Transmission electron microscopy in structural Biology

From specimen preparation to data collection

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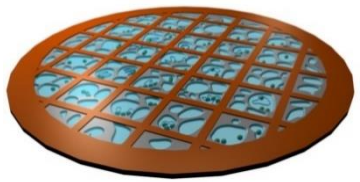
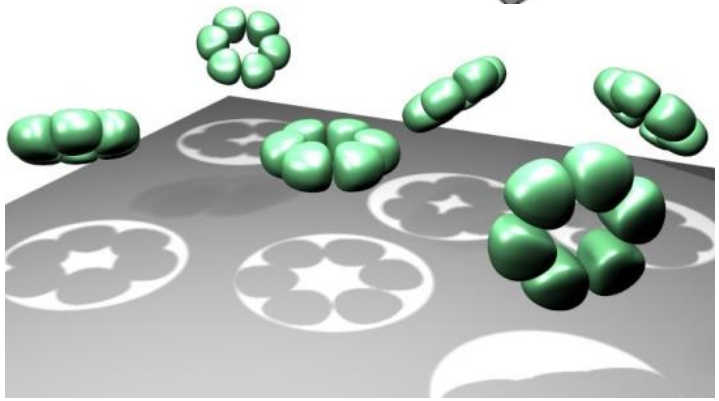
Specimen preparation



Frozen-hydrated Molecules on EM grid

Data collection with an Electron Microscope

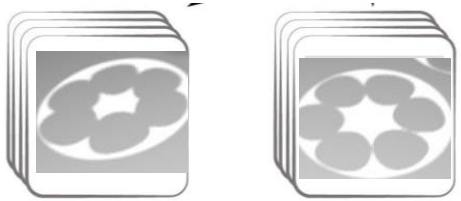
Electron Beam molecules



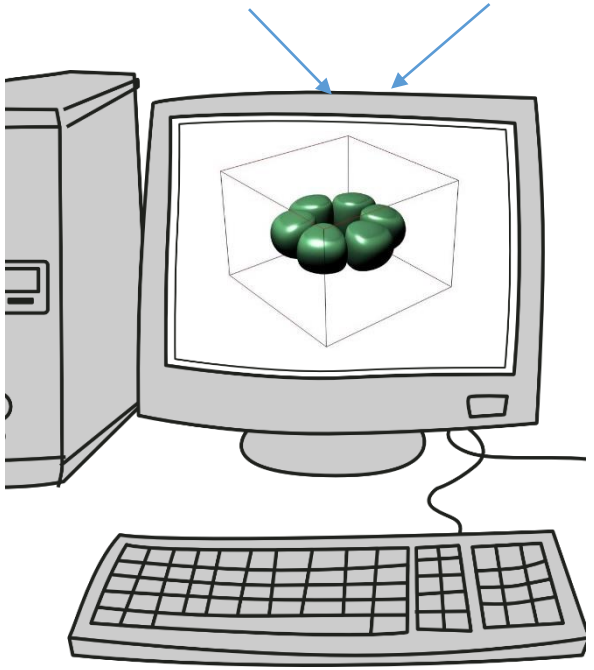
Direct electron detector
Captures projection image of of each molecule

Image Analysis

2D classes



3D reconstruction



Model building & validation
Structure deposition PDB

IDH
93 kDa
3.8 Å

GDH
334 kDa
1.8 Å

p97
540 kDa
2.3 Å

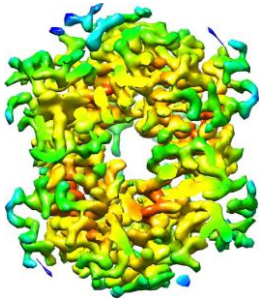
LDH
145 kDa
2.8 Å

β-galactosidase
465 kDa
2.2 Å

Ribosome
2,300 kDa
2.9 Å

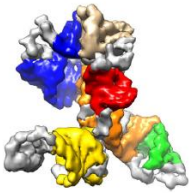
Dengue Virus
11,200 kDa
3.6 Å

Merk, 2016



Hemoglobin
64 kDa 3.2 A
R Danev, 2017

Streptavidin
52 kDa 3.2 A
X Fan…. H-W Wang,
2019 Nat Commun



Riboswitch RNA
40 kDa 3.7 A
Zhang…. W Chiu,
2019 Nat Commun

	PAC1R		*GLP-1R-TAS*	
	+VPP	**-VPP**	**+ZLF**	**-ZLF**
Data processing				
Micrographs	4,032	3,617	5,508	3,251
Micrographs after CTF fits (retention)	3,761 (93%)	3,553 (98%)	4,839 (88%)	2,638 (81%)
Measured defocus [µm]	0.4 – 1.2	0.8 – 1.7	0.7 – 1.8	0.7 – 1.7
Picked particles [x10^3]	2,446	3,012	3,246	1,490
(per micrograph)	(650)	(848)	(671)	(565)
Particles after classification [x10^3] (retention) (per micrograph)	553 (23%) (147)	607 (20%) (171)	181 (5.6%) (37)	140 (9.4%) (53)
Resolution after CTF refinement [Å]	3.12	2.83	2.83	2.99
B-factor after CTF refinement [Å^2]	137.5 (6.2)	120.1 (5.3)	124.0 (2.4)	124.3 (6.9)
Particles to reach 3 Å after CTF refinement [x10^3]	958.0 (532)	241.5 (127)	78.0 (15)	150.1 (91)
Resolution from 100k particles after CTF refinement [Å]	3.58 (0.18)	3.22 (0.14)	2.95 (0.04)	3.09 (0.14)
Resolution after polishing [Å]	**2.99**	**2.69**	**2.72**	**2.87**
B-factor after polishing [Å^2]	**146.5 (3.4)**	**121.3 (2.8)**	**122.4 (4.9)**	**123.0 (3.5)**
Particles to reach 3 Å after polishing [x10^3]	487.0 (132)	116.7 (30)	41.1 (17)	71.9 (21)
Resolution from 100k particles after polishing [Å]	3.34 (0.07)	3.04 (0.06)	2.82 (0.07)	2.93 (0.06)

% particles needed for 3 A is less than 4%

Danev et al BioRxiv, 2020

How to reach high resolution in cryoEM with high efficacy?

and without spoiling money

Optimize Specimen preparation

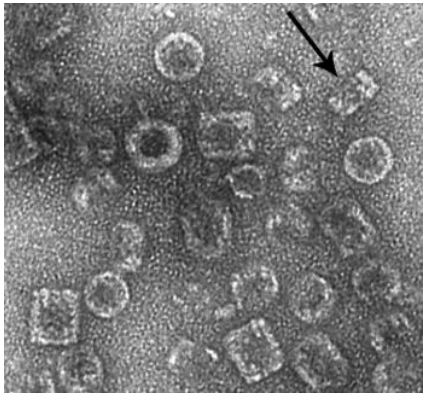
Optimize data collection (Hardware and software)

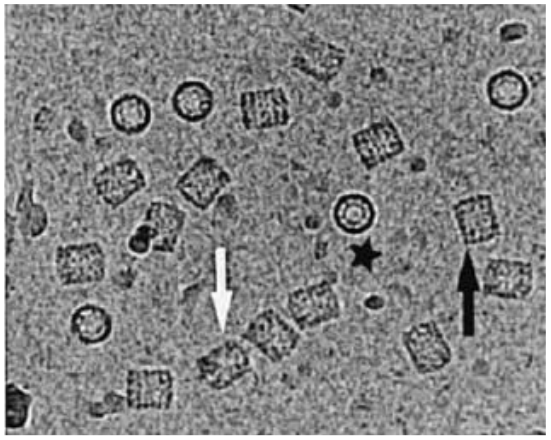
Optimize image processing (software)

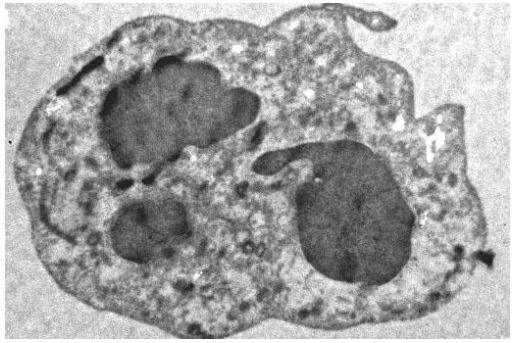
Specimen preparation

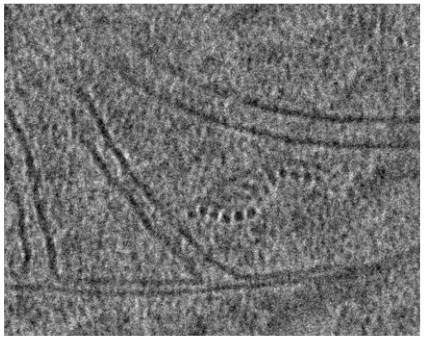
Preparation regarding the size of biological samples

	Dehydrated / stained specimen	**Frozen hydrated/ unstained specimen**
Thin Specimen :	**negative staining**	**freeze-plunging** SPA -cryoEM
Thick Specimen :	**Plastic section**	**cryosection/FIB**

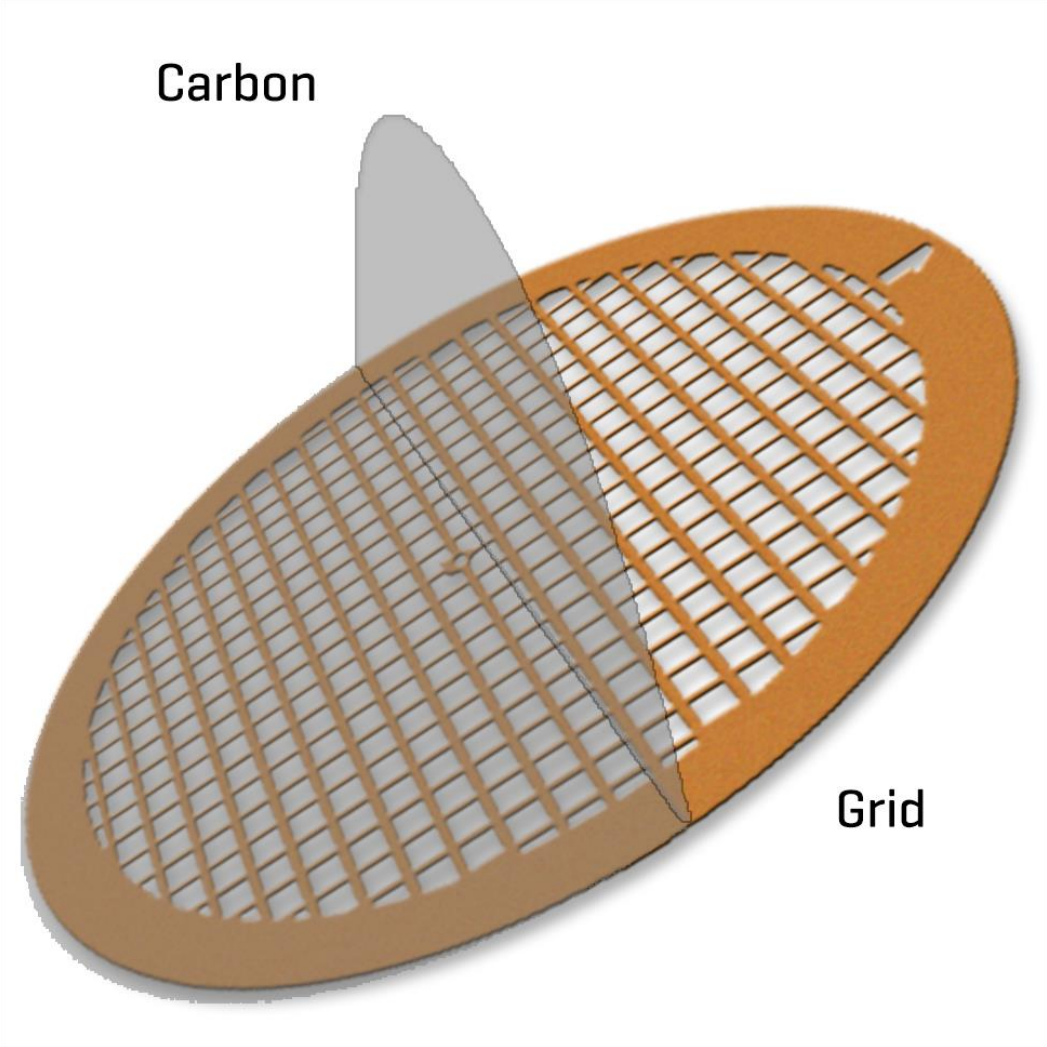




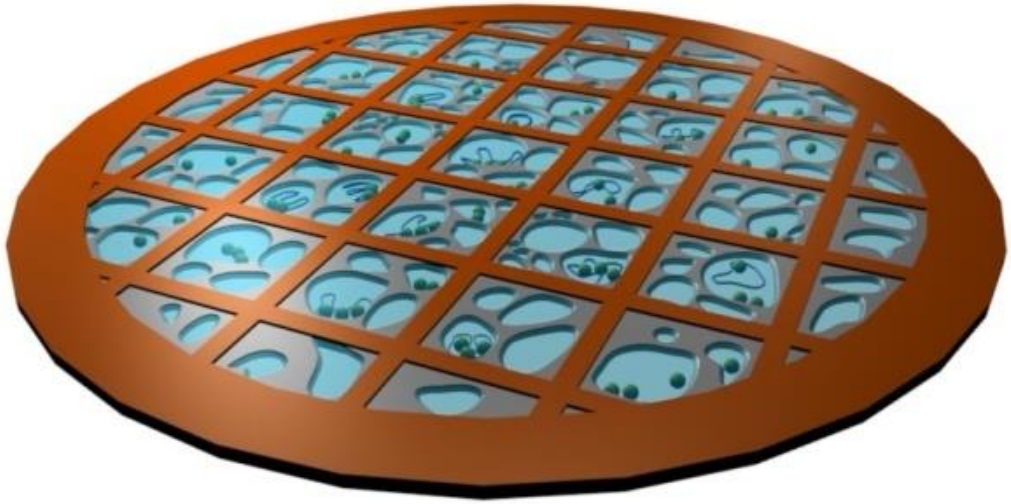


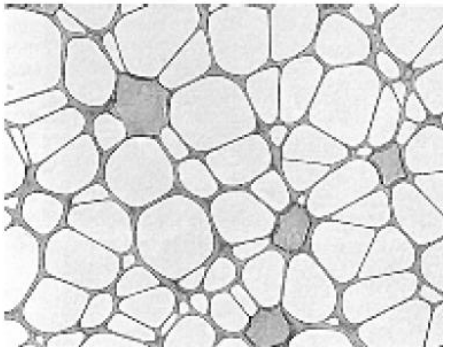


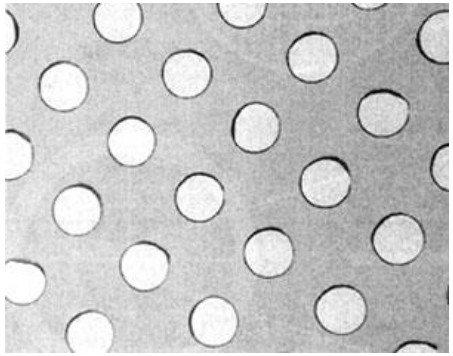
Support : grid coated with thin amorphous carbon

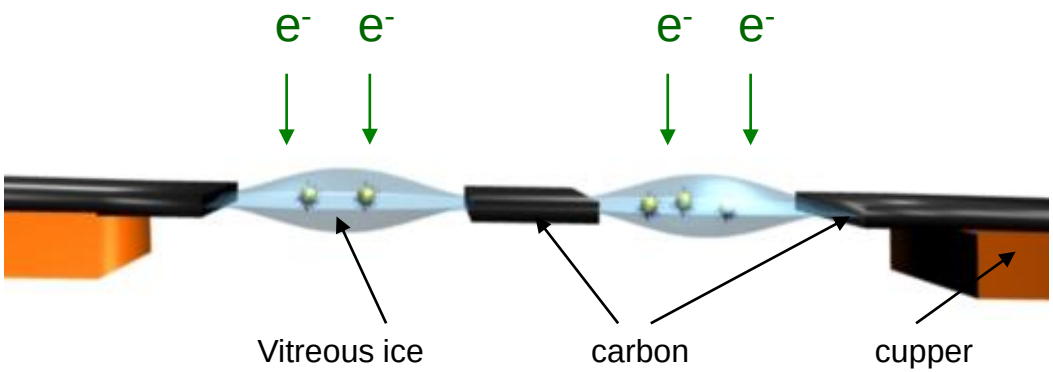


Cryo EM: Holey carbon grid



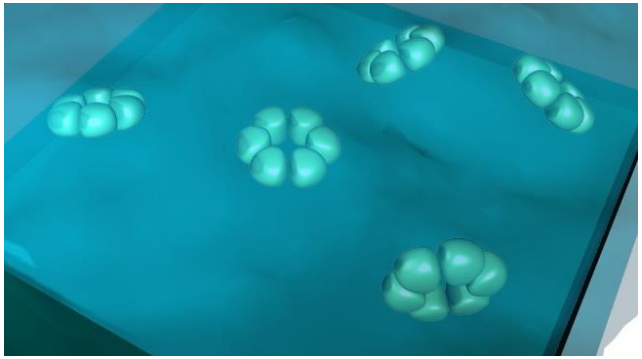






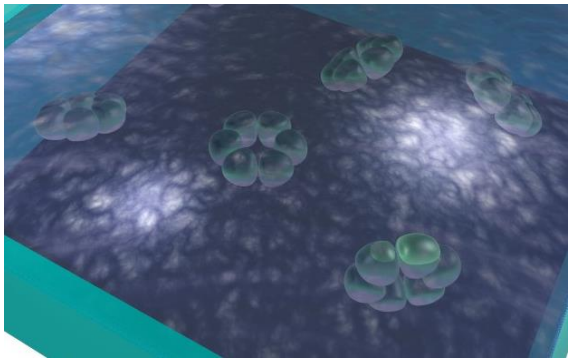
Because the microscope column is under vacuum to maintain a coherent electron beam, WATER is forbidden.
Specimen must be introduced into EM either in a dehydrated form or in a “solid water” form

Liquid

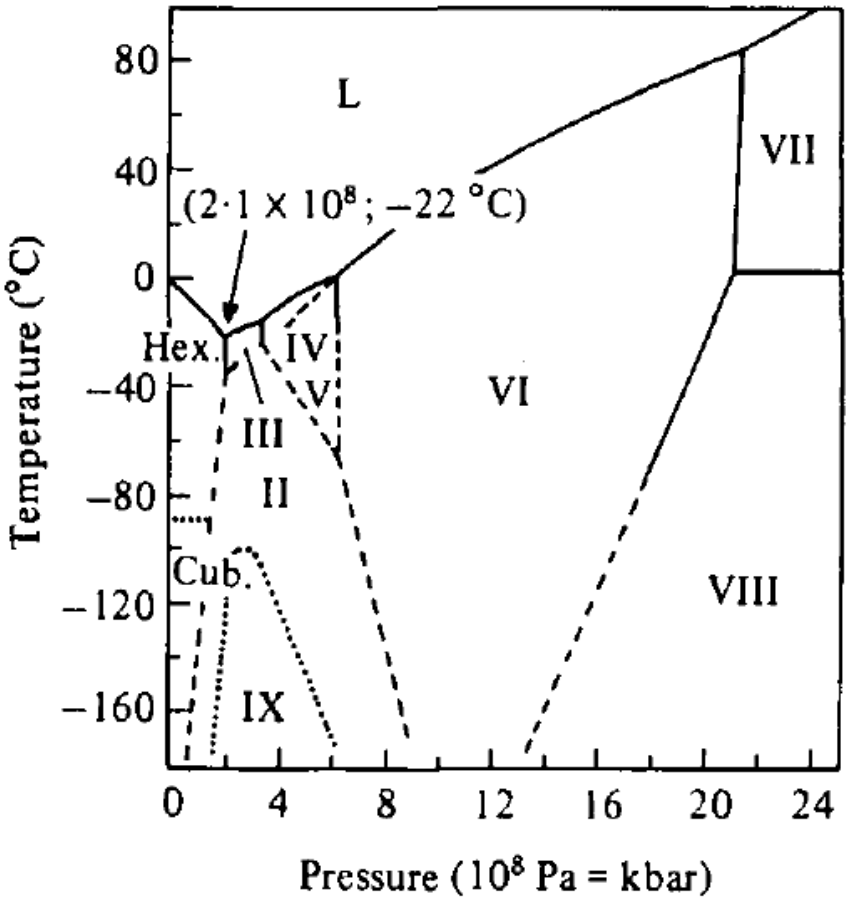


Vitrification

Solid



Phase Diagram of Water



Quarterly Review of Biophysics **21**, 2 (1988), pp. 129–228
Printed in Great Britain

Cryo-electron microscopy of vitrified specimens

JACQUES DUBOCHET[1], MARC ADRIAN[2], JIIN-JU CHANG[3], JEAN-CLAUDE HOMO[4], JEAN LEPAULT[5], ALASDAIR W. McDOWALL[6] AND PATRICK SCHULTZ[4]

European Molecular Biology Laboratory (EMBL), Postfach 10. 2209, D-6900 Heidelberg, FRG

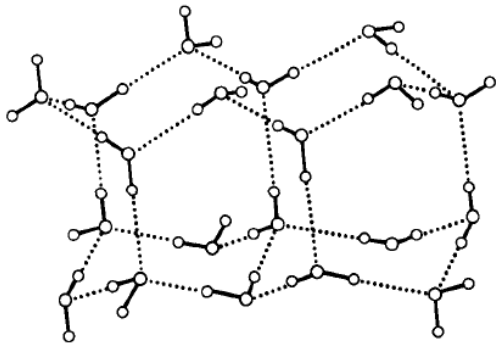
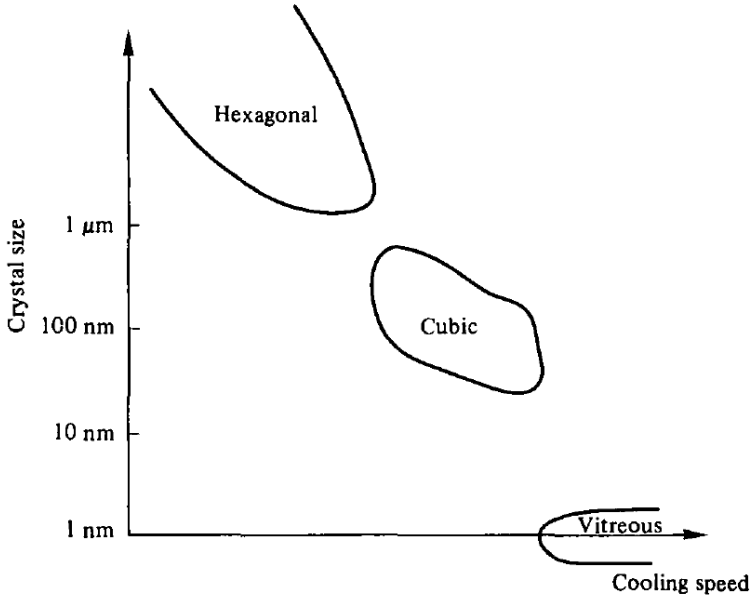
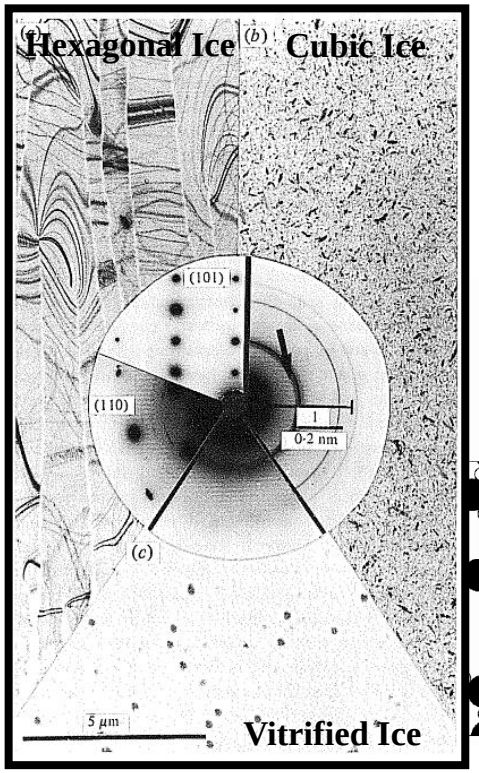


Fig. 2. Schematic view of a small part of a hexagonal ice crystal.

Vitrification of specimens:

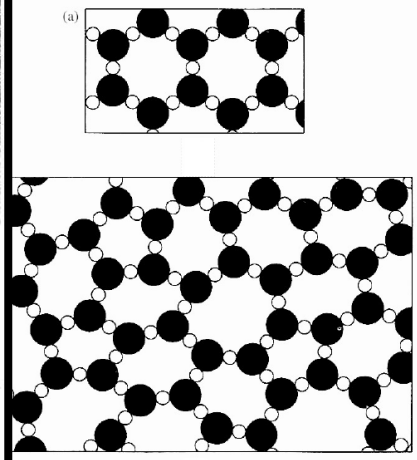
Relationship between cooling speed and the size of the ice crystals





Dubochet *et al.*, 1988

crystalline



amorphous

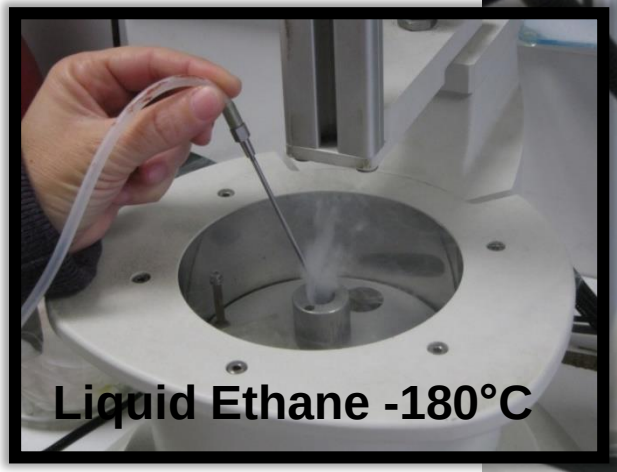
Vitrification : at a high cooling rate : ~10^4 K / s
It is the state in which immobilization is achieved before nucleation

Density = 933 kg/m3 :Water expands during vitrification

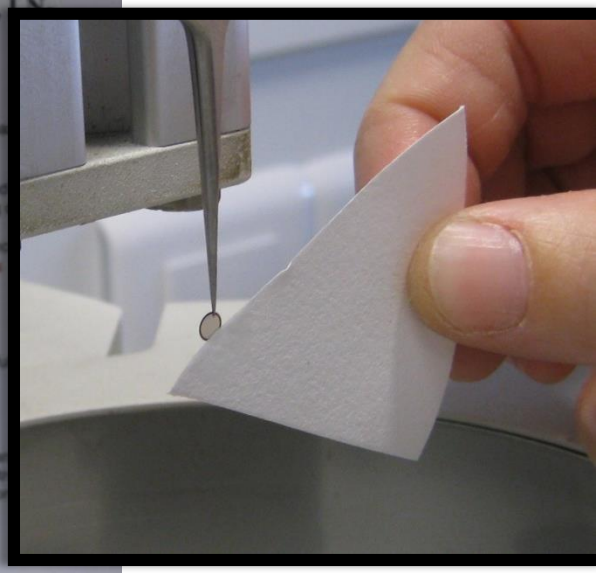
Boiling /melting points
liquid ethane : -88.7 °C / -183.3 °C
Nitrogen: -196 °C / -210 °C

Vitreous ice forms by flash-cooling, is metastable and converts to crystalline ice:

- cubic ice, forms when vitreous ice is warmed up above -135°C → keep samples below ~-135°C

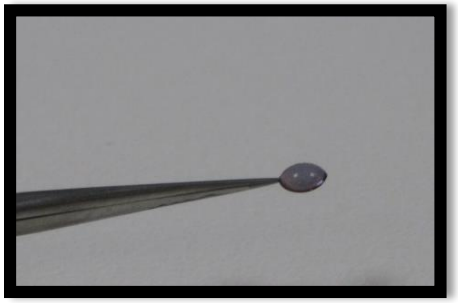


filling



"Blotting« step

5 µl of sample



Grid mounted on the Guillotine

Improve the sample distribution/stability

Plunging system

Home made

Control of temperature and humidity

Gatan

Leica

FEI

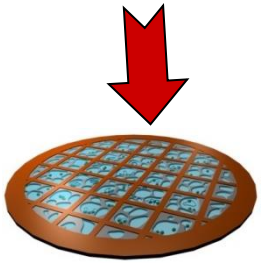
Freezing
-blotting time
-single or double side blotting
-waiting time
-multiple sample application

Transfer into cryoTEM

observation

Cryo-holder

specimen

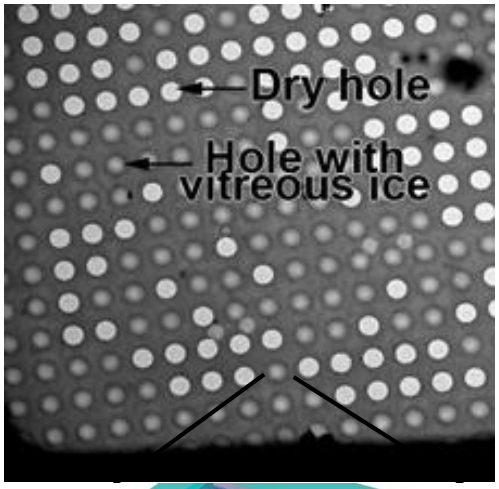


EM grid

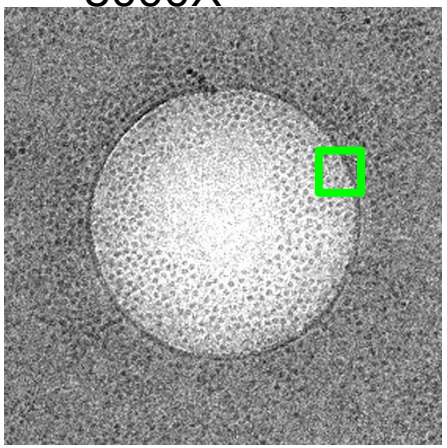
Autoloader equipped instrument

Cryo EM: What do you expect ?

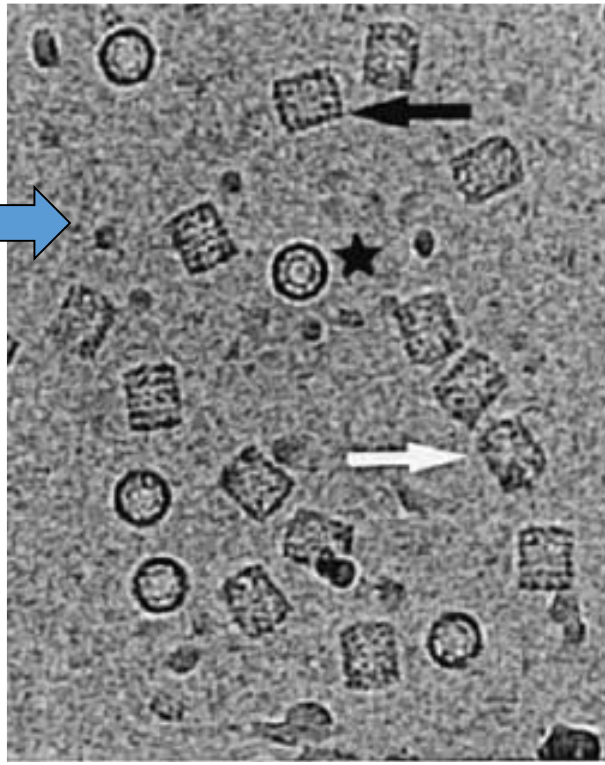
500X

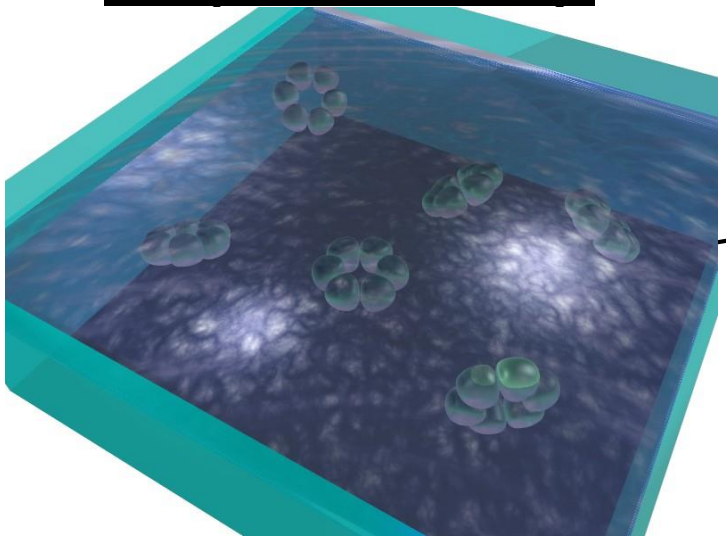


5000X



50000X





Particles in different (random) orientations

Vitreous ice

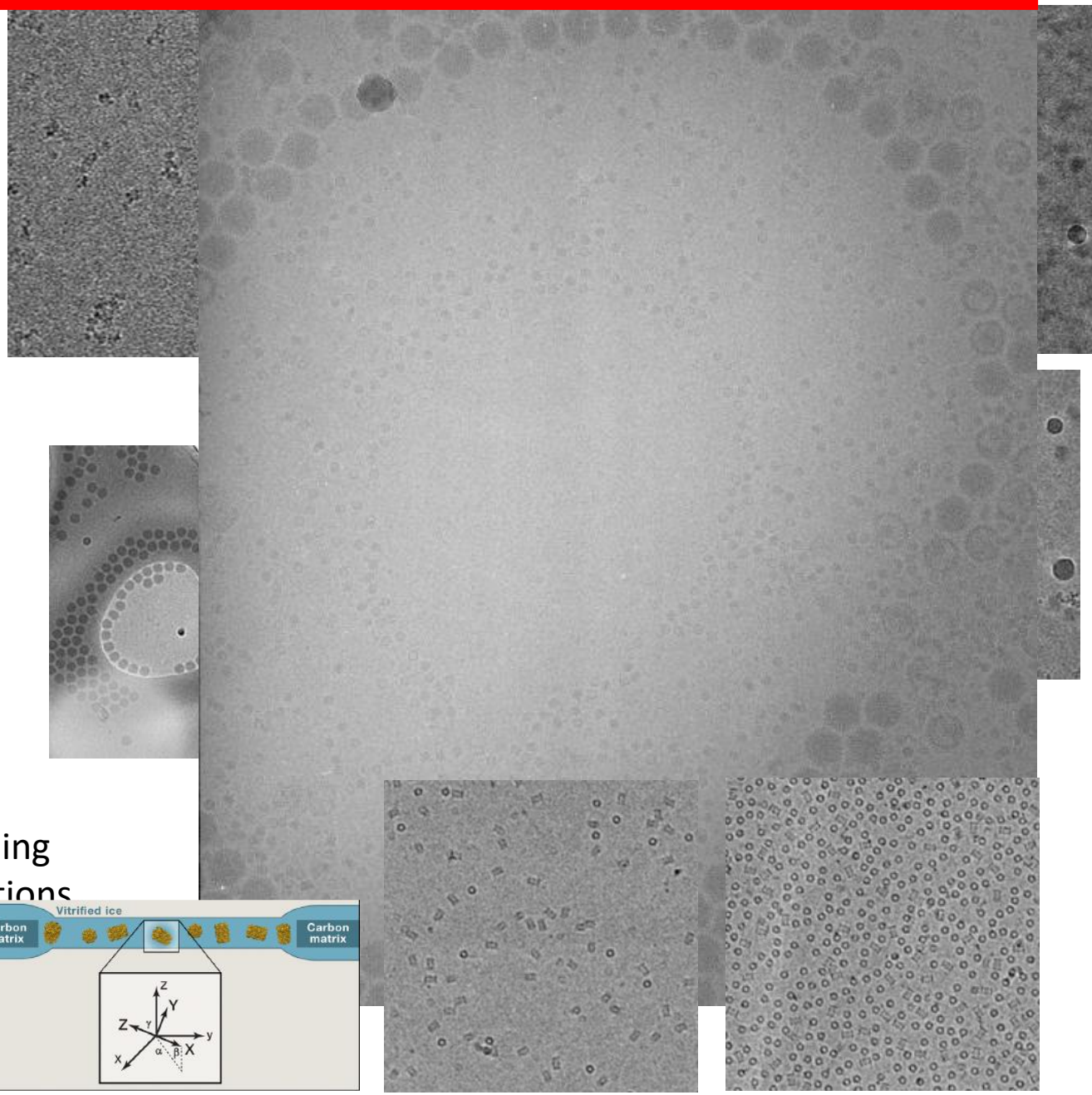
Cryo EM: What is a good cryo grid ?

Good amorphous ice
-not crystalline ice
-no « leopard skin »pattern
-no contamination

Appropriate ice thickness
-typically as thin as possible

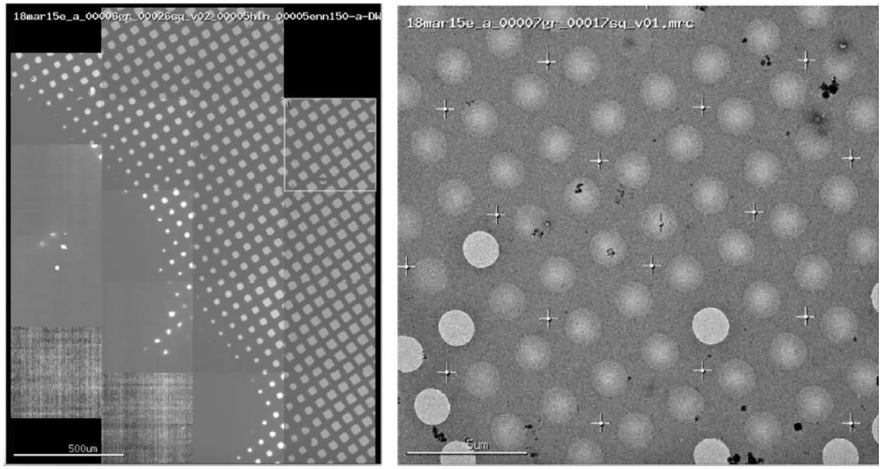
Clearly visible particles
-particle size and shape
-buffer composition
-defocus,

Good particle distribution
-in holes
-dense but particles not touching
-randomly distributed orientations

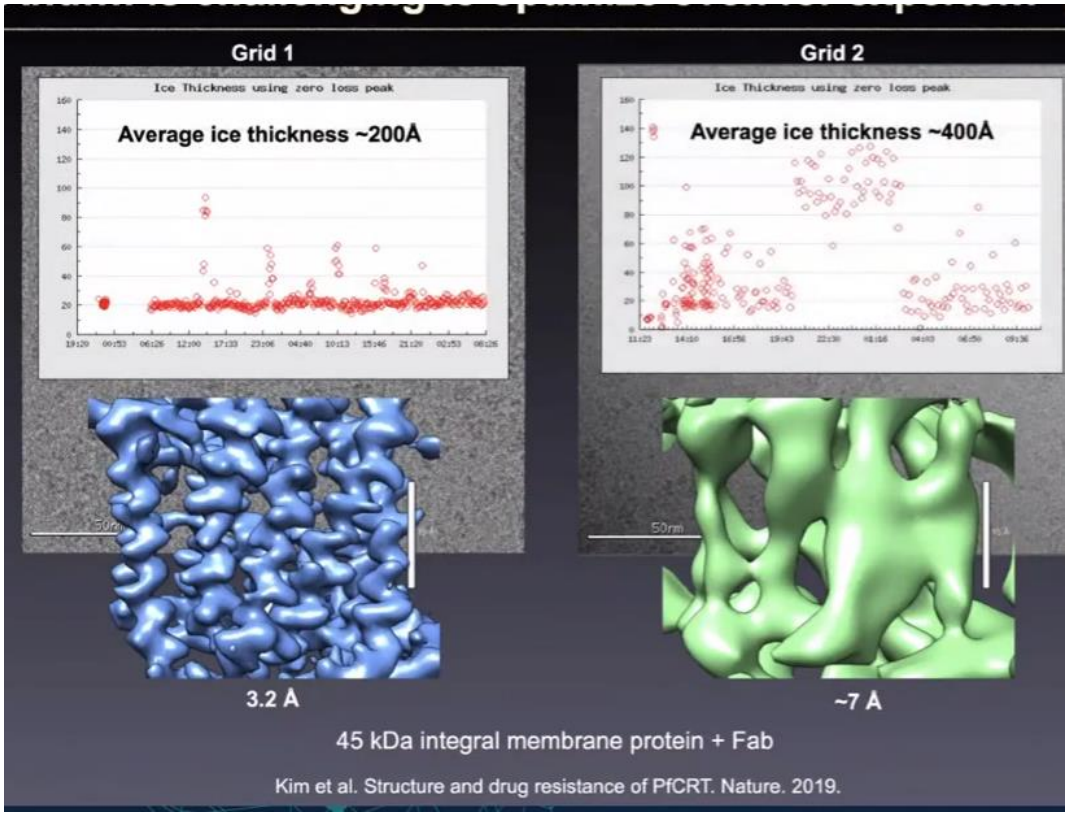


Some points of vigilance

Uneven Ice thickness

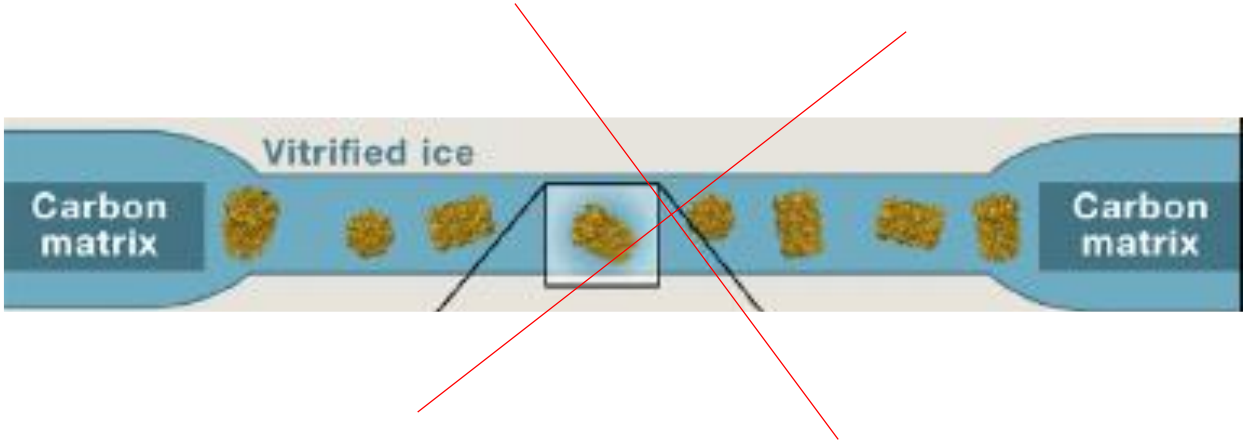


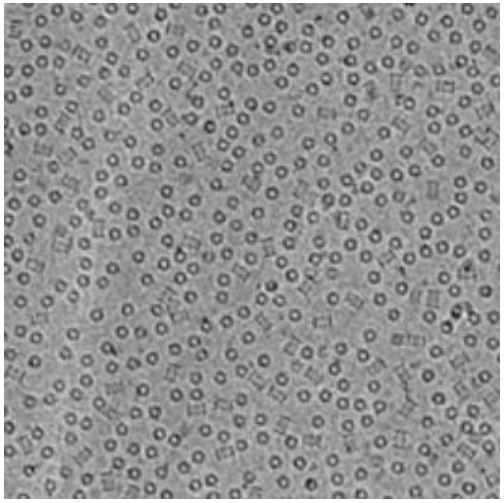
Rice et al,
J struct Biol, 2018

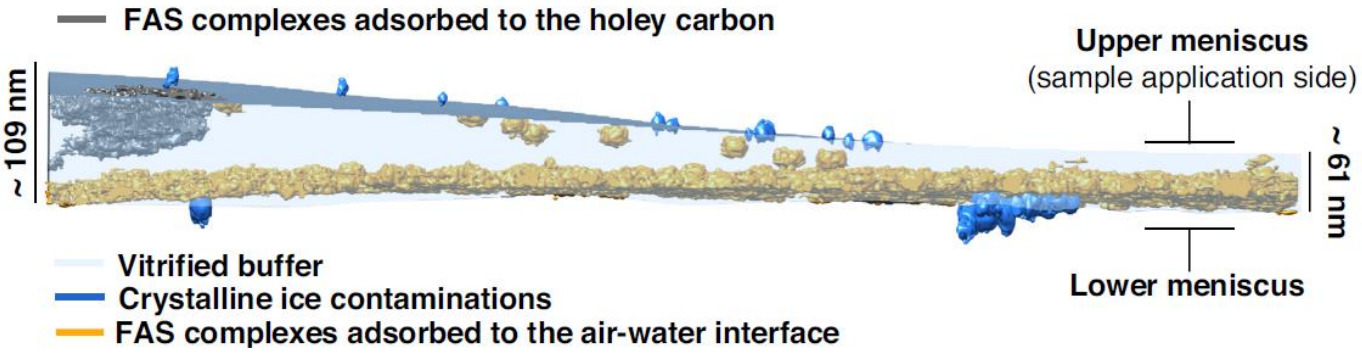


Bridget Carragher
Riccem2021

Particle distribution within the ice layer explored by tomography

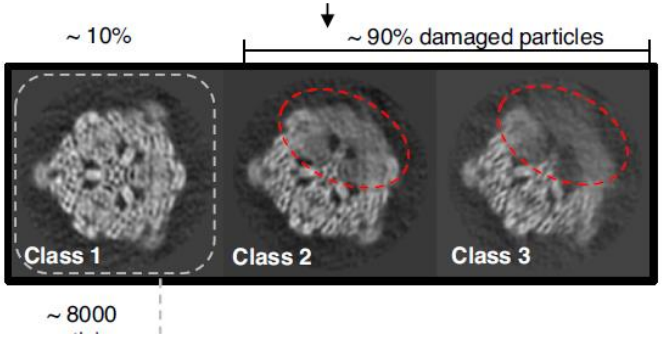




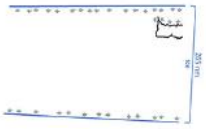


Noble et al, Elife, 2018
D'Imprima et al, Elife, 2019

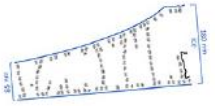
The vast majority of particles are localized to the air-water interface



At the air water interface, particles are damaged

Sample # Name	Example cross-sectional schematic diagram
1* 32 kDa Kinase	
4*† Hemagglutinin	
5* HIV-1 Trimer Complex 1	
6* HIV-1 Trimer Complex 1	
7* HIV-1 Trimer Complex 2	
10* Stick-like Protein 1	
12* Stick-like Protein 2	
13* Neural Receptor	

Sample # Name	Example cross-sectional schematic diagram
14* Neural Receptor	
17* Protein with Bound Lipids (deglycosylated)	
18 Protein with Bound Lipids (glycosylated)	
19* Lipo-protein	
20 GPCR	
21*† Rabbit Muscle Aldolase (1mg/mL)	
22*† Rabbit Muscle Aldolase (6mg/mL)	
25* Protein in Nanodisc (0.58 mg/mL)	

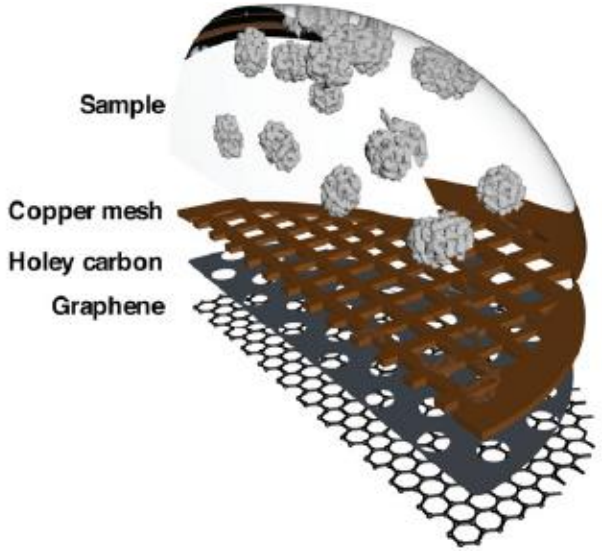
Sample # Name	Example cross-sectional schematic diagram
27* IDE	
30*† GDH	
31*† GDH	
32*† GDH + 0.001% DDM (2.5 mg/mL)	
33*† DnaB Helicase-helicase Loader	
34*† Apoferritin	
35*† Apoferritin	
36*† Apoferritin	
37*† Apoferritin (1.25 mg/mL)	

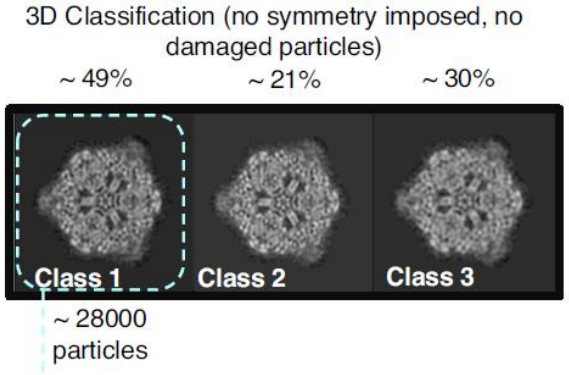
Sample # Name	Example cross-sectional schematic diagram
38*† Apoferritin (0.5 mg/mL)	
39*† Apoferritin with 0.5 mM TCEP	
40 Protein with Carbon Over Holes	
41 Protein and DNA Strands with Carbon Over Holes	
42*† T20S Proteasome	
43*† T20S Proteasome	
44*† T20S Proteasome	
45*† Mtb Proteasome	
46 Protein on Streptavidin	

Noble et al. Elife 2018

Need to keep our particles away from the air water interface

Support film (thin carbon, graphene)



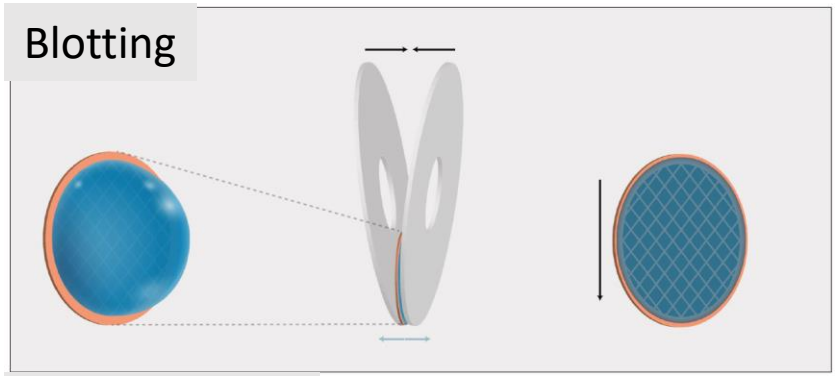


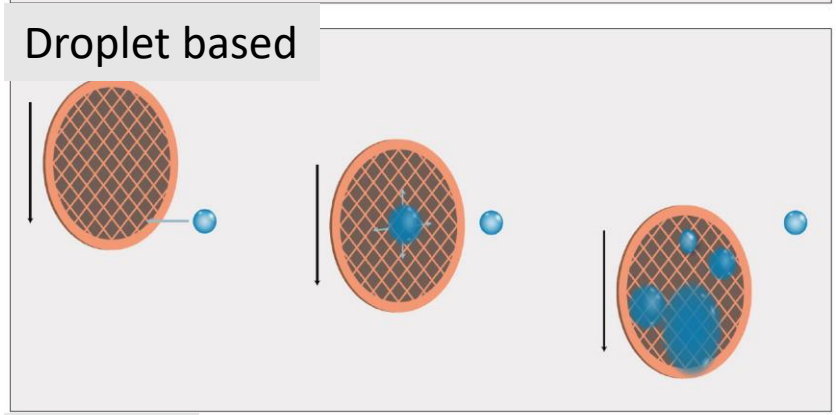
D'Imprima et al, Elife, 2019

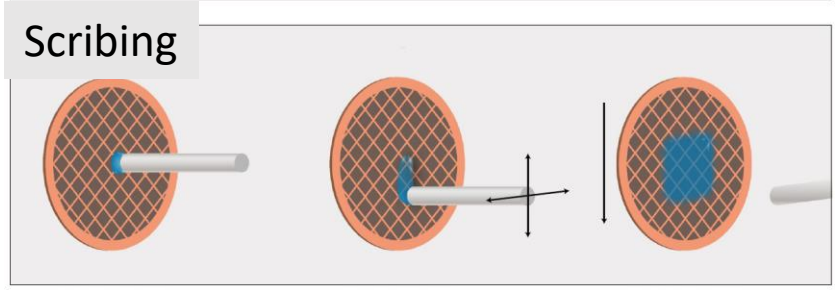
Detergents or similar layers

Other freezing machines

Diversification of methods for preparing grids







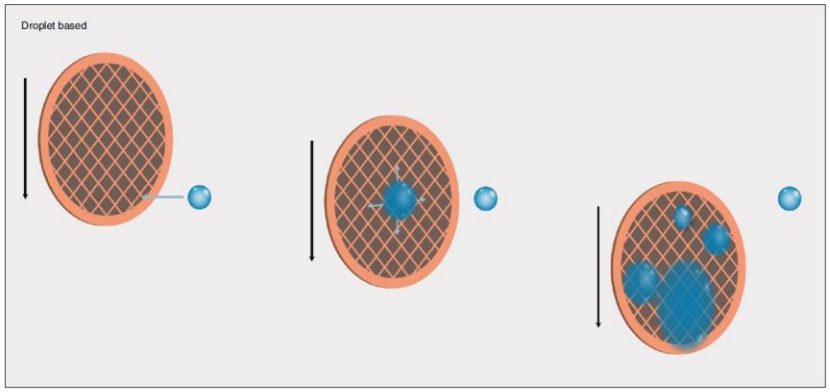
Ultrasonic spray 1-10µm (Rubenstein group)
Gas pressurized spray (Muench group, Franck group
Electrostatic spray 0.25-0.5 µm (Trinick group)

Inkjet printing 25µm Spotiton, Chameleon

Pin printing with a metal pen Vitrojet (Peters group)

Capillary writing Cryowritter (Braun group Nuonex)

Weissenberger G, 2021
Nature Methods



nature methods

BRIEF COMMUNICATION

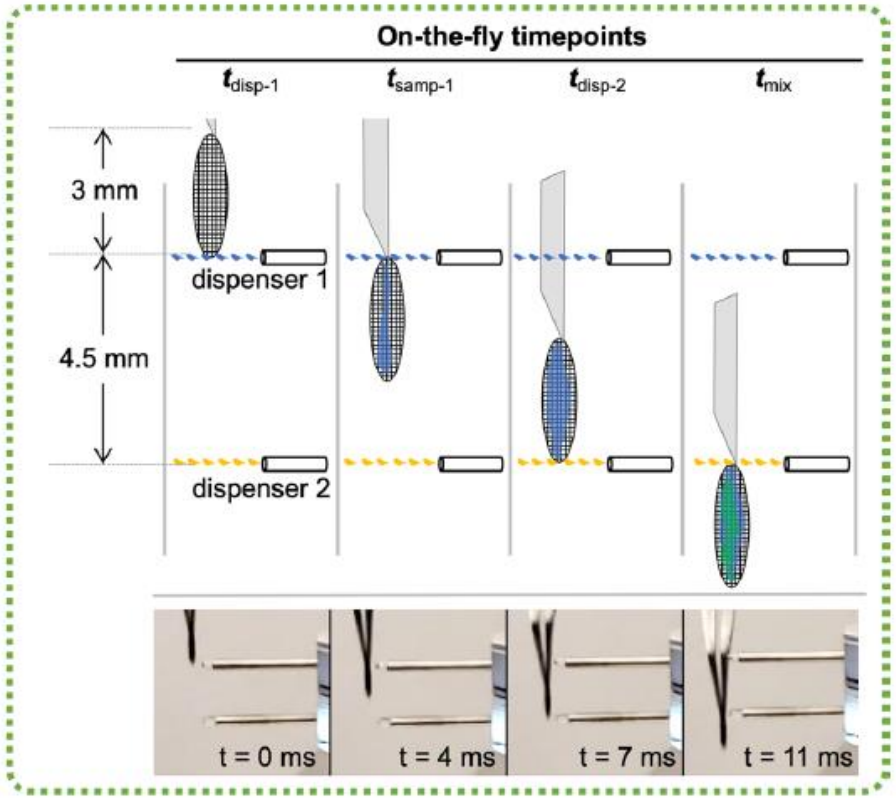
https://doi.org/10.1038/s41592-020-0925-6

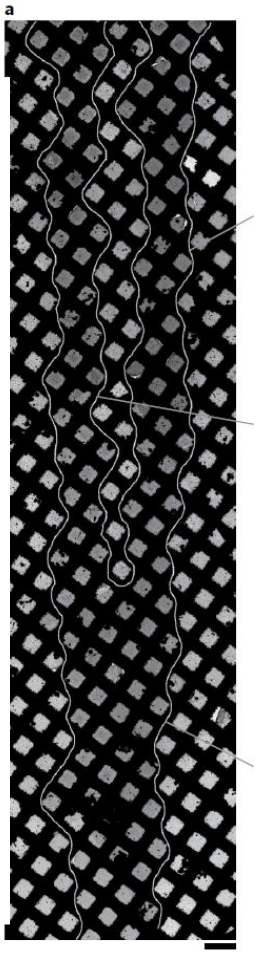
Check for updates

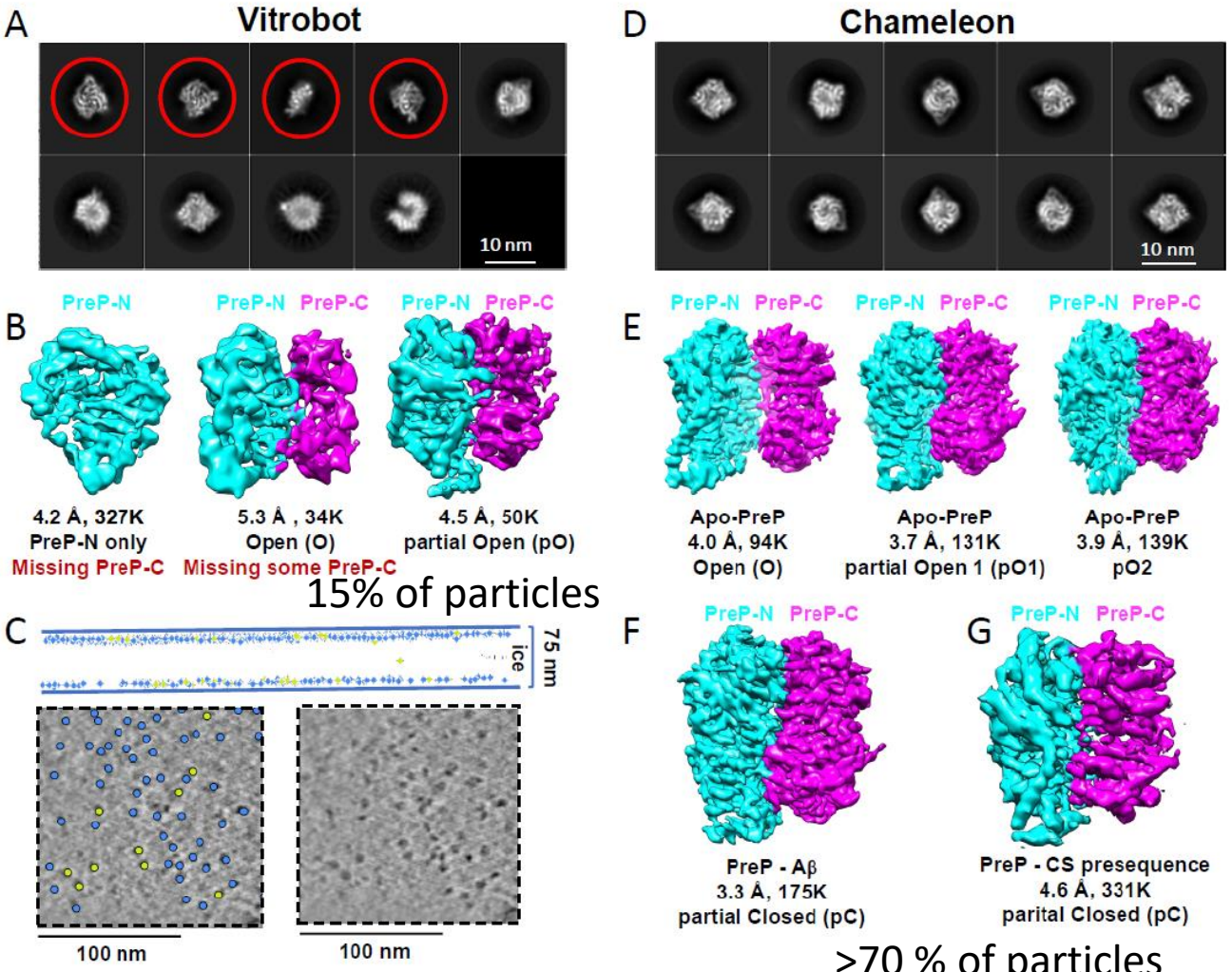
Time-resolved cryo-EM using Spotiton

Venkata P. Dandey[1,7], William C. Budell[1,7], Hui Wei[1], Daija Bobe[1], Kashyap Maruthi[1], Mykhailo Kopylov[1], Edward T. Eng[1], Peter A. Kahn[2], Jenny E. Hinshaw[3], Nidhi Kundu[3], Crina M. Nimigean[4], Chen Fan[4], Nattakan Sukomon[4], Seth A. Darst[5], Ruth M. Saecker[5], James Chen[5], Brandon Malone[5], Clinton S. Potter[1,6] and Bridget Carragher[1,6]

NATURE METHODS | VOL 17 | SEPTEMBER 2020 | 897–900 |





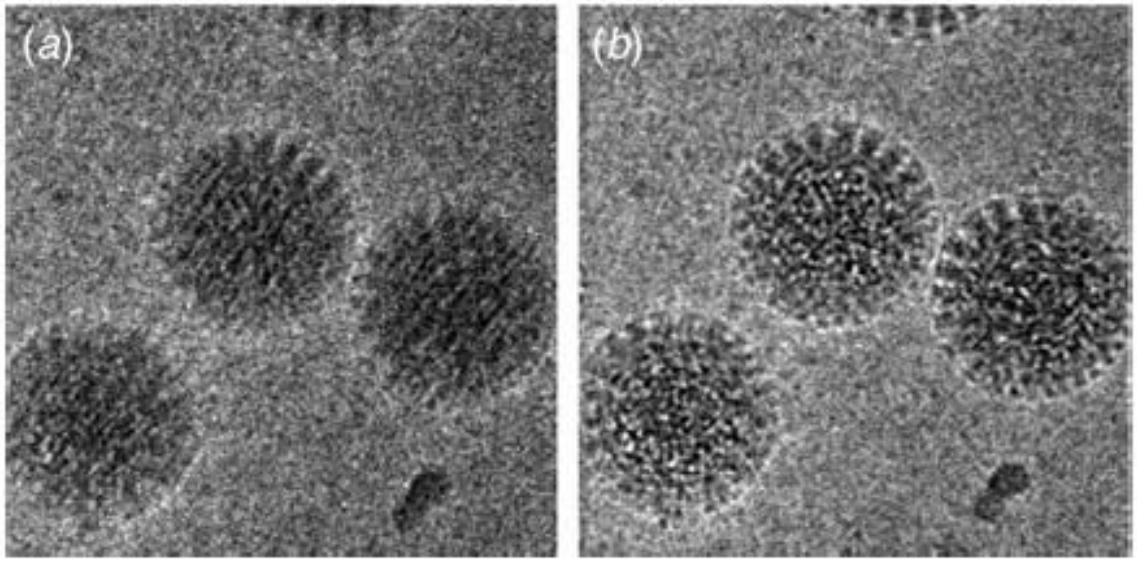


Liang, et al. Structural basis for the mechanisms of human presequence protease conformational switch and substrate recognition. Research square 2020

Beam-induced movement: Image blurring

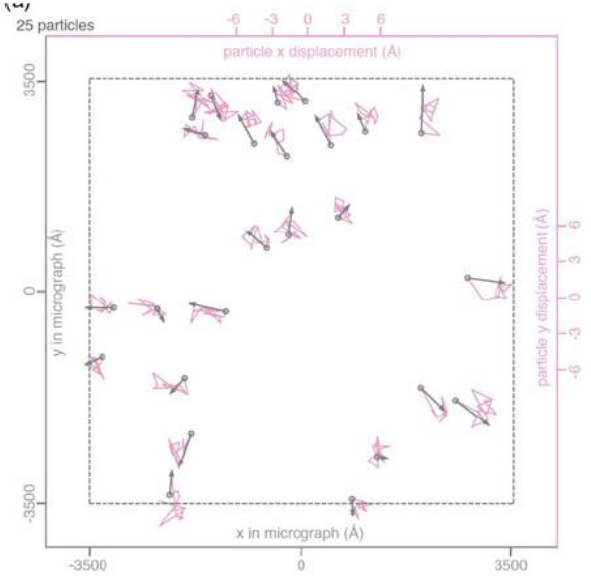
Average of 60 frames

Average of 60 frames aligned in translation

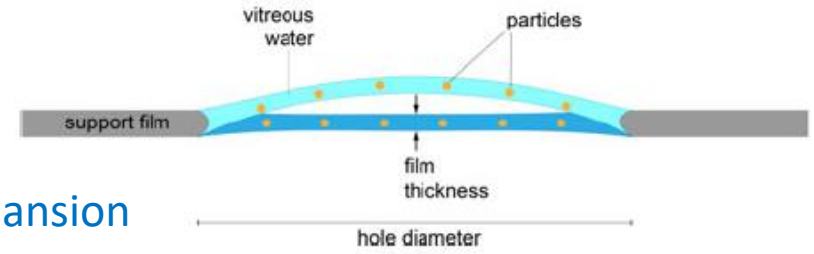


Brilot et al., 2012
J. Struct. Biol

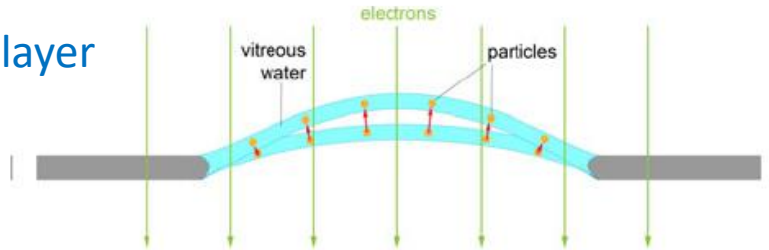
Particle trajectories



Dome formation
due to water expansion



During irradiation, layer
becomes instable



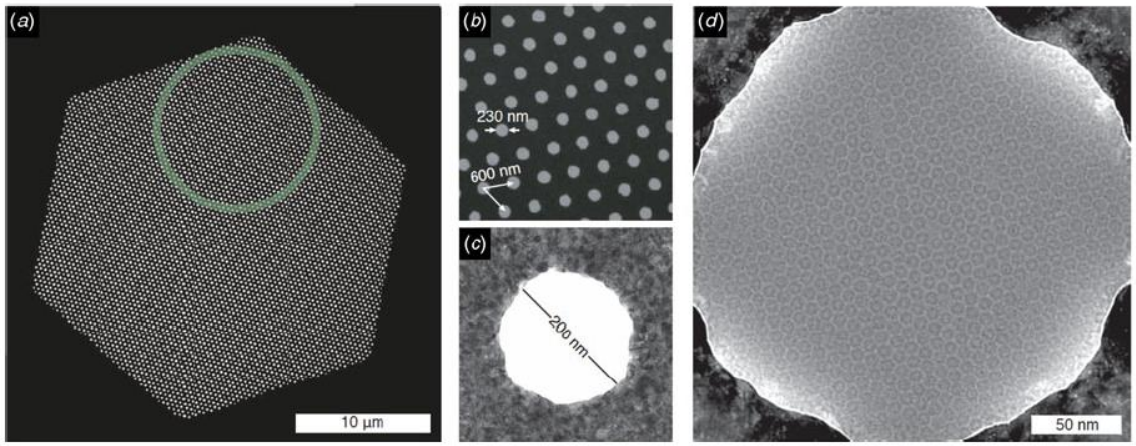
D'imprima, 2021, Naydenova, 2020

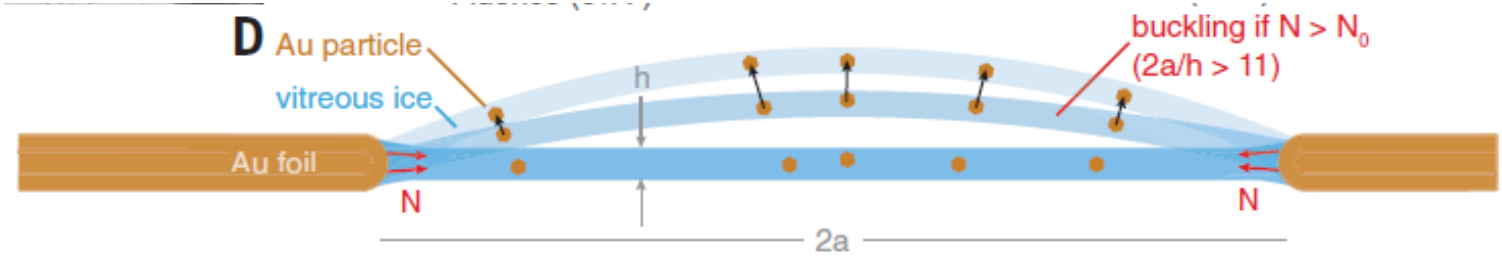
ELECTRON MICROSCOPY

Cryo-EM with sub–1 Å specimen movement

Katerina Naydenova[1], Peipei Jia[1,2]*, Christopher J. Russo[1]†

Science **370**, 223–226 (2020) 9 October 2020





holes 330 nm in diameter for 300-Å-thick ice

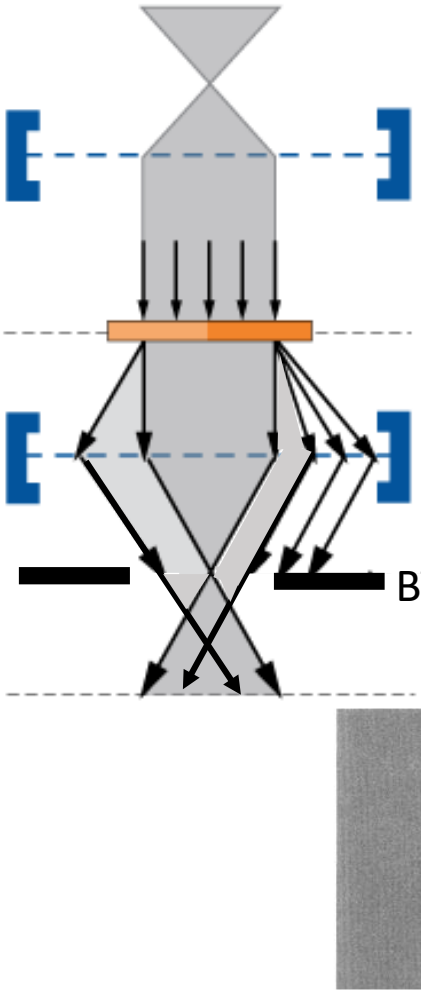
the ice never builds up enough stress to buckle during freezing or to deform under irradiation

Data collection

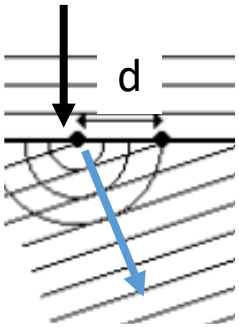
Electron microscopy in few words

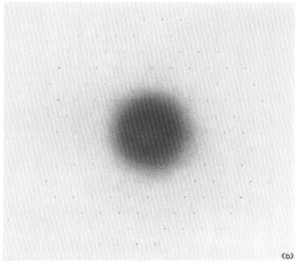
- Electrons have much shorter wavelength than x-rays (~ 2pm~ 0.022 Å for 300 kV electrons
- Resolution not limited by wavelength
- **But resolution is degraded by lens defects, radiation damage, mechanical drift, specimen motion**

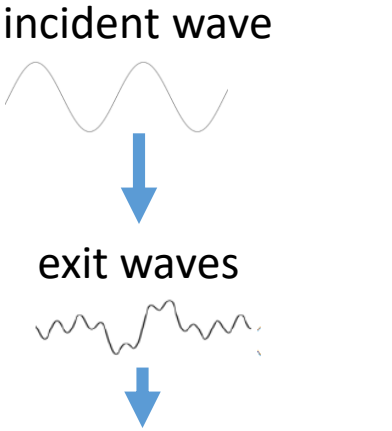
Electron microscope : Principle



Abbe' s theory : **A convergent lens** produces at its *back* focal plane the Fourier transform of the wave distribution at its *front* focal plane







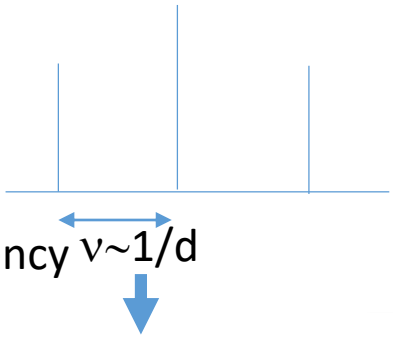
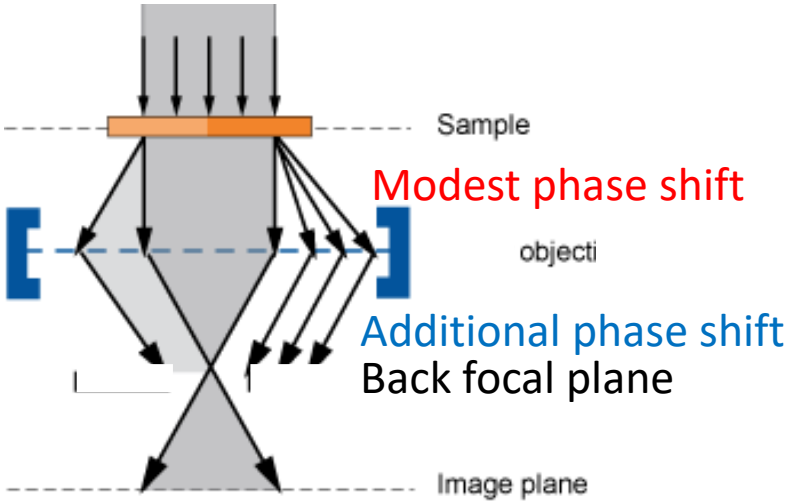
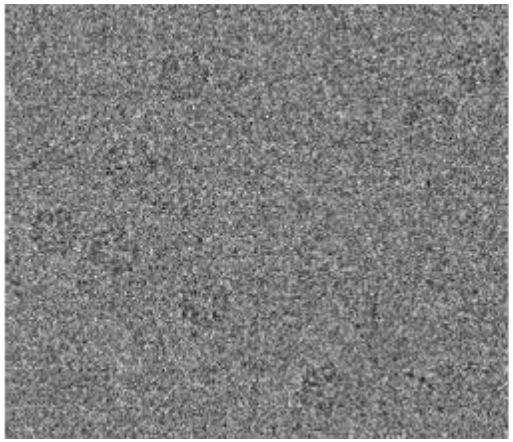


Image Formation: Phase contrast

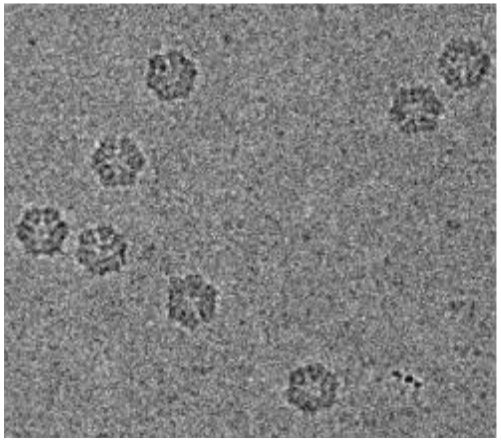
Ice-embedded specimens of biological macro-molecules is well described by the "weak-phase object" approximation The electron beam passing thorough the object (thin specimen) only suffers a modest phase shift, but its amplitude is effectively unchanged



Close to **focus**



-2 µm defocus

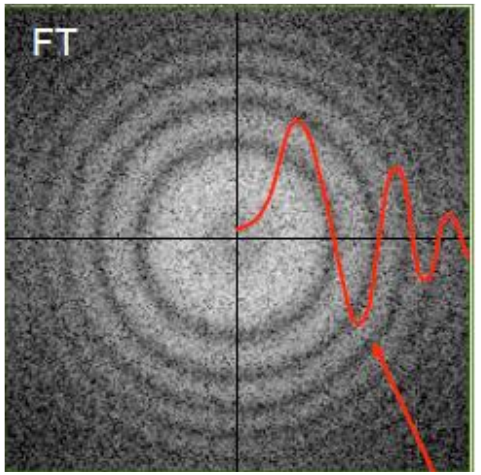


Images are intentionally taken out-of-focus in order to generate contrast (additional phase shift by changing the focal length)

- Contrast is modulated by defocus and lens aberrations

Spatial frequency dependent phase shift $\gamma(\nu)$ introduced by objective lens: Phase Contrast Transfer Fonction, Scherzer , (1949)

$$\sin\left[\frac{2\pi}{\lambda}\left(\frac{Cs\cdot\lambda^4\cdot\nu^4}{4}-\frac{\Delta f\cdot\lambda^2\cdot\nu^2}{2}\right)\right]$$

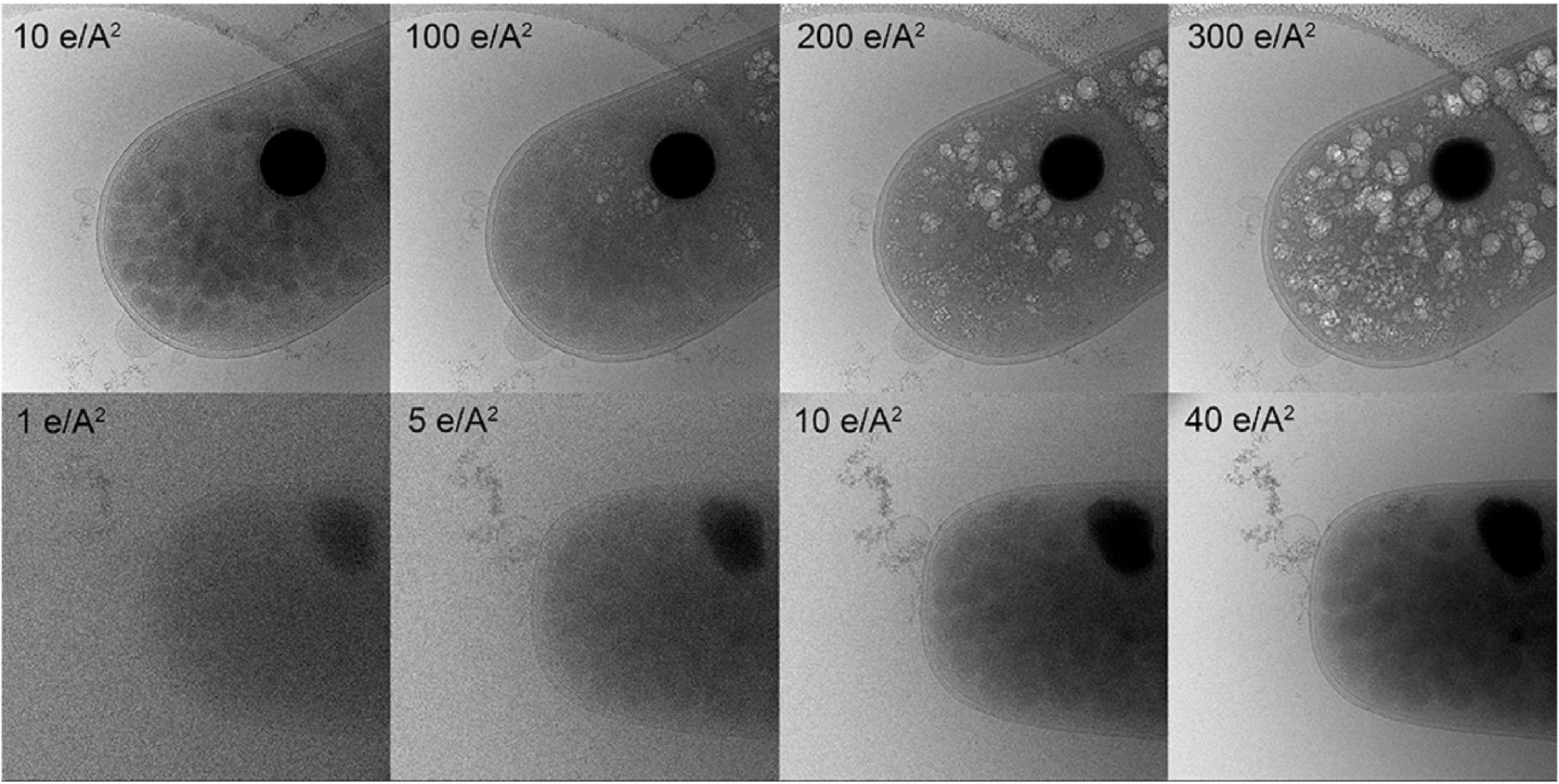


CTF

Cryo EM: Advantages and Drawbacks

Advantages : Hydrated state, High resolution, Small amount of sample

Drawbacks : Low contrast , Highly sensitive to electron dose



Konig R et al., Annals of Anatomy, 2018, 217 82

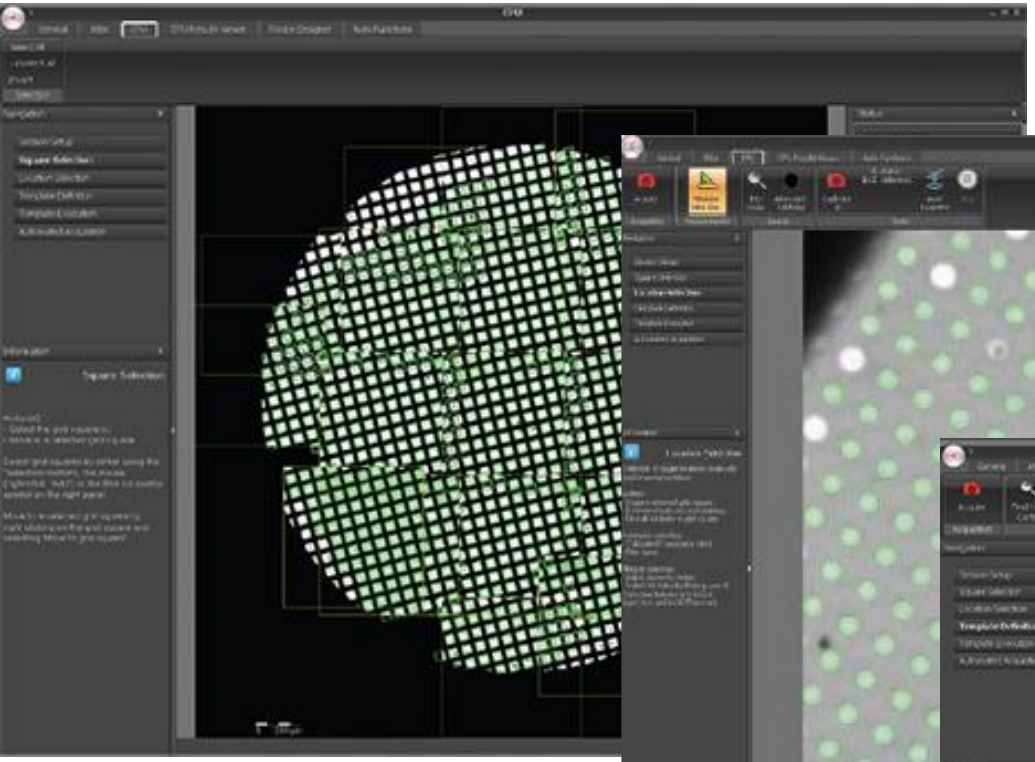
Cryo EM: reference

300 kV FEG TITAN Krios , Parallel illumination, Automated collection, very stable stage, autoloader (12 grids)

Cryo EM: Automated data acquisition

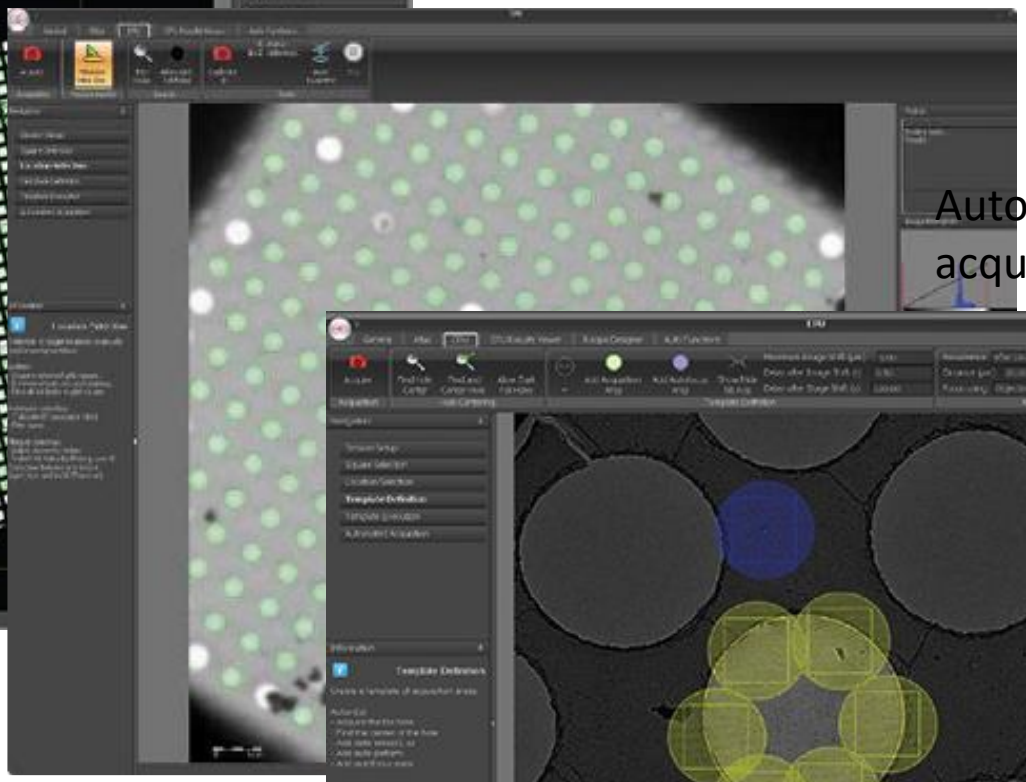
Example : Automated data acquisition software EPU (FEI)

Atlas = image of the EM grid

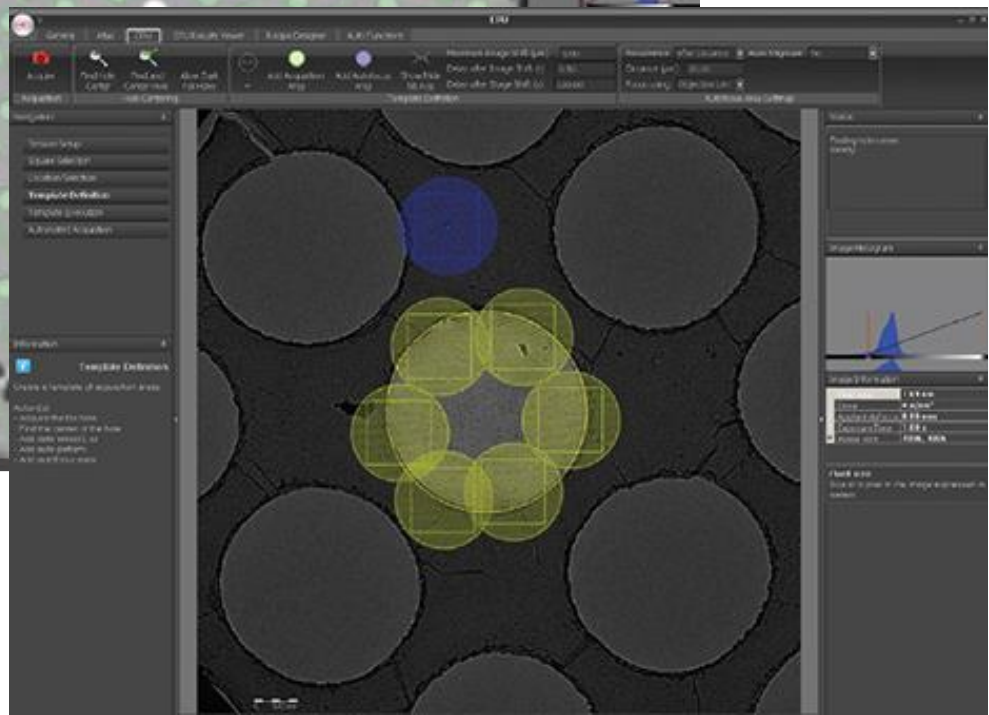


Settings

Hole coordinates, ice thickness, dose…

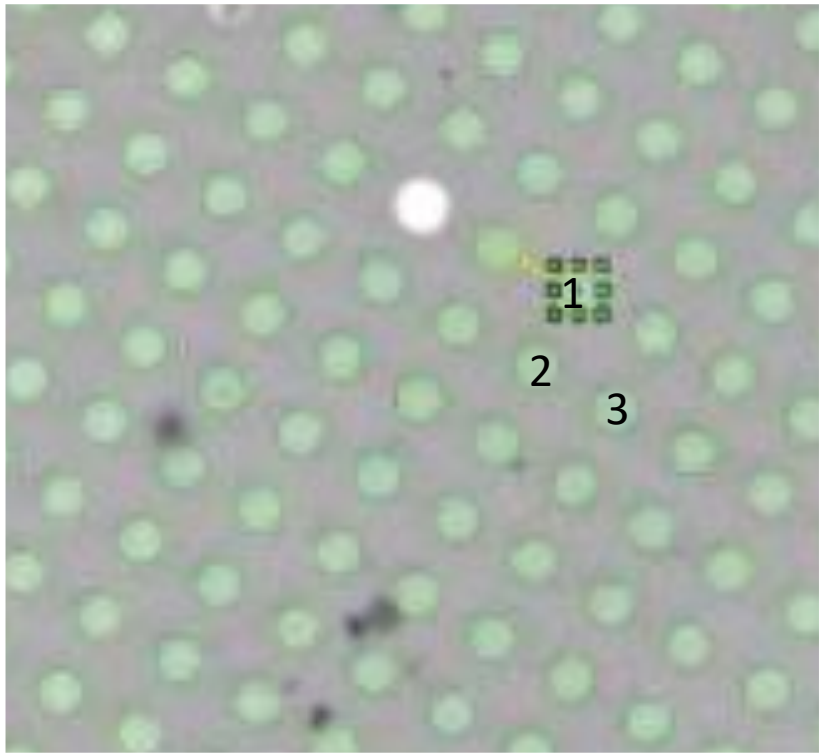


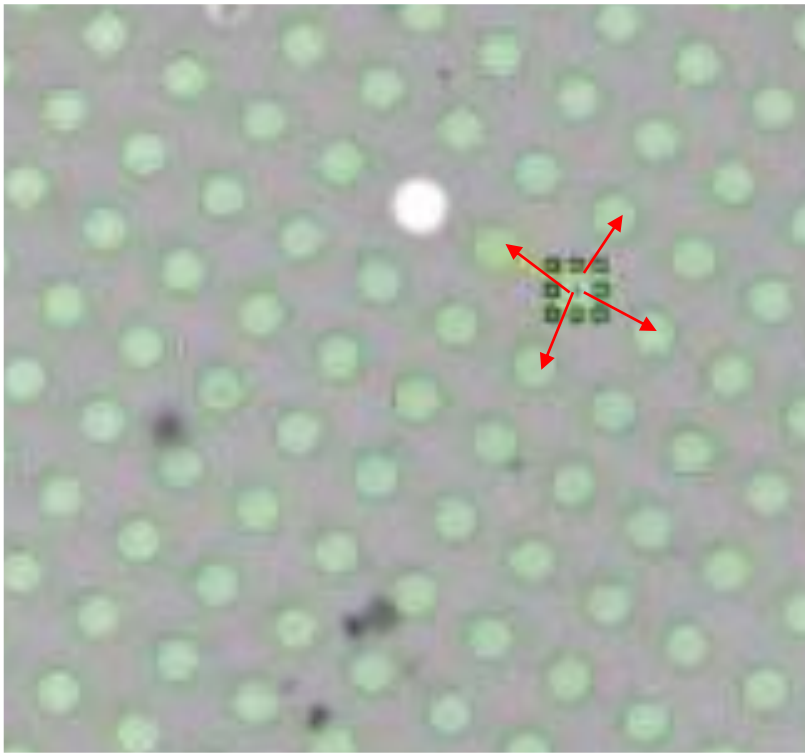
Automatic acquisition areas



24 hours
3000 images
100000-300000 particles

Improve collect speed: Fewer stage moves





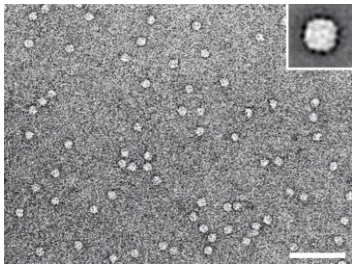
Setup
30s stage move and settling
30s focus and other setting
5s image recording

AFIS (Aberration-Free image shift)
EPU TFS

Reduce total stage settling time

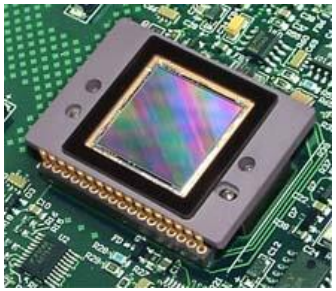
Conversion electron into images

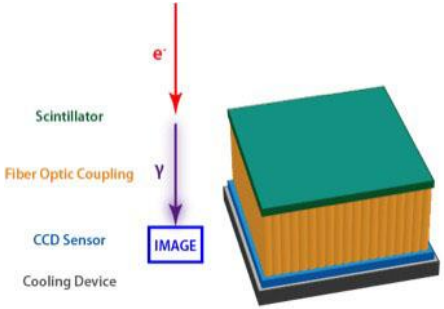
Film sensitive to electrons



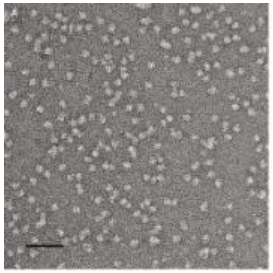
Pixel size = 8 µm
1 image / s

CCD





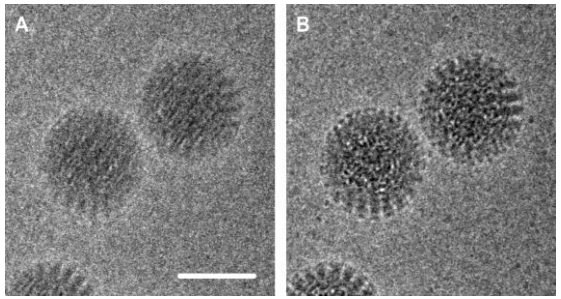
CCD Camera



14 µm 1 image / s

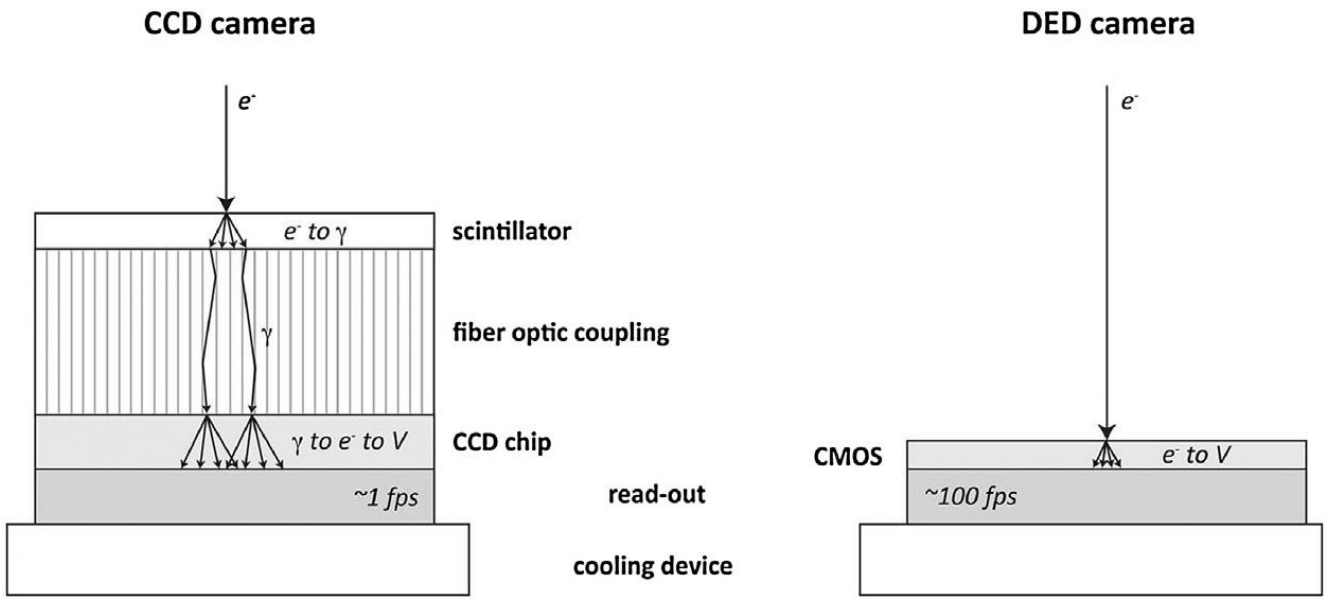
Direct Electron Detector CMOS

(complementary metal oxide semiconductor)

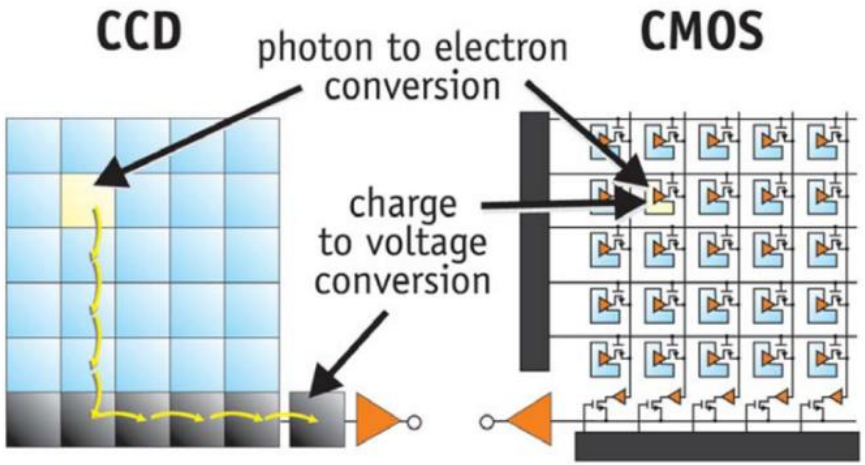


5 µm
20 images / s

Advantage of Direct Electron Detector vs CCD camera



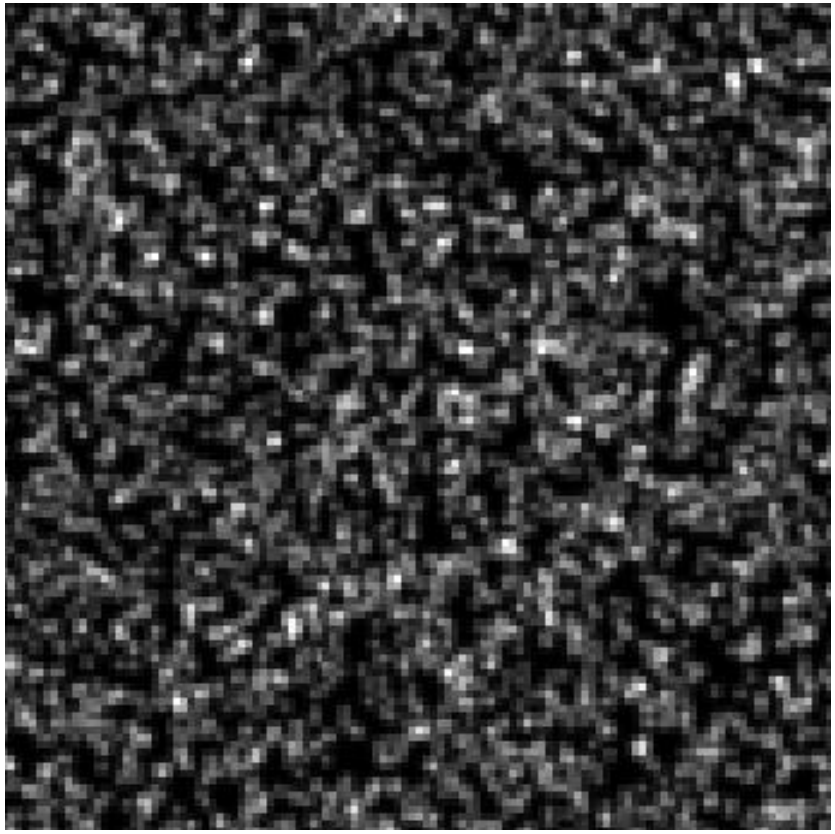
Konig R et al., Annals of Anatomy, 2018, 217 82



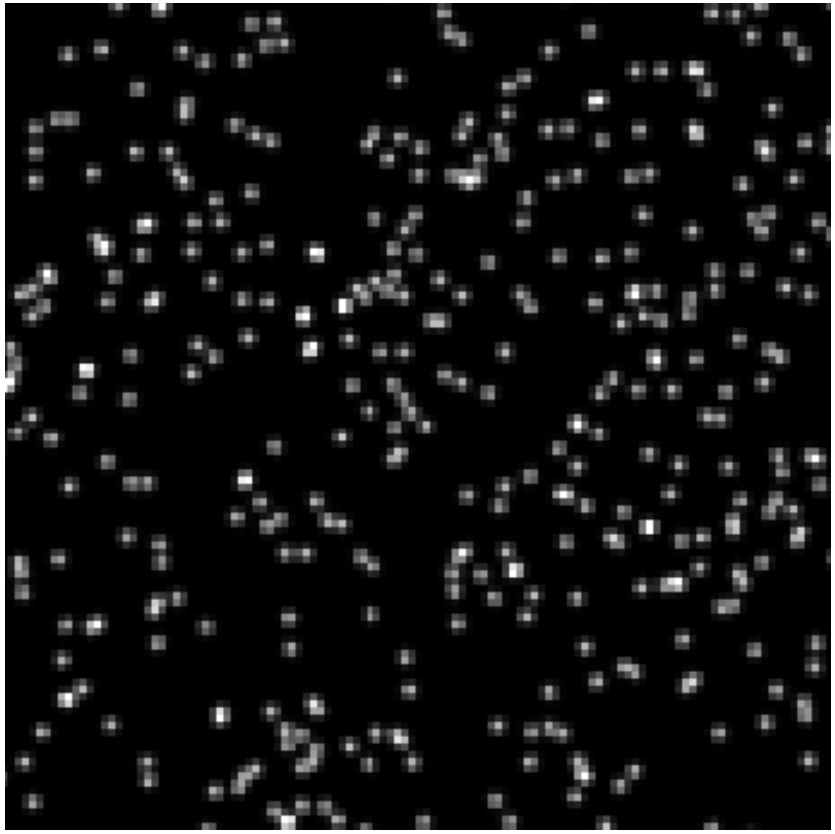
CCDs move photogenerated charge from pixel to pixel and convert it to voltage at an output node. CMOS imagers convert charge to voltage inside each pixel.

Counting requires speed

Typical dose rate of 10 e^-/pix/s.



40 frames per second: events overlap and cannot be resolved.

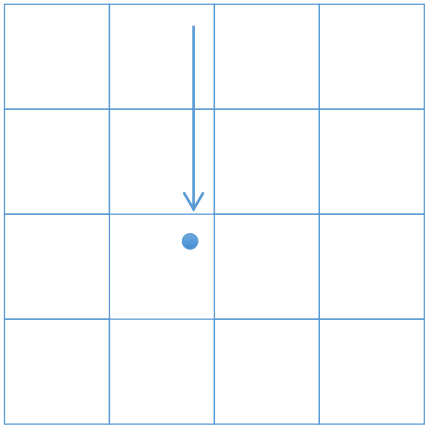


400 frames per second: events are resolved.

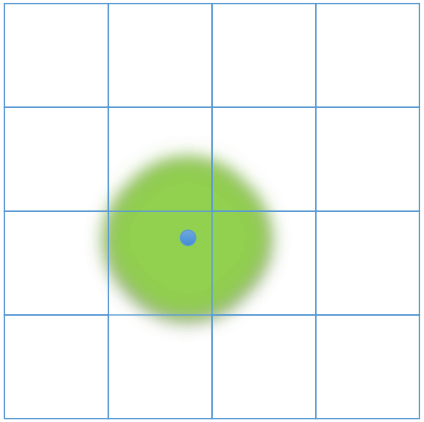
It takes 400 fps to resolve electrons at a dose rate of 10 e^-/pix/s.

Counting mode

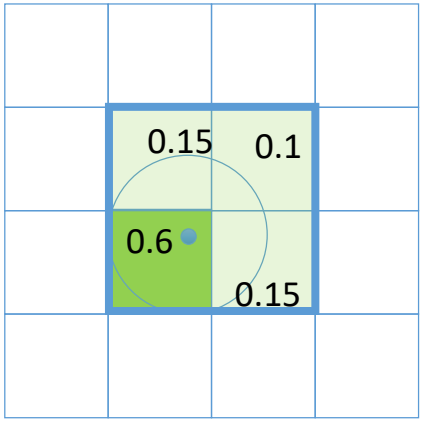
1. Electron enters detector



2. Signal is scattered

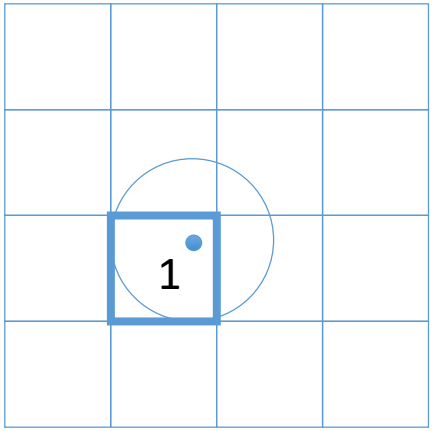


3. Charge collects in each pixel



Integration mode

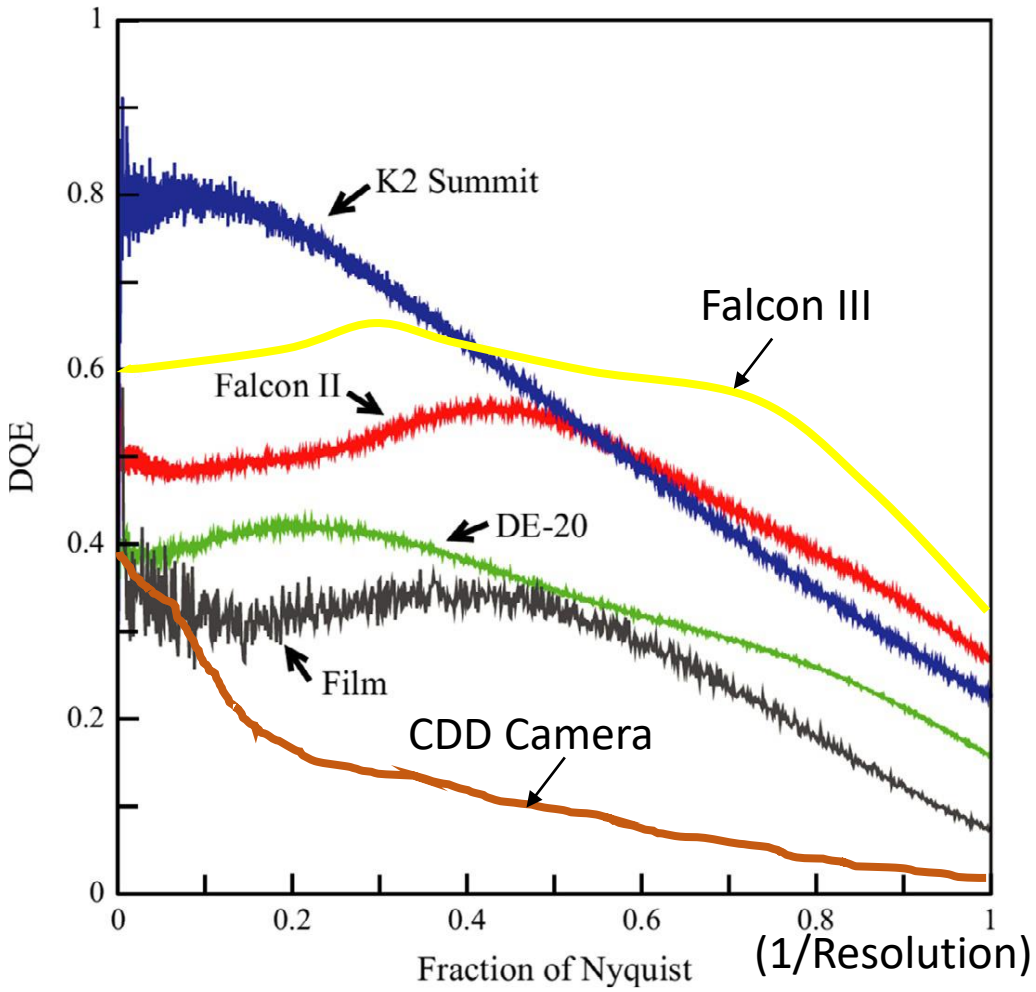
4. Events reduced to the highest charge pixels



Counting mode

Improved DQE

Detective quantum efficiency ≈ Sensitivity
% incident electron converted in signal



(1/Resolution)

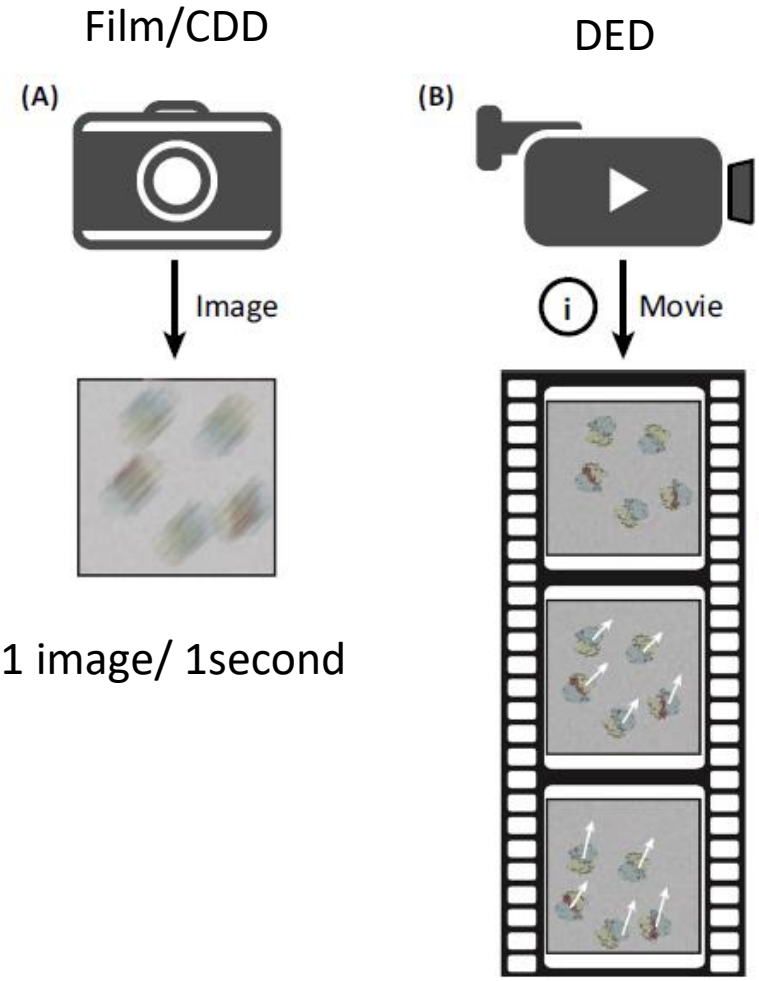
$$\mathrm{DQE} = (S/N)^2_{\mathrm{out}}/(S/N)^2_{\mathrm{in}}$$

Higher DQE for DED
K2 summit (Gatan)
Falcon II and III (FEI)
DE-20 (Direct electron)

McMullan et al, 2014, and 2016

Movie acquisition

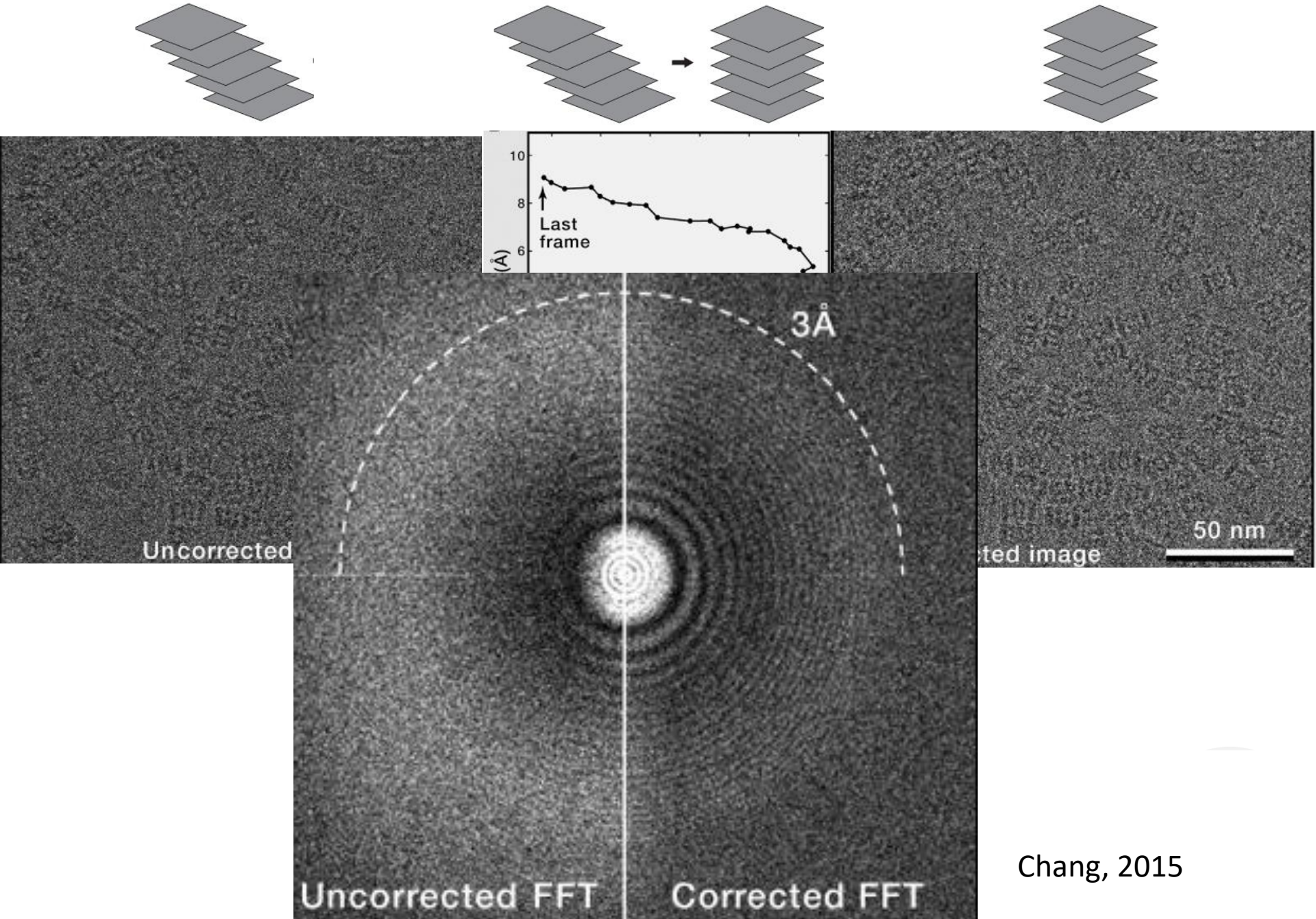
CMOS characteristic Rolling shutter : high speed read out
400 frames per second (for K2 camera)



40 e/A2 in 1 image/ 1second

Typically a movie of 40 frames
1 e/A2 /0.4 s
40 e/A2
Total 16 second
Dose fractioning

Advantage of Movie acquisition: Correction for mechanical motion

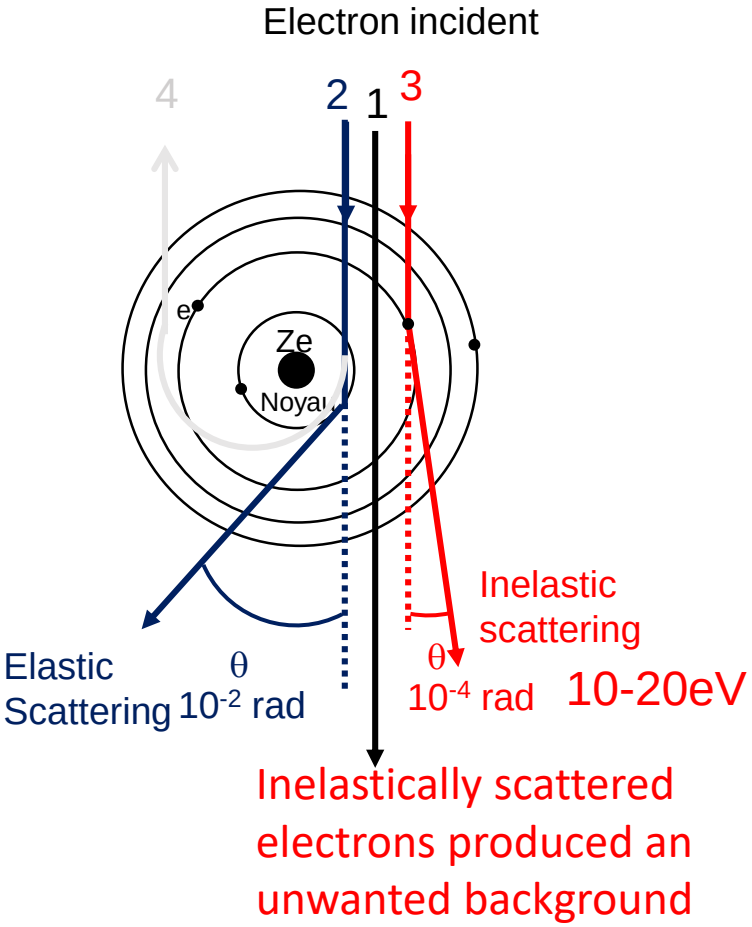


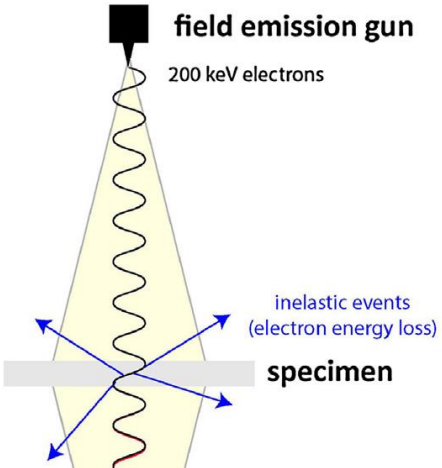
Chang, 2015

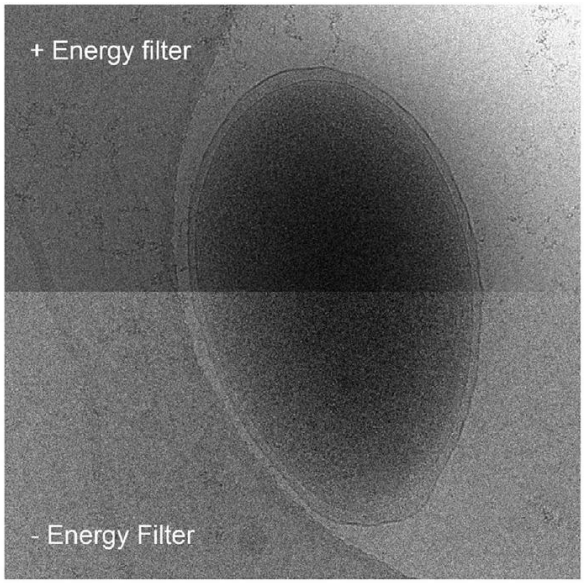
Accessory equipments improving image quality and contrast

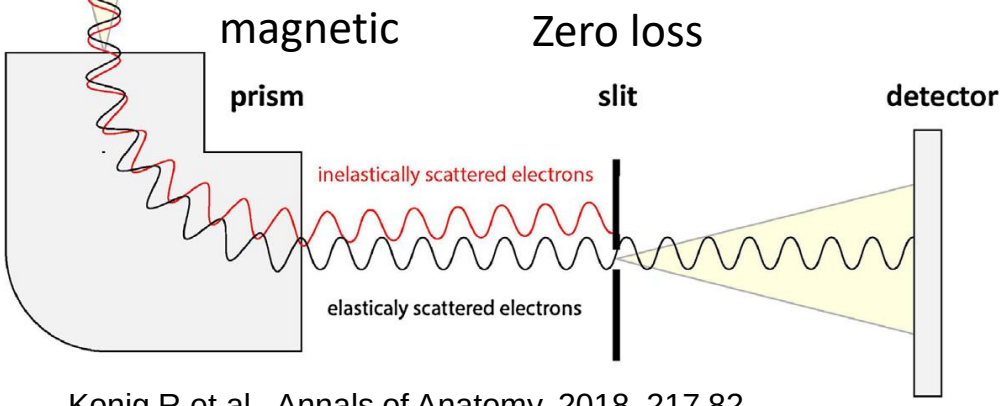
Monochromator, Cs corrector
Energy filter
Volta Phase Plate

Energy Filtered TEM: contrast enhancement







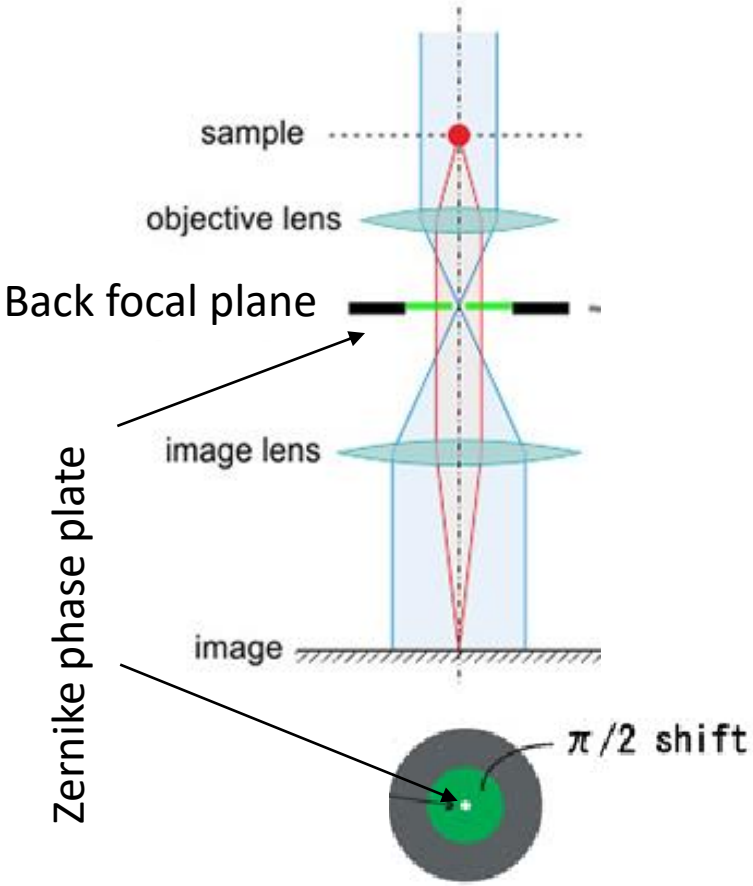


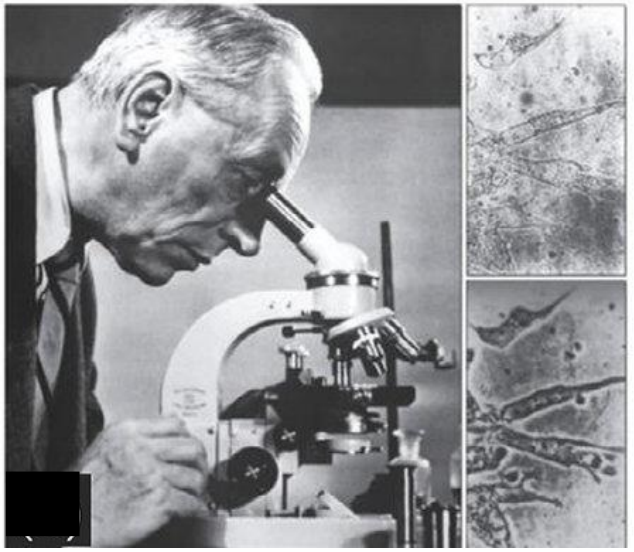
Konig R et al., Annals of Anatomy, 2018, 217 82

zero-loss imaging mode removes the background noise due to inelastically scattered electrons

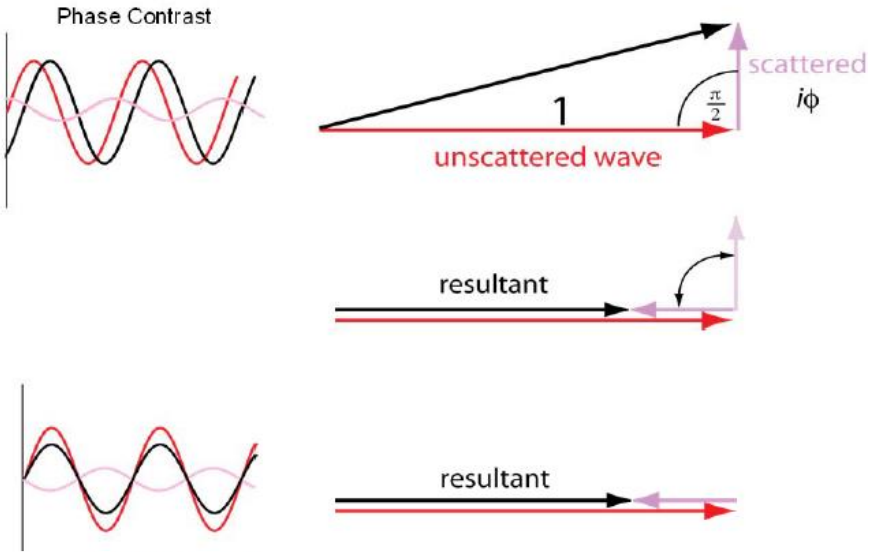
GIF Quantum Gatan
Selectris TFS

Image contrast modulated by spatial filter



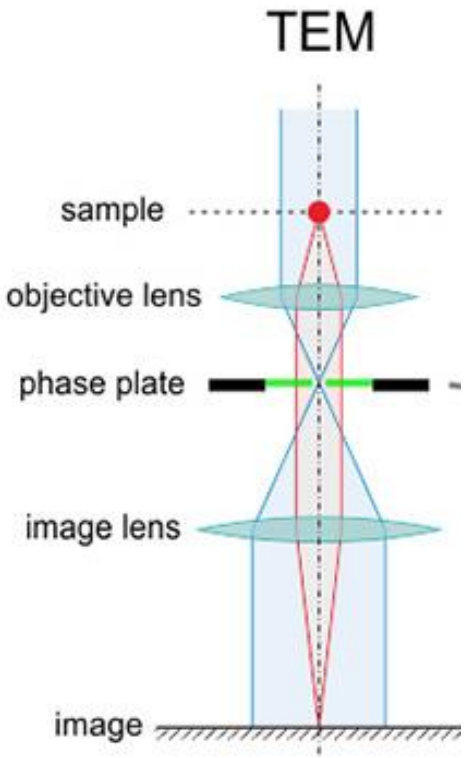


Frits Zernike (Wikipedia)

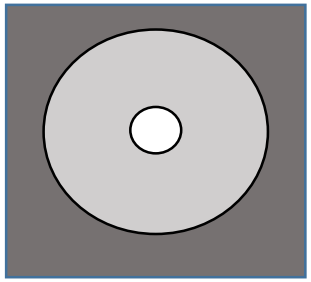


Volta Phase Plate

Radostin Danev, 2009 Ultramicroscopy , 2014 PNAS



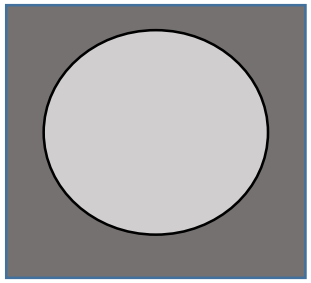
ZPP (2009)



Small (1µm) hole

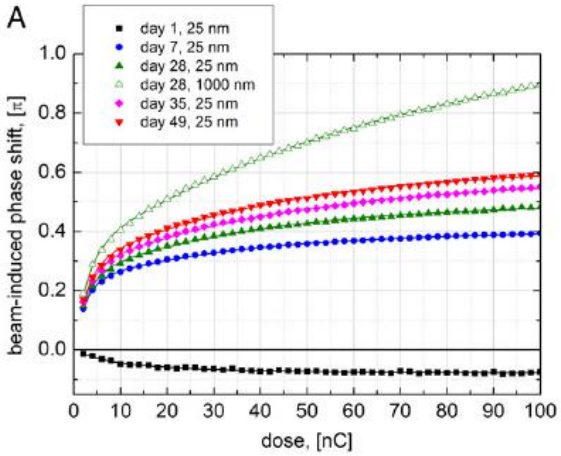
Thin amorphous carbon 20 nm that creates a $\frac{\pi}{2}$ shift

VPP Volta Phase Plate (2014)



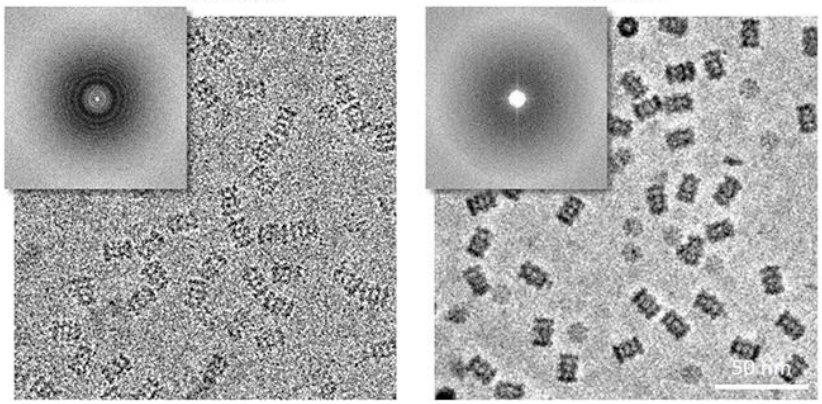
Thin amorphous carbon 12 nm

Beam induced phase shift over time controlled by temperature



Conventional cryo-EM
1.5 um defocus

VPP cryo-EM
in-focus



Local changes in the properties of the carbon film that lead to changes in the inner and/or surface potentials (Volta Potential)

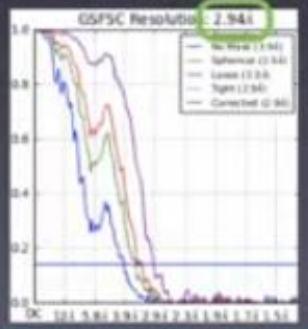
Sample preservation may be key to data collection speed…

How many images do we need under "ideal" circumstances?

1	2	3	4	5
6	7	8	9	10

For a "very good" sample, 10 images is sufficient for a 3Å map

At ~10s/image that is ~2 minutes of data acquisition



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