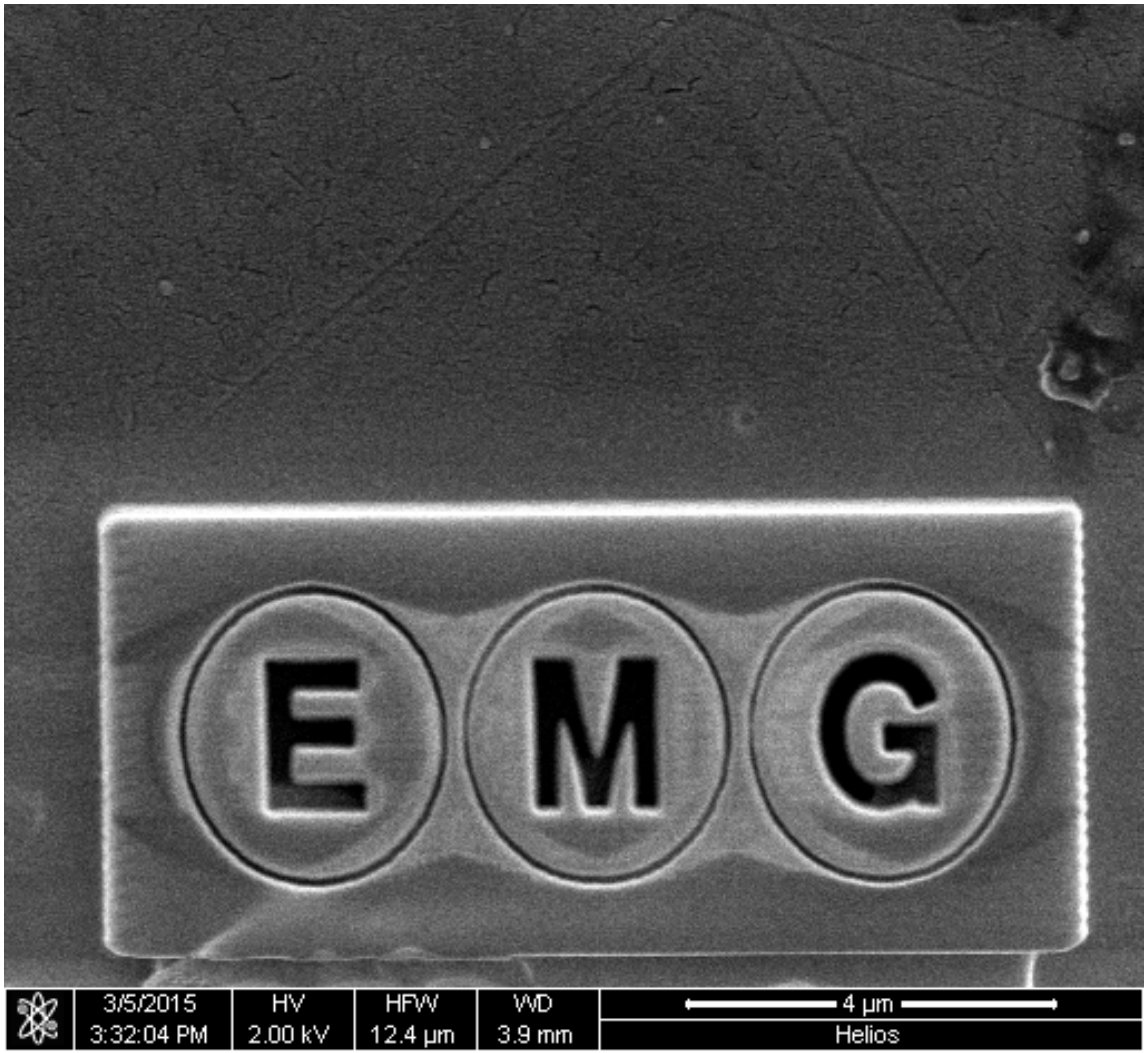
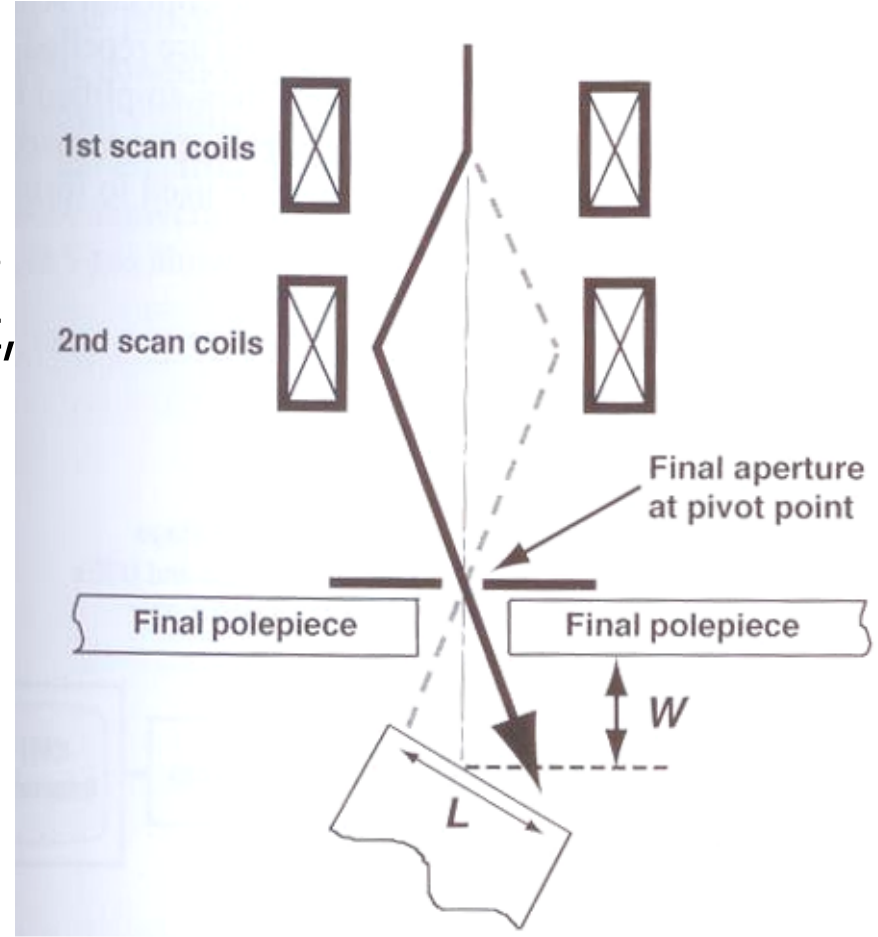
# FIB/SEM
# Feb. 13, 2017



# SEM Basics

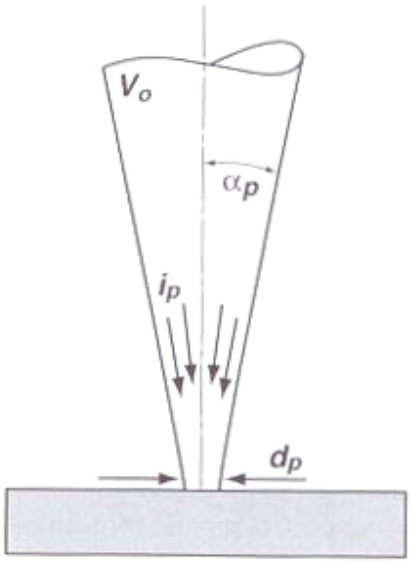
- Electron probe is focused to a sharp point
- The probe is scanned across the specimen point by point, with each point producing signal
- Scan coils deflect beam to move across sample




Goldstein et al, 2003

# SEM Beam: probe size

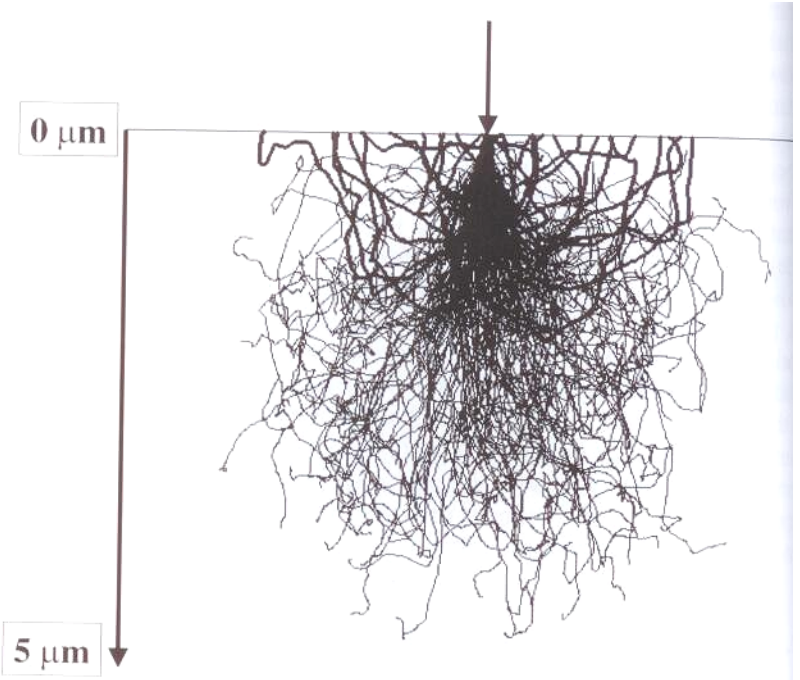
- Ideally want as small a probe as possible, relative to pixel size
- Probe size is determined by voltage, current, divergence angle
- Lens distortions
  - Spherical aberration (focus different at center and edge of lens) – instrument dependent
  - Aperture diffraction
  - Astigmatism (user correctable)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




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

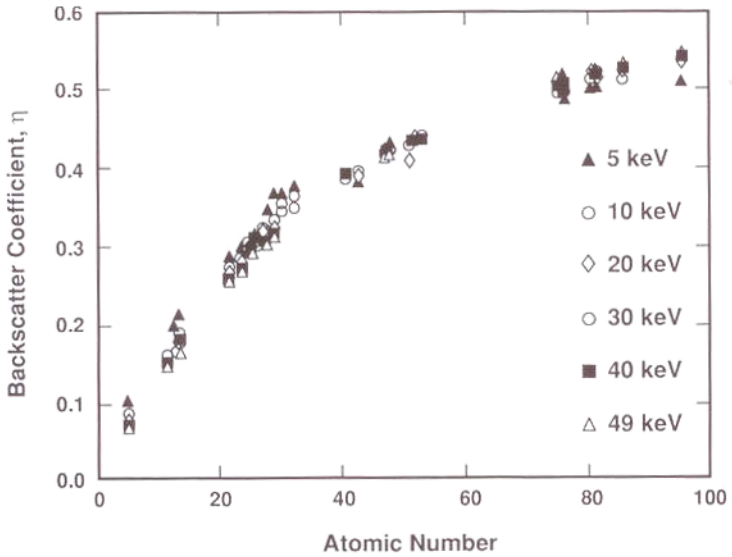



Goldstein et al, 2003

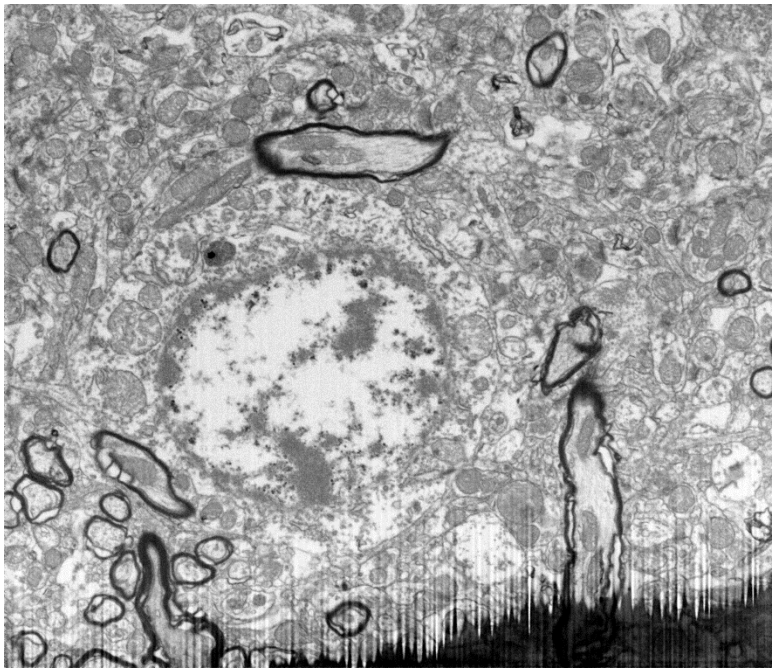
# BSE's and SE's

# BSE efficiency is material dependent, voltage independent

Fraction of
e's that backscatter

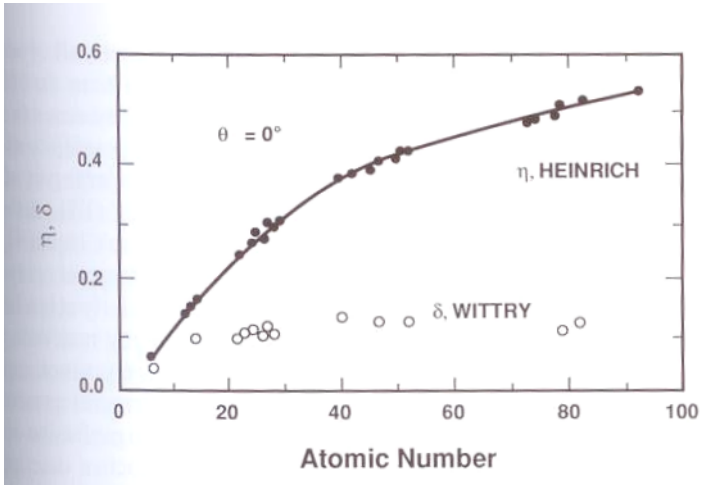



Goldstein et al, 2003



BSE's give contrast between light and heavy elements

# Specimen Dependence of BSE, SE

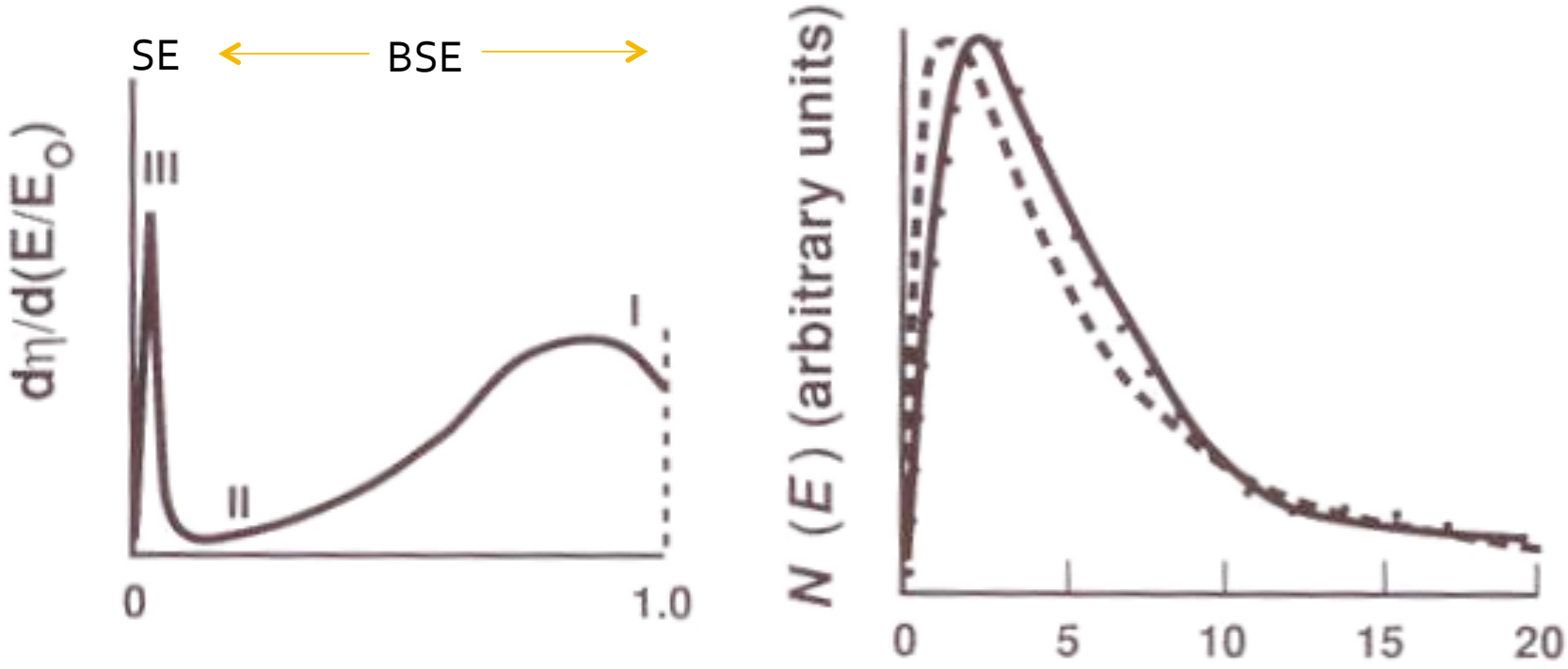



30 keV

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

Goldstein et al, 2003

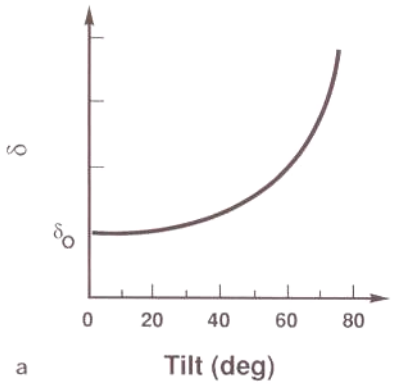
# Energies of SE and BSE



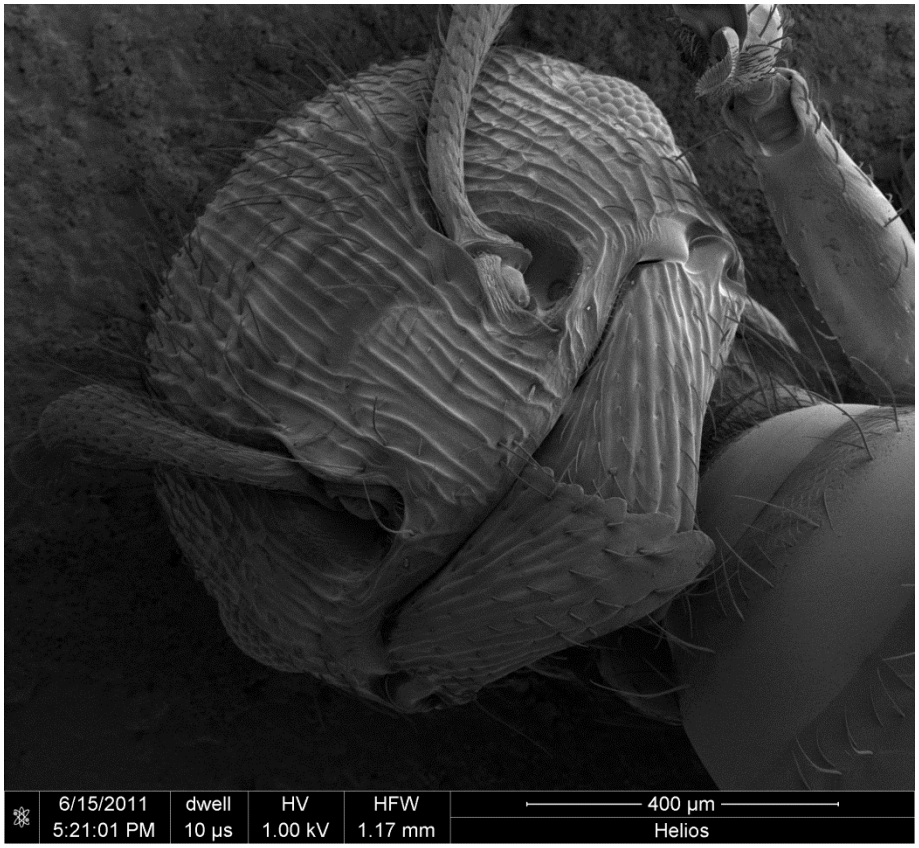

SE's have much lower energy (majority < 20 eV)

Goldstein et al, 2003

# Angular Dependence of SE's



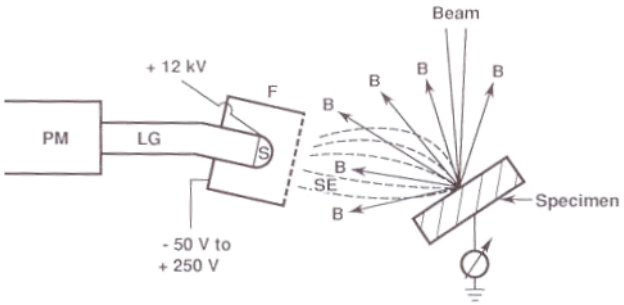

Goldstein et al, 2003




SE's also give topographic information

# Detection of BSE's, SE's

# Everhart-Thornley (ET) Detector

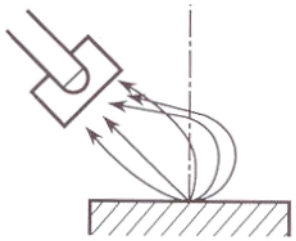



Electrons strike scintillator, releasing photons
Photons travel to photomultiplier tube
Eventually converted to electric signal, storing intensity values
Combined SE/BSE detector

# Apply bias

- Bias can be applied to detector, directing electrons toward or away from it
  - Negative bias (< -50 V): detect only BSE's
  - Positive bias: collect more SE's, indirect BSE's : greater total signal

Positive bias



Goldstein et al, 2003

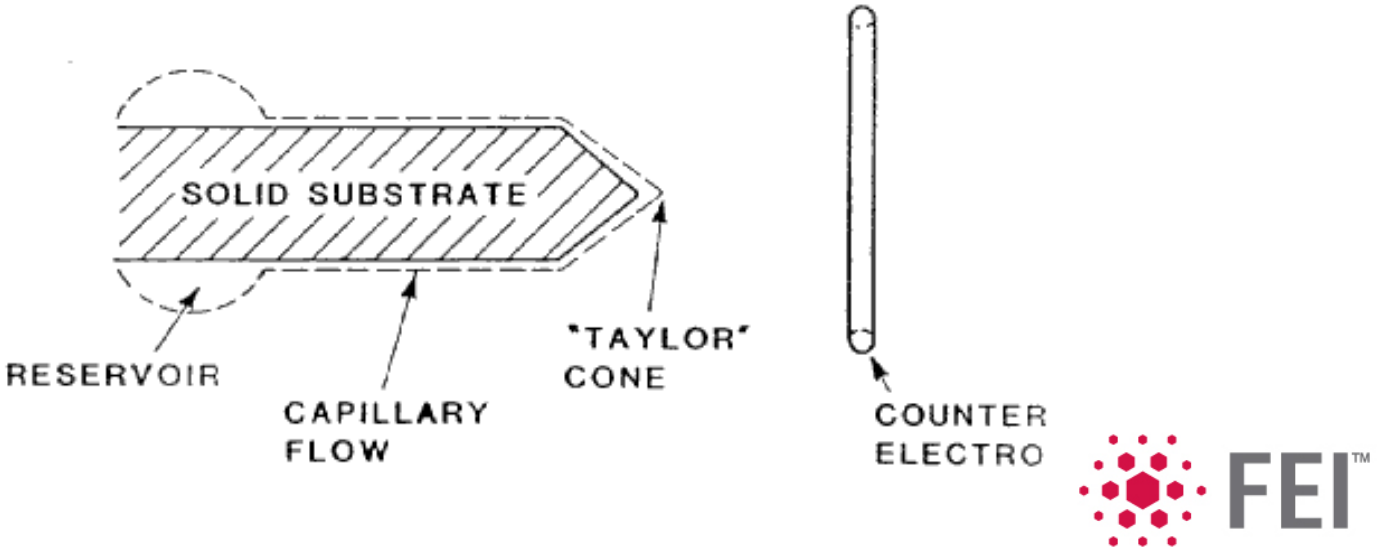
# SEM summary

- Images formed by scanning points across sample
- For higher resolution, want to minimize both probe size and interaction volume
  - Low voltage operation
  - But still need enough signal for detection
- Use Through-lens detector for higher resolution imaging
- Apply negative bias to detect mainly BSE's

# FIB Operation

# Basic Mechanism

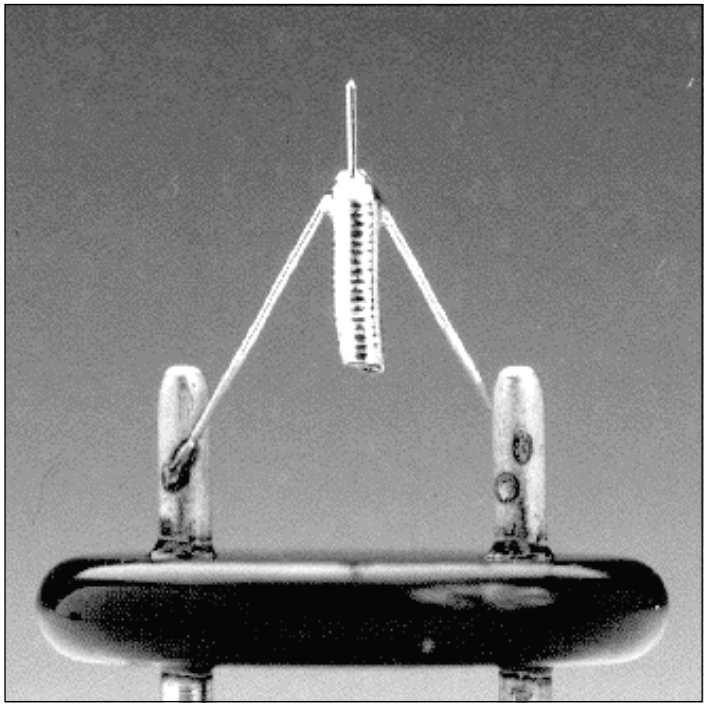
- Liquid Flow from Reservoir
- Ion Formation
- External Beam Interactions




FEI™

# 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



FEI™

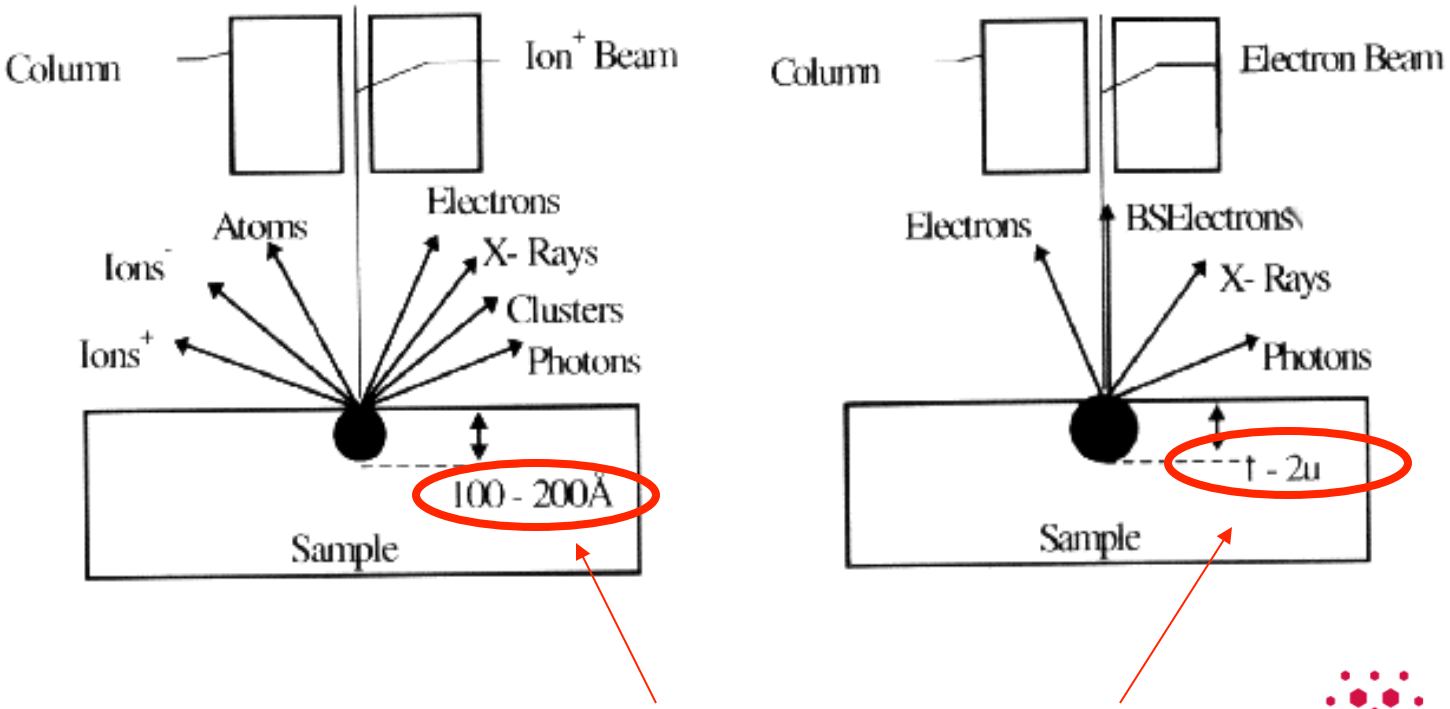
# Ion Column

- 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

FEI™

# Using the System

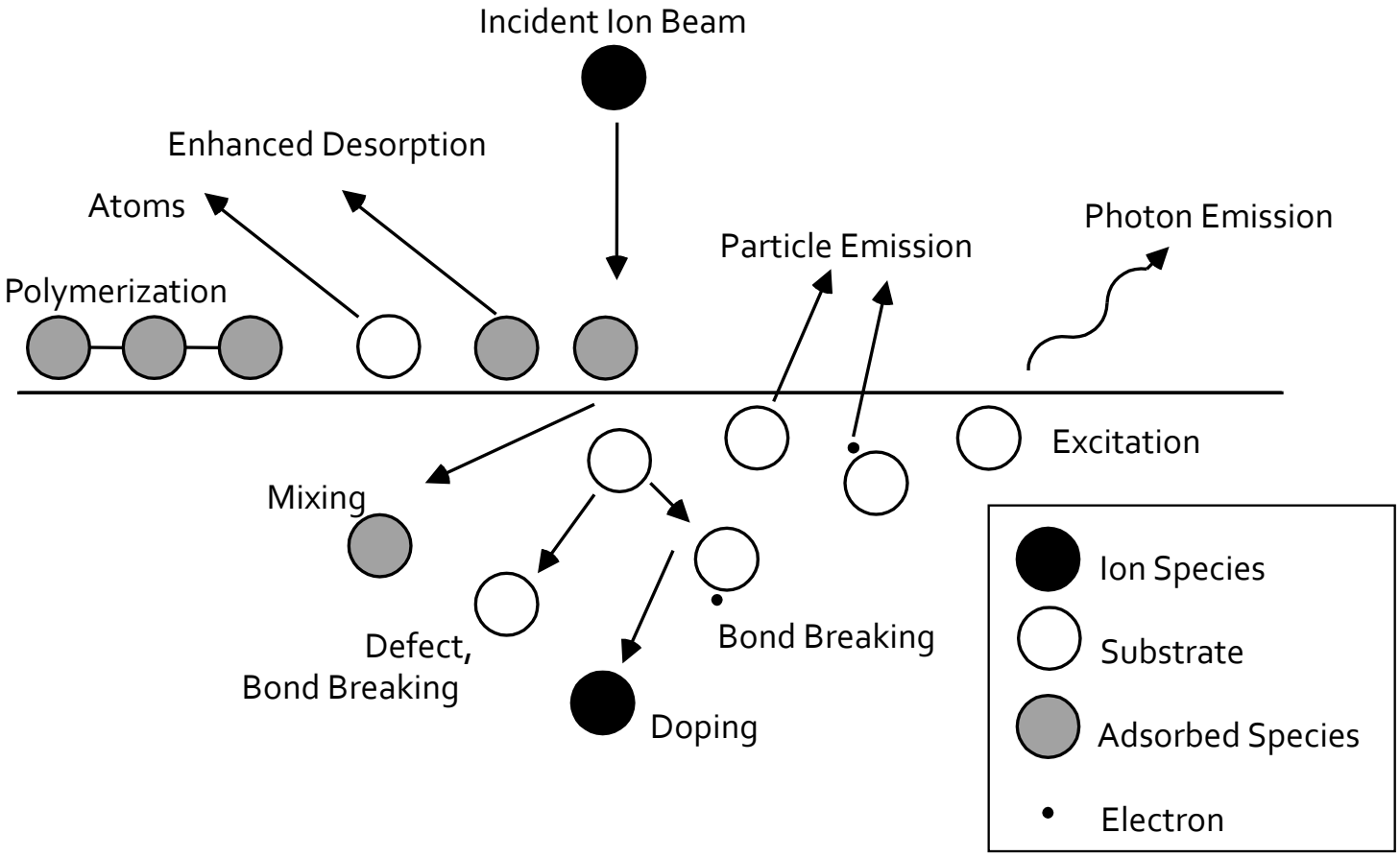
- Beam Interactions




*Note difference in interaction volume*

FEI™

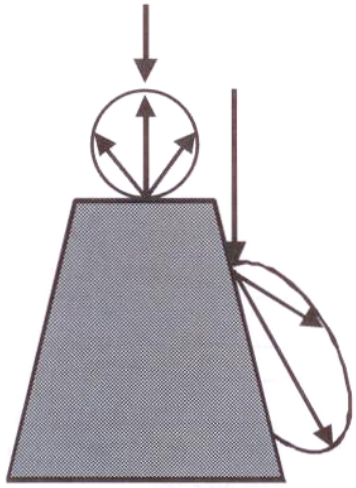
# Ion Beam to Sample Interactions




FEI™

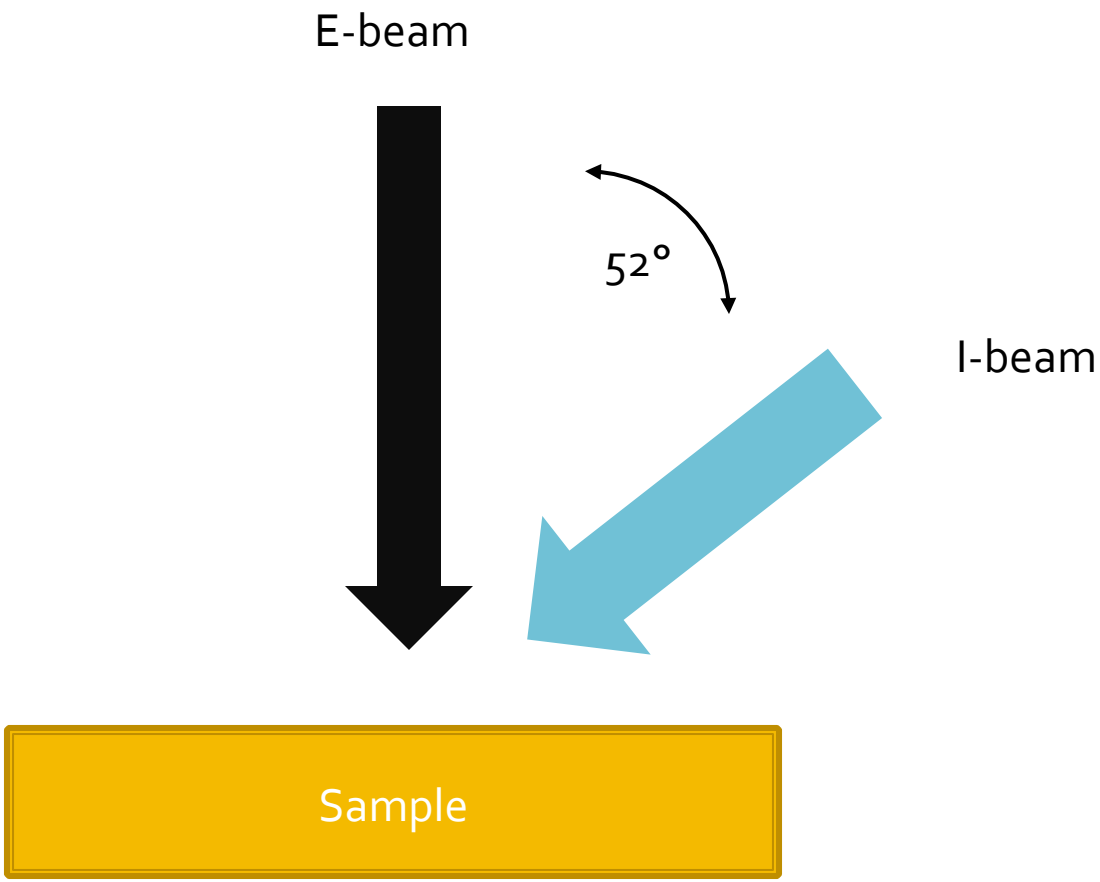
# Sputtered Particles

Sputtered Particle Ejection Behavior



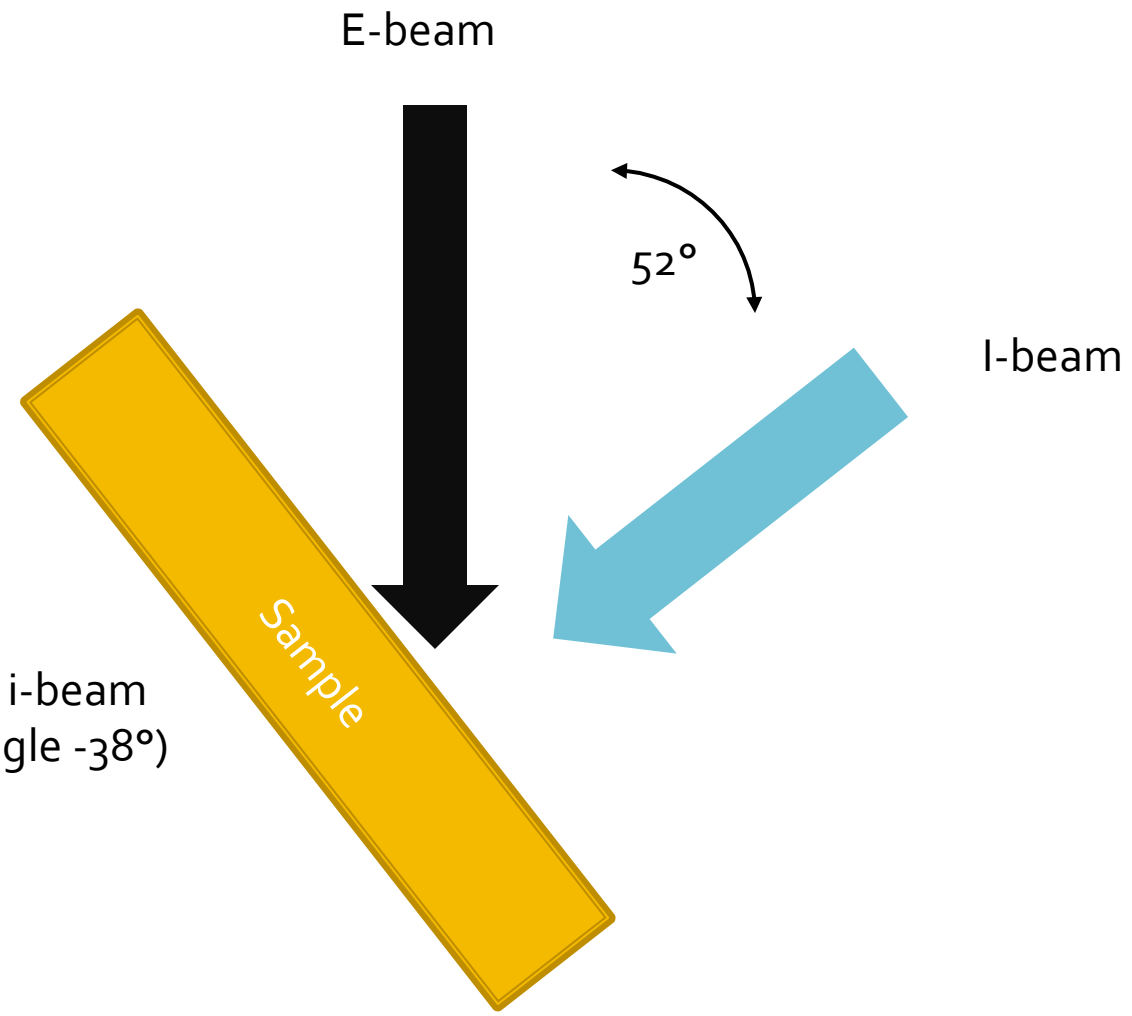
FEI™

# Geometry




Sample:
Tilt from -10° to +70°

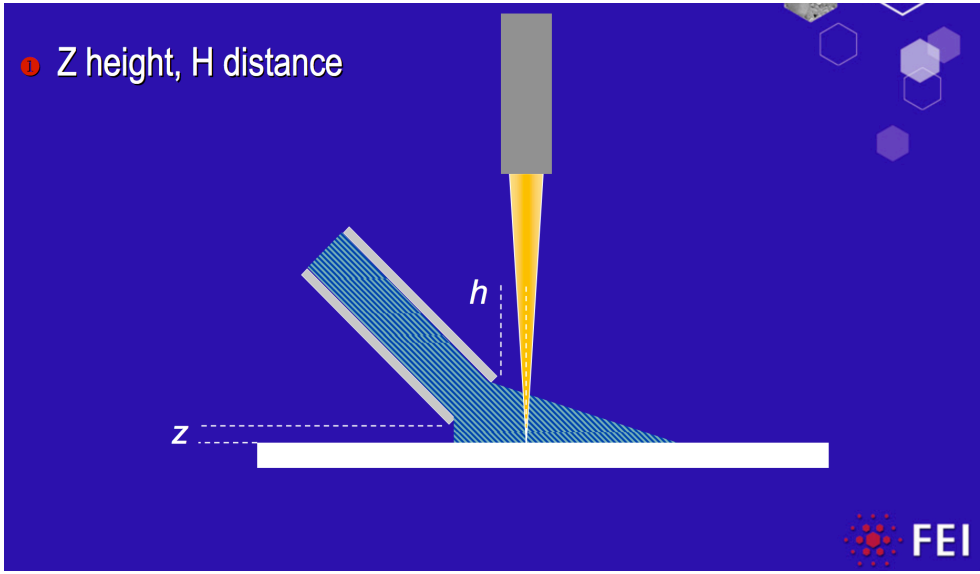
# Geometry




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

# Deposition

- (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



# Applications to Resin-embedded tissue

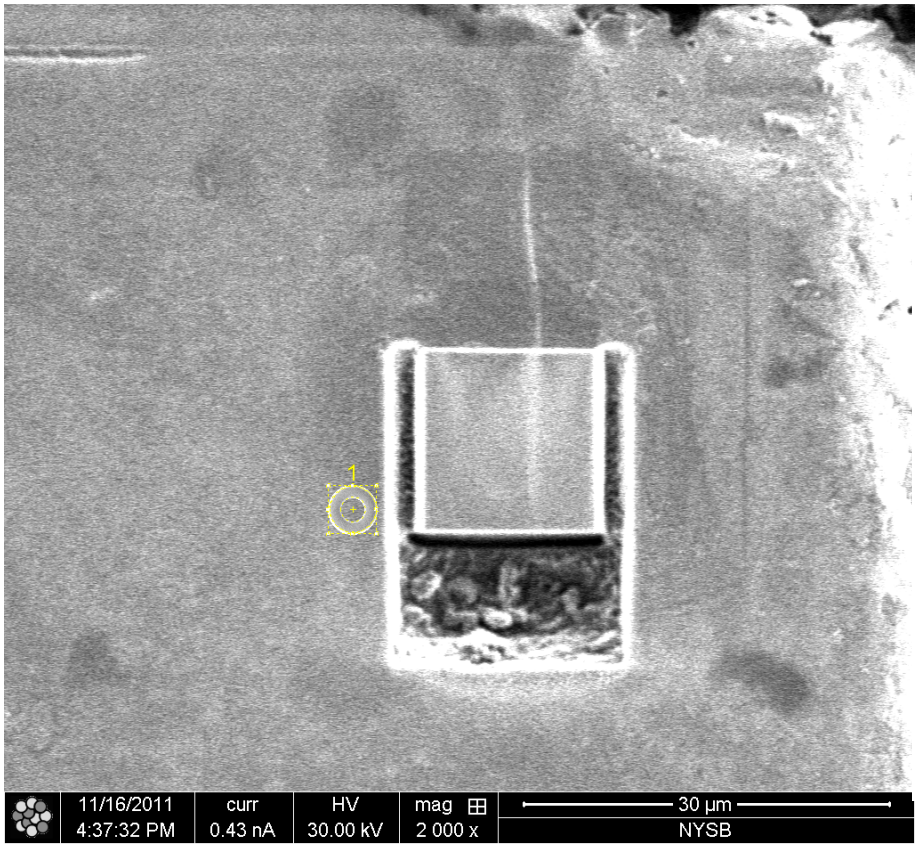
# Tissue or Cells

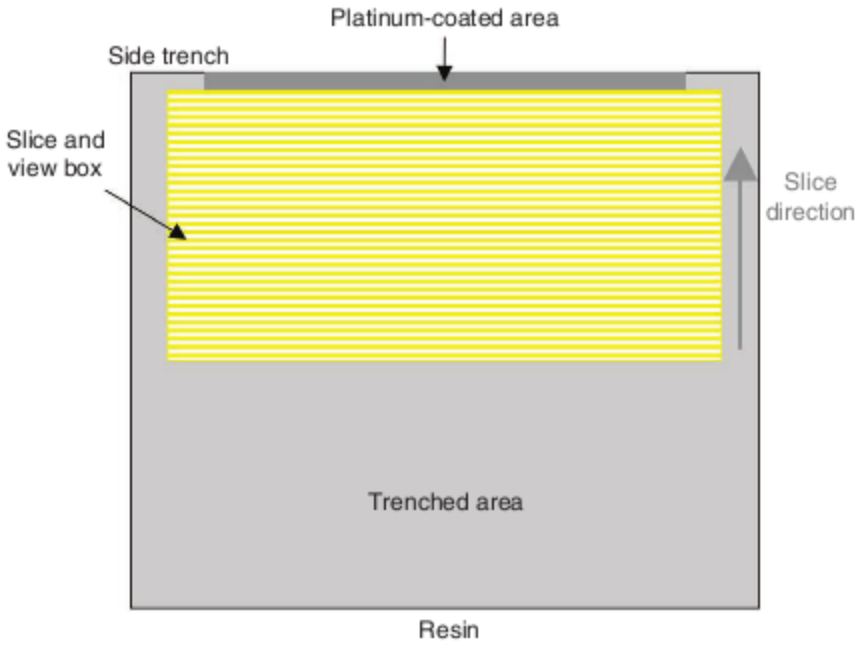
- Sample Prep
  - High Pressure frozen (optional)
  - Chemically fixed, freeze substituted
  - Resin embedded
  - En bloc staining
    - $OsO_4$, U Ac, Pb citrate
    - Osmium impregnation (OTO)
    - Want to make samples more conductive, more heavily stained
  - Thin conductive layer (C, Pt, Au-Pd) coated just before insertion

# Imaging conditions

- Low voltage (2 keV or less)
  - Want to image only the surface
  - Minimal depth penetration (slice as thin as 10 nm)
  - No topographic information
  - Elemental contrast (C vs Os)
  - Through-lens detector for highest resolution
  - BSE mode (positive bias)
  - Stained parts will show up as bright on dark

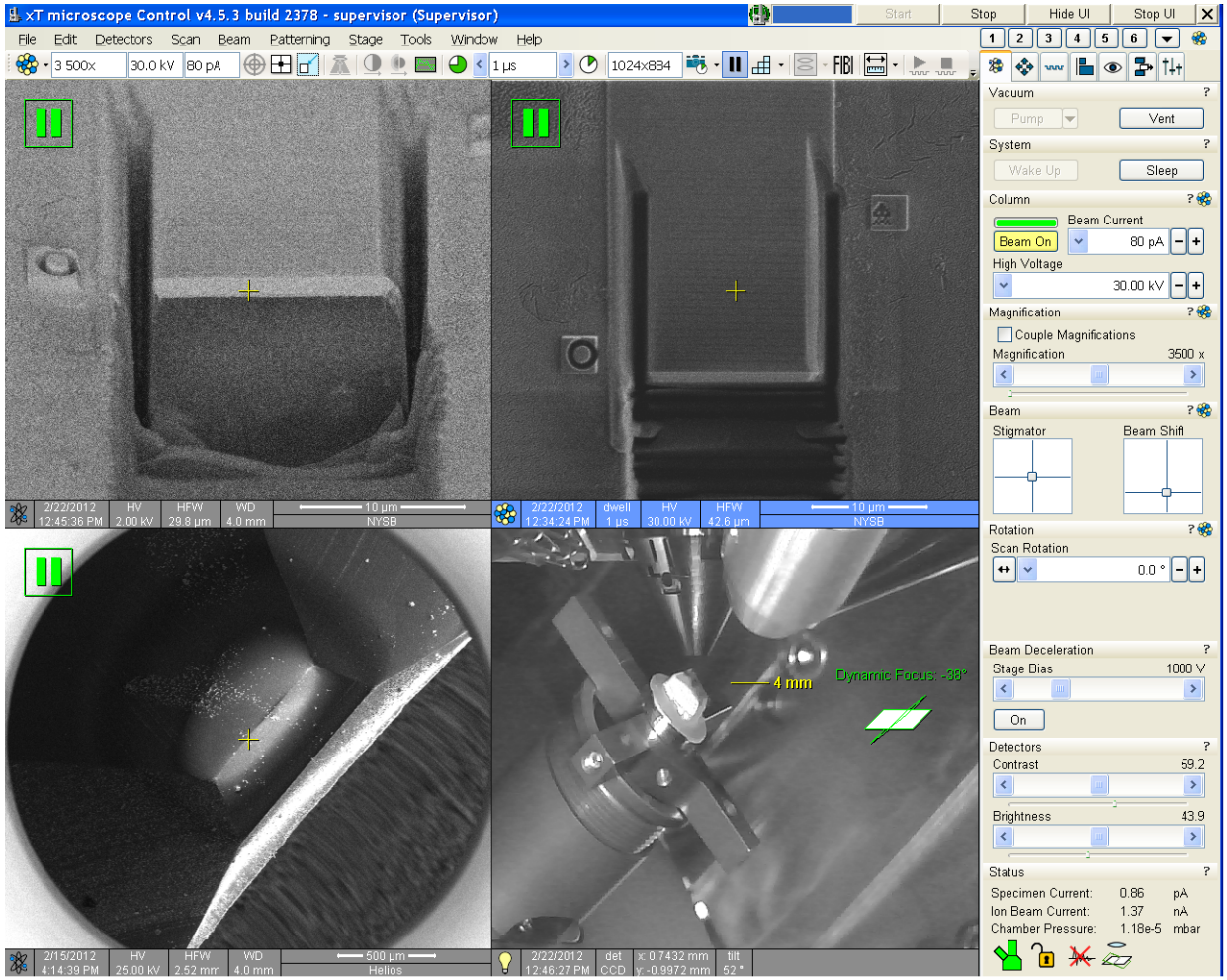
# Milling Samples



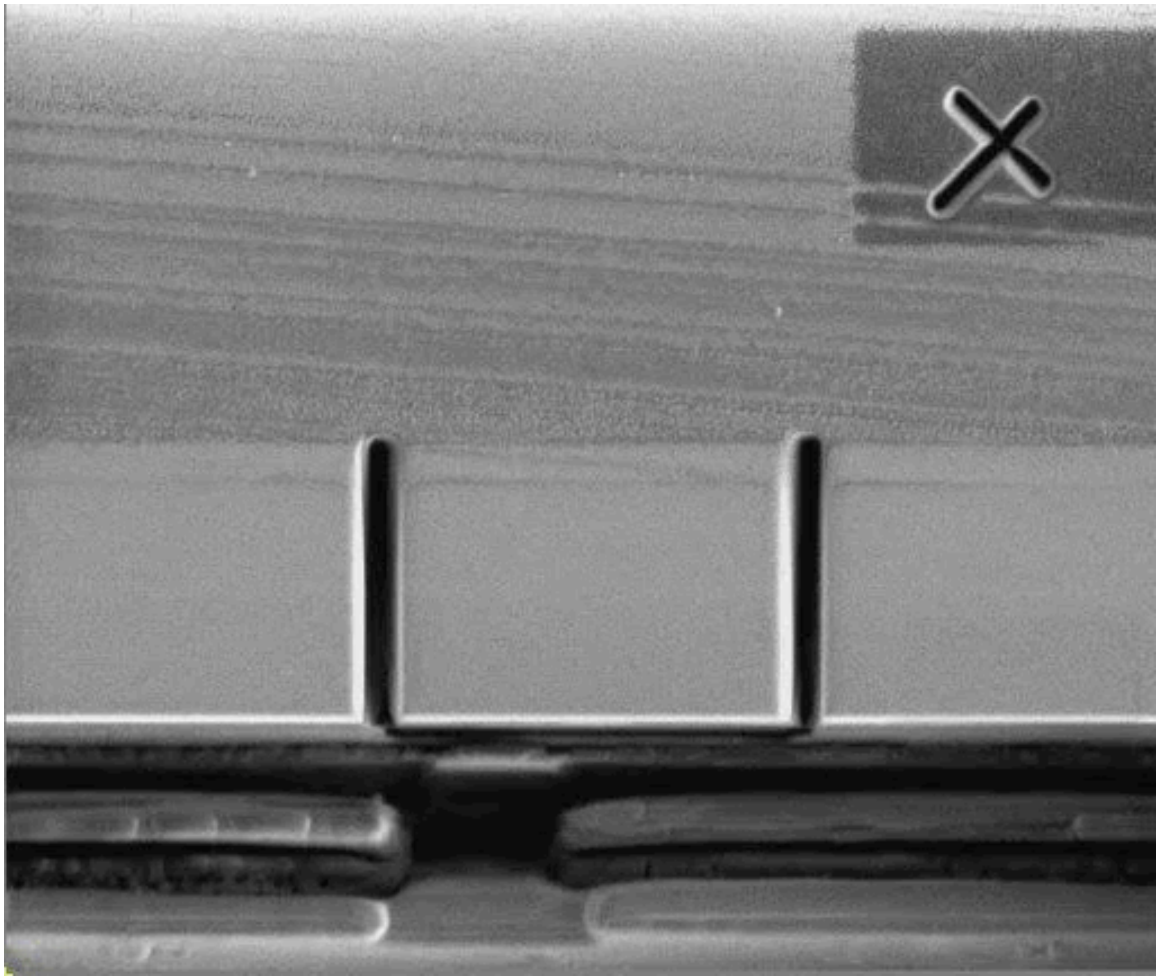




Bushby et al, 2011

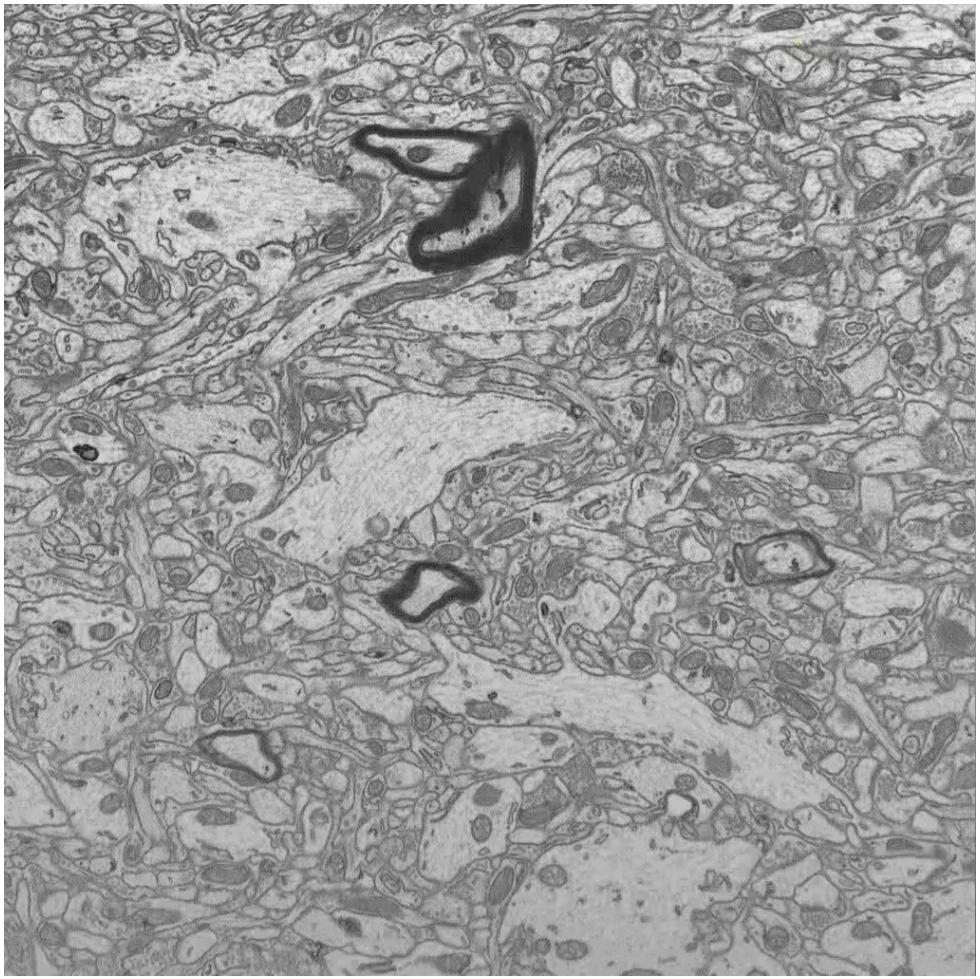
# Set up for Slice and View



# Milling: i-beam view



# Example Movie: Neural Tissue

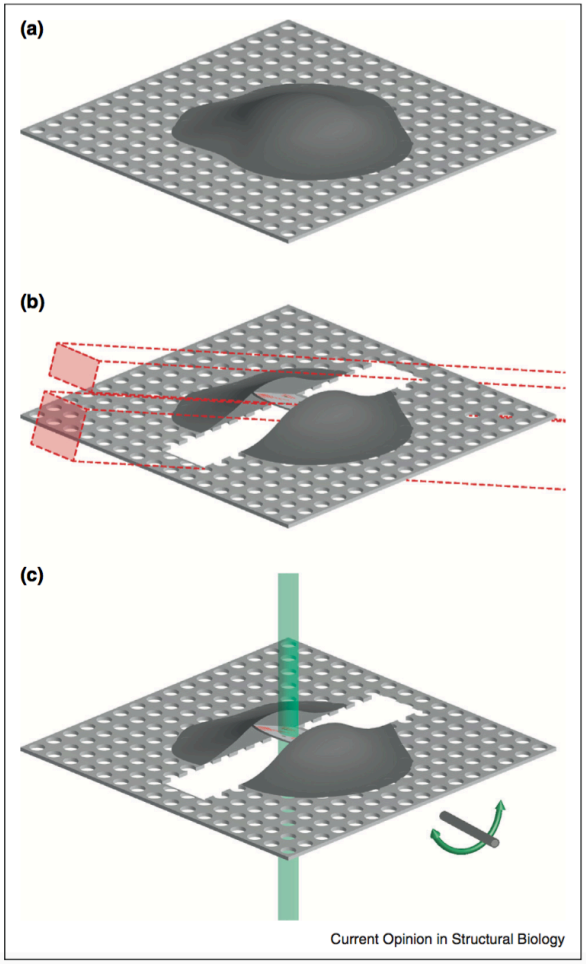


# Ideal workflow

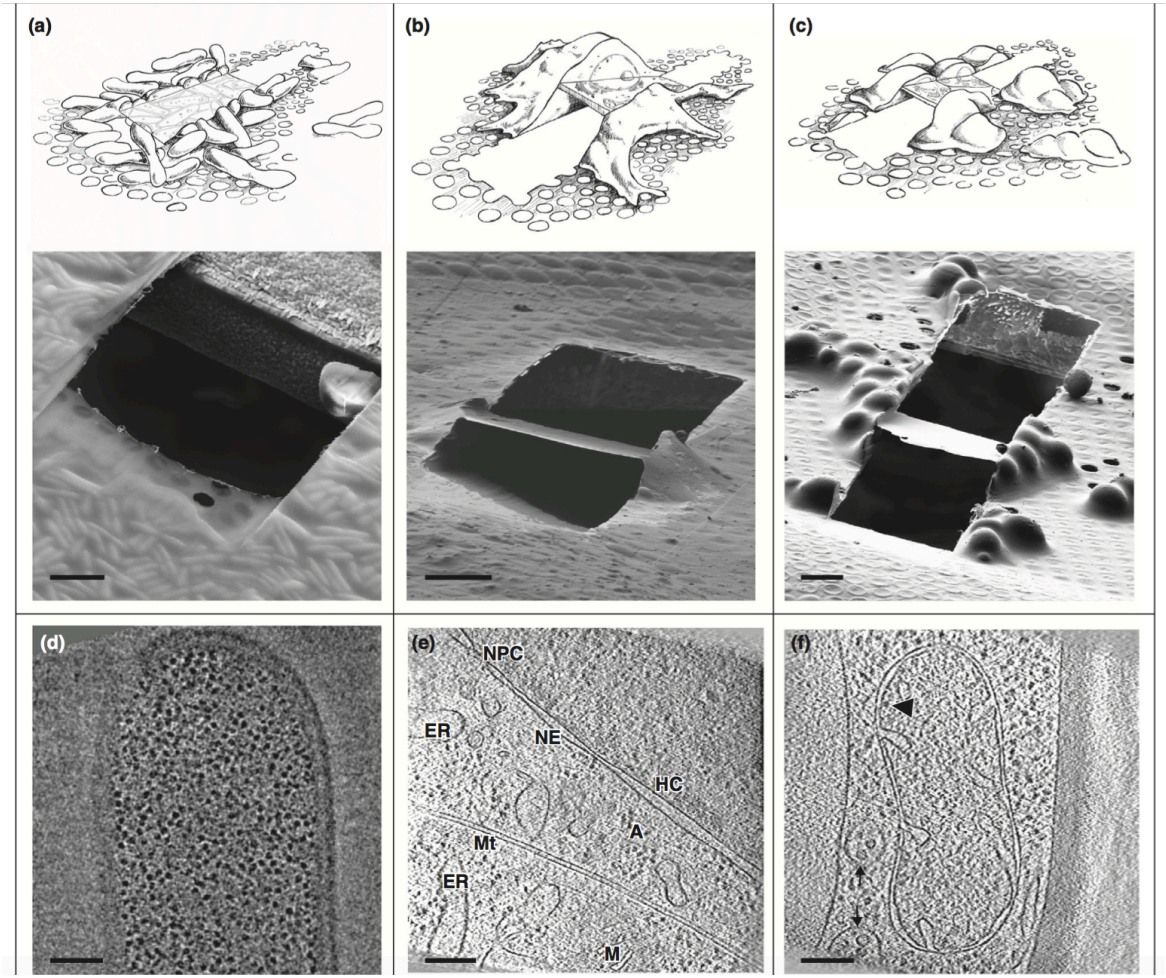
- Samples stained and embedded
- Thin slice for overall map – make easier to find features (LM or ultrathin EM)
- Face of block polished
- Set up for slice and view (1 day)
- Collect slices (1-5 days)
- Align, process (IMOD, Amira) (1 day)
- Segmentation (IMOD, Amira) (weeks-months)

# FIB/SEM for Cryo Prep

# Mill a thin slice through a cell



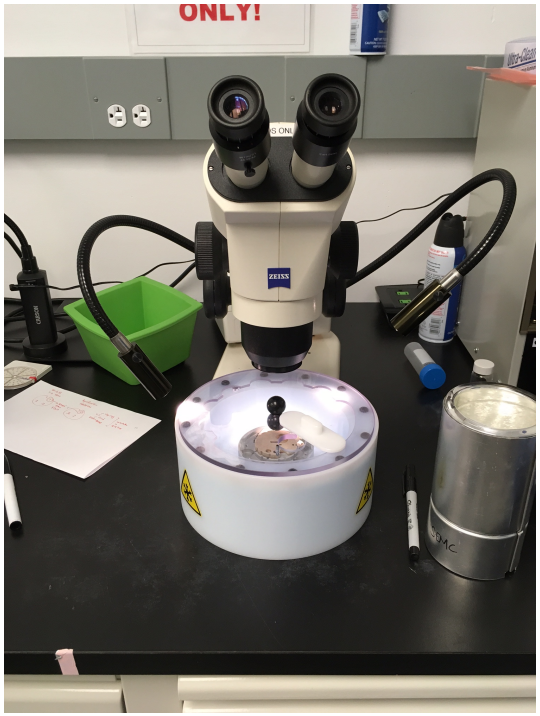

1: Villa E, Schaffer M, Plitzko JM, Baumeister W. Opening windows into the cell: focused-ion-beam milling for cryo-electron tomography. Curr Opin Struct Biol. 2013 Oct;23(5):771-7. doi: 10.1016/j.sbi.2013.08.006. Review. PubMed PMID: 24090931.




1: Villa E, Schaffer M, Plitzko JM, Baumeister W. Opening windows into the cell: focused-ion-beam milling for cryo-electron tomography. Curr Opin Struct Biol. 2013 Oct;23(5):771-7. doi: 10.1016/j.sbi.2013.08.006. Review. PubMed PMID: 24090931.

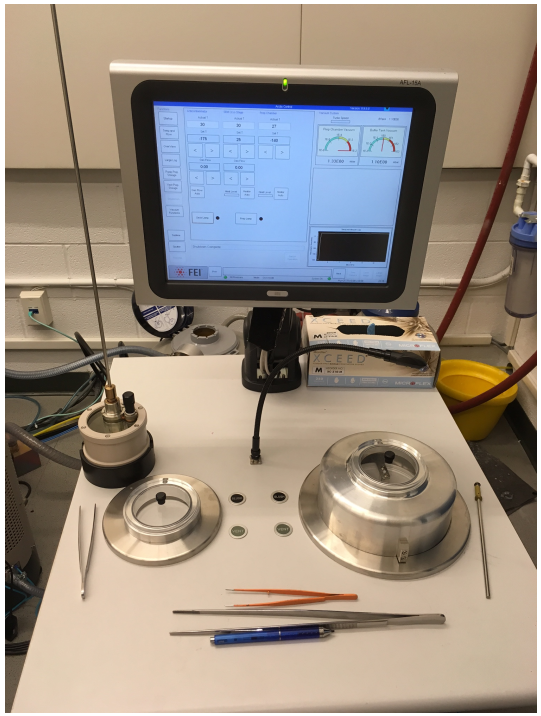
# Setup for standard Lamellae Preparation

FEI autogrid loading station with stereo microscope



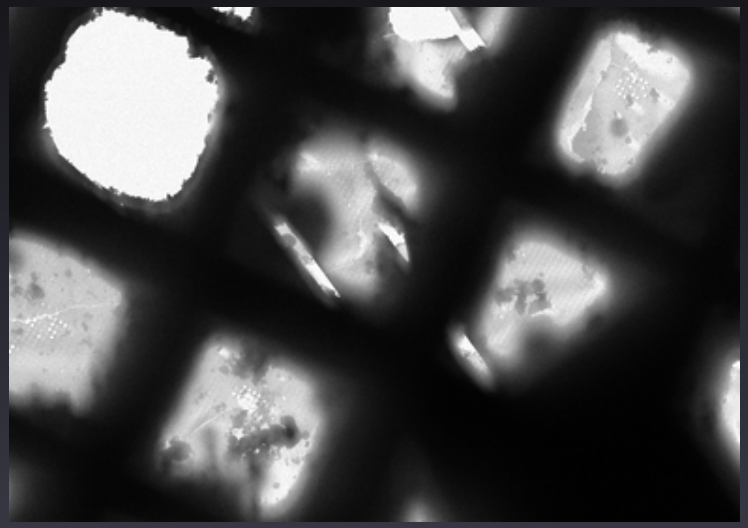

Shuttle with 2 grids

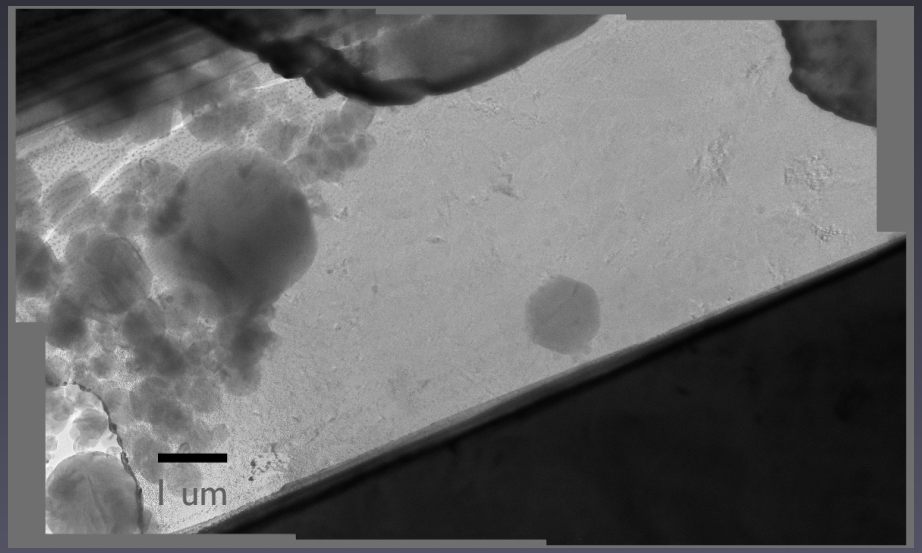
Quorum cryo loading station



# Cutting windows into cells and tissues

## Find lamella in TEM





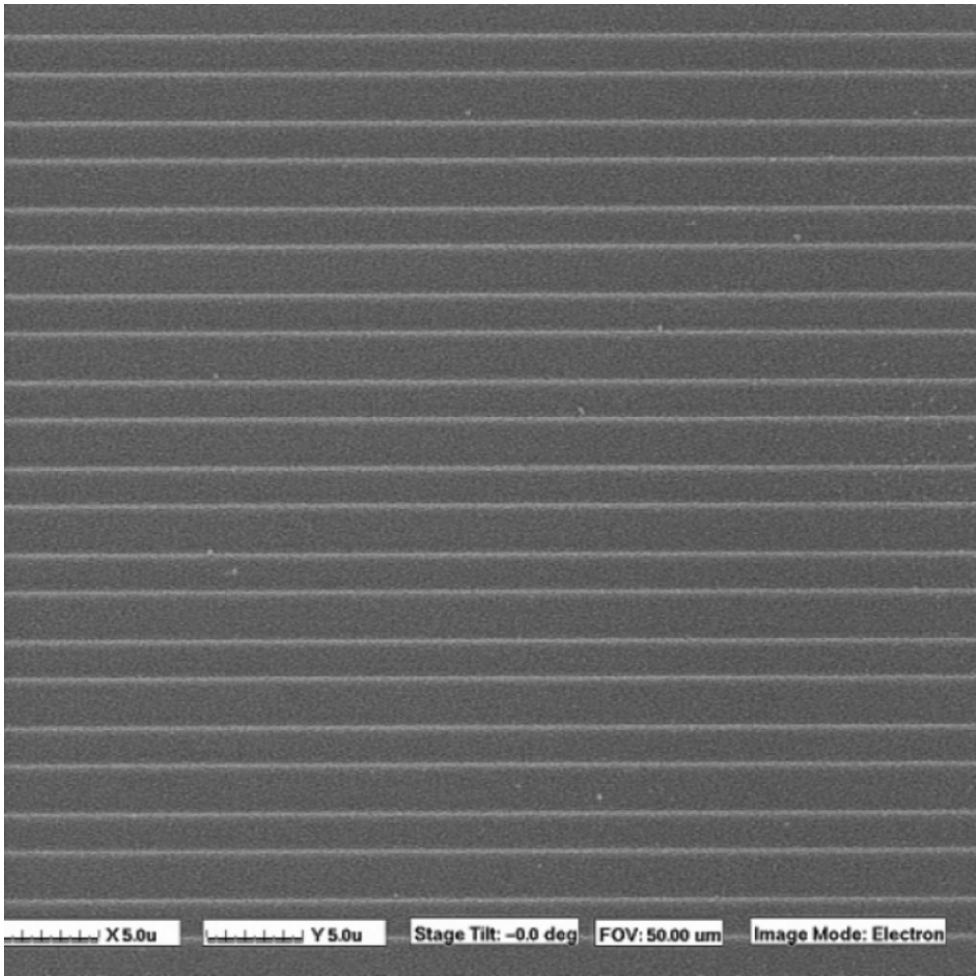
# Ideal workflow

- Cells grown on gold grid, then plunge frozen
- Image by cryo-LM to find features (1 day)
- Load into FIB/SEM, mill slices (1 day)
- Load into TEM, collect tomograms (1-2 days)
- Align, process (Protomo,IMOD) (1 day)
- Segmentation (IMOD, Amira)
- Sub-tomogram averaging

# Lift Out

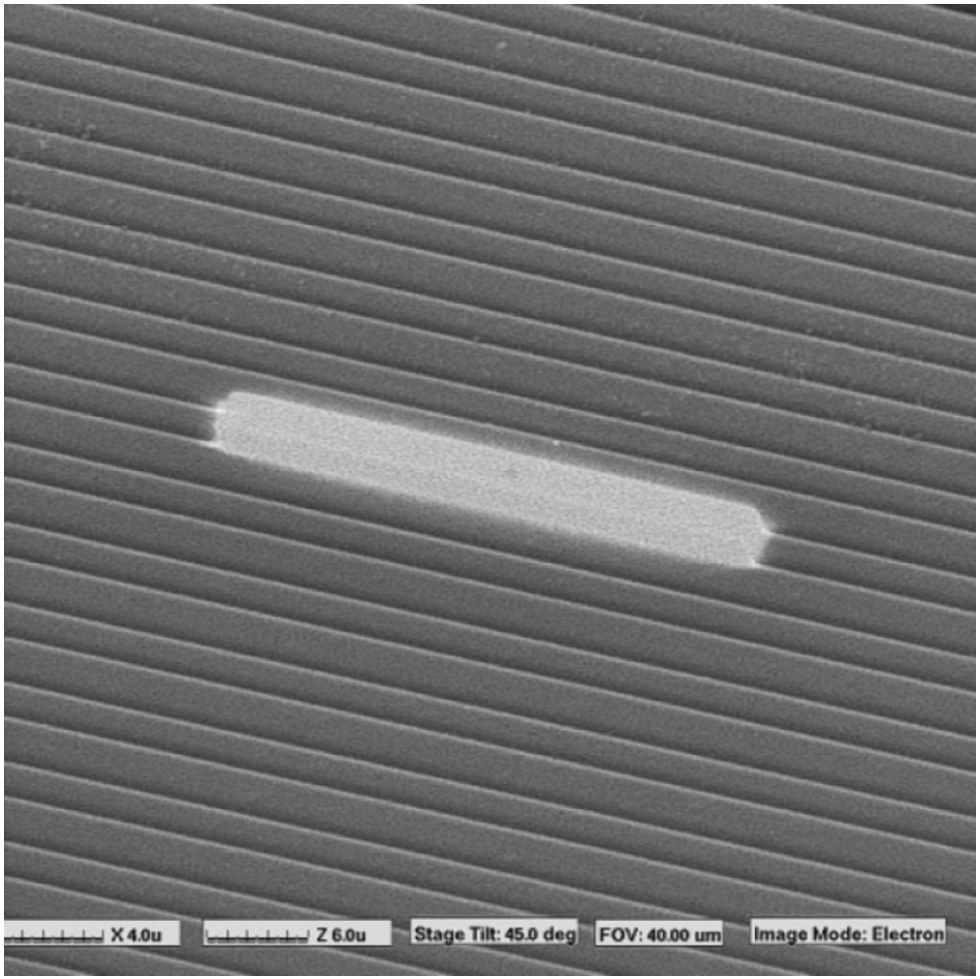
Use of FIB/SEM to prepare bulk material for TEM imaging

# Area of Interest

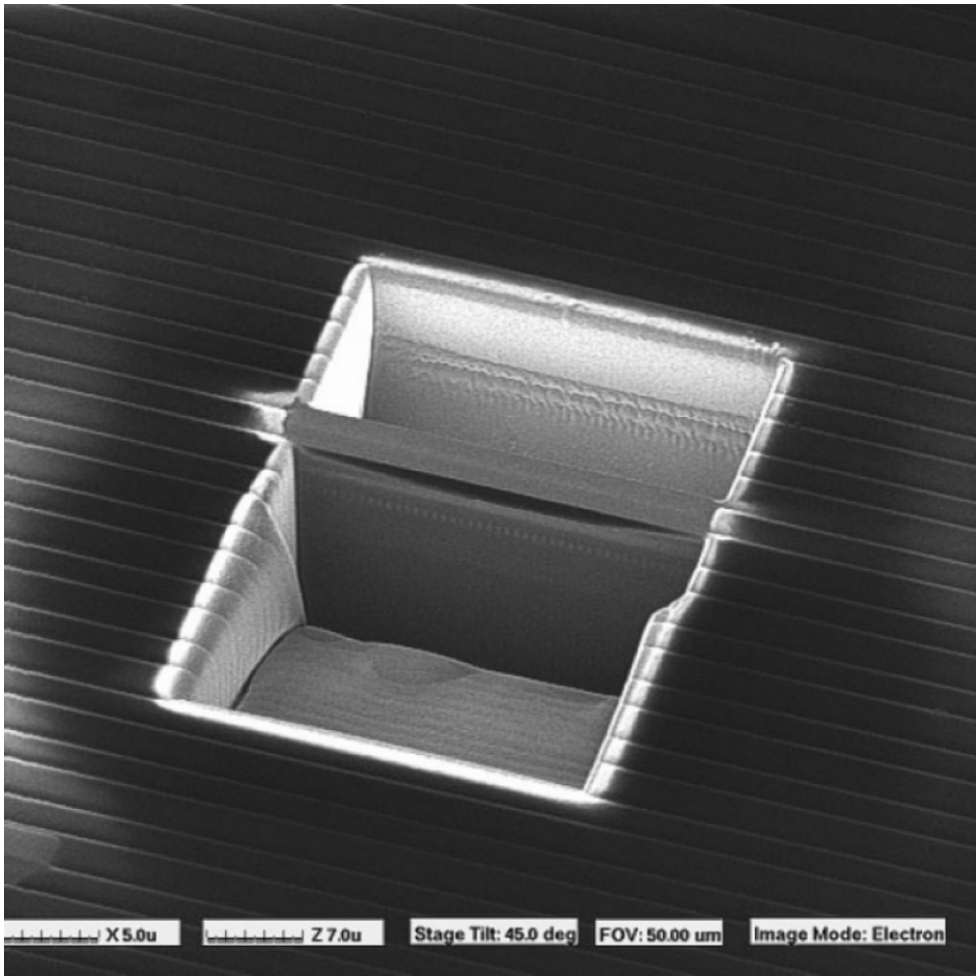



http://www.fibics.com/fib/application/steps-in-tem-specimens-preparation-by-lift-out-method-/24/

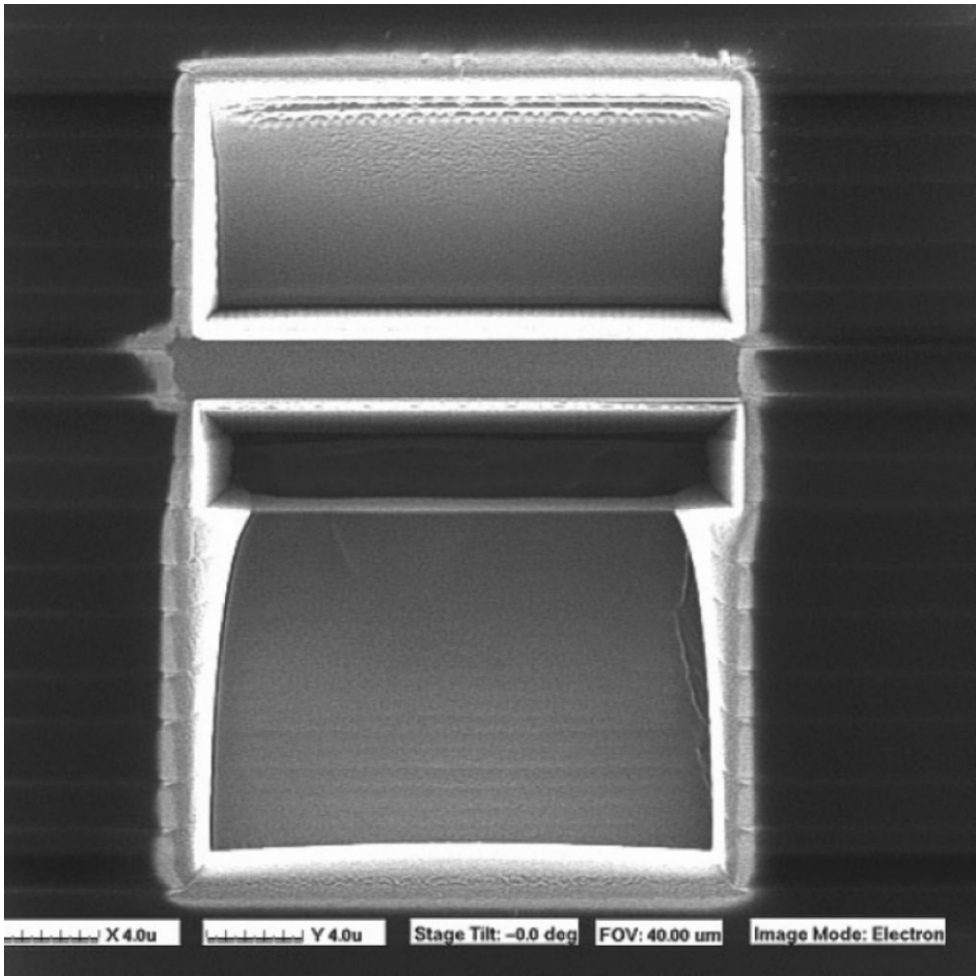
# Protect Area of Interest



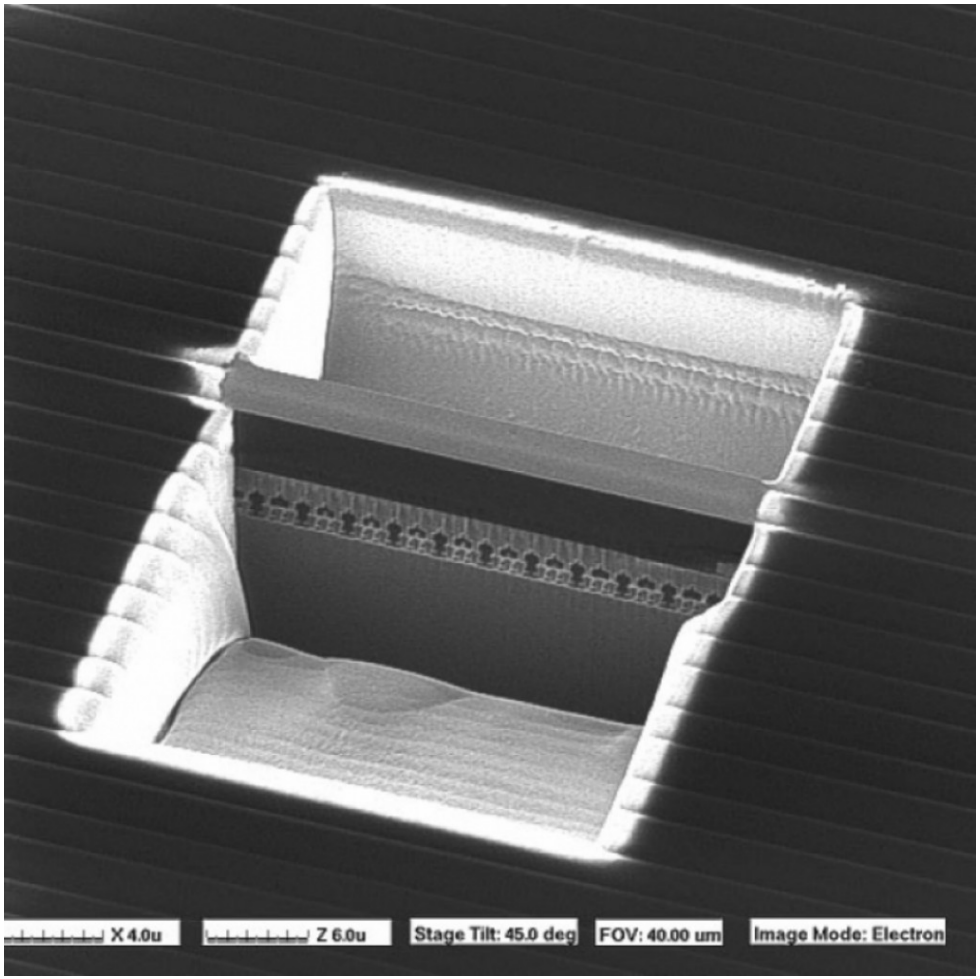
# Mill Trenches around area



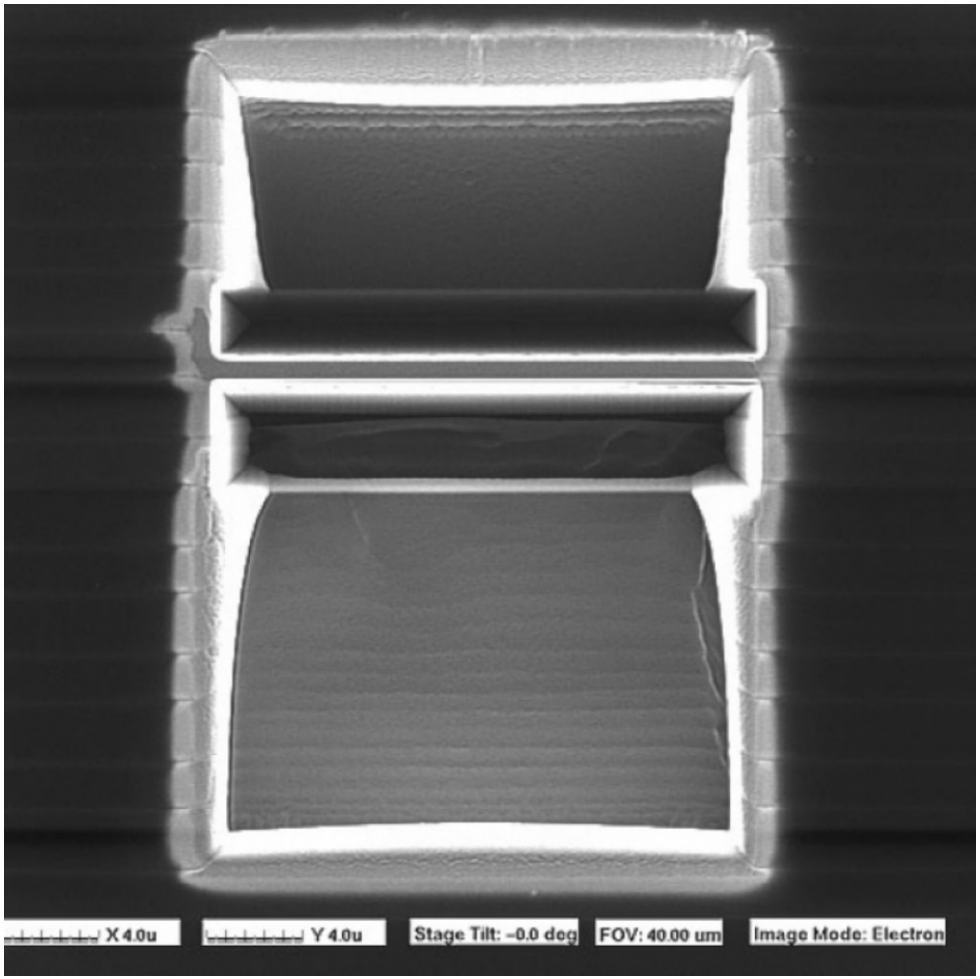
# Top View of Trench



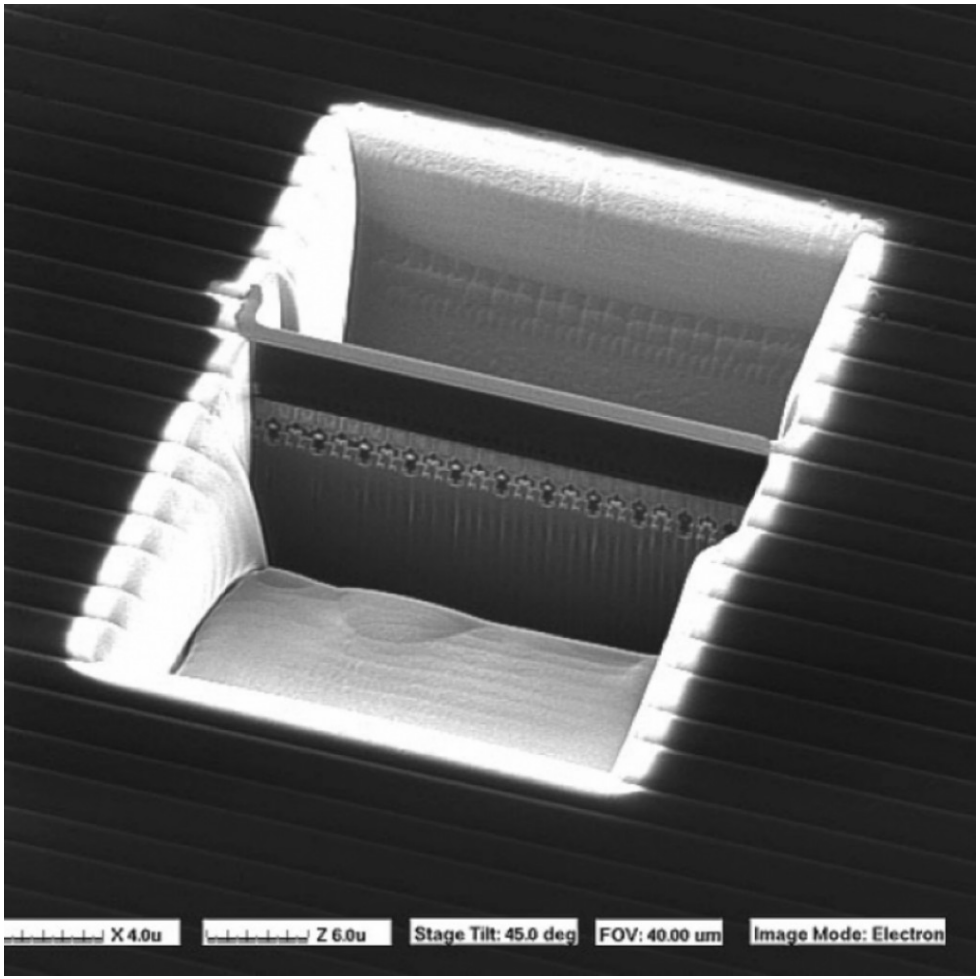
# Polish Section



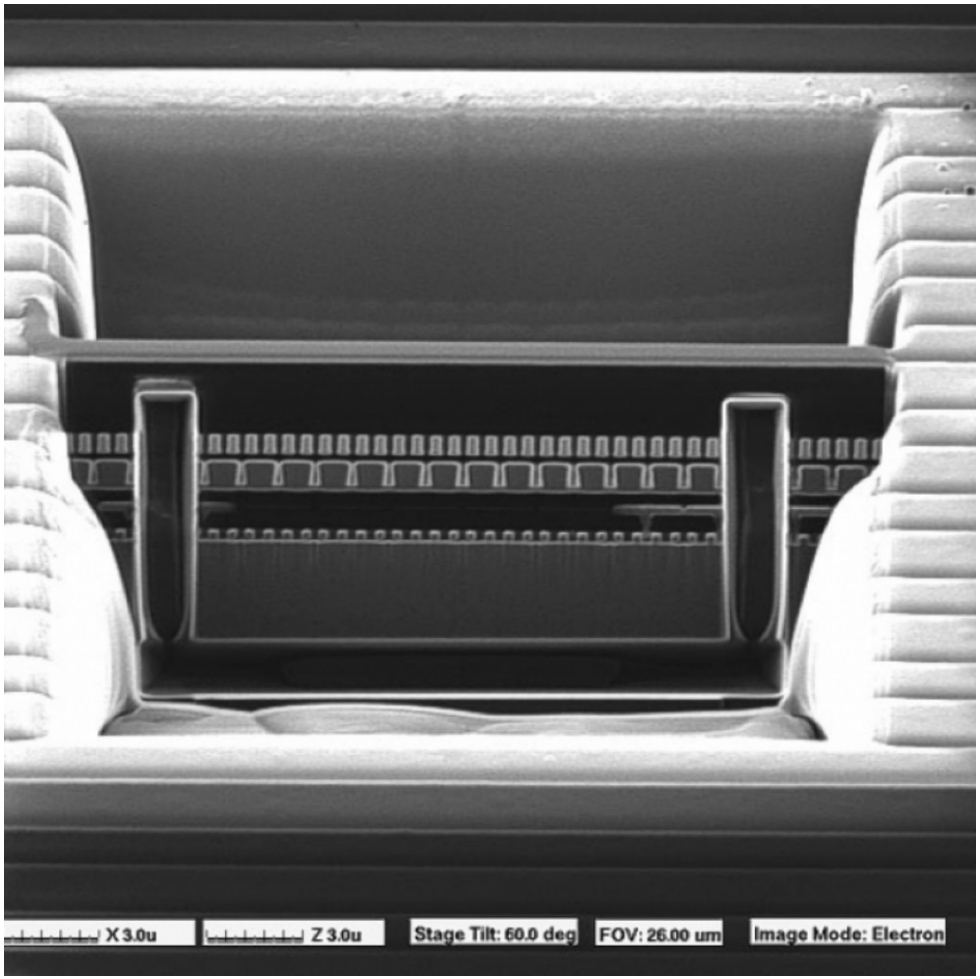
# Further Polishing



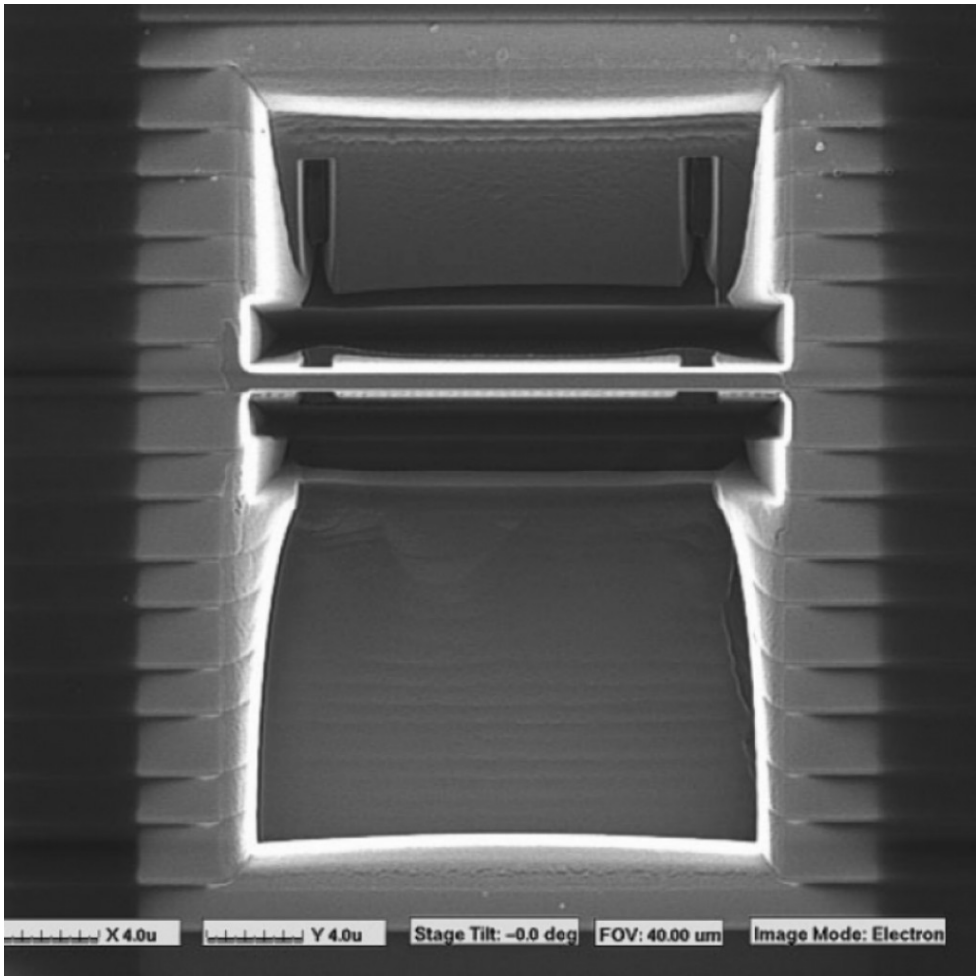
# Iso-View of Second Polish



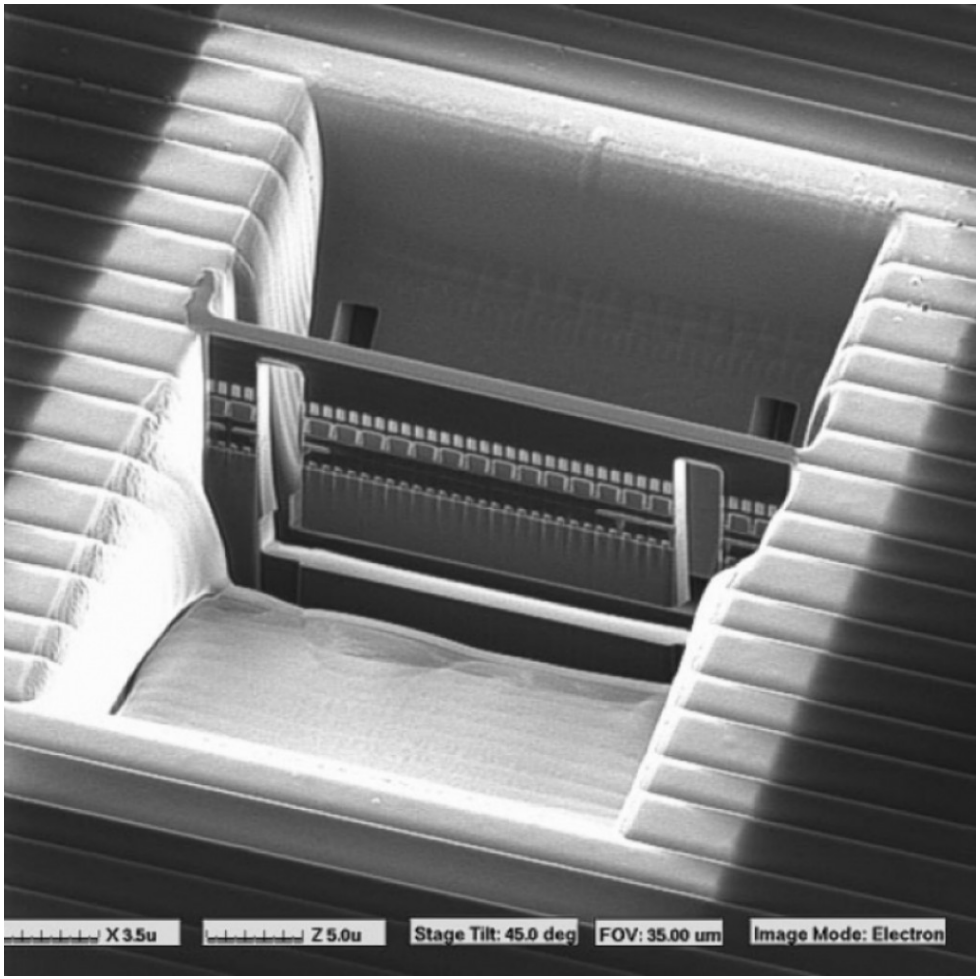
# Frame Cuts to Define Area for Removal



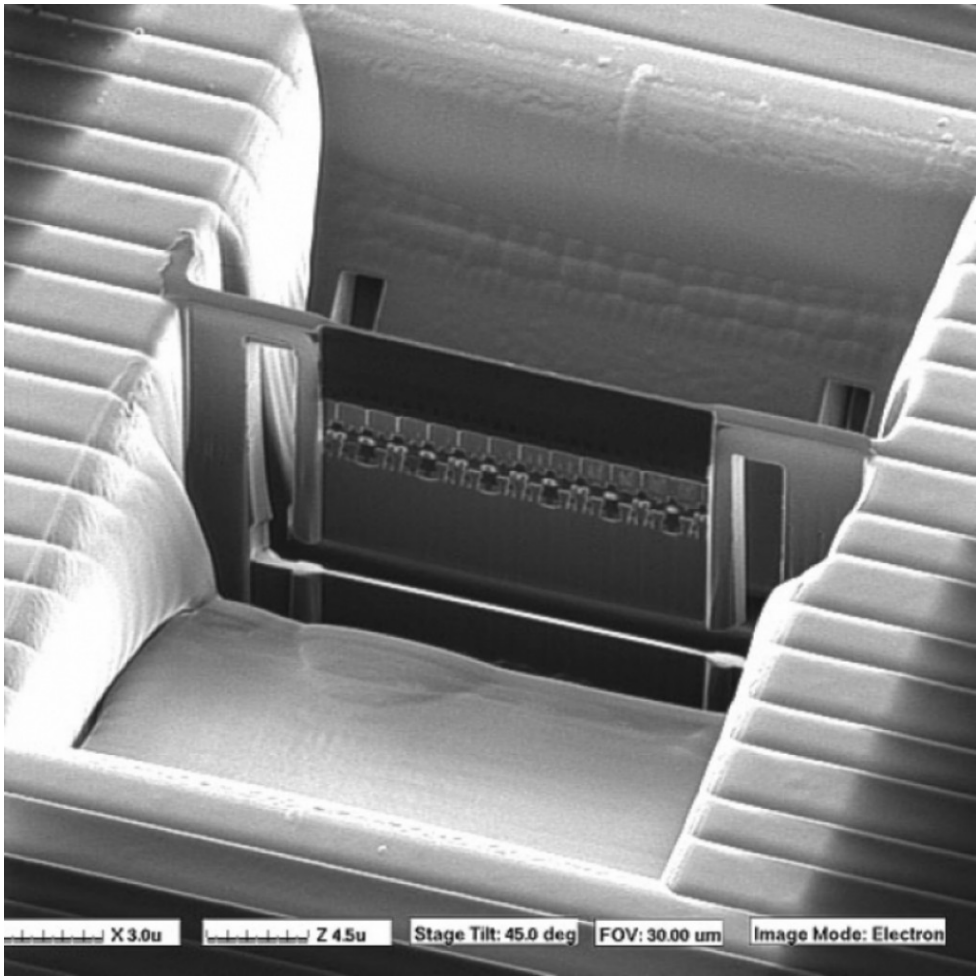
# Top View of Frame Cut



# Iso-View of Frame Cut

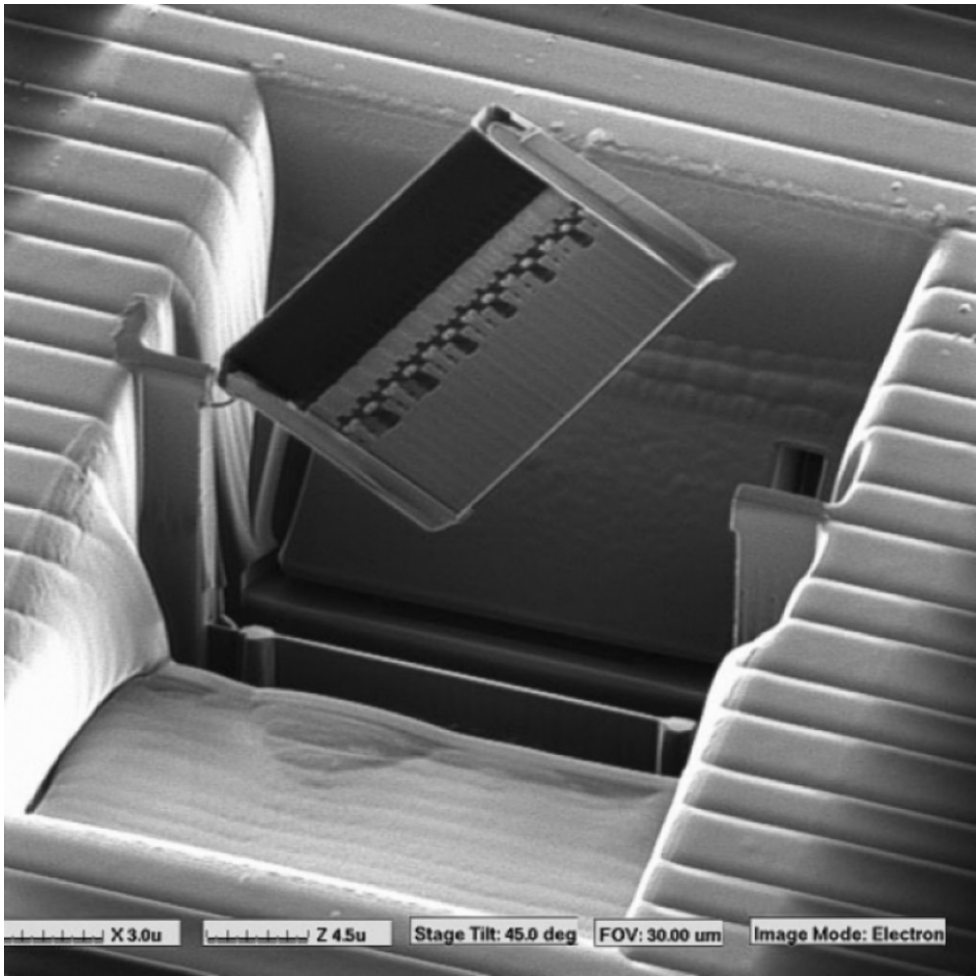


# Thin to Electron Transparency



# Top View just before Removal

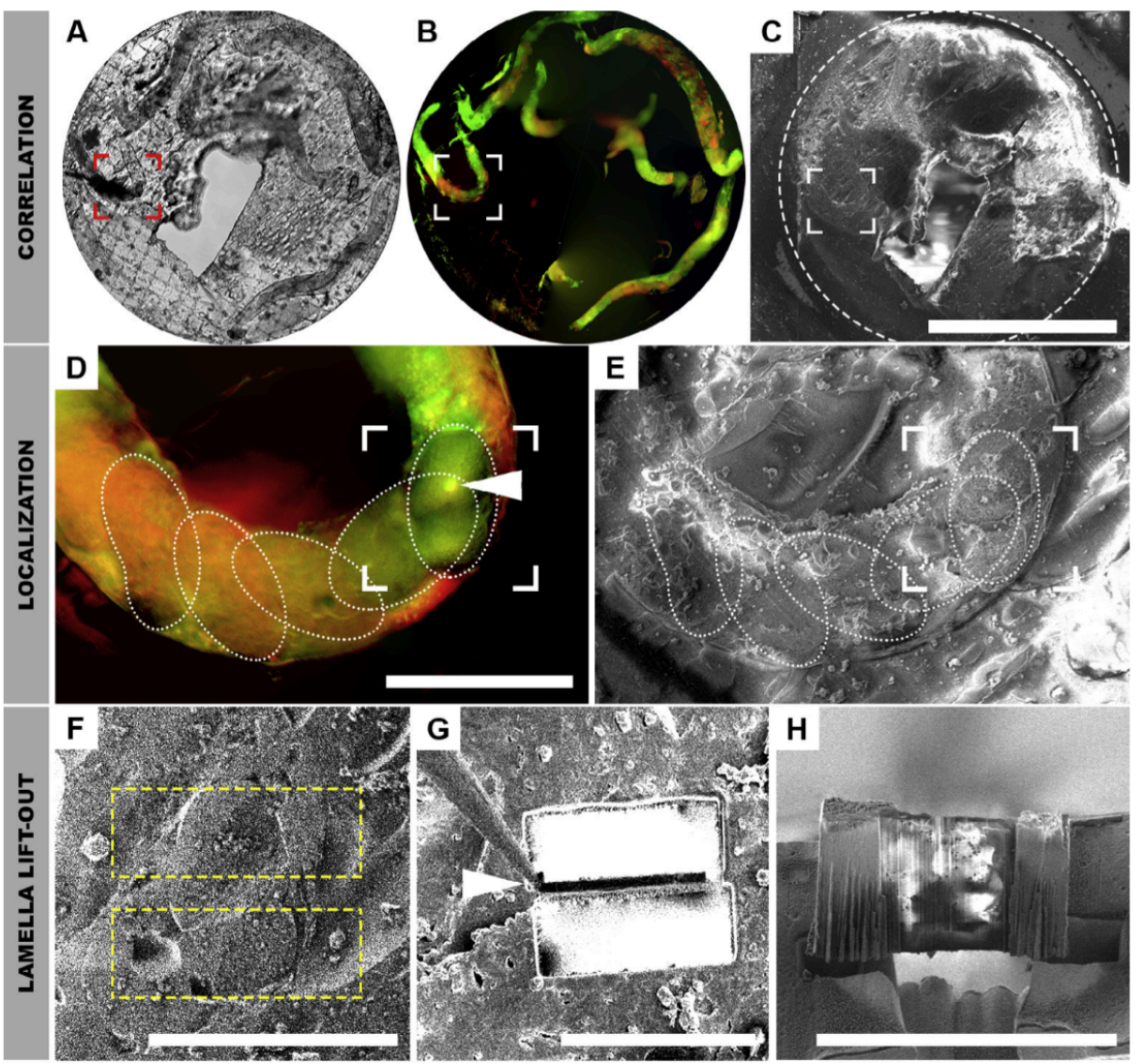
# Remove Section and attach to manipulator (not shown)



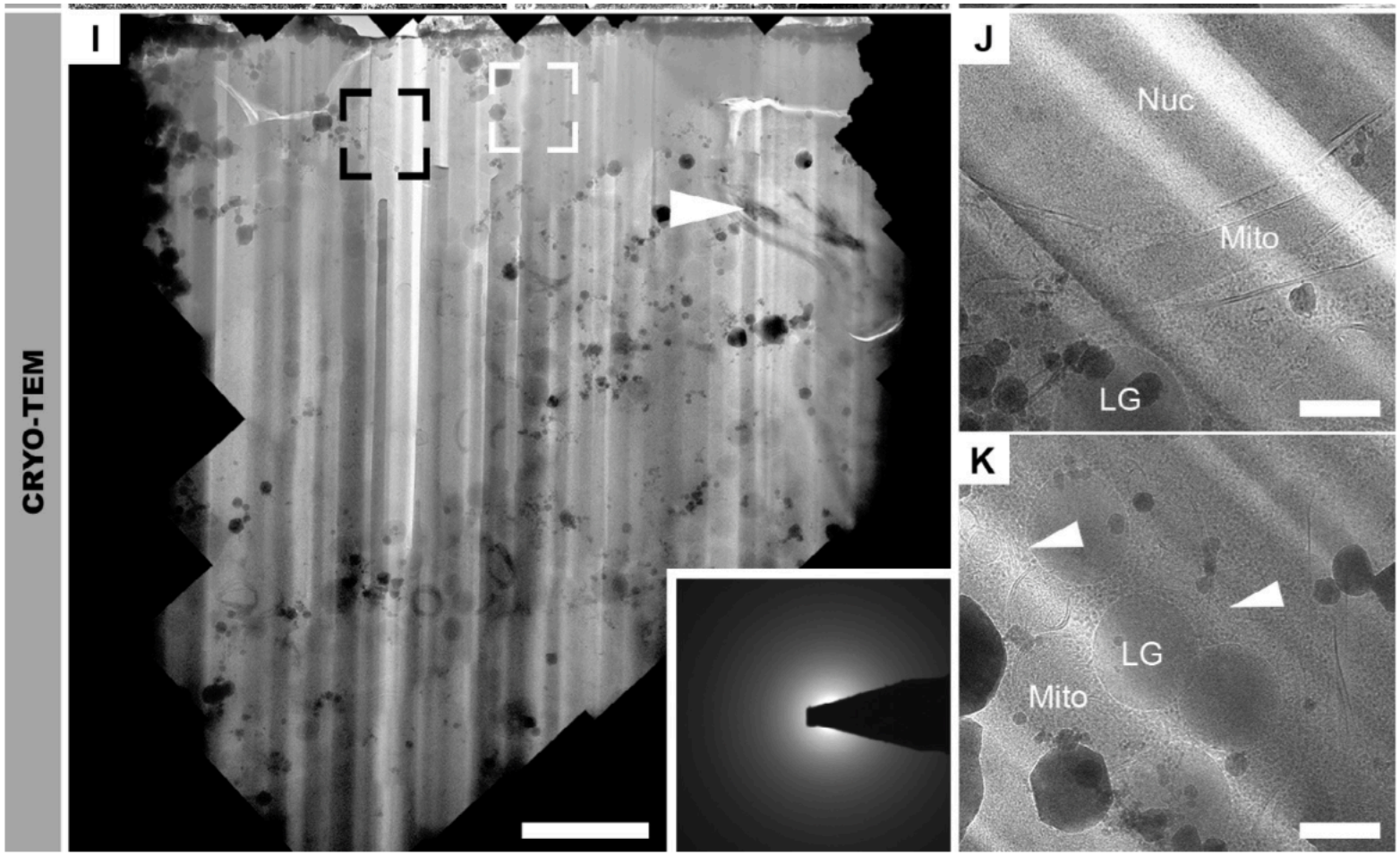
# Applications to Cryo

- Mahamid J, Schampers R, Persoon H, Hyman AA, Baumeister W, Plitzko JM. A focused ion beam milling and lift-out approach for site-specific preparation of frozen-hydrated lamellas from multicellular organisms. J Struct Biol. 2015 Nov;192(2):262-9. doi: 10.1016/j.jsb.2015.07.012. PubMed PMID: 26216184.

# C Elegans Embryo HPF on grid



# TEM



# Equipment Needed