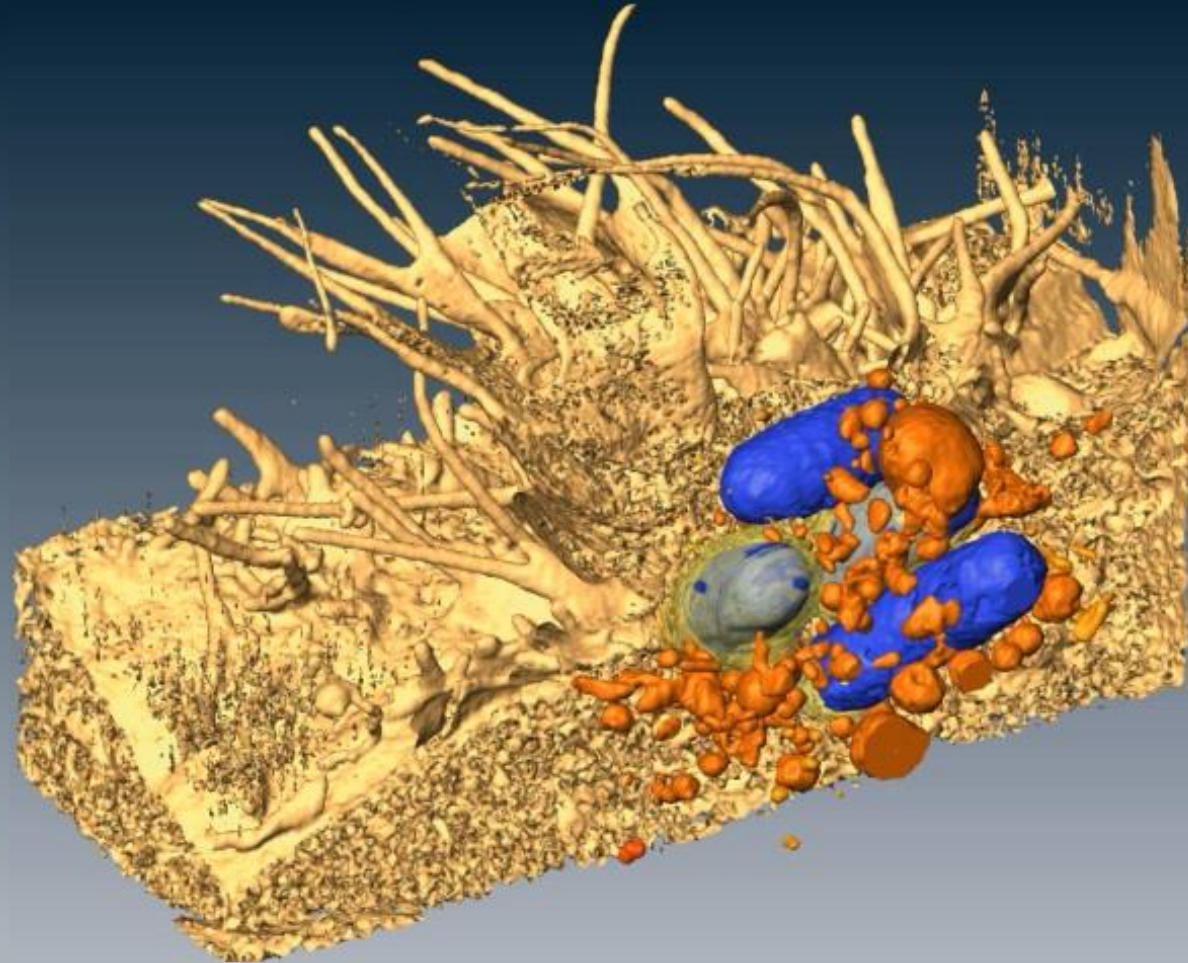


FIB-SEM Tomography



Volume Imaging and more

Katya Rechav

Electron Tomography
Virtual Workshop
23-26 November, 2020

Four applications. Two devices. One system



Scanning Electron Microscope

Focused Ion Beam

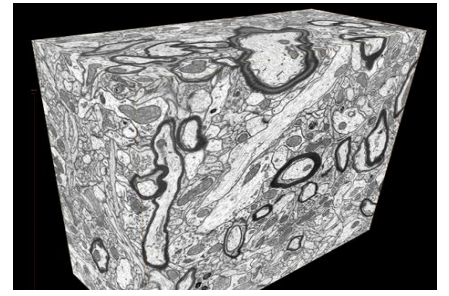
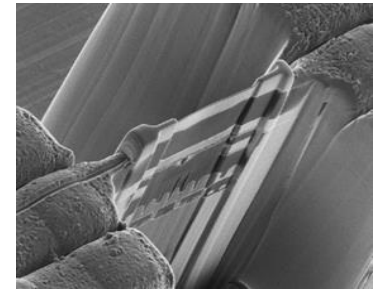
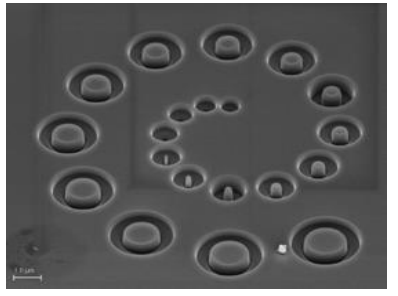
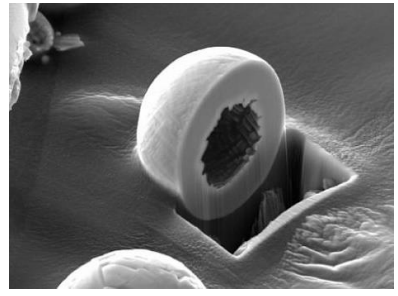
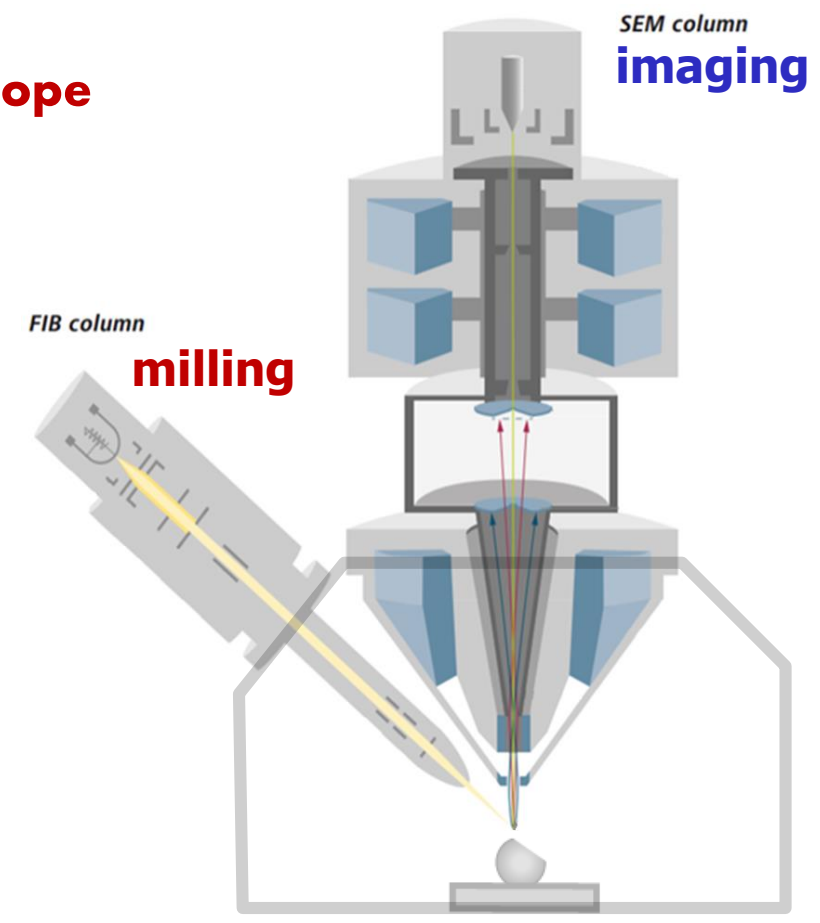
Gas Injection System

Micromanipulator

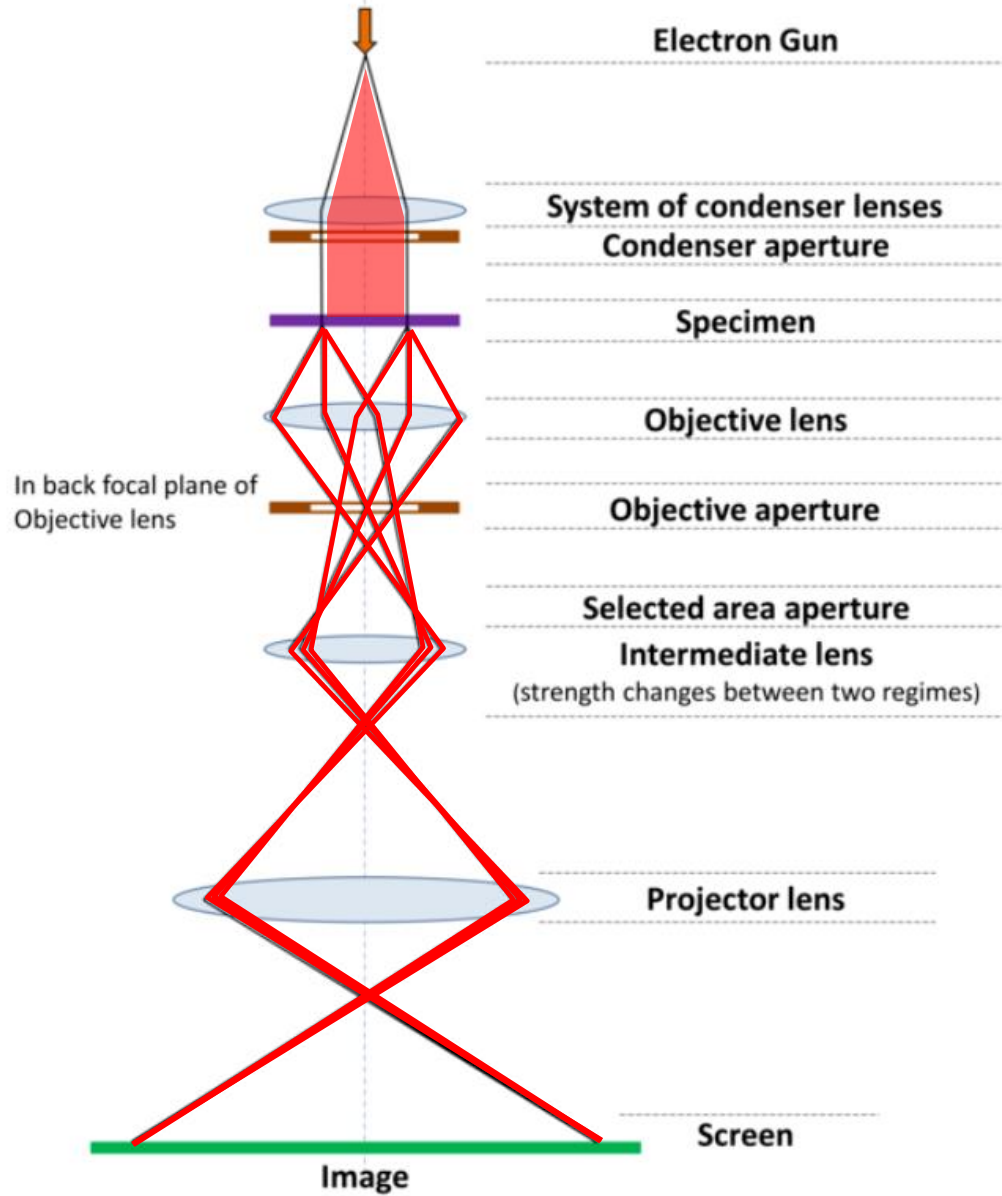
EDX spectrometer

STEM detector

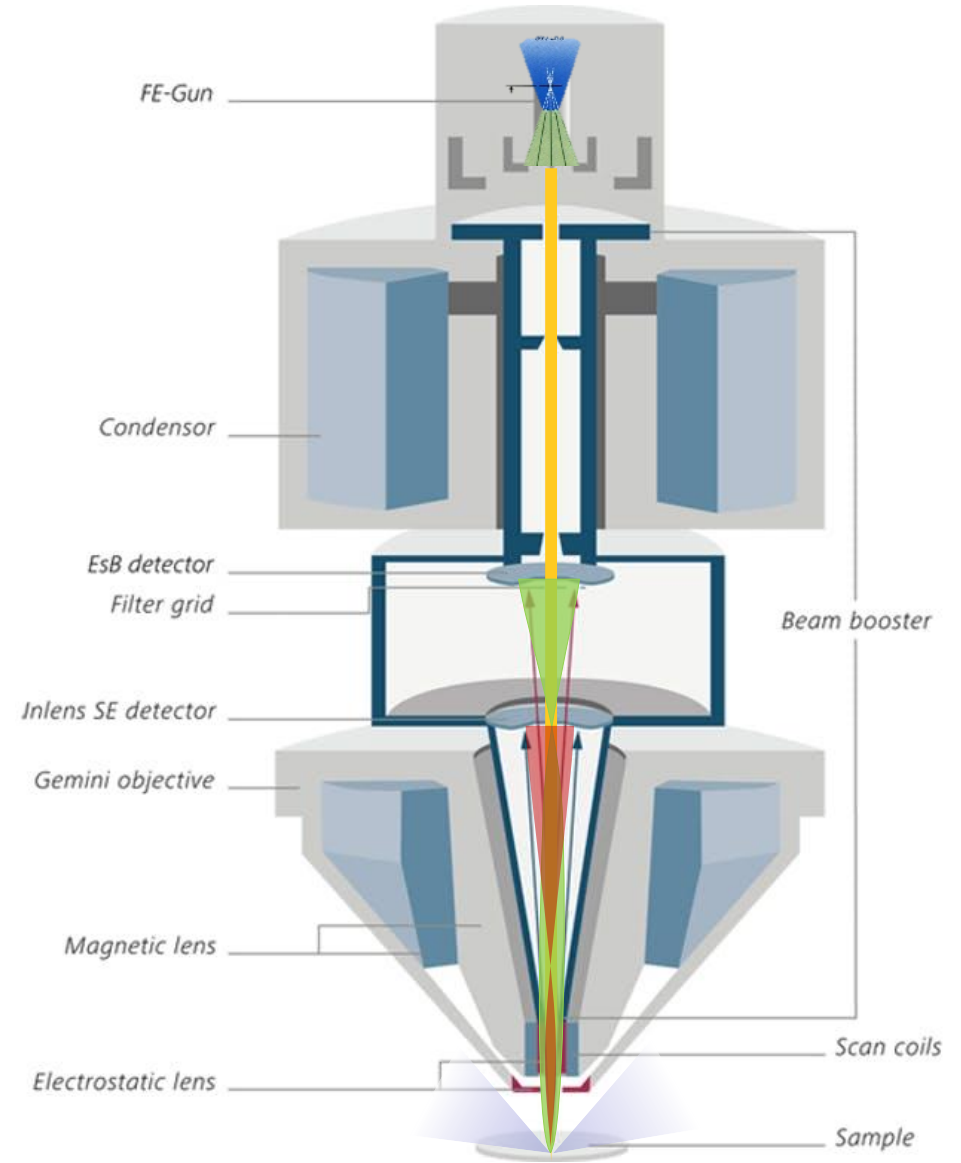
Cryo stage



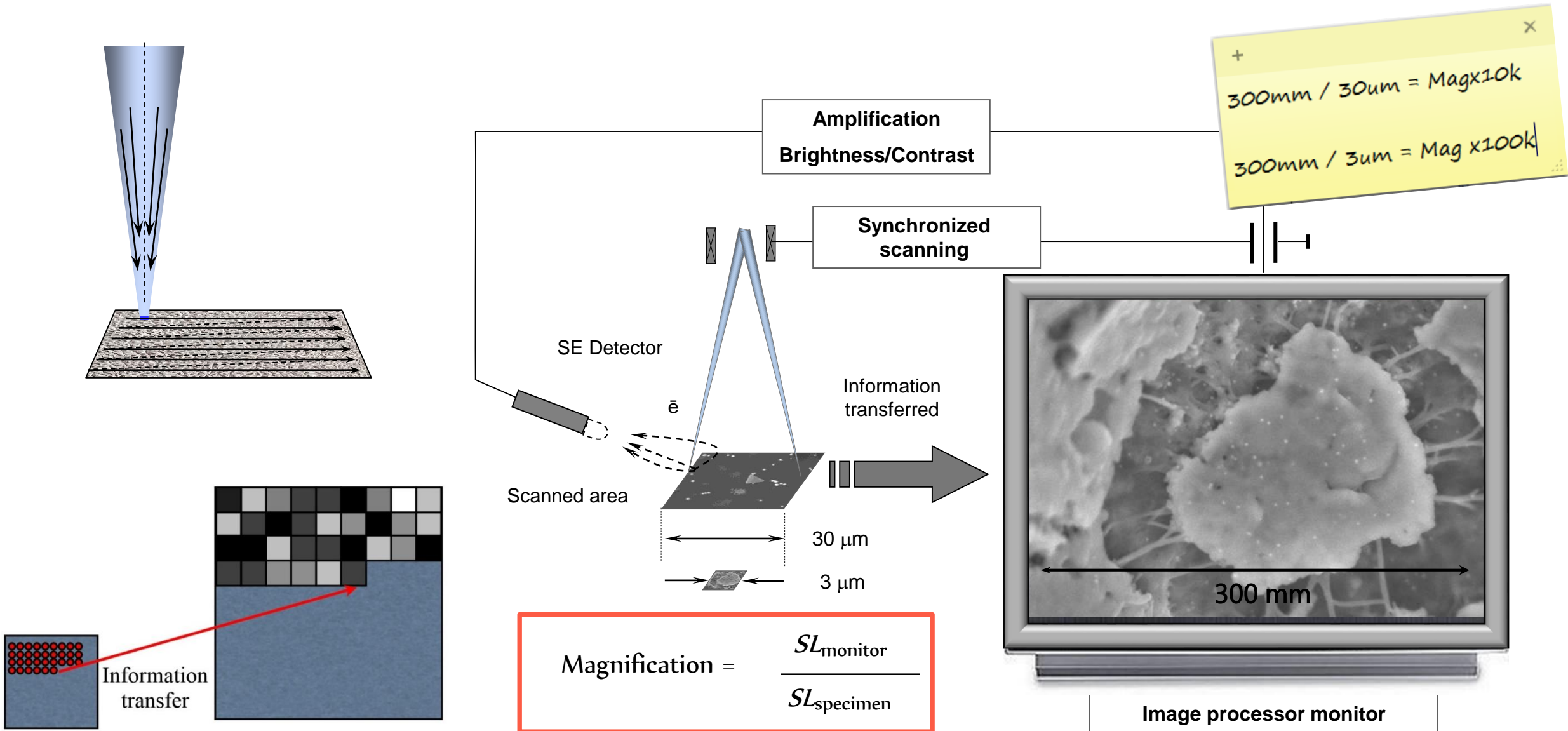
TEM BF imaging mode



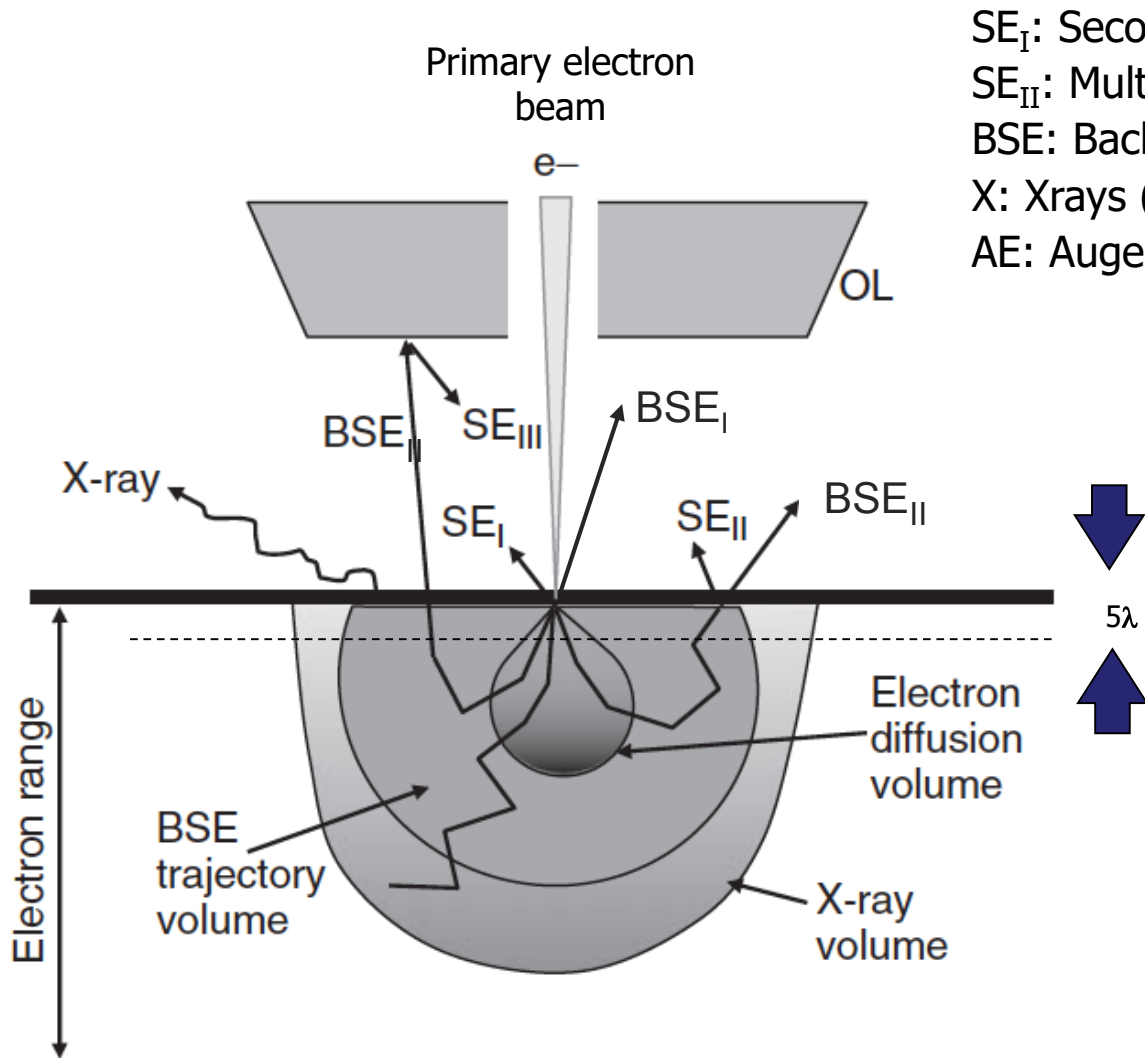
SEM



SEM image formation

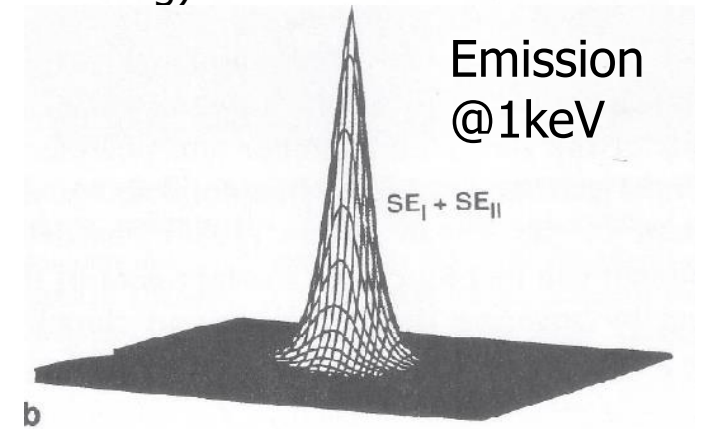


Interaction volume and emission region



- SE_I: Secondary electrons, "single scattering"
- SE_{II}: Multiple scattering
- BSE: Backscattered electrons
- X: X-rays (characteristic and bremsstrahlung)
- AE: Auger electrons

Region of secondary electron emission



Goldstein III edition

Image resolution depends on the electron beam size
AND
 on the interaction volume

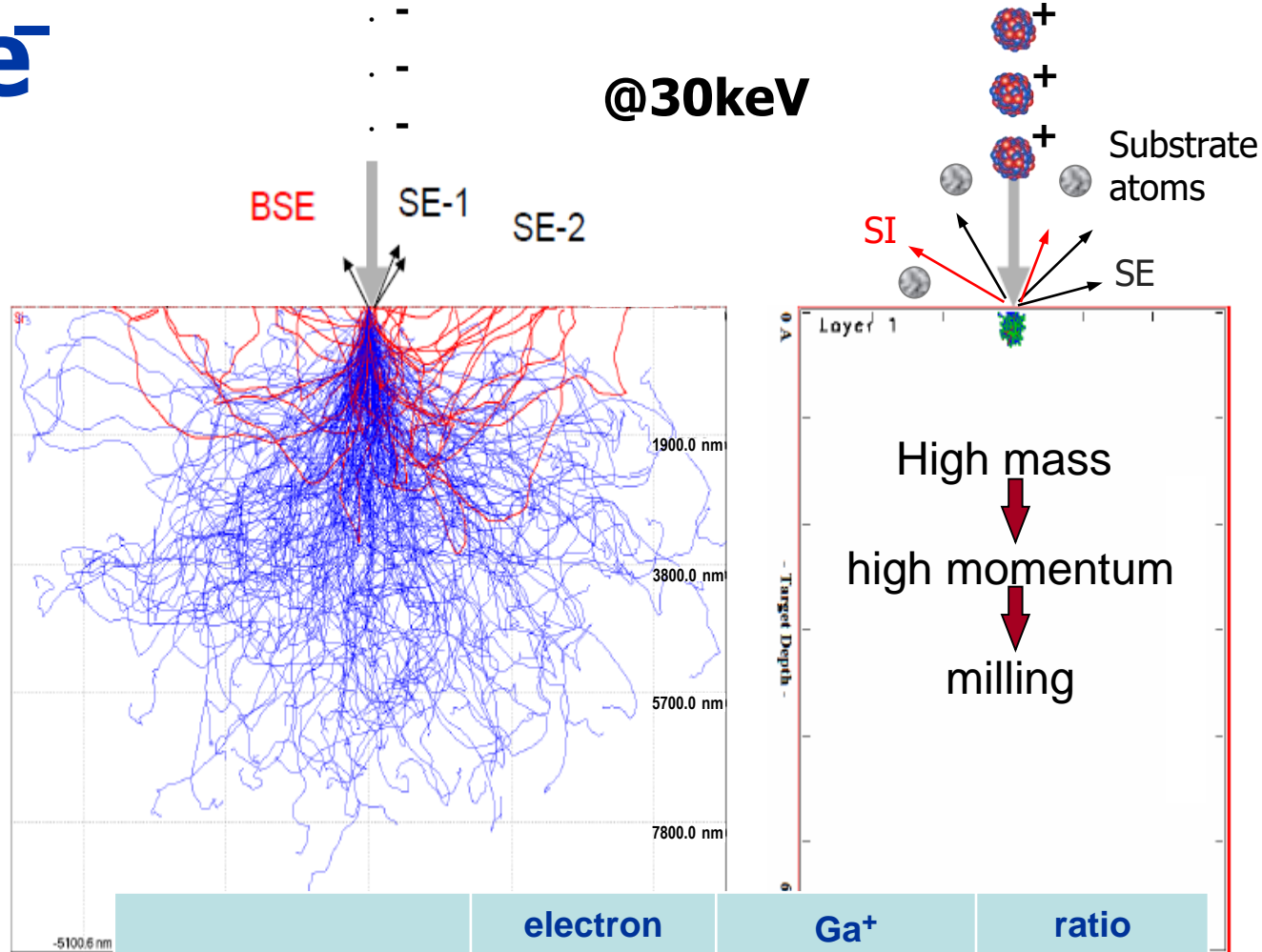
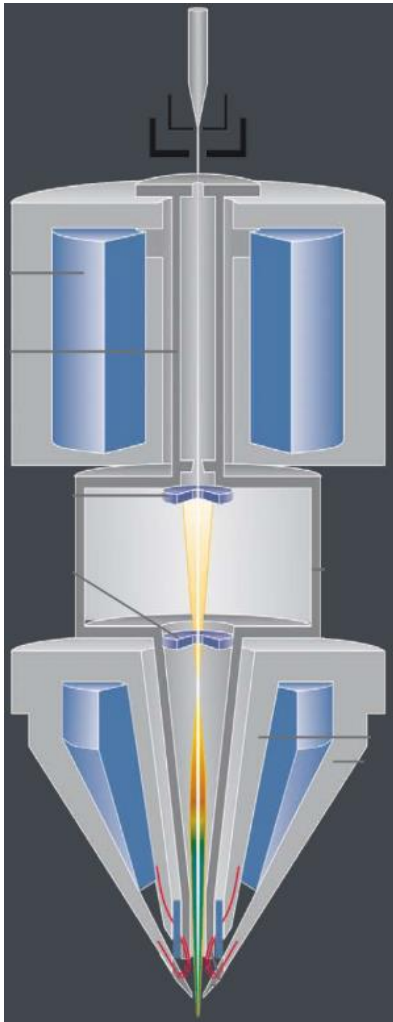
30kV electrons Vs Ga⁺ ions

0.6 - 30keV

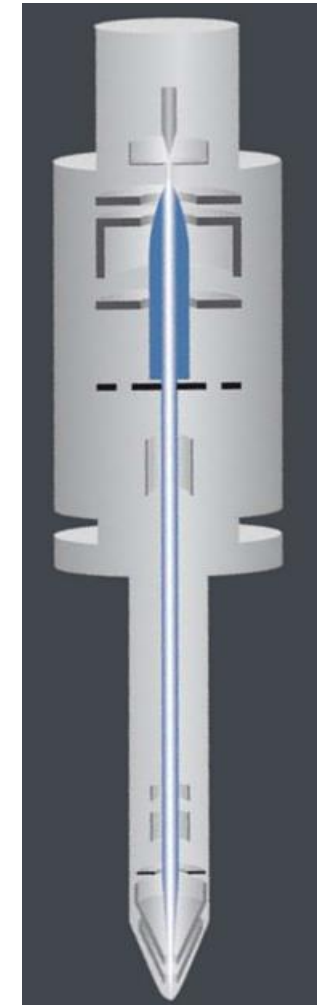
e⁻

@30keV

Ga⁺ 30keV



Monte-Carlo Simulation Casino v2.42

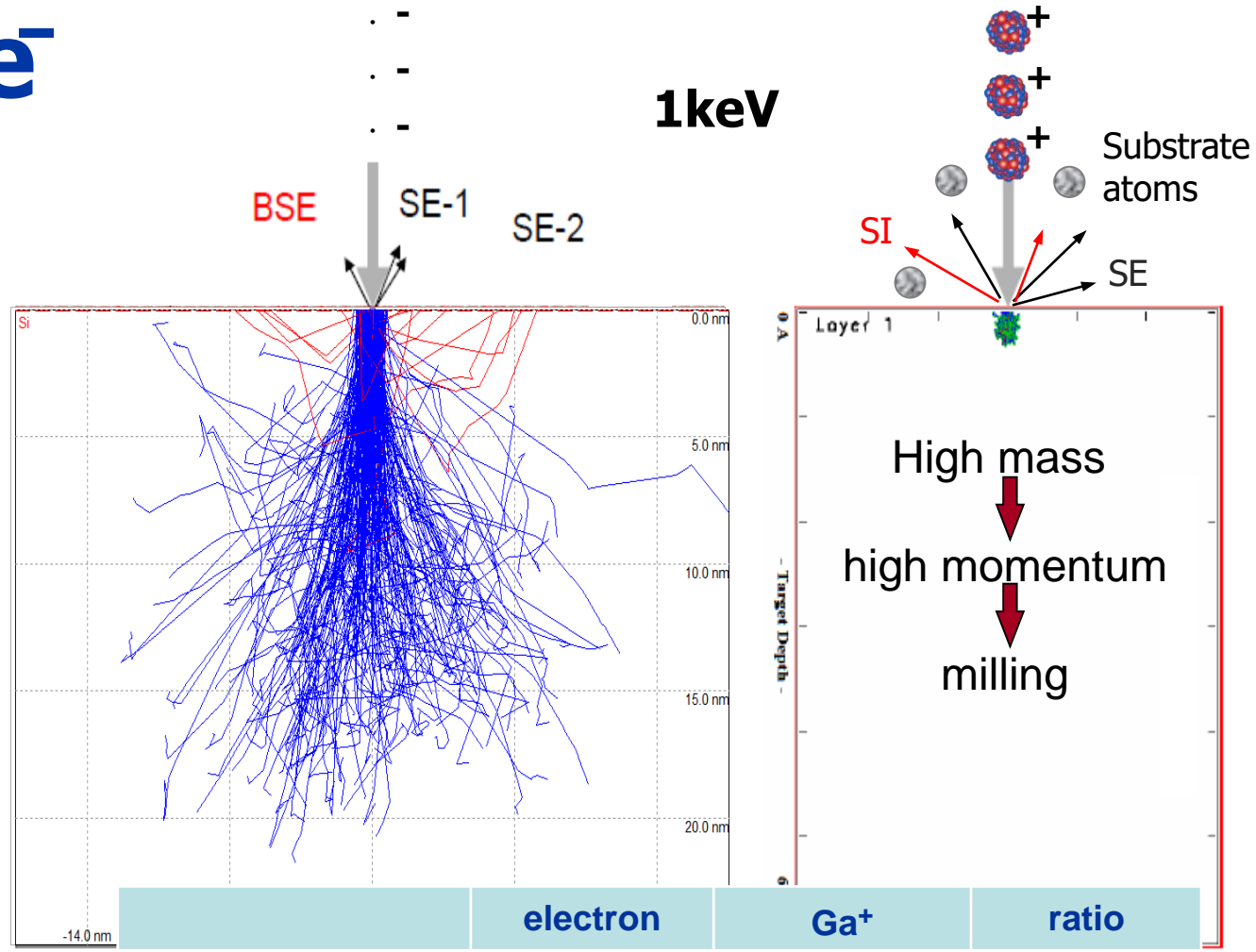
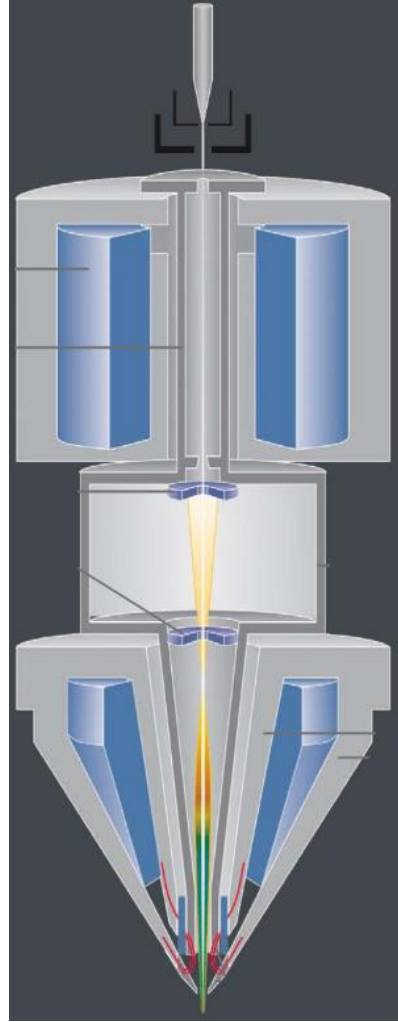


30kV electrons Vs Ga⁺ ions

e⁻

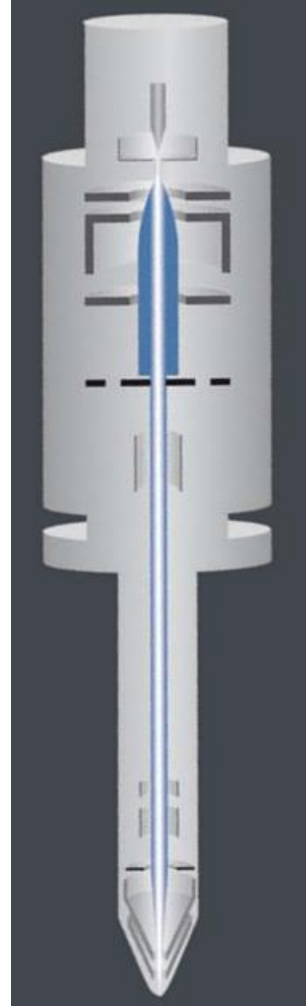
1keV

Ga⁺ 30kV

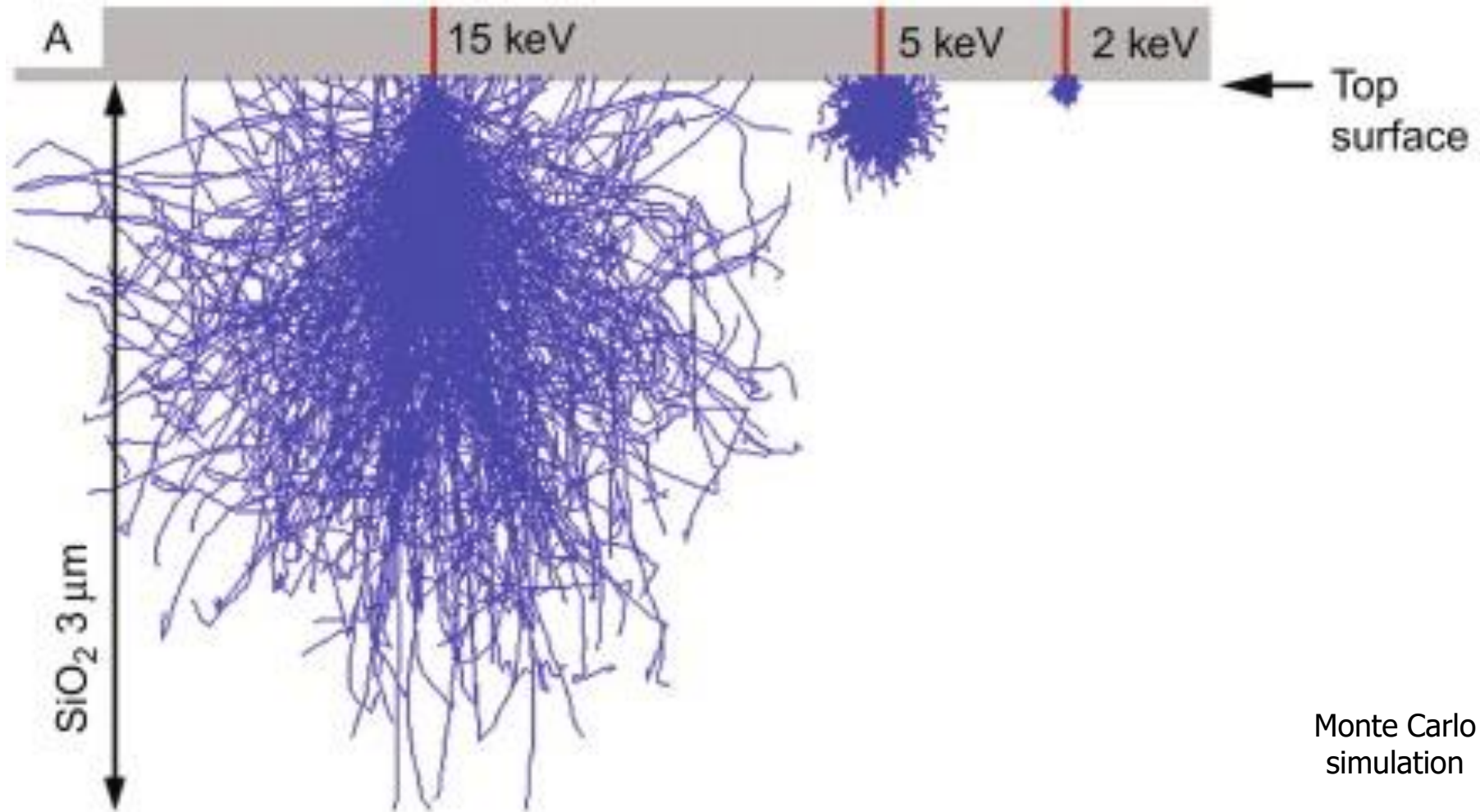


| | electron | Ga ⁺ | ratio |
|---------------|--------------------------|--------------------------|---------|
| particle size | 0.00001 nm | 0.2 nm | 20'000 |
| mass | 9.1×10 ⁻²⁰ kg | 1.2×10 ⁻²⁵ kg | 130'000 |

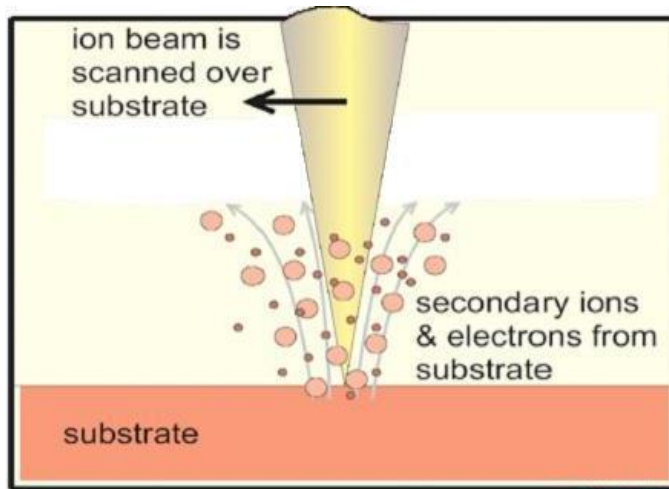
Monte-Carlo Simulation Casino v2.42



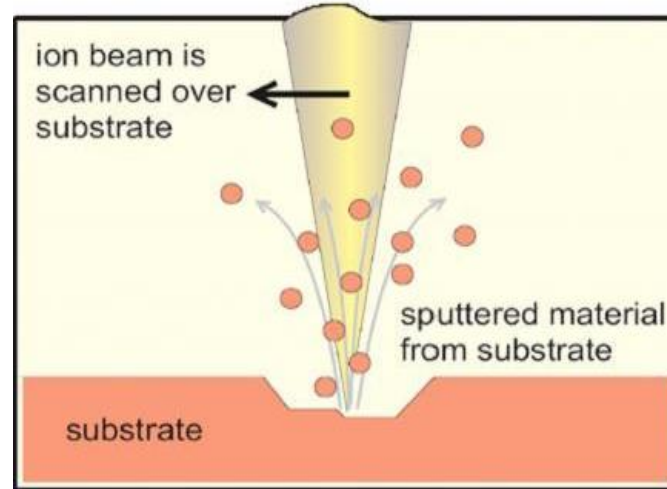
Interaction volume at different electron energy



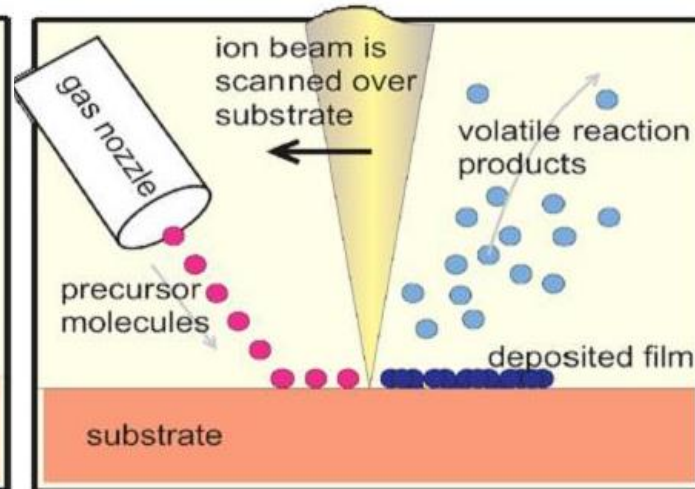
FIB working modes



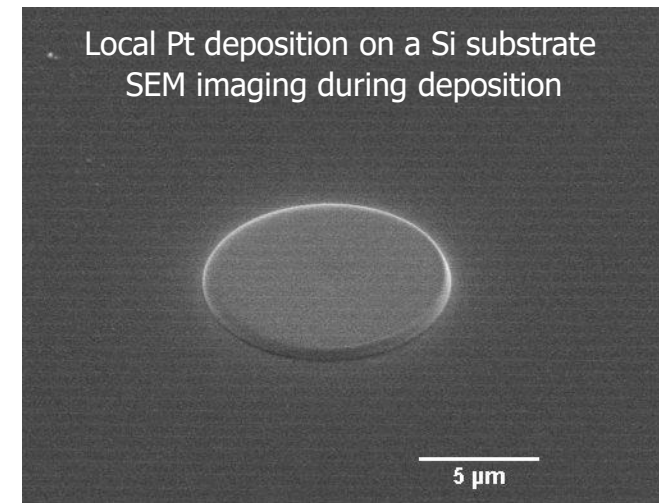
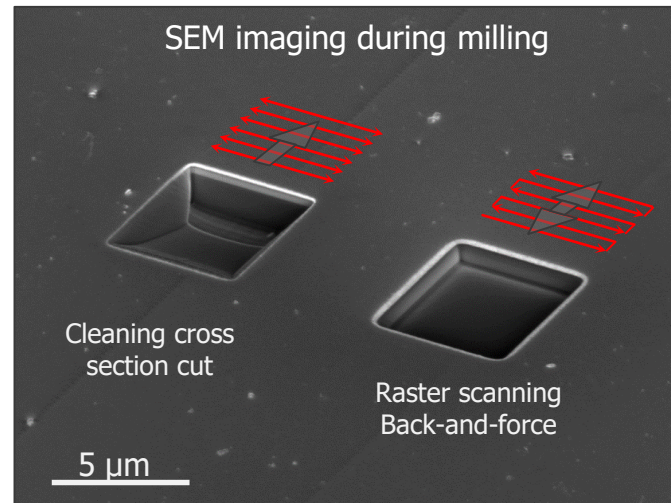
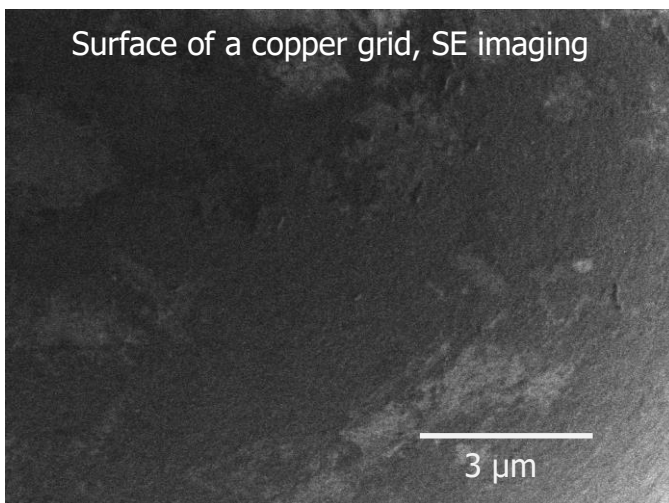
Emission of secondary ions and electrons
– FIB **imaging** - low ion current



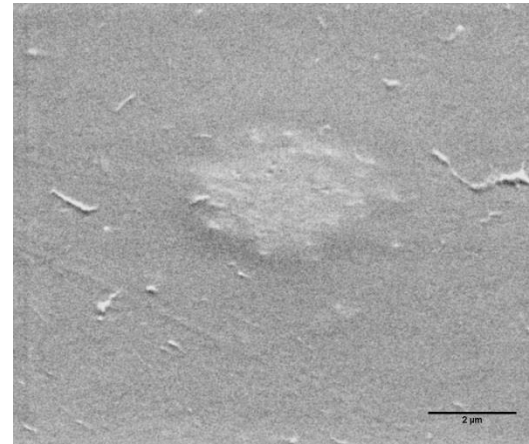
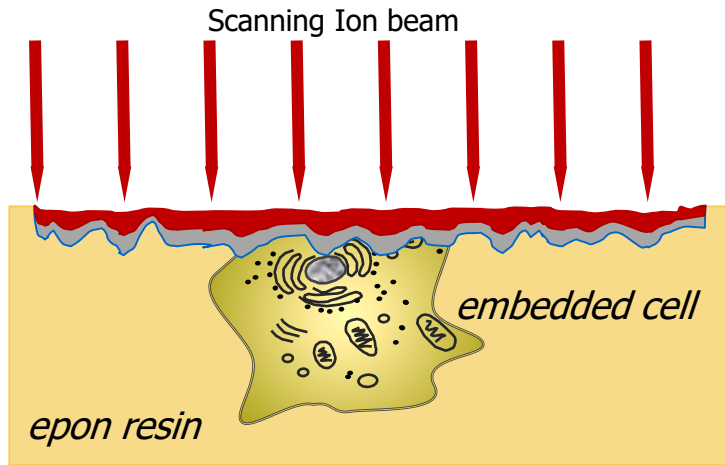
Sputtering of substrate atoms
– FIB **milling** - high ion current



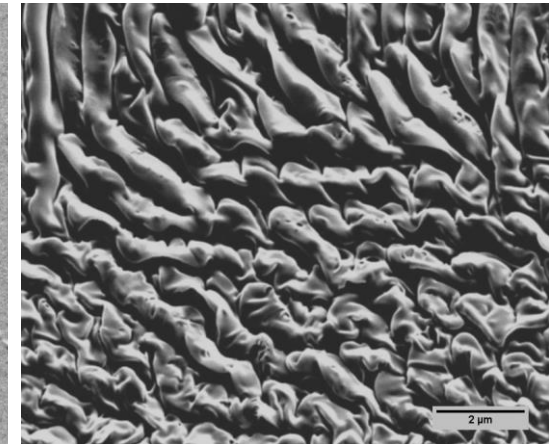
Chemical Vapor Deposition (gas assisted)
– FIB induced Pt **deposition**



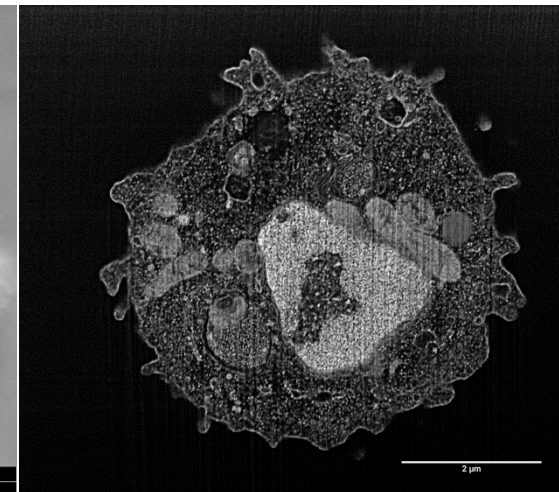
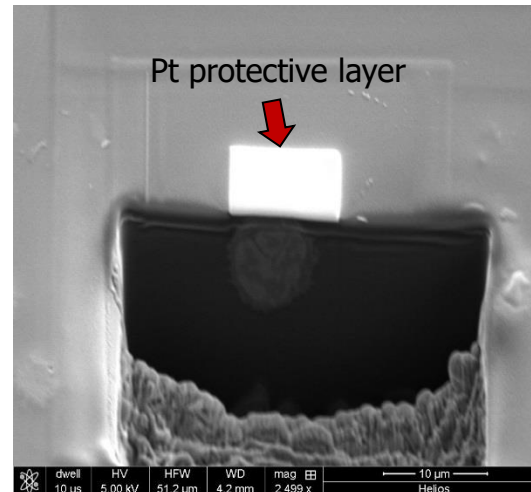
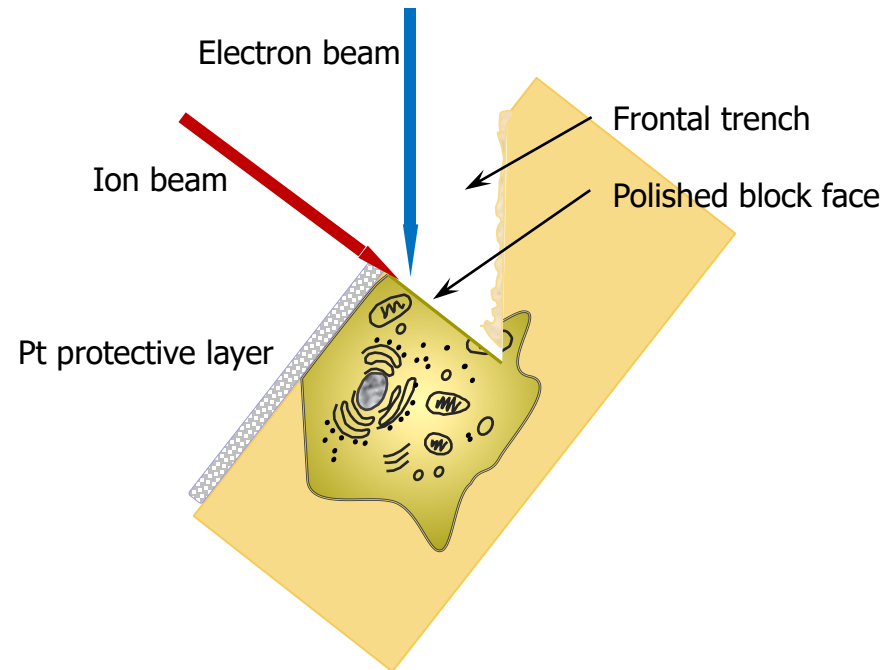
Dual beam FIB - SEM geometry



Before FIB exposure



After exposure of 30s. $I_i = 340\text{pA}$



SEM images of FIB prepared cross section of T-cell embedded into epon resin

Types of Ion Irradiation Damage

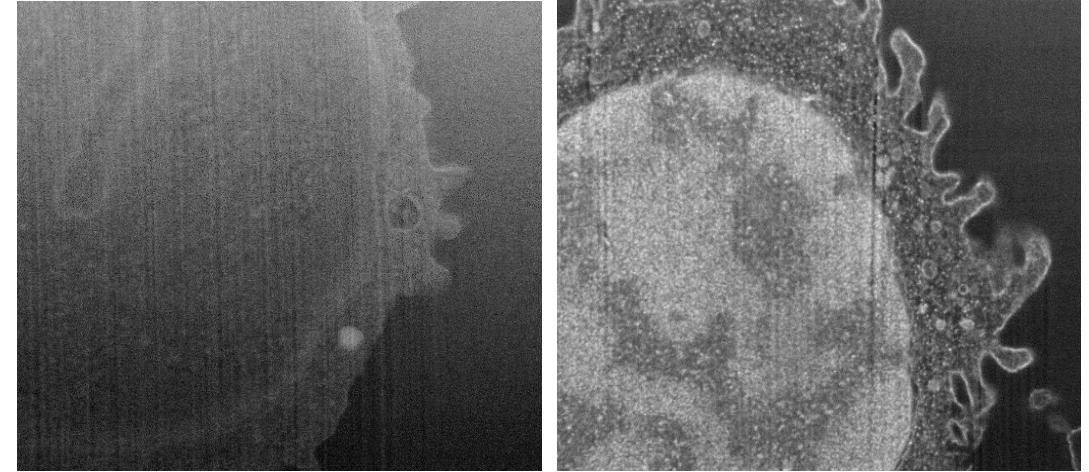
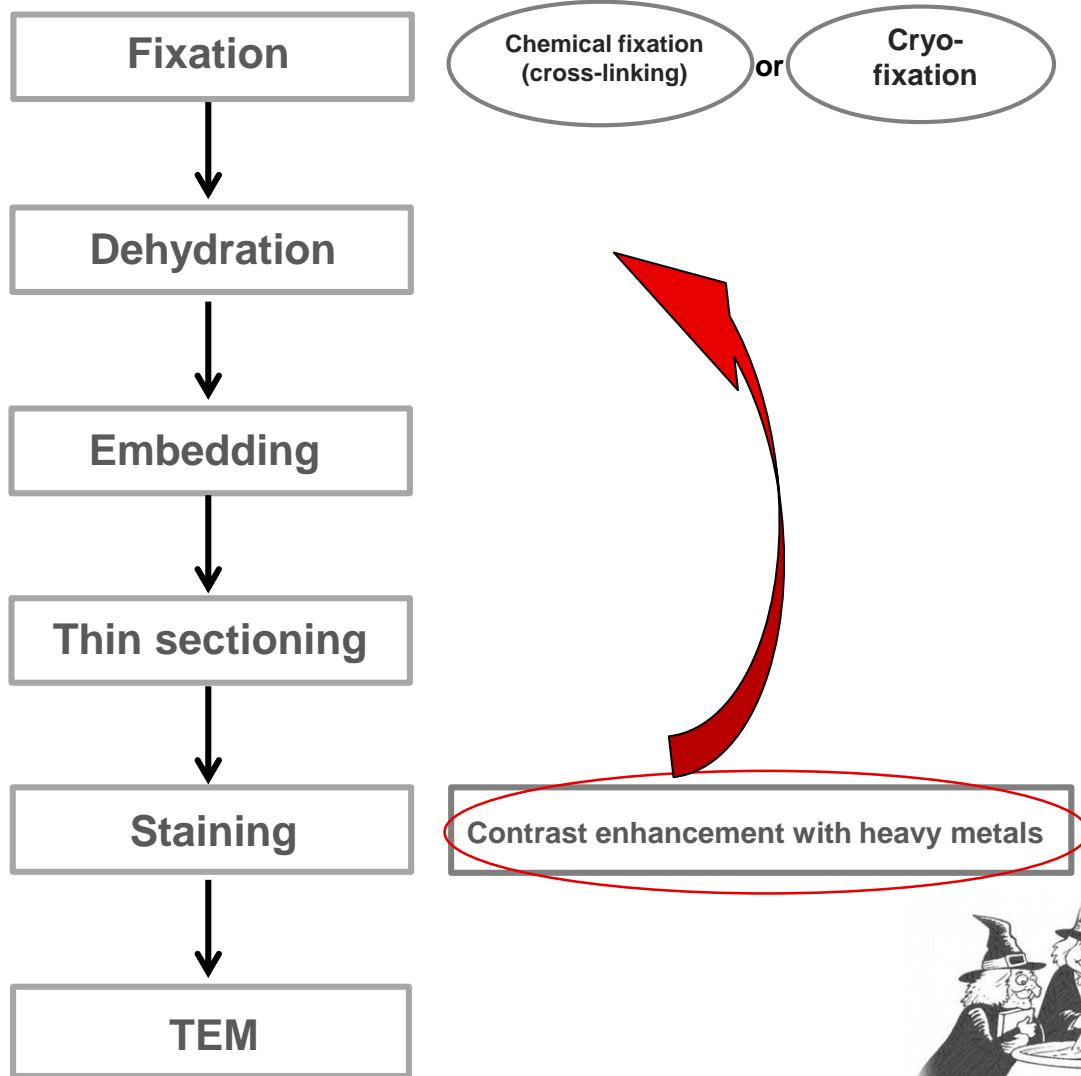


- **Amorphization of surface – loss of crystallinity**
- **Lattice Defects – Vacancies / Interstitials / Dislocations**
- **Ion Implantation – Ga⁺ atoms remain in the sample target**
- **Local heating**
 - **Melting-like effect**
 - **Morphological deformations**

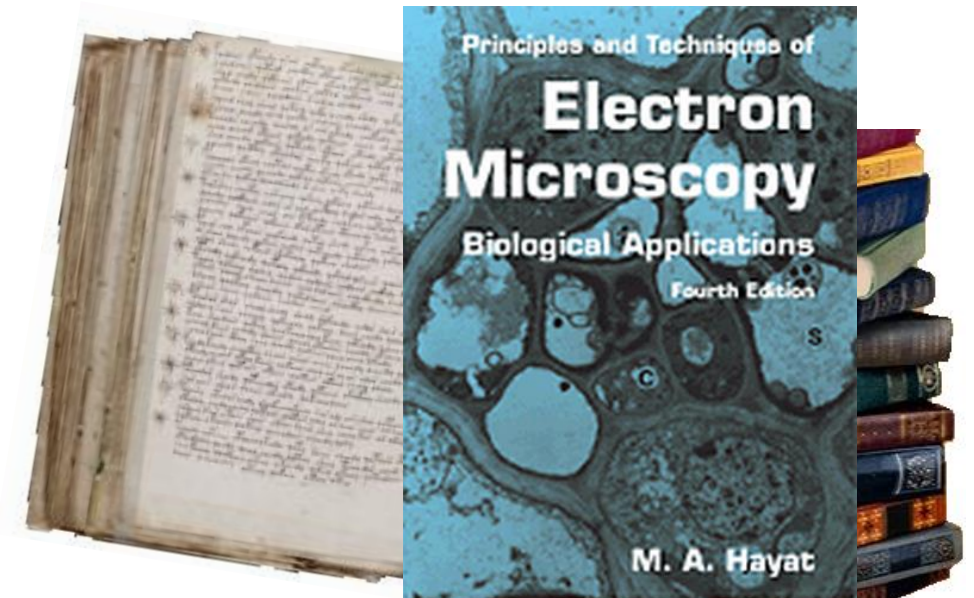
Nanofabrication: principles, capabilities and limits, by Zheng, Cui, 2008

Surface damage induced by FIB milling and imaging of biological samples is controllable. Drobne D., Microsc Res Tech. 2007 Oct;70(10):895-903.

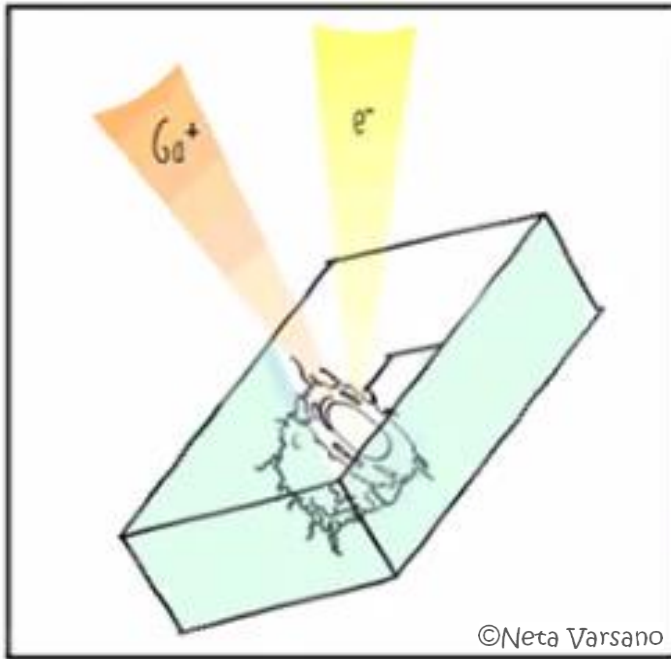
60 seconds about preparation



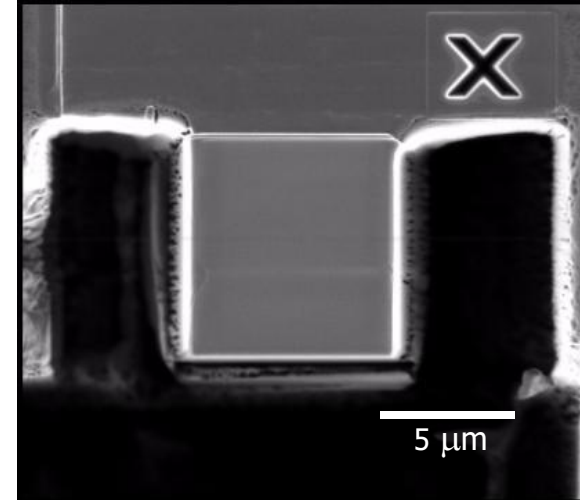
FIB milled cross section of T-cell.
 (a) stained with 1% OsO₄, (b) 1%OsO₄ + 2%UrAc



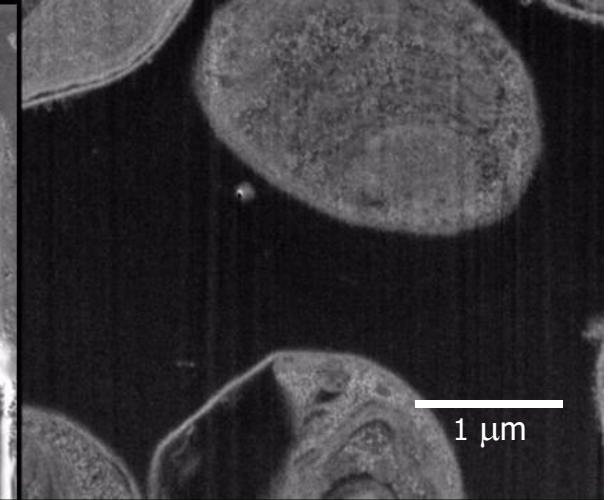
FIB – SEM Volume imaging



Ion Beam view – slicing
-block surface-

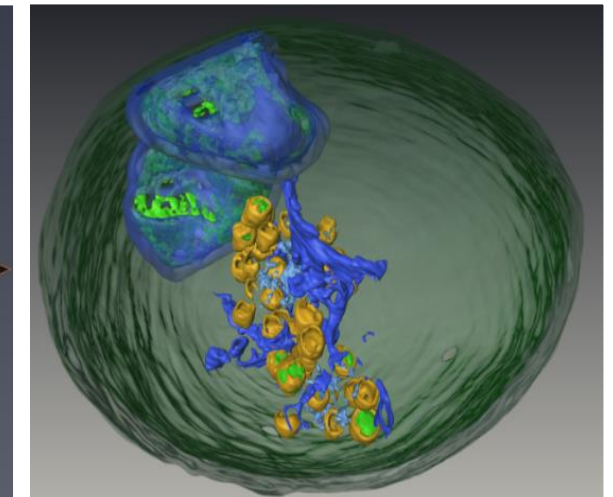
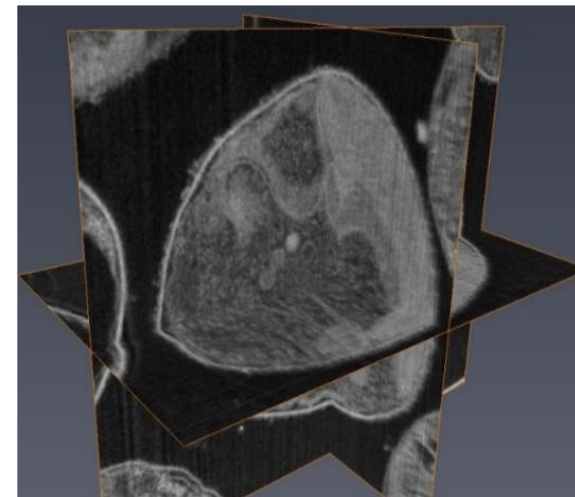


SEM view – imaging
-block cross section-



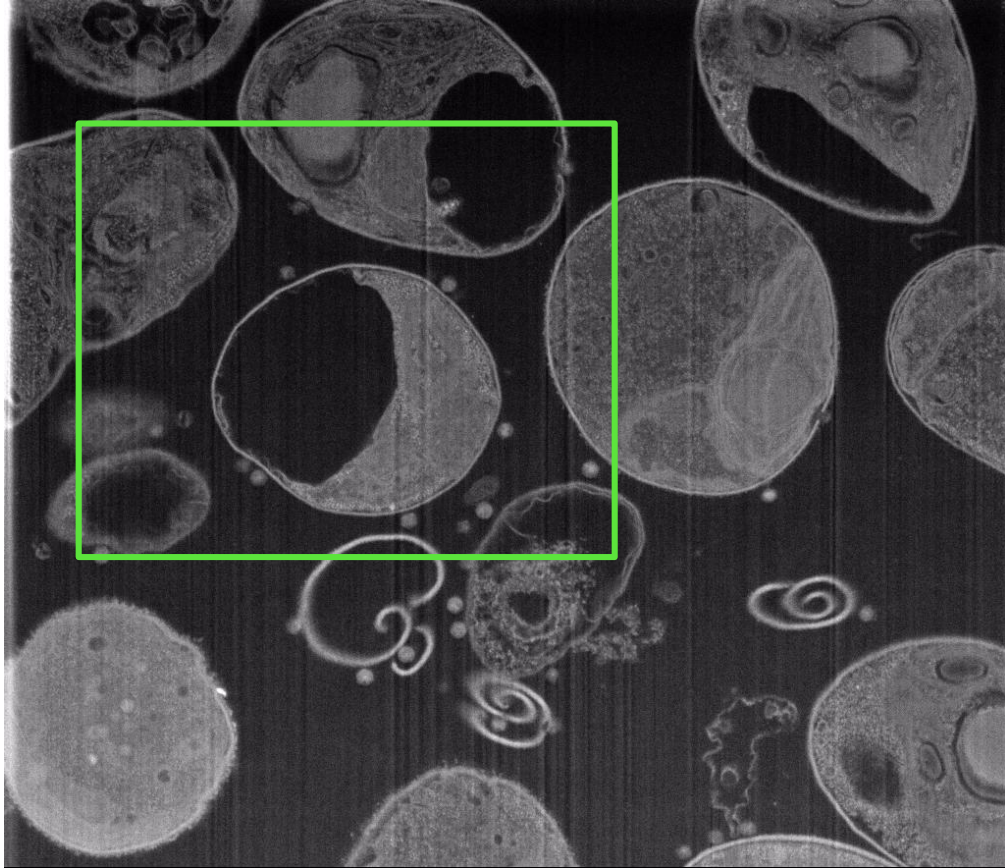
Chlorella alga infected by PBCV-1

The Viral Factory spatial organization and its replication cycle

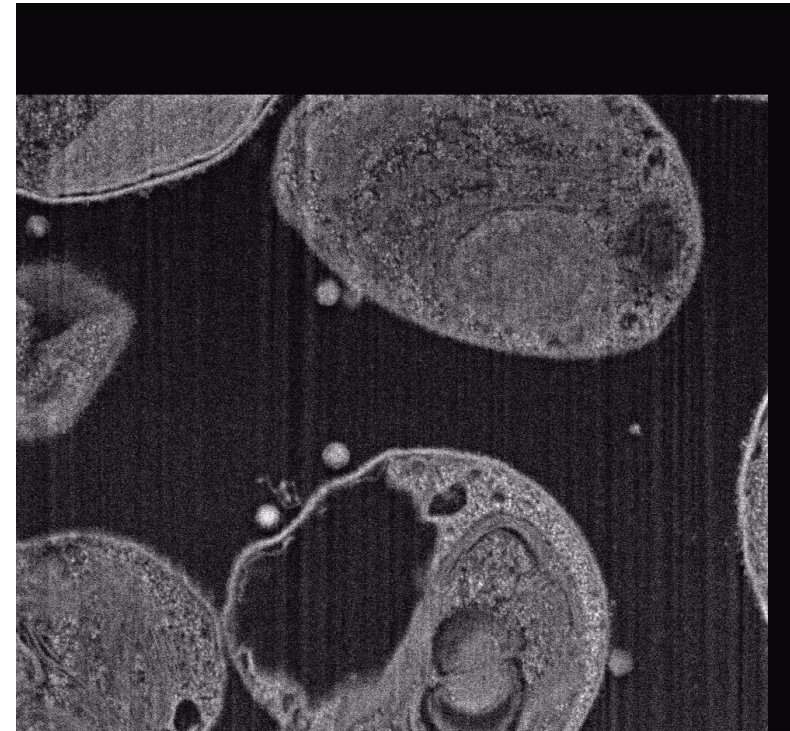


SEM images stack alignment

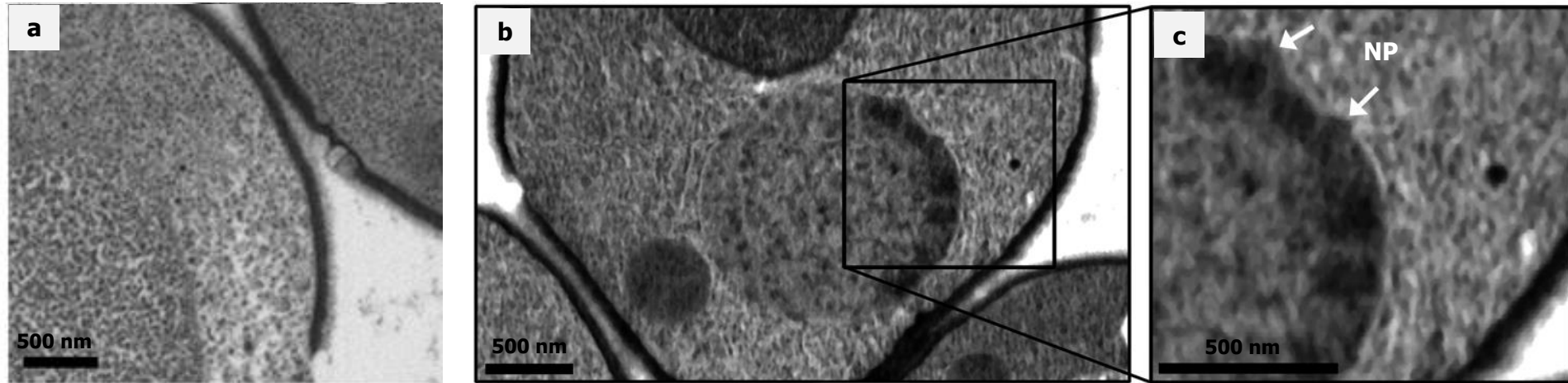
Raw 3D stack



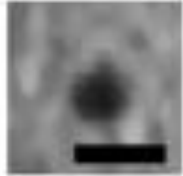
X – Y translation
Image processing



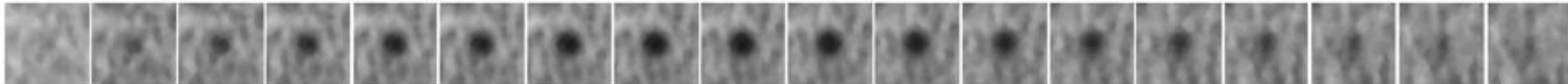
FIB-SEM dataset reconstruction



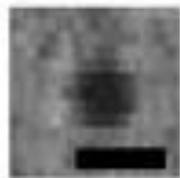
X-Z



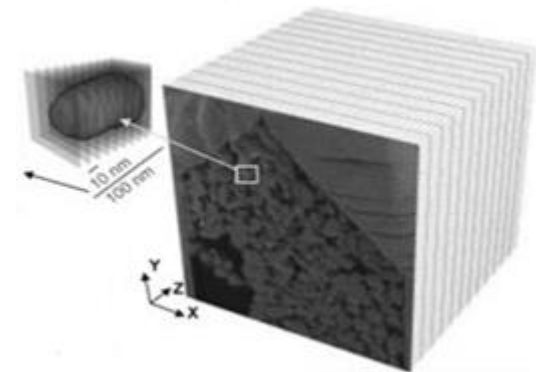
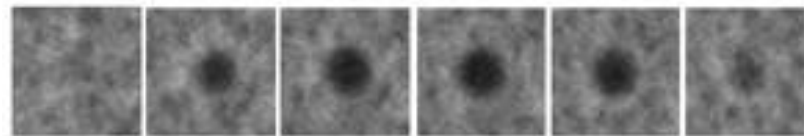
61 nm vesicle at 3 nm z-interval. **18 serial X-Y images**



X-Z



74 nm vesicle at 15 nm z-interval. **6 serial X-Y images**



Scale bars = 100 nm.

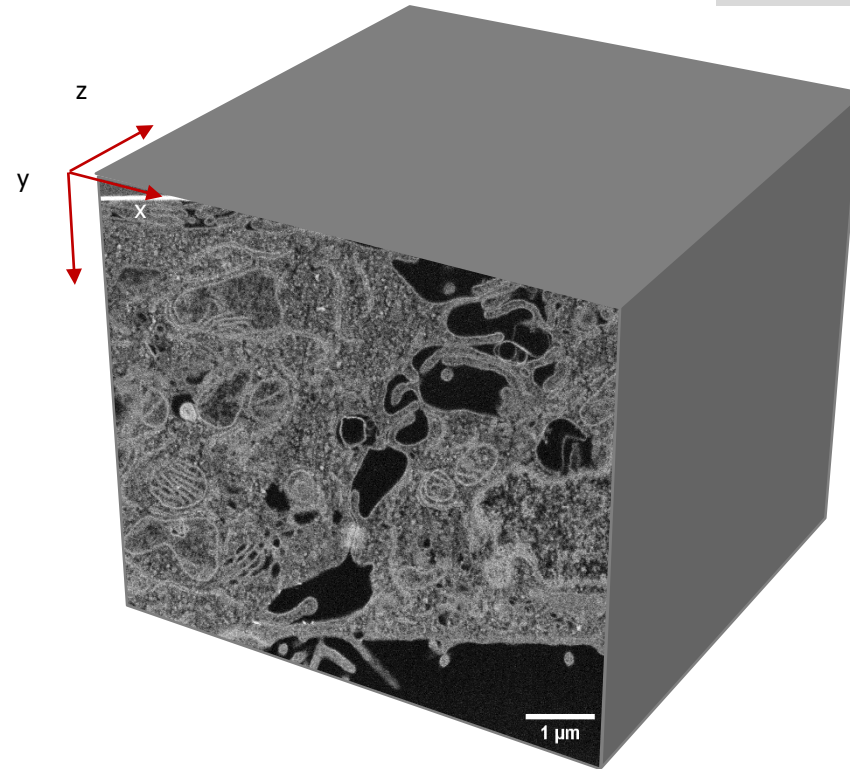
Saccharomyces cerevisiae yeast cell

Optimizing acquisition parameters



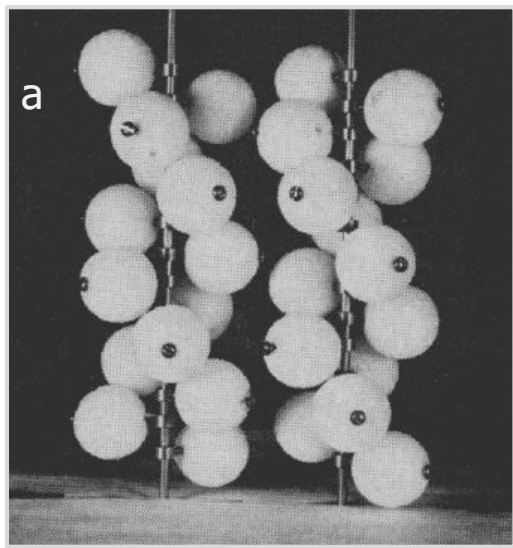
- Smallest feature size
- SNR vs damage
- Acquisition time
- Data size
- System stability

Aspiration: Isometric voxel size
Slice thickness (z) \approx image pixel size (x,y)

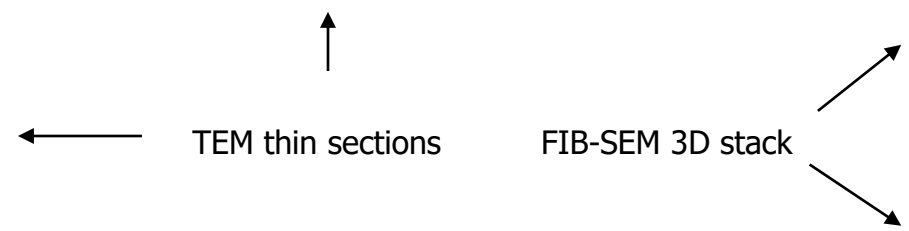
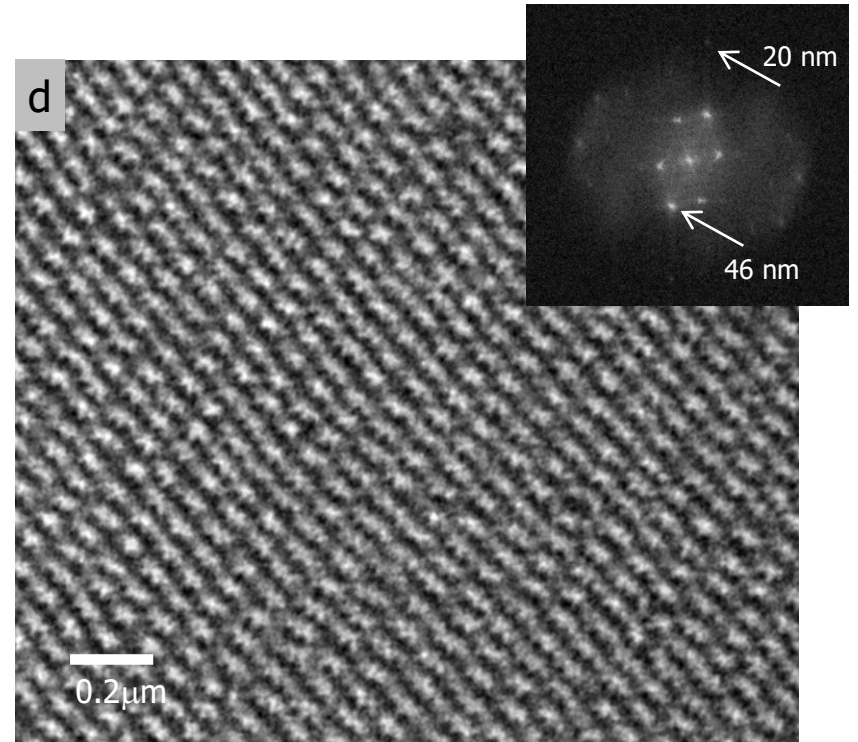
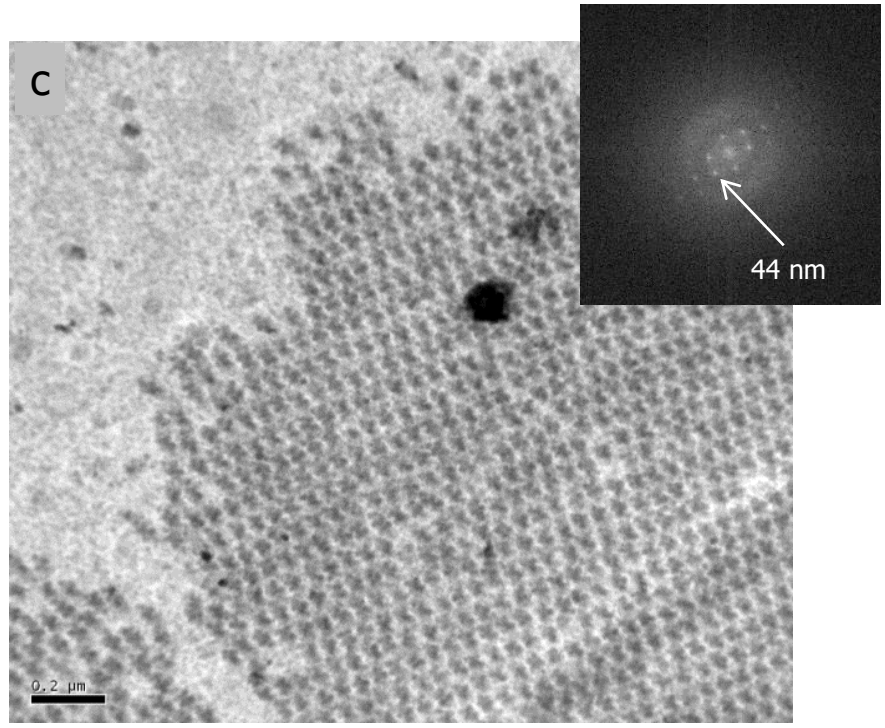
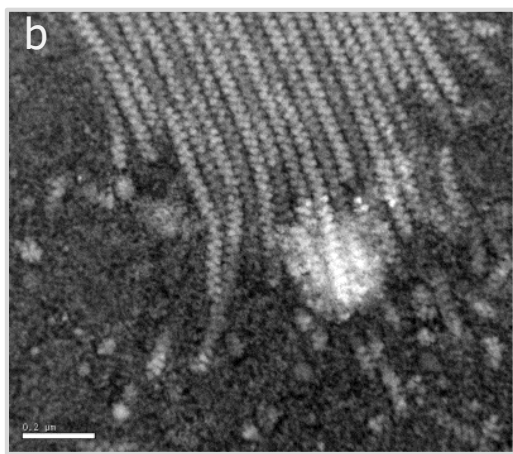


| FOV | pixel size | pix in frame | pix num | frame time |
|--------------|------------|--------------|----------|------------|
| 10um x10um | 10nm | 1000x1000 | 1million | 30s |
| 100um x100um | 10nm | 10kx10k | 100m | 5 min |

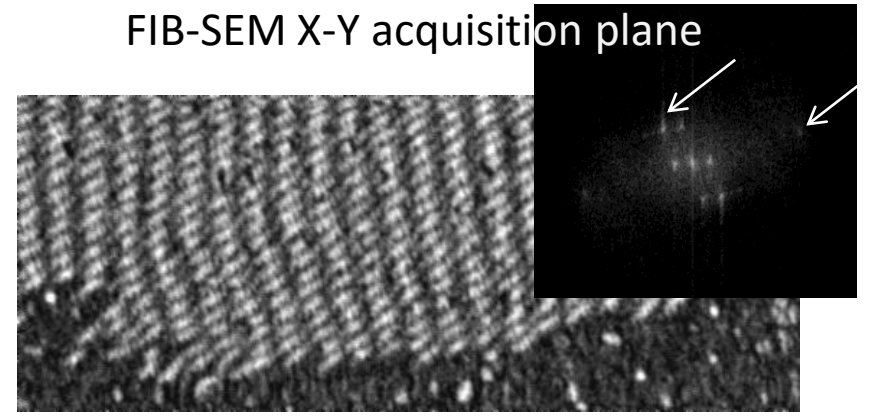
Resolution measurement



(a) Regular array of ribosomes model. RS Morgan. Science 1965

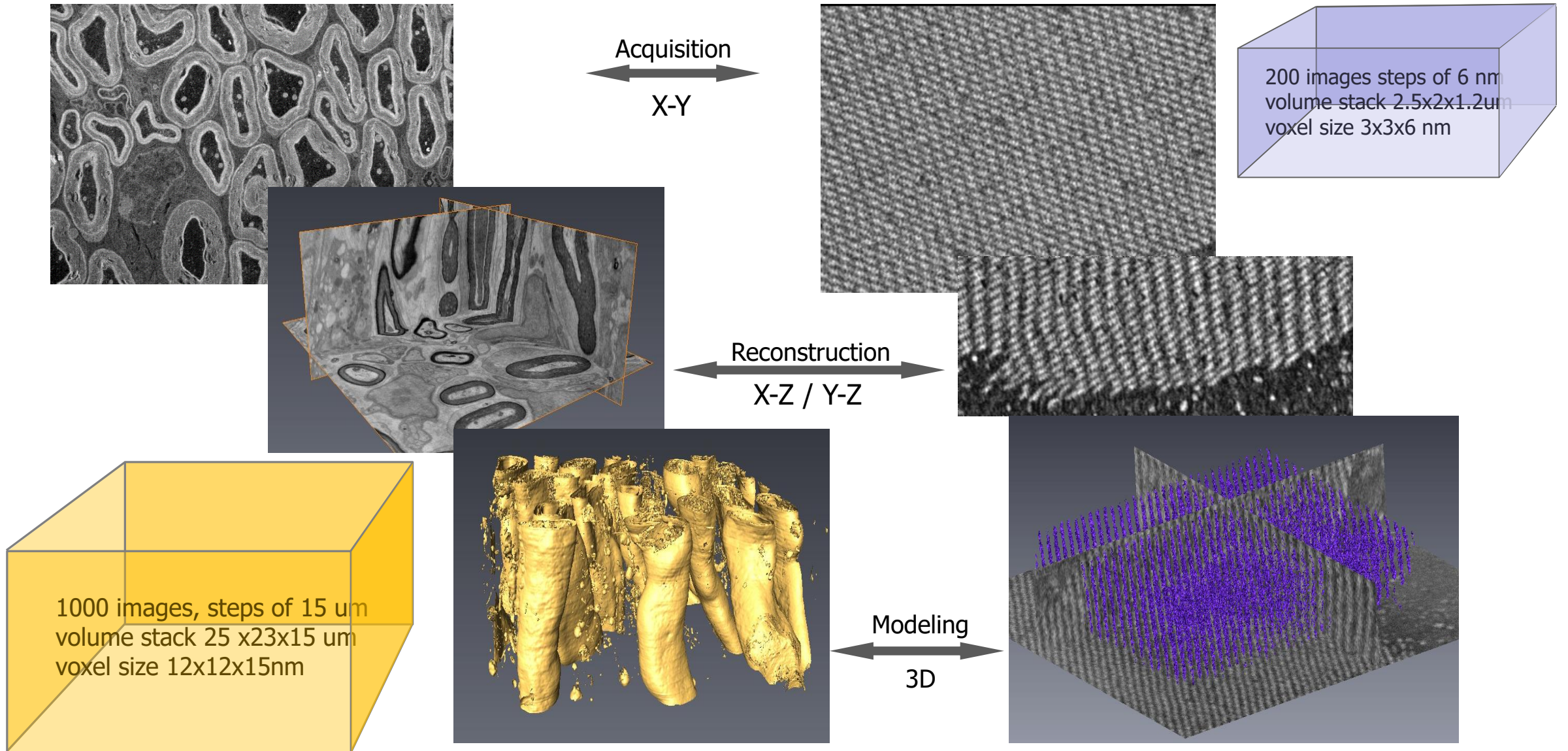


Chromatoid body in *Entamoeba invadens* cyst
 The distance between neighboring chains is 440 Å

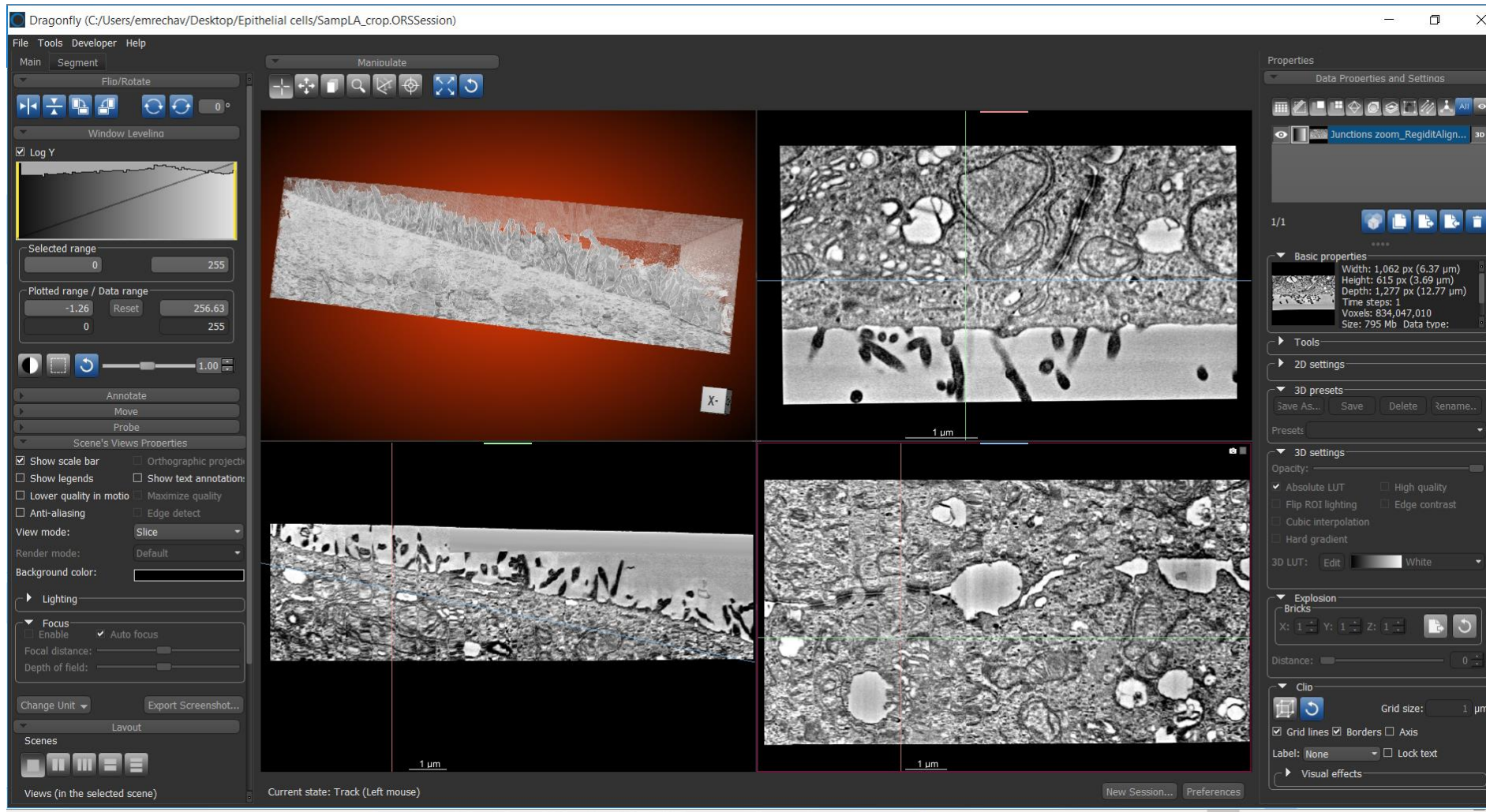


Y-Z plane of aligned stack

FIB – SEM Volume imaging



3D editor



Dragonfly

Object Research Systems,
Montreal, Canada

Epithelia cells interface.

With:

Nili Dezorella

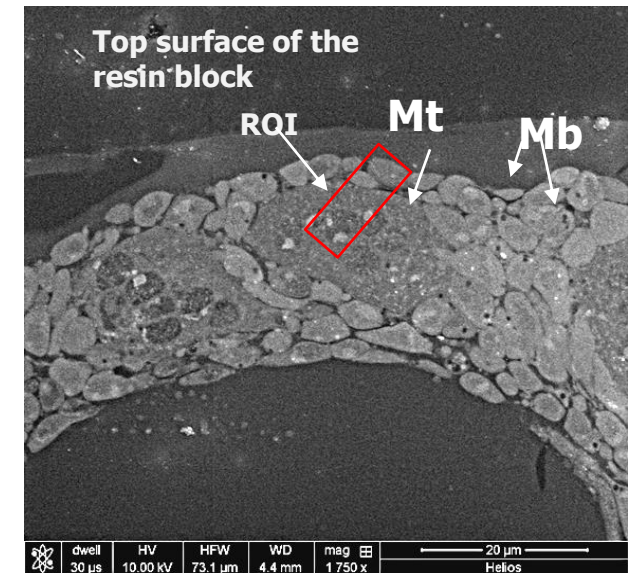
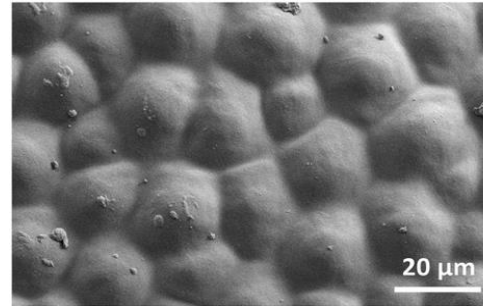
Melanie Bokstad

Inna Grosheva

(Geiger's Lab, WIS)

Defining the area of interest

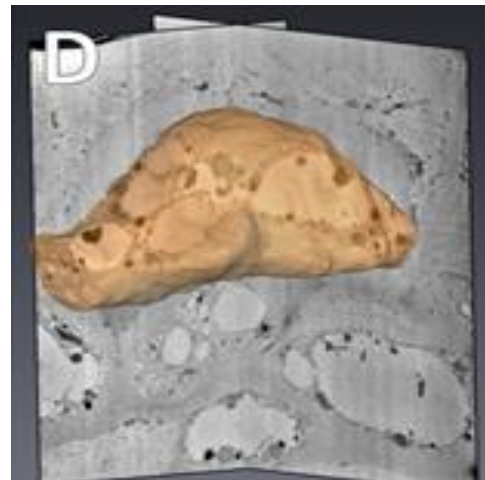
You don't really see the object before cutting



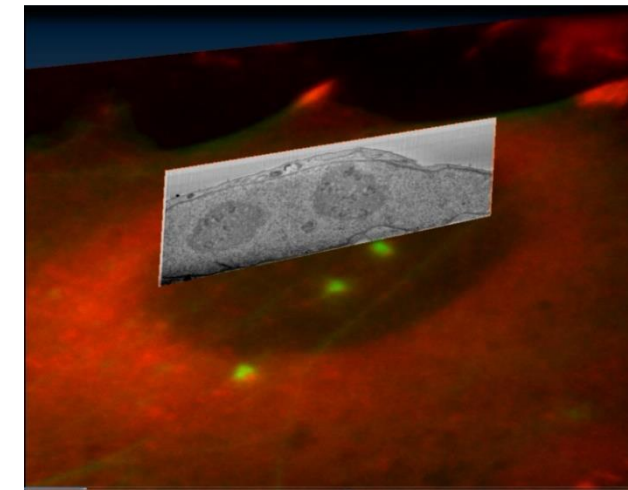
Dhanyasi et al. J Cell Biology 2015

The challenge of ROI identification

- “in blind”
- acquisition of large volume
- topography block face relief
- BSE image of the sample surface
- correlative with TEM, MicroCT, LM...



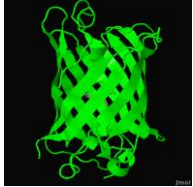
Lashbrooke et al., Plant physiology 2015



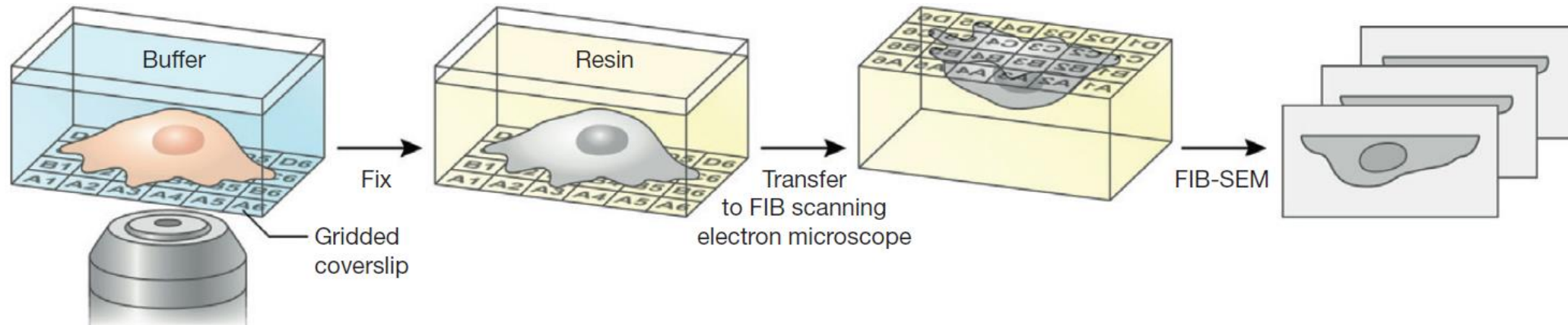
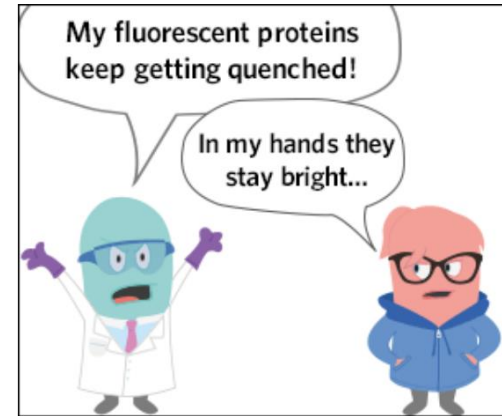
Revach et al., SCIENTIFIC REPORTS, 2015

Correlative Fluorescent Microscopy

fluorescent proteins or synthetic fluorophores



Green
Fluorescent
Protein



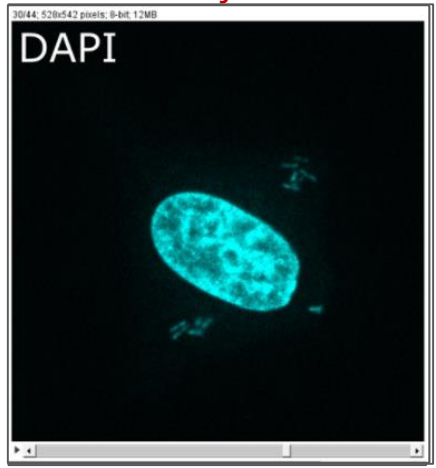
Focused ion beams in biology.

Kedar Narayan and Sriram Subramaniam.
NATURE METHODS, 2015.

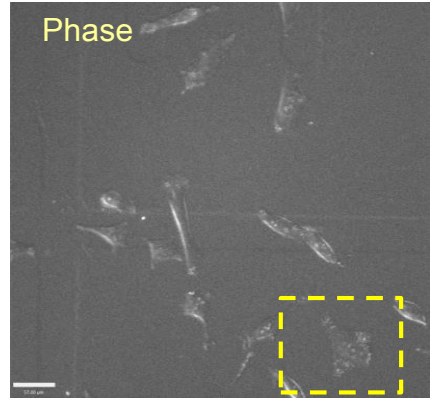
Correlative FM-FIB (confocal)

acquisition

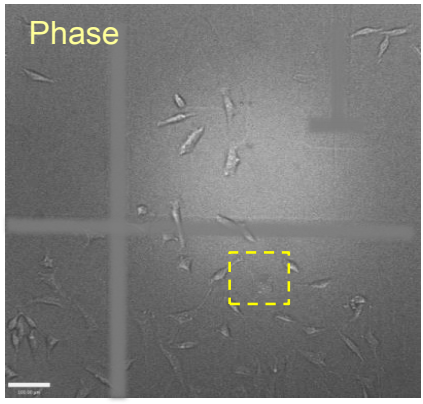
60x objective



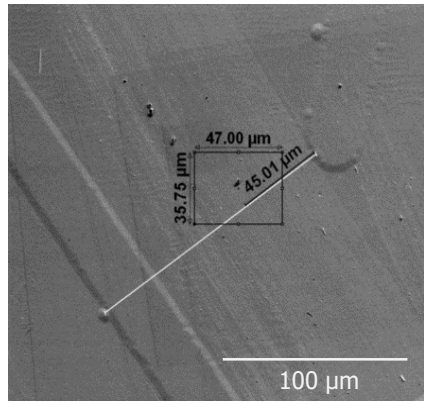
20x objective



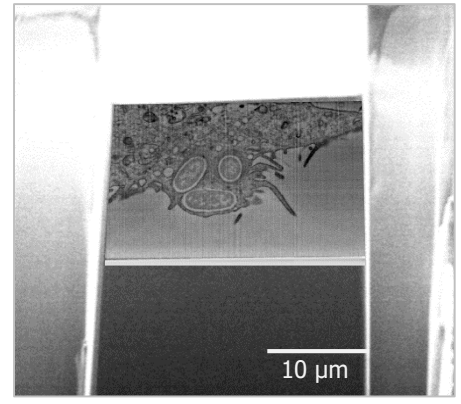
10x objective



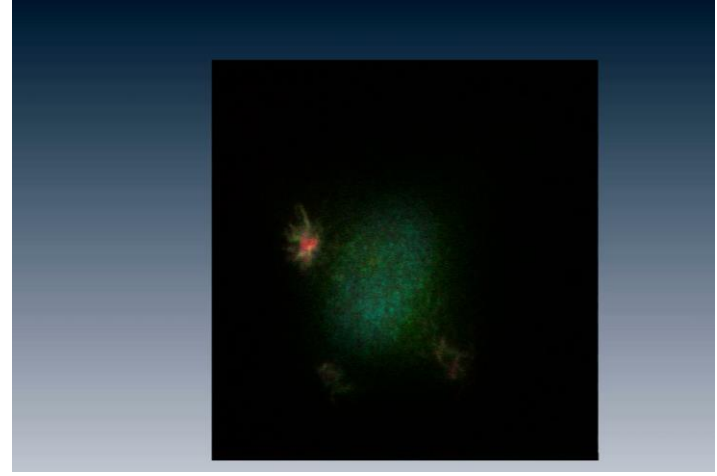
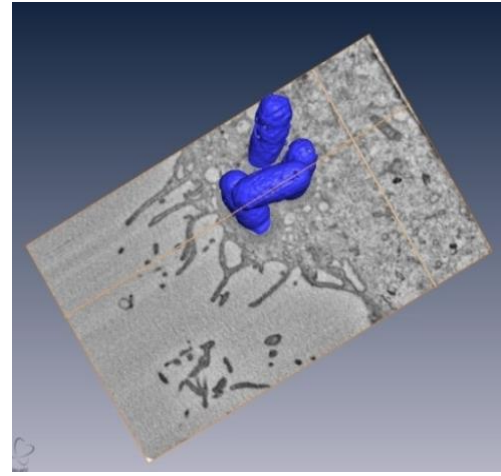
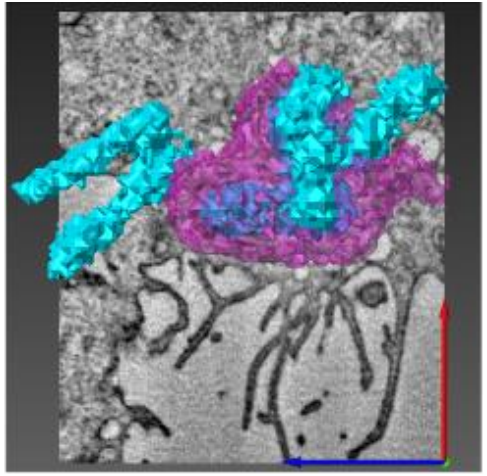
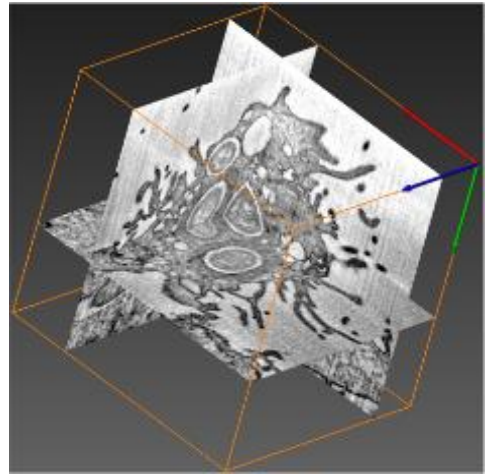
FIB-SEM low mag



FIB-SEM acquisition



processing



Macropinosomes are Key Players in Early Shigella Invasion and Vacuolar Escape in Epithelial Cells. Weiner et al., PLoS Pathogens 2016

Movie: courtesy of Allon Weiner Cimi-Paris, Faculty of Medicine, Sorbonne University

Cryo FIB applications

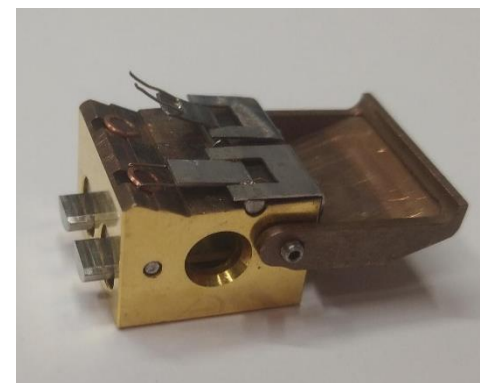
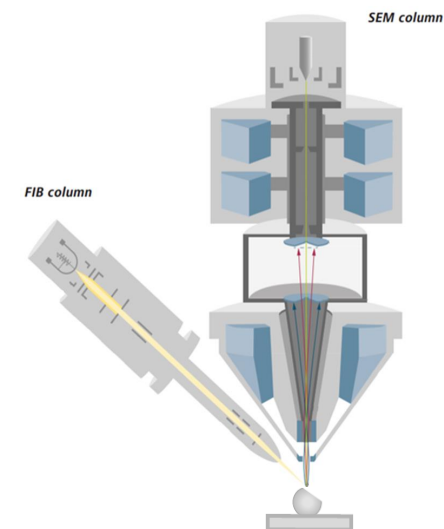
Cryo stage (Leica)

+

CrossBeam 550 (Zeiss)



Cryo shuttle

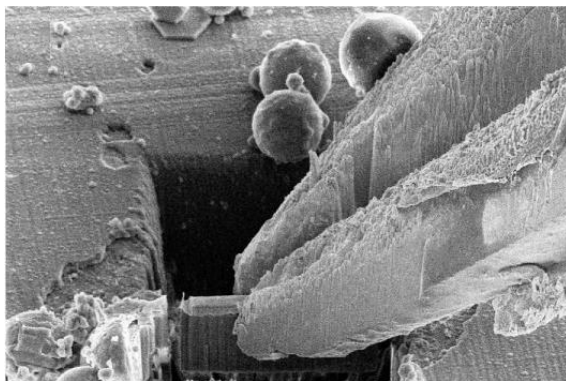


Cryo holder (modified)



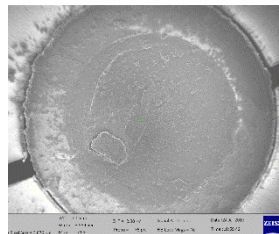
Loading station (modified)

Cryo FIB applications

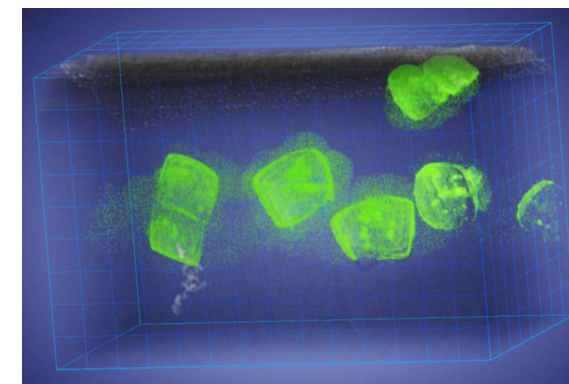


In-situ Lift-Out cryo-FIB lamella

M. Schaffer, Nature Methods 2019

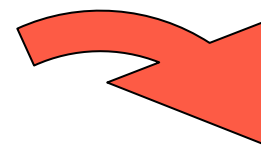
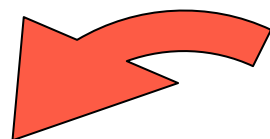


HPF



HPF tissue or "big" cells

S Kumar et al., Science advances, 2020

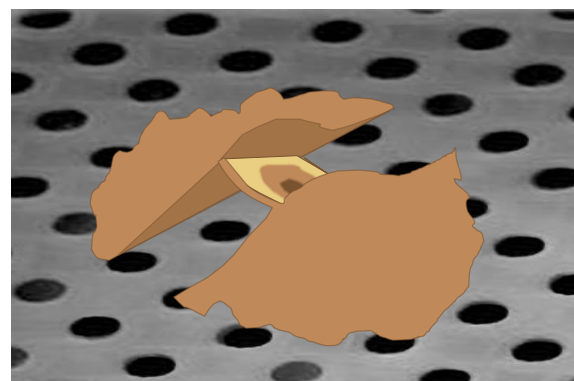
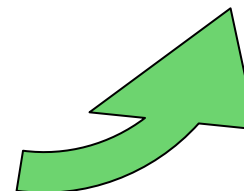
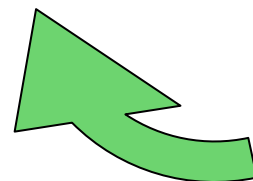
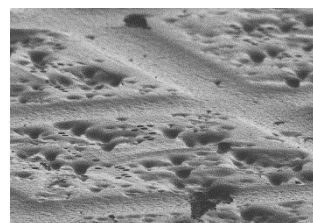


Cryo lamellae

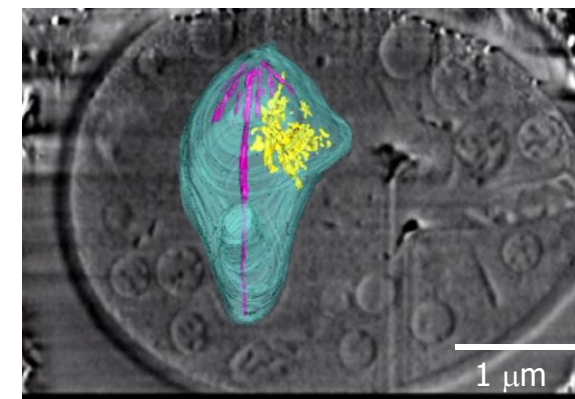


Volume imaging

Plunged grids



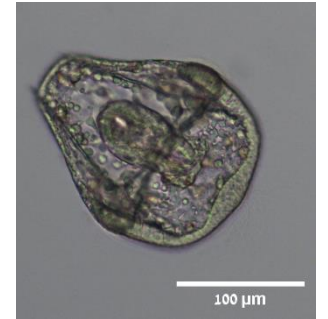
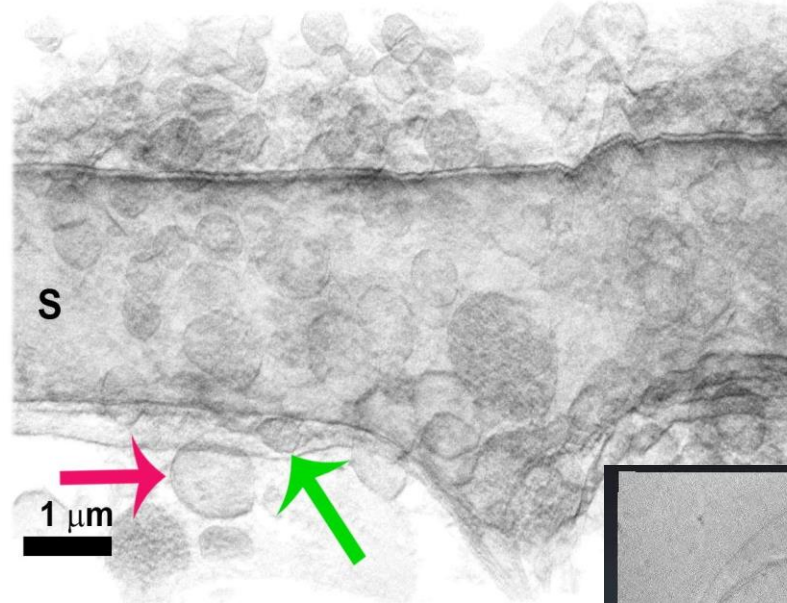
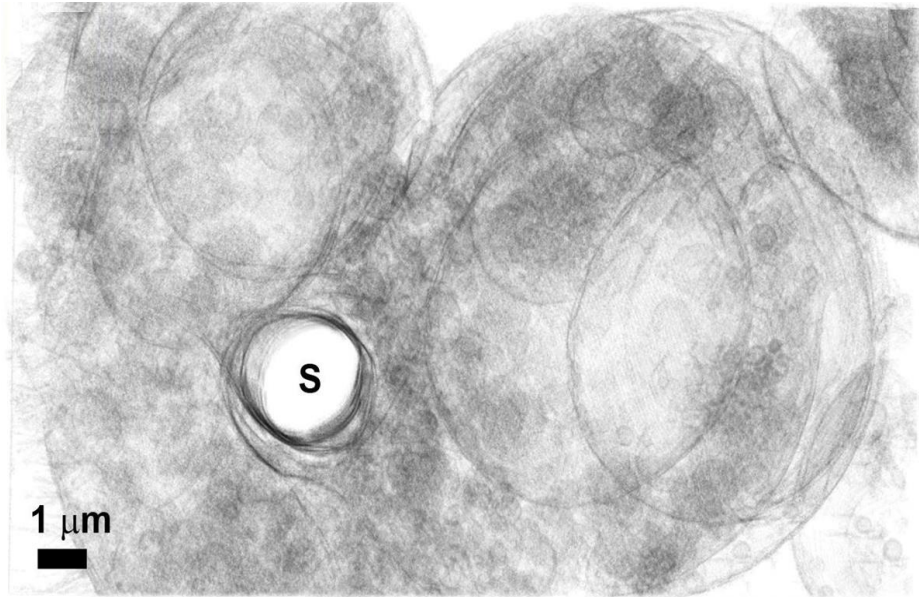
On grid cryo-FIB prepared lamella



Plunge-frozen cells

With I. Pereman, Elbaum's lab

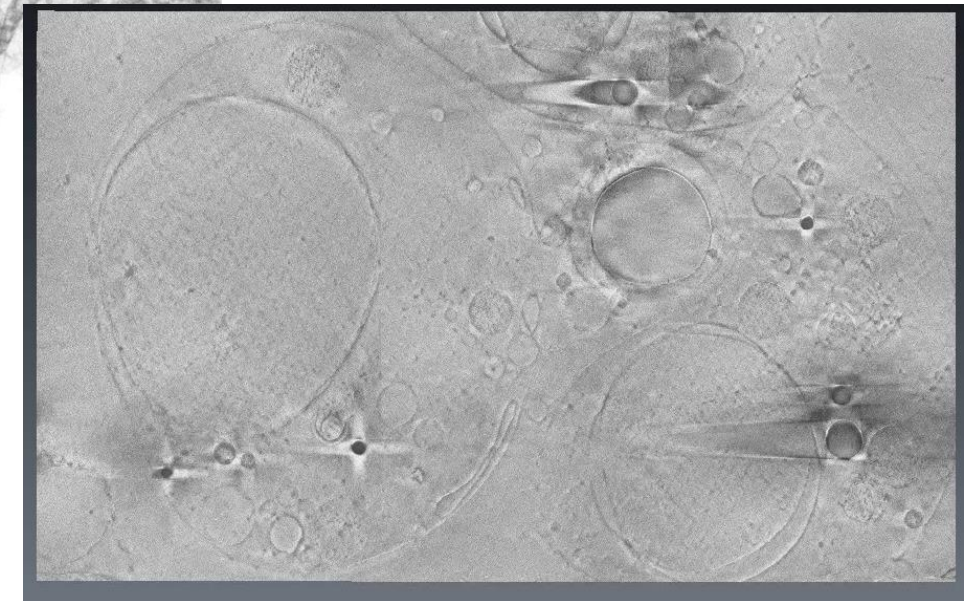
Volume Imaging (HPF)



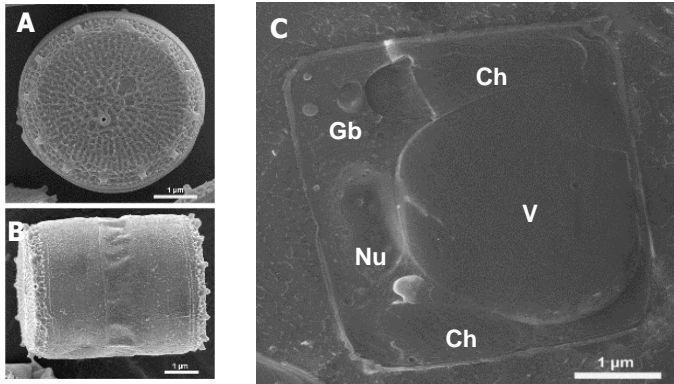
sea urchin embryo

Cryo-FIB-SEM Large volume structural analysis of HPF sea urchin embryo

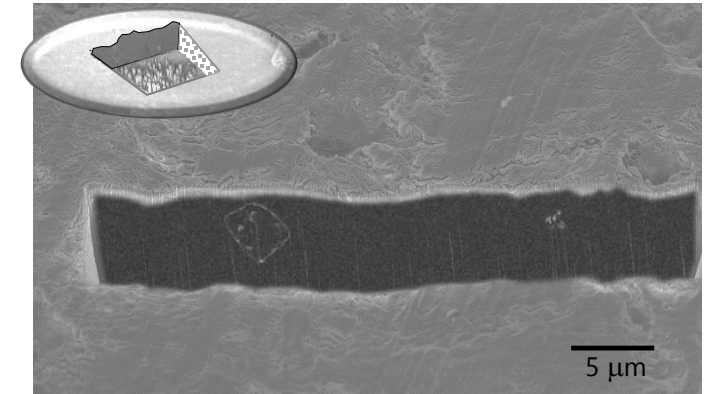
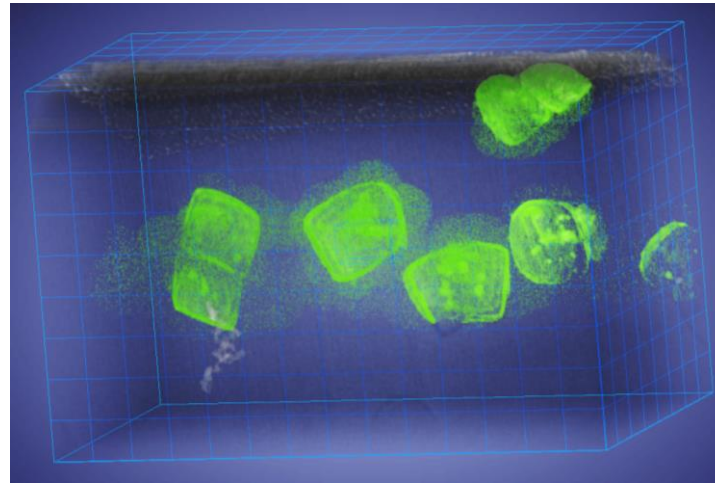
3D reconstruction of the stack
 $X = 20 \mu\text{m}$, $Y = 12 \mu\text{m}$ $Z = 11 \mu\text{m}$



Cryo volume imaging + EDS characterization

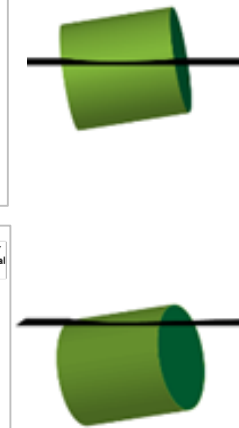
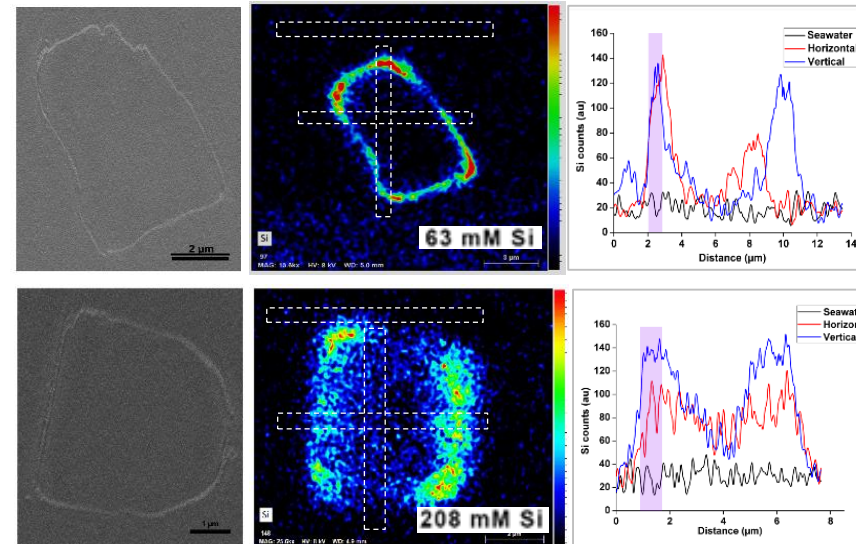


Thalassiosira pseudonana cells in (A) valve view, (B) girdle band view. (C) Freeze-fractured *T. pseudonana* cell showing internal organelles.



BSE image of a cryo-FIB milled cross section of HPF pellet containing *T. pseudonana* cells

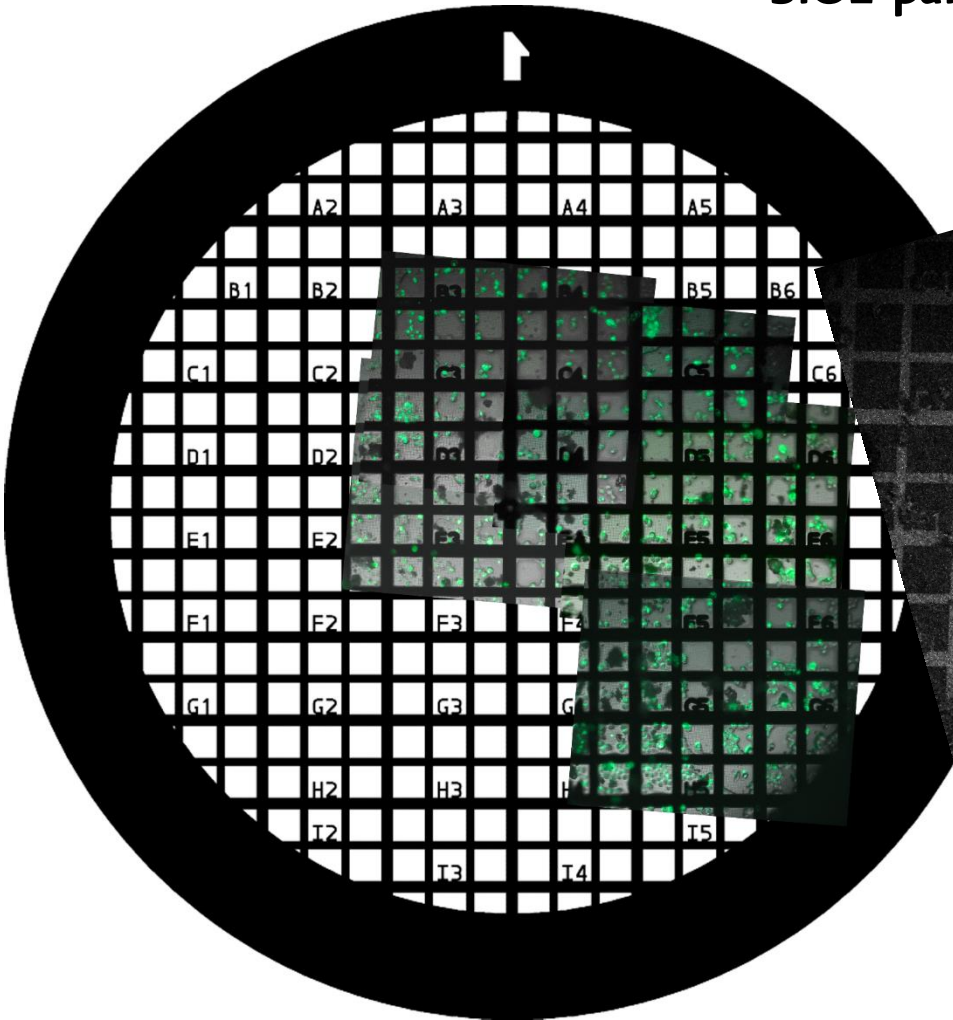
Intracellular silicon concentrations in unsynchronized *T. pseudonana* cells as revealed by cryo-focused ion beam milling followed by energy-dispersive X-ray spectroscopy. (upper row: cell with low concentration Si-pool, bottom row: cell with high concentration Si-pool)



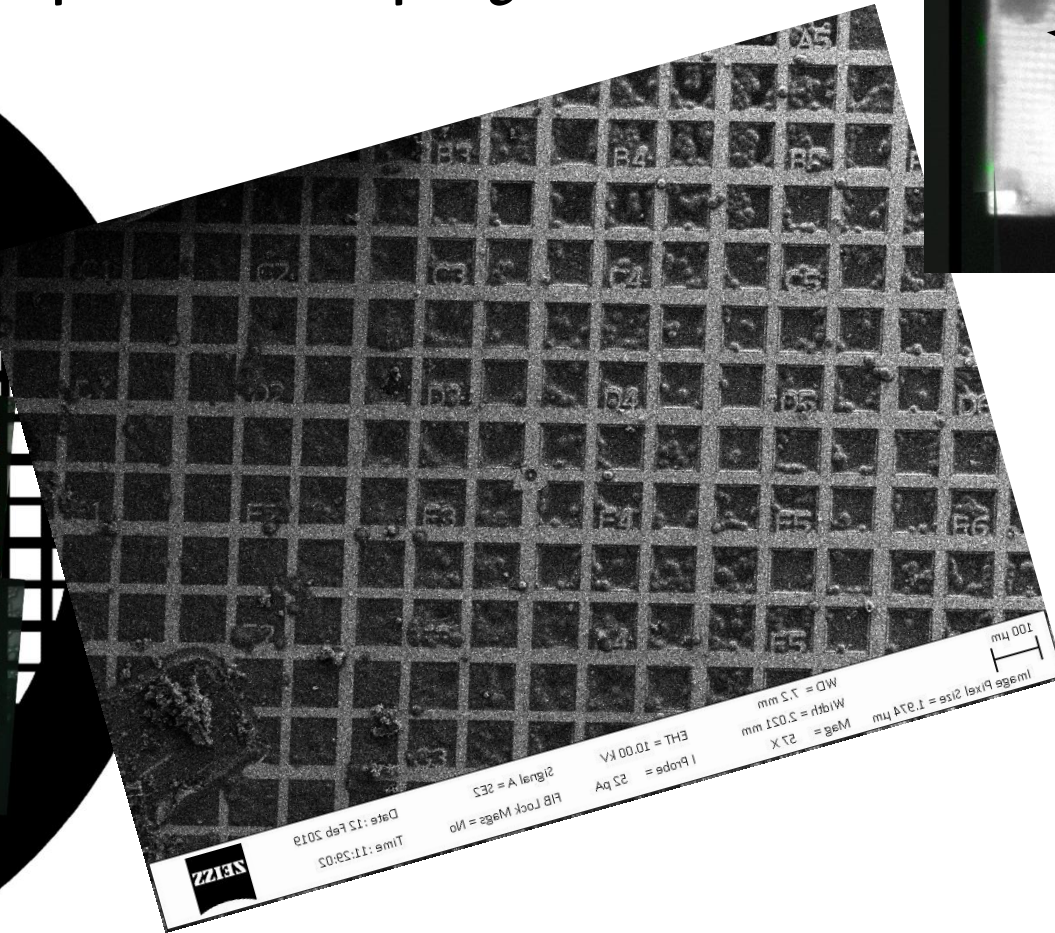
3D animation of *T. pseudonana* cell. From FIB-SEM acquisition at cryo-conditions.

Imaging and quantifying homeostatic levels of intracellular silicon in diatoms. S Kumar et al., Science advances, 2020

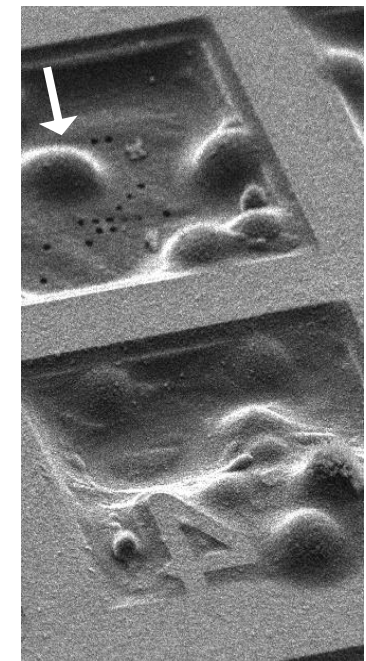
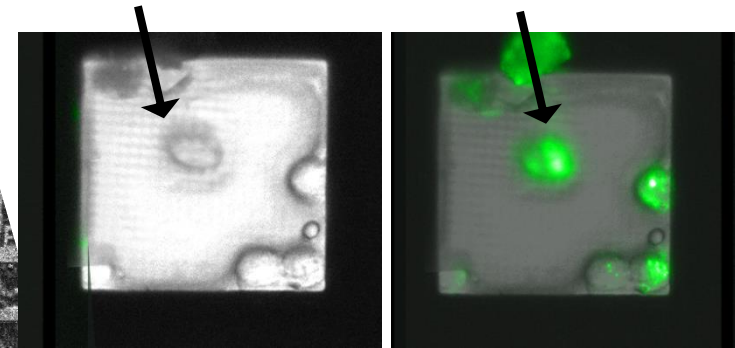
SiO₂ particles inside plunged cell



Fluorescence signal under cryo-light
Objective Olympus x40, cryo stage Linkam



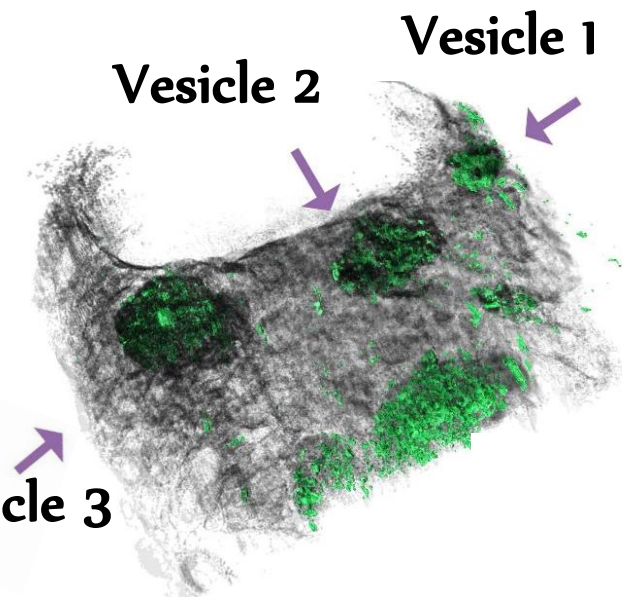
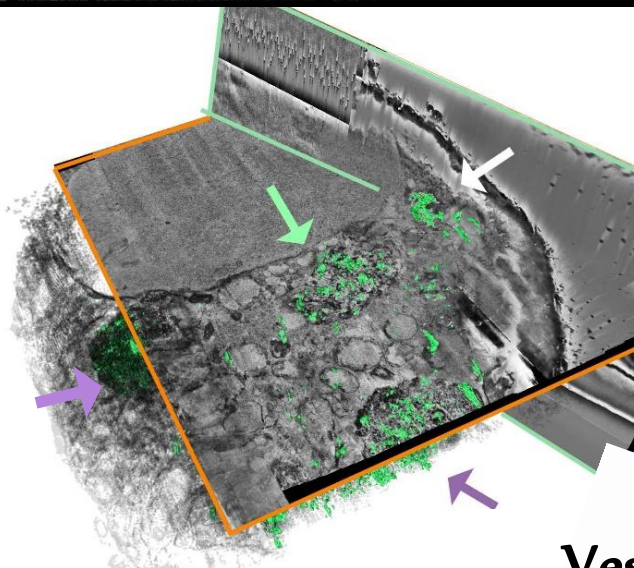
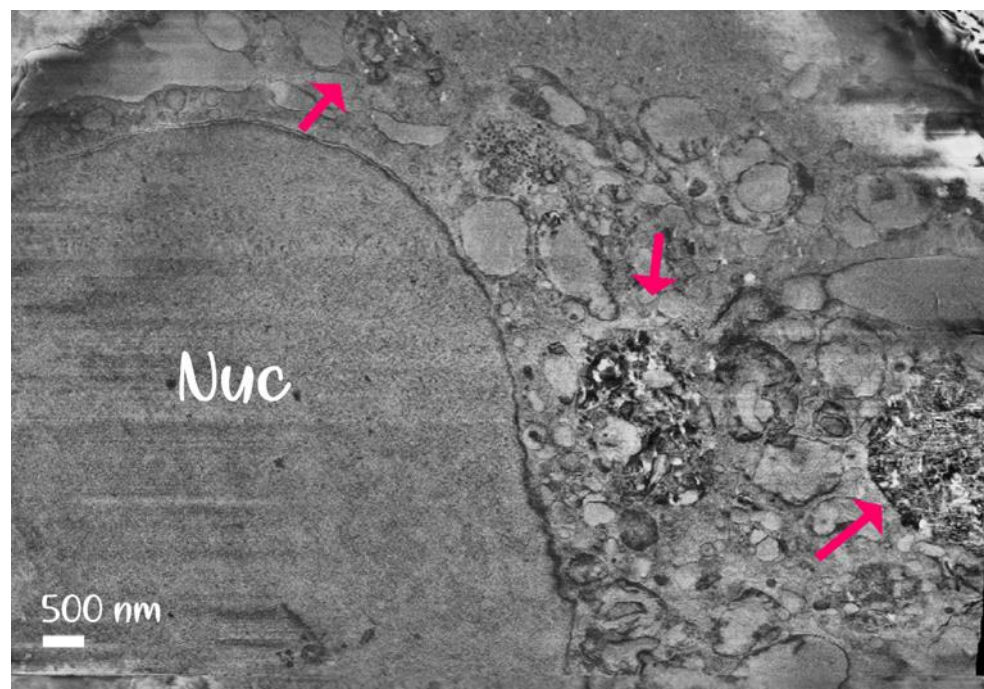
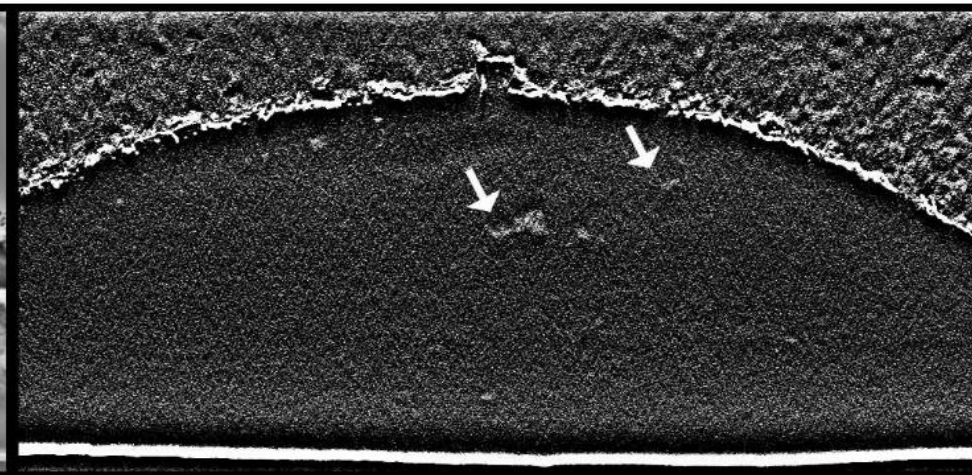
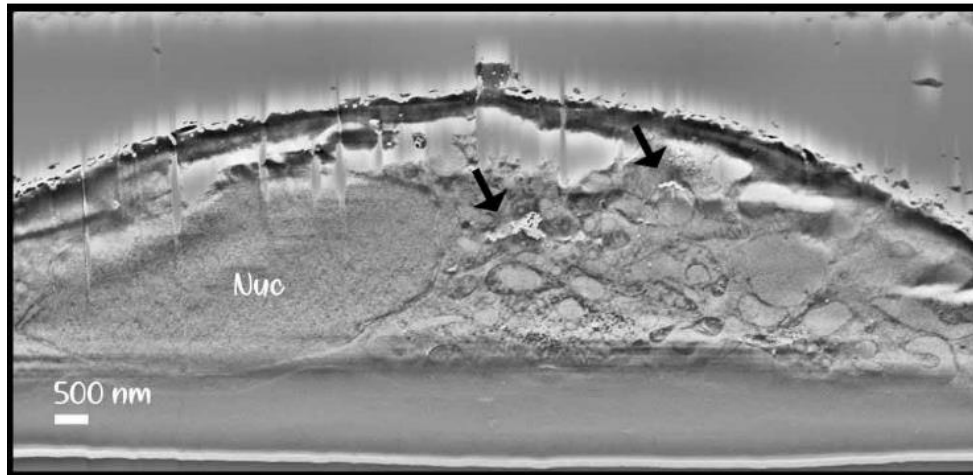
MDA-MB-231 Human metastatic breast cancer cells



20 μm InLens

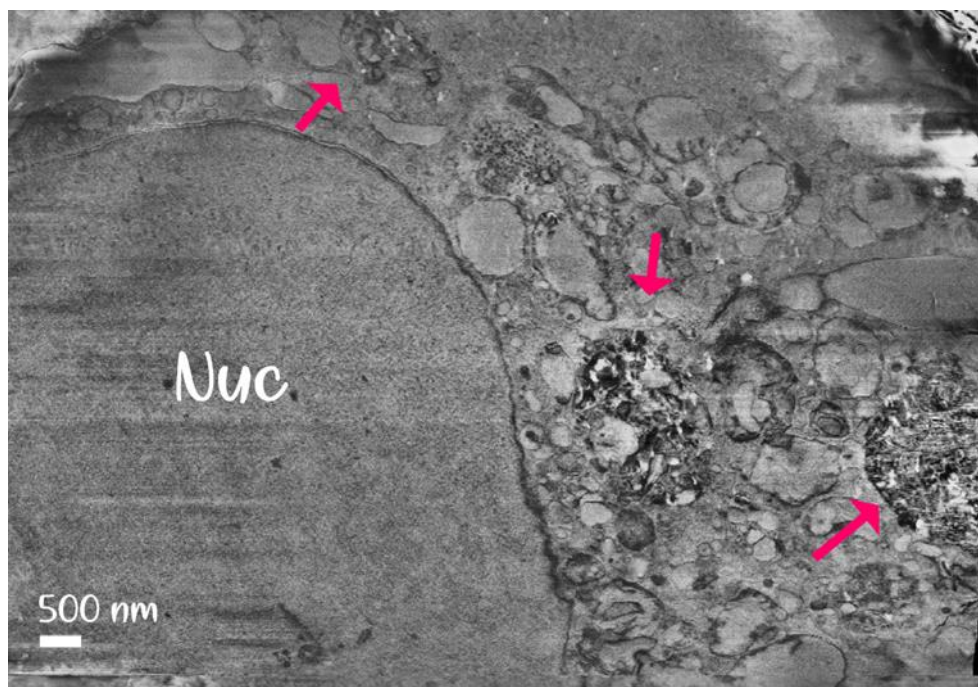
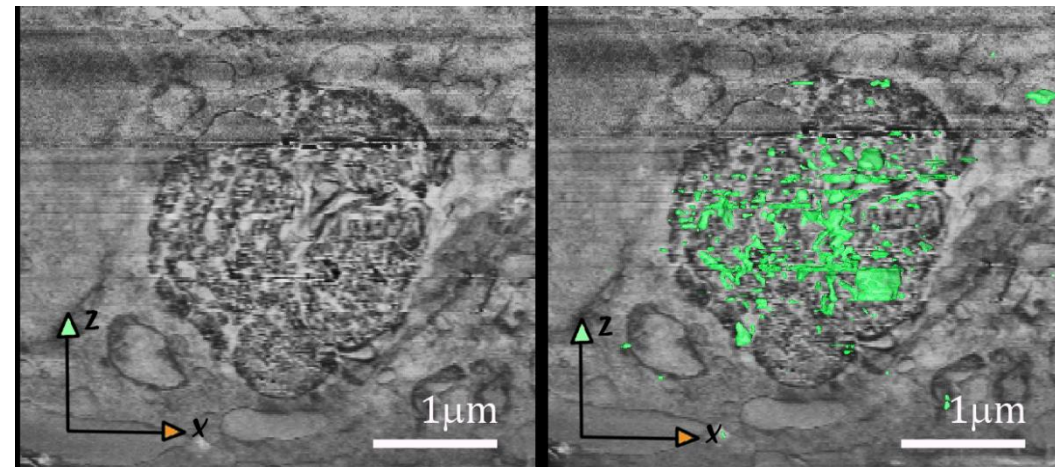
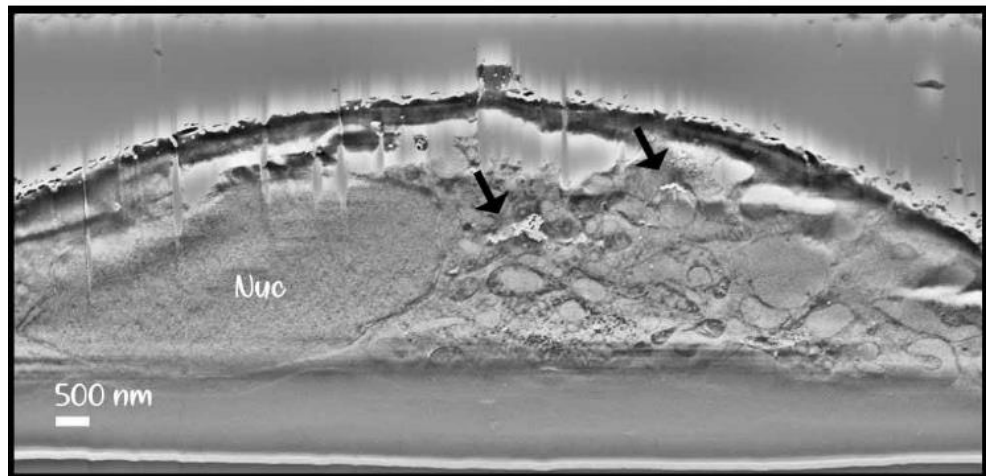


SiO₂ particles are detectable inside plunged cell



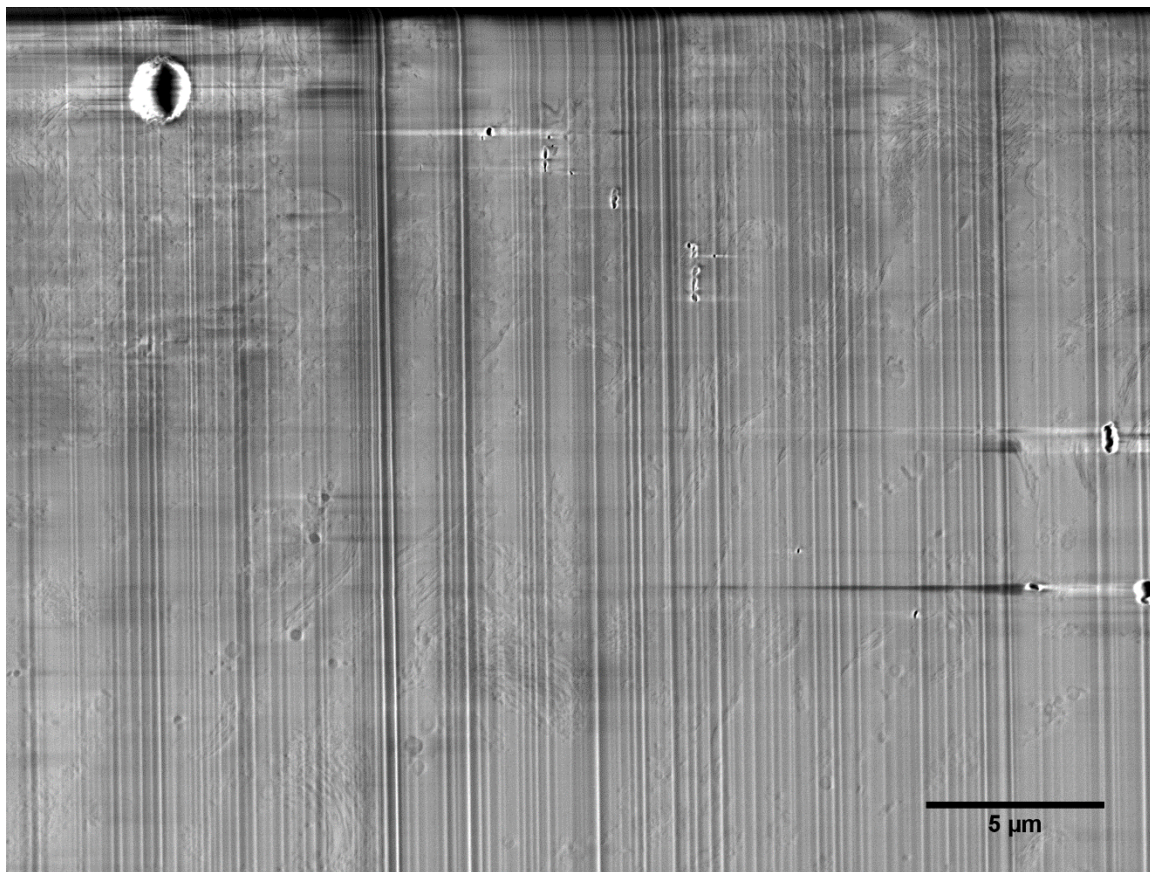


SiO₂ particles are detectable inside plunged cell

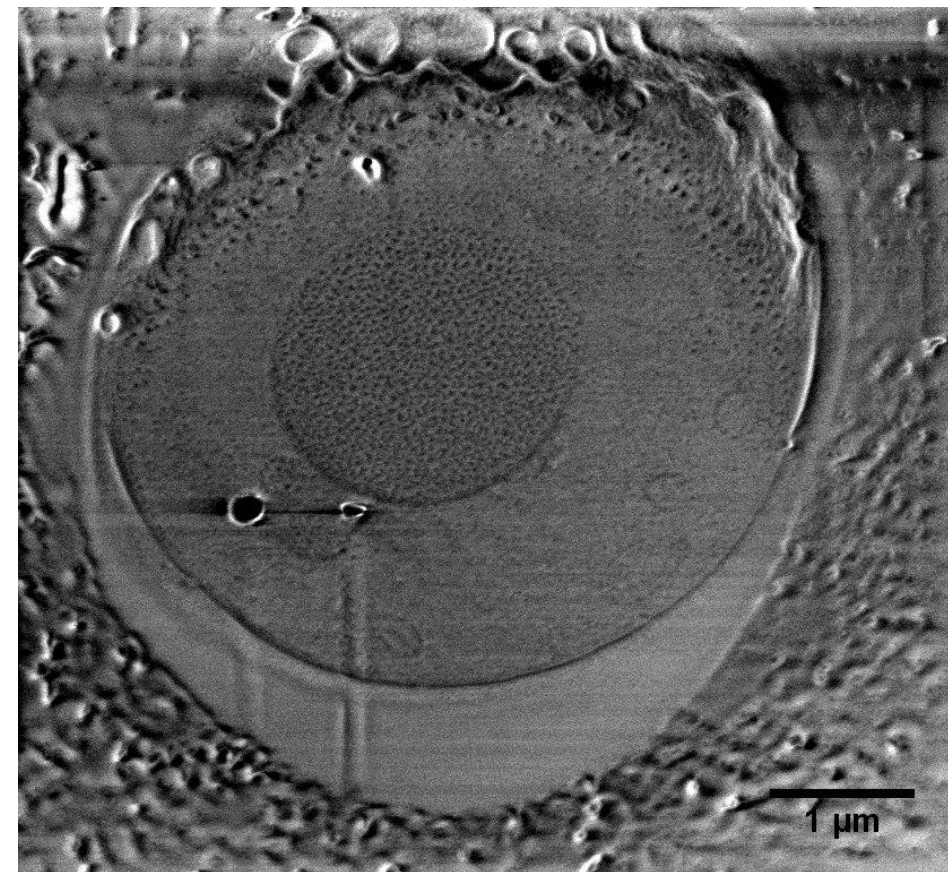


Pixel Size: 14.28 nm
Slice thickness: 17.5 nm

Cryo volume imaging



HPF bone part with surrounding tissue.
The Pectoral Fin Rays of Mudskipper
With Lihi Vevin, Addady's lab

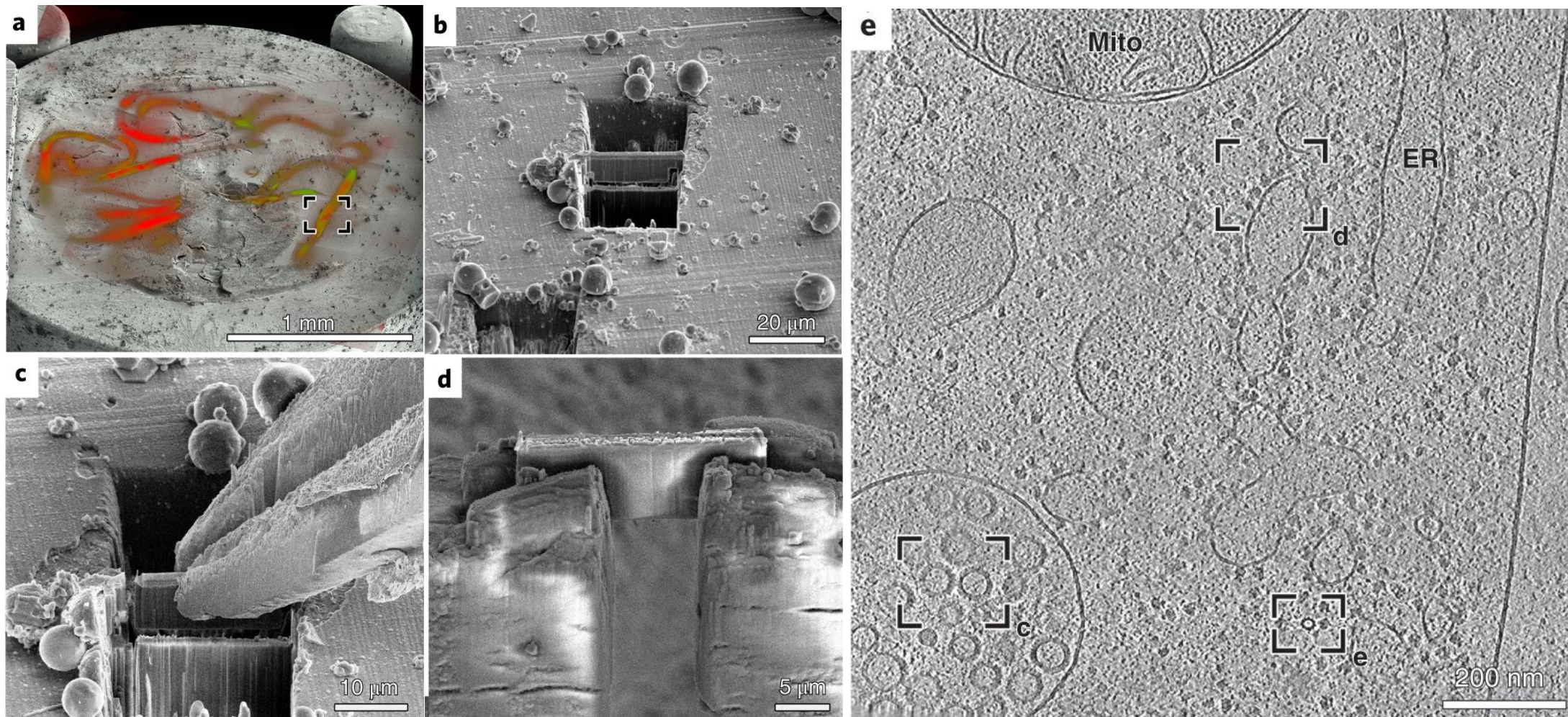


Plunged yeast cell.

With Idan Pereman, Elbaum's lab

- ☹️ **Low contrast**
- ☹️ **Charging artifacts**
- ☹️ **Topographical artifacts - curtaining**
- ☹️ **ROI definition for HPF prepared samples**
- ☹️ **Beam damage**

cryo-FIB lift-out lamella



Cryo lamellae workflow



1
Cells growing



2
Plunging



3
Transfer to cryo LM



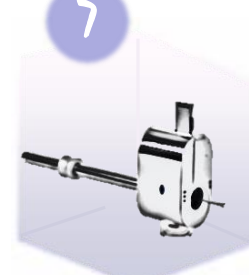
4
Imaging in cryo LM



5
Clipping to AutoGrid rim



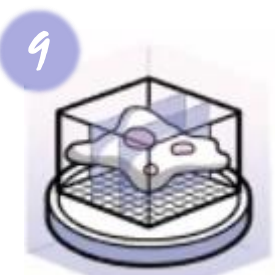
6
Transfer to FIB cryo holder



7
Transfer to FIB/SEM



8
Loading in FIB/SEM



9
Mapping ROIs on sample



10
Serial imaging / FIB lamellae cutting



11
Unloading from FIB/SEM



12
Transfer to LN2 storage



13
Transfer to the Krios autoloader

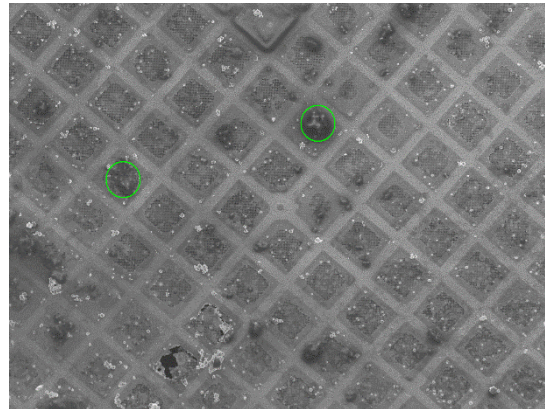
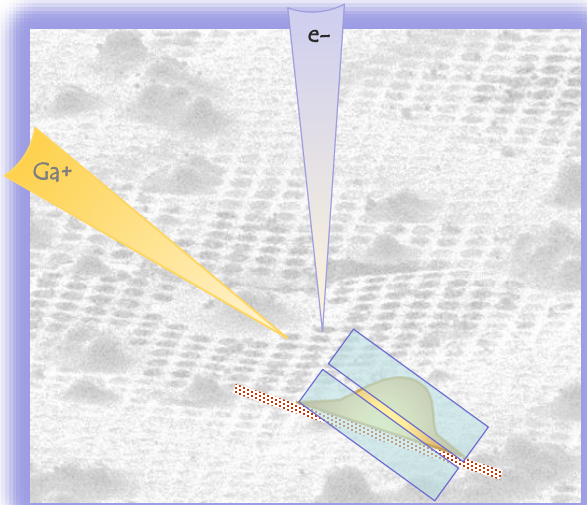


14
TEM/STEM analysis

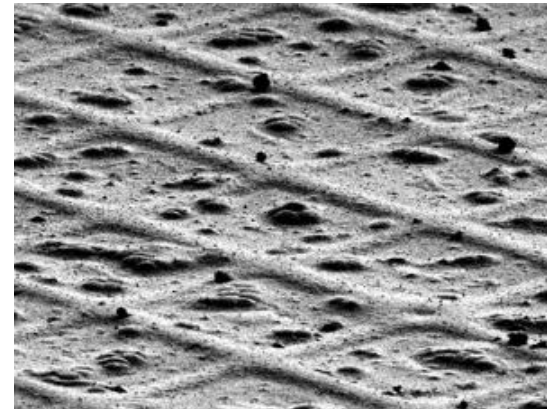
Illustration: from [delmic](#) product presentation

Risks: Samples damage due to devitrification / ice contamination / mechanical damage
Milling “blind” and missing the structure of interest in the lamella due to low z-resolution of FM

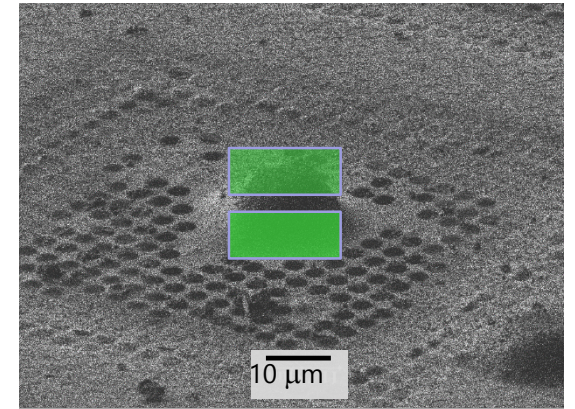
Cryo lamellae preparation



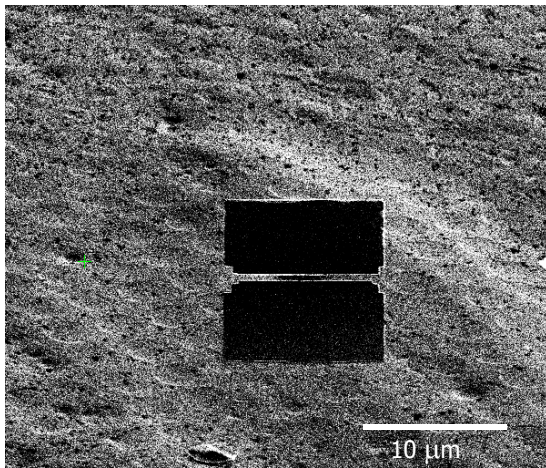
Plunged grid. SEM view



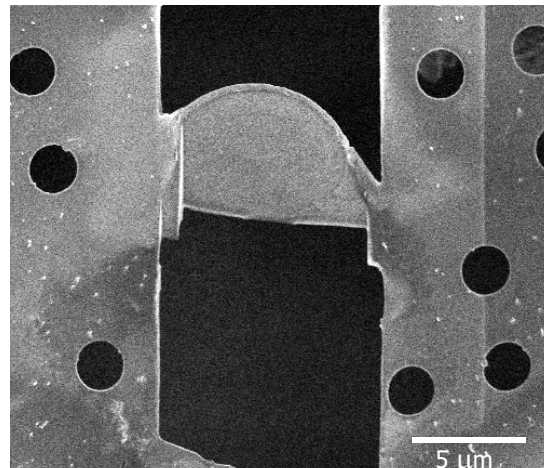
Plunged grid. FIB view



ROI. FIB view



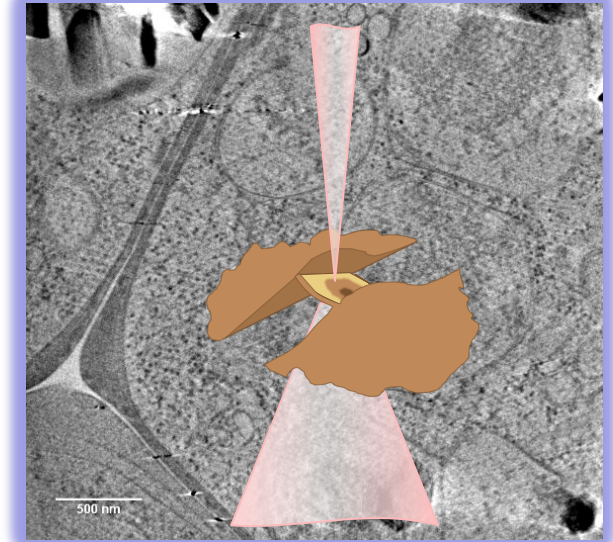
Window opening. FIB view



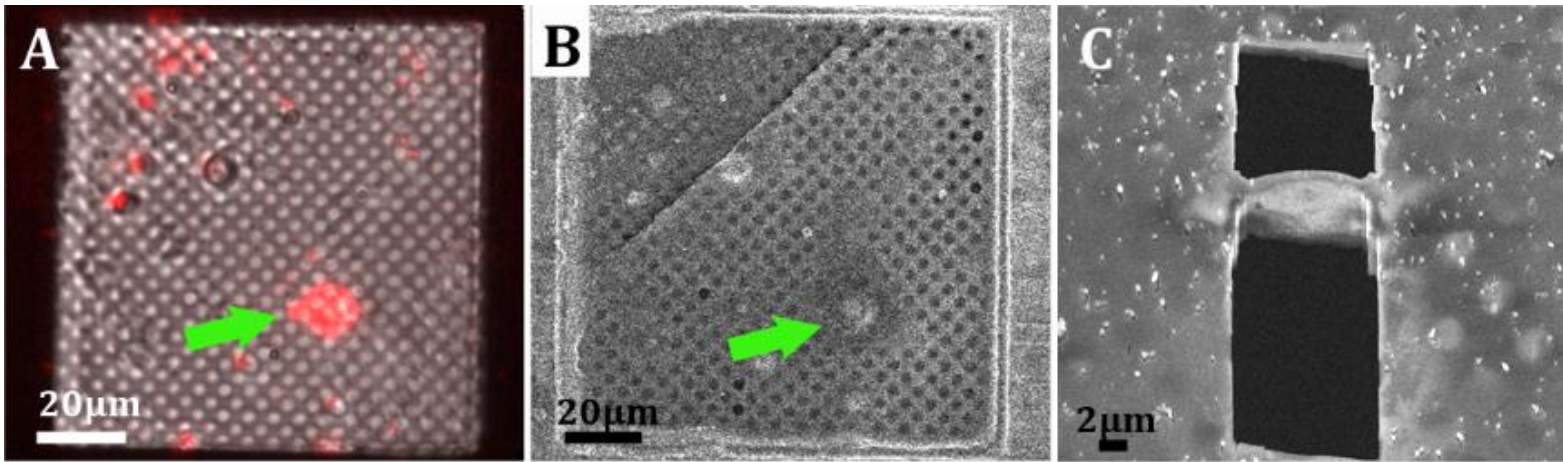
Lamella face. SEM view



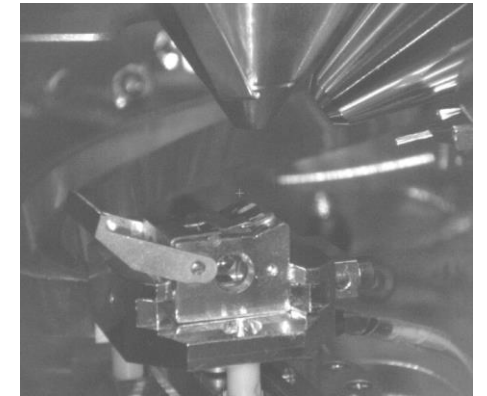
STEM Tomography



Biom mineralization – calcium transport mechanism

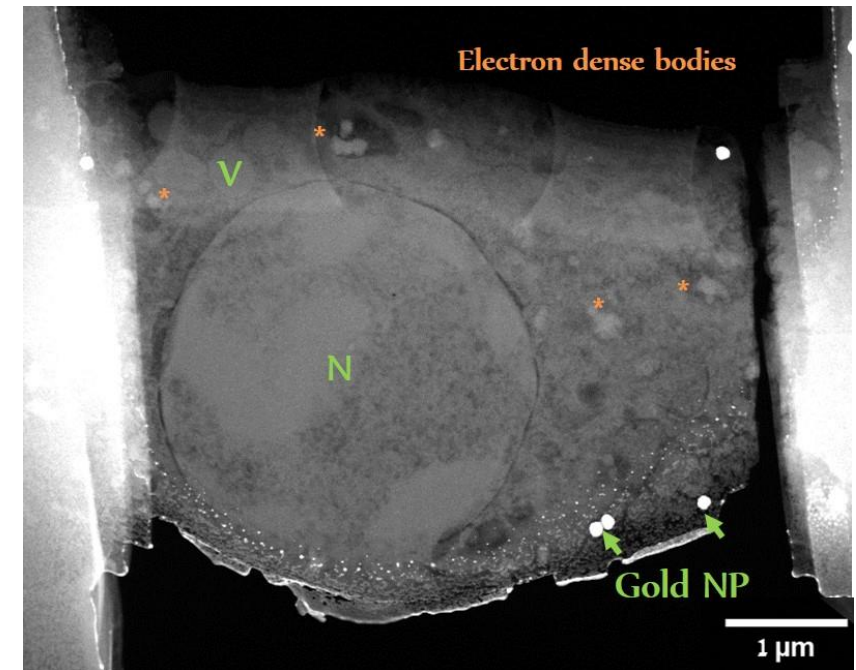


Cryo
FIB
←

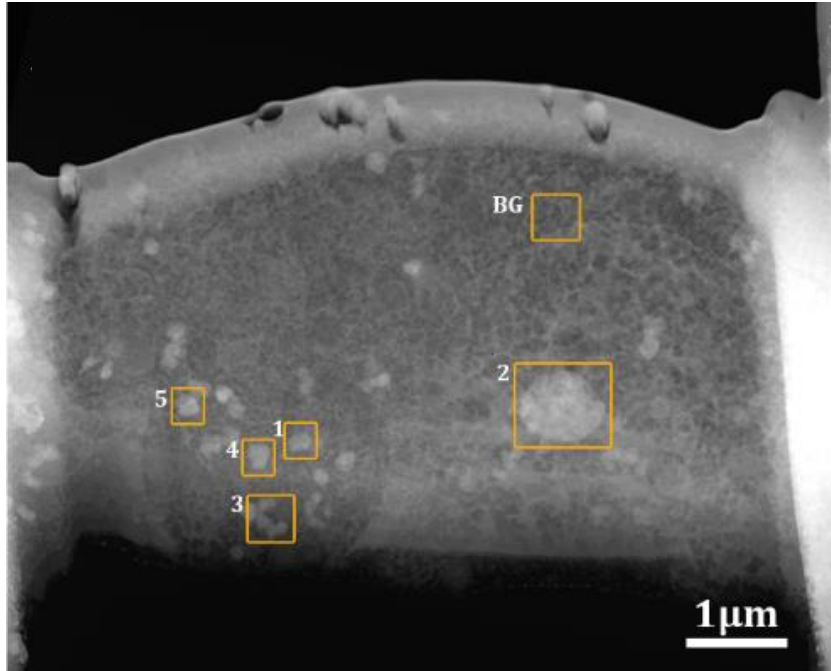


Cryo FIB prepared on-grid lamella of PMC (skeleton forming cell)

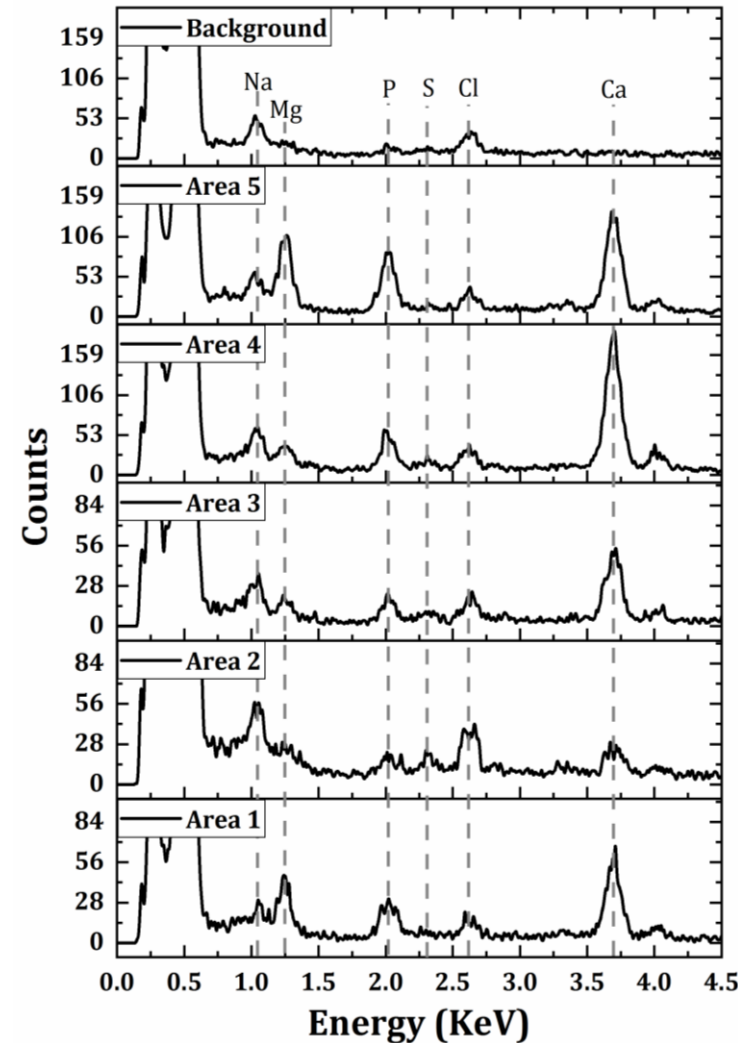
Cryo
TEM
→



Biom mineralization – calcium transport mechanism



Cryo FIB lamella + TEM EDS analysis



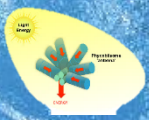
Ca/Mg= 1
Ca/P= 1.5

Ca/Mg= 3.7
Ca/P= 2.6

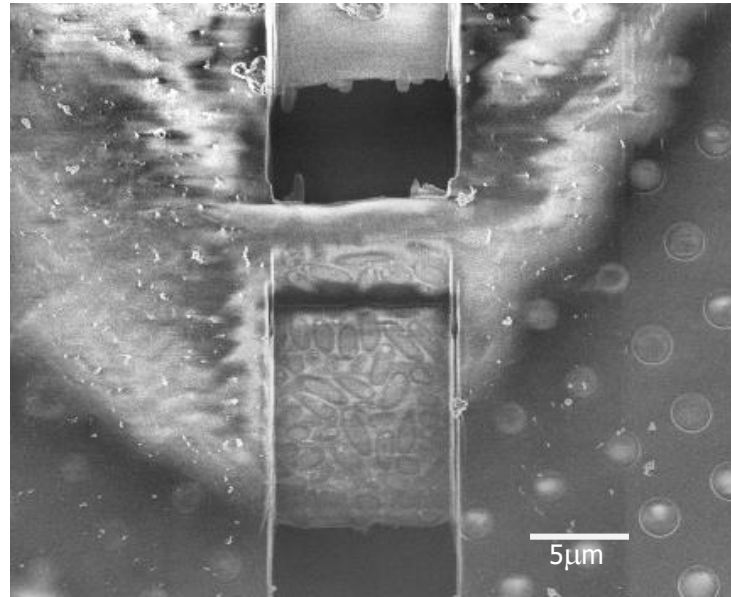
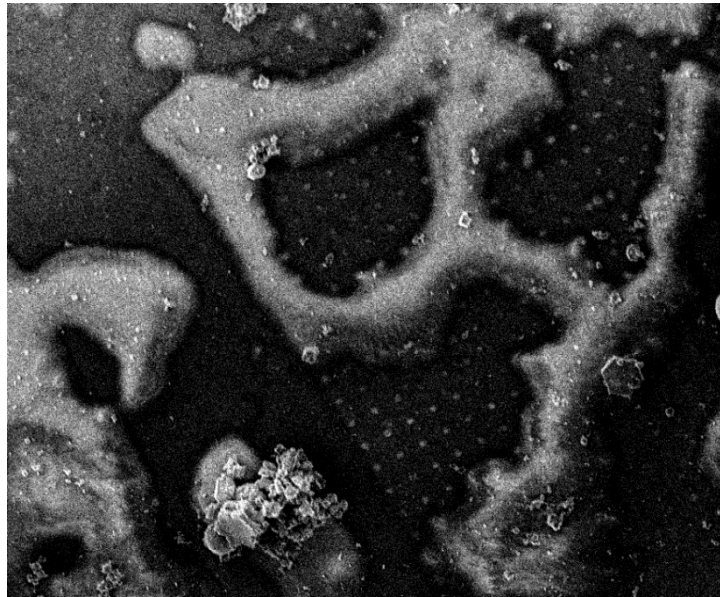
Ca/Mg= 1.7
Ca/P= 2.1

Ca/Mg= 0.9
Ca/P= 1.5

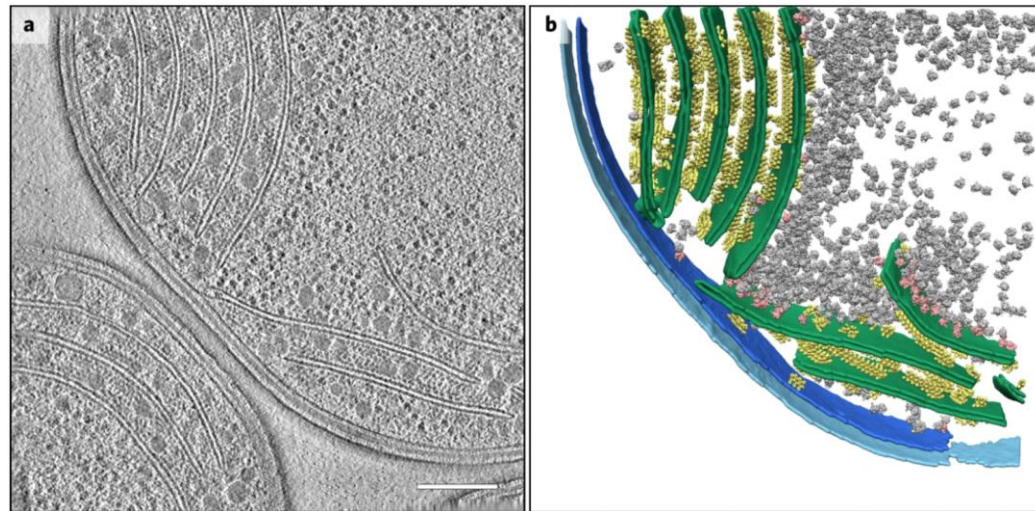
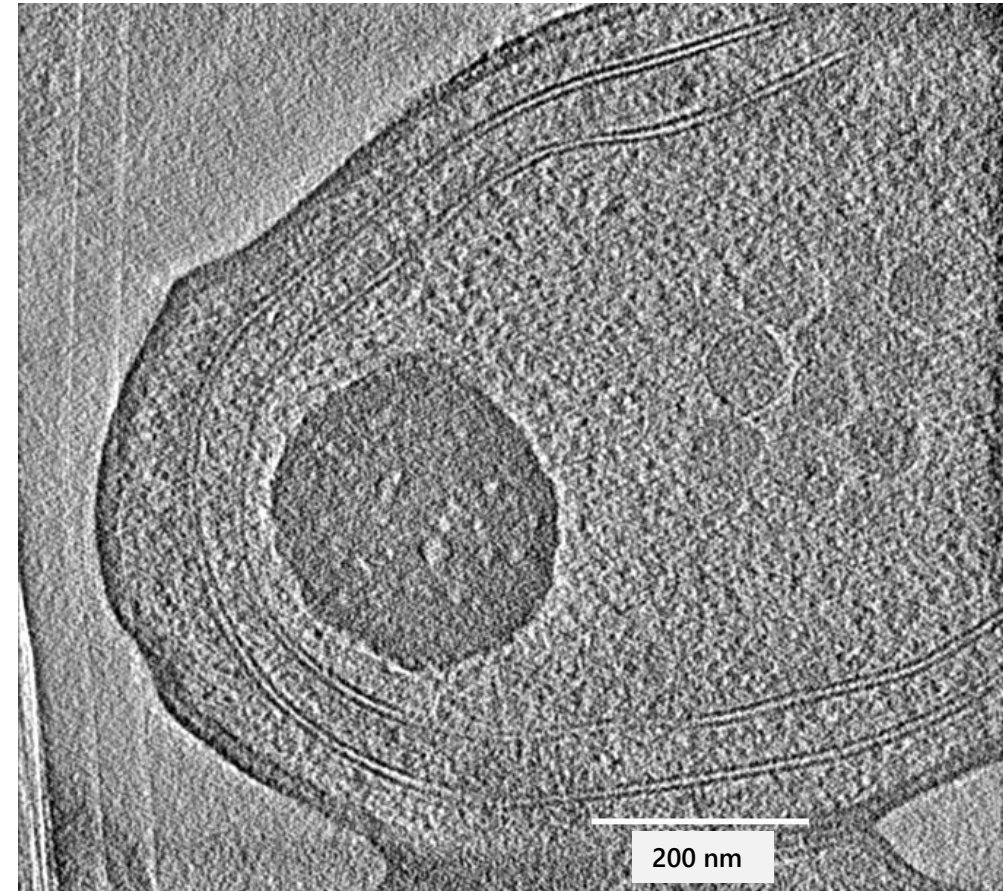
Ca/Mg= 1
Ca/P= 2.3



Structure & functionality of photosynthetic bio-machineries



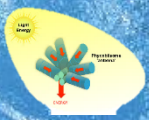
Lamella 350 nm



Biogenic regions of cyanobacterial thylakoids form contact sites with the plasma membrane. Rast A *et al.* Nat. Plants (2019)

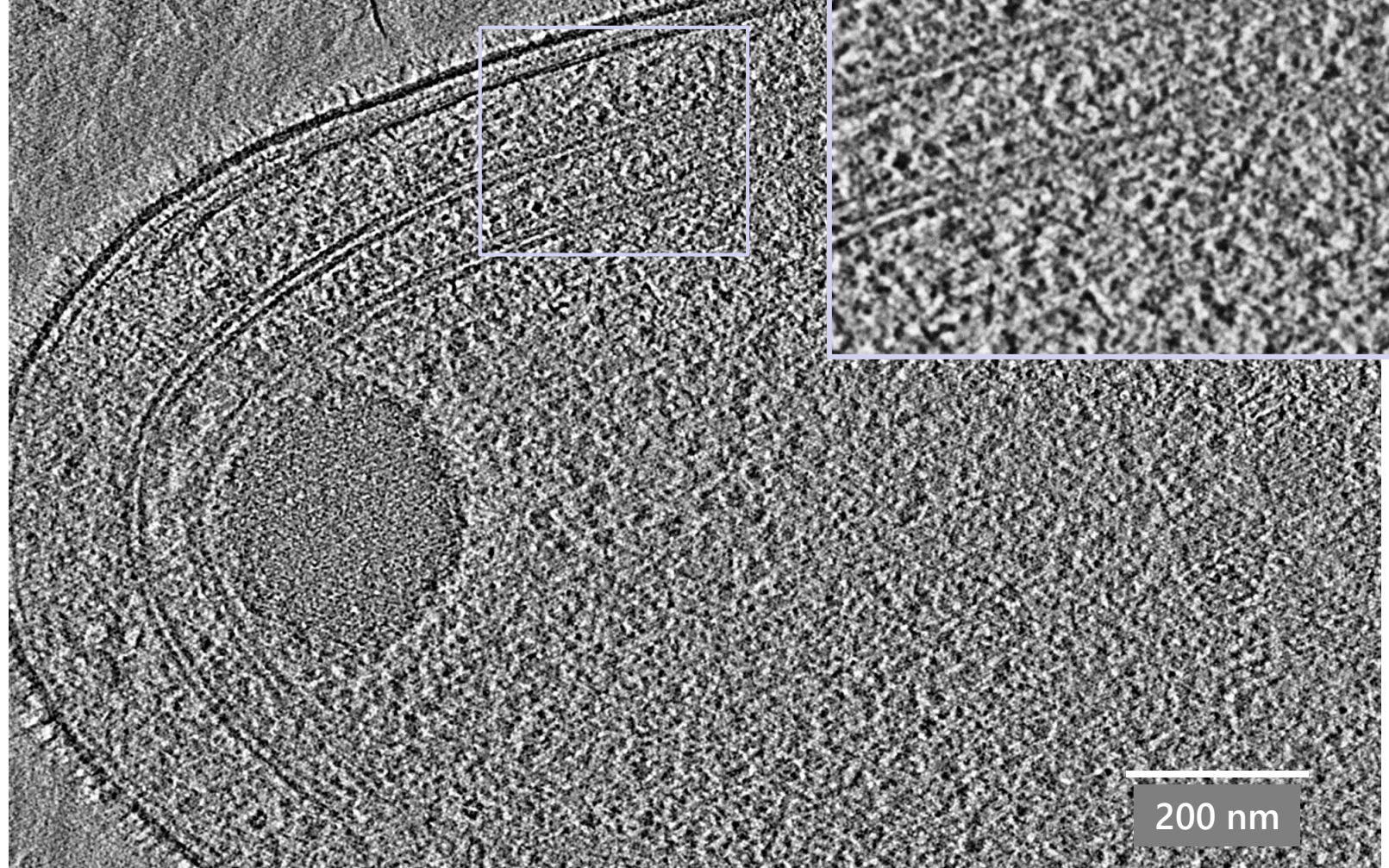
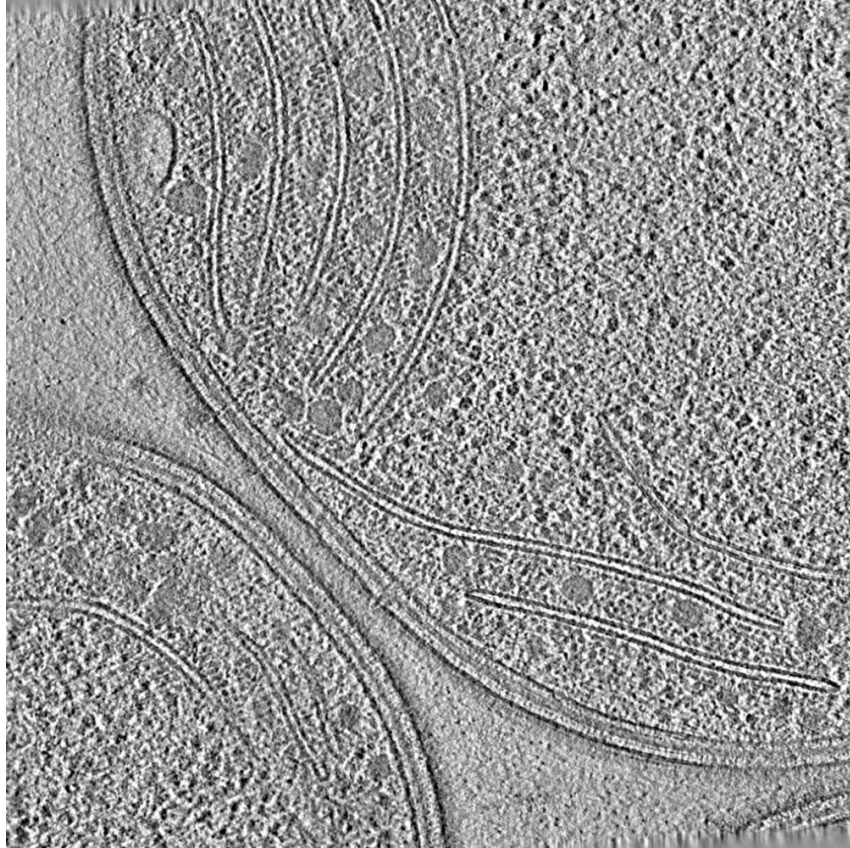
Cryo lamella prepared on plunge frozen *cyanobacteria*

With: Nir Kedem, Hebrew University
Ziv Reich, WIS



Structure & functionality of photosynthetic bio-machineries

Lamella 190 nm



Biogenic regions of cyanobacterial thylakoids form contact sites with the plasma membrane. Rast A *et al.* Nat. Plants (2019)

Thanks to my colleagues and friends

Eyal Shimoni

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Lothar Houben

Olga Brontvein

Anna Kossoy

Smadar Zaidman

Orna Yeger

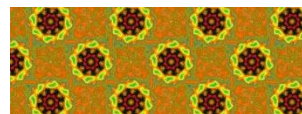
Elena Kartvelishvily

Konstantin Blinder

Sergey Kapishnikov



Michael Elbaum – WIS



Julia Mahamid – EMBL, Heidelberg

EMBL



Andreas Schertel – Zeiss Microscopy, Oberkochen



Early microscope

Thank You

==== *For Your Attention* ====

