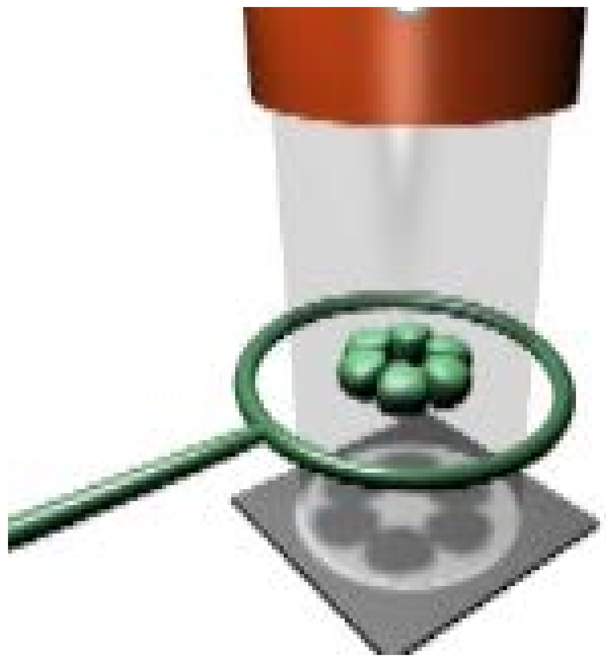
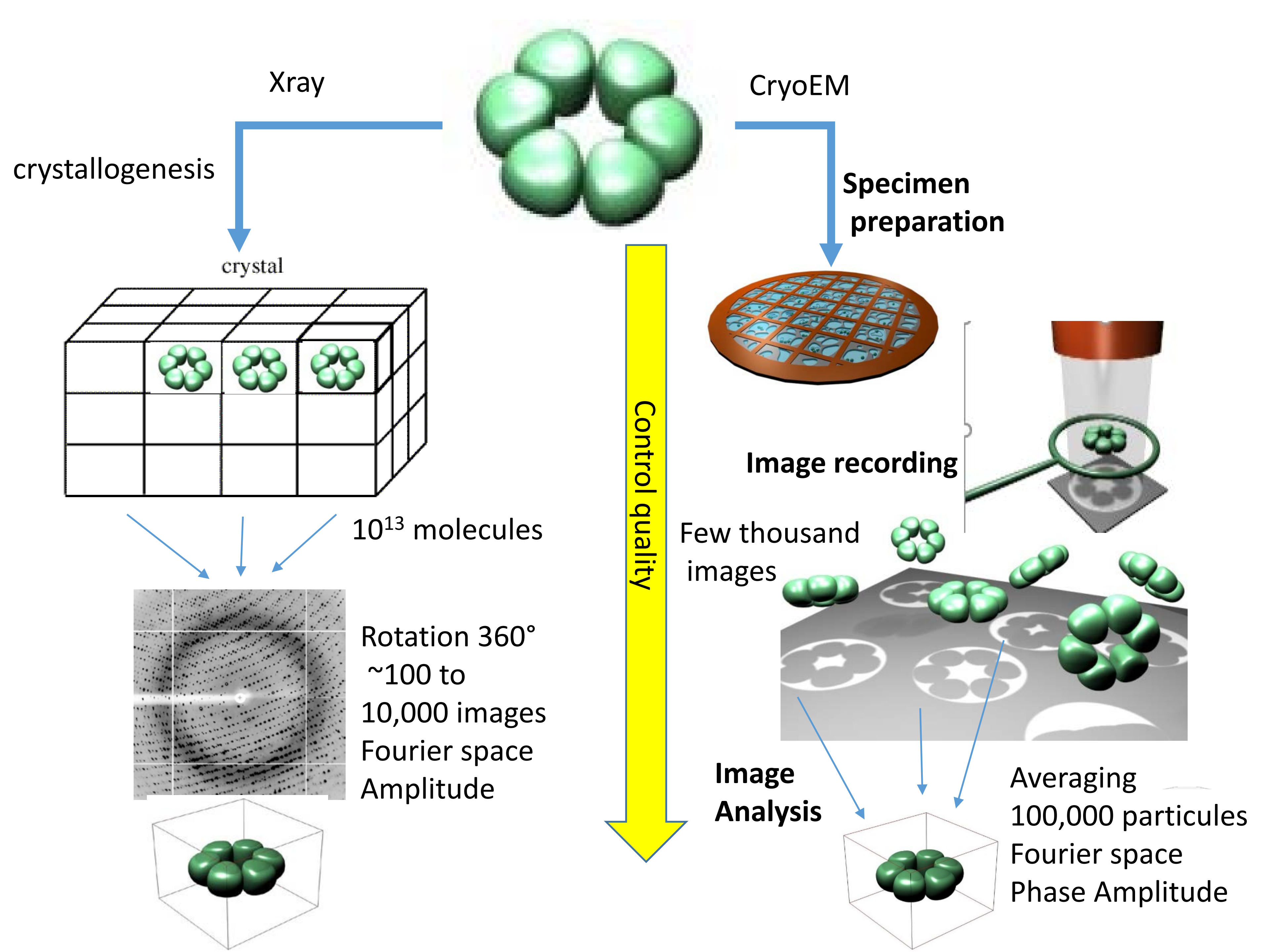
Transmission electron microscopy in structural Biology

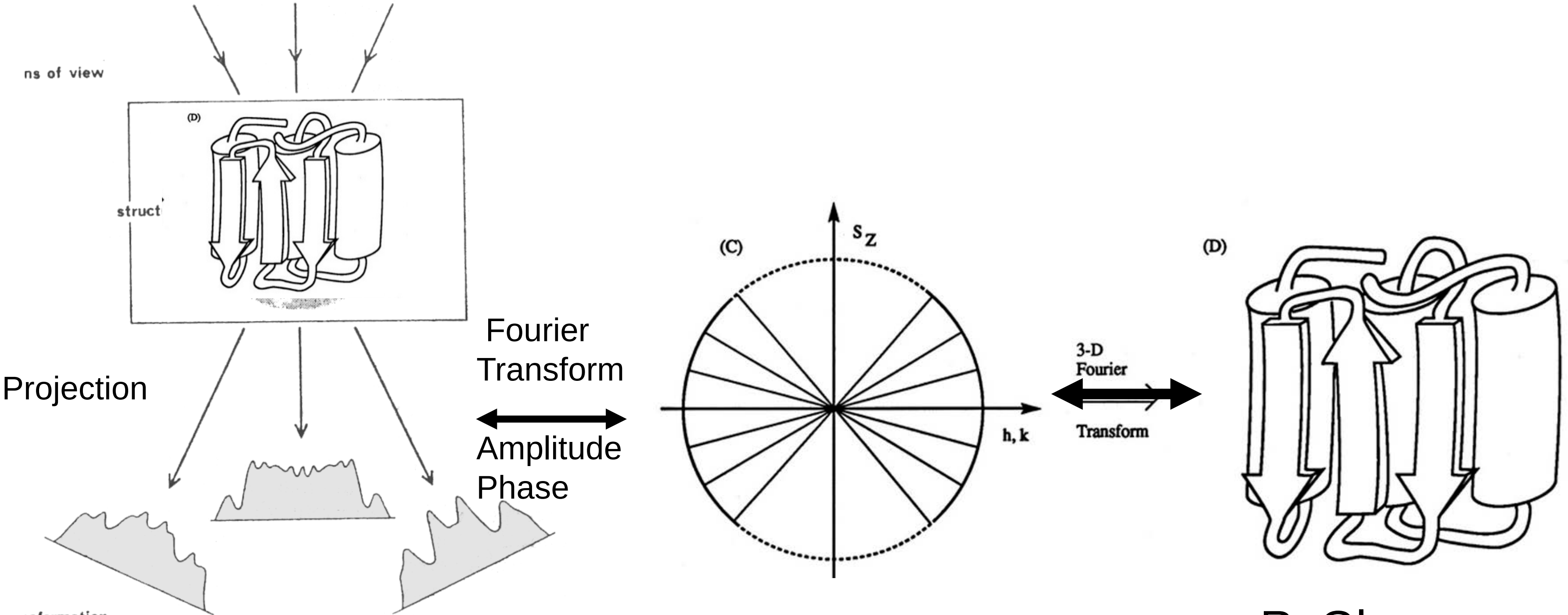
From specimen preparation to data collection



o.lambert@cbmn.u-bordeaux.fr

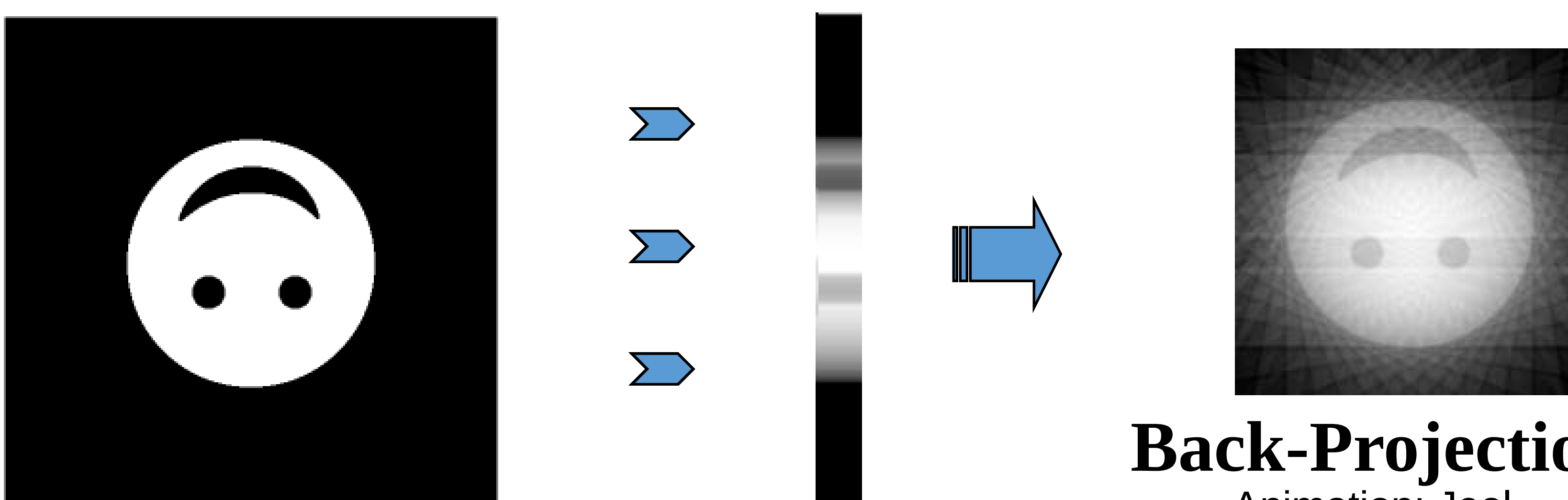


Xray
CryoEM
crystallogenesis
Specimen preparation
crystal
Control quality
Image recording
10^13 molecules
Few thousand images
Rotation 360°
~100 to
10,000 images
Fourier space
Amplitude
Image Analysis
Averaging
100,000 particules
Fourier space
Phase Amplitude



Real Space **Fourier (reciprocal) Space**

R. Glaeser



Back-Projection

Animation: Jeol

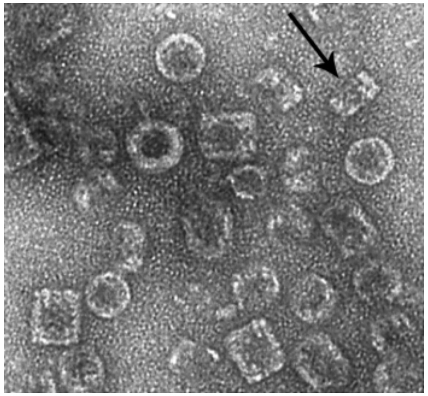
Specimen prepartion

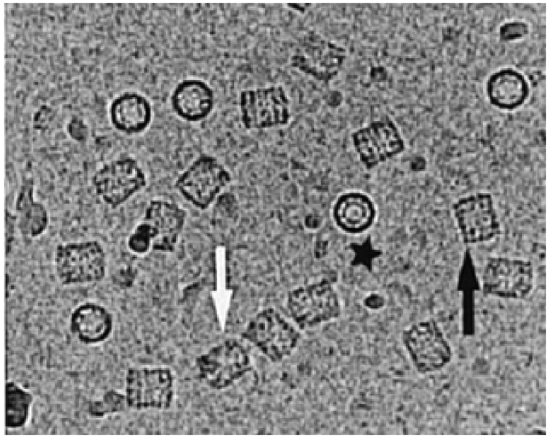
Electron microscopy in few words

- Electrons are energy-rich elementary particles
- Electrons have much shorter wavelength than x-rays (~ 3 pm = 3 x 10^{-12} m for 100kV electrons)
- Resolution not limited by wavelength
- **But resolution is degraded by lens defects, radiation damage, mechanical drift, specimen motion**
- High vacuum is essential - no live specimens!

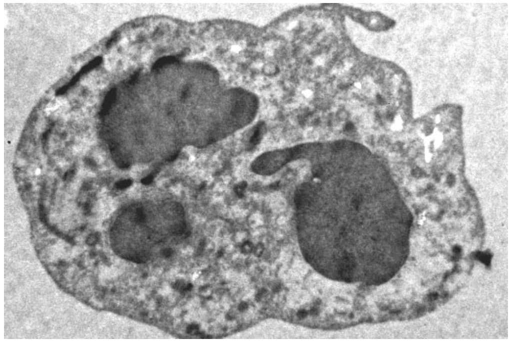
Preparation regarding the size of biological samples

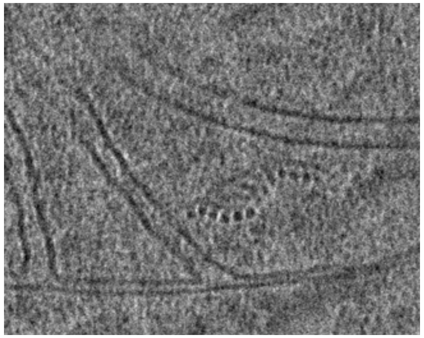
	Dehydrated / stained specimen	**Frozen hydrated/ unstained specimen**
Thin Specimen :	**negative staining**	**freeze-plunging** Cryo-electron microscopy



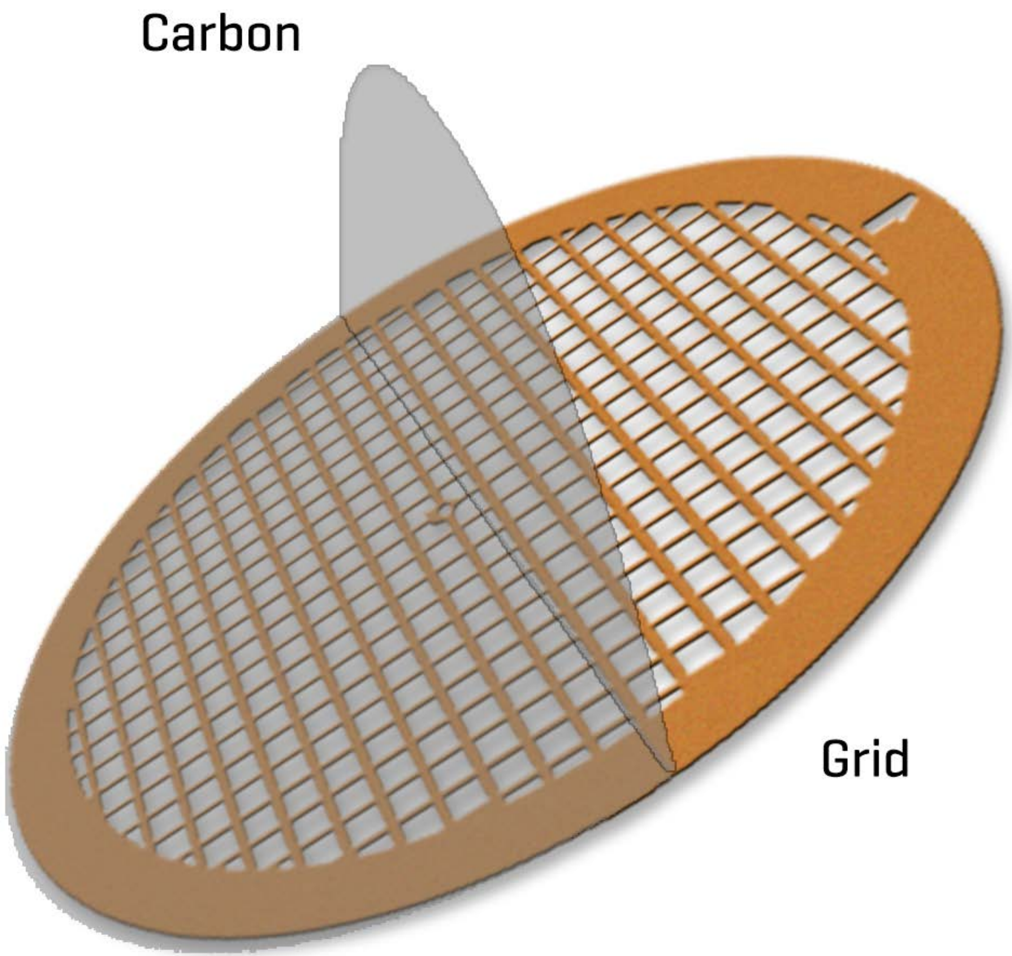


Thick Specimen :	**Plastic section**	**cryosection**

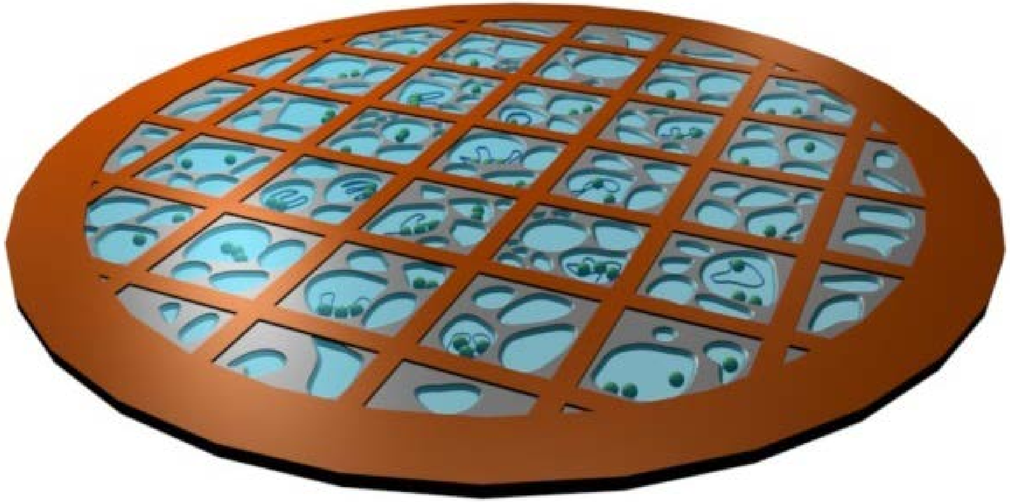


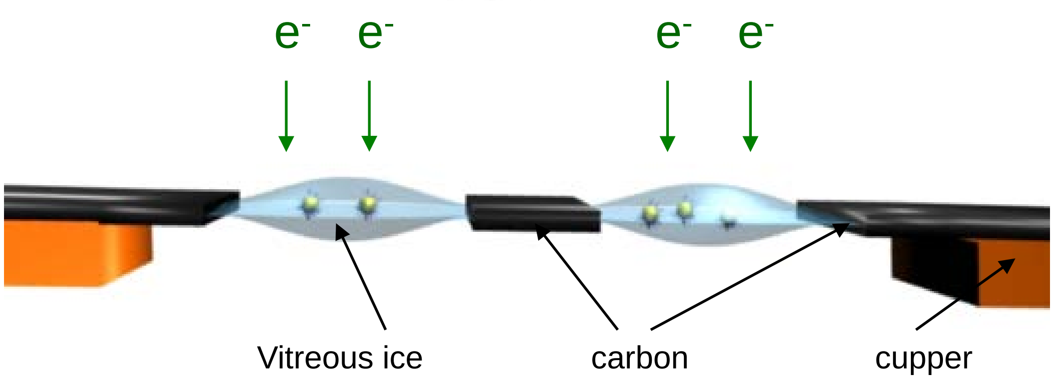


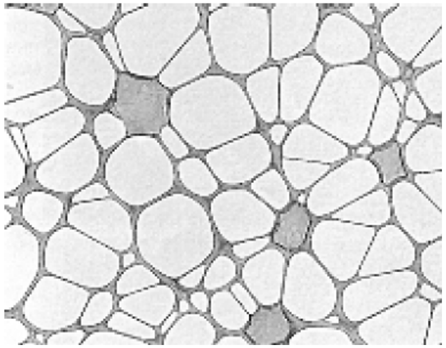
Support : grid coated with thin amorphous carbon

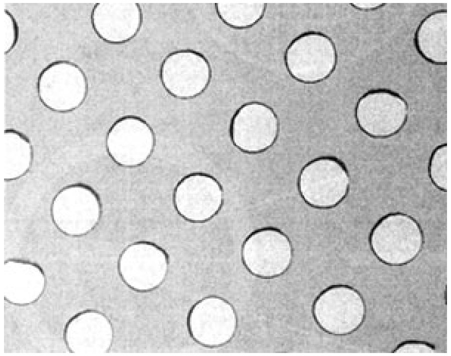


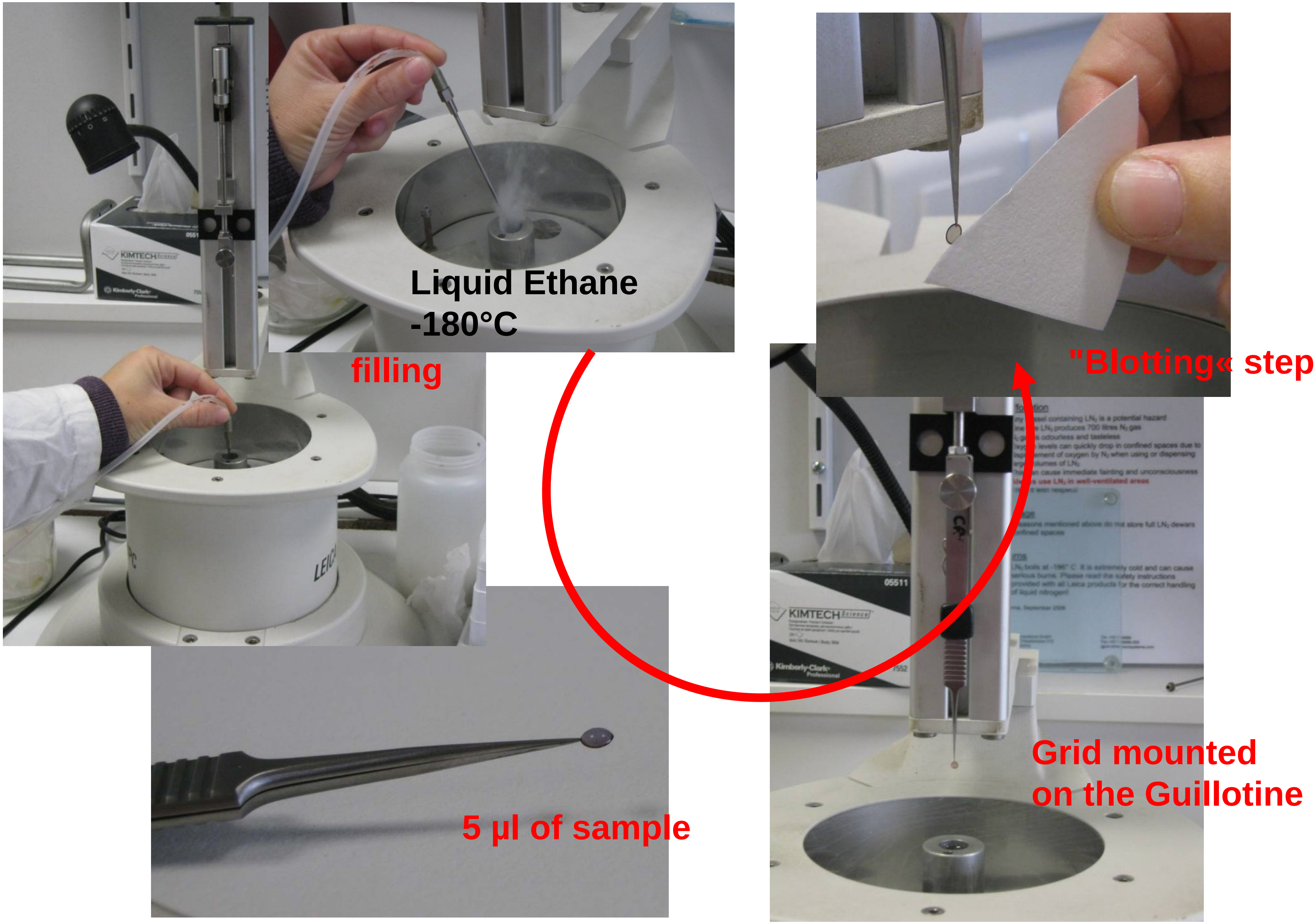
Cryo electron microscopy: Holey carbon grid



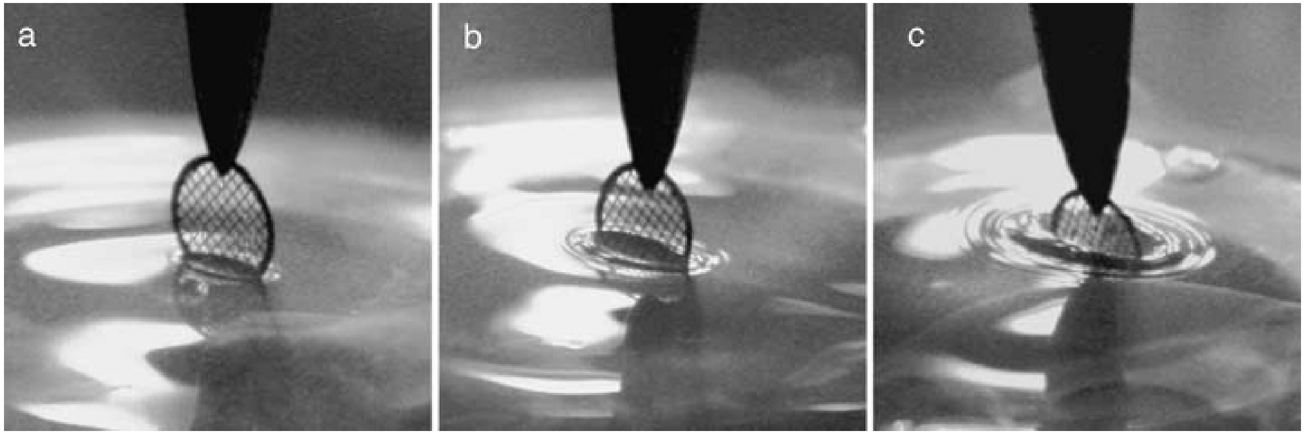


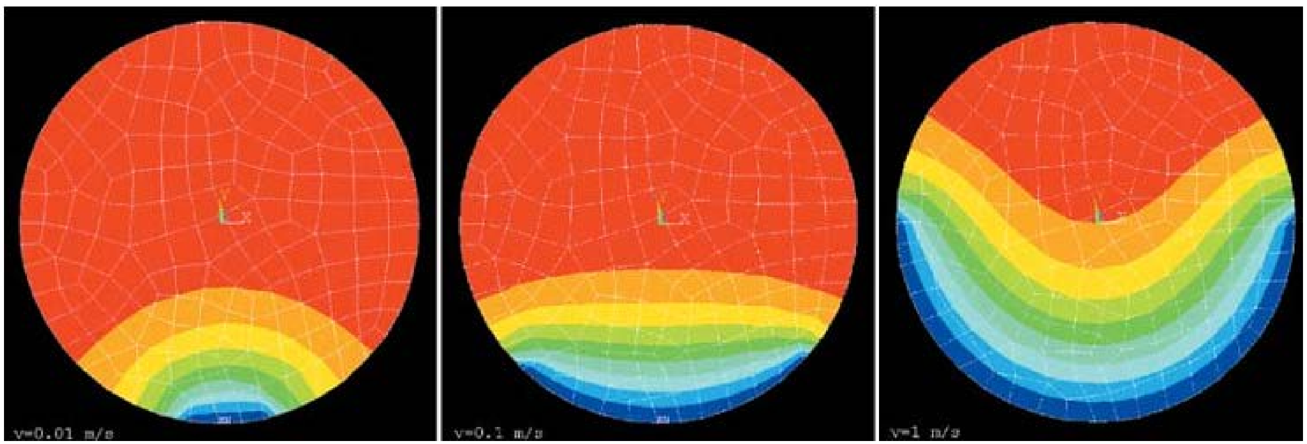


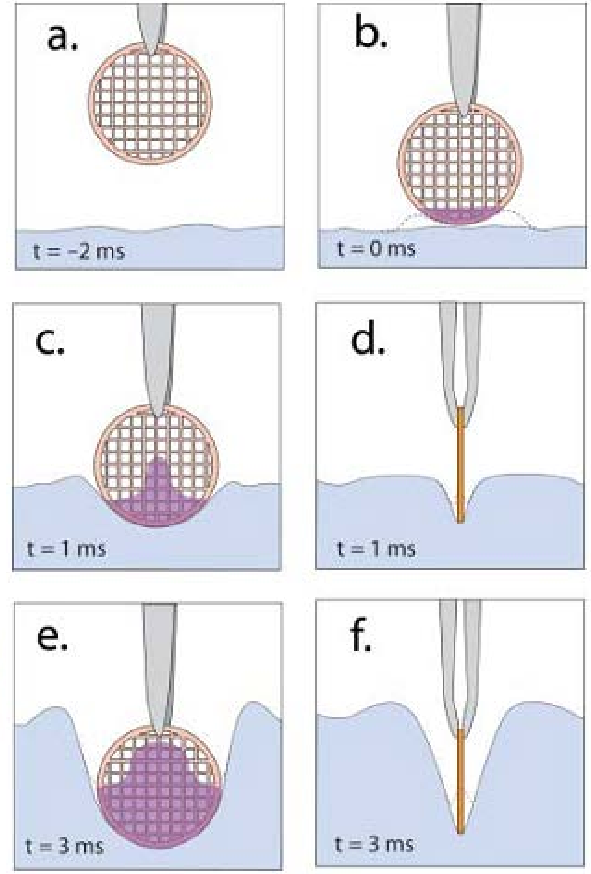



Liquid Ethane
-180°C
filling
"Blotting« step
Grid mounted
on the Guillotine
5 µl of sample

The importance of cryo-approaches in cryo-EM







Kasas et al. & Adrian,
Journal of Microscopy, 2003

Advantages of flash-freezing:
- → vitrified water (amorphous ice)
- → specimen conservation (frozen-hydrated)
- → very weak ice sublimation in the vacuum of the microscope
- → fixation of particle orientations

The importance of cryo-approaches

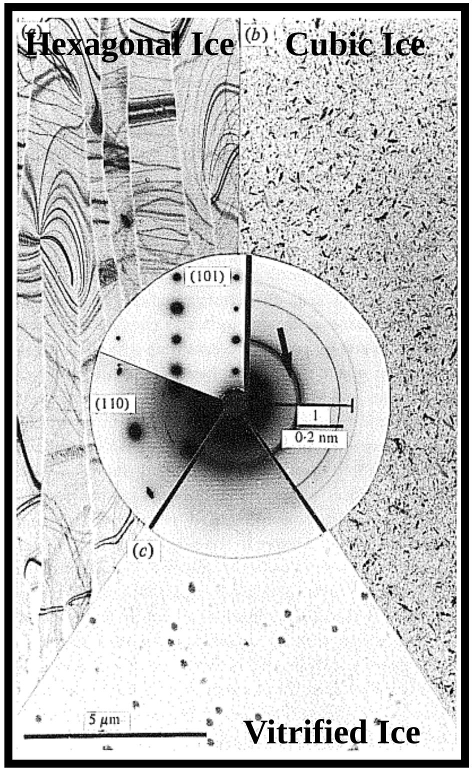
Vitreous ice:

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

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

- hexagonal ice, forms when water is (relatively slowly) cooled down at atmospheric pressure

(is typical source of contamination in cryo-EM)



Dubochet *et al.*, 1988

cooling rate required to obtain vitreous ice: ~10^4 K / s

Boiling and melting points of liquid ethane: <u>-88.7 °C / -183.3 °C</u>,
Boiling and melting points of Nitrogen: <u>-196 °C / -210 °C</u>,

Improve the sample distribution/stability

Plunging system

Home made

Control of the temperature and humidity

Gatan

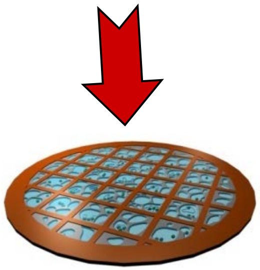
Leica

FEI

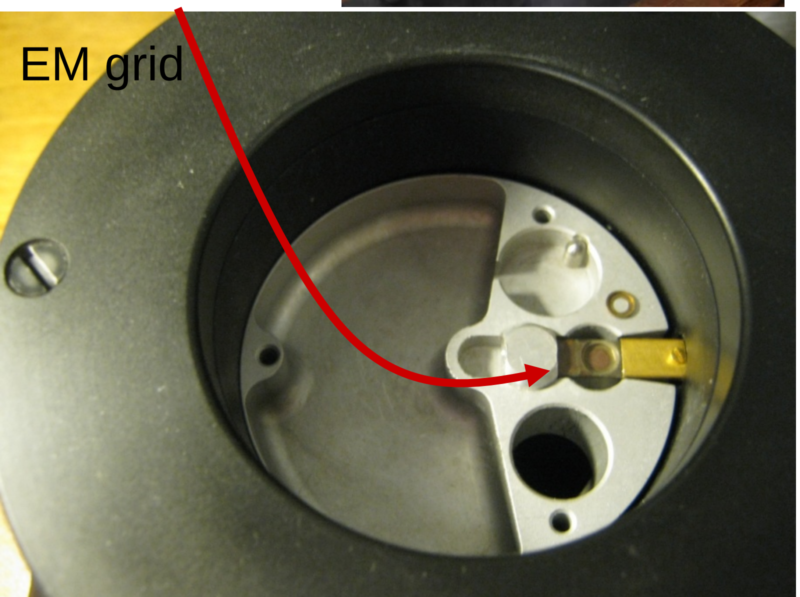
Freezing
- -blotting time
- -single or double side blotting
- -waiting time
- -multiple sample application

Transfer into cryoTEM

specimen

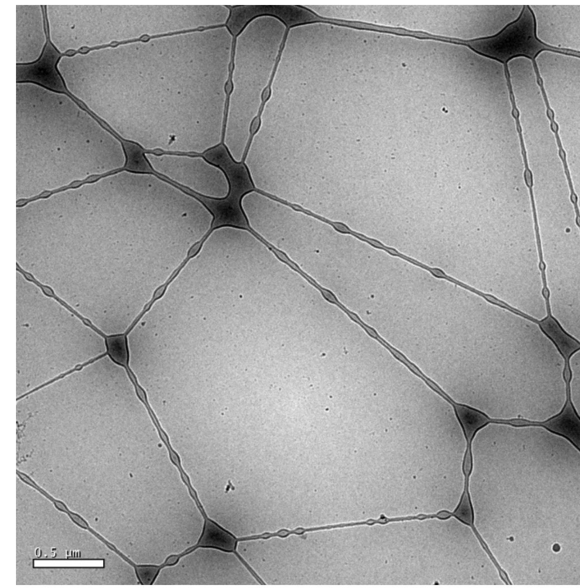


EM grid



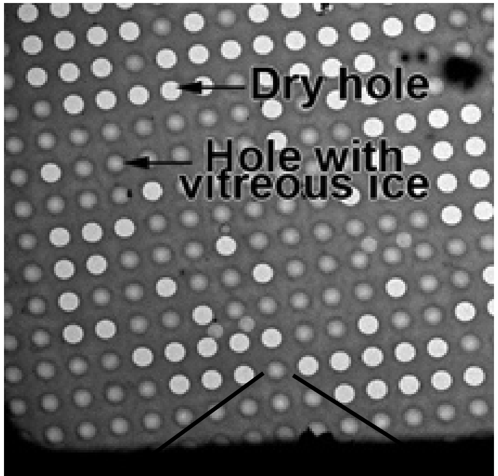
Cryo-holder

observation

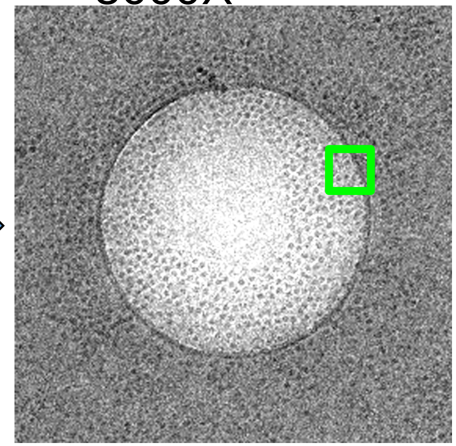


Cryo electron microscopy: What do you expect ?

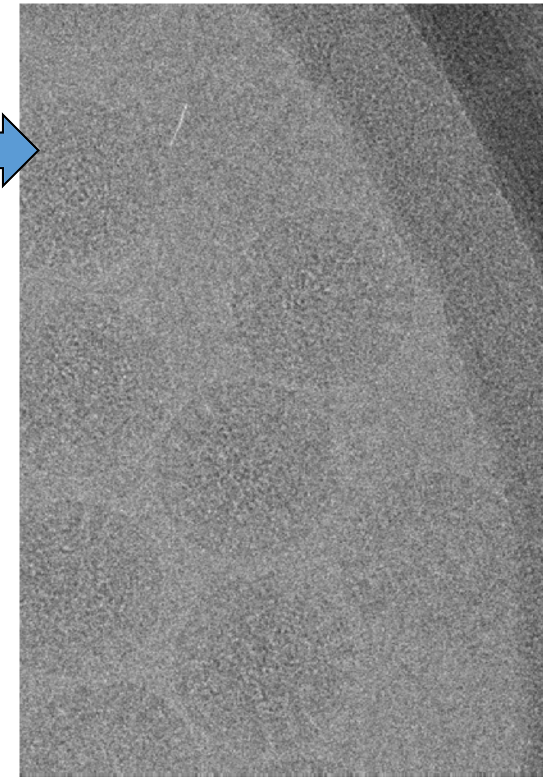
500X

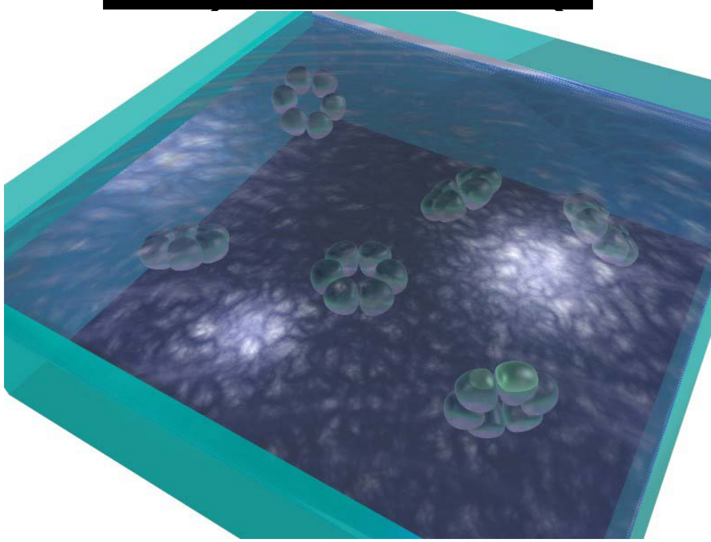


5000X



50000X

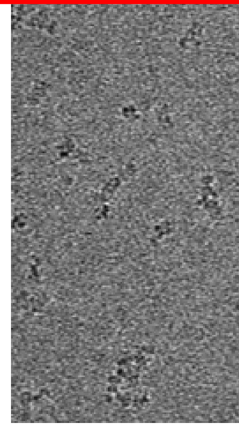


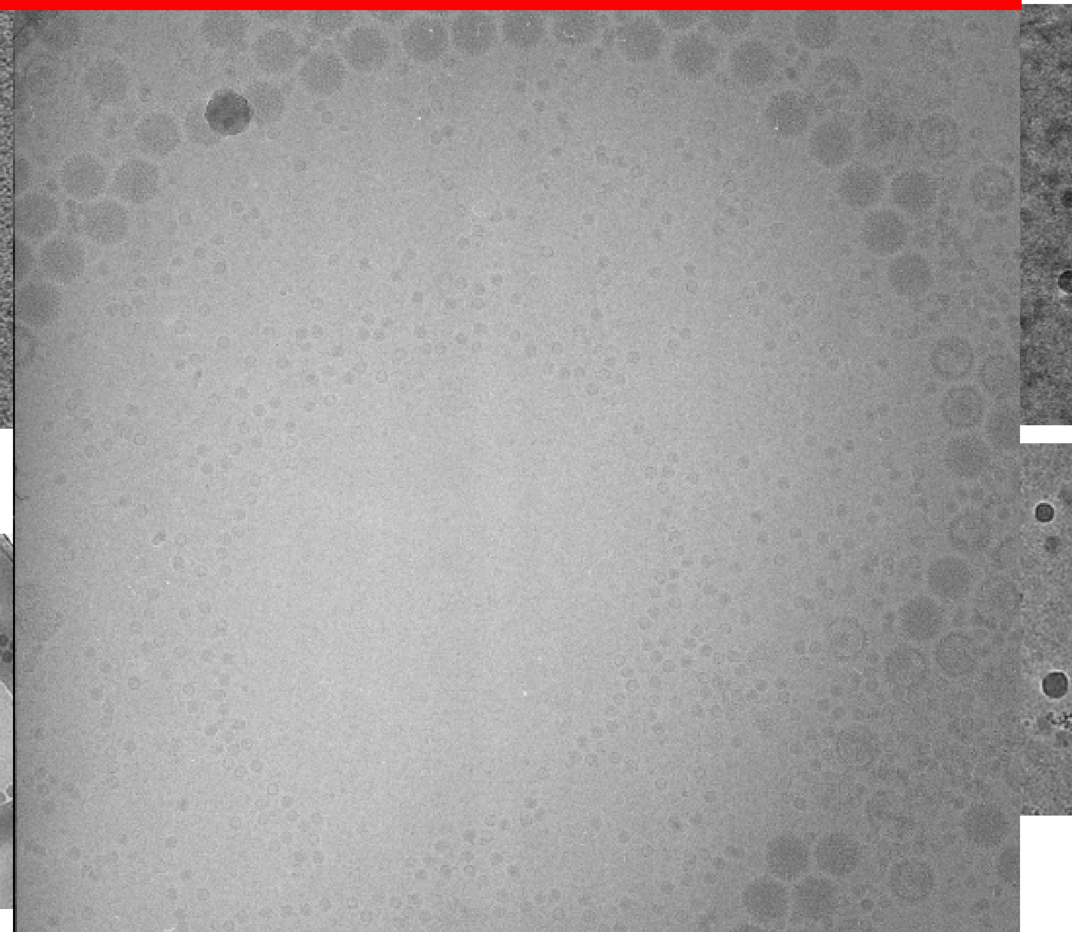


Vitreous ice

Particles in different (random) orientations

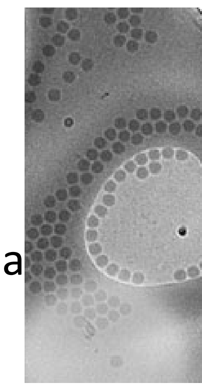
Cryo electron microscopy: What is a good cryo grid ?





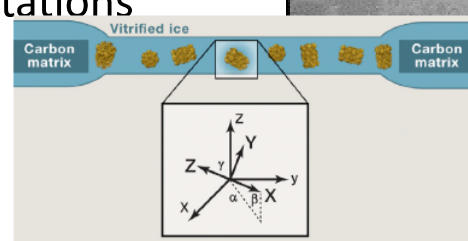
Good amorphous ice
-not crystalline ice
-no « leopard skin »pattern
-no contamination

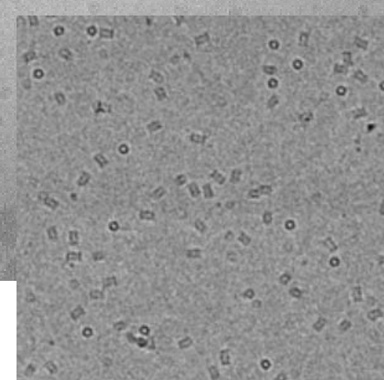
Appropriate ice thickness
-typically as thin as possible

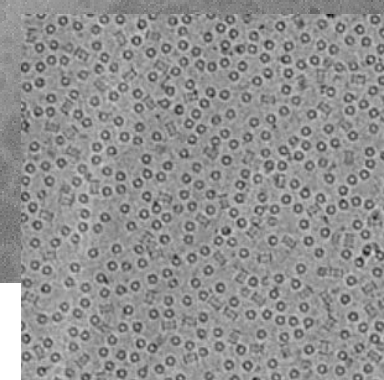


Clearly visible particles
-particle size and shape
-buffer composition
-defocus, movie mode, phase pla

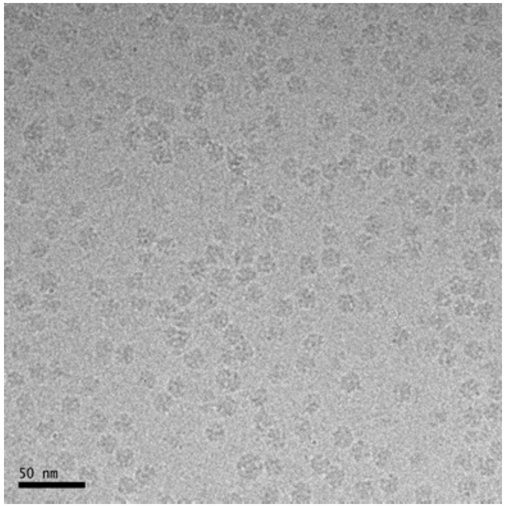
Good particle distribution
-in holes
-dense but particles not touching
-randomly distributed orientations

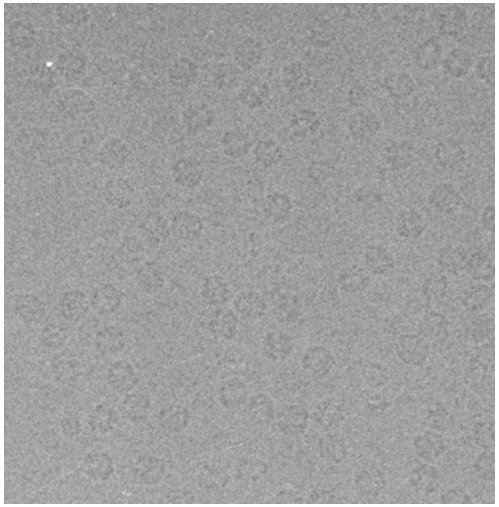






Cryo electron microscopy: Advantages and drawbacks





Advantages :

hydrated state of the sample

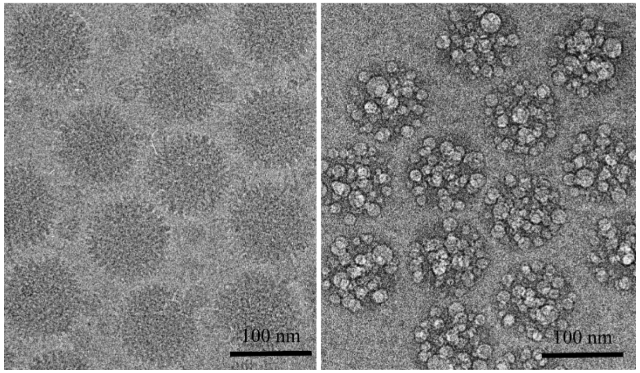
High resolution

Small amount of sample

Drawbacks :

Low contrast

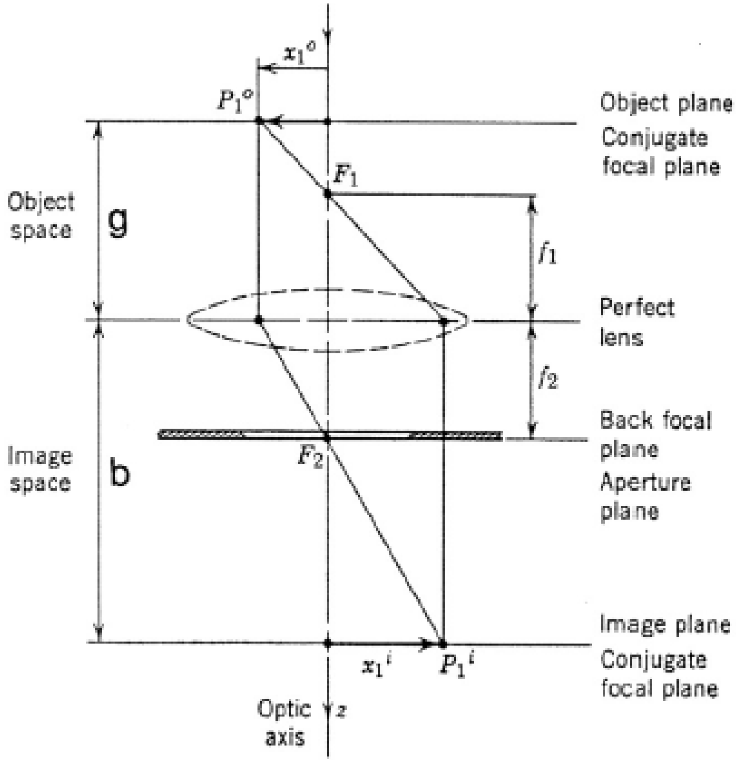
Highly sensitive to electron dose

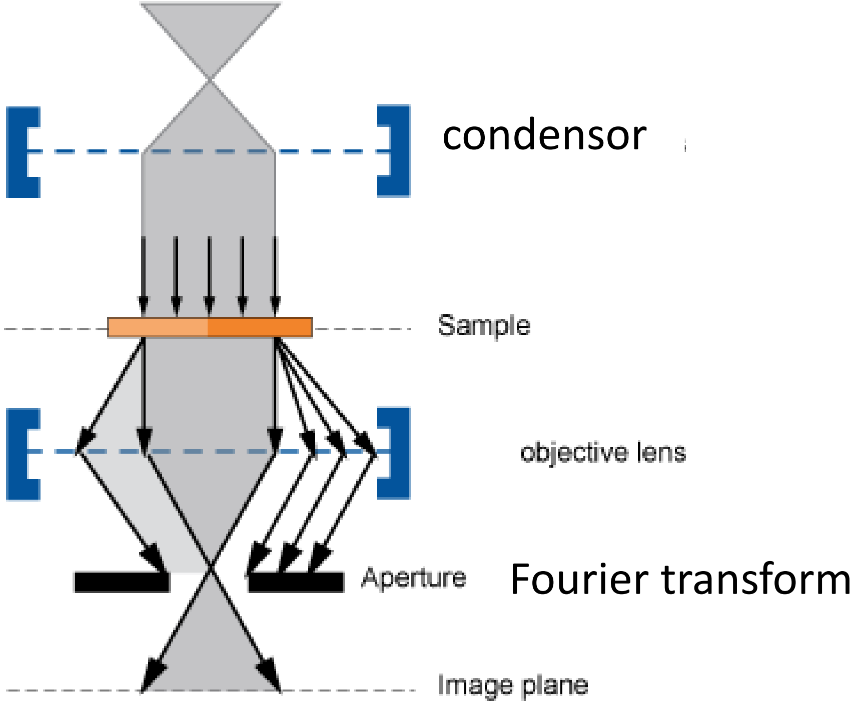


Data collection

Cryo electron microscope : reference

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





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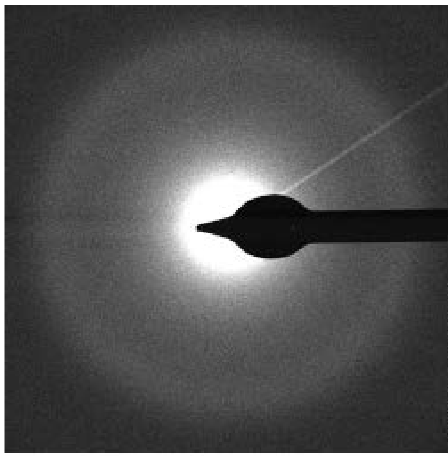
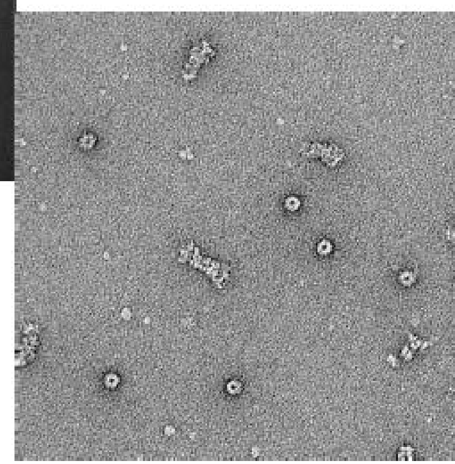


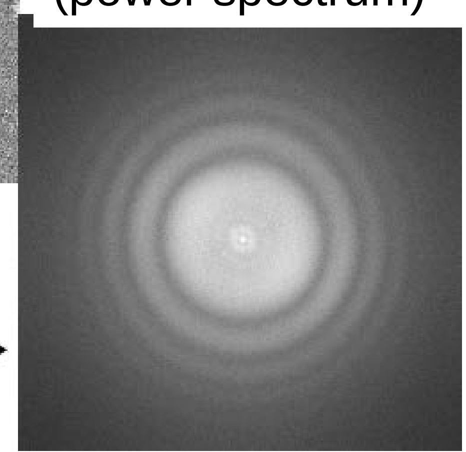
Diffraction (Fourier transform)

diffraction



Image

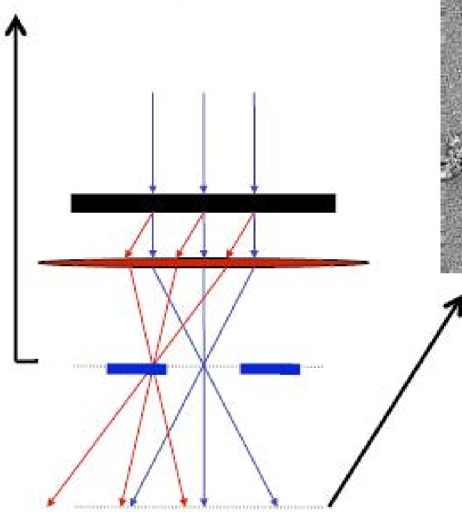
Fourier Transform (power spectrum)



specimen

lens

Back focal plane

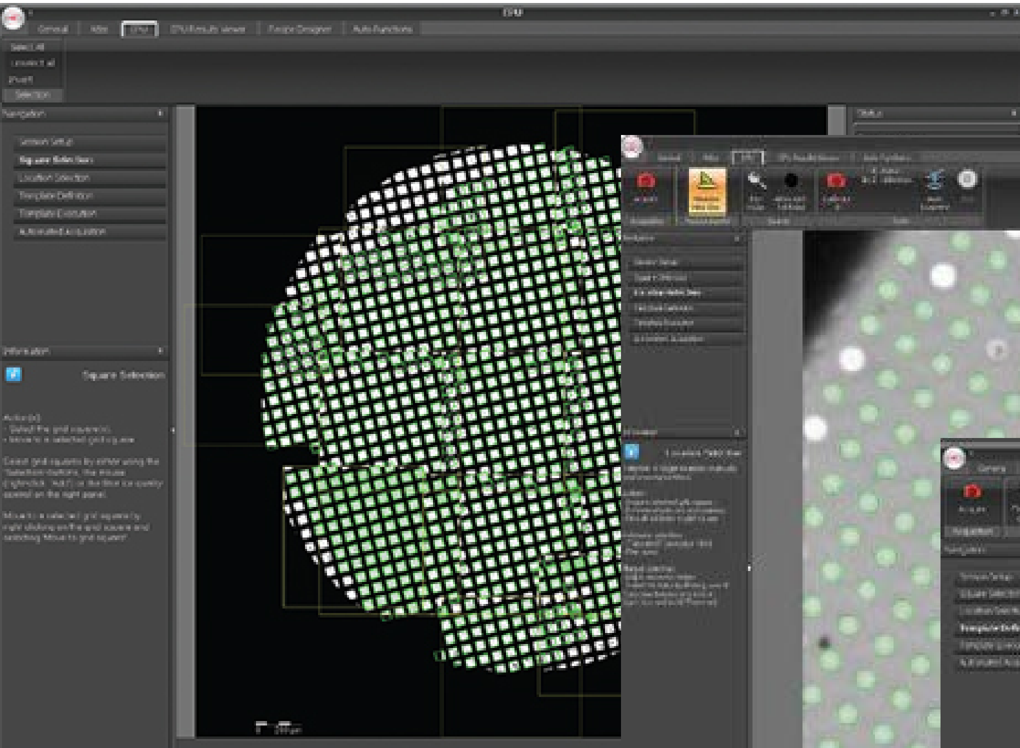


FT

Cryo electron microscopy: Automated data acquisition

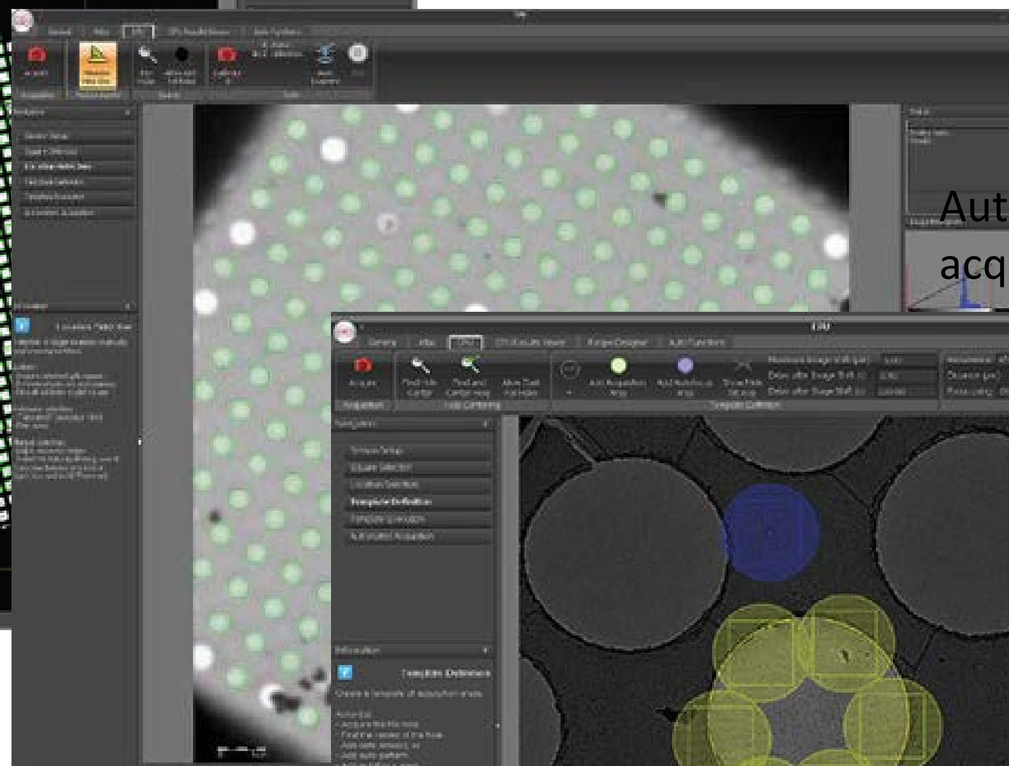
Example : Automated data acquisition software EPU (FEI)

Atlas = image of the EM grid

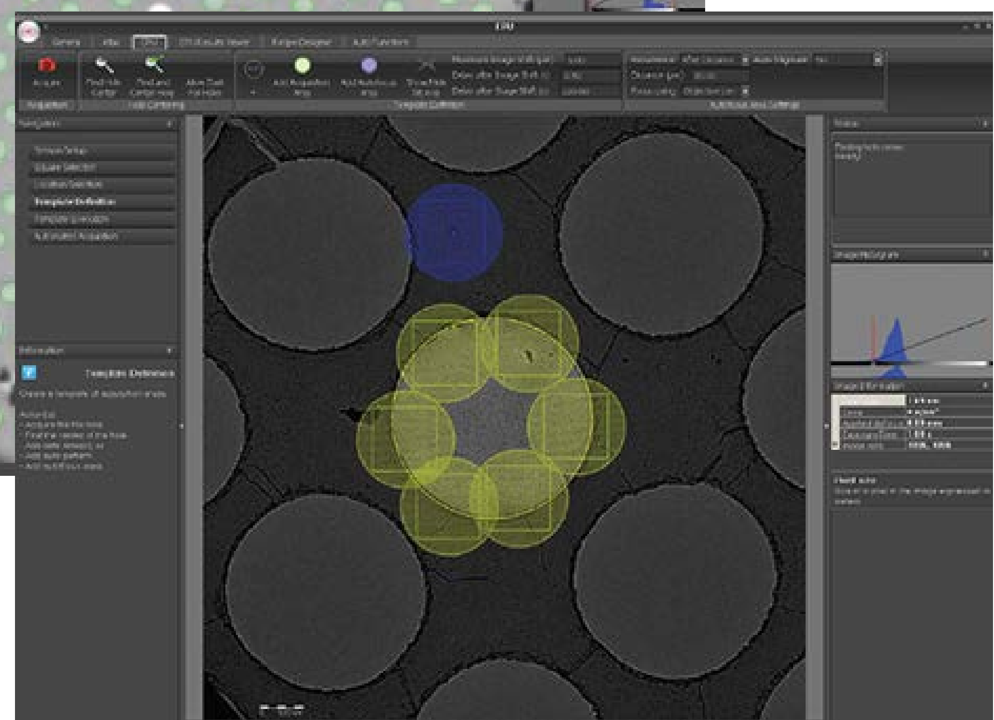


Settings

Hole coordinates, ice thickness, dose…



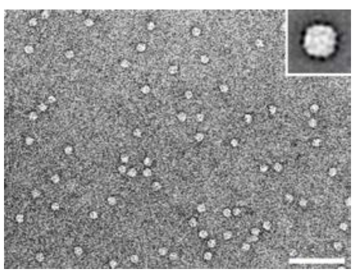
Automatic acquisition areas



24-72 hours
1000-3000 images
100000-300000 particles

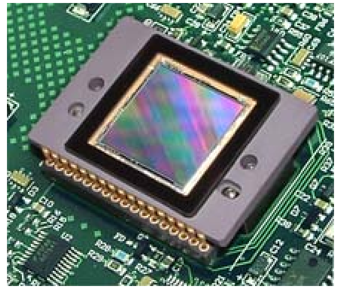
Conversion electron into images

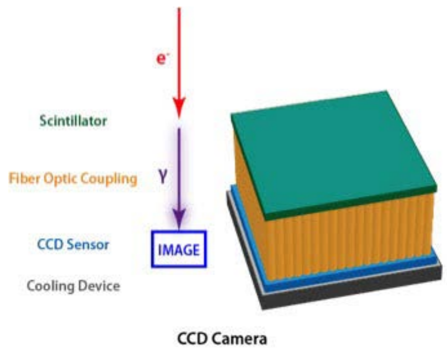
Film sensitive to electrons

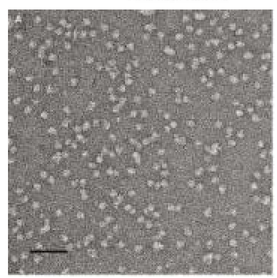


Pixel size = 8 µm
1 image / s

CCD

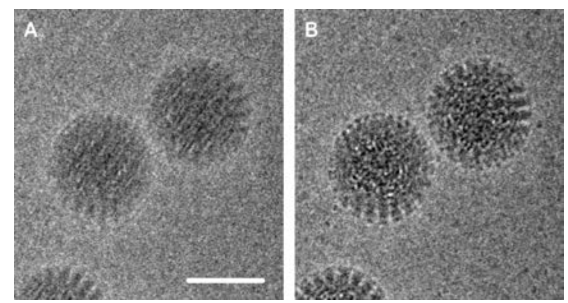






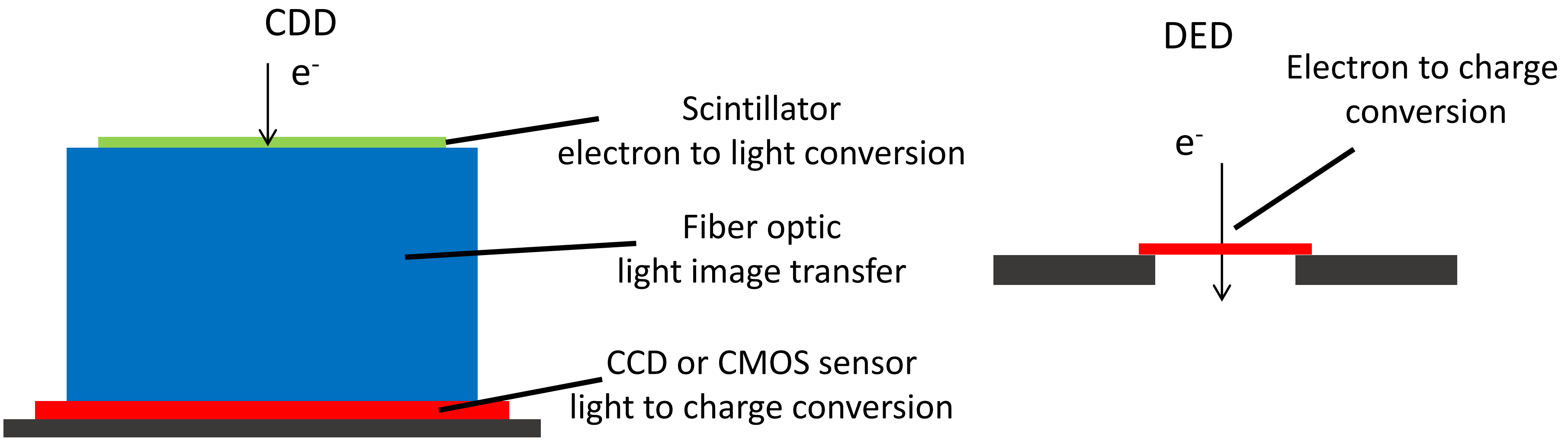
14 µm 1 image / s

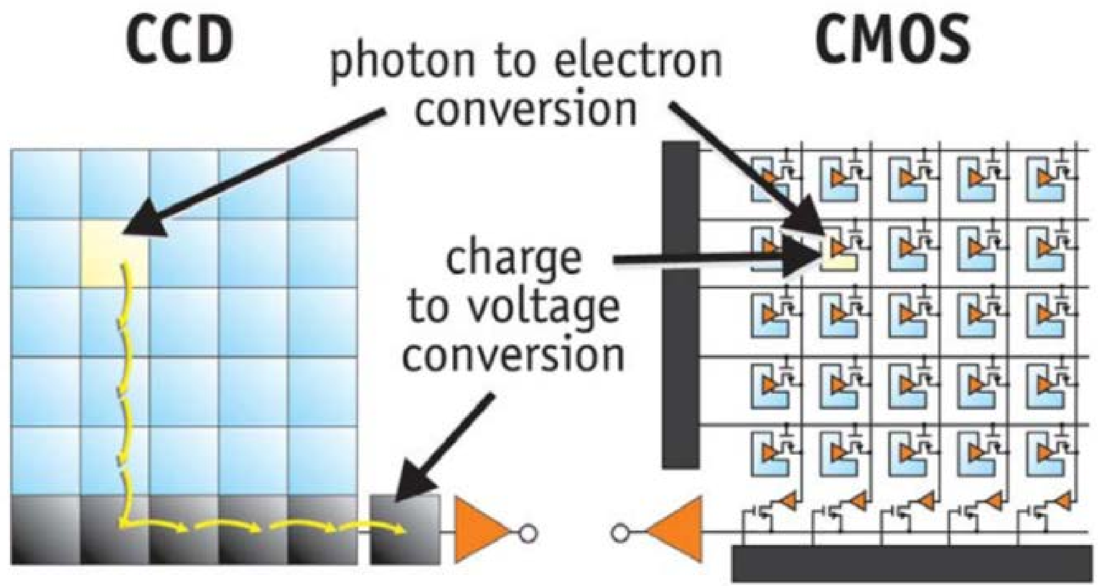
Direct Electron Detector CMOS
(complementary metal oxide semiconductor)



5 µm
20 images / s

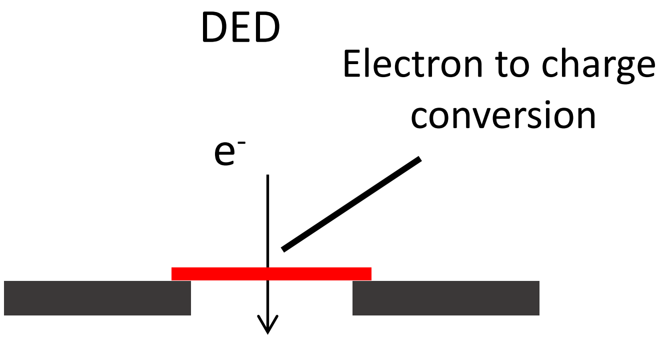
Advantage of Direct Electron Detector vs CCD camera

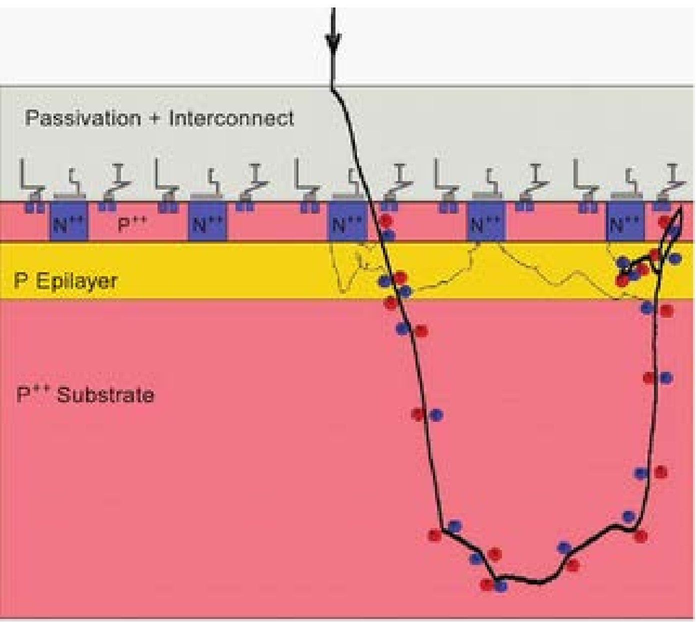


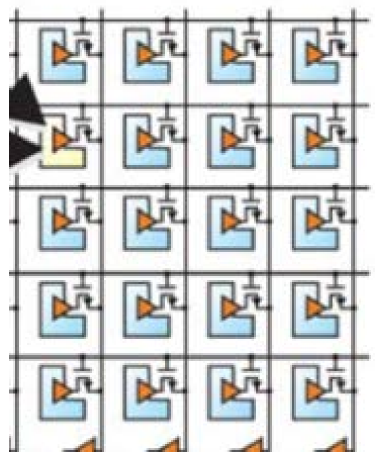


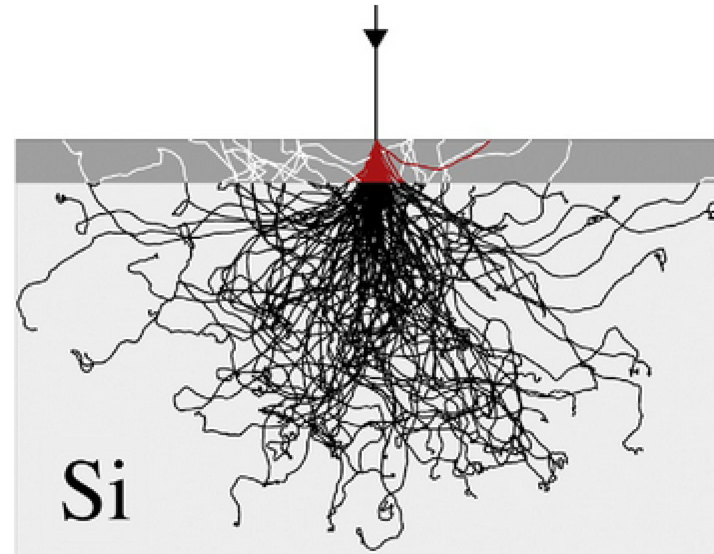
CCDs move photogenerated charge from pixel to pixel and convert it to voltage at an output node. CMOS imagers convert charge to voltage inside each pixel.

Advantage of Direct Electron Detector vs CCD camera







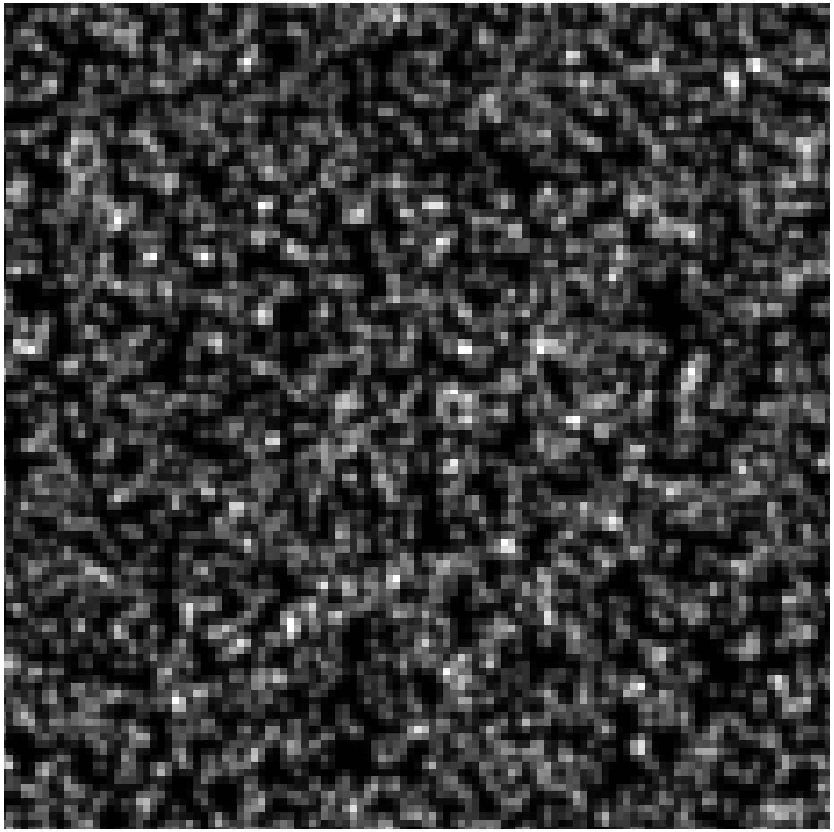


1 electron creates ~80 electron-hole pairs per micron (Faruqi, 2013)

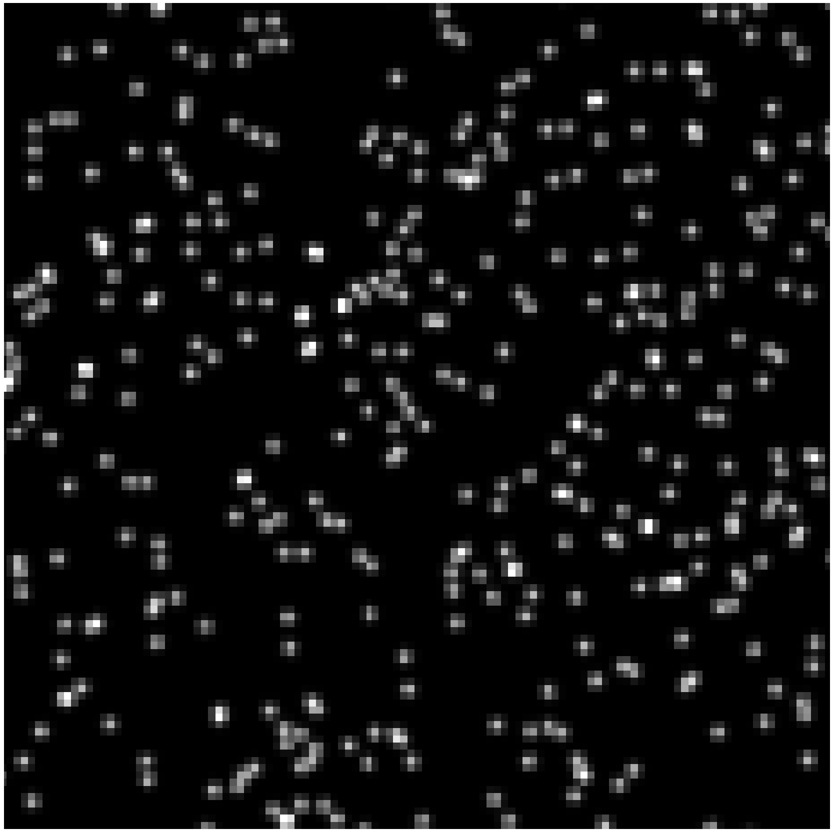
Reduction of electronic noise
Reduction of electron scattering in the sensor

Counting requires speed

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



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

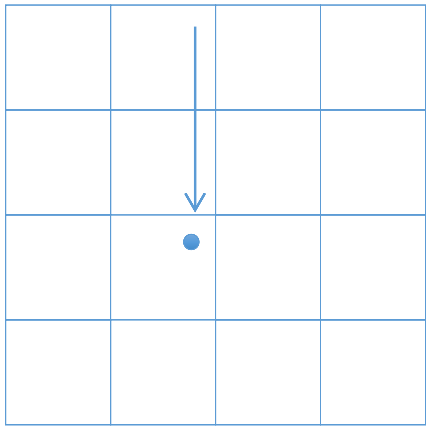


400 frames per second: events are resolved.

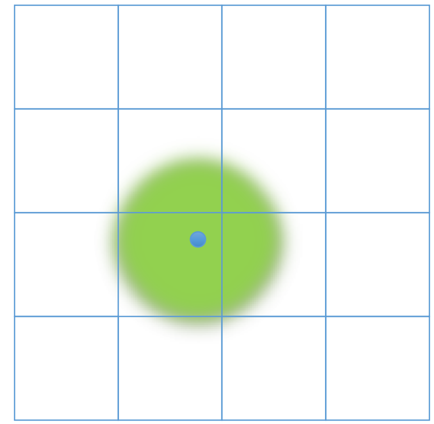
It takes 400 fps to resolve electrons at a dose rate of 10 e^-/pix/s.

Counting mode

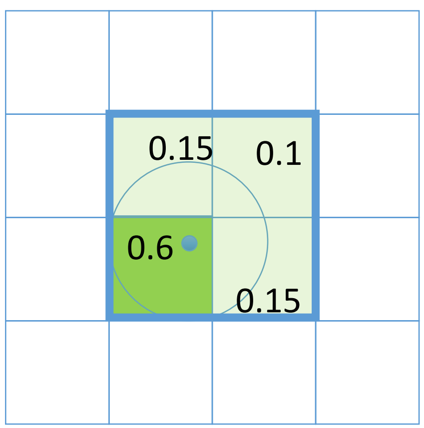
1. Electron enters detector



2. Signal is scattered

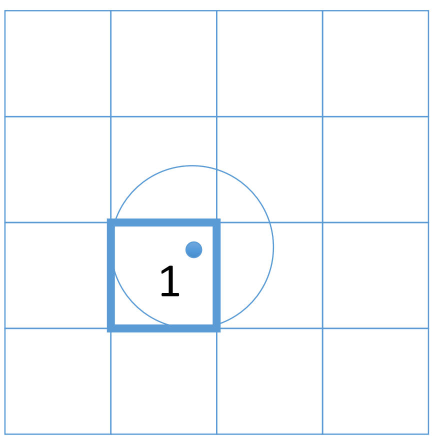


3. Charge collects in each pixel



Integration mode

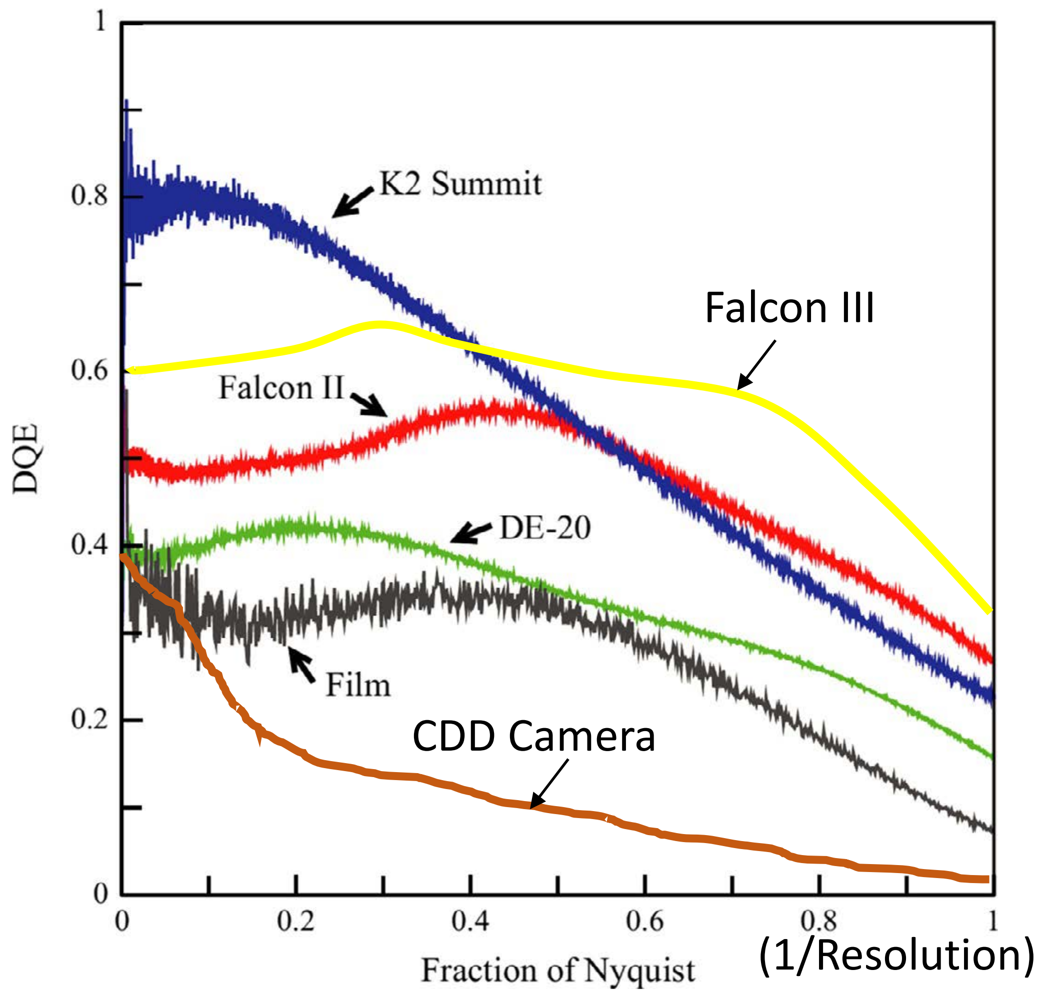
4. Events reduced to the highest charge pixels



Counting mode

Improved DQE

Detective quantum efficiency ≈ Sensitivity
% incident electron converted in signal

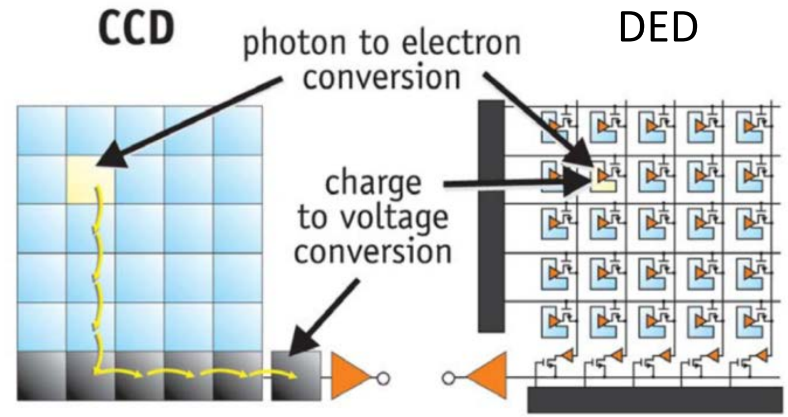


$$DQE = (S/N)^2_{out}/(S/N)^2_{in}$$

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

McMullan et al, 2014, and 2016

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



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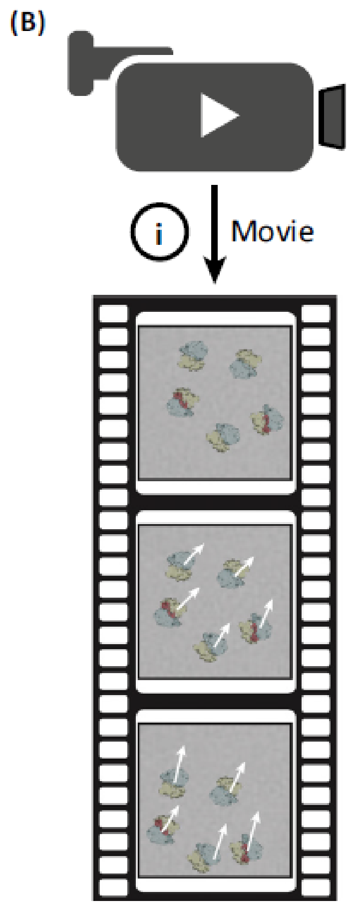
Film/CDD

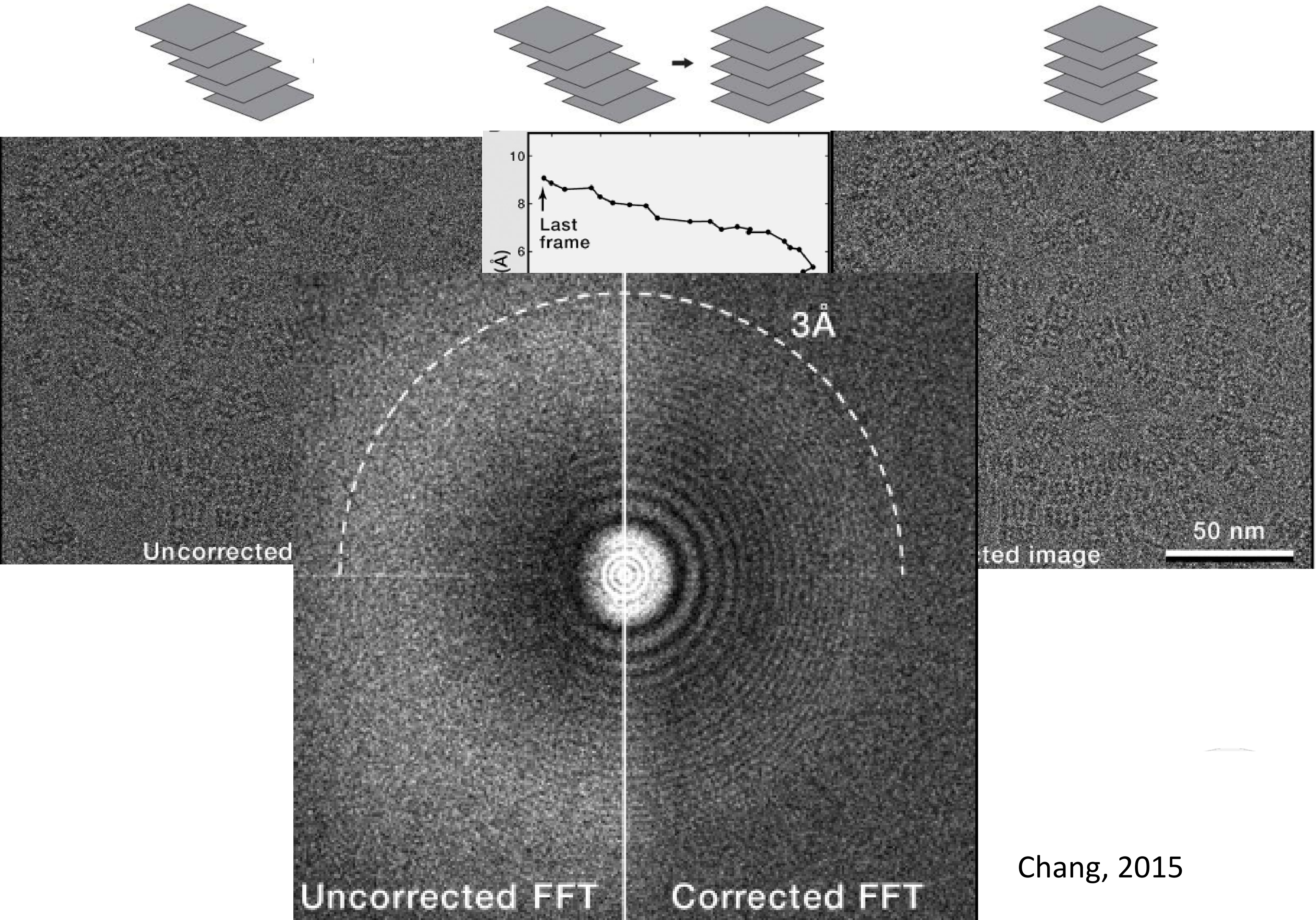
DED



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

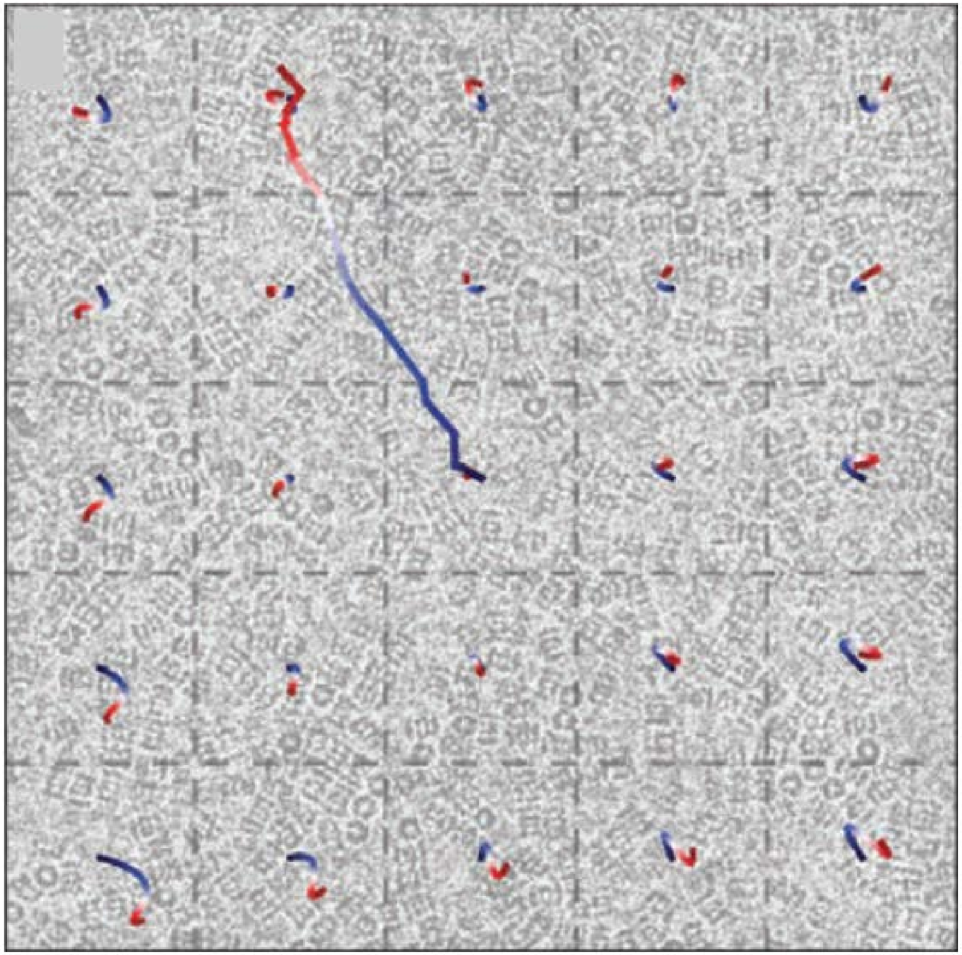
Advantage of Movie acquisition: Correction for mechanical motion



Chang, 2015

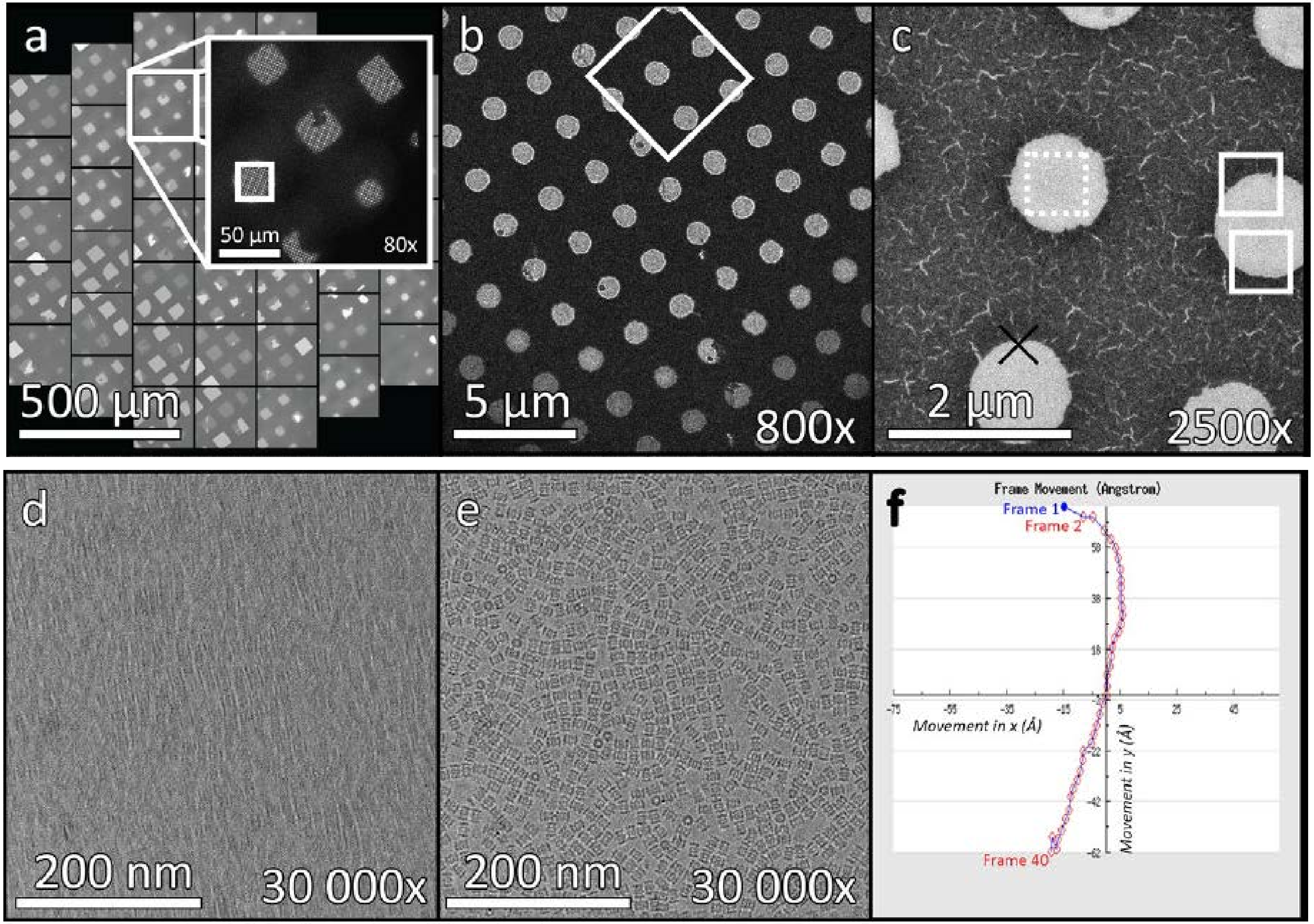
Correction for beam-induced motion

Cryospecimens exhibit beam-induced movement when irradiated at low electron doses



Zheng SQ, Palovcak E, Armache JP, Verba KA, Cheng YF, Agard DA. 2017. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat. Methods* 14:331–32

Automated CryoEM Data Collection Using Direct Detectors

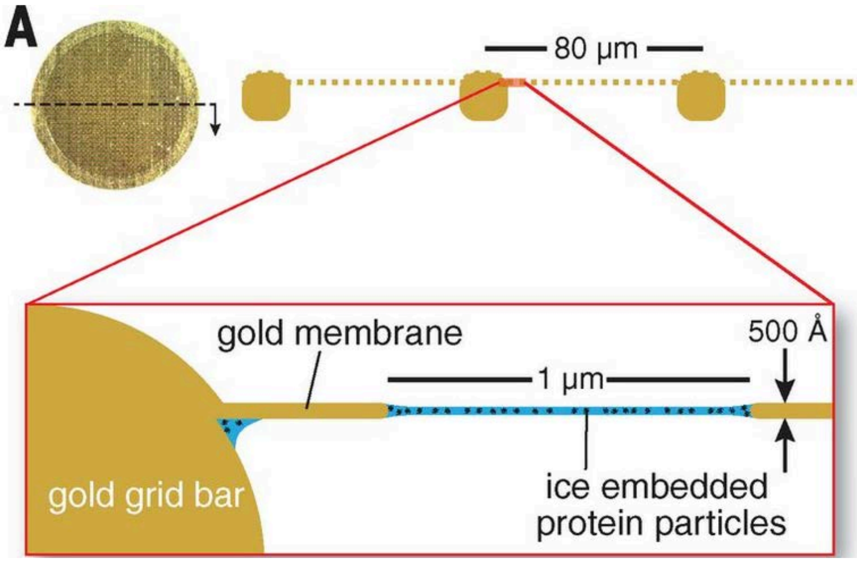


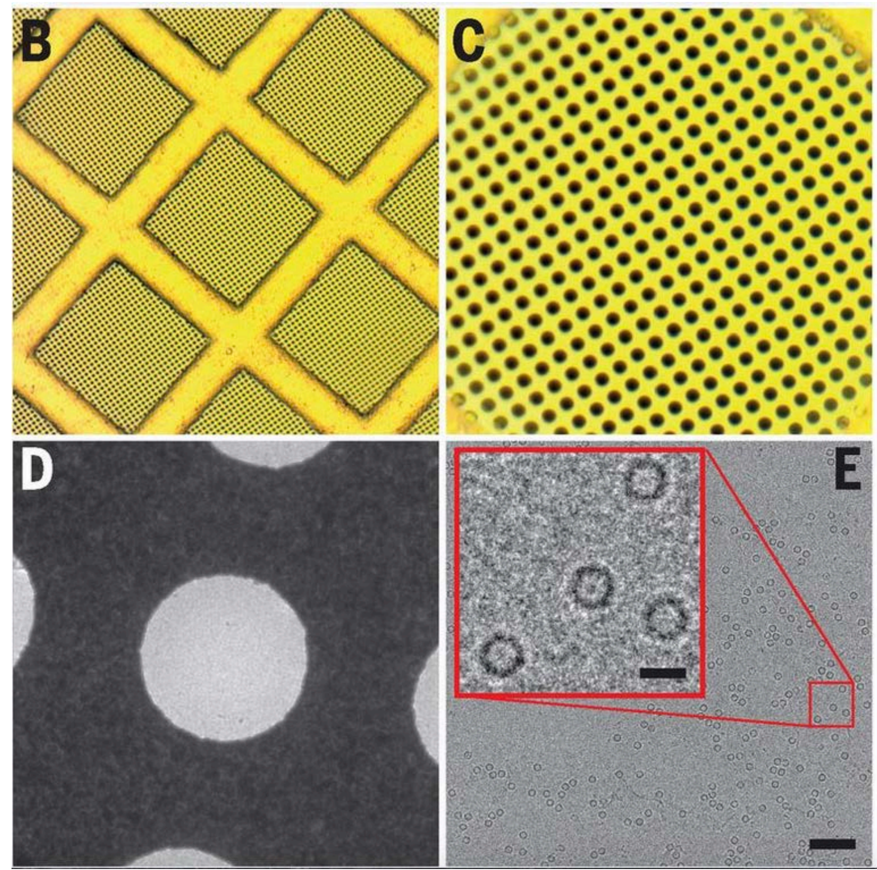
Chang, 2015

Improve the stability of the substate under electron beam

Use a gold grid

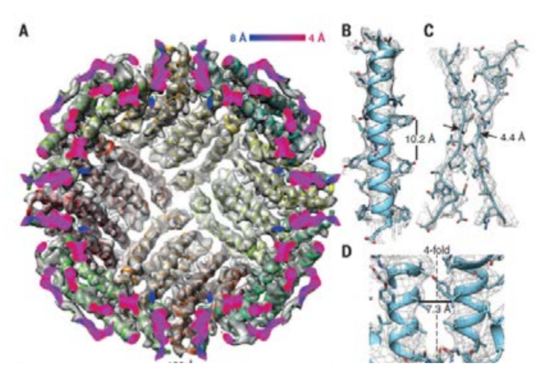
a gold specimen support
nearly eliminates substrate motion during irradiation





Russo & Passmore Science 2014

Compared with commercial am-C supports with nearly identical geometry, there was a 40-fold reduction movement. Apoferritin, 483 images , resolution 4.7 A.



ESTIMATING THE PHYSICAL LIMITS OF WHAT SINGLE-PARTICLE CRYO-EM CAN DO

Glaeser 2019

their molecular orientation can be determined with sufficient accuracy. With these points in mind, Henderson (34) estimated that the particle weights could be as low as about 40 kDa and that data need to be merged from only as few as 12,400 identical particles. Glaeser (24) proposed that these limits might actually be too conservative and that as few as 1,400 particles would be needed, while their molecular weights might be as low as 17 kDa. A subsequent analysis (56) reduced these

24. Glaeser RM. 1999. Review: electron crystallography: present excitement, a nod to the past, anticipating the future. *J. Struct. Biol.* 128:3–14

Improve Camera with a better DQE at high resolution
Improve image contrast (Volta phase plate)
Improve high-resolution information recovery