

FIB SEM

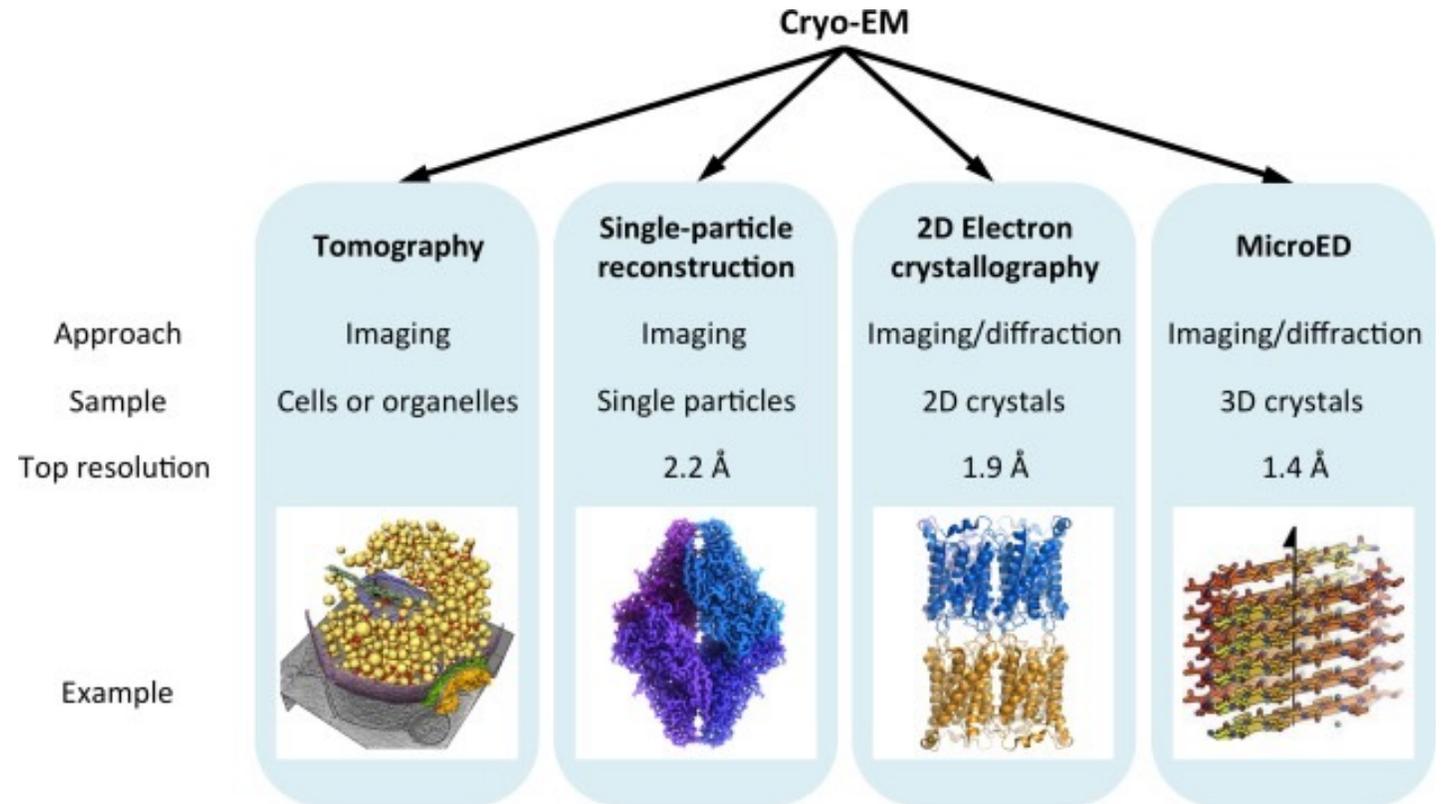


March 9, 2026

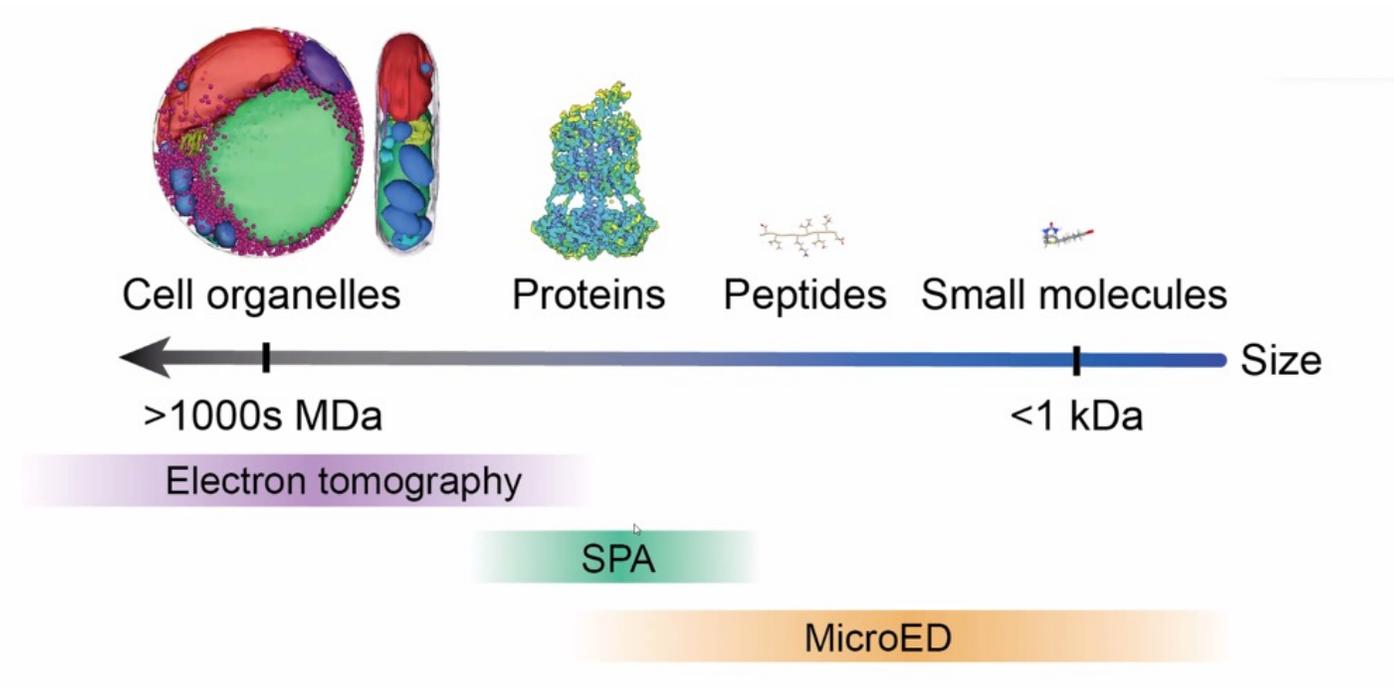
William Rice, NYU Langone Health

	3/5/2015	HV	HFWD	WD	 4 μm
	3:32:04 PM	2.00 kV	12.4 μm	3.9 mm	

Cryo-EM Imaging Modalities



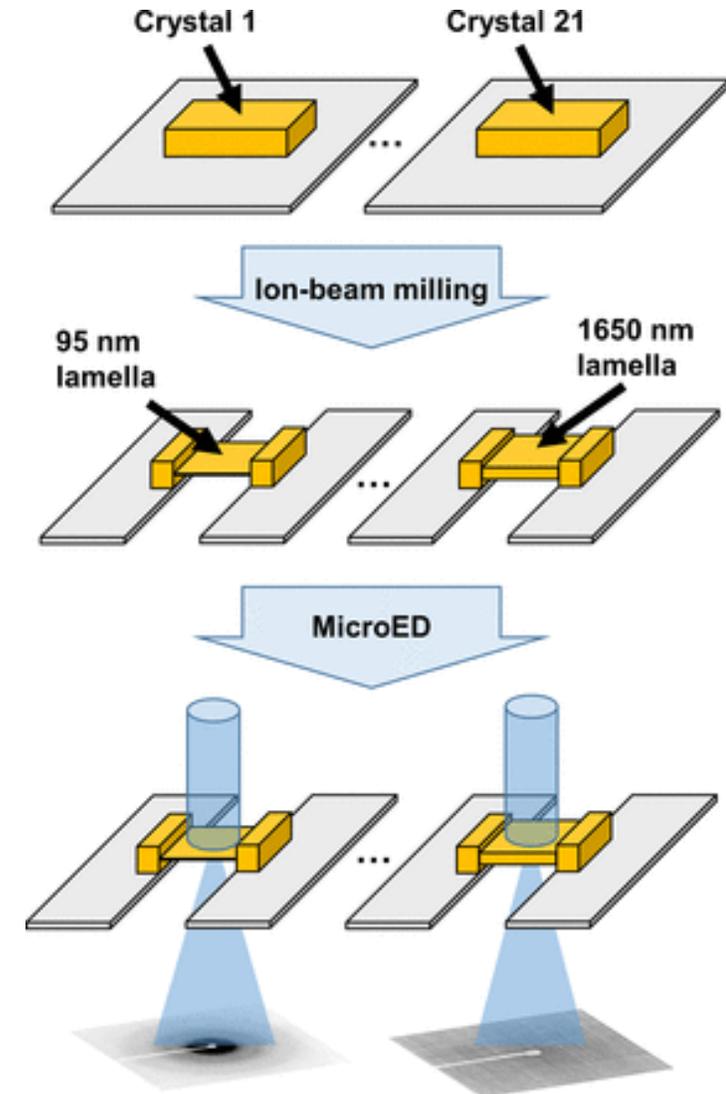
Target Sizes



Sample thickness for TEM

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

Thickness increases by a factor of $1/\cos(\theta)$
60° tilt: twice as thick as nominal untilted

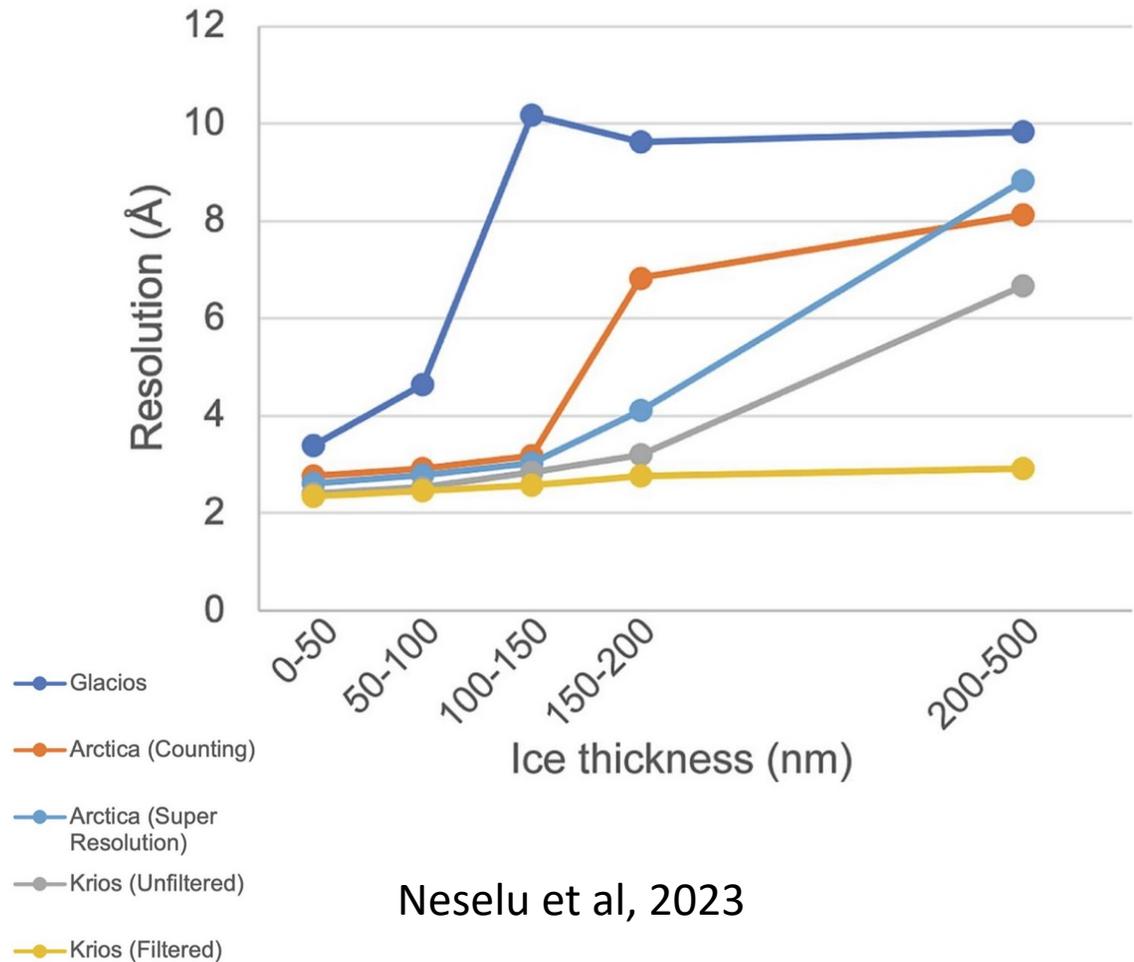


Typical sample thickness

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

(A)

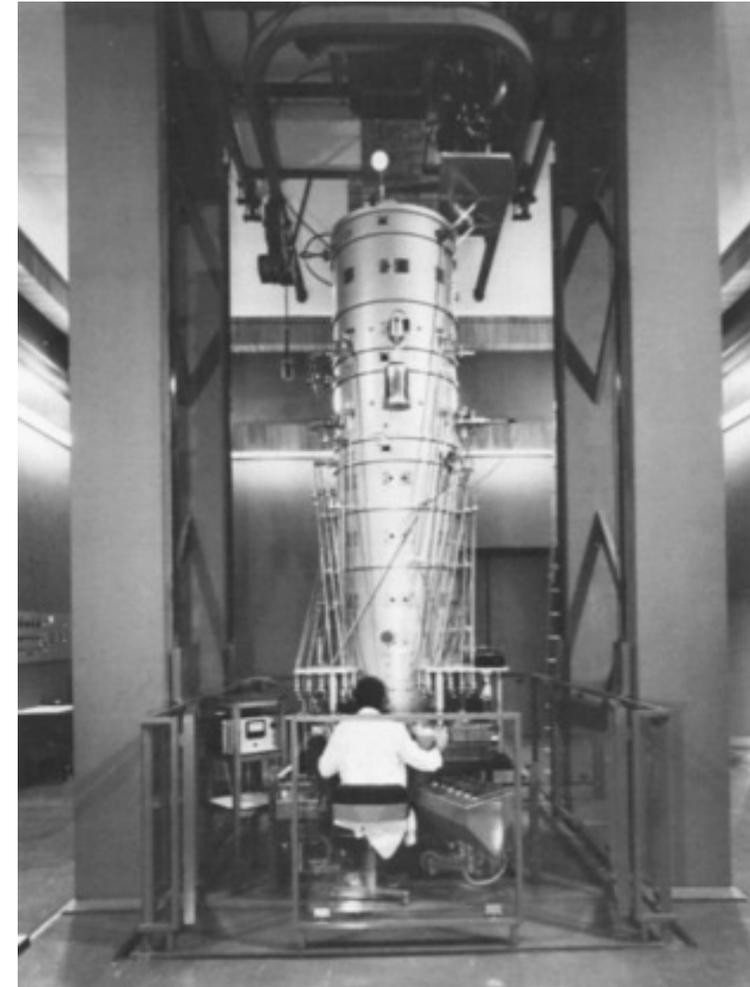
Resolution vs ice thickness



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

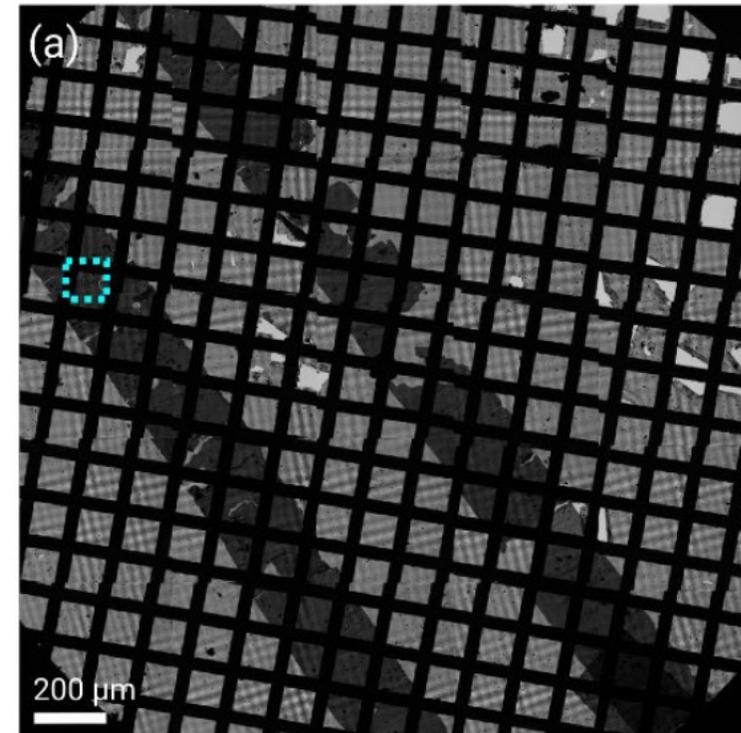


<https://doi.org/10.1016/bs.aiep.2021.08.008>.

(<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

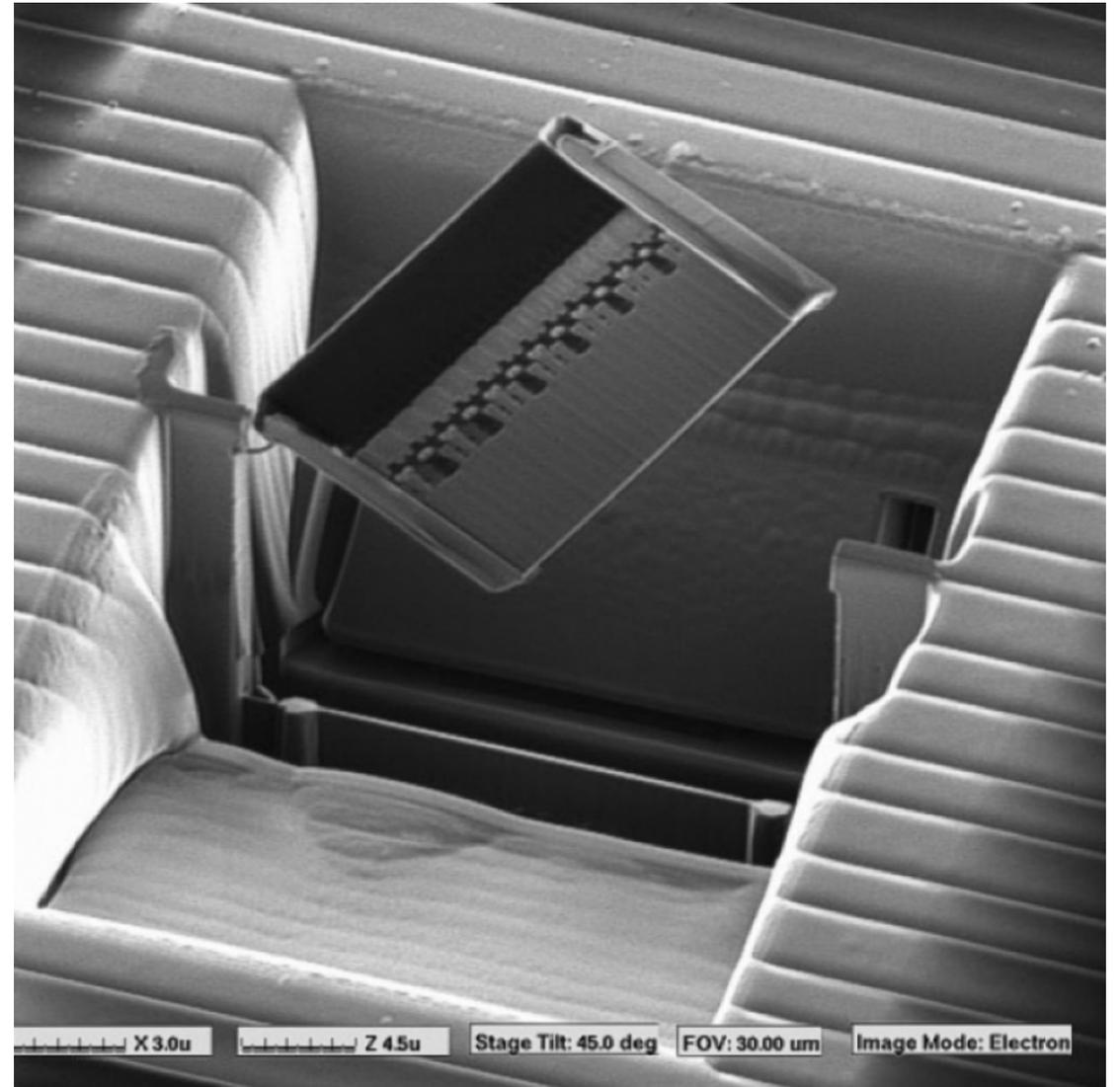
 Johannes Eiferich,  Marek Kaminek,  Lingli Kong,  Adolfo Odriozola,  Wanda Kukulska,  Benoît Zuber,  Nikolaus Grigorieff

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

This article is a preprint and has not been certified by peer review [what does this mean?].

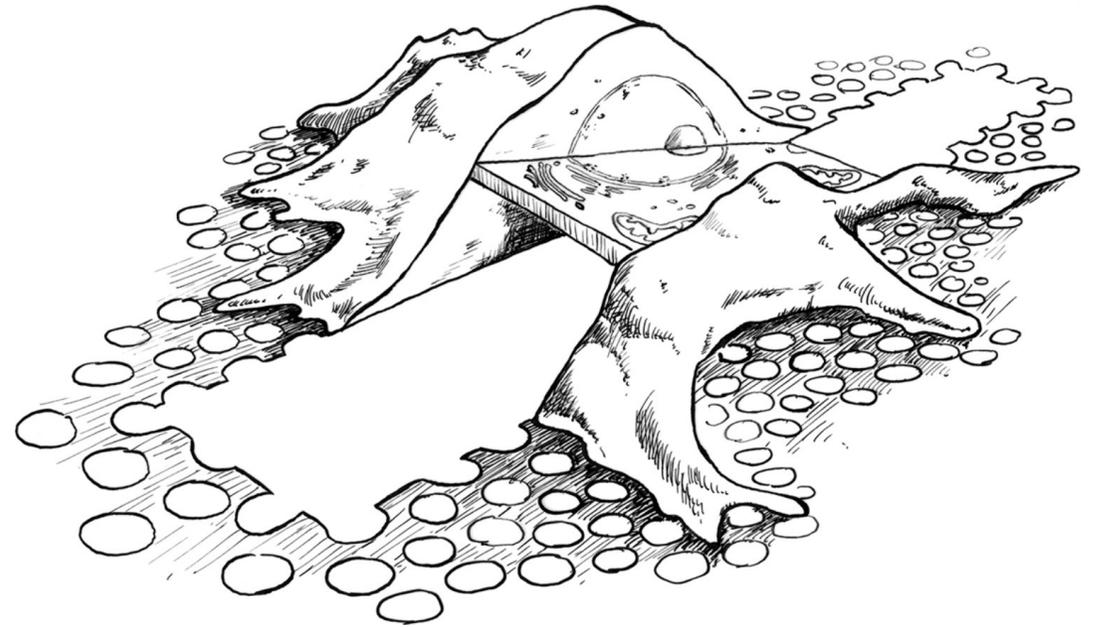
FIB SEM

- 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



Freezing thin samples on grids

Leica EM GP single sided blotting

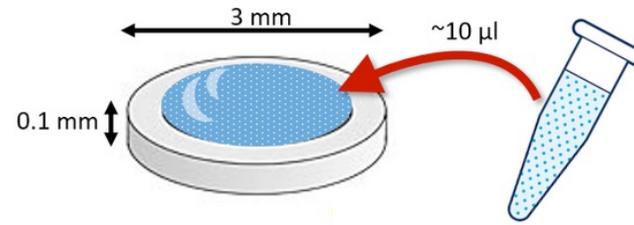


Vitrobot, can be set up for single sided blotting

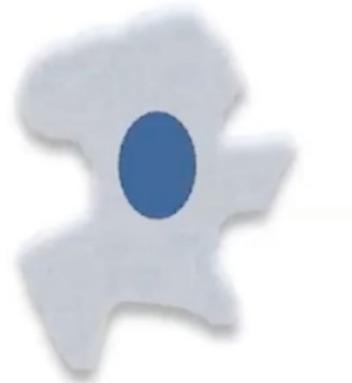
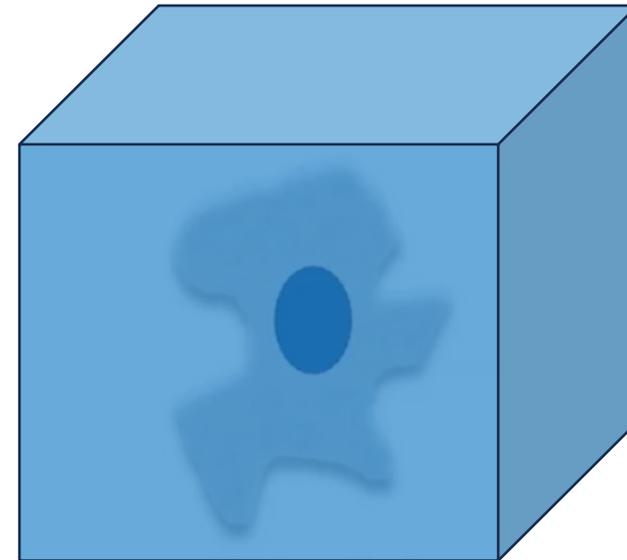
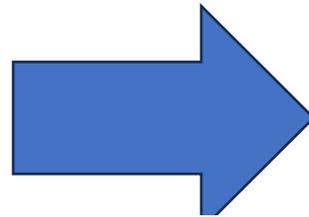


Freezing thick ($> 5-10 \mu\text{m}$) Samples

Frozen Samples



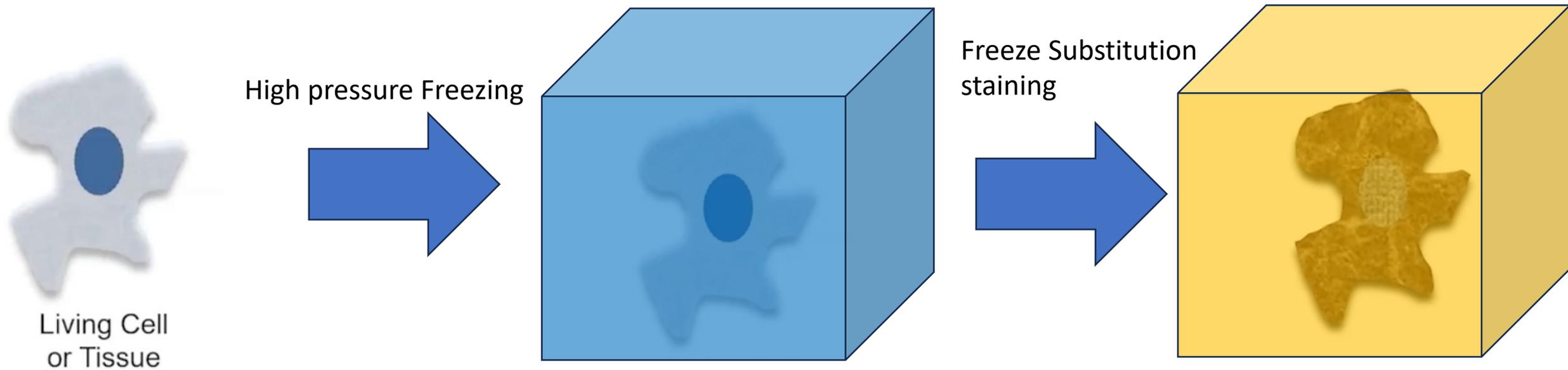
High pressure Freezing



Living Cell
or Tissue



Freeze Substituted Samples



Living Cell
or Tissue

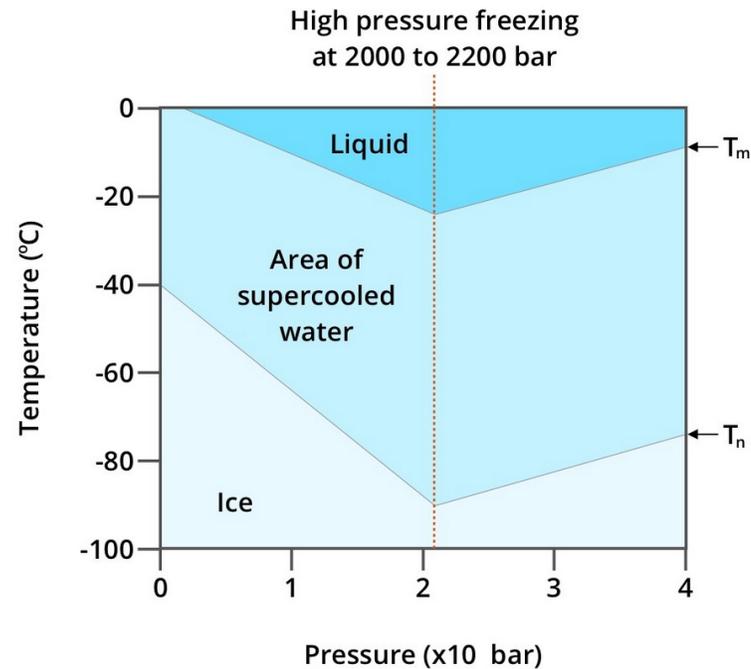
High pressure Freezing

Freeze Substitution
staining

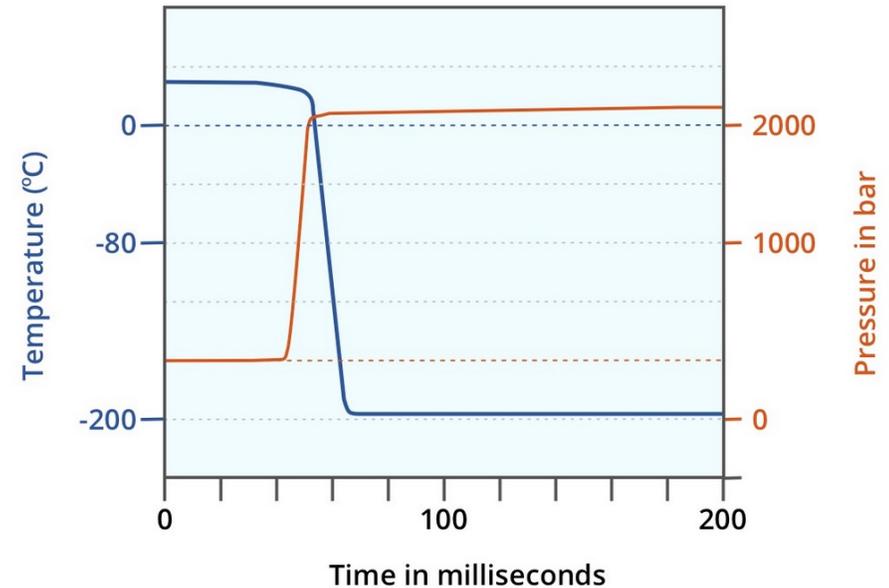
Size limit: $\sim 200 \mu\text{m}$ thick

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.

High Pressure Freezing: Planchettes

https://myscope.training/CRYO_High_pressure_freezing

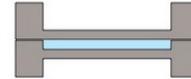
100/200 μm^*



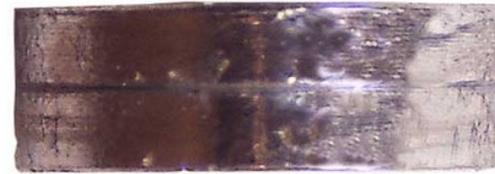
300 μm /flat*



100 μm
assembled



300 μm
assembled

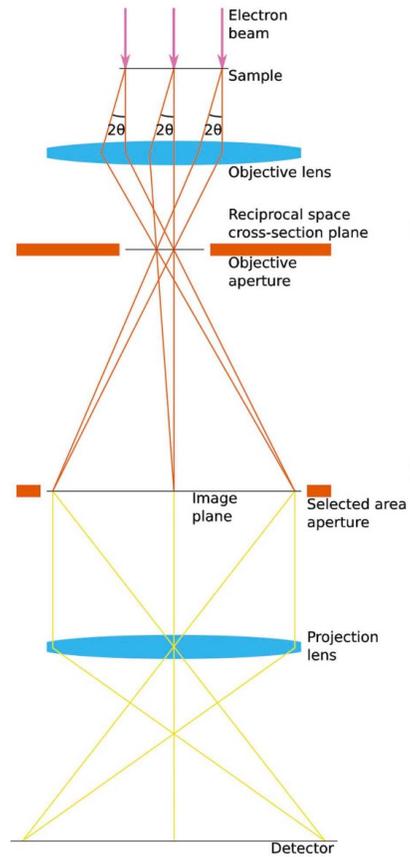


FIB SEM:

Start with
SEM Basics

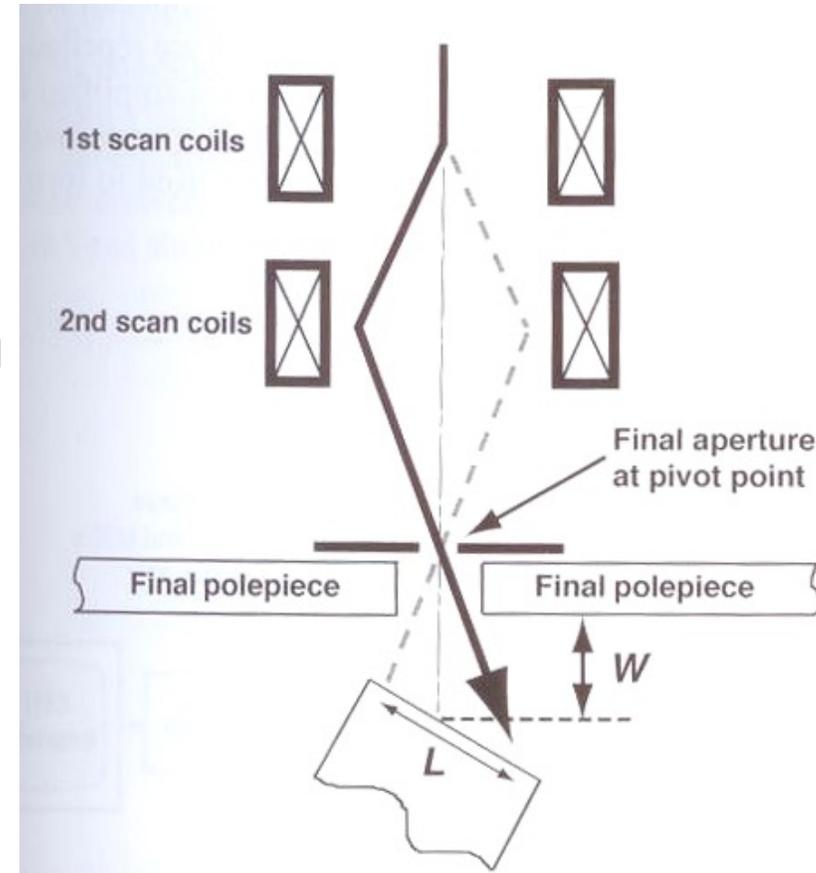
SEM versus TEM

TEM



Projection through sample

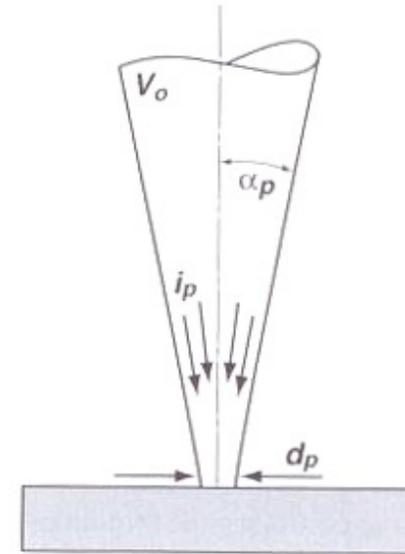
SEM



Surface imaging

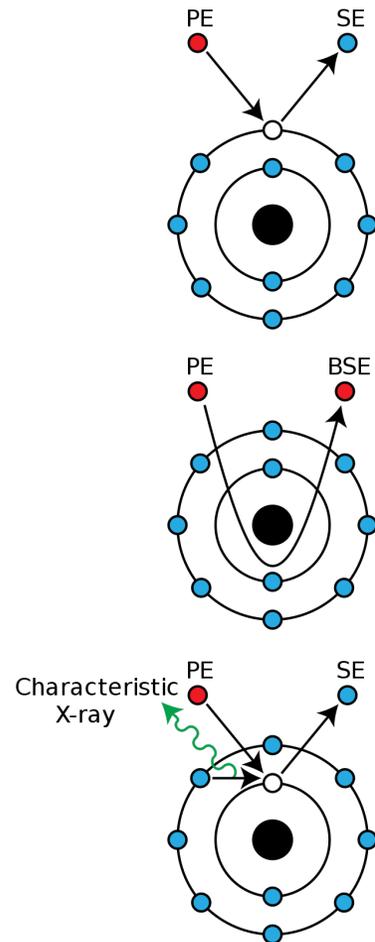
SEM Beam: probe size

- ◆ 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)

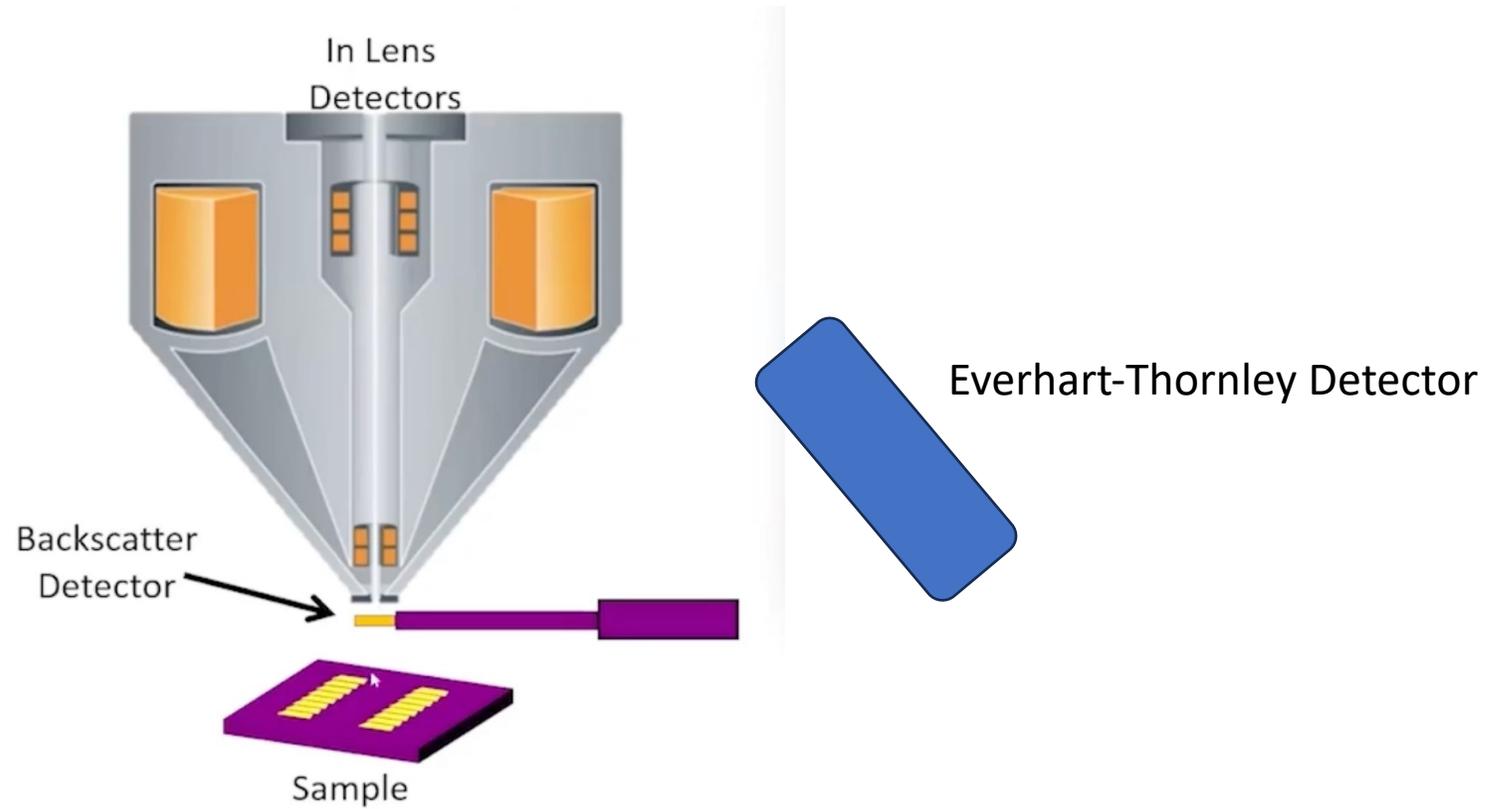


Goldstein et al, 2003

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

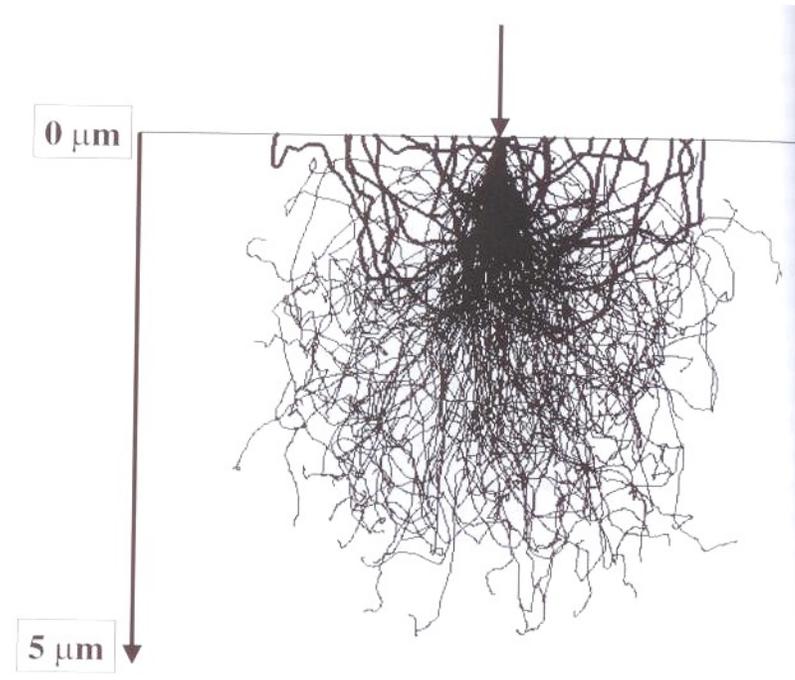


Detector Setup for SEM



Beam-Specimen Interaction

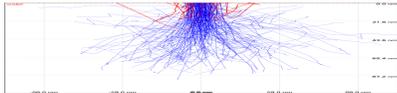
- Monte Carlo simulation of a 20 keV beam in Si
 - Dark traces: electrons which left the sample (BSE's)
- Electrons may be scattered elastically or inelastically
- Probability of elastic scattering $\sim Z^2$
- Inelastic scattering:
 - Secondary electrons
 - X-rays



Goldstein et al, 2003

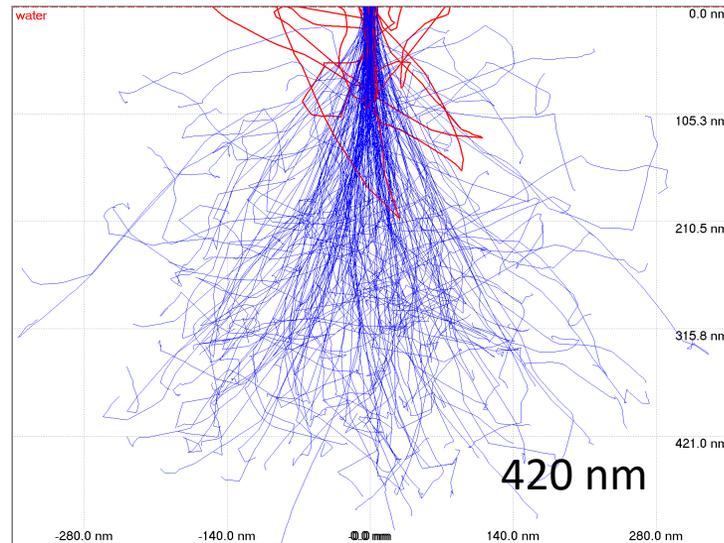
Monte Carlo simulation: water

1 KEV



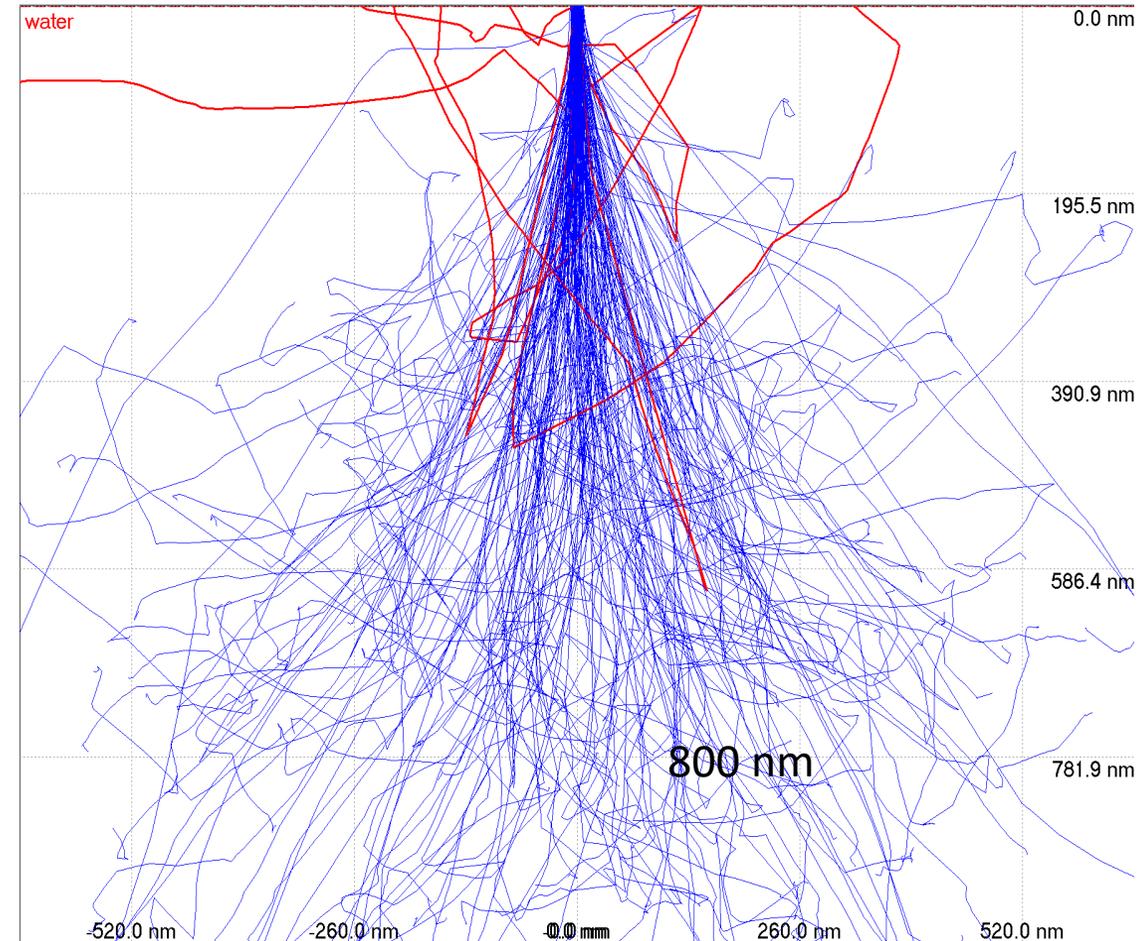
87 nm

3 KEV



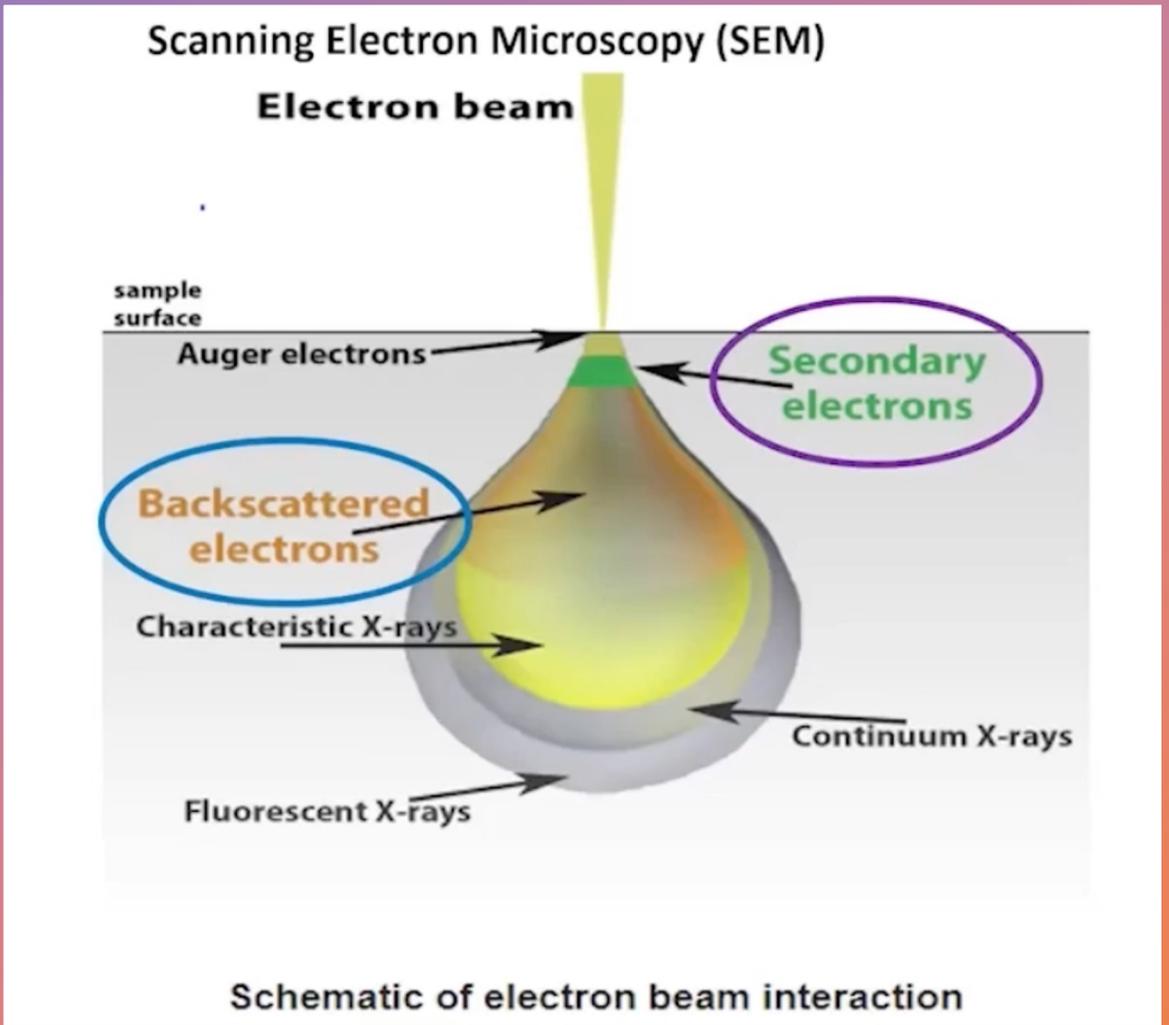
420 nm

5 KEV

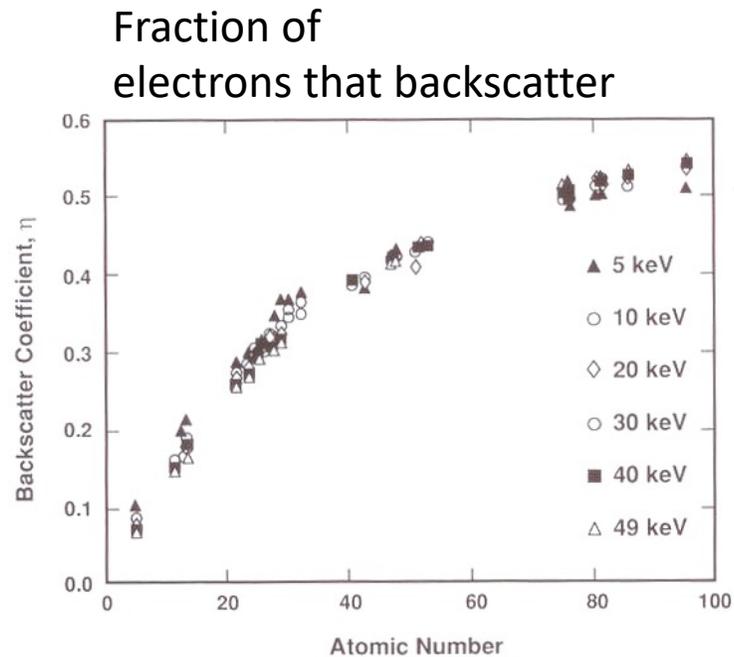


800 nm

Interaction Volume

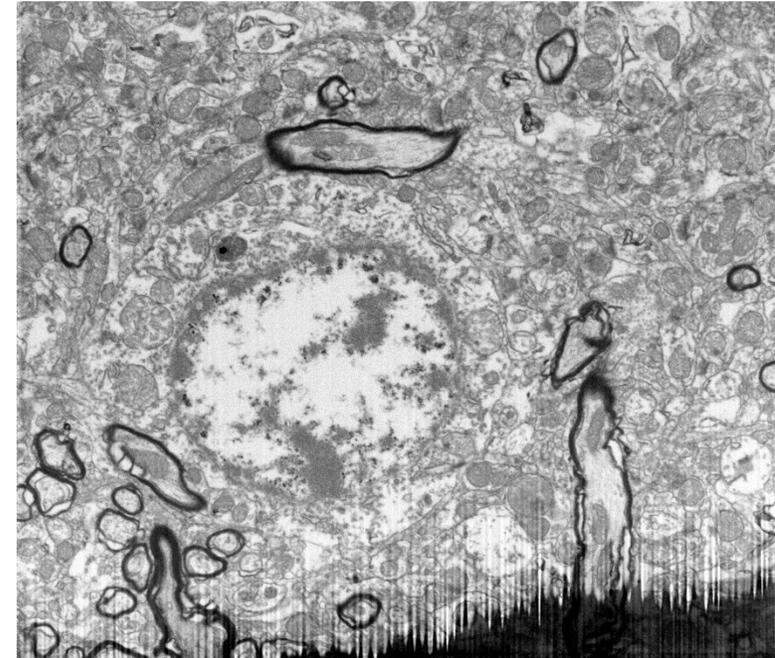


BSE efficiency is material dependent, voltage independent



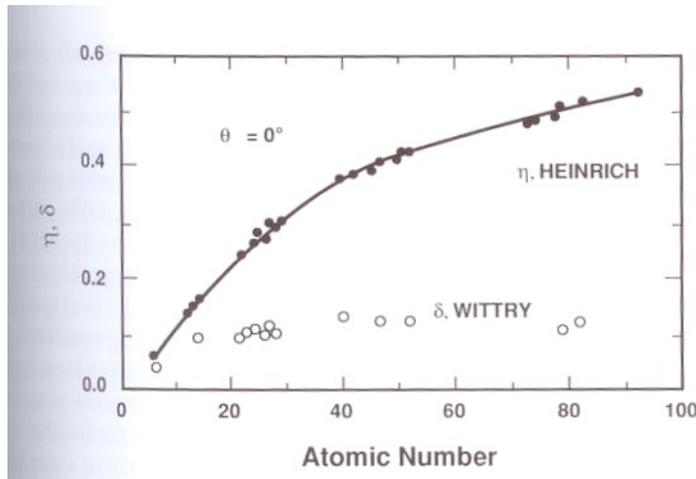
Goldstein et al, 2003

BSE's give contrast between light and heavy elements

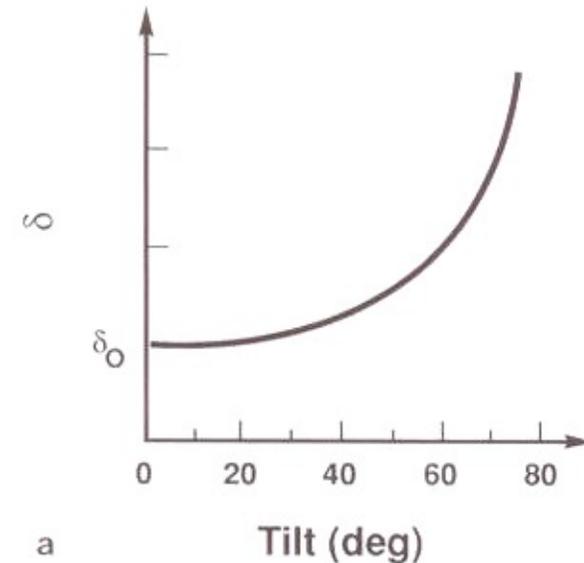


Osmium stained, resin-embedded tissue

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



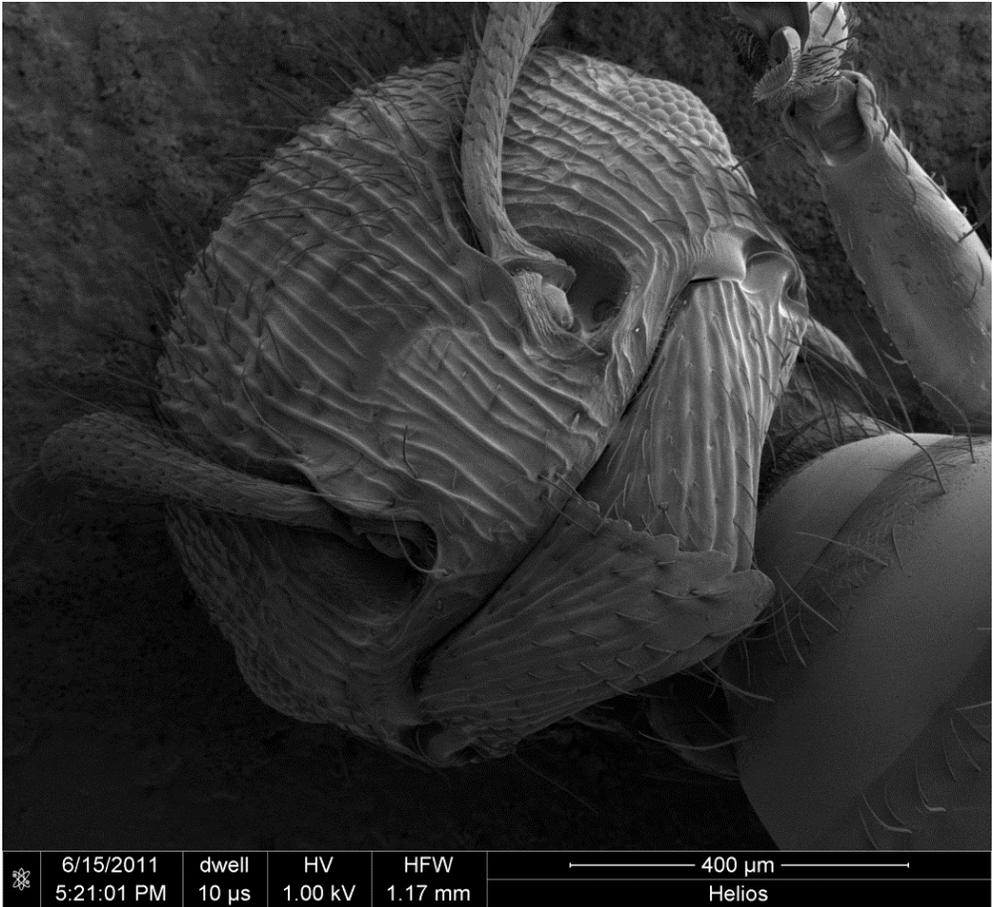
30 keV



Signal is strongly dependent on viewing direction

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

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

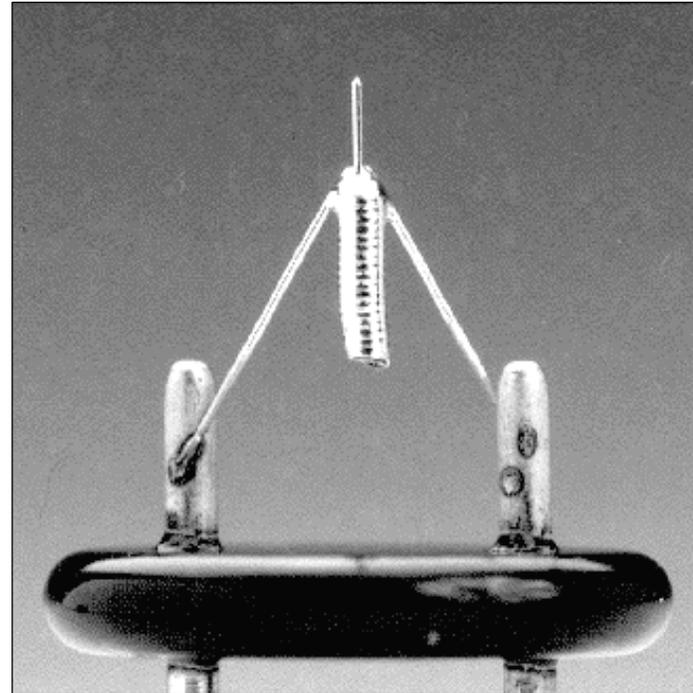
FIB Operation

Ion Column

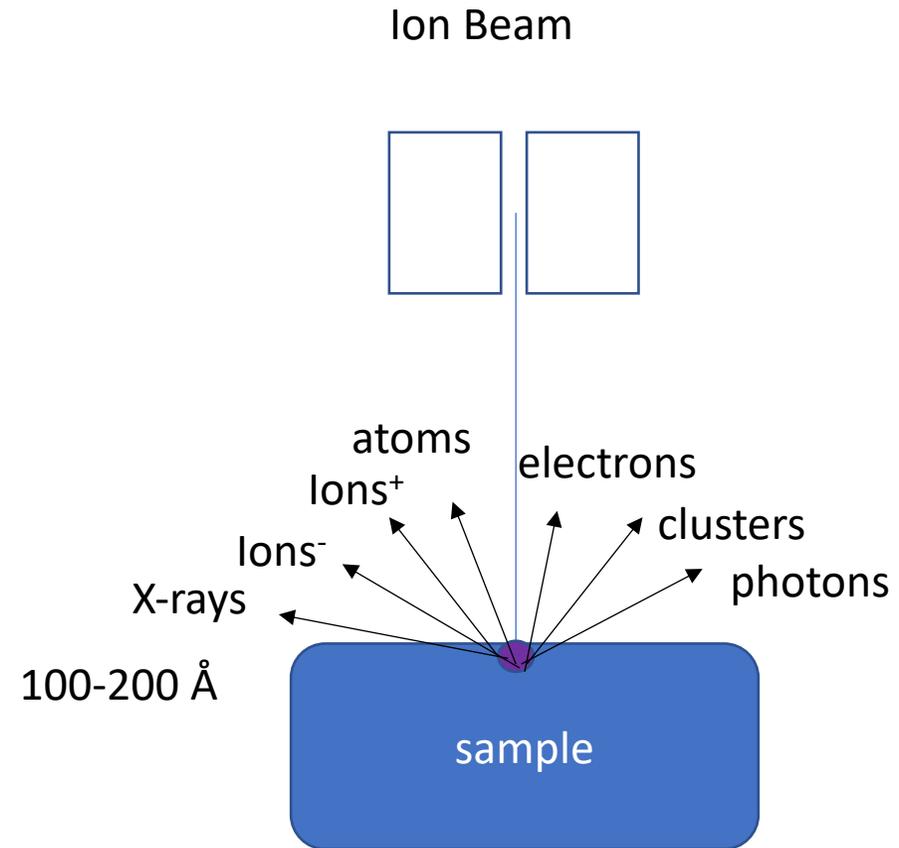
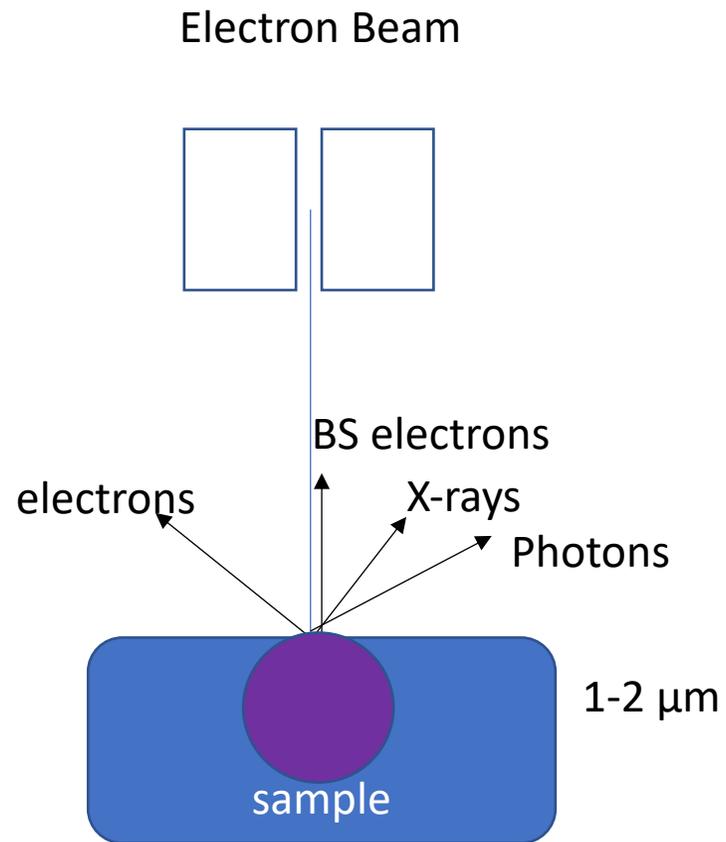
- ◆ Source - LMIS at top
- ◆ Focusing Optics
 - Use Electrostatic lenses since ions are heavier than electrons.
- ◆ Deflection Electronics/Pattern Board
- ◆ High-speed Blanking
 - Need to prevent milling while blanking
- Current is controlled by apertures
 - Apertures wear out over time and must be replaced!
- 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

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

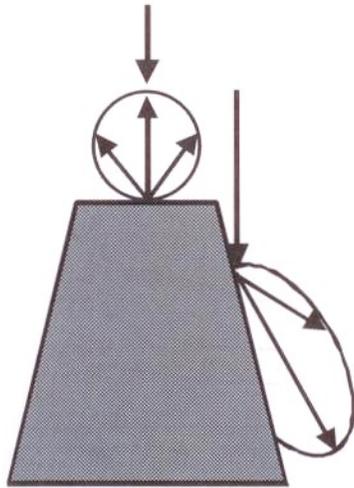


Beam Interactions with Specimens

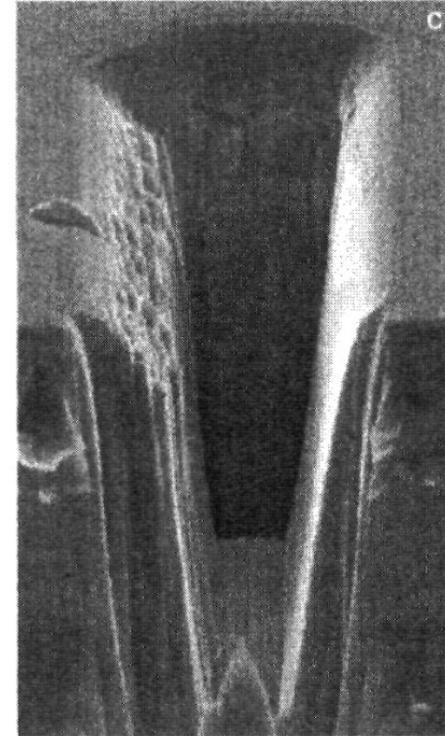


Common Use: Sputtering particles from substrate

Sputtered Particle Ejection Behavior

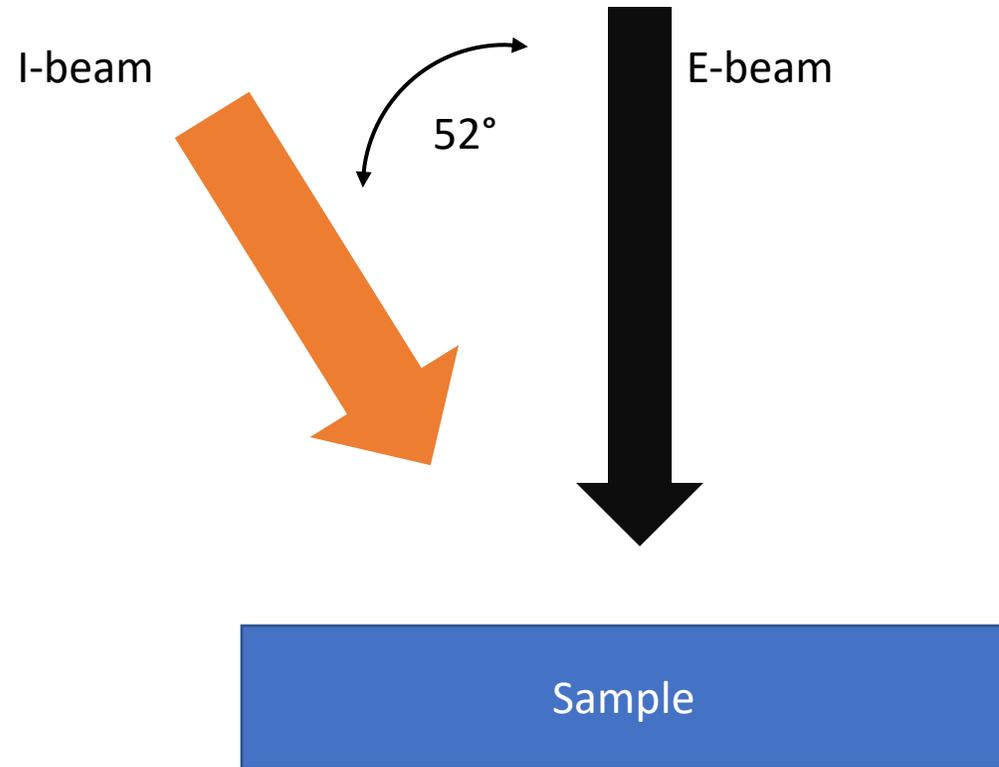


More efficient milling at edge than in bulk



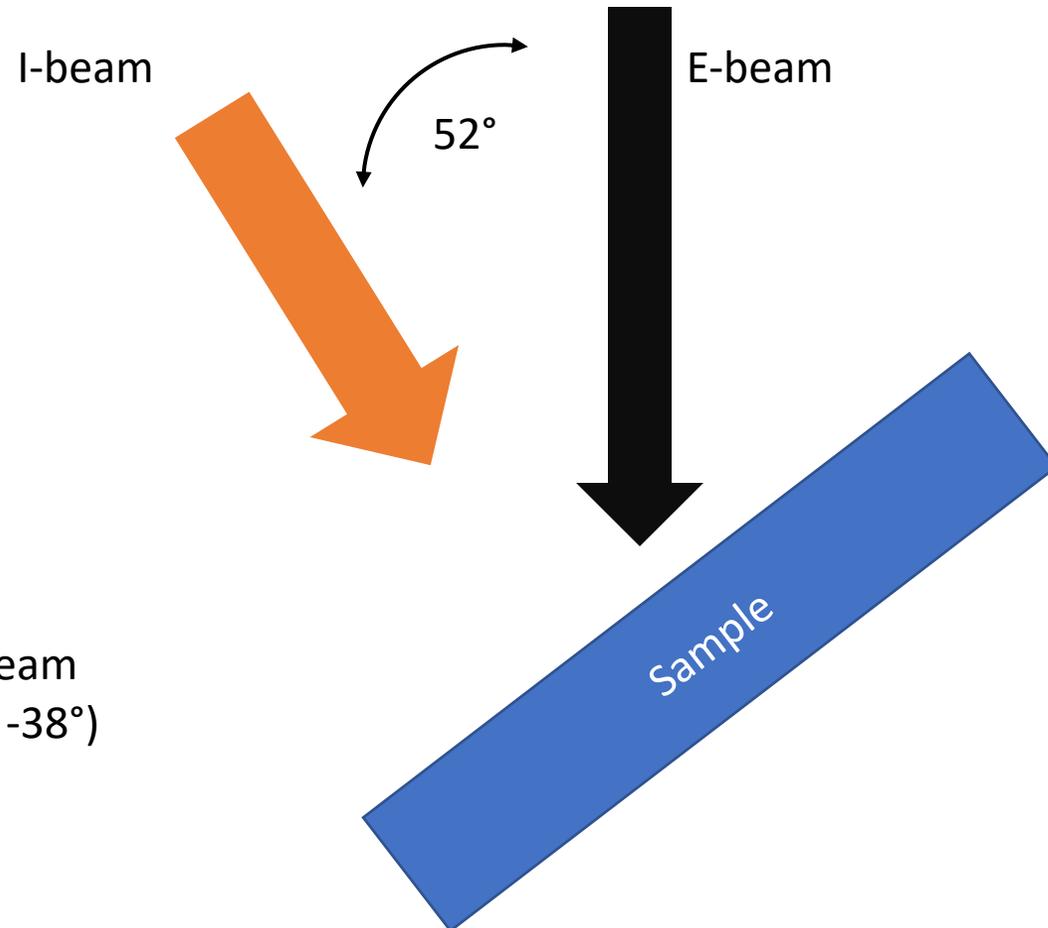
Redeposition limits trench depth

Geometry



Sample:
Tilt from -10° to $+70^\circ$

Geometry



Sample:

Tilt from -10° to $+70^\circ$

Tilt to 52° for orthogonal i-beam

(cross-section viewing angle -38°)

Geometry

The screenshot displays the xT microscope Control v4.5.3 build 2378 - supervisor (Supervisor) interface. The main window is divided into four quadrants, each showing a different view of a specimen. The top-left and top-right quadrants show a close-up of a rectangular feature with a yellow crosshair. The bottom-left quadrant shows a similar view with a yellow crosshair. The bottom-right quadrant shows a wider view of the specimen with a yellow crosshair and a green arrow pointing to a feature labeled "4 mm" and "Dynamic Focus: -38°".

The control panel on the right side of the interface includes the following sections:

- Vacuum:** Pump, Vent
- System:** Wake Up, 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 (59.2), Brightness (43.9)
- Status:** Specimen Current (0.86 pA), Ion Beam Current (1.37 nA), Chamber Pressure (1.18e-5 mbar)

The status bar at the bottom of the interface provides technical details for each quadrant:

- Top-left: 2/22/2012 12:45:36 PM, HV 2.00 kV, HFW 29.8 μm, WD 4.0 mm, 10 μm scale, NYSB
- Top-right: 2/22/2012 12:34:24 PM, dwell 1 μs, HV 30.00 kV, HFW 42.5 μm, 10 μm scale, NYSB
- Bottom-left: 2/15/2012 4:14:39 PM, HV 25.00 kV, HFW 2.52 mm, WD 4.0 mm, 500 μm scale, Helios
- Bottom-right: 2/22/2012 12:46:27 PM, det CCD, x: 0.7432 mm, tilt y: -0.9972 mm, 52°

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”

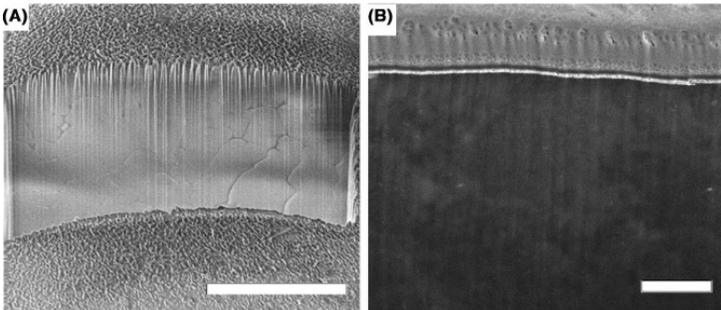
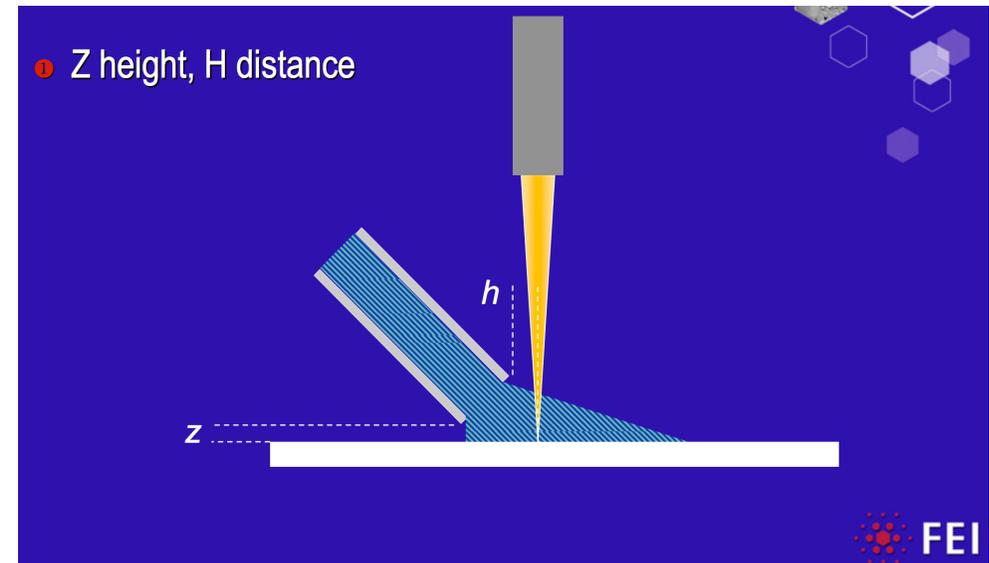
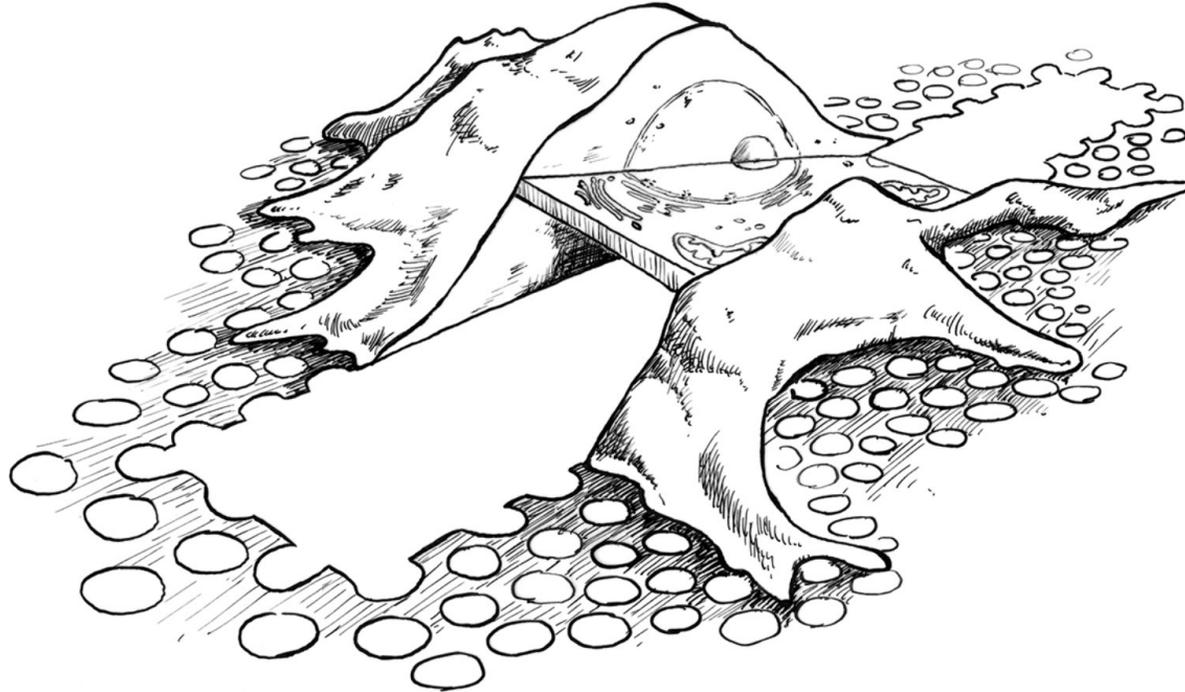


Image: Hayles and Winter, 2021



Cryo FIB/SEM for tomographic sample preparation



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

M. MARKO, C. HSIEH, W. MOBERLYCHAN*,
C. A. MANNELLA & J. FRANK*†

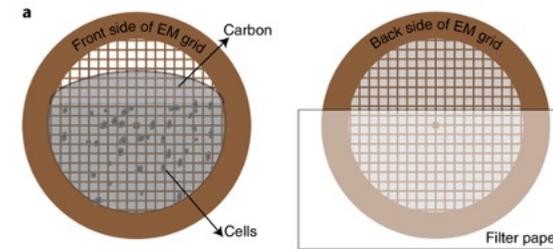
Resource for Visualization of Biological Complexity, †Howard Hughes Medical Institute, Wadsworth Center, Empire State Plaza, Albany, NY 12201, U.S.A.

*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.

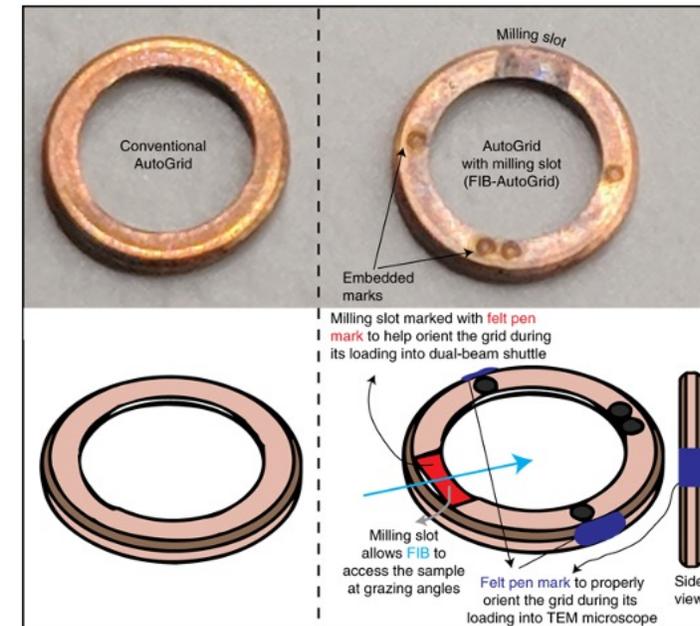
Place cells on Grids

- 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



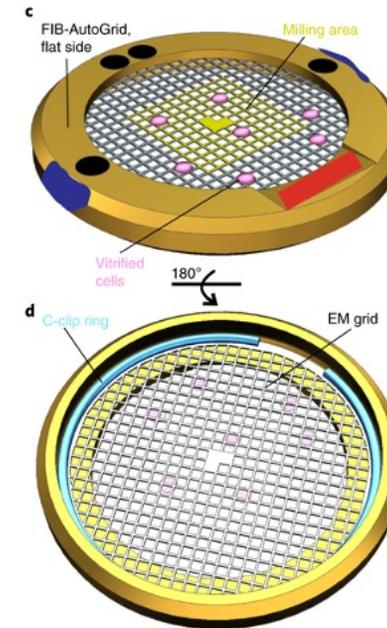
Grid Geometry

- 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

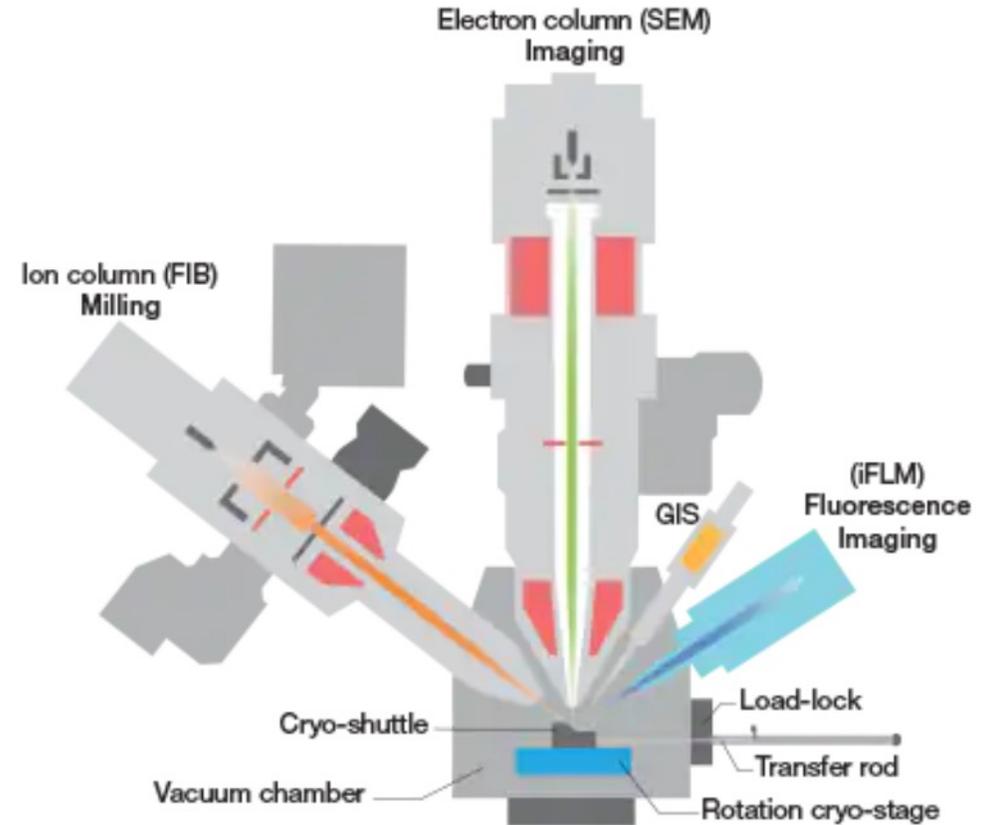


Grid Geometry

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



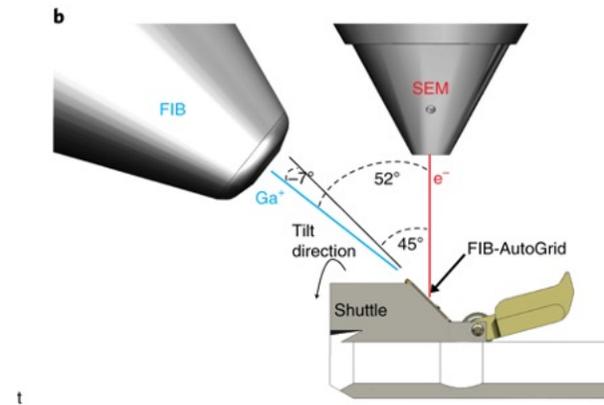
Specialized Microscopes: Aquilos 2 Cryo FIB



Shuttle holds 2 grids

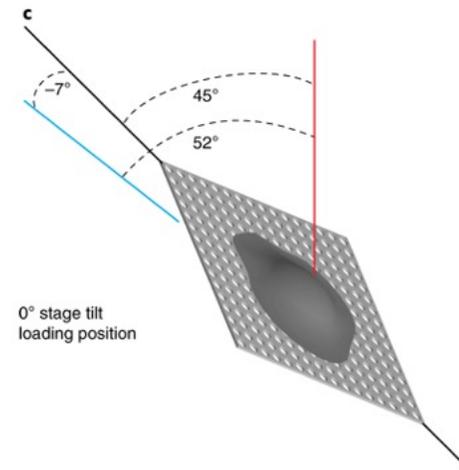
Geometry

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



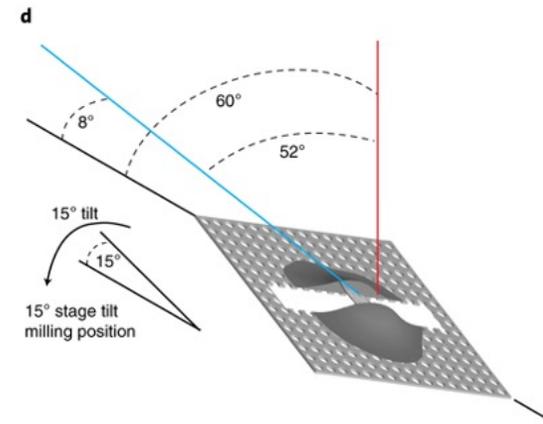
Geometry: Untilted

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



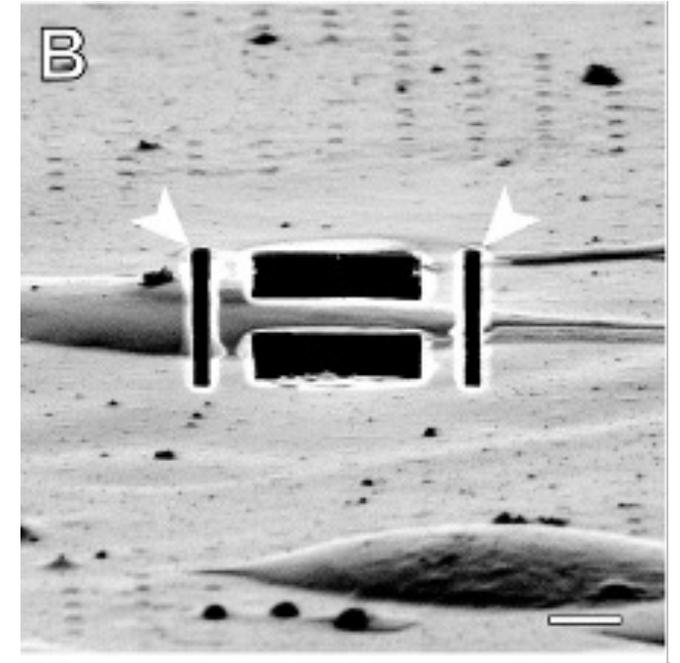
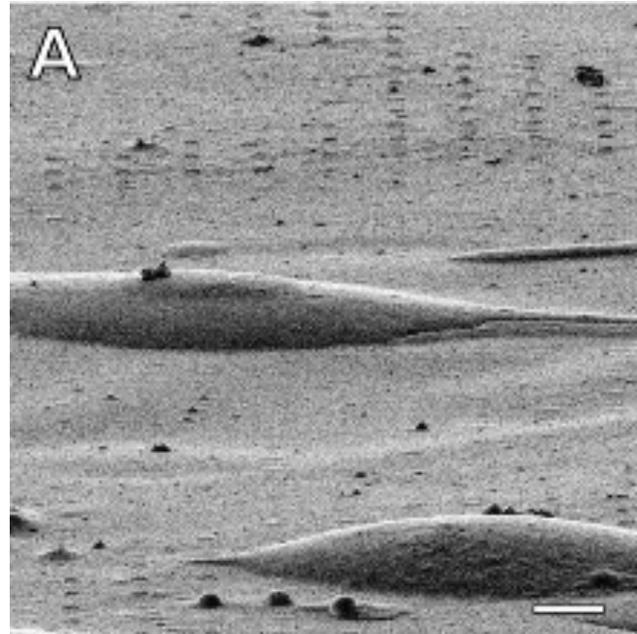
Geometry: Tilted

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

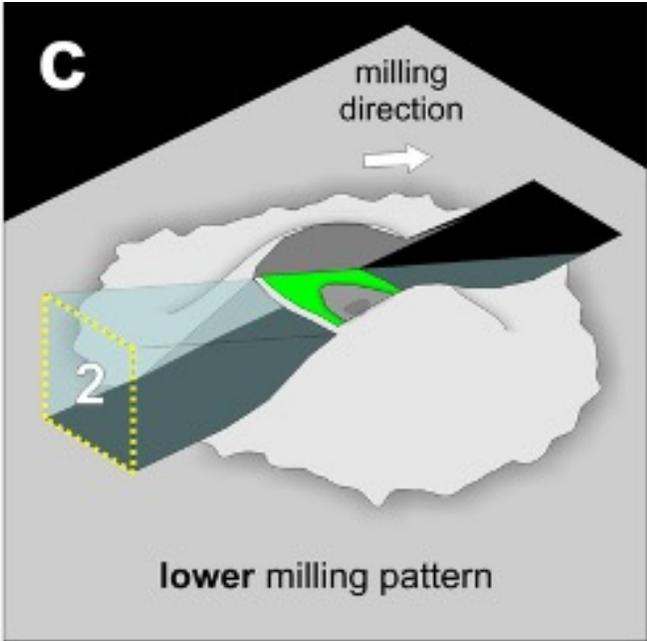
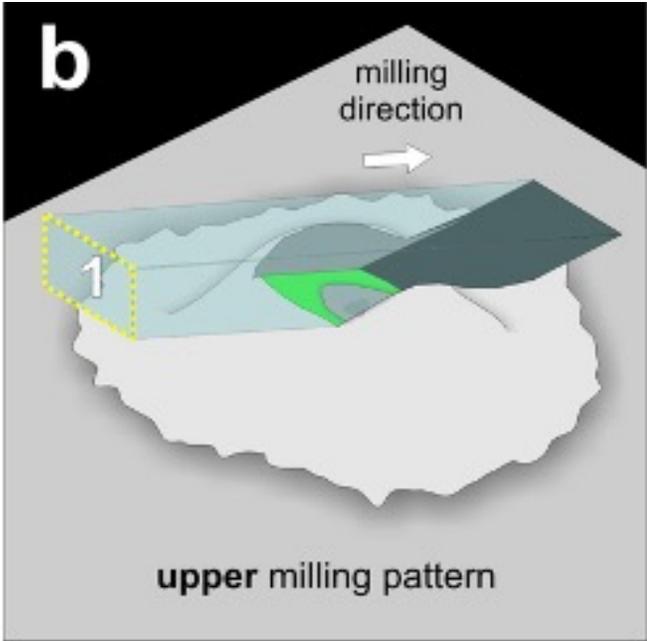
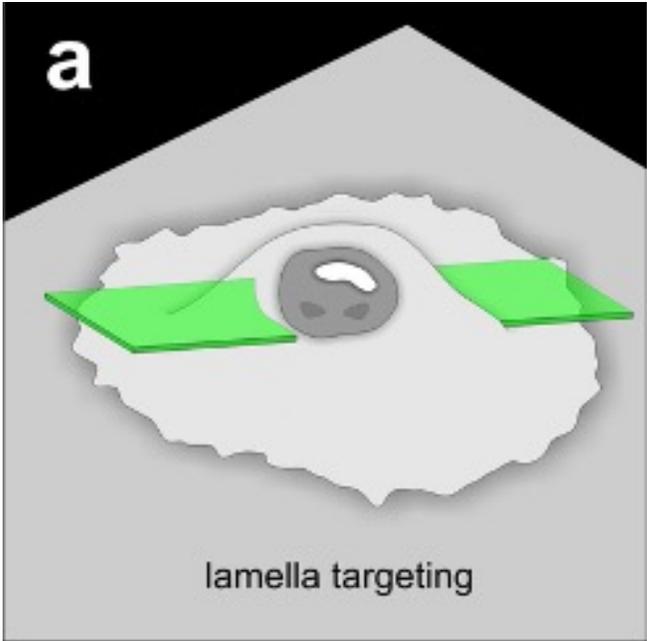


Imaging cells with ion beam

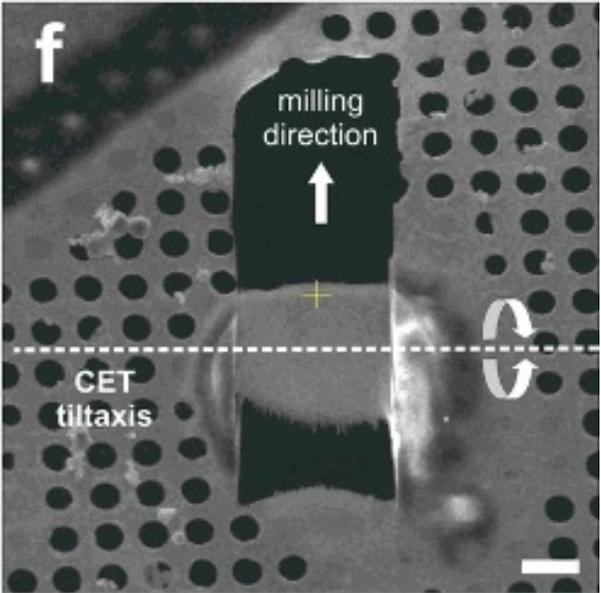
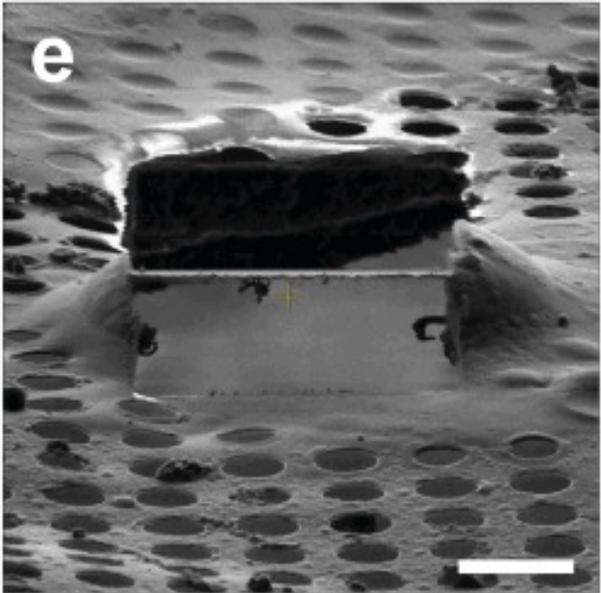
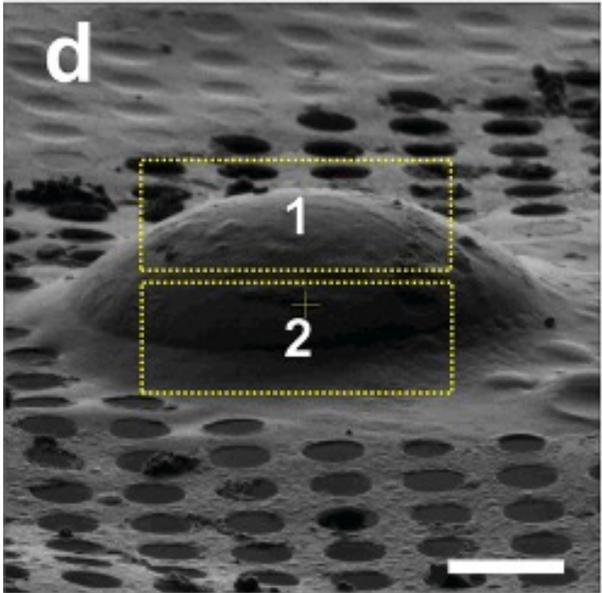
- A: Ion-beam view of cells
- B: Cells after milling, showing position of micro-expansion joints for stress relief



Targeting of Milling Regions



Targeting of Milling Regions



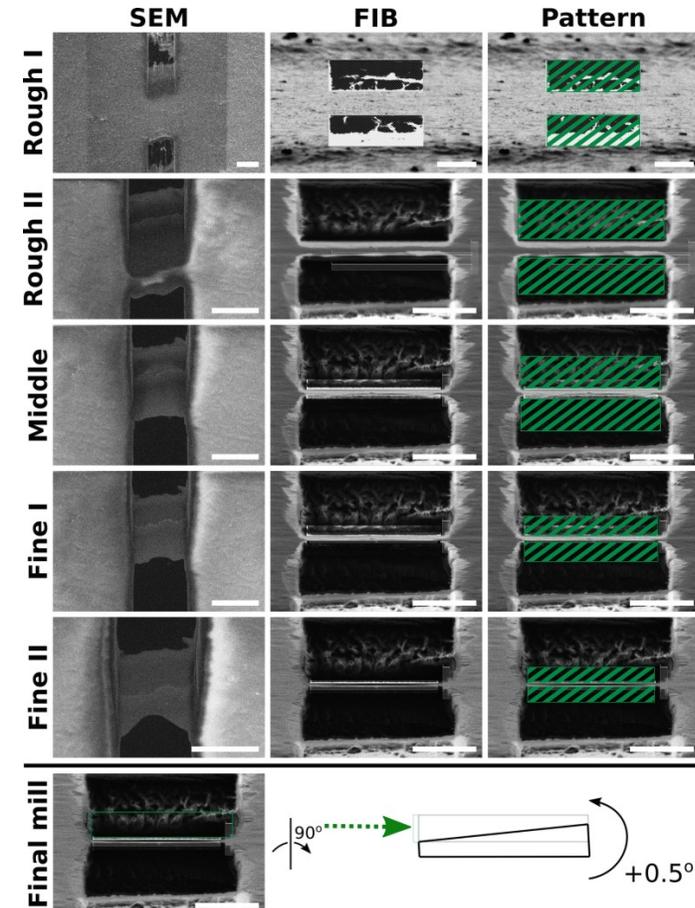
Milling

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

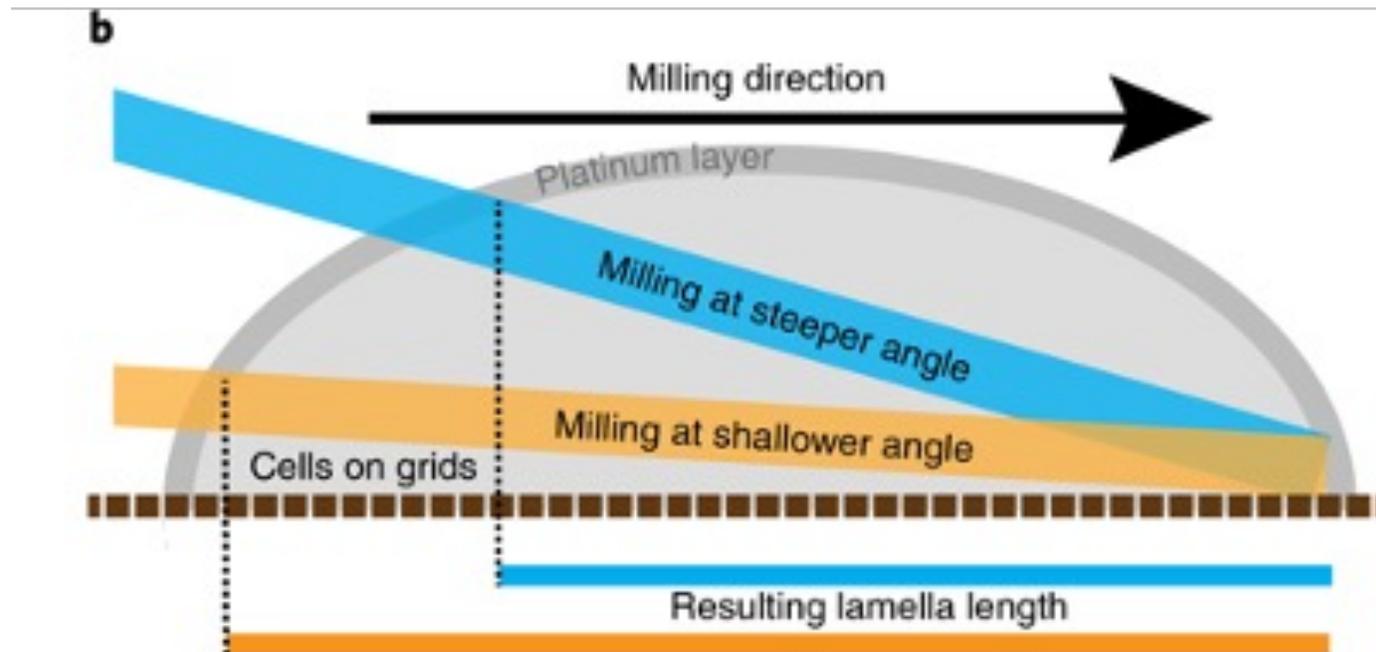
Higher throughput

- Target several regions and do rough mills
- After all rough work is done, do final polishing and remove from SEM



These steps are now generally automated

Milling at as shallow an angle as possible



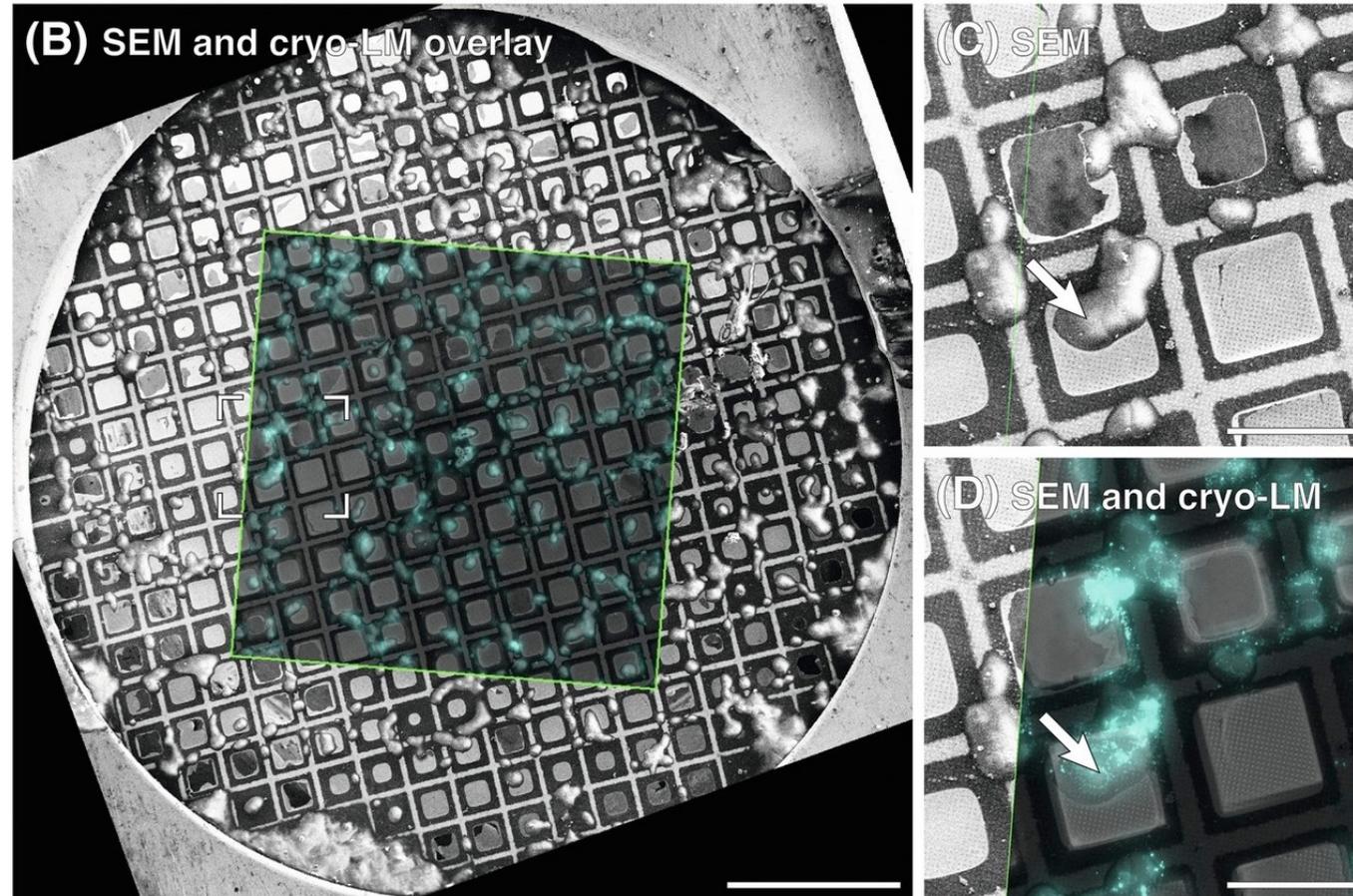
Where to mill?

- 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

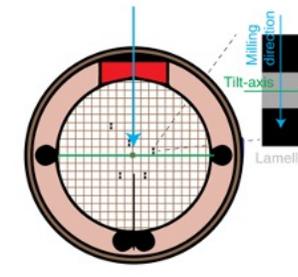


Cryo-CLEM: Overlay



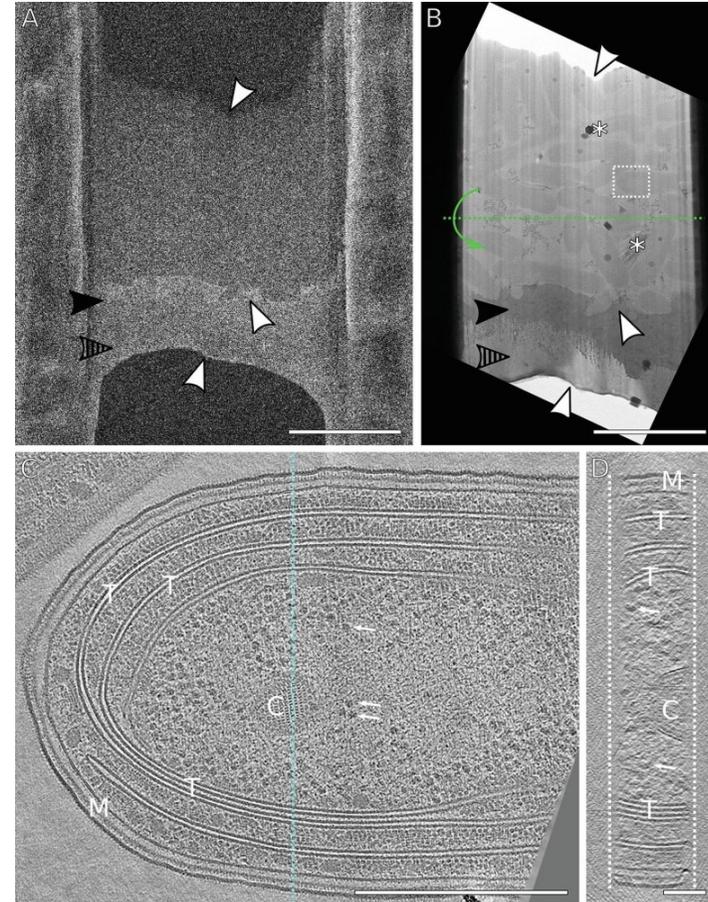
Post-milling geometry: Loading into TEM

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

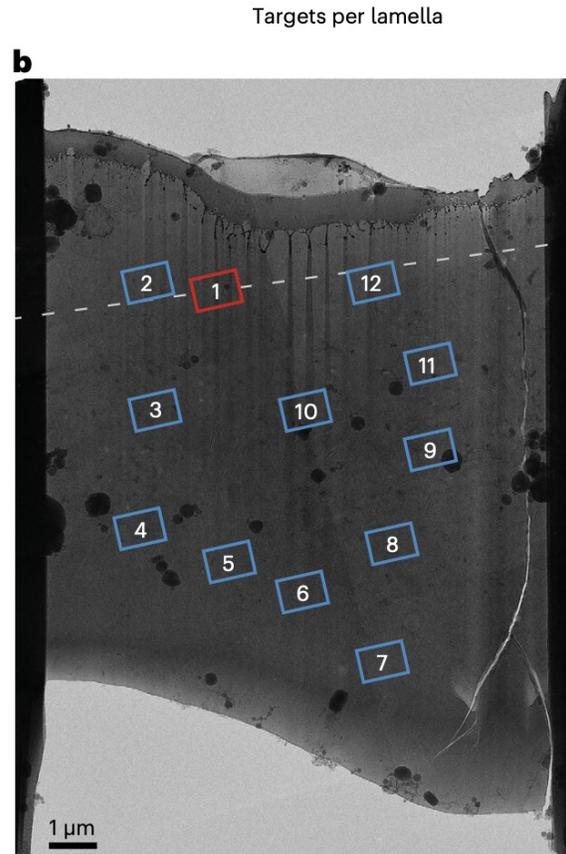


Ideal Result

- 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.



Many areas can be collected on a single lamella



Article

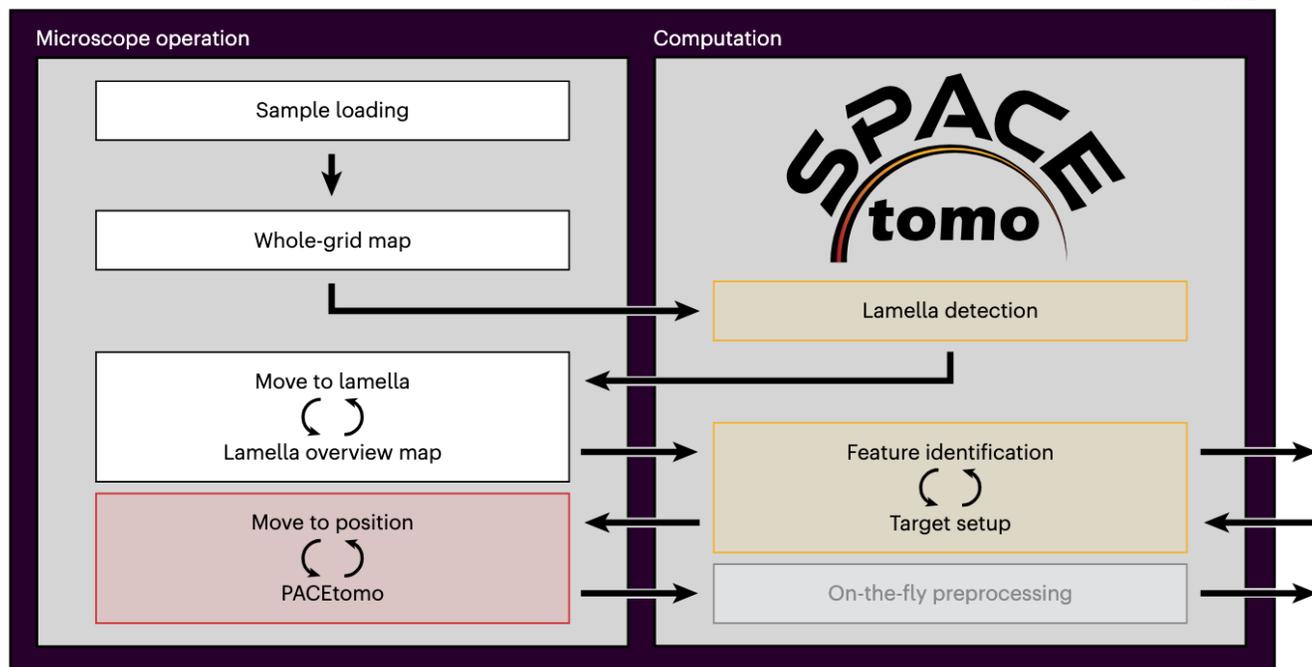
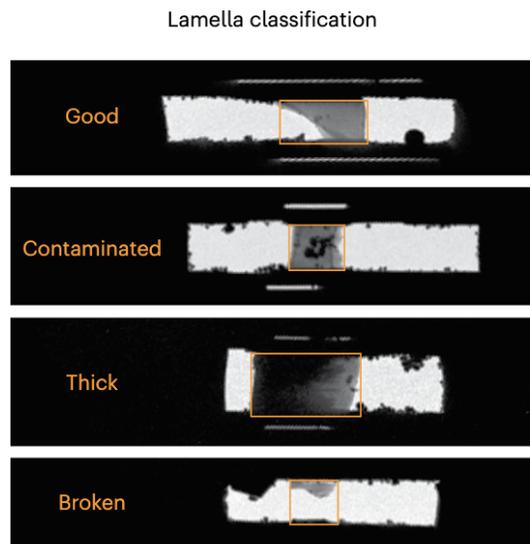
<https://doi.org/10.1038/s41592-022-01690-1>

Parallel cryo electron tomography on in situ lamellae

Received: 22 March 2022

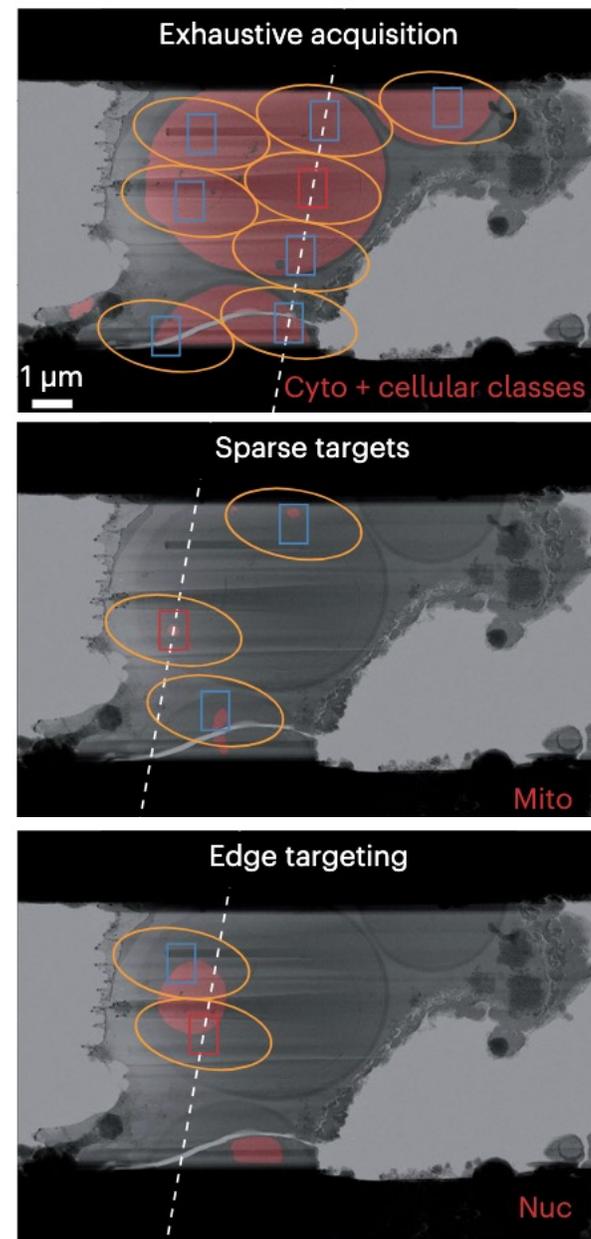
Accepted: 28 September 2022

Fabian Eisenstein¹, Haruaki Yanagisawa¹, Hiroka Kashihara²,
Masahide Kikkawa¹, Sachiko Tsukita² & Radostin Danev¹✉

**b****c**

Lamella detection		Ground truth ($n = 85$)				False positive ($n = 1$)
		Good ($n = 60$)	Cont. ($n = 8$)	Thick ($n = 5$)	Broken ($n = 12$)	
Prediction ($n = 81$)	Good ($n = 60$)	98%				2%
	Contaminated ($n = 8$)		100%			
	Thick ($n = 4$)			100%		
	Broken ($n = 9$)	11%			89%	
False negative ($n = 5$)				20%	80%	

Precision: 99% ($n = 80/81$)
Recall: 94% ($n = 80/85$)

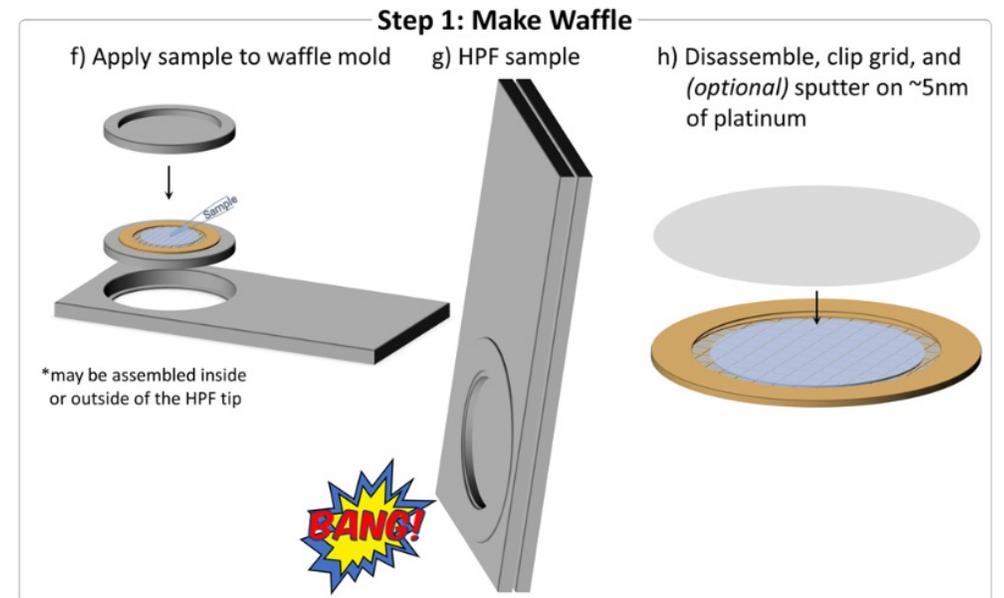
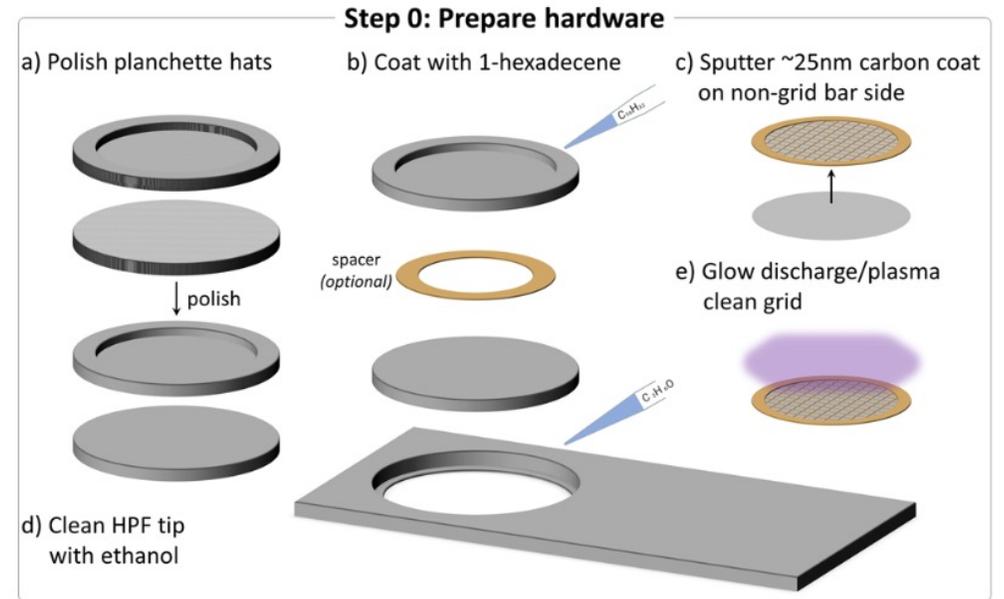
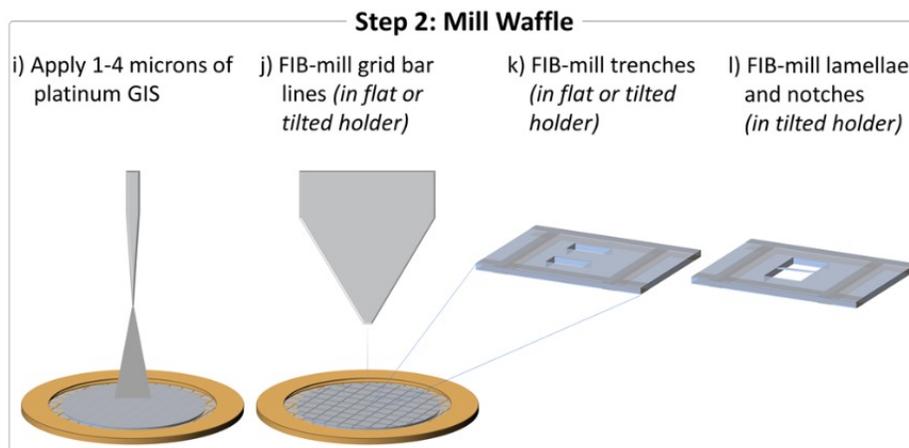


Eisenstein, F., Fukuda, Y. & Danev, R. Smart parallel automated cryo-electron tomography. *Nat Methods* **21**, 1612–1615 (2024). <https://doi.org/10.1038/s41592-024-02373-9>

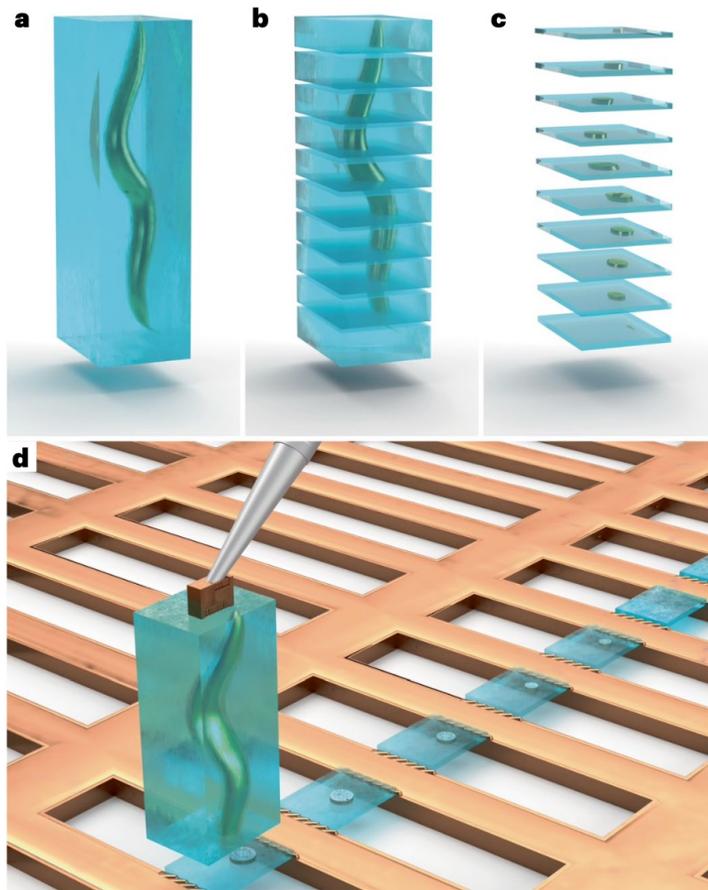
HPF on grids

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

Kotaro Kelley^{1,6}, Ashleigh M. Raczowski^{1,4,5}, Oleg Klykov^{1,2,5}, Pattana Jaroenlak^{3,5}, Daija Bobe^{1,5}, Mykhailo Kopylov¹, Edward T. Eng¹, Gira Bhabha³, Clinton S. Potter^{1,2}, Bridget Carragher^{1,3} & Alex J. Noble¹



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

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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 Poege³, Jonathan Schneider³, Florian Beck¹, David P. Klebl², Christopher Thompson⁴ & Jürgen M. Plitzko¹✉

Serial Lift-out

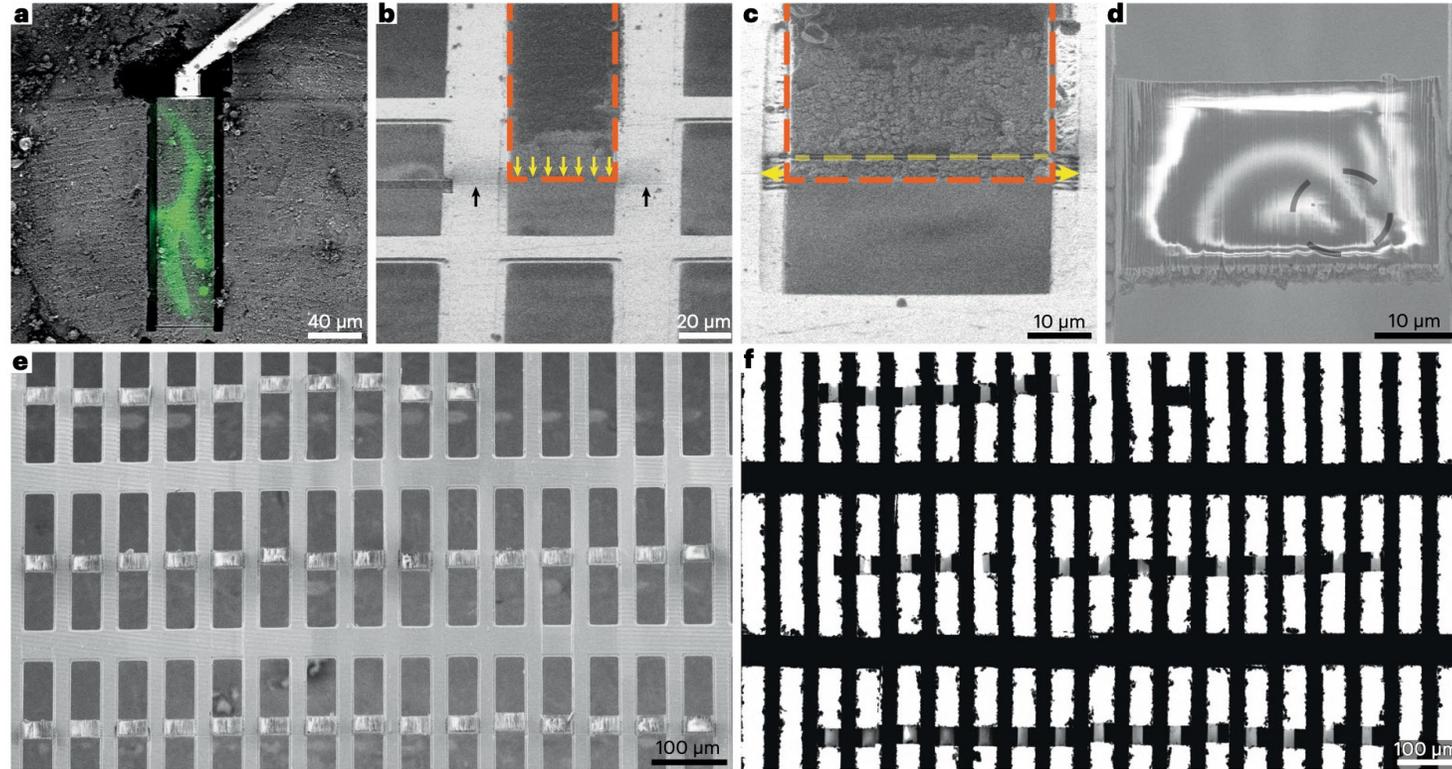
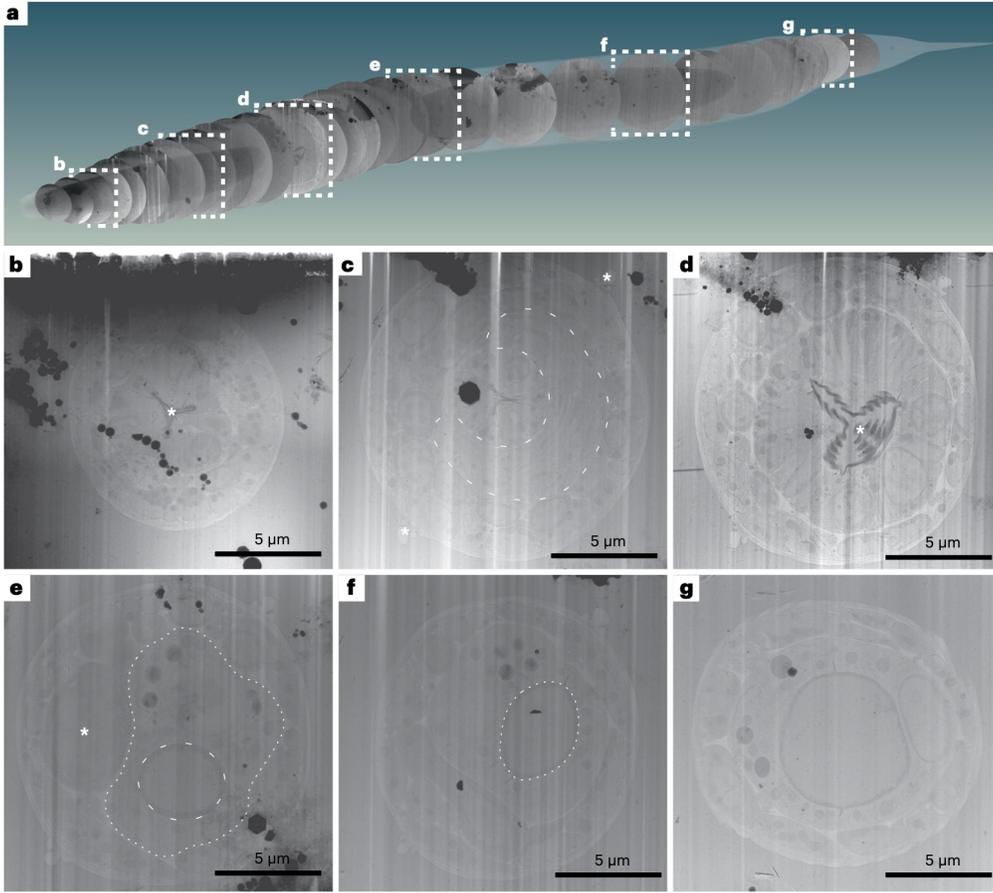


Fig. 2 | A workflow for double-sided attachment Serial Lift-Out. **a**, FIB image of the prepared extraction site with overlaid correlated fluorescence data (green) indicating the larva being targeted. The micromanipulator is attached to the extraction volume by redeposition from the copper adaptor (trench-milling 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

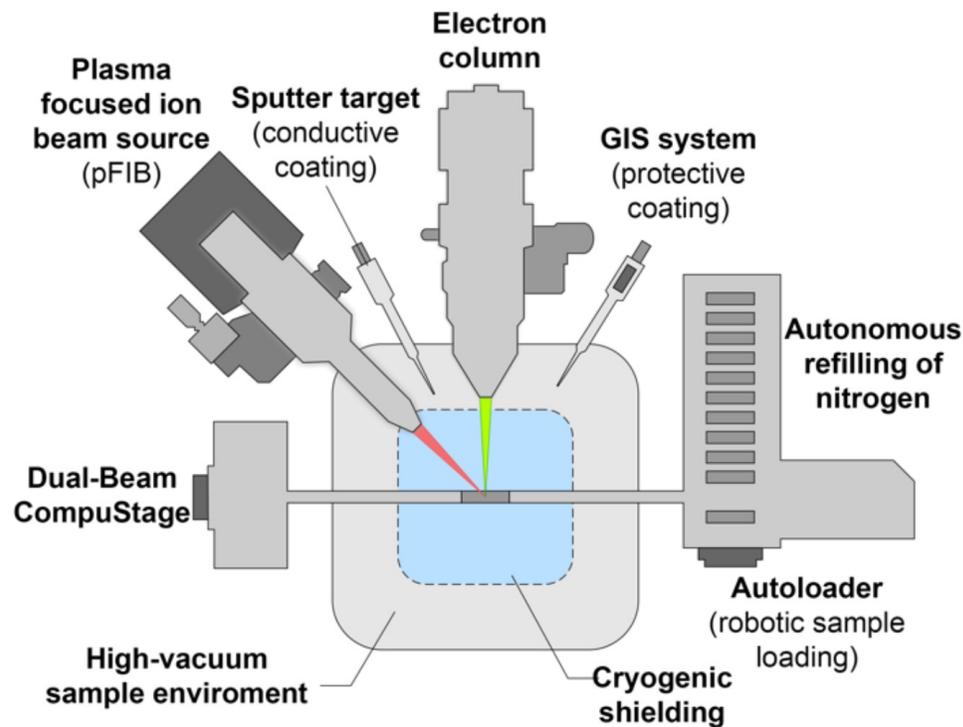
Received: 9 August 2022

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Casper Berger ^{1,2,5}, Maud Dumoux ^{1,5}, Thomas Glen^{1,5}, Neville B.-y. Yee³,
John M. Mitchels⁴, Zuzana Patáková ⁴, Michele C. Darrow³,
James H. Naismith^{1,2} & Michael Grange ^{1,2} 

Plasma instead of Ga



Berger et al, 2023

- Typical Gases: Ar, Xe, O₂, N₂
 - Ar, Xe seem to be most useful
- Faster bulk material removal
 - Due to higher currents
- Potentially thinner damaged region than with Ga
 - Needs further study
- More expensive instrument
 - But perhaps lower consumable cost (Ga needs to be replaced 2-3X annually)

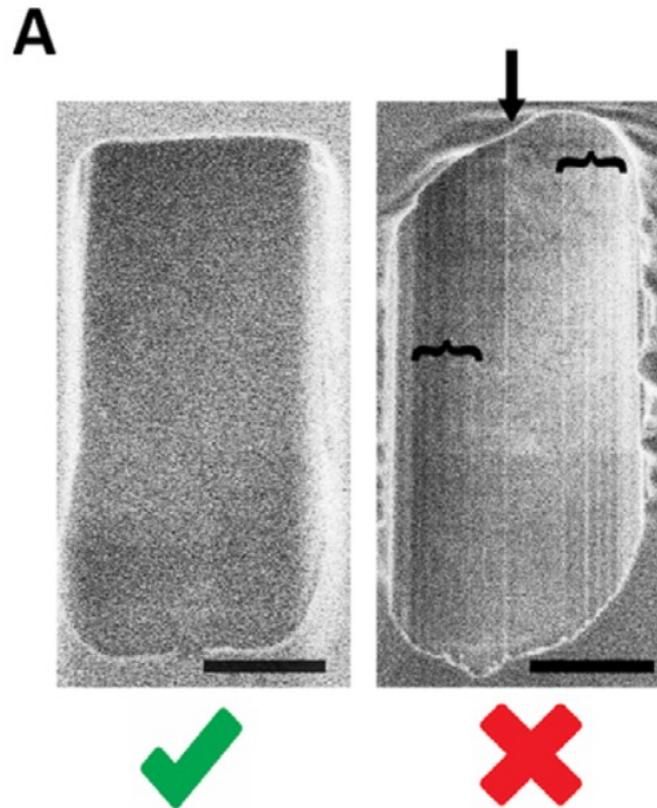
Milling Rates for Plasma (Berger et al, 2023)

Beam	Xe	N	O	Ar	Ga
Milling rate ($\mu\text{m}^3/\text{nC}$)	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 with 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

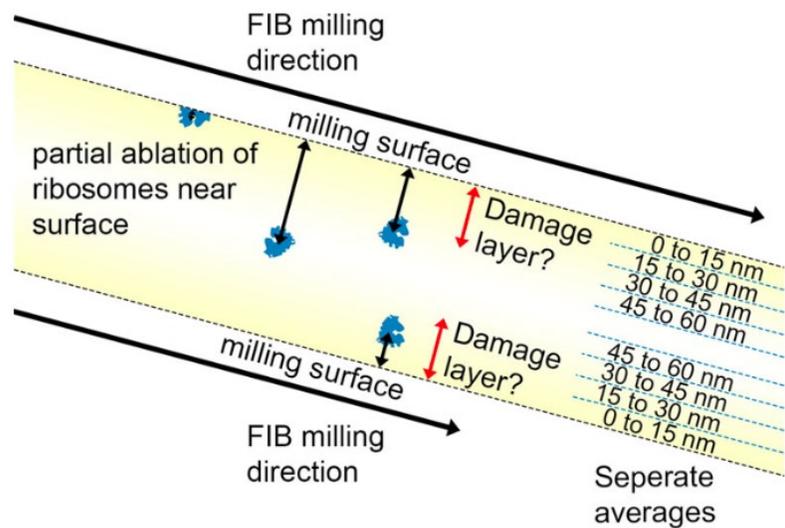
Curtaining



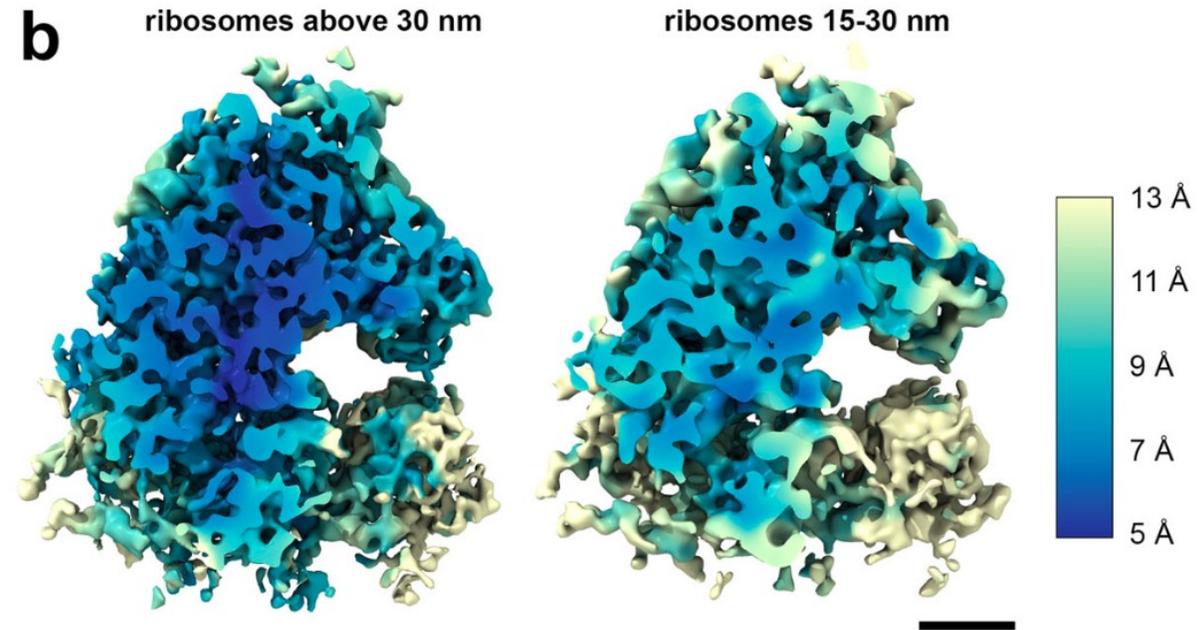
- Ideally the cutting from the ion beam will leave a perfectly flat face
- Uneven interactions with the surface can result in uneven milling which shows up as “curtains”

Damage near the lamella surface

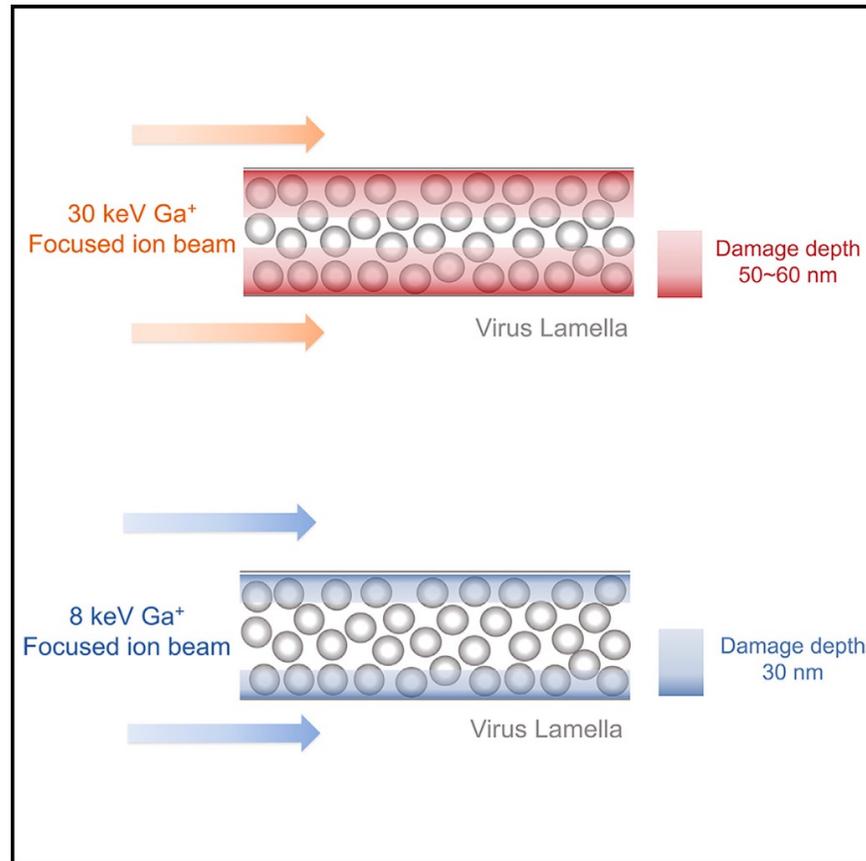
a



b



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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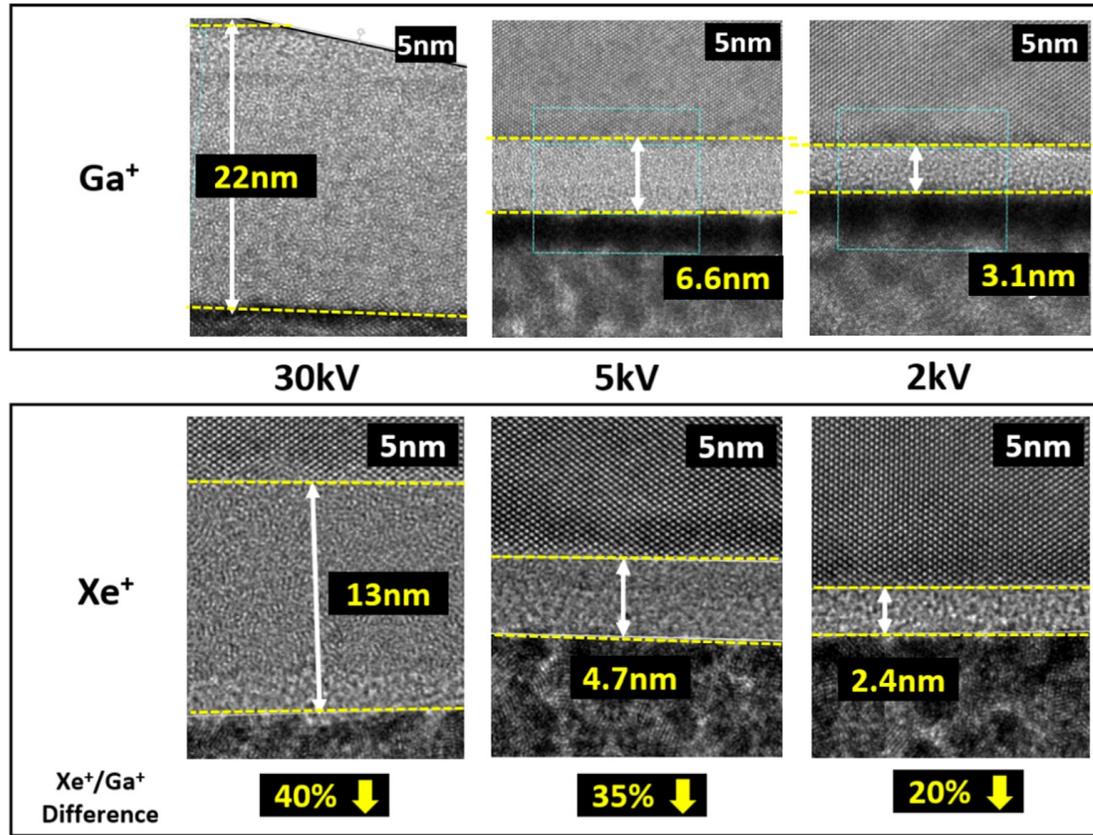
³These authors contributed equally

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*Correspondence: xzzhang@ibp.ac.cn

<https://doi.org/10.1016/j.str.2023.07.002>

Damage near surface: materials science



Full Length Article

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



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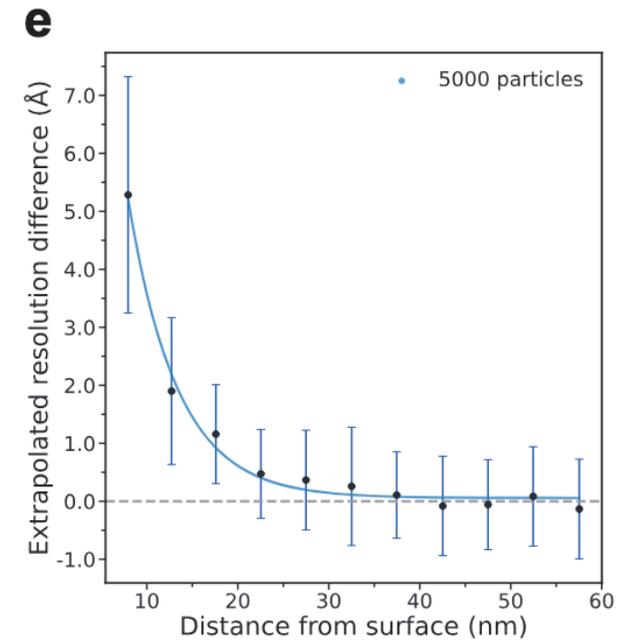
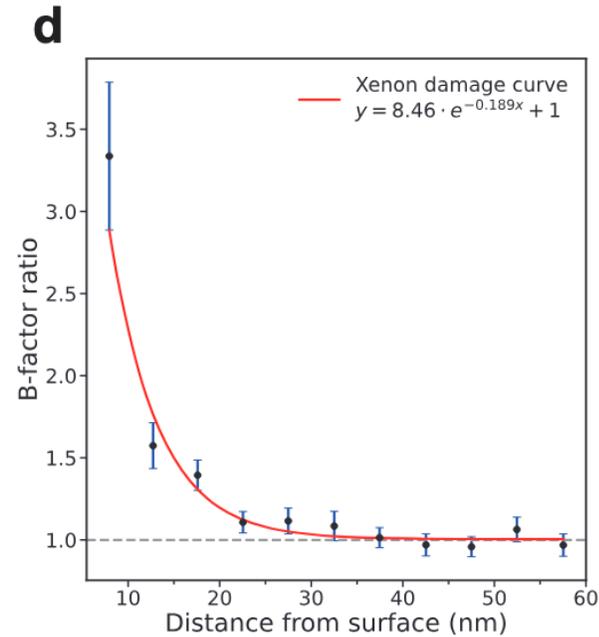
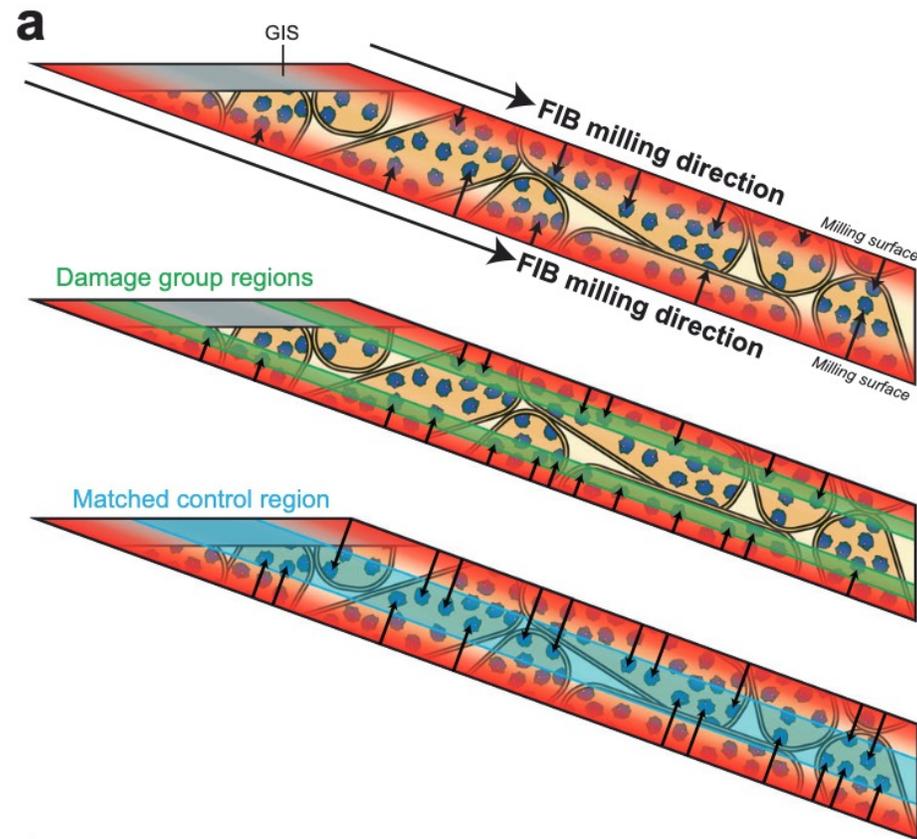
^b FEI Company, Achtseweg Noord 5, Bldg, 5651 GG, Eindhoven, The Netherlands

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^d BP International Centre for Advanced Materials, University of Manchester, Manchester M13 9PL, UK

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].

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

Casper Berger^{1,5}, Helena Watson^{1,2,5}, James H. Naismith^{1,3},
Maud Dumoux¹ & Michael Grange^{1,4}✉

Accepted: 24 February 2025

Summary: Equipment and expertise needed

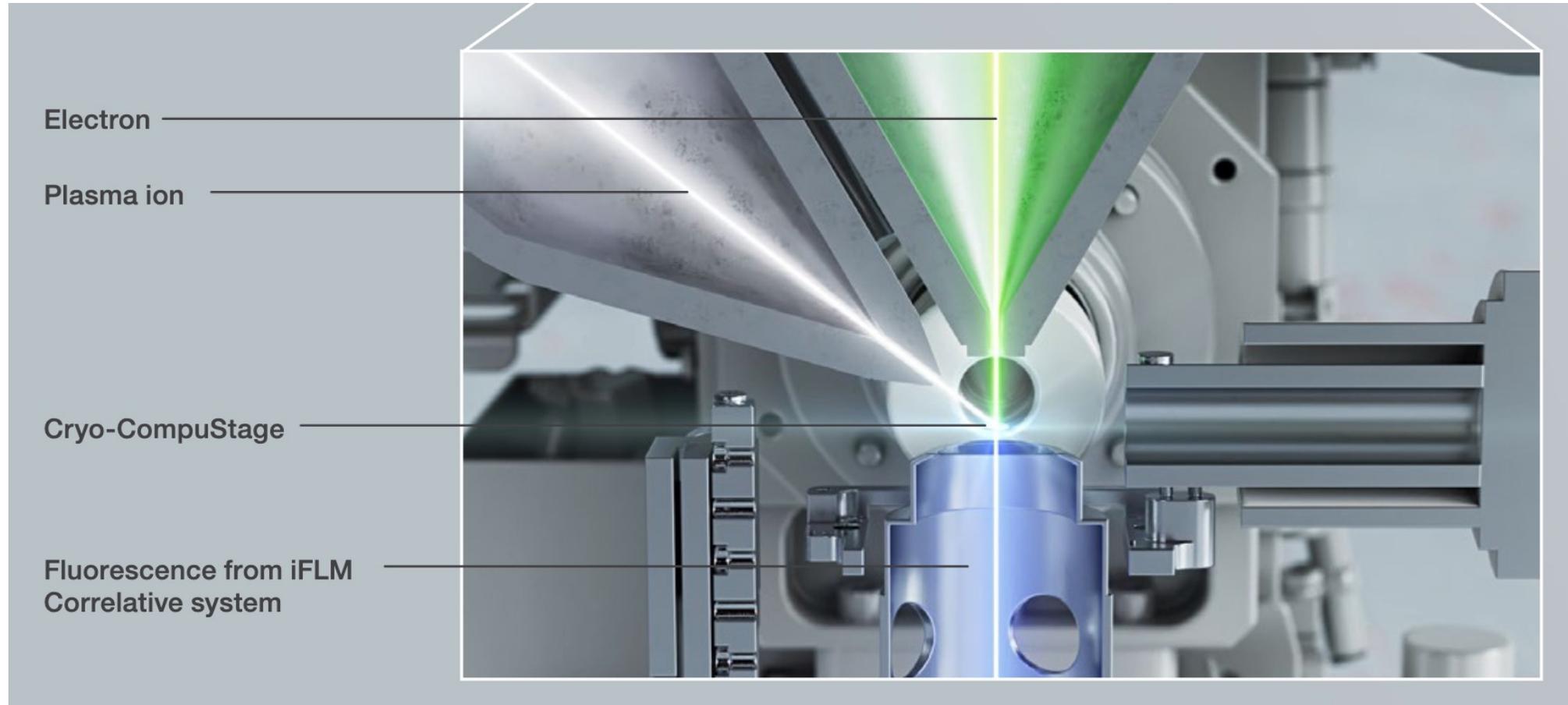
- Sample preparation
 - Grow cells on grids
 - Plunge freezing
 - High pressure freezing for waffle method or tissues
- FIB SEM
 - Cryo stage with full rotation
 - Ga or plasma (Ar/Xe/O₂/N₂) source
 - Gas injection system (Pt coating)
 - Sputter coater in loading station
 - Shuttles and transfer equipment
 - 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: High-throughput lamellae



Questions

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