RUTGERS

SEMC Winter CryoEM Course

Introduction to Cryo-electron Tomography

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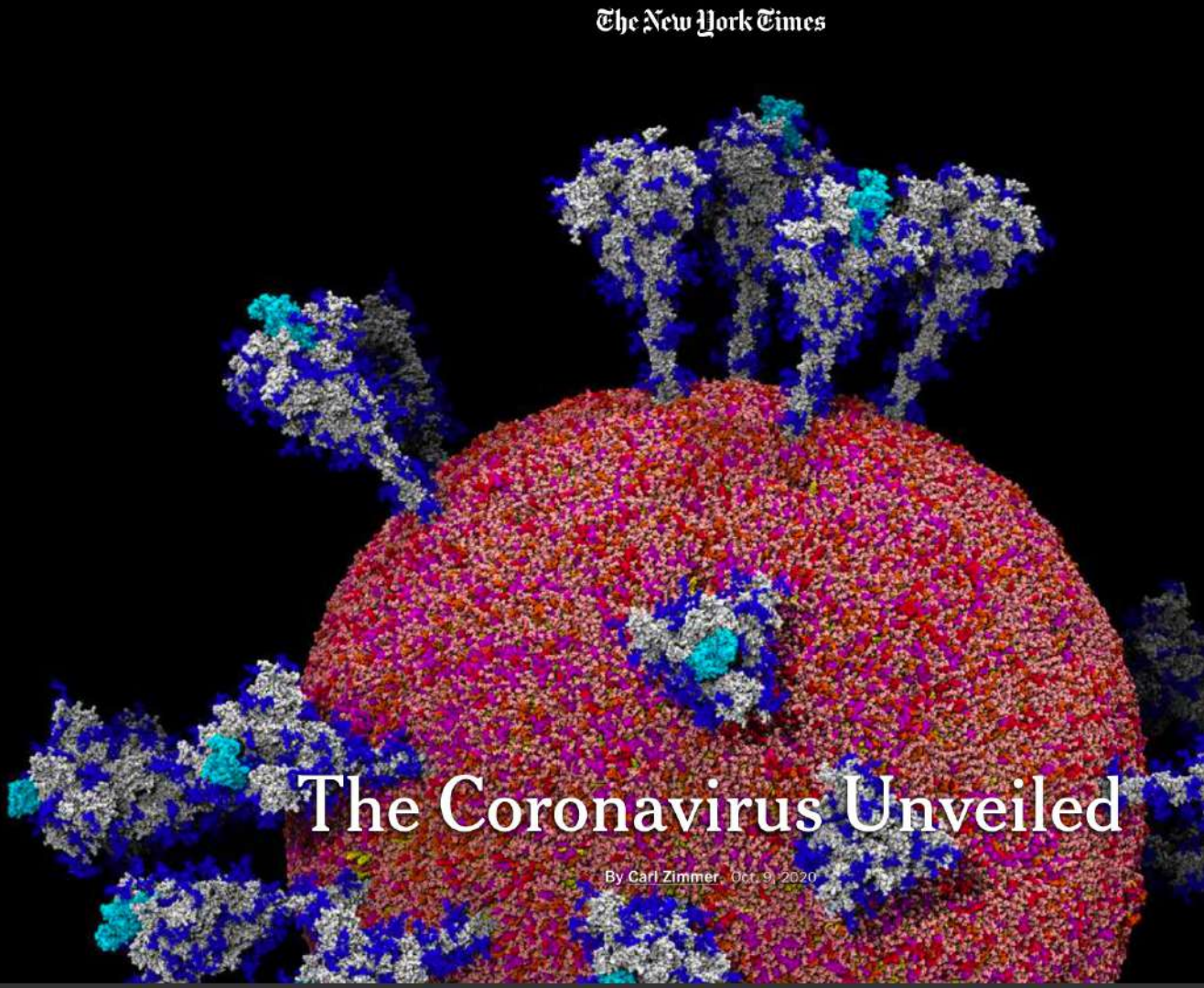
Learning Goals

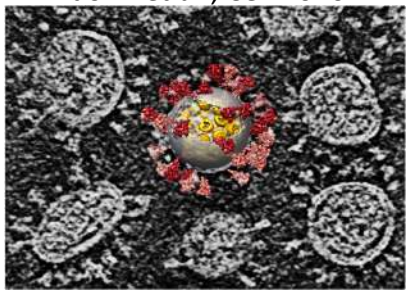
- To have a good understanding of the capabilities, potential, and limitations of cryo-electron microscopy and tomography in biomedical research
- To be able to make an educated decision whether cryoEM or cryoET will contribute to their research and thesis.

Resolving Structures to Drive Scientific Discoveries during the Pandemic



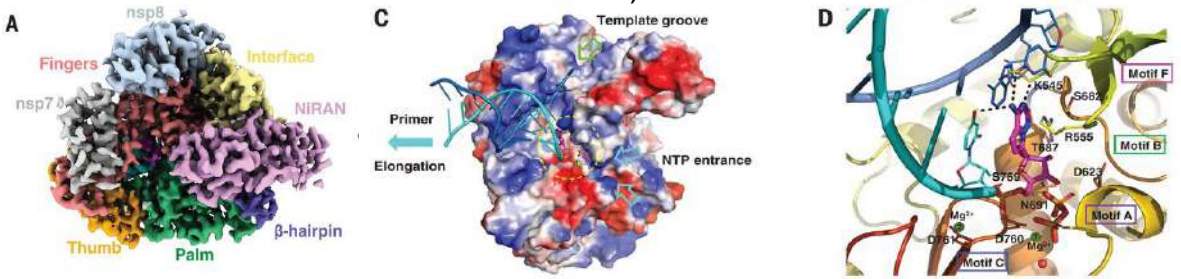
How does the virus look like?

Yao H. *et al.*, Cell 2020



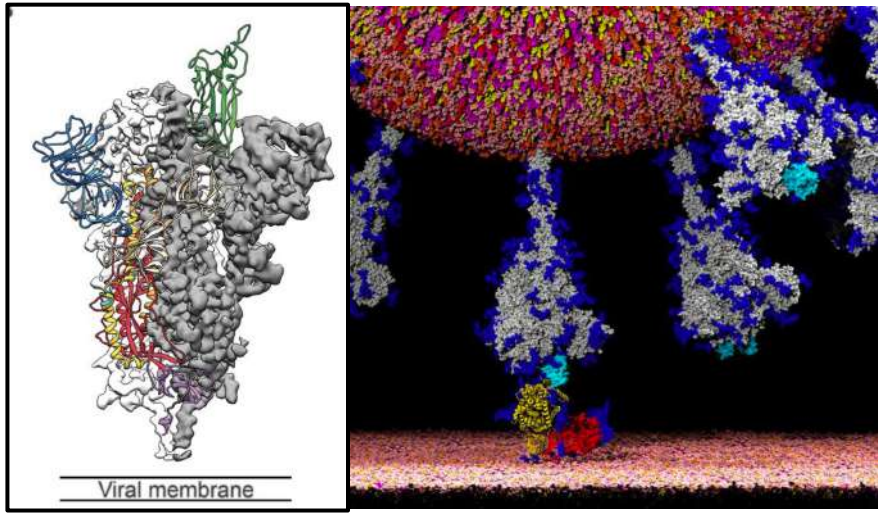
How does Remdesivir inhibit SARS-CoV-2 infection?

Yin W. *et al.*, Science 2020



How does SARS-CoV-2 enter human cells?

Wrapp D. *et al.*, Science 2020; Simulation by Amaro lab, UCSD



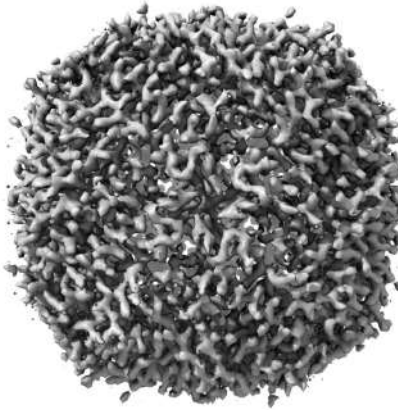
How does SARS-CoV-2 assemble inside cells?

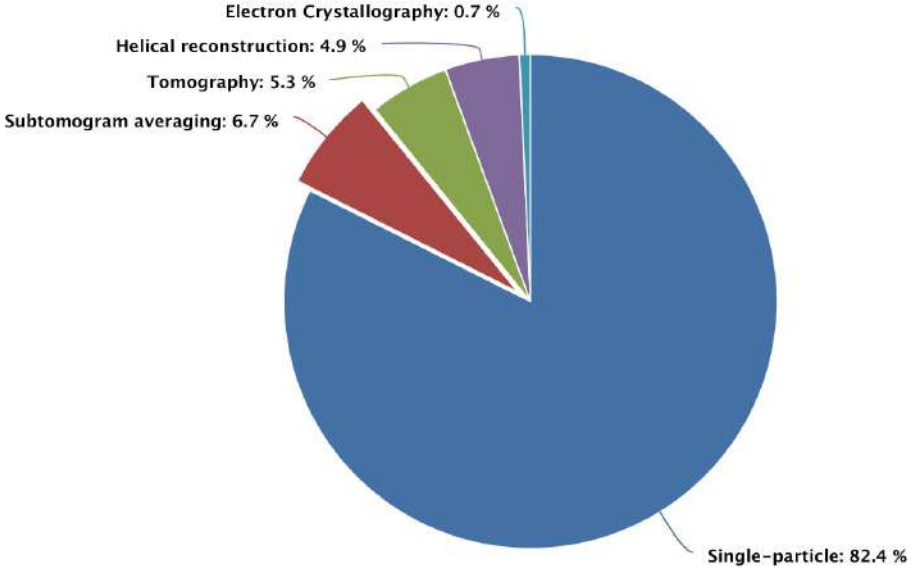
Klein S. *et al.*, Nature Communications 2020

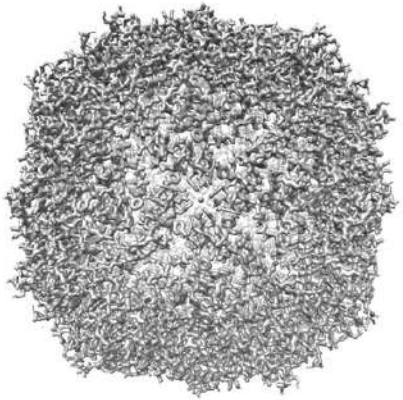
Outline

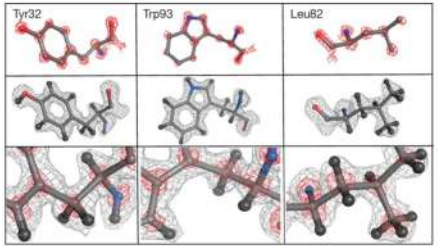
- What is cryo-electron tomography, and how is it different from cryoEM single particle analysis?
- Sample preparation: special considerations
- Data collection, alignment, and reconstruction
- Application of cryo-electron tomography in cell biology (structural cell biology)

Structures Resolved by Single Particle Analysis & Tomography in EMDB





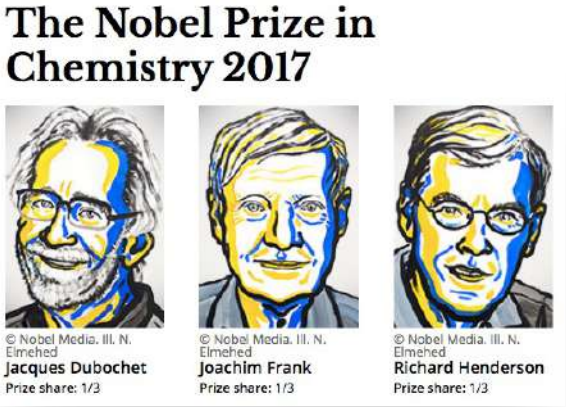


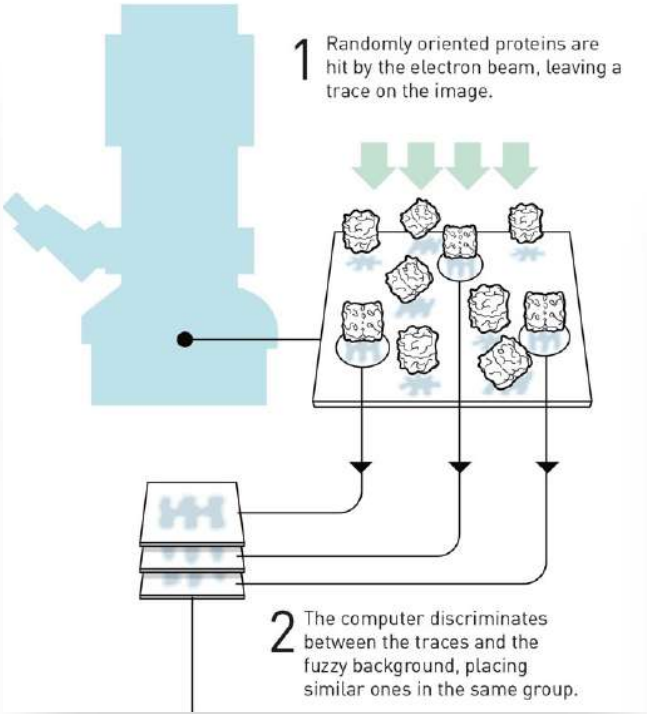


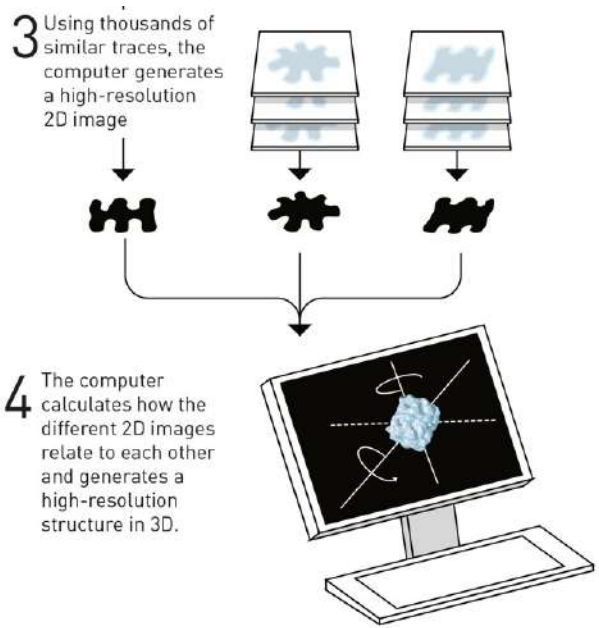
Apoferritin at 2.86Å by
subtomogram averaging
Ni T. *et al*., Nat. Protoc (2022)

Human apoferritin at 1.15Å
by single particle analysis
Yip K. *et al*., Nature (2020)

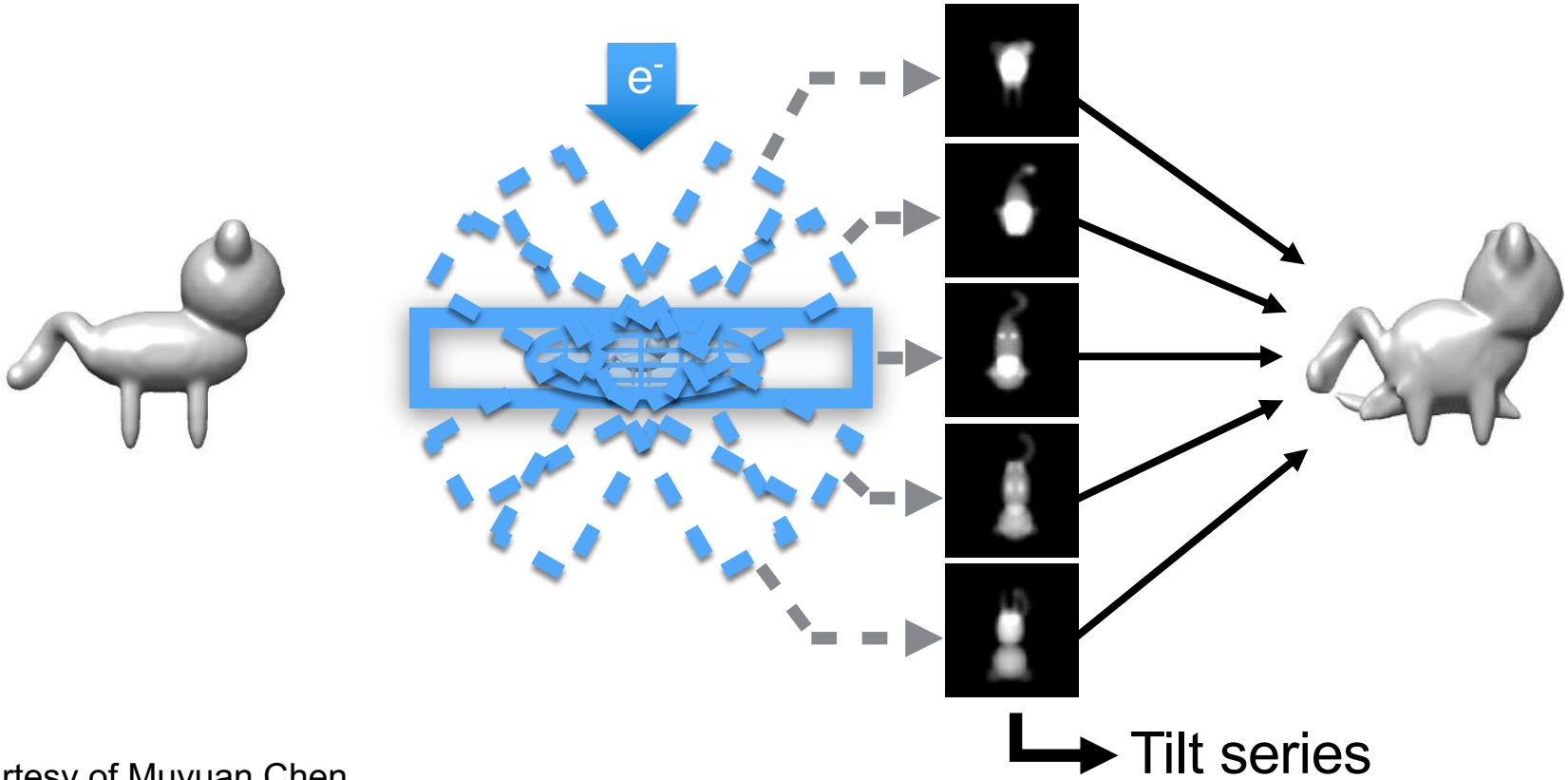
Cryo-EM Single Particle Analysis







What is Cryo-electron Tomography - CryoET



Courtesy of Muyuan Chen

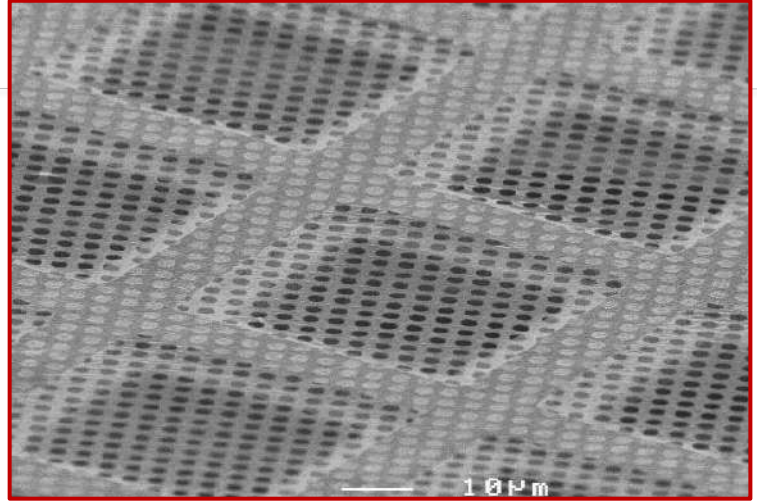
Why Cryo-electron Tomography (Cryo-ET)?

- Visualize dynamics (structure and distribution) of protein complexes or organelles involved in fundamental biological processes
- Resolve *in situ* structures under physiological conditions
- Provide a structure determination option for challenging samples

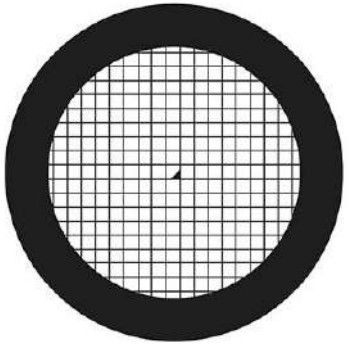
Sample Preparation

- Preserving the specimen in native conformation in aqueous solution
- Good concentration
- Good thickness & good contrast
- Target tracking in their native environment

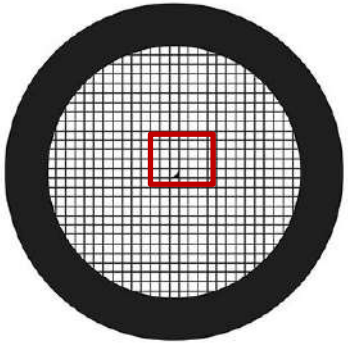
EM Grids



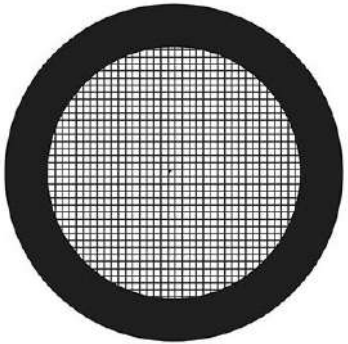
- Material:
  - Copper
  - Gold
- Thickness: 10 – 25 um
- Mesh: define the square size and number on the grid
- Usually have an additional layer of continuous/perforated support film



200 mesh

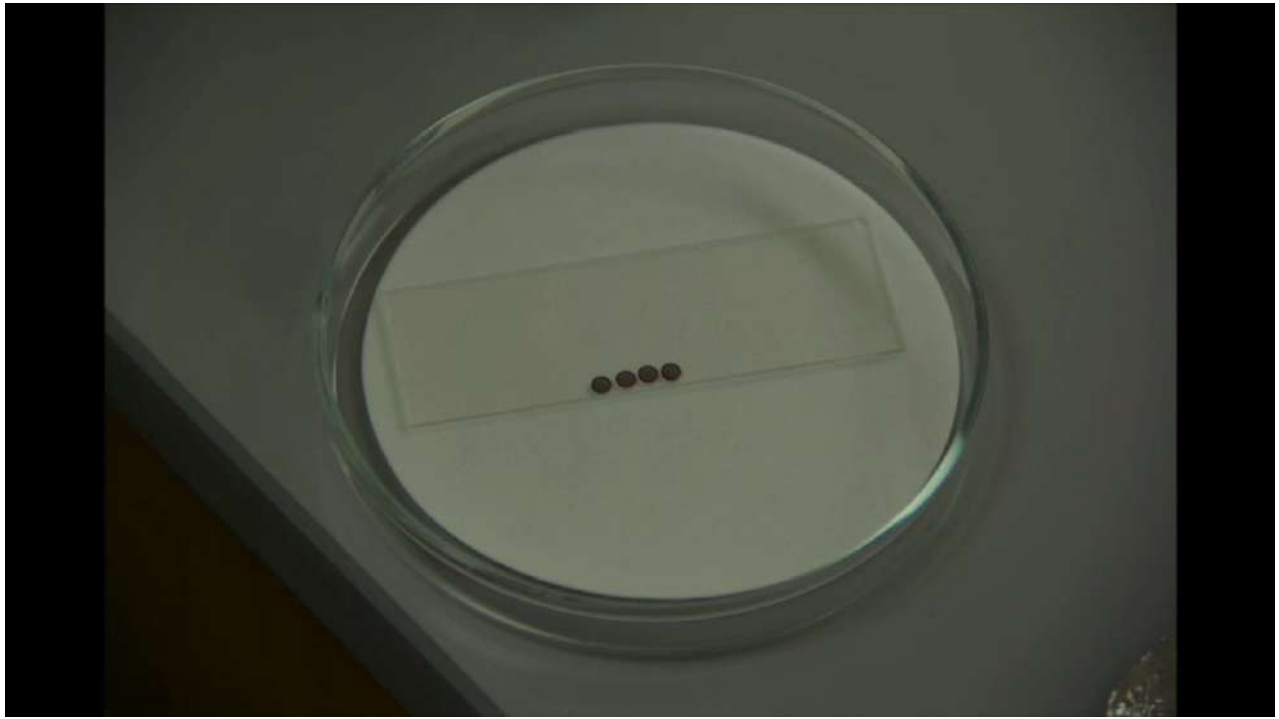


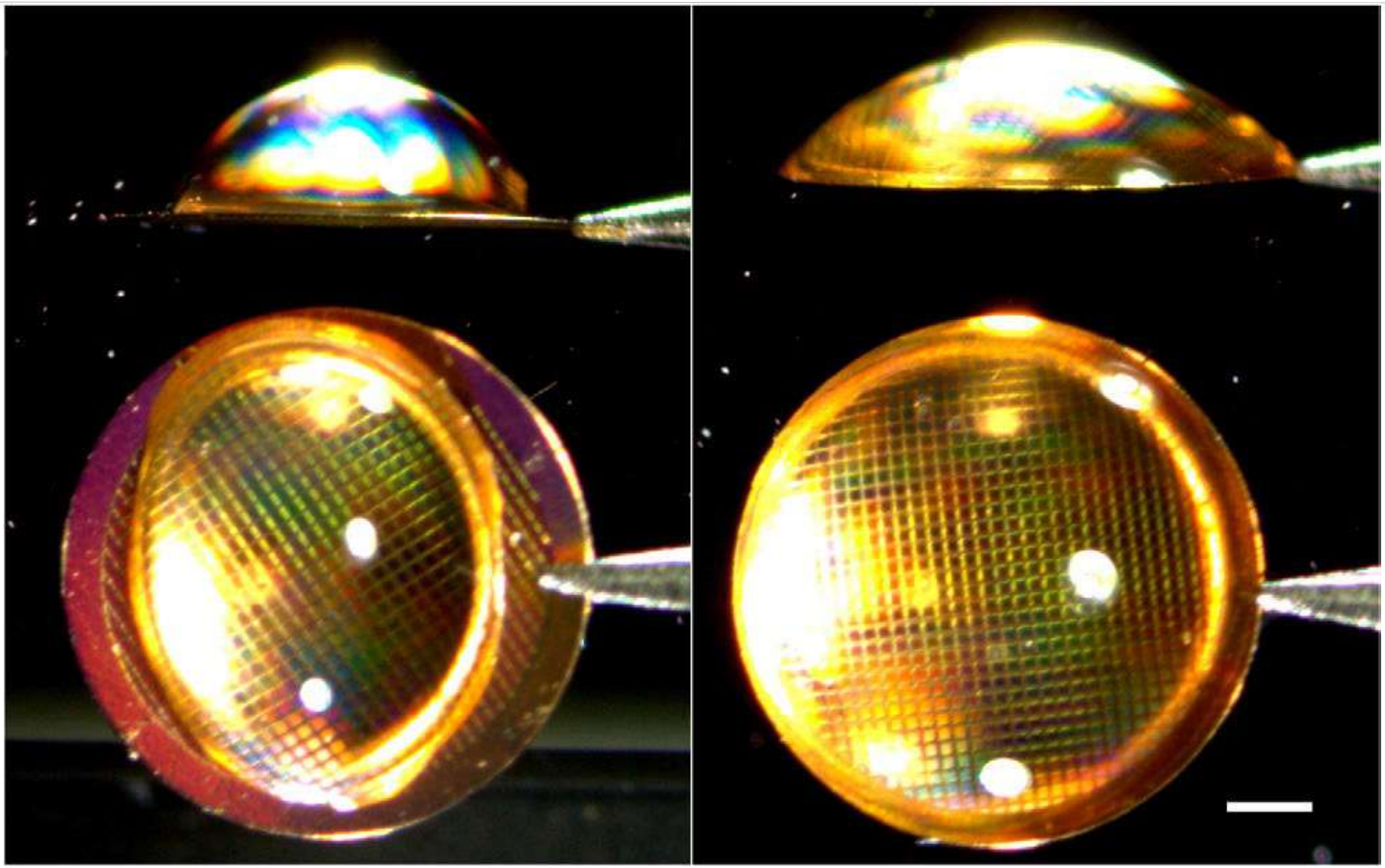
300 mesh



400 mesh

Glow Discharge





Before After

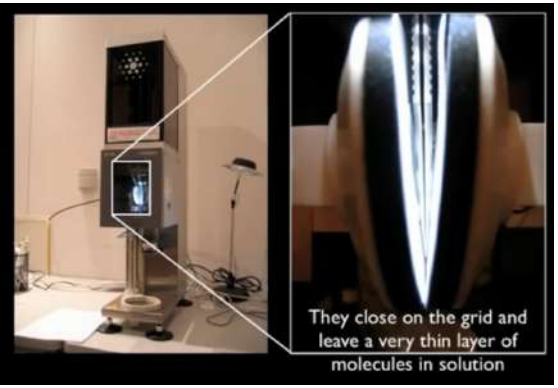
C. J Russo, MRC Laboratory of Molecular Biology, 2016

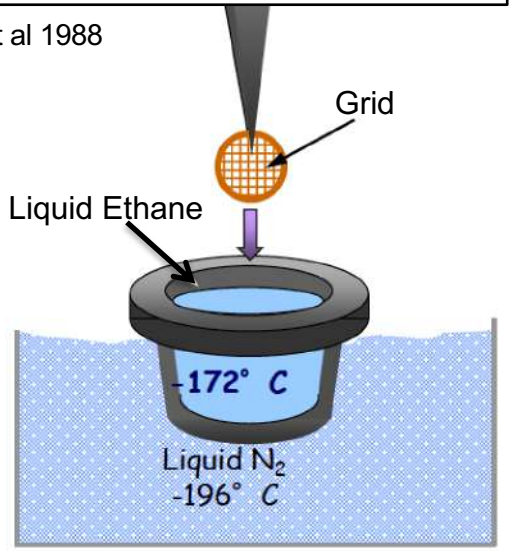
Plunge Freezing

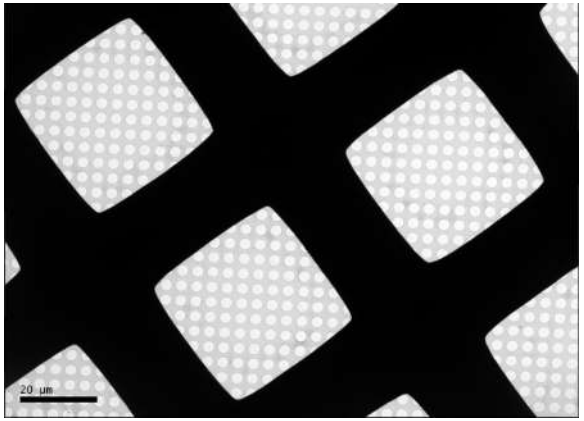
To vitrify water:

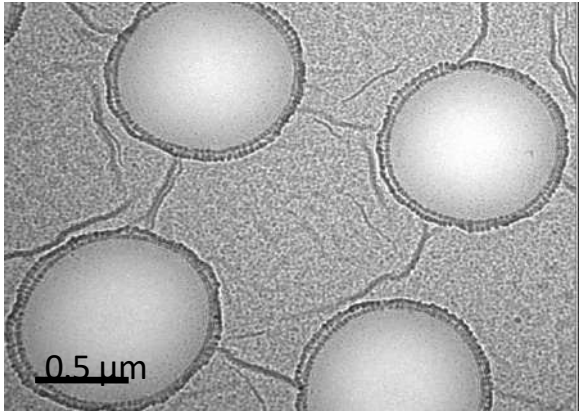
- Liquid ethane → good heat conductivity with a cooling rate of > 10^5-10^6 K/s
  - Liquid N_2 has poor cooling capacity
  - Water is a poor thermal conductor so sample thickness < 5 µm
- Gravity plunge at > 1 m/s

Dubochet et al 1988









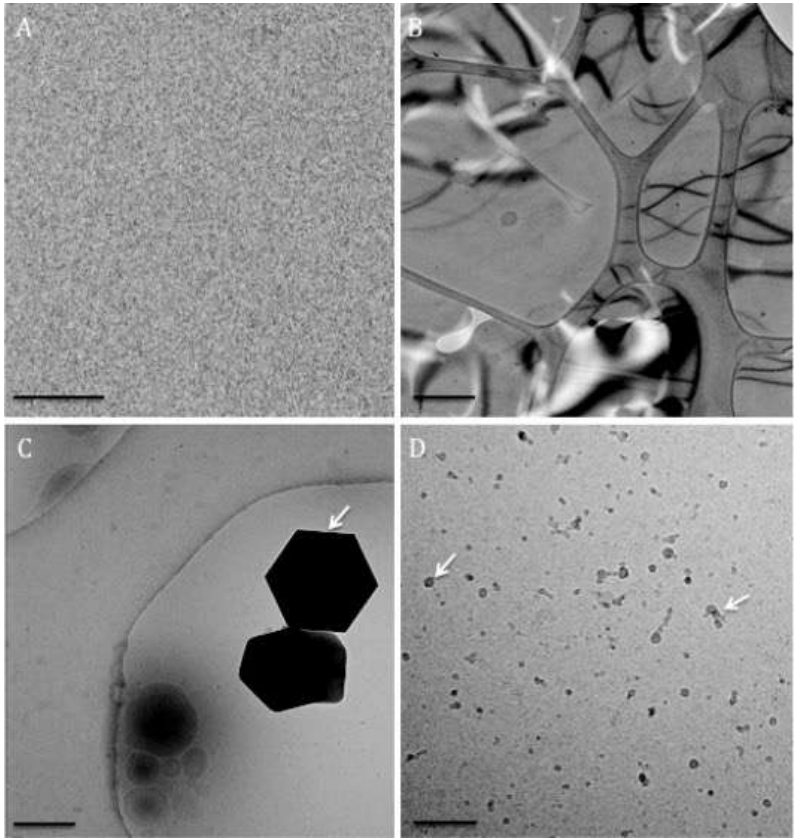
Ice Quality: Vitreous vs Non-vitreous Ice

A: Vitreous ice

B: Hexagonal ice

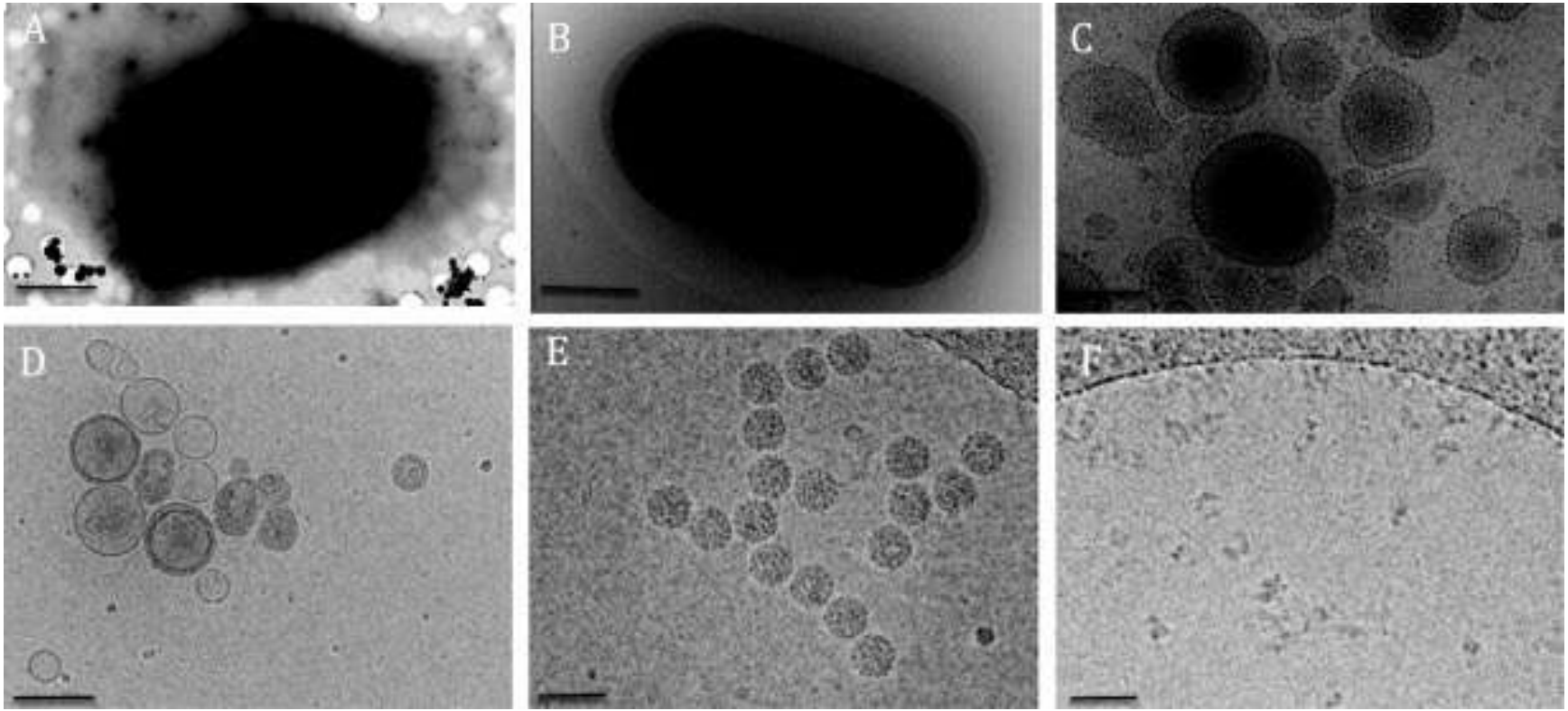
C: Large ice crystal

D: Ethane contamination



R. F. Thompson, et al, 2016
Methods, Vol 100 , 3-15

Specimen Preserved by Plunge Freezing



R. F. Thompson, *et al.*, 2016 Methods, Vol. 100 , 3-15

Preparing Intact Mammalian Cells For Cellular Tomography

Prepare EM Grids
- Material: gold
- Mesh: 200 mesh
- Supporting film: carbon or SIO_2
- Hole size: support *vs* contrast

↓

Plate Cells on EM Grids
- Coating protein
- Plating density

↓

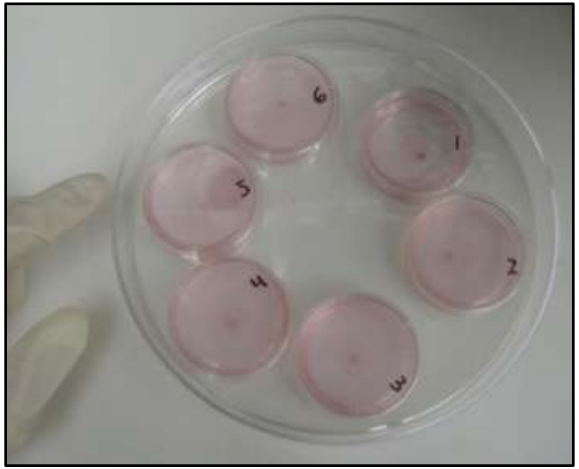
Allow Cells to Adhere/Grow

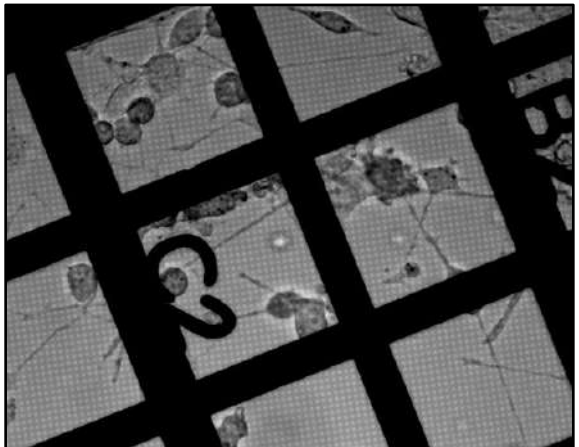
↓

Plunge Freeze Grids

↓

Imaging



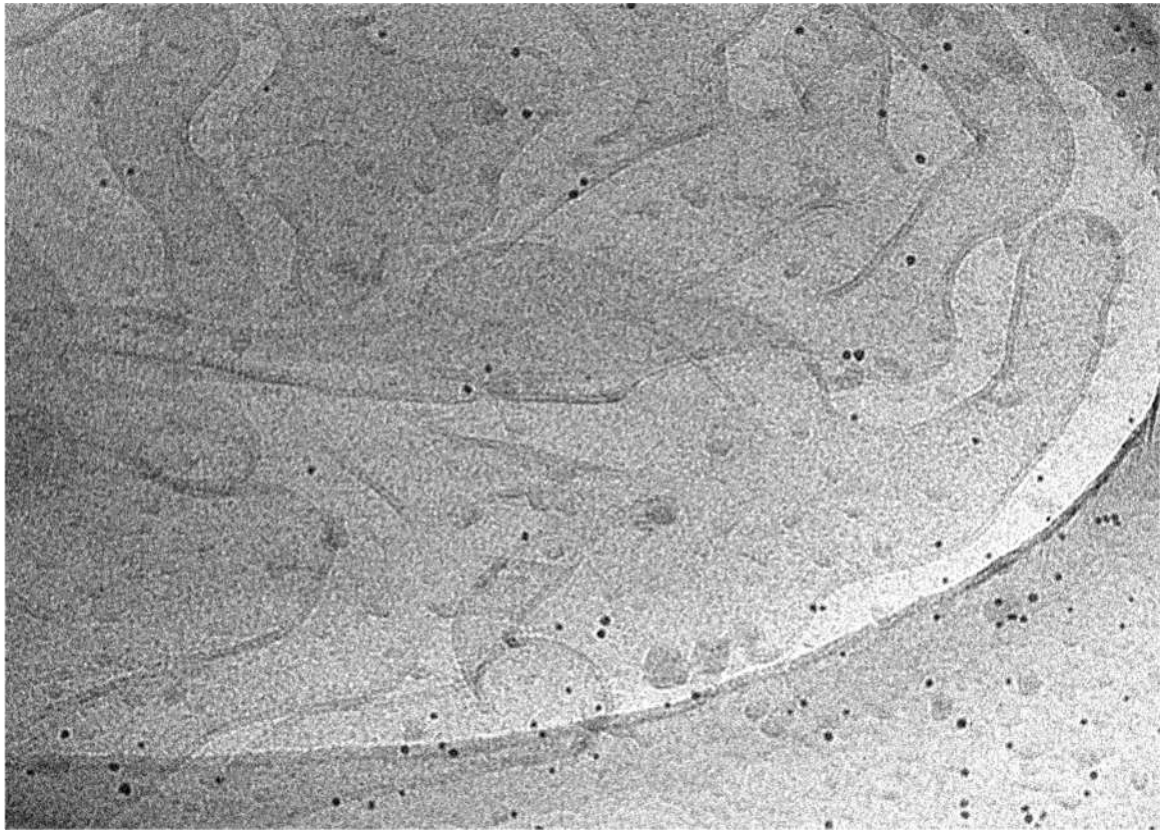


- Data Collection

CryoET Data Collection

What is a “tilt-series”?

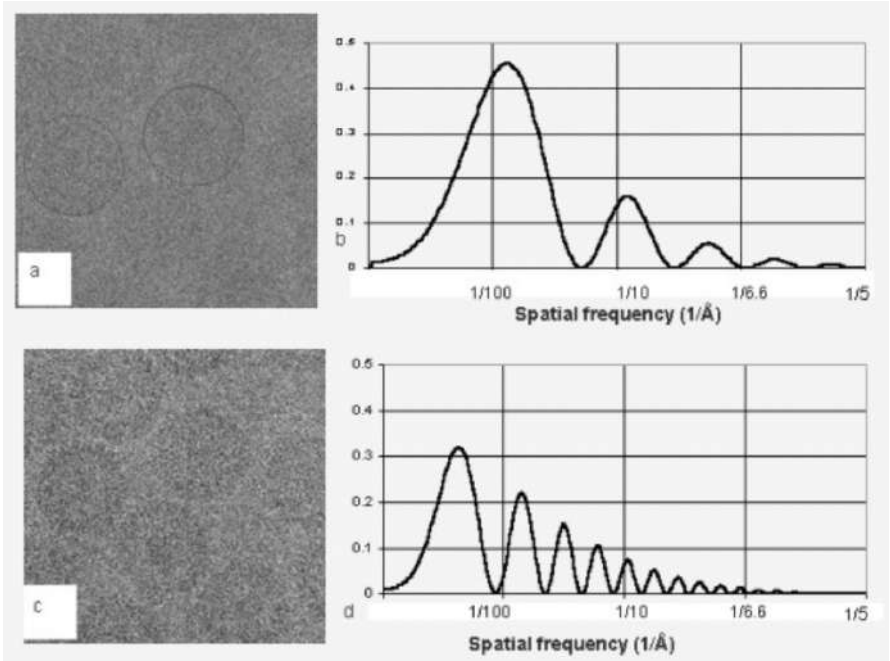
- Images taken when the sample is tilting about the tilt axis.



Tilt series of *C. glabrata* plasma membranes

CryoET Data Collection

- There are many configuration parameters involved in data collection. Each is a balance between opposing considerations.
  - Defocus: contrast vs resolution

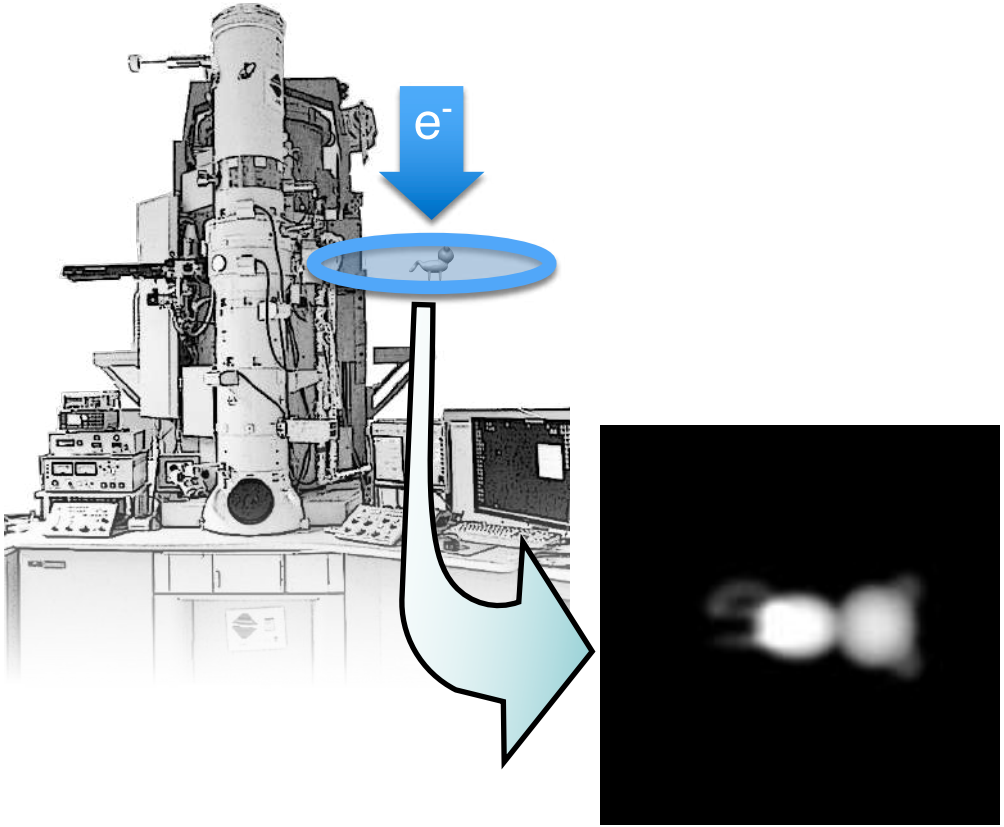


A. S. Saad, *BMC Struct. Biol.* 2005.

CryoET Data Collection

- There are many configuration parameters involved in data collection. Each is a balance between opposing considerations.
  - Defocus: contrast vs resolution
  - Total dose: signal vs radiation damage

Signal to noise ratio (SNR)



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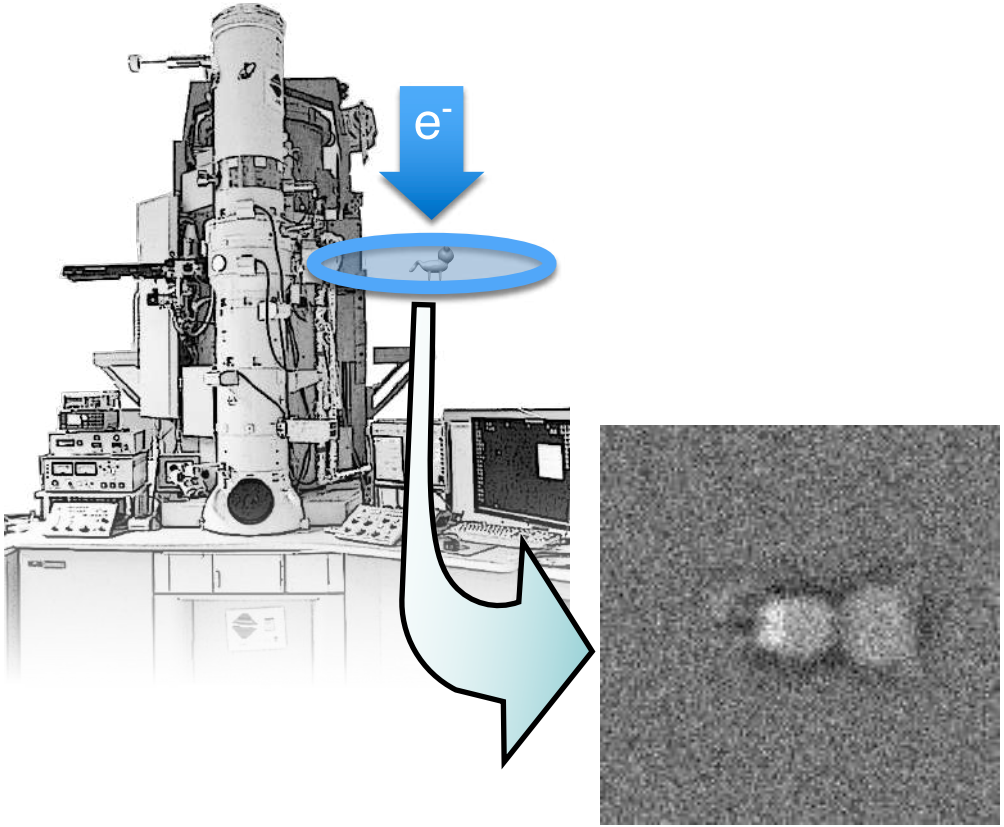
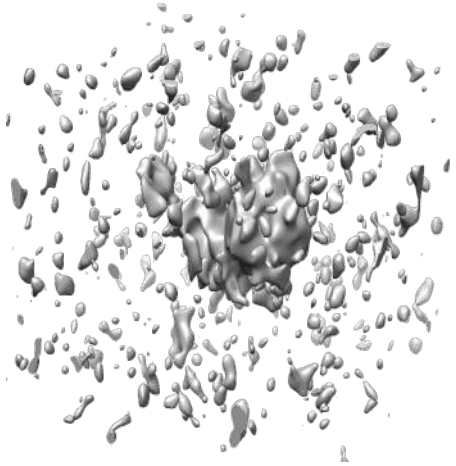
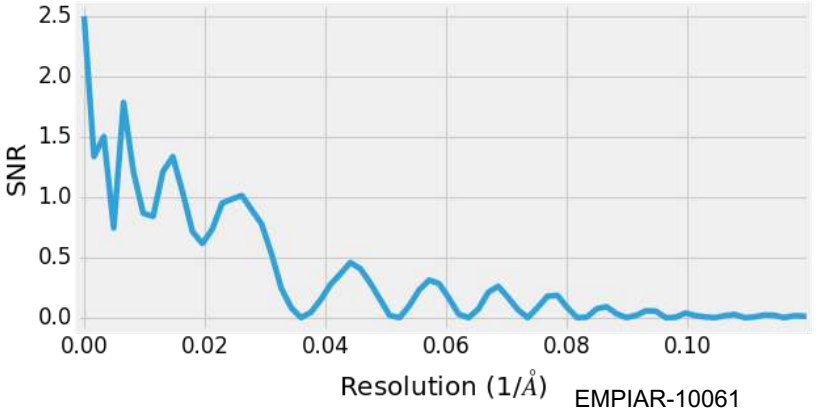
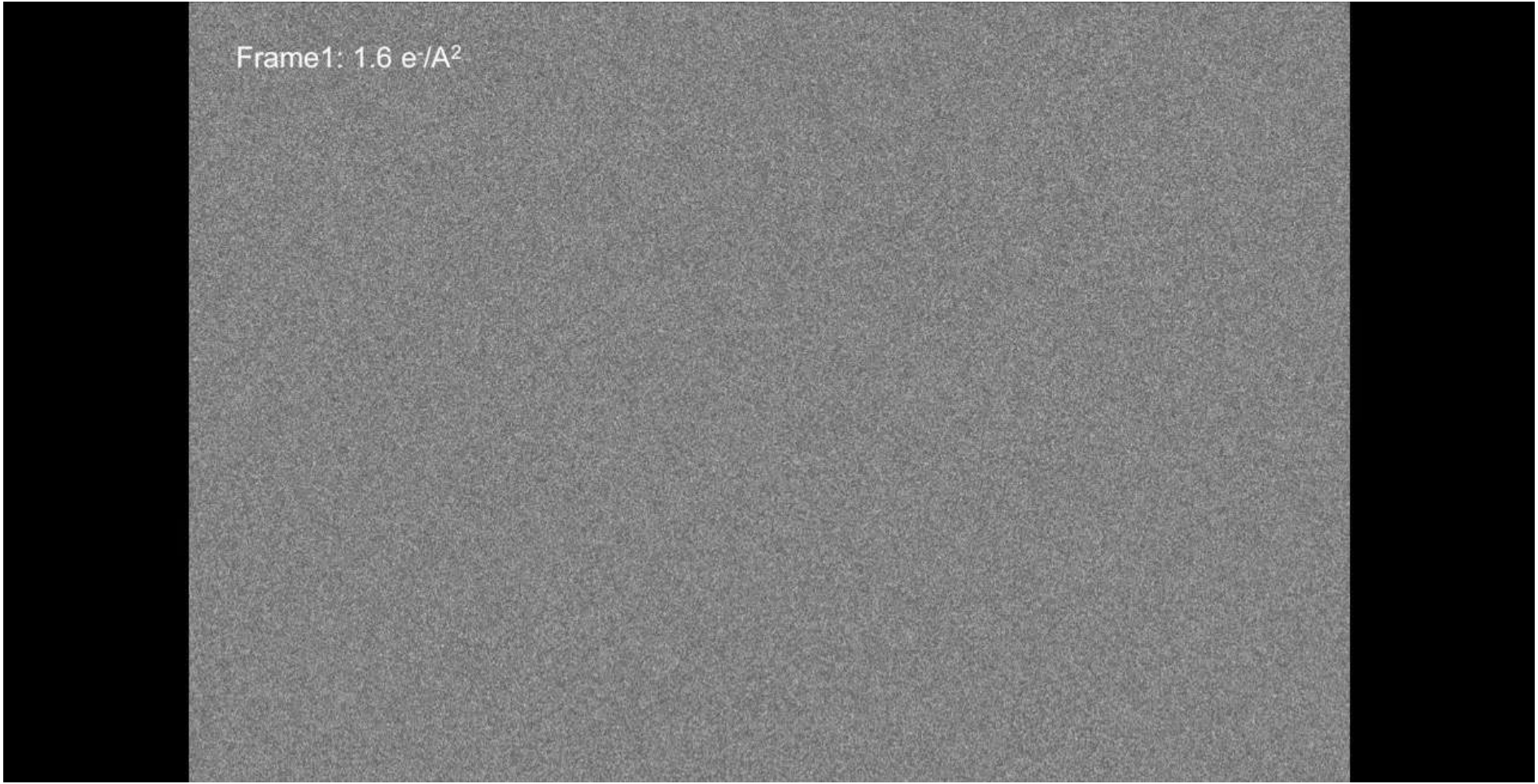


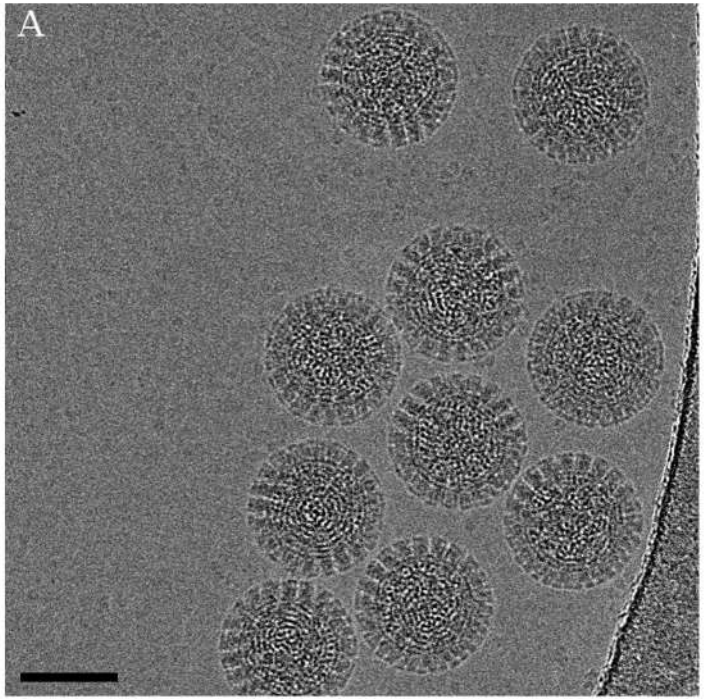


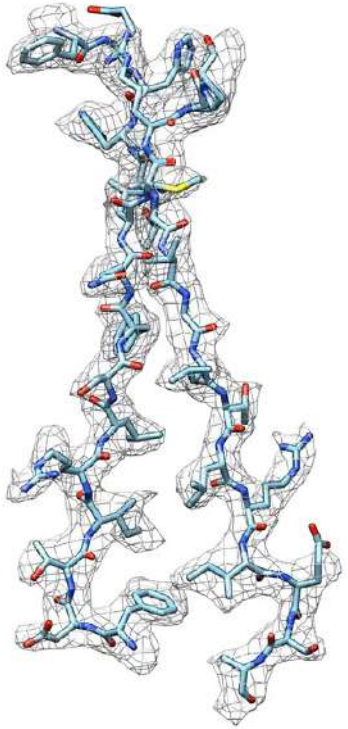


EMPIAR-10061


Frame1: 1.6 e-/A2

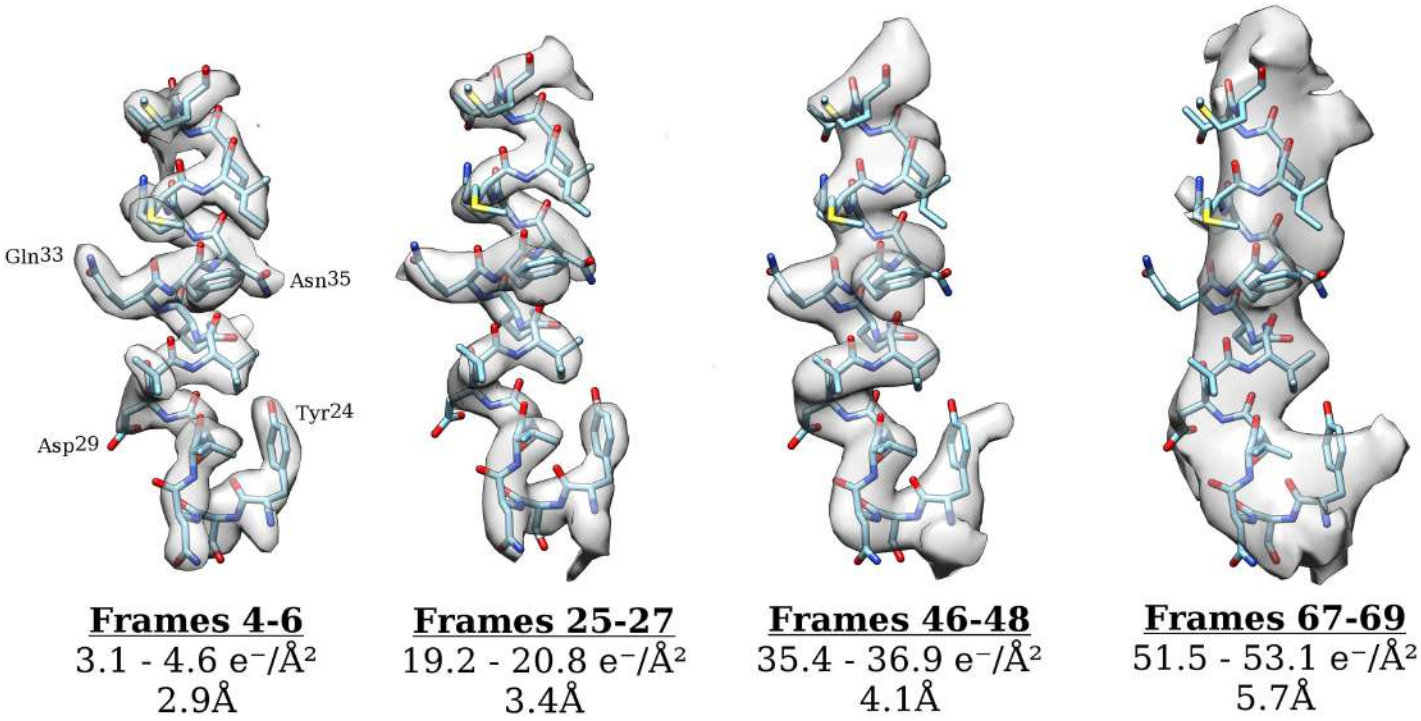
Why Can’t We Just Shed Lots of Electrons





T. Grant, et al, 2015 Elife. DOI: 10.7554/eLife.06980

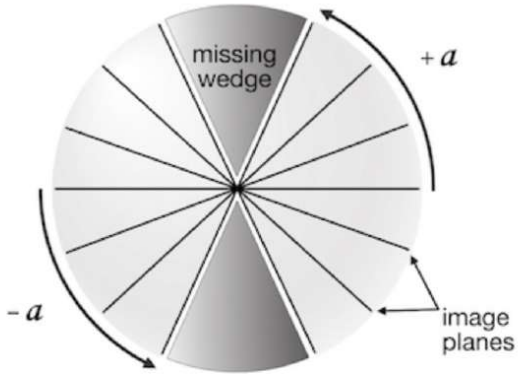
Why Can't We Just Shed Lots of Electrons



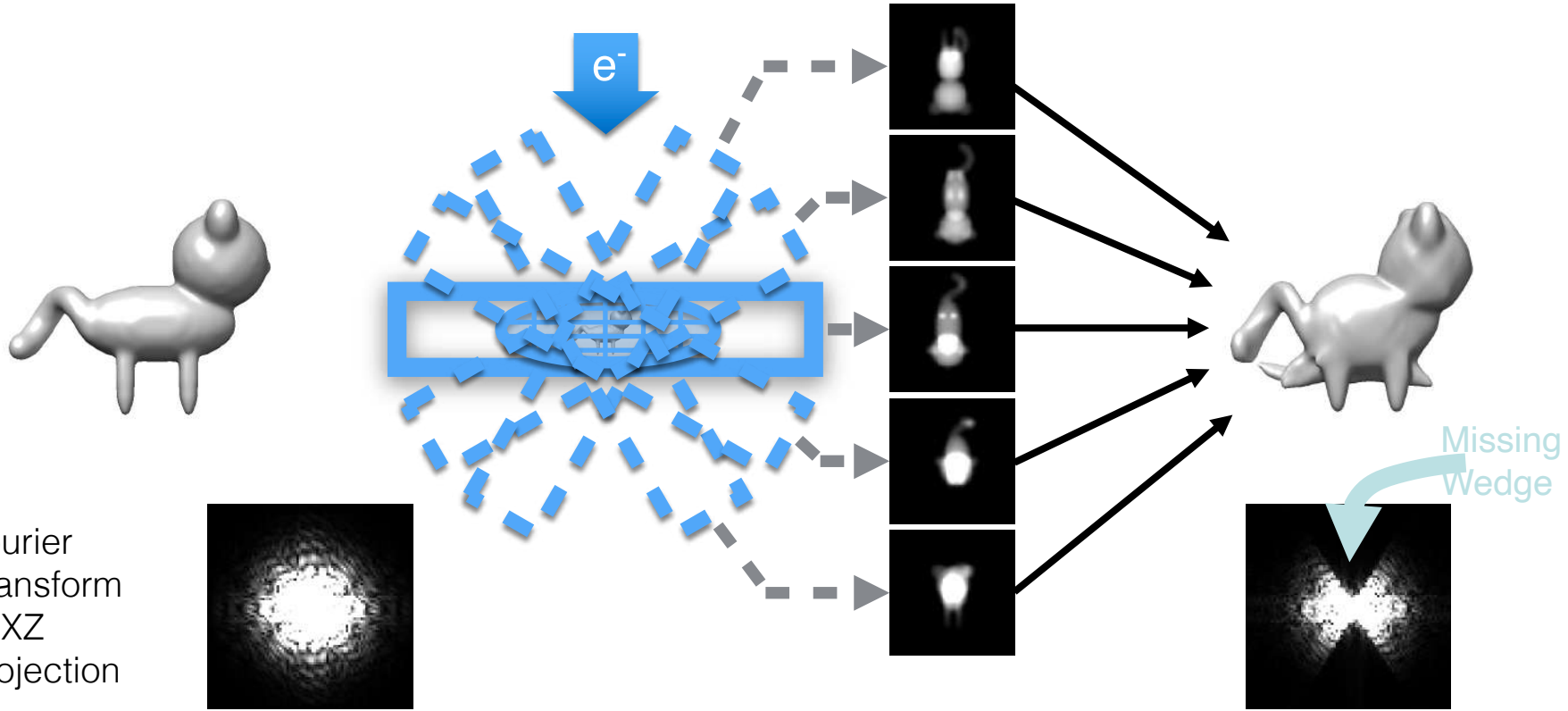
T. Grant, et al, 2015 Elife. DOI: 10.7554/eLife.06980

CryoET Data Collection

- There are many configuration parameters involved in data collection. Each is a balance between opposing considerations.
  - Defocus: contrast vs resolution
  - Total dose: signal vs radiation damage
  - Tilt range and increment:
    - Goniometer mechanical limit (-70 <->+70)
    - Larger increment → less images in a series
      → Better contrast
    - Dose allocation in a tilt series

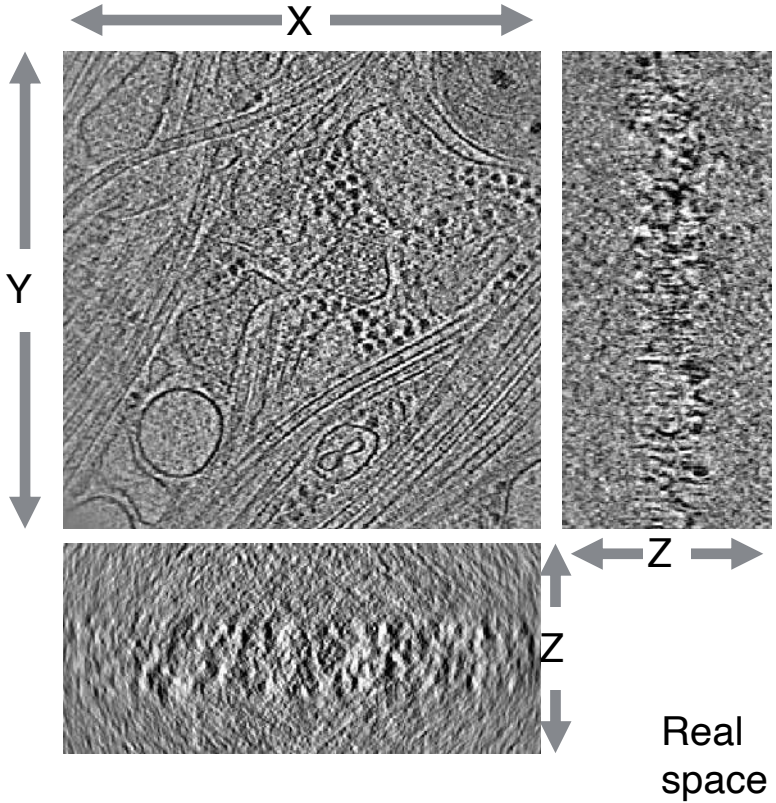


The Missing Wedge Artifact

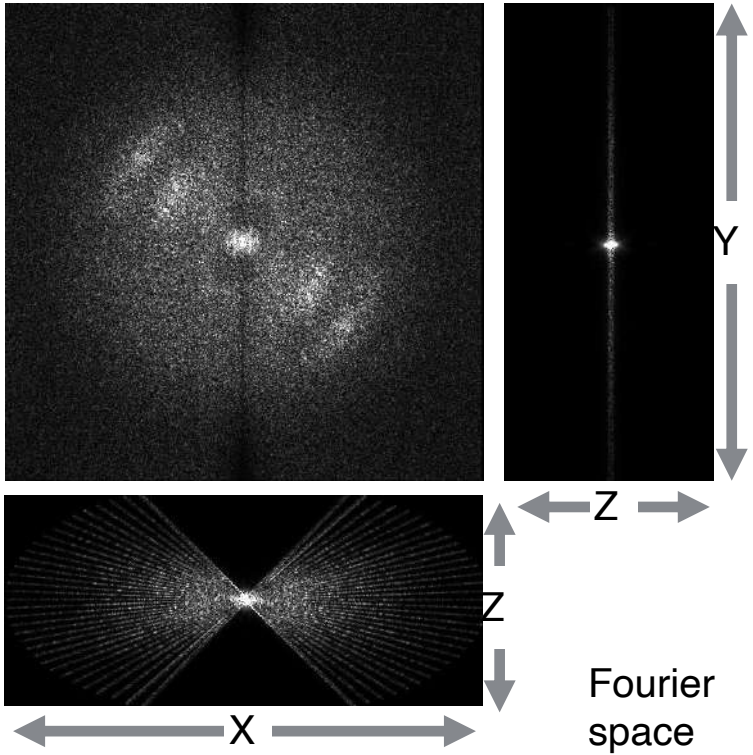


Fourier Transform of XZ projection

The Missing Wedge Artifact



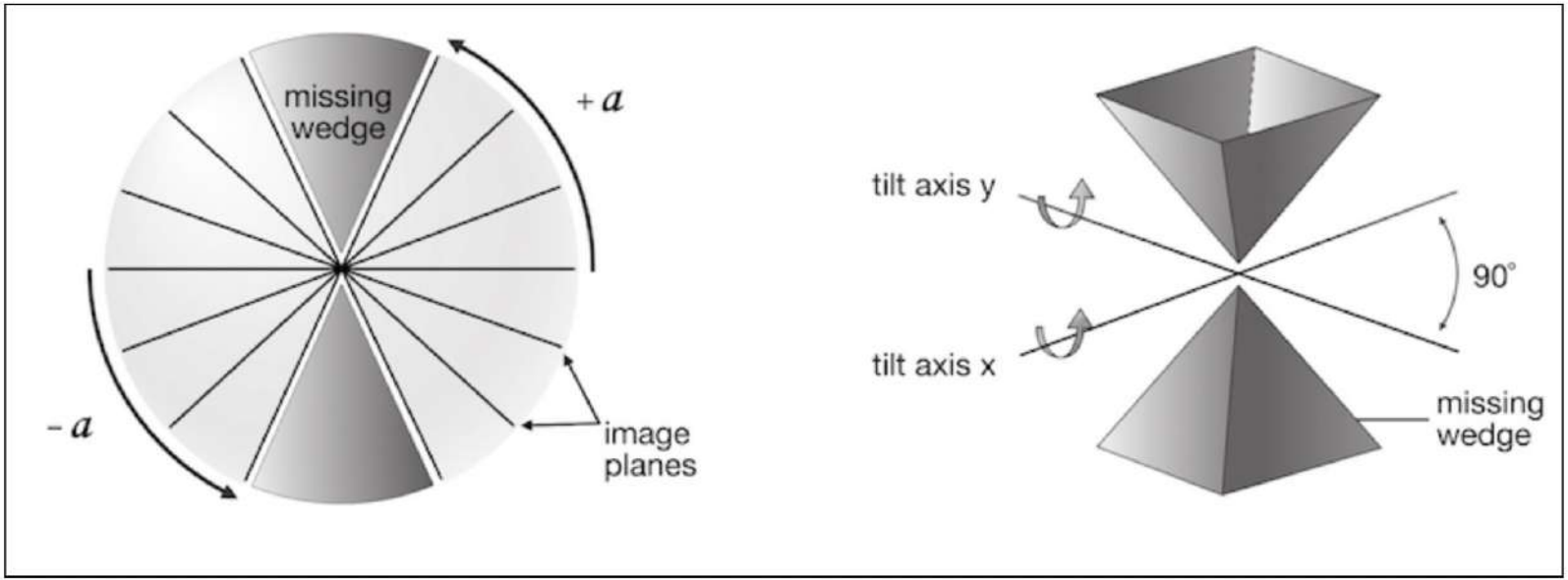
Real space



Fourier space

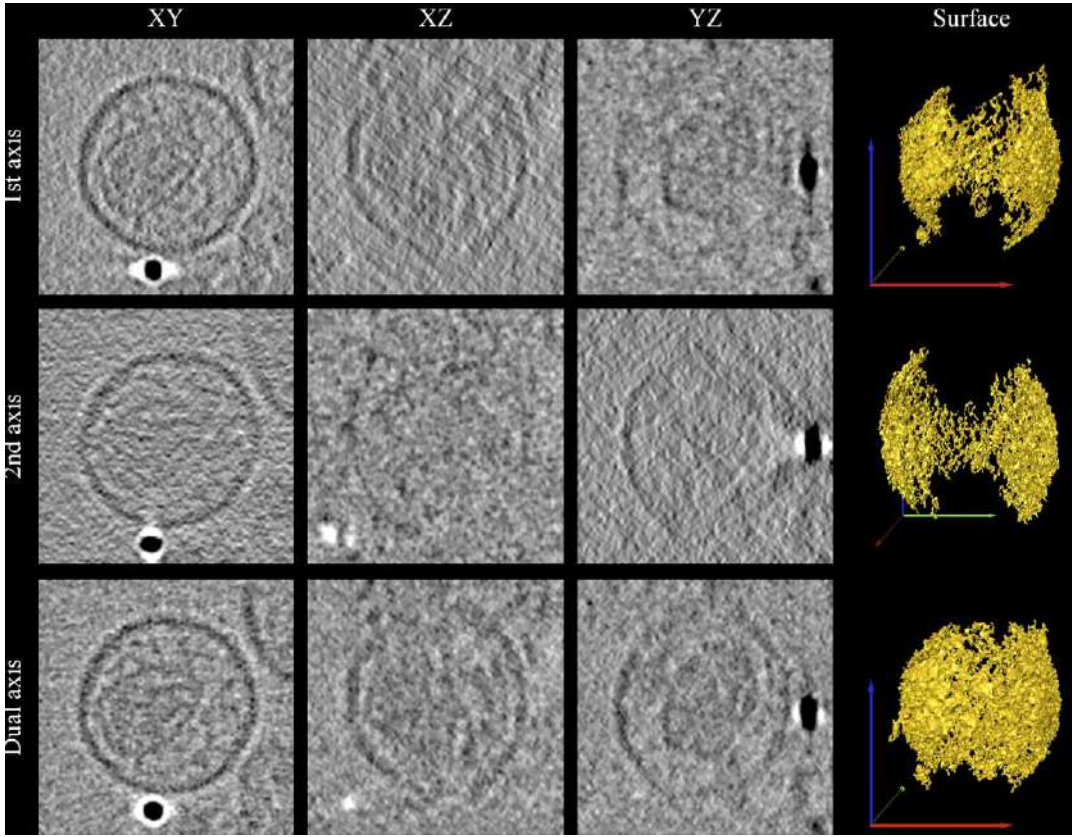
Dual tilt reduces the missing wedge

A holder that “flips” in the microscope so x-tilt will image a second axis



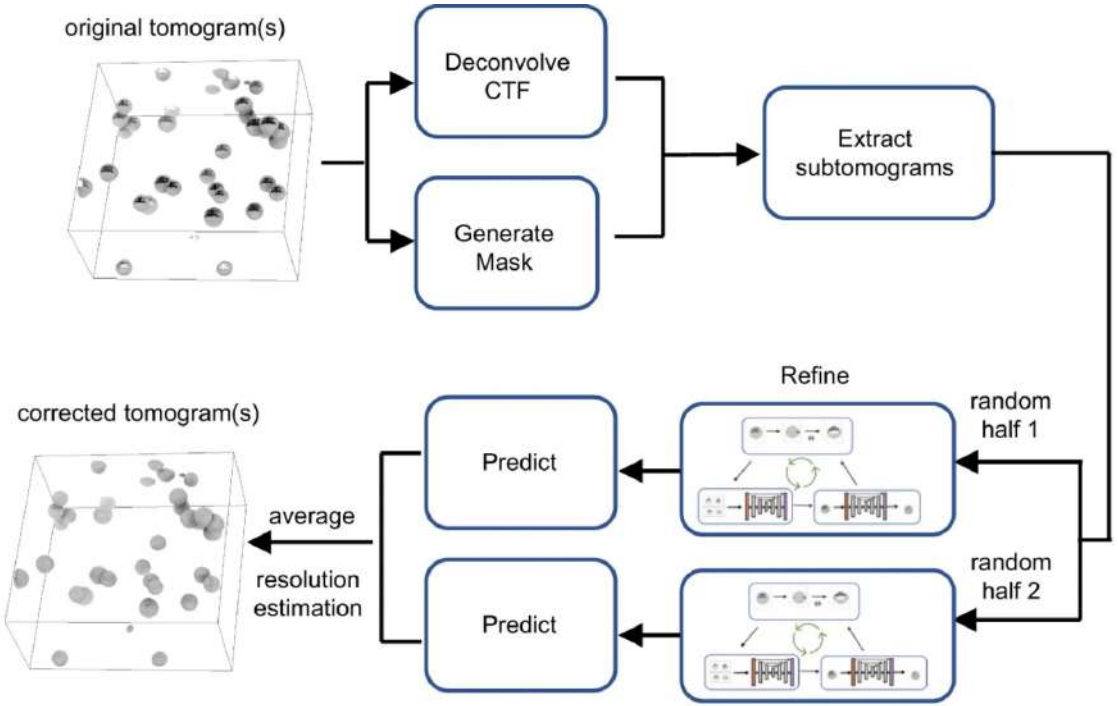
https://www.wormatlas.org/EMmethods/ETmethods.htm

Dual tilt reduces the missing wedge



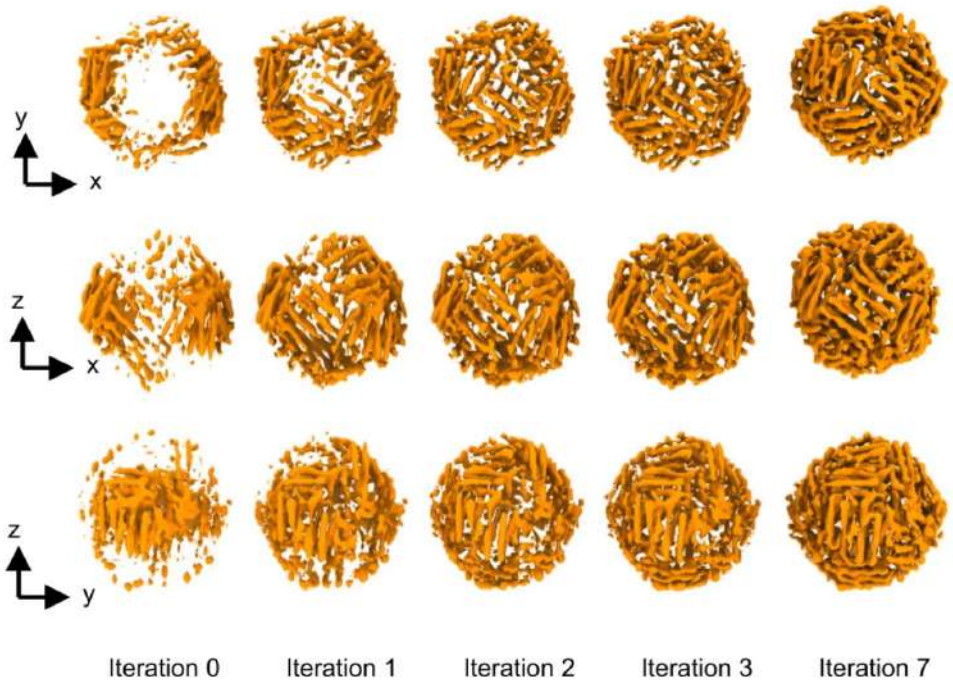
Iancu, Wright &al. *JSB* 2005

Deep-learning based approach to reconstruct the missing wedge



Y. Liu, et al, 2022 Nature Communications. 13, 6482

Deep-learning based approach to reconstruct the missing wedge

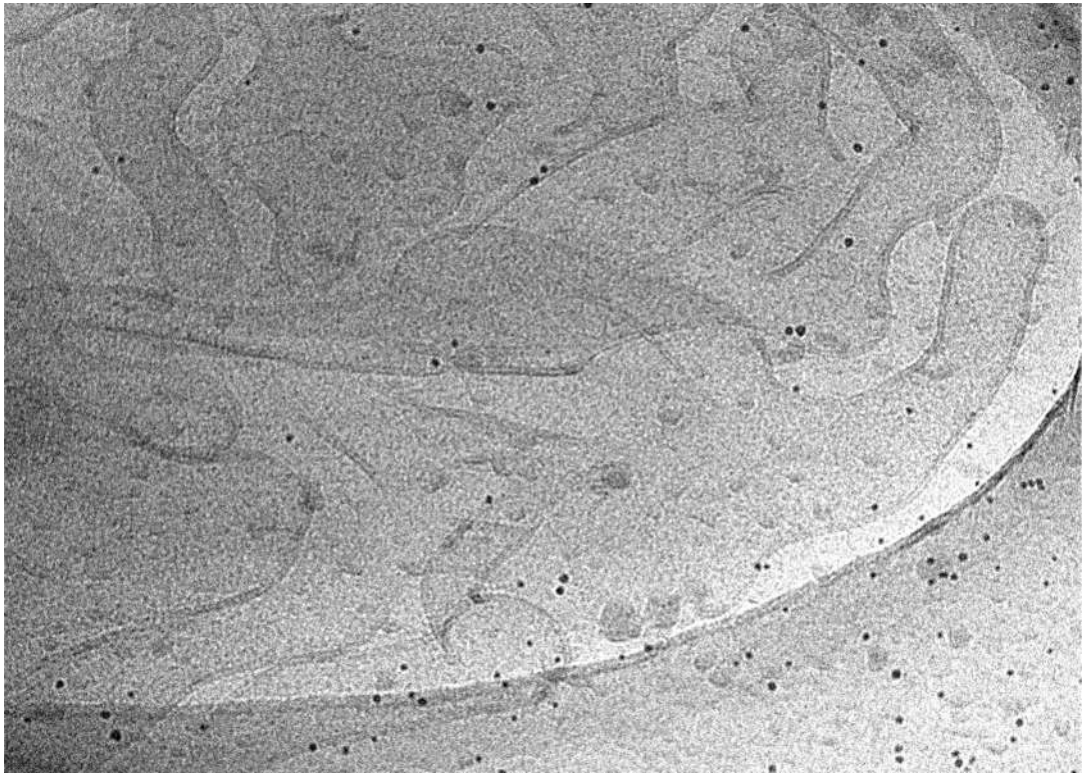


Y. Liu, et al, 2022 Nature Communications. 13, 6482

- Data Processing

Data alignment and reconstruction

- Each image in a tilt series has to be “aligned”
  - x, y shift
  - rotation (position of tilt axis)
  - tilt angle
  - defocus



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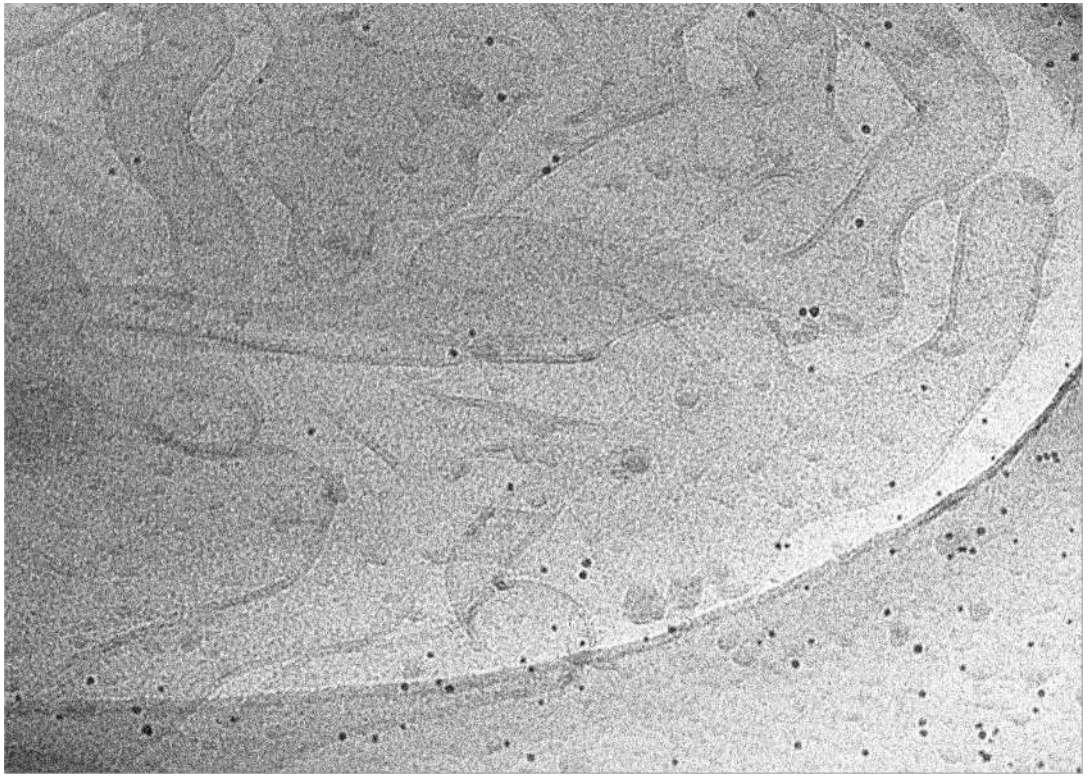

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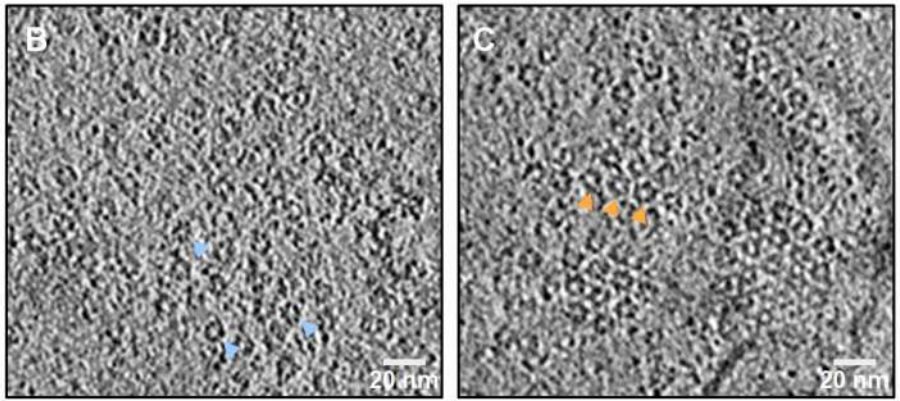
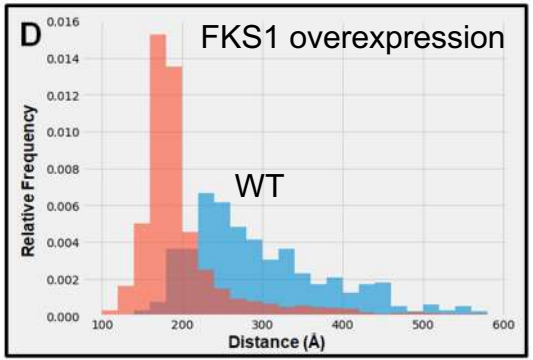
Data alignment and reconstruction

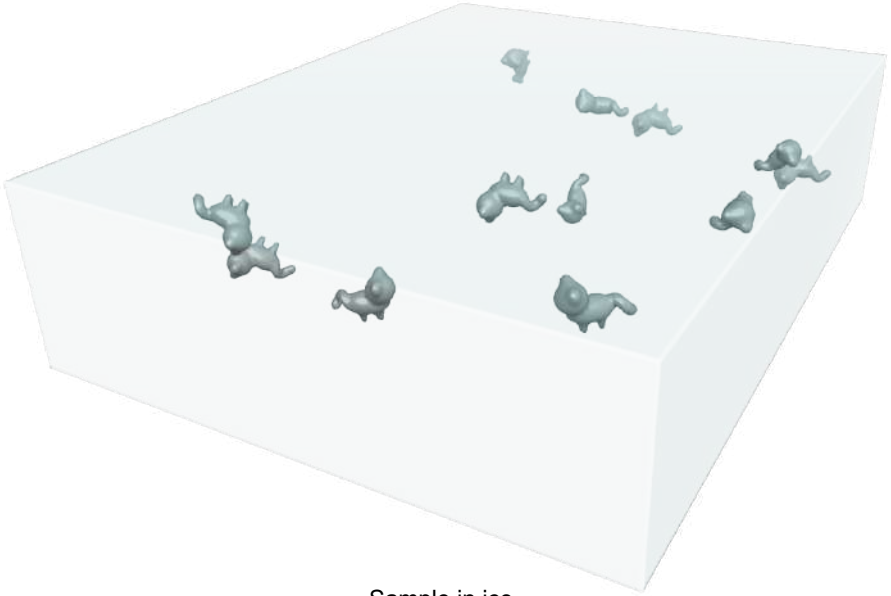
Data visualization, analysis and subtomogram averaging



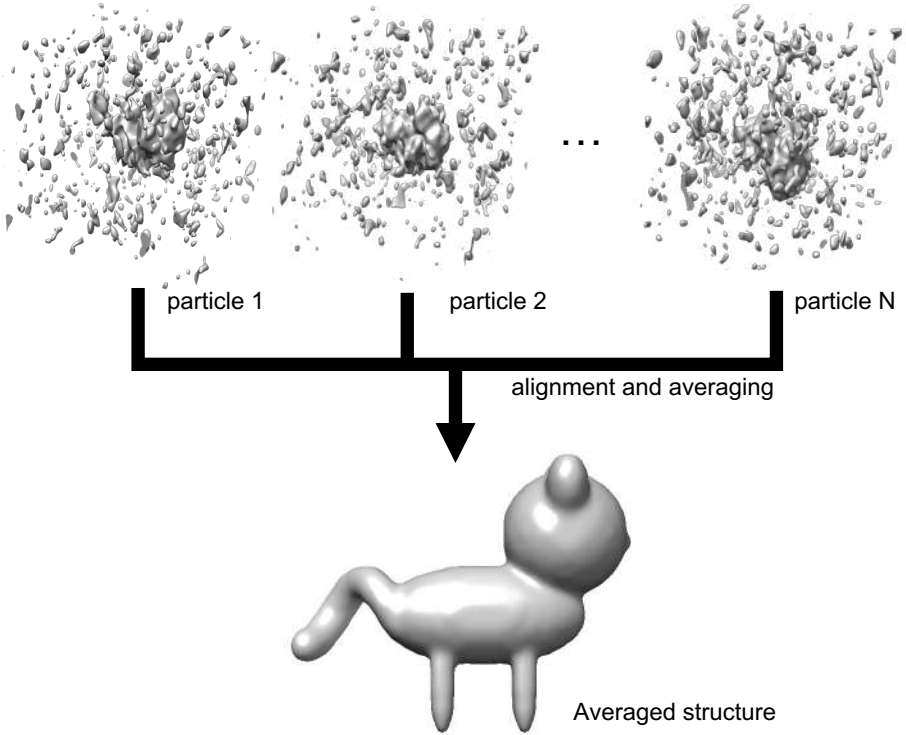
WT *FKS1* overexpression



Subtomogram averaging

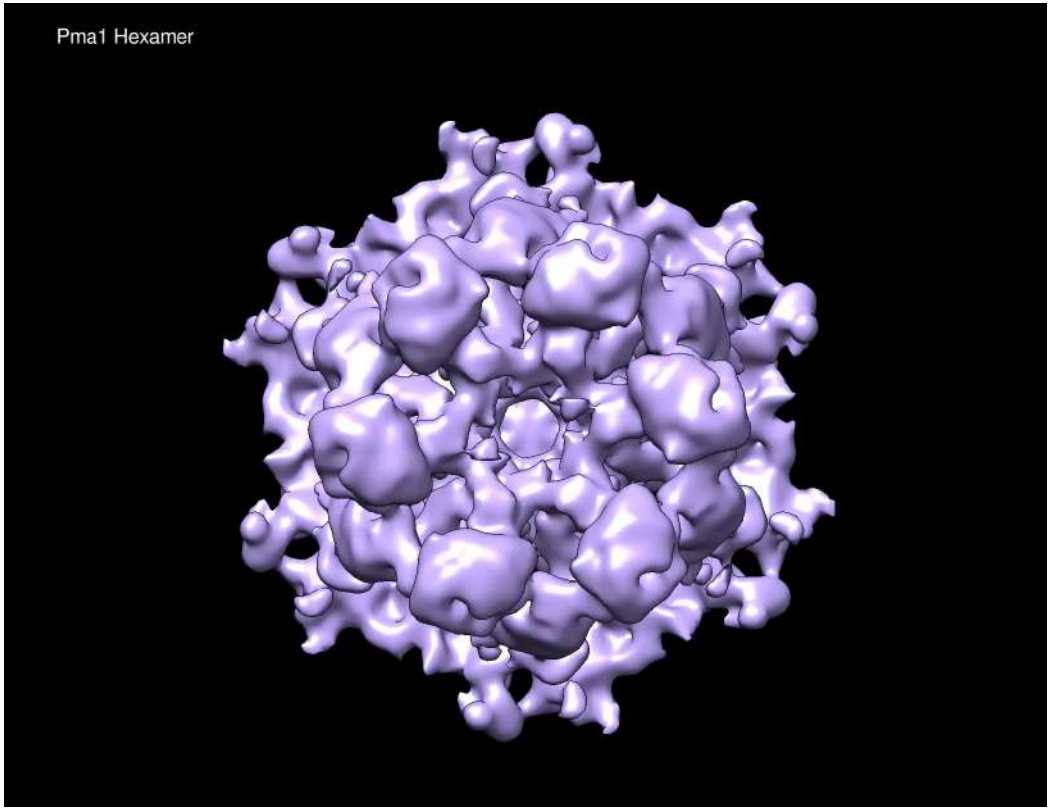


Sample in ice

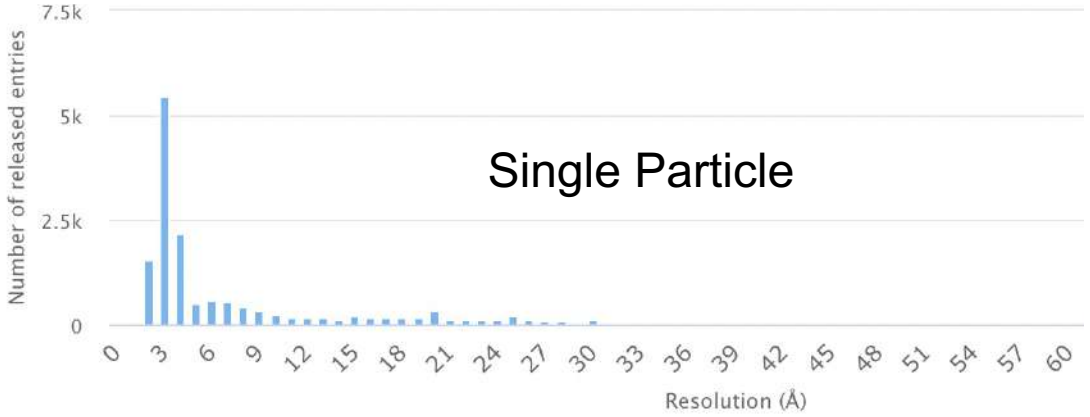


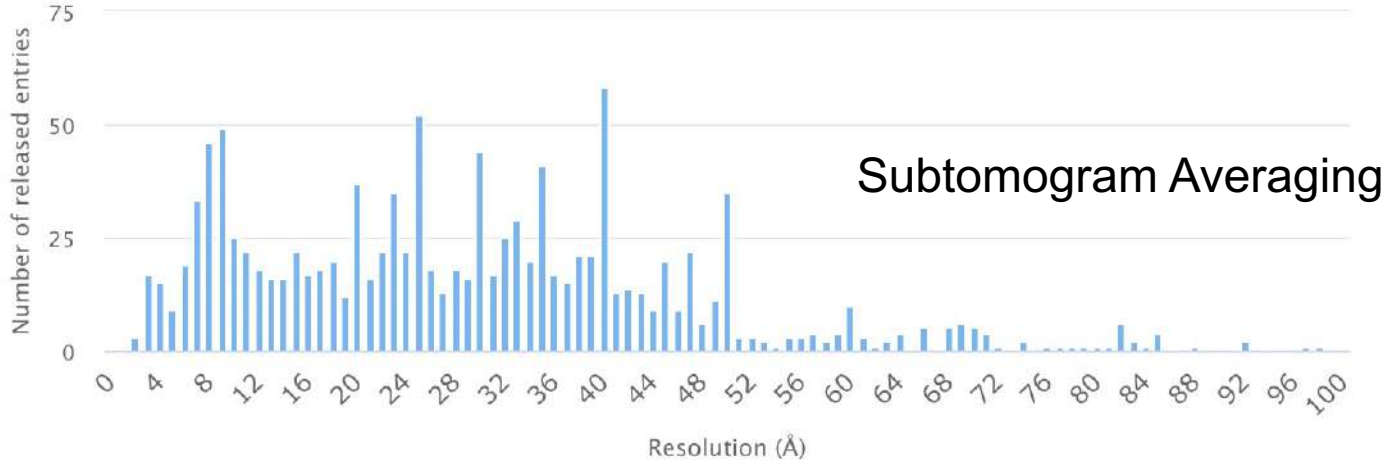
Averaged structure

Data visualization, analysis and subtomogram averaging



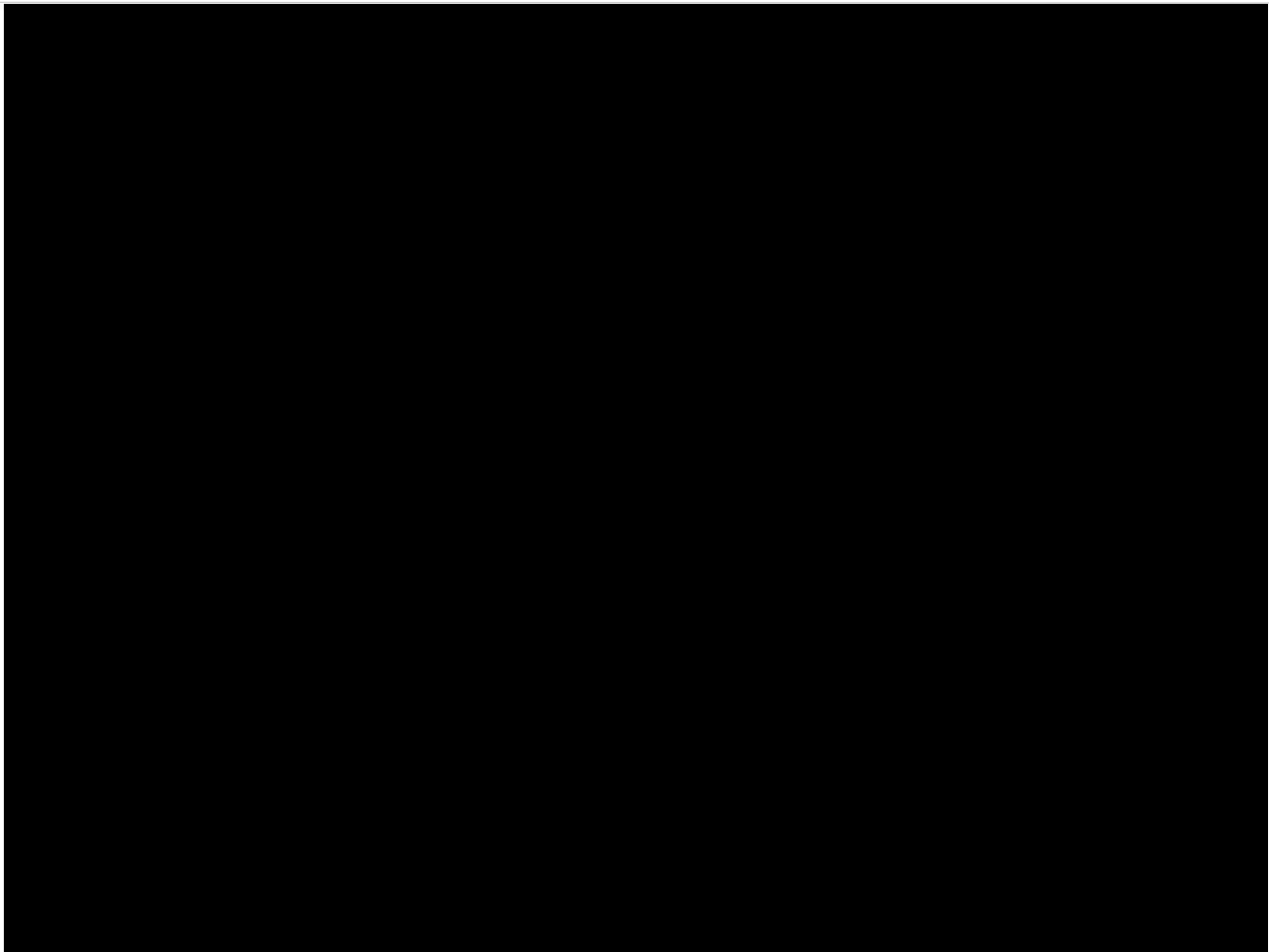
Factors limiting subtomogram average resolution?





Annotation and Visualization

- How is “volume rendering” different than showing an “isosurface” or single slice?
  - Volume rendering: see through the 3D object, all voxels.
  - Isosurface: one surface based on a threshold.

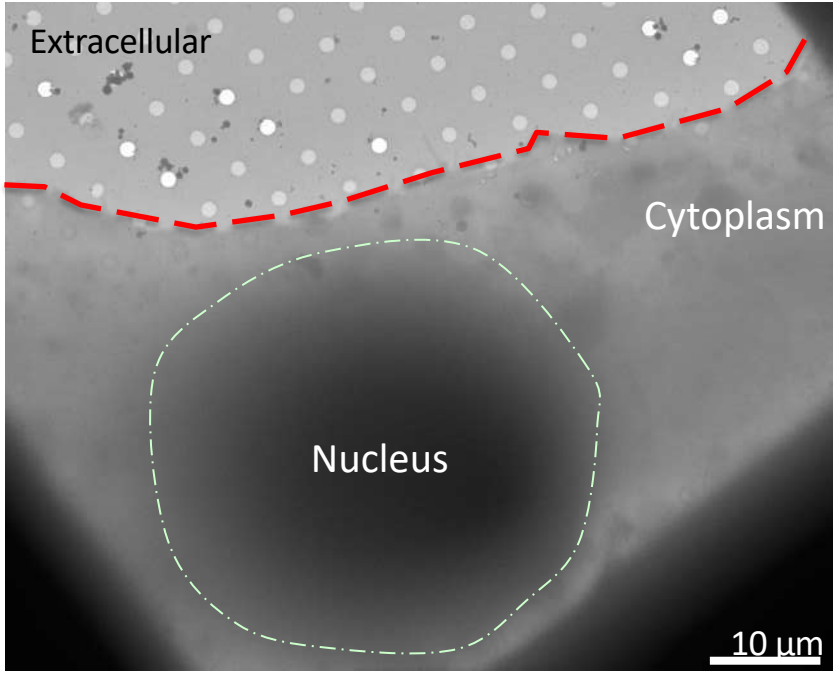


Outline

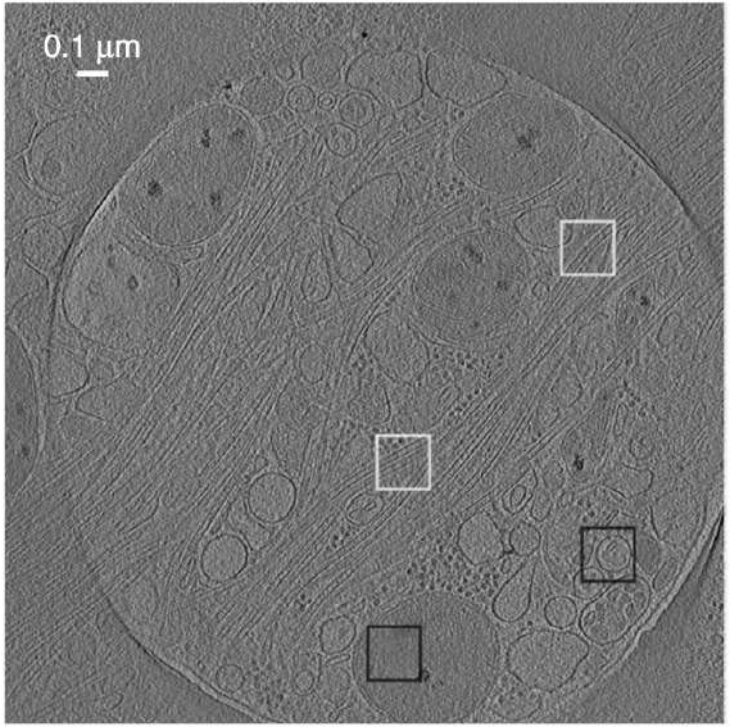
- What is cryo-electron tomography, and how is it different from cryoEM single particle analysis?
- Sample preparation: special considerations
- Data collection, alignment, and reconstruction
- Application of cryo-electron tomography in cell biology (structural cell biology)

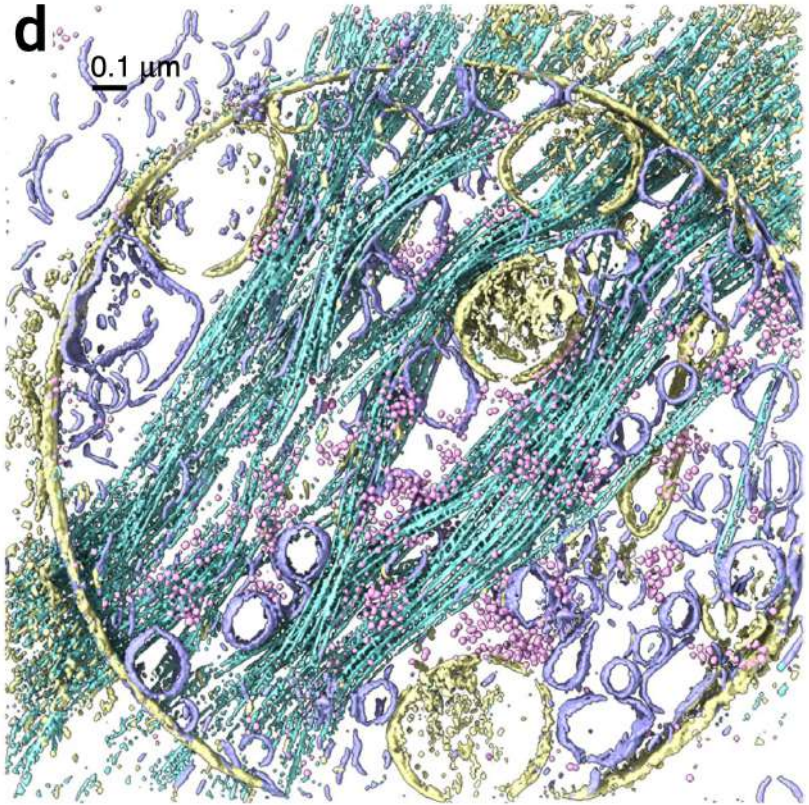
Cellular Tomography:

- Cytoplasm: too thick for electrons to penetrate – Focused ion beam milling (FIB)
- How to find your targets within a crowded cell

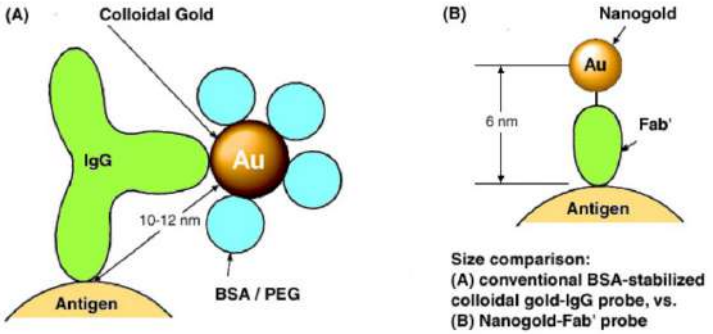


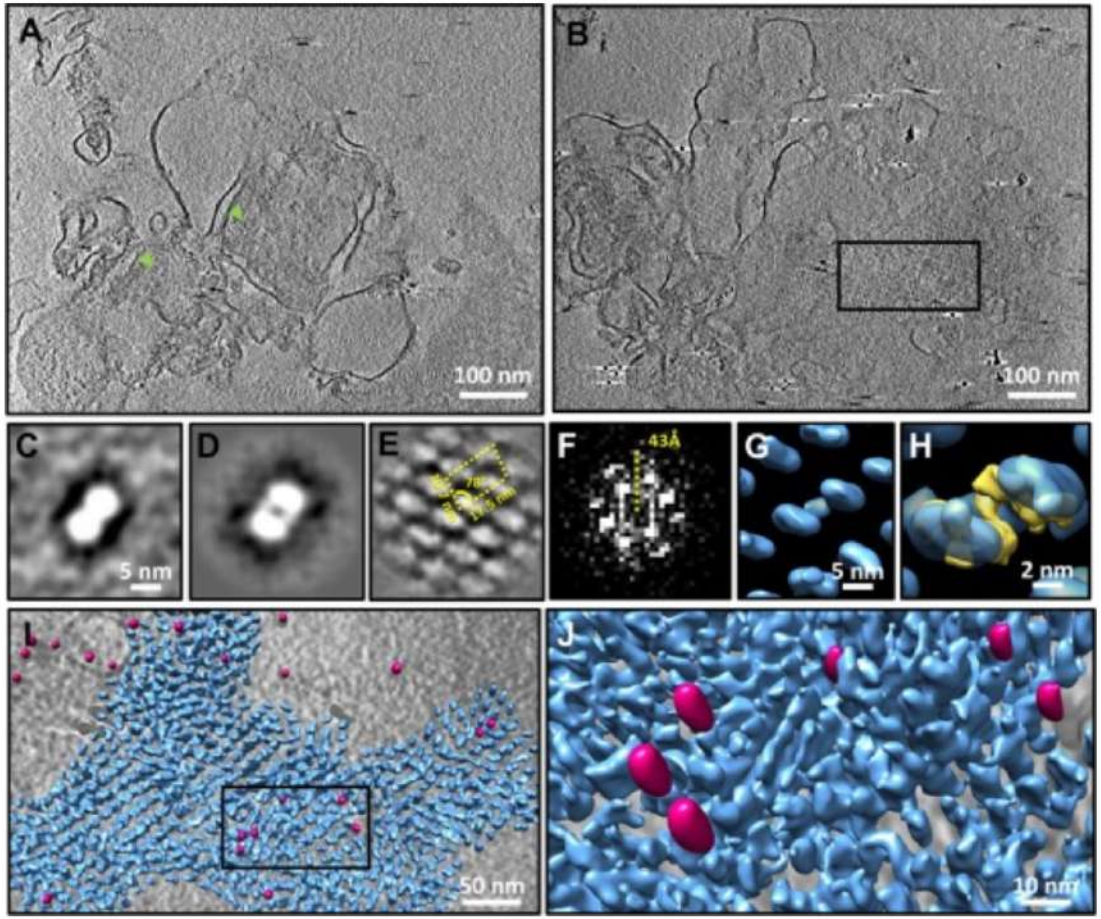
Structure characteristics





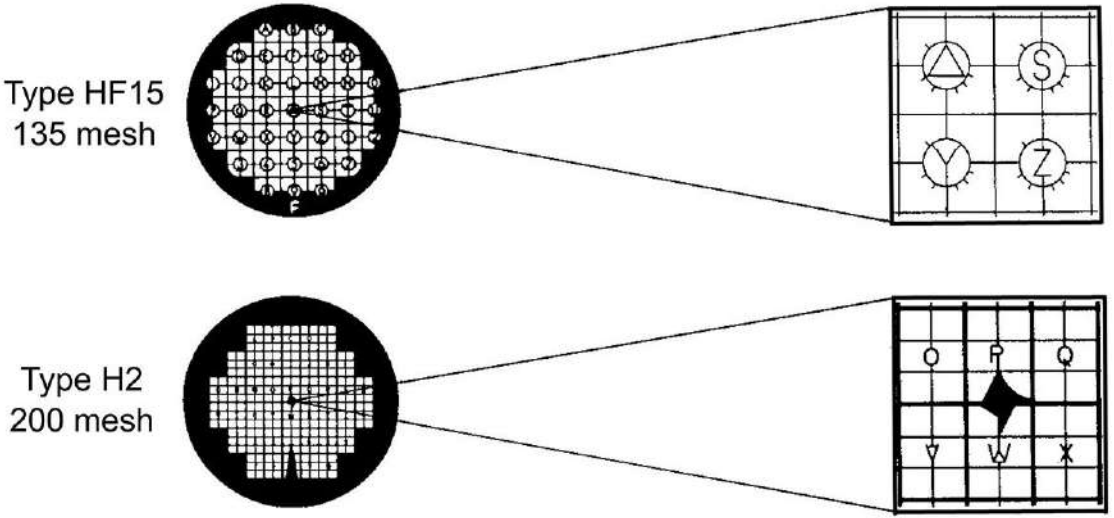
Immunolabeling





Cryo-correlative light and Electron Microscopy (Cryo-CLEM)

- Targets are fluorescence-labeled
- Use special finder grids



CLEM: Bridging Fluorescence (Dynamics) & Electron Microscopy (Structure)

- Target proteins are located by light microscopy operated under cryogenic conditions
- The corresponding positions are imaged by cryo-ET

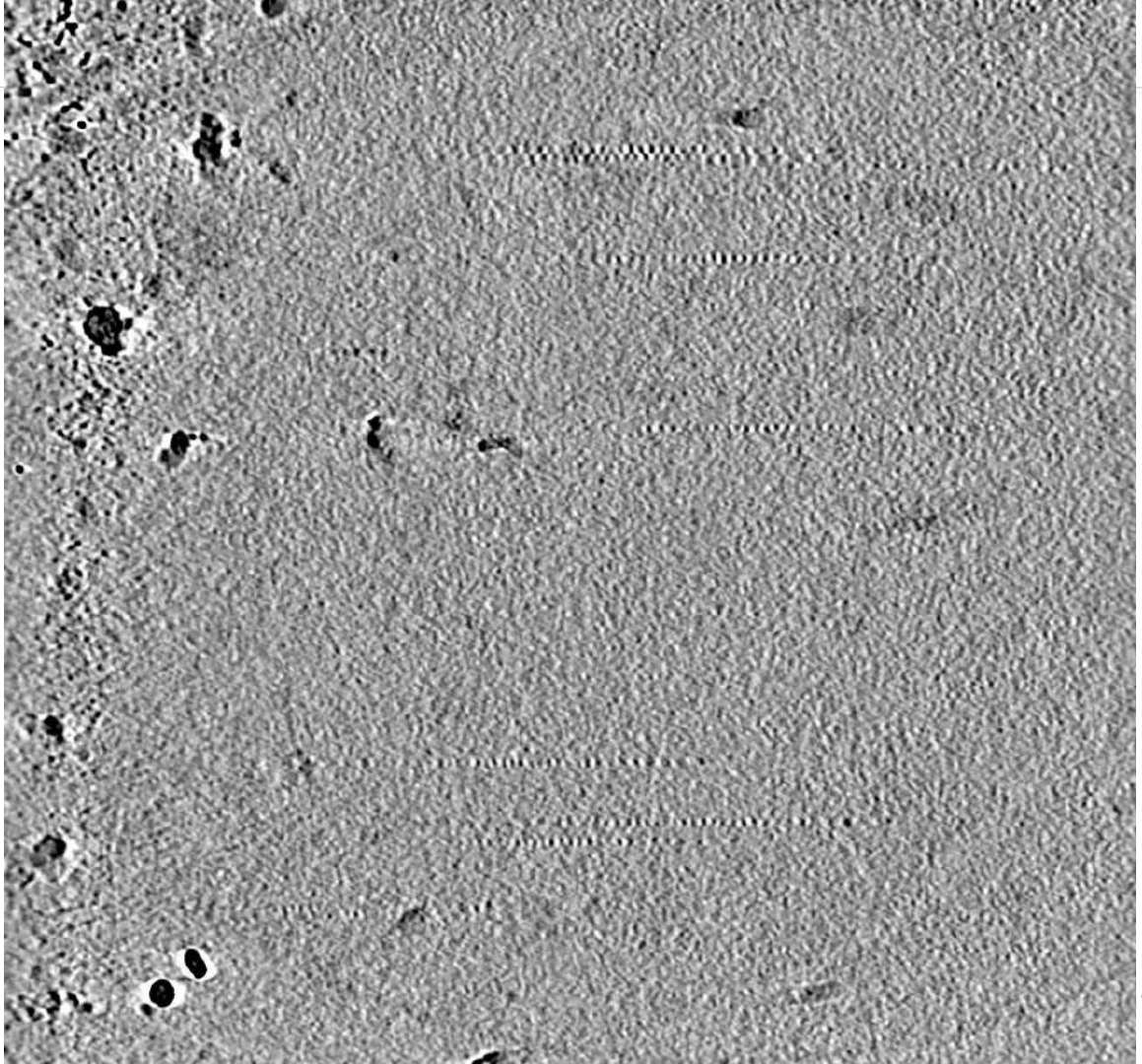
Targeting mHTT-EGF Inclusions Using CLEM

Zooming in on mHTT Inclusions by Cryo-ET

AI-based automated annotation

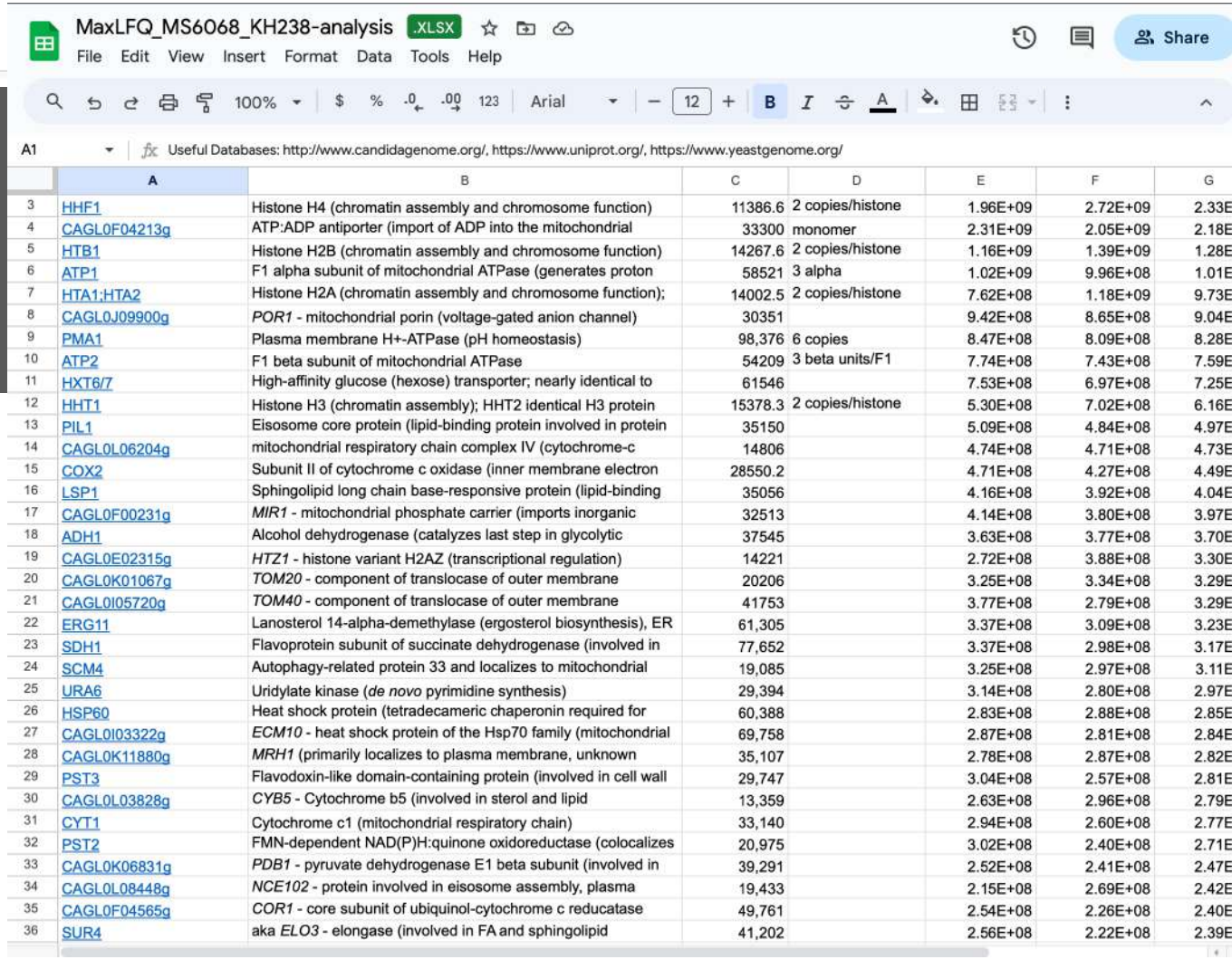
RUTGERS

Tomograms of *C. glabrata* Plasma Membrane



RUTGERS

Detectable Protein Complexes on Fungal Plasma Membrane



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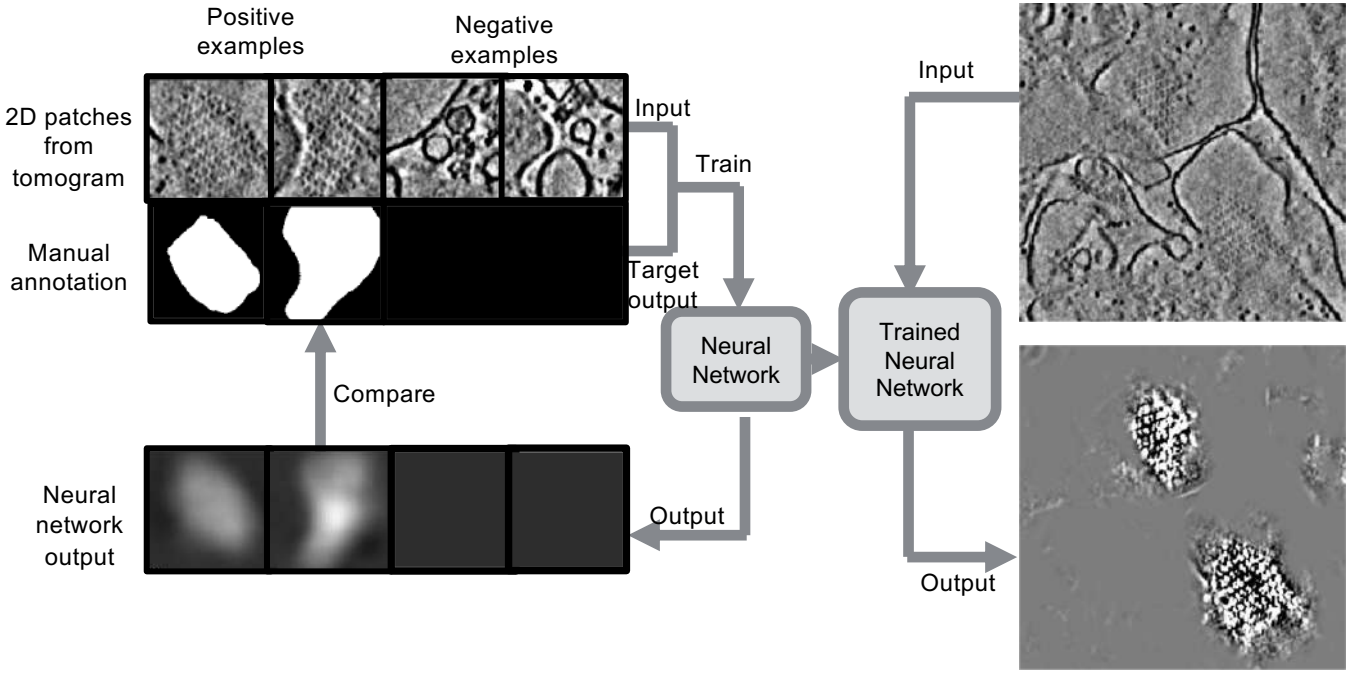
A1 Useful Databases: http://www.candidagenome.org/, https://www.uniprot.org/, https://www.yeastgenome.org/

	A	B	C	D	E	F	G
3	HHF1	Histone H4 (chromatin assembly and chromosome function)	11386.6	2 copies/histone	1.96E+09	2.72E+09	2.33E
4	CAGL0F04213g	ATP:ADP antiporter (import of ADP into the mitochondrial	33300	monomer	2.31E+09	2.05E+09	2.18E
5	HTB1	Histone H2B (chromatin assembly and chromosome function)	14267.6	2 copies/histone	1.16E+09	1.39E+09	1.28E
6	ATP1	F1 alpha subunit of mitochondrial ATPase (generates proton	58521	3 alpha	1.02E+09	9.96E+08	1.01E
7	HTA1;HTA2	Histone H2A (chromatin assembly and chromosome function);	14002.5	2 copies/histone	7.62E+08	1.18E+09	9.73E
8	CAGL0J09900g	*POR1* - mitochondrial porin (voltage-gated anion channel)	30351		9.42E+08	8.65E+08	9.04E
9	PMA1	Plasma membrane H+-ATPase (pH homeostasis)	98,376	6 copies	8.47E+08	8.09E+08	8.28E
10	ATP2	F1 beta subunit of mitochondrial ATPase	54209	3 beta units/F1	7.74E+08	7.43E+08	7.59E
11	HXT6/7	High-affinity glucose (hexose) transporter; nearly identical to	61546		7.53E+08	6.97E+08	7.25E
12	HHT1	Histone H3 (chromatin assembly); HHT2 identical H3 protein	15378.3	2 copies/histone	5.30E+08	7.02E+08	6.16E
13	PIL1	Eisosome core protein (lipid-binding protein involved in protein	35150		5.09E+08	4.84E+08	4.97E
14	CAGL0L06204g	mitochondrial respiratory chain complex IV (cytochrome-c	14806		4.74E+08	4.71E+08	4.73E
15	COX2	Subunit II of cytochrome c oxidase (inner membrane electron	28550.2		4.71E+08	4.27E+08	4.49E
16	LSP1	Sphingolipid long chain base-responsive protein (lipid-binding	35056		4.16E+08	3.92E+08	4.04E
17	CAGL0F00231g	*MIR1* - mitochondrial phosphate carrier (imports inorganic	32513		4.14E+08	3.80E+08	3.97E
18	ADH1	Alcohol dehydrogenase (catalyzes last step in glycolytic	37545		3.63E+08	3.77E+08	3.70E
19	CAGL0E02315g	*HTZ1* - histone variant H2AZ (transcriptional regulation)	14221		2.72E+08	3.88E+08	3.30E
20	CAGL0K01067g	*TOM20* - component of translocase of outer membrane	20206		3.25E+08	3.34E+08	3.29E
21	CAGL0I05720g	*TOM40* - component of translocase of outer membrane	41753		3.77E+08	2.79E+08	3.29E
22	ERG11	Lanosterol 14-alpha-demethylase (ergosterol biosynthesis), ER	61,305		3.37E+08	3.09E+08	3.23E
23	SDH1	Flavoprotein subunit of succinate dehydrogenase (involved in	77,652		3.37E+08	2.98E+08	3.17E
24	SCM4	Autophagy-related protein 33 and localizes to mitochondrial	19,085		3.25E+08	2.97E+08	3.11E
25	URA6	Uridylate kinase (*de novo* pyrimidine synthesis)	29,394		3.14E+08	2.80E+08	2.97E
26	HSP60	Heat shock protein (tetradecameric chaperonin required for	60,388		2.83E+08	2.88E+08	2.85E
27	CAGL0I03322g	*ECM10* - heat shock protein of the Hsp70 family (mitochondrial	69,758		2.87E+08	2.81E+08	2.84E
28	CAGL0K11880g	*MRH1* (primarily localizes to plasma membrane, unknown	35,107		2.78E+08	2.87E+08	2.82E
29	PST3	Flavodoxin-like domain-containing protein (involved in cell wall	29,747		3.04E+08	2.57E+08	2.81E
30	CAGL0L03828g	*CYB5* - Cytochrome b5 (involved in sterol and lipid	13,359		2.63E+08	2.96E+08	2.79E
31	CYT1	Cytochrome c1 (mitochondrial respiratory chain)	33,140		2.94E+08	2.60E+08	2.77E
32	PST2	FMN-dependent NAD(P)H:quinone oxidoreductase (colocalizes	20,975		3.02E+08	2.40E+08	2.71E
33	CAGL0K06831g	*PDB1* - pyruvate dehydrogenase E1 beta subunit (involved in	39,291		2.52E+08	2.41E+08	2.47E
34	CAGL0L08448g	*NCE102* - protein involved in eisosome assembly, plasma	19,433		2.15E+08	2.69E+08	2.42E
35	CAGL0F04565g	*COR1* - core subunit of ubiquinol-cytochrome c reducatase	49,761		2.54E+08	2.26E+08	2.40E
36	SUR4	aka *ELO3* - elongase (involved in FA and sphingolipid	41,202		2.56E+08	2.22E+08	2.39E

Detectable Protein Complexes on Fungal Plasma Membrane

Protein	Relative Abundance
H^+-ATPase (MCP landmark protein)	8.47E+08
Glucose transporter	7.53E+08
Lipid binding protein	4.16E+08
Glucan synthase	5.64E+07
ABC multidrug transporter	5.27E+07

Automated Annotation by Convolutional Neural Network



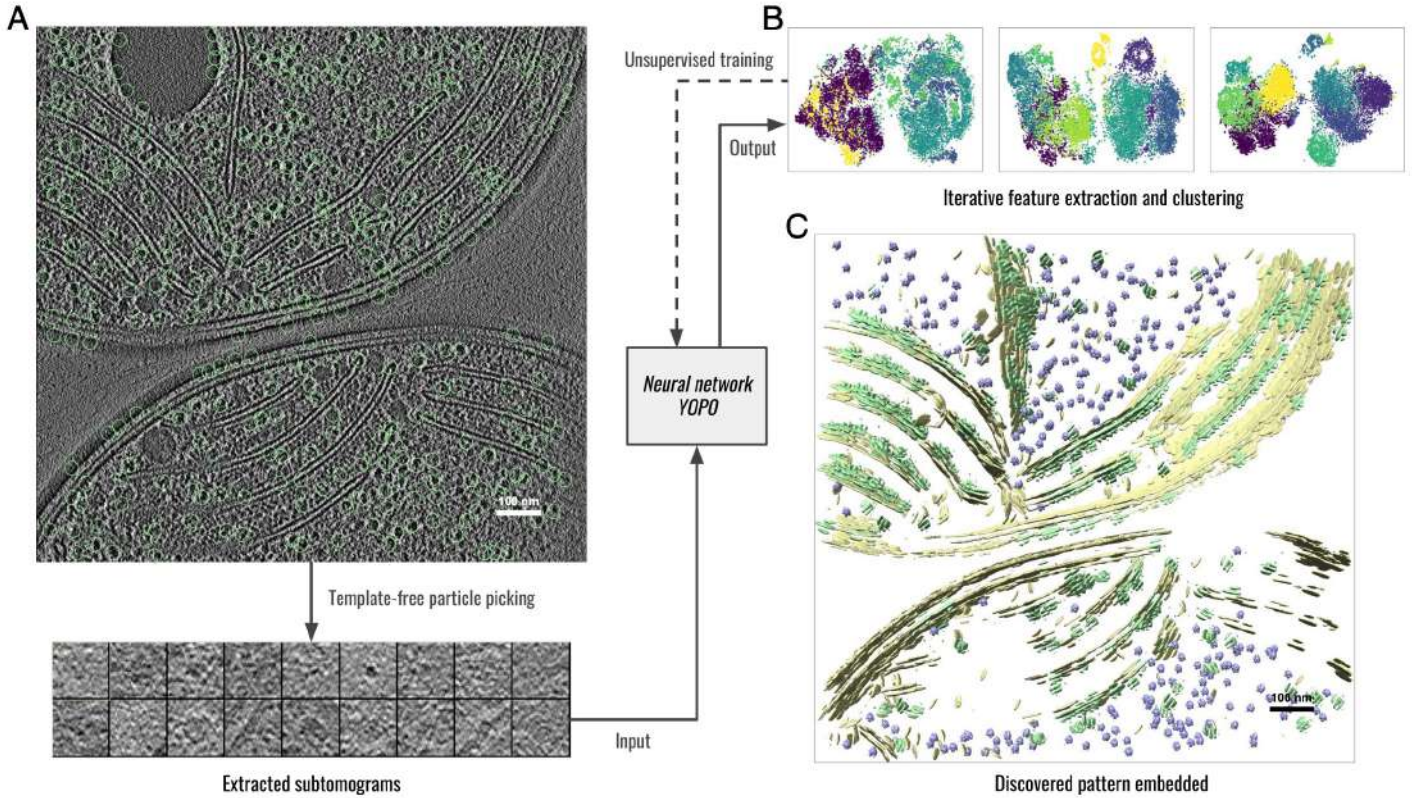
- Easily trained with a small number of regions (~10 positive and ~100 negative samples)
- Duplicate training samples by random rotation
- One network per feature

Courtesy of Dr. Muyuan Chen

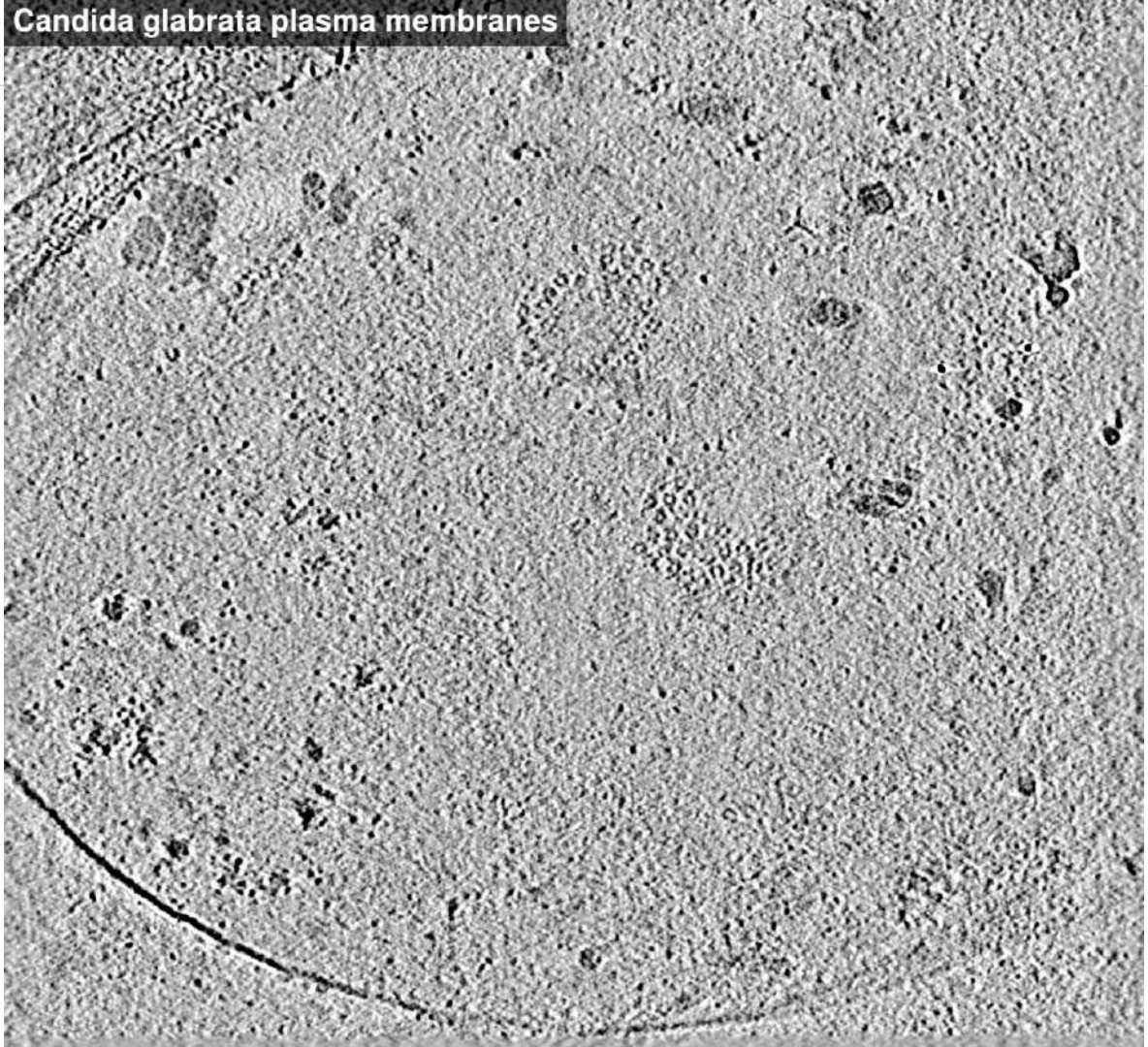
Training of neural network

Annotation by applying trained network

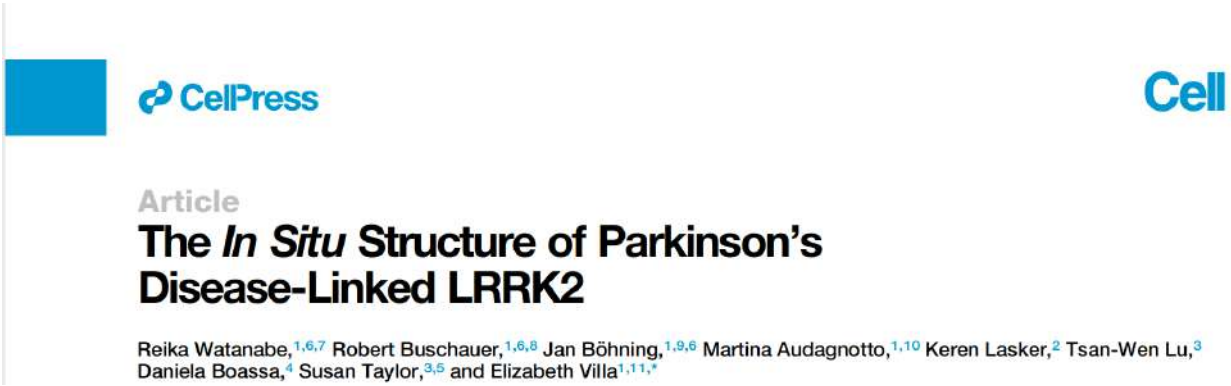
Unsupervised, Deep-learning Based Annotation -DISCA



Zeng *et al*. (2023) *PNAS*


Candida glabrata plasma membranes

Applying Cryo-ET to Reveal Protein Structure *in situ* – The Workflow



CellPress

Cell

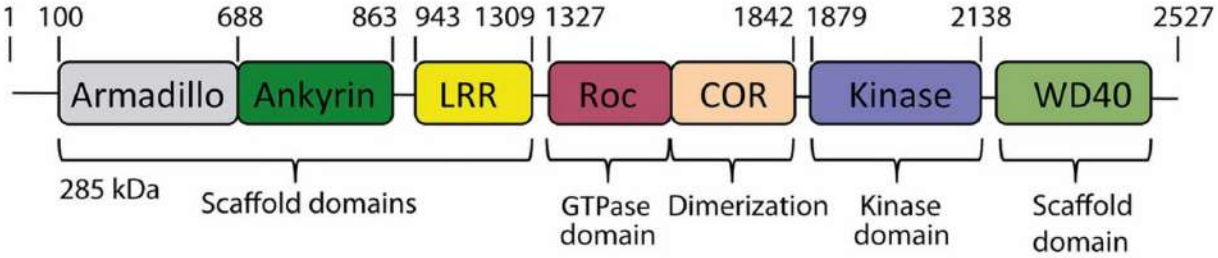
Article

The *In Situ* Structure of Parkinson's Disease-Linked LRRK2

Reika Watanabe,[1,6,7] Robert Buschauer,[1,6,8] Jan Böhning,[1,9,6] Martina Audagnotto,[1,10] Keren Lasker,[2] Tsan-Wen Lu,[3] Daniela Boassa,[4] Susan Taylor,[3,5] and Elizabeth Villa[1,11,*]

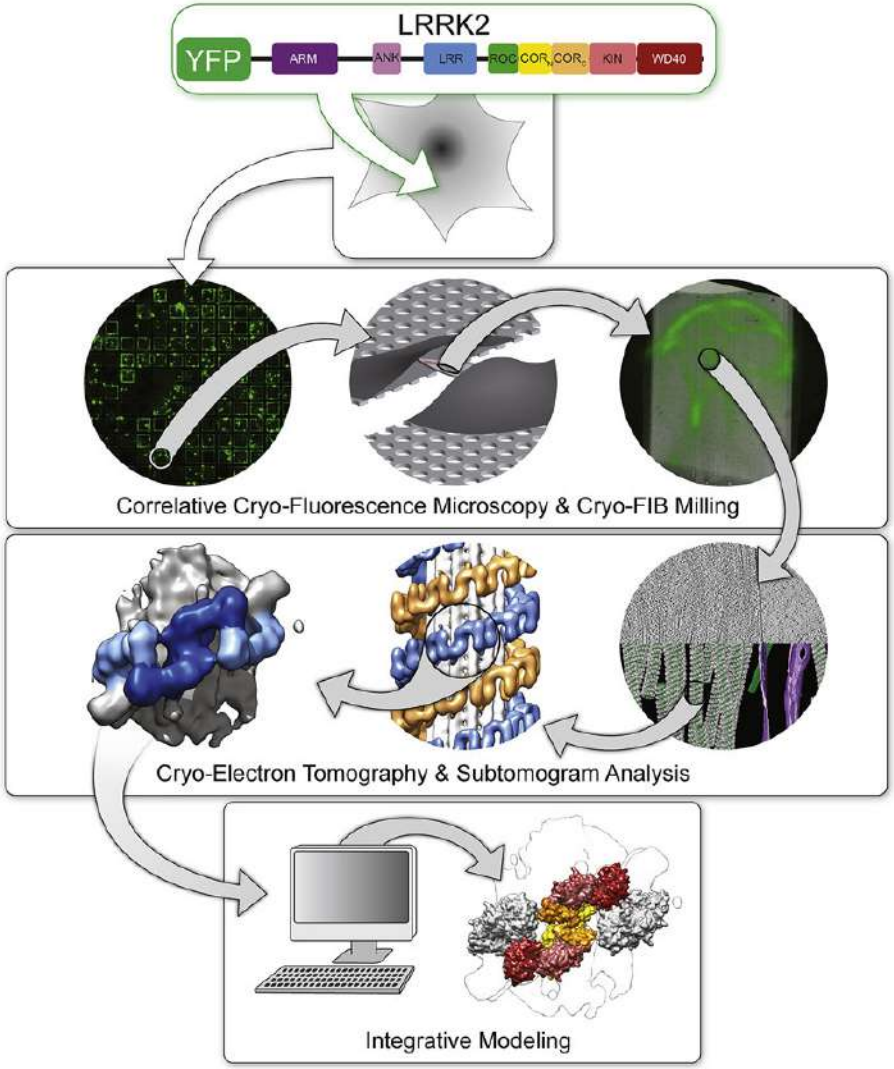
Structure of LRRK2

- LRRK2: (Leucine-rich repeat kinase 2) the most mutated gene in familial Parkinson's disease
- Functions in neurite outgrowth, membrane trafficking, autophagy
- Mutations or pharmacological inhibition of kinase activity recruit LRRK2 to microtubules
- Multi-domain protein; structure of the full-length protein is not available.



Guaitoli, G. *et al.*, PNAS 2016

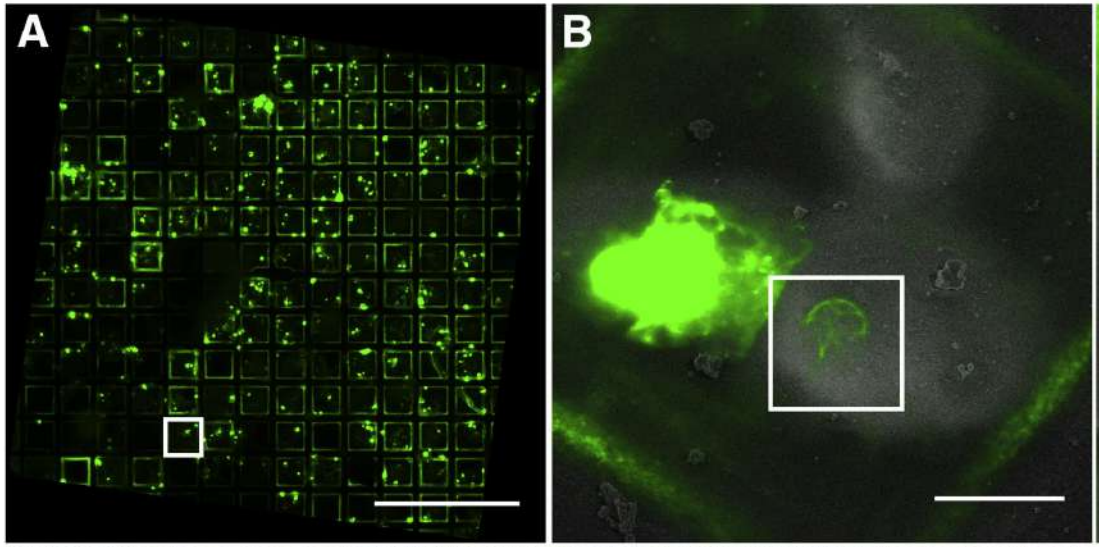
Workflow



Watanabe, R. *et al*., Cell 2020

Step 1: Design and Prepare Cells to Allow Detection of Targets in the Crowded Environment

- Add fluorescence tag; Increasing abundance for easy detection
- Correlative Light and Electron Microscopy (CLEM)



Watanabe, R. *et al*., Cell 2020

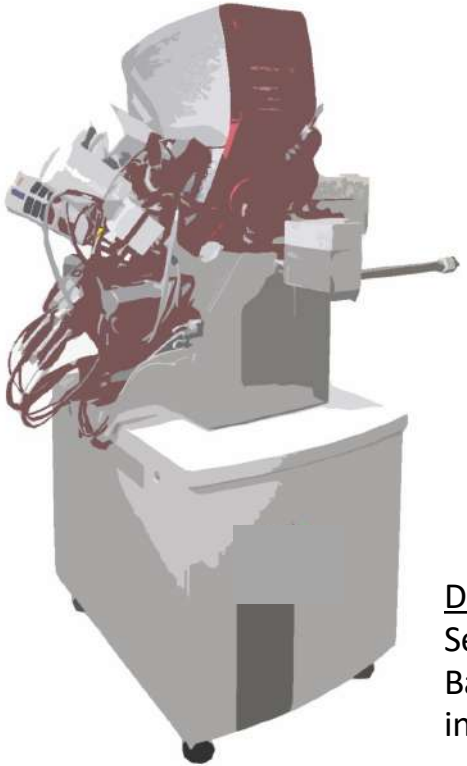
Step 2: Focused Ion Beam Milling to Generate Thin Cell Lamella for Cryo-ET

- Cells on grids: 1 - 5 µm in thickness
- Electron penetration power: 100– 300 nm

Watanabe, R. *et al*., Cell 2020

Focused Ion Beam

SEM column
~1nm resolution
Beam deceleration

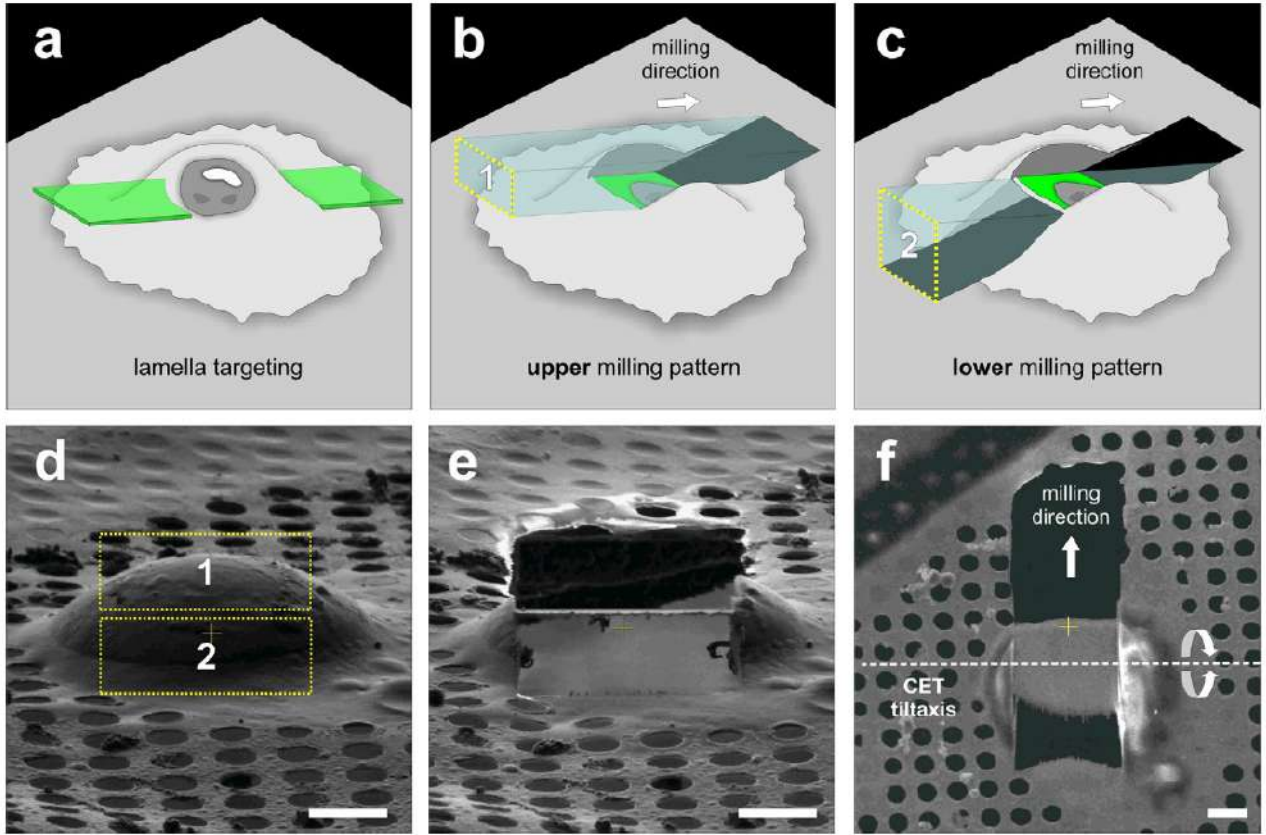


Gallium ion milling capability
20 nm milling precision
Fine milling (<1pA) to preserve specimen and high-current (>100nA) for large areas

Cryostage/cryotransfer
Accommodates autogrid cartridges for integration with cryoCLEM & cryoTEM
Stable operation below the devitrification point of water
Approaches liquid nitrogen temperature
Airlock for loading/unloading under cryo-conditions

Detectors
Secondary electron
Back-scattered electron
incl. in-lens detectors

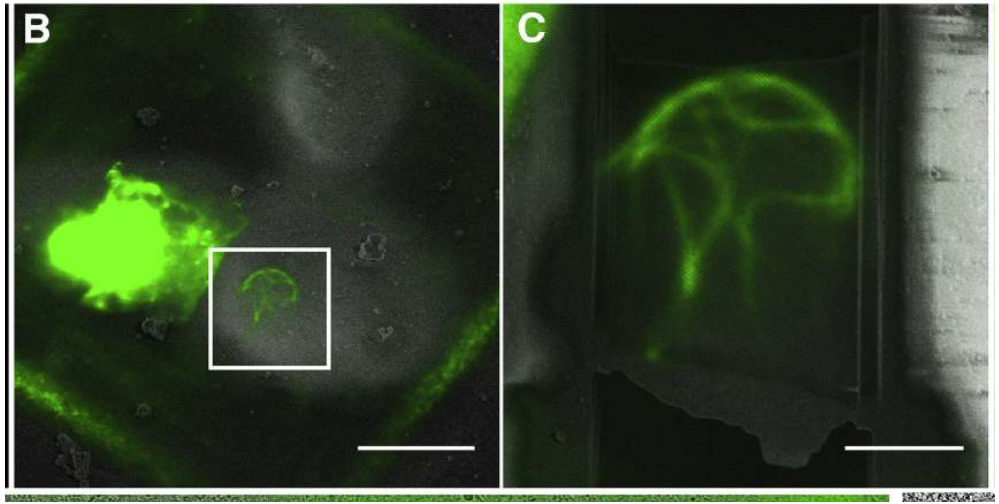
Focused Ion Beam



Rigort and Plitzko, 2015 *Arch Biochem Biophys.* 581: 122-130

Step 2: Focused Ion Beam Milling to Generate Thin Cell Lamella for Cryo-ET

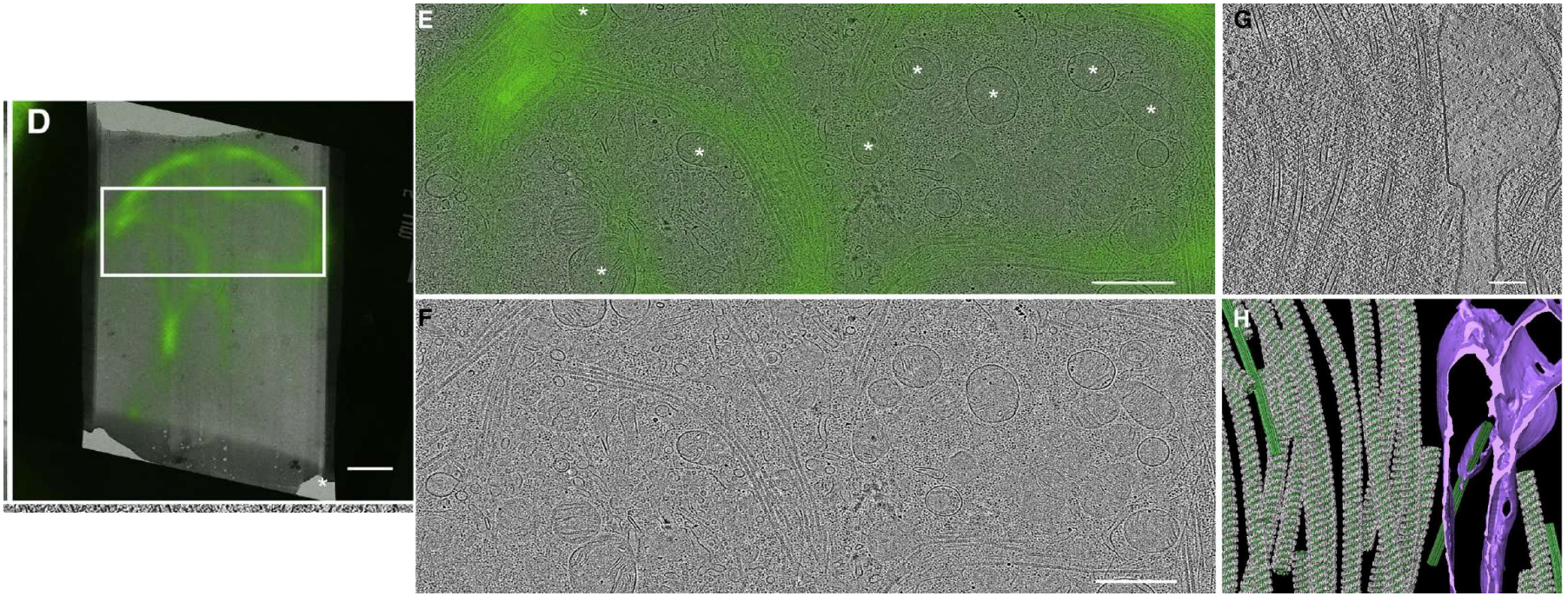
- Cells on grids: 1 - 5 µm
- Lamella: 100– 150 nm



Watanabe, R. *et al*., Cell 2020

Step 3: Cryo-ET Imaging and Tomogram Reconstruction

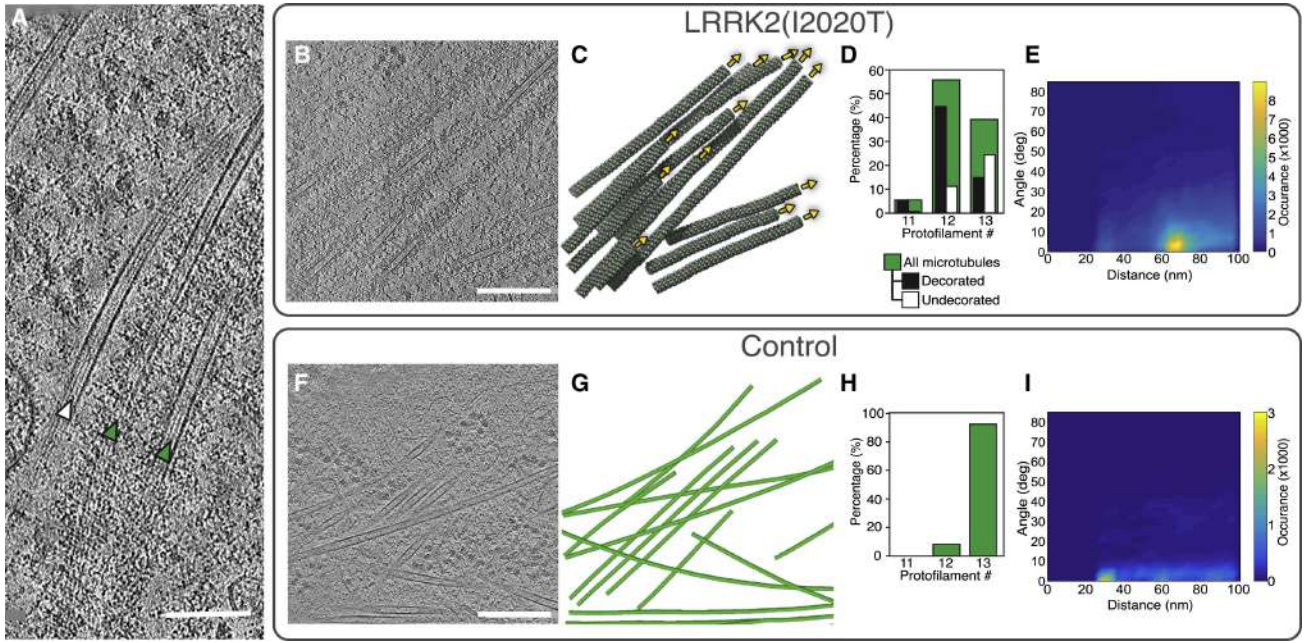
- Use CLEM to guide tilt series data collection



Watanabe, R. *et al*., Cell 2020

Step 4: *In situ* Structure Analysis

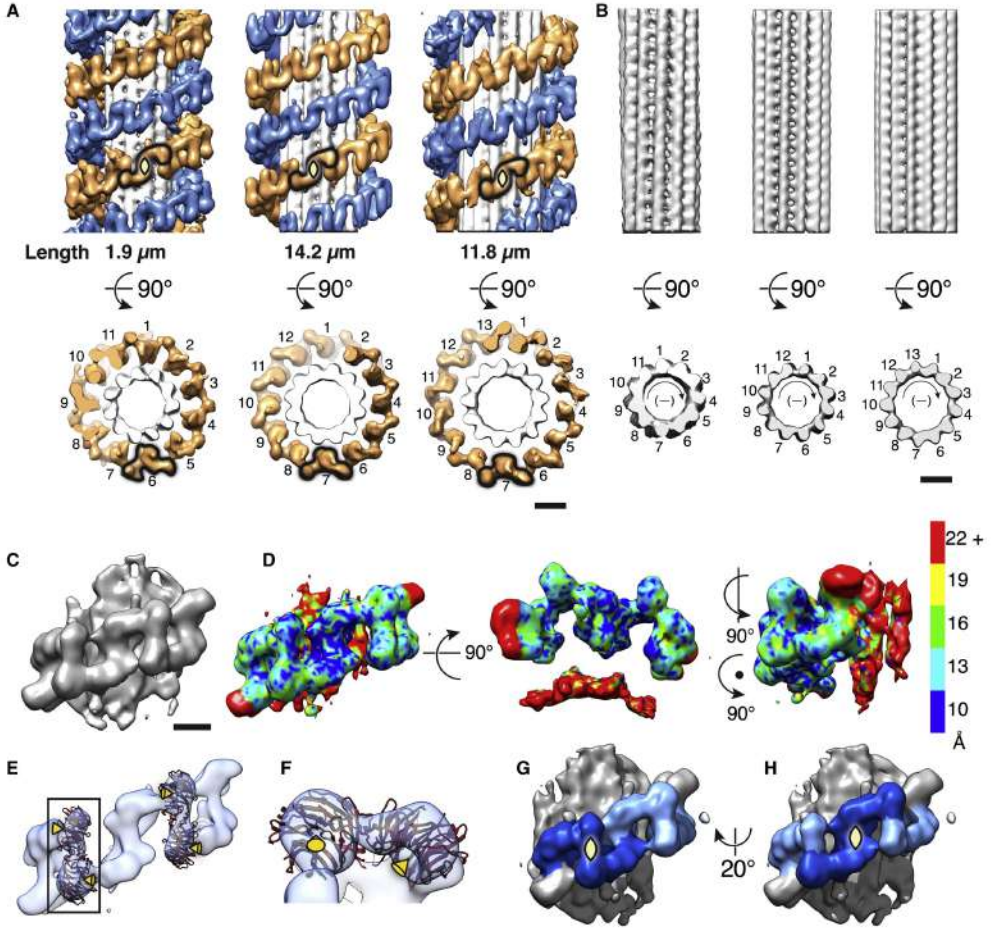
- Distribution and dynamics in cells



Watanabe, R. *et al*., Cell 2020

Step 5: Subtomogram Analysis

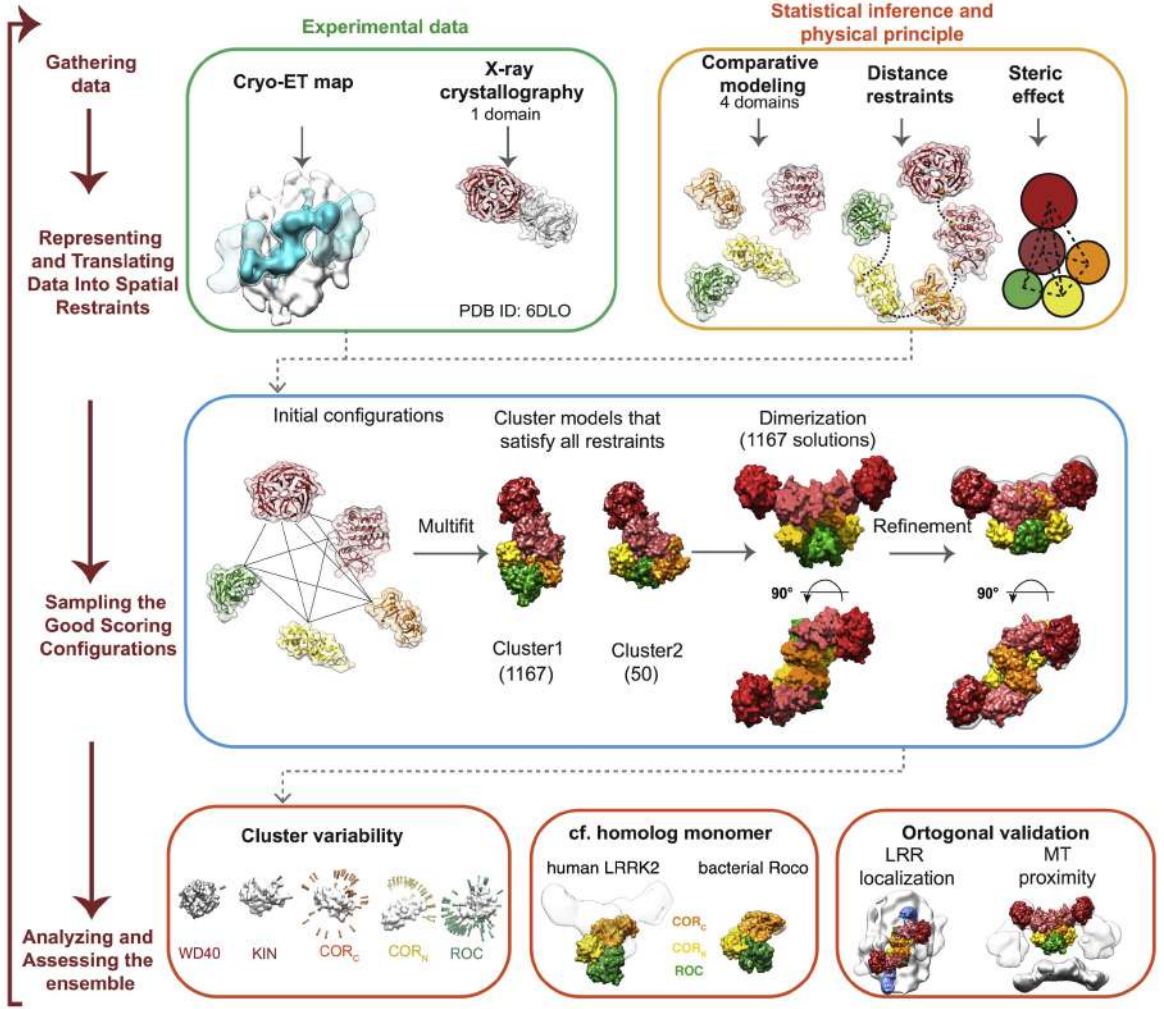
- Extraction
- Classification
- Averaging
- Model fitting



Watanabe, R. *et al*., Cell 2020

Step 6: Integrative Modeling

- Details in domain organization can be deduced from nanometer resolution maps



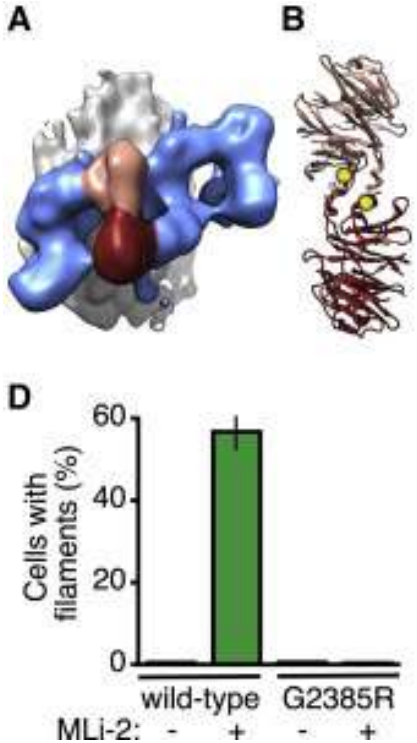
Watanabe, R. *et al*., Cell 2020

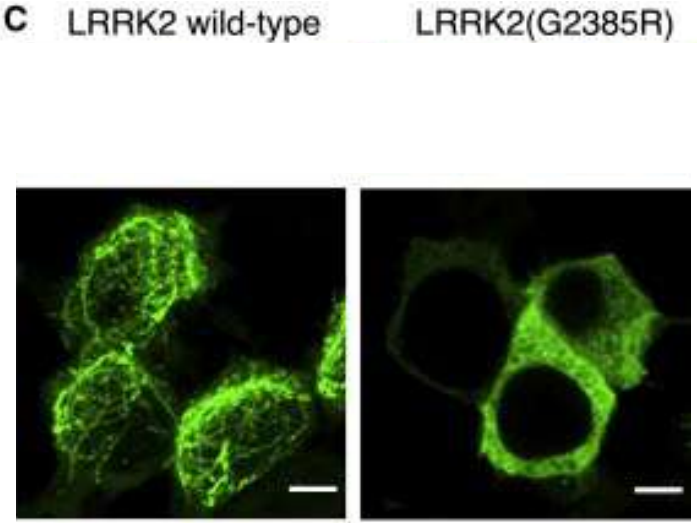
Step 7: Functional Analysis

Disturbing structure

↕

Variations of functions



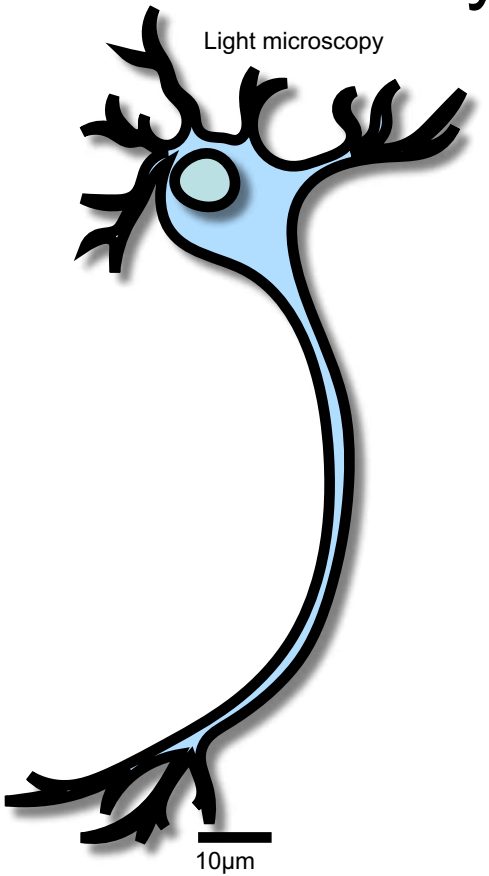


Watanabe, R. *et al*., Cell 2020

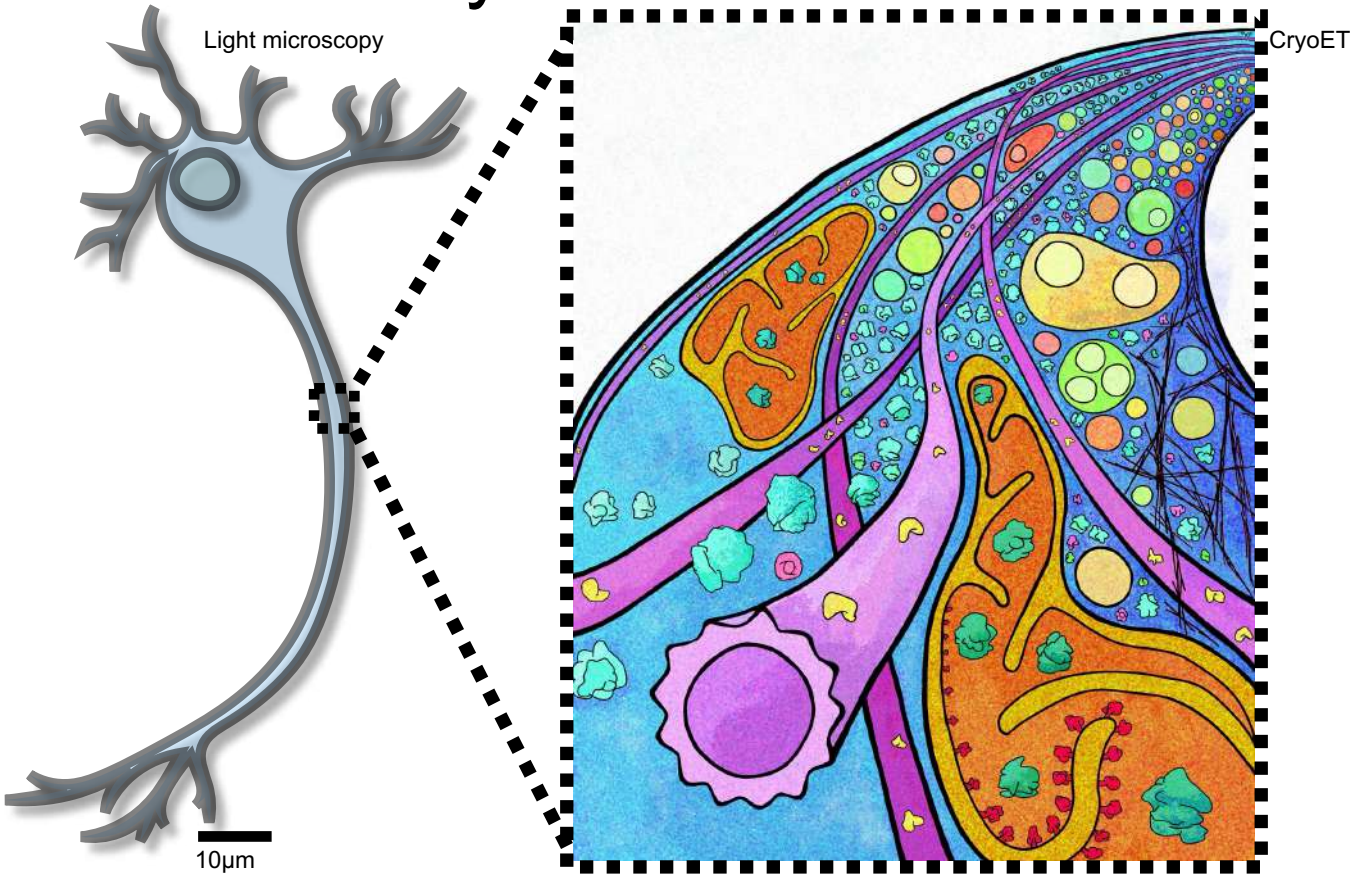
Summary

- What is cryo-electron tomography, what can we learn from tomograms?
- Sample preparation: type of samples and special considerations
- Data collection, alignment, and reconstruction
- Application of cryo-electron tomography in cell biology (structural cell biology)

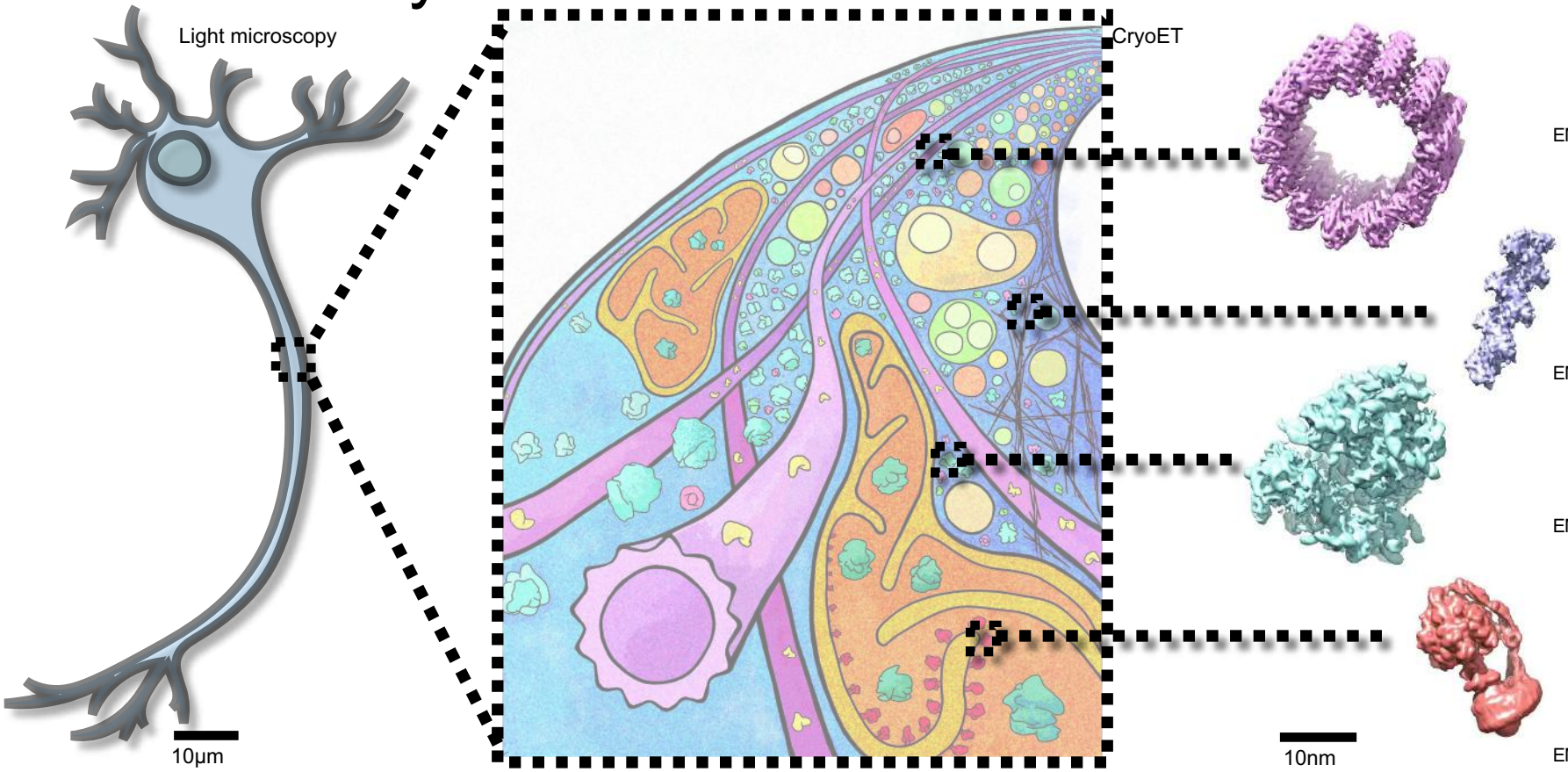
Scale of CryoET Studies



Scale of CryoET Studies



Scale of CryoET Studies



References

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2. Yao, H. *et al.* Molecular Architecture of the SARS-CoV-2 Virus. *Cell* **183**, 730-738 e713, doi:10.1016/j.cell.2020.09.018 (2020).
3. Klein, S. *et al.* SARS-CoV-2 structure and replication characterized by in situ cryo-electron tomography. *Nat Commun* **11**, 5885, doi:10.1038/s41467-020-19619-7 (2020).
4. Deniston, C. K. *et al.* Structure of LRRK2 in Parkinson's disease and model for microtubule interaction. *Nature* **588**, 344-349, doi:10.1038/s41586-020-2673-2 (2020).
5. Watanabe, R. *et al.* The In Situ Structure of Parkinson's Disease-Linked LRRK2. *Cell* **182**, 1508-1518 e1516, doi:10.1016/j.cell.2020.08.004 (2020).