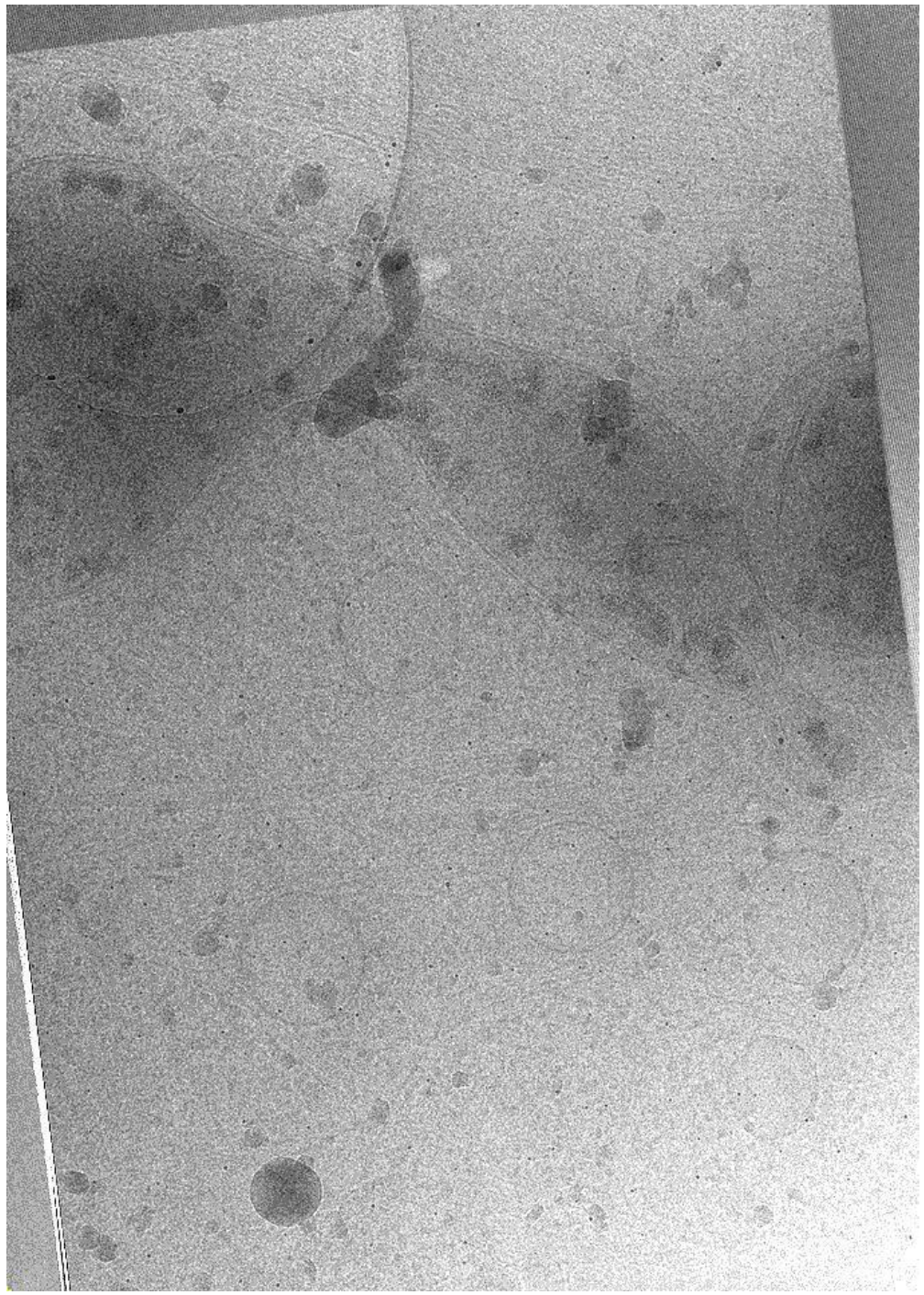
Segmentation and Sub-tomogram Averaging Pipelines

April 2nd, 2025

New York Structural Biology Center
Columbia University
Jake Johnston

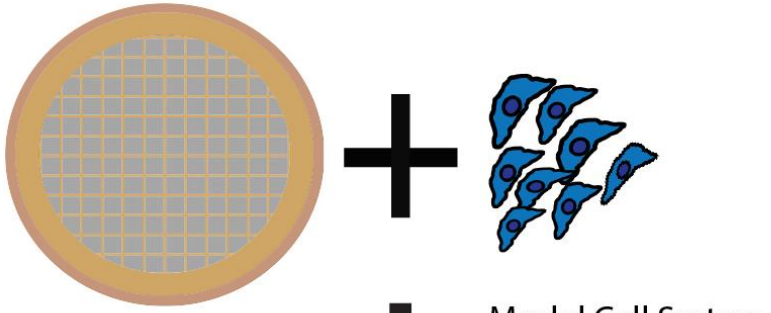


Today's goals

- Discuss the several software/pipelines available for cryo-ET processing

- Approaching biological questions using sub-tomogram averaging or segmentation workflows

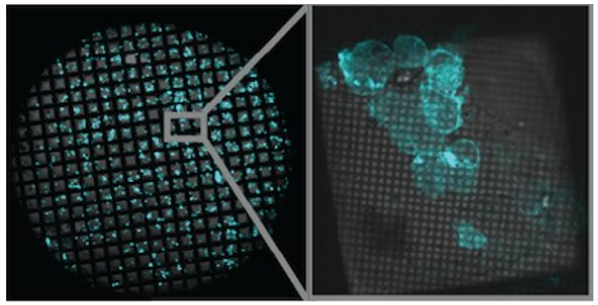
Cryo-ET Workflow Summary



EM Grid

Model Cell System

Plunge Freezing



Fluorescence Screening
Fluorsecence guided data collection/Milling

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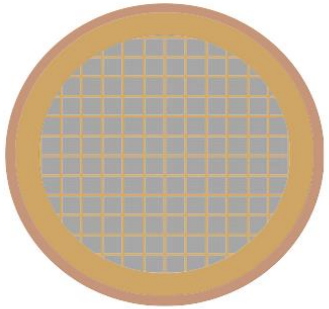

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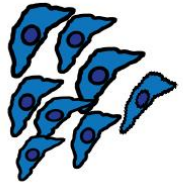

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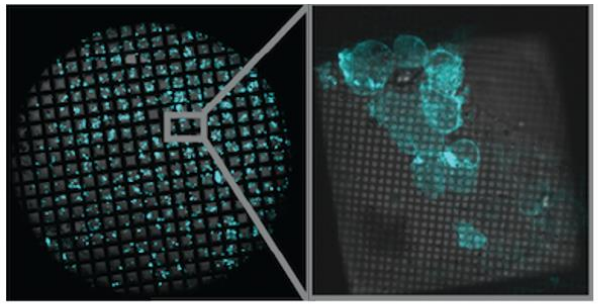


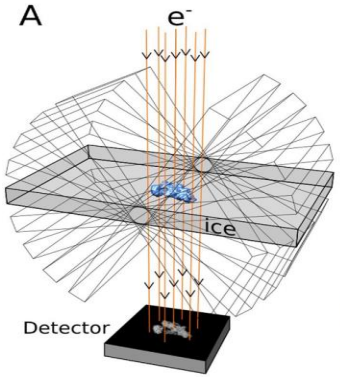
Fluorescence Screening
Fluorsecence guided data collection/Milling

Samples thinner then 350 nm or less!

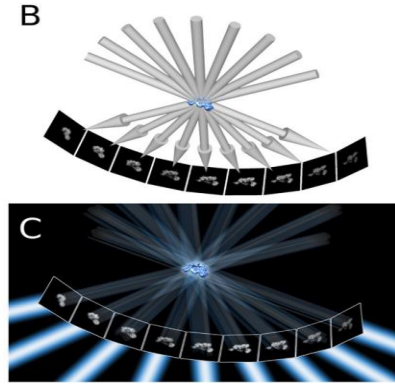
Tomography Data Collection

Krios 300 kv microscope

For Thin Samples, such as thin edges of cells, and purified samples



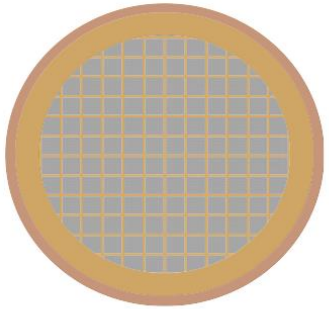
Tilt-series Data Collection



Tomogram Reconstruction

Koning et al., Annals of Anatomy, 2018

Cryo-ET Workflow Summary



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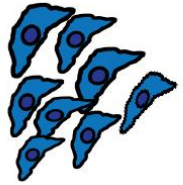

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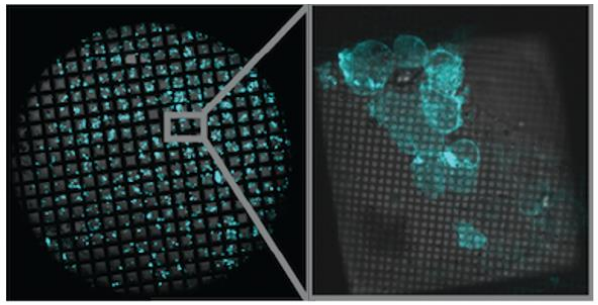

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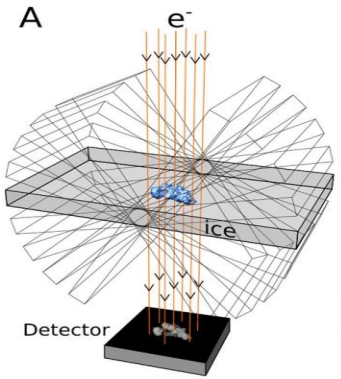

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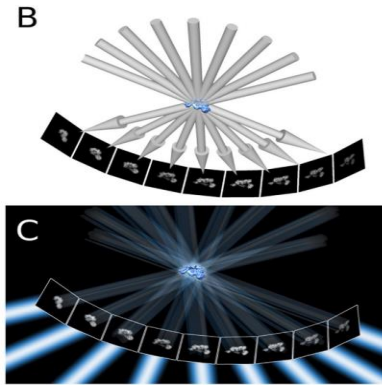

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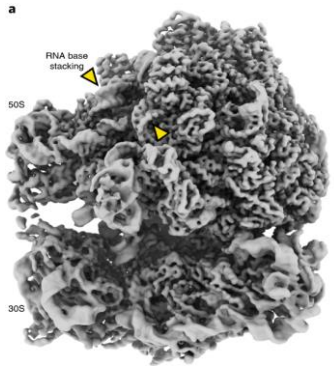
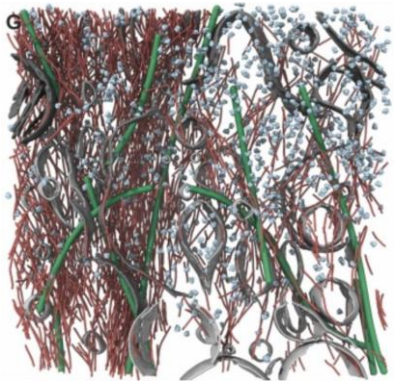


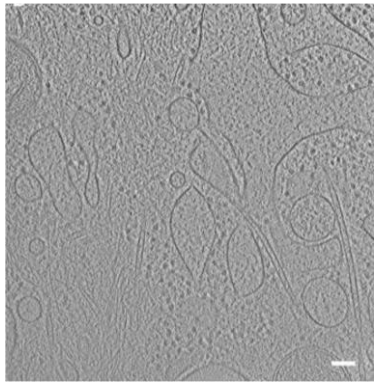
3D maps In-situ

3D Particle Picking
Subtomogram Averaging



Segmentations

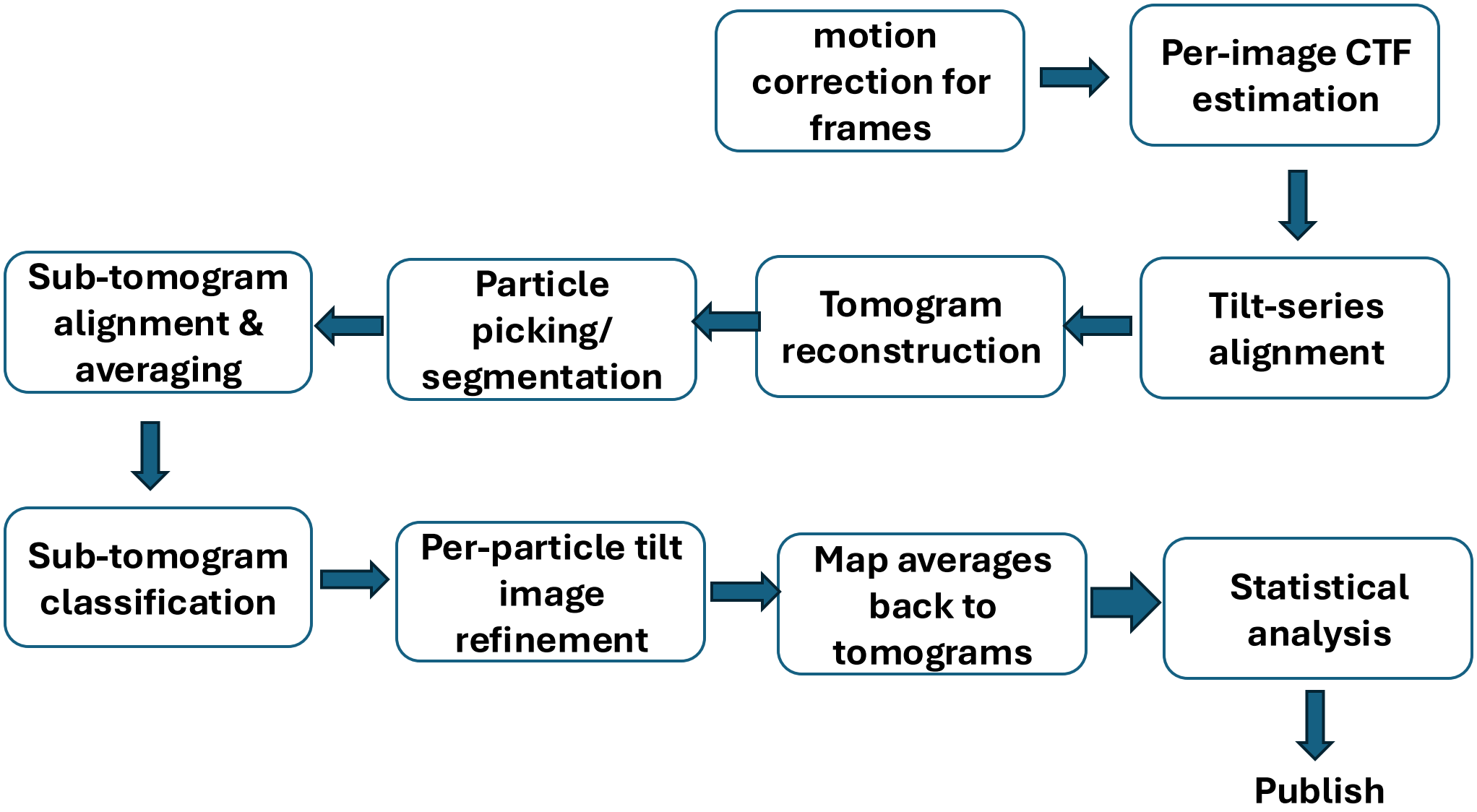
Morphology Analysis



Tomographic Slice of a Thin Edge Sample

Koning et al., Annals of Anatomy, 2018

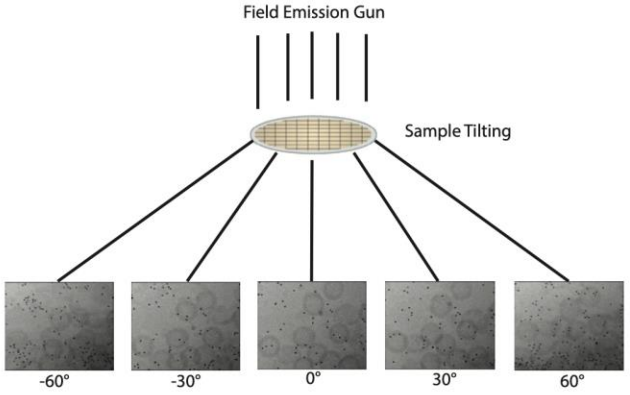
Generalized Workflow for Cryo-ET Processing



Adapted from Leigh et al., 2019

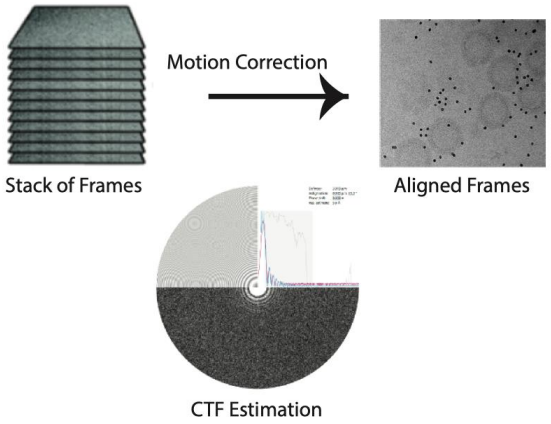
Tomography Software Packages

TS Acquisition



Serial EM, Tomo5

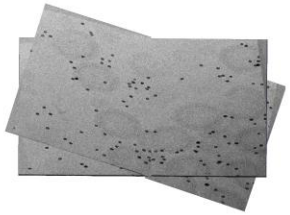
Motion Correction/CTF Estimation



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

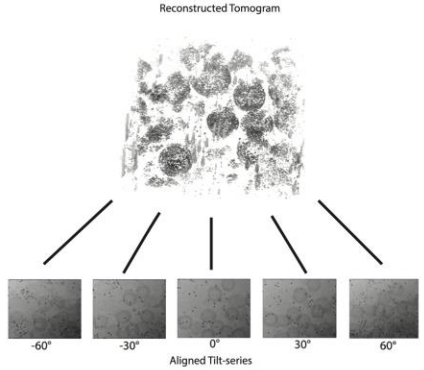
CTFFIND4/5,Gctf, IMOD (CTFPlotter), Warp, emClarity

Tilt-Series Alignment



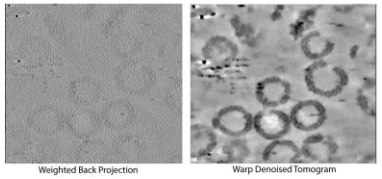
Imod, Raptor, EMAN2, Dynamo, UCSF tomo, Protomo, TomoAlign, AreTomo, Xmip

Tomogram Reconstruction



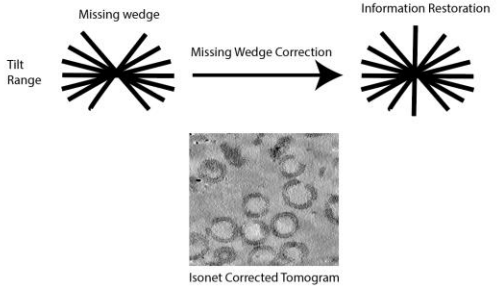
Imod, Raptor, AuTom, EMAN2, Dynamo, UCSF tomo, Protomo, TomoAlign, AreTomo, TOMO3D

Denoising



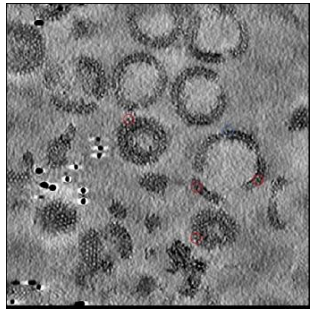
Warp-denoise, Topaz Denoise, CryoCare

Missing Wedge Correction



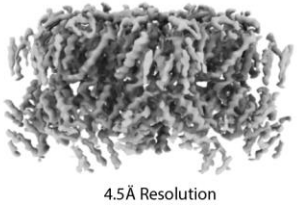
IsoNet, REST

3D-Particle Picking



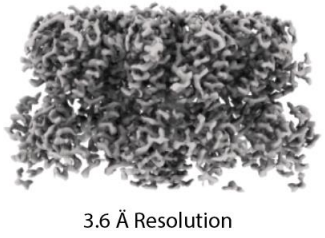
Cryolo, EMAN2, PyTom, Dynamo, StopGap, Relion5, NextPYP, Scipion, DeepETPicker, DeepFinder, TomoNet, AiS, TomoTwin, DeePict, PySeg

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

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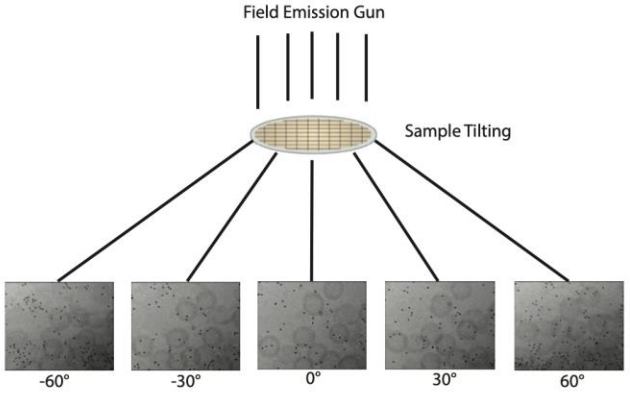

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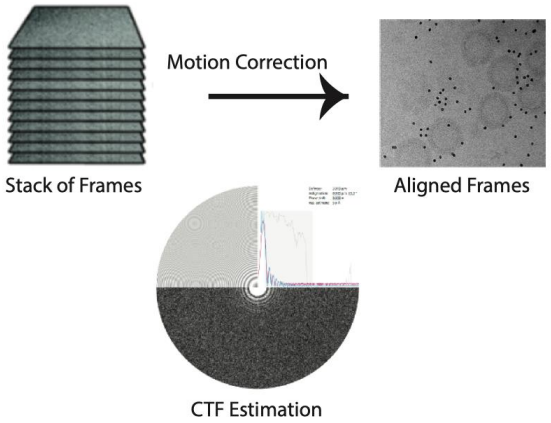

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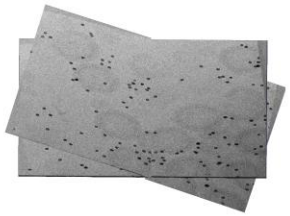

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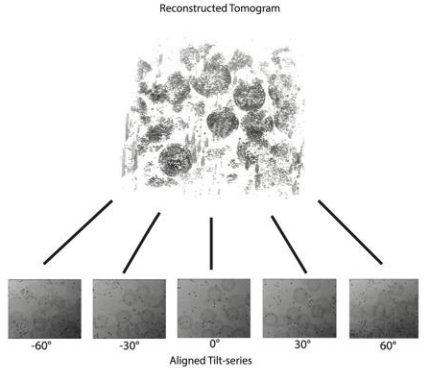
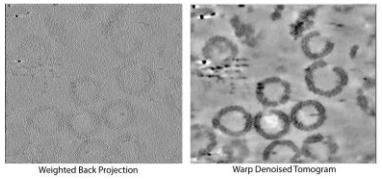


Imod, Raptor, AuTom, EMAN2, Dynamo, UCSF tomo, Protomo, TomoAlign, AreTomo, TOMO3D

Denoising



Warp-denoise, Topaz Denoise, CryoCare

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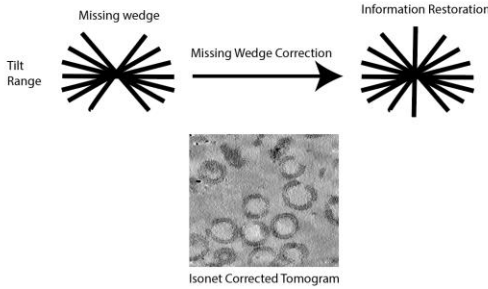


IsoNet, REST

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

Motion Correction

All:

- Global and local
- Near real-time with collection
- Dose weighting

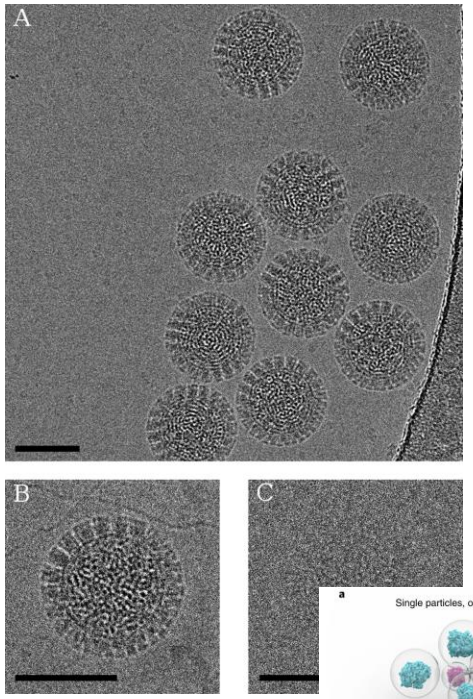
Motioncor & Warp:

- GPU
- CTF estimation

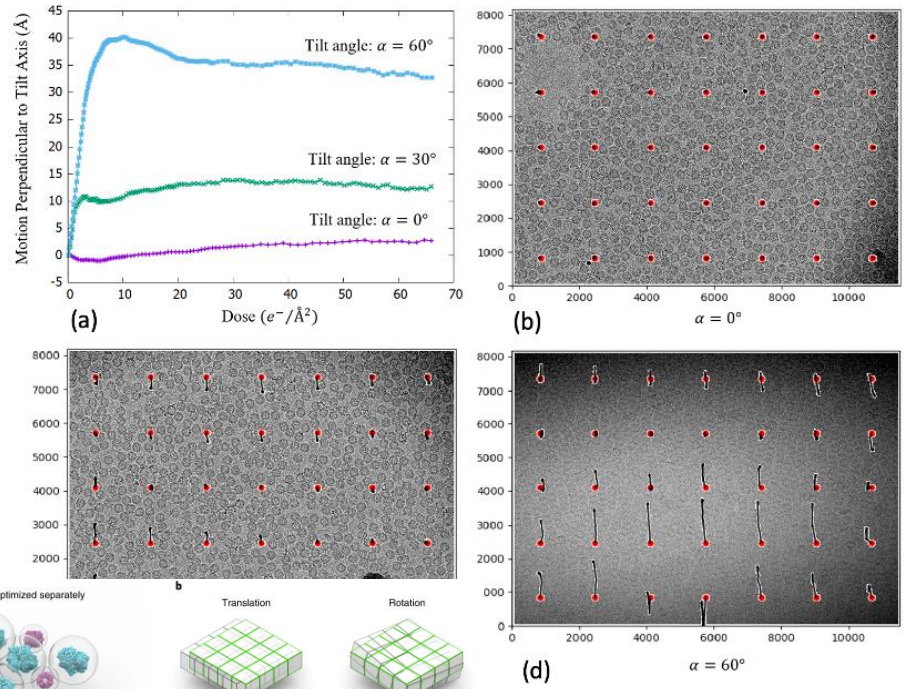
Warp:

- 3D modeling

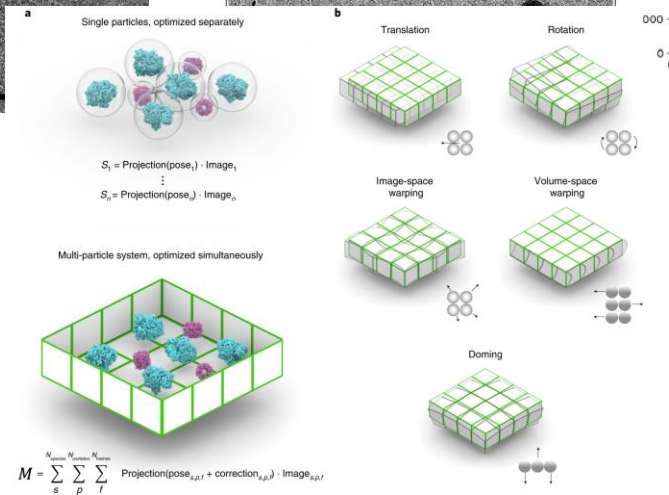
Unblur



Motioncor2/3



Warp

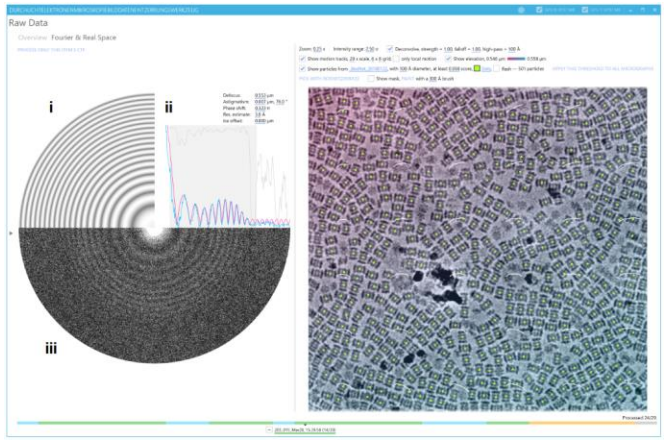


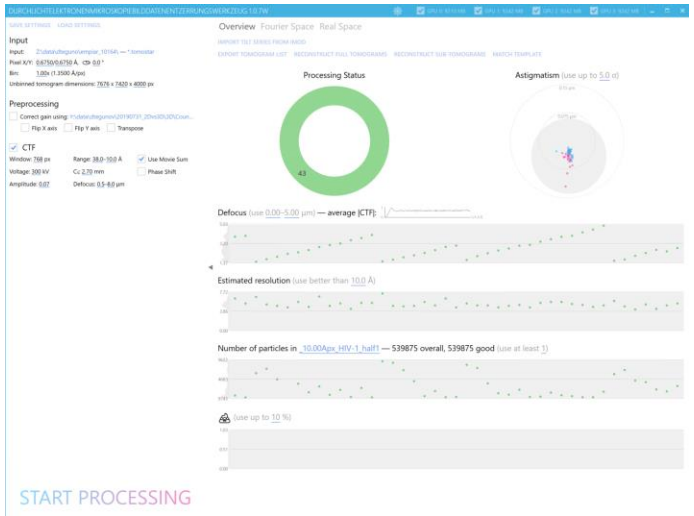
CTF Estimation for Tilt-Series

- Ideally, Accurate high-resolution estimation (3-4 angstroms) should be seen

Warp

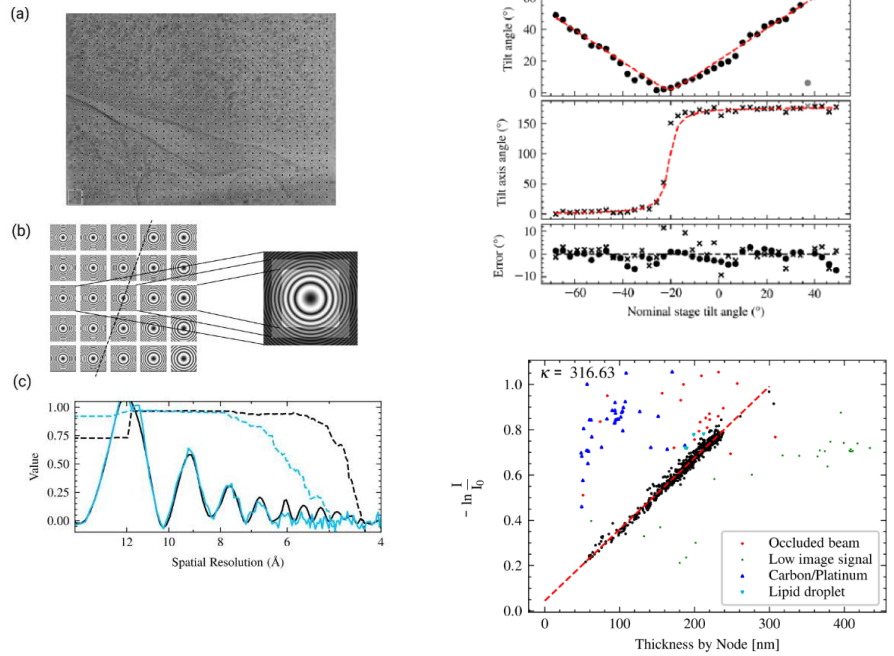
- Local CTF estimation
- Estimates tilt axis angle per image
- Determines handedness
- Refines based on whole tilt-series
- Corrects for CTF
- Local CTF refinement





Tegunov et al., 2019, 2021

CTFFIND 4/5

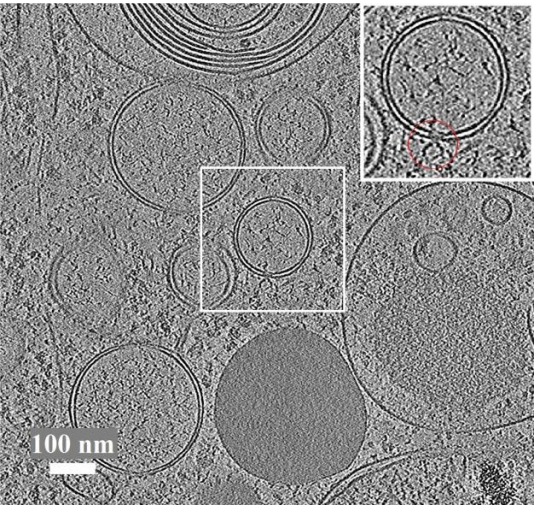


- Local CTF estimation
- Estimates tilt axis angle per image
- Estimates local ice thickness
- Corrects for CTF

Elferich et al., 2023

Tilt-Series Alignments

Fiducial-less

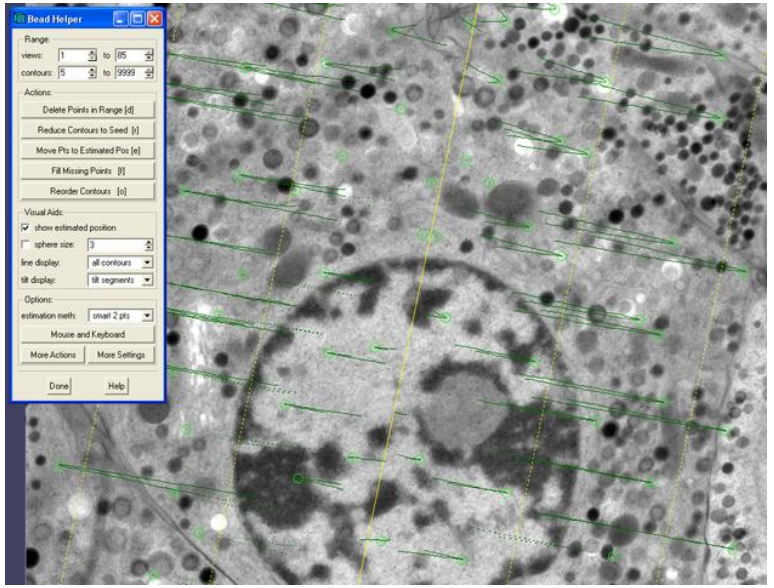


Zheng et al., 2022

- 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

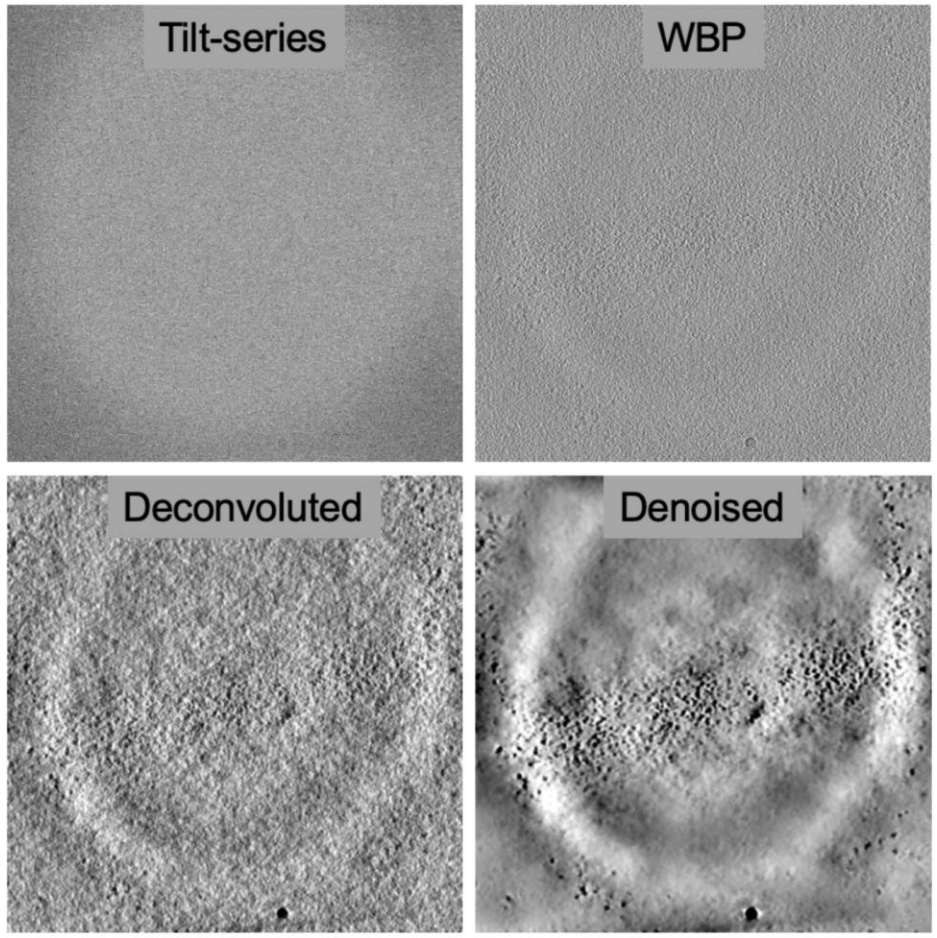
- Requires a sufficient number of well-behaved gold beads
  - Sample prep optimization
- Semi-automated in IMOD
- Automated in other workflows

Tomography Particle Picking/Segmentation

Several different approaches can be taken depending on the target.....

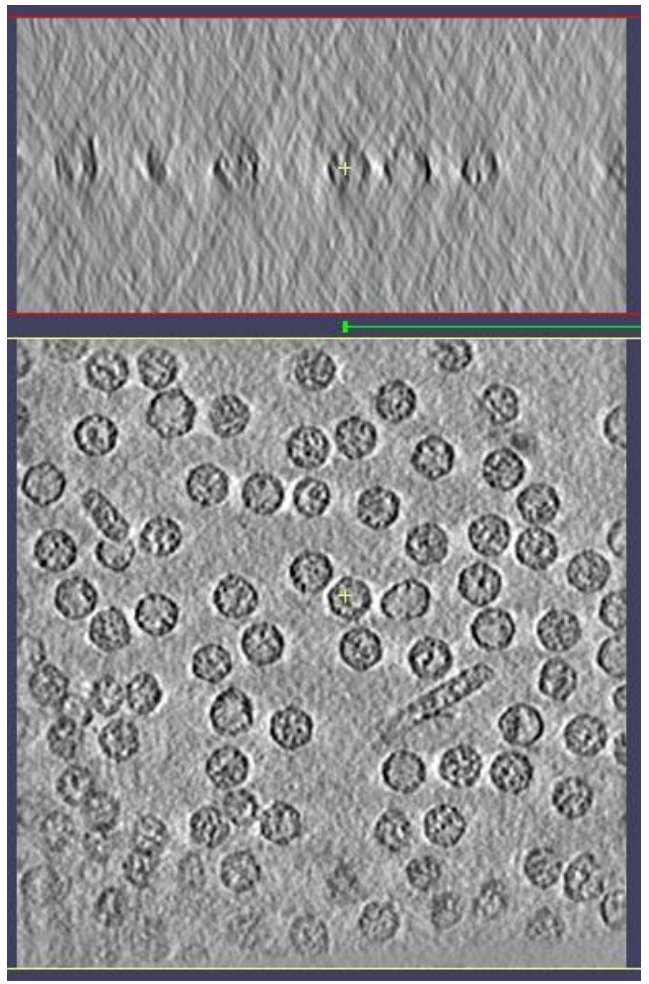
- Geometry-based approaches
- Template Matching
- Machine Learning
- Iterative Approaches
- Manual
- Denoise before picking or segmenting, it helps!

Tomogram Denoising Approaches



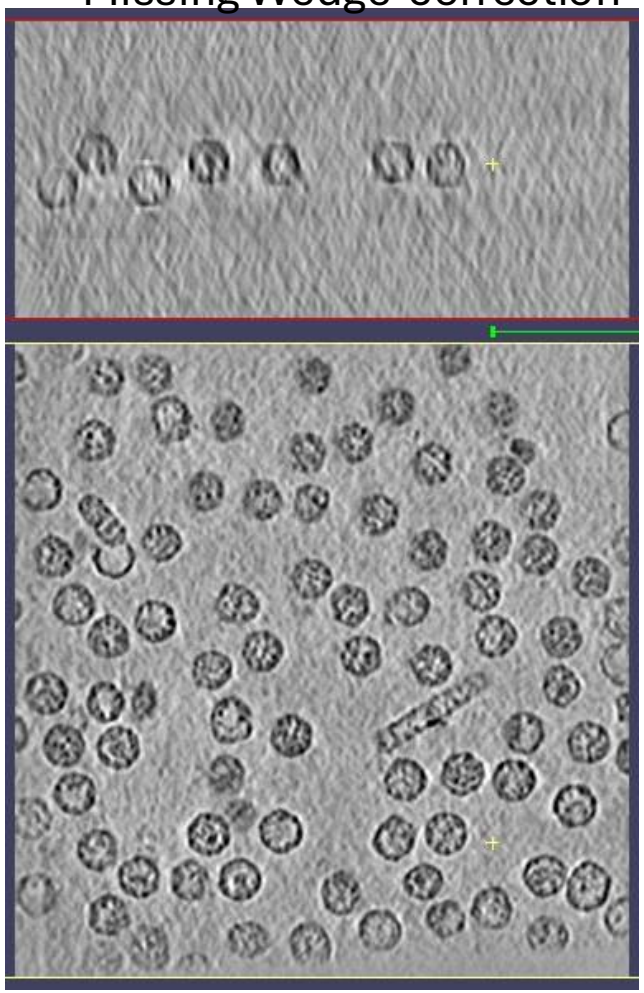
- Warp-Denoise
- Purified hemoglobin

Deconvolution



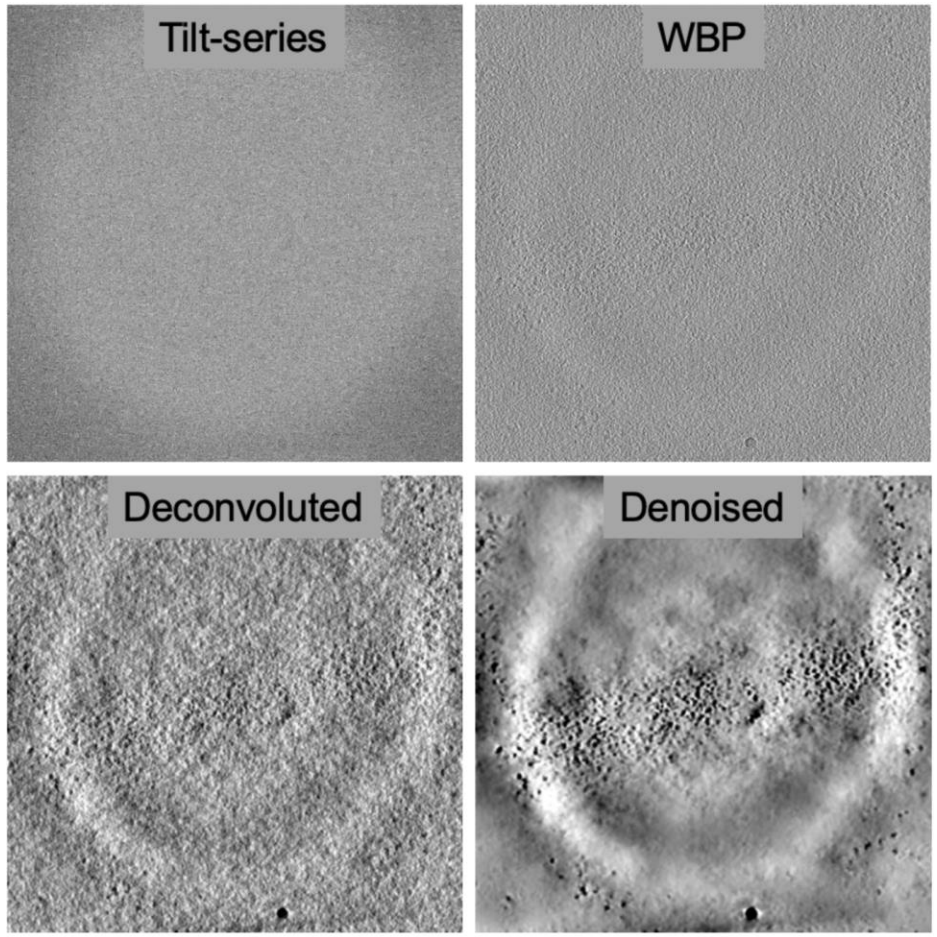
- Makes annotating tomograms much easier!

Deconvolution
Missing Wedge-correction



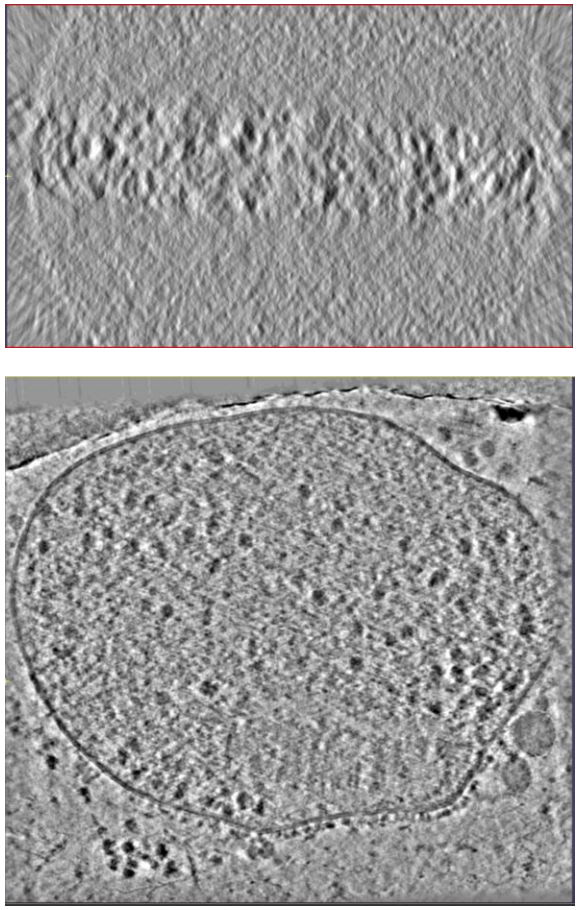
- IsoNet Missing Wedge Correction
- Purified Virus-like particles
- https://github.com/IsoNet-cryoET/IsoNet

Tomogram Denoising Approaches



- Warp-Denoise
- Noise2Noise
- Purified hemoglobin

Deconvolution



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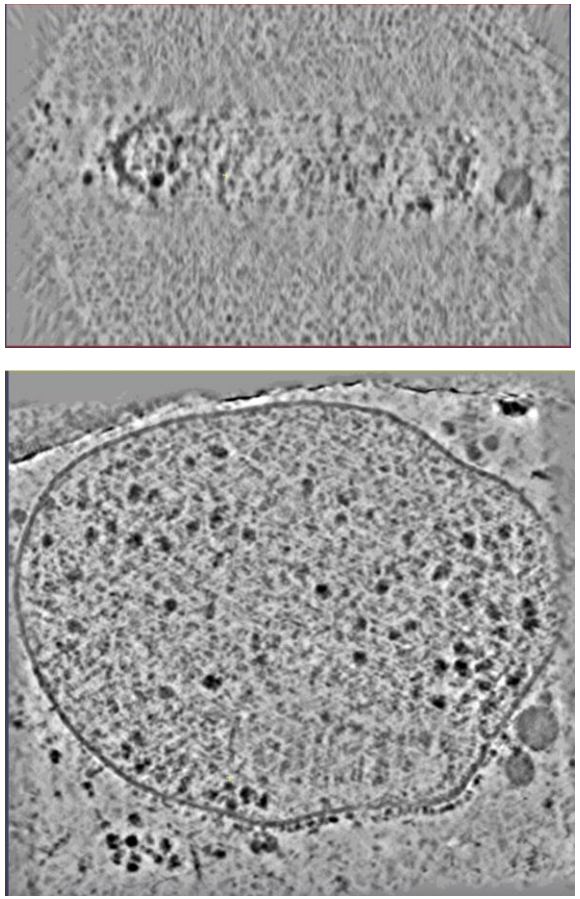

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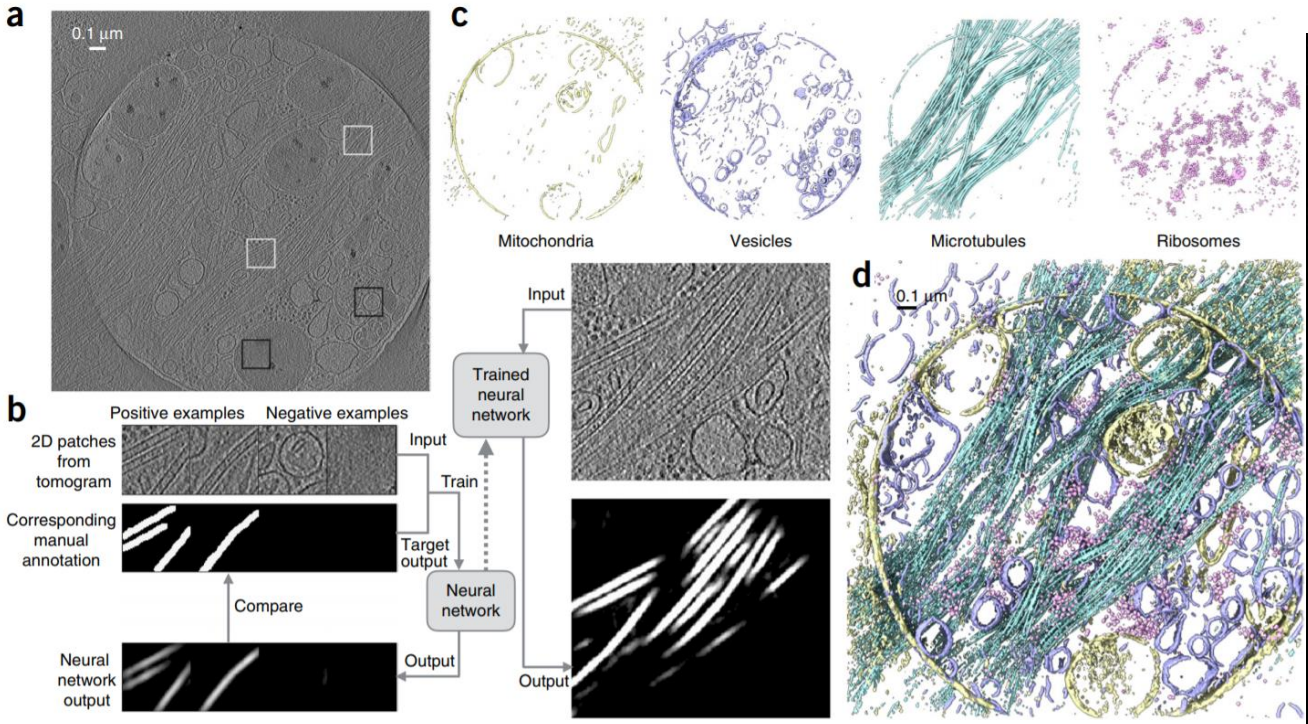
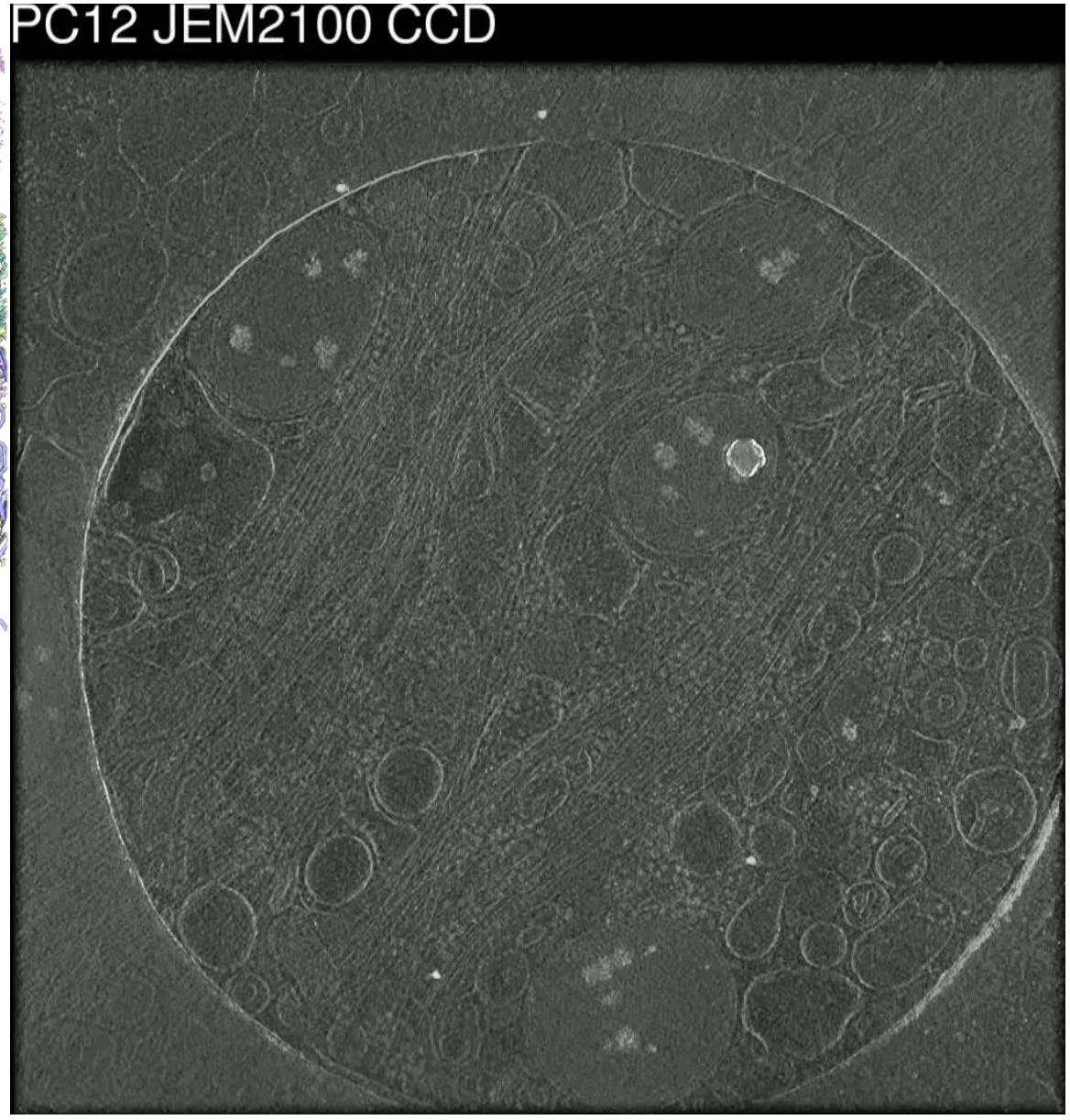
Tomography Particle Picking/Segmentation

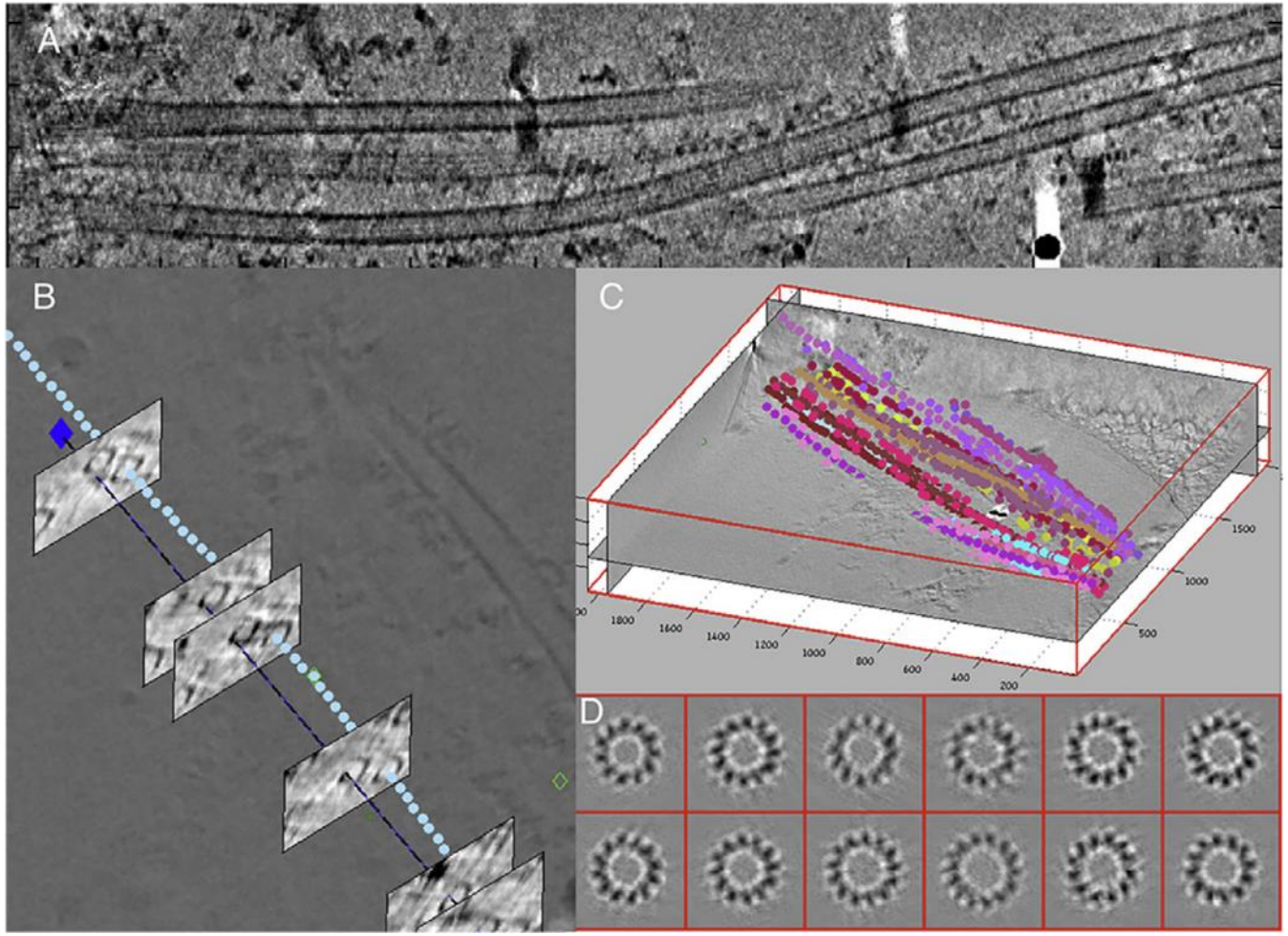




- Machine learning
- Train with positives and negatives
- Softwares such as **Tardis** and **Membrain-seg** provide automatic segmentations but do require manual clean and potentially labeling
- Github: Tardis: https://github.com/SMLC-NYSBC/TARDIS
- Github: Membrain-seg: https://github.com/teamtomo/membrain-seg

Tomography Particle Picking/Segmentation

Dynamo

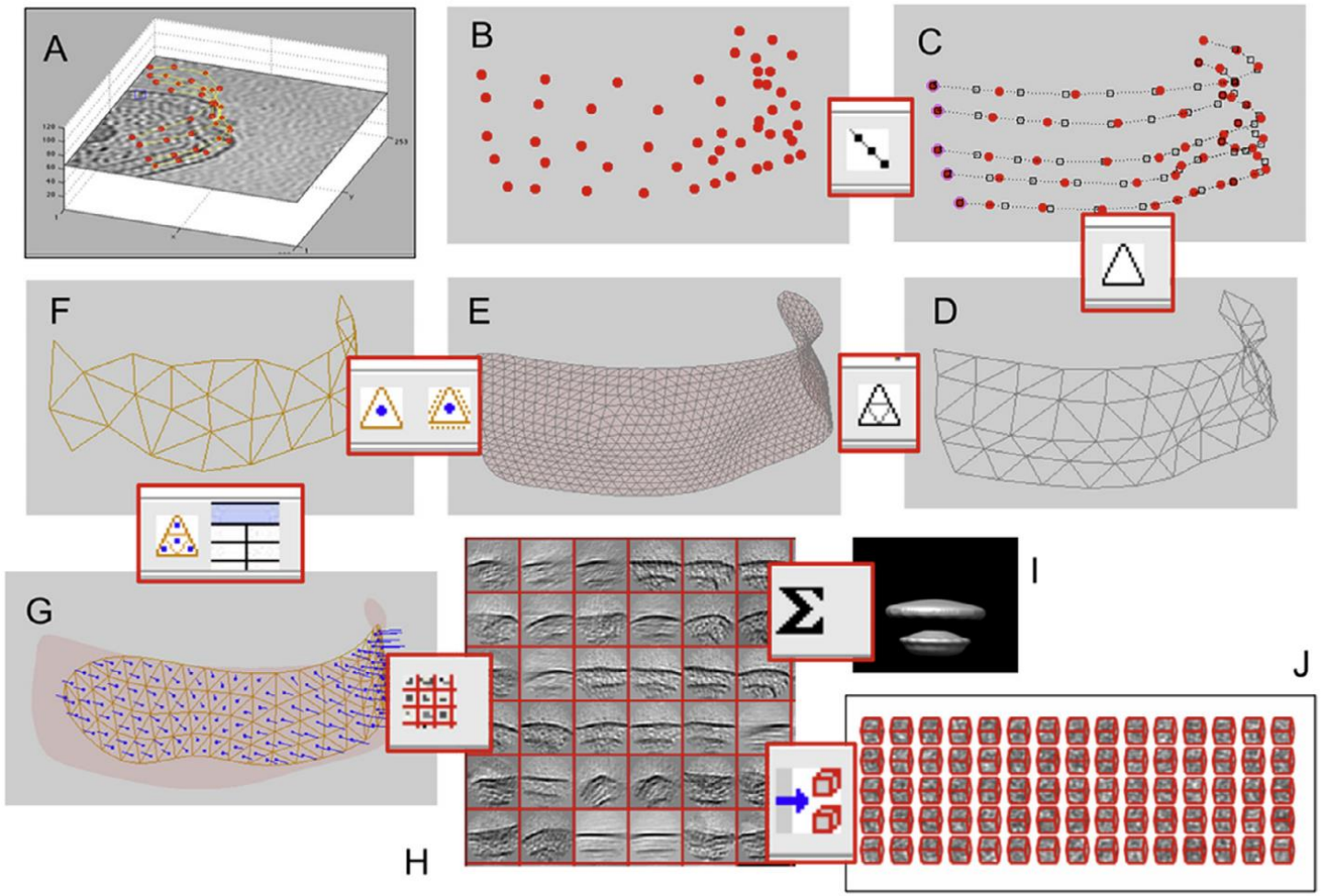


- Semi-automated
- Backbone, helical, and circumferential picking
- Helical symmetry determination

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

Tomography Particle Picking/Segmentation

Dynamo

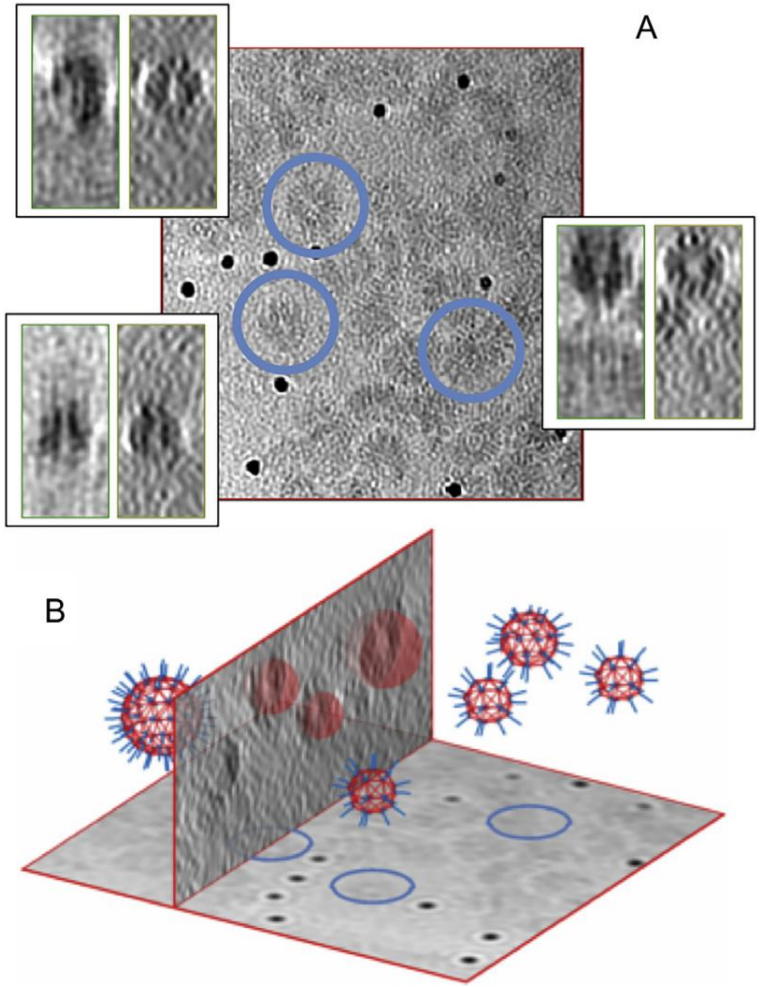


- Create meshes to pick on any shape membrane

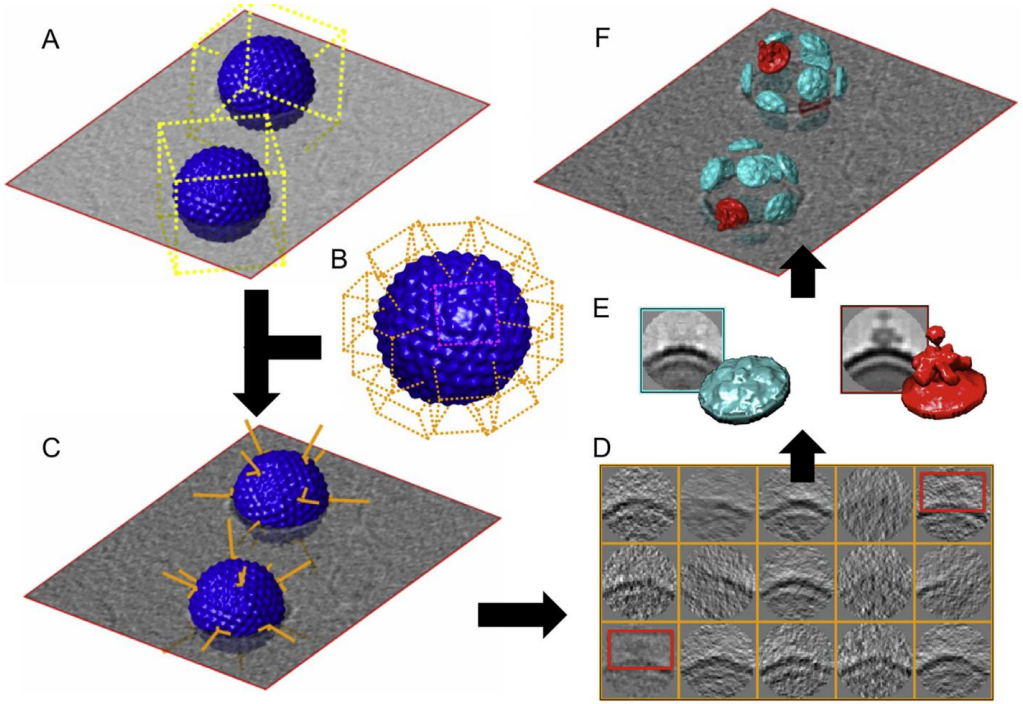
Castaño-Díez et al., 2012, 2017, 1018, 2021

Tomography Particle Picking/Segmentation

Dynamo



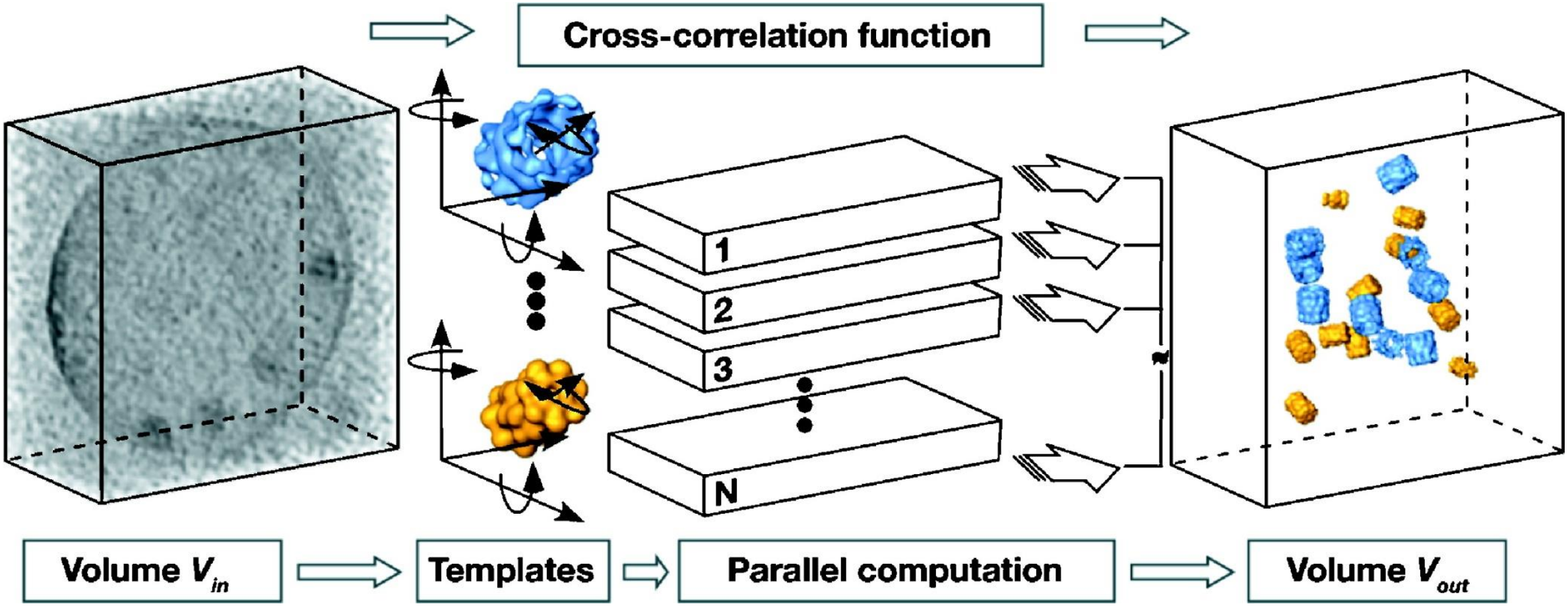
- Pick vesicles and pick around vesicles



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

Tomography Particle Picking/Segmentation

Template matching

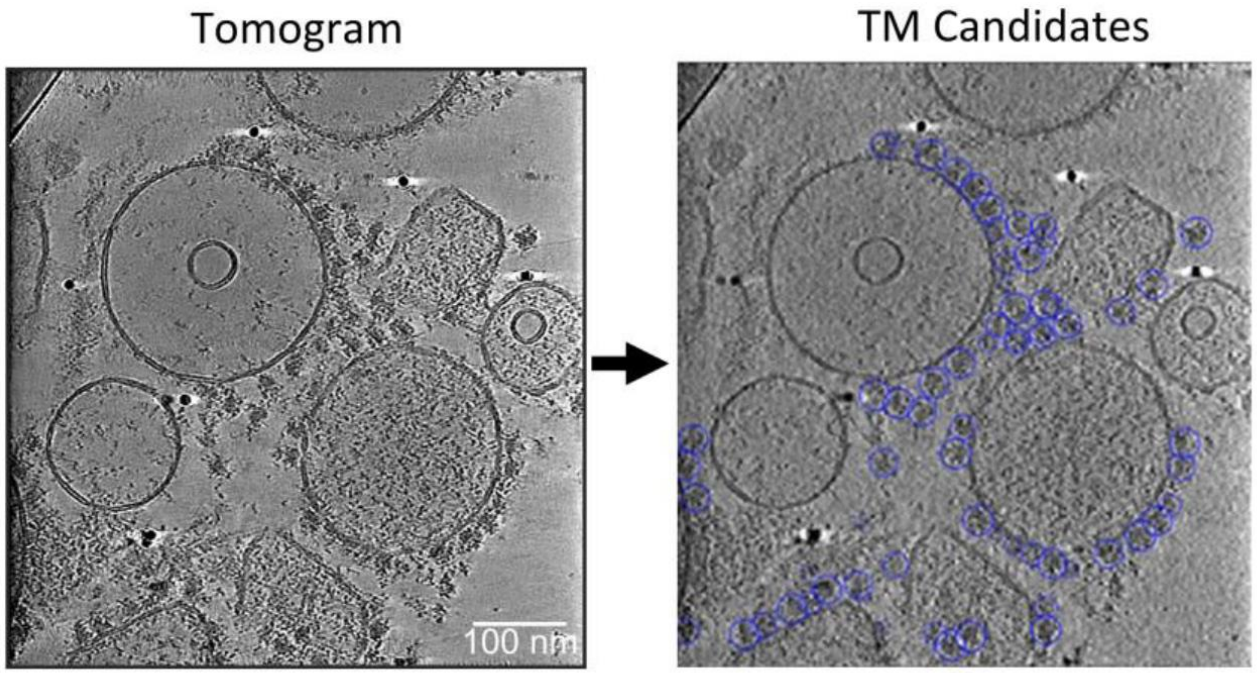


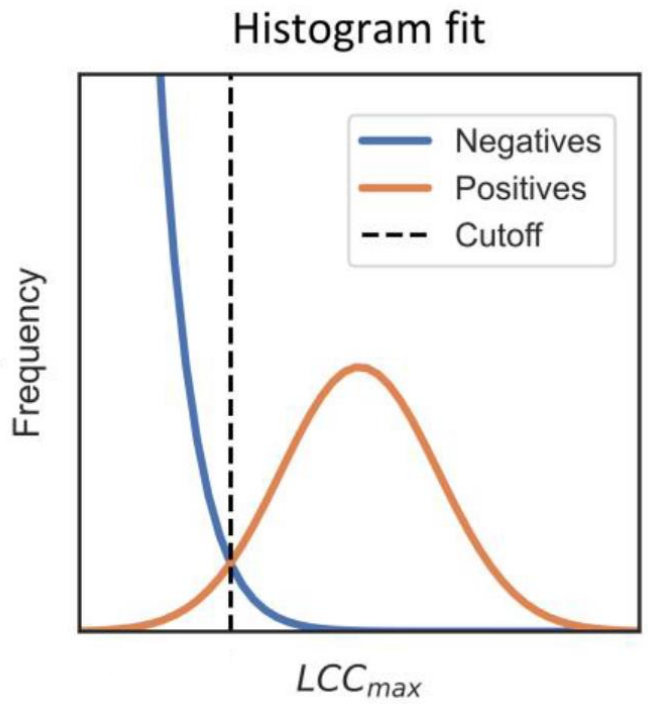
- Pick based on size and shape

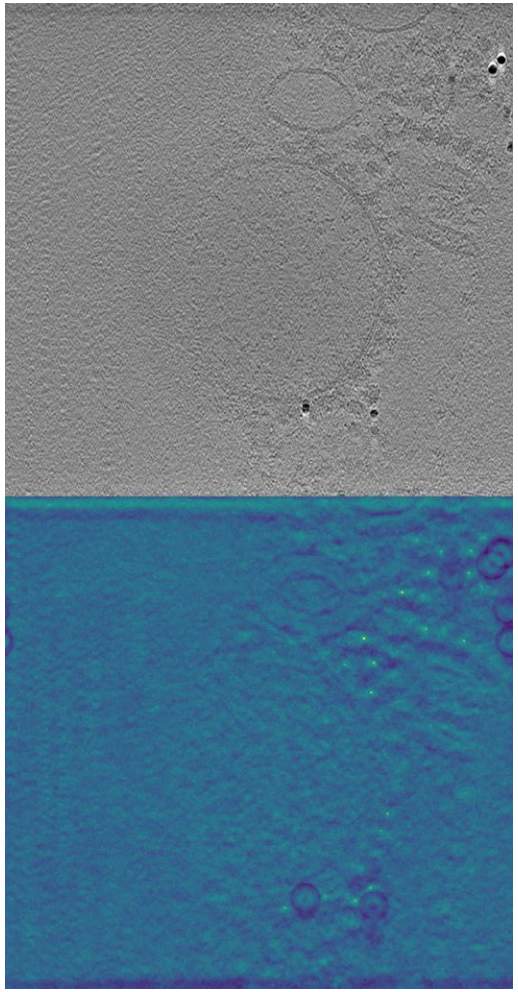
Castaño-Díez et al., 2012, 2017, 1018, 2021

Tomography Particle Picking/Segmentation

Template matching with PyTom

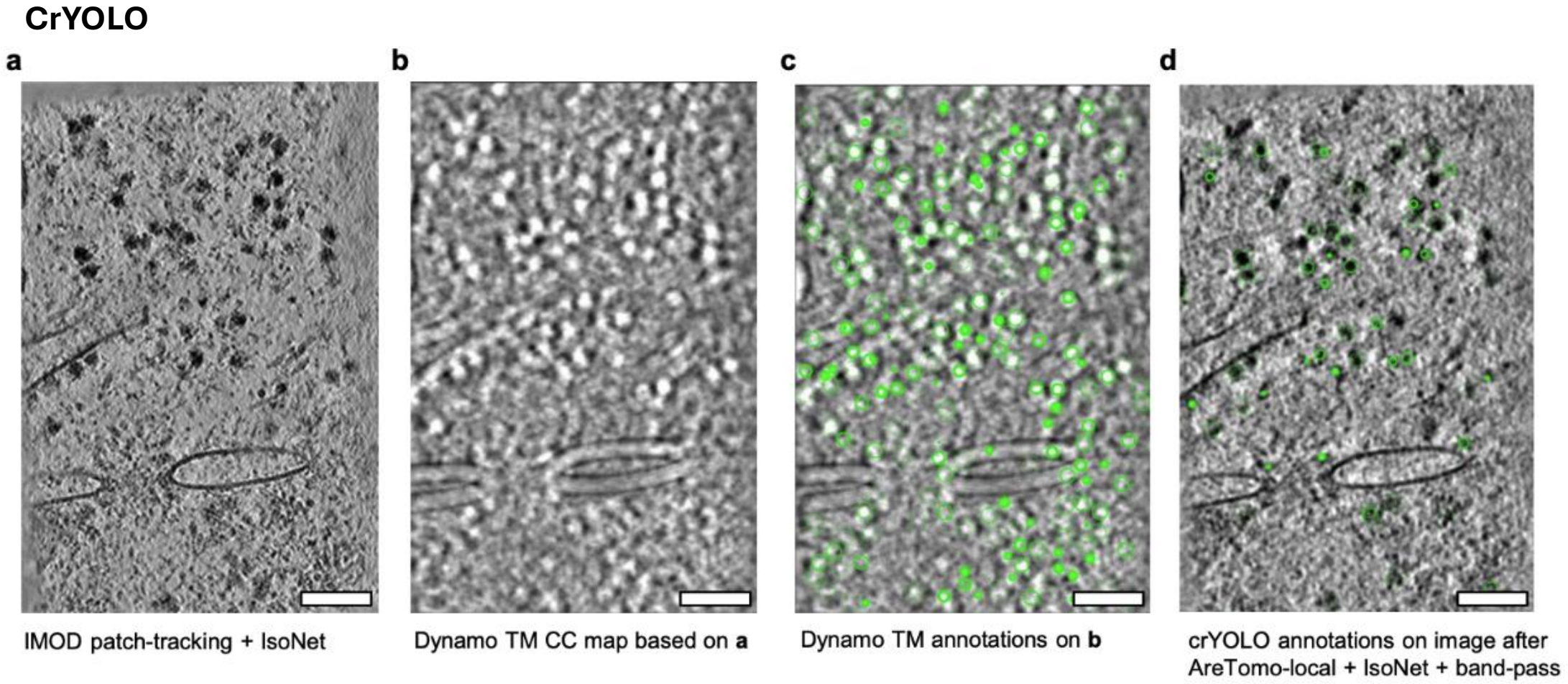






Chaillet et al., 2023

Tomography Particle Picking/Segmentation

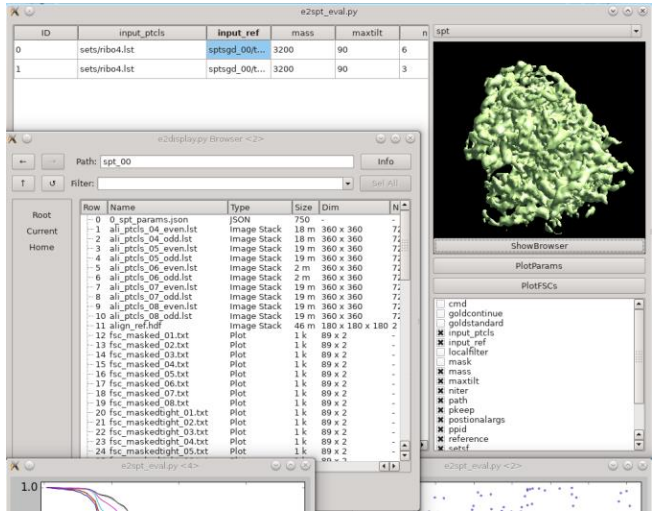


- Machine learning
  - Requires many positive and negative labels

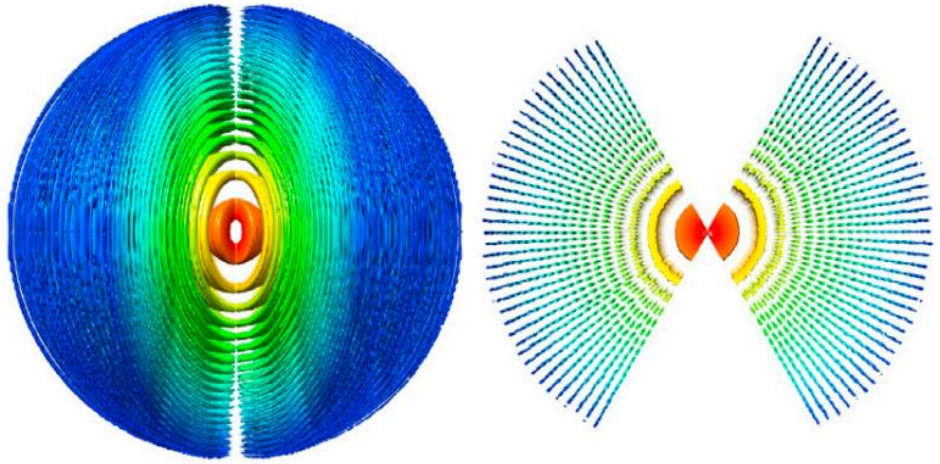
Wagner et al., 2019
Balyschew et al., 2023

Software for Subtomogram-Averaging

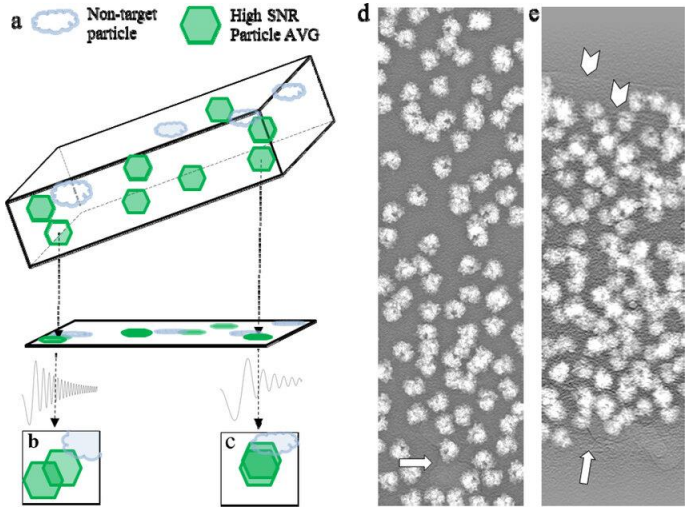
EMAN2



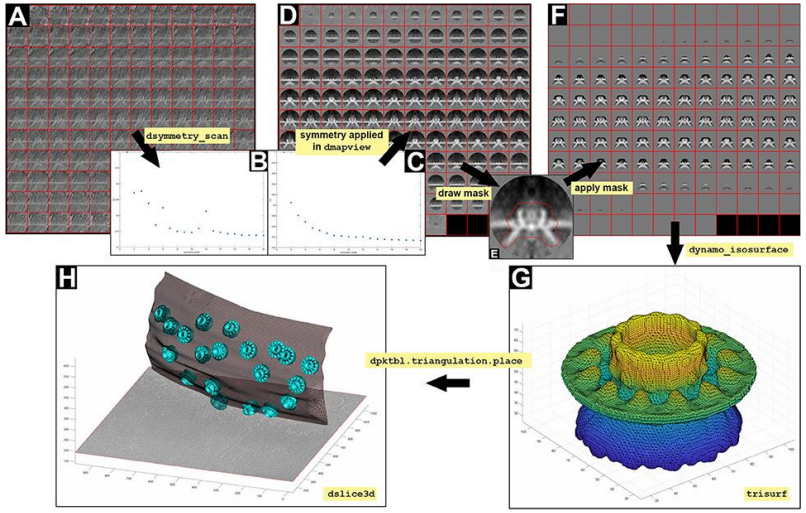
Relion



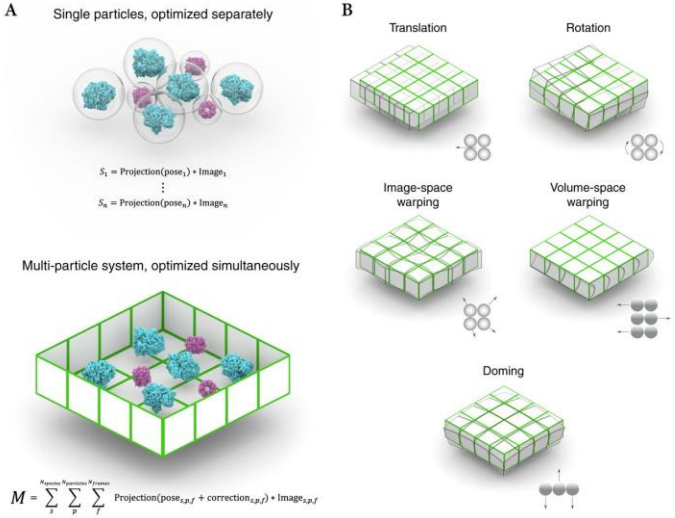
EMClarity



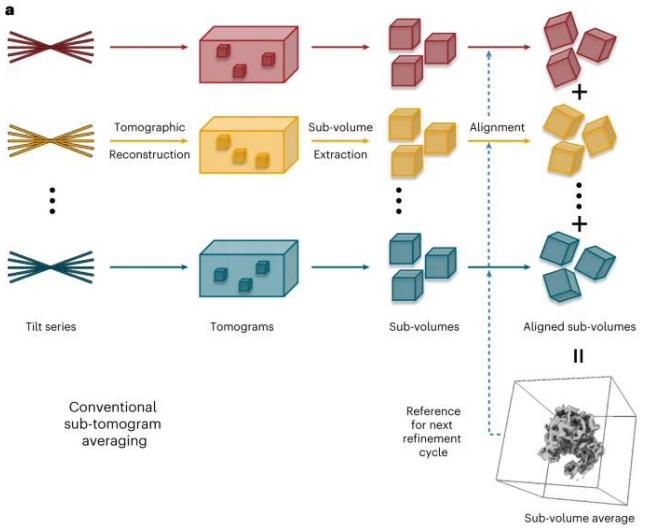
Dynamo



Warp



NextPYP



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

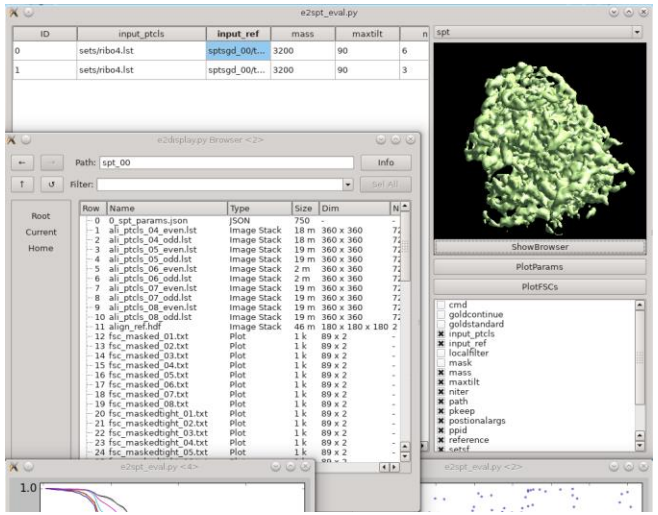
Subtomogram-Classification

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

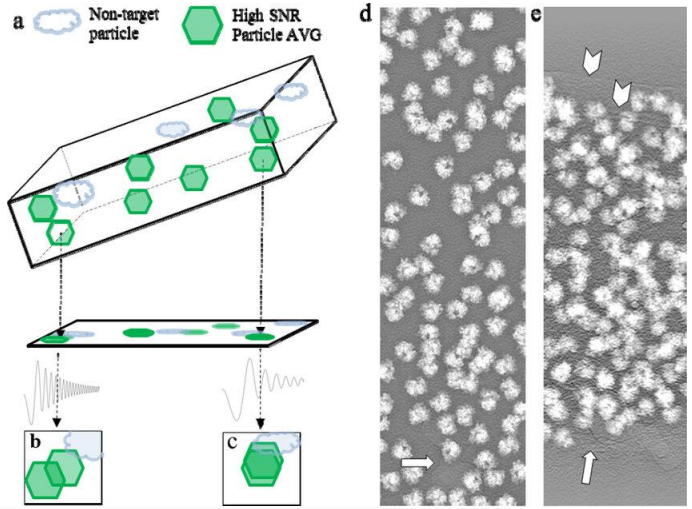
- Speed is often an issue – 3D data takes long to process, i.e, classify using extracted 3D subtomos

Sub-Tilt Refinement Software

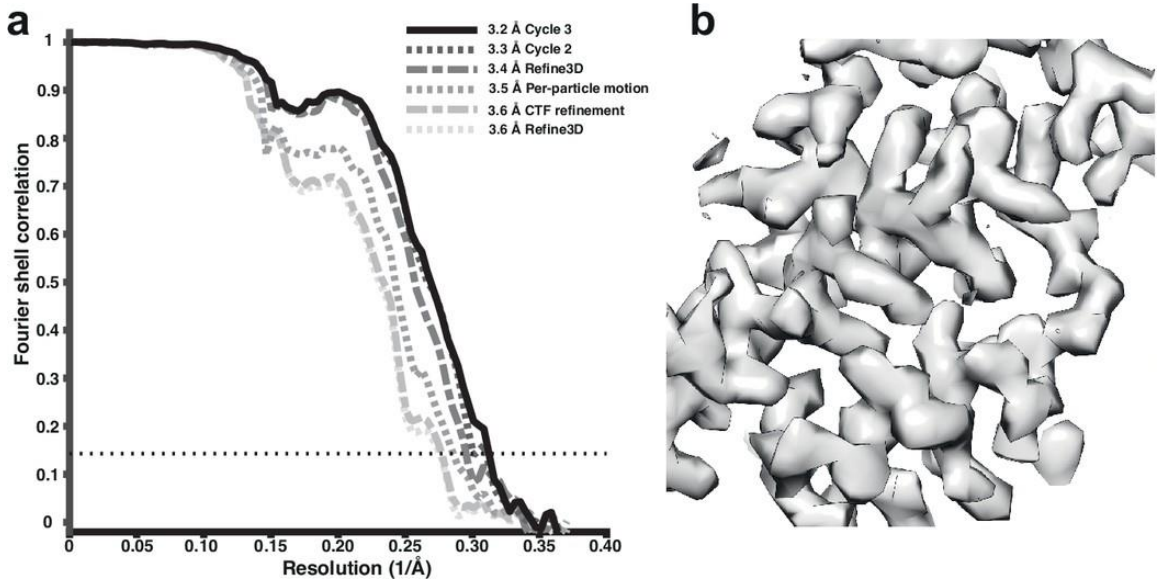
EMAN2



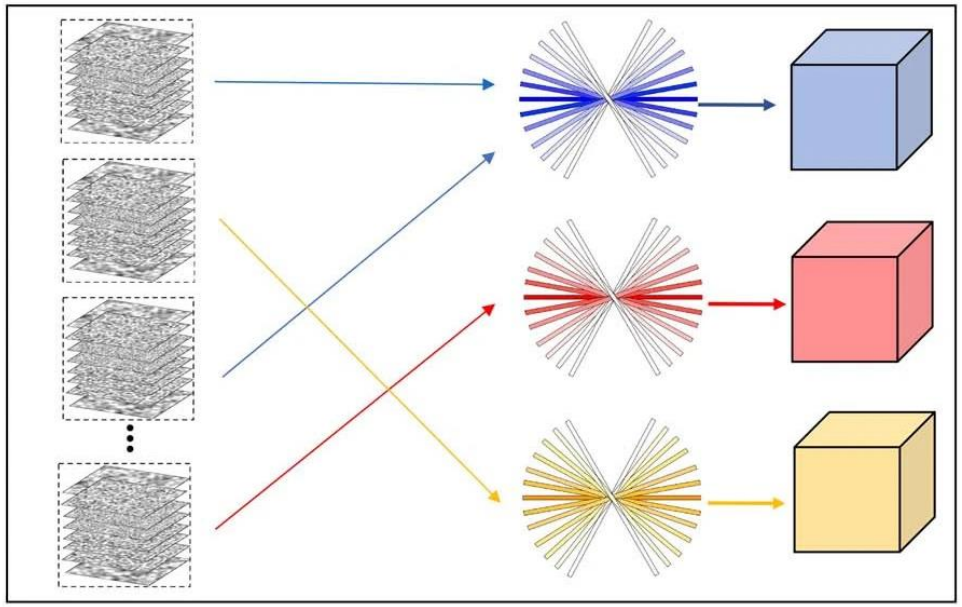
EMClarity



Relion



NextPYP

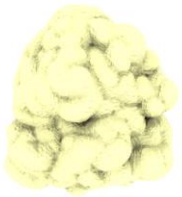


Sub-Tilt Refinement

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

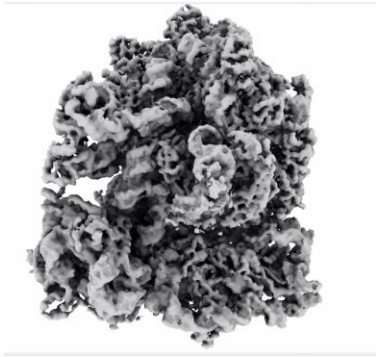
5.1 angstrom map
296 box
Unbinned (pixel size 1.7005 A/pix)
MCore --population m/10499_round1.population --refine_imagewarp 5x5 --refine_particles --ctf_defocus --ctf_defocusexhaustive --perdevice_refine 4

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

Processed with WarpTools, Relion4, to M in linux☺

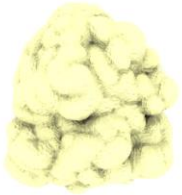
M sub-tilt refinement
EMPIAR-10499

Sub-Tilt Refinement

Total Particles ==23307

M Refinement
low-pass filter 15A
masked refinement

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



Mask used

4th/5th round

3.69 angstrom map
296 box
Unbinned (pixel size 1.7005 A/pix)
MCore \
--population m/10499_round1.population \
--refine_imagewarp 5x5 \
--refine_particles \
--refine_mag \
--ctf_cs \
--ctf_defocus \
--ctf_zernike3

Alister will talk more about WarpTools, Relion, and M right after this!

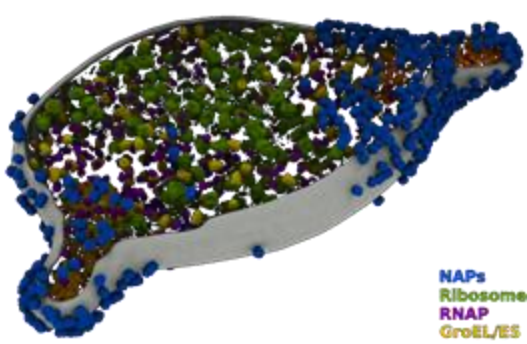
M sub-tilt refinement
EMPIAR-10499

Processed with WarpTools, Relion4, to M in linux☺

Mapping Averages back for Cellular Context

- 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

ArtiaX



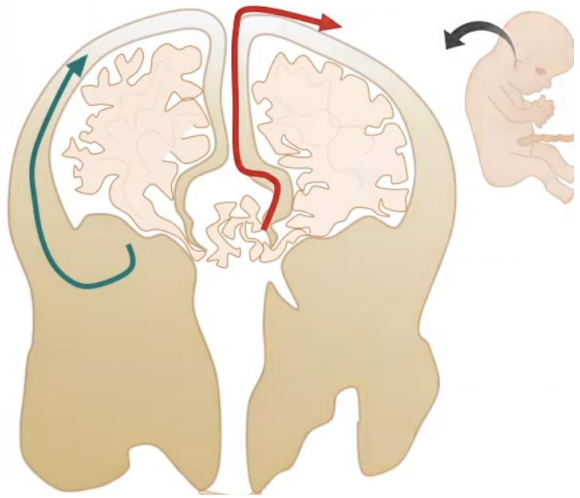
Ermel et al., 2022

Test Cases

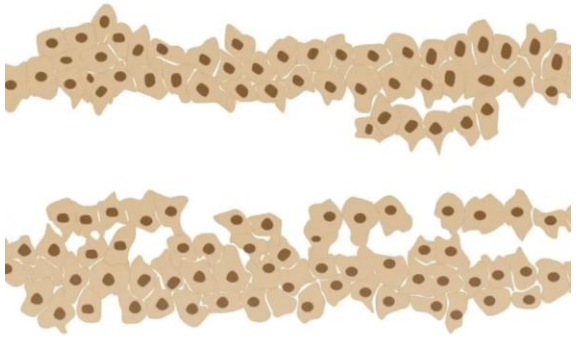
- Too many Software, and how do we apply these different software to our datasets?
- It depends on the biological question you are answering and the sample type
- What you need can dictate how the data collection is done…
- Test Case, looking at ER ultrastructure in migrating cells, Segmentation pipeline

Directed Cell Migration is Critical for Several Physiological and Pathological Processes

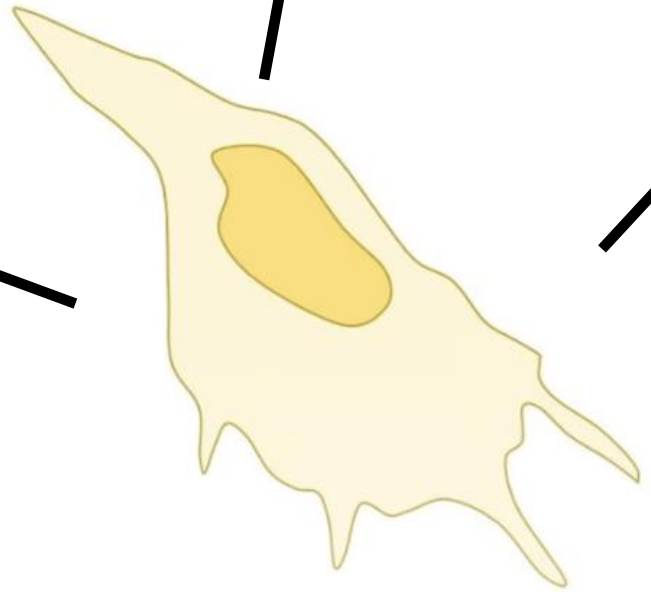
Embryonic Development



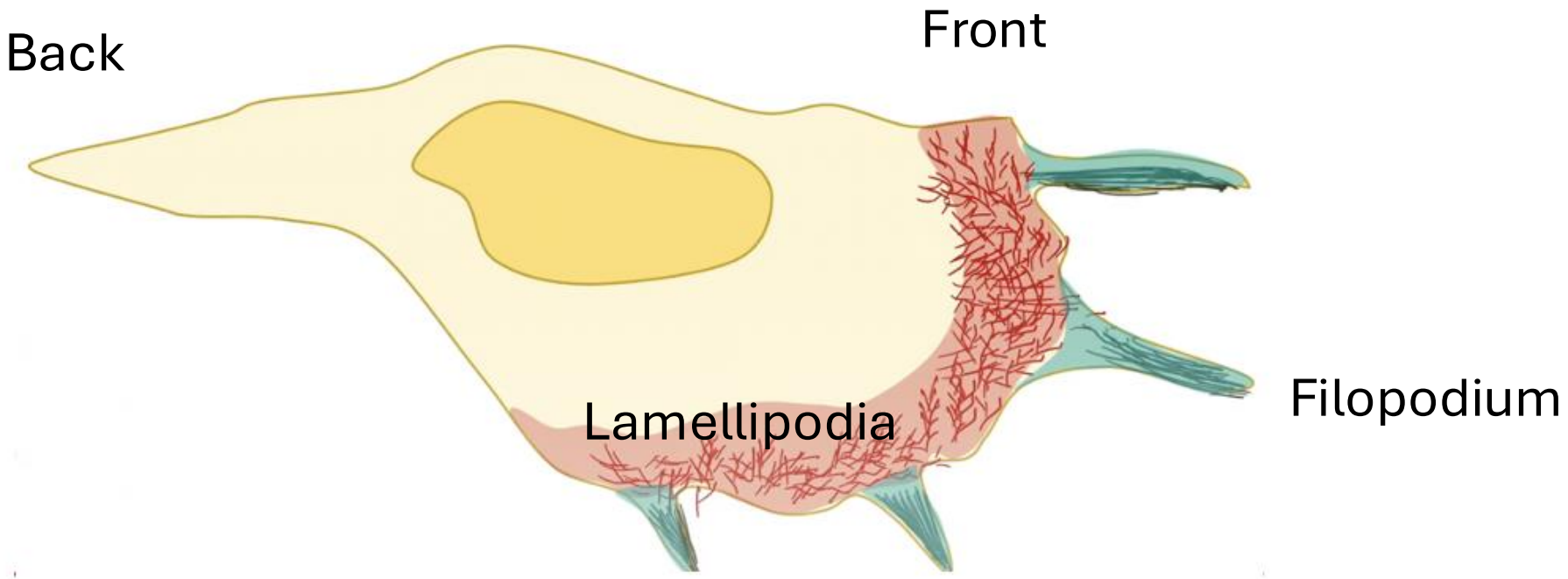
Wound Healing



Neutrophil extravasation

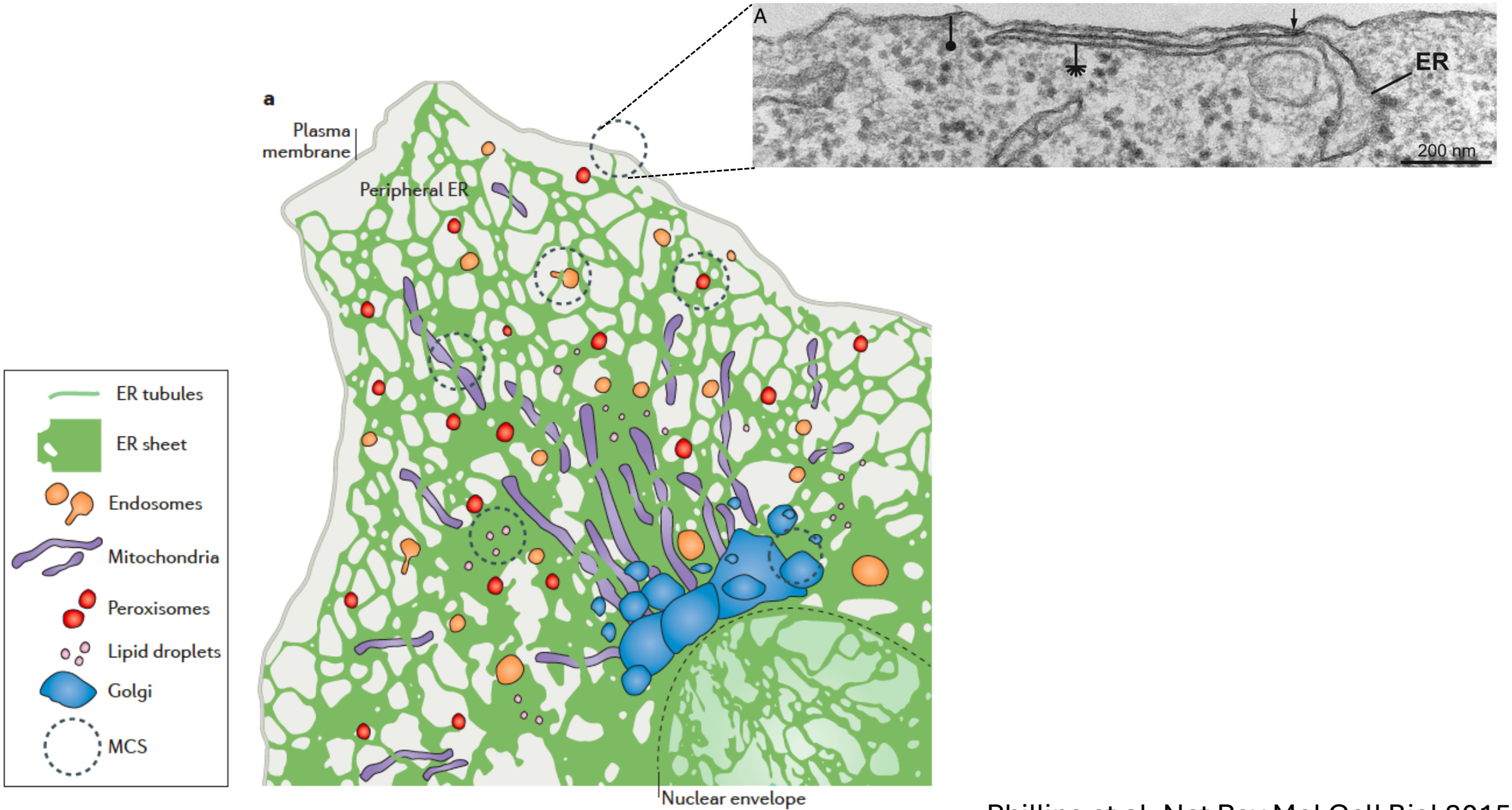


Cell Polarity is present in all Migrating Cells



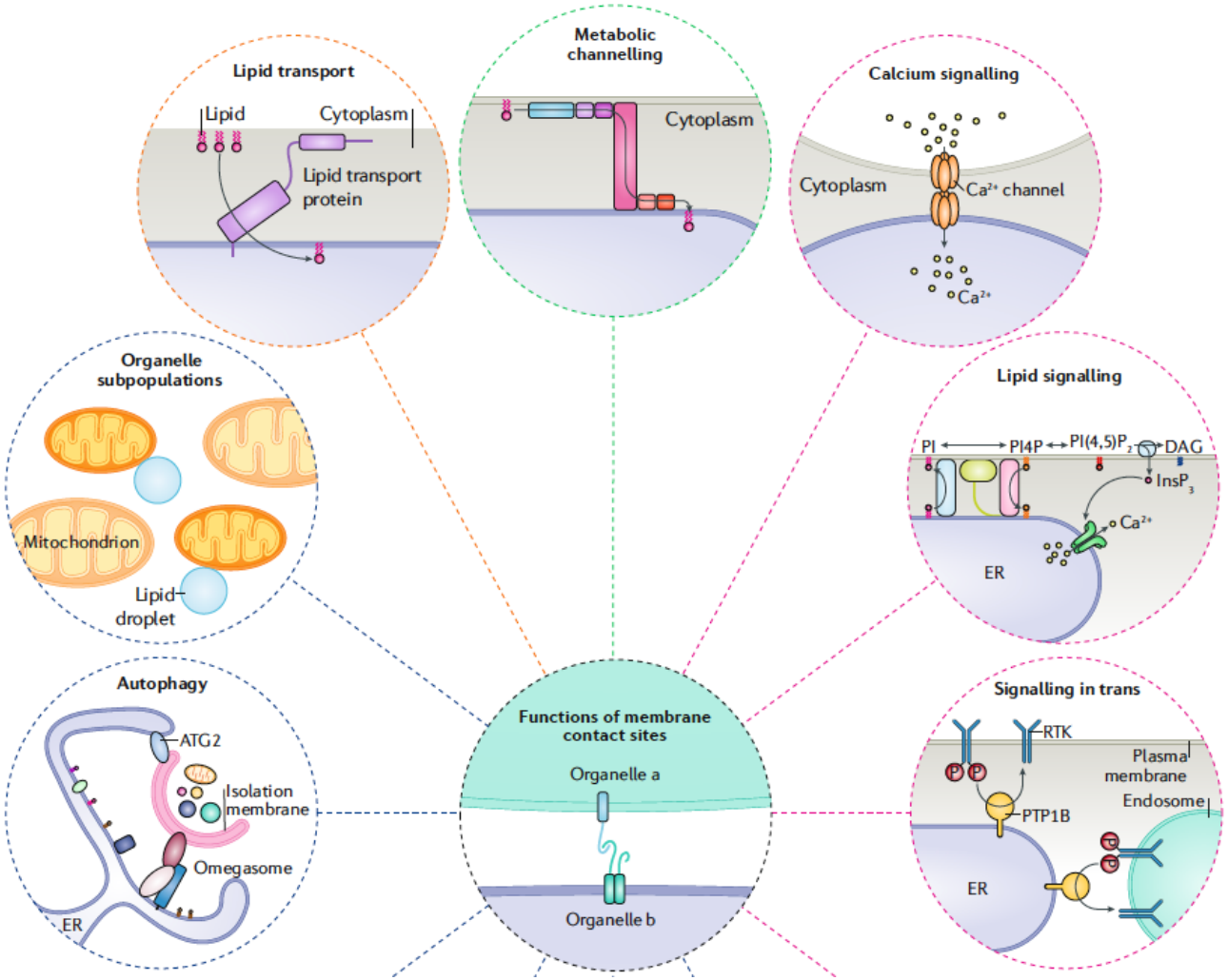
Certain Structures always form at the front of the cell, and others in the back!

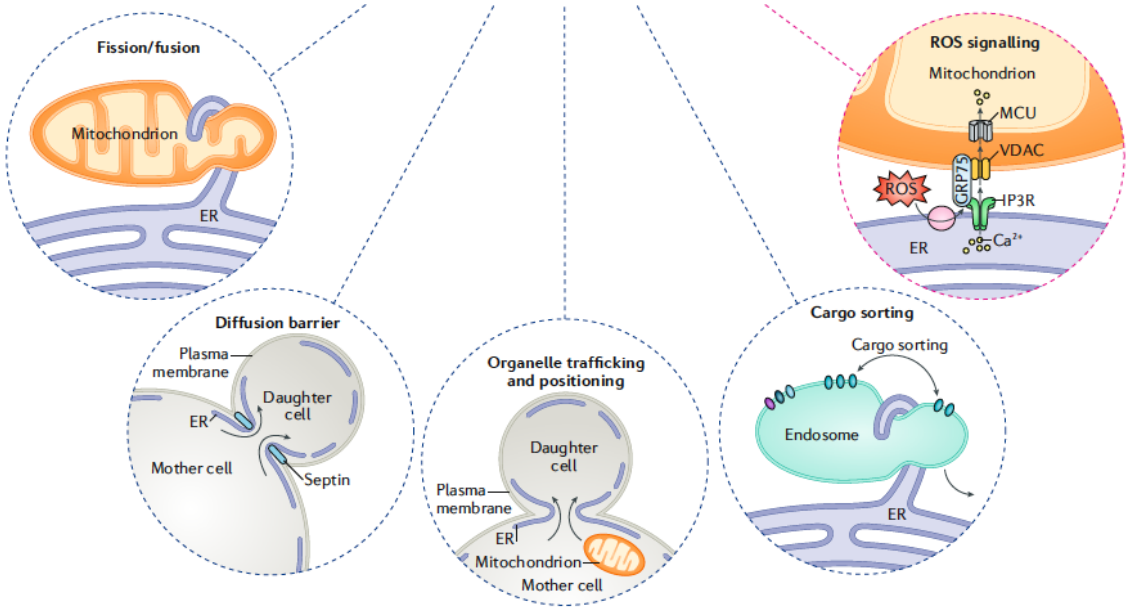
Membrane Contact Sites



Phillips et al, Nat Rev Mol Cell Biol 2015

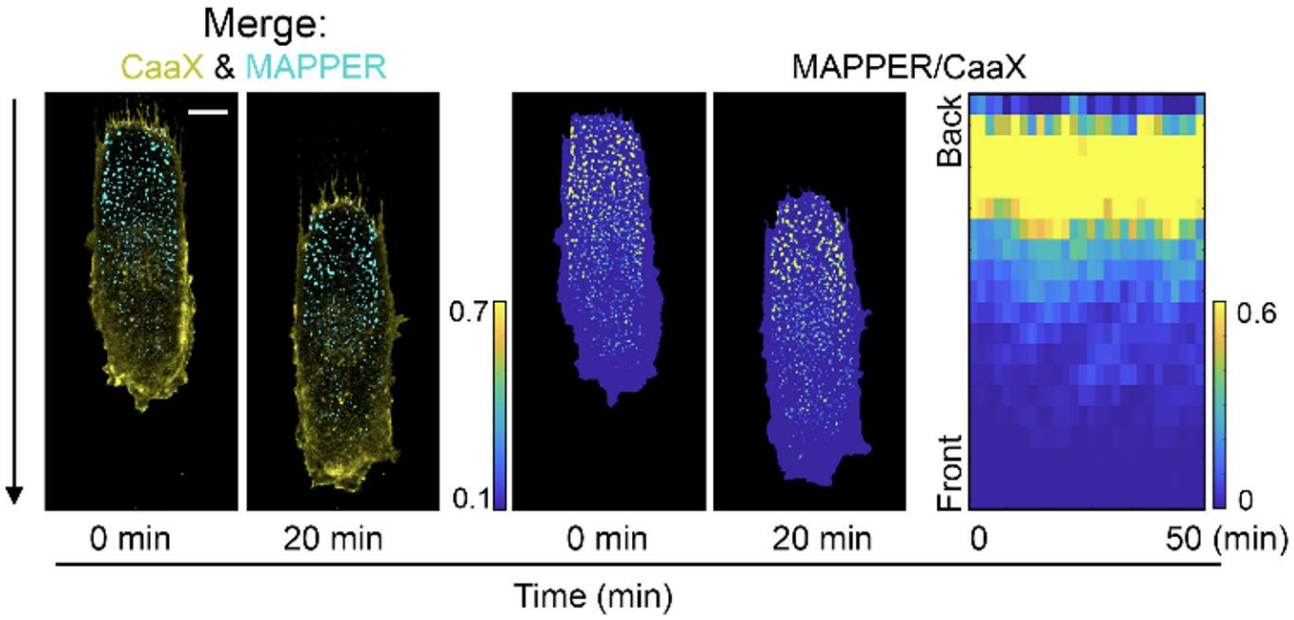
The Functional Diversity of Membrane Contact Sites

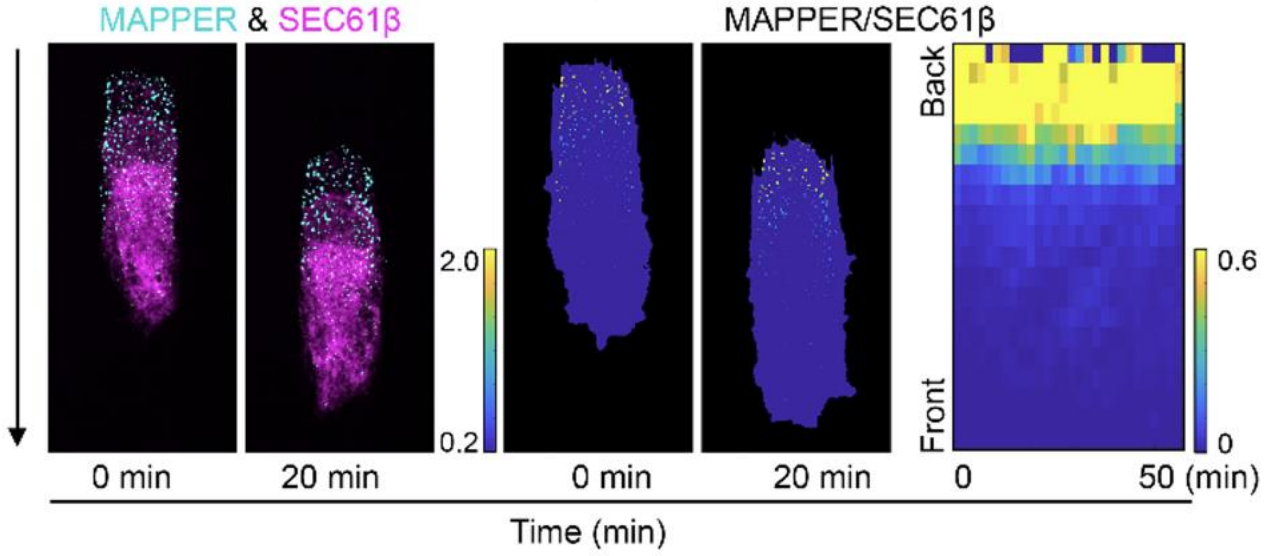


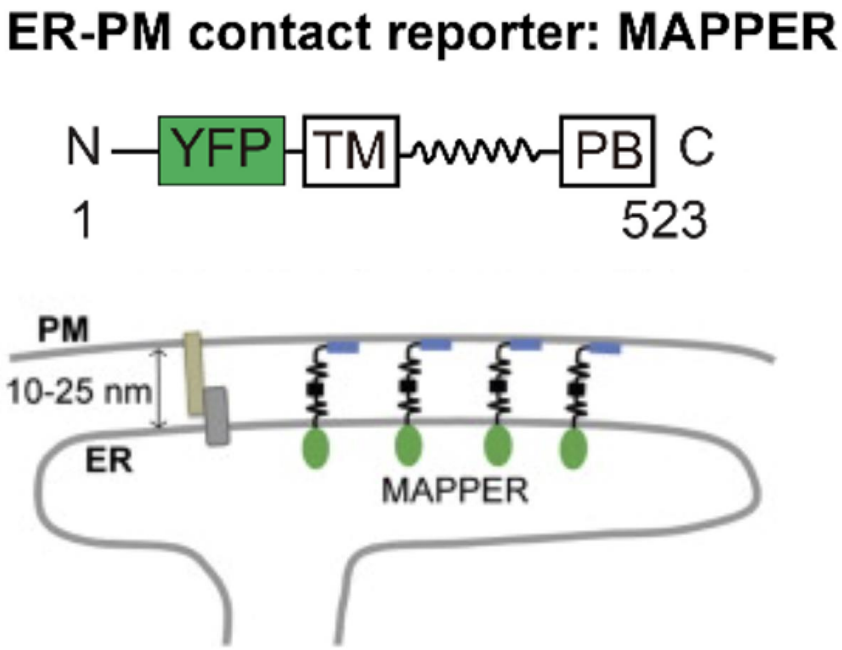


Prinz et al, Nat Rev Mol Cell Biol 2020

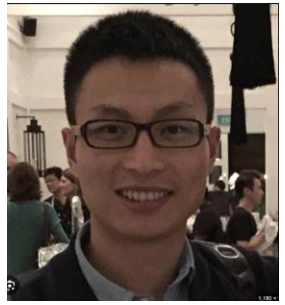
Endoplasmic Reticulum Contact Sites are Polarized in Migrating Cells







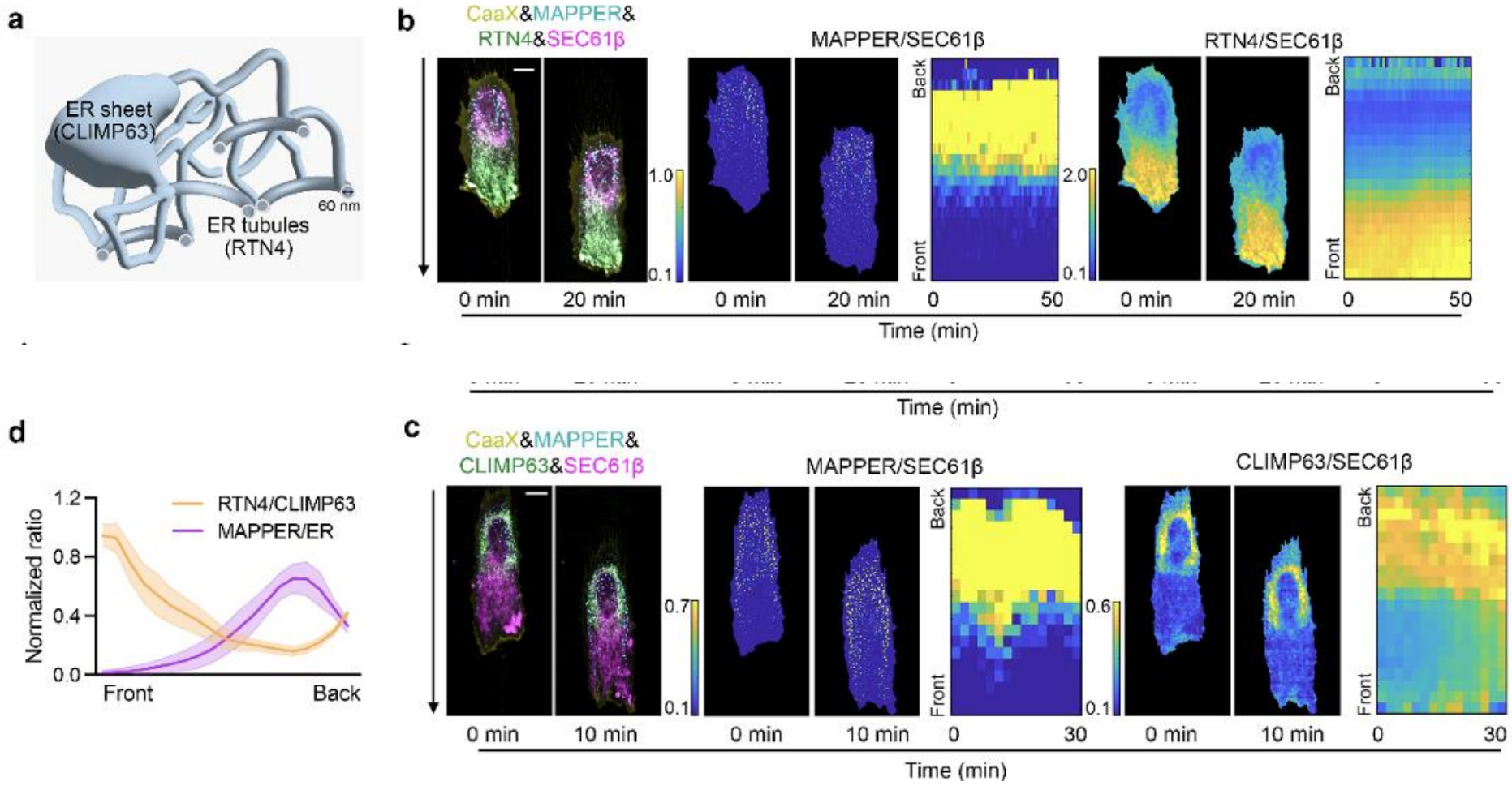
Chang et al, Cell Reports, 2013

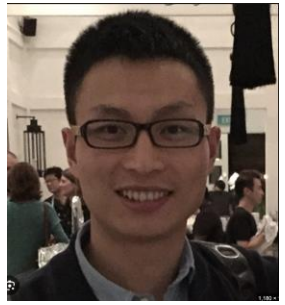


Data from Bo Gong, Weill Cornell Medicine

Gong, B., **Johnston, J.D.**, Thernicke, A., DeMarco, A. & Meyer, T. (2024) Endoplasmic Reticulum-Plasma Membrane contact gradients direct cell migration. **Just accepted to Nature!**

Polarized Cells Exhibit Endoplasmic Curvature Gradients





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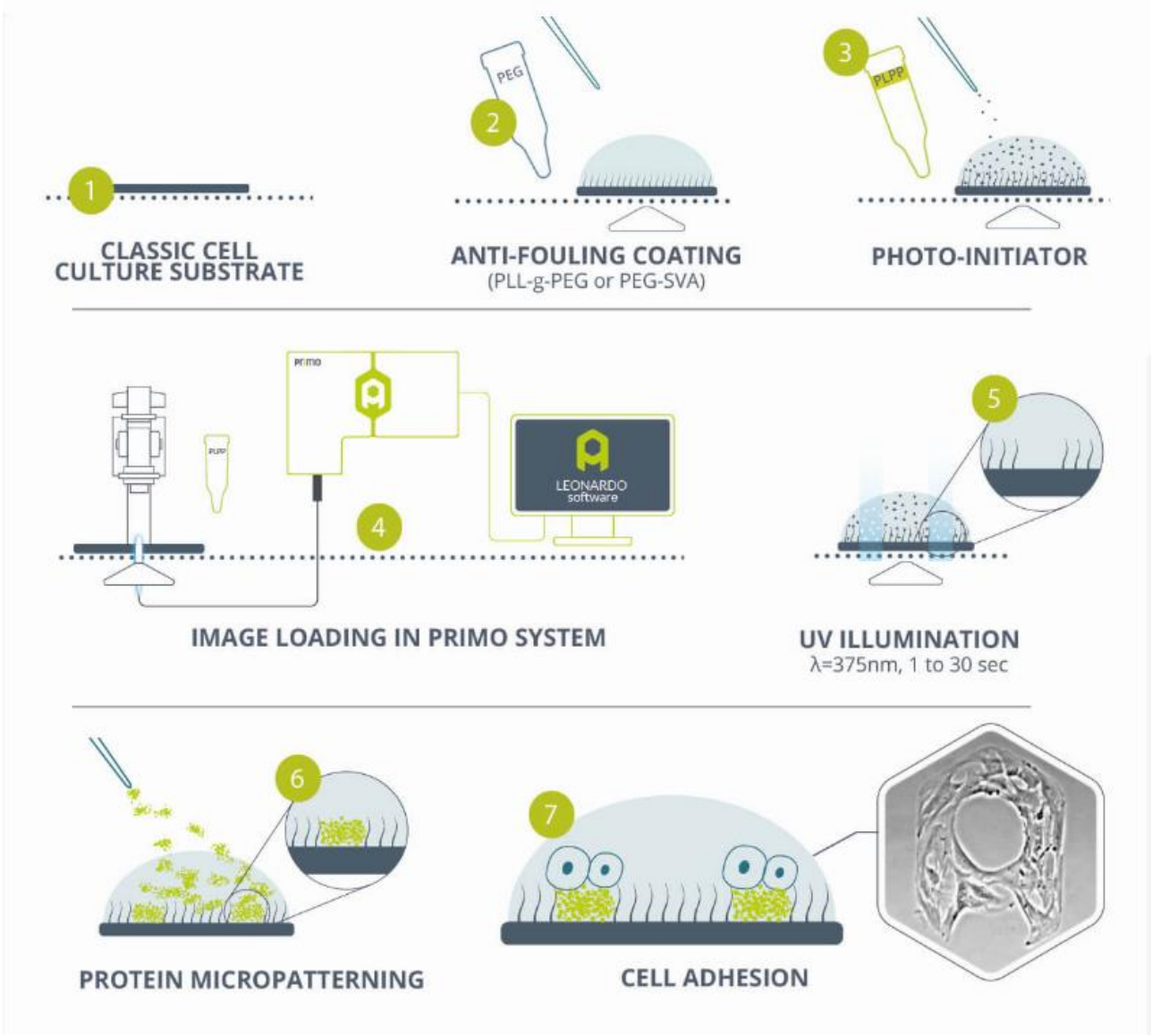
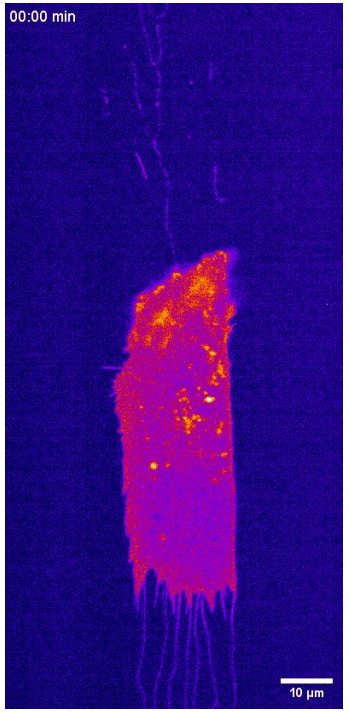
Goals of the Project

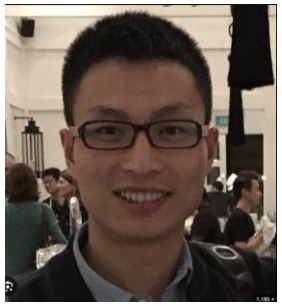
- **Determine the high-resolution endoplasmic reticulum architecture in polarized cells using cryo-electron tomography**

- **Comparative analysis of endoplasmic reticulum to plasma membrane contact sites at the front and back of polarized cells at high-resolution using cryo-electron tomography**

Forced cell polarization using Micropatterning Approach

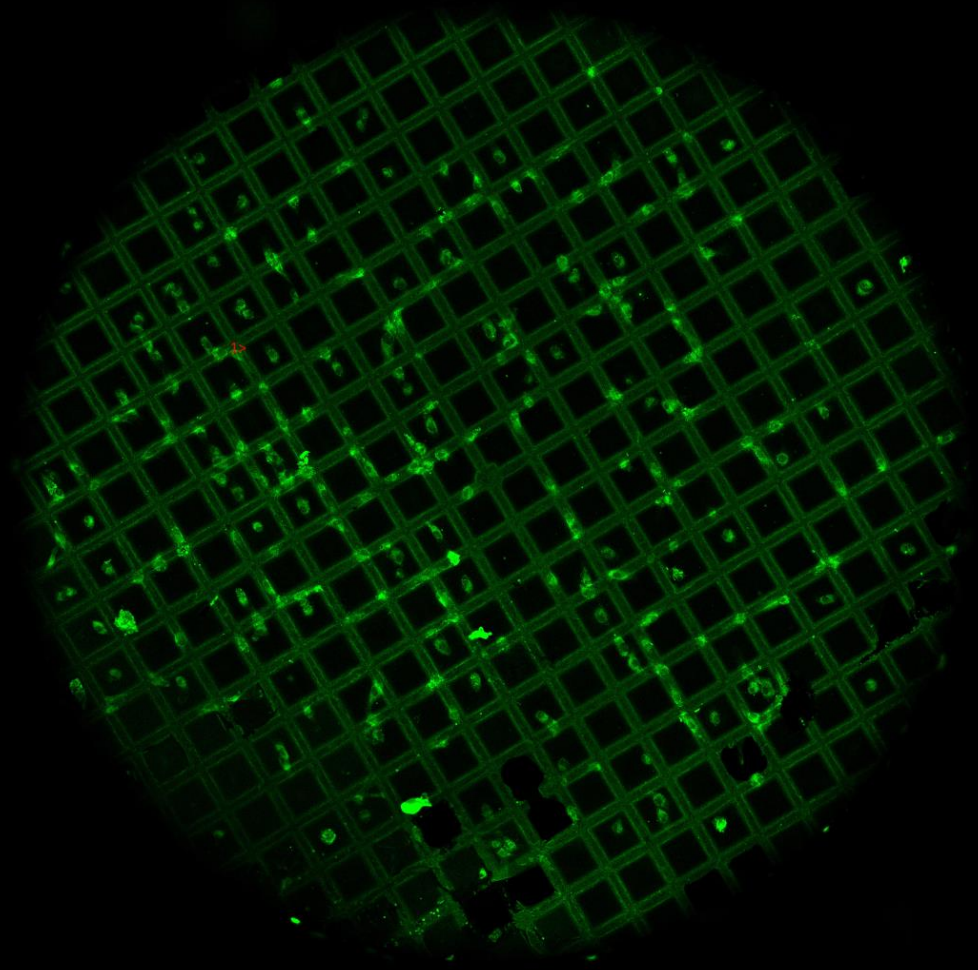






Data from Bo Gong, Weill Cornell Medicine

Photo-Micropatterning to Induce Cell-Polarization on Electron Microscopy Grids



5X Grid Overview
RPE1 cells Venus-Mapper

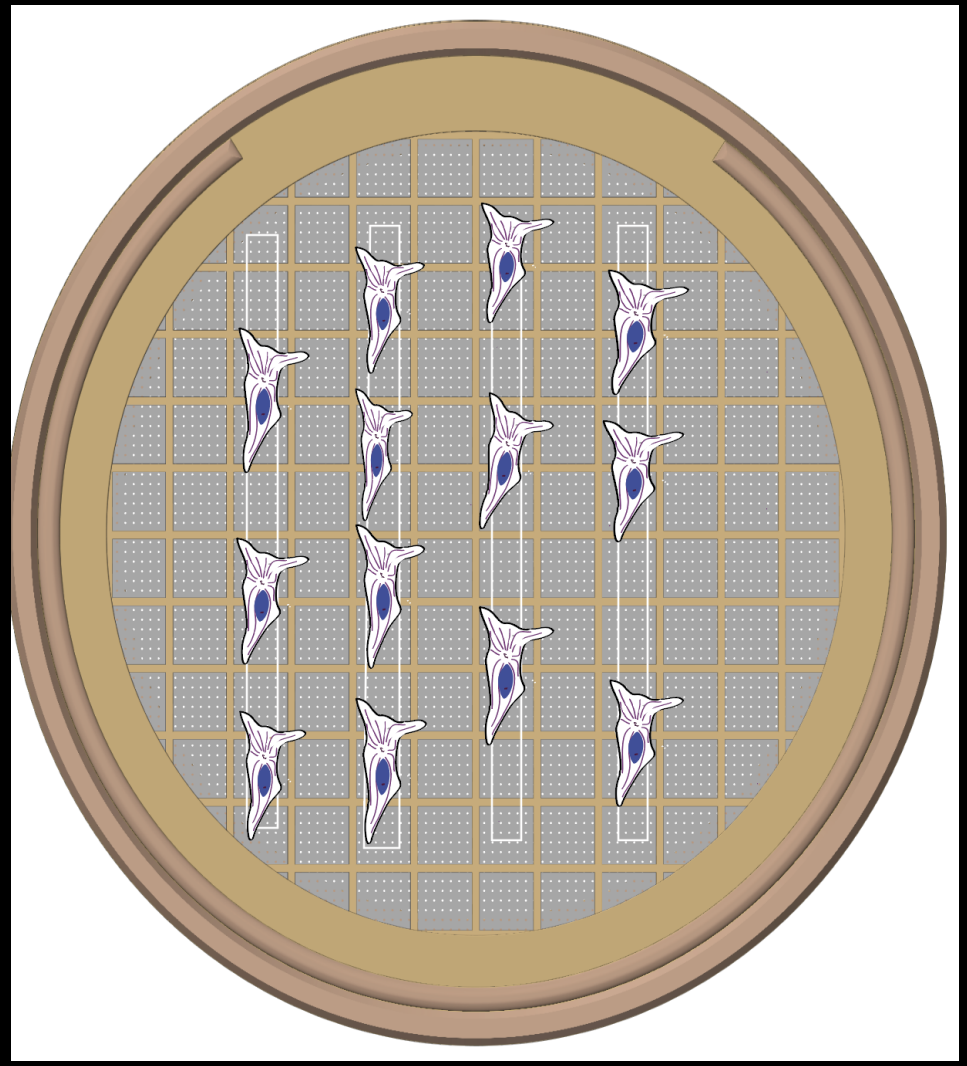
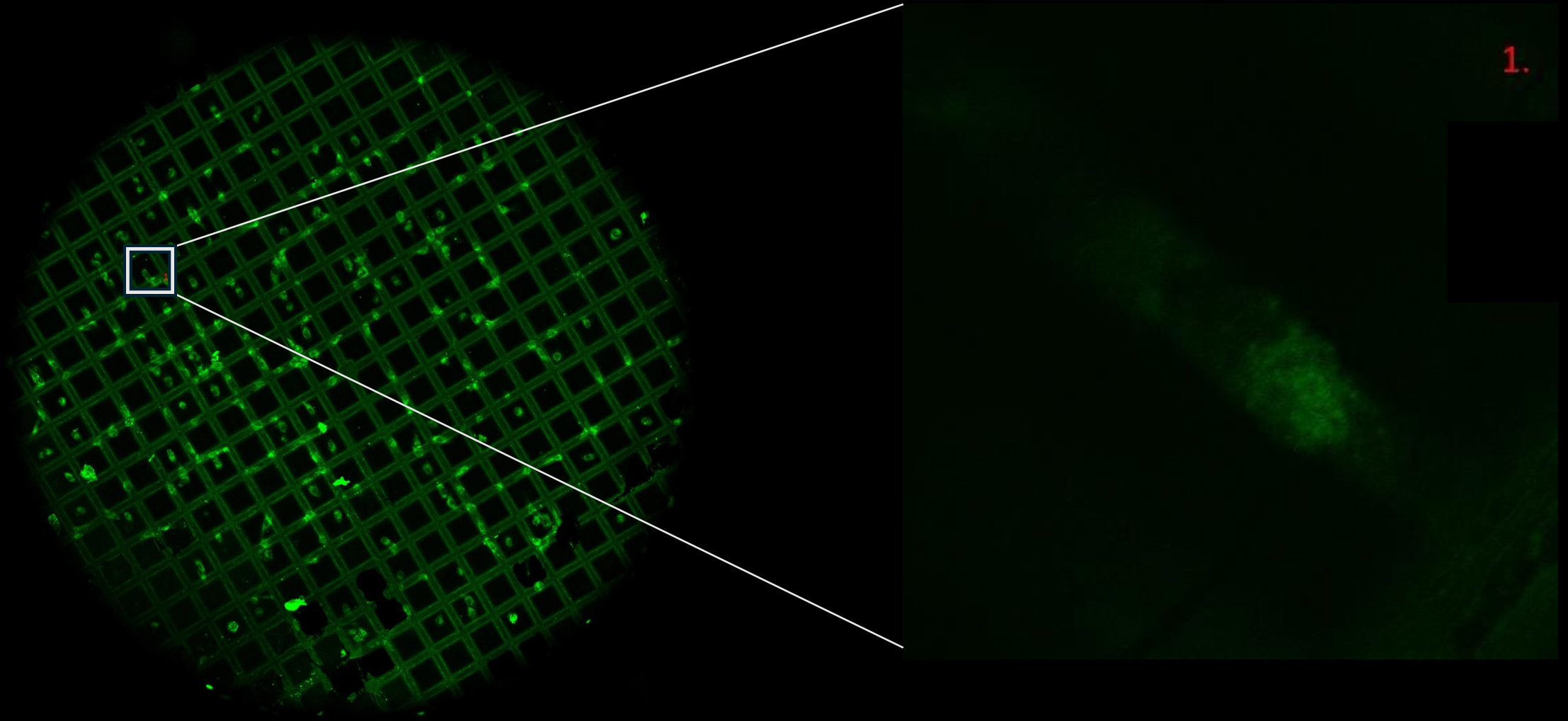


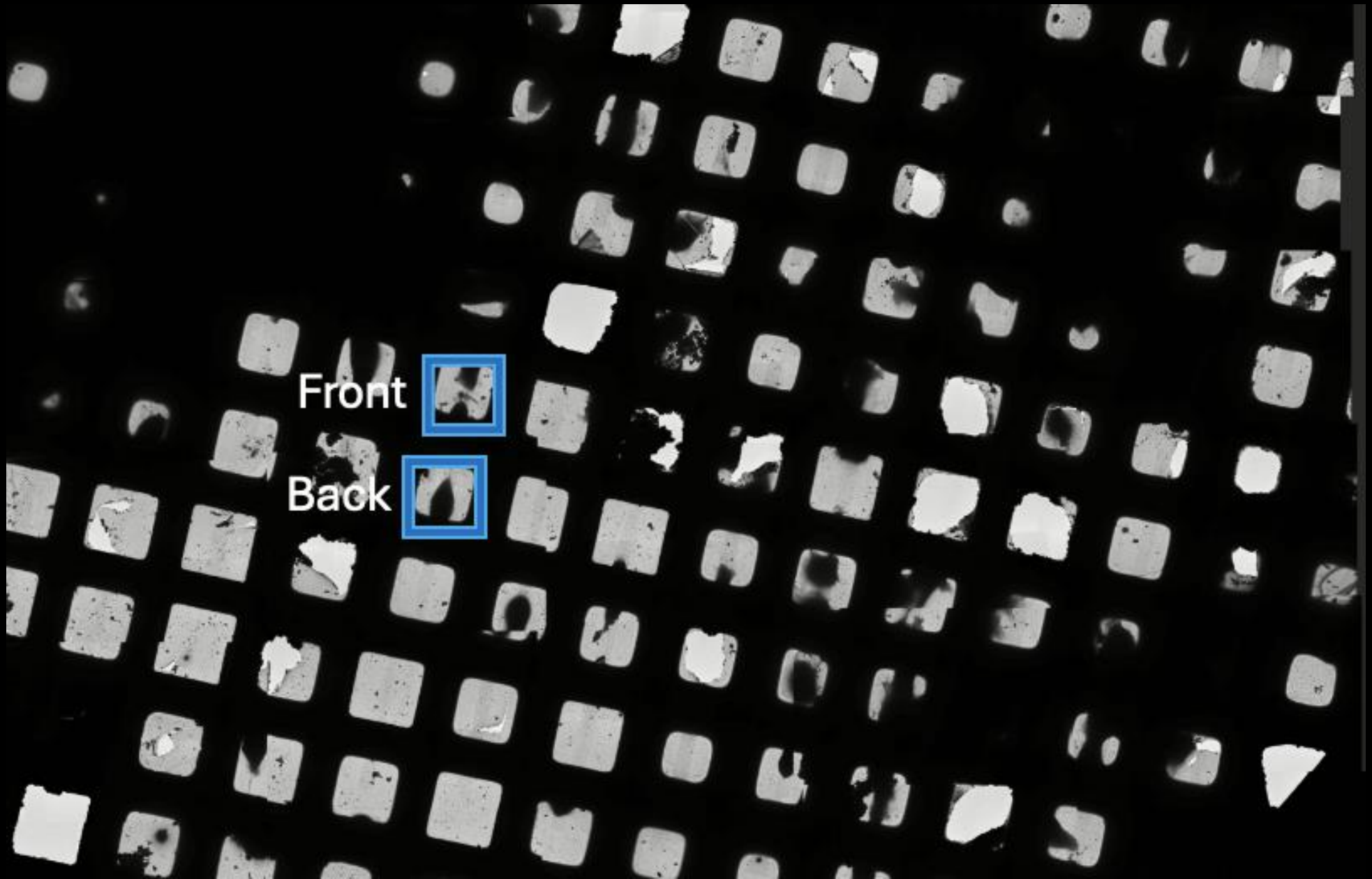
Photo-Micropatterning to Induce Cell-Polarization on Electron Microscopy Grids



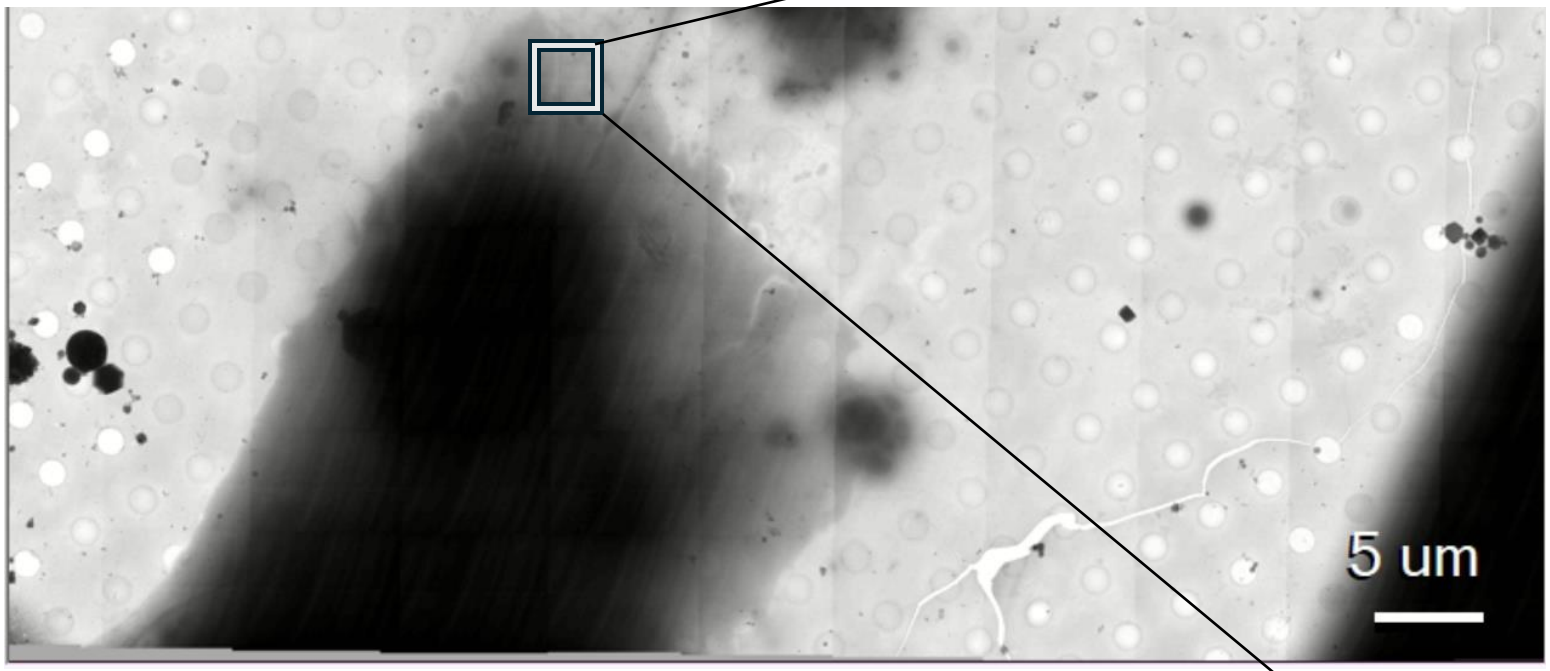
5X Grid Overview
RPE1 cells Venus-Mapper

100X Grid Overview
Z-stack RPE1 cells Venus-Mapper

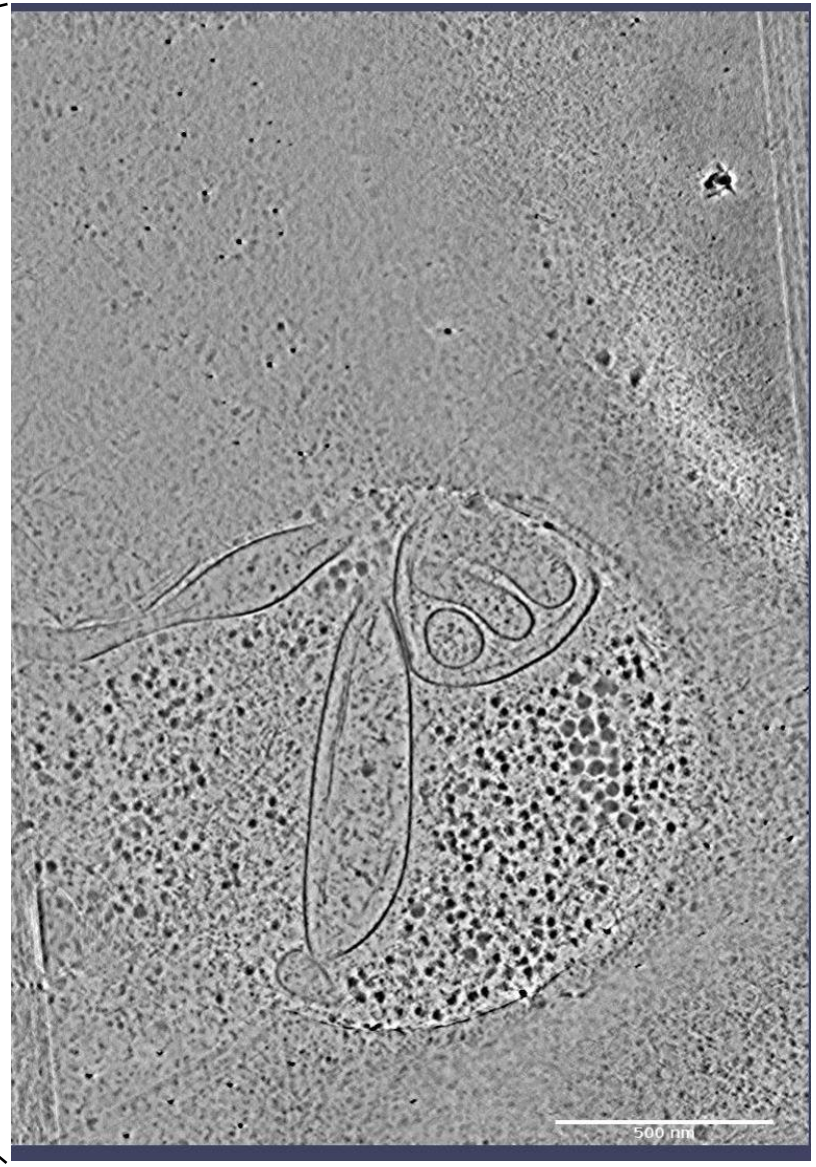
Low magnification Overview of Micro-Patterned Grid



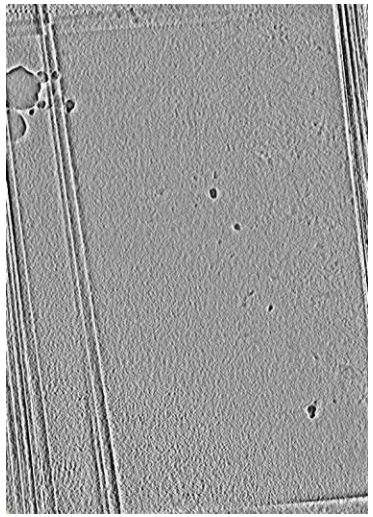
Front of the Polarized Cell Exhibits Tubular or Smooth Endoplasmic Reticulum



WT RPE1 Cells



Membrane-Segmentation Workflow



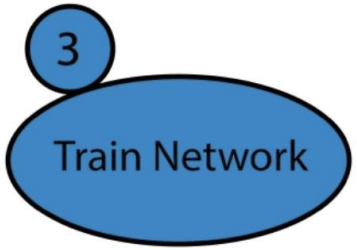
IsoNet-Corrected Tomogram

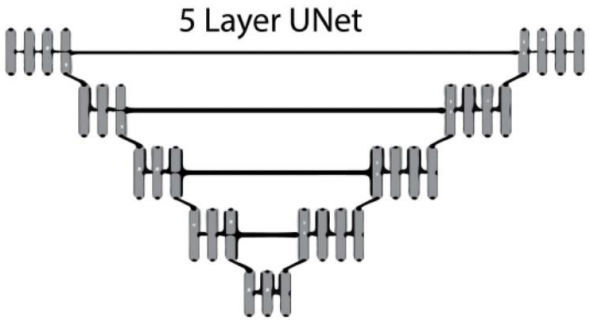
Initial Segmentation with Tardis/ and or TomomemsegTV

Binary Mask segmentation

Import into DragonFly
Manually Clean up ~ 5 2-D Tomographic slices
Train

ORSDragonFly Training

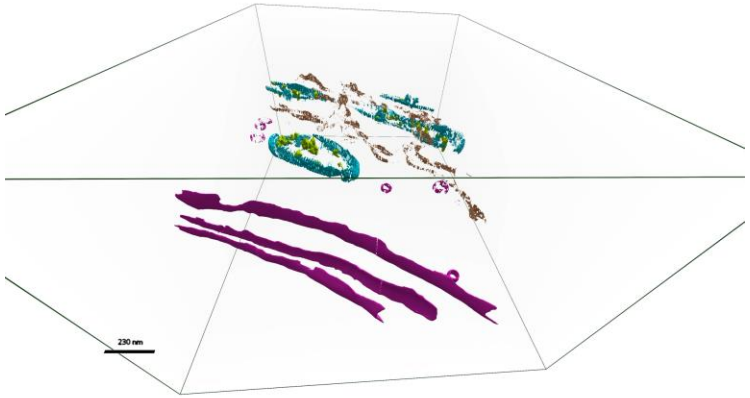




Iterate

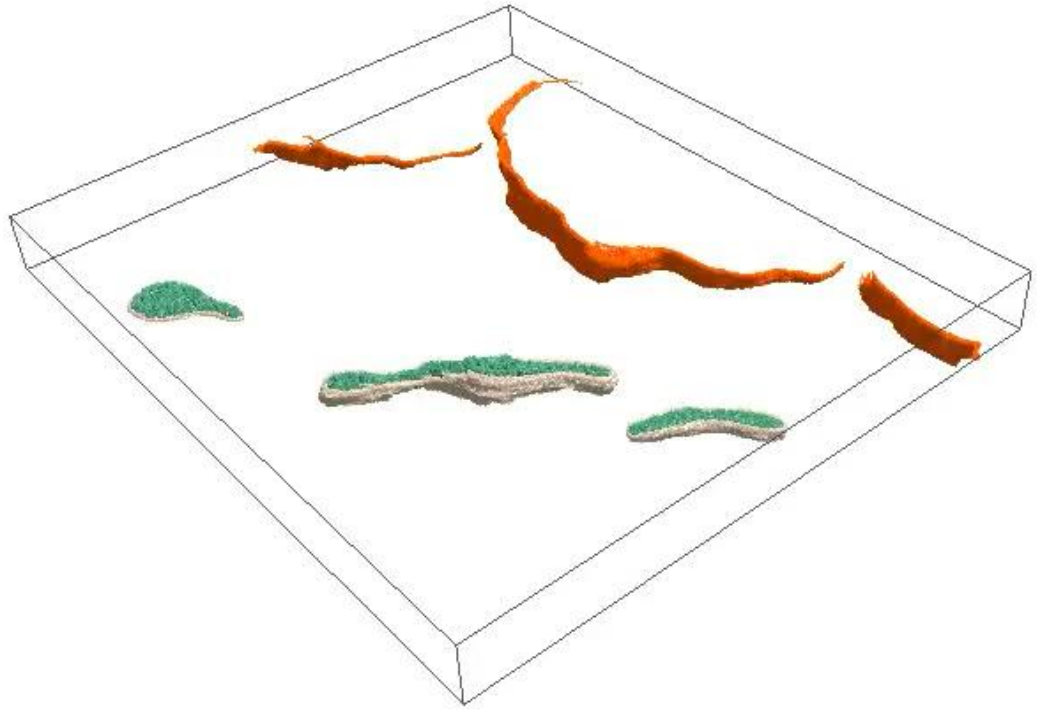
Train Tardis with Clean Segmentations

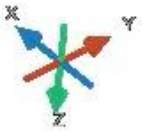
Final clean segmentation



3d surface rendering

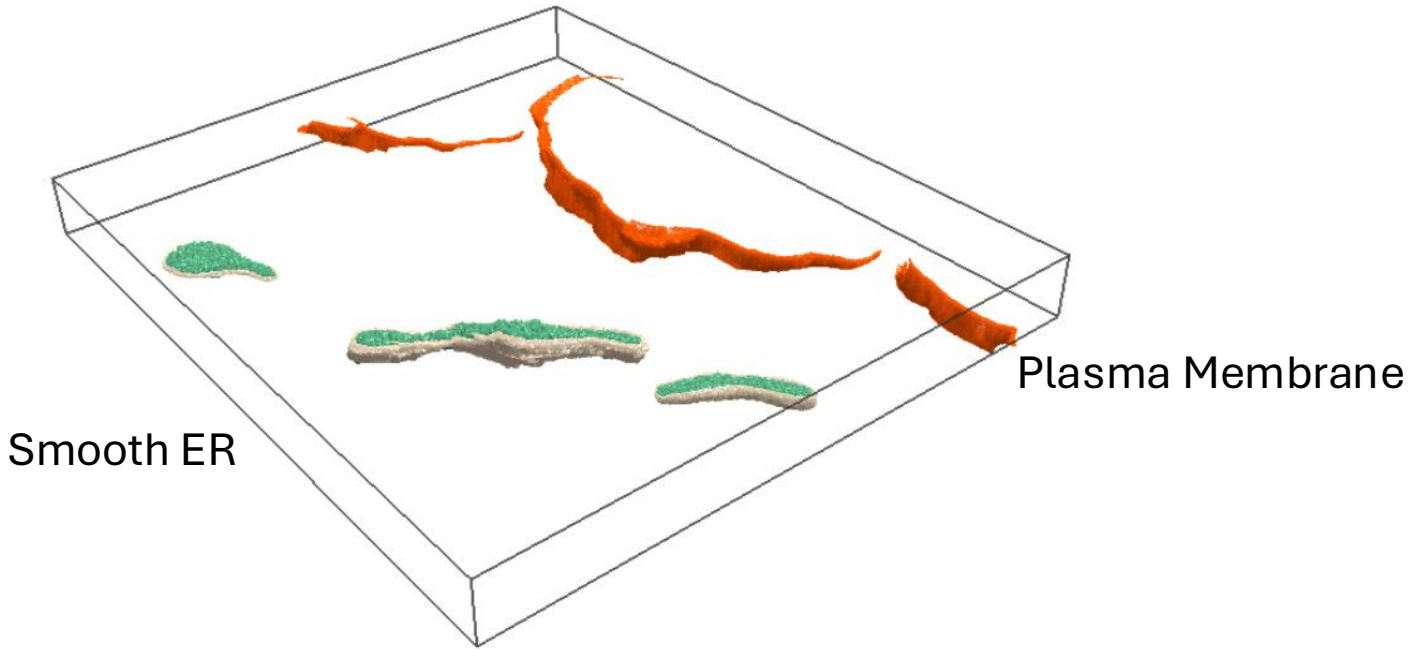
Front of the Polarized Cell Exhibits Tubular or Smooth Endoplasmic Reticulum





Gong, B., **Johnston, J.D.**, Thernicke, A., DeMarco, A. & Meyer, T. (2024) Endoplasmic Reticulum-Plasma Membrane contact gradients direct cell migration. **Just accepted to Nature!**

Front of the Polarized Cell Exhibits Tubular or Smooth Endoplasmic Reticulum



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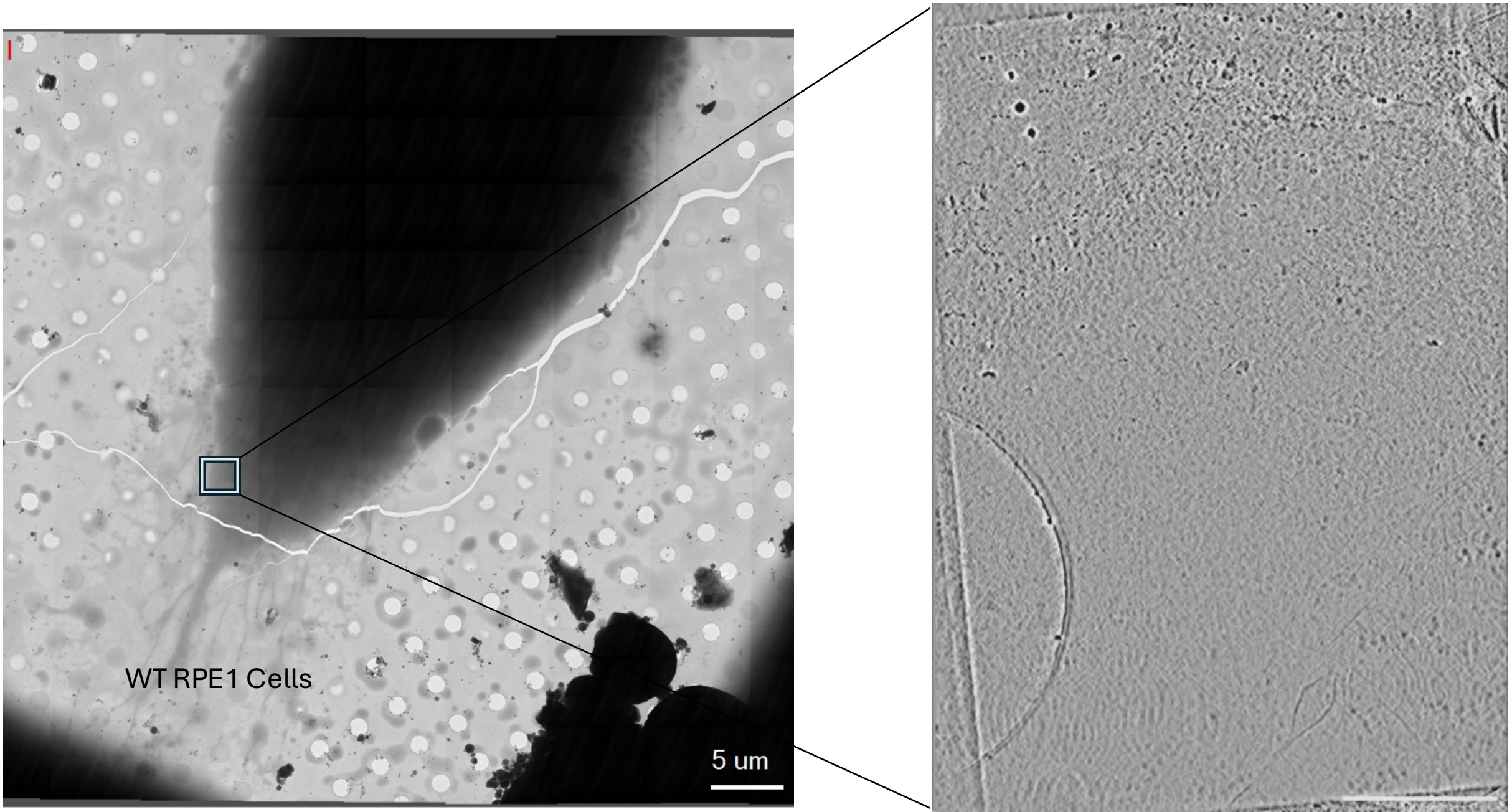
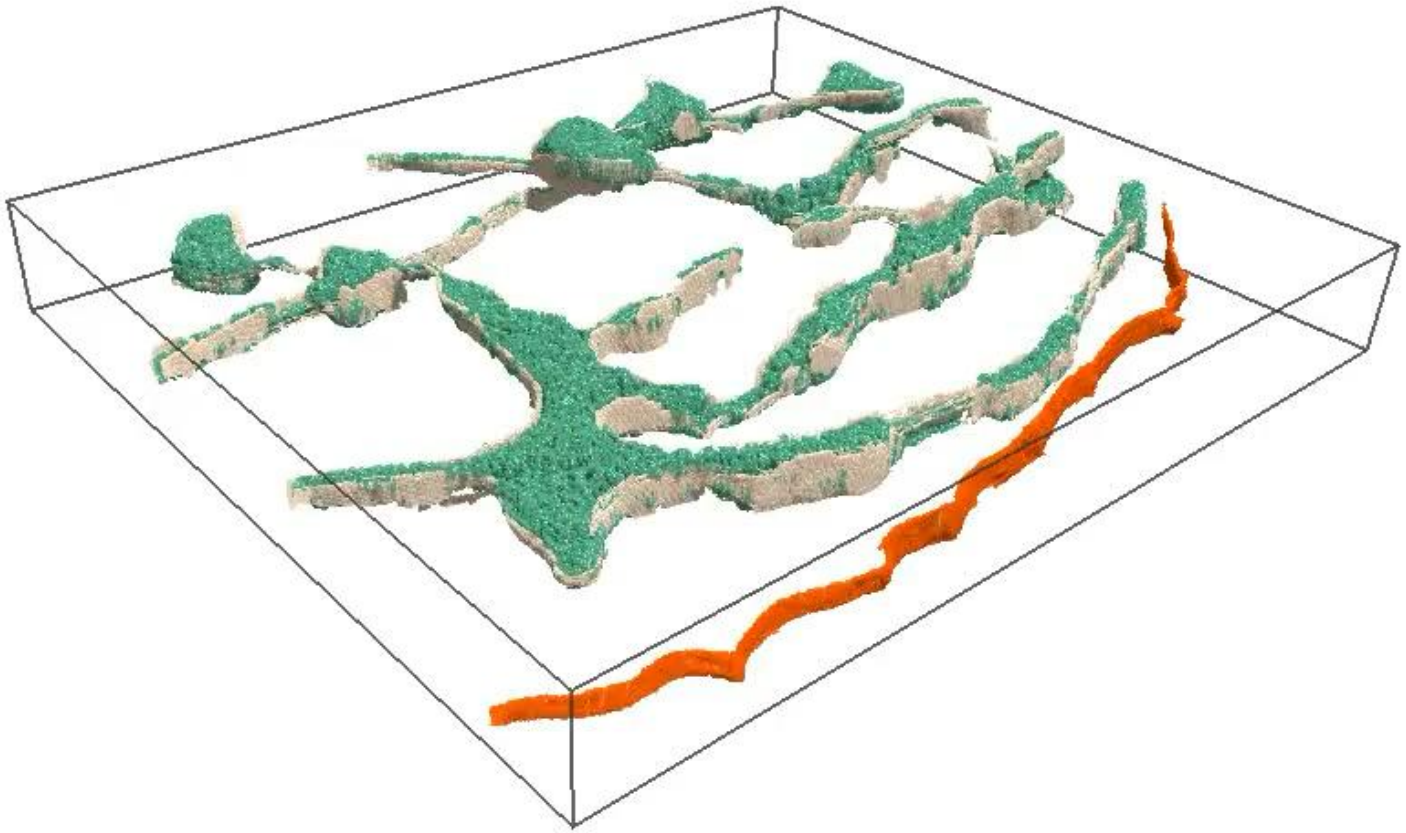
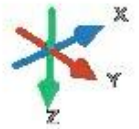
Back of the Polarized Cell Exhibits Rough or Sheet-Like Endoplasmic Reticulum



Back of the Polarized Cell Exhibits Rough or Sheet-Like Endoplasmic Reticulum





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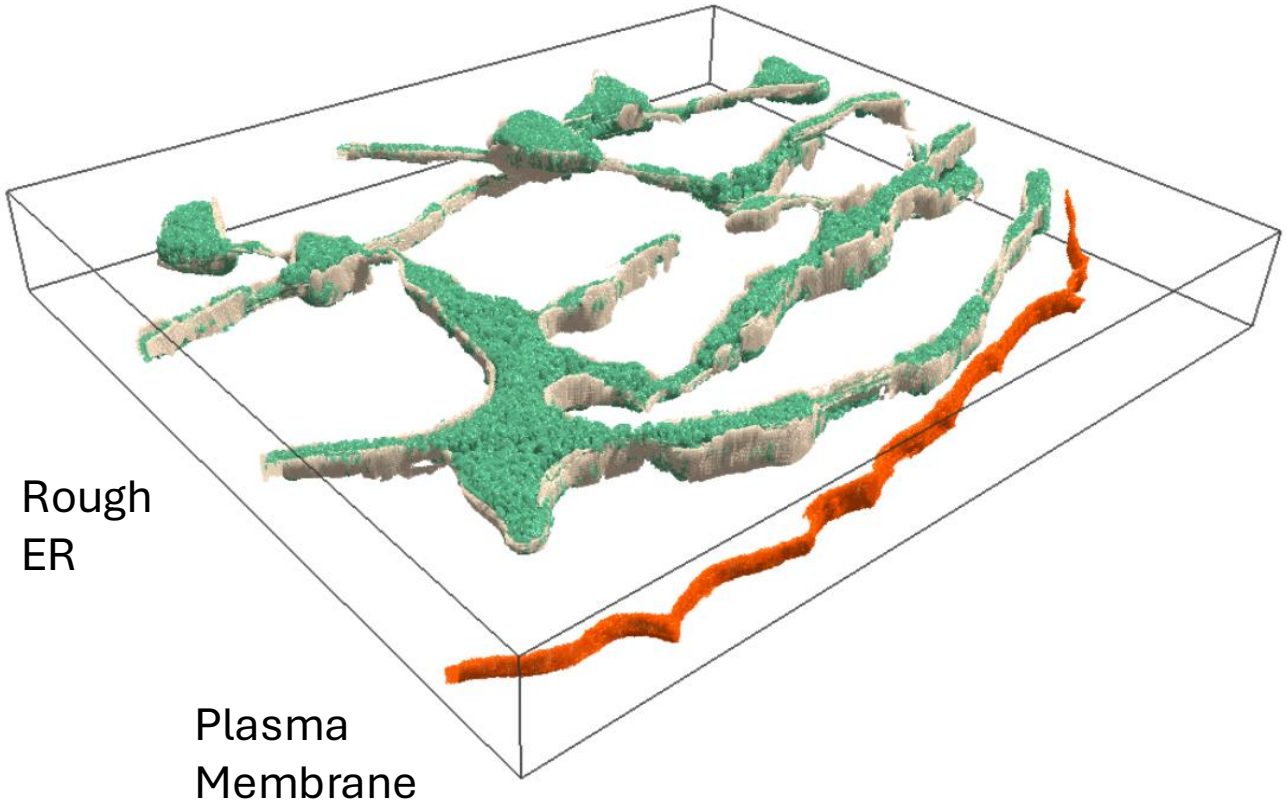

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Surface Morphometrics Analysis to Quantify Segmentations

Quantifying organellar ultrastructure in cryo-electron tomography using a surface morphometrics pipeline

Benjamin A. Barad[1,†], Michaela Medina[1,†], Daniel Fuentes[1,2], R. Luke Wiseman[2], and Danielle A Grotjahn[1,*]

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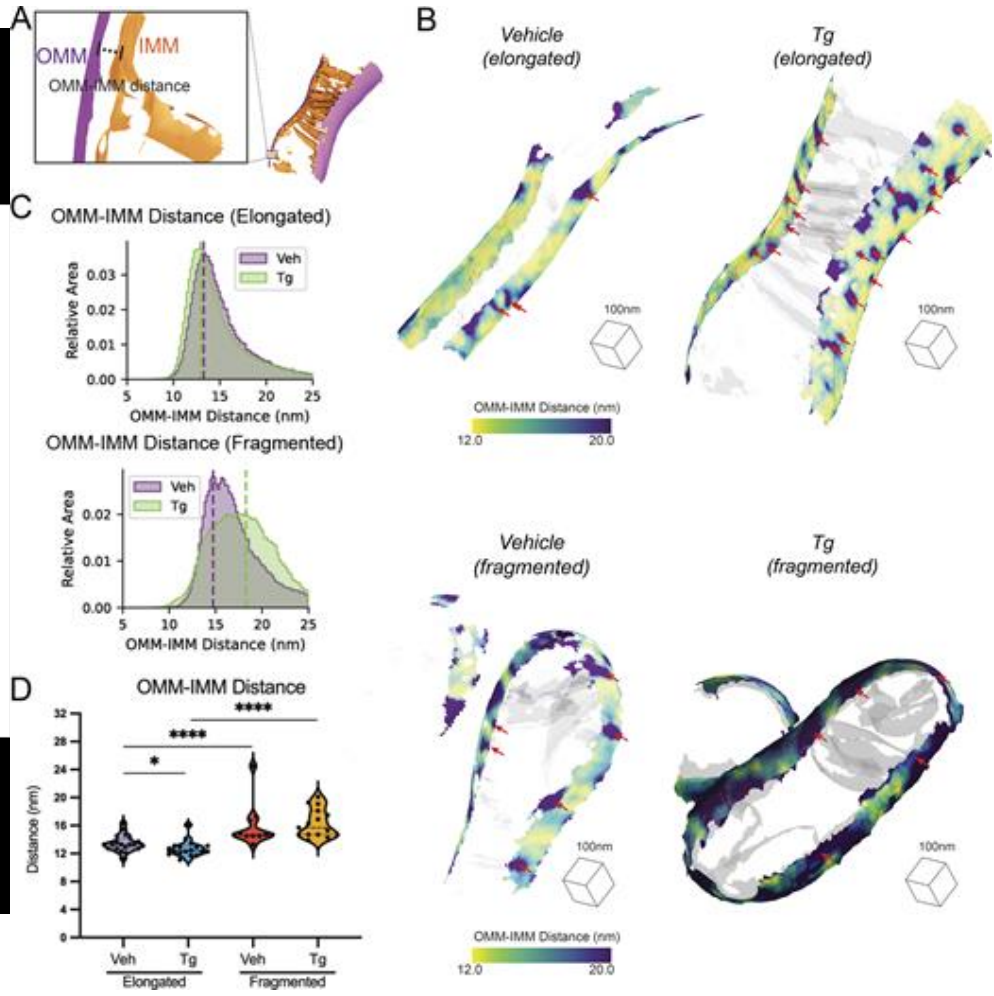
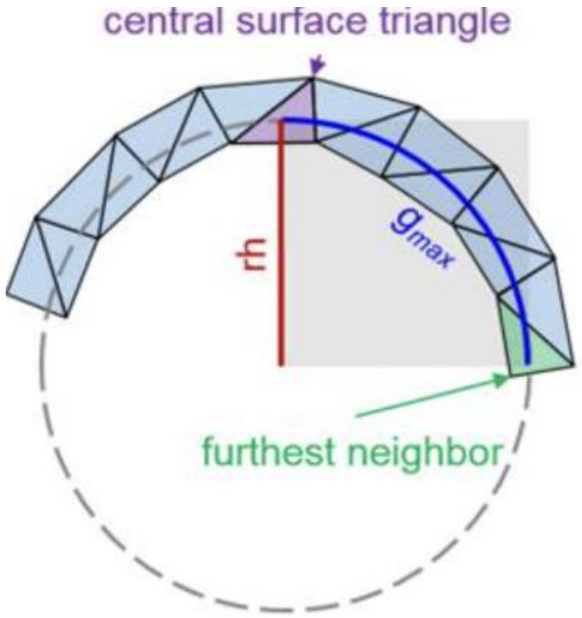
[2]Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA 92037
[†]These authors contributed equally.
[*]Corresponding Author

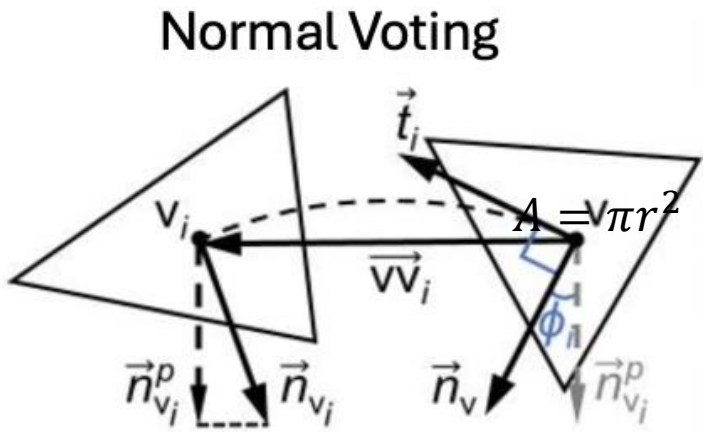


Baraad et al, Journal of cell biology 2023

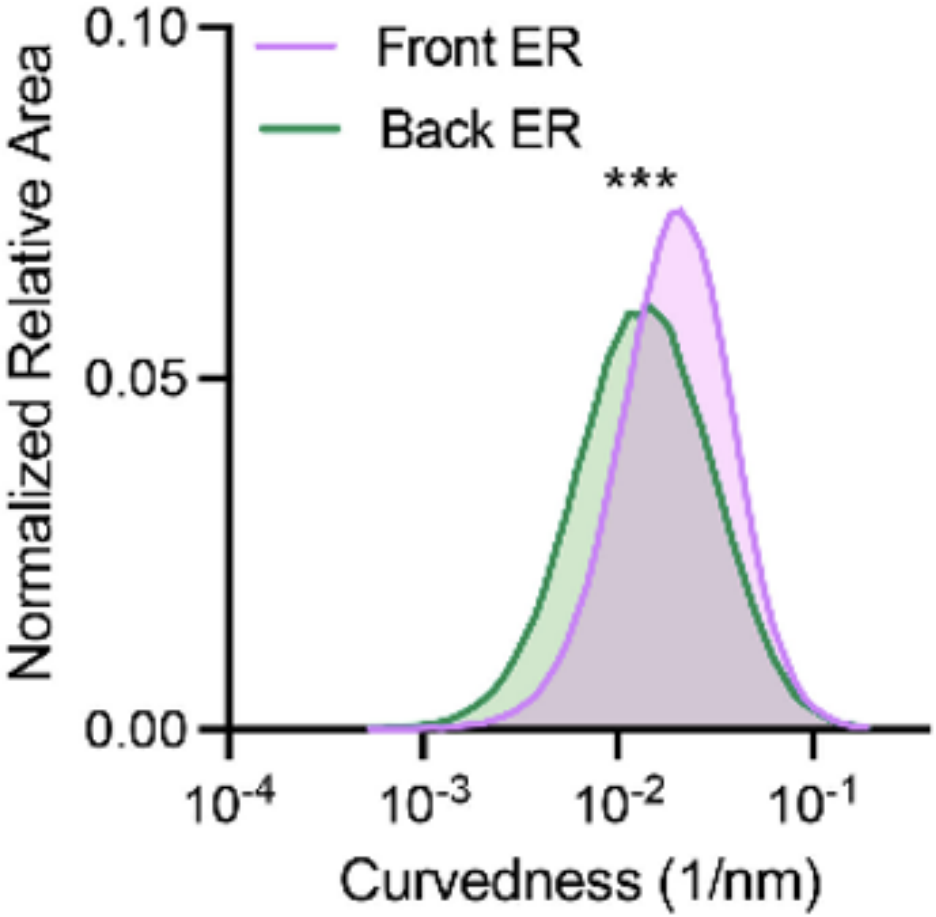
Tubular ER in the Front of Migrating Cells Exhibits Higher Curvature then the Sheet-like ER in the Back

$$g_{max} = \frac{\pi \cdot rh}{2}$$





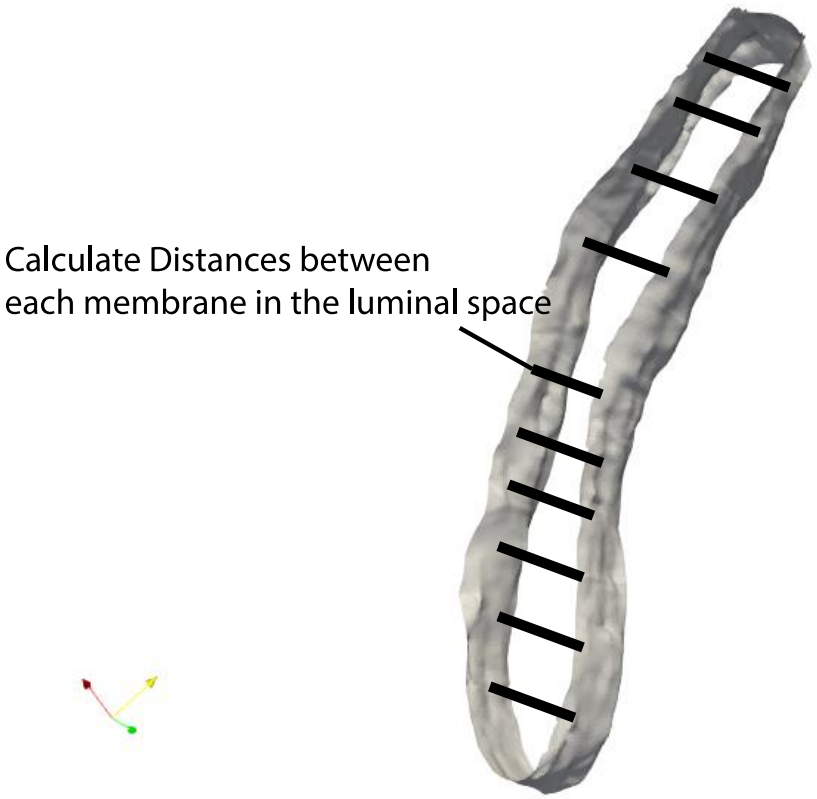
Salfer M, et al. Reliable estimation of membrane curvature for cryo-electron tomography. PLoS Comput Biol. 2020 Aug 10.



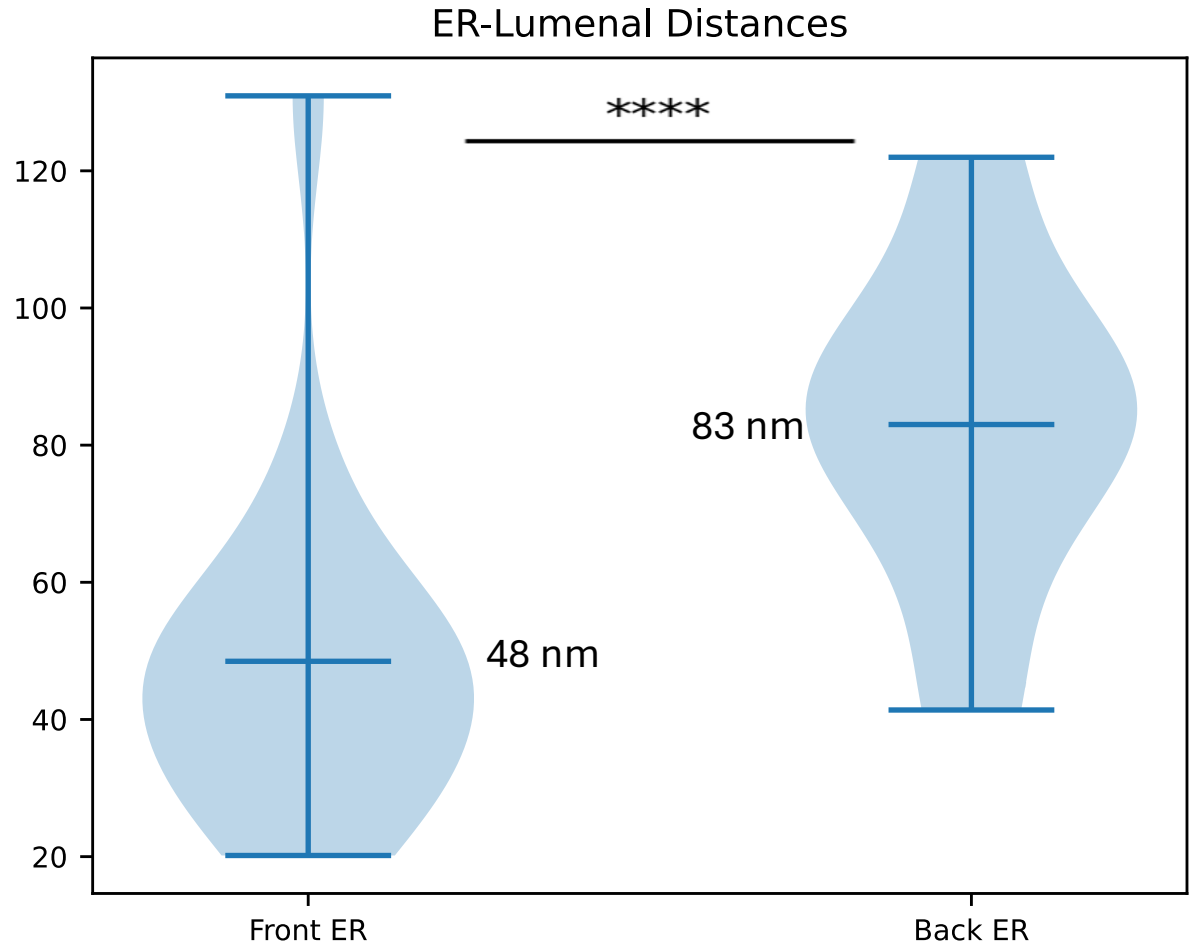
Area-Weighted Histograms (smoothed for visuals)
Unpaired student t-tails test (P***<0.006)
N = 7 tomograms

Gong, B., **Johnston, J.D.**, Thiemicke, A. *et al.* Endoplasmic reticulum–plasma membrane contact gradients direct cell migration. ***Nature* 631**, 415–423 (2024). https://doi.org/10.1038/s41586-024-07527-5

Tubular ER in the Front of Polarized Cells Exhibit Smaller ER-Luminal Widths then the Sheet-like ER in the Back



Segmented ER in Front of Cell



Mann-Whitney U-Test for Segmented ERs
N=7 Tomograms for Front and Back

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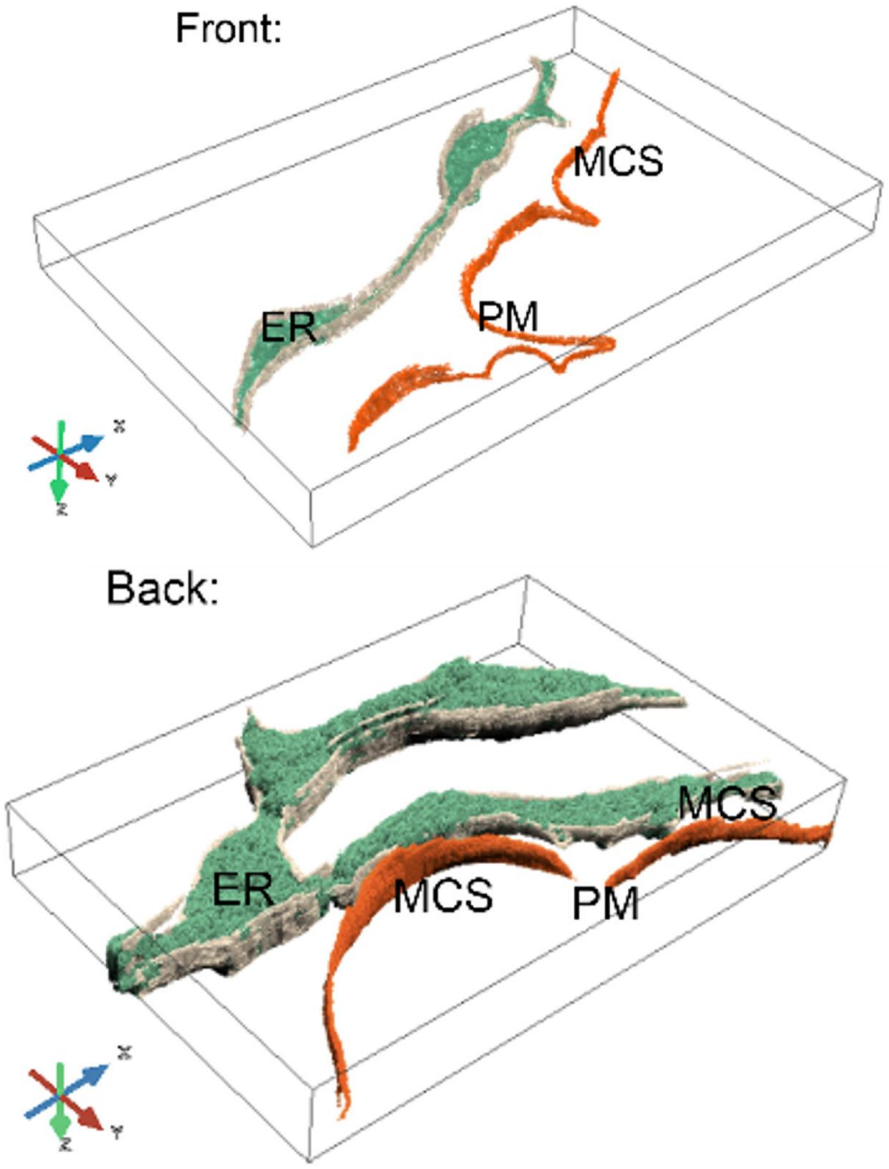
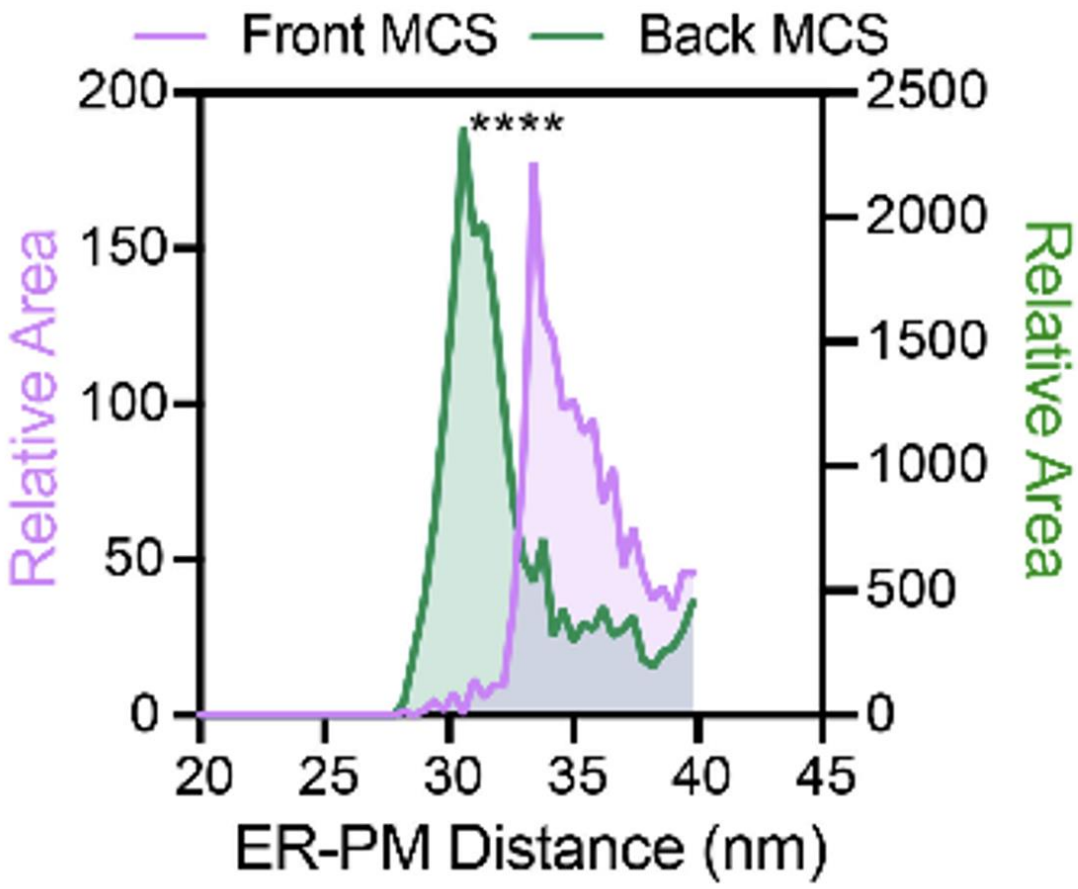
Contact-site Arrangement in the Front and the Back of Polarized Cells





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Conclusions from this Work

- Polarization of ER in migrating cells is comprised of tubular ER in the Front and rough-ER or sheet-like ER in the back

- ER and plasma membrane contact site gradients are driven by this to form larger more stable contacts in the back and weaker contact sites in the front of the cell

Thank you!

Questions/ Discussion?