The Challenge of Doing Structural Biology *in situ*

EMBO Global Lectures

EMBO

About EMBO

EMBO stands for excellence in the life sciences.

EMBO is an organization of more than 1500 leading researchers that promotes excellence in the life sciences. The major goals of the organization are to support talented researchers at all stages of their careers, stimulate the exchange of scientific information, and help build a European research environment where scientists can achieve their best work.

EMBO helps young scientists to advance their research, promote their international reputations and ensure their mobility. Courses, workshops, conferences and scientific publications disseminate the latest research and offer training in techniques to maintain high standards of excellence in research practice. EMBO helps to shape science policy by seeking input and feedback from our community and by following closely the trends in science in Europe.

EMBO supports talented researchers, selected through impartial evaluation processes, to allow them to do great science. The wide scientific scope across the full range of life science research coupled with the broad geographical reach of more than 1500 members and associate members – some of the best researchers in Europe and around the world – positions EMBO optimally to serve Europe's life science community.

Funding and awards

Access to funding and awards is essential for young scientists to excel in their research careers. EMBO offers awards and financial support for scientists at all stages of their careers that help to create an environment where researchers can achieve their best work.

EMBO Fellowship

Support postdoctoral and predoctoral research experience in laboratories in Europe and around the world. EMBO Fellowships have supported thousands of talented young scientists since they were first offered in the 1960s.

EMBO Young Investigators

Talented young group leaders in the first years of establishing independent research laboratories. The first programme of its kind in Europe, young investigators receive financial, academic and practical support and participate in a network of more than 300 young scientists.

EMBO Installation Grants

Help promising scientists relocate and set up their labs in participating countries. Installation Grantees are integrated into the EMBO Young Investigators network to benefit from the offered support.

EMBO Courses & Workshops

Funding and support for life scientists to organize meetings. Funding is available for conferences, symposia, workshops, lecture courses and practical courses, as well as for plenary lectures.

Lecture Grants

Support and promote the active participation of researchers at national and international meetings.

Travel Grants

A limited number of travel grants are available for eligible participants who are selected to attend EMBO Practical Courses, EMBO | FEBS Lecture Courses, ESF | EMBO Symposia, EMBO | EMBL Symposia and selected other EMBO events, including *The EMBO Meeting*.

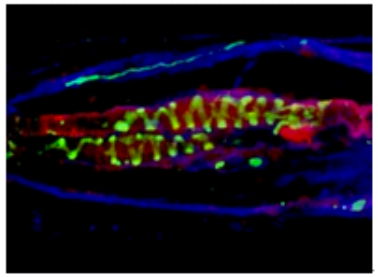
Gold Medal

Awarded annually to scientists under the age of 40 for outstanding contributions to molecular biology in Europe.

Women in Science Award

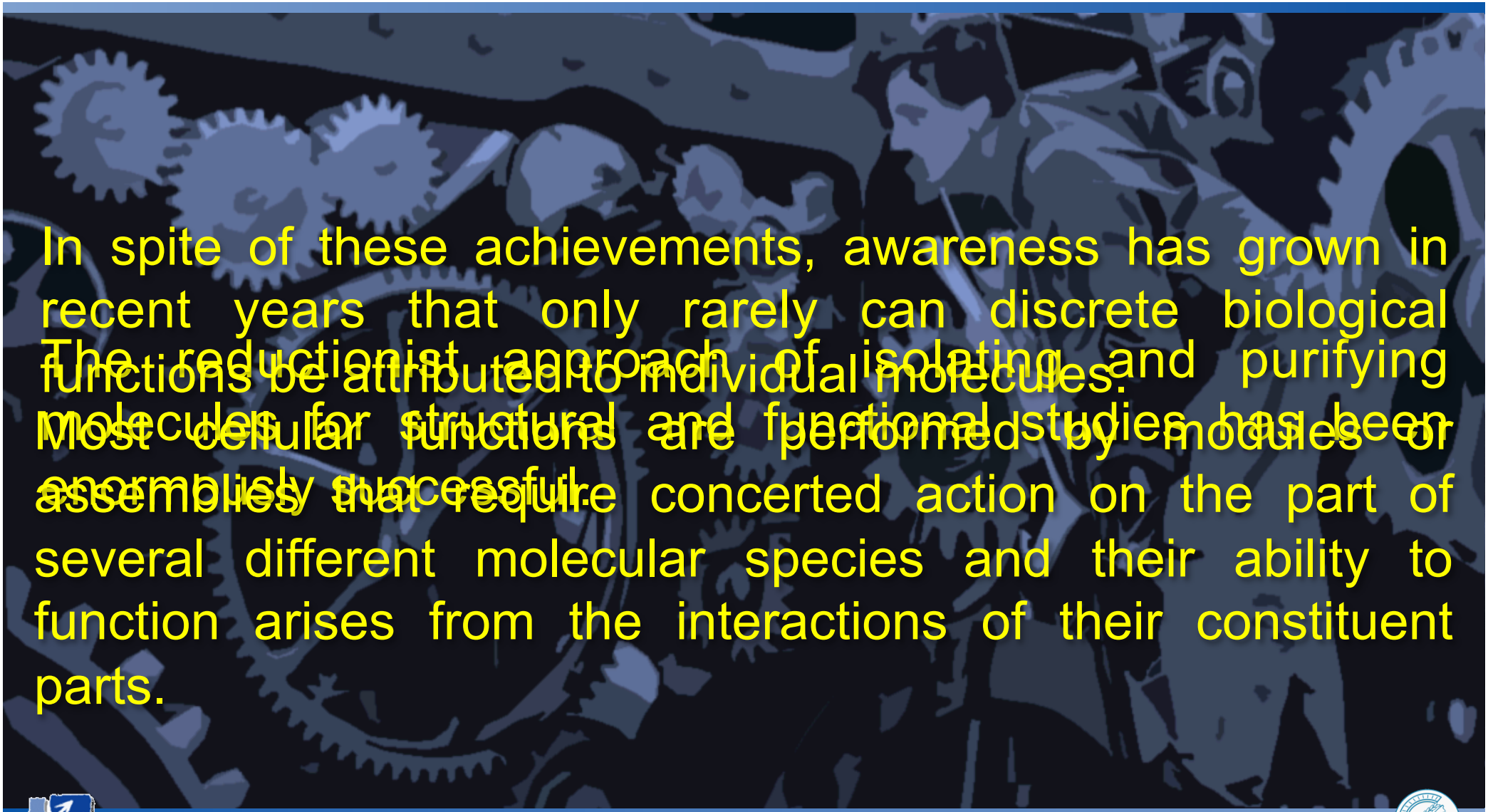
Awarded annually to female researchers who have made exceptional contributions to molecular biology and who have been role models to other scientists.

NEWS GALLERY



News: Alzheimer's protein controls movement in mice

Why Structural Biology *in situ* ?



In spite of these achievements, awareness has grown in recent years that only rarely can discrete biological functions be attributed to individual molecules. Most cellular functions are performed by modules of assemblies that require concerted action on the part of several different molecular species and their ability to function arises from the interactions of their constituent parts.

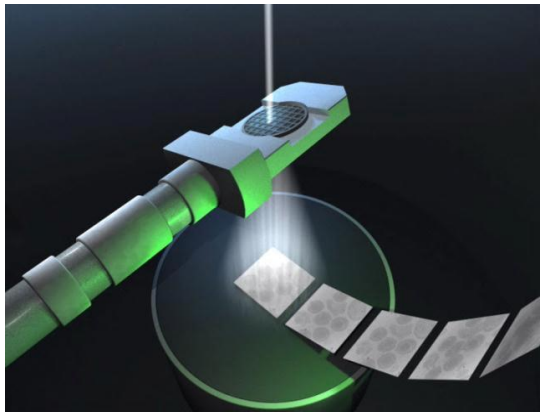
The reductionist approach of isolating and purifying molecules for structural and functional studies has been enormously successful.

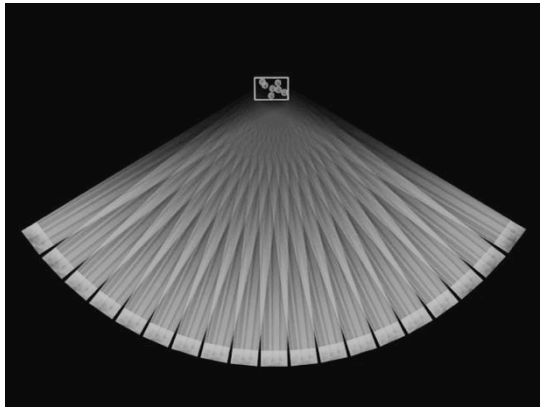
A Definition of Structural Biology *in situ*

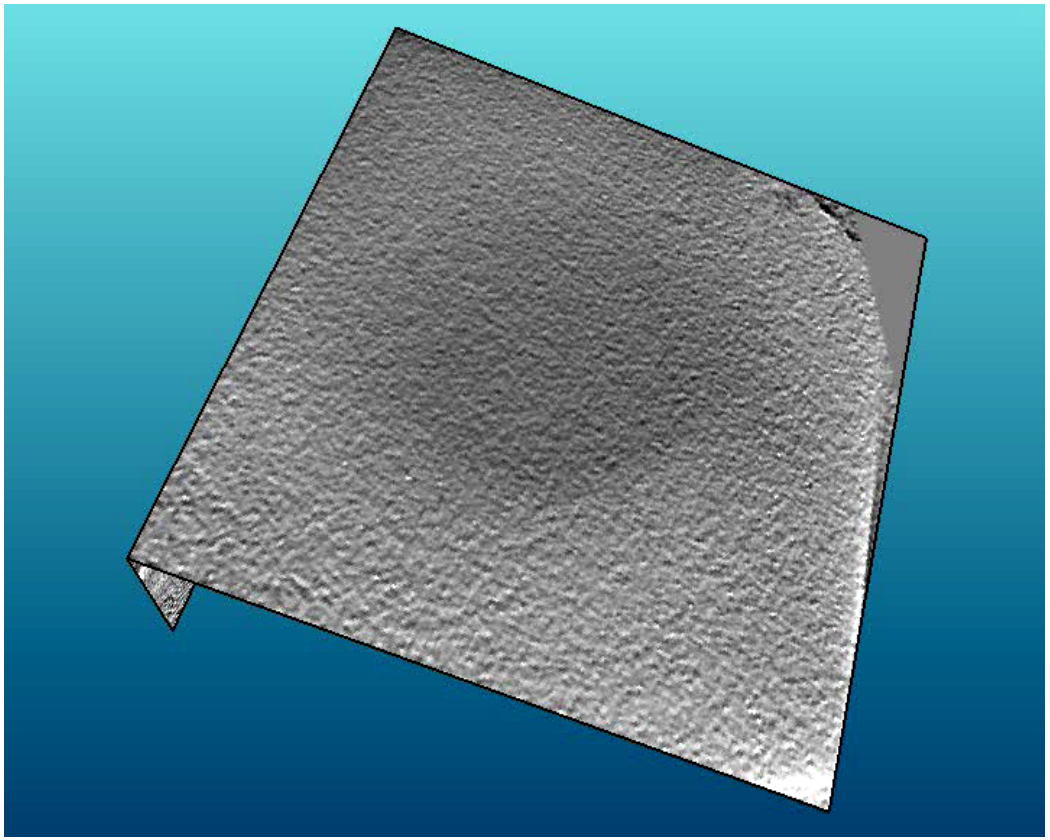
Sensu stricto the term should apply only to a scenario in which the cellular environment is preserved in its entirety, i.e. all organelles and supramolecular assemblies are kept at their native place.

Sensu largo it may refer to situations where the local environment of a macromolecular assembly is either preserved or reconstituted such that some functionality is maintained.

Electron Cryotomography







ECT combines the best possible structural preservation of cellular structures with the power of three-dimensional imaging

Electron tomography – The idea was there …

Electron Microscopy of Unstained Biological Material: The Polytropic Montage

Abstract. *With use of an electronic picture-scanning device and a digital computer, electron micrographs taken of a specimen along several different directions can be superimposed to form a montage that is more informative than the component images. Preliminary results indicate that one may thus study unstained, unshadowed biological material at high resolution.*

R.G. Hart, Science 159 (1968) 1464-1467

Hydrated Protein Crystals Diffract to High Resolution

Electron Diffraction of Wet Proteins: Catalase

Abstract. *Electron diffraction patterns having 3500 reflections out to 2 angstroms were obtained from wet microcrystals of catalase. No diffraction was obtained if the water vapor pressure was set below 90 percent of the equilibrium value.*

VICTOR R. MATRICARDI
ROGER C. MORETZ
DONALD F. PARSONS
Electron Optics Laboratory, Roswell Park Memorial Institute, Buffalo, New York 14203

Science. 1972 Jul 21;177:268-70.

Electron diffraction pattern of a catalase crystal which was frozen in liquid nitrogen

Electron Diffraction of Frozen, Hydrated Protein Crystals

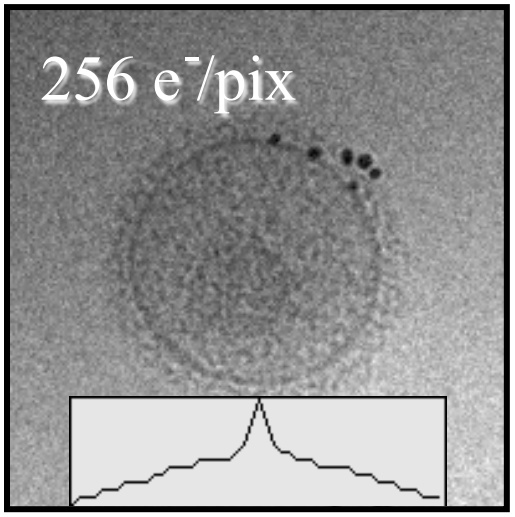
Abstract. *High-resolution electron diffraction patterns have been obtained from frozen, hydrated catalase crystals to demonstrate the feasibility of using a frozen-specimen hydration technique. The use of frozen specimens to maintain the hydration of complex biological structures has certain advantages over previously developed liquid hydration techniques.*

KENNETH A. TAYLOR
ROBERT M. GLAESER
Division of Medical Physics, Donner Laboratory, and Lawrence Berkeley Laboratory, University of California, Berkeley 94720

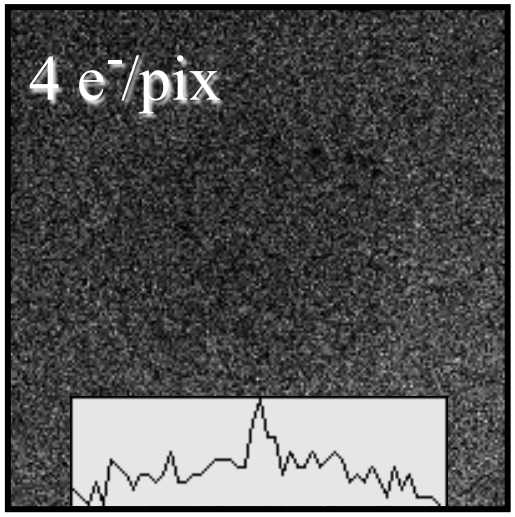
13 DECEMBER 1974

Science. 1974 Dec 13;186:1036-7

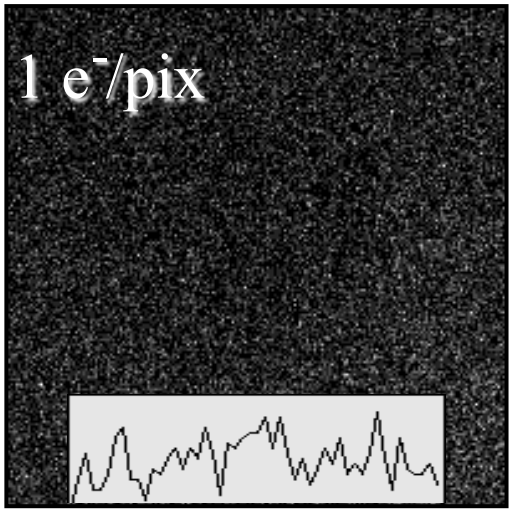
The solution:
Dose fractionation and automation



1 projection
(TD = 256 e^-/pix)
SNR = 6.1



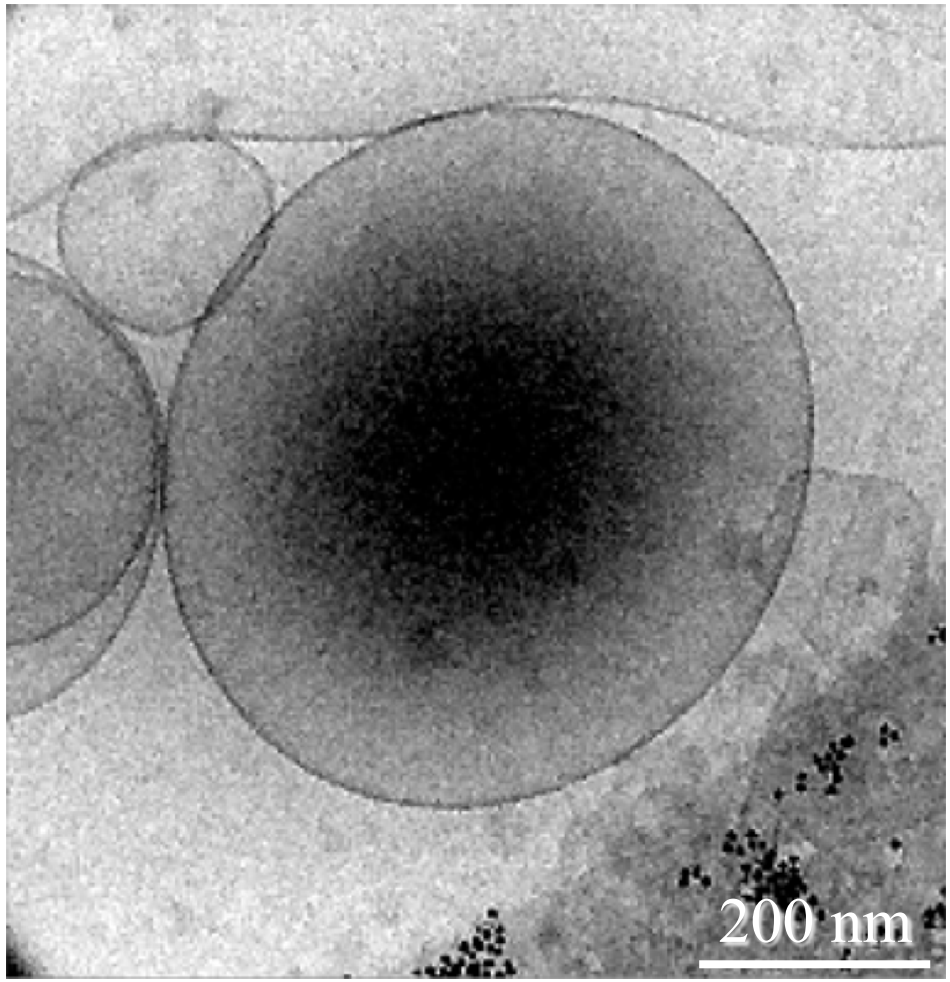
64 projections
(TD = 256 e^-/pix)
SNR = 0.14

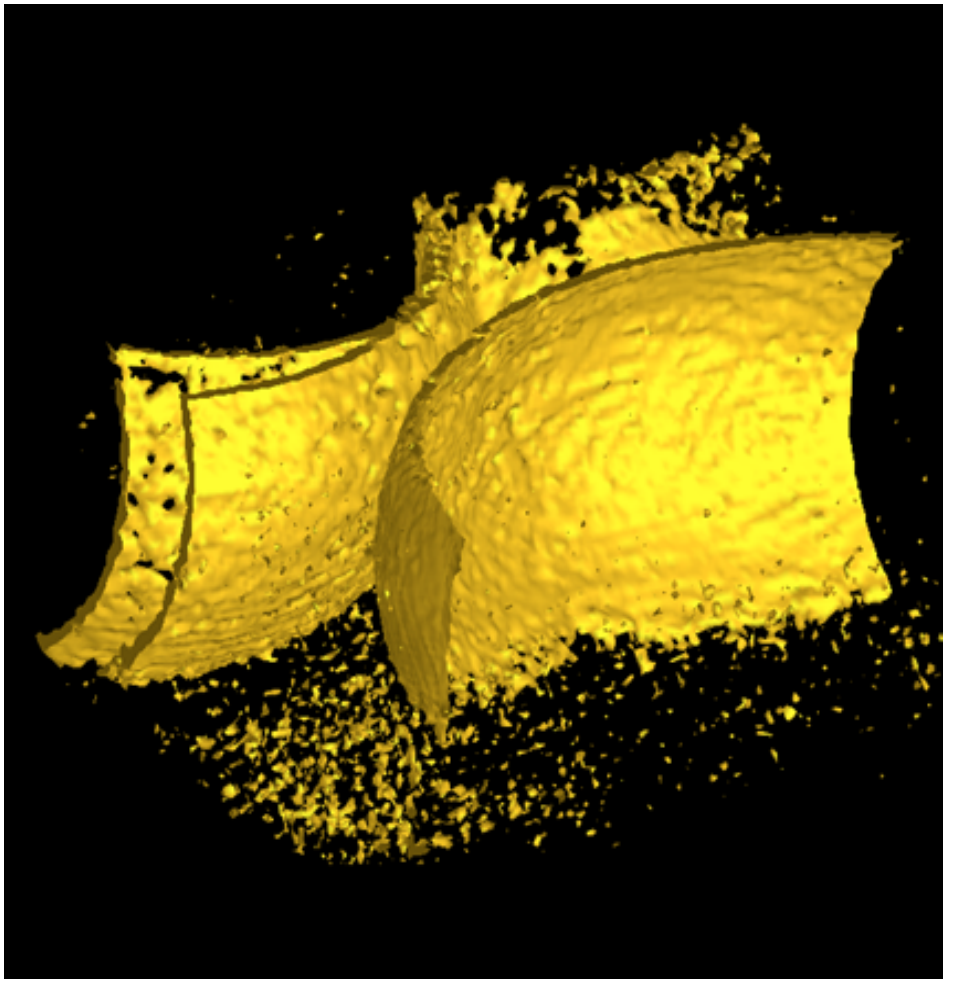


256 projections
(TD = 256 e^-/pix)
SNR = 0.036

$$SNR = \frac{S^2}{N^2} = \frac{\text{variance (msd) of noise free image}}{\text{variance (msd) of noise}}$$

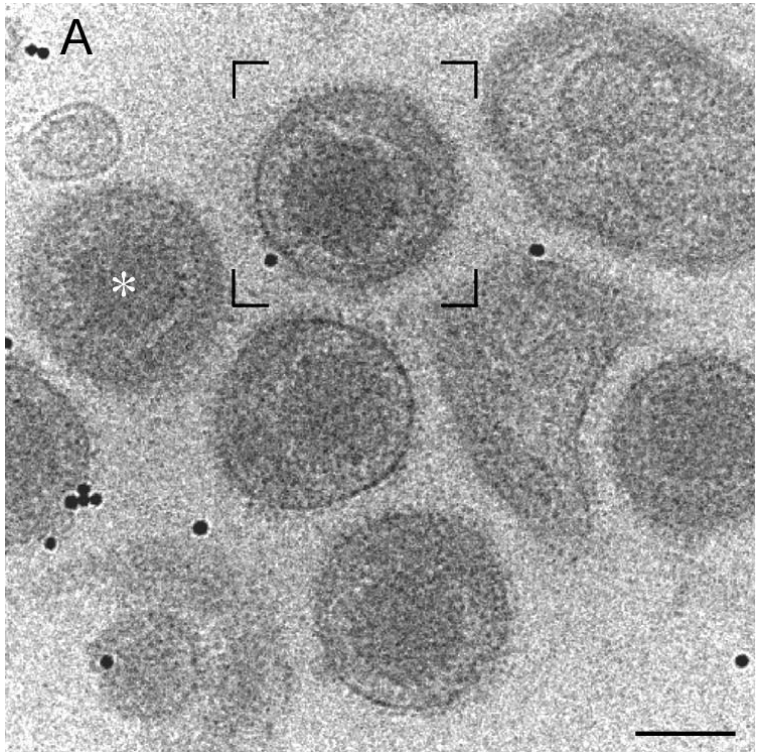
Reconstruction of two vesicles in contact



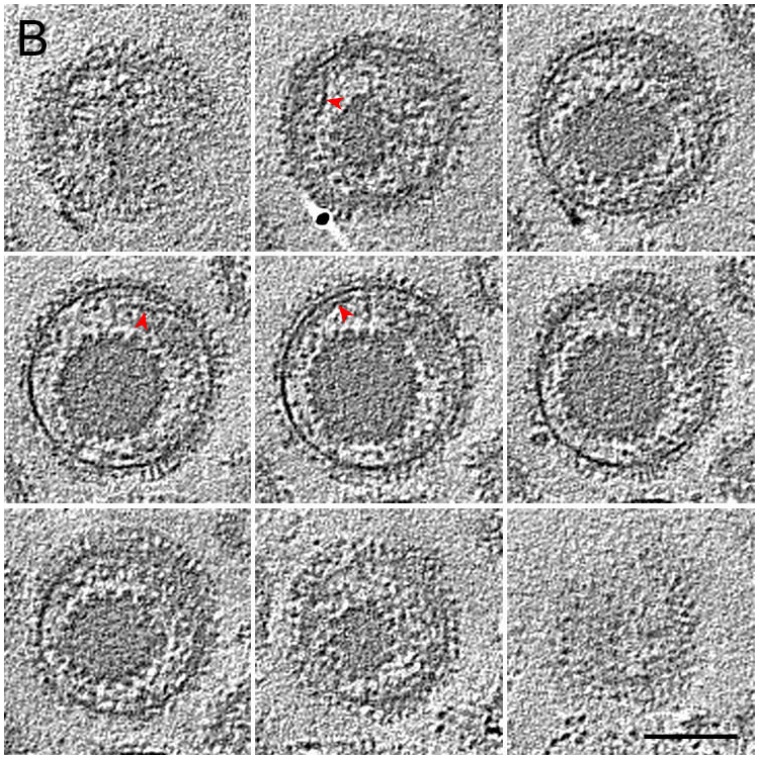


K. Dierksen et al., Biophys. J. 68 (1995) 1416-1422

Tomography of HSV-1 virions in vitreous ice



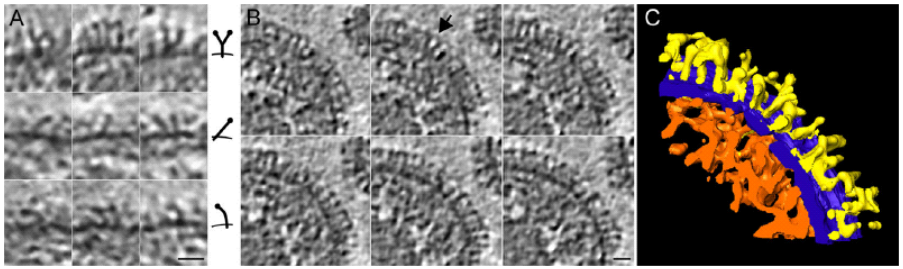
Zero degree projection
from the tilt series

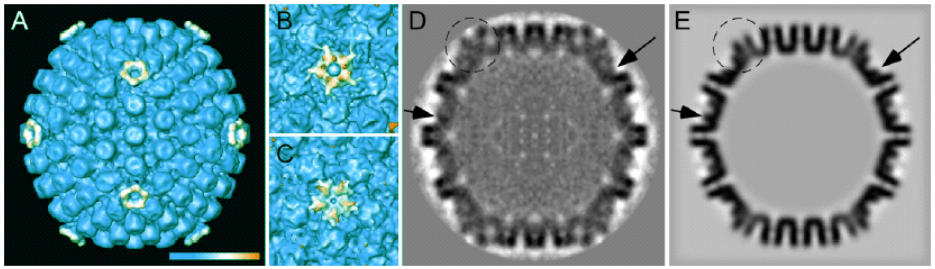


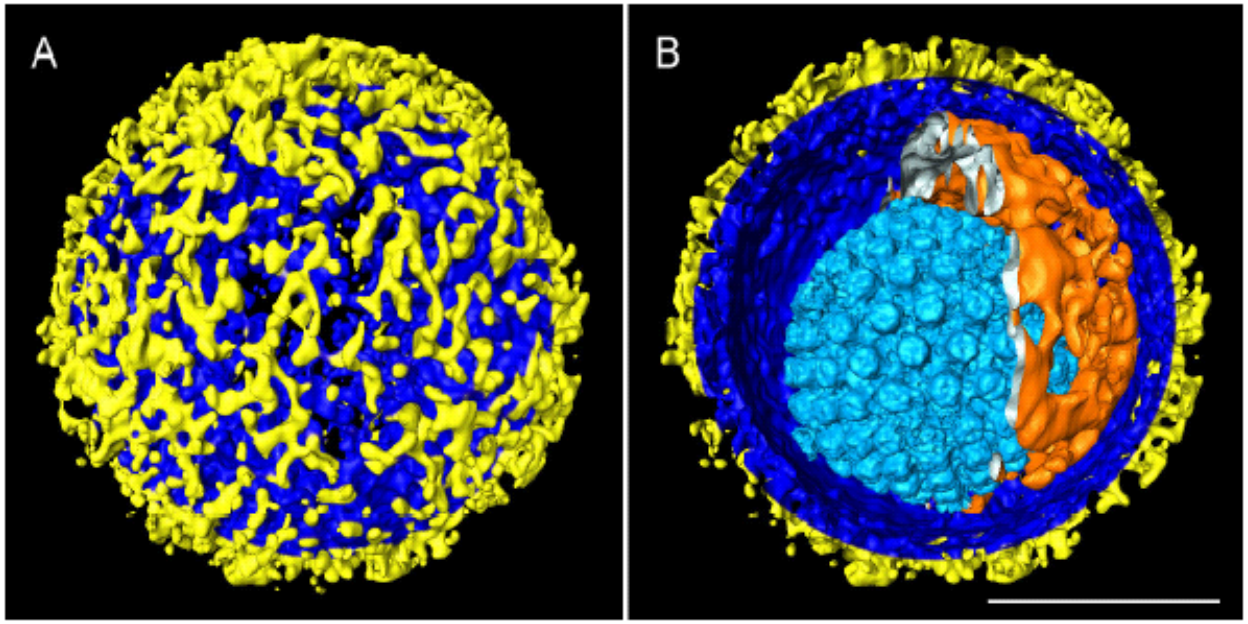
Equidistant slices (15Å thick)
of the framed virion

K. Grünewald, P. Desai, D.C. Winkler, J.B. Heymann, D.M. Belnap, W. Baumeister and A.C. Steven:
Science 302 (2003) 1396-1398

Herpes simplex virus

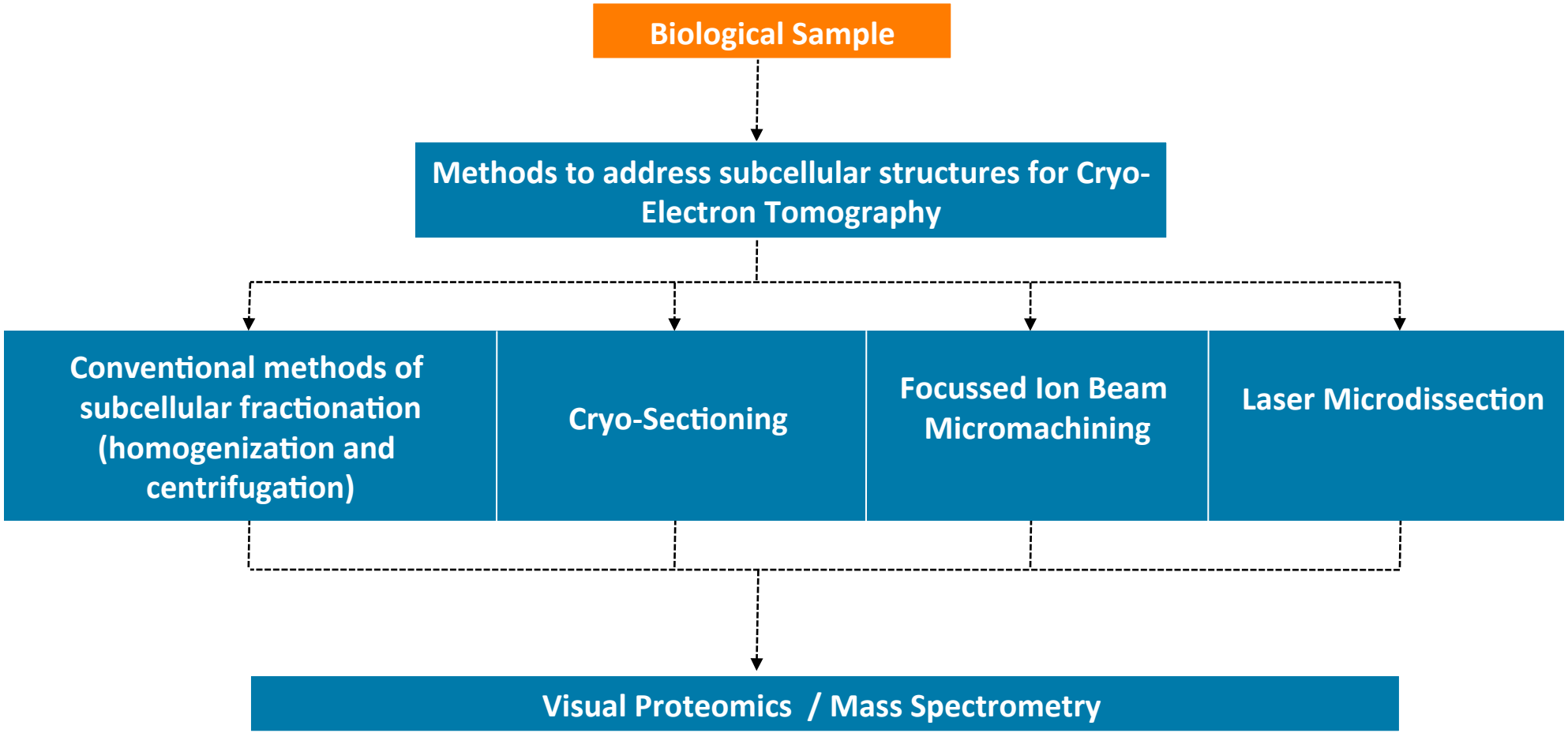




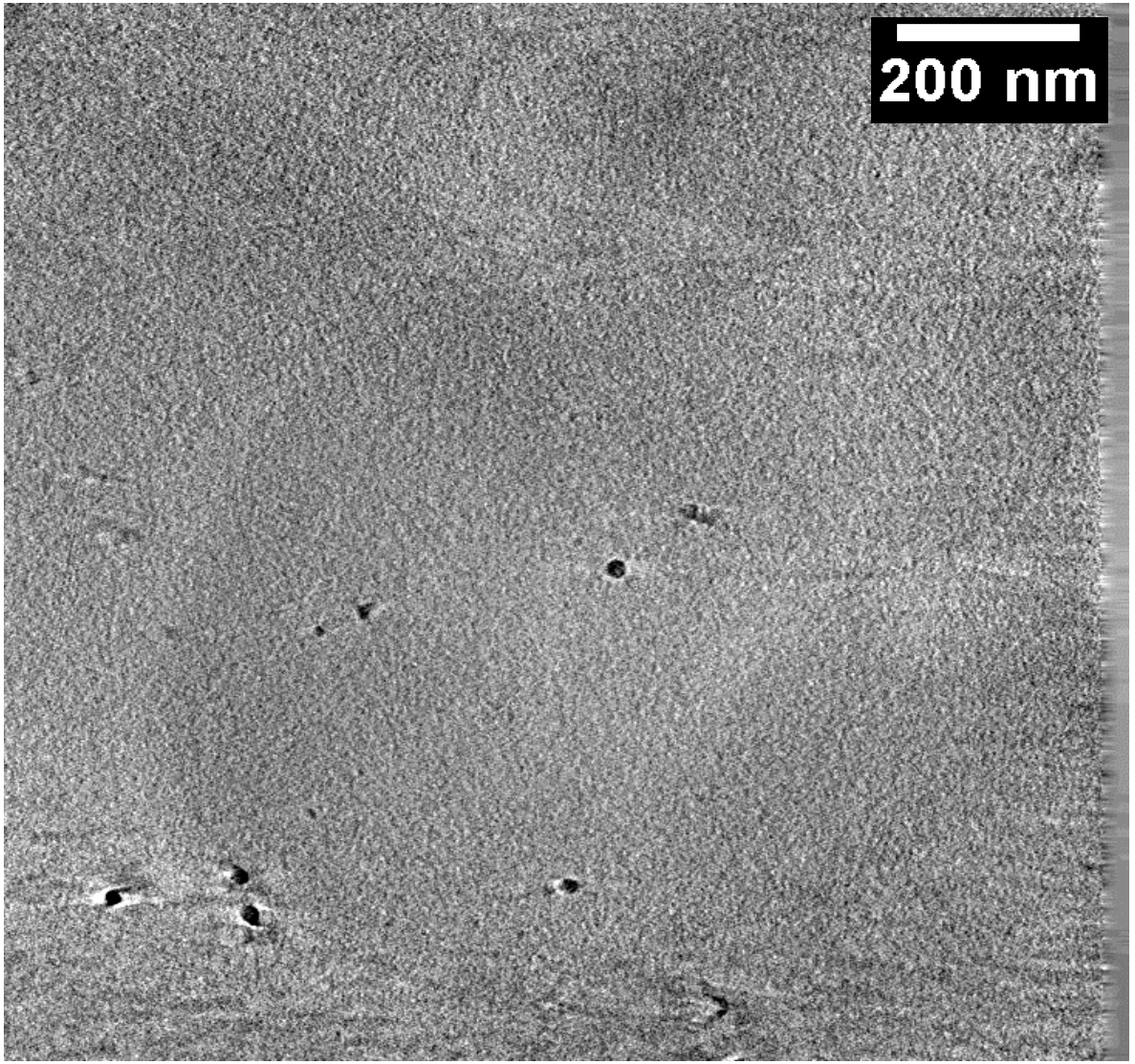


K. Grünewald, P. Desai, D.C. Winkler, J.B. Heymann, D.M. Belnap, W. Baumeister and A.C. Steven:
Science 302 (2003) 1396-1398

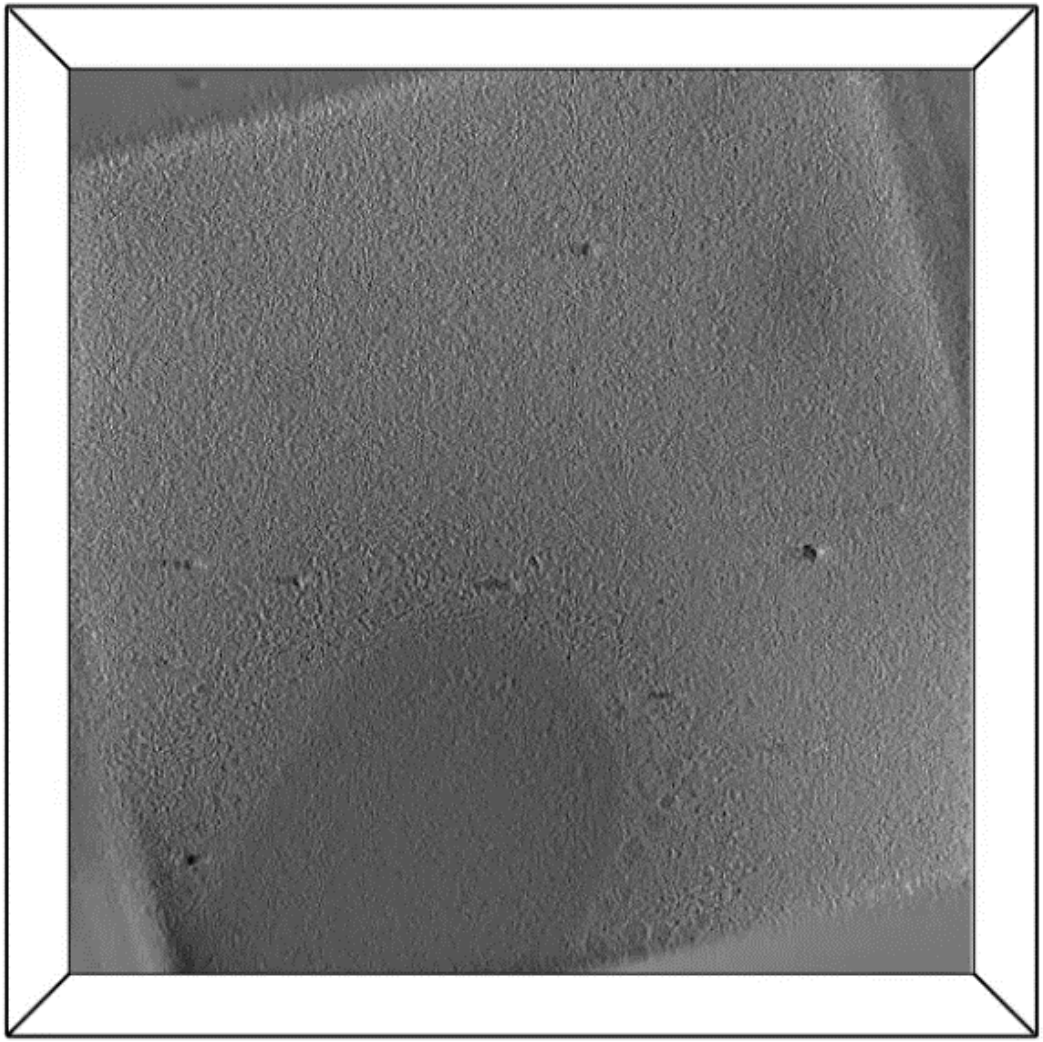
Key to attaining ‘high resolution’ in Cryo-Electron Tomography is the preparation of thin samples (< 1 µm)!



Synaptosome Tomogram



3D structure of an early tail in *Xenopus* egg extracts



FEI Polara G2 @ 300kV

Defocus -12

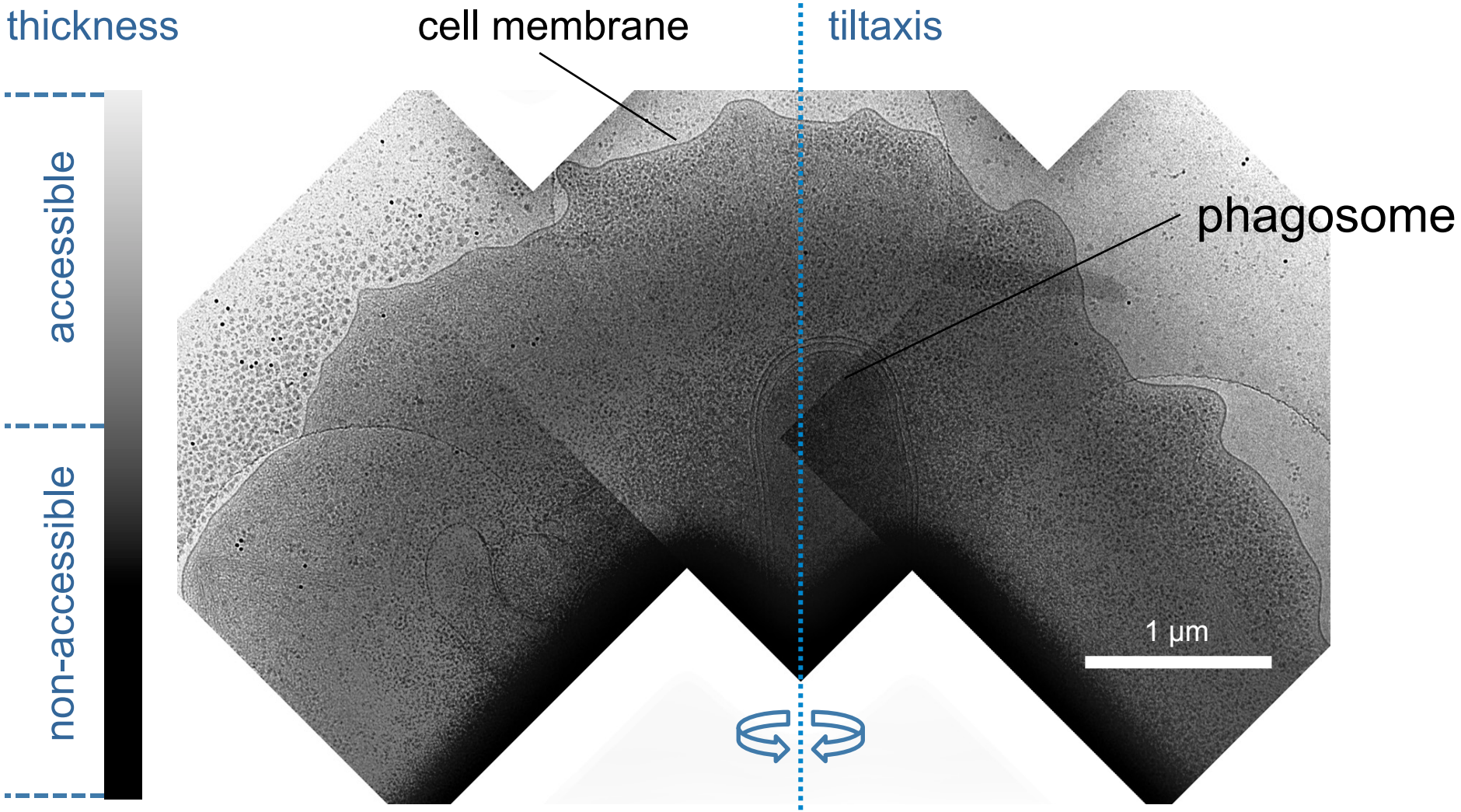
-42 to 60°, 1.5° increment

1.42 nm/pixel

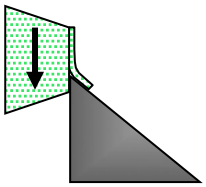
Sample thickness = 320 nm

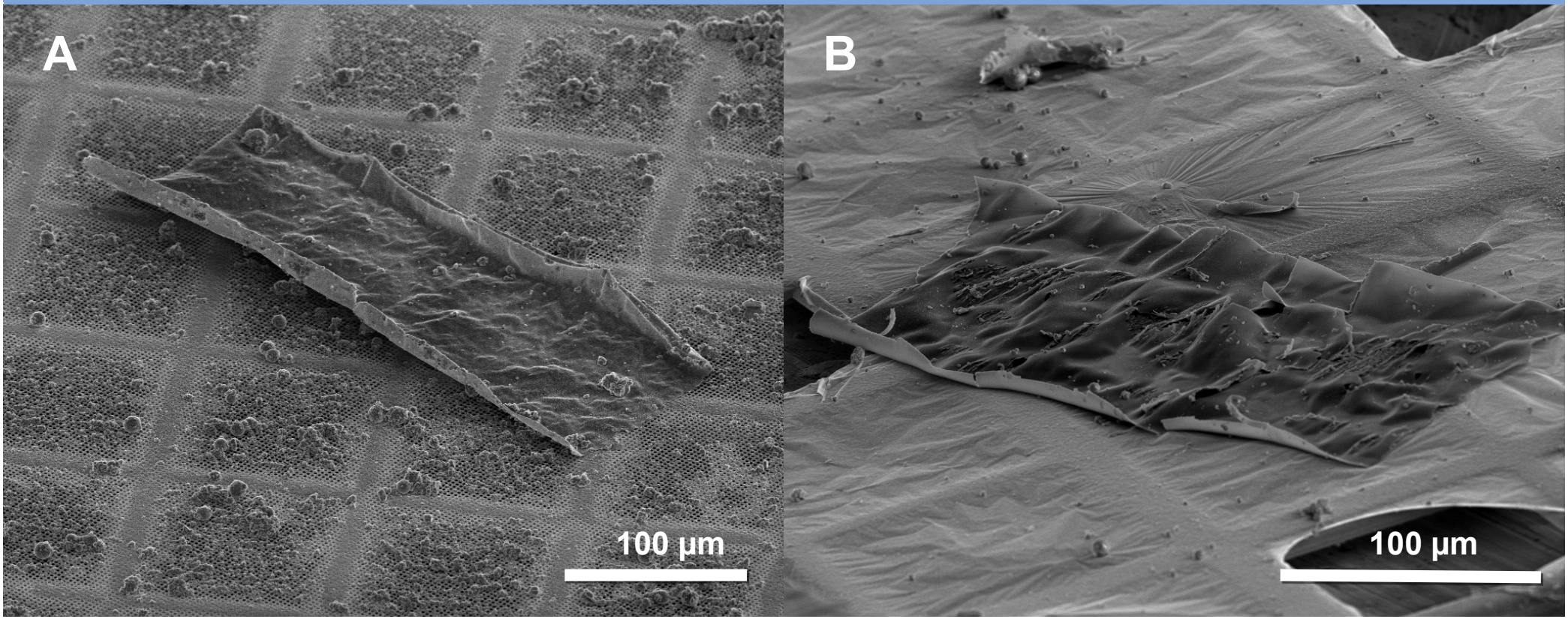
Automated filament segmentation (Amira)

Cellular Tomography



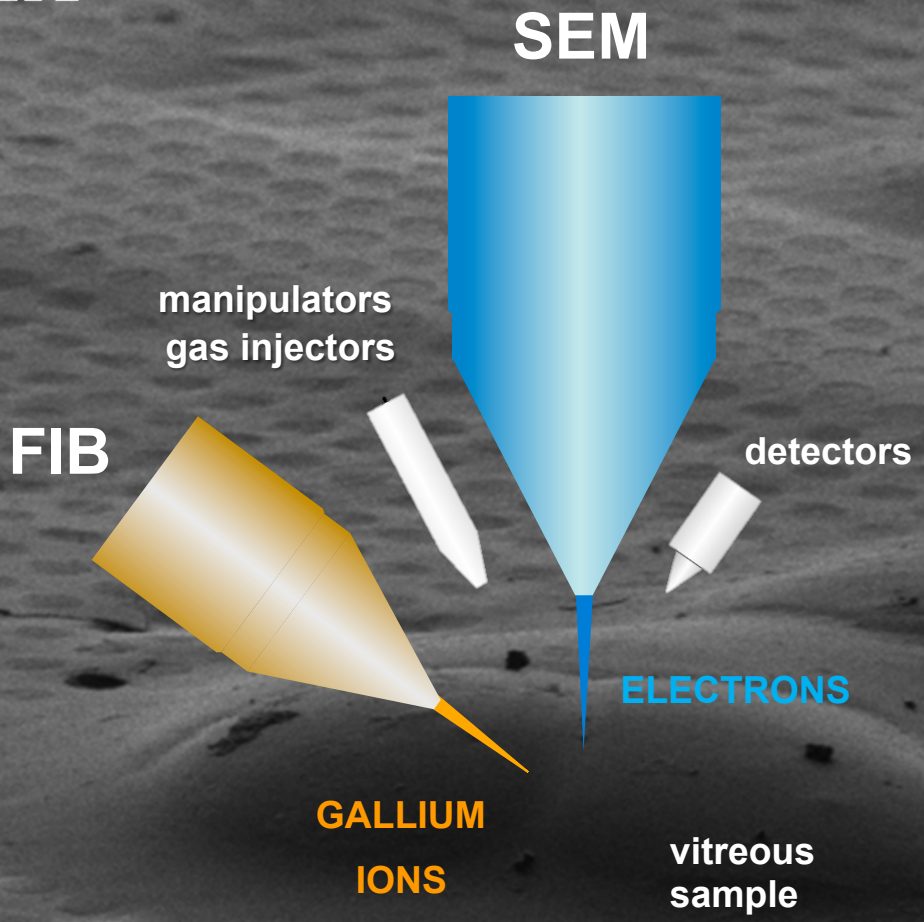
Cryo-ultramicrotomy

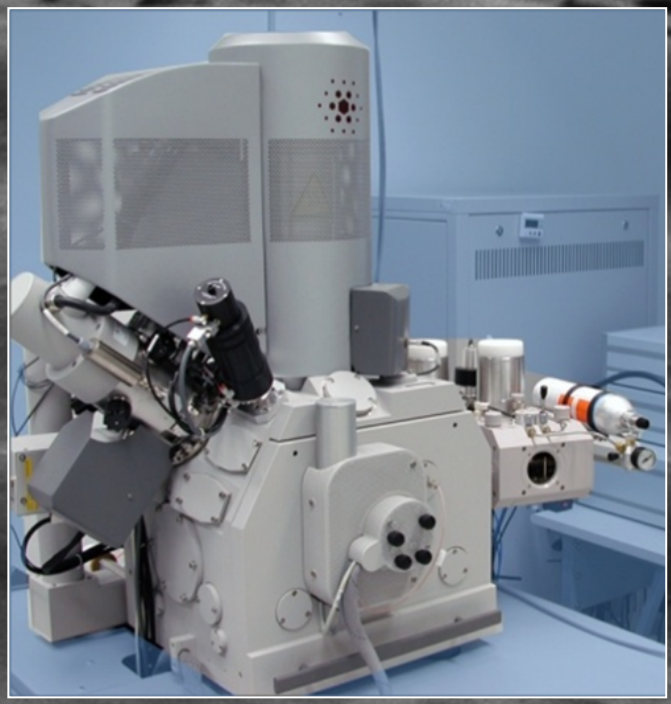




- waviness of sections on the grid
- crevasses/knife marks
- attachment to the grid
- compression

Focused Ion Beam Milling





…allows the making of thin and compression-free ‘windows‘ into cells

Cryo-FIB: The Challenges

- **Ion interaction**

  minimize depth of ion penetration (~10-20 nm)

- **Temperature**

  specimen must remain below devitrification temperature at all times
  ion milling should not cause significant heating

- **Contamination**

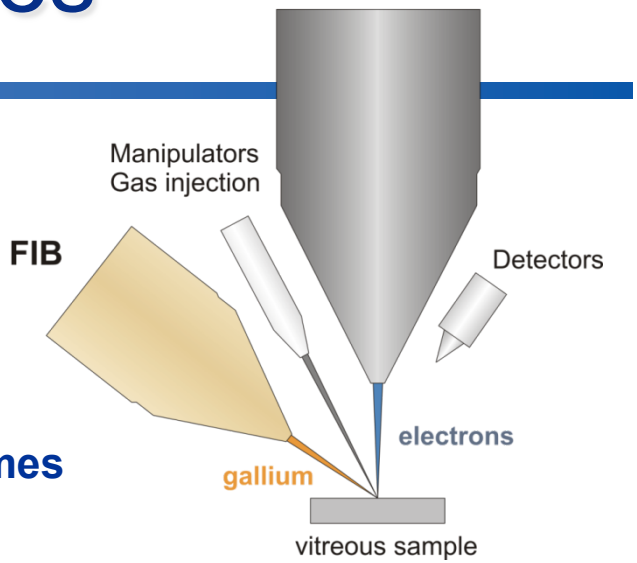
  specimen must remain frost free during all transfer steps

- **Milling**

  thinned area must be suitably oriented for TEM imaging

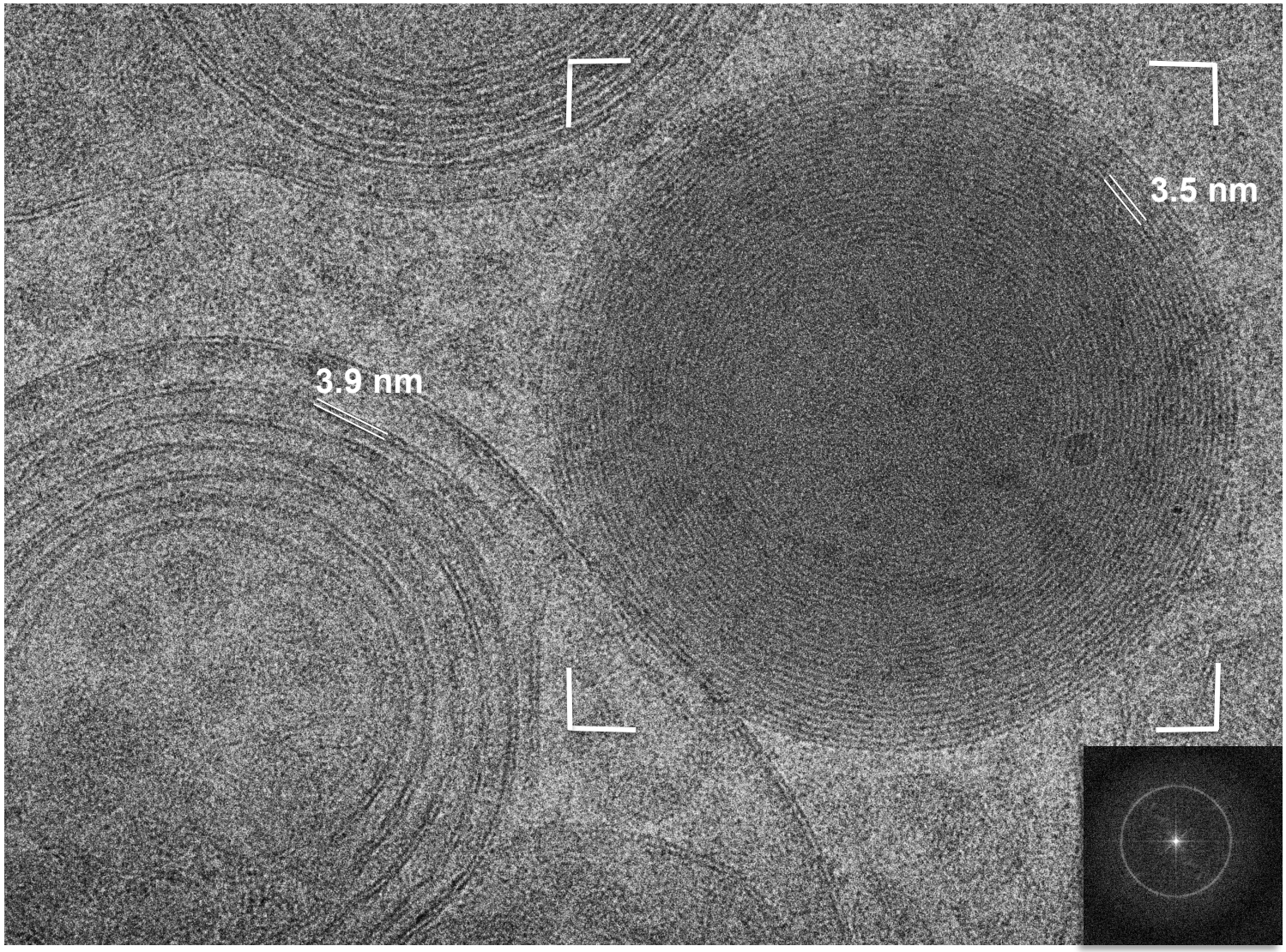
- **Specimen navigation**

  appropriate milling sites must be identified and targeted



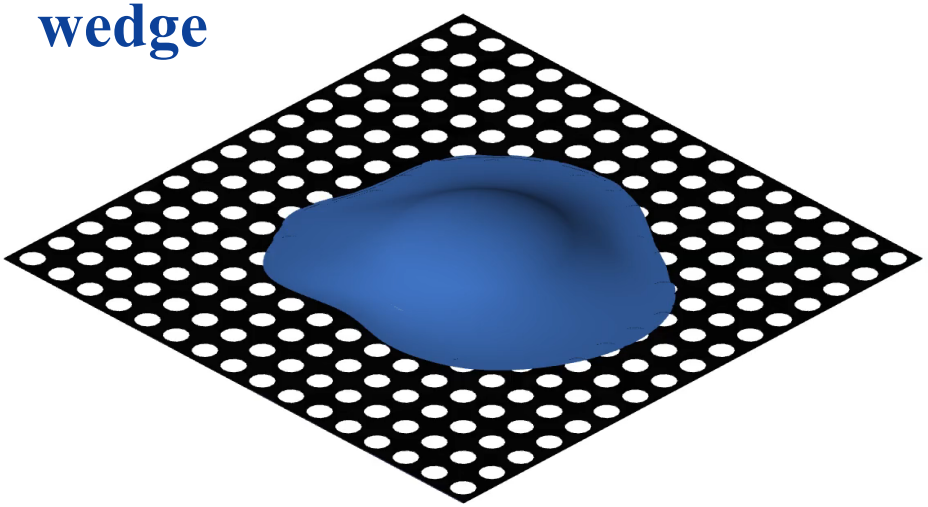
Lipid Droplets in a HeLa Cell

High dose single projection

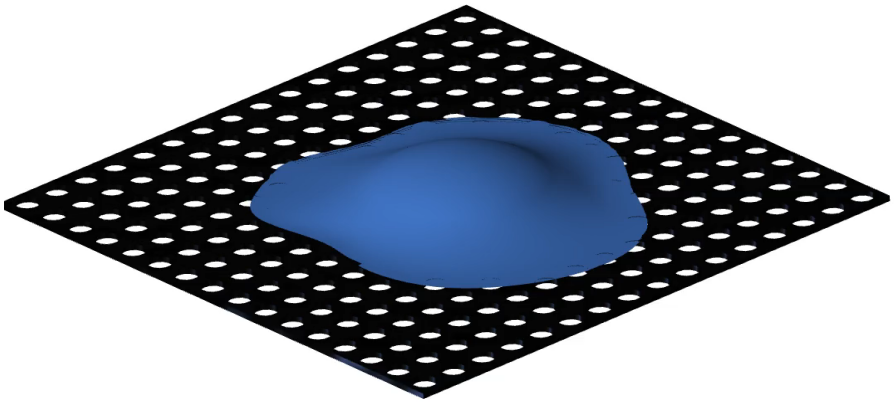


Cryo-FIB milling

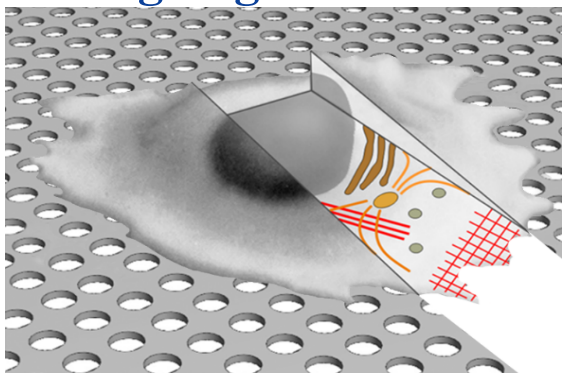
wedge

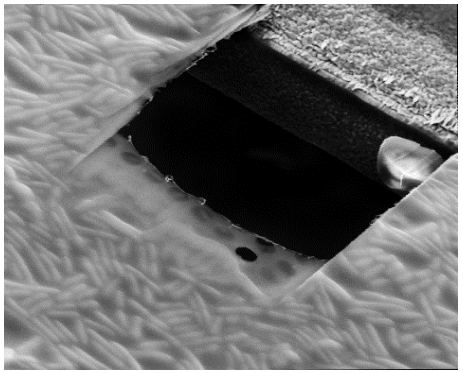


lamella

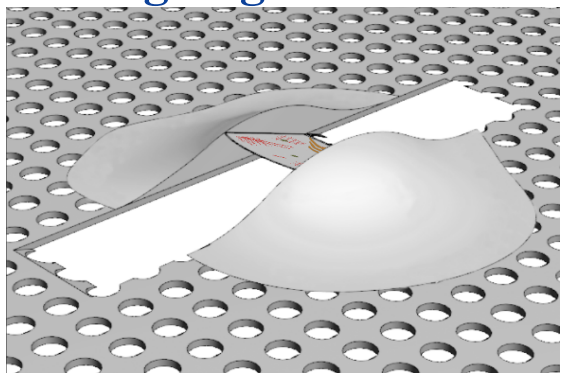


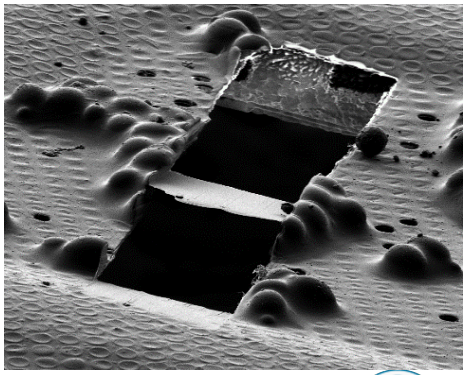
milling angle: 3 to 7°





milling angle: 7 to 15°

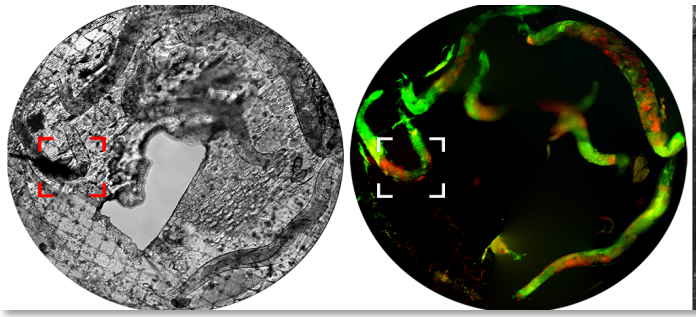




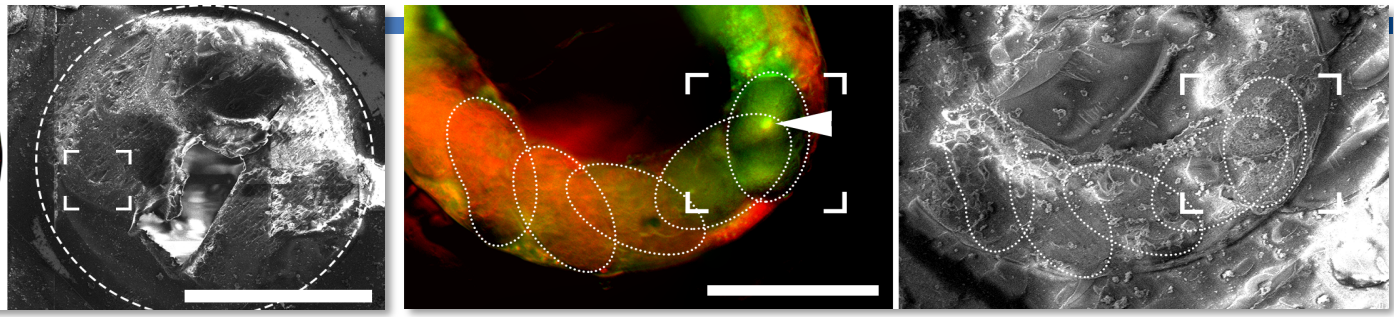
[1] E Villa et al., COSTBI 23(5) (2013) p.771–777

The Next Step:
Cryo-FIB and Lift-out for Site-specific Preparation of Frozen-hydrated Volumes

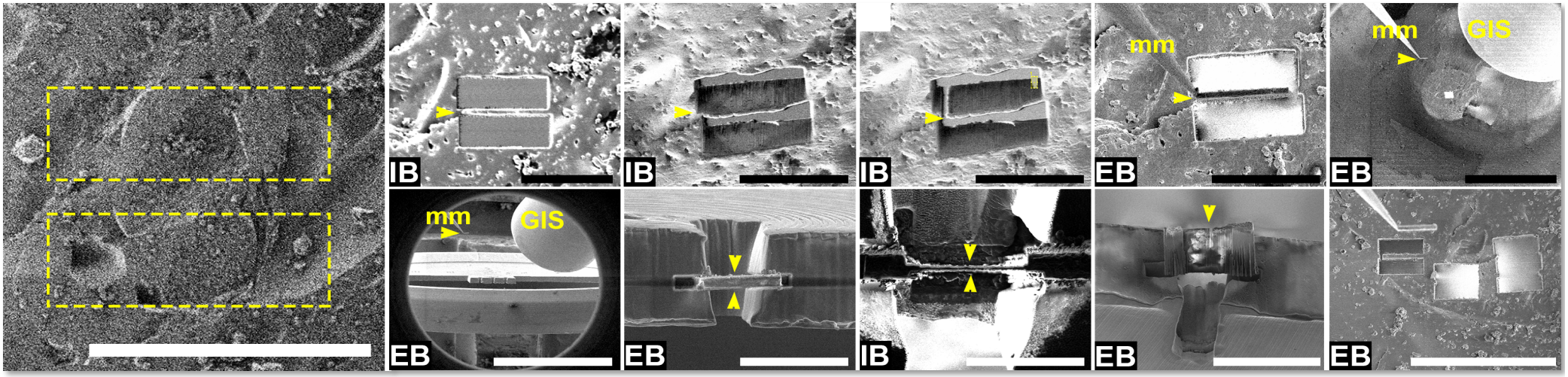
Correlation



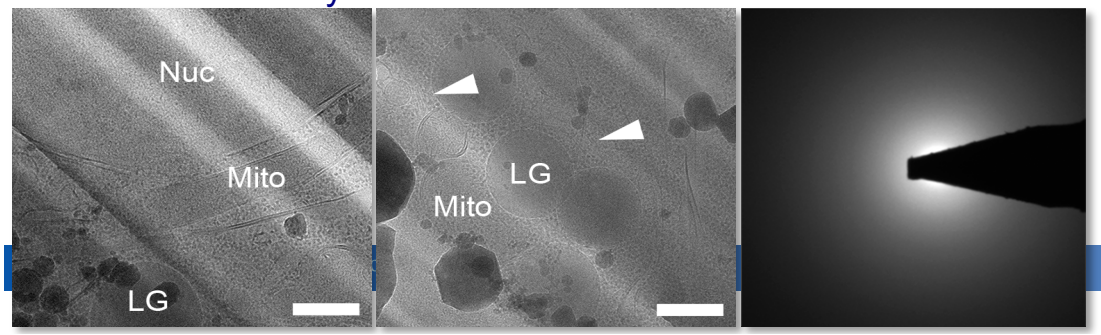
Localization



Site-specific cryo-FIB lift-out



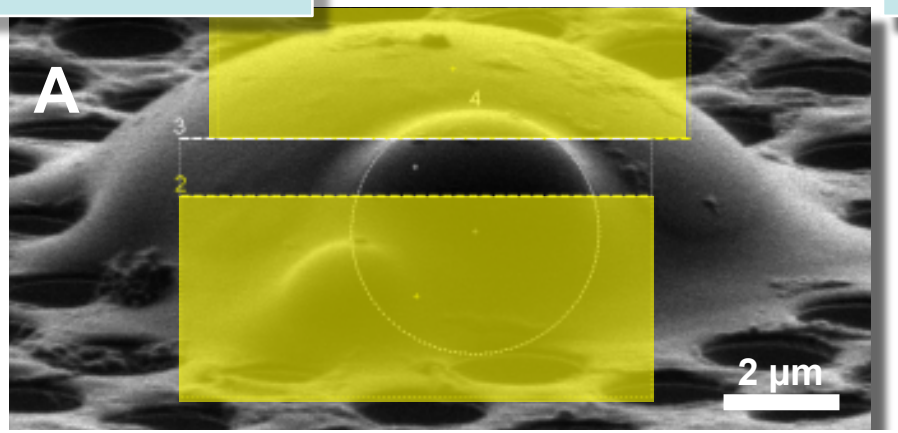
Cryo-TEM & electron diffraction



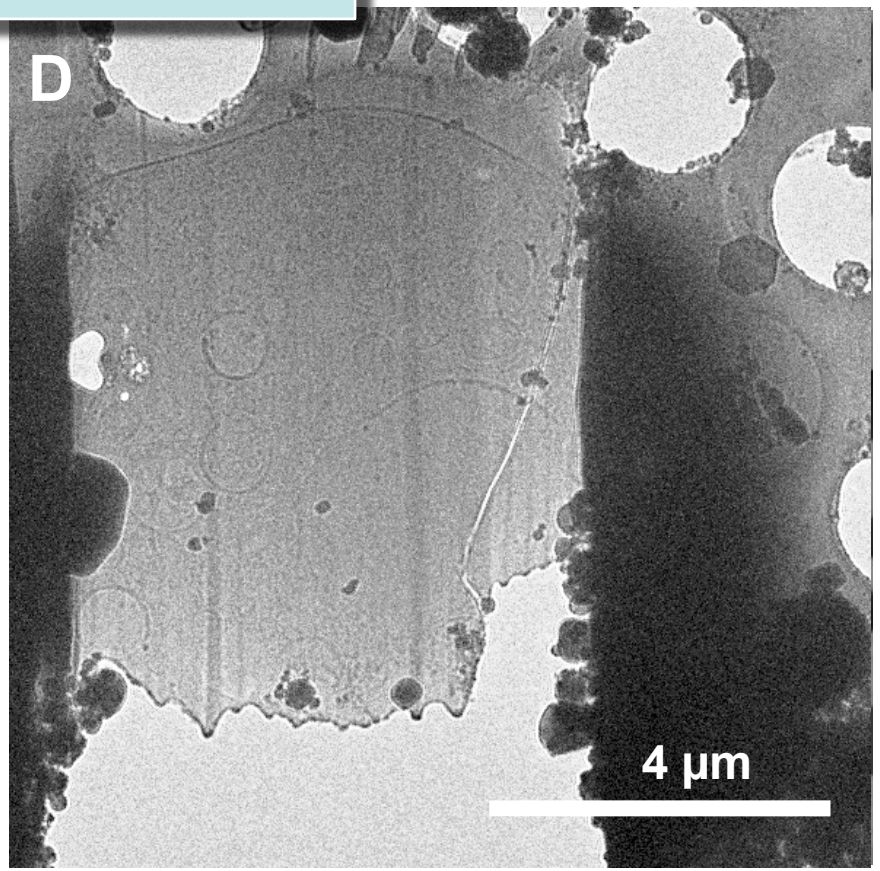
Julia Mahamid, Ruud Schampers, Hans Persoon, Jürgen Plitzko

Cryo-FIB Lamella Preparation

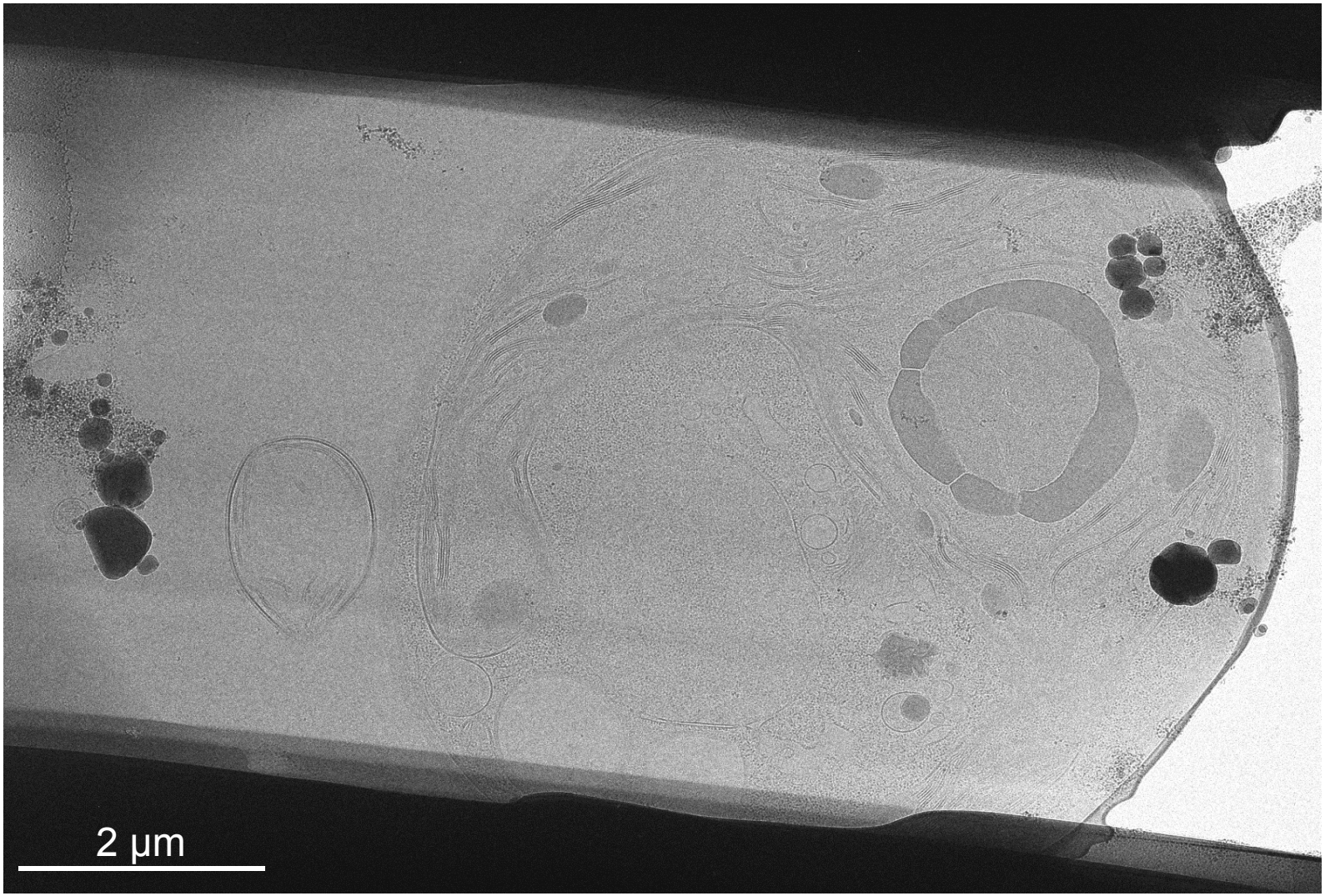
FIB milling
(**A** before; **B** after)



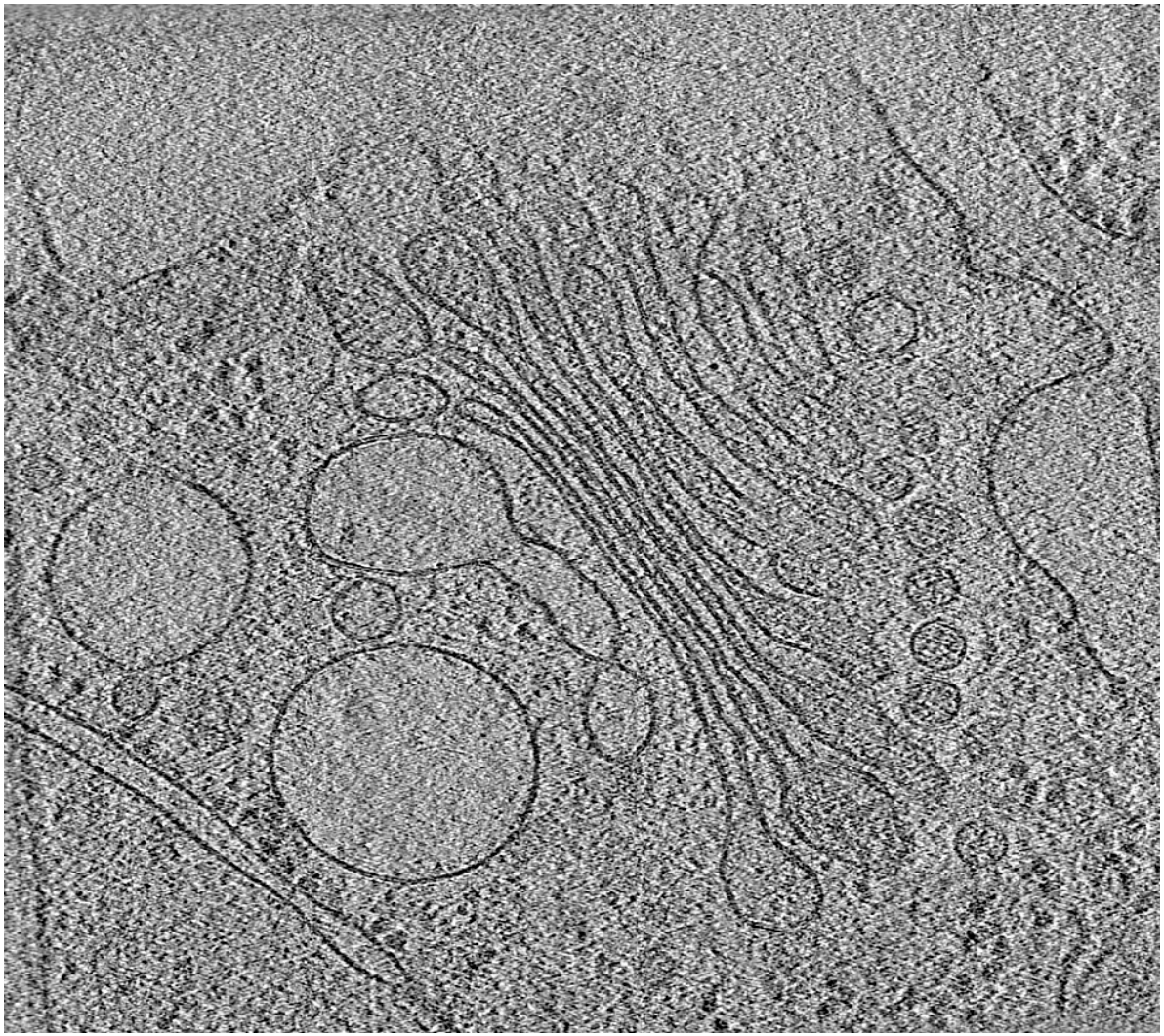
Thinned region
(Cryo-TEM)

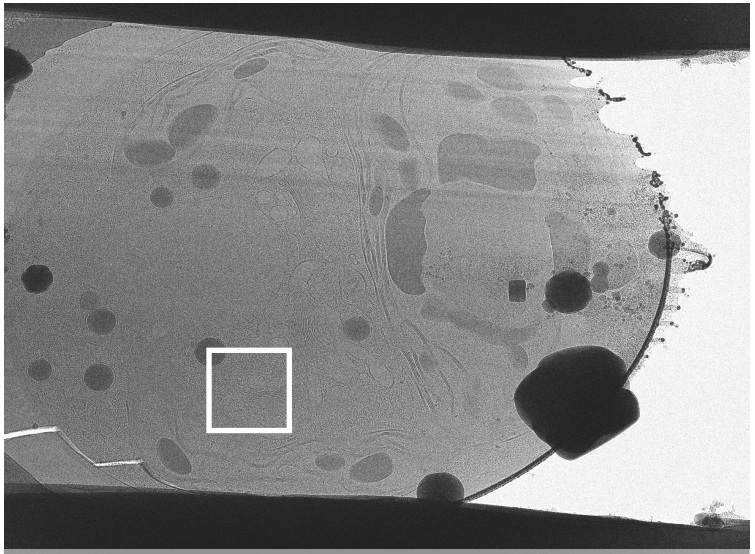


Chlamydomonas reinhardtii



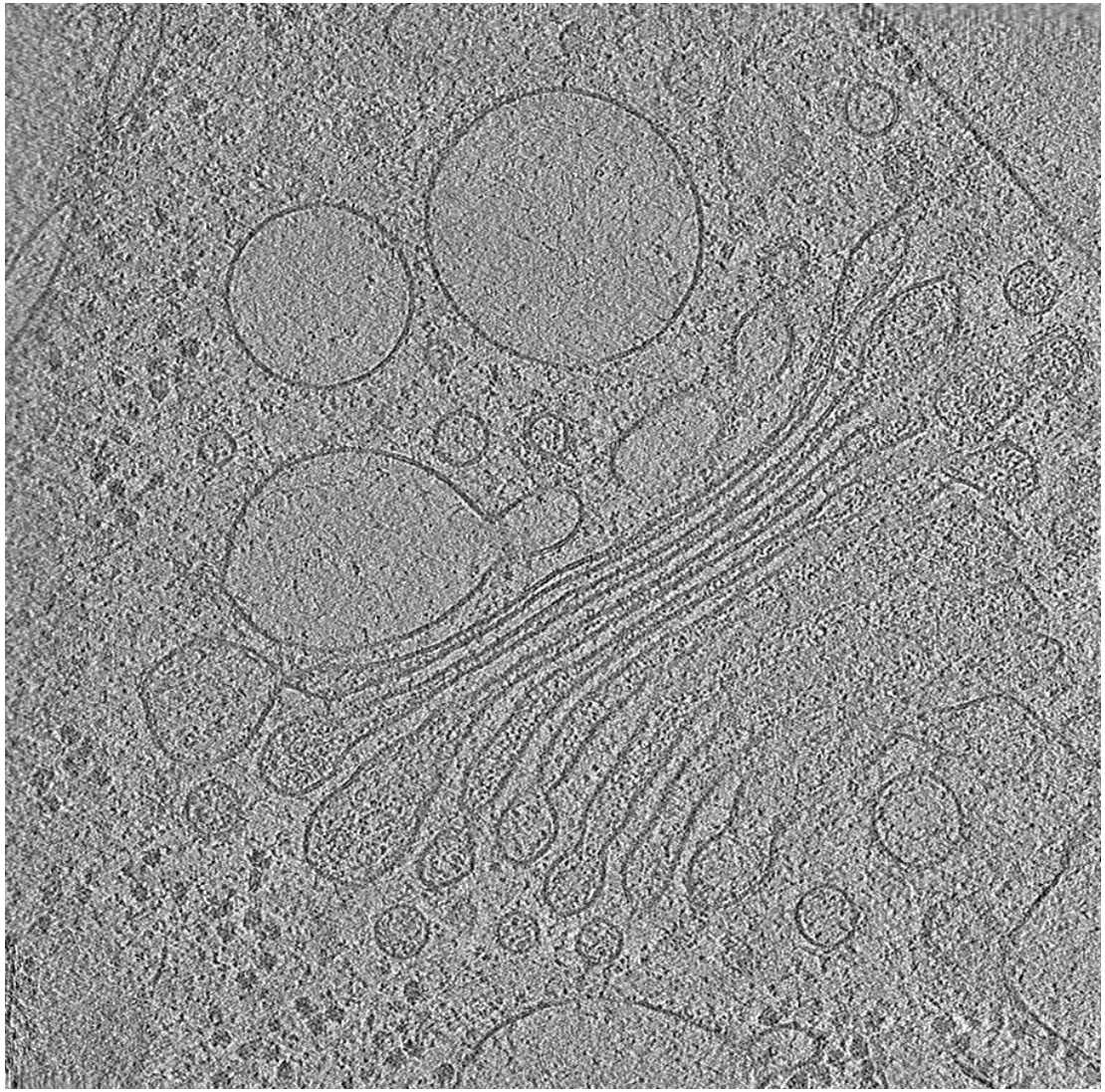
Golgi apparatus





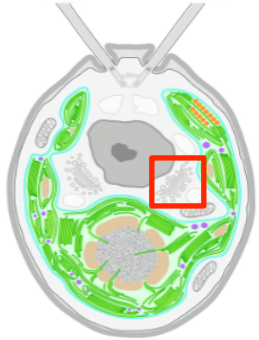
total dose: ~ 90 e/A^2
Defocus = - 4µm
~200-nm FIB lamella

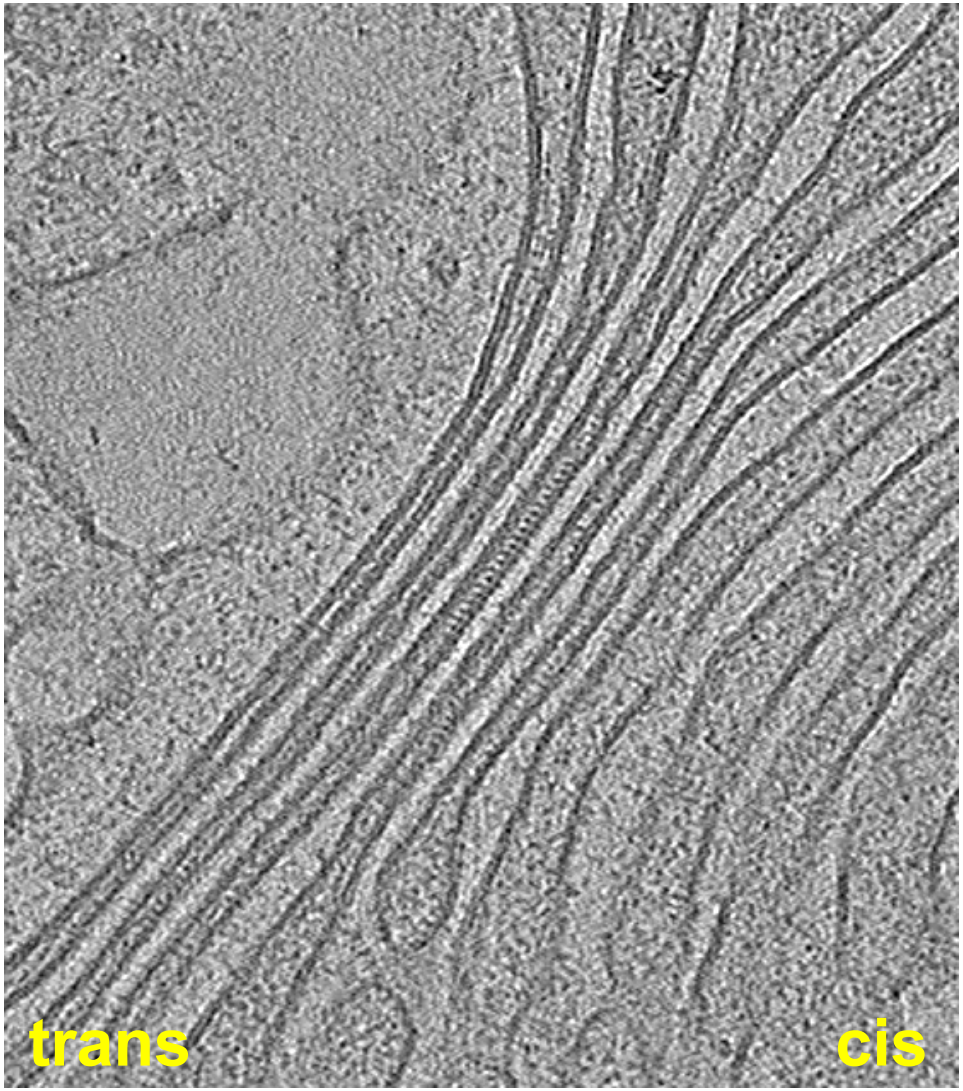
Golgi apparatus

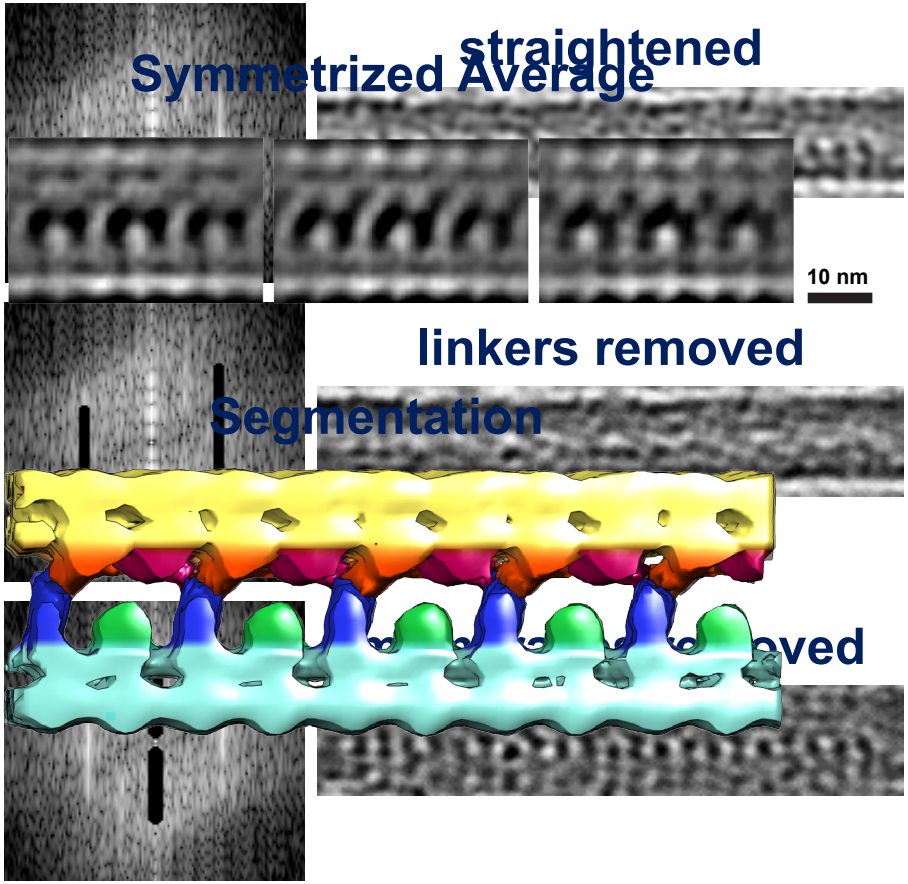


total dose: ~ 90 e/A^2
Defocus = - 4µm
~200-nm FIB lamella

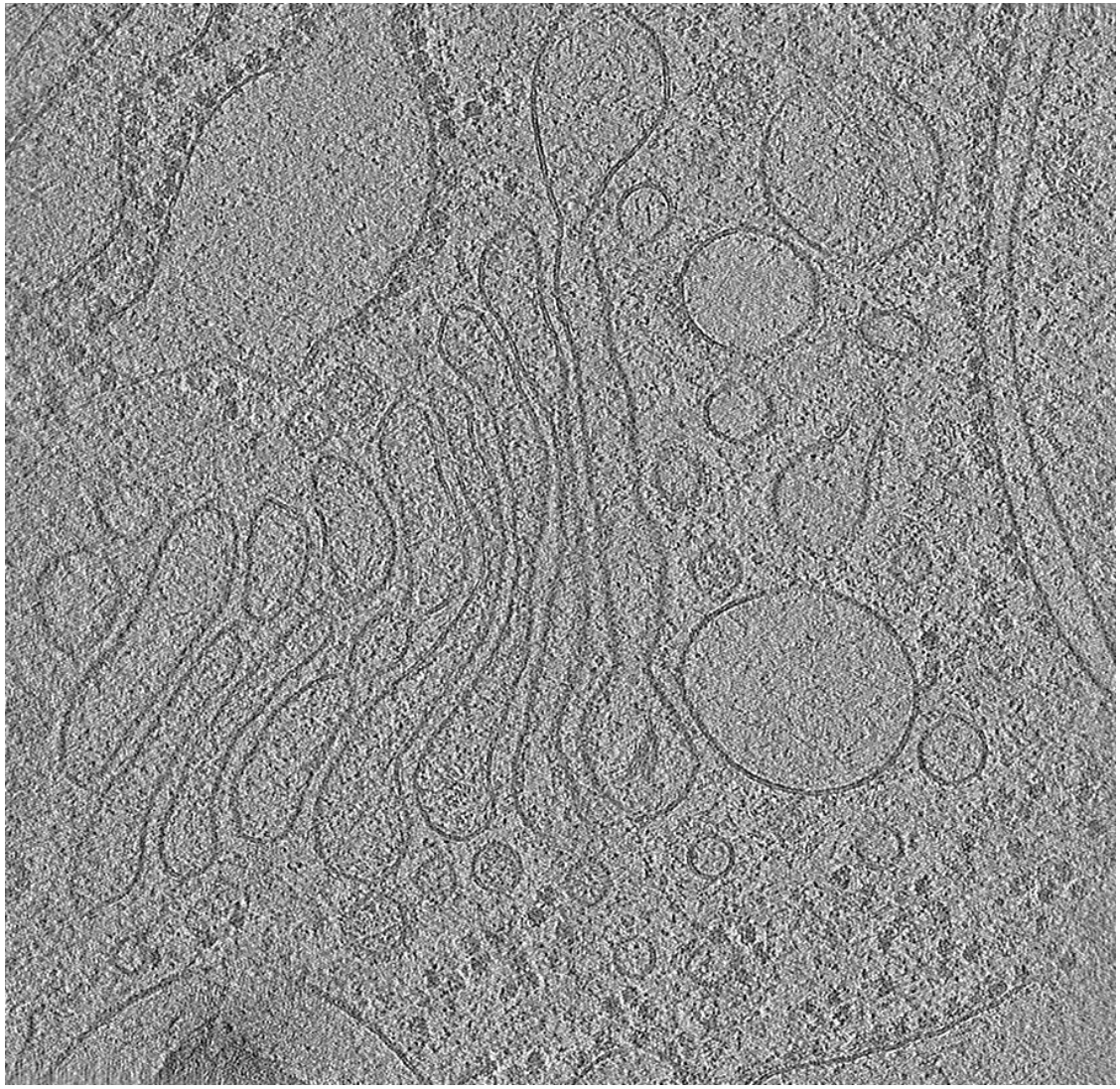
Golgi: Linkers with 11.8 nm repeat within thin stacks





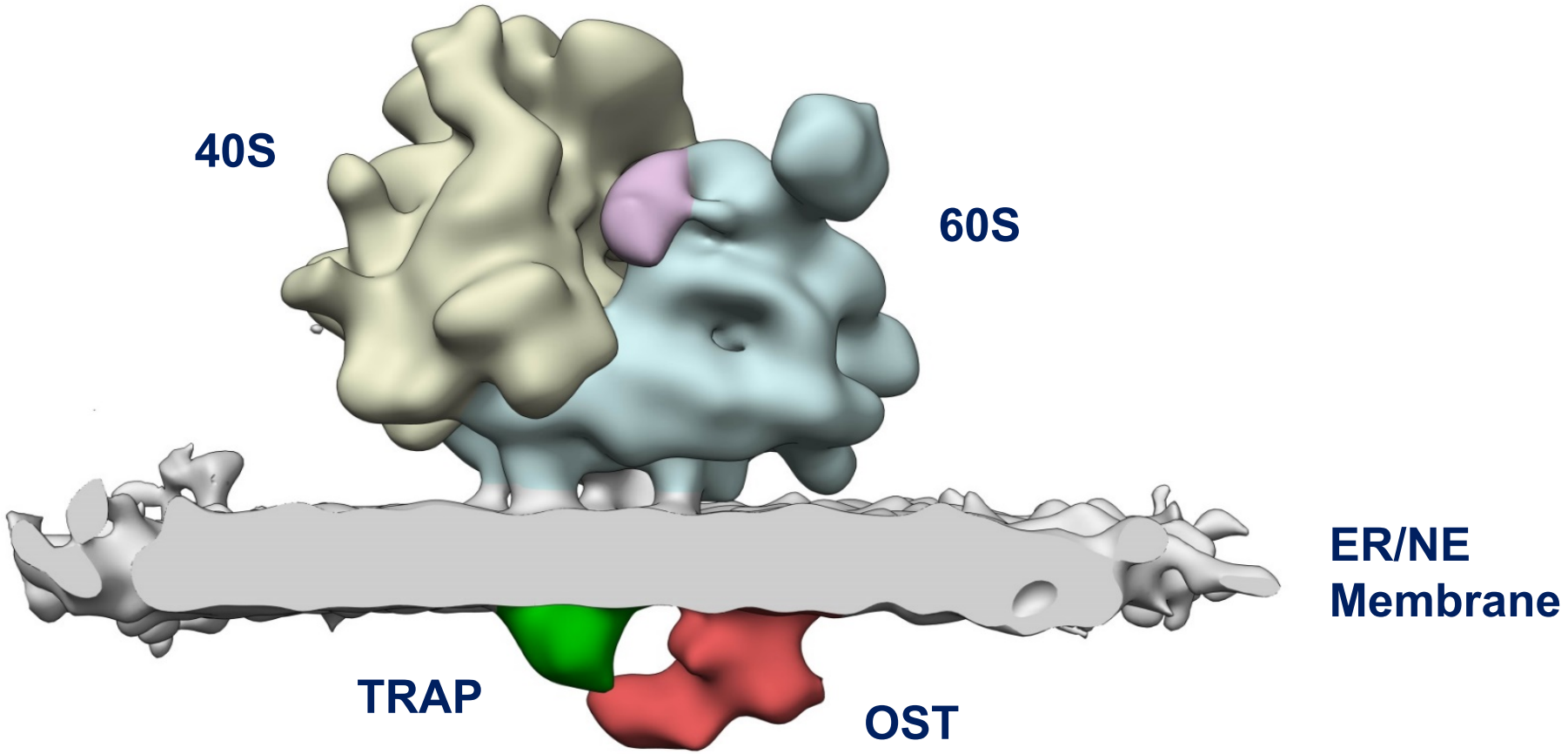


Golgi apparatus, ER and Nucleus

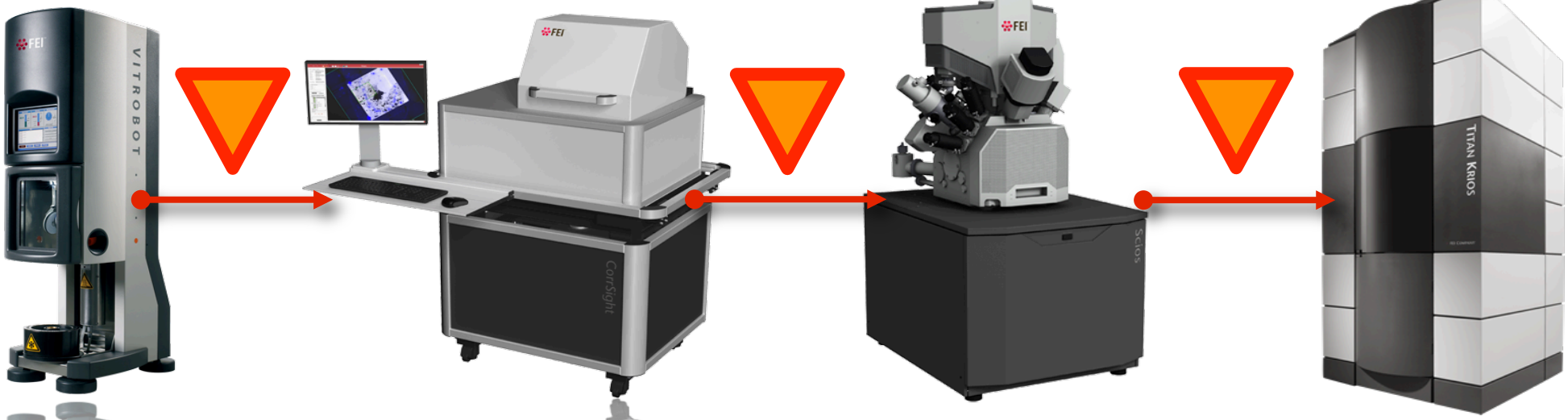


total dose: ~ 90 e/A^2
Defocus = - 4µm
~200-nm FIB lamella

In situ structure of ER and NE membrane-bound ribosomes and translocon



Cellular Cryo-Electron Tomography: Sample Preparation Workflow



Vitrobot IV

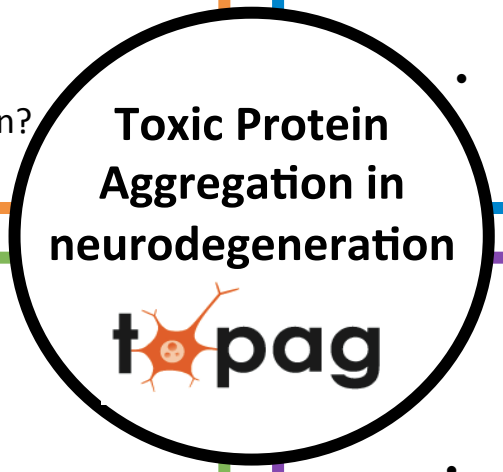
CorrSight

Quanta 3D
Quorum system

Titan Krios
Energy filter
K2 direct detector
Volta phase plate
(Danev et al. 2015)

Plitzko JM, Rigort A and Leis A. Current Opinion in Biotechnology 2009

The Topag Project



F.U. Hartl
MPI Biochemistry

- How do aggregates affect the proteostasis network?
- Can we boost the cellular defenses against aggregation?

W. Baumeister
MPI Biochemistry

- What is the in situ structure of the aggregates?
- How do they interact with the cellular environment?

R. Klein
MPI Neurobiology

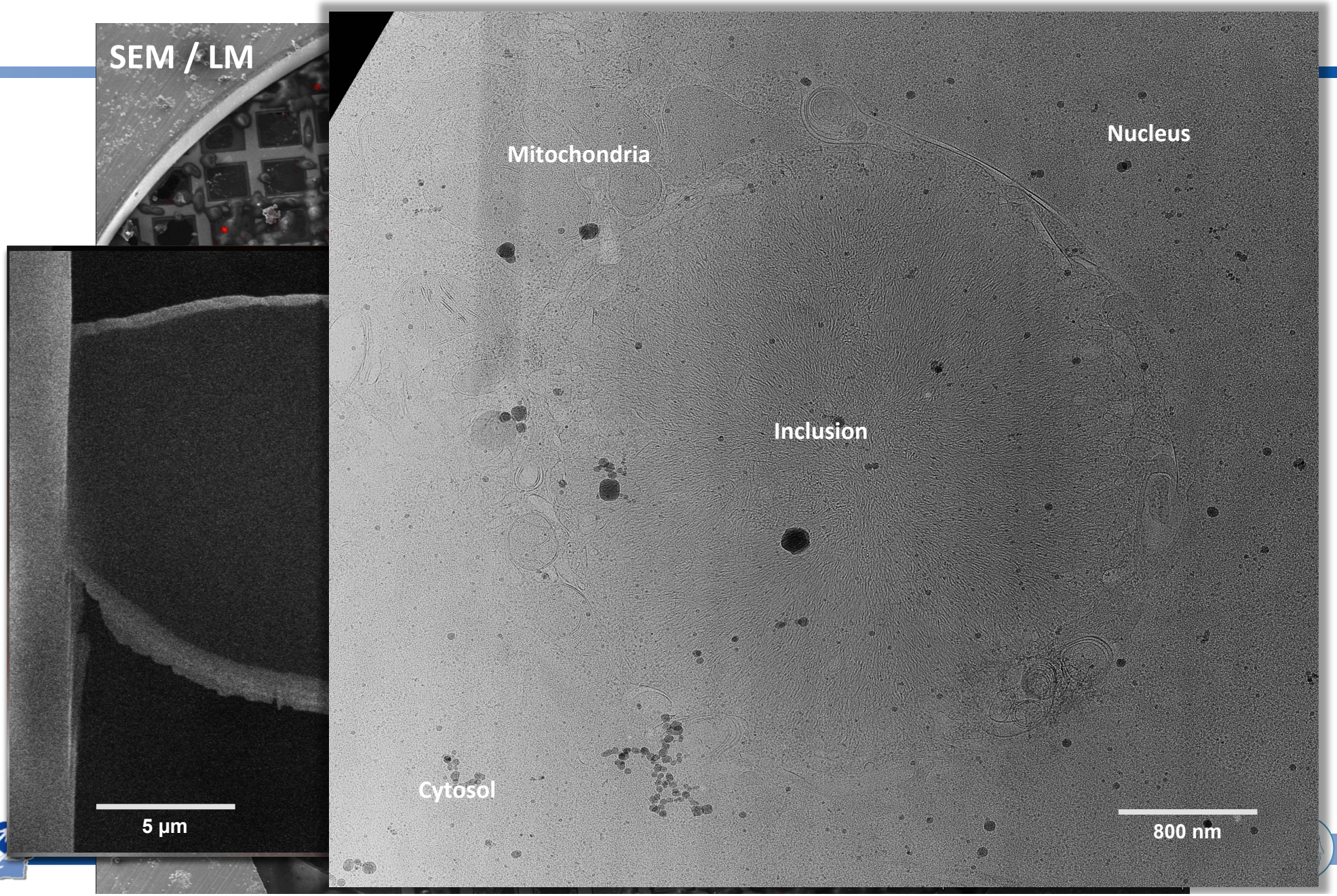
- How do aggregates cause neurodegeneration in vivo?
- How does the state of the proteostasis network affect neuronal function?

M. Mann
MPI Biochemistry

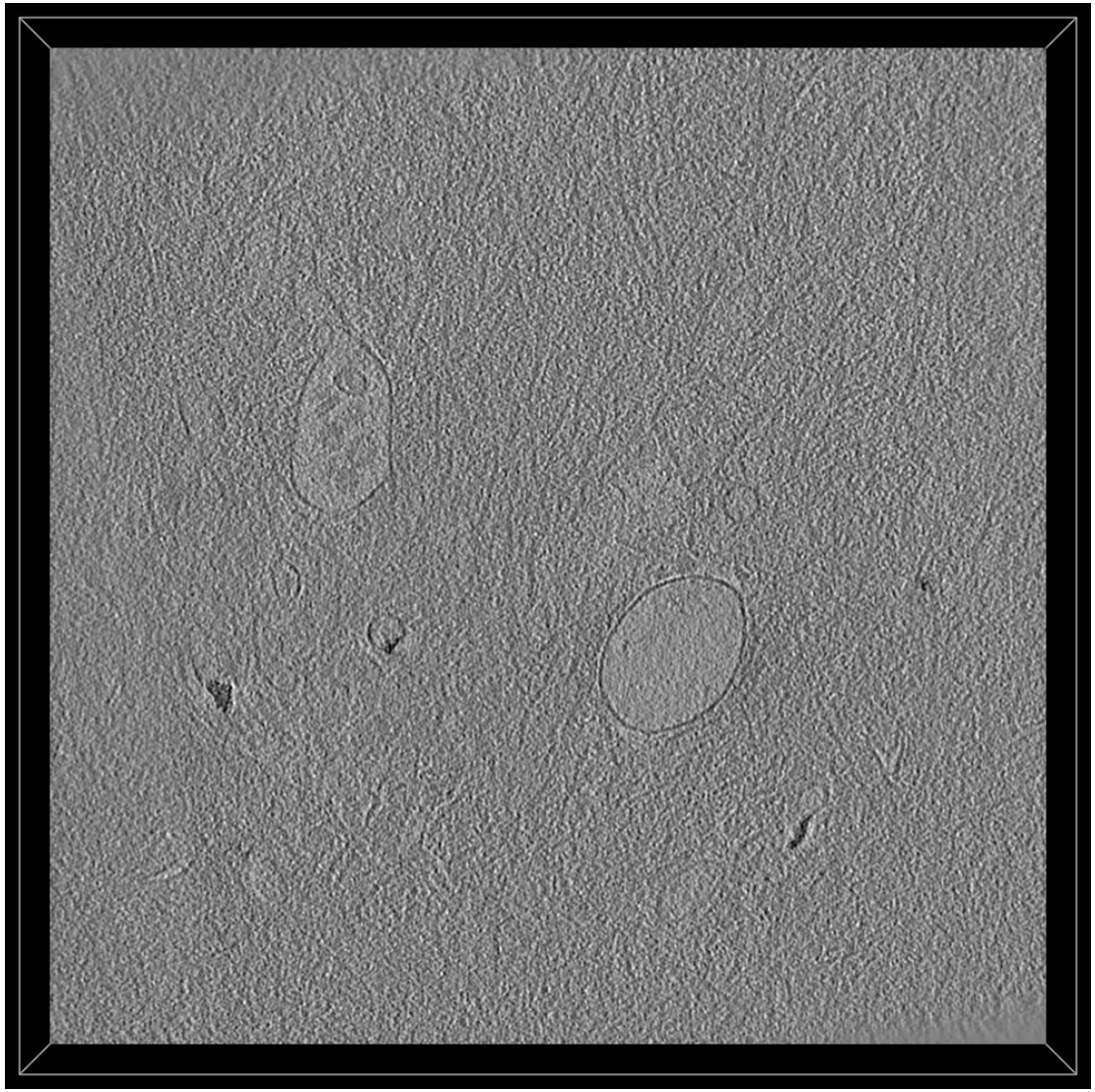
- What is the composition of the aggregates?
- How does the cellular proteome change in response to toxic aggregation?

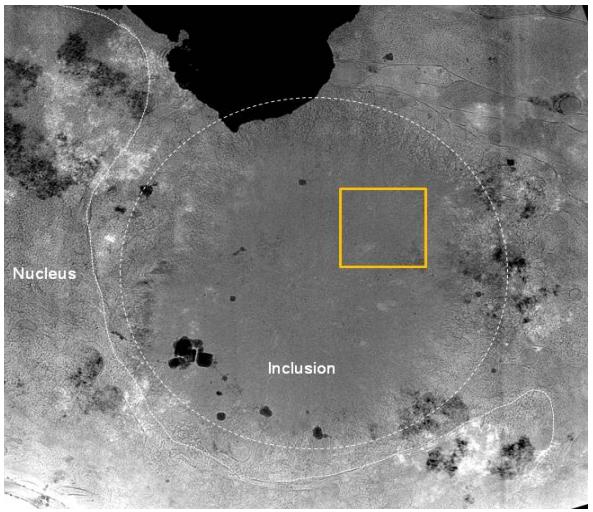
erc

Correlative Microscopy of Htt-Inclusions



Tomography of Intracellular Huntingtin Inclusions

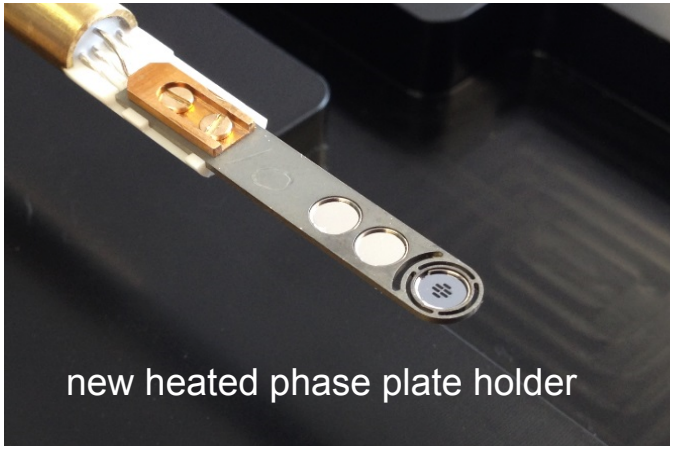




250 nm

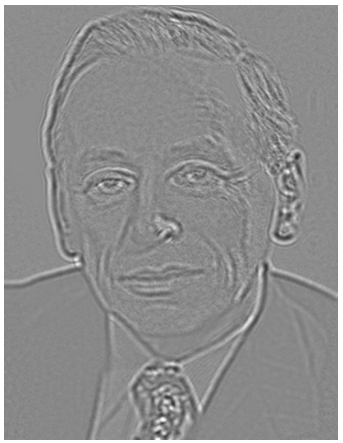
The Volta phase plate

- The Volta phase plate (VPP) enables in-focus phase contrast in TEM.
- It consists of a thin (~10 nm) continuous carbon film positioned at the back focal plane of the objective lens.
- The phase shift is generated by the beam-induced Volta potential on both sides of the film.
- Compared to previous phase plate designs the VPP is easy to use and has a long service life.



conventional EM in-focus

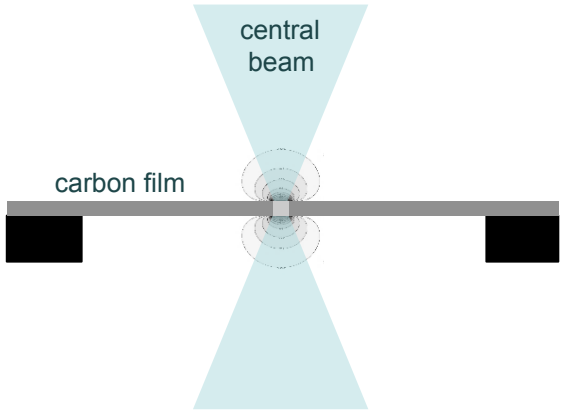
conventional EM 1.5 um defocus



VPP EM in-focus

Frits Zernike 1888-1966

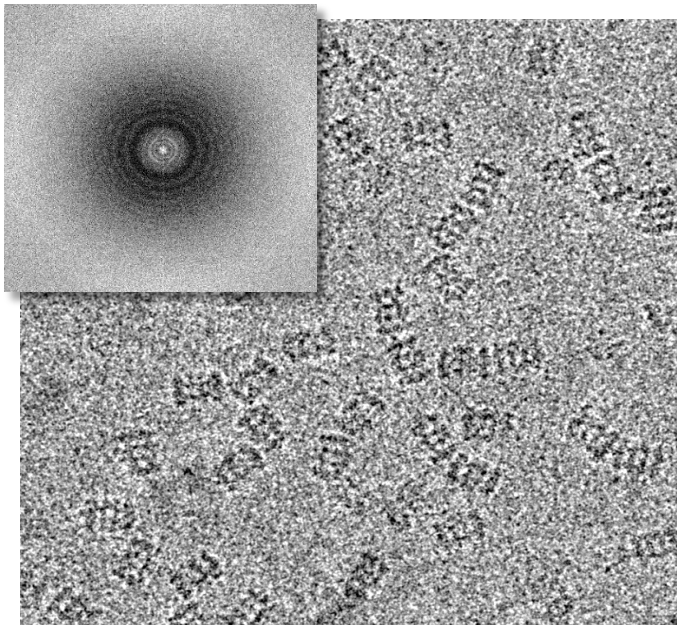
Volta Phase Plate



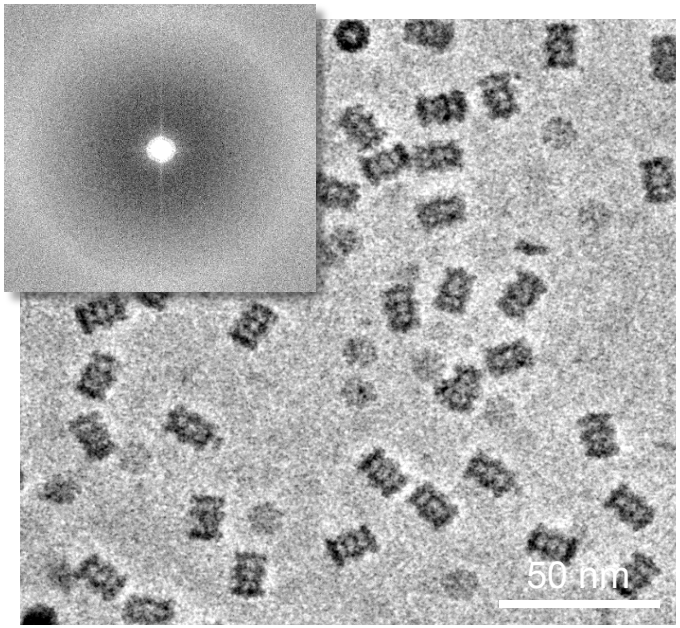
Danev et al., 2014. Volta potential phase plate for in-focus phase contrast transmission electron microscopy. PNAS 111, 15635-15640.

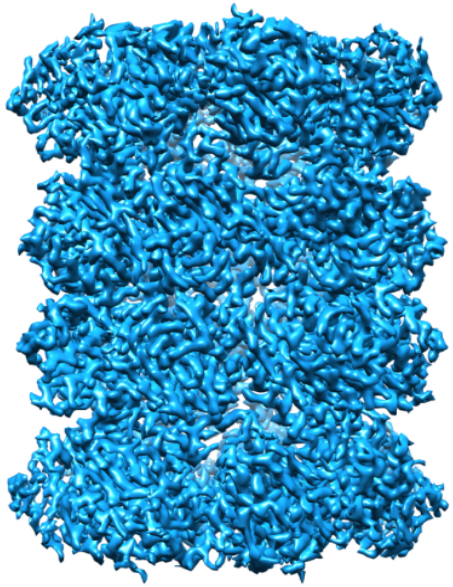
VPP cryo-EM single particle analysis

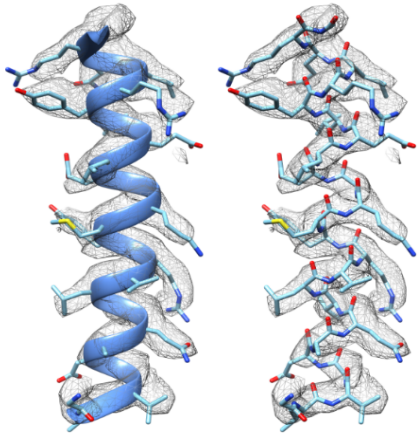
Conventional cryo-EM
1.5 um defocus



VPP cryo-EM
in-focus



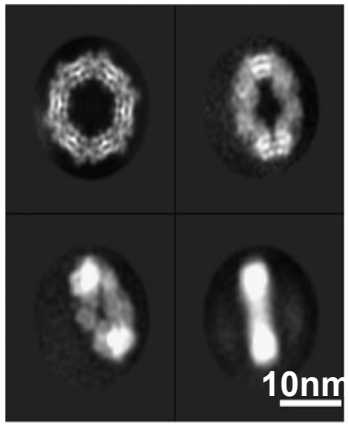


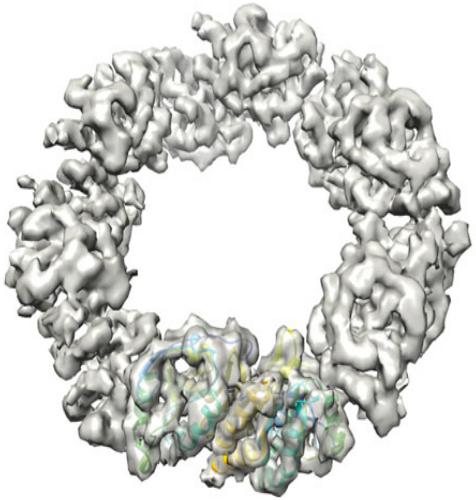


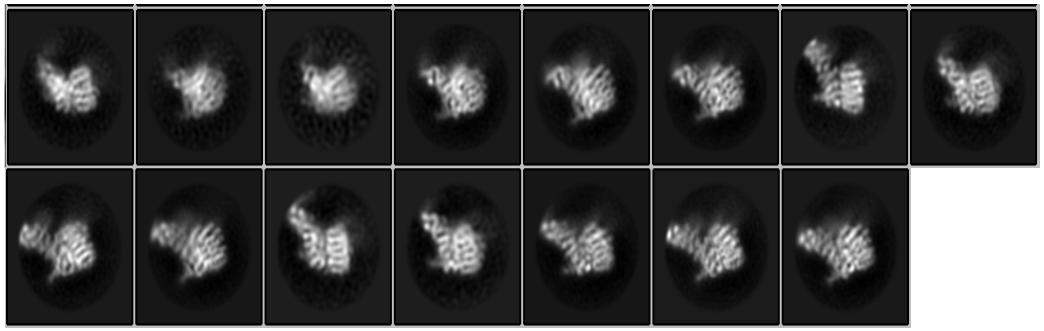
- We evaluated the performance of the VPP for cryo-EM single particle analysis with the *Thermoplasma Acidophilum* 20S proteasome.
- The in-focus VPP dataset reached 3.2 Å resolution with ~13,000 particles.

Danev, R., Baumeister, W. 2016. Cryo-EM single particle analysis with the Volta phase plate. eLife, doi:10.7554/eLife.13046

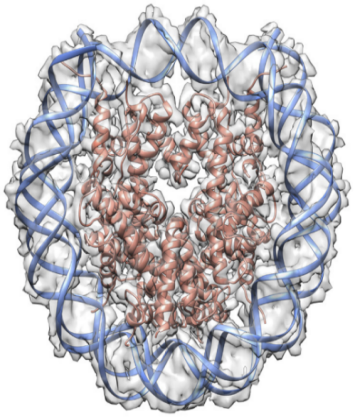
Volta phase plate Cryo-EM of small protein complexes

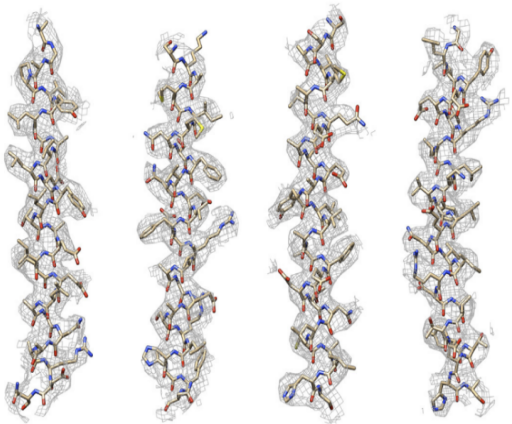




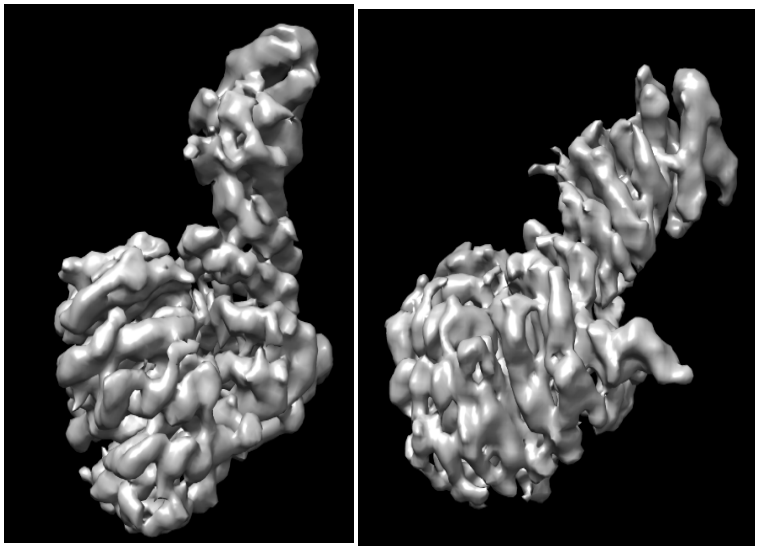


Human peroxiredoxin-3
Molecular weight: 257 kDa



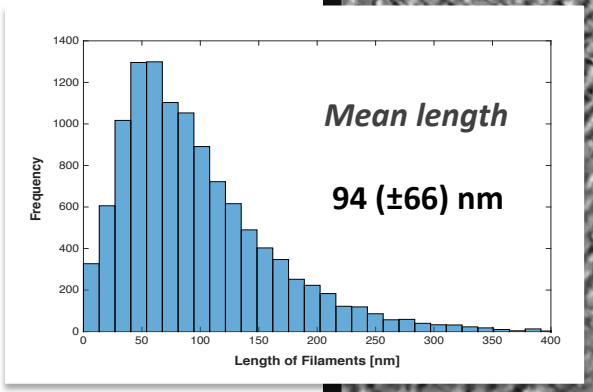


Nucleosome
Molecular weight: 200 kDa



Yeast Rpn1
Molecular weight: 109 kDa

Filament Network



Persistence length

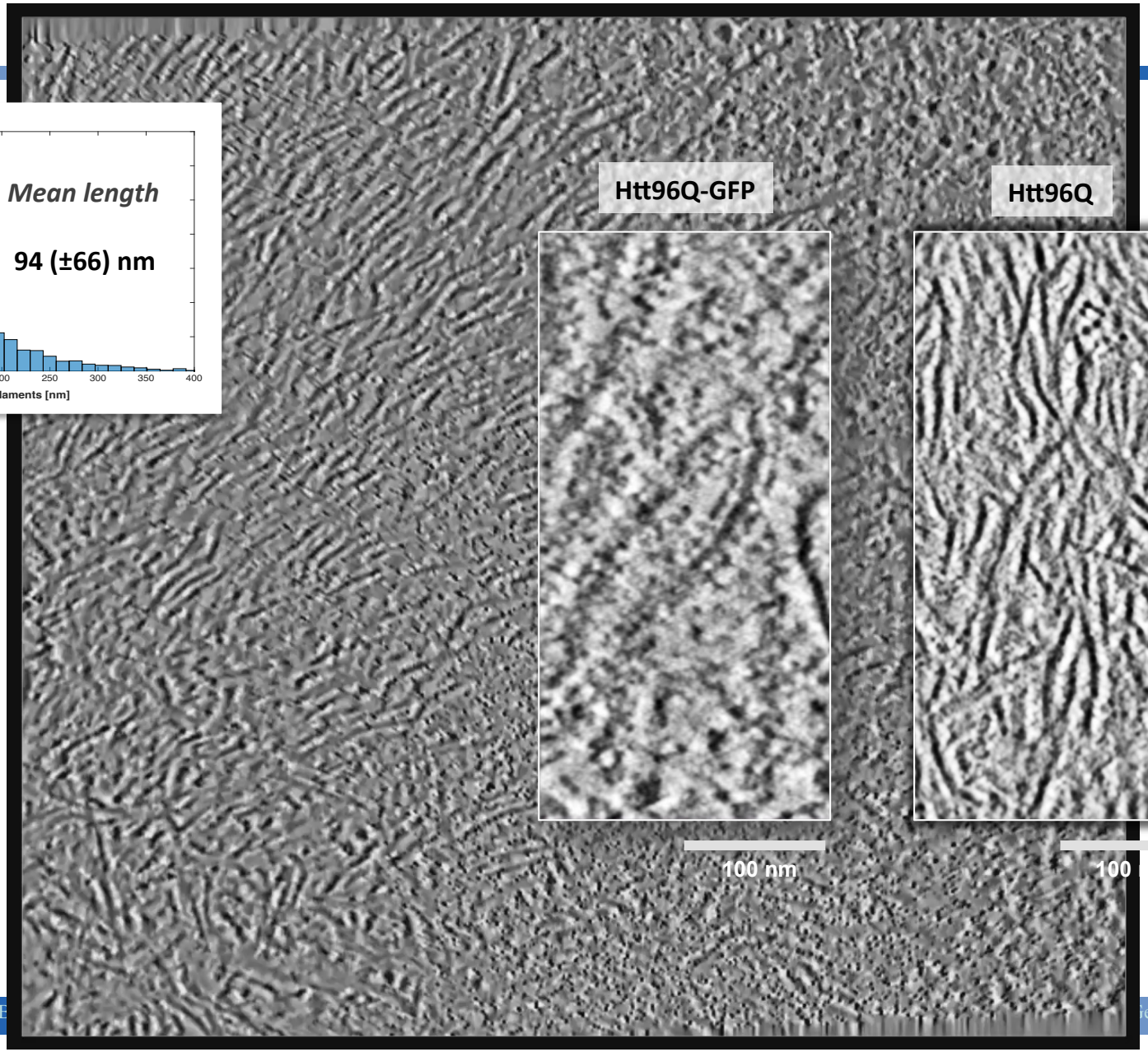
818 (±101) nm

DNA	*50 nm*
Actin	*3 µm*
MT	*1 mm*

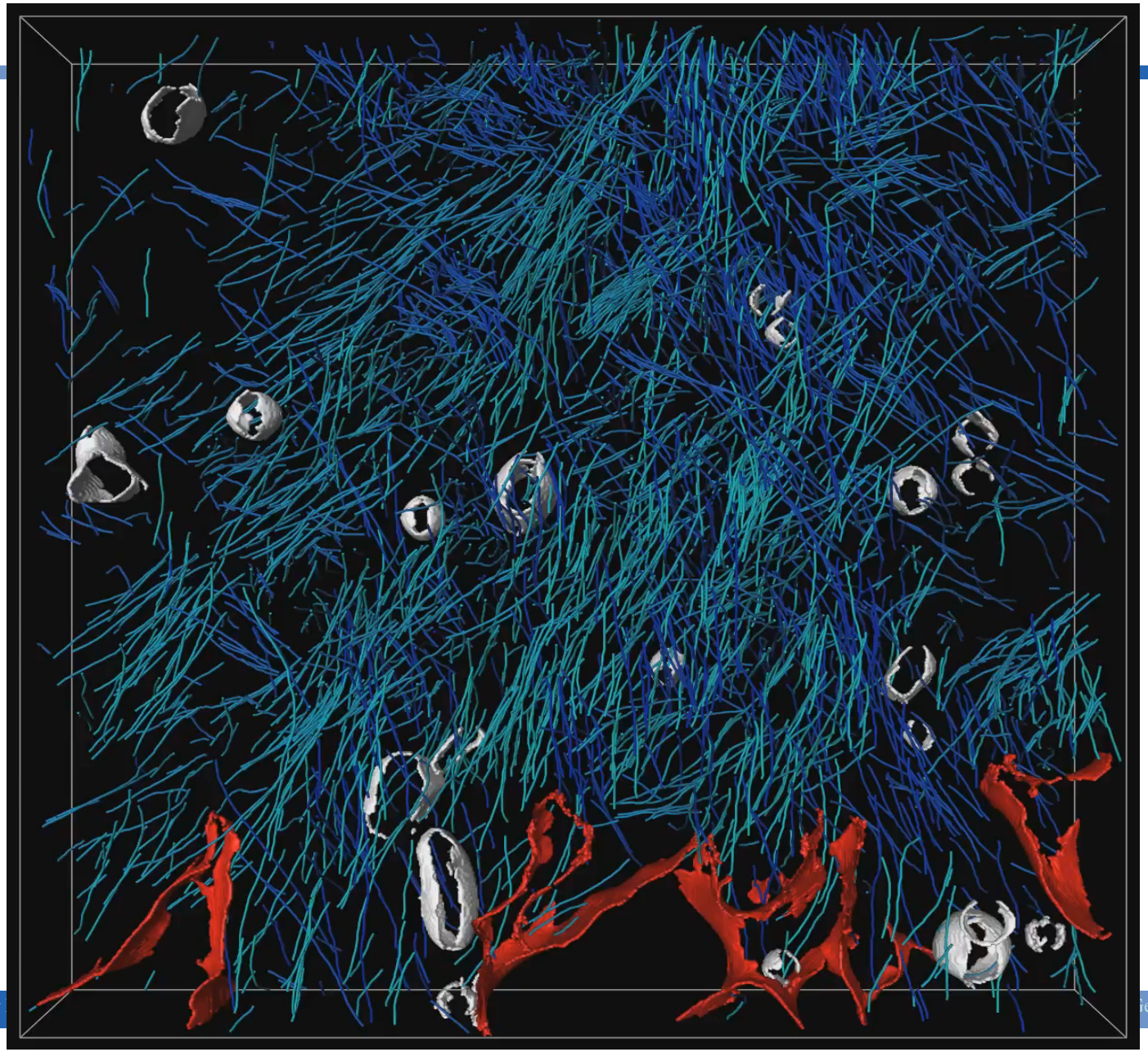
Young Modulus

17 • 10^6 N/m^2

Titan II
Phase Plate
K2 Summit
Mag 33.000
OPS 0.42 nm

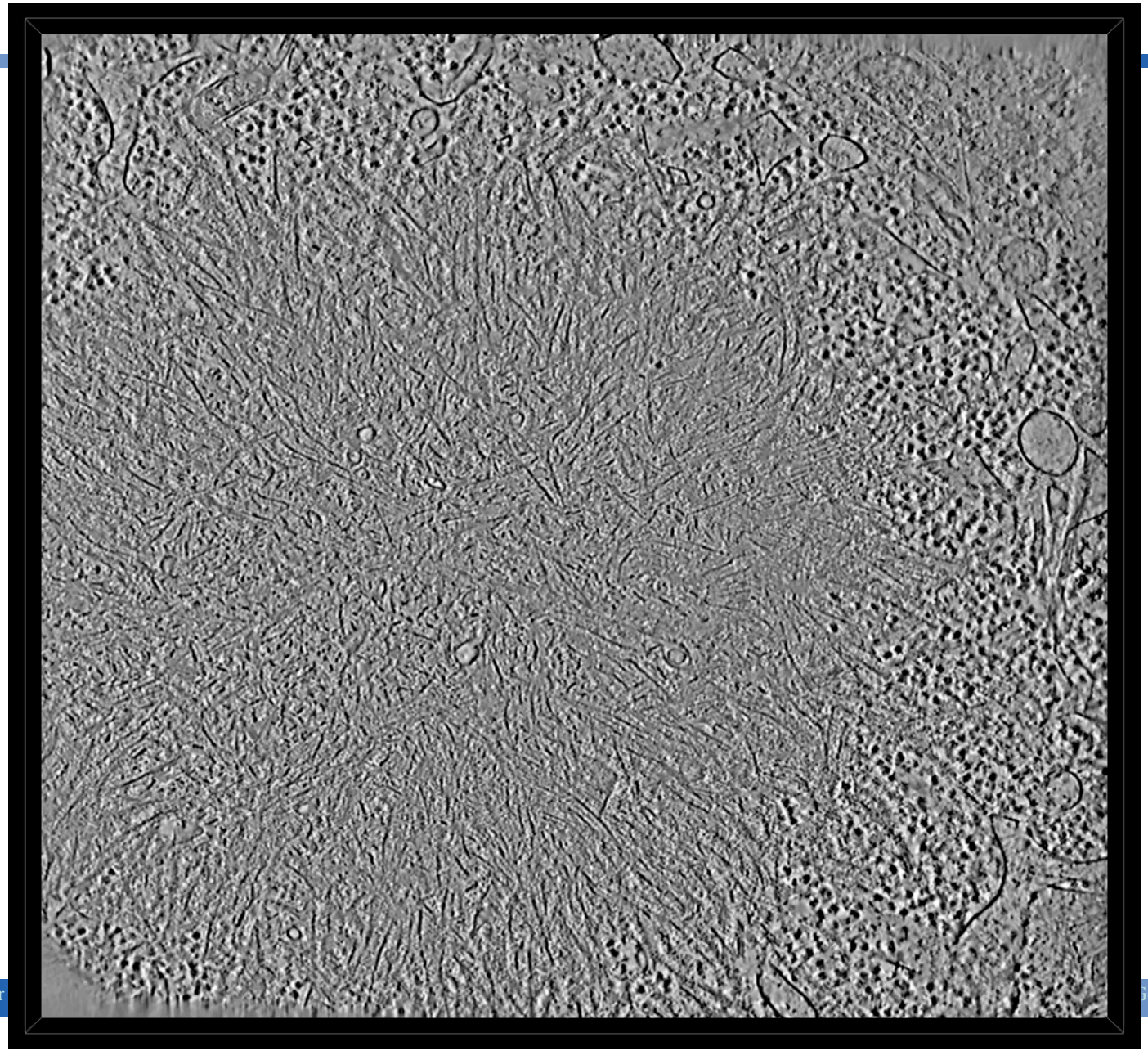


ER Interaction at Inclusions Periphery



Titan II
Phase Plate
K2 Summit
Mag 33.000
POPS 0.42 nm

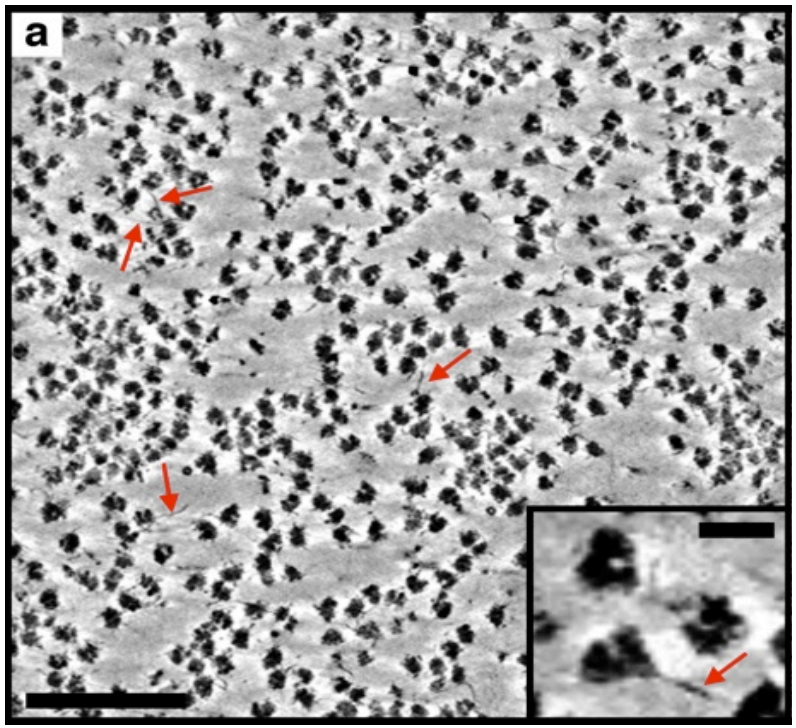
Htt-Aggregation in Primary Neurons (Mouse)

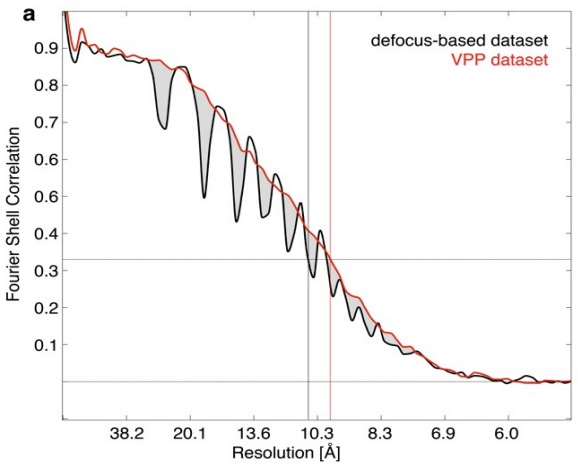


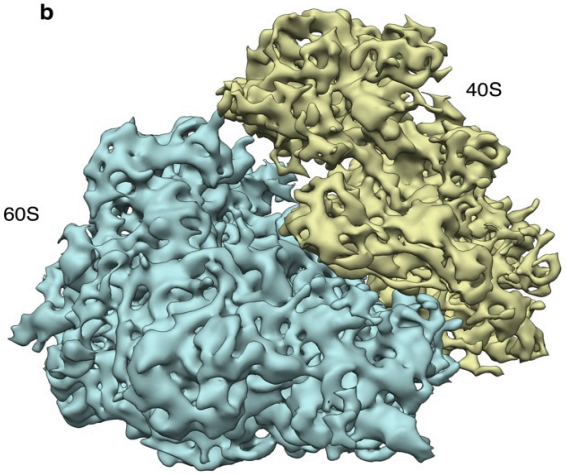
Titan II
Phase Plate
K2 Summit
Mag 19.500
DPS 0.71 nm

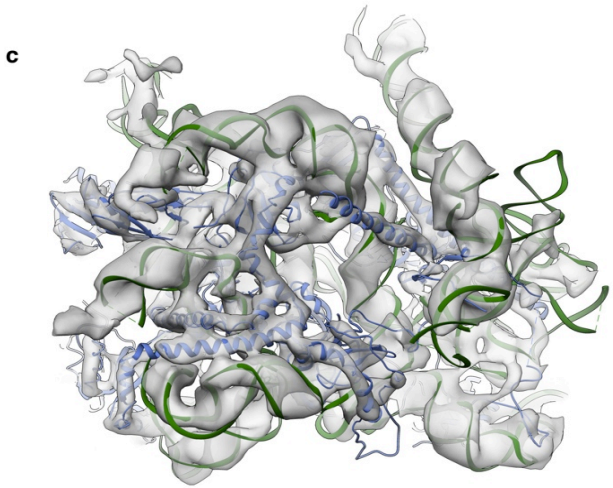
VPP subtomogram analysis

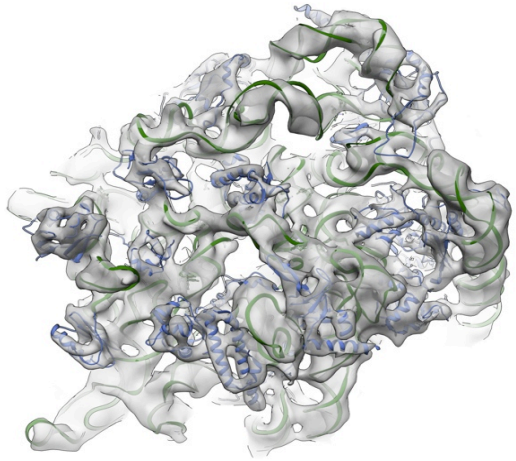
A test case of subtomogram analysis with the mammalian 80S ribosome.







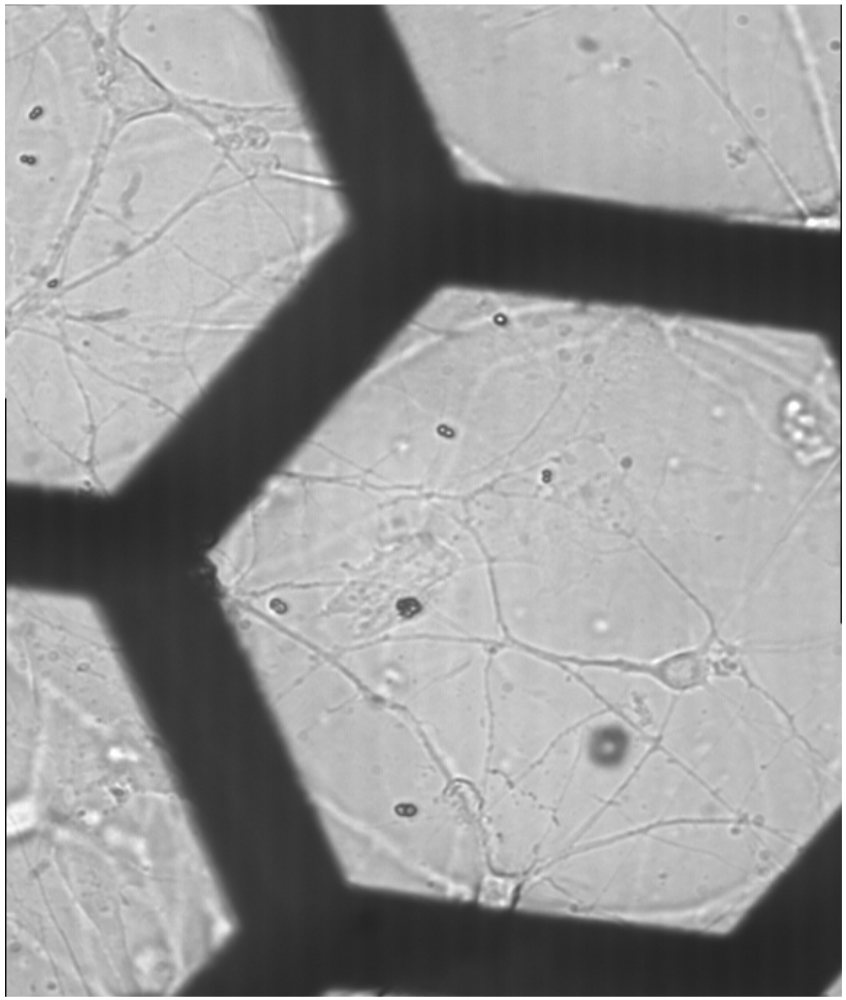


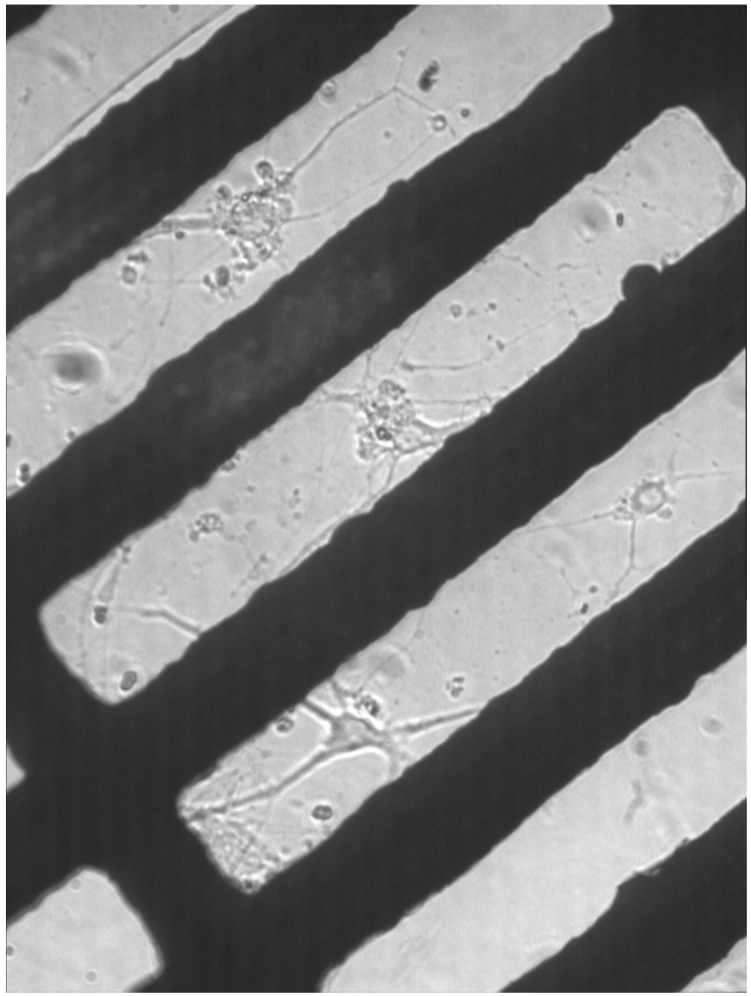


Khoshouei et al. 2016. Subtomogram analysis using the Volta phase plate. JSB, submitted.

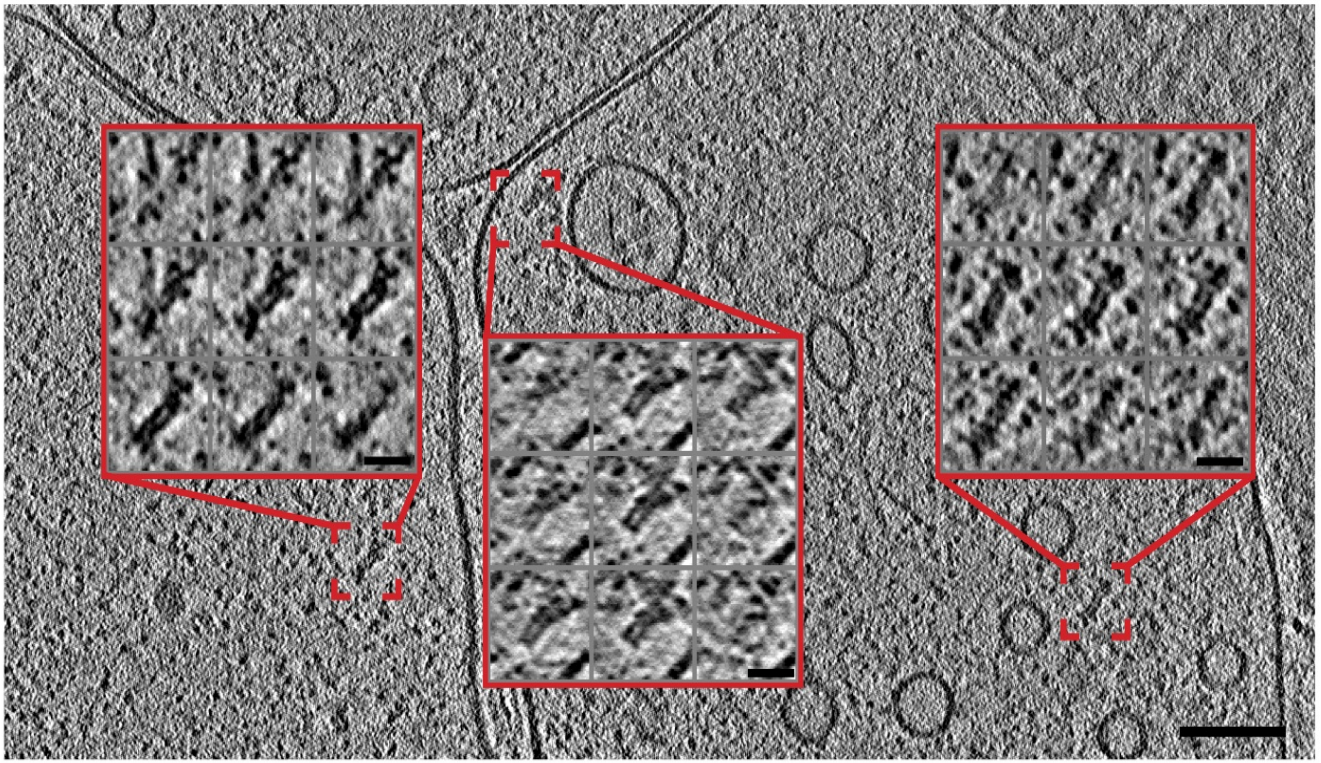
Structural Studies of the 26S Proteasome *in situ*

Electron cryotomography of intact rat hippocampal neurons



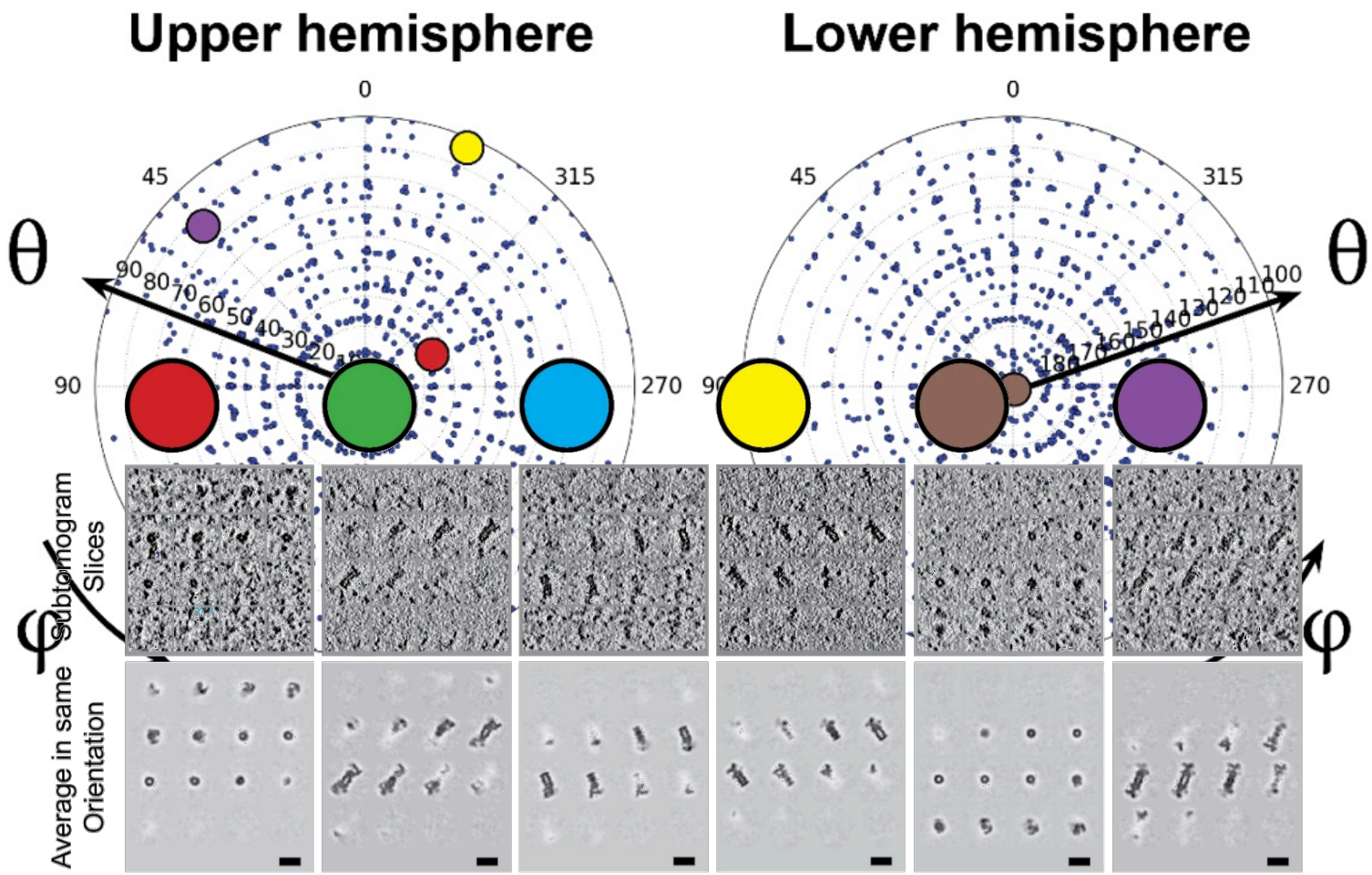


26S Proteasome in Situ



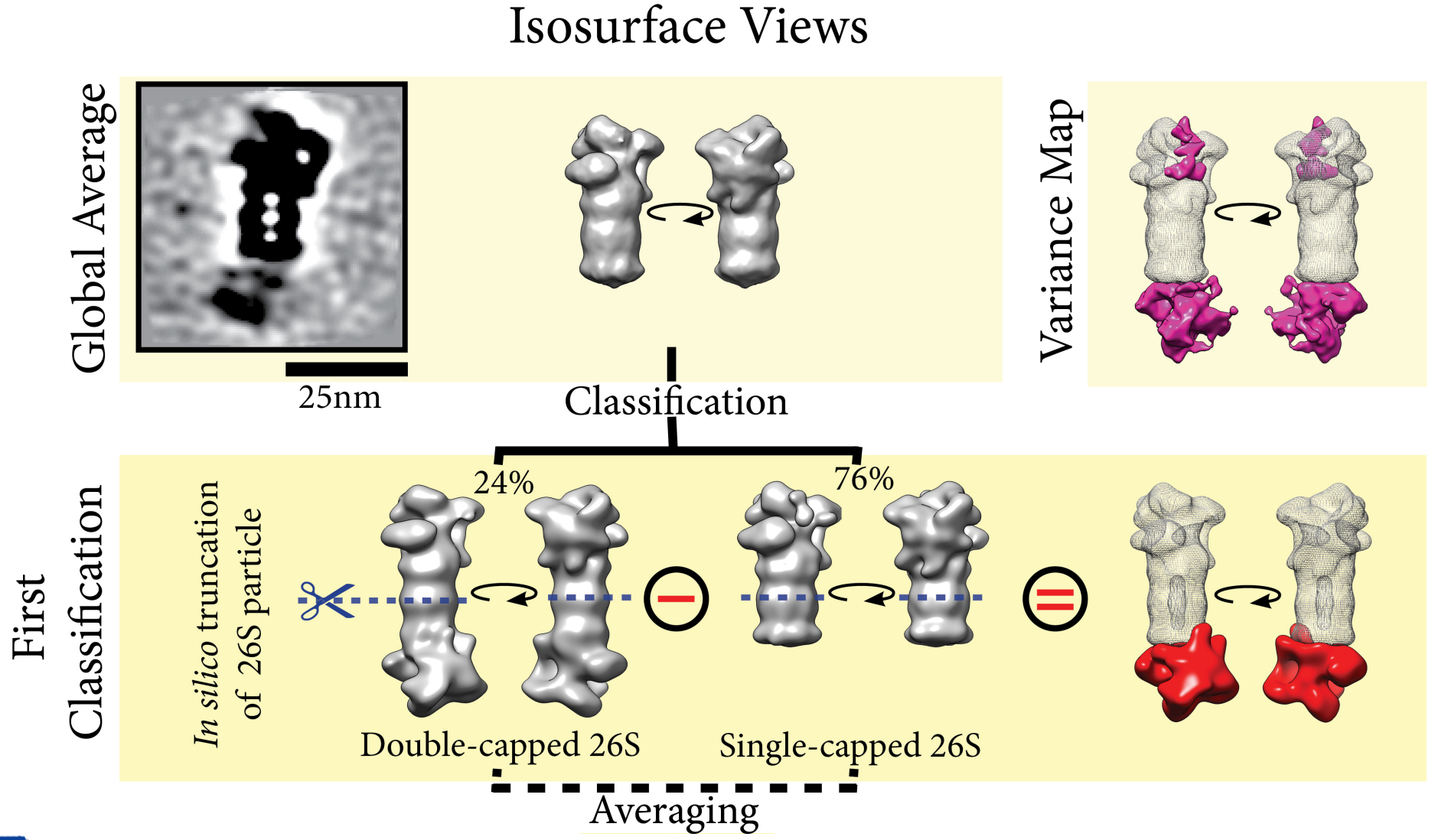
Automatic Detection

Subtomogram Orientation

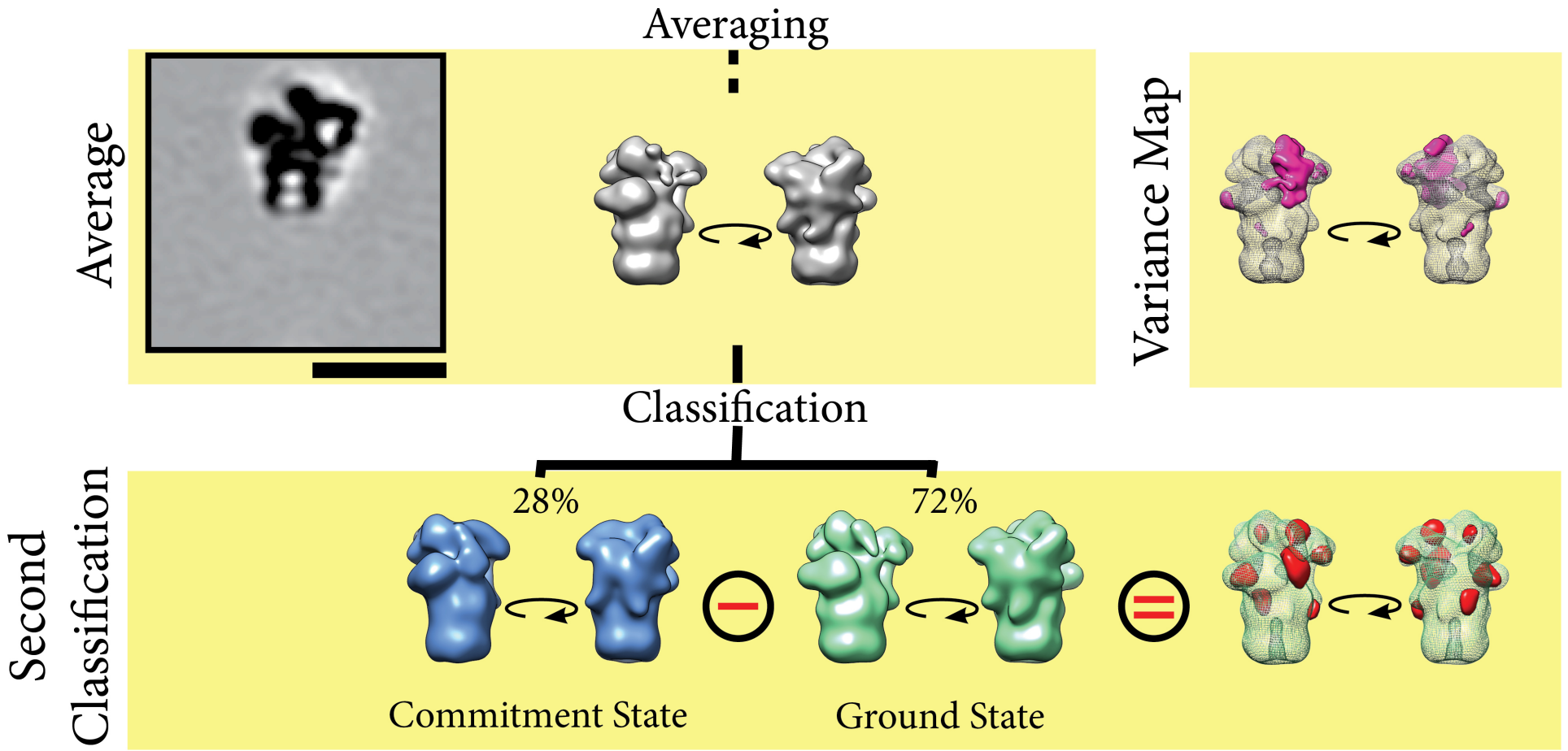


26S Proteasomes in situ

Assembly Status

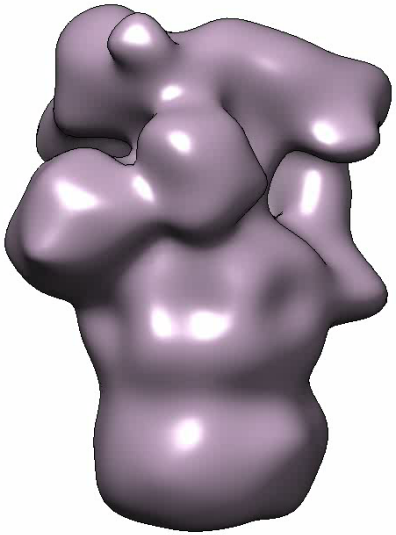


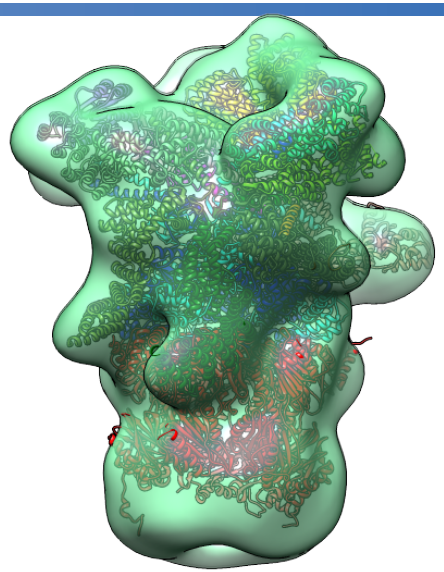
26S Proteasomes in situ
Conformational States

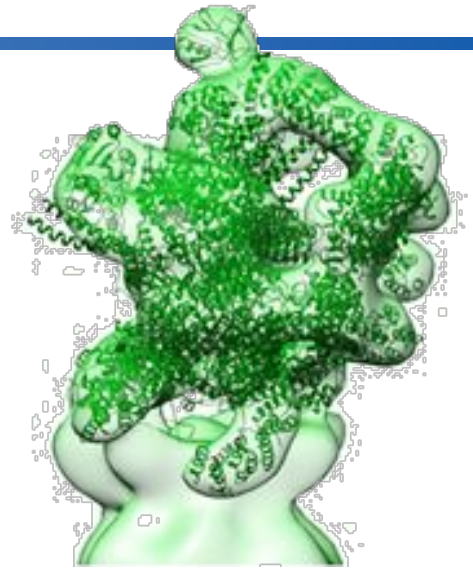


26S Proteasomes in situ

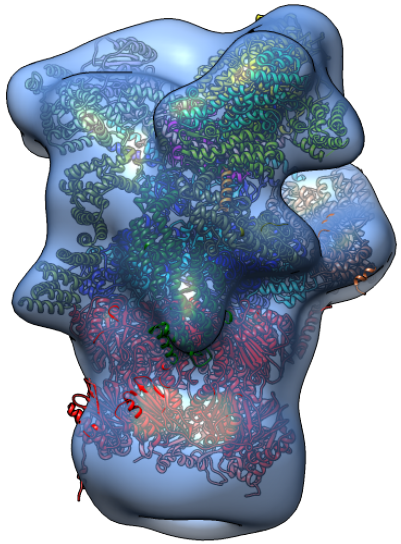
Ground State



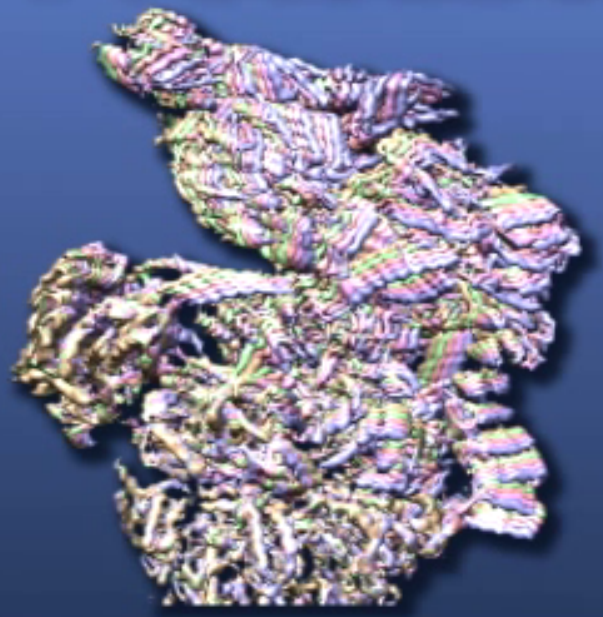




Commitment State



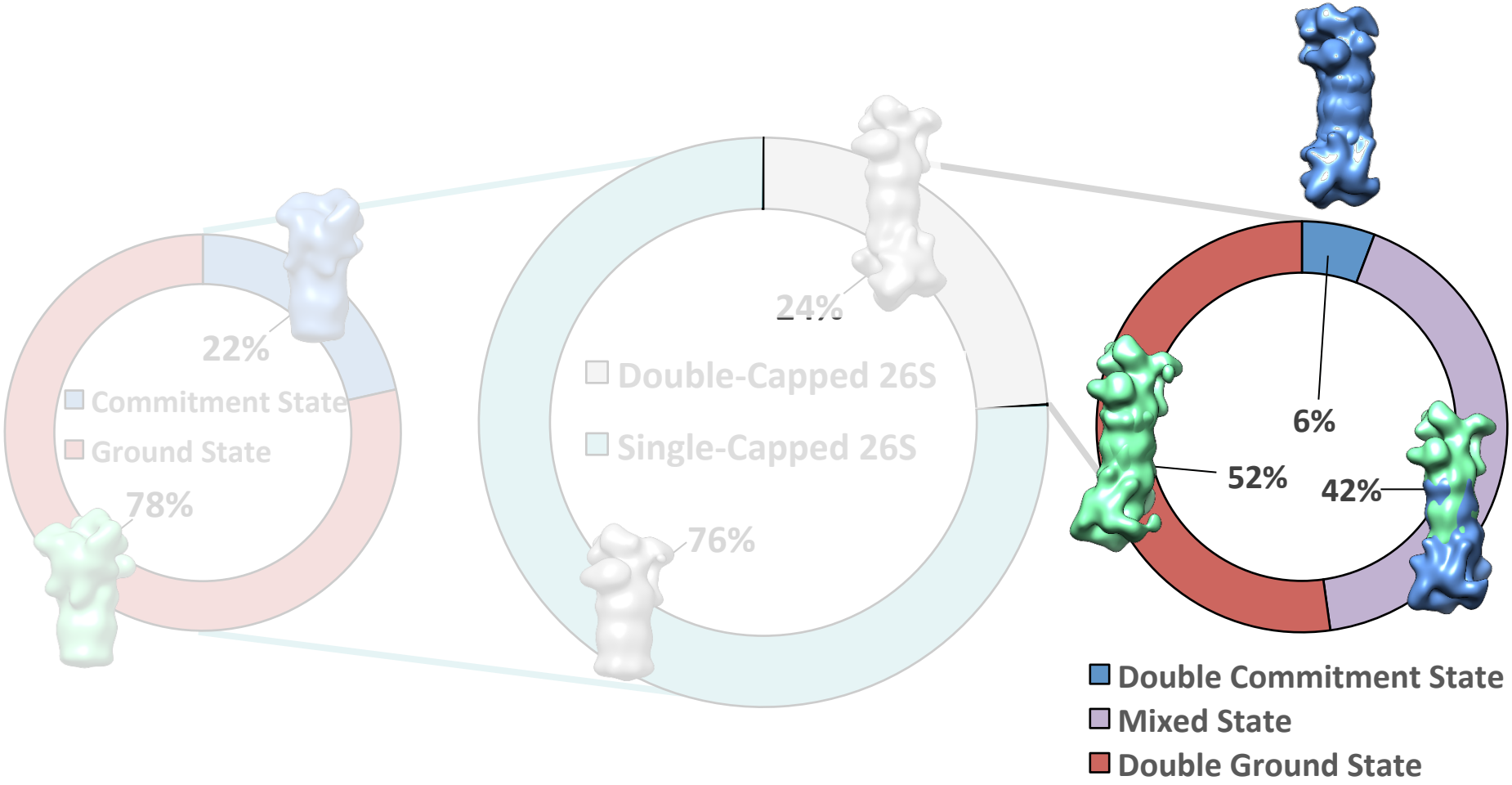
The Conformational Landscape of the 26S Proteasome



Unverdorben, P., et al. PNAS 2014

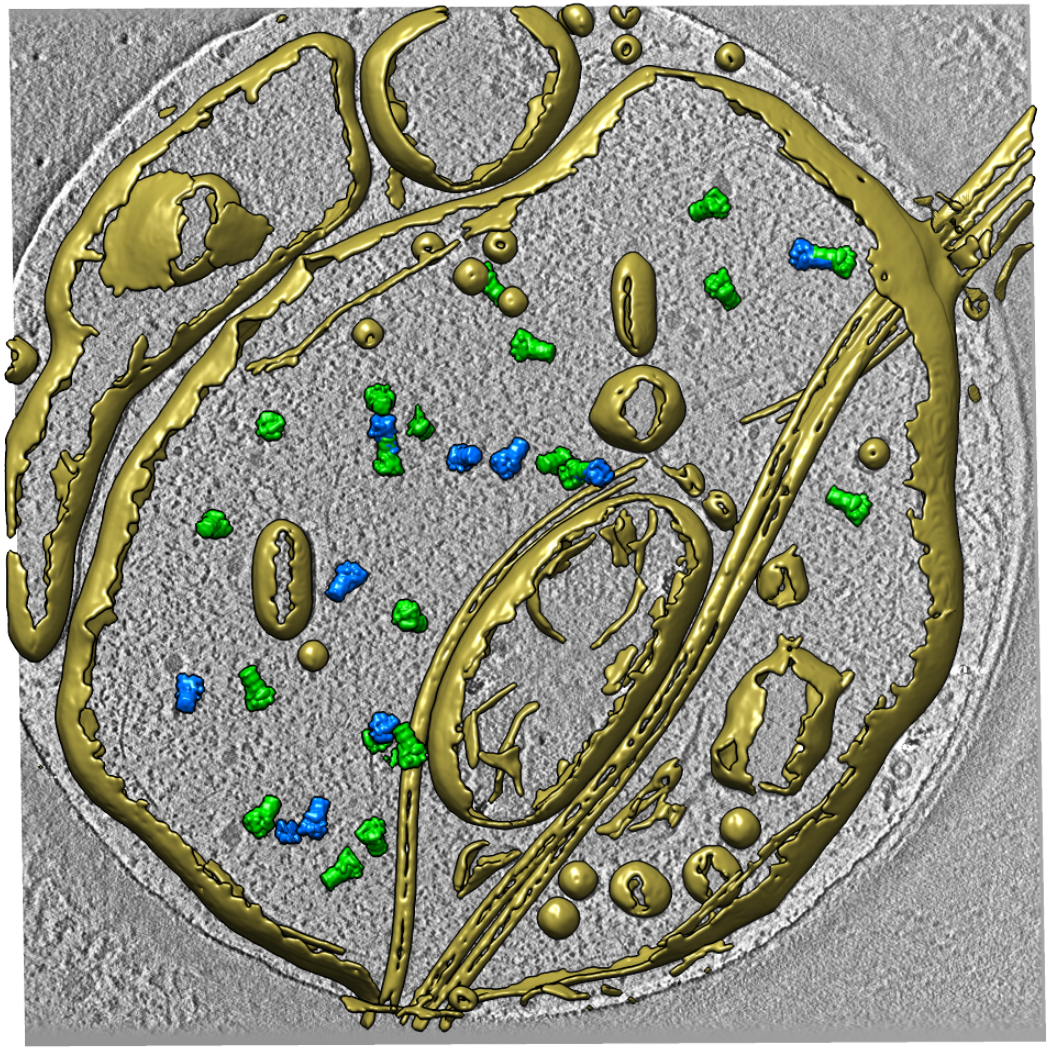
animated by Julio Ortiz

26S Proteasomes in situ



Asano S., Fukuda Y. Beck F., Aufderheide A., Förster F., Danev R., Baumeister W.: Science 347 (2015) 439-442

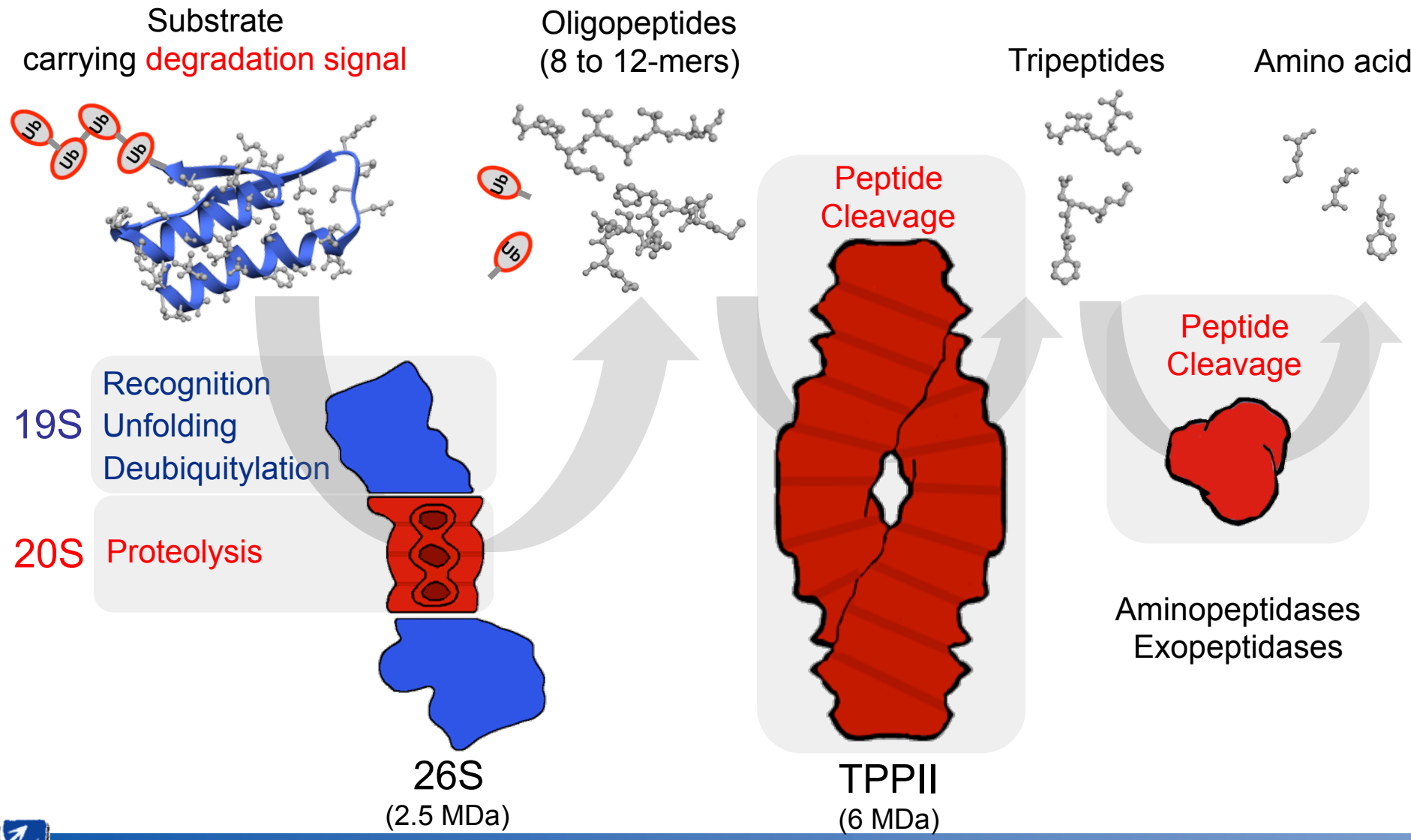
26S Proteasomes in situ



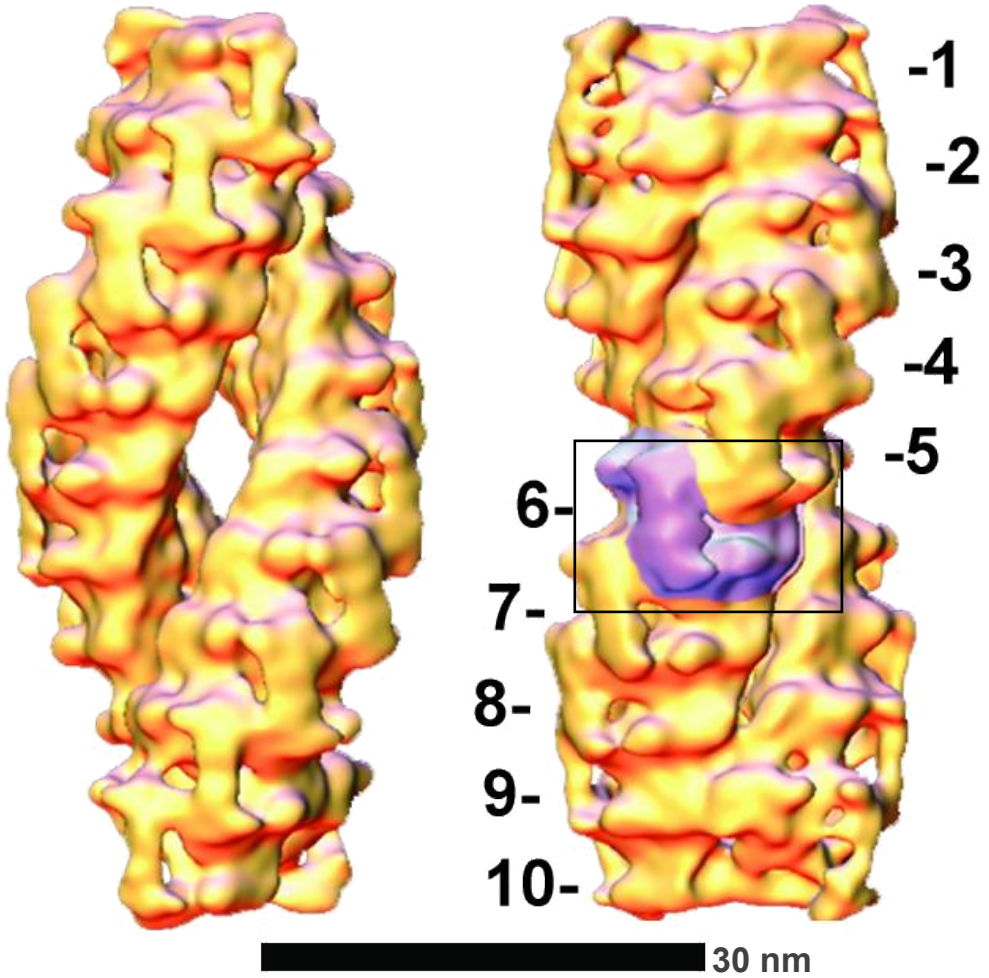
Ground State

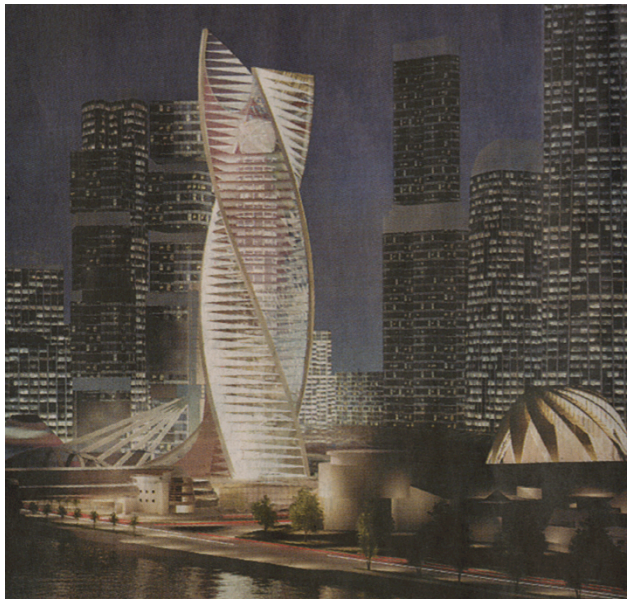
Commitment State

Eukaryotic Protein Degradation Pathway



Three-dimensional Structure of TPPII





"Tower of two halves"

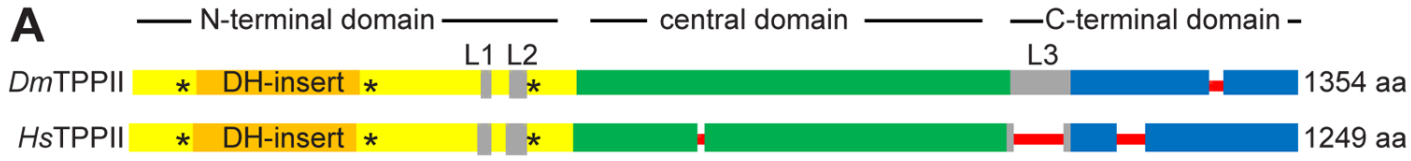
Total Mass (STEM): 5.7 +/- 1 MDa

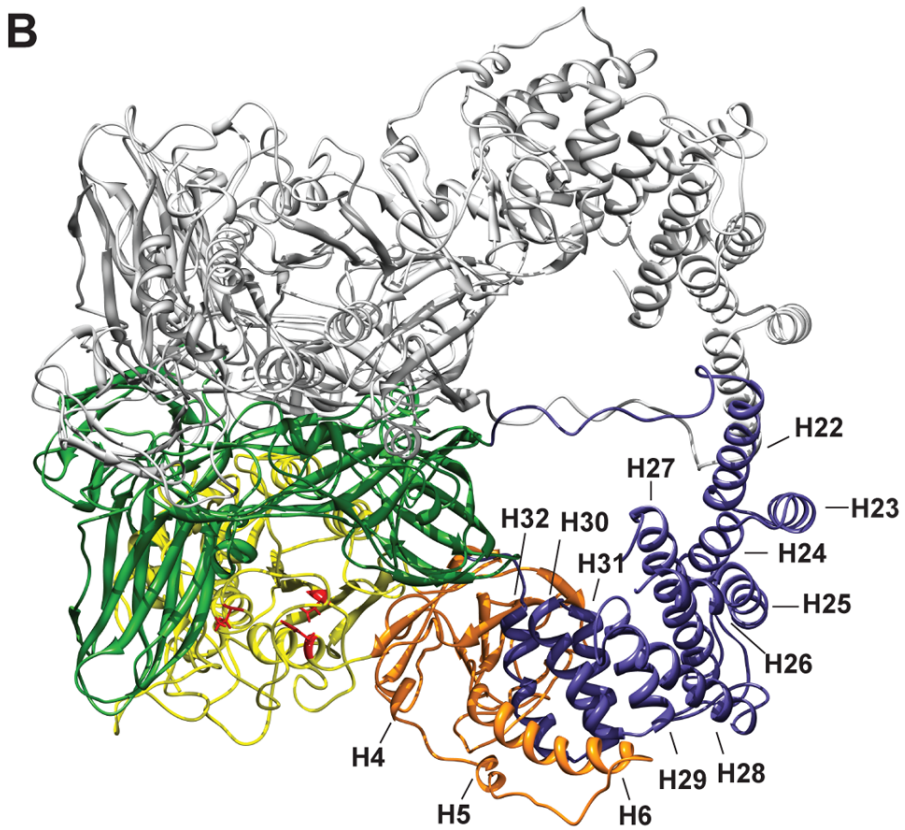
20 Segments per 'Spindle':
1 Segment approx. 290 kDa

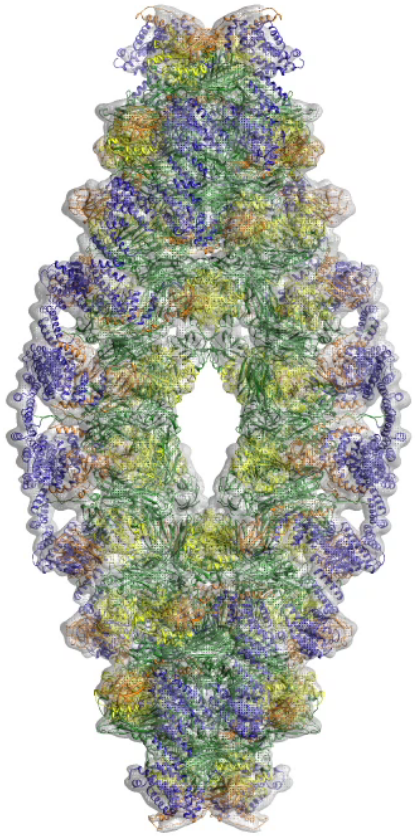
Segments are Dimers (2 x 145 kDa)

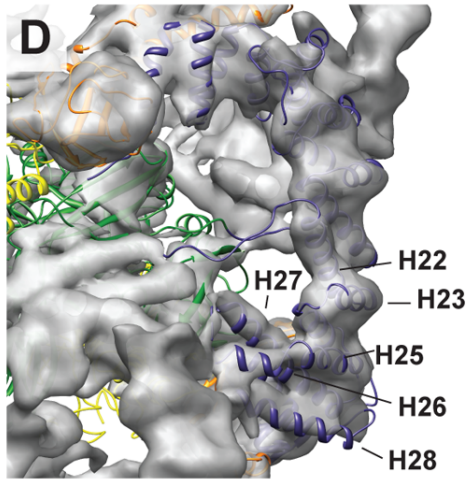
B. Rockel, J. Peters, S.A. Müller, G. Seyit, P. Ringler, R. Hegerl, R.M. Glaeser, W. Baumeister: PNAS 102 (2005) 10135-10140

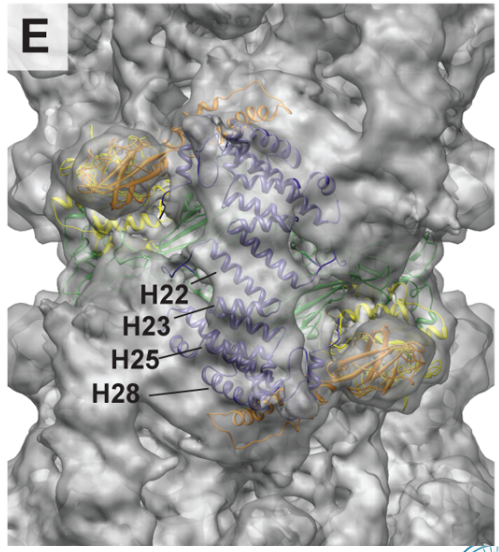
Hybrid Structure of Human TPPII





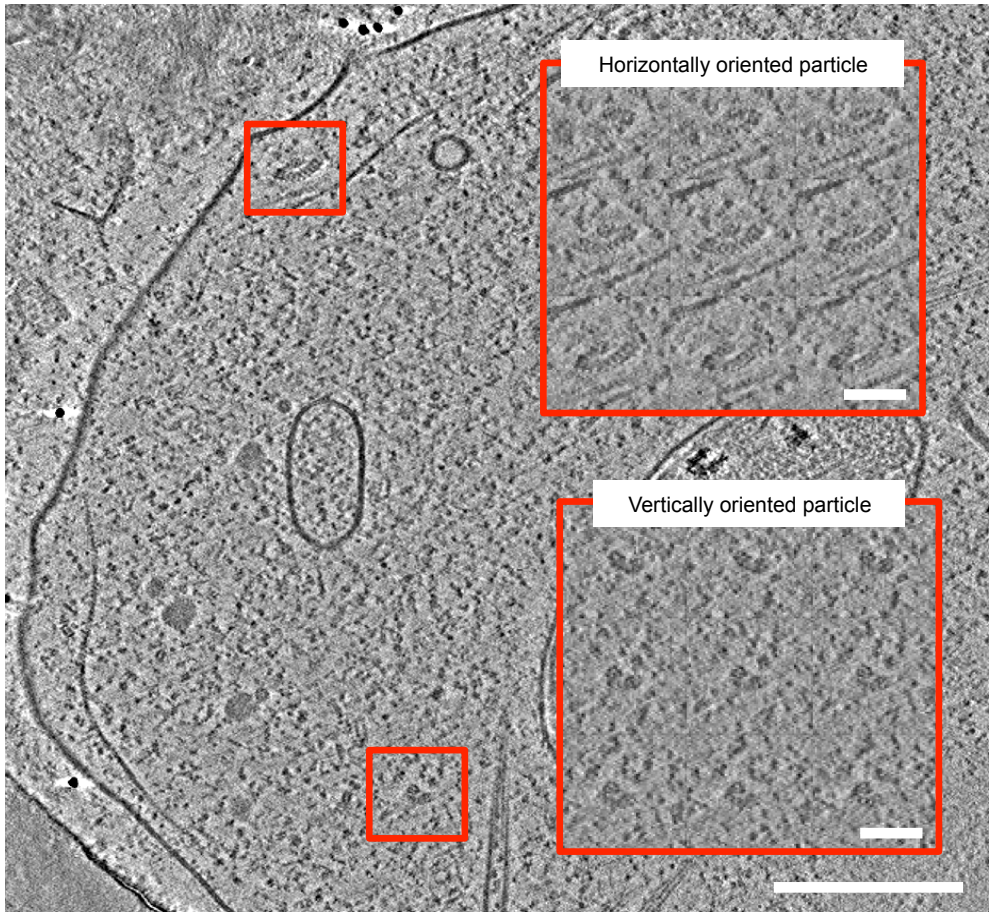






In situ structural studies of Tripeptidyl peptidase II by cryo-electron tomography

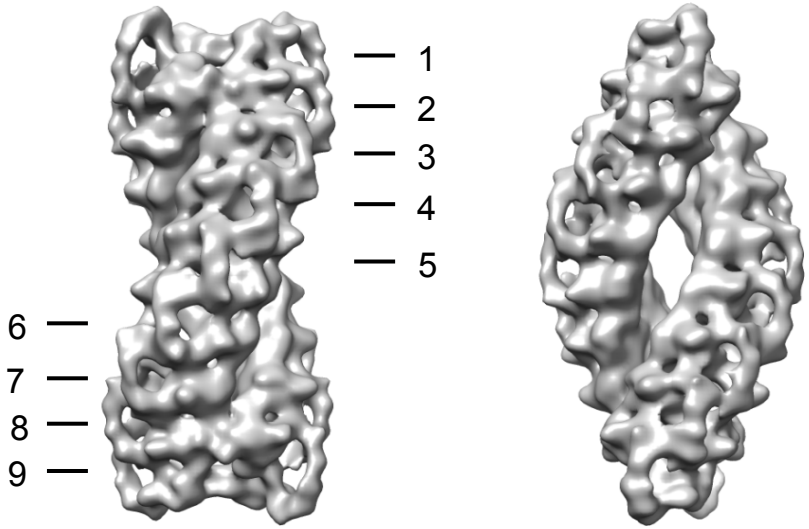
Detection of TPPII complexes *in situ*

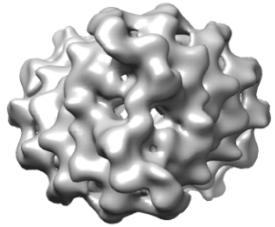


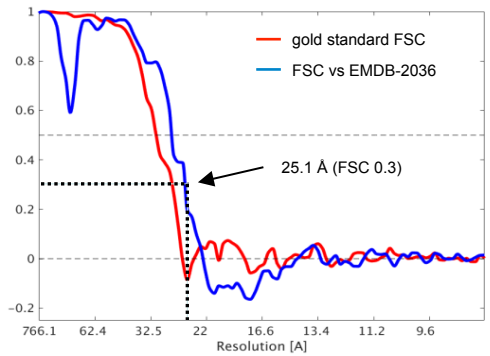
Scale bars 200 nm, inset 50 nm

Averaging of TPPII complexes

93 particles from 70 tomograms

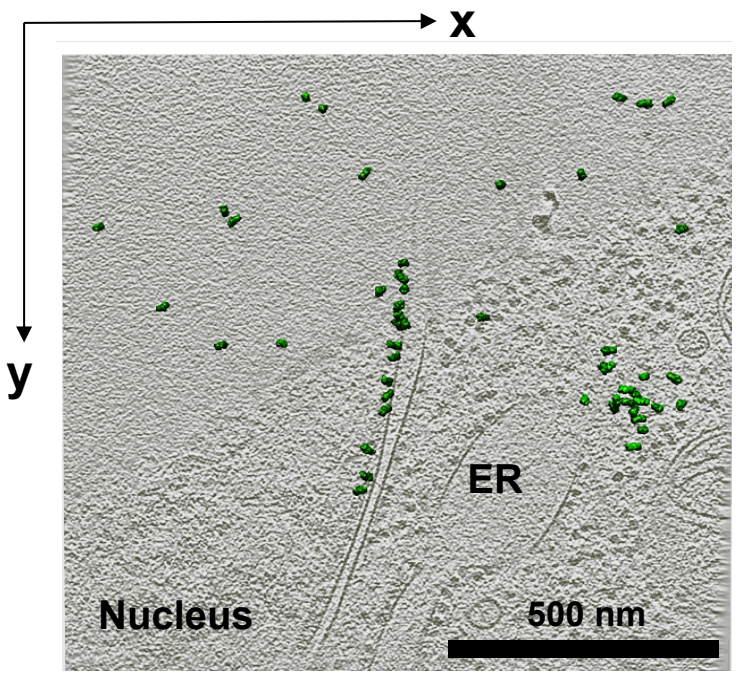


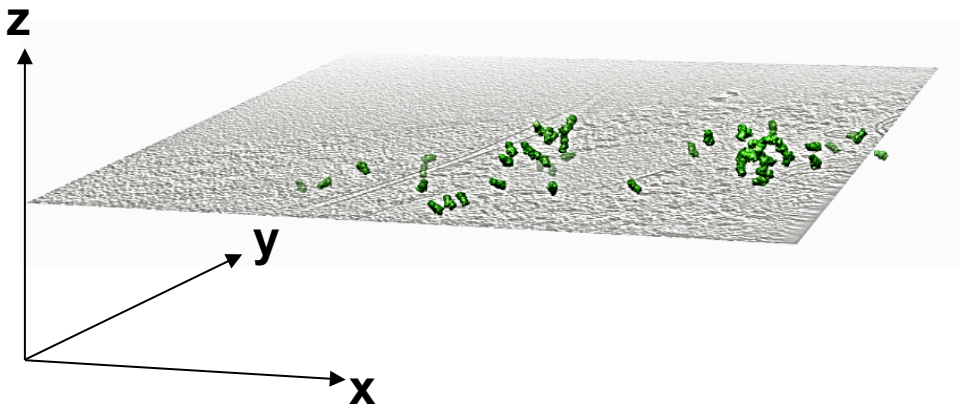




Proteasome Clusters in the Cytosol and Nucleus

- Proteasomes

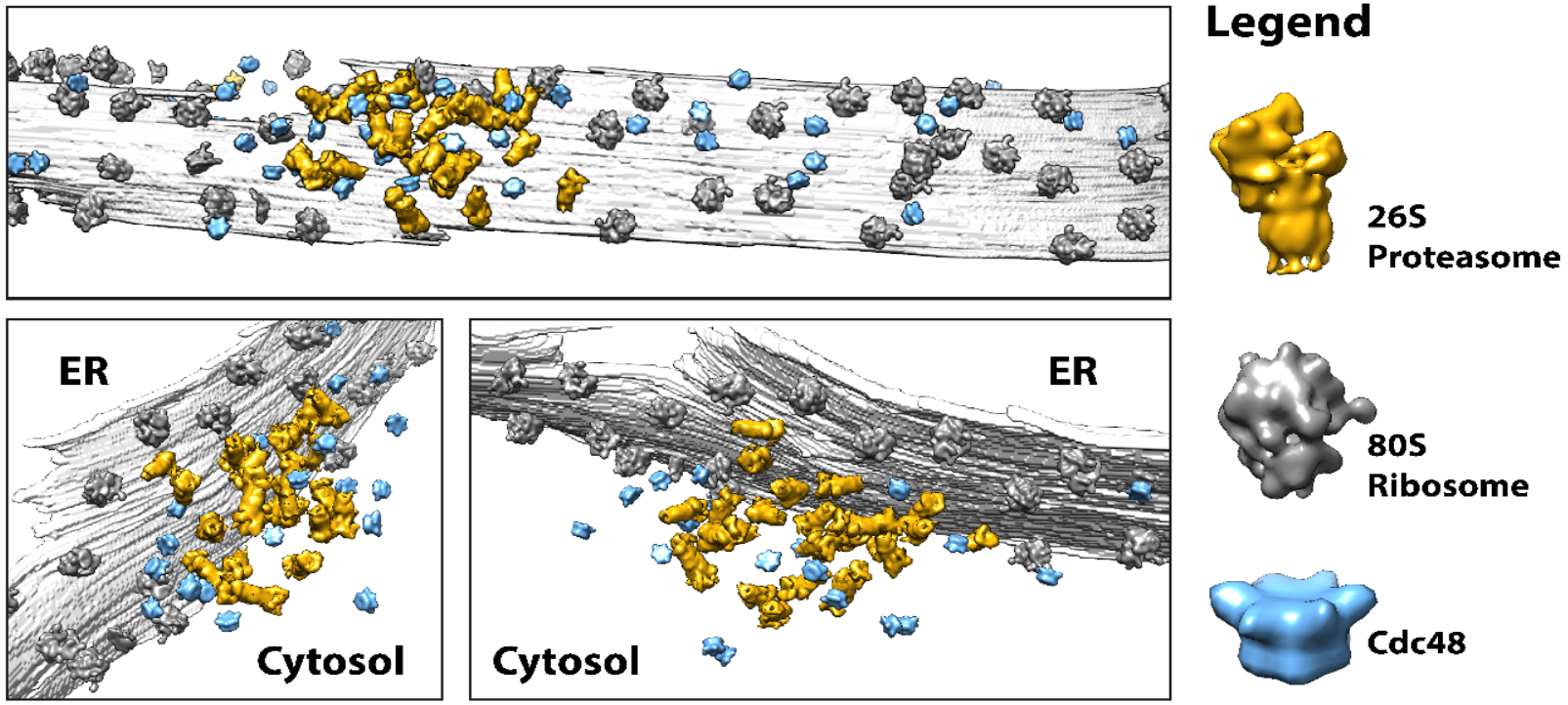




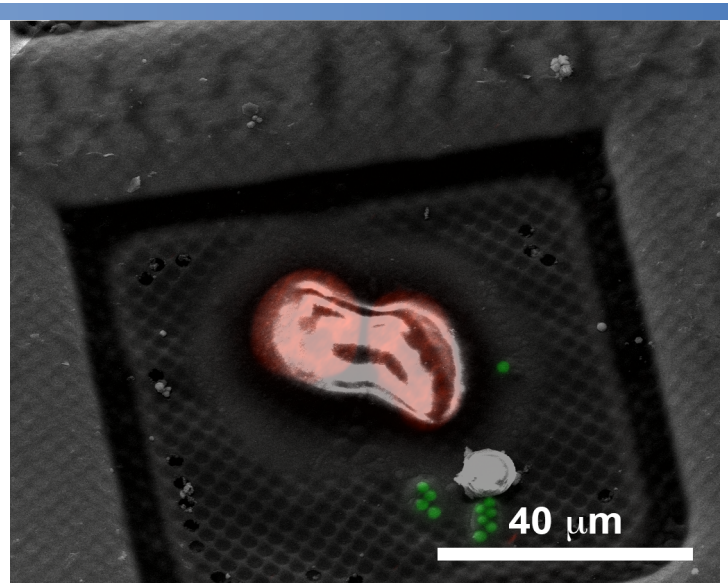
Several Clusters of Cytoplasmic Proteasomes were found close to the ER

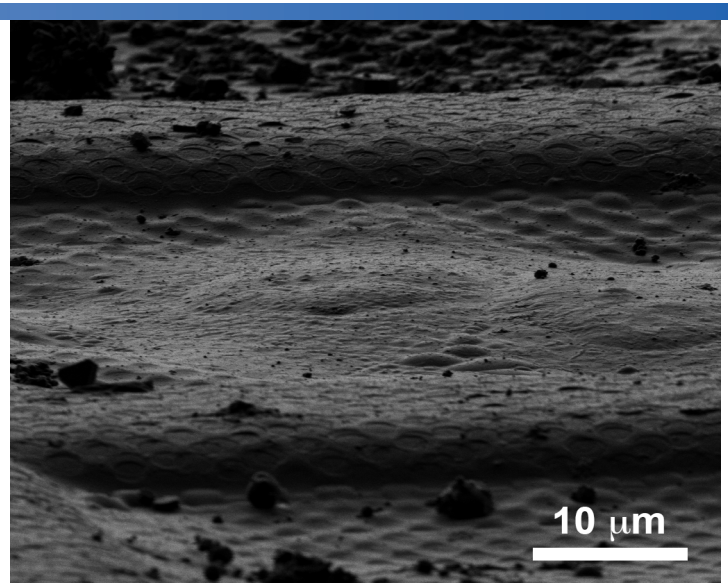
Degradation center at the ER

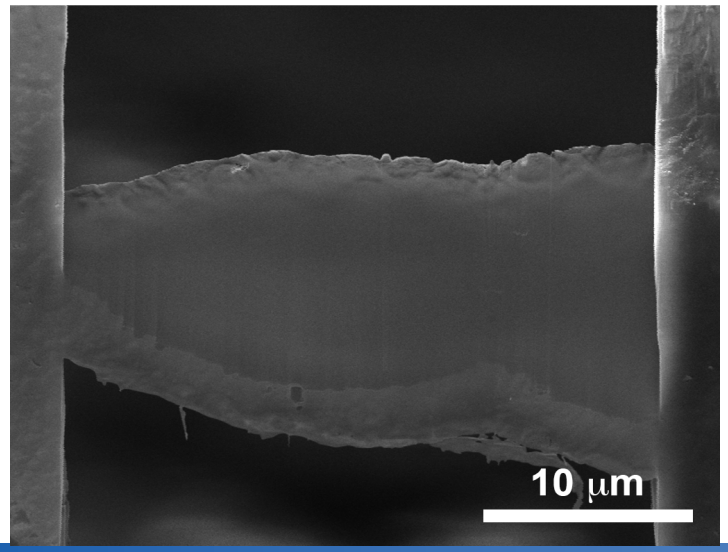
Proteasomes form Clusters at the ER

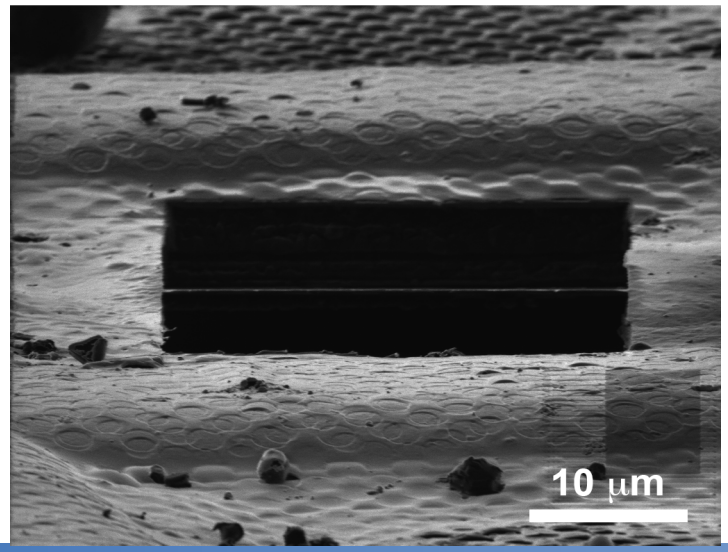


Preparing FIB Lamella of Targeted Volumes in HeLa cell nuclei









Julia Mahamid

Visualizing the Molecular Sociology at the HeLa Cell Nuclear Periphery

Nucleus

Nucleosome chains

Nuclear Lamina

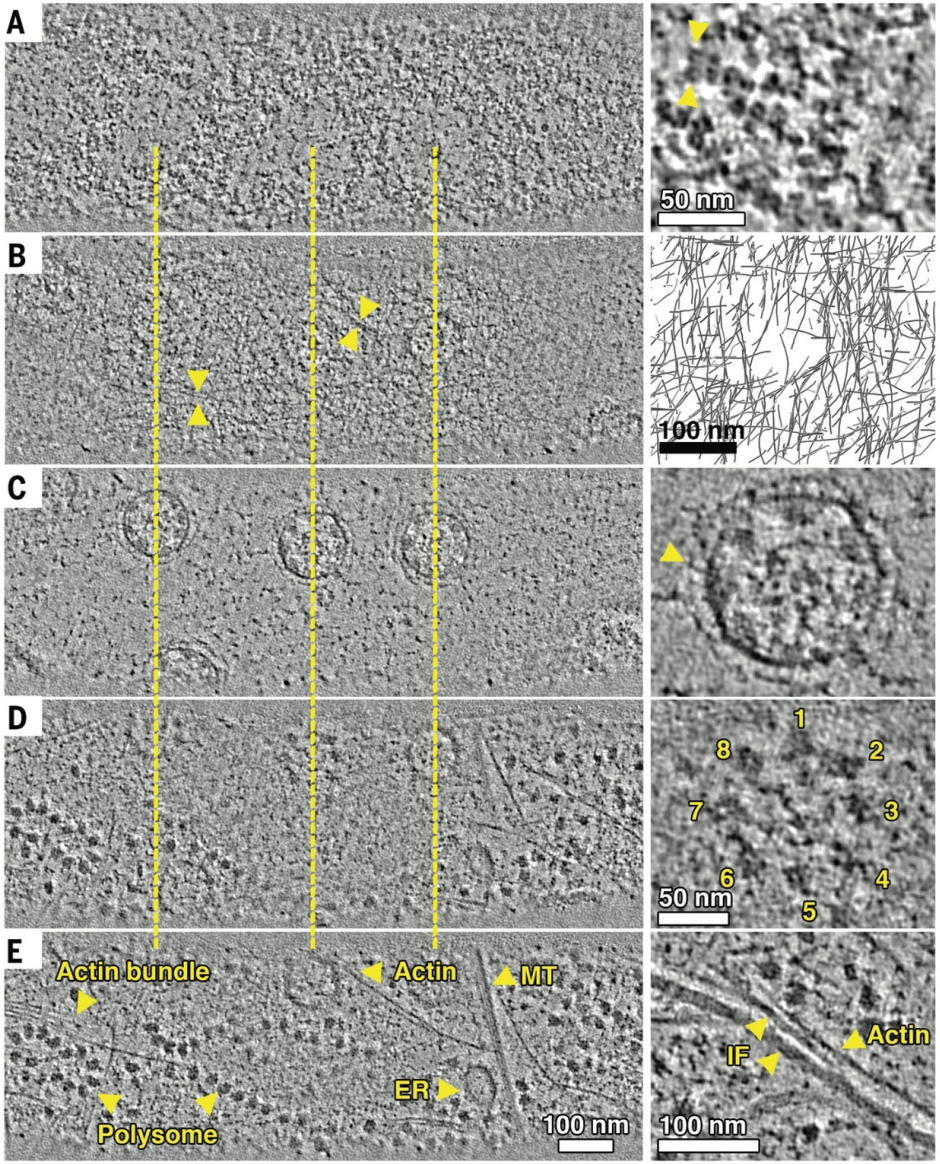
Nuclear Envelope

Nuclear Pore Complex: spoke ring

Nuclear Pore Complex: cytoplasmic ring

Cytoplasm

Nuclear Envelope polysomes and cytoskeletal filaments



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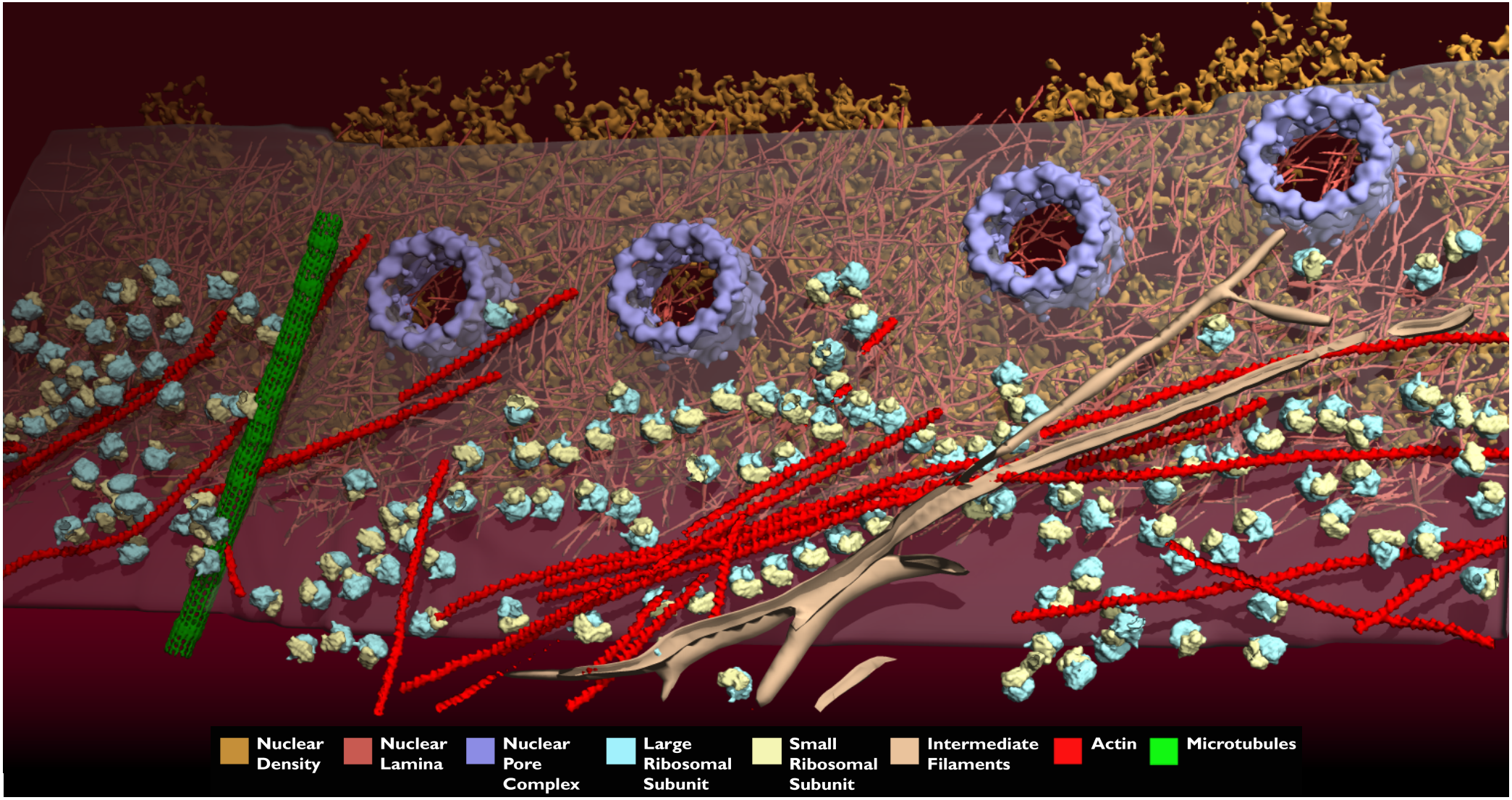

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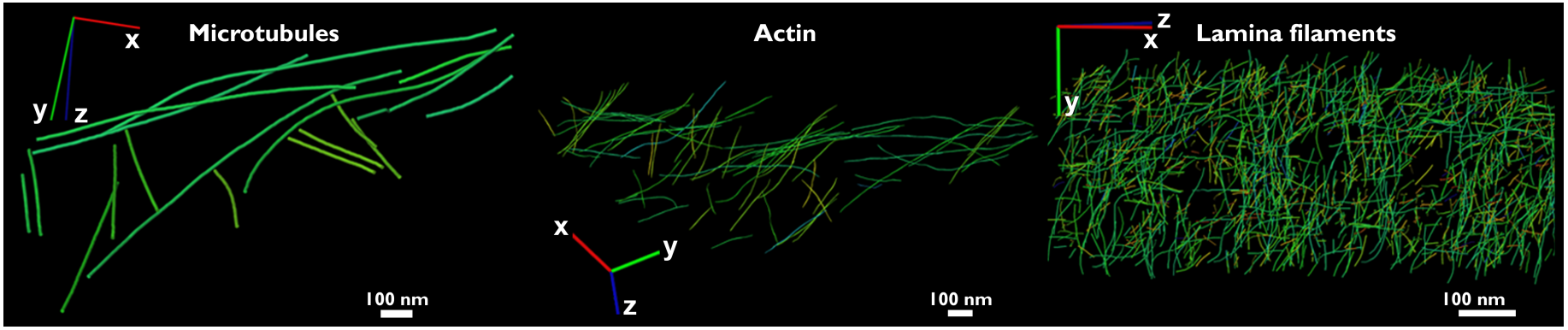
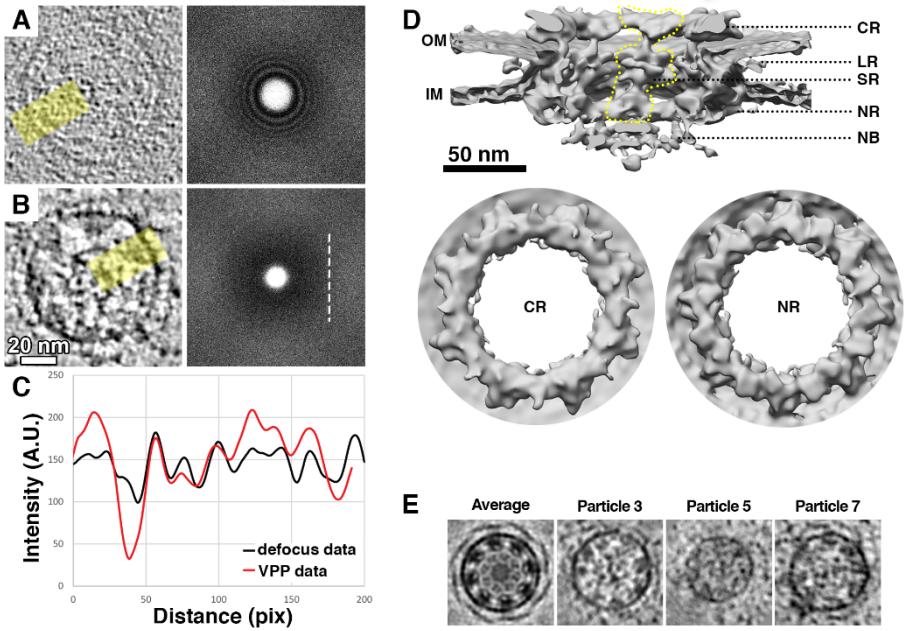
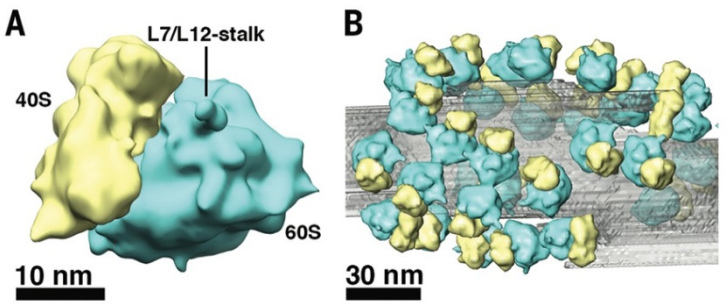


Plasticity of the Nuclear Pore Complex

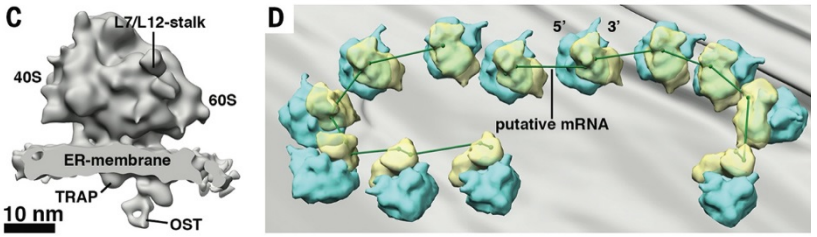


3D analysis of Ribosomes and Polysomes

Cytoplasmic Ribosomes, 900 particles: 28 Å @ 0.33 FC



ER-bound Ribosomes.140 particles: 35 Å @ 0.33



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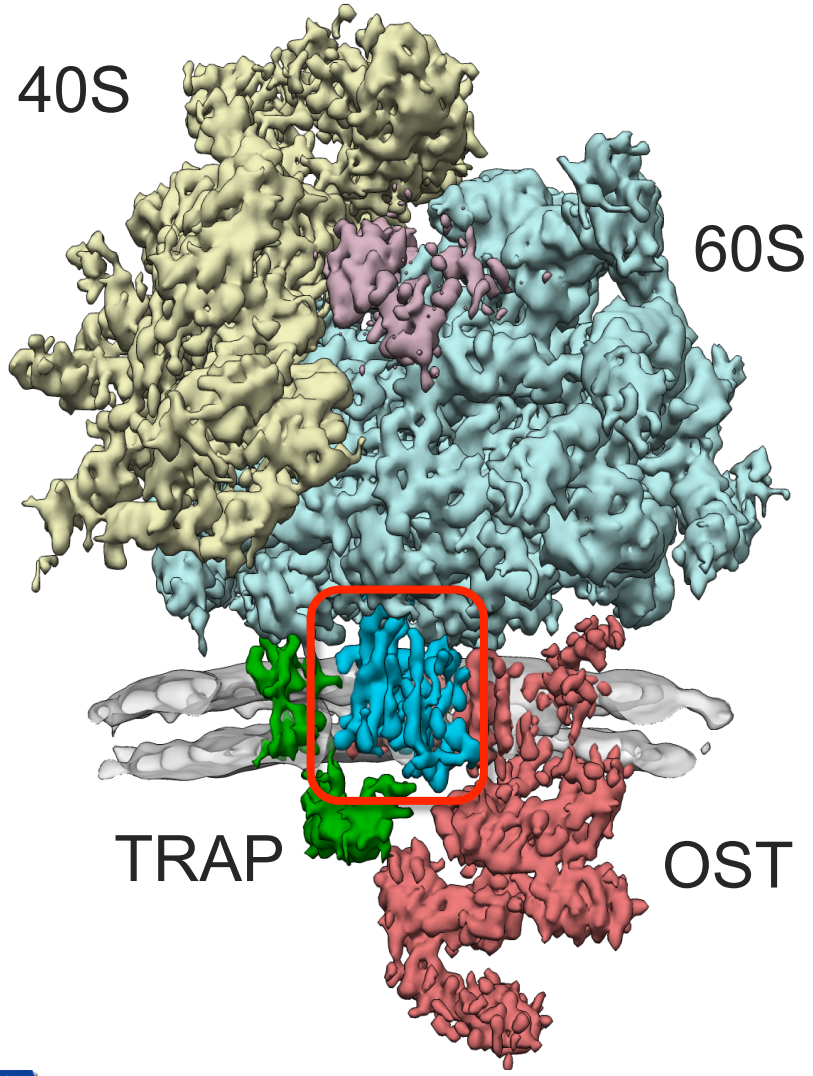
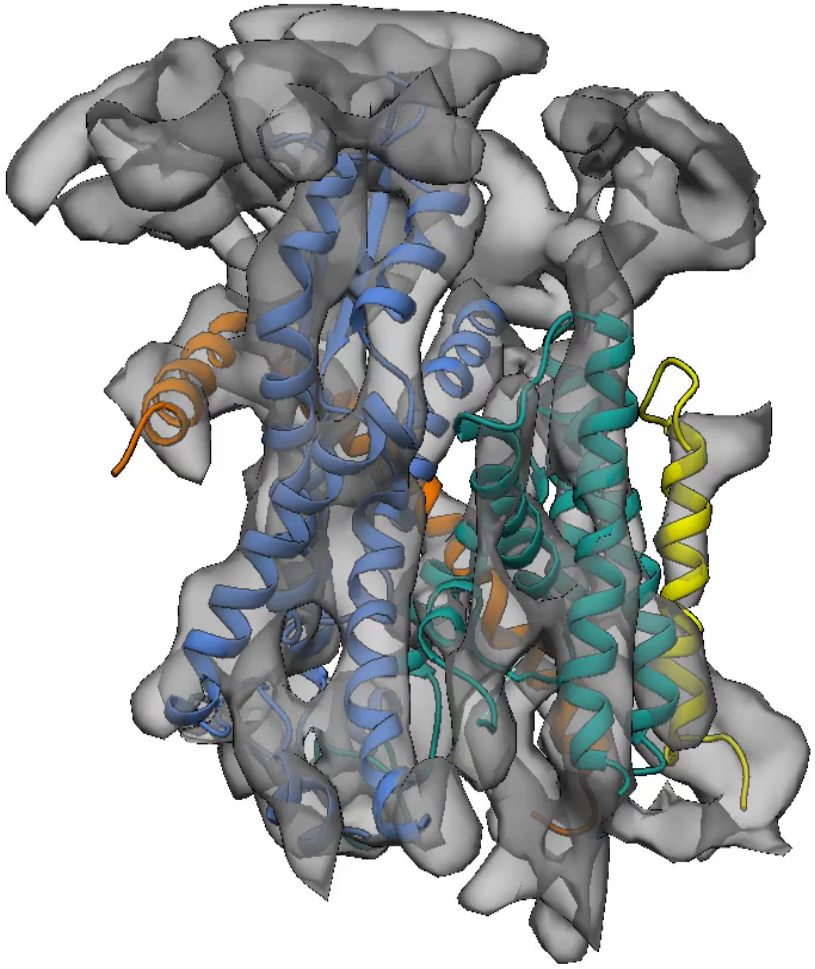
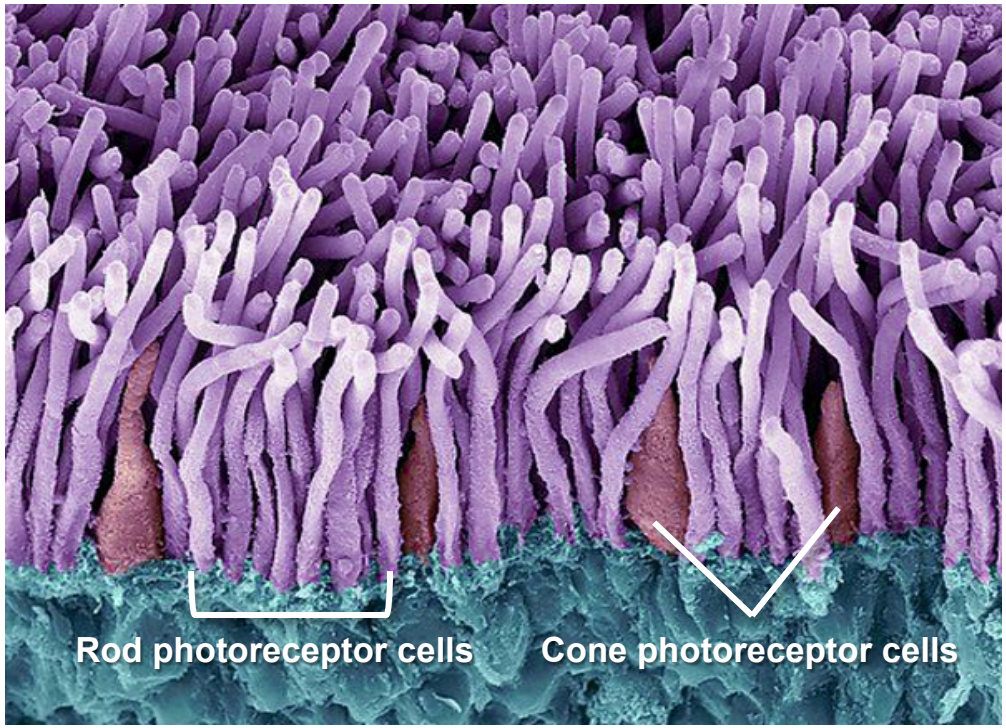
Structure of the native Sec61 protein-conducting channel



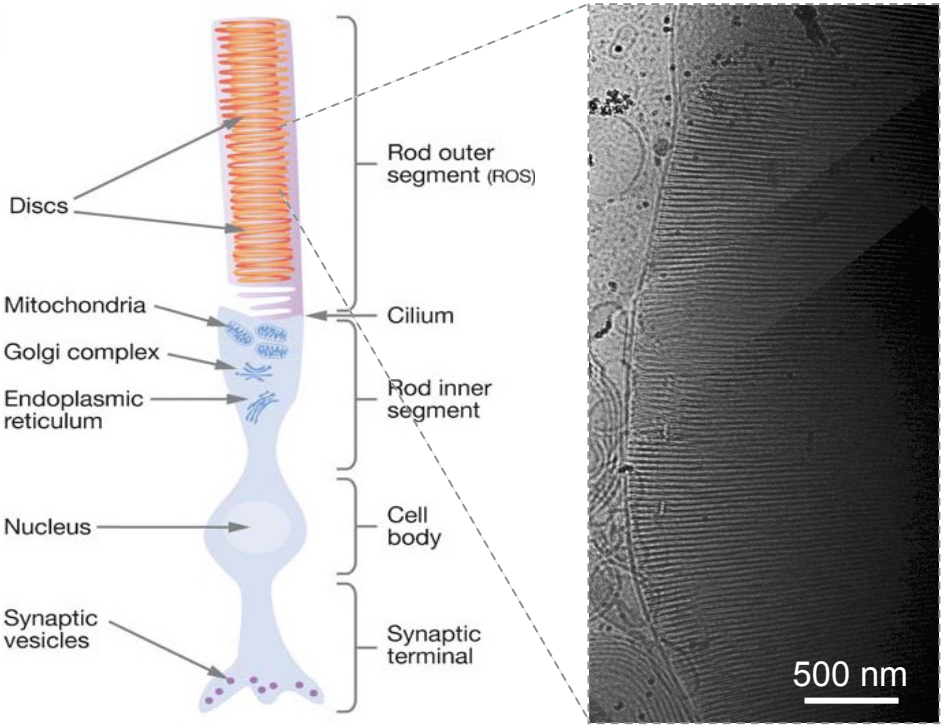


Pfeffer S, Burbaum L, Unverdorben P, Pech M, Chen Y, Zimmermann R, Beckmann R & Förster F. *Nat. Comm*. 6:8403 (2015).

Cellular Cryo-Electron Tomography: Mouse Retina Rod Outer Segment



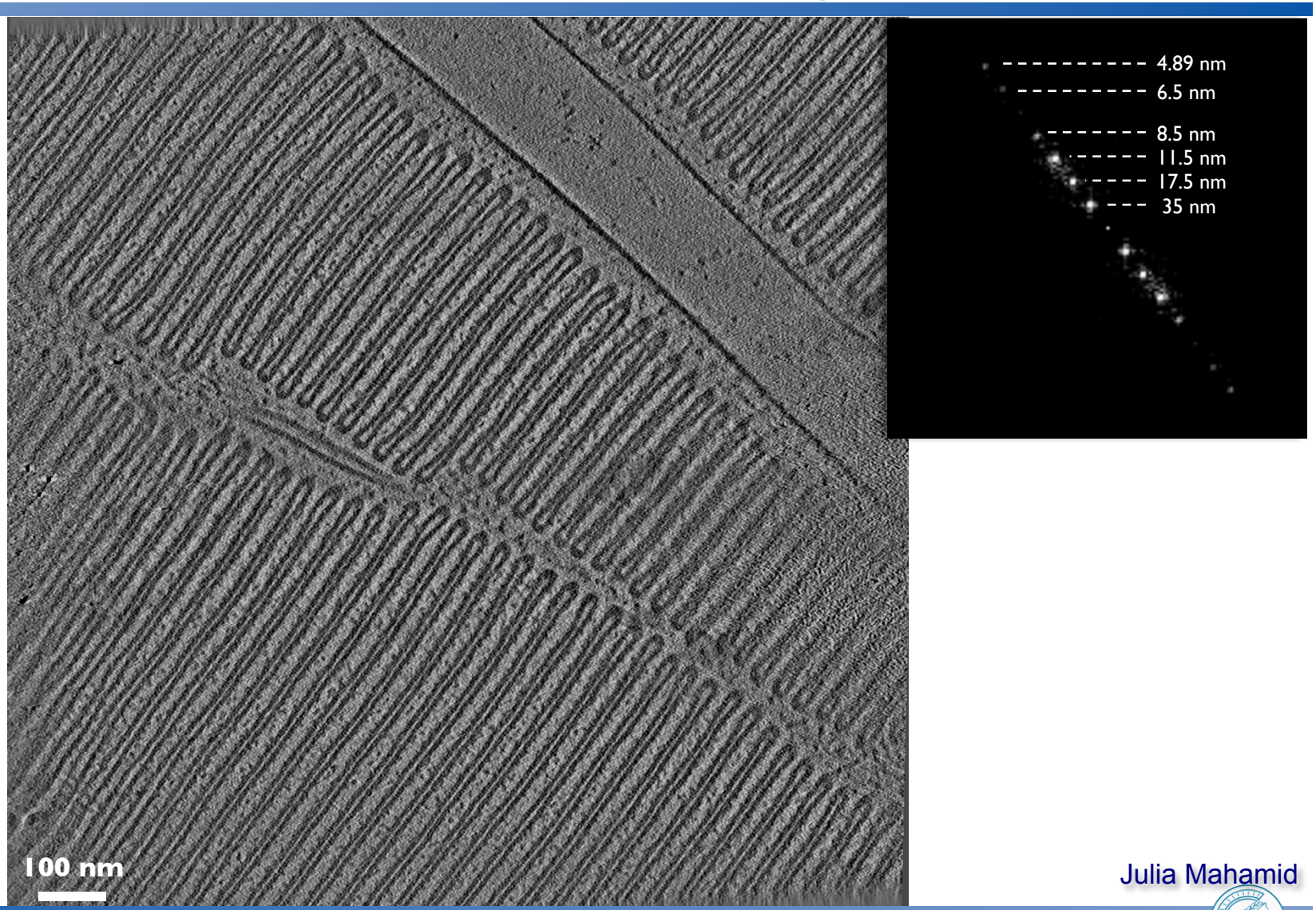
False color SEM of Retina



Rod photoreceptor cells

Nickell et al. JCB 2007

Cellular Cryo-Electron Tomography: Mouse Retina Rod Outer Segment



Julia Mahamid

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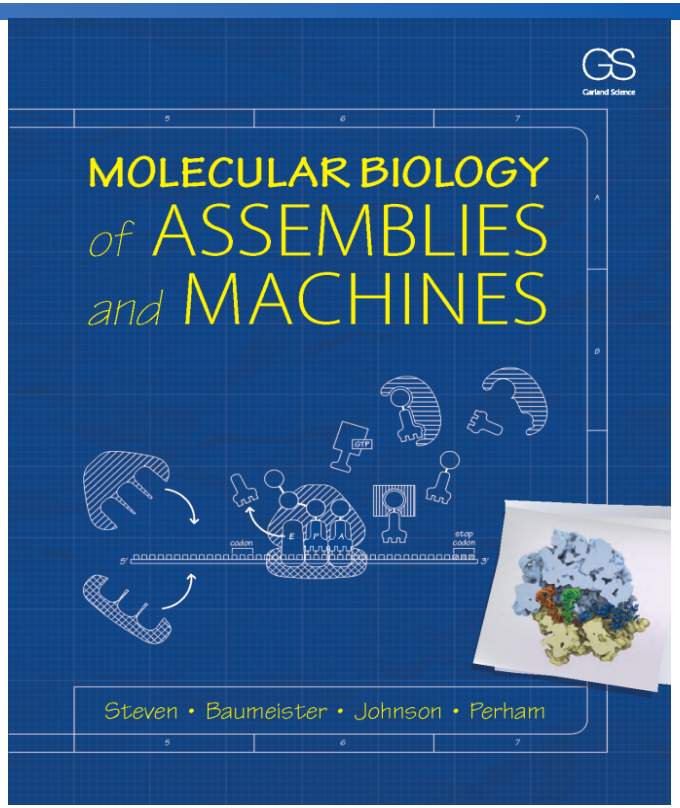

KEY FEATURES

- Comprehensive narrative covers eukaryotic, bacterial, and archaeal systems
- Vivid illustrations portray the structures of macromolecular complexes and how they assemble and interact
- Relates certain diseases to mutations or malfunctions affecting macromolecular assemblies

FROM THE FOREWORD

"The book covers almost every basic biological topic at the level that will allow the inquisitive reader to quickly absorb the fundamentals in terms of the currently available structural information".

Michael G. Rossmann

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