# Advanced electron microscopy techniques

**Vlada Filimonenko**

Institute of Molecular Genetics

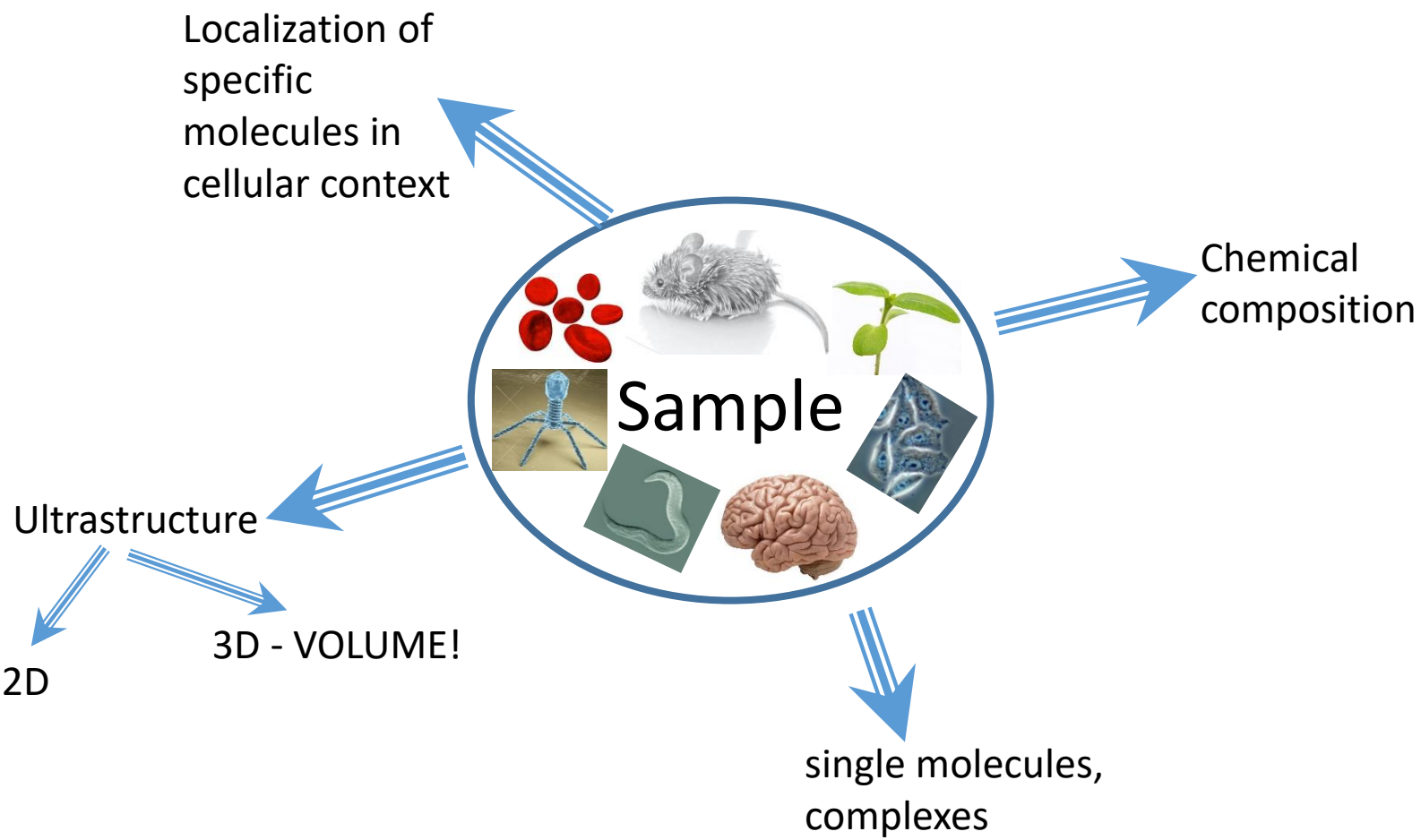
Electron Microscopy Core Facility

IMG

CZECH BIOIMAGING

Imaging principles of life

EURO-BIOIMAGING

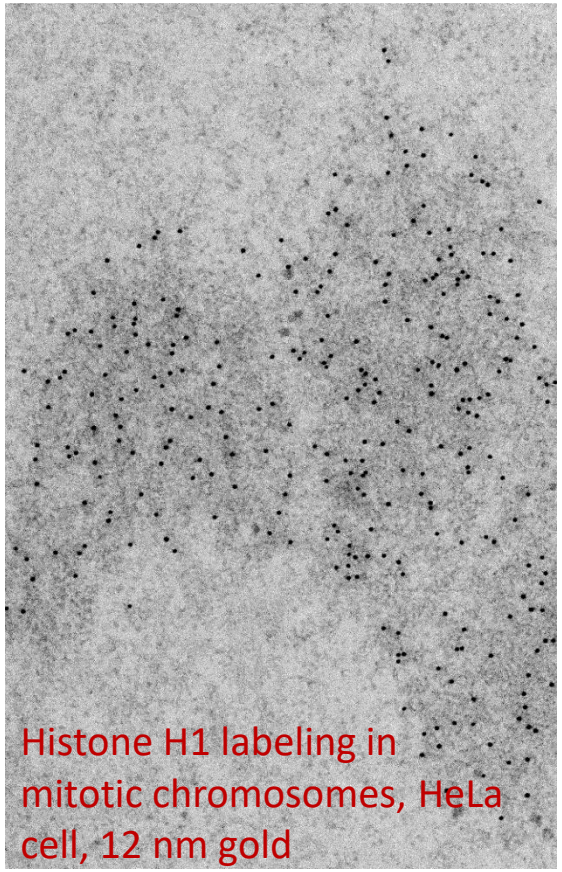



**Correlative microscopy**

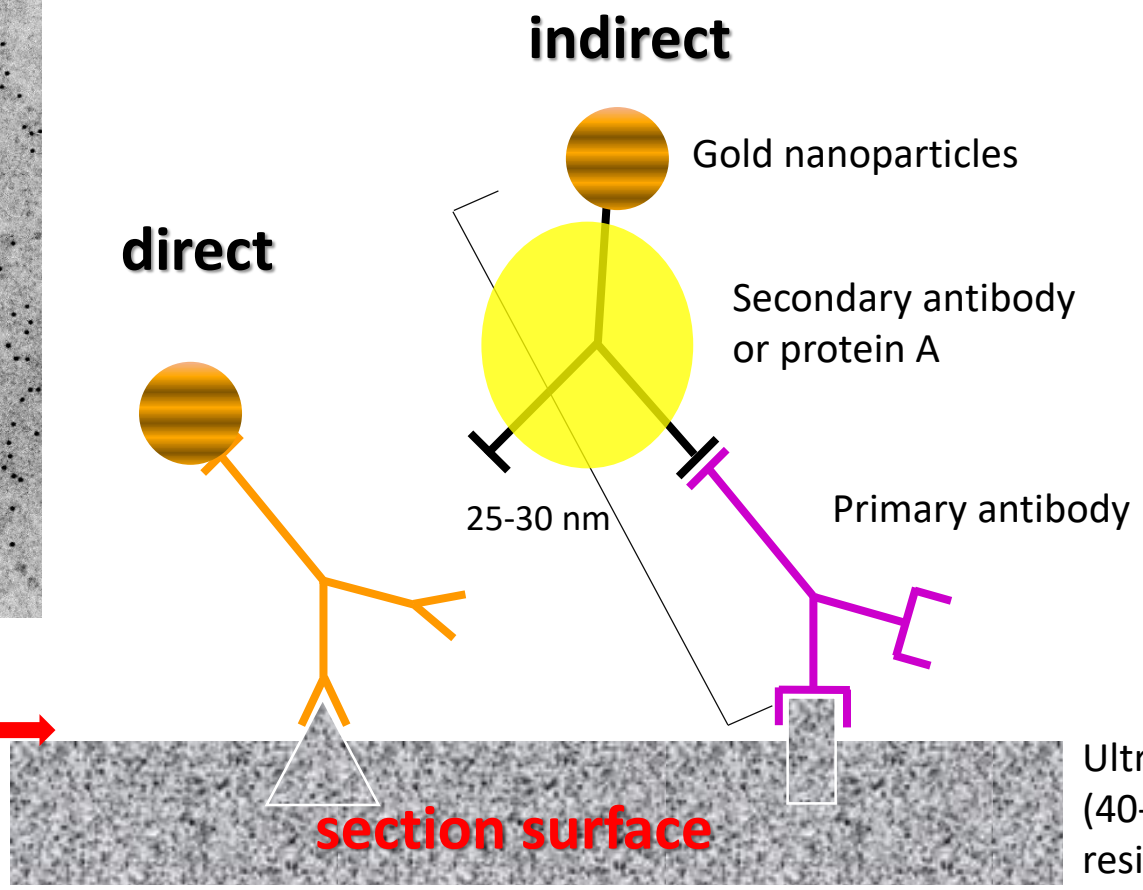
CLEM, cryo-CLEM

# Labelling of specific molecules in cellular context

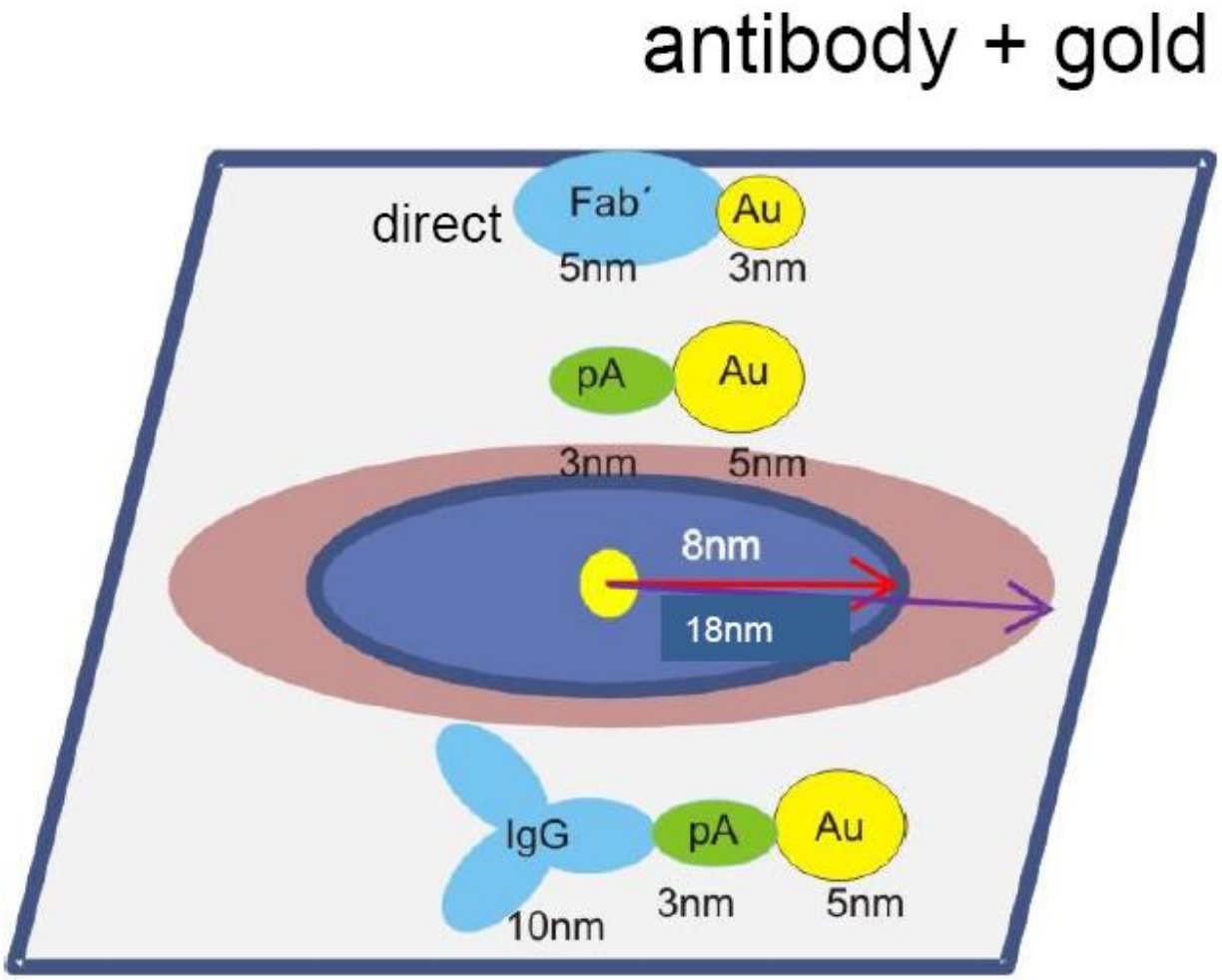
# Immunogold labeling for electron microscopy

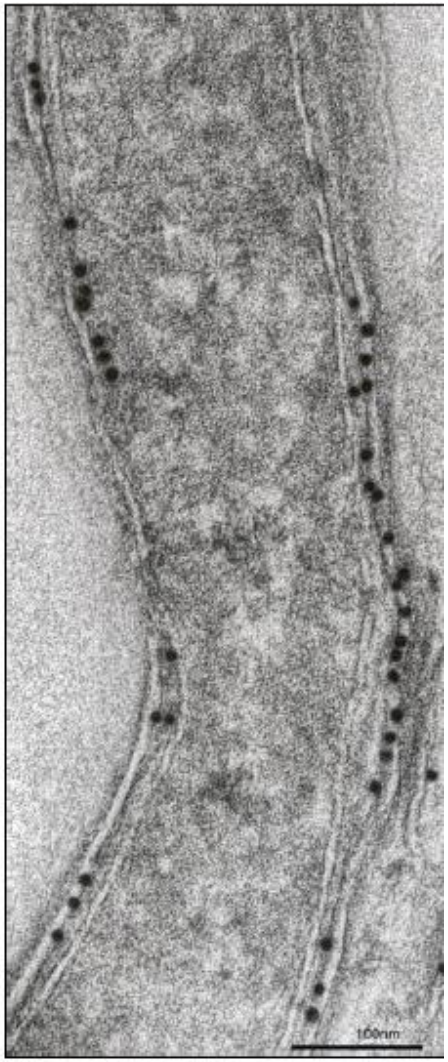


Histone H1 labeling in mitotic chromosomes, HeLa cell, 12 nm gold



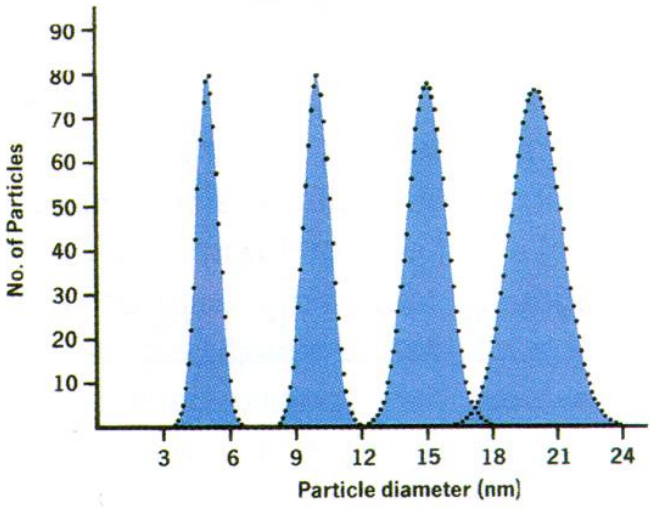
# How can we improve the resolution?

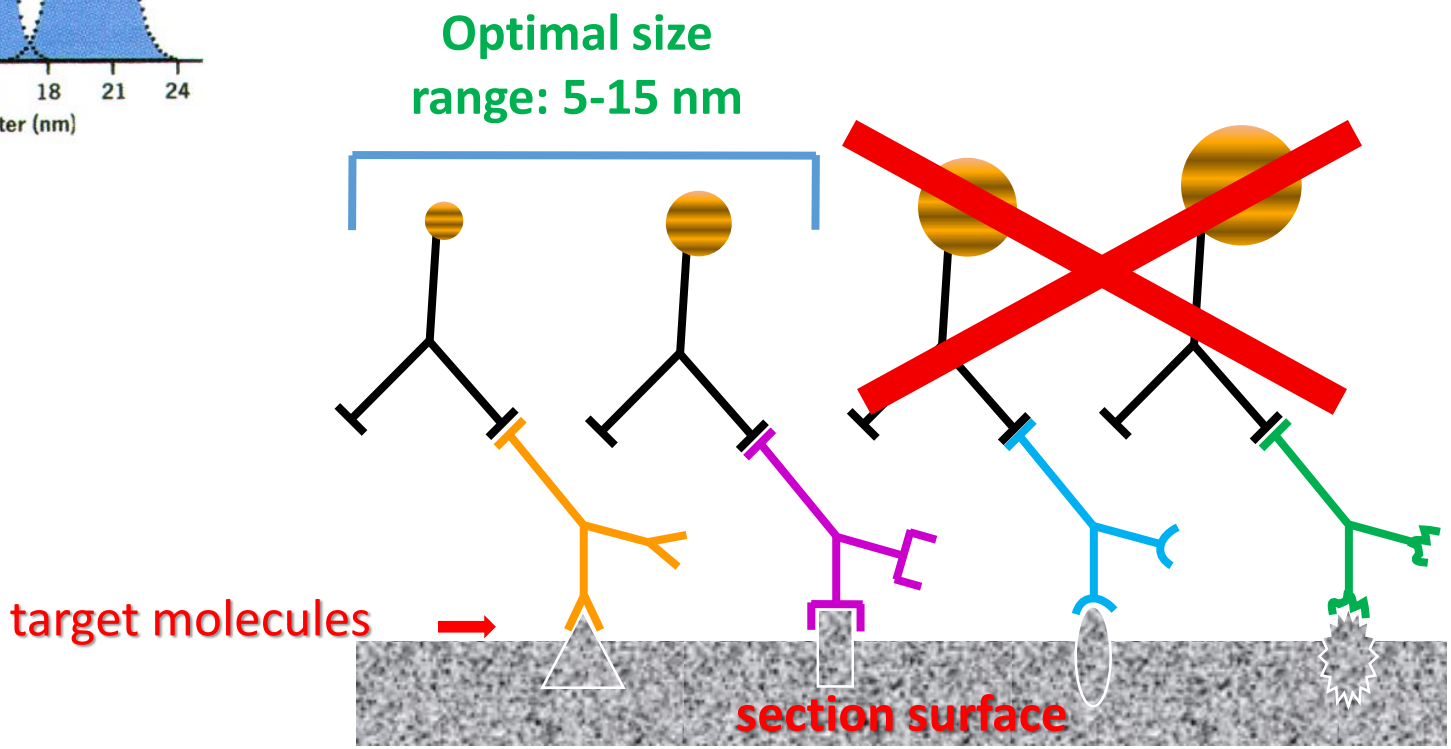


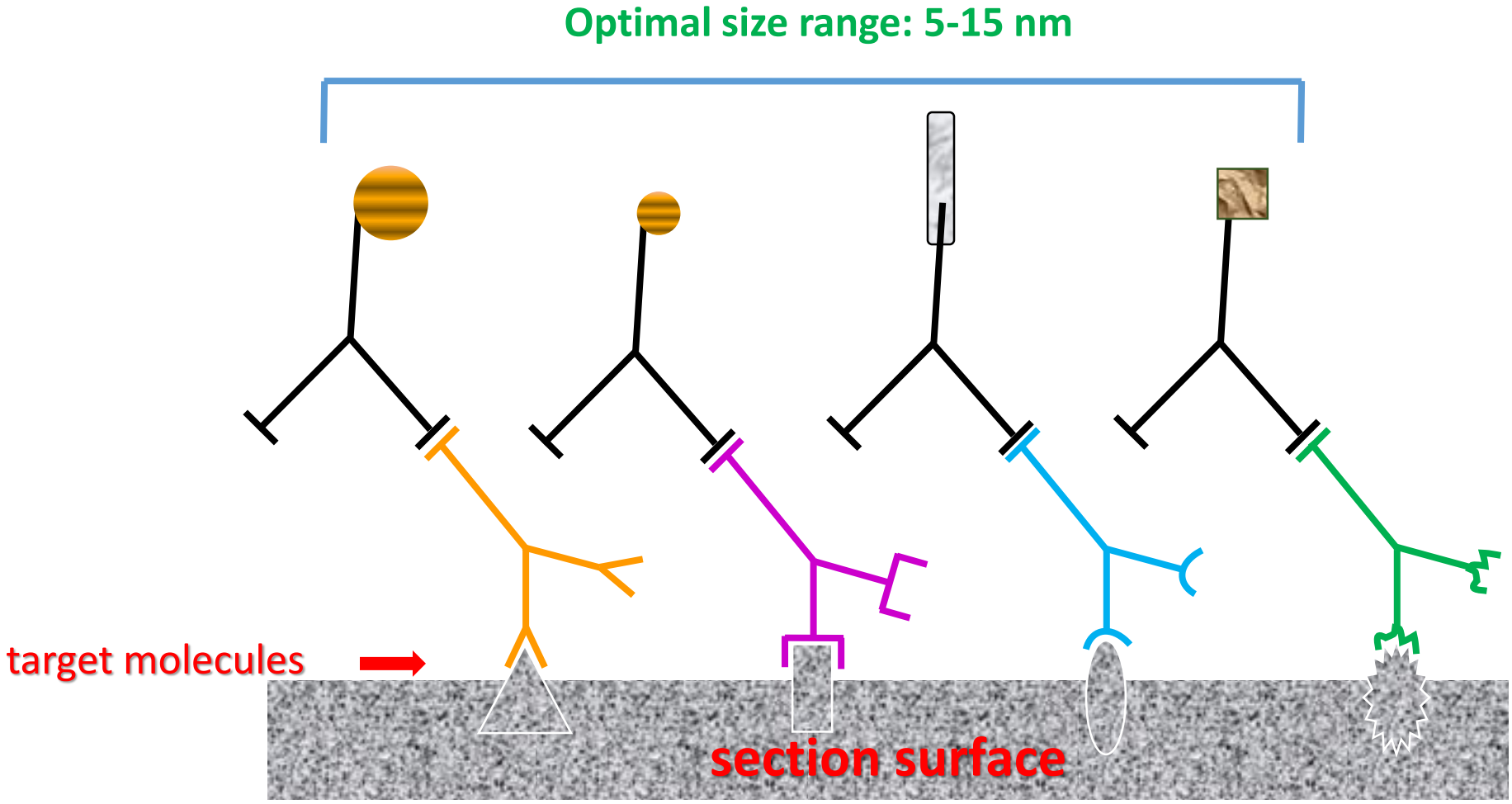
**The best theoretical resolution:** Fab fragments conjugated directly to very small Au particles giving „worstcase“- resolution of 8 nm radius
Specific IgG+ 5 nmAu/protein A = 18 nm

# Gold particle size and multiple immunolabeling

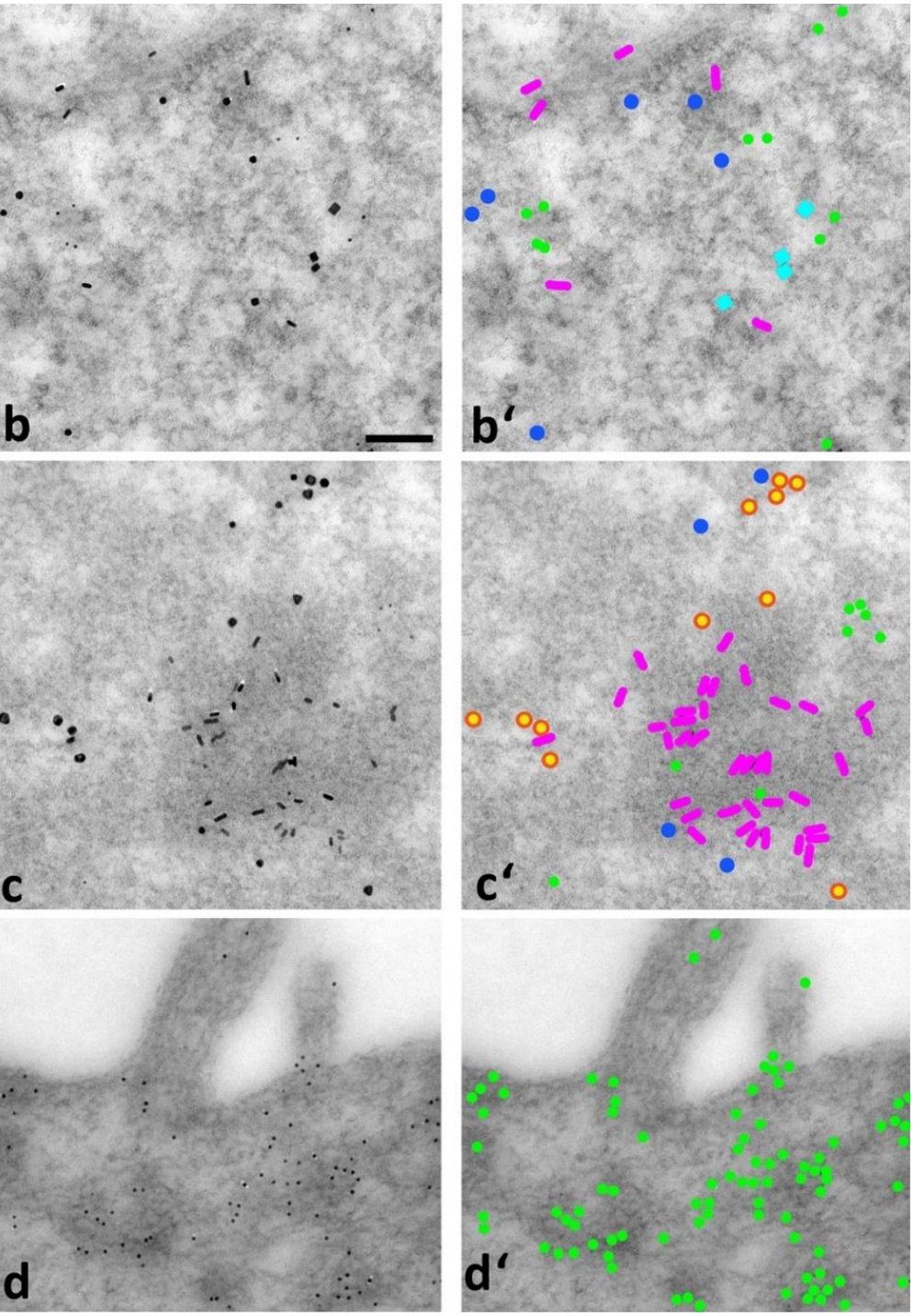


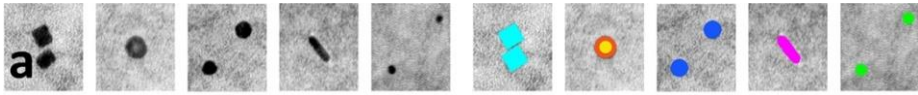



# We need nanoparticles differeing by other parameters



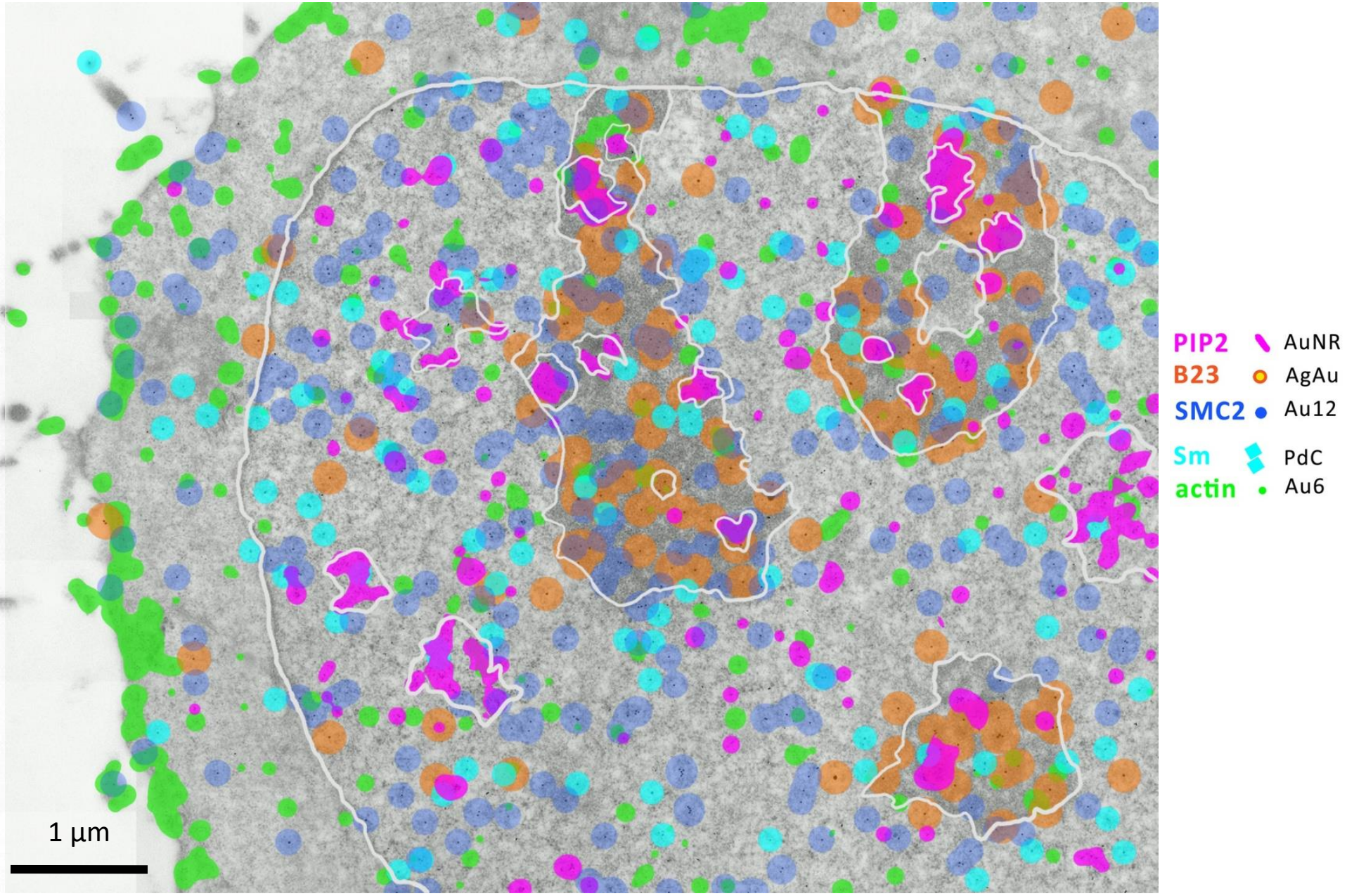
# Immunolabeling of a biological sample




PIP2 AuNR
B23 AgAu
SMC2 Au12
Sm PdC
actin Au6

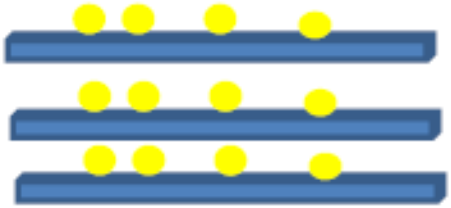
V. V. Philimonenko, A. A. Philimonenko, I. Šloufová, M. Hrubý, F. Novotný, Z. Halbhuber, M. Krivjanská, J. Nebesářová, M. Šlouf, and P. Hozák. Simultaneous detection of multiple targets for ultrastructural immunocytochemistry. Histochem Cell Biol. 2014; 141(3): 229–239




Philimonenko et al., Histochem Cell Biol. 2014; 141(3): 229–239

# Methods of immunolabeling

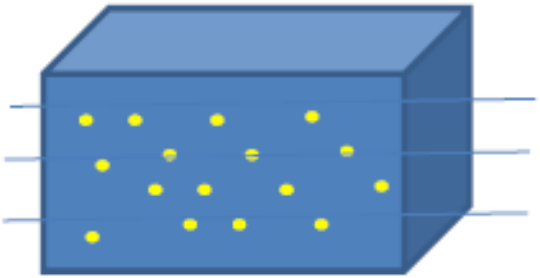
On-section



**Post-embedding** – on plastic section – lower efficiency

**Tokuyasu method** – thawed cryosections

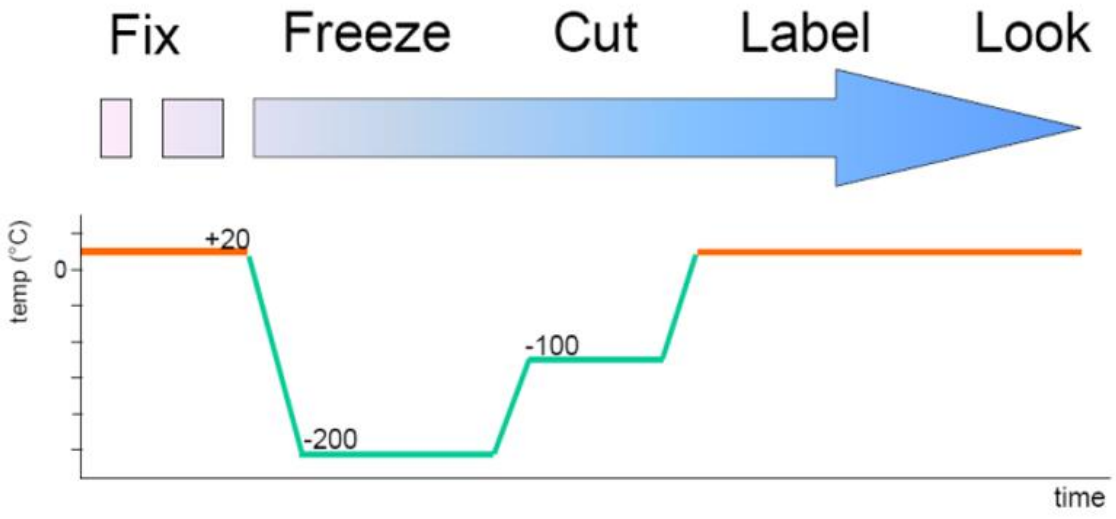
In the volume

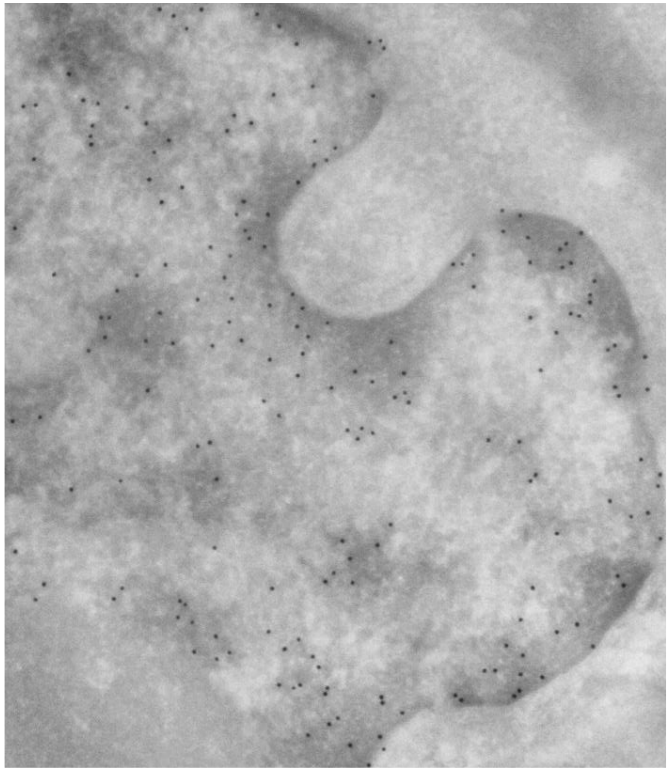


**Pre-embedding** – before embedding into the resin

# Tokuaysu technique

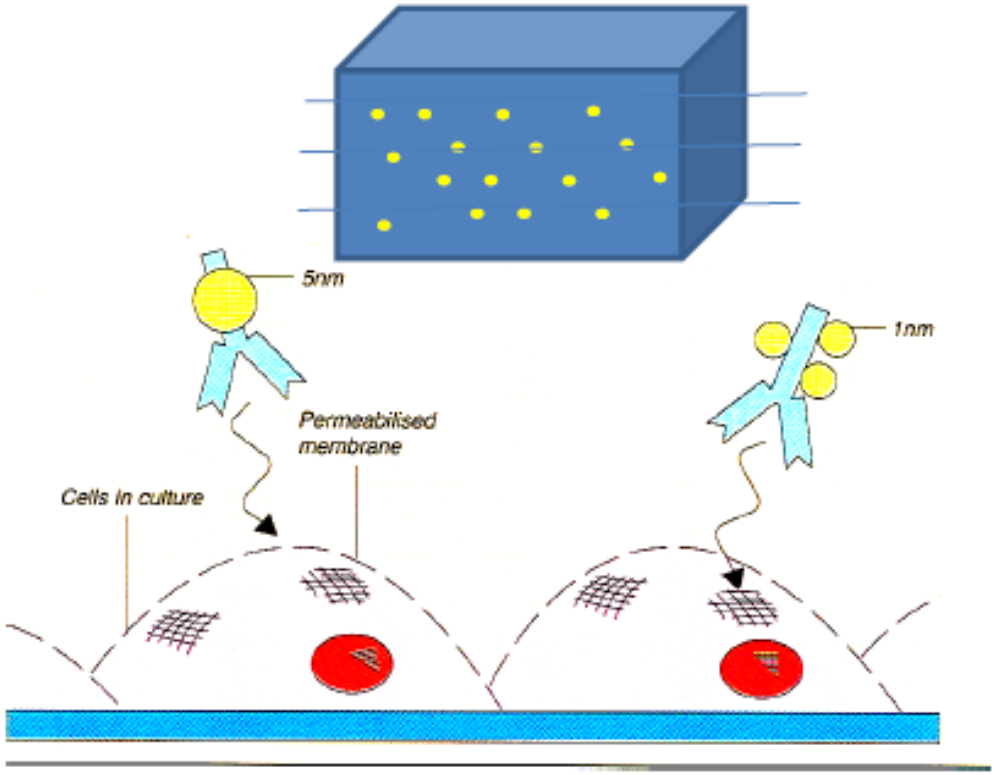
Tokuyasy, KT. 1973: A technique for ultracryotomy of cell suspensions and tissues. J Cell Biol. May;57(2):551-65,



# Labelling of intracellular antigens

## Pre-embedding



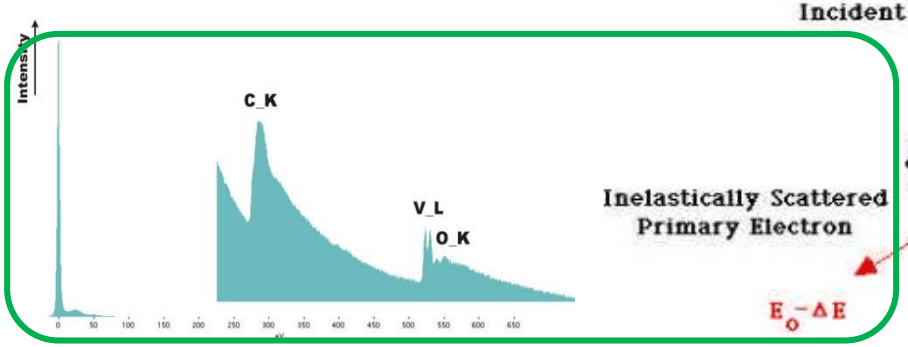

+ labelling throughout the whole section thickness
+antigens are not denatured prior labelling

- antigen accessibility, permeabilization → cell ultrastructure, extraction of many components and risk of false negative results
- Larger gold conjugates (>5nm) difuse unefficiently

HRP-coupled antibodies, GFP-anti GFP antibodies, fluorochrom/Ab, fluorescently conjugated Nanogold antibodies, ultrasmall gold conjugated / Ag enhancement

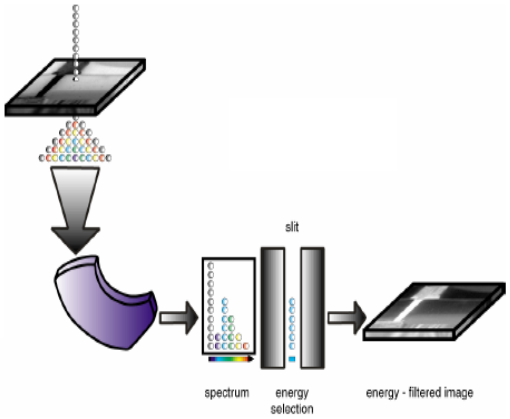
# Analytical EM

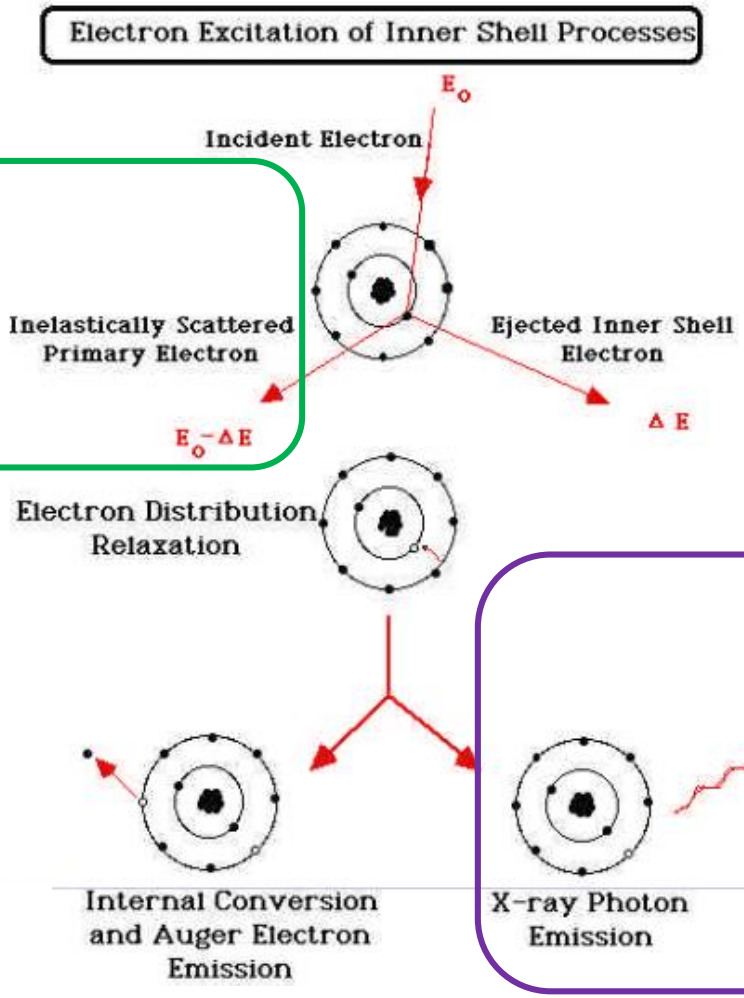
# Electron Microscopy: element specific signals

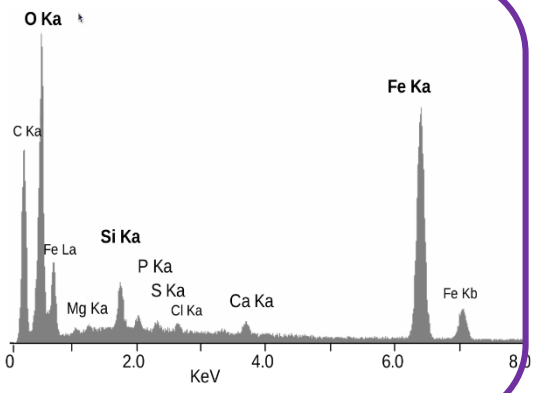



**EELS**

Electron-energy loss spectroscopy



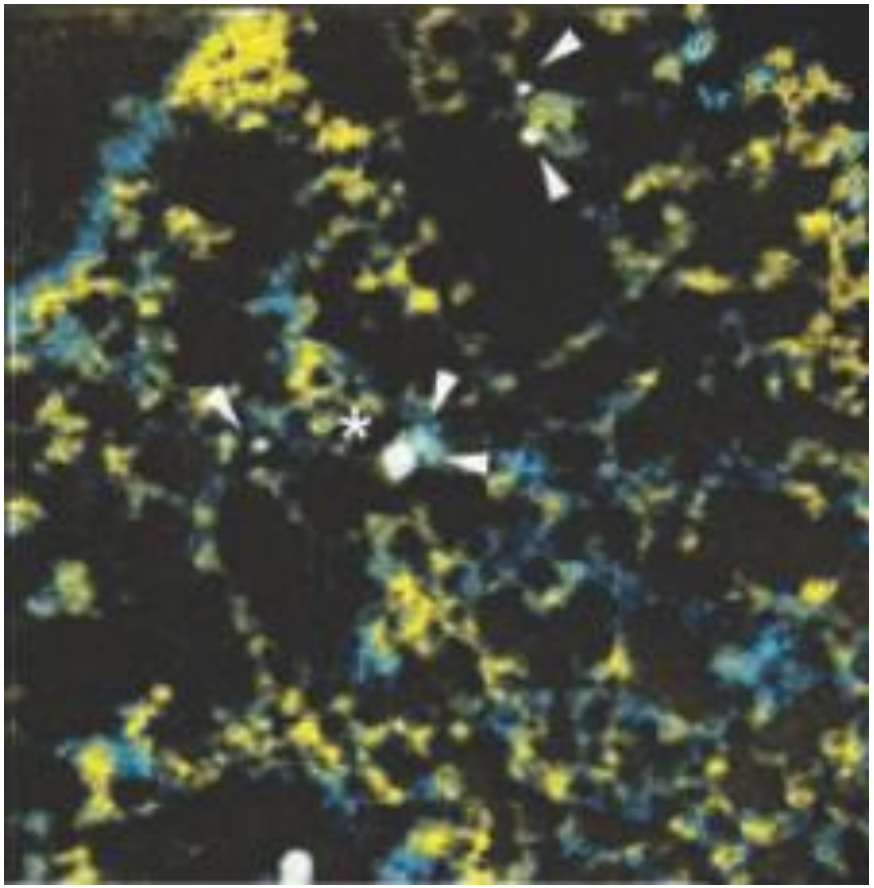




**EDS**

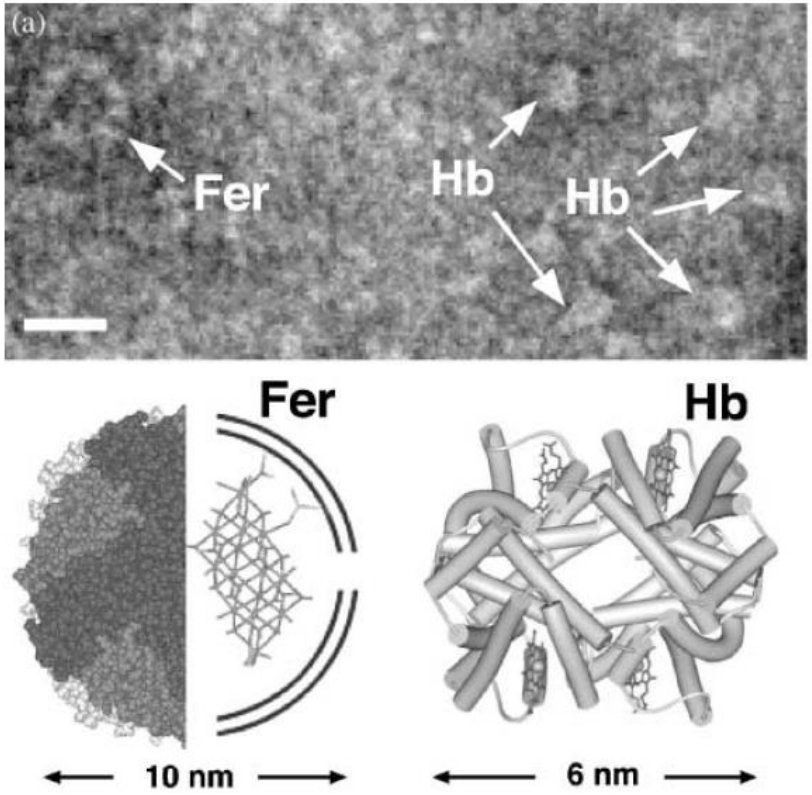
Energy-dispersive X-ray spectroscopy

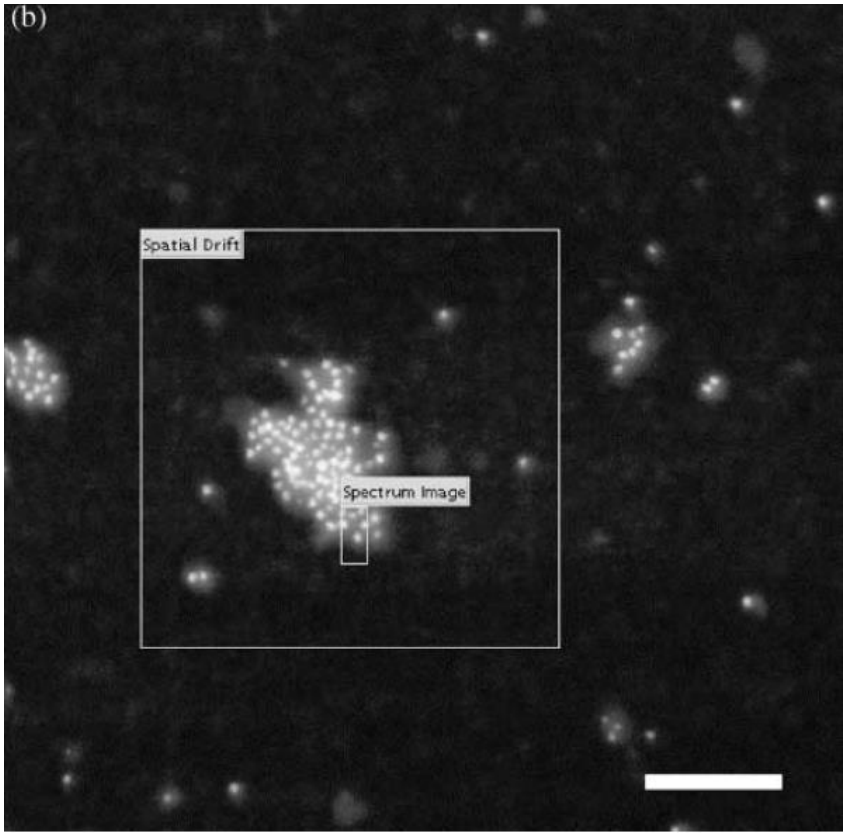
# Mapping of protein-rich and nucleic-acid rich structures in the cell using EFTEM



Ahmed K et al. PLoS One. 2010;5(5):e10531.

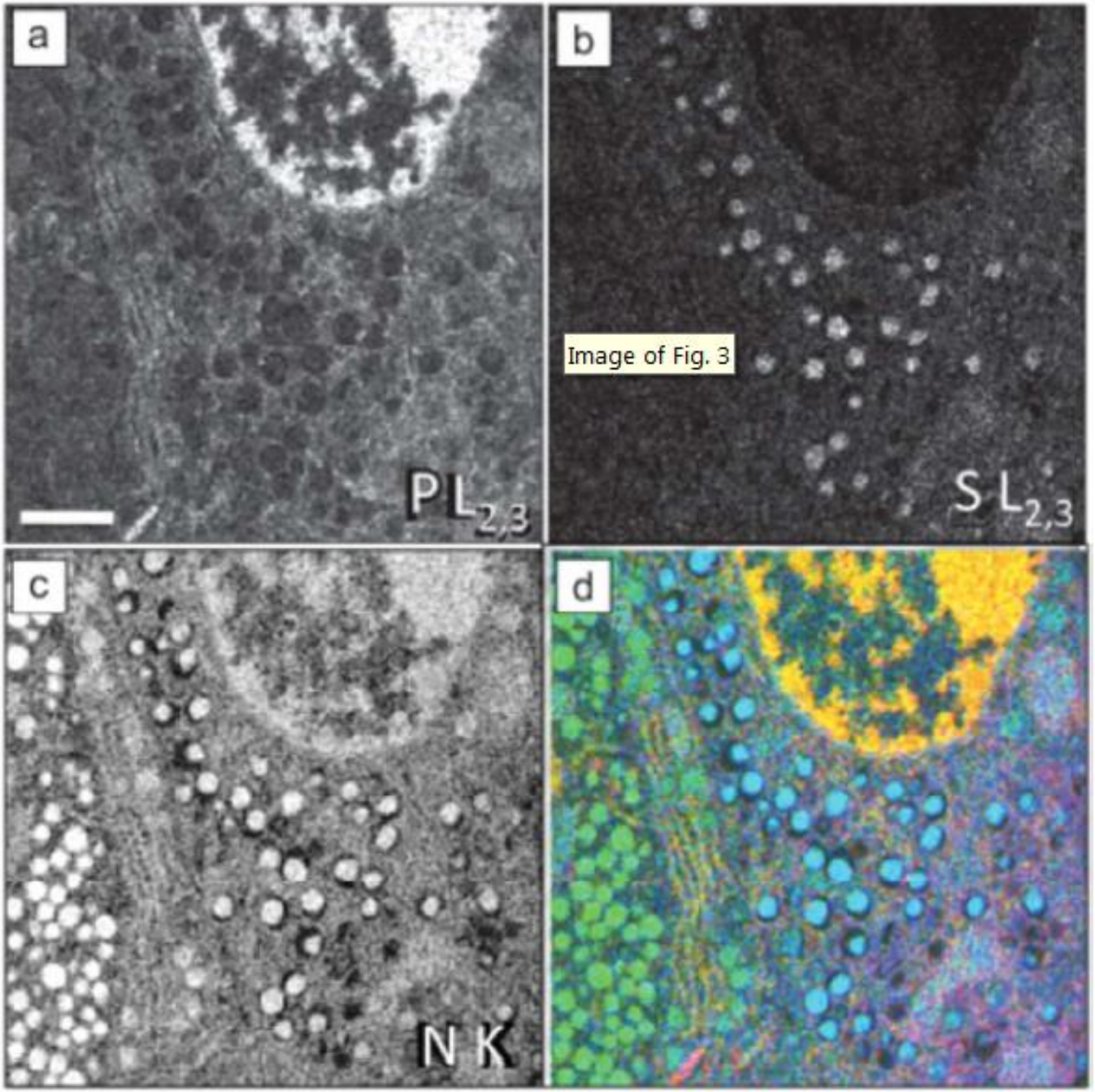
# EELS mapping of single iron atoms in haemoglobin



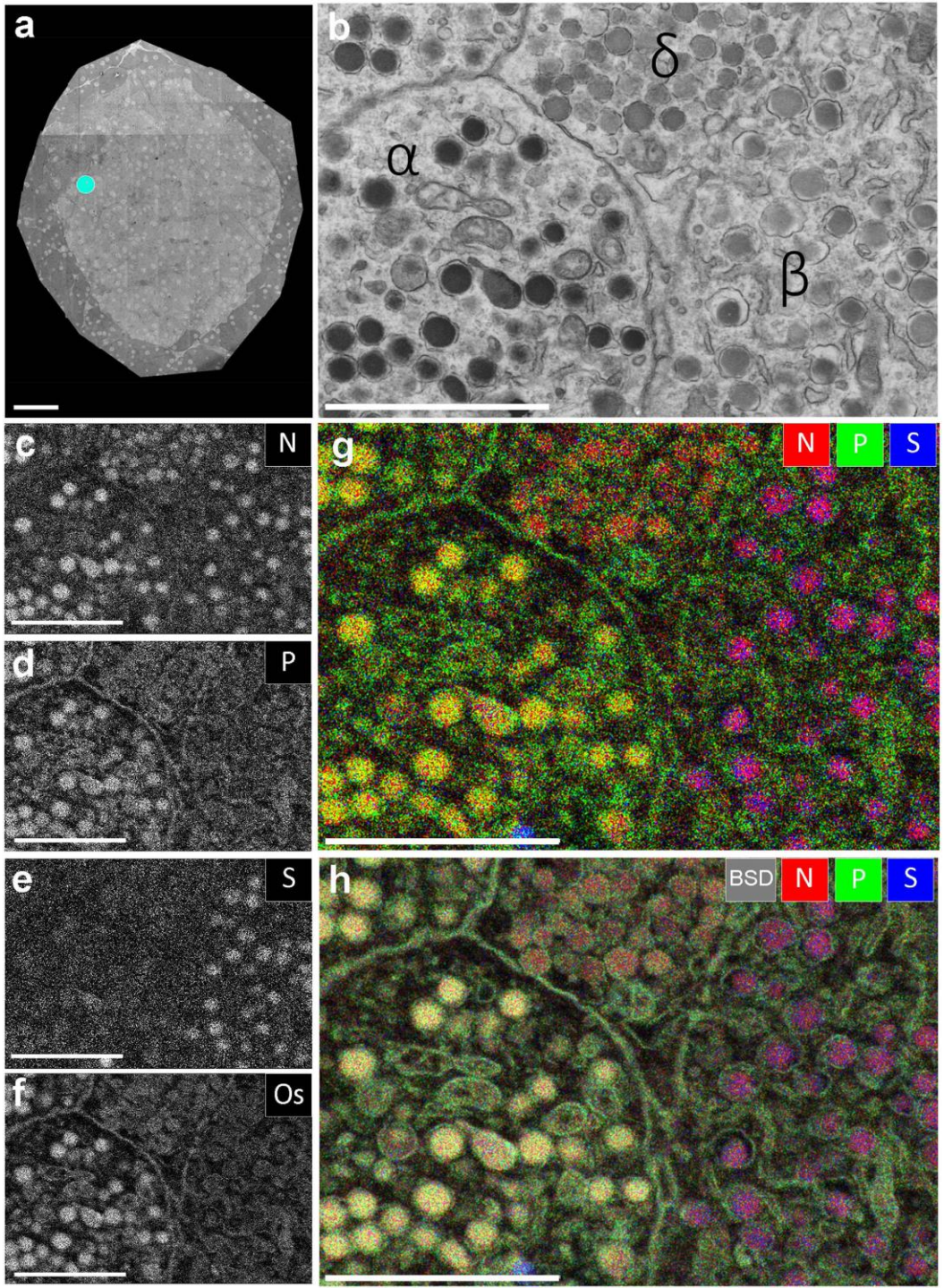

Leapman R.D., J Micros 2003

# EFTEM spectrum-imaging of unstained section of mouse pancreatic islet of Langerhans




Goping et al.,
Micros Res Techn 2003

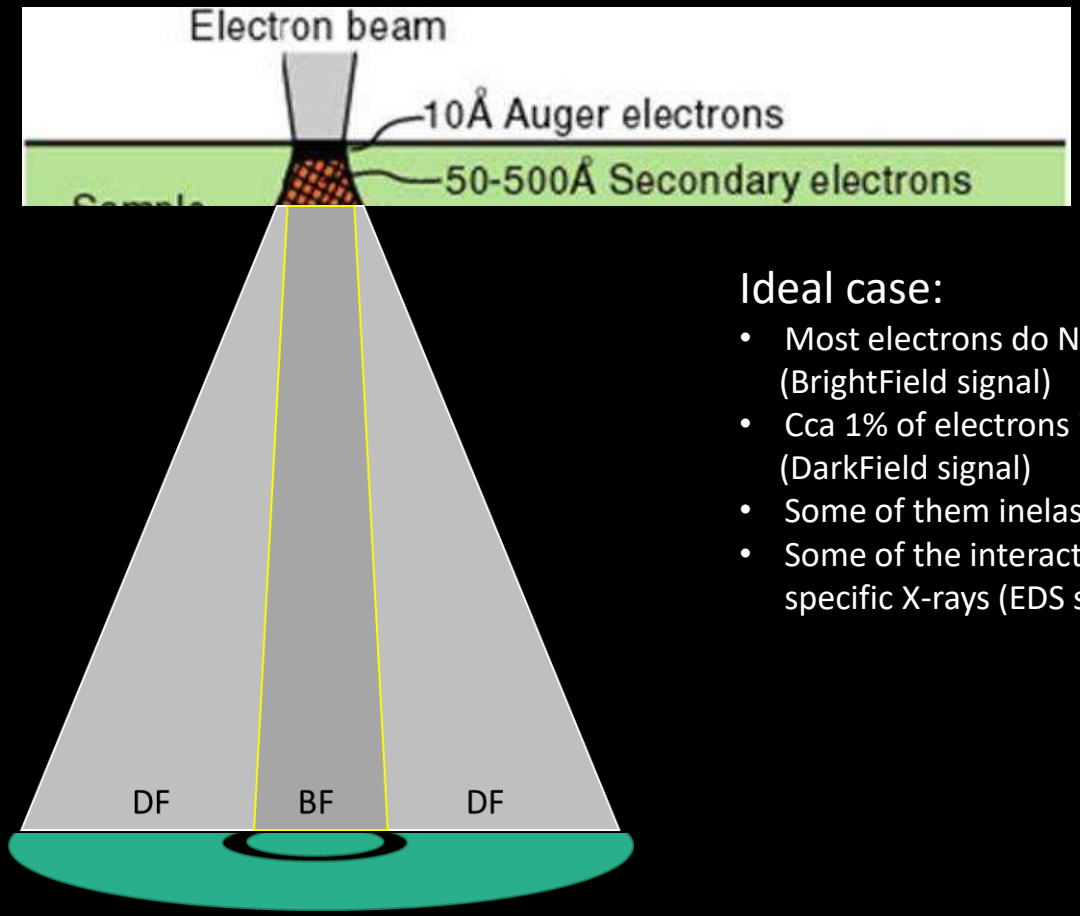
# EDS mapping




mouse pancreatic islet of Langerhans

Scotuzzi et al., Sci Rep 2017

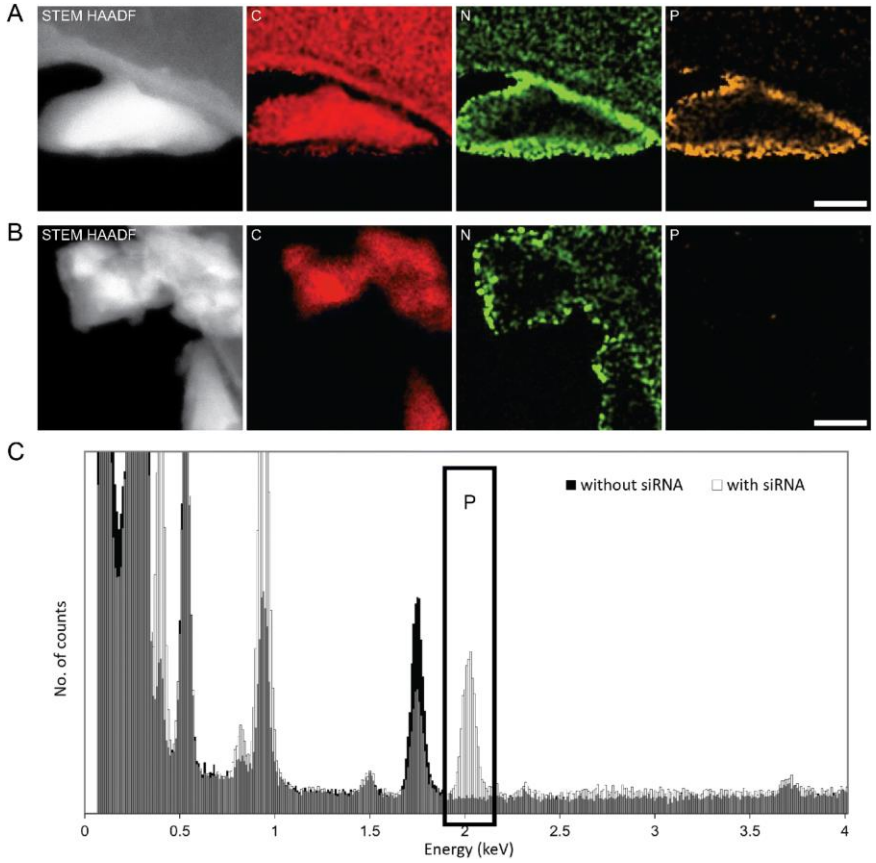
# Spatial resolution of EDS




Ideal case:

- Most electrons do NOT interact at all (BrightField signal)
- Cca 1% of electrons interact once (DarkField signal)
- Some of them inelastically
- Some of the interactions produce specific X-rays (EDS signal)

# Nanoparticle composition analysis by EDS




Nanodiamond particles with polymer coating to serve as siRNA delivery system

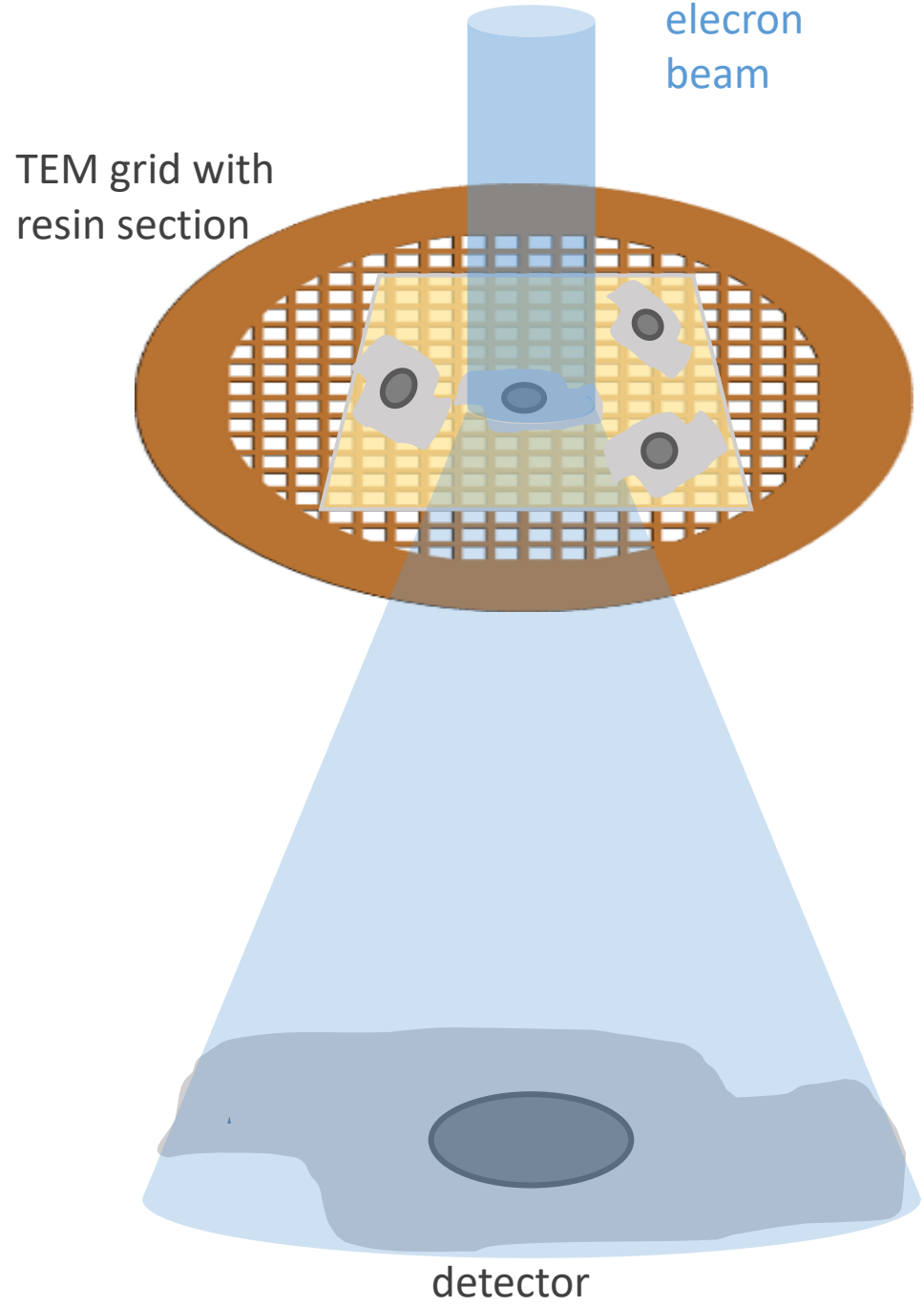
- Lacking direct proof for RNA binding to the polymer layer

EDS elemental mapping reveals:

- Core-shell structure with N-containing polymer on surface
- Increase in N and presence of P after siRNA binding

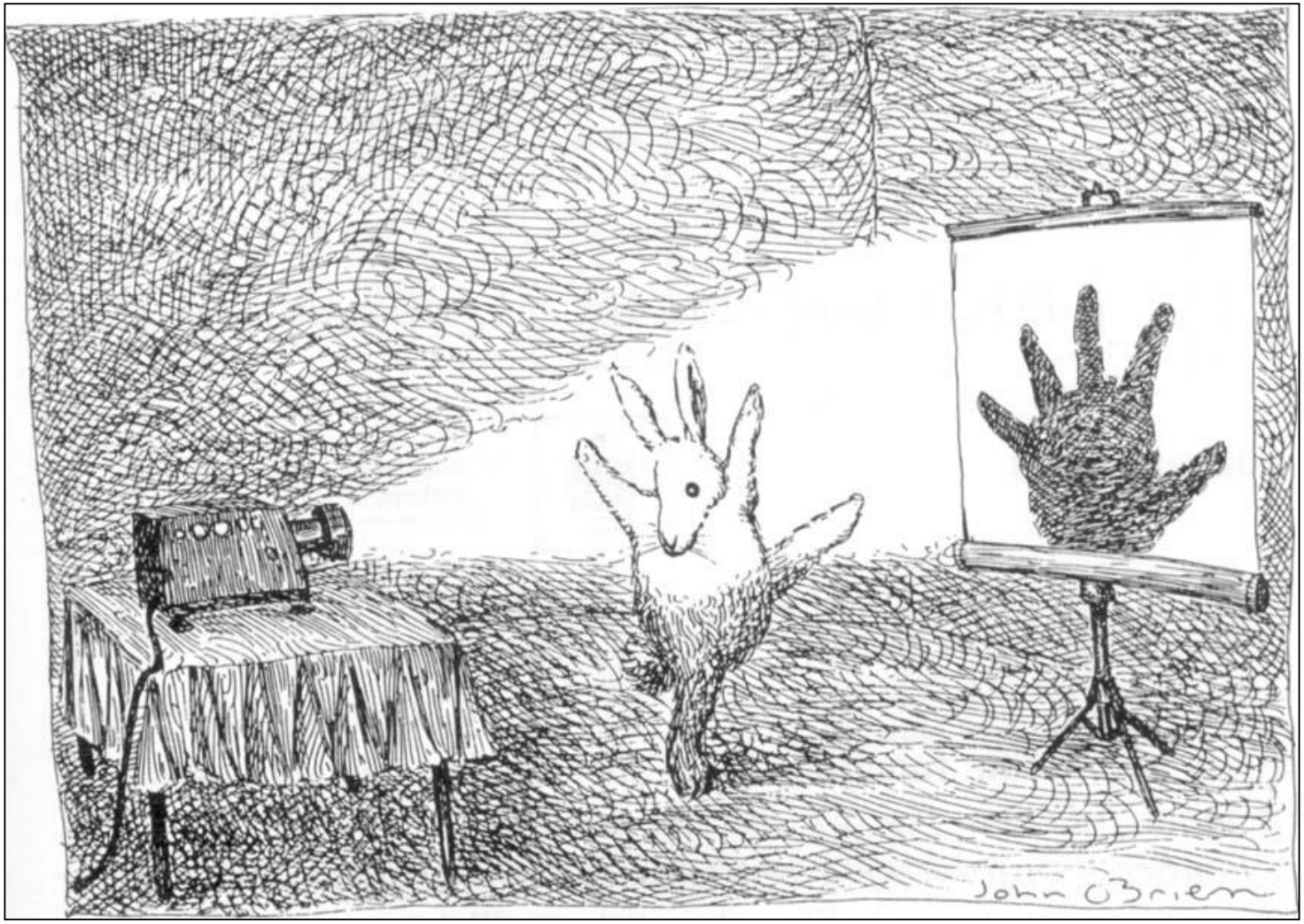
Majer, J., M. Kindermann, D. Pinkas, D. Chvatil, P. Cigler and L. Libusova (2024). "**Cellular uptake and fate of cationic polymer-coated nanodiamonds delivering siRNA: a mechanistic study**." Nanoscale **16**(5): 2490-2503.

# Volume electron microscopy


elecron
beam
TEM grid with
resin section
detector

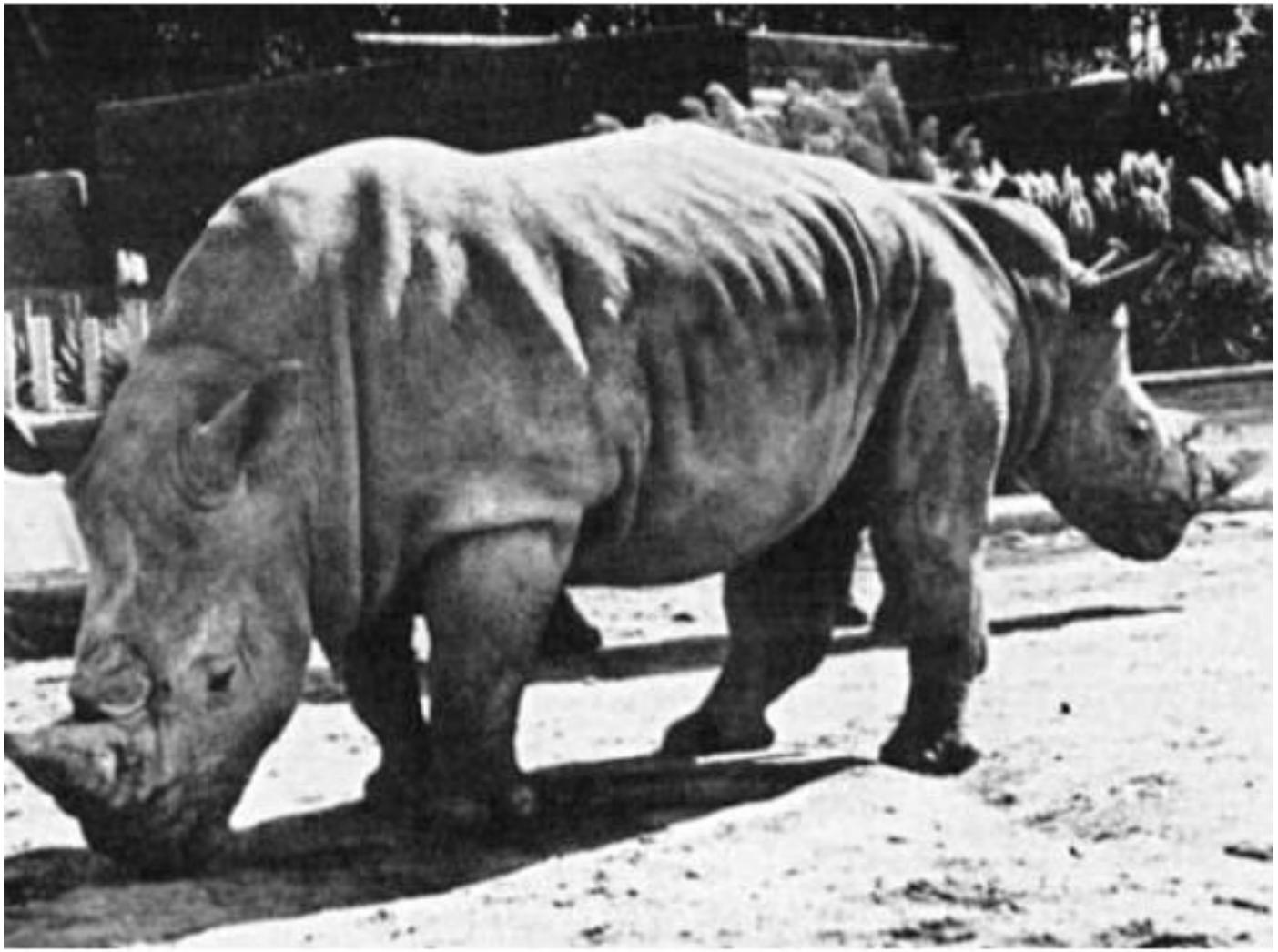
# Why do we need 3D information?

- Every TEM image is a 2D projection of a 3D/4D object



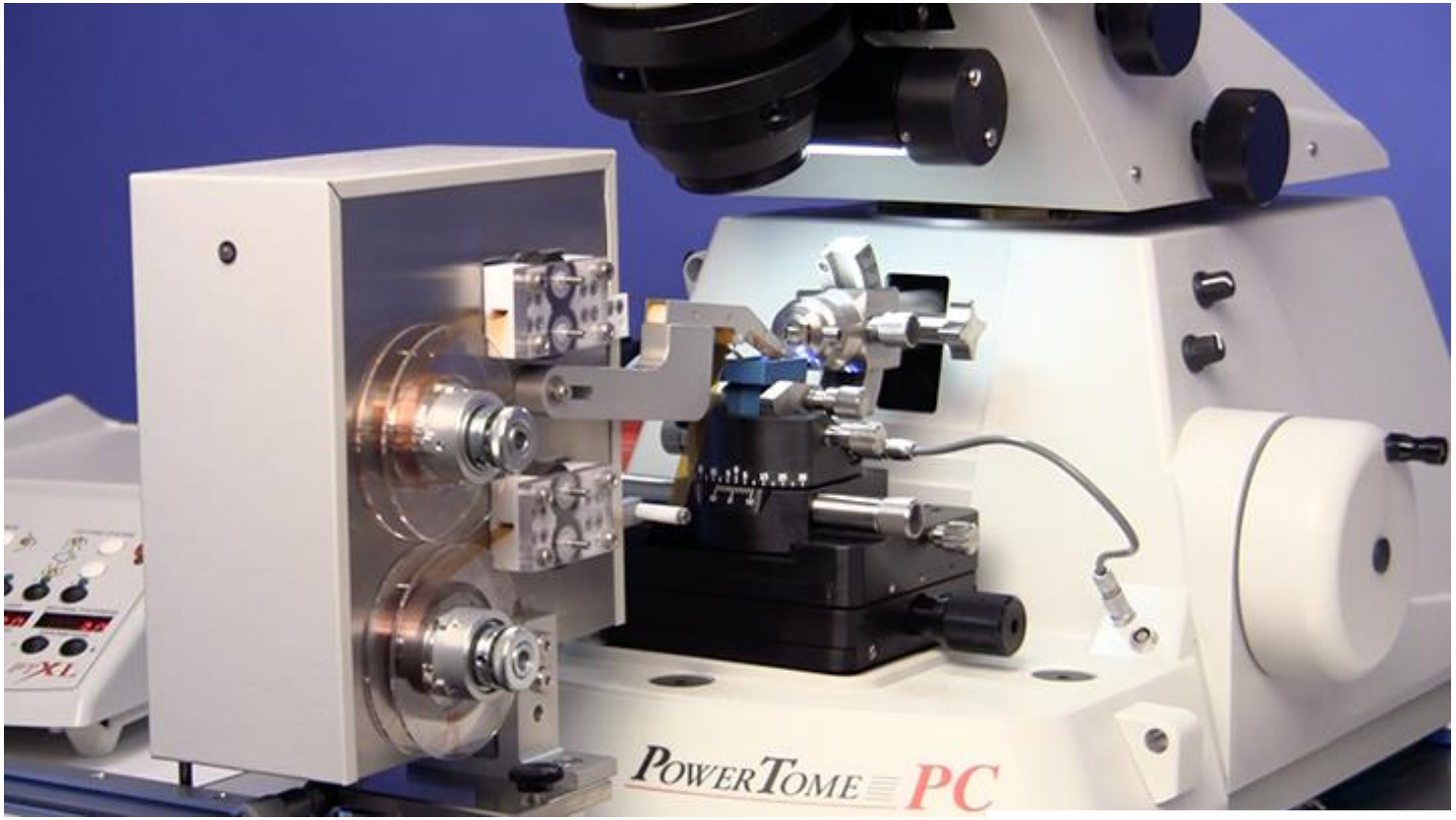

Drawing by John O'Brien, The New Yorker Magazine (1991)

The TEM presents us with 2D images of 3D specimens, viewed in transmission. Our eyes and brain routinely understand reflected light images but are ill-equipped to interpret TEM images and so we must be cautious

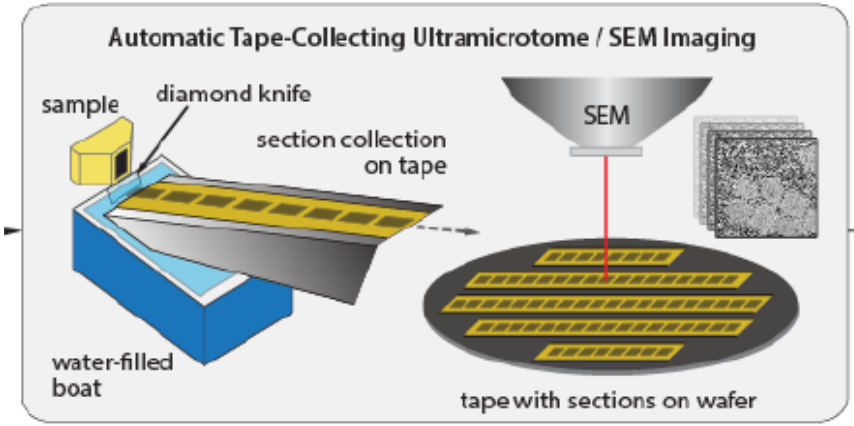


**Photograph of two rhinos taken so that, in projection, they appear as one two-headed beast. Such projection artifacts in reflected-light images are easily discernible to the human eye but similar artifacts in TEM images are easily mistaken for 'real' features.**

# Array tomography

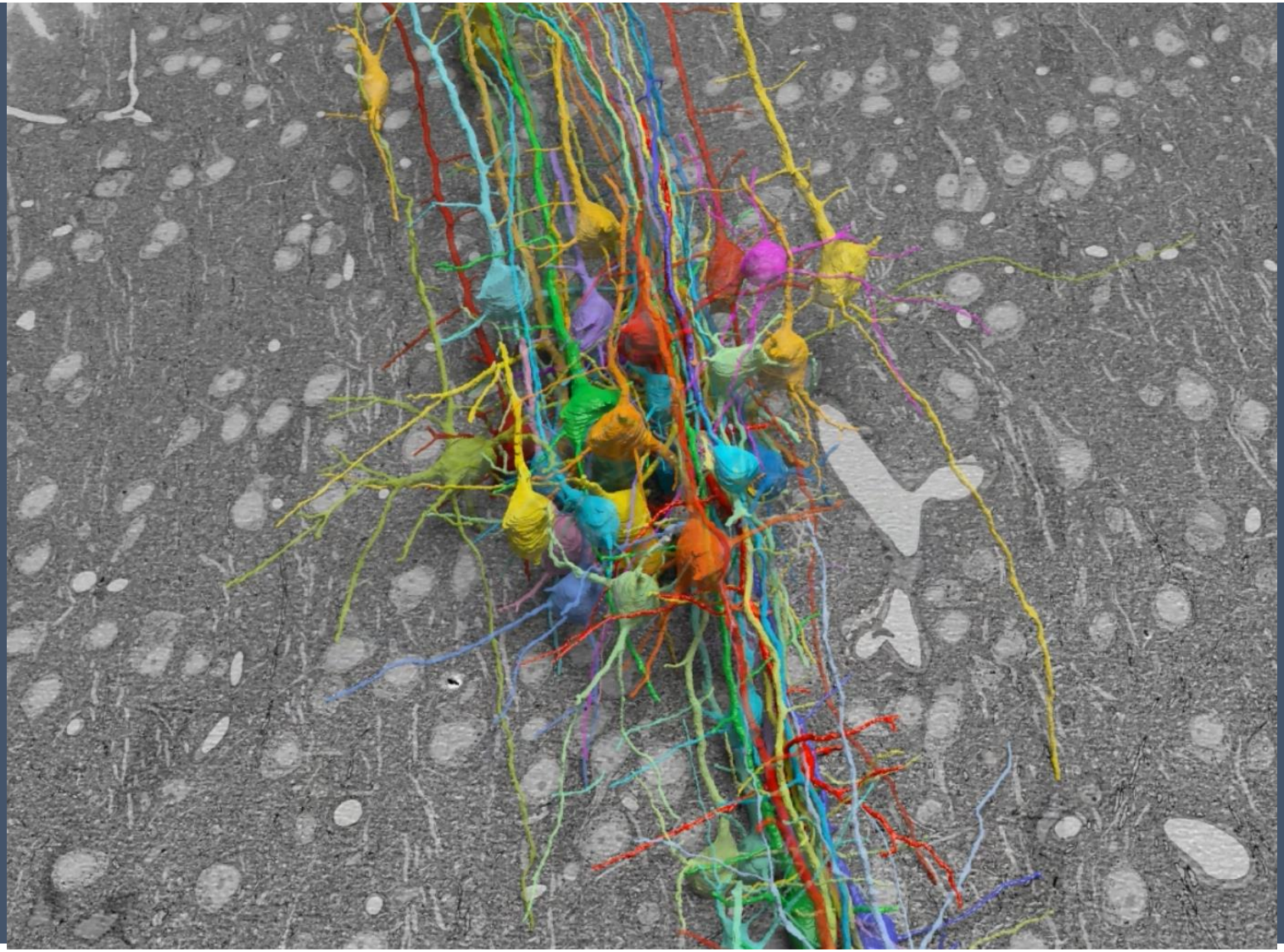



Thousands of utra-thin sections with a thickness of 30nm can be automatically collected on 8mm wide Kapton tape for SEM imaging and sebsequent 3-D reconstruction.

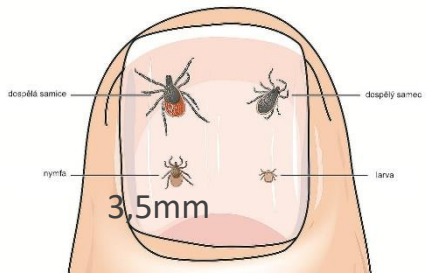


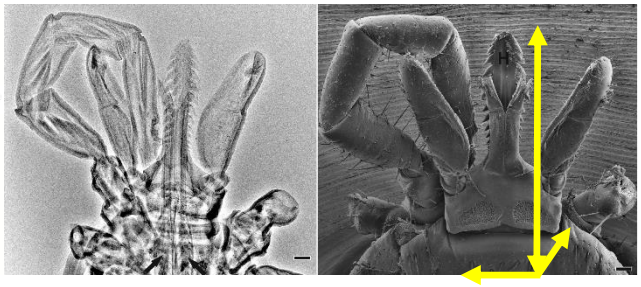
# Large Volume High Resolution 3D SEM

ATUMtome und Atlas Array Tomography
the perfect team for large Volume SEM Imaging



**455 µm** (5 µm/ 500nm)

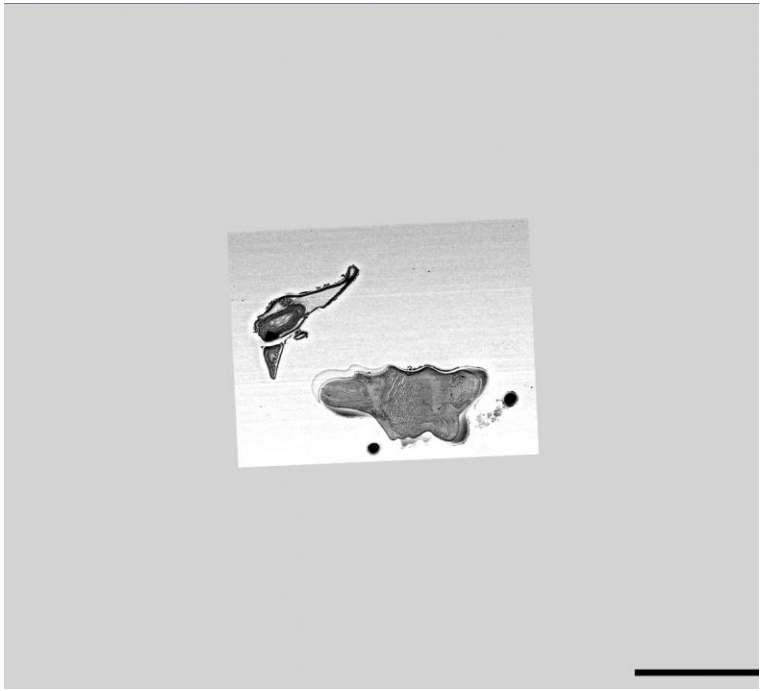


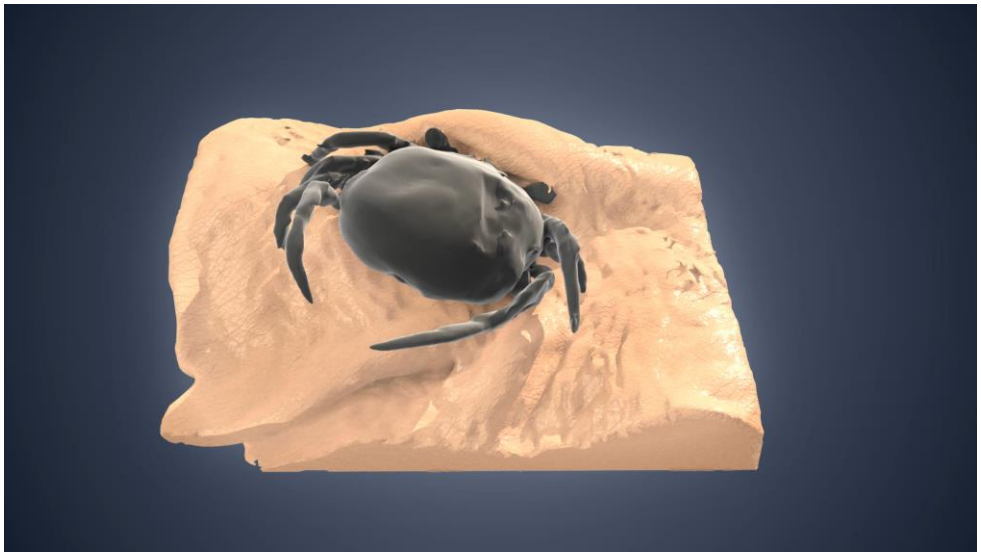
**156x136 µm** (39 nm)

ARRAY TOMOGRAPHY

Reconstruction of the feeding apparatus, unfed nymph *Ixodes ricinus*

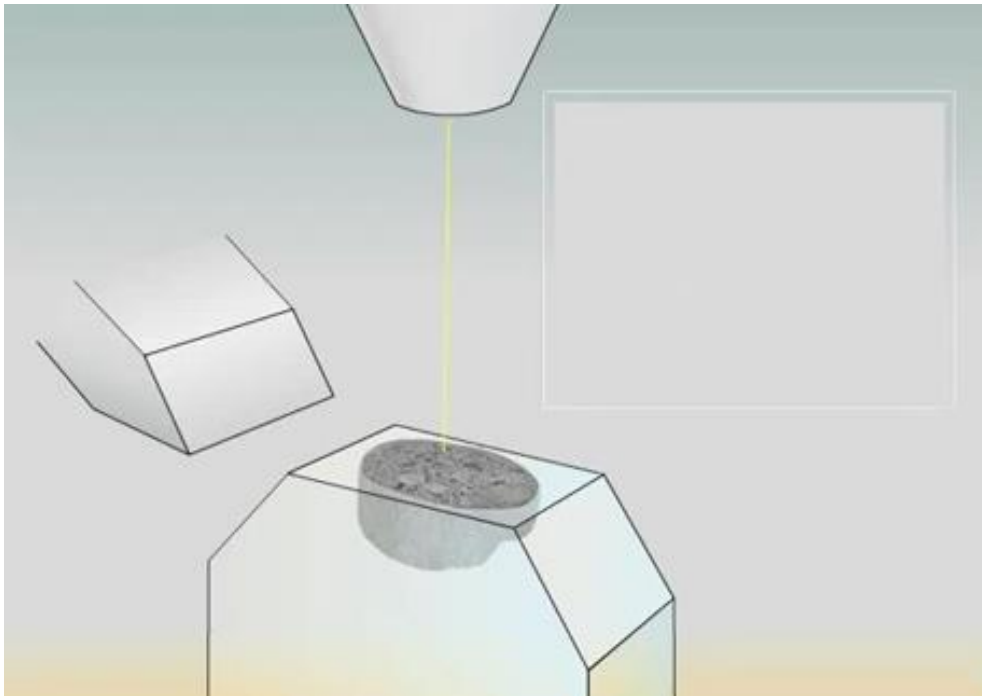


Vancova et al. 2020 SciRep

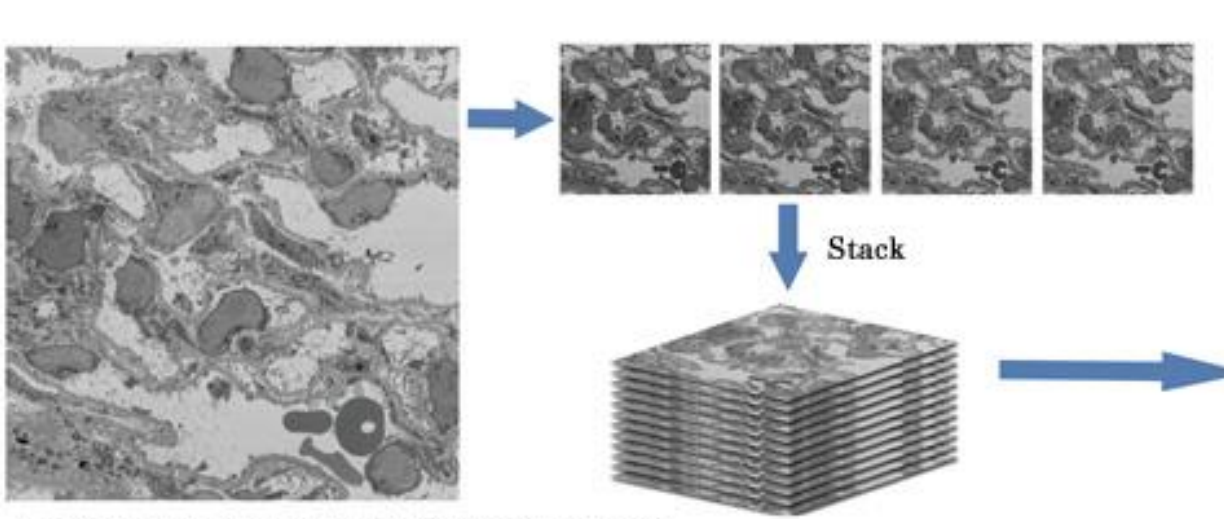


M. Vancova, LEM BC

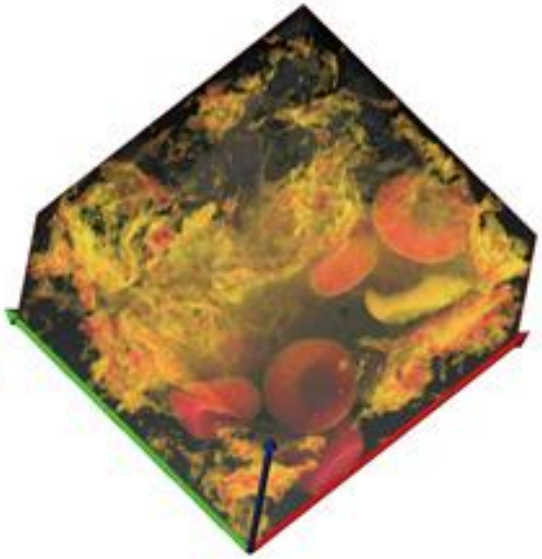
# Serial block face SEM



GATAN website
3View System




Acquired image (backscattered electron image)
Specimen: Mouse kidney glomerular cells



Jeol company website; JSM-7200F/ 7800F

# Large excretory organs of springtail – Minimal resin

SERIAL-BLOCK FACE SEM

SBEM strength:

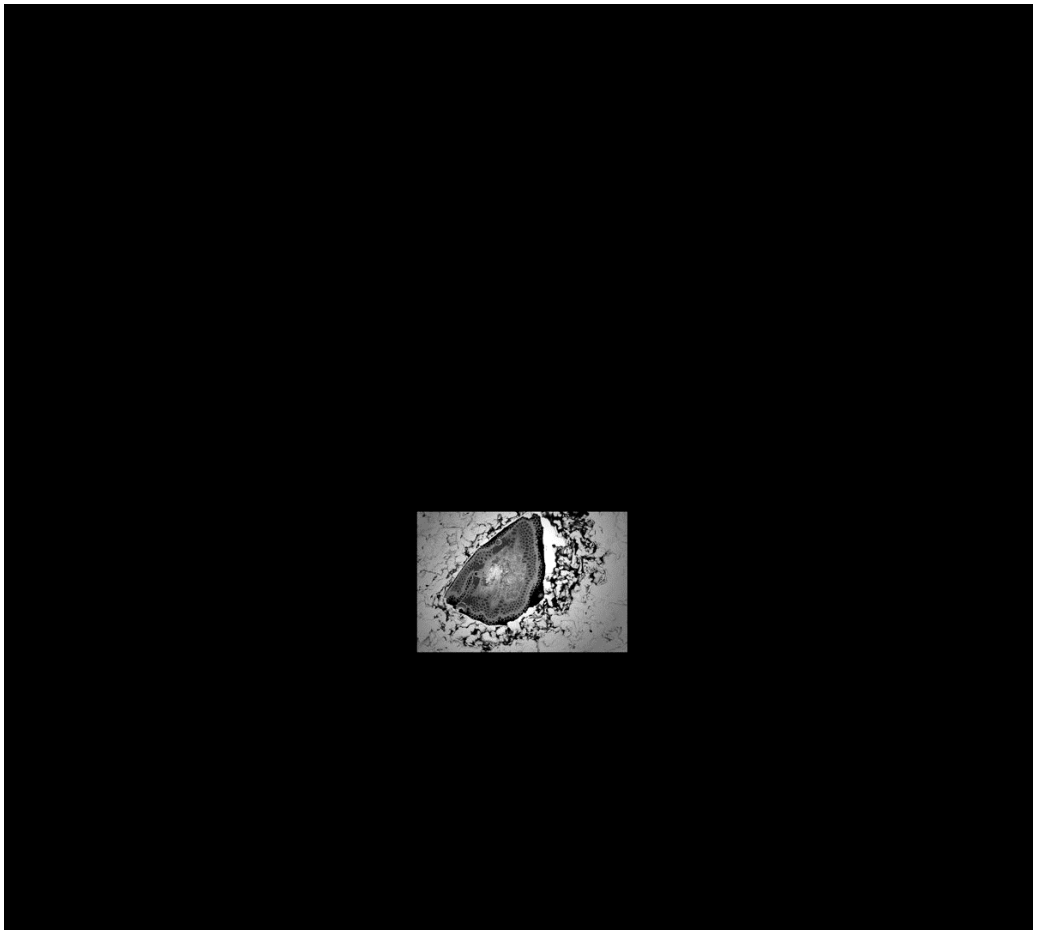
- Larger ROI
- Tiles can be adjusted during the run
  - moved around
  - expanded
  - collapsed

To note:

Acquisition time
Amount of data

Video shows approx every 5$^{th}$ slice (250 nm)

2 jumps to speed it up

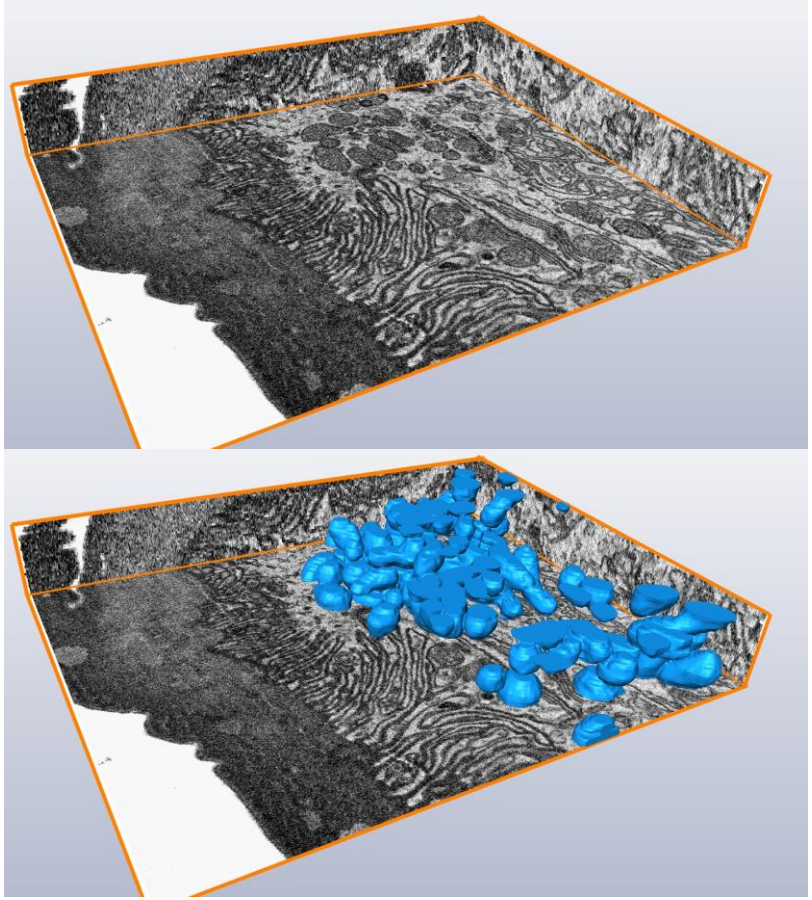


Konopová, B., Týč, J. Minimal resin embedding of SBF-SEM samples reduces charging and facilitates finding a surface-linked region of interest. *Front Zool* **20**, 29 (2023).

M. Vancova, LEM BC

# 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** |



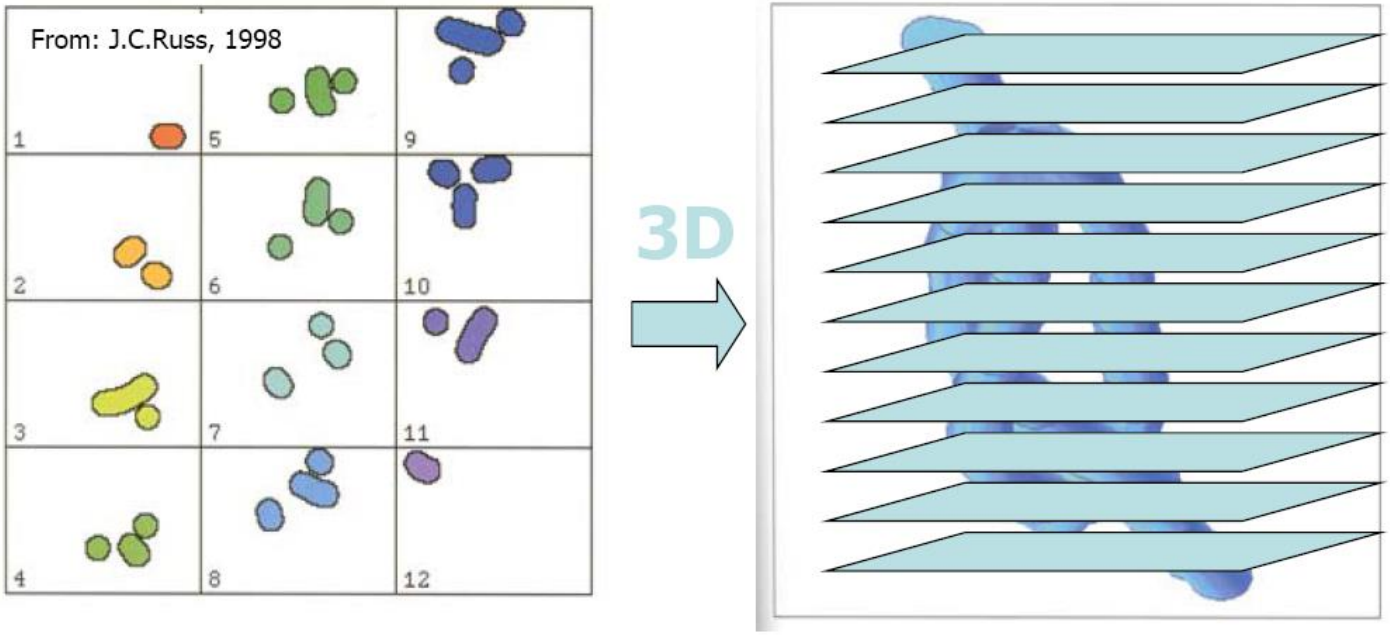
SERIAL-BLOCK FACE SEM

**Take home messages:**

**Large volumes are time and data storage demanding**
**Resolution is enough to distinguish mitochondrial cristae and other fine membranous details**

M. Vancova, LEM BC

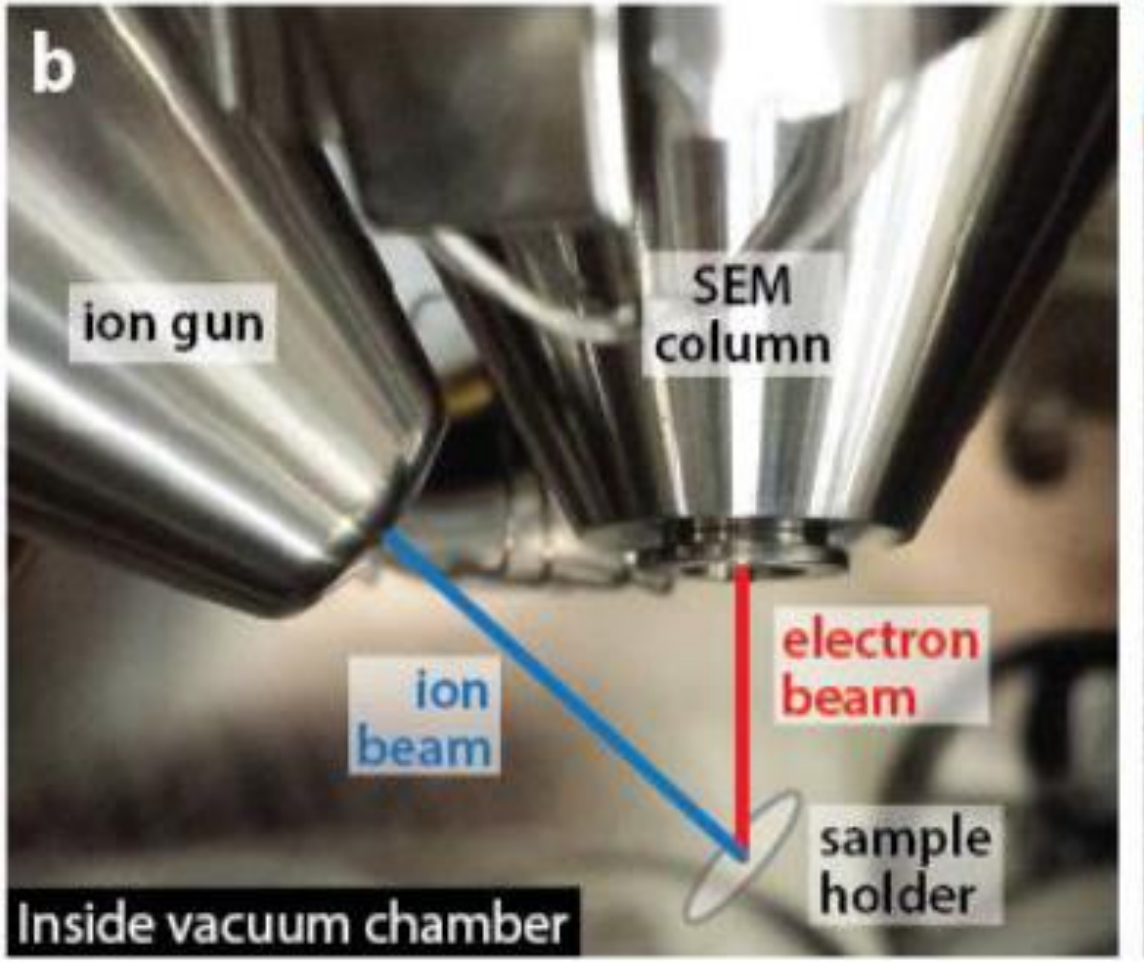
# Problem of 3D serial sectioning: reconstruction of disordered microstructures




2D volume fraction

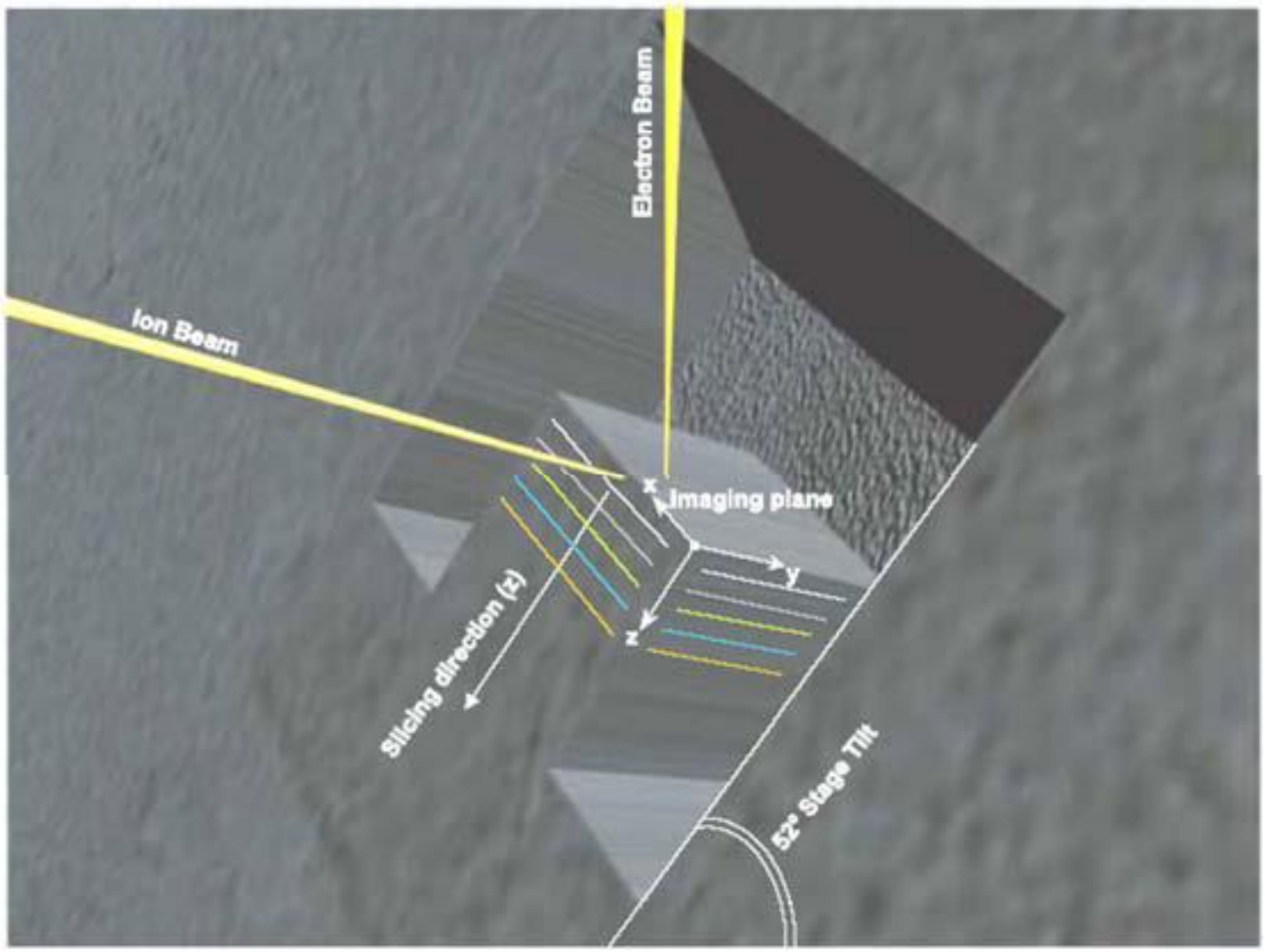
How many particles?
Shape?

# FIB SEM = dual beam

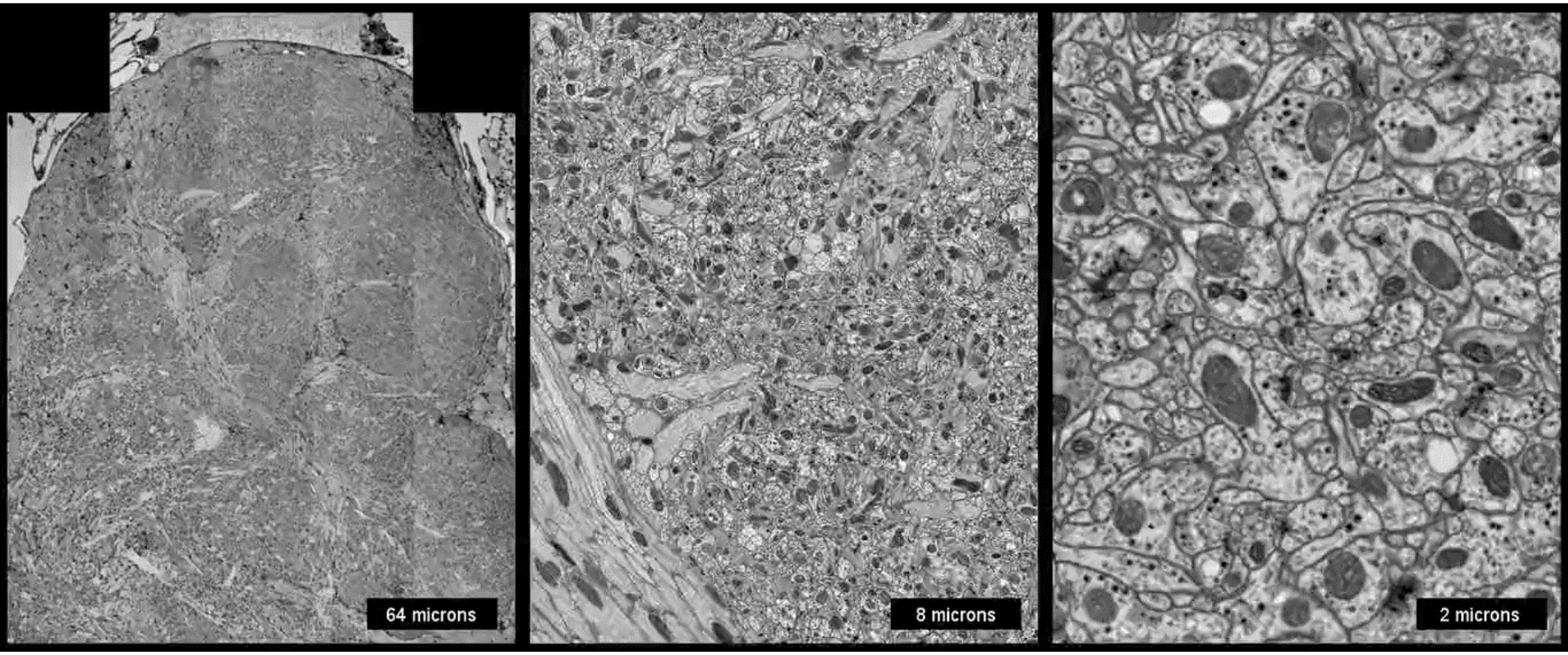



The sectioning is done by means of the focused ion beam working in the similar way as the scanning electron microscope.

# FIB SEM = dual beam

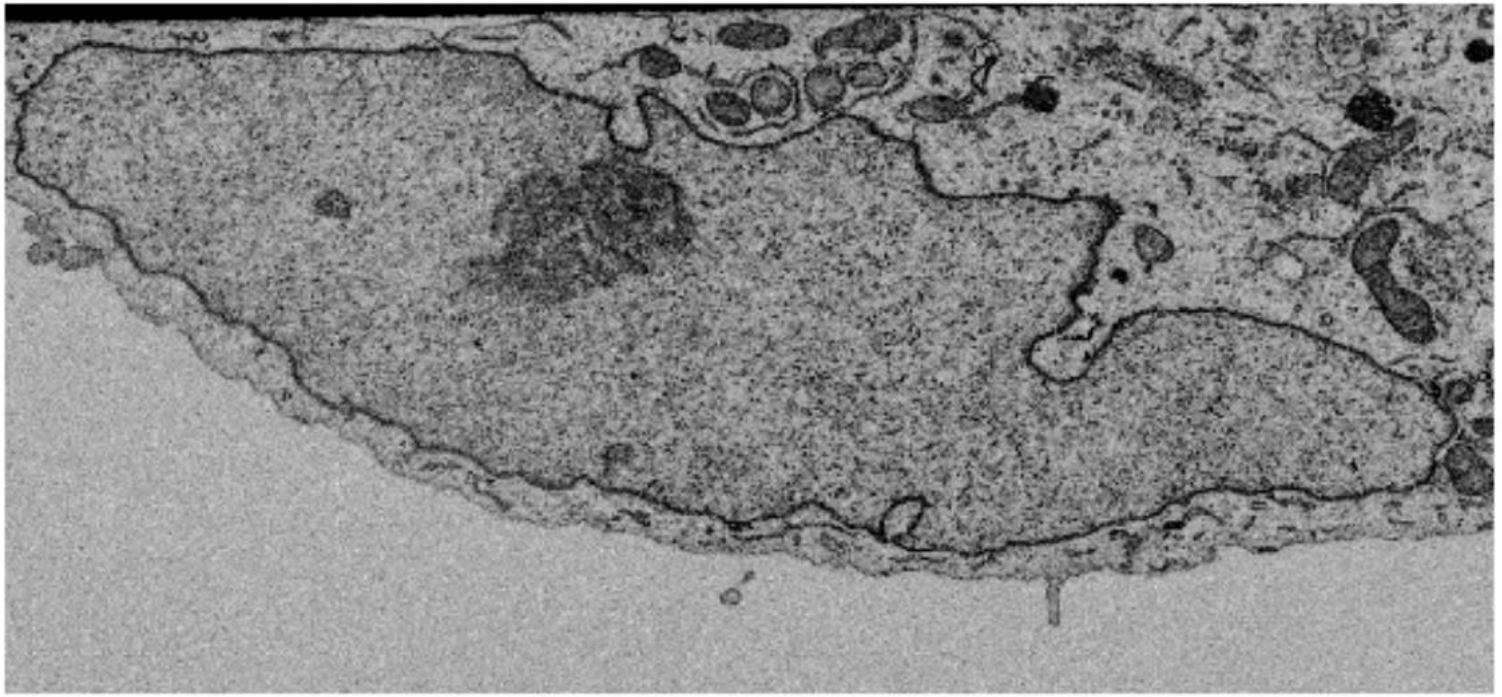


# Drosophila brain imaged by FIB-SEM

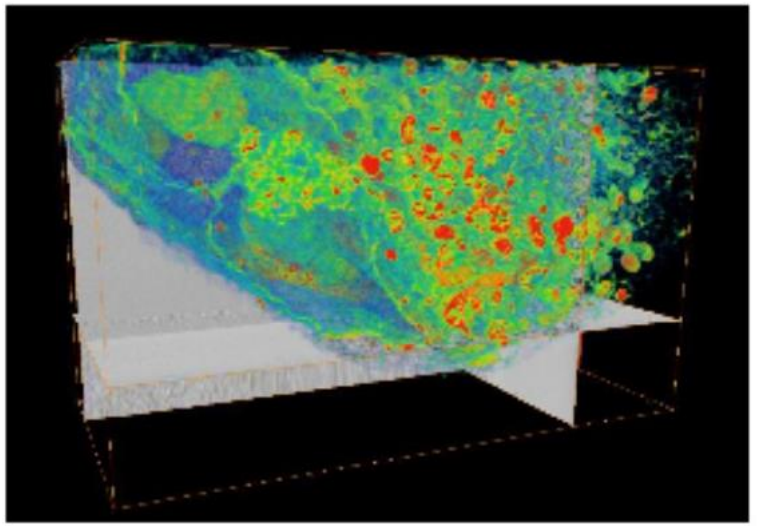



pixel sizes: 512nm, 64nm, and 16nm

Hess Lab, Janelia Campus, HHMI



A single slice image and a 3D visualization of the HT1080 cell. The data were acquired by **Dual-beam FEI Helios Nanolab 660 G3 UC** with in-lens backscatter electron detector (ICD) at 5 nm slice thickness and 3 nm pixel size. The dataset was post-processed with **Amira Software 6.2**. The sample was provided by **Dušan Cmarko (FFM CU)**.

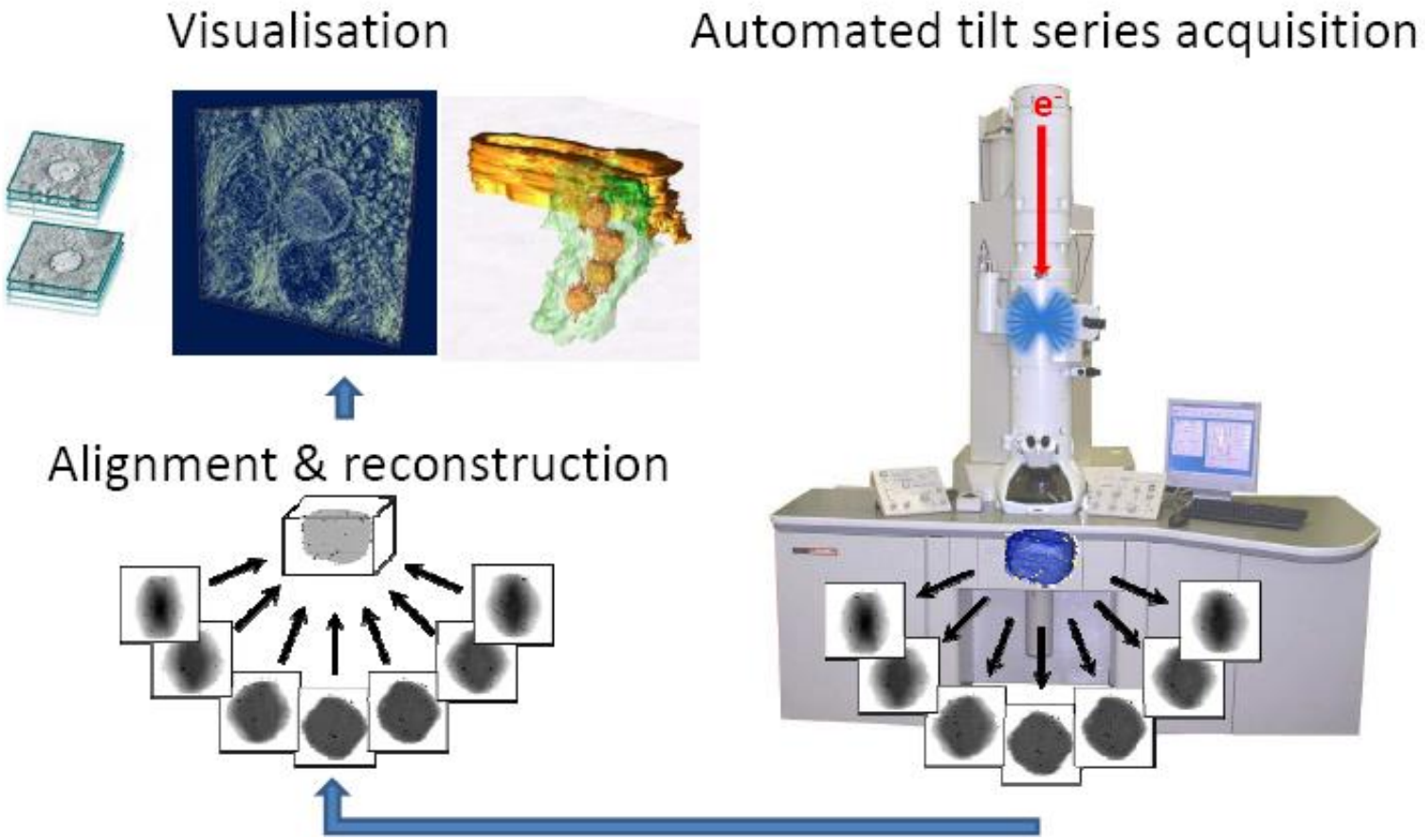


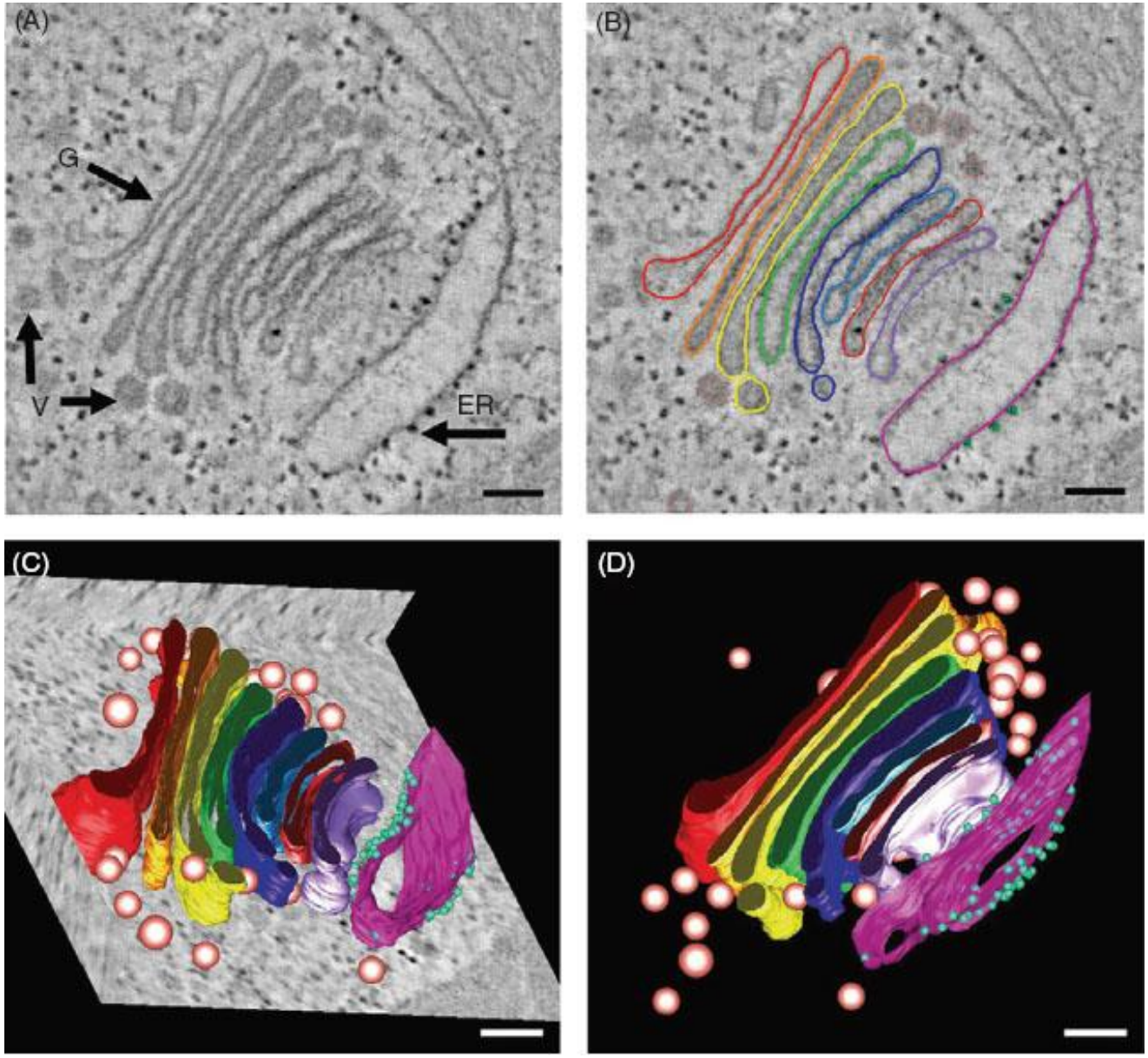
BIOCEV

M. Dalecká, currently IMG EM

# Electron tomography

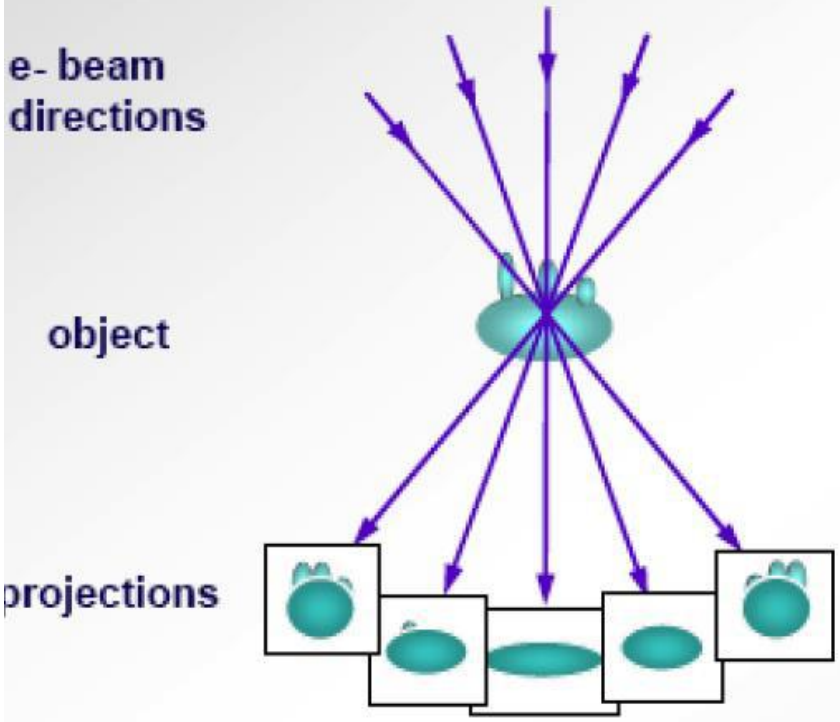
Is a method for volume reconstruction of an object from its projections






Cellular membranes in 3D
O´Toole 2010, Met. Cell Biol.

# Electron tomography




IDEAL SITUATION:

As many images as possible from ALL orientations (-90° to 90°)

RESOLUTION, *d*

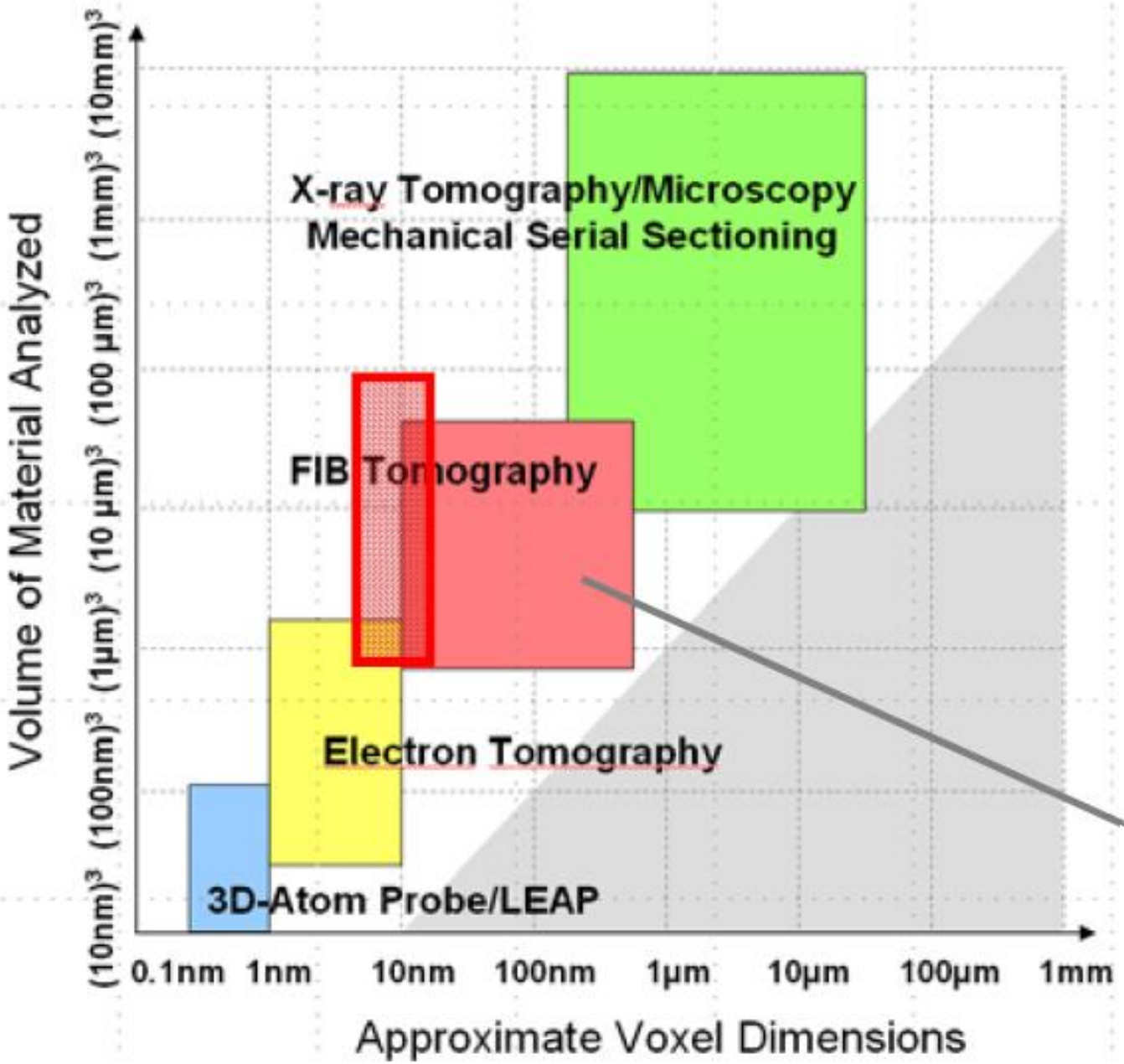
Crowther, DeRosier, and Klug, 1970

$$d = \pi \frac{D}{N}$$

*D*, diameter of the object
*N*, number of projections

2 nm with 141 images of a 100 nm object

# Comparison of various 3D-techniques




New possibilities in 3D-microscopy: Combination with quantitative analytical SEM techniques: EDX, EBSD

From: Uchic, Holzer and Inkson Mat. Res. Bul.,

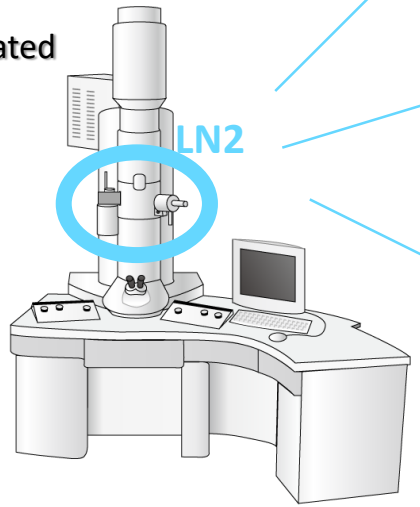
# Cryo-TEM – low dose

# Cryo electron miroscopy

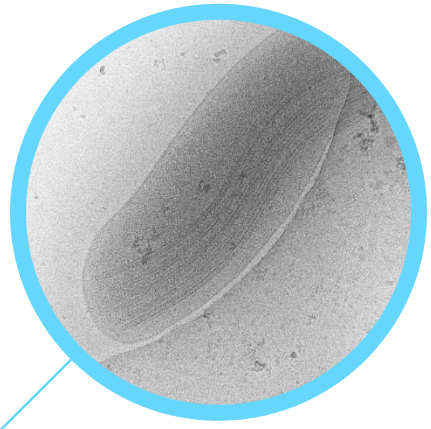
LN2

**Cryo-immobilization – vitrification**

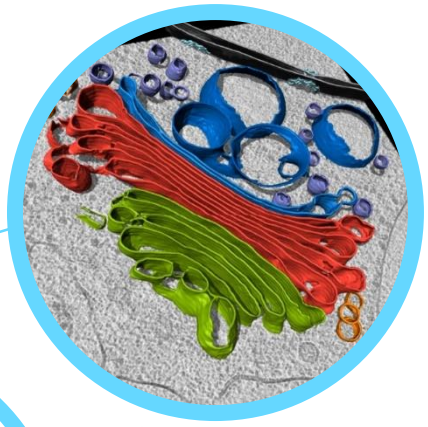
preserves the sample in hydrated close-to-native state



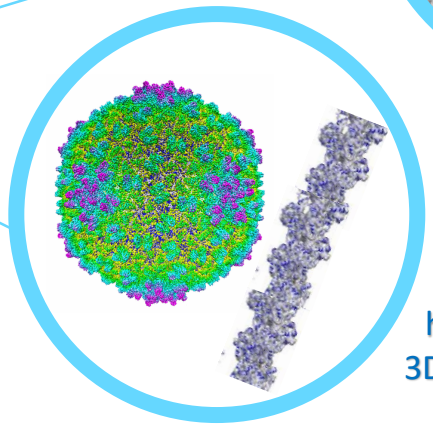
LN2



2D cryo TEM

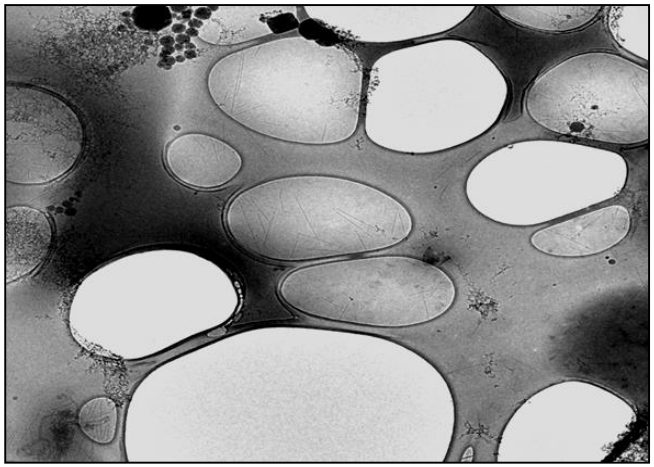


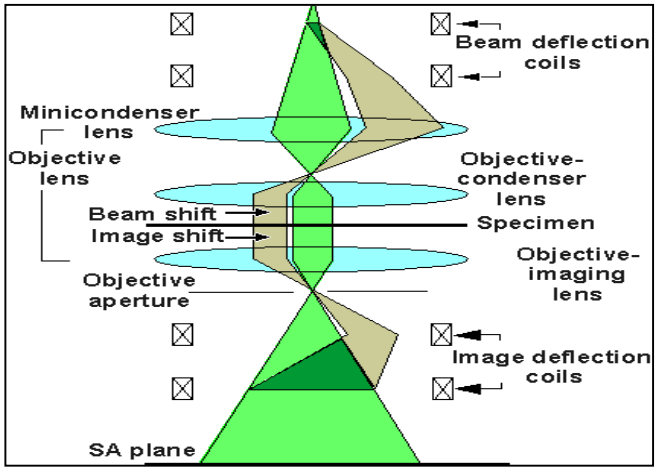
volume cryo EM

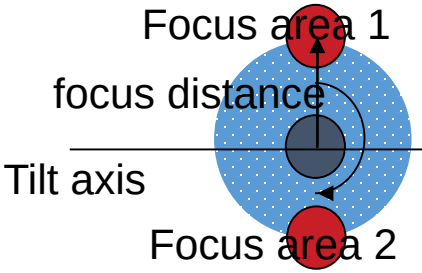


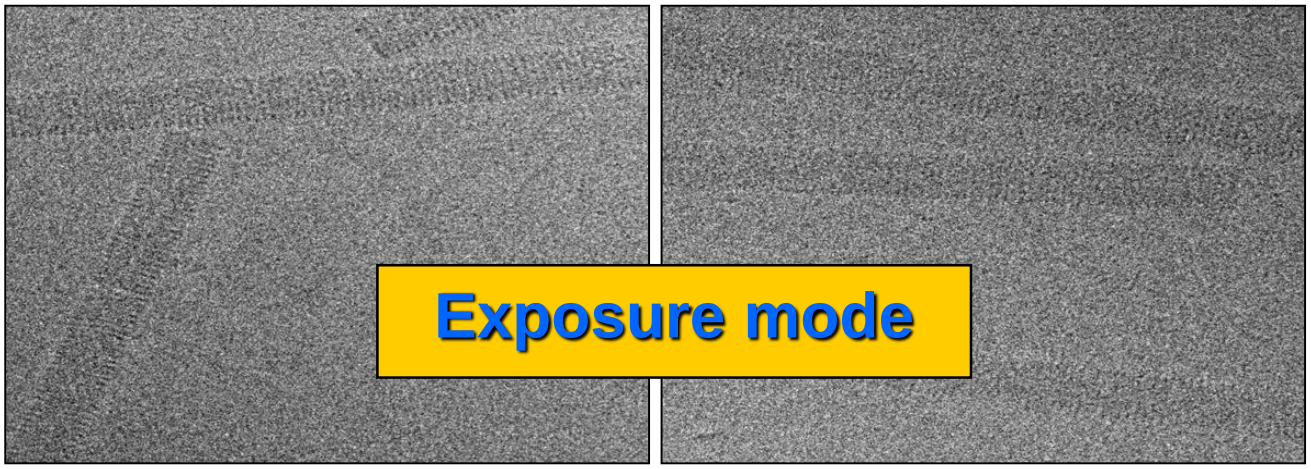
high resolution 3D reconstruction of molecular complexes

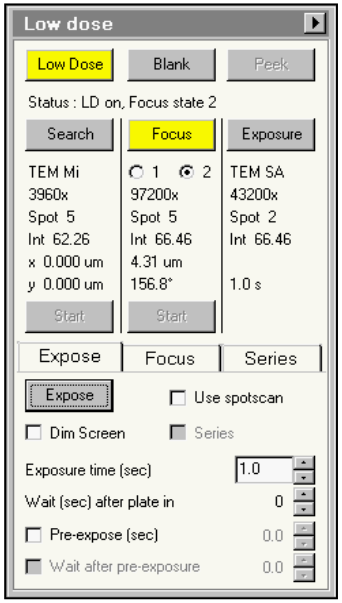
# Low-Dose / Cryo-TEM Principle





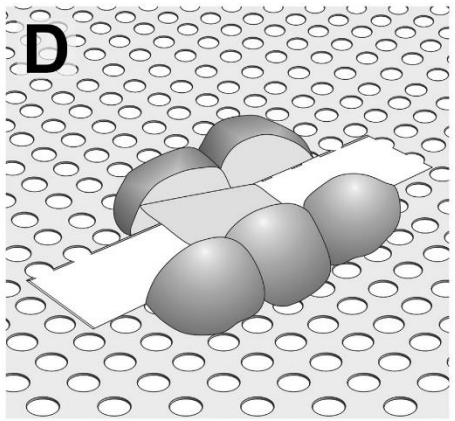



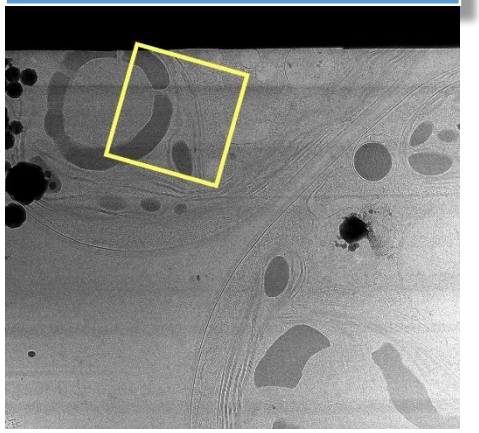

Tobacco Mosaic Virus (TMV)
Recorded on Slow-scan CCD using Low-Dose mode

# Cryo-ET of FIB-milled Lamella

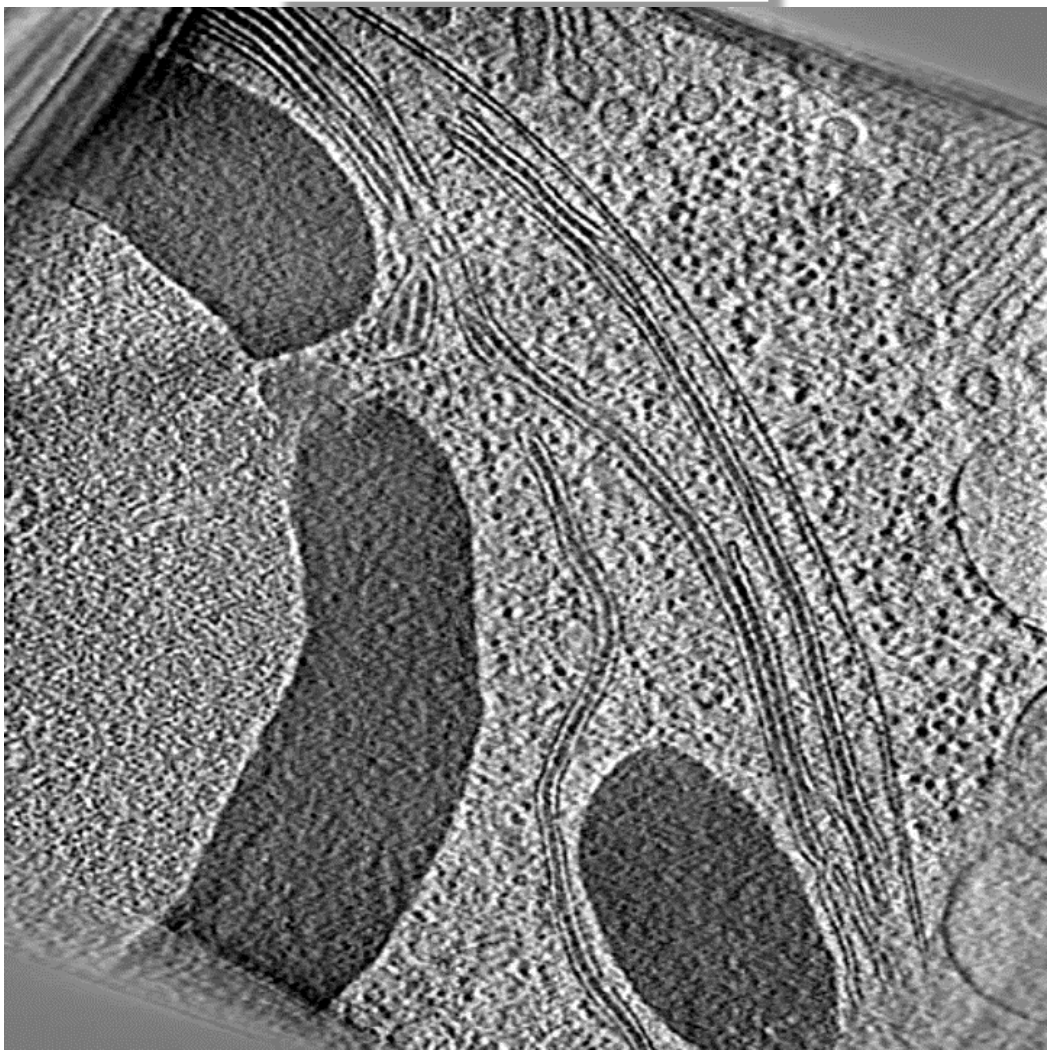
lamella preparation by FIB




ROI selection in Cryo TEM - Tomography



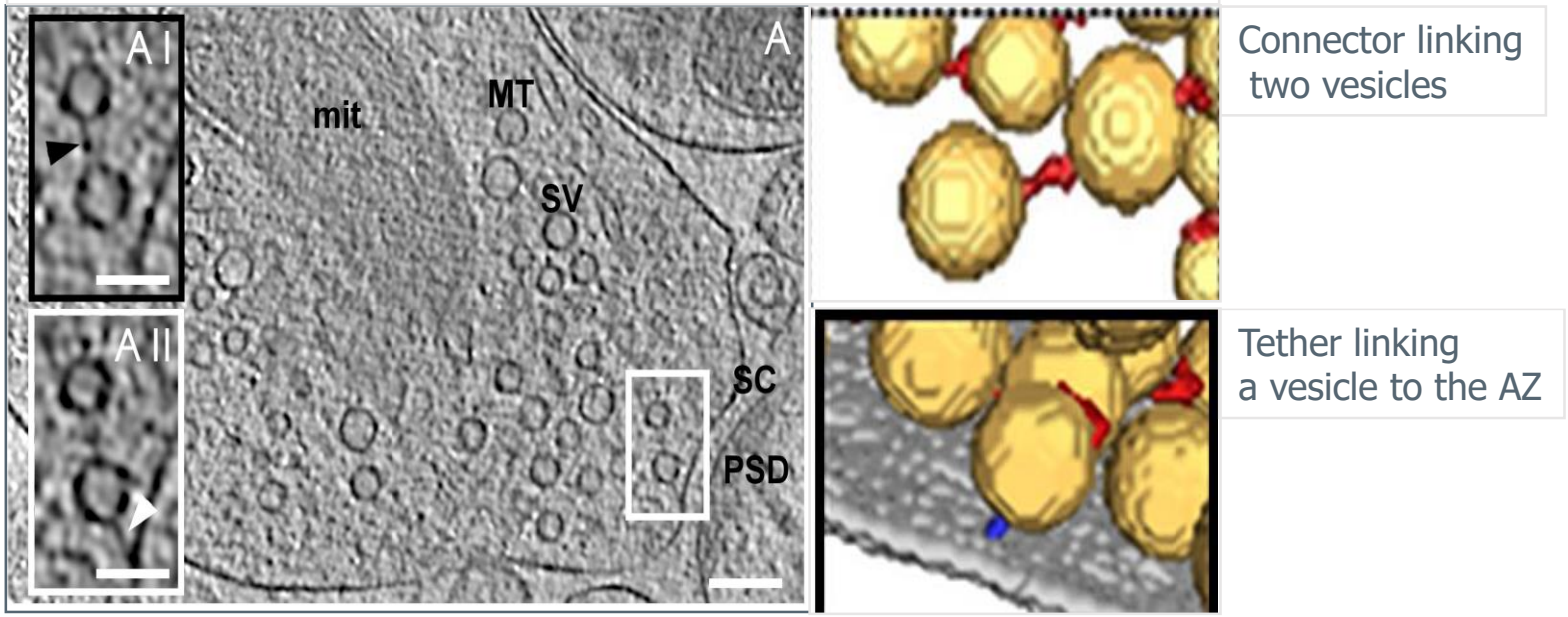
3D Reconstruction



Engel et al. eLife 2015;4:e04889

# Structures visualized by Cryo-ET

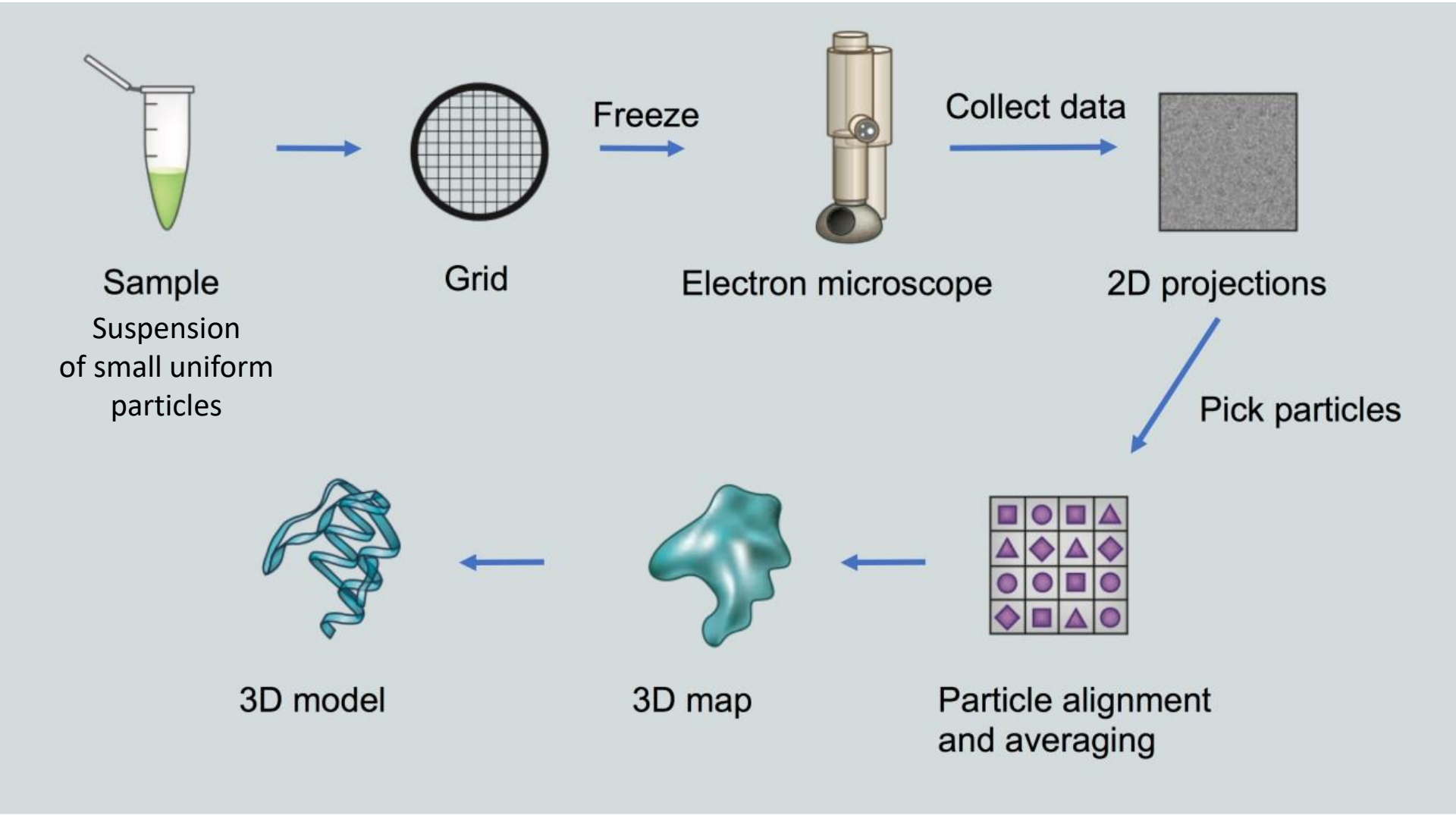
Presynaptic morphology of frozen-hydrated synapses visualized by CET




Fernández-Busnadiego,R., Zuber B., Maurer,U.E., Cyrklaff, M., Baumeister, W., Lučić, V.,2010 *J. Cell Biol. Vol. 188*

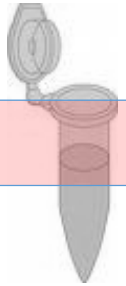
# Single particle analysis

# SPA workflow




Source: Creative Biostructure website

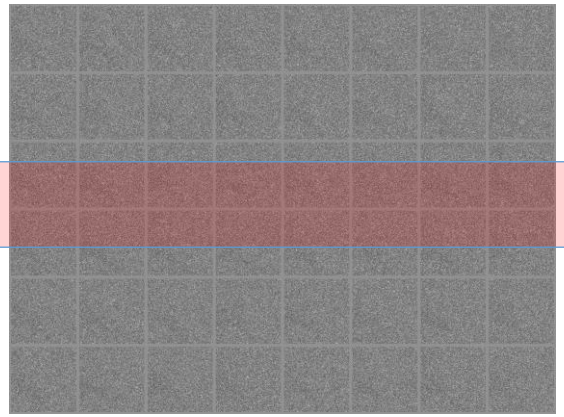
# SPA workflow



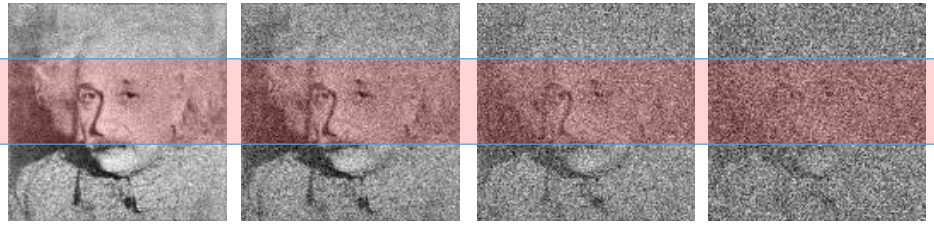
Protein purification

Protein vitrification


Automated image acquisition

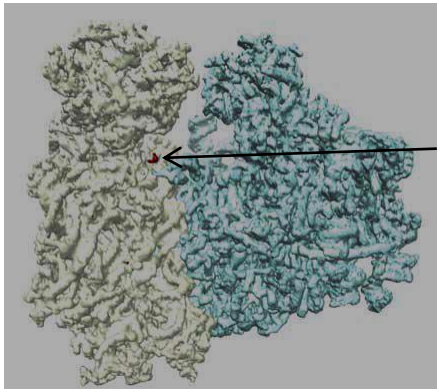


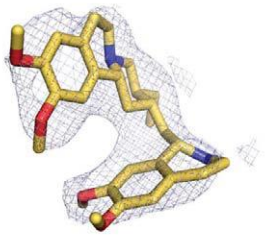
Particle classification

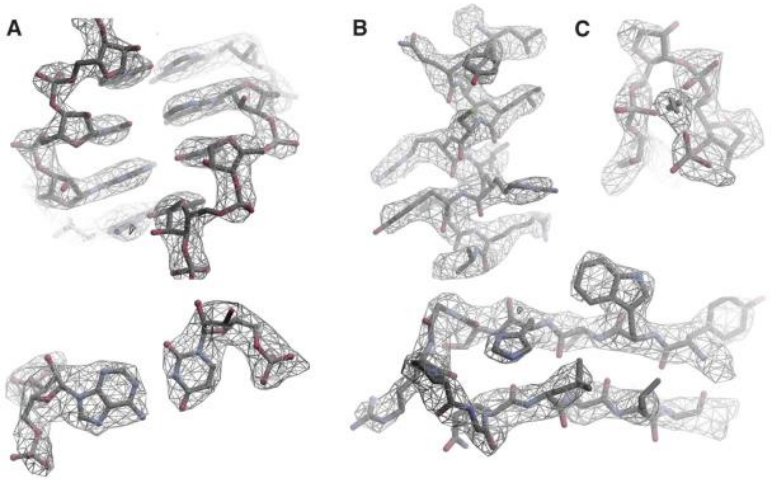


Iterative image processing

# Ribosome (2014):

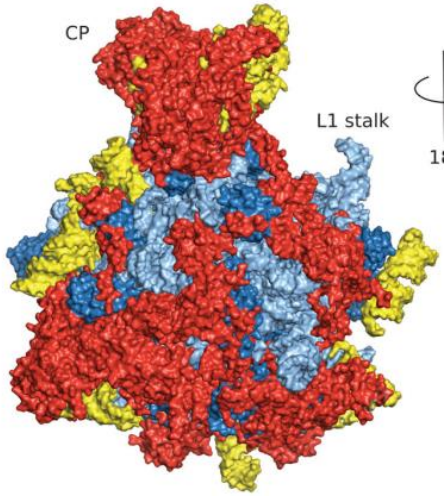






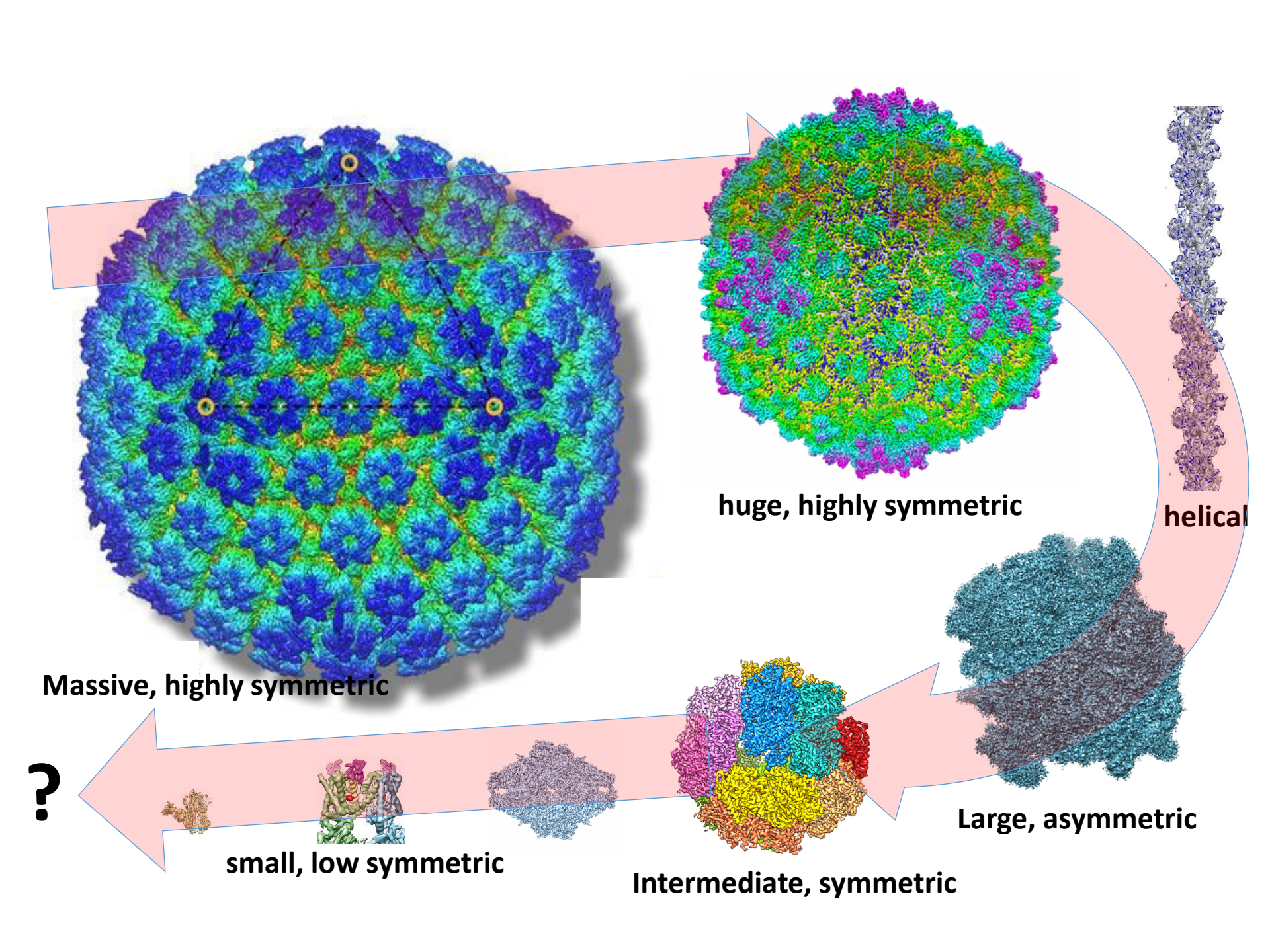

**Full *de novo* model build**

**Routinely ≤4Å**

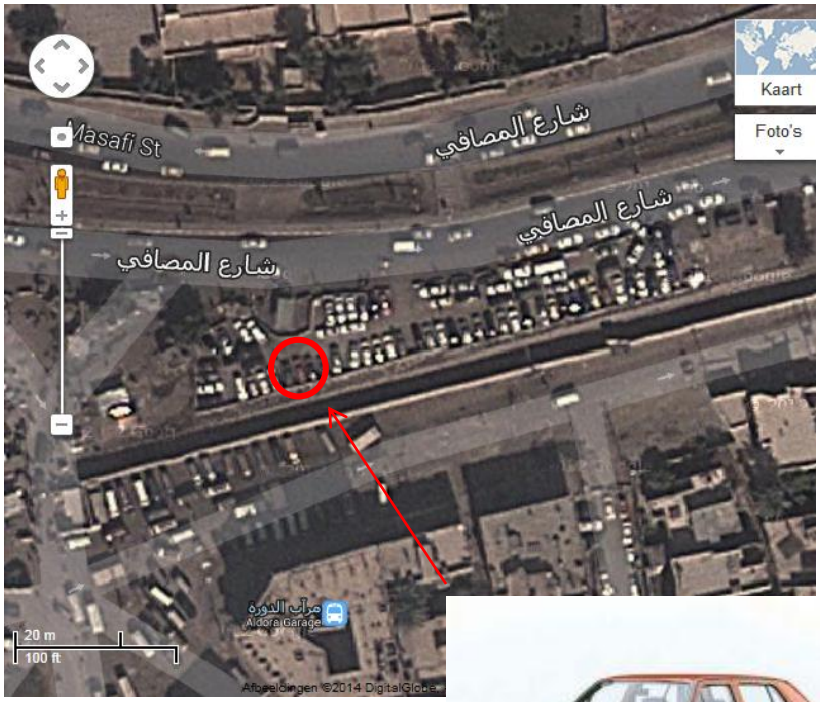


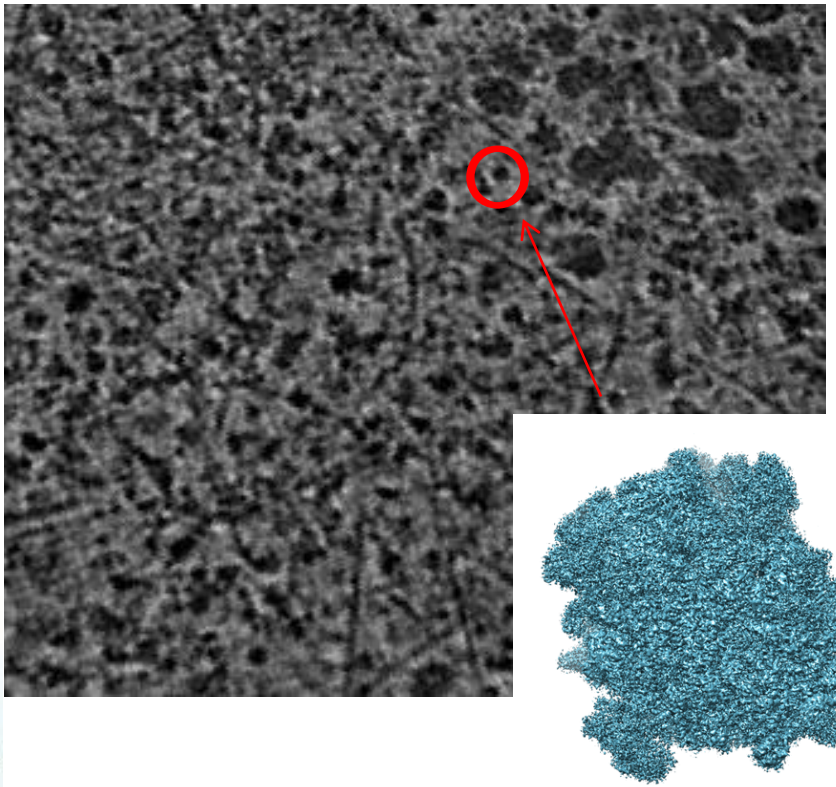


Hussain T, et al. **Cell** (2014) 159 pp. 597-607 (falcon)
Bischoff L, et al. **Cell Rep**. (2014) 9 pp. 469-475 (Falcon)
Arenz S, et al. **Molecular Cell** (2014) (falcon)
Brown A, et al. **Science** (2014) 346 pp. 718-722 (Falcon)
Greber BJ, et. Al. **Nature** (2014) (Falcon)
Shao S, et al. **Molecular Cell** (2014) 55 pp. 880-890 (Falcon)
Voorhees RM, et al. **Cell** (2014) 157 pp. 1632-1643 (Falcon)
Wong W, et al. **eLife** (2014) 3 (Falcon)
Fernandez IS, **Cell** (2014) 157 pp. 823-831 (Falcon)
Amunts A, **Science** (2014) 343 pp. 1485-1489 (Falcon)
Greber BJ, et al. **Nature** (2014) 505 pp. 515-519 (cover) (Falcon)


huge, highly symmetric
helical
Massive, highly symmetric
Large, asymmetric
?
small, low symmetric
Intermediate, symmetric

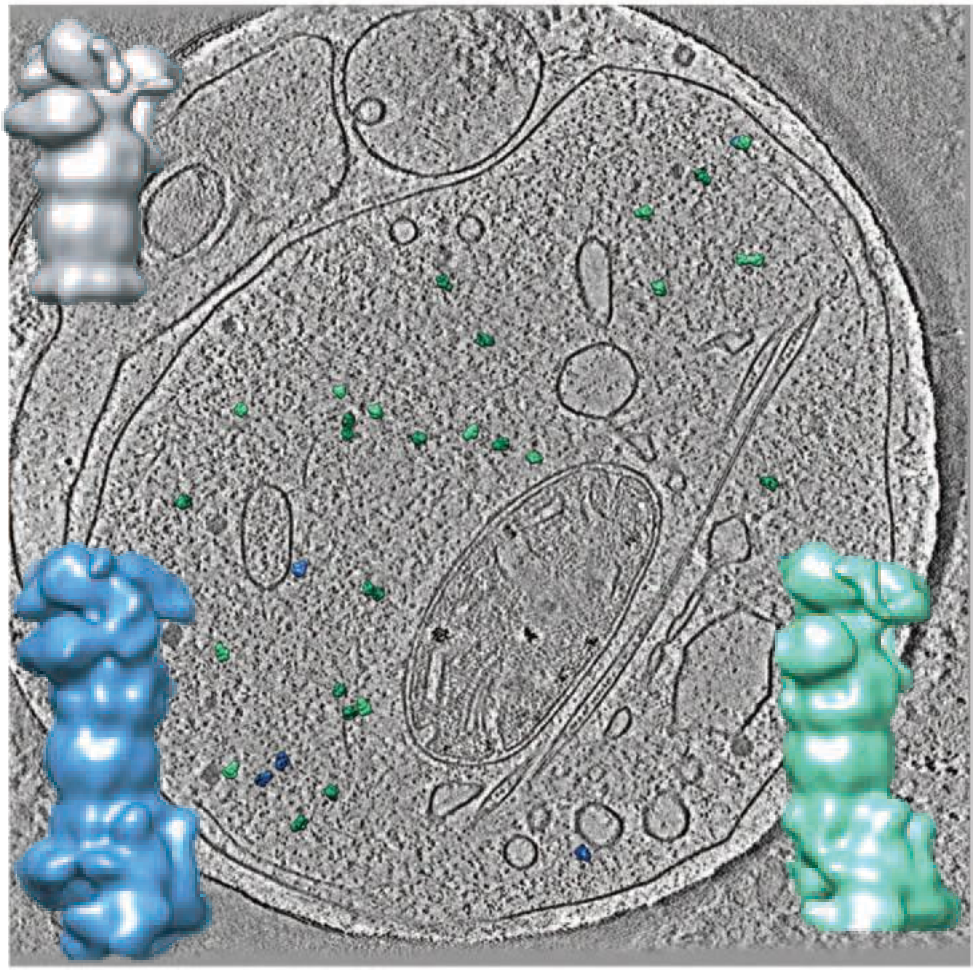
# Protein in the context of the cell at intermediate resolution



**Docking SPA protein into a tomogram**

# Cryo-ET of a neuron



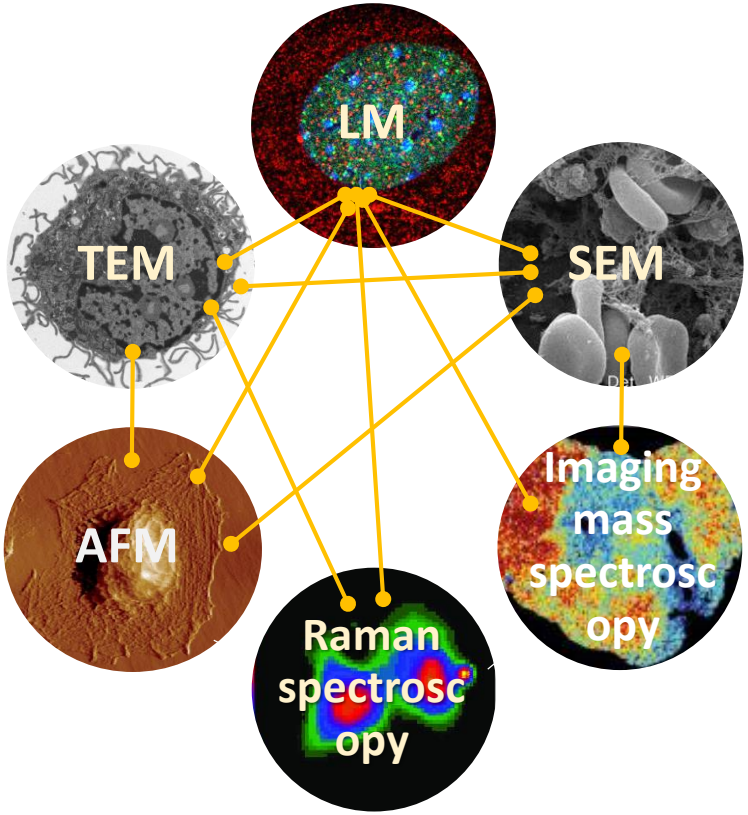
**Phase plate assisted sub-tomo averaging, conformation classification and quantification INSIDE the cell!**

Asano S, et al. **Science** (2015) 440

# Correlative microscopy

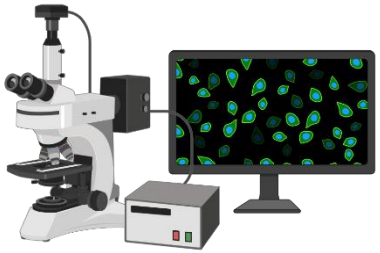
# Correlative microscopy

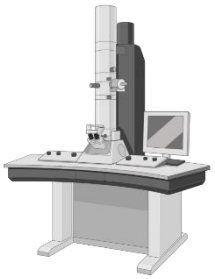
**combination** of two or more **microscopic techniques** applied to **the same region** in a specimen




Examples:
LM +TEM
LM + SEM
AFM + TEM
LM + AFM
…

# **C**orrelative **l**ight and **e**lectron **m**icroscopy (**CLEM**)





**Light Microscopy** (widely used in biology)

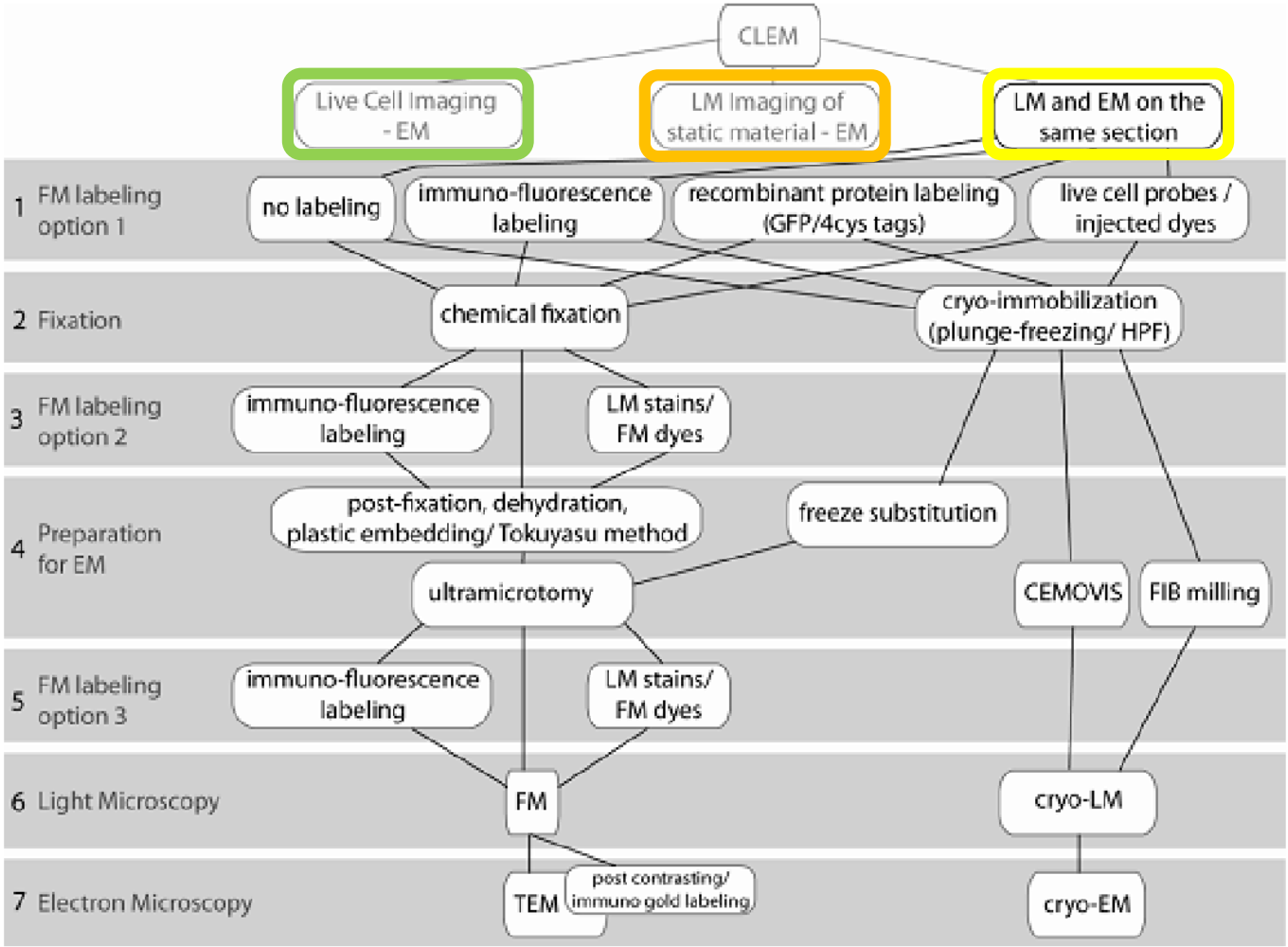
- Large field of view/overview
- Live cell imaging
- Short or no sample preparation
- Combination with fluorescent labels
- Resolution 200 – 20 nm
- Little cellular context

**Electron Microscopy**

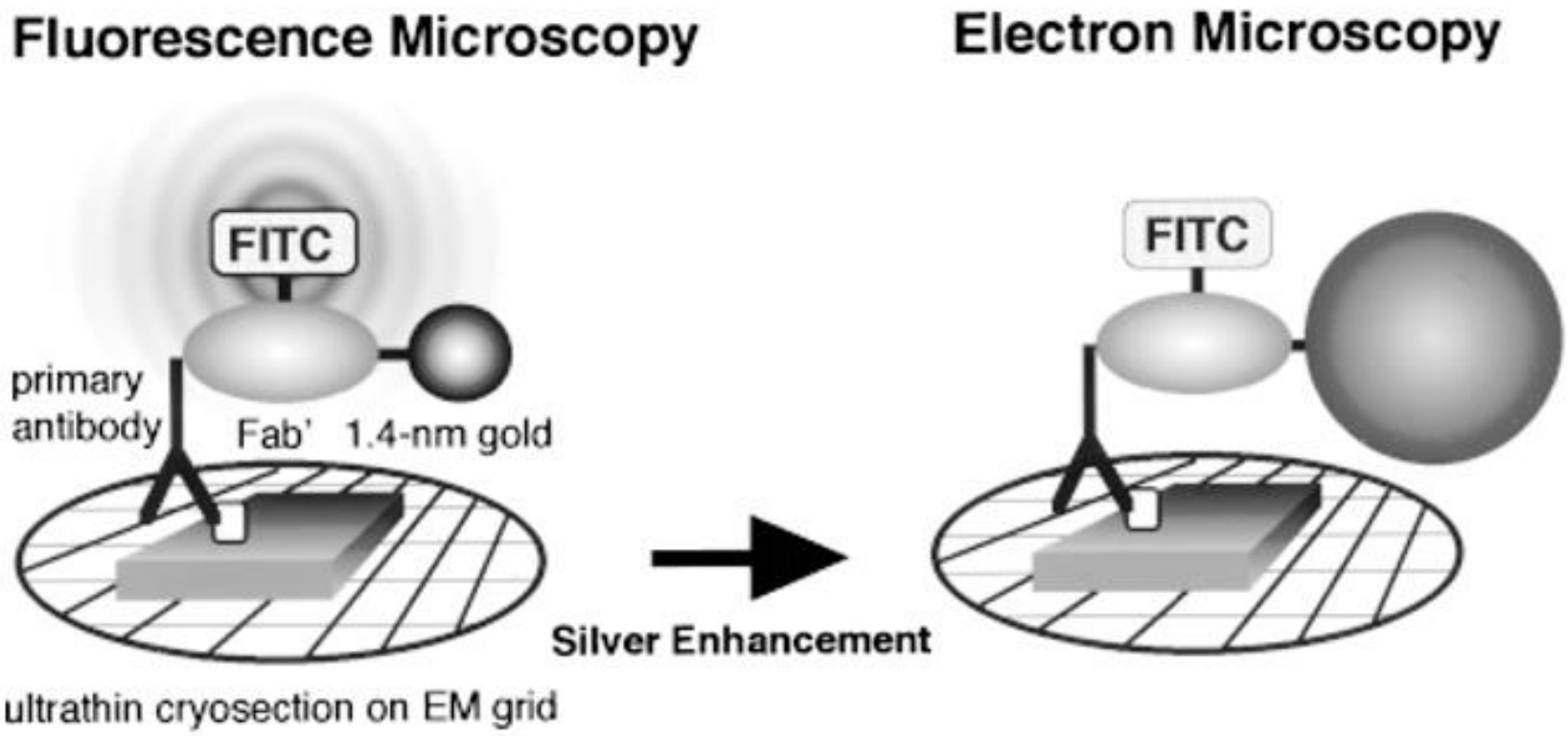
- Excellent spatial resolution
- Provides the cellular context
- Extensive sample preparation

**Challenges:**

- Defining the precise position on the sample both in LM and EM
- Tailored sample preparation for the EM (preserve ultrastructure, fluorescence, antigenicity…)
- Fluorescent labels in LM to electron-dense labels in EM



# Immunolabeling using dual label




Takizava and Robinson, J Cytochem Histochem 2000

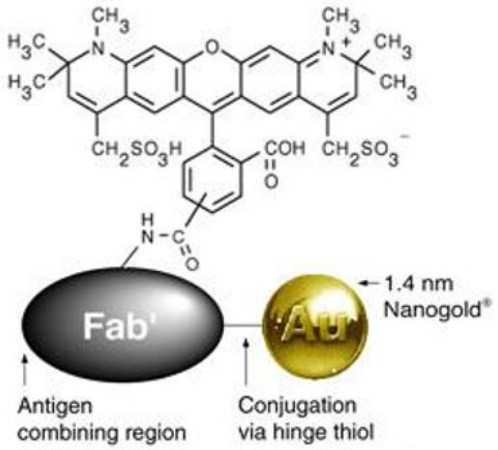
**Fluorescent labels** → **Electron-dense labels**

LM and TEM on the same section

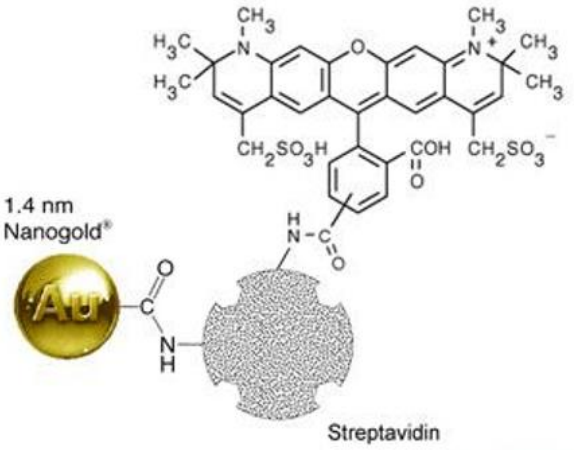
## IMMUNOLABELING

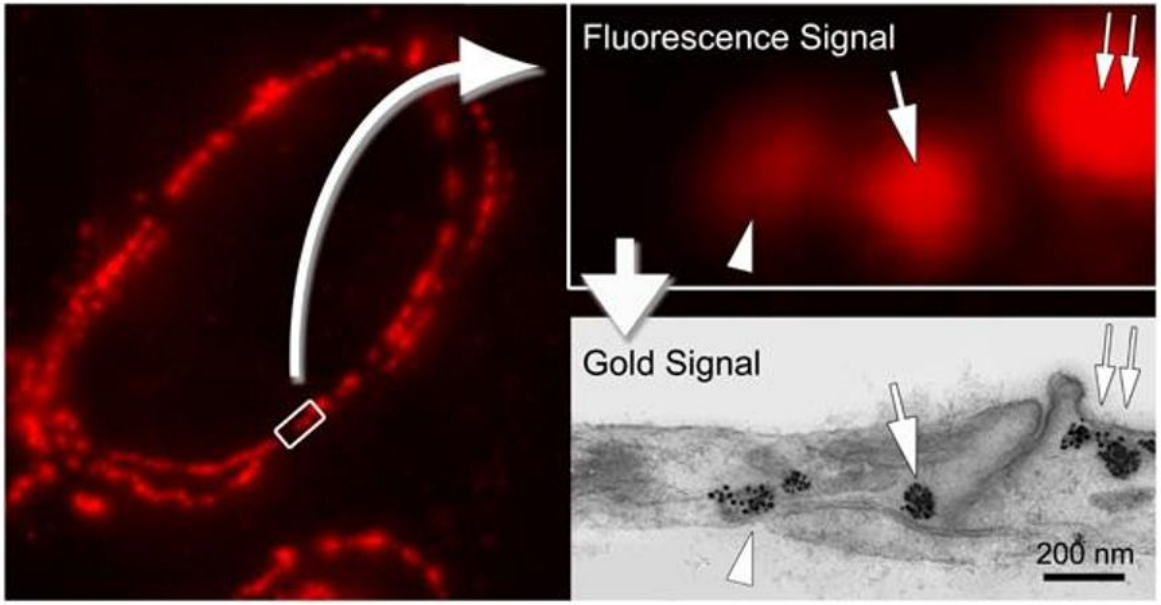
**Cav-1 localisation on the same ultrathin cryosection**

**Alexa Fluor® 594 FluoroNanogold-Fab'**




**Alexa Fluor® 594 FluoroNanogold-Streptavidin**




Correlative fluorescence and electron microscopic immunolocalisation of Caveolin 1 on the same ultrathin cryosection.

Labeled with Nanoprobes Alex Fluro 594 FluroNanogold.

Silver enhancement of immunogold is REQUIRED

*From Takizawa T, Robinson JM (2003) Correlative microscopy of ultrathin cryosections is a powerful tool for placental research. Placenta 24: 557 - 565*

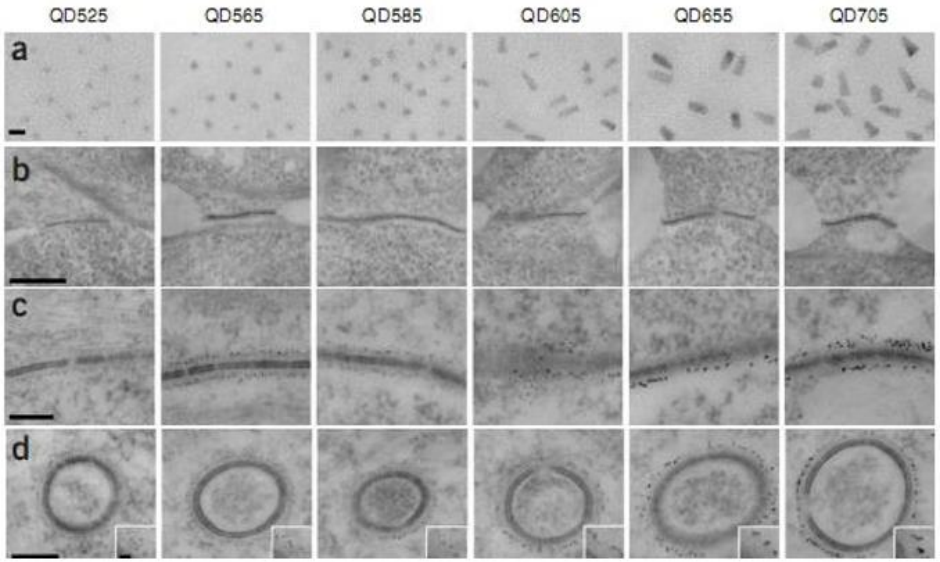
# Quantum dots

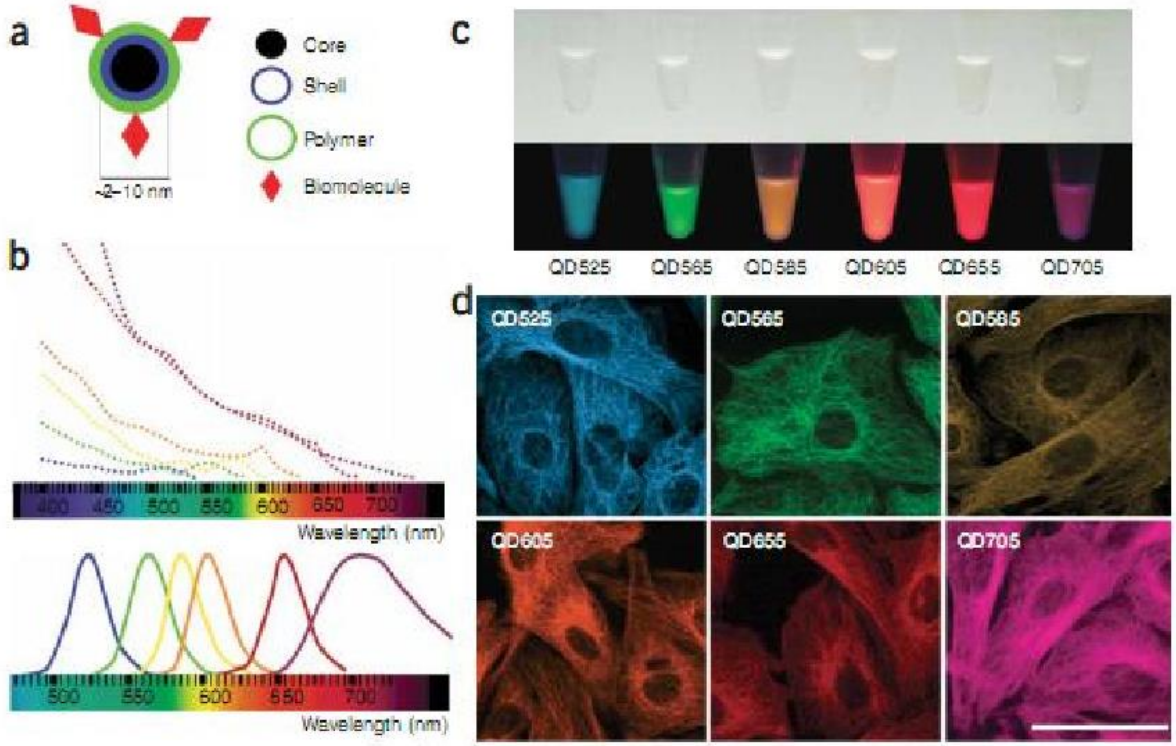
- both fluorescent and electron dense

- different emission wavelength

- multilabeling analysis

- pre-embedding labeling (requires permeabilization)

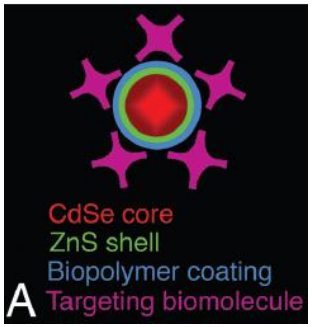



RFL6 fibroblasts, tubulin, biotin-conjugated sec. Abs, streptavidin-conjugated QDs

*Giepmans et al., Nature Methods, 2(10), 2005*

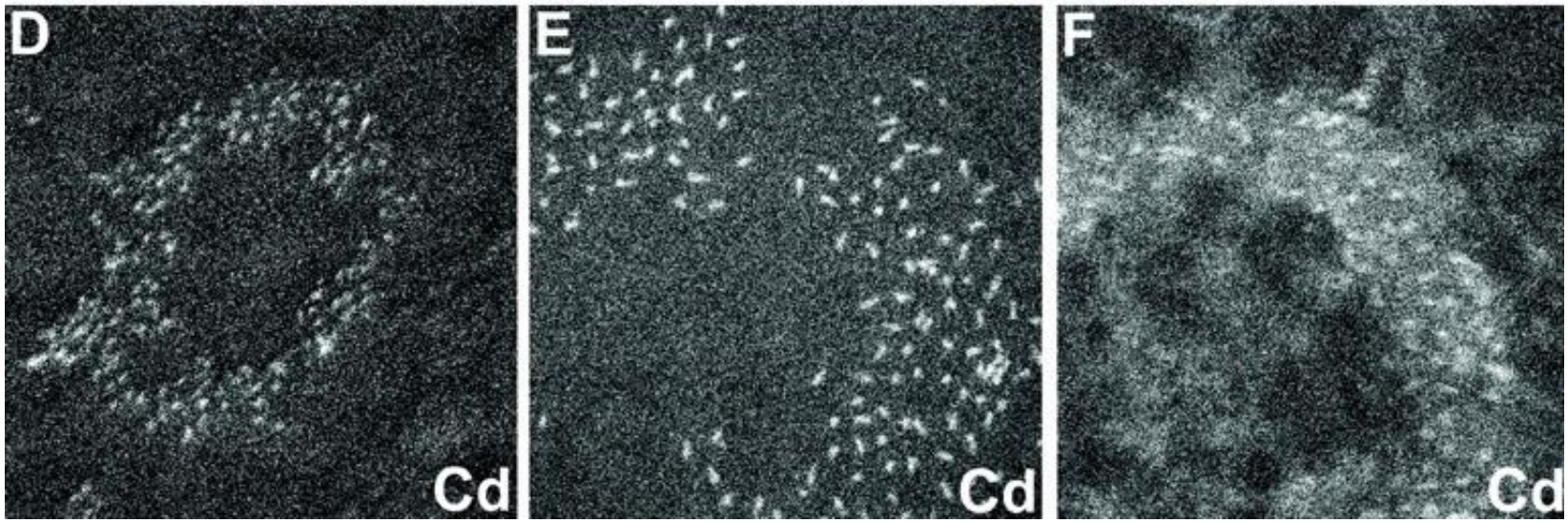
# Q-dots visualization by elemental mapping




| | |
|---|---|
| Cd – M 4,5 403.7 eV | N – K 401.6 eV |
| Se – L 2,3 1436 eV | |
| Te – M 4,5 577 eV | |
| Zn – L 2,3 1020 eV | |
| S – L 2,3 164.8 eV | |

N: 385 and 415 eV, Cd: 415 and 510 eV

**EFTEM**

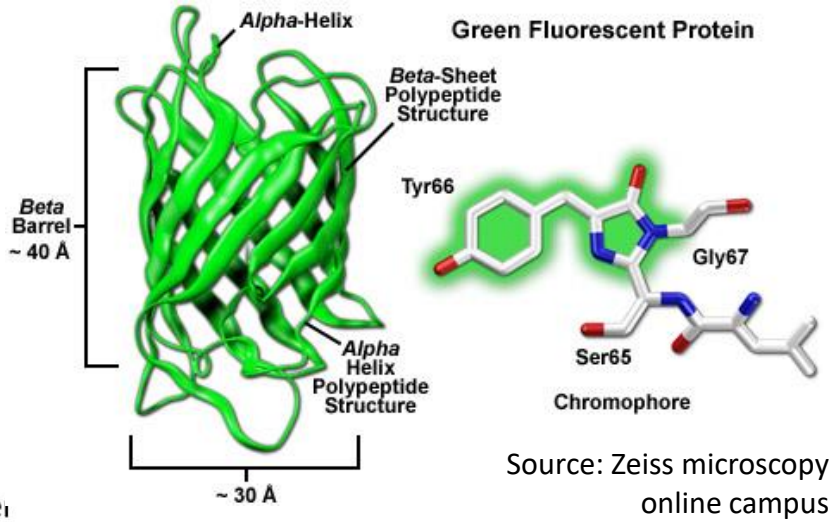



Nisman R. et al., 2004. J Histochem Cytochem 52: 13-18

## Genetically encoded fluorescent tags photooxidation

Green Fluorescent Protein (GFP)

Drawback: size (~30 kDa)




Source: Zeiss microscopy online campus

Bright fluorescence, well-established technology

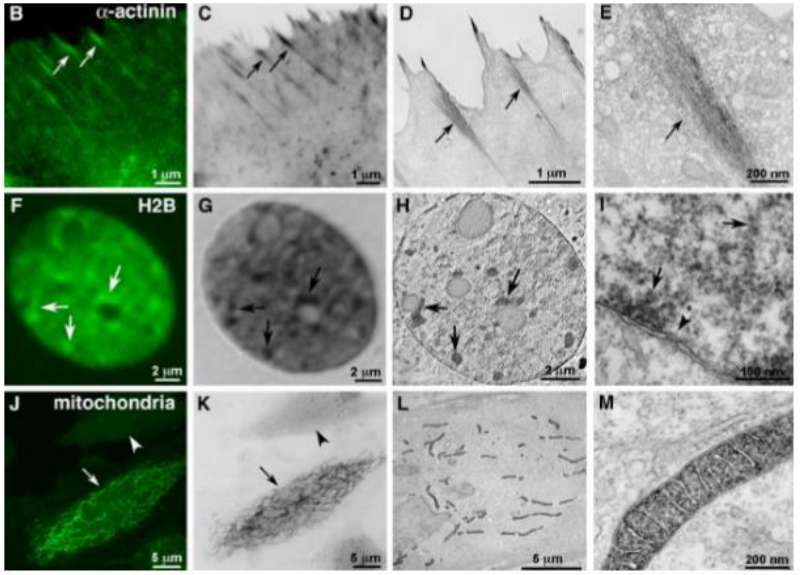
Poor photo-oxidizer
Very low reactive oxygen yield
Size (~30 kDa)

MiniSOG

size (~15 kDa)

Very good singlet oxygen generator; Strong EM signal after photo-oxidation.

Weak fluorescence
Bleaches rapidly

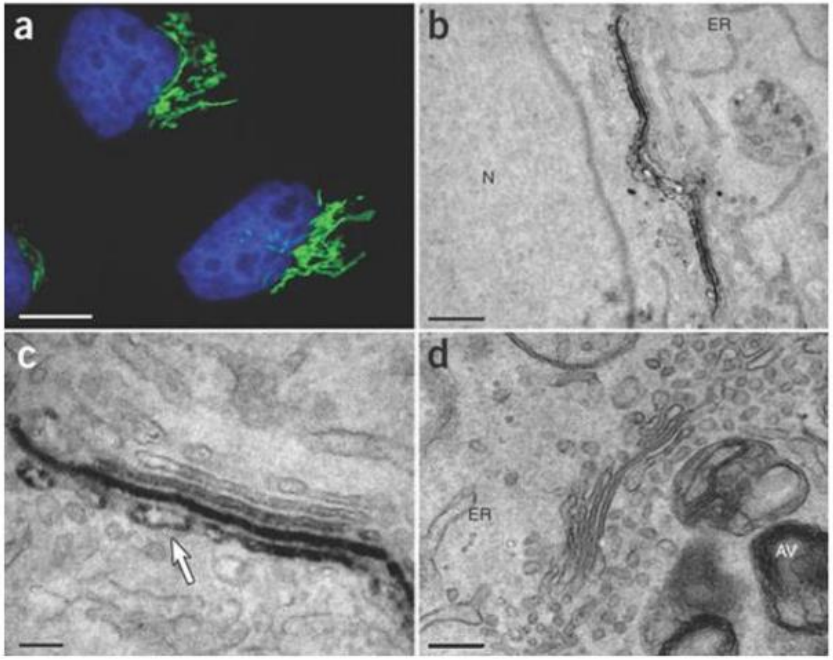



EM using the genetic tags described below has the potential for significantly improved preservation of ultrastructure. Since no permeabilization step is necessary
d because the label is intrinsically incorporated into the sample, strong fixatives such as 1–2% glutaraldehyde can be used.

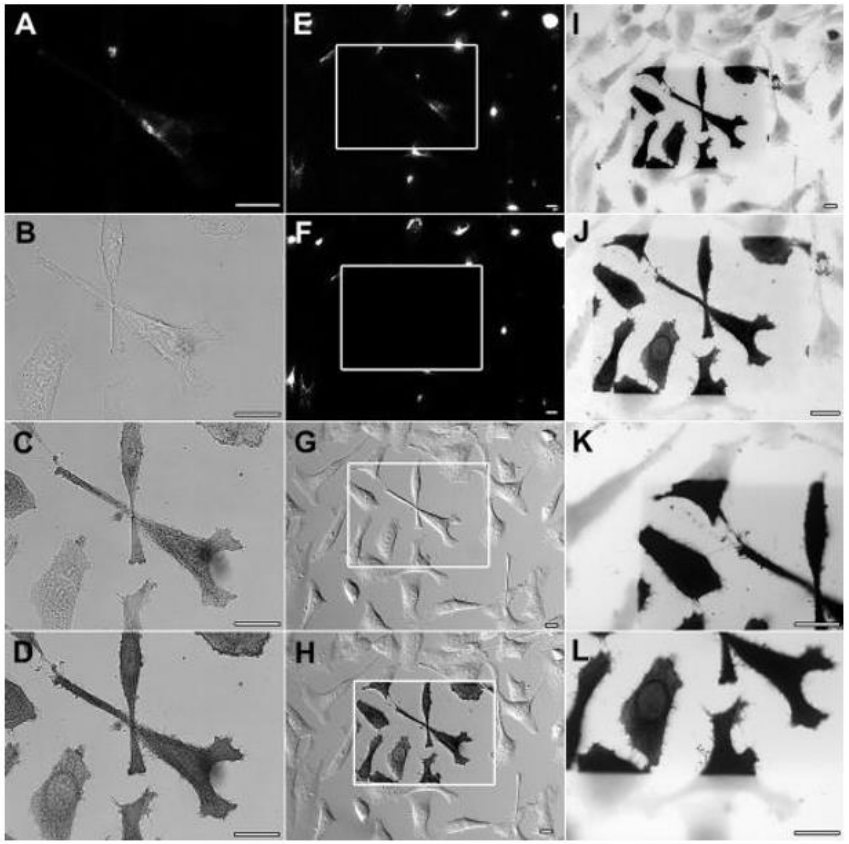
Shu et al., PLoS Biol 2011

# Photo-oxidation of GFP

+ DAB + light = precipitation




Correlative microscopy of GFP through photo-oxidation. HeLa cells expressing the Golgi-resident enzyme GalNAc-T2 fused to EGFP.



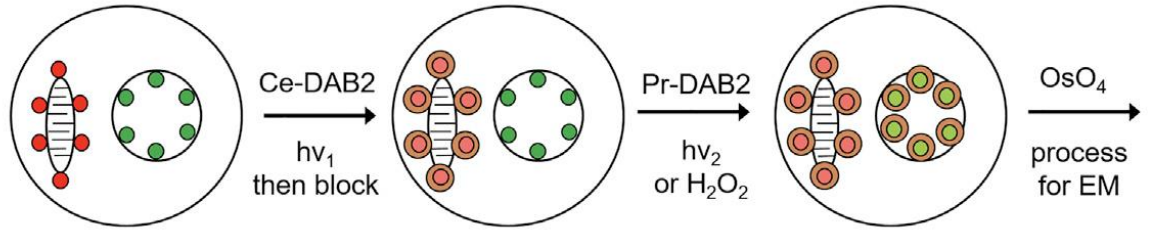

Bleaching process in higher (A–D) and lower magnification (E–H), and the same area after osmification and Epon embedding (I–L).

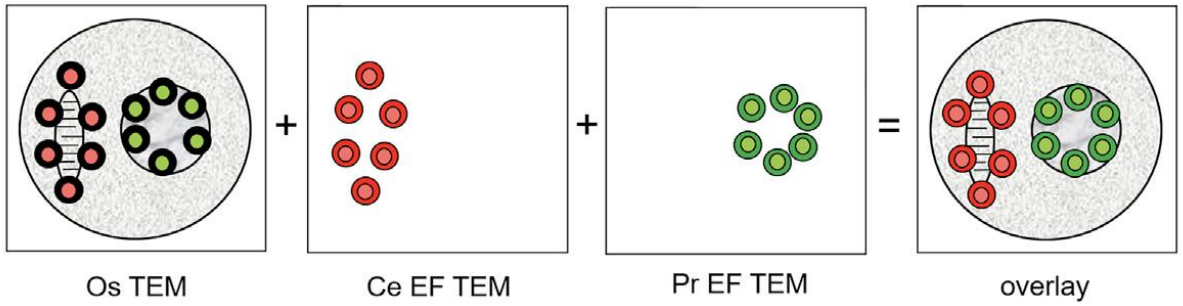
Grabenbauer et al., 2007/2012

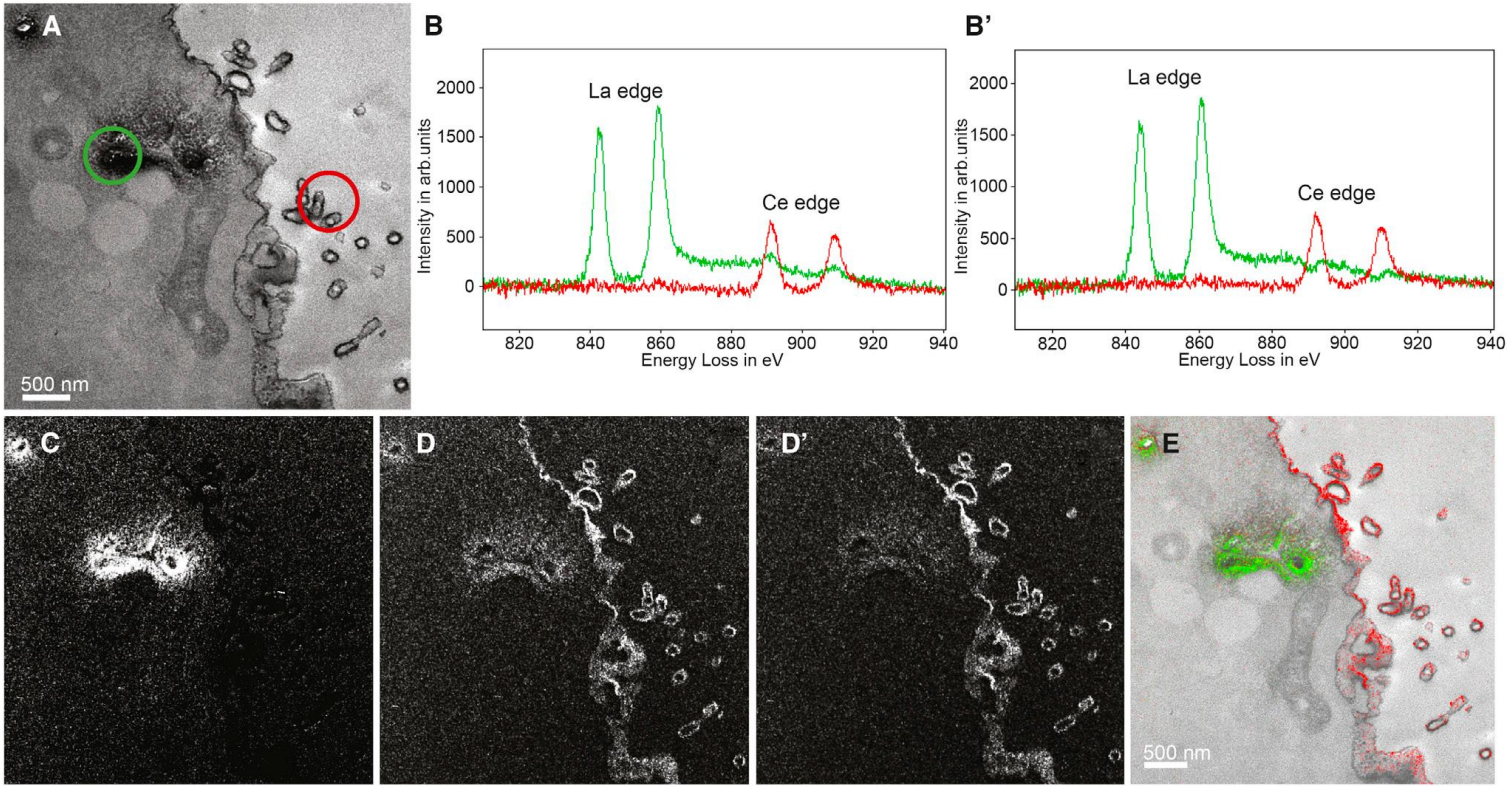
**Fluorescent labels**

↓

**Electron-dense labels**



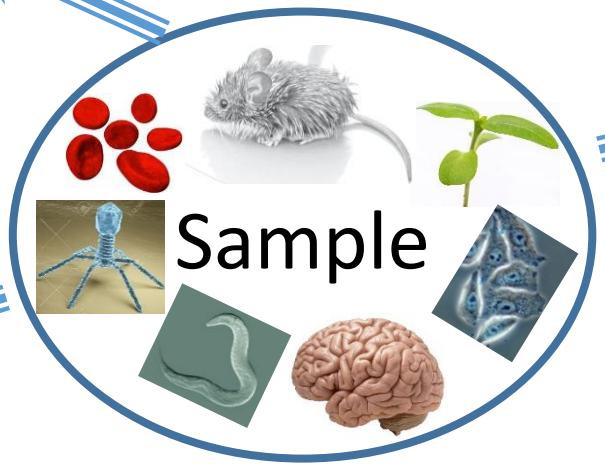

Adams et al., Cell Chemical Biology 2016

# Why electron microscopy?

Localization of specific molecules in cellular context

*Immunolabeling*
*Genetic tags*
*Docking SPA structures into tomograms*

**Subnanometer resolution**




Chemical composition

*EELS*
*EFTEM*
*ESI*
*EDS*

Ultrastructure

2D

*Conventional TEM, SEM*

3D - VOLUME!

*Serial imaging*
*Electron tomography*

Single molecules, complexes

*Single particle analysis*

**Imaging of hydrated specimens in cryo conditions!**

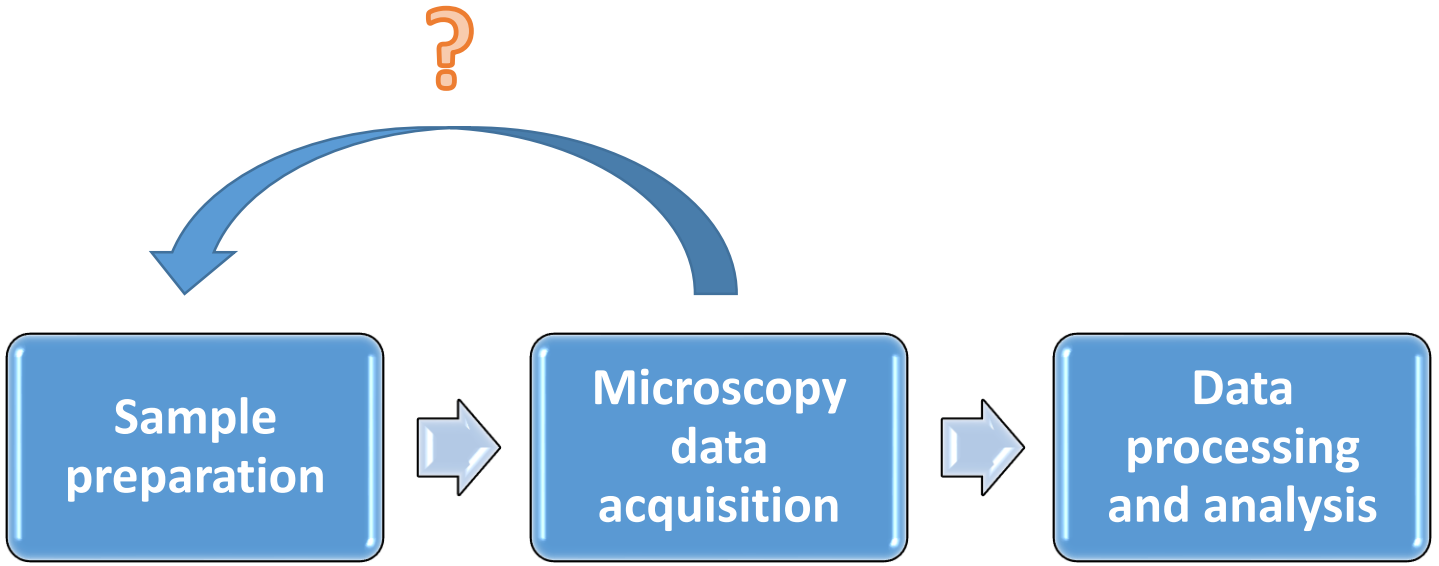
**Correlative microscopy**

**multimodal**
**multiscale**

CLEM, cryo-CLEM

Functional studies: light, electrical stimulation before HPF

- When you have several options, consider their advantages and disadvantages (quality of obtained information, cost, time and labor investments).
- Good to start with a more simple method for first tests, screenings



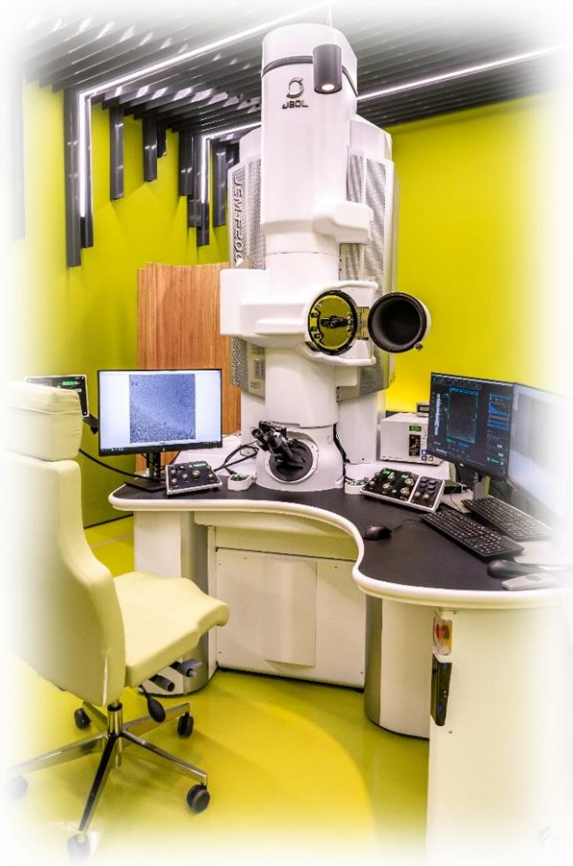

**Select suitable microscopy technique(s)**

**To address my scientific question:**

- what features am I going to observe in my sample? (surface, internal structures, whole mount, atomic composition, specific molecules in context, molecular structures?)
- 2D or 3D?
- What is my optimal balance between resolution, size of acquired area/volume, and throughput?
- Do I need to observe my sample in hydrated state?
- Do I need prior/additional targeting of my structures by ancillary techniques?

# The Electron Microscopy Core Facility

Contact: Vlada Filimonenko
*vlada@img.cas.cz*



**CzBI Call for Financial Support**

- **Call opens: 12 September 2024**
- **Application deadline: 15 October 2024**
- **Result announcement: by 30 December 2024**
- **Project execution period: 1 January 2025 - 31 December 2025**

*https://www.czech-bioimaging.cz/czbi-user-support*

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