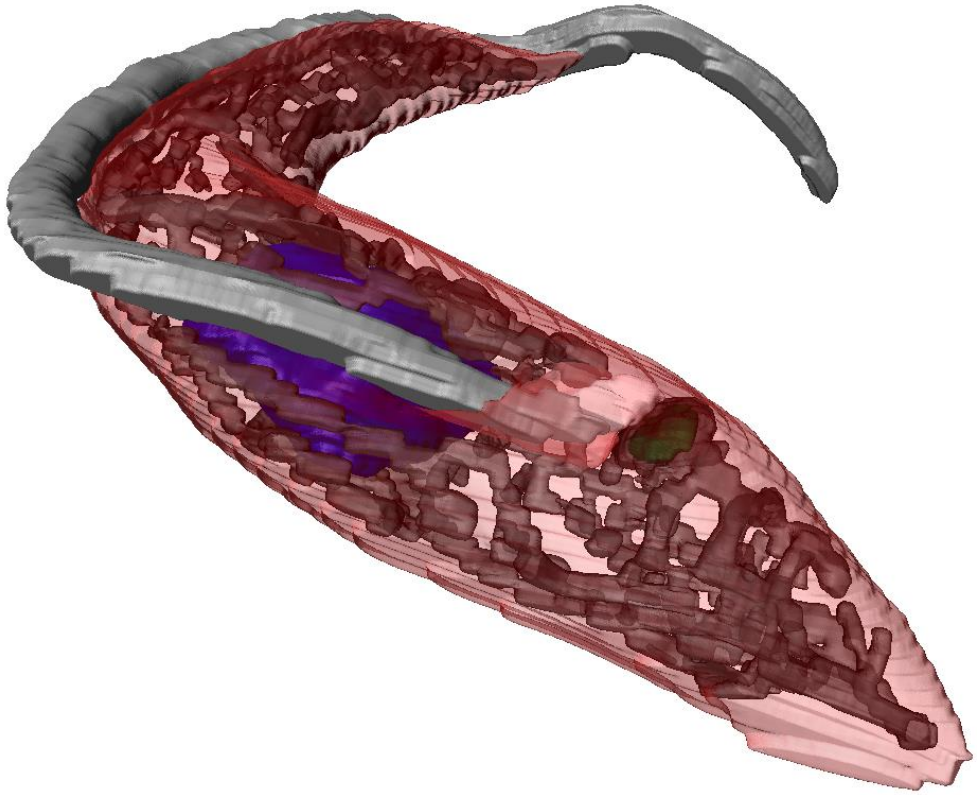

SBF SEM and Array Tomography

Jiří Týč

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CZECH BIOIMAGING
Imaging principles of life

EURO BIOIMAGING
Image life, discover the future

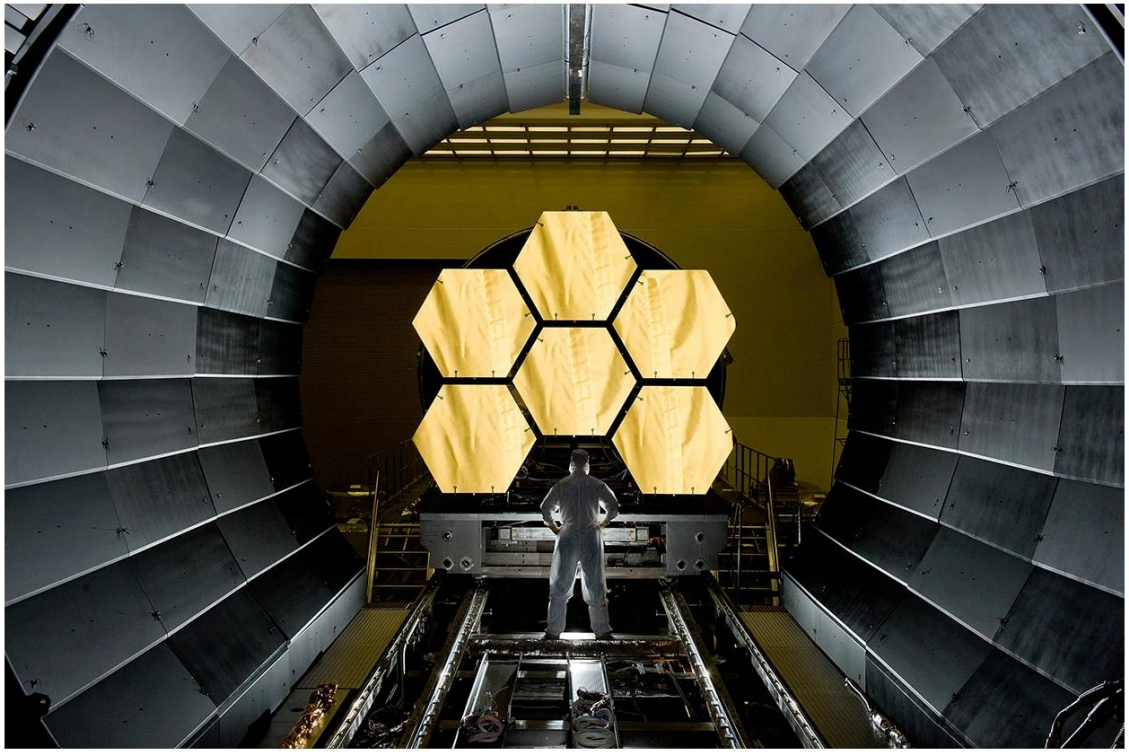
Volume electron microscopy (for 3D reconstructions), was named by the journal Nature as one of the "Seven technologies to watch in 2023" alongside the James Webb Space Telescope, CRISPR, and others.

TECHNOLOGY FEATURE | 23 January 2023

Seven technologies to watch in 2023

***Nature*'s pick of tools and techniques that are poised to have an outsized impact on science in the coming year.**

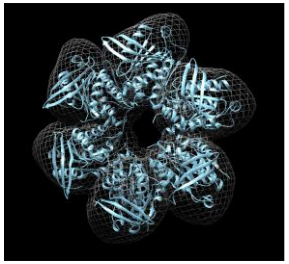
Michael Eisenstein

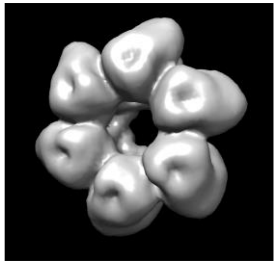


The James Webb Space Telescope's 6.5-metre primary mirror (6 of 18 segments shown) can detect objects billions of light years away. Credit: NASA/MSFC/David Higginbotham

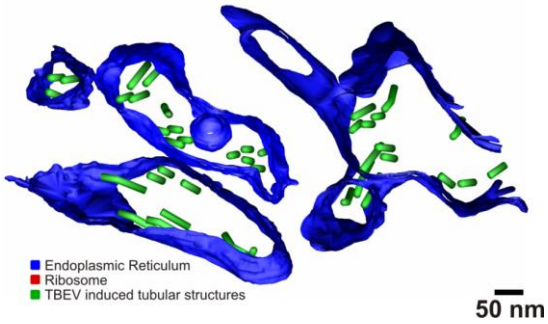
3D TEM

Single particle analysis
resolution in angstrems





Electron tomography
resolution in nm

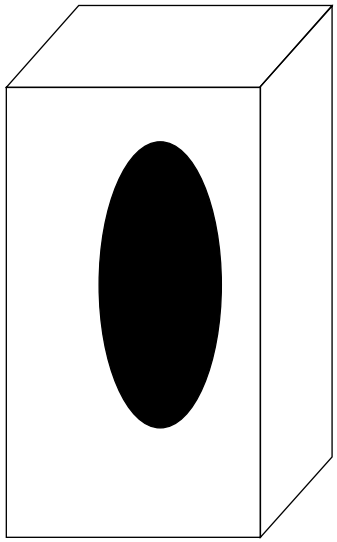


Advantage: really good resolution
Disadvantage: really small volumes – macromolecules, part of the organelle

ssTEM (serial section TEM)
Really laborious – SEM provides automatization of data acquisition

Principle of imaging in 3D SEM

Serial sections



Simple concept:

Trim the resin block

↓

Cut consecutive sections

↓

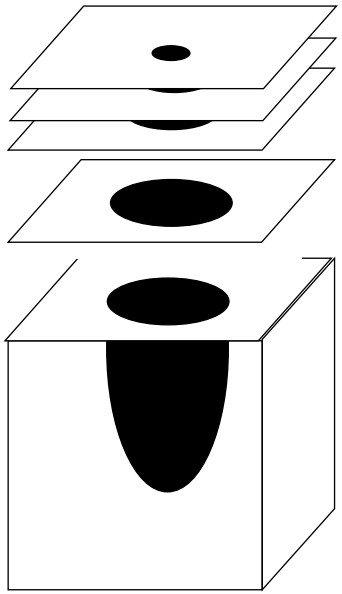
Image consecutive section

↓

Reconstruct in 3D

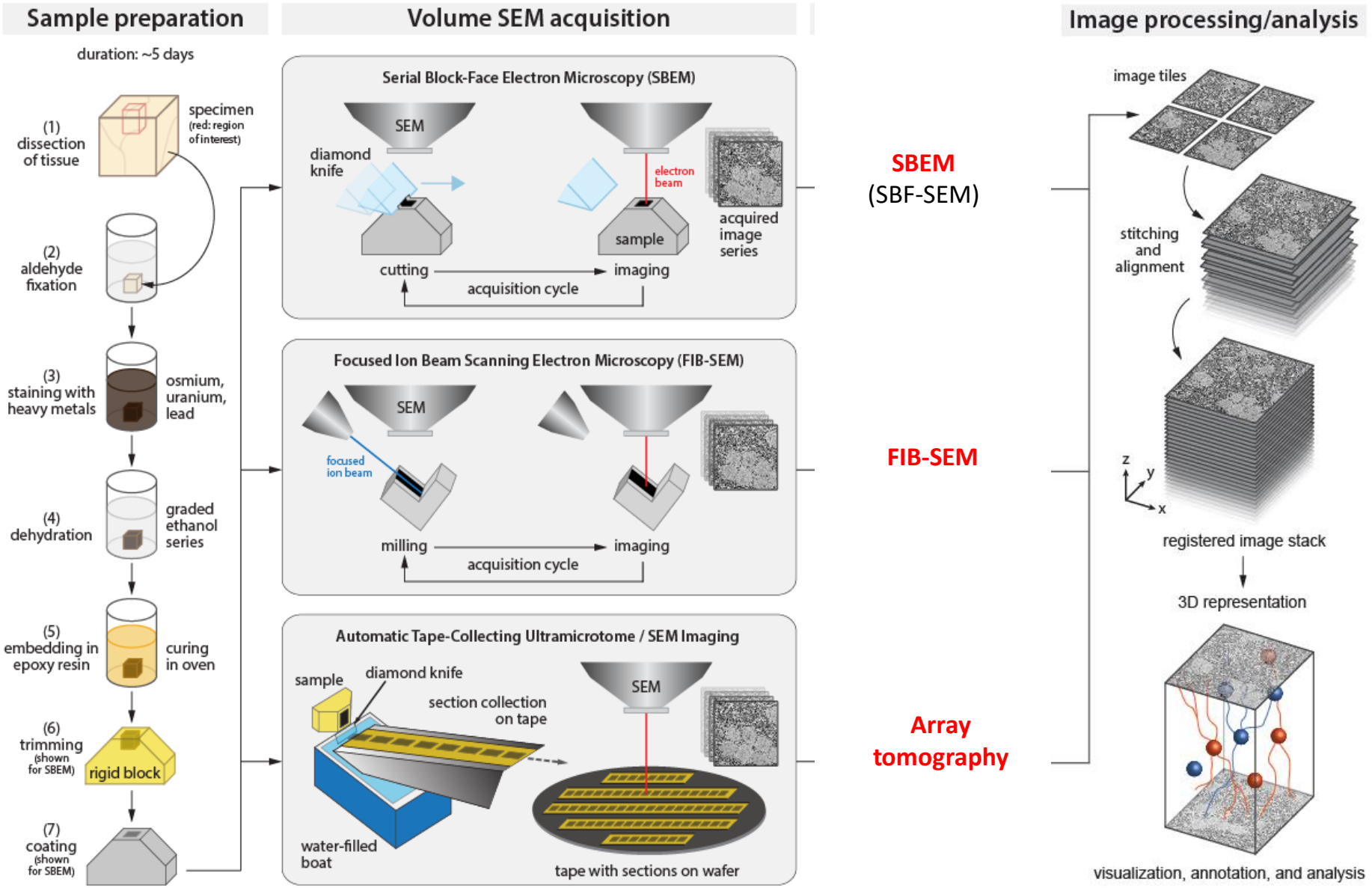
↓

Model or measure features



Modified from Yannick Schwab

Principle and Methods of imaging in 3D using SEM

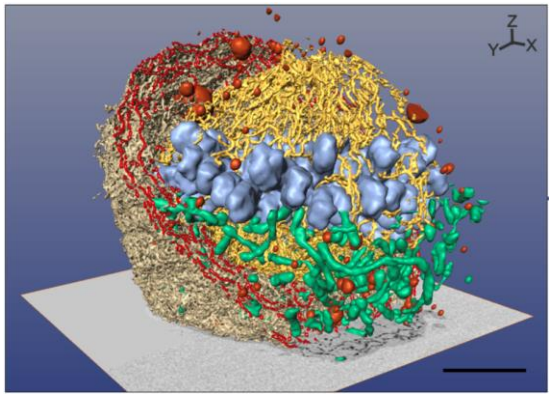


Titze B., Genoud C. (2016) Volume scanning electron microscopy for imaging biological ultrastructure. *Biology of the Cell*, 1–17.

Modified from Yannick Schwab

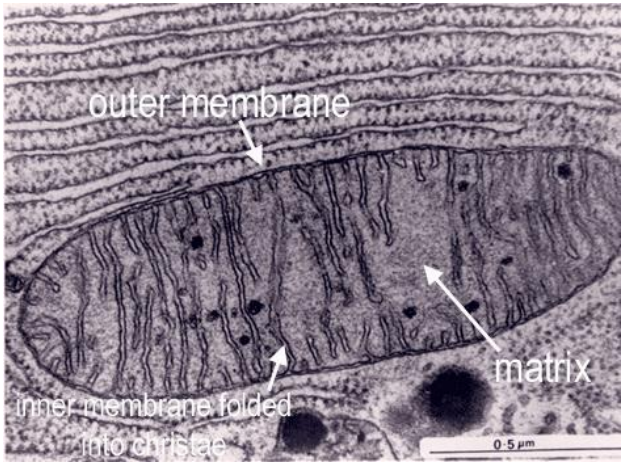
Why to image in 3D SEM? What benefits it offers?

Bigger volumes!! (compare to 3D TEM)

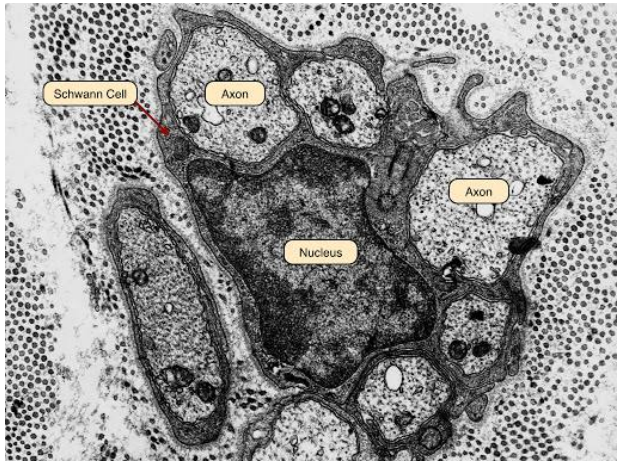


Belevich, I. *et al.*. Microscopy Image Browser: A Platform for Segmentation and Analysis of Multidimensional Datasets. *PLoS Biol* **14,** e1002340–e1002340 (2016).

Cellullar level:
Shape, volume and amount of organelles, interactions of organelles, spatial organization of the cell.

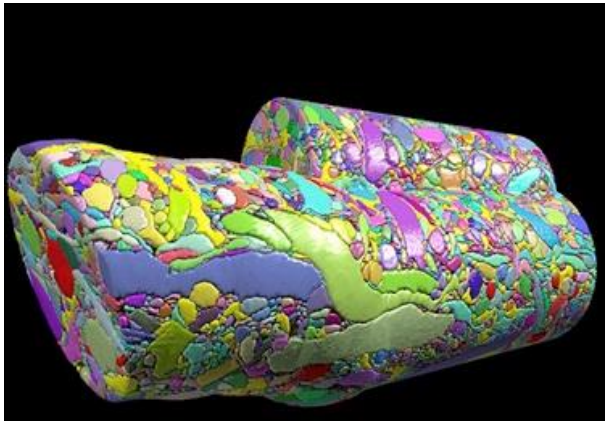


https://www.histology.leeds.ac.uk/cell/cell_organelles.php



http://medcell.med.yale.edu/systems_cell_biology/nervous_system_lab.php

Tissue level:
Cell shape, cell to cell interactions, connectivity, connections (tight junctions etc.)

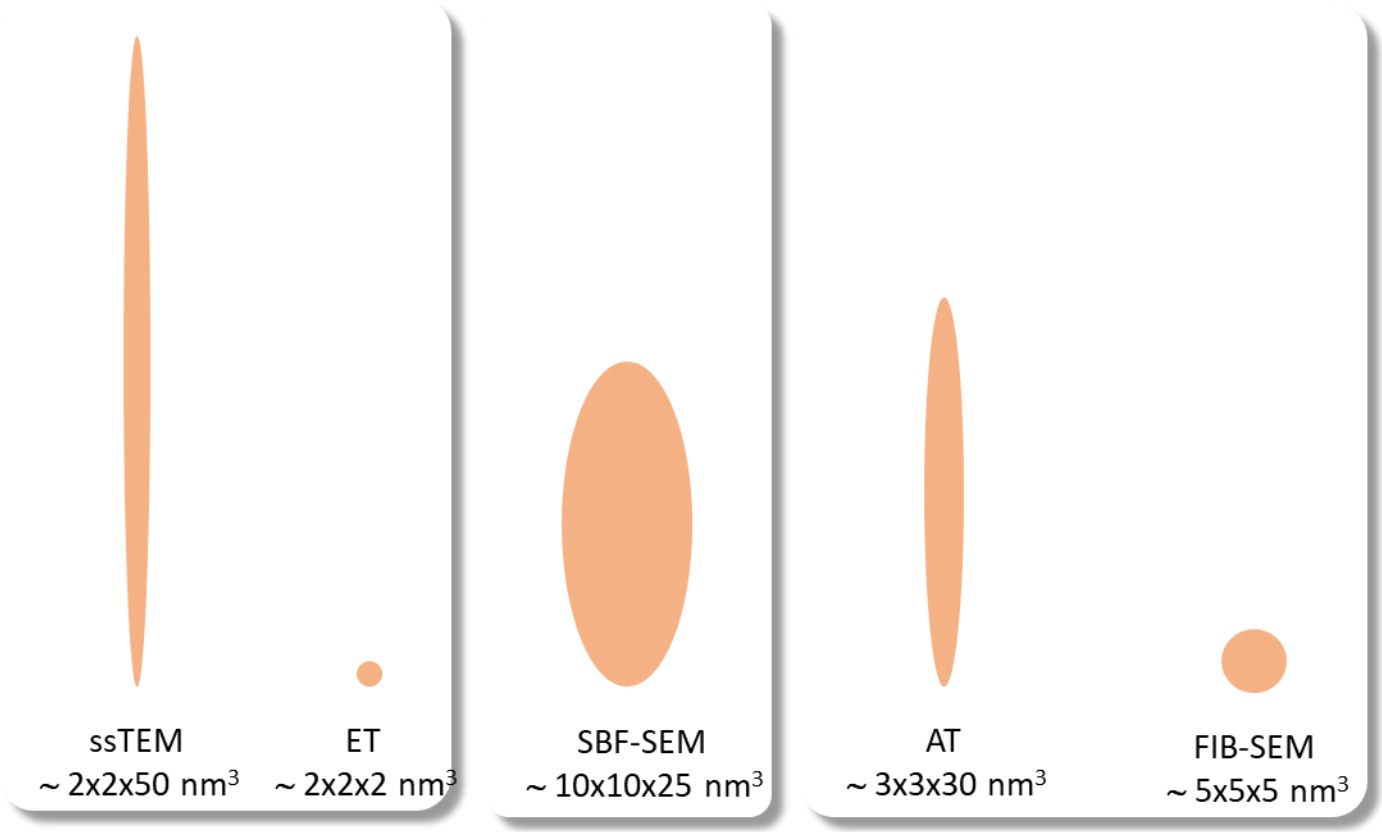


Kasthuri, N. *et al.* Saturated Reconstruction of a Volume of Neocortex. *Cell* **162,** 648–661 (2015).

Comparisons of 3D SEM methods

	SBEM	FIB-SEM	Array Tomography
Fully automated data collection	YES	YES	NO??
The sample is left intact and can be reimaged	NO	NO	YES
Best achievable resolution in 3D (x,y,z)	10 x 10 x25 nm3 *	5 x 5 x 5 nm3	3 x 3 x 30 nm3
Maximal width of ROI (region of interest)	1 mm	20-100 µm #	3 mm
Problems specific for given technology	Surface charging, sensitivity to electron dose	Redepostion of material	Damage, compression or loss of some sections
Labelling	Only in whole volume (en bloc)	Only in whole volume (en bloc)	en bloc as well as labelling of individual sections
Stitching and alignment of acquired images	Usually just lateral shift	Usually just lateral shift	More difficult – rotation, damage, compression
Approximate time and dataset size for given volume			
10 × 10 × 10 µm3	2 h, 0.4 GB	39 h, 8 GB	23 h, 3.7 GB
20 × 20 × 20 µm3	4 h, 3.2 GB	10 days, 64 GB	2 days, 30 GB
50 × 50 × 50 µm3	22 h, 50 GB	4 months, 1 TB	6 days, 460 GB
100 × 100 × 100 µm3	5 days, 400 GB	-	15 days, 3.7 TB
200 × 200 × 200 µm3	5 weeks, 3.2 TB	-	8 weeks, 30 TB
1000 × 1000 × 1000 µm3 (= 1 mm3)	13 years, 400 TB	-	12 years, 3700 TB
*** Better resolution in X and Y is achievable by sacrificing thicker sections in Z**			
# 20 µm ROI is a limit for which the best resolution is possible			

Visualization of voxel dimensions of 3D SEM methods



if the voxel is not <u>isometric</u>

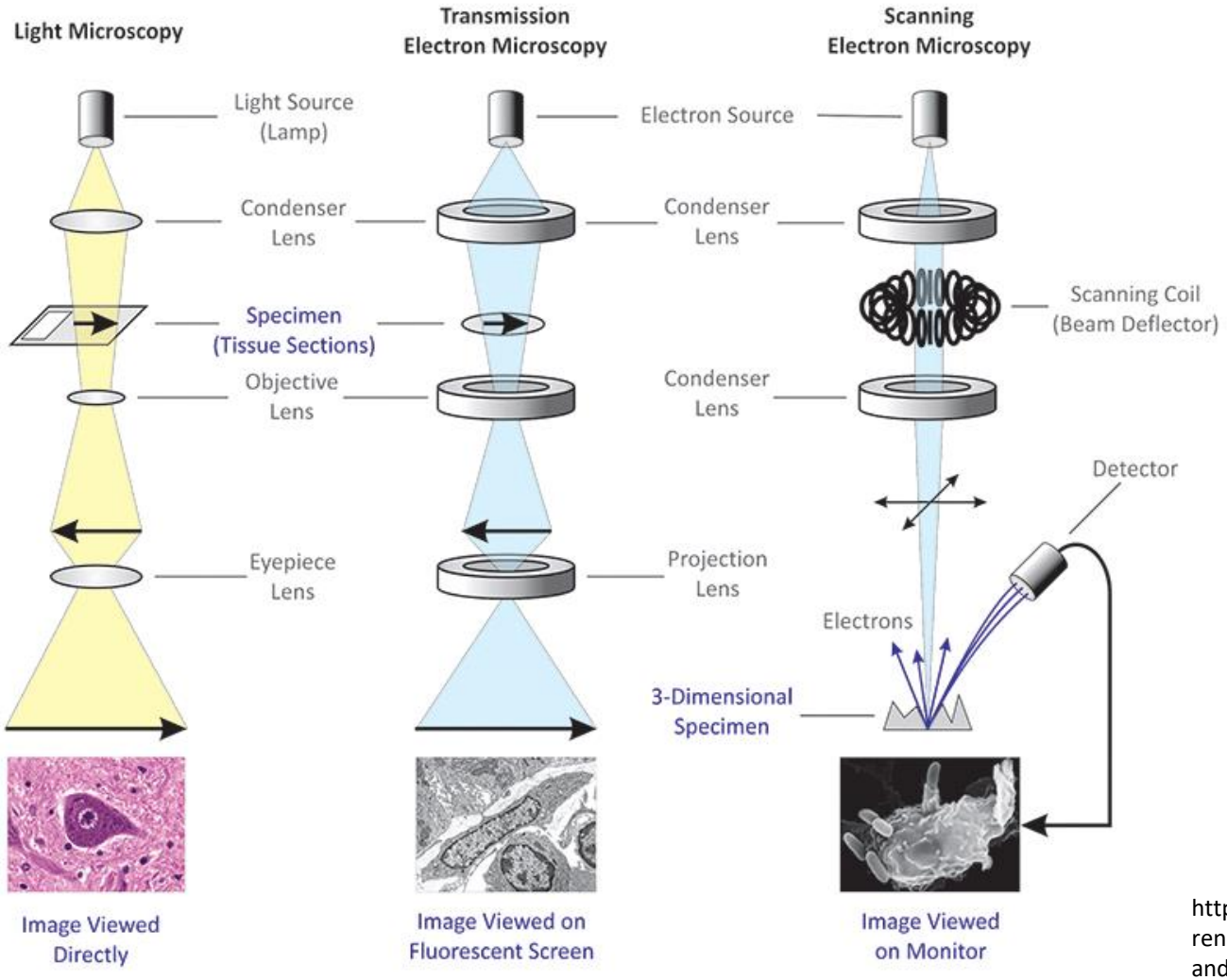
➔ Distortion in one dimension

<u>Z resolution in SEM is kV dependent</u>

Modified from Yannick Schwab

Principle of SEM – comparison to other microscopes

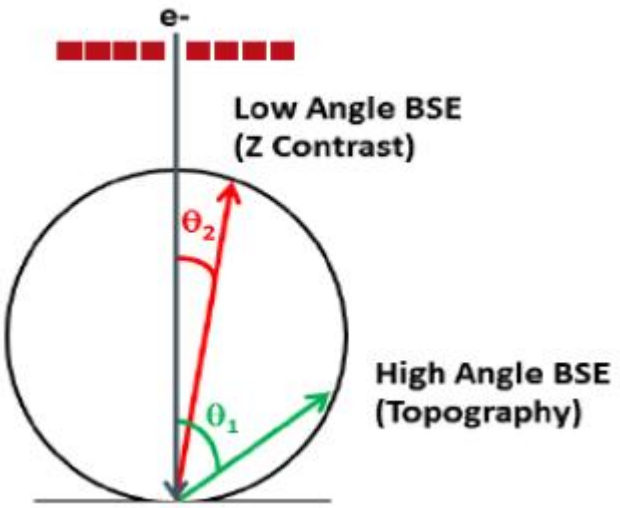
- In the SEM we detect electrons that bounced BACK from the sample
- There is no camera in the SEM

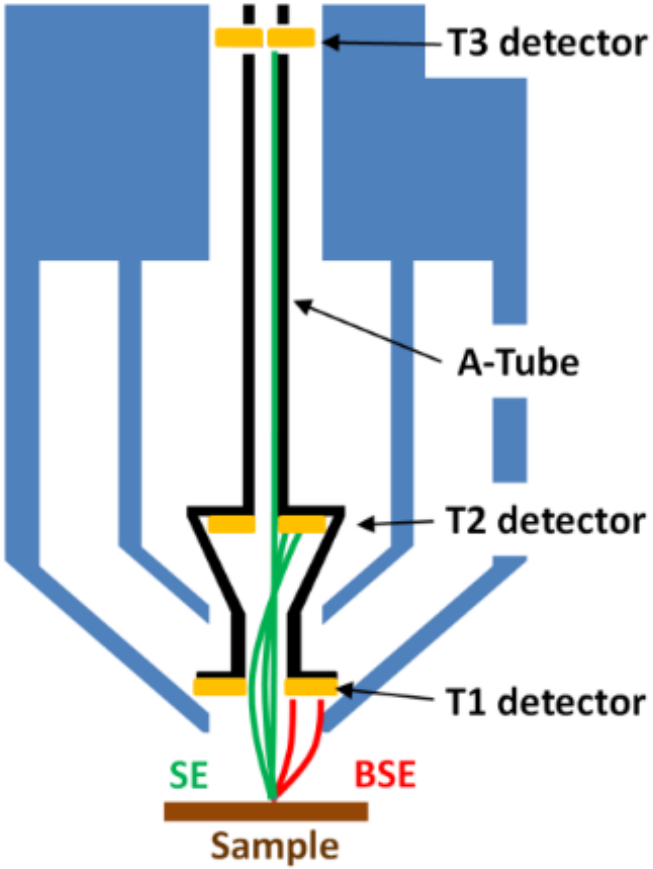


https://microbiologyinfo.com/differences-between-light-microscope-and-electron-microscope/

Imaging in 3D SEM

We are using mostly Back scattered electrons (BSE)
Highest energy (less noise), information about sample composition + highest contrast
Secondary and low energy electrons – info about topology (but we are imaging a FLAT surface)





Elastic scattering in the sample
Backscattering is dependant of the atomic number: heavier the element, more backscattered e-

Imaging in 3D SEM

Image from TEM

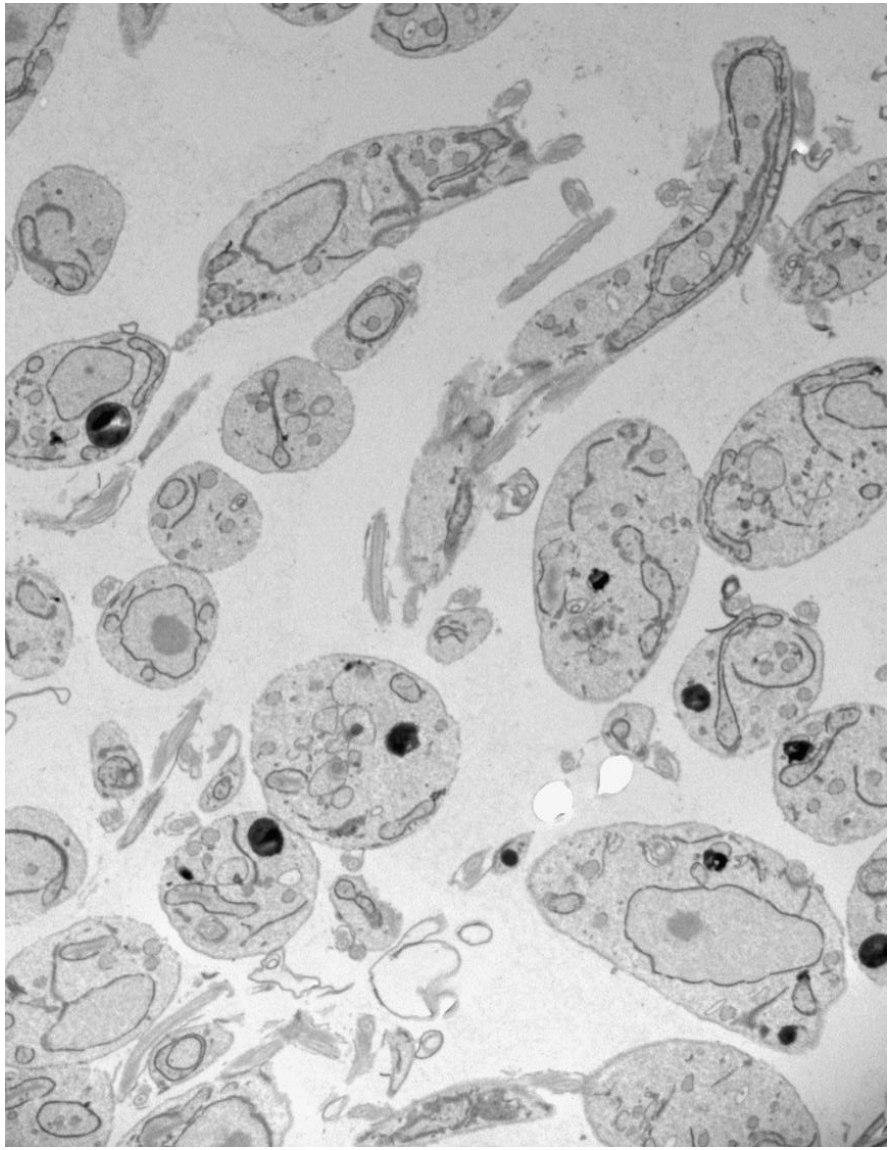
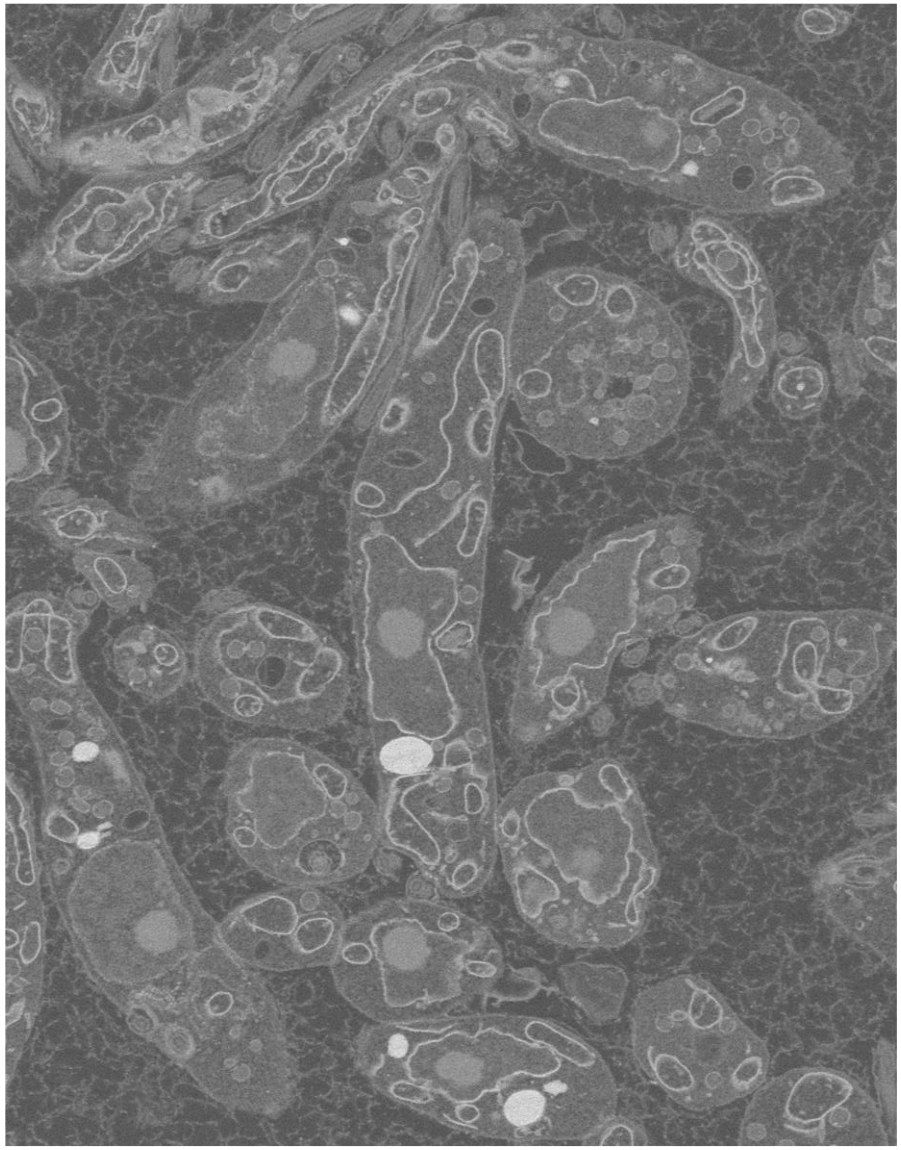
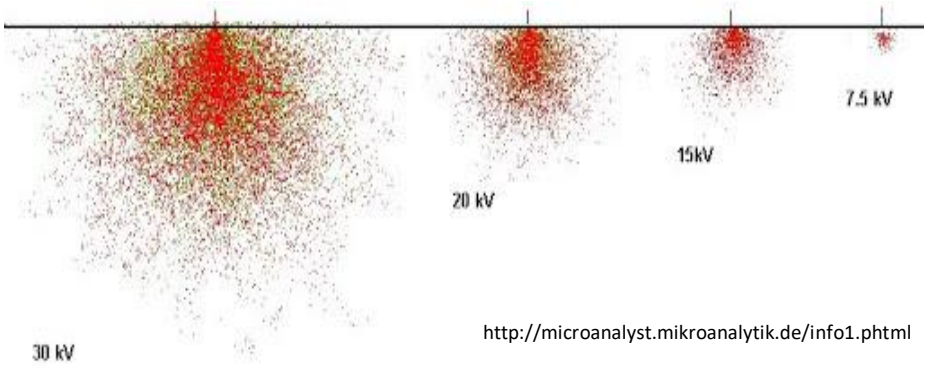


Image from SEM



Parameters used that makes a difference

Voltage - the energy of landing electrons
(and the depth from which we obtain the signal)



http://microanalyst.mikroanalytik.de/info1.phtml

2kV	24nm
3kV	48nm
4kV	77nm
5kV	112nm
6kV	152nm
7kV	196nm
8kV	245nm
9kV	299nm
10kV	356nm

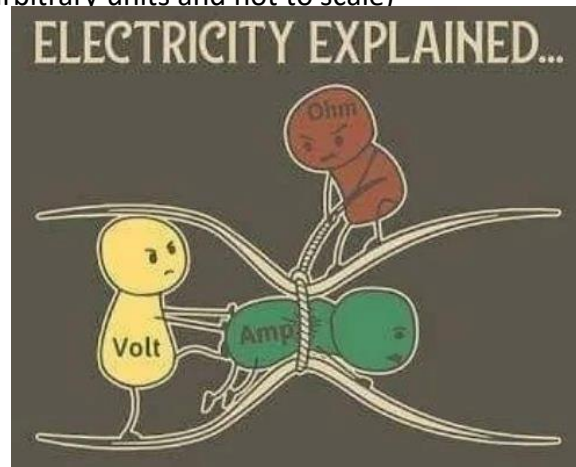
Current (spot size) - the amount of electrons that hit the sample and can result in a signal,
- the bigger the current the bigger "the footprint" of the beam

(arbitrary units and not to scale)

Dwell time - time spent per pixel – affects noise

Pixel size

Electron dose: calculated from the above-mentioned parameters – results in beam damage, important especially for SBEM



Parameters used that makes a difference

We always search for parameters with the best ratio of signal, resolution and speed of acquisition + minimal or no beam damage.

Acquisition parameters:

3kV, 50pA, 2us, 5nm	3kV, 50pA, 1us, 5nm	3kV, 50pA, 2us, 10nm

Acquisition time per image (slice):

4 min	2 min	1min

Acquisition time per run (1000 slices):

67 hrs (2,8 days)	33,5 hrs (1,4 days)	16,7 hrs (<day)

Total cost of the run (500 CZK per hour)

33 500 CZK	16 750 CZK	8 350 CZK

PS:

usually you need to image at least 2 samples (one being control)

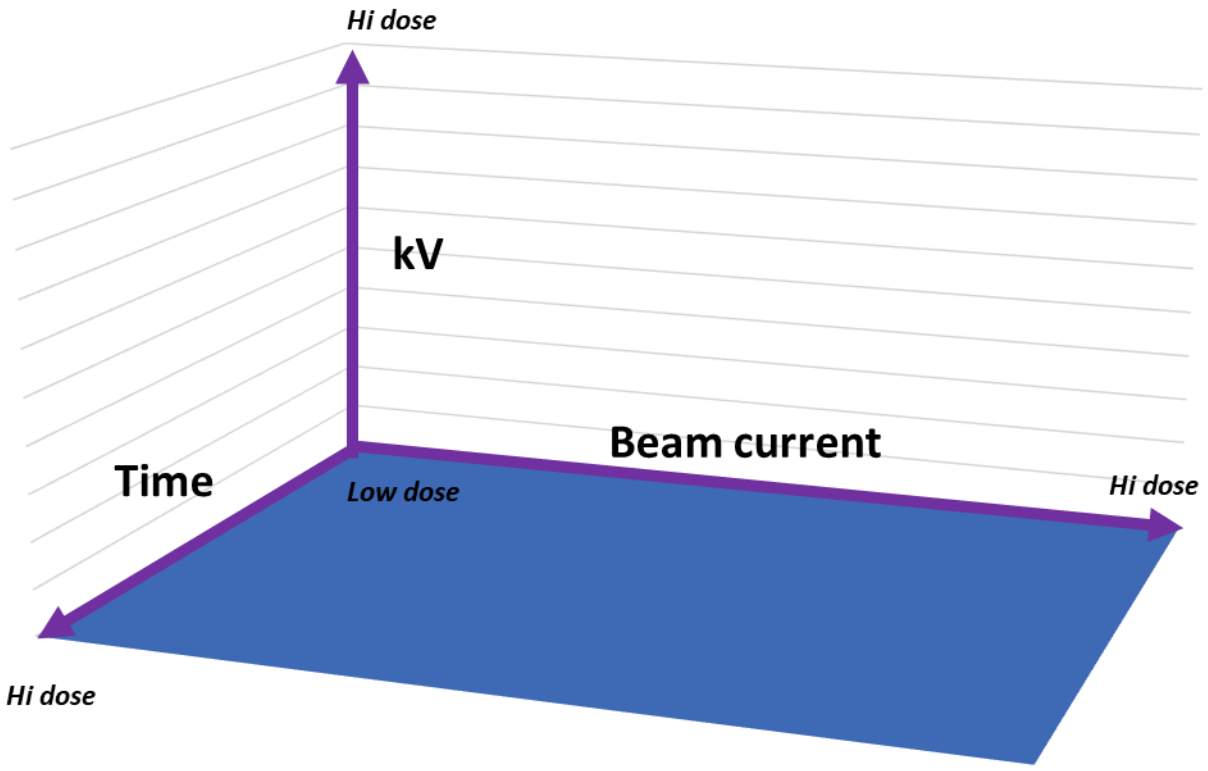
pixelsize 5 nm = 25 nm²

vs

10 nm = 100 nm²)

Shorter time can be to some extend compensated by increase of current, but that lowers the resolution ☺

Electron dose per surface

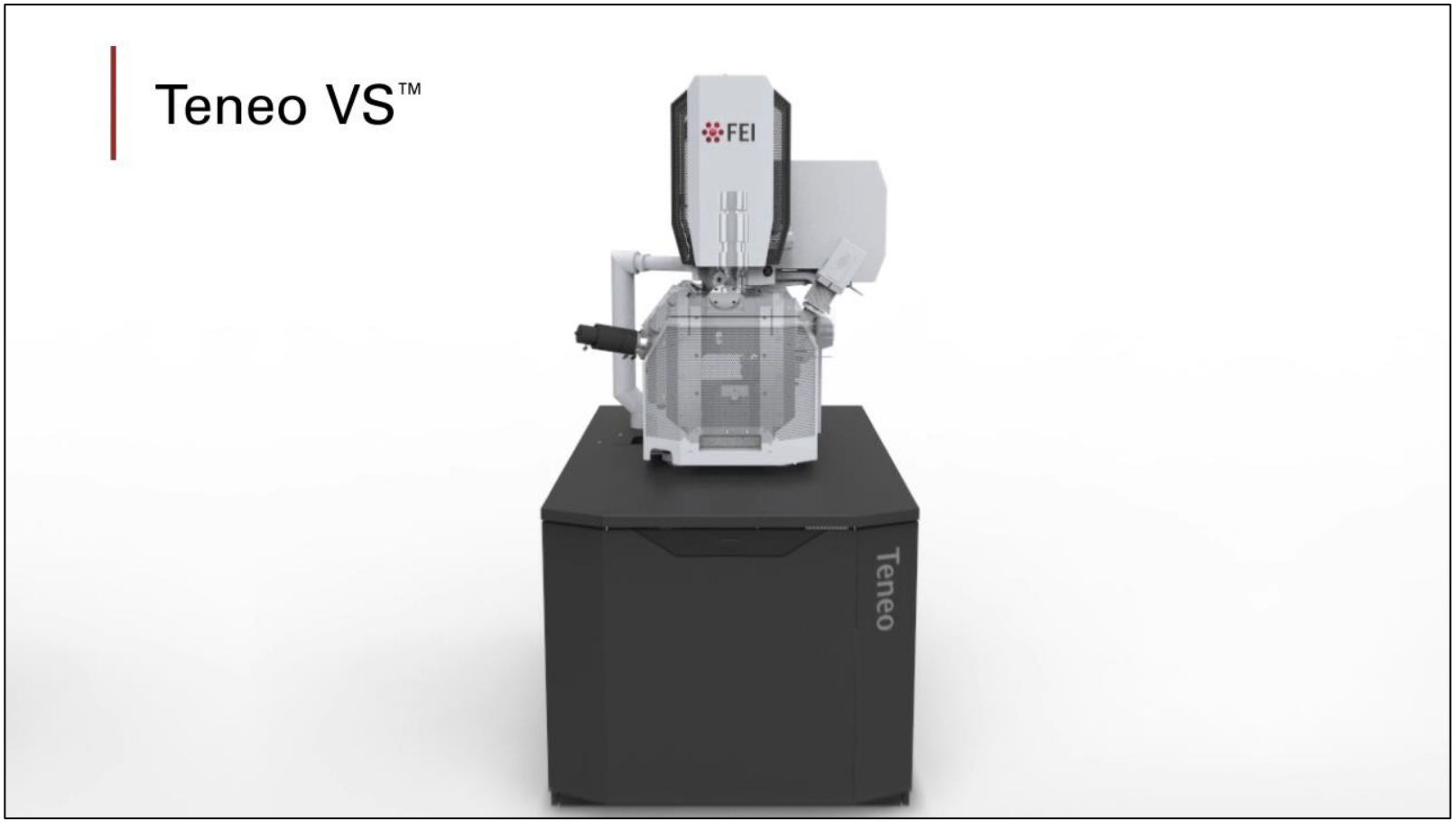


Each sample has a limit of electron dose

- Above this experimental limit, the sample is damaged by the beam and is charging
  + in SBEM it is not possible to reliably cut sections
- Below a given threshold, the electron dose is too low to generate a signal to detect

SBF-SEM
SBEM

SBEM technology overview



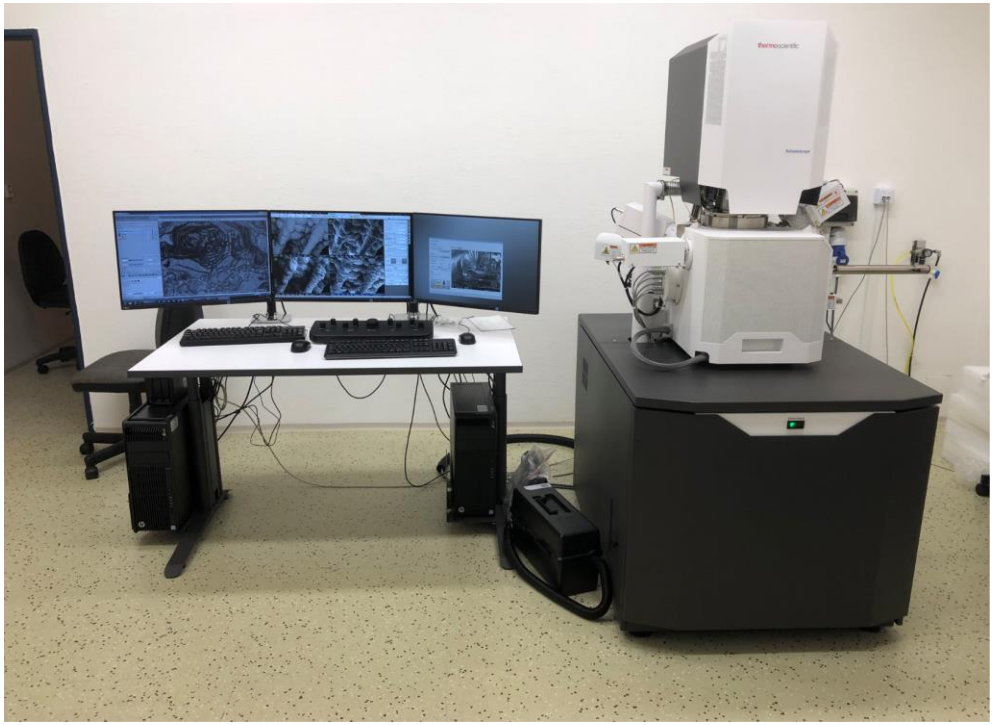
Take home message:

<u>There is a ultramicrotome in the SEM chamber that allows to collect serial images</u>

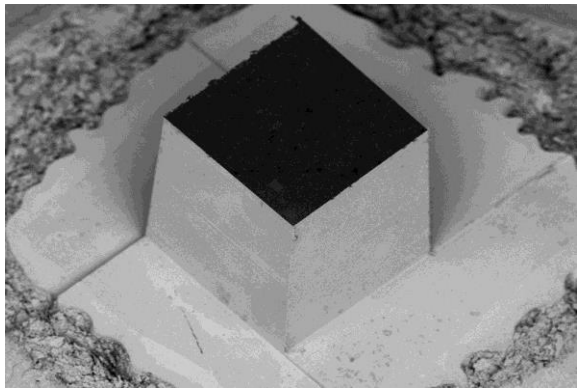
Solution used in the Laboratory of Electron Microscopy České Budějovice

Apreo SEM equipped with Volumescope from

ThermoFisher SCIENTIFIC



Typical sample size:
0,5 mm^3



Maximum sample size:
1,1 mm^3

Typical imaged volume:
tens – lower hundreds of cubic microns

Summary SBEM:

Issues

- Charging
- Sample prep is more difficult
- Sections are lost and can not be reimaged
- Lower resolution compare to FIB and Aarray Tomography (AT)

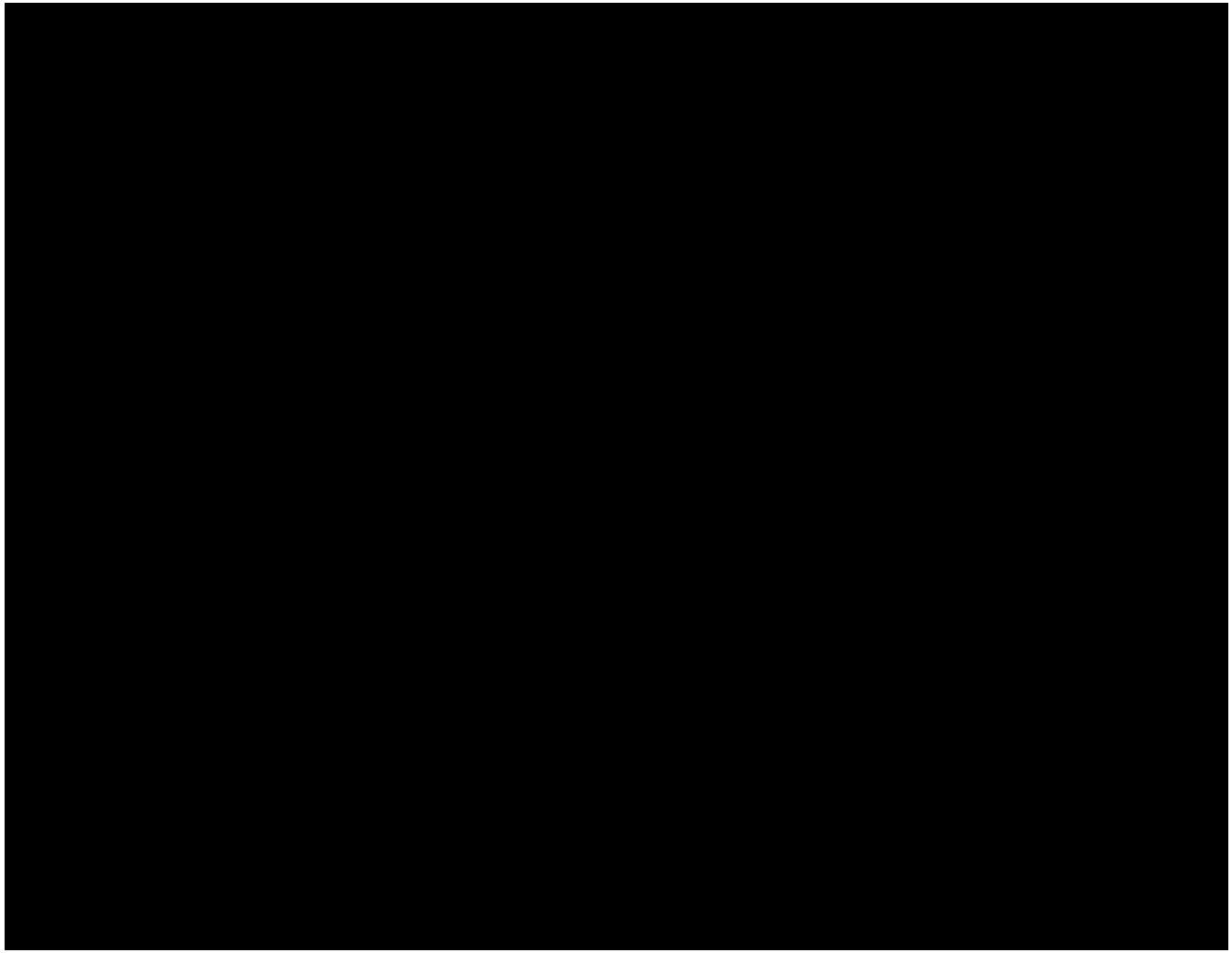
Advantages

- Stable run and automatic collection of images compare to FIB and AT (usually)
- Many sections and **larger volumes** compare to FIB and AT
    - Even part of tissues can be imaged, not just single cells
- Lower Z resolution – larger volumes and less data for processing ☺

What works really well

Nervous tissue – rat spinal cord

Homogenous quite well conductive sample.
It can be imaged in Hi-Vac, with high details and almost no limitations (high contrast, enough signal)

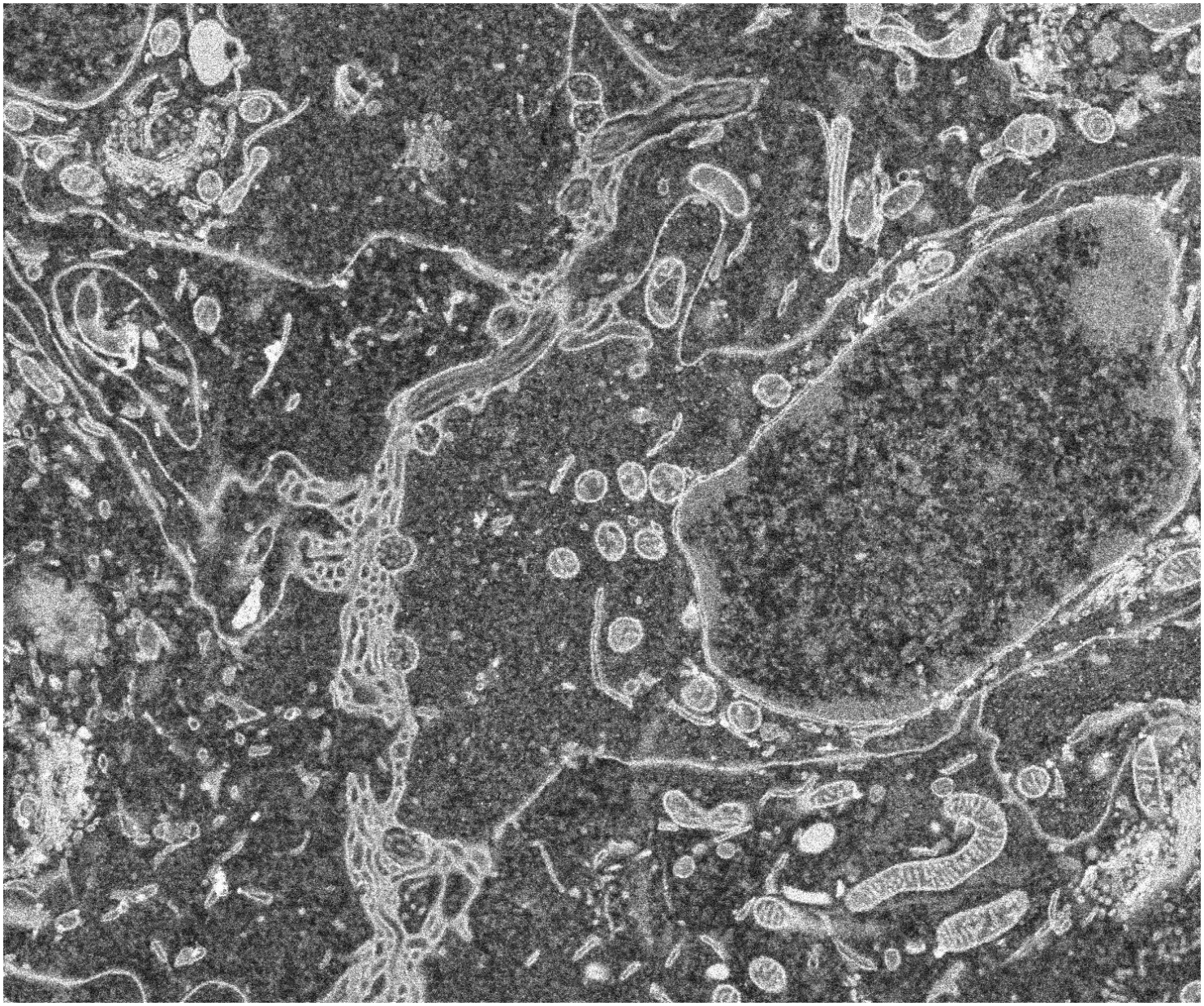


Video shows 200 slices (per 50 nm)

Nervous tissue – rat spinal cord

Resolution good enough to distinguish:

- 9 tubules in the cilium
- Golgi vesicles
- Nuclear double membrane
- Mitochondrial cristae



Take home message: <u>Larger areas can be acquired in several days in high resolution</u>

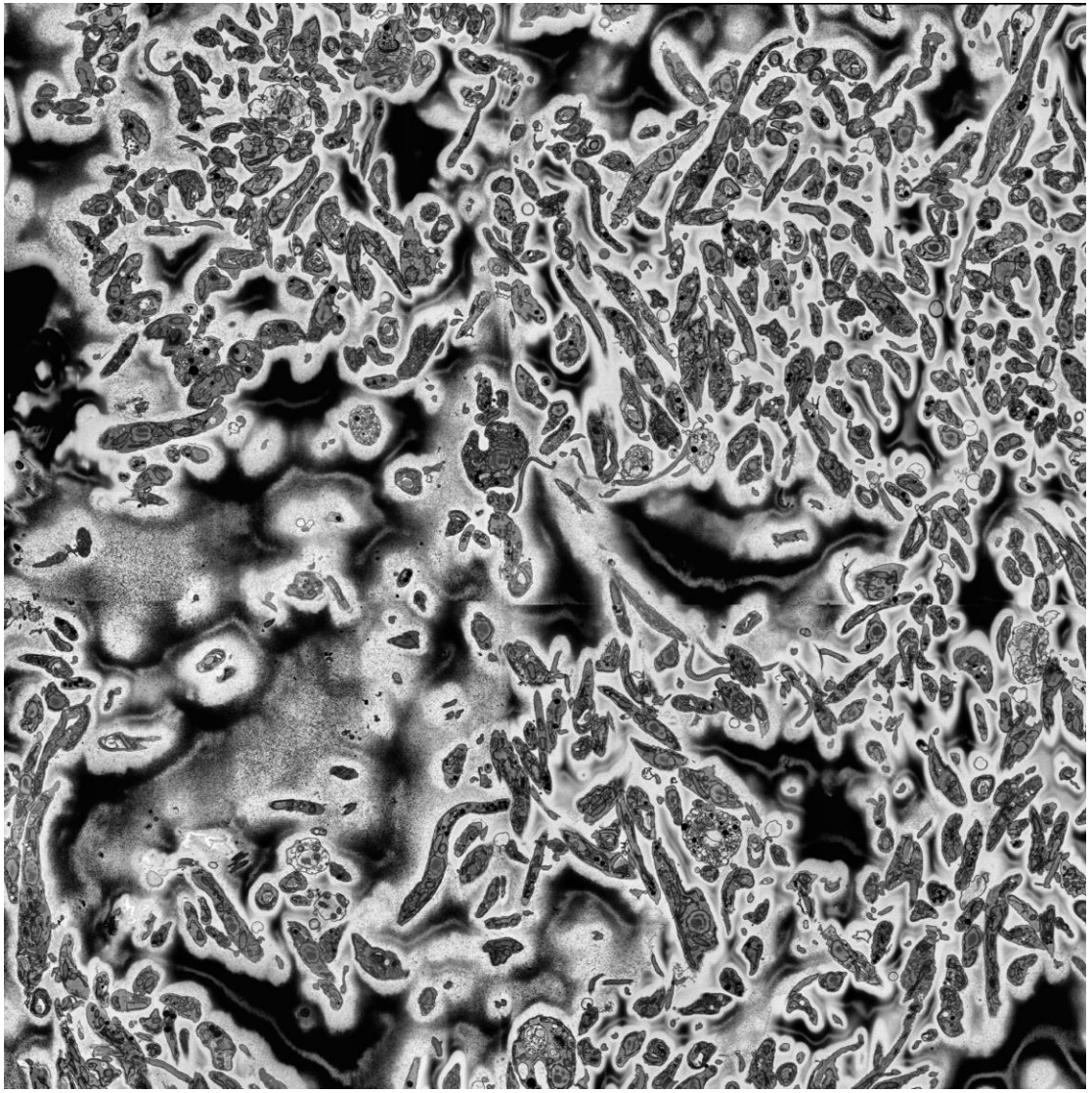
What does not work that well

Pretty much any other sample ☺

Especially those where is a lot of empty resin.

- Single-cell cultures
- Tissues with empty spaces (lungs, fish roe, invertebrate haemolymph etc.)

- Charging can be overcome by using low vac, works pretty well, but lowers contrast and resolution, electron dose applied must be increased – limitations for the reliability of cutting,
- There have to be pauses during the run for the vacuum recovery

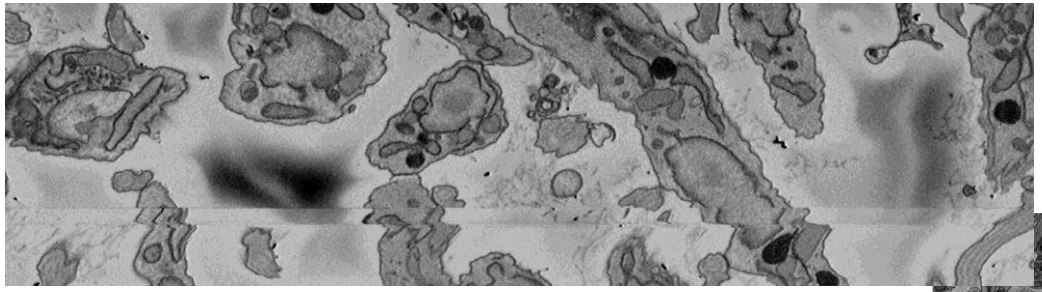


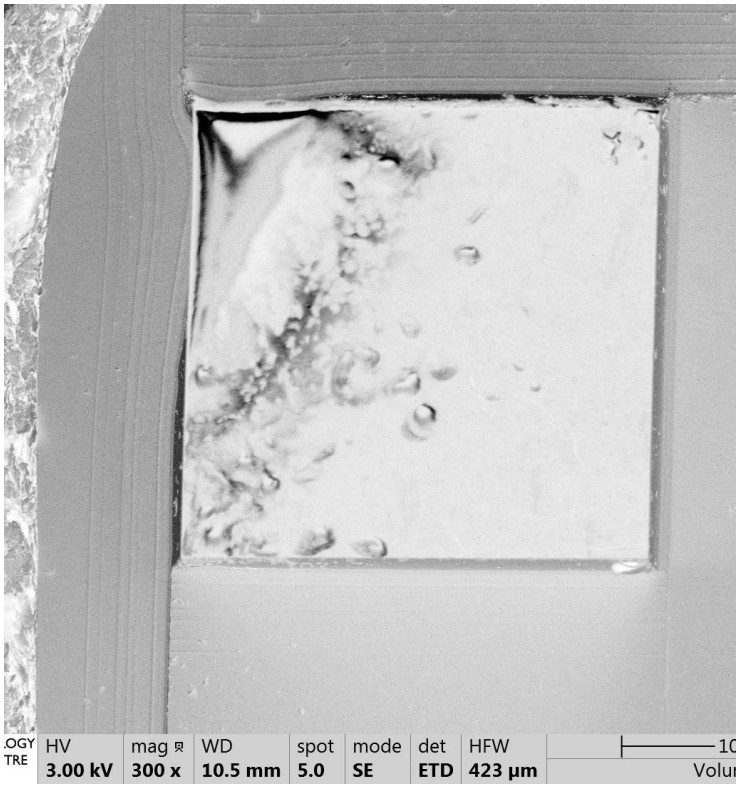
Novymonas, unicellular protist 40µm

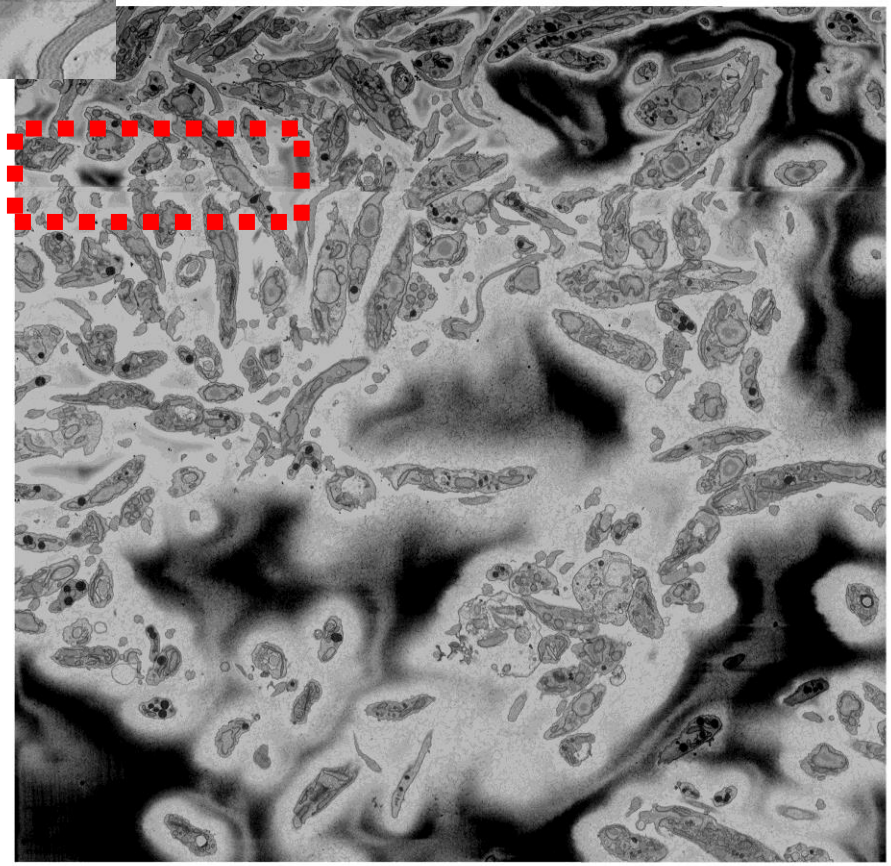
What are the limiting factors for SBEM? – A) Charging

What does it look like?

Solutions:
Lower the dose (not always possible)
Variable pressure
Sample prep (more stain)



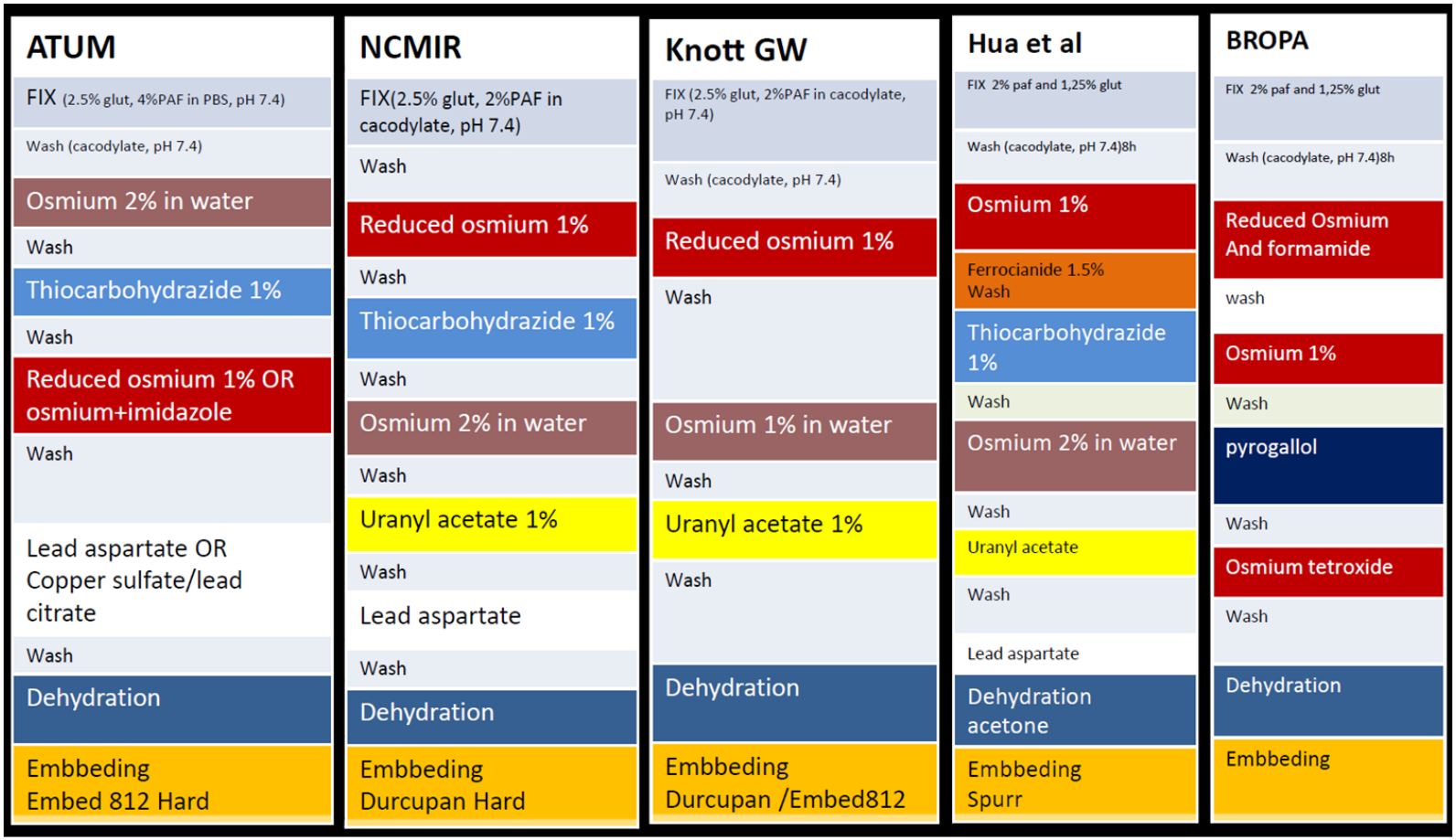




What are the limiting factors for SBEM? – A) Charging

solution:

1) Making sample more conductive – STAINING

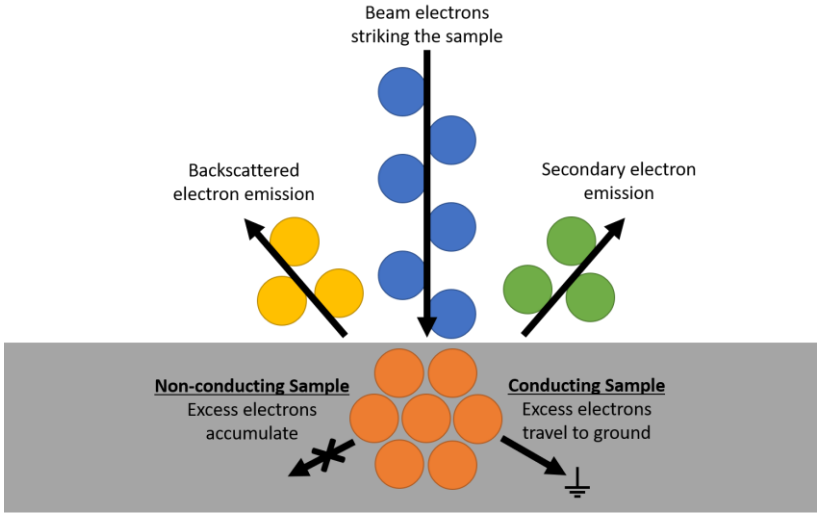


Standard TEM prep: Osmium + poststaining Uranyl acetate and lead citrate

Modified from Christel Genoud

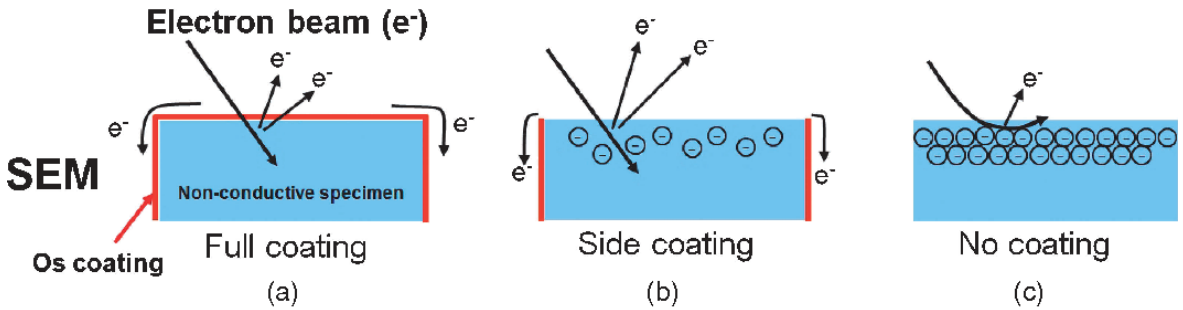
What are the limiting factors for SBEM? – A) Charging

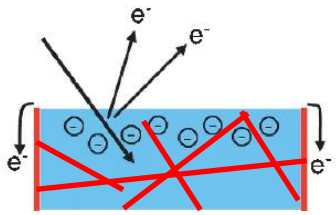
How does it work? (1) - Staining)



https://www.nanoscience.com/applications/materials-science/reduce-charging-in-sem-using-low-voltage-imaging/

Filling (mainly) membranes with heavy atoms make them conductive so the charge can dissipate





Membrane staining
d)

https://www.semanticscholar.org/paper/Charging-Effects-on-SEM%2FSIM-Contrast-of-System-in-Kim-Akase/7c241ea00bea131799b232509449a2d4eb3b4206/figure/1

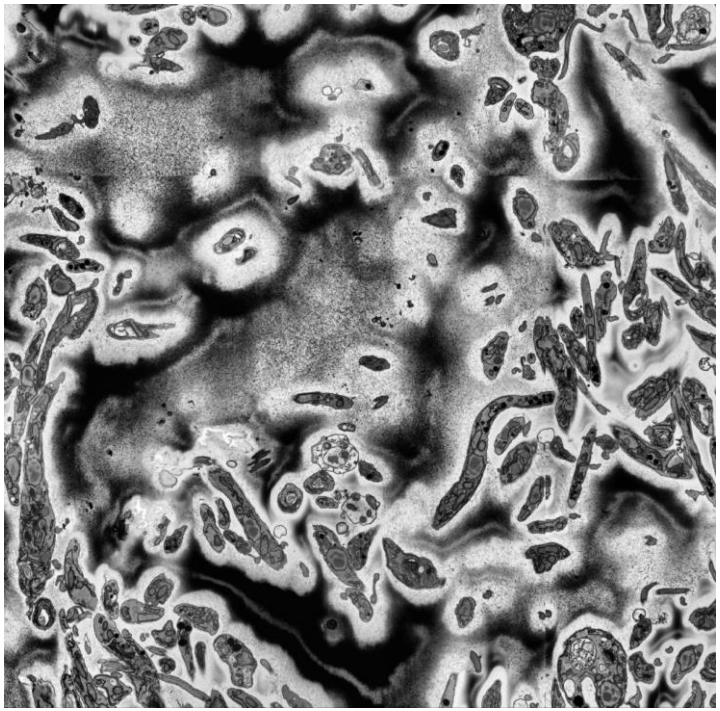
What are the limiting factors for SBEM? – A) Charging

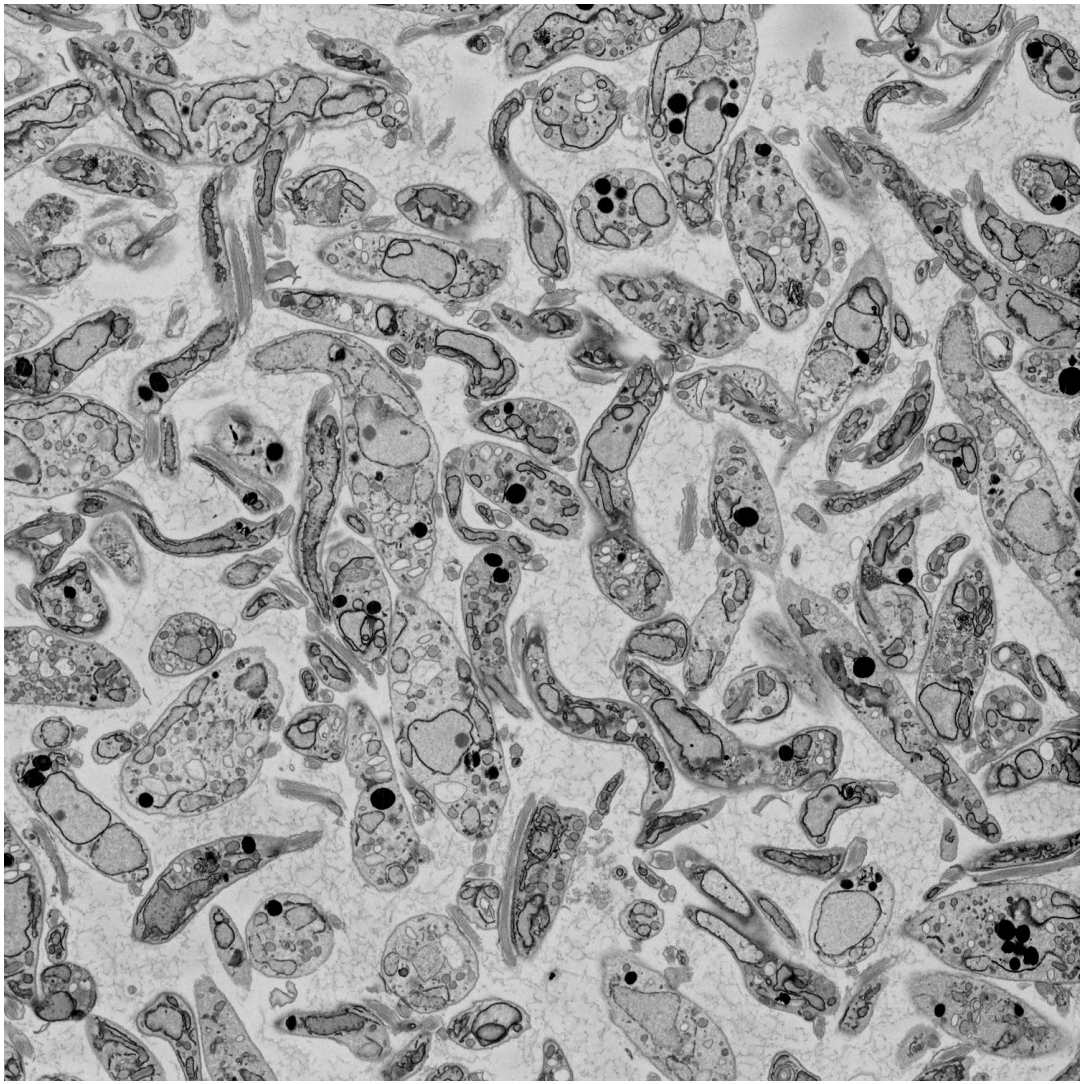
solution:

2) Variable pressure (low vacuum mode)

Cons:

- lower resolution
- more energy needed



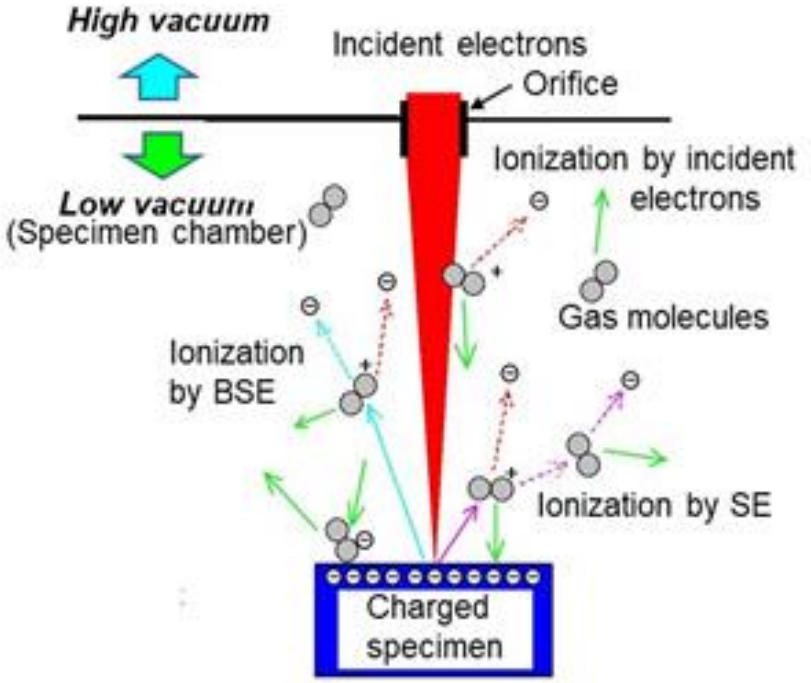


What are the limiting factors for SBEM? – A) Charging

How does it work? (2) – Variable pressure)

Gas released into the SEM chamber (water, nitrogen) gets ionised by the electron beam. The positively charged Ions remove the negative charge from the sample. Still, extra molecules result in the scattering of the electrons inside the chamber, leading to a lower resolution and less signal.

https://www.stinstruments.com/materials-science/overcome-charge-up-effects-in-scanning-electron-microscopes-sems/



https://www.jeol.co.jp/en/words/semterms/search_result.html

What are the limiting factors for SBEM? – A) Charging

solution:

2) Variable pressure (low vacuum mode)

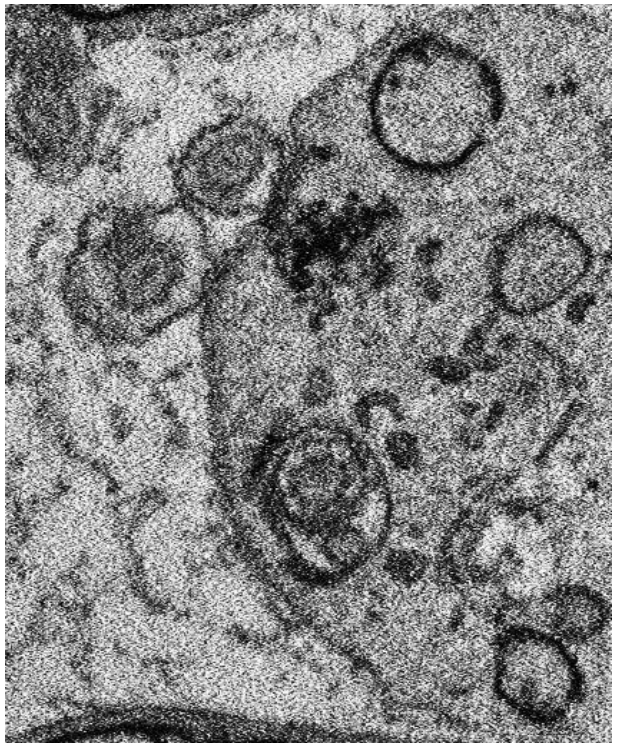
Cons:

lower resolution

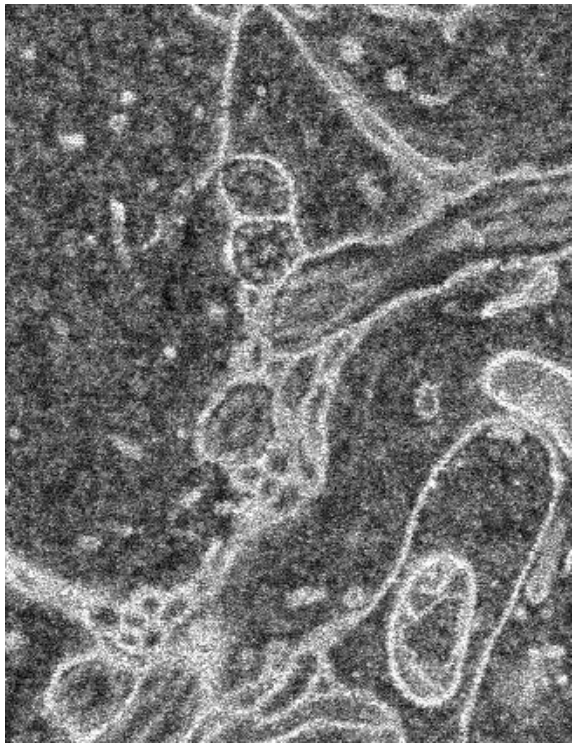
more energy needed

Comparison of Vacuum and methodology settings on the resolution

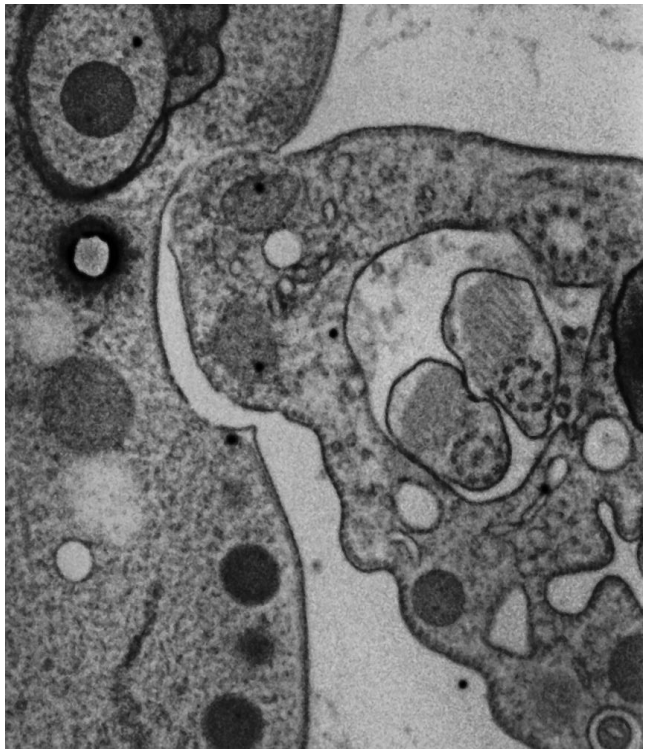
Images SBEM – **Low Vac Mode** (trypanosoma):



Images SBEM – **Hi Vac Mode** (spine cord):



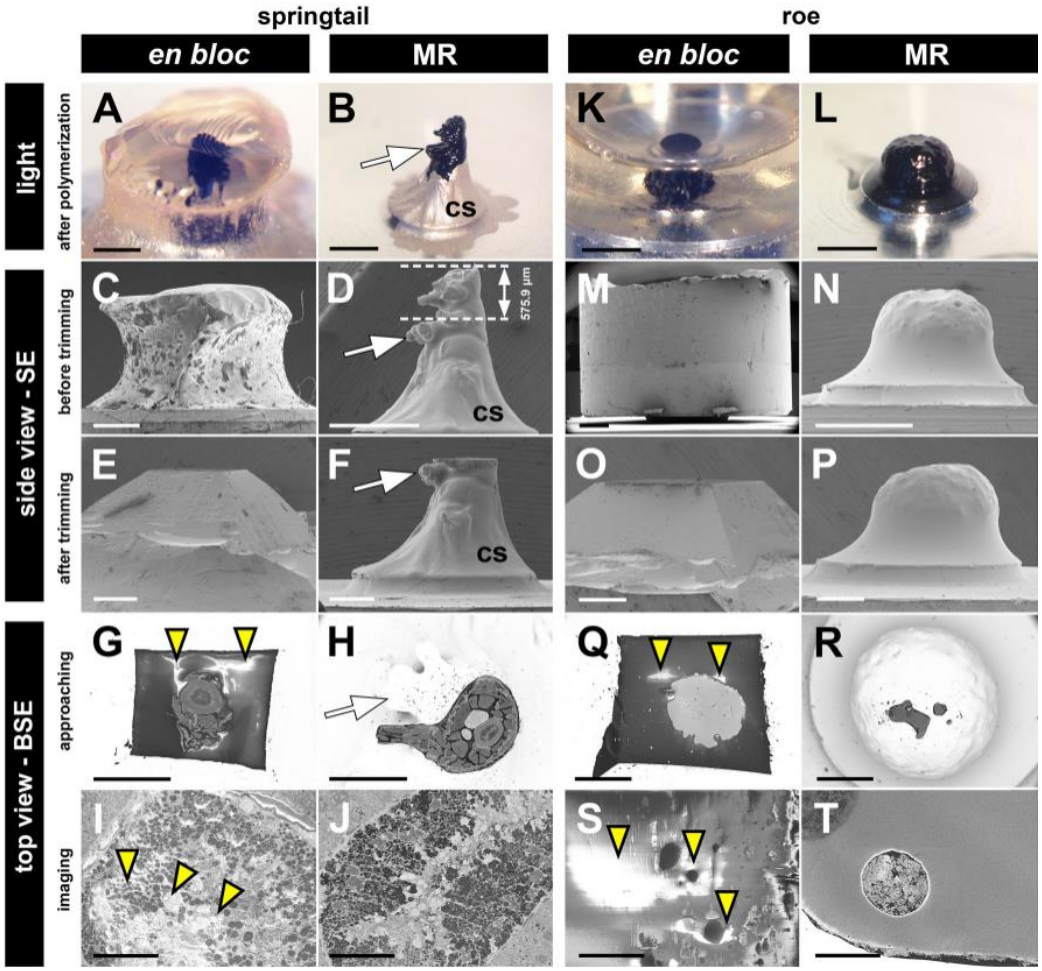
Images Array Tomo – **Hi Vac Mode** (trypanosoma):



What are the limiting factors for SBEM? – A) Charging

How does it work? (3) – Minimal resin)

No (or minimal) resin insulation layer
<u>also helps with the ROI (region of interest) finding</u>



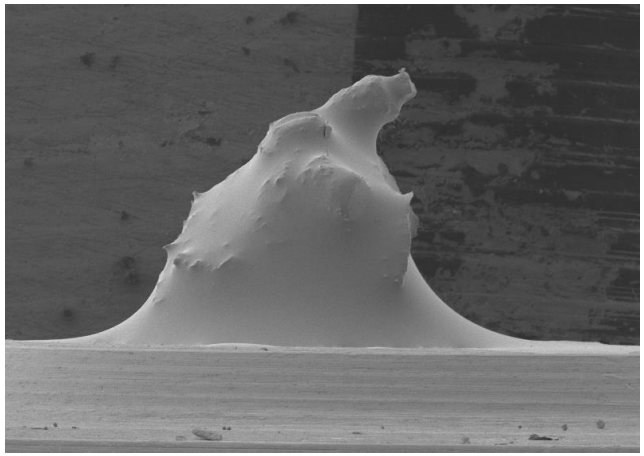
Konopová and Týč *Frontiers in Zoology* (2023) 20:29
https://doi.org/10.1186/s12983-023-00507-x

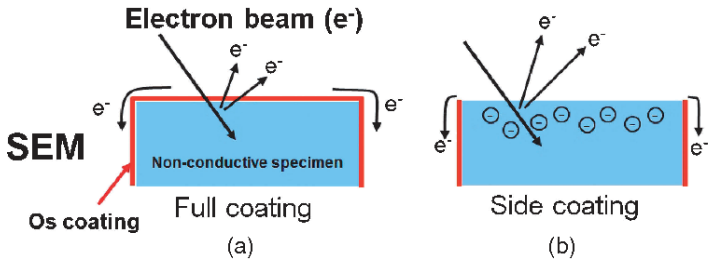
Frontiers in Zoology

METHODOLOGY Open Access

Minimal resin embedding of SBF-SEM samples reduces charging and facilitates finding a surface-linked region of interest

Barbora Konopová[1,2*†] and Jiří Týč[3*†]





Large excretory organs of springtail – Minimal resin, Hi Vac Mode

SBEM strength:

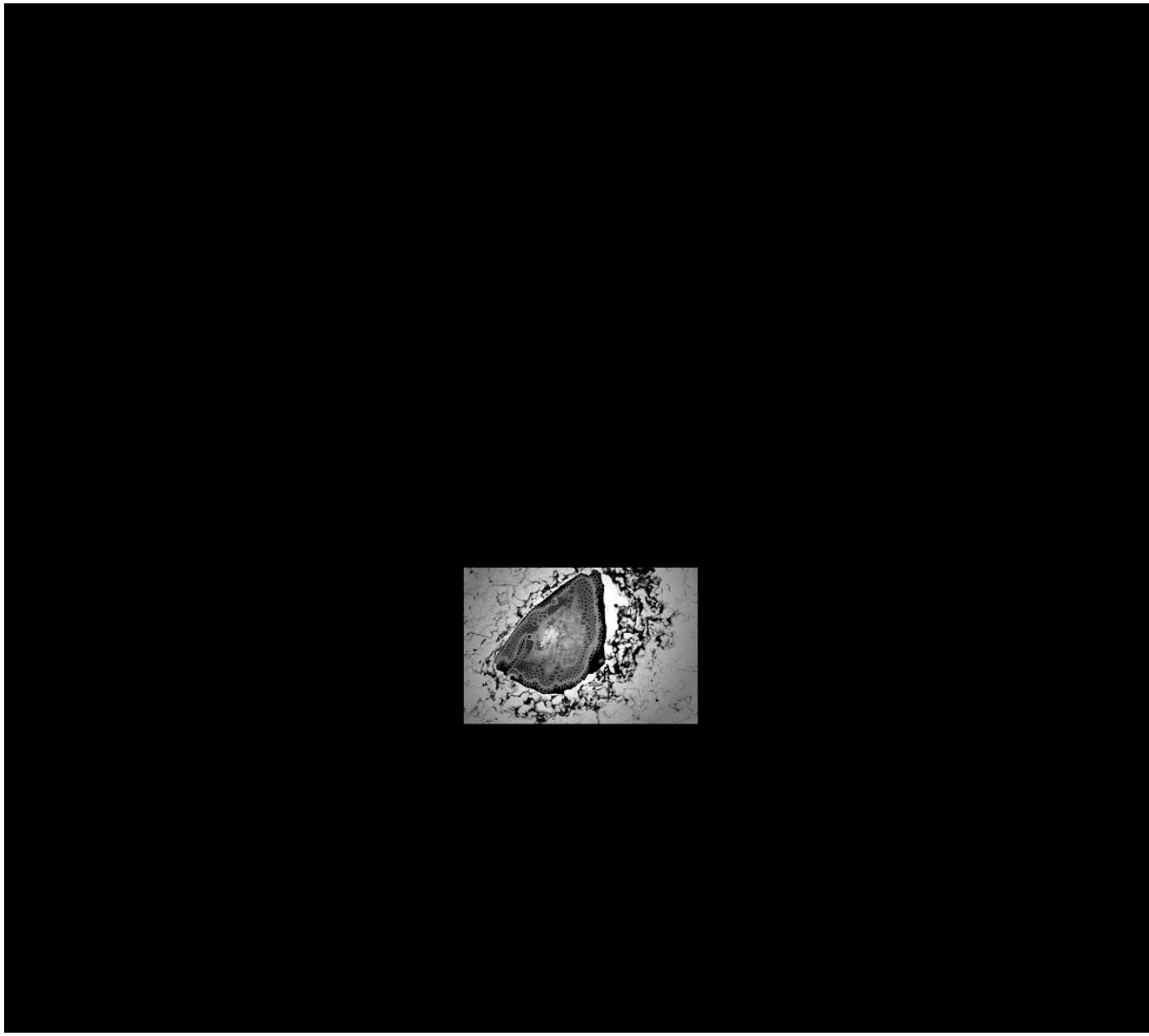
- Larger ROI
- Tiles can be adjusted during the run
  - moved around
  - expanded
  - collapsed

To note:

Acquisition time
Amount of data

Video shows approx every 5th slice (250 nm)

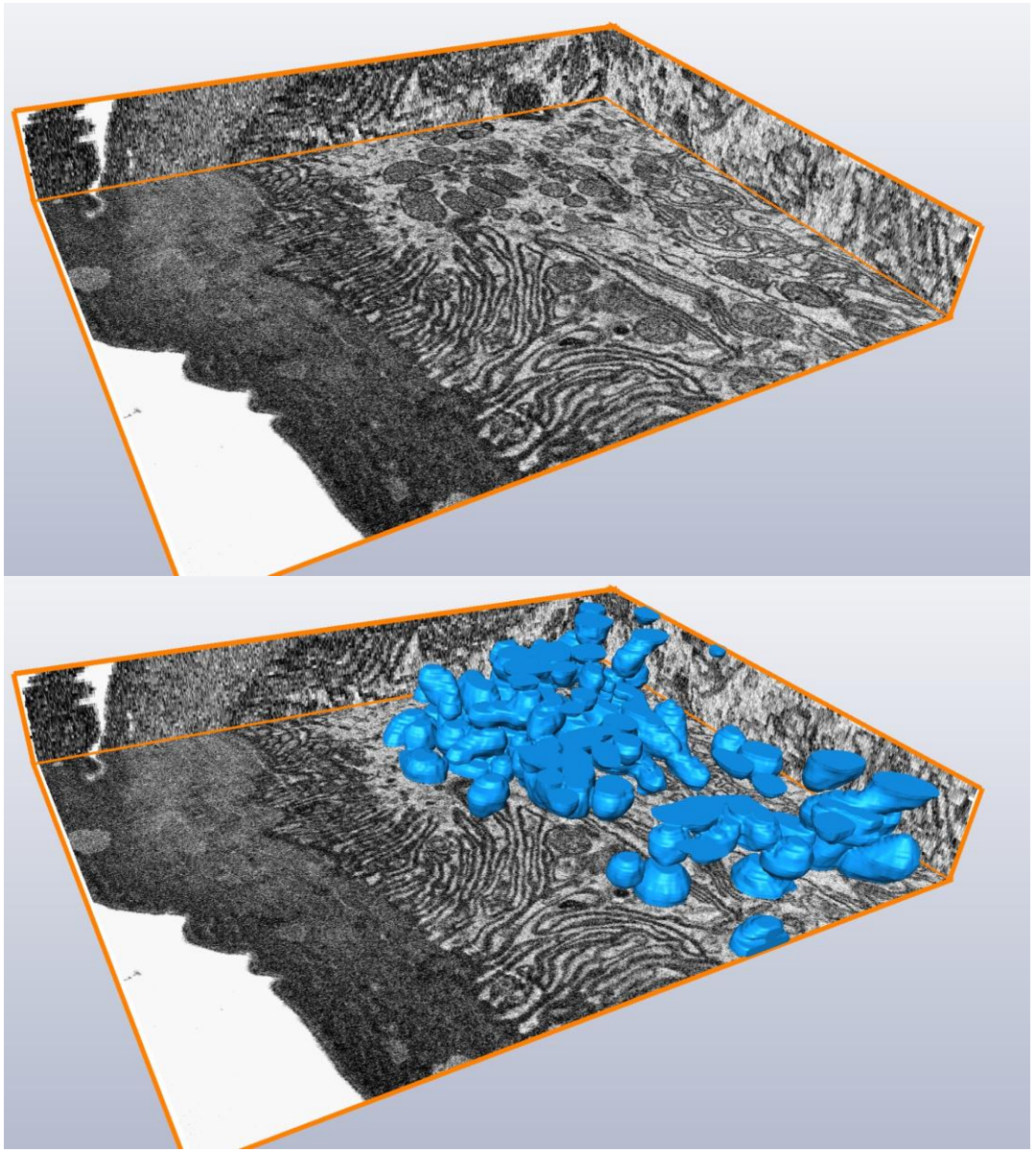
2 jumps to speed it up



Take home message: <u>Imaged region can be freely adjusted during the run</u>

Large excretory organs of springtail

Run parametres	value
Pixel size	8 nm
Slice thickness	50 nm (part 25 nm)
Imaged area	170 x 170 µm
Total slices	2500
Total volume	0,002 mm^3
Total acquition time	**Approx. 1 month**
Amount of Data acquired	**8,2 Tb**



Take home messages:

Large volumes are time and data storage demanding

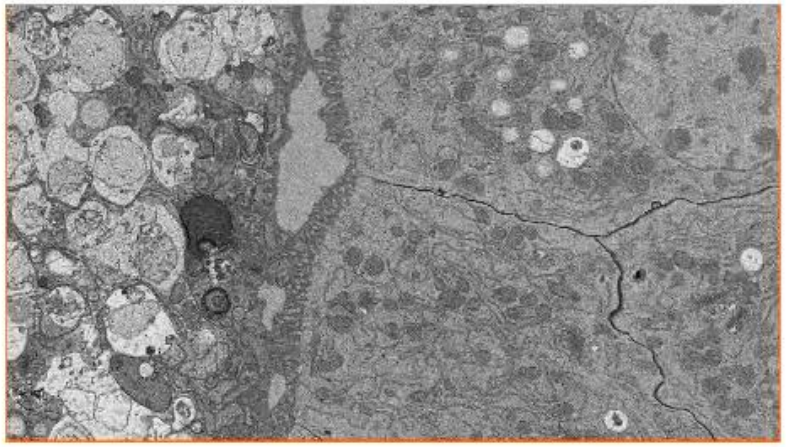
Resolution is enough to distinguish mitochondrial cristae and other fine membranous details

What are the limiting factors for SBEM? – A) Charging

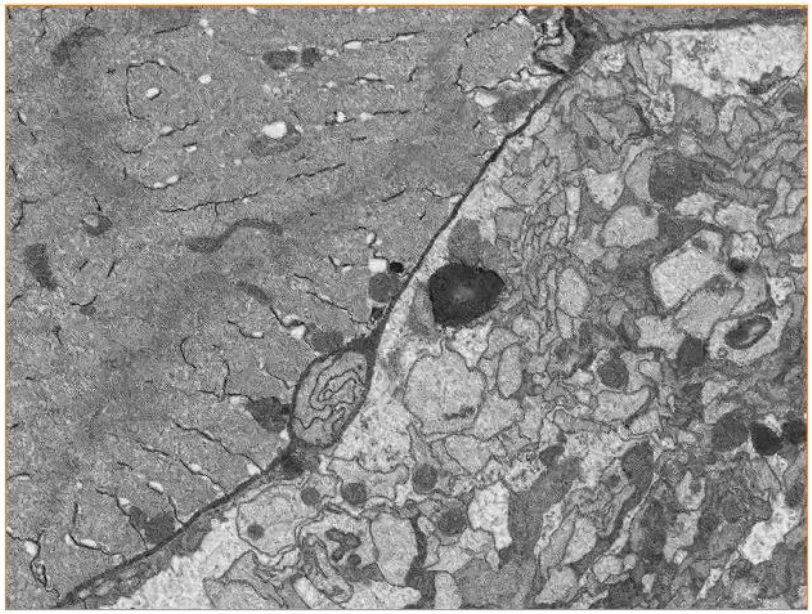
solution:

3) Minimal resin – no charging, more signal, lower electron dose – smaller voxel size achievable

Gut tissue
Voxel size isometric 10 x 10 x 10 nm



Muscle and adjacent tissue
Voxel size 8 x 8 x 10 nm



Finding the ROI when we could not use the minimal resin for navigation - the digestive tract of tick

Possible problem:

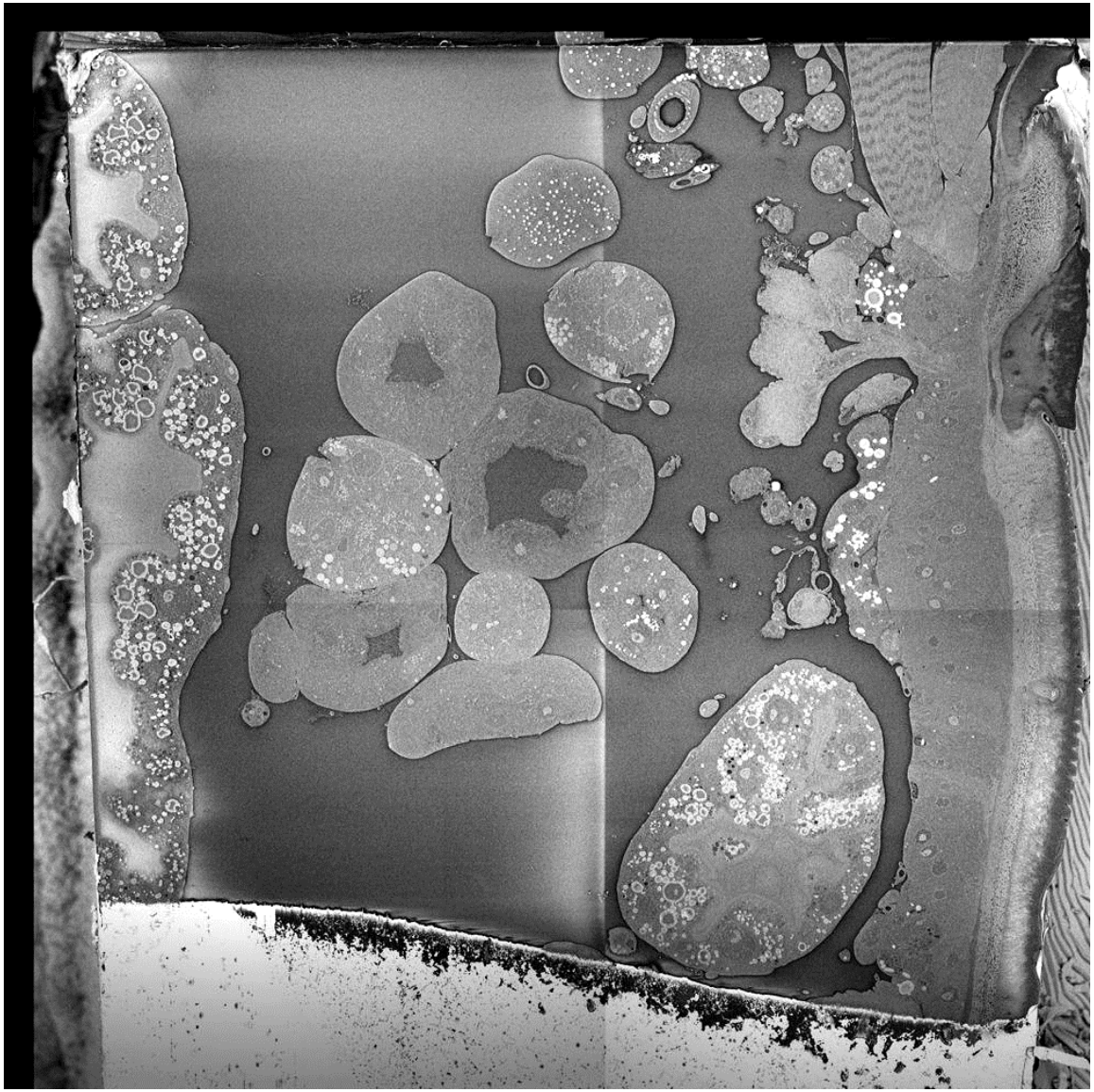
Finding the digestive tract

Solution:

Using SBEM as an expensive microtome

Video shows approx every 7th slice (490 nm)

Run parametres	value
Pixel size	25 nm
Slice thickness	490 nm
Imaged area	460 x 460 µm
Total slices	460
Total volume	0,047 8mm^3
Total acquition time	Approx 2 weeks



Finding the spirochetes in the digestive tract of tick

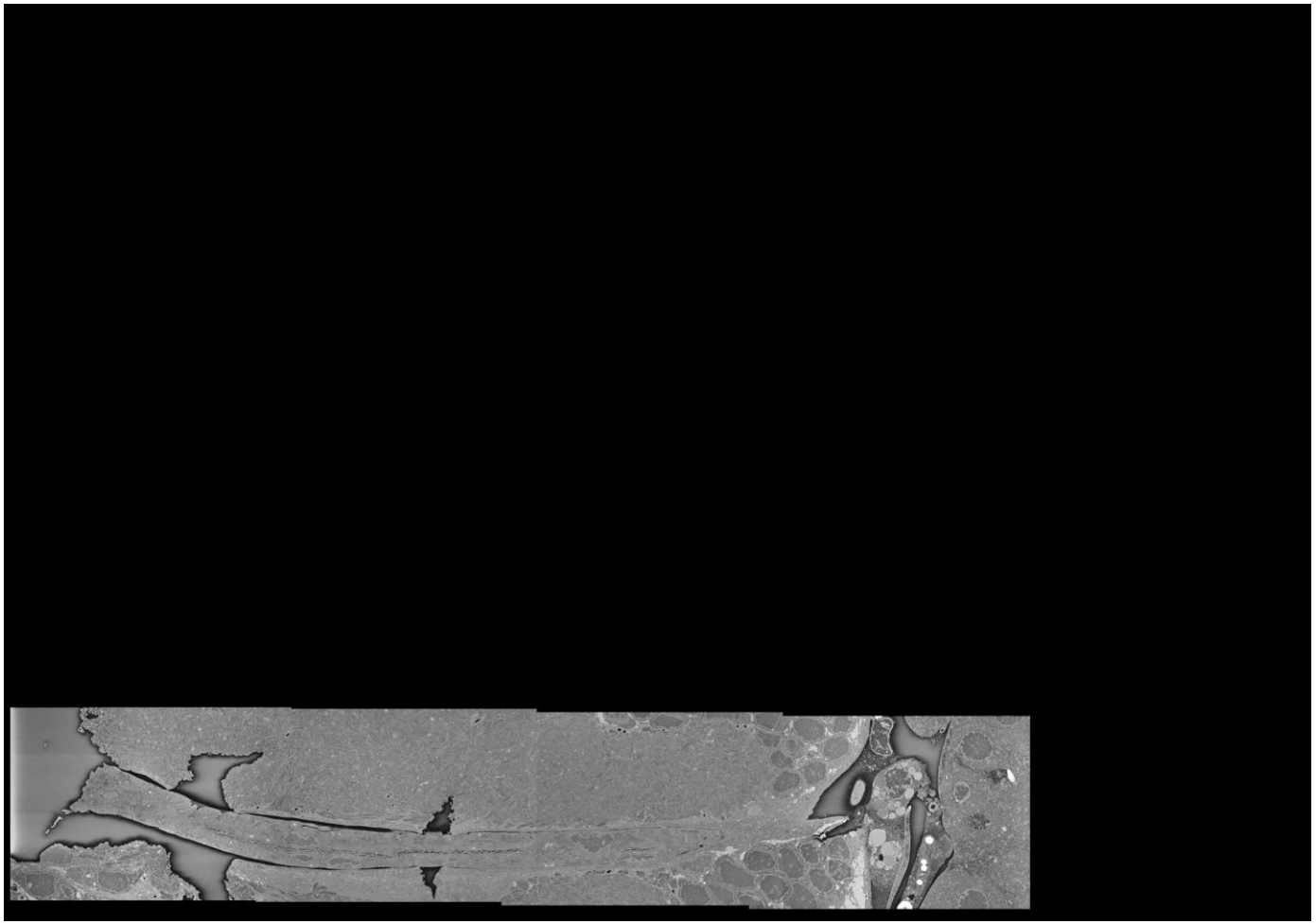
To note:

Acquisition time
Amount of data

SBEM strength:

- Larger ROI
- Tiles can be adjusted during the run
  - ➢ moved around
  - ➢ expanded
  - ➢ collapsed

Run parametres	value
Pixel size	10 nm
Slice thickness	70 nm
Total slices	1131



Take home messages:

<u>- Only the specified interesting region will be acquired in high resolution</u>

<u>- Multiple ROIs can be acquired simultaniously</u>

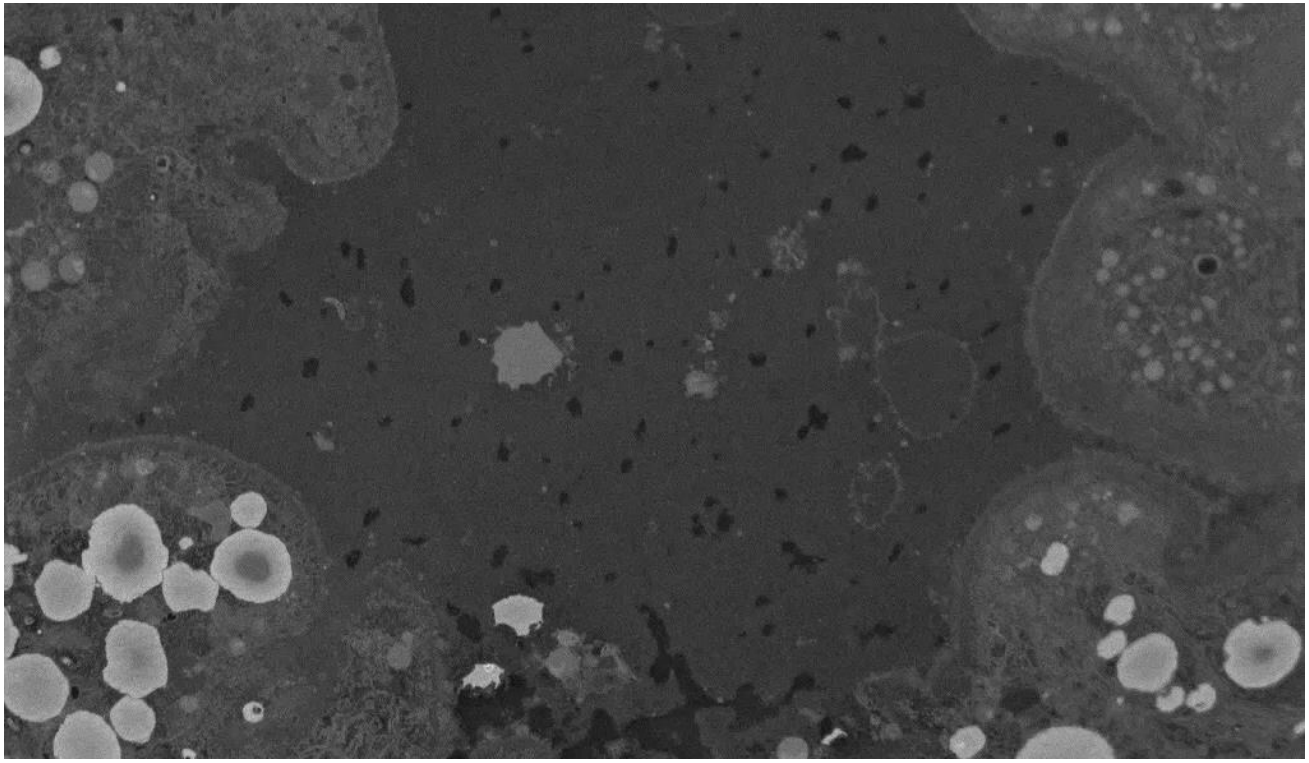
Finding the spirochetes in the digestive tract of tick

To note:

Acquisition time
Amount of data

SBEM strength:

- Larger ROI
- Tiles can be adjusted during the run
  - ➢ moved around
  - ➢ expanded
  - ➢ collapsed



Run parametres	value
Pixel size	10 nm
Slice thickness	70 nm

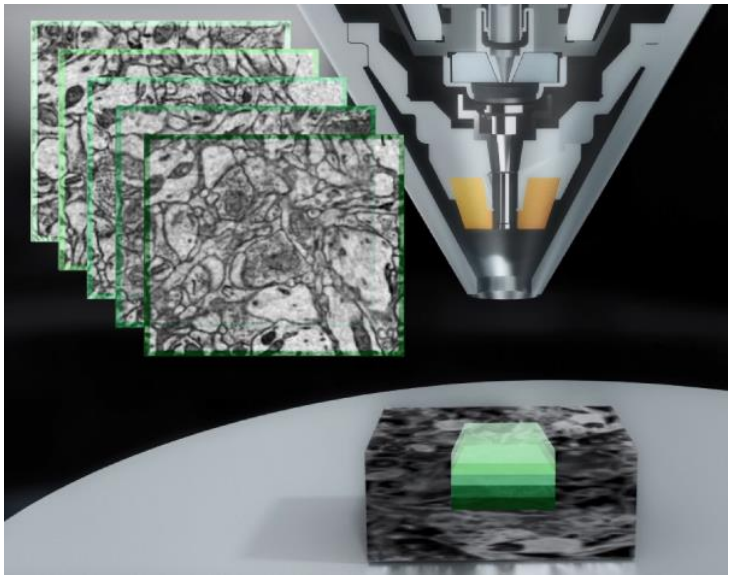
Take home message:

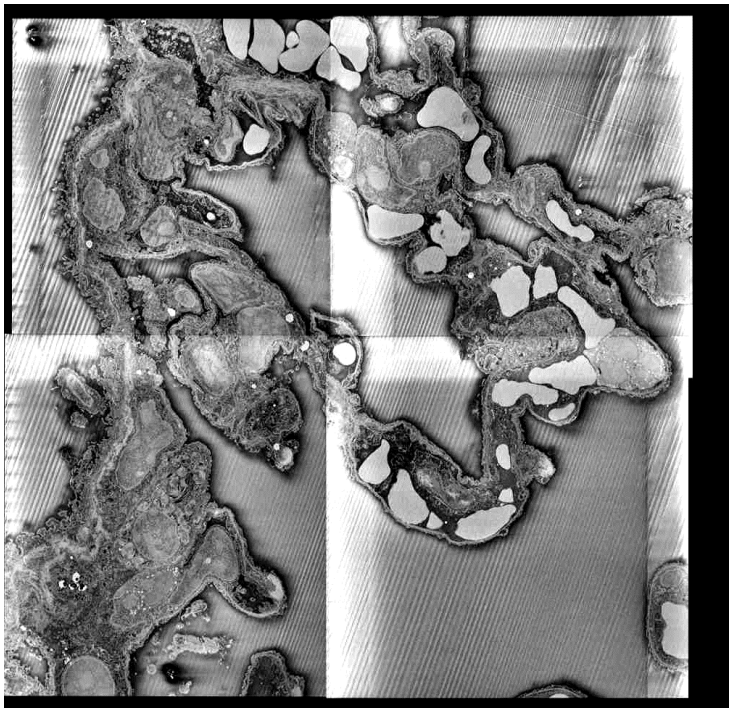
<u>Resolution is enough to distinguish bacterial cells in larger fields of view</u>

What is the limiting factor?

B) Electron dose = physical properties of the resin/s

- Beam damage (and heat damage) to the sample has a limit of 15-19e/nm2 (for optical sectioning it means total dose)
- With really good samples I can achieve that. With many samples, I have to go over, usually up to 40-60 e/nm2. Mostly it is still OK for cutting thicker sections like 100nm.
- Higher dose means more information, less noise. Nicer image. (especially in low vac mode)
- Stitching actually locally increases the dose (overlapping regions are scanned twice)
- **Too high dose results in shrinkage of the sample, and some irregularities in cutting. Some sections are recorded twice (or more) or there is a bigger jump.**



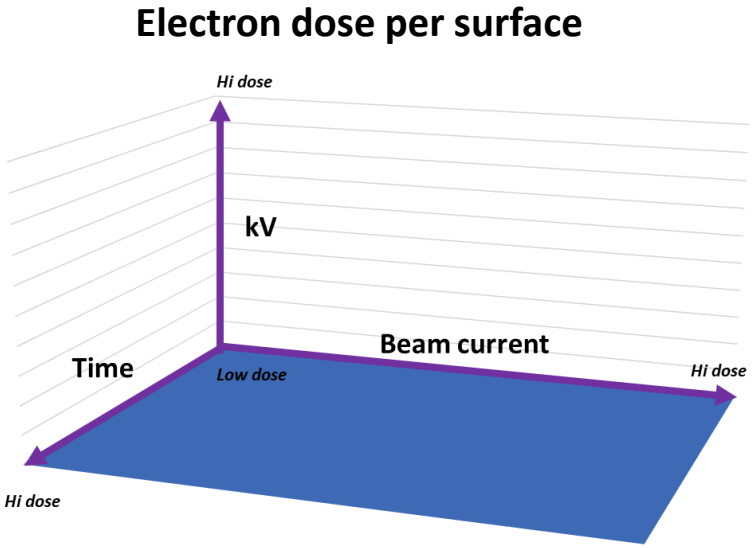


What are the limiting factors for SBEM? – B) Electron dose

solution:

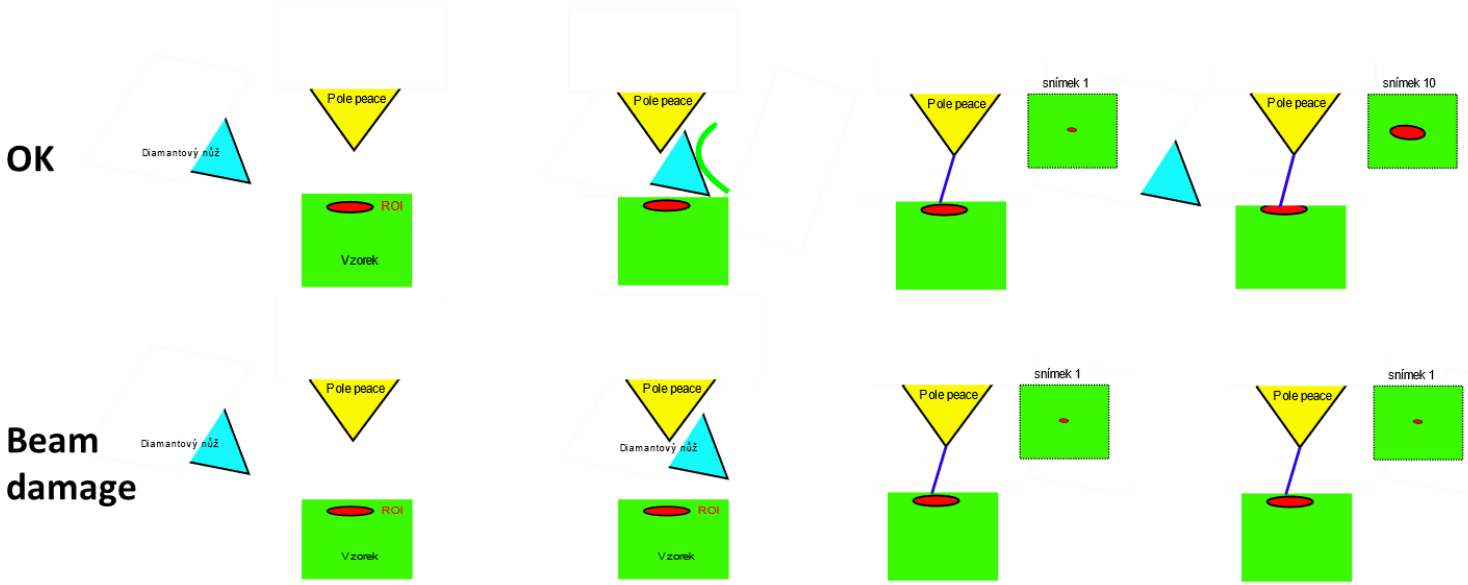
2) Lower the dose

- Shorter dwell time
- Bigger pixels
- Smaller kV, current



Problem:

Lower does mean less signal, so poorer resolution, less contrast…



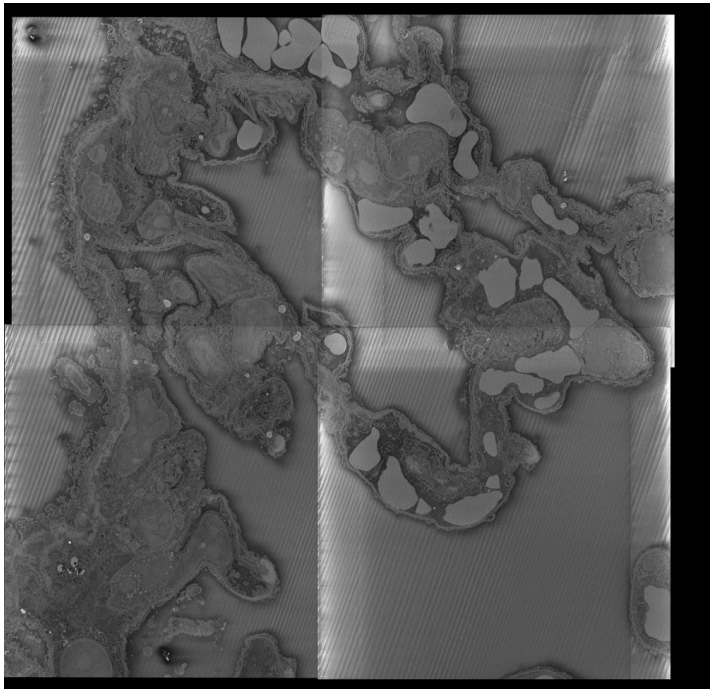
What is the limiting factor?
C) Contrast

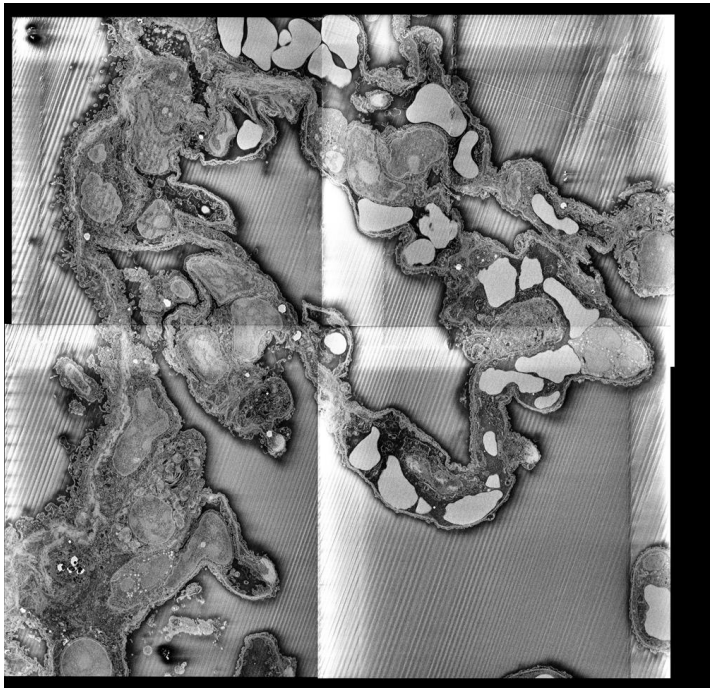
- increasing contrast would decrease the dwell time, resulting in a lower electron dose. Higher contrast also brings more information for stitching, alignment…

- There is some progress with staining protocols, **is there any other way to increase the contrast? – resins, instrumentation?**

- - So far increase of contrast using staining protocols works at least partly as increasing extraction of material, therefore resulting in a decrease in details visible.

ATUM	NCMIR	Knott GW	Hua et al	BROPA
FIX (2.5% glut, 4%PAF in PBS, pH 7.4)	FIX(2.5% glut, 2%PAF in cacodylate, pH 7.4)	FIX (2.5% glut, 2%PAF in cacodylate, pH 7.4)	FIX 2% paf and 1,25% glut	FIX 2% paf and 1,25% glut
Wash (cacodylate, pH 7.4)	Wash	Wash (cacodylate, pH 7.4)	Wash (cacodylate, pH 7.4)8h	Wash (cacodylate, pH 7.4)8h
Osmium 2% in water	Reduced osmium 1%	Reduced osmium 1%	Osmium 1%	Reduced Osmium And formamide
Wash	Wash	Wash	Ferrocianide 1.5% Wash	wash
Thiocarbohydrazide 1%	Thiocarbohydrazide 1%	Osmium 1% in water	Thiocarbohydrazide 1%	Osmium 1%
Wash	Wash	Wash	Wash	Wash
Reduced osmium 1% OR osmium+imidazole	Osmium 2% in water	Uranyl acetate 1%	Osmium 2% in water	pyrogallol
Wash	Wash	Wash	Wash	Wash
Lead aspartate OR Copper sulfate/lead citrate	Uranyl acetate 1%	Dehydration	Uranyl acetate	Osmium tetroxide
Wash	Wash	Embbeding Durcupan /Embed812	Wash	Wash
Dehydration	Lead aspartate		Lead aspartate	Dehydration
Embbeding Embed 812 Hard	Dehydration		Dehydration acetone	Embbeding
	Embbeding Durcupan Hard		Embbeding Spurr	

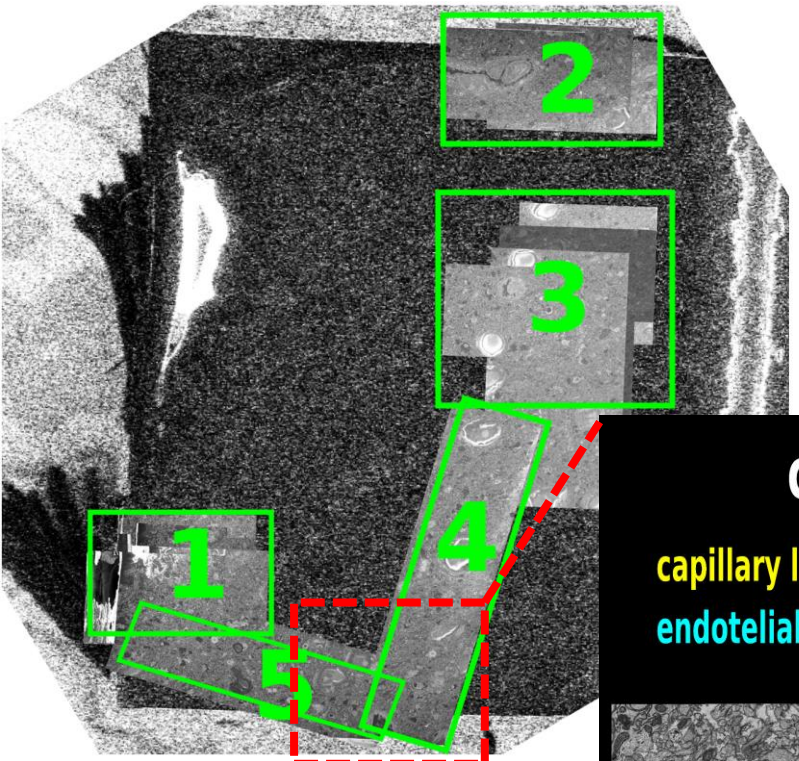


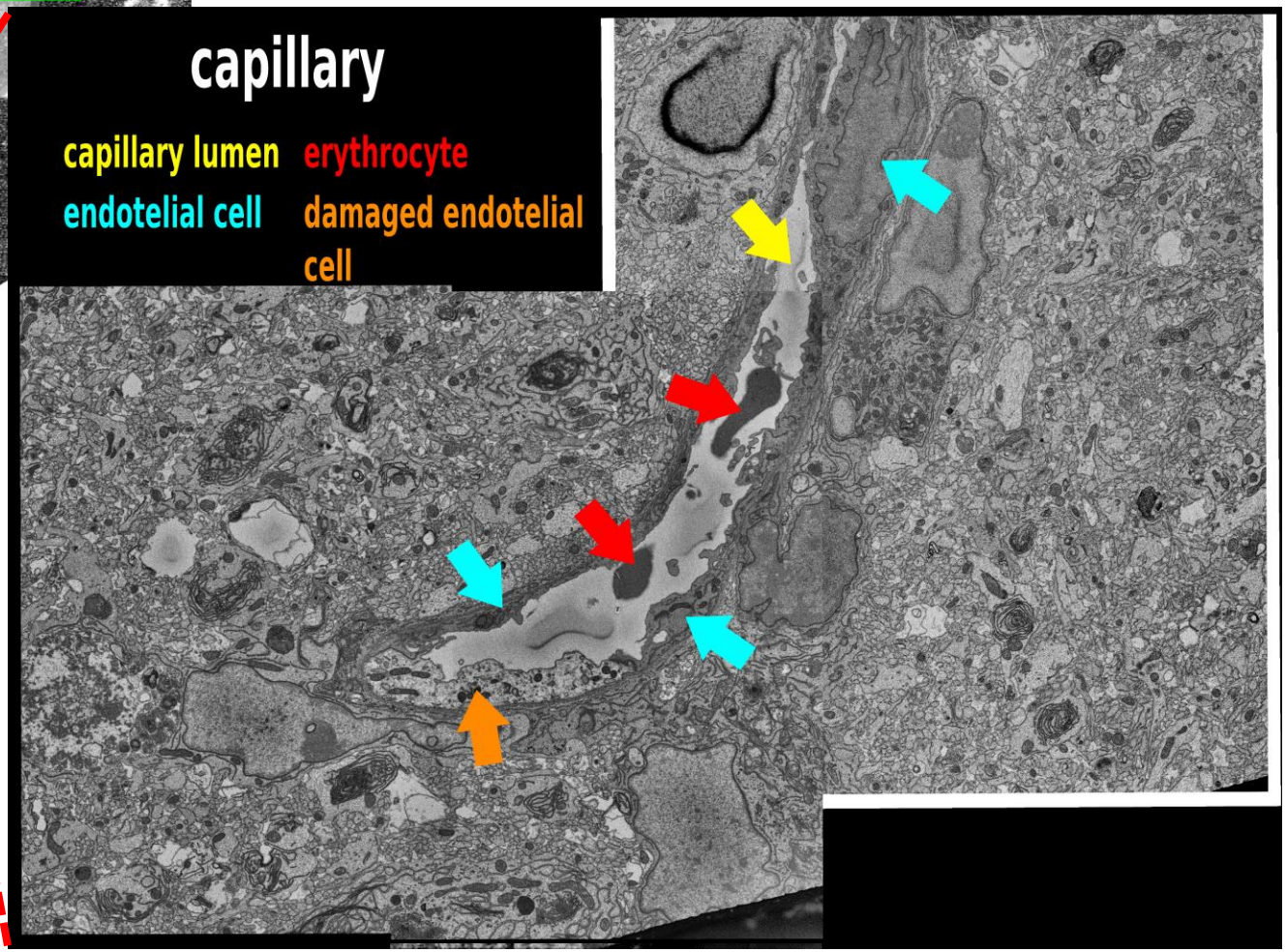


SBF-SEM

What can be used for?

SBEM dataset can be quite large, and multiple ROIs can be acquired simultaneously





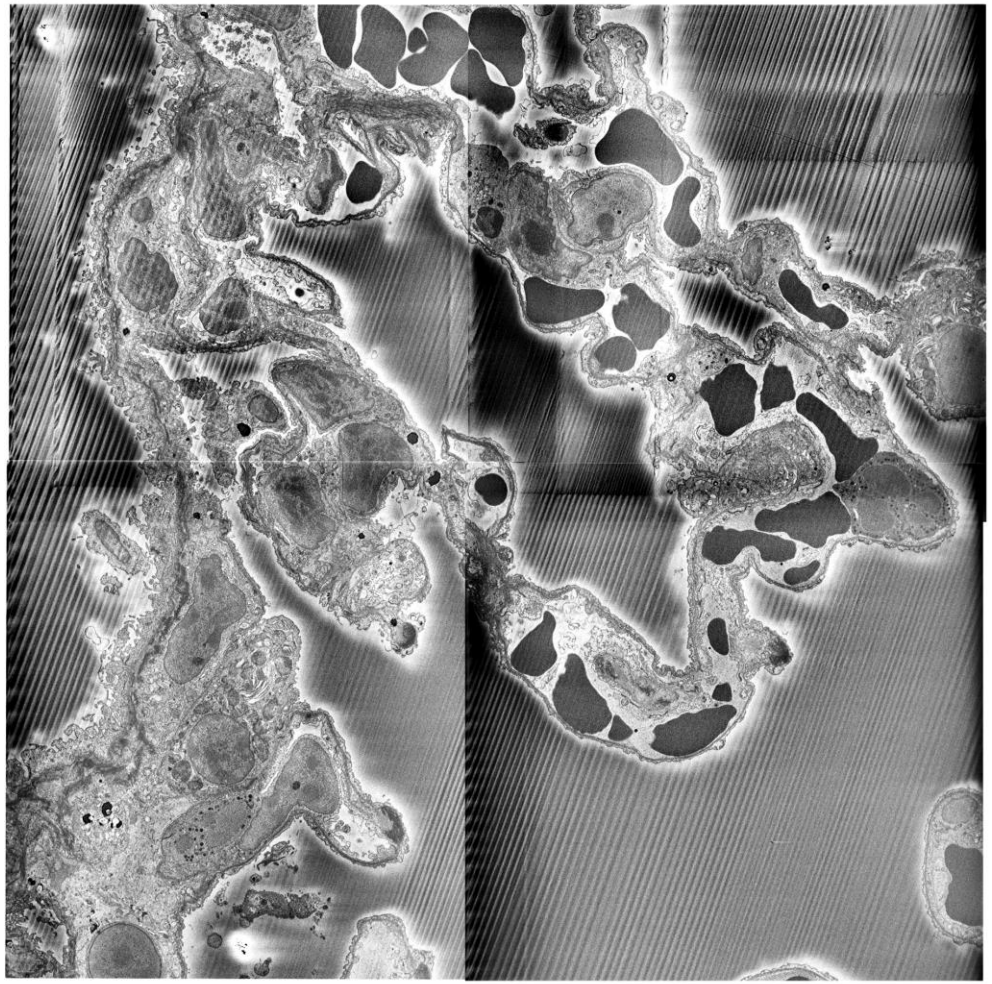
Voxel dimensions 4x4x50nm

Unpublished data, collaboration with Martin Palus

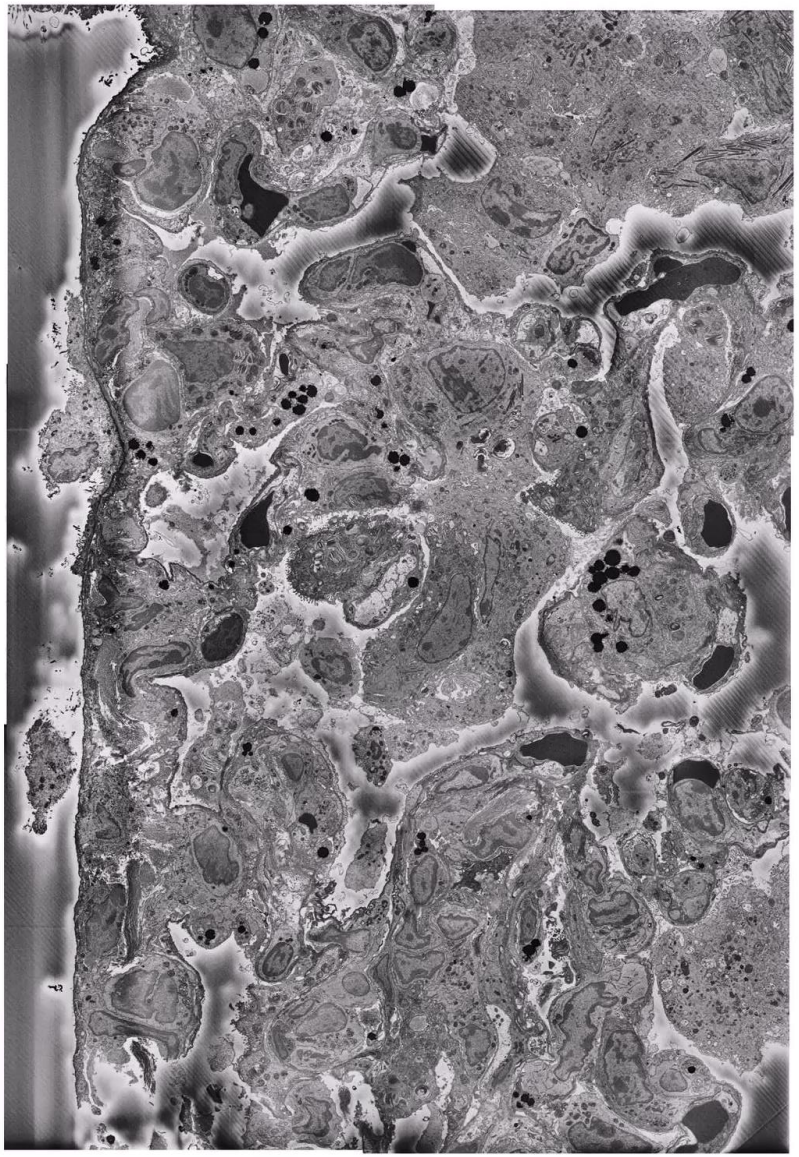
SARS-CoV-2 - Quantification

We can image the lung tissue with sufficient details in SBFSEM.

Healthy mouse lungs



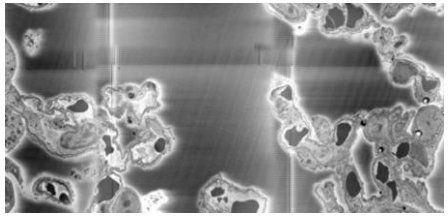
Mouse lungs After Sars-CoV-2 infection
(5 days post-infection)



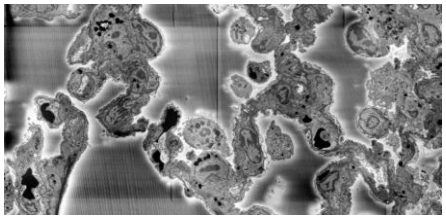
Unpublished data, collaboration with
Martin Palus and Daniel Růžek

QUANTIFICATION - We were able to see changes during the disease progression.

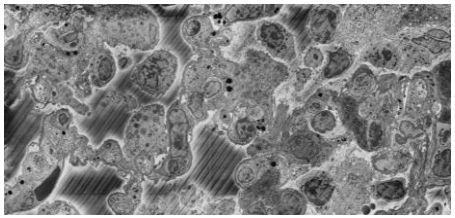
No infection

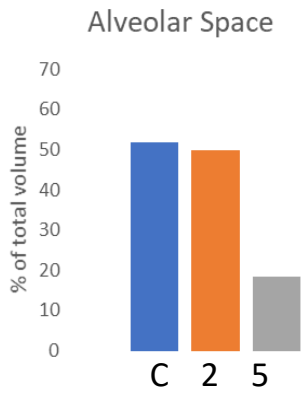


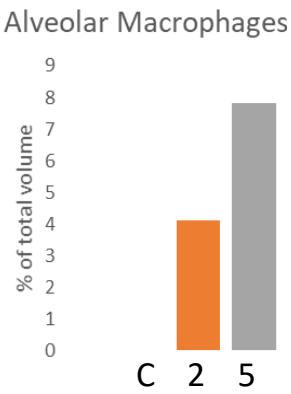
2 DPI

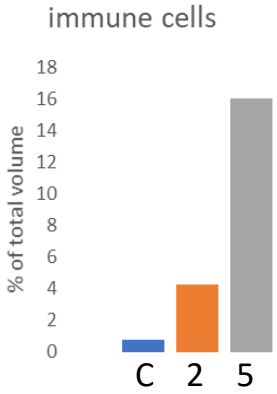


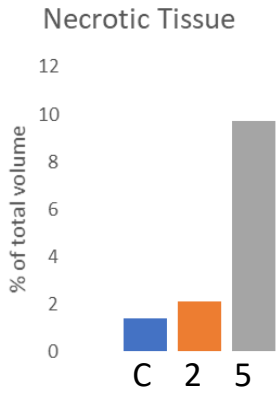
5 DPI

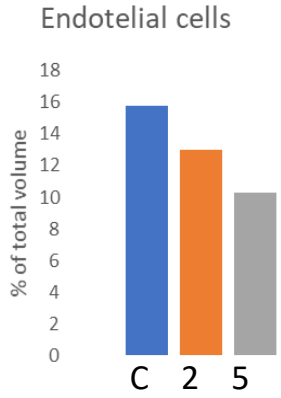


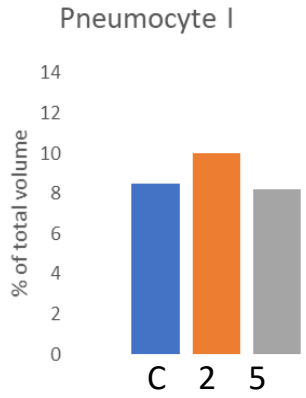


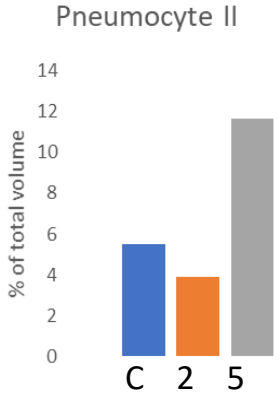


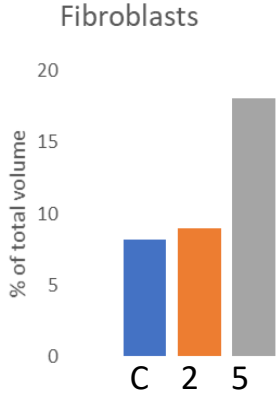










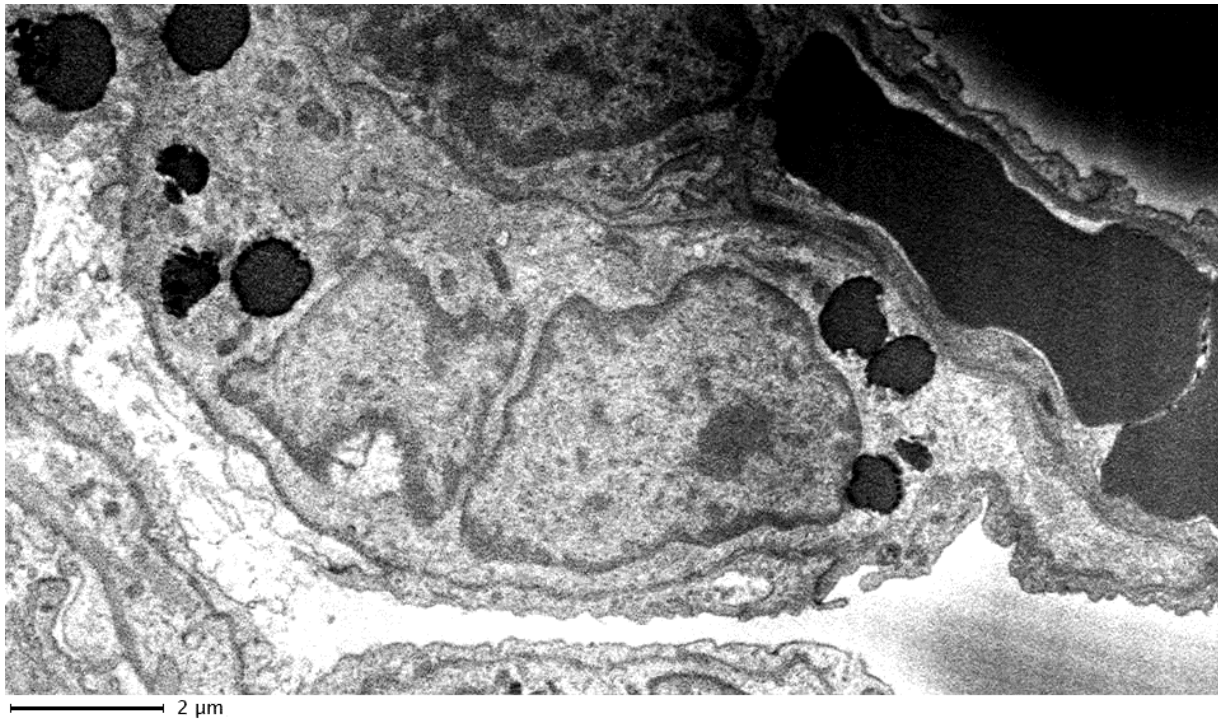


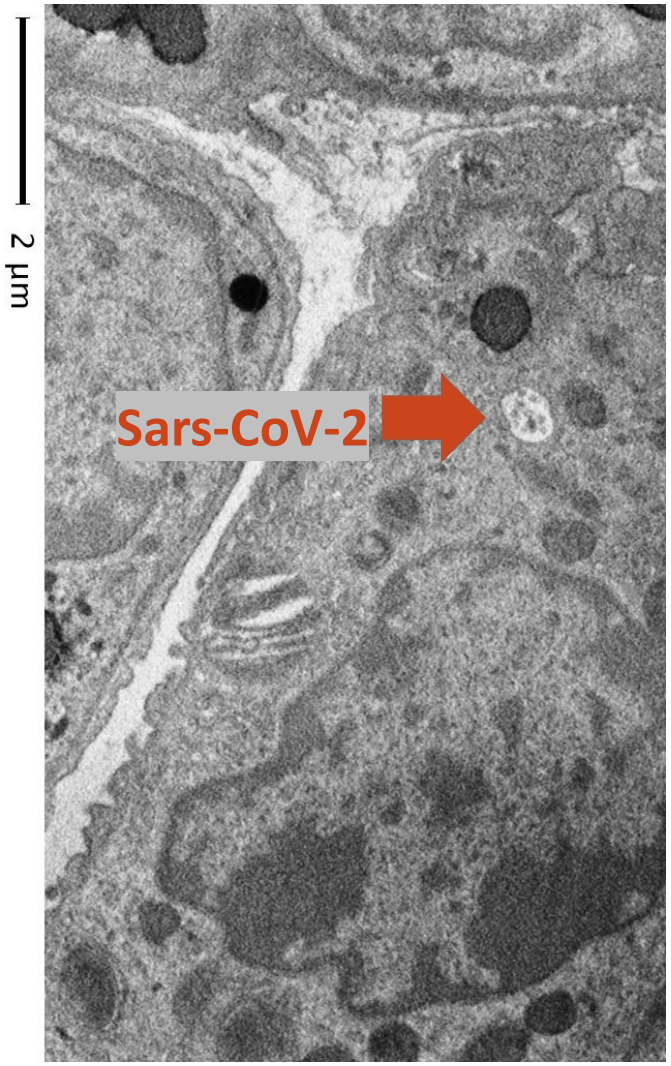
Unpublished data, collaboration with
Martin Palus and Daniel Růžek

We have enough resolution to see the viral particles and we were able to identify the virus.

Pneumocyte I, Pneumocyte II and alveolar macrophages were infected with the Sars-CoV-2 virus. We did not find the infection in other cell types

Sars-CoV-2 infection of Pneumocyte II





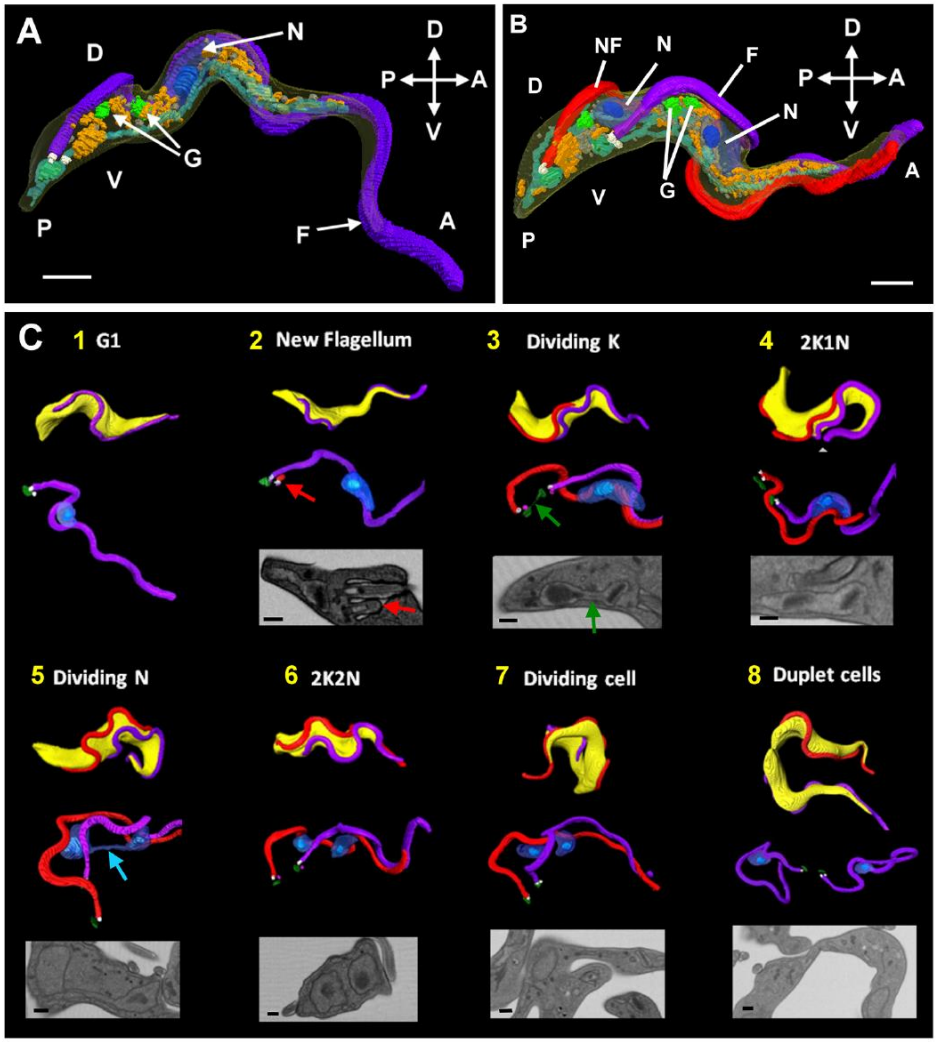
Unpublished data, collaboration with
Martin Palus and Daniel Růžek

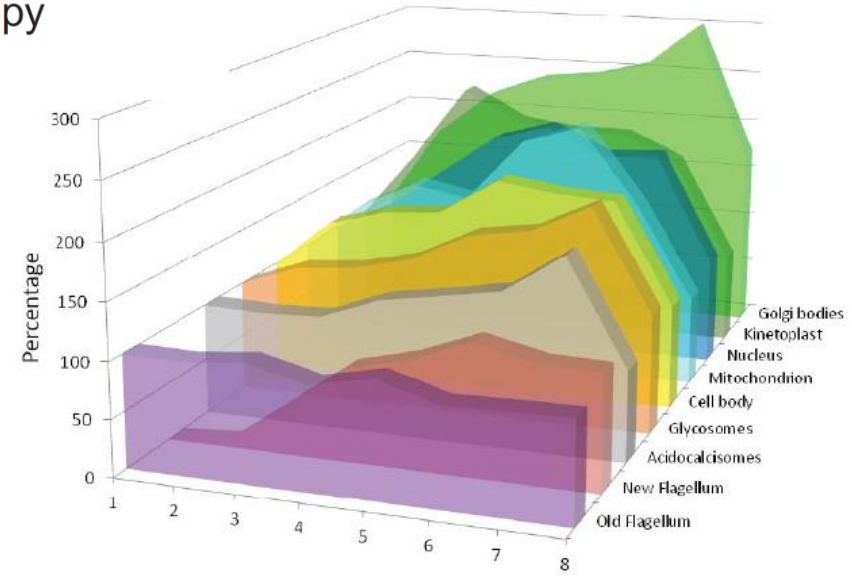
© 2017. Published by The Company of Biologists Ltd | Journal of Cell Science (2017) 130, 637-647 doi:10.1242/jcs.198887

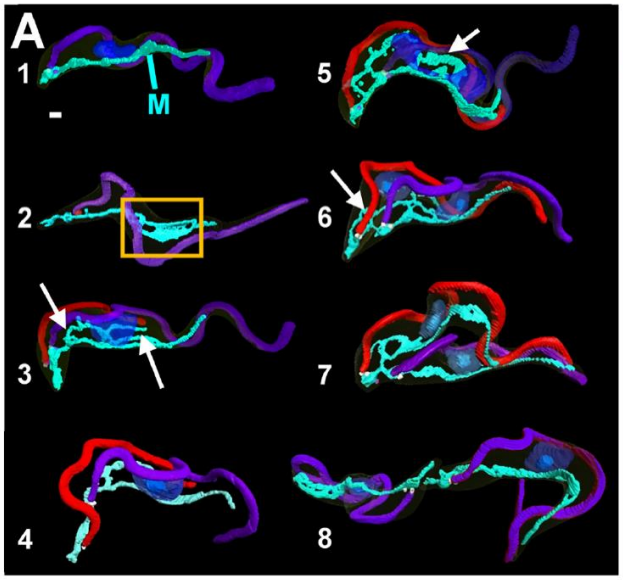
TOOLS AND TECHNIQUES

Patterns of organelle ontogeny through a cell cycle revealed by whole-cell reconstructions using 3D electron microscopy

Louise Hughes[1], Samantha Borrett[1], Katie Towers[1], Tobias Starborg[2] and Sue Vaughan[1,*]







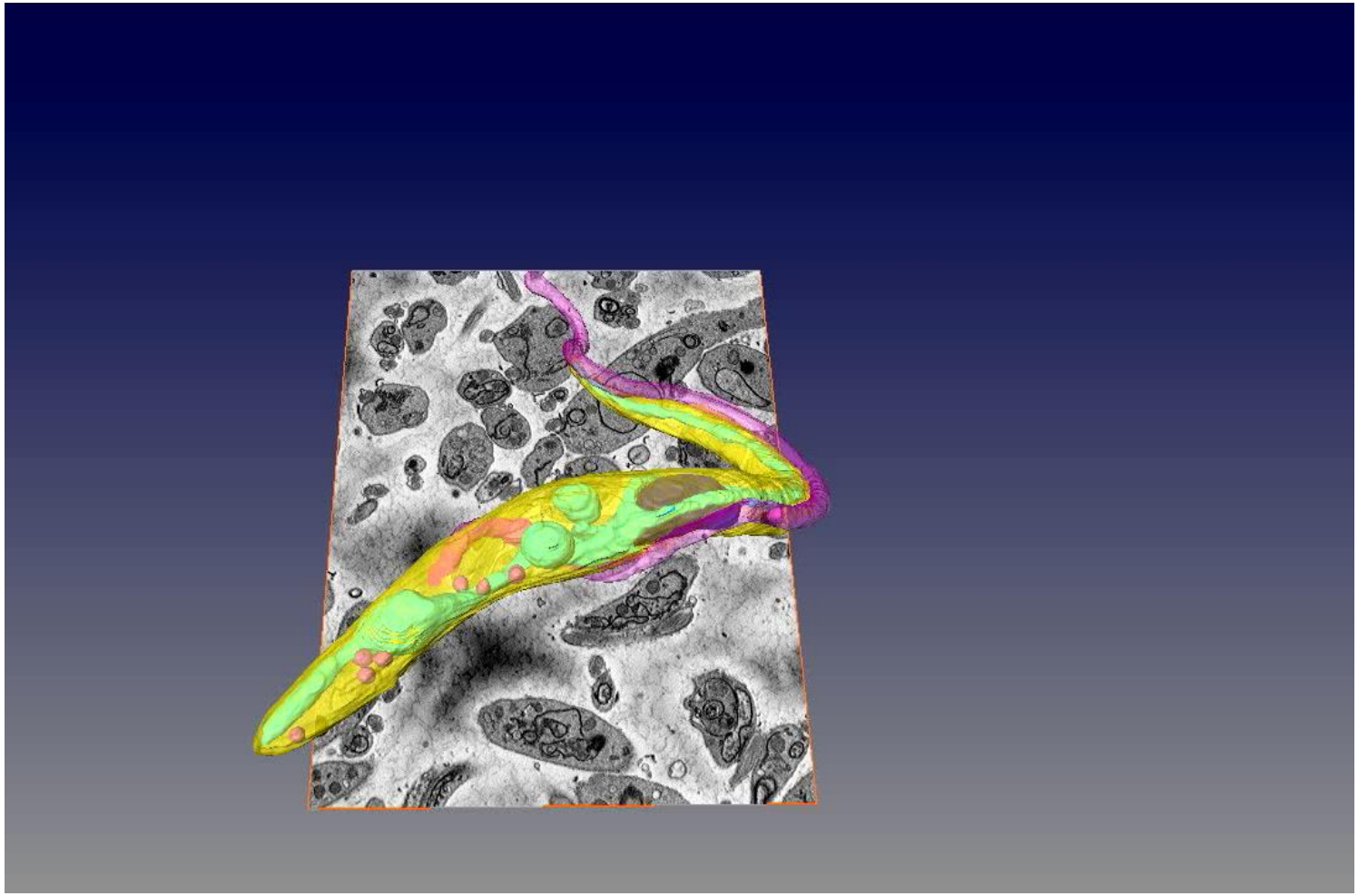
JOURNAL ARTICLE

A Novel Group of Dynamin-Related Proteins Shared by Eukaryotes and Giant Viruses Is Able to Remodel Mitochondria From Within the Matrix

Shaghayegh Sheikh, Tomáš Pánek, Ondřej Gahura, Jiří Týč, Kristína Záhonová, Julius Lukeš, Marek Eliáš ✉, Hassan Hashimi ✉

Molecular Biology and Evolution, Volume 40, Issue 6, June 2023, msad134, https://doi.org/10.1093/molbev/msad134

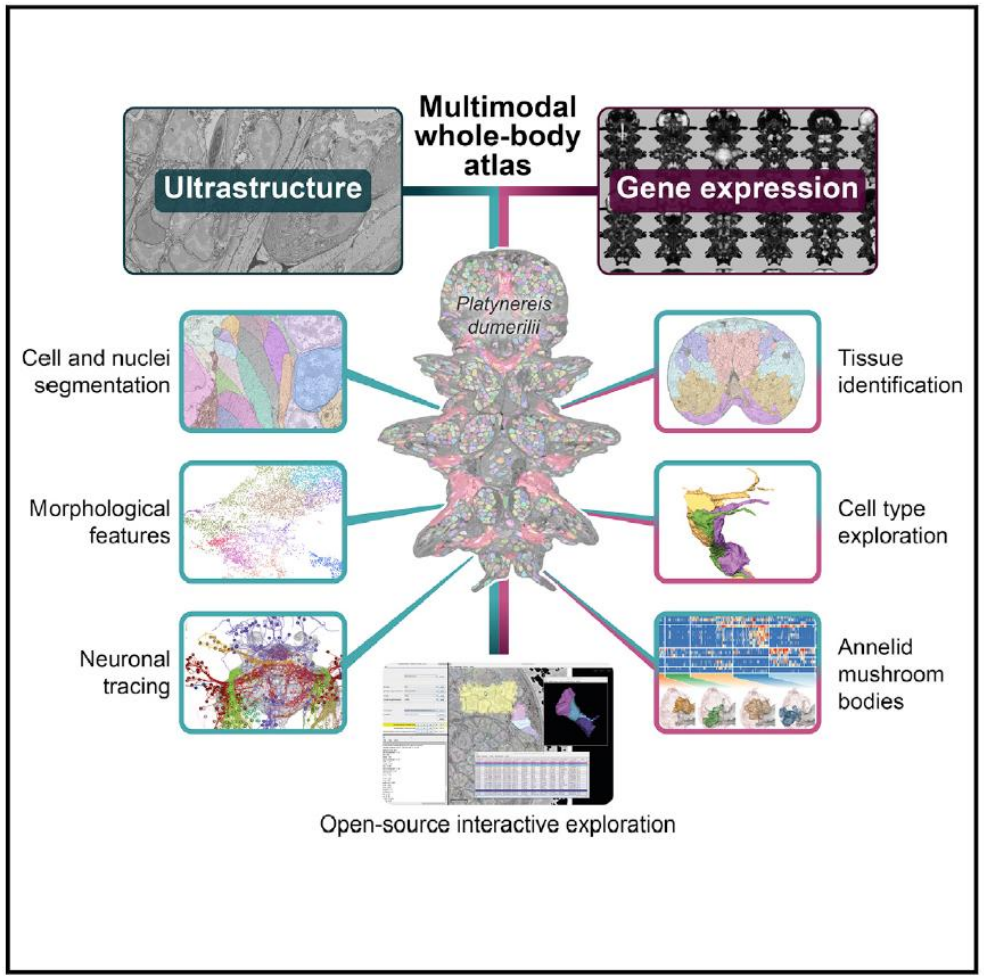
Published: 06 June 2023



Cell

Whole-body integration of gene expression and single-cell morphology

Graphical abstract



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Hernando M. Vergara, Constantin Pape, Kimberly I. Meechan, ..., Anna Kreshuk, Christian Tischer, Detlev Arendt

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christian.tischer@embl.de (C.T.),
arendt@embl.de (D.A.)

In brief

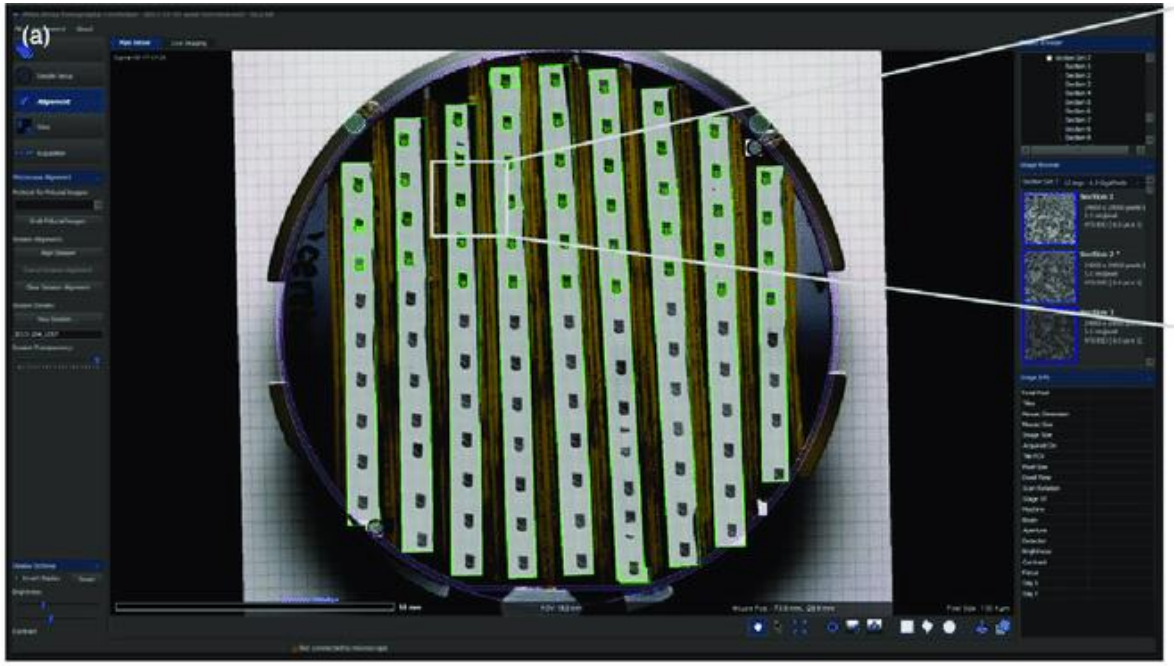
A framework for integrating cellular-resolution gene expression and cell morphological information at full-organism scale is provided for the marine annelid Platynereis dumerilii

pixel size (x/y) of 10 nm and 25 nm section thickness (z), resulting in 11,416 planar images made of >200,000 tiles for a total size of 2.5 TB.

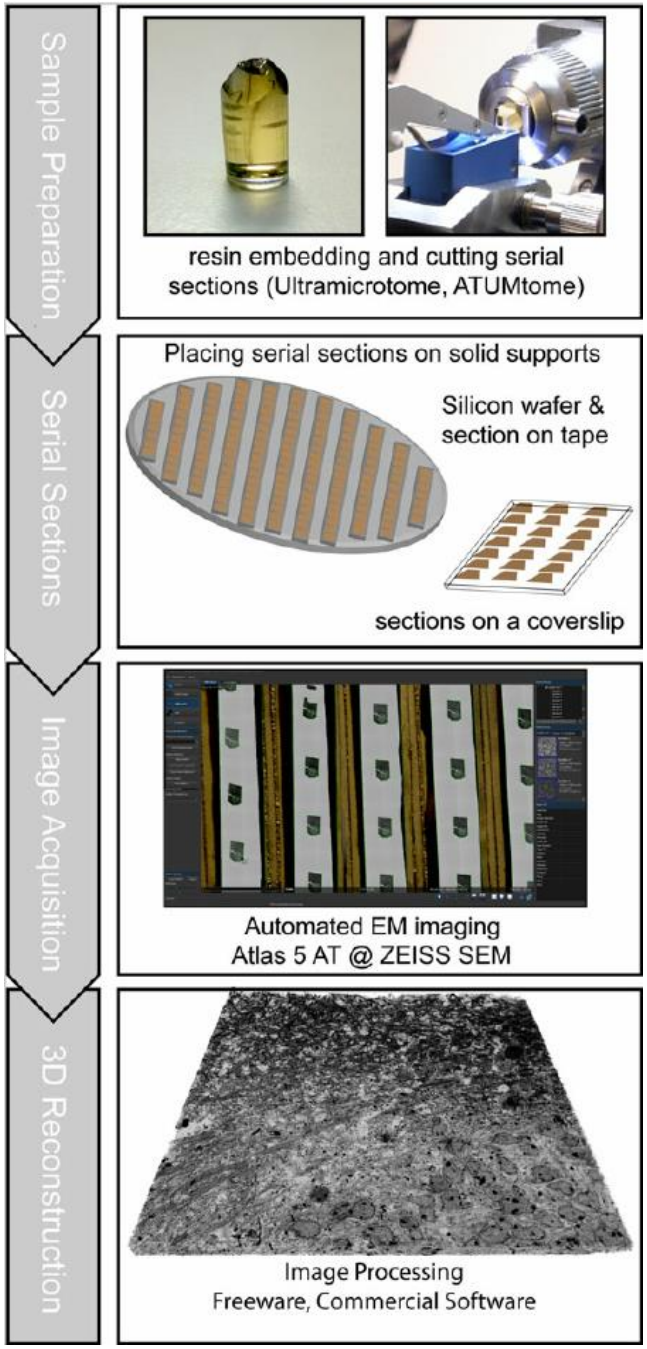
Array Tomography

(not an electron tomography which is an TEM based method)

Array Tomography technology overview



Eberle et al., 2014

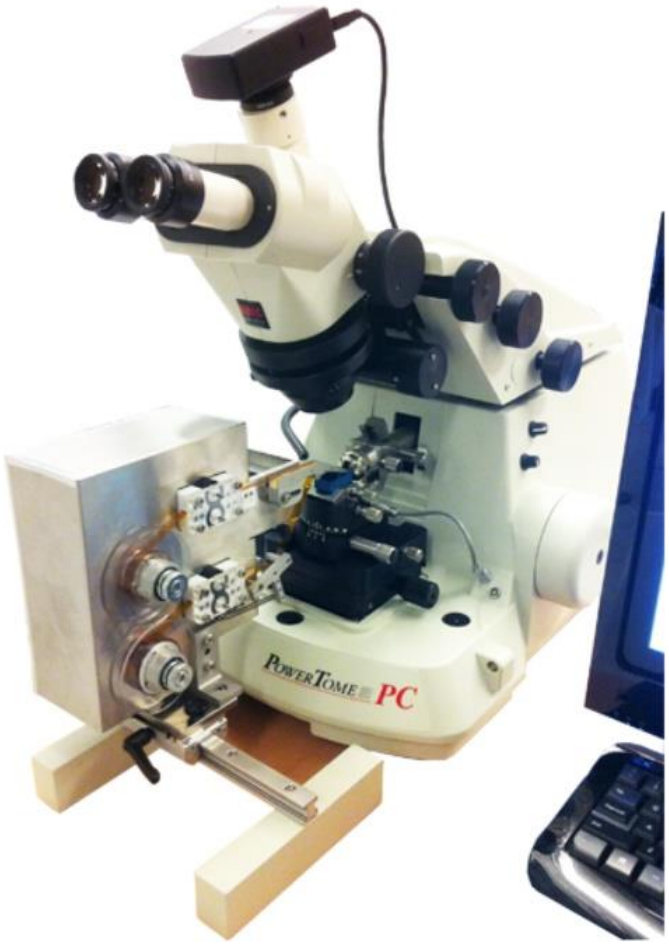


Take home message:
<u>Serial sections are collected by ultramicrotome first and then imaged in SEM</u>

Solutions for collecting sections for Array-tomography ATUMTOME (RMC)

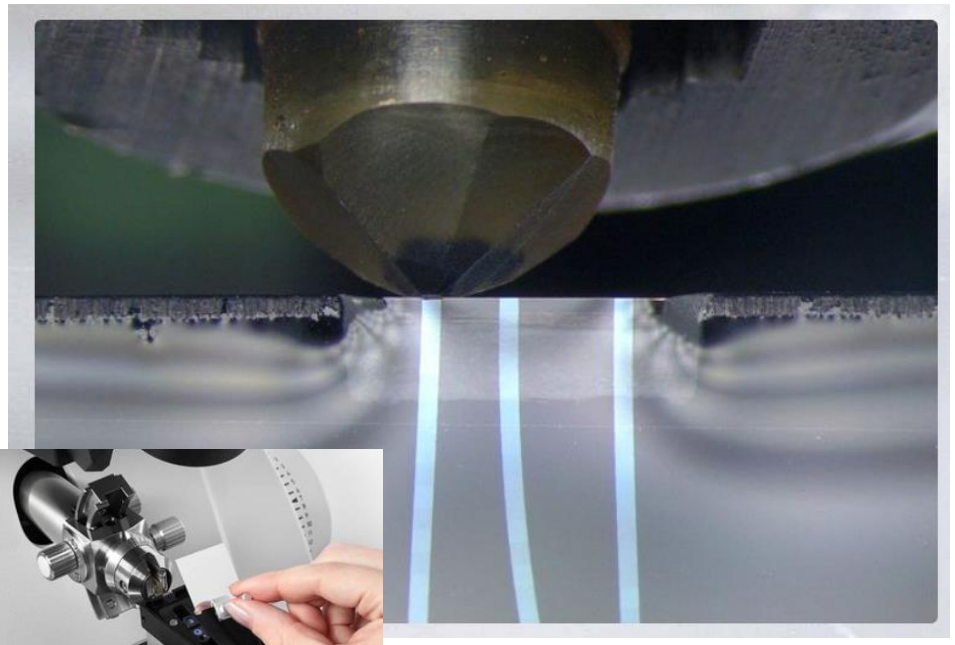
https://www.eden-instruments.com/en/ex-situ-equipments/rmc-em-sample-prep-solutions/atumtome/

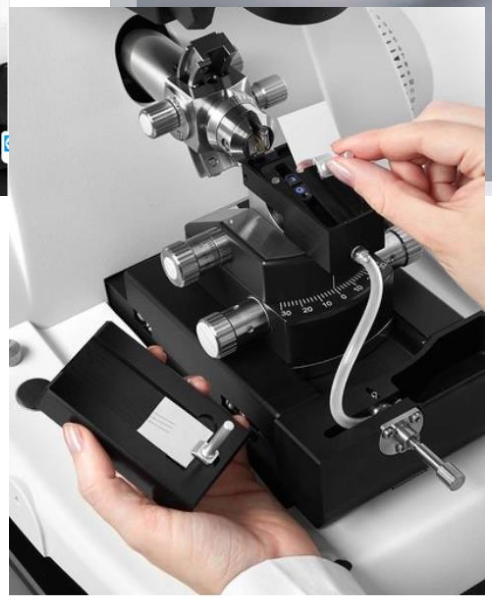
https://youtu.be/IVtqFSDPQqU



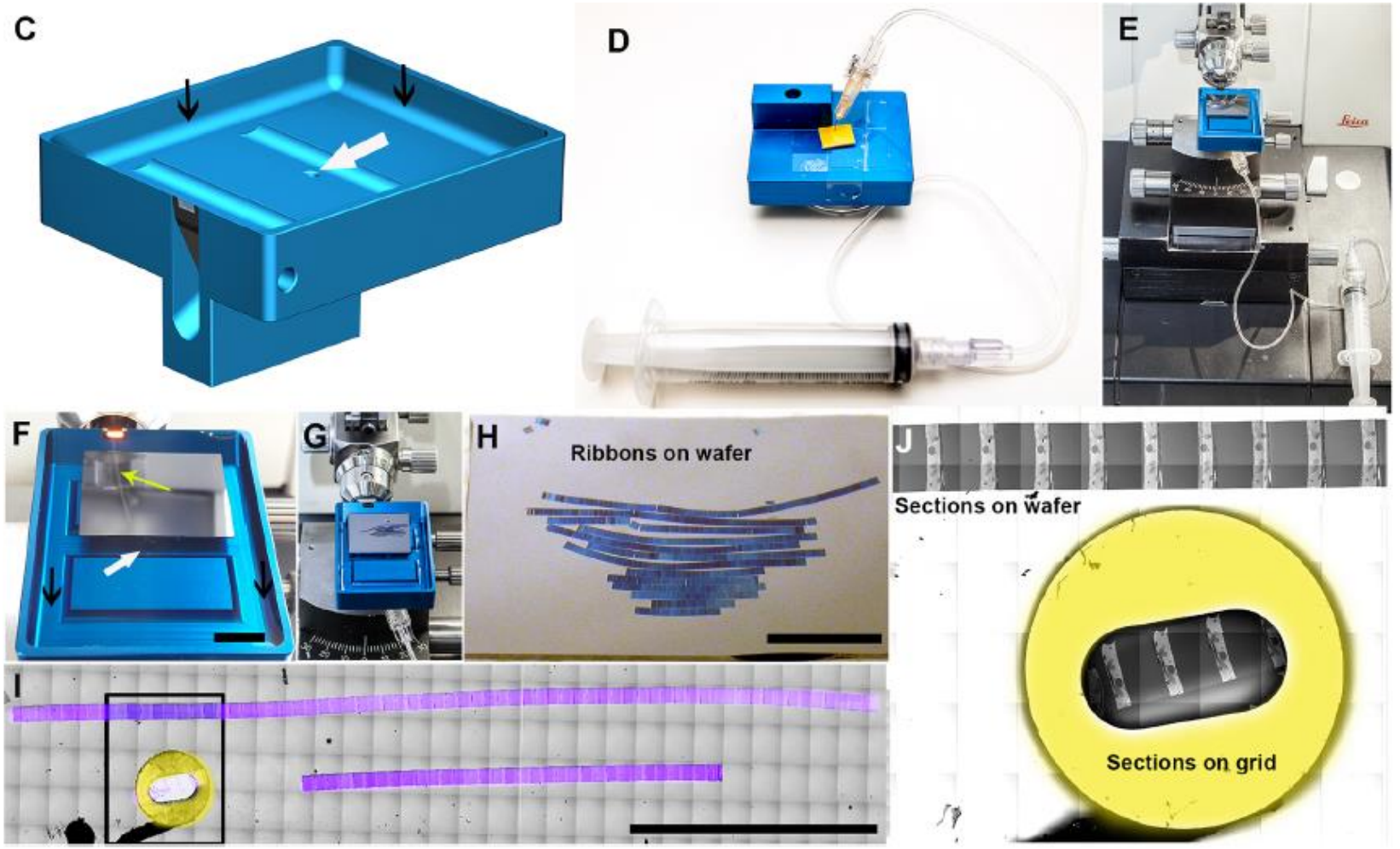
Solutions for collecting sections for Array-tomography
Artos (Leica)

https://www.leica-microsystems.com/products/sample-preparation-for-electron-microscopy/p/artos-3d/
https://www.youtube.com/watch?v=V4XIqdRjc28



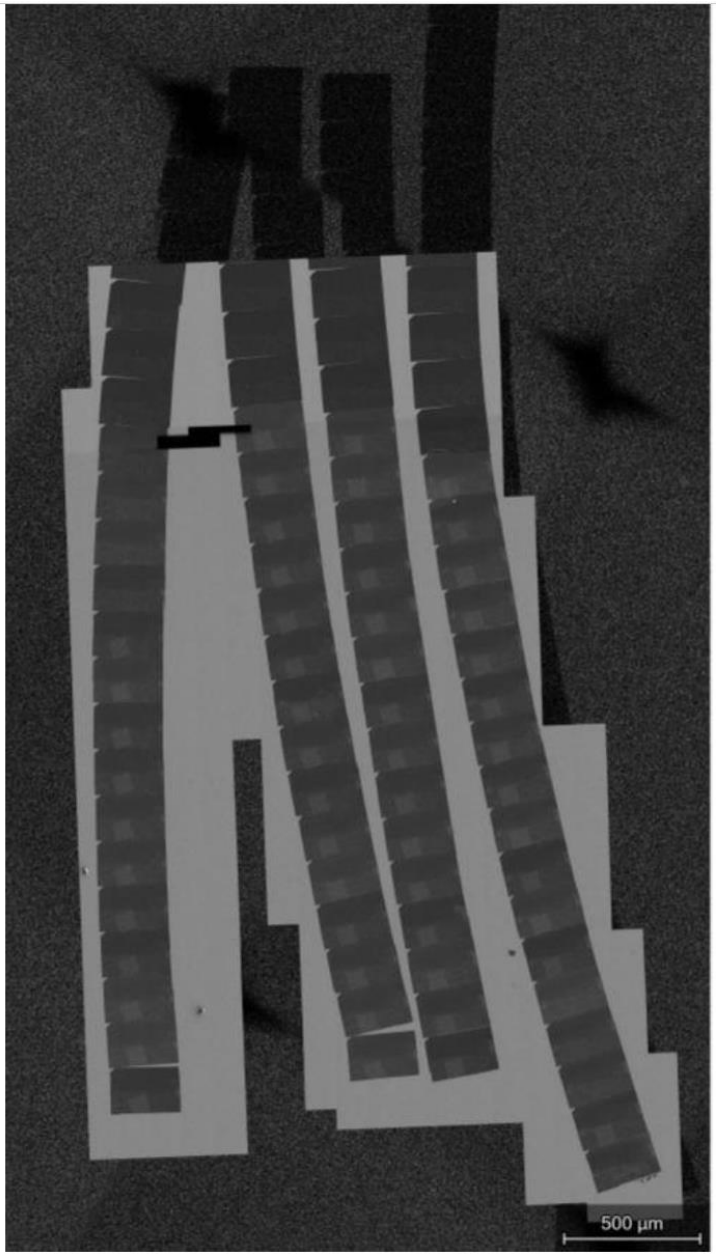


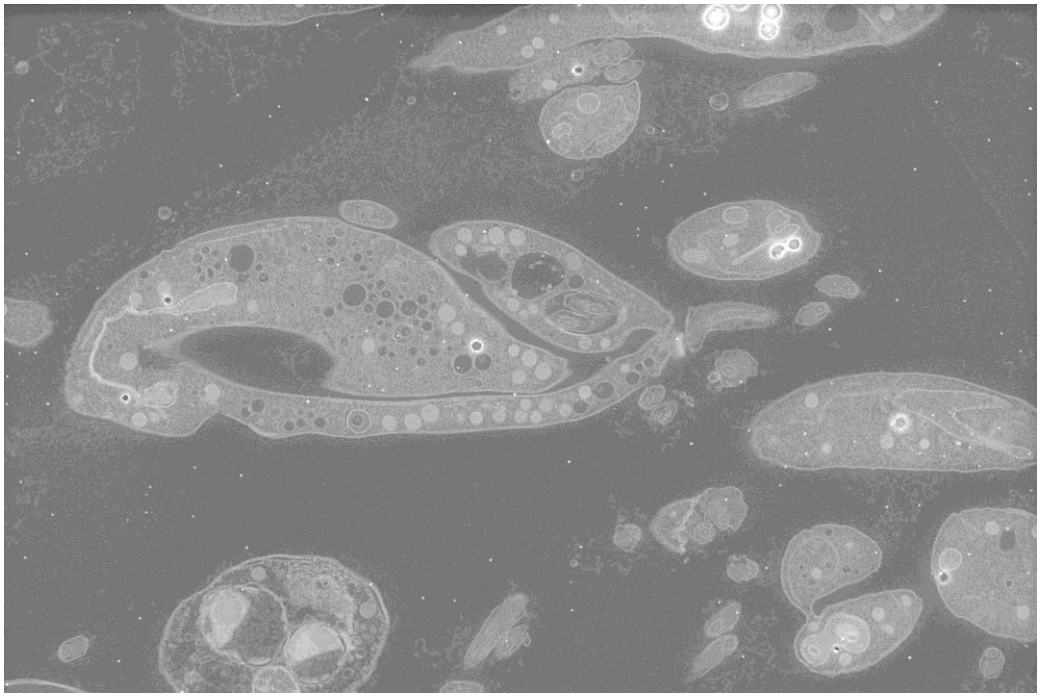
Solutions for collecting sections for Array-tomography
Specialized diamond knife (Diatome)

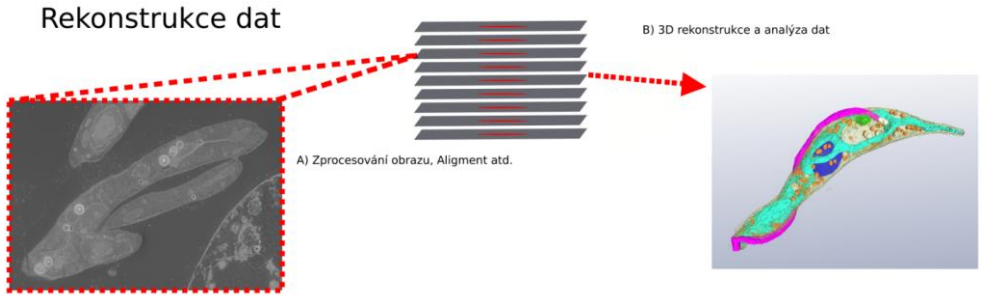


Burel et al., 2018

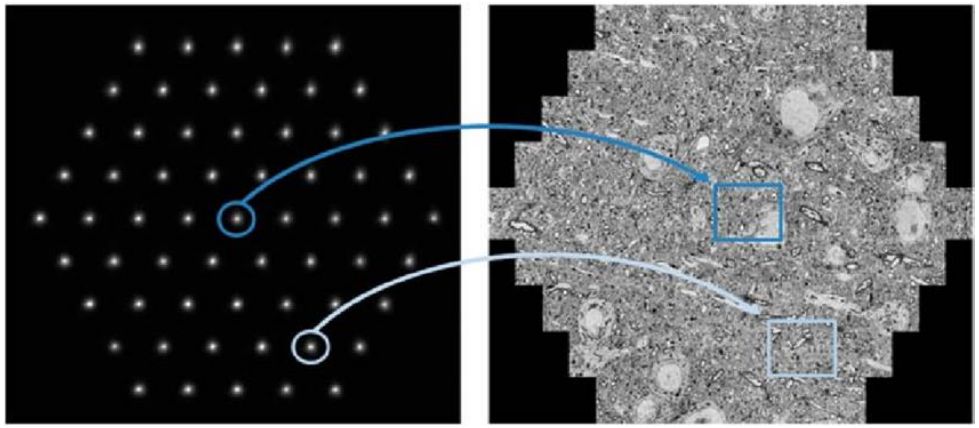
Array tomography imaging workflow





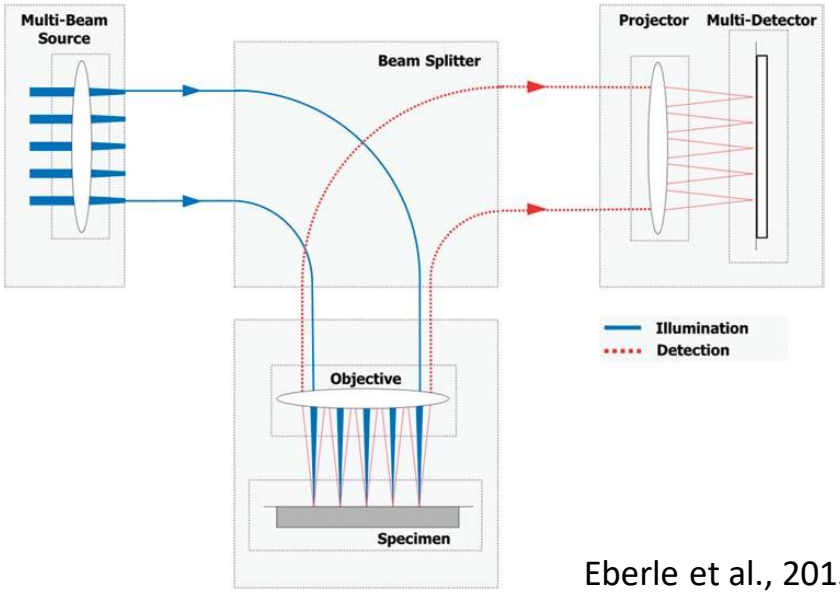


How to speed up the acquisition?



Multibeam SEM

61-92 beams



Eberle et al., 2015

Fast - EM

64 parallel electron beams

High image acquisition with 64 parallel electron beams and short dwell times

Rigid uniform substrate

The scintillator screen allows the loading of up to nine substrates at the same time

STEM imaging

Collect nanoscale detail while retaining the larger context of the sample

Robust automation software

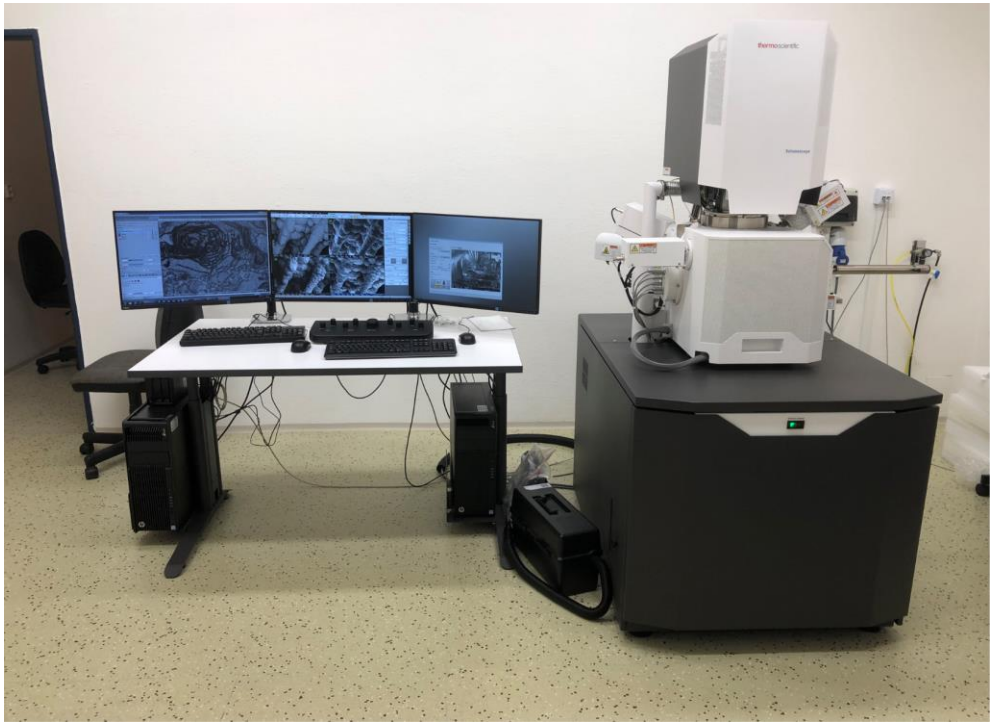
Leave the system to automatically acquire complex datasets without constant supervision

https://www.delmic.com/en/products/fast-imaging/fast-em

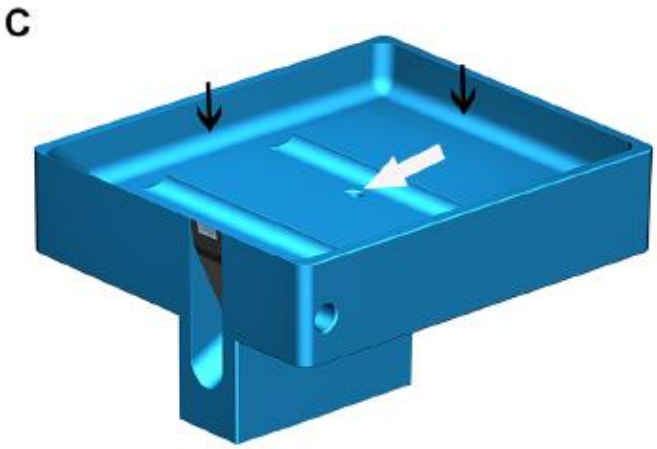
Solution used in the Laboratory of Electron Microscopy České Budějovice

Apreo SEM equipped with Volumescope from

ThermoFisher SCIENTIFIC



Typical sample size:
Couple of hundreds to ten of thousands sections
Depending on the technology used for collecting the sections.



Summary Array Tomography:

- Sections are collected first

Issues

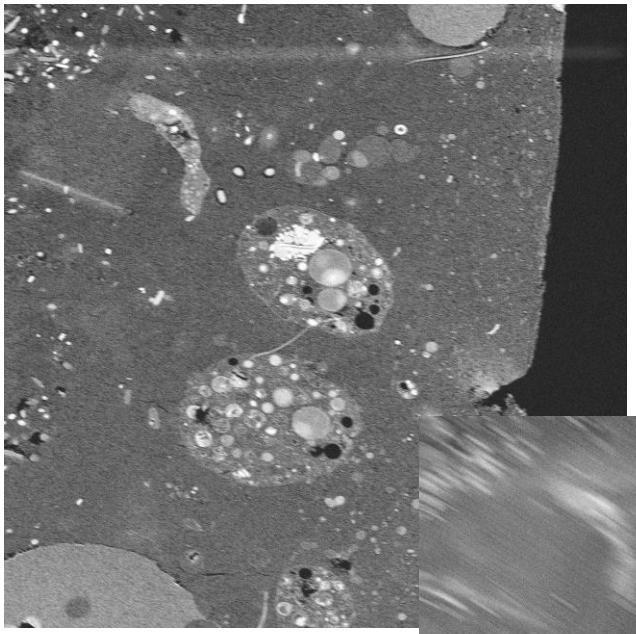
- More difficult to obtain and process sections
- Individual Sections can be lost or damaged (so there is a gap in the data)
- Image likely is a bit distorted (compression like when you are cutting slice of bread)
- Much more difficult to obtain the data and to process and ALIGN them – sections can a rotate a little bit in respect to previous one. (special software needed)
- In general you do not have than many sections as in SBEM

Advantages

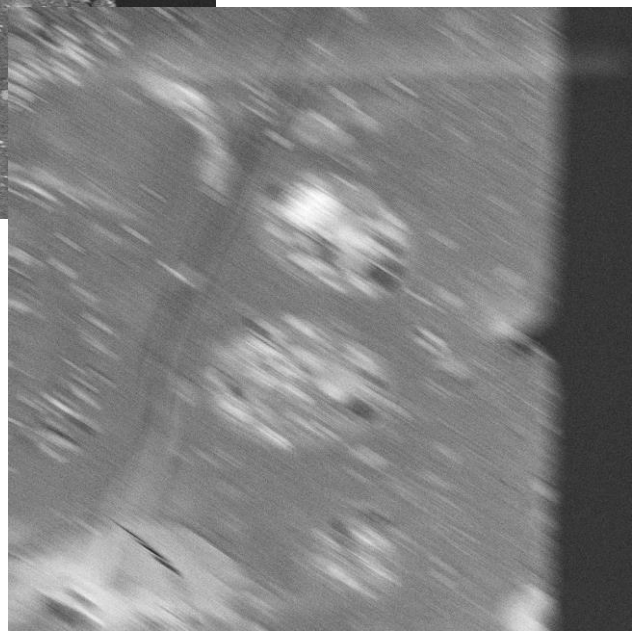
- SECTIONS are NOT LOST – can be reimaged (even in better resolution) – you can screen the data first with poorer resolution
- In theory can be much faster than SBFSEM (scan only the ROI you want in Hi Res)
- In theory every SEM can do it, but that would be more manual and slow
- Really good resolution in X and Y as you can use other electromagnetic tools in SEM + shorter Working distance (no knife above it etc.)
- You can process the sections for other methods
  - poststaining (so you can use any sample for TEM)
  - CLEM, immunolabelling (but with specialized resins)
- NO charging issues as the surface can be carbon coated and is fully conductive
- Sample prep can be simple TEM prep

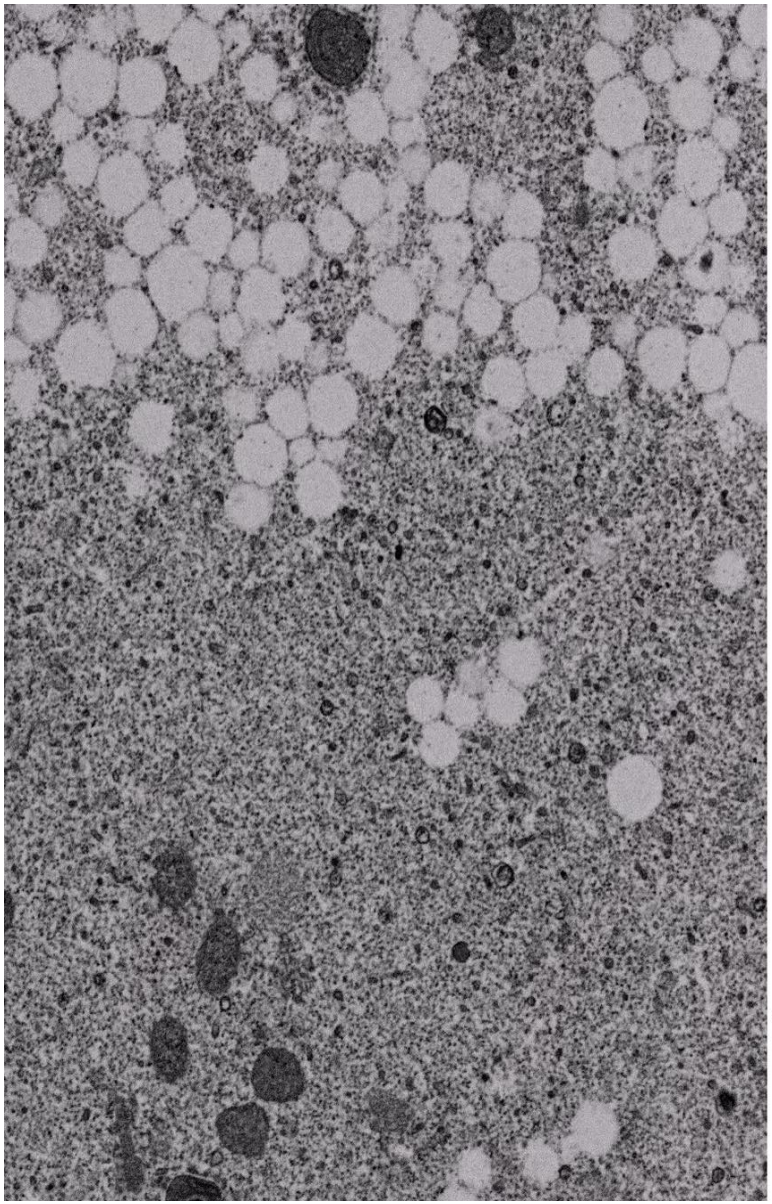
What are the limiting factors for Array Tomo? – A) Focus, stigmator issues

What does it look like?



Next section





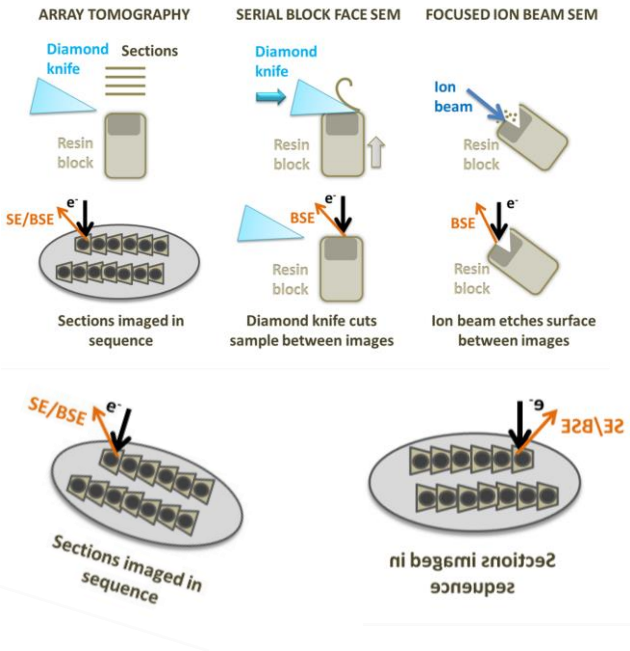
What are the limiting factors for Array Tomo? – A) Focus, stigmator issues

How does it work?

<u>The sections are not always on the exact same focal plane</u>

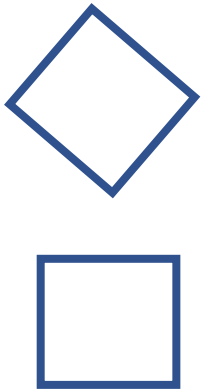
<u>- The whole plate can be mounted on an angle (it is fairly large – cm, dm), the sections are wrinkled…</u>

<u>solution: automatic focus, alignment, keeping the ribbons as straight as possible</u>

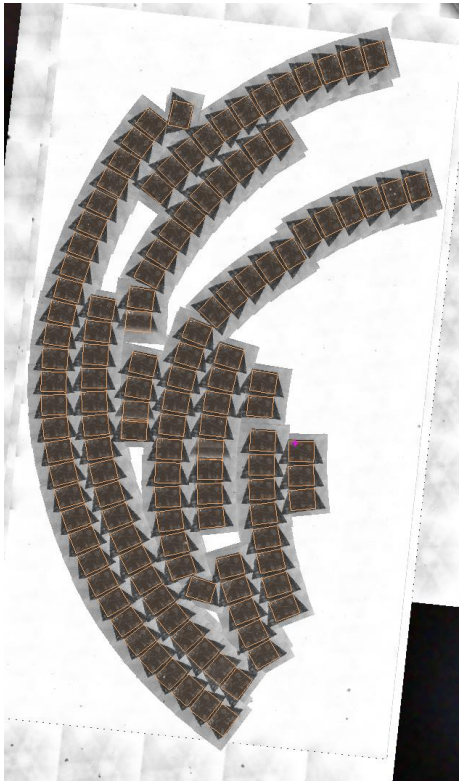


Different WD

Different rotation



ROI rotates, the beam has to rotate – should be realigned



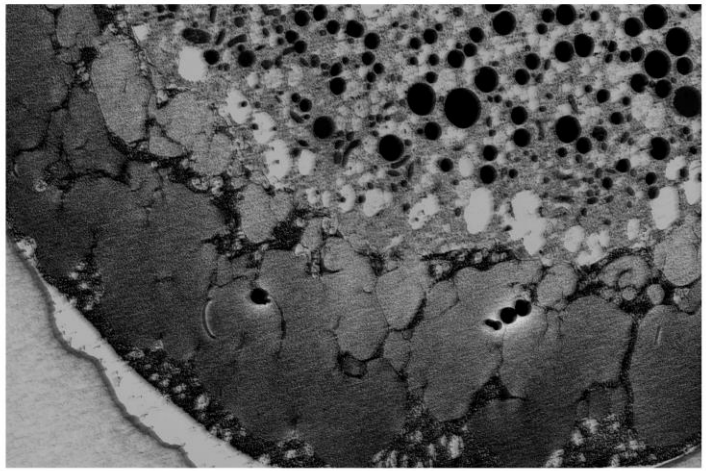
https://nano.oxinst.com/library/blog/3d-scanning-electron-microscopy-for-biology

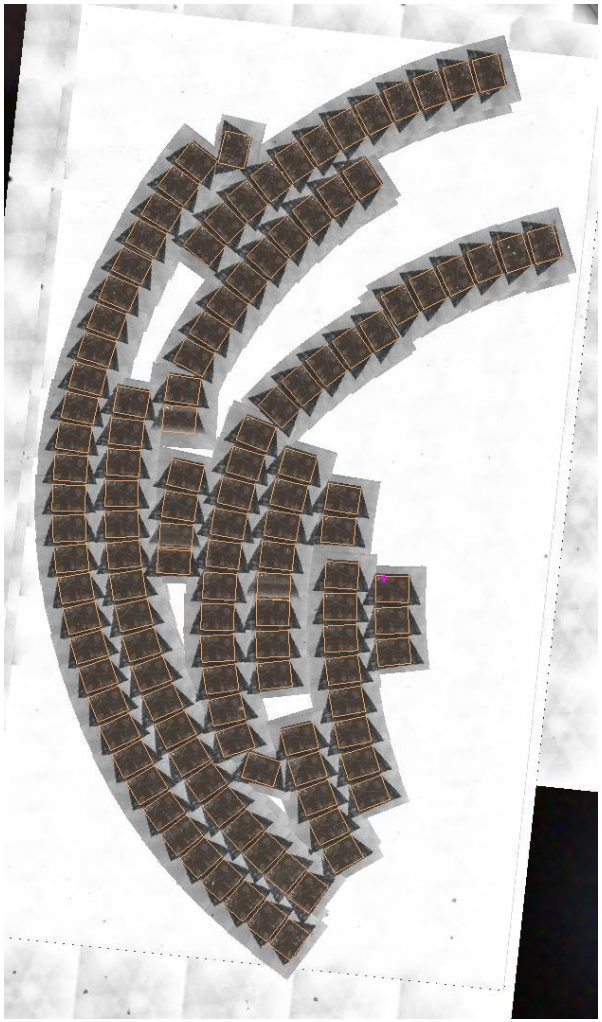
What are the limiting factors for Array Tomo? – B) targetting and imaging the ROI precisely

How does it work?

The problem for acquiring images – finding ROI on subsequent section

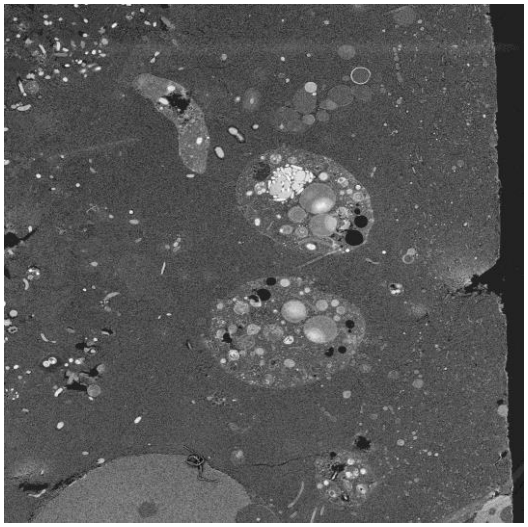
solution: good acquisition software ☺, manual check and corrections, keeping the ribbons as straight and regullar as possible

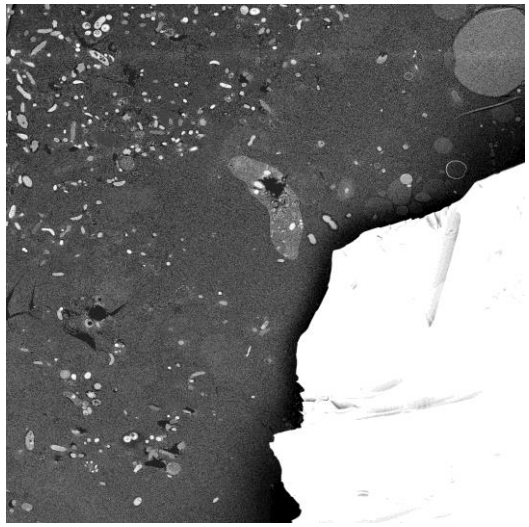


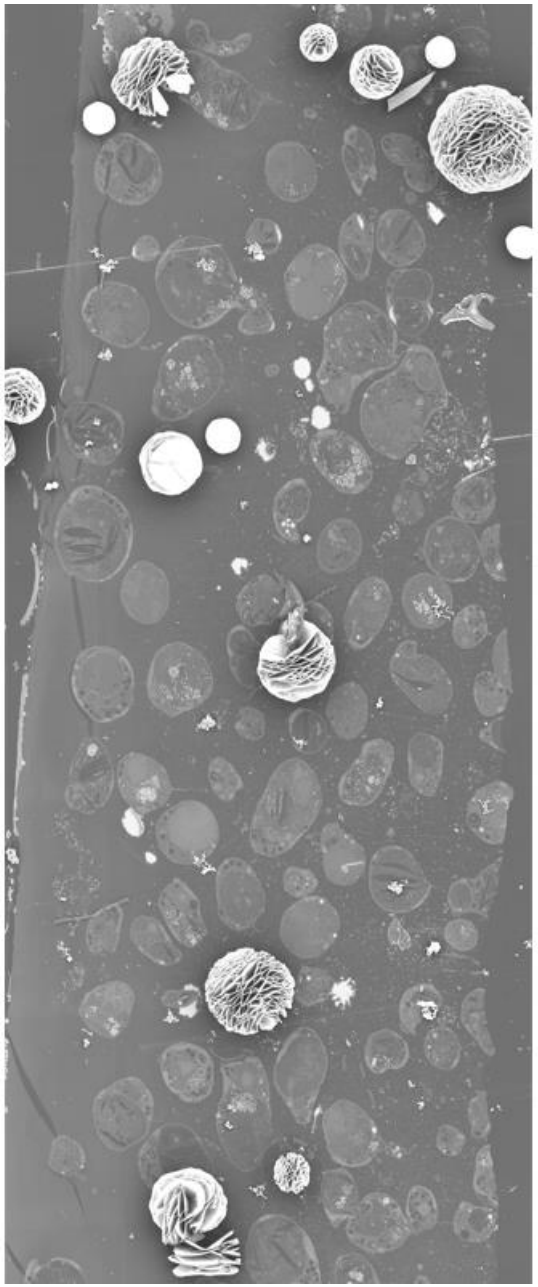


What are the limiting factors for Array Tomo? – C) Debris

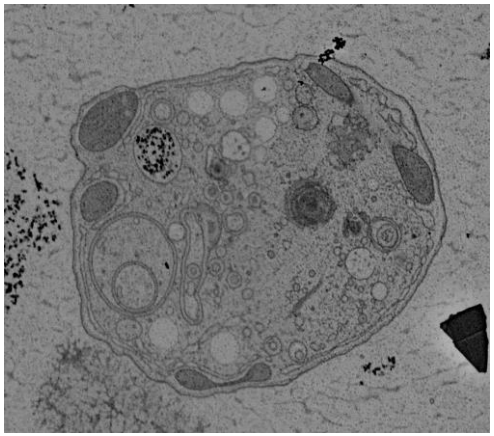
What does it look like?

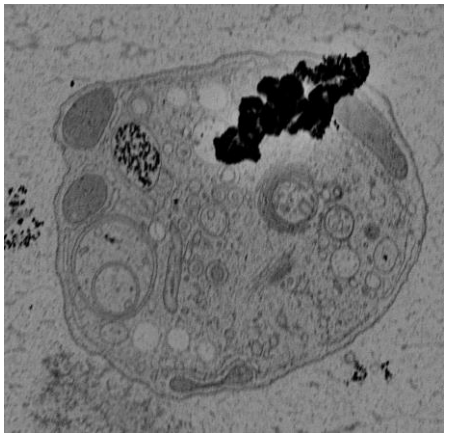






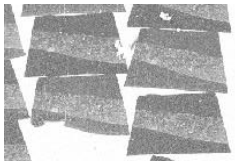
On some sections, it can completely mask the ROI.

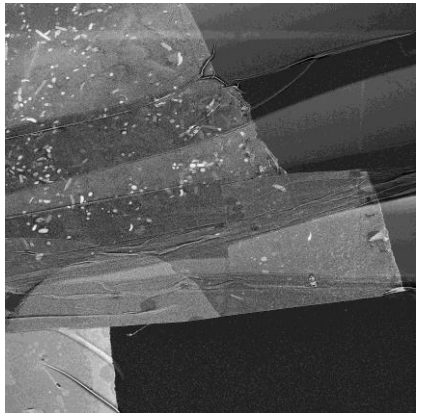




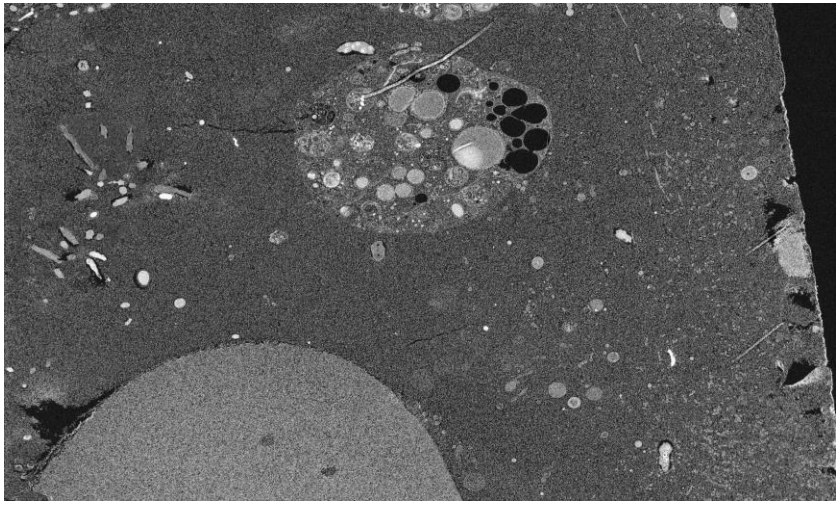
<u>solution: Be super careful while preparing and HANDLING the sample</u>
<u>Post-staining can be an issue</u>

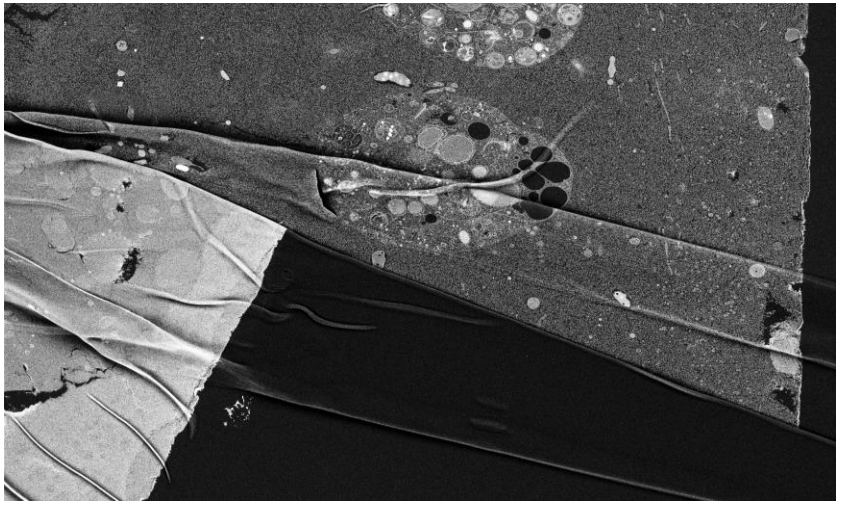
What are the limiting factors for Array Tomo? – E) Sections <u>folds, are being damaged</u>, squeezed - compression

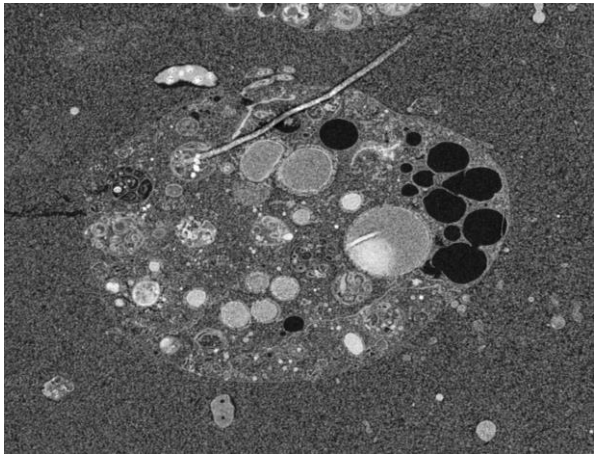




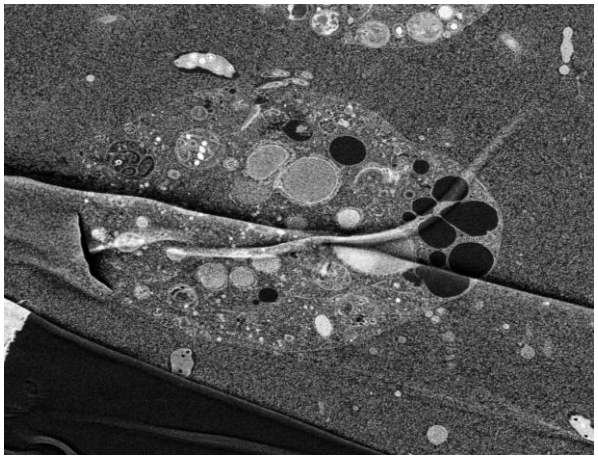
What does it look like?







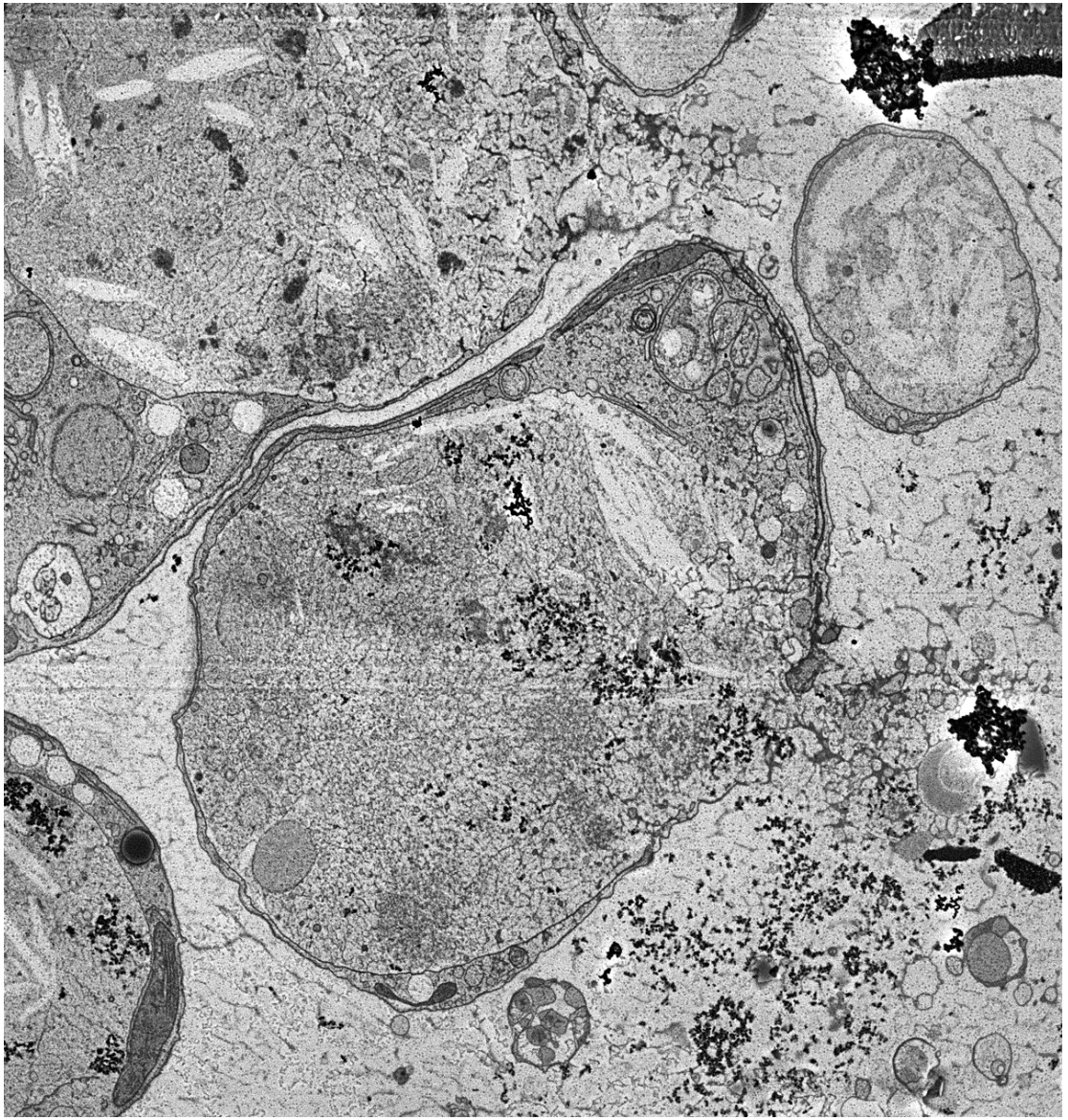
Folds
Significant distortion, some information (parts) are invisible/missing. (partly or completely – on the whole section)



What are the limiting factors for Array Tomo? – E) Sections folds, are being damaged, squeezed - compression

What does it look like?

Sections are compressed by the knife
It is the same as when you are slicing bread



What are the limiting factors for Array Tomo? – E) Sections folds, are being damaged, squeezed - compression

solution:

It is just a fact ☺

Very often occurs when being picked up onto the wafer

What are the limiting factors for Array Tomo? – F) Sections are lost

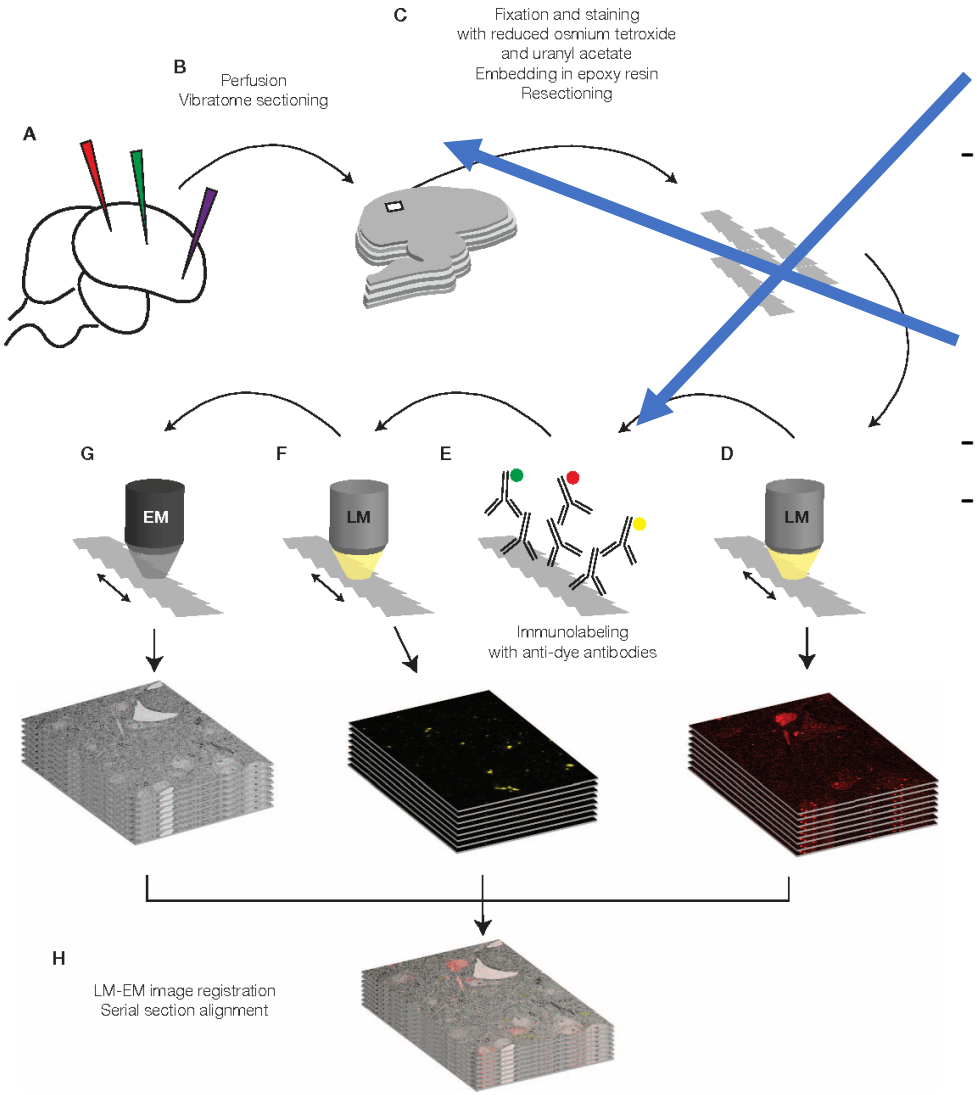
What does it look like?

Solution:
you need to remember which section is missing and how many – for data processing and reconstruction

Array Tomography

What can be used for?

CLEM and Immunolabelling

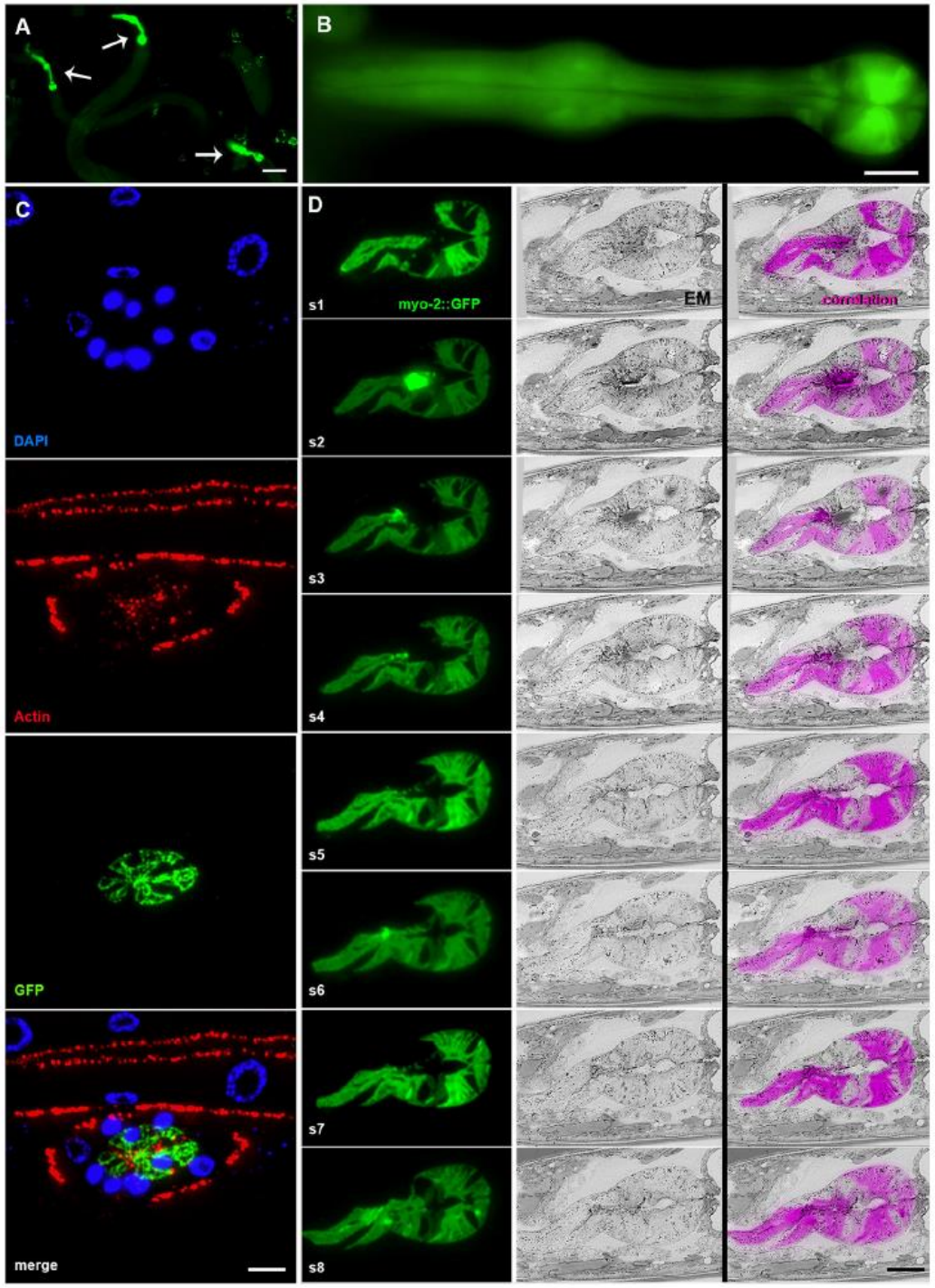


Obertl et al., 2011

- For Array tomography you first embed the sample, cut the sections and use antibodies (gold beads or fluorescence labelled on sections)
- For SBEM and FIB-SEM you have to work **EN-BLOC** everything has to be labelled before embedding
- With immunolabelling you can use just EM
- Fluorescence has to be imaged by light microscope and be correlated

Usage:
A) for targeting
B) for localization within image

Fluorescence and immunolabelling is compatible only with certain type of resins. There is a trade of as in these resins usually the ultrastructure is not superb and is a bit compromised.



CLEM

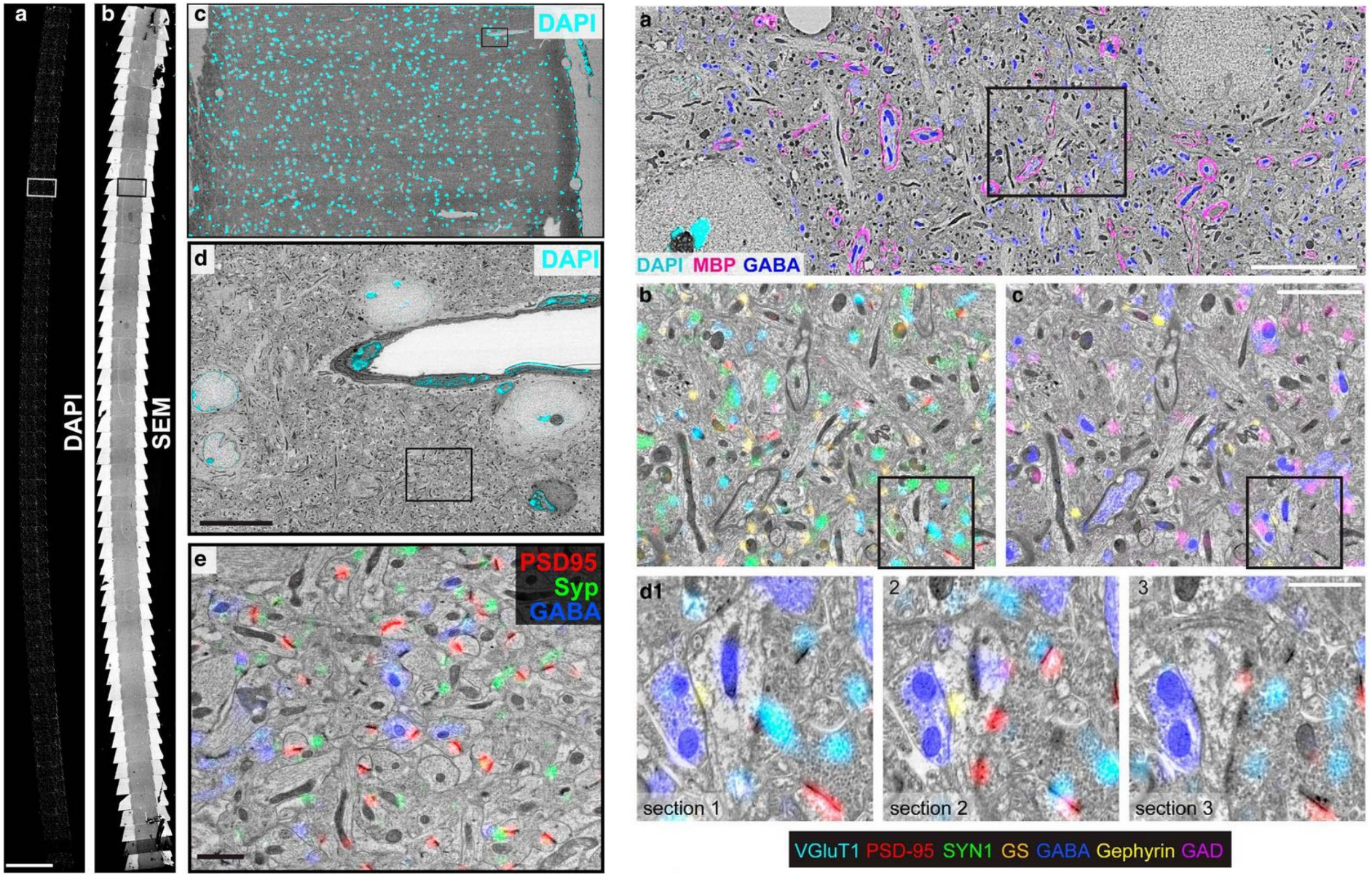
- Example for Array tomography workflow
- From Burel et al., 2018

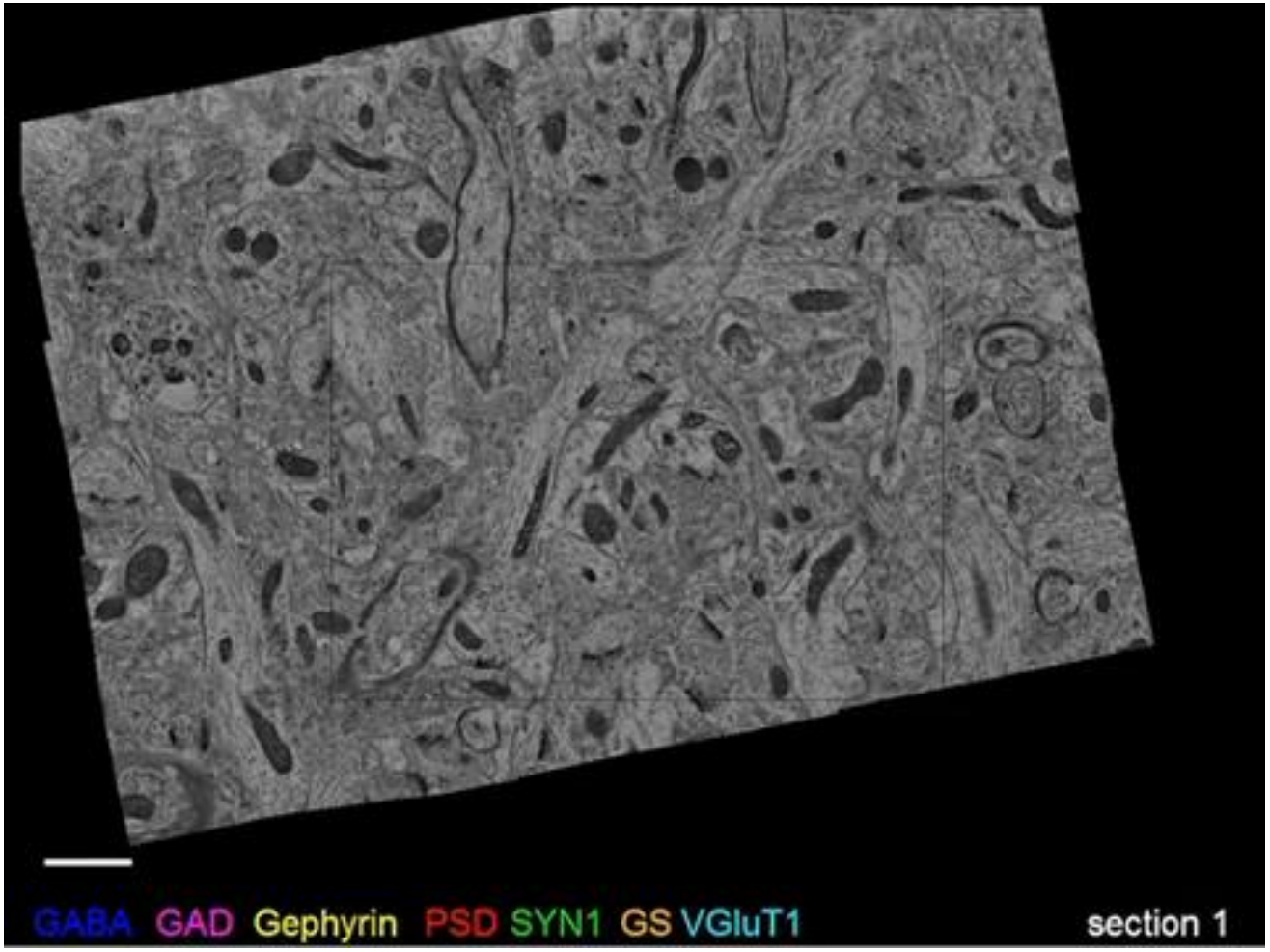
A targeted 3D EM and correlative microscopy method using SEM array tomography

Mapping Synapses by Conjugate Light-Electron Array Tomography

Forrest Collman, JoAnn Buchanan, Kristen D. Phend, Kristina D. Micheva, Richard J. Weinberg, and Stephen J Smith

Journal of Neuroscience 8 April 2015, 35 (14) 5792-5807; DOI: https://doi.org/10.1523/JNEUROSCI.4274-14.2015

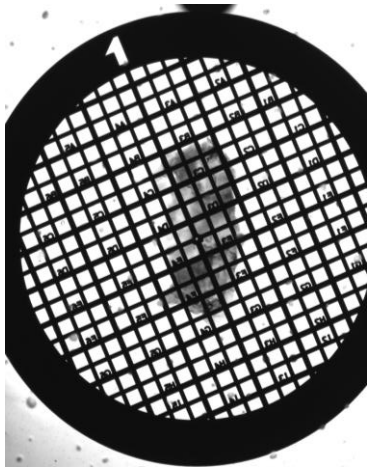



GABA GAD Gephyrin PSD SYN1 GS VGluT1
section 1

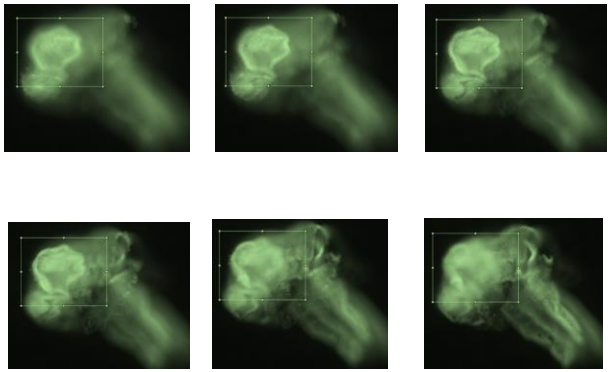
Hi-resolution imaging of the needle in a haystack

CLEM workflow used for finding *Plasmodium* in the mosquito intestine for Array tomography imaging

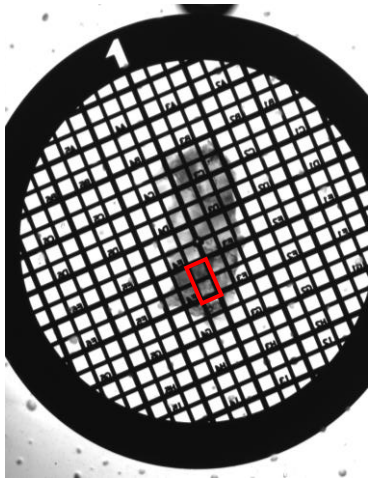
Mosquito intestine



Finding ROI using IF, motorized stage, to map also Z coordinates

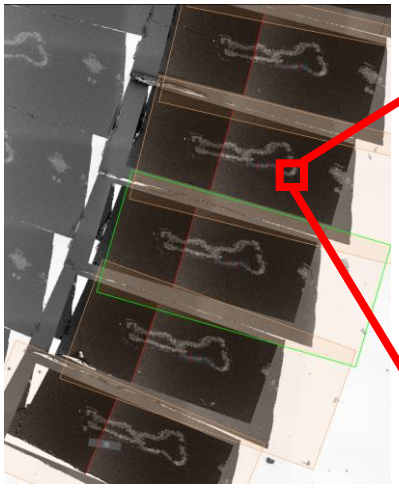


Map the ROI onto the original sample



Process the sample for the AT (staining, embedding into the resin)

Prepare wafer with sections



Find ROI

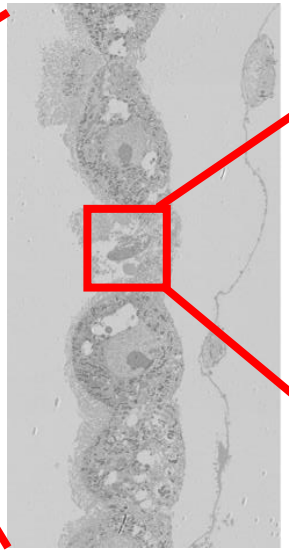
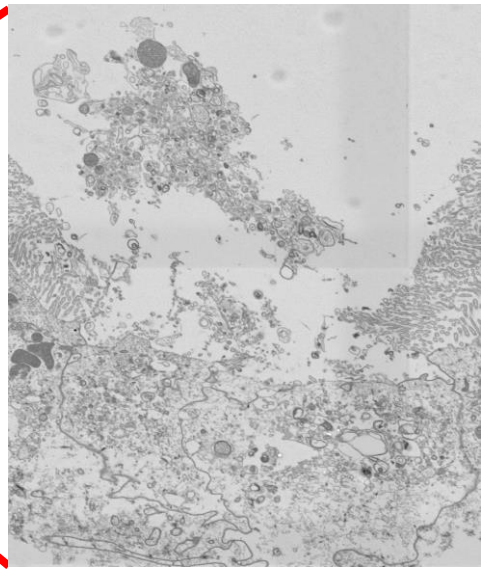


Image ROI



Process and analyse the data

Unpublished data, collaboration with Pablo Suárez-Cortés

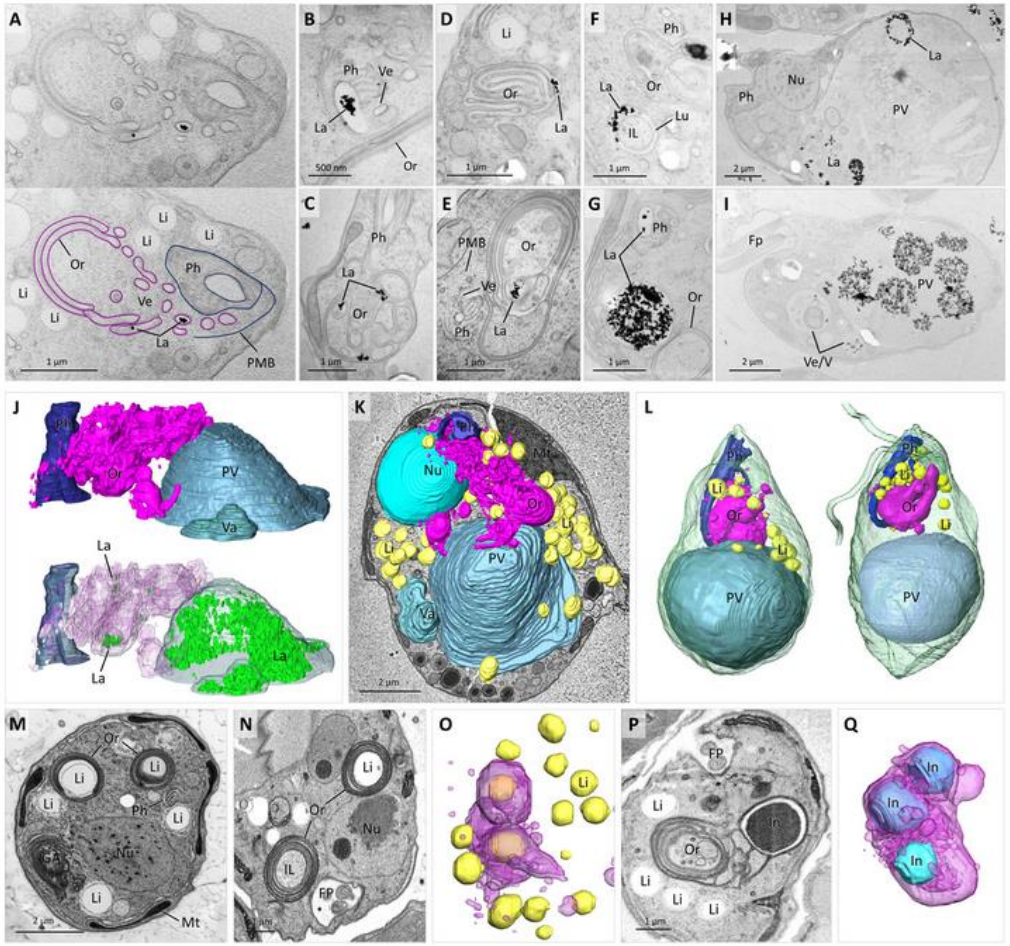
Voxel dimensions 2,5 x 2,5 x 90 nm

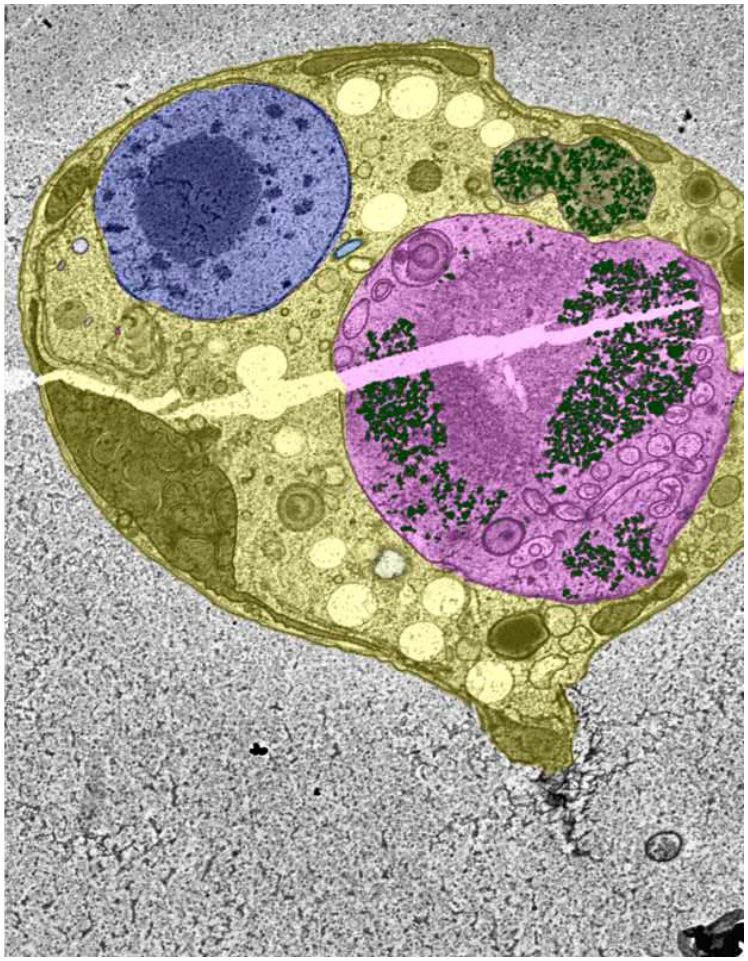
| Eukaryotic Cells | Research Article | 22 September 2023

Ultrastructure and 3D reconstruction of a diplonemid protist (Diplonemea) and its novel membranous organelle

Authors: Daria Tashyreva, Jiří Týč, Aleš Horák, Julius Lukeš | AUTHORS INFO & AFFILIATIONS

DOI: https://doi.org/10.1128/mbio.01921-23 · Check for updates





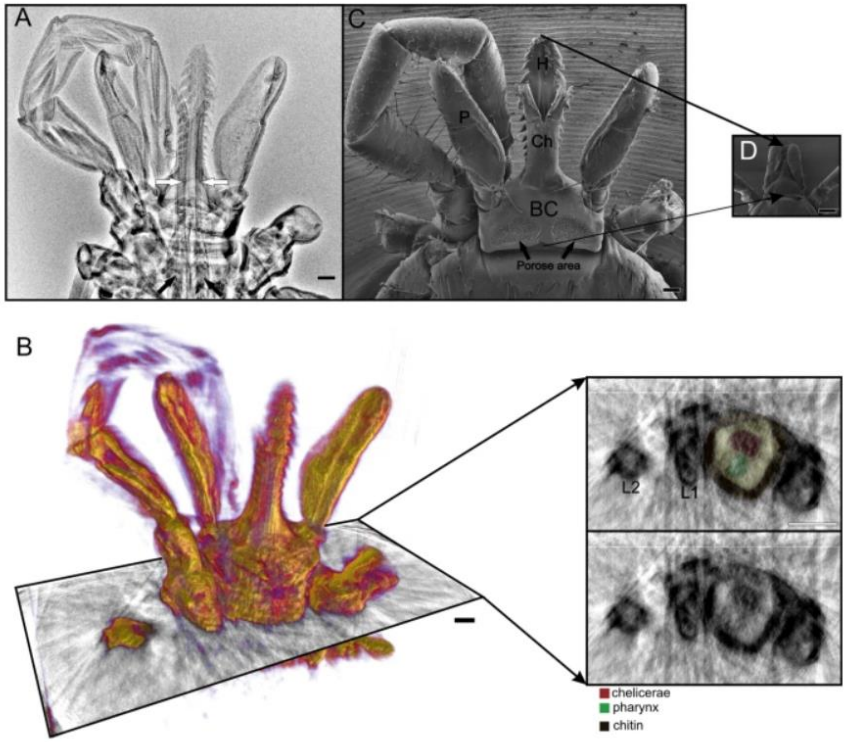
Imaging large structures

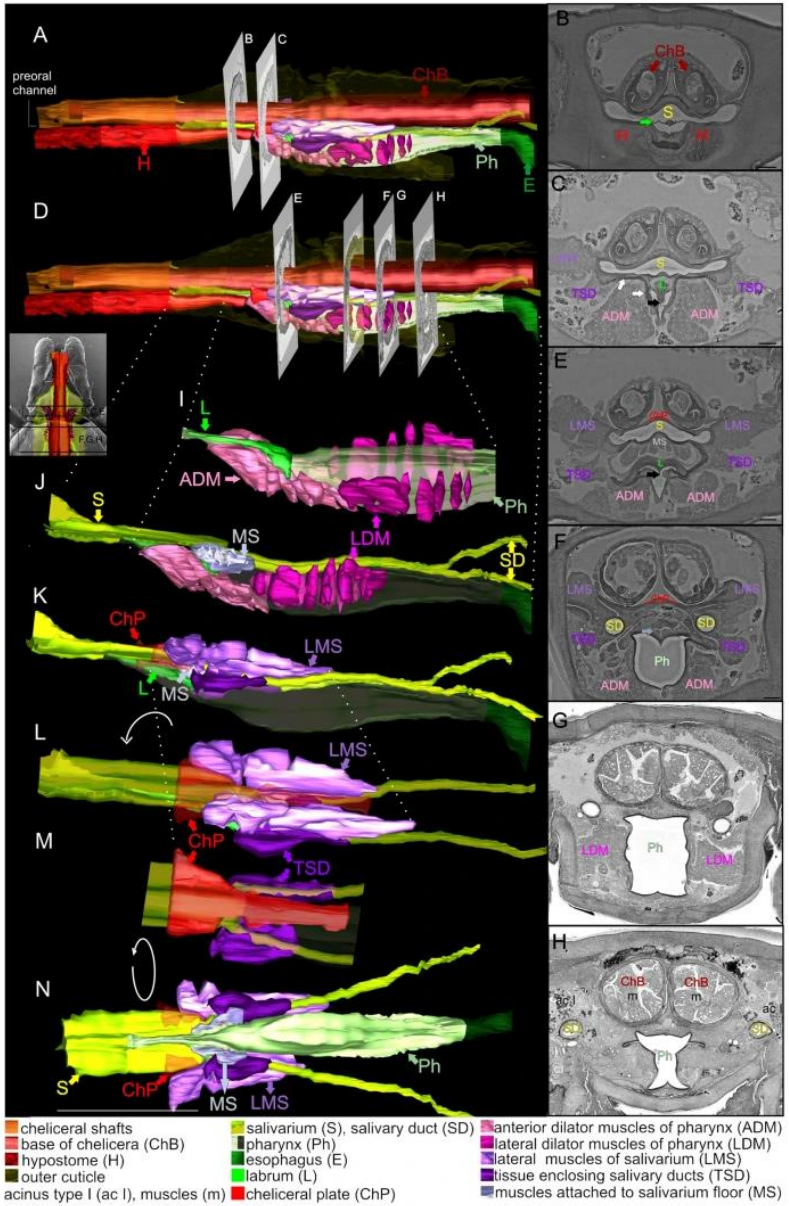
Article | Open Access | Published: 13 January 2020

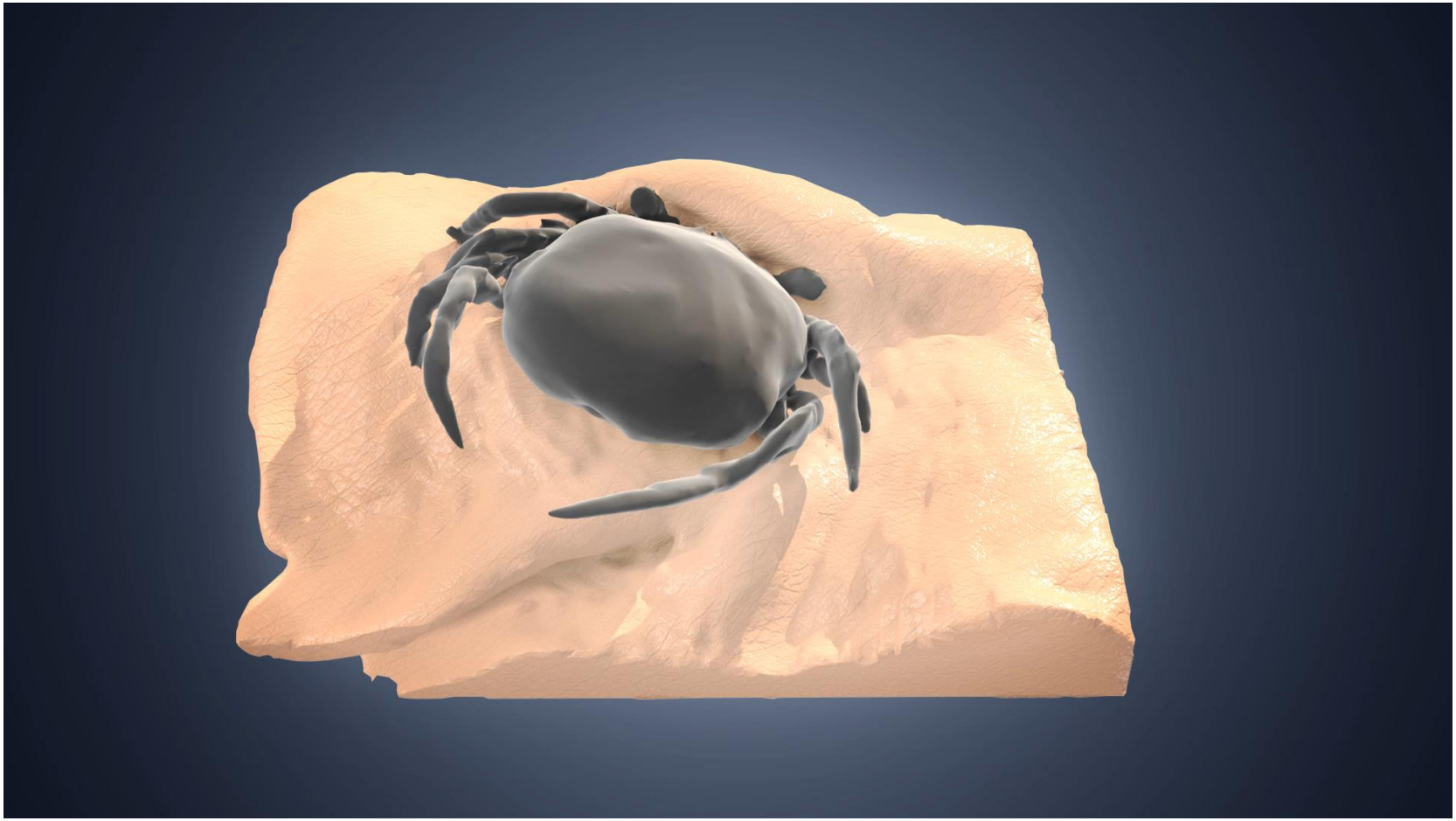
Three-dimensional reconstruction of the feeding apparatus of the tick *Ixodes ricinus* (Acari: Ixodidae): a new insight into the mechanism of blood-feeding

Marie Vancová, Tomáš Bílý, Ladislav Šimo, Jan Touš, Petr Horodyský, Daniel Růžek, Adam Novobilský, Jiří Salát, Martin Strnad, Daniel E. Sonenshine, Libor Grubhoffer & Jana Nebesářová

Scientific Reports **10**, Article number: 165 (2020) | Cite this article







Data processing and visualization

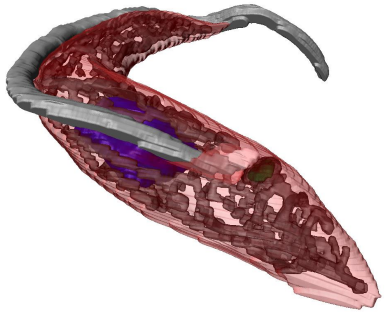
- Stitching and aligning
- Noise reduction and signal enhancement
  - Filters as Gausian, Median, Perona-Malic, contrast adjustment etc.
- Finding ROI, cropping it out of the big dataset (if possible)
  - Reduces the amount of data that has to be handled by the computer
- Analysis and measurement
  - Segmentation, 3D visualization

- **Image processing and analysis is by far the LONGEST part**
  - Sample prep:
    - up to couple of days (weeks)
  - Data acquisition in the microscope:
    - up to couple of days (weeks)
  - Data processing
    - At least couple of days, mostly weeks, easily several months
    - Basically the whole Bc, Msc even PhD thesis ☺

Programs in use:
MAPS, Amira (Thermo Fisher Scientific)
MIB (Microscopy image browser), Fiji – ImageJ (free software)

Summary 3D SEM:

- 3D EM are a cool powerful, and versatile techniques
- Quantitative (volume, distances measurements)
- Time and storage demanding (tens to hundreds of GB)
- Full datasets processing requires powerful gaming stations (or servers)
- Works really well with certain samples (such as neural tissue).
- Some workarounds and bypasses work pretty well for the rest (low vacuum etc.), so we can successfully image and analyse pretty much anything, but we need to know the weaker points



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