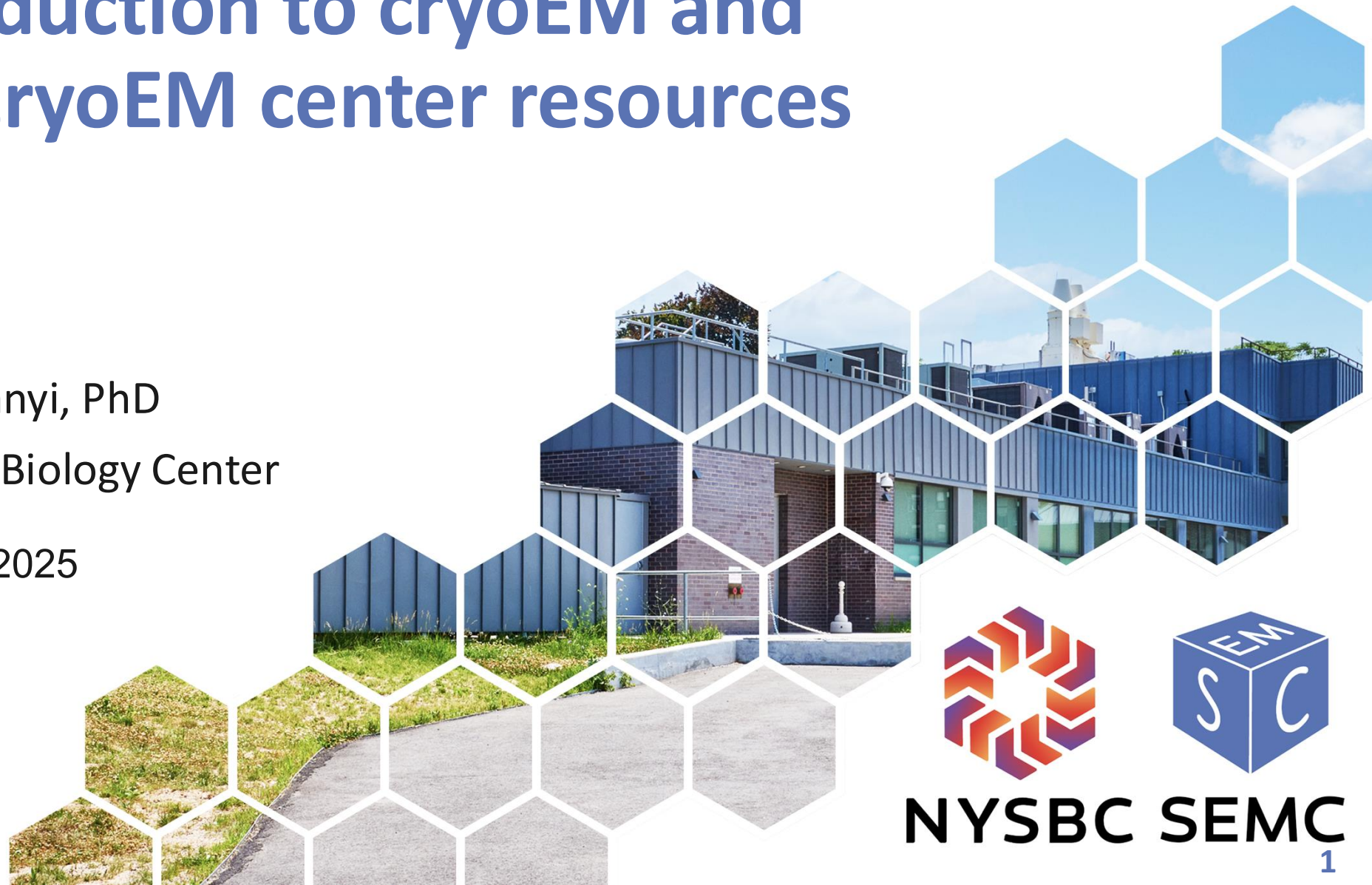
An introduction to cryoEM and national cryoEM center resources

Christina Zimanyi, PhD

New York Structural Biology Center

Friday, May 9, 2025



NCCAT

NYSBC SEMC

Workshop has three components

Friday May 9, 2025. Introduction to cryoEM.
Goal: Introduce cryoEM for biomolecules. Introduce National Center & local resources.

10:00 – 11:00 Talk: CryoEM and the national centers. Brown Laboratory Rm 219
12:30 – 3:00 Office Hours: Small group discussions (by appointment – check your email)
4:00 – 5:00 Seminar: Recent advances in cryoEM. Brown Laboratory Rm 219

Monday May 19, 2025. Hands-on grid preparation workshop. 12 participants.
Goal: Demystify the grid making process.

9:30 – 4:00 Hands-on: Grid handling, negative stain grid prep & room temperature screening, and cryo grid prep at the BioImaging Center.

Friday May 30, 2025. Evaluation of cryoEM grids
Goal: Gain experience evaluating micrographs and see outcomes of the grid making session.

10:00 – 12:00 Live demo of cryoEM screening remotely on NCCAT's Glacios
12:00 – 1:00 Break
1:00 – 3:00 Review of cryoEM images from the workshop samples

The teams who made this workshop possible

NCCAT @ NYSBC

Eugene Chua

Mahira Aragon

Elina Kopylov

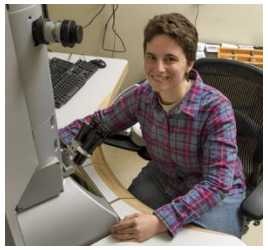
Ed Eng

Alex de Marco

Jeff Kieft

DELAWARE BIOTECHNOLOGY INSTITUTE® | BIOIMAGING CENTER

Jeffrey Caplan



Shannon Modla

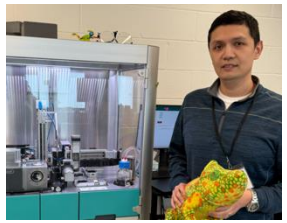
University of Delaware CryoEM champions

Fabio Gonzalez

Sharon Rozovsky

Yujue Liu

Henry Nwaora

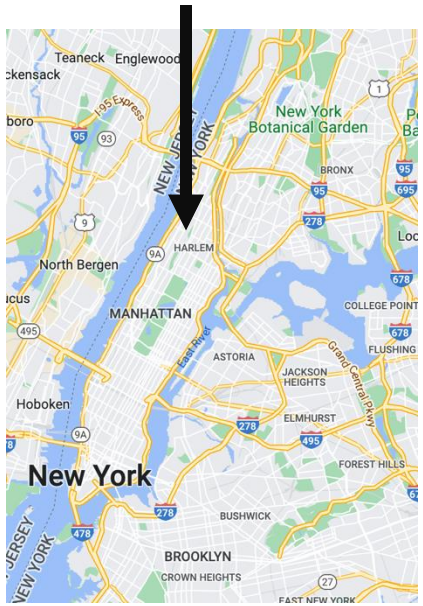


Sadik Sattar

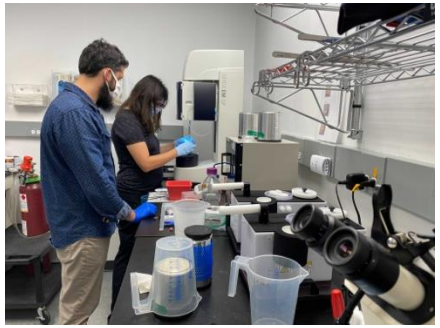
National Center for CryoEM Access and Training: NCCAT

National Center for Cryo-EM Access & Training

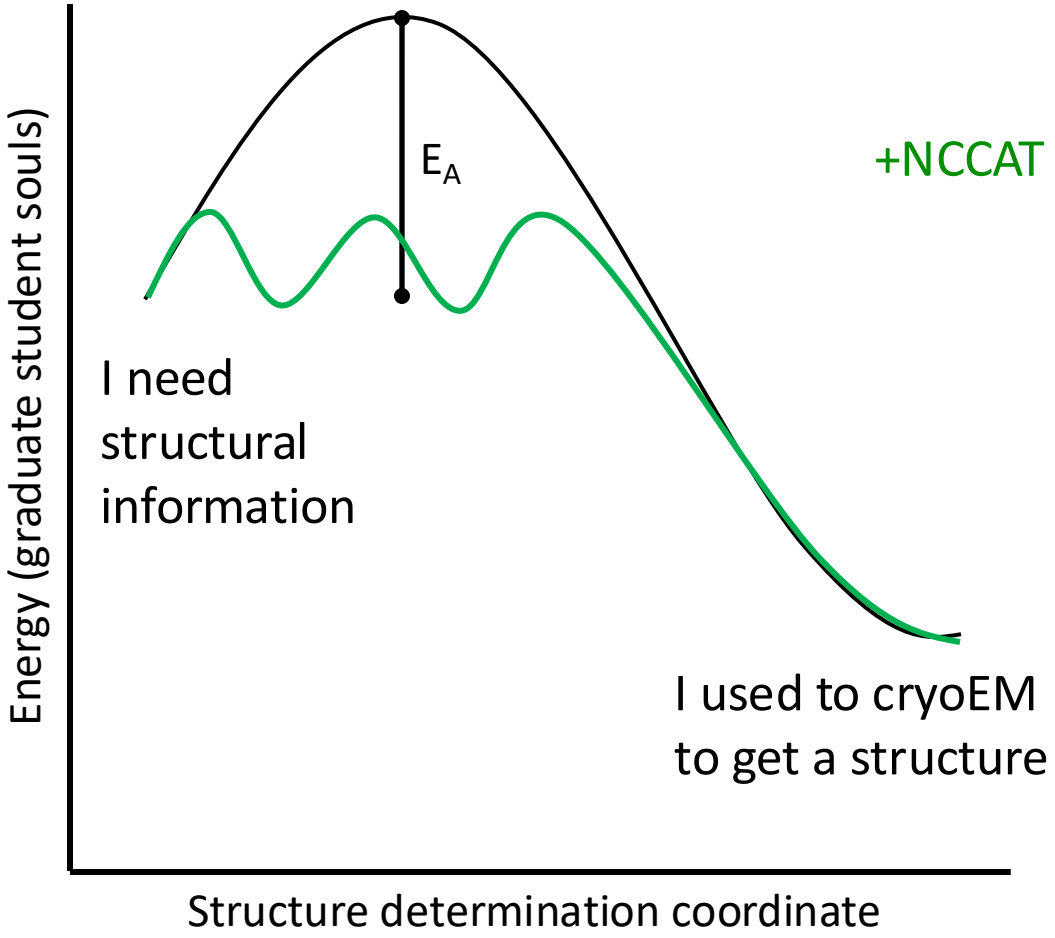
NIH Aim: broaden access to high-resolution cryoelectron microscopy for biomedical researchers, and cultivate a skilled workforce through the development and implementation of cryoEM training material



+ very cool people



NCCAT's mission is to lower barriers for cryoEM use



E_A = Samples + Instrumentation + Expertise + $$

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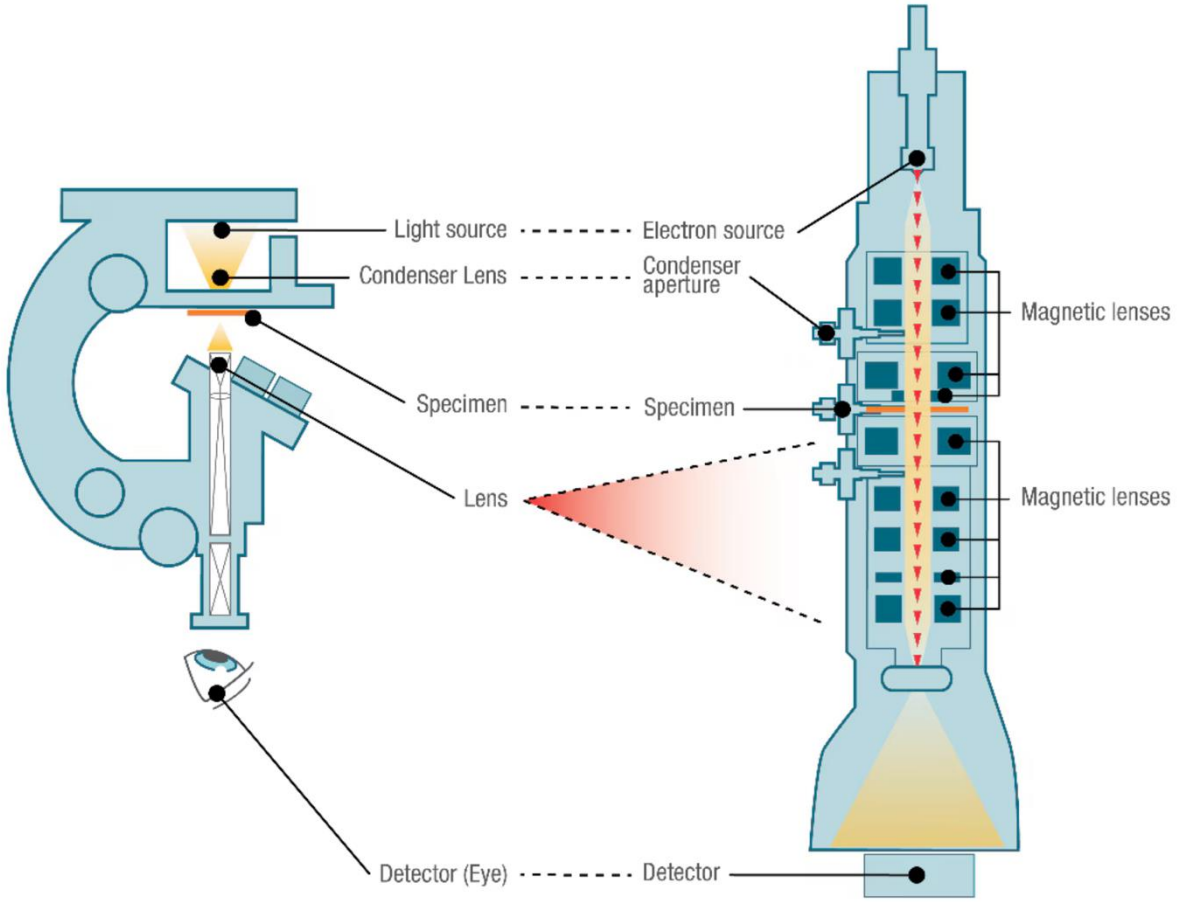
Workshops

Roadmap for the talk: a practical guide to cryoEM samples

- CryoTEM basics and challenges for imaging biomolecules
- Single Particle Analysis (SPA) workflow
- SPA practical sample preparation tips
- How the National Centers can help

Transmission electron microscopy in one slide



ThermoFisher

$$d = \frac{\lambda}{2NA}$$

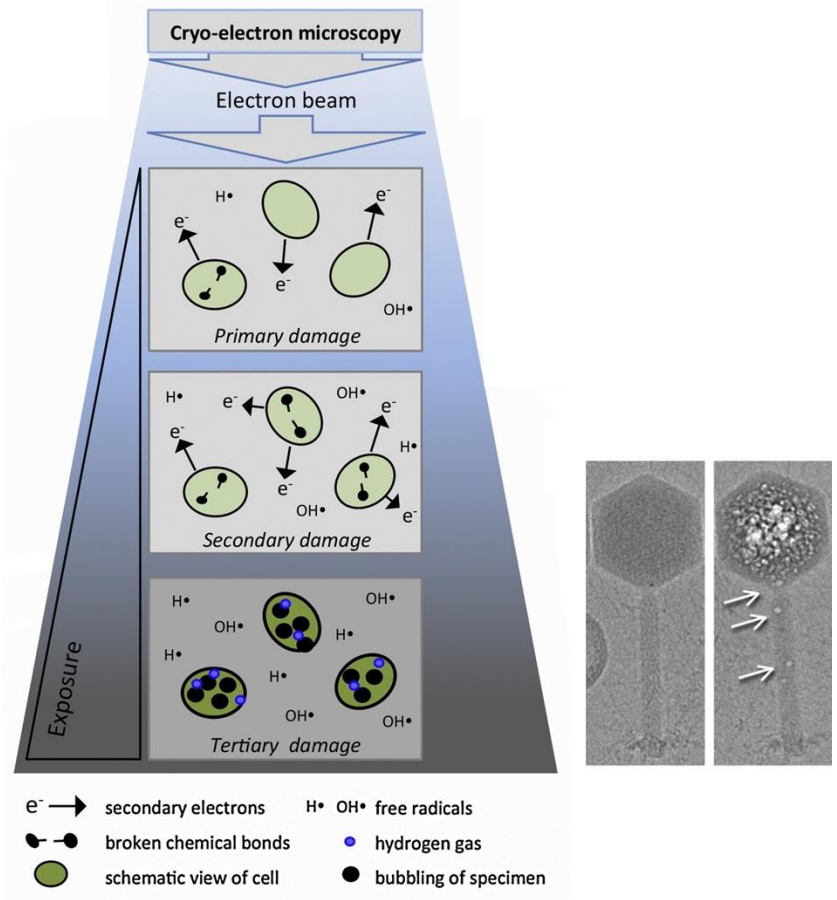
e^- @ 80-300 keV → λ = 2-4 pm

Theoretical resolution limit ~ Å

Sample requirements:

- Thin (~100 nm)
- Stable under vacuum

Electron microscopy on biological samples has unique challenge



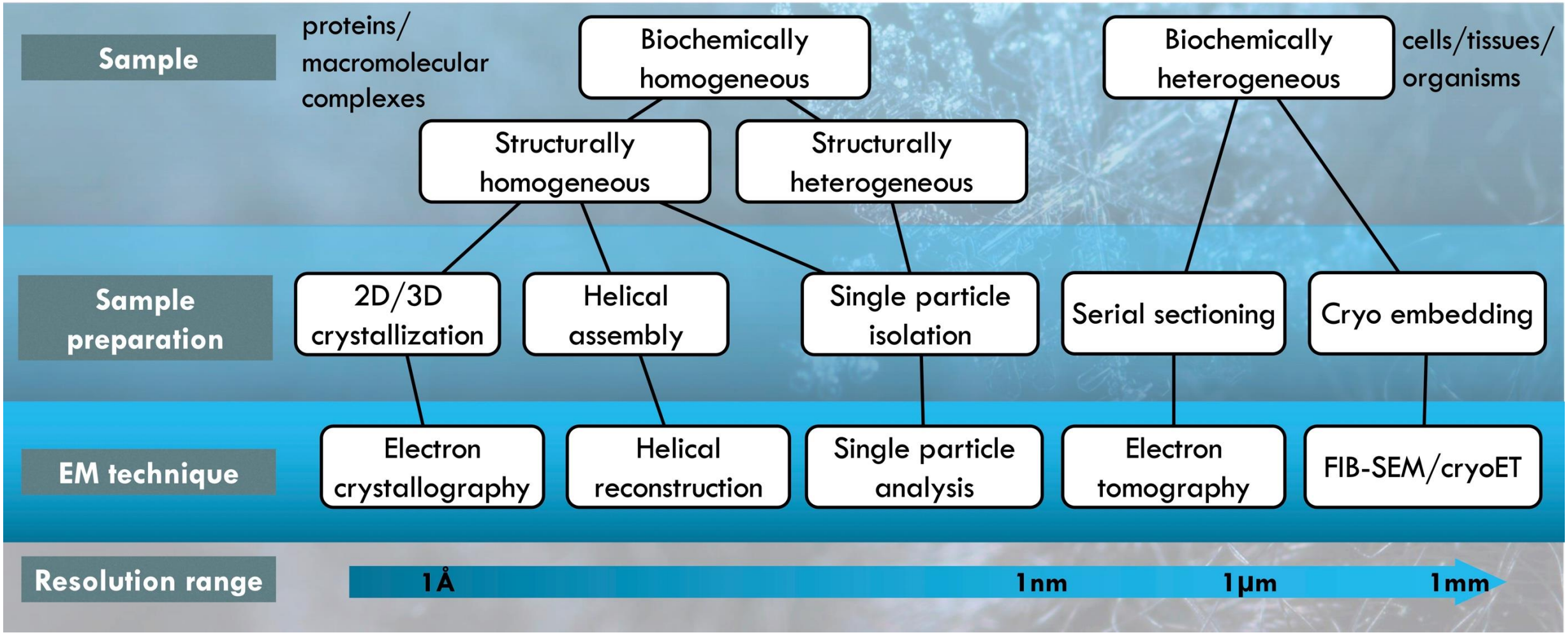
- Radiation sensitivity requires low dose
- Objects are low contrast
- Low **signal-to-noise**

But we can get high-resolution reconstructions!

- Better detectors
- Better processing

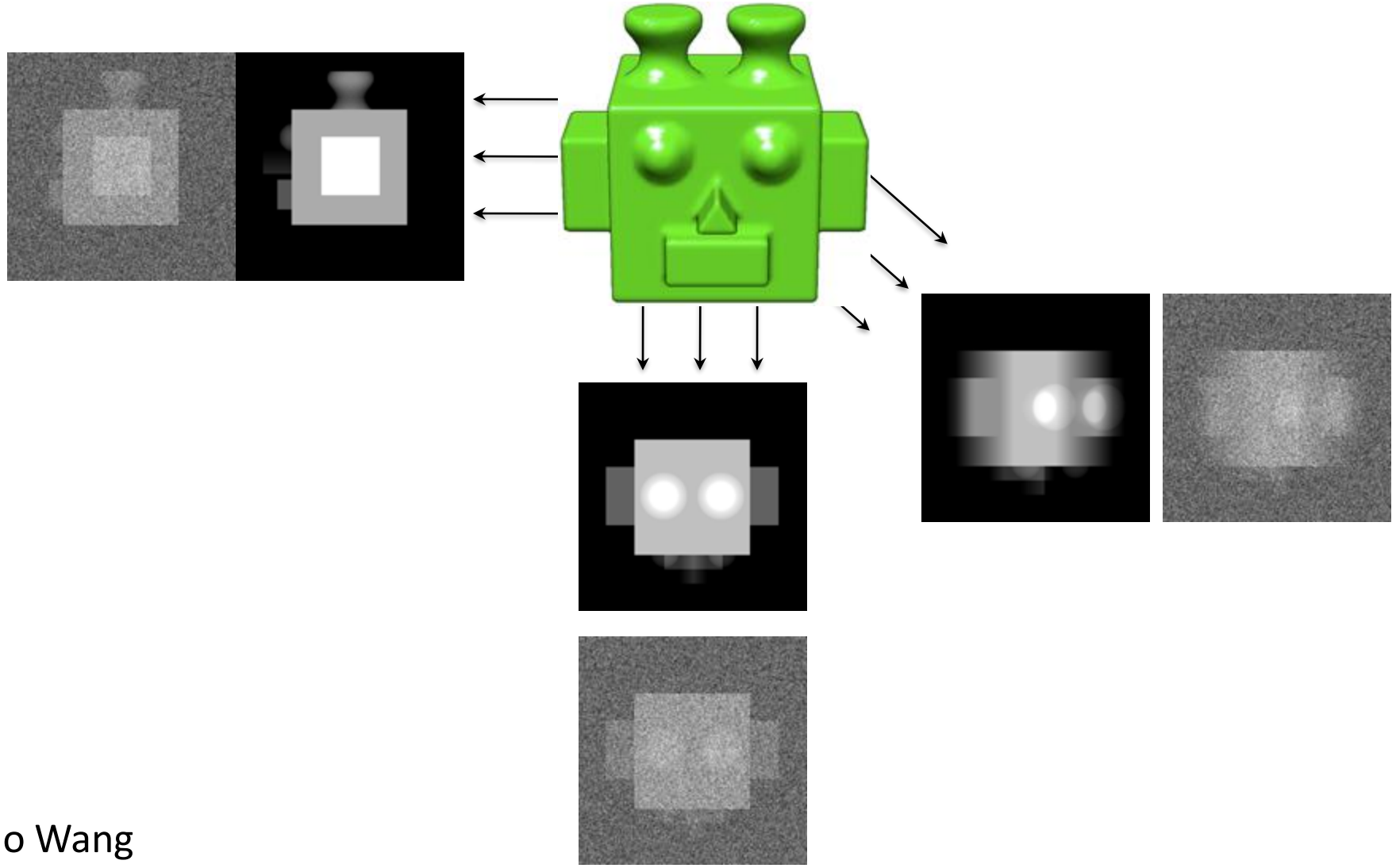
Mishyna et al. (2017) Micron 96: 57-64

Sample preparation depends on the system of interest



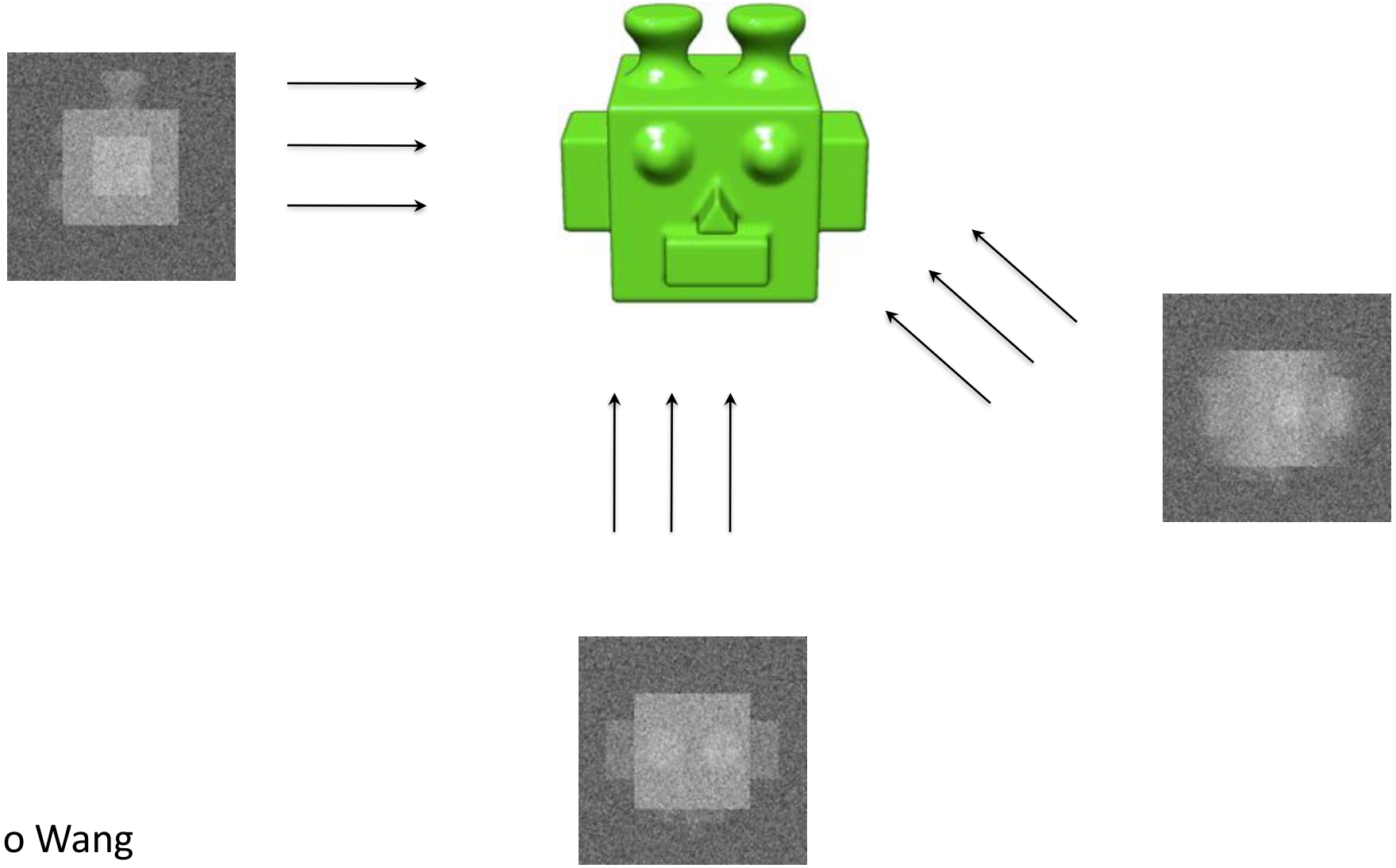
Slide credit: Ed Eng

Image formation and 3D reconstruction



Slide credit: Liguo Wang

Image formation and 3D reconstruction



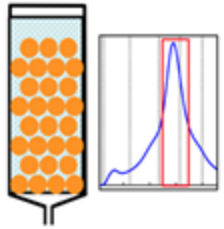
Slide credit: Liguo Wang

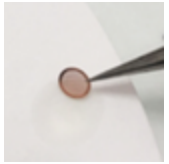
The typical SPA workflow has many steps

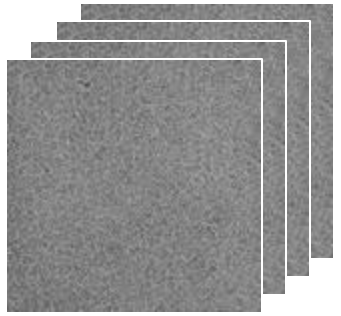
Sample preparation and screening

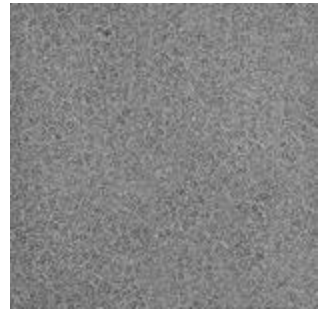
Data collection

Biochemical preparation → EM specimen preparation → Sample screening → High resolution data collection → Frame stacks → Micrographs



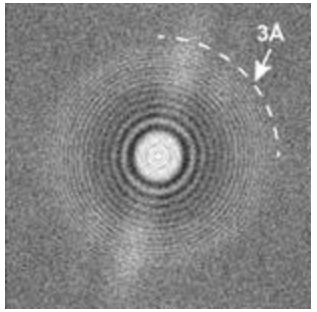


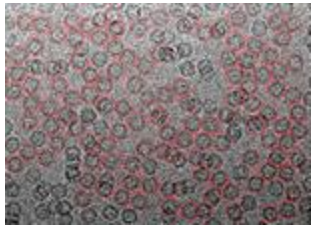


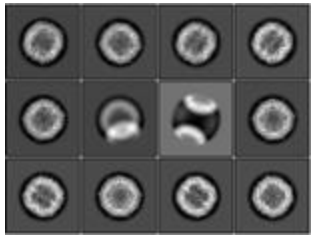


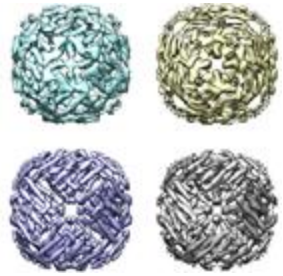
Data processing

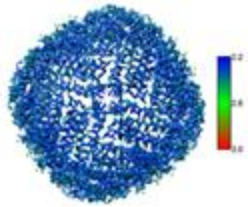
Image Evaluation → Protein particles → 2D classification → 3D classification → 3D reconstruction → Data interpretation

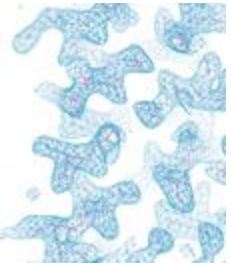




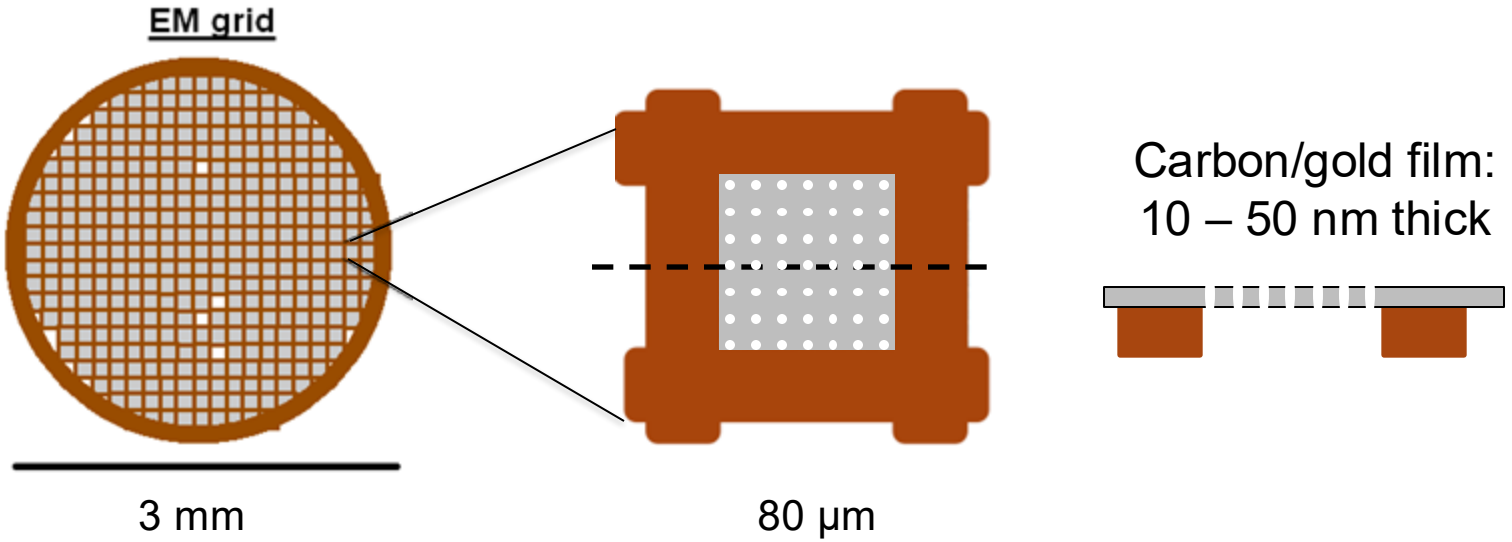


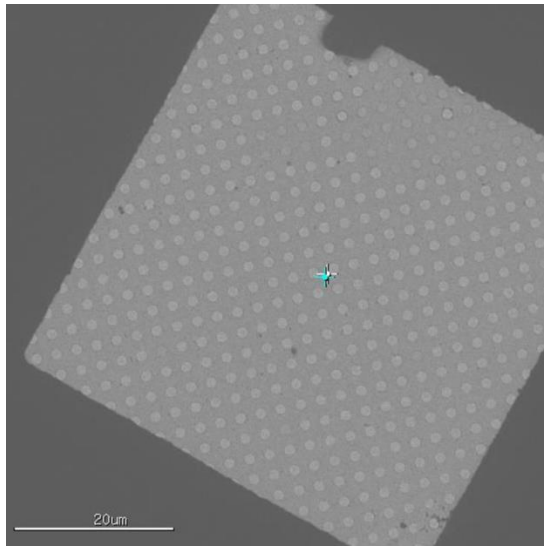


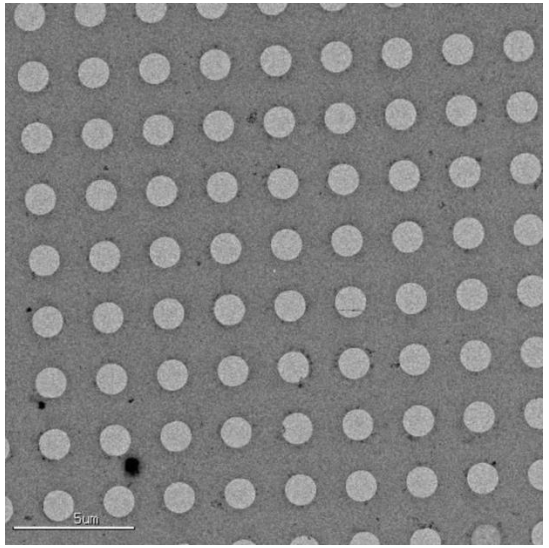




Holey TEM grids are used for SPA cryoEM



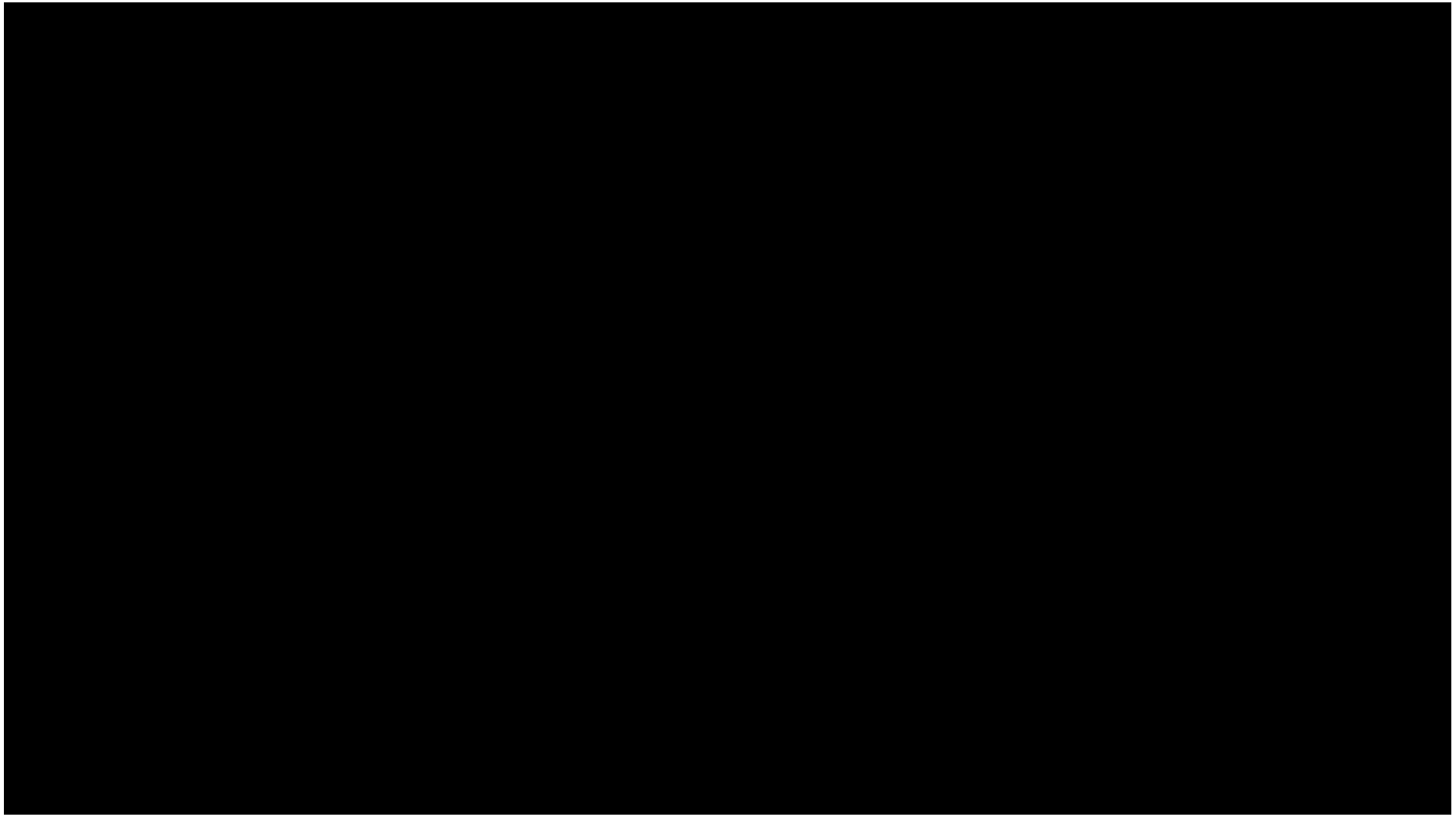




- An EM grid: a circular metal mesh (Cu or Au).
- A thin film sits across the mesh.
- The thin film has holes (0.6-2 µM diameter) where an aqueous sample is suspended.

Mainly purchased from companies.

Single particle cryoEM specimens are prepared on grids



CryoEM 101 (cryoem101.org)

There are two major hurdles for practical cryoEM sample preparation

- Optimizing the sample before it goes onto a grid.

  For averaging, particles must be homogeneous (conformational and compositional) or if not, then well characterized.

- Getting the sample nicely frozen in a thin film on a grid.

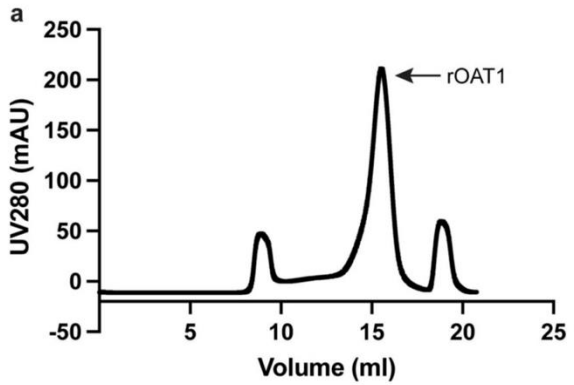
Assessing sample quality before freezing is vital

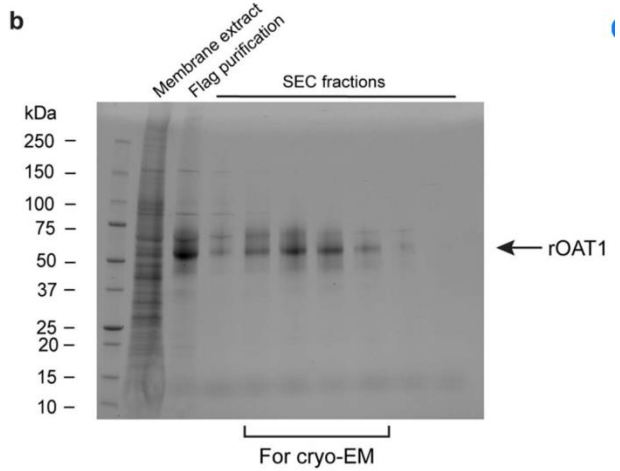
Assess **purity**: SDS-PAGE, mass spectrometry

Assess **homogeneity:** size exclusion, SEC-MALS, mass photometry, SAXS, <u>negative stain EM</u>

Don’t start vitrification until you know you have a good sample.

Garbage in -------> Garbage out





Dou *et al. Nat Struct Mol Biol* (2023) **30:** 1794–1805

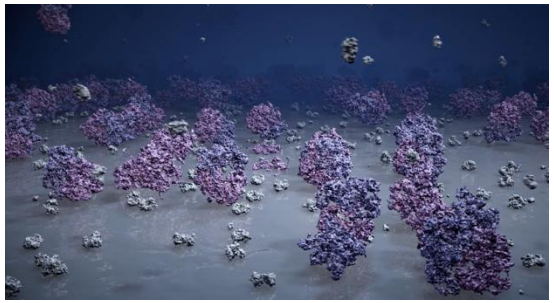
Negative stain EM is a useful tool for lower resolution sample characterization

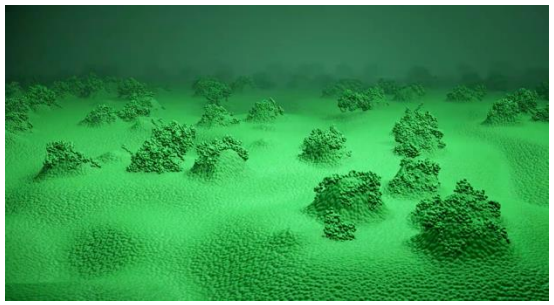
Negative Stain EM:

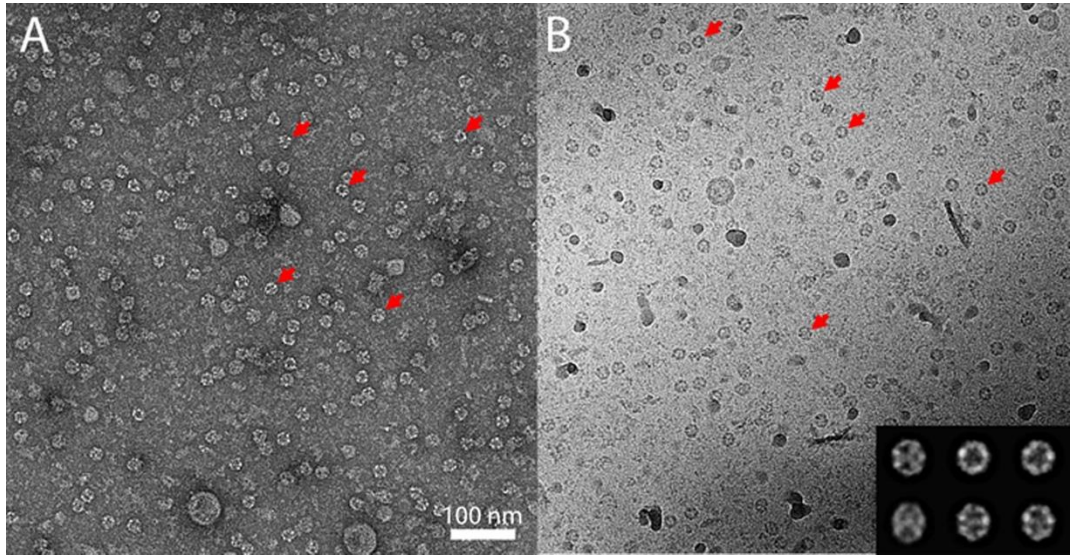
- Sample adsorbed to thin support
- Contrast comes from stain
- Resolution limited by size of stain grains (10s of Å)
- Lower sample concentration (µg/mL)
- Room temp imaging

Cryo-EM:

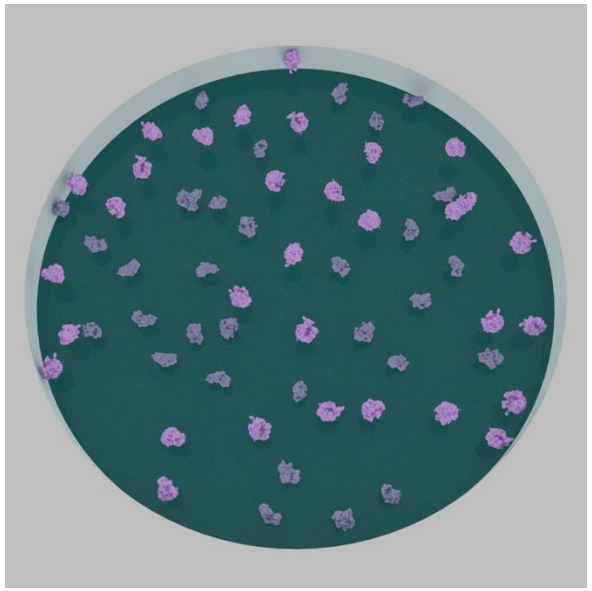
- Sample suspended in liquid and frozen
- Contrast comes from sample itself
- Resolution limited by sample/data processing (1s of Å)
- Higher sample concentration (mg/mL)
- Requires cryo



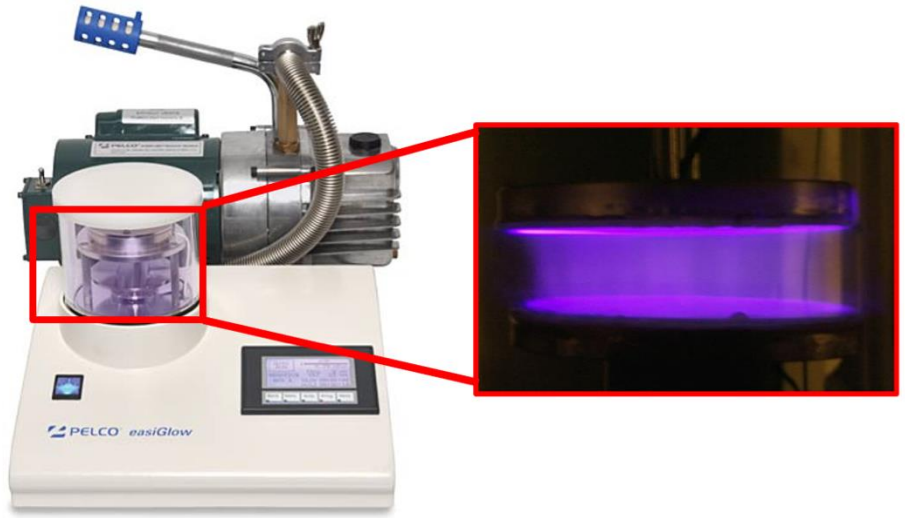




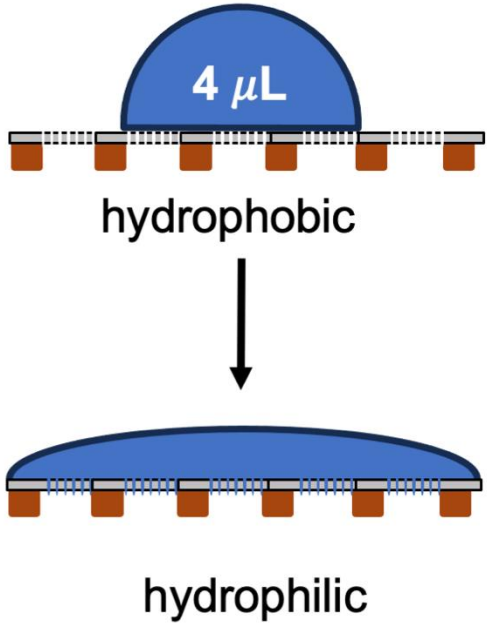
Baron S, et al. (2018) PLOS ONE 13(9): e0204457.

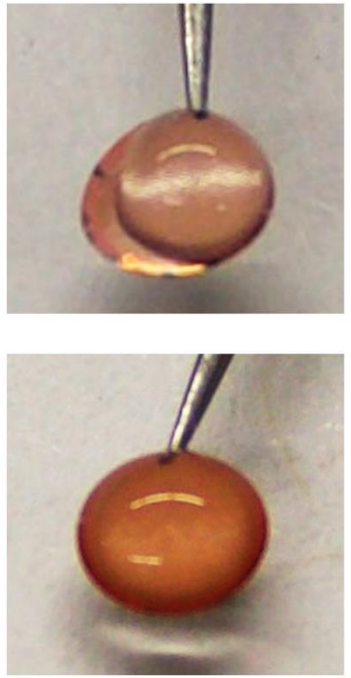


Grids are glow discharged to change surface hydrophobicity

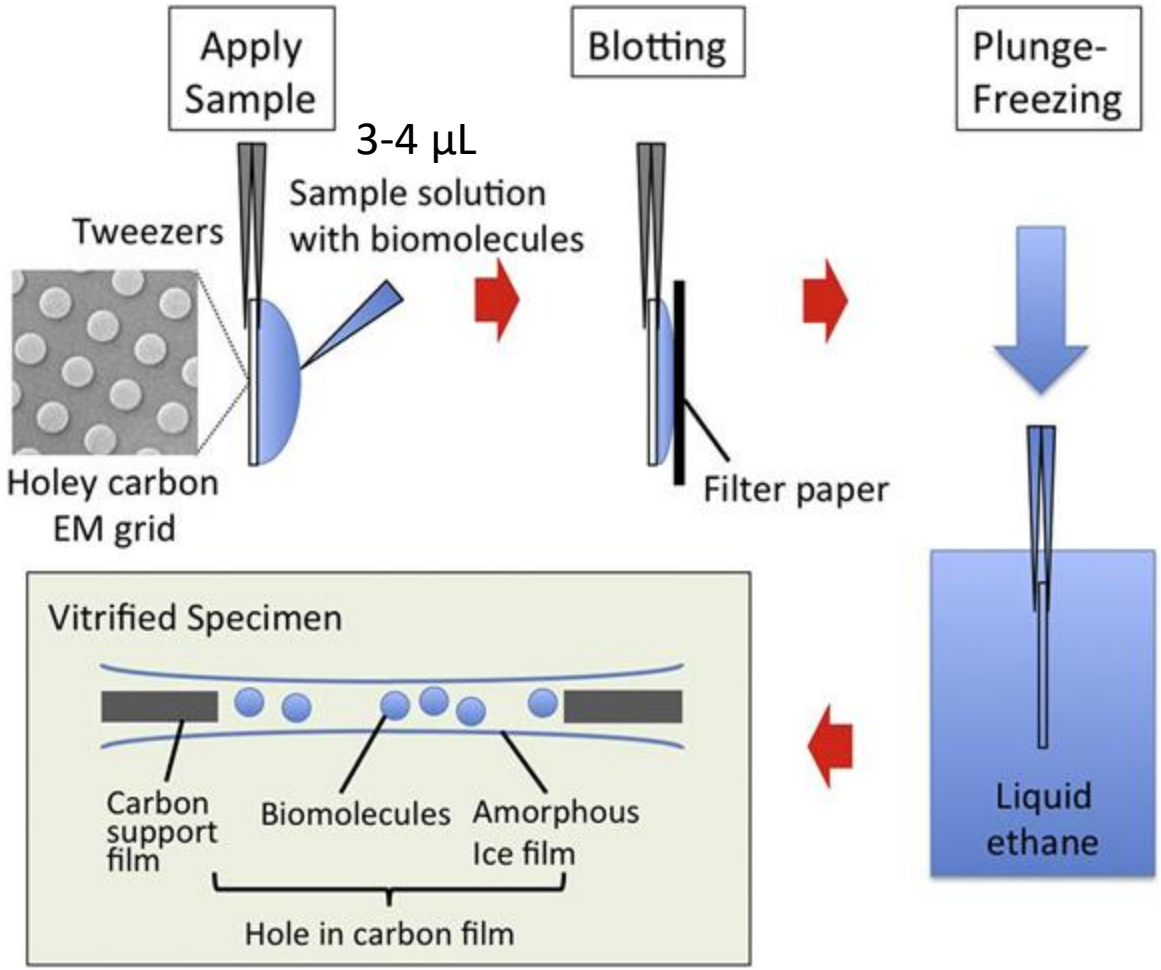


Pelco Easiglow
Glow Discharge Cleaning System





The most common method for making frozen thin films is plunge freezing



Murata & Wolf, 2018

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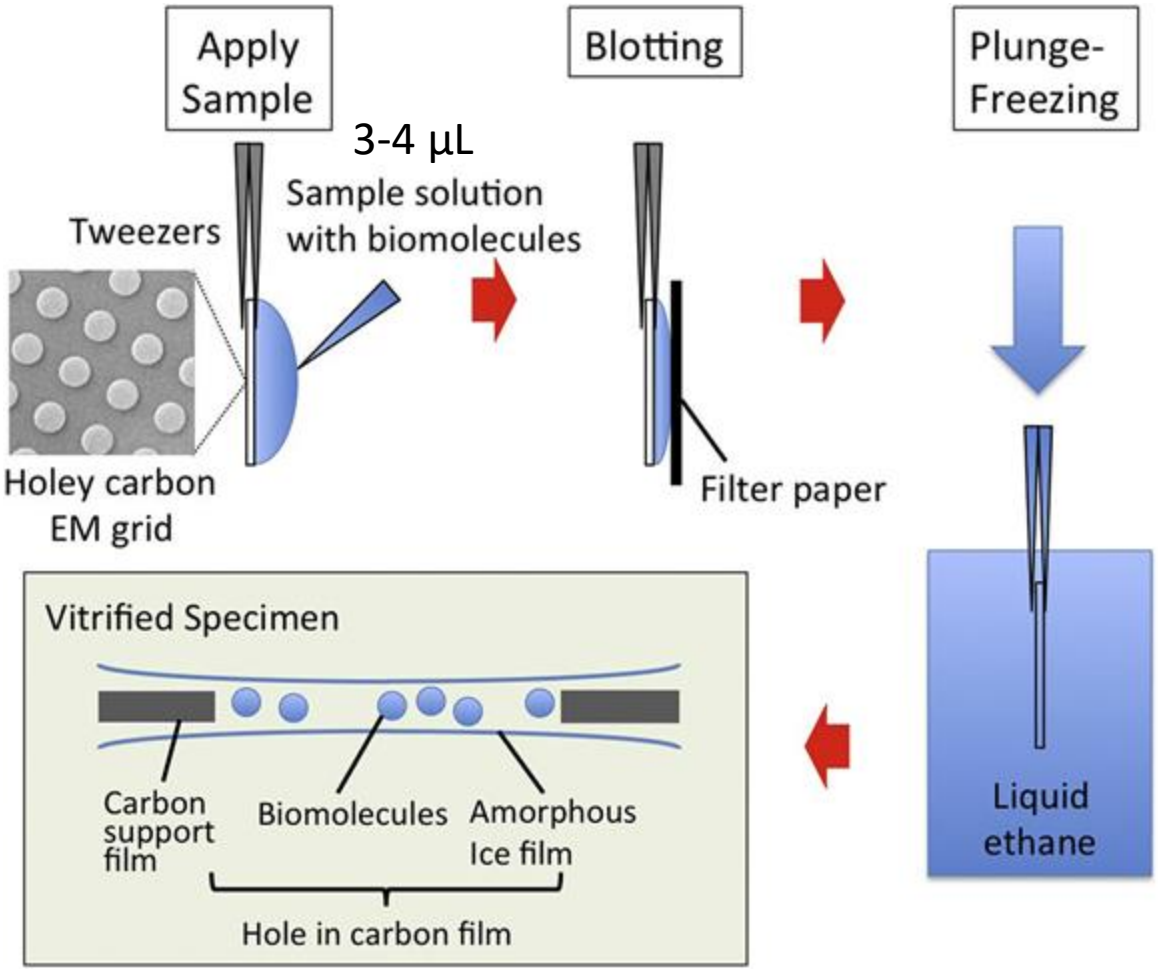
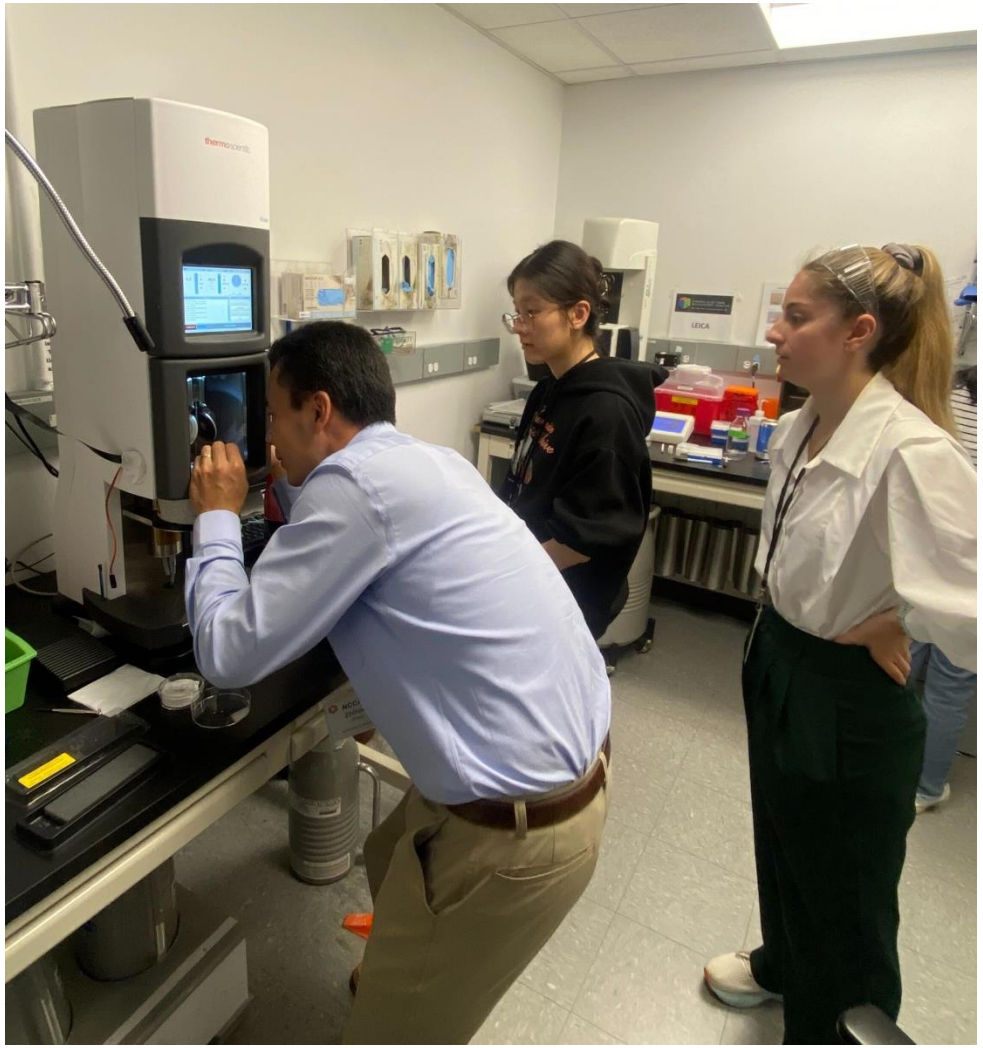
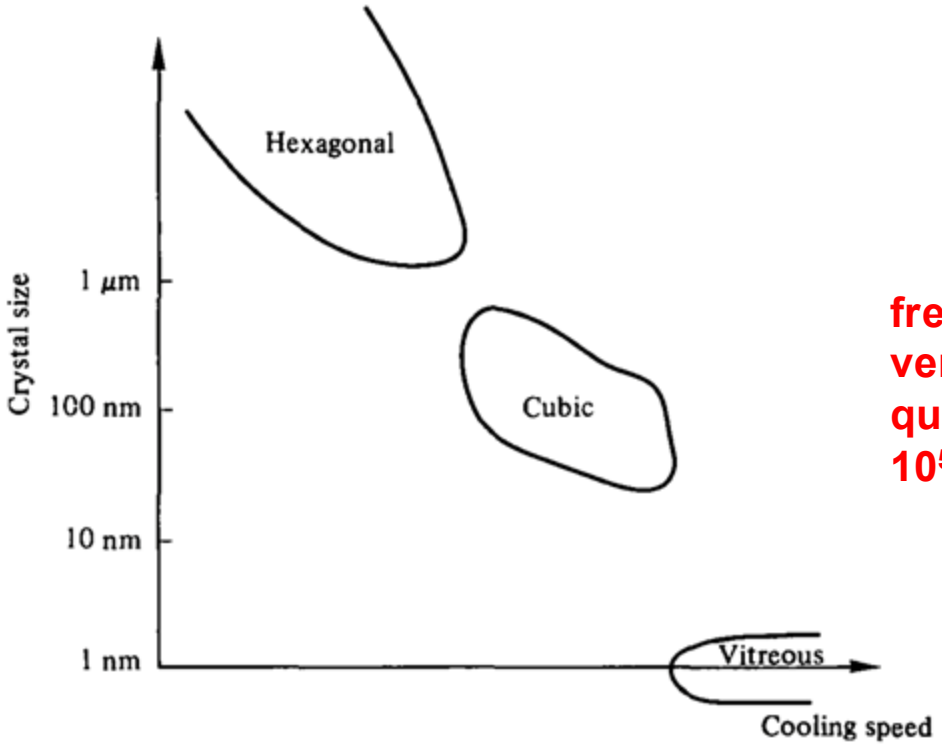


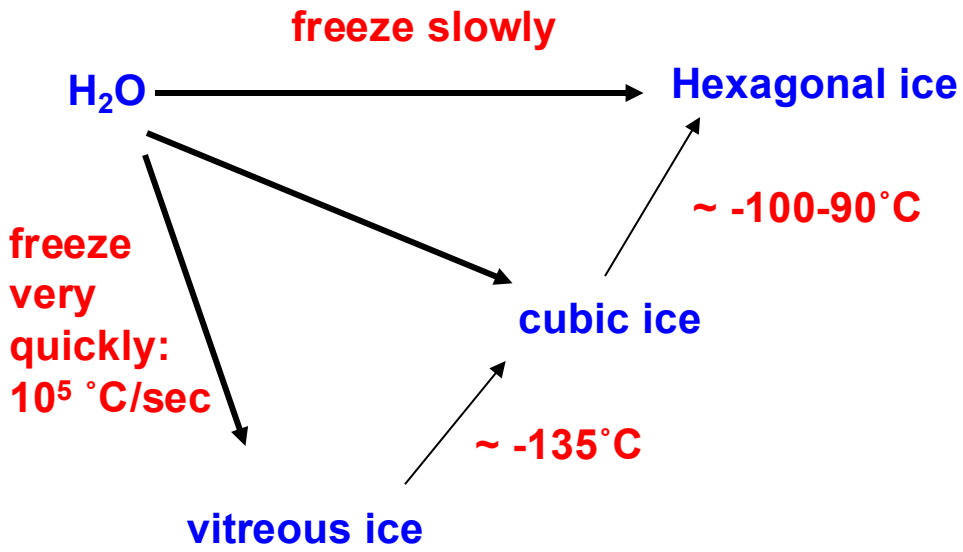


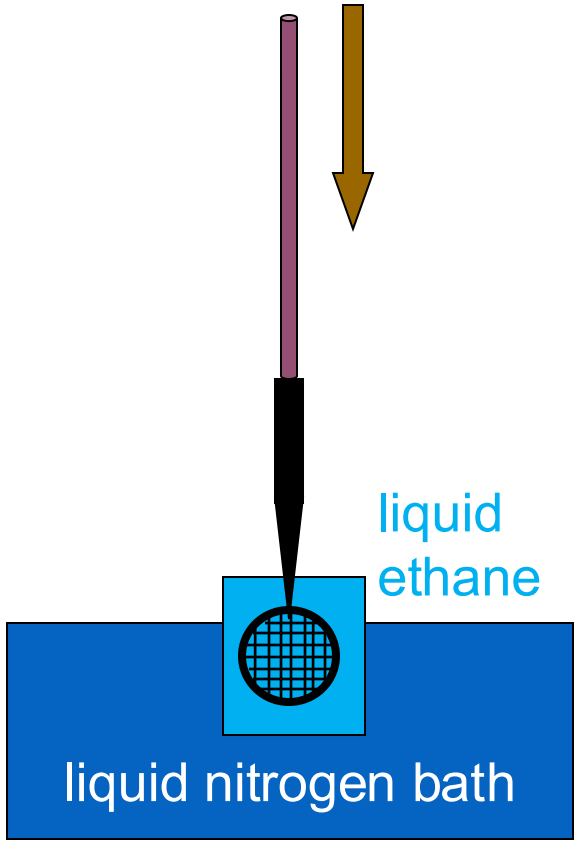
Murata & Wolf, 2018

Fast freezing allows for the vitrification of water

Vitreous ice: An amorphous solid state in which water is frozen without adopting a crystalline structure

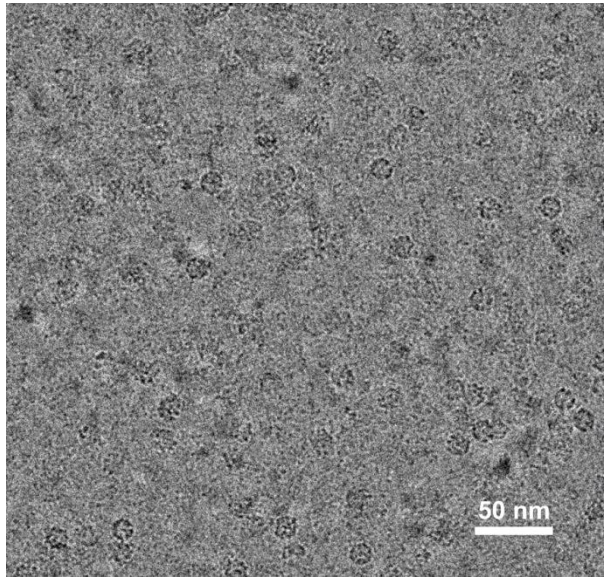






Dubochet, et. al. (1988). Quarterly Reviews of Biophysics, 21(2), 129-228

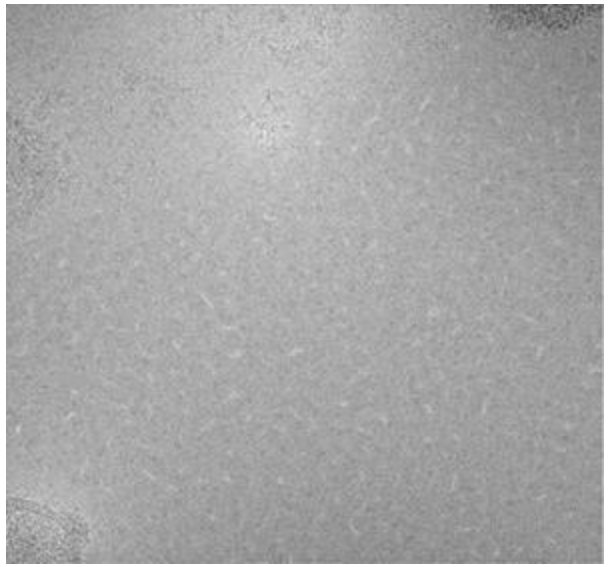
CryoEM practitioners have seen many kinds of “bad ice”



Crystaline ice

Potential sources:

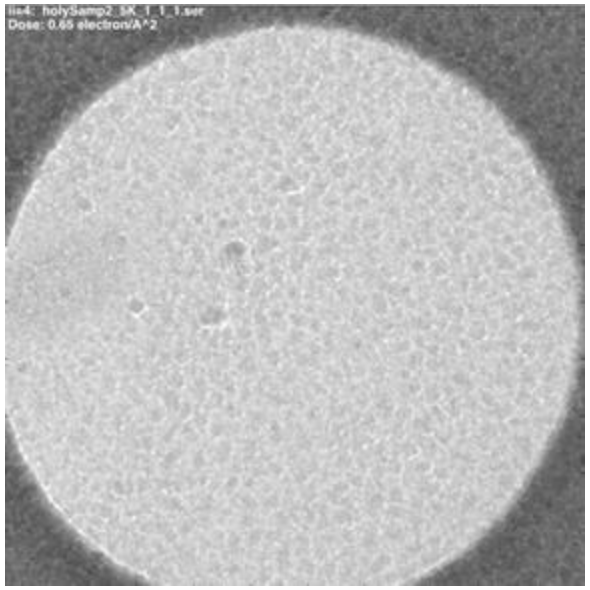
- Freezing not fast enough
- Ice is too thick

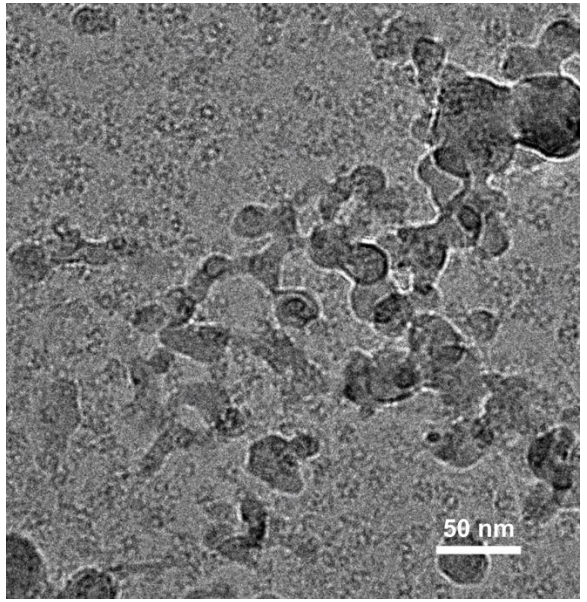


“Leopard skin” usually from a grid warming up and refreezing

Potential sources:

- Grid comes out of cryogen during transfer
- Grid warms on microscope stage





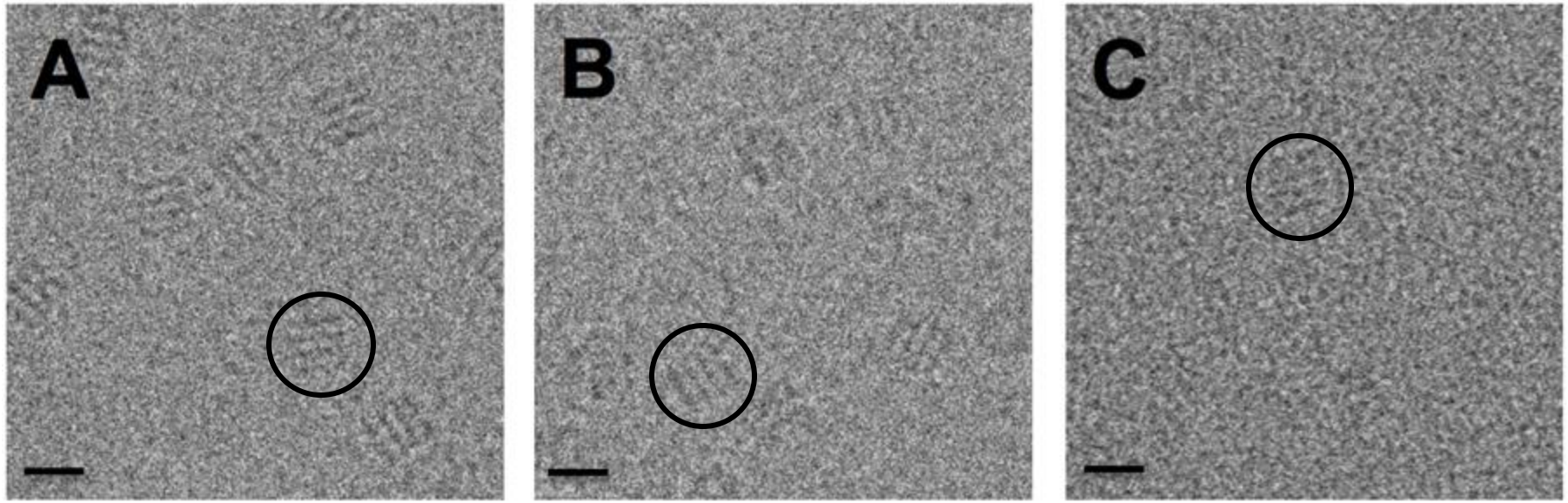
Surface contaminating ice

Potential sources:

- Dirty nitrogen

Ice thickness affects image contrast significantly

Cryo-EM images of GroEL

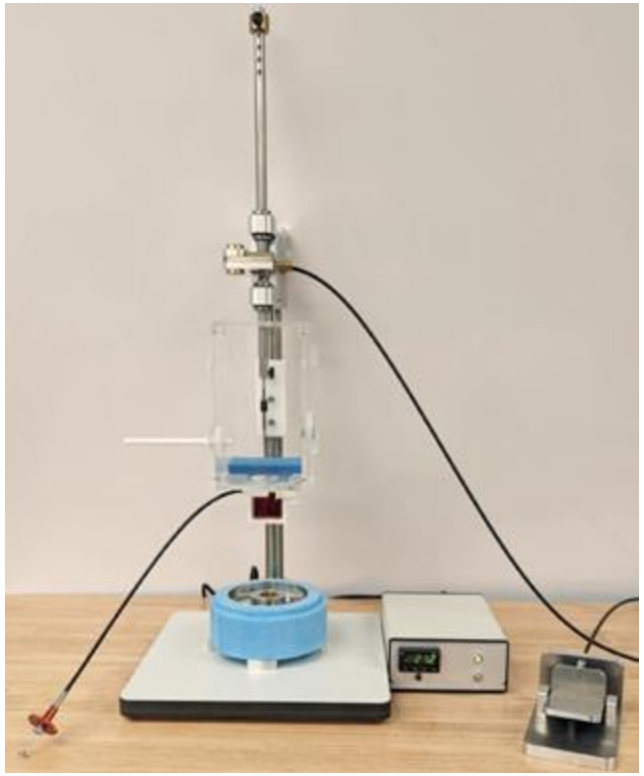


Relative ice thickness:
A = 1.0X
B = 1.5X
C = 2X

- Goal: as thin as possible
- How to control it: Blot parameters, grid type, buffer additives

http://nramm.scripps.edu/data/05may19a

Commercial plunge freezers provide environmental and timing control. Reproducability is still hard.

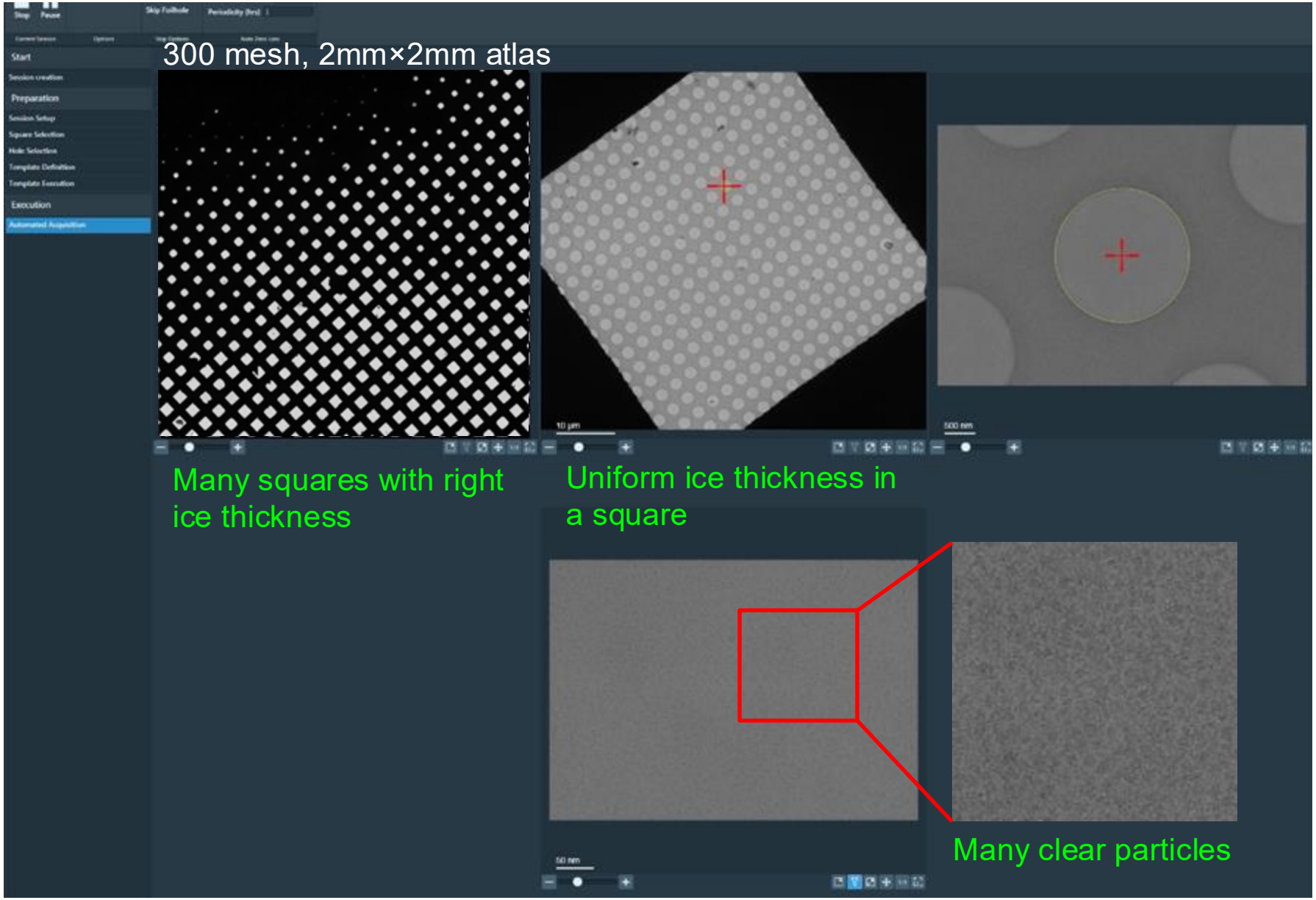


There are two levels of quality control for a “good” grid

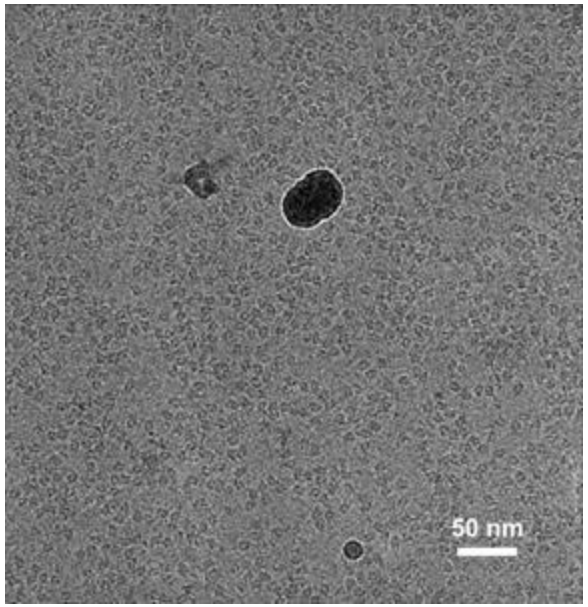
1. A sufficient area of uniformly distributed vitreous ice

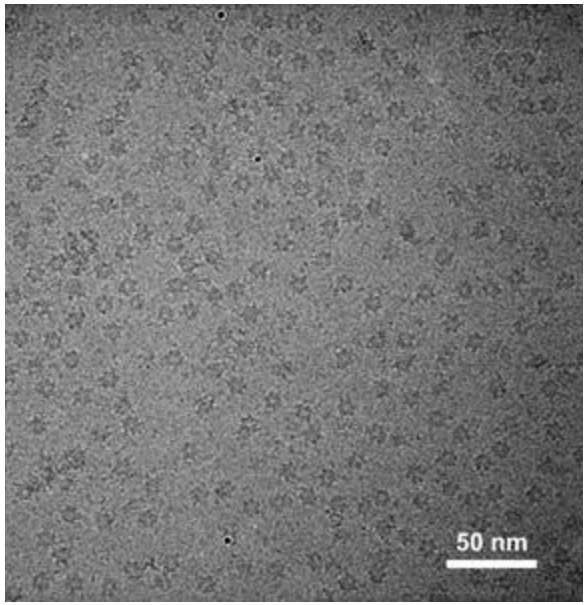
2. Well distributed particles randomly oriented in the vitreous ice

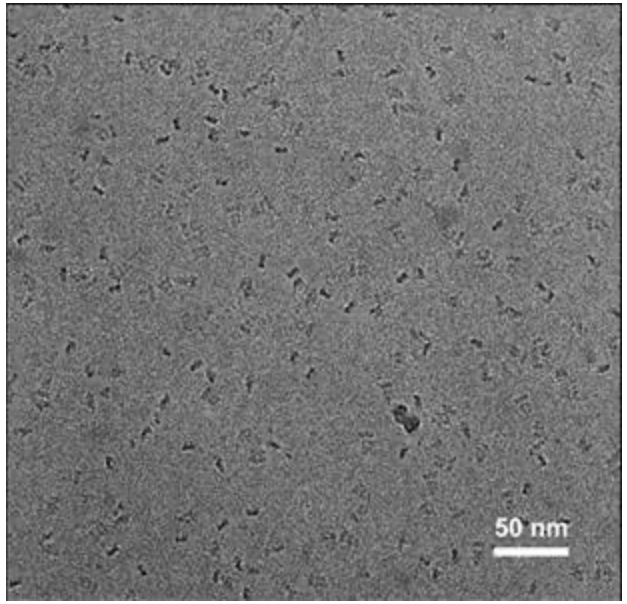
Ideal example: Nice distribution of ice and sample

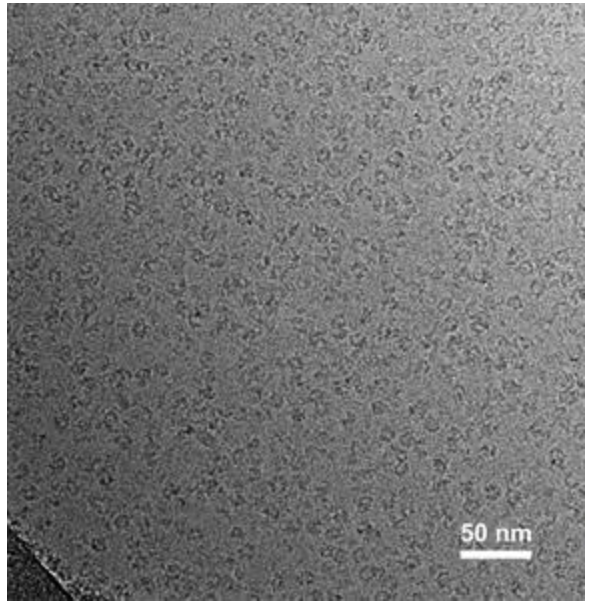


Good particle distribution is easy to spot









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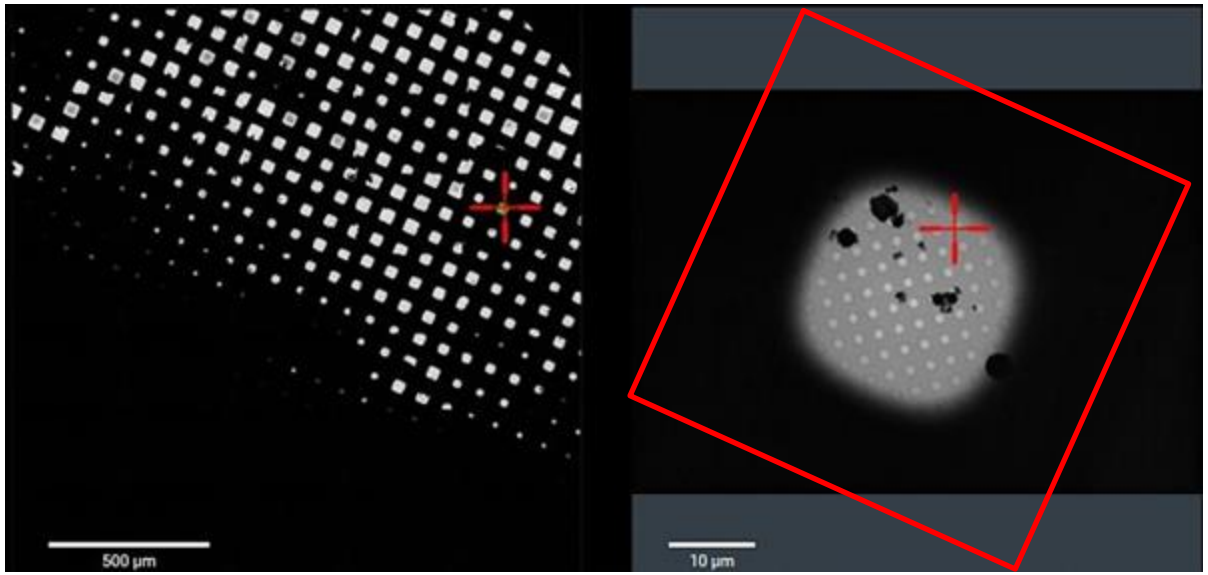
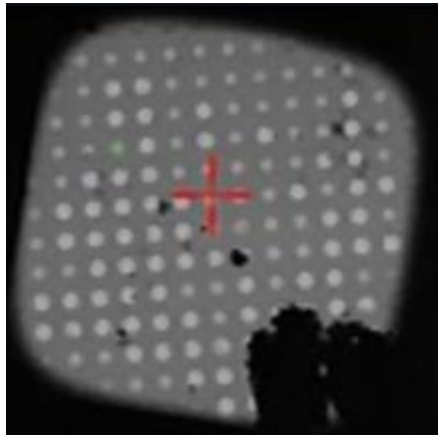
Not-ideal (and common) example: Poor ice quality

2mmx2mm atlas



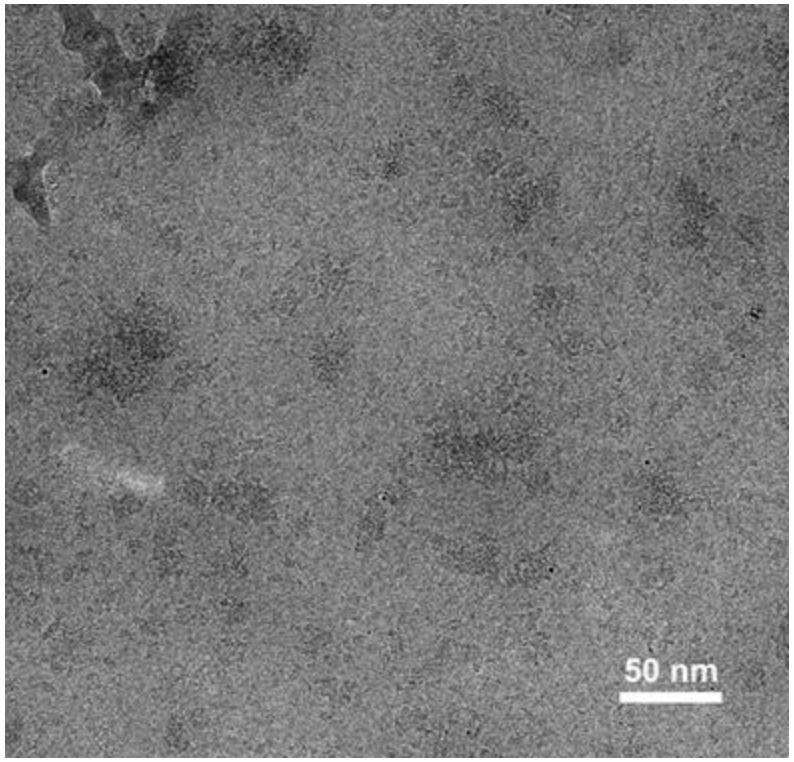


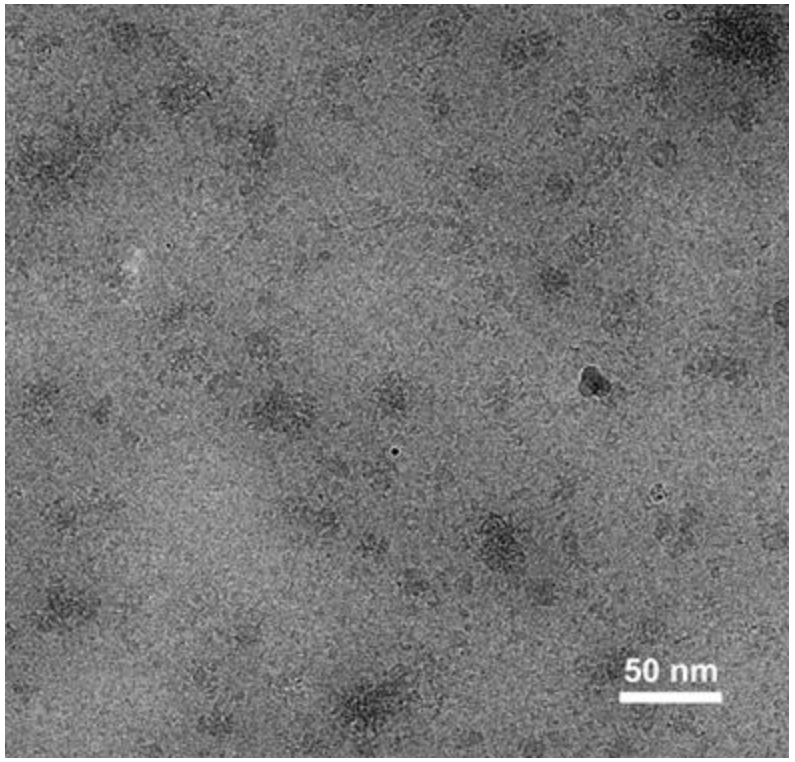
Too few squares with thin ice

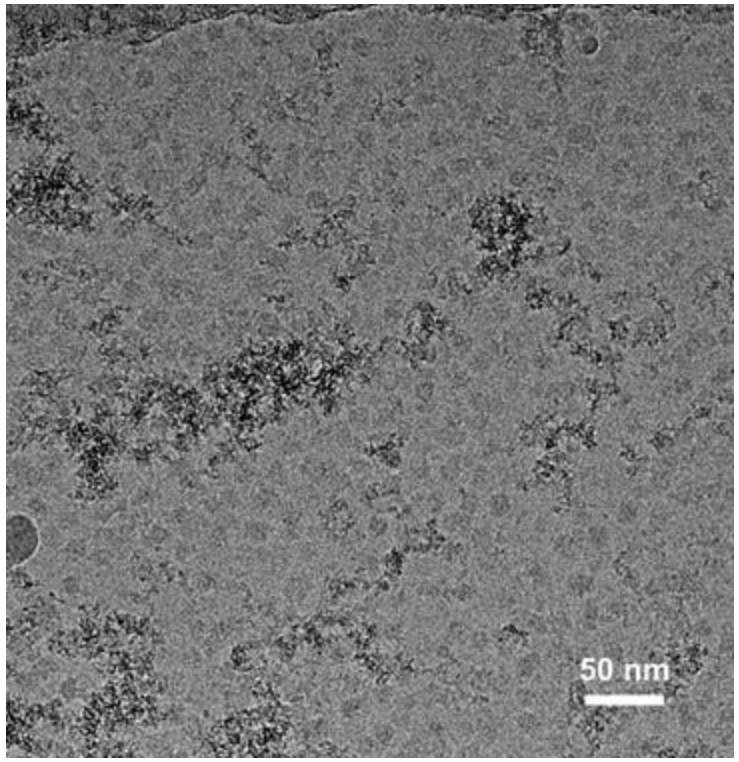
Too thick ice around bars

Non-uniform ice thickness

Aggregation happens, even in good ice







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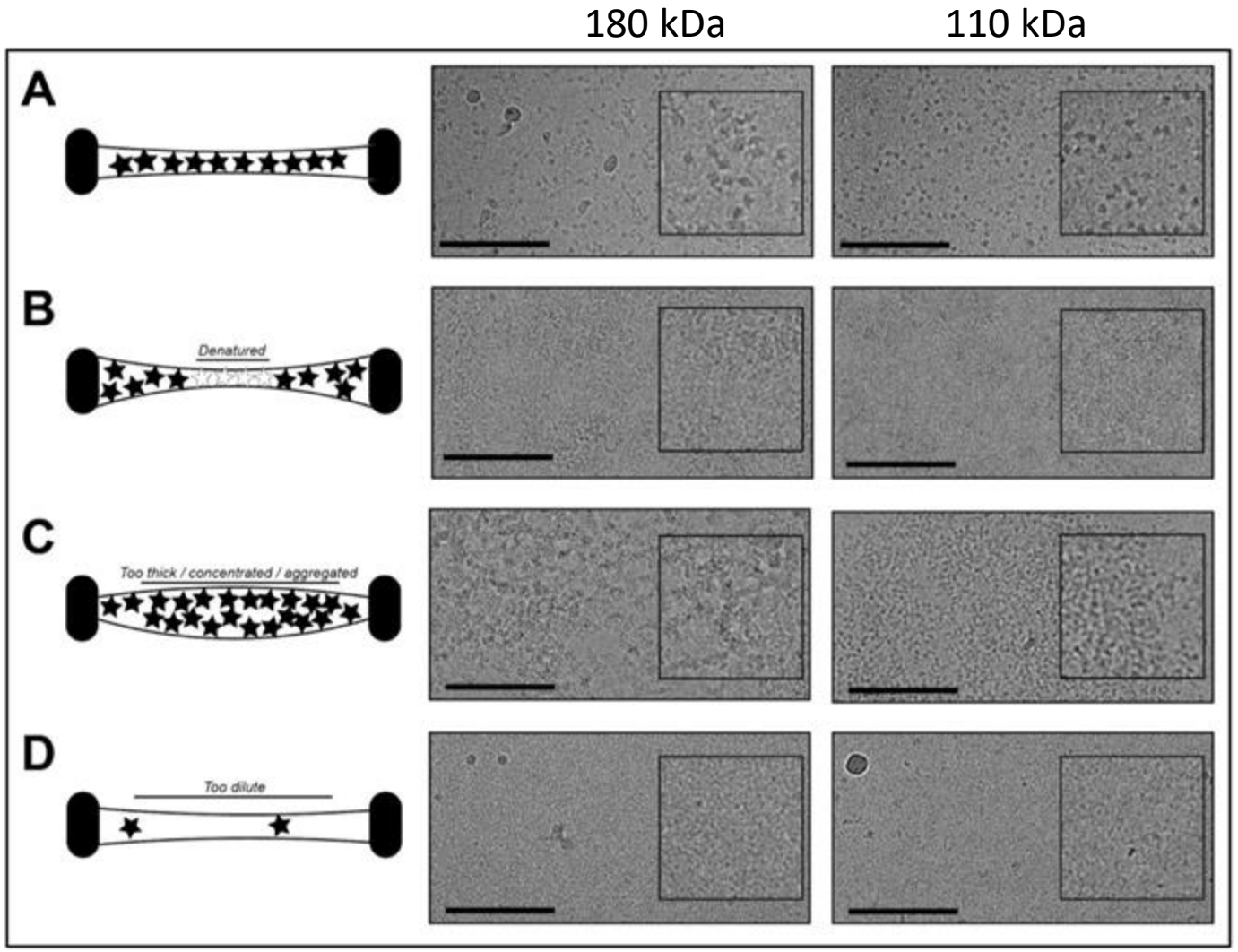
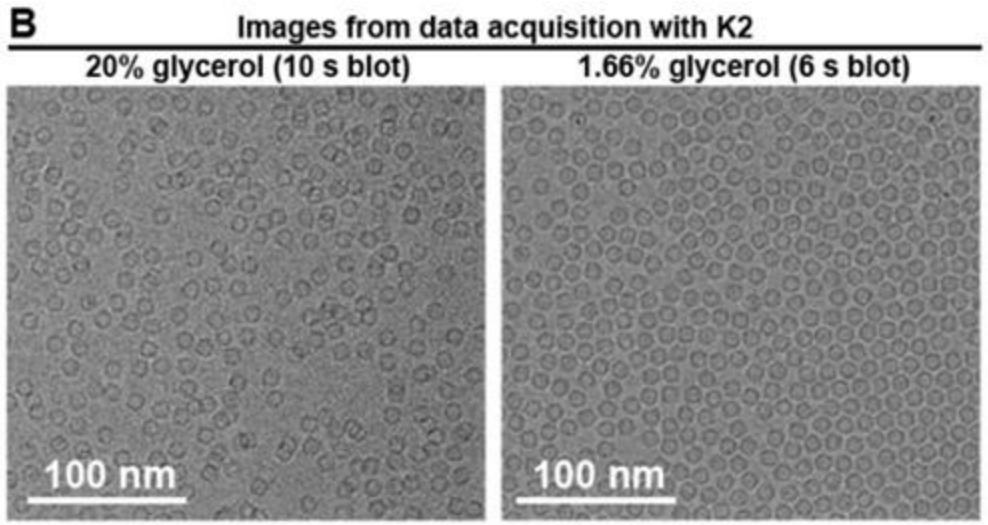
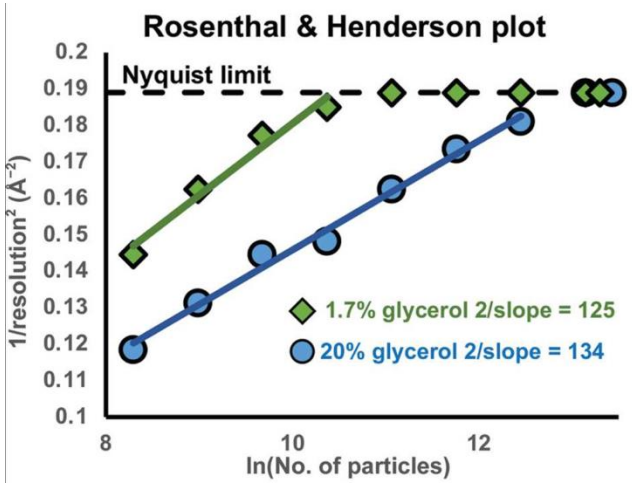
Biomolecules don’t like to be in thin films

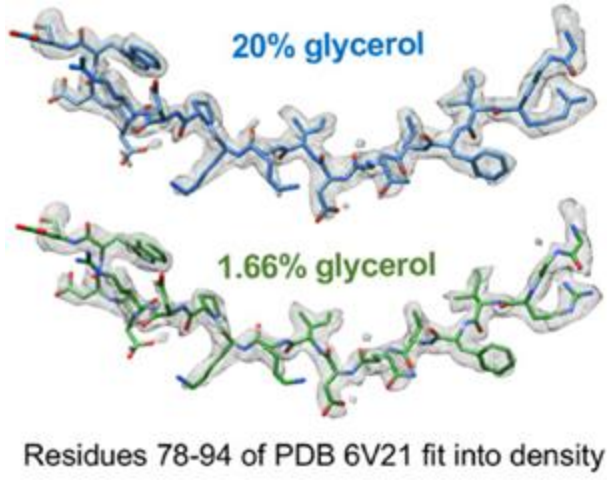


Cianfrocco & Kellogg. J Chem Inf Model. 2020

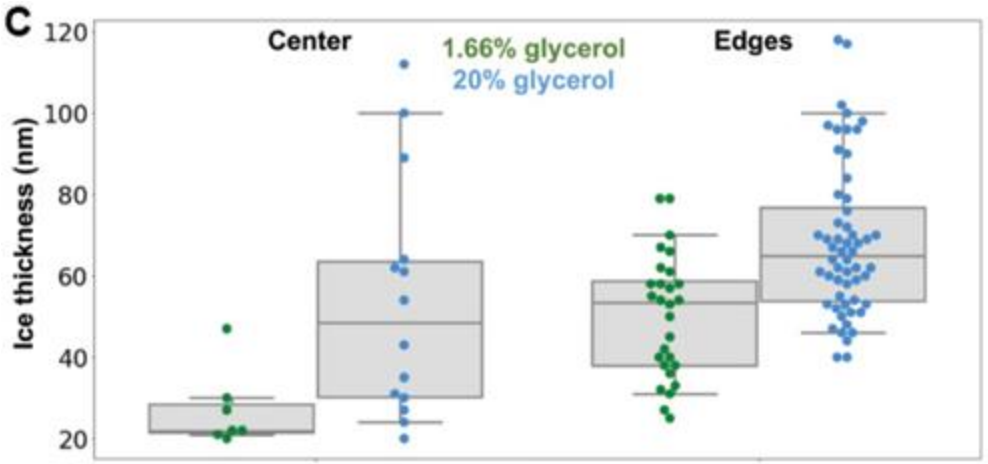
Buffer also matters: What about glycerol?







Residues 78-94 of PDB 6V21 fit into density



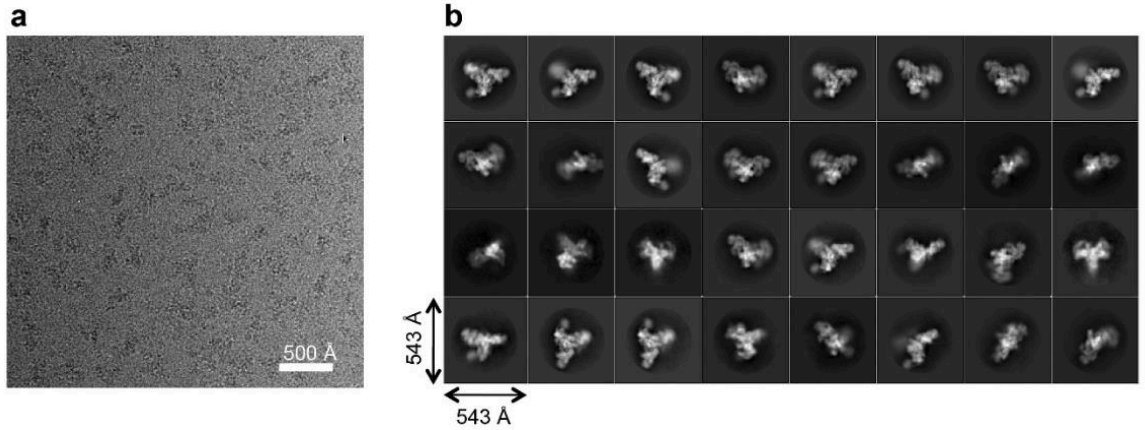
Balance between advice for “ideal” and what keeps your sample well behaved.

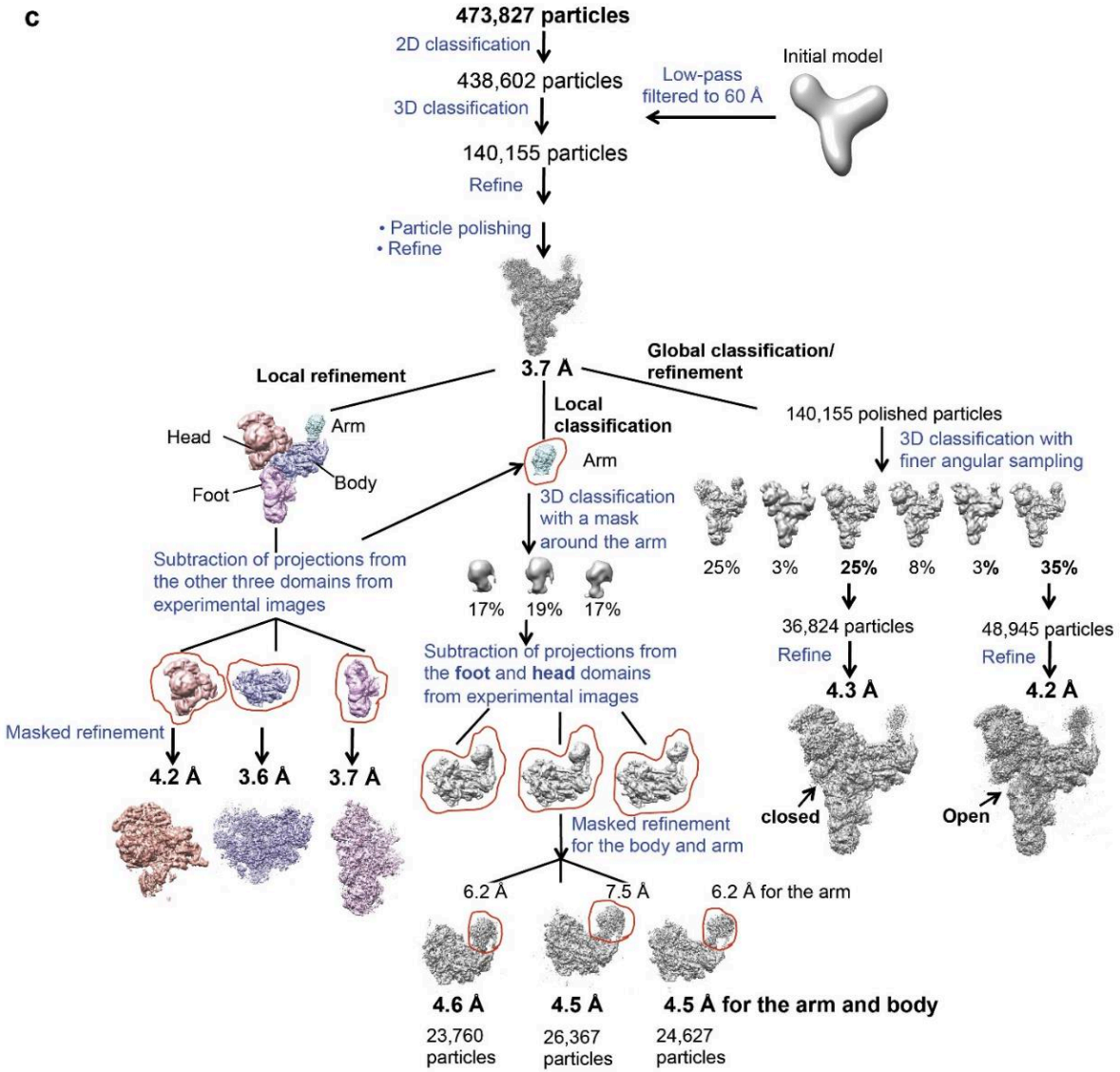
Basanta et al. (2022). Acta Cryst. D78: 124-135

There are multiple ways to troubleshoot particle distribution/ice thickness on grids

- Check the literature for other samples similar to yours
- Grids: mesh size, foil type (carbon/Au/NiTi), additional support film (thin amorphous carbon, graphene, graphene oxide, streptavidin 2D crystals)
- Buffers: Detergents or additives (make sure your sample likes them)
- Ligands/binding partners: but validate your biochemistry
- Alternative instruments: Chameleon, VitroJet, home built solutions

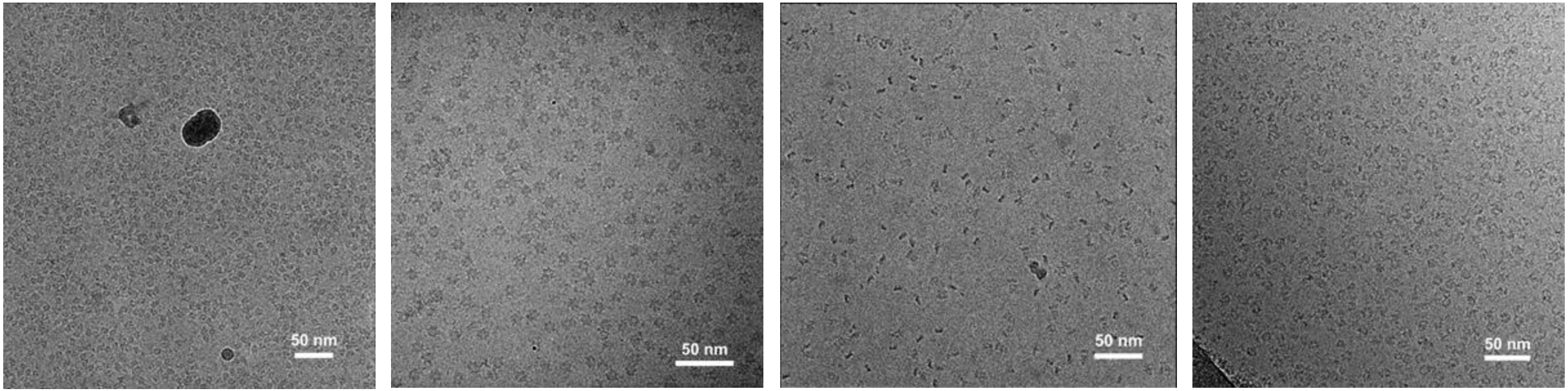
Not every particle needs to be perfect





Nguyen T. *et al.* (2016). *Nature* **530**, 298–302

Good particle distribution is easy to spot!



But other issues may become apparent later in processing:

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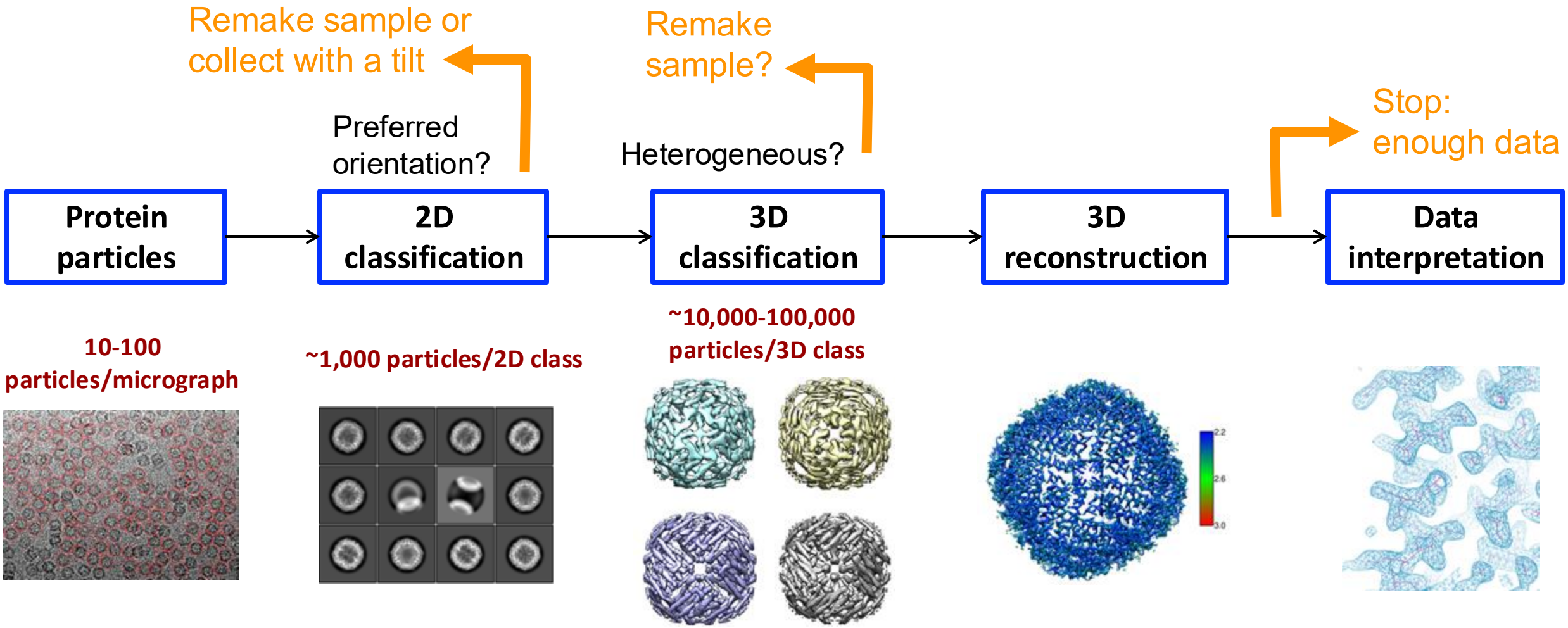
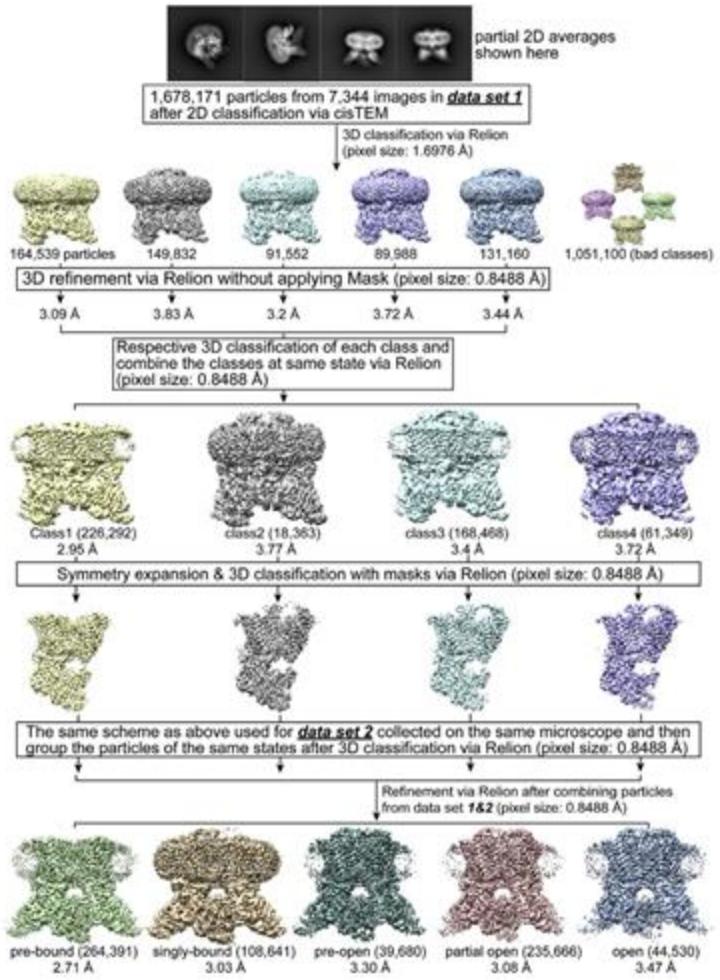
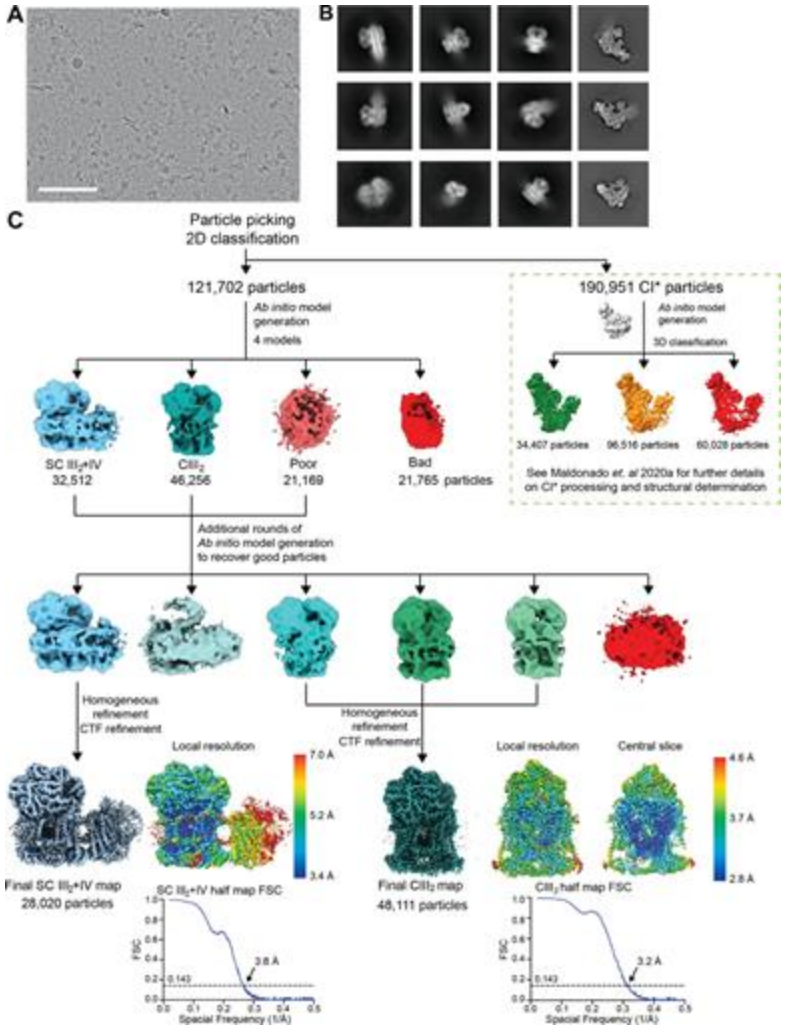
Some issues that limit resolution are only apparent during downstream processing



Heterogeneous samples can be characterized with advanced processing





Zhang et. al. (2021) *Cell.* 184(20): 5138 - 5150

Maldonado et. al. (2021) eLife. 10:e62047

There are multiple strategies for stabilizing a sample

Particle	Resolution (Å)	Stabilization method	EMDB Accession No.
β-galactosidase	1.9	Small molecule inhibitor (PETG)	EMD-7770
Vps4	3.2	Non-hydrolyzable nucleotide analog (ADP·BeFx)	EMD-8887
Ribosome Quality Control Complex	8.2	Catalytic inactive mutant	EMD-6170
Insulin degrading enzyme	3.7	Fab antibody fragment	EMD-7062
ABCG2	3.1	Catalytic inactive mutant	EMD-0190
IGPD2	3.1	Small molecule inhibitor (C384)	EMD-3999

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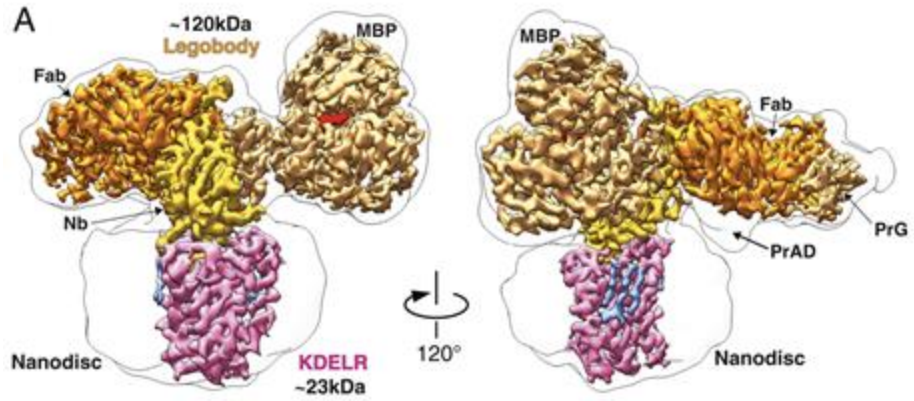
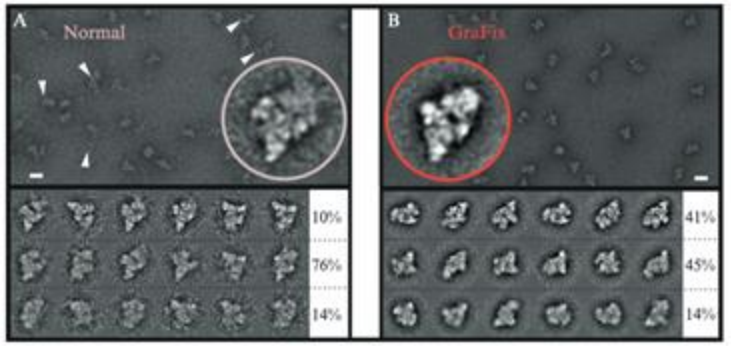
A balance between size and heterogeneity can help you choose the best strategy

Fab, nanobodies, megabodies, DARPins, etc.



Wu & Rapoport (2021) PNAS 118 (41): e2115001118

Cross-linking



Stark (2010) Methods Enzymol. 481:109-26

Purification tags:

His_{10}-

Epitope

Protein

To cleave or not to cleave…

What is the research question you are trying to address??

Every sample must be optimized but here's where you can start:

- For SPA cryoEM: ~1-5 mg/mL (or ~100x the concentration that looks good in negative stain).
- 3-4 µL per grid. Plan on needing ~a dozen grids to make progress (testing grid type/concentration/blot parameters/etc).
- As homogeneous as possible, measured by non-EM techniques!
- Proteins smaller than 100 kDa are challenging.

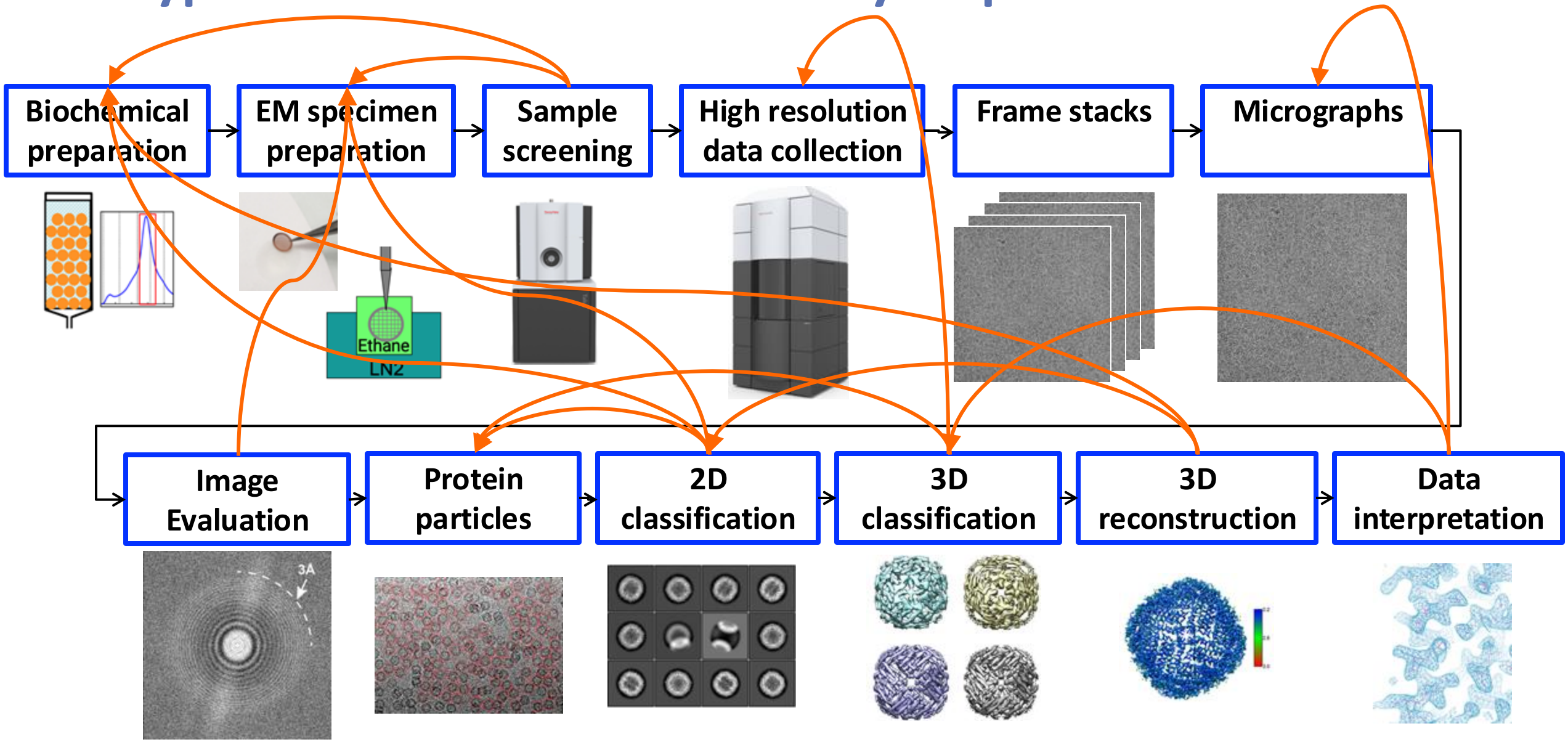
Cryo-sample preparation tips from the experts

- Plan your freezing session in advance!
- Reduce contamination: Get everything ready before pouring LN_2
- Check ethane level to ensure immersion of the grid – be fast and careful during grid transfer steps
- Grids: 400 mesh grids for NS
  300 mesh grids for cryo-samples
- Glow discharge:
  20-30 mA, 20 s for carbon / 120 s for gold;
  10 mA, 5s for ultrathin carbon coated holey grids
- Reproducibility is hand-to-hand dependent
- Working fast is helpful - generally improves with experience

Cryo-sample preparation tips from the experts

- Trial and error and a little bit of luck are all part of cryo-EM sample preparation.
- Ask advice of more experienced groups or instrumentation facility staff.
- Time spent doing biochemistry to make a sample as homogeneous as possible can save a significant amount of time during grid preparation and data processing.
- Time spent at the grid-preparation stage can make data collection on high-demand instruments more efficient.

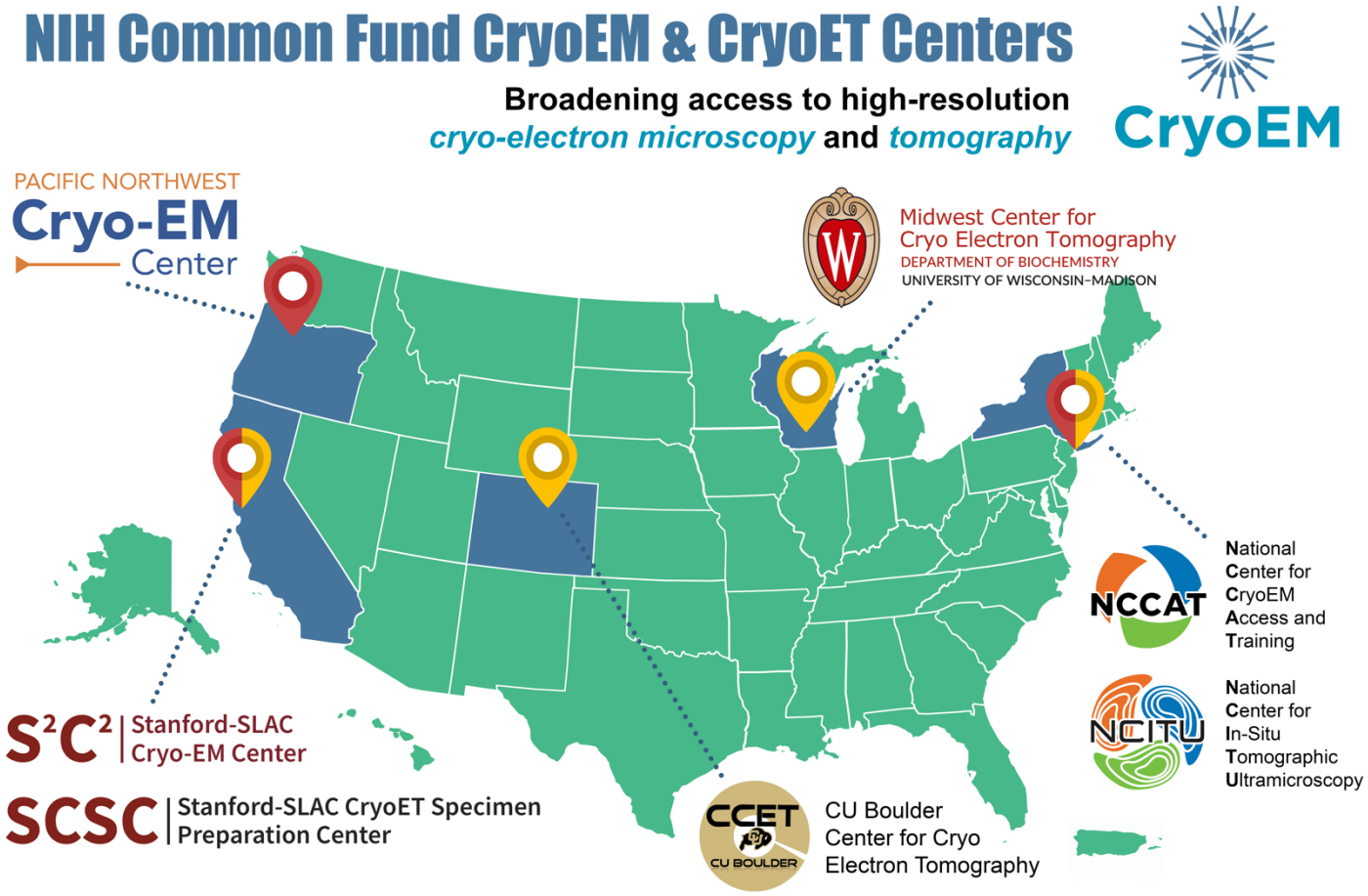
The typical SPA workflow has many steps



Multiple centers share a mission make cryoEM accessible

https://cryoemcenters.org

- **Funded in 2018:**
  - **CryoEM Centers & Curriculum Development**
- **Funded in 2020:**
  - **CryoET Network & Curriculum Development**



Open Curriculum

PURDUE UNIVERSITY CryoVR

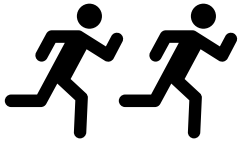
THE UNIVERSITY OF UTAH CryoEM 101 CryoET 101

Caltech | Getting Started in Cryo-EM

Yale Cryo-EM Principles

UNIVERSITY OF MICHIGAN cryoEDU

NCCAT has five avenues of access

General User Proposal 1 (**GUP1**): Data collection on Krios TEMs for ready samples	General User Proposal 2 (**GUP2**): Blot-free vitrification and screening	General User Proposal 3 (**GUP3**): Grid preparation and screening	Training Proposal 1 (**TP1**): Training researchers to become independent cryoEM users	Training Proposal 2 (**TP2**): Training facility managers
				
	 2-3 days	 1-4 days	 2-10 weeks	 3-5 days

- All access categories require submission of a proposal that is scored by an external review committee to determine priority and duration of scheduling.
- Instrument & training time is at no-cost to the user.

Accessing NIH-funded resources is the same but different

Single particle focus

Multiple independent centers. Apply to each separately.

NCCAT

nccat.nysbc.org

PACIFIC NORTHWEST Cryo-EM Center

pncc.labworks.org

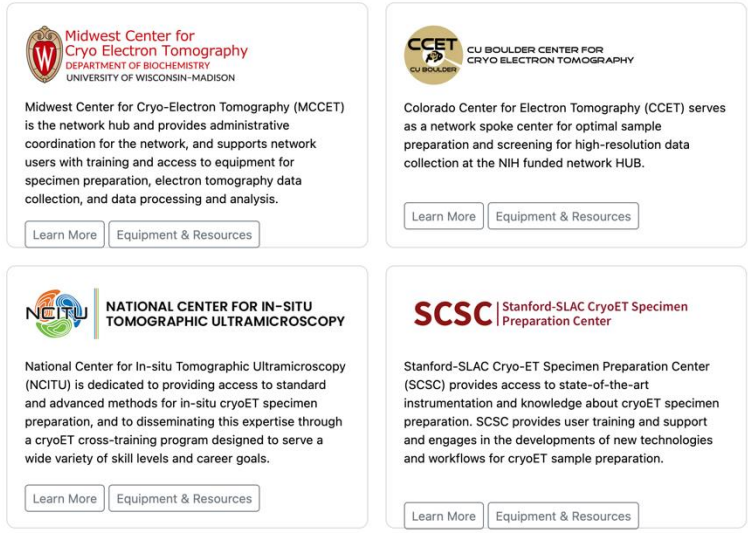
S²C² | Stanford-SLAC CryoEM Center

s2c2.slac.stanford.edu/

High-resolution data collection at the NCI National CryoEM Facility (NCEF)
https://www.cancer.gov/research/resources/cryoem

Cryo-electron tomography focus

One stop shop.
Proposals assigned by the hub.



https://www.cryoetportal.org/

cryoemcenters.org

Bio-Imaging Center

Located in the Ammon Pinizzotto Biopharmaceutical Innovation Center:

590 Avenue 1743, Suite 141
Newark, DE 19713

https://bioimaging.dbi.udel.edu/

Need cryo-TEM help? Contact Us!

Shannon Modla,
smodla@udel.edu

Our center is equipped with a Thermo Talos L120 TEM with a Ceta 16M camera, a Thermo Vitrobot Mark IV, and a side-entry Gatan Elsa cryo-transfer holder.

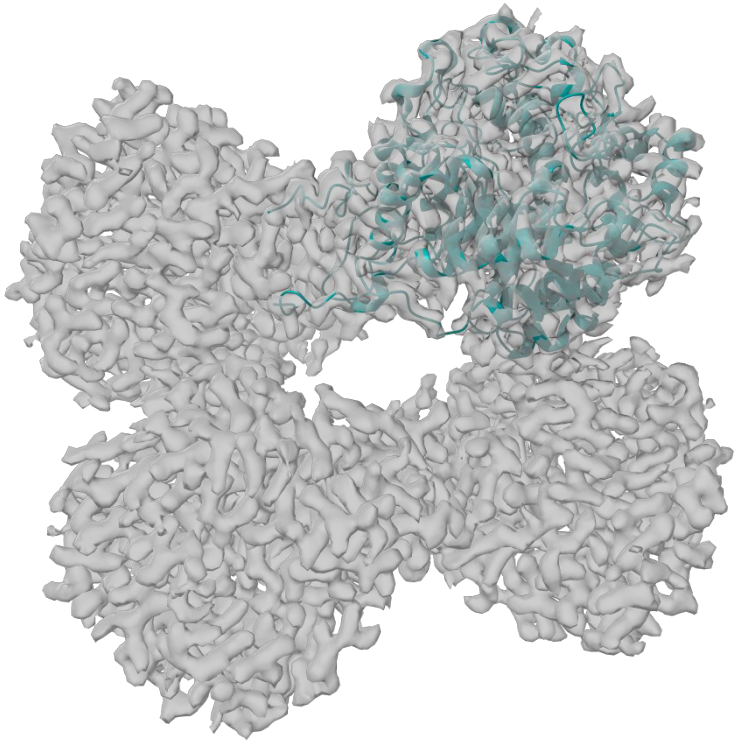
We also have a Pelco easiGlow glow discharge system, a cryo-storage dewar, and dry shipper with a Mitegen puck storage system.

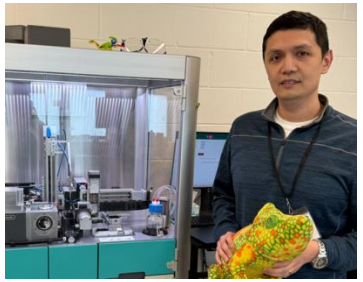
CryoEM is easiest in a community

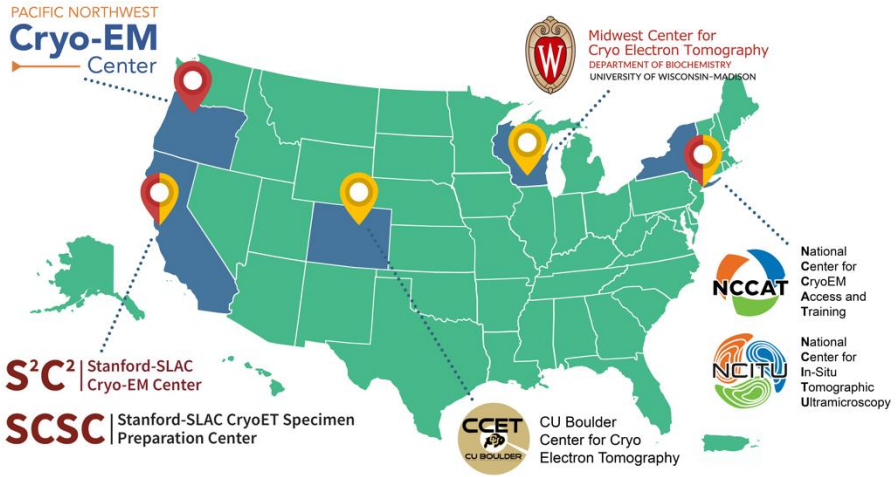
The Delaware Biotechnology Institute's

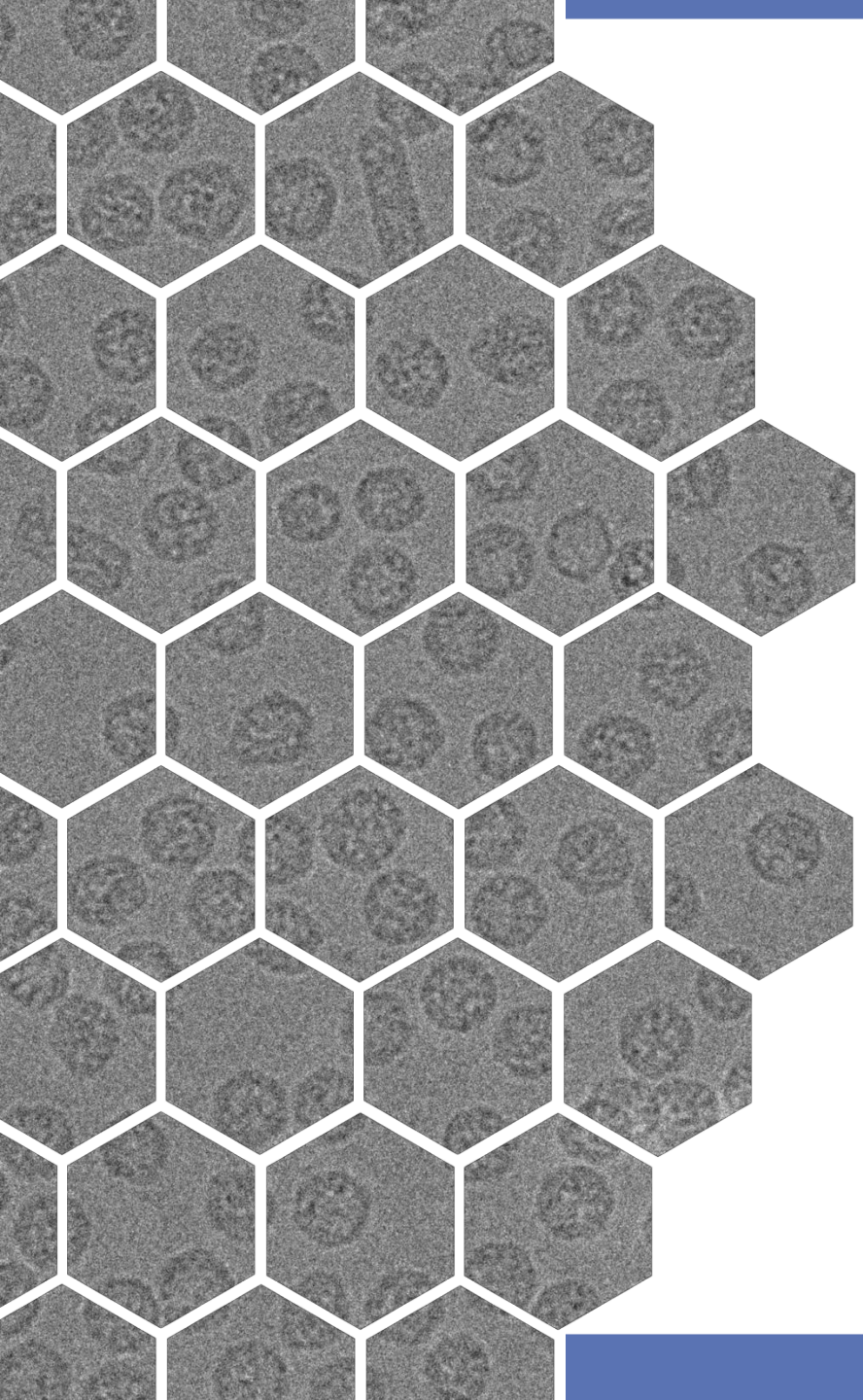
BIO-IMAGING CENTER

Bringing your research into focus









Let’s make some grids!

Website: nccat.nysbc.org

E-mail: nccatinfo@nysbc.org