Electron Microscopy

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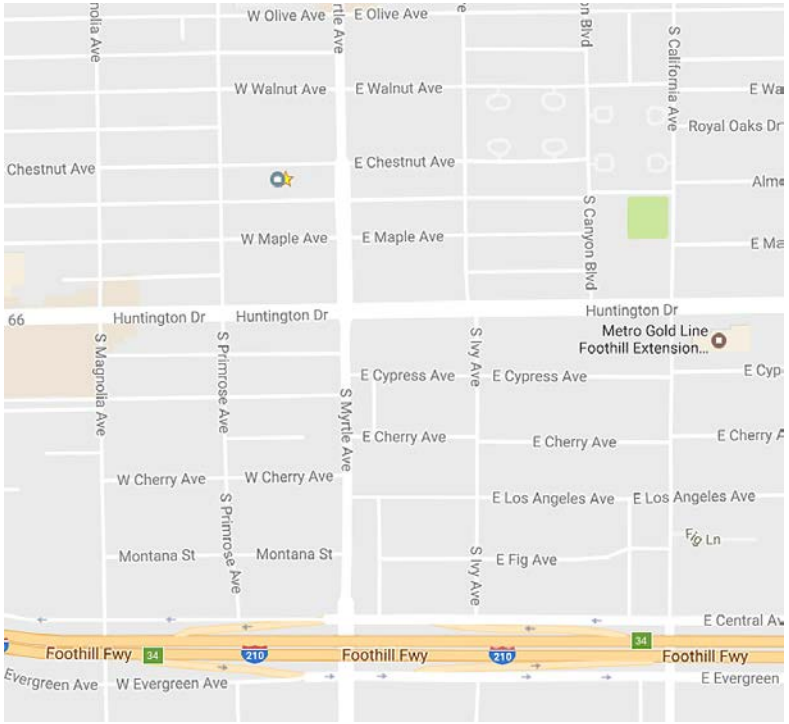
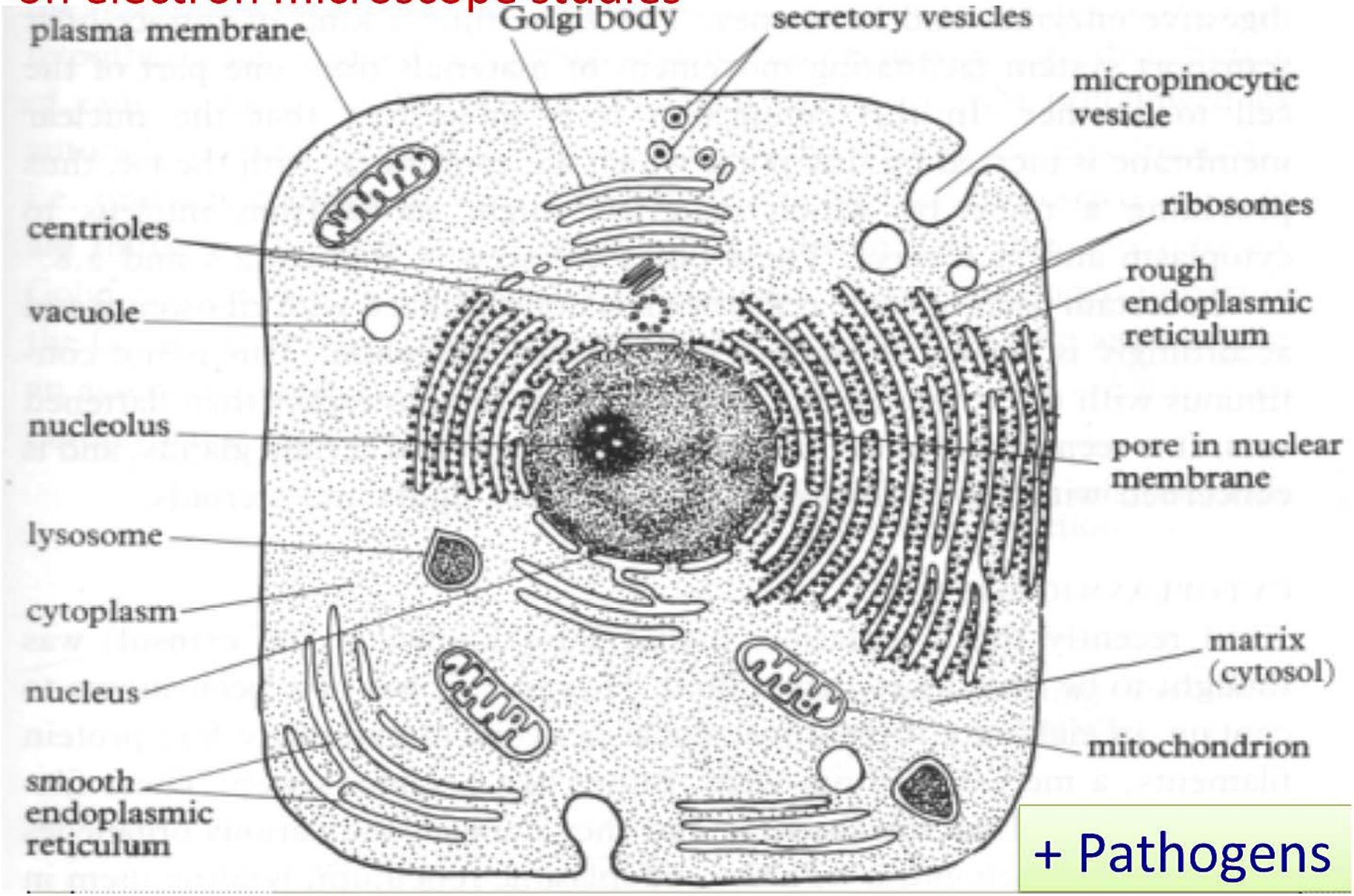
Oak Crest Institute of Science

- 132 W. Chestnut Avenue, Monrovia, CA 91016



- An innovative chemistry research and education center that provides community college and high school students with first hand experience of scientific research in an academic environment.

Our knowledge of the ultrastructure of an animal cell- based on electron microscope studies



Who “invented” the electron microscope?

- 1897: J.J. Thompson
  - Electrons (cathode rays, electricity, atoms)

J.J. Thompson

- 1924: Luise de Broglie
  - Wave nature of electrons ($\lambda = h/mv$)

Luise de Broglie

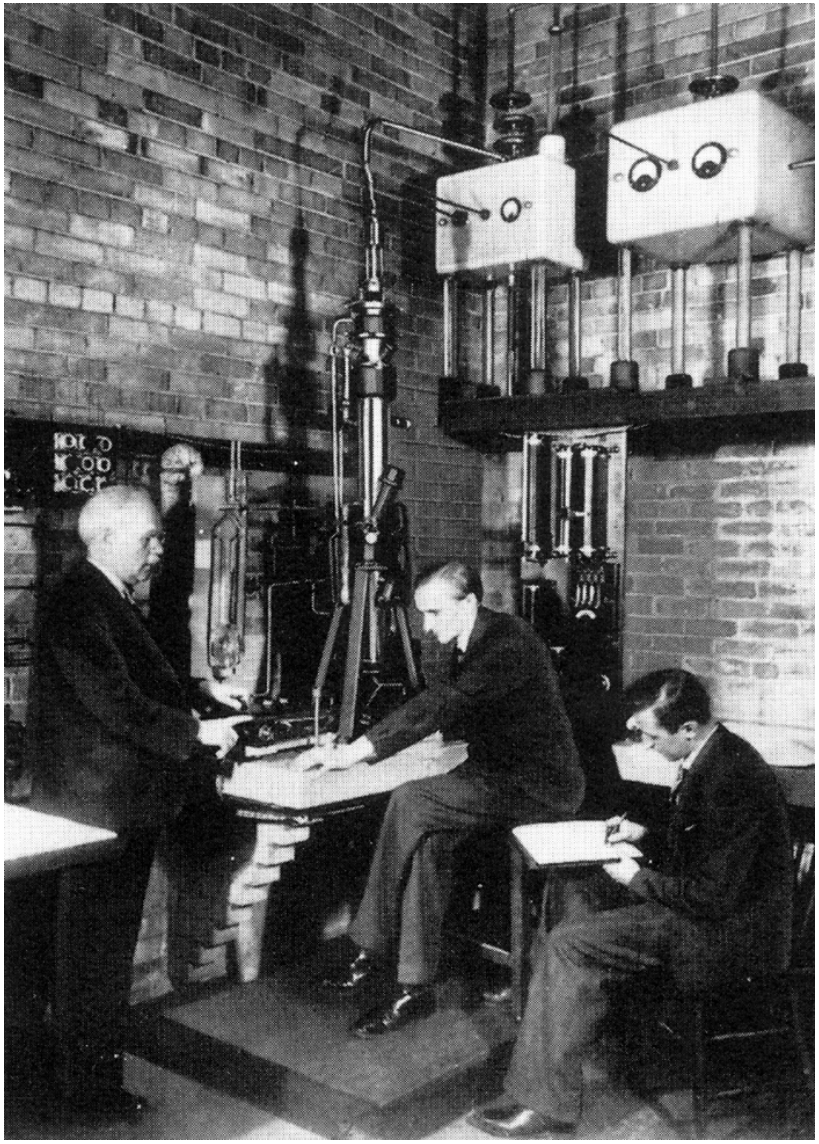
Thomson: 1906 Nobel Prize in Physics
De Broglie: 1924 PhD thesis – 1929 Nobel Prize in Physics

Building an electron microscope

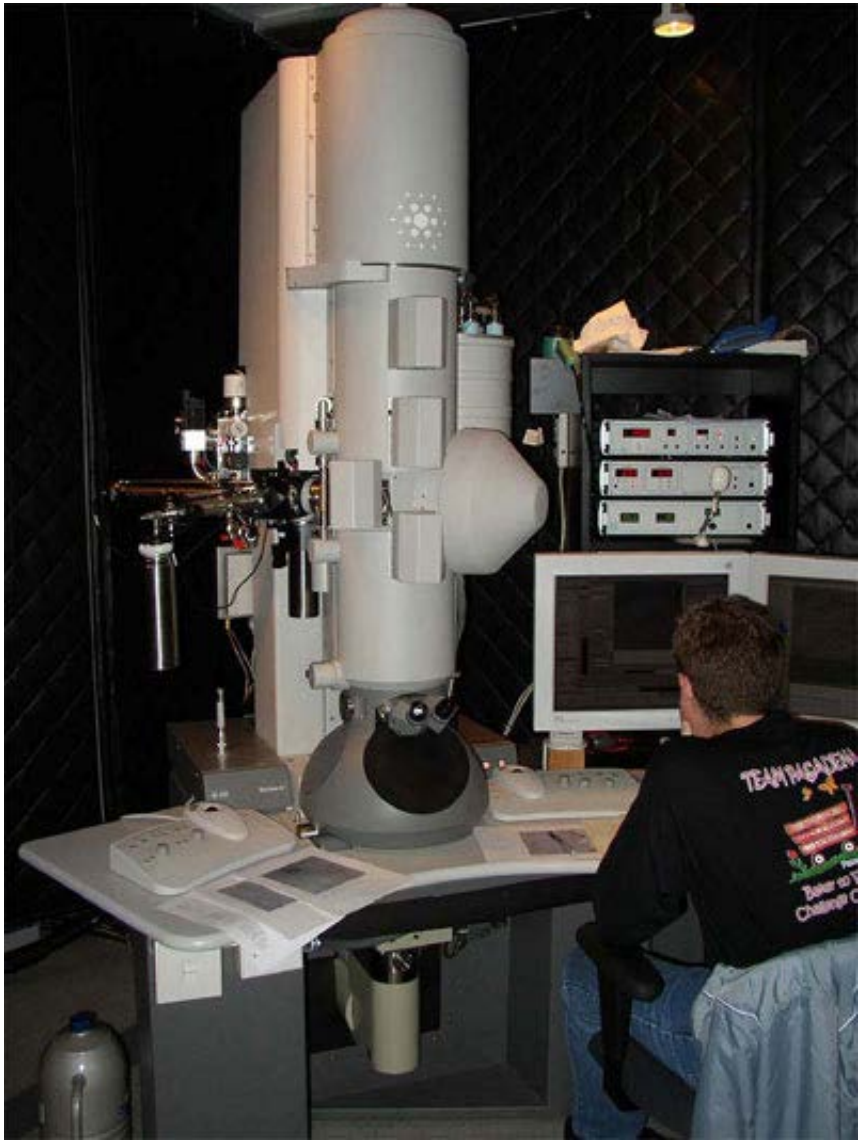
Ernst Ruska & Max Knoll
built the first EM in 1931

Ruska: Nobel Prize 1986

Electron microscopes have changed…



1940's



2015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Electron Microscopy

- It's all about the resolution

Abbe's equation:

$$d = \lambda/(2n \sin \theta)$$

Abbe limit is approx

$$\lambda/2$$

Green light λ = 500nm d = 250nm

TEM resolution

$$l = \frac{h}{m * v}$$

l = wavelength
h = Planck's constant (6.6×10^{-27})
m = mass of the particle (9.1×10^{-28})
v = velocity of the particle

De Broglie equation

$$d = \frac{0.753}{a\, V^{1/2}}$$

d = resolution in nm
a = half aperture angle
V = accelerating velocity

Abbe's equation

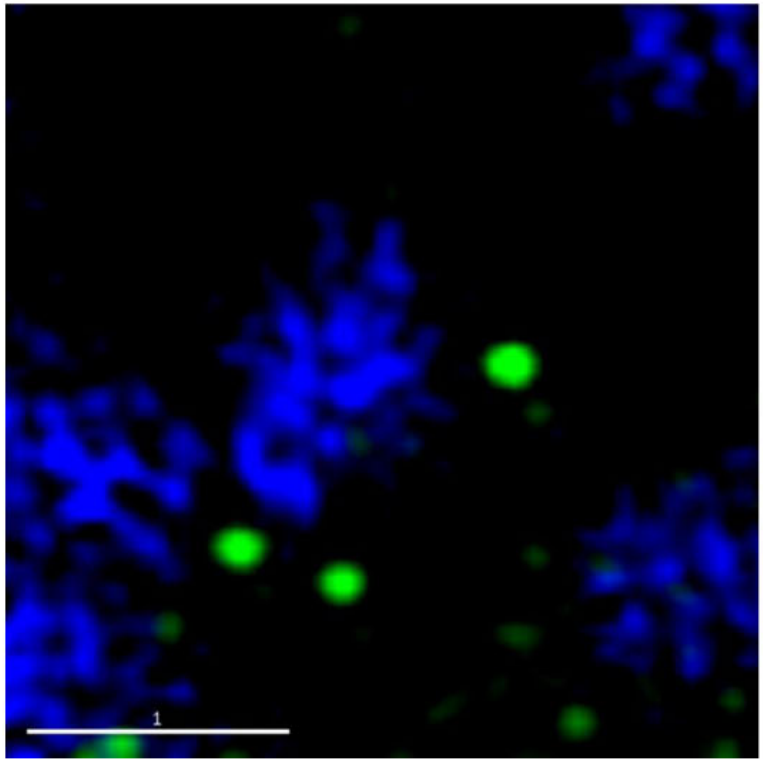
Translation:
at 100kV accelerating voltage resolution is 0.24nm

Higher accelerating voltage : better resolution

But, is it all about the resolution?

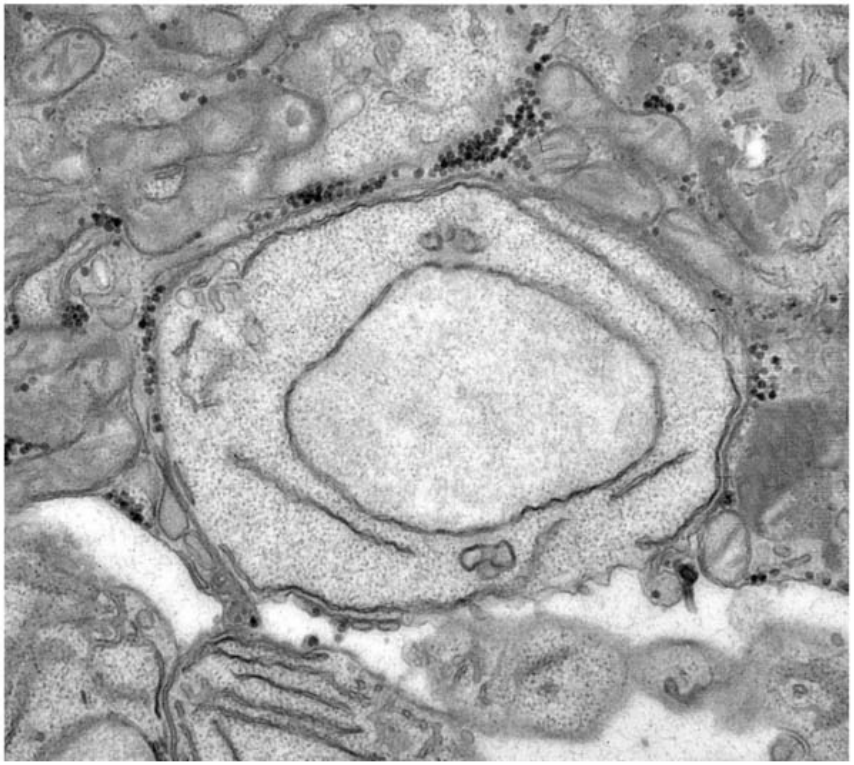
Reference space is important too

50 – 200 nm resolution



Superresolution OMX

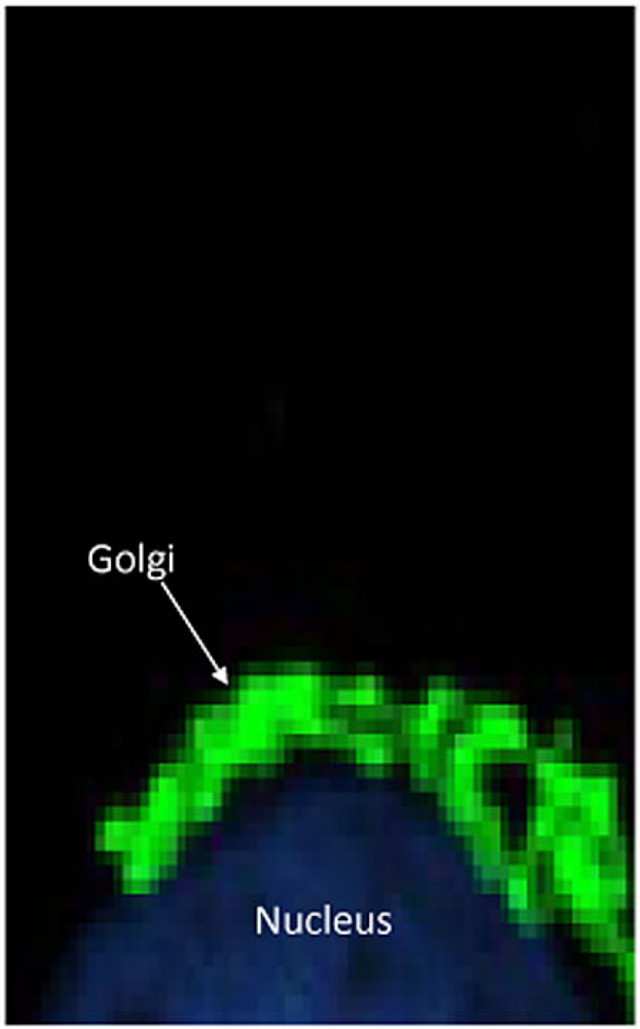
nm resolution

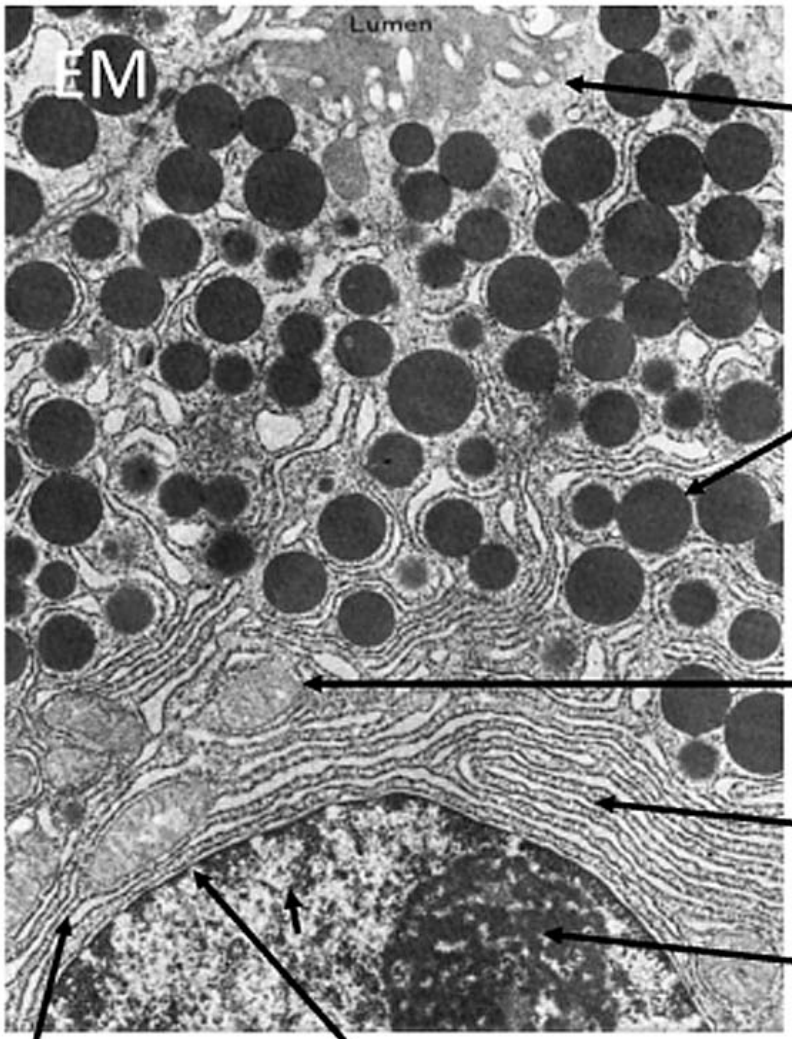


TEM, resin-embedded material

But, is it all about the resolution?

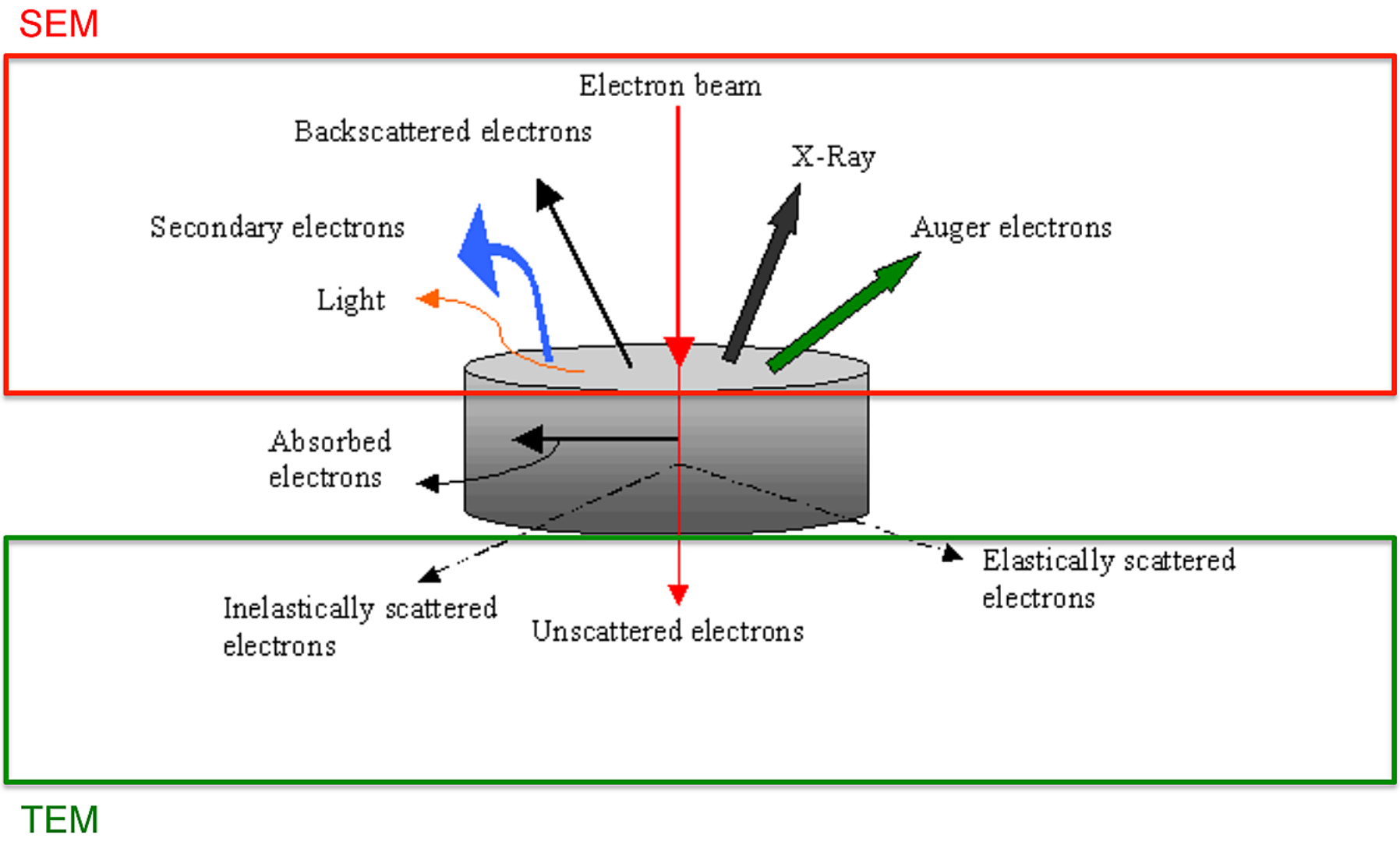
Reference space is important too





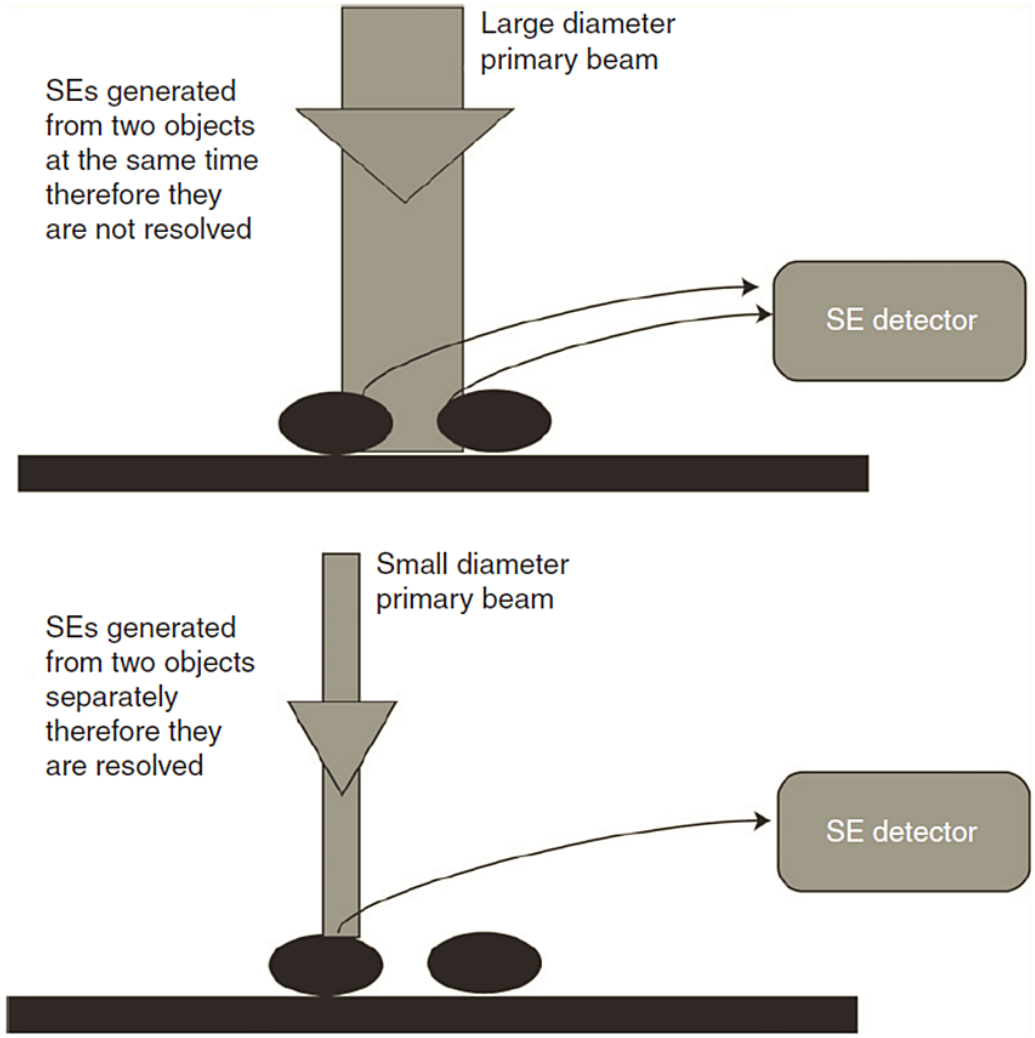
Pictures from "The Cell" by Don Fawcett

Electron–specimen interactions



http://infohost.nmt.edu

Scanning Electron Microscope

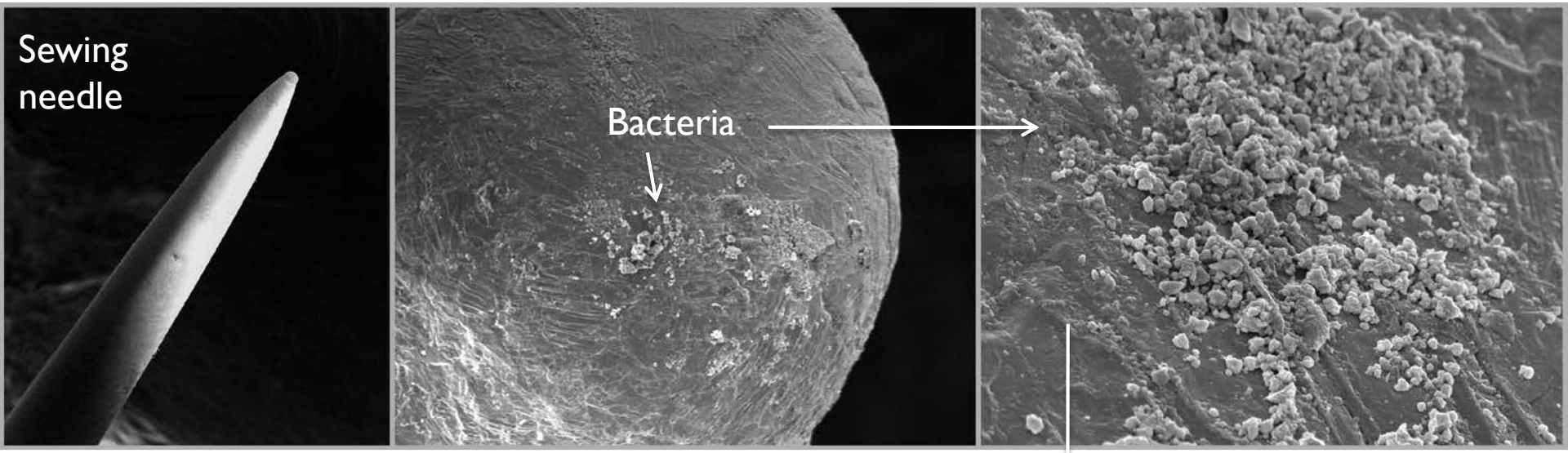


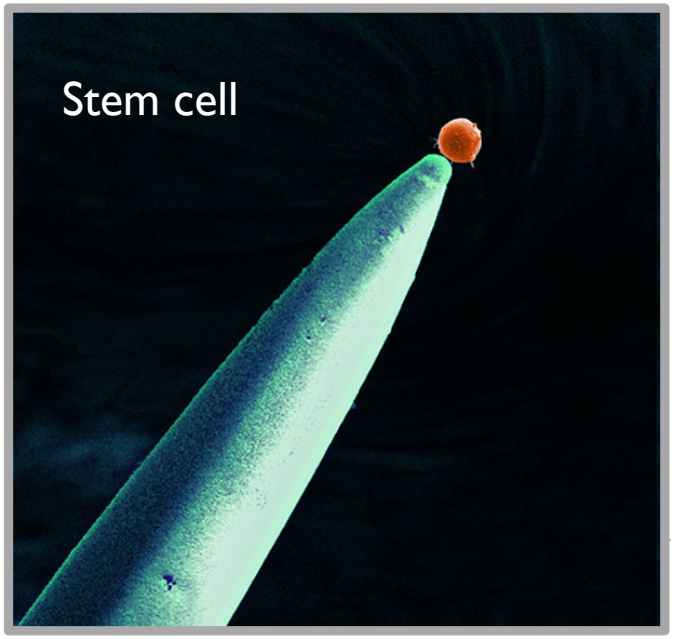
Resolution

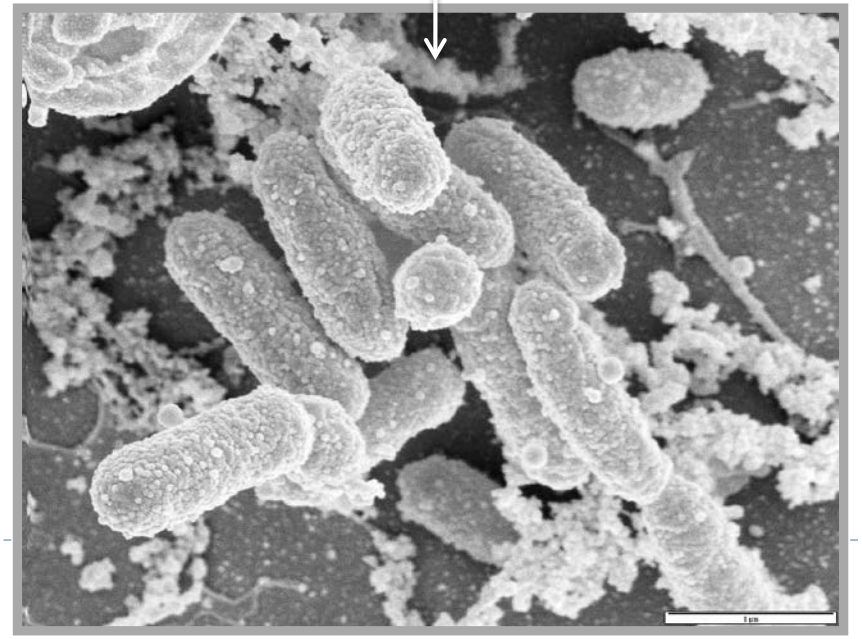
Determined by the diameter (d) of the primary electron beam

Resolution = 1-15 nm

Scanning electron microscopy (SE imaging)







Cells & Electron Microscopes: a paradox

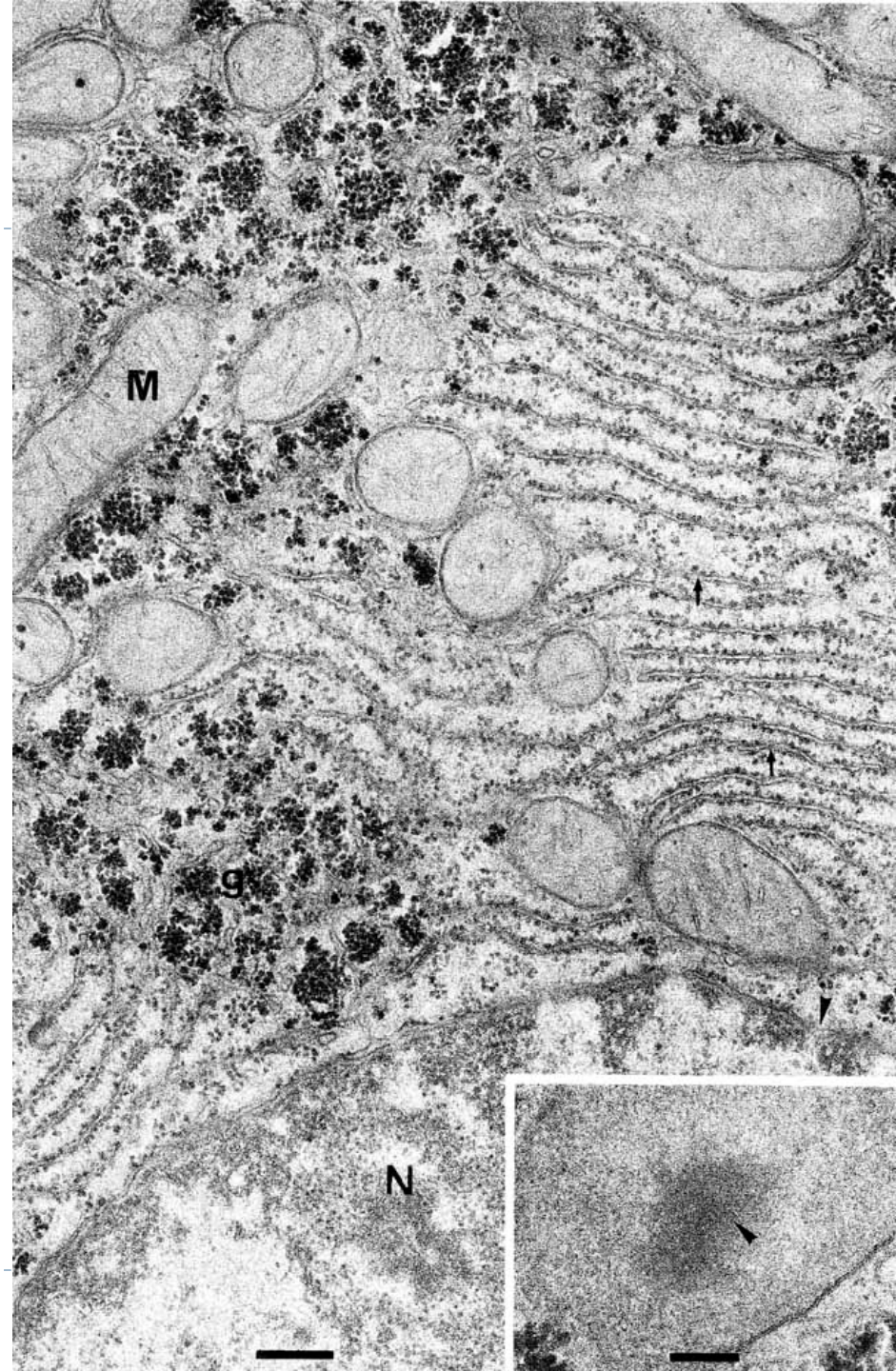
CELLS	ELECTRON MICROSCOPES
Mostly water	Work under vacuum
Mostly C, O, N (low atomic #)	Work best with high atomic #
3-dimensional	Work best with thin samples

<u>Initial Solution: 1945:</u>
Dry cells
Heavy metal processing
Image thin parts

Porter, Claude & Fullam 1945 J. Exp. Med

Specimen Preparation

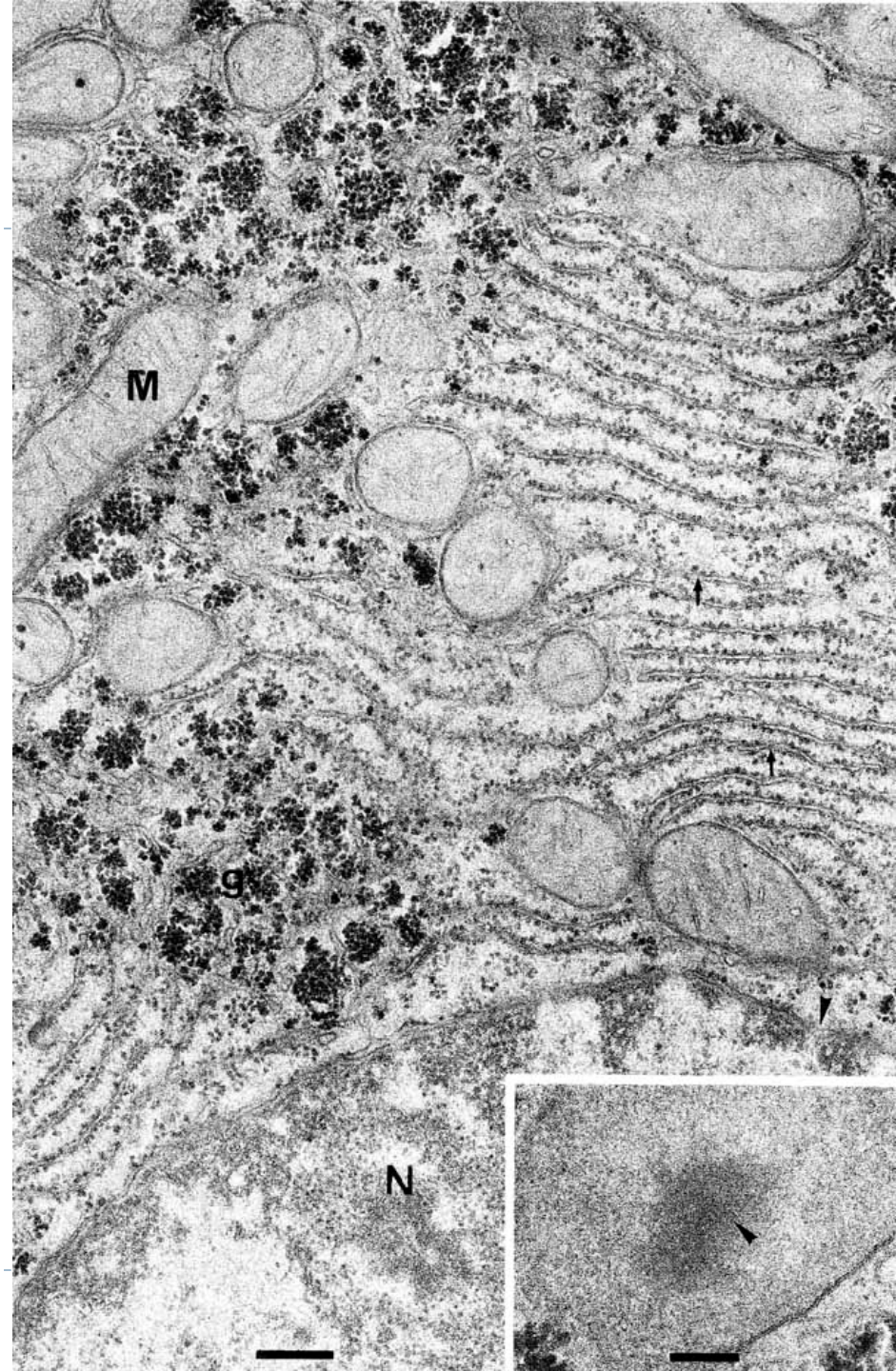
- **Conventional EM preparation**
  - Chemical crosslinking (fixation)
  - Heavy metal salts
  - Drying with ethanol or acetone
  - Infiltration in epoxy resin
  - Polymerization at 60°C
  - Thin slicing



Specimen Preparation

- **Conventional EM preparation**

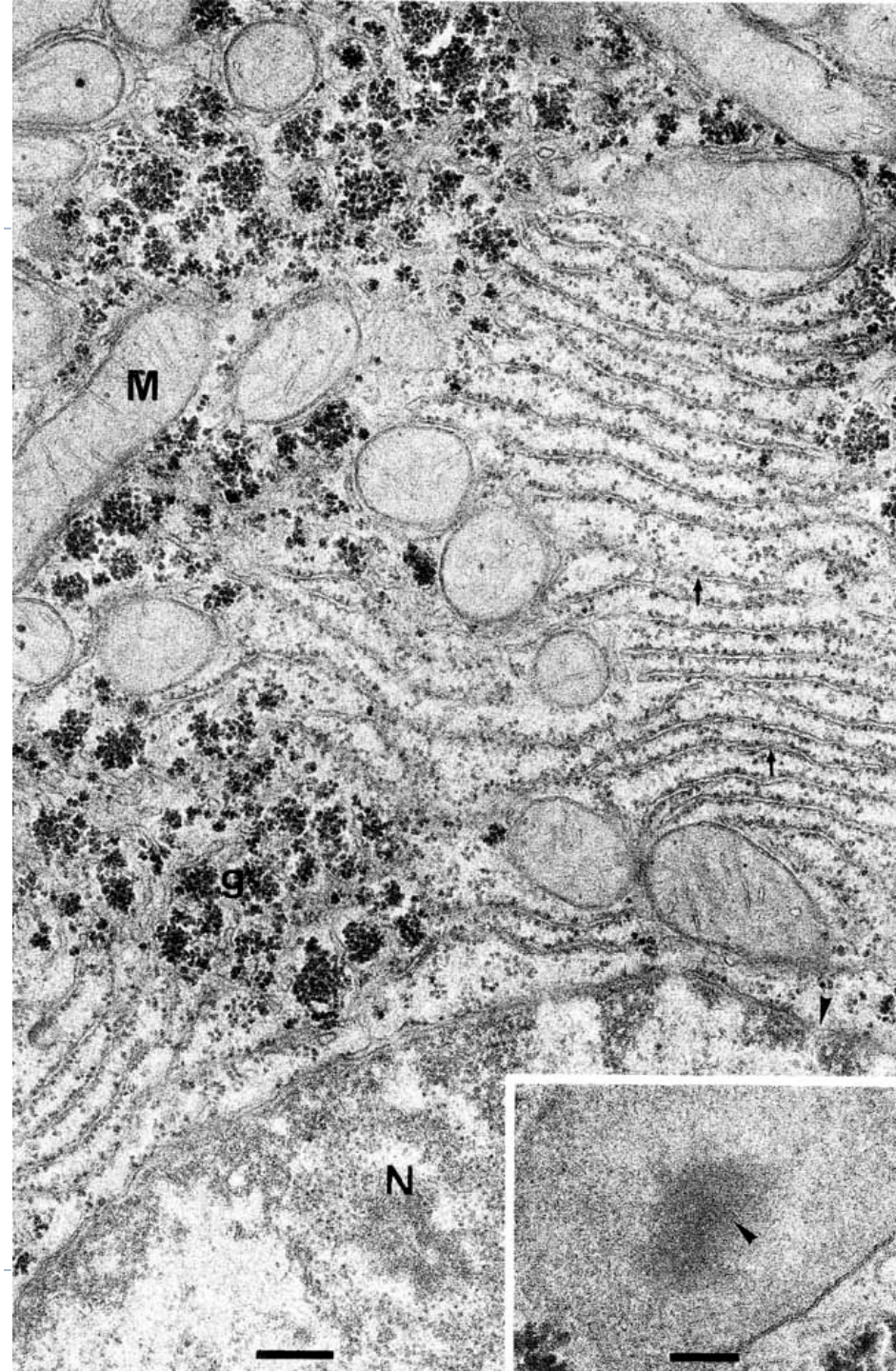
- <u>Advantages</u>
  - Convenient
  - Relatively simple
  - Well known
  - Images match textbooks
  - Organelles easily identified



Specimen Preparation

- **Conventional EM preparation**

- Disadvantages
  - Is it alive?
  - Does specimen change?
  - Is fixation rapid?
  - 2-dimensional
  - Temporal event
  - Is high-resolution detail preserved?
  - Immunolabel?



Is specimen resolution affected by preparation?

Chemical fixation is not rapid

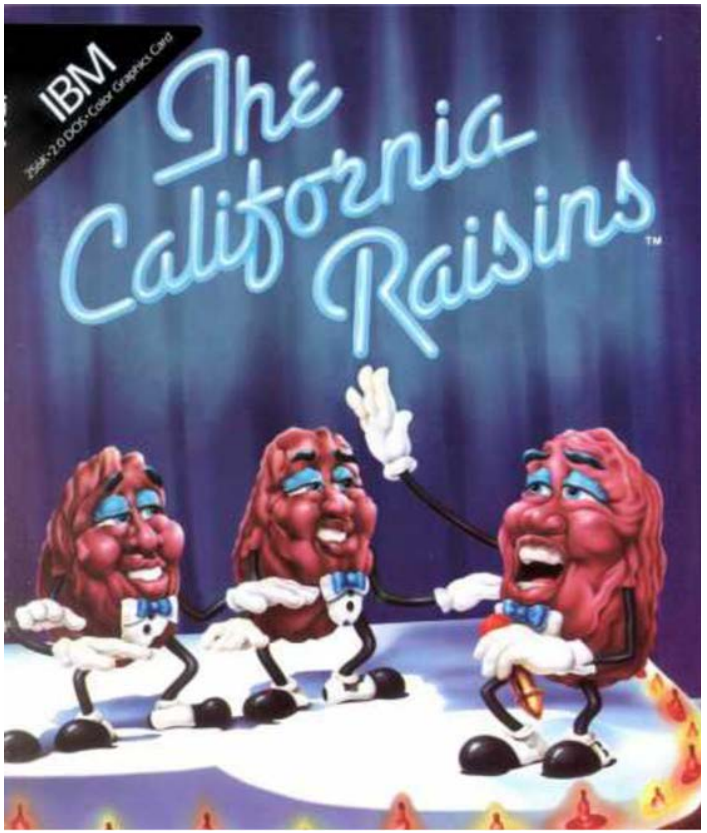
C. elegans immersed in 2.5% glutaraldehyde

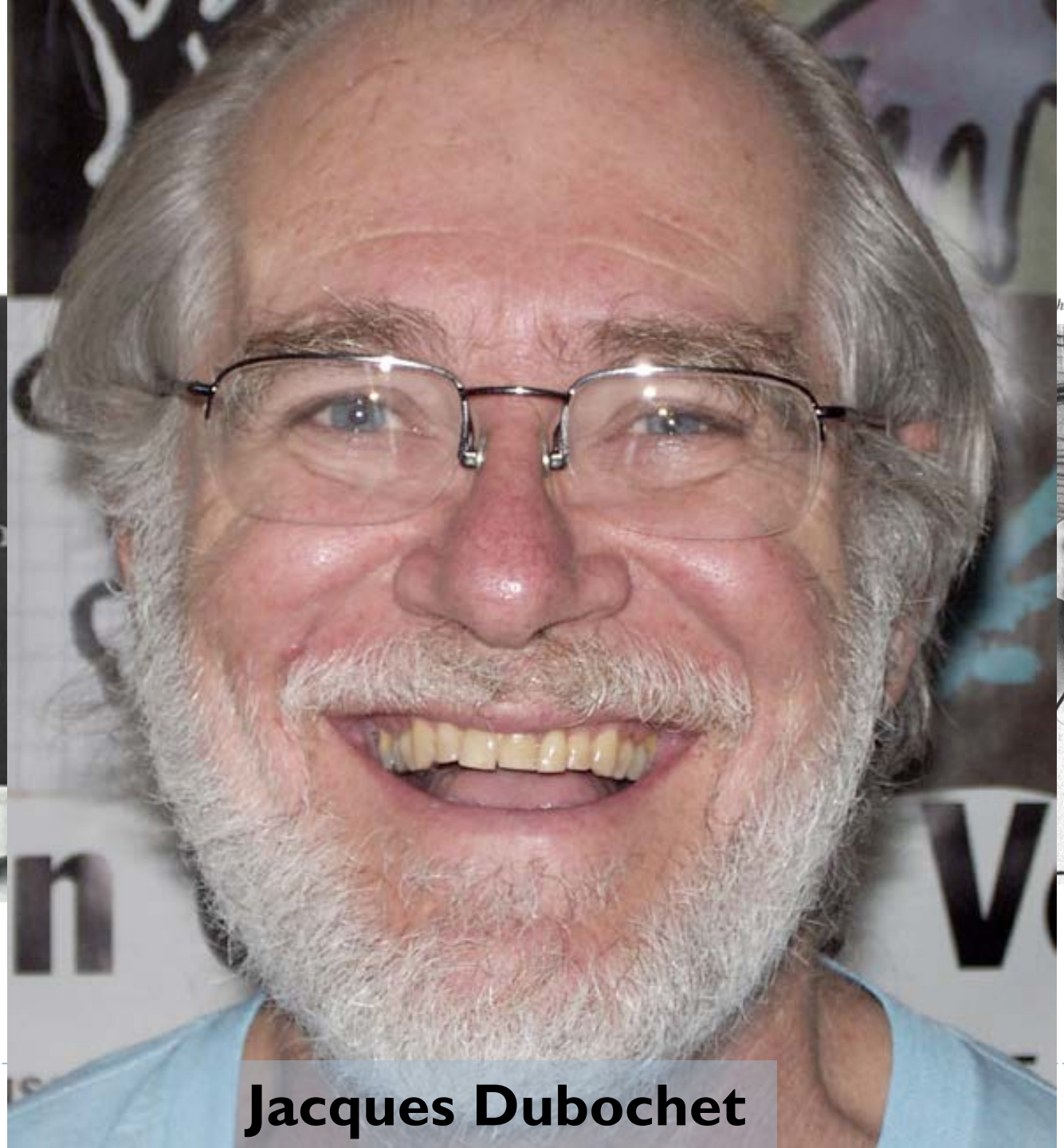
Live worms | 10 min | 2 hours

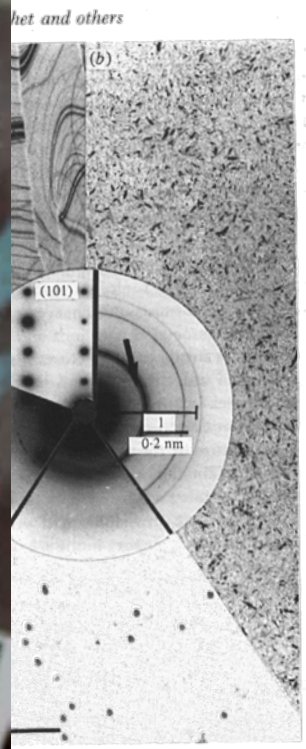
Videos: Kent McDonald, UC Berkeley

Is specimen resolution affected by preparation?

- Drying alters structure:





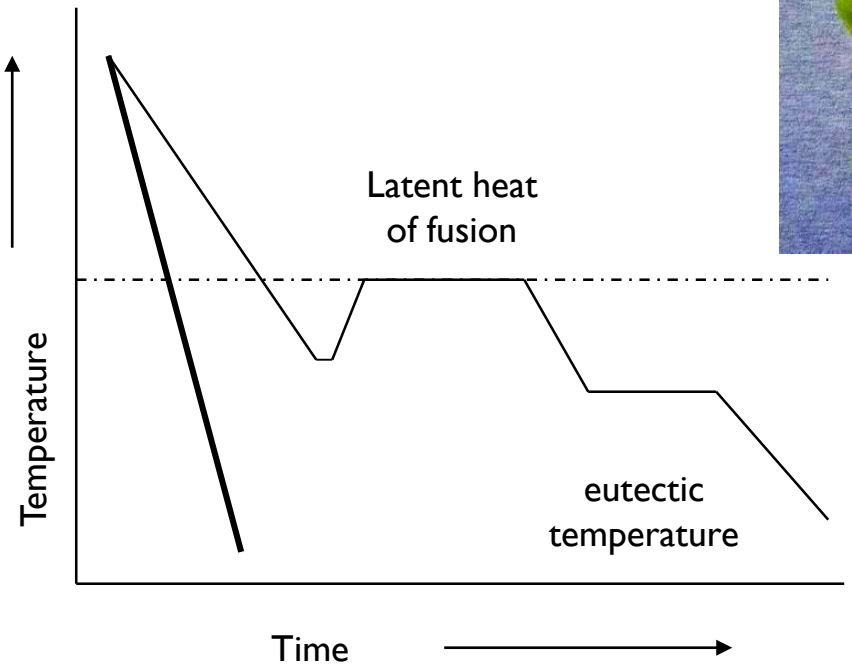


Jacques Dubochet

Cryo electron microscopy: 1980's

- Specimen preparation using rapid freezing (cryofixation)

  NO ICE CRYSTAL FORMATION



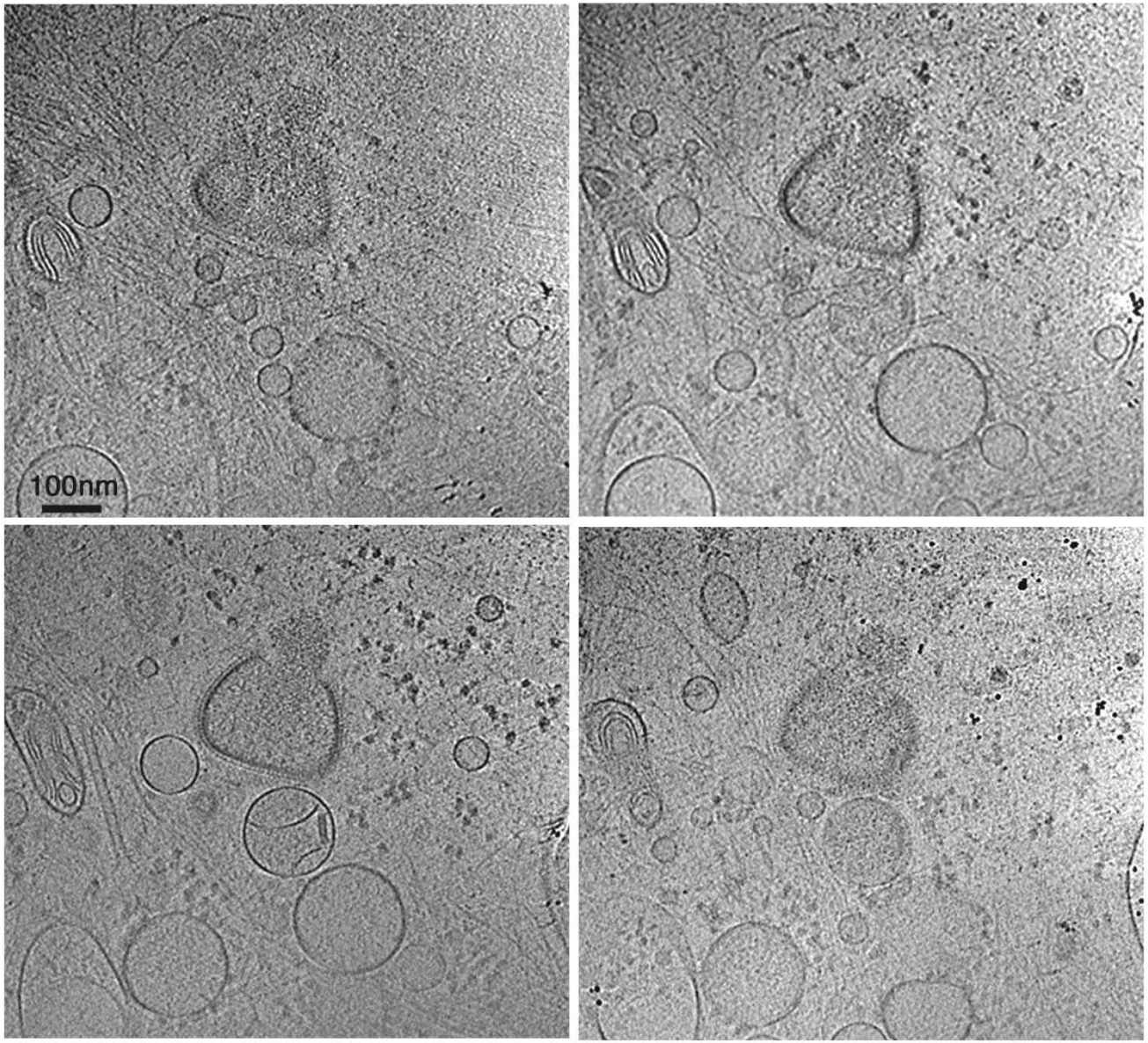
Theory:
Vitrification – no ice crystals

Practice:
rapid freezing, cryo-specimen stage, cryo-EM

Cryo-electron microscopy

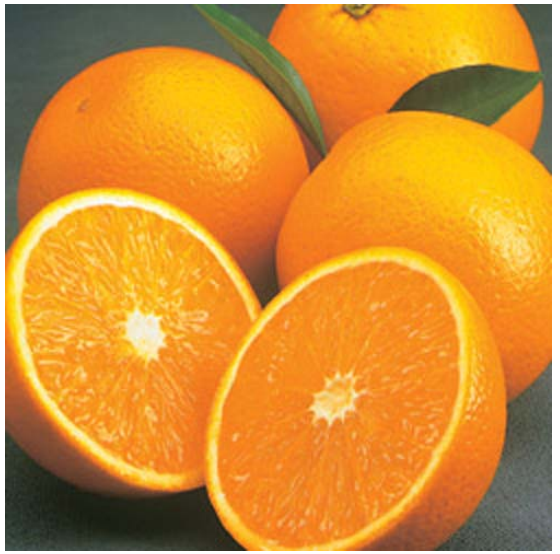
Intracellular virus

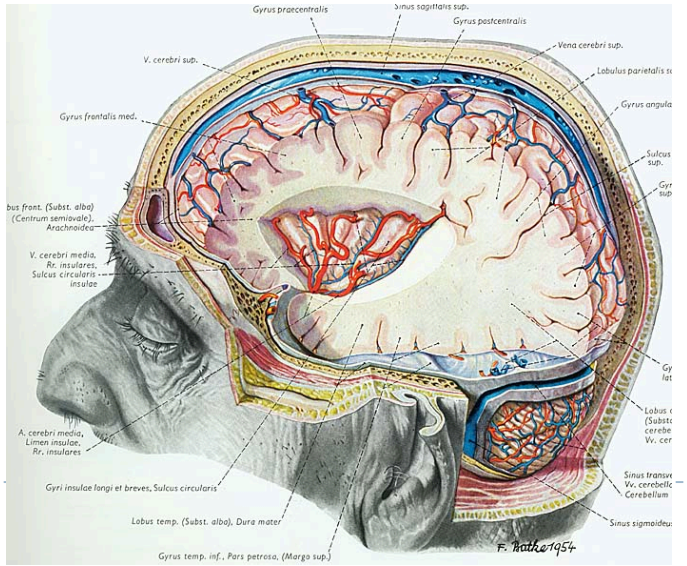
1940's



Sectioning

- For TEM sectioning is a prerequisite
- Gain access to
  - Morphology
  - Antigens
- Preserve
  - Morphology
  - Antigenicity
  - High resolution detail





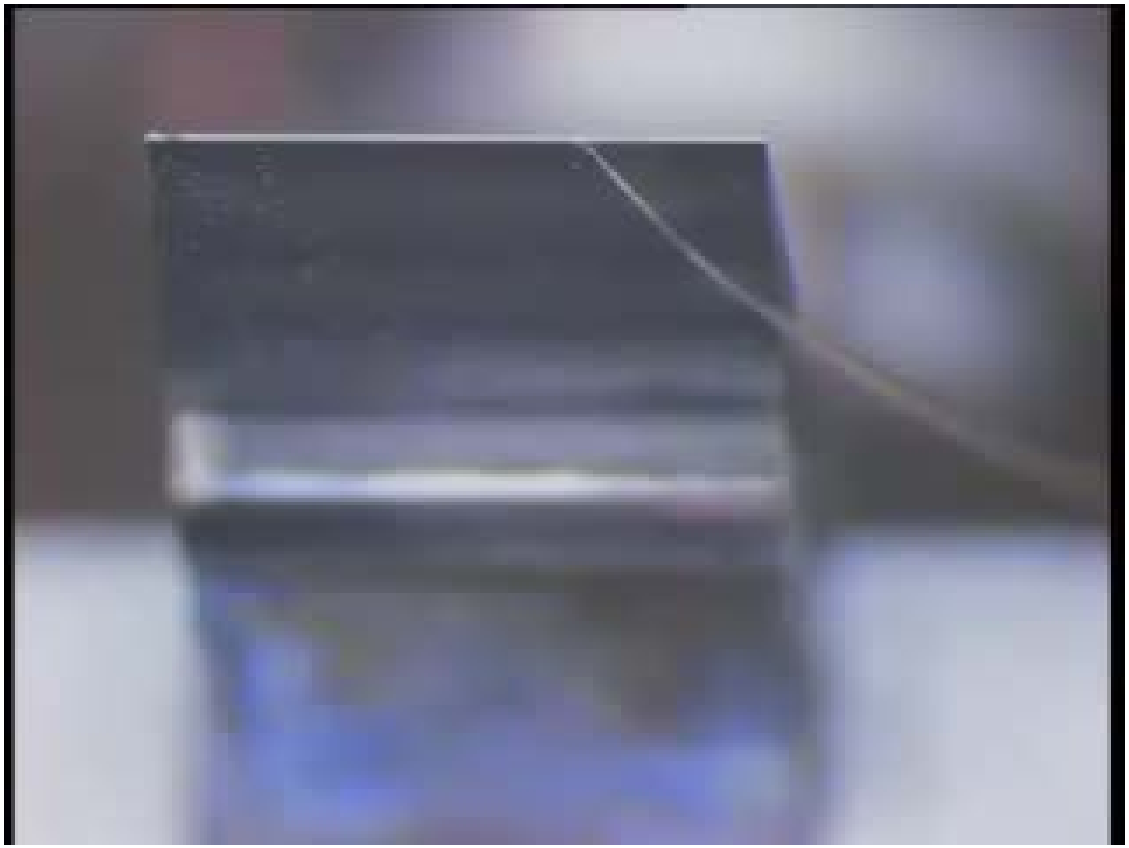
Imaging larger cells & tissues - cryosectioning

- CEMOVIS
  - **C**ryo-**E**lectron **M**icroscopy of **VI**trified **S**ections

Freeze

Section

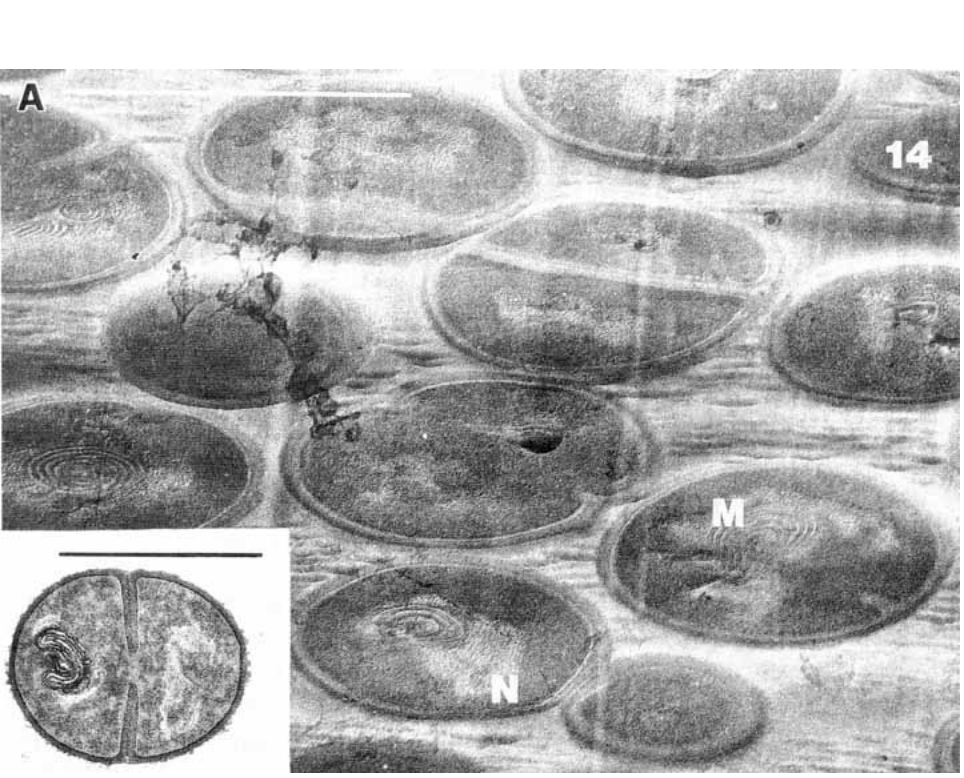
Image

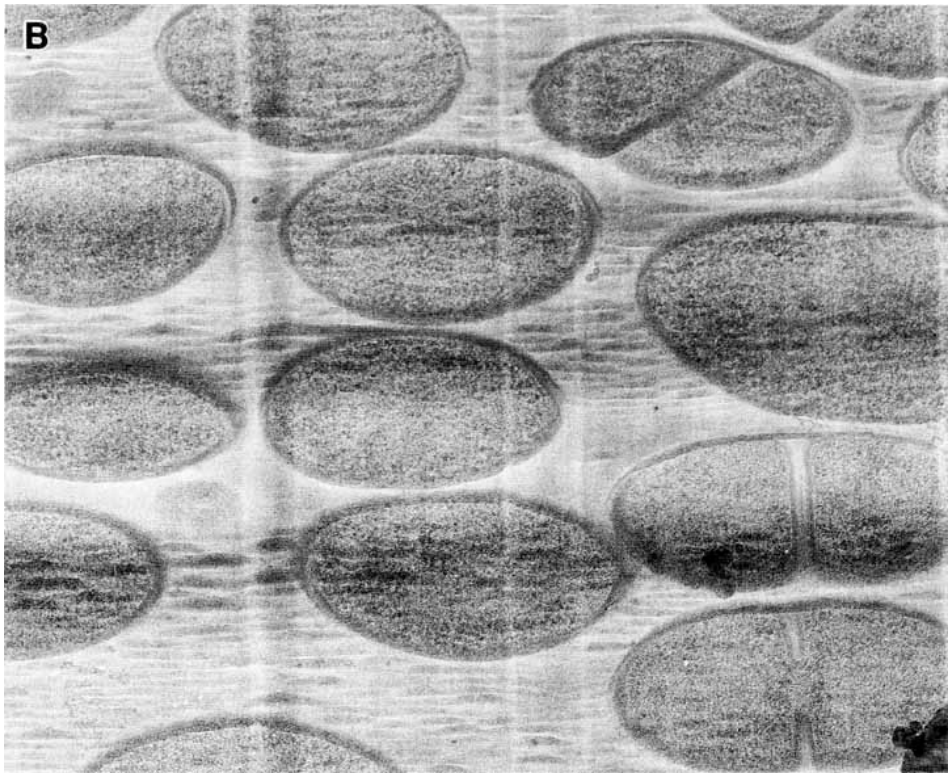


Chemical fixation compared with cryo-immobilization

The bacterial "mesosome" is a structure that appears as a result of chemical fixation

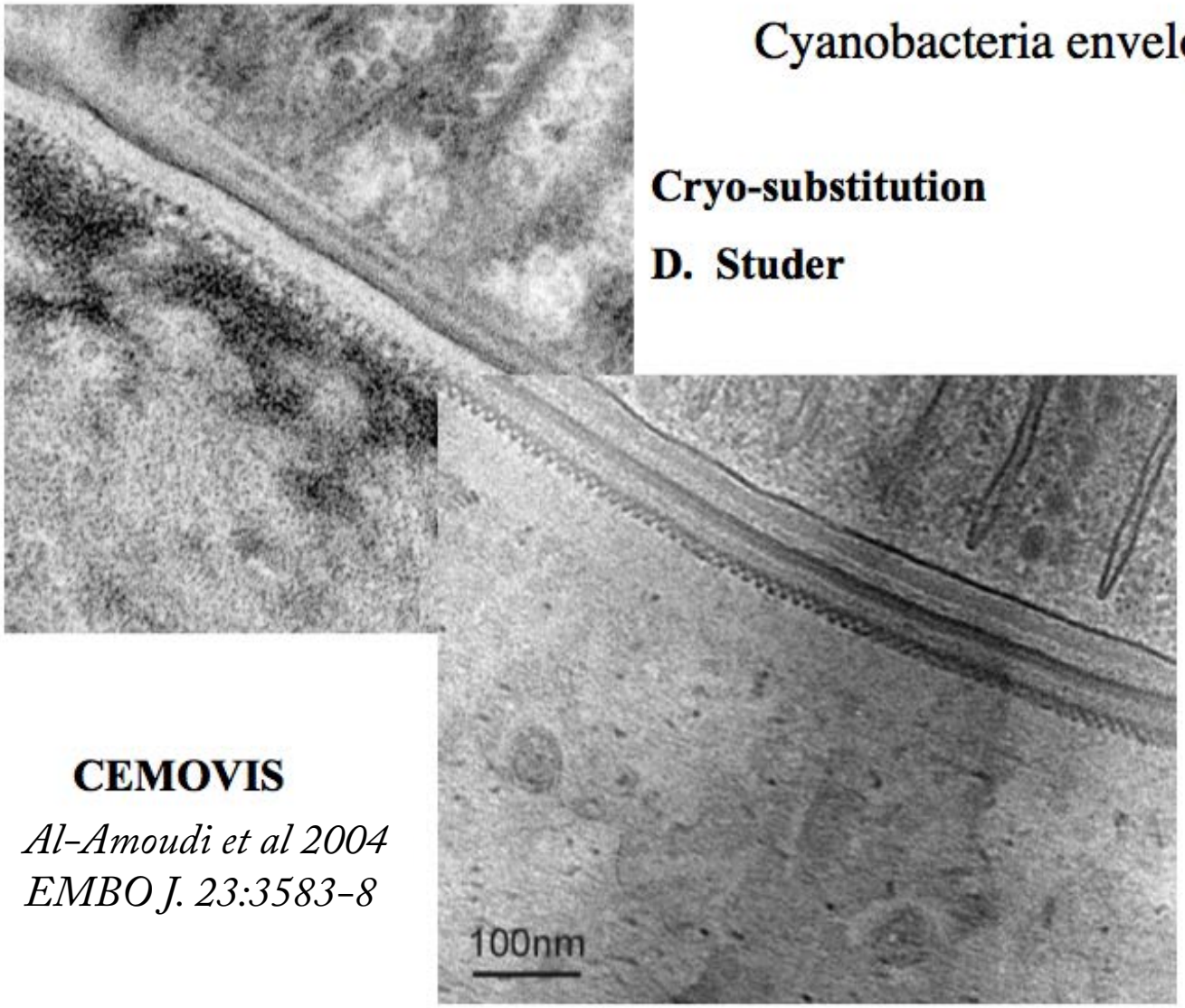
(a fixation artifact)





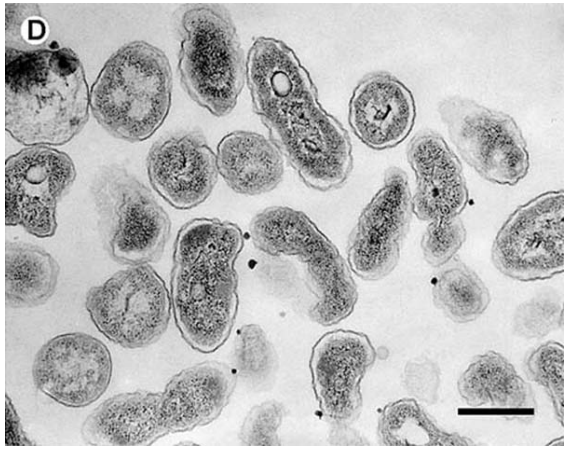
McDowall & Dubochet 1983 J. Bact. 155:381-390

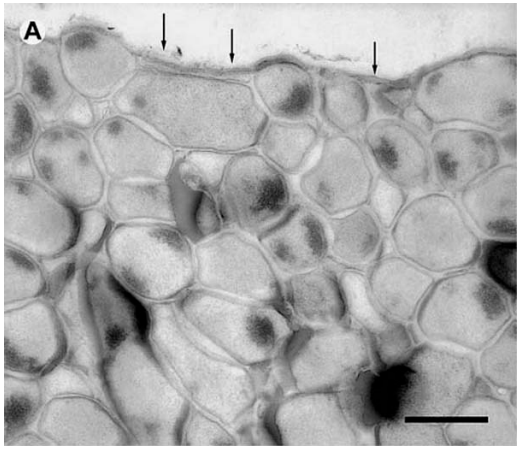
CEMOVIS


Cyanobacteria envelope
Cryo-substitution
D. Studer
CEMOVIS
Al-Amoudi et al 2004
EMBO J. 23:3583-8
100nm

Cryofixation

- Small specimens freeze best
- Subsequent processing:
  - Frozen specimen imaged in frozen state
    - tomography
  - Dehydrate specimen in frozen state (freeze substitution)
    - resin embedding
    - sectioning
    - immunolabeling
    - 3-D reconstruction

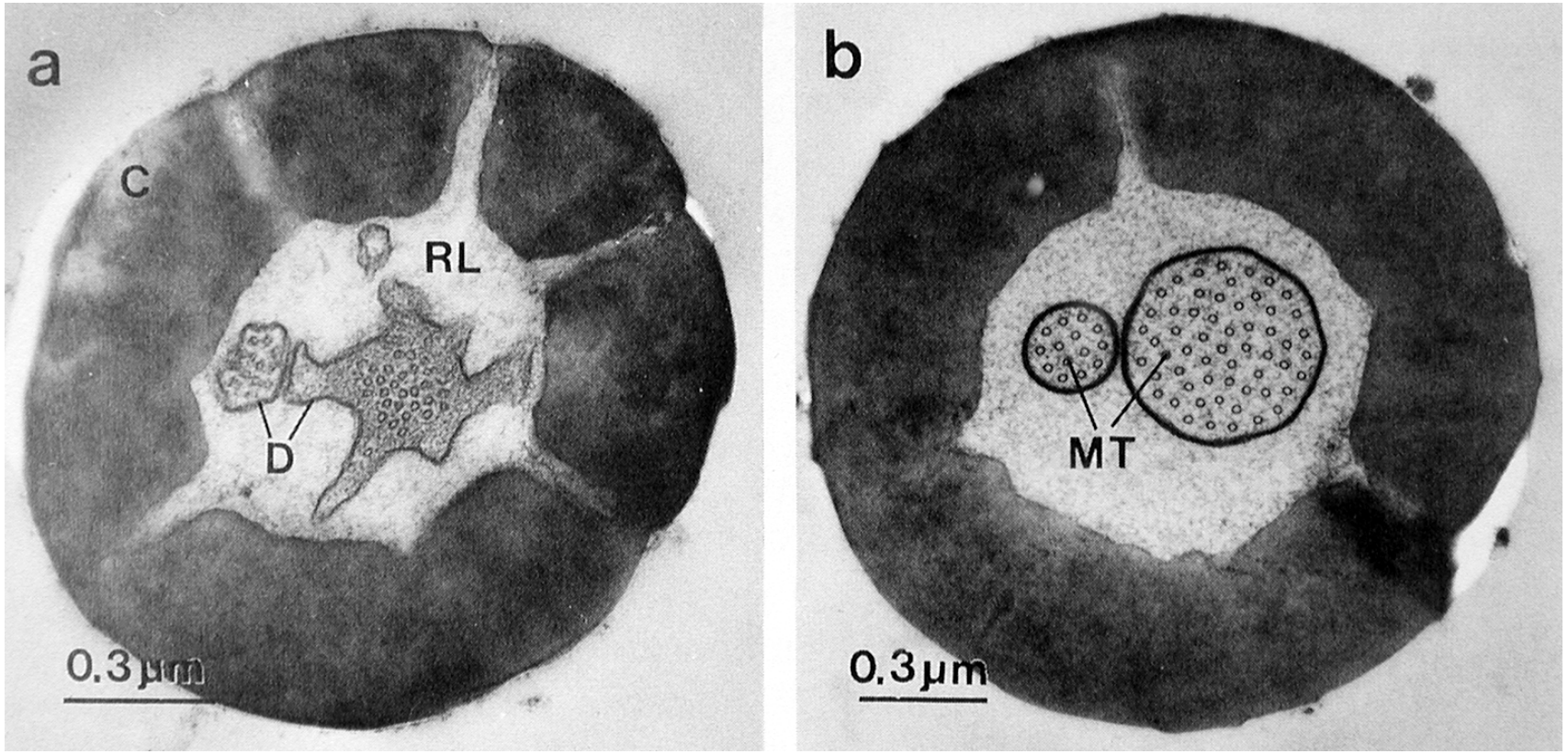




Cryomethods

Conventional processing

Rapid freeze, freeze-substitution



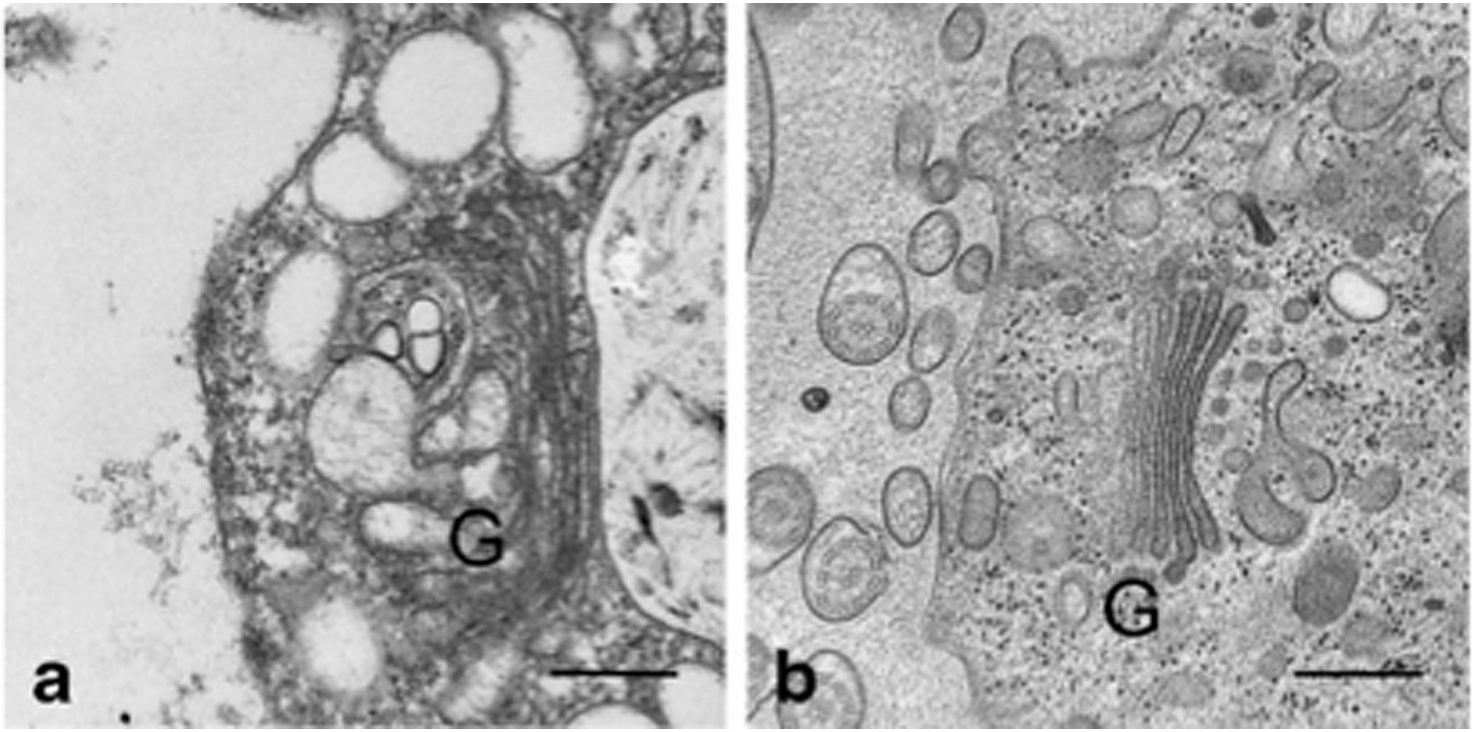
Sensory hairs *Bombyx mori (*silkworm moth)

From: Steinbrecht, R.A. and M. Müller. 1987. Freeze-Substitution and Freeze-Drying. In, Cryotechniques in Biological Electron Microscopy, R.A.Steinbrecht and K. Zierold (eds.), pp. 149-172. Springer-Verlag, Berlin.

Cryomethods

Conventional processing

Rapid freeze, freeze-substitution



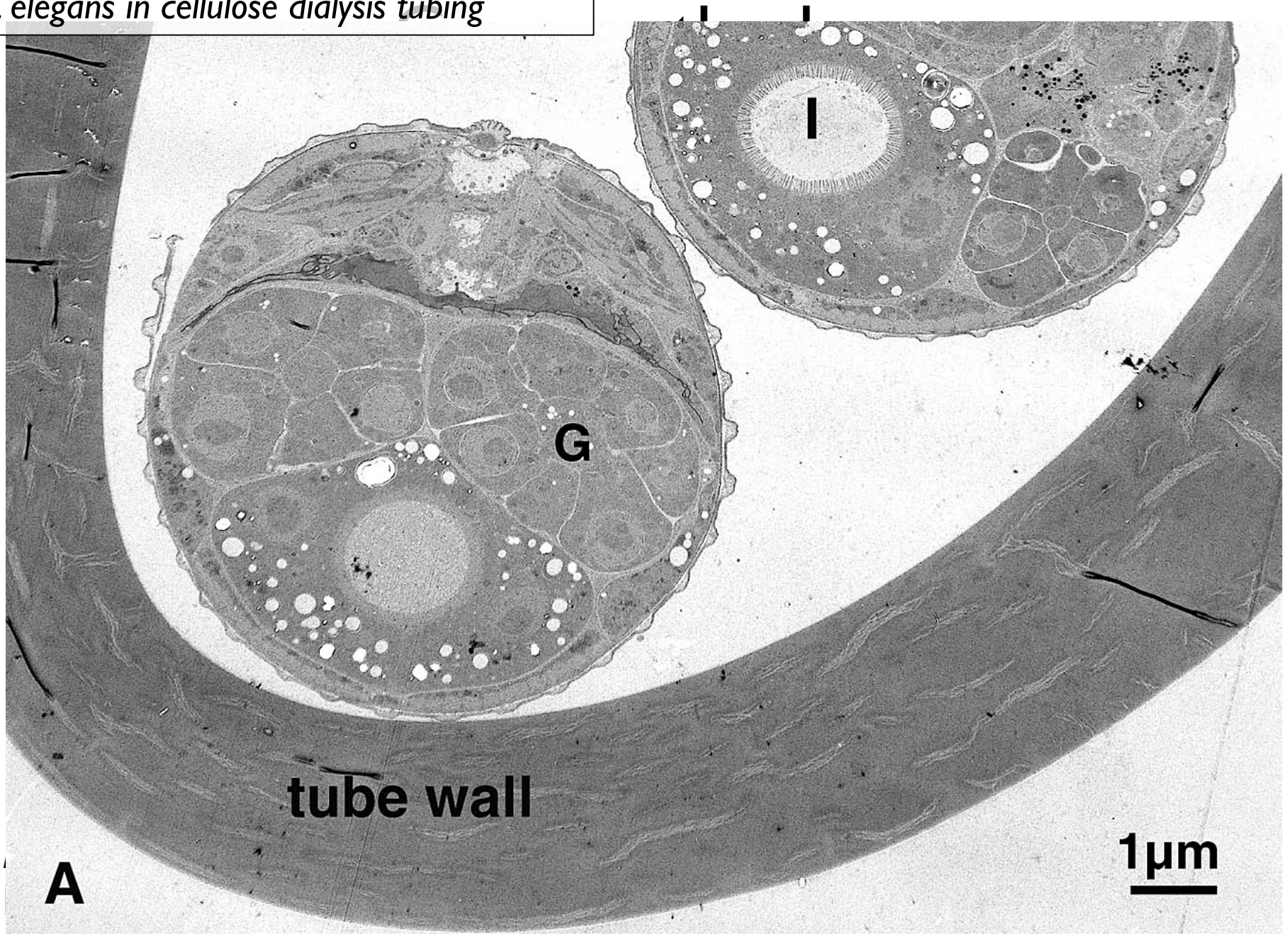
Oscarella carmela
(slime sponge)

HPF	30 ms
FS	2.5 hr
Infiltration	40 min
Polymerization	2 hr

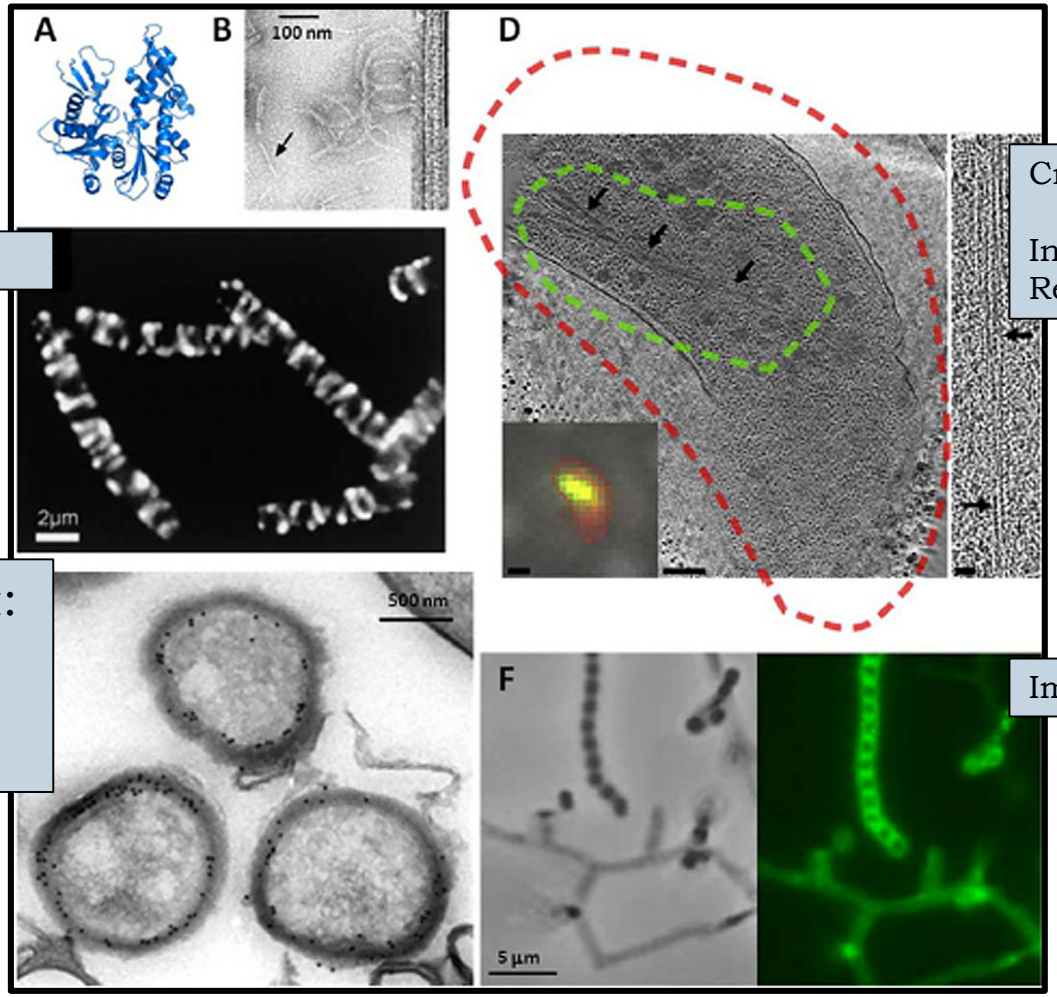
McDonald 2014 Protoplasma 251:429–448

HPF: freeze substitution: epoxy resin

C. elegans in cellulose dialysis tubing



Immunocytochemistry



GFP-fusion protein

Cryo-tomography

Insert:
Red membrane dye and GFP

Immuno-EM:

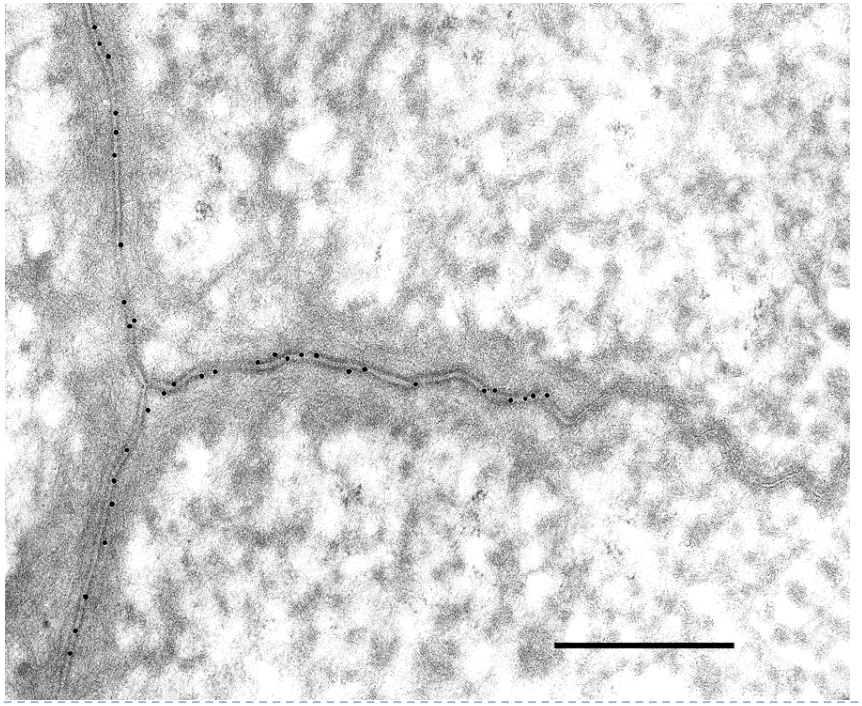
On resin-embedded cells

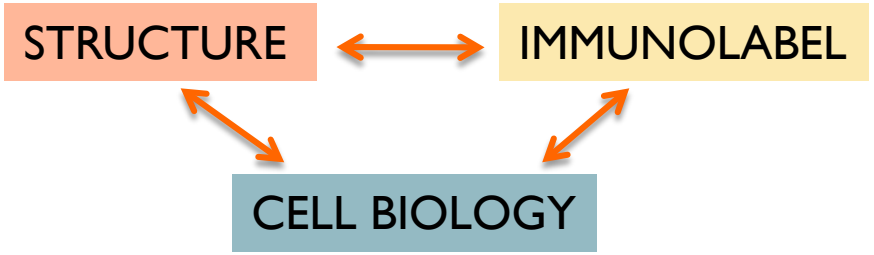
Immunofluorescence

Celler et al 2013 J. Bact. 195:1627-1636

Immunocytochemistry

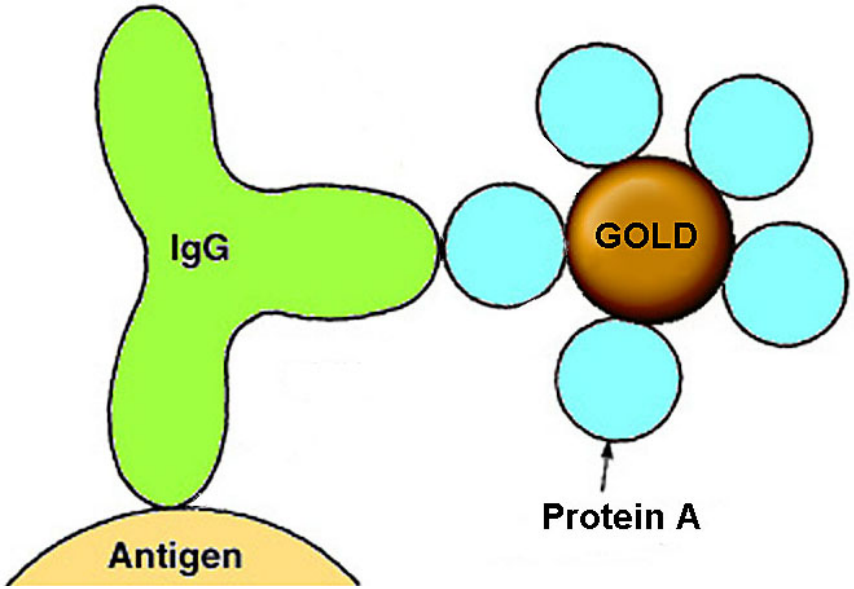
- Locating antigens using specific antibodies
  - Best performed on sections
  - High resolution signal preferred
  - Compromises necessary

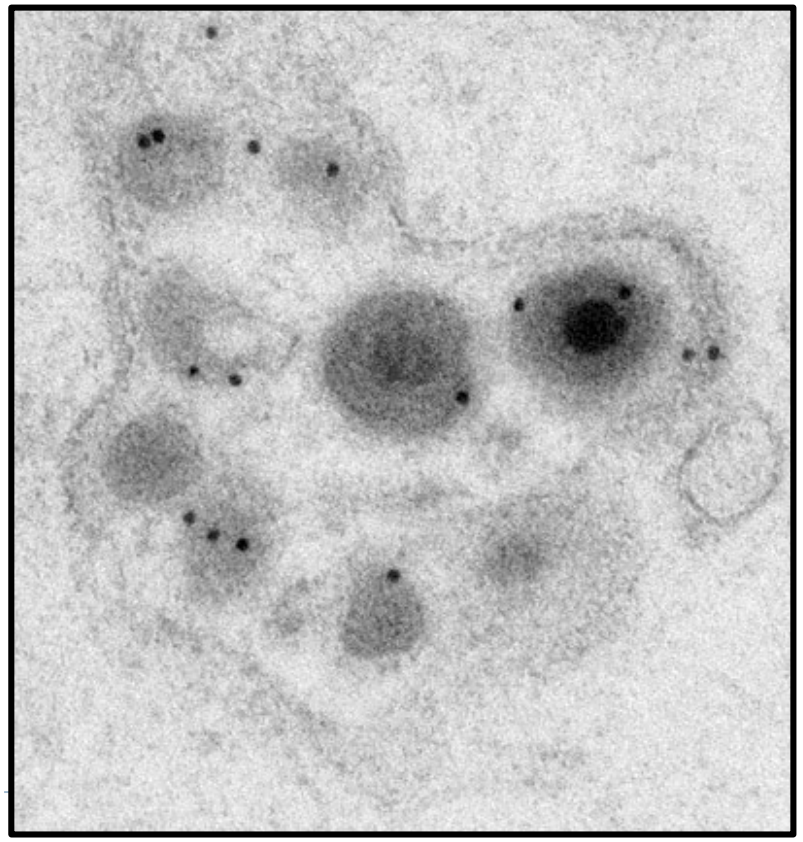




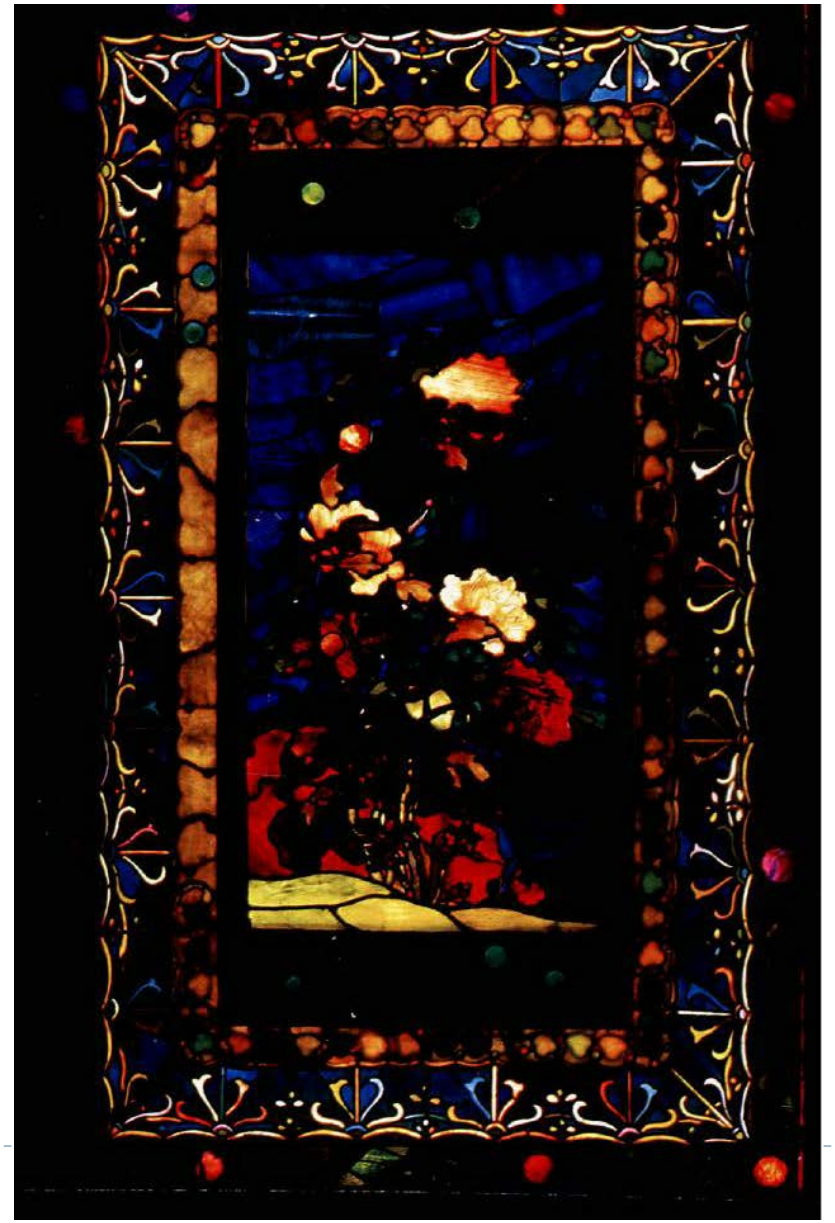
High resolution signal

- Antibodies applied to thin sections:
  - *Bind to specific proteins*
  - *Protein A gold "locates" bound antibody*





Colloidal gold



Colloidal Gold

Becomes hydrophobic and negatively charged

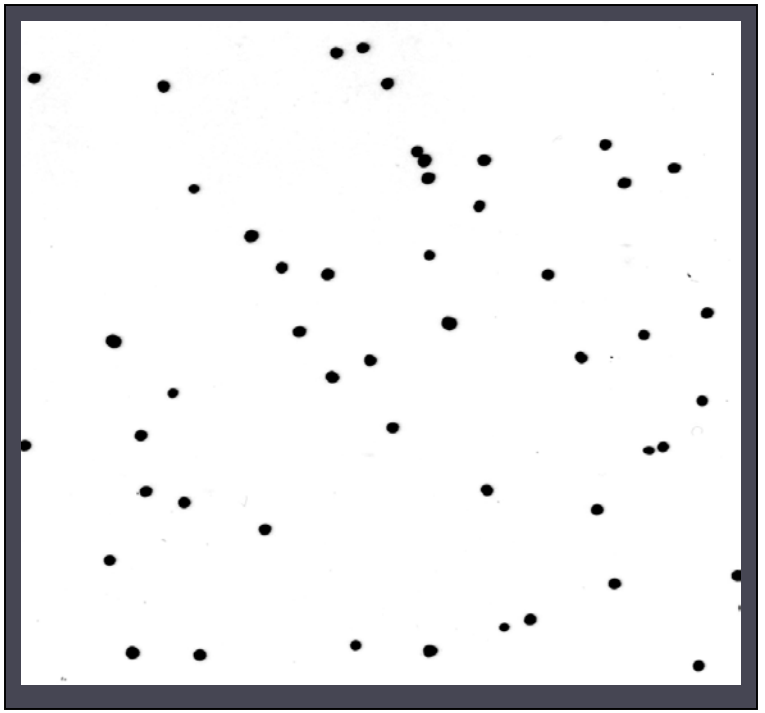
Negative charge stabilises the colloid by electrostatic screening

Stable for years (hundreds of years)

(*1856 Ri, London*)

Spontaneous complexing with proteins & biopolymers

High resolution signal



Does not occur naturally

Complexed proteins retain biological activity

PARTICULATE - Points can be counted

Imaging volumes

- Tomography
  - *"missing wedge"*
- In situ FIB-SEM serial section tomography
  - *Automated*
  - *Destructive*
  - *Small block face*
- Serial block face imaging – 3-View
  - *Automated*
  - *Destructive*
- Array tomography
  - *Serial sections can be re-used*
  - *Useful for light microscopy*

Tomography

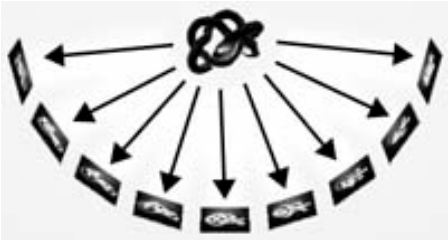
Frozen hydrated specimen

Rapid freezing

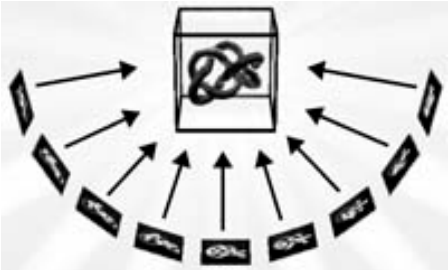
TEM examination

Multiple image scan

(tilting goniometer)

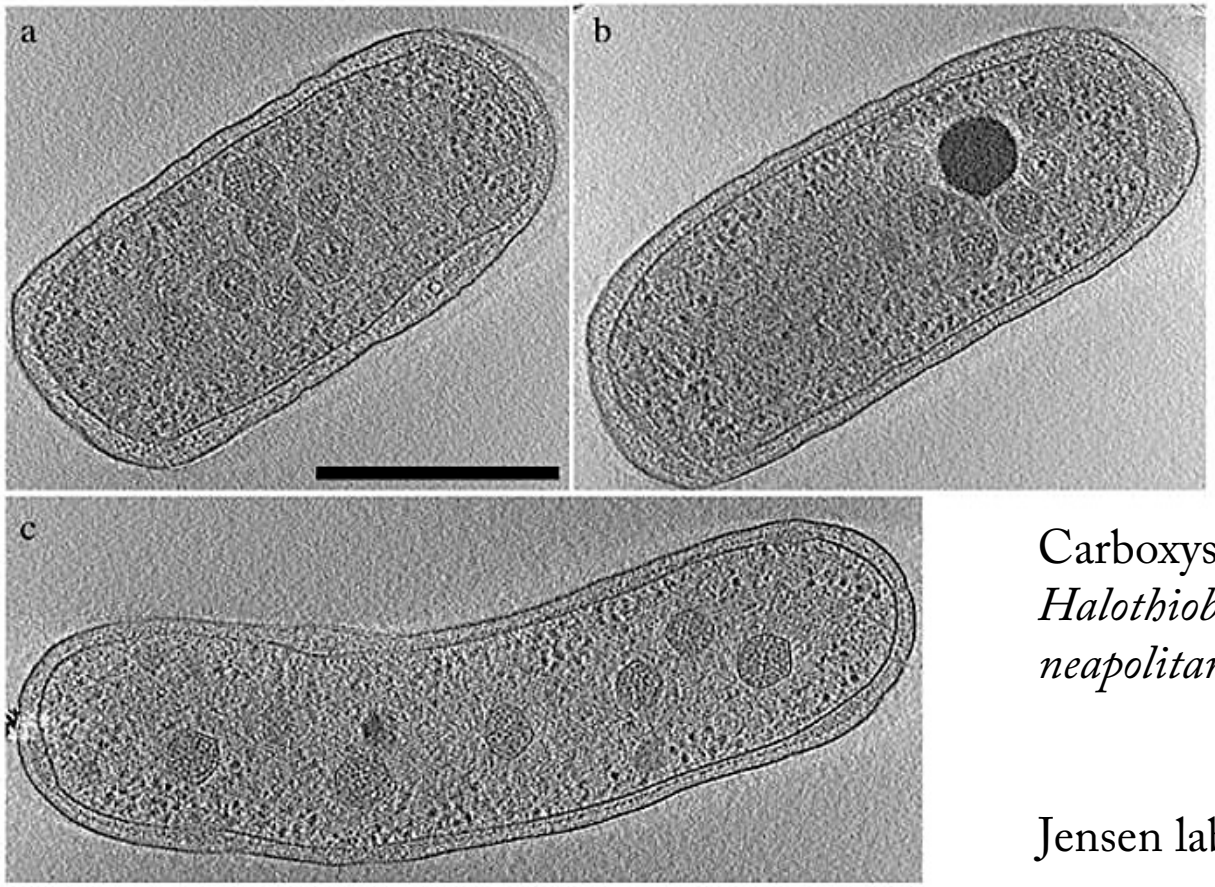


3-D reconstruction (TOMOGRAPHY)



Imaging Whole Cells

Fully hydrated, frozen cells



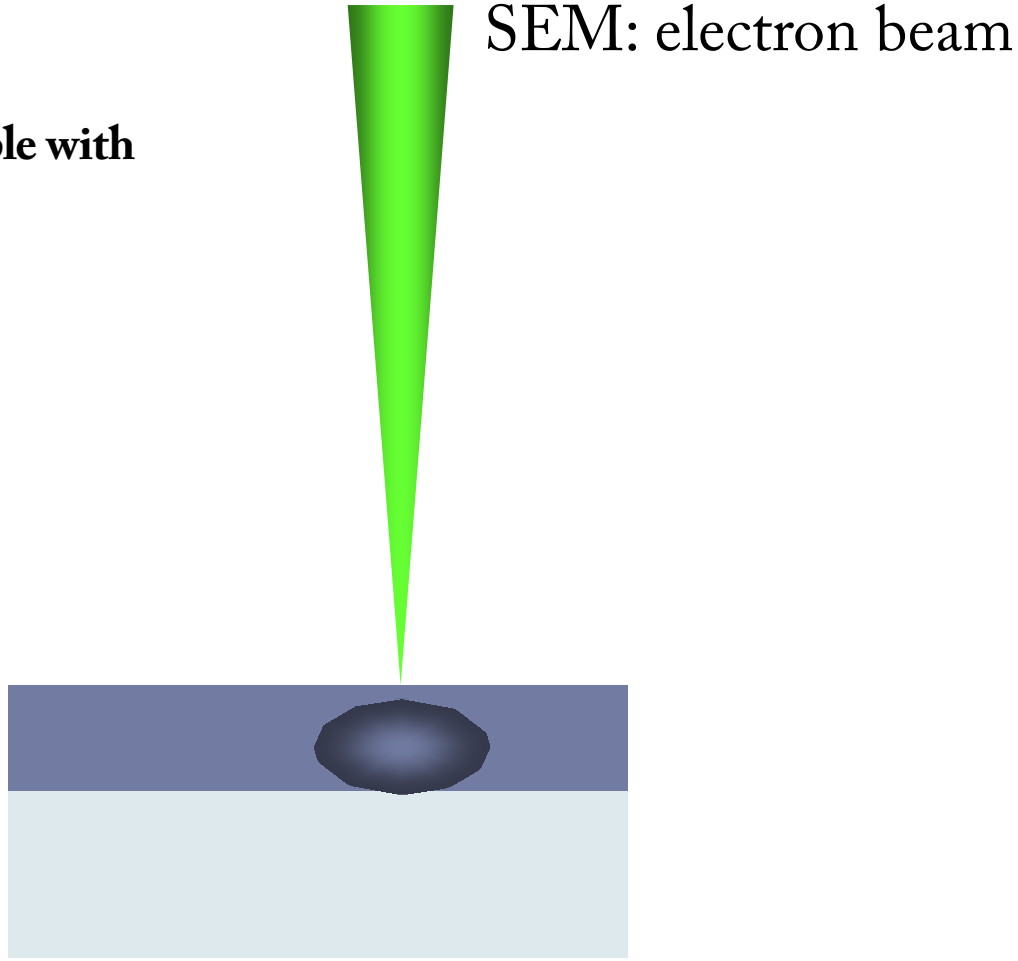
Carboxysomes in *Halothiobacillus neapolitanus* cells

Jensen lab, Caltech

Iancu et al 2010 J Mol Biol. 2010 February 12; 396(1): 105–117.

Focused ion beam/SEM

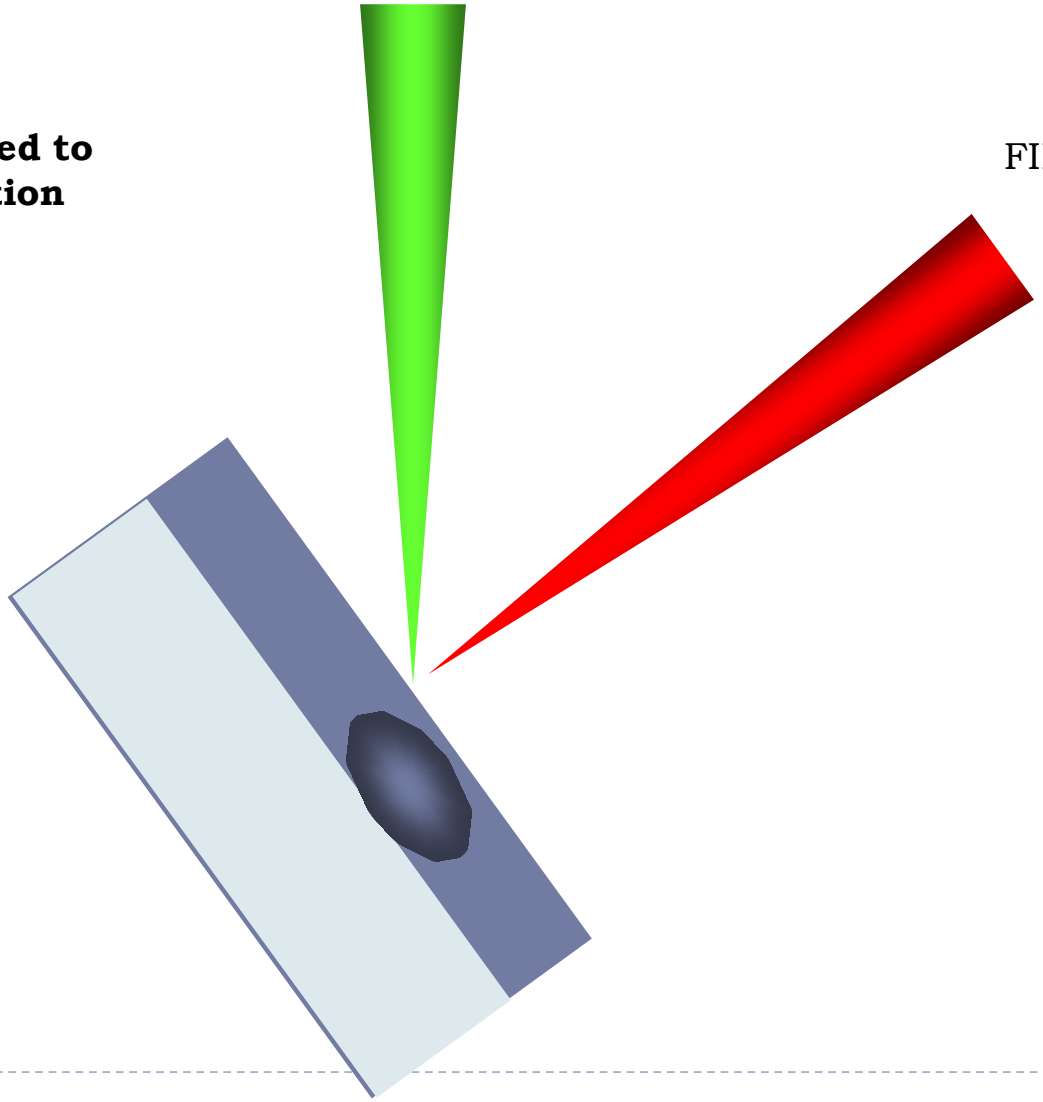
1. **Image sample with e-beam**



Focused ion beam/SEM

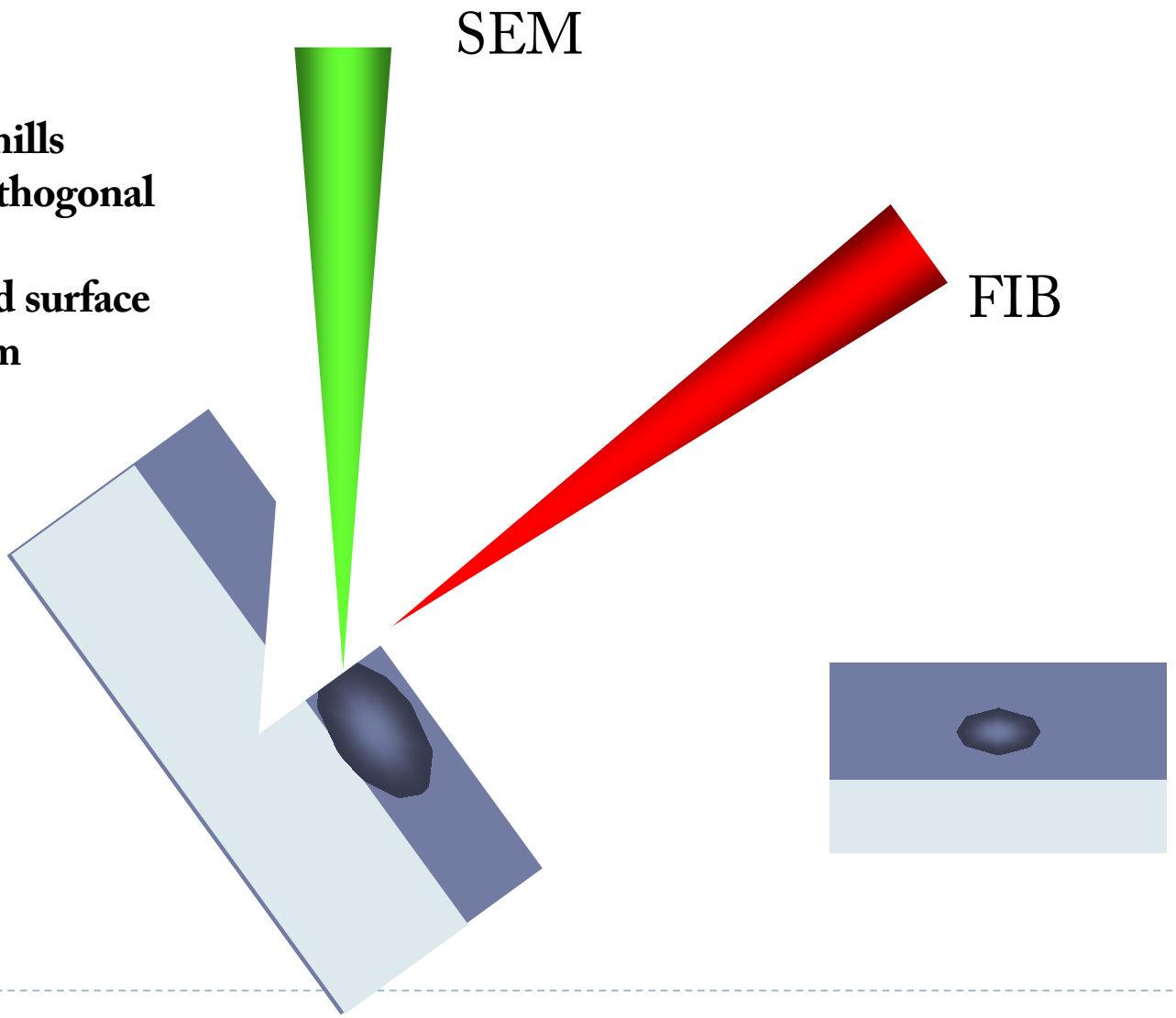
2. Sample moved to milling position

FIB: gallium ion source



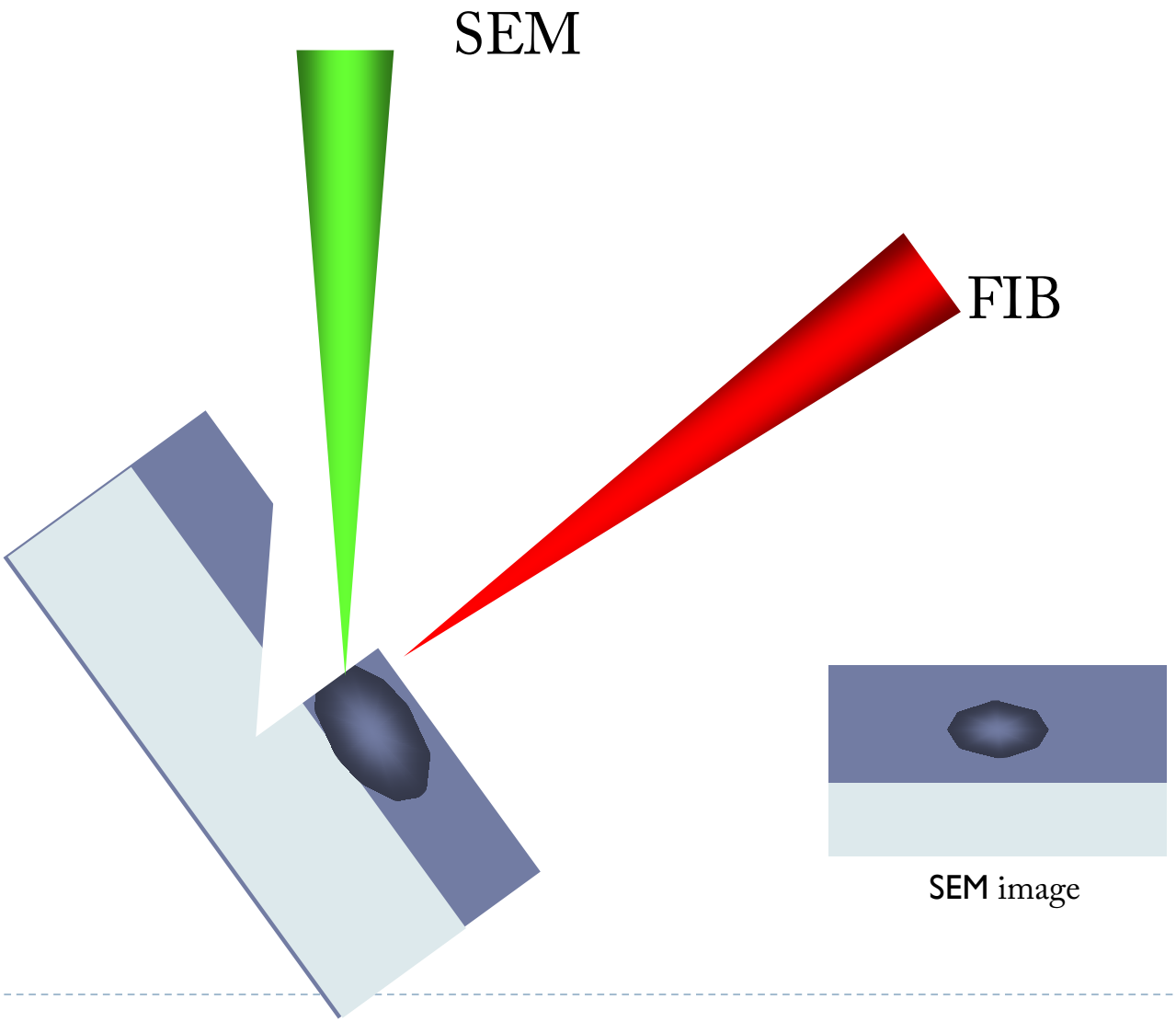
Focused ion beam/SEM

3. **Ion beam mills material orthogonal to surface**
4. **Image tilted surface with e-beam**



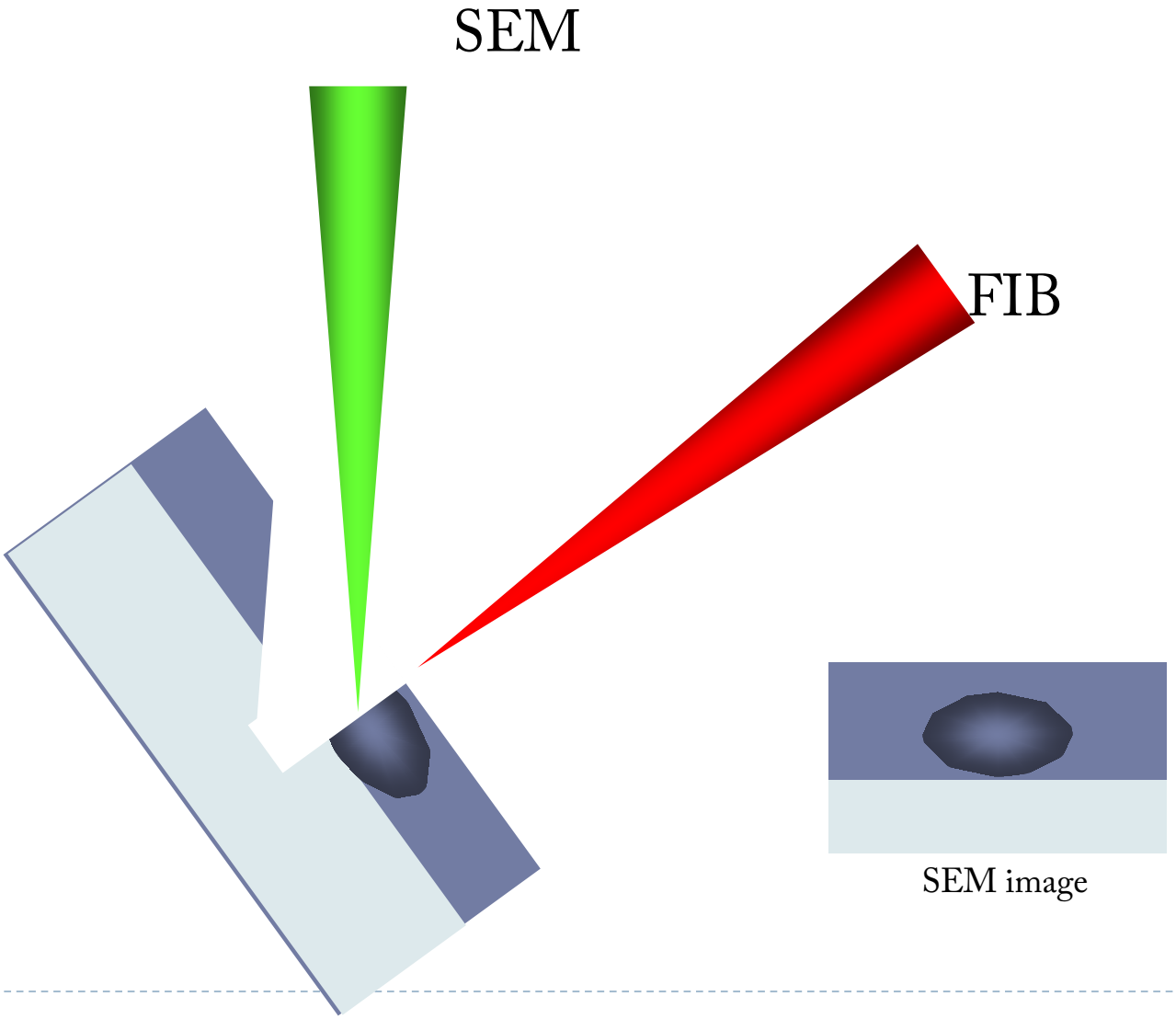
Focused ion beam/SEM

5. **Repeat...**



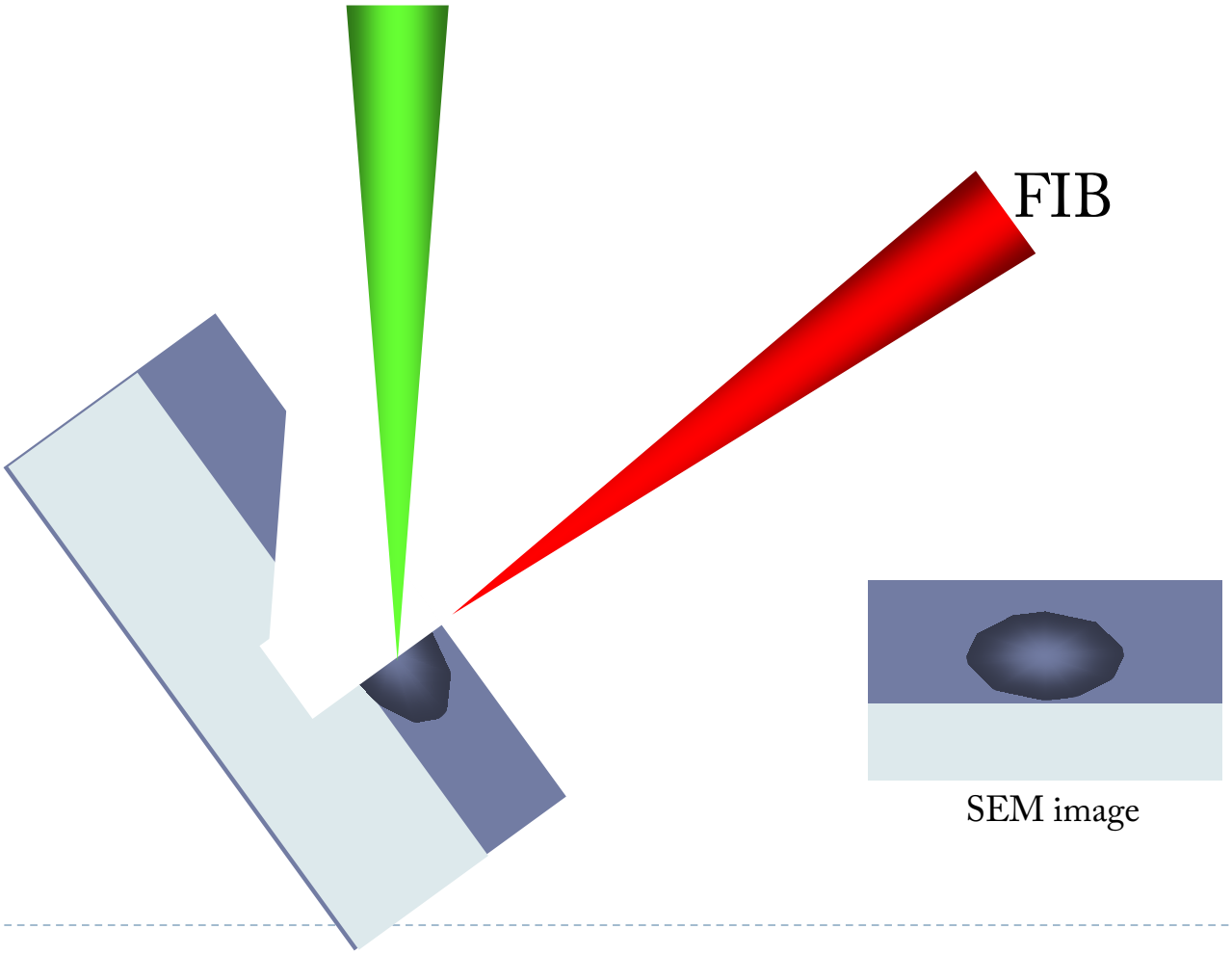
Focused ion beam/SEM

6. **Repeat...**



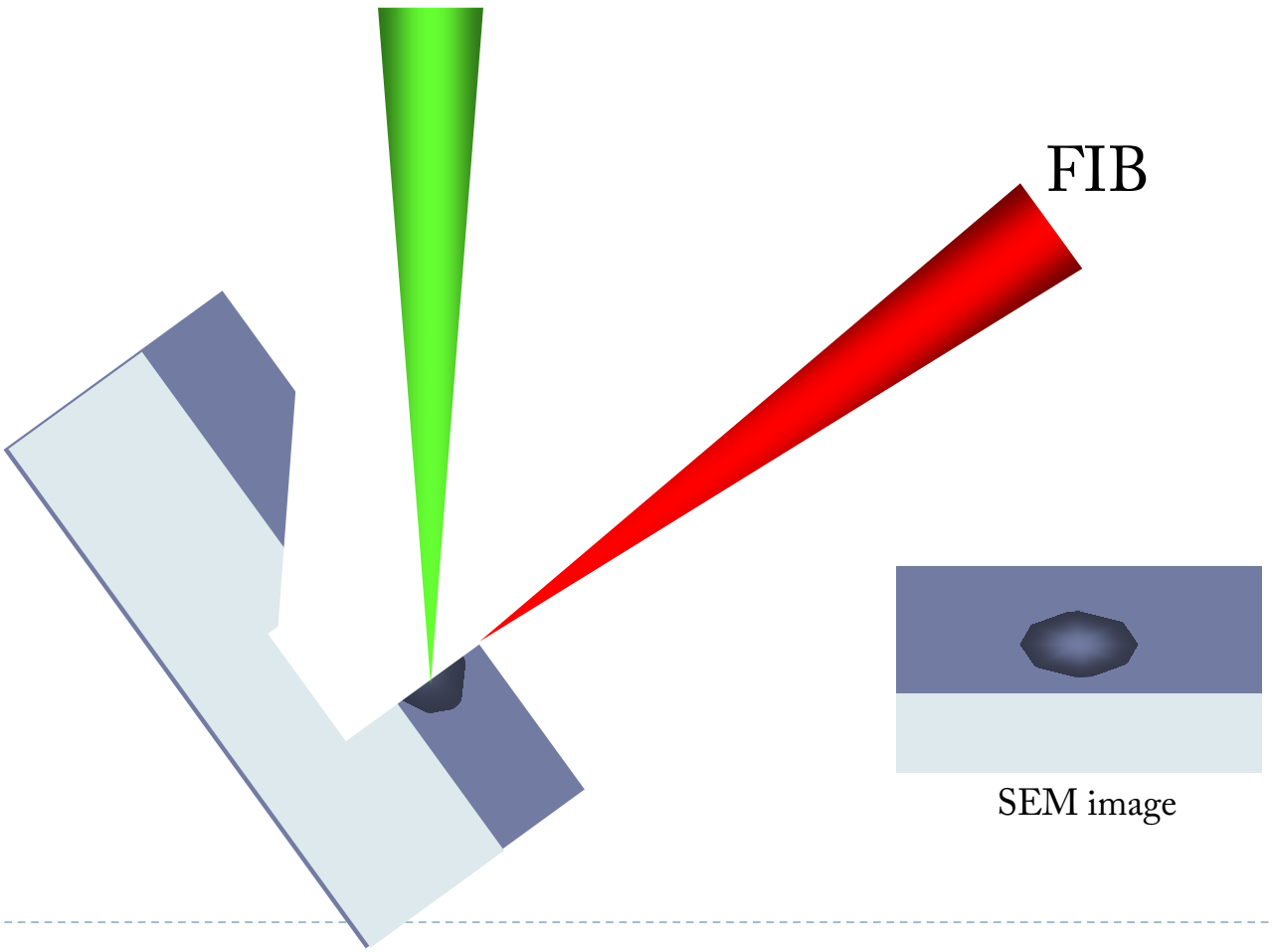
Focused ion beam/SEM

7. **Repeat...**

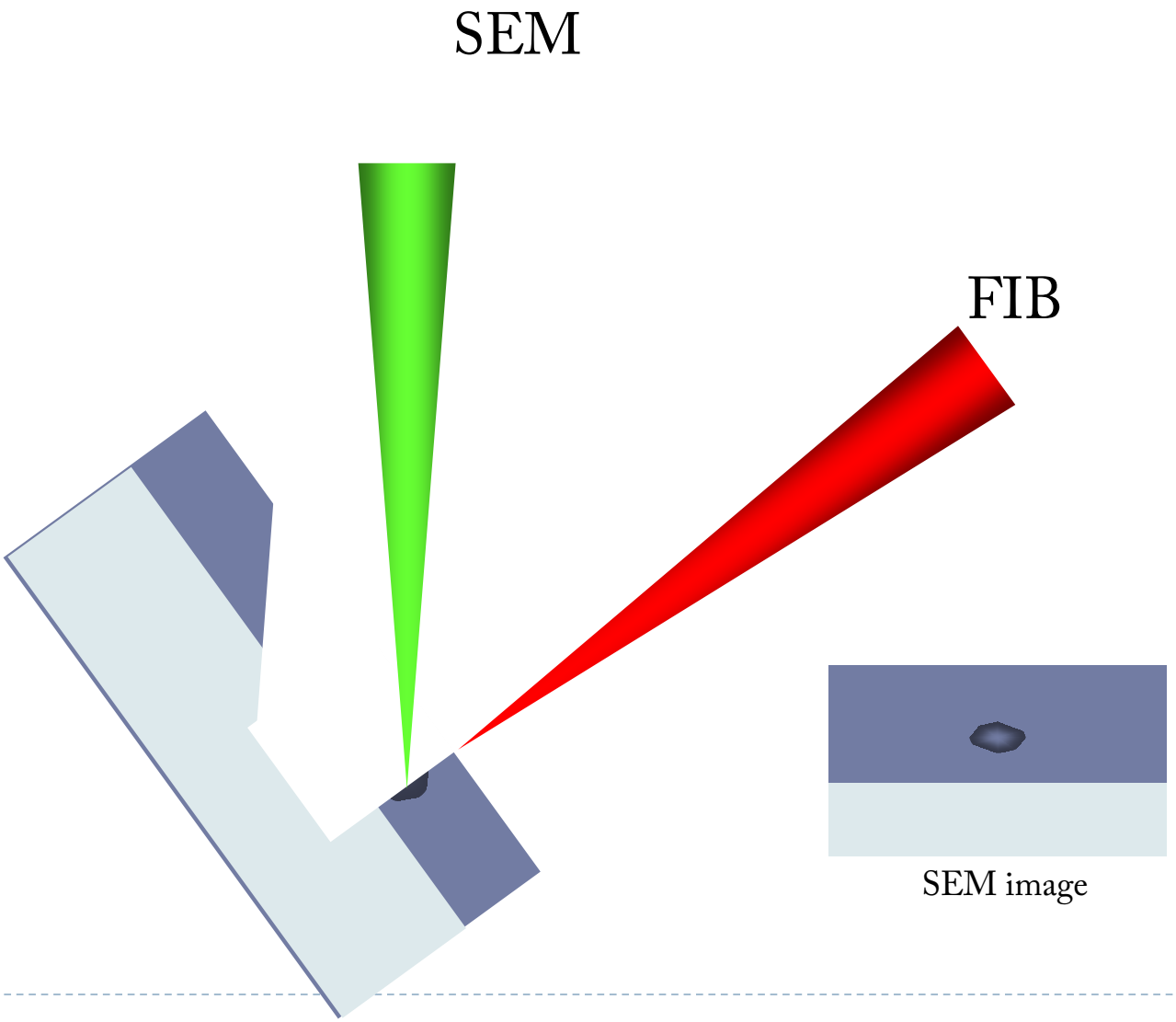


Focused ion beam/SEM

8. **Repeat...**

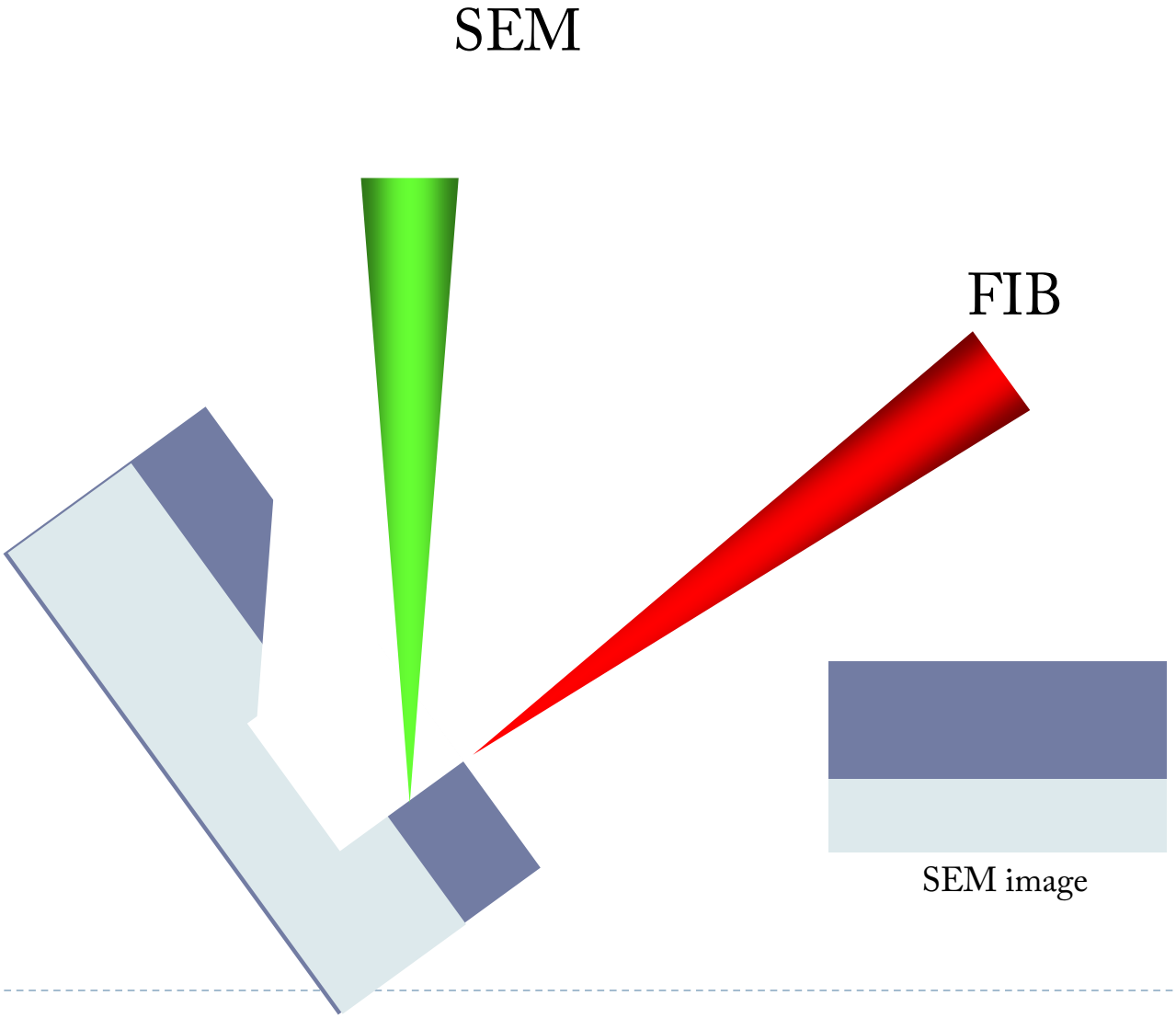


Focused ion beam/SEM



9. **Repeat...**

Focused ion beam/SEM

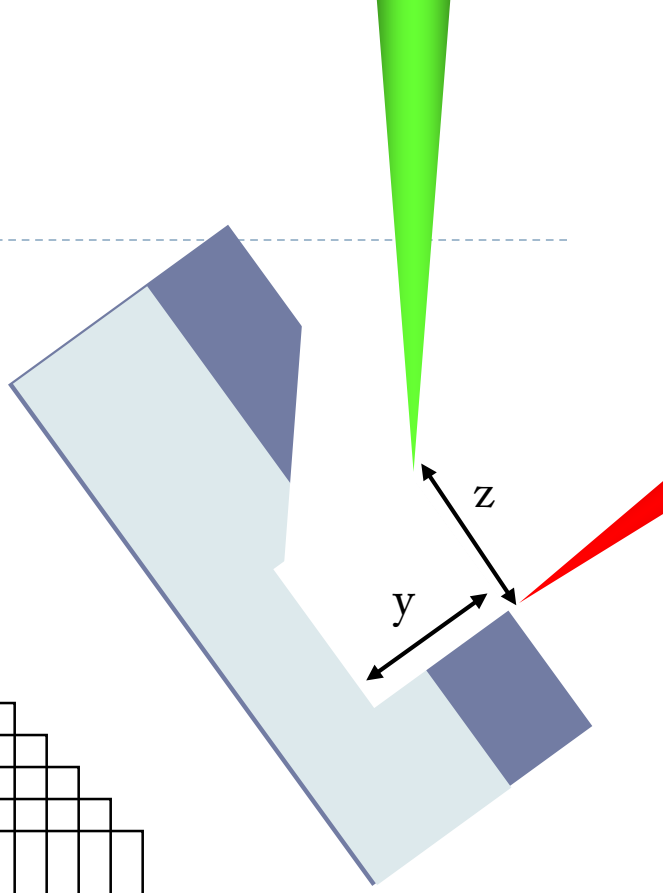


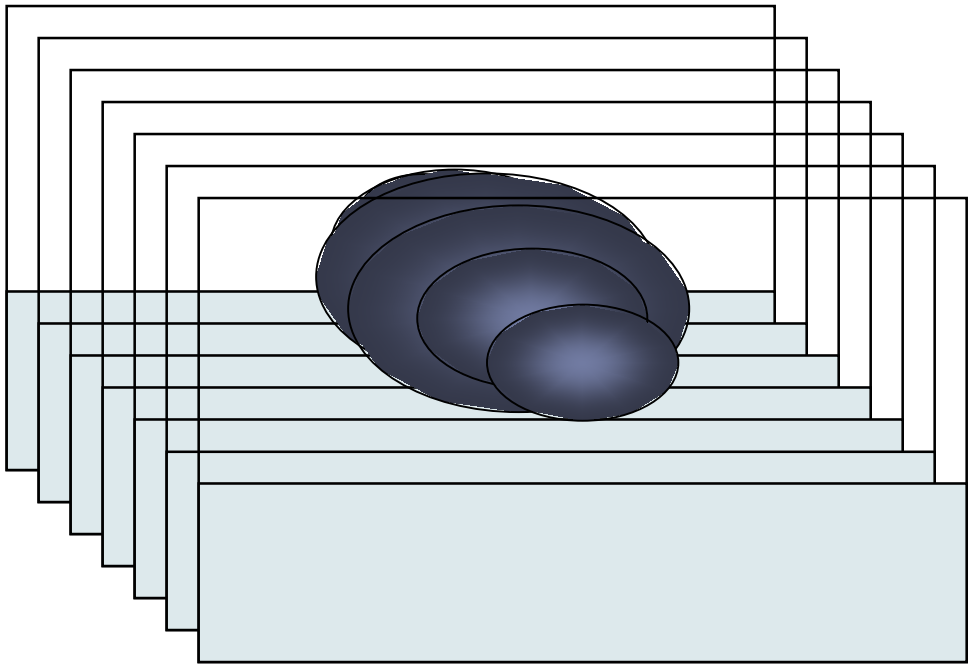
10. **Repeat...**

Focused ion beam/SEM

Reconstruction:

1. **Align slices in x,y (cross correlation)**
2. **Align in z**
3. **Correct tilt angle**



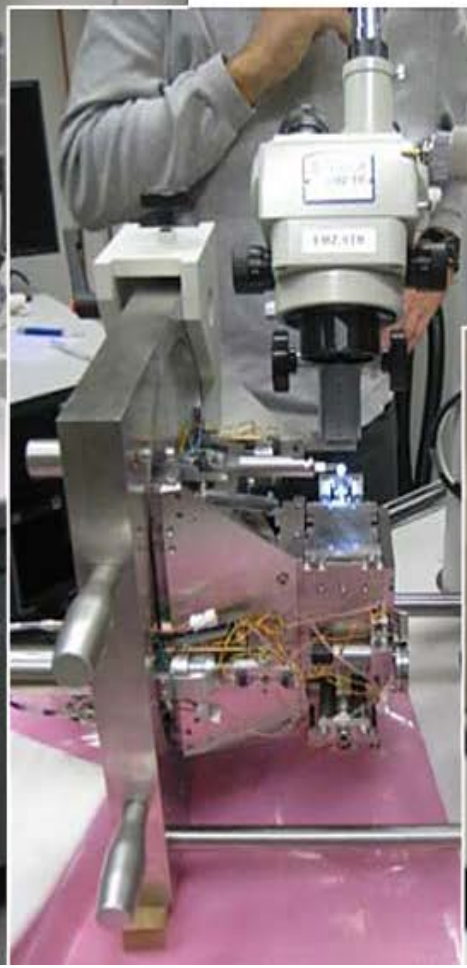


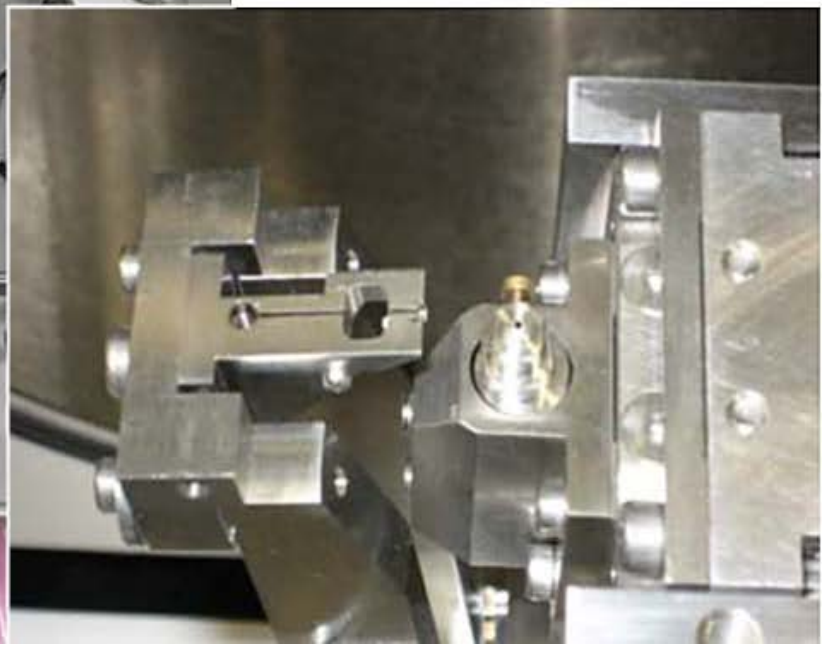
Focused ion beam/SEM

- **Advantages of FIB-SEM serial sectioning**
  - Uniformly thin sections ~ 3nm slices
  - Resolution the same in X,Y & Z (Square voxel potential)
  - No missing wedge related artifacts

- **But:**
  - **FIB is destructive**
  - Need automated FIB-SEM
  - Small specimen area
  - Distorted image
  - Immunolabeling not practical

Serial block face imaging

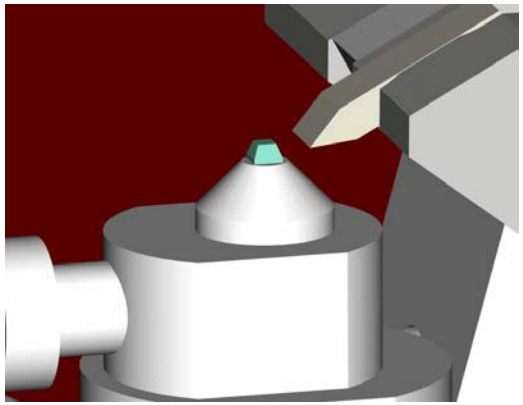




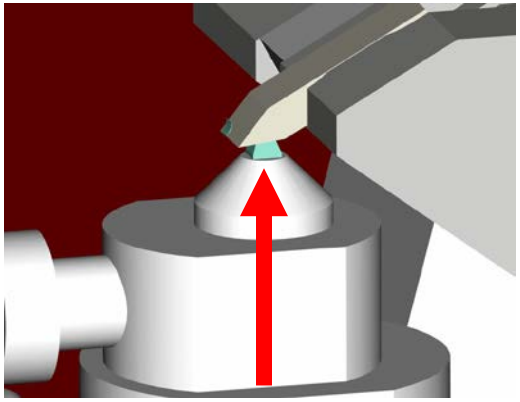
Gatan:
3-View

How it Works: Serial Block Face

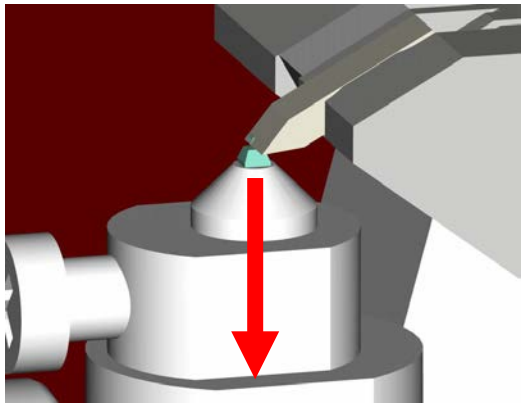
Resin specimen is squat flat topped pyramid.



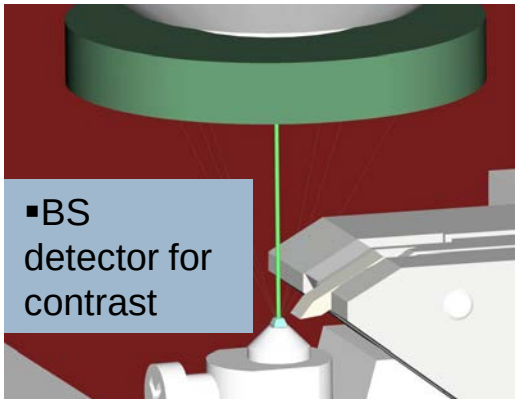
Specimen is pushed up and desired thickness shaved off.



Specimen lowers on knife retraction. Cut and retract takes seconds.

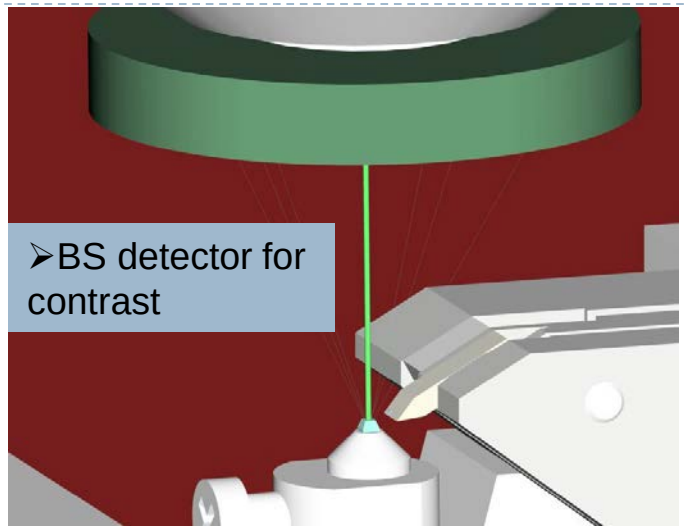


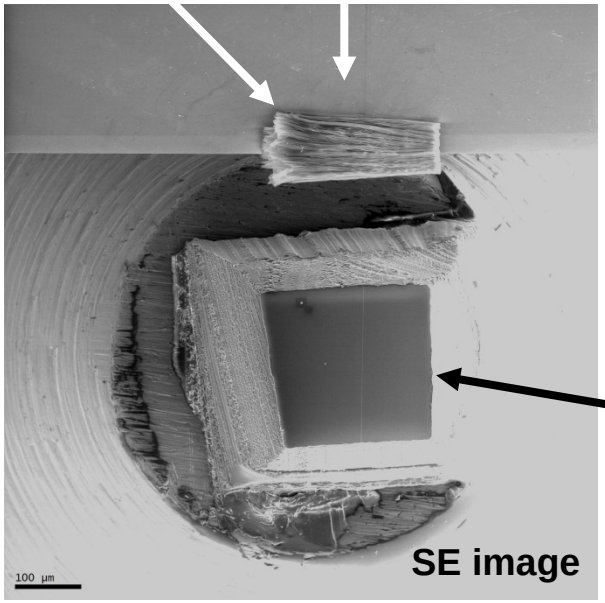
Block raised and Image acquired. Repeat.

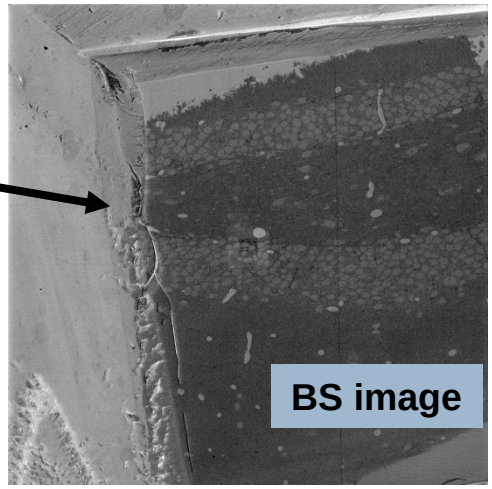


How it Works: Serial Block Face

➢**Shaved material stays on knife till cleaned.**



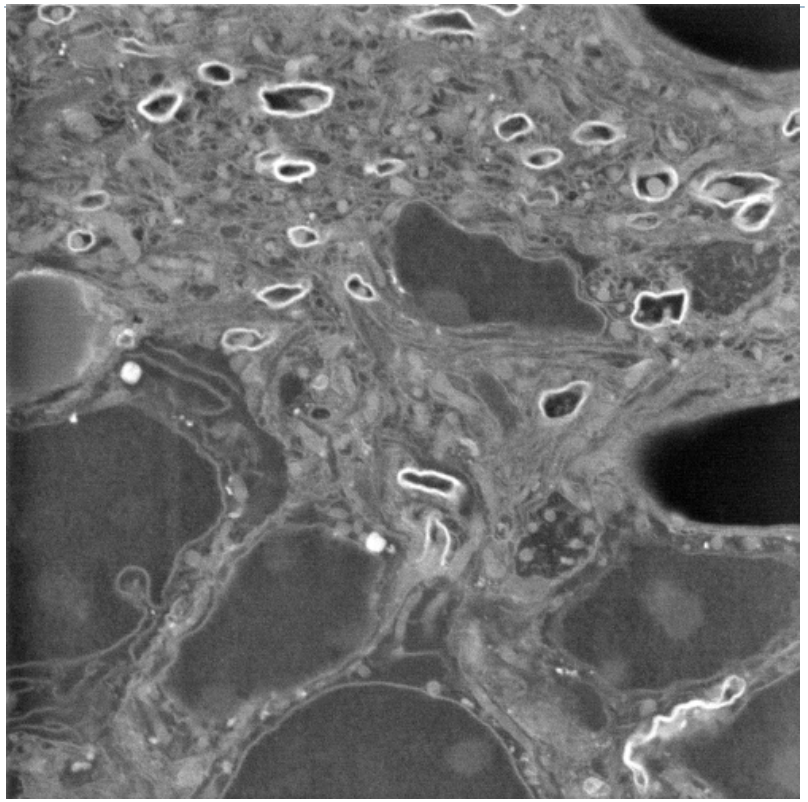




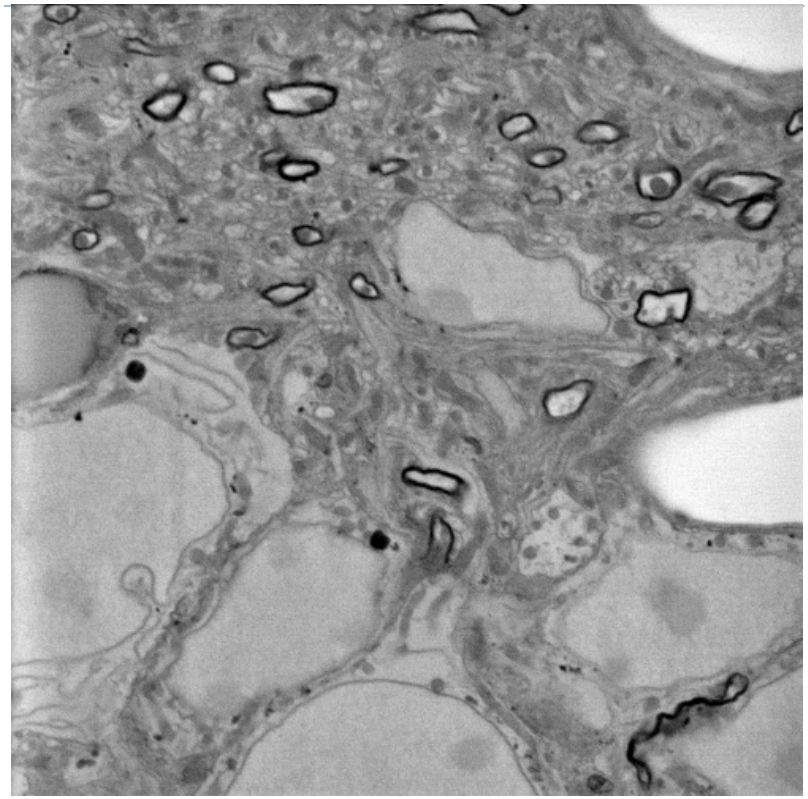
➢Freshly cut resin block face.

➢No topography contrast

How it Works: Serial Block Face



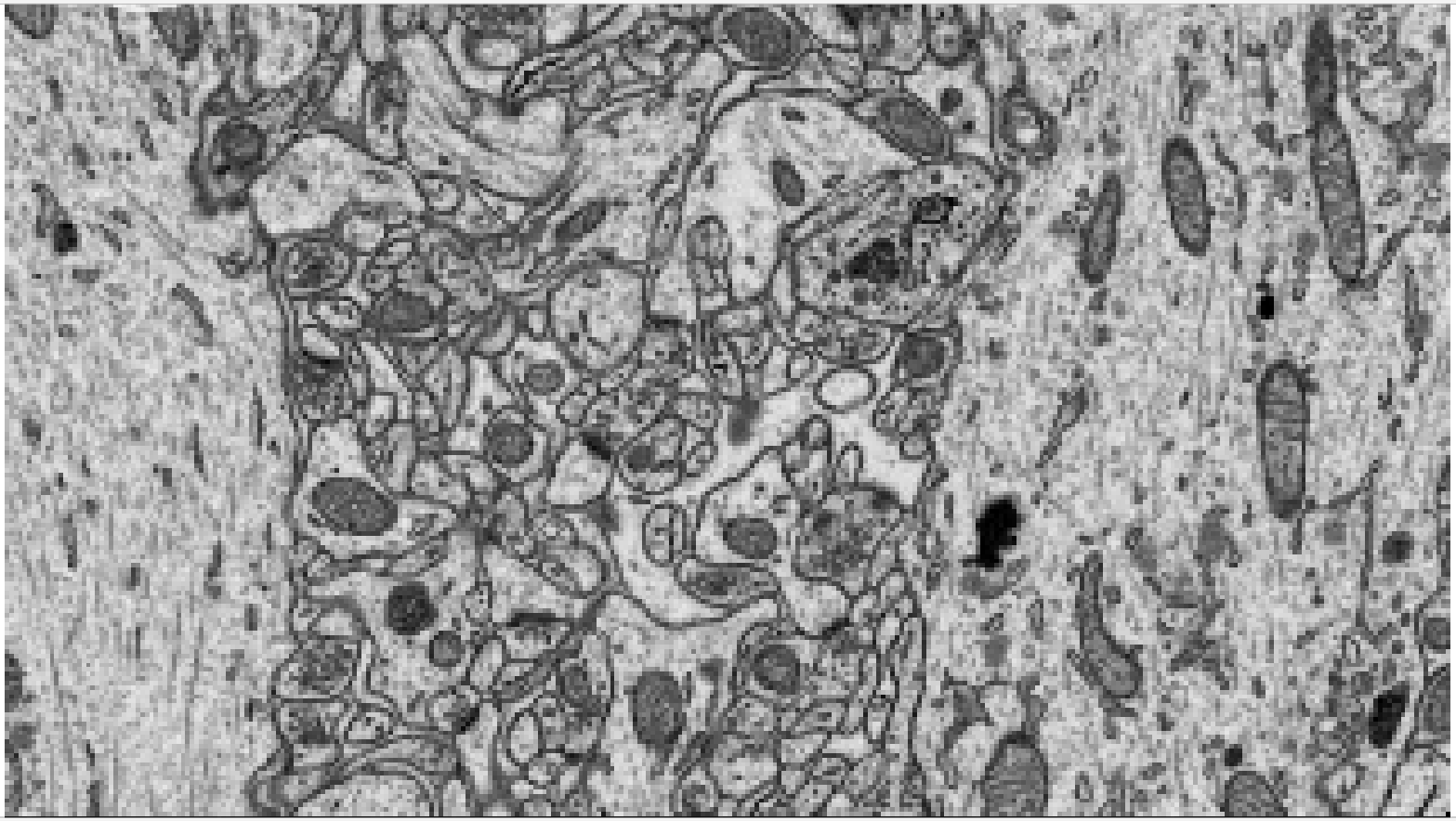
Raw BS signal. Brighter = denser area, more signal.



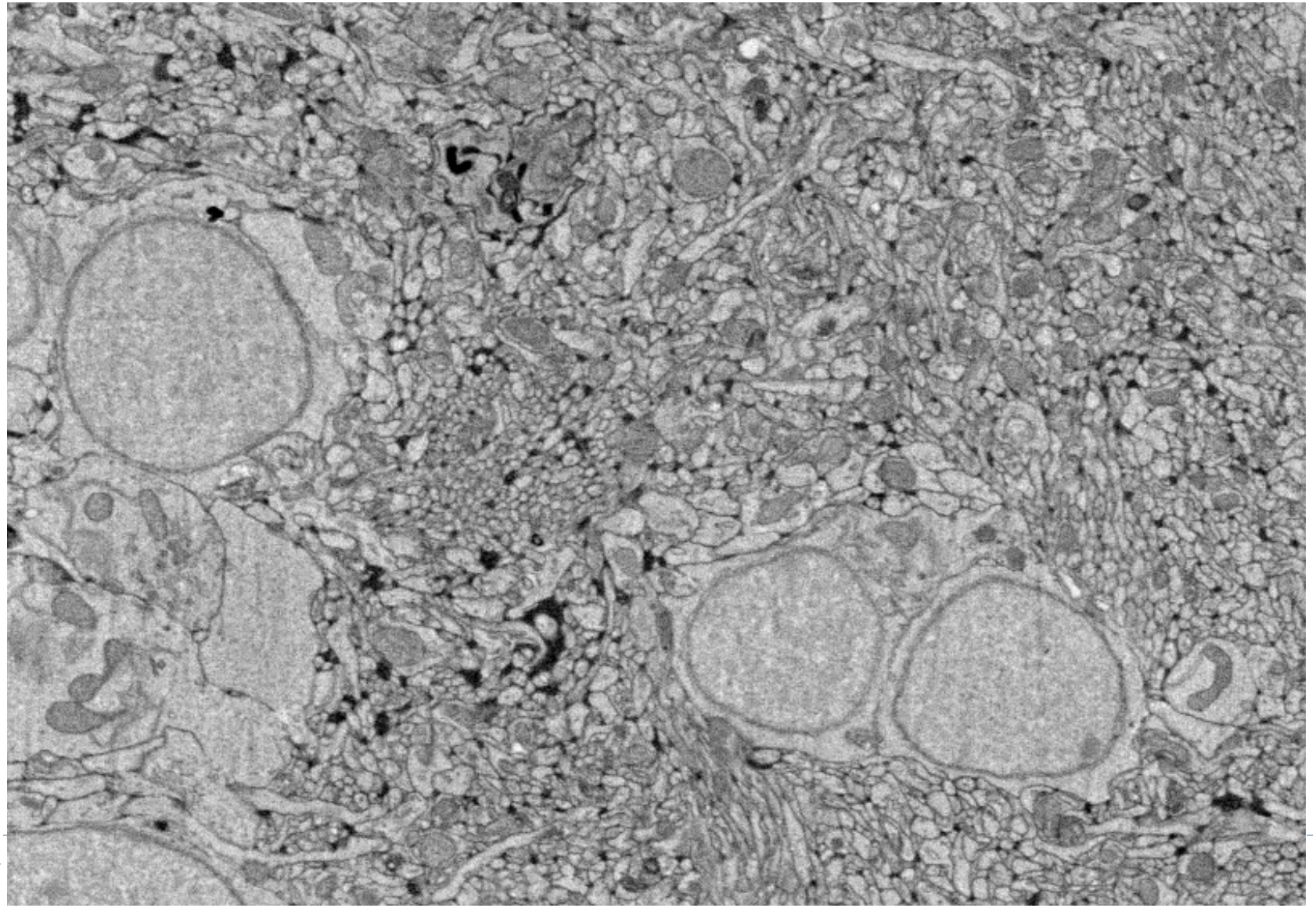
Reverse Contrast.

Reverse contrast similar to traditional TEM images, so easier to interpret.

Serial block face imaging



Serial block face imaging

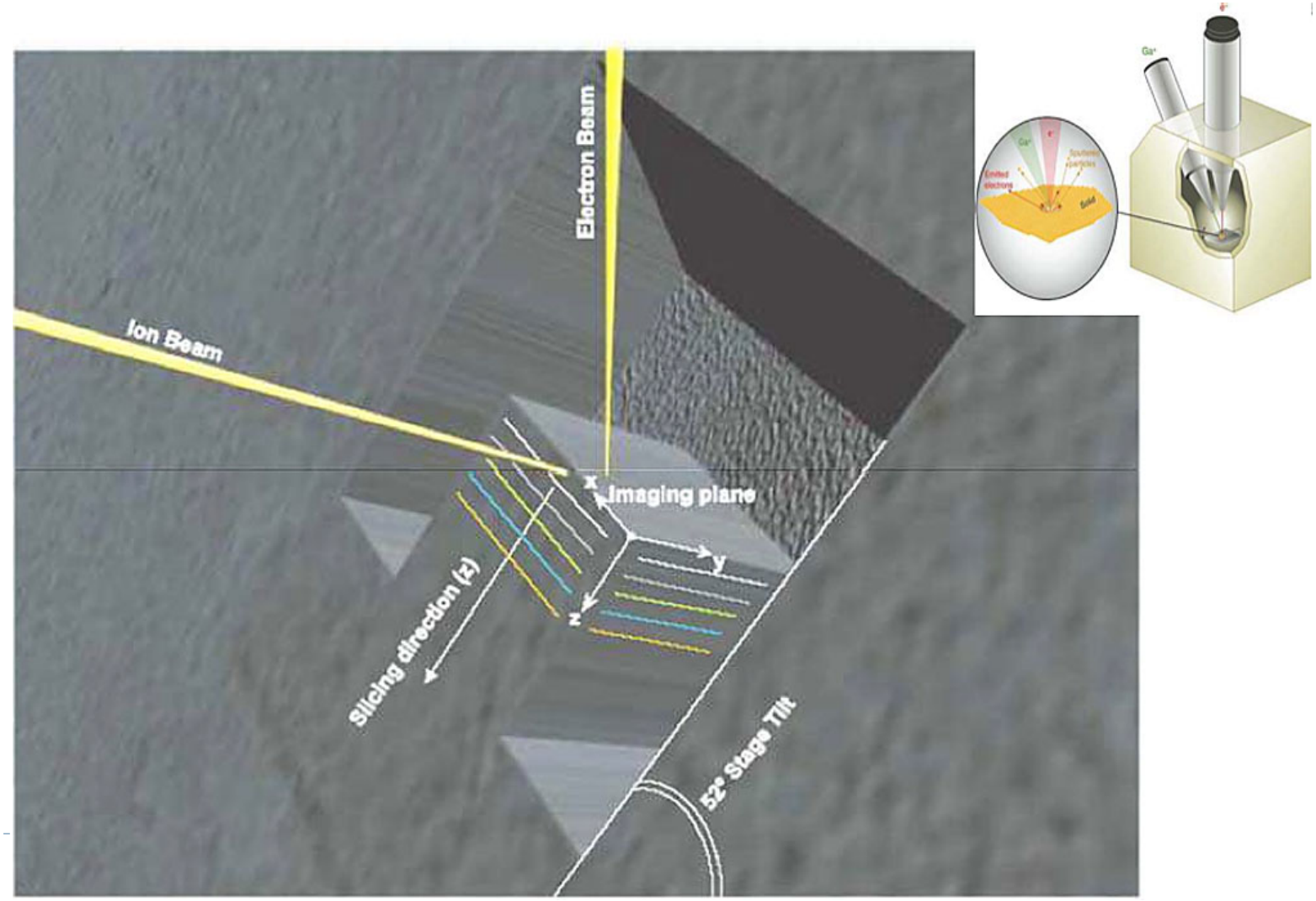


Serial Block Face Tomography

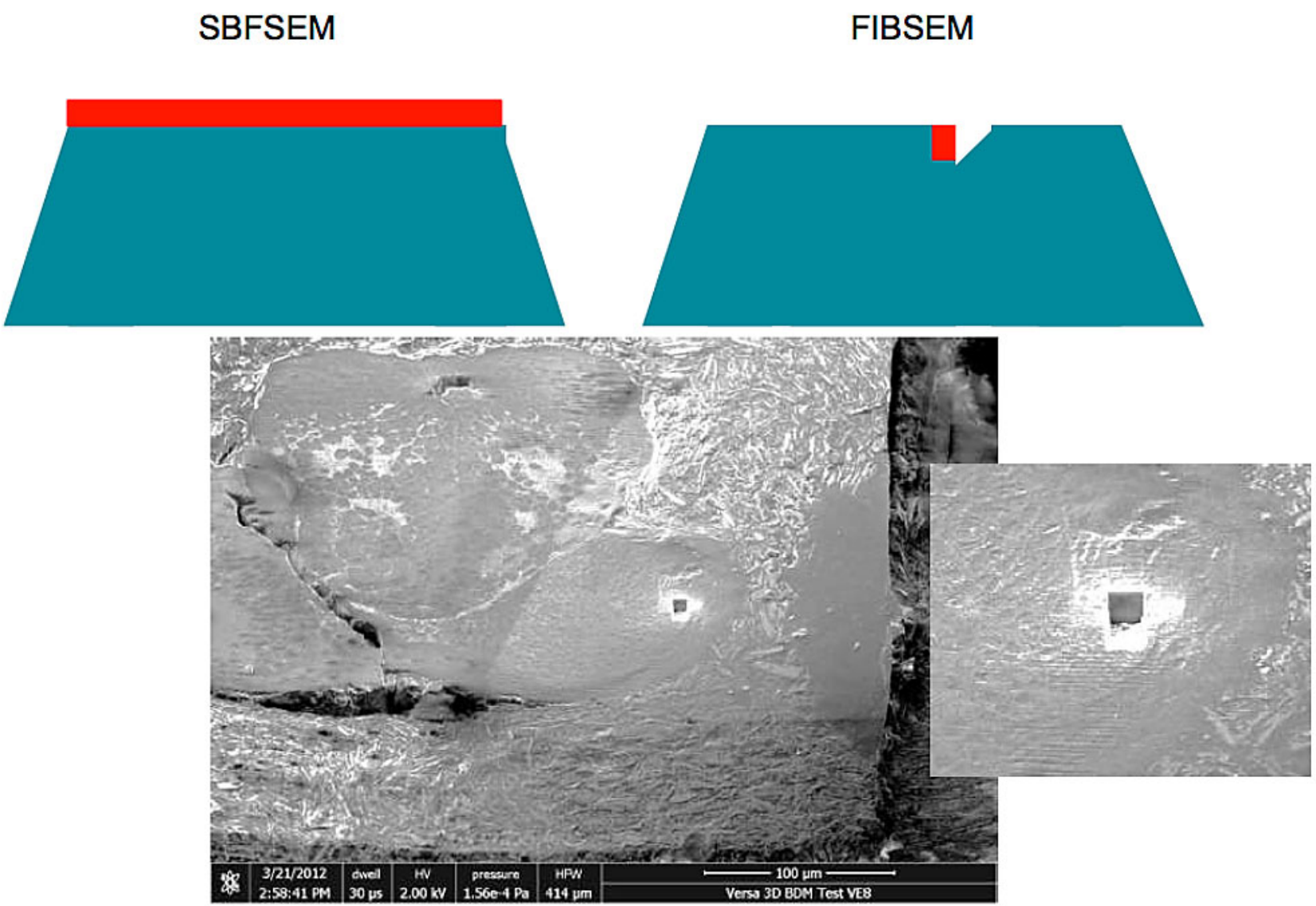
- **Advantages of 3-View**
  - Automated
  - Large specimen face imaged
  - Large volume imaging
  - Perfect specimen alignment
  - No missing wedge related artifacts

- **But:**
  - **SBF tomography is destructive**
  - Need 3-View and automated SEM
  - Changes due to prolonged exposure to electrons
  - What about immunolabeling?

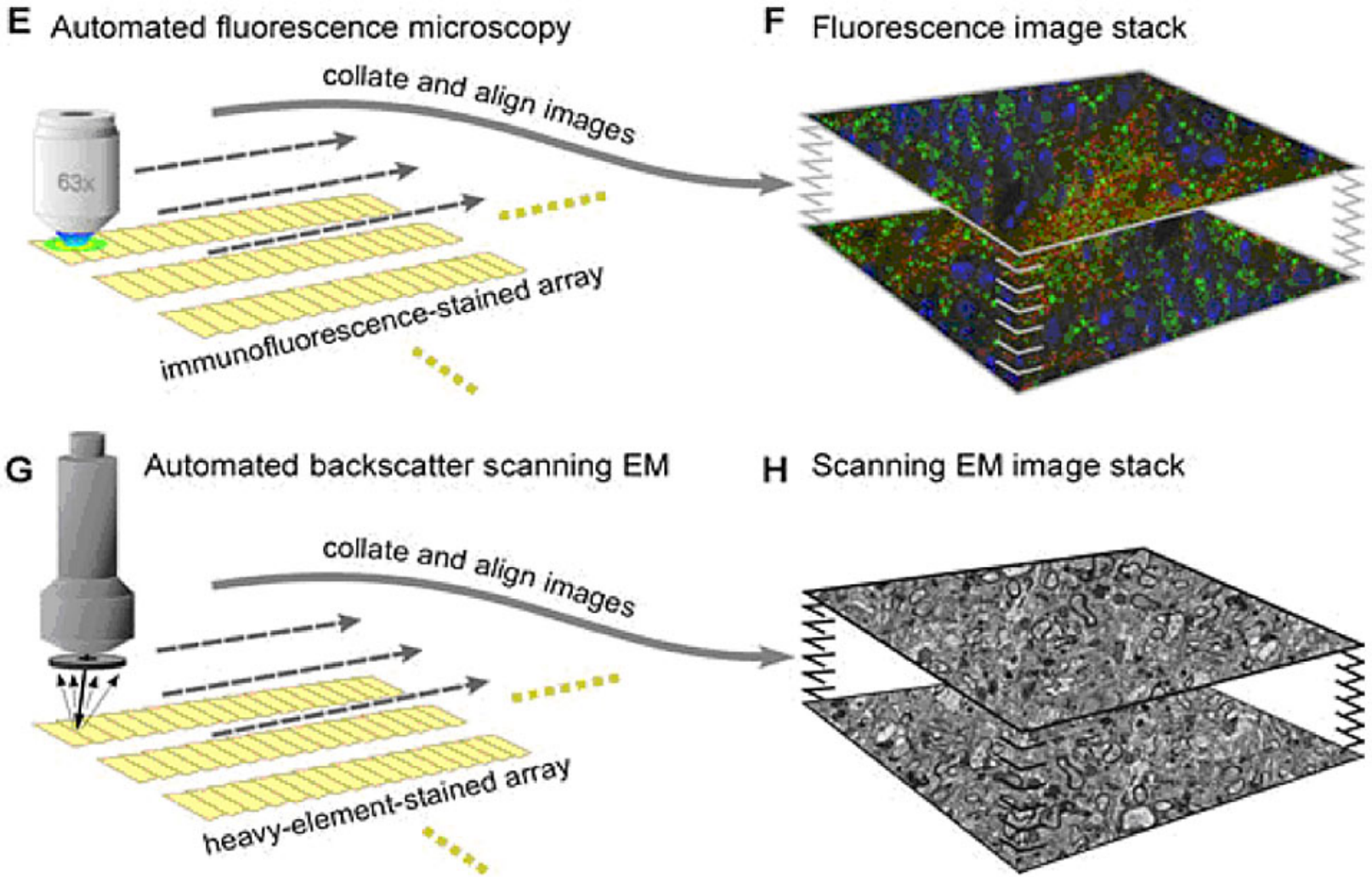
Focused Ion Beam-SEM (FIB-SEM)



FIB-SEM *v* 3-View

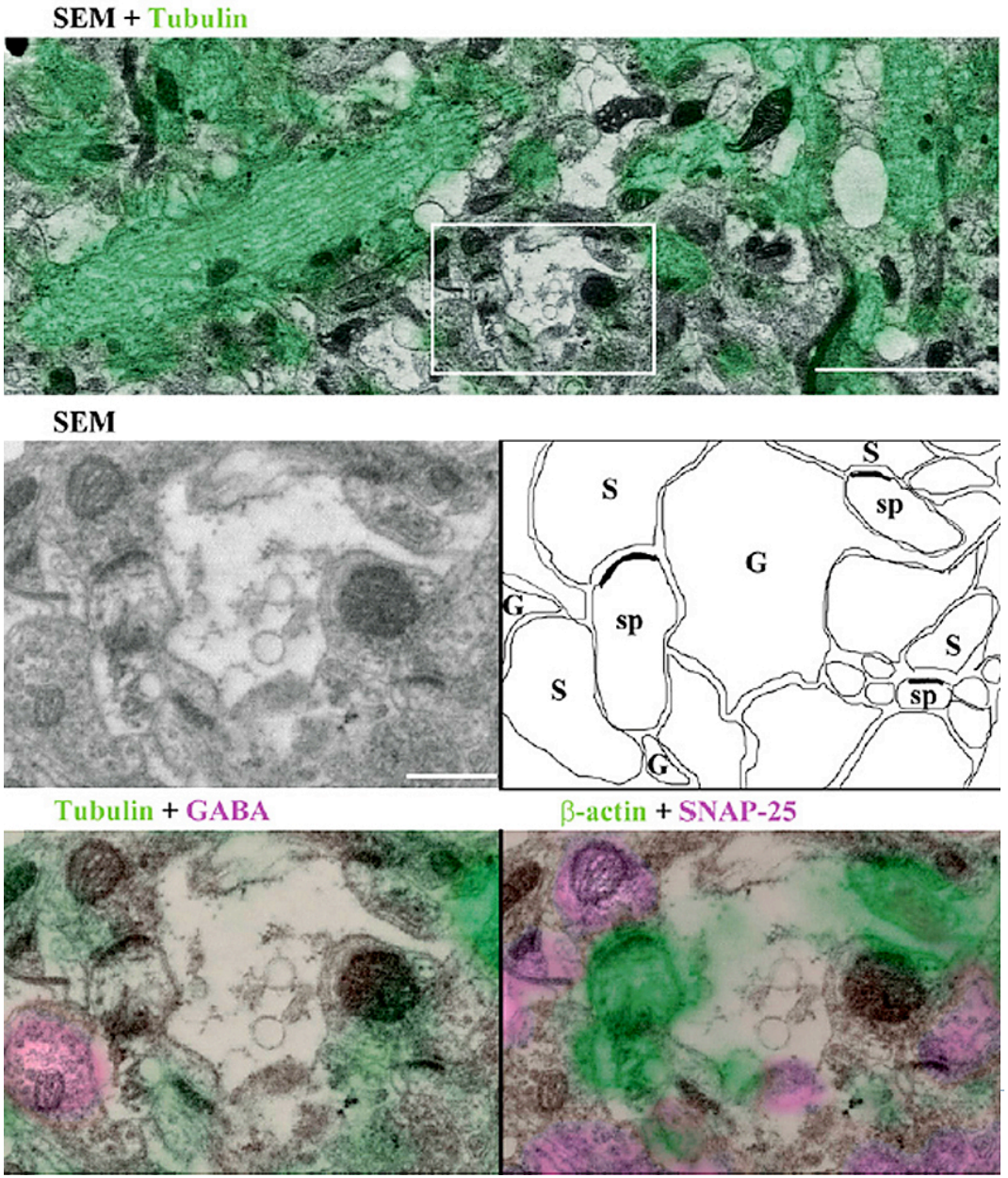


Array Tomography



Micheva, KD & Smith SJ. 2007 Neuron 55:25-36

Array Tomography

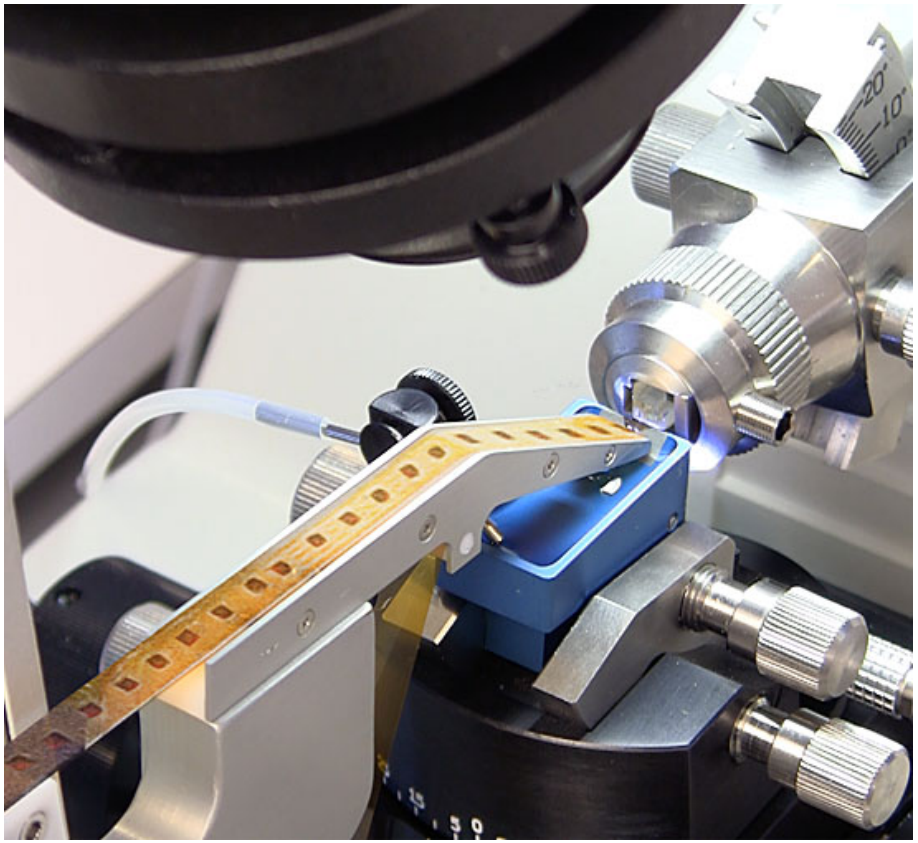


Micheva, KD & Smith SJ. 2007
Neuron 55:25-36

and

Micheva KD, O'Rourke N, Busse B, Smith SJ. 2010
Cold Spring Harb Protoc. Nov 1

Array Tomography



ATUMtome:

Automatic **T**ape **U**ltra**M**icrotomy

(*Jeff Lichtman, Harvard U*)

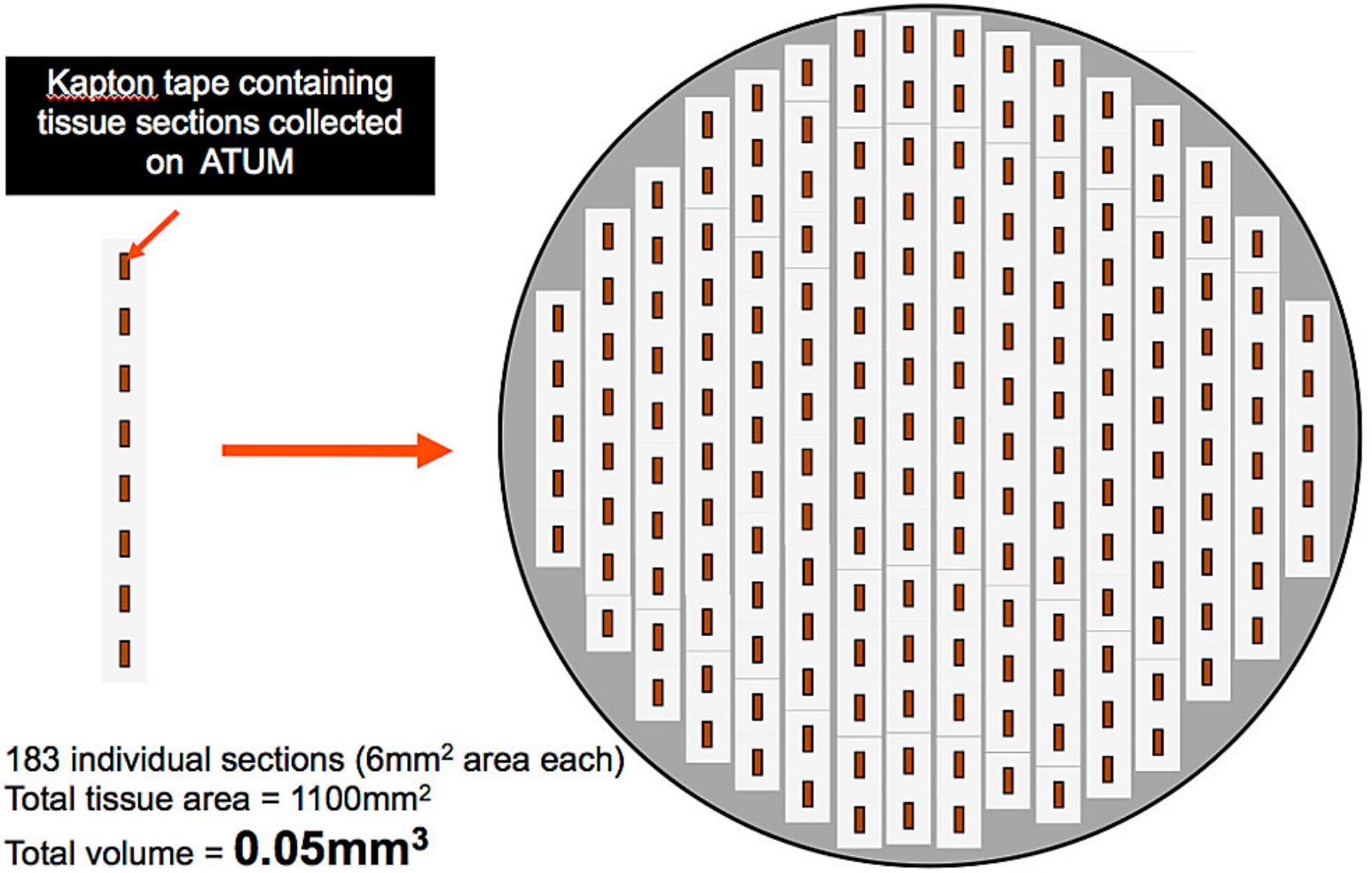
ATUM:

Attached to ultramicrotome

Sections collected on tape

LM and SEM (BSE) imaging

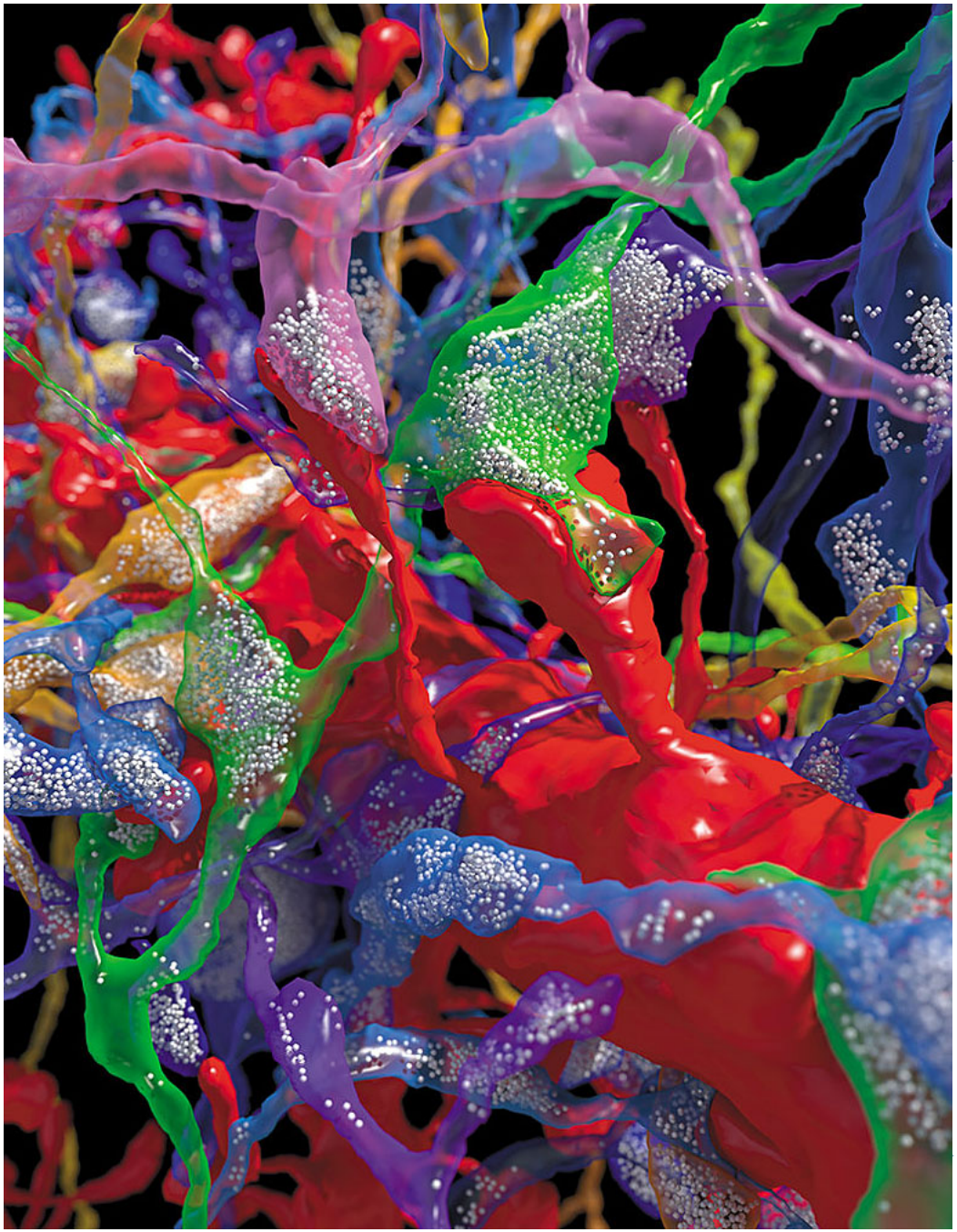
Array Tomography



Array Tomography

- **Advantages of ATUM**
  - Automated sectioning and section collection
  - Large specimen block-face
  - Immunocytochemistry possible (repeatedly)
  - Reusable sections
  - No missing wedge related artifacts
  - Useful for light microscopy (Z-axis resolution control)

- **But:**
  - **Time consuming**
  - Complicated data collection & reconstruction

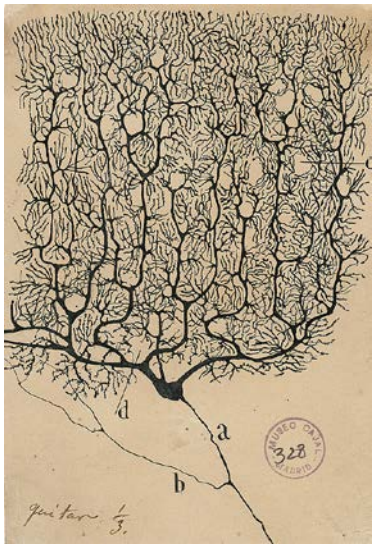


Dendrite reconstruction

From work by:
D. Berger, N. Kasthuri and J.W. Lichtman

Dendrite (red) and axons (multicolored)

1890's Ramón y Cajal: neuron doctrine



Summary

- Resolution
  - Instrument
  - Specimen
    - Preparation protocols
      - Chemical fixation, dehydration
      - Rapid freezing
      - Rapid freezing, dehydration
- Reference space
  - Context
- Cell components
  - Immunolabeling
- Volumes
  - 3-D relationships