LECTURE 2

Cryo-Electron Tomography

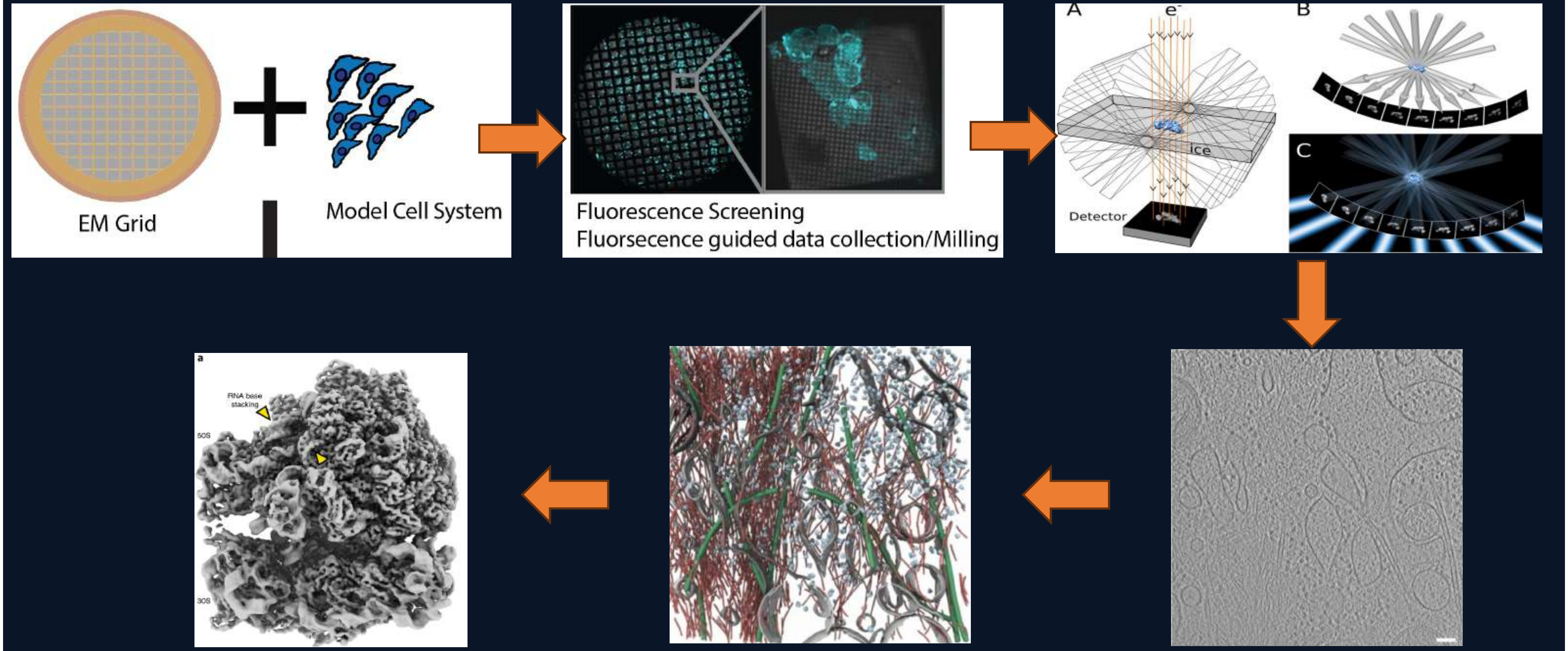
Image Processing Pipeline

Motion Correction · CTF · Reconstruction · Denoising · Missing Wedge · STA · Segmentation

Lecture Overview

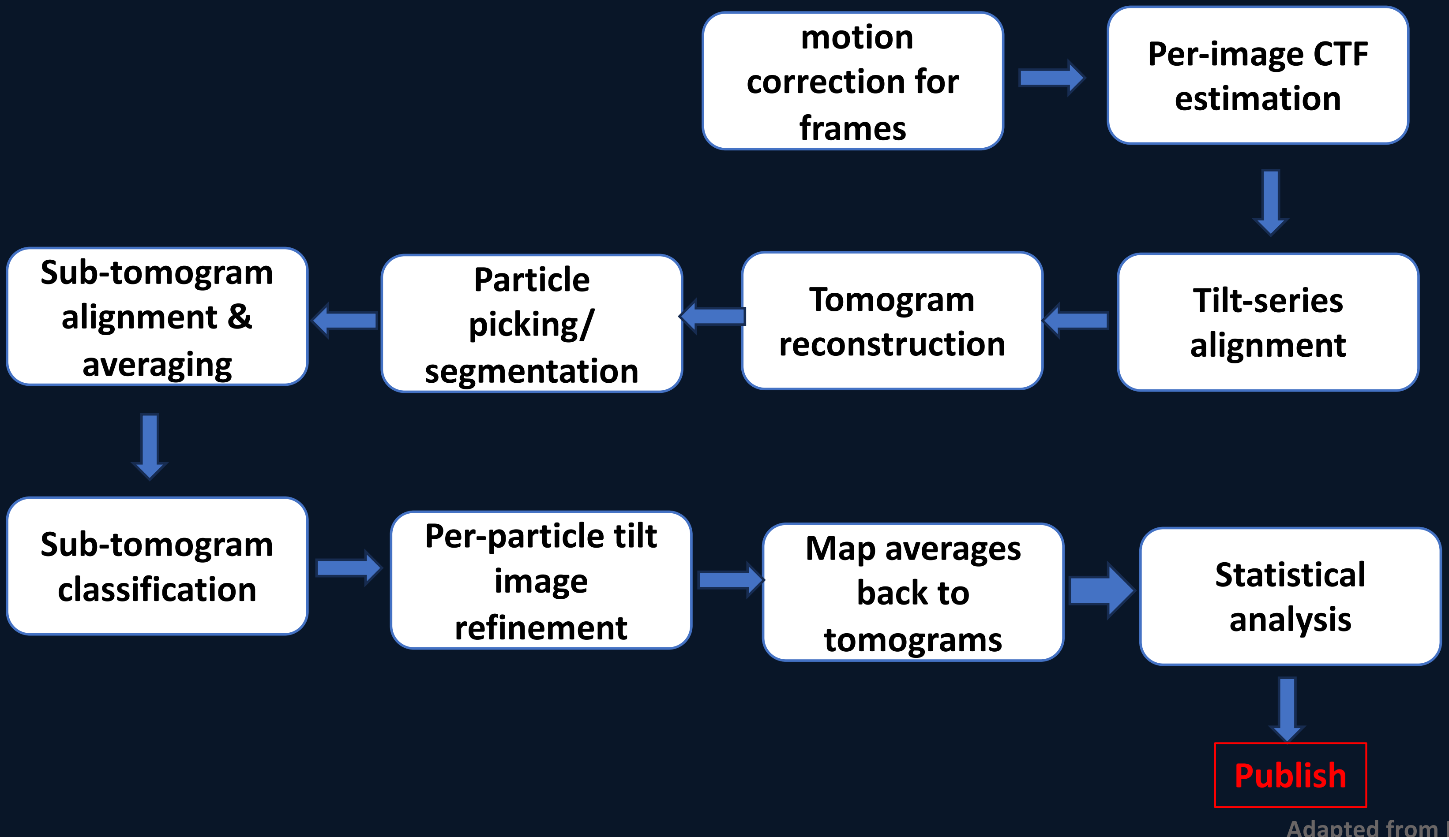
01	**From Tilt Series to Data**	Motion correction & preprocessing	~8 min
02	**CTF Estimation & Correction**	Contrast transfer function	~10 min
03	**Tomogram Reconstruction**	WBP, SIRT & iterative methods	~12 min
04	**Denoising**	Filtering, DL & cryo-CARE	~10 min
05	**Missing Wedge Correction**	Fourier constraints & AI methods	~8 min
06	**Subtomogram Averaging**	Alignment, classification & resolution	~15 min
07	**Segmentation**	Manual, automated & deep learning	~10 min
08	**Software Landscape**	Handoff & practical guide	~5 min

Tomography workflow



Fluorescence Screening
Fluorsecence guided data collection/Milling

Generalized Workflow for Cryo-ET Processing



motion correction for frames

Per-image CTF estimation

Tilt-series alignment

Tomogram reconstruction

Particle picking/ segmentation

Sub-tomogram alignment & averaging

Sub-tomogram classification

Per-particle tilt image refinement

Map averages back to tomograms

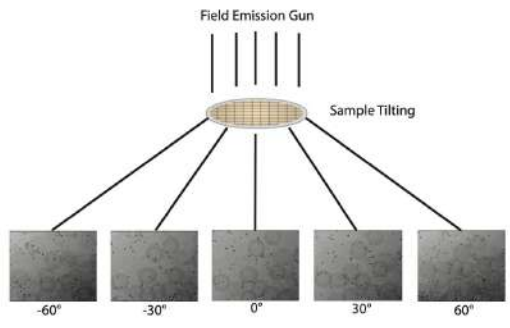
Statistical analysis

Publish

Adapted from Leigh et al., 2019

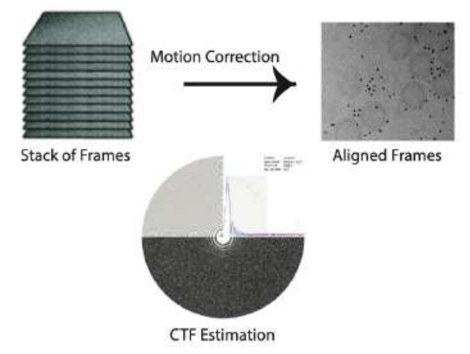
Tomography Software Packages

TS Acquisition



Serial EM, Tomo5

Motion Correction/CTF Estimation



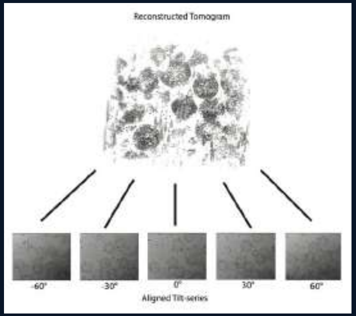
MotionCorr, UnBlur, MotionCor2, alignparts_lmbfgs, Zorro / Xmipp, CTFplotter

CTFFIND4/5,Gctf, IMOD (CTFPlotter), Warp, emClarity

Tilt-Series Alignment

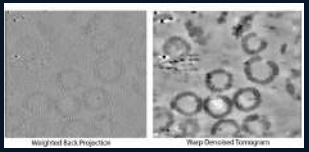
Imod, Raptor, EMAN2, Dynamo, UCSF tomo, Protomo, TomoAlign, AreTomo, Xmip

Tomogram Reconstruction



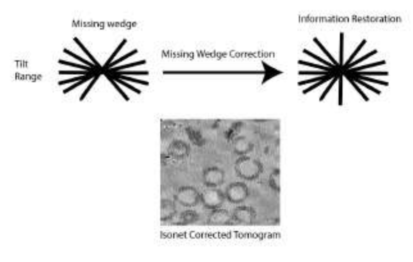
Imod, Raptor, AuTom, EMAN2, Dynamo, UCSF tomo, Protomo, TomoAlign, AreTomo, TOMO3D

Denoising



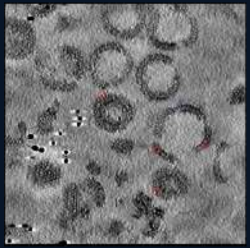
Warp-denoise, Topaz Denoise, CryoCare

Missing Wedge Correction



IsoNet, REST

3D-Particle Picking

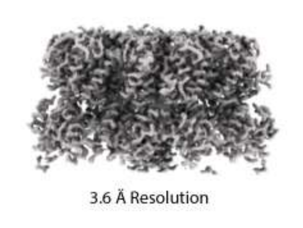


Cryolo, EMAN2, PyTom, Dynamo, StopGap, Relion5, NextPYP, Scipion, DeepETPicker, DeepFinder, TomoNet, AiS, TomoTwin, DeePict, PySeg

Sub-tomogram Ab-initio-Averaging/Classification

Dynamo, Relion, PEET, NextPYP, EmClarity

Sub-Tilt Refinement

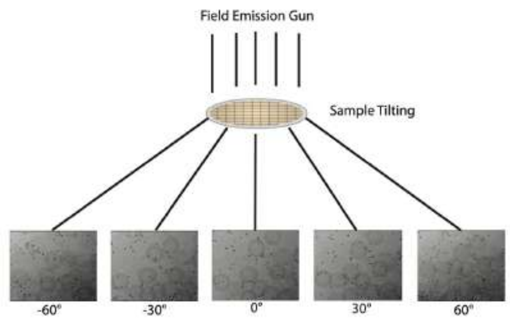


M, Relion4/5, NextPYP, EmClarity

A localized list of software packages available for each step
https://github.com/phonchi/Computational-CryoET

Tomography Software Packages

TS Acquisition



Serial EM, Tomo5

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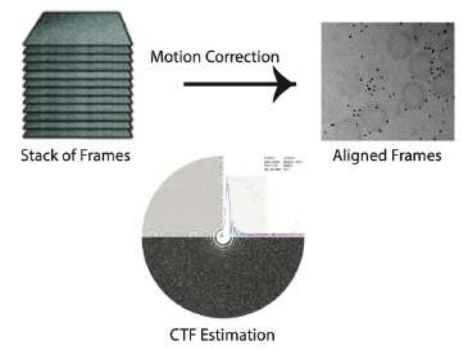

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CTFFIND4/5,Gctf, IMOD (CTFPlotter), Warp, emClarity

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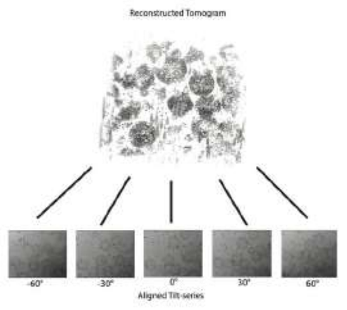
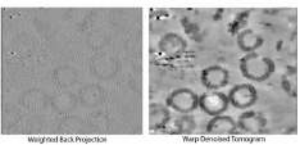
Tomogram Reconstruction



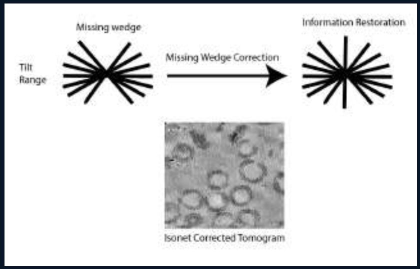
Imod, Raptor, AuTom, EMAN2, Dynamo, UCSF tomo, Protomo, TomoAlign, AreTomo, TOMO3D

Denoising



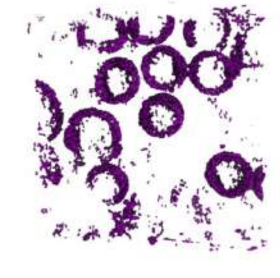
Warp-denoise, Topaz Denoise, CryoCare

Missing Wedge Correction



IsoNet, REST

Segmentation



TomomemSegTV, MemBrain, Tardis, Amira, OrsDragonfly, EMAN2

Software containing full/almost full sub-tomogram averaging workflows

Warp/Relion/M, Relion4/5 Tomography, TomoBear, NextPYP, EMAN2

A localized list of most of the software packages available for each step
https://github.com/phonchi/Computational-CryoET

01

Motion Correction & Preprocessing

From raw tilt frames to an aligned tilt series

Motion Correction

Beam-Induced Motion

Electrons deposit energy → specimen moves during exposure. Each frame slightly shifted relative to others.

Frame-Level Correction

Each tilt position acquired as a movie stack (10–40 frames). Frames aligned and averaged → motion-corrected image per tilt.

Tools

MotionCor2 (GPU-accelerated), IMOD alignframes, Relion own implementation. Patch-based correction handles local beam-induced motion.

3-6

Frames per tilt

~1–50 Å

Typical drift magnitude

2–3 $e^-/Å^2$

Dose per frame stack

Dose Weighting

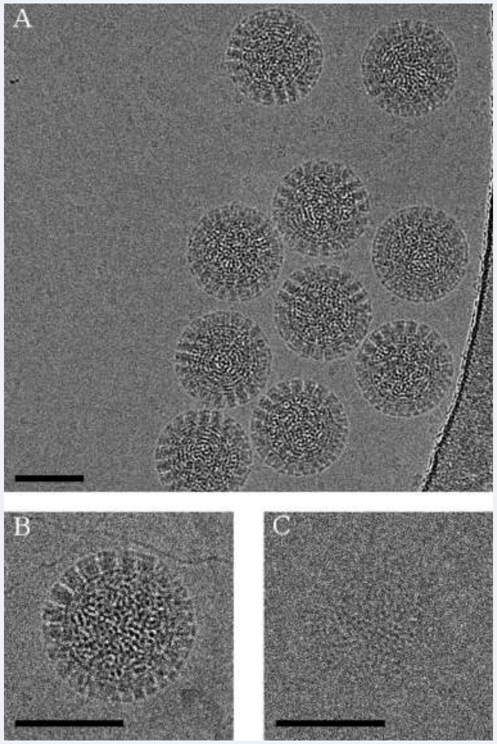
Critical for cryo-ET: early frames are least damaged, so they carry more structural information. Dose weighting applies a resolution-dependent filter (Grant & Grigorieff 2015) to weight frames by their accumulated dose before summation.

Tilt-Series Alignment

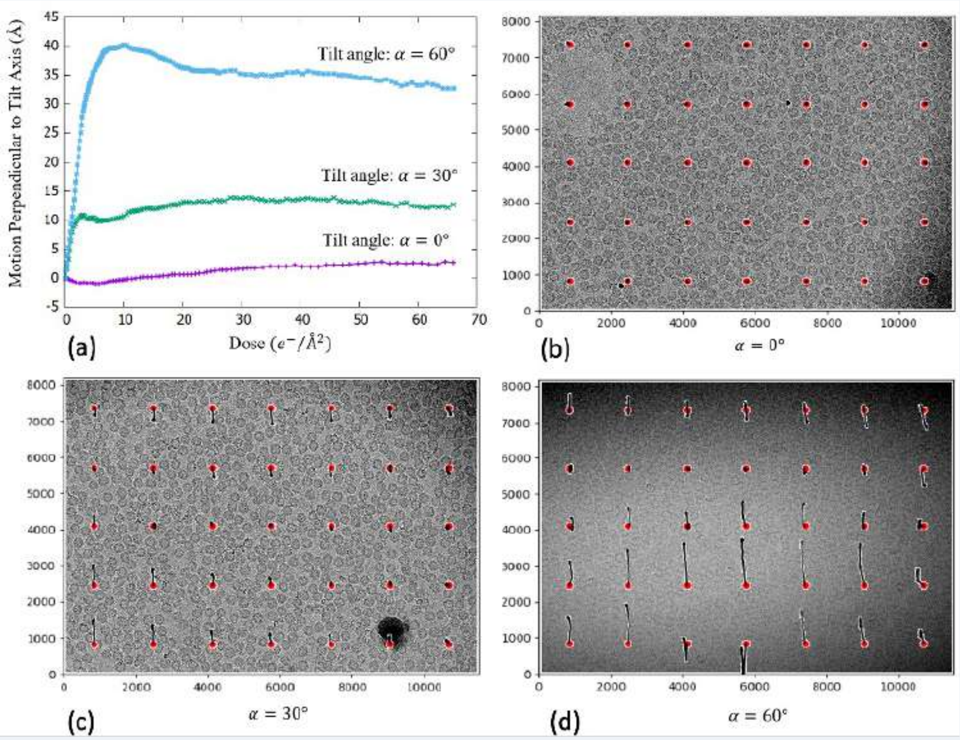
After per-tilt motion correction: fiducial-based (gold beads) or fiducial-free (cross-correlation) alignment of the full tilt series using IMOD or AreTomo.

Motion Correction

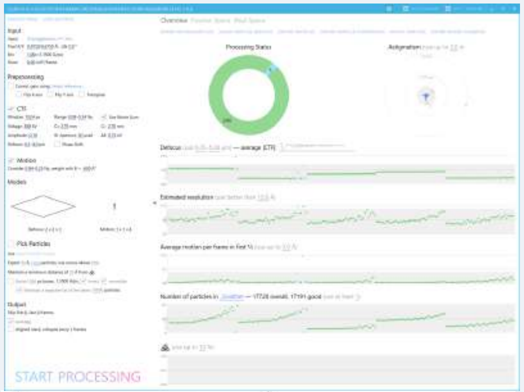
Unblur

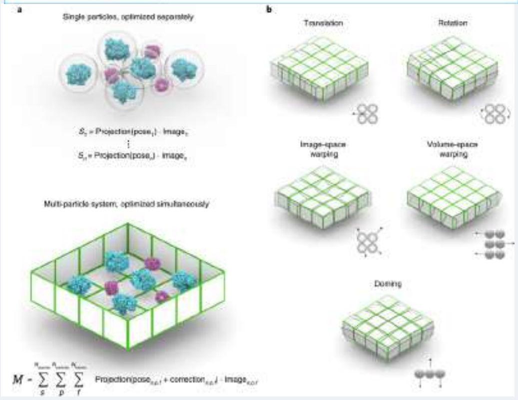


Motioncorr



(Warp)





02

CTF Estimation & Correction

Restoring information lost to lens aberrations

Contrast Transfer Function (CTF)

What is the CTF?

The CTF describes how the electron microscope modulates image contrast as a function of spatial frequency. It oscillates between positive and negative, causing some frequencies to be enhanced and others suppressed or reversed.

Why is it worse in cryo-ET?

Thick specimens require high defocus (−3 to −6 µm) for contrast — but this pushes the first zero of the CTF to lower resolution, limiting interpretable information. Each tilt angle also has a slightly different defocus.

Per-Tilt CTF Estimation

CTFFIND4, Warp, Imod, or Gctf estimates defocus per tilt image. High tilt angles are particularly challenging due to specimen tilt and astigmatism changes. 3D CTF models account for defocus gradient through the lamella depth.

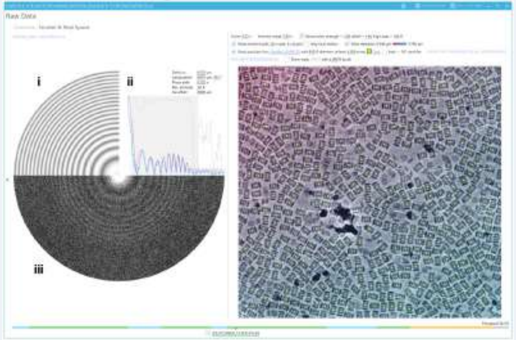
CTF Correction Strategies

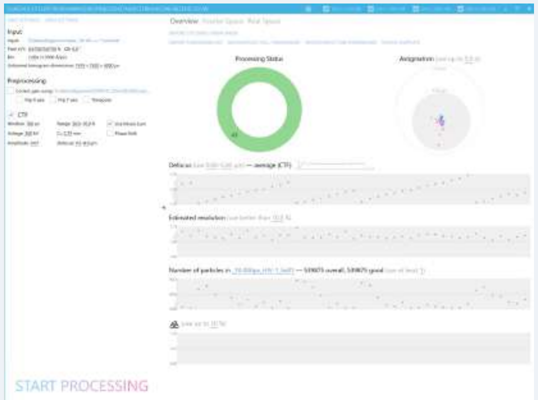
Phase flipping (sign correction only) or Wiener filtering (amplitude restoration). Applied before or during reconstruction. CTF correction is essential for subtomogram averaging beyond ~10 Å resolution. One can use Warp, Aretomo, IMOD or Dynamo

Contrast Transfer Function (CTF) – tilt-series

- Ideally, Accurate high-resolution estimation (3-4 angstroms) should be seen
- Local CTF estimation
- Estimates tilt axis angle per image
- Determines handedness
- Refines based on whole tilt-series
- Corrects for CTF
- Local CTF refinement

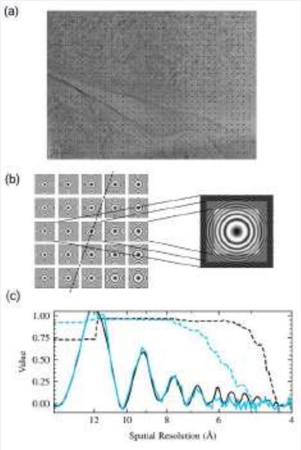
(Warp)

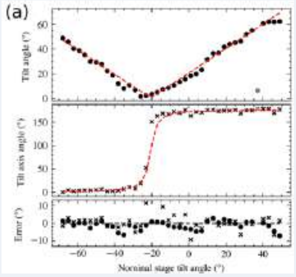


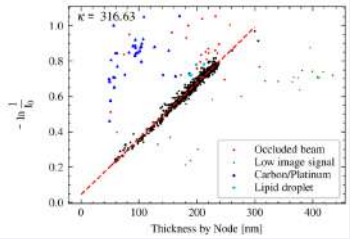


Tegunov et al., 2019, 2021

CTFFIND 4/5







Elferich et al., 2023

03

Tomogram Reconstruction

From a tilt series to a 3D volume

Reconstruction Algorithms

Standard

Weighted Back-Projection (WBP)

- Project each tilt image back into 3D volume along acquisition angle
- Apply ramp filter in Fourier space to compensate for uneven sampling density
- Fast — minutes on modern hardware
- Sensitive to missing wedge and noise
- Default in IMOD; still widely used

Better quality

SIRT / Iterative Methods

- Simultaneous Iterative Reconstruction Technique
- Forward-project current estimate → compare to real data → update
- Significantly suppresses streak artifacts
- Computationally expensive (many iterations)
- Implemented in IMOD, Tomo3D, ASTRA

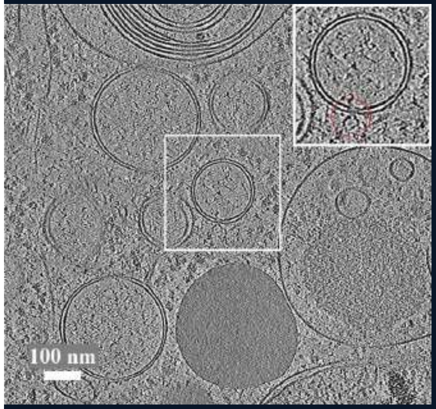
Emerging

Deep Learning Reconstruction

- IsoNet: reconstructs missing wedge using learned priors
- Neural network trained on same tomogram (self-supervised)
- DeepETPicker, TomoTwin for integrated pipelines
- Dramatically improved contrast and isotropy
- Requires GPU; still maturing for routine use

Tilt-series alignment

Fiducial-less

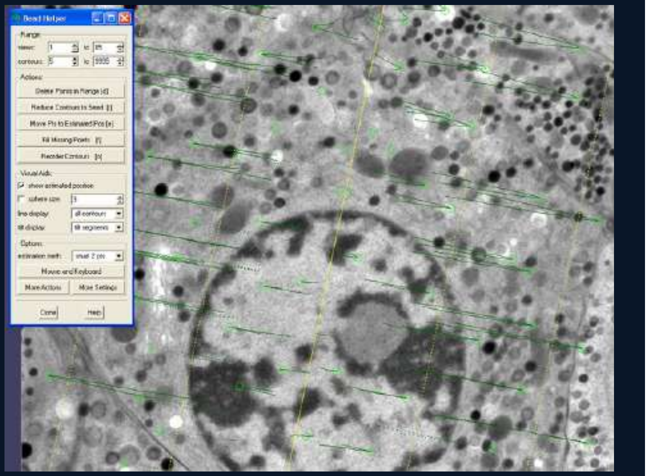


Zheng et al., 2022

- No gold beads
- AreTomo utilizes projection method for alignment
- Local alignment
- Aretomo3 includes CTF estimation, and frame alignment
- Fast – a couple minutes, does not require powerful Gpus
- Critical Parameters to tune– AlignZ, increase or decrease based on sample thickness
- Some software, such as imod, utilize patch tracking for tiltseries alignment

Fiducial Alignments

IMOD (and other gold bead tracking software)



David Mastronarde

- Requires a sufficient number of well-behaved gold beads
    - Sample prep optimization
- Semi-automated in IMOD
- Automated in other workflows

04

Denoising

Recovering signal from the noise floor

Denoising Approaches

~2–3 $e^-/Å^2$ per tilt image → each tomogram is extremely noisy by design. SNR ≈ 0.01–0.1 before denoising. All downstream analysis depends on quality of denoising.

Classical Filters

- Gaussian blur: fast but destroys edges
- Median filter: good for salt-and-pepper noise
- Anisotropic diffusion: edge-preserving
- Non-local means (NLM): patch-based averaging
- **Best for: quick visualization only**

cryo-CARE (Content-Aware Restoration)

- Self-supervised deep learning (Buchholz 2019)
- Split each tilt movie into odd/even frames → two independent tomograms
- Neural network (U-Net) trained to predict one from the other
- Learns signal structure without clean reference
- **State of the art for cryo-ET; now standard practice**

Topaz-Denoise

- Generalizable neural network pre-trained on cryo-EM data
- Works without paired training data
- Applies noise2noise principle across different micrograph regions
- Fast inference; can run on single tomogram
- **Available in standalone or within EMAN2/Topaz pipeline**

IsoNet

- Simultaneously denoises AND corrects missing wedge
- Self-supervised training on the tomogram itself
- Iteratively fills in missing Fourier information
- Most computationally expensive option (~hours on GPU)
- **Yields near-isotropic resolution; major improvement for STA**

05

Missing Wedge Correction

Recovering unsampled Fourier space

Missing Wedge: Problem & Solutions

The Problem

- Max tilt ±60–70° → ~40–60° of Fourier space never sampled
- Elongation artifact along beam axis (z-stretching ~30%)
- Anisotropic resolution: ~2–3× worse in Z than XY
- Flat membranes parallel to beam: worst affected
- Template matching biased toward features sampled in wedge

Constrained Optimization

PPCT, ART: use prior information (non-negativity, support constraints) to iteratively fill missing wedge. Limited success for biological specimens.

IsoNet (Deep Learning)

Self-supervised CNN trained on the tomogram. Learns volumetric features and extrapolates missing Fourier data. Near-isotropic results; best available method.

Symmetry Exploitation

When particle or structure has symmetry, multiple orientations fill the wedge during subtomogram averaging. Most powerful

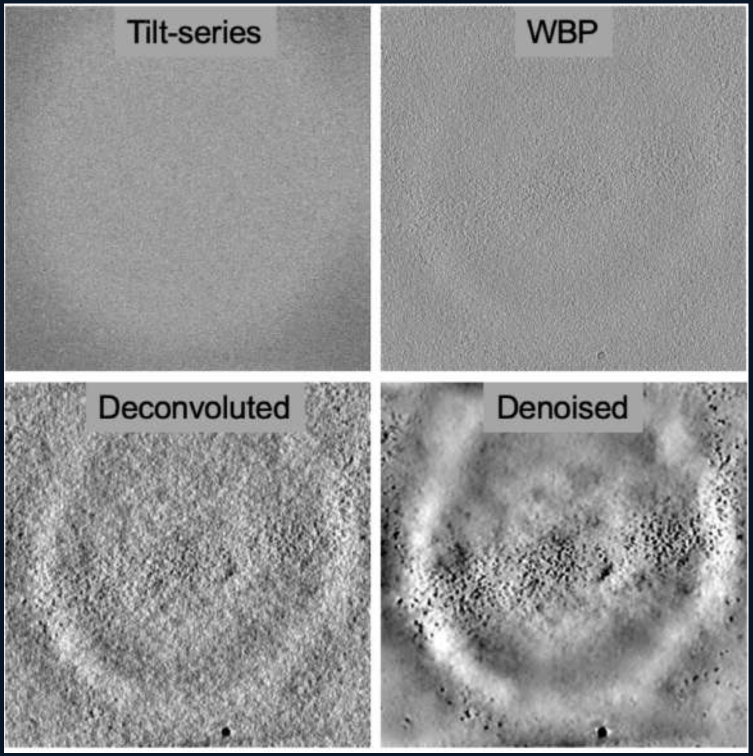
Dual-Axis Tomography

Collect two tilt series perpendicular to each other → combine to reduce missing region to a 'missing pyramid'. Doubles acquisition time;

Impact on Subtomogram Averaging

During STA, particles are collected in many orientations. When averaged, different particles fill each other's missing wedge — this is the primary mechanism for recovering near-isotropic resolution. Wedge masking in alignment and averaging is critical: without it, the missing wedge biases cross-correlation and introduces alignment errors.

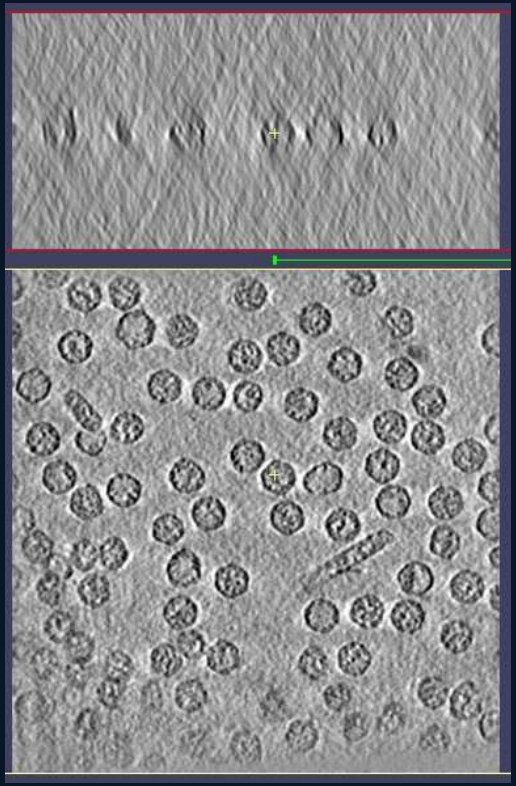
Tomogram Denoising Approaches



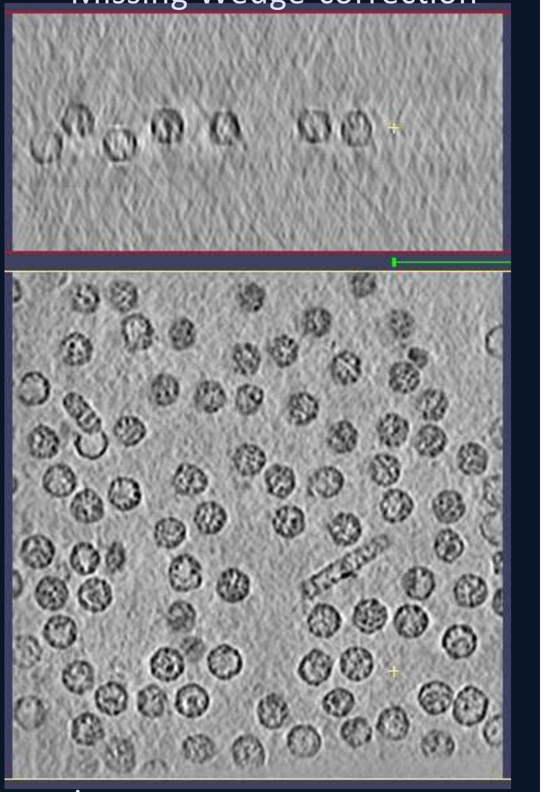
- Warp-Denoise
- Purified hemoglobin

- Makes annotating tomograms much easier!

Deconvolution

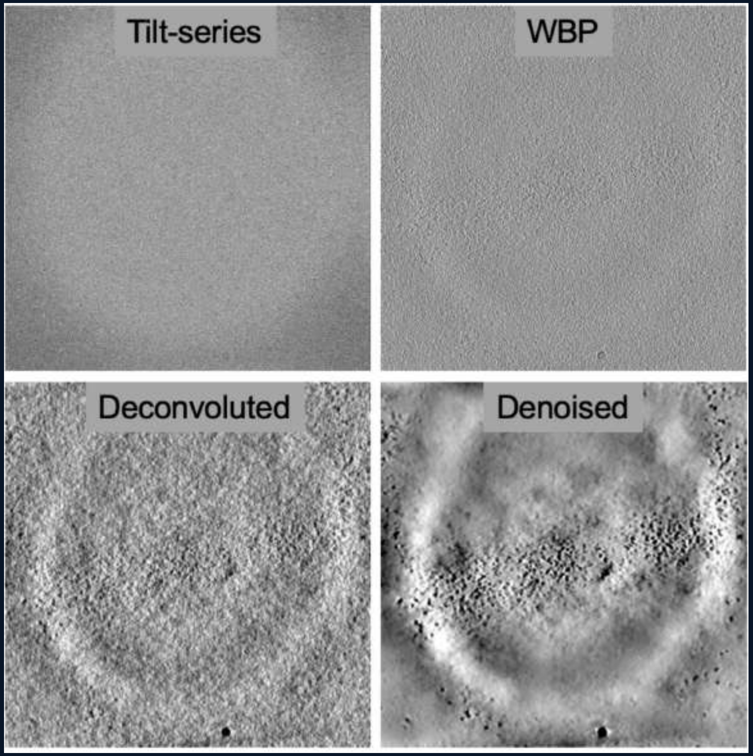


Deconvolution
Missing Wedge-correction



- IsoNet Missing Wedge Correction
- Purified Virus-like particles
- https://github.com/IsoNet-cryoET/IsoNet

Tomogram Denoising Approaches



- Warp-Denoise
- Noise2Noise
- Purified hemoglobin

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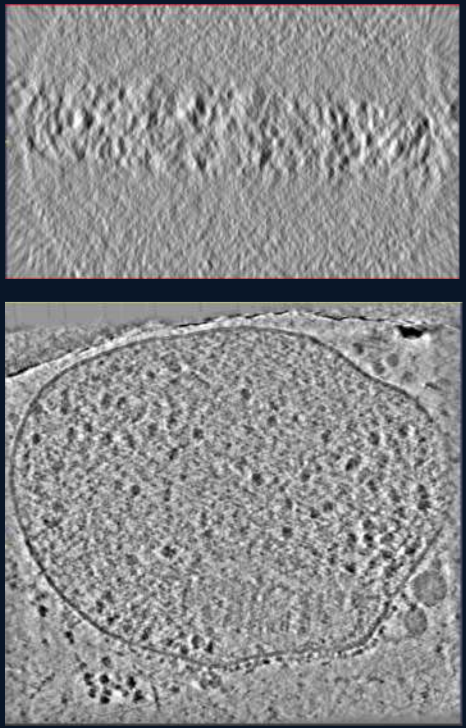

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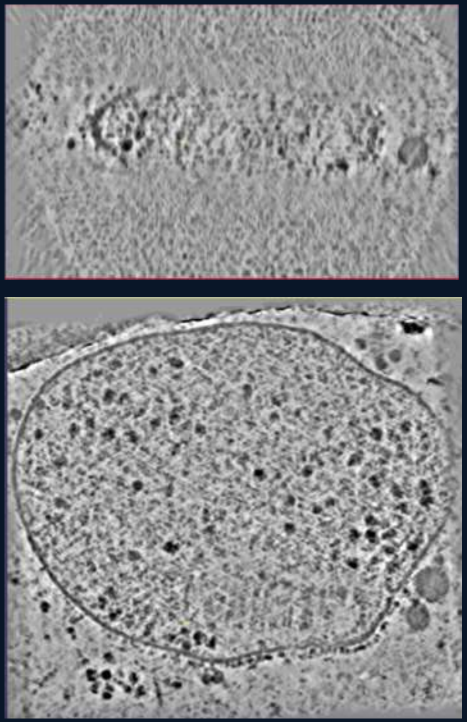


- IsoNet Missing Wedge Correction
- Empiar-10499
- https://github.com/IsoNet-cryoET/IsoNet

07

Segmentation

Annotating 3D volumes: membranes, organelles & macromolecules

Segmentation Approaches

Manual Segmentation

Gold standard

Slice-by-slice annotation in Amira, IMOD 3dmod, Dragonfly, or FIJI. Expert annotator traces membranes, organelles, and filaments. Extremely time-consuming (~days per tomogram) but highest fidelity. Required for ground-truth training data.

nnU-Net / Deep Learning

State of the art

Self-configuring neural network framework. Trained on annotated cryo-ET data; achieves near-human accuracy with sufficient training data. CryoSeg, membrain-seg (membrane specialist), organelle-specific models. Requires GPU and training annotations.

Threshold & Classical

Fast & simple

Global threshold + connected components for high-contrast objects. Watershed for separating adjacent regions. Filtered back-projection + morphological operations. Effective for lipid membranes and dense organelles; fails for low-contrast structures.

membrain-seg

Membrane specialist

Purpose-built transformer network (Lamm 2024) for cryo-ET membrane segmentation. Generalizes across datasets without retraining. Outputs membrane coordinates usable directly for STA. Now the standard approach for membrane-bound complexes.

06

Subtomogram Averaging

High-resolution structures from the crowded cellular interior · ~15 min

Subtomogram Averaging Pipeline

1

Particle Picking

Template matching using a known reference, manual picking, or deep learning (TomoTwin, DeepETPicker). Coordinates extracted for each candidate particle.

2

Subtomogram Extraction

3D subvolumes extracted around each coordinate. Size chosen to encompass particle plus some context (~1.5–2× particle diameter).

3

Initial Reference

Low-pass filtered known structure (PDB/SPA map), random conical tilt average, or direct classification of raw subvolumes. Reference bias is a major concern.

4

Alignment & Averaging

Each subtomogram aligned in 3D (6 degrees of freedom: 3 translations, 3 Euler angles) against reference. Cross-correlation with missing wedge mask. Aligned particles averaged.

5

Classification

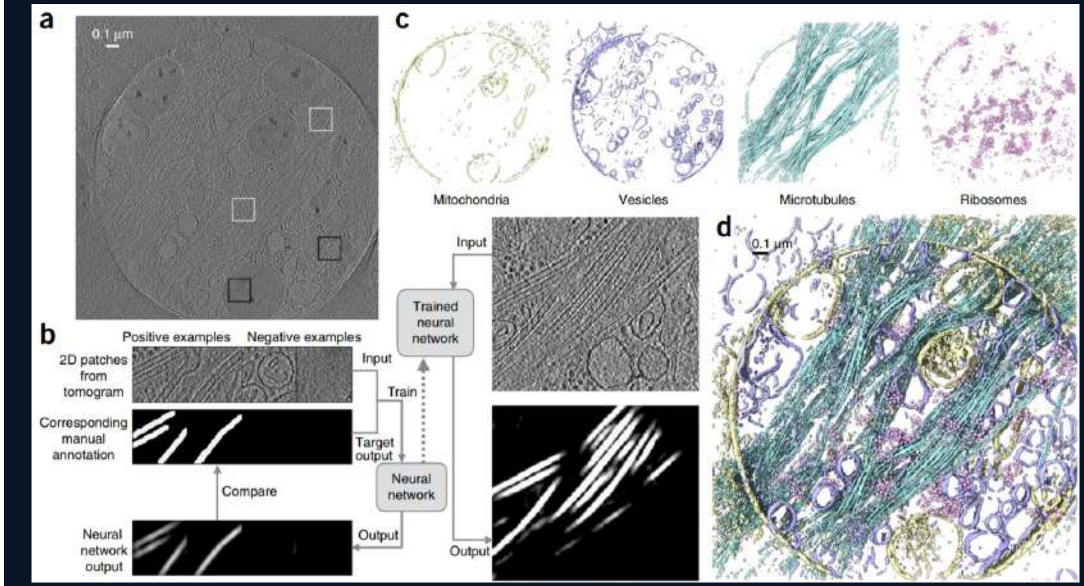
3D classification removes bad particles and separates conformational states. Principal component analysis or multi-reference alignment. Critical for heterogeneous datasets.

6

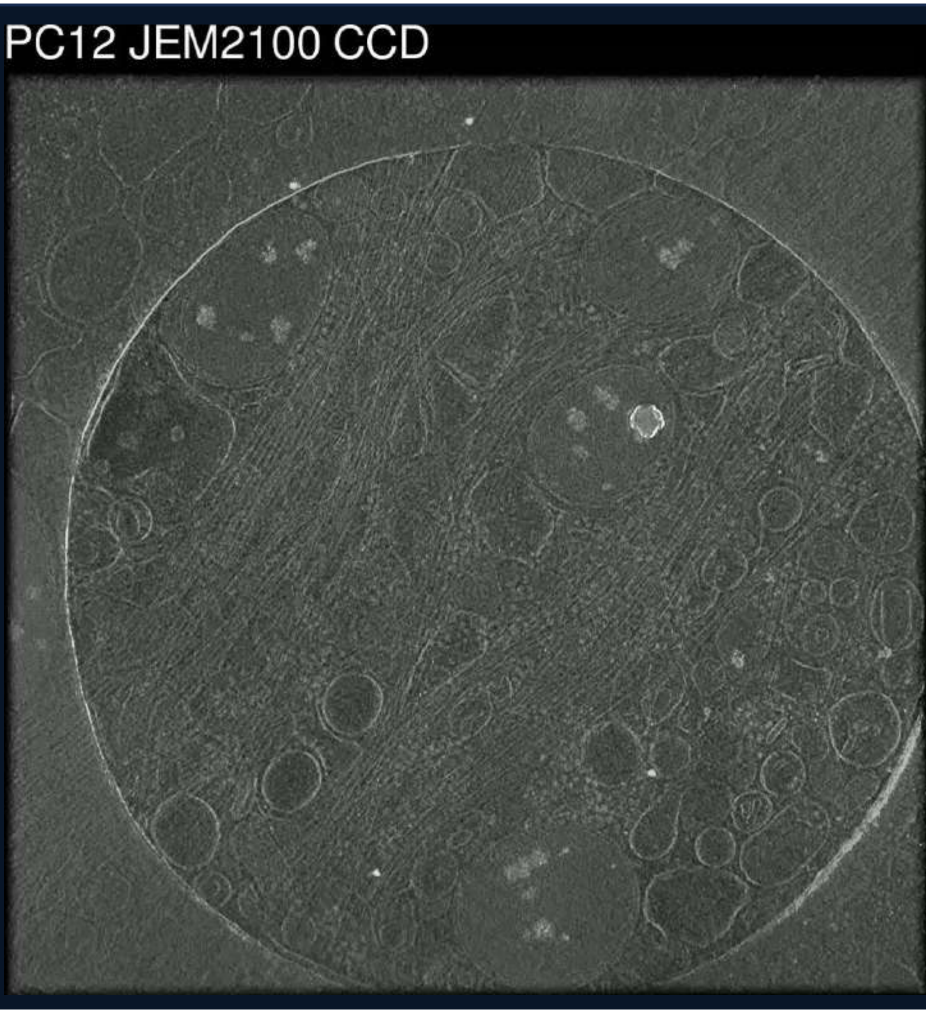
Iterative Refinement

Alternate alignment → average → new reference until convergence. Resolution assessed by gold-standard FSC (0.143 criterion). State-of-the-art: <10 Å routinely, <4 Å for favorable targets.

Tomography Particle Picking/Segmentation

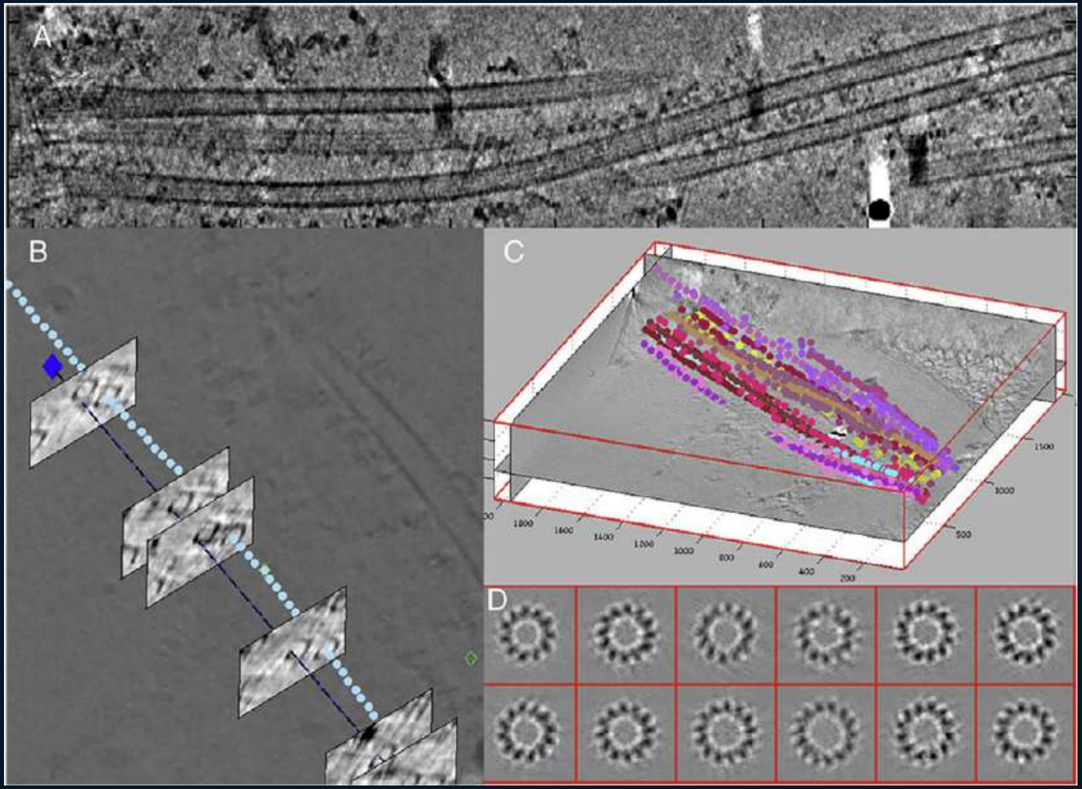


- Machine learning
- Train with positives and negatives
- Softwares such as **Tardis** and **Membrain-seg** provide automatic segmentations but do require manual clean and potentially labeling
- Github: Tardis: https://github.com/SMLC-NYSBC/TARDIS
- Github: Membrain-seg: https://github.com/teamtomo/membrain-seg



Tomography Particle Picking/Segmentation

Dynamo

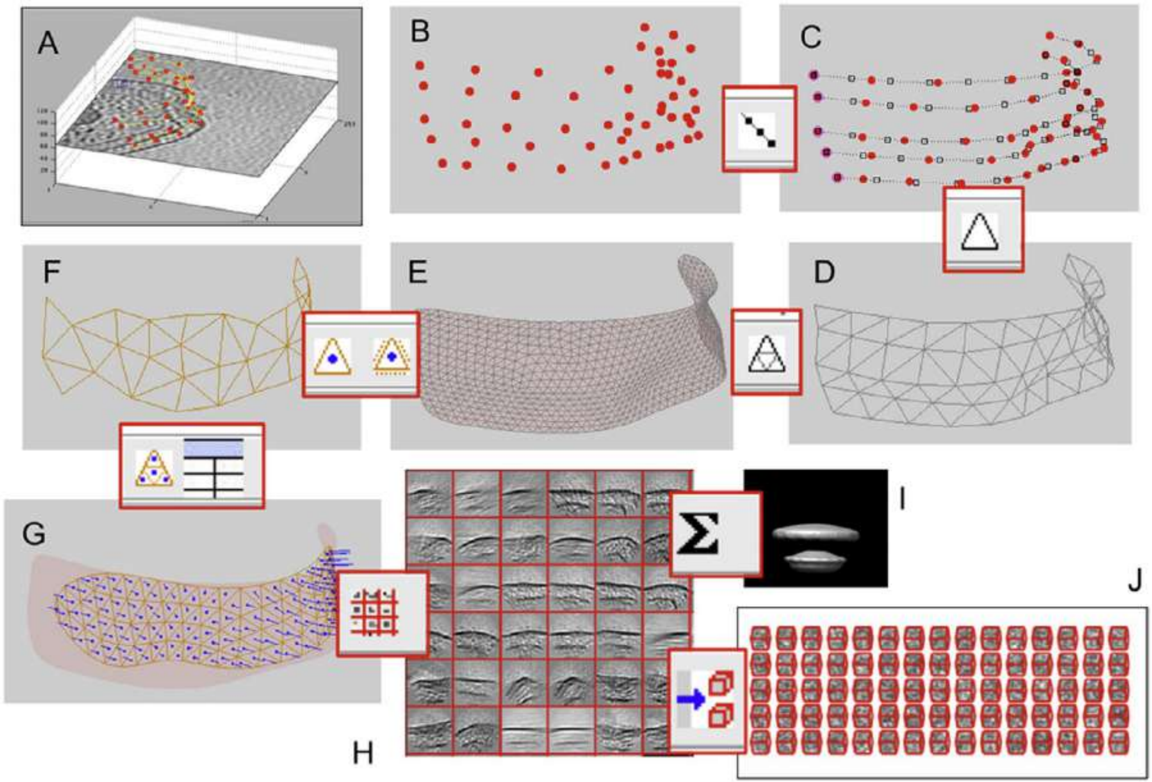


- Semi-automated
- Backbone, helical, and circumferential picking
- Helical symmetry determination

Castaño-Díez et al., 2012, 2017, 1018, 2021

Tomography Particle Picking/Segmentation

Dynamo

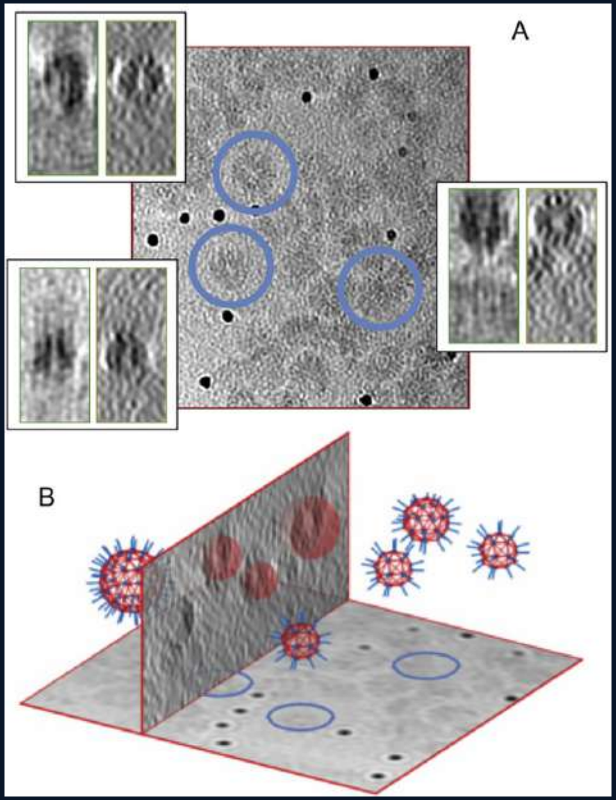


Create meshes to pick on any shape membrane

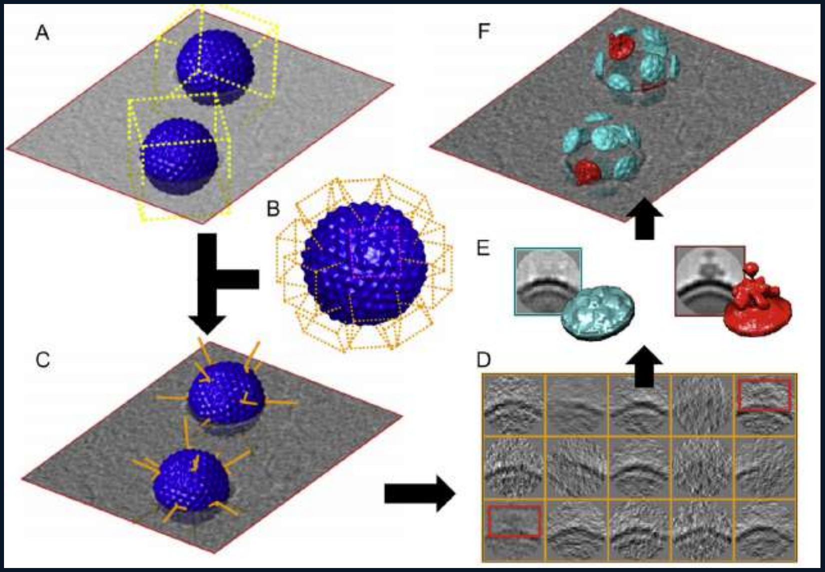
Castaño-Díez et al., 2012, 2017, 1018, 2021

Tomography Particle Picking/Segmentation

Dynamo



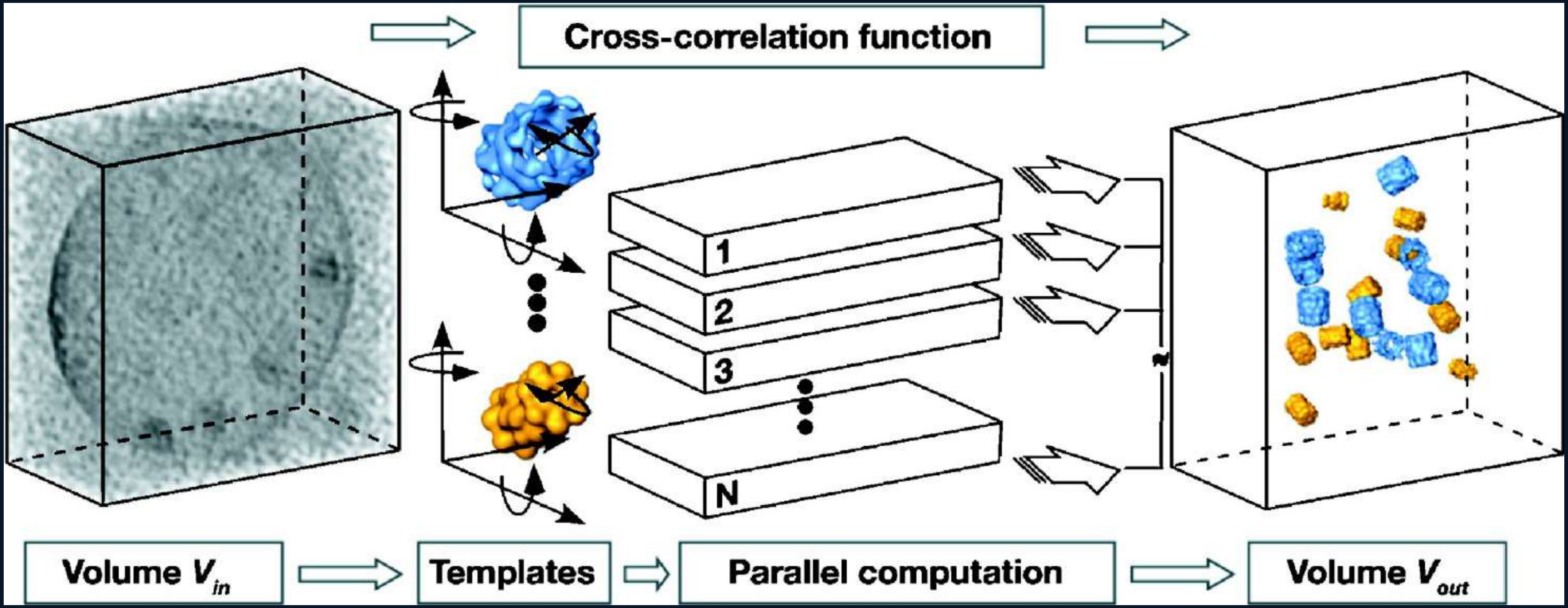
- Pick vesicles and pick around vesicles



Castaño-Díez et al., 2012, 2017, 1018, 2021

Tomography Particle Picking/Segmentation

Template matching

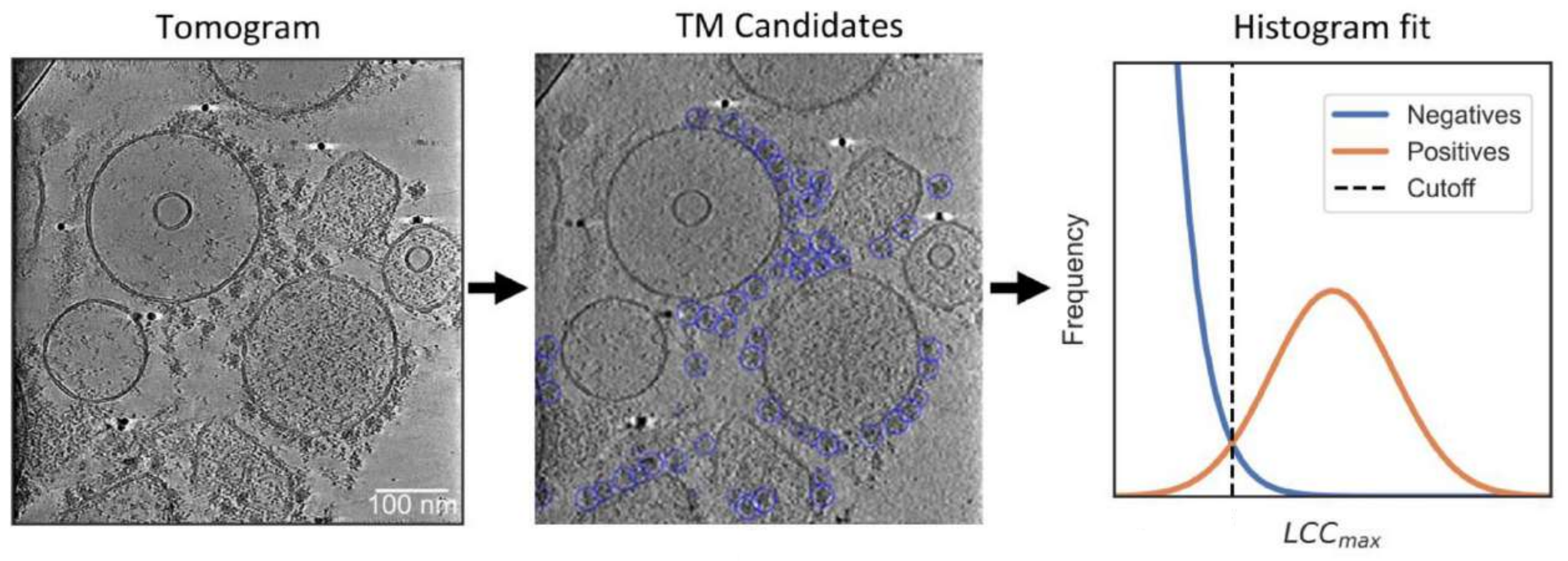


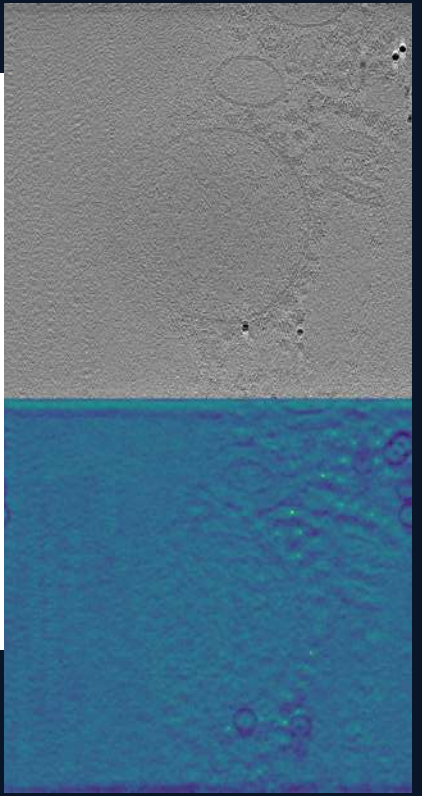
- Pick based on size and shape

Castaño-Díez et al., 2012, 2017, 1018, 2021

Tomography Particle Picking/Segmentation

Template matching with PyTom

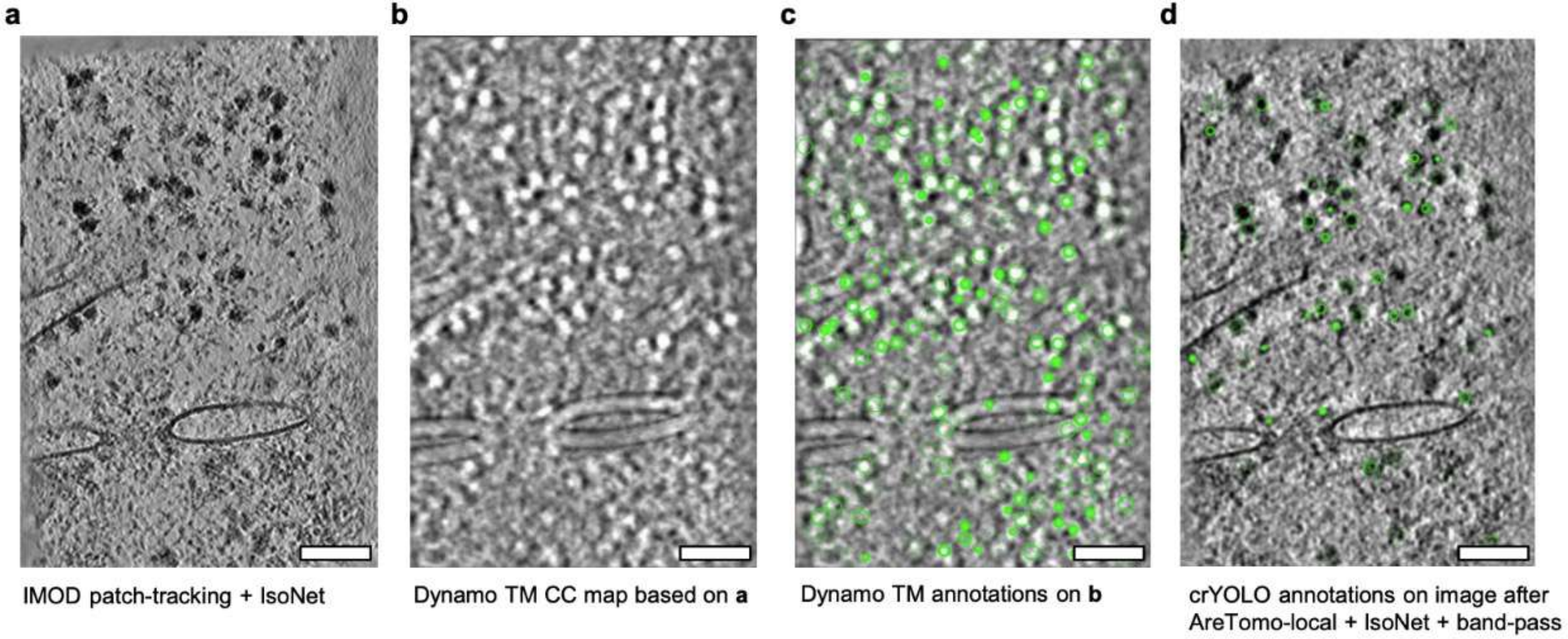




Chaillet et al., 2023

Tomography Particle Picking/Segmentation

CrYOLO



IMOD patch-tracking + IsoNet

Dynamo TM CC map based on **a**

Dynamo TM annotations on **b**

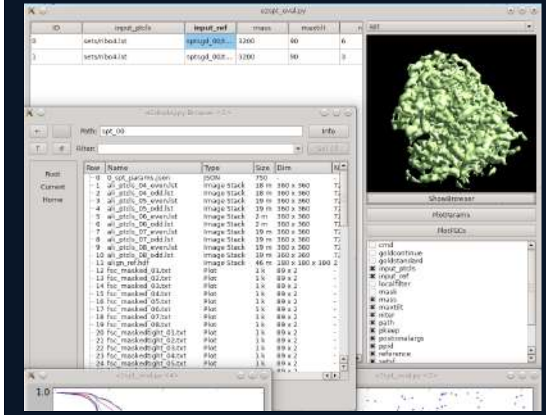
crYOLO annotations on image after AreTomo-local + IsoNet + band-pass

- Machine learning
- Requires many positive and negative labels

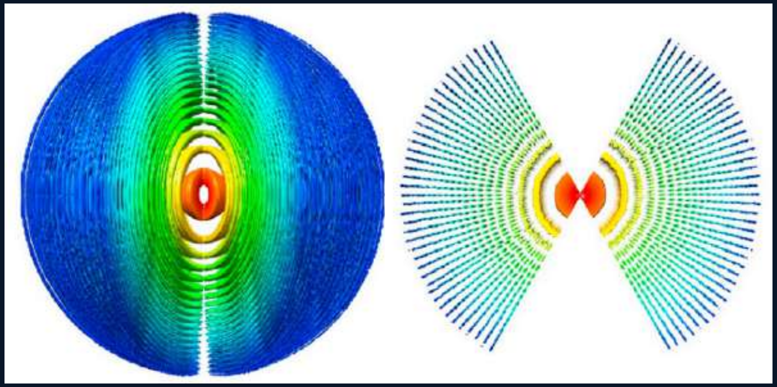
Wagner et al., 2019
Balyschew et al., 2023

Software for Subtomogram-Averaging

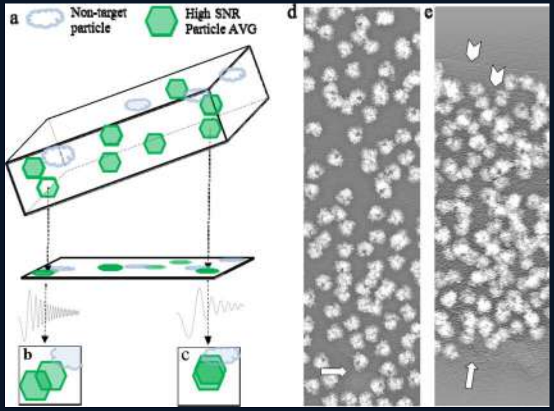
EMAN2



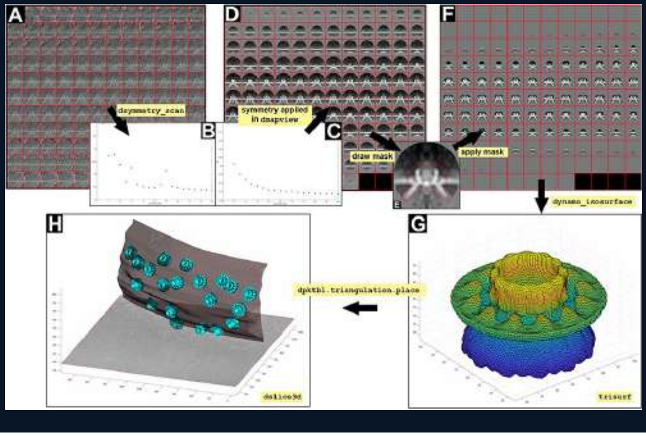
Relion



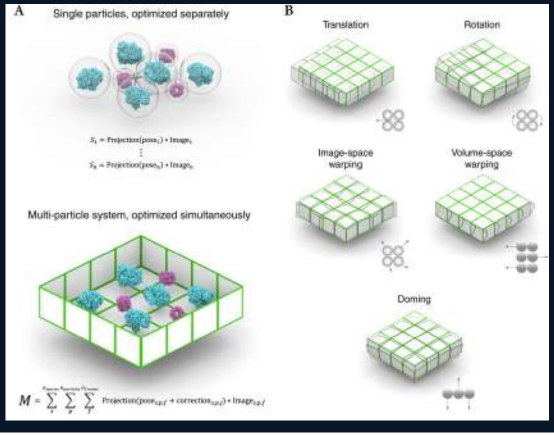
EMClarity



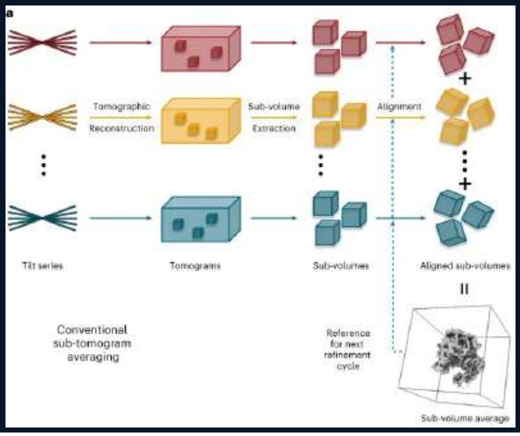
Dynamo



Warp



NextPYP



08

Software Landscape

Tools, pipelines & practical guide

Key Software by Pipeline Stage

Motion Correction	MotionCor2, AreTomo, IMOD alignframes
CTF Estimation	CTFFIND4, Gctf, AreTomo (integrated)
Tilt-Series Alignment	IMOD (fiducial), AreTomo (fiducial-free), eTomo
Reconstruction	IMOD WBP, Tomo3D SIRT, ASTRA, IsoNet (DL)
Denoising	cryo-CARE, Topaz-Denoise, IsoNet
Missing Wedge	IsoNet, STA-averaging effect, ART
Particle Picking	TomoTwin, DeepETPicker, crYOLO, template match
Subtomogram Averaging	RELION-5, EMAN2, Dynamo, STOPGAP, M
Segmentation	membrain-seg, nnU-Net, Amira, EMAN2, DragonFly
Visualization	UCSF ChimeraX, Dragonfly, Napari, IMOD 3dmod

Software for Subtomogram-Averaging

- 3D alignment: Can’t refine image angles
- 2D alignment: Can refine image angles – higher resolution
- Common workflow: Start with 3D alignment and high binning (account for missing wedges properly), then go to 2D refinement

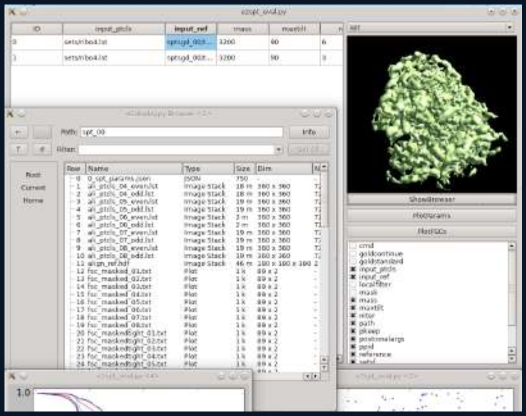
Subtomogram-Classification

- Can the software separate real features-cleaner picks to start with make classification much easier

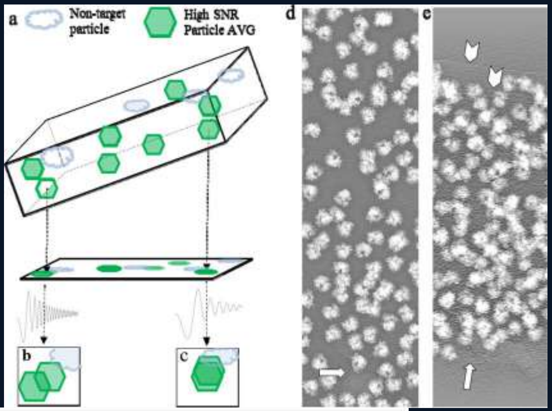
- Speed is often an issue – 3D data takes long to process, i.e, classify using extracted 3D subtomos

Sub-Tilt Refinement Software

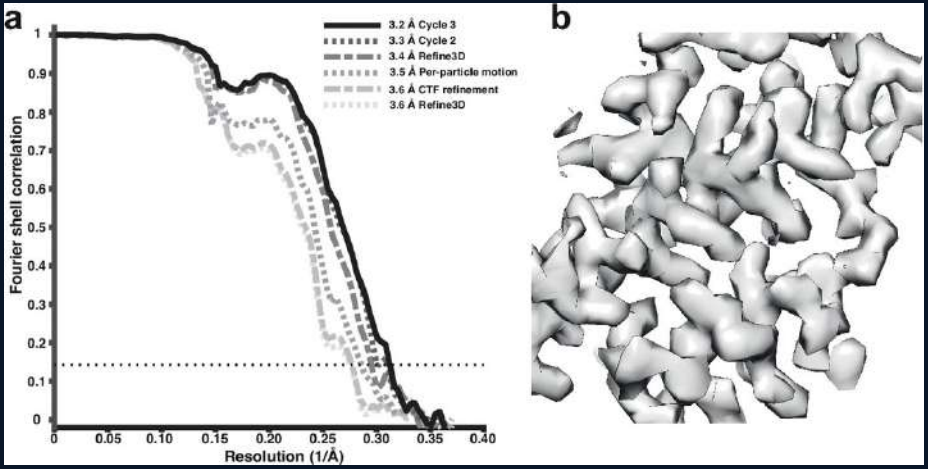
EMAN2



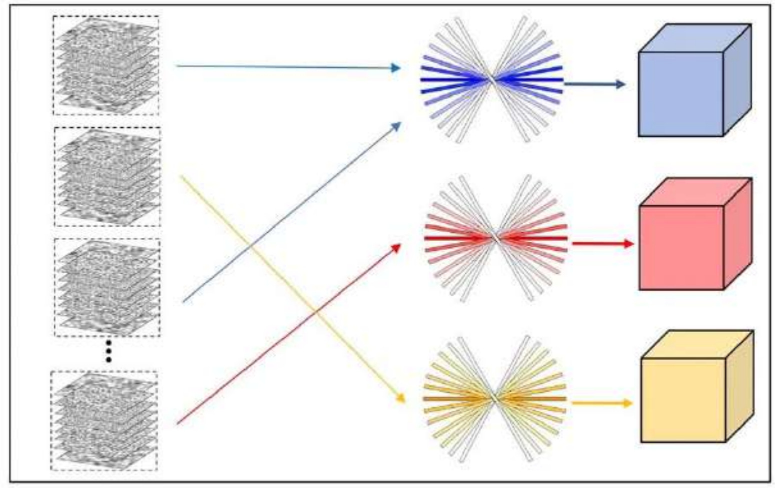
EMClarity



Relion



NextPYP



Sub-Tilt Refinement

Total Particles ==23307

10 angstrom map
256 box
Unbinned (pixel size
1.7005 A/pix)

M Refinement
low-pass filter 15A
masked refinement

Mask used

1st_round

5.1 angstrom map
296 box
Unbinned (pixel size 1.7005 A/pix)

```
 MCore --population
m/10499_round1.population --
refine_imagewarp 5x5 --
refine_particles --ctf_defocus --
ctf_defocusexhaustive --
perdevice_refine 4
```

2nd_round

4.0 angstrom map
296 box
Unbinned (pixel size 1.7005 A/pix)

```
MCore \
--population
m/10499_round1.population \
--refine_imagewarp 5x5 \
--refine_particles \
--ctf_defocus
```

3rd_round

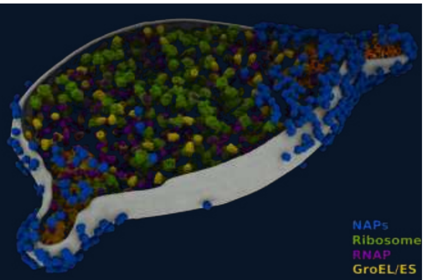
3.87 angstrom map
296 box
Unbinned (pixel size 1.7005 A/pix)

```
 MCore \
--population
m/10499_round1.population \
--refine_imagewarp 5x5 \
--refine_particles \
--refine_stageangles
```

M sub-tilt refinement
EMPIAR-10499

Processed with WarpTools, Relion4,
to M in linux☺

Mapping Averages back for Cellular Context



ArtiaX

- Takes alignment/ segmentation files from various software, imports the averages in a point cloud in ChimeraX
- Only viewable in ChimeraX
- Can overlay with the tomogram
- Can manipulate objects in real-time and in VR
- Scriptable in ChimeraX

Ermel et al., 2022

Key Takeaways

01 Motion correction and tilt-series alignment are the foundation — errors here propagate through every downstream step and cannot be recovered.

02 CTF correction is essential for high-resolution subtomogram averaging. Per-tilt estimation with 3D CTF models is now the standard.

03 WBP is fast and standard; SIRT reduces artifacts; deep learning reconstruction (IsoNet) gives near-isotropic volumes but at high computational cost.

04 cryo-CARE has transformed data quality — self-supervised denoising routinely reveals features invisible in raw tomograms. Now considered essential.

05 The missing wedge is inherently corrected during STA through angular averaging — explicit correction methods (IsoNet) are most useful before picking/segmentation.

06 Subtomogram averaging can reach sub-nanometer and even near-atomic resolution in favorable cases. Gold-standard FSC (0.143) is the resolution criterion.

07 Segmentation is rapidly being transformed by deep learning. membrain-seg and nnU-Net are enabling quantitative analysis of entire tomographic volumes.

Next: Hands-on data processing workshop