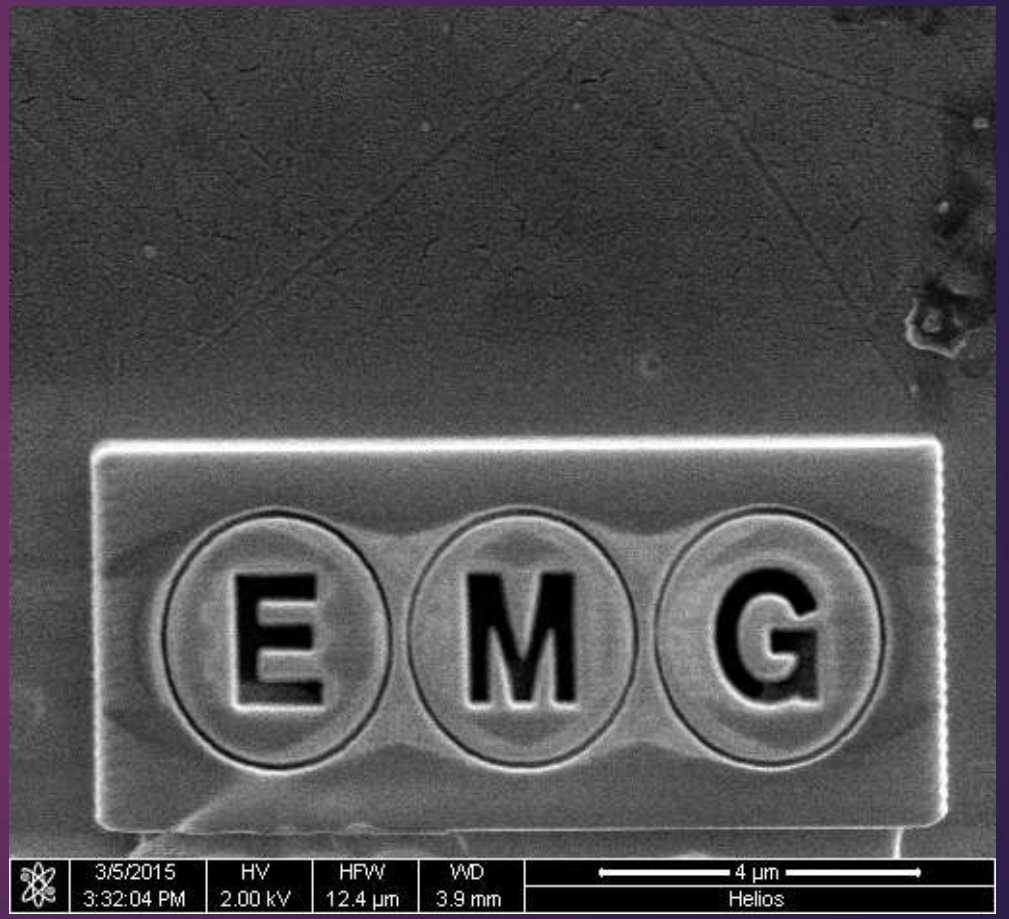
FIB SEM

TOMOGRAPHY SHORT COURSE

APRIL 2021

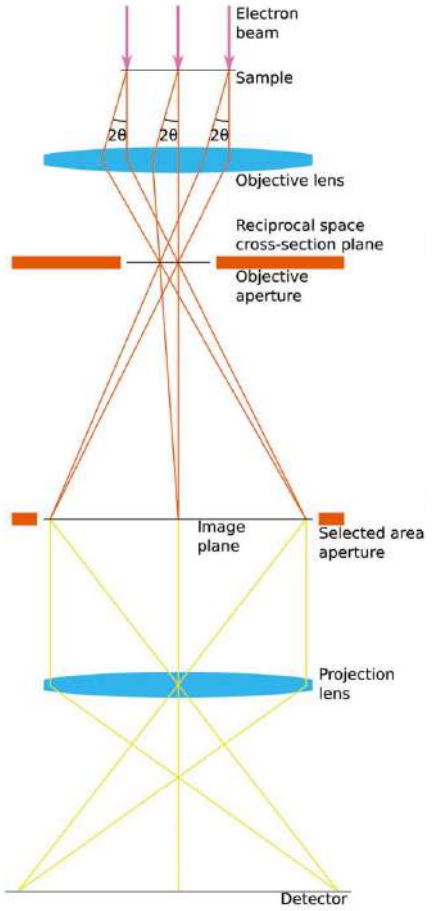
WILLIAM RICE, NYU SCHOOL OF MEDICINE



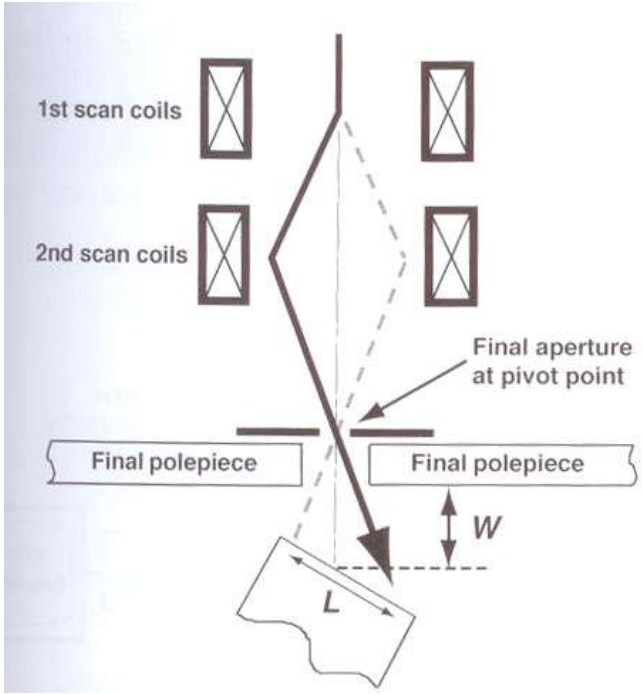
SEM Basics

SEM versus TEM

TEM

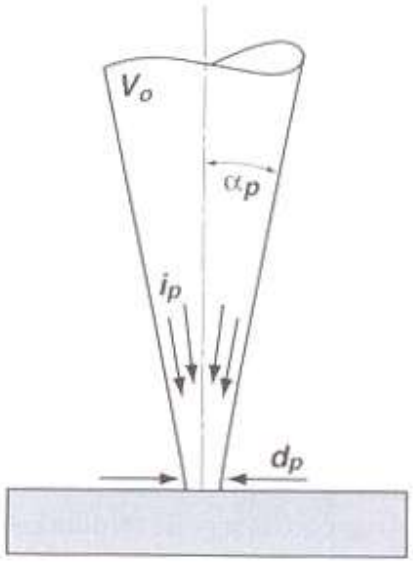


SEM



SEM Beam: probe size

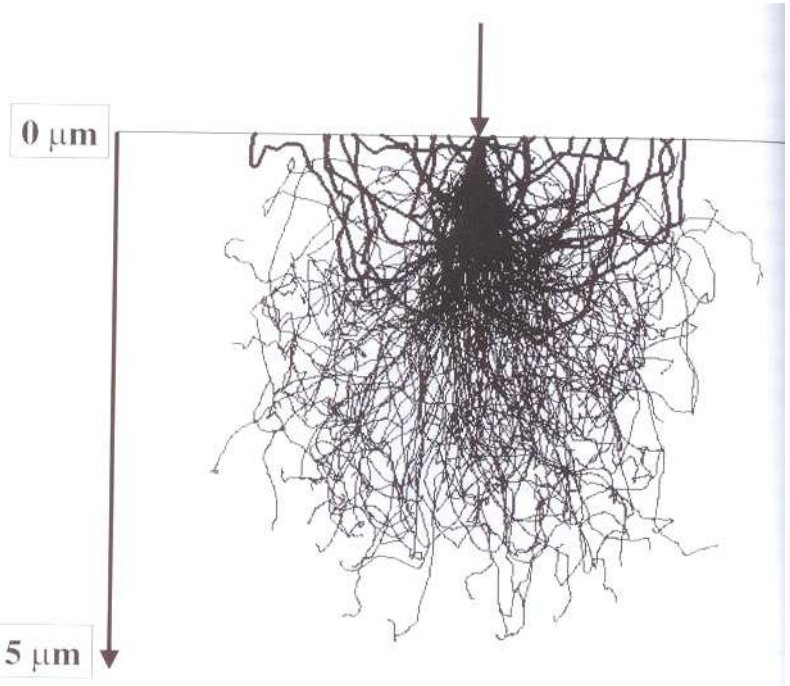
- Ideally want as small a probe as possible, relative to pixel size
- Probe size is determined by voltage, current, divergence angle
- Lens distortions
  - Spherical aberration (focus different at center and edge of lens) – proportional to focal length (working distance)
  - Aperture diffraction
  -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



Goldstein et al, 2003

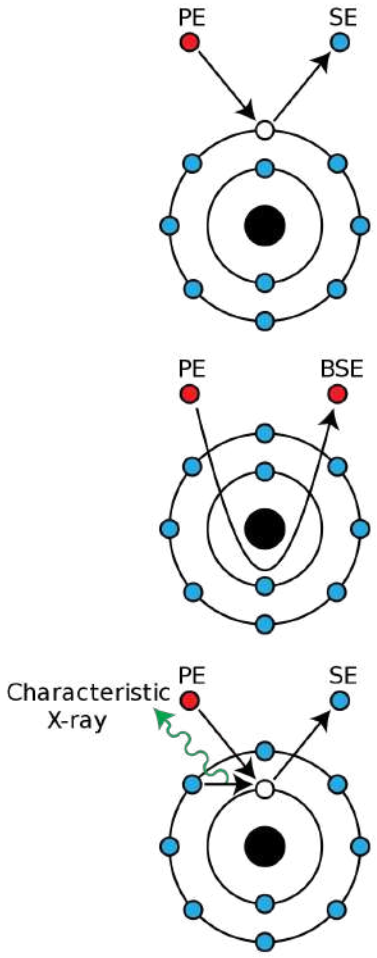
Beam-Specimen Interaction

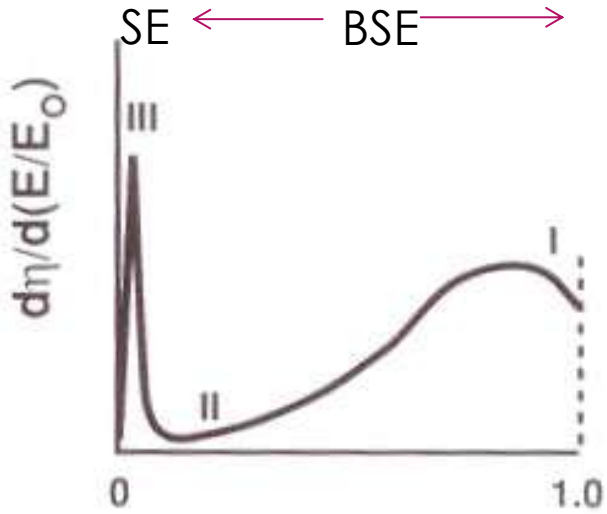
- Monte Carlo simulation of a 20 keV beam in Si
  - Dark traces: electrons which left the sample (BSE's)
- Electrons may be scattered elastically or inelastically
- Probability of elastic scattering ~ Z^2
- Inelastic scattering:
  - Secondary electrons
  - X-rays



Goldstein et al, 2003

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



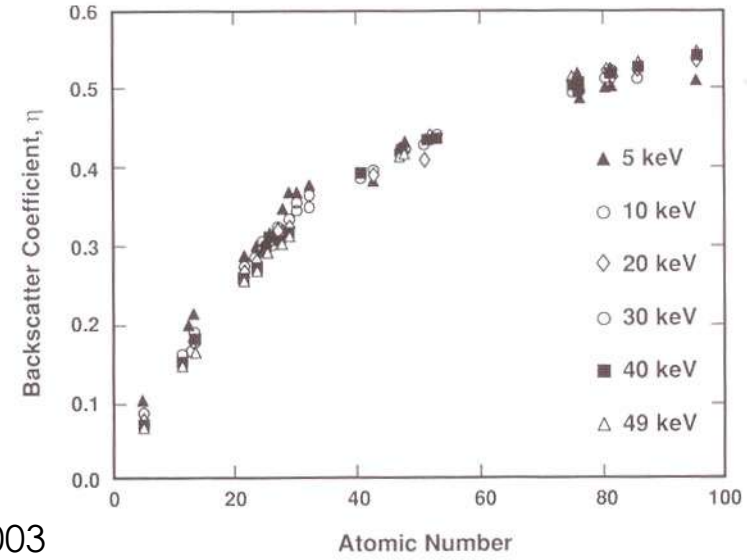


SE: <50 eV

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

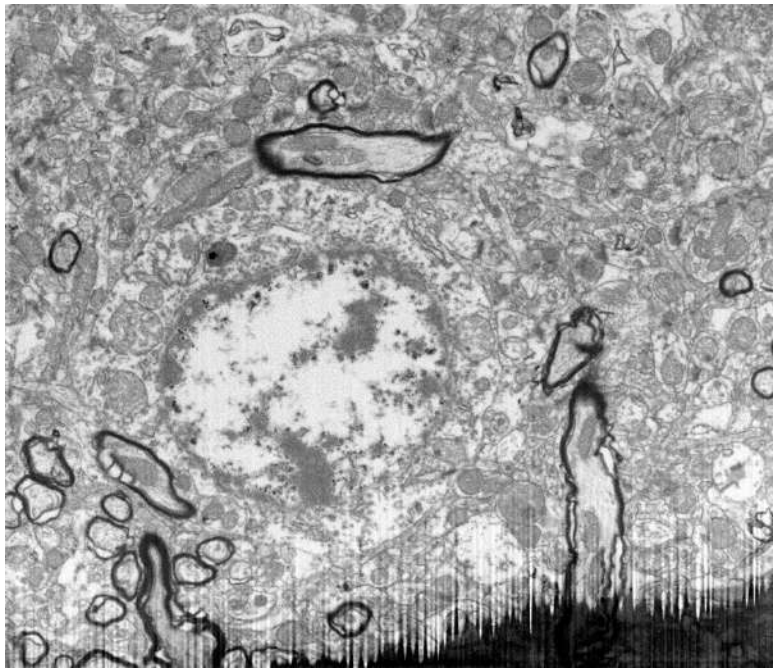
BSE efficiency is material dependent, voltage independent

Fraction of electrons that backscatter



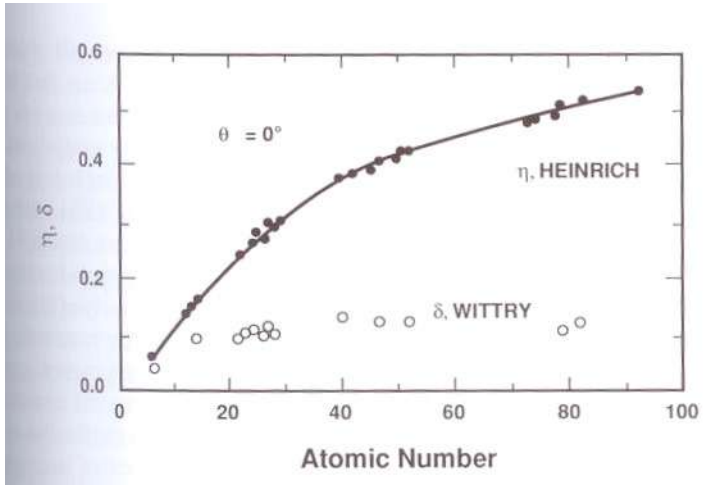
Goldstein et al, 2003

BSE's give contrast between light and heavy elements



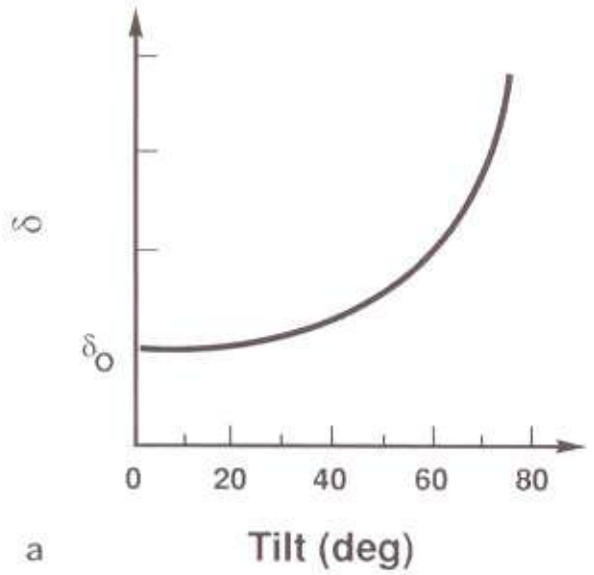
Osmium stained, resin-embedded tissue

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



30 keV

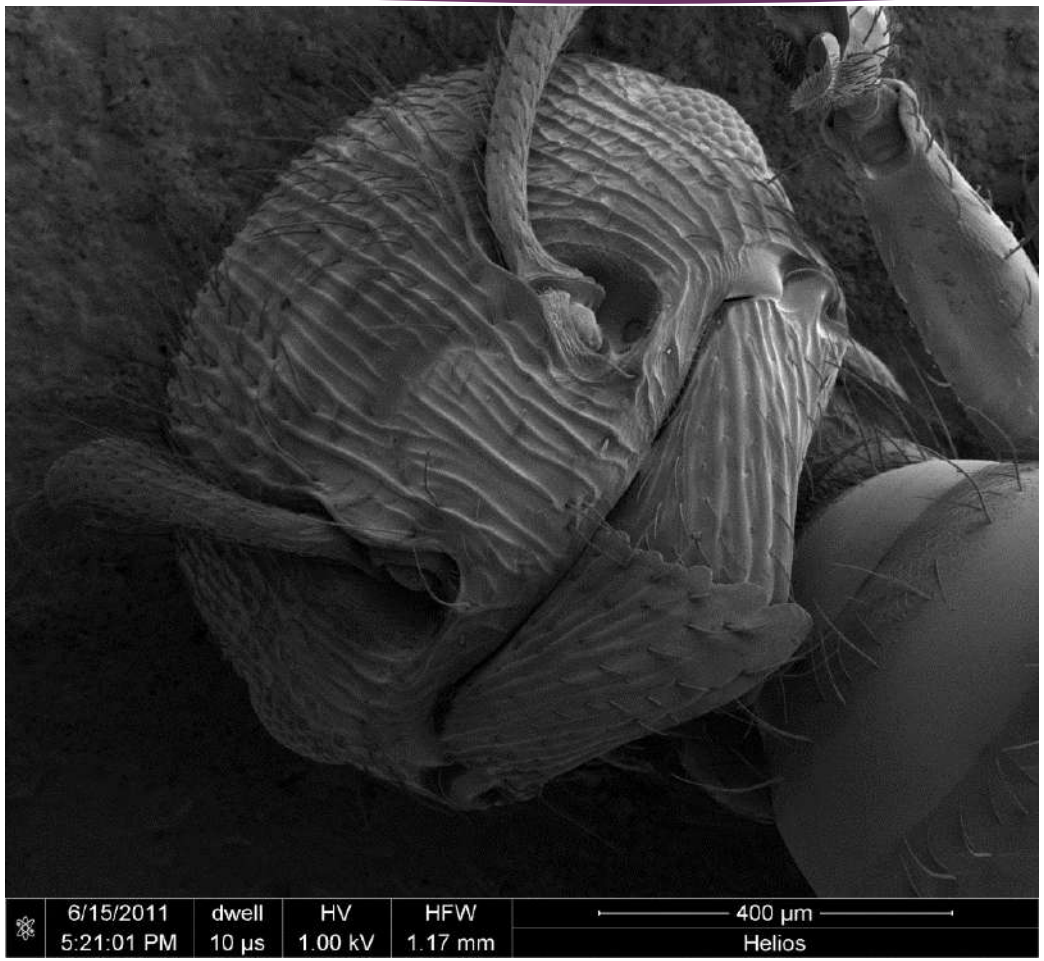
SE's are less sensitive to atomic number than BSE's (may be more sensitive at lower beam energies)



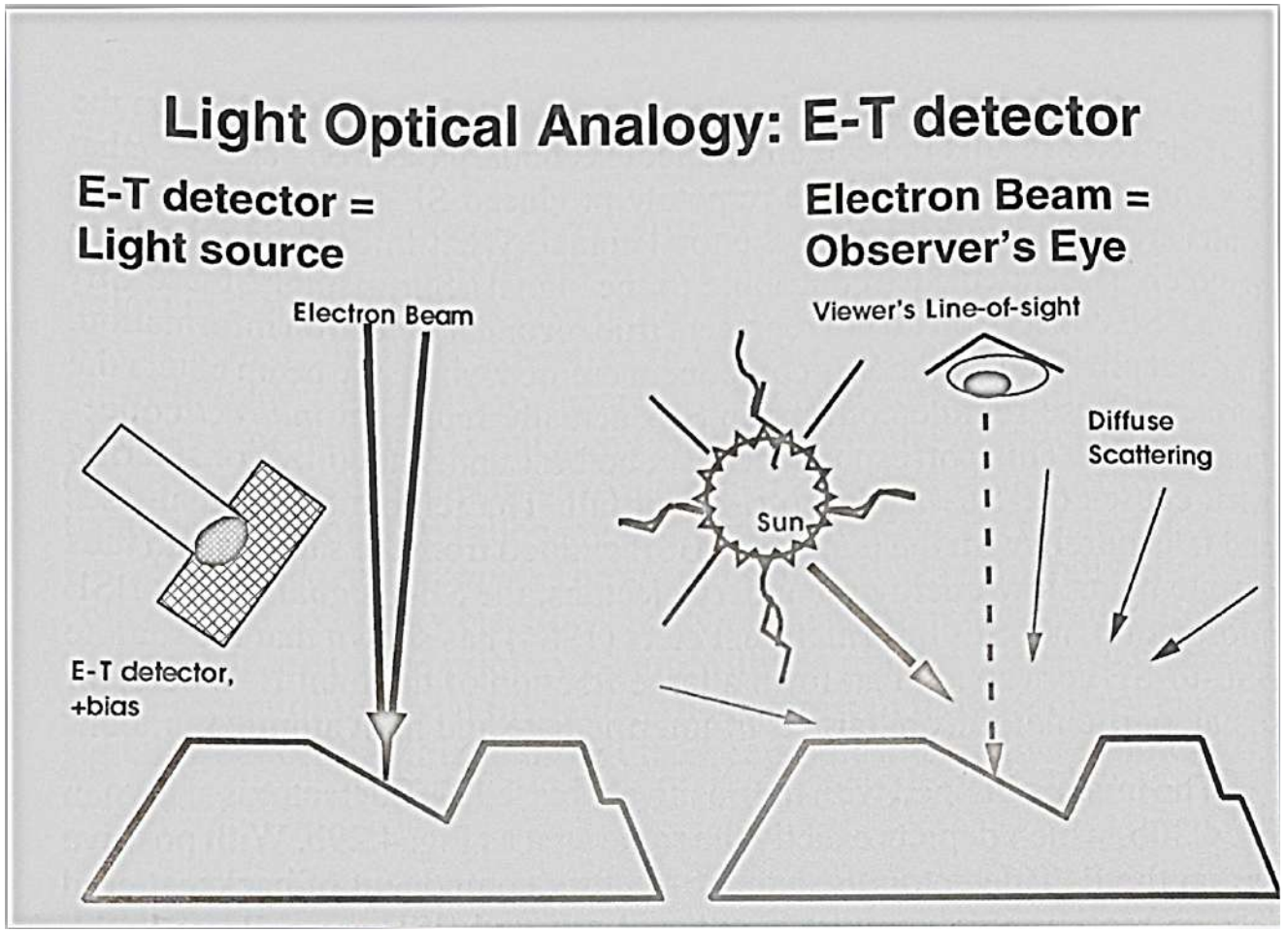
Signal is strongly dependent on viewing direction

Goldstein et al, 2003

SE's give excellent topographic information



Light-optical analogy



Goldstein et al, 2003

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

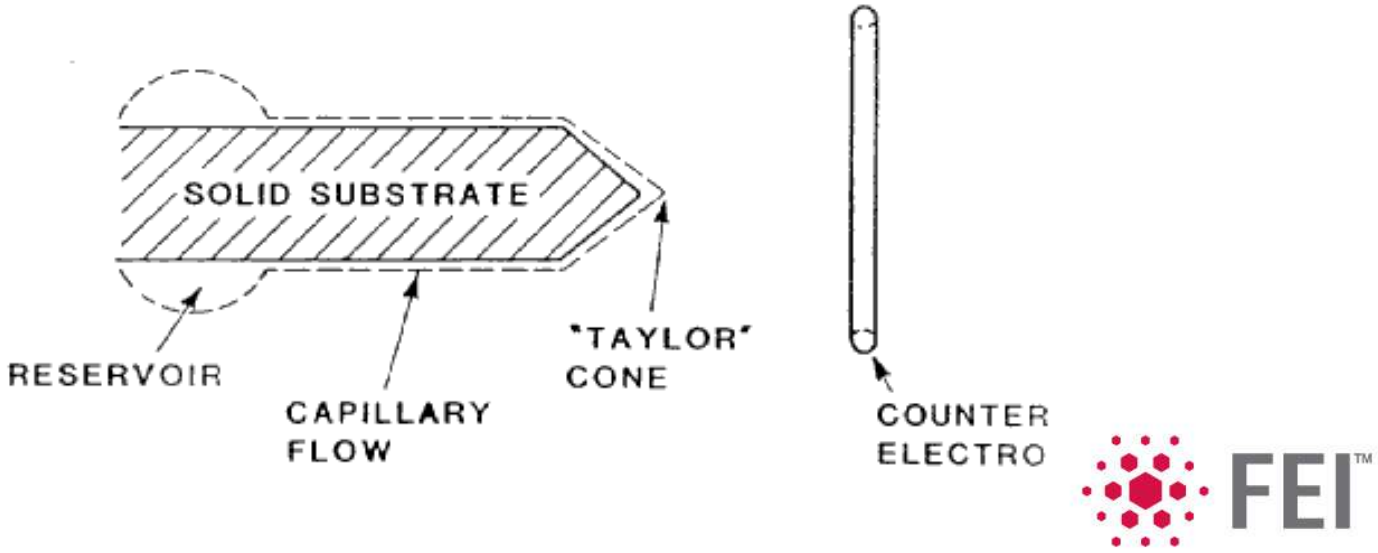
TEM

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

FIB Operation

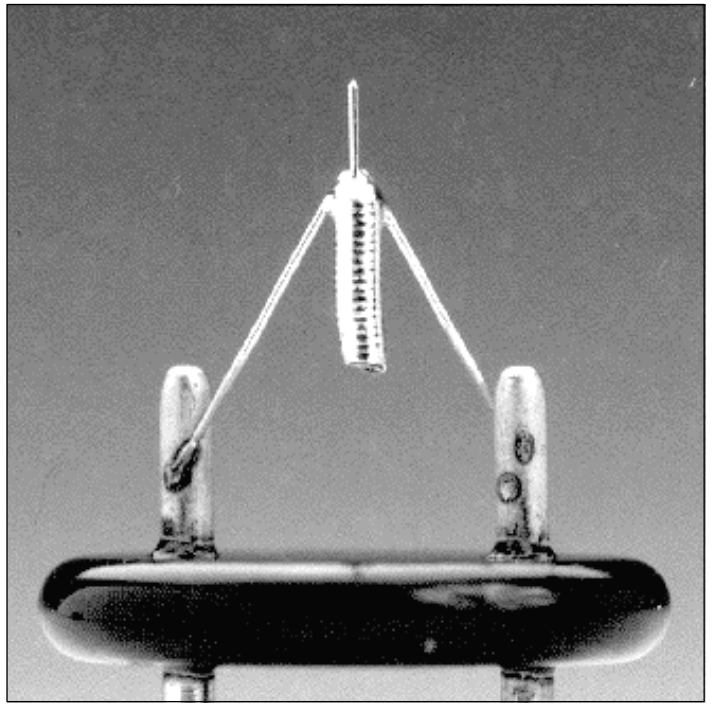
Basic Mechanism

- Liquid Flow from Reservoir
- Ion Formation
- External Beam Interactions



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



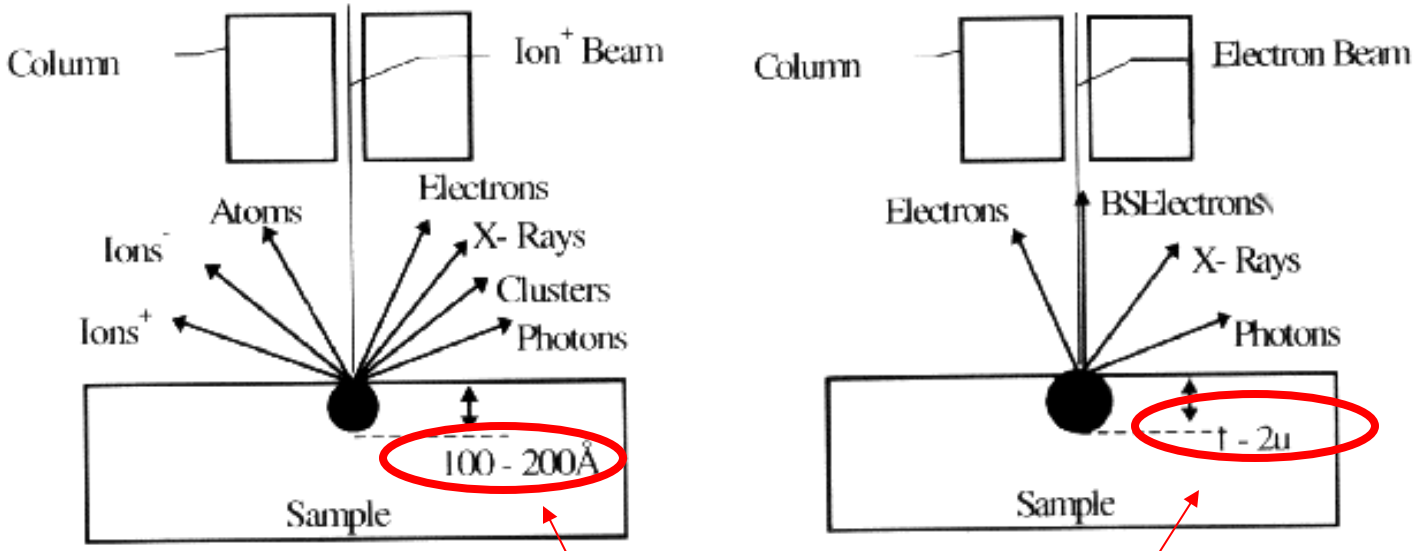
FEI™

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

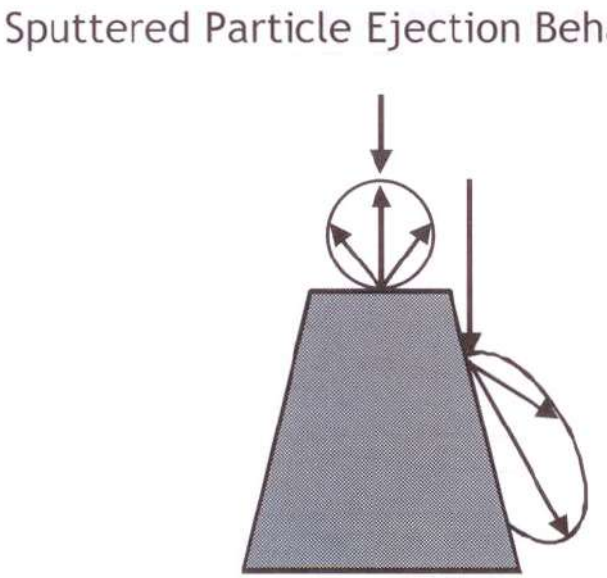
Using the System

- Beam Interactions



Note difference in interaction volume

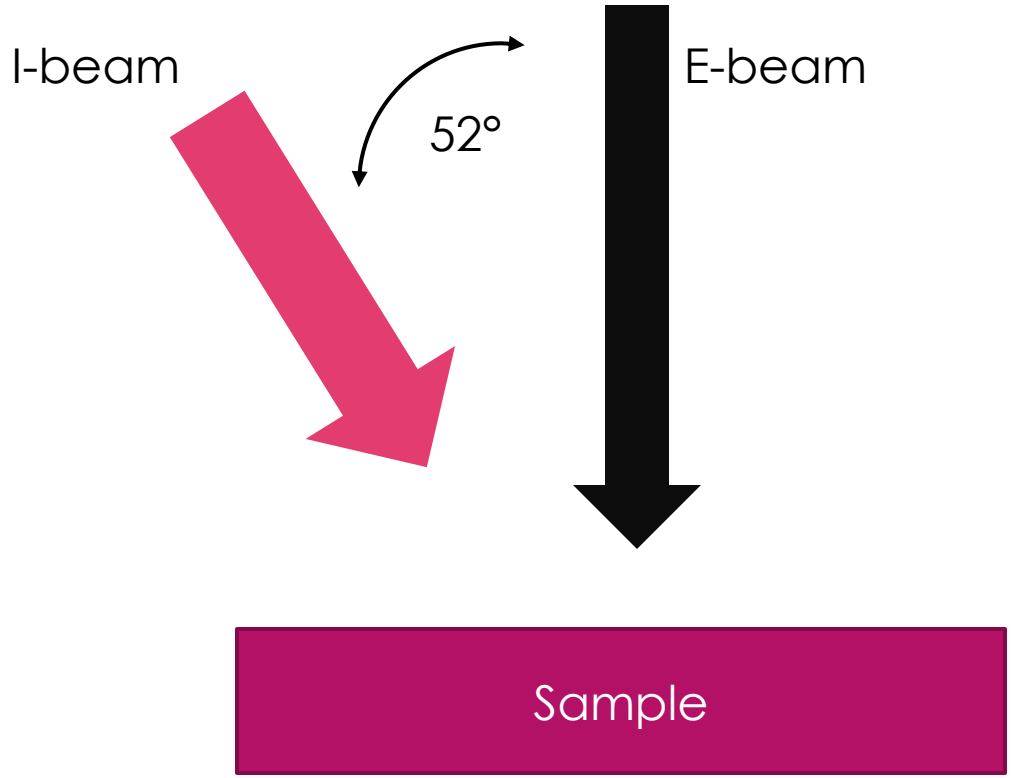
Common Use: Sputtering particles from substrate



More efficient milling at edge than in bulk

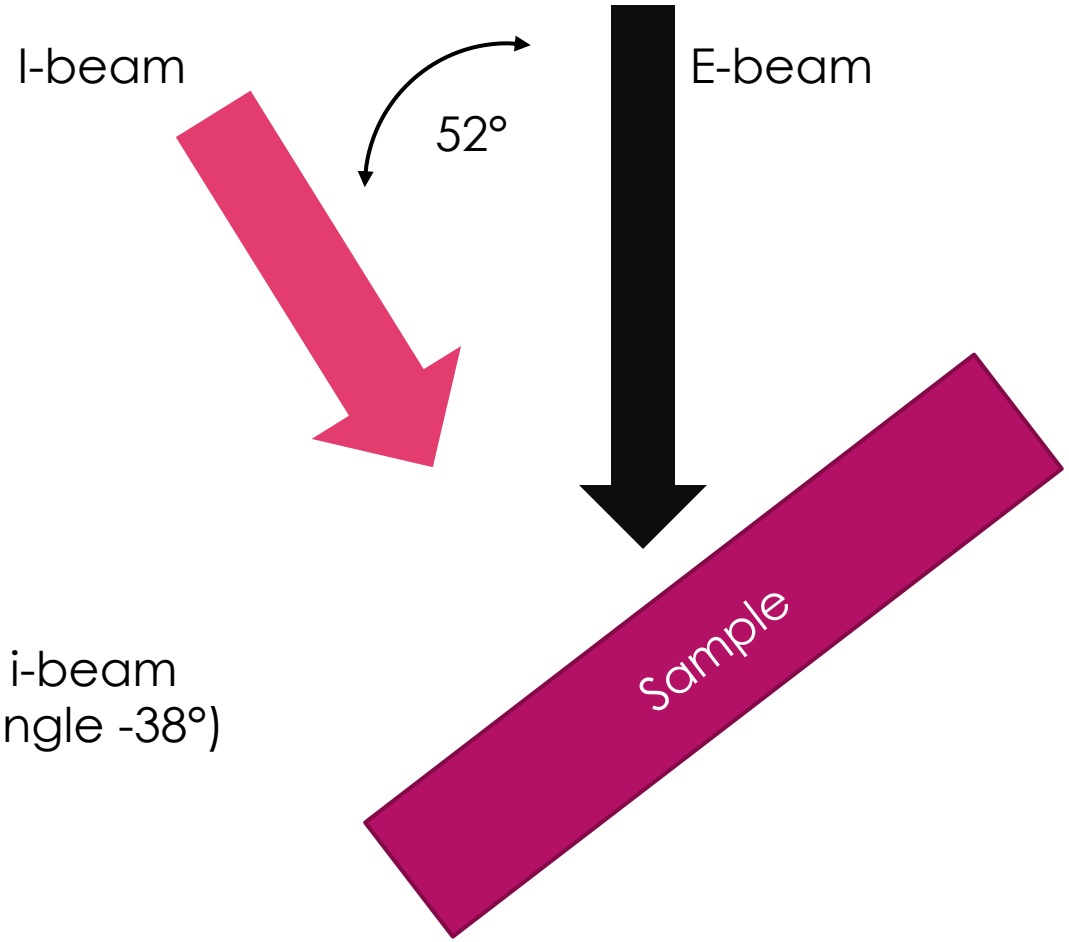
FEI™

Geometry



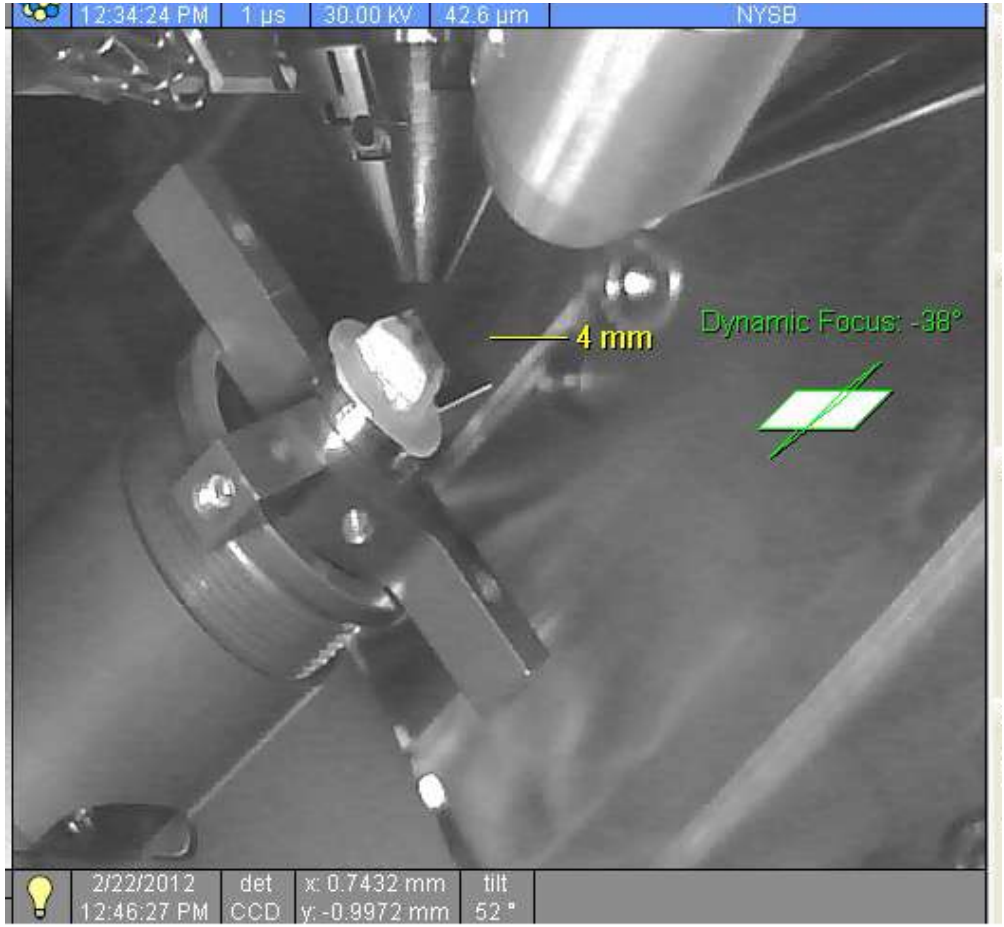
Sample:
Tilt from -10° to +70°

Geometry



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

Geometry


12:34:24 PM
1 µs
30.00 kV
42.6 µm
NYSB
4 mm
Dynamic Focus: -38°
2/22/2012
12:46:27 PM
det
CCD
x: 0.7432 mm
y: -0.9972 mm
tilt
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"

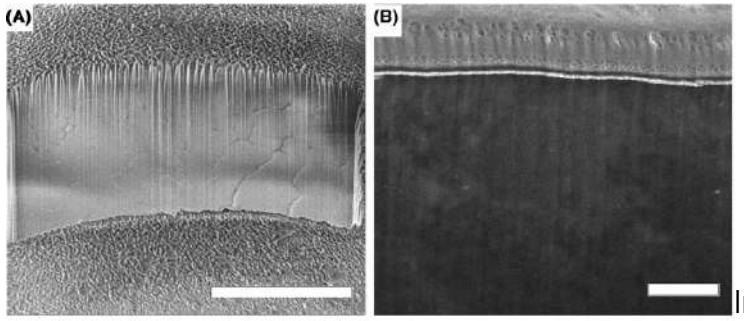
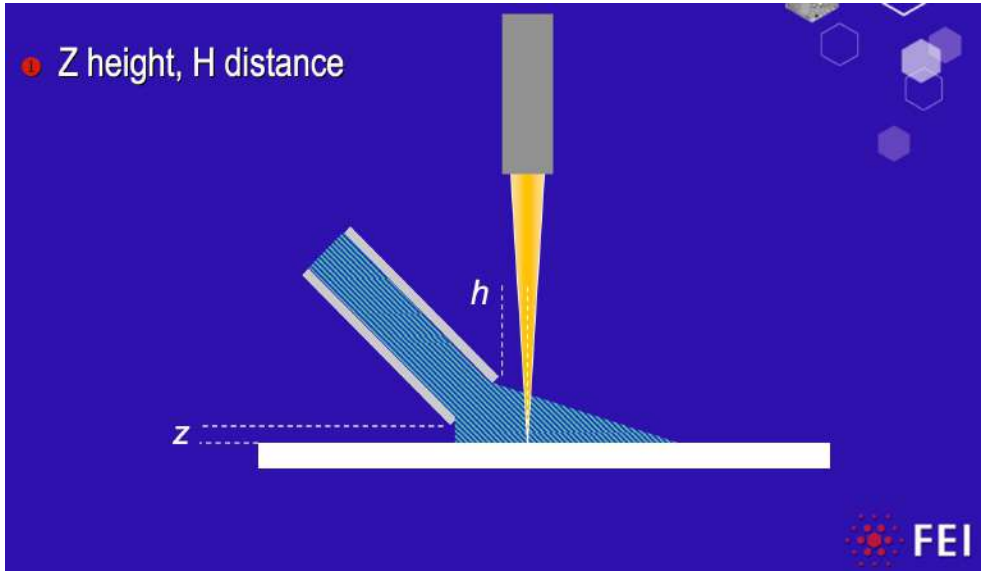
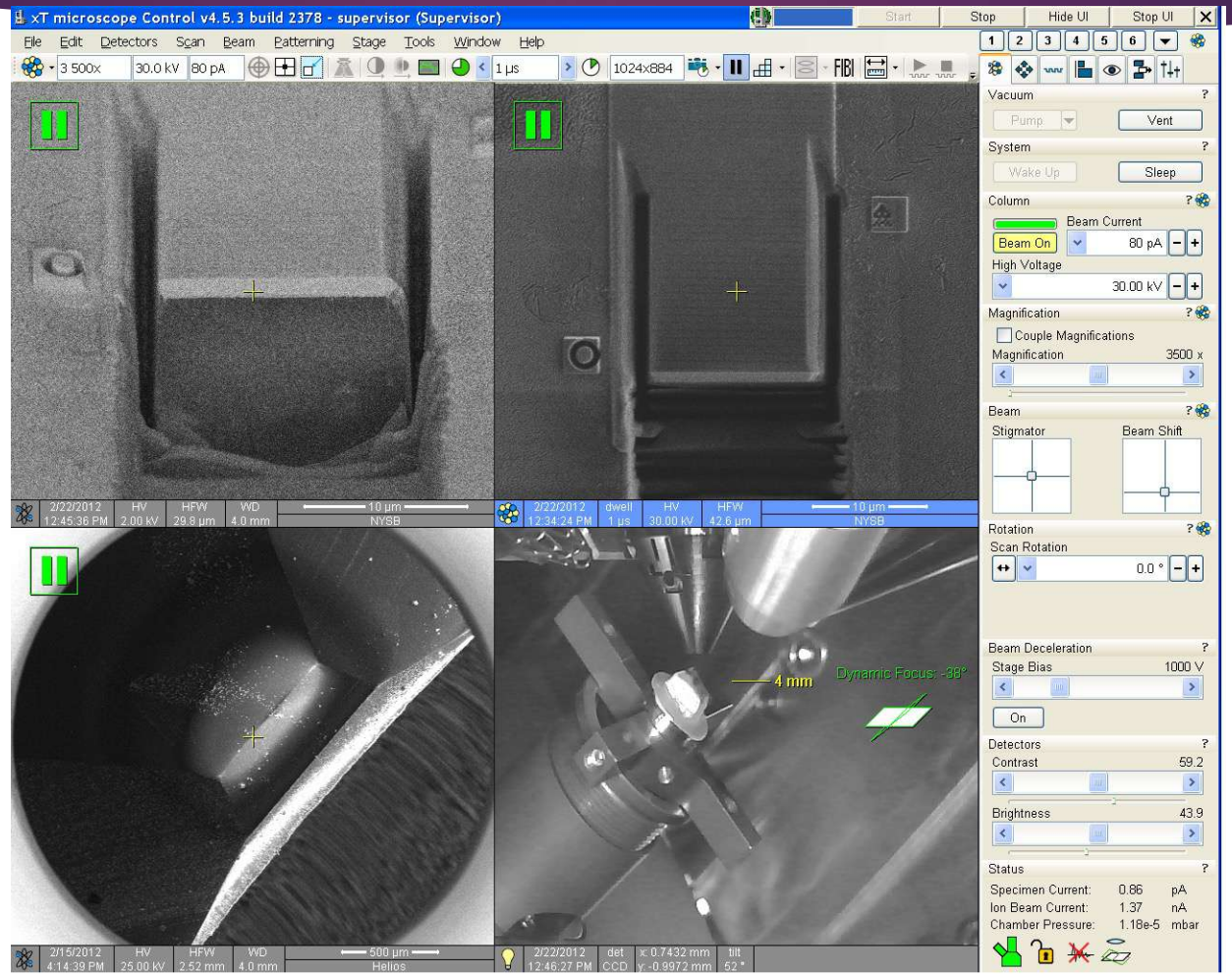


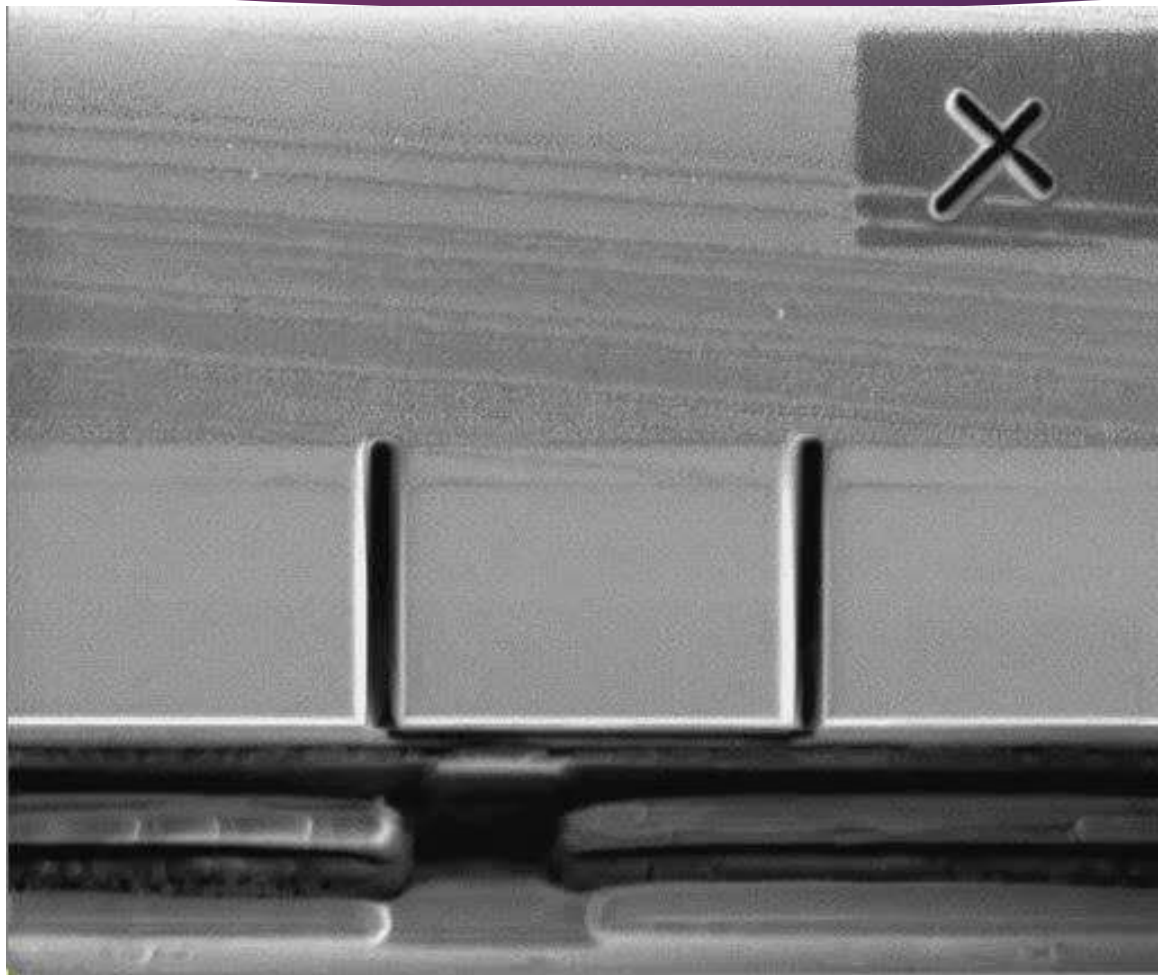
Image: Hayles and Winter, 2021



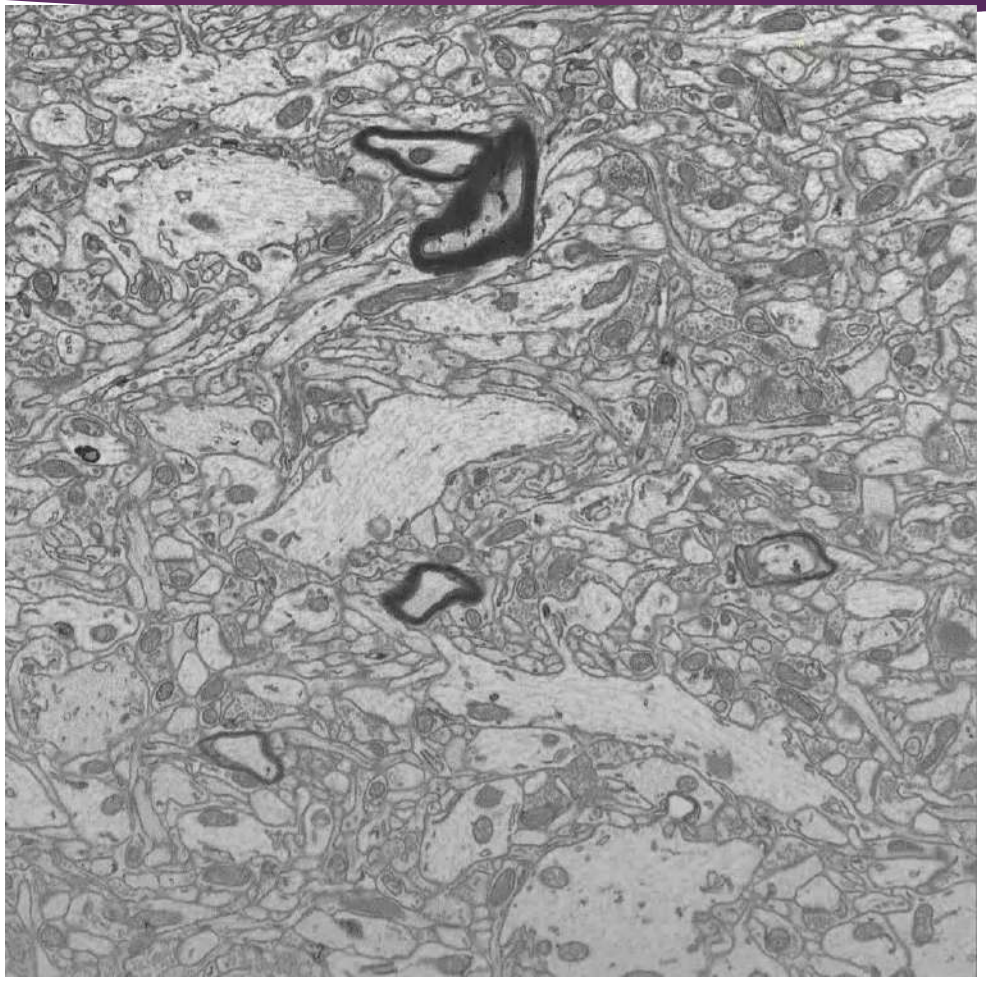
Application: 3D reconstruction of stained, resin-embedded tissue



Milling: i-beam view



Example Movie: Neural Tissue



Cryo FIB/SEM for tomographic sample preparation

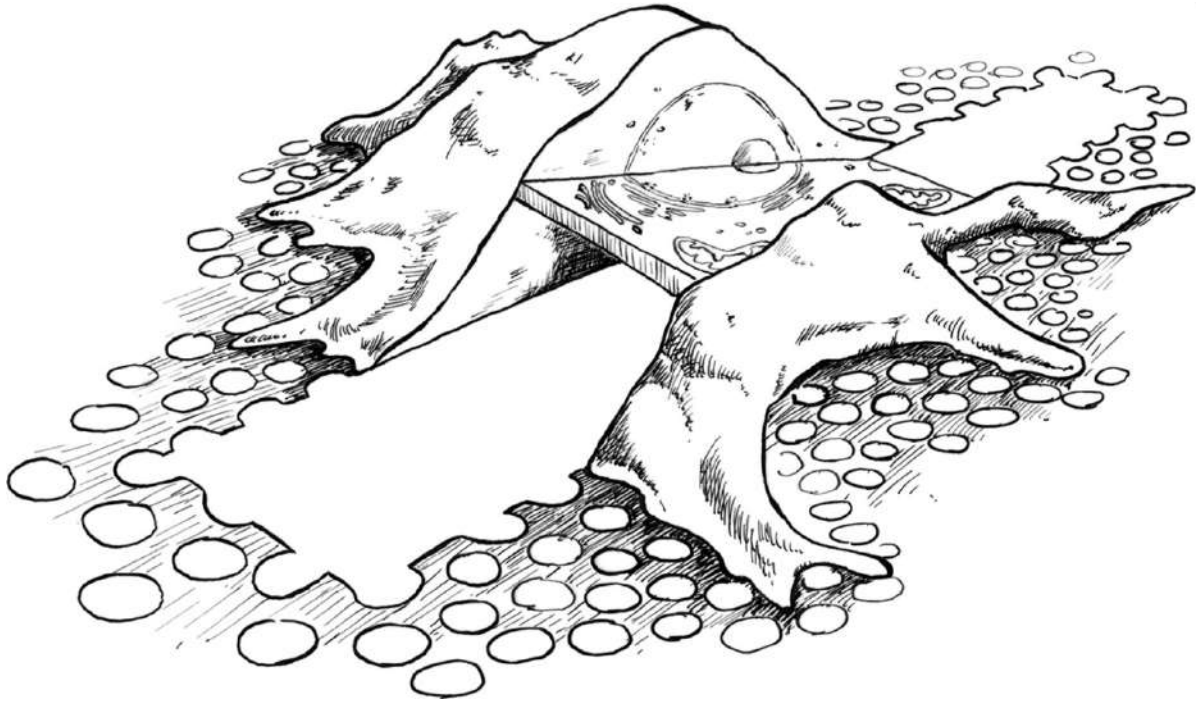


Image: Villa *et al*, 2013

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

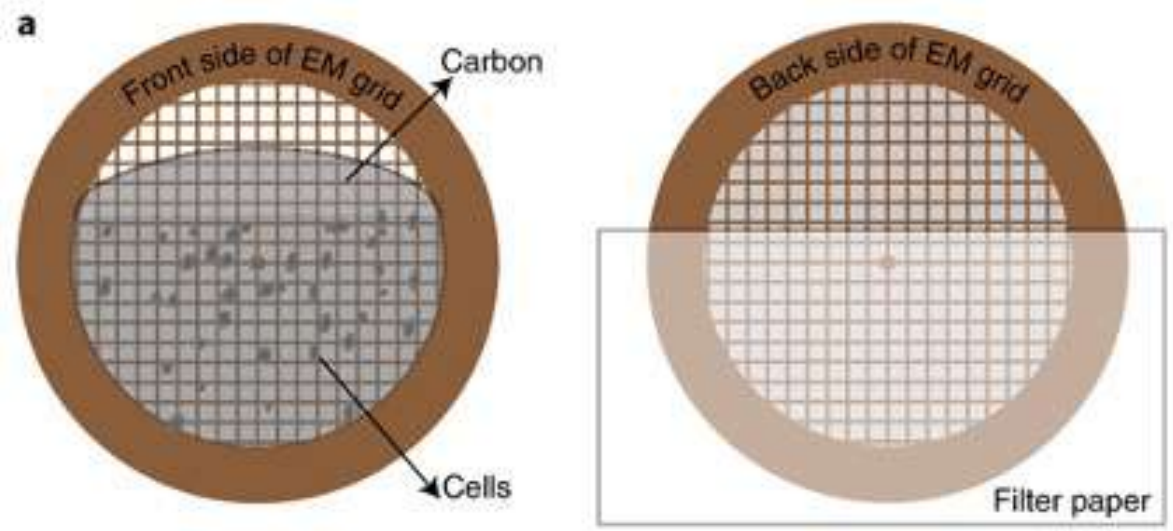


Image: Wagner *et al*, 2020

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

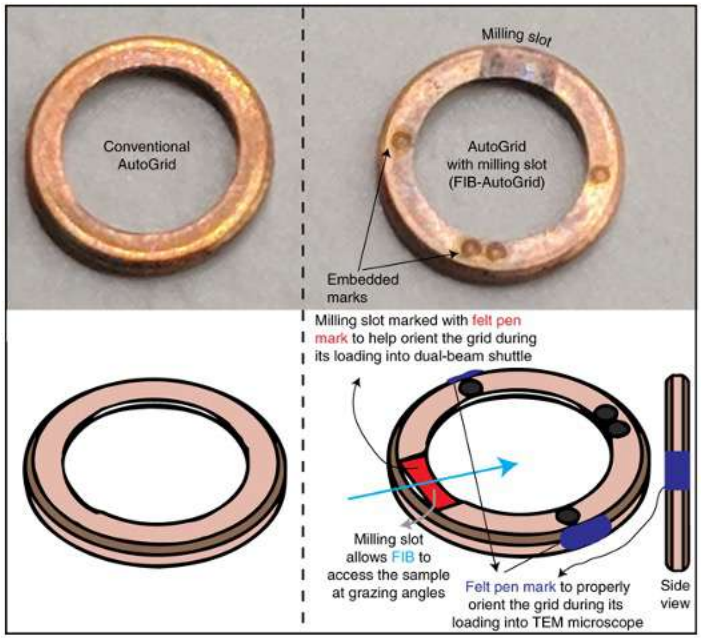


Image: Wagner *et al*, 2020

Grid Geometry

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

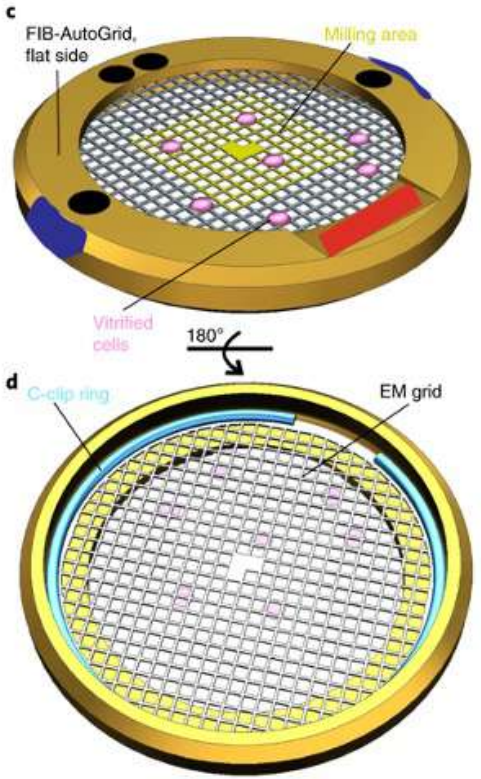


Image: Wagner *et al*, 2020

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

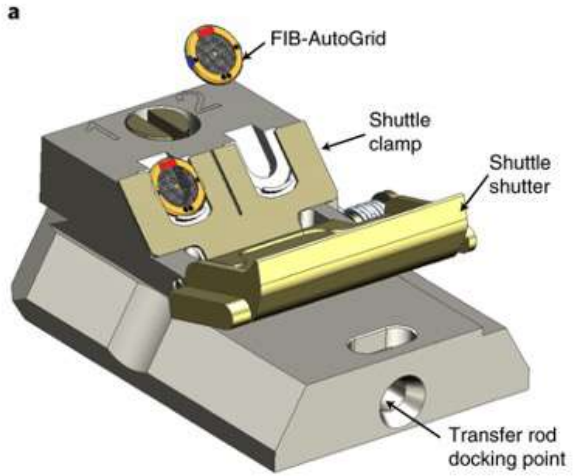
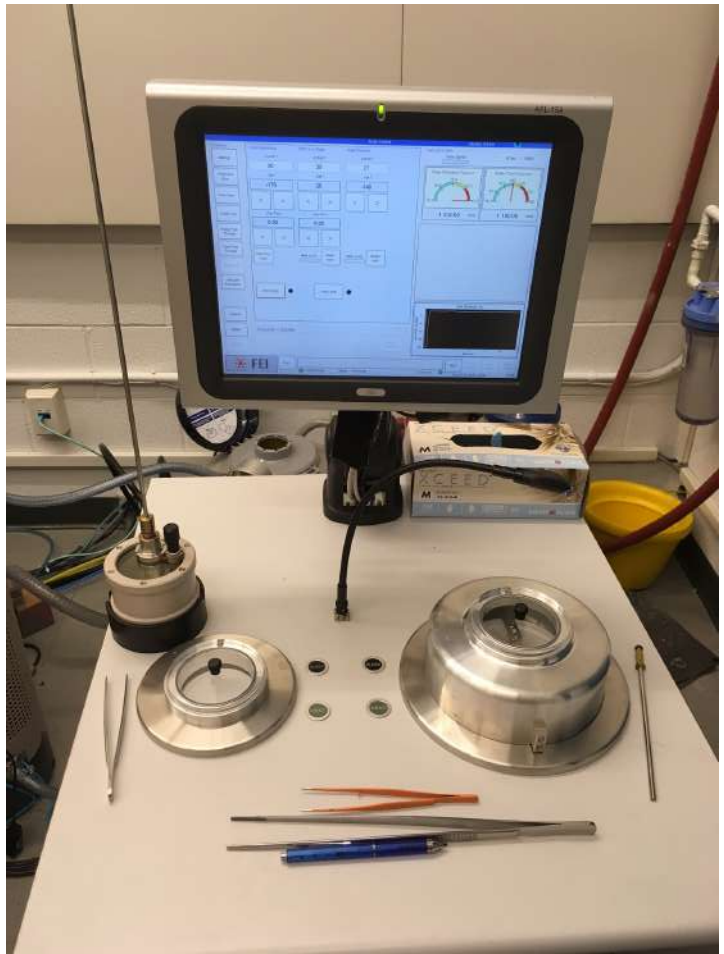


Image: Wagner *et al*, 2020

Transfer Rod for Loading

Image: Wagner *et al*, 2020

Older Loading Station (Quorum)



Geometry

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

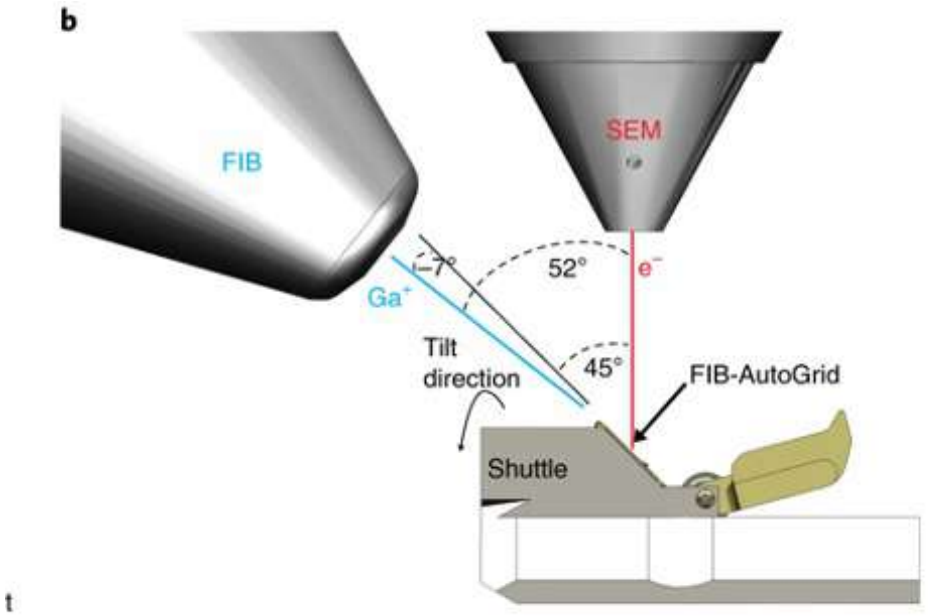


Image: Wagner *et al*, 2020

Geometry: Untilted

- Untilted stage:
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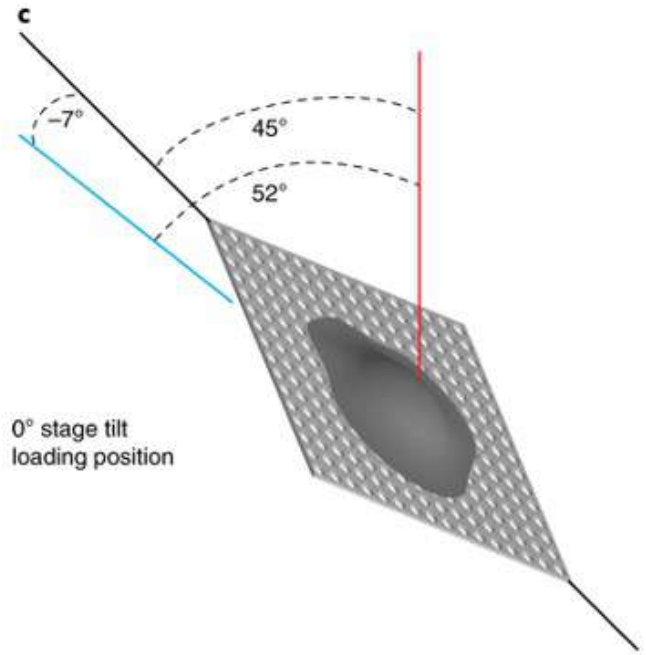


Image: Wagner *et al*, 2020

Geometry: Tilted

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

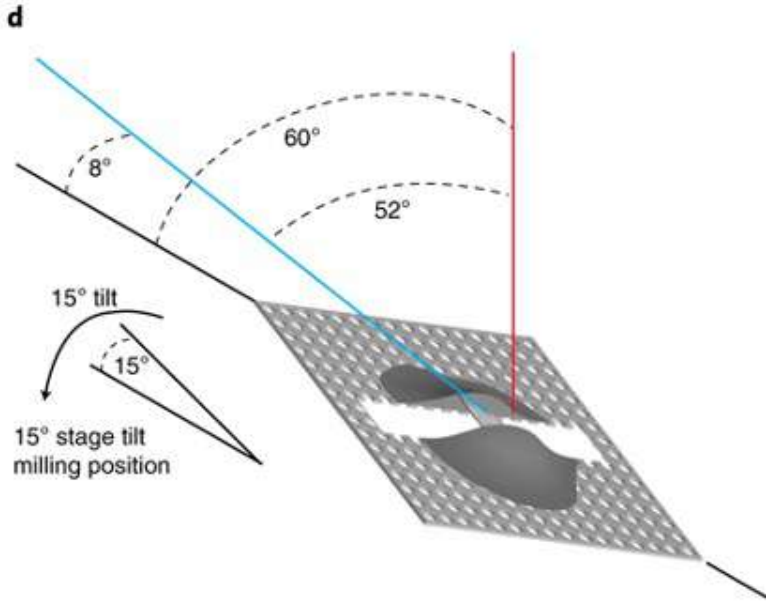


Image: Wagner *et al*, 2020

Imaging cells with ion beam

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

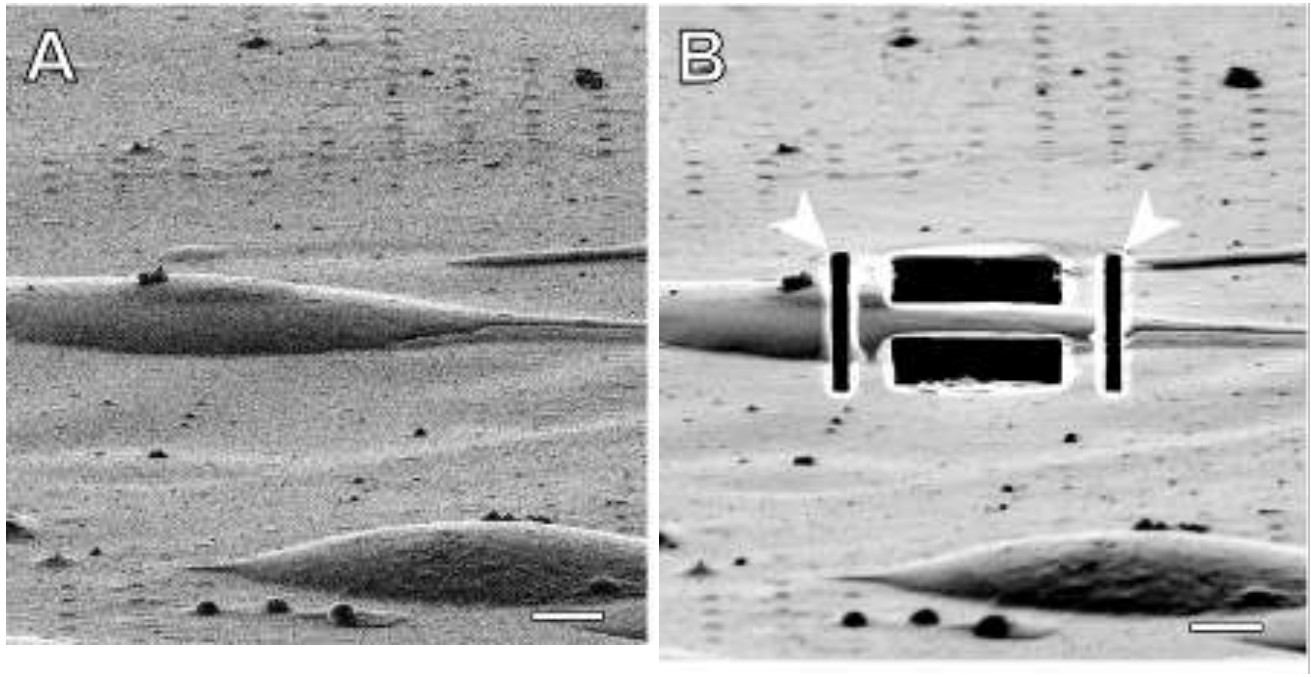


Image: Wolff *et al*, 2019

Targeting of Milling Regions

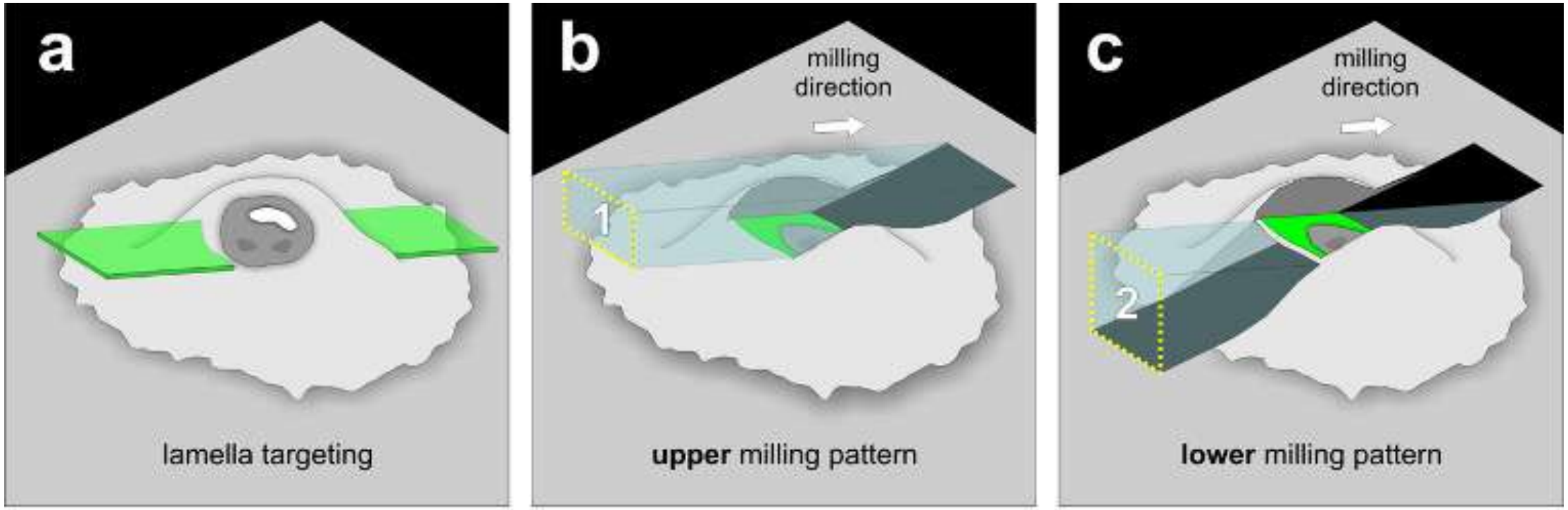


Image: Rigort and Plitzko, 2015

Targeting of Milling Regions

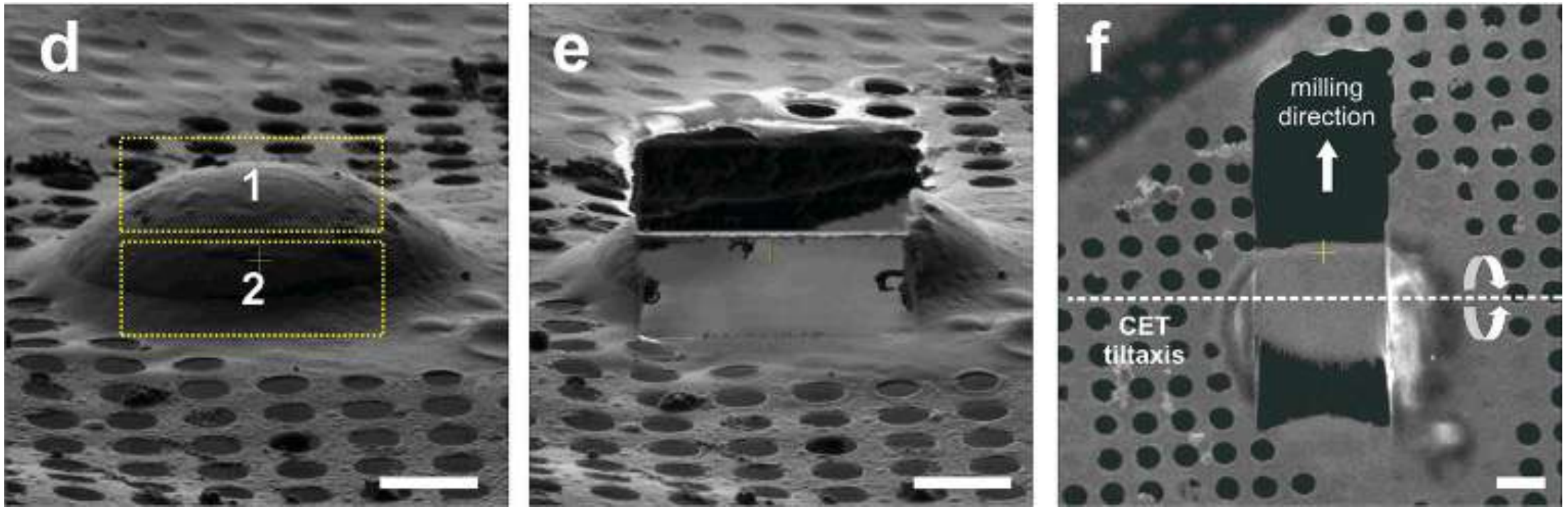


Image: Rigort and Plitzko, 2015

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

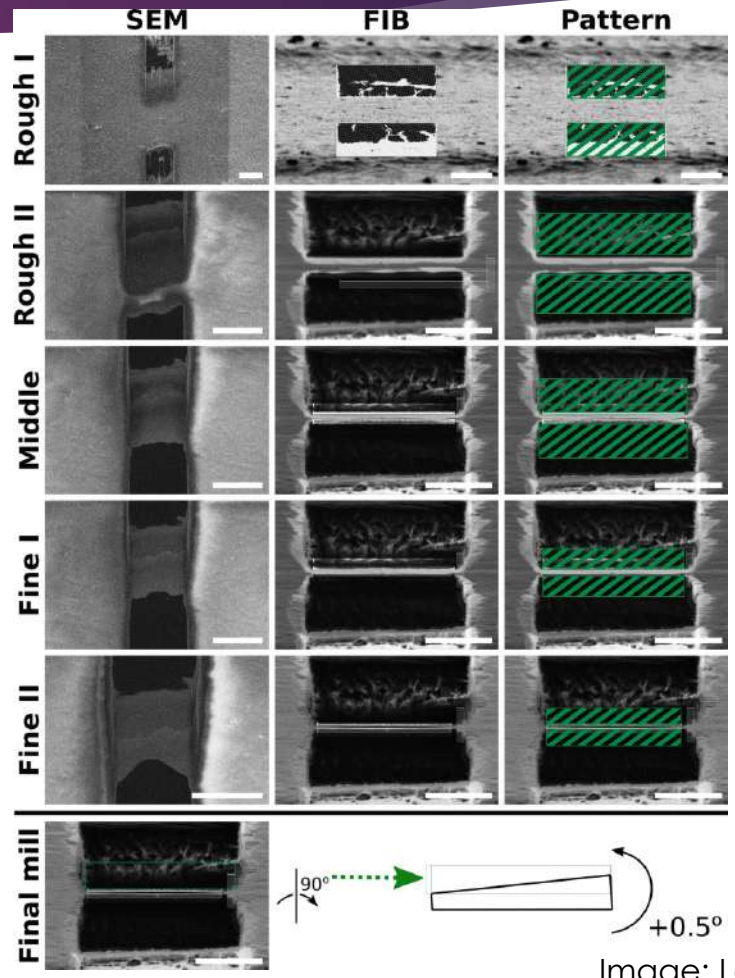


Image: Lam and Villa, 2021

Milling at as shallow an angle as possible

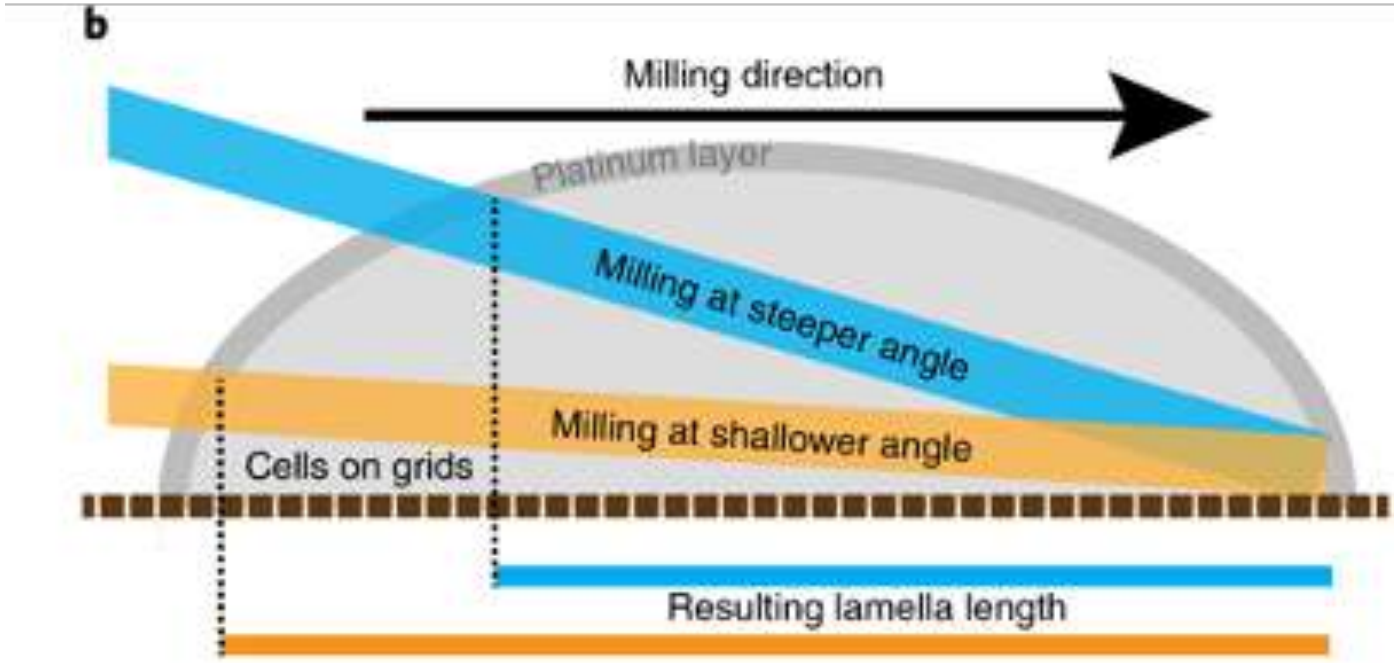


Image: Wagner *et al*, 2020

Geometry: Loading into TEM

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

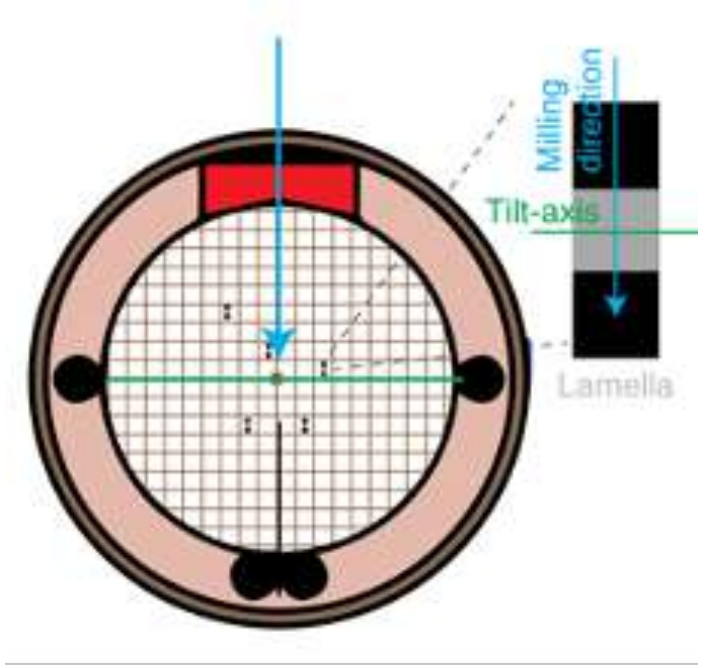


Image: Wagner *et al*, 2020

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.

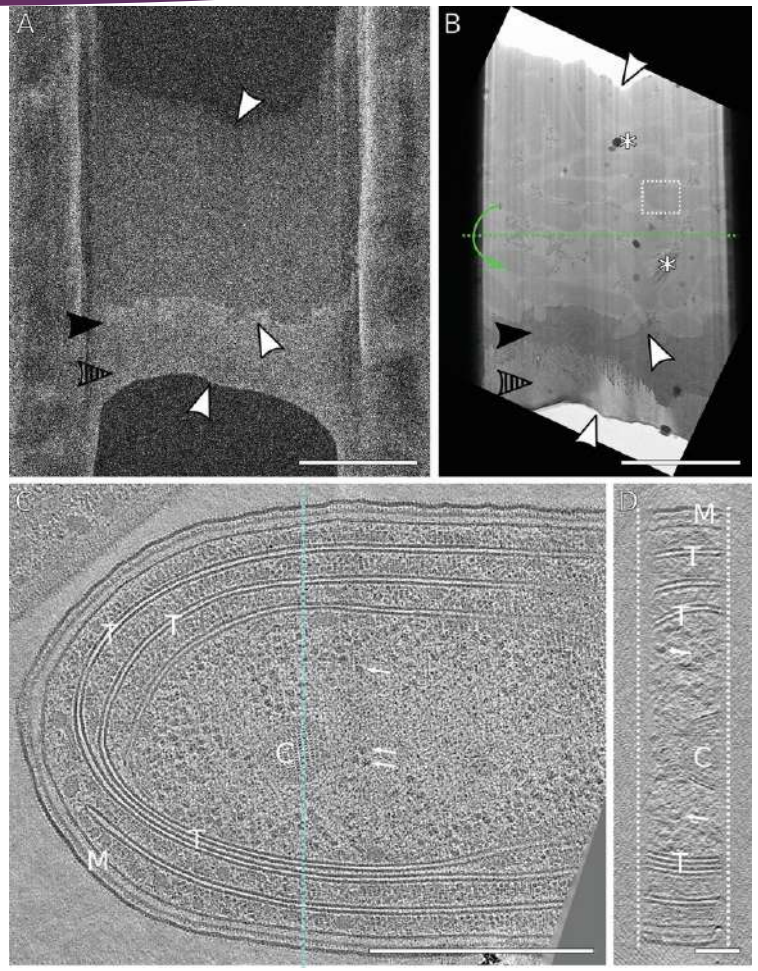


Image: Lam and Villa, 2021

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
- All sample transfer steps have the danger of adding contamination

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 LM inside SEM chamber?

Cryo-CLEM: Correlate points between images

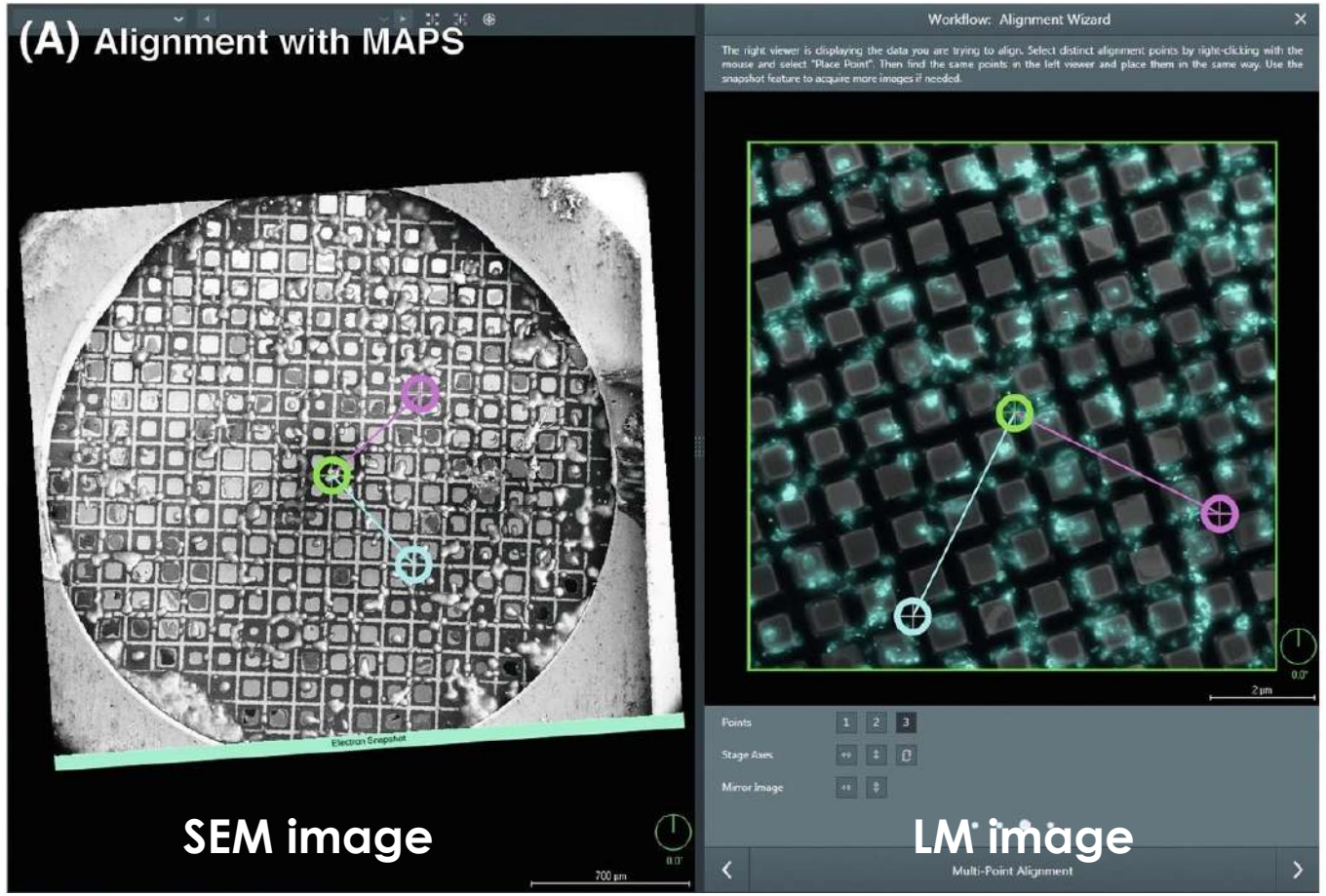


Image: Klein *et al*, 2021

Cryo-CLEM: Overlay

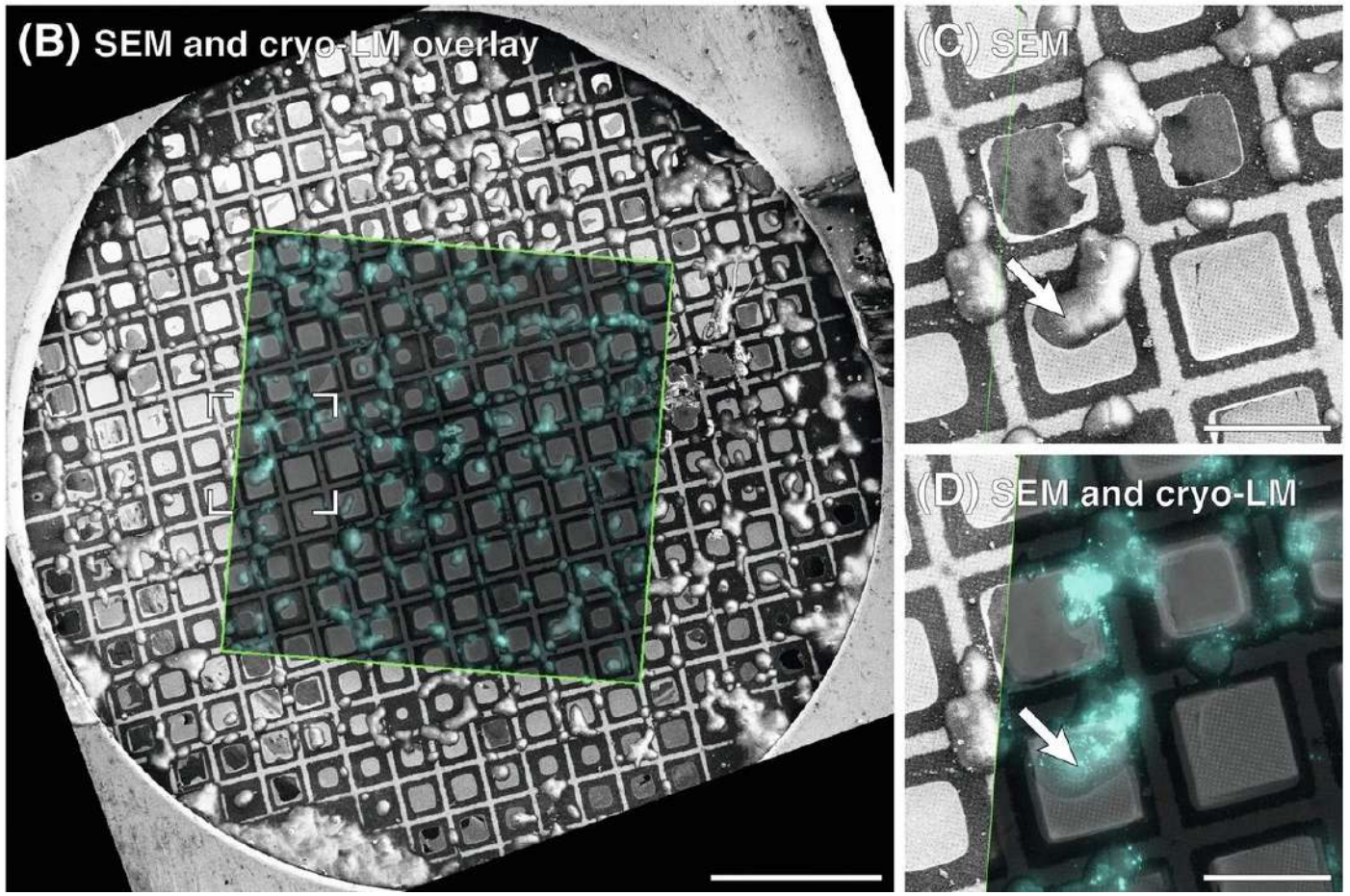


Image: Klein *et al*, 2021

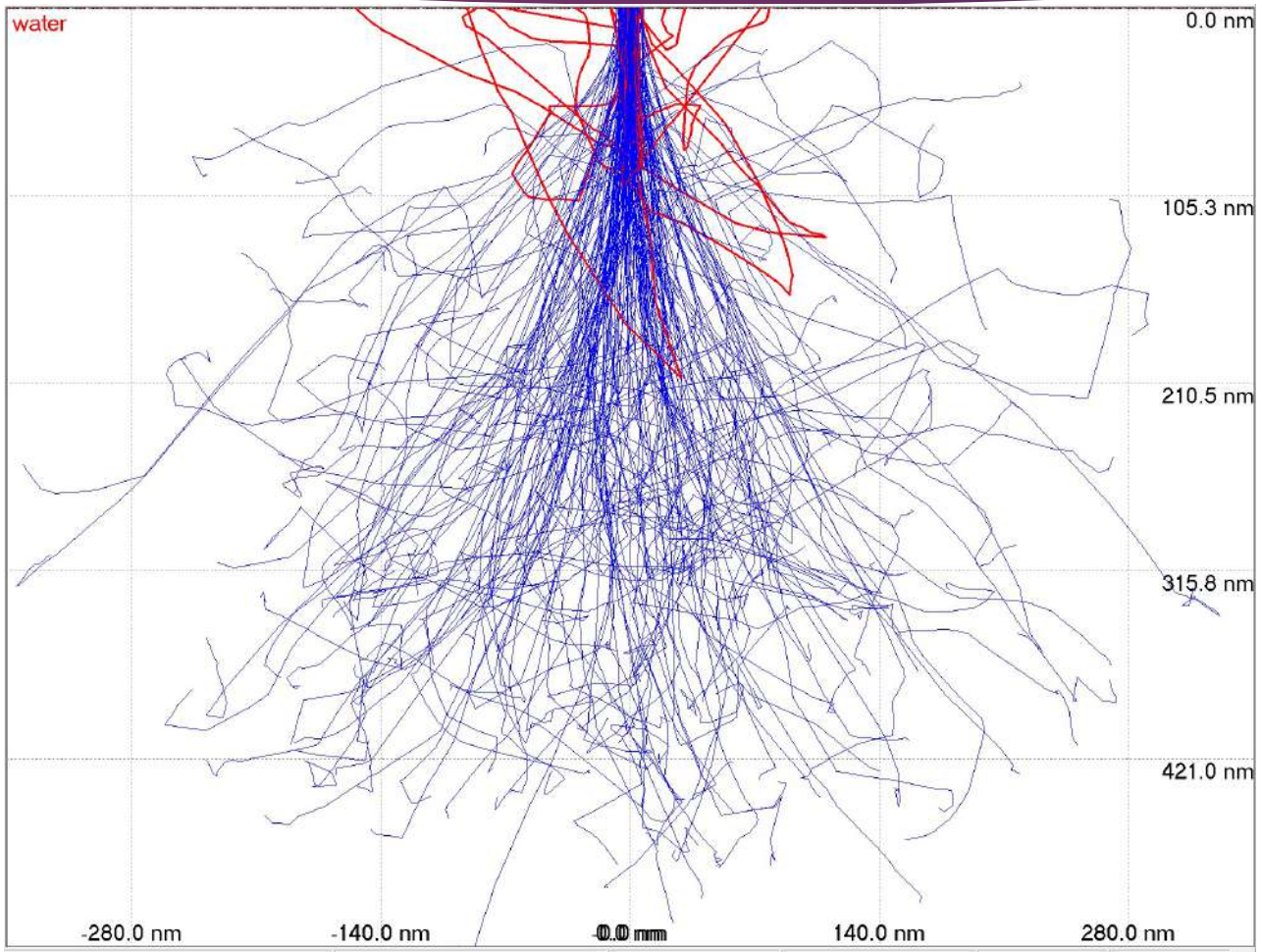
Summary: Equipment and expertise needed

- FIB SEM
  - Cryo stage with full rotation
  - GIS
  - Sputter coater
  - Shuttles and transfer equipment
  - Software for mapping and overlaying signals
- Cryo LM
  - Compatible cryo stage
  - Fluorescent signal detection
  - Shuttles and transfer equipment
- TEM
  - Suitable for high resolution tomography

References

- Buckley, G., G. Gervinskas, C. Taveneau, H. Venugopal, J. C. Whisstock and A. de Marco (2020). "Automated cryo-lamella preparation for high-throughput in-situ structural biology." J Struct Biol **210**(2): 107488.
- Goldstein, J. I., D. C. Joy, J. R. Michael, D. E. Newbury, N. W. M. Ritchie and J. H. J. Scott (2018). Scanning Electron Microscopy and X-Ray Microanalysis. New York, NY, Springer New York : Imprint: Springer,: 1 online resource (XXIII, 550 pages 546 illustrations, 409 illustrations in color.
- Hayles, M. F. and D. E. W. DAM (2021). "An introduction to cryo-FIB-SEM cross-sectioning of frozen, hydrated Life Science samples." J Microsc **281**(2): 138-156.
- He, J., C. Hsieh, Y. Wu, T. Schmelzer, P. Wang, Y. Lin, M. Marko and H. Sui (2017). "Cryo-FIB specimen preparation for use in a cartridge-type cryo-TEM." J Struct Biol **199**(2): 114-119.
- Klein, S., M. Wachsmuth-Melm, S. L. Winter, A. Kolovou and P. Chlanda (2021). "Cryo-correlative light and electron microscopy workflow for cryo-focused ion beam milled adherent cells." Methods Cell Biol **162**: 273-302.
- Lam, V. and E. Villa (2021). "Practical Approaches for Cryo-FIB Milling and Applications for Cellular Cryo-Electron Tomography." Methods Mol Biol **2215**: 49-82.
- Rigort, A. and J. M. Plitzko (2015). "Cryo-focused-ion-beam applications in structural biology." Arch Biochem Biophys **581**: 122-130.
- Villa, E., M. Schaffer, J. M. Plitzko and W. Baumeister (2013). "Opening windows into the cell: focused-ion-beam milling for cryo-electron tomography." Curr Opin Struct Biol **23**(5): 771-777.
- Wagner, F. R., R. Watanabe, R. Schampers, D. Singh, H. Persoon, M. Schaffer, P. Fruhstorfer, J. Plitzko and E. Villa (2020). "Preparing samples from whole cells using focused-ion-beam milling for cryo-electron tomography." Nat Protoc **15**(6): 2041-2070.
- Wolff, G., R. Limpens, S. Zheng, E. J. Snijder, D. A. Agard, A. J. Koster and M. Barcena (2019). "Mind the gap: Micro-expansion joints drastically decrease the bending of FIB-milled cryo-lamellae." J Struct Biol **208**(3): 107389.

Monte Carlo simulation: water at 3 keV



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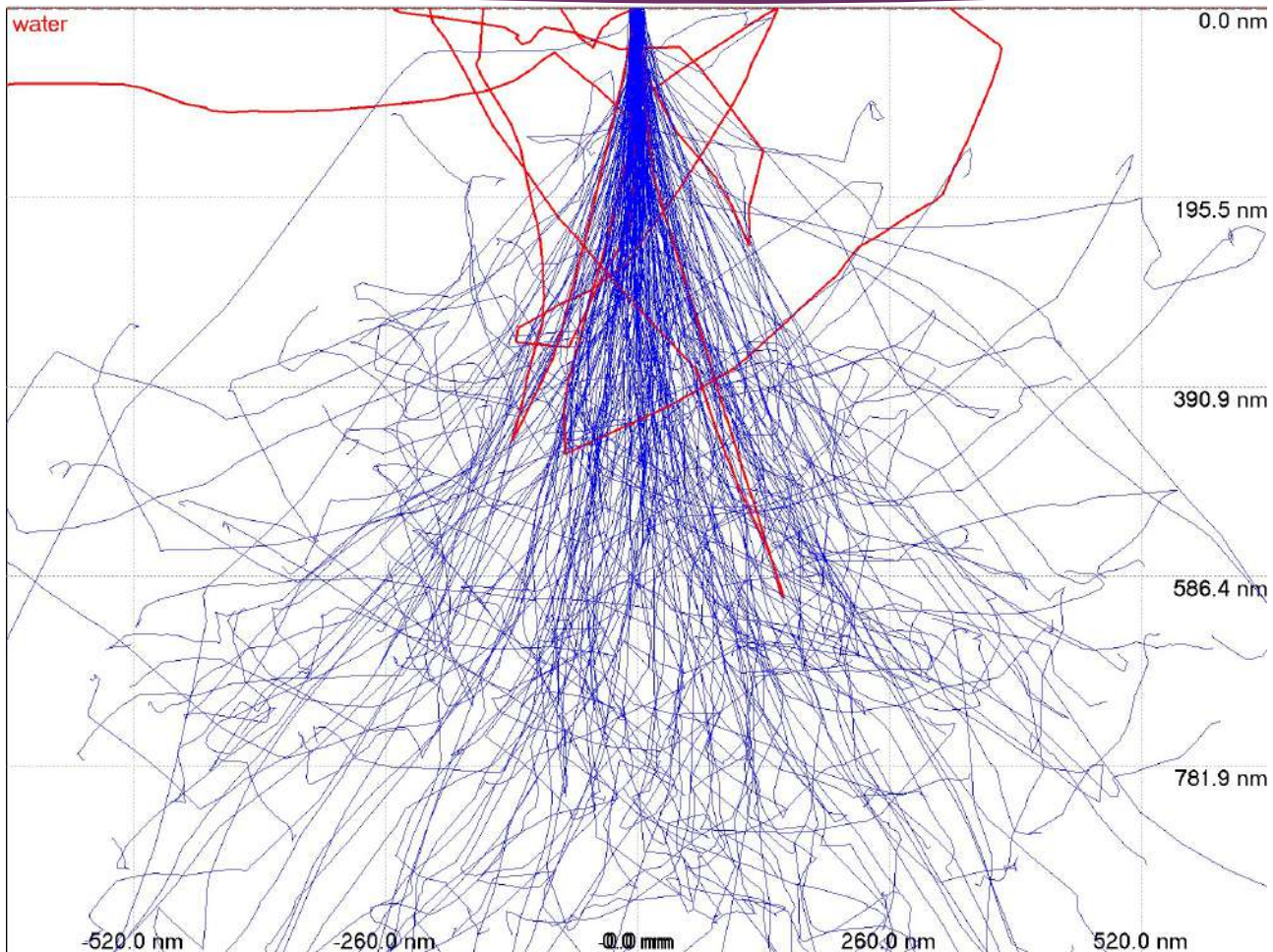
Monte Carlo simulation: water at 5 keV



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