ThermoFisher SCIENTIFIC

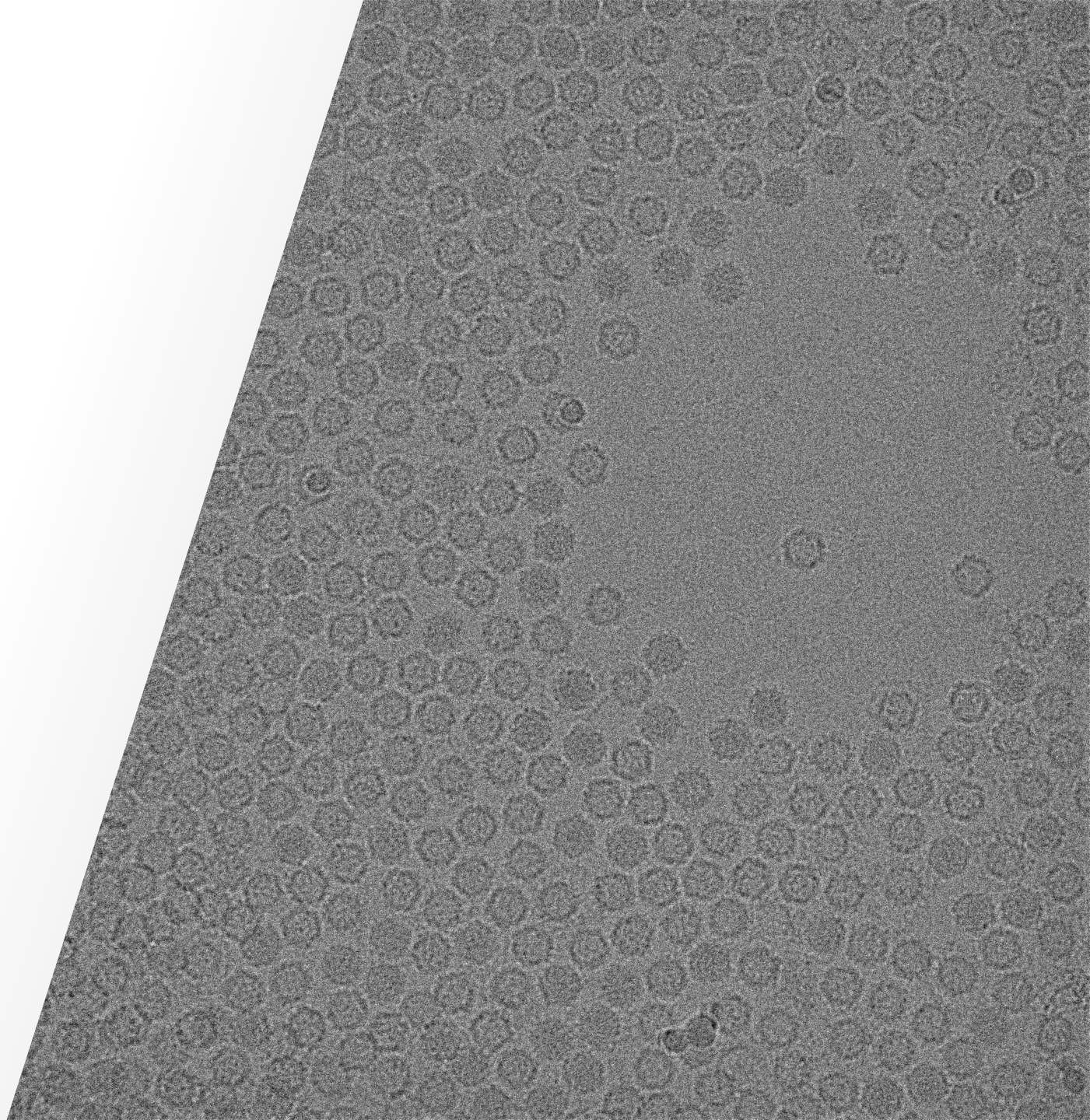
TEM Sample Preparation

Electron Cryo Microscopy in Structural Biology

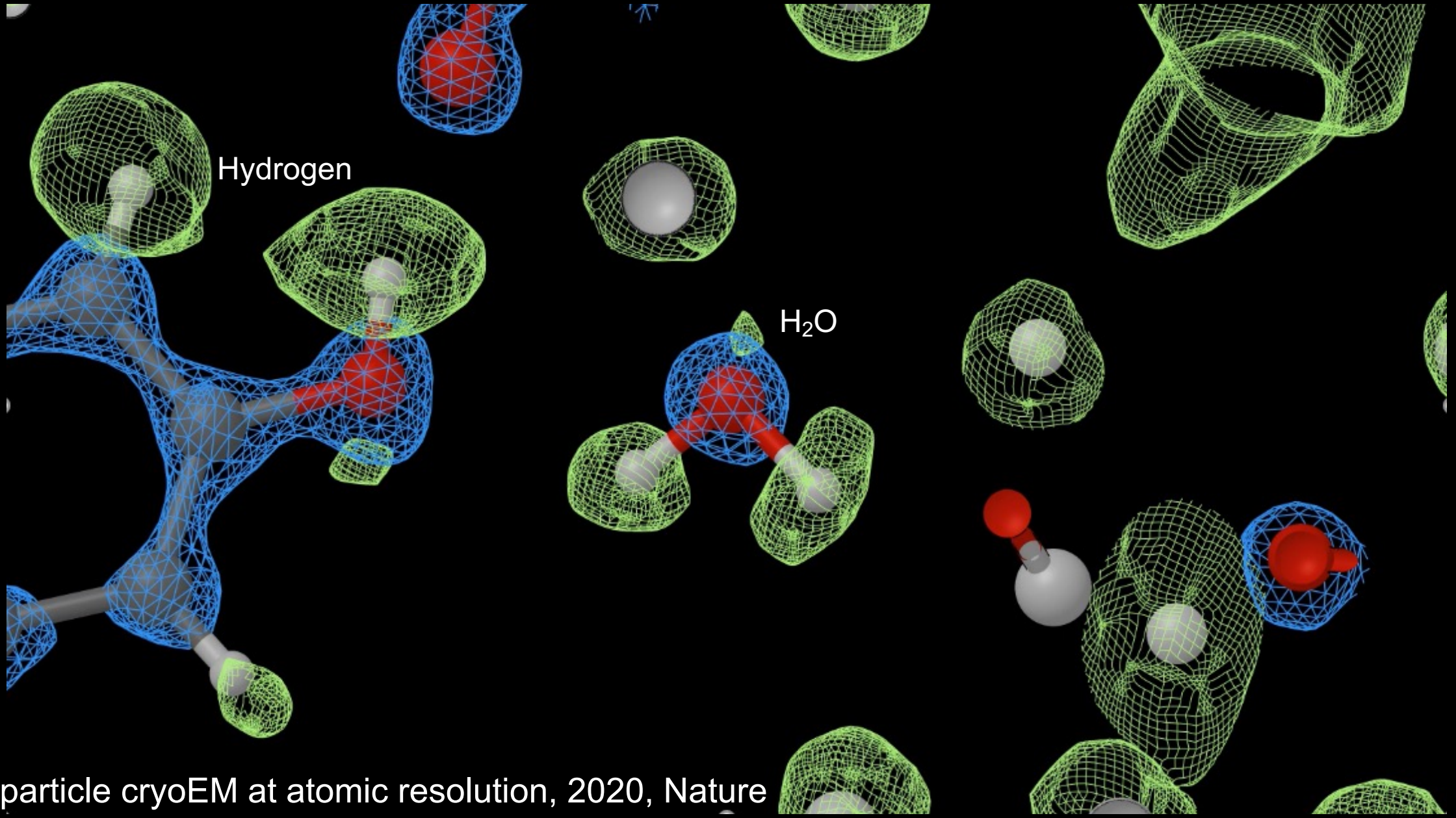
26th -29th April, 2021

eBIC- Diamond Light Source

Dimple Karia



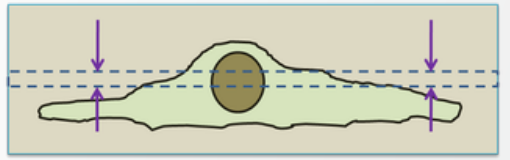
1.22 Å – ApoF Map: B-factor 32.5 Å^2



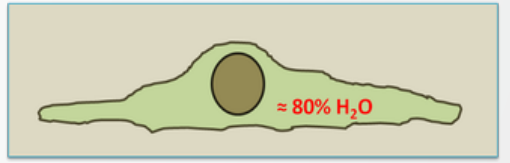
Single-particle cryoEM at atomic resolution, 2020, Nature

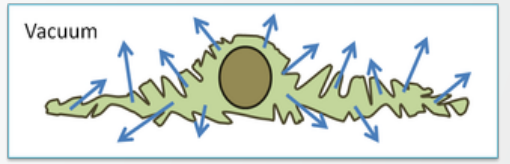
Requirements for TEM samples

- Thickness is limited



- Samples need to be able to withstand the conditions inside the TEM- vacuum and electron beam

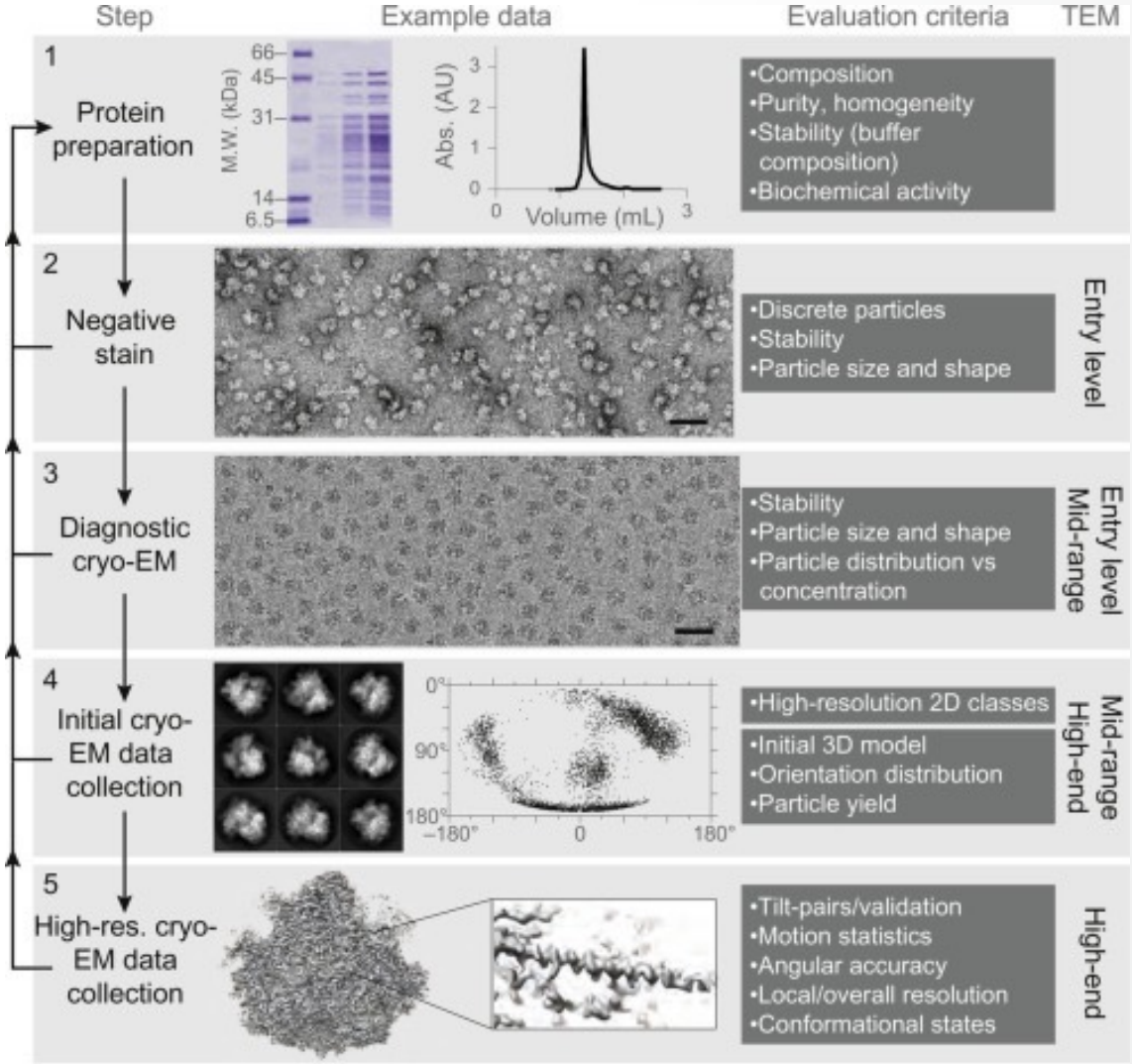




- Biological samples need special treatment with electron dense stains - unless working with cryo samples.

(Images courtesy Dr. Louise Hughes)

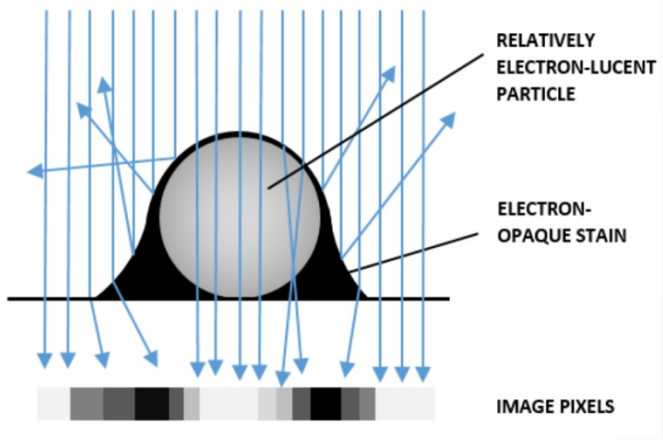
Structure Determination by cryoEM

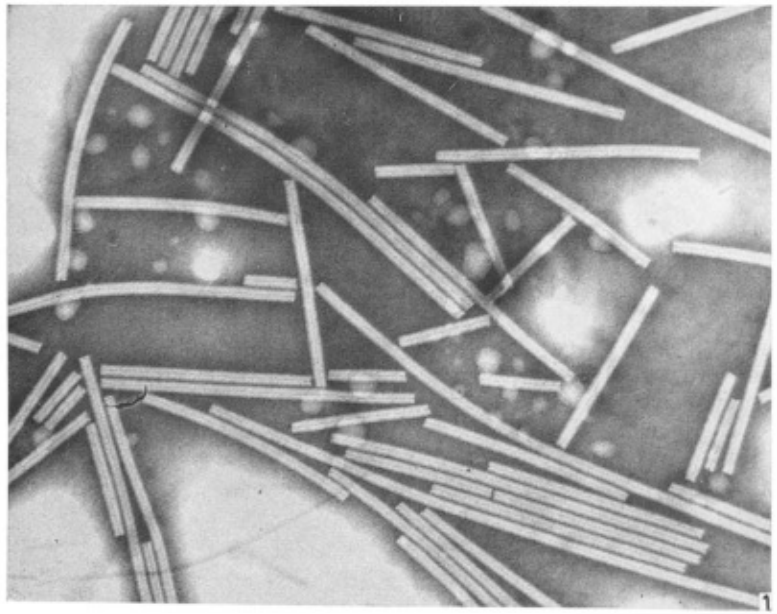


(Passmore & Russo , Methods in Enz 2016)

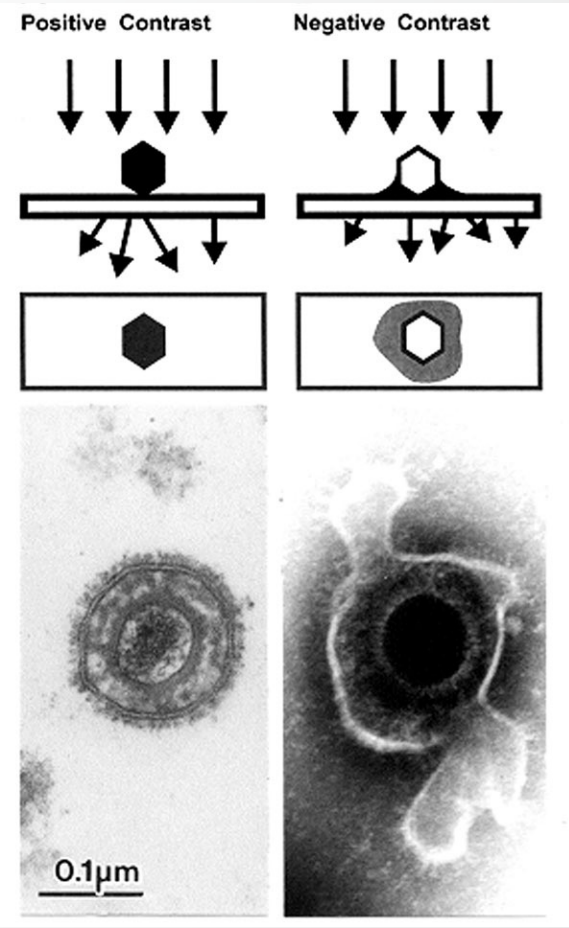
Negative Staining

Uranyl acetate (pH 4)
Uranyl formate (pH 4, finer grain)
Tungstate (neutral pH) [NanoW]
Ammonium molybdate (neutral pH)





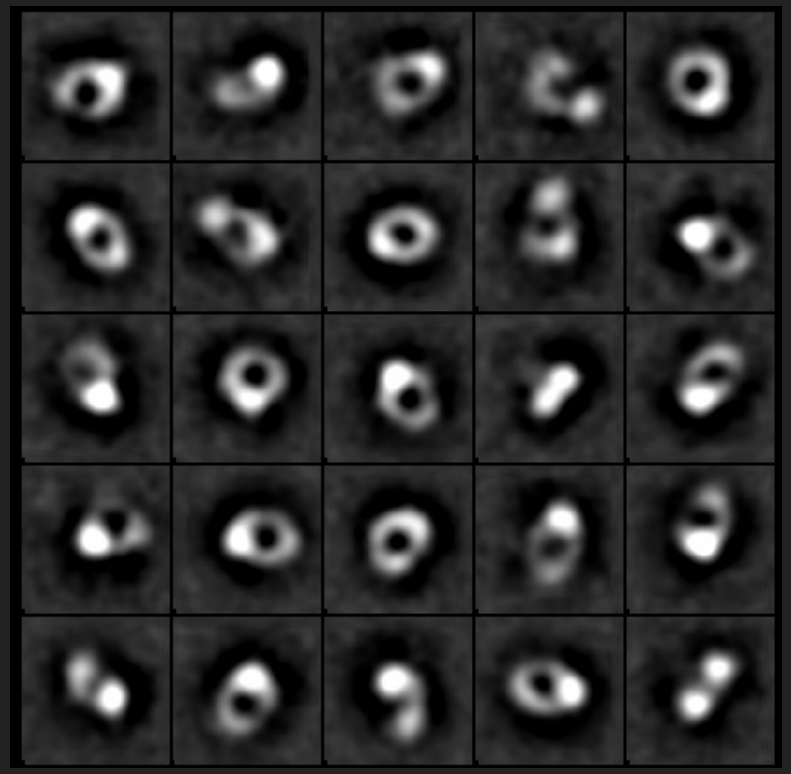
(Brenner & Horne, Biochemica ET Biophysica Acta, 1958)



(Hazelton & Gelderblom , Emerging infectious Diseases 2003)

Negative Staining

Sample quality
Particle shape/size
Distribution/conc



Negative Staining

Pros

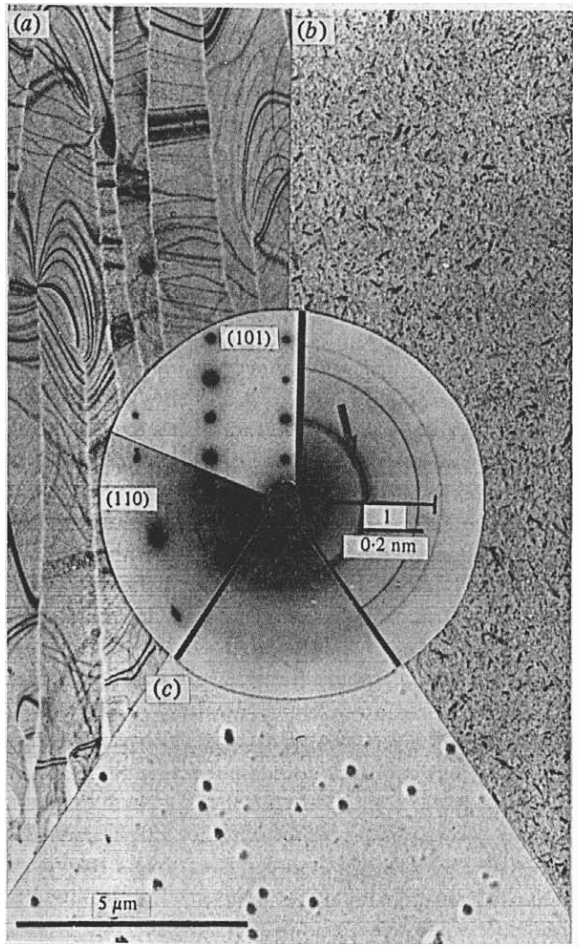
- Grids are easy and quick to prepare and image
- Contrast is very high, allows visualization of small particles
- Radiation damage is not an issue
- Stain helps to stabilize the particles (fixative effect of heavy metals)

Cons

- Resolution is limited (20-25 Å in optimal cases)
- The protein is not imaged directly (instead a shell of stain around the protein)
- Particles may become distorted/flattened due to dehydration
- Uneven staining may cause problems in image processing

Vitrification of Biological samples

Hexagonal ice



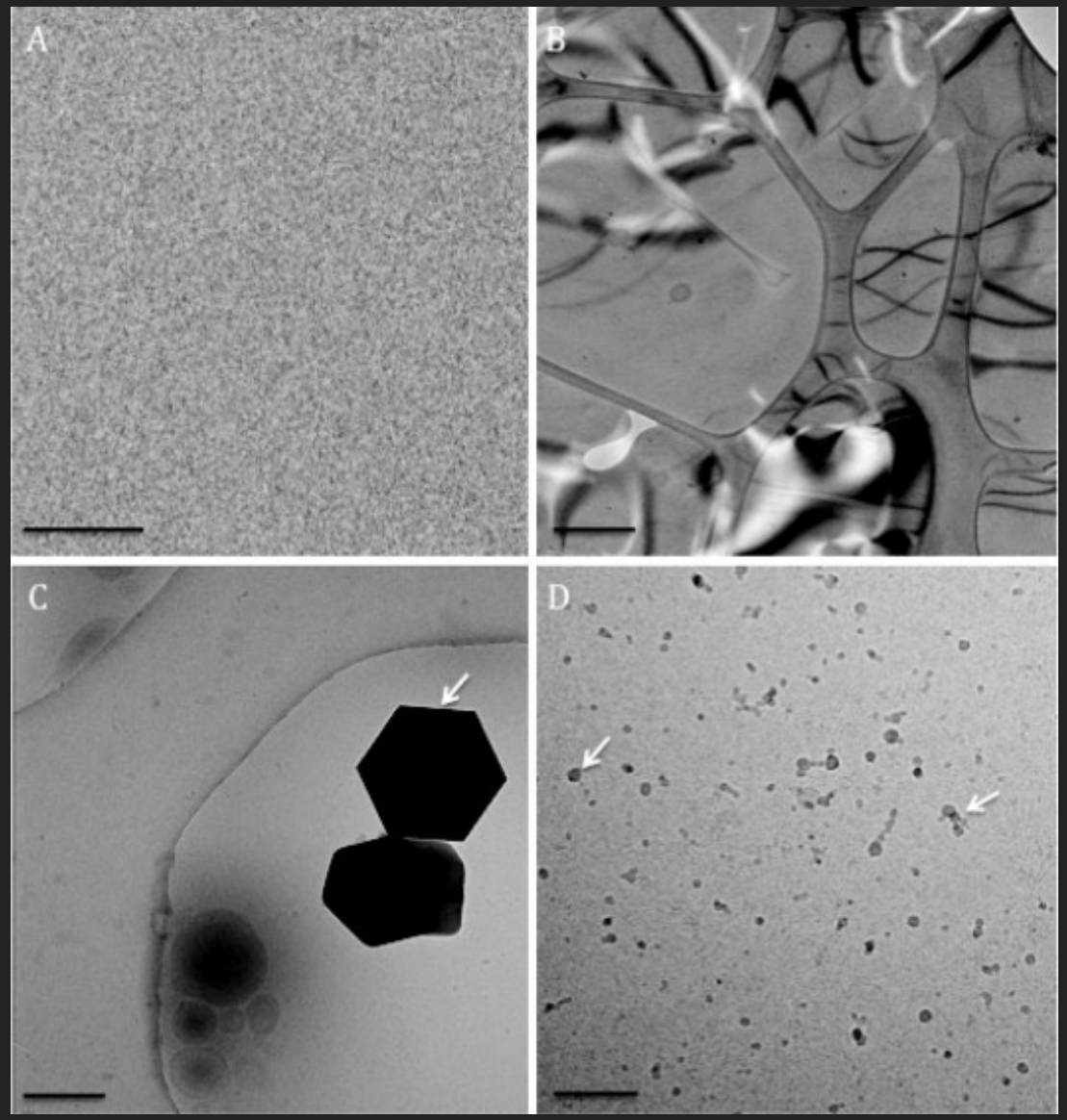
Cubic ice

Vitreous ice

(Dubochet *et al,* QRB 1958)

Jacques Dubochet
(2017 Noble Prize in Chemistry)

Vitreous/non-vitreous ice

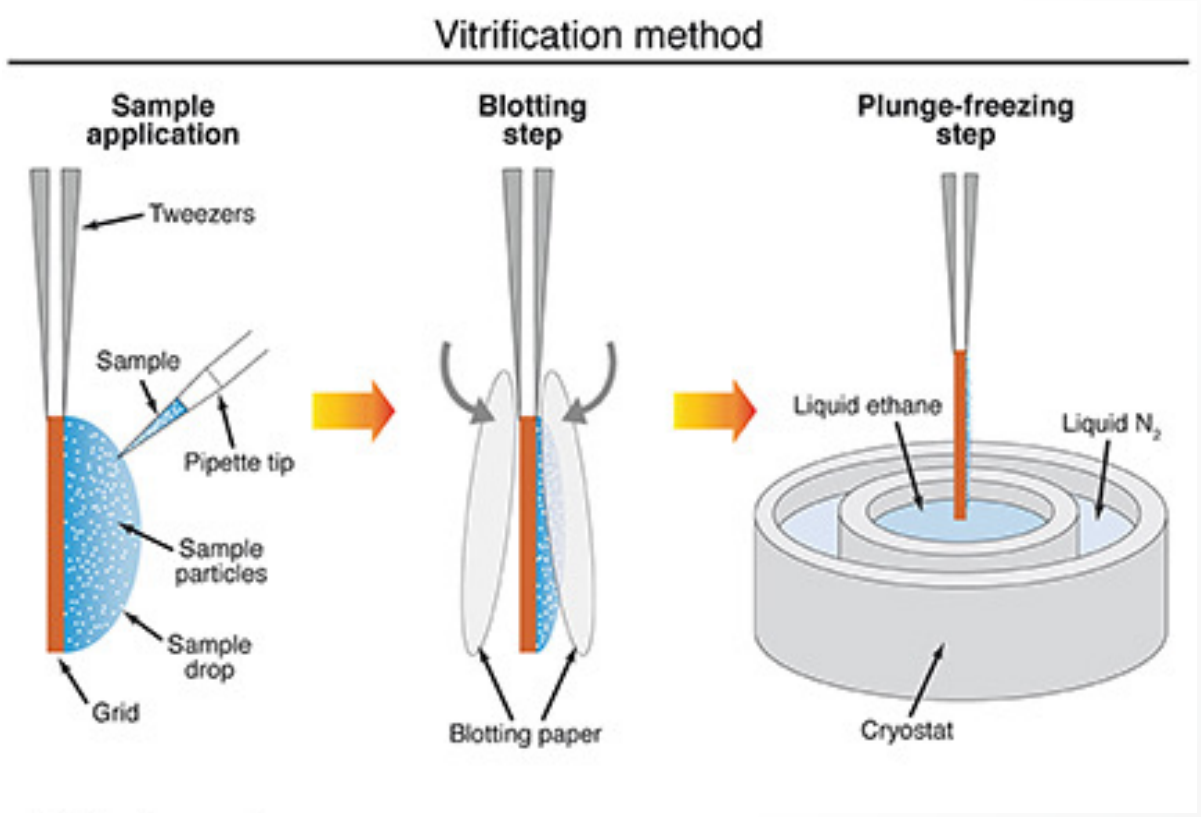


(Thompson *et al,* Methods 2016)

Cryo Samples

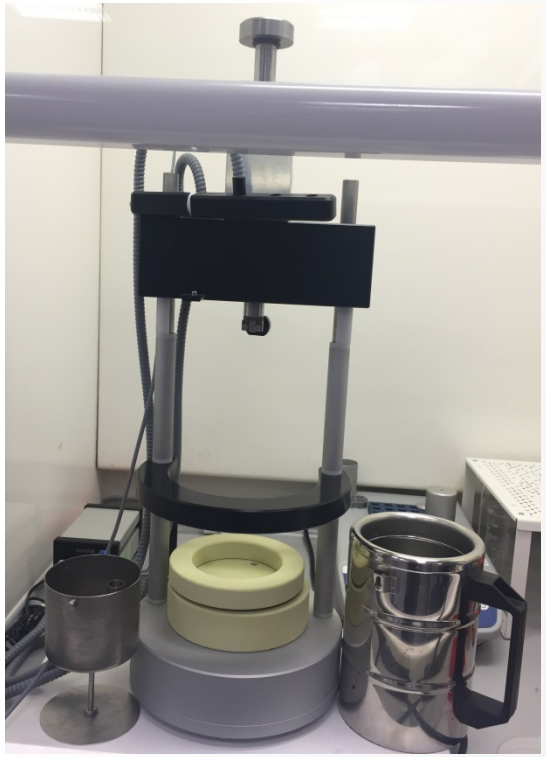
- Allow observation in near native state – no artefacts through chemical fixation or dehydration
- No staining involved - enough density difference in scattering properties of sample and ice
- Several methods can be used to obtain vitrified samples
- Grid preparation takes longer and generally involves the use of highly specialized and expensive equipment
- Need low dose imaging due to susceptibility to radiation damage (but: low temperatures can protect against damage)

cryoEM sample preparation

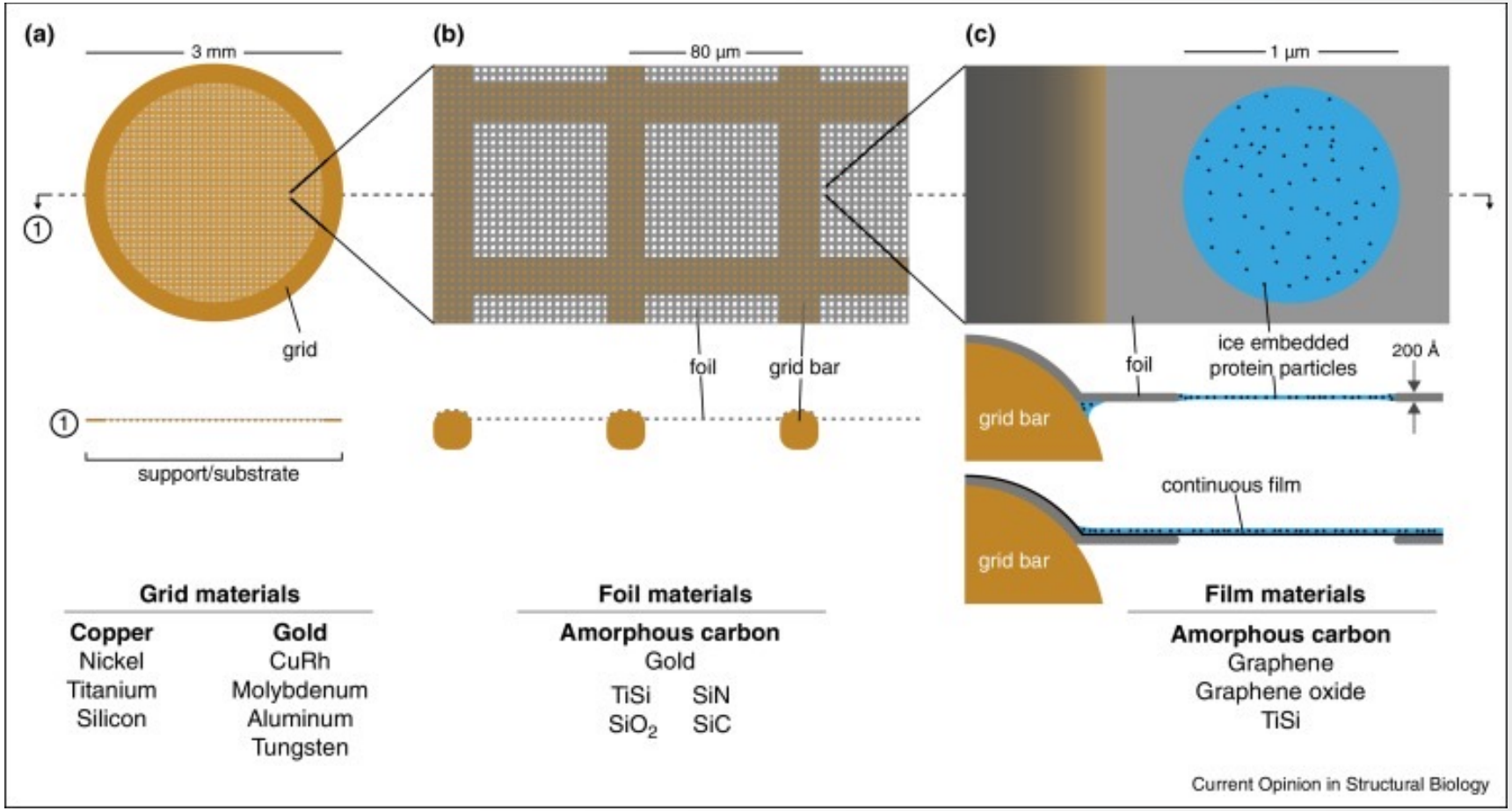


(Sgro & Costa, Front. Mol. Biosci., 2018)

Plunging instruments

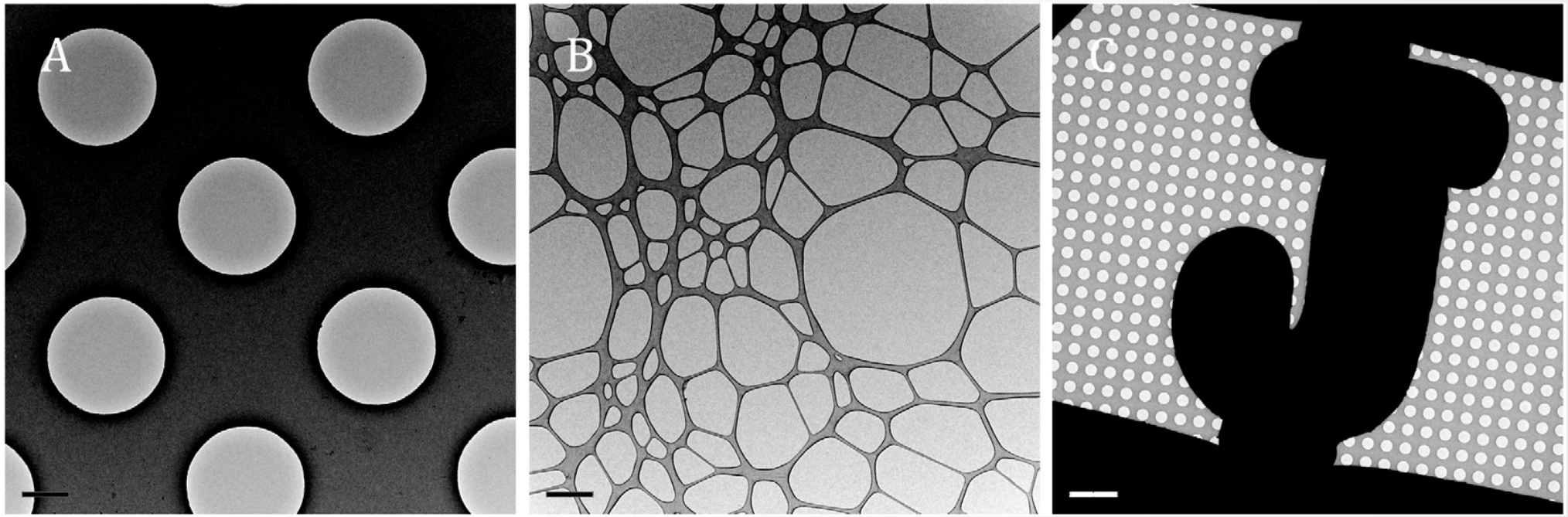


EM Grids



(Russo & Passmore, Current Opinion in Str Biology 2016)

EM Support films



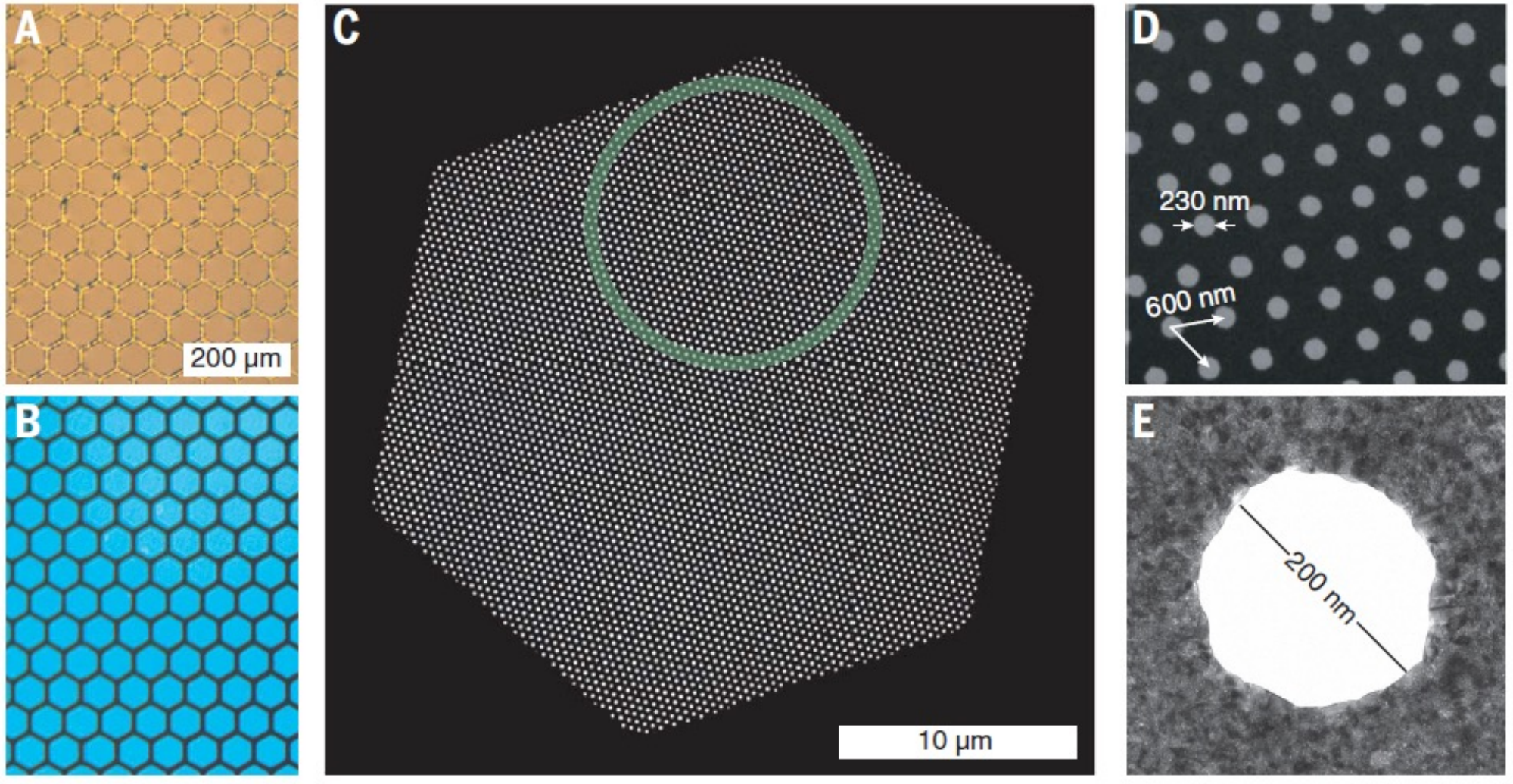
Holey

Lacey

Finder

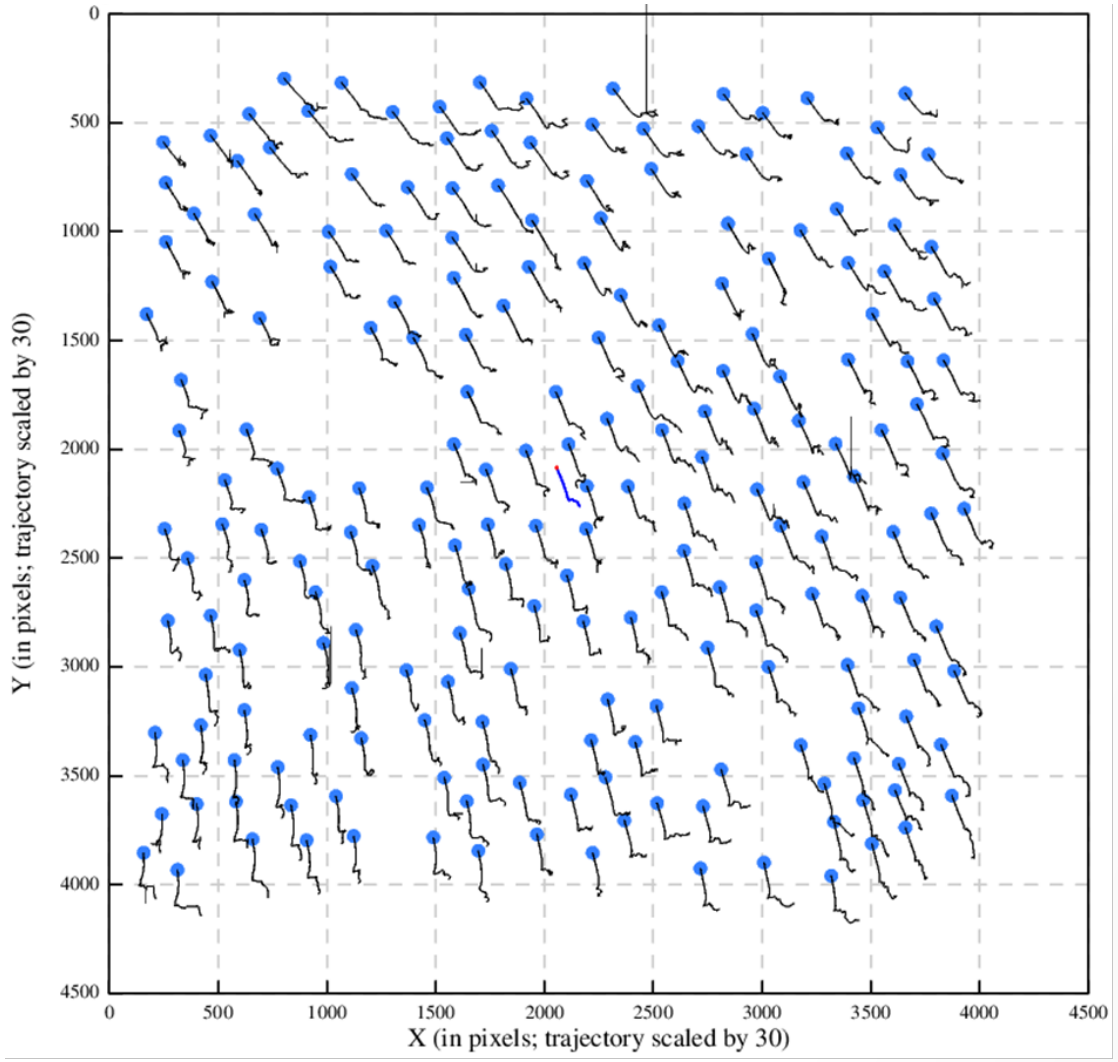
(Thompson *et al*, Methods 2016)

Hex Au Foil grids- reduced beam induced motion

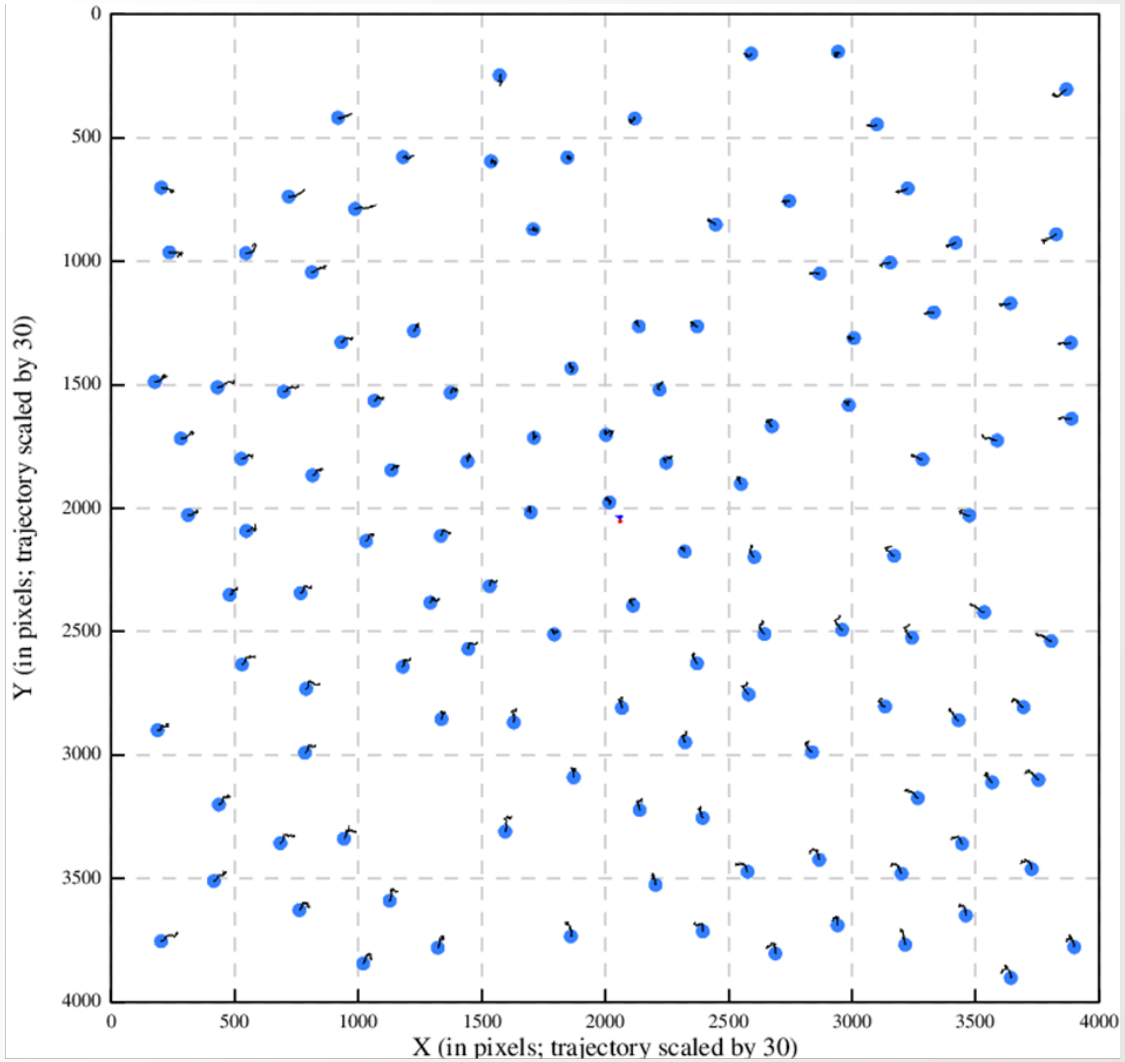


(Naydenova *et al,* Science 2020)

Motion Tracks



UltraAu 1.2/1.3um grid



HexAu 0.29/0.6um grid (Russo)

Motion in Early Frames 0.1e/Å^2

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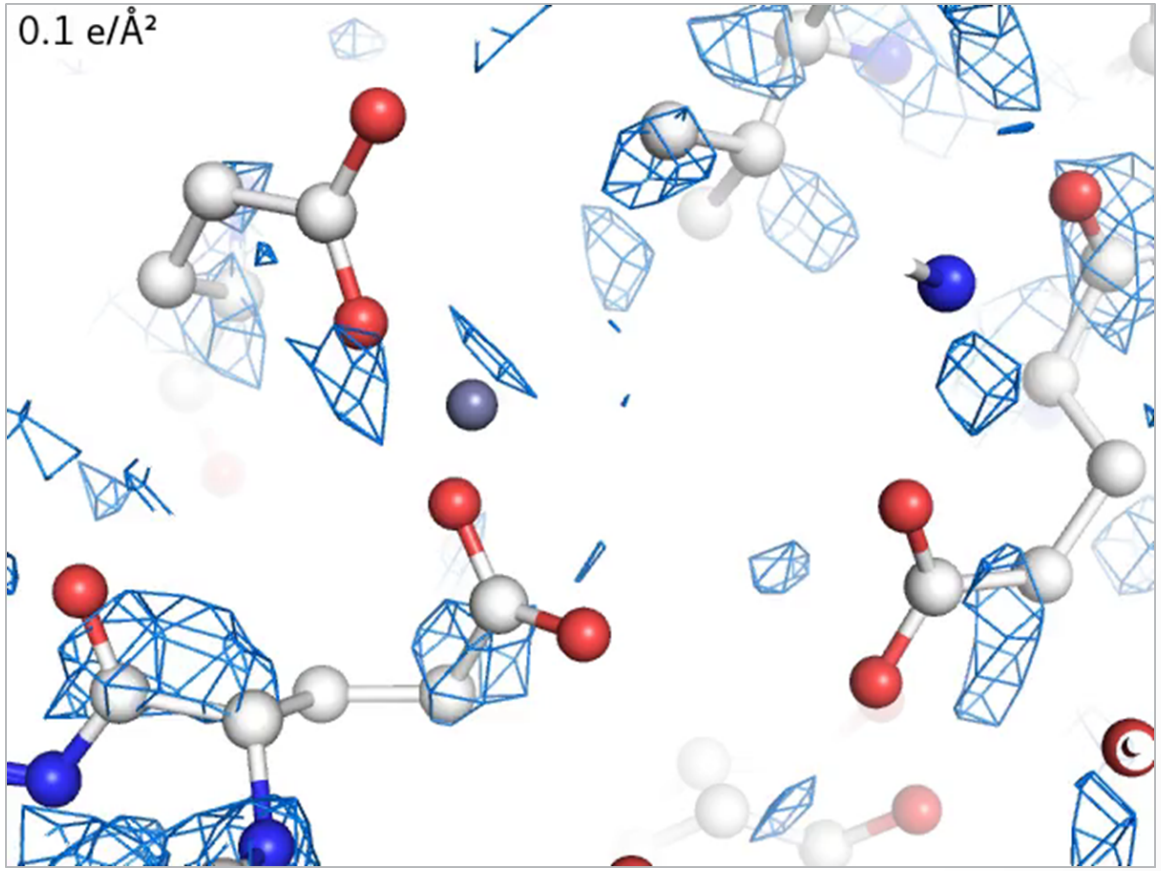
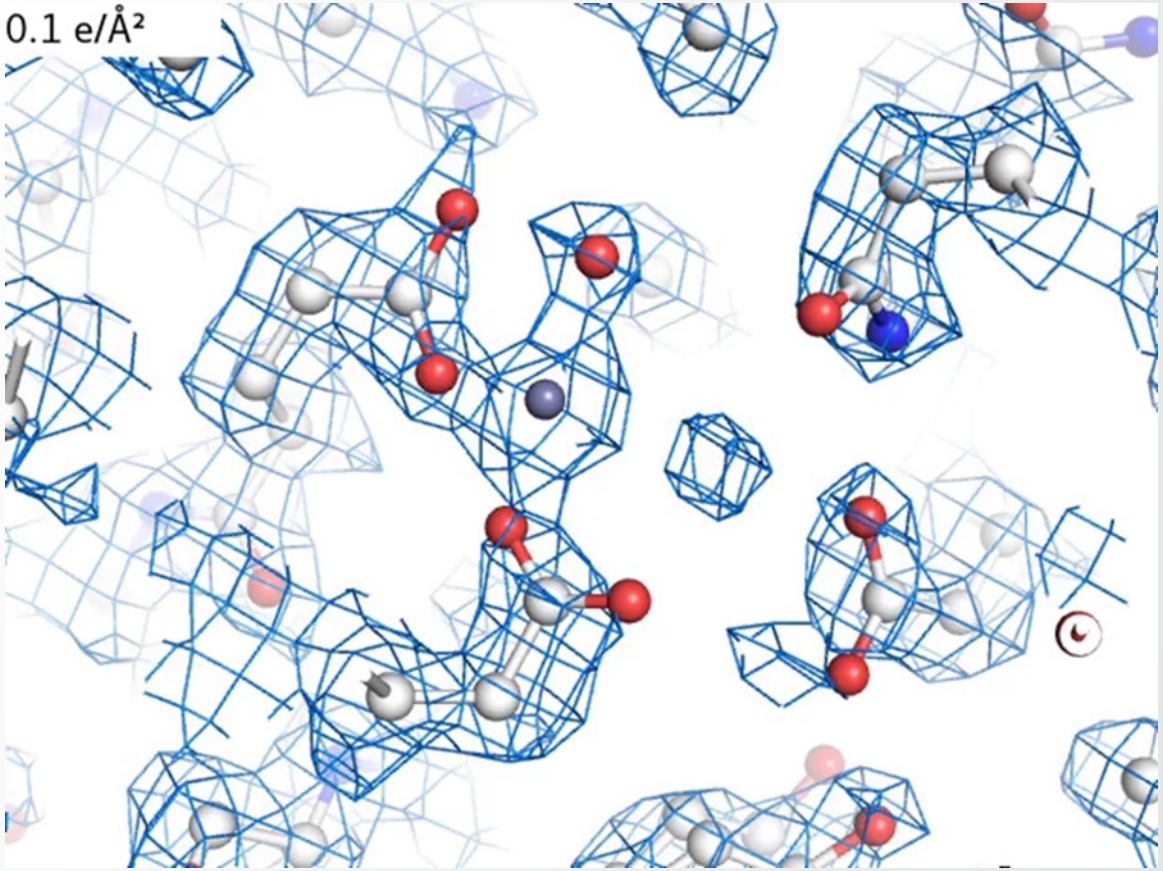
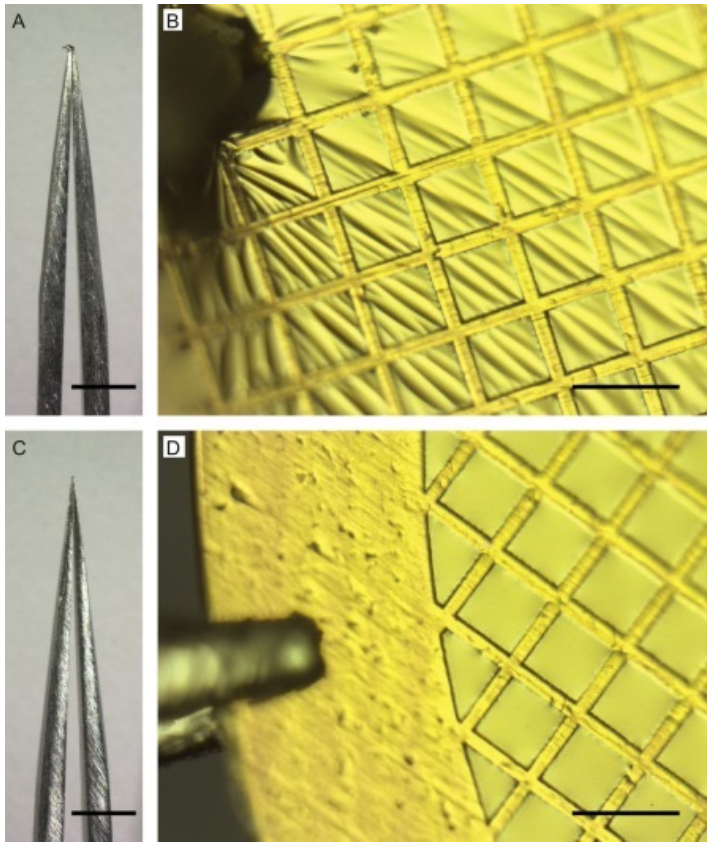
UltraAu 1.2/1.3um grid

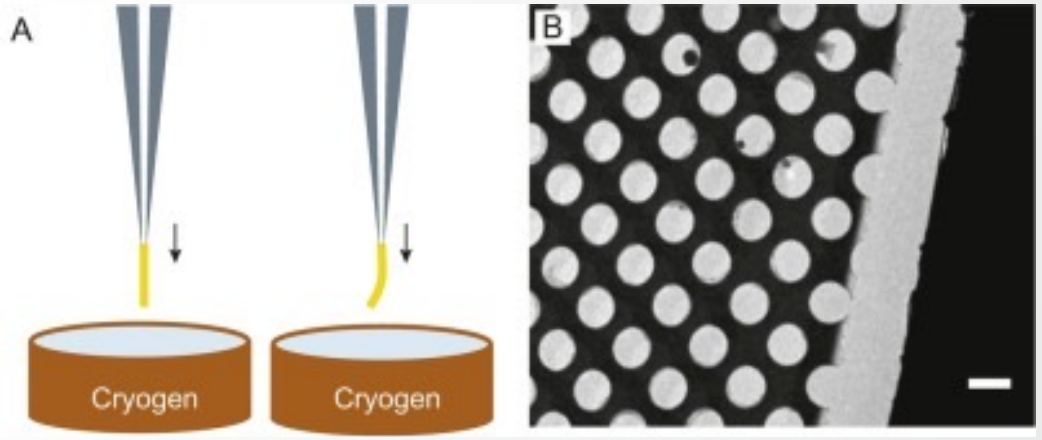


HexAu 0.29/0.6um grid



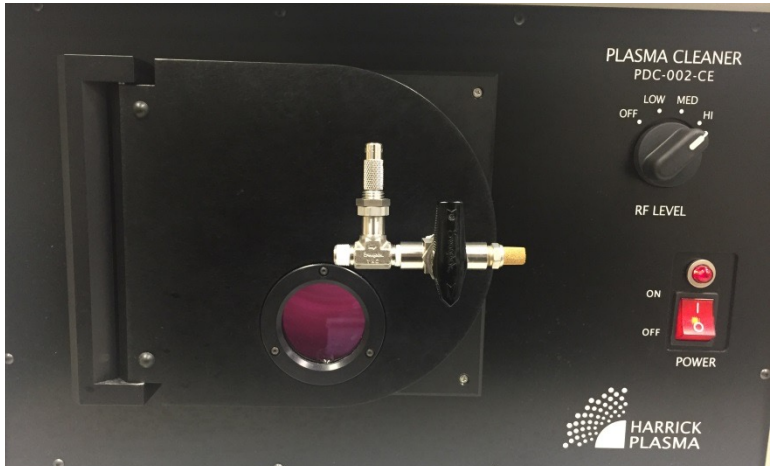
Handling of EM grids



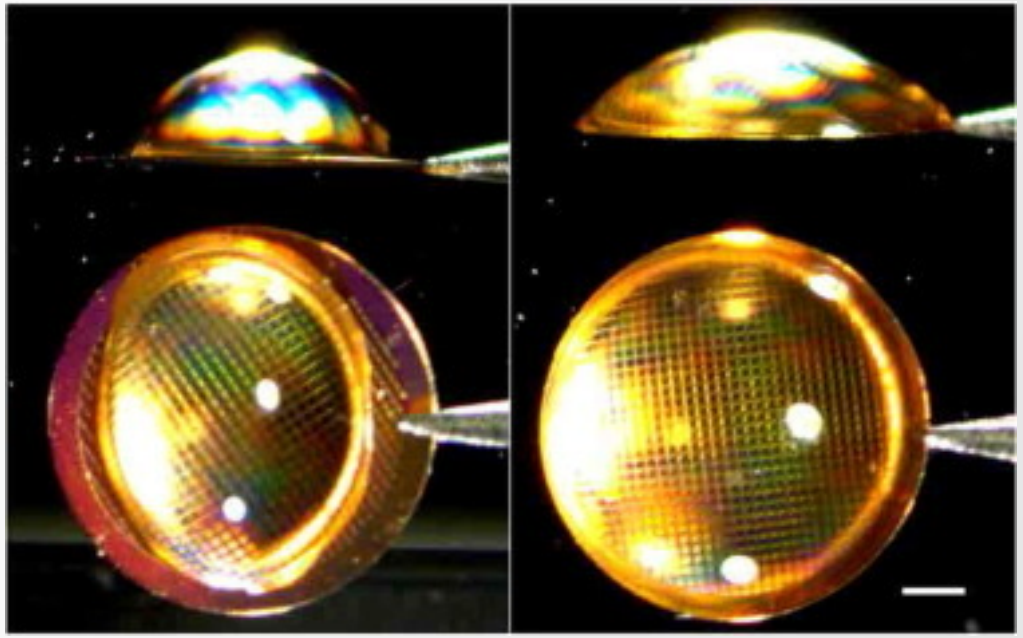


(Passmore & Russo , Methods in Enz 2016)

Plasma cleaning

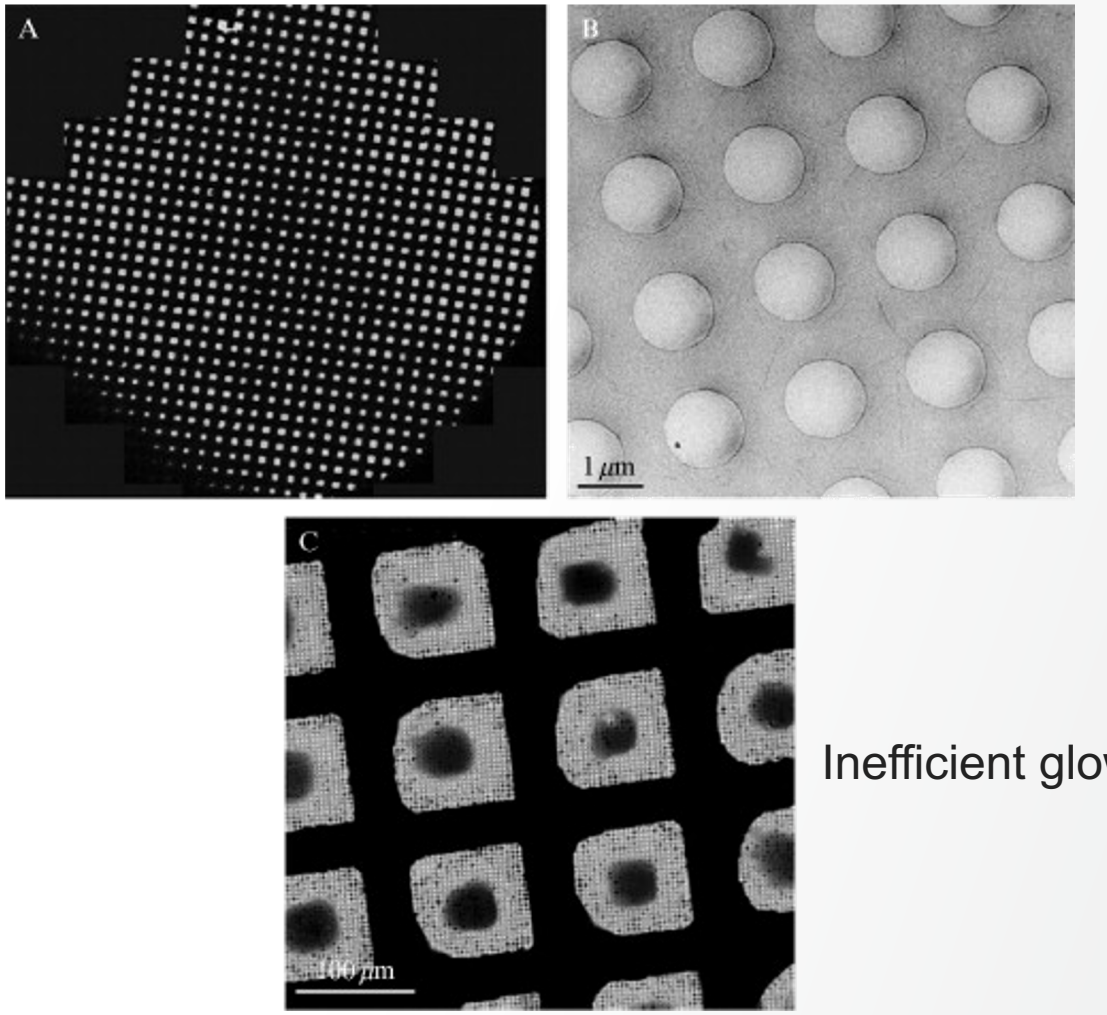


Air (glow discharge)
Oxygen
Argon
Hydrogen



(Russo & Passmore, J Struct Biol 2016)

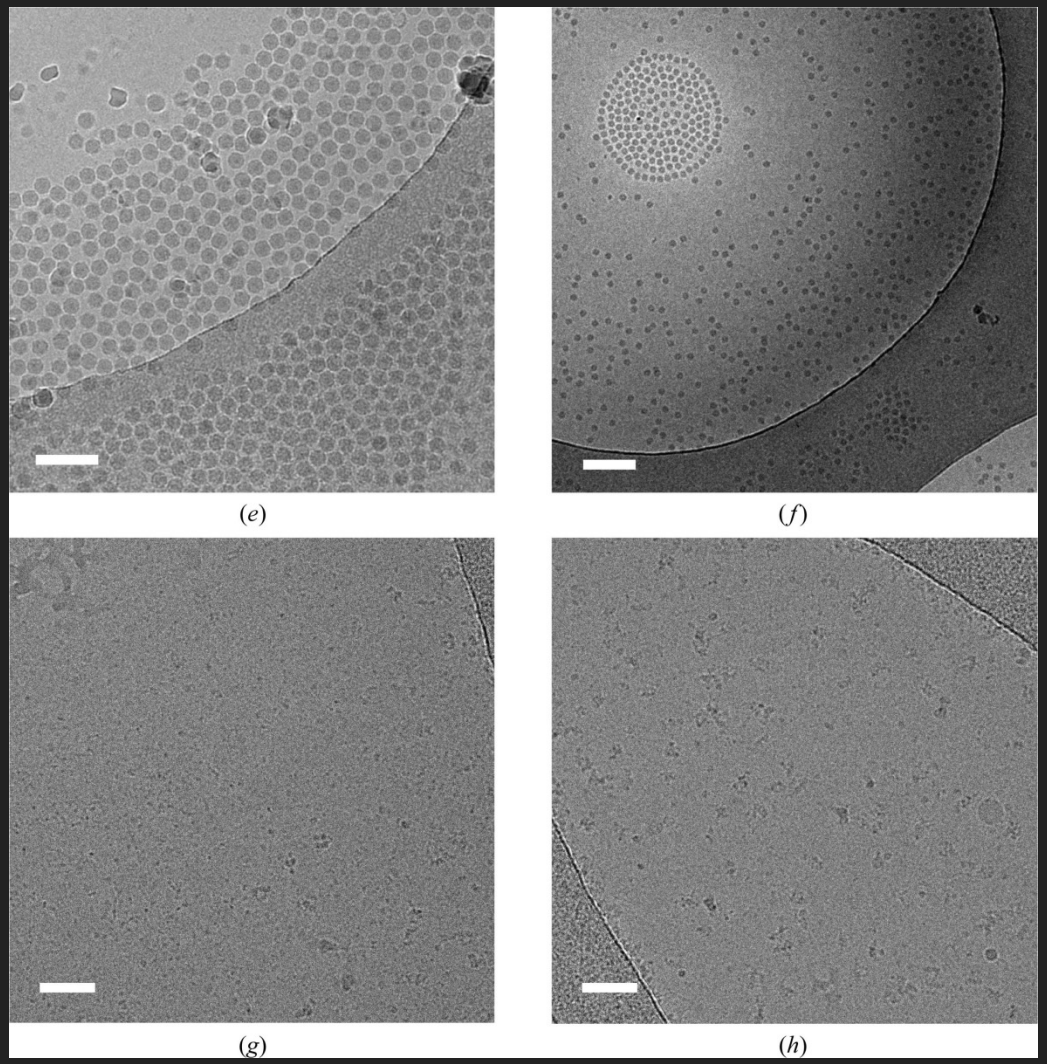
Effect of Plasma cleaning on ice quality



Inefficient glow discharge

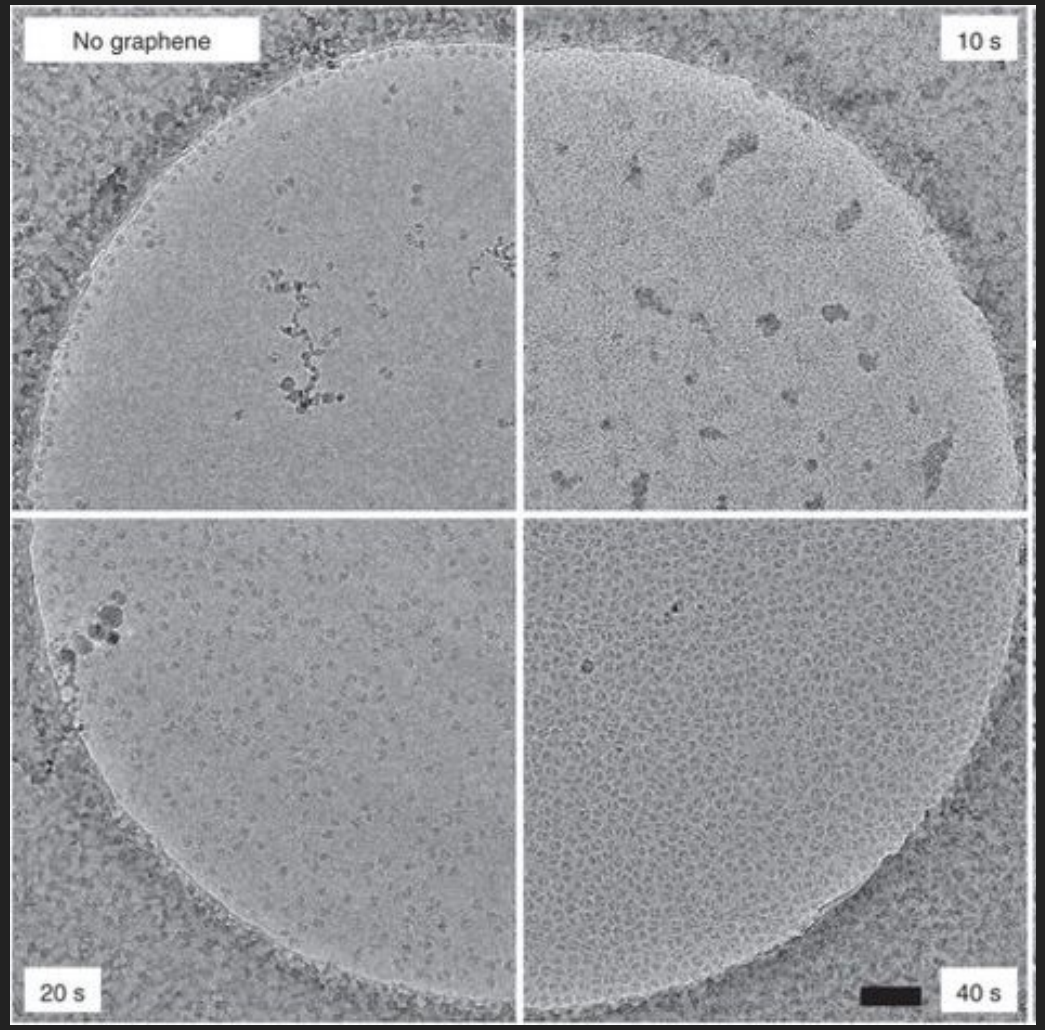
(Dobro *et al* , Methods in Enz 2010)

Particle distribution in ice



(Drulyte *et al,* Acta Crystallogr D 2018)

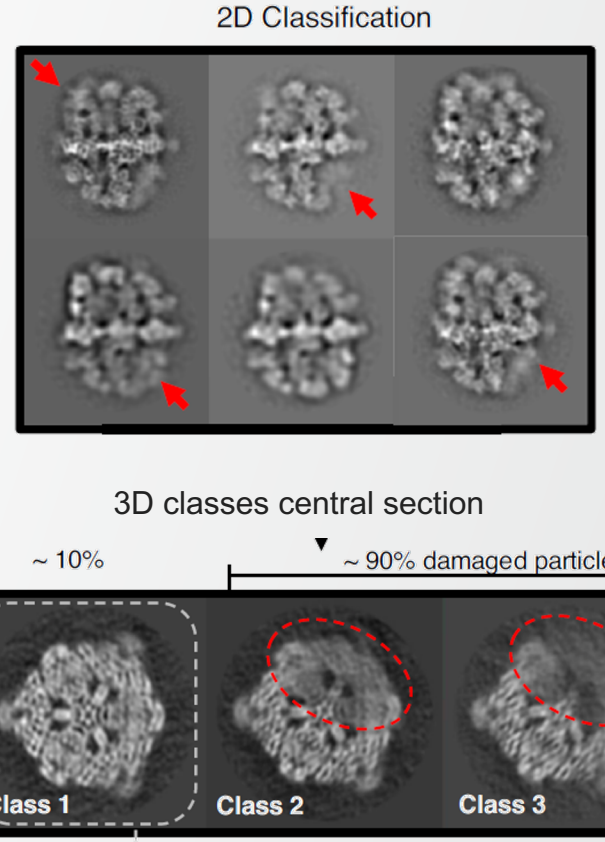
Particle distribution on support film



(Russo & Passmore, Nat Methods 2016)

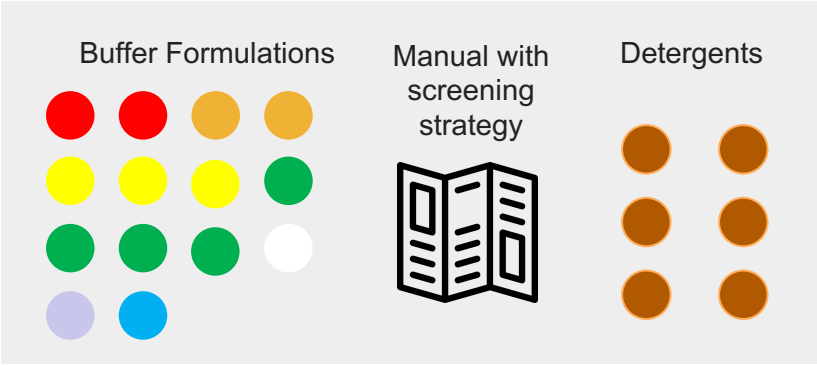
Challenges of cryoEM sample preparation

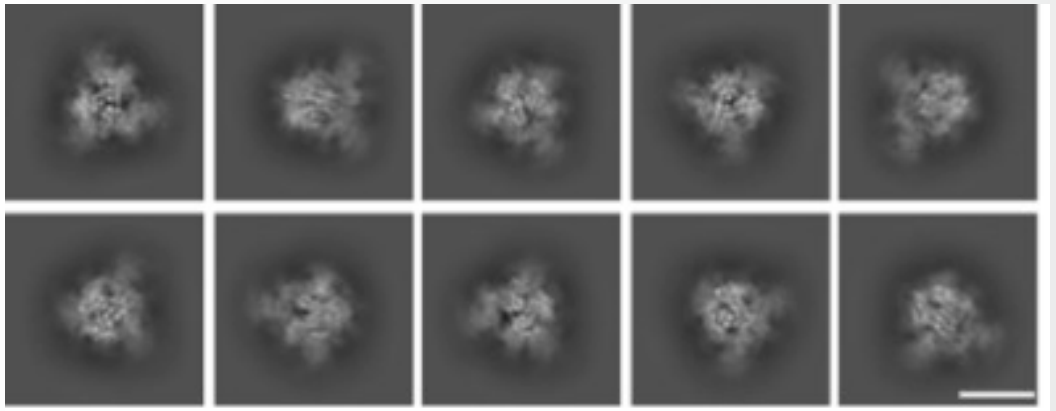
- Sample sticking to foil material → Grid PEGylation
- Heterogeneity → crosslinking for multi-protein complexes
- Low particle density → Support films (carbon, graphene)
- Low image contrast → optimize buffer conditions
- Sample aggregation/denaturation on grid → optimize buffer conditions/use support films
- Preferential orientation → Use detergents/Plasma clean in presence of pentylamine/collect tilted dataset

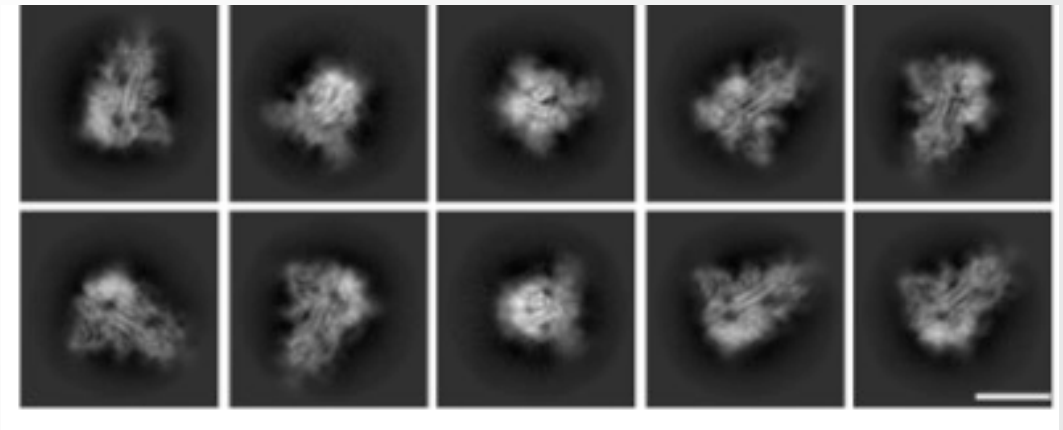


(D'Imprima *et al* , eLife 2019)

Vitroease™ Buffer Screening Kit







https://www.thermofisher.com/order/catalog/product/A49856?SID=srch-srp-A49856#/A49856?SID=srch-srp-A49856

Particle distribution Vs concentration

Number of particles in projection/μm^2 in 800 Å thick ice film (separation)

	Concentration				
M.W.	10mg/ml	2mg/ml	0.5mg/ml	0.1mg/ml	20µg/ml
10 kD	48000 (45Å)	10000 (100Å)	2500 (200Å)	500 (450 Å)	100 (1000 Å)
50 kD	10000 (100Å)	2000 (220Å)	500 (400Å)	100 (1000Å)	20 (0.2µm)
250kD	2000 (220Å)	400 (500 Å)	100 (1000 Å)	20 (0.2µm)	4 (0.5µm)
1 MD	500 (400Å)	100 (1000Å)	25 (0.2µm)	5 (0.4µm)	1 (1µm)
5 MD	100 (1000Å)	20 (0.2µm)	5 (0.4µm)	1 (1µm)	0.2 (2.2µm)
25 MD	20 (0.2µm)	4 (0.5µm)	1 (1µm)	0.2 (2.2µm)	0.04 (5µm)

(Vinothkumar & Henderson, QRB 2016)

New Developments

Spot-it-on

Vitrojet

Pros and Cons of cryoEM

Pros

- No fixation, dehydration or staining artefacts
- Native conformation is preserved
- Allows for more random orientation
- Resolution information is higher than in negative stain

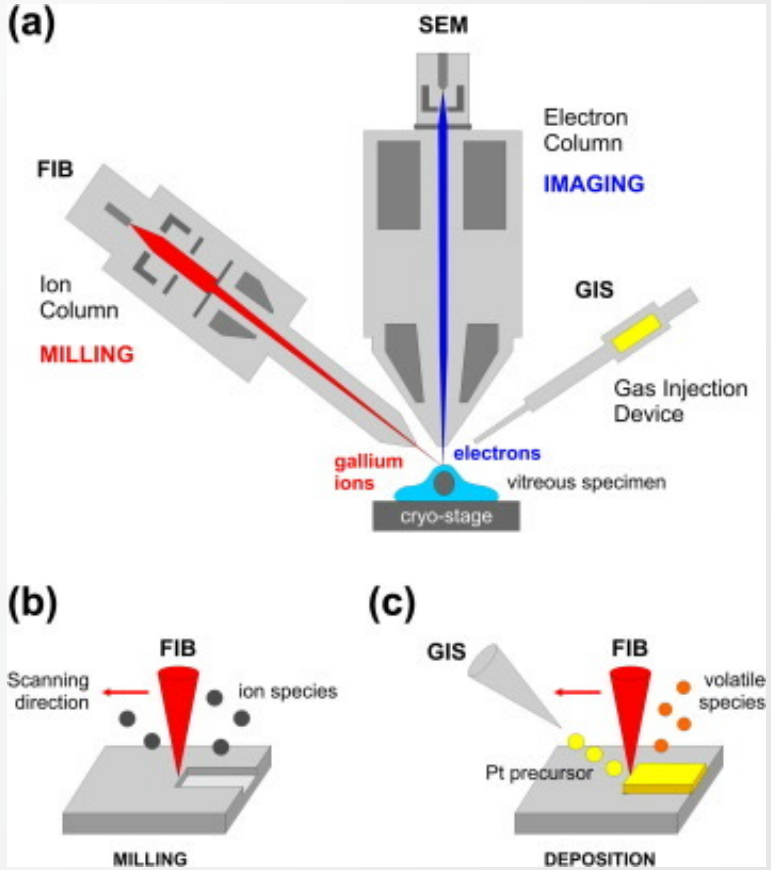
Cons

- The contrast is lower than in negative stain samples
- Signal-to-noise-ratio is low – collecting a large number of images can overcome this problem
- It is more difficult to obtain good quality grids
- Risk of contamination/warm up

FIB SEM preparation

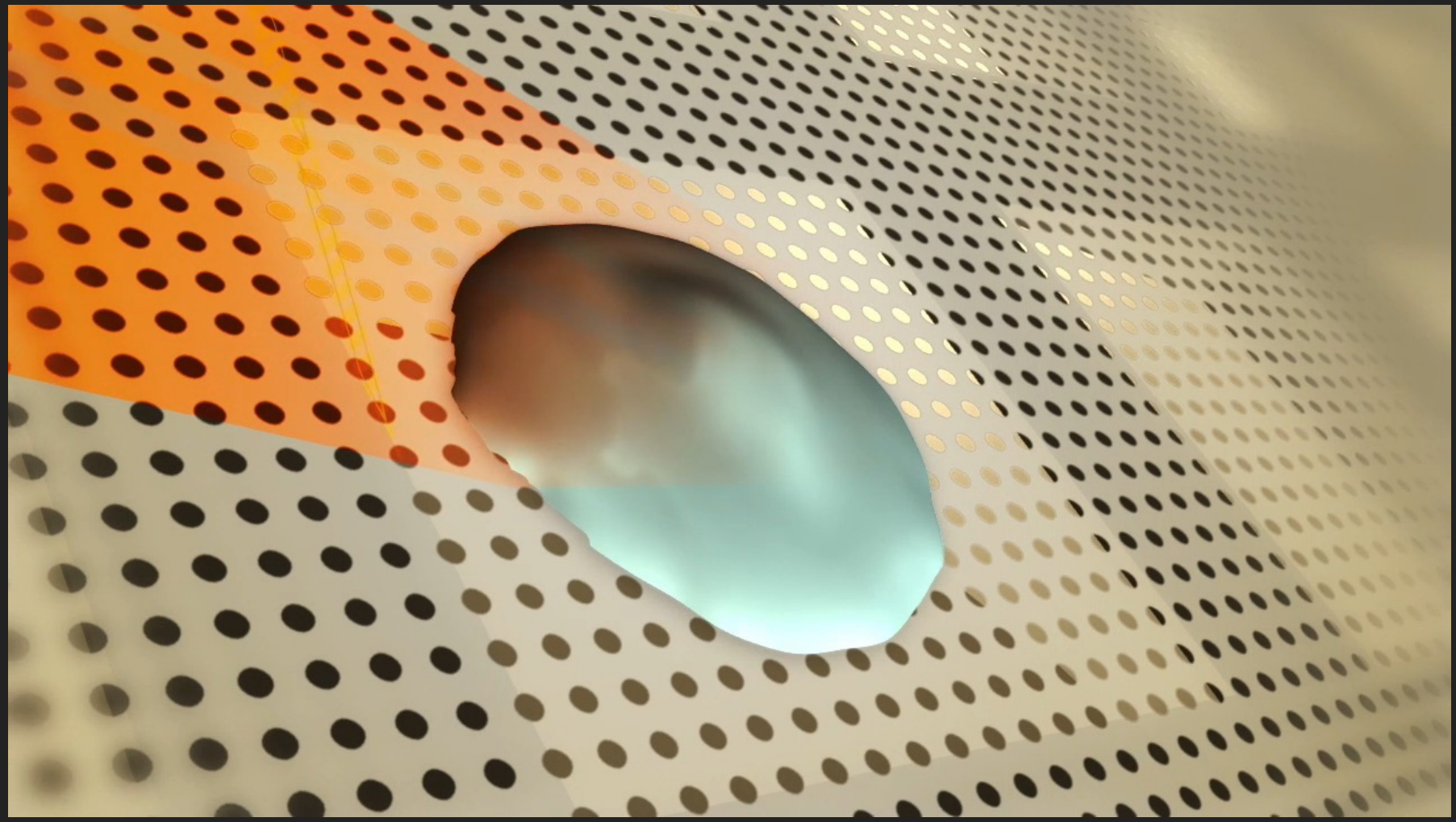
Operating principle of a FIB/SEM microscope.

(a) A focused gallium ion beam removes material from the sample in a process called milling. Non-destructive imaging is performed with the electron beam.
(b) Ion beam milling: kinetic energy transfer during multiple ion-atom collisions causes surface atoms to overcome their surface binding energy and to become ejected as a sputtered species. Scanning the ion beam multiple times over the target surface leads to progressive removal of material.
(c) Ion beam-assisted deposition: the ion beam is used to deposit molecules released from a gas-injection needle onto the specimen surface.

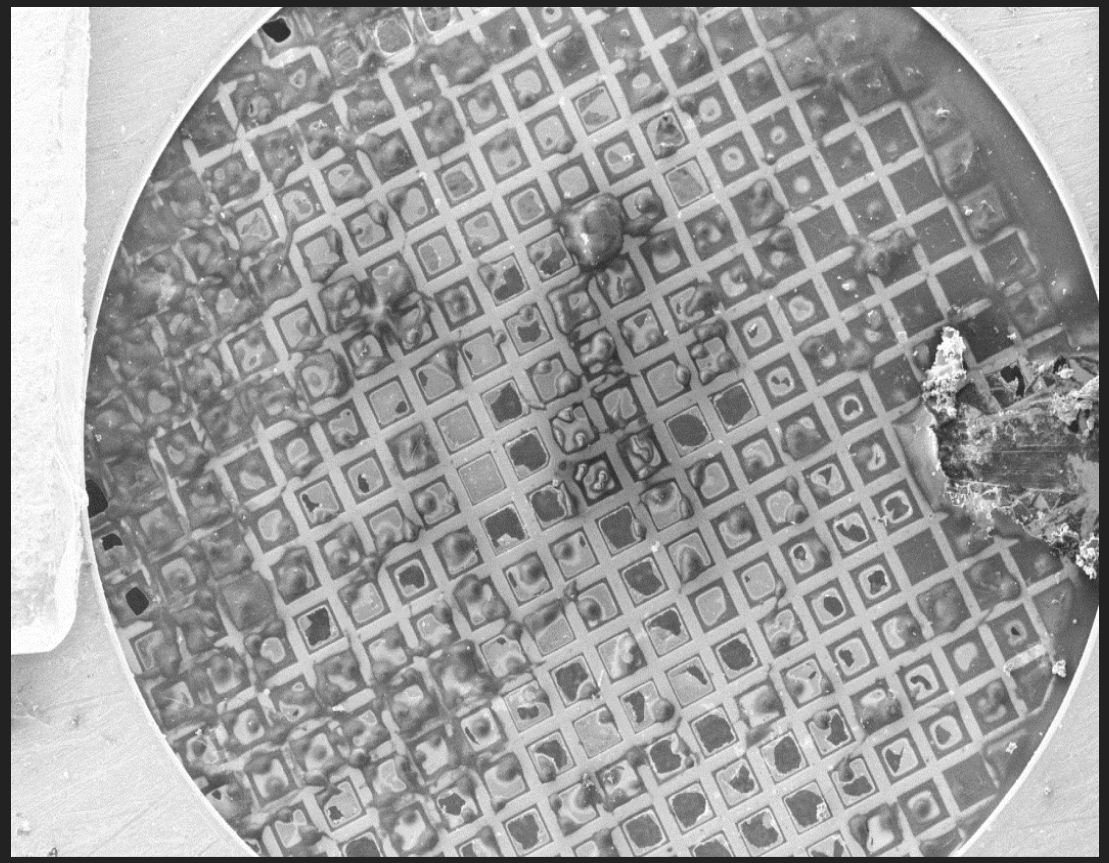


(Rigort *et al* , Archives of Biochem and Biophy 2015)

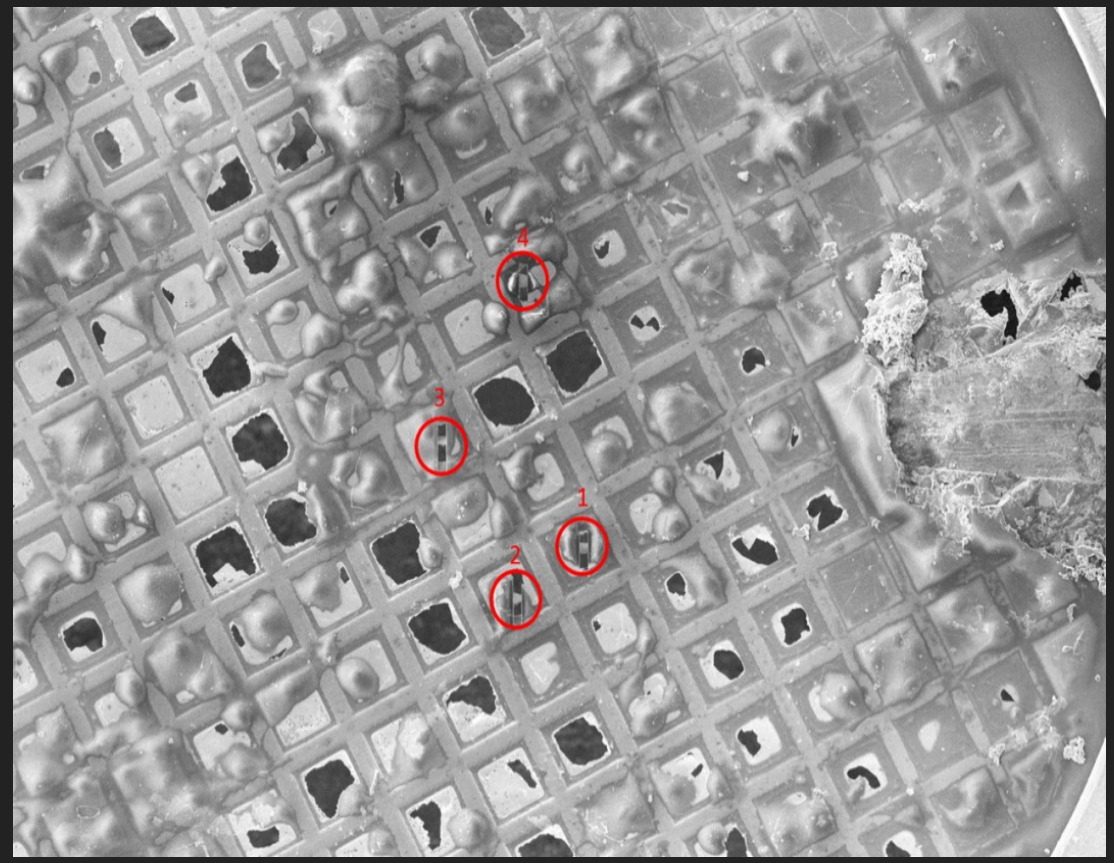
In-Situ Lamella Milling



FIB milling for cellular tomography

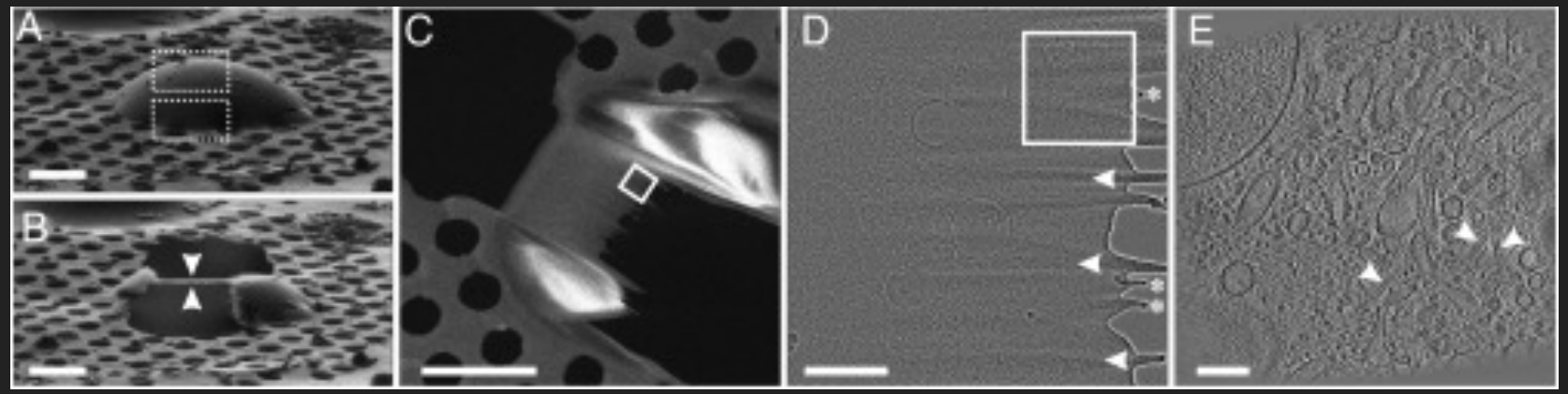


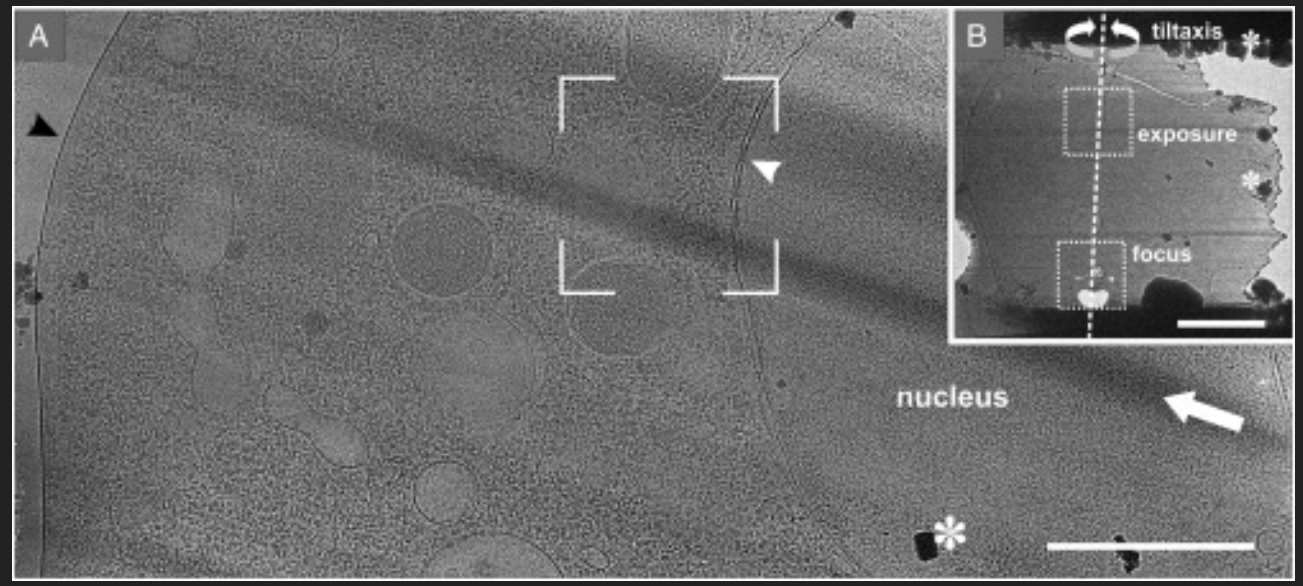
(Before)



(After)

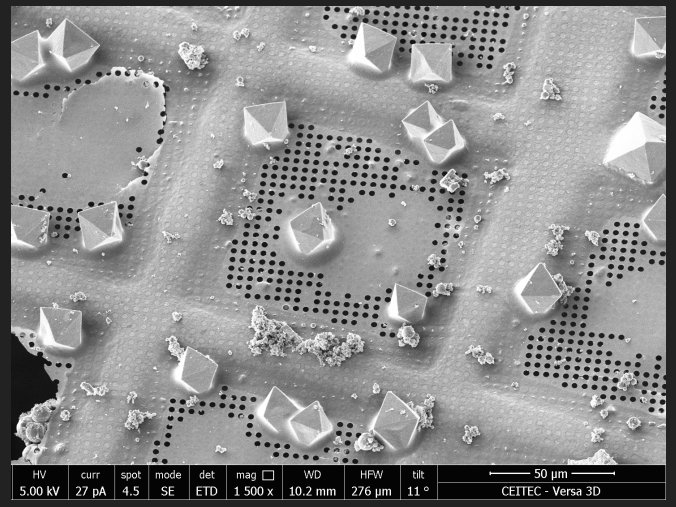
FIB milling for cellular tomography

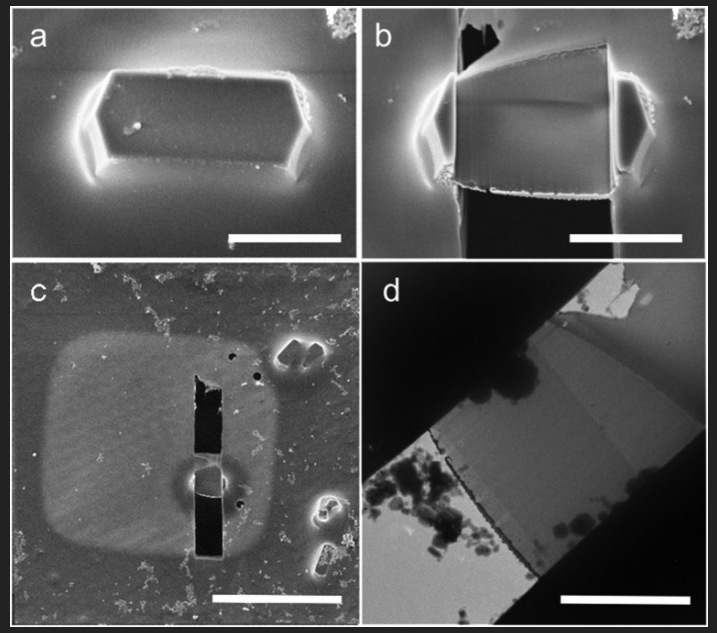




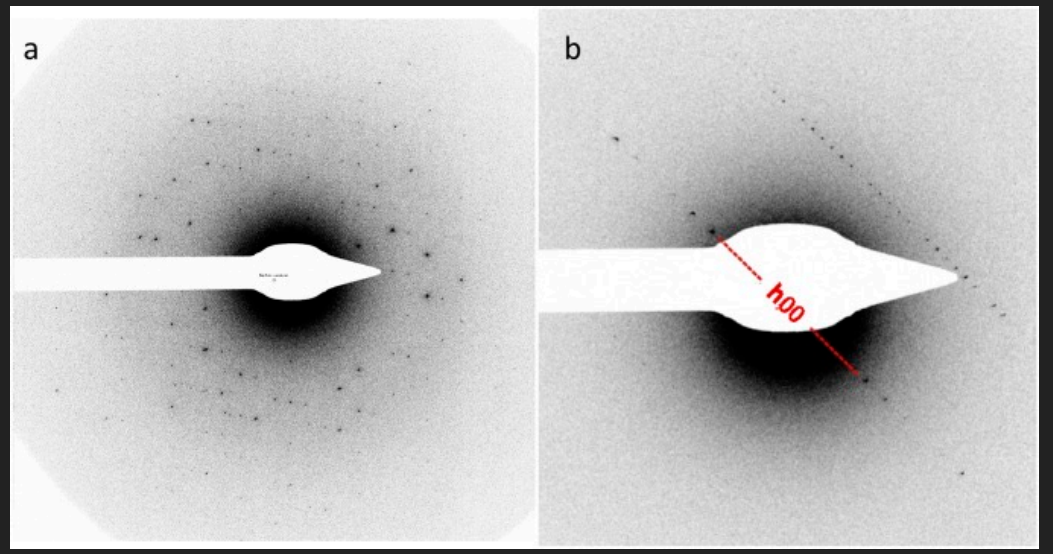
(Rigort *et al*, PNAS 2012)

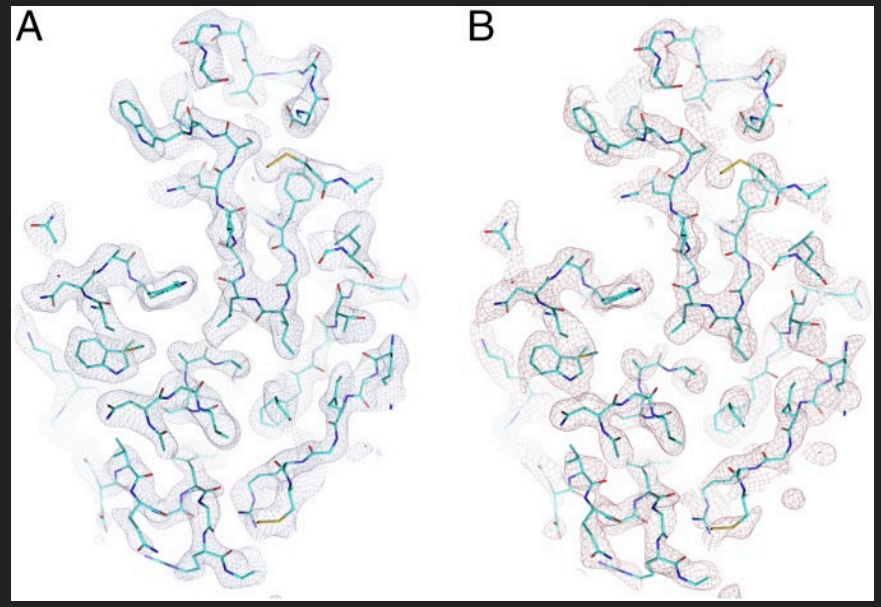
FIB milling for microED





(Duyvesteyn & Kotecha *et al* , PNAS 2018)





Take Home message!

Optimize your sample well!