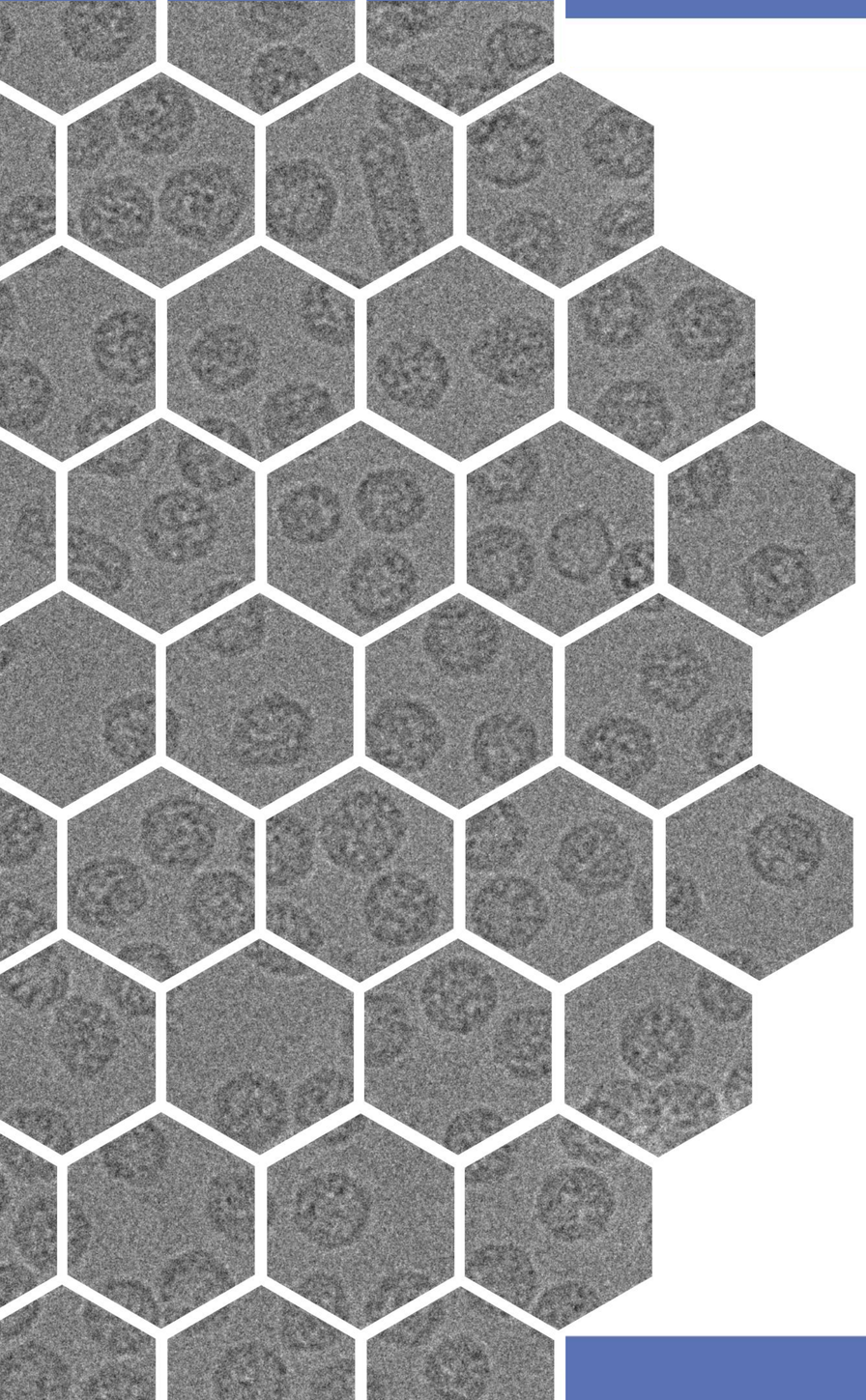
2024 Winter cryoEM course

Considerations for biological cryoEM

January 29, 2024

NYSBC SEMC



- Journal club and practical recap
- Considerations for biological cryoEM
  - Overview
  - Grids
  - What happens to a sample
  - Newer methods

Course logistics: main topics

Section I : EM fundamentals

Section 2 : EM crystallography

Section 3 : Tomography

Section 4 : SPA short course*
March 11-15

Section 5 : Future perspectives

Course logistics: Wednesday practical

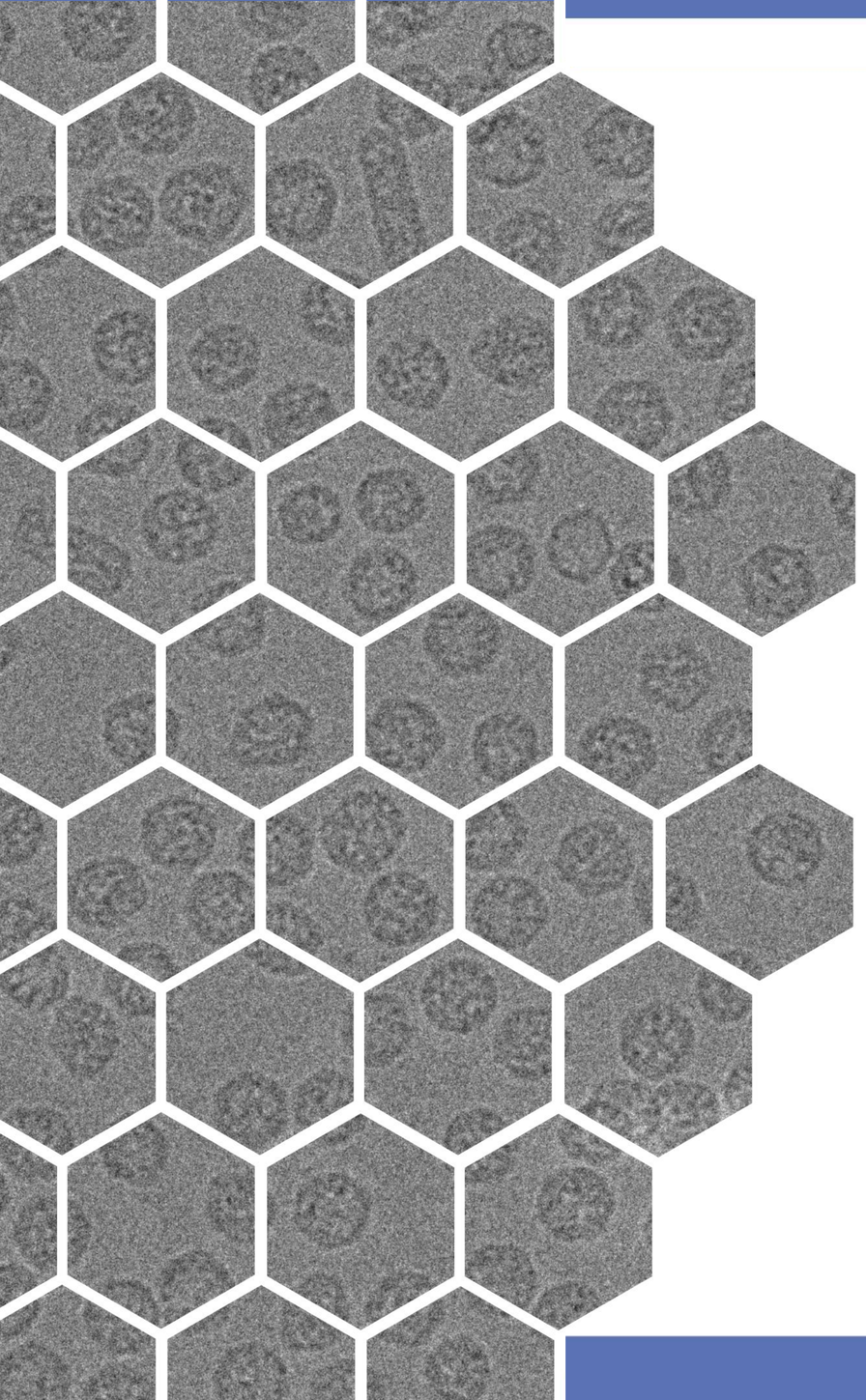
January 31, 2024

Sample preparation practical

-standard vitrification

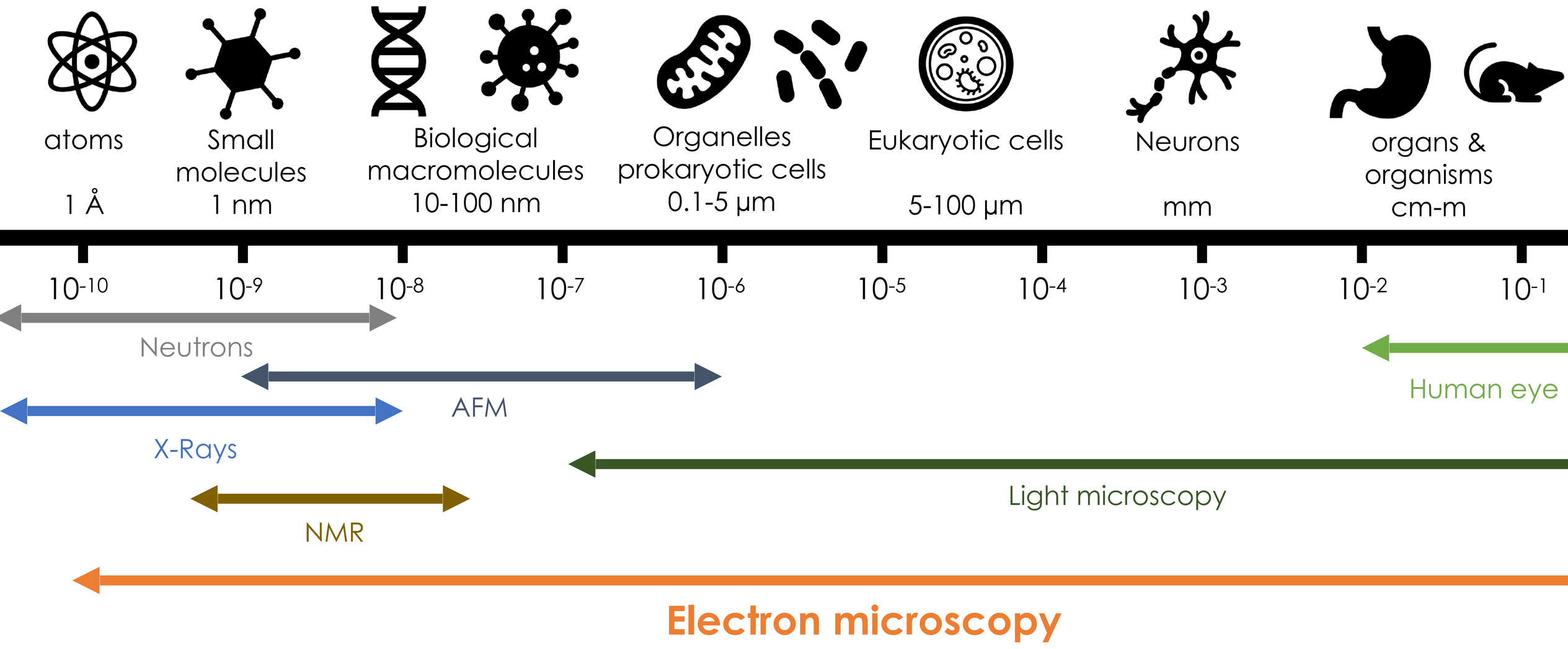
-45 min first session

-30 min second session



- Journal club and practical recap
- Considerations for biological cryoEM
  - Overview
  - Grids
  - What happens to a sample
  - Newer methods

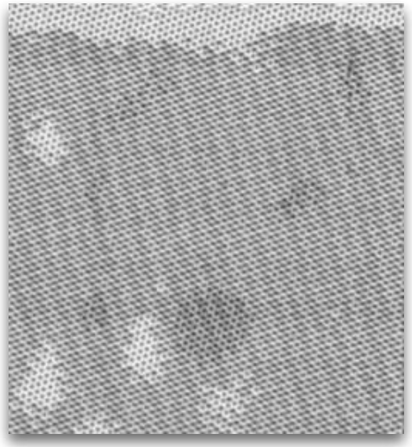
Scale of biology

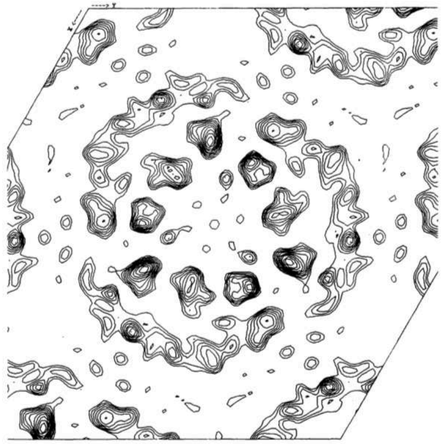


doi: 10.2210/rcsb_pdb/goodsell-gallery-014

cryoEM: technology on the rise

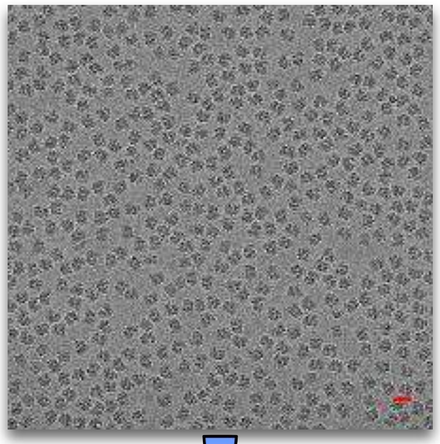
1986

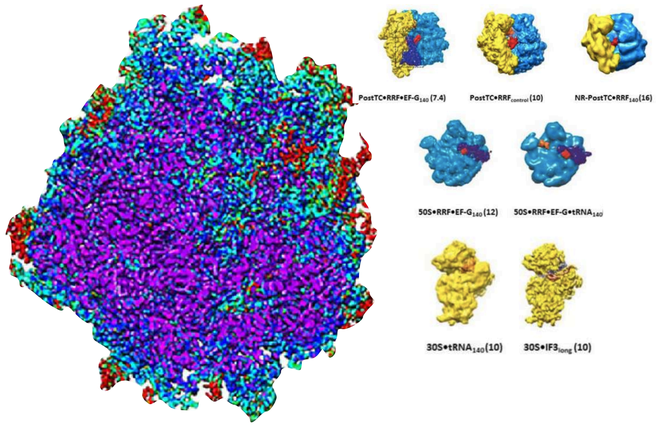




Henderson et al. (1986)

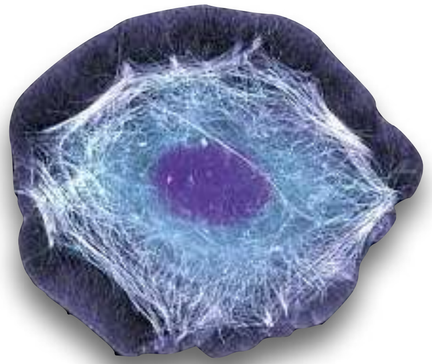
2017

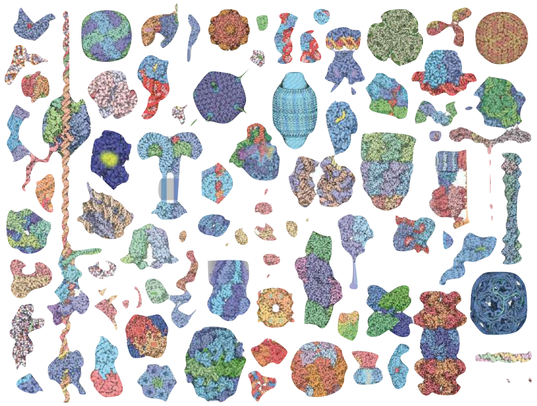




Frank et al. (2017)

in progress

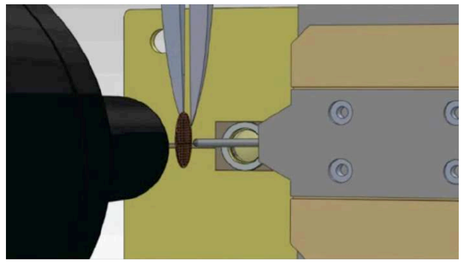
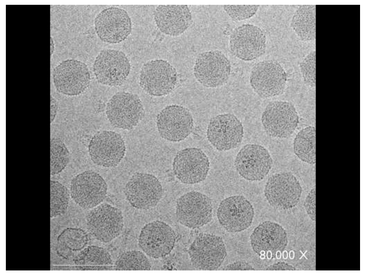


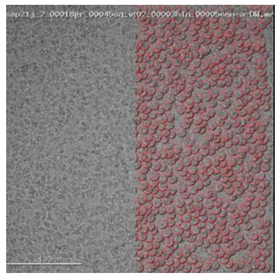
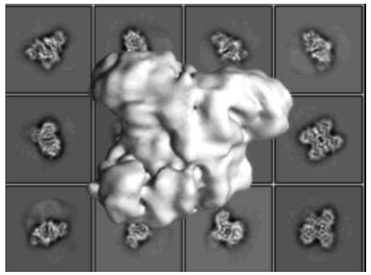
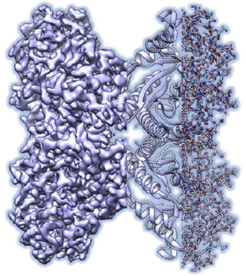


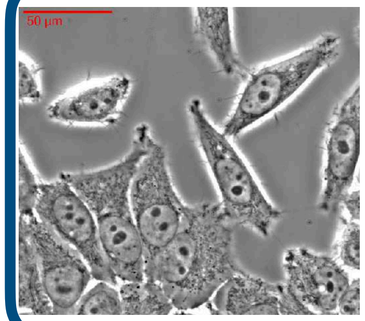
the next chapter

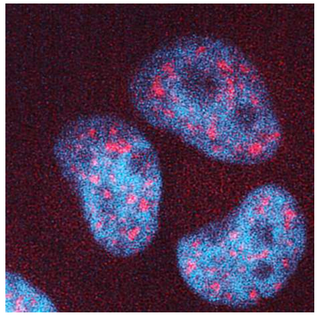
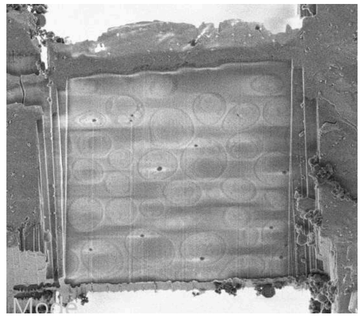
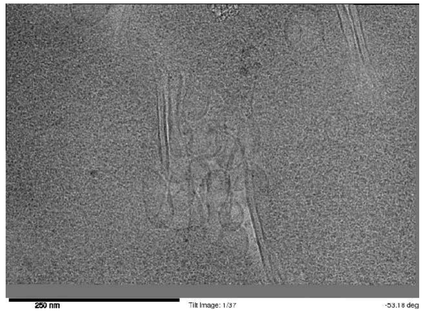
cryoEM: a technology on the rise

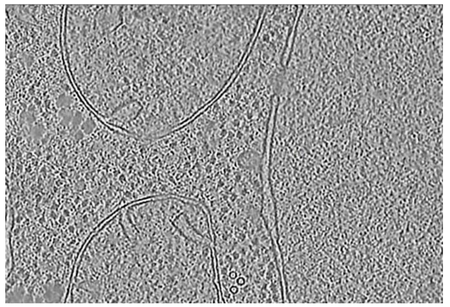
Single particle cryoEM

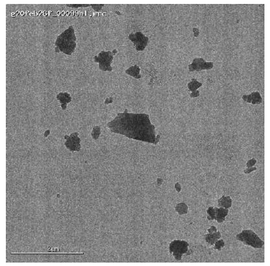
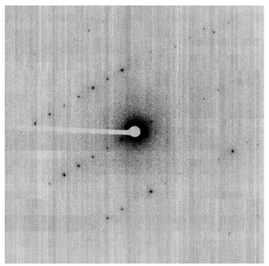
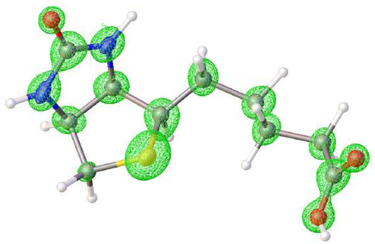
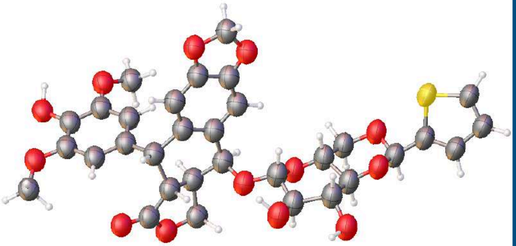


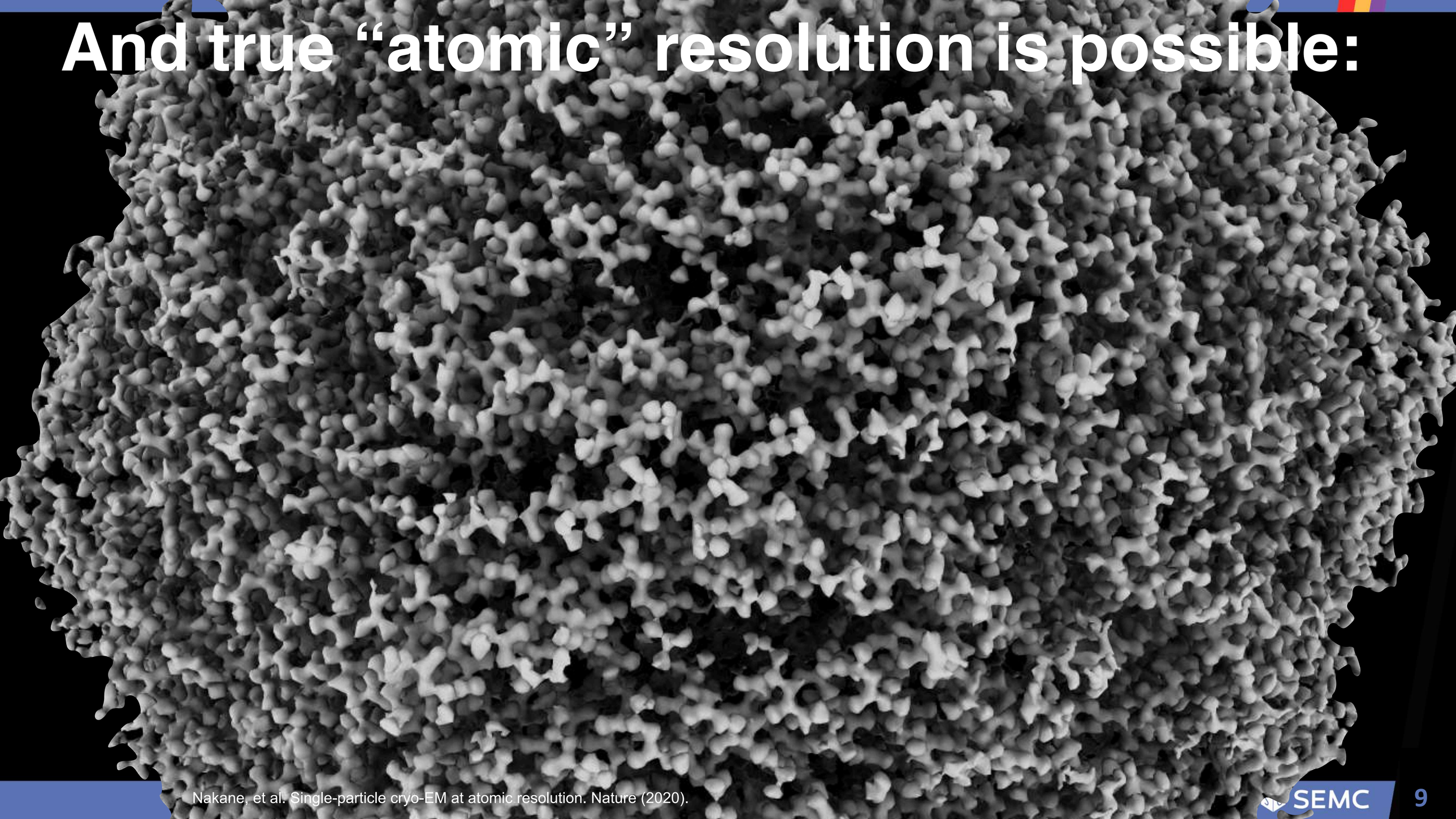


Cryo Electron Tomography (cryoET)

Micro crystal electron diffraction (microED)

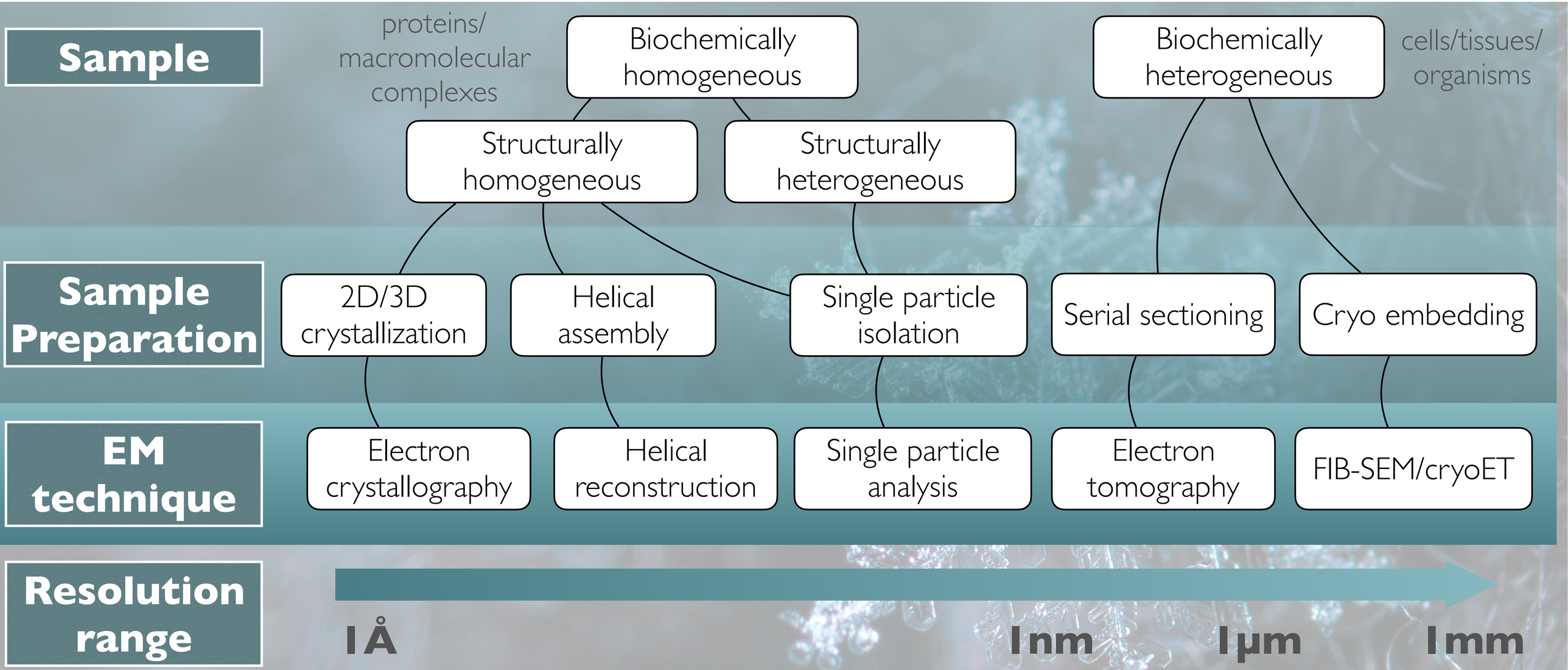





And true “atomic” resolution is possible:

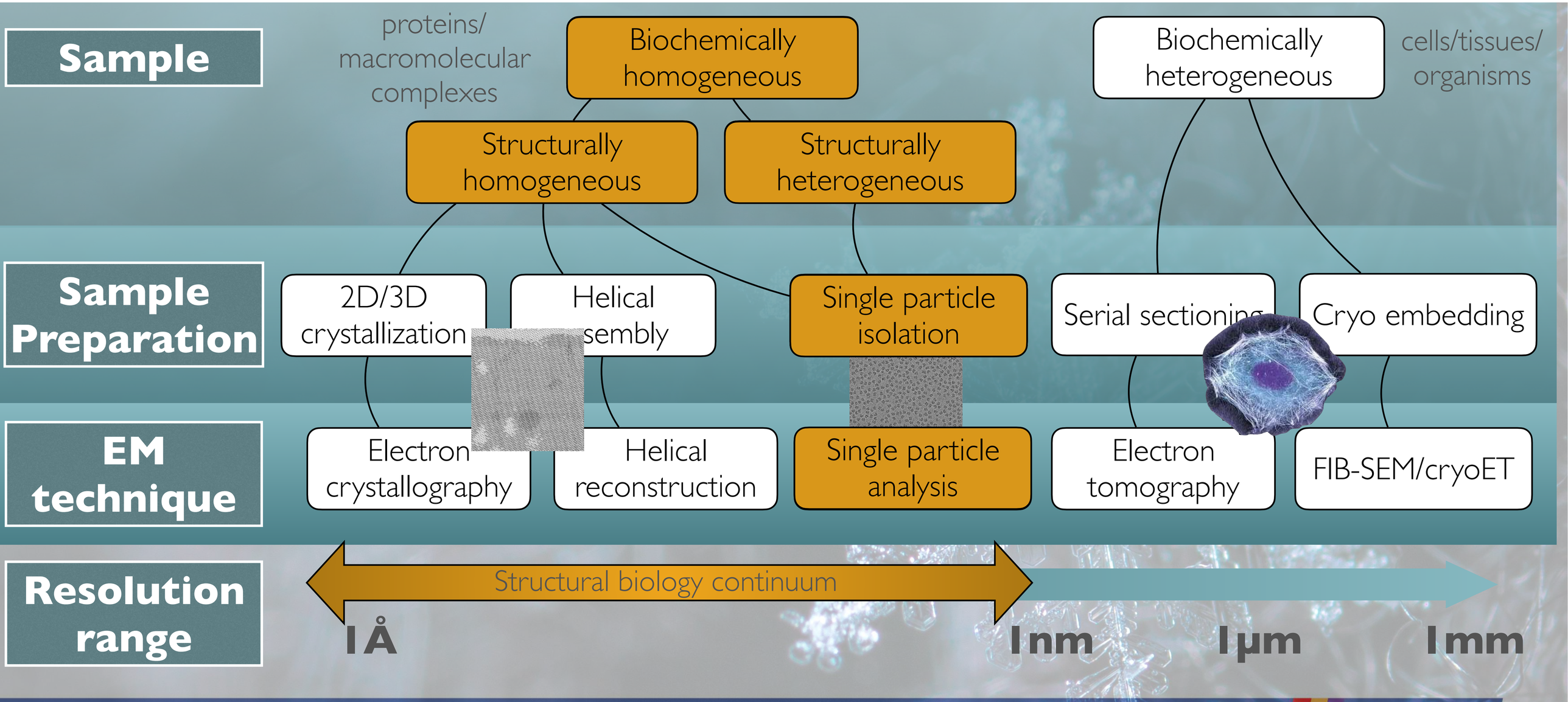


Nakane, et al. Single-particle cryo-EM at atomic resolution. Nature (2020).

How are samples prepared for cryoEM?

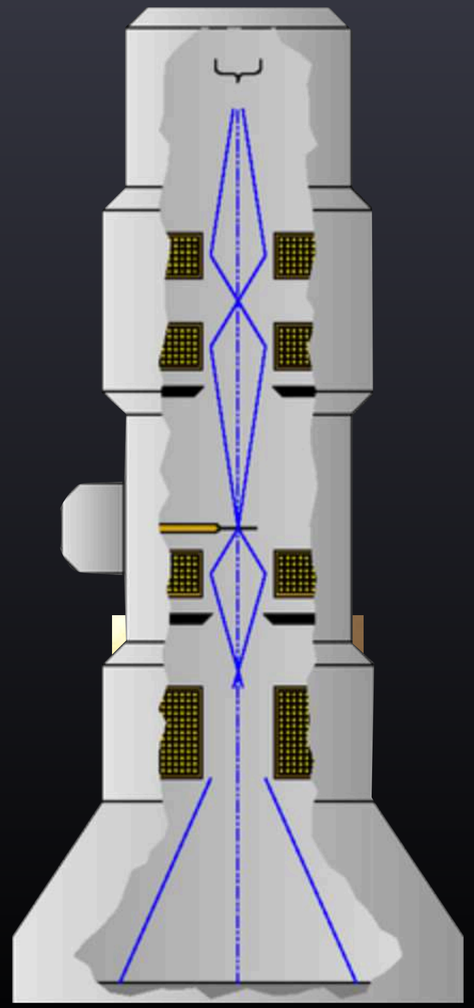


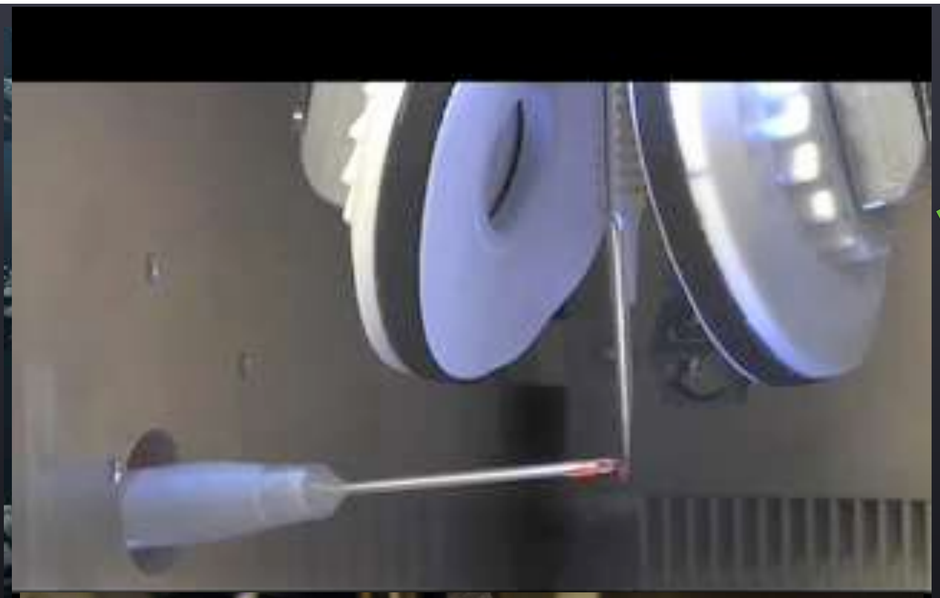
How are samples prepared for cryoEM?



How are samples prepared for cryoEM?

Vitrifying a biological sample



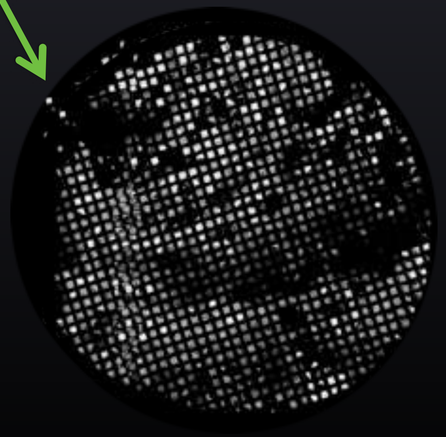


>99.999%

<0.001%

~3µl

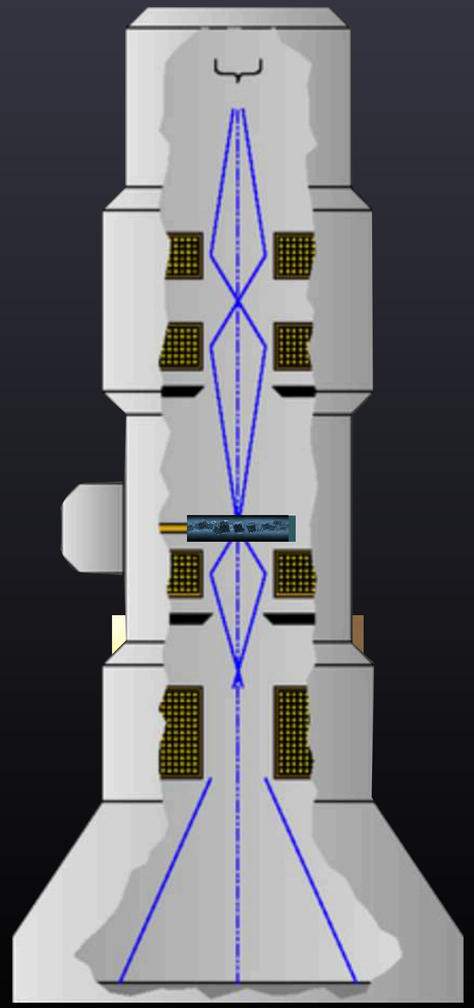
<3nl

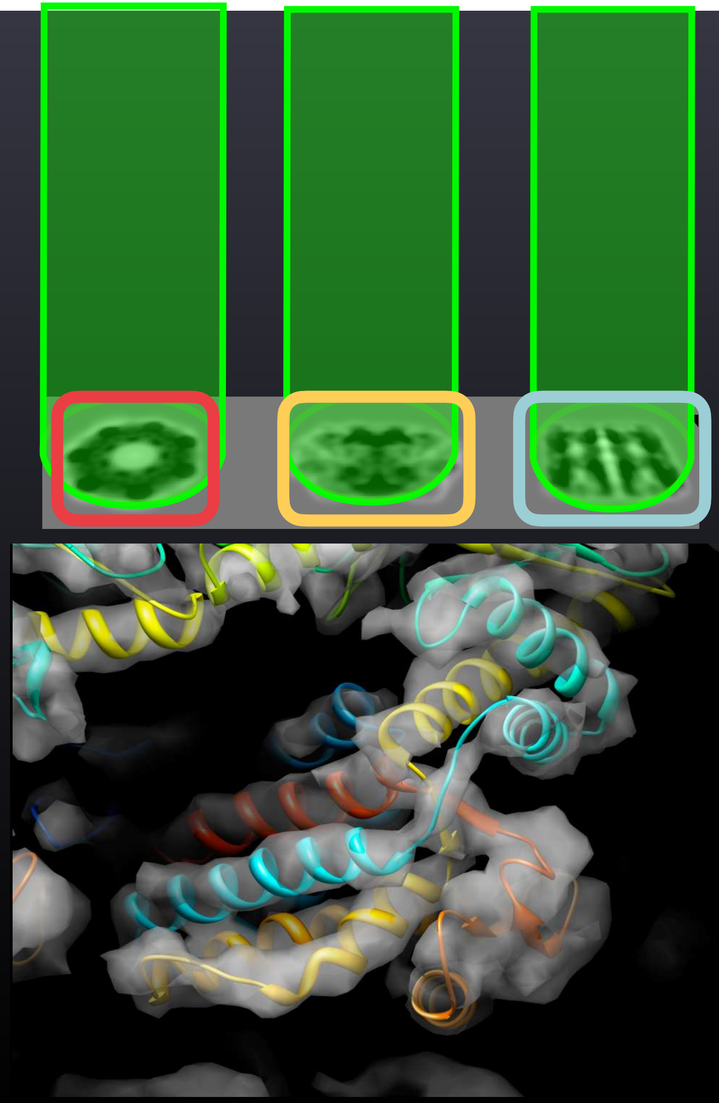


>100,000 potential imaging targets; most of them are not usable.

How are samples prepared for cryoEM?

Vitrifying a biological sample

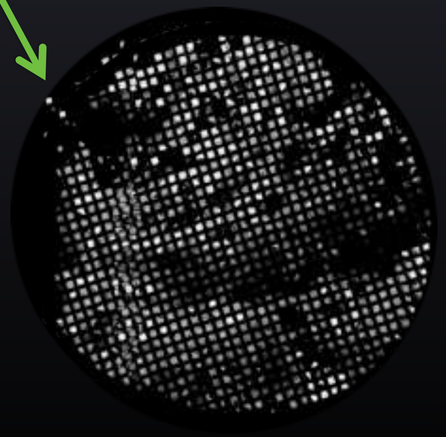




>99.999%

<0.001%

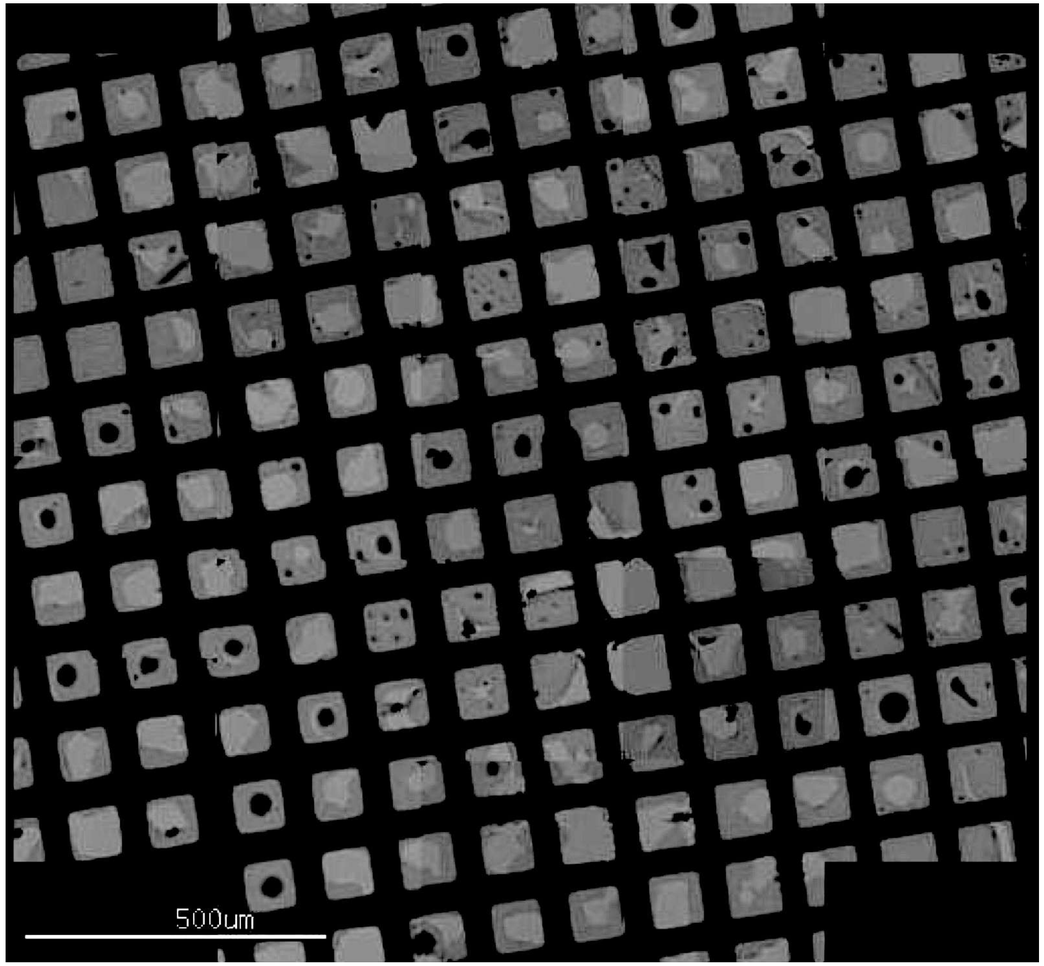
~3µl

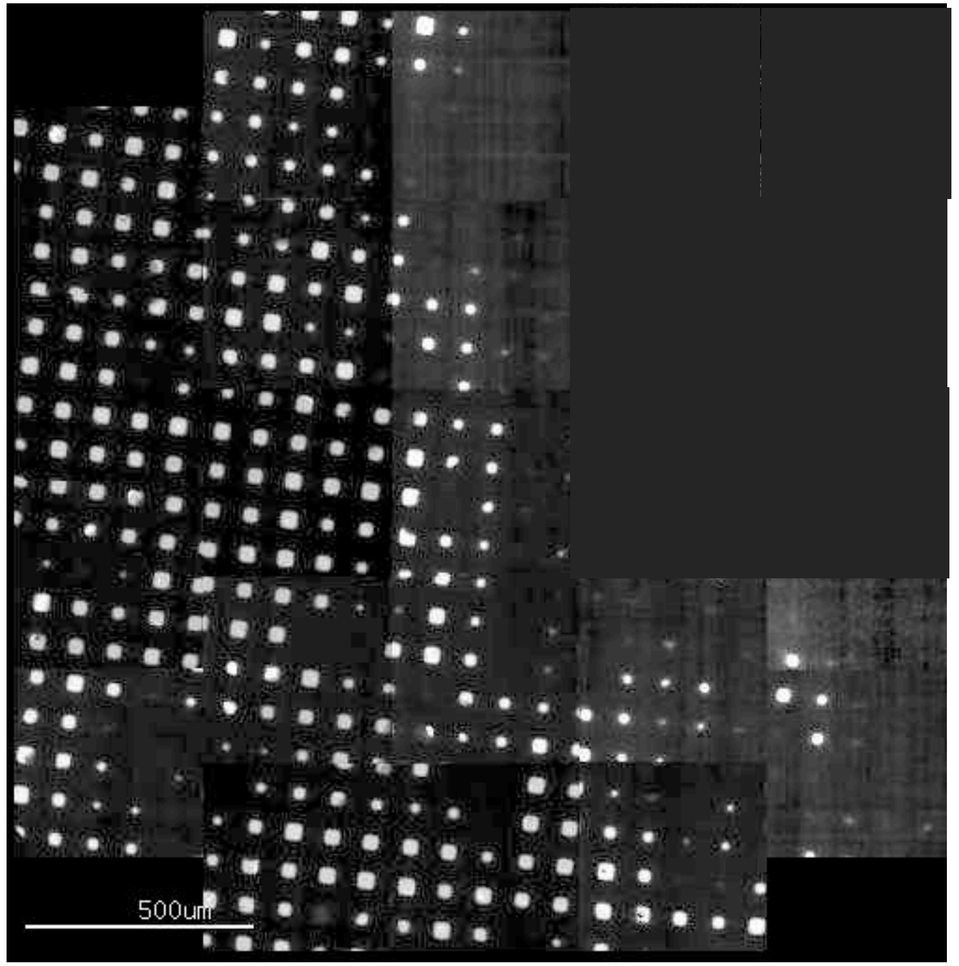


<3nl

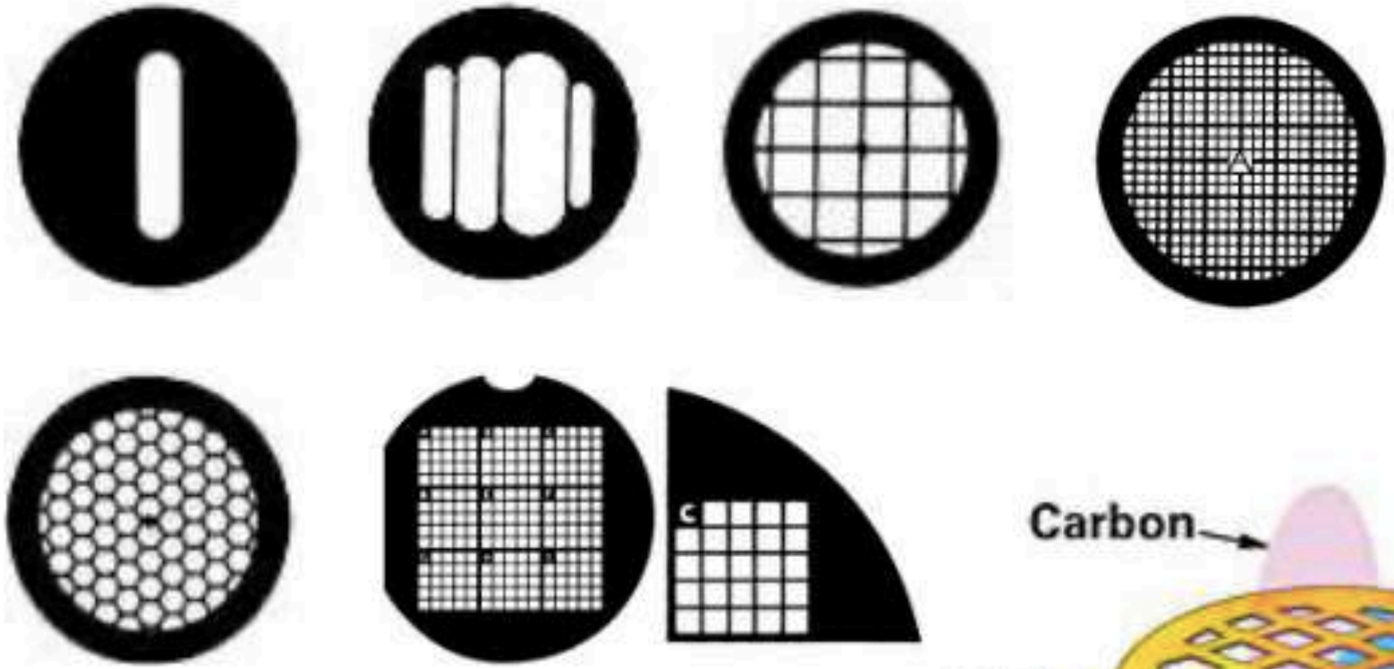
>100,000 potential imaging targets; most of them are not usable.

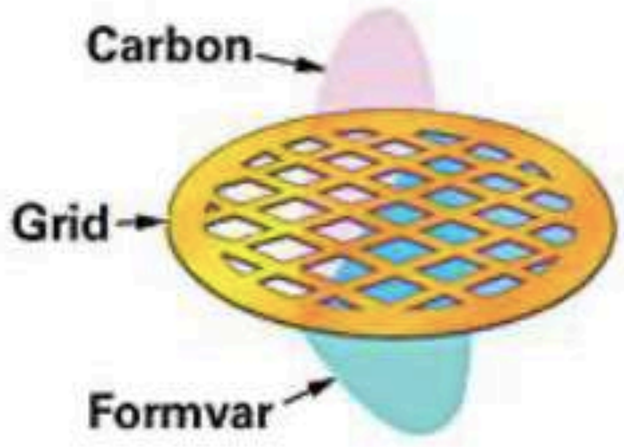
What do EM grids look like?





What do EM grids look like?





Common Materials

Copper

Nickel

Gold

Aluminum

Molybdenum

Titanium

Stainless Steel

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

What do EM grids look like?

Rough grid parameters

Rim Width: 350-400µm.

Thickness: approximately 25µm thick.

Diameter: 3.0 to 3.05mm

Pitch: Is 1"/mesh or 25.4mm/mesh

Example 200 mesh pitch = 25.4/200 = 127µm

PELCO® Grid Size

Square Mesh	Pitch µm	Hole µm	Bar µm	% Trans-mission		
50		508		425	83	70
75		339		284	55	70
100		254		204	50	65
150		169		125	44	60
200		127		90	37	50
300		85		54	31	40
400		64		38	26	35
500		51		28	23	30

What do EM grids look like?

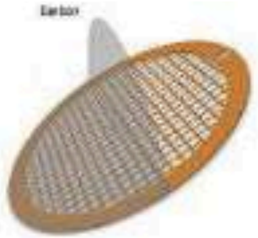
TERMINOLOGY

Grid (Cu, Au, Mo, etc…)

- mesh

Foil (C, Au, etc…)

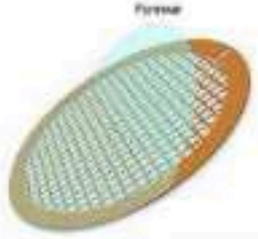
- Continuous
- lacy
- holey (hole size and spacing)



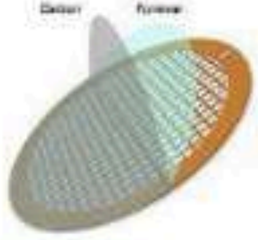
CARBON ONLY SUPPORT FILMS

HOLEY CARBON SUPPORT FILMS

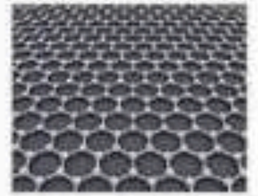
LACEY CARBON SUPPORT FILMS



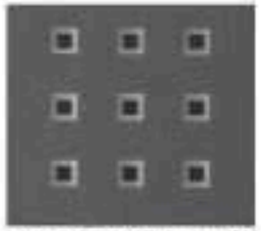
FORMVAR ONLY SUPPORT FILMS



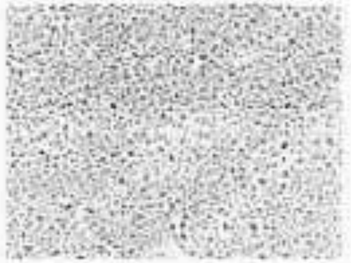
FORMVAR / CARBON SUPPORT FILMS



EM-TEC GRAPHENE SUPPORT FILMS



EM-TEC SILICON NITRIDE SUPPORT FILMS



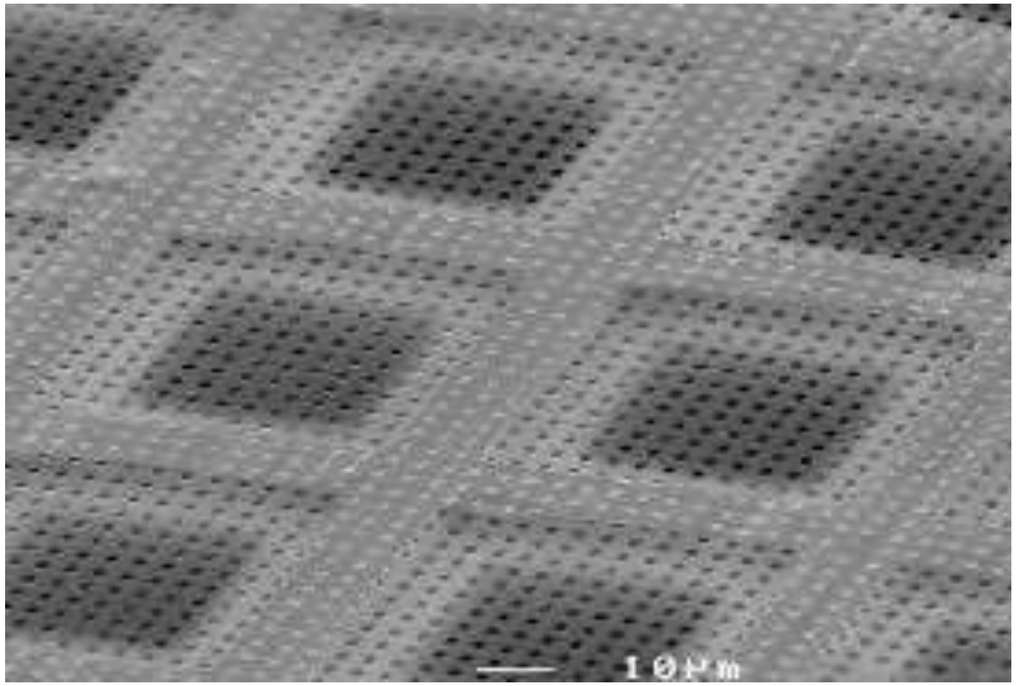
TEM CALIBRATION & TEST STANDARDS

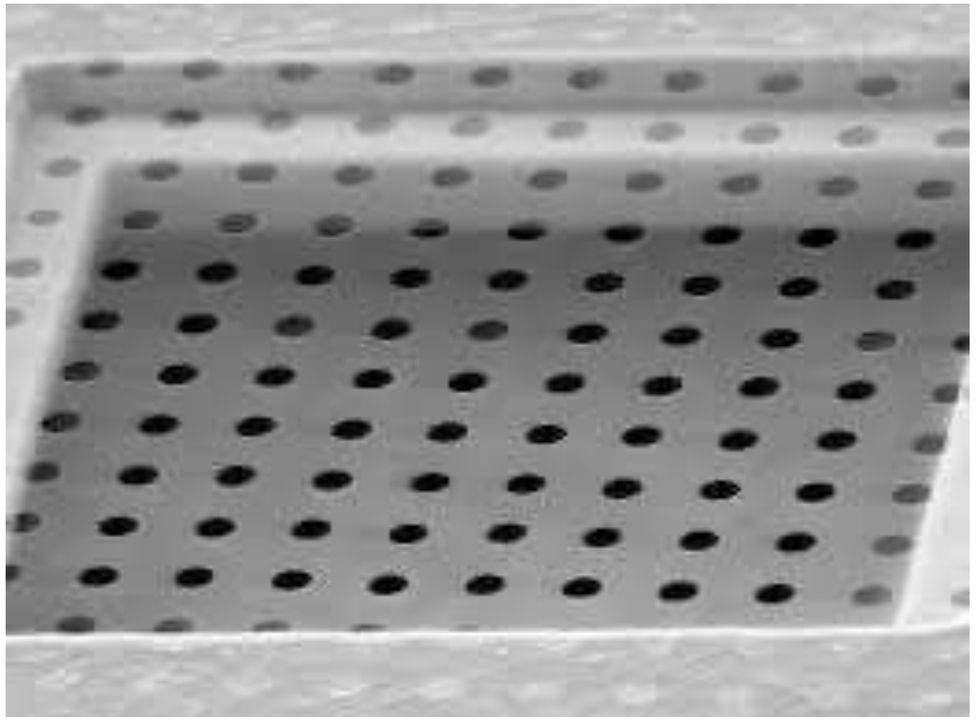
TEM GRID STORAGE BOXES

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

What do EM grids look like?

TERMINOLOGY





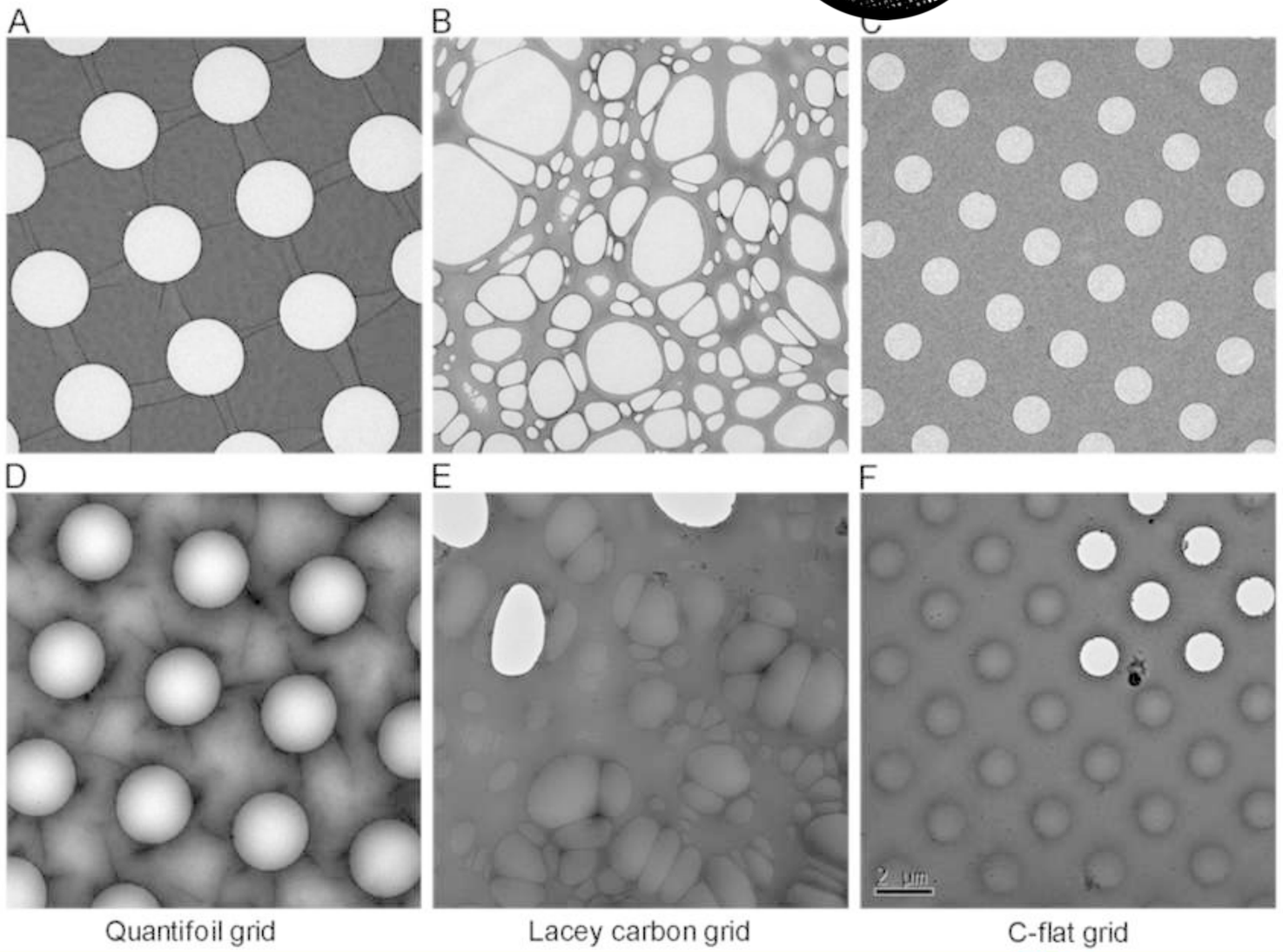
- Protochips.com

Quantifoil.com

What do EM grids look like?

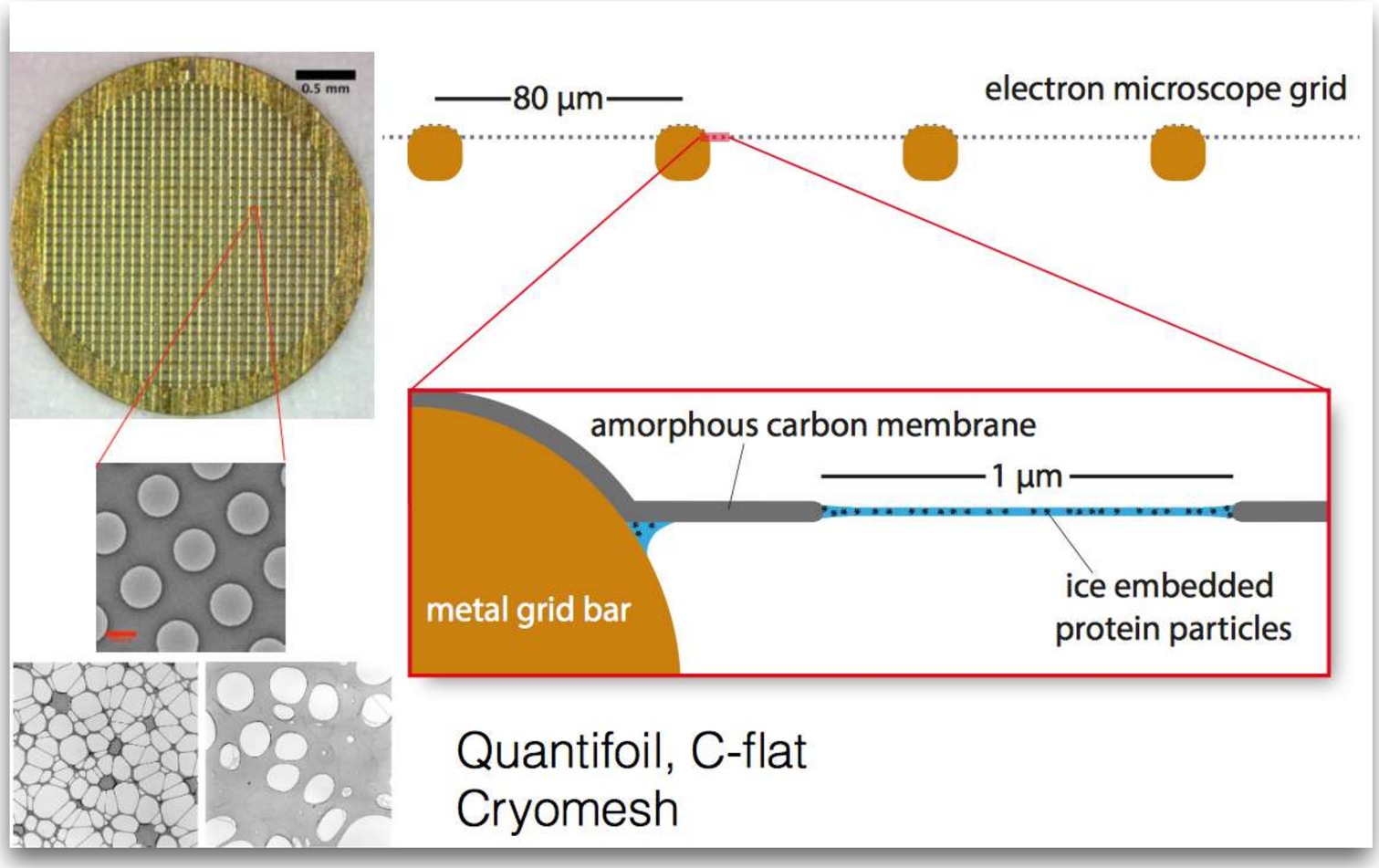
TERMINOLOGY

Cho, Hye-Jin & Hyun, Jae-Kyung & Kim, Jin-Gyu & Jeong, Hyeong & Park, Hyo & You, Dong-Ju & Jung, Hyun. (2013). Measurement of ice thickness on vitreous ice embedded cryo-EM grids: investigation of optimizing condition for visualizing macromolecules. Journal of Analytical Science and Technology. 4. 10.1186/2093-3371-4-7.



What do EM grids look like?

TERMINOLOGY



Russo & Passmore, 2015

What do EM grids look like?

TERMINOLOGY

- Holey gold foil on gold mesh grid

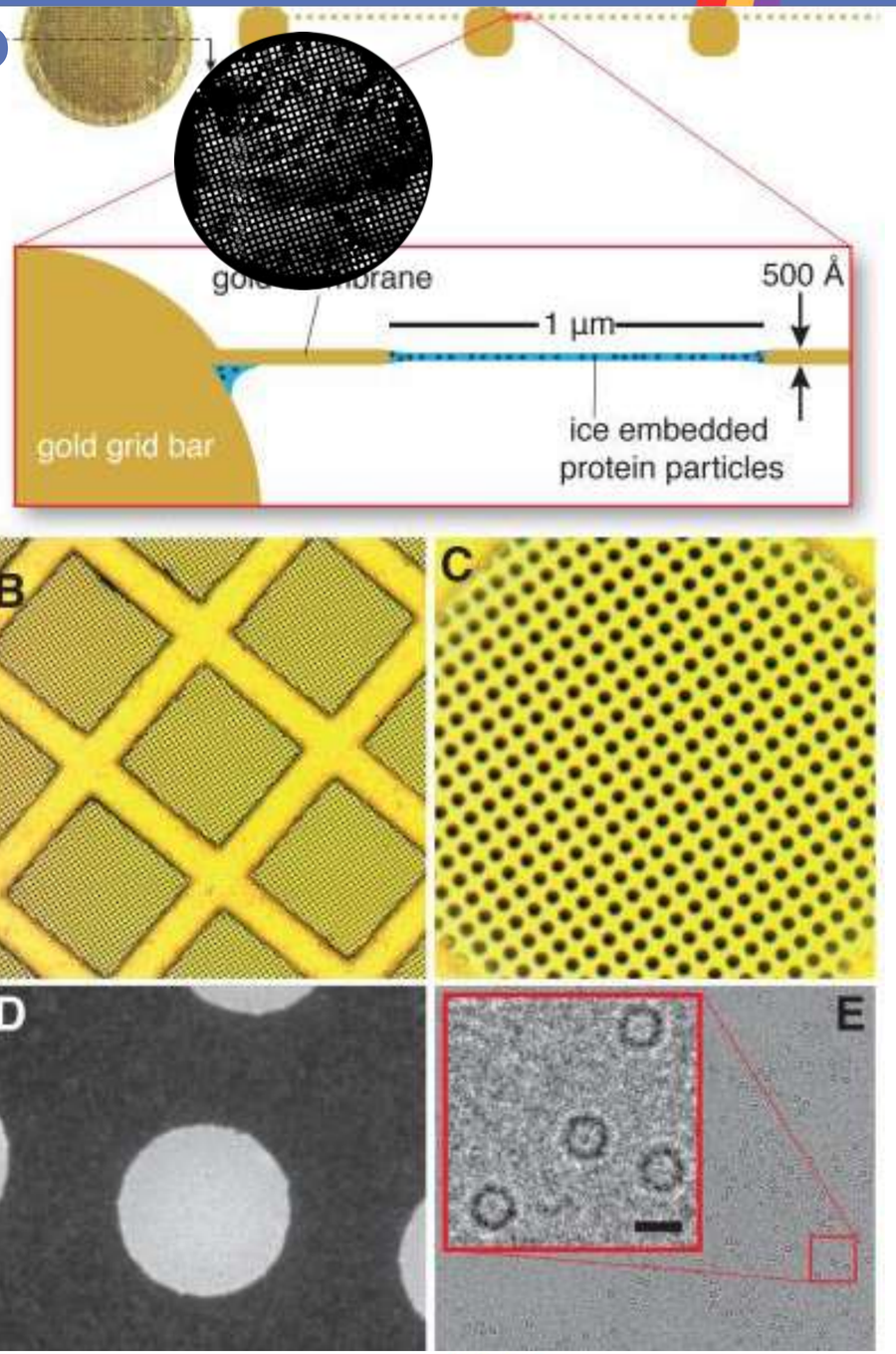
Advantages:

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

Disadvantages:

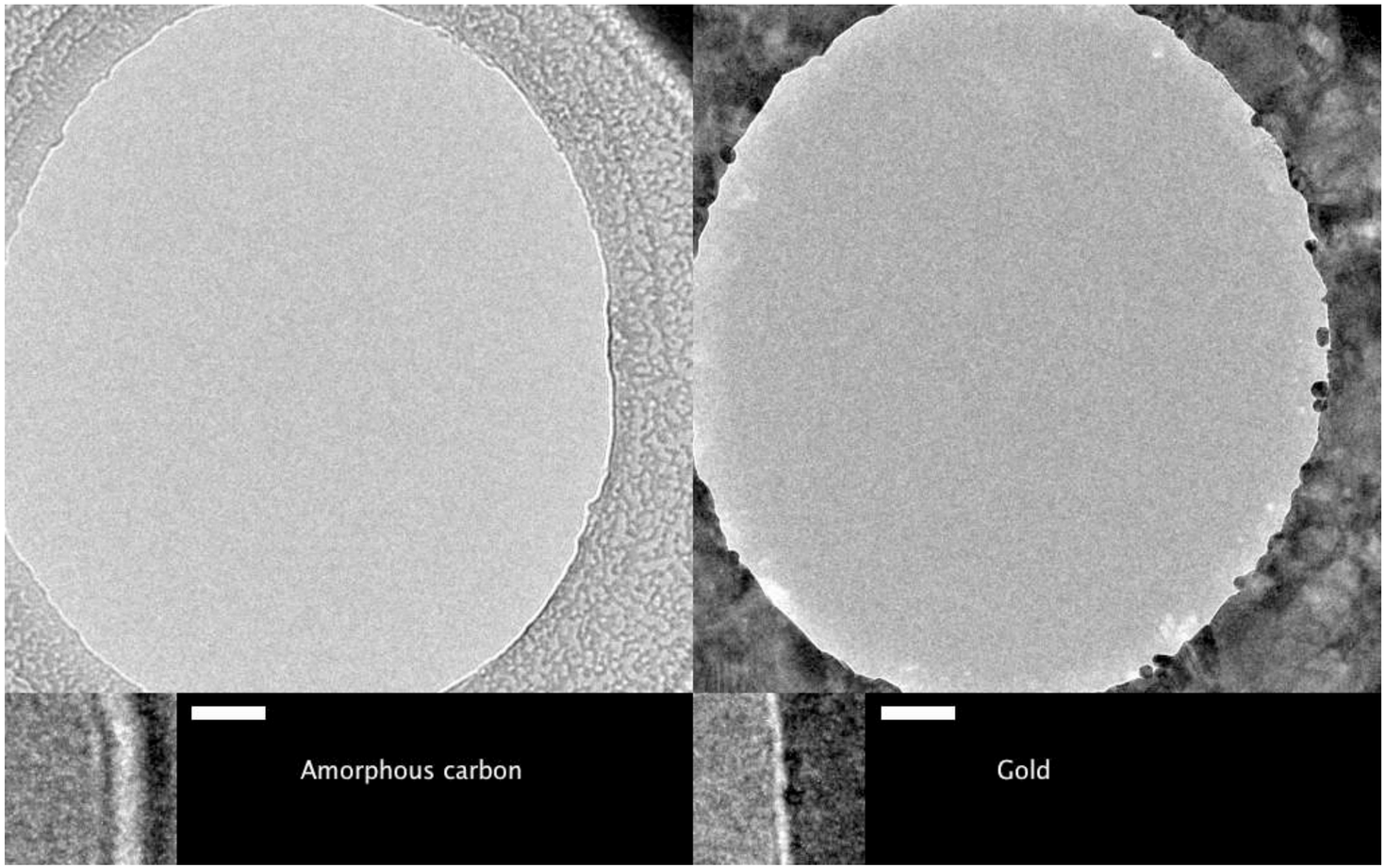
- Difficult to find focus due to lack of amorphous substrate

Russo & Passmore, 2015



What do EM grids look like?

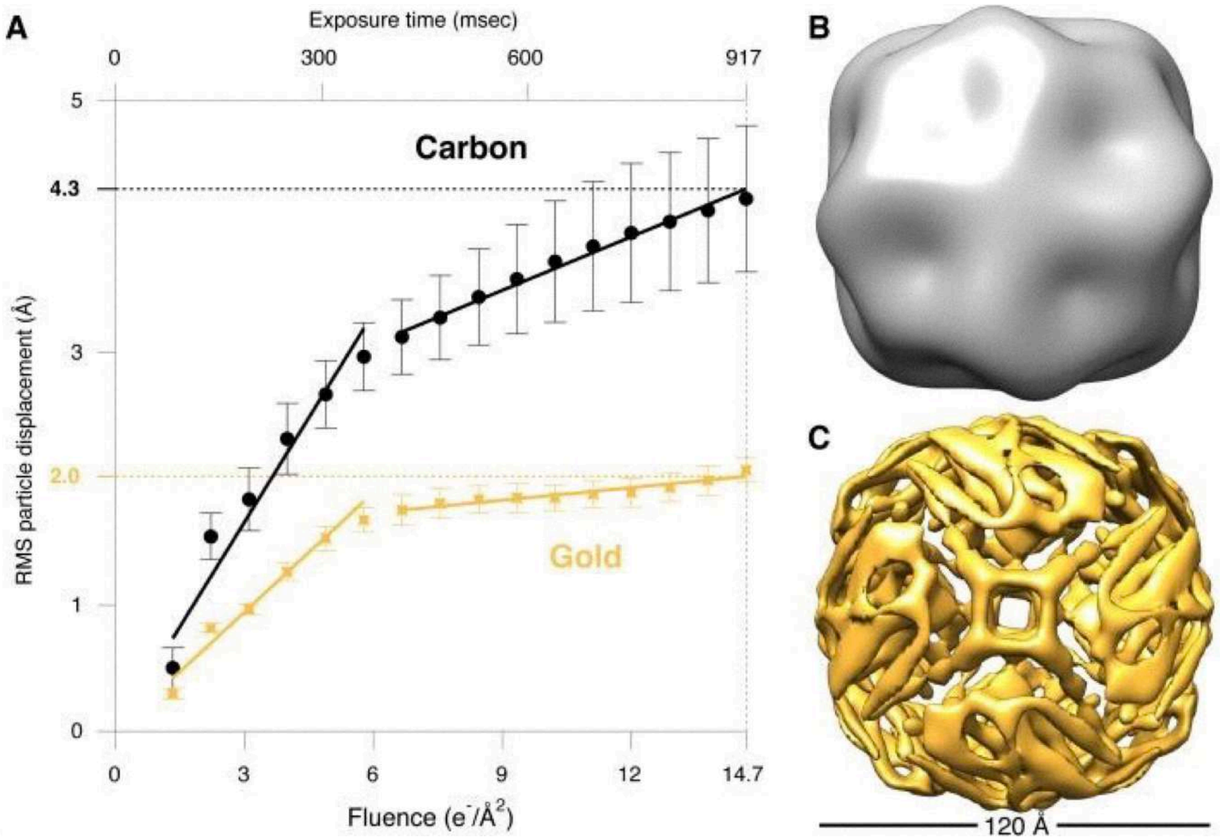
Gold grids



Russo & Passmore, 2015

What do EM grids look like?

Gold grids



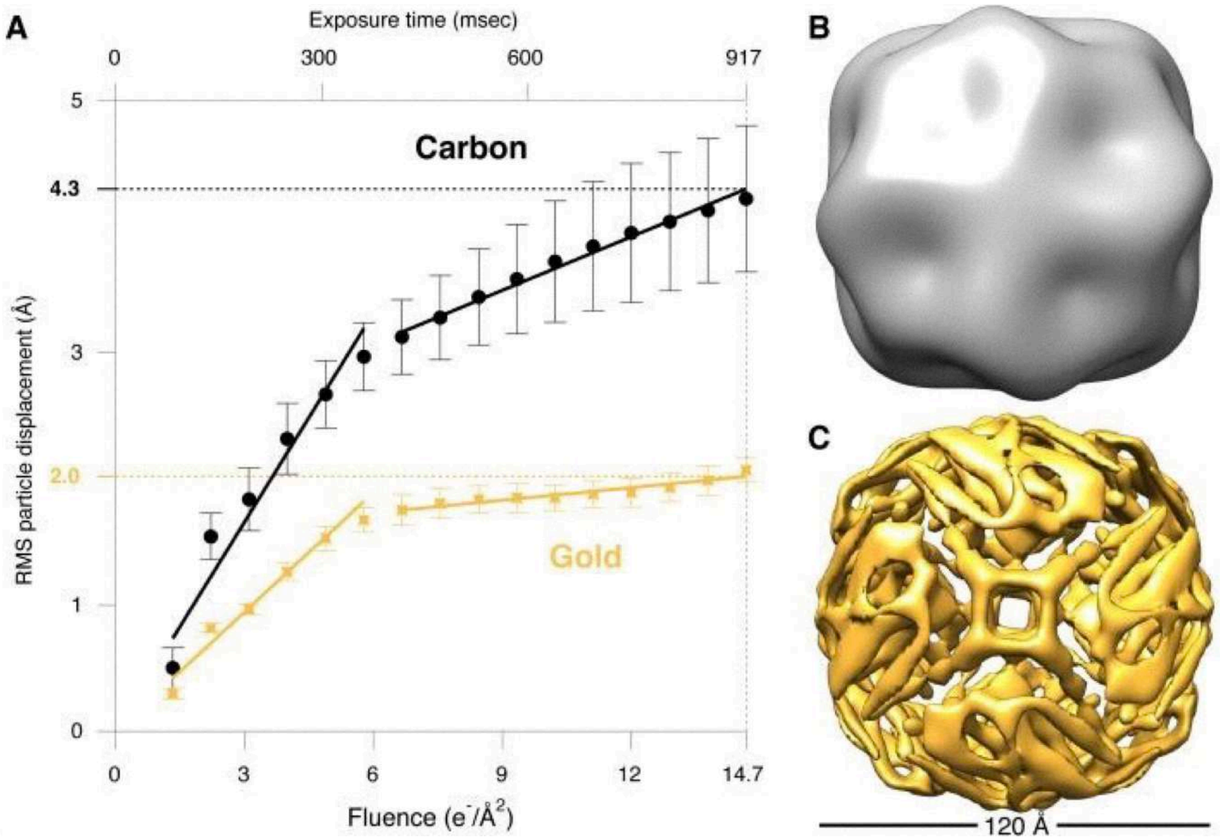
A. 80S ribosome movement during irradiation supported by amorphous carbon and gold using same imaging conditions.

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

What do EM grids look like?

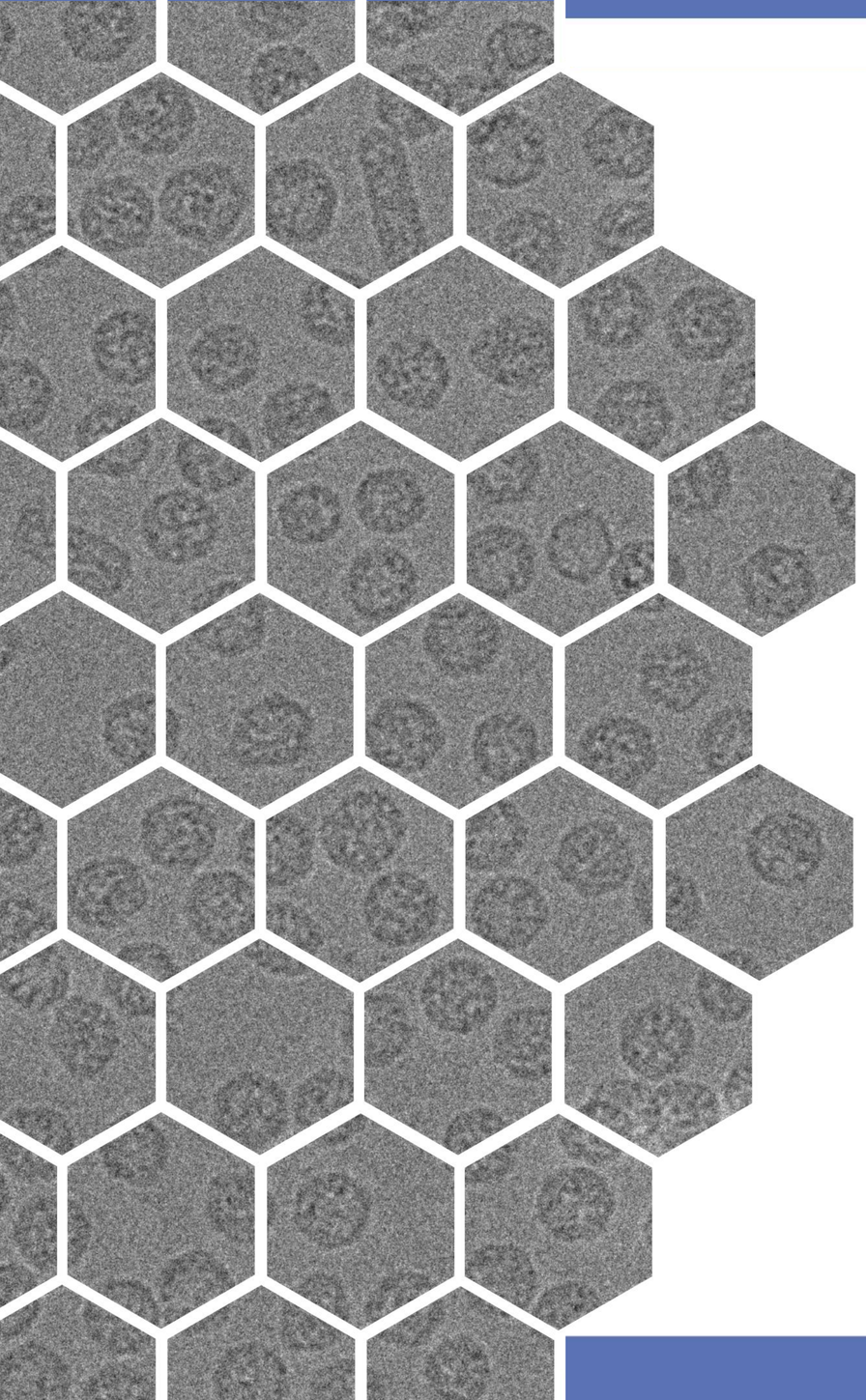
Gold grids



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

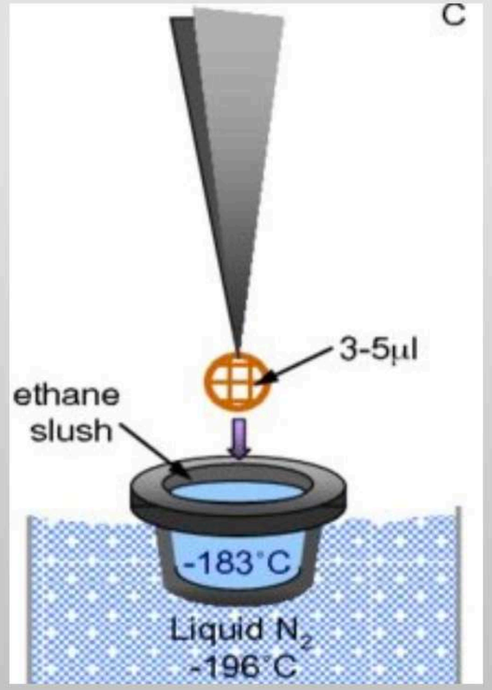


- Journal club and practical recap
- Considerations for biological cryoEM
  - Overview
  - Grids
  - What happens to a sample
  - Newer methods

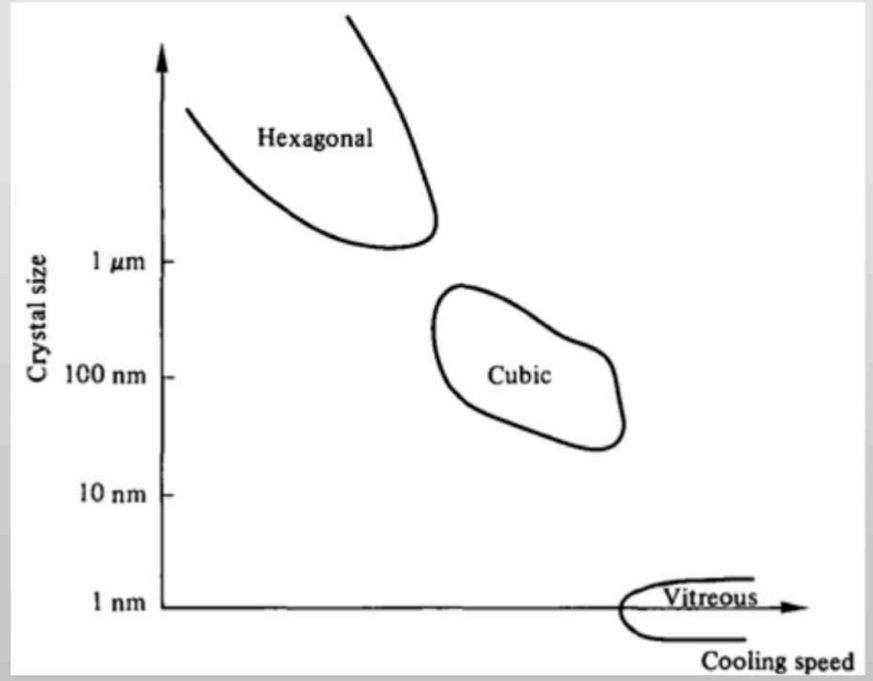
How are samples prepared for cryoEM?

Vitrification process

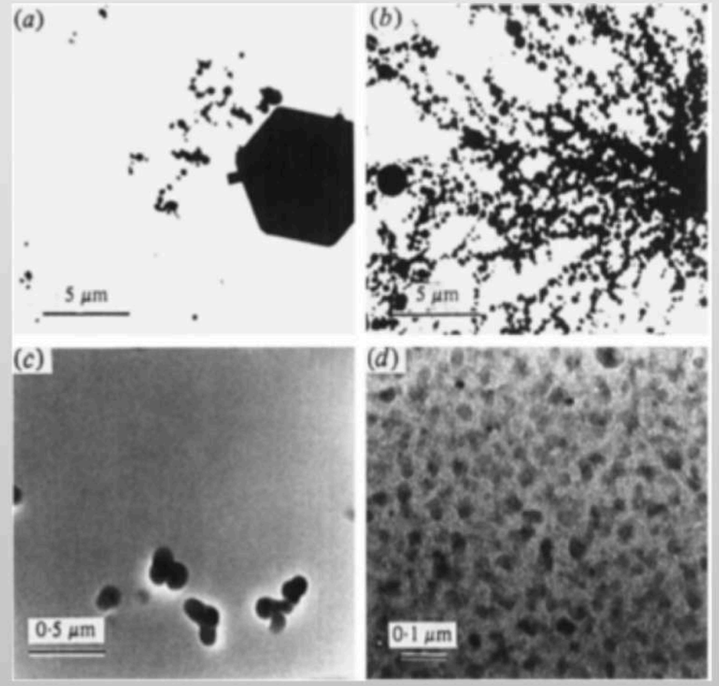
- Liquid ethane is a suitable coolant.
- Liquid nitrogen boils on contact, which makes it a poor coolant for cryo-EM.
- Cooling speed faster than 10^5-10^6 K/s ensure the formation of vitrified ice.



Setup of liquid ethane
(Image from Wen Jiang)



Cooling speed &
forms of ice

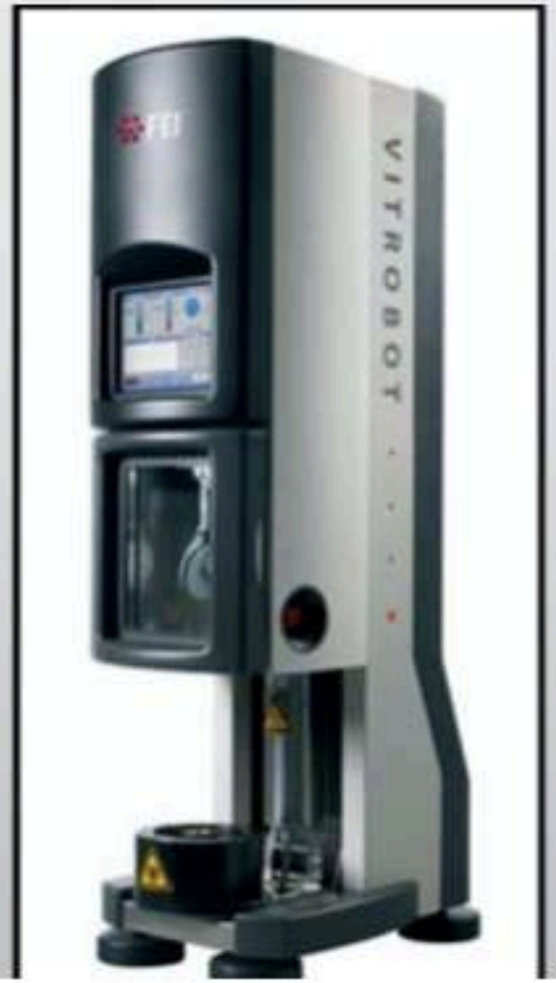


Different forms of ice contamination

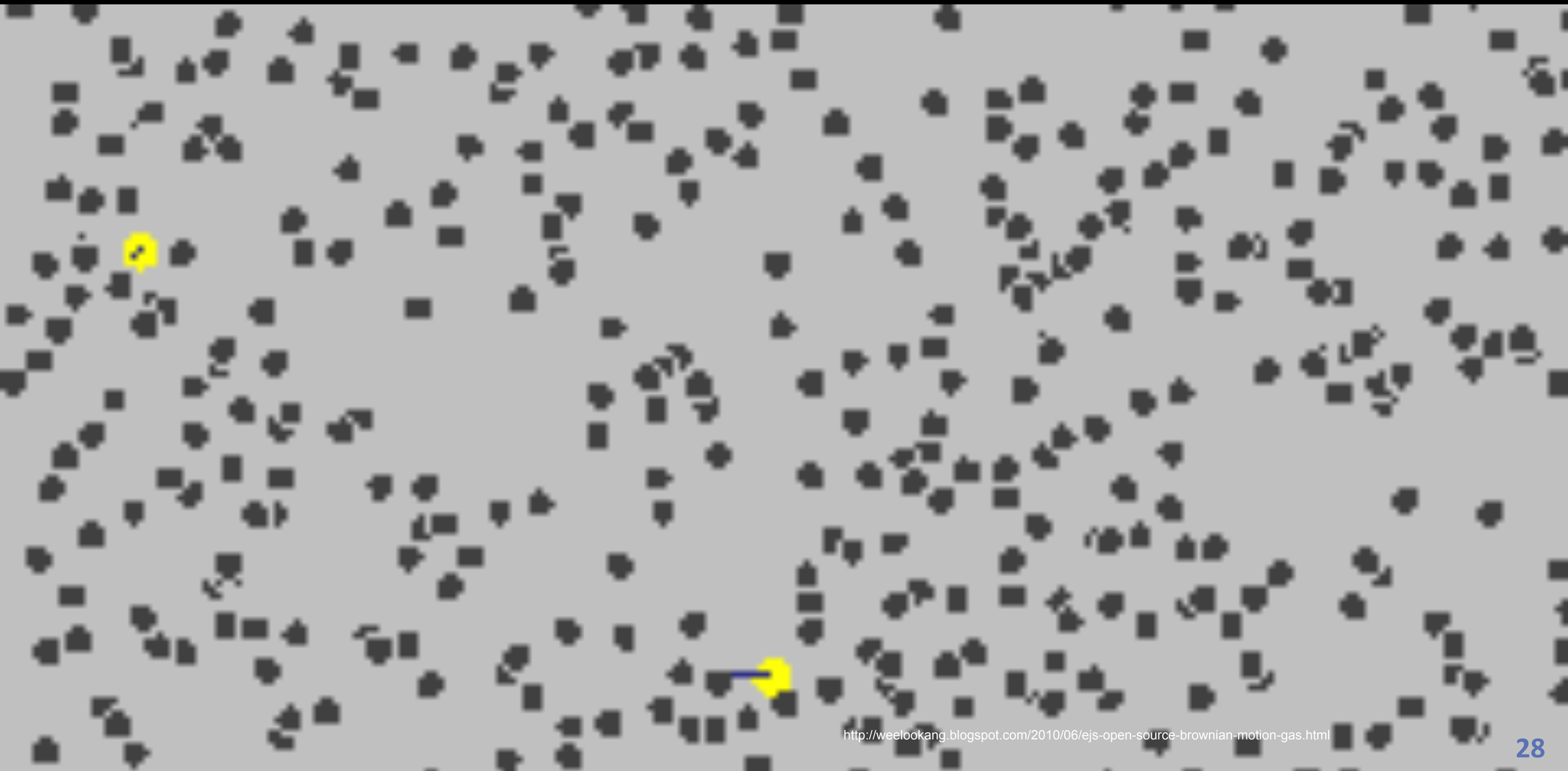
Jacques Dubochet et al., 1988

How are samples prepared for cryoEM?

Vitrification process



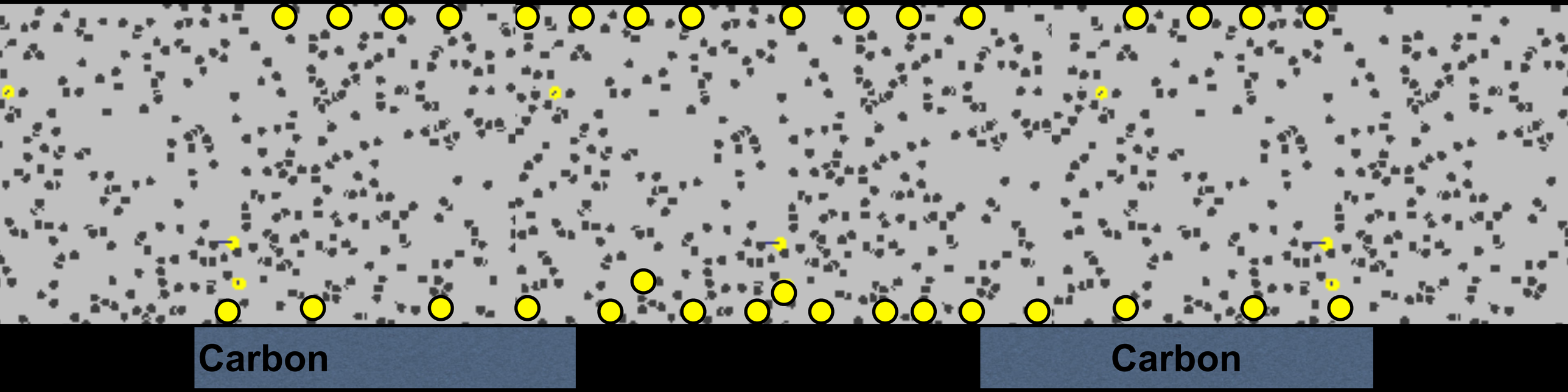
What happens to samples during vitrification?



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

What happens to samples during vitrification?

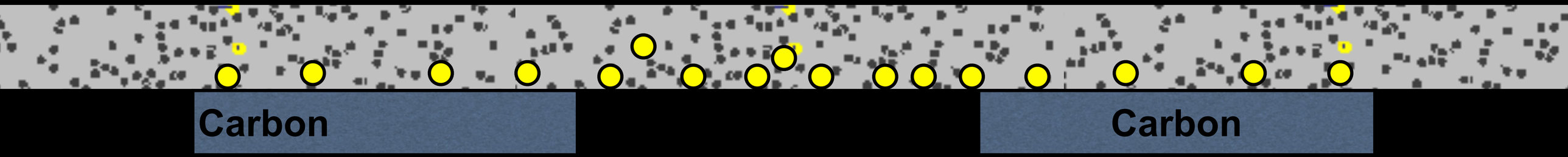
A hypothetical scenario during cryoEM grid preparation



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

What happens to samples during vitrification?

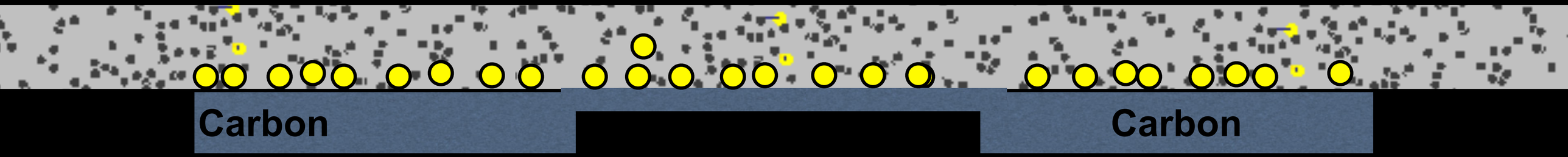
A hypothetical scenario during cryoEM grid preparation



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

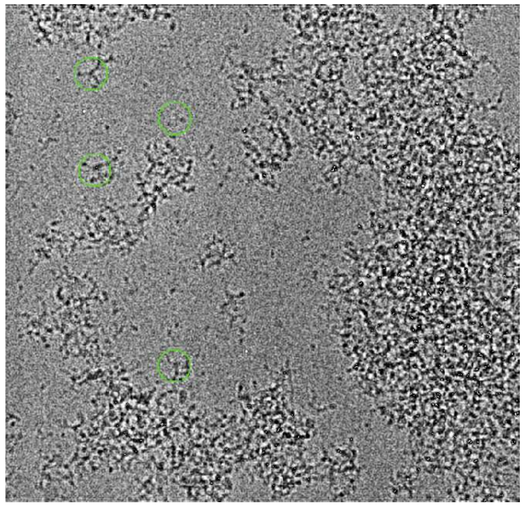
What happens to samples during vitrification?

A hypothetical scenario during cryoEM grid preparation

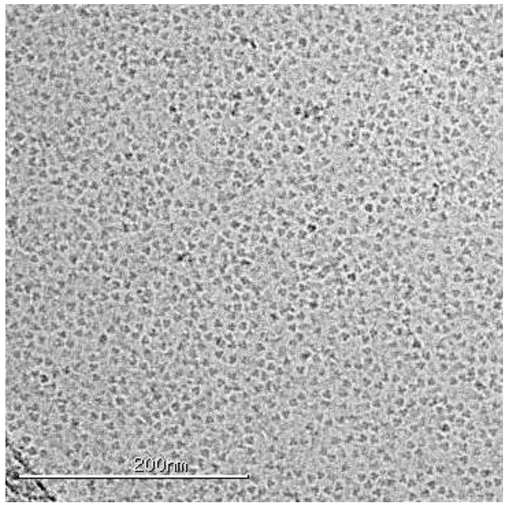


http://weelookang.blogspot.com/2010/06/ejs-open-source-brownian-motion-gas.html

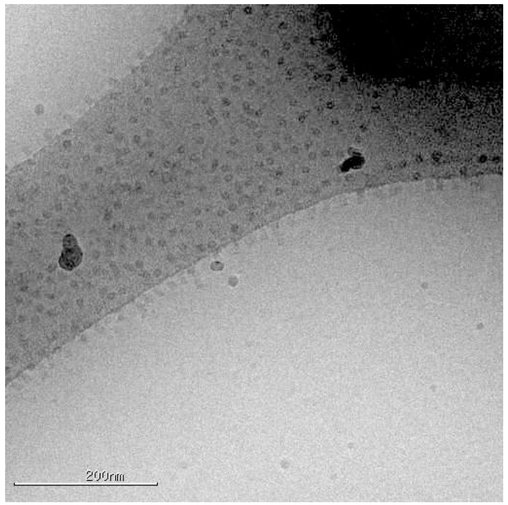
What issues arise?



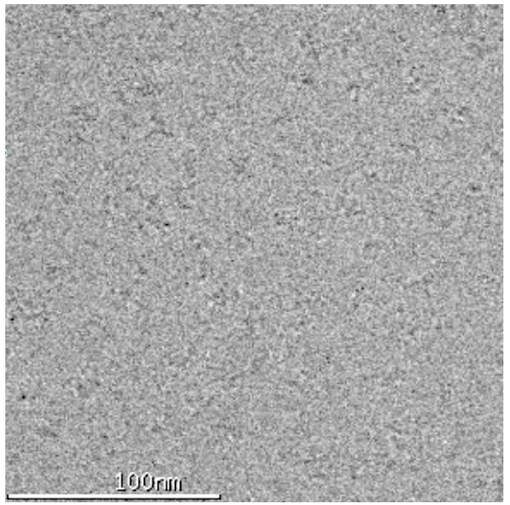
Aggregating in ice



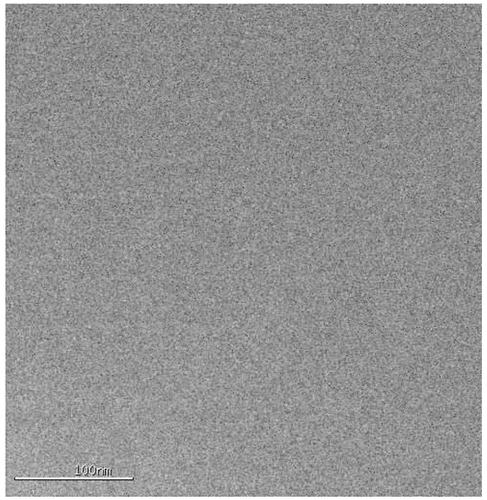
Preferred orientation



Particles not going into holes

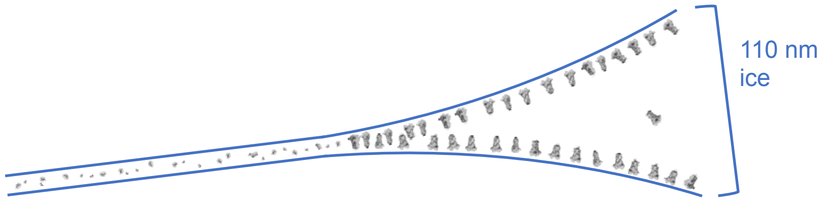


Rejecting 90% of particles



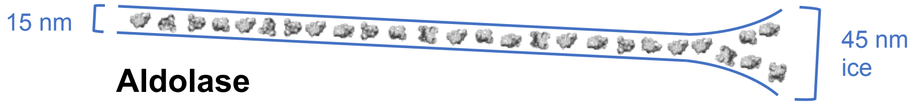
Particles disappearing in ice

What issues arise?

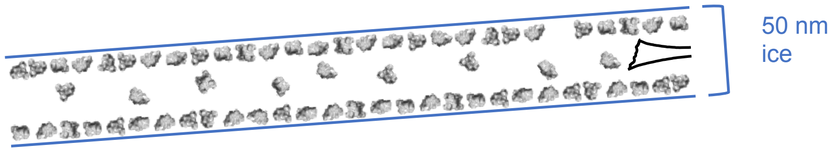


Hemagglutinin

Hemagglutinin



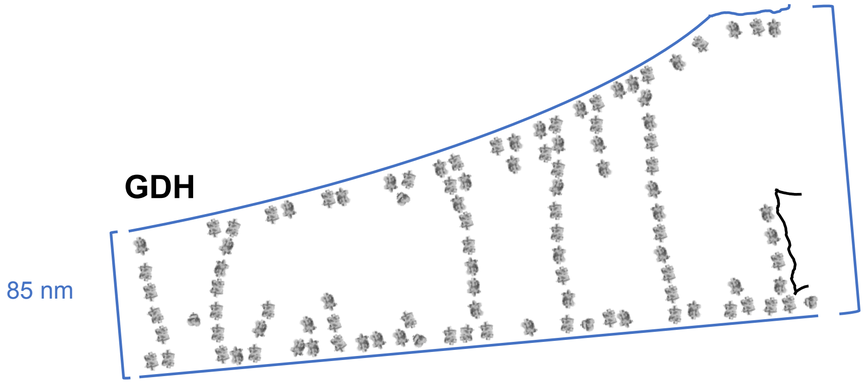
Aldolase

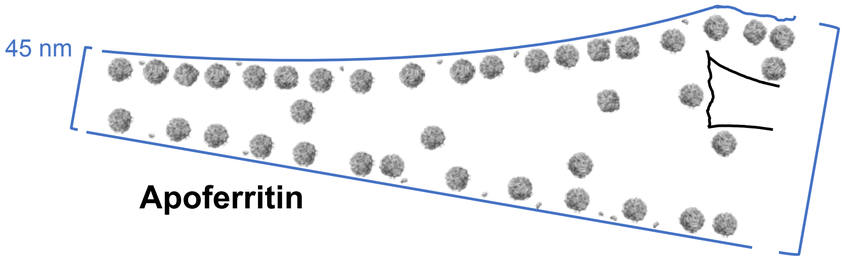


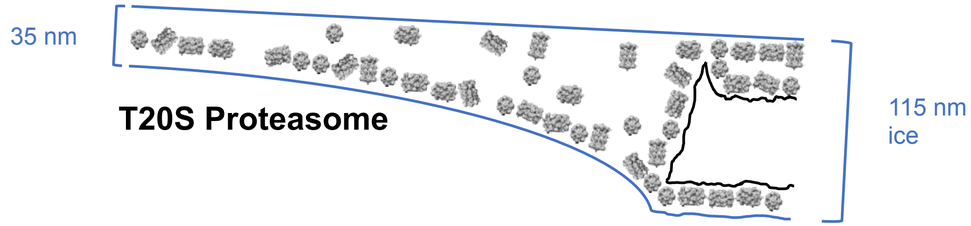
Aldolase

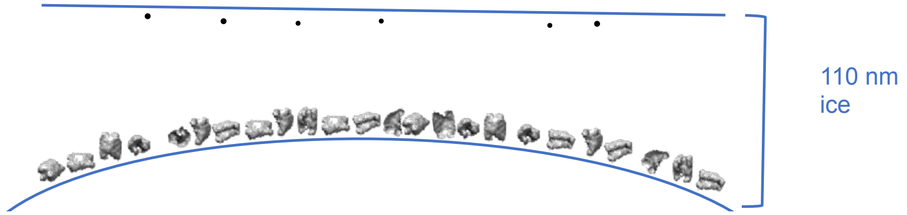
Noble AJ, et al. Routine single particle CryoEM sample and grid characterization by tomography. Elife. 2018;7.

Alex Noble









What issues arise?

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

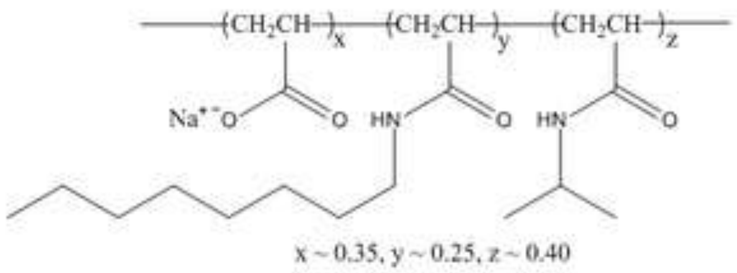
Filamentous protein

- 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.

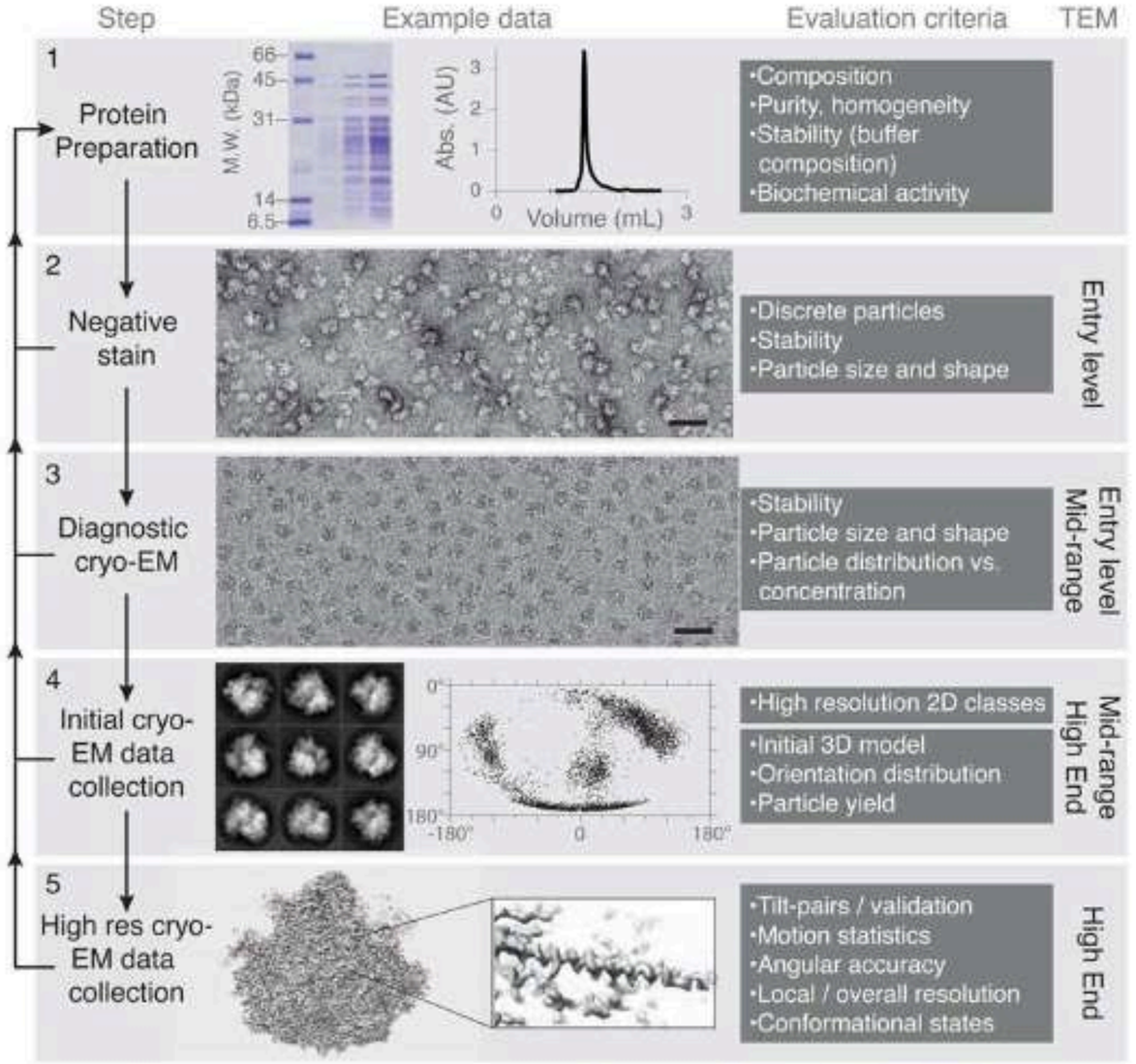
Drulyte, I et al. (2018) Acta Crystallogr D Struct Biol 74(Pt 6), 560-571.

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

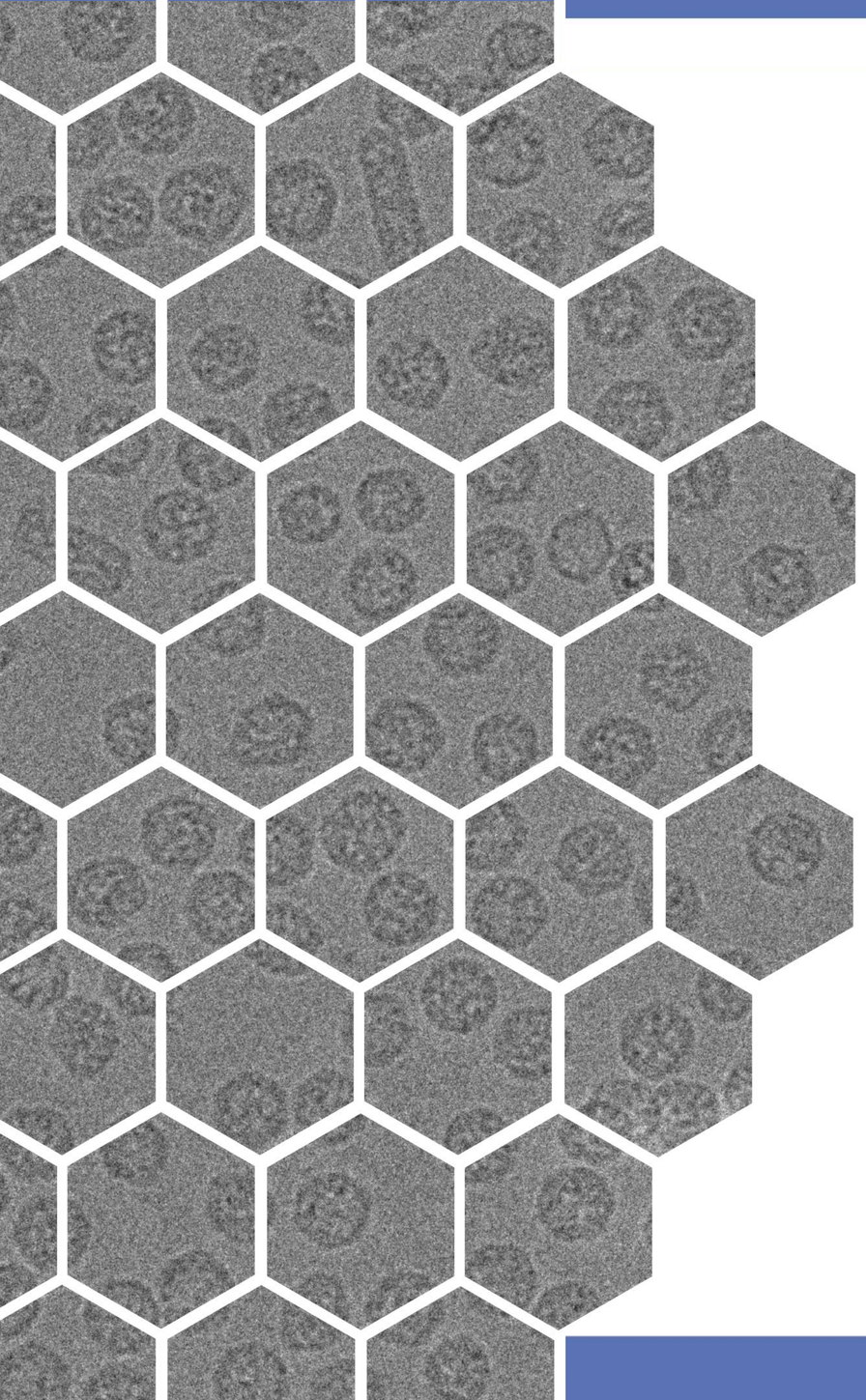
Preparing EM ready samples

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/



- Journal club and practical recap
- Considerations for biological cryoEM
  - Overview
  - Grids
  - What happens to a sample
  - Newer methods

Other methods

Spotiton

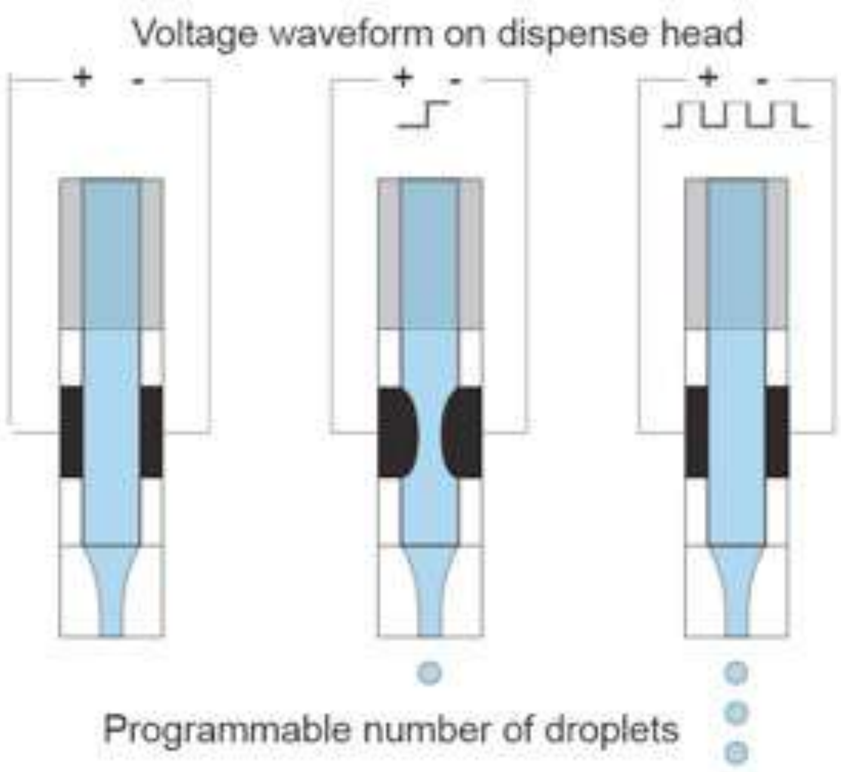
Improving Current CryoTEM Grid Preparation Methods

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

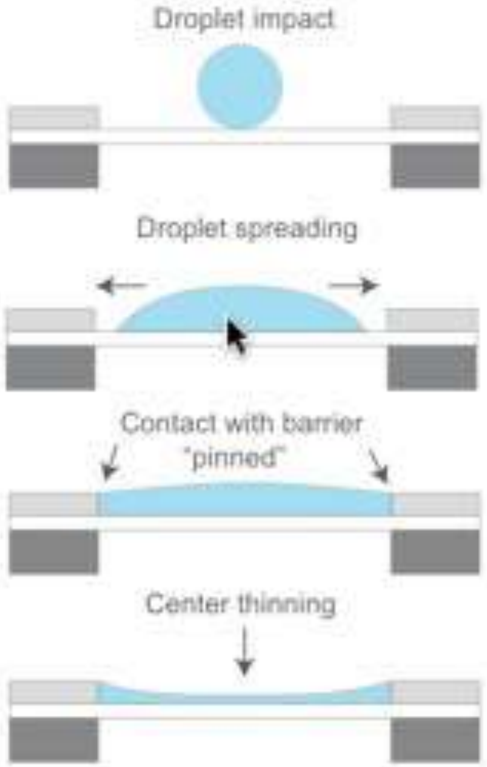
Venkat Dandey

Hui Wei

Accurate pL dispensing



Thin films without blotting



Other methods

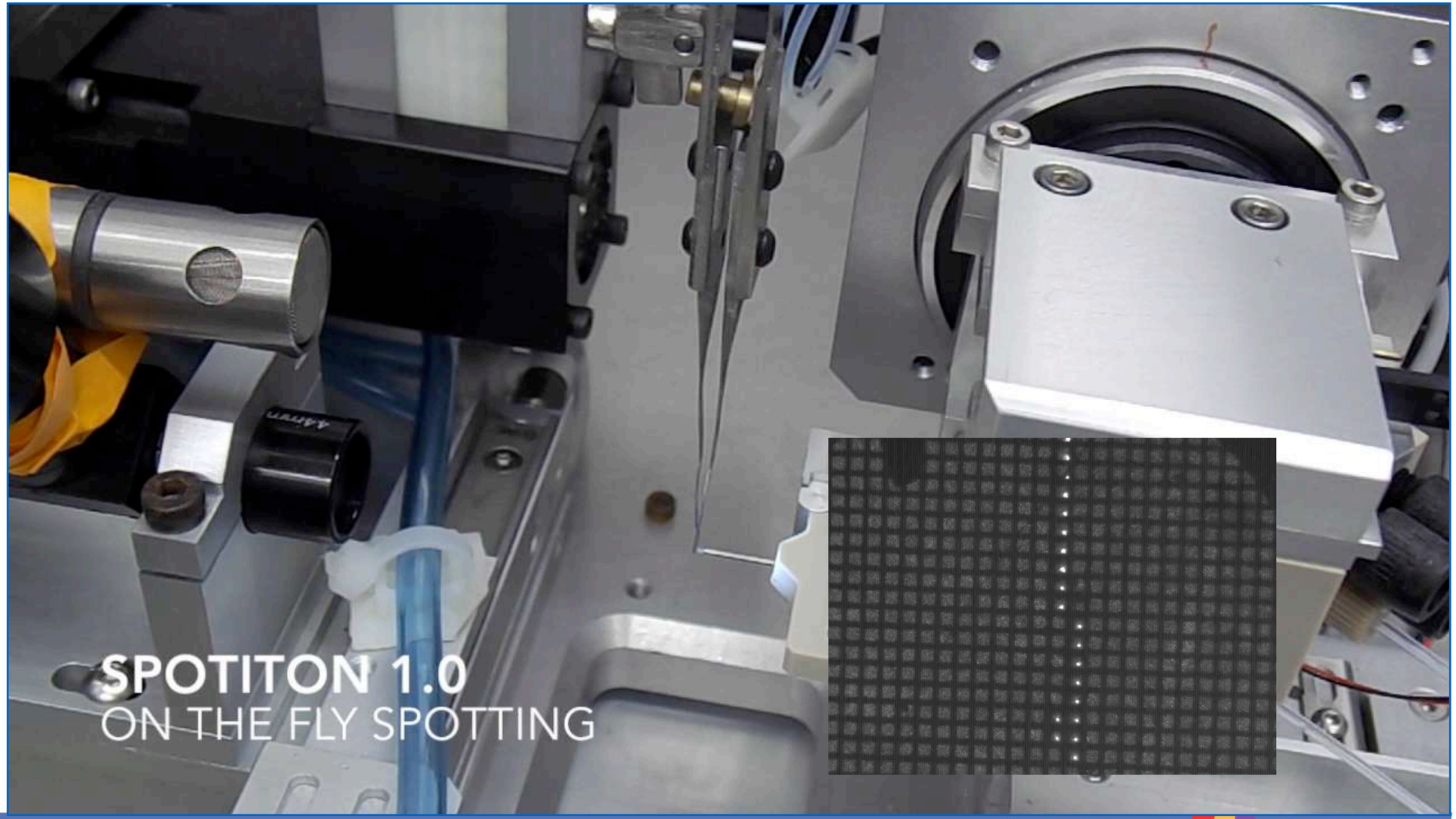
Spotiton

Improving Current CryoTEM Grid Preparation Methods

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



Other methods

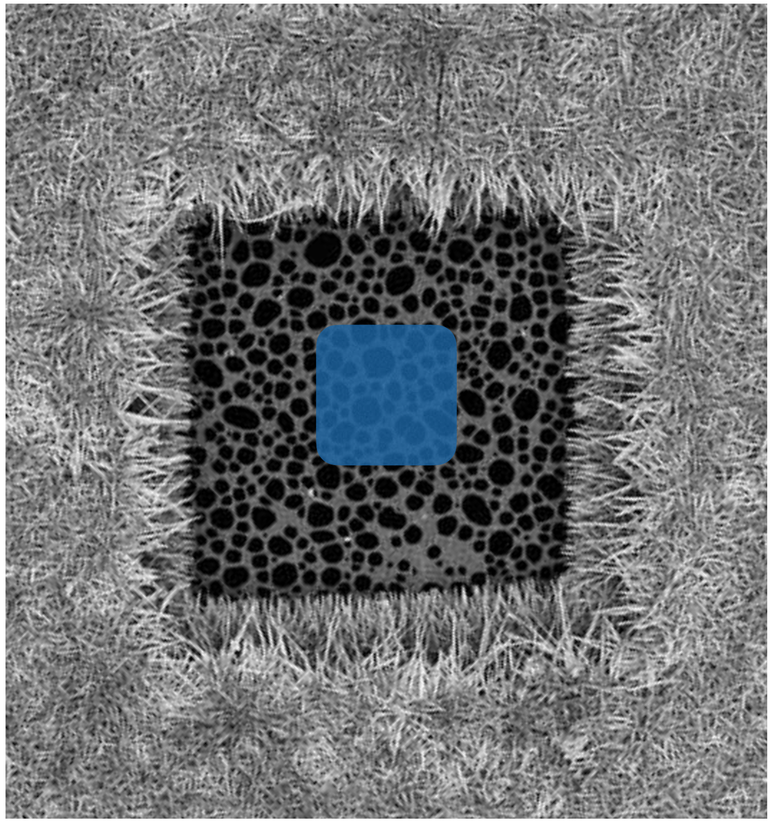
Spotiton

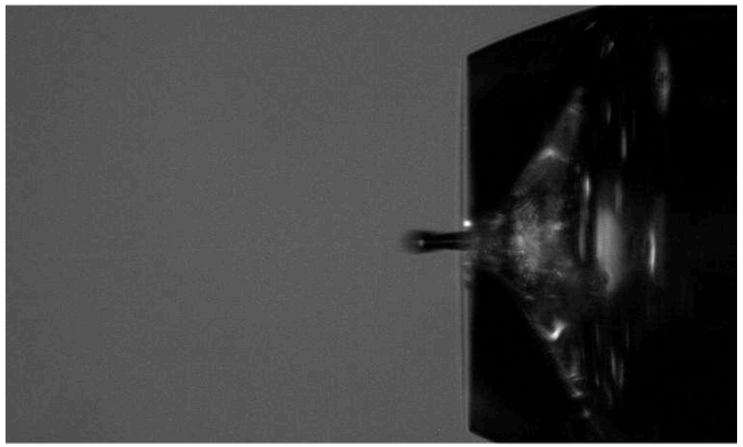
Improving Current CryoTEM Grid Preparation Methods

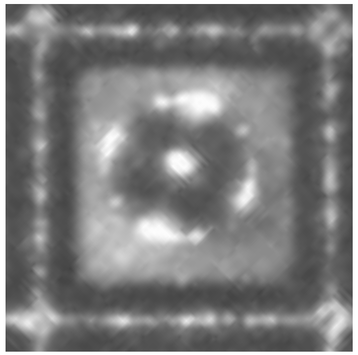
Wei H, Dandey VP, Zhang Z, Raczkowski A, Rice WJ, Carragher B, Potter CS. Optimizing "self-wicking" nanowire grids. J Struct Biol. 2018;202(2):170-4.

Venkat Dandey

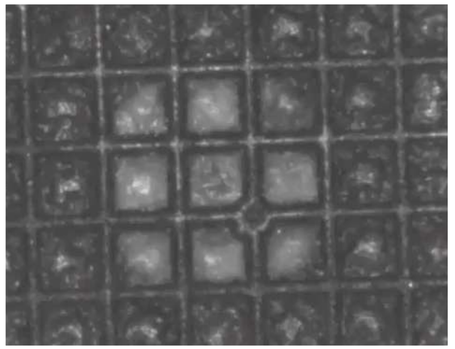
Hui Wei







Single frame from loop



Video loop

Other methods

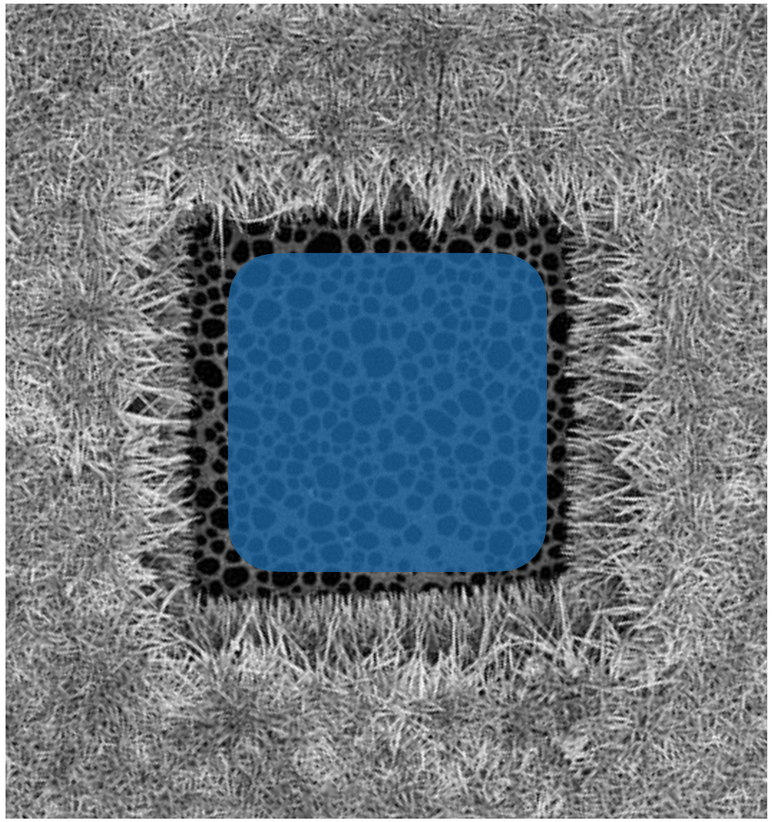
Improving Current CryoTEM Grid Preparation Methods

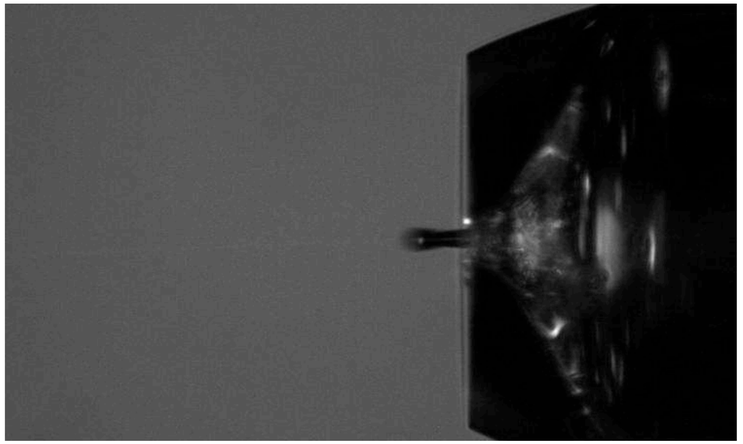
Spotiton

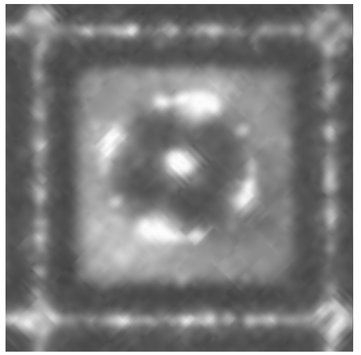
Wei H, Dandey VP, Zhang Z, Raczkowski A, Rice WJ, Carragher B, Potter CS. Optimizing "self-wicking" nanowire grids. J Struct Biol. 2018;202(2):170-4.

Venkat Dandey

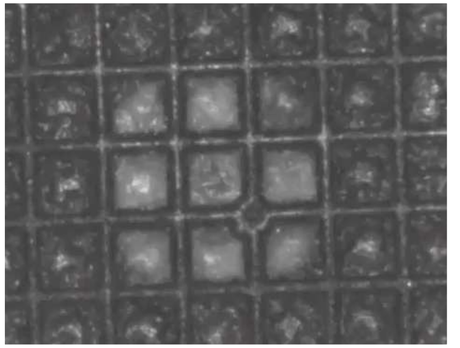
Hui Wei







Single frame from loop



Video loop

Other methods

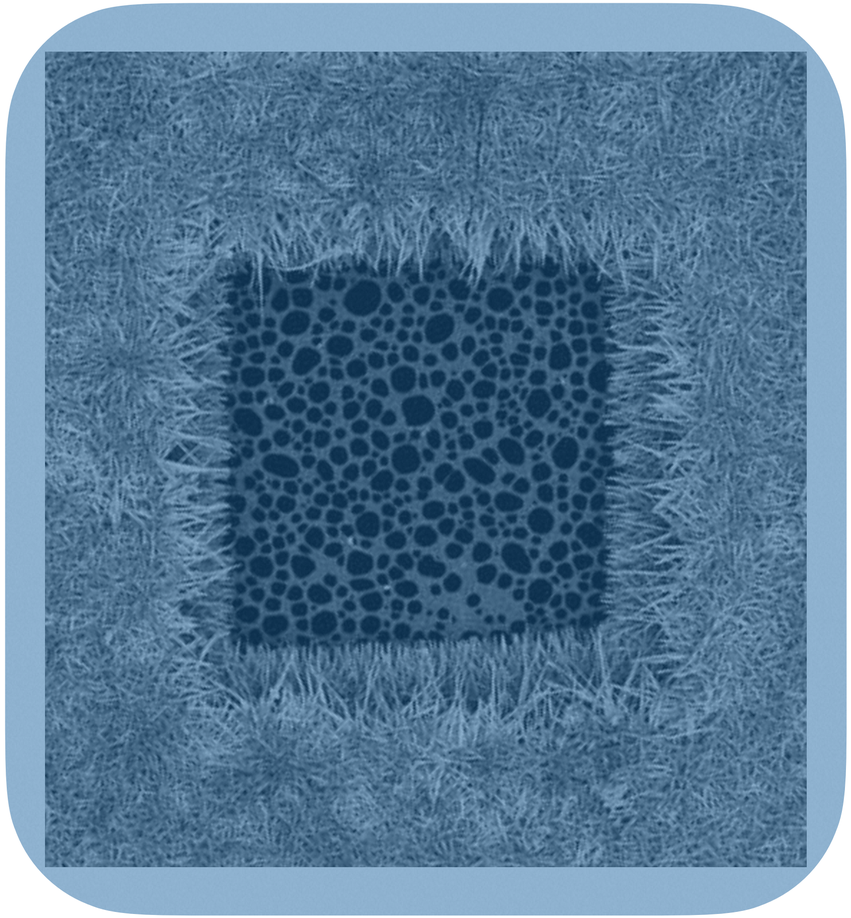
Spotiton

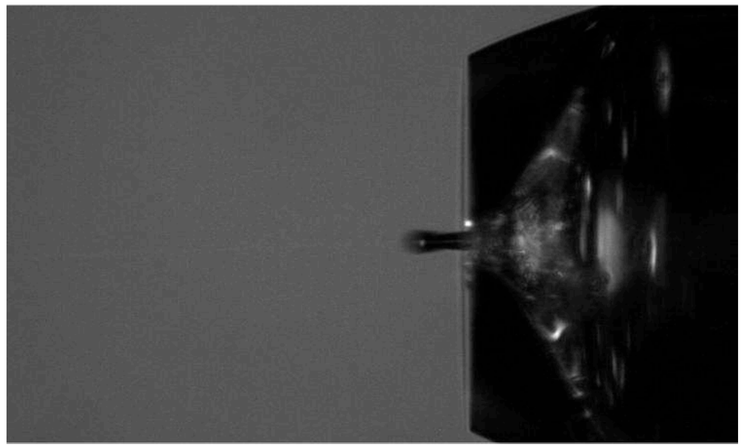
Improving Current CryoTEM Grid Preparation Methods

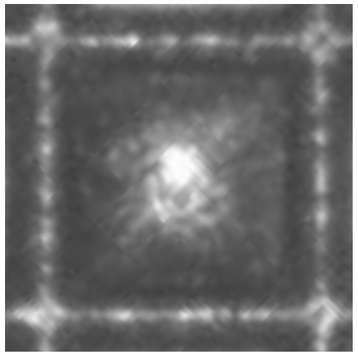
Wei H, Dandey VP, Zhang Z, Raczkowski A, Rice WJ, Carragher B, Potter CS. Optimizing "self-wicking" nanowire grids. J Struct Biol. 2018;202(2):170-4.

Venkat Dandey

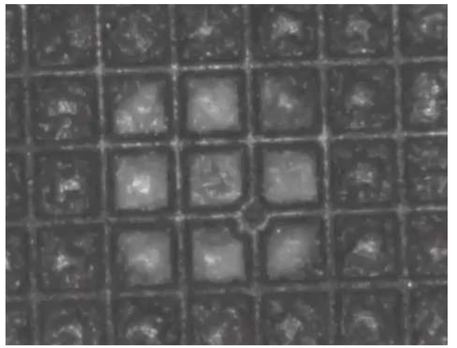
Hui Wei







Single frame from loop



Video loop

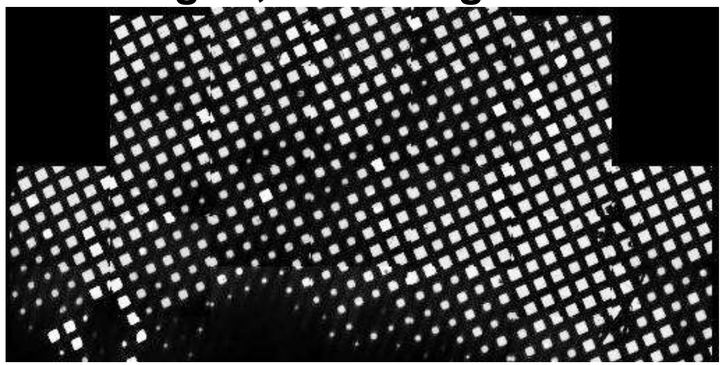
Other methods

Spotiton

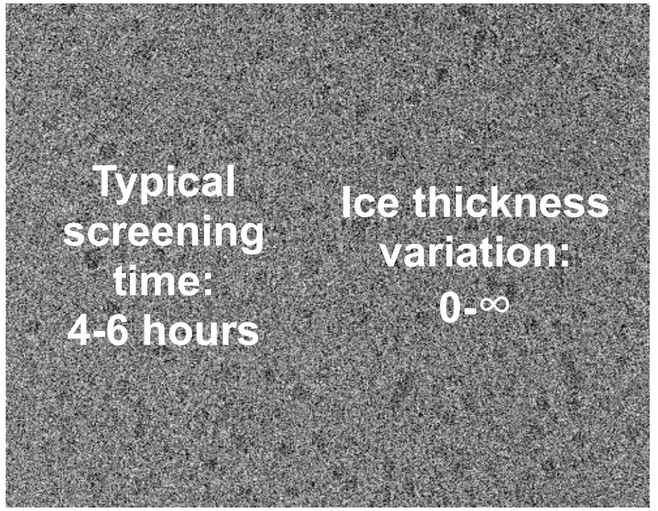
Improving Current CryoTEM Grid Preparation Methods

Vitrobot

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

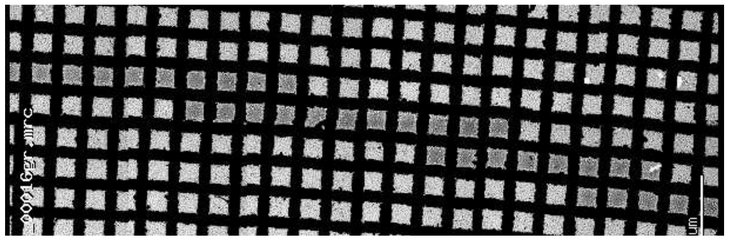


Usable area: ~0-10%

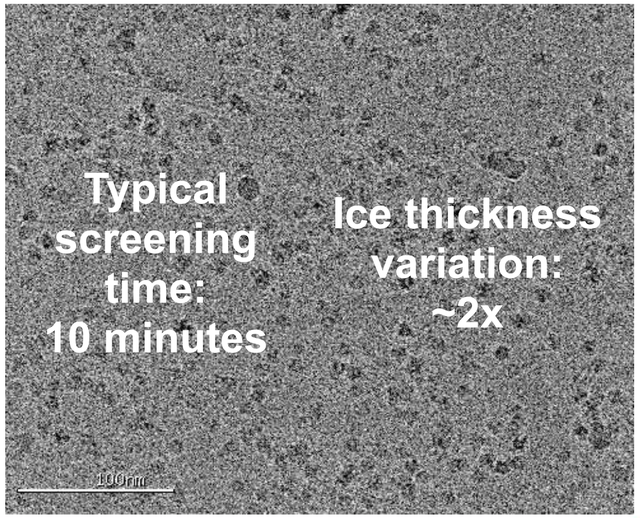


Spotiton

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.

Venkat Dandey

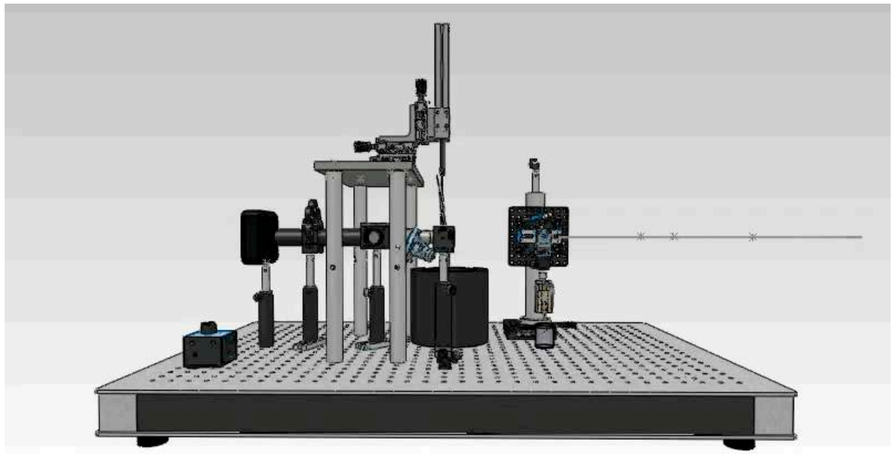
Hui Wei

What is chameleon?

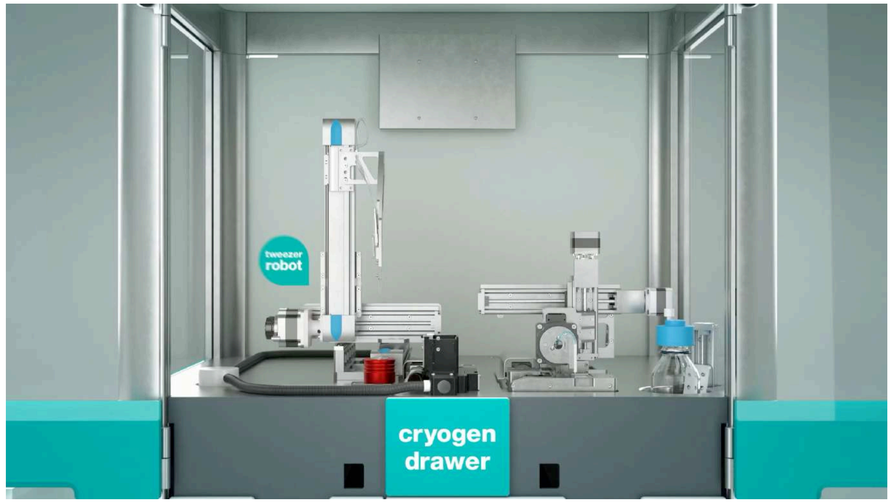
Spotiton

The Spotiton Project: Commercialization

Spotiton concept: 2011



Chameleon: 2019



sptlabtech

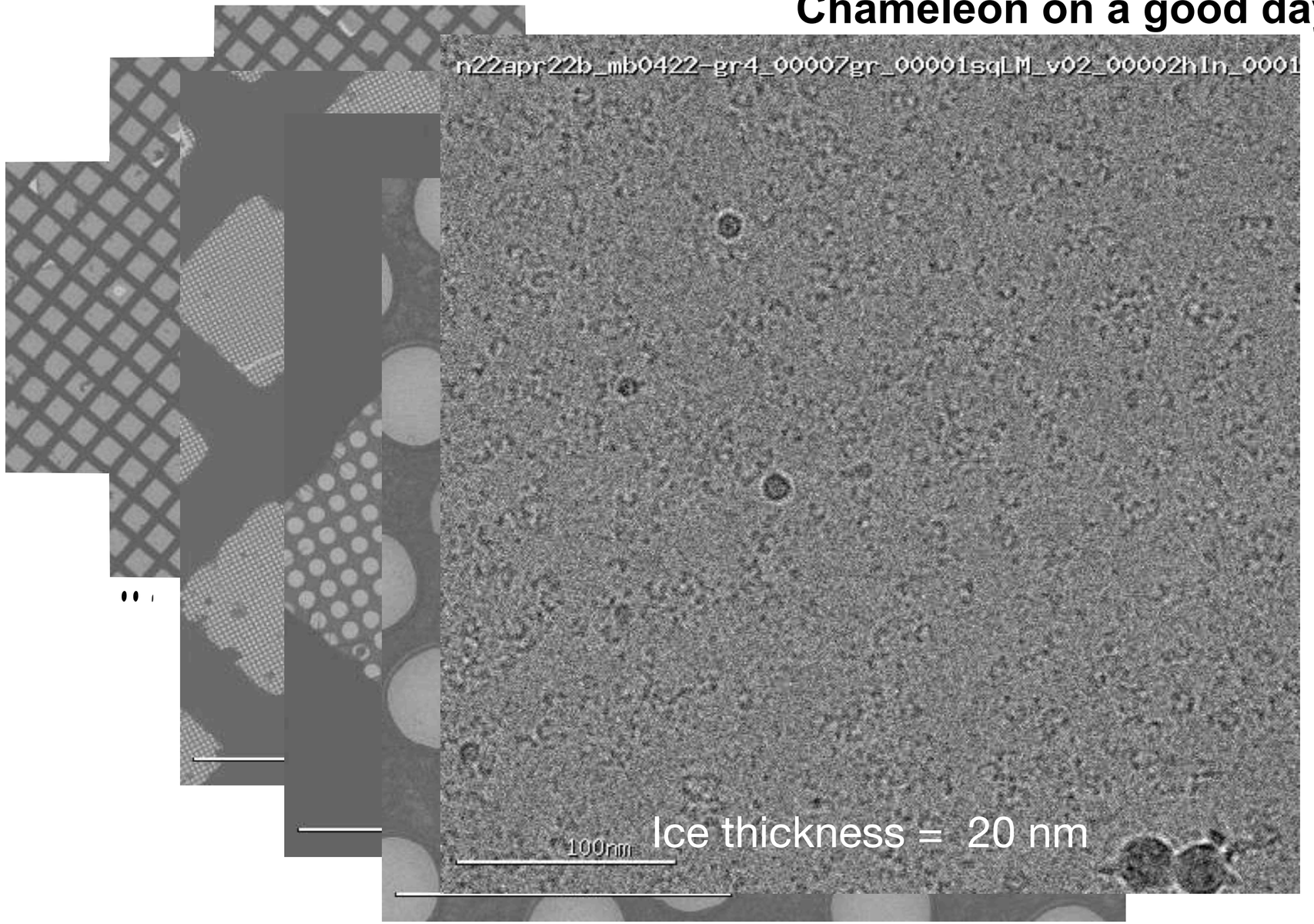

Venkat Dandey


Alex Wei


Chase Budell

What is chameleon?

Chameleon on a good day



Ice thickness = 20 nm

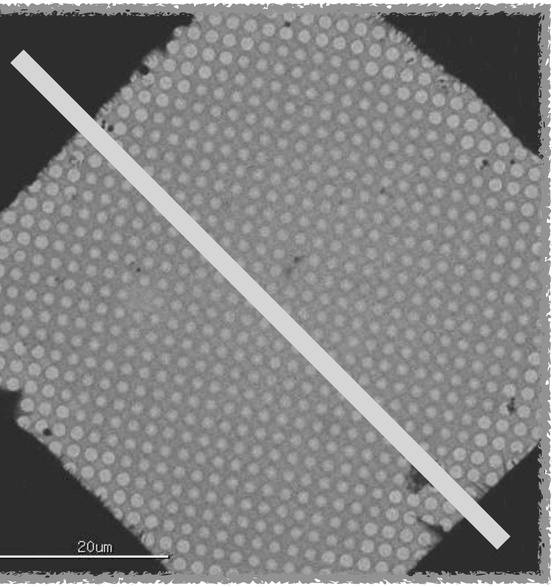
Spotiton

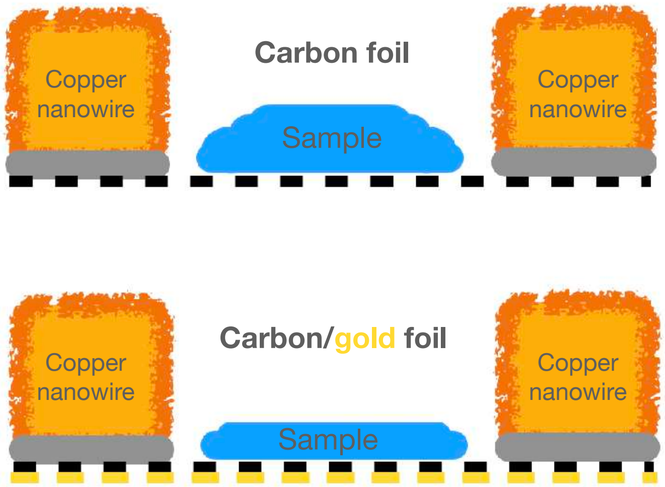

Mahira Aragon

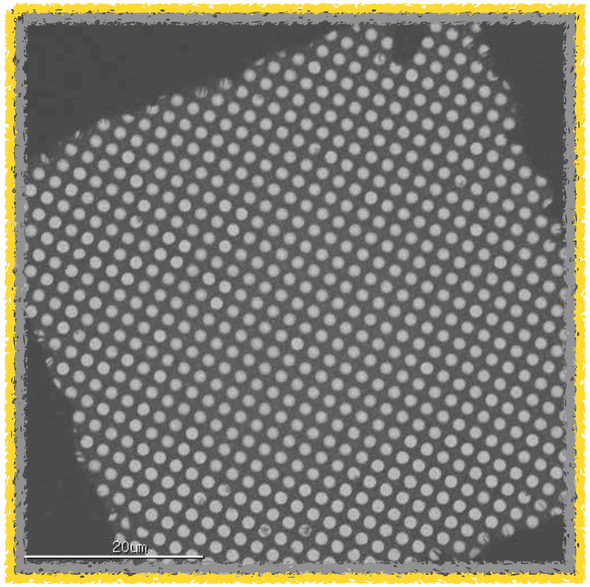

Alex Wei


Chase Budell

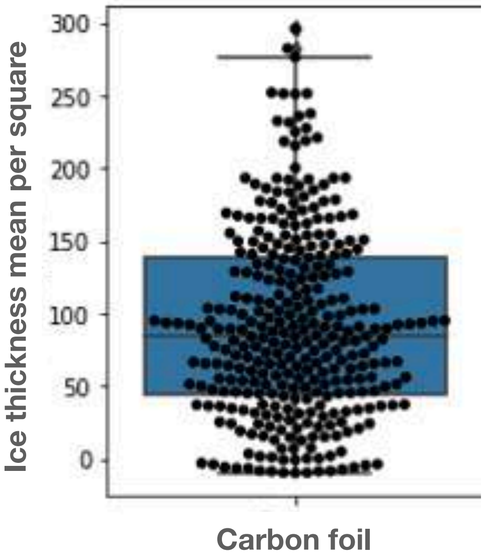
What is chameleon?

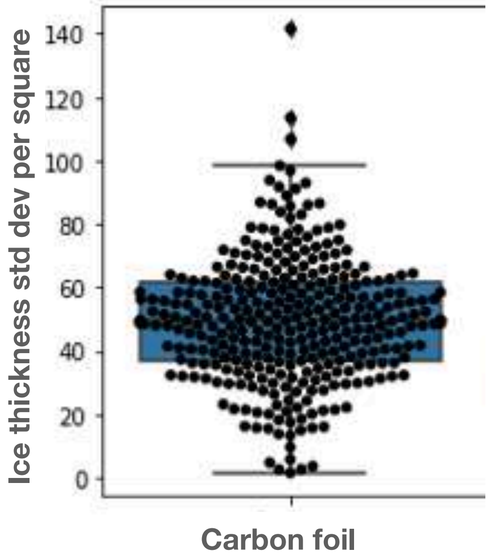






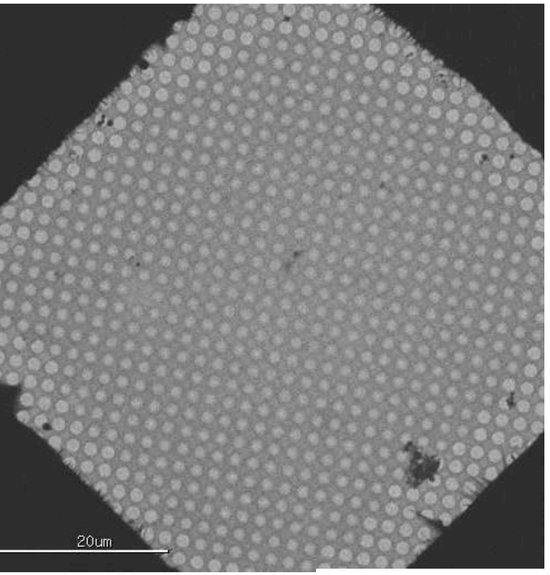
Spotiton

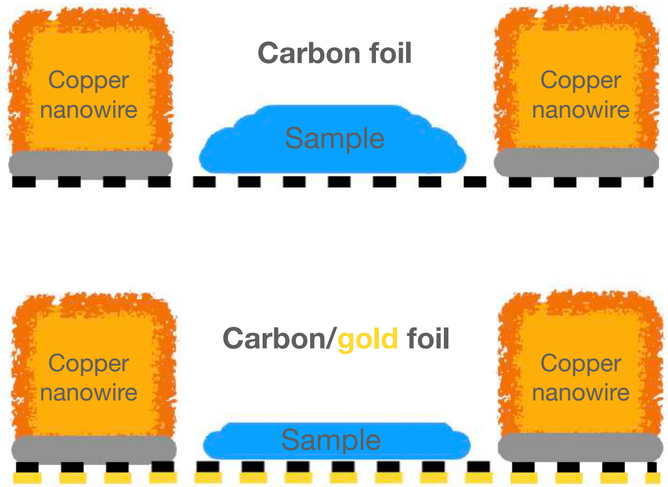


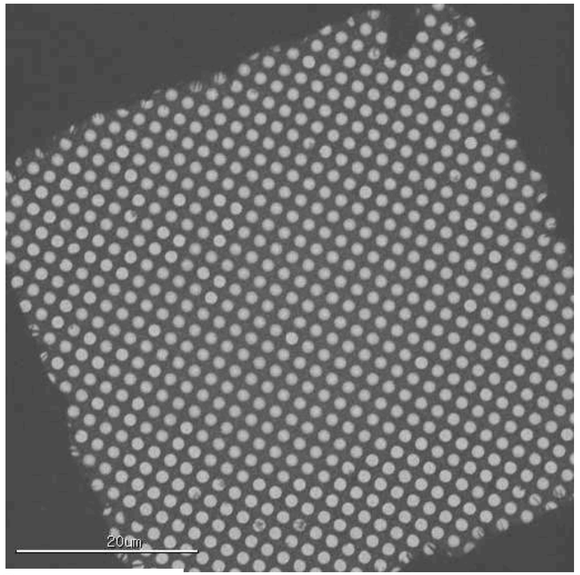


Alex Wei Eugene Chua

What is chameleon?

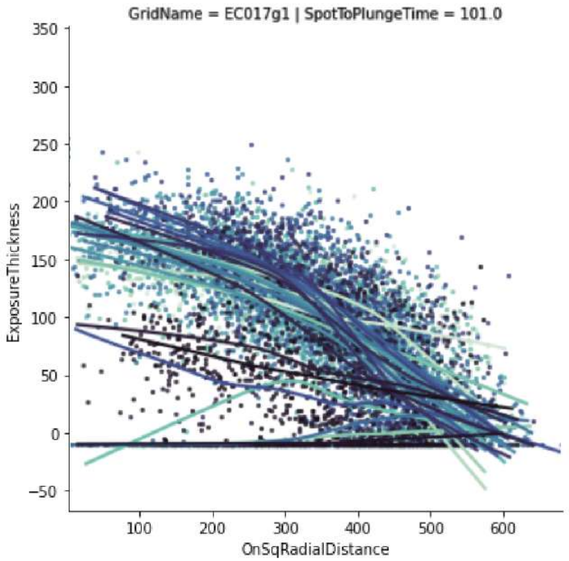




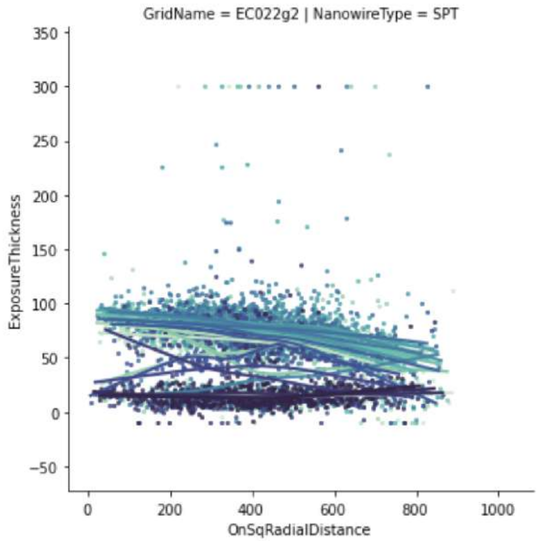


Spotiton

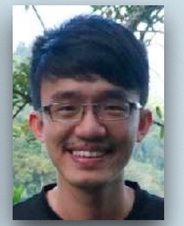
Batch 413



Batch 392



Alex Wei



Eugene Chua

What is chameleon?

Where is the ice relative to the grid?

Spotiton

Grid geometry

Vitreous ice ideal -> typical thickness

What is chameleon?

Where is the ice relative to the grid?

Spotiton

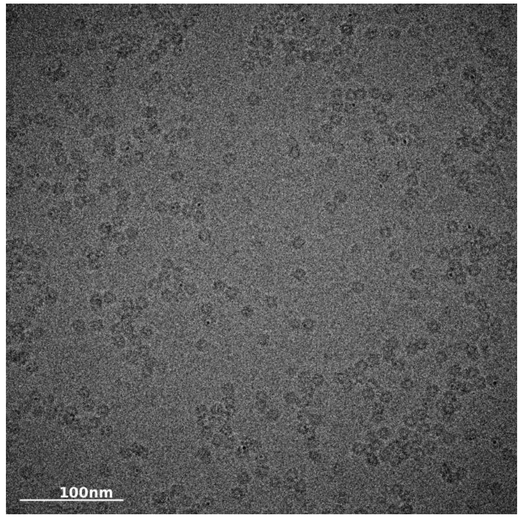
Does the substate determine this?

What is chameleon?

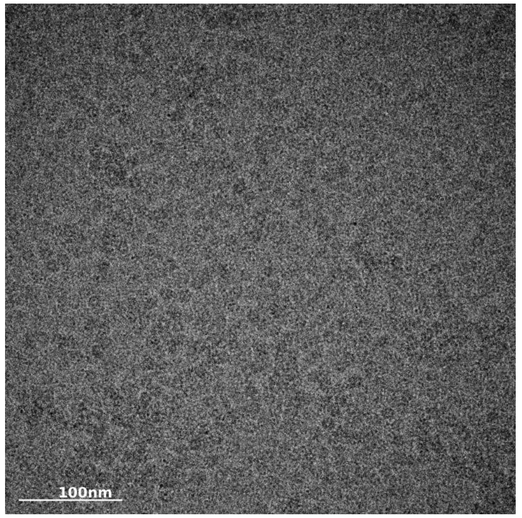
Where is the ice relative to the grid?

Spotiton

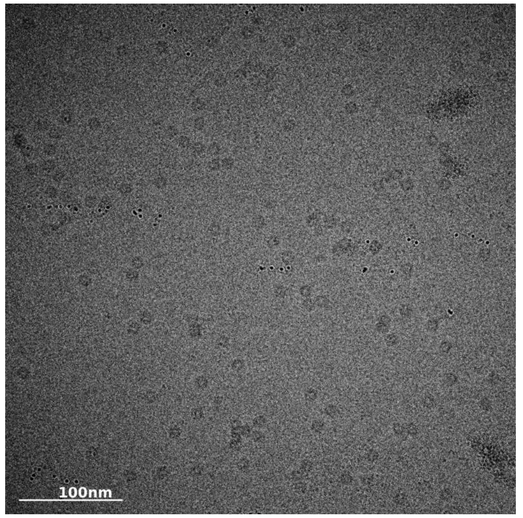
Add a graphene layer under the substrate



No graphene (8 mg/ml)



Graphene (8mg/ml)



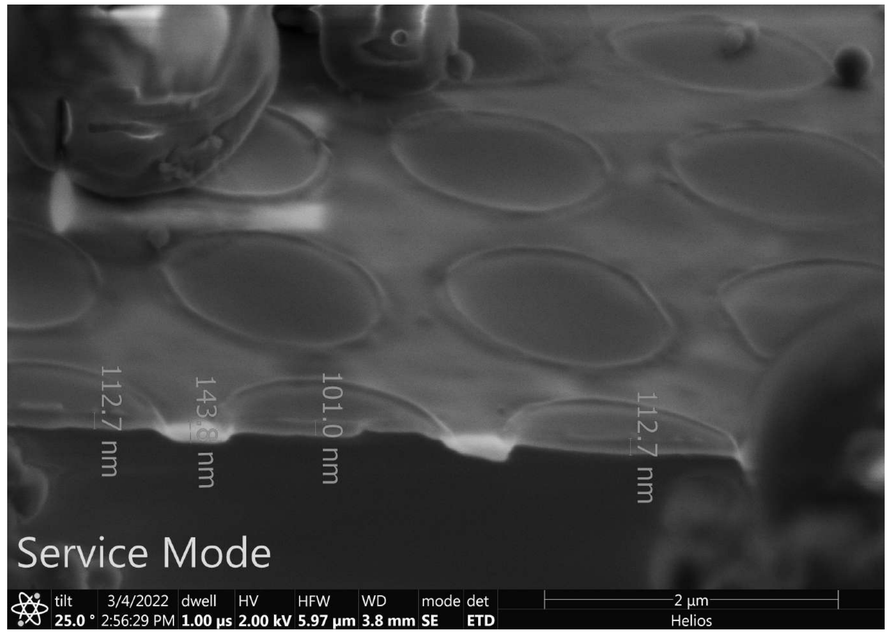
Graphene (0.8 mg/ml)

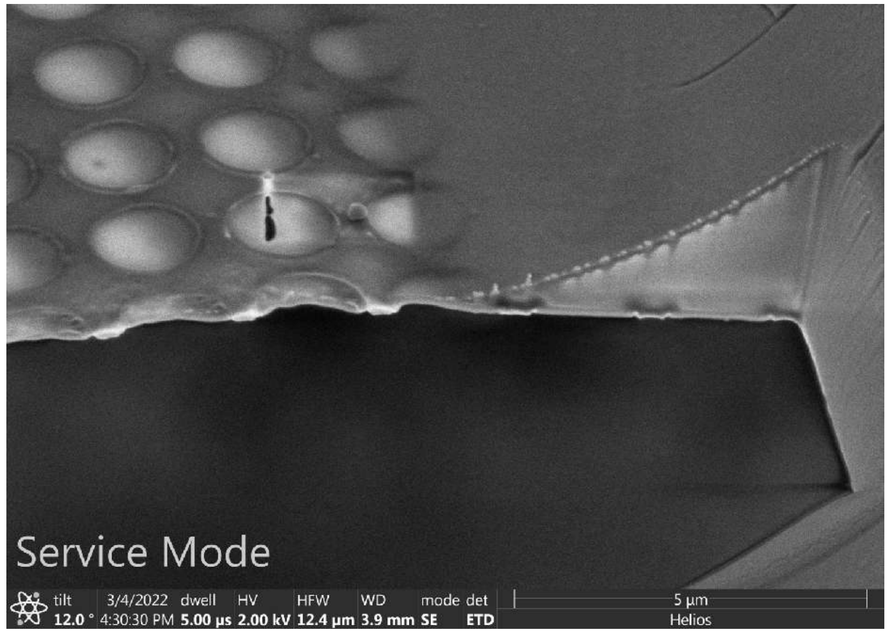
What is chameleon?

Where is the ice relative to the grid?

Spotiton

Cryo FIB-SEM



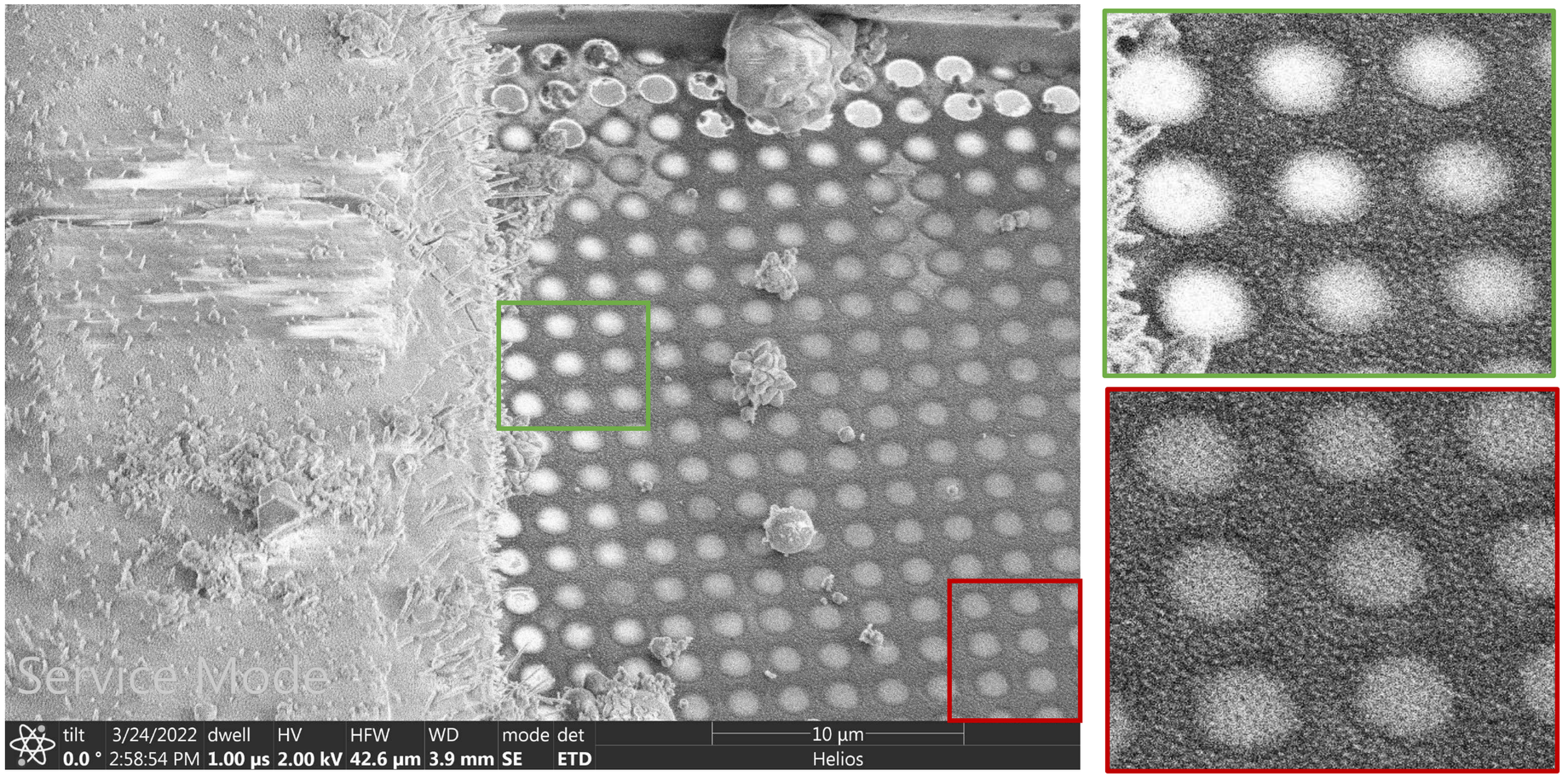


What is chameleon?

Where is the ice relative to the grid?

Spotiton

Sample application side of grid

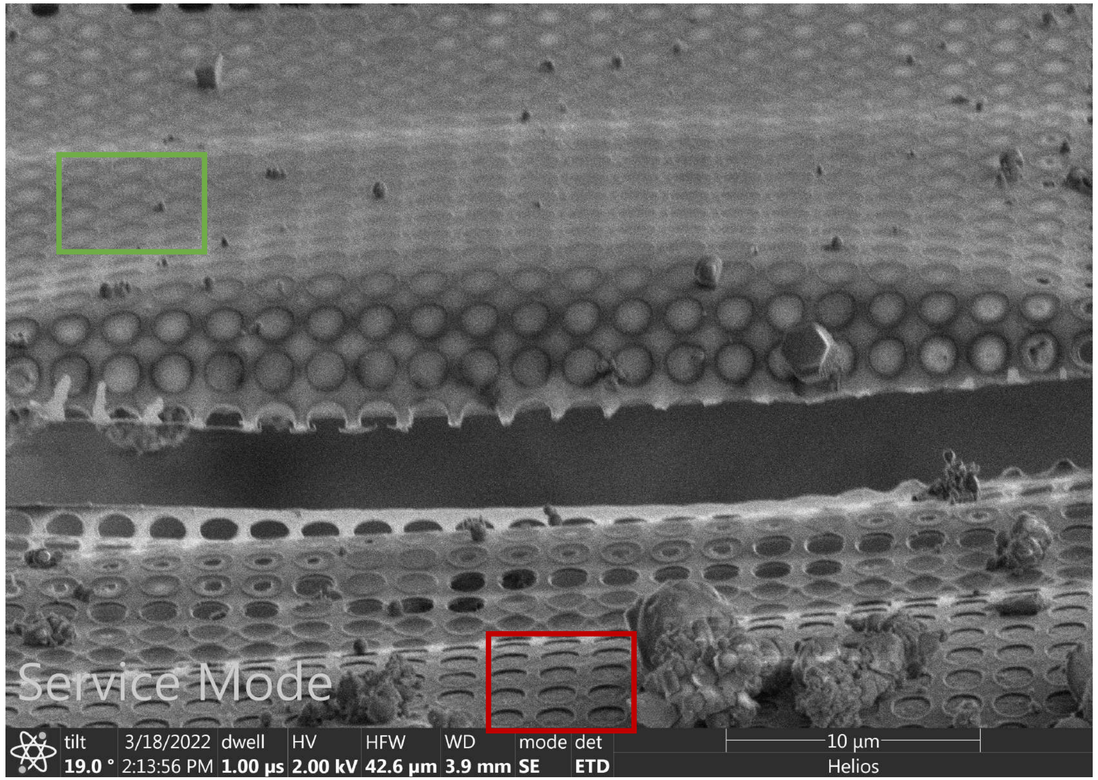


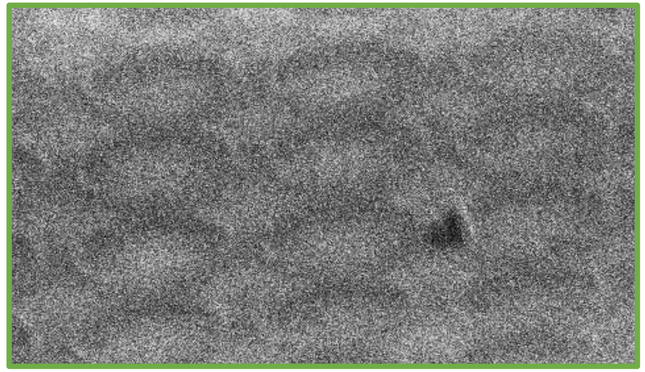
What is chameleon?

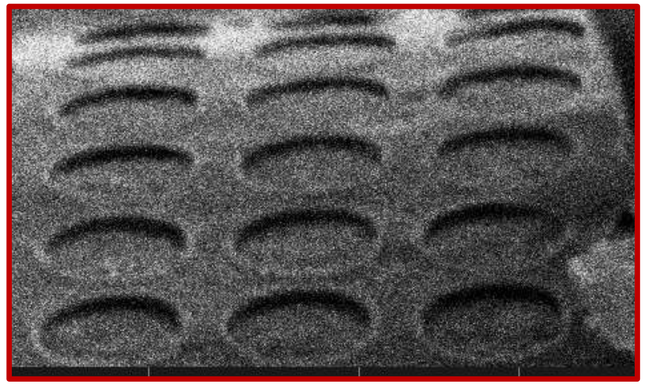
Where is the ice relative to the grid?

Spotiton

Opposite side of sample application





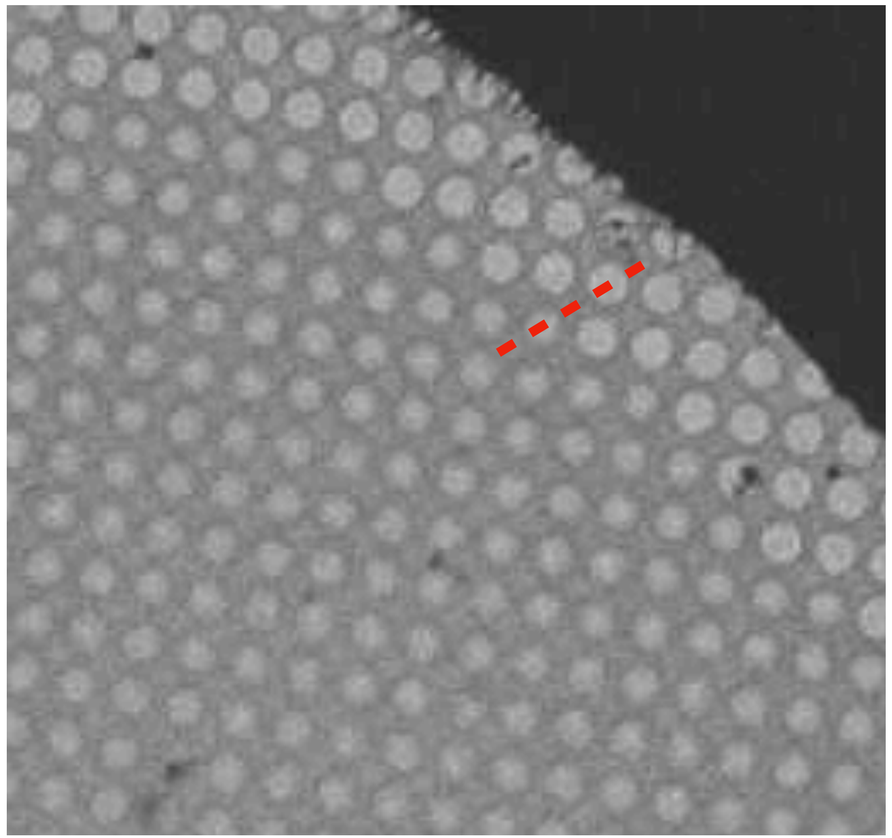


What is chameleon?

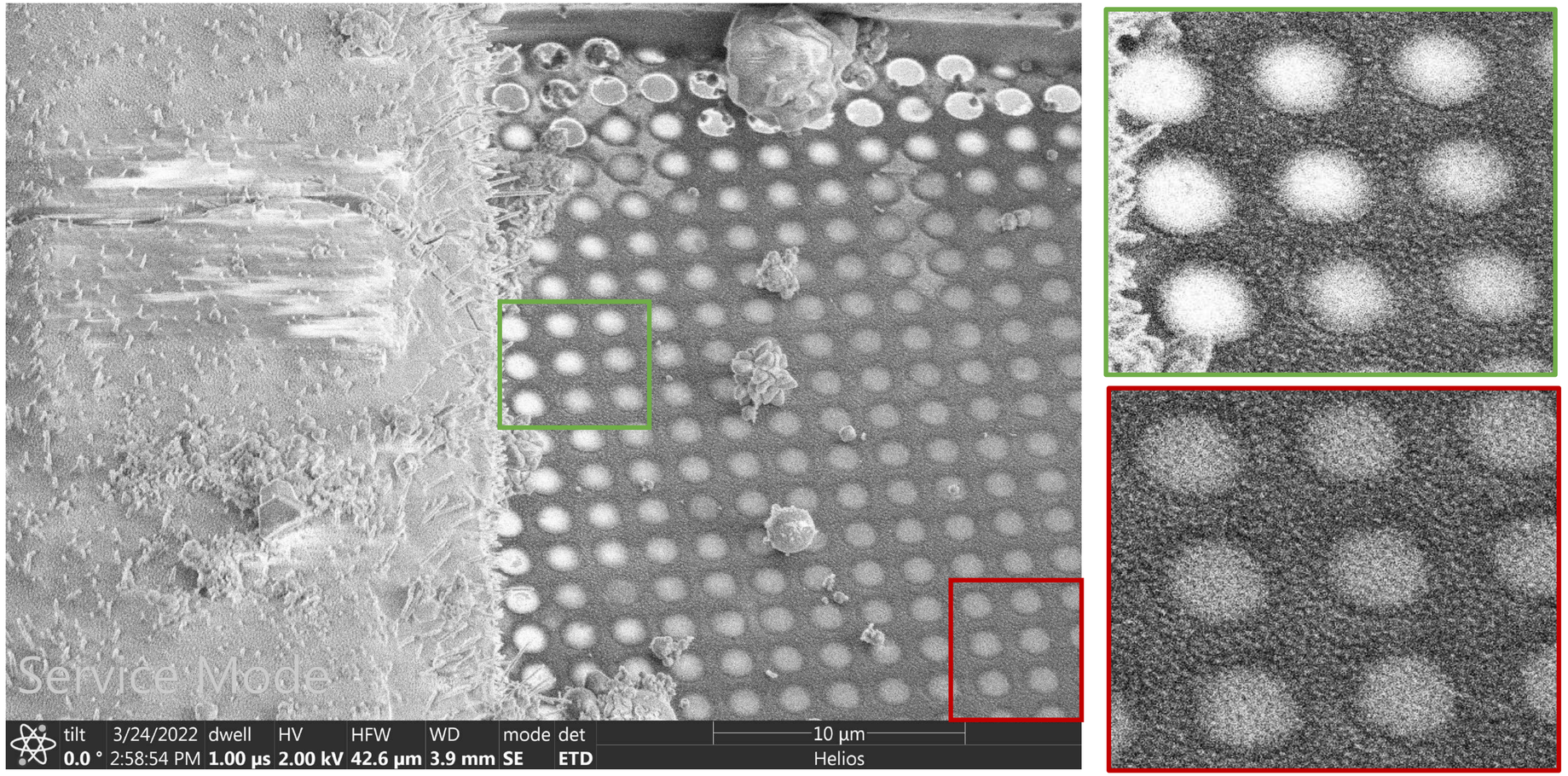
Where is the ice relative to the grid?

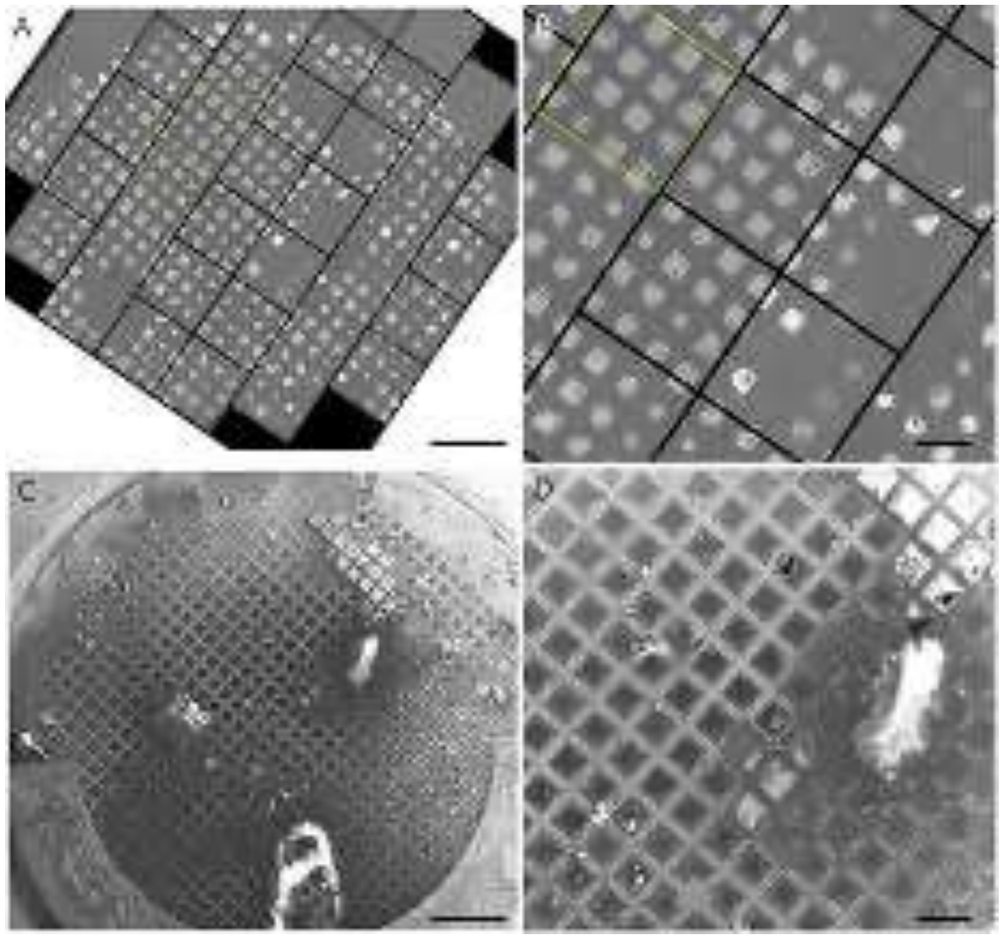
Spotiton

And why does this happen?

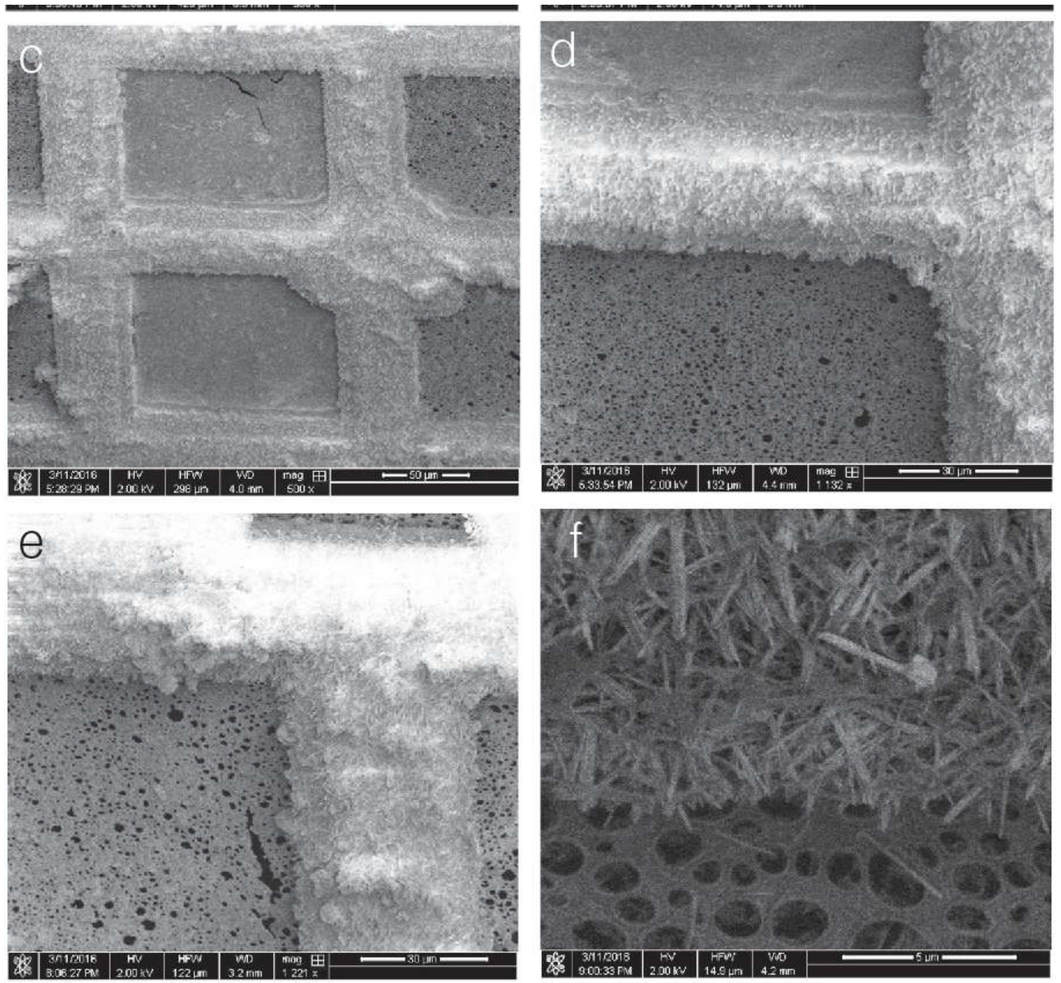


WHAT DOES A GRID LOOK LIKE?



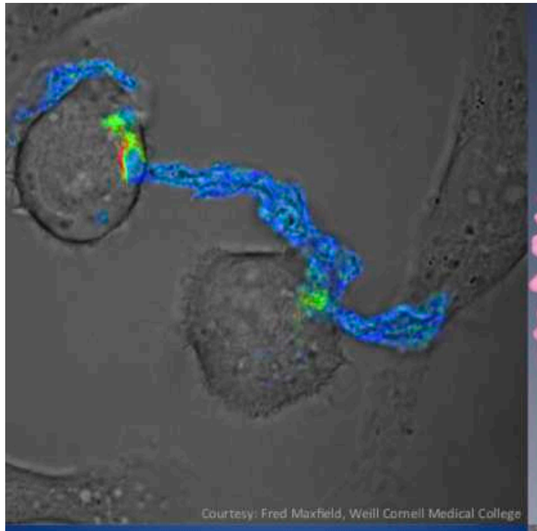


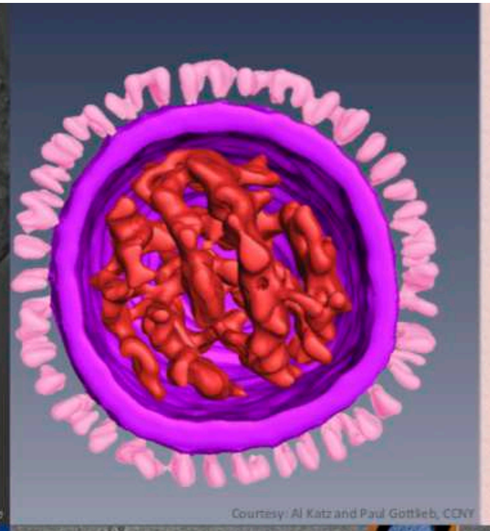
- Schmidli, Claudio & Rima, Luca & Arnold, Stefan & Stohler, Thomas & Syntychaki, Anastasia & Bieri, Andrej & Albiez, Stefan & Goldie, Kenneth & Chami, Mohamed & Stahlberg, Henning & Braun, Thomas. (2018). Miniaturized Sample Preparation for Transmission Electron Microscopy. Journal of Visualized Experiments. 2018. 10.3791/57310.

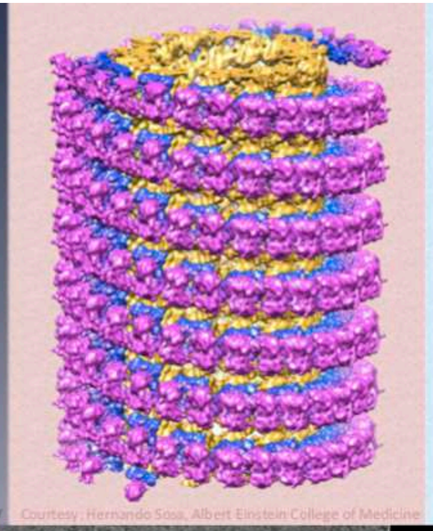


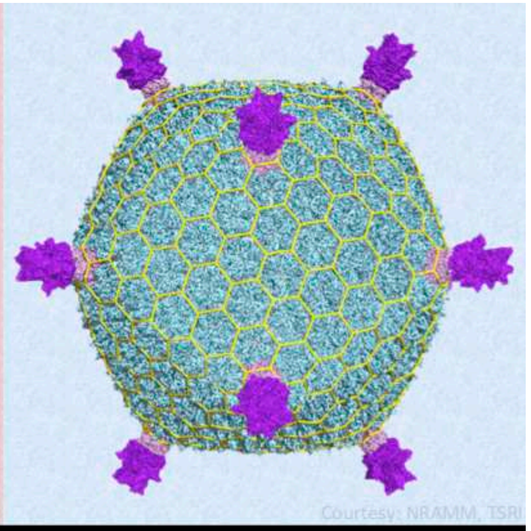
- Razinkov, I., Venkata P. Dandey, Hui Wei, Z. Zhang, D. Melnekoff, W. Rice, Christoph Wigge, C. S. Potter and B. Carragher. “A new method for vitrifying samples for cryoEM.” *Journal of structural biology* 195 2 (2016): 190-198 .

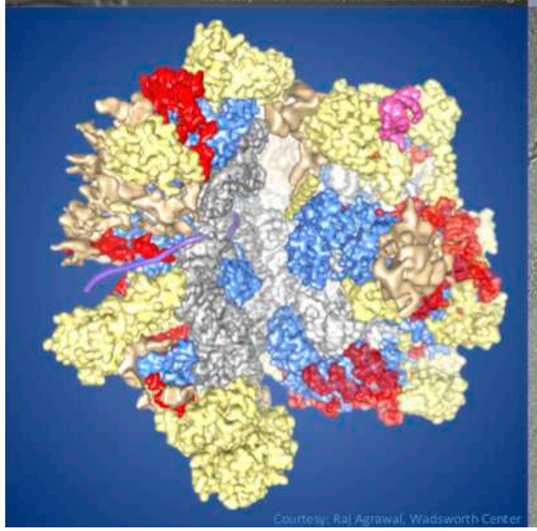
How are samples prepared for cryoEM?

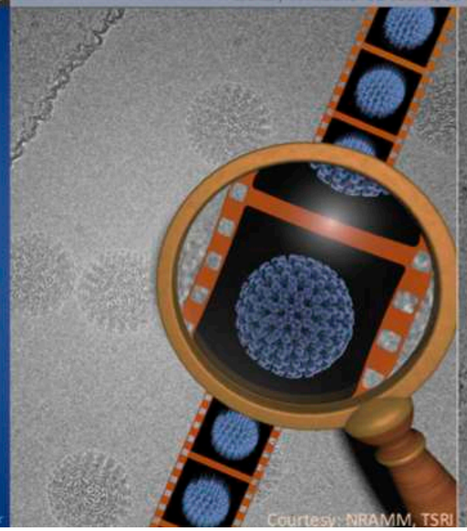


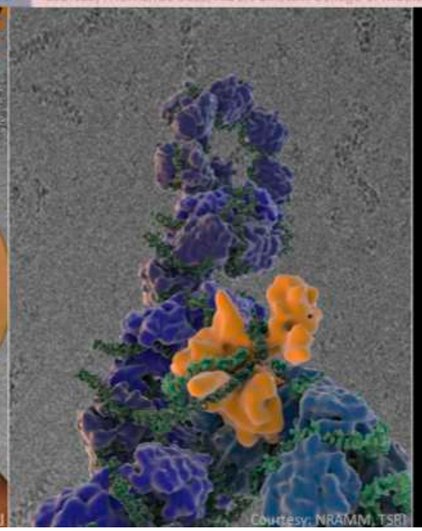


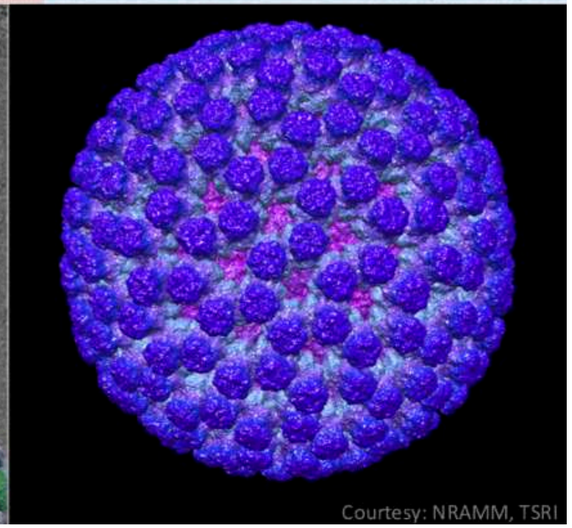




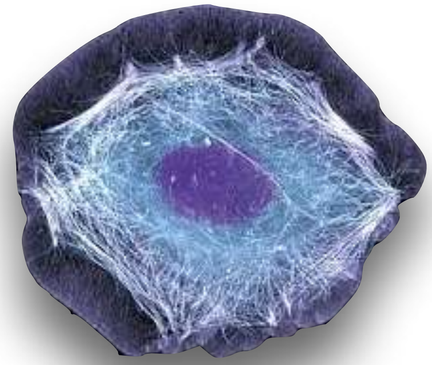


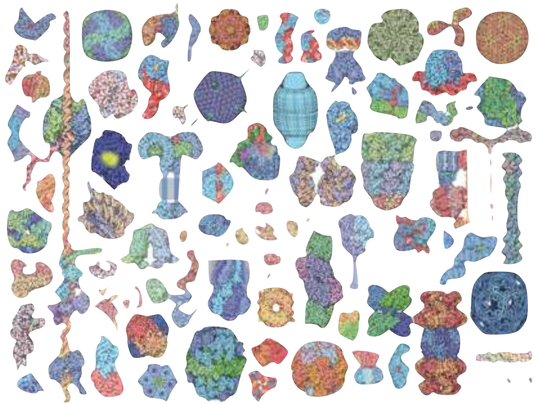






What about thicker samples?



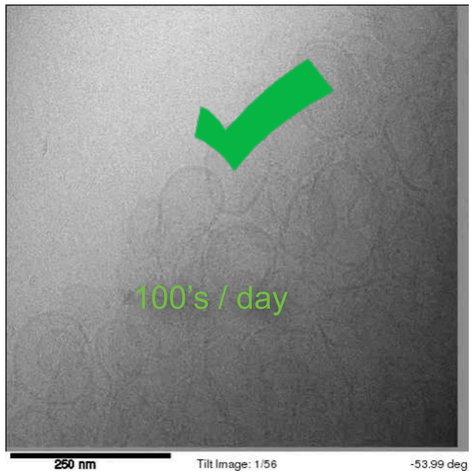


...

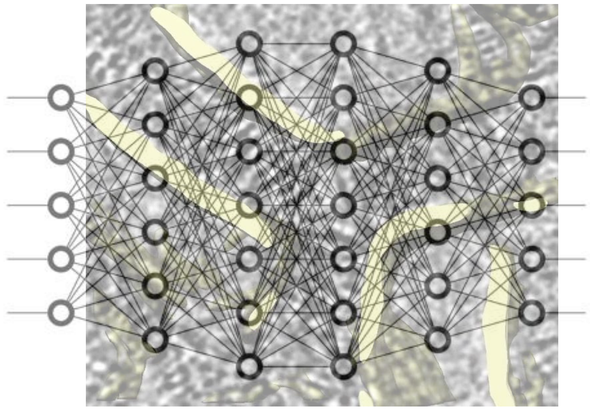
How are samples prepared for cryoEM?

Towards Automation for
In Situ CryoEM

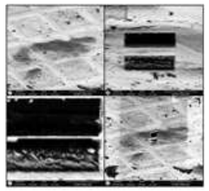
Sample

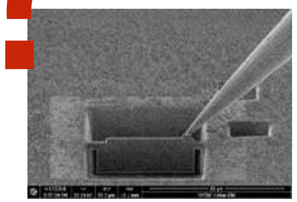


Automated Data Collection
(Leginon, etc.)



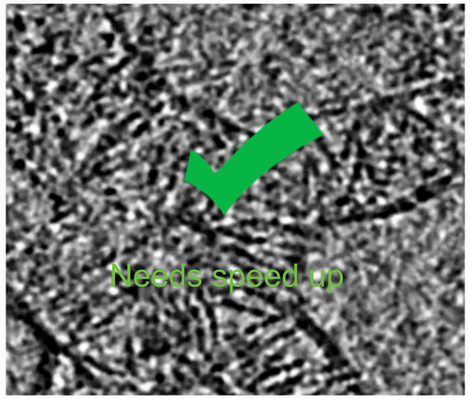
Deep learning?





Milling
Grid preparation

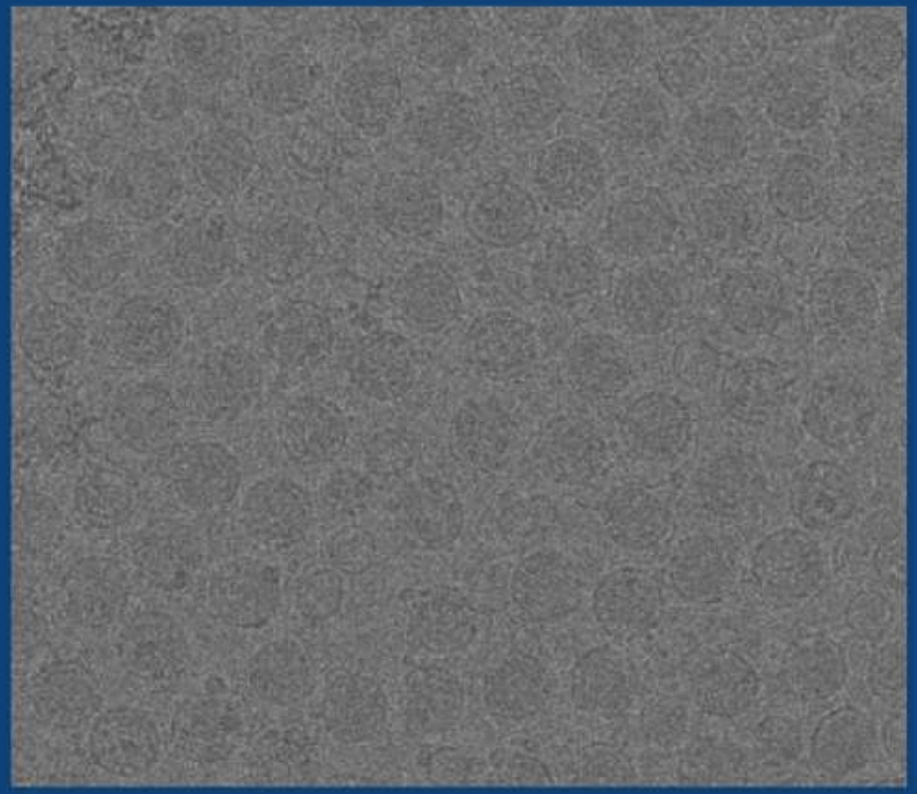
Lift out



Streamlined Processing
(Appion Protomo)

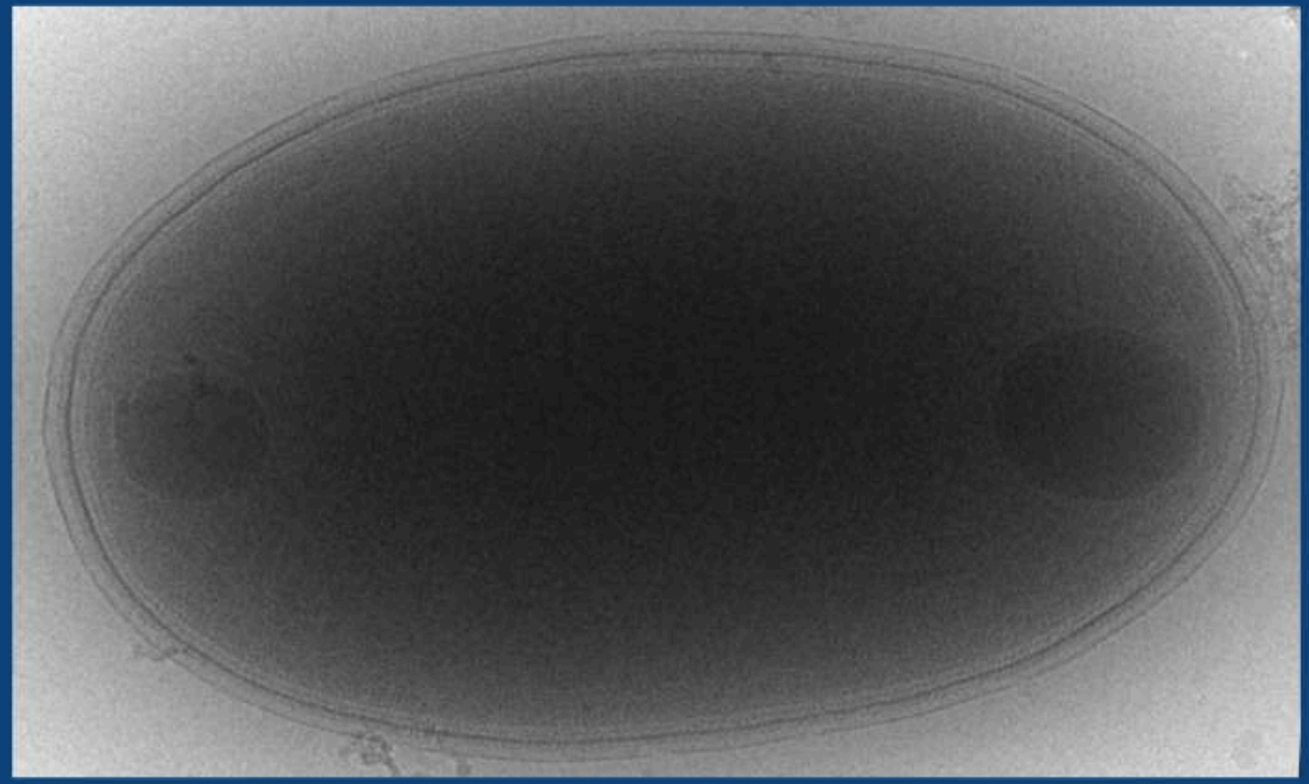
How are samples prepared for cryoEM?

HOW THIN DOES THE SAMPLE NEED TO BE?



50 nm

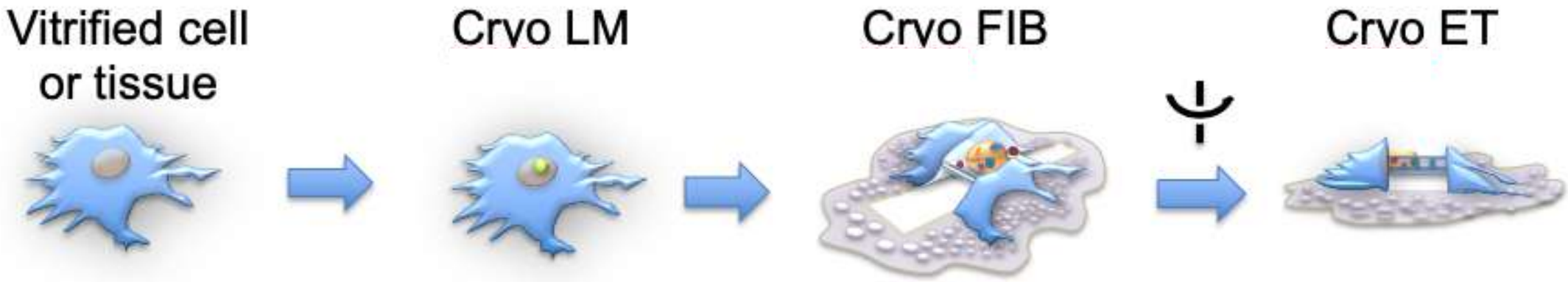
Bacteriophage (ϕ12)



E. coli, Salmonella, Cyanobacteria

How are samples prepared for cryoEM?

CLEM workflow



How are samples prepared for cryoEM?

STEP 1: Vitrify sample

Plunge Freezing

-Rapid freezing in liquid nitrogen (LN2)-cooled liquid ethane

Leica Microsystems

High Pressure Freezing (HPF)

-Rapid freezing at LN2 temps and high pressure

How are samples prepared for cryoEM?

STEP 1: Vitrify sample

Sample: cells (adherent, suspension)

SEMC Equipment: Leica EM-GP, Gatan CP3, manual plunger

Bottlenecks: sample concentration, sample buffer, preferred orientation of sample on grid, plunge freezing parameters, grid mesh, support film, warming up, ice thickness, vitrification

Takeaways

-Vitrify up to ~10 um of sample

-One-sided blotting

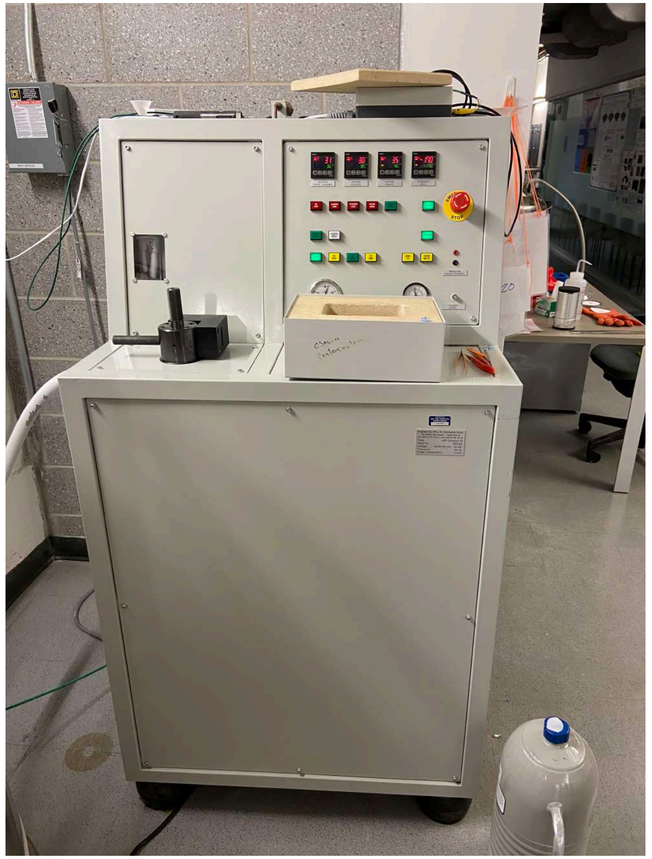
How are samples prepared for cryoEM?

STEP 1: Vitrify sample

Sample: cells, tissue

SEMC Equipment: Wohlwend HPF Compact 01

Bottlenecks: sample amount, sample concentration, sample buffer, ice thickness, warming up, pressure, vitrification



How are samples prepared for cryoEM?

STEP 2: Cryo-LM

Takeaways

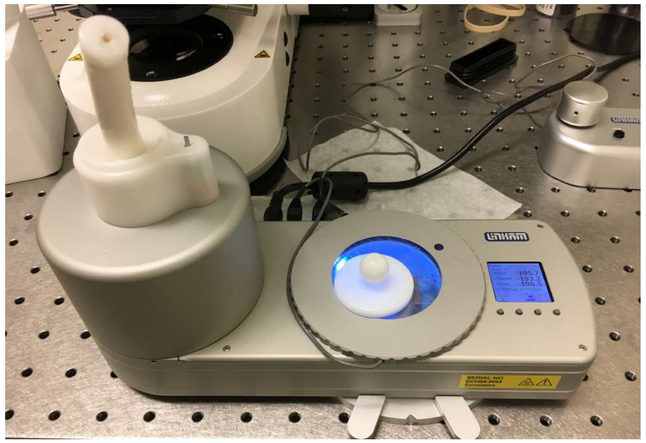
-Do I have cells? Where?

-Is my target fluorescing?

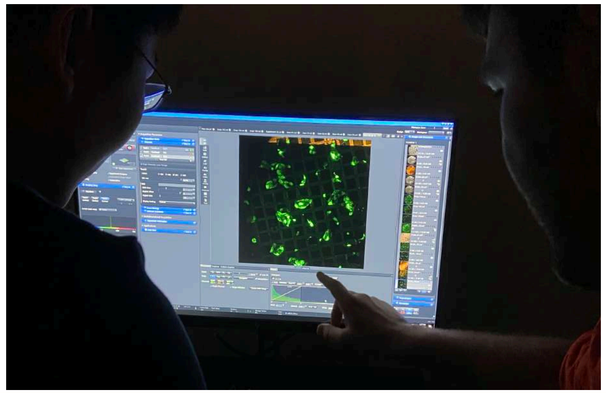
Sample: vitrified cells on bare or clipped grid

SEMC Equipment: Zeiss LSM 900 with Airyscan with Linkam Cryostage

Zeiss



Bottlenecks: sample concentration, sample location, support film, laser damage, ice thickness, autofluorescence, warming up



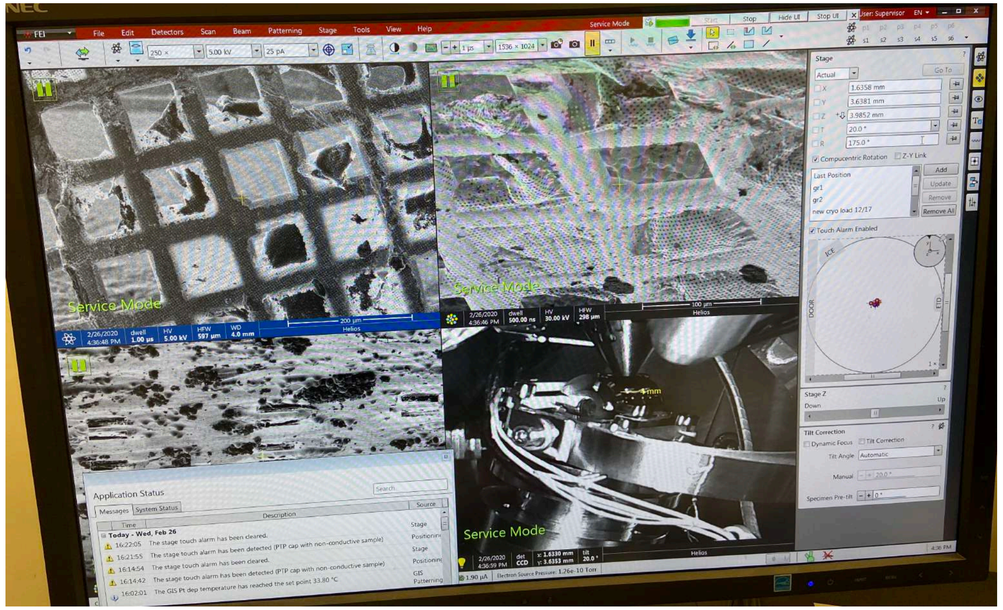
How are samples prepared for cryoEM?

STEP 3: Cryo-FIB milling

Sample: vitrified cells on a clipped grid

SEMC Equipment: Helios Nanolab G3 FIB-SEM, Leica VCT, VCM, & ACE

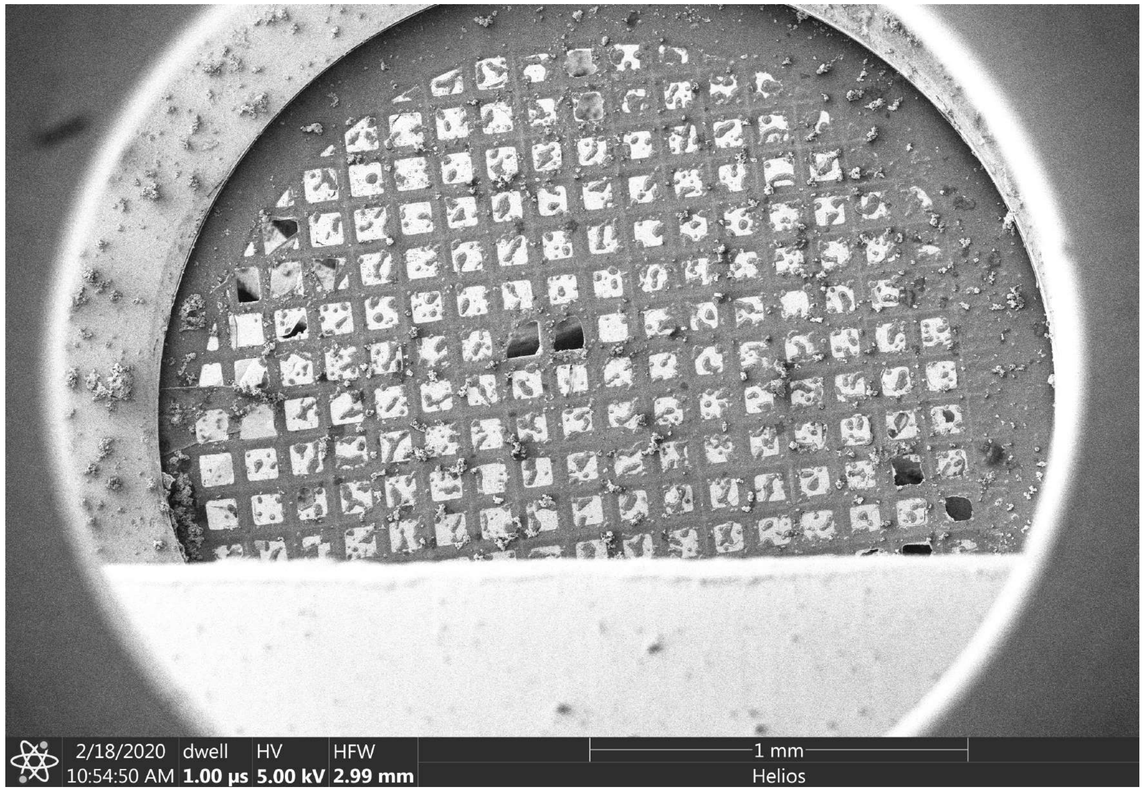
Bottlenecks: sample concentration, preferred orientation, imaging & milling parameters, grid mesh, support film, grid orientation, beam damage, charging, pole touches, dropping holder, image correlation, warming up, catching fire, …



How are samples prepared for cryoEM?

STEP 3: Cryo-FIB milling

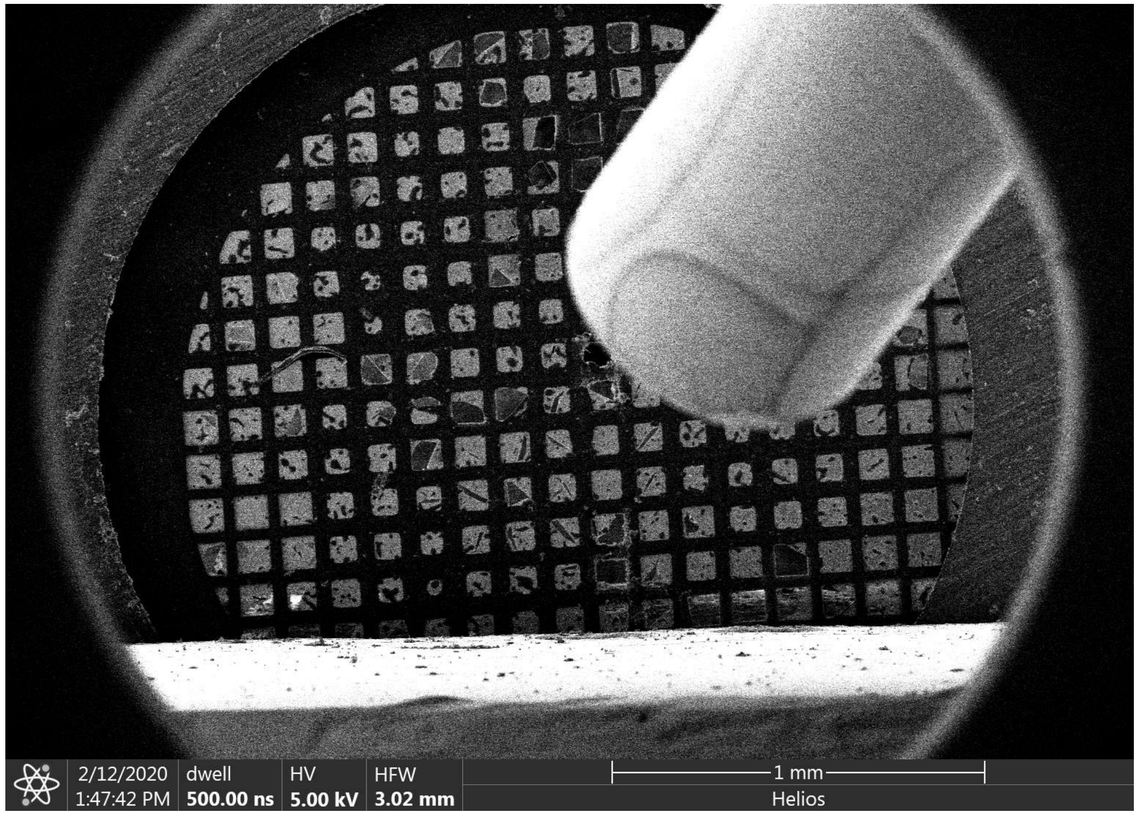
1) Grid overview/atlas images with electron and ion beam
2) GIS Platinum coat for 5 seconds
3) Mill large rectangular trenches on either side of each lamella site
4) Continue thinning lamella by milling on each side until <300 nm thickness



How are samples prepared for cryoEM?

STEP 3: Cryo-FIB milling

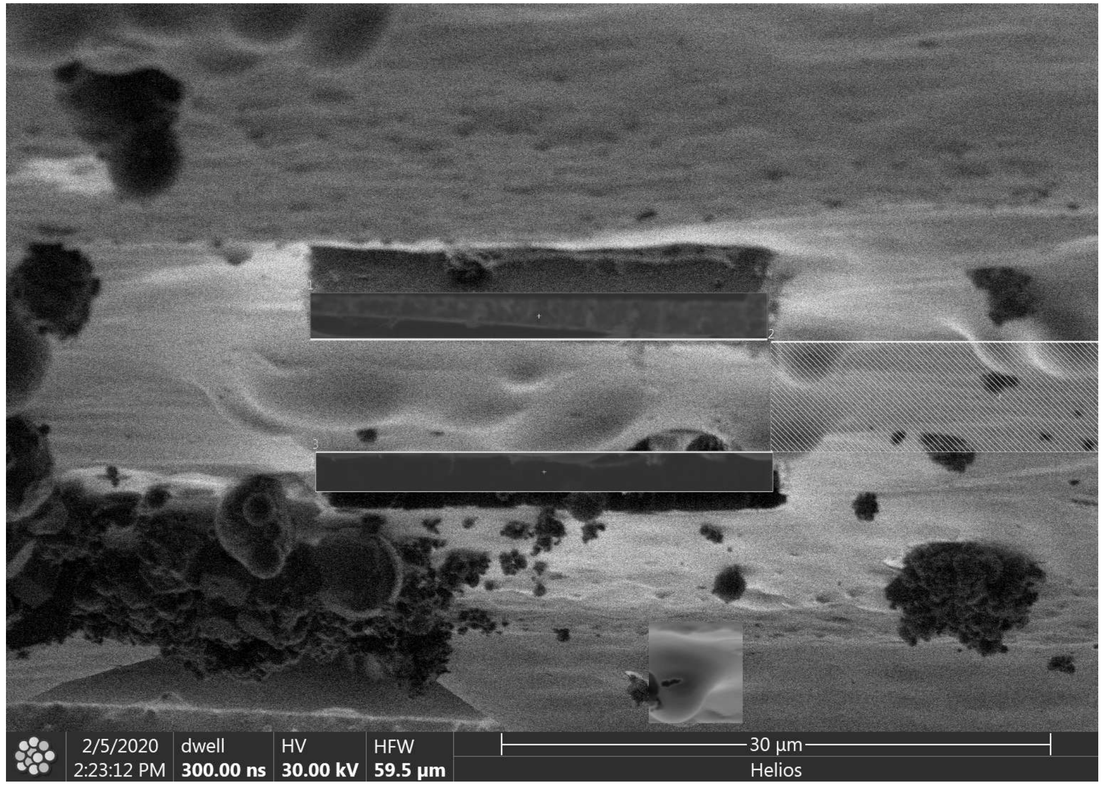
1) Grid overview/atlas images with electron and ion beam
2) GIS Platinum coat for 5 seconds
3) Mill large rectangular trenches on either side of each lamella site
4) Continue thinning lamella by milling on each side until <300 nm thickness



How are samples prepared for cryoEM?

STEP 3: Cryo-FIB milling

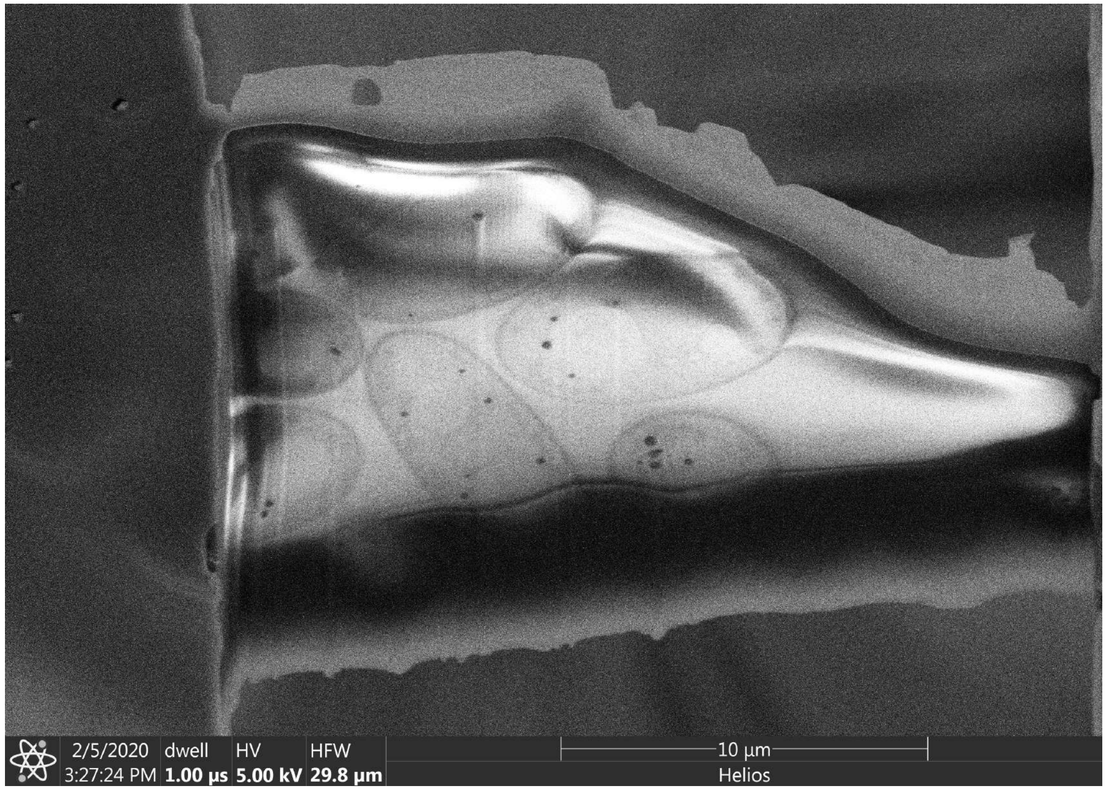
1) Grid overview/atlas images with electron and ion beam
2) GIS Platinum coat for 5 seconds
3) Mill large rectangular trenches on either side of each lamella site
4) Continue thinning lamella by milling on each side until <300 nm thickness



How are samples prepared for cryoEM?

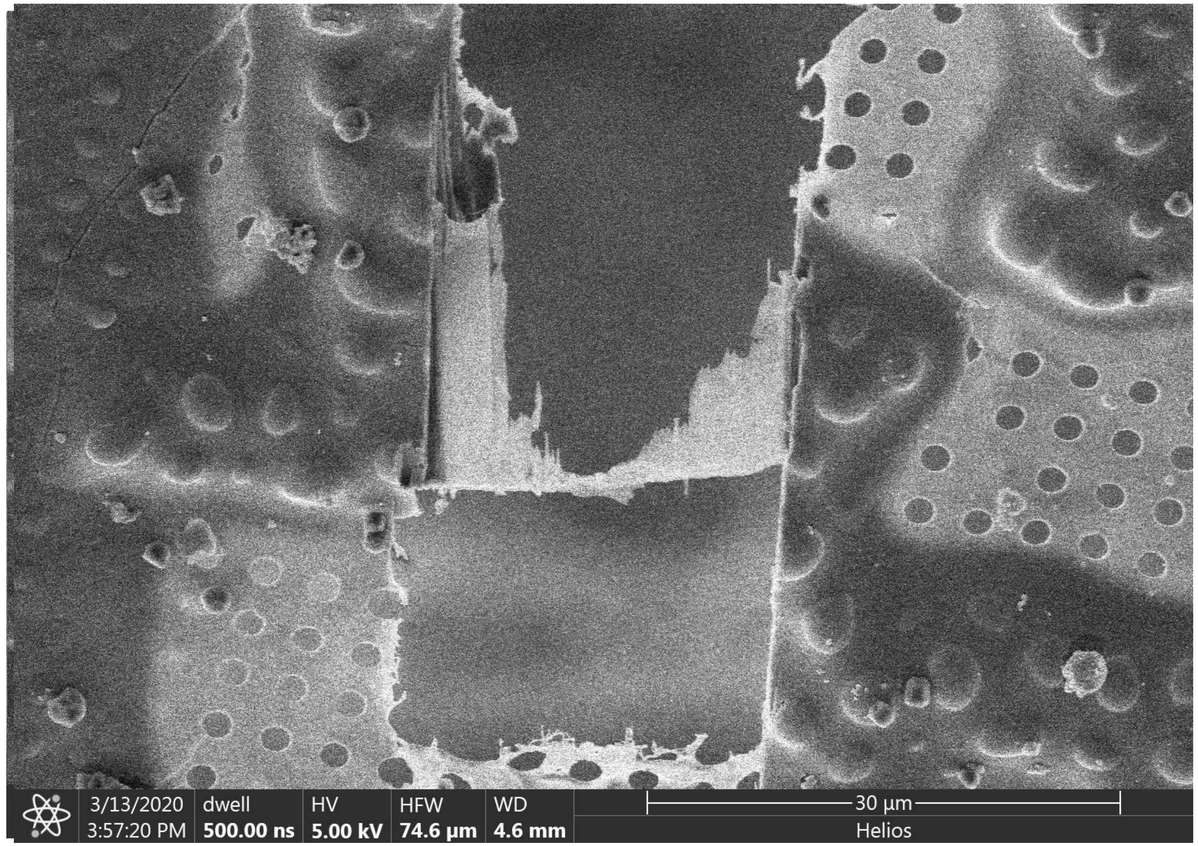
STEP 3: Cryo-FIB milling

1) Grid overview/atlas images with electron and ion beam
2) GIS Platinum coat for 5 seconds
3) Mill large rectangular trenches on either side of each lamella site
4) Continue thinning lamella by milling on each side until <300 nm thickness



How are samples prepared for cryoEM?

STEP 3: Cryo-FIB milling



Takeaways

-Large learning curve if done manually

-Many, many steps that all need to be completed successfully for experiment to work

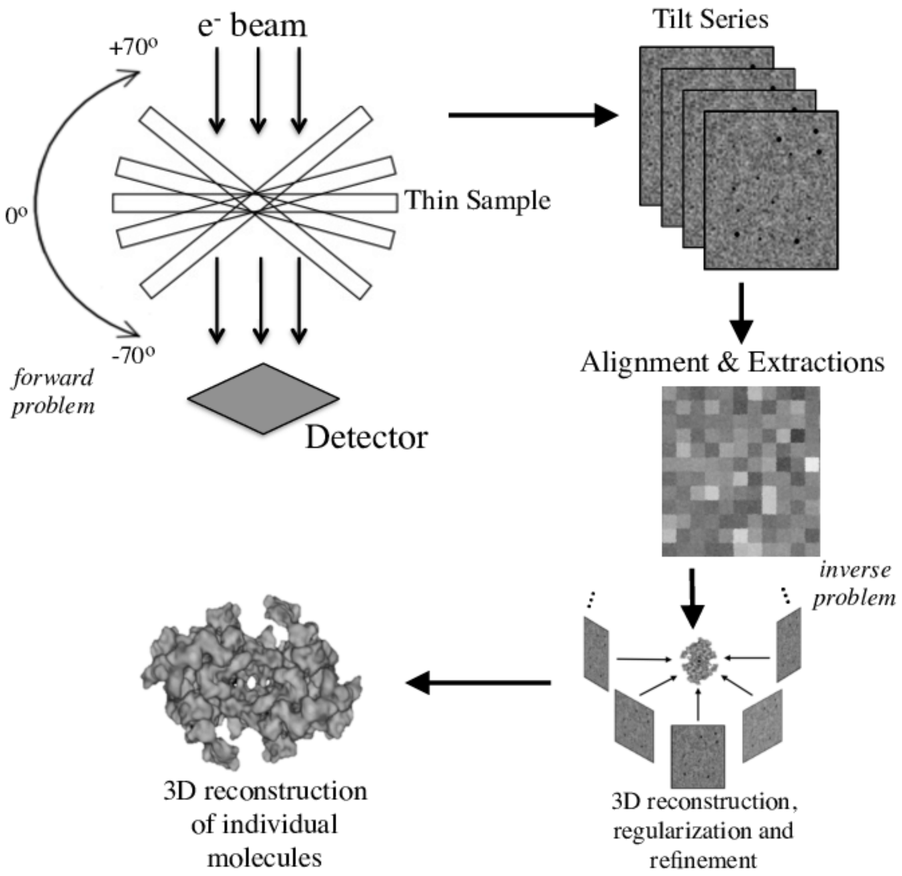
How are samples prepared for cryoEM?

STEP 4: Cryo-ET

Sample: whole cells or lamella on clipped grid

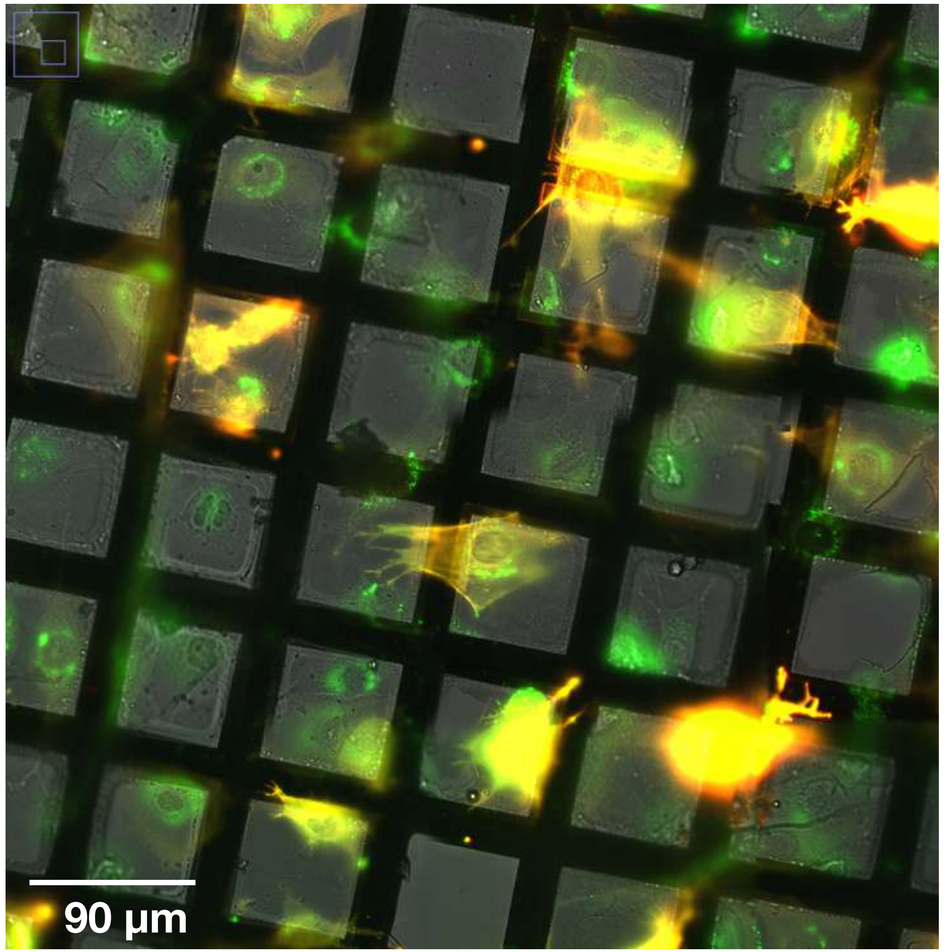
SEMC Equipment: TFS Titan Krios 300kV TEM with Energy filter + direct detector

Bottlenecks: sample concentration, sample buffer, preferred orientation, grid mesh, grid orientation, support film, warming up, ice thickness, vitrification, stage stability, beam damage, contamination, …



How are samples prepared for cryoEM?

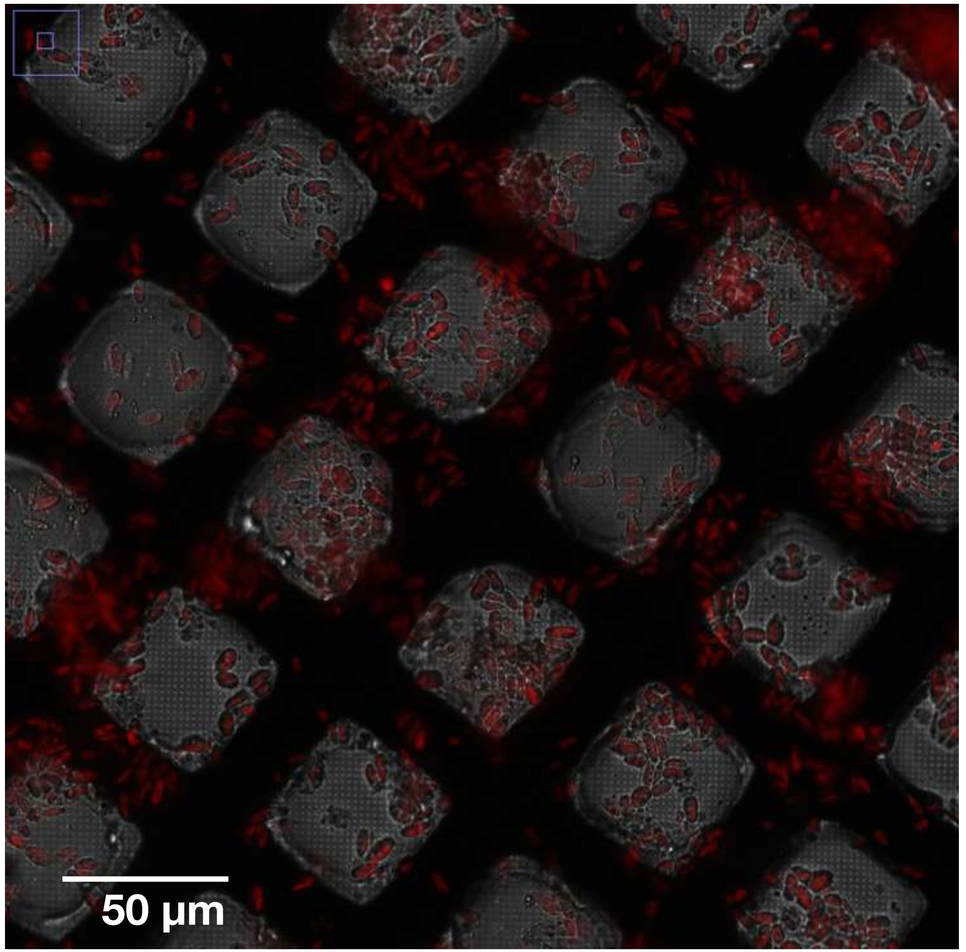
Mouse fibroblasts



Transfected adhesion signaling protein tagged with GFP (green) and F-tractin marker with mApple (red).

Greg Alushin (RU)

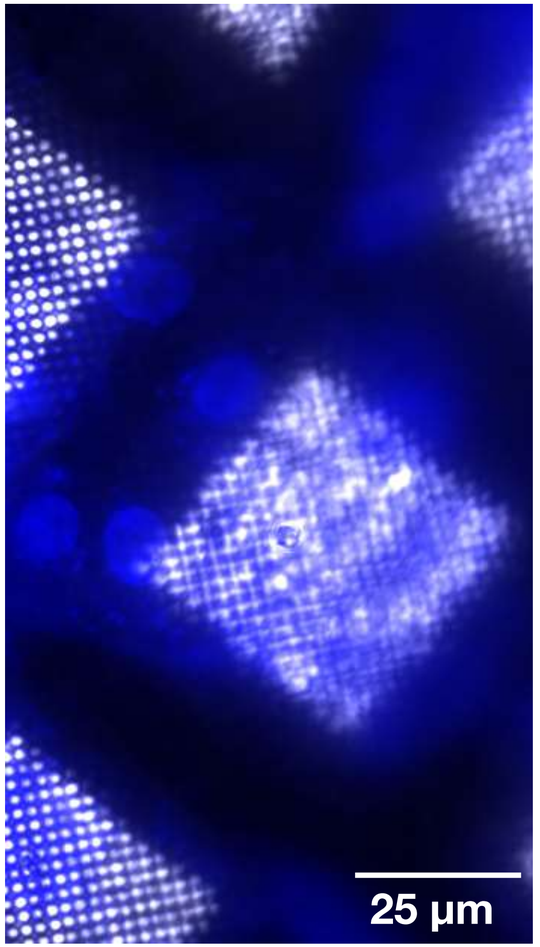
Diatoms



Auto-fluorescence.

Wei Dai (Rutgers)

Microsporidia

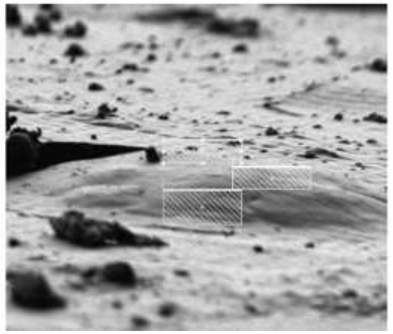


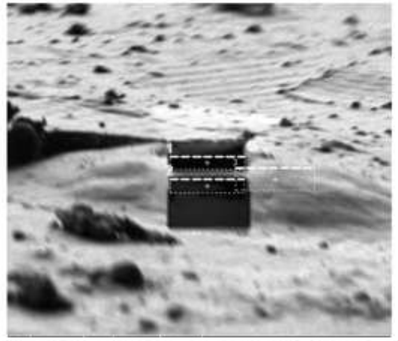
DAPI.

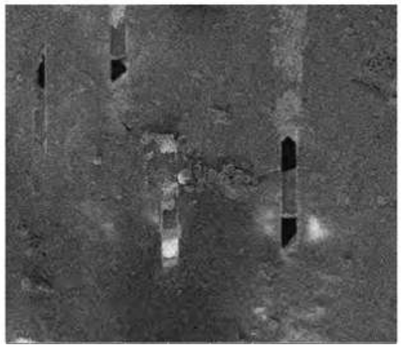
Gira Bhabha (NYU)

How are samples prepared for cryoEM?

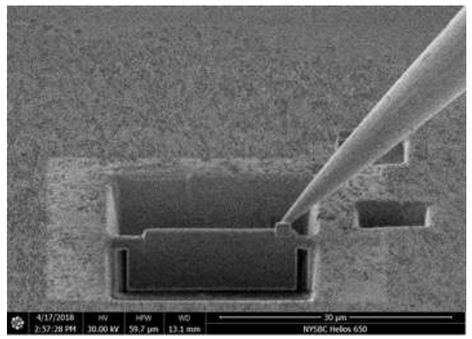
Lamella



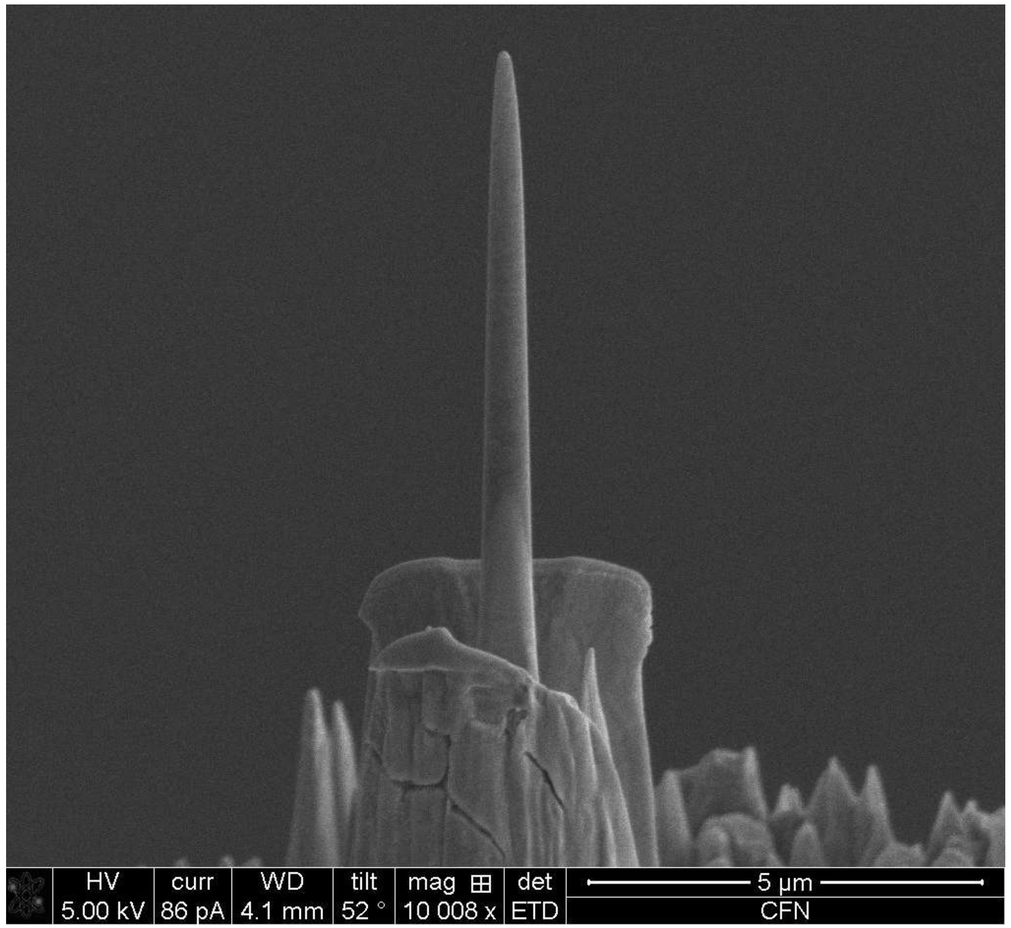




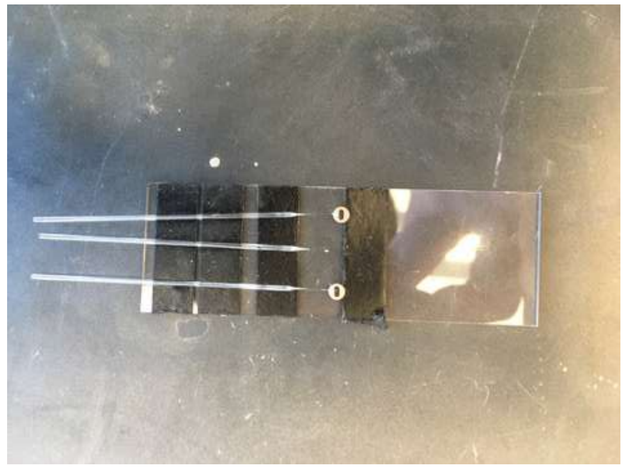
with lift-out

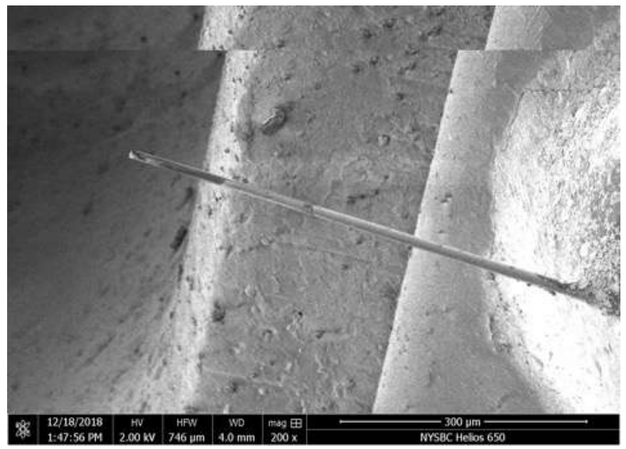


Rods



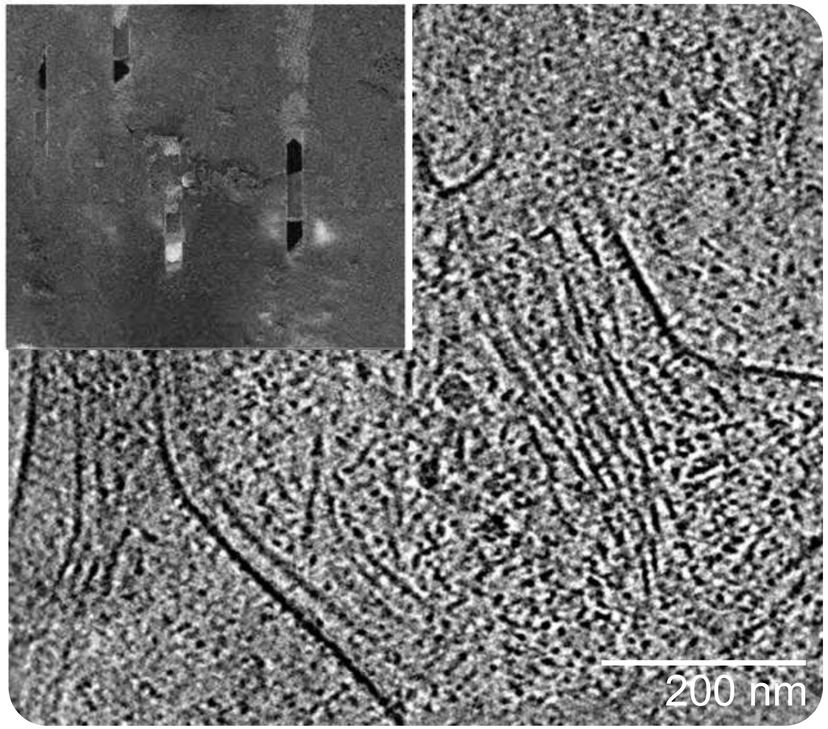
Capillaries





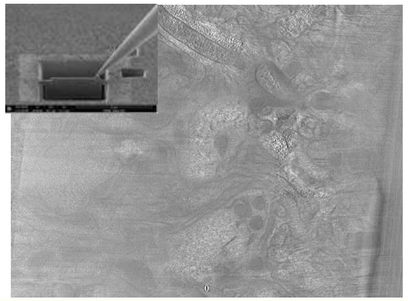
How are samples prepared for cryoEM?

Lamella



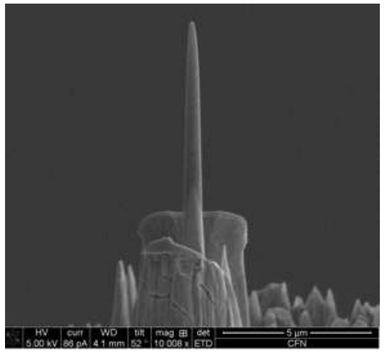
Zach Freyberg,
(Univ. Pittsburg)

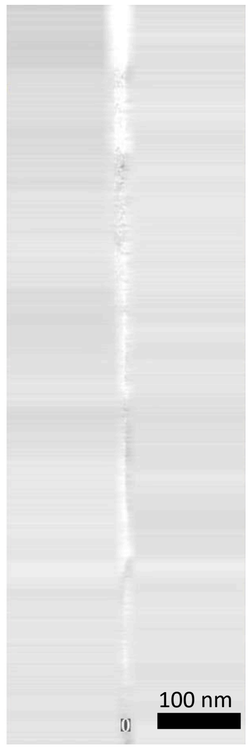
with lift-out



Kotaro
Kelley
(NRAMM)

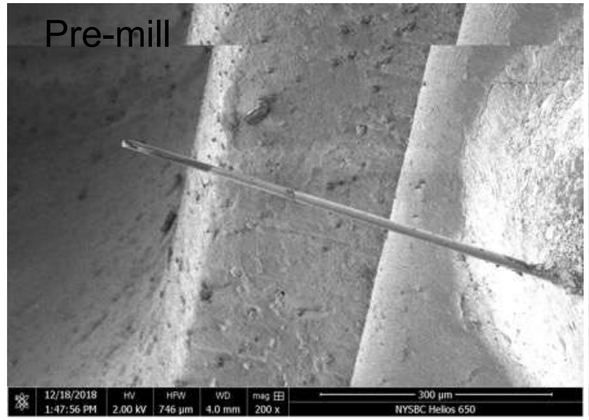
Rods

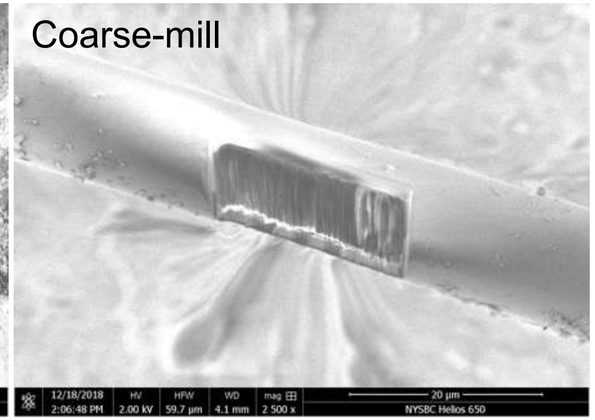


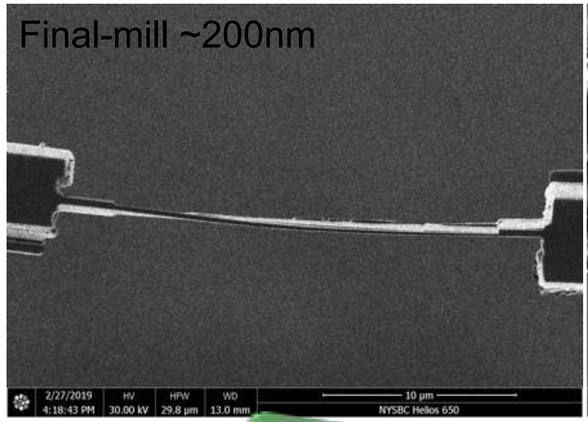


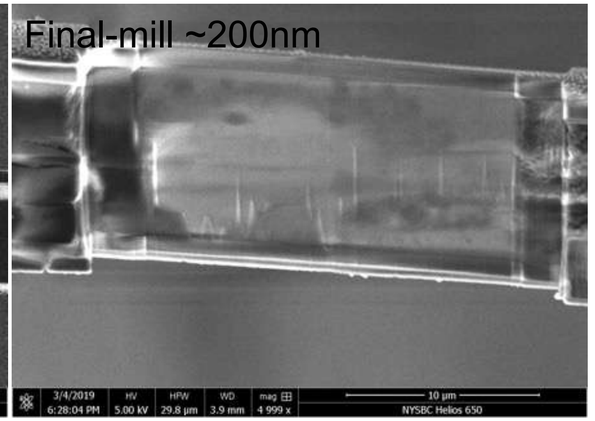
with Xin
Group
(BNL)

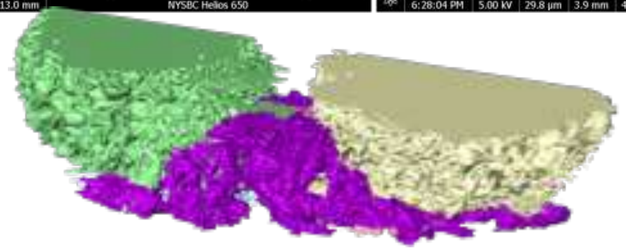
Capillaries





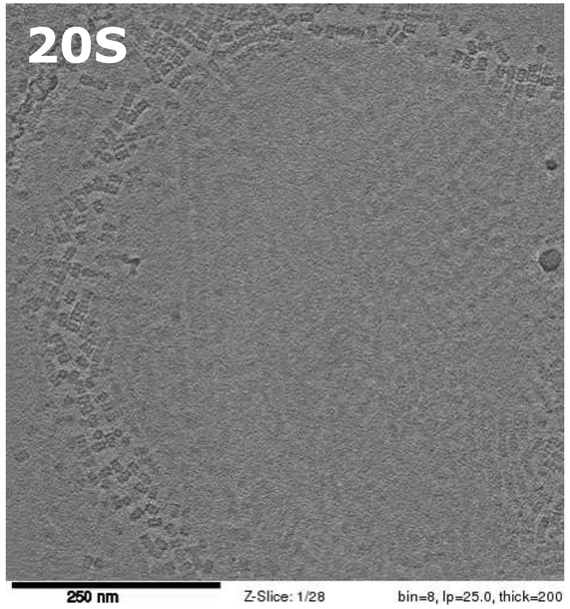




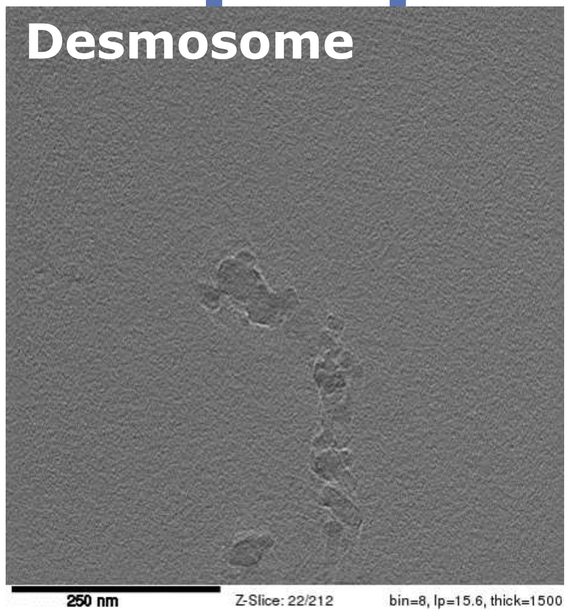


Kotaro
Kelley
(NRAMM)

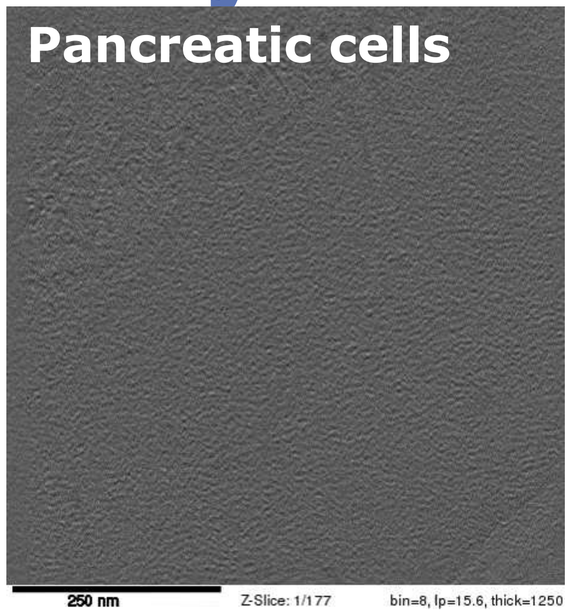
How are samples prepared for cryoEM?



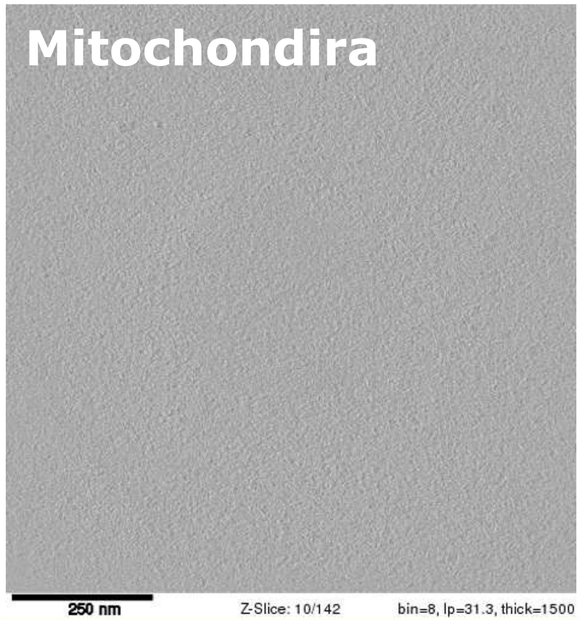
Alex Noble (NYSBC), Radostin Danev (MPI)



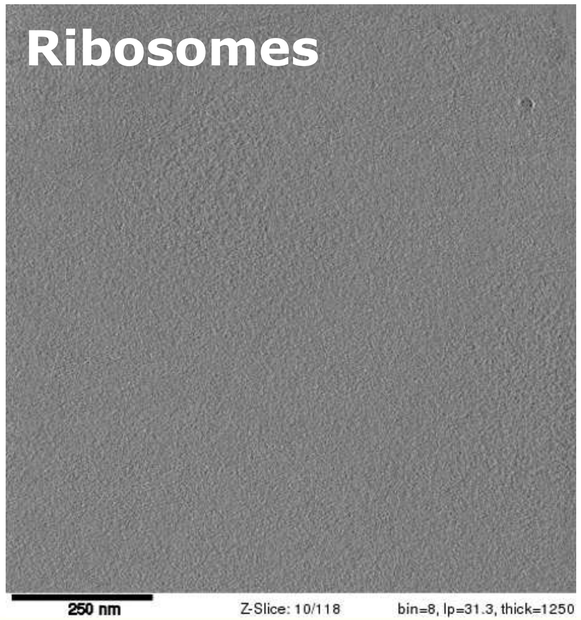
Julia Brasch (COLU)



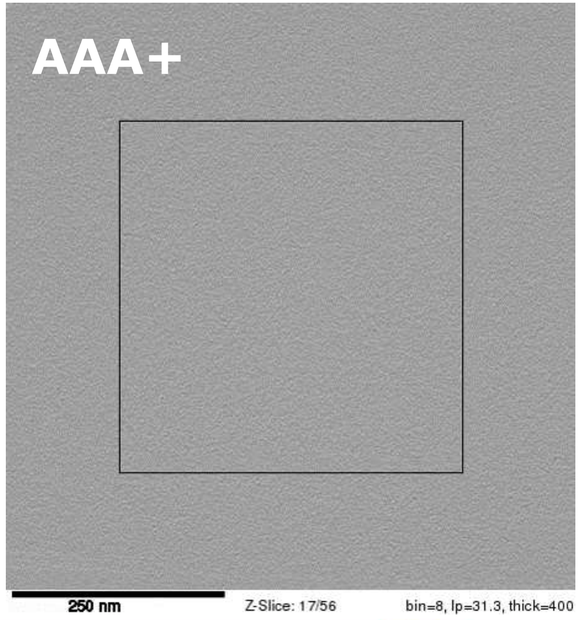
Zachary Freyberg (U. Pitt.)



Stephanie Siegmund (COLU)

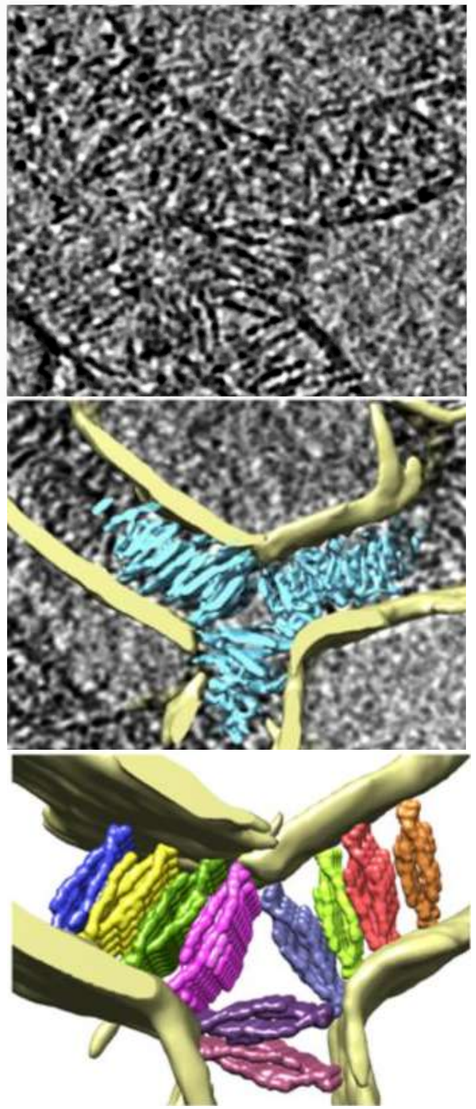


Stephanie Siegmund (COLU)

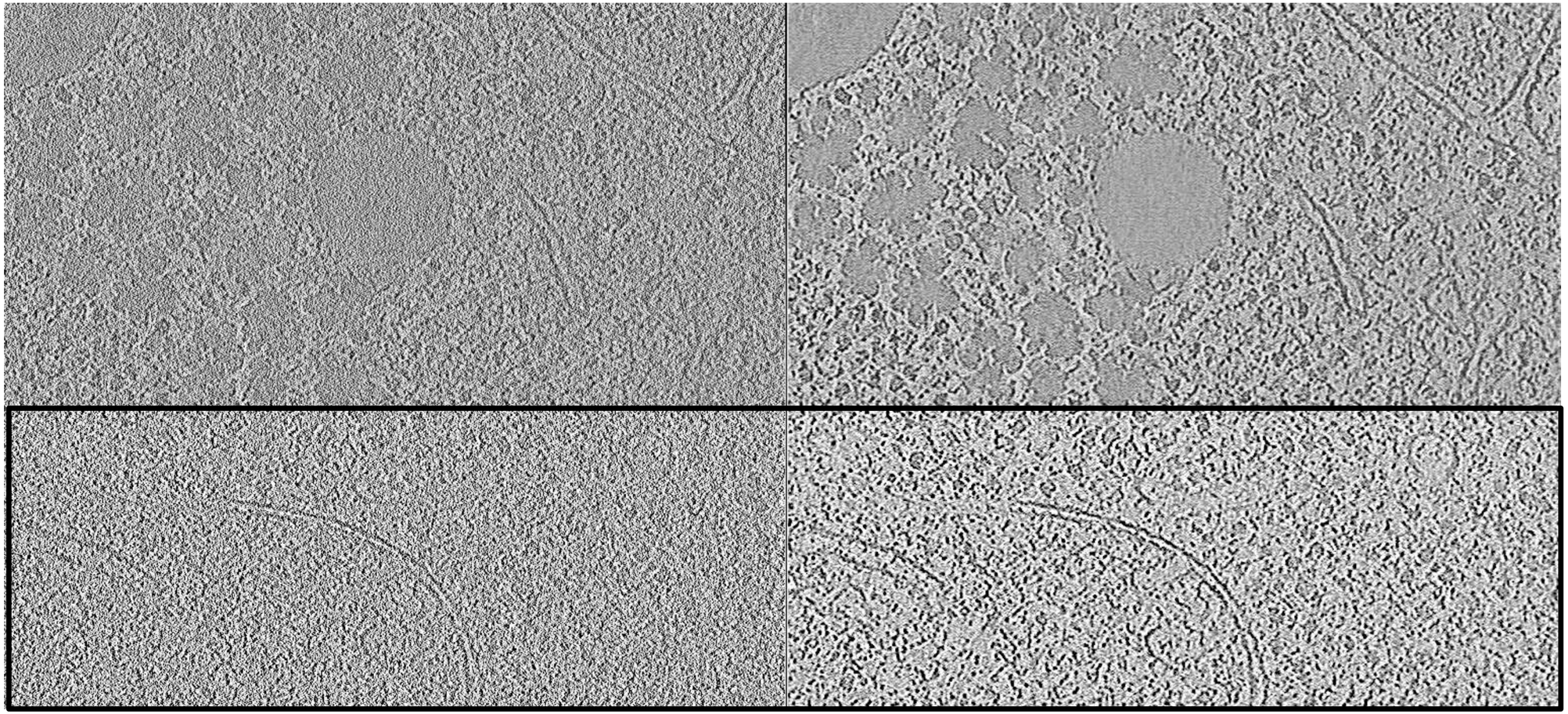


Jillian Chase (CUNY)

How are samples prepared for cryoEM?



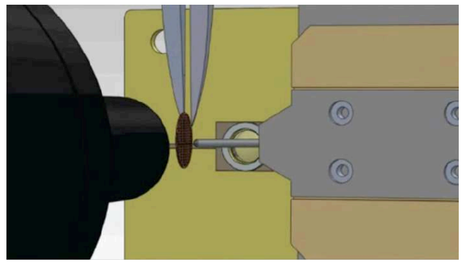
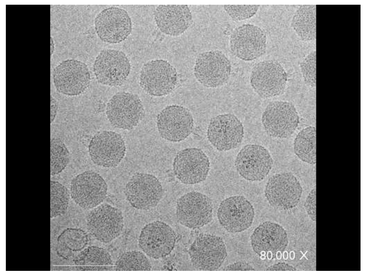
Micah Rapp (COLU)

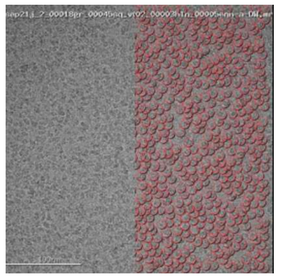
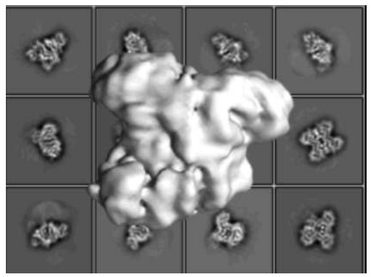
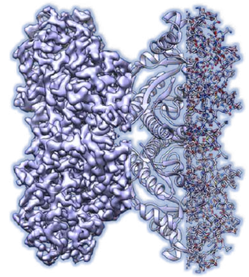


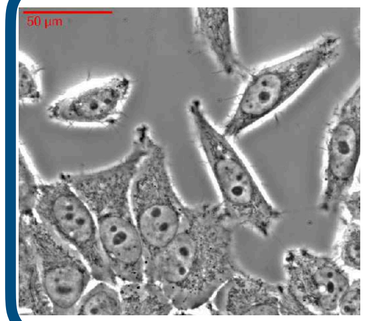
Kotaro Kelley (NRAMM)

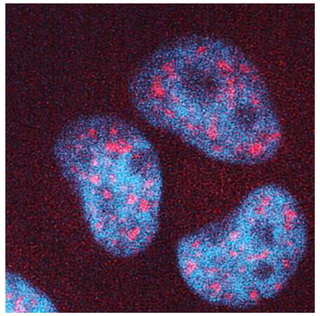
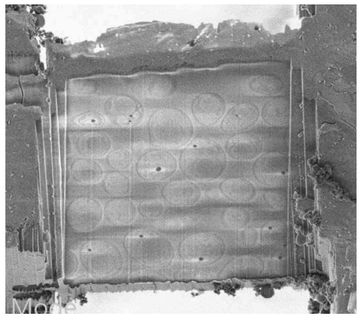
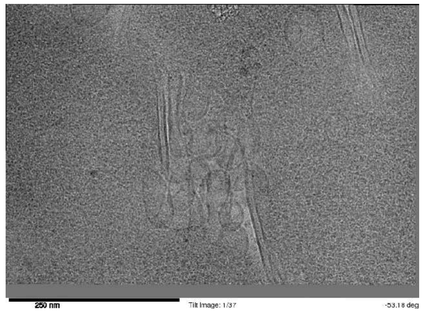
cryoEM: technology on the rise

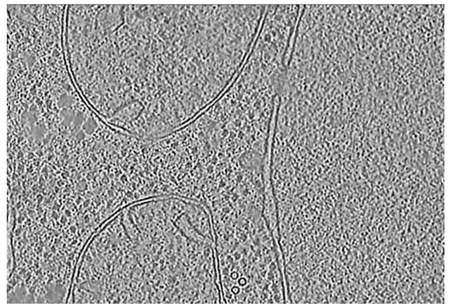
Single particle cryoEM

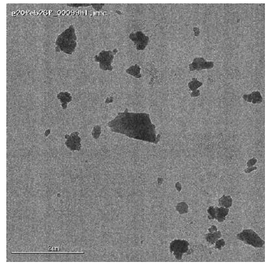
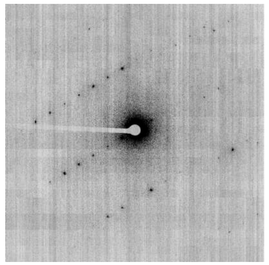
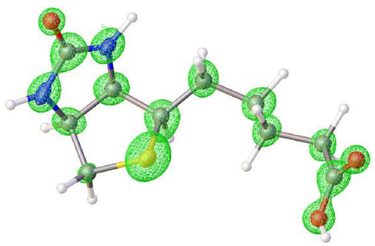
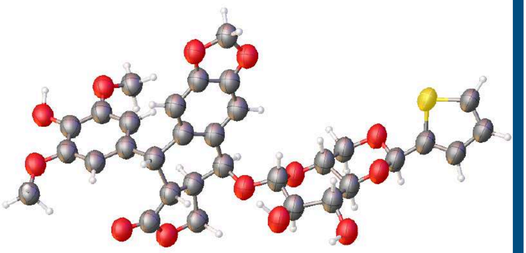




Cryo Electron Tomography (cryoET)

Micro crystal electron diffraction (microED)

TO BE CONTINUED

Questions?