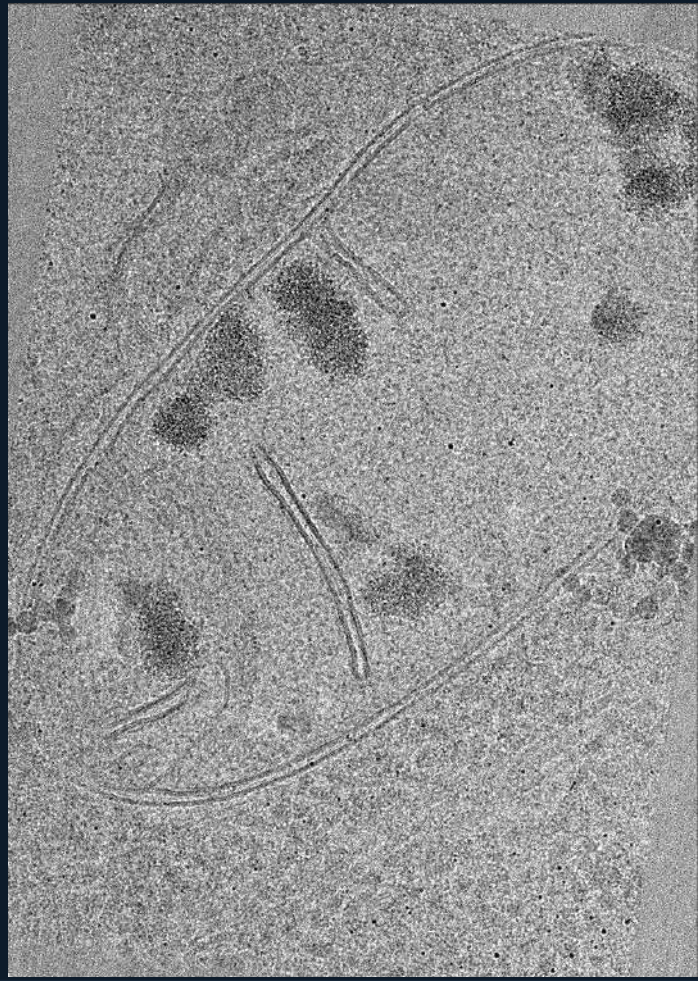
# Introduction to Cryo-Electron Tomography

Principles, Acquisition Physics & Sample Preparation
2026 winter cryo-EM course
Jake Johnston



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Motivation & biological context

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Projection theorem & missing wedge

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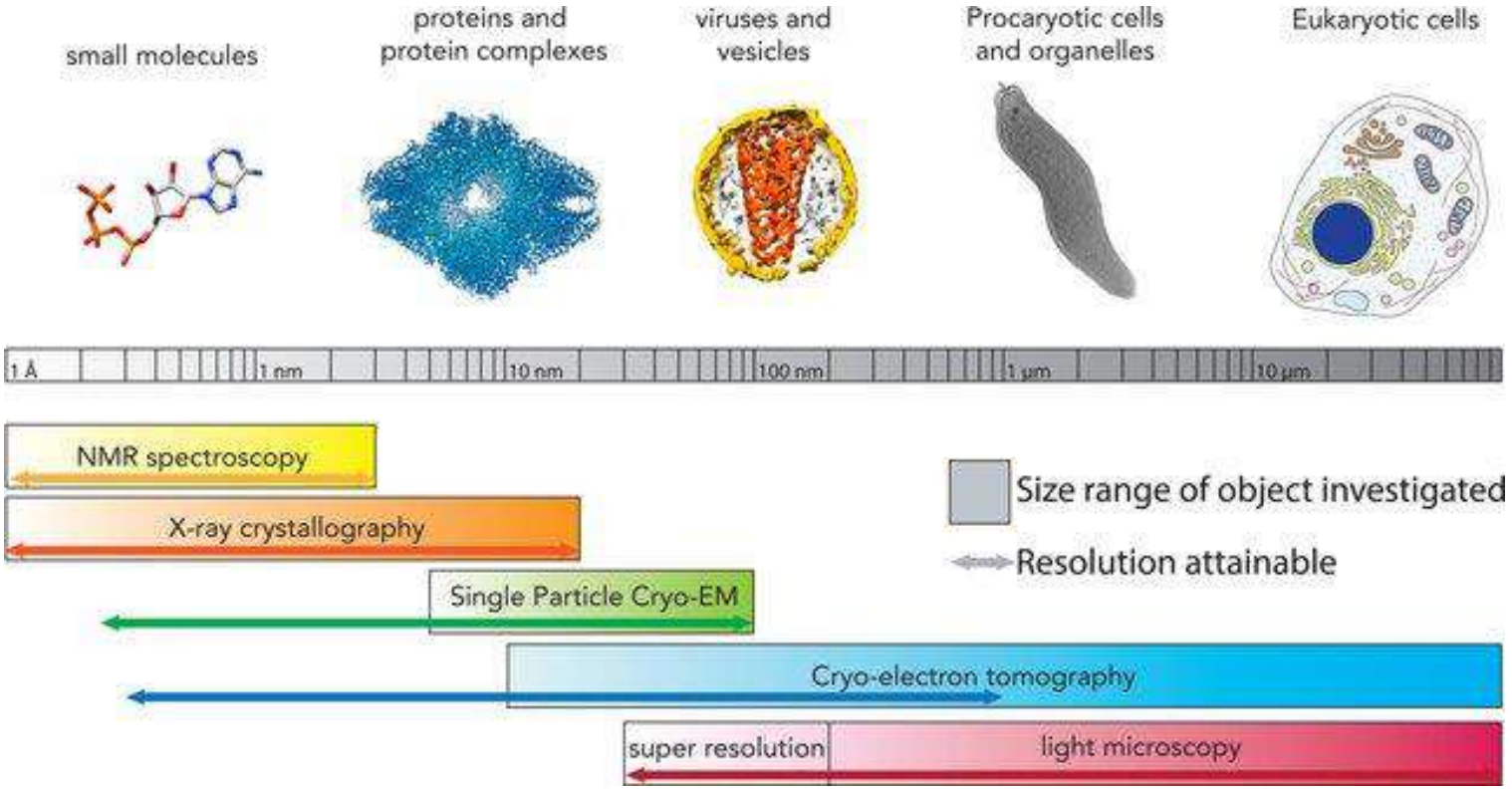
01

# Why Tomography?

Motivation & biological context

# Cryo-ET as a Tool for Structural Biology





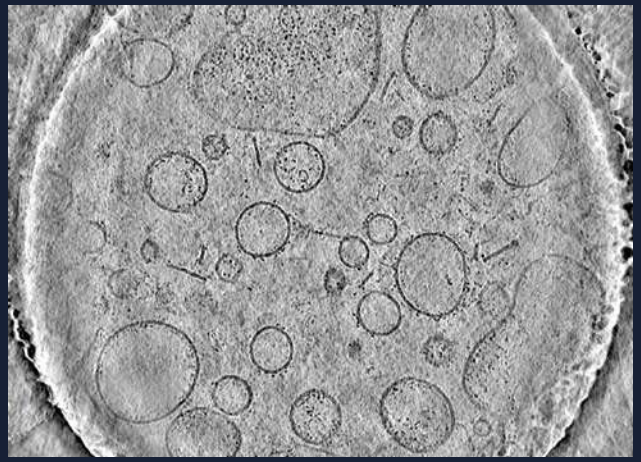



Hutchings and Zanetti, Biochemical Society Transactions, 2018

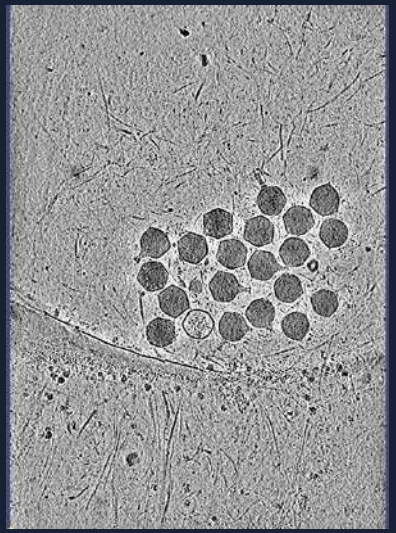
# Why Cryo-ET?



Cryo-ET is the **highest resolution method** for analyzing **native specimens**



Pleomorphic samples:
Protein-coated vesicles purified from human blood



Pleomorphic samples:
Brighton Beach ocean water

Pleomorphic samples:
Cellular lamella mitochondria

# The Limit of Single-Particle Cryo-EM



### Single-Particle EM

- **Resolves atomic structures of isolated complexes**
- Requires purified, conformationally homogeneous sample
- Averages thousands of particles → high SNR
- Loses cellular environment entirely
- Conformational states biased by purification

### What SPA Cannot Tell You

- Where is the complex in the cell?
- Who are its interaction partners in context?
- How is it organized natively within organelles?
- What is its native stoichiometry and local density?
- How do macromolecules interact with each other?

# Cryo-ET: Structure in Native Cellular Context

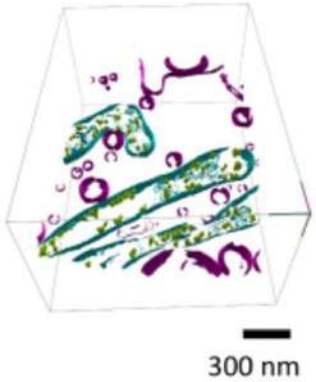


*"Molecular Sociology" — Baumeister (2002)*

## In Situ

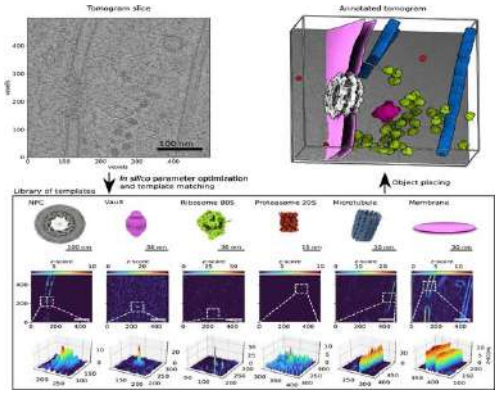

Macromolecular complexes imaged inside the cell, in their native lipid environment, without purification or chemical fixation

## Spatial Context




Reveals where complexes are located, how they cluster, and their relationships to membranes, organelles, and other macromolecules

## Visual Proteomics




Template-matching identifies and localizes many complex types simultaneously across the entire tomogram volume

*Cruz-León et al., Nat. Comms, 2024*

# Landmark Results — What Cryo-ET Has Revealed



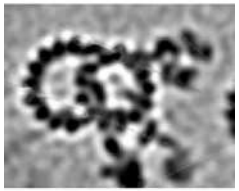
## Nuclear Pore Complex

Full architecture mapped in situ; ~110 MDa, 8-fold symmetry resolved in intact nuclei


*von Appen et al., Nature, 2015*

## Flagellar Motor

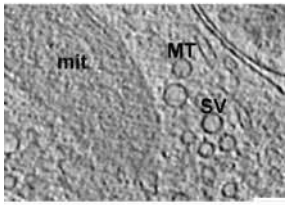
9-fold symmetry, conformational states of dynein arms during power stroke



*Nicastro et al., Science, 2006*

## Synaptic Vesicle Fusion

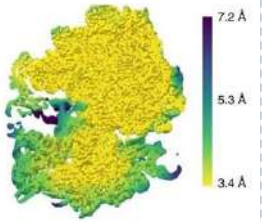
SNARE complexes, tethering factors mapped at the active zone in neurons




*Fernández-Busnadiego et al., JCB, 2010*

## Ribosome Landscape

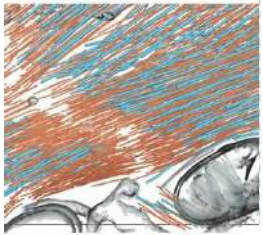
All ribosomes on ER membrane located and averaged simultaneously — 'ribosome atlas'




*Tegunov et al., Nat. Methods, 2021*

## Cytoskeletal Networks

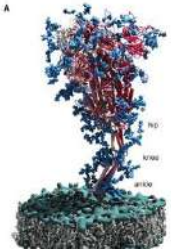
Actin-myosin organization in sarcomeres; MT-associated protein decoration in cilia



*Burbaum et al., Nat. Comms, 2021*

## Viral Entry

SARS-CoV-2 spike flexibility via three hinges; HIV spike conformations on intact virions



*Turoňová et al., Science, 2020*

# High-Resolution Tomography



Apoferritin

In-house sample

~15,000 particles
Octahedral symmetry

2.8 Å

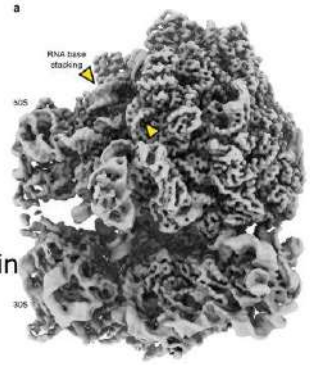
EMPIAR-10164 HIV

~13,000 particles, C6 symmetry

3.5 Å

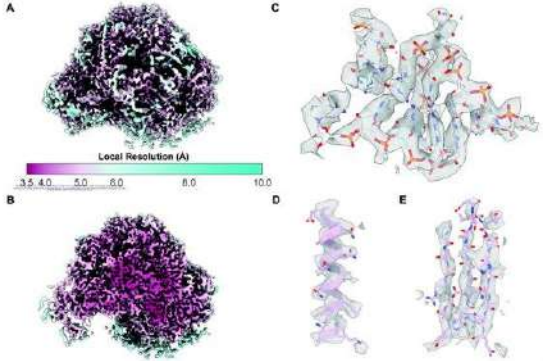
In-situ thin-cell

~18,000 particles
3.4 megadalton protein




3.4 Å

In-situ fib-milled



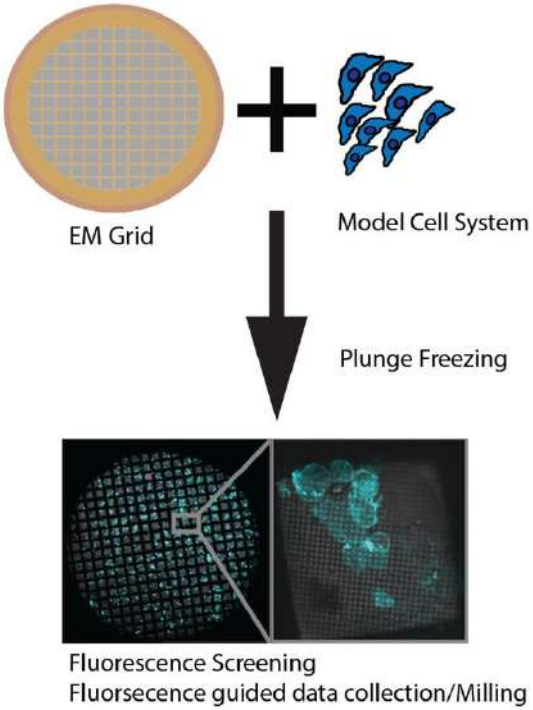

~120,000 particles
3.4 megadalton protein

3.6 Å

Tegunov et al., Nature Methods, 2021
Khavnekar et al., Biorxiv, 2022

# Cryo-ET Workflow





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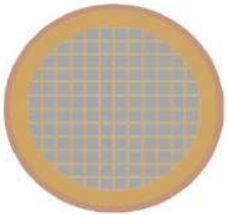
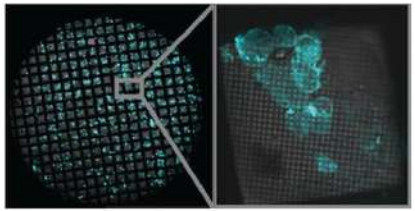


EM Grid

Model Cell System

Plunge Freezing

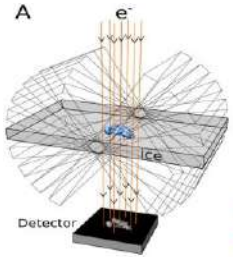


Fluorescence Screening
Fluorsecence guided data collection/Milling

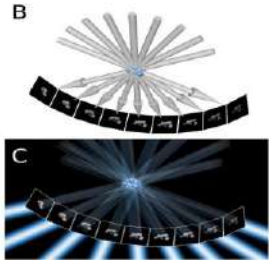
Samples thinner then
350 nm or less!

Tomography Data Collection

Krios 300 kv microscope



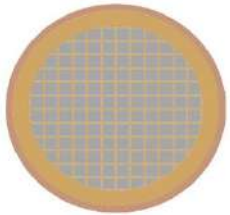

Tilt-series
Data Collection




Tomogram Reconstruction

# Cryo-ET Workflow

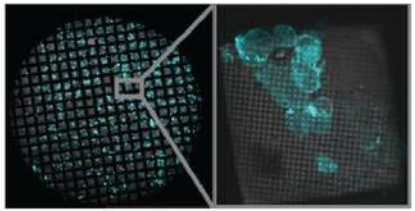




EM Grid

Model Cell System

Plunge Freezing



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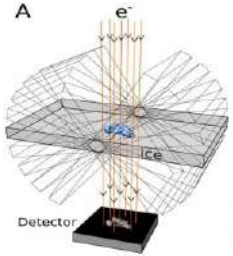
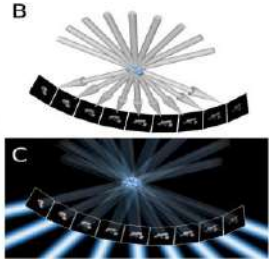
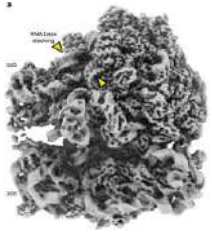



Tilt-series
Data Collection



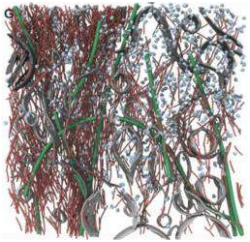

Tomogram Reconstruction

3D maps In-situ

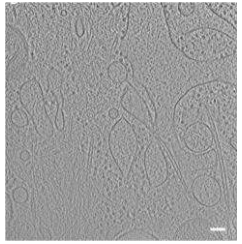


3D Particle Picking
Subtomogram Averaging

Segmentations



Morphology Analysis



Tomographic Slice of a Thin
Edge Sample

02

# Core Geometry

From 2D projection to 3D volume · ~12 min

# The Projection Theorem



- **A TEM image is a 2D projection of a 3D object**

  All depth information is collapsed into one plane

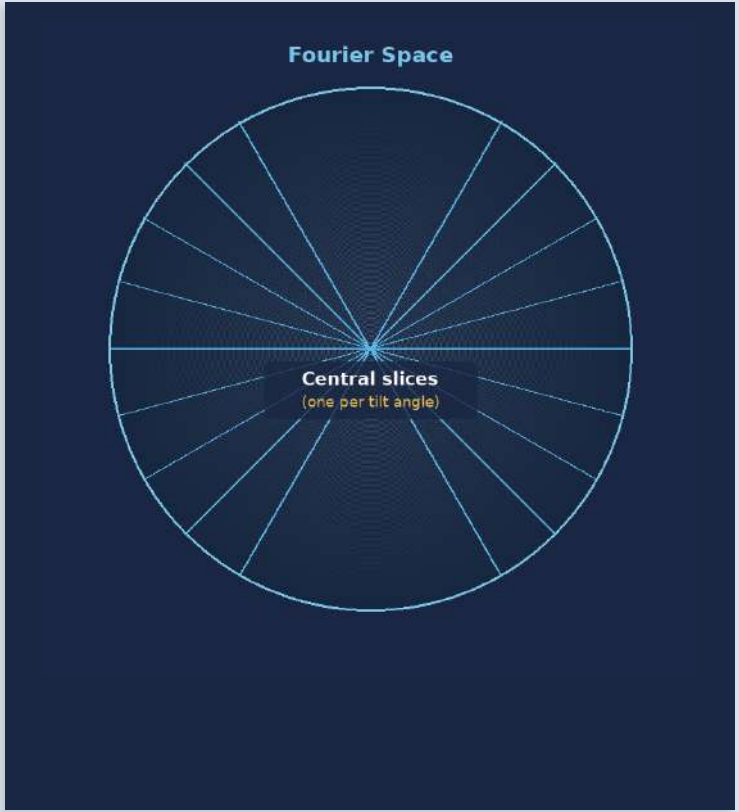
- **Projection theorem (central slice theorem):**

  A projection at angle θ = a central slice through the 3D Fourier transform at angle θ

  Rotating the specimen samples different slices of Fourier space

- **Therefore:**

  Collect many projections at different tilt angles
  Combine slices in Fourier space
  Inverse transform → 3D reconstruction



# The Missing Wedge

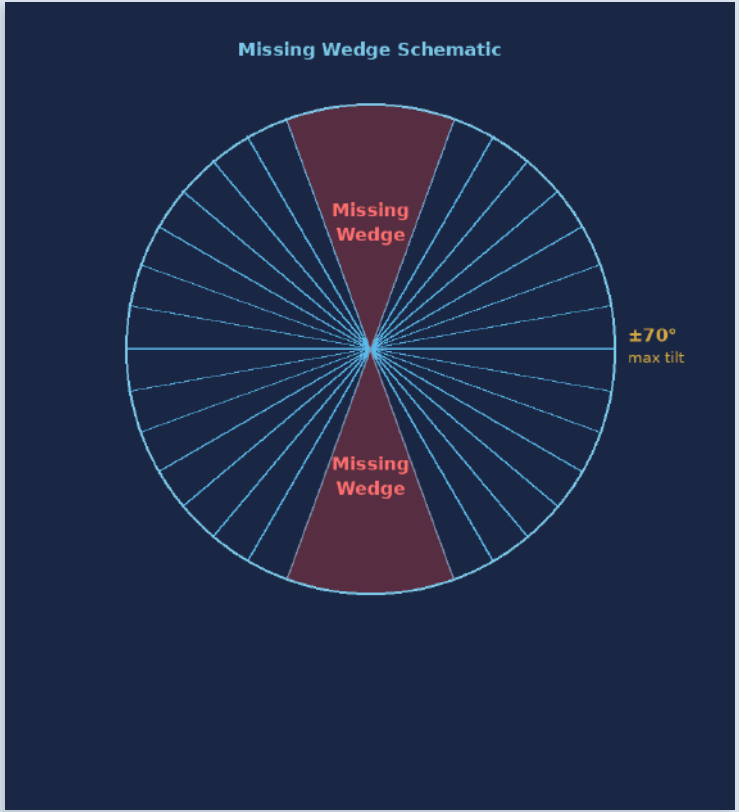


## Origin of the Problem

- Maximum tilt angle limited mechanically to ±60–70° by the pole-piece geometry and specimen holder
- A 'wedge' of Fourier space is never sampled — the missing wedge
- Larger tilt increments = coarser sampling but lower dose per image

## Consequences for Reconstructions

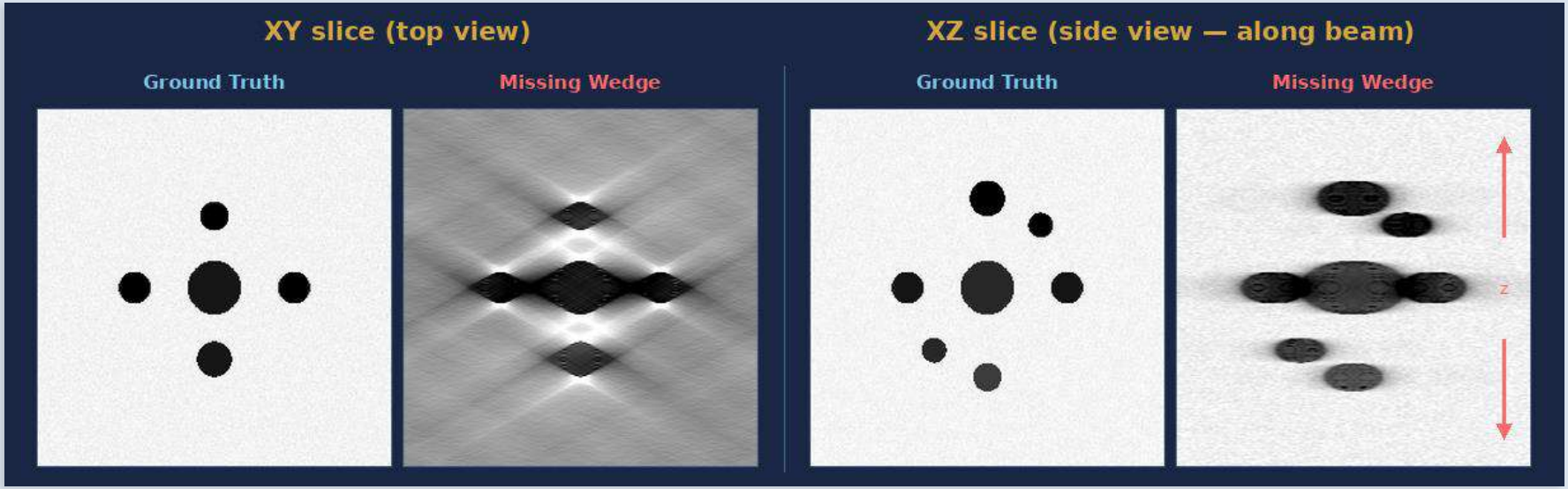
- Elongation artifact along the beam axis (z-axis stretching)
- Anisotropic resolution — worse in z than x/y
- Features parallel to the beam poorly represented
- Flat membranes perpendicular to tilt axis most affected



# Missing Wedge Effect on Reconstruction



*Simulated phantom: discrete spheres reconstructed with ±60° tilt range*

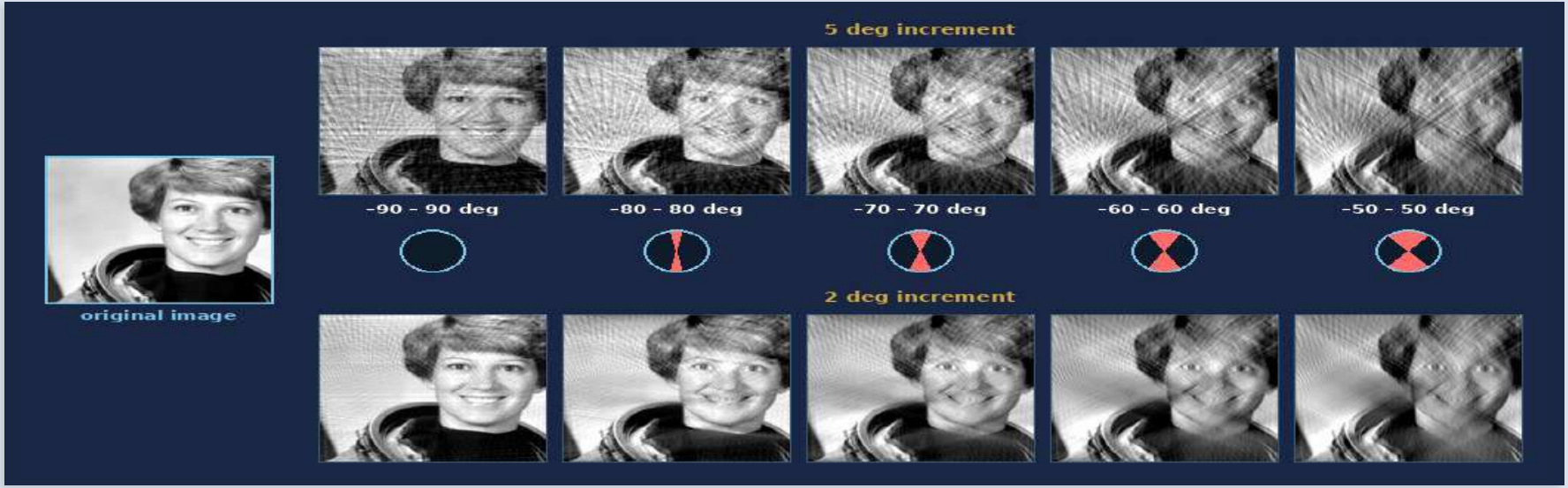



**Left:** XY slice shows characteristic diamond-shaped streaking artifacts. **Right:** XZ slice reveals dramatic z-axis elongation — spheres become eggs. Both artifacts arise from the same unsampled Fourier region.

# Missing Wedge Artifacts vs. Tilt Range



*Reconstructions at different tilt ranges and angular increments*




**Two effects compete:** Reducing tilt range increases the missing wedge and degrades resolution (left → right). Finer angular increments improve sampling within the accessible range (top → bottom). In cryo-ET, practical tilt range is ±60–70° with 2–3° steps.

# Cryo-ET vs. Medical CT

*Same principle, very different constraints*

| | Medical CT | Cryo-ET |
|---|---|---|
| **Radiation** | X-rays | Electrons |
| **Rotation** | Full 360° | ±60–70° max |
| **Dose limit** | None (relatively) | ~100–120 $e^{-}$/Å² total |
| **Specimen size** | Human body | ~100–300 nm lamella |
| **Images per volume** | 1000s of projections | 40–60 tilts |
| **Temperature** | Room temperature | –180°C (cryo) |
| **Resolution** | ~1 mm | ~1–5 nm (subtomogram avg) |

03

# Radiation Damage & the Dose Budget

The master constraint governing all acquisition decisions

# The Dose Budget: A Fixed and Precious Resource



- **Electrons are ionizing radiation**
  - Break chemical bonds
  - Generate free radicals
  - Cause mass loss and structural rearrangement
- **Total usable dose is fixed:**
  - ~100–120 $e^-/Å^2$ before information is irretrievably lost
- In SPA: entire budget on one image per particle → high SNR per micrograph
- **In cryo-ET: dose divided across every tilt angle in the series**

**~110 $e^-/Å^2$**
Total dose budget

**41–61**
Typical number of tilts

**~2–3 $e^-/Å^2$**
Dose per tilt image

*→ Each image is extremely noisy by design*

# What Low Dose Means in Practice



***~3 $e^-/Å^2$ per tilt image — compared to ~50 $e^-/Å^2$ for a typical SPA micrograph***

**Raw tilt images look terrible**

Individual frames appear almost entirely noise — features invisible without processing. This is normal and expected.

**SNR scales with dose**

Every tilt has the same low dose. High-tilt images are harder to align because features are foreshortened AND noisy.

**Early tilts are more informative**

Dose damage accumulates through the series. Images acquired later represent a more damaged specimen.

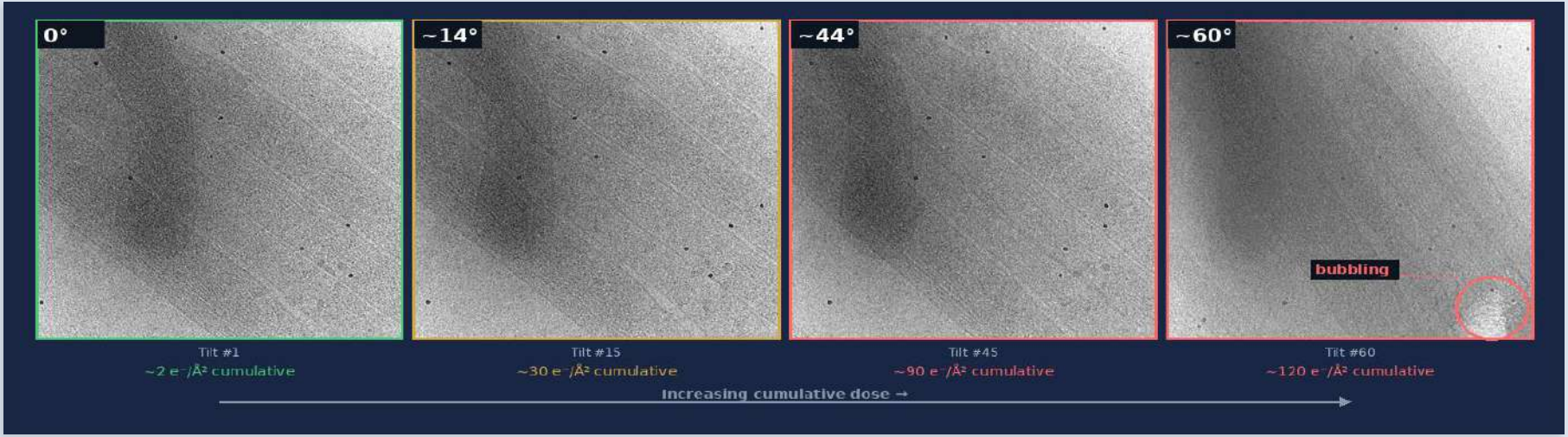
**Every acquisition parameter matters**

Tilt scheme, increment, defocus, magnification all affect how dose is spent. There are no free choices.

# Radiation Damage in a Real Tilt Series



*Dose-symmetric tilt series, 2.0 e⁻/Å² per tilt — same field of view at increasing cumulative exposure*




**What to notice:** Contrast decreases progressively as dose accumulates. By tilt #60 (~120 e⁻/Å² total), bubbling from radiolysis becomes visible: hydrogen gas from broken water molecules nucleates within the vitreous ice. This is irreversible structural damage, which is why every electron counts.

04

# Tilt-Series Acquisition

Physics, tilt schemes & practical parameters

# Tilt Schemes: How You Spend the Dose Budget



### Continuous

*–60° → 0° → +60°*

- One sweep, simple
- 0° imaged mid-series (damaged)

✗ **Not recommended**

### Bi-directional

*0° → +60°, then –3° → –60°*

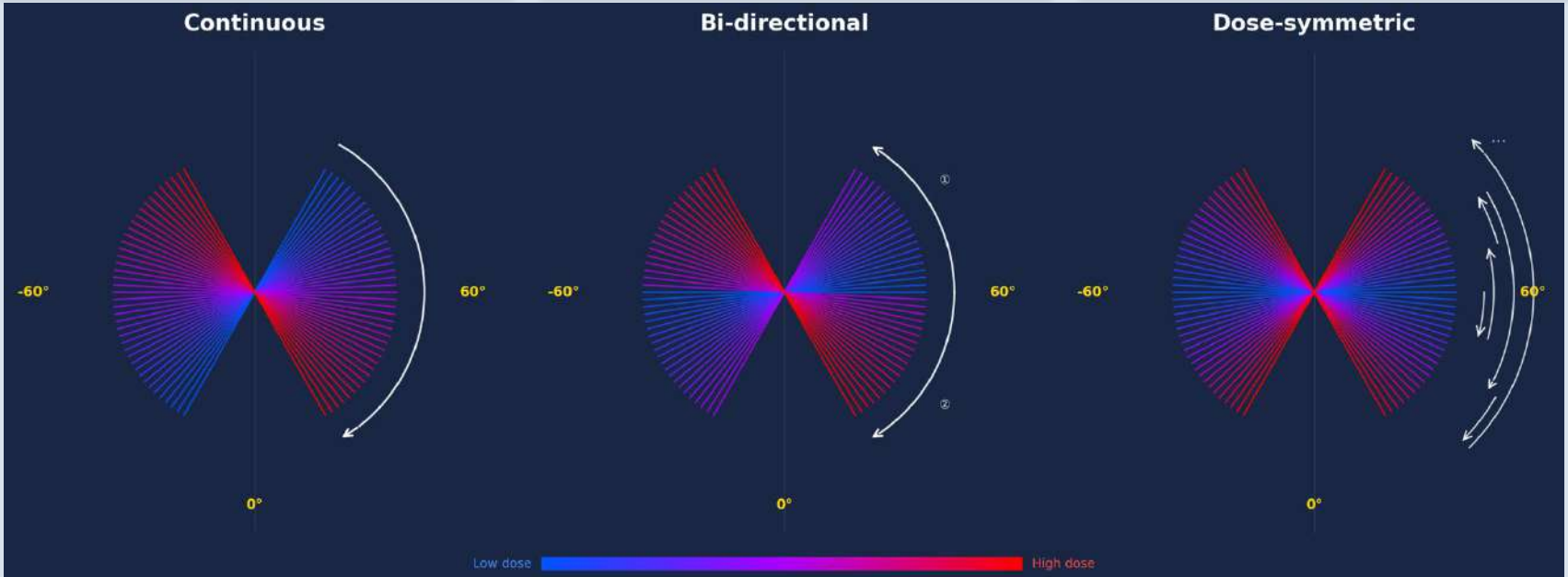
- Starts at 0°, two half-sweeps
- Better than continuous
- Second branch starts damaged

### Dose-Symmetric (Hagen 2017)

*0°, +3°, –3°, +6°, –6°, ...*

- Alternates ± around zero
- Low tilts get lowest dose

✓ **Current best practice**



# Key Acquisition Parameters



| | |
|---|---|
| Tilt Range<br>**±60–70°** | Limited by pole-piece geometry; beyond ~70° the specimen holder shadows the beam |
| Angular Increment<br>**2–3°** | Finer steps = better Fourier sampling but more images = less dose per image. Crowther criterion sets the minimum. |
| Total Tilts<br>**41–61** | Depends on tilt range and increment. 3° steps over ±60° = 41 images. |
| Defocus<br>**–2 to –4 µm** | Higher than SPA — needed for contrast in thick specimens. Pays a cost in CTF zero positions. |
| Pixel Size<br>**2–5 Å/px** | Higher magnification → finer sampling but smaller field of view. Balance with lamella size. |
| Total Dose<br>**100–120 $e^-/Å^2$** | Fixed biological constraint. Distribute evenly or weight toward low tilts. |

# Detectors & Energy Filtering



## Direct Electron Detectors

- Essential for low-dose cryo-ET
- Electron counting mode — detect individual electron events
- High DQE at low electron doses vs. film or CCD
- Fast readout: capture multiple frames per tilt position (movie mode)
- Motion correction on frame stacks within each tilt

## Energy Filtering (EFTEM / EF-TEM)

- **Far more important in cryo-ET than SPA**
- Thick specimens (~150–300 nm) produce abundant inelastic scattering
- Inelastic electrons carry no useful structural information — only add noise and blur
- Zero-loss peak (ZLP) filtering selects only elastically scattered electrons
- Can double effective contrast in lamella-thickness specimens
- Now standard on most cryo-ET microscopes (Falcon 4, K3, BioQuantum)

# Automation & High-Throughput Acquisition



*Historically: one tilt series per hour, manually supervised. Today: 50–70+ per day at modern facilities.*

1

**Grid Screening & Square Selection**

Automated quality assessment of ice thickness, contamination, and lamella integrity across the grid

2

**Target Identification**

PACE-tomo, tomo5, and SerialEM scripts identify multiple acquisition positions per stage location

3

**Beam-Image Shift Acquisition**

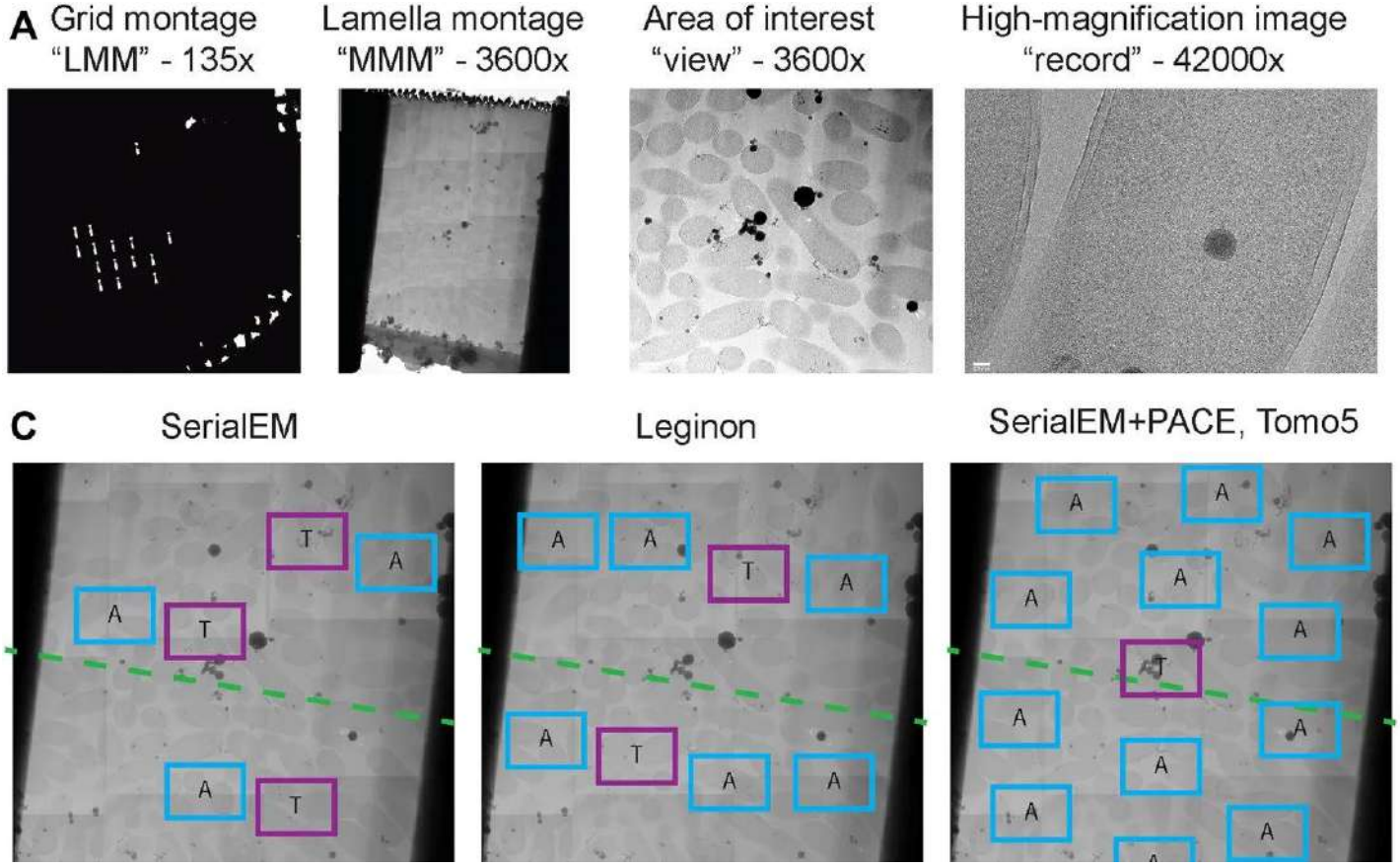
Multiple tilt series acquired per stage move using beam deflection — avoids slow stage repositioning between targets

4

**On-the-Fly Preprocessing**

Motion correction, CTF estimation, and tilt-series alignment begin during acquisition for rapid quality control

# Data Collection Overview




Single tracking/focusing area for each acquisition area

Single tracking/focusing area for a group of acquisition areas along the tilt axis

Single tracking/focusing area for multiple acquisition areas

05

# Sample Preparation

Vitrification, FIB-milling & grid considerations

# The Thickness Problem: The Central Constraint



**Inelastic scattering increases with specimen thickness. Beyond ~300–400 nm, contrast is destroyed.**

| | |
|---|---|
| **Purified complex on grid**<br>5–15 nm | ✅ Ideal for cryo-ET |
| **Thin bacterial cell**<br>~200–500 nm | ⚠️ Borderline — depends on region |
| **Yeast / small cell**<br>~2–5 µm | ❌ Too thick → FIB-milling required |
| **Mammalian cell**<br>~5–15 µm | ❌ Way too thick → FIB-milling required |
| **Tissue section**<br>10–100 µm | ❌ FIB-liftout or cryo-sectioning |

# Plunge Freezing: Thin Specimens



## When Plunge Freezing Works

- Purified macromolecular complexes
- Small bacteria (mycoplasma, thin E. coli edges)
- Thin cellular extensions: axons, lamellipodia, filopodia
- Viruses and virus-like particles
- Extracellular vesicles

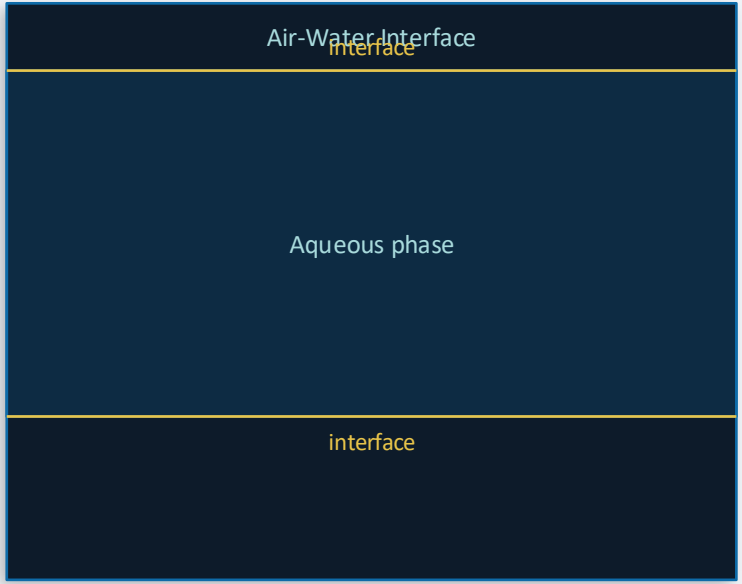
## What's Different vs. SPA

- **Gold fiducials required for tilt-series alignment**
  - 10 nm colloidal gold beads added to sample or grid
  - Provide landmarks for accurate alignment in 3D
- Grid stability matters more
  - Repeated electron exposure across full tilt series stresses the support film
  - Gold foil (Au Quantifoil) preferred over carbon
- Ice thickness is more critical
  - Must be thin enough across the full tilt range, including at high tilt angles where path length increases

# The Air-Water Interface Problem



- **During blot-and-plunge, the thin film forms an air-water interface on both surfaces**
- Proteins adsorb to and partially denature at the interface
  - Hydrophobic patches exposed at the interface
  - Partial unfolding and conformational heterogeneity
  - Preferred orientation artifacts — same problem in SPA
- Structural damage is proportional to time at the interface
- Relevant in cryo-ET for in vitro reconstituted systems placed on grids




Encapsulation strategies address this directly — nanocrate technology (MS2 viral capsids) sequesters proteins in an aqueous interior, shielded from the air-water interface. This is particularly powerful for labile complexes and in cryo-ET sample prep where the interface damage is often severe.

# Cryo-FIB Milling: The Transformative Technique



***FIB-milling allows cryo-ET of any cell type by thinning the specimen to electron-transparency in situ***

1

**Load cryogenic grid into FIB-SEM**

Cryo-transfer under vacuum and cold conditions. SEM provides surface imaging for navigation.

2

**Coat specimen surface**

Organometallic platinum deposited by GIS (gas injection system). Protects surface, reduces charging.

3

**Rough milling — remove bulk material**

$Ga^+$ or Xe plasma ion beam removes material above and below the ROI. Trenches formed on both sides.

4

**Fine milling — thin the lamella**

Progressive thinning to 100–200 nm. Requires skill — too thin = fragile; too thick = poor contrast.

5

**Transfer to TEM for cryo-ET**

Cryo-transfer the grid. The lamella is now electron-transparent and ready for tilt-series acquisition.

# FIB-Milling: Artifacts & Targeting with Cryo-CLEM



## Common FIB Artifacts

- Curtaining
  - Parallel striping pattern along milling direction
  - Caused by density variations deflecting ion beam
  - Reduced by tilting stage and using platinum coat
- Amorphous surface damage layer
  - ~10–20 nm of ion-beam-amorphized material on both faces
  - Not vitreous ice — limits interpretable depth
- Redeposition
  - Milled material re-deposits on the lamella surface
  - Managed by sputter coat and cleaning steps
- Charging
  - Insulating biological material accumulates charge
  - Causes image drift and beam deflection

## Targeting with Cryo-CLEM

- **Cryo-fluorescence microscopy identifies ROI before FIB milling**
- Fluorescently labeled protein/organelle → pinpoint location in cell
- Coordinate transfer: overlay fluorescence on SEM image
- Mill exactly the right cell region — critical for rare events or specific organelles
- Integrated cryo-CLEM systems
  - FLM stage in FIB-SEM (e.g., SECOM, Meteor)
  - Separate cryo-LM → FIB-SEM workflow
- Without CLEM: milling is often blind — high failure rate for targeting

06

# What Comes Next

A tilt series → handoff to the processing lecture

# What You Get: A Tilt Series



- **A tilt series is a stack of ~41–61 images at different angles**
- Each individual frame looks almost entirely like noise — ~2–3 $e^{-}/Å^{2}$ per image
- Features only become visible after alignment and reconstruction
- The raw data is the input to everything that comes next

## What Processing Will Do (Next Lecture)

1. Tilt-series alignment & fiducial tracking
2. CTF estimation & correction
3. Weighted back-projection / SIRT reconstruction
4. Denoising & segmentation
5. Subtomogram averaging → high-resolution structures

# Key Takeaways

01 Cryo-ET is unique in its ability to image macromolecular complexes in their native cellular environment — no purification, no fixation

02 The projection theorem provides the mathematical foundation — tilting samples Fourier space, but the missing wedge imposes fundamental limits on 3D information

03 The dose budget (~110 $e^-/Å^2$ total) is the master constraint — divide across 41–61 tilts means ~2–3 $e^-/Å^2$ per image. Raw data will look terrible.

04 Tilt scheme, detector, and energy filter choices directly determine the quality of information recoverable from the data

05 Sample thickness is the central sample prep challenge — FIB-milling revolutionized the field by making any cell type accessible

*Next lecture: Tilt-series processing pipelines — alignment, reconstruction, CTF correction, subtomogram averaging*