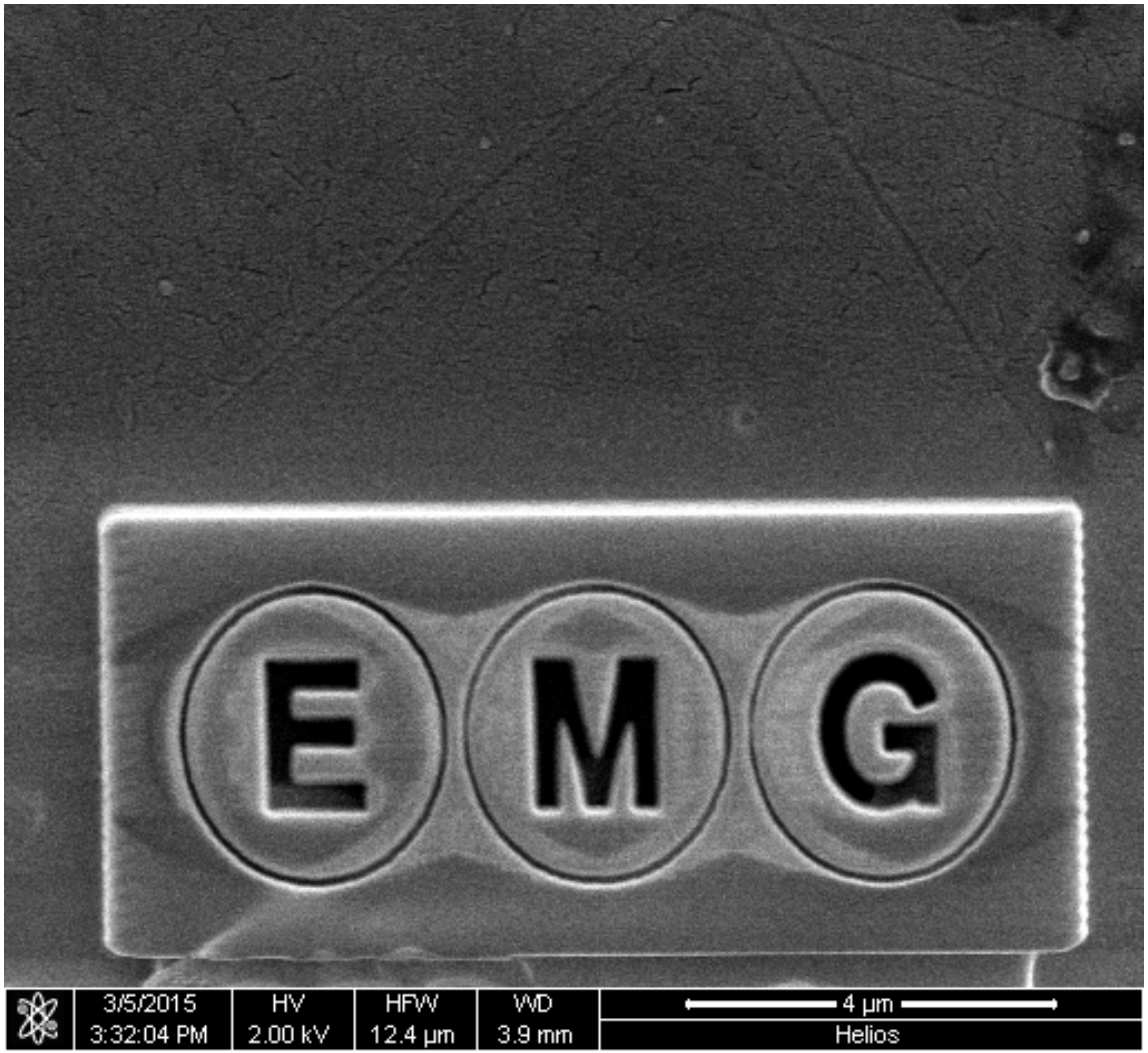
FIB-SEM
Feb. 4, 2019

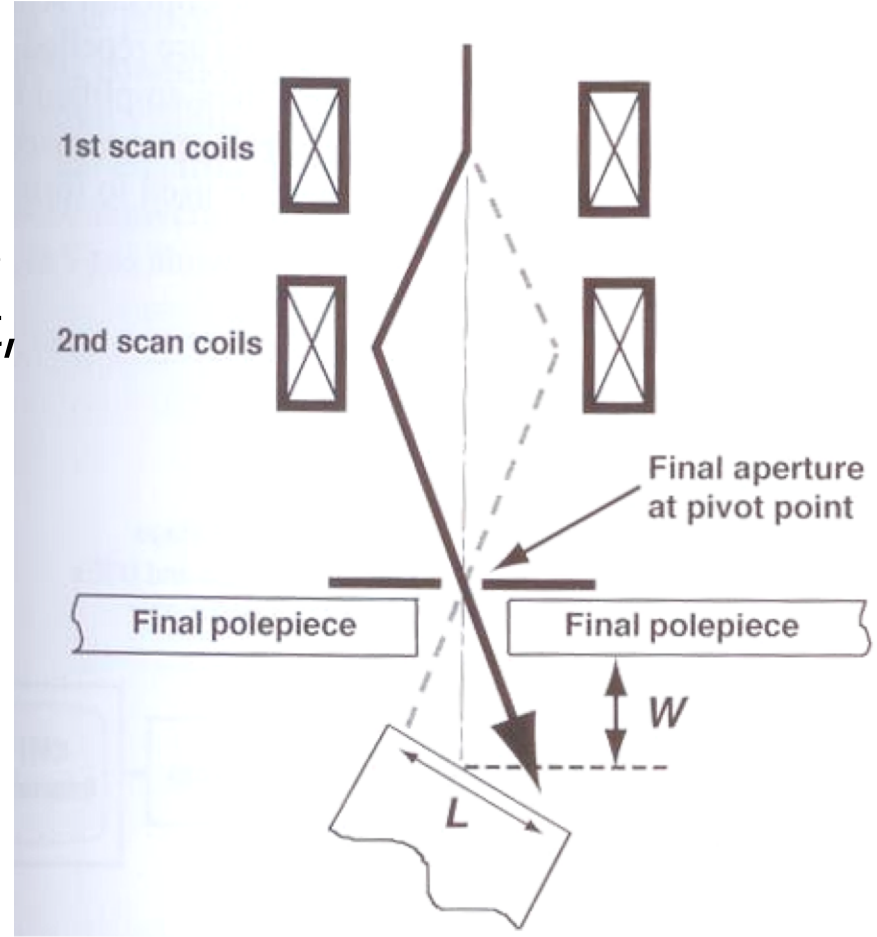


Outline

- SEM basics
- FIB Basics
- Application to room-temperature (conventional) specimens
- Use as a cryo-prep tool for cryo-TEM
- Use as a general prep tool (liftout) for TEM

SEM Basics

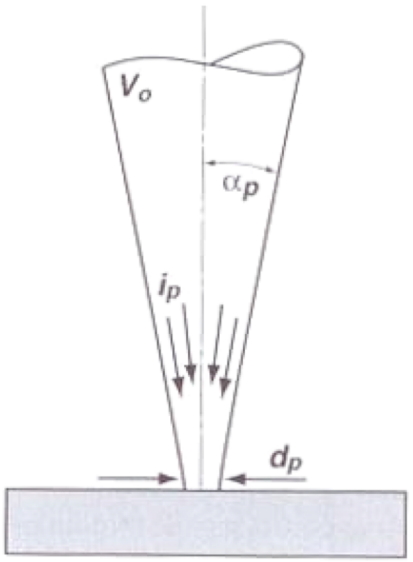
- Electron probe is focused to a sharp point
- The probe is scanned across the specimen point by point, with each point producing signal
- Scan coils deflect beam to move across sample



Goldstein et al, 2003

SEM Beam: probe size

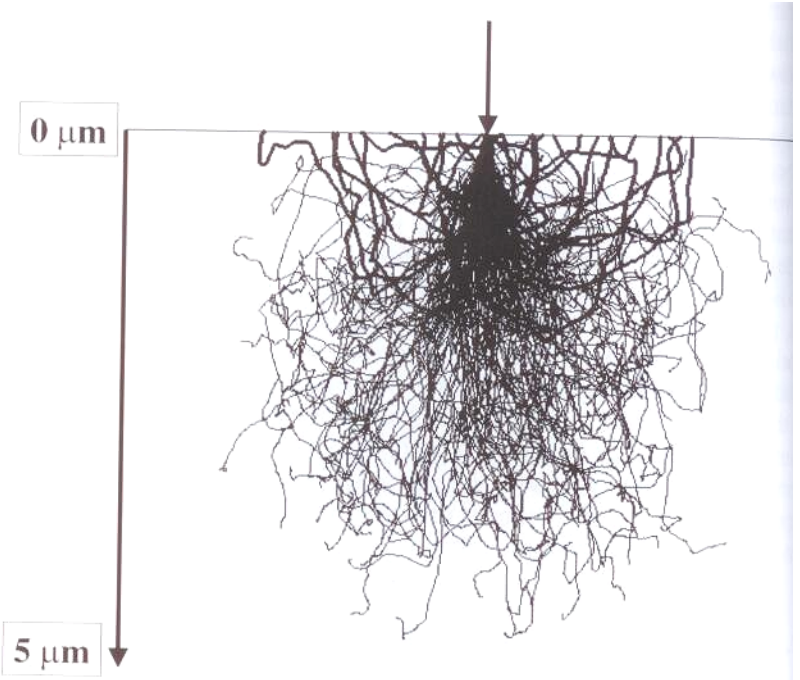
- Ideally want as small a probe as possible, relative to pixel size
- Probe size is determined by voltage, current, divergence angle
- Lens distortions
  - Spherical aberration (focus different at center and edge of lens) – instrument dependent
  - Aperture diffraction
  - Astigmatism (user correctable)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



Goldstein et al, 2003

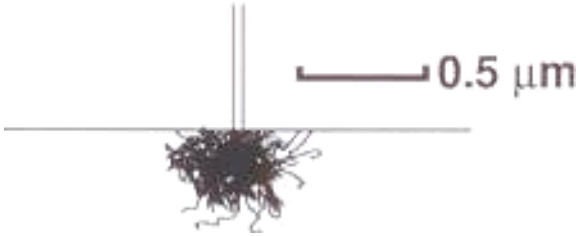
Beam-Specimen Interaction

- 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

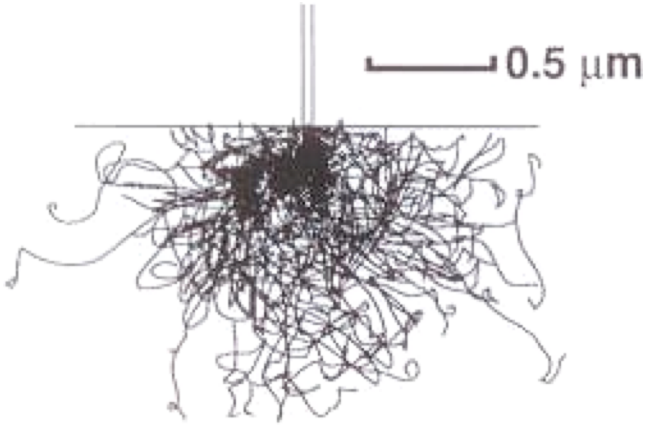


Goldstein et al, 2003

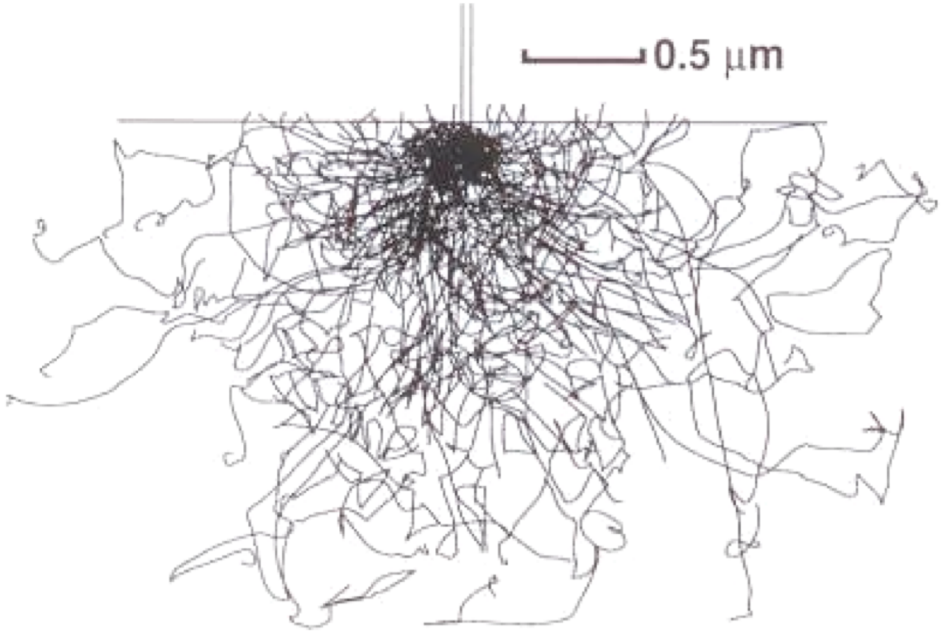
Simulations at different voltages



10 keV



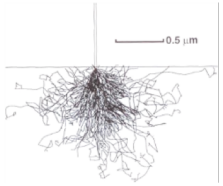
20 keV



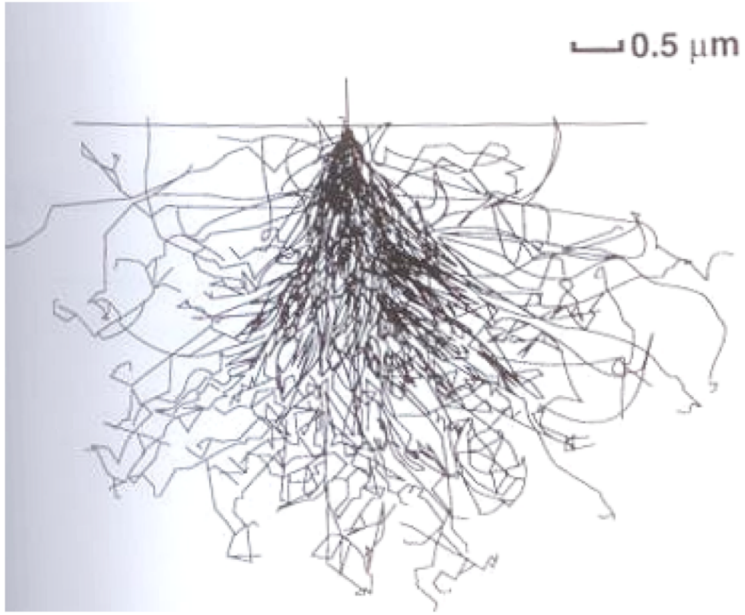
30 keV

Goldstein et al, 2003

Material Dependence of Interaction Volume



Iron, 20 keV



Carbon, 20 keV

Goldstein et al, 2003

Calculation of Beam Penetration

$$r\ (\mu m) = \frac{2.76 \times 10^{-2}\, A\, E_o^{1.67}}{\rho\, Z^{0.89}}$$

where ρ = density of the material (g/cm^3),
Z = atomic number,
A = atomic mass,
and E_o = accelerating voltage.

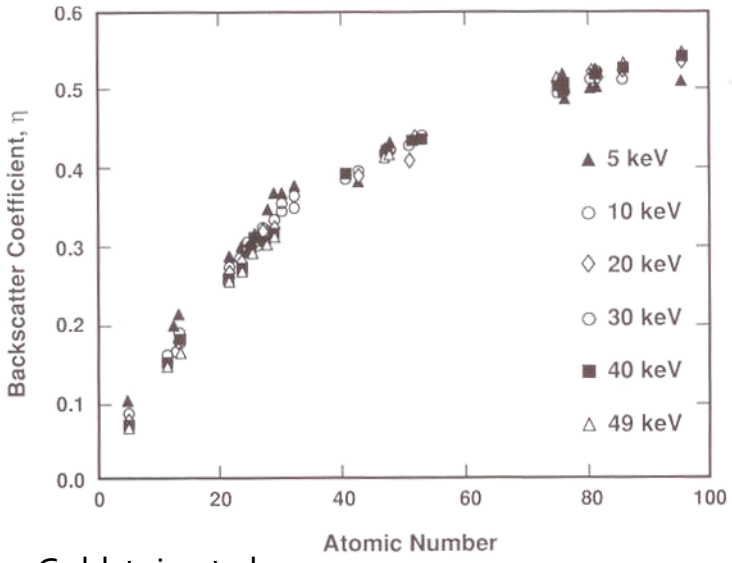
Goldstein et al, 2003

Back Scattered Electrons and Secondary Electrons

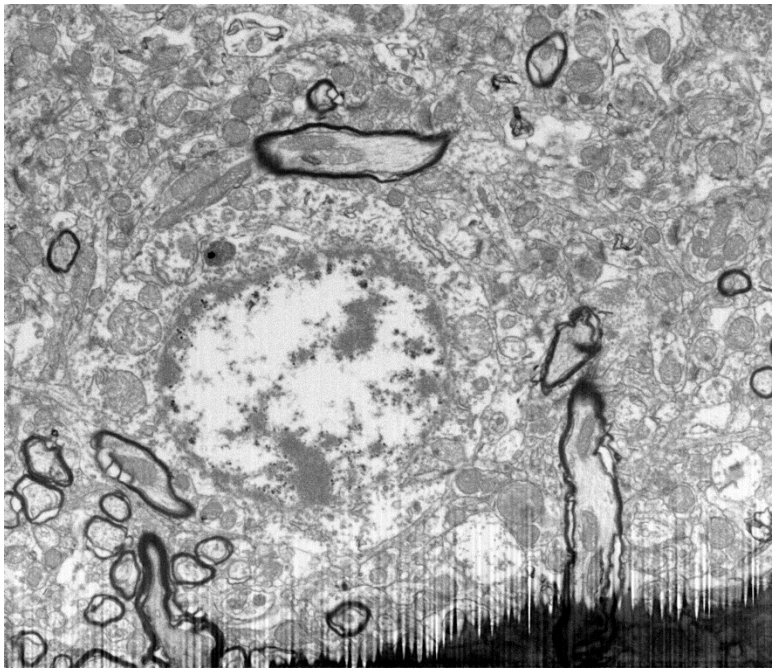
Detectors can be tuned for either one, or for both

BSE efficiency is material dependent, voltage independent

Fraction of
e's that backscatter

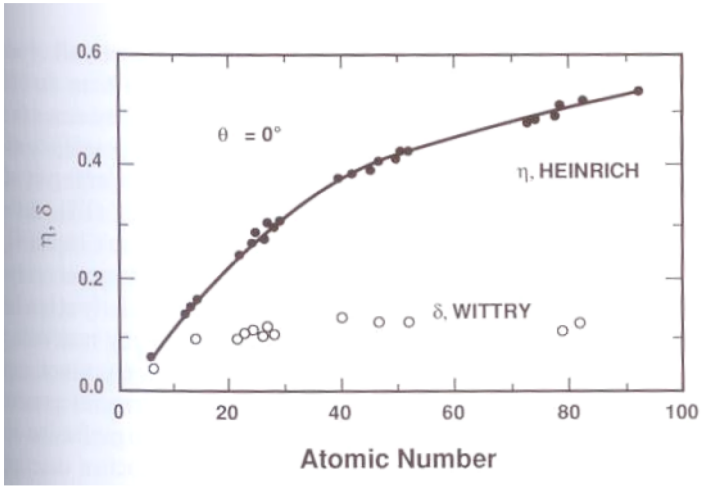


Goldstein et al, 2003



BSE's give contrast between light and heavy elements

Specimen Dependence of BSE, SE

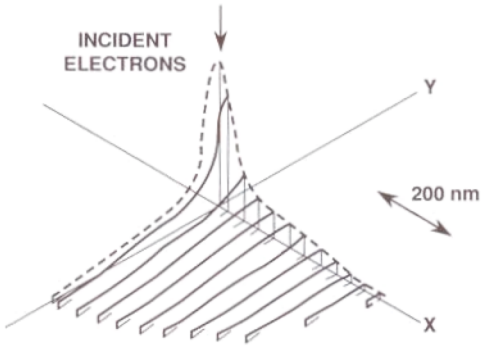


30 keV

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

Goldstein et al, 2003

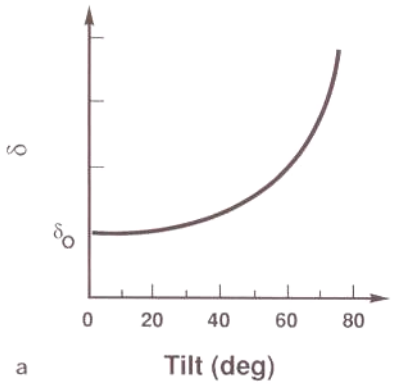
Emission shape of BSE's



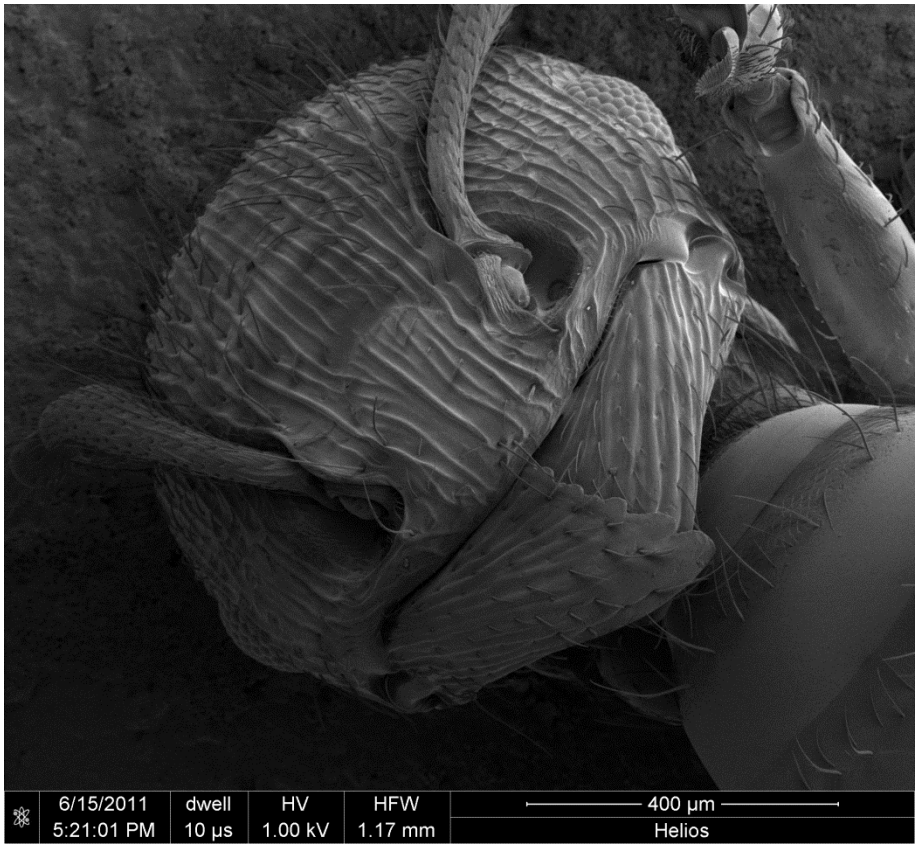
Most BSE's are released close to origin ("high quality" BSE's)
Higher atomic number elements have sharper central peak

Goldstein et al, 2003

Angular Dependence of SE's

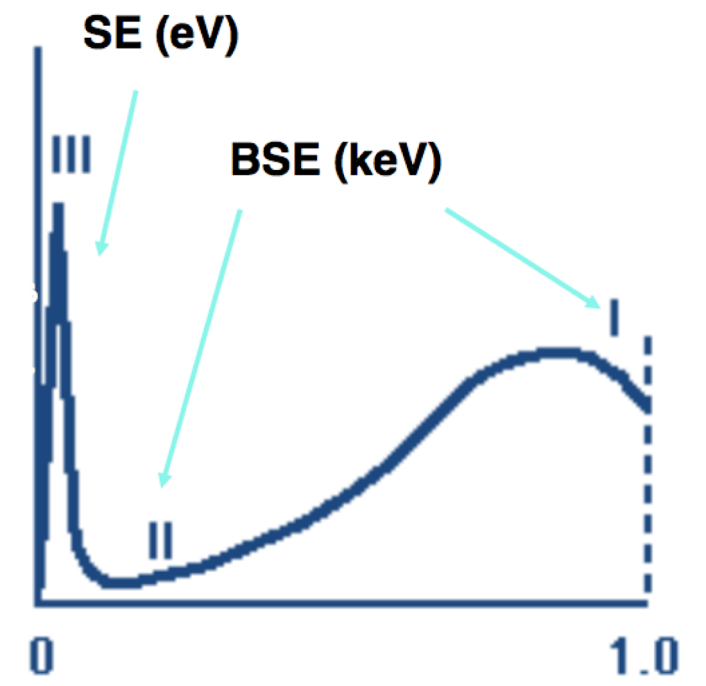


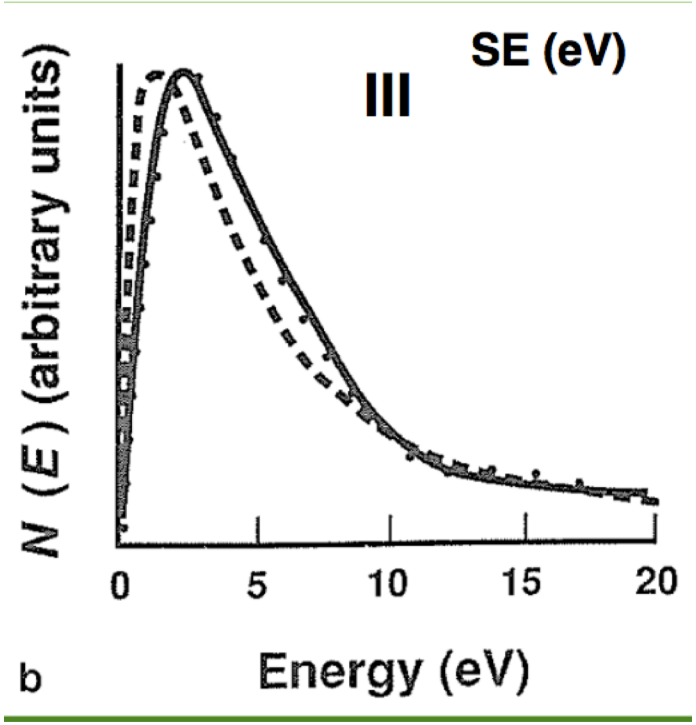
Goldstein et al, 2003



SE's also give topographic information

Secondary electrons are low voltage



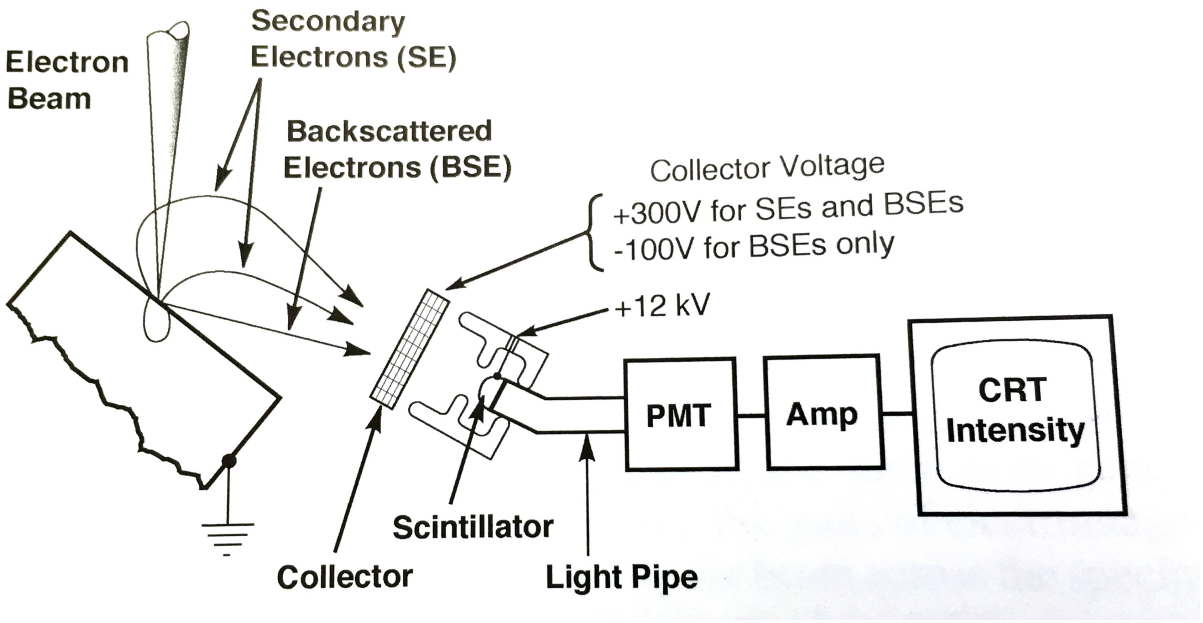


Bombardment with keV beam

Goldstein et al, 2003

Detection of BSE's, SE's

Everhart-Thornley (ET) Detector



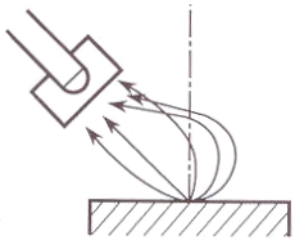
Electrons strike scintillator, releasing photons
Photons travel to photomultiplier tube
Eventually converted to electric signal, storing intensity values
Combined SE/BSE detector

Goldstein et al, 2003

Apply bias

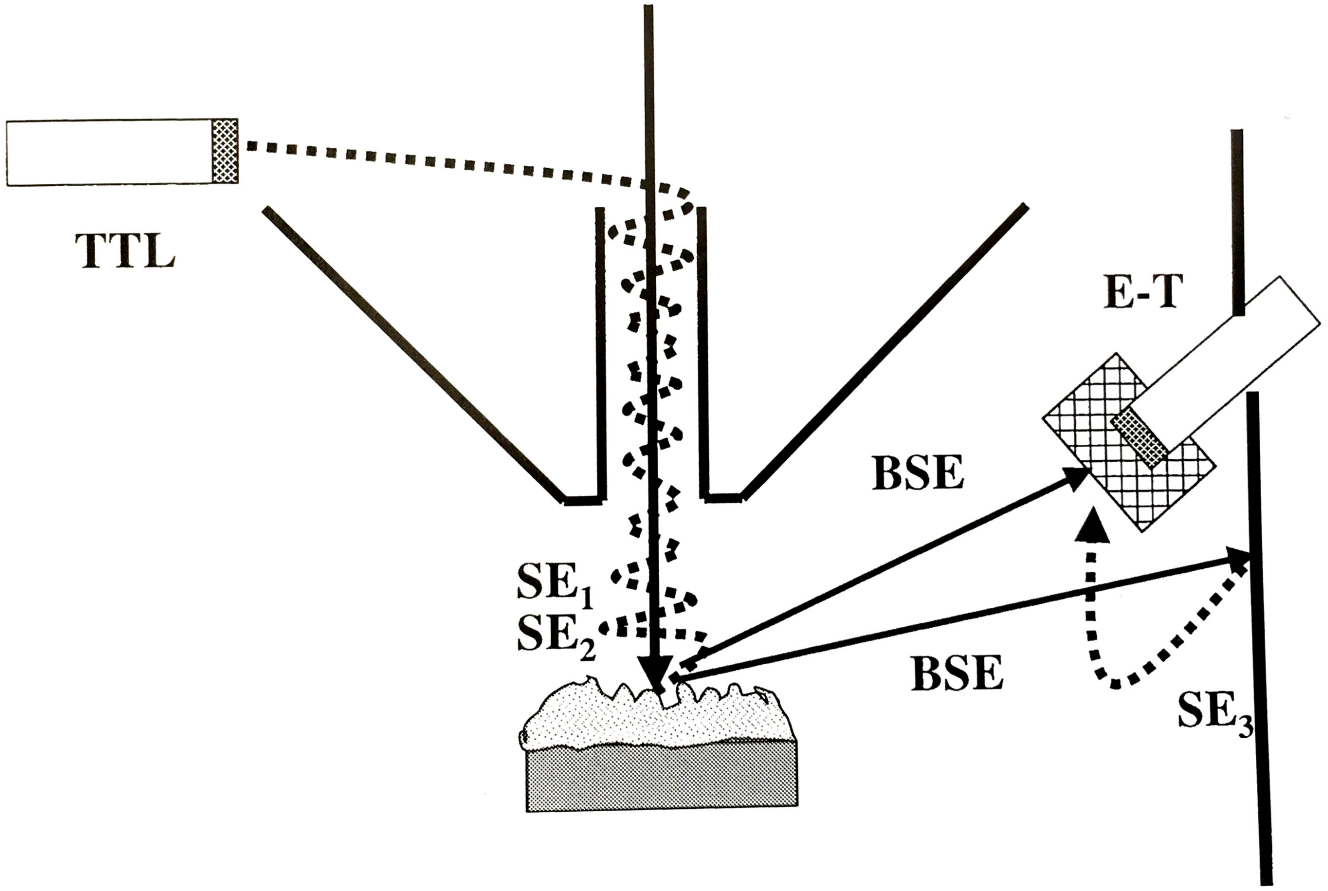
- Bias can be applied to detector, directing electrons toward or away from it
  - Negative bias (< -50 V): detect only BSE's
  - Positive bias: collect more SE's, indirect BSE's : greater total signal

Positive bias



Goldstein et al, 2003

Through-Lens Detector (TTL)



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
- Apply negative bias to detect mainly BSE's
- Backscattered imaging gives elemental contrast
- Secondary imaging gives more signal and topographic images
- Through Lens Detector for better resolution

Parameters for Optimal Imaging

- Voltage: lower voltage for less penetrance (but less signal)
  - Higher quality optics needed for very low voltage
- Current: Higher current gives more signal but a larger probe size
- Working distance: decreasing distance from lens increases signal
- Field of view (magnification): pixel size
- Dwell time per pixel
- Number of scans
- Detector type
  - EVT, TLD, ICE (SE's and ions)
  - SE , BS or mixed mode

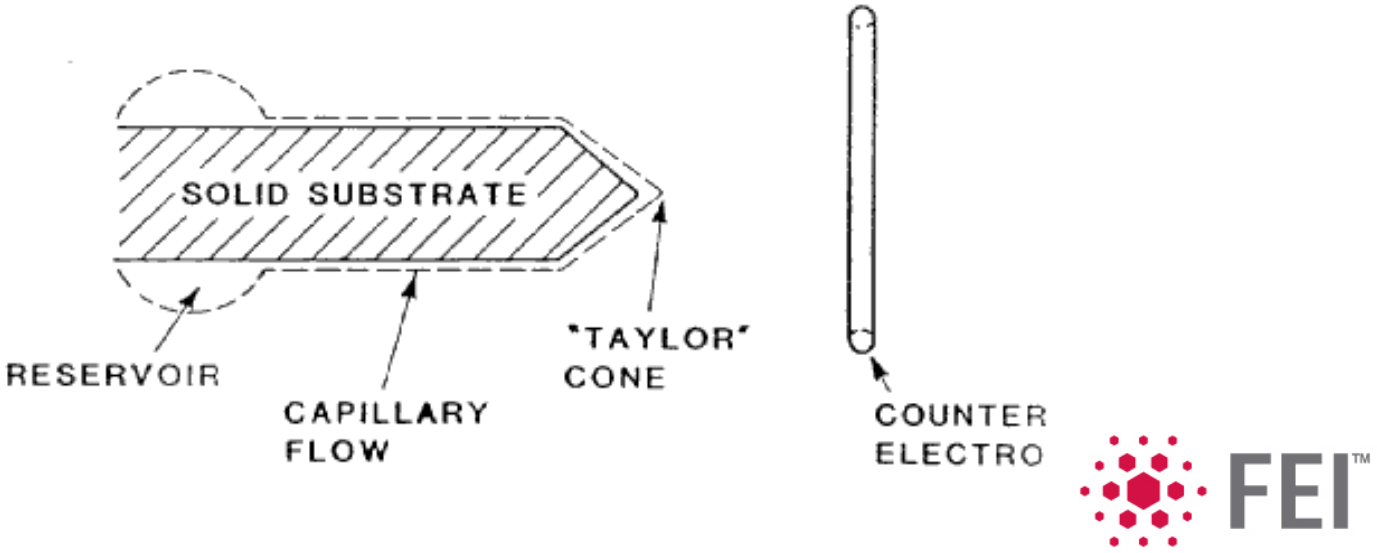
Biological Imaging

- Biological specimens are mostly light elements: little elemental contrast
- Standard procedure, as with negative stain TEM, is to stain with heavy metal salts (lead, uranium, tungsten) and look at the stain
- Long procedure involving:
  - Tissue fixation
  - Substitution of water with organic solvent
  - Infiltration with resin
  - Staining of biological components
  - Polymerization of resin
- Worked out over past 50 years, many protocols for different cells, tissues, organelles
- Can get very fine ultrastructural detail

FIB Operation

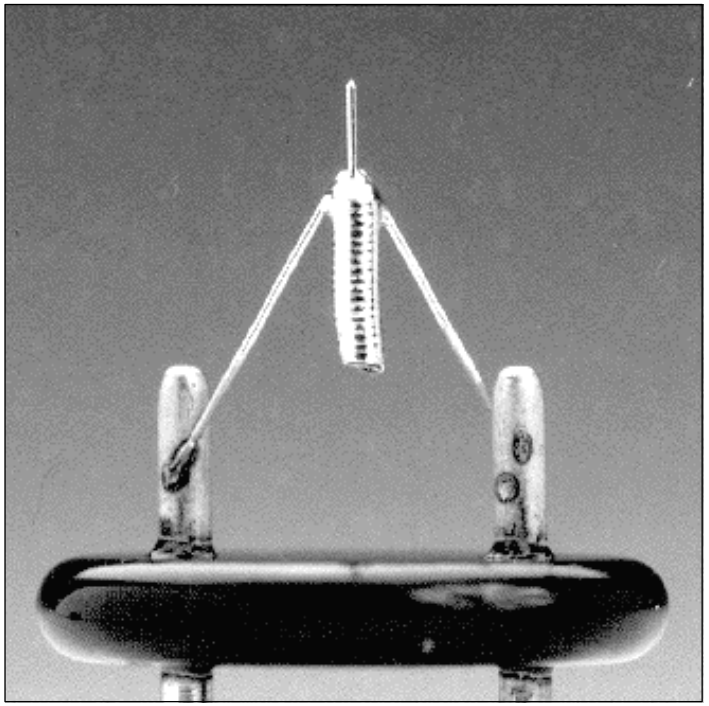
Basic Mechanism

- 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



FEI™

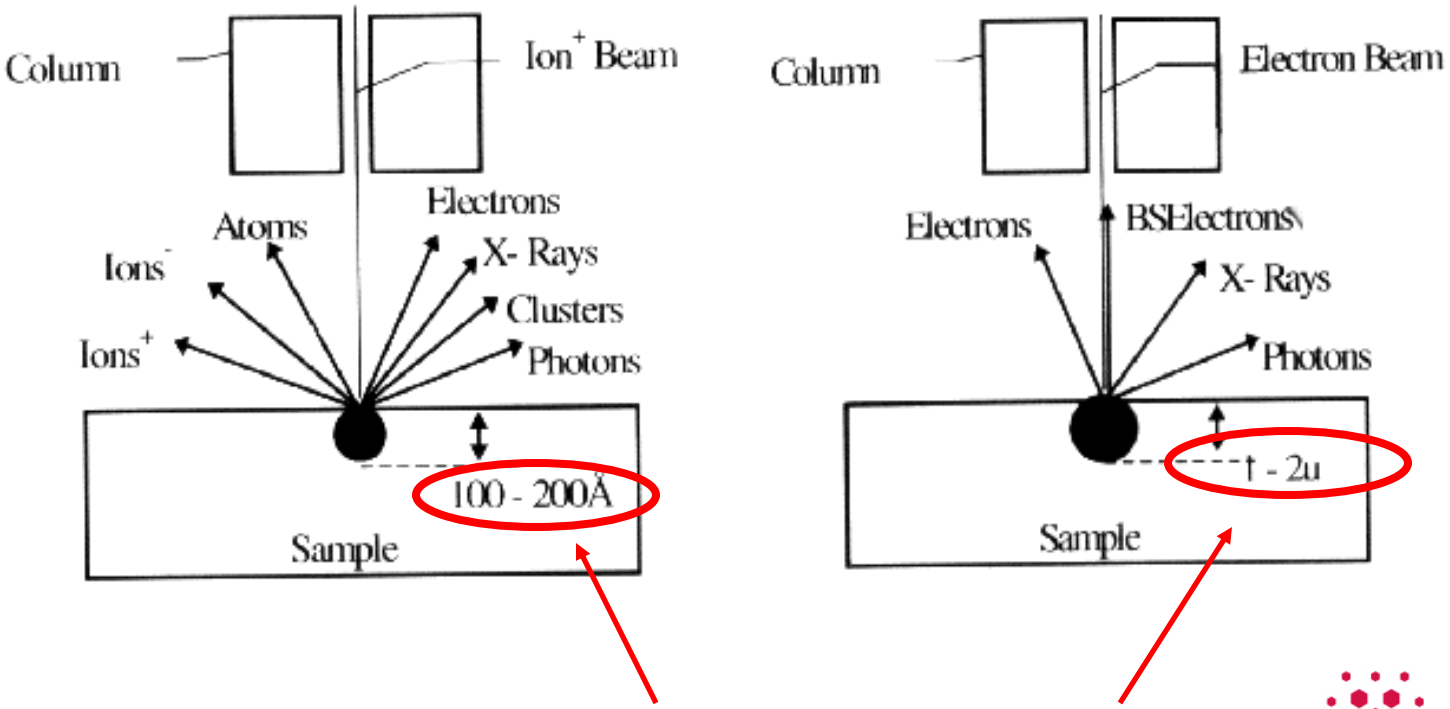
Ion Column

- 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

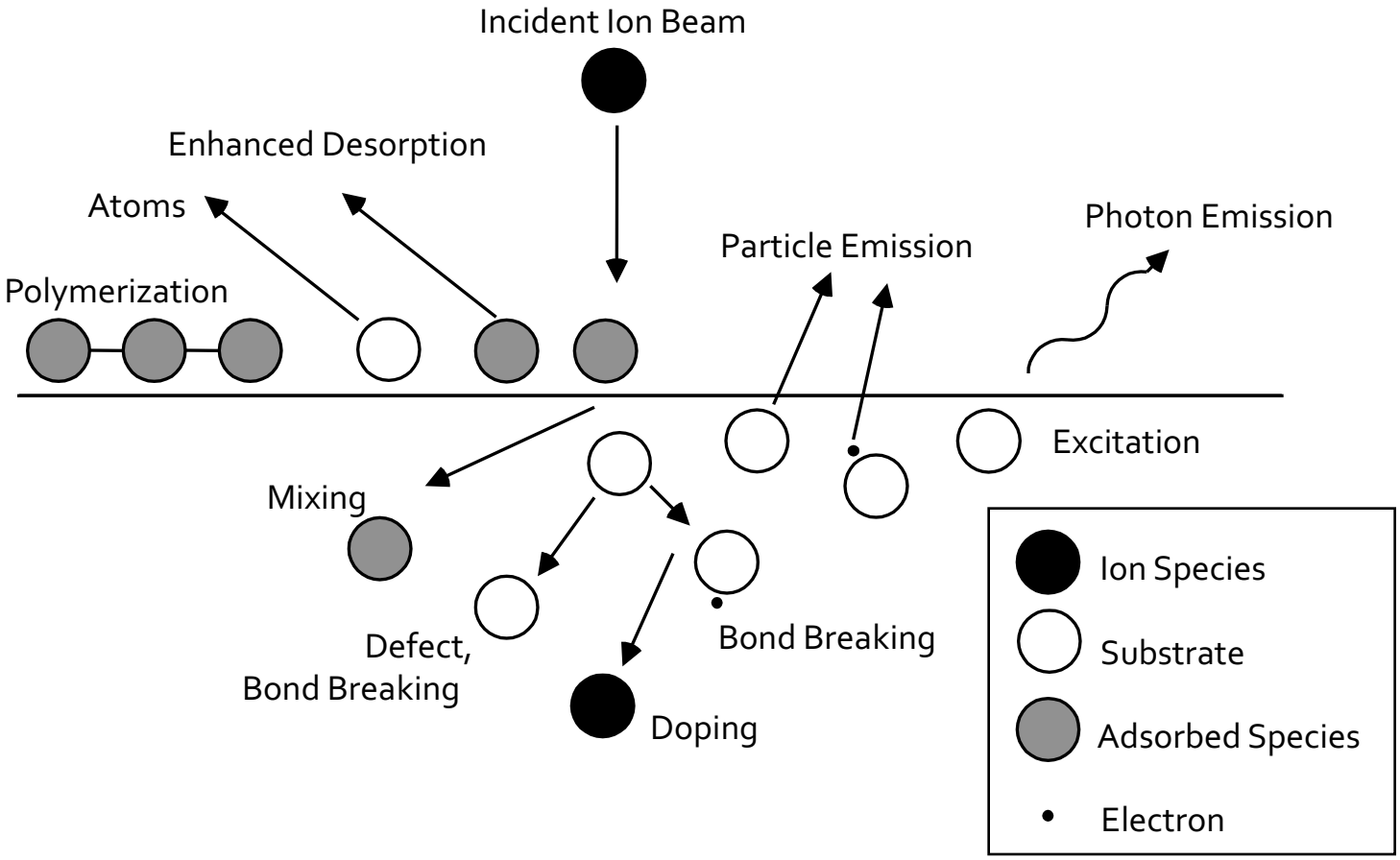
- Beam Interactions



Note difference in interaction volume

FEI™

Ion Beam to Sample Interactions



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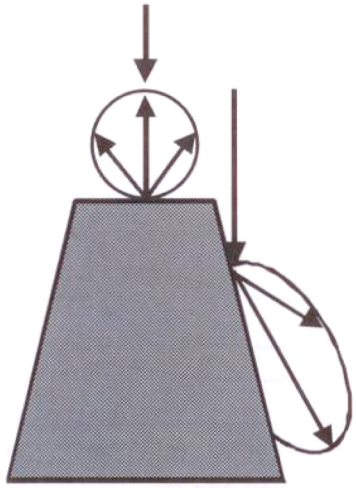
Sputtered Particles

Sputtered Particle Ejection Behavior



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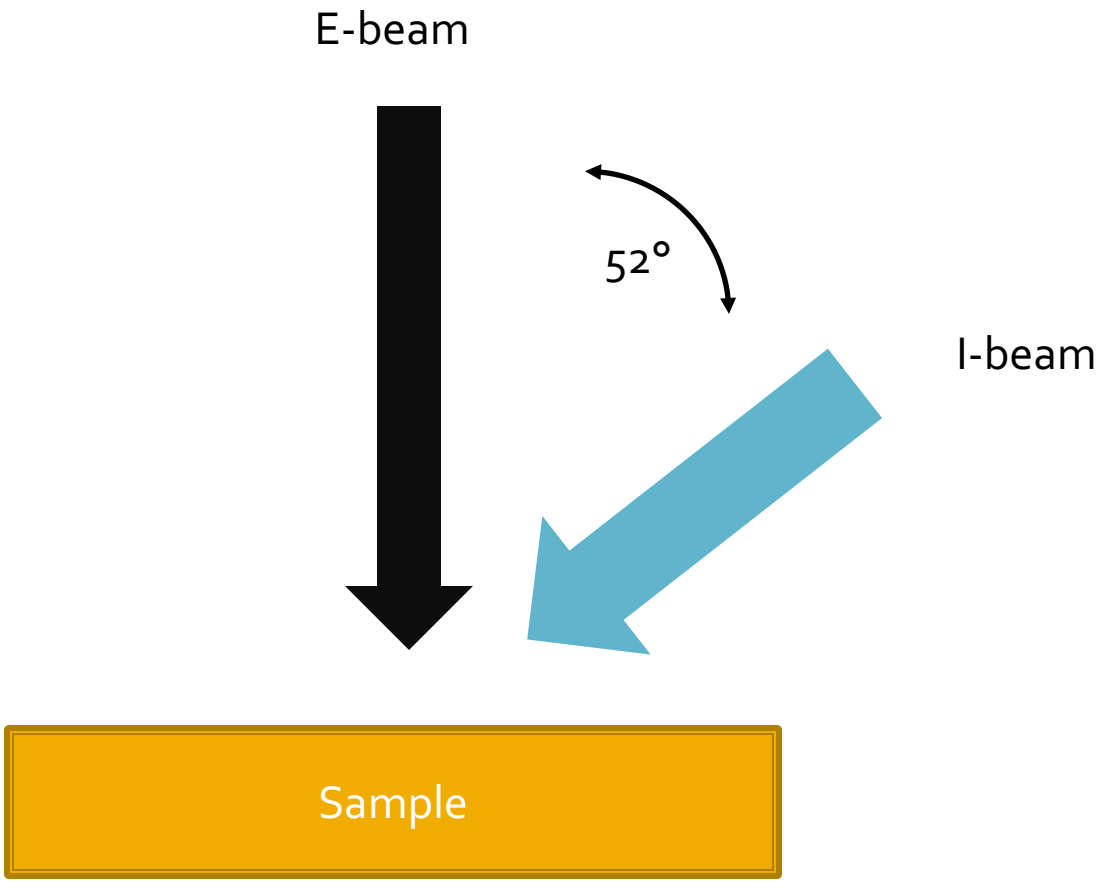
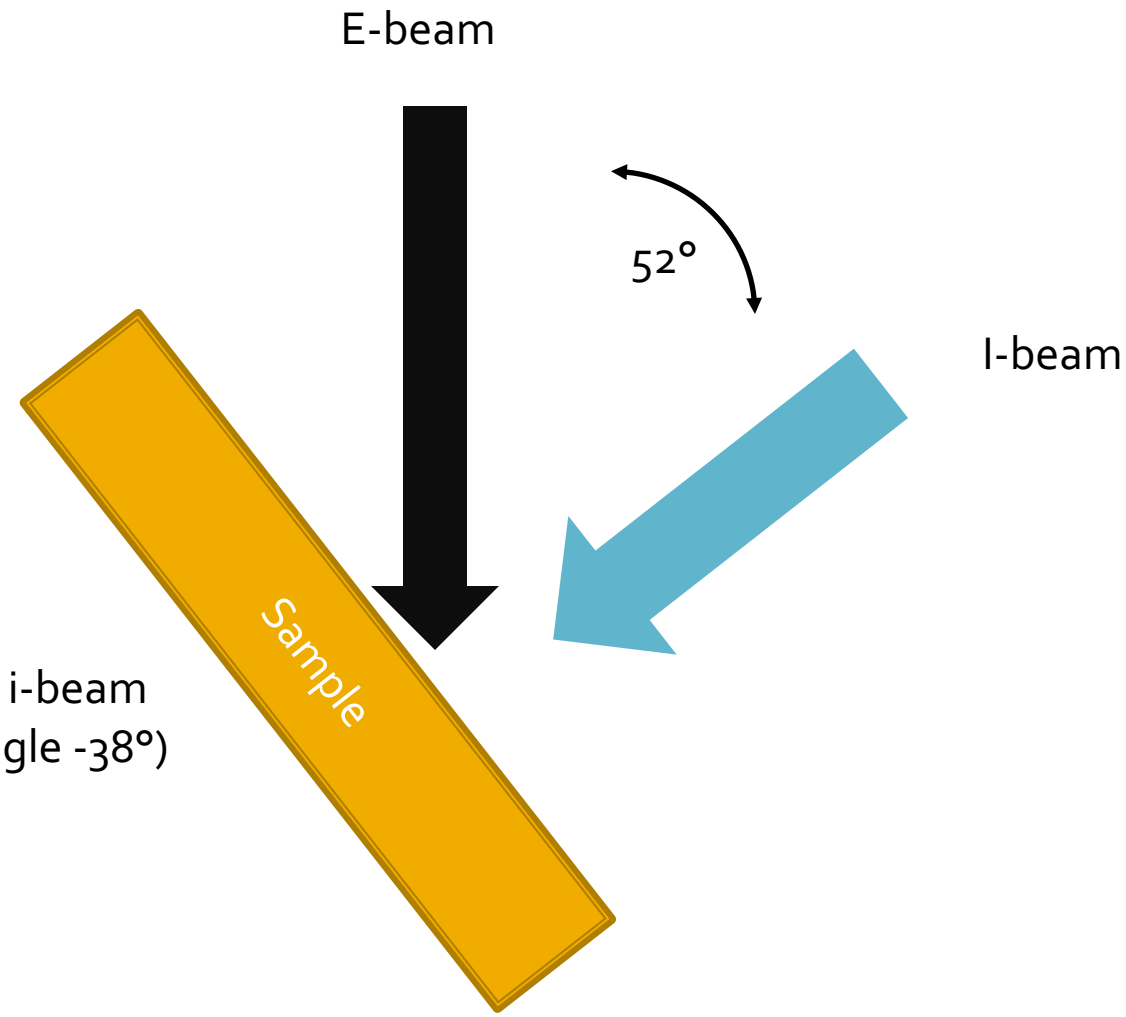
Geometry



Sample:
Tilt from -10° to +70°

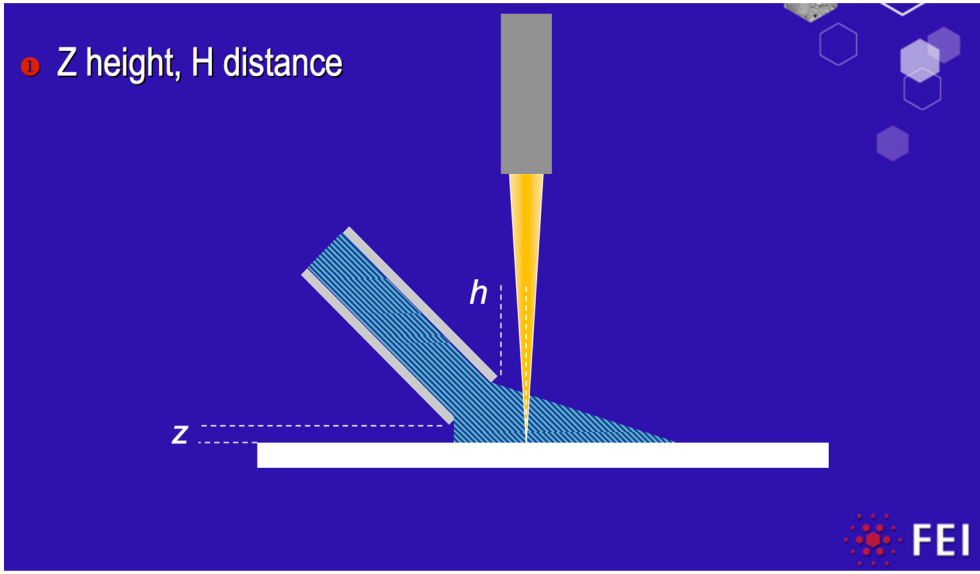
Geometry



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

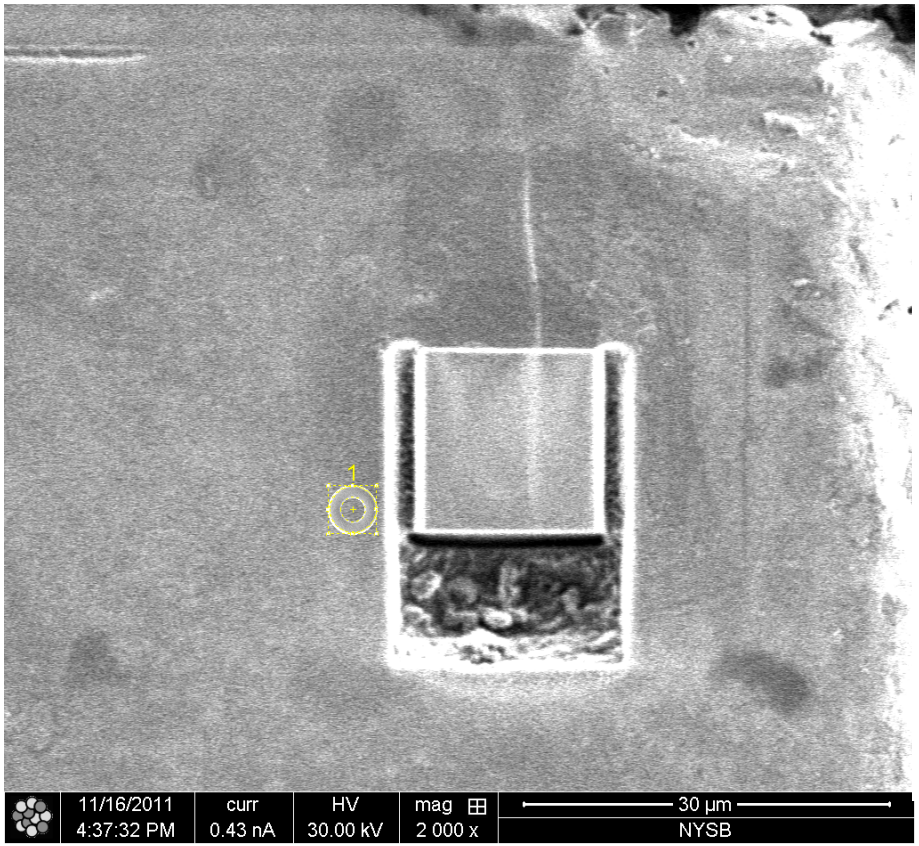
Tissue or Cells

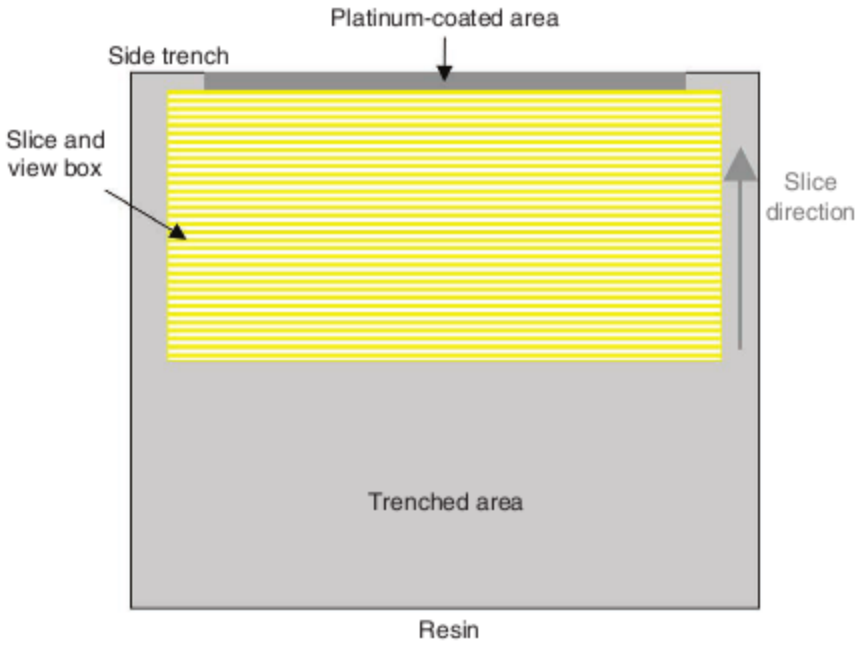
- Sample Prep
  - High Pressure frozen (optional)
  - Chemically fixed, freeze substituted
  - Resin embedded
  - En bloc staining
    - OsO_4, U Ac, Pb citrate
    - Osmium impregnation (OTO)
    - Want to make samples more conductive, more heavily stained
  - Thin conductive layer (C, Pt, Au-Pd) coated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

Imaging conditions

- Low voltage (2 keV or less)
  - Want to image only the surface
  - Minimal depth penetration (slice as thin as 5 nm)
  - No topographic information
  - Elemental contrast (C vs Os/Pb/U)
  - Through-lens detector for highest resolution
  - BSE mode (positive bias)
  - Stained parts will show up as bright on dark

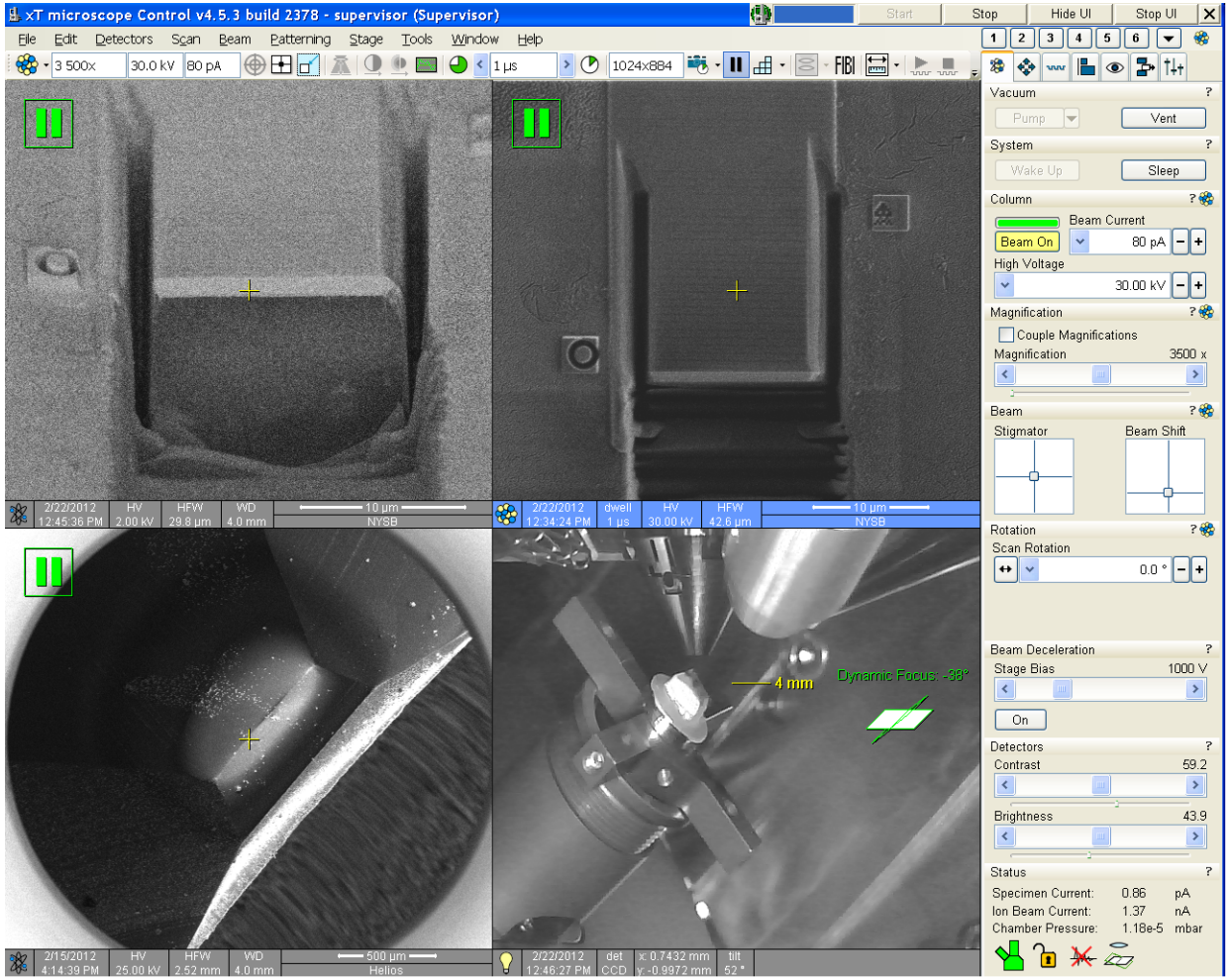
Milling Samples



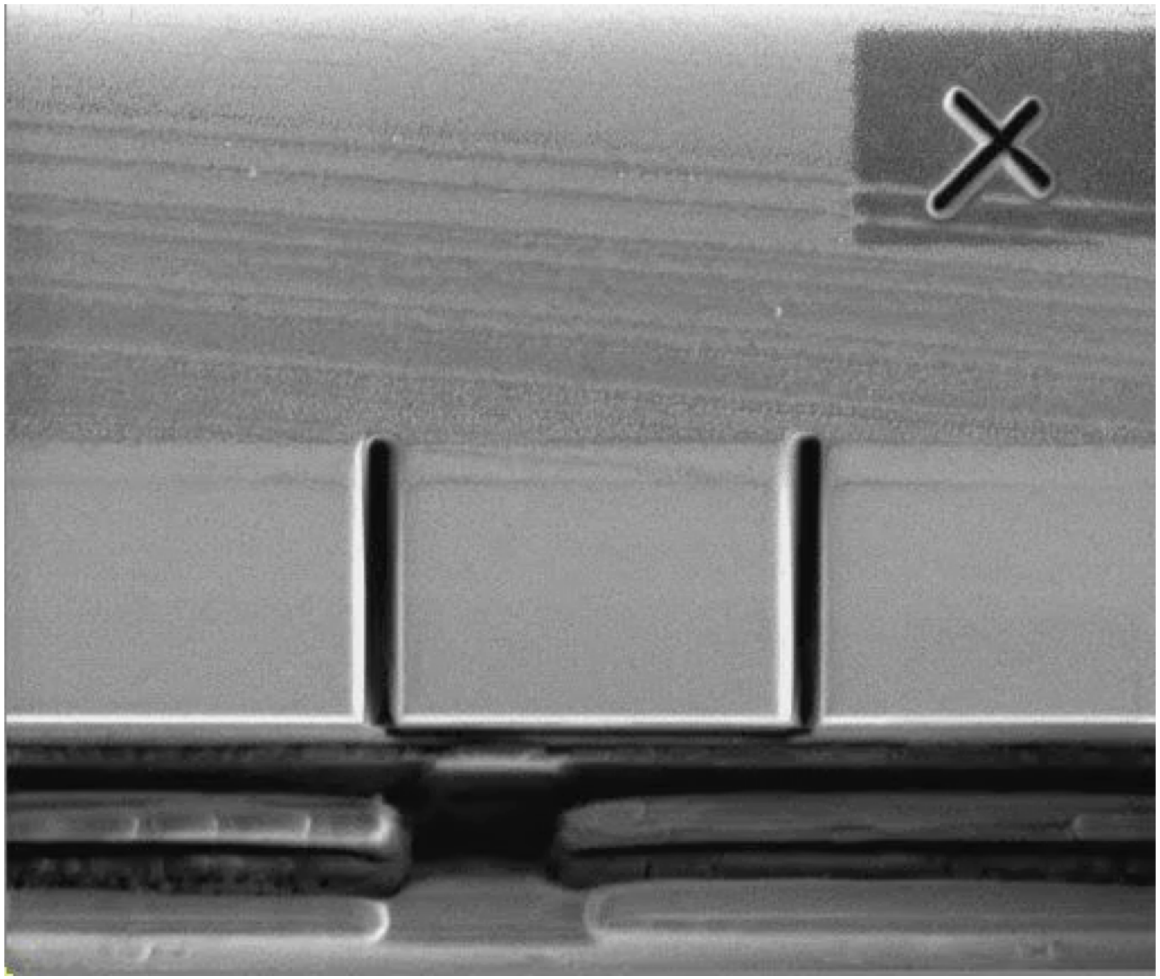


Bushby et al, 2011

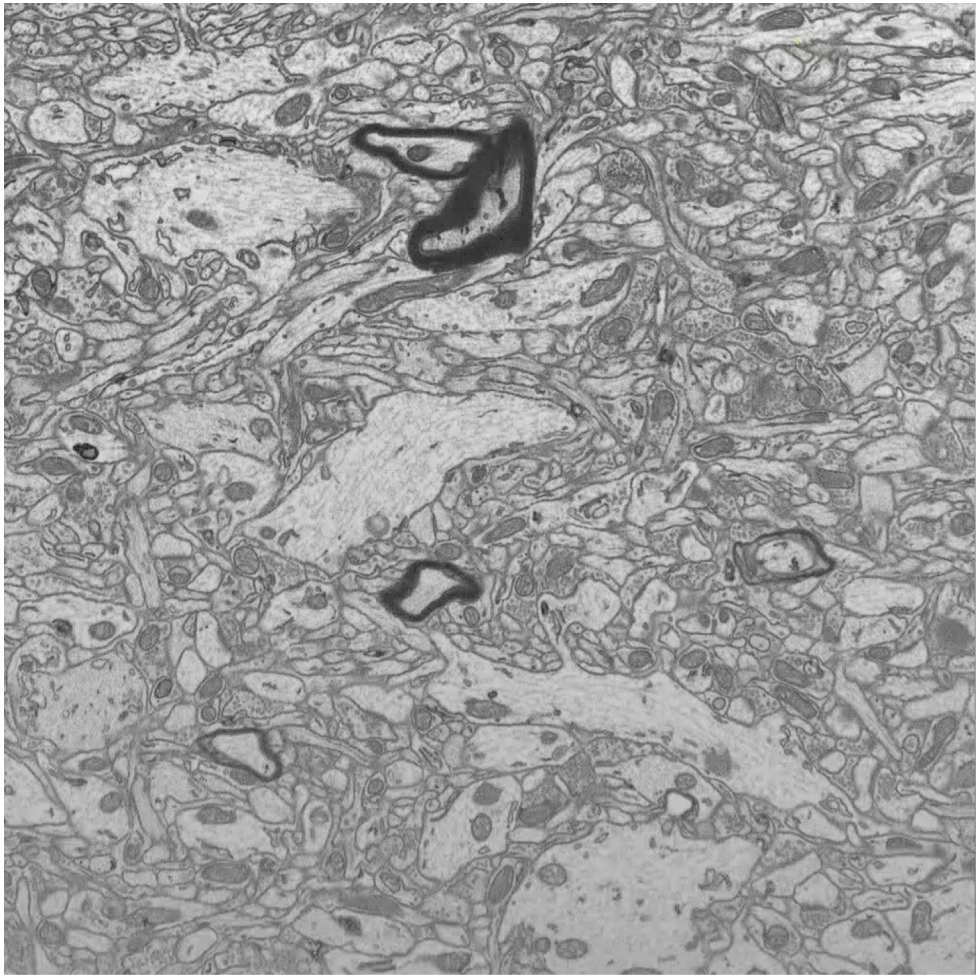
Set up for Slice and View



Milling: i-beam view



Example Movie: Neural Tissue

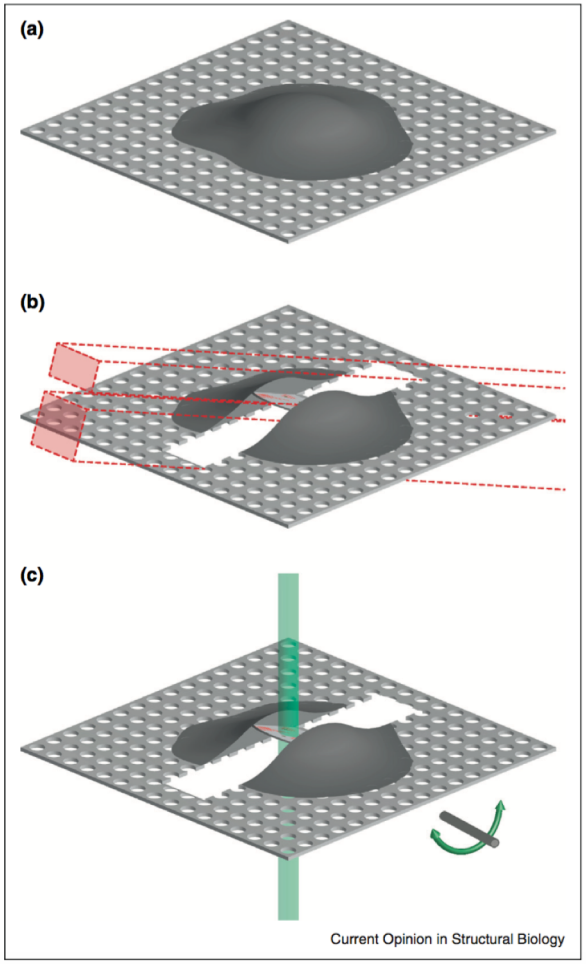


Ideal workflow

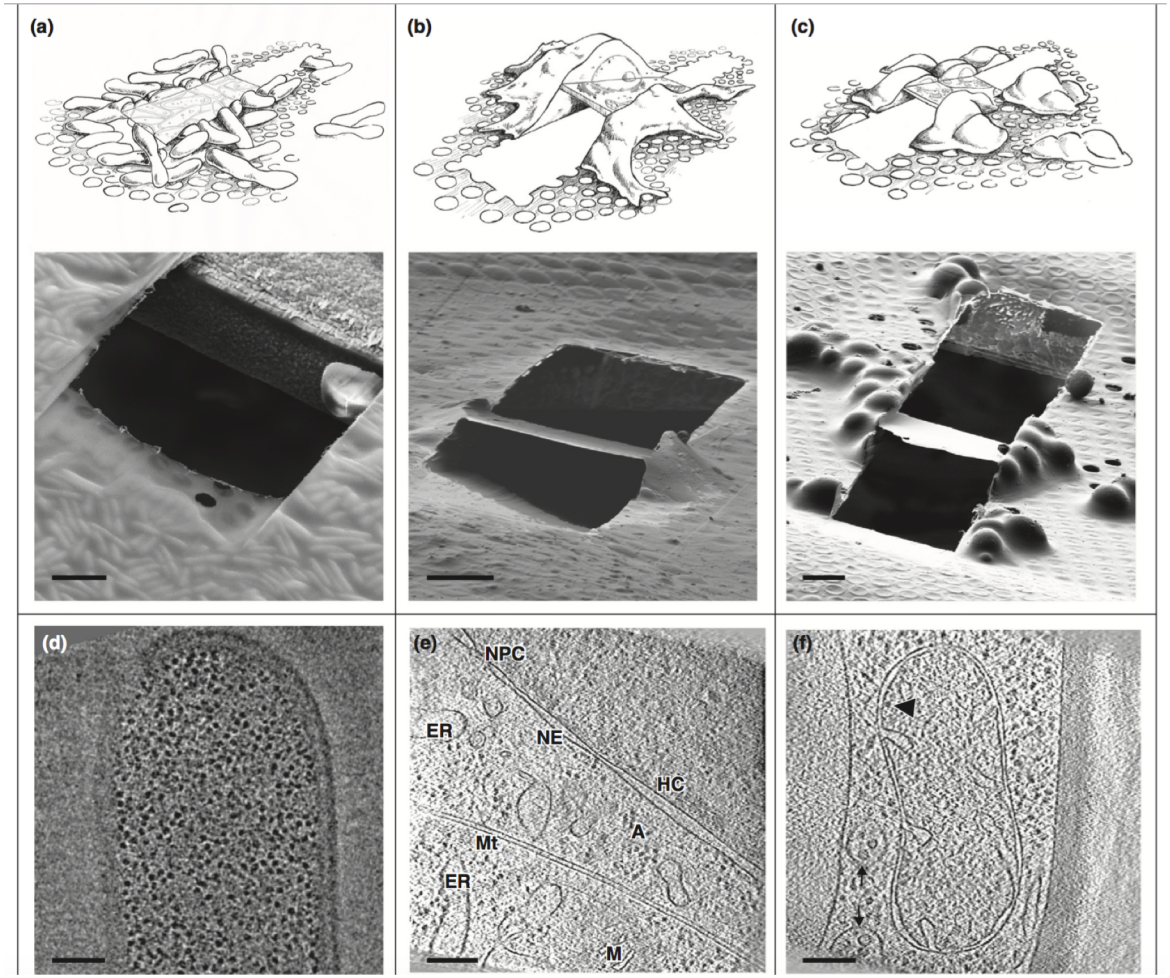
- Samples stained and embedded
- Thin slice for overall map – make easier to find features (LM or ultrathin EM section)
- Face of block polished
- Set up for slice and view (1 day)
- Collect slices (1-5 days)
- Align, process (IMOD, Amira) (1 day)
- Segmentation (IMOD, Amira) (weeks-months)
  - Neural network automation : EMAN 2.2

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.



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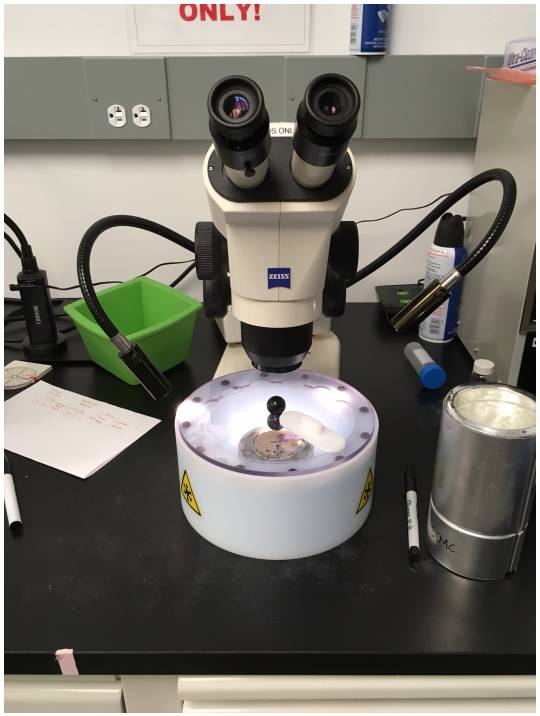
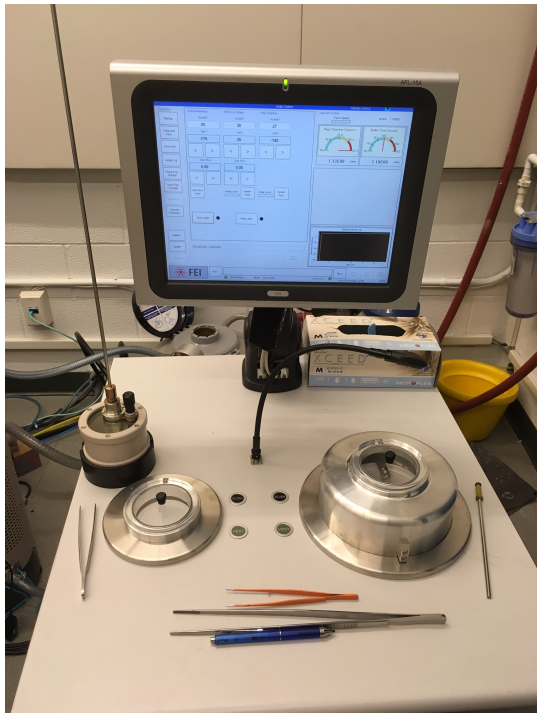
Setup for standard Lamellae Preparation

FEI autogrid loading station with stereo microscope



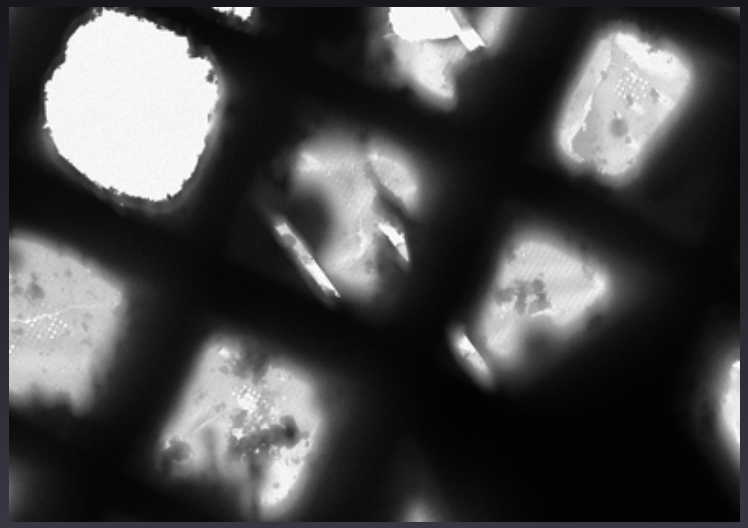
Shuttle with 2 grids

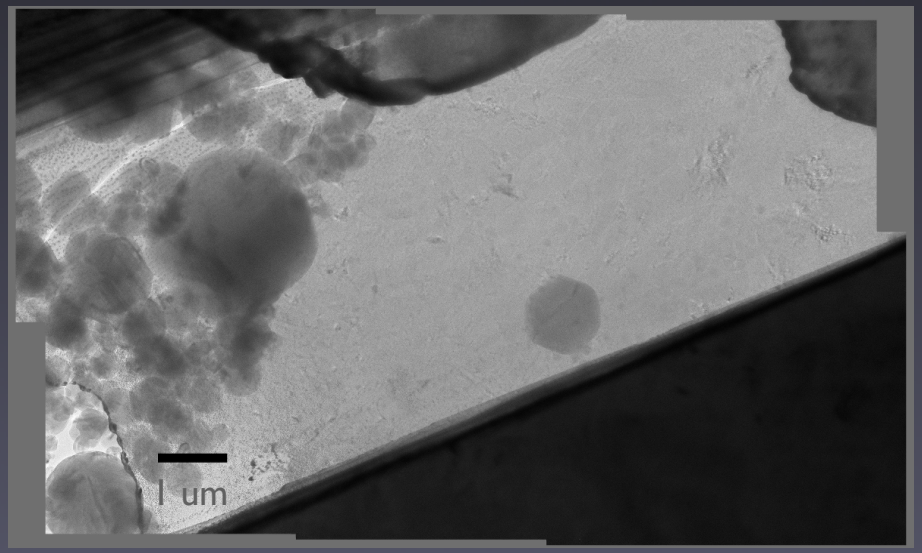
Quorum cryo loading station



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

Cryo-SEM imaging

Technical Note

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

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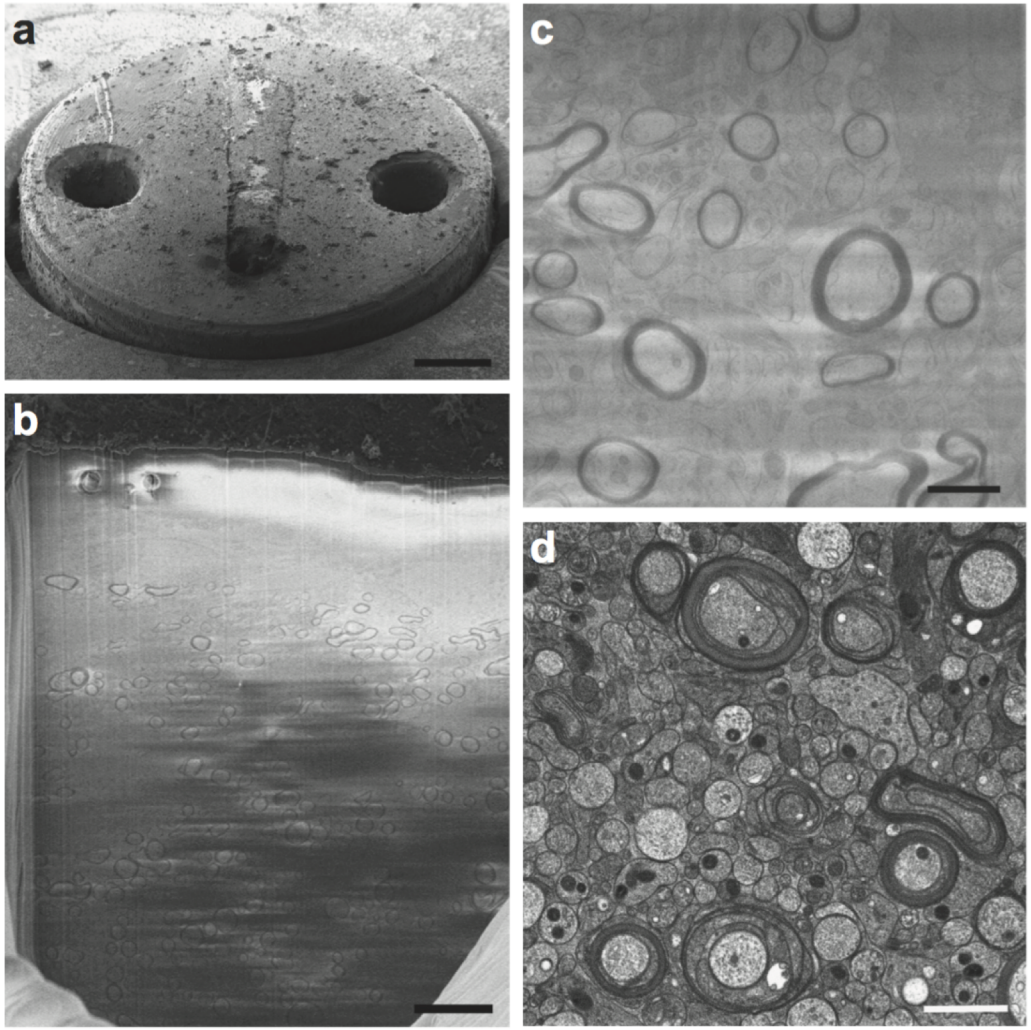
[f] Center for Nanoscale Microscopy and Molecular Physiology of the Brain (CNMPB), Göttingen, Germany

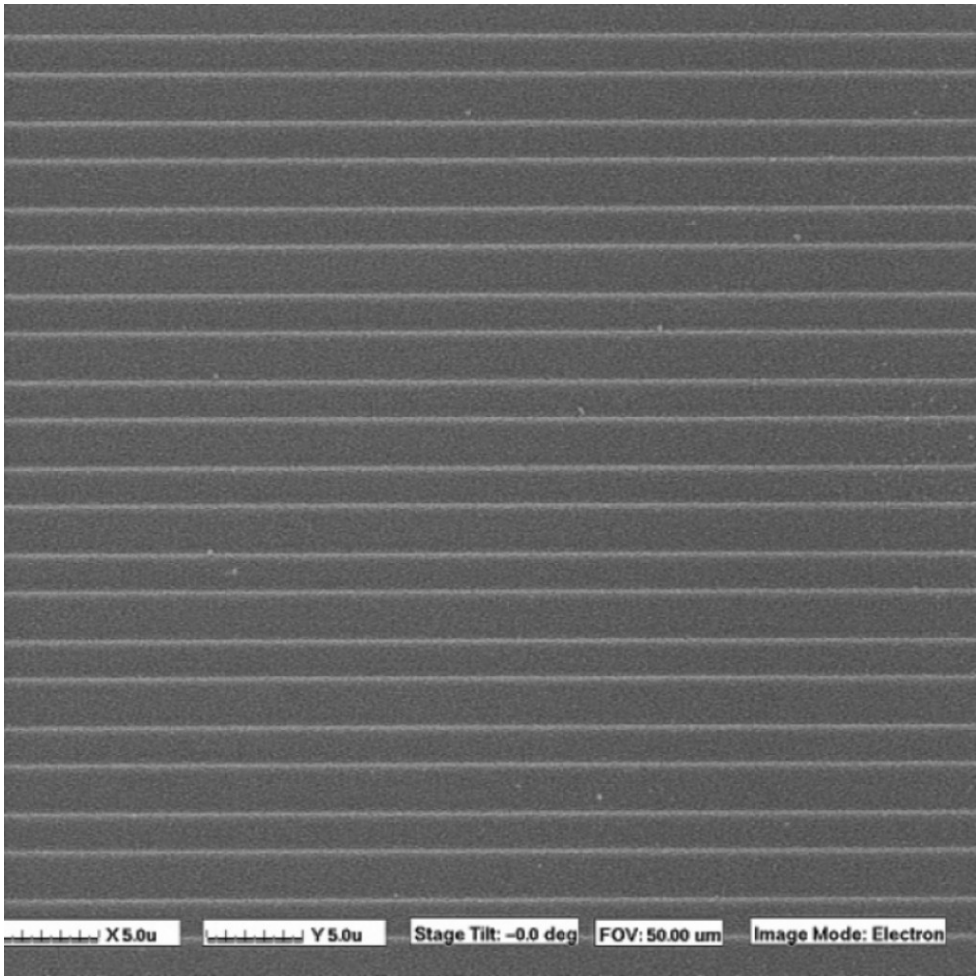
In-lens SE detector at 2.33 kV



Lift Out

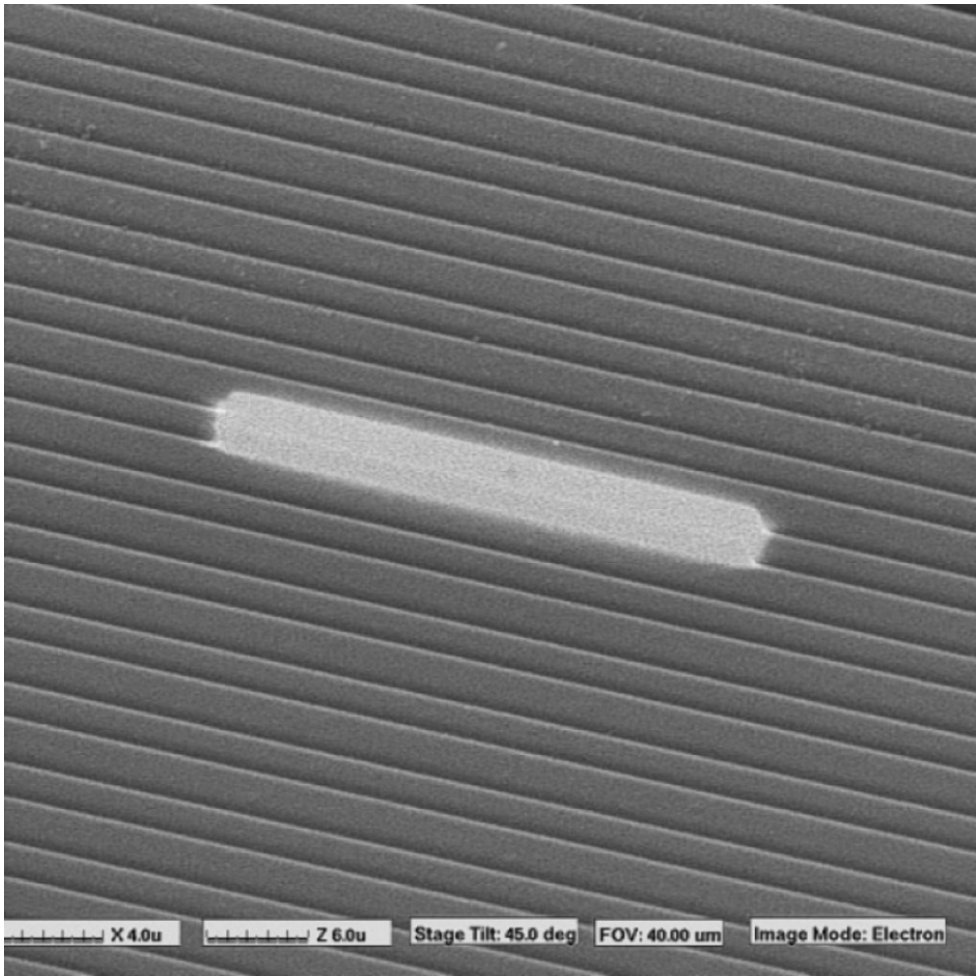
Use of FIB/SEM to prepare bulk material for TEM imaging

Area of Interest

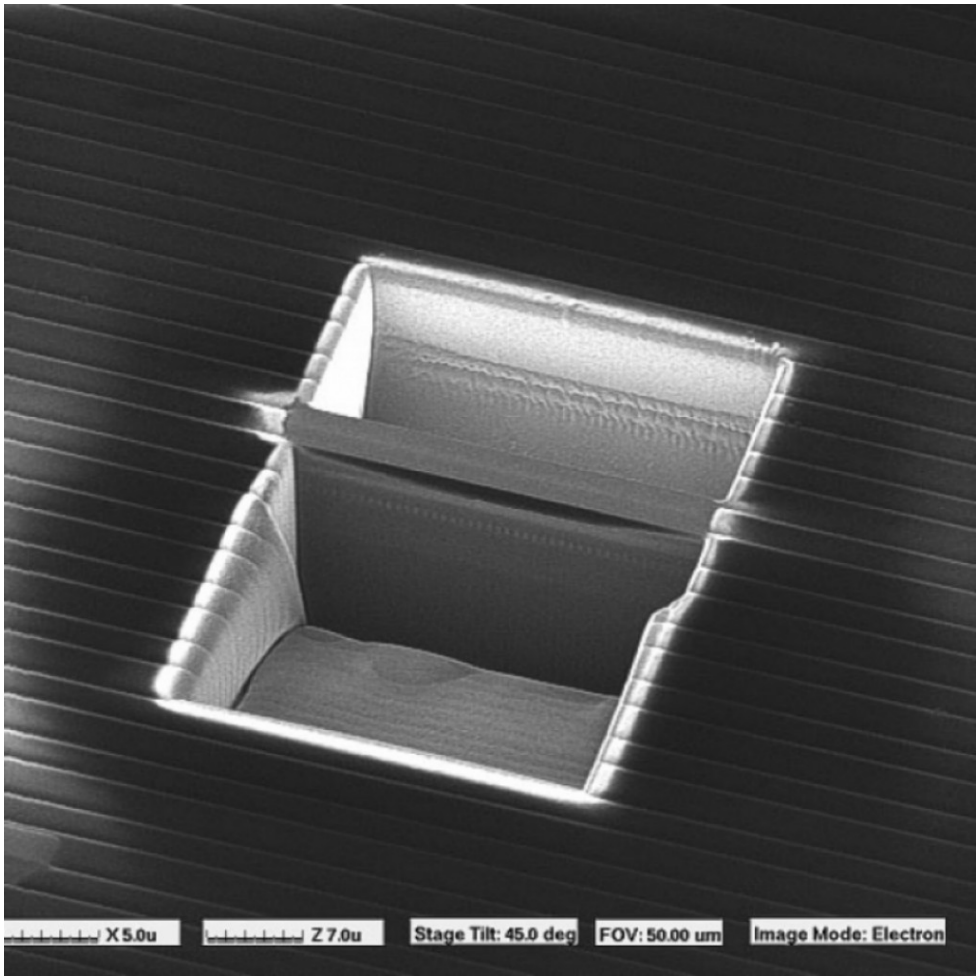


http://www.fibics.com/fib/application/steps-in-tem-specimens-preparation-by-lift-out-method-/24/

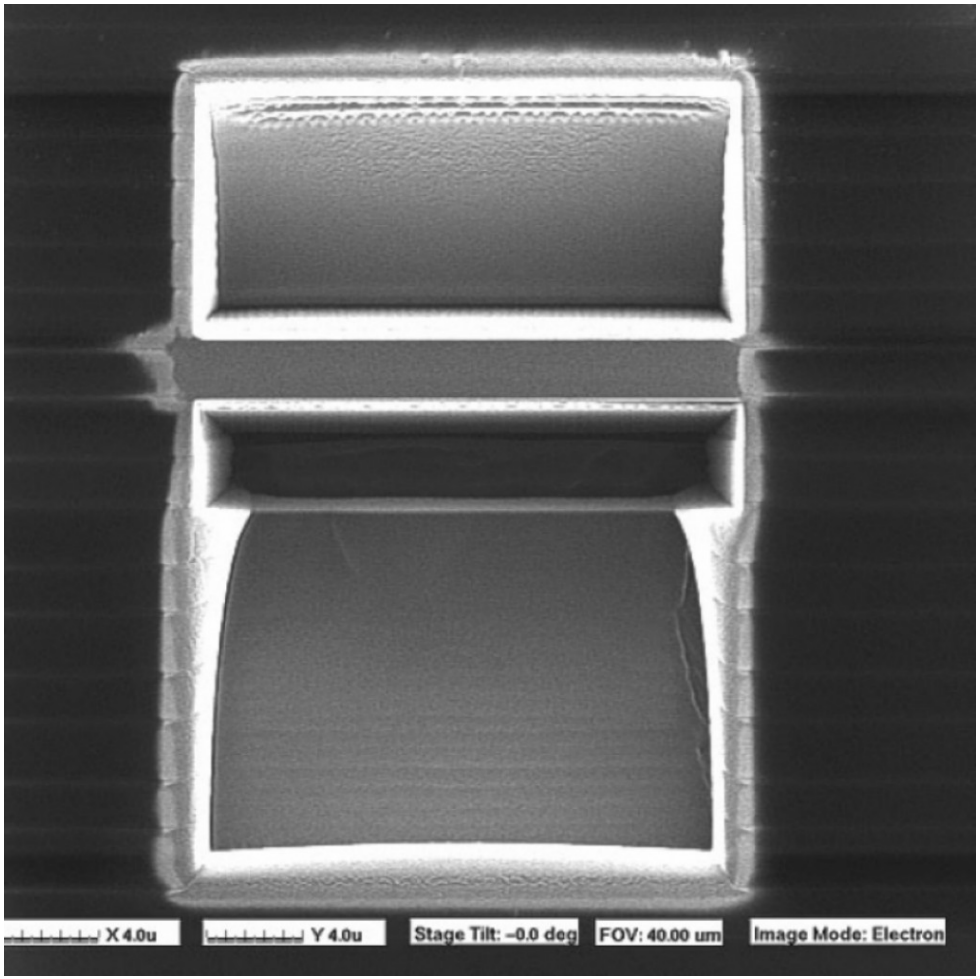
Protect Area of Interest



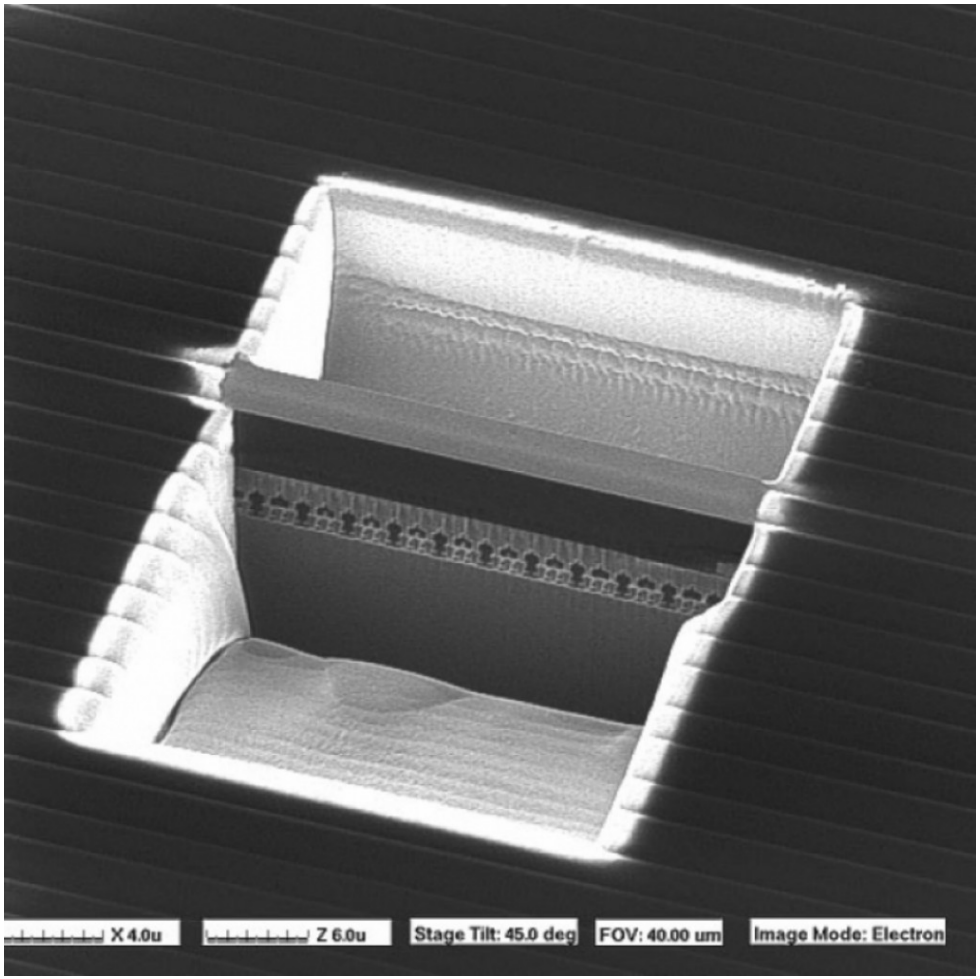
Mill Trenches around area



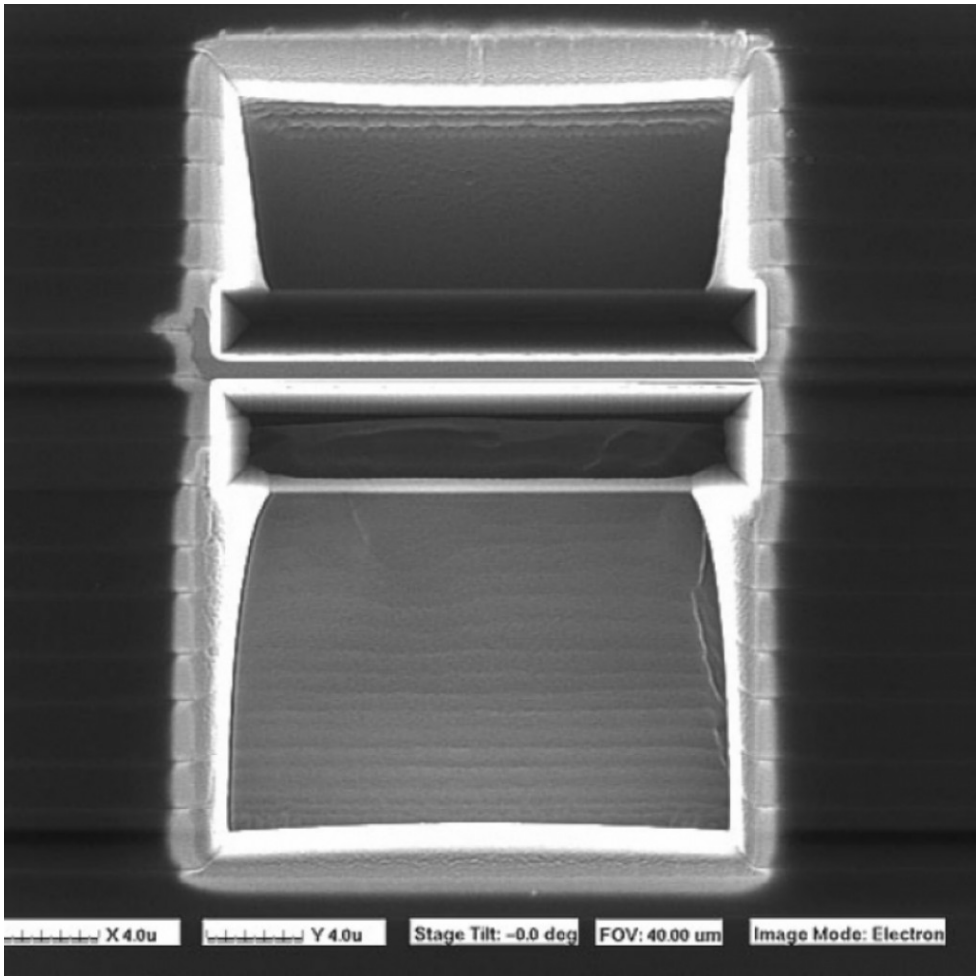
Top View of Trench



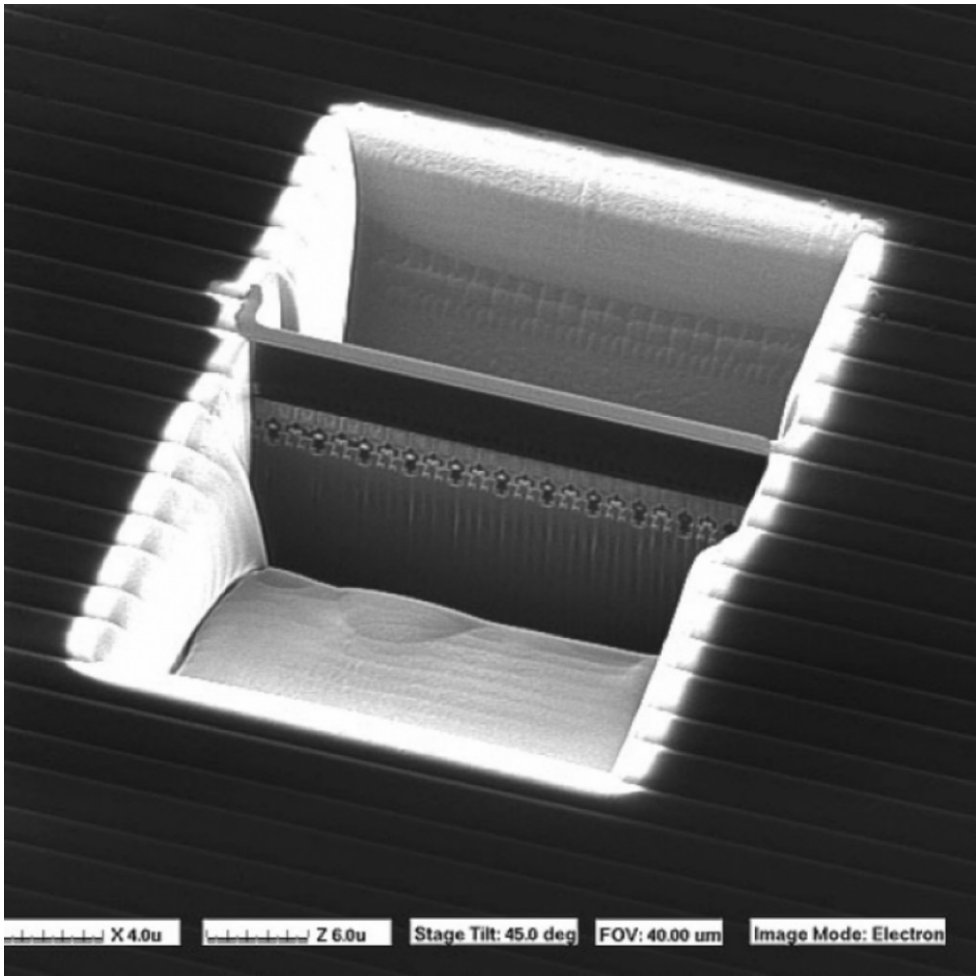
Polish Section



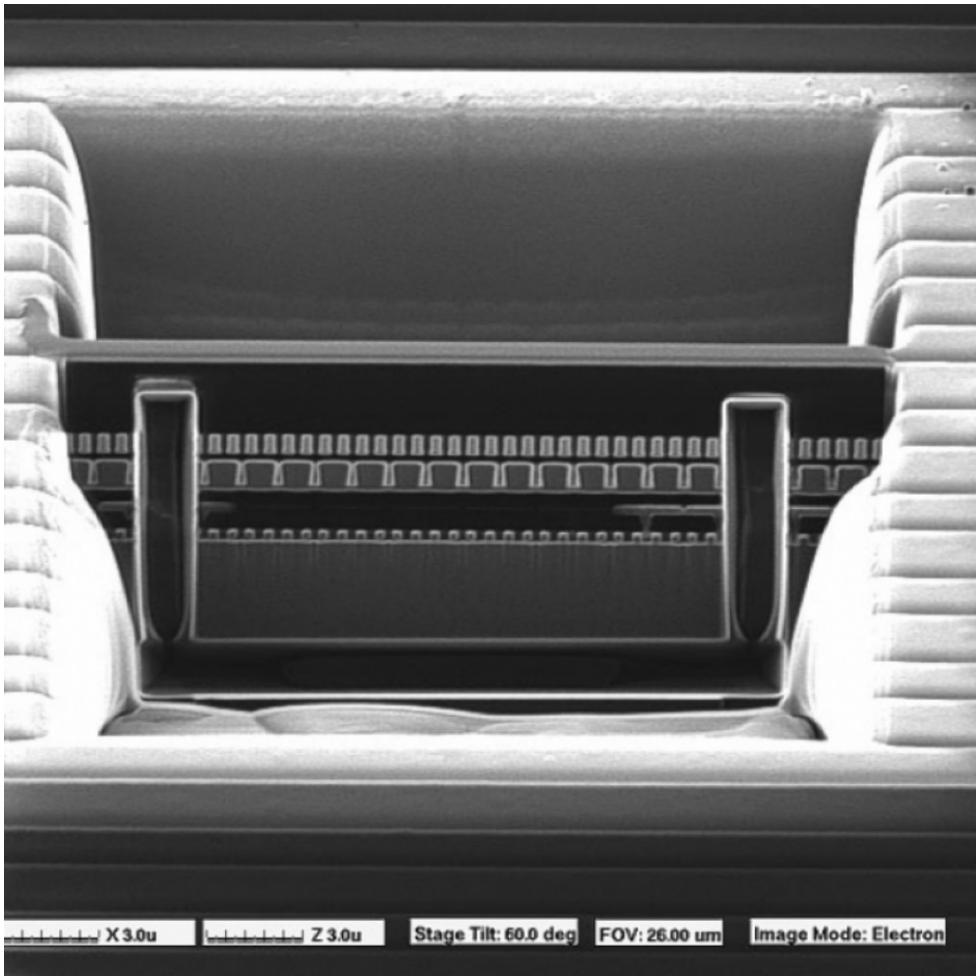
Further Polishing



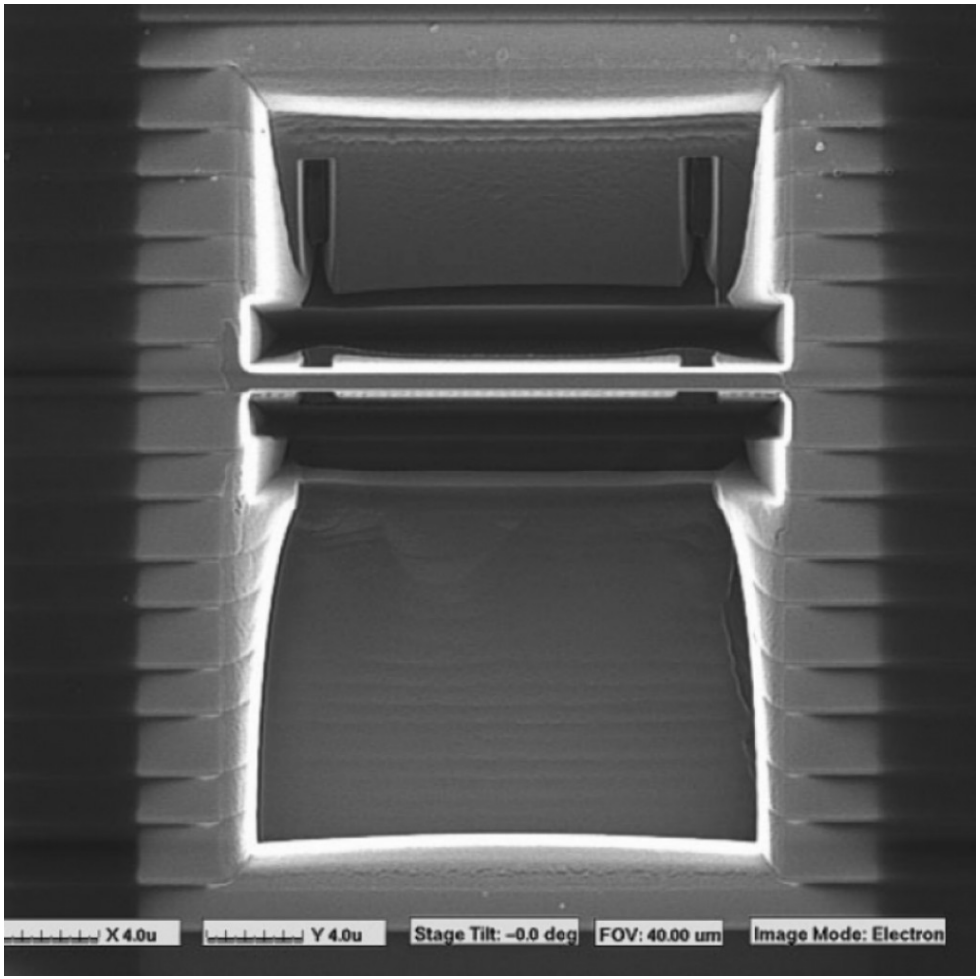
Iso-View of Second Polish



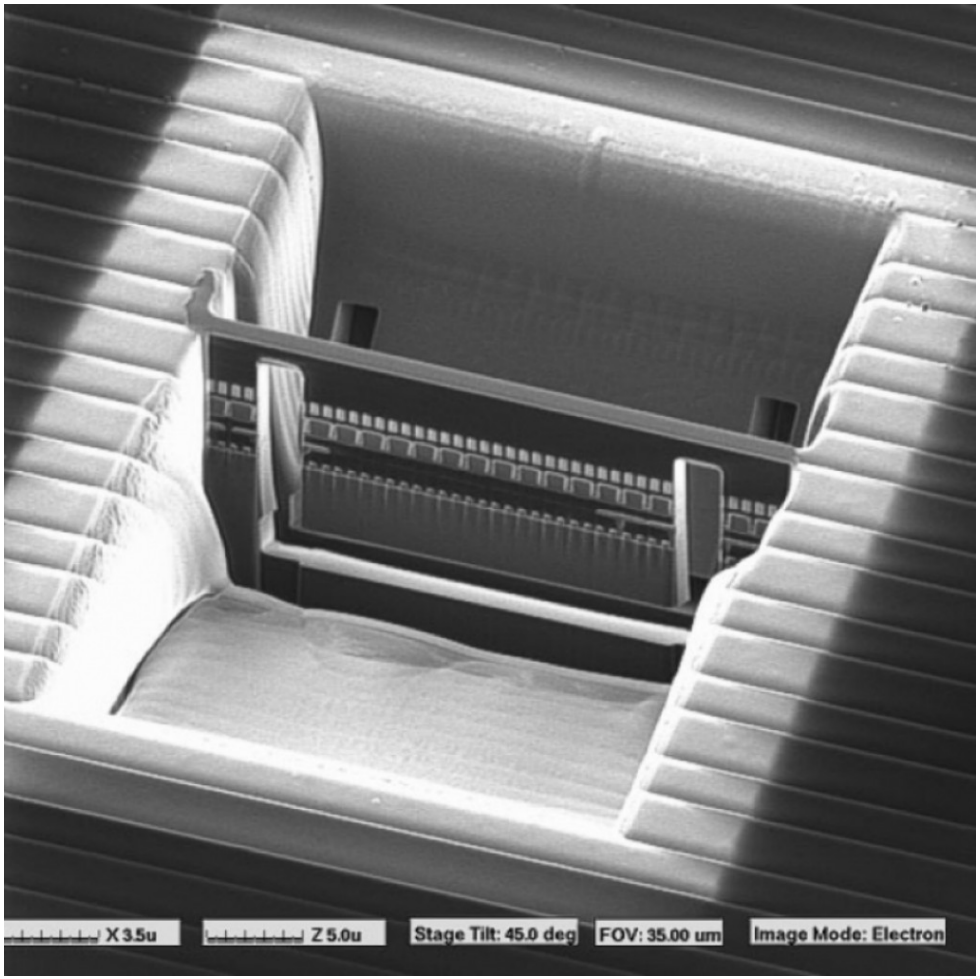
Frame Cuts to Define Area for Removal



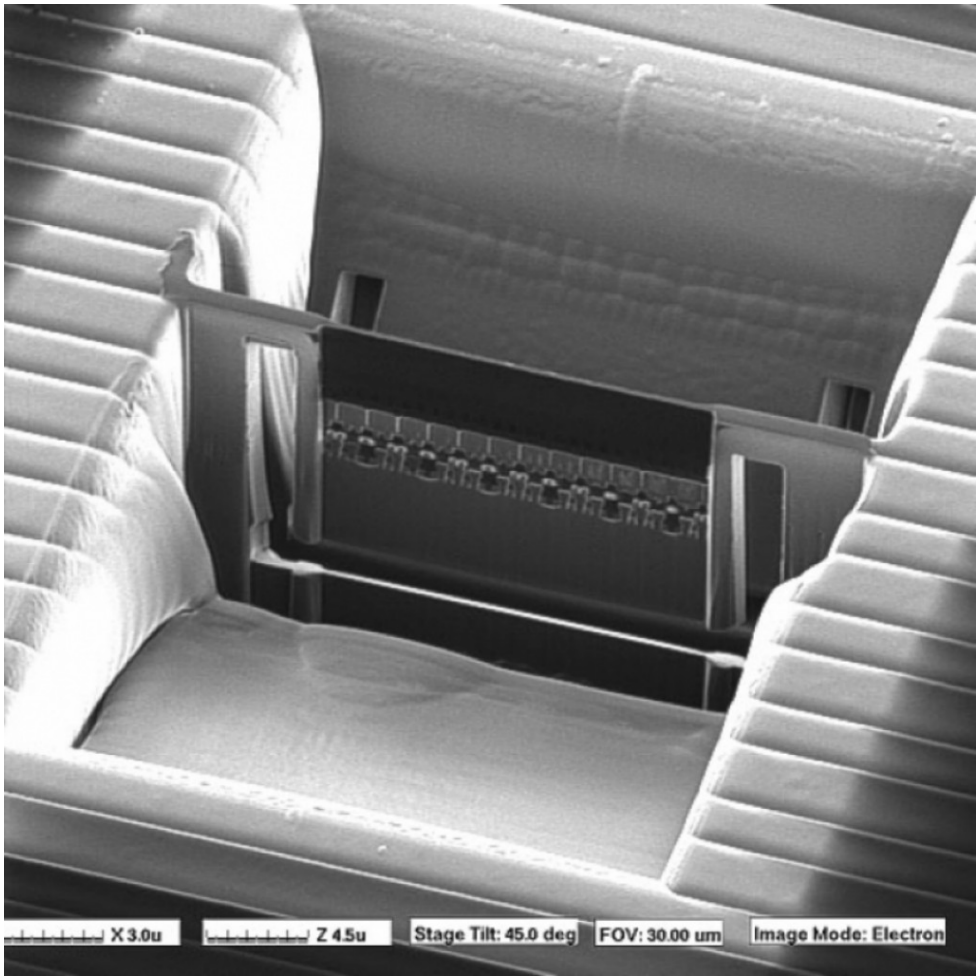
Top View of Frame Cut



Iso-View of Frame Cut

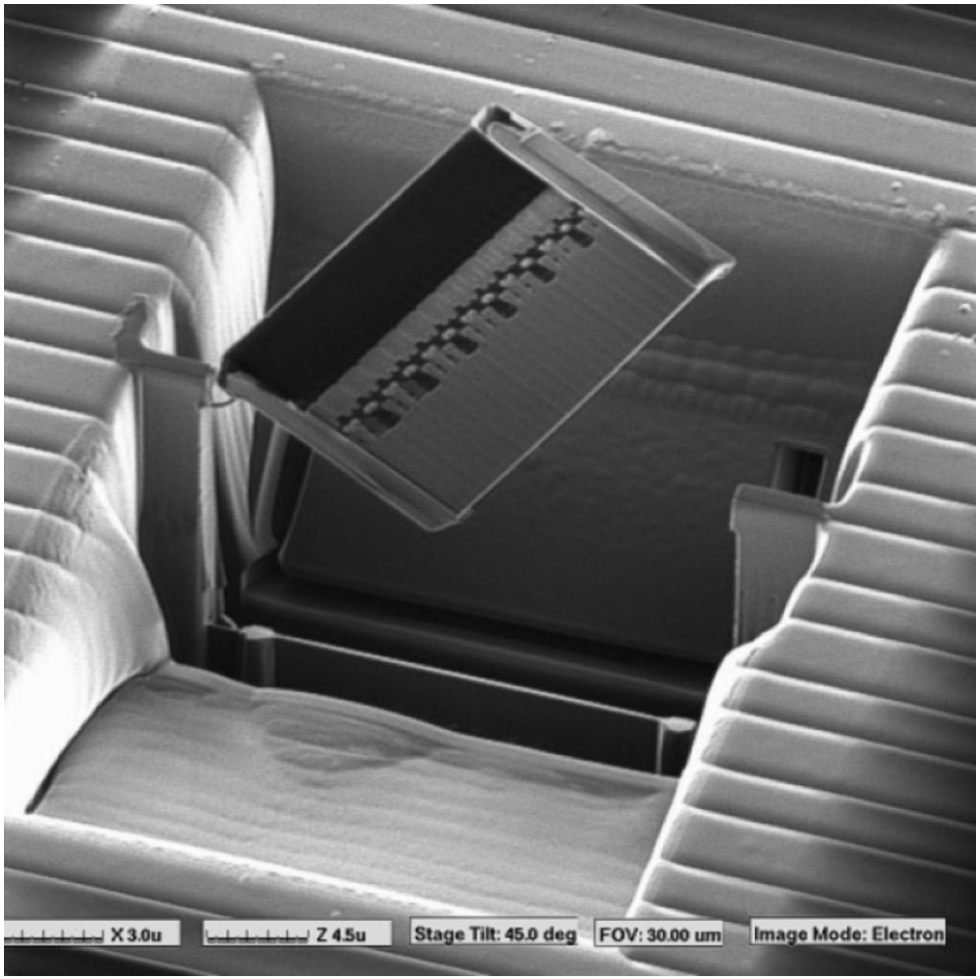


Thin to Electron Transparency



Top View just before Removal

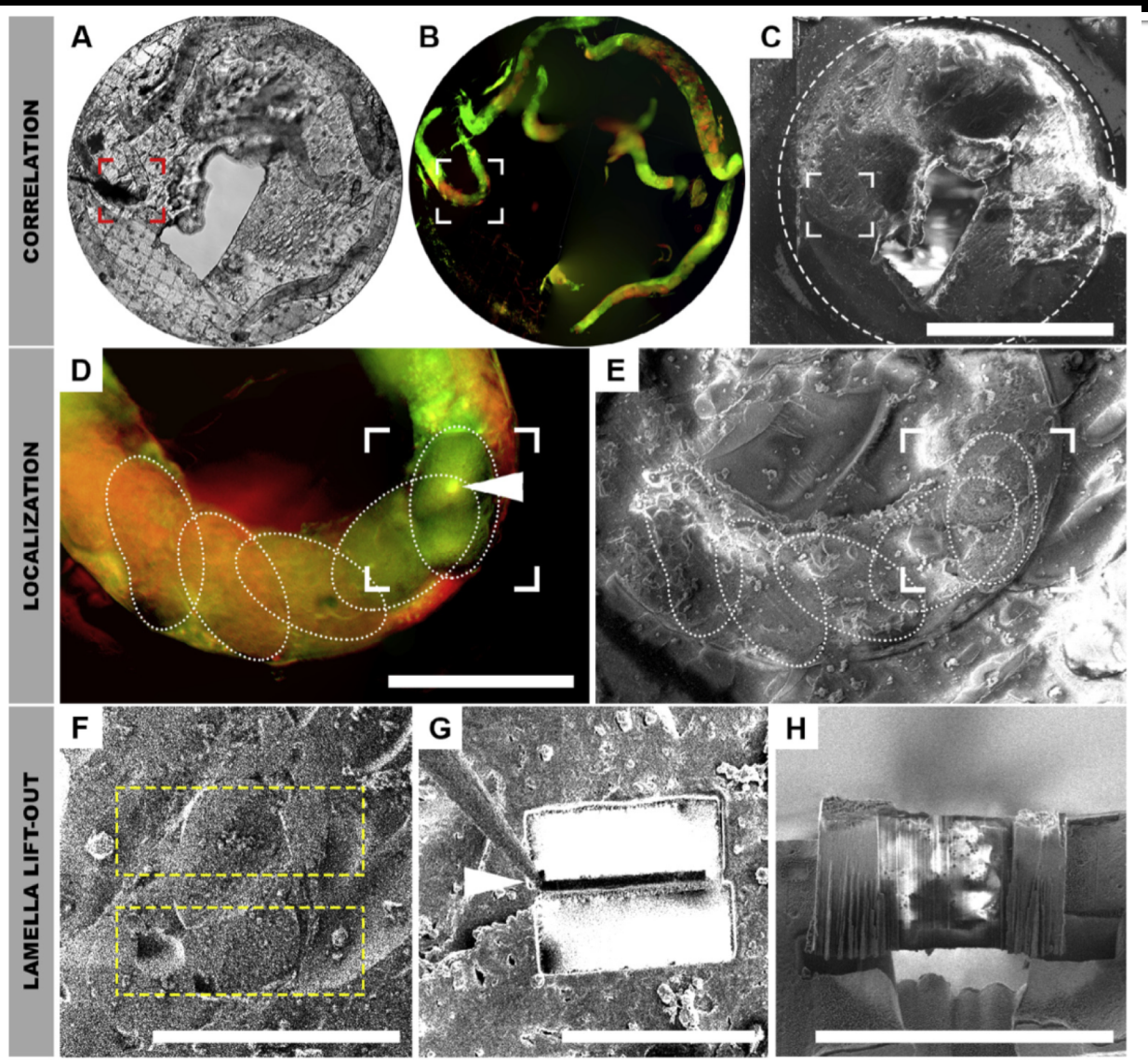
Remove Section and attach to manipulator (not shown)



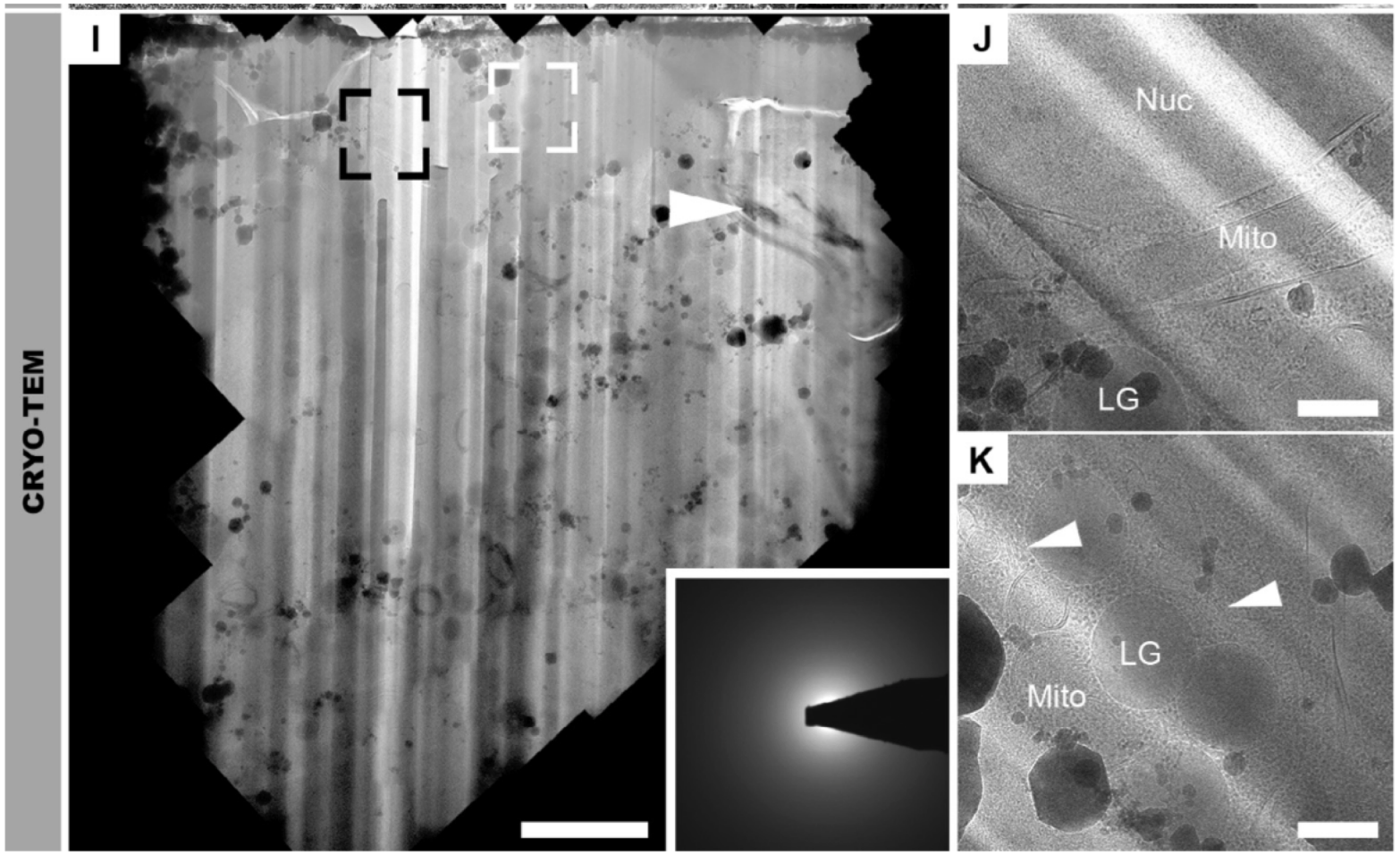
Applications to Cryo

- 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.

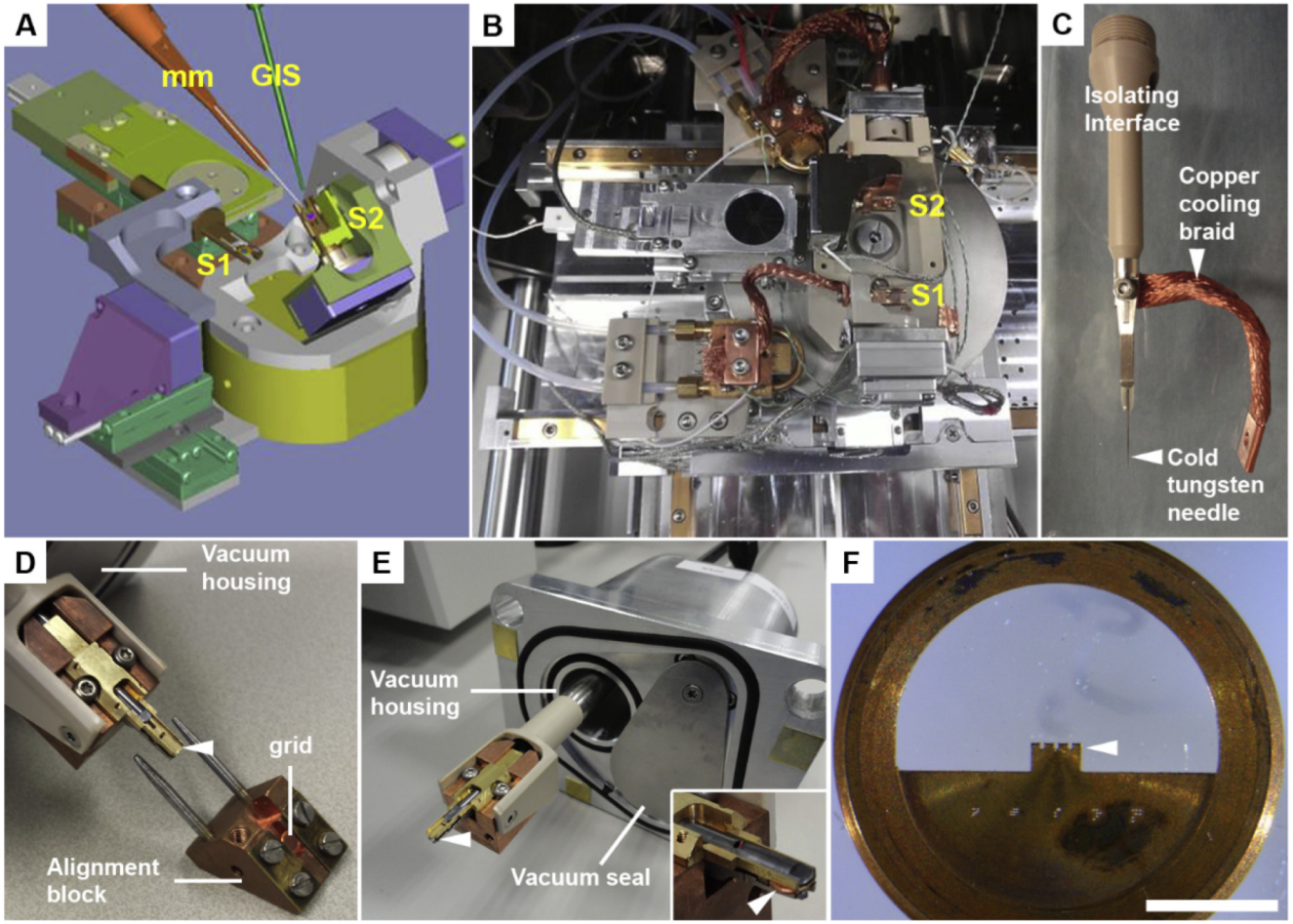
C Elegans Embryo HPF on grid



TEM



Equipment Needed



Cryo-Liftout

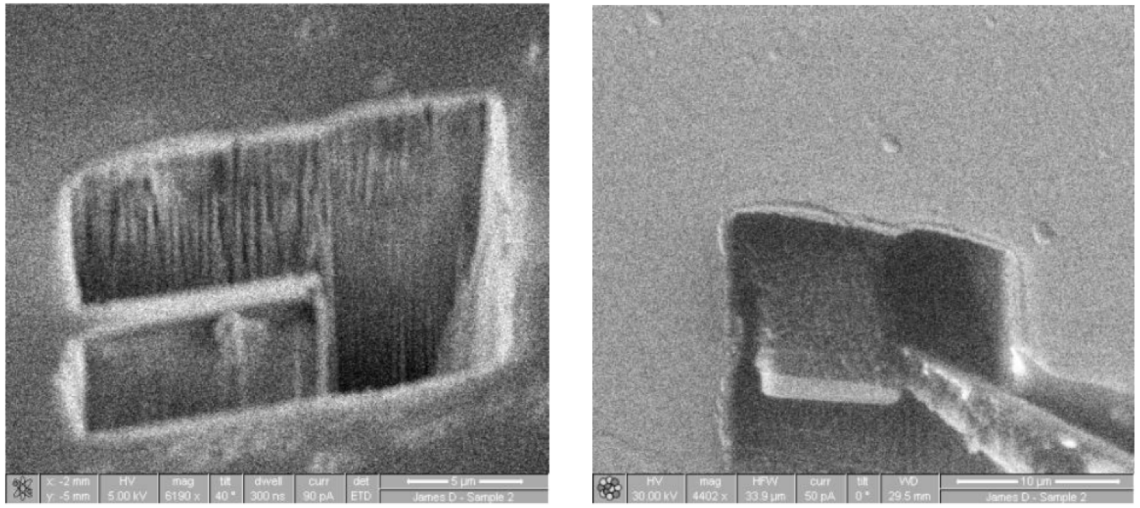


Figure 1. (Left) Cryo-FIB milling of a bulk sample to prepare a thin lamella, scale bar 5 µm. (Right) extraction of the lamella by the cooled manipulator after attachment and release of lamella, scale bar 10 µm.

Parmenter et al Microscopy Microanal (2014)

Cryo-Liftout

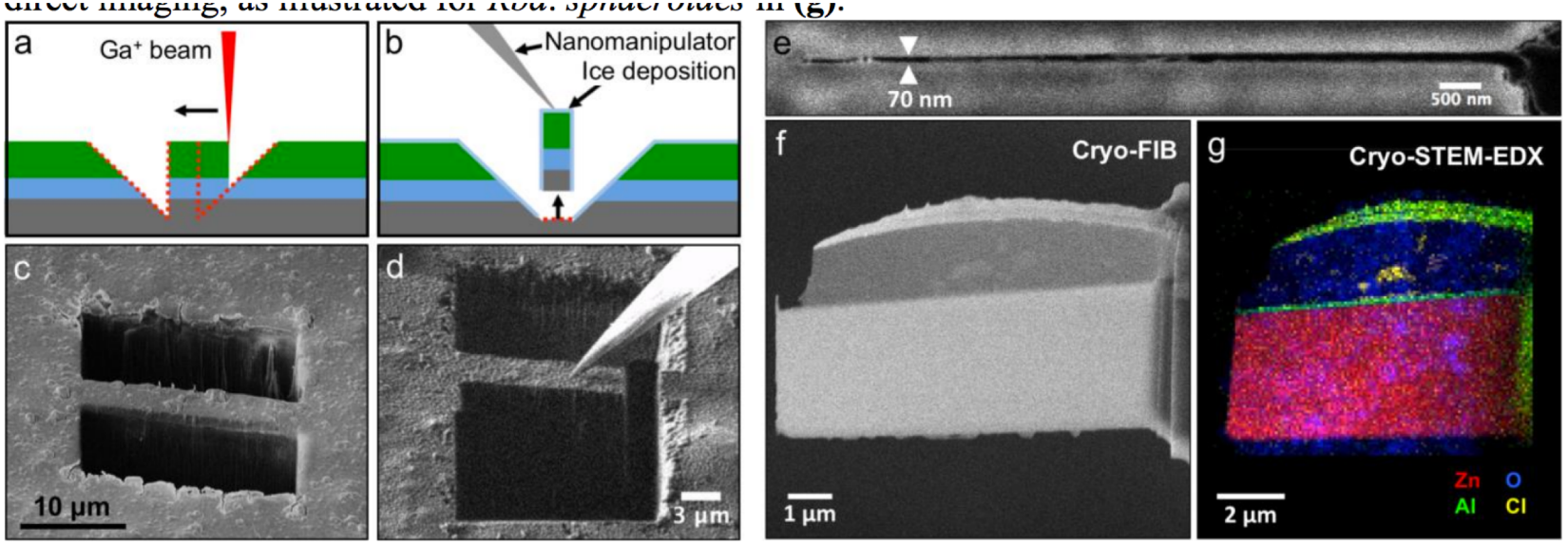


Figure 2. Preparation of electron transparent lamellas of frozen-hydrated specimens by cryo-FIB lift-out. **(a, c)** Site-specific milling of a lamella from the vitrified sample. **(b, d)** Attachment of the frozen lamella to a liquid nitrogen cooled nanomanipulator using water vapor. After similar attachment to a cooled TEM grid, the lamella is thinned to electron transparency. **(e)** A top-down image of a lamella created from a solid–liquid interface shows sample thinning to <100 nm. The final sample **(f)** was subsequently transferred to the cryo-STEM for nanoscale chemical analysis by EDX **(g)**.

Questions