# CRYOEM 001 :
# EM SAMPLE PREPARATION

NCCAT Embedded Training – Master Class series

September 30, 2020

NATIONAL CENTER FOR
CRYOEM ACCESS & TRAINING

New York Structural
Biology Center

SIMONS ELECTRON
MICROSCOPY CENTER

# CRYOEM 001 : SINGLE PARTICLE MASTERCLASS

Introduction to cryoEM: SPA

Building a cryoEM toolkit

EM compatible samples

EM support films and grids

Sample preparation

Tools of the trade:

- microscopes and detectors

Microscope operations

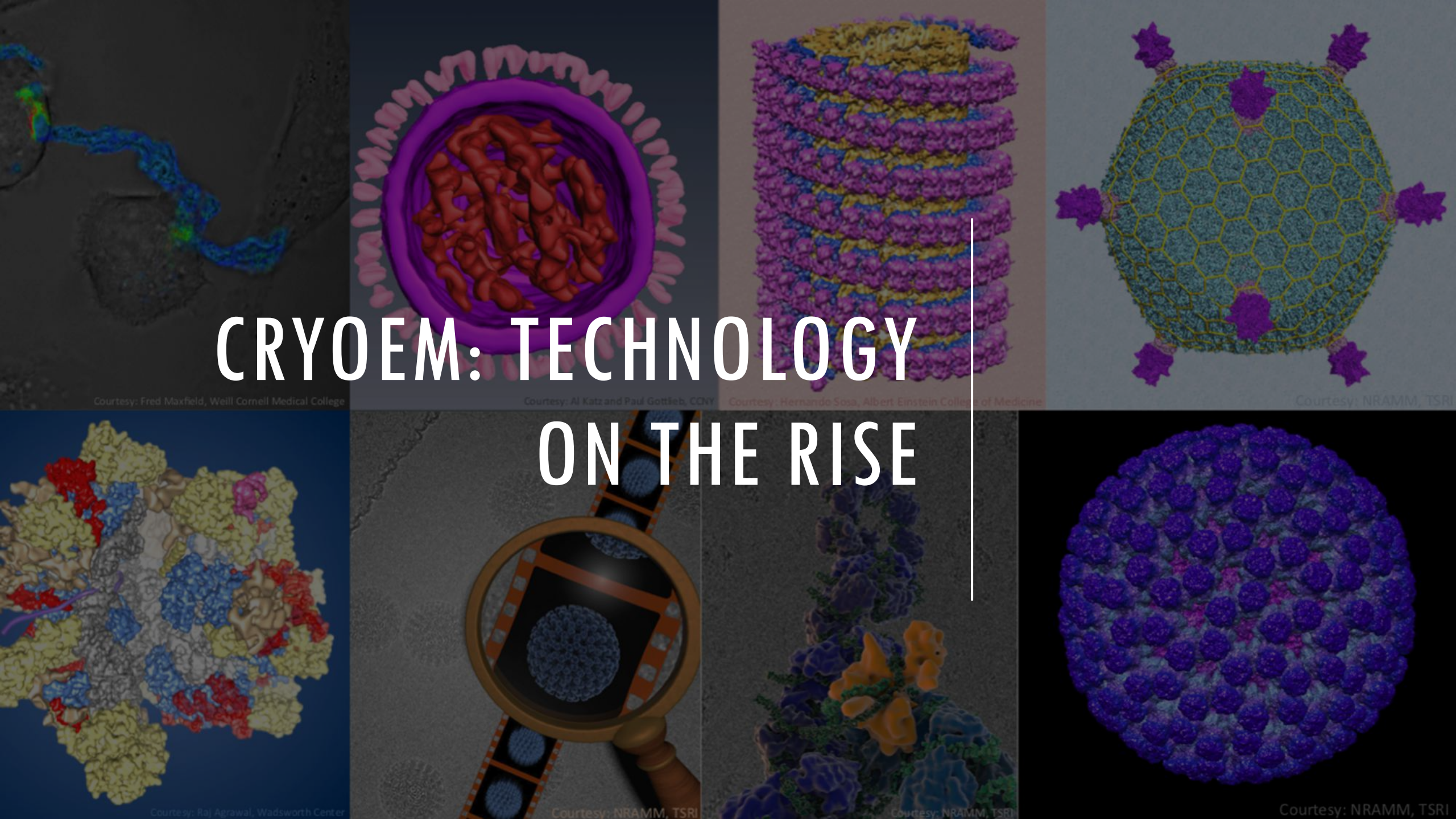
Data collection strategies

Data assessment & QC

Data processing:

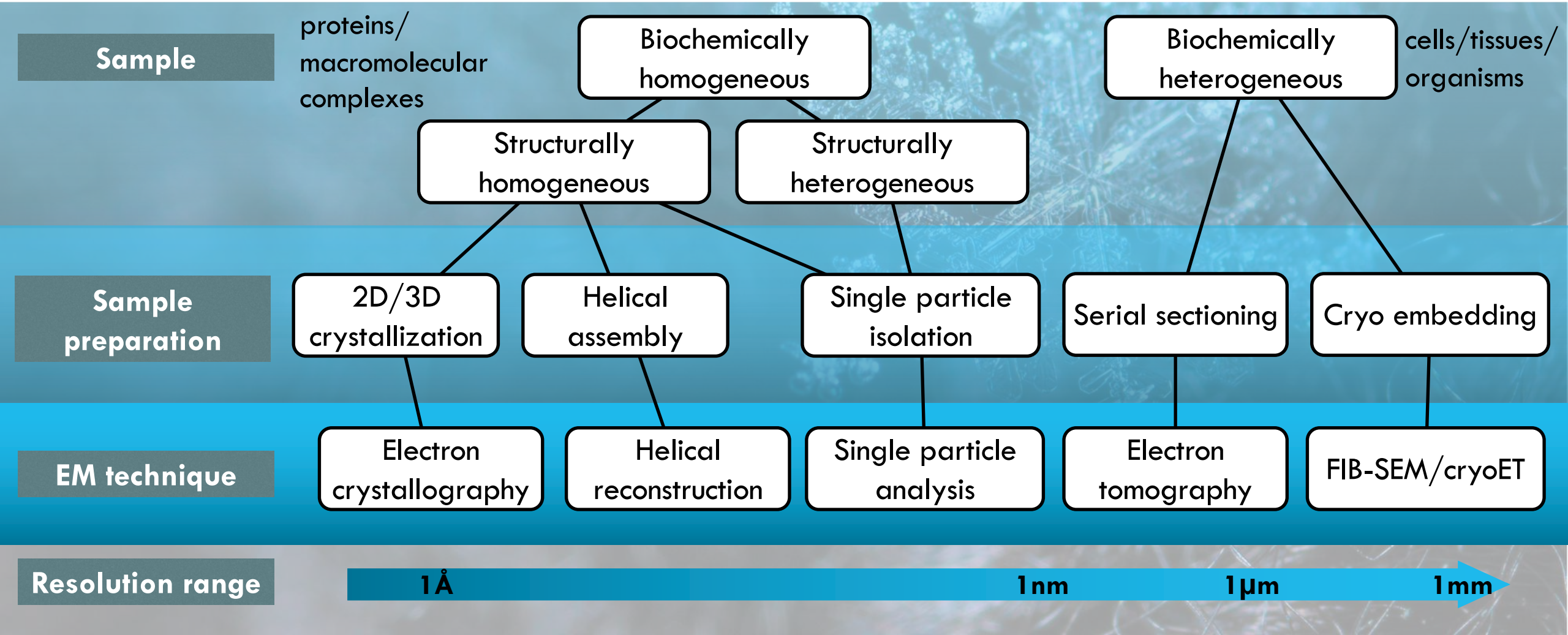
- cryoEM IT infrastructure
- On-the-fly feedback
- 3D Reconstruction

Visualization and validation




# CRYOEM: TECHNOLOGY ON THE RISE

# HOW ARE SAMPLES PREPARED?



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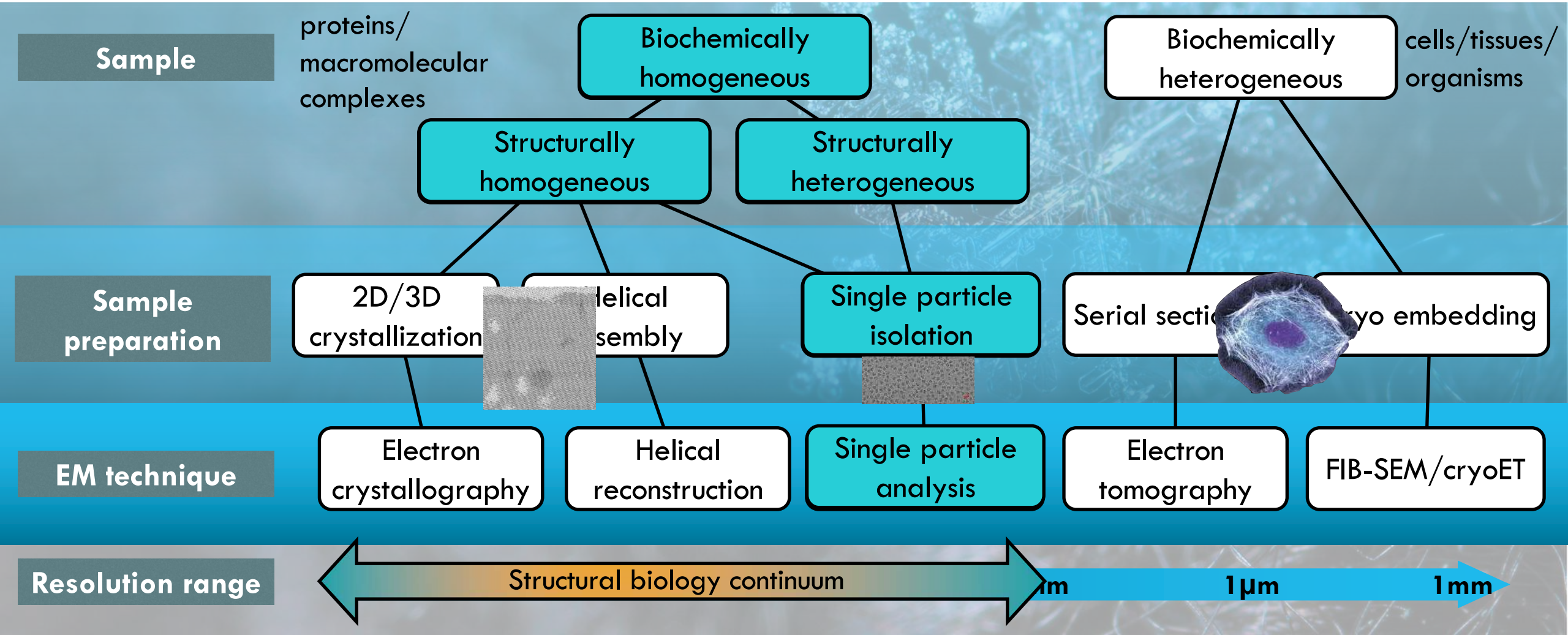
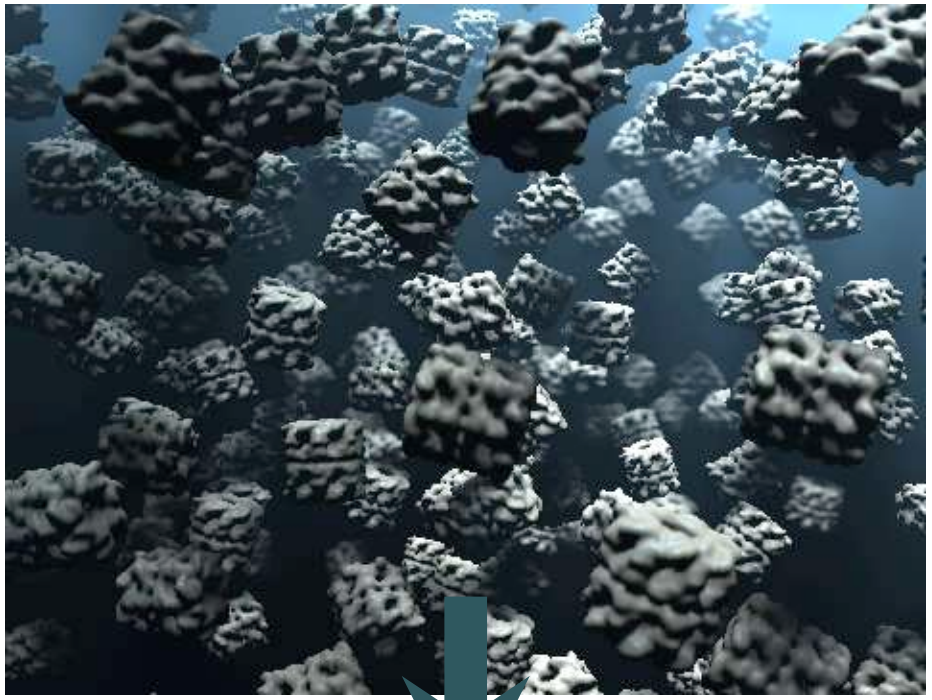
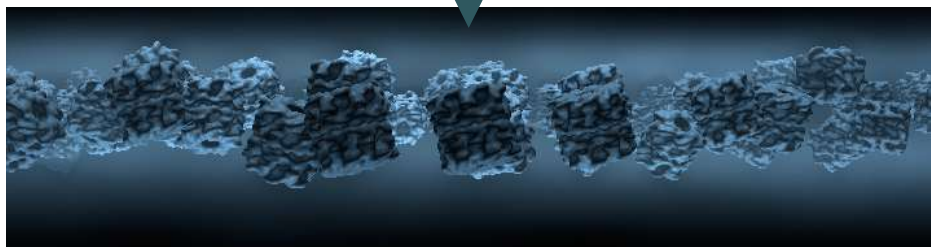
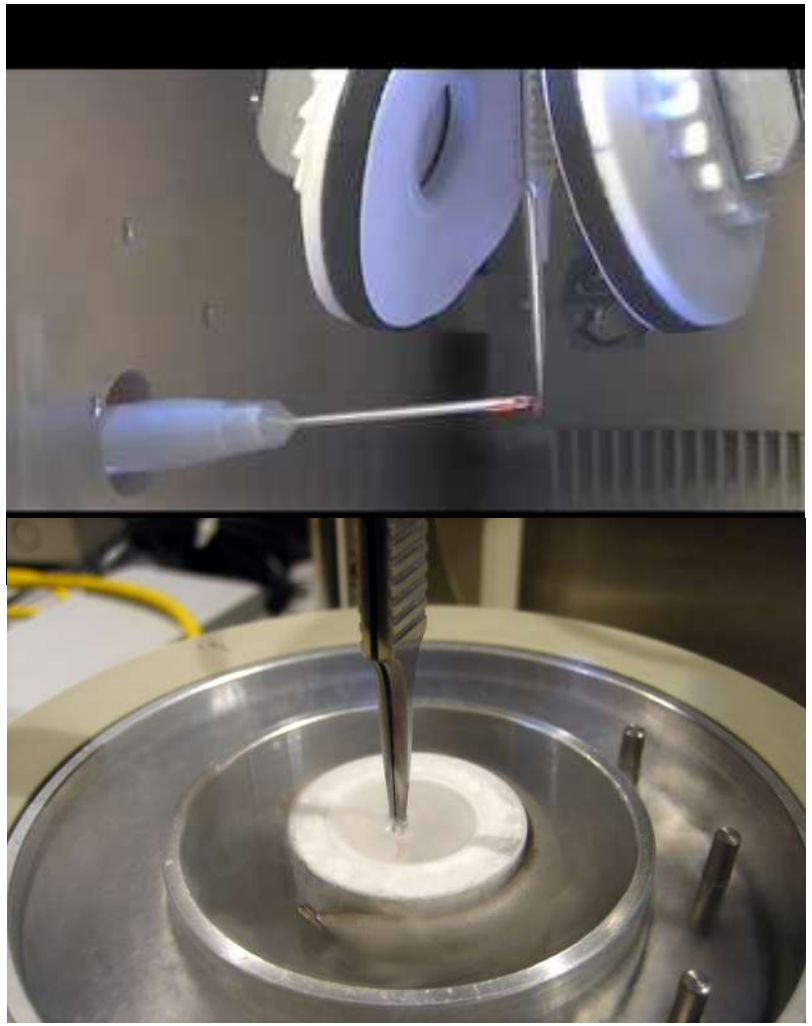


# PLUNGE FREEZING

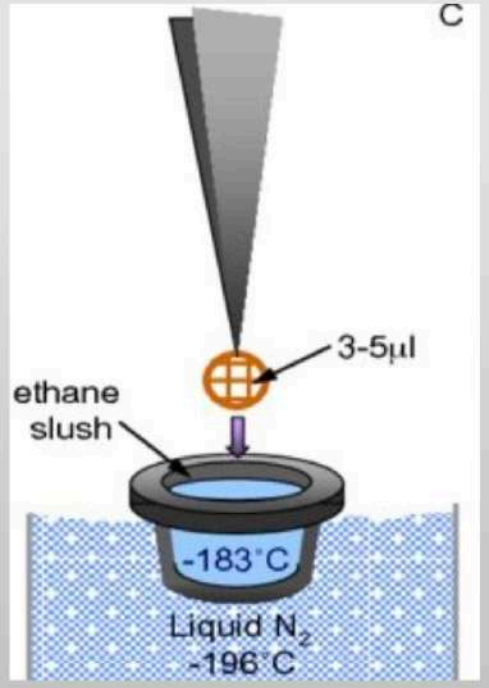




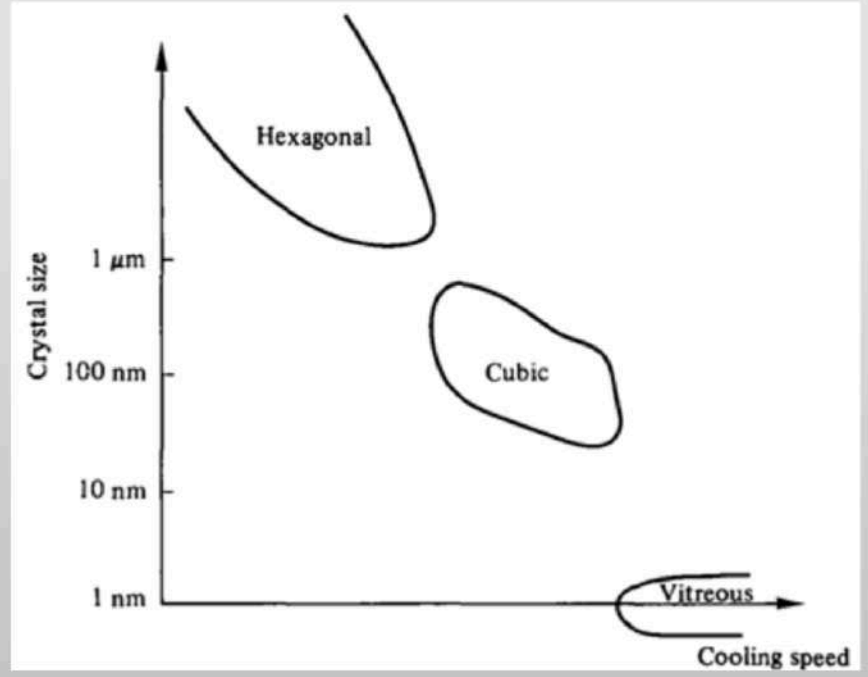


# PLUNGE FREEZING

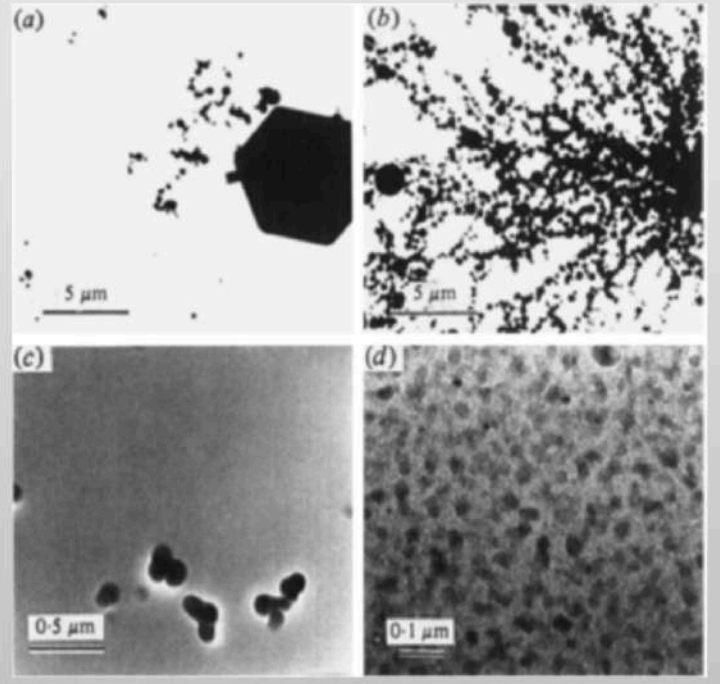
- Liquid ethane is a suitable coolant.
- Liquid nitrogen boils on contact, which makes it a poor coolant for cryo-EM.
- Cooling speed faster than $10^5$-$10^6$ K/s ensure the formation of vitrified ice.




Setup of liquid ethane
(Image from Wen Jiang)

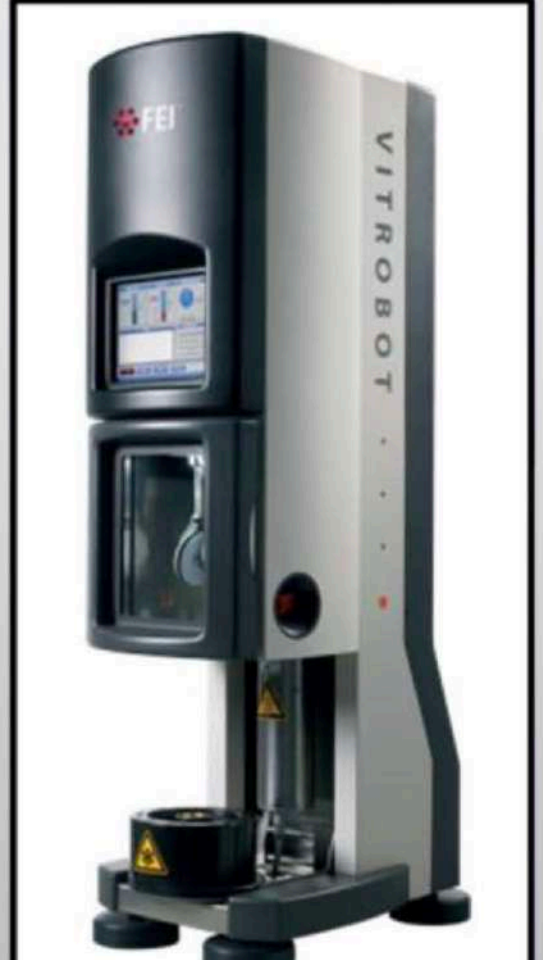



Cooling speed &
forms of ice



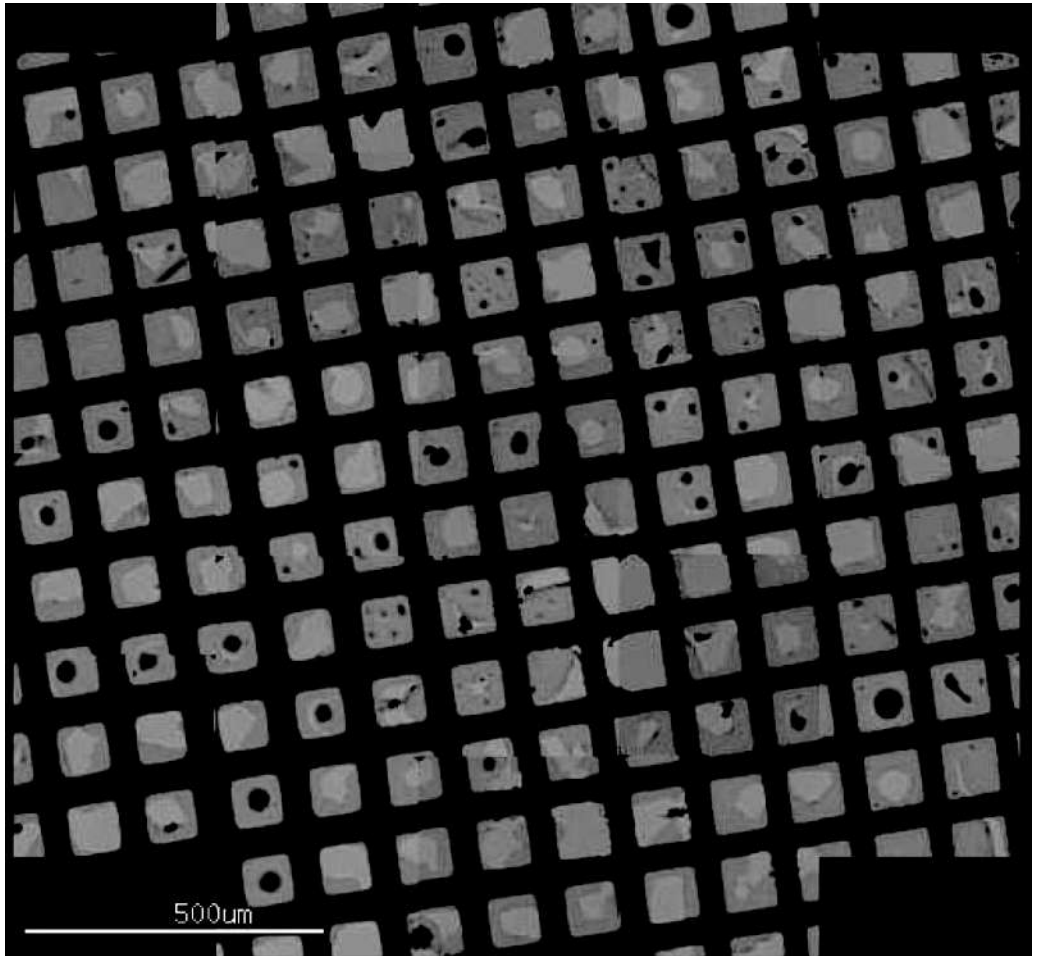

Different forms of ice contamination

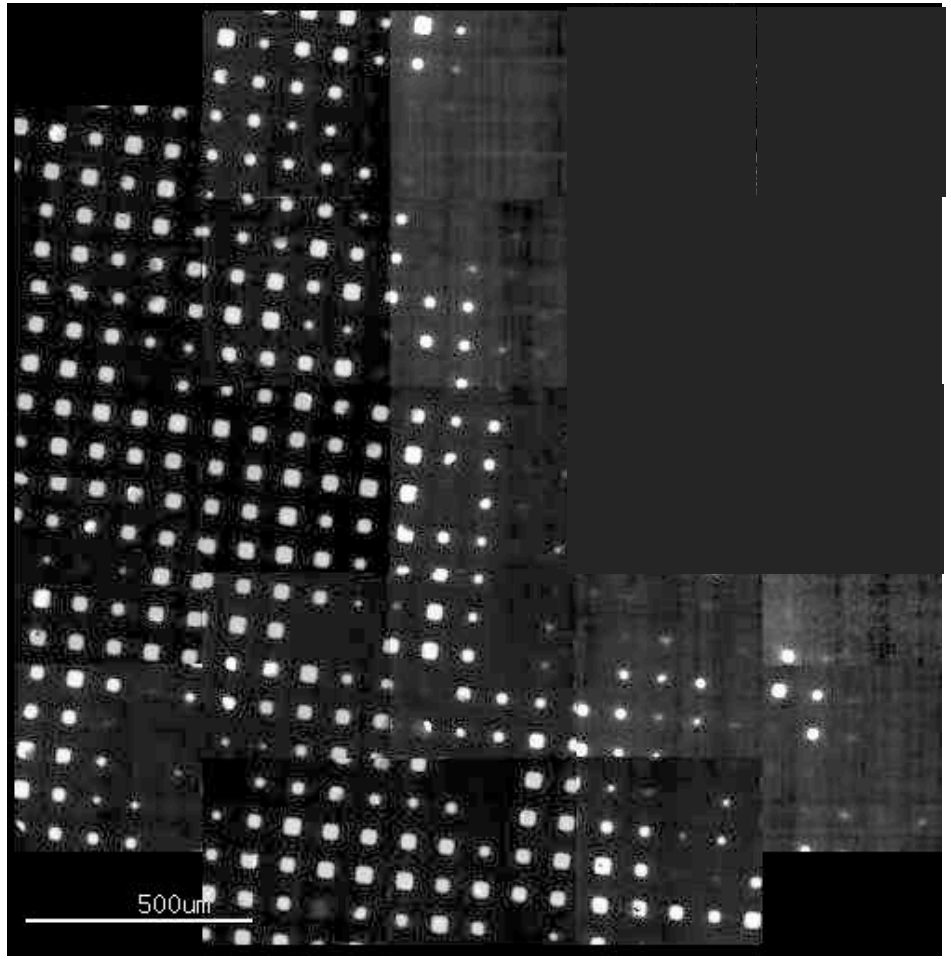
Jacques Dubochet et al., 1988




# PLUNGE FREEZING

# WHAT DO GRIDS LOOK LIKE?



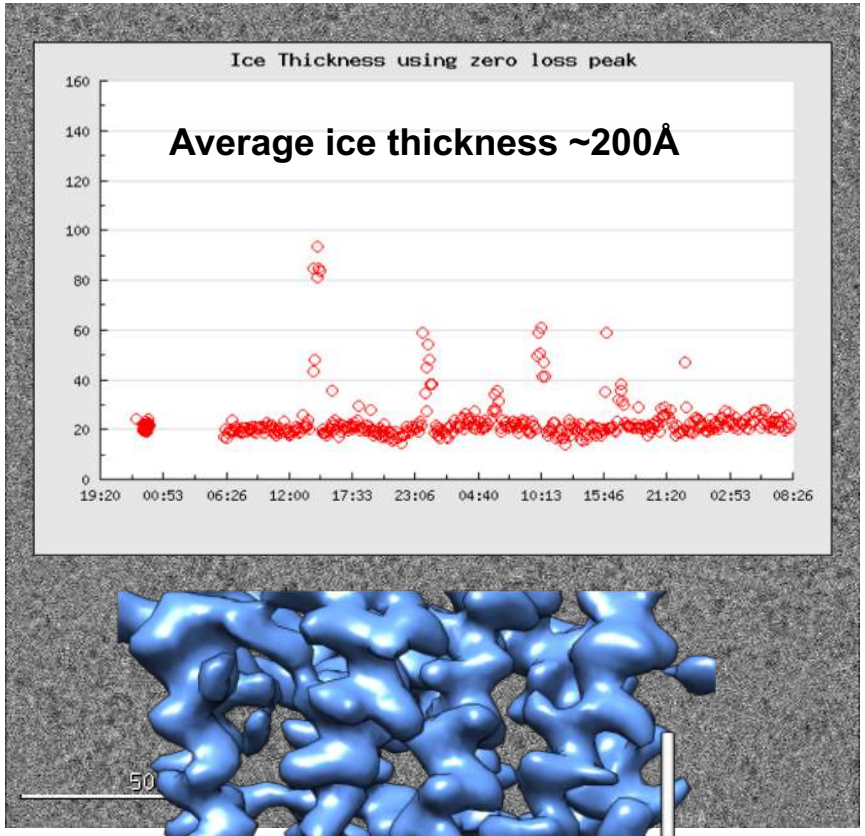



# GRID PREPARATION IS A CHALLENGE

Yong Zi Tan

45 kDa integral membrane protein + Fab

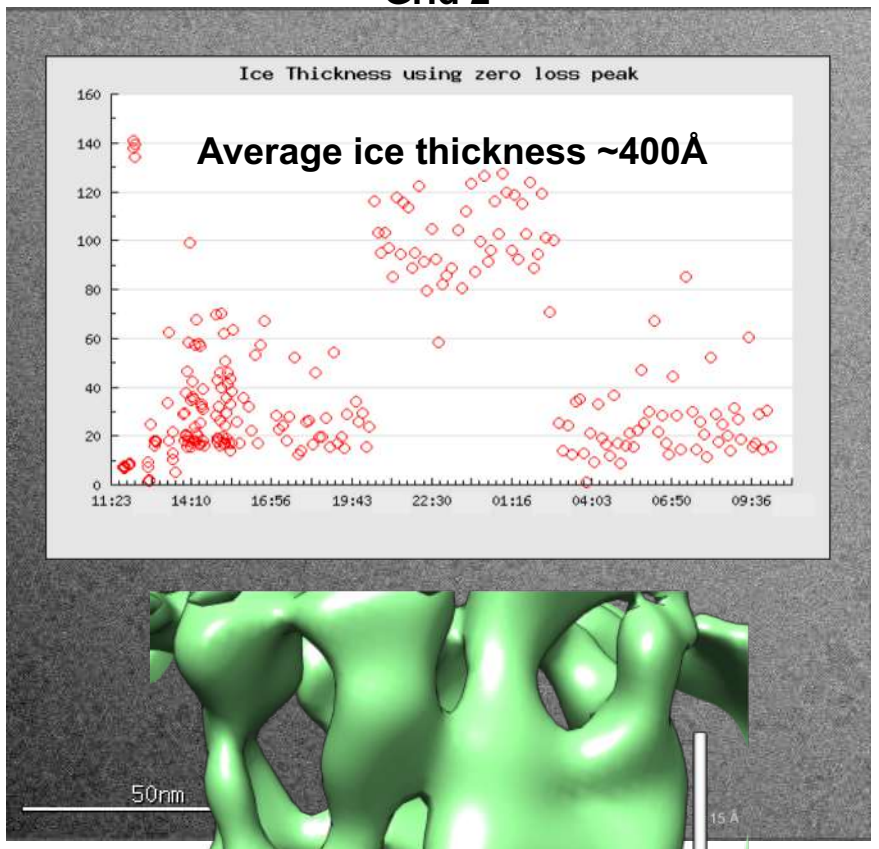
**Grid 1**






**3.2 Å**

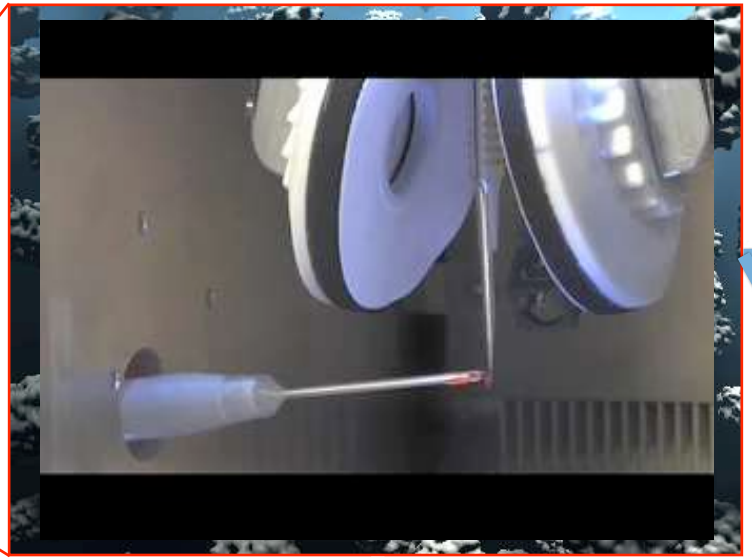
**Grid 2**



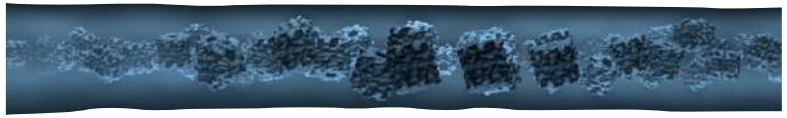



**~7 Å**

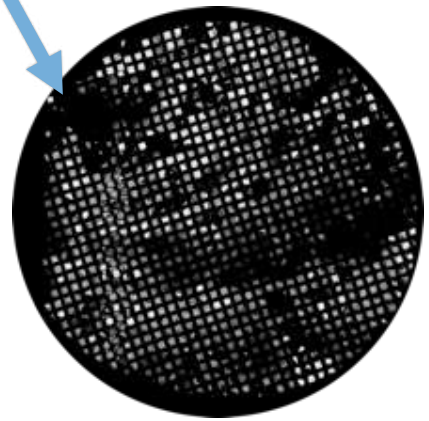
# GRID PREPARATION IS A CHALLENGE



~3µl



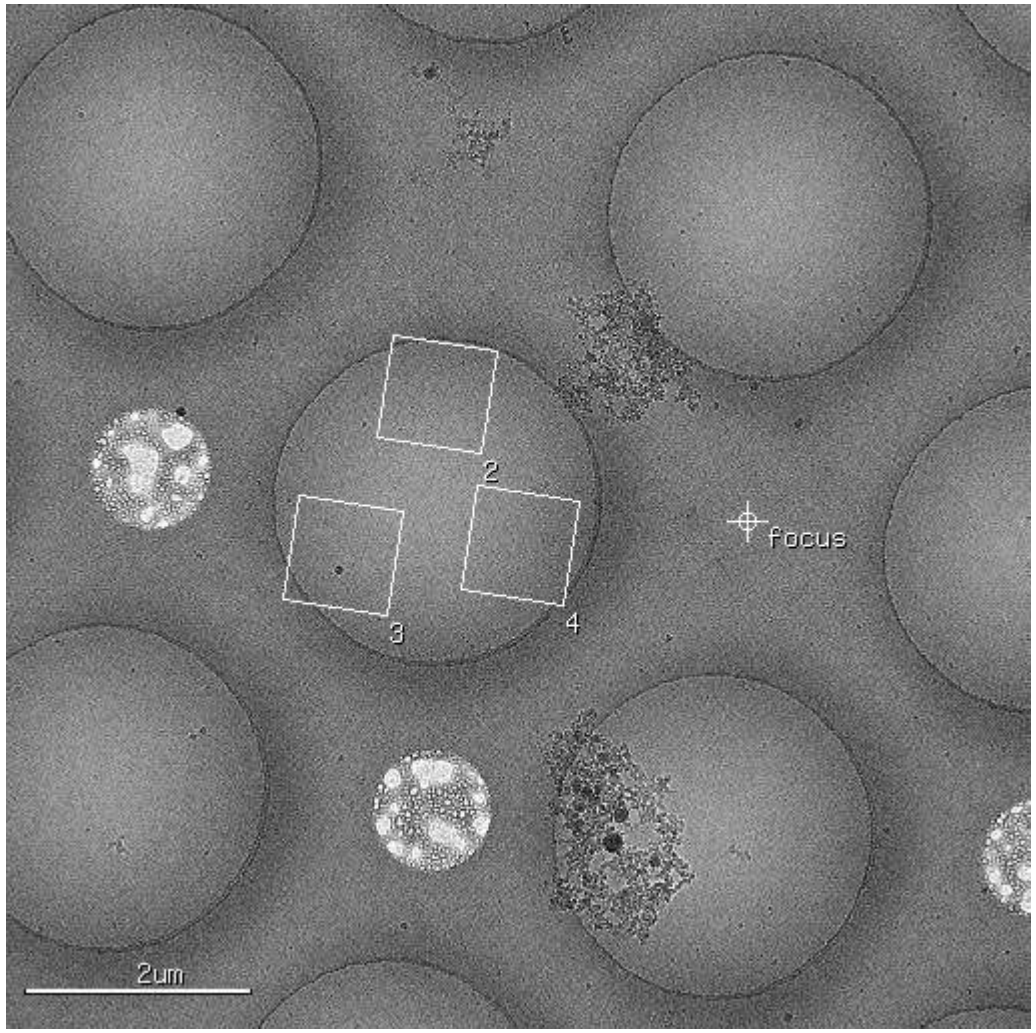
<3nl

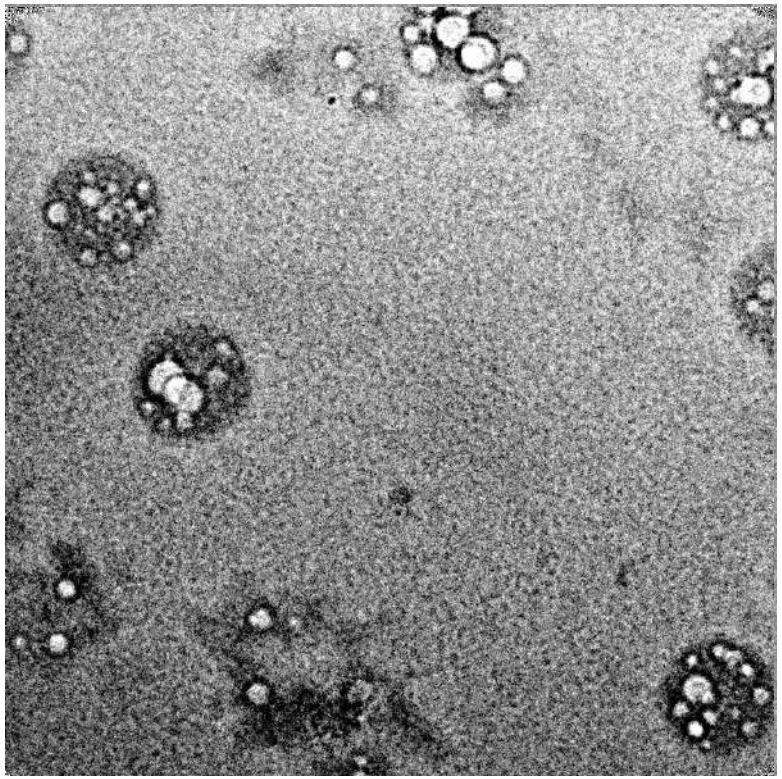


Graphics courtesy Gabe Lander

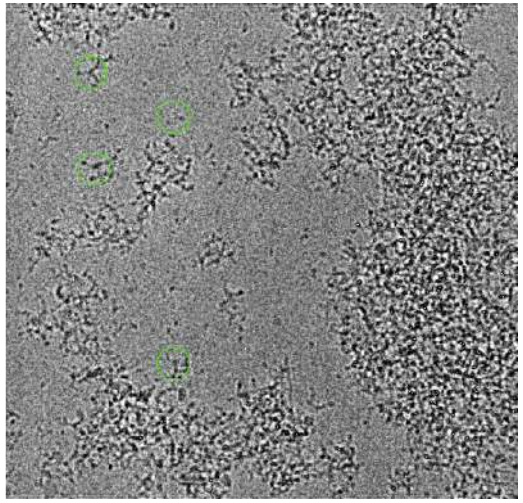
>100,000 potential imaging targets; most of them are not usable.

# LOW DOSE IMAGING

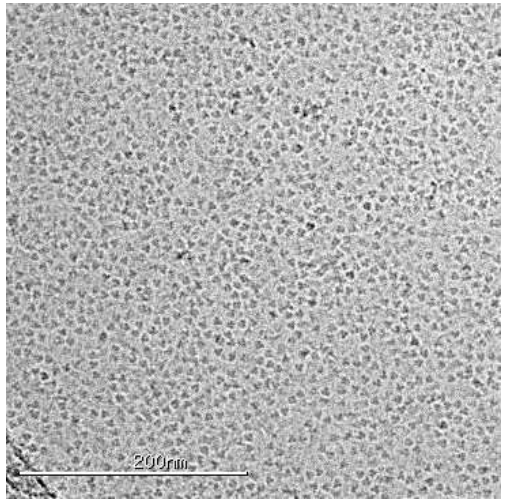


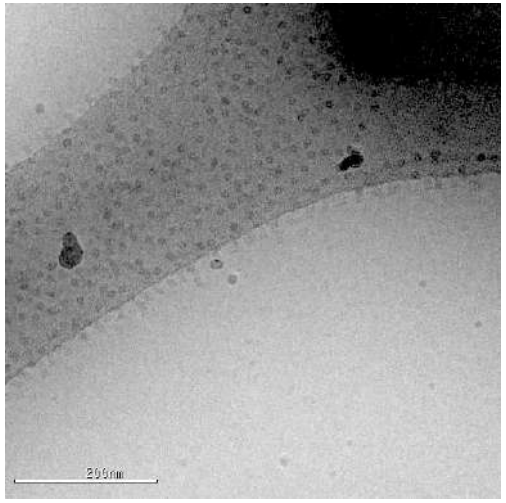
# WHAT ISSUES ARISE?



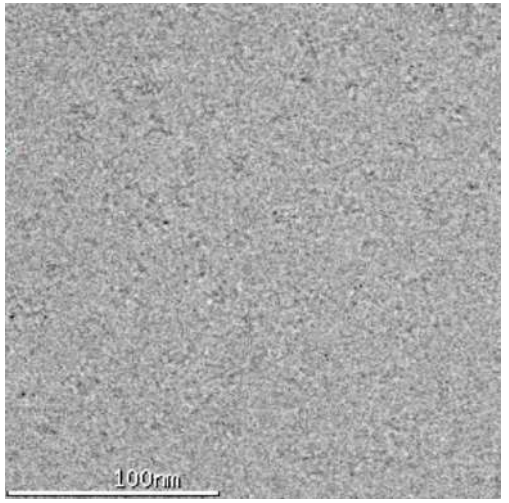
Aggregating in ice



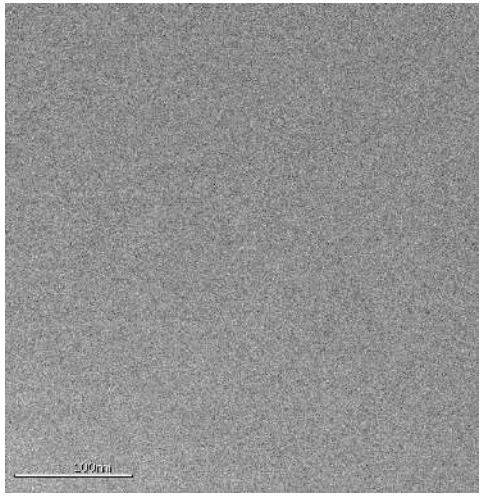

Preferred orientation



Particles not going into holes




Rejecting 90% of particles

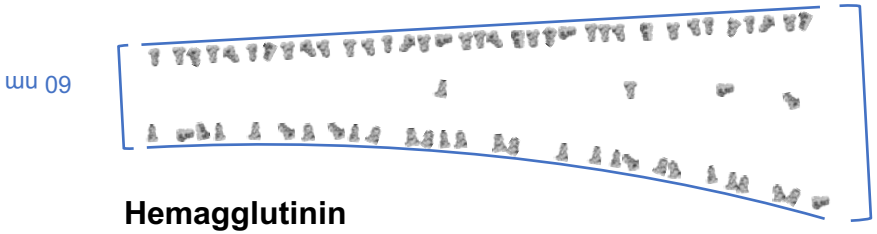


Particles disappearing in ice

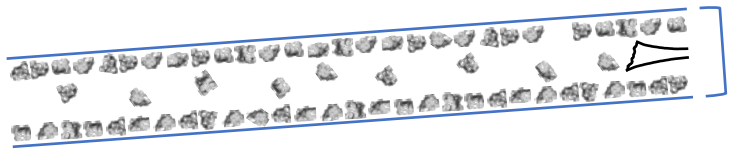
# WHAT ISSUES ARISE?


Hemagglutinin

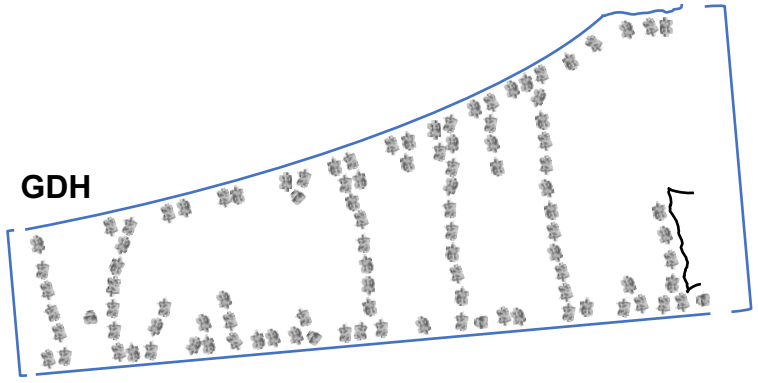

Aldolase

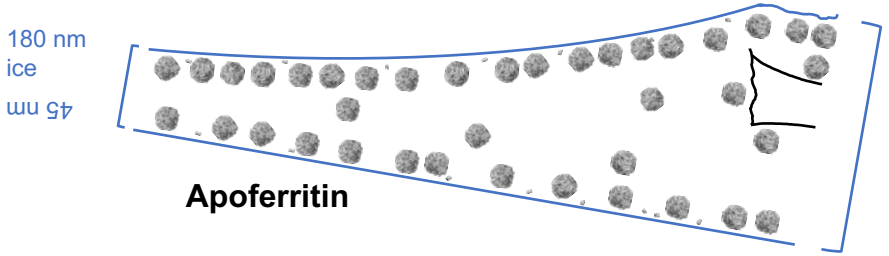



Hemagglutinin

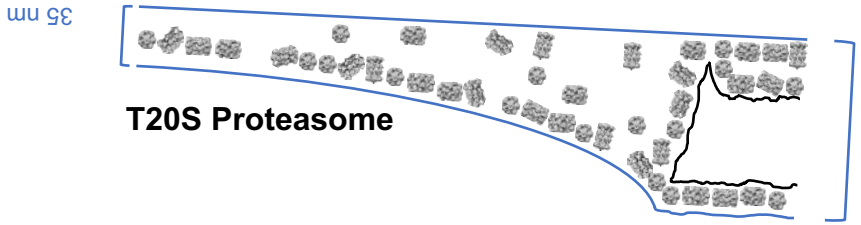



Aldolase

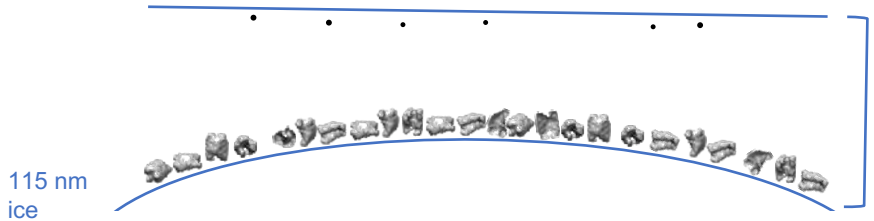



Apoferritin




T20S Proteasome

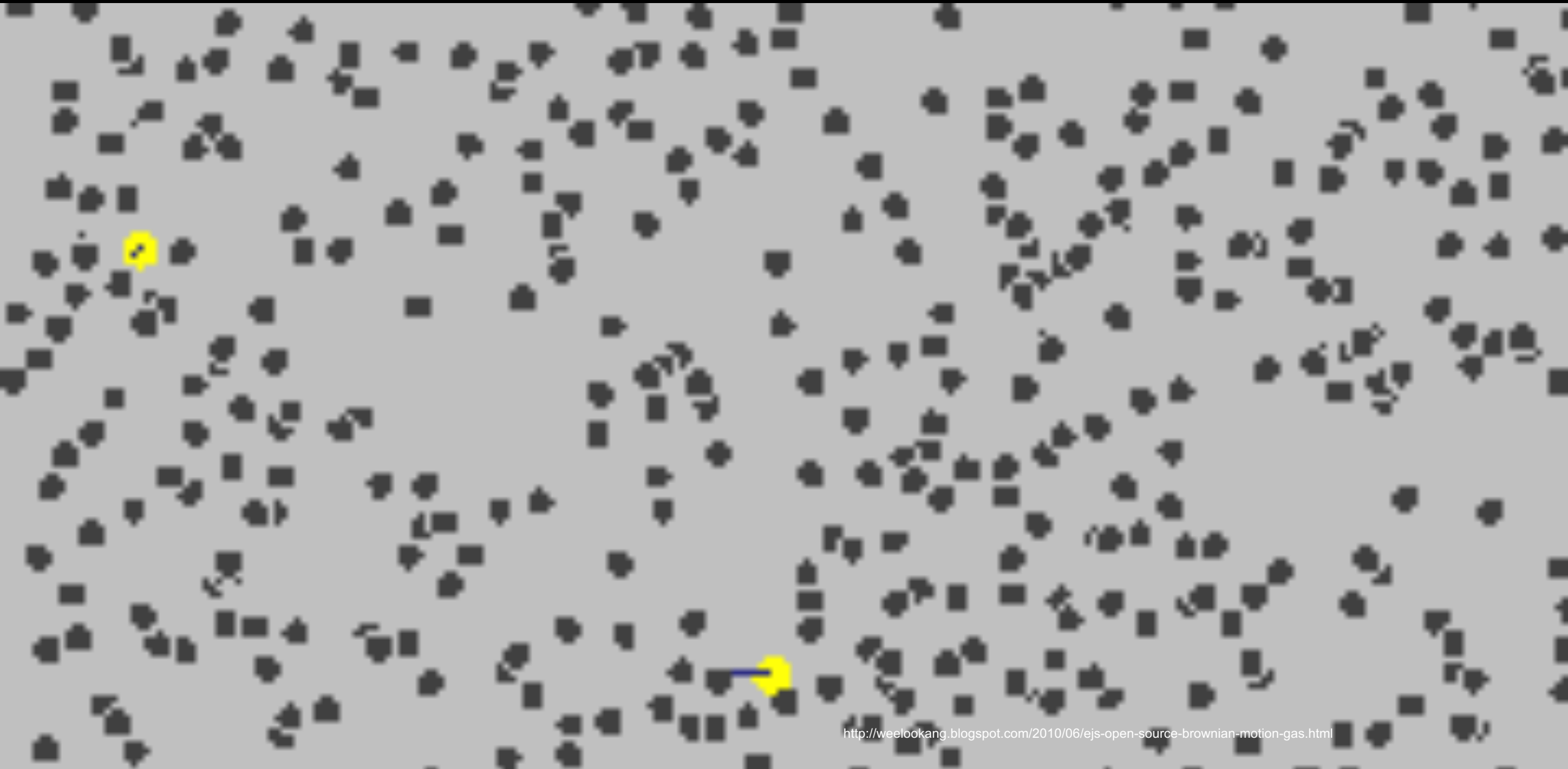



DNAB Helices

Noble AJ, et al. Routine single particle CryoEM sample and grid characterization by tomography. Elife. 2018;7.

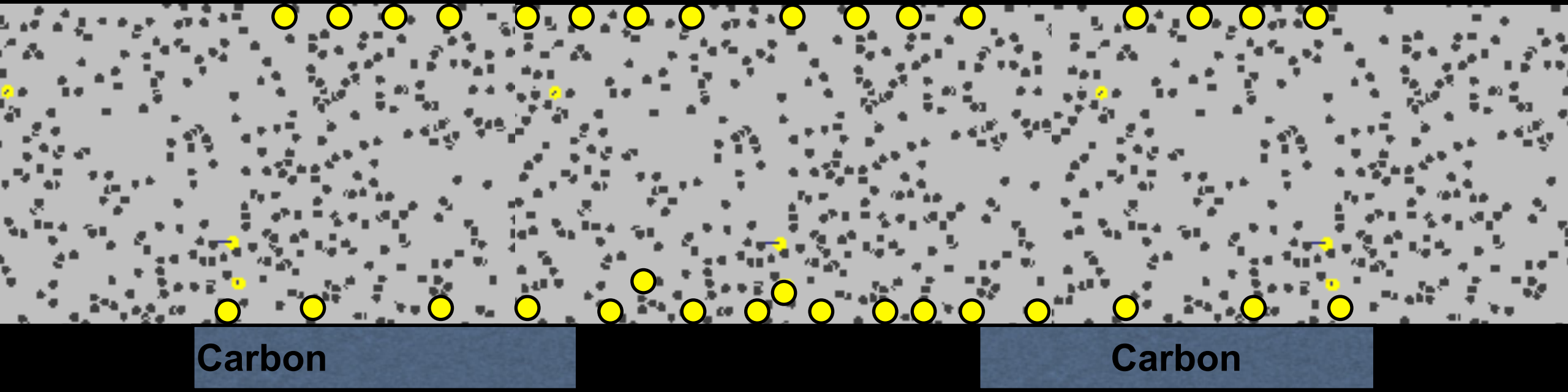
Alex Noble

# What happens to samples during vitrification?



# What happens to samples during vitrification?

A hypothetical scenario during cryoEM grid preparation




http://weelookang.blogspot.com/2010/06/ejs-open-source-brownian-motion-gas.html

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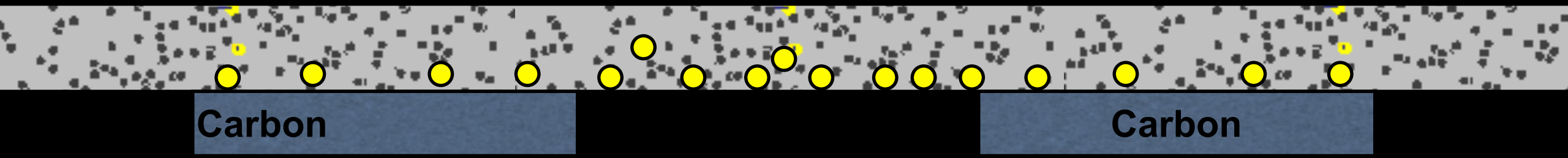
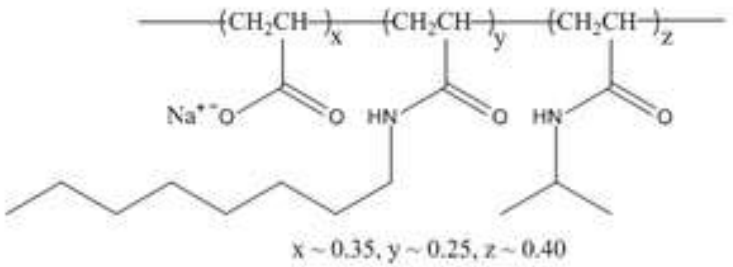



http://weelookang.blogspot.com/2010/06/ejs-open-source-brownian-motion-gas.html

# REAGENTS FOR IMPROVING VITRIFICATION OF CRYO-EM GRIDS USED IN SINGLE PARTICLE ANALYSIS.




Molecular Formula:
(C6.2H10.3O1.35N0.65Na0.35)35

Molecular Weight:
approx. 8 kDa

CAS#: 1423685-21-5

Amphipol A8-35

A short amphipathic polymer that is specifically designed for membrane protein stabilization. The surfactant possesses a very high affinity for the transmembrane surfaces and allows to solubilize membrane proteins in a detergent-free aqueous solution

# REAGENTS FOR IMPROVING VITRIFICATION OF CRYO-EM GRIDS USED IN SINGLE PARTICLE ANALYSIS.

| Surfactants and Cryoprotectants | Amount | Conc. | CMC | Class |
|---|---|---|---|---|
| Fluorinated Octyl Maltoside (FOM) | 100 µl | 0.41% (w/v) | 0.07% (w/v) | non-ionic detergent |
| Hexadecyl-trimethyl-ammonium Bromide (CTAB) | 100 µl | 0.34% (w/v) | 0.03% (w/v) | cationic detergent |
| n-Decyl-ß-D-Maltoside (DM) | 100 µl | 0.87% (w/v) | 0.09% (w/v) | non-ionic detergent |
| n-Decyl-α-D-Maltoside (DαM) | 100 µl | 0.46% (w/v) | 0.08% (w/v) | non-ionic detergent |
| n-Dodecyl-ß-D-Maltoside (DDM) | 100 µl | 0.09% (w/v) | 0.01% (w/v) | non-ionic detergent |
| Sodium Deoxycholate | 100 µl | 1.66% (w/v) | 0.17% (w/v) | anionic detergent |
| Triton X-100 | 100 µl | 0.15% (w/v) | 0.01% (w/v) | non-ionic detergent |
| Tween 20 | 100 µl | 1% (w/v) | 0.01% (w/v) | non-ionic detergent |
| CHAPSO | 100 µl | 2.5% (w/v) | 0.5% (w/v) | zwitterionic detergent |
| Amphipol A8-35 | 100 µl | 5% (w/v) | | anionic surfactant |
| Glycerol | 1 ml | 30% (w/v) | | cryoprotectant |

https://www.mitegen.com/product/cryo-em-vitrification-starter-kit/

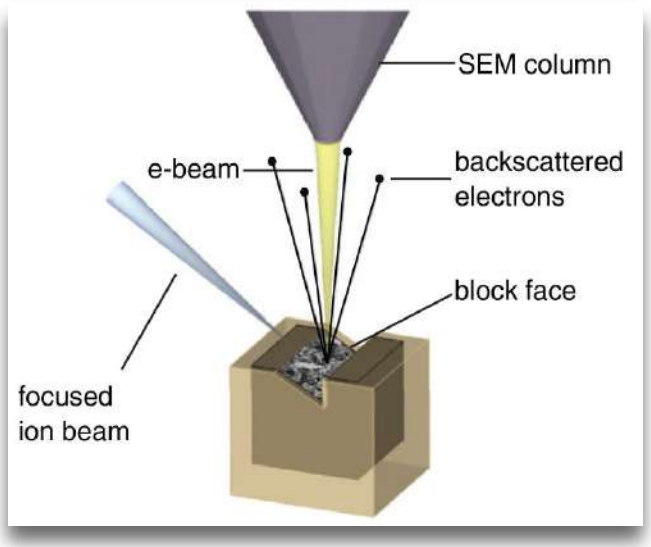

[1] Noble *et al.* (2018) Routine Single Particle CryoEM Sample and Grid Characterization by Tomography. DOI: 10.7554/eLife.34257.
[2] Thonghin *et al.* (2018) Cryo-electron microscopy of membrane proteins. *Methods* **147**:176.
[3] Drulyte *et al.* (2018) Approaches to altering particle distributions in cryo-electron microscopy sample preparation. *Acta Cryst. D* **74**:560.
[4] Glaeser *et al.* (2017) Opinion: hazards faced by macromolecules when confined to thin aqueous films. *Biophys Rep* **3**:1.
[5] Gatsogiannis *et al.* (2016). Membrane insertion of a Tc toxin in near-atomic detail. *Nat. Struct. Mol. Biol.* **23**:884.
[6] Efremov *et al.* (2015) Architecture and conformational switch mechanism of the ryanodine receptor. *Nature* **517**:39.

# FIB/SEM VS THIN SECTION SAMPLE PREP

- Chemical fixation
- Staining
  En bloc, enhanced contrast and electrical conductivity
- Dehydration
- Embedding

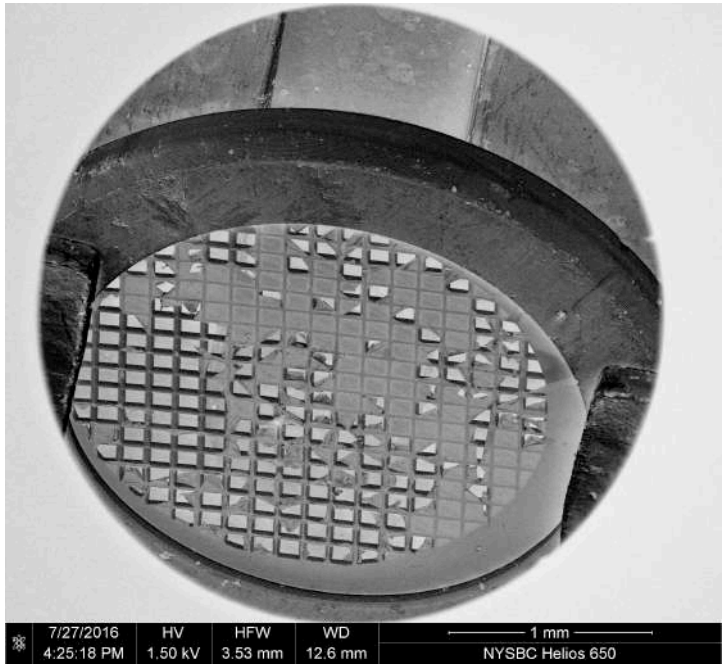
- Au/Pd coat
  Conductivity

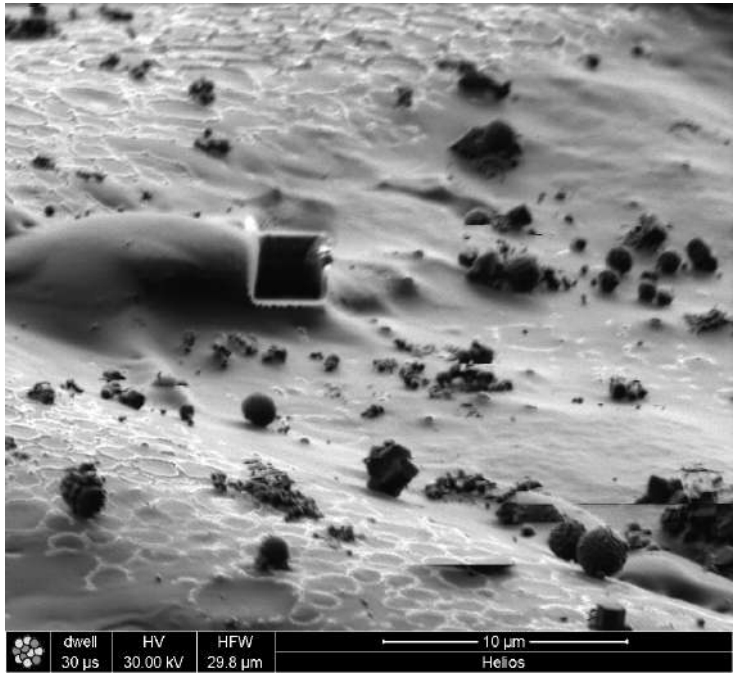
Cryofixation: High pressure freezing
Dehydration: Freeze substitution



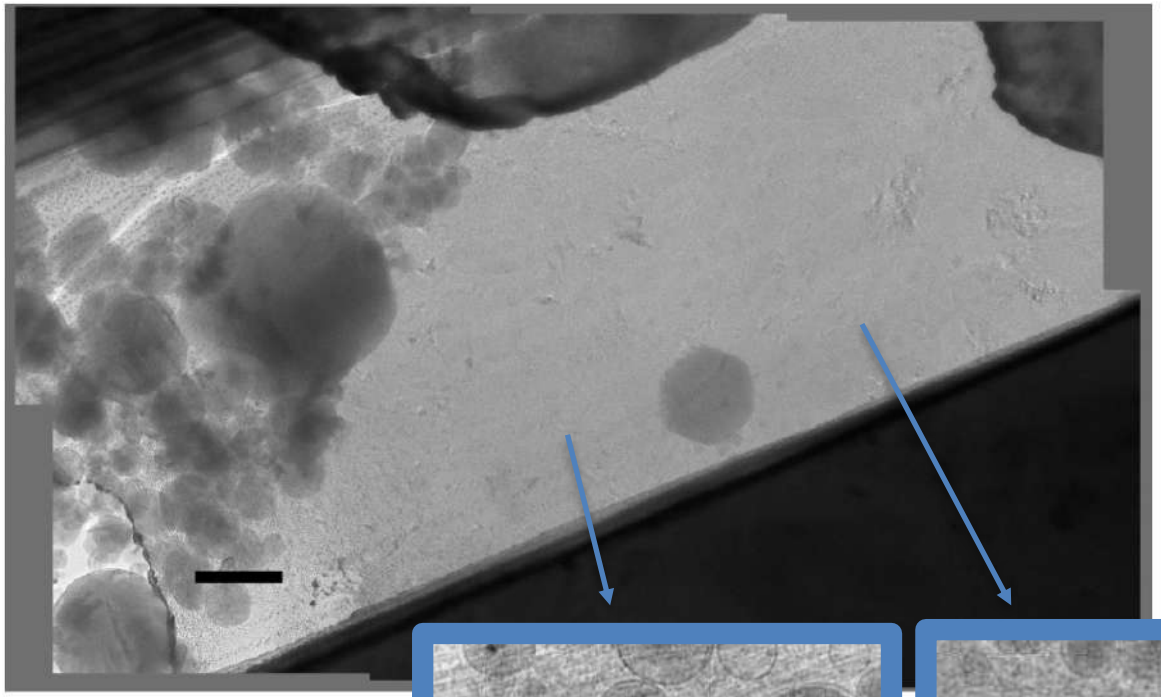

- Chemical fixation
- Dehydration
- Embedding
- Sectioning
- Staining

# CRYO FIB MILLING

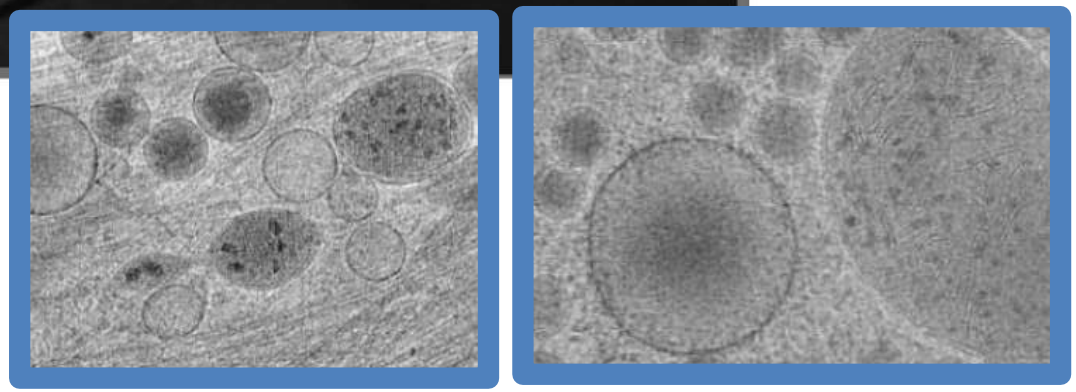


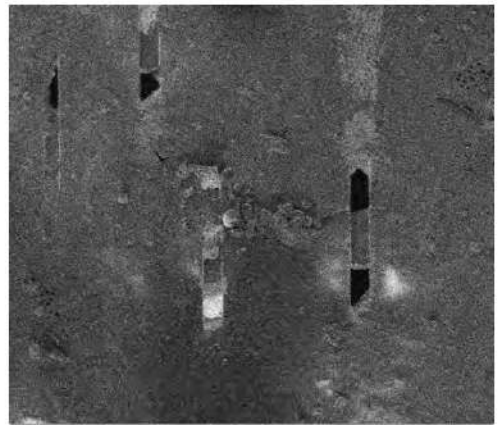



# CRYO FIB MILLING

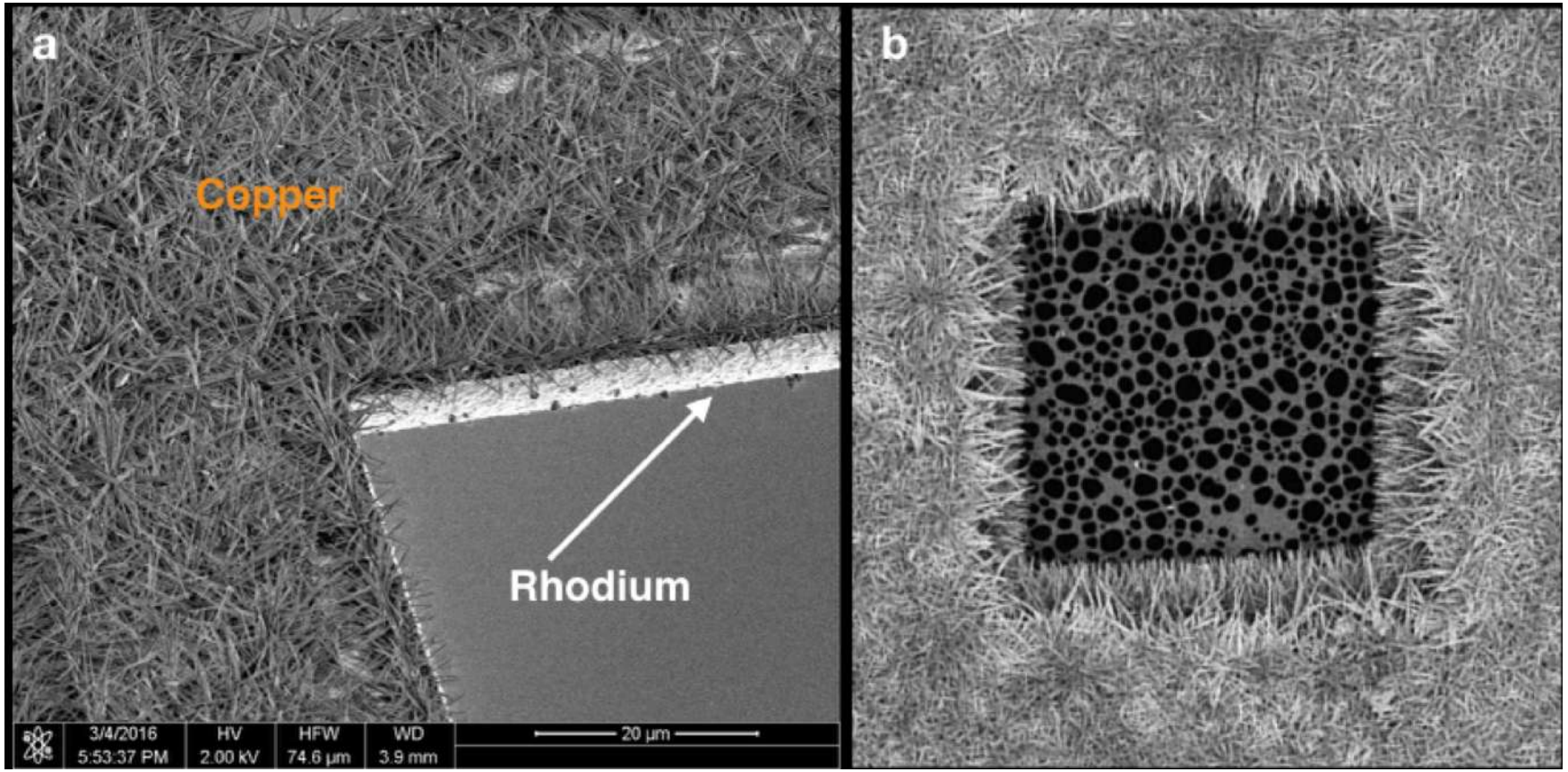


Scale bar: 1 µm





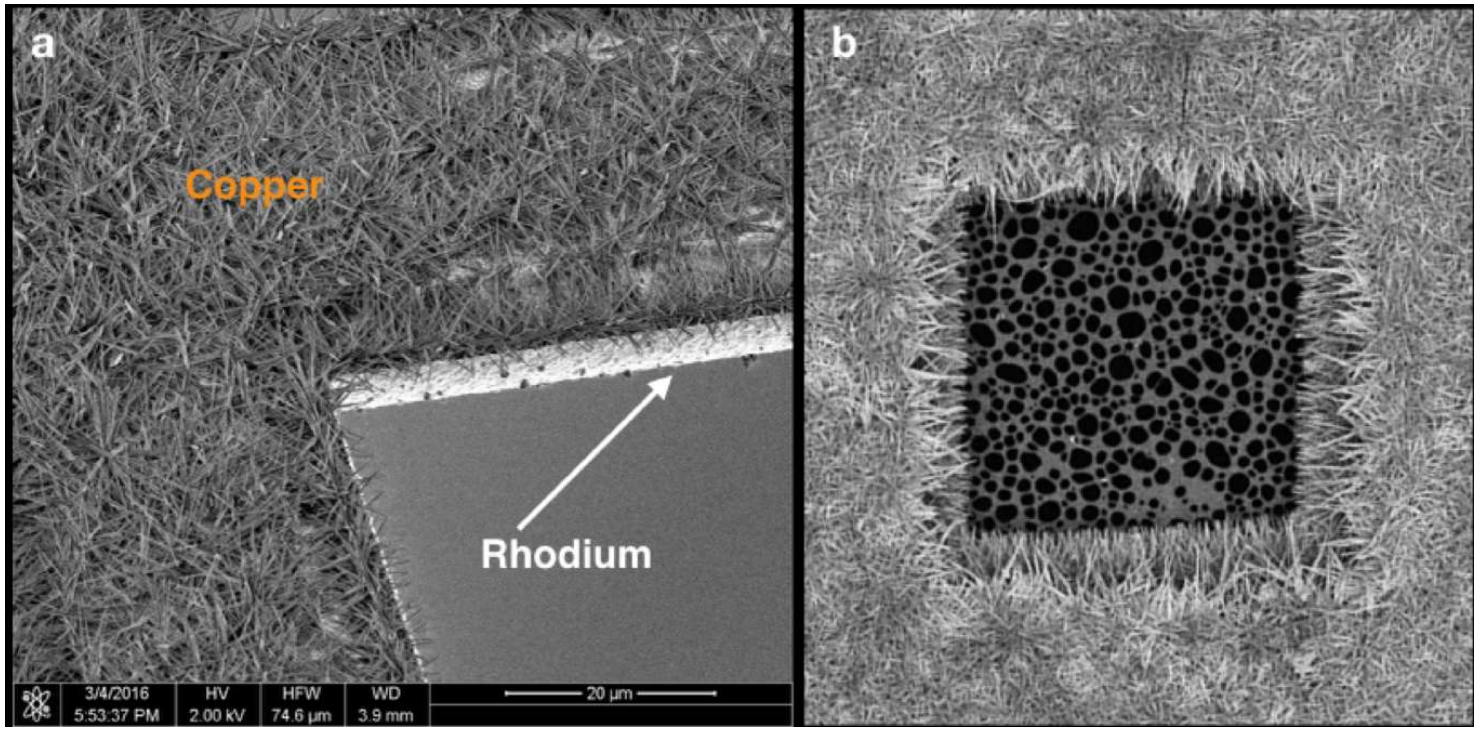
# BLOT FREE VITRIFICATION



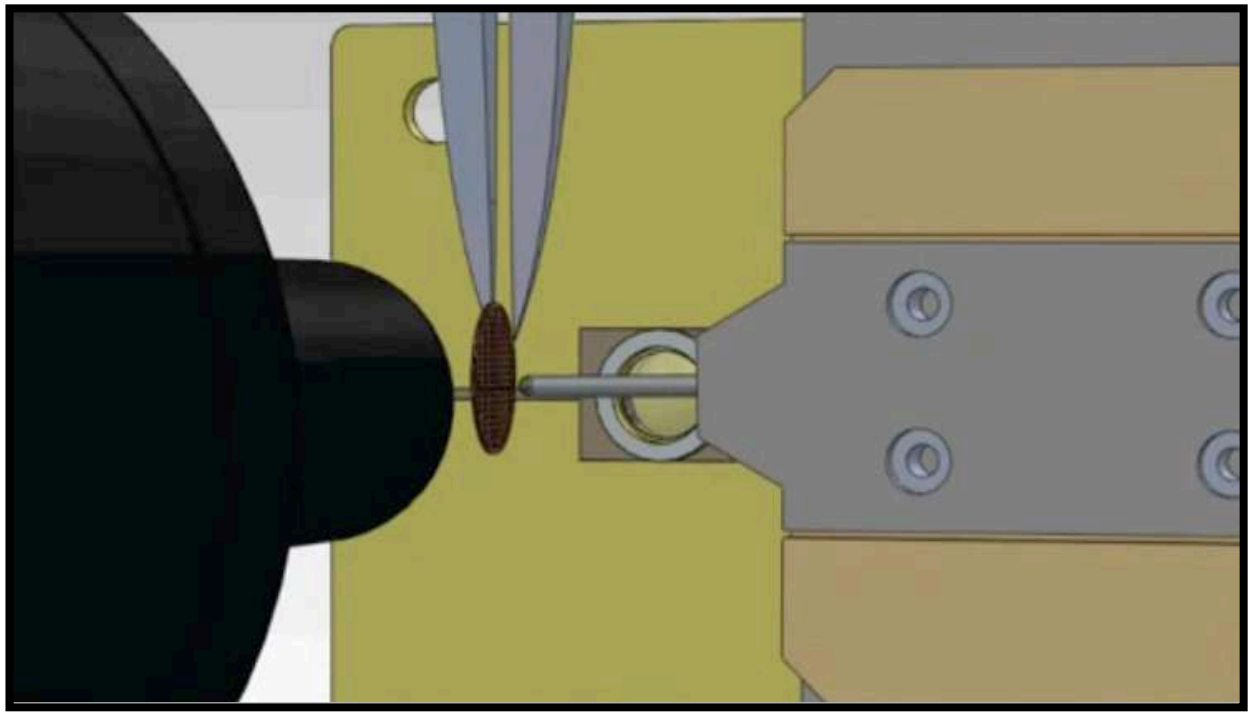

nramm.nysbc.org

# SPOTITON | CHAMELEON

Spotiton



# SPOTITON | CHAMELEON

# WHAT NEXT?

## cryoEM 001 : Single Particle Masterclass

1. Building a cryoEM toolkit
2. EM compatible samples
3. EM support films and grids
4. Sample preparation
5. Tools of the trade: microscopes and detectors
6. Microscope operations
7. Data collection strategies
8. Data assessment & QC
9. Data processing:
   - cryoEM IT infrastructure
   - On-the-fly feedback
   - 3D Reconstruction
10. Visualization and validation

# DIFFICULT SPECIMENS

**Small protein**

- VPP
- Thinner ice

**Protein denaturation/Dissociation of protein complex**

**Continuous carbon film**

**Graphene oxide**

**Cross-linking (GraFix)**

**Preferred orientation**

**Tilt stage**

**Cross-linking**

**Detergent**

**Glow-discharging conditions**

**Support film (Graphene oxide)**

**Image analysis (3D classification)**

**Flexibility**

**Focused classification (subtraction)**

**Multibody refinement**

**Filamentous protein**

- Segmented analysis

**VI. Low concentration**

Multiple blots

Affinity grids