2023 Winter-Spring EM Course

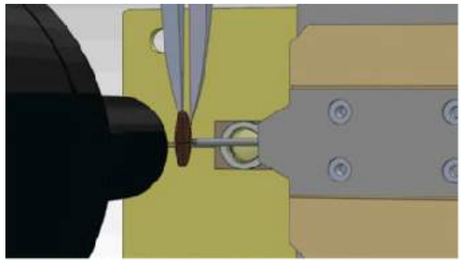
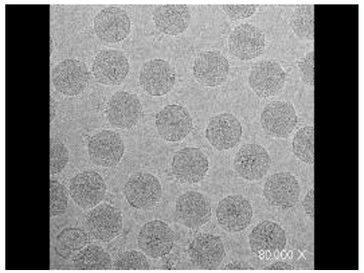
Sample preparation for cryoEM

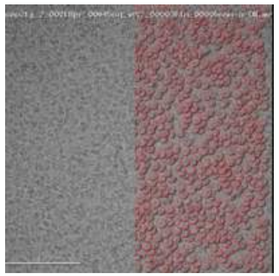
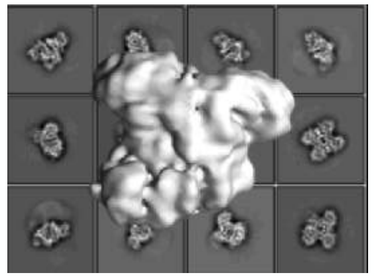
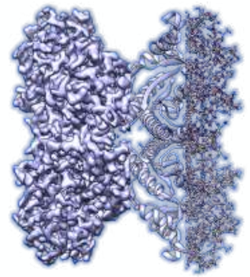
JANUARY 25, 2023

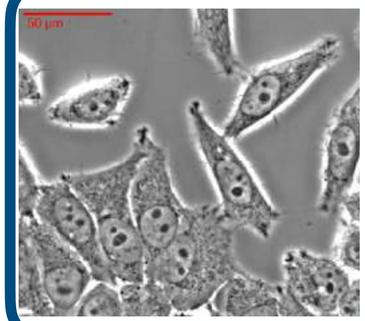
EDWARD T ENG

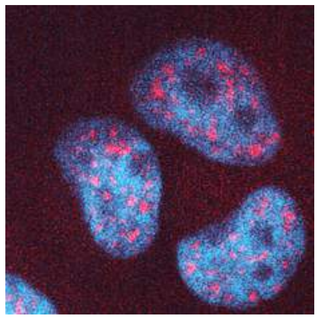
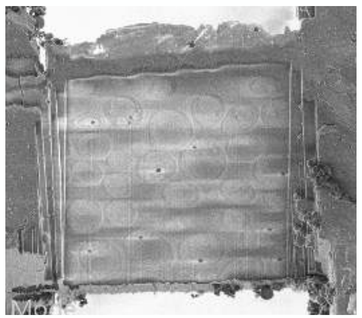
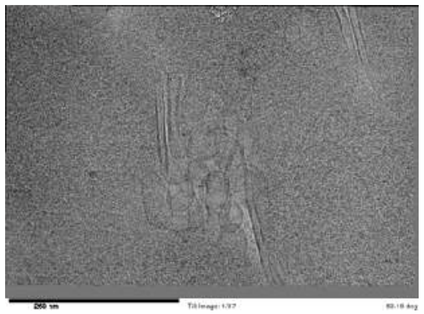
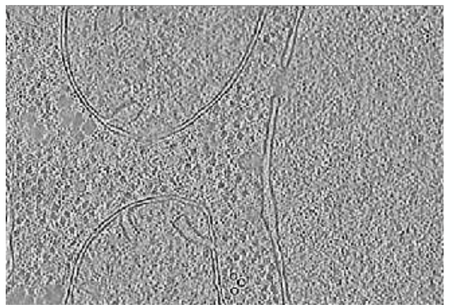
CRYOEM: TECHNOLOGY ON THE RISE

Single particle cryoEM

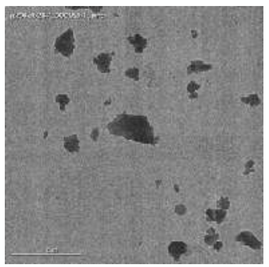
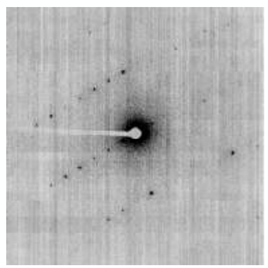
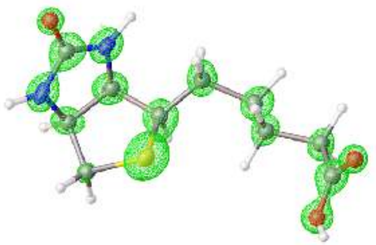
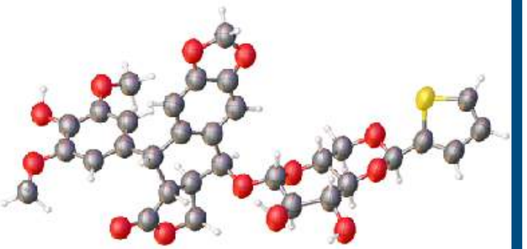






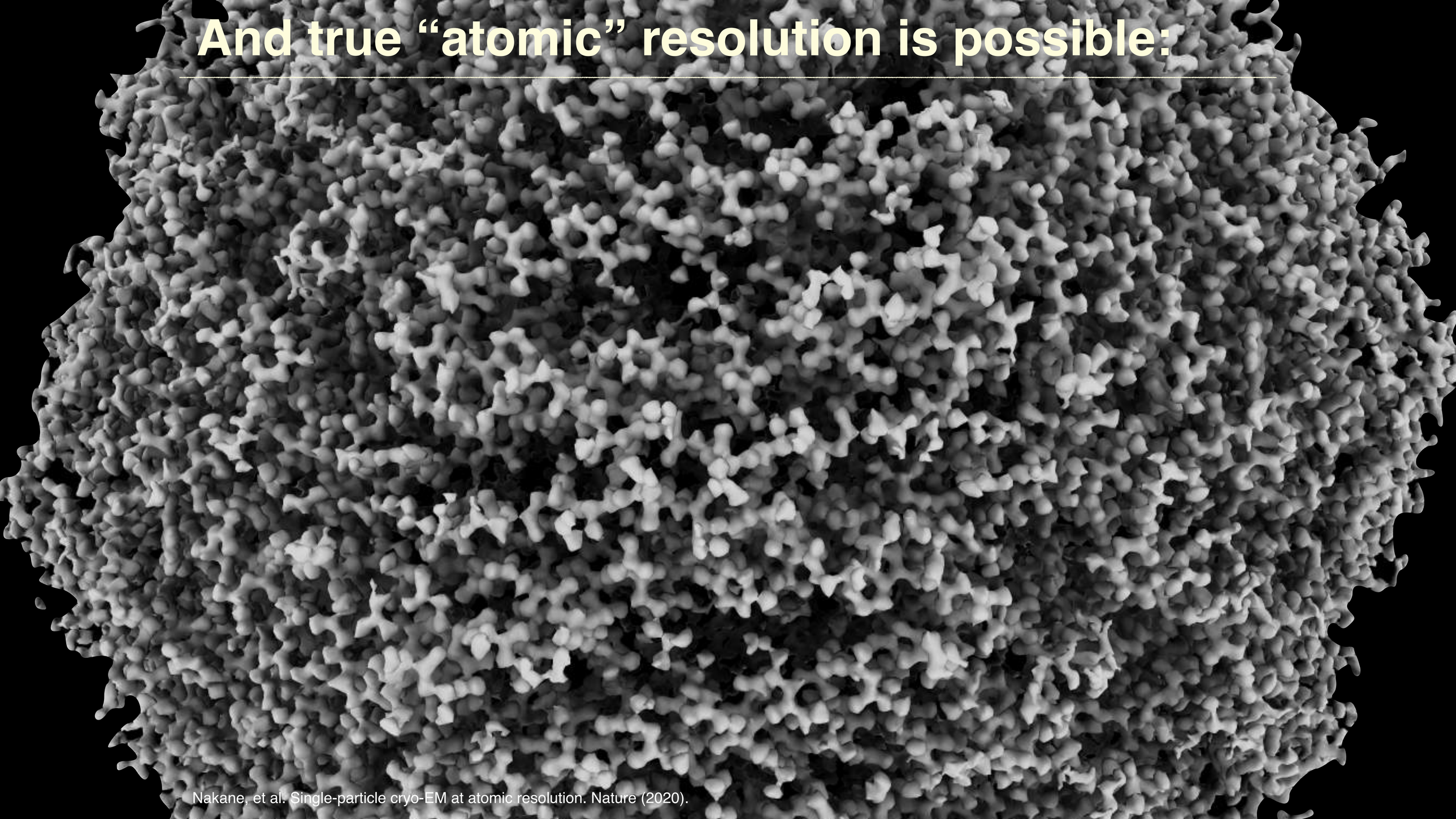





Cryo Electron Tomography (cryoET)

Micro crystal electron diffraction (microED)

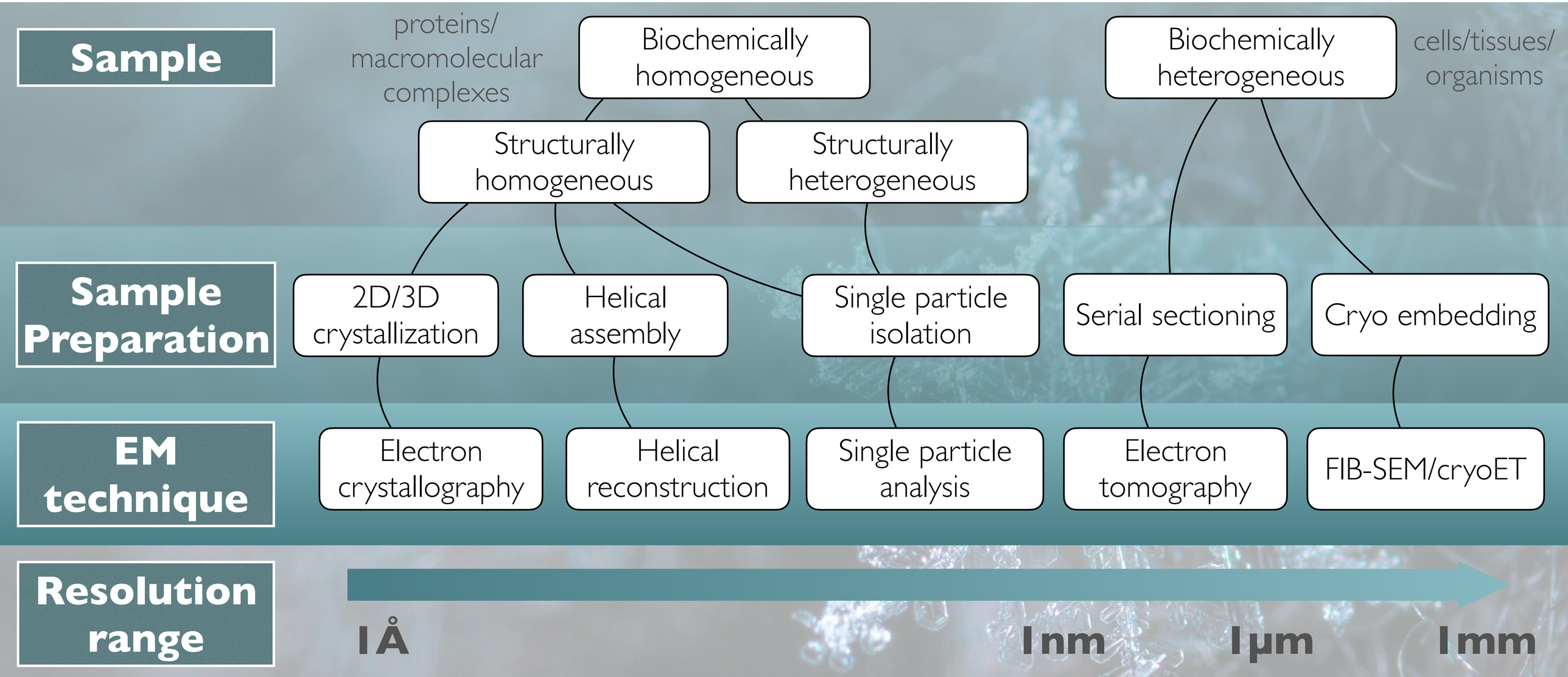





And true “atomic” resolution is possible:



Nakane, et al. Single-particle cryo-EM at atomic resolution. Nature (2020).

HOW ARE SAMPLES PREPARED?



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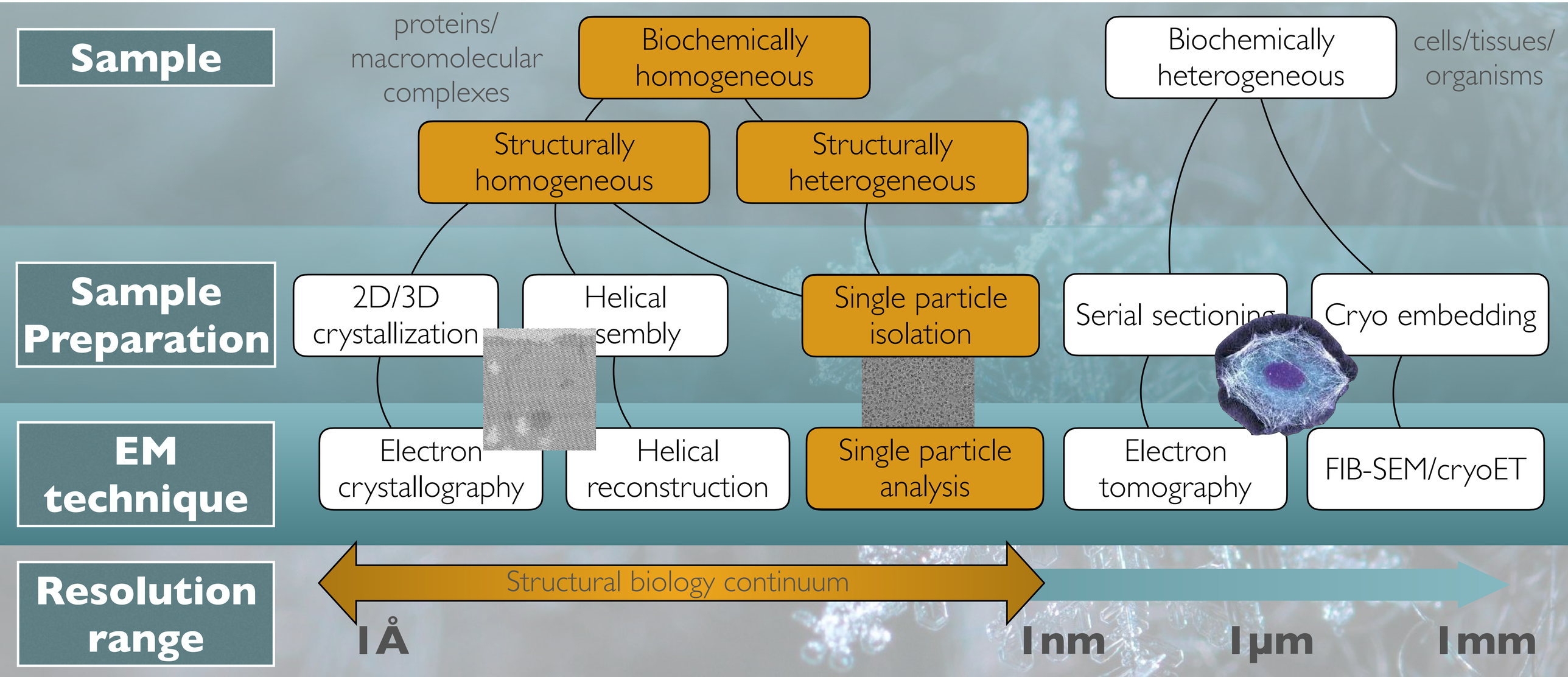
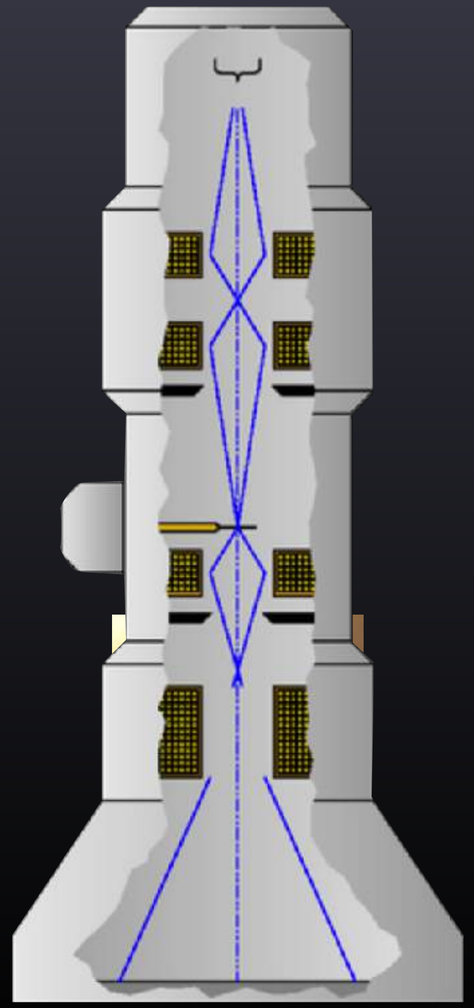
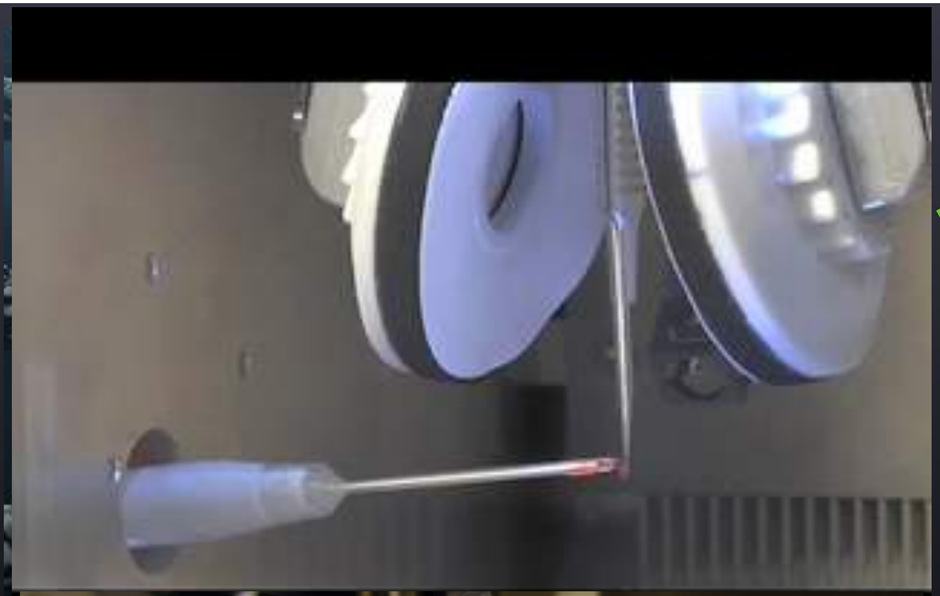


HOW ARE SAMPLES PREPARED?

Vitrifying a biological sample



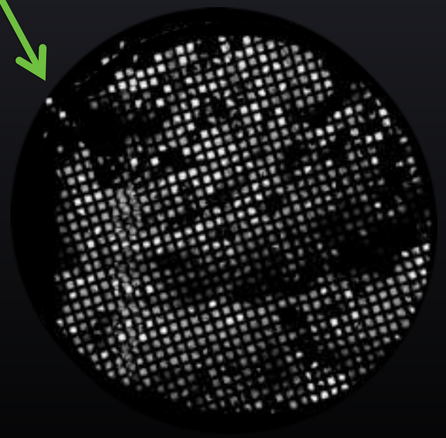


~3μl

<3nl

>99.999%

<0.001%



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

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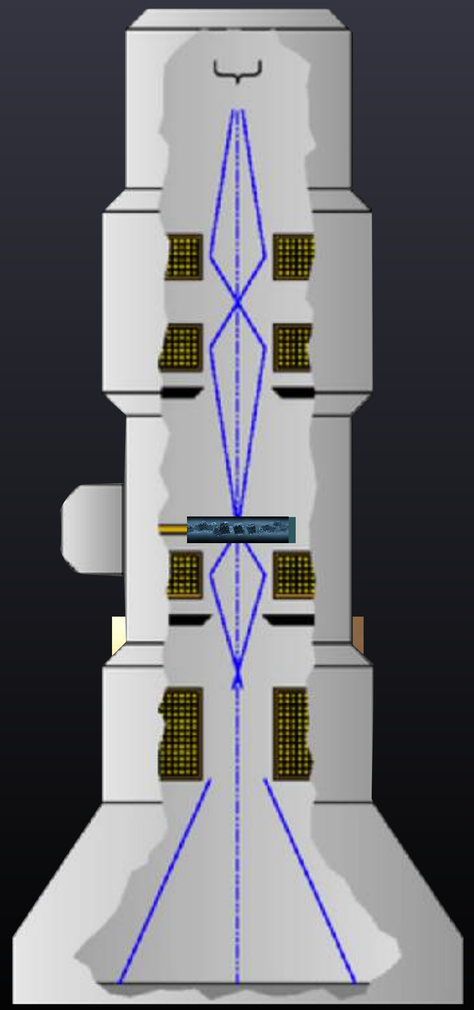

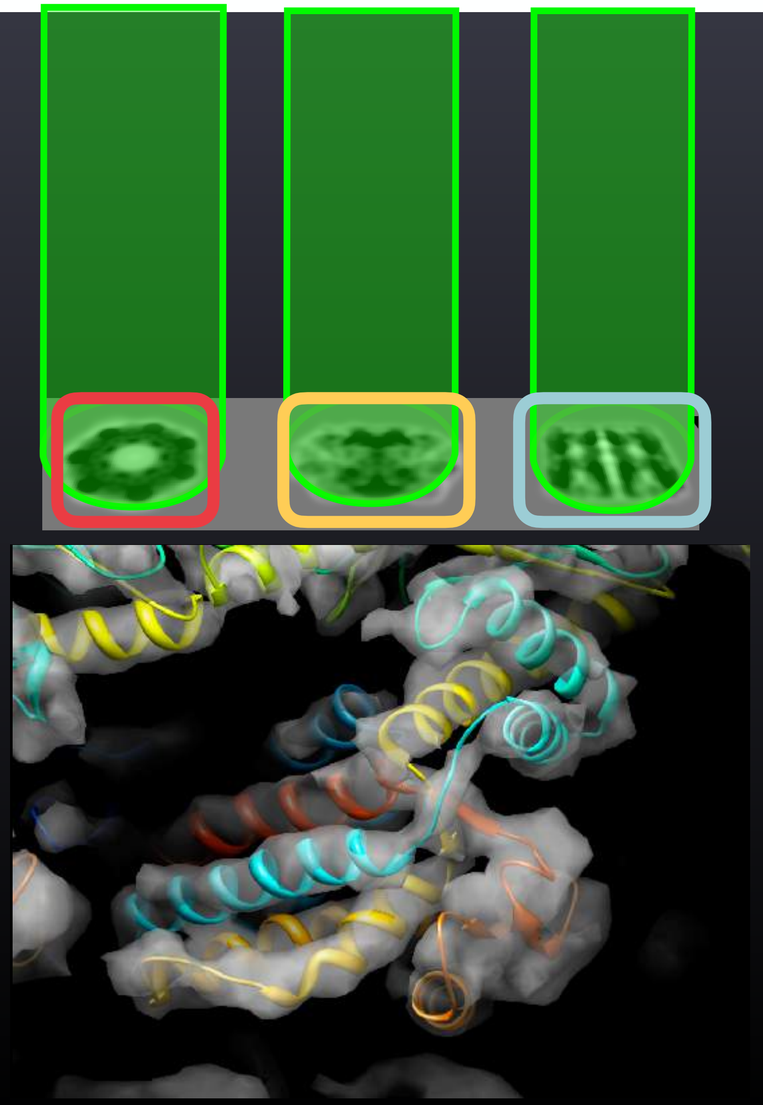

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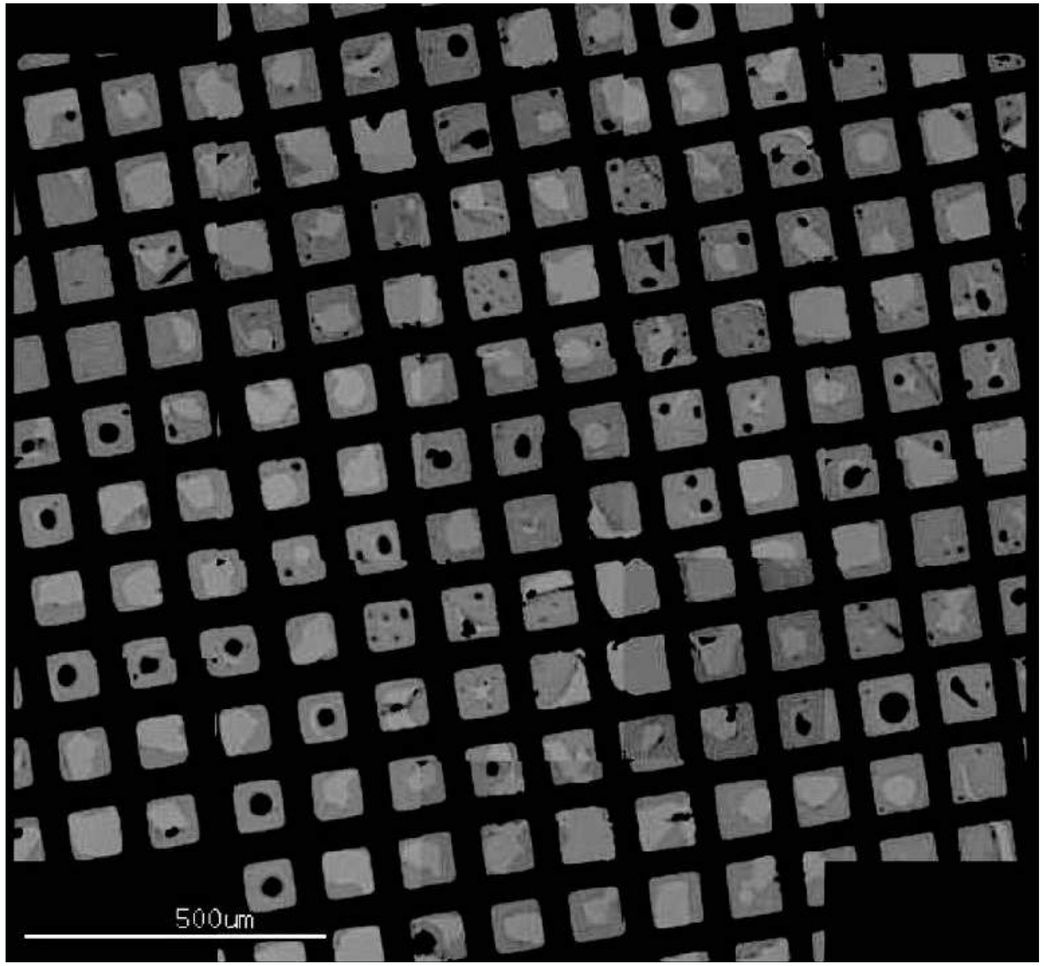
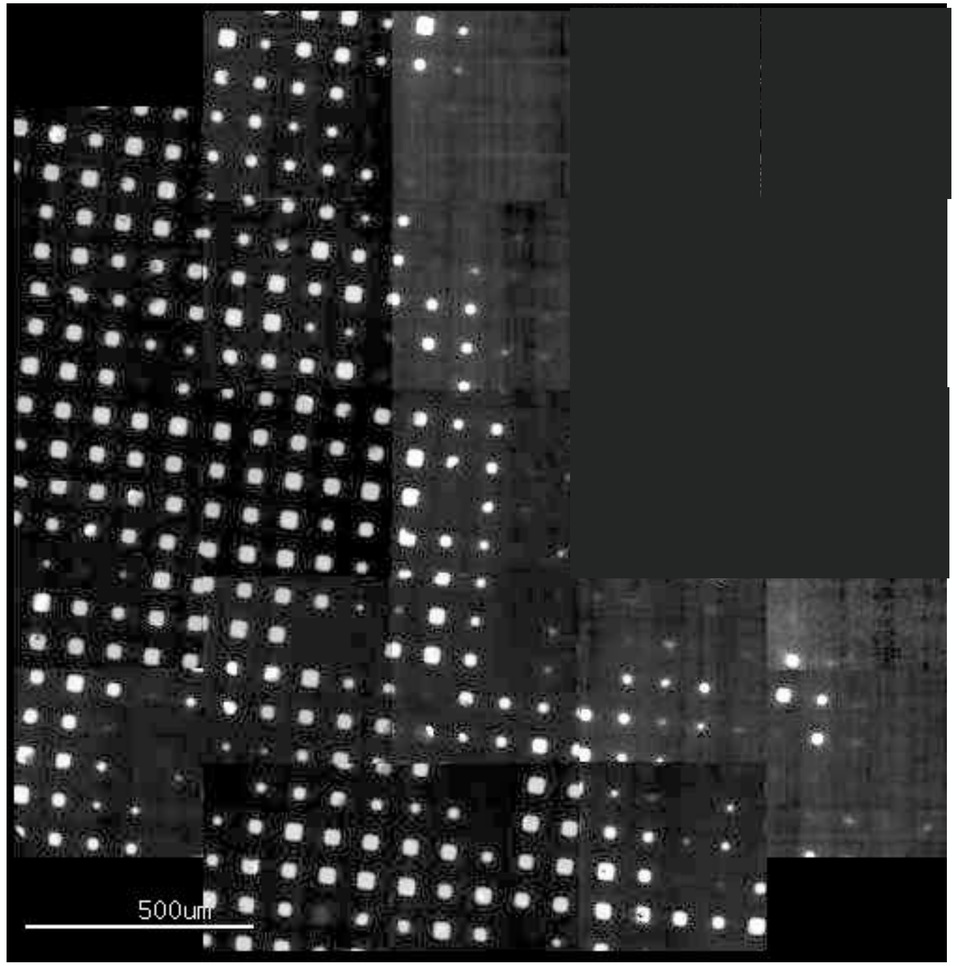
WHAT DO GRIDS LOOK LIKE?





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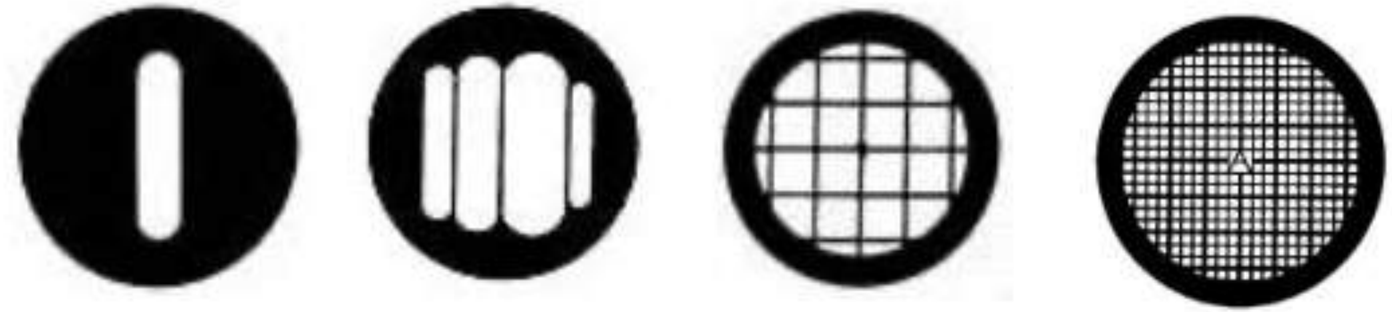
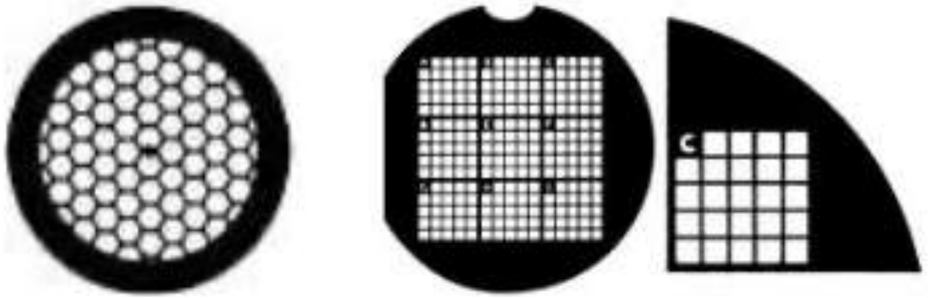
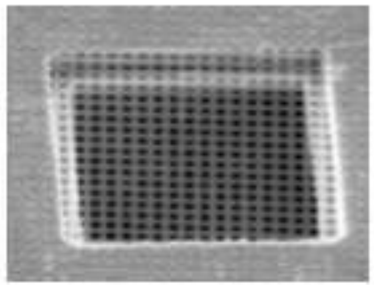
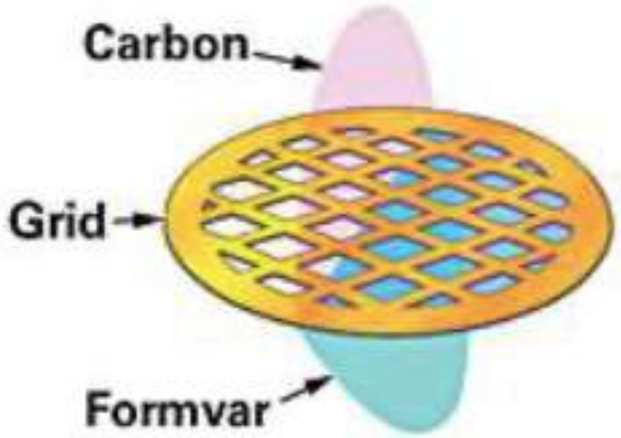








Common Materials

Copper

Nickel

Gold

Aluminum

Molybdenum

Titanium

Stainless Steel

https://www.tedpella.com/grids_html/

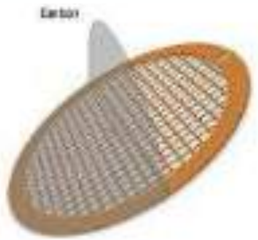
TERMINOLOGY

Grid (Cu, Au, Mo, etc…)

- mesh

Foil (C, Au, etc…)

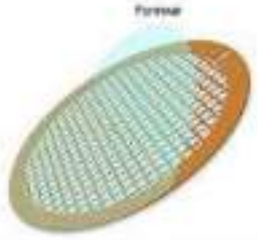
- Continuous
- lacy
- holey (hole size and spacing)



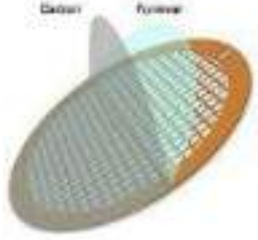
CARBON ONLY SUPPORT FILMS

HOLEY CARBON SUPPORT FILMS

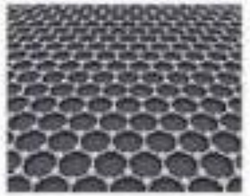
LACEY CARBON SUPPORT FILMS



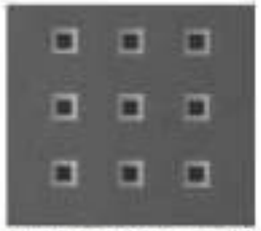
FORMVAR ONLY SUPPORT FILMS



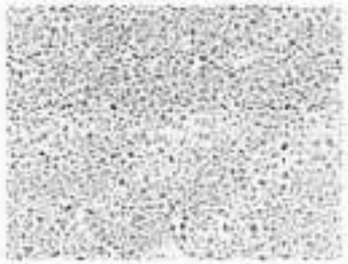
FORMVAR / CARBON SUPPORT FILMS



EM-TEC GRAPHENE SUPPORT FILMS



EM-TEC SILICON NITRIDE SUPPORT FILMS

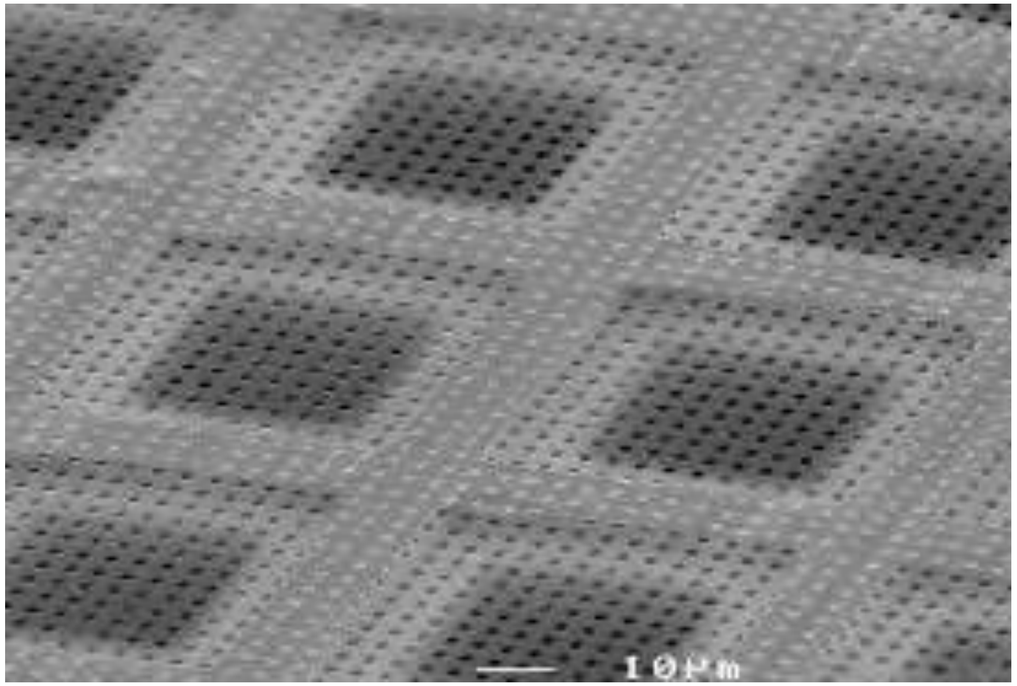


TEM CALIBRATION & TEST STANDARDS

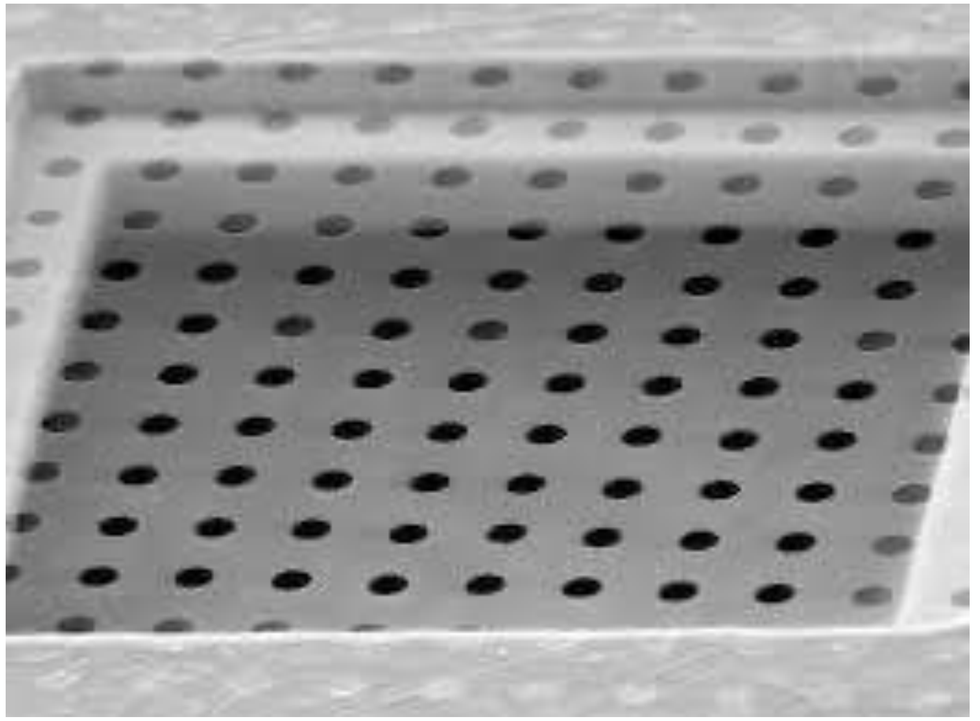
TEM GRID STORAGE BOXES

https://edgescientific.com/product-category/tem-supplies/tem-support-films/

WHAT DOES A GRID LOOK LIKE?

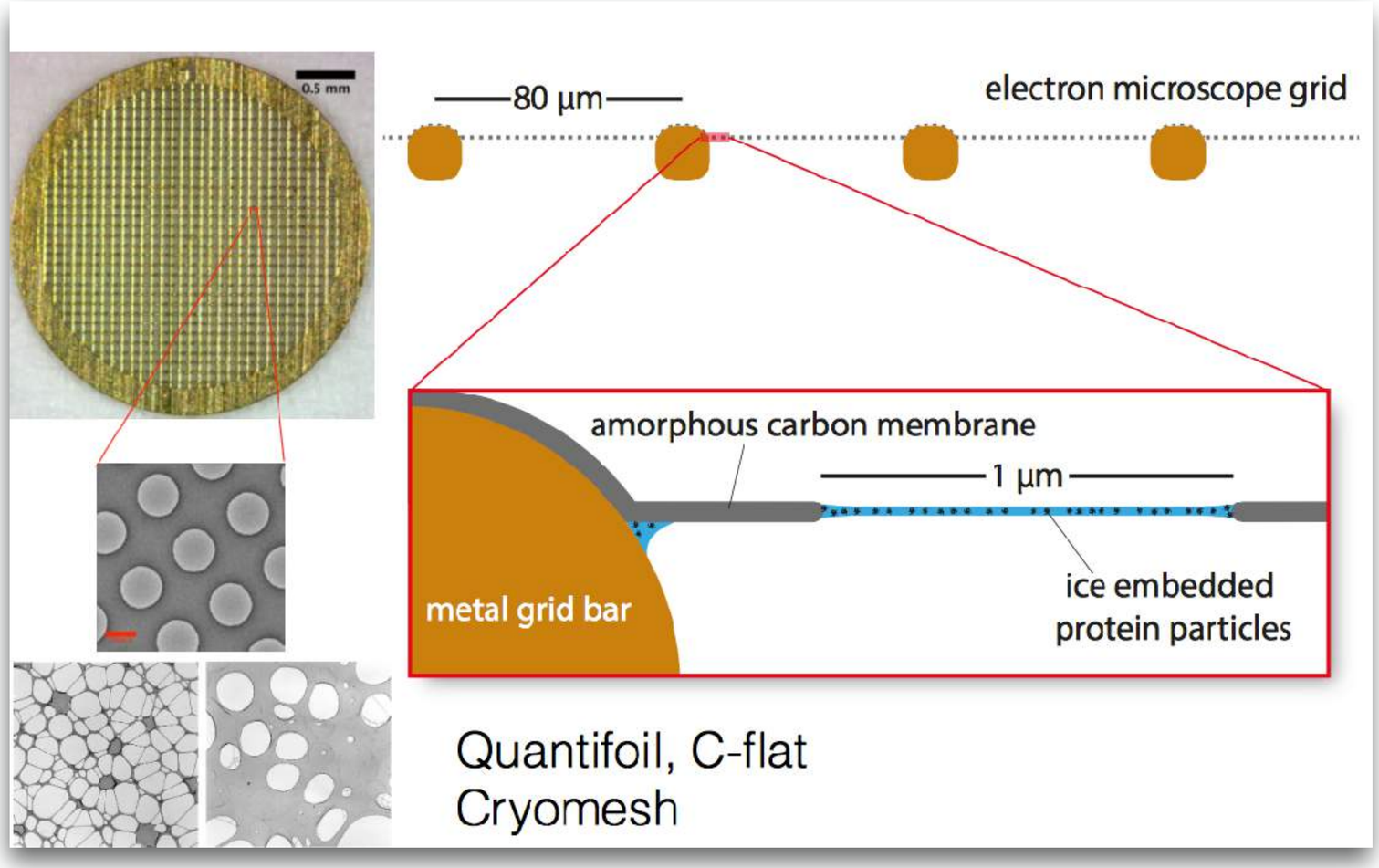


- Protochips.com



Quantifoil.com

TERMINOLOGY



Russo & Passmore, 2015

GOLD GRIDS

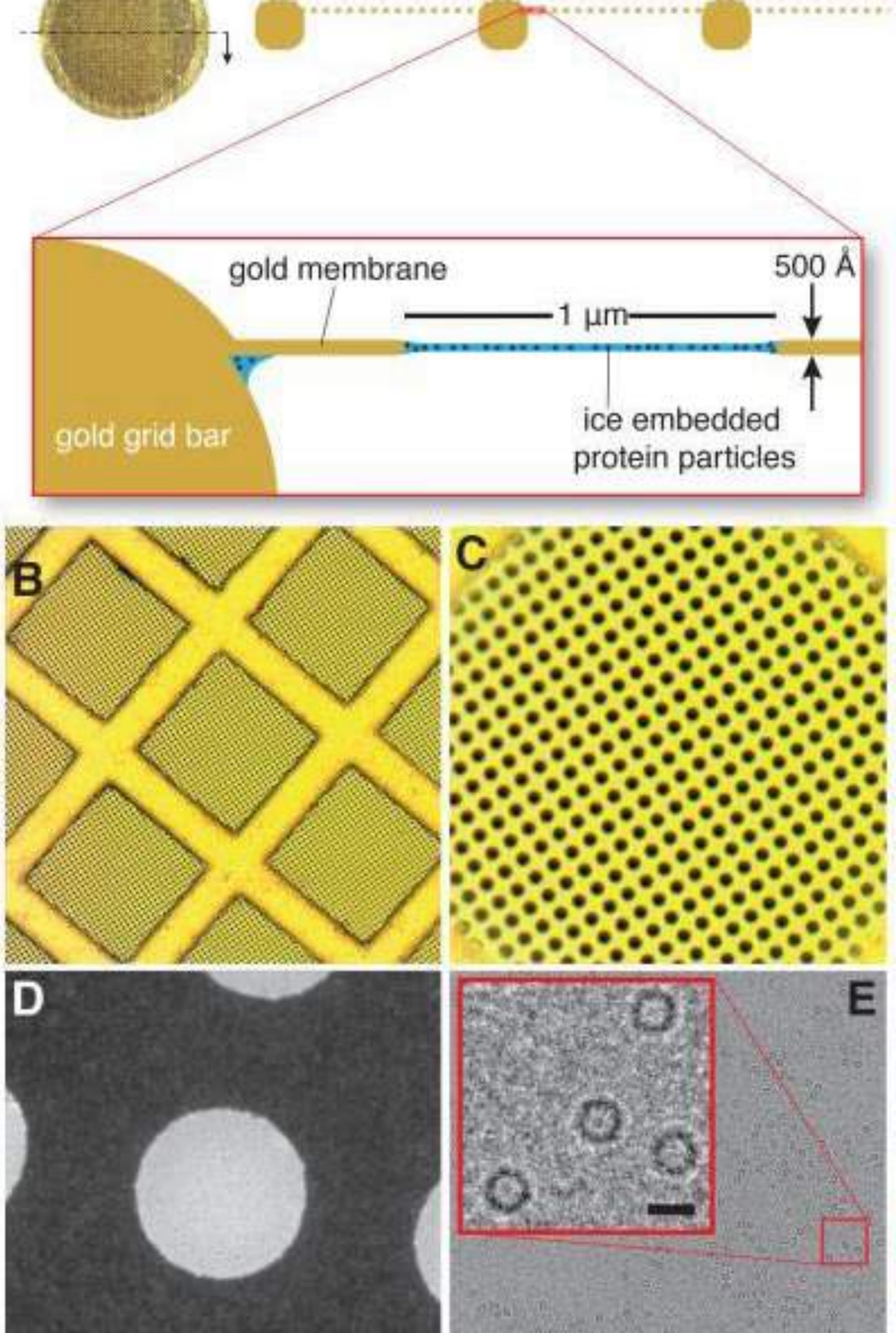
- Holey gold foil on gold mesh grid

Advantages:

- Prevents differential thermal contraction when freezing
- Reduces beam-induced specimen movement
- Combined with direct detector technology allows for near atomic resolution

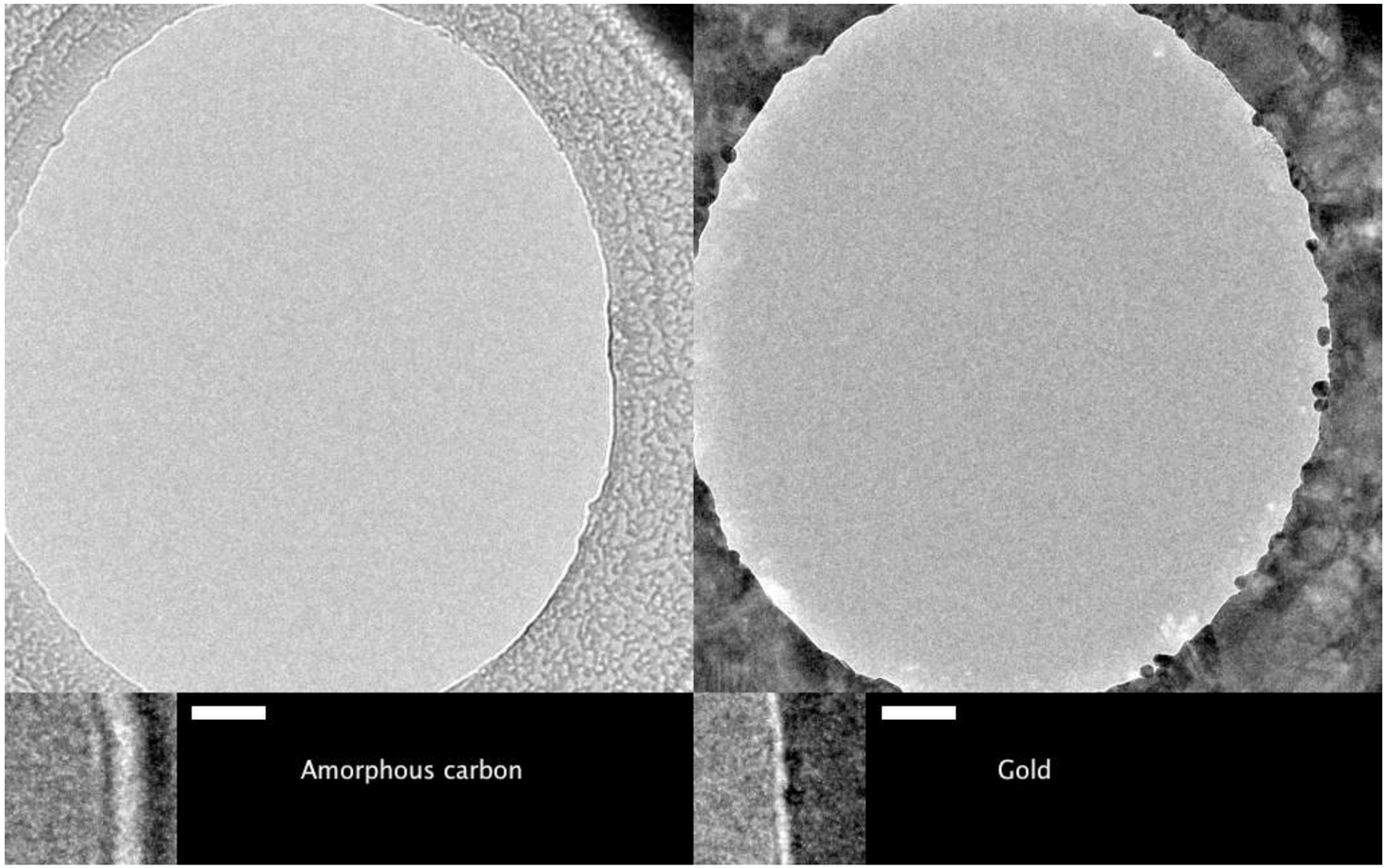
Disadvantages:

- Difficult to find focus due to lack of amorphous substrate



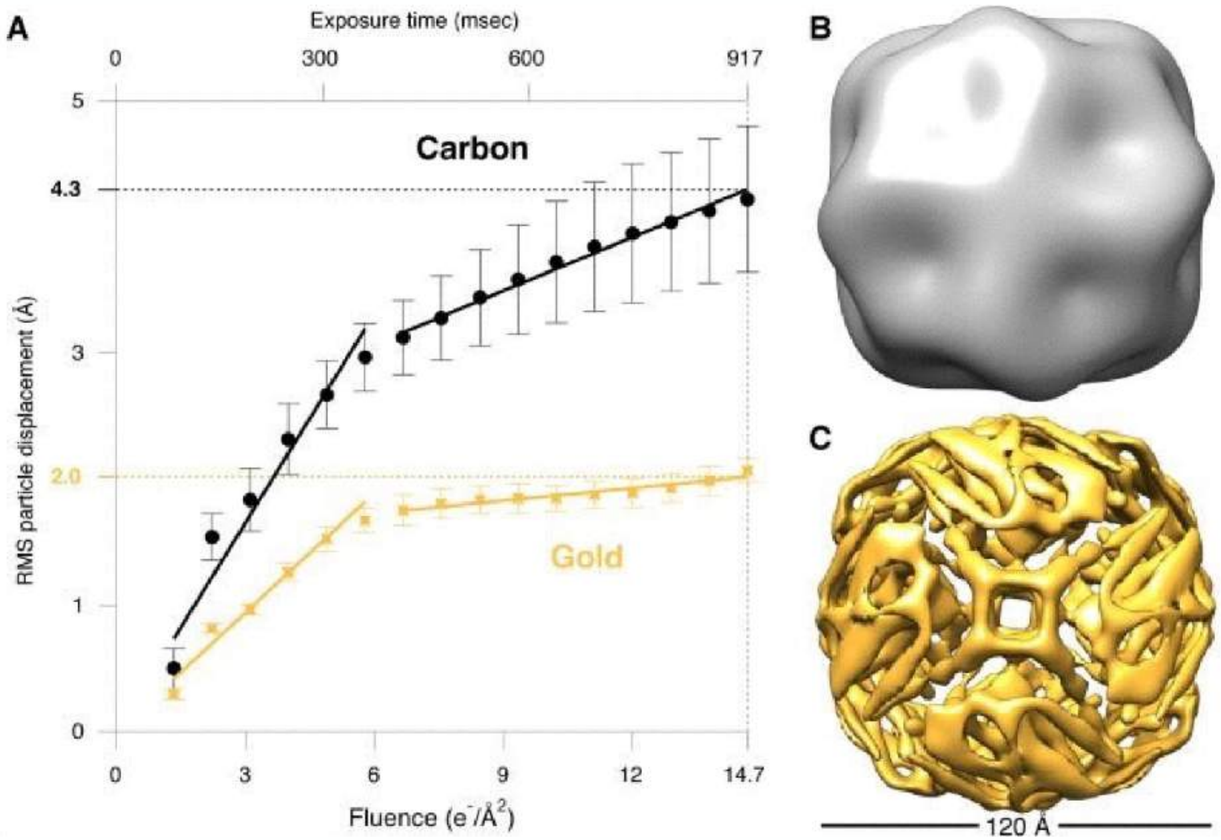
Russo & Passmore, 2015

GOLD GRIDS



Russo & Passmore, 2015

GOLD GRIDS - how much better are they?



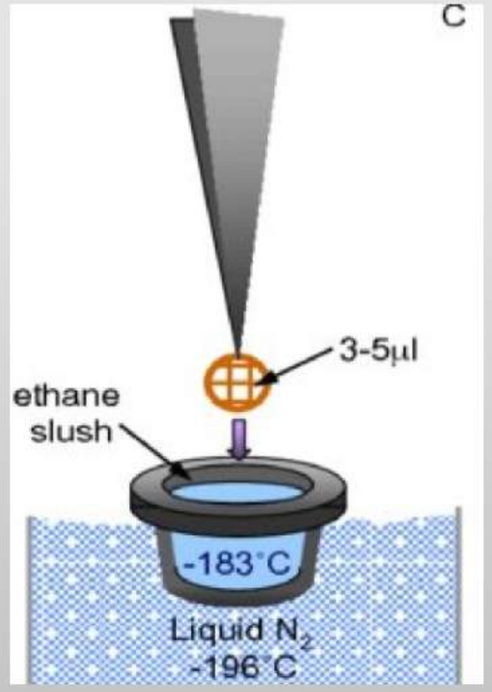
A. 80S ribosome movement during irradiation supported by amorphous carbon and gold using same imaging conditions.

Apoferritin density maps using same imaging conditions and identical processing for **B.** carbon and **C.** gold substrates. **B.** is at 25 Å and **C.** 8 Å resolution.

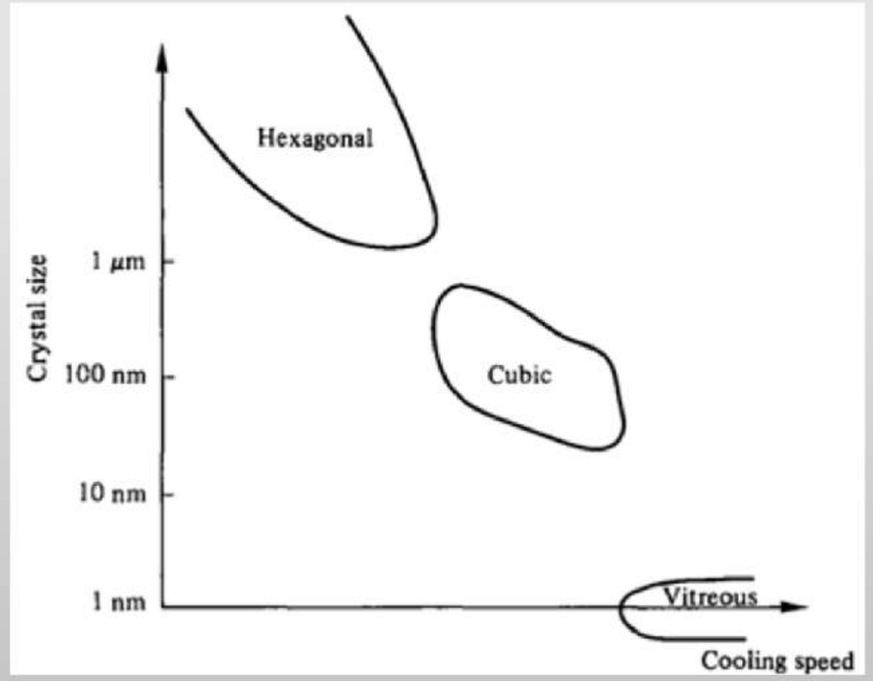
Russo & Passmore, 2015

VITRIFICATION PROCESS

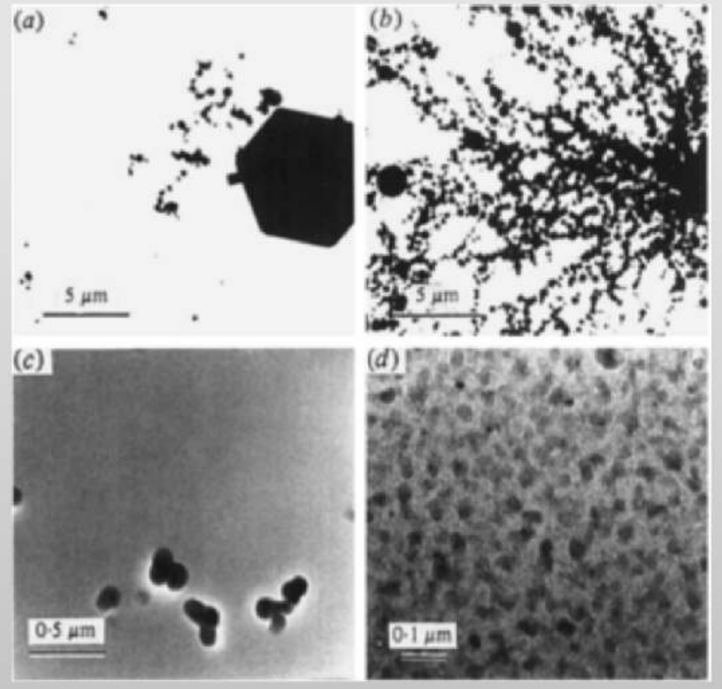
- Liquid ethane is a suitable coolant.
- Liquid nitrogen boils on contact, which makes it a poor coolant for cryo-EM.
- Cooling speed faster than 10^5-10^6 K/s ensure the formation of vitrified ice.



Setup of liquid ethane
(Image from Wen Jiang)



Cooling speed &
forms of ice

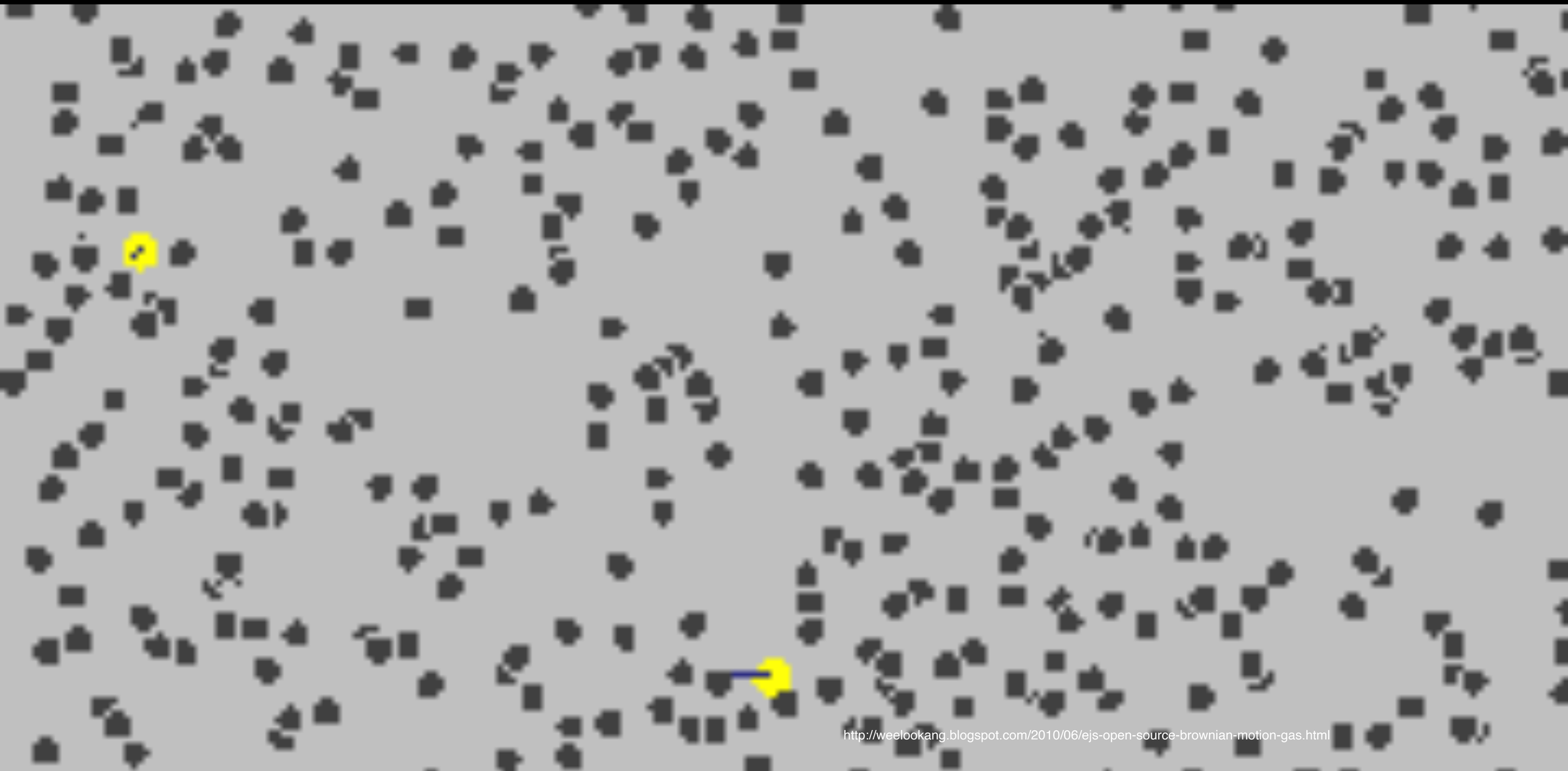


Different forms of ice contamination

Jacques Dubochet et al., 1988

VITRIFICATION PROCESS

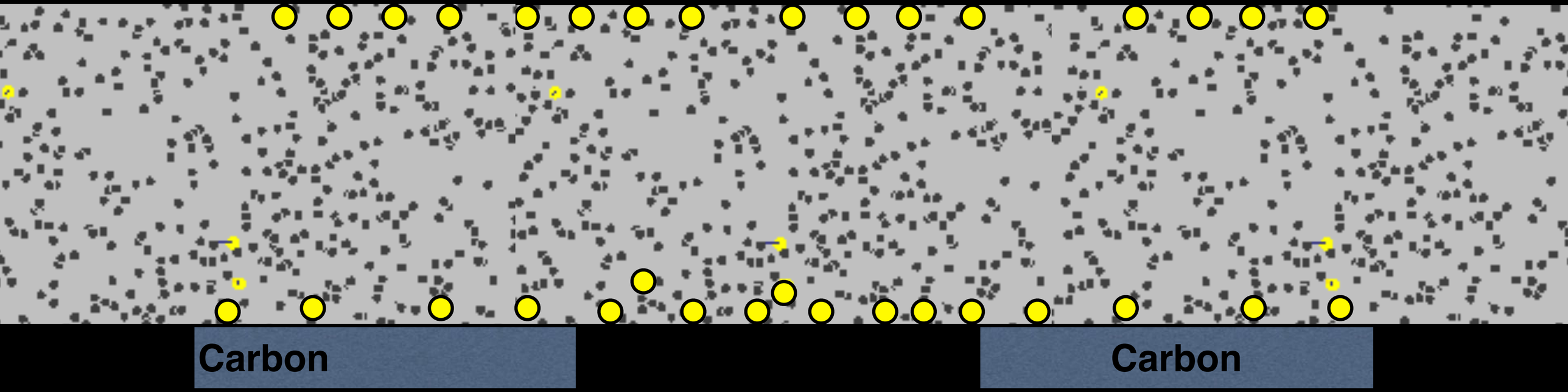
What happens to samples during vitrification?



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

What happens to samples during vitrification?

A hypothetical scenario during cryoEM grid preparation



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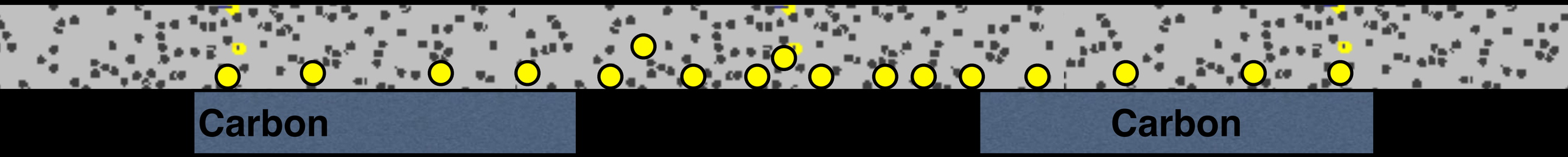

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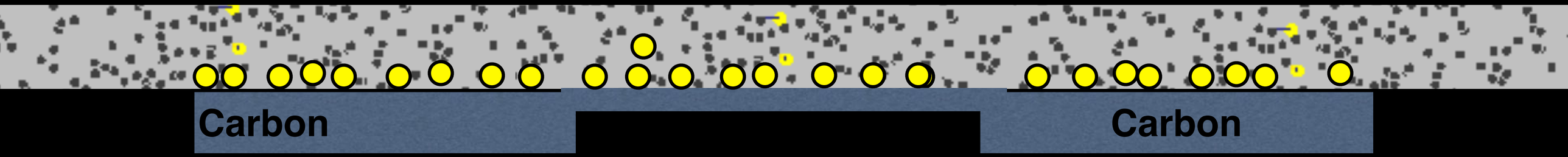

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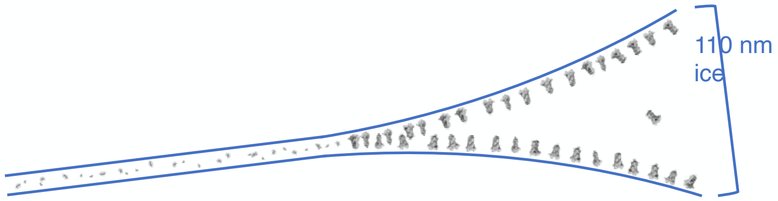
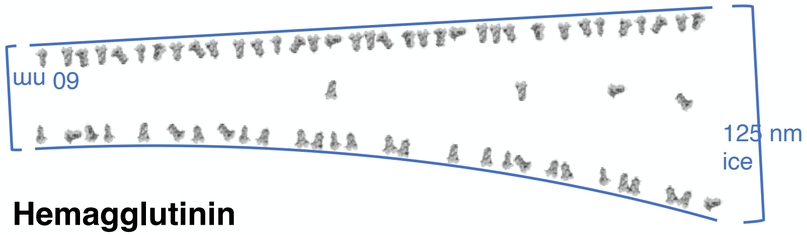
What issues arise?



Hemagglutinin



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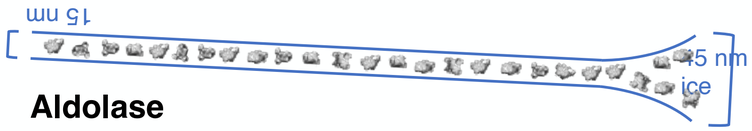
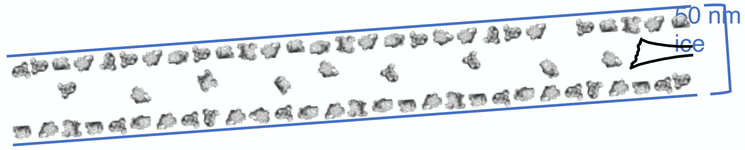
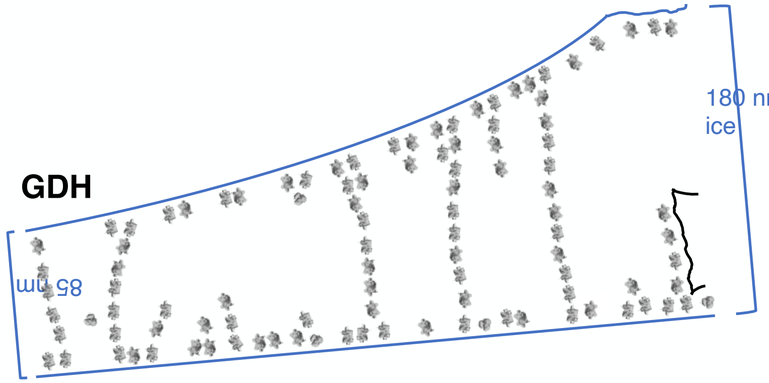


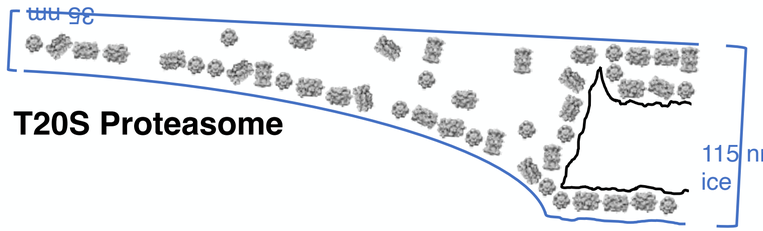
Aldolase



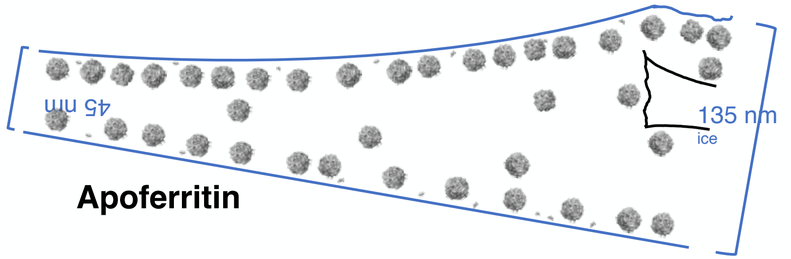
Aldolase



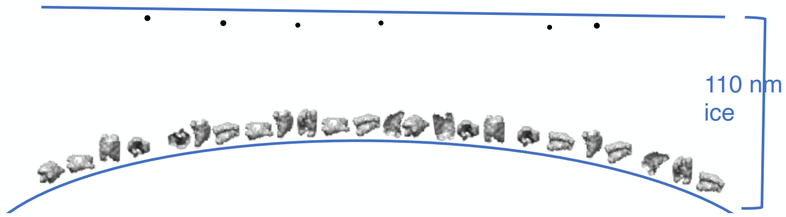
GDH



T20S Proteasome



Apoferritin



DNAB Helices

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

Alex Noble

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

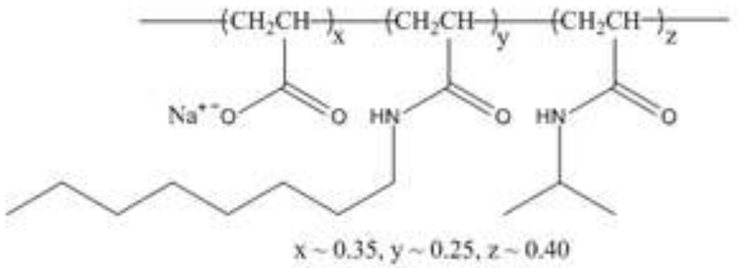
Filamentous protein

- Segmented analysis

Low concentration

- Multiple blots
- Affinity grids

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.

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

PDB Release Date	PDB	Protein	Additive
2020-01-08	6PWN	MscS mechanosensitive channel	0.01% f-OM
2019-09-04	6KG7	Piezo2 mechanosensitive channel	0.65 mM f-FC8
2019-08-28	6QTI	Nicotinamide nucleotide proton channel	0.05% CHAPS
2019-08-07	6R7L	SecYEG translocon	0.2% f-OM
2019-02-06	6E0H	TMEM16 scramblase	3 mM f-FC8
2018-12-19	6N3Q	Sec protein-translocation channel complex	3 mM f-FC8
2018-11-07	6H3I	Type 9 secretion system translocon	1.5 mM f-FC8 or 0.7 mM f-OM
2018-10-24	6DMR	TRPV5 ion channel	3 mM f-FC8
2018-10-17	6D3R	CFTR	3 mM f-FC8
2018-09-26	6HJR	Influenza Hemagglutinin	2% Octyl Glucoside
2018-08-08	6FOO	Ryanodine receptor 1	0.2% f-OM
2018-08-01	6CJQ	SthK CNG Potassium channel	3 mM f-FC8
2018-05-23	5YX9	TRPC6 ion channel	0.5 mM f-OM
2018-01-31	6C0V	P-Glycoprotein transporter ABCB1	3 mM f-FC8
2017-12-27	6B5V	TRPV5 ion channel	3 mM f-FC8
2017-12-13	6BPQ	TRPM8 channel	2% DMSO

https://www.anatrace.com/Landing/2020/Mar20-Newsletter

Glaeser, RM, et al. (2017) Biophys Rep 3(1), 1-7.

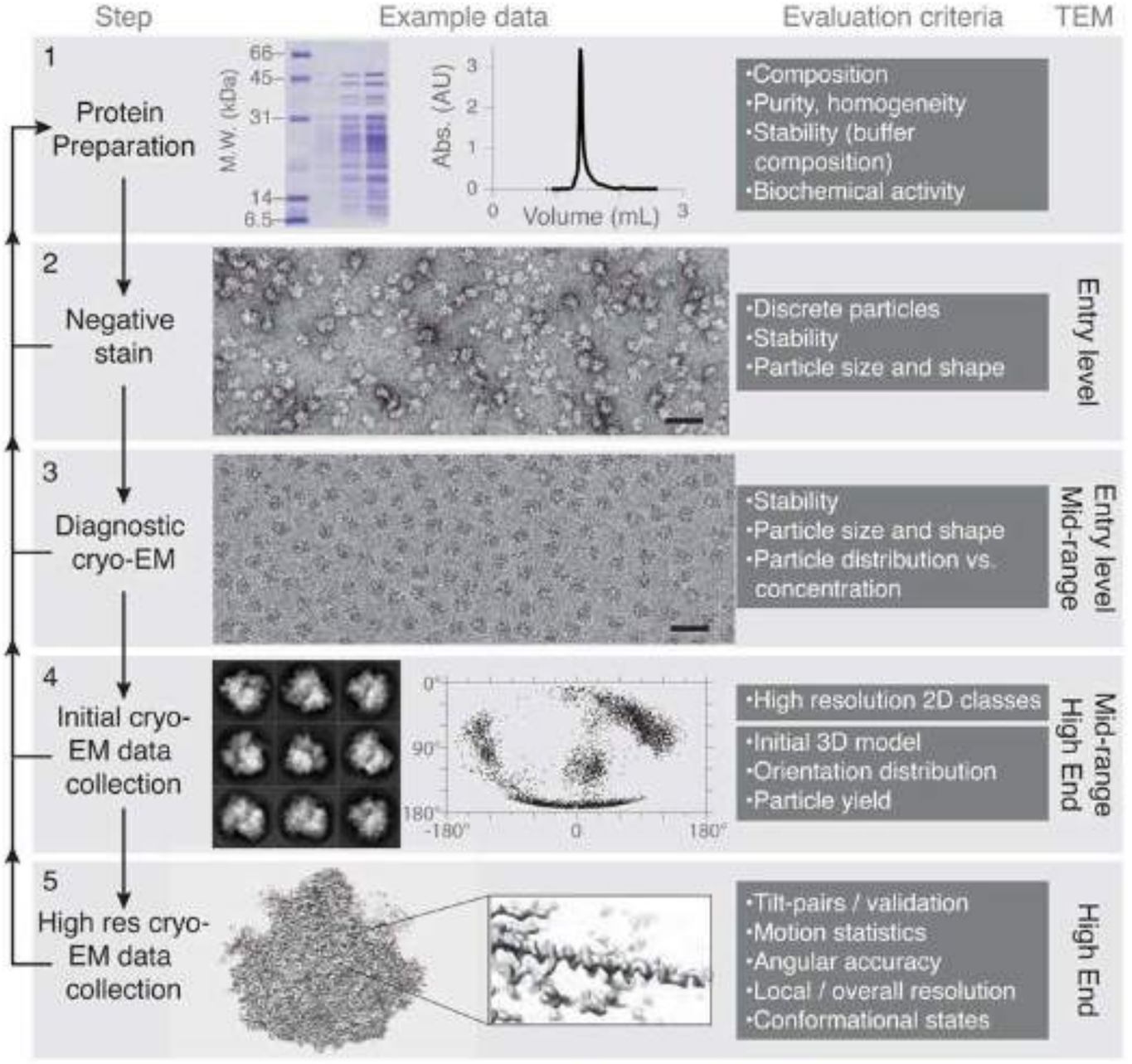
Noble, AJ, et al. (2018) Nat Methods 15(10), 793-795.

Drulyte, I et al. (2018) Acta Crystallogr D Struct Biol 74(Pt 6), 560-571.

Chen, J, et al. (2019) J Struct Biol X Volume 1. DOI: 10.1016/j.yjsbx.2019.100005

THE OPTIMIZATION WORKFLOW

Structure determination by cryo-EM. A systematic approach to 3D structure determination is shown. In the left column, the major steps are listed. Each step should be performed successively and only after one has been completed successfully should the scientist move onto the next step. In the second column, example data are shown for ribosomes (details in text). Scale bars on the micrographs are 500 Å. Each step should be evaluated with the criteria listed in the third column, returning to earlier steps for troubleshooting.



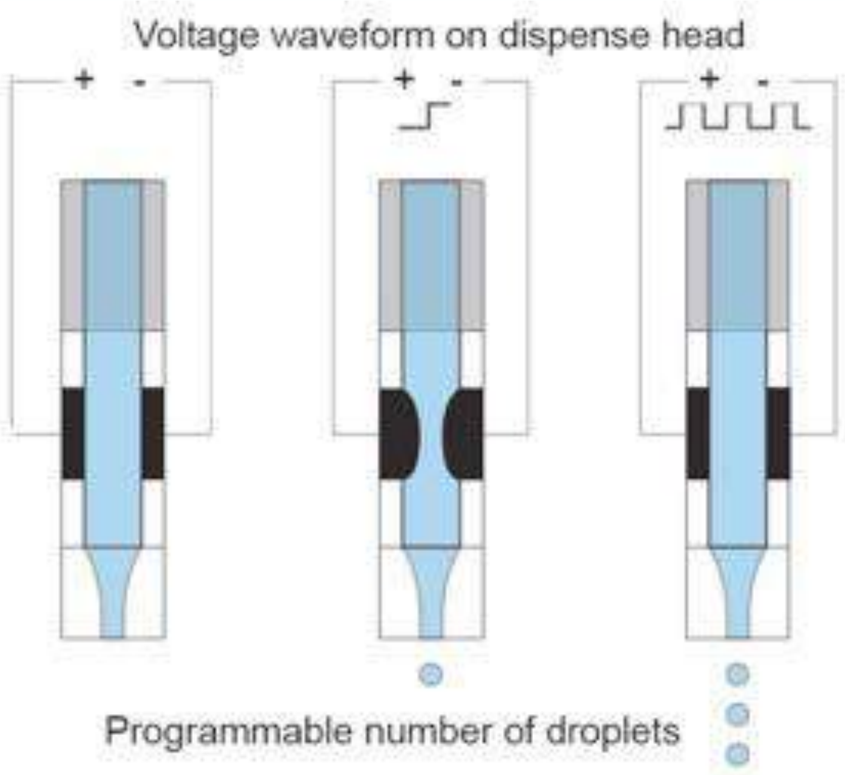
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5140023/

WHAT IS SPOTITON?

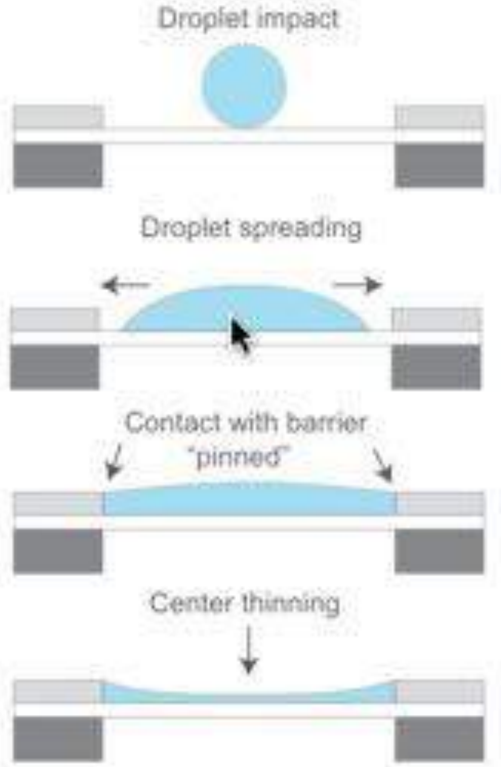
Improving Current CryoTEM Grid Preparation Methods

Spotiton

Accurate pL dispensing



Thin films without blotting



Dandey VP, Wei H, Zhang Z, Tan YZ, Acharya P, Eng ET, Rice WJ, Kahn PA, Potter CS, Carragher B. Spotiton: New features and applications. Journal of structural biology. 2018;202(2):161-9

Venkat Dandey

Hui Wei

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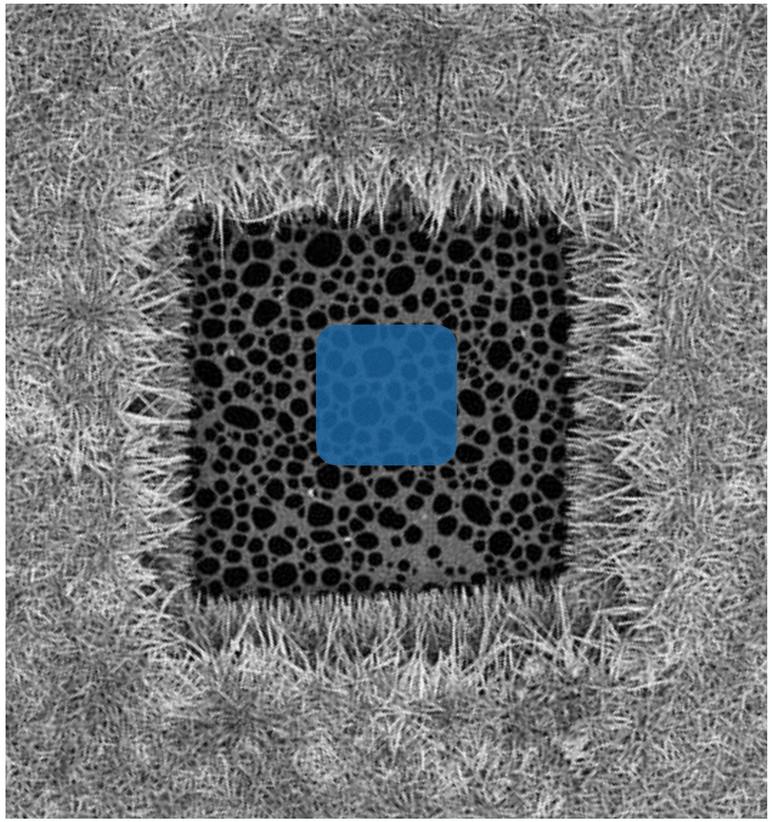
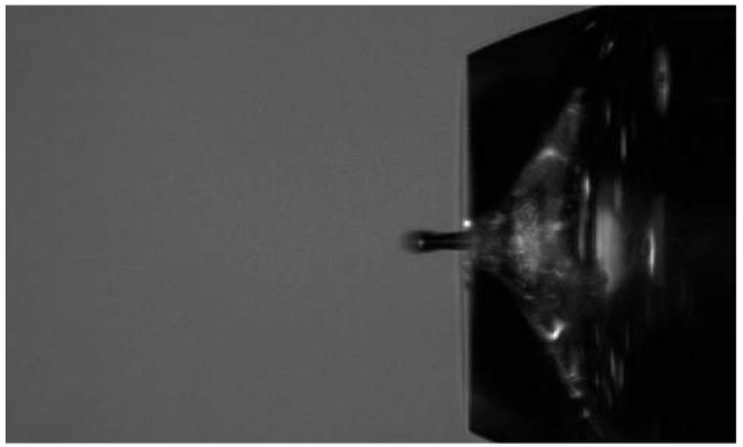
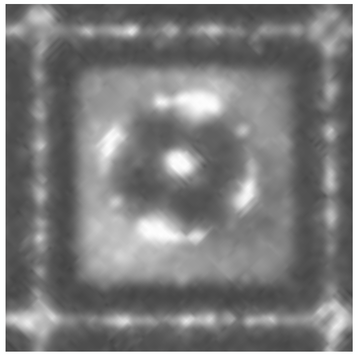
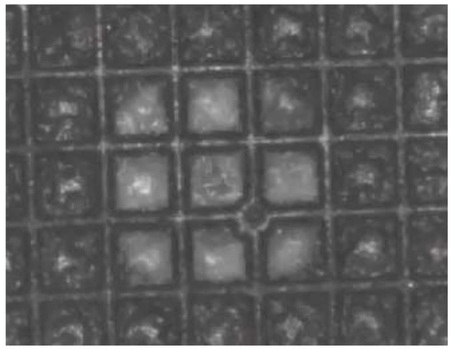
Hui Wei







Single frame from loop



Video loop

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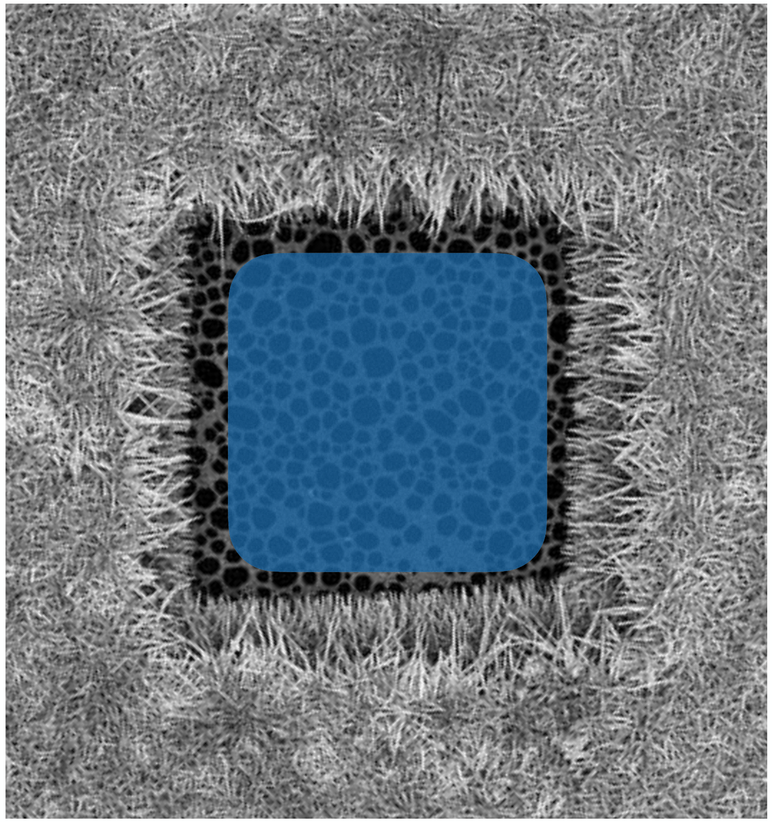
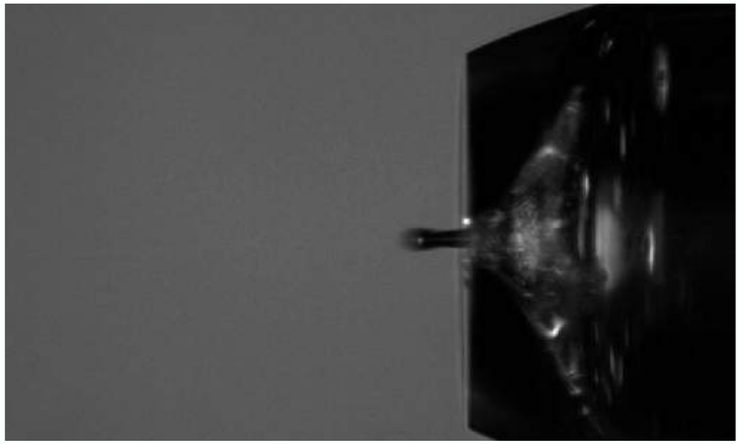
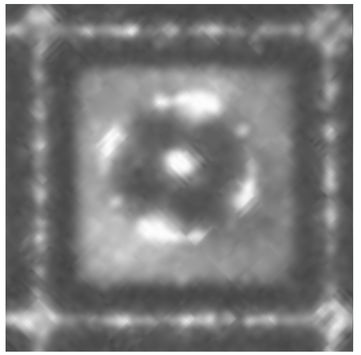
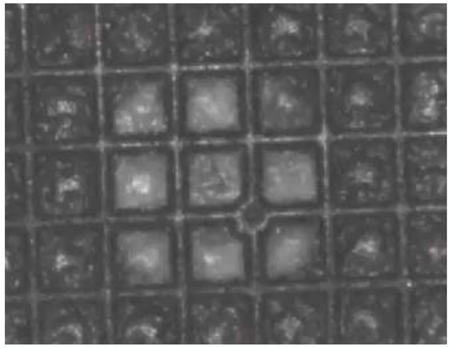






Single frame from loop



Video loop

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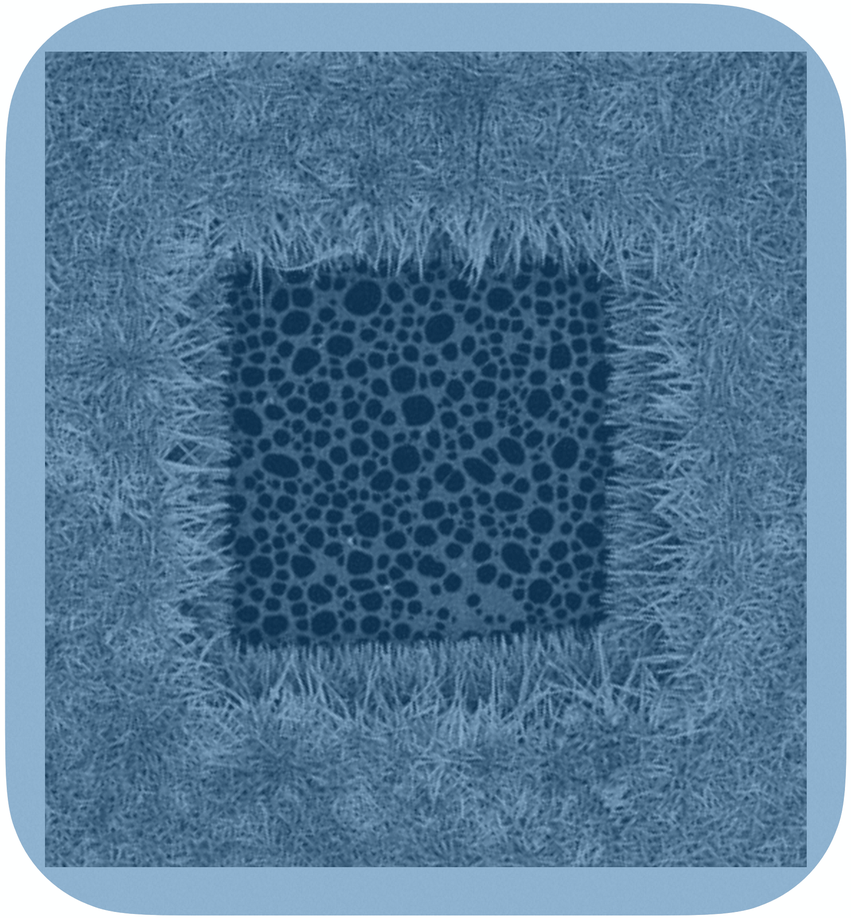
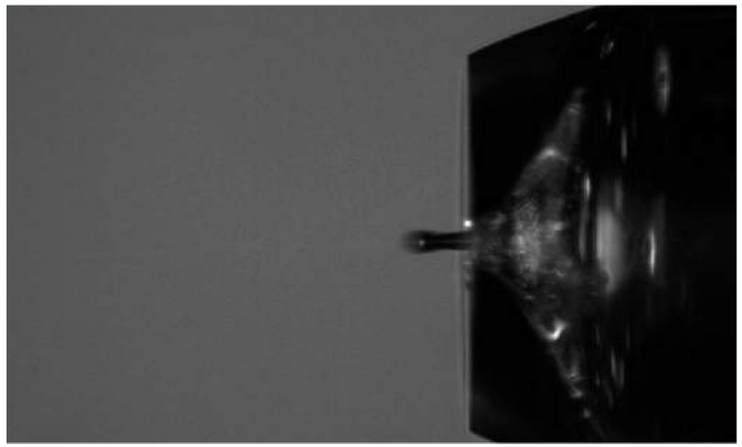
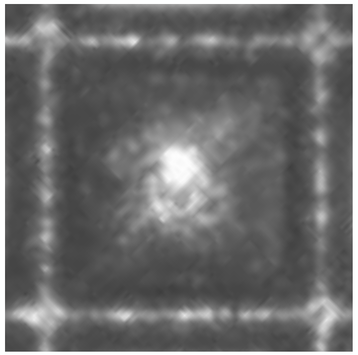
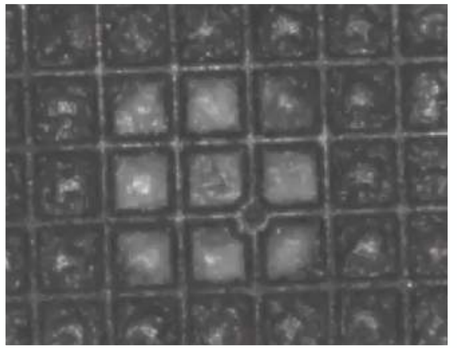






Single frame from loop



Video loop

Wei H, Dandey VP, Zhang Z, Raczkowski A, Rice WJ, Carragher B, Potter CS. Optimizing "self-wicking" nanowire grids. J Struct Biol. 2018;202(2):170-4.

Hui Wei

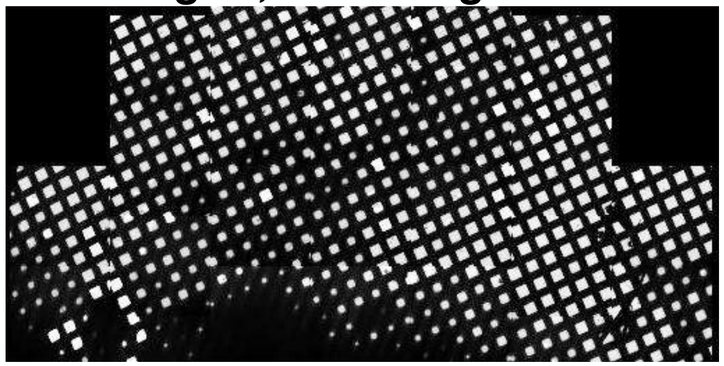
WHAT IS SPOTITON?

Improving Current CryoTEM Grid Preparation Methods

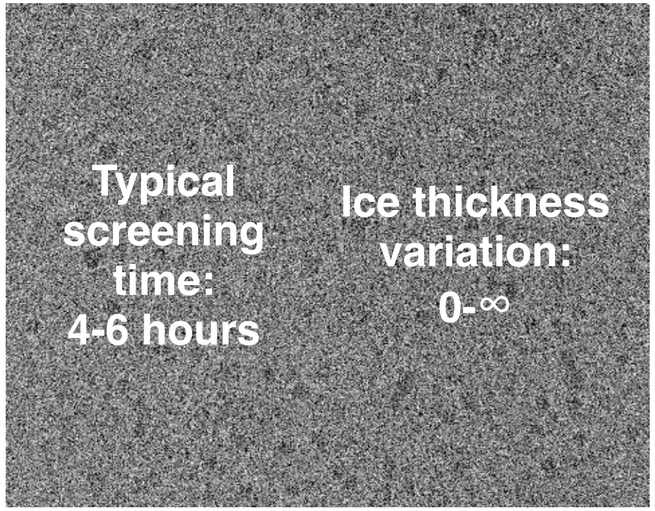
Spotiton

Vitrobot

3 uL of sample required for each grid; ~2nL on grid

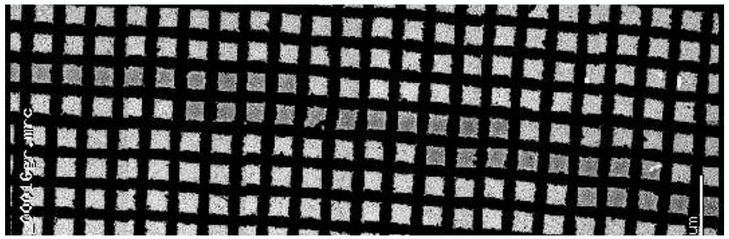


Usable area: ~0-10%

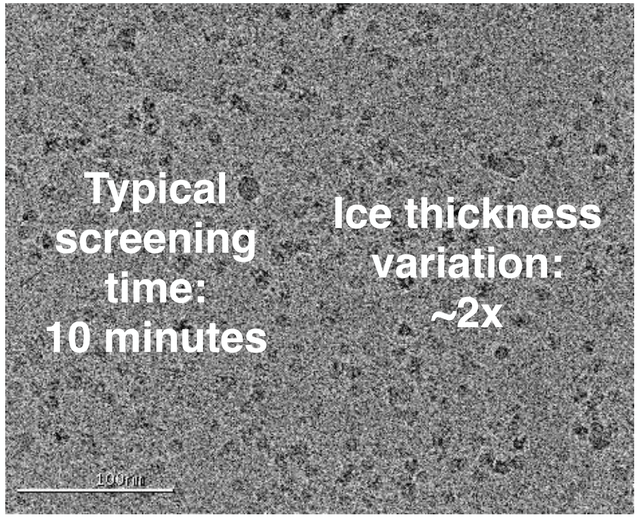


Spotiton

3 uL of sample enough for >100 grids; ~500pL on grid



Usable area: ~100%



Wei H, Dandey VP, Zhang Z, Raczkowski A, Rice WJ, Carragher B, Potter CS. Optimizing "self-wicking" nanowire grids. J Struct Biol. 2018;202(2):170-4.

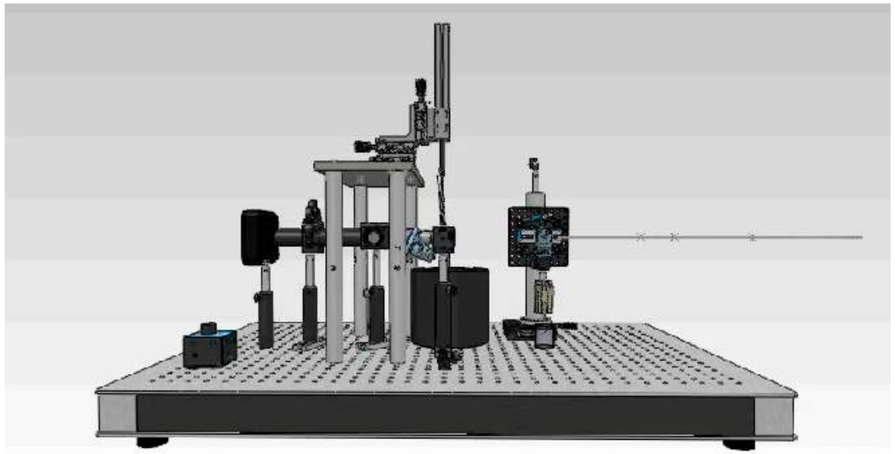
Hui Wei

WHAT IS CHAMELEON?

Spotiton

The Spotiton Project: Commercialization

Spotiton concept: 2011



Chameleon: 2019

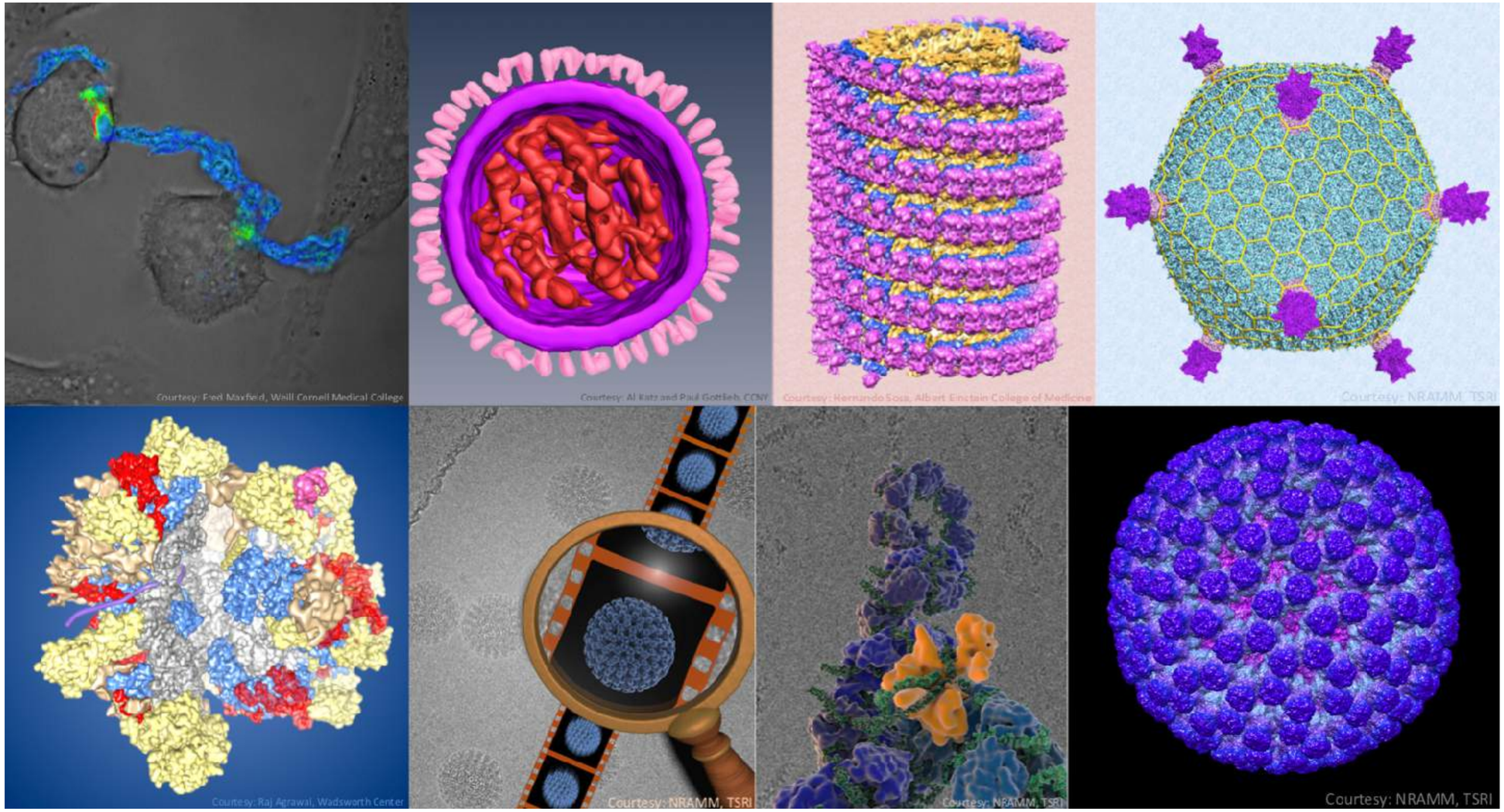
sptlabtech

Venkat Dandey

Alex Wei

Chase Budell

CRYOEM: TECHNOLOGY ON THE RISE



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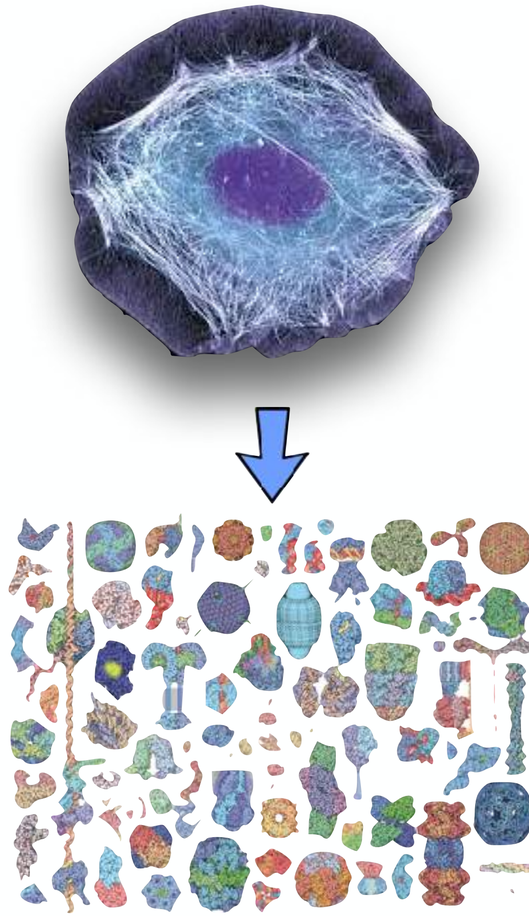
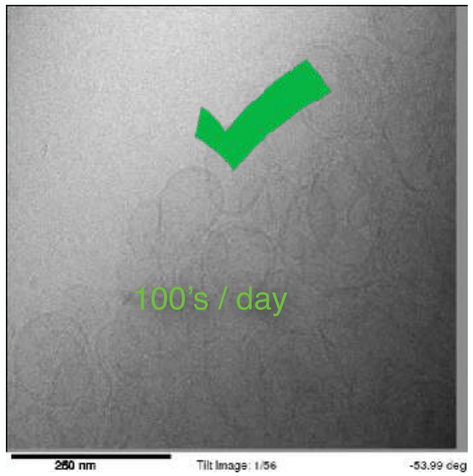


TBD (20??)

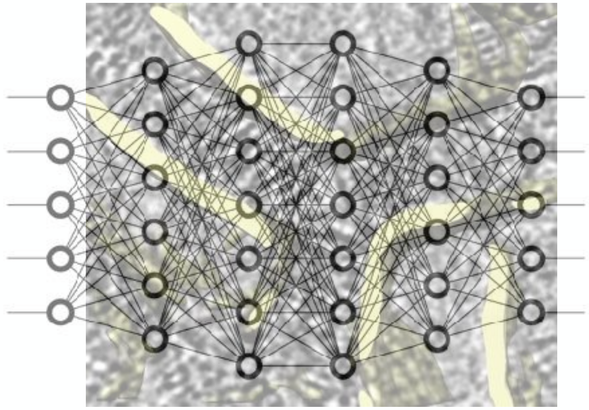
WHERE ARE WE HEADING?

Towards Automation for In Situ CryoEM

Sample



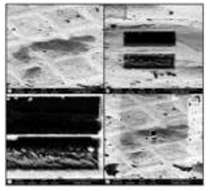
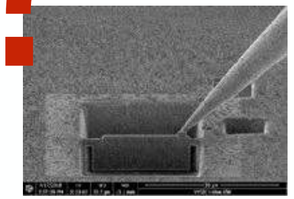
Automated Data Collection
(Leginon, etc.)



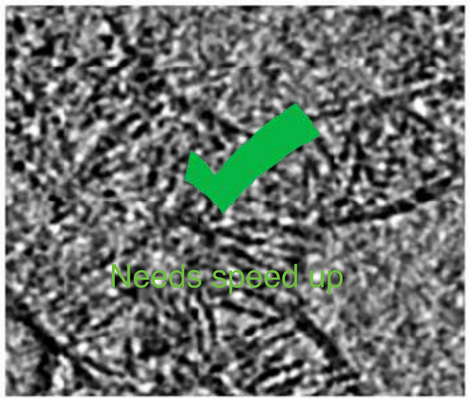
Deep learning?



?

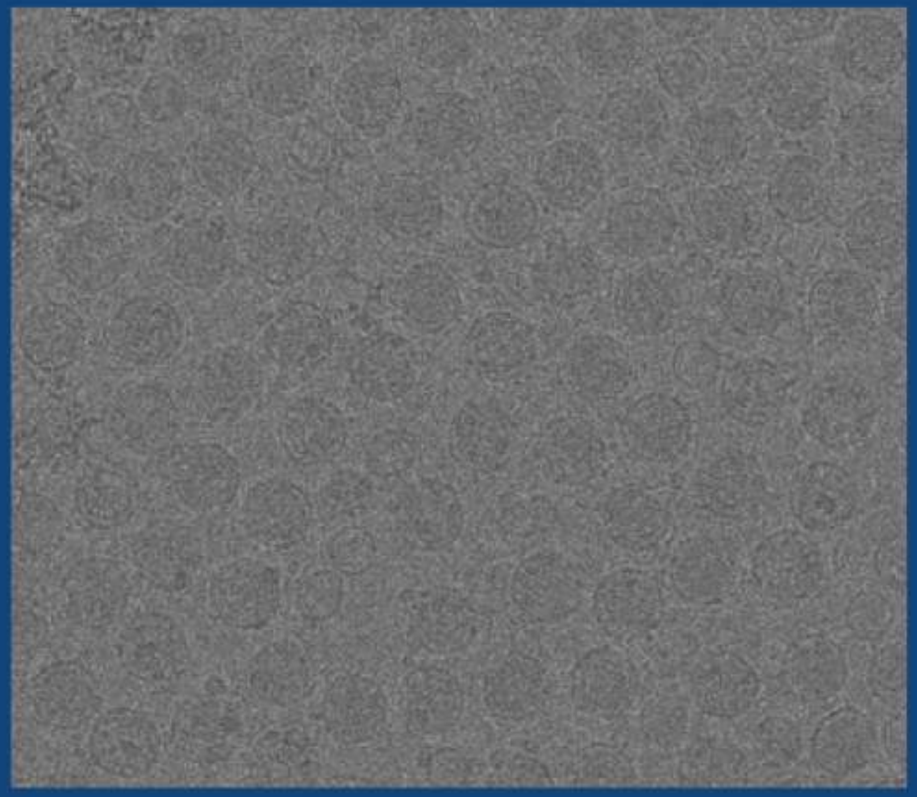



Grid preparation



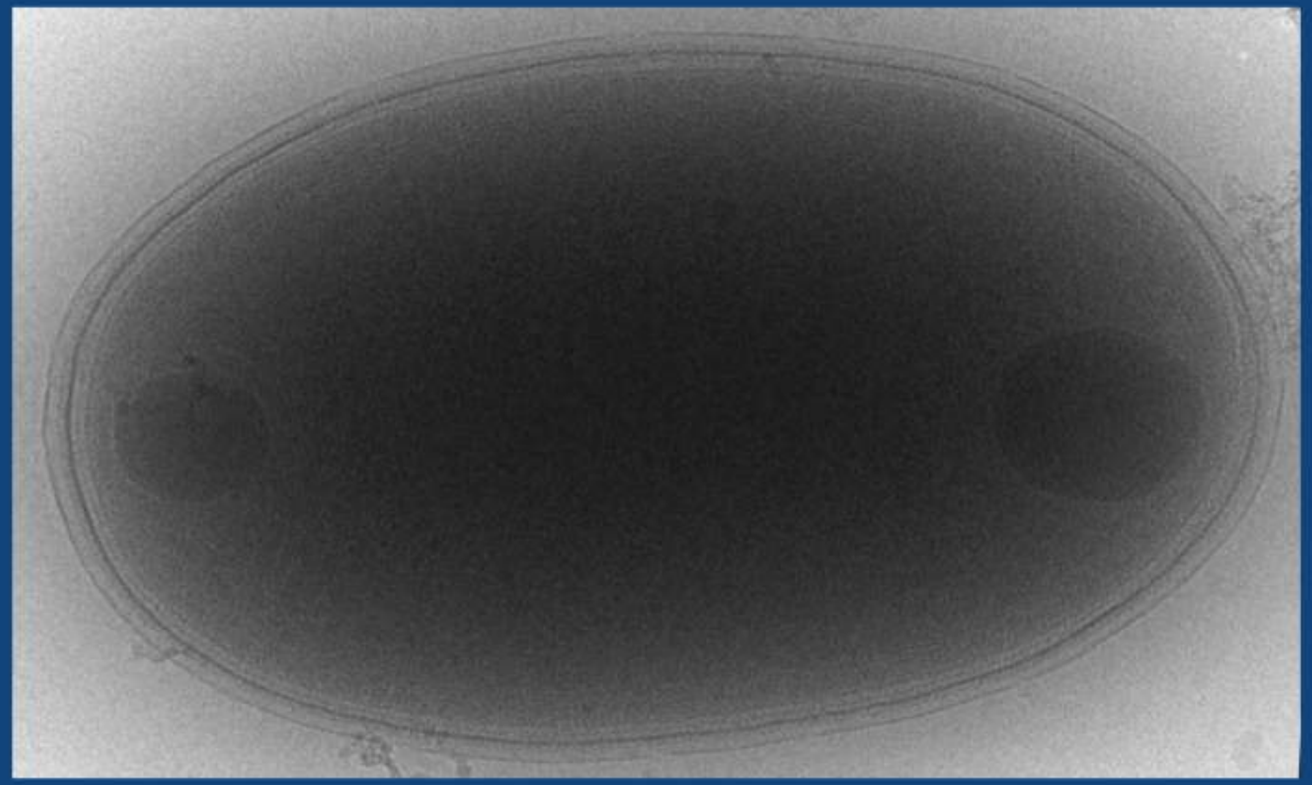
Streamlined Processing
(Appion Protomo)

HOW THIN DOES THE SAMPLE NEED TO BE?



50 nm

Bacteriophage (ϕ12)



E. coli, Salmonella, Cyanobacteria

TO BE CONTINUED

Questions?