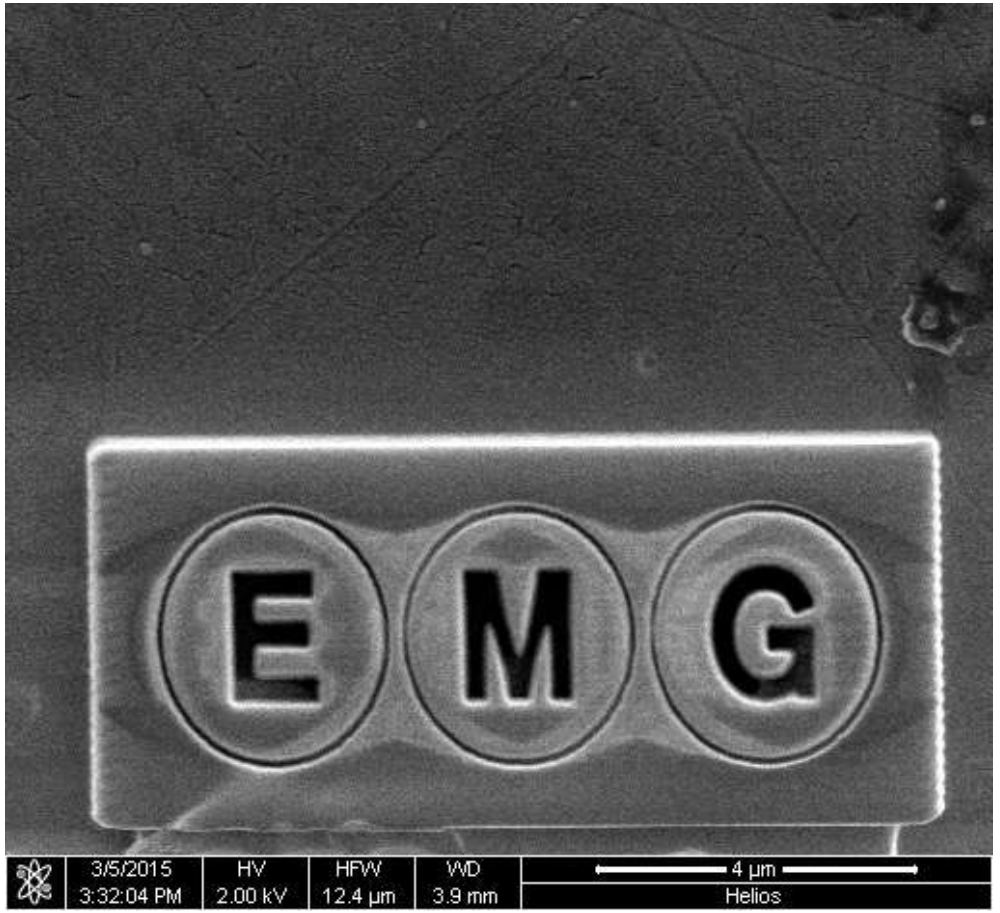
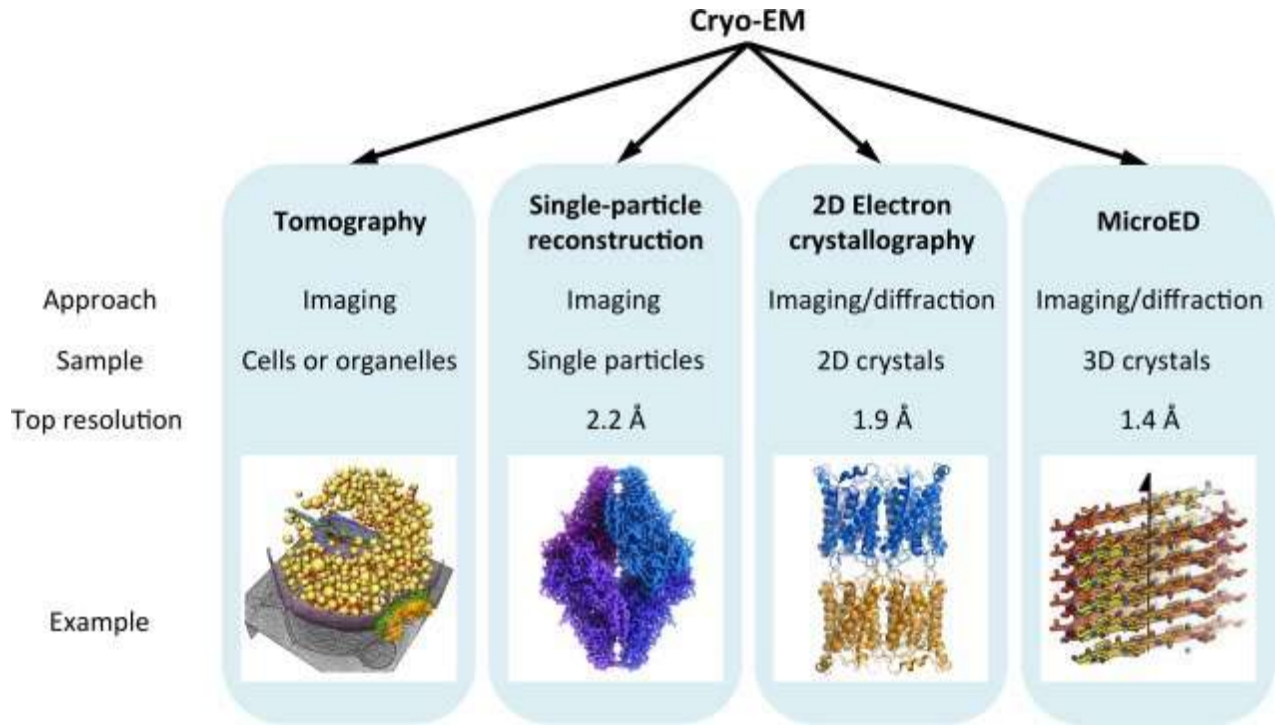
FIB SEM

March 4, 2024

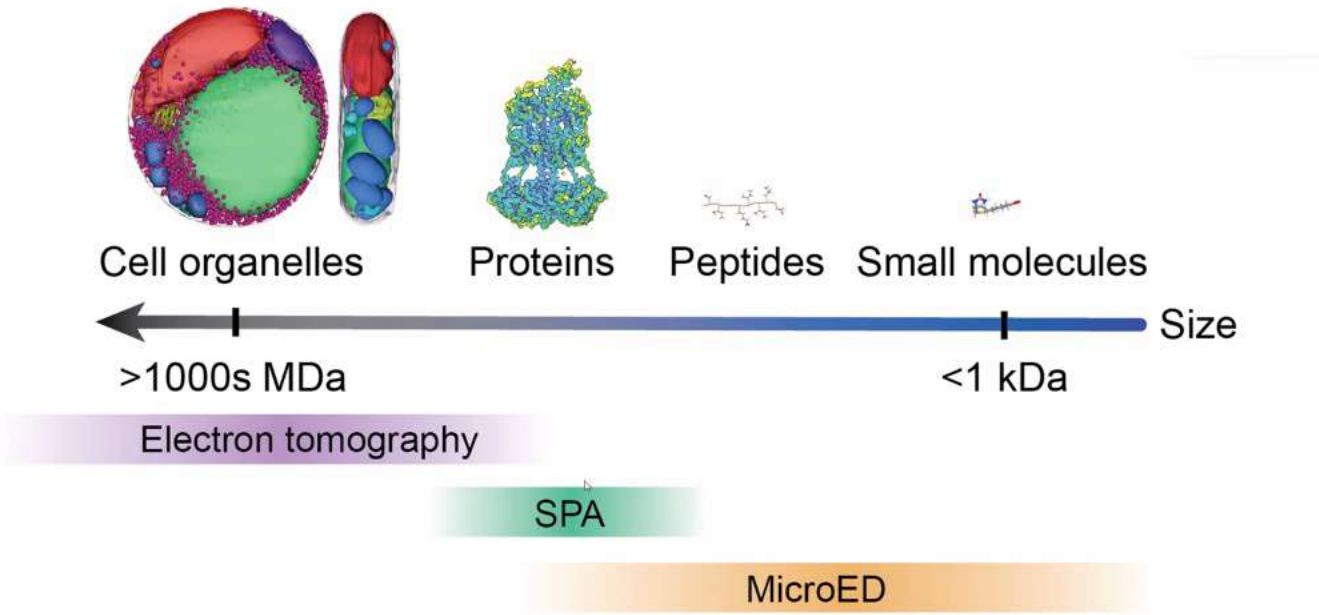
William Rice, NYU School of Medicine



Cryo-electron Imaging Modalities



Target Sizes

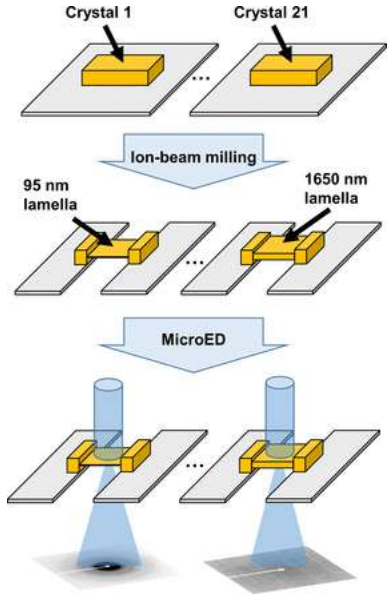


Sample thickness for TEM

- 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

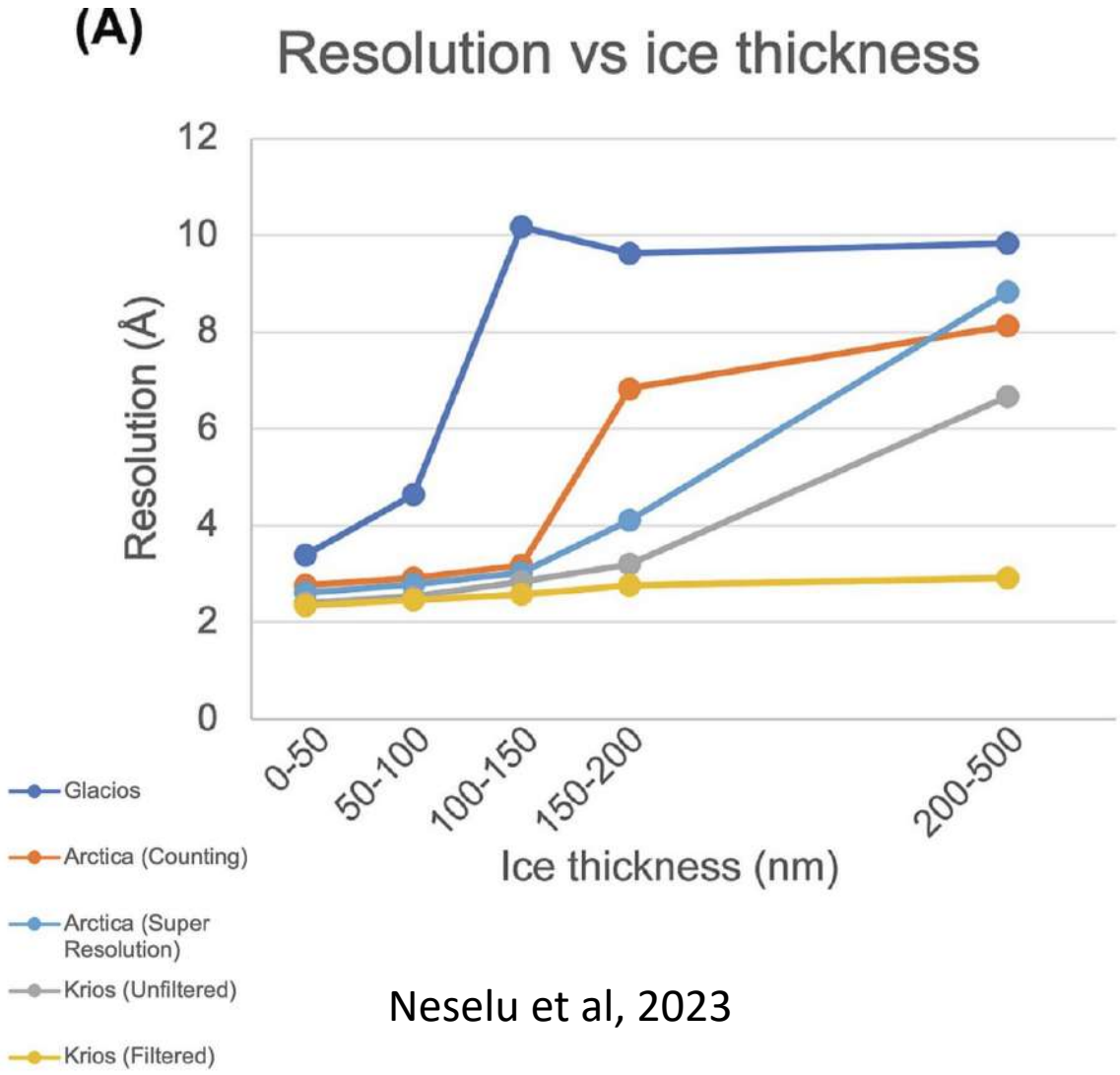
Thickness increases by a factor of 1/cos(θ)

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

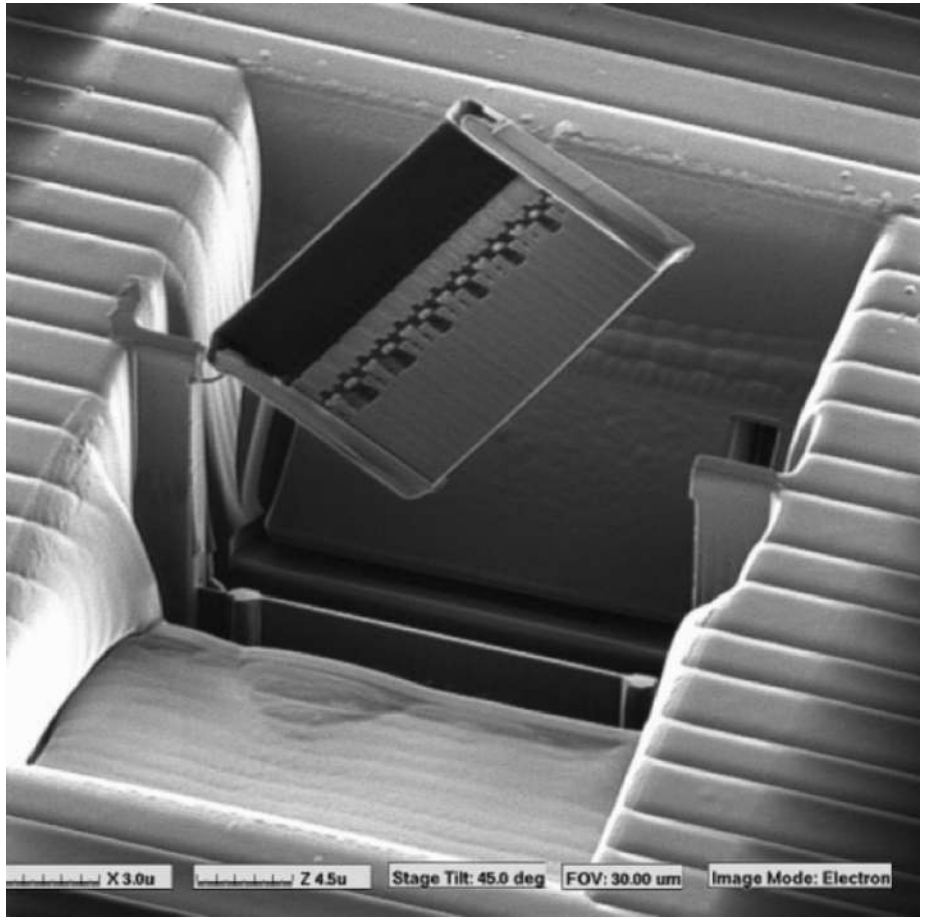
Solutions

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

- Microtomy
- Cryomicrotomy (CEMOVIS) is difficult
  - Sections hard to pick up
  - May be difficult to place on grids
  - Compression and knife artifacts

Solutions

- 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

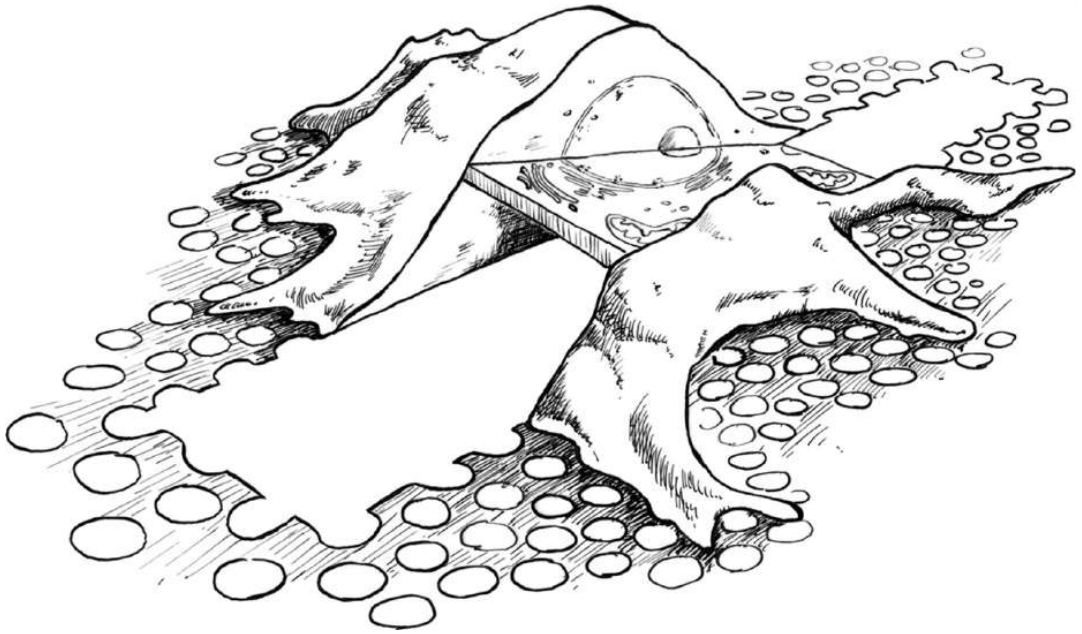
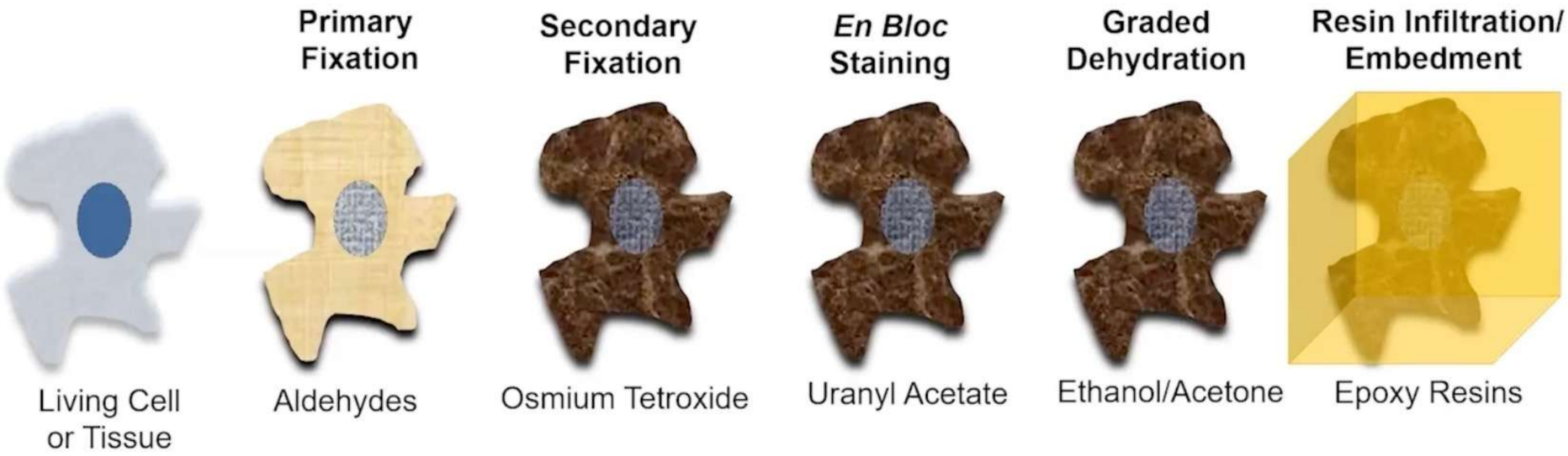


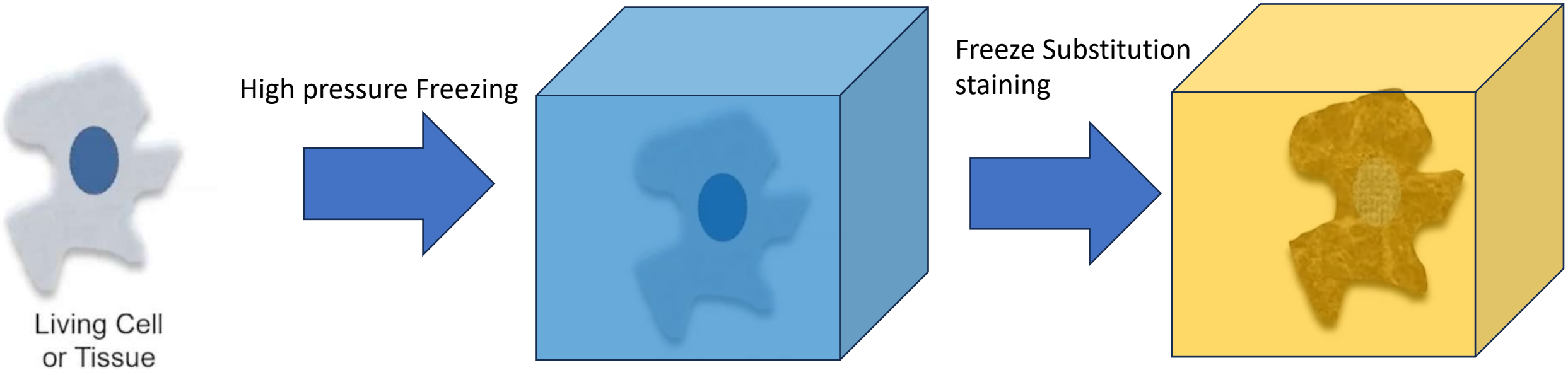
Image: Villa *et al*, 2013

Types of Samples

Room temperature Samples



Freeze Substituted Samples

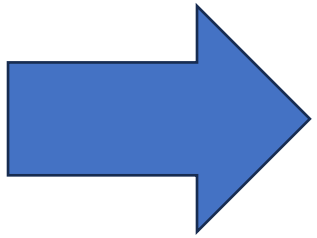


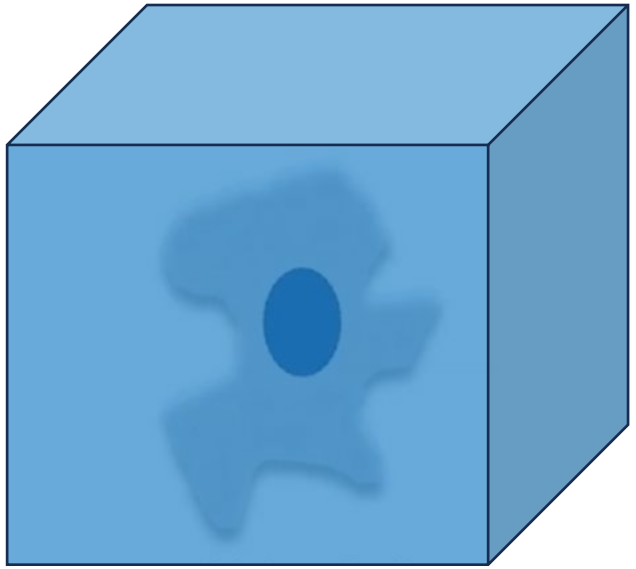
Size limit: ~200 µm thick

Frozen Samples

Living Cell
or Tissue

High pressure Freezing

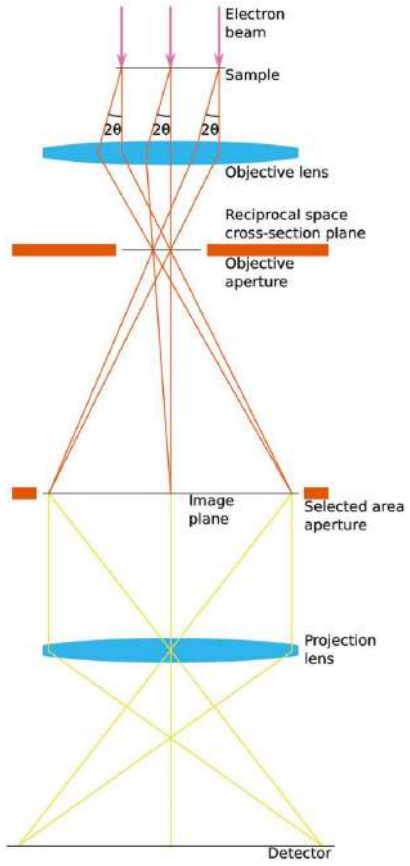




SEM Basics

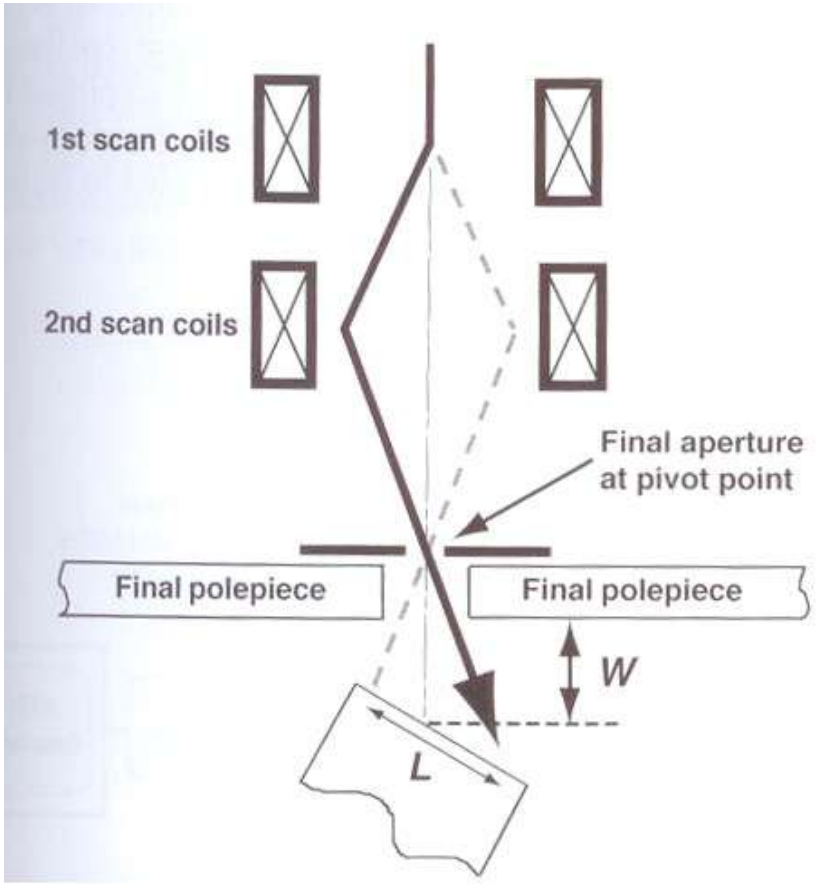
SEM versus TEM

TEM



Projection through sample

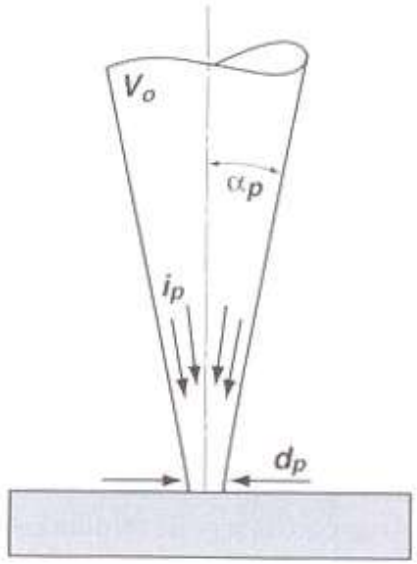
SEM



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)

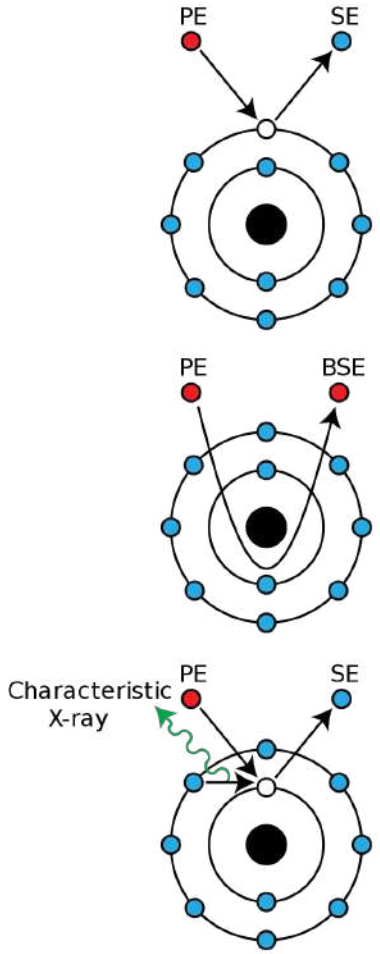
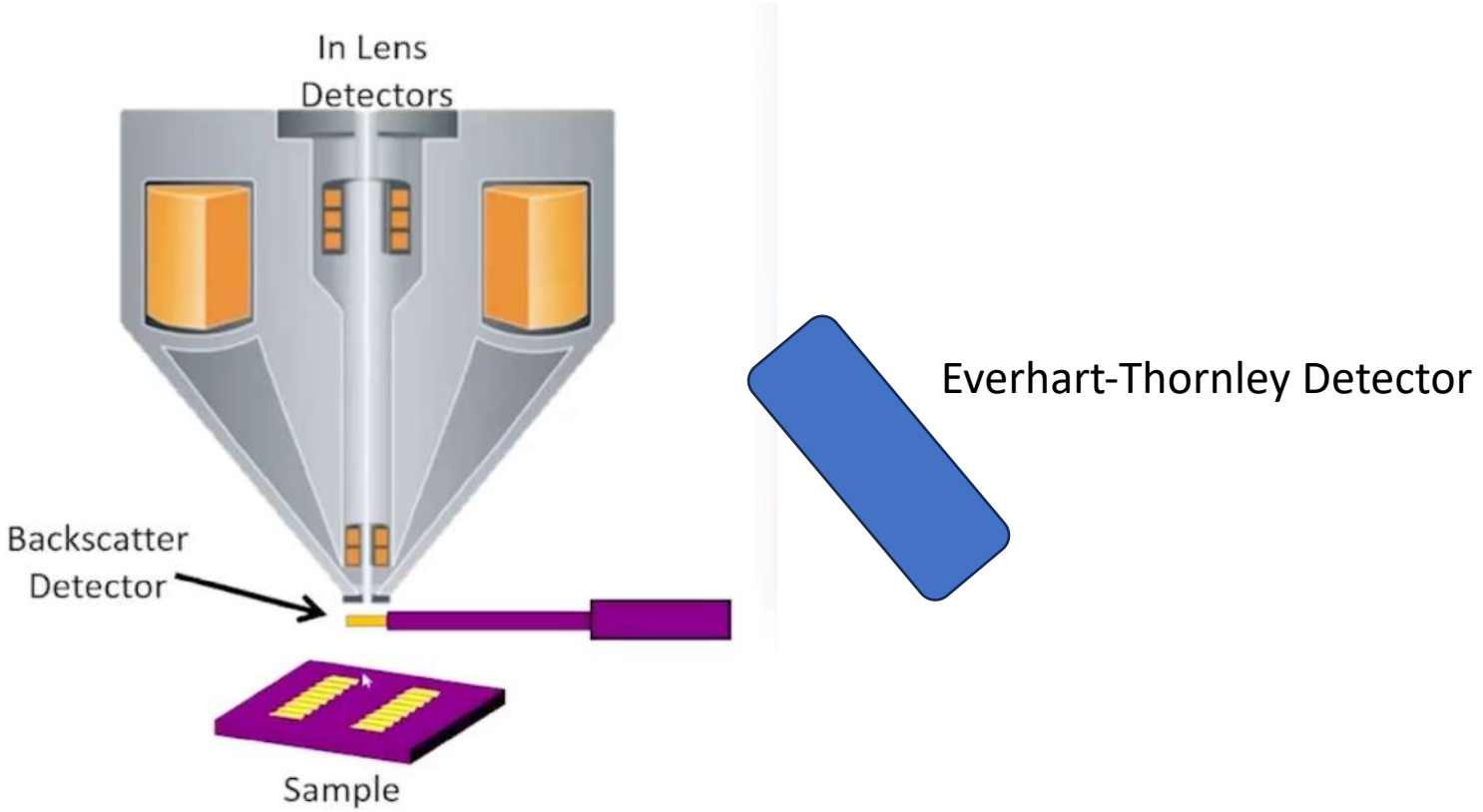


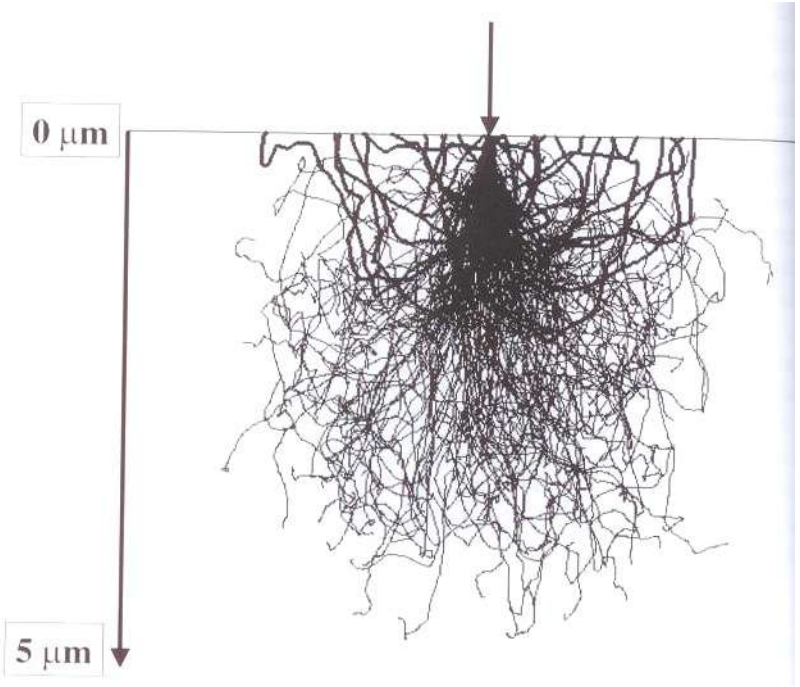
Image Source: Rob Hurt - Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=50931451

Detector Setup for SEM



Beam-Specimen Interaction

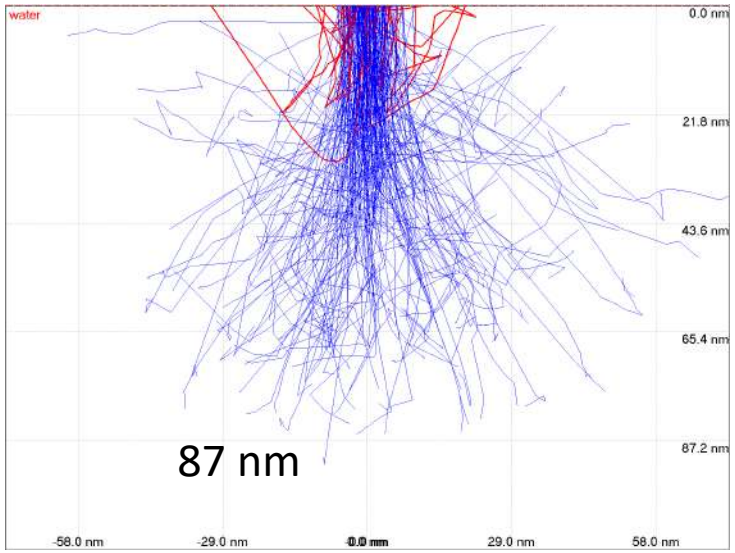
- Monte Carlo simulation of a 20 keV beam in Si
  - Dark traces: electrons which left the sample (BSE's)
- Electrons may be scattered elastically or inelastically
- Probability of elastic scattering ~ Z^2
- Inelastic scattering:
  - Secondary electrons
  - X-rays



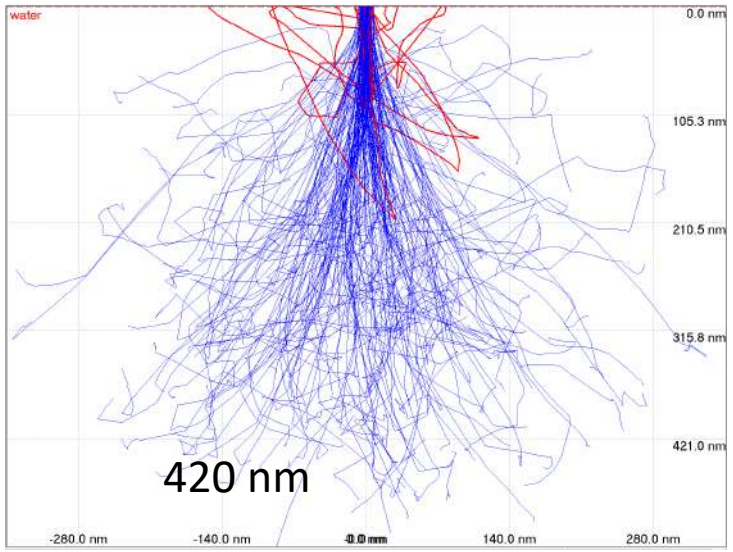
Goldstein et al, 2003

Monte Carlo simulation: water

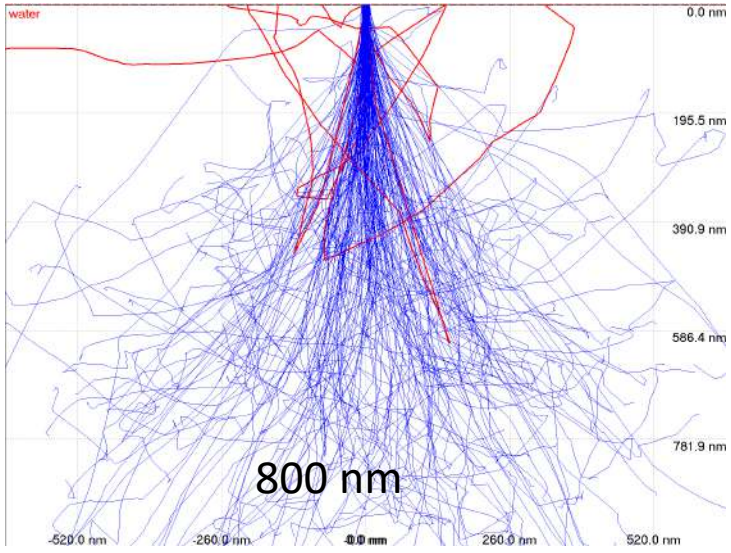
1 KEV



3 KEV

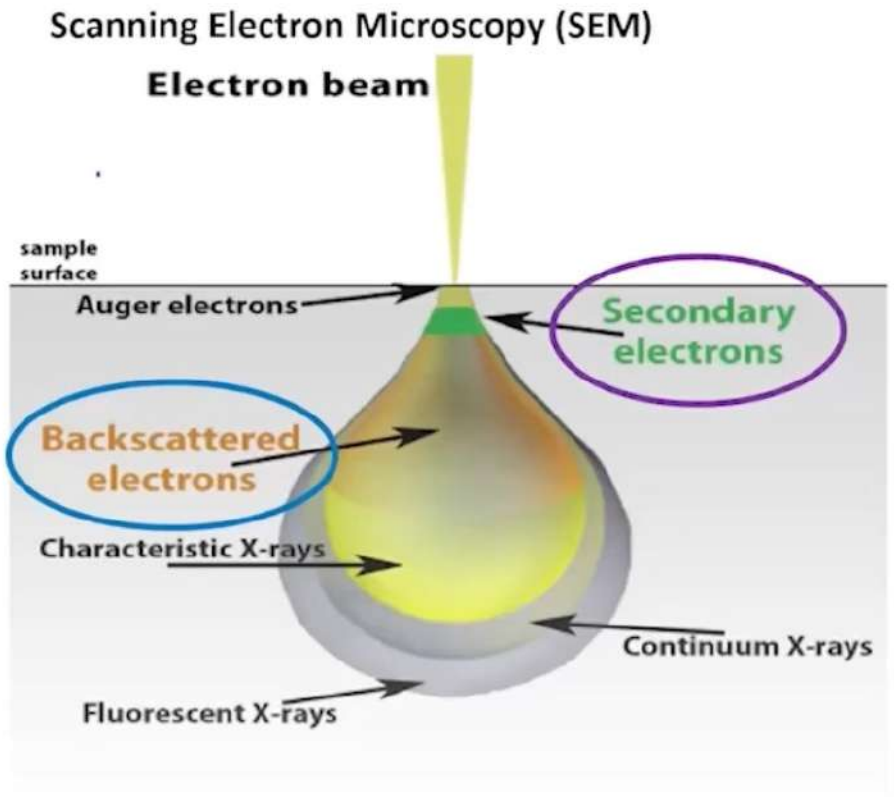


5 KEV



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

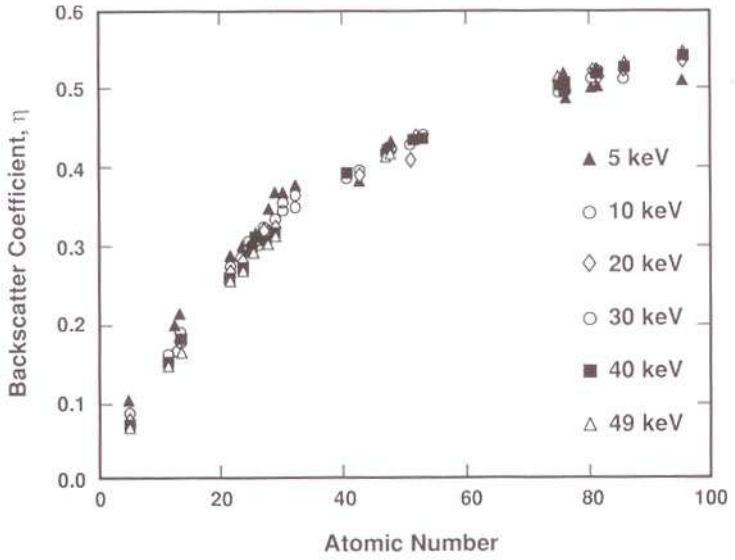
Interaction Volume



Schematic of electron beam interaction

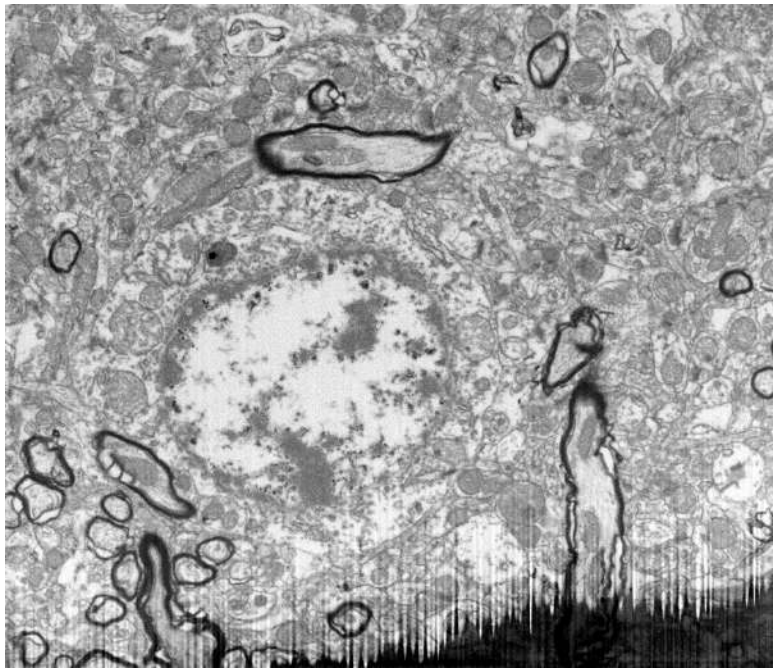
BSE efficiency is material dependent, voltage independent

Fraction of
electrons that backscatter



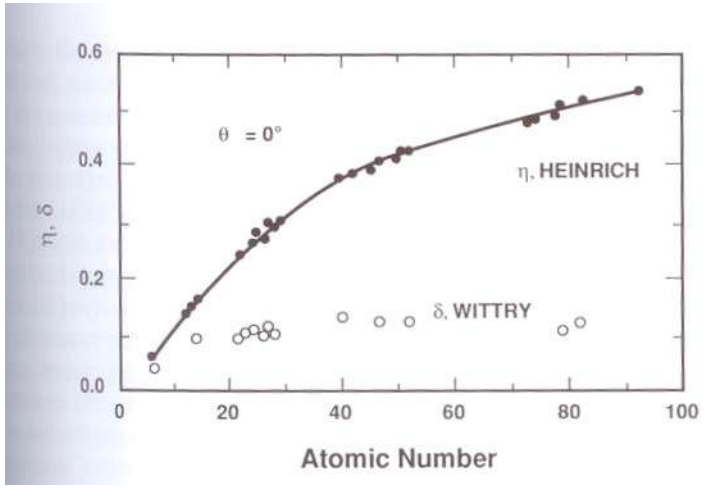
Goldstein et al, 2003

BSE's give contrast between
light and heavy elements



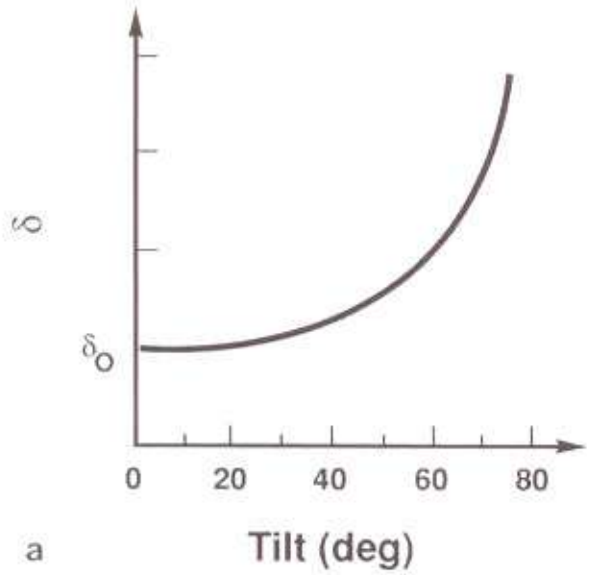
Osmium stained, resin-embedded tissue

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



30 keV

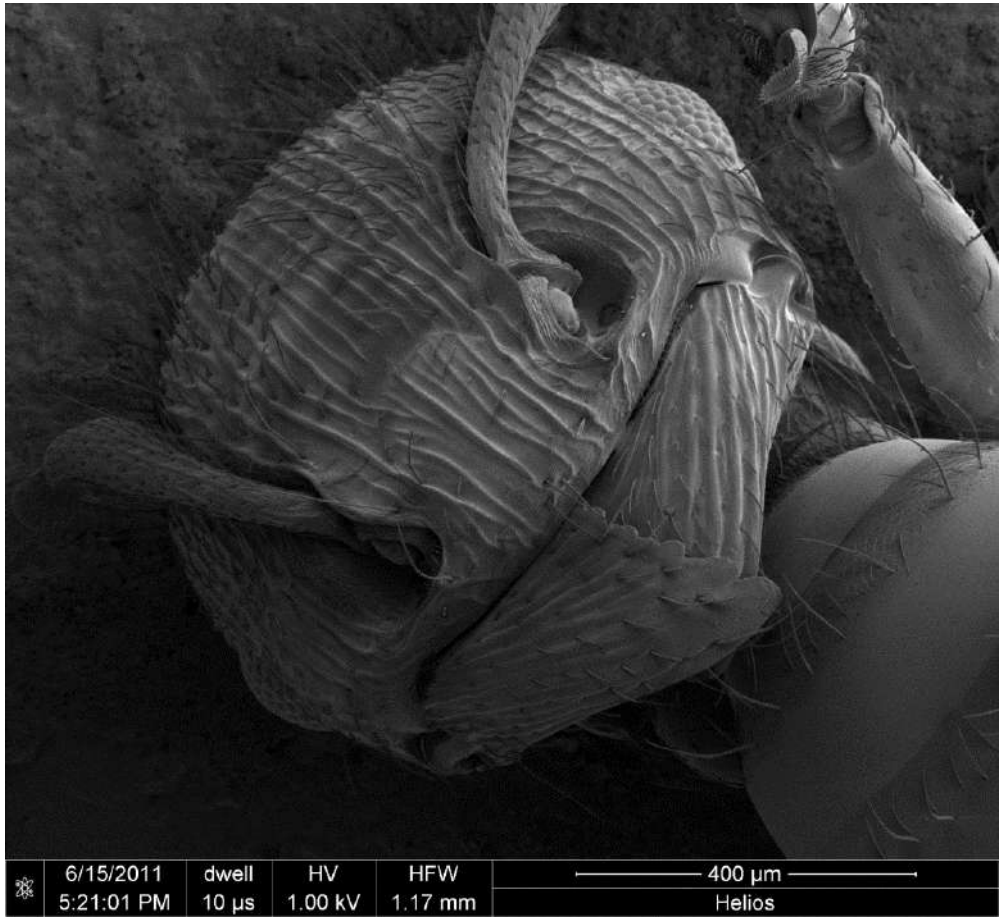
SE's are less sensitive to atomic number than BSE's (may be more sensitive at lower beam energies)



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

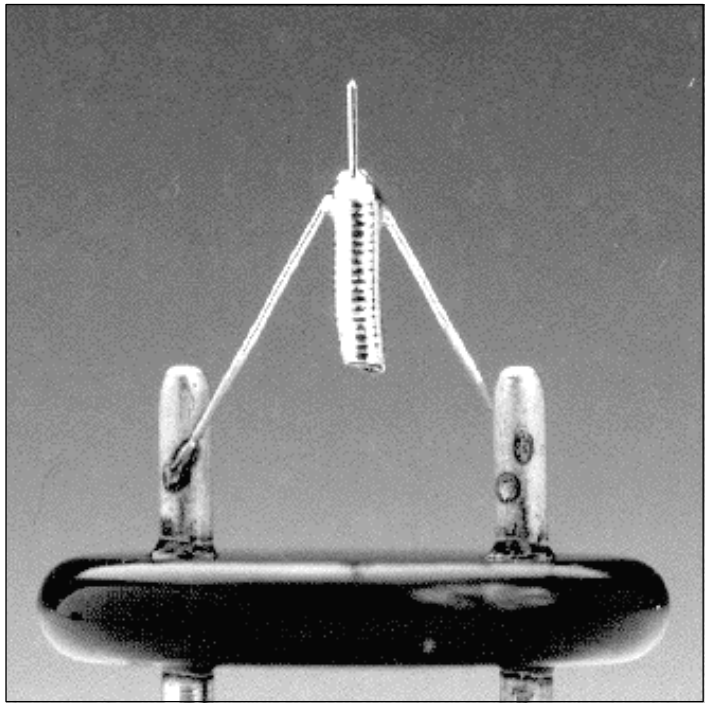
TEM

- 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

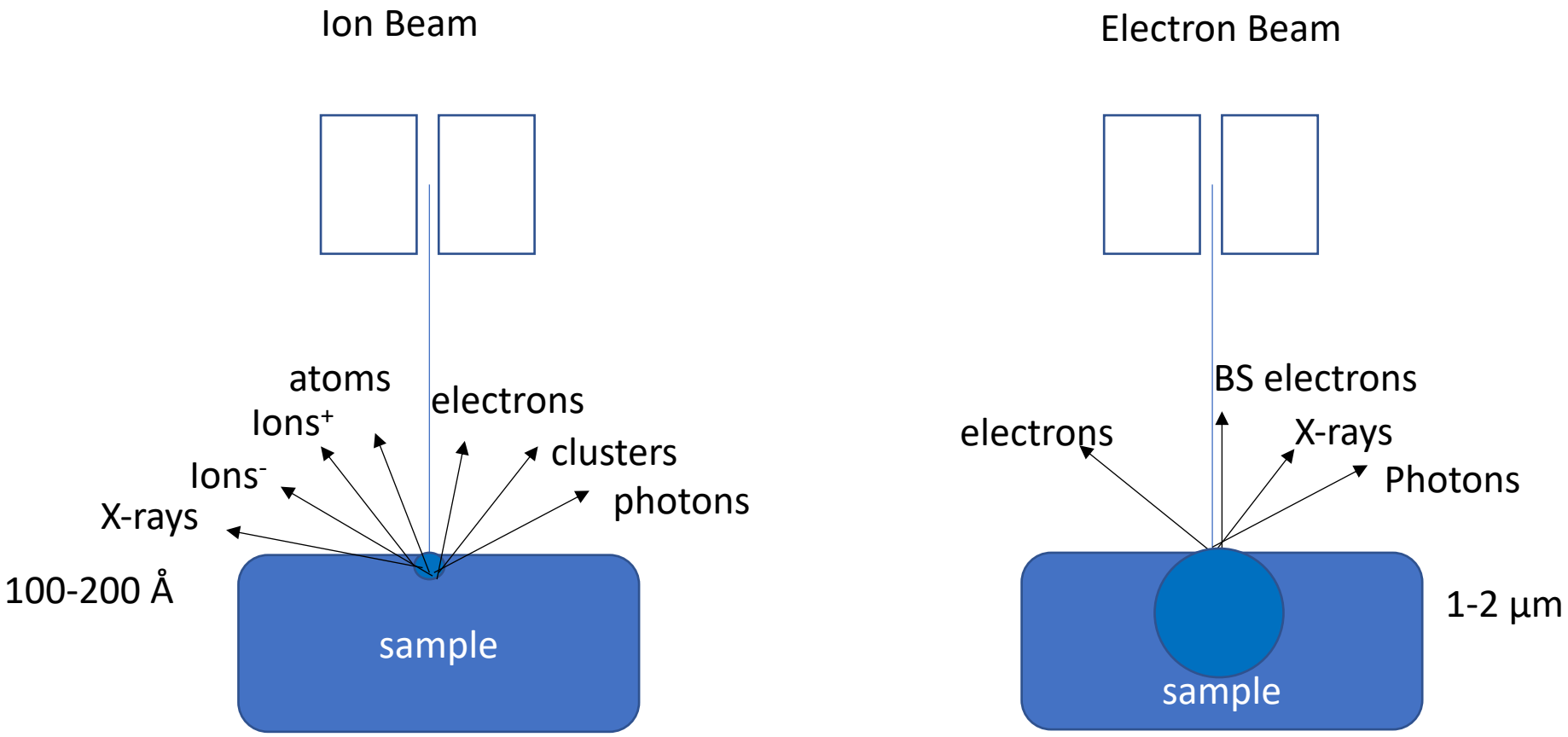


FEI™

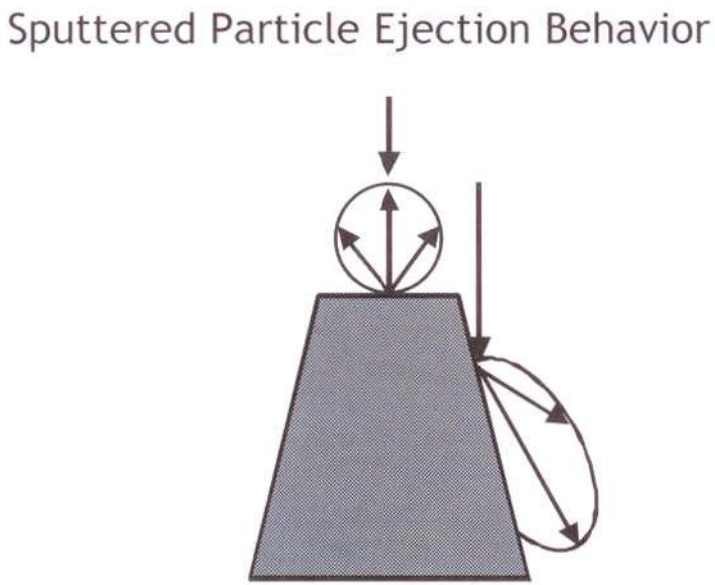
Ion Column

- ◆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



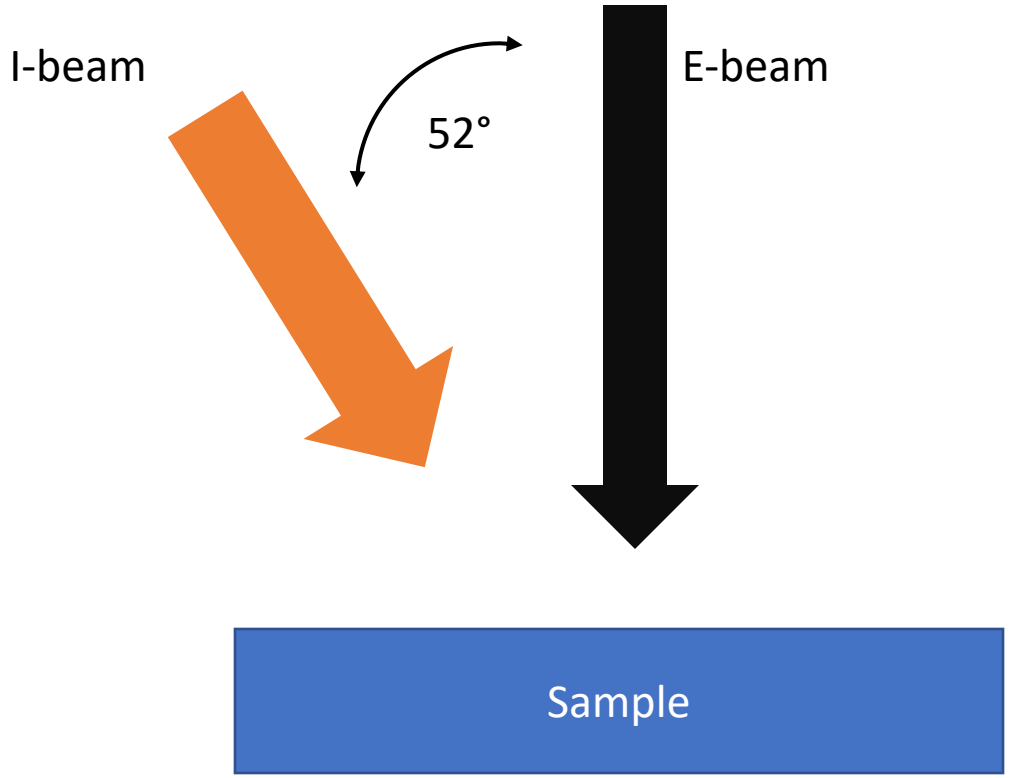
Common Use: Sputtering particles from substrate



More efficient milling at edge than in bulk

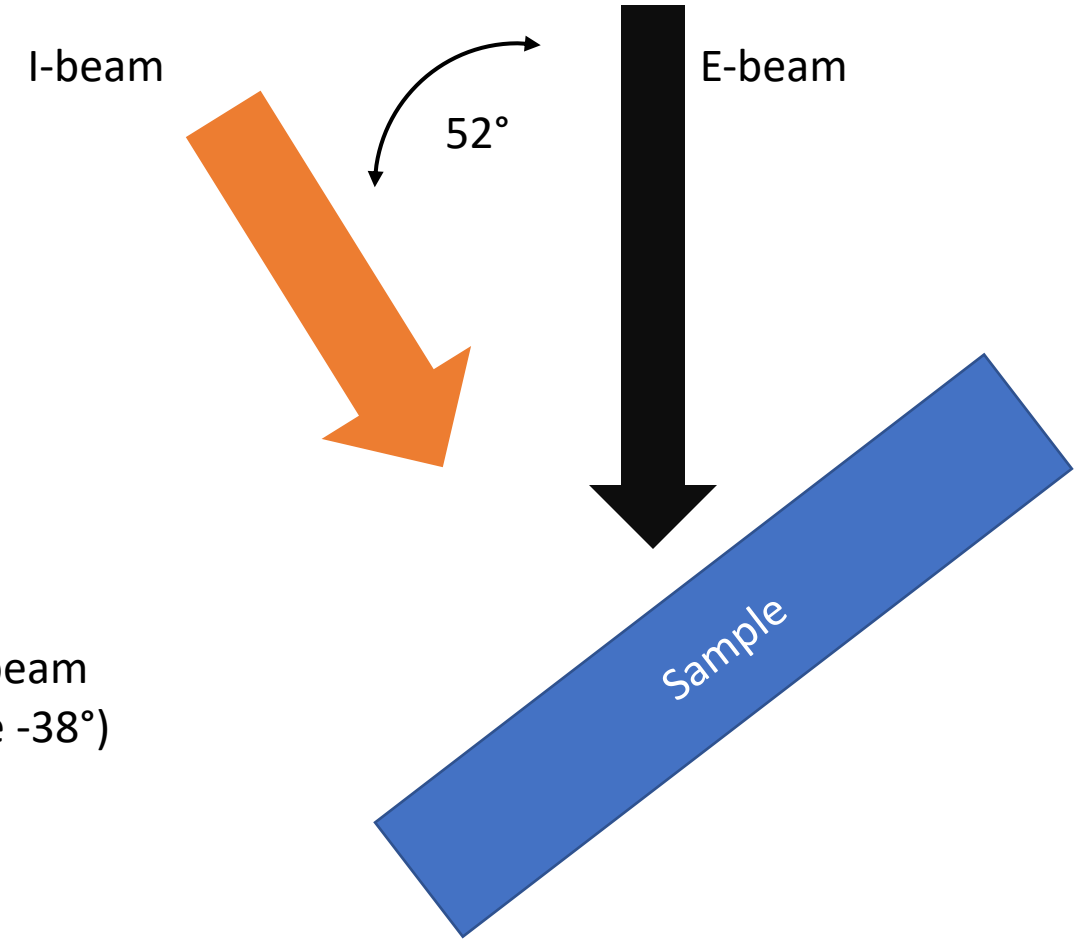
FEI™

Geometry



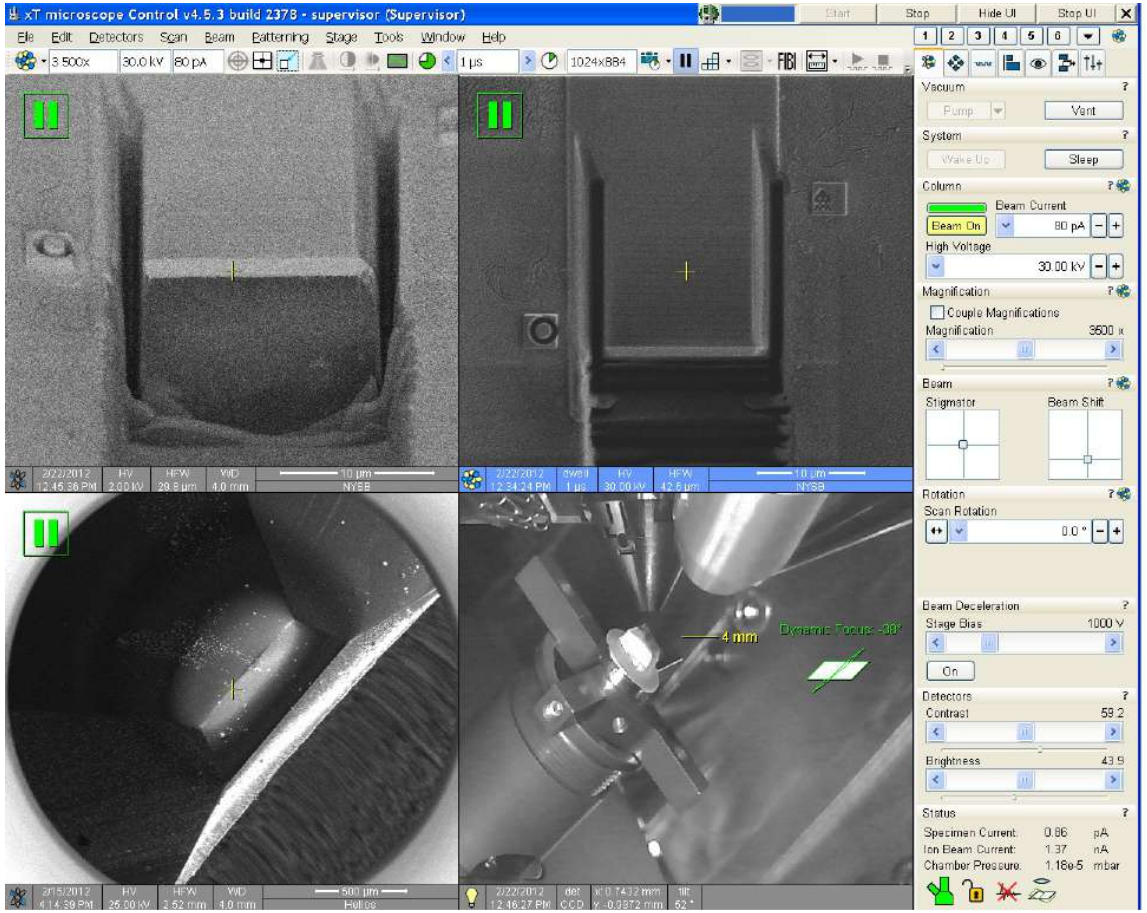
Sample:
Tilt from -10° to +70°

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)
Start
Stop
Hide UI
Stop UI
File
Edit
Detectors
Scan
Beam
Patterning
Stage
Tools
Window
Help
Vacuum
Pump
Vent
System
Wake Up
Sleep
Column
Beam Current
Beam On
80 pA
High Voltage
30.00 kV
Magnification
Couple Magnifications
Magnification
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
4 mm
Dynamic Focus: -38°

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”

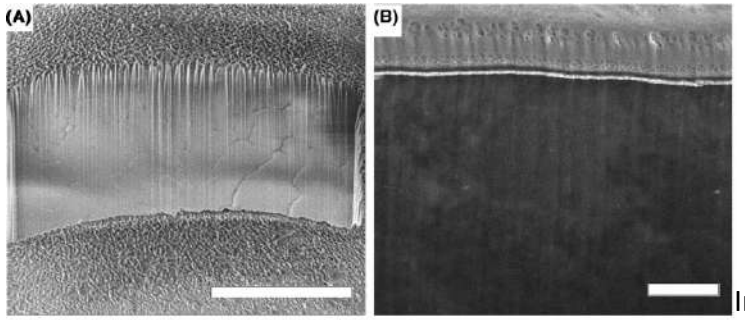
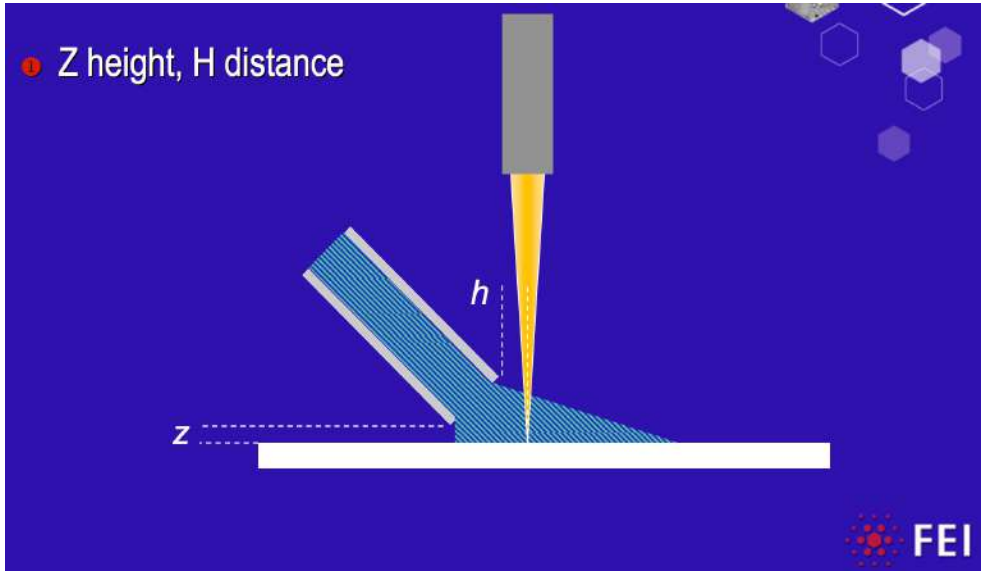
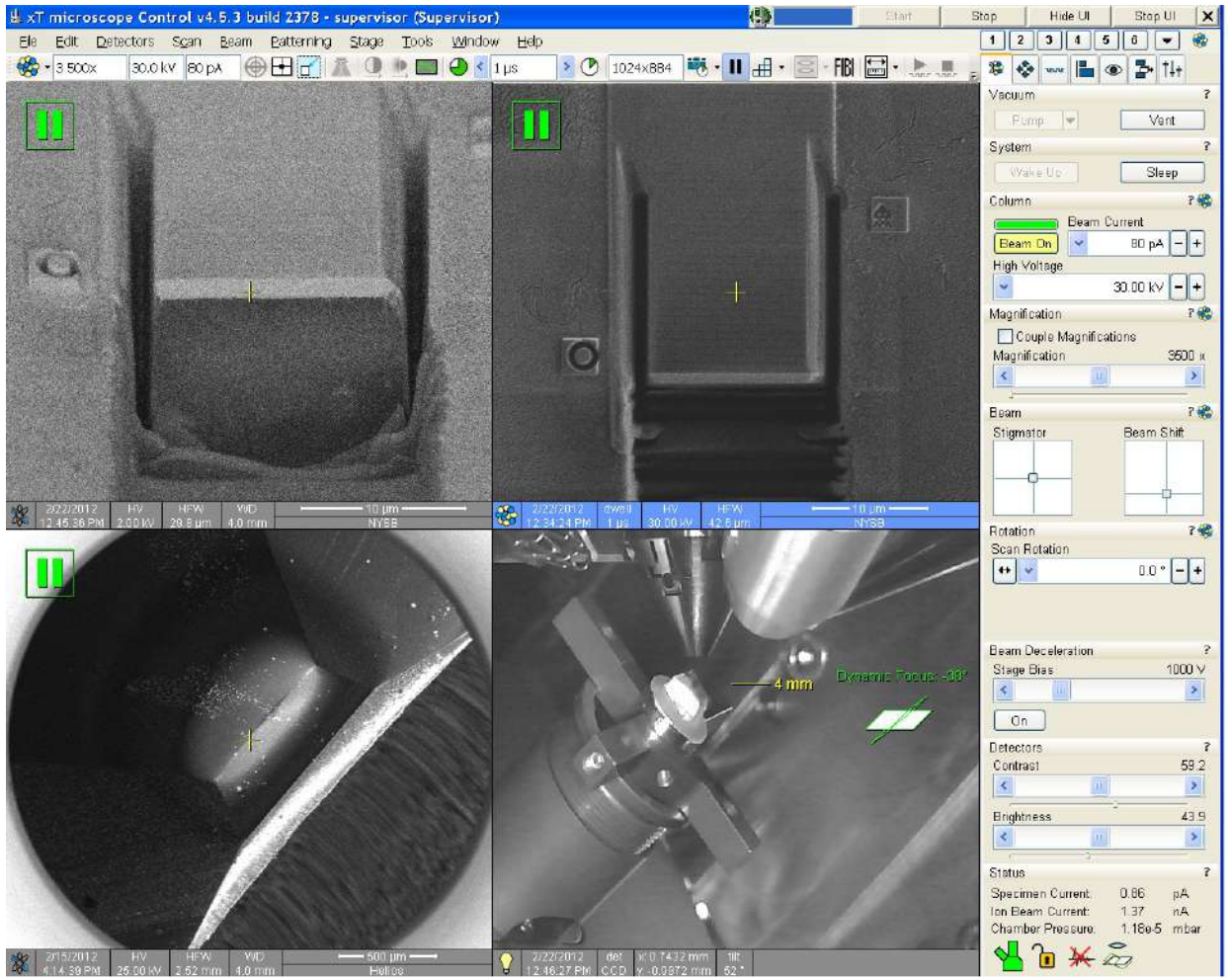


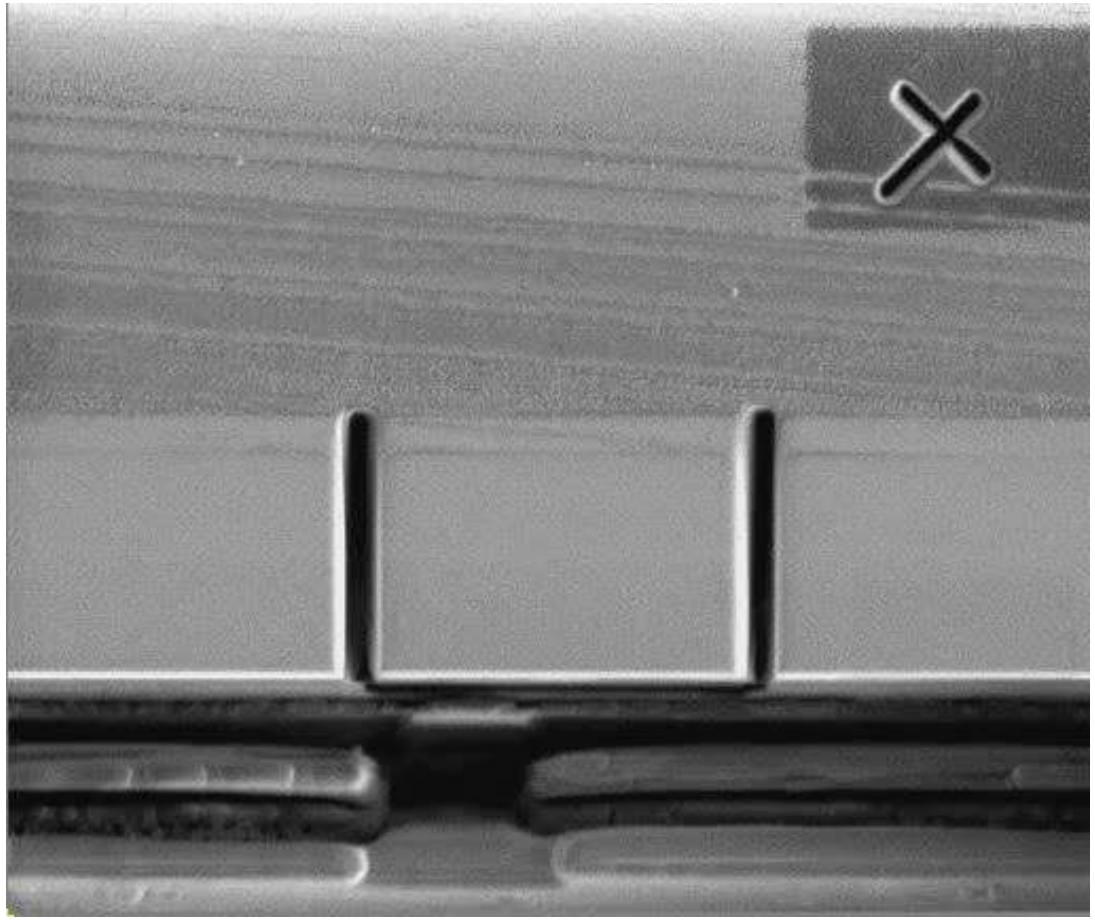
Image: Hayles and Winter, 2021



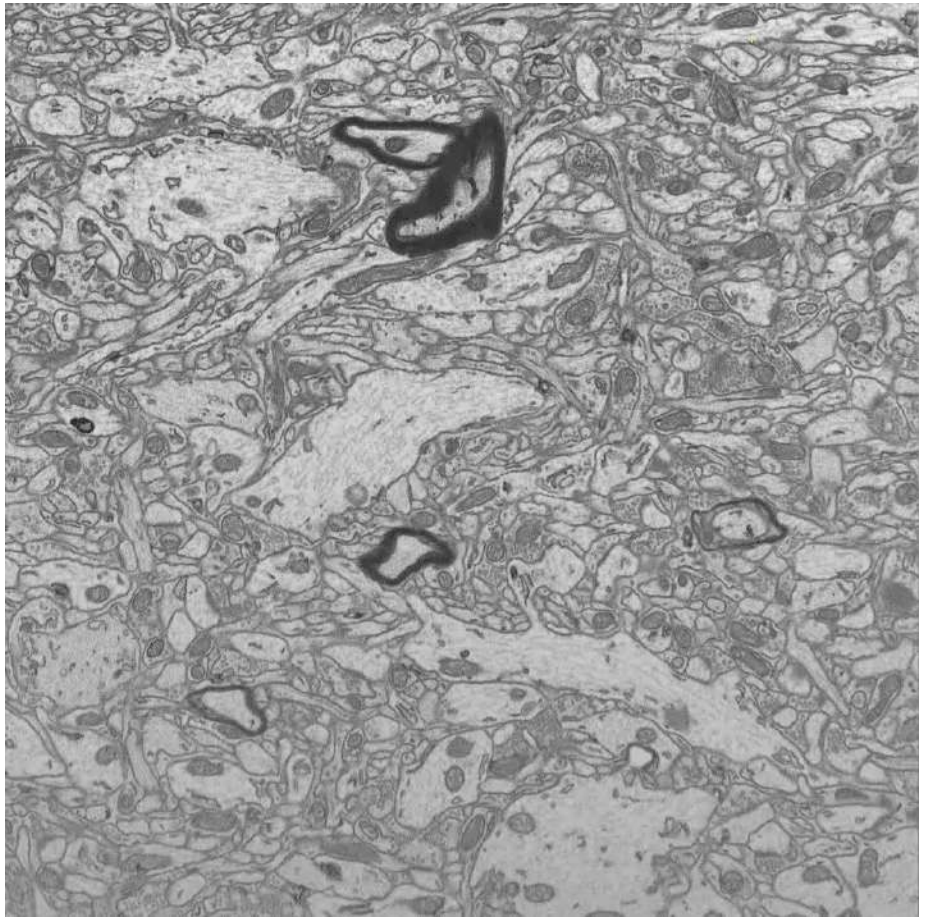
Application: 3D reconstruction of stained, freeze substituted resin-embedded tissue



Milling: i-beam view



Example Movie: Neural Tissue



Volumetric imaging with
nearly isotropic pixels

Cryo-SEM imaging

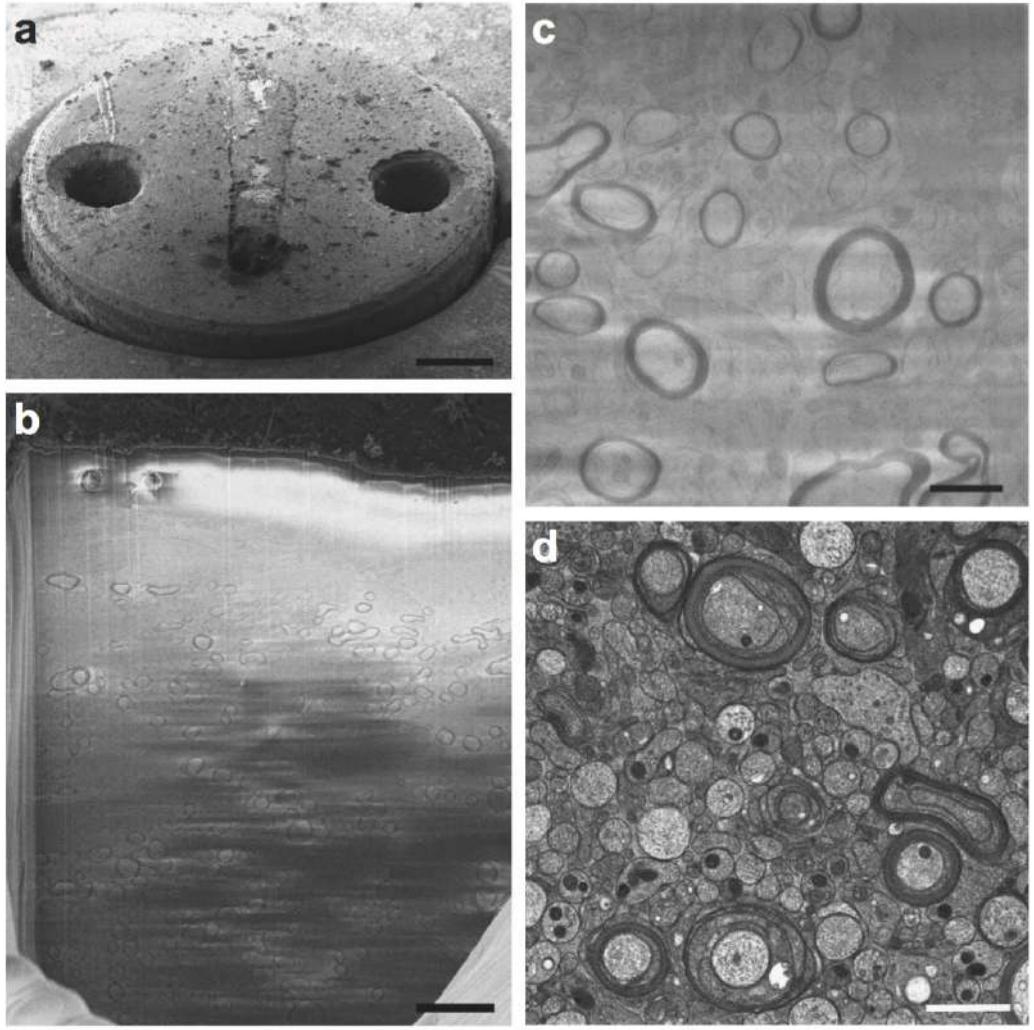
Technical Note

Cryo FIB-SEM: Volume imaging of cellular ultrastructure in native frozen specimens

Andreas Schertel [a,1], Nicolas Snaidero [b,1], Hong-Mei Han [c], Torben Ruhwedel [d], Michael Laue [e], Markus Grabenbauer [c,2], Wiebke Möbius [d,f,*]

[a] Carl Zeiss Microscopy GmbH, Training, Application and Support Center (TASC), Carl-Zeiss-Straße 22, D-73447 Oberkochen, Germany
[b] Cellular Neuroscience, Max Planck Institute of Experimental Medicine, Hermann-Rein-Straße 3, D-37075 Göttingen, Germany
[c] Department of Systemic Cell Biology, Max-Planck-Institute of Molecular Physiology, Otto-Hahn-Straße 11, D-44227 Dortmund, Germany
[d] Department of Neurogenetics, Electron Microscopy Facility, Max-Planck-Institute of Experimental Medicine, Hermann-Rein-Straße 3, D-37075 Göttingen, Germany
[e] Advanced Light and Electron Microscopy, Centre for Biological Threats and Special Pathogens, Robert Koch Institute, Nordufer 20, D-13353 Berlin, Germany
[f] Center for Nanoscale Microscopy and Molecular Physiology of the Brain (CNMPB), Göttingen, Germany

In-lens SE detector at 2.33 kV



Technical Note

Cryo FIB-SEM: Volume imaging of cellular ultrastructure in native frozen specimens

Andreas Schertel [a,1], Nicolas Snaidero [b,1], Hong-Mei Han [c], Torben Ruhwedel [d], Michael Laue [e], Markus Grabenbauer [c,2], Wiebke Möbius [d,f,*]

[a] Carl Zeiss Microscopy GmbH, Training, Application and Support Center (TASC), Carl-Zeiss-Straße 22, D-73447 Oberkochen, Germany
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Cryo FIB/SEM for tomographic sample preparation

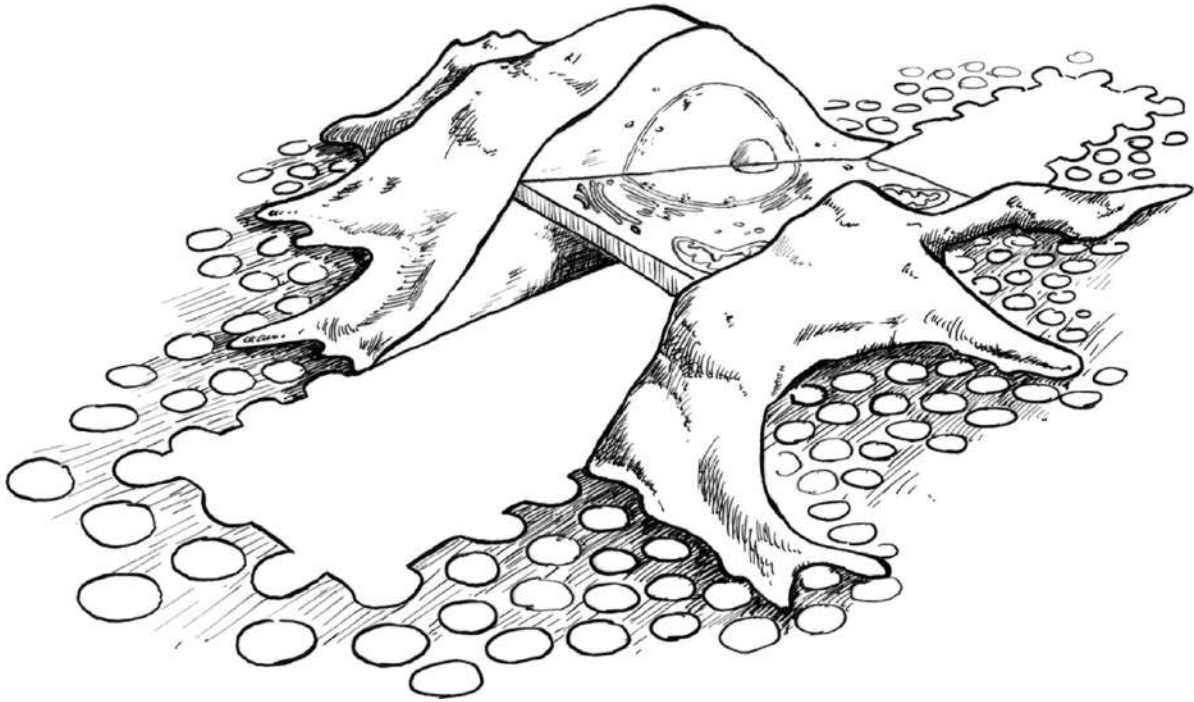


Image: Villa *et al*, 2013

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

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

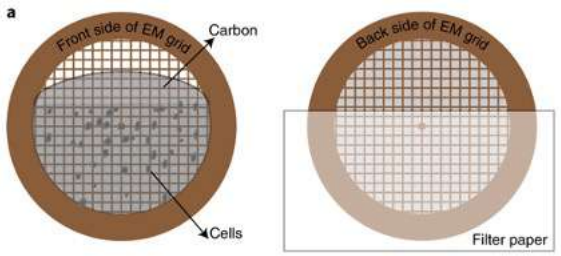
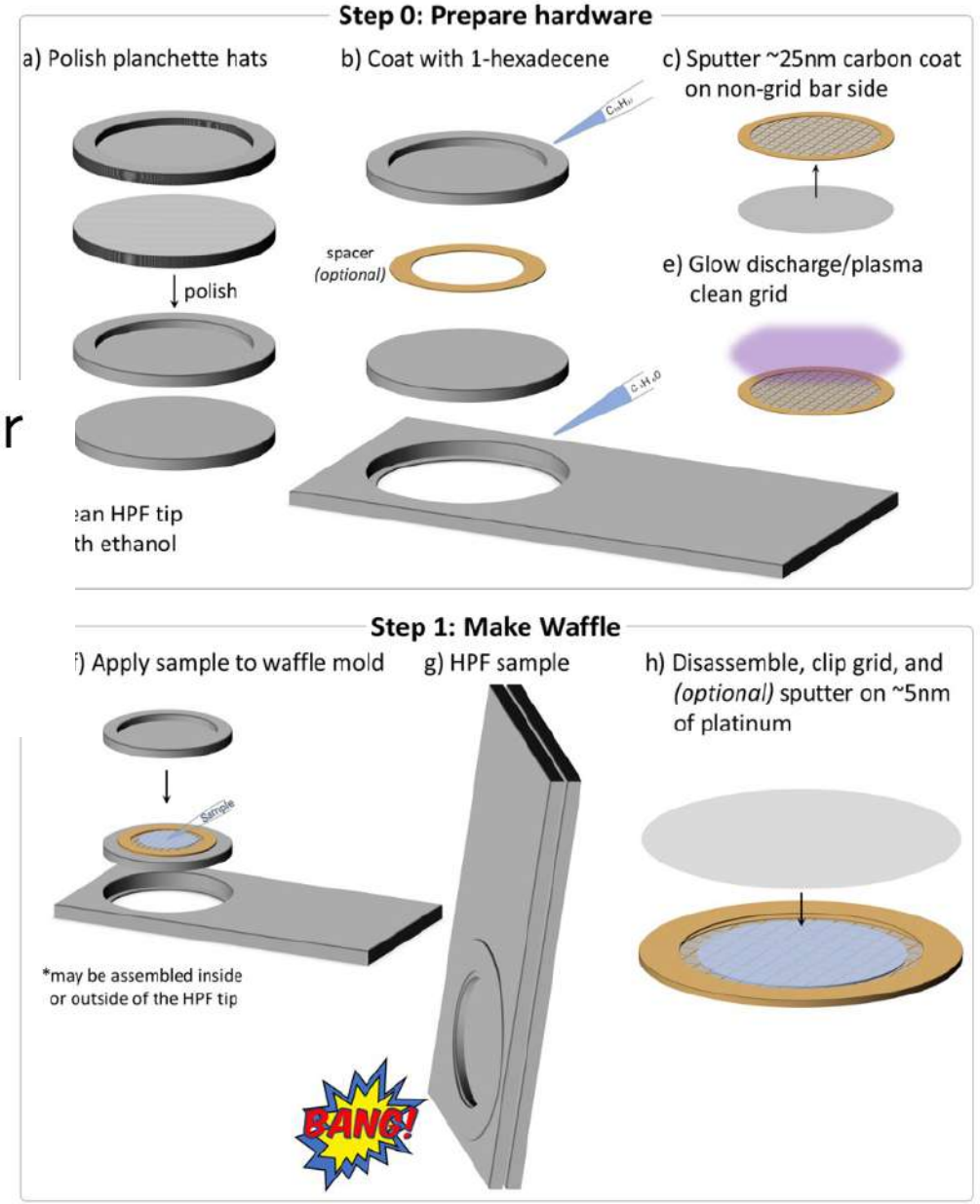


Image: Wagner *et al*, 2020

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✉]



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

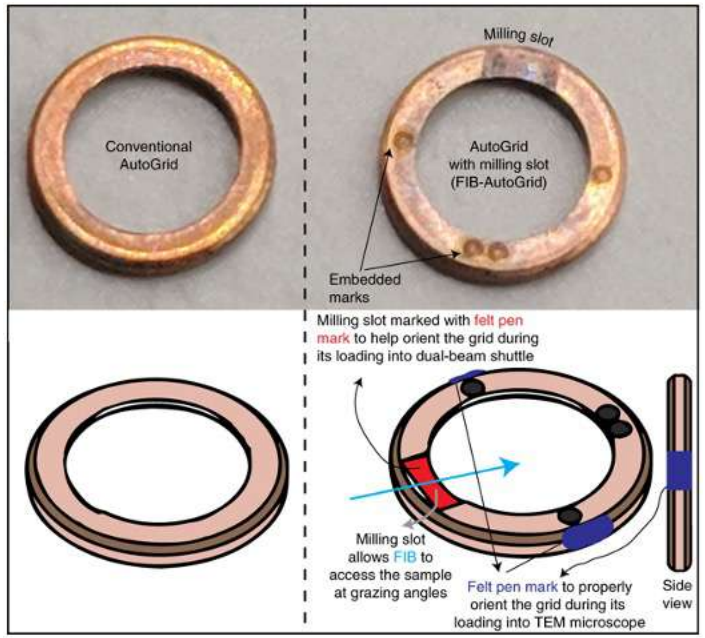


Image: Wagner *et al*, 2020

Grid Geometry

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

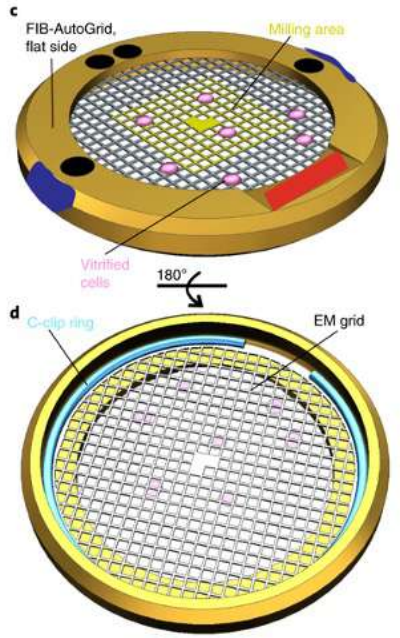


Image: Wagner *et al*, 2020

Sample Shuttle

- Shuttle for loading grids into FIB SEM
- 2 grids at a time
- Geometry needs to be known
- Grids are pre-tilted 45°
- Shutter to protect grids

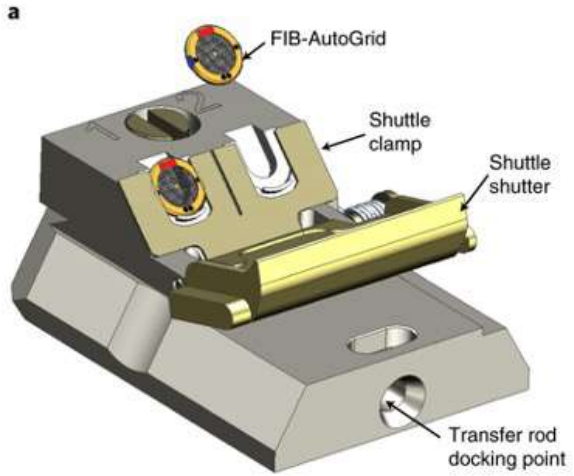
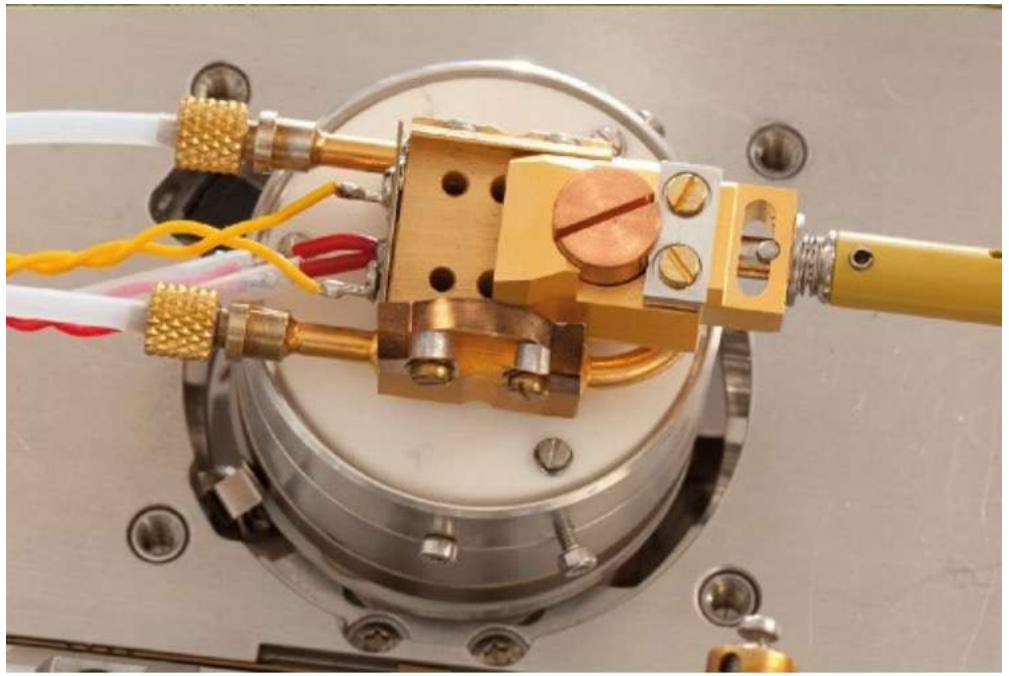


Image: Wagner *et al*, 2020

Transfer Shuttle and Cryostage on Fib SEM

Leica EM VCT500 transfer shuttle

Quorum 3010 stage



Geometry

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

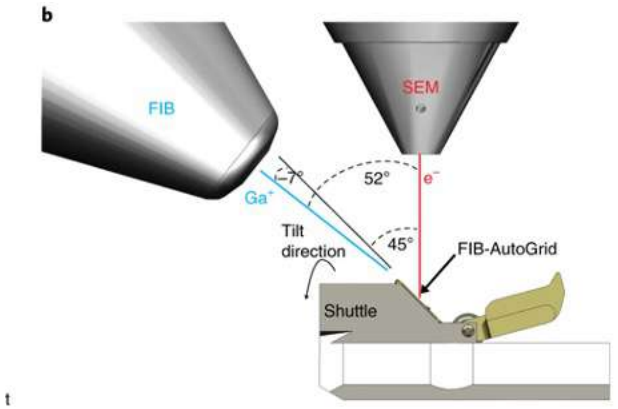


Image: Wagner *et al*, 2020

Geometry: Untilted

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

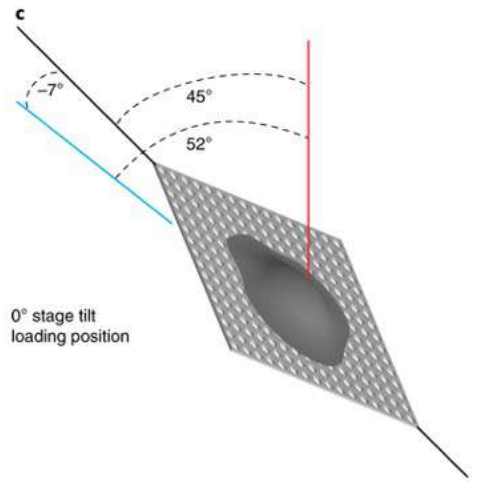


Image: Wagner *et al*, 2020

Geometry: Tilted

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

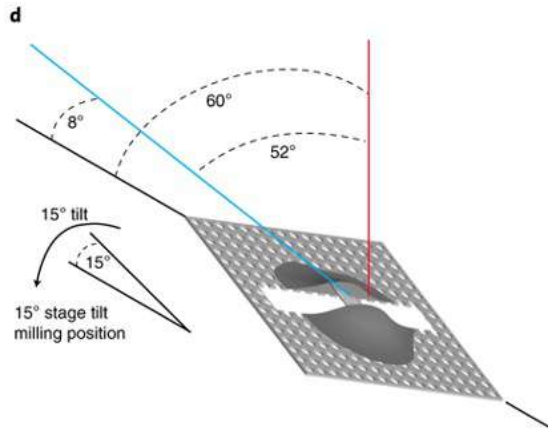


Image: Wagner *et al*, 2020

Imaging cells with ion beam

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

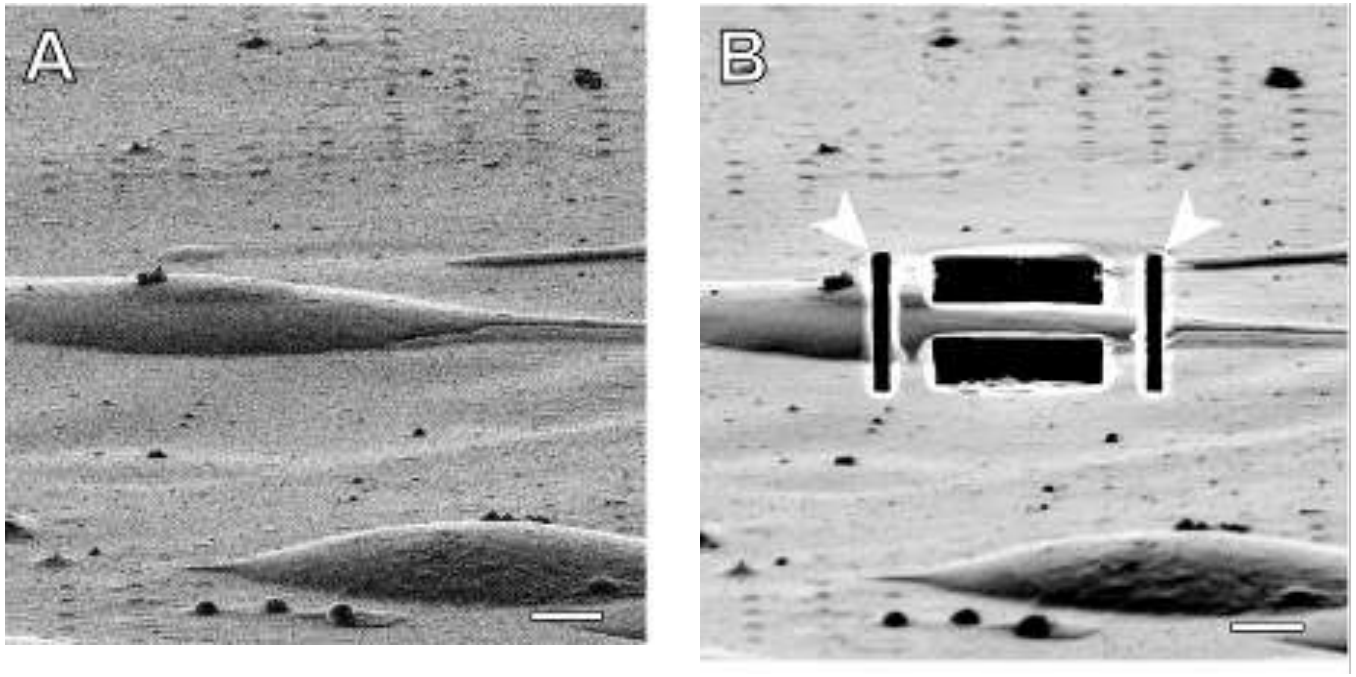


Image: Wolff *et al*, 2019

Targeting of Milling Regions

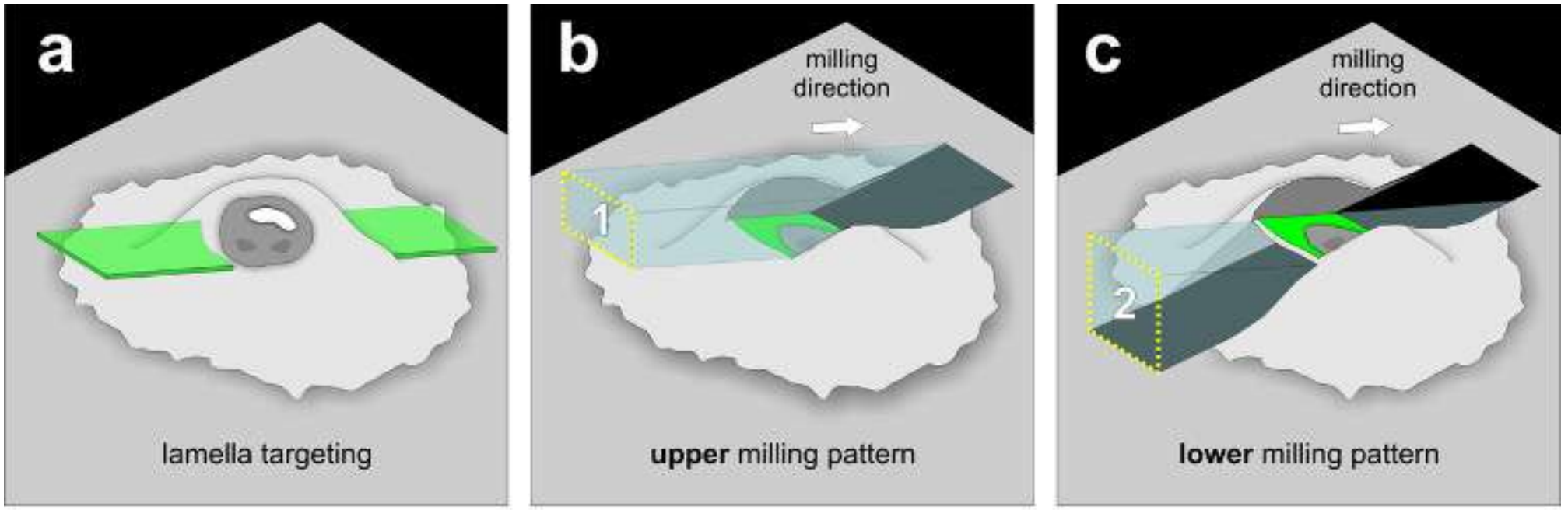


Image: Rigort and Plitzko, 2015

Targeting of Milling Regions

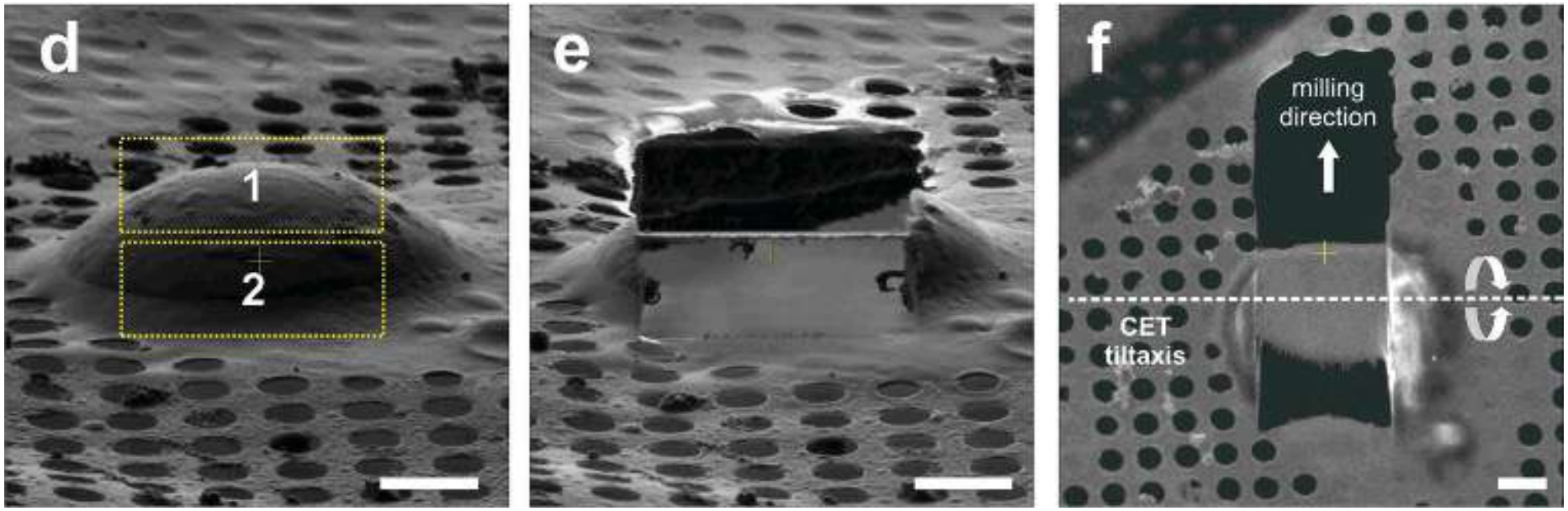


Image: Rigort and Plitzko, 2015

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

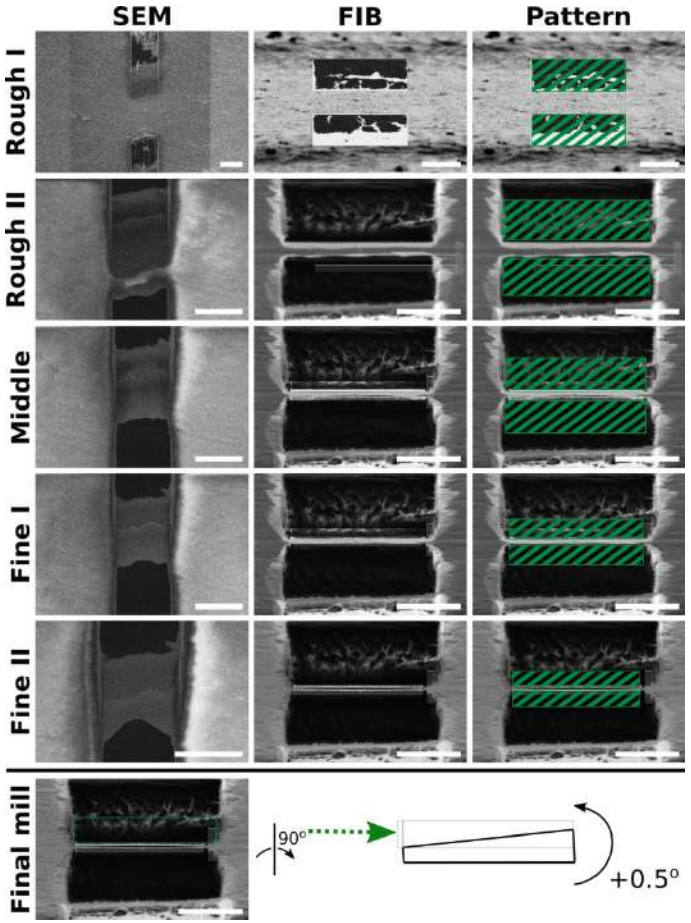
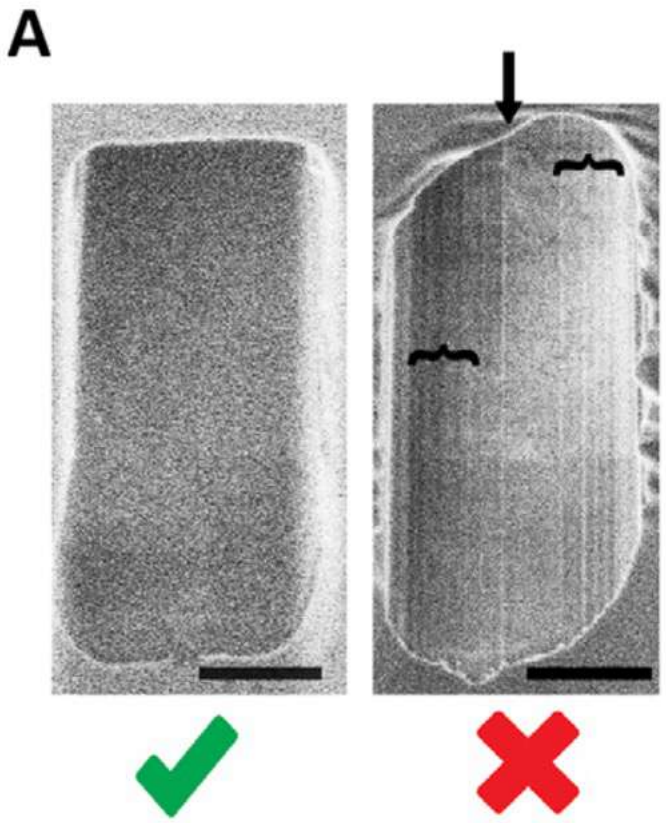


Image: Lam and Villa, 2021

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”

Milling at as shallow an angle as possible

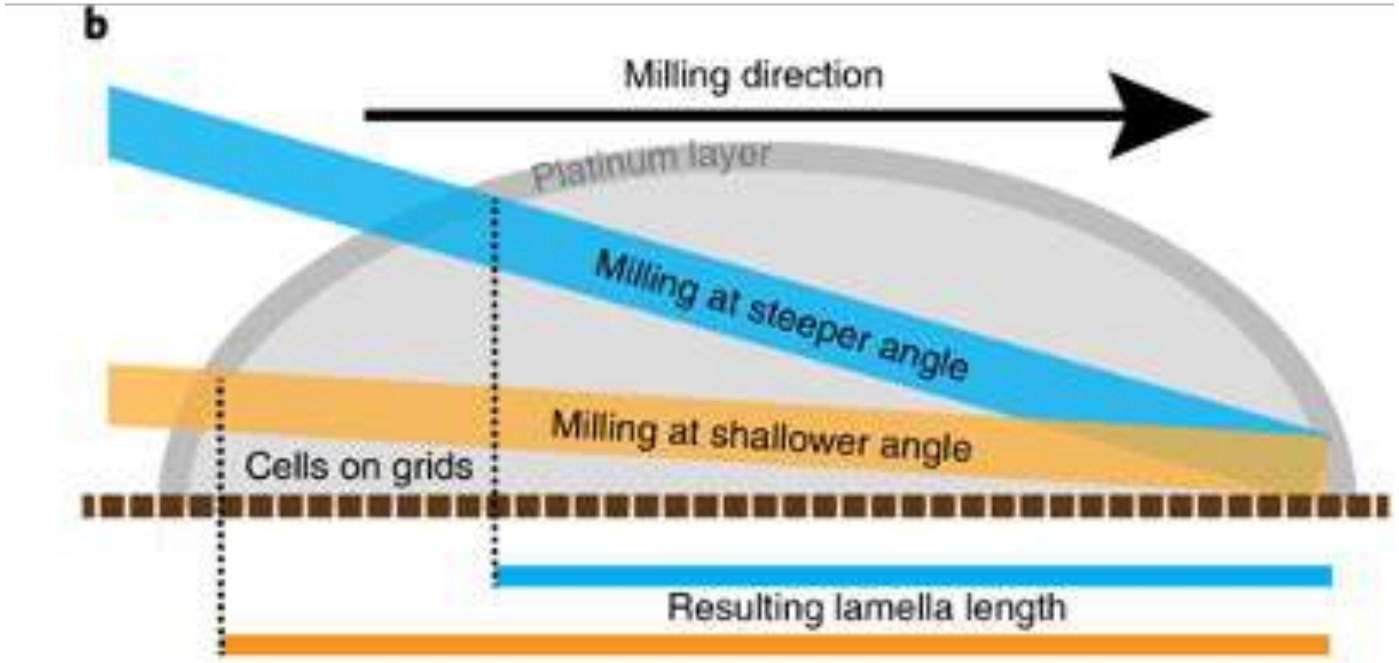


Image: Wagner *et al*, 2020

Geometry: Loading into TEM

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

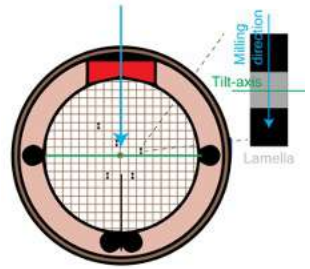


Image: Wagner *et al*, 2020

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.

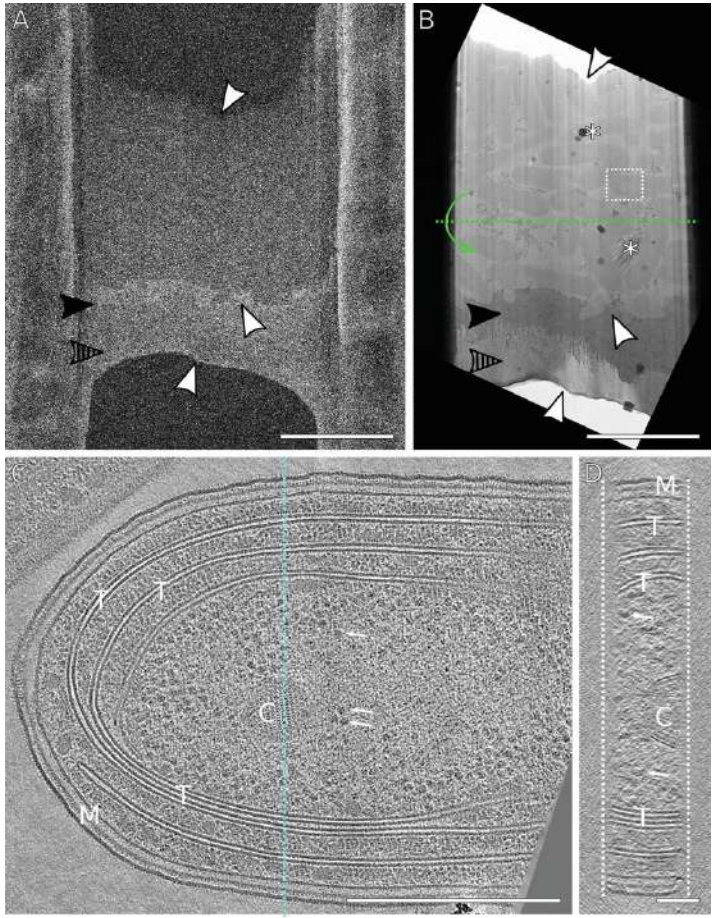


Image: Lam and Villa, 2021

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

Article

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

Plasma FIB milling for the determination of structures in situ

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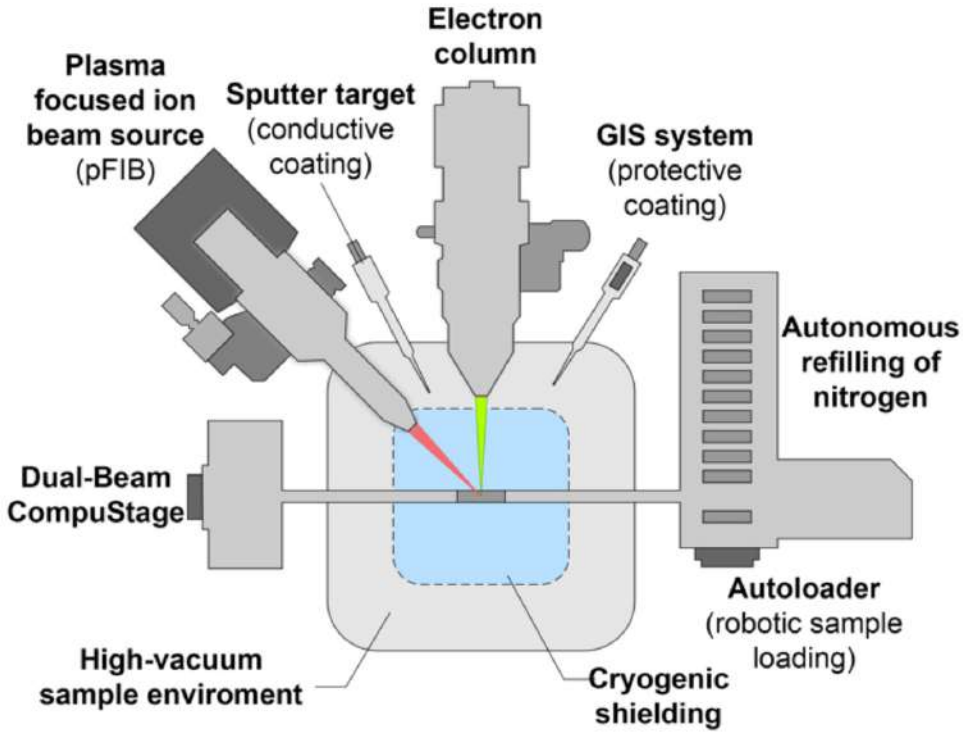
Casper Berger[1,2,5], Maud Dumoux[1,5], Thomas Glen[1,5], Neville B.-y. Yee[3], John M. Mitchels[4], Zuzana Patáková[4], Michele C. Darrow[3], James H. Naismith[1,2] & Michael Grange[1,2]

Plasma instead of Ga



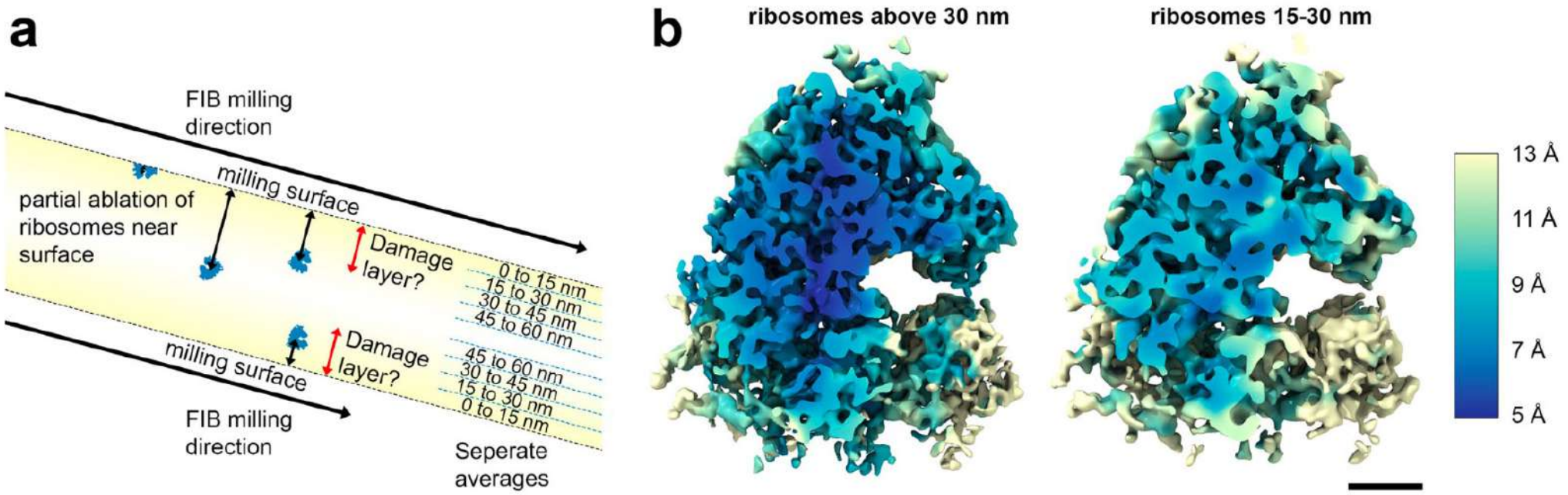
Berger et al, 2023

- 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

Damage near the lamella surface



Berger et al, 2023

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

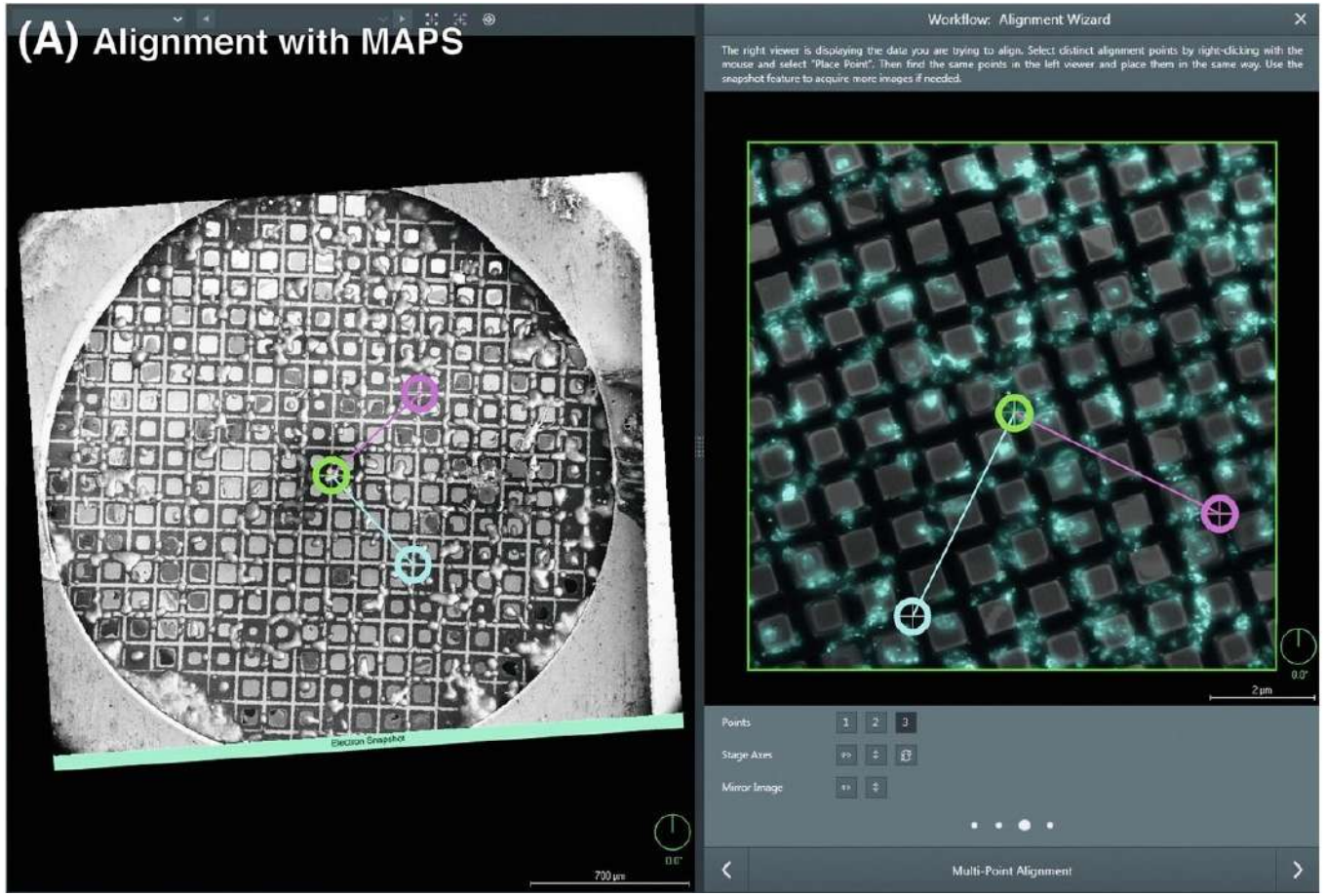


Image: Klein *et al*, 2021

Cryo-CLEM: Overlay

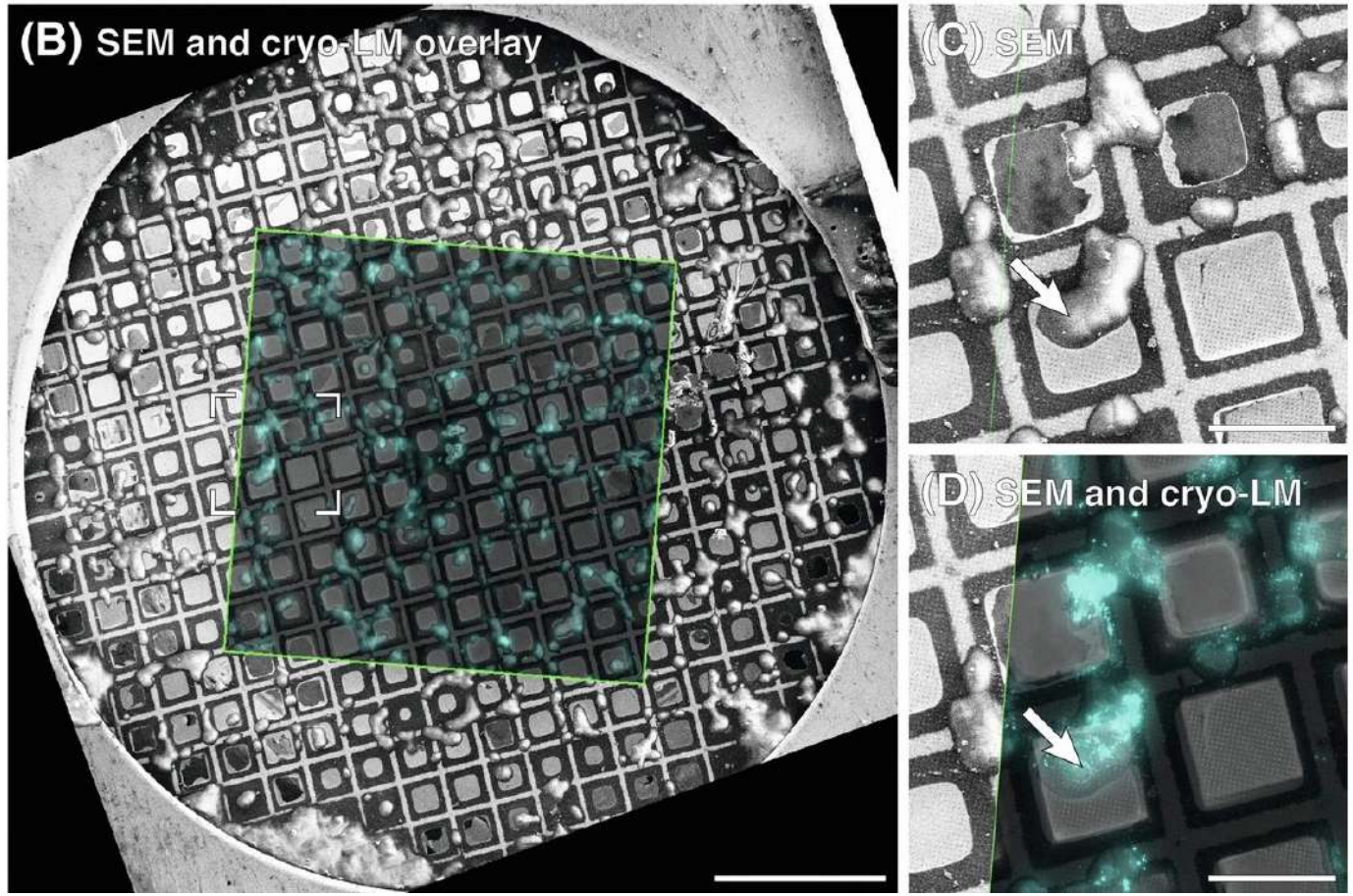
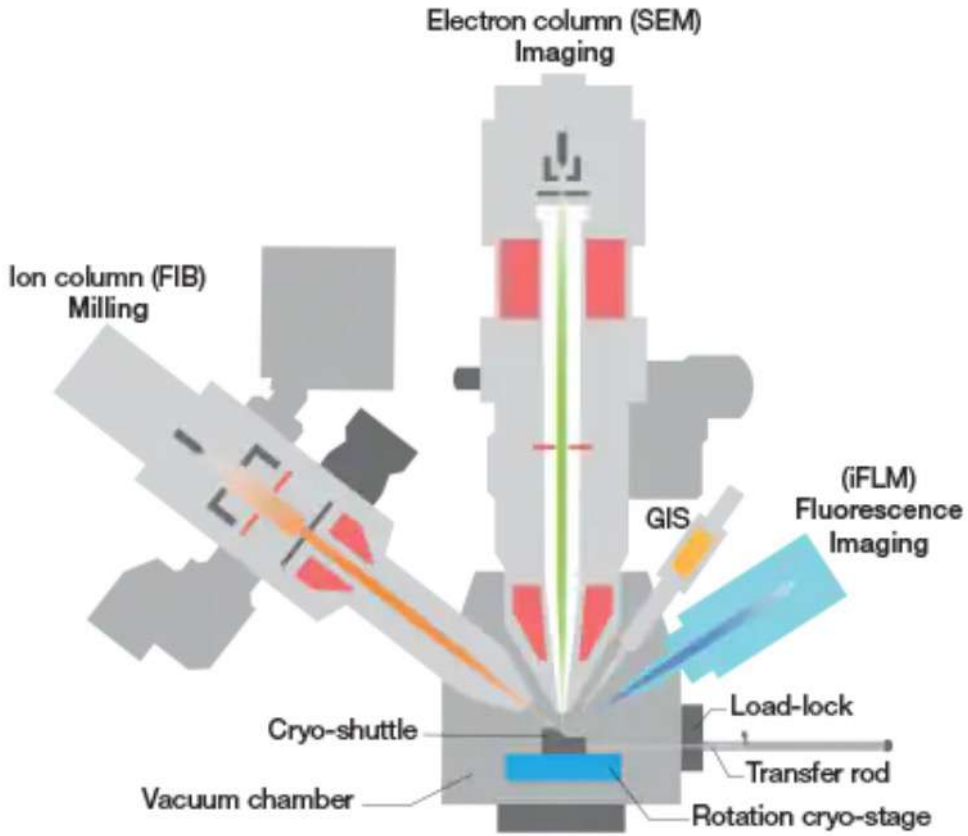


Image: Klein *et al*, 2021

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

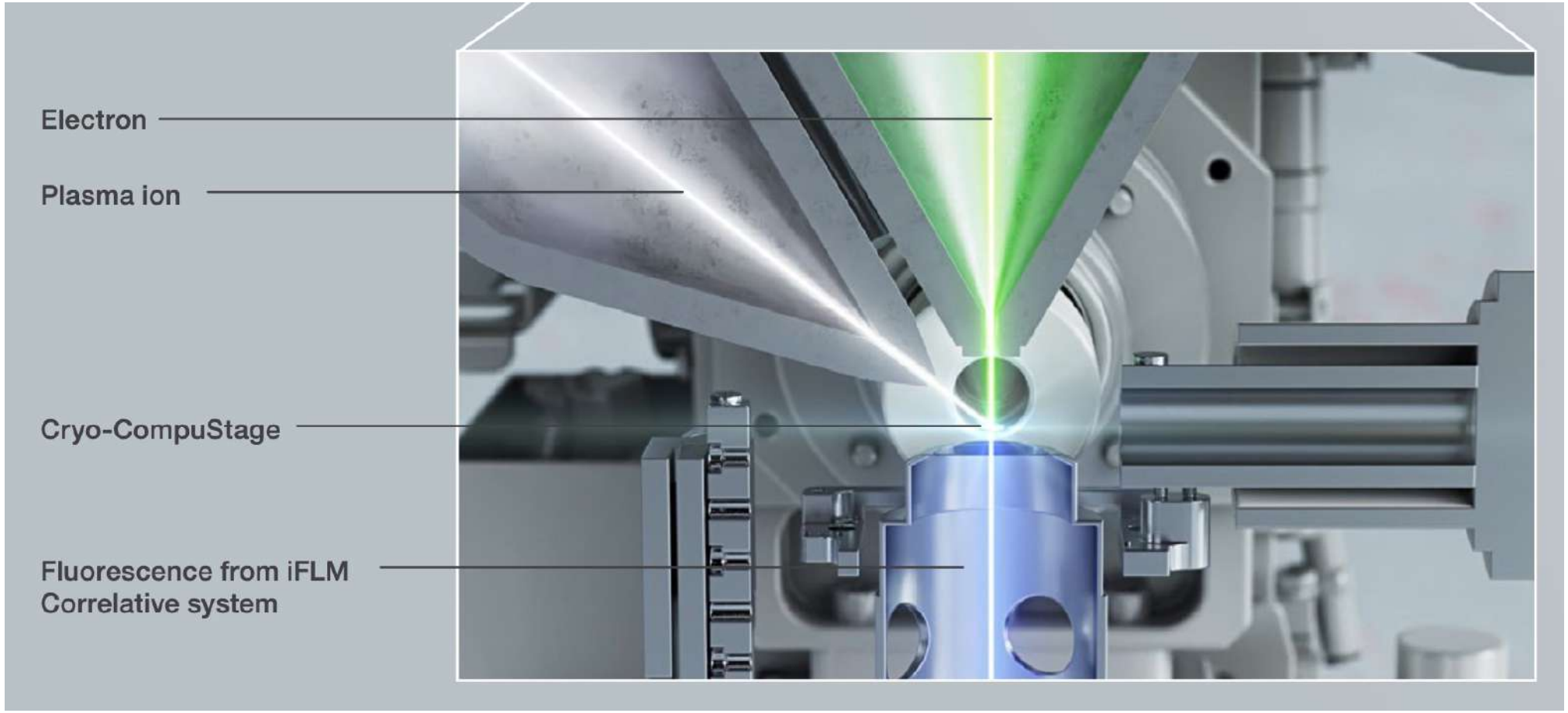
Specialized Microscopes: Aquilos 2 Cryo FIB



Specialized Microscopes: Arctis Plasma FIB

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

Arctis Plasma FIB



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