Biological Materials in Transmission Electron Microscopy

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What are we talking about?

1. **Sample Preparation**
   - Considerations for Biological Samples
   - Biological Sample Preparation for Conventional TEM
     - Small Particles
     - Tissues
   - Drawbacks of Conventional TEM

2. **Introduction to CryoTEM**
   - What is CryoTEM?
   - Biological Sample Preparation for CryoTEM
   - CryoTEM Techniques
     - Single-Particle Analysis
     - Cryo-electron Tomography
     - Micro-Electron Diffraction

Considerations for Biological Samples in TEM

Biological Samples

Mostly water

Temperature sensitive

Composed of “light” elements

Star Trek:The Next Generation, “Home Soil” (episode 18)

The Enterprise UT description of human physiology

1. TEM column is under high vacuum.

Water evaporates immediately in a vacuum

2. Electrons are extremely high energy.

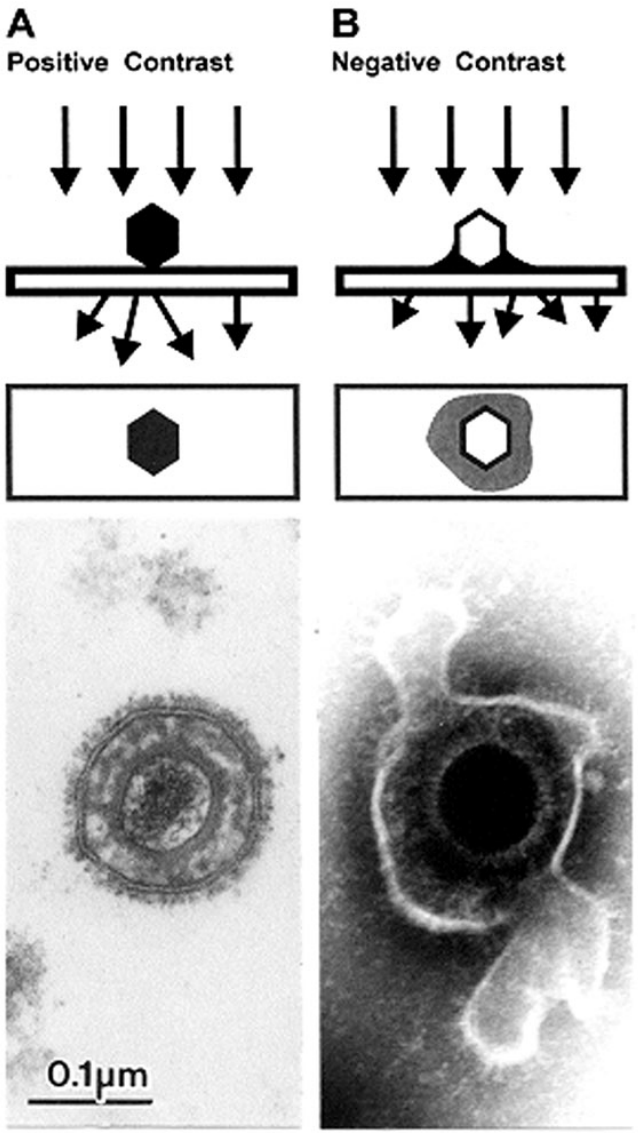
Temperatures can reach ~150°C (302°F) where the beam hits the sample

3. Contrast is generated by e^- interactions with the sample

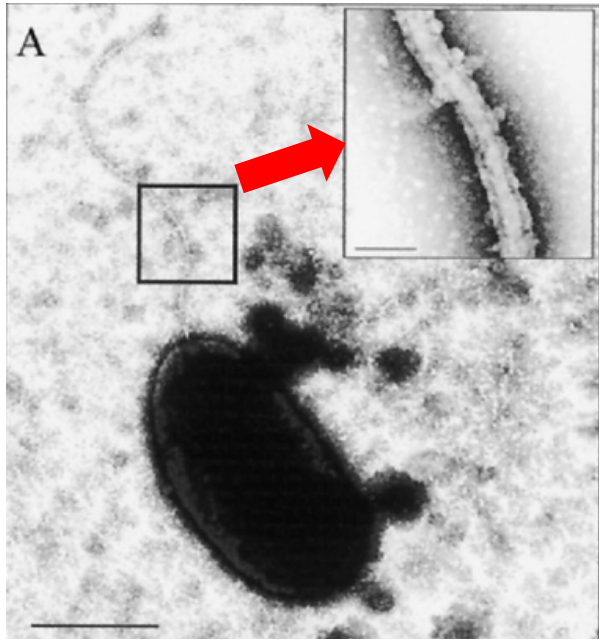
Biological samples are composed of mostly carbon, nitrogen, and oxygen.

Biological samples need to be processed to be compatible with TEM imaging

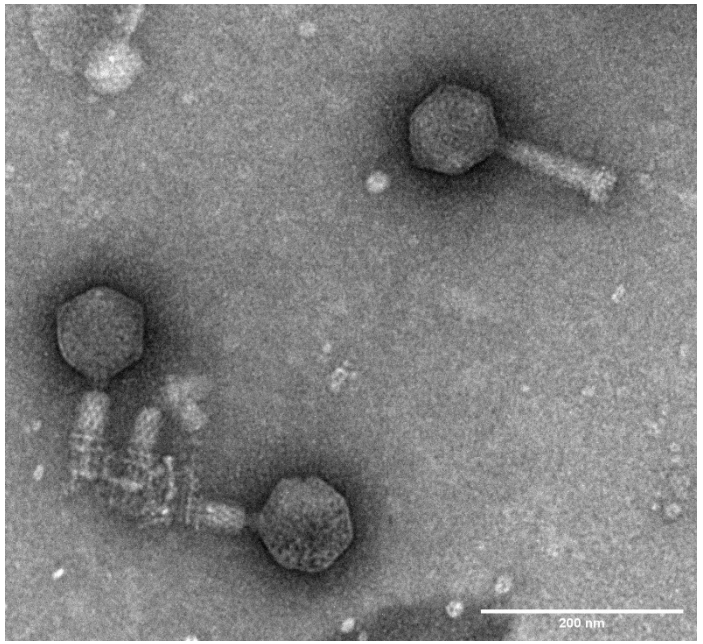
Negative Staining for Small Structures



https://wwwnc.cdc.gov/eid/article/9/3/02-0327-f5



Bacterial flagellum, PTA
Kirov et al., 2002



Bacteriophage, UA
Hatoum Group, UIUC

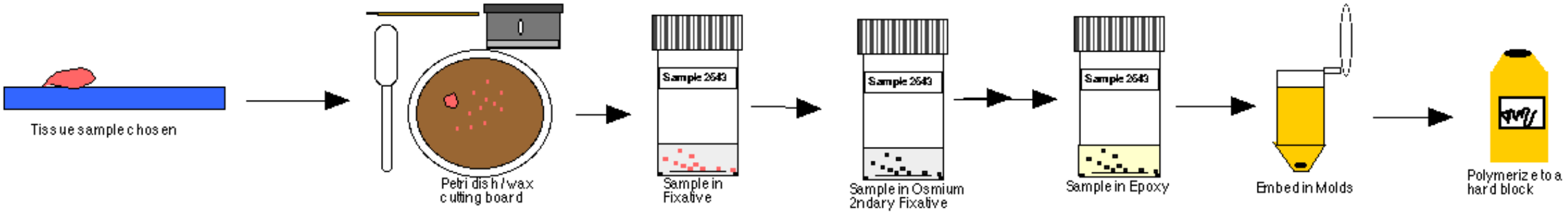
Negative staining **PROS**:

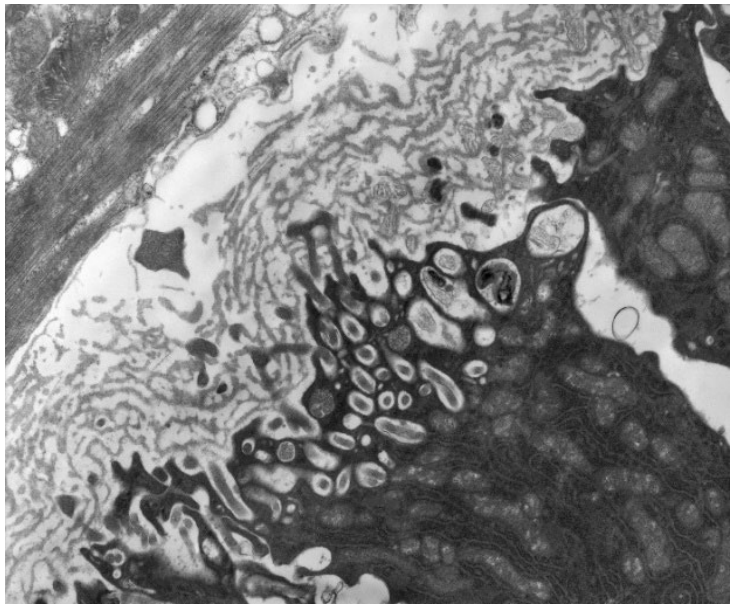
- Very quick and simple setup
- Rapid results
- Provides sample size and morphology data
- Great for diagnostic work and pre-screening

Negative Staining **CONS**:

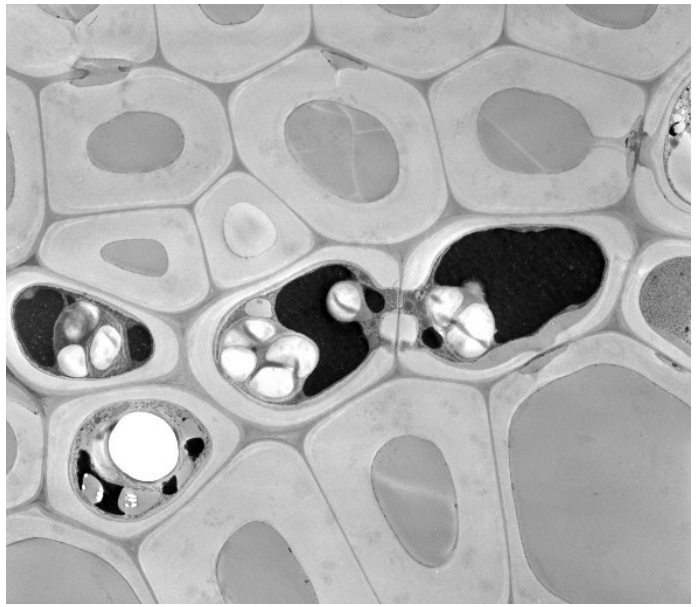
- Only works well for small, thin samples
- Ideal for proteins/viruses
- Does not readily penetrate cells

Biological Sample Preparation for Conventional TEM

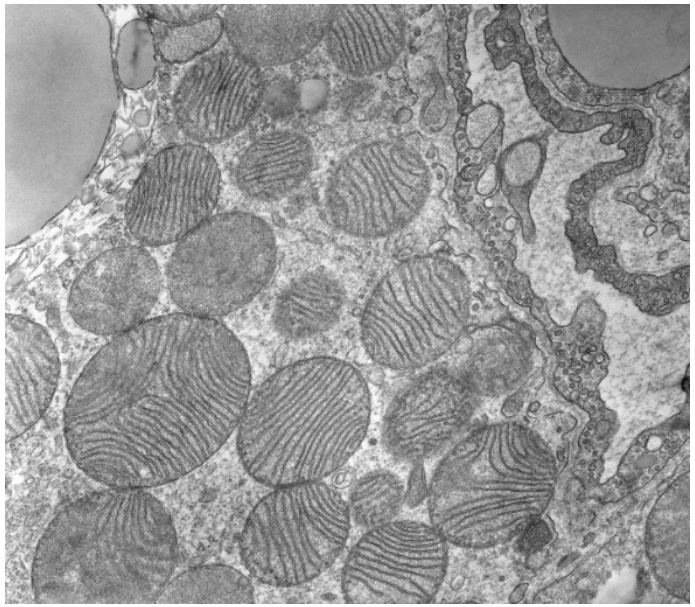




Millipede Hindgut, 10kX



Cherry Stem, 3kX



Mitochondria, 10kX

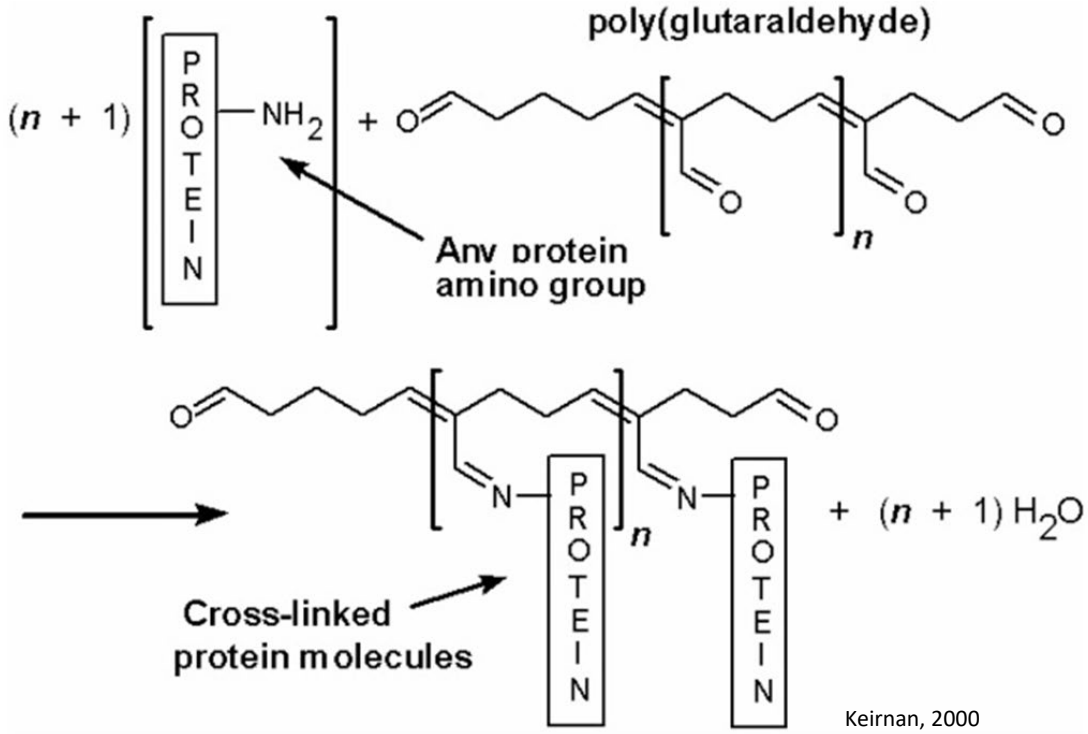
Biological Sample Preparation for Conventional TEM

1. PRIMARY FIXATION

-Stabilizes and preserves tissue ultrastructure

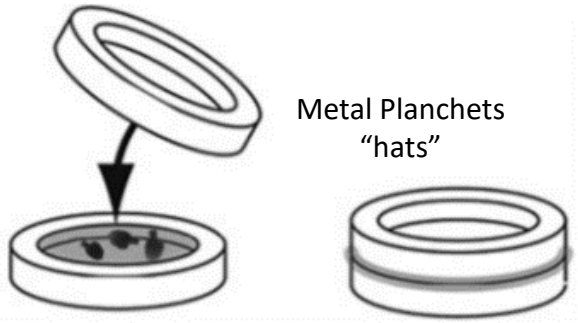
-Can be chemical or cryogenic

Chemical Fixation



Keirnan, 2000

CryoFixation by High-Pressure Freezing

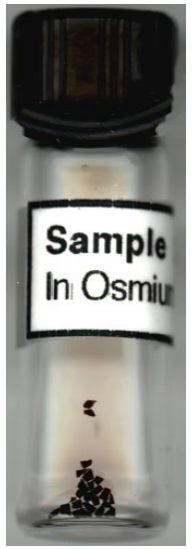


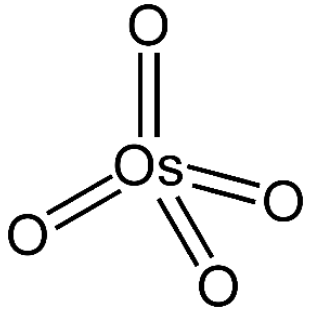
Susaki et al., 2006

2100 bar = ~30,500 psi

Biological Sample Preparation for Conventional TEM

2. **SECONDARY FIXATION (& *en bloc* stain)**
   - Osmium Tetroxide and Uranyl Acetate
   - Stabilization and contrast enhancement





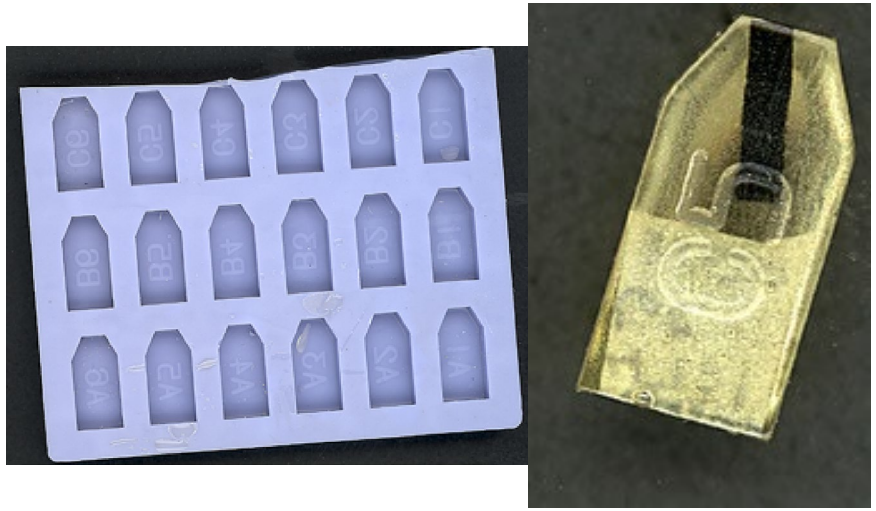
3. **DEHYDRATION**
   - Removing water from sample
   - Replaces water with organic solvents
     (typically acetone or ethanol)

10% → 100%

Biological Sample Preparation for Conventional TEM

4. INFILTRATION AND EMBEDDING

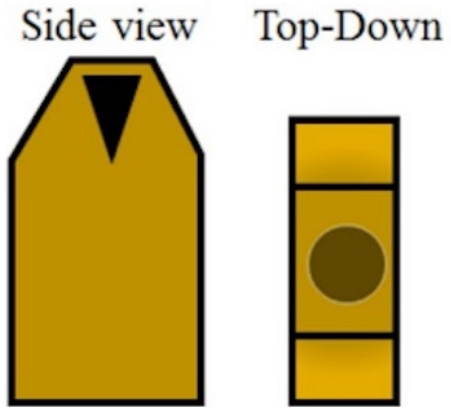
- Use epoxy resin to stabilize samples
- Gradually increase concentration until the samples are in 100% resin



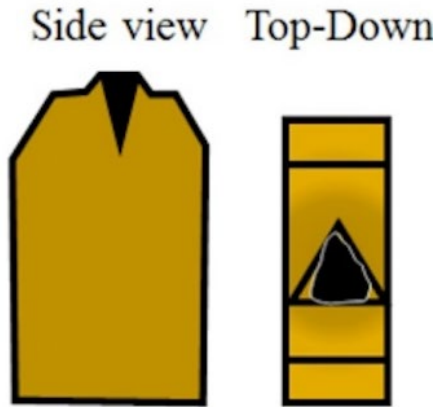
5. BLOCK TRIMMING

- Remove excess resin around sample

Before Trimming

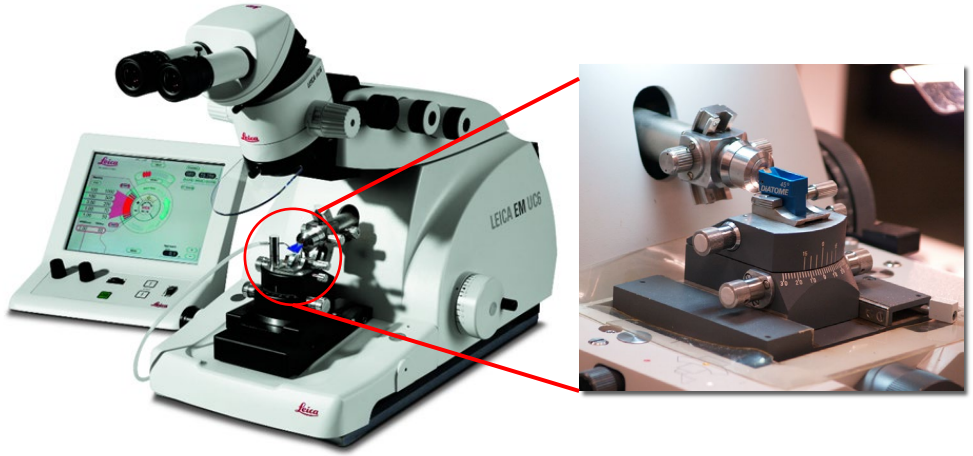


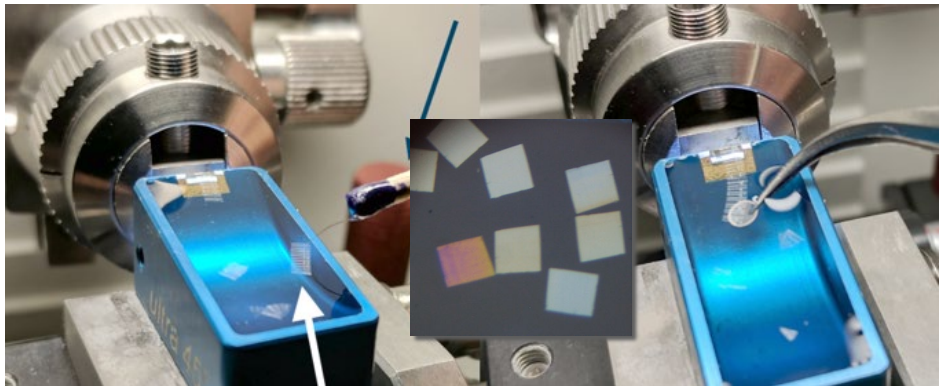
After Trimming



Biological Sample Preparation for Conventional TEM

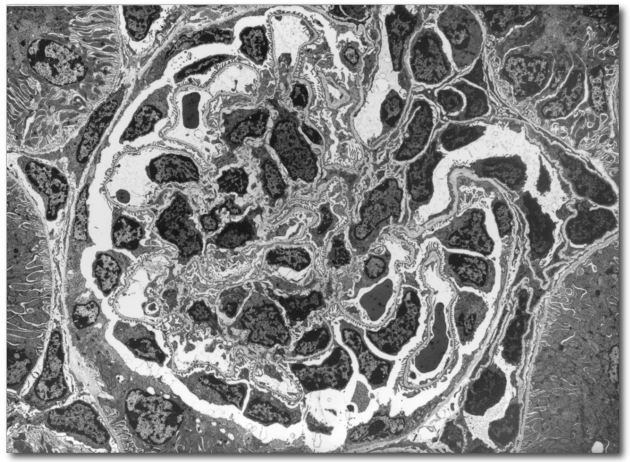
Ultramicrotomy is the process by which a sample is cut (sectioned) into very thin slices (sections) for imaging





Thin Sections for Conventional TEM

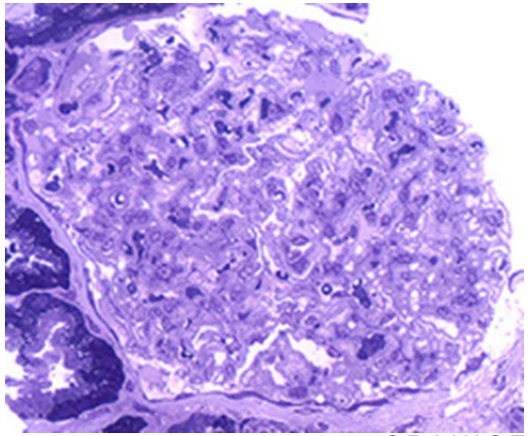
50 – 100 nm



Kidney Biopsy

Thick sections for Light Microscopy

200 – 500 nm



Kidney Biopsy

Post-Staining for Contrast in Conventional TEM

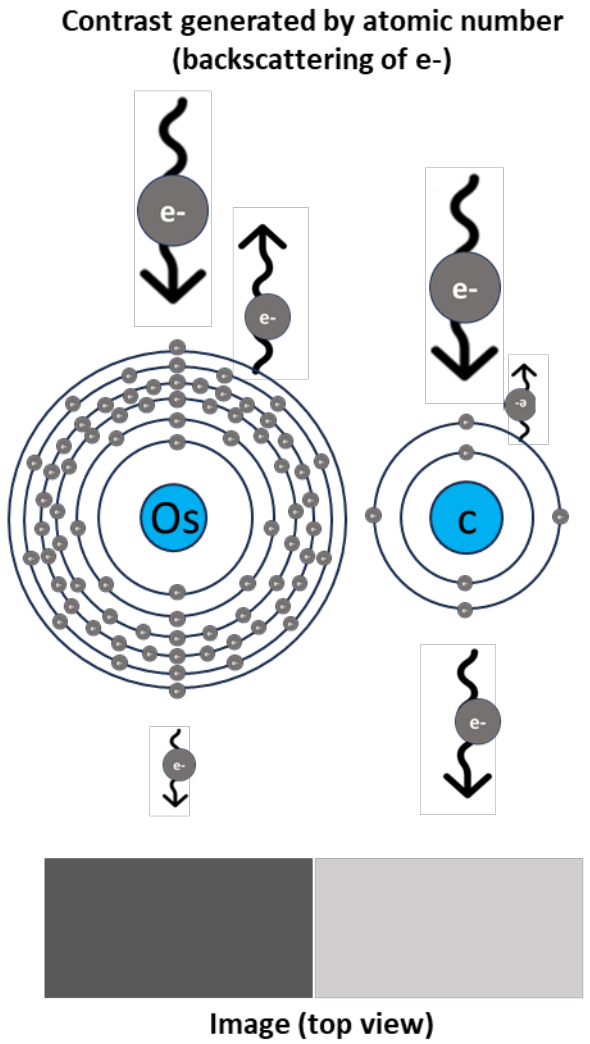
Contrast in the electron microscope is dependent on the differences in electron density of the organic molecules within the tissues.

We use the **heavy metals** to add contrast to EM sections

Uranyl Acetate stains proteins, lipid membranes and nucleic acids.

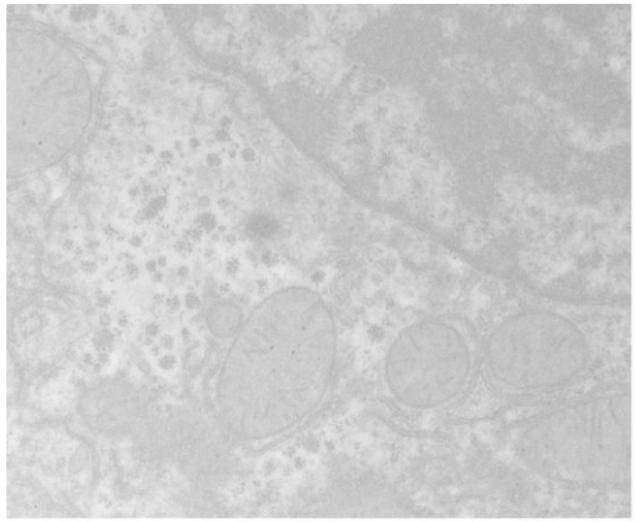
Lead Citrate stains ribosomes, lipid membranes, cytoskeleton and various other components of tissues.

CAUTION: Both Uranyl Acetate and Lead Citrate are extremely toxic. Use extreme care when handling.

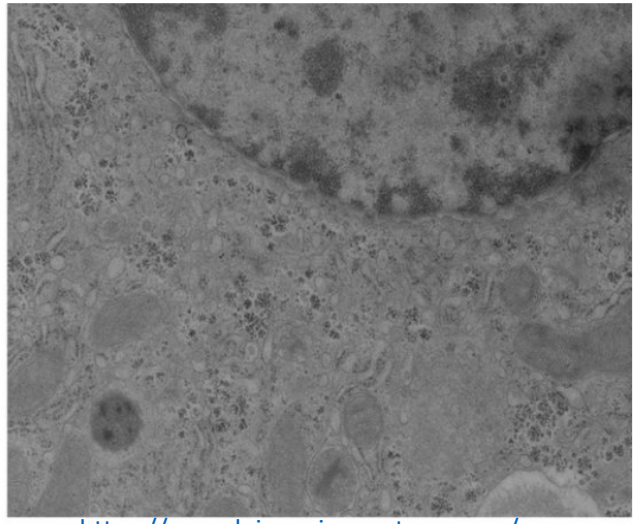


Adapted from: https://advanced-microscopy.utah.edu/education/electron-micro/

No post stain



Post-stained



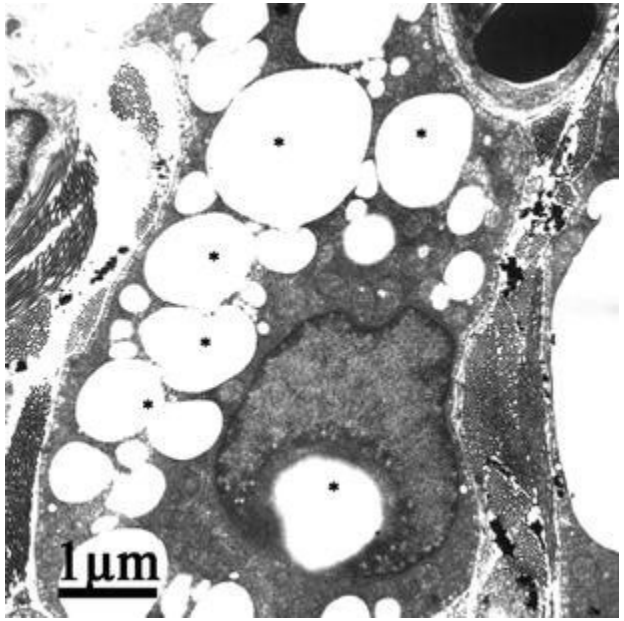
https://www.leica-microsystems.com/

Artifacts in Embedded TEM Samples

Conventional TEM sample preparation can cause **artifacts** in the samples at virtually ANY STEP

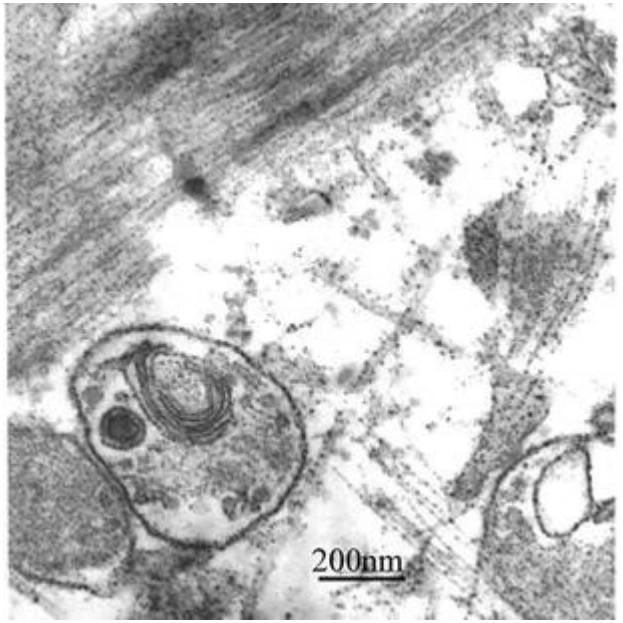
Artifacts are damage caused by preparation techniques that can easily be confused with microstructure

Chemical Fixation Artifact



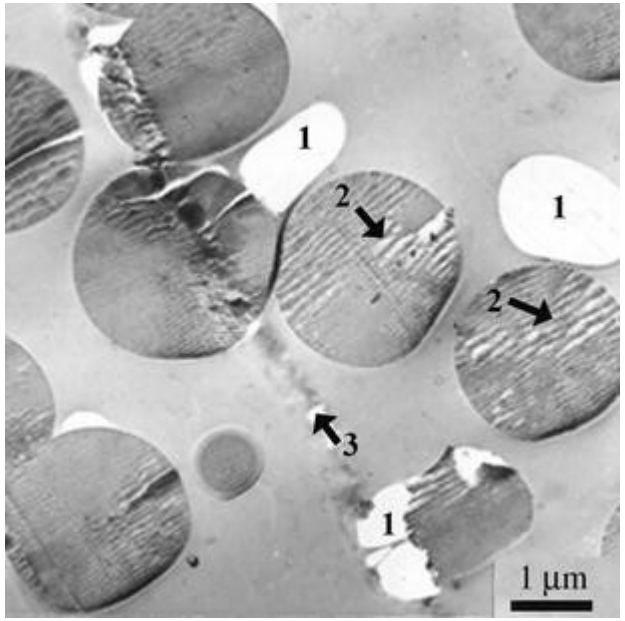
Loss of lipids due to poor fixation

***En bloc* stain artifacts**



Osmium Precipitation

Microtomy Artifacts



Knife marks, tearing, compression

Is there a way to avoid these artifacts??

https://link.springer.com/chapter/10.1007/978-0-387-98182-6_6

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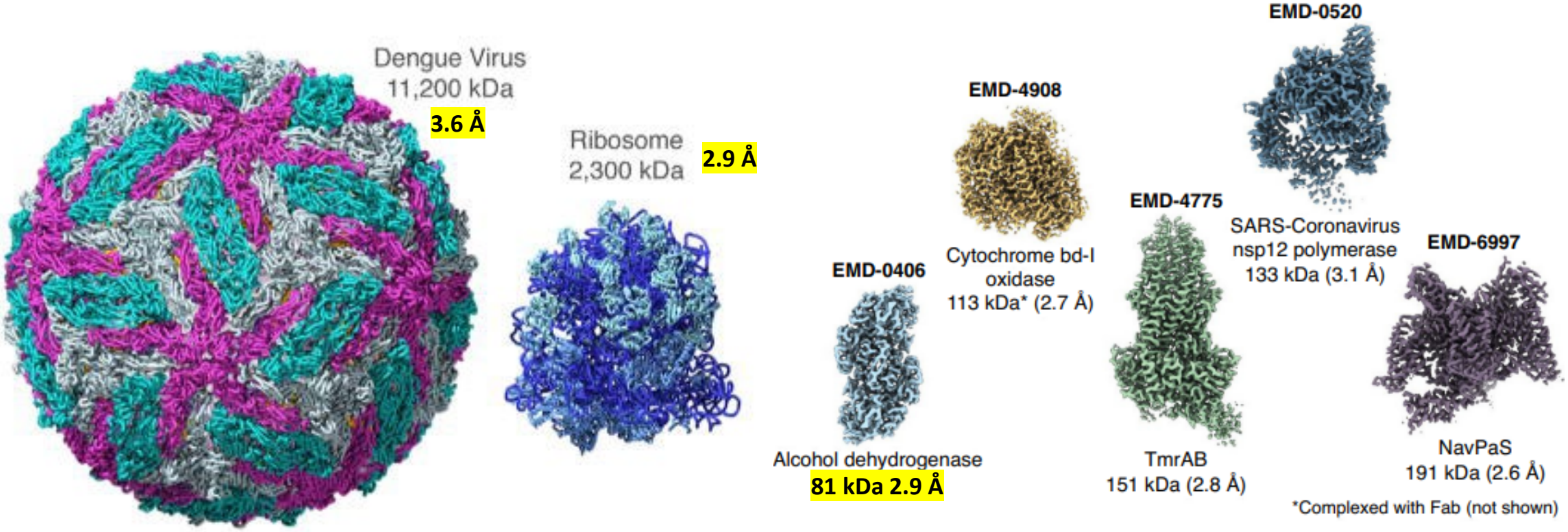
- CryoTEM Techniques
  - Single-Particle Analysis
  - Cryo-electron Tomography

Cryogenic Transmission Electron Microscopy (CryoTEM)

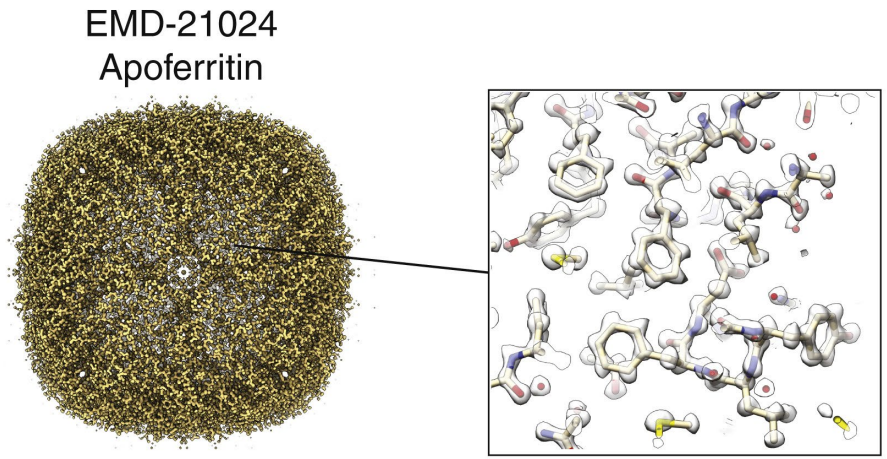
Cryo-TEM allows us to view samples at near atomic resolution in their native, hydrated state without the use of chemical fixatives or stains.



Merk et al., 2016

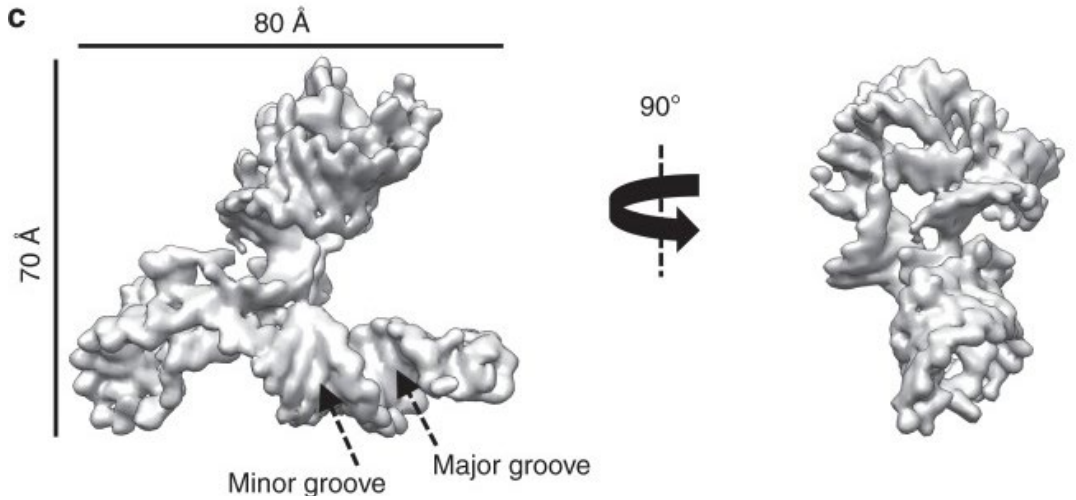
Wu & Lander, 2020

How Low Can We Go?



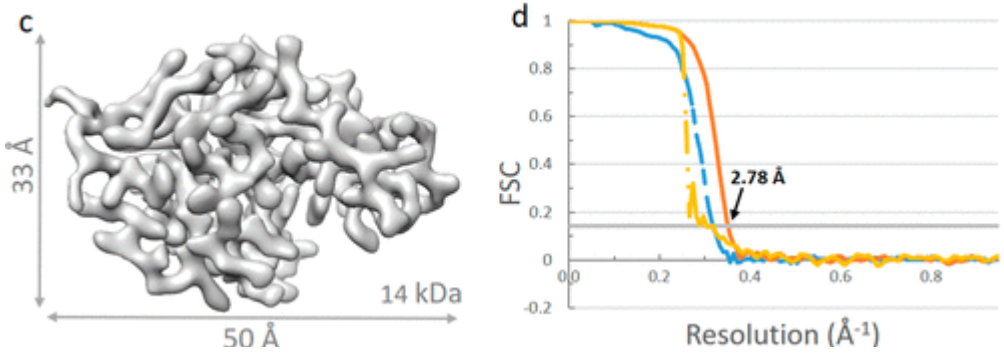
Mouse apoferritin, 1.2Å

Nakane et al., 2020



~40-kDa SAM-IV riboswitch, 3.7Å

K. Zhang et al., 2019



14-kDa hen egg white lysozyme, 2.8Å (Simulated)

Y. Zhang et al., 2020

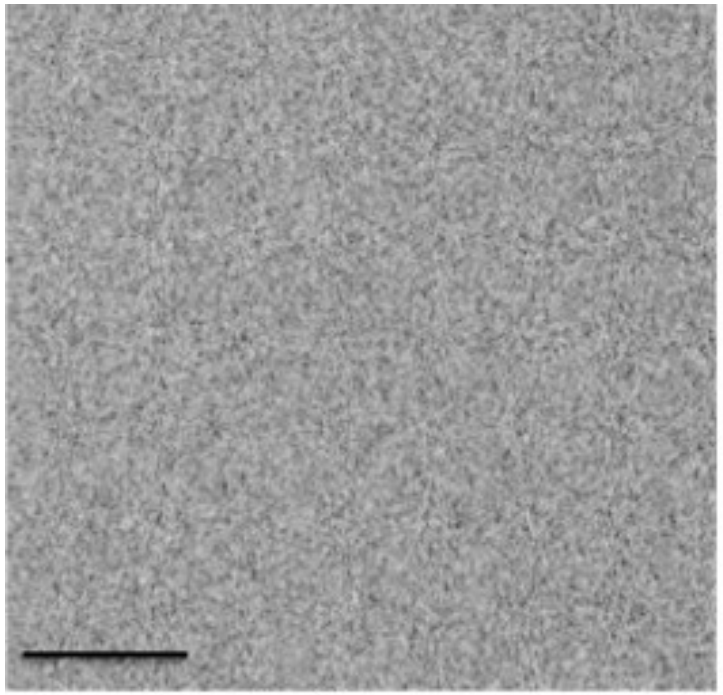
Sample Preparation for CryoTEM: Vitrification

Vitrification marks the transition of water from a liquid into an amorphous solid phase while avoiding formation of ice crystals

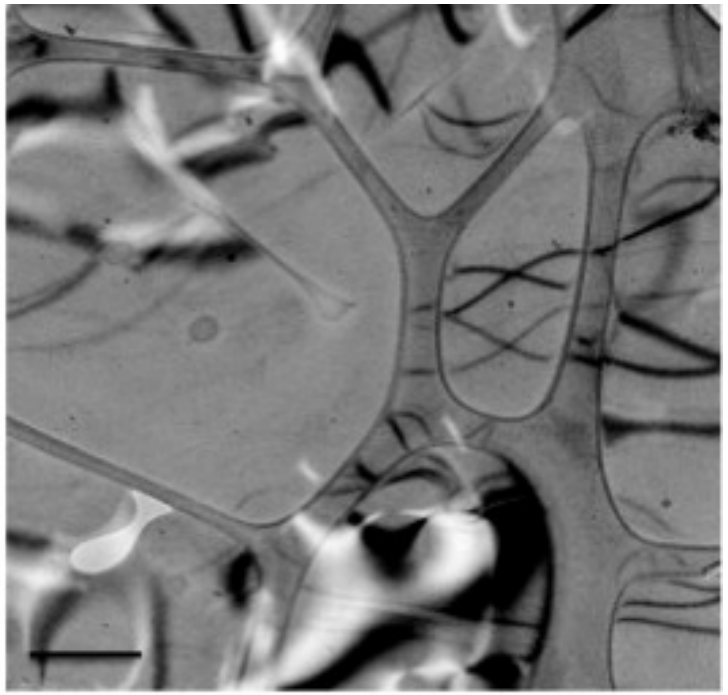
Vitrification requires a rapid cooling rate:

~10^6 °C/second per micron

Vitreous Ice



Crystalline Ice



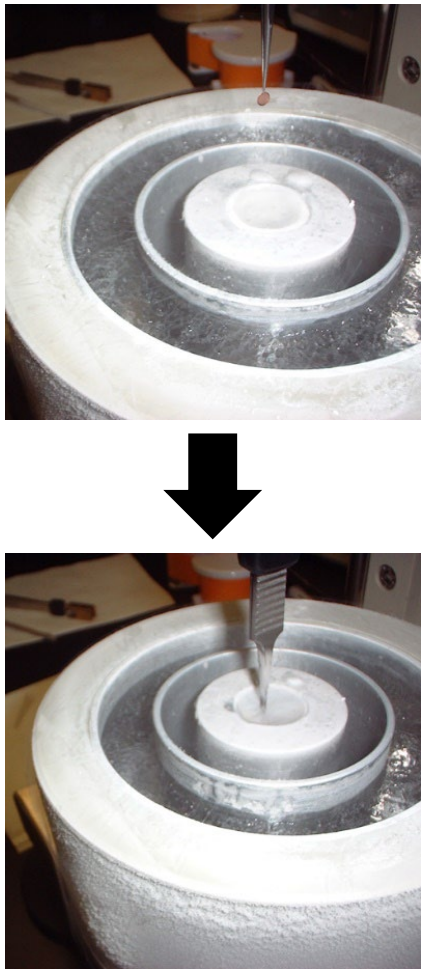
Adapted from Thompson et al., 2016

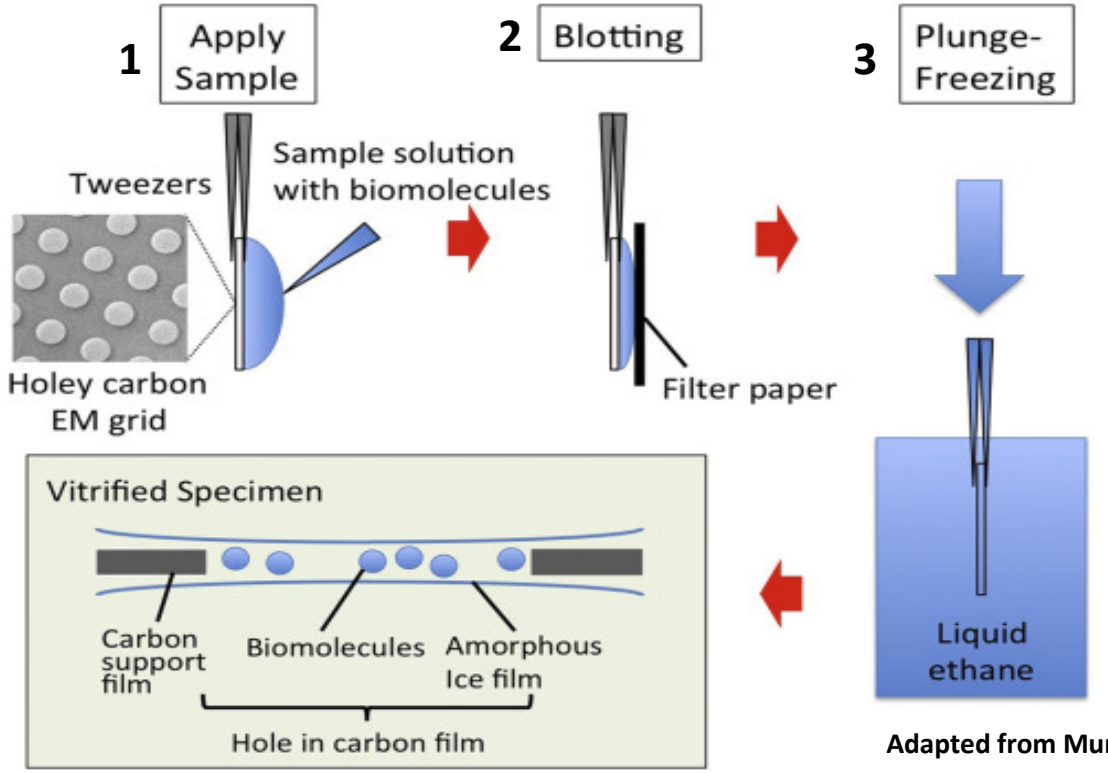
Vitrification by Plunge Freezing

Plunge Freezing

Samples up to **1-2µm** thick

FEI Vitrobot

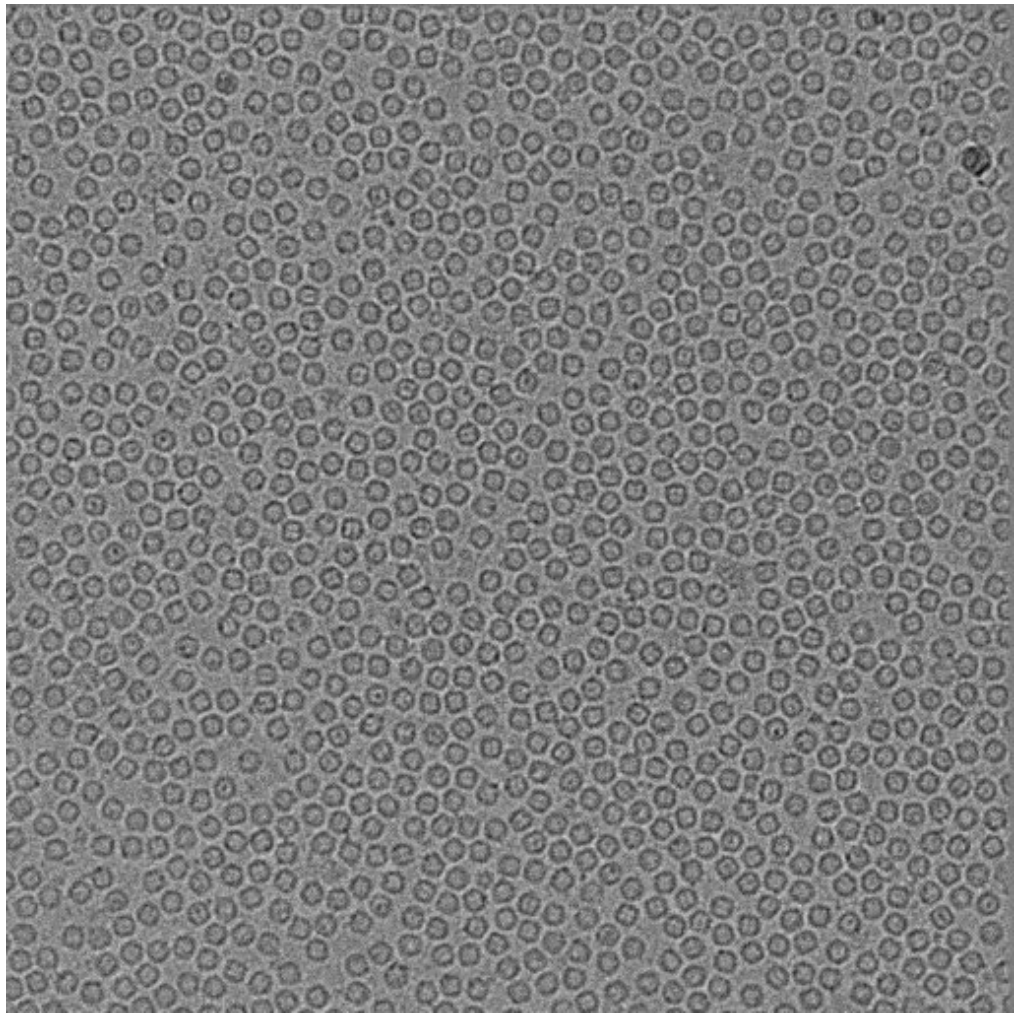




Adapted from Murata and Wolf, 2018

	Melting Point (°C)	Boiling Point (°C)
Propane	- 188°C	- 44°C
Ethane	- 183°C	- 89°C
Nitrogen	- 210°C	- 196°C

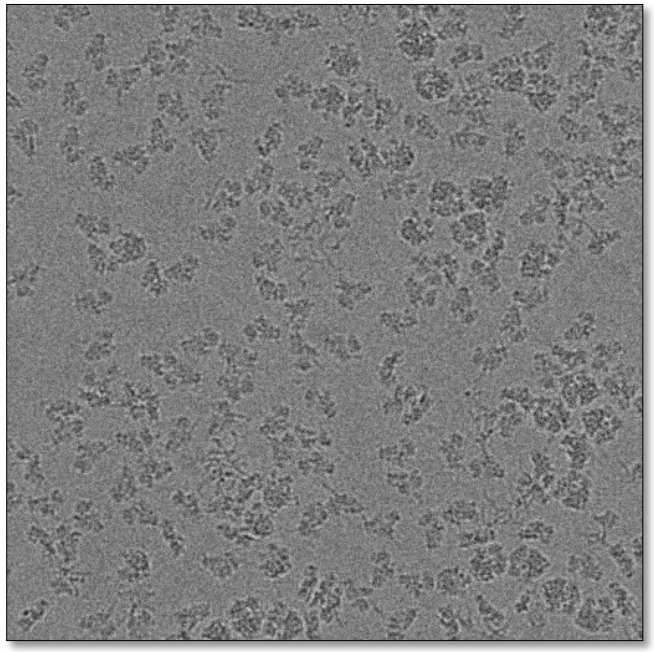
2D Imaging of Small Particles in the CryoTEM



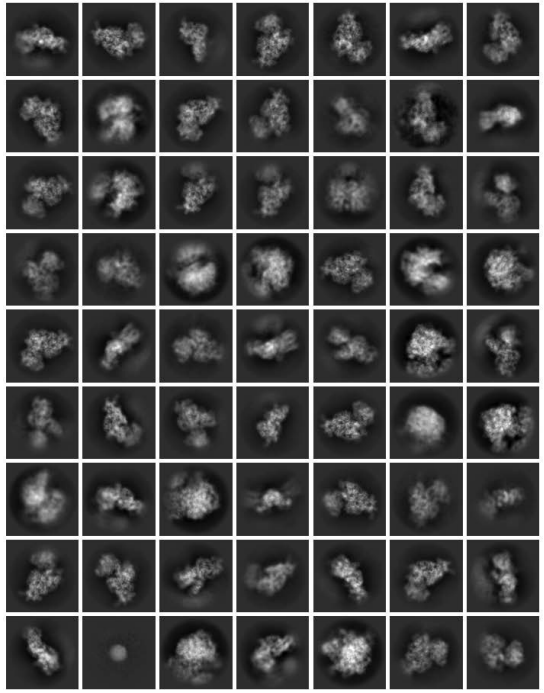
2D image of **Apoferritin** (~480 kDA), 120kX

Single-Particle Analysis (SPA)

Single-Particle Analysis is an imaging technique that combines several TEM images of small particles to give an image with more easily interpretable features, or 3D reconstruction.



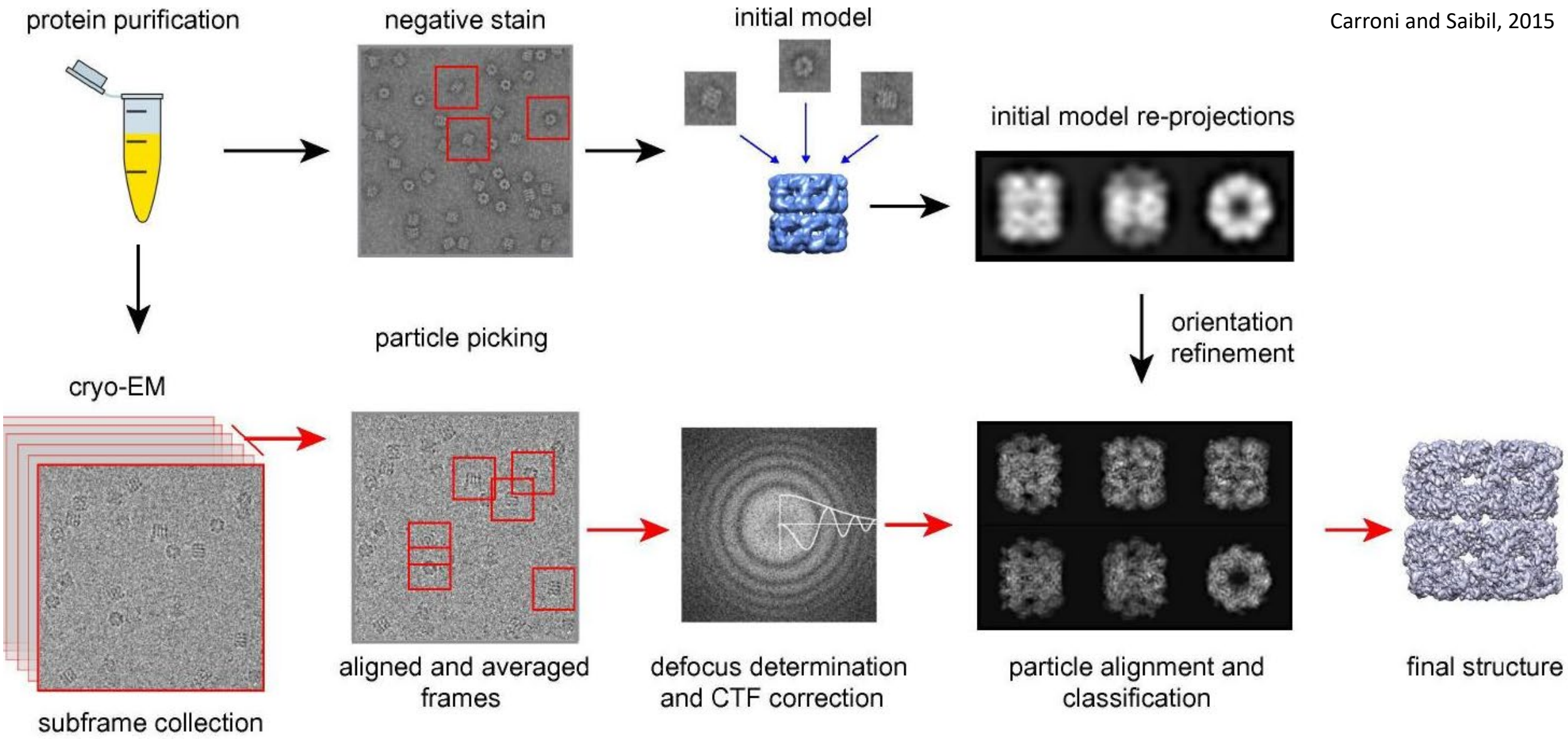
2D Image Acquisition



Alignment and Classification

3D Reconstruction

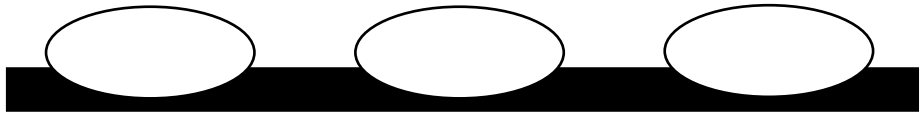
SPA Workflow



Carroni and Saibil, 2015

Negative Staining vs. CryoTEM SPA

Particles in Negative Stain

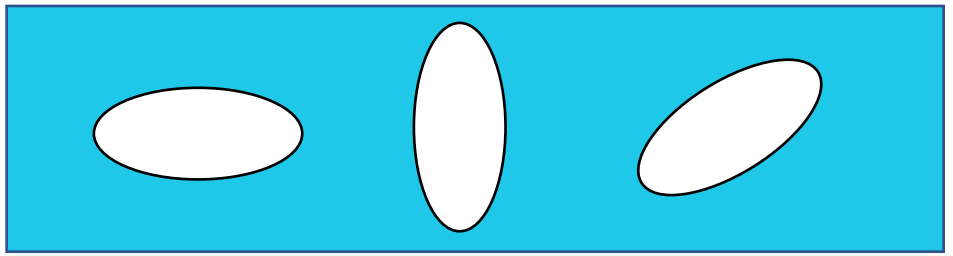


- High contrast image
- Room temperature imaging
- Resistant to beam damage

- Particles often distorted
- Preferred orientation on substrate
- Imaging stain “shell” around particles
- LOW RESOLUTION METHOD: 15-20 Å

Good choice for initial sample screening

Particle in Vitreous Ice



- Low Contrast Images*
- Must be held at cryo-temps (-160°C)
- Very sensitive to beam damage

- Particles undistorted
- Random orientations
- Image is of actual particle
- HIGH RESOLUTION METHOD: 1.5 - 15 Å

Best choice for high resolution reconstructions

CryoTEM Sample Preparation: Thick Specimens (>1-2µm)

High-Pressure Freezing

Samples up to **200µm** thickness

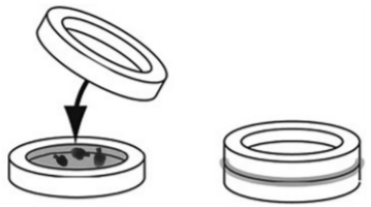
Whole Cells

Whole Organisms

Tissues

2100 bar = ~30,500 psi

Metal Planchets (“Hats”)

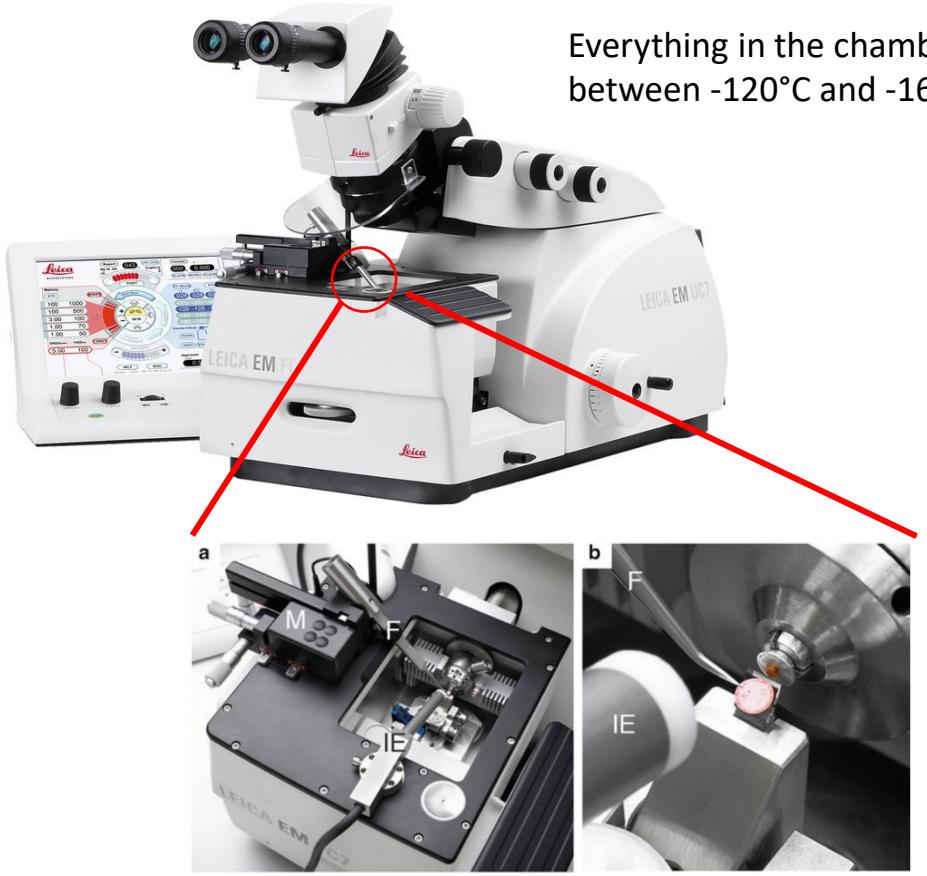


Susaki et al., 2006

Copper capillary tubing

CryoTEM Sample Preparation: Thick Specimens (>1-2µm)

Cryo-Ultramicrotomy

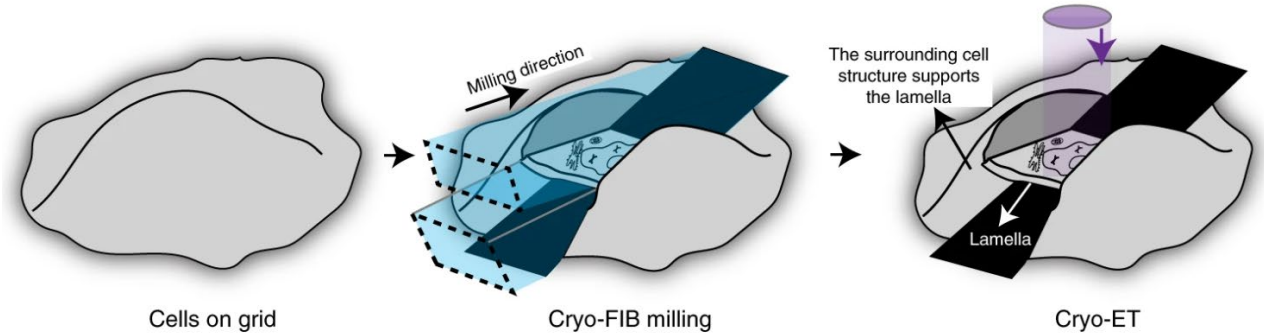


Everything in the chamber is kept between -120°C and -160°C

Chlanda and Sachse, 2014

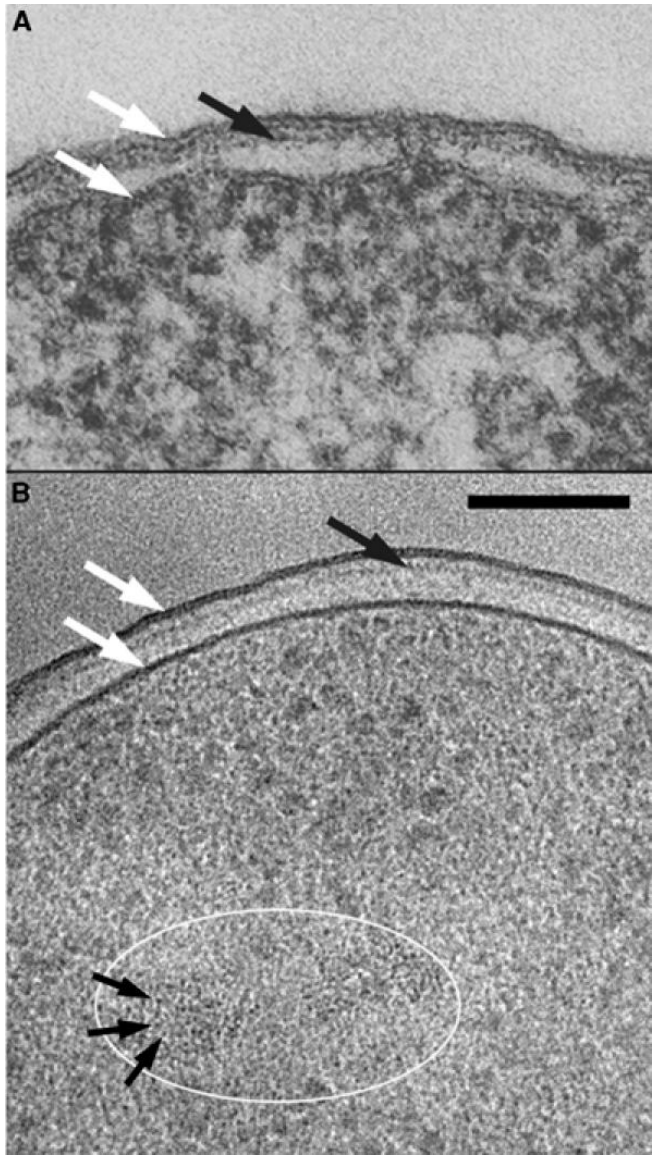
CryoFIB-milling

Sample is frozen onto TEM grid and transferred to a Focused-Ion-Beam Scanning Electron Microscope (FIB-SEM). The ion beam “mills” the sample into a lamella that contains the region of interest.

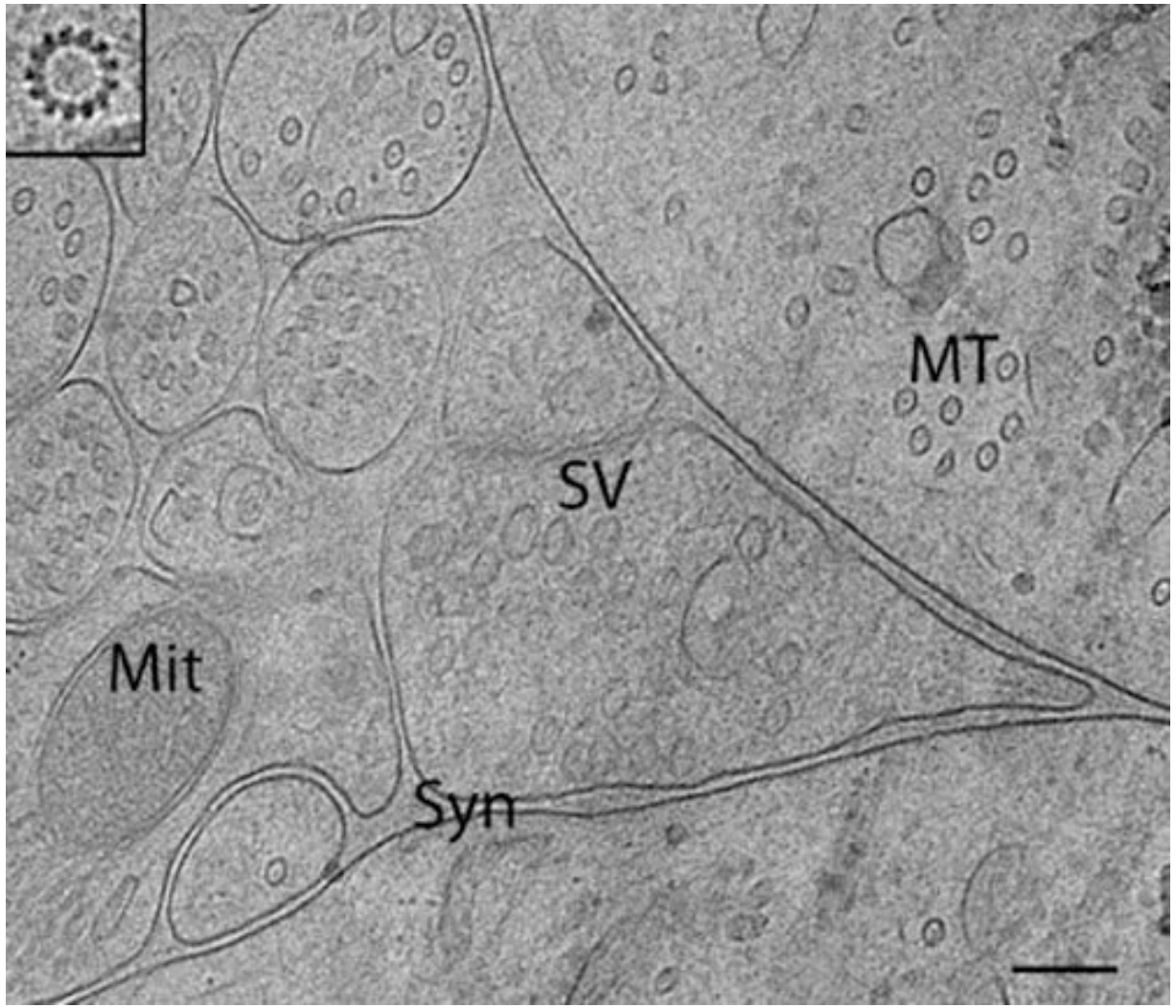


Adapted from Wagner et al., 2020

Cryo-Electron Microscopy of Vitreous Sections



Al-Amoudi et al. 2004

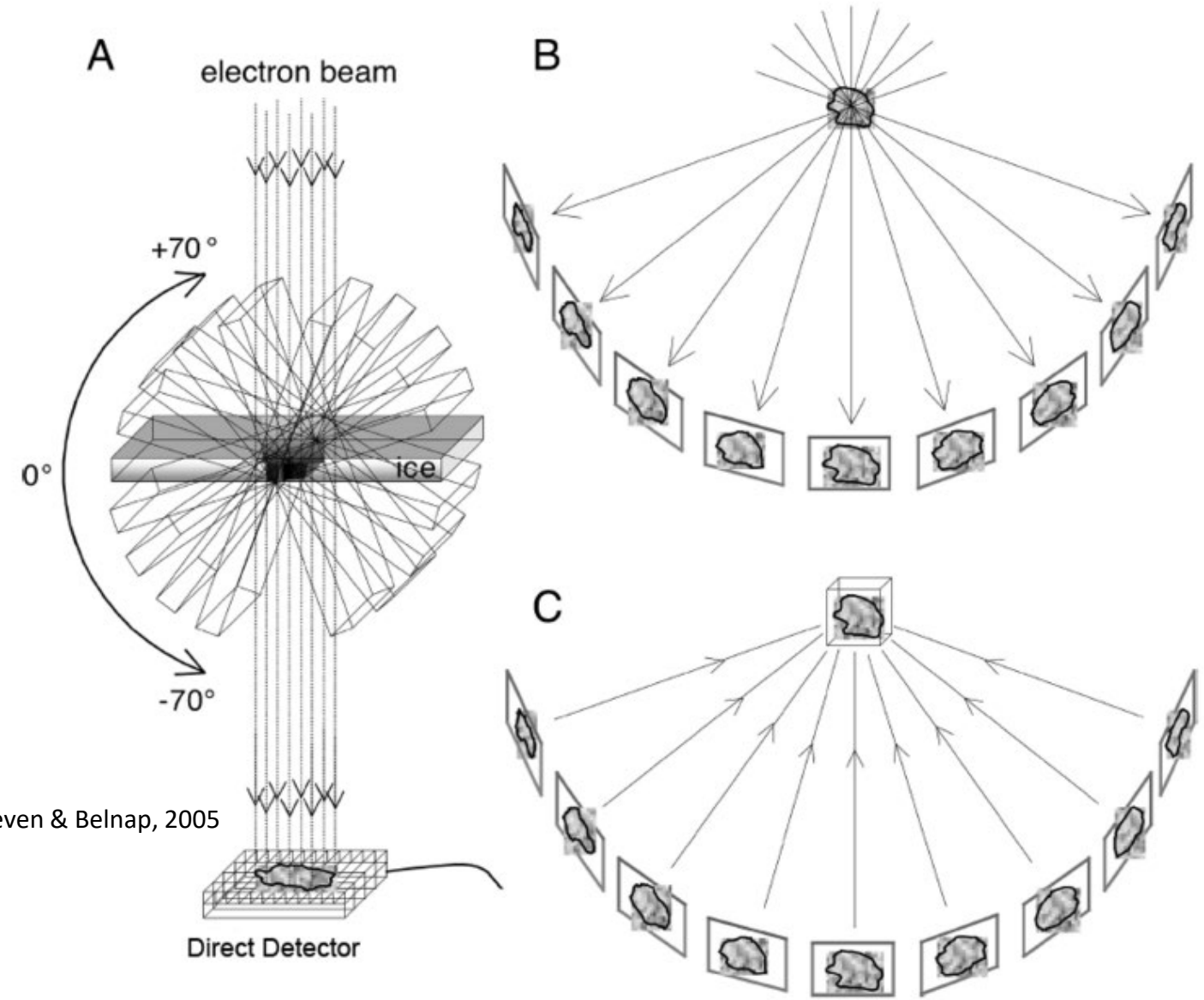


https://www.ana.unibe.ch/research/microscopic_anatomy_and_structural_biology/index_eng.html

Mit – Mitochondria
Syn – Synapse
SV – Synaptic Vesicles
MT - Microtubules

Cryo-Electron Tomography (CryoET)

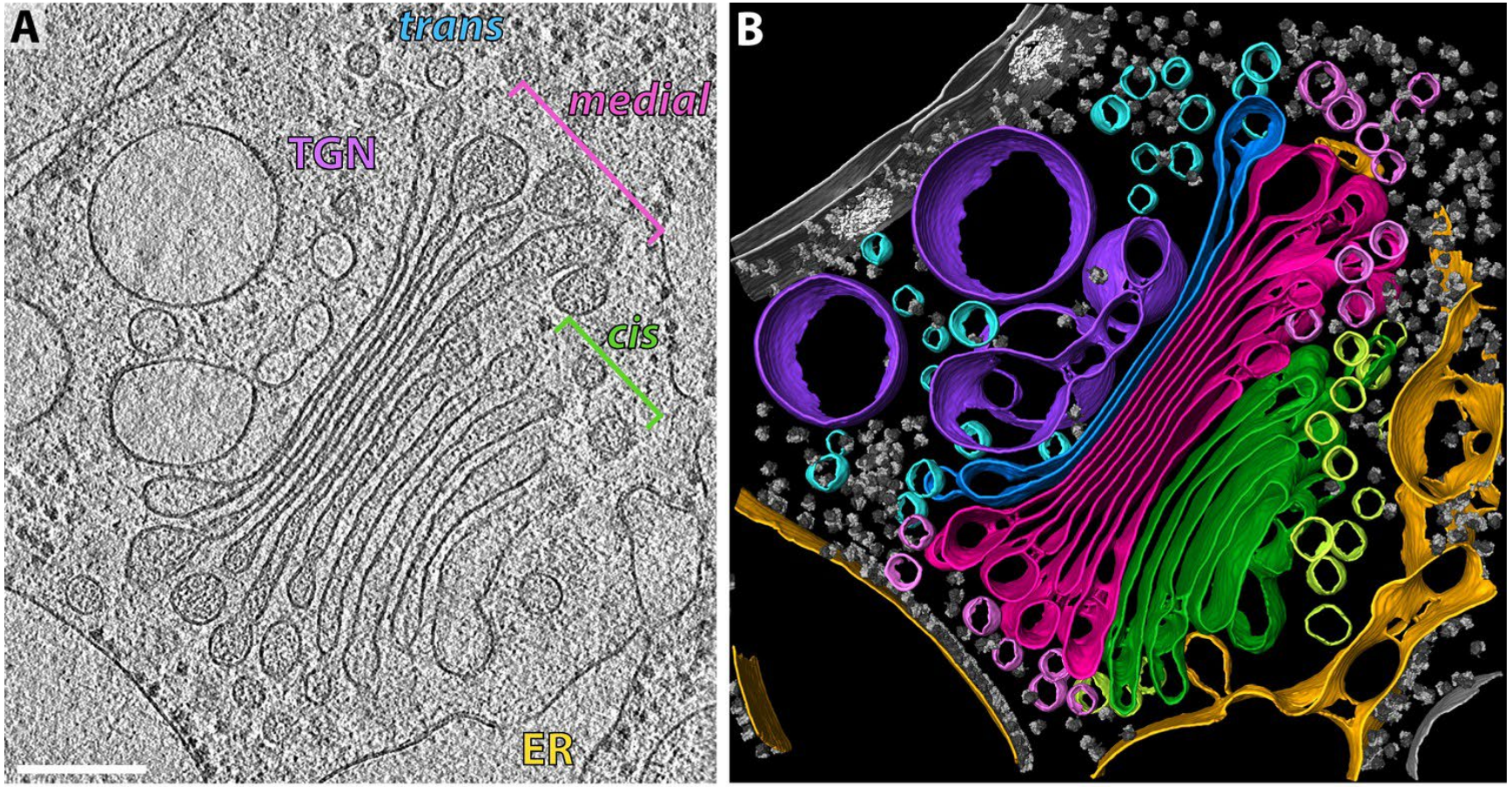
CryoET collects a series of 2-dimensional images while a sample, held at cryogenic temperatures, is tilted. The 2D images are then aligned to yield a 3D reconstruction.



Steven & Belnap, 2005

CryoET - Reconstruction

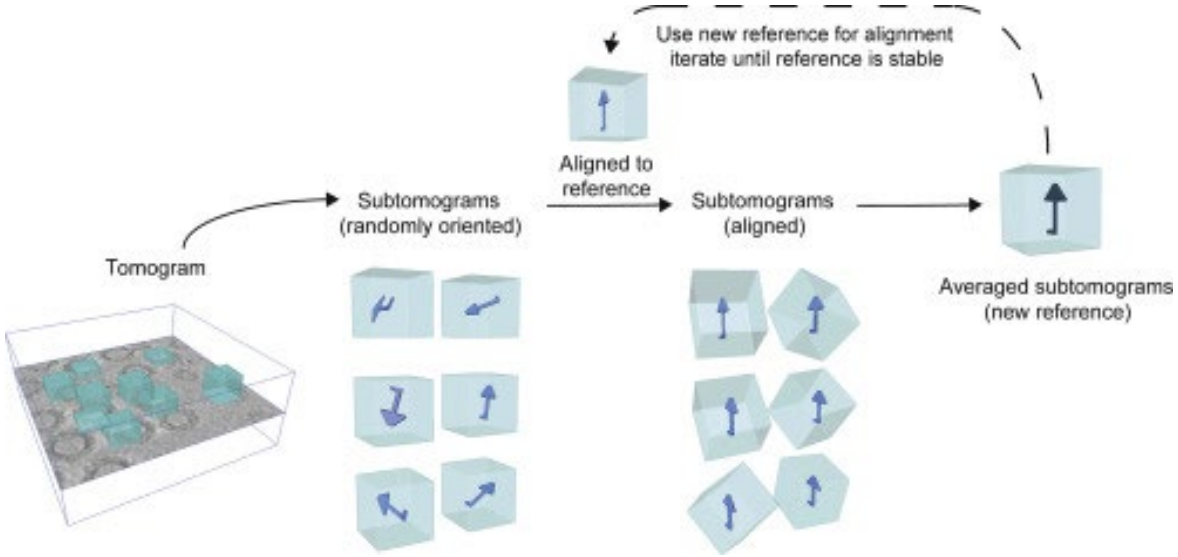
Molecular architecture of the *Chlamydomonas* Golgi apparatus and transport vesicles revealed by *in situ* cryo-ET.



Adapted from
Bykov et al., 2017

Sub-Tomogram Averaging (STA)

SubTomogram Averaging is analogous to SPA, with the key distinction that STA particles are represented by 3D volumes (tomograms) rather than 2D projections



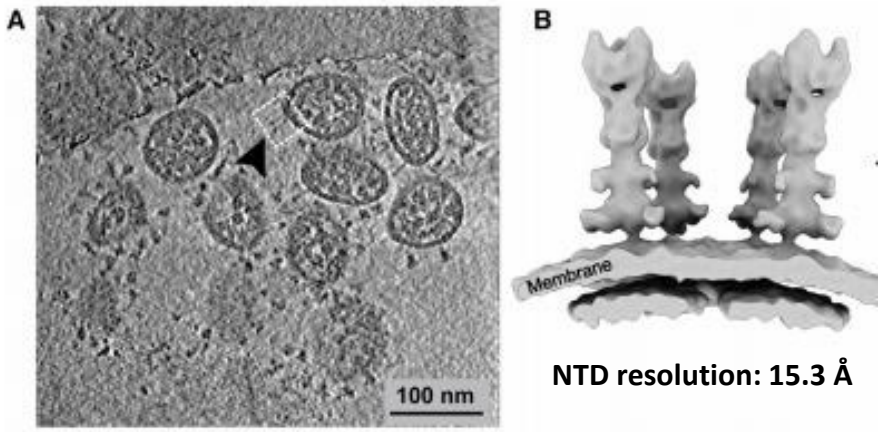
Wan & Briggs, 2016

"**Subtomograms** are cubes extracted from the full tomogram; each subtomogram contains a randomly oriented copy of the molecule of interest.

Subtomograms are aligned to the reference and a new reference is generated from the aligned particles. This process is iterated until the alignment converges to a stable reference…"

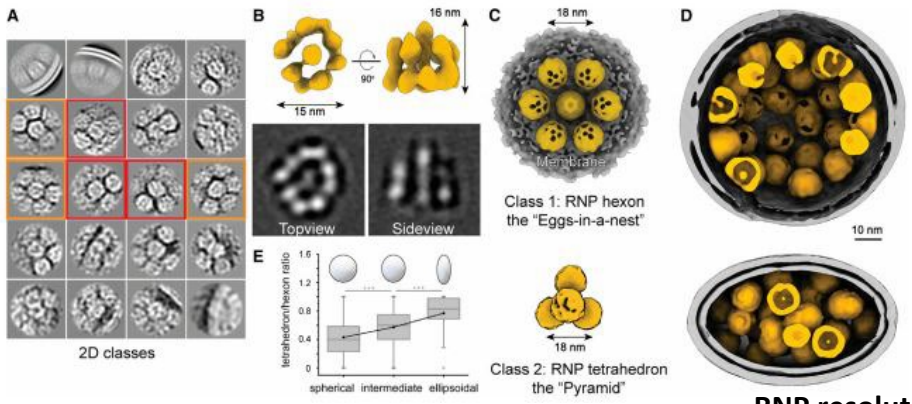
SubTomogram Averaging

STA used to determine structures of **spike proteins** on OUTSIDE of virion



NTD resolution: 15.3 Å

STA used to determine structures of **ribonucleoproteins** INSIDE virion



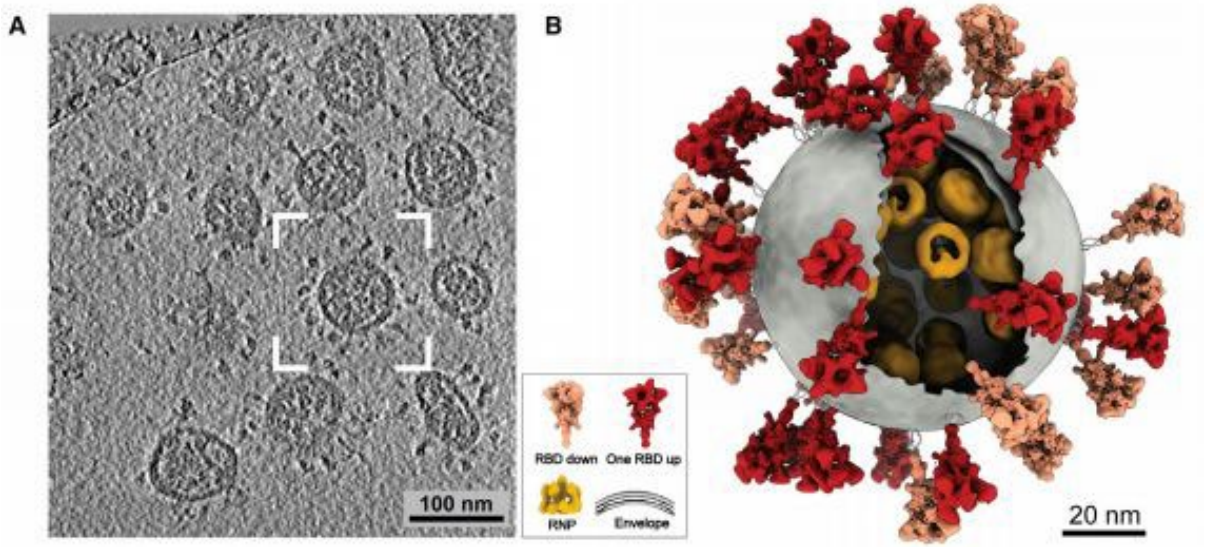
RNP resolution: 13.1Å

Article

Molecular Architecture of the SARS-CoV-2 Virus

Hangping Yao,[1,2,9] Yutong Song,[3,4,9] Yong Chen,[3,4,9] Nanping Wu,[1,2,9] Jialu Xu,[3,4,5,9] Chujie Sun,[3,4,5] Jiaxing Zhang,[3,4] Tianhao Weng,[1,2] Zheyuan Zhang,[3,4] Zhigang Wu,[1,2] Linfang Cheng,[1,2] Danrong Shi,[1,2] Xiangyun Lu,[1,2] Jianlin Lei,[3,4] Max Crispin,[6] Yigong Shi,[3,4,5,7,8] Lanjuan Li,[1,2,*] and Sai Li[3,4,5,10,*]

Representative Viral Structure



CryoET and STA combined to reveal the molecular architecture of SARS-CoV-2

Sub-Tomogram Averaging vs. Single-Particle Analysis

Single-Particle Analysis

Purified, homogenous samples

Single layer on grid

2D images

Super high-resolution reconstructions

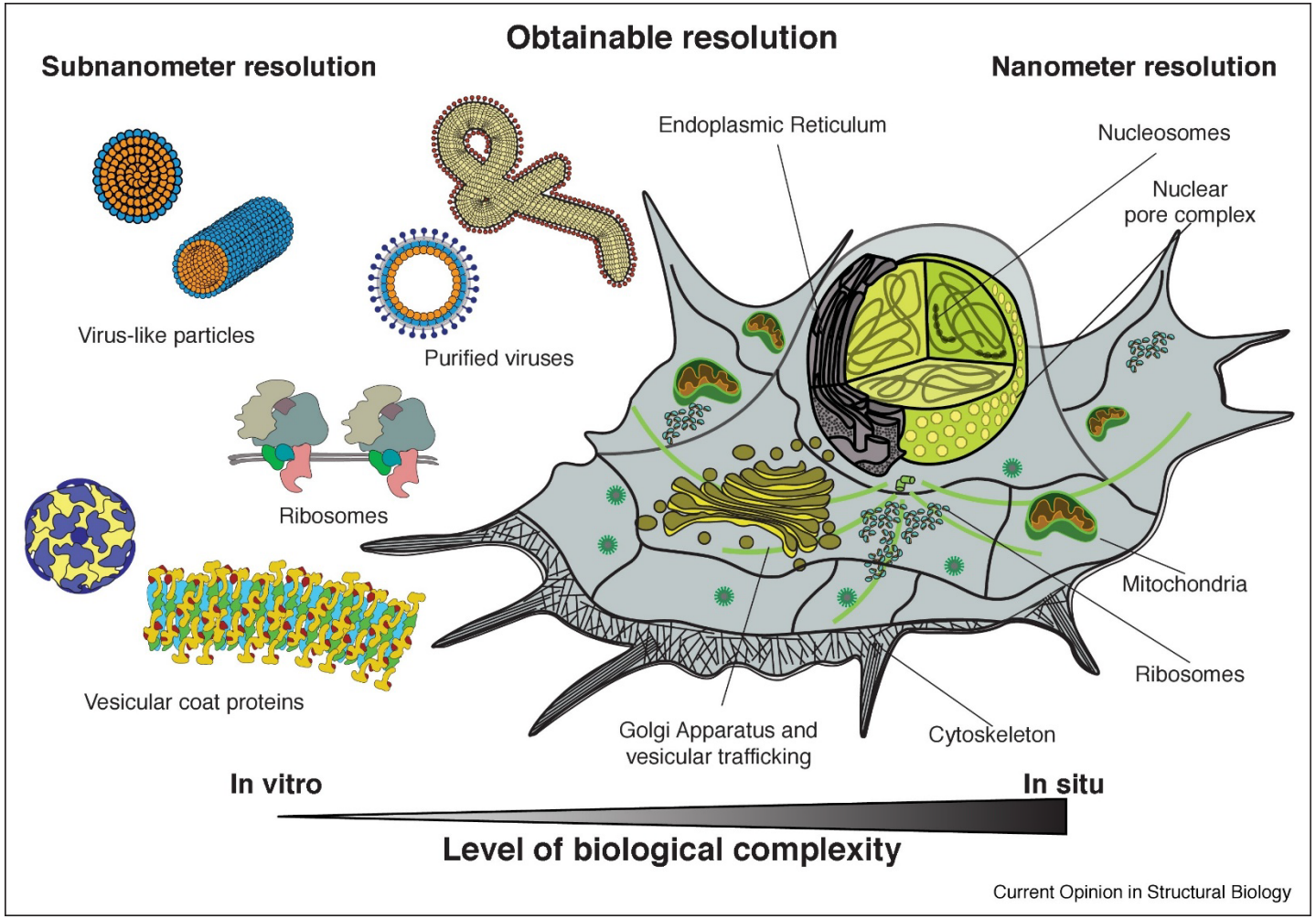
SubTomogram Averaging

Samples *in situ*

Other cellular components

3D “boxes”

Mid-to-low resolution reconstructions

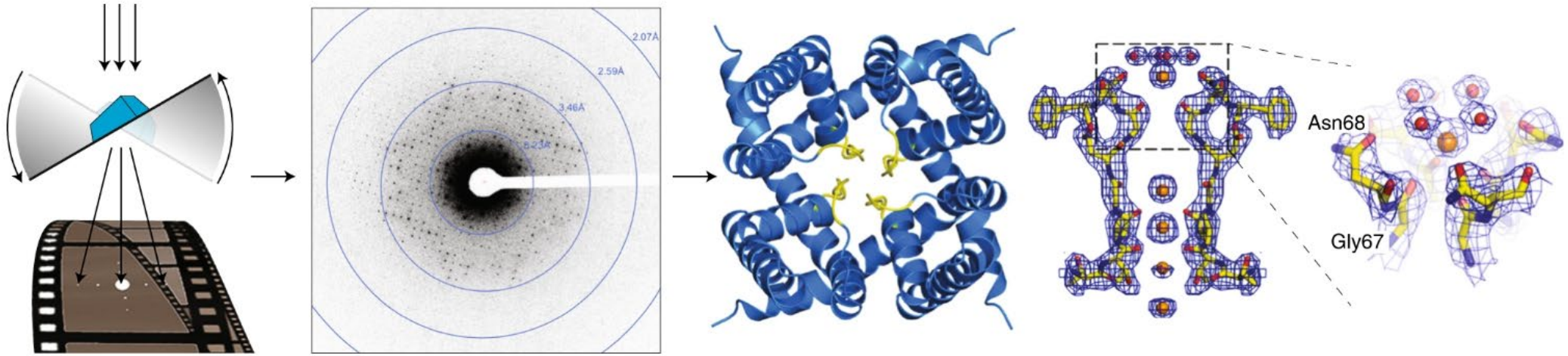


Schur, 2019

Micro-Electron Diffraction (MicroED)

MicroED is a form of electron crystallography that uses very thin 3D crystals for structural determination by electron diffraction.

- **Useful for crystals that are too small for XRC (~100 nm minimum crystal size)**

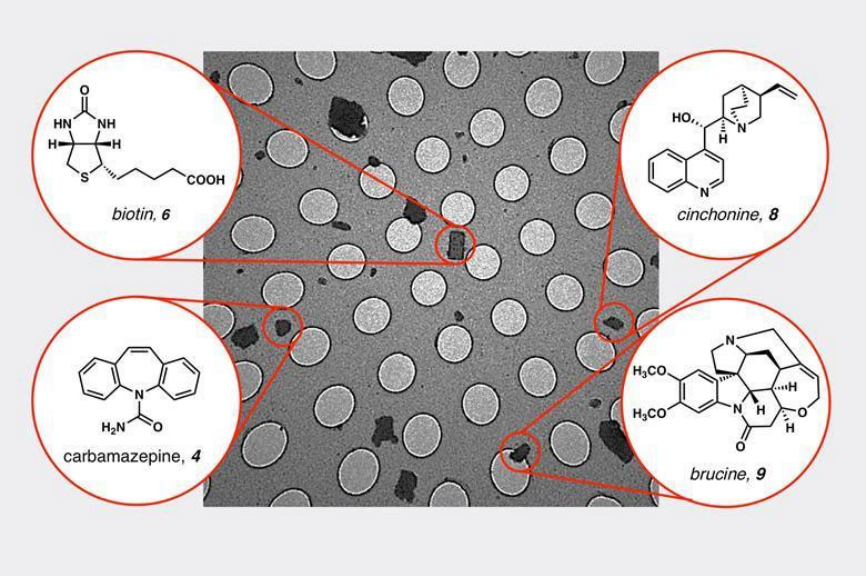


Gonan et al., 2019

3D microcrystals are exposed to the diffracting electron beam while being constantly rotated, and diffraction patterns are recorded on the detector as a movie

MicroED Applications

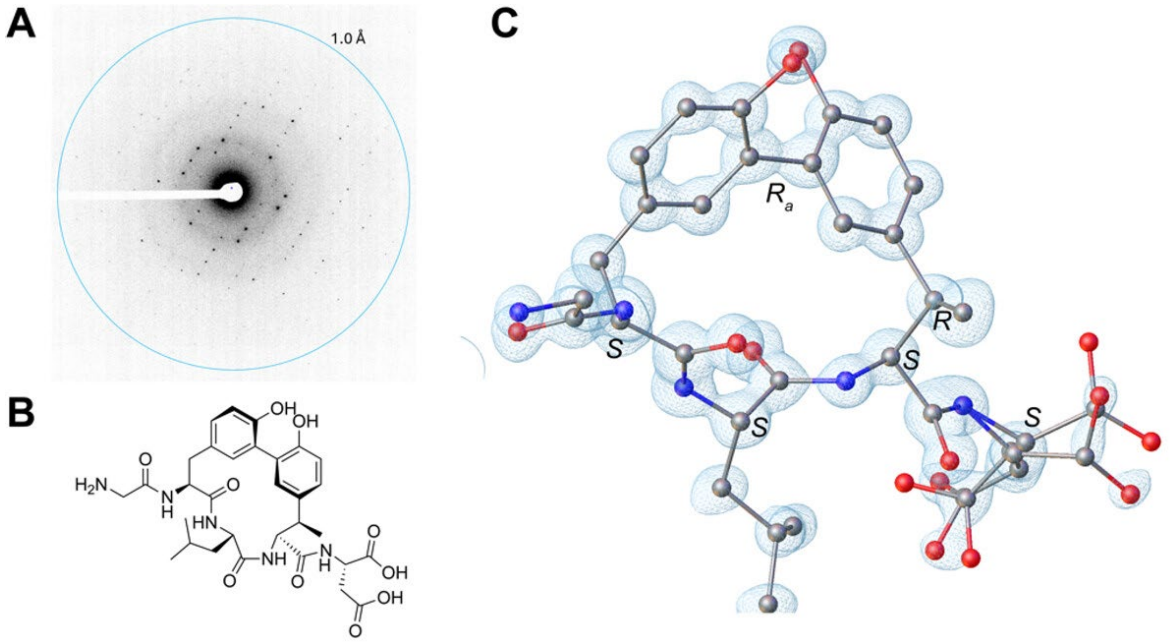
Identifying Compounds from Heterogeneous Mixtures



"MicroED data were collected from several nanocrystals, and the identity of each species was resolved within minutes by confirmation of unit cell parameters […] All structures were solved to ~1 Å resolution..." – Jones et al., 2018

Biosynthesis of Macrocyclic Peptides with C-Terminal β-Amino-α-keto Acid Groups by Three Different Metalloenzymes

ACS Cent. Sci. 2024, 10, 5, 1022-1032



"MicroED structure of ApyD- and ApyO-modified ApyA pentapeptide at 1.0 Å resolution. CCDC ID 2324739. The blue mesh represents the observed electron density map (F_{obs}). The full structure comprises two complete peptide molecules with a Zn atom and water molecules. […] Here, only one peptide molecule is shown, and hydrogen atoms are not shown for clarity." – Nguyen et al., 2024 (van der Donk Lab, UIUC)

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