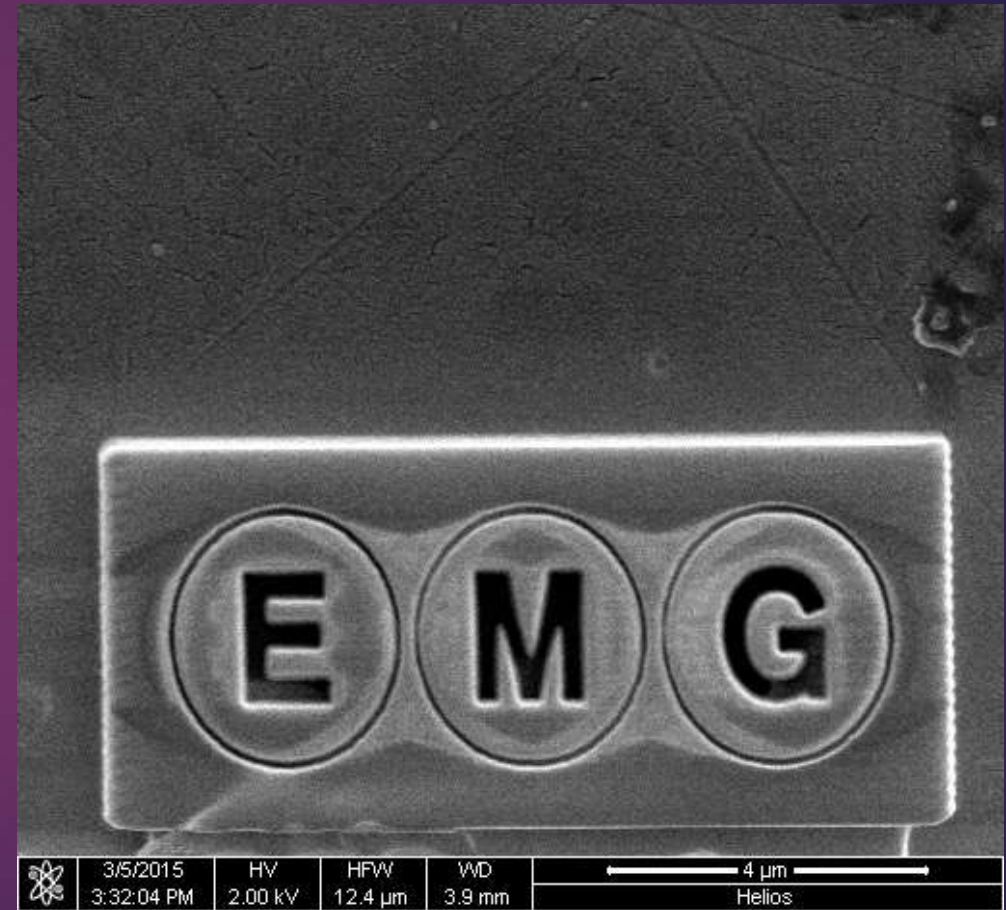


# FIB SEM

TOMOGRAPHY SHORT COURSE

FEBRUARY 2022

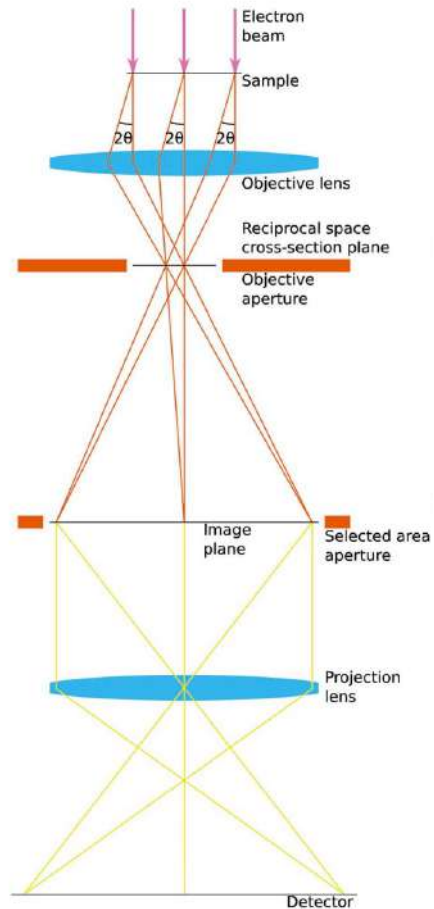
WILLIAM RICE, NYU SCHOOL OF MEDICINE



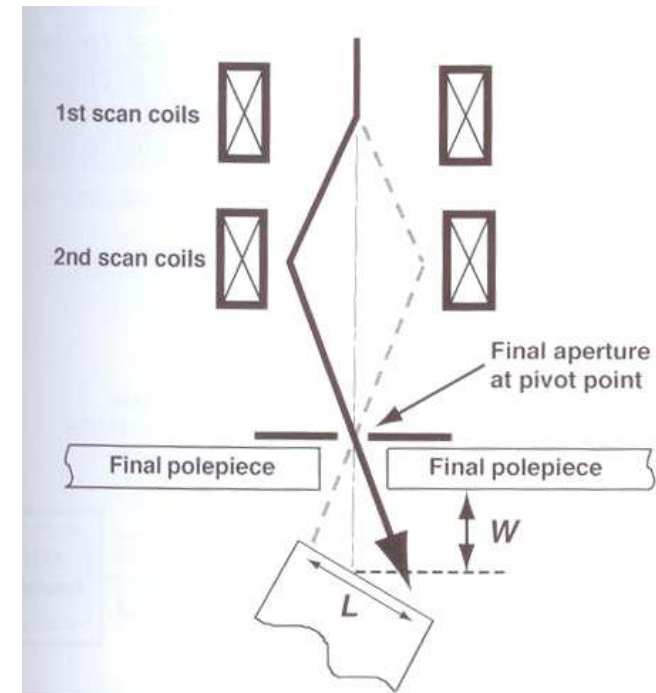
# SEM Basics

# SEM versus TEM

TEM

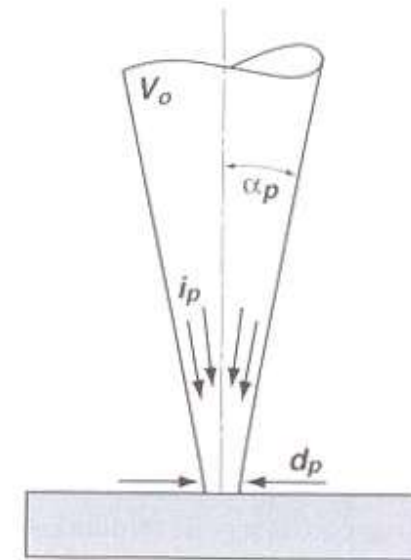


SEM



# SEM Beam: probe size

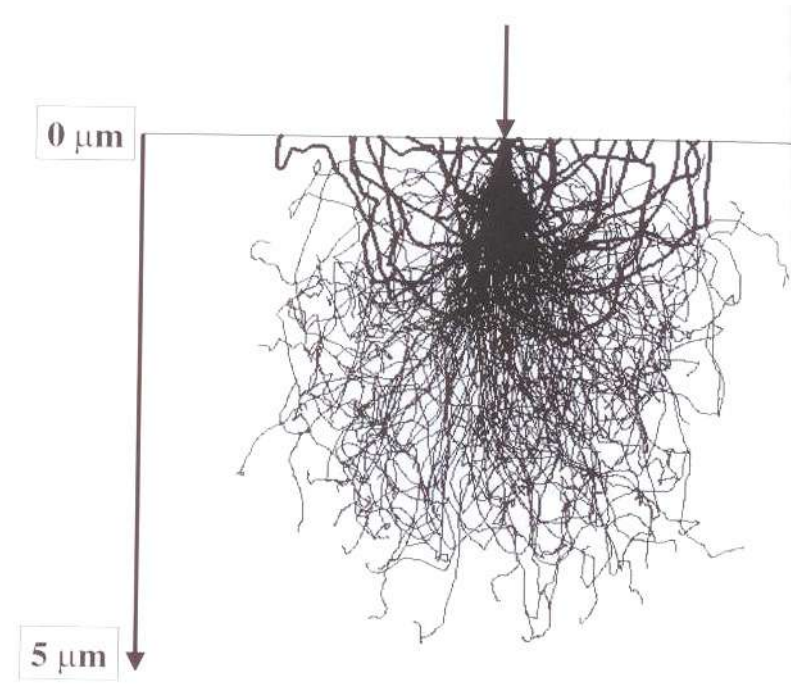
- ◆ Ideally want as small a probe as possible, relative to pixel size
- ◆ Probe size is determined by voltage, current, divergence angle
- ◆ Lens distortions
  - ◆ Spherical aberration (focus different at center and edge of lens) – proportional to focal length (working distance)
  - ◆ Aperture diffraction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



Goldstein et al, 2003

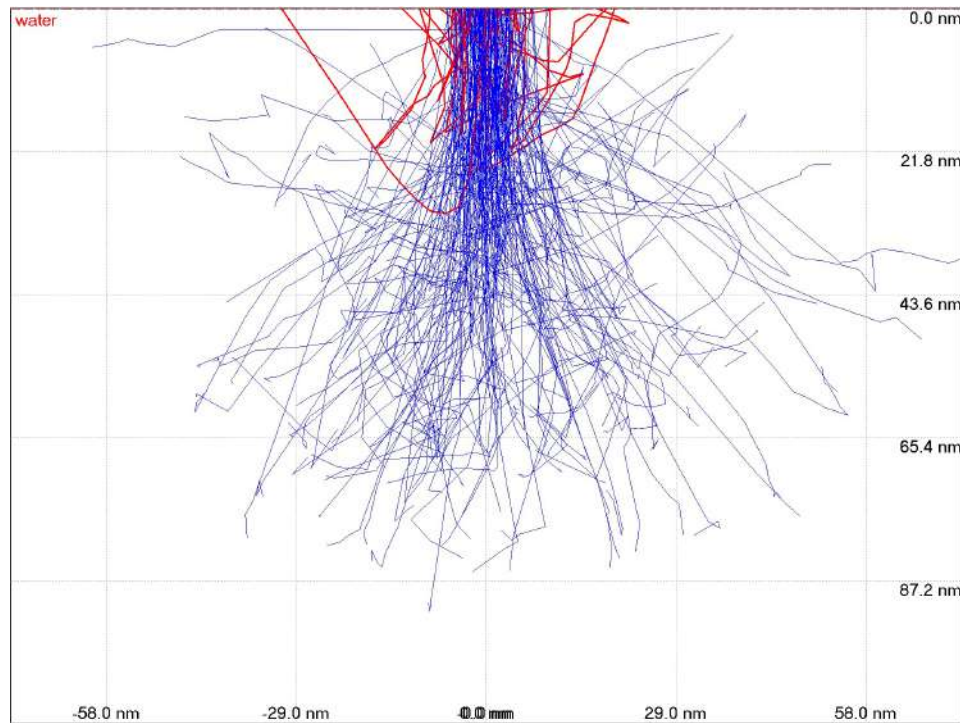
# Beam-Specimen Interaction

- 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  $\sim Z^2$
- Inelastic scattering:
  - Secondary electrons
  - X-rays



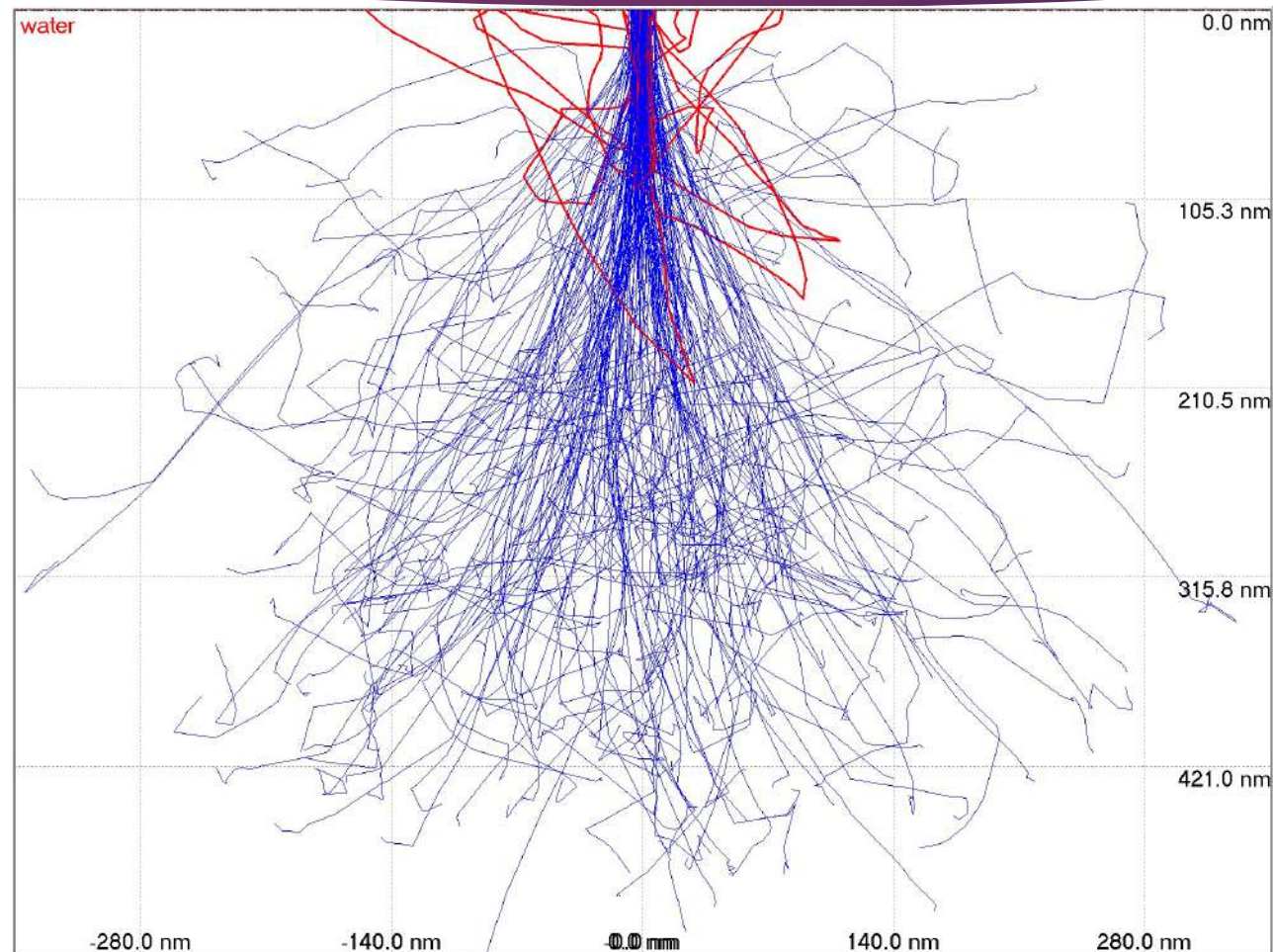
Goldstein et al, 2003

# Monte Carlo simulation: water at 1 keV

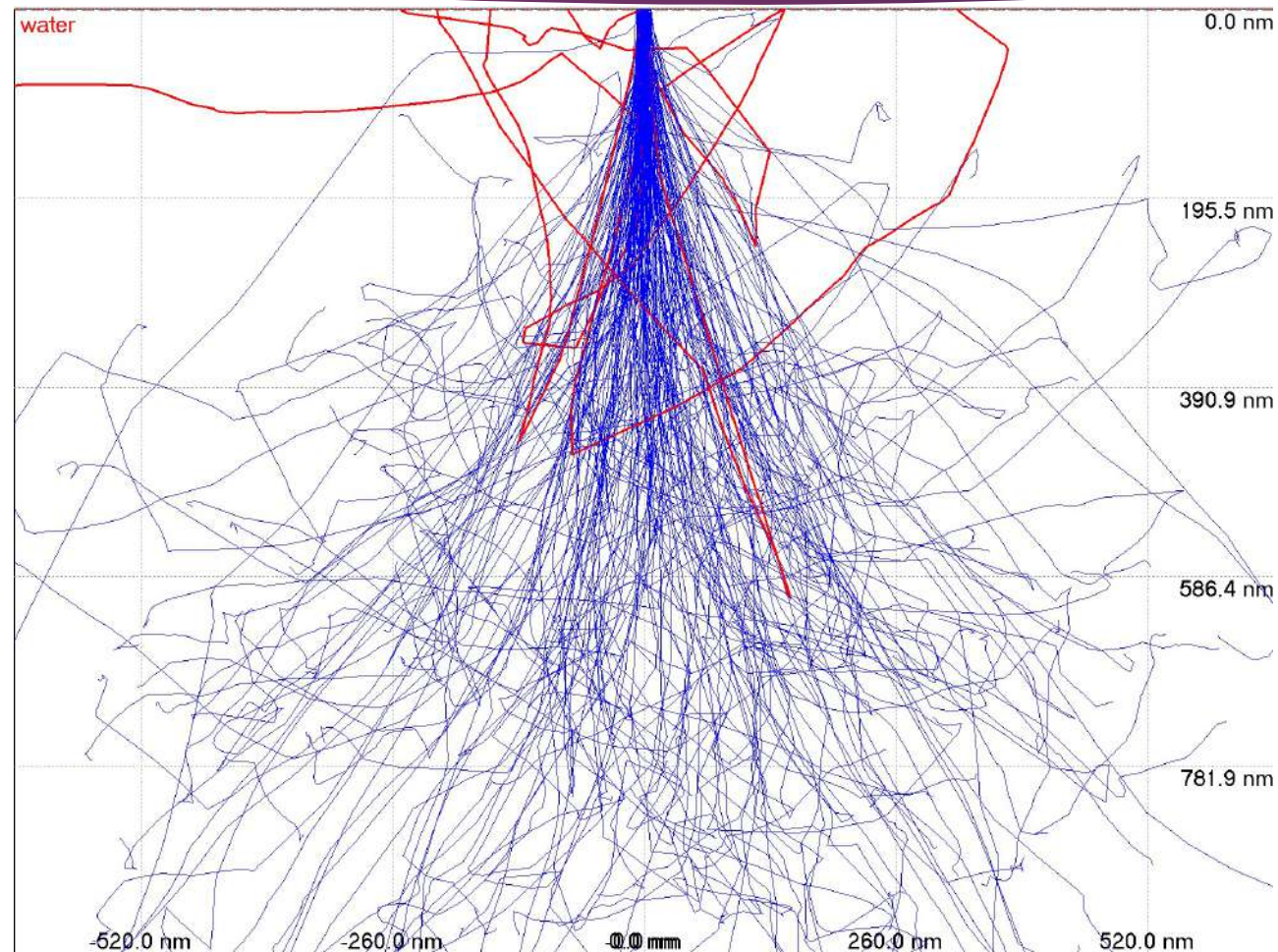




# Monte Carlo simulation: water at 3 keV

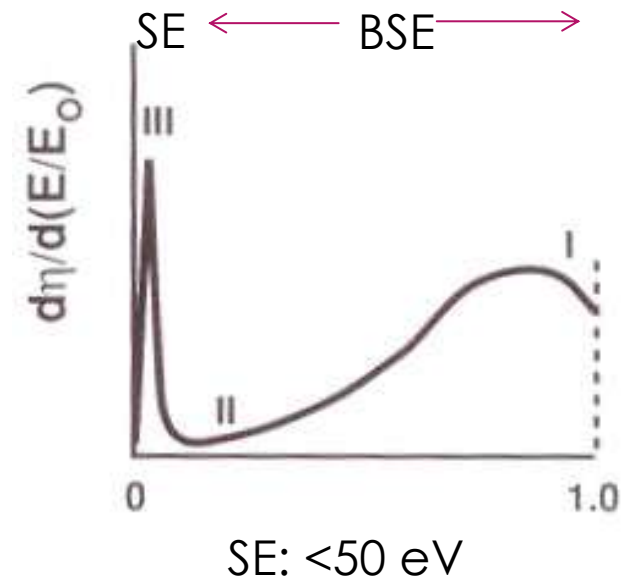
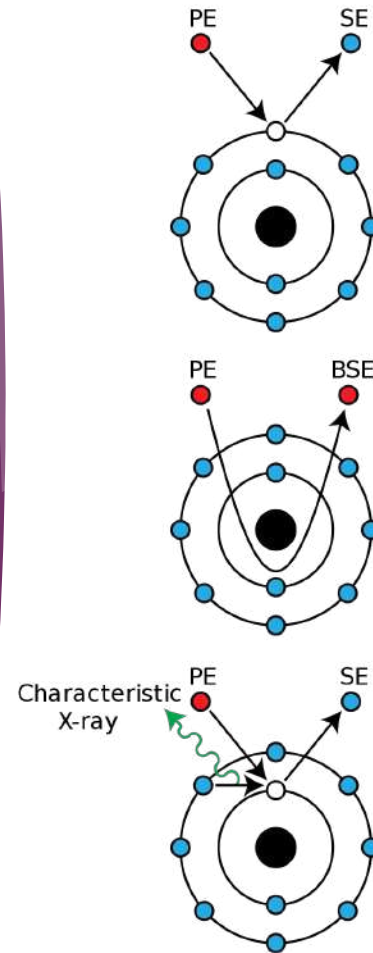


# Monte Carlo simulation: water at 5 keV

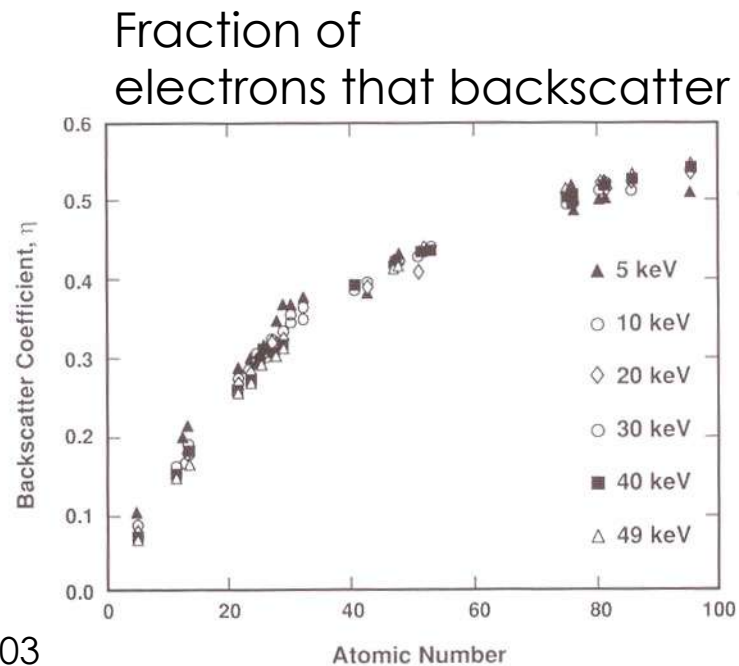




# Signal: Back Scattered Electrons (BSE's) and Secondary Electrons (SE's)

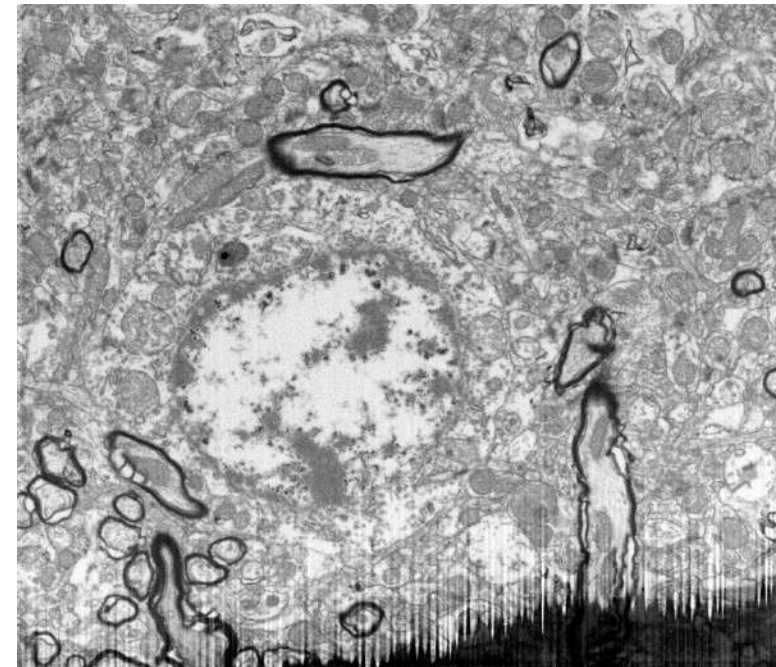


# BSE efficiency is material dependent, voltage independent



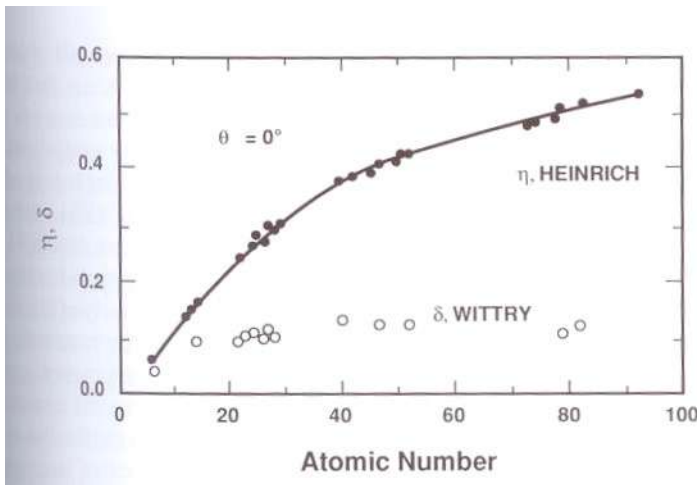
Goldstein et al, 2003

BSE's give contrast  
between light and  
heavy elements



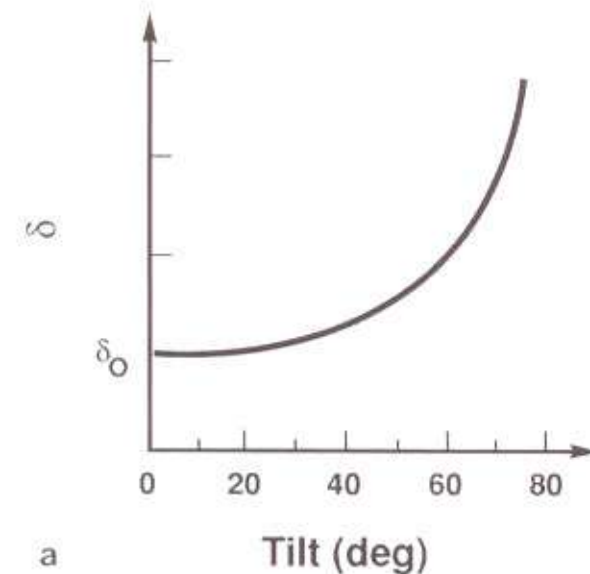
Osmium stained, resin-embedded tissue

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



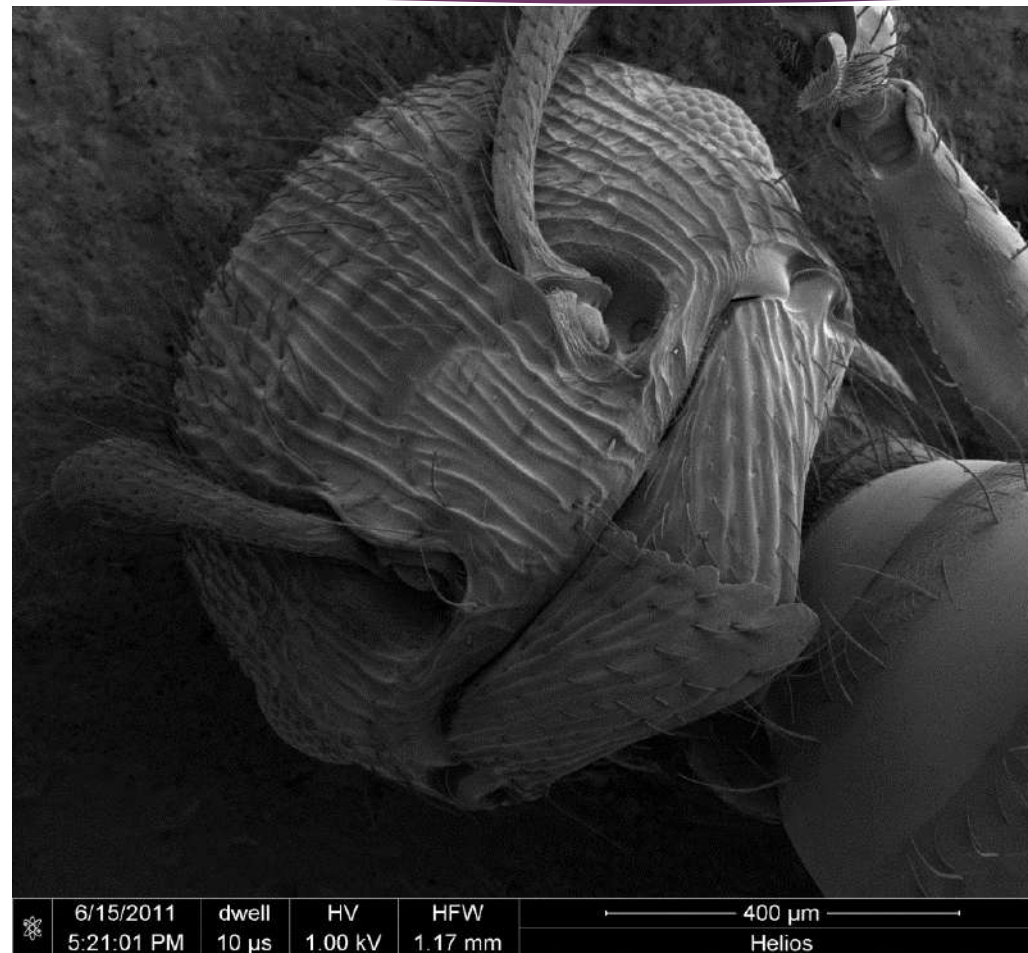
30 keV

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

# 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

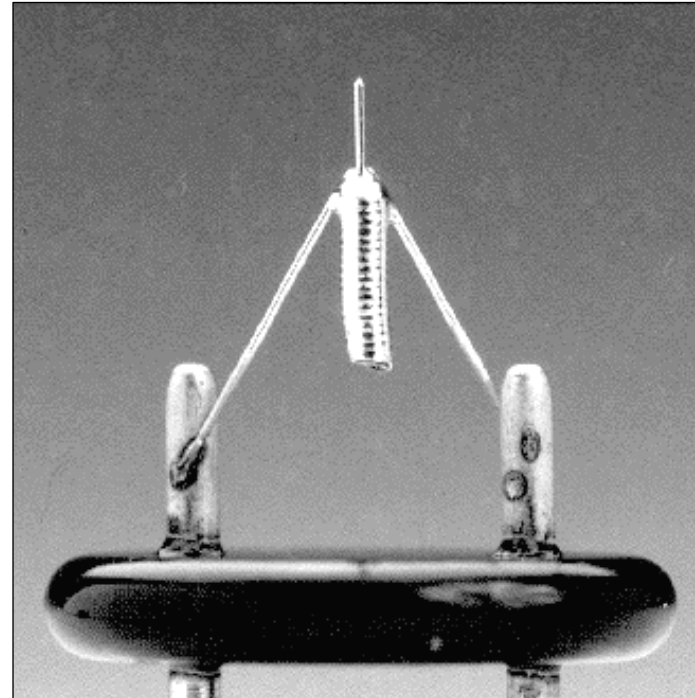
## TEM

- ▶ Small Chamber
  - ▶ Easier to reach very high vacuum
  - ▶ Few ports for add-ons
- ▶ Voltage: 80-300 keV
  - ▶ 300 keV for highest resolution
  - ▶ 200 keV minimum for DED
- ▶ 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

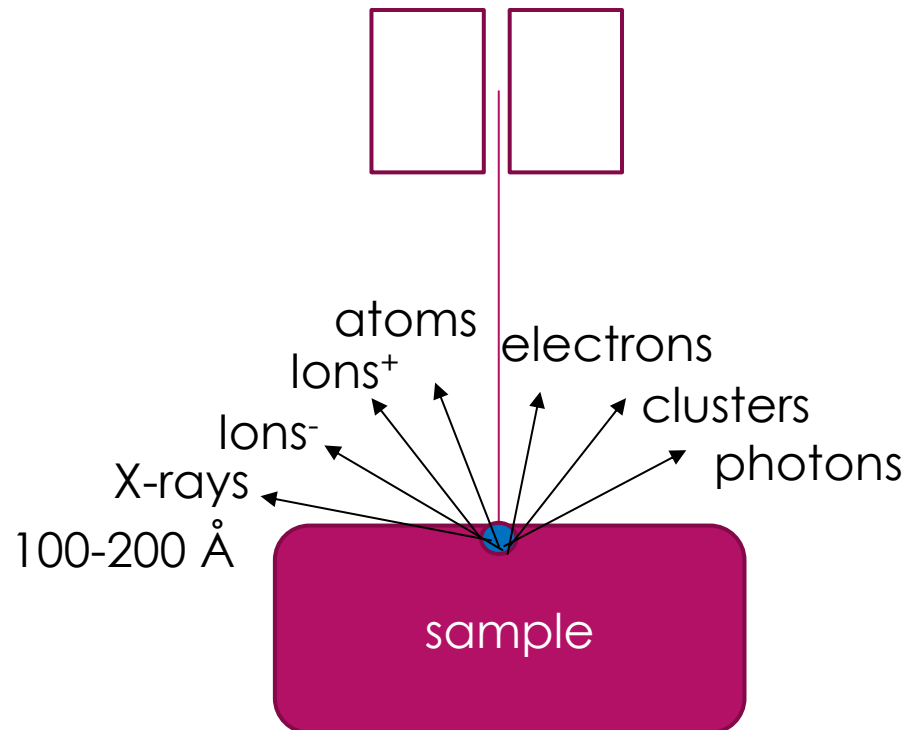


# Ion Column

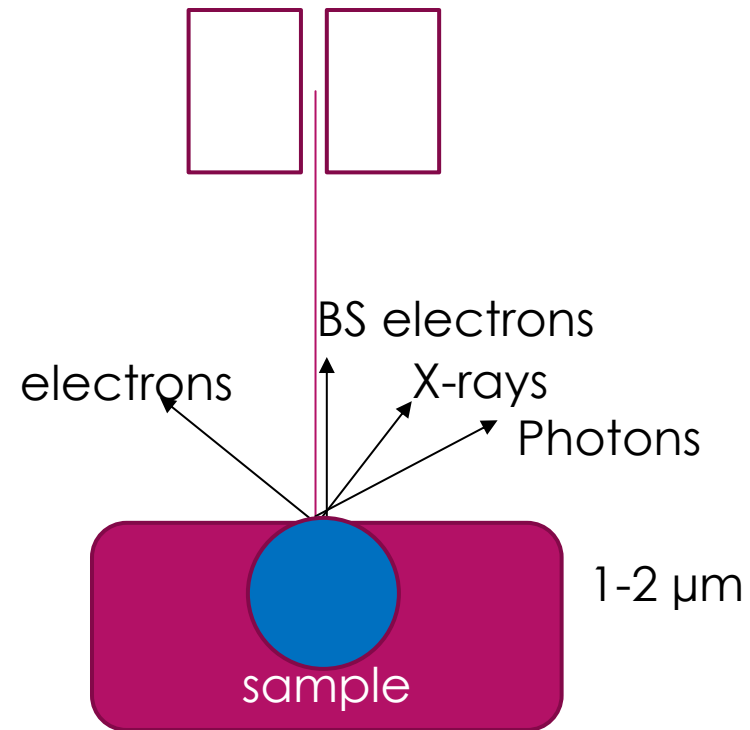
- ◆ Source - LMIS at top
- ◆ Focusing Optics
  - ▶ Use Electrostatic lenses since ions are heavier than electrons.
- ◆ Deflection Electronics/Pattern Board
- ◆ High-speed Blanking
  - ▶ Need to prevent milling while blanking
- ▶ Current is controlled by apertures
  - ▶ Apertures wear out over time and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

Ion Beam



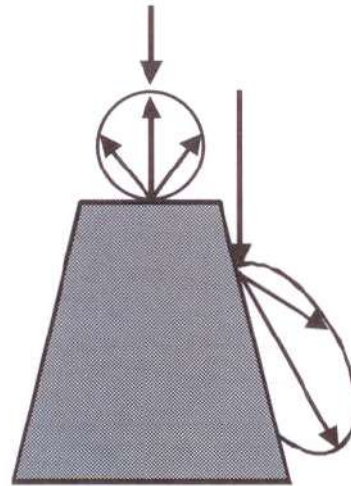
Electron Beam





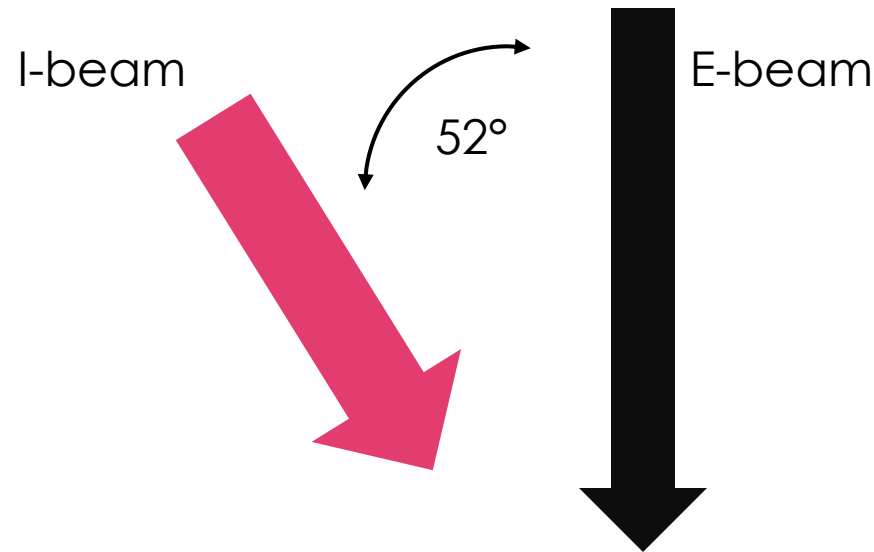
# Common Use: Sputtering particles from substrate

Sputtered Particle Ejection Behavior



More efficient milling at edge than in bulk

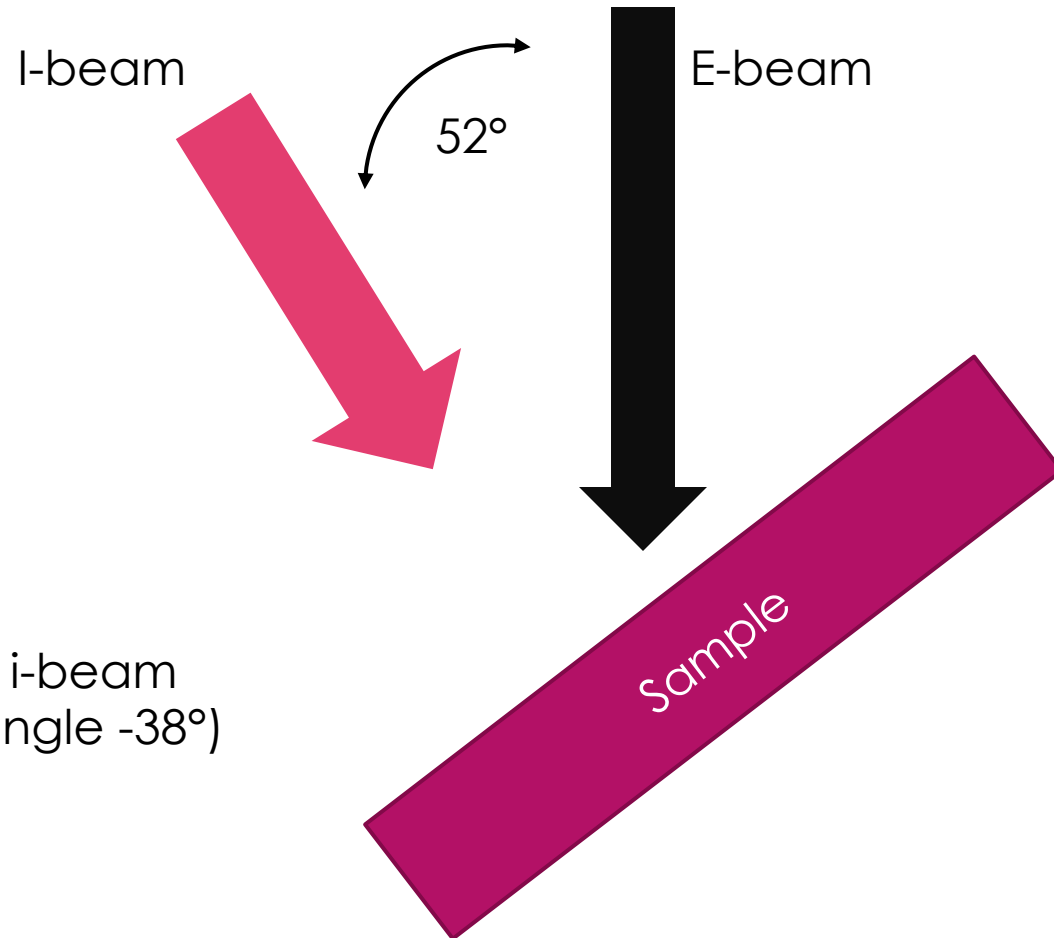
# Geometry



Sample:  
Tilt from  $-10^\circ$  to  $+70^\circ$

Sample

# Geometry



Sample:

Tilt from  $-10^\circ$  to  $+70^\circ$

Tilt to  $52^\circ$  for orthogonal i-beam  
(cross-section viewing angle  $-38^\circ$ )

# Geometry



# 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”

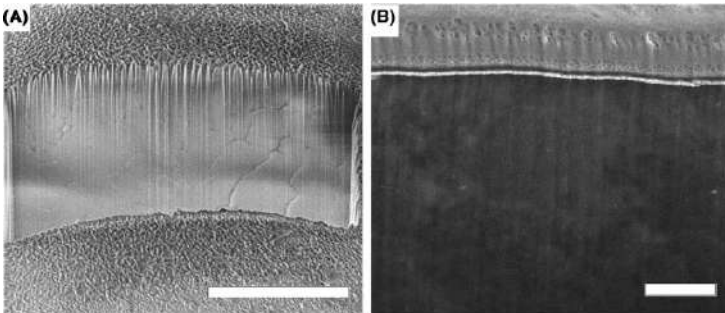
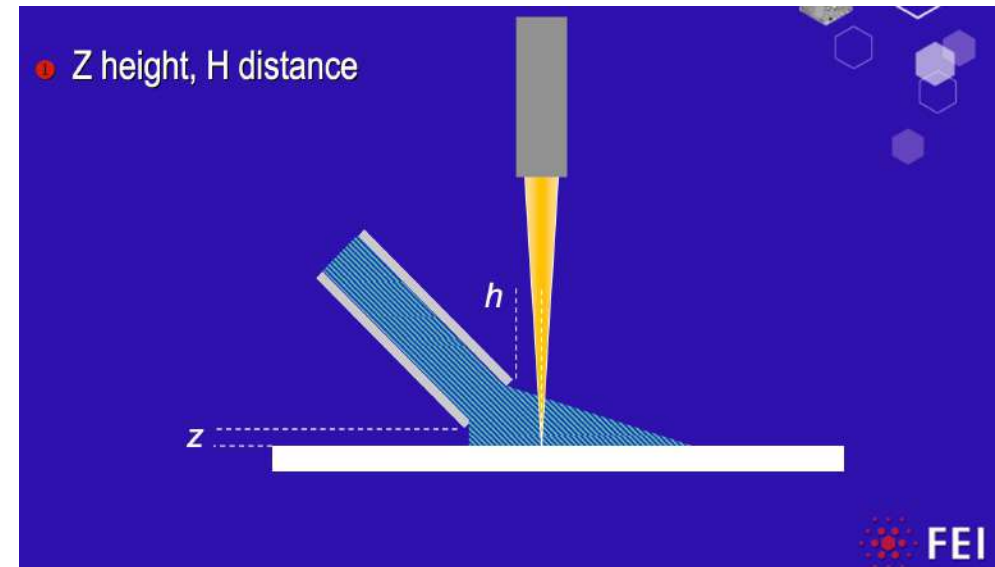
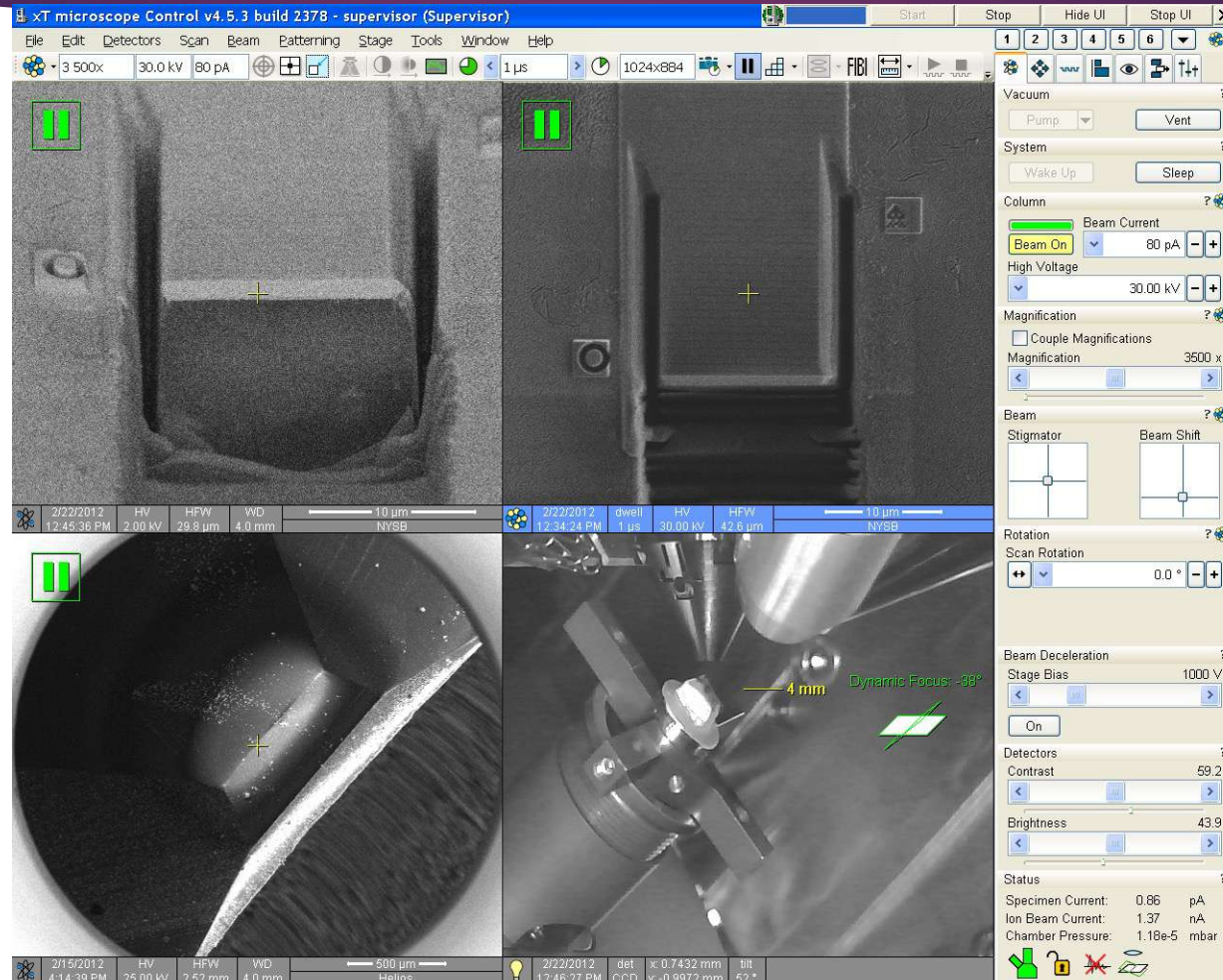


Image: Hayles and Winter, 2021

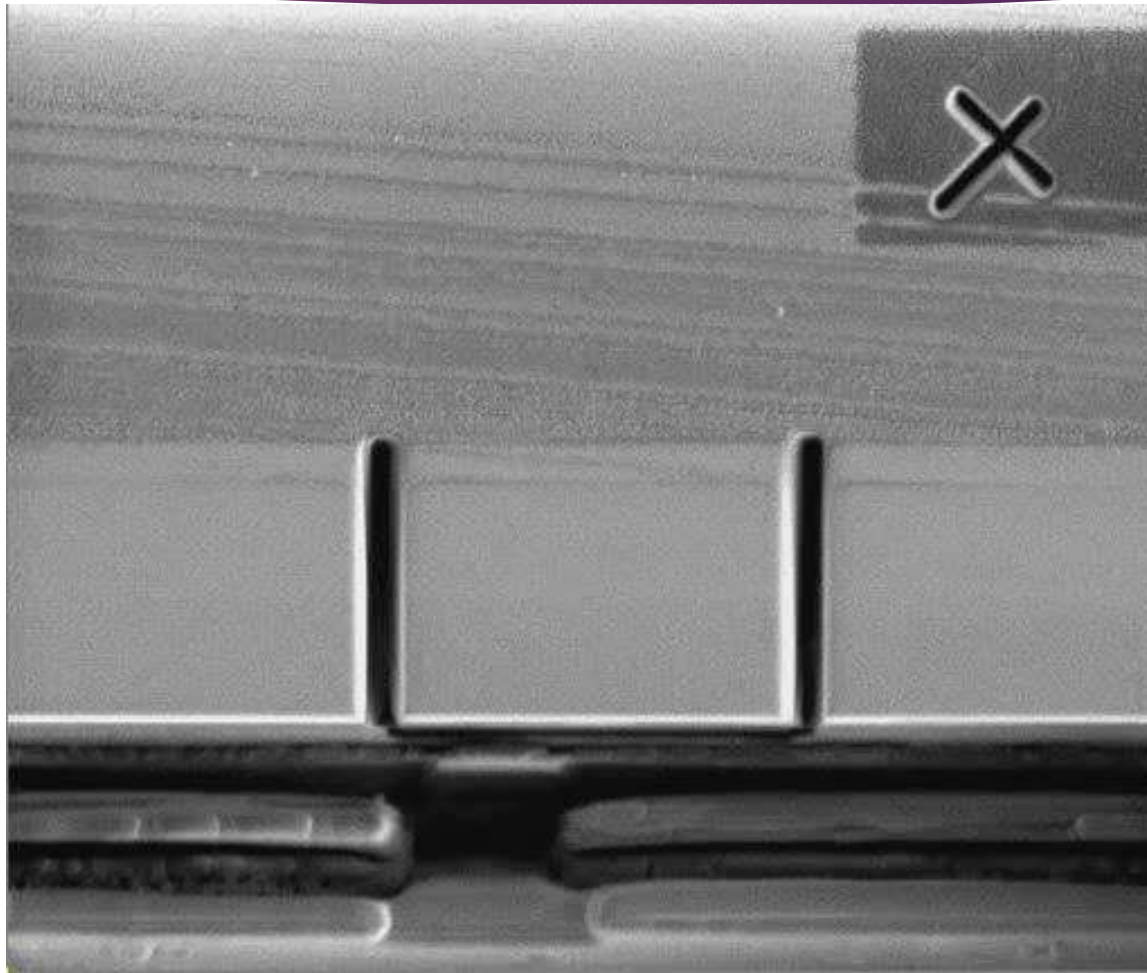




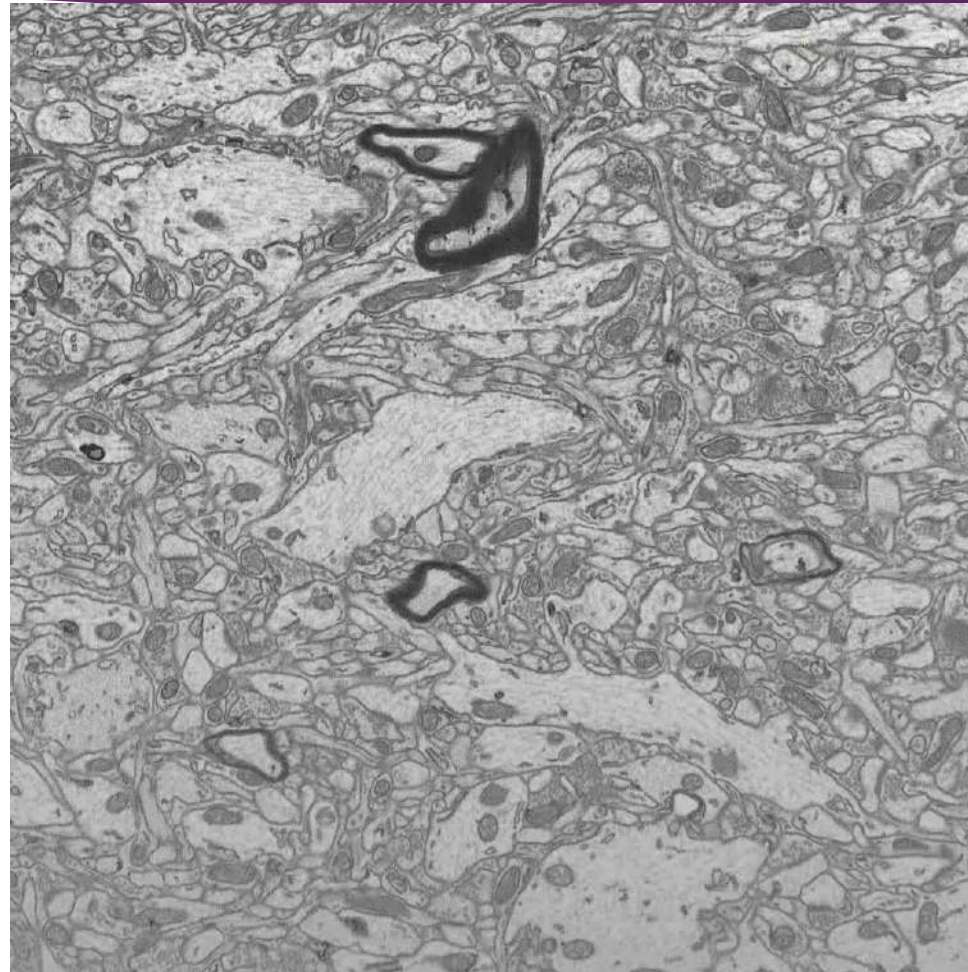
# Application: 3D reconstruction of stained, resin-embedded tissue



# Milling: i-beam view

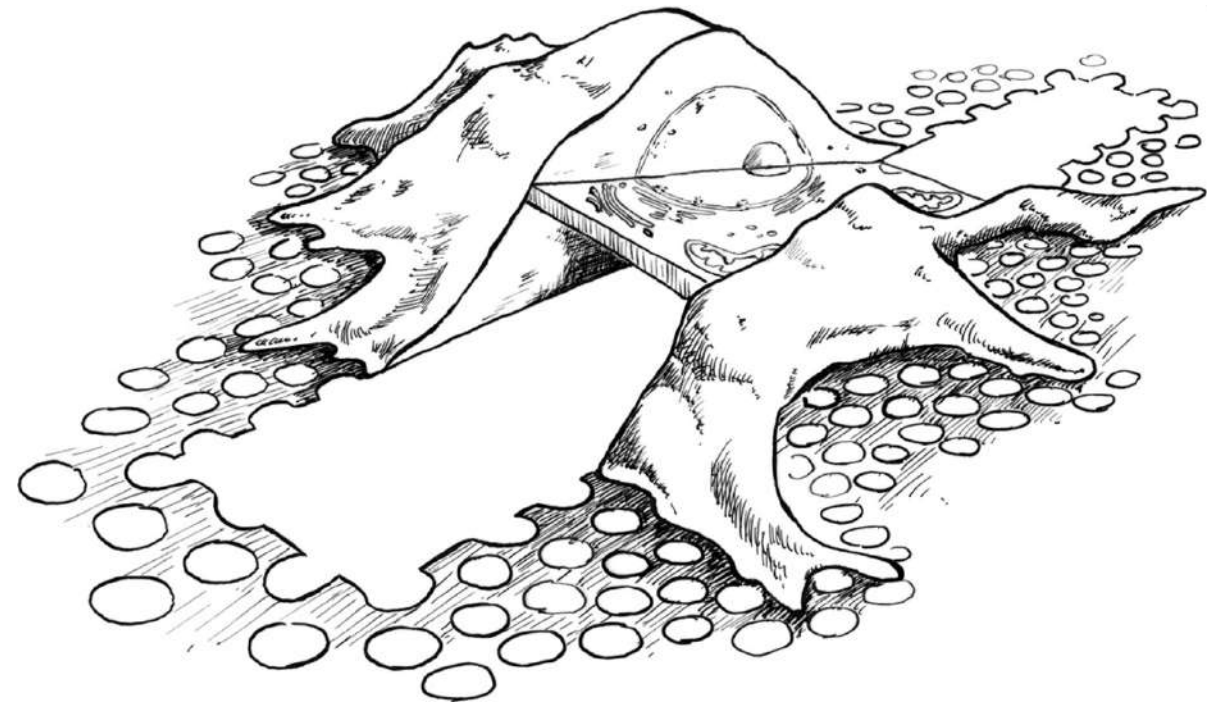


# Example Movie: Neural Tissue





# Cryo FIB/SEM for tomographic sample preparation



# 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.

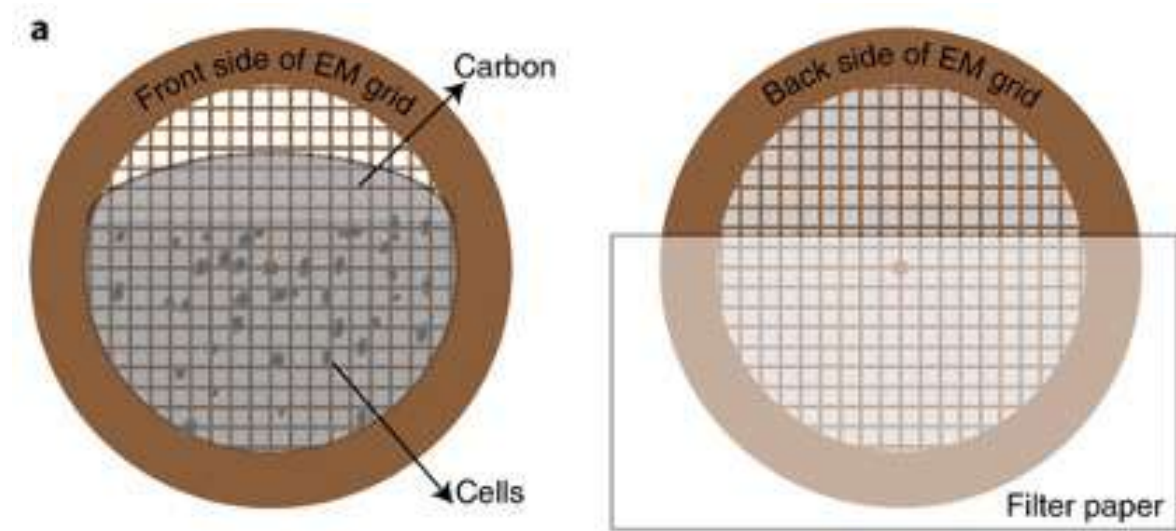
\*Center for Nanoscale Systems, Harvard University, 17 Oxford St., Cambridge, MA 02138, U.S.A.

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



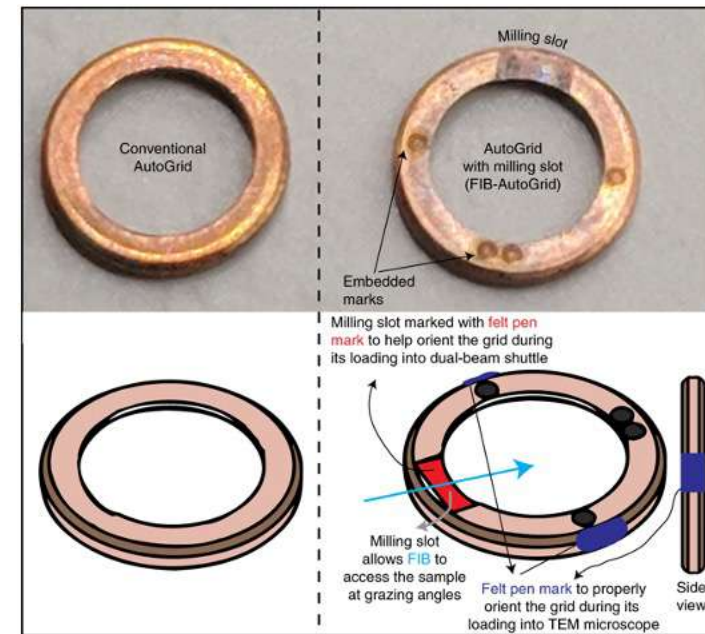
# Place cells on Grids

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



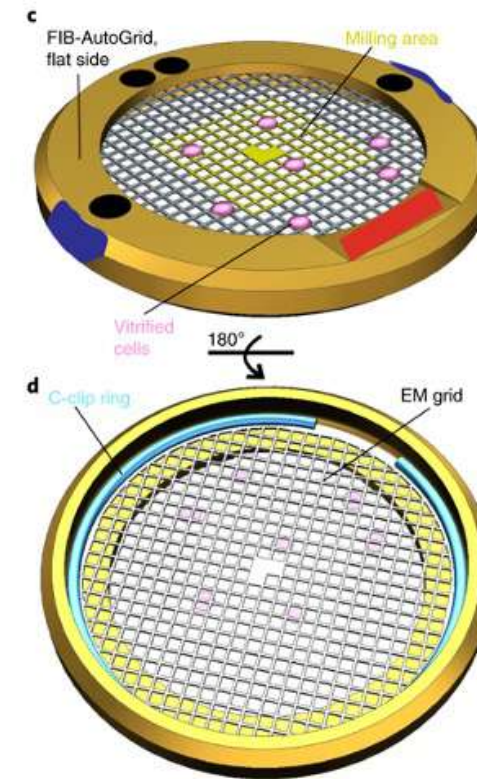
# Grid Geometry

- ▶ 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



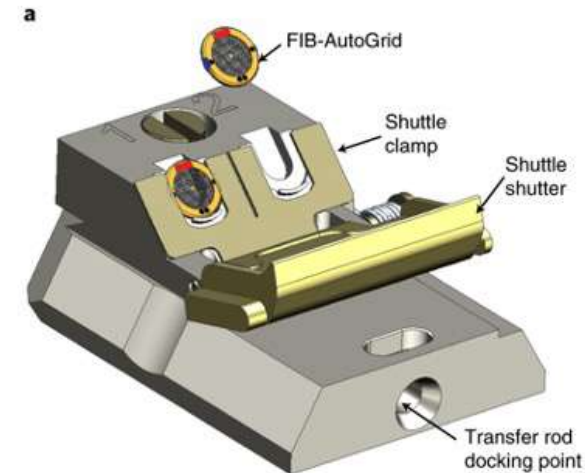
# Grid Geometry

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



# 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



# Transfer Rod for Loading



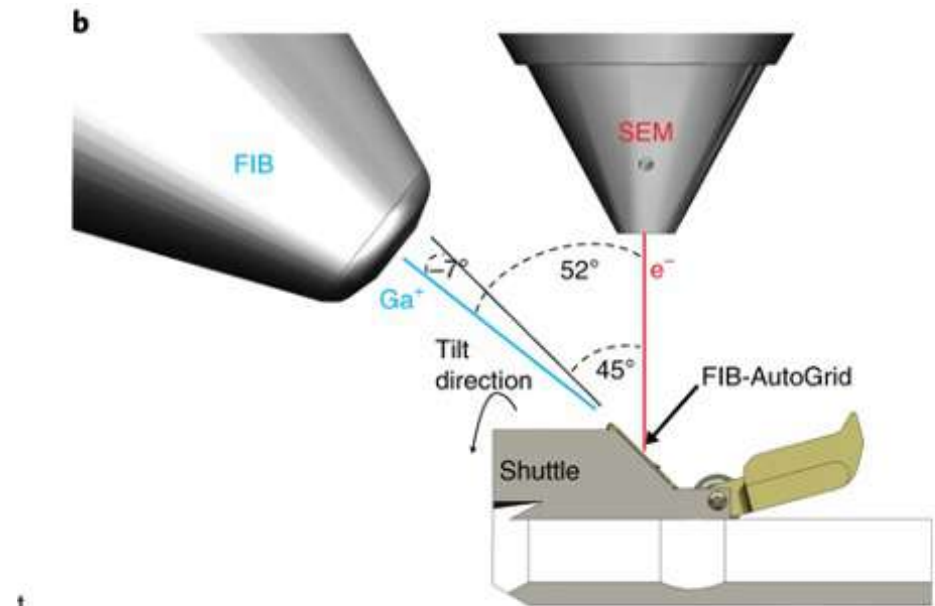


# Older Loading Station (Quorum)



# Geometry

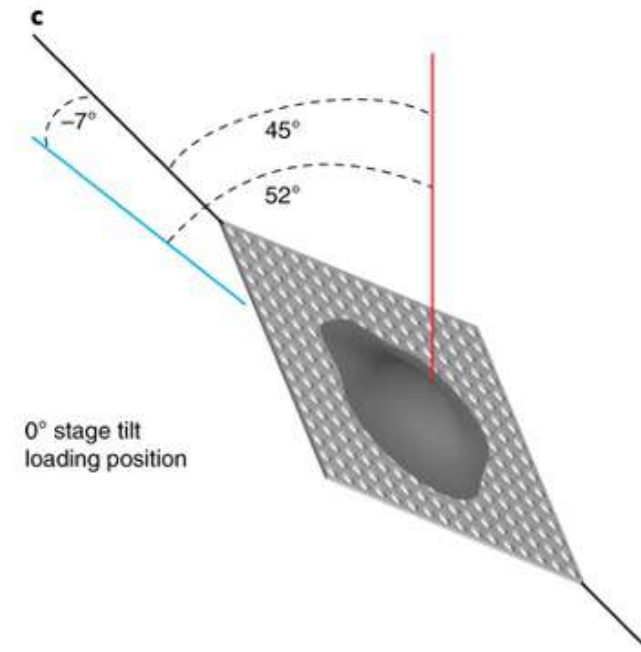
- ▶ Untilted stage:
  - ▶ Ga beam at  $-7^\circ$  angle to grid surface
  - ▶ E-beam at  $45^\circ$  angle to grid surface





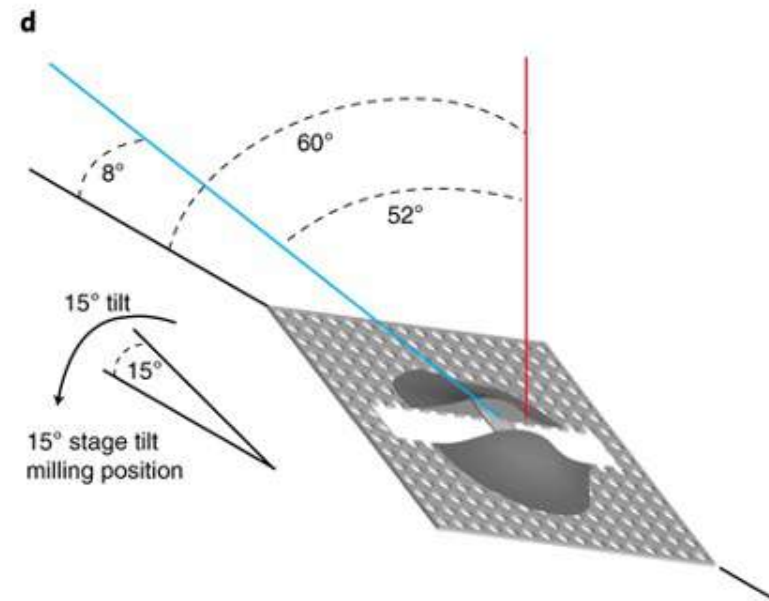
# Geometry: Untilted

- ▶ Untilted stage:
  - ▶ Ga beam at  $-7^\circ$  angle to grid surface
  - ▶ E-beam at  $45^\circ$  angle to grid surface



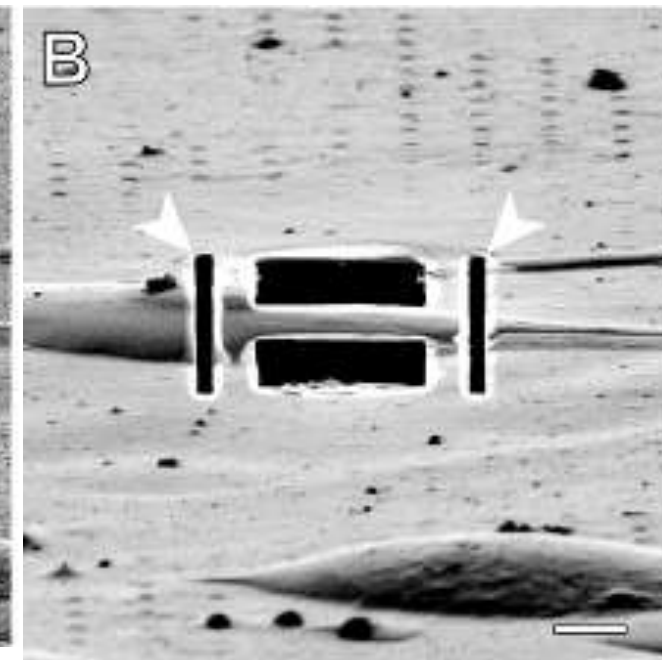
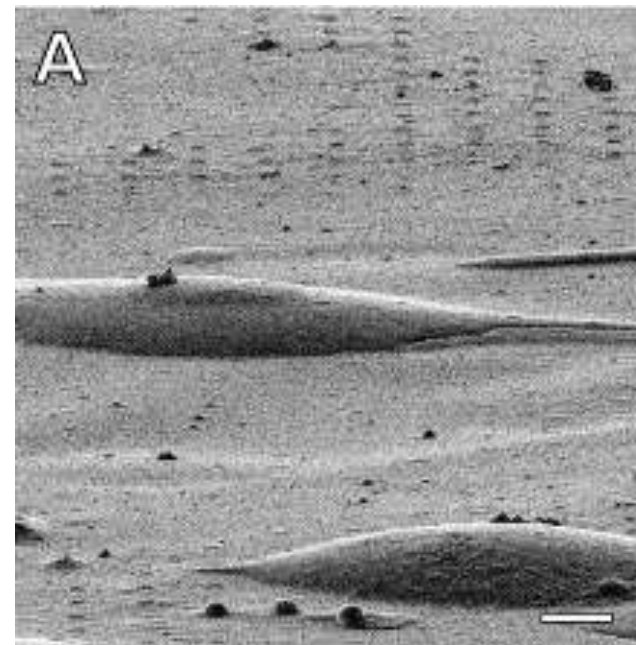
# Geometry: Tilted

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

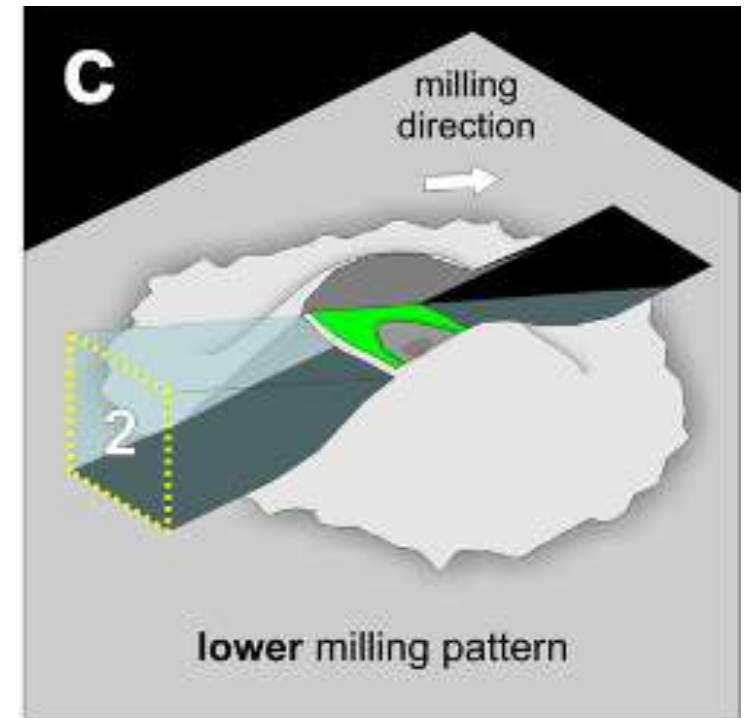
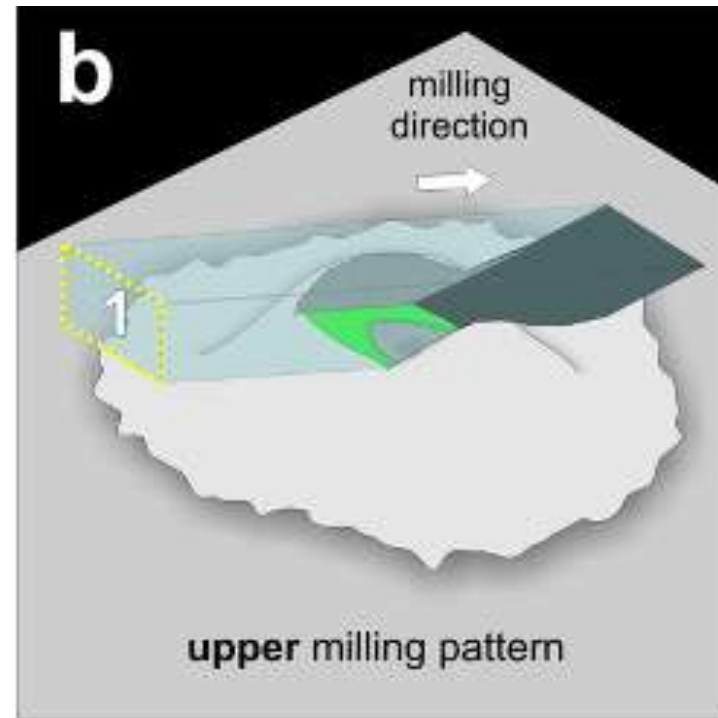
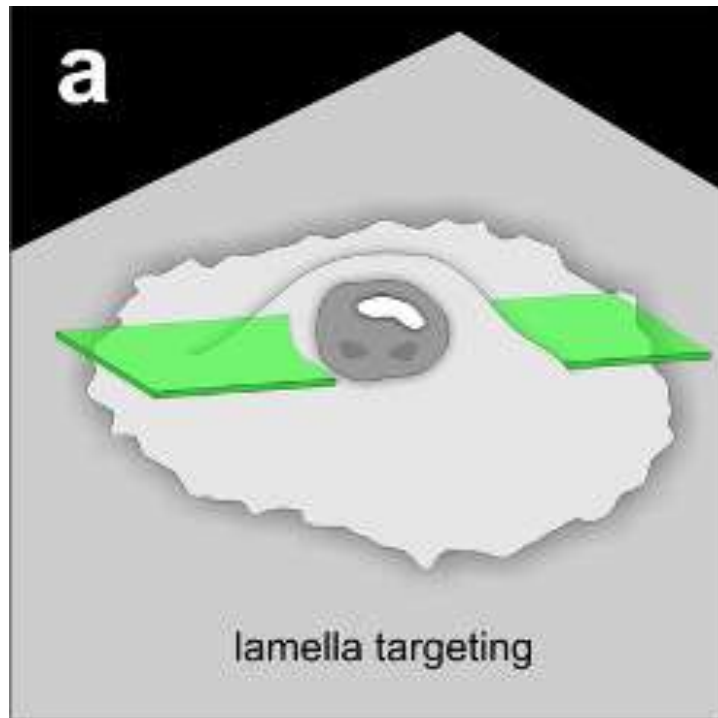


# Imaging cells with ion beam

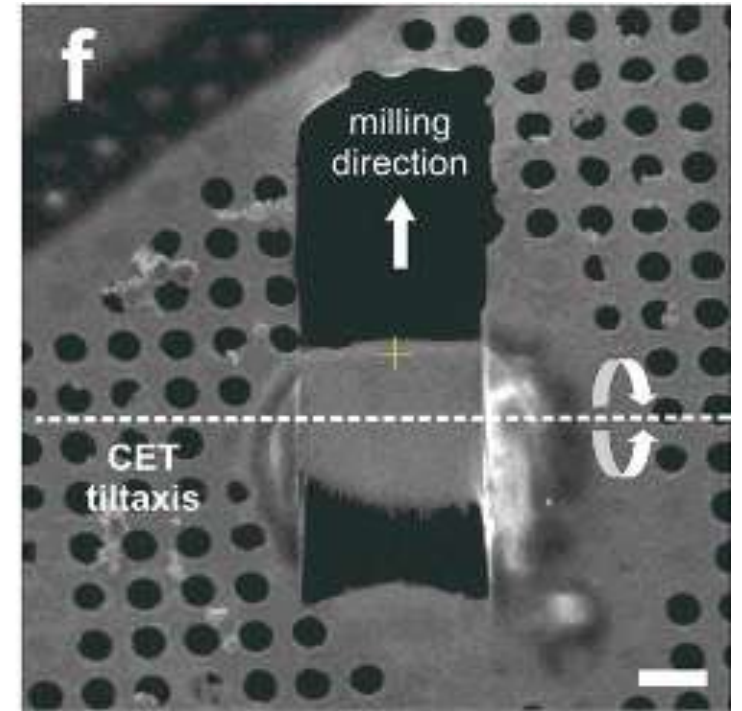
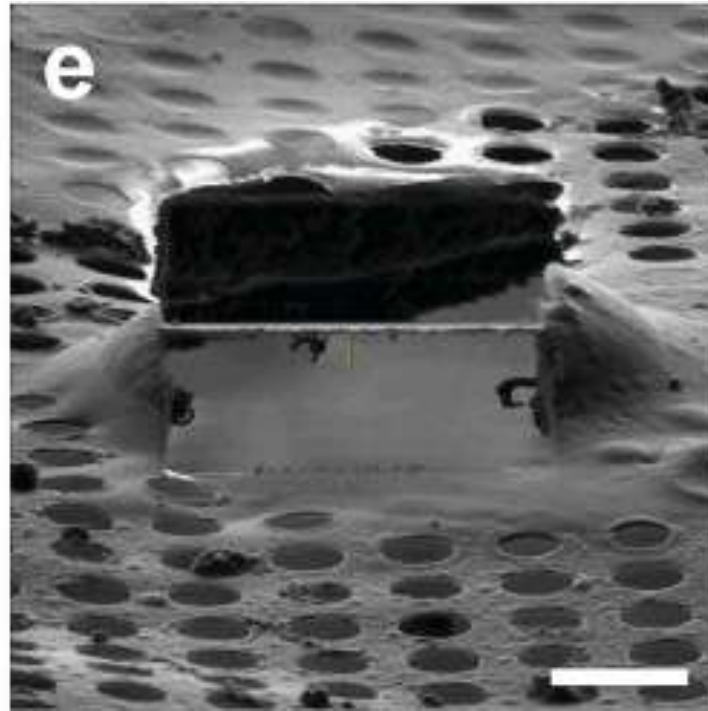
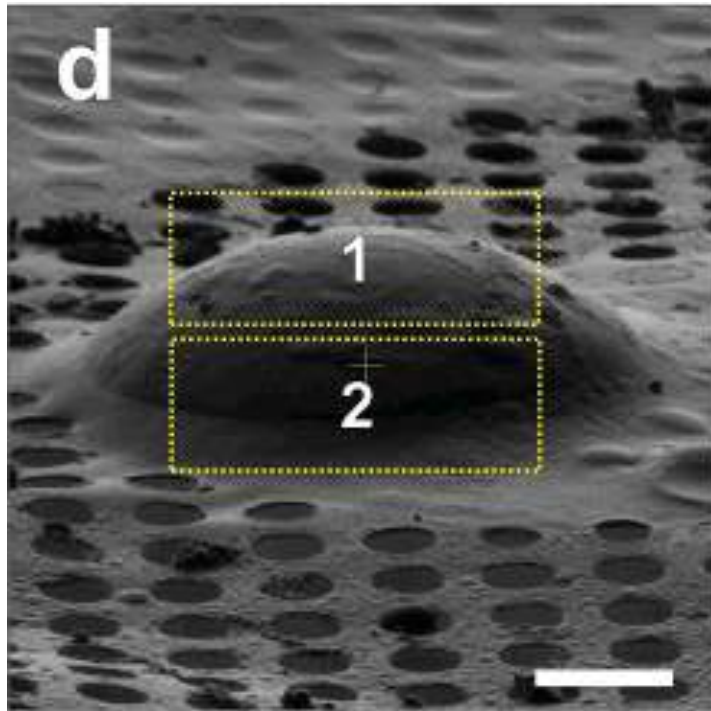
- ▶ A: Ion-beam view of cells
- ▶ B: Cells after milling, showing position of micro-expansion joints



# Targeting of Milling Regions



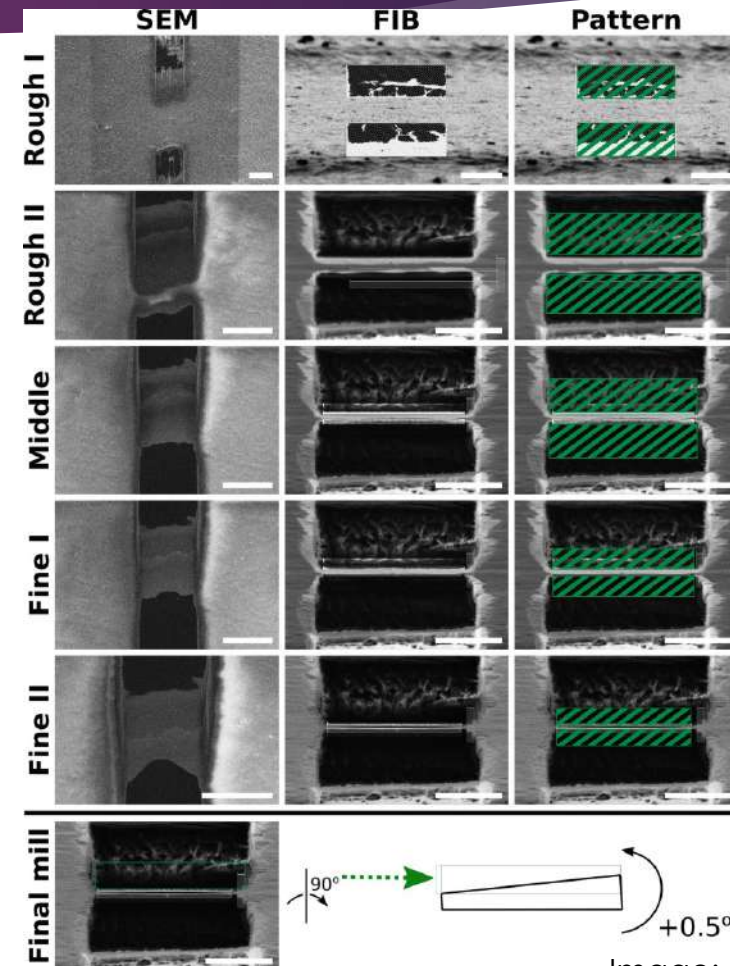
# Targeting of Milling Regions



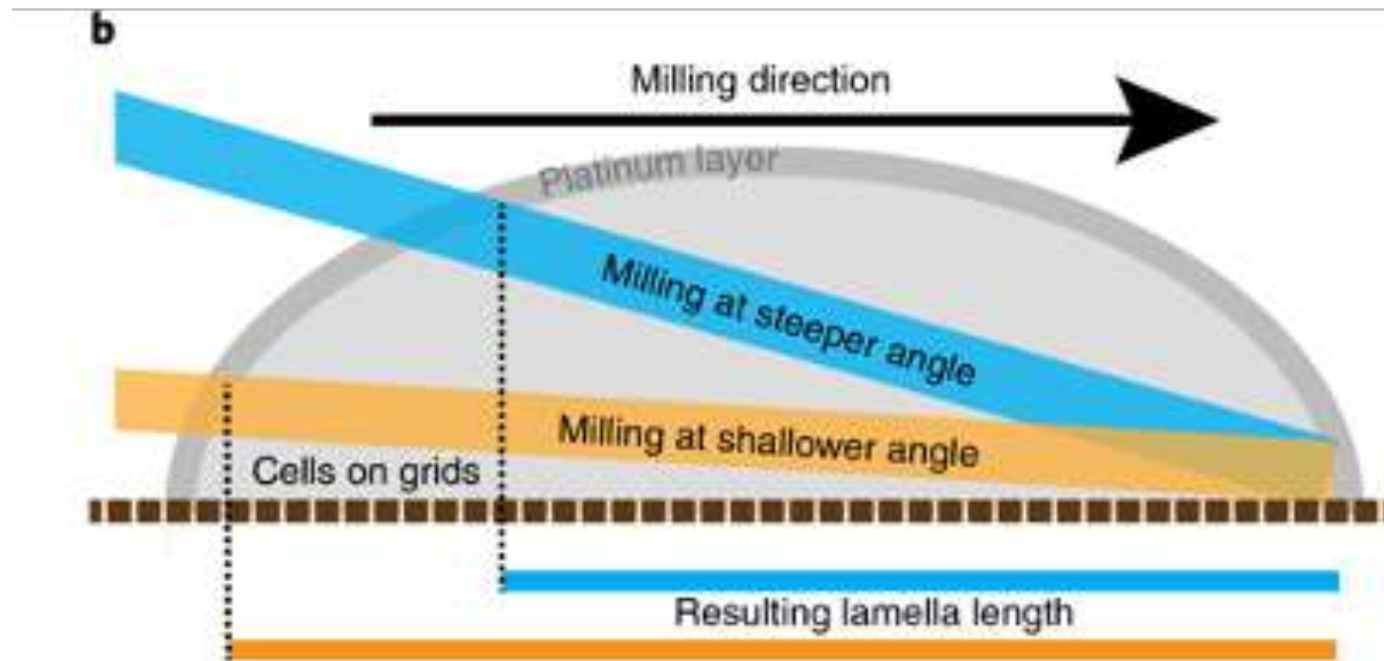


# Milling

- ▶ 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  $0.5^\circ$  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



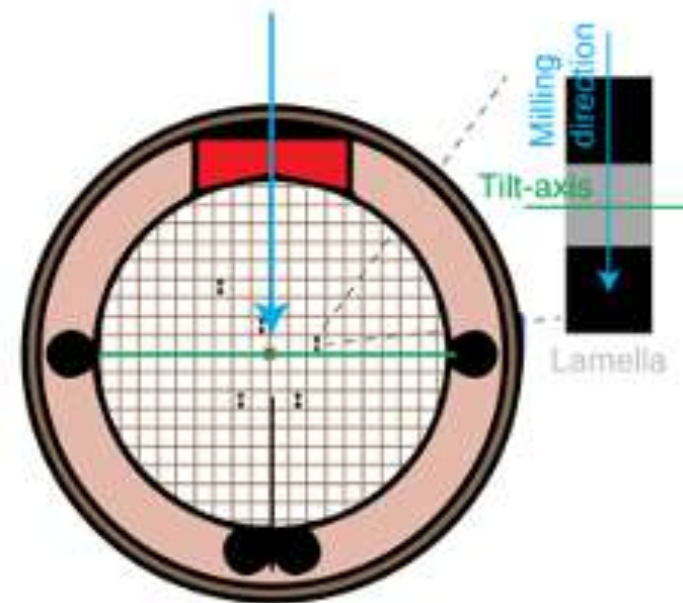
# Milling at as shallow an angle as possible





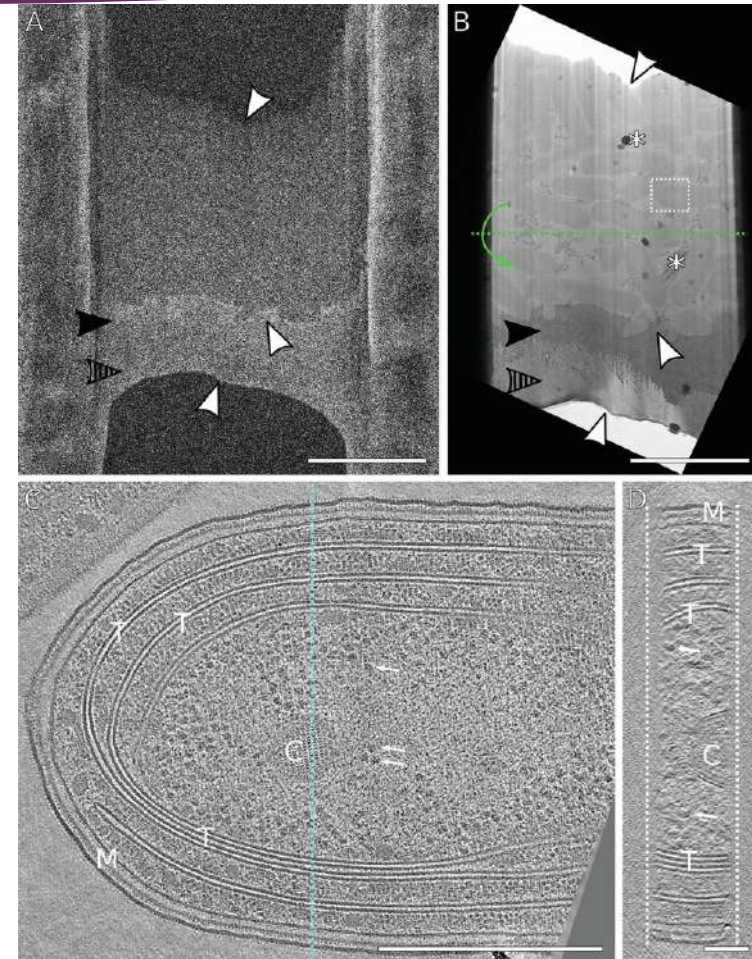
# Geometry: Loading into TEM

- ▶ 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.



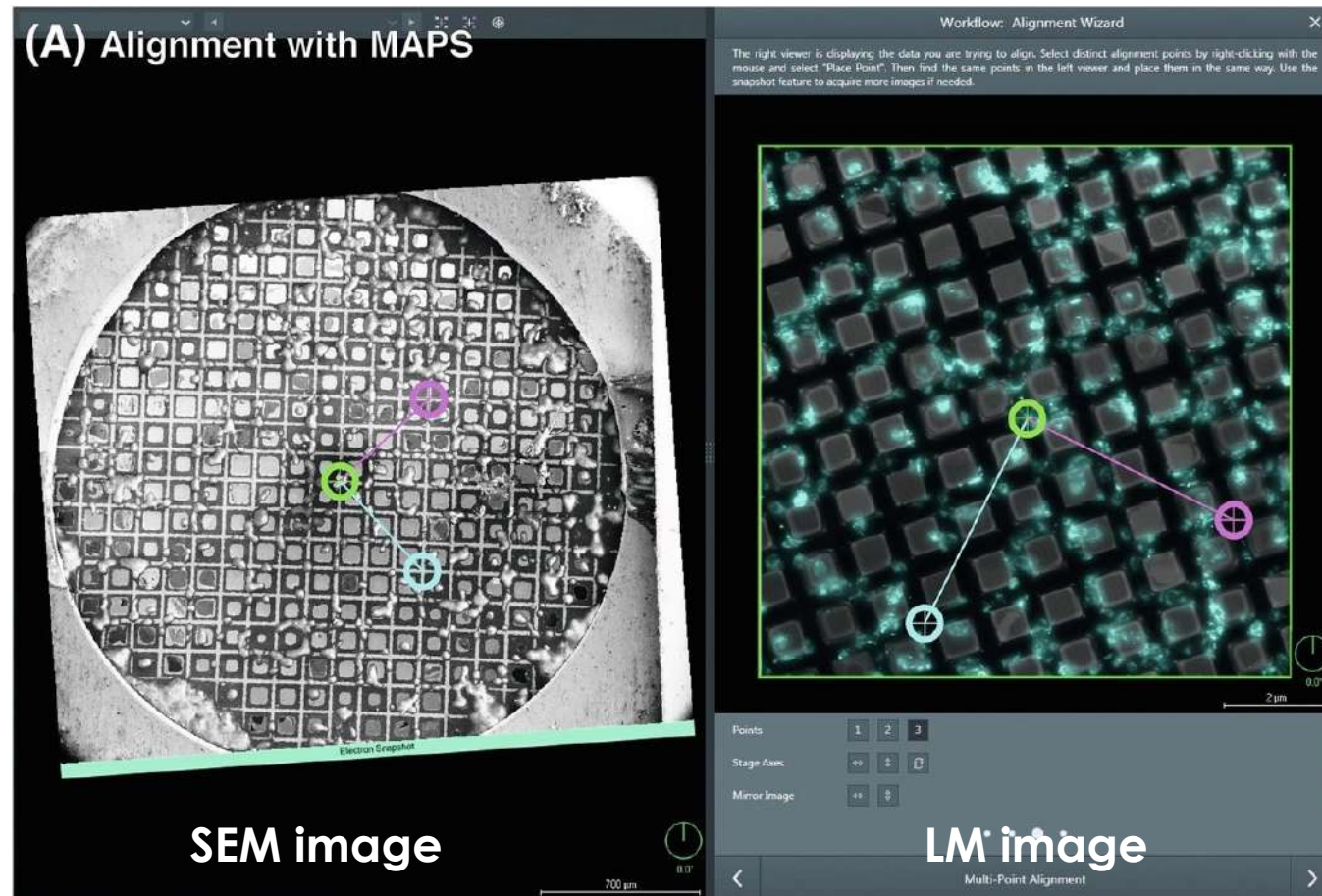
# Difficulties / Issues

- ▶ 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

# Where to mill?

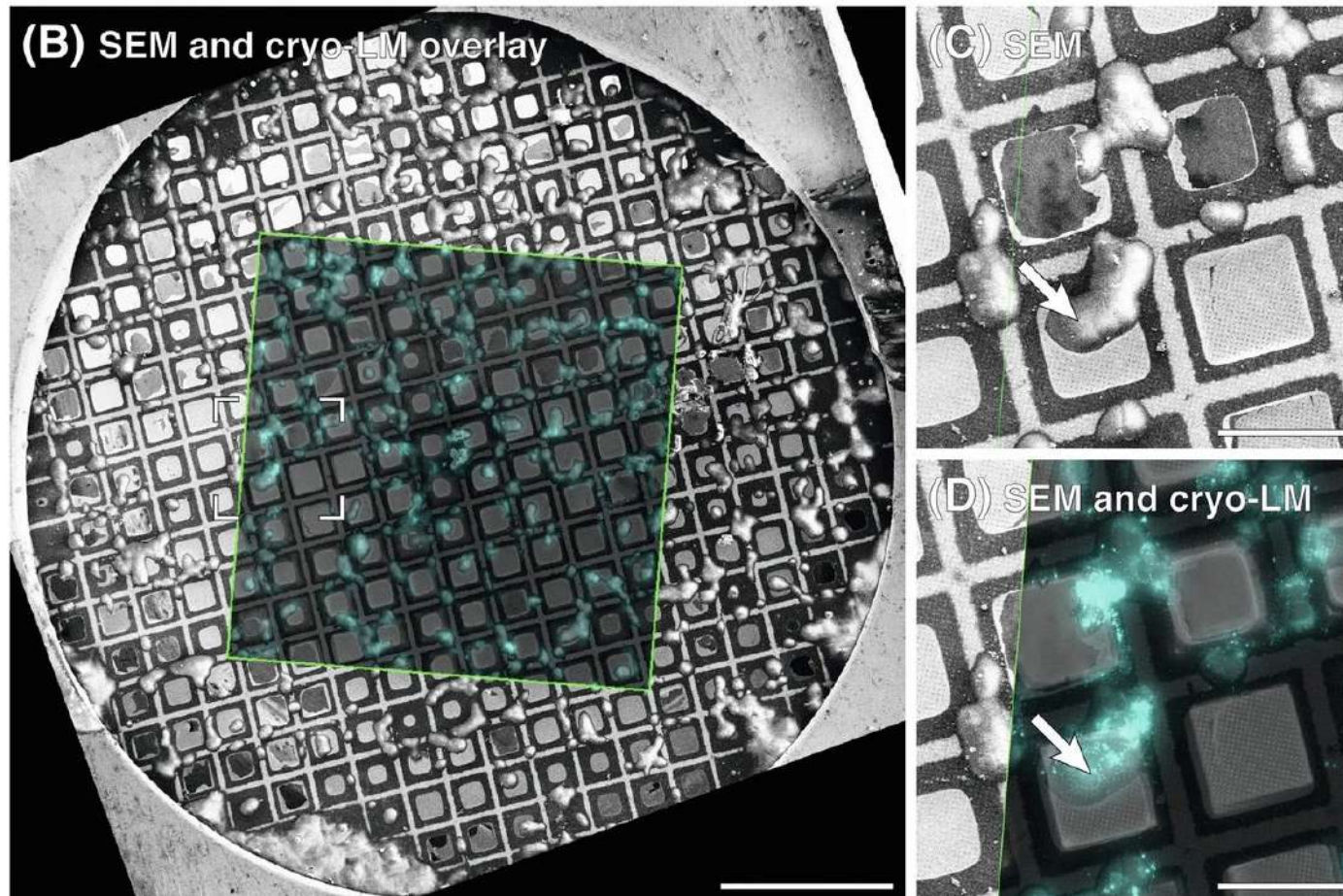
- ▶ Unless all cells are the same, you need to be able to determine which are the target cells
- ▶ Also which part of the cell to keep
- ▶ Solution: Another microscope!
  - ▶ Fluorescent light microscopes with cryo stages are available
  - ▶ Need to have a long working distance, cannot use oil immersion, 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



# 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
  - ▶ 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



# References

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