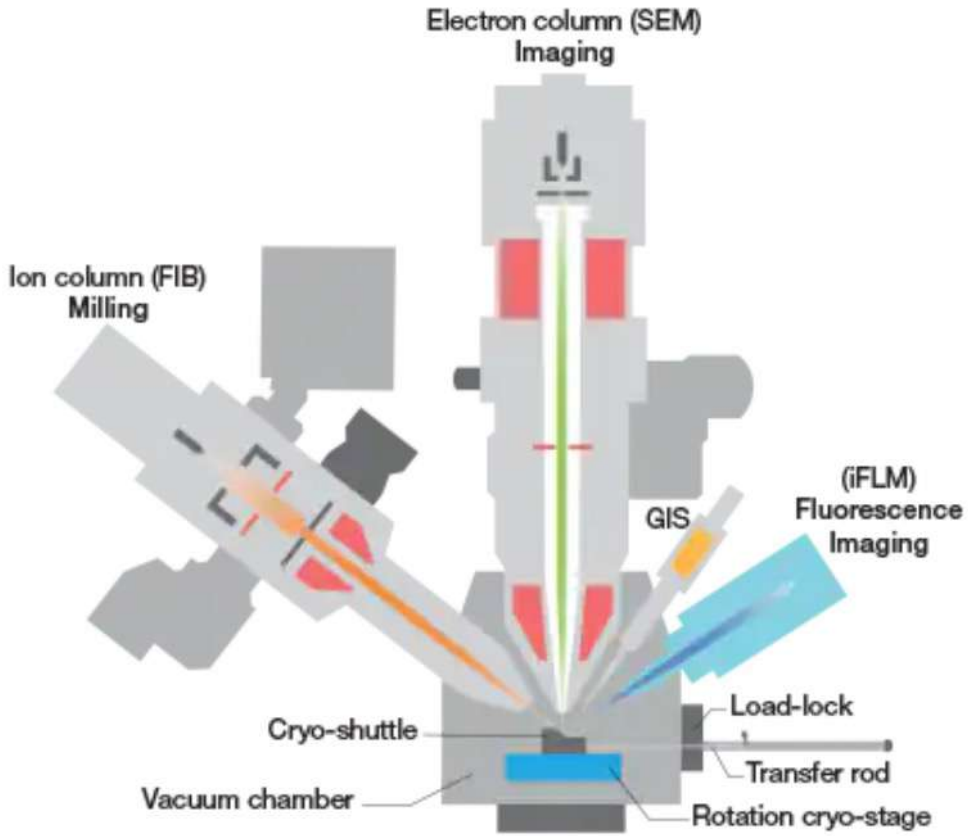


Image: Wagner *et al*, 2020

Specialized Microscopes: Aquilos 2 Cryo FIB



Geometry

- Untilted stage:
  - Ga beam at -7° angle to grid surface
  - E-beam at 45° angle to grid surface

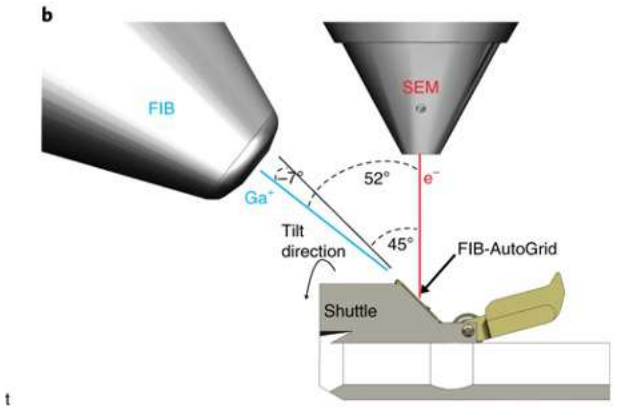


Image: Wagner *et al*, 2020

Geometry: Untilted

- Untilted stage:
  - Ga beam at -7° angle to grid surface
  - E-beam at 45° angle to grid surface

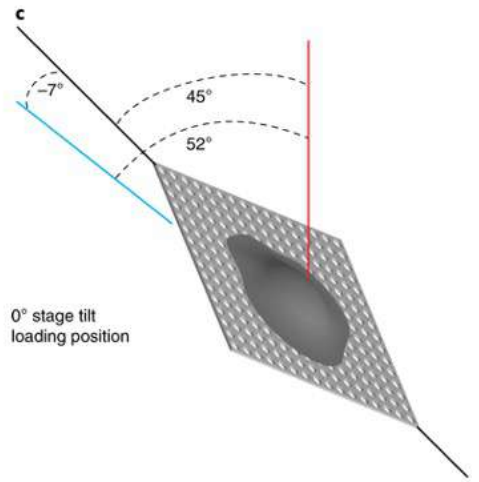


Image: Wagner *et al*, 2020

Geometry: Tilted

- Tilt stage +15°
  - Ga beam at +8° angle to grid surface
  - E-beam at 60° angle to grid surface

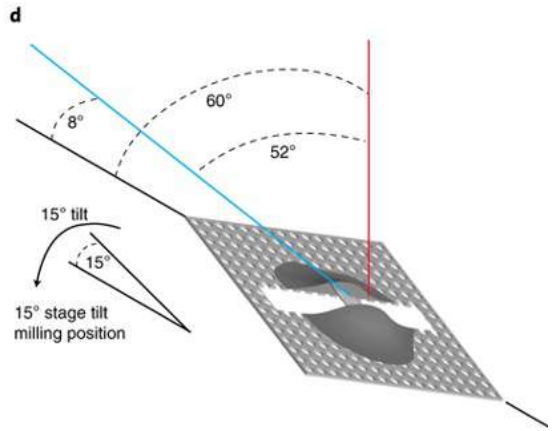


Image: Wagner *et al*, 2020

Imaging cells with ion beam

- A: Ion-beam view of cells
- B: Cells after milling, showing position of micro-expansion joints

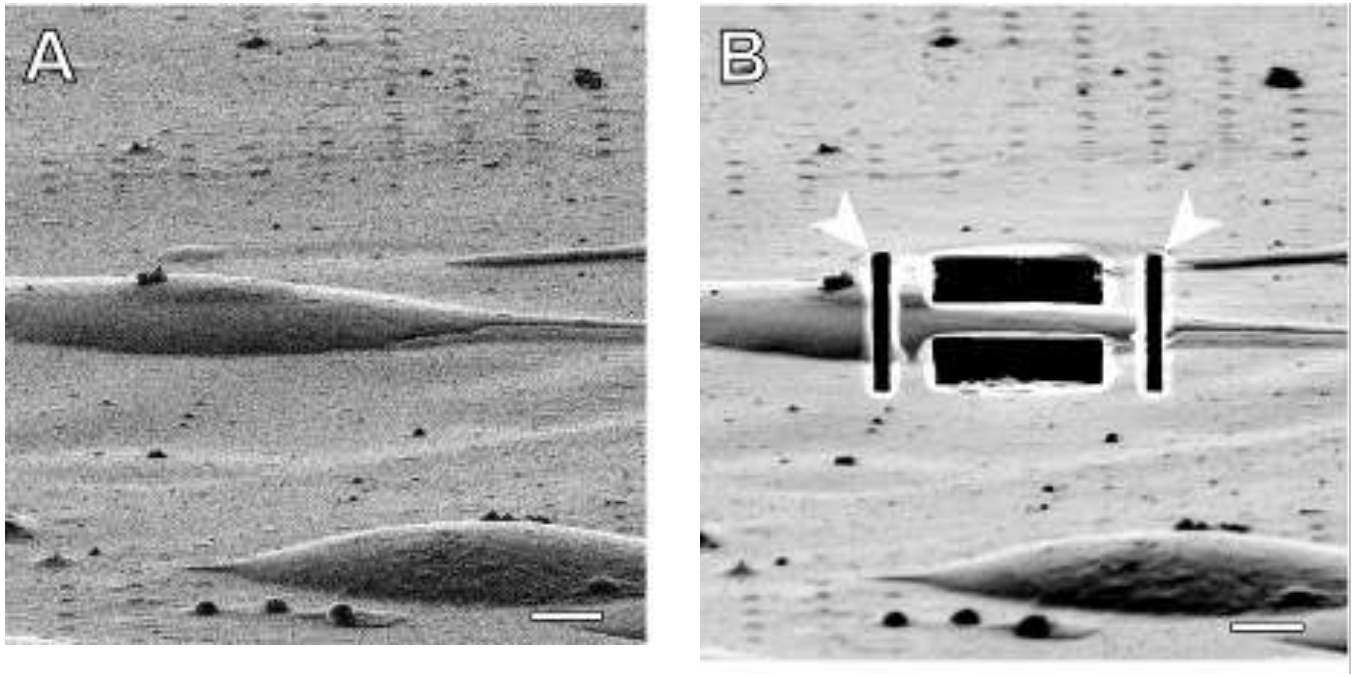


Image: Wolff *et al*, 2019

Targeting of Milling Regions

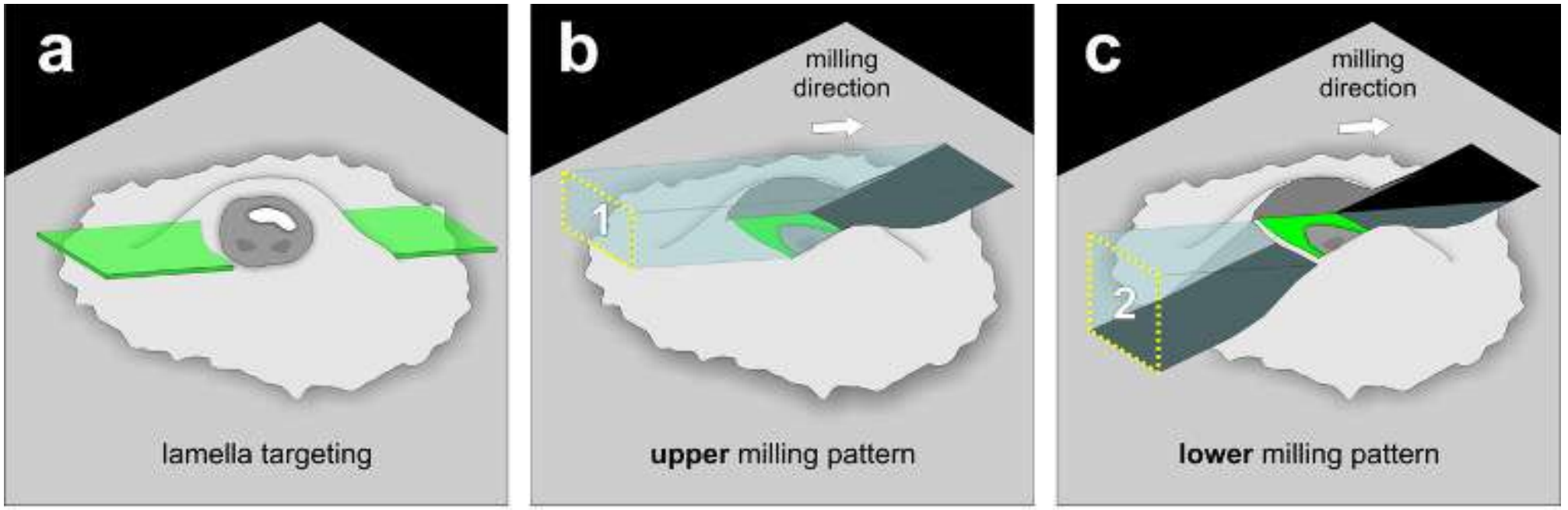


Image: Rigort and Plitzko, 2015

Targeting of Milling Regions

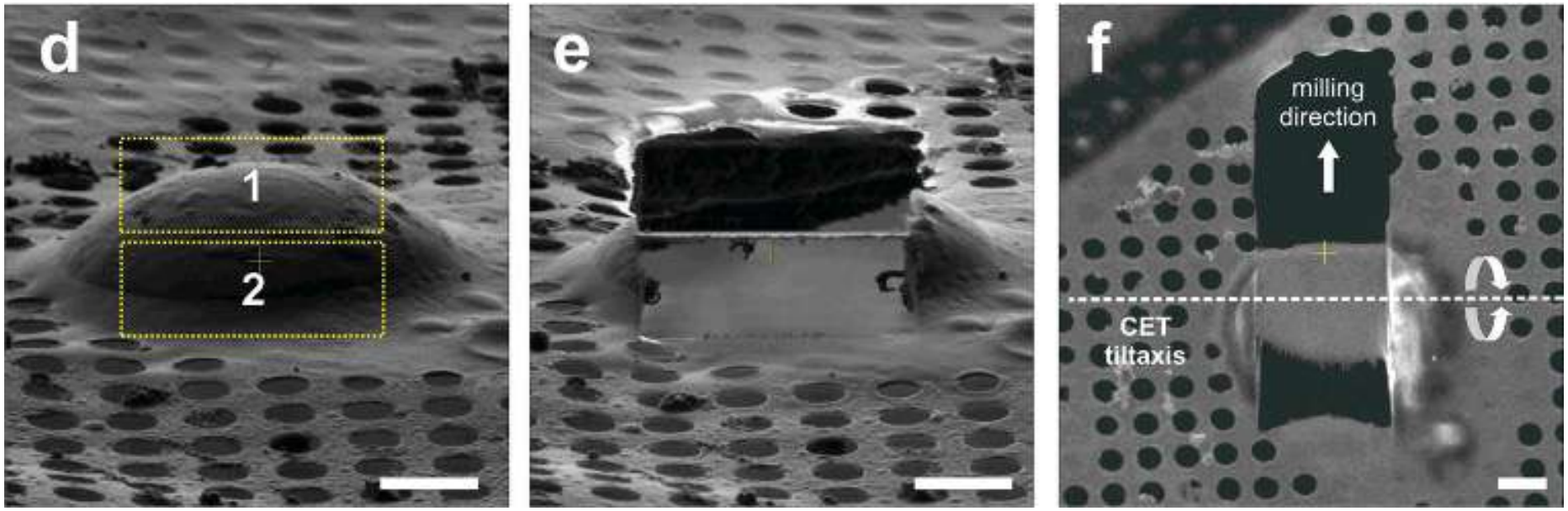


Image: Rigort and Plitzko, 2015

Milling

- In practice, milling is done in several steps
  - Rough cuts
  - Finer and finer polishing steps
  - Start at high current, finish at low current
  - Final step: additional 0.5° tilt to make lamellae even thickness throughout section
- Higher throughput
  - Target several regions and do rough mills
  - After all rough work is done, do final polishing and remove from SEM

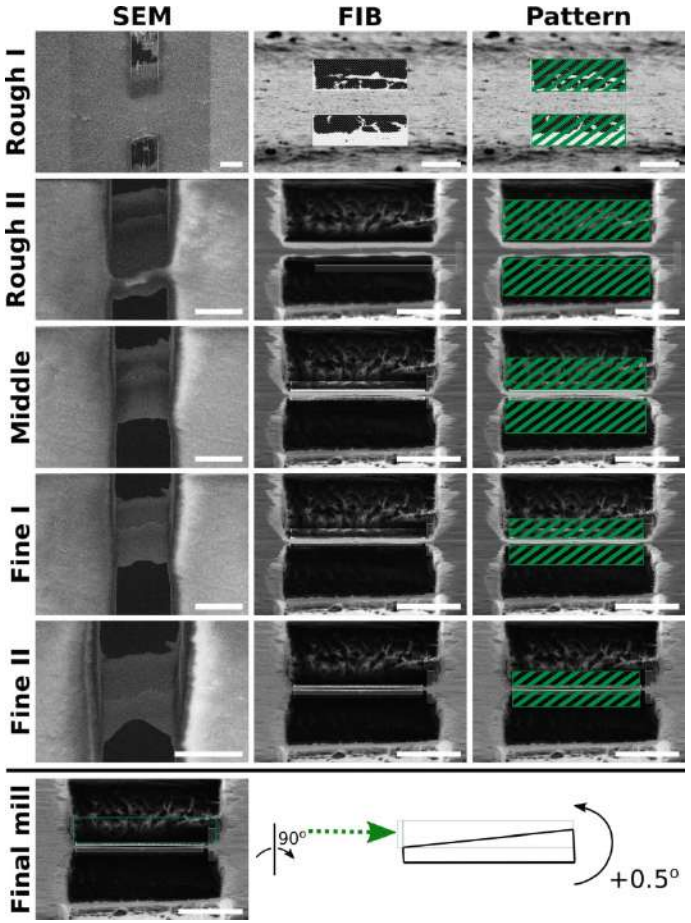


Image: Lam and Villa, 2021

Milling at as shallow an angle as possible

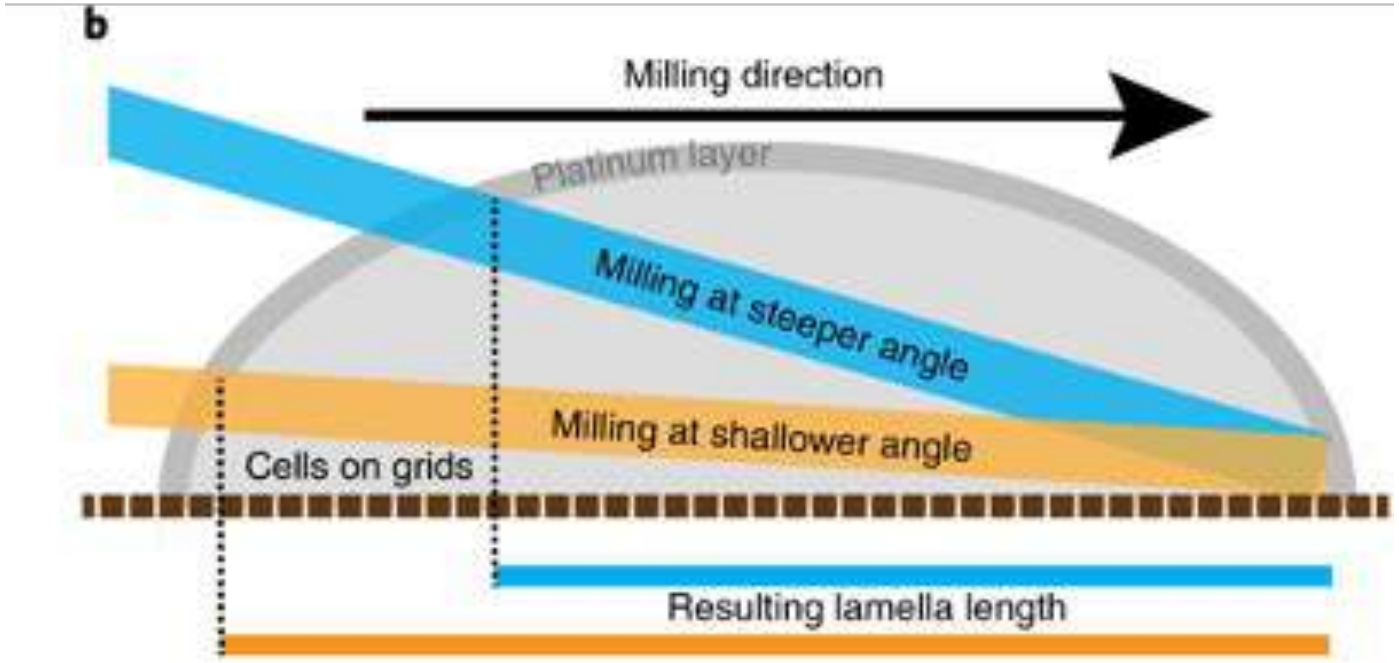


Image: Wagner *et al*, 2020

Geometry: Loading into TEM

- Sample needs to be loaded such that milling axis is perpendicular to microscope tilt axis

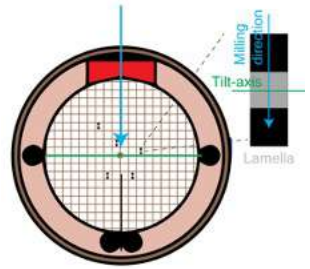


Image: Wagner *et al*, 2020

Ideal Result

- A: Image of prepared lamella using e-beam in FIB SEM
- B: Image of same region taken in Titan Krios. White arrows mark areas of correlation between (A) and (B). Solid black arrowhead: Pt from sputtering. Striped arrowhead: Pt from GIS. Green line shows the TEM tilt axis. White box: area for tilt-series acquisition. Asterisk: poor vitrification or contamination
- C: XY view of a reconstructed tomogram of a single cyanobacterium from the lamella.

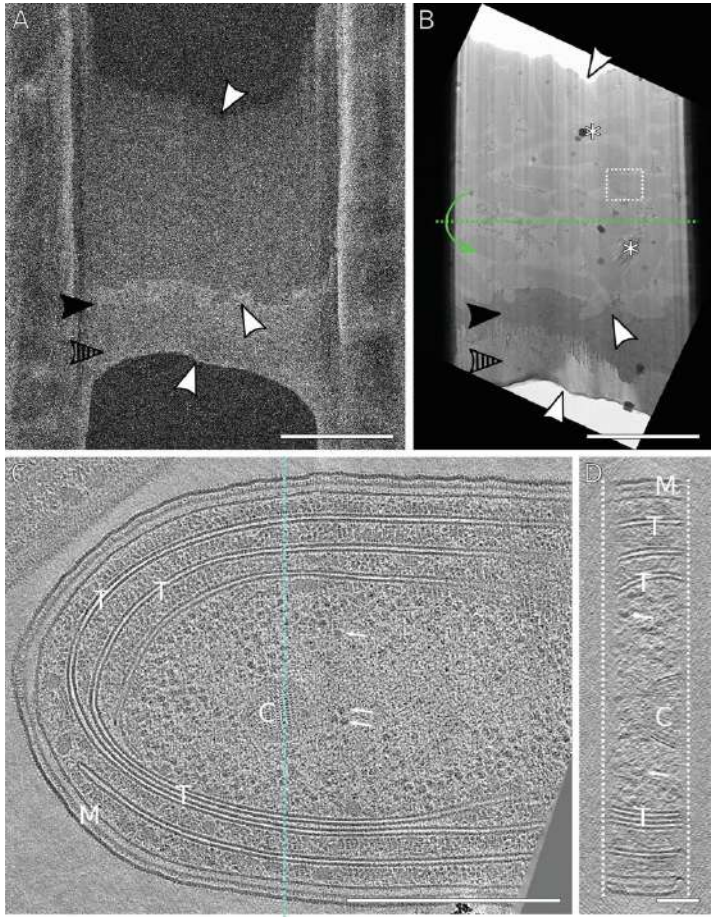
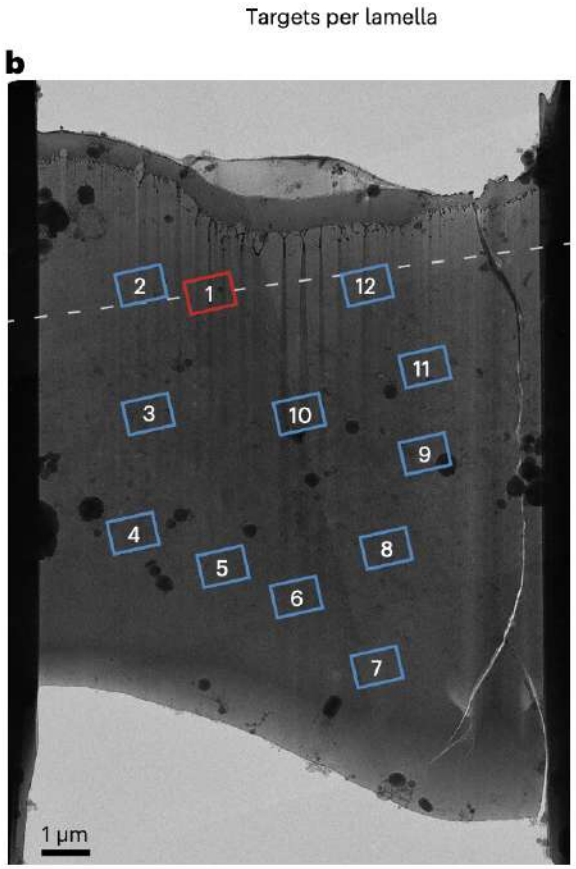


Image: Lam and Villa, 2021

Many areas can be collected on a single lamella



Article

https://doi.org/10.1038/s41592-022-01690-1

Parallel cryo electron tomography on in situ lamellae

Received: 22 March 2022

Accepted: 28 September 2022

Fabian Eisenstein[1], Haruaki Yanagisawa[1], Hiroka Kashihara[2], Masahide Kikkawa[1], Sachiko Tsukita[2] & Radostin Danev[1]

Where to mill?

- Unless all cells are the same, you need to be able to determine which are the target cells
- Also which part of the cell to keep
- Solution: Another microscope!
  - Fluorescent light microscopes with cryo stages are available
  - Need to have a long working distance, cannot use oil immersion, relatively high NA
  - Z signal is lowest resolution, confocal not available
  - Latest microscopes have software to import and correlate LM images with SEM images for localization
  - More transfers lead to increased danger of contamination / damage
  - Place FLM inside SEM chamber

Cryo-CLEM: Correlate points between images

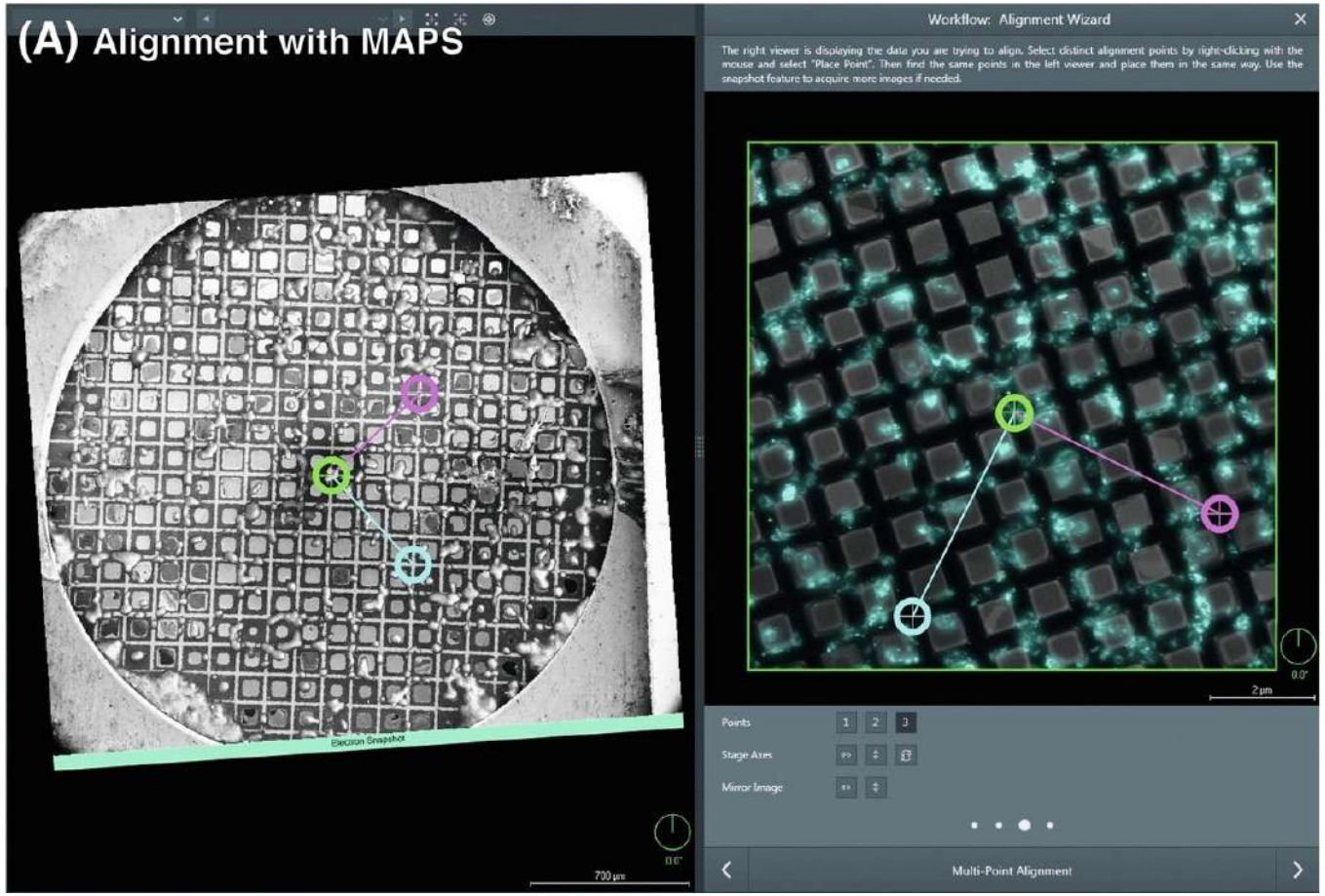


Image: Klein *et al*, 2021

Cryo-CLEM: Overlay

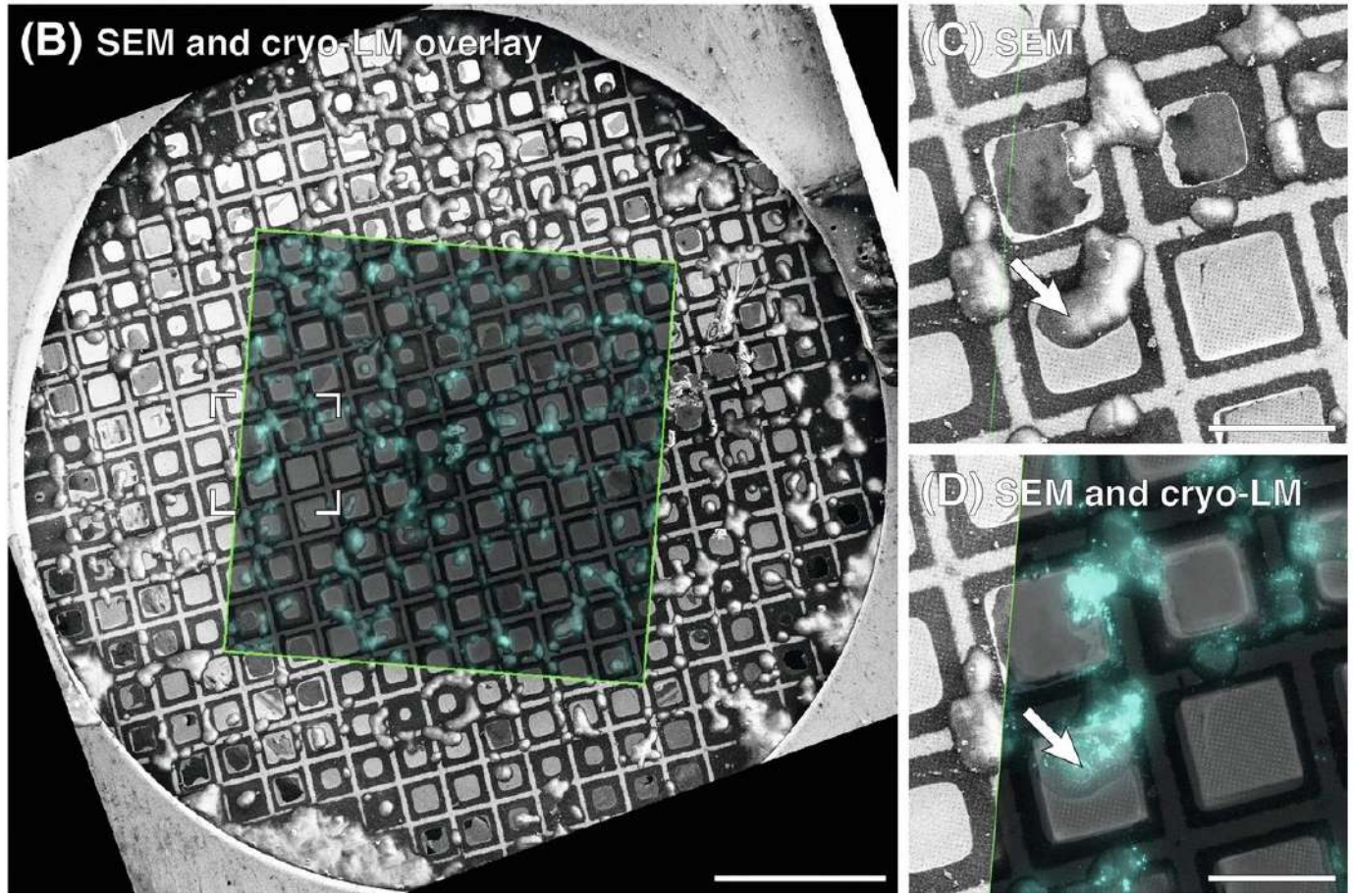
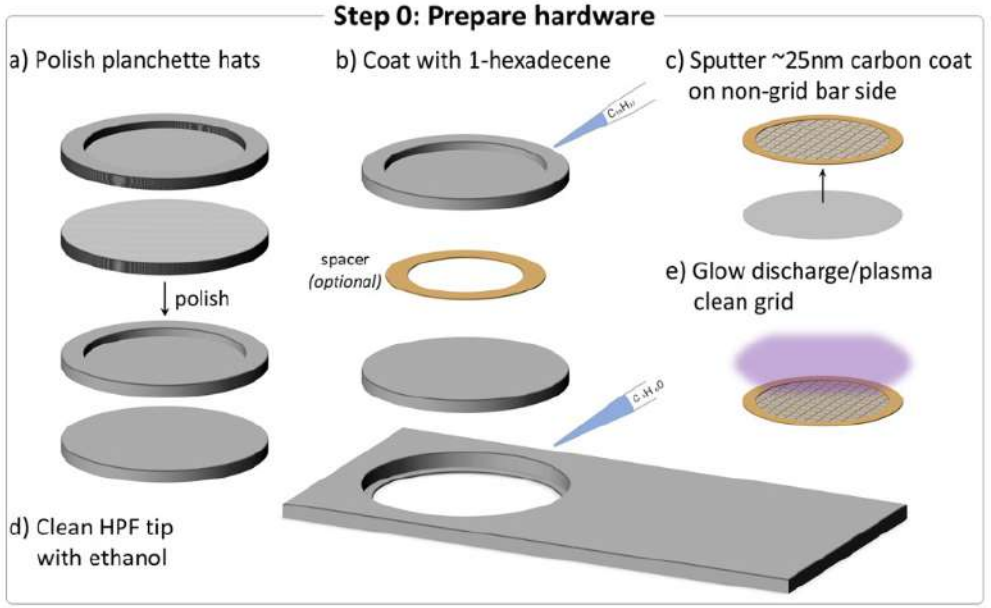


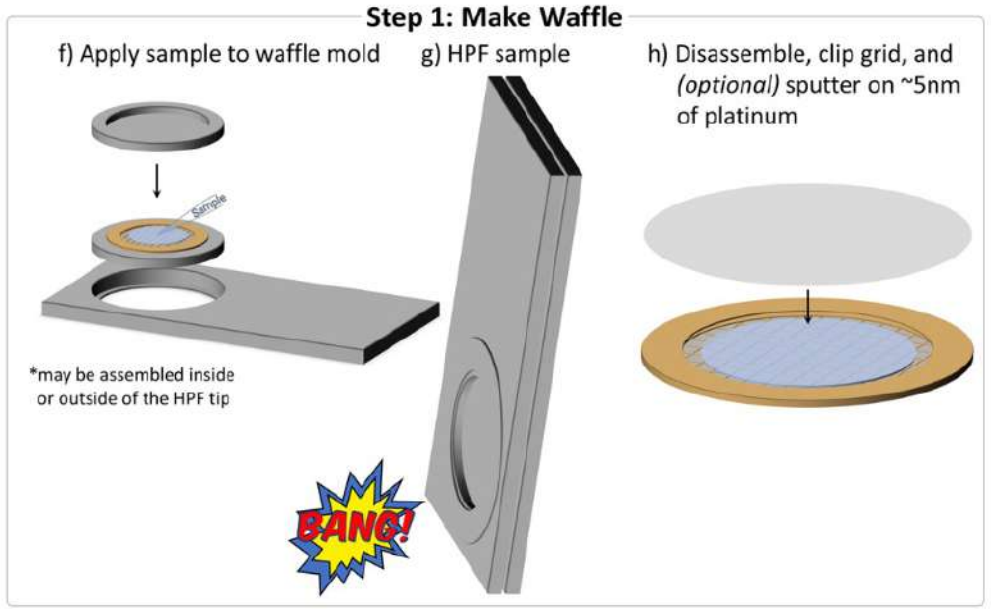
Image: Klein *et al*, 2021

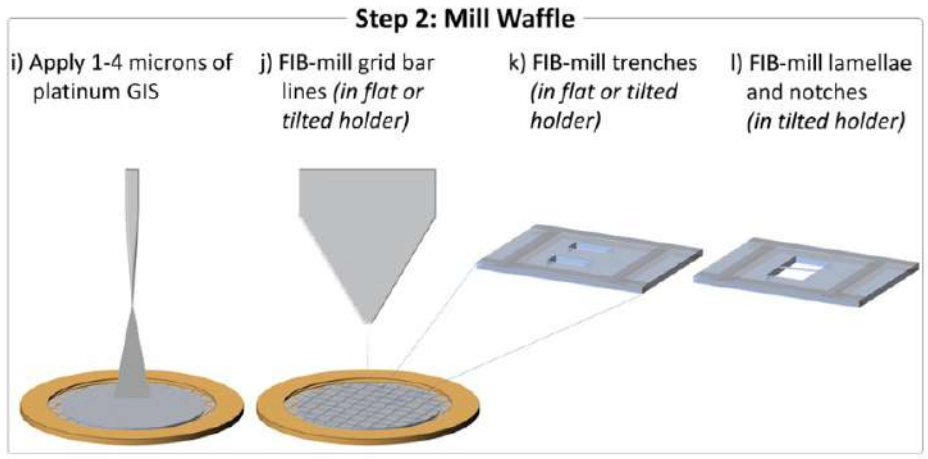
HPF on grids

Waffle Method: A general and flexible approach for improving throughput in FIB-milling

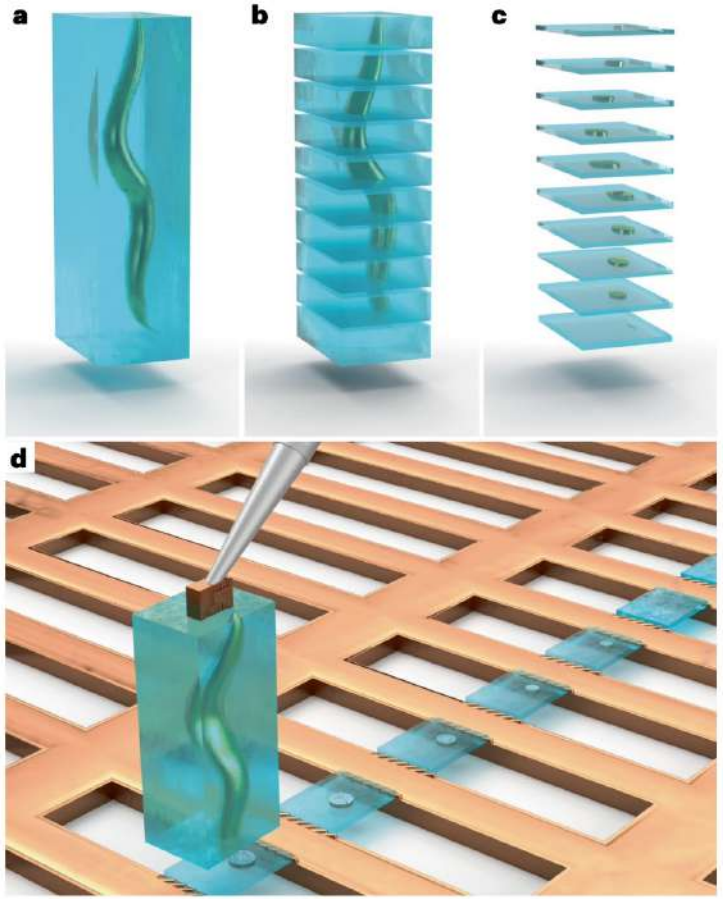
Kotaro Kelley[1,6], Ashleigh M. Raczkowski[1,4,5], Oleg Klykov[1,2,5], Pattana Jaroenlak[3,5], Daija Bobe[1,5], Mykhailo Kopylov[1], Edward T. Eng[1], Gira Bhabha[3], Clinton S. Potter[1,2], Bridget Carragher[1,3✉] & Alex J. Noble[1✉]







Serial Lift-out



nature methods

Article

https://doi.org/10.1038/s41592-023-02113-5

Serial Lift-Out: sampling the molecular anatomy of whole organisms

Received: 28 April 2023

Accepted: 25 October 2023

Published online: 18 December 2023

Oda Helene Schiøtz[1,6], Christoph J. O. Kaiser[1,6], Sven Klumpe[1,6] ✉, Dustin R. Morado[2,5], Matthias Poege[3], Jonathan Schneider[3], Florian Beck[1], David P. Klebl[2], Christopher Thompson[4] & Jürgen M. Plitzko[1] ✉

Serial Lift-out

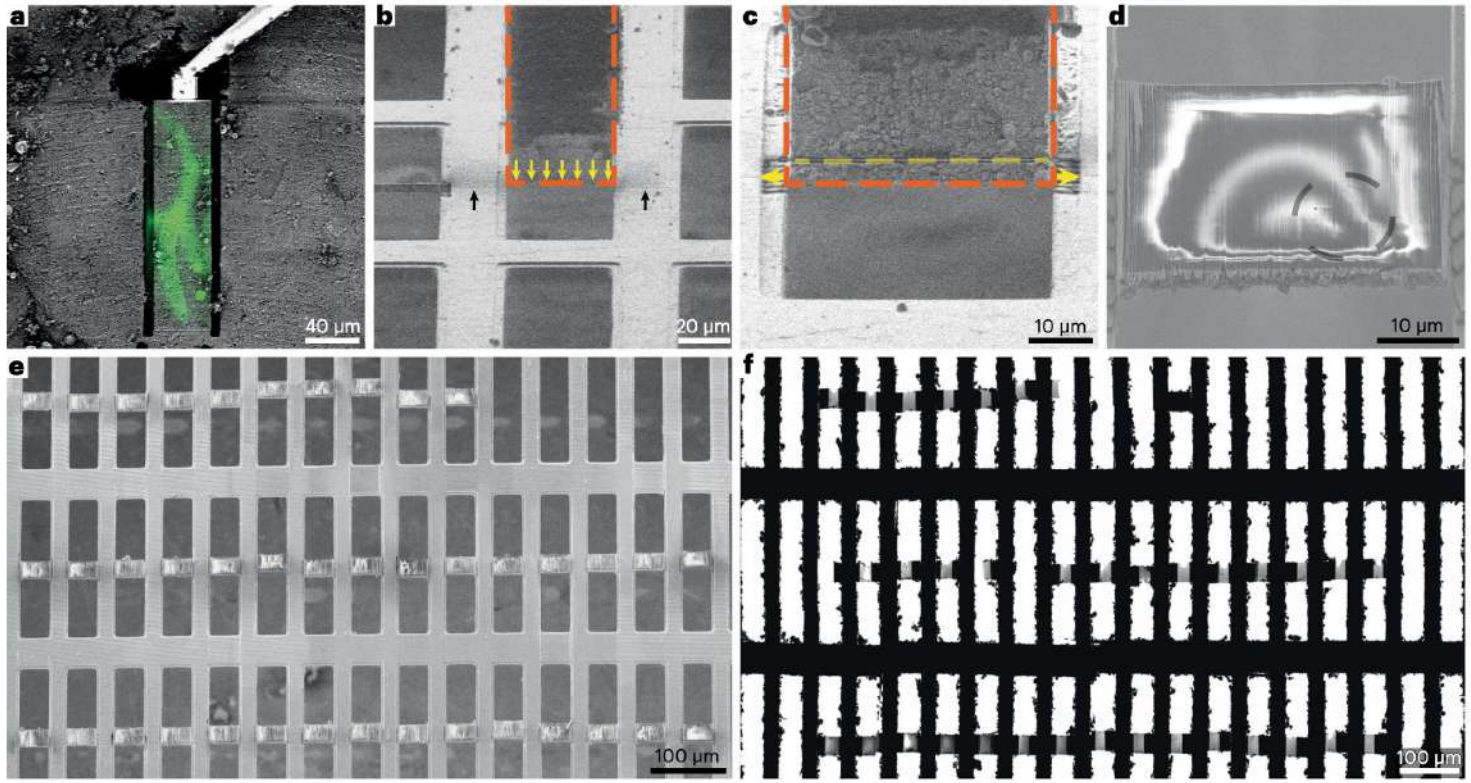
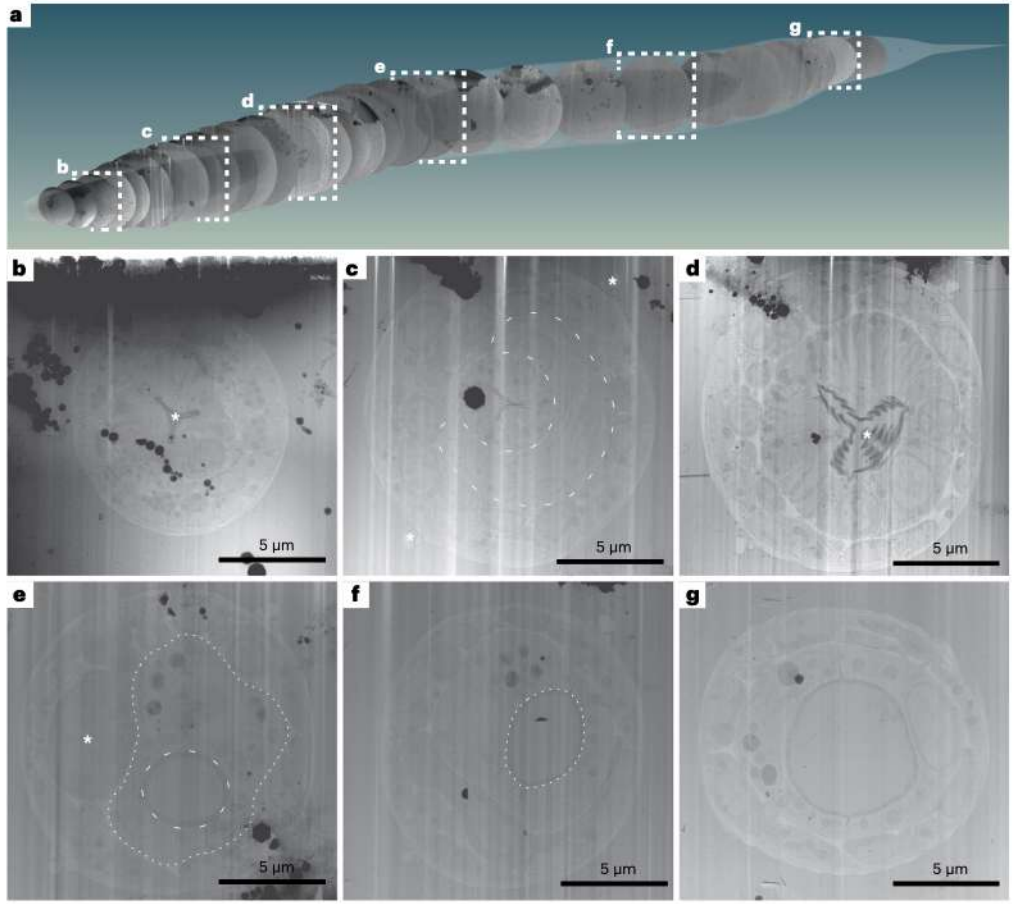


Fig. 2 | A workflow for double-sided attachment Serial Lift-Out. a, FIB image of the prepared extraction site with overlaid correlated fluorescence data (green) indicating the larva being targeted. The micromanipulator is attached to the extraction volume by redeposition from the copper adaptor (trench-milling orientation). **b**, The extracted volume (orange dashed line) is lowered into position between two grid bars in lamella-milling orientation. The lower front edge of the volume (yellow arrows) is aligned to the pre-milled line mark (black arrows). **c**, Double-sided attachment by redeposition from the grid bars (yellow arrows indicate direction of milling), followed by line pattern milling, releasing the section of a desired thickness (dashed yellow line). The orange dashed line indicates the outline of the extracted volume. **d**, SEM image of a typical section after being released from the extracted volume. The black dashed line indicates the outline of the worm cross-section. **e**,**f**, SEM (**e**) and TEM (**f**) overview images of the 40 double-sided attached sections and the resulting lamellae. Supplementary Video 1 summarizes the process. The data presented in this figure stem from experiment 2.

Serial Lift-out



Using plasma instead of Ga ions

Article

https://doi.org/10.1038/s41467-023-36372-9

Plasma FIB milling for the determination of structures in situ

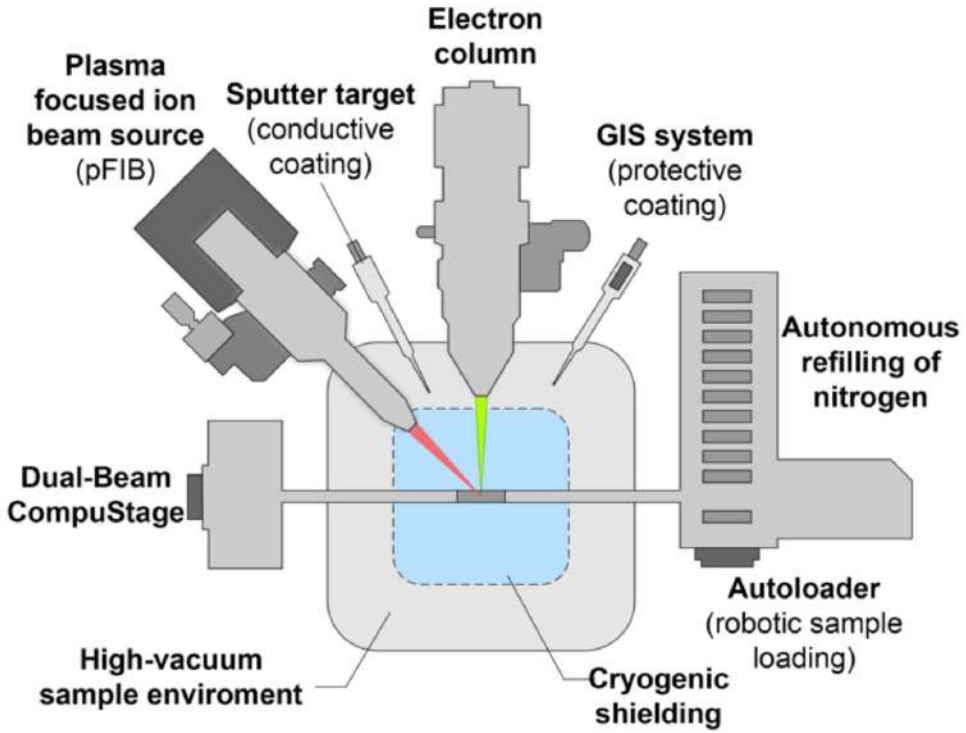
Received: 9 August 2022

Accepted: 26 January 2023

Published online: 06 February 2023

Casper Berger[1,2,5], Maud Dumoux[1,5], Thomas Glen[1,5], Neville B.-y. Yee[3], John M. Mitchels[4], Zuzana Patáková[4], Michele C. Darrow[3], James H. Naismith[1,2] & Michael Grange[1,2]

Plasma instead of Ga



Berger et al, 2023

- Typical Gases: Ar, Xe, O_2, N_2
- No worry about deposition/implantation
- Faster bulk material removal
- More expensive instrument

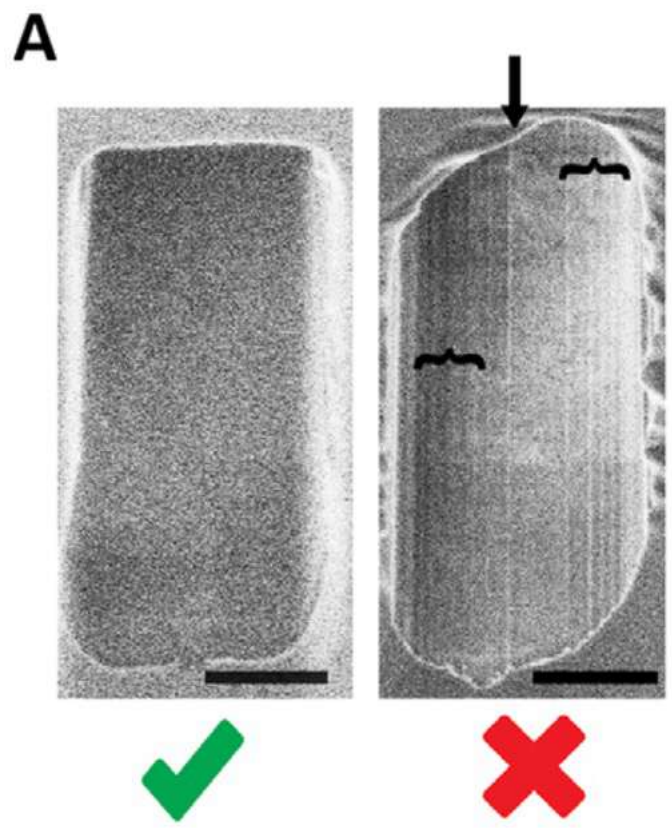
Milling Rates for Plasma (Berger et al, 2023)

Beam	Xe	N	O	Ar	Ga
Milling rate (μm^3/nC)	16.7 +/- 0.2	10.6 +/- 0.2	10.0 +/- 0.4	4.3 +/- 0.1	7.7

Difficulties / Issues

- Geometry: Need a cryo stage which will rotate and tilt with as much freedom as possible
- Sample Charging
  - Pre-coat with Pt Sputter coat
  - Perhaps post-coat wth PT sputter as well
- Curtaining due to uneven milling
  - Cover with organic Pt layer to provide even surface
- Lamella Bending
  - Cut notches for stress relief
- Contamination
  - Vacuum is much worse than inside a TEM, contamination buildup limits the number of lamella which can be produced
  - All sample transfer steps have the danger of adding contamination

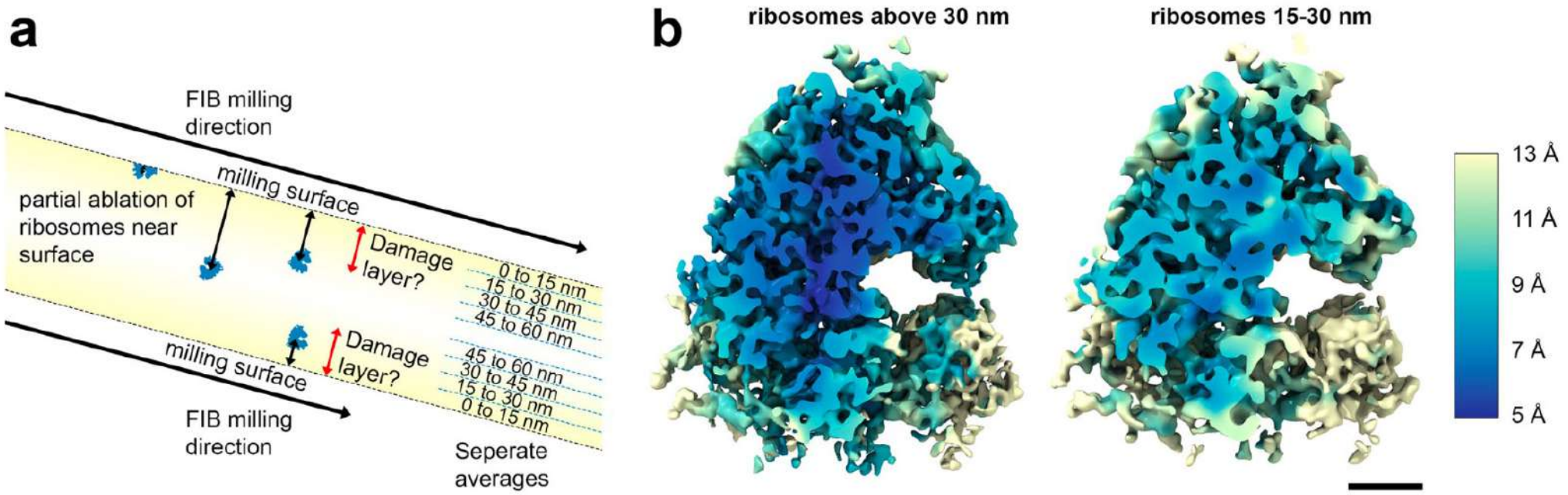
Curtaining



Dumoux et al, elife 2023

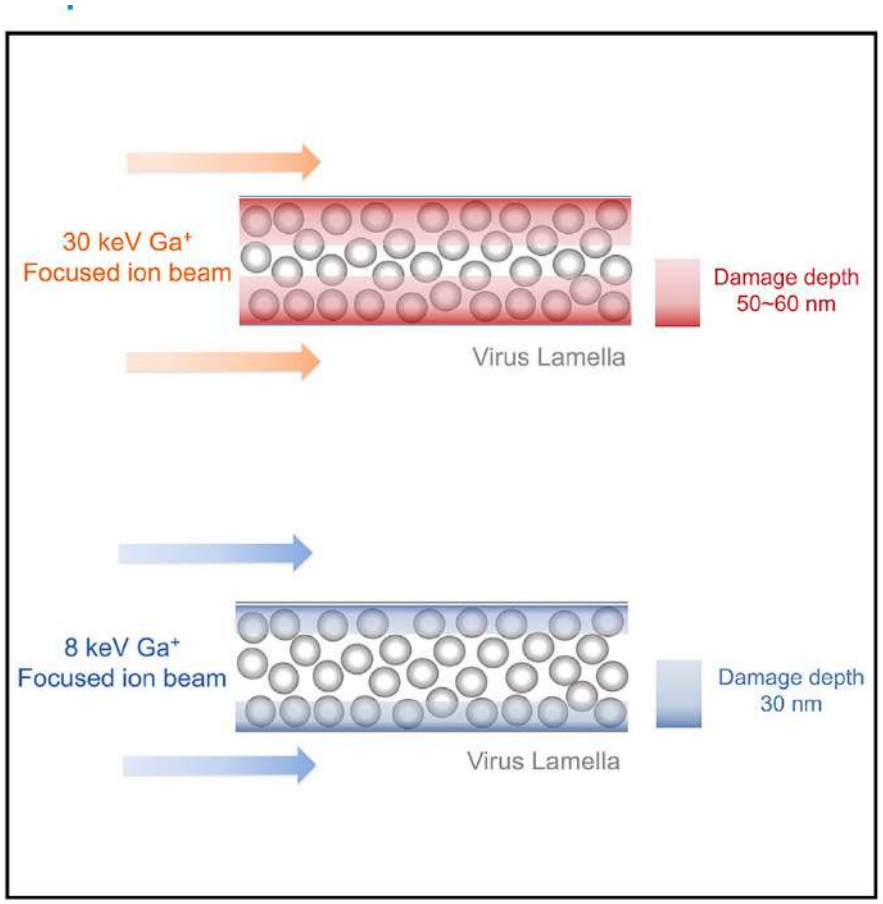
- Ideally the cutting from the ion beam will leave a perfectly flat face
- Uneven interactions with the surface can result in uneven milling which shows up as “curtains”

Damage near the lamella surface



Berger et al, 2023

Lower energy beams may cause less damage near surface



Article

The reduction of FIB damage on cryo-lamella by lowering energy of ion beam revealed by a quantitative analysis

Qi Yang,[1,2,3] Chunling Wu,[1,3] Dongjie Zhu,[1] Junxi Li,[1,2] Jing Cheng,[1] and Xinzheng Zhang[1,4,*]
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https://doi.org/10.1016/j.str.2023.07.002

Damage near surface: materials science

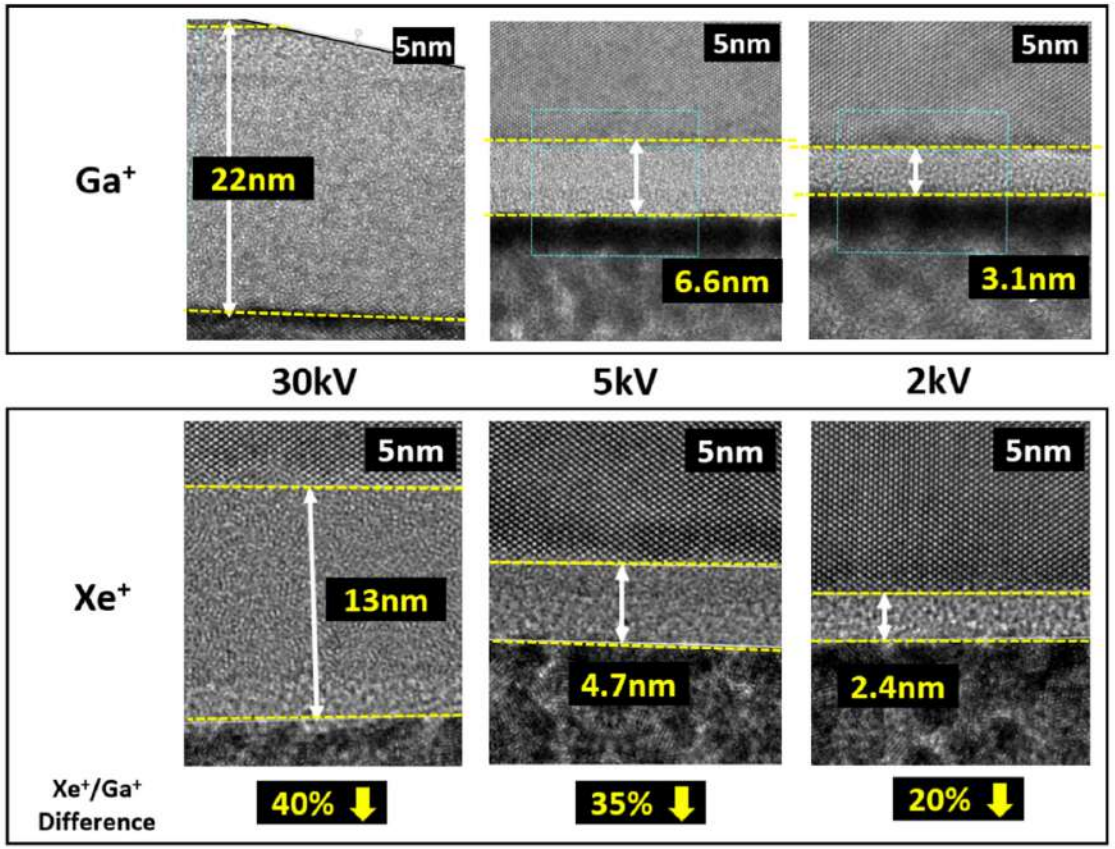

Fig. 3. TEM images showing the depth of amorphization of Si prepared at 30 kV, 5 kV and 2 kV comparing results from Xe^+ and Ga^+ FIB at 30 kV, 5 kV and 2 kV [35].

Full Length Article

Large volume serial section tomography by Xe Plasma FIB dual beam microscopy

T.L. Burnett [a,b], R. Kelley [c], B. Winiarski [a,b], L. Contreras [c], M. Daly [a], A. Gholinia [a], M.G. Burke [a], P.J. Withers [a,d,*]

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[b] FEI Company, Achtseweg Noord 5, Bldg, 5651 GG, Eindhoven, The Netherlands
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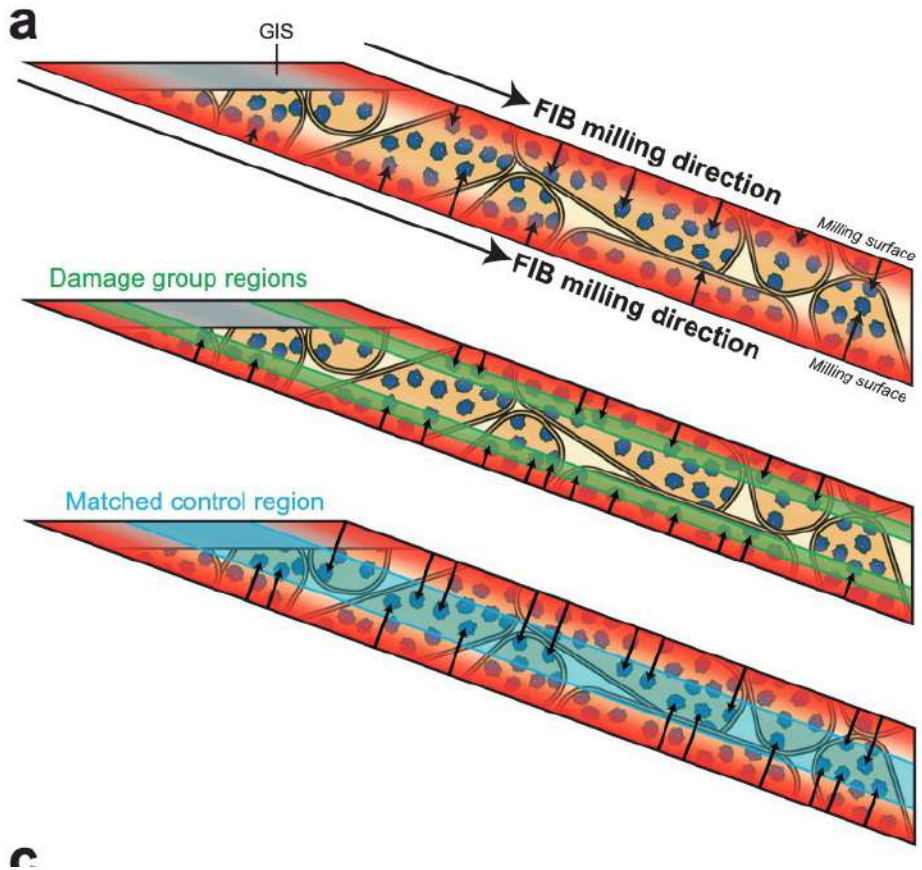
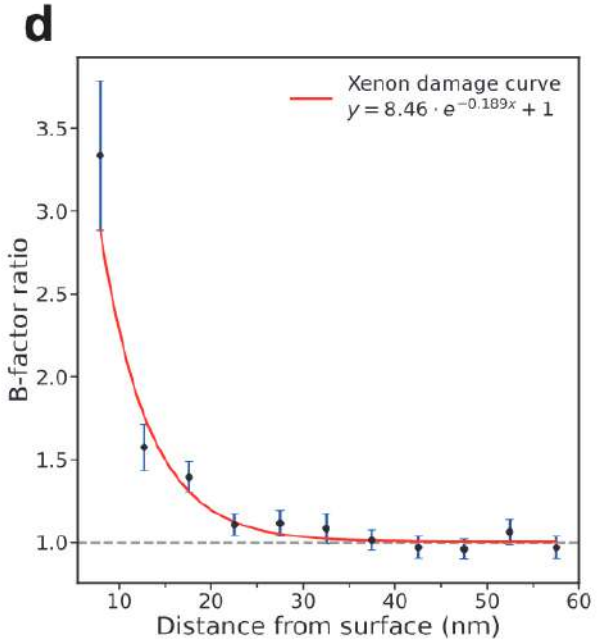
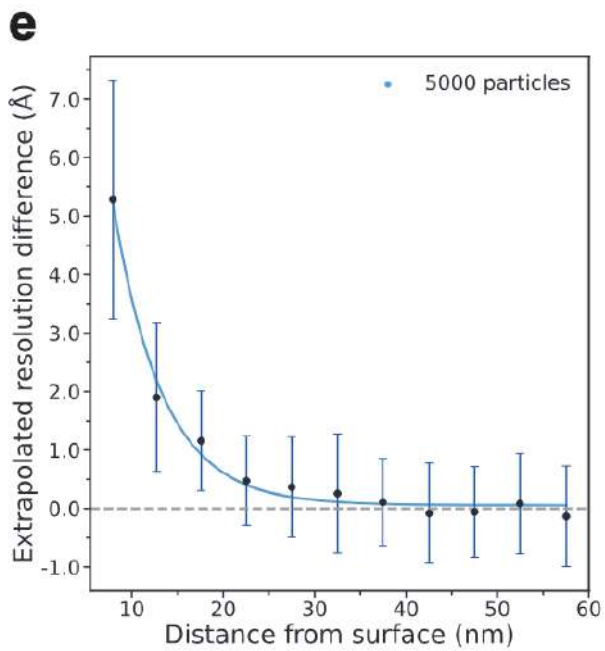
[d] BP International Centre for Advanced Materials, University of Manchester, Manchester M13 9PL, UK

Damage near surface: Xe plasma beam







Article
https://doi.org/10.1038/s41467-025-57493-3

Xenon plasma focused ion beam lamella fabrication on high-pressure frozen specimens for structural cell biology

Received: 18 June 2024
Accepted: 24 February 2025

Casper Berger[1,5], Helena Watson[1,2,5], James H. Naismith[1,3], Maud Dumoux[1] & Michael Grange[1,4]

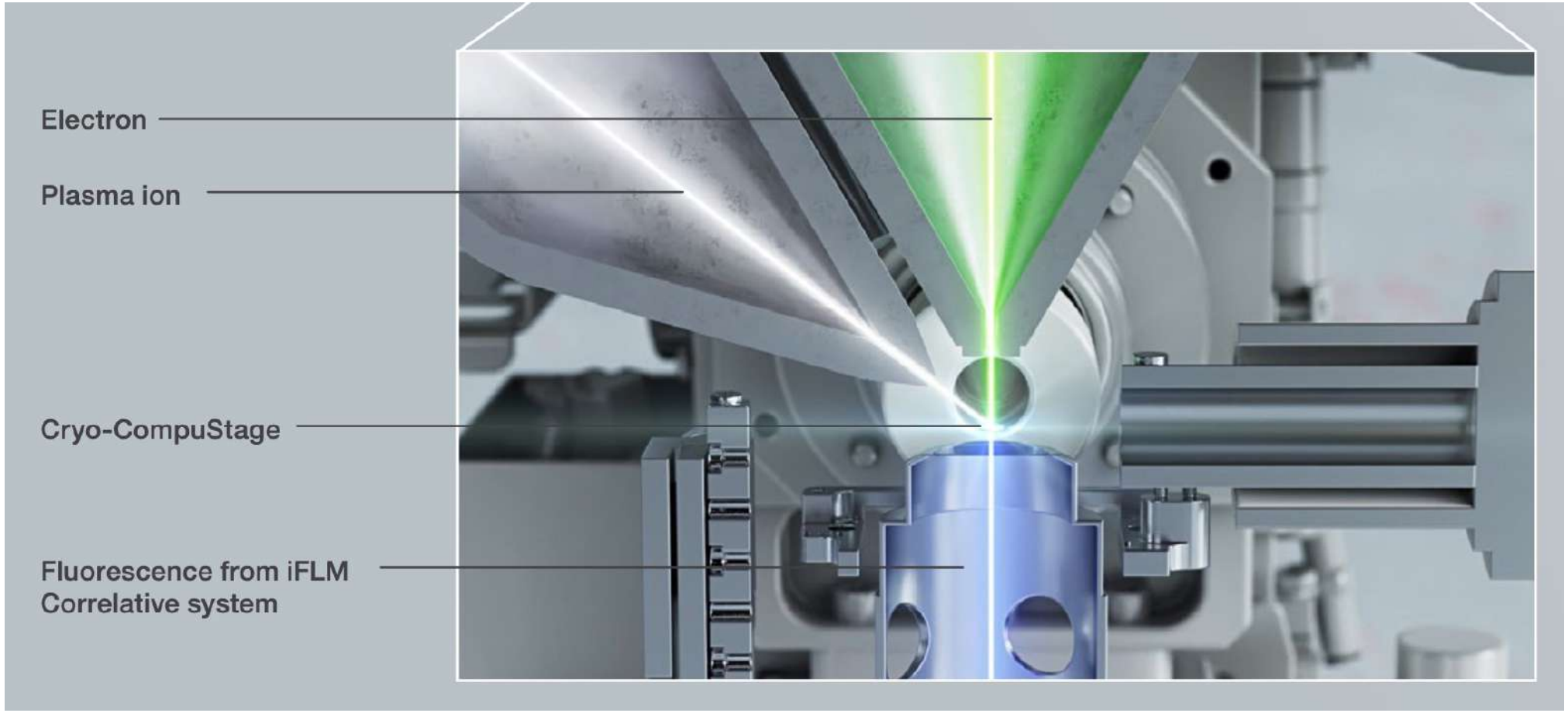
Summary: Equipment and expertise needed

- FIB SEM
  - Cryo stage with full rotation
  - GIS or plasma (Ar/Xe/O_2/N_2) source
  - Sputter coater
  - Shuttles and transfer equipment
  - Software for mapping and overlaying signals
  - Integrated FLM
- Cryo LM
  - Compatible cryo stage
  - Fluorescent signal detection
  - Shuttles and transfer equipment
- TEM
  - Suitable for high resolution tomography
  - 300 keV, direct detector, energy filter

Specialized Microscopes: Arctis Plasma FIB

- Dedicated to lamella generation only
- Autoloader system
- Small chamber
- Plasma FIB instead of Ga

Arctis Plasma FIB



Questions

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