New EM Technologies:

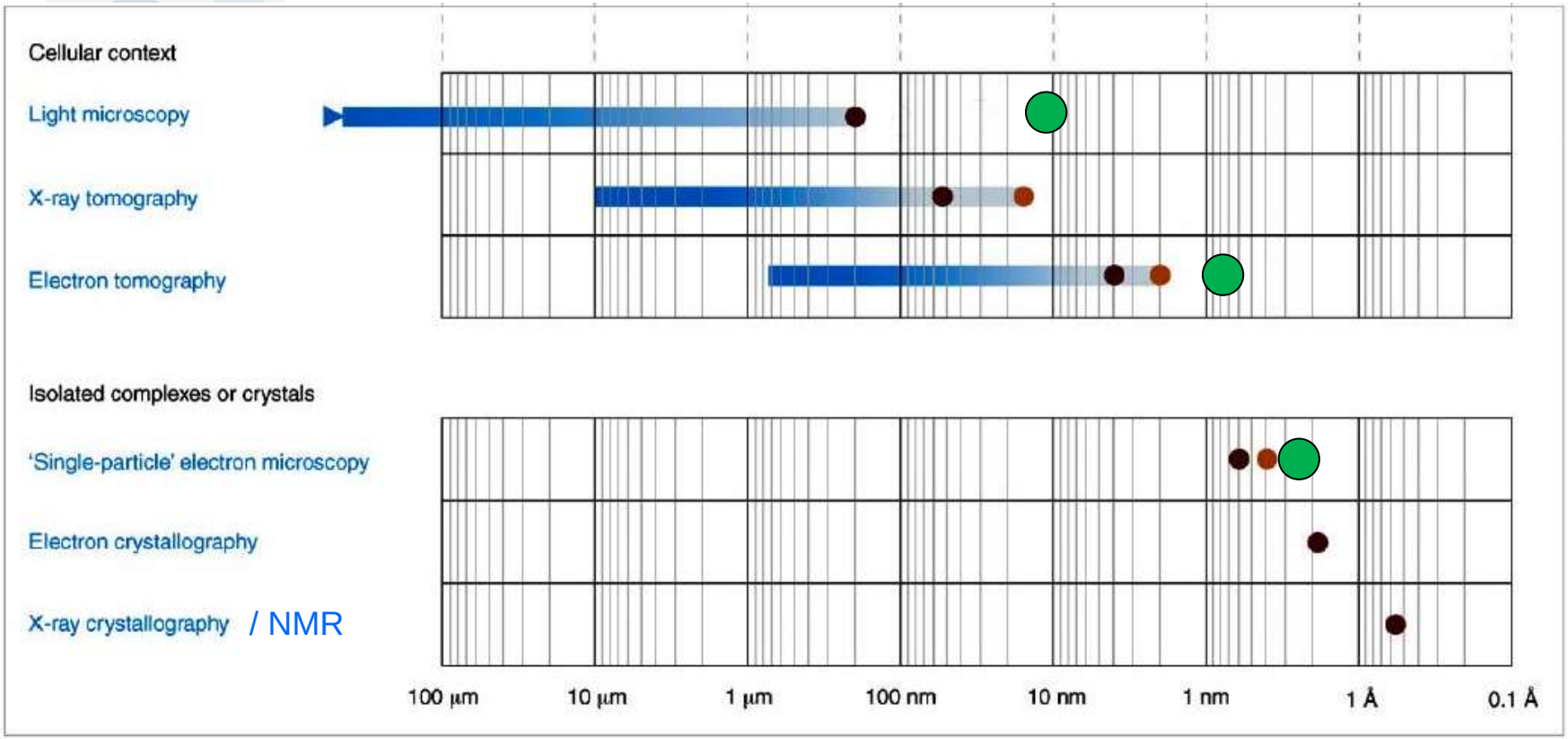
unveiling the wonders of nature at the molecular level.

CEITEC Winter School on Structural Cell Biology
February 9-13, 2015

Dr. Sacha De Carlo
Applications Lab
FEI Company - Eindhoven

The Never-Ending Quest for Higher Resolution in EM

Limits of Resolution of Various Imaging Technologies



- Currently achieved resolution
- Prediction for resolution improvement
- 2015

Leis, *et al.* (2009)

Current state-of-the-art

Review

Xiao-chen Bai, Greg McMullan & Sjors Scheres *Trends in Biochemical Sciences* January 2015, Vol. 40, No. 1

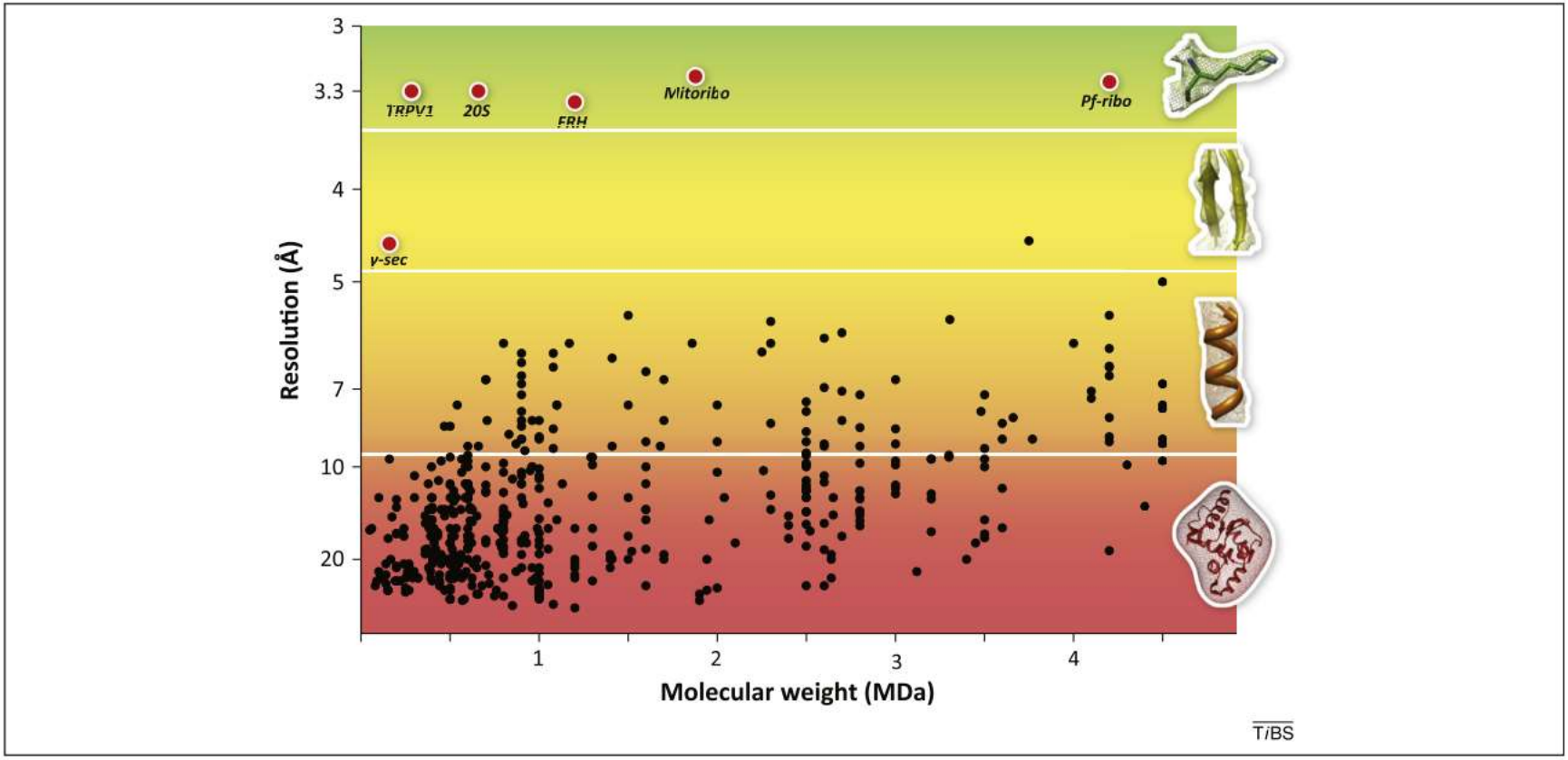
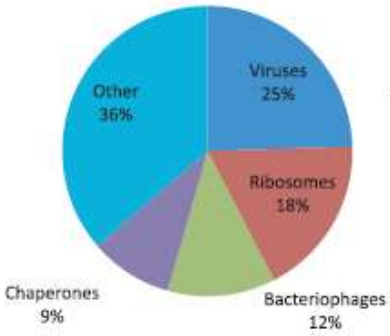


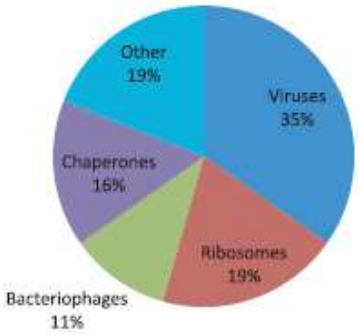
Figure 1. Revolutionary progress in cryo-electron microscopy (EM) single-particle analysis. The black dots represent single-particle cryo-EM structures that were released from the Electron Microscopy Data Bank (EMDB) between 2000 and 2012. The red dots are examples of recent progress in the field: γ-secretase (γ-sec), the transient receptor potential cation channel subfamily V member 1 (TRPV1), the 20S proteasome (20S), F420-reducing [NiFe] hydrogenase (FRH), the large subunit of the yeast mitochondrial ribosome (mitoribo), and the cytoplasmic ribosome of *Plasmodium falciparum* in complex with emetine (Pf-ribo). Whereas previously many structures only resolved protein domains (red area) or α helices (orange area), recent structures are detailed enough to distinguish β strands (yellow area) or even amino acid side-chains (green area).

2011-2012: challenges

Released "single particle" and "icosahedral" EMDB entries

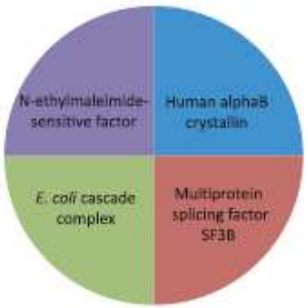


Total number of entries: 1146



Number of entries with resolution better than 10 Å: 285

2015: >700



Categories of proteins with MW < 500 kDa with structure derived by single particle cryo-EM at resolutions better than 10 Å : 4

2015: 35

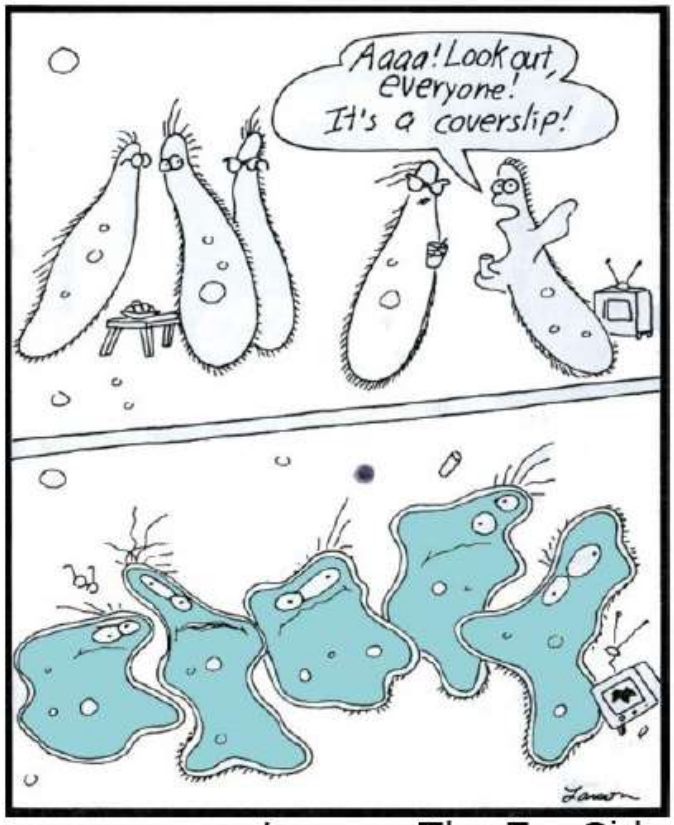
"Smaller (<500kDa), potentially dynamic, protein complexes are at the heart of cell function, and understanding how they work will generate fundamental insights that will be key to the development of biomedical therapeutics in the coming decades."

Milne *et al.* (2012) Cryo-EM: a primer for the non-microscopist. *FEBS Journal* **280**

FEI™

Sample Limitations

- Sample heterogeneity/stability
  - biochemistry
  - new algorithms
- Transient complexes
  - affinity grids, streptavidin crystals
- Detergent and lipid
  - amphipol, GLC/GDN
  - amphiphilic β-strand peptides
- Low molecular weight



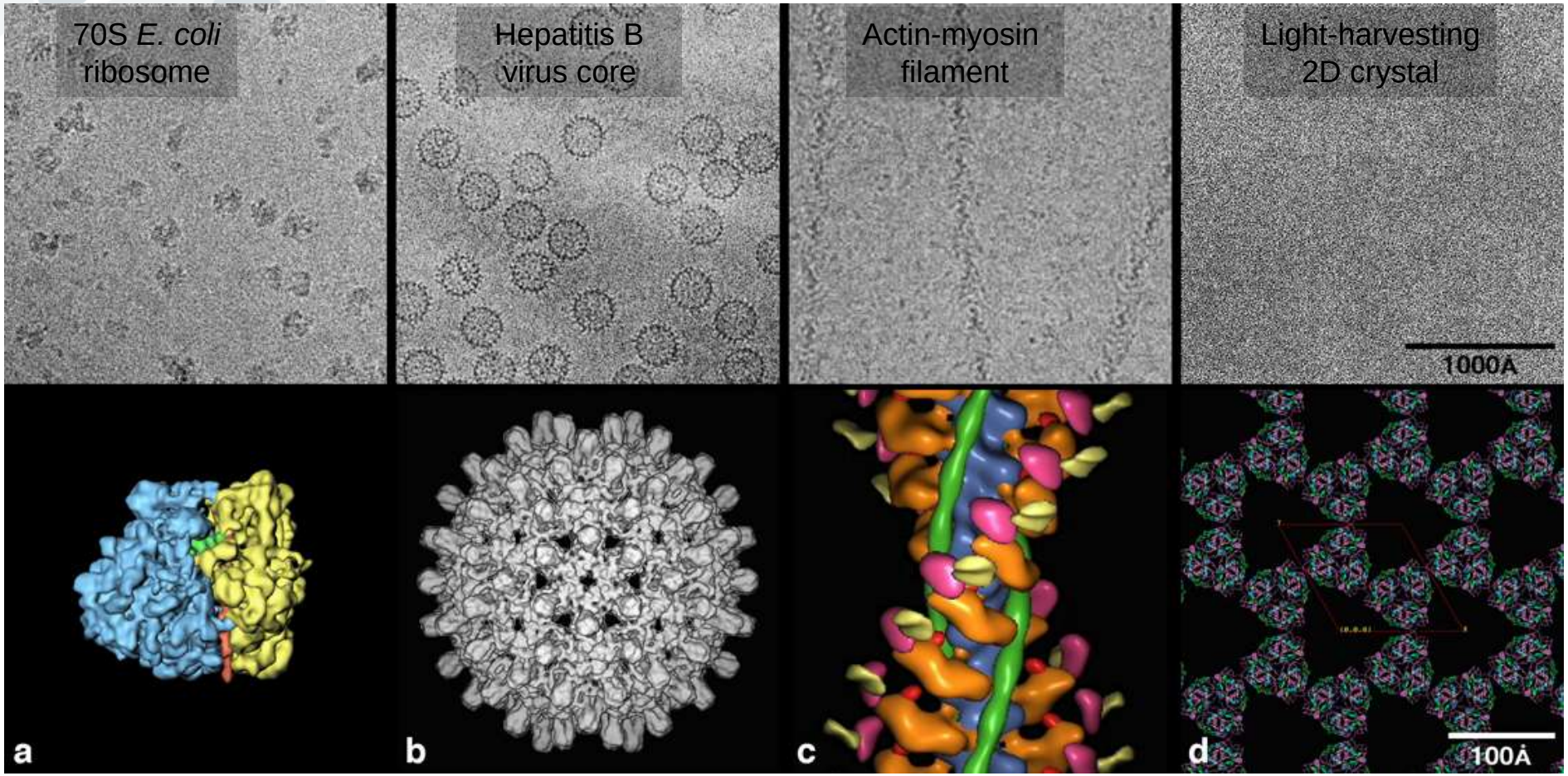
Larson, The Far Side

State-of-the-Art

Current and future challenges

Sample size, quality *(bottleneck)*
Beam Damage *(dose fractionation)*
Better Detectors *(fast, counting)*
Better Hardware *(PP, aberrations)*

Types of Samples Studied by Cryo-EM



Single particles with little or no symmetry

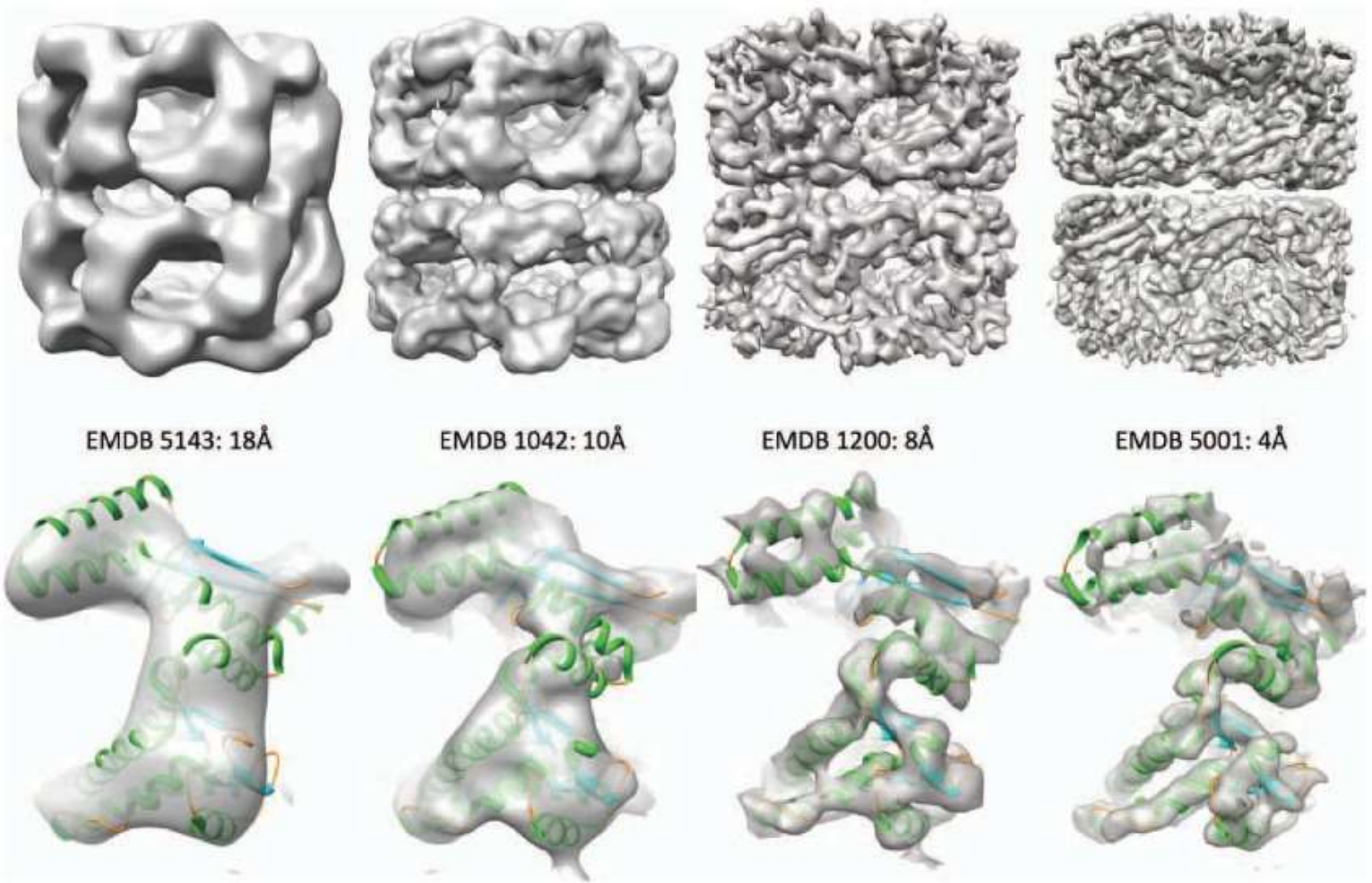
Single particles with icosahedral or other symmetries

Helical symmetry

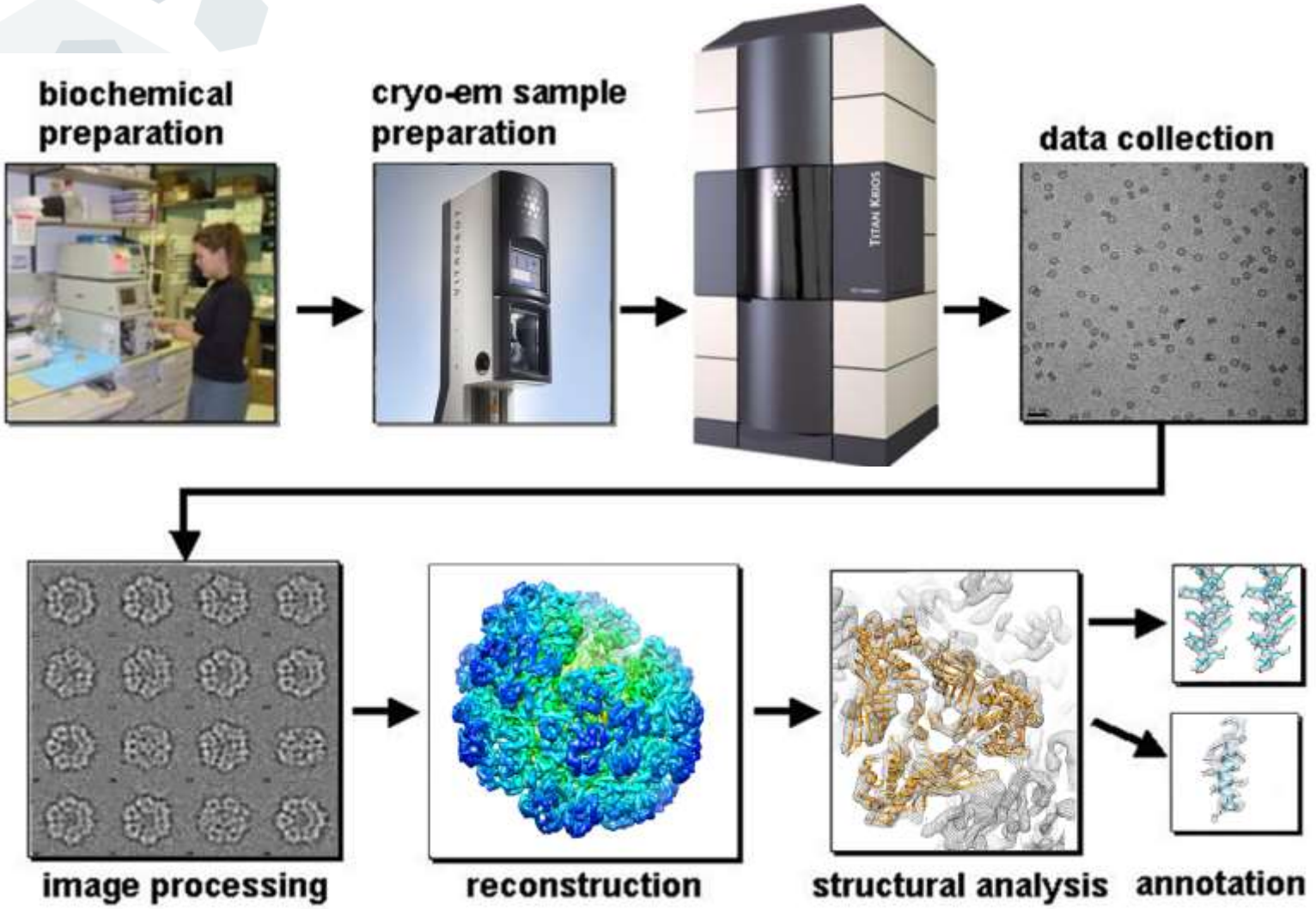
Two-dimensional crystals

Baker and Henderson (2001)

Sample Types



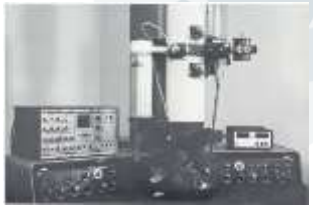
From Sample to Structure (SPA workflow)



Slide adapted from De Carlo & Rémigy, Biophysics in drug discovery (April 2015)

Automation = High Throughput

Single particle analysis (SPA)

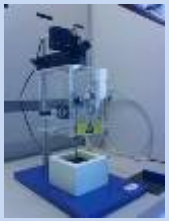


FEG

Krios

Manual

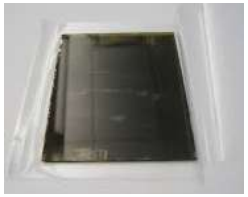
70’s



Automation

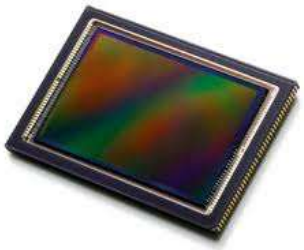
10’s

Film

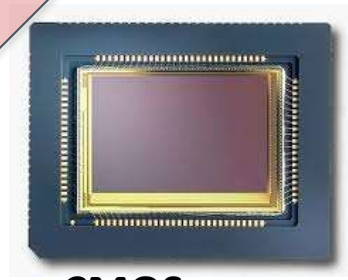


scanner

Digital CCD



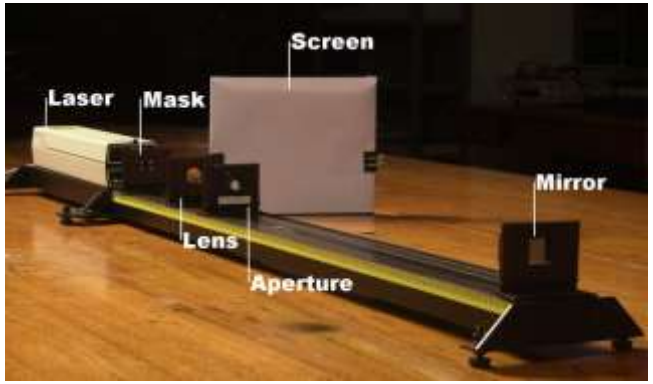
CMOS

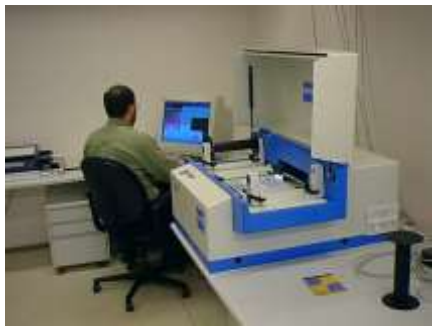


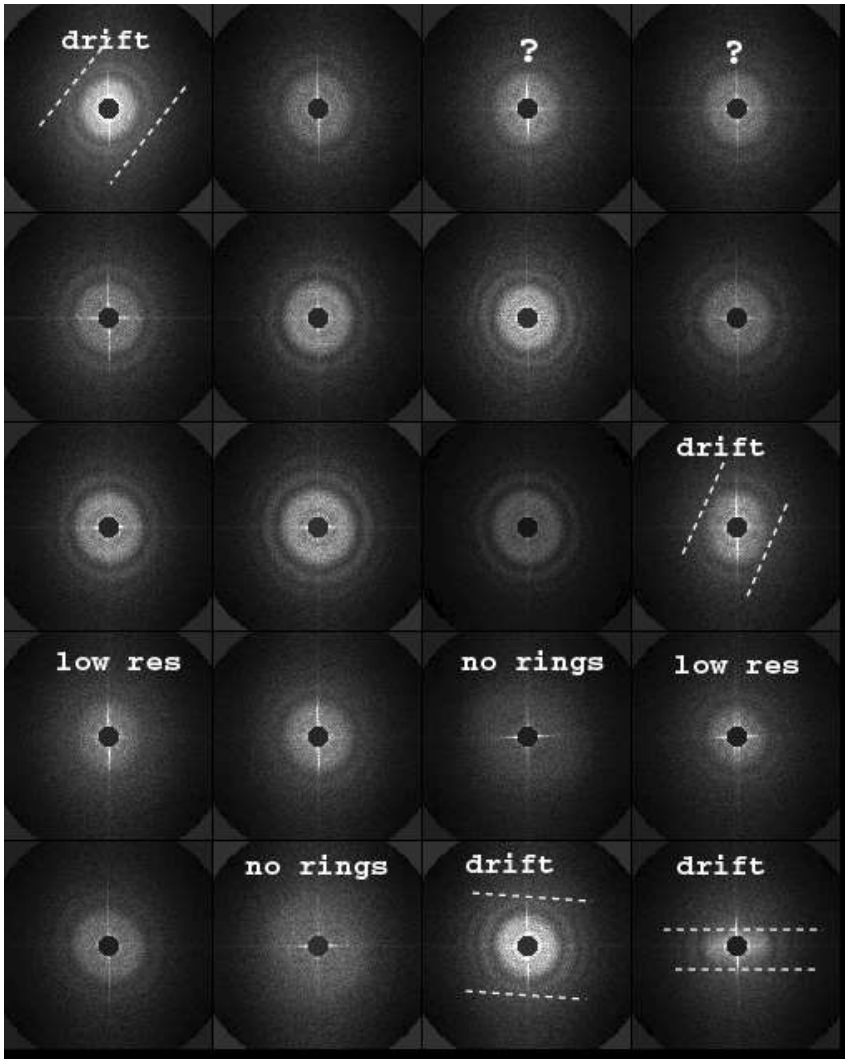
Explore. Discover. Resolve.

FEI™

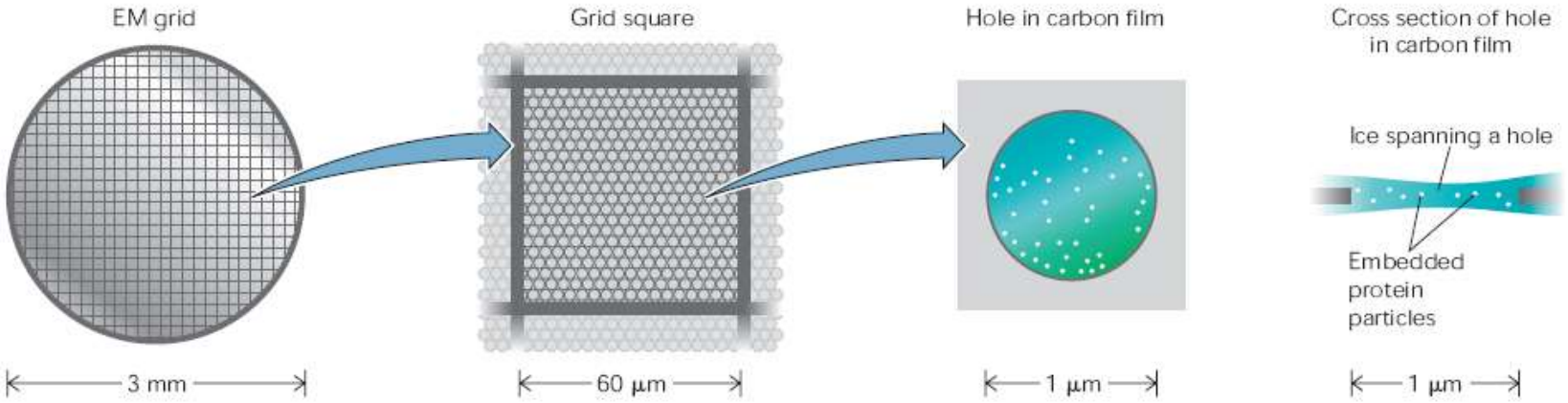
Automation -> electronic detectors



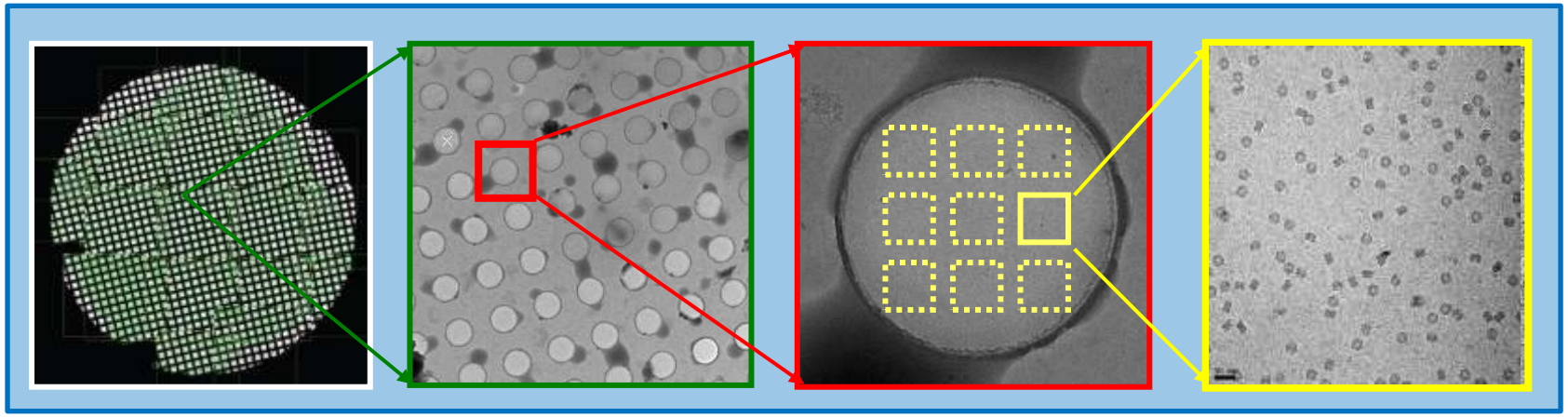




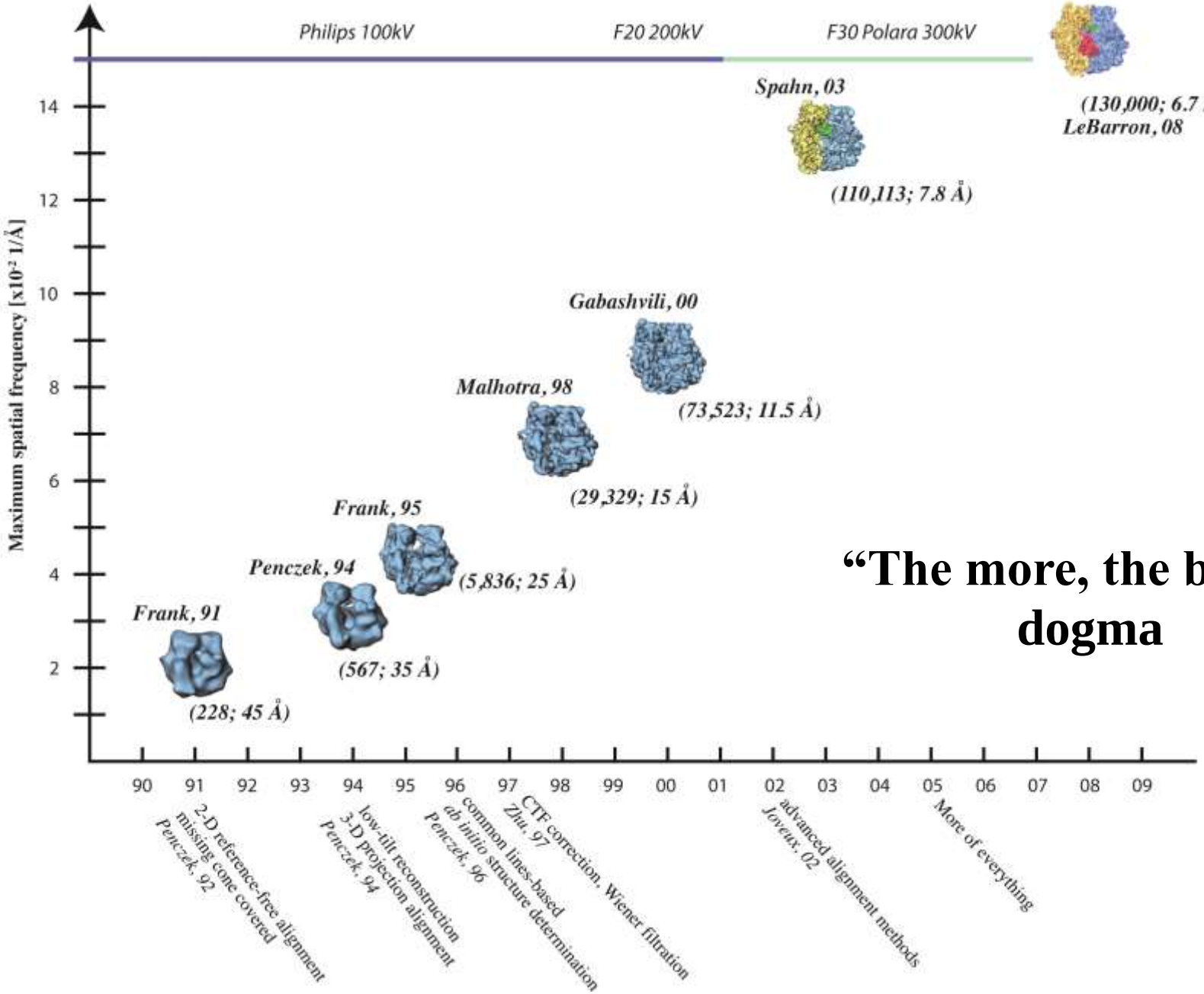
FEI™



Automated image acquisition software – FEI EPU™



Potter *et al.* (1999). Leginon: … 1000 images a day. *Ultramicroscopy* **77**, 153-161



?

FEI Titan Krios
2,504,547; ?? Å

“The more, the better” dogma

FEI™

Direct electron detectors

The Resolution Revolution

BIOCHEMISTRY

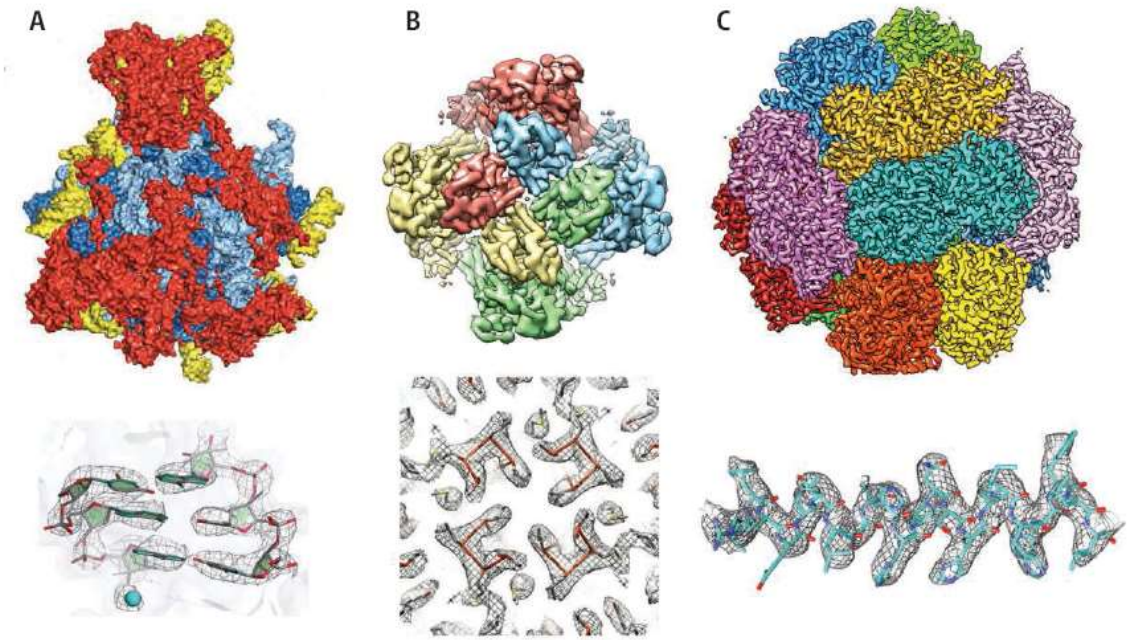
The Resolution Revolution

Advances in detector technology and image processing are yielding high-resolution electron cryo-microscopy structures of biomolecules.

Werner Kühlbrandt

Precise knowledge of the structure of macromolecules in the cell is essential for understanding how they function. Structures of large macromolecules can now be obtained at near-atomic resolution by averaging thousands of electron microscope images recorded before radiation damage accumulates. This is what Amunts *et al*. have done in their research article on page 1485 of this issue (*1*), reporting the structure of the large subunit of the mitochondrial ribosome at 3.2 Å resolution by electron cryo-microscopy (cryo-EM). Together with other recent high-resolution cryo-EM structures (*2*–*4*) (see the figure), this achievement heralds the beginning of a new era in molecular biology, where structures at near-atomic resolution are no longer the prerogative of x-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy.

Ribosomes are ancient, massive protein-RNA complexes that translate the linear genetic code into three-dimensional proteins. Mitochondria—semi-autonomous organelles



Near-atomic resolution with cryo-EM. (**A**) The large subunit of the yeast mitochondrial ribosome at 3.2 Å reported by Amunts *et al*. In the detailed view below, the base pairs of an RNA double helix and a magnesium ion (blue) are clearly resolved. (**B**) TRPV1 ion channel at 3.4 Å (*2*), with a detailed view of residues lining the ion pore on the four-fold axis of the tetrameric channel. (**C**) F_{420}-reducing [NiFe] hydrogenase at 3.36 Å (*3*). The detail shows an α helix in the FrhA subunit with resolved side chains. The maps are not drawn to scale.

Science **343** (2014)

BIOCHEMISTRY

The Resolution Revolution

Advances in detector technology and image processing are yielding high-resolution electron cryo-microscopy structures of biomolecules.

Pieces of the puzzle. These individual proteins form part of the yeast mitochondrial large ribosomal subunit (*9*). Amunts *et al.* built the proteins one by one using maps derived from cryo-EM images of large ribosomal subunits and intact ribosomes. The results, once combined and refined further, are equivalent to an atomic model from x-ray crystallography.

STRUCTURAL BIOLOGY

Beyond blob-ology

Advanced cryo–electron microscopy yields high-resolution structures of proteins

By **Martin T. J. Smith**[1] *and* **John L. Rubinstein**[1,2,3]

Structural biology aims to obtain high-resolution snapshots of the macromolecules that make up living cells. Most have come from x-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy. However, x-ray crystallography is limited by the ability to grow well-ordered three-dimensional (3D) crystals, whereas NMR spectroscopy requires highly concentrated samples with nonoverlapping spectral peaks. Many macromolecular assemblies are too large, scarce, or unstable for analysis by these techniques. Single-particle electron microscopy (EM) offers an alternative, allowing high-resolution structures to be determined from small quantities of noncrystalline material.

In single-particle EM, images are obtained from preparations of randomly oriented molecular complexes. Individual assemblies (particles) are selected from each image and their orientations determined computationally. A 3D map of the particle is calculated by combining information from images showing different views. However, the highly energetic electron beam destroys biological molecules, breaking covalent bonds and creating a cascade of damaging chemical reactions. This radiation damage places strict limitations on the electron exposure that can be used to record images.

In the 1980s, Dubochet *et al.* reported the innovation that set single-particle EM on the path to high-resolution structure determination. They prepared specimens in a thin layer of vitreous ice for imaging under cryogenic conditions (*1*). This method, known as

CREDITS: FIRST FIGURE, ALEXEY AMUNTS AND ALAN BROWN/MRC-LMB, CAMBRIDGE

Science **343** (2014)

Explore. Discover. Resolve.

FEI™

Review

CellPress

How cryo-EM is revolutionizing structural biology

Xiao-chen Bai, Greg McMullan, and Sjors H.W Scheres

MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge Biomedical Campus, Cambridge, CB2 0QH, UK

For many years, structure determination of biological macromolecules by cryo-electron microscopy (cryo-EM) was limited to large complexes or low-resolution models. With recent advances in electron detection and image processing, the resolution by cryo-EM is now beginning to rival X-ray crystallography. A new generation of electron detectors record images with unprecedented quality, while new image-processing tools correct for sample movements and classify images according to different structural states. Combined, these advances yield density maps with sufficient detail to deduce the atomic structure for a range of specimens.

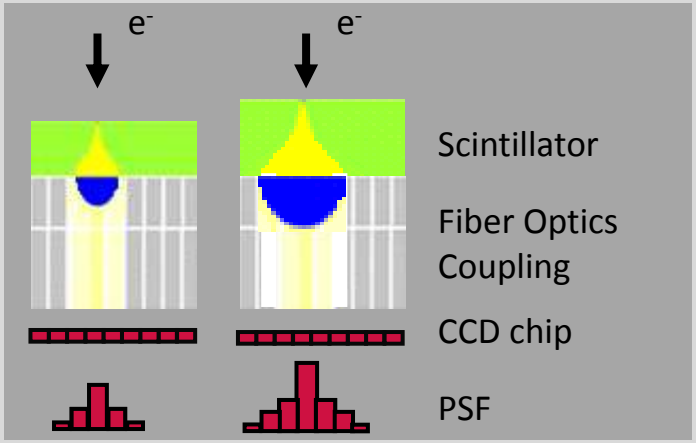
by air molecules, EM requires a high vacuum in the beam path, which compromises preservation of liquid aqueous samples. Even more importantly, biological macromolecules are susceptible to radiation damage through the breakage of chemical bonds by energy that is deposited in the sample by the electron beam.

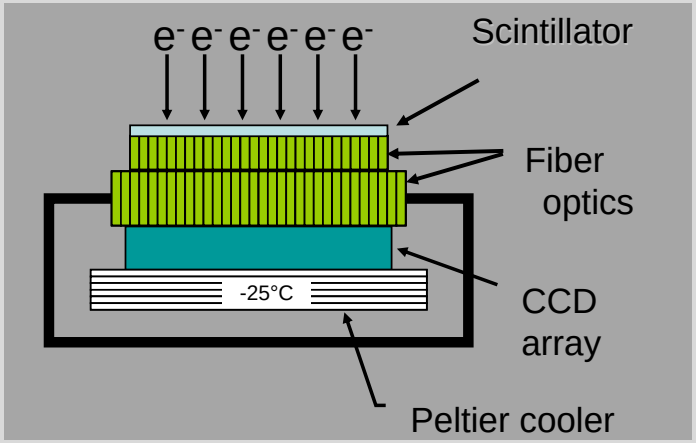
Initial EM studies of biological samples used dehydrated samples or fixation techniques, many of which introduced artifacts in the structures. In particular, negative staining [3], where the water that surrounds the macromolecules is replaced by a dried solution of heavy-metal salt, became popular during the early 1960s and

Improving detection: “seeing electrons”

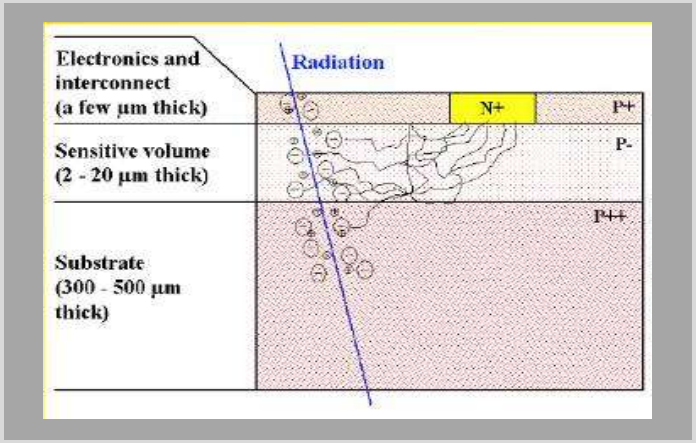
Improved Detective Quantum Efficiency (DQE)

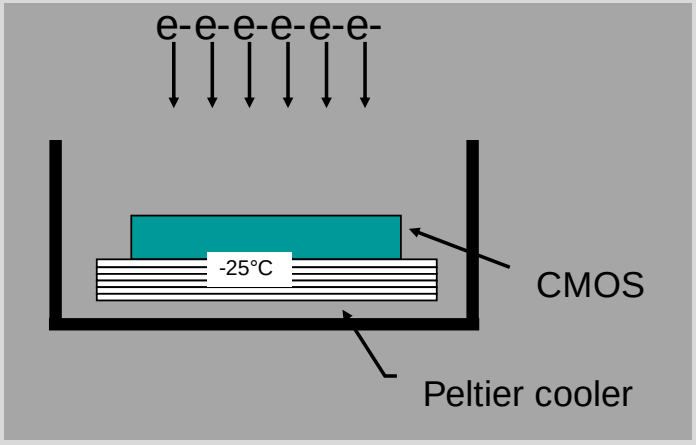
CCD: multi stage conversion of electron energy via fiber or lens optics





CMOS: direct conversion of electron energy without fiber or lens optics



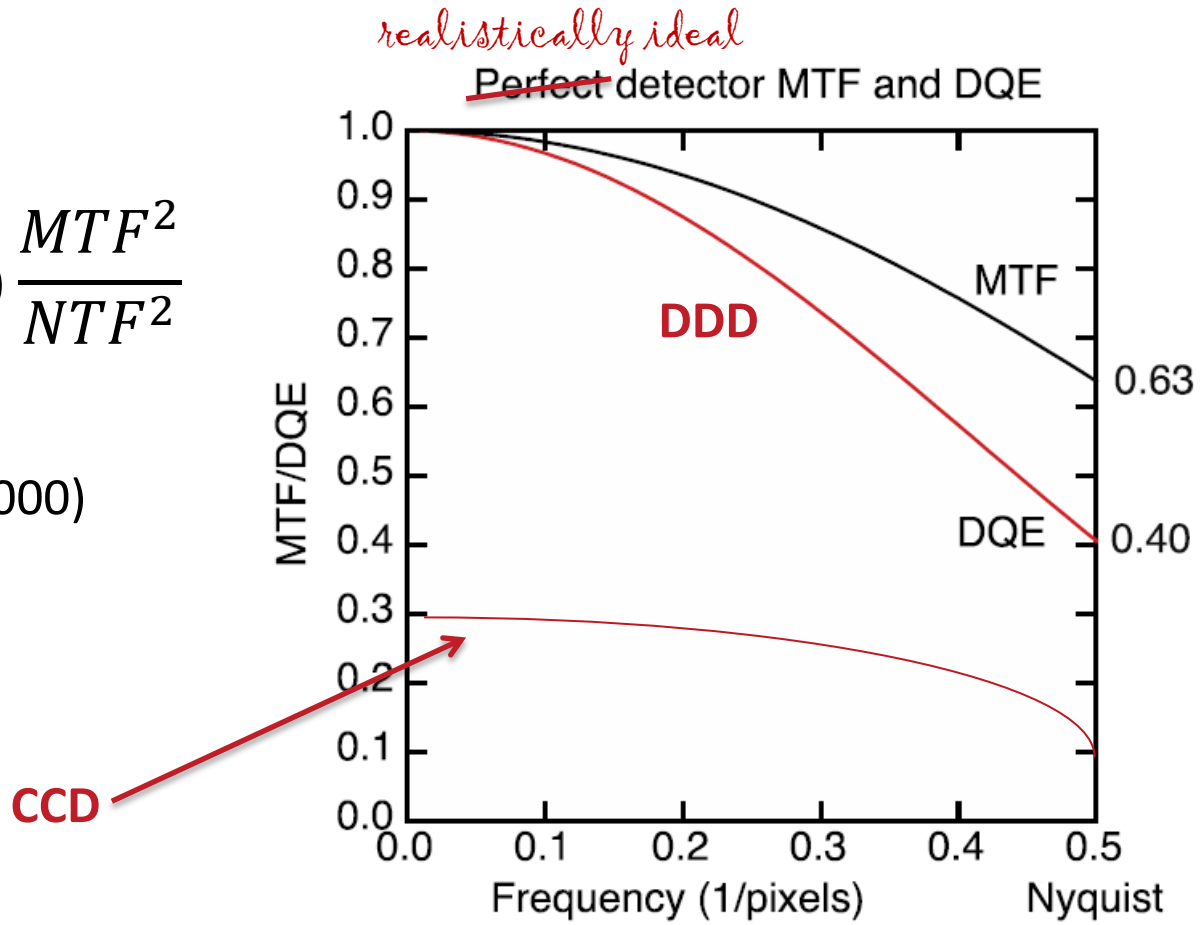


FEI™

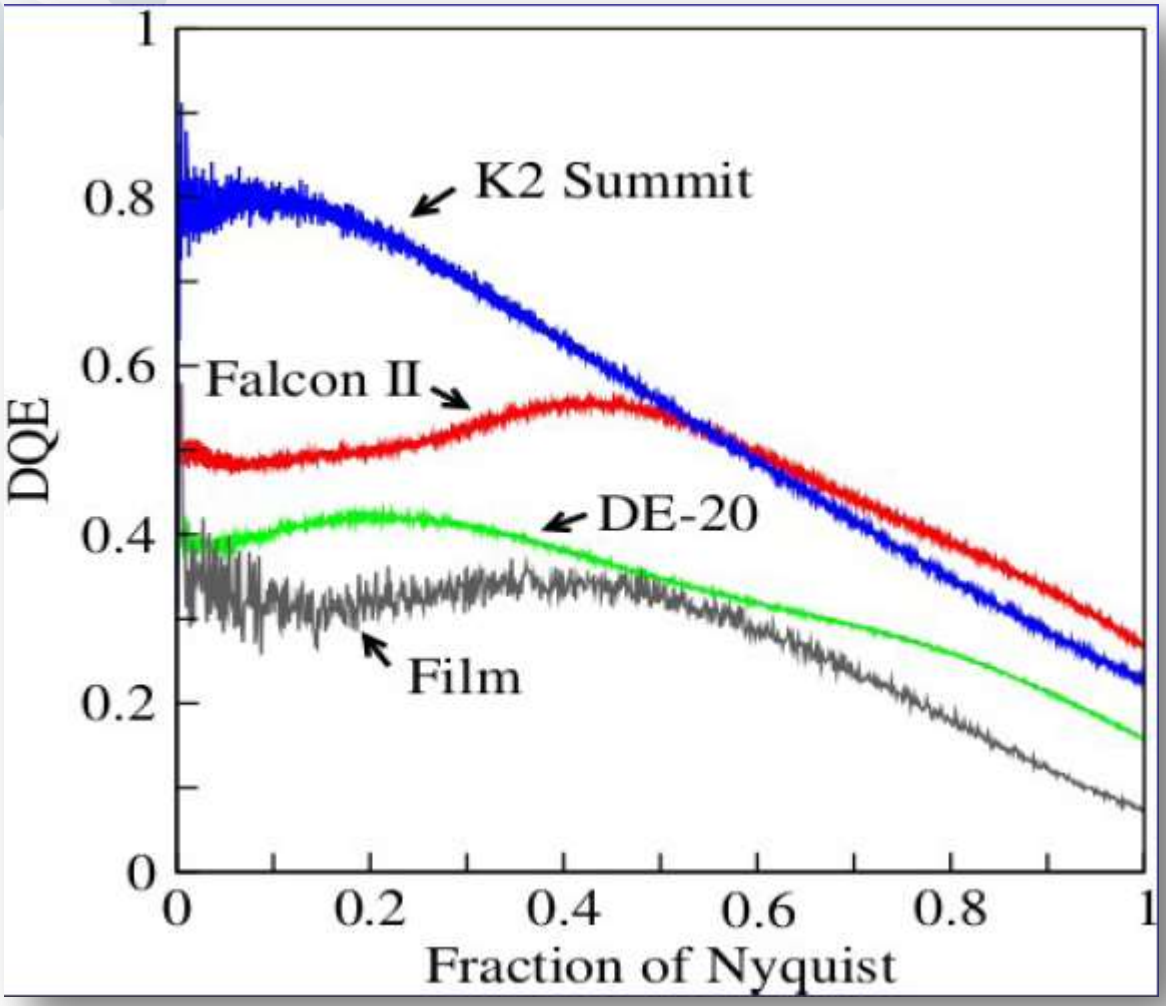
Assessing the quality of a detector

$$DQE(k) = DQE(0)\frac{MTF^2}{NTF^2}$$

Meyer & Kirkland (2000)
De Ruijter (1995)



DQE comparison for various detectors at 300kV

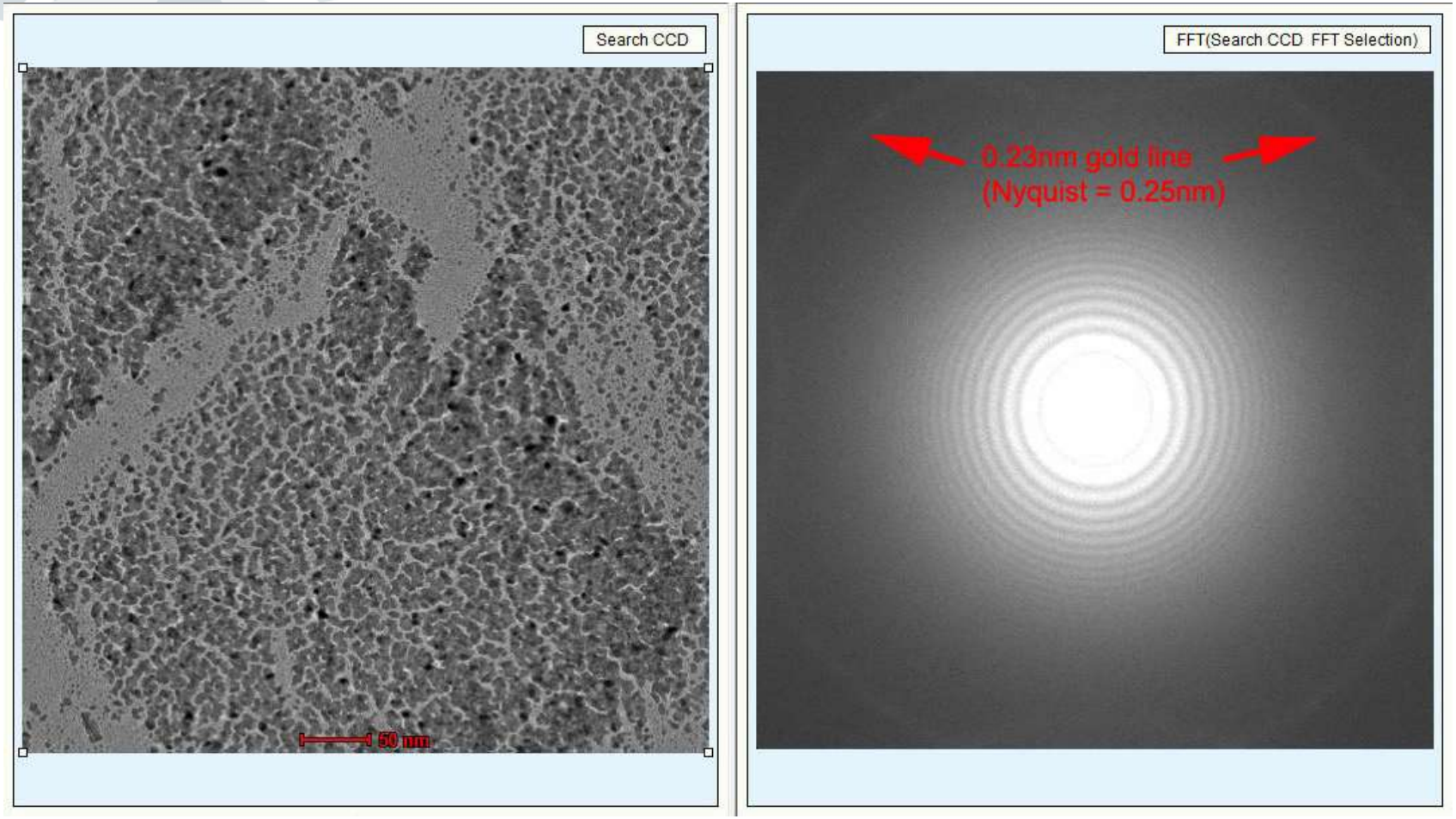


McMullan *et al.* (2014) *Ultramicroscopy* **147**, 156-163

Assessing detector performance

Comparison tests with Gatan US-4000 CCD mounted on the F20; same FOV and same illumination conditions

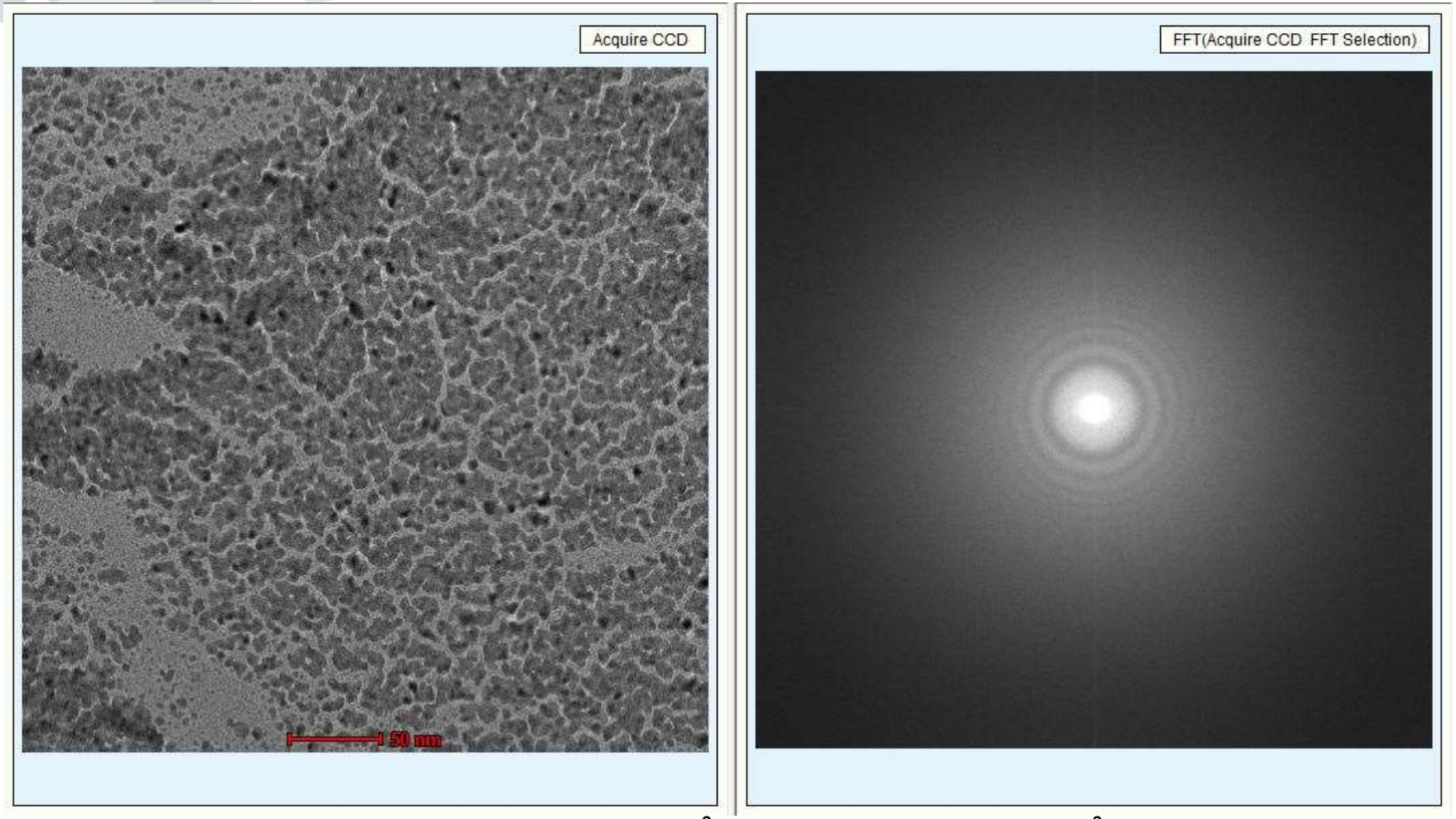
Cross-grating gold replica: Falcon2



80kX nominal mag (1.25Å pix size); Nyquist = 2.5Å

2.3Å gold reflection line seen past Nyquist !!

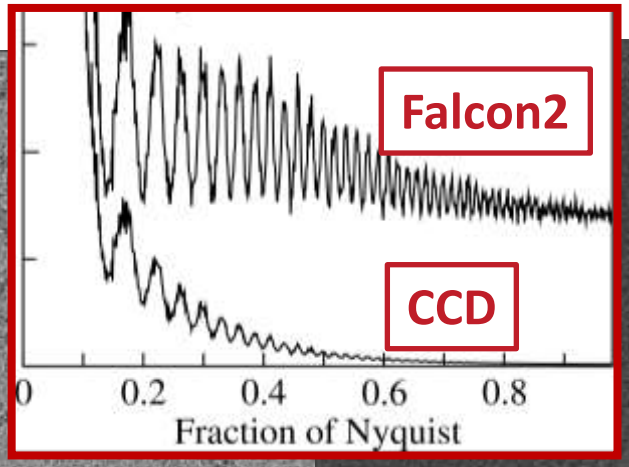
Cross-grating gold replica: Gatan CCD



100kX nominal mag (0.9 Å pix size); Nyquist = 1.8Å

Nothing visible besides a few Thon rings in the center

Ribosome (Krios, 300kV, Falcon2)



Thon rings visible to 4/5th Nyquist [1/3.1Å]

Movie mode

(aka dose fractionation)

Movie processing (Motioncorr, Relion, …)

Review

Trends in Biochemical Sciences January 2015, Vol. 40, No. 1

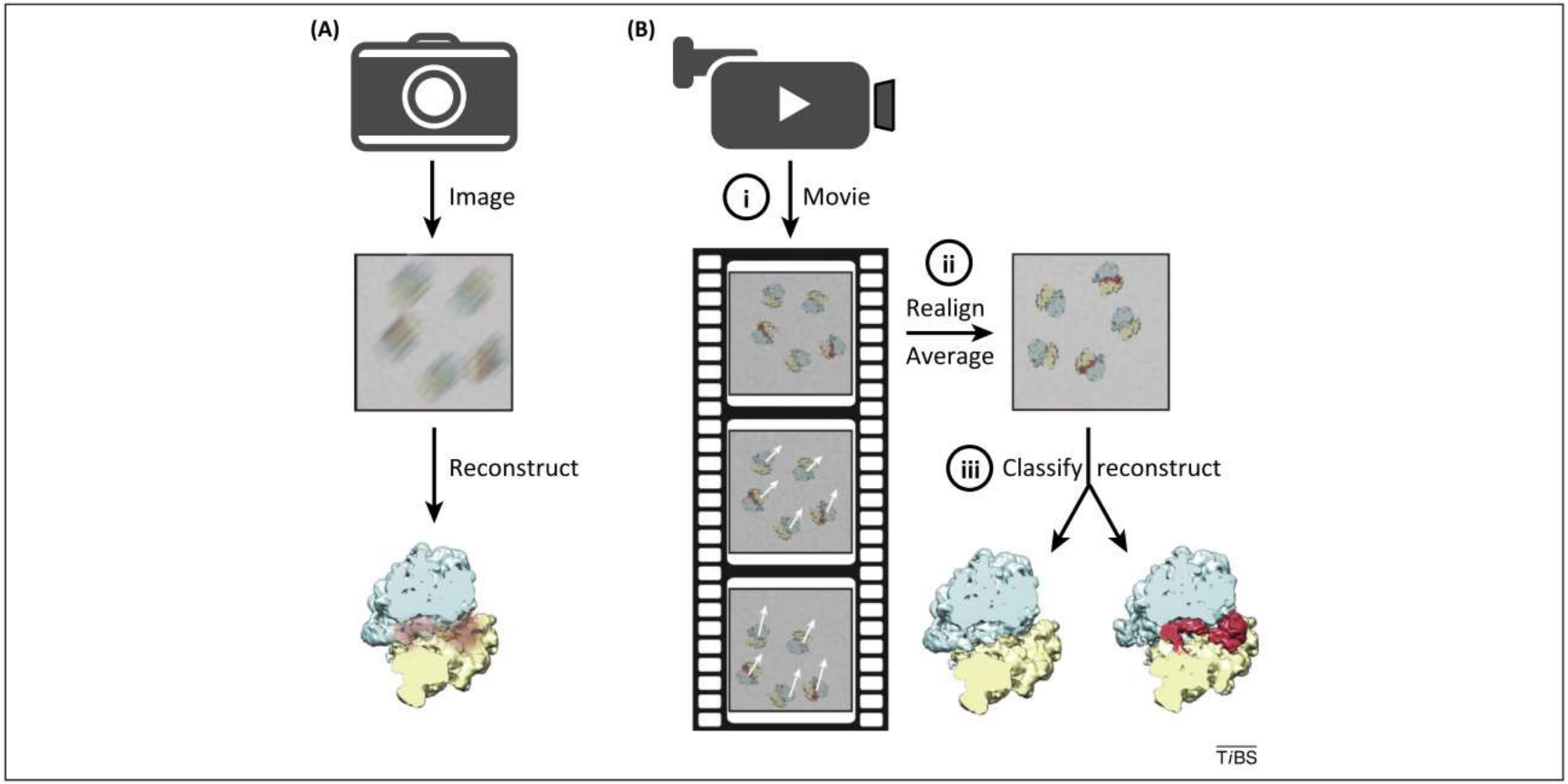
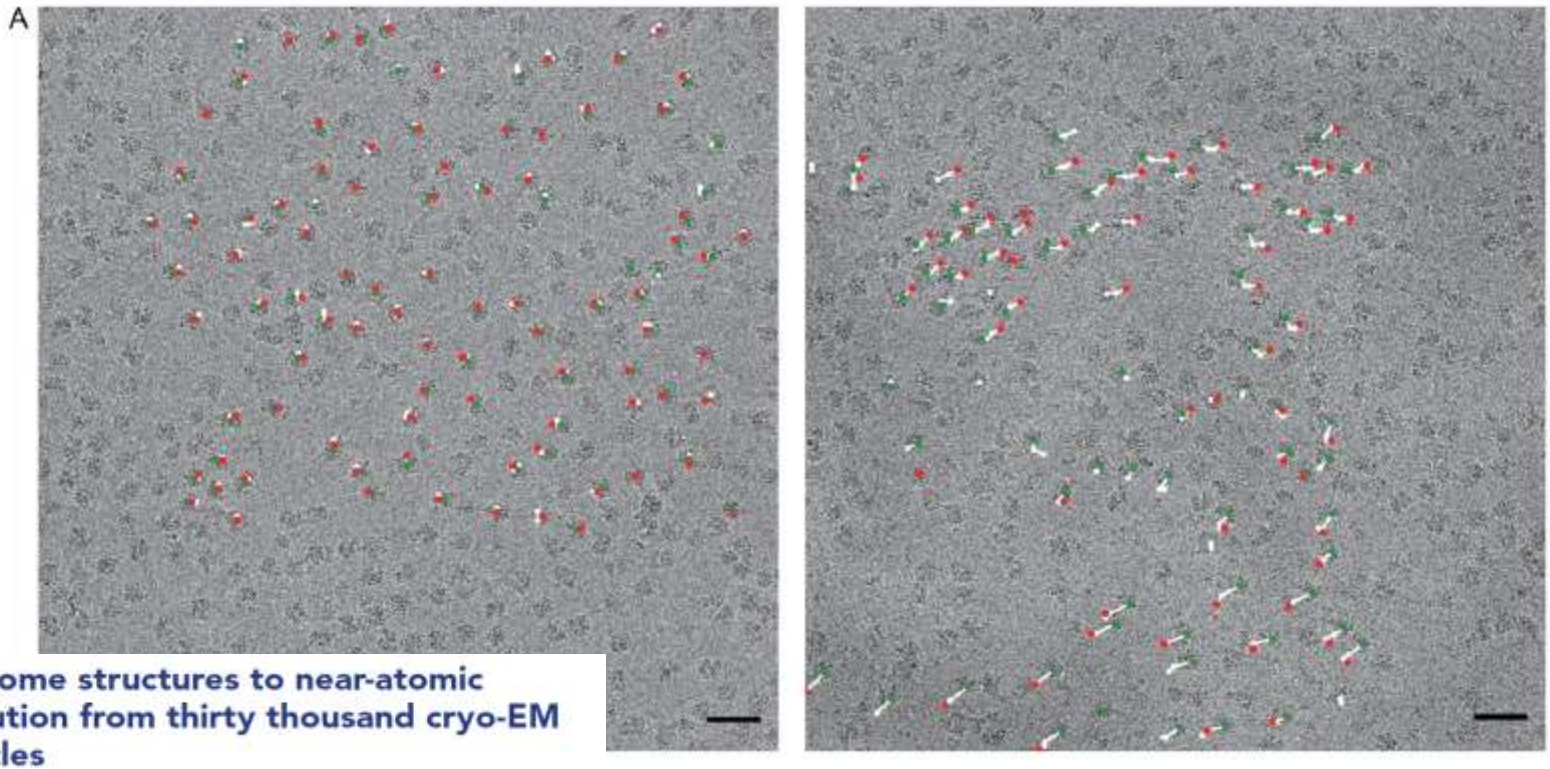


Figure 2. Recent technological advances. **(A)** Previously, noisier images were recorded on photographic film, beam-induced sample motion led to image blurring, and structurally different particles were often mixed in a single reconstruction. **(B)** Three recent advances yield better reconstructions: (i) digital direct-electron detectors yield data of unprecedented quality and allow recording movies during exposure; (ii) computer programs to realign the movie frames may correct for sample movements that are induced by the electron beam; and (iii) powerful classification methods lead to multiple structures from a sample mixture.

Motion correction: “movie mode”

Beam-induced movements



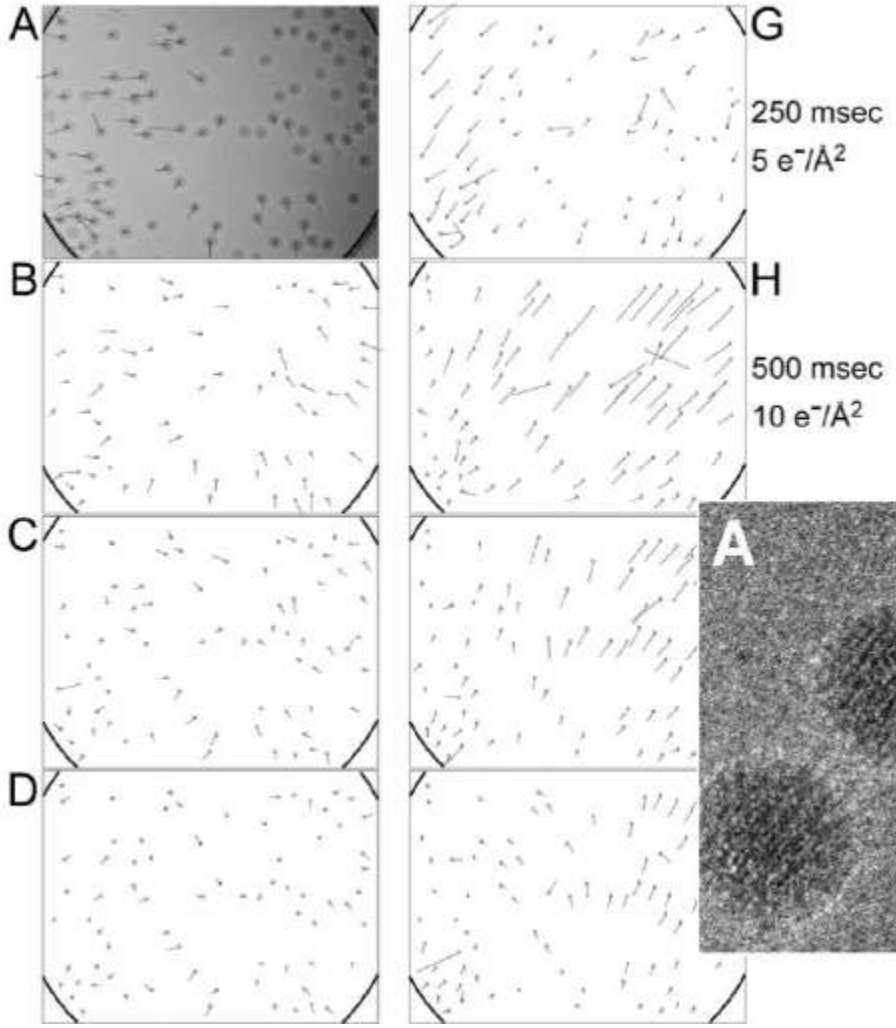
Ribosome structures to near-atomic resolution from thirty thousand cryo-EM particles

Xiao-chen Bai, Israel S Fernandez, Greg McMullan, Sjors HW Scheres*

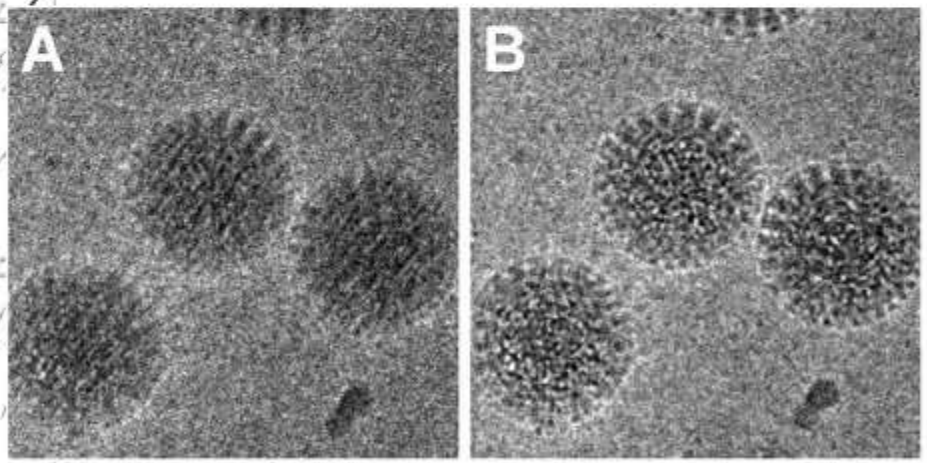
Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom

Bai *et al* (2013). eLife **2**

Motion correction: Rotavirus particles



Brilot, … , Potter, Carragher, … , Grigorieff (2012) *J.Struct.Biol.*



DED Cameras – Gatan K2

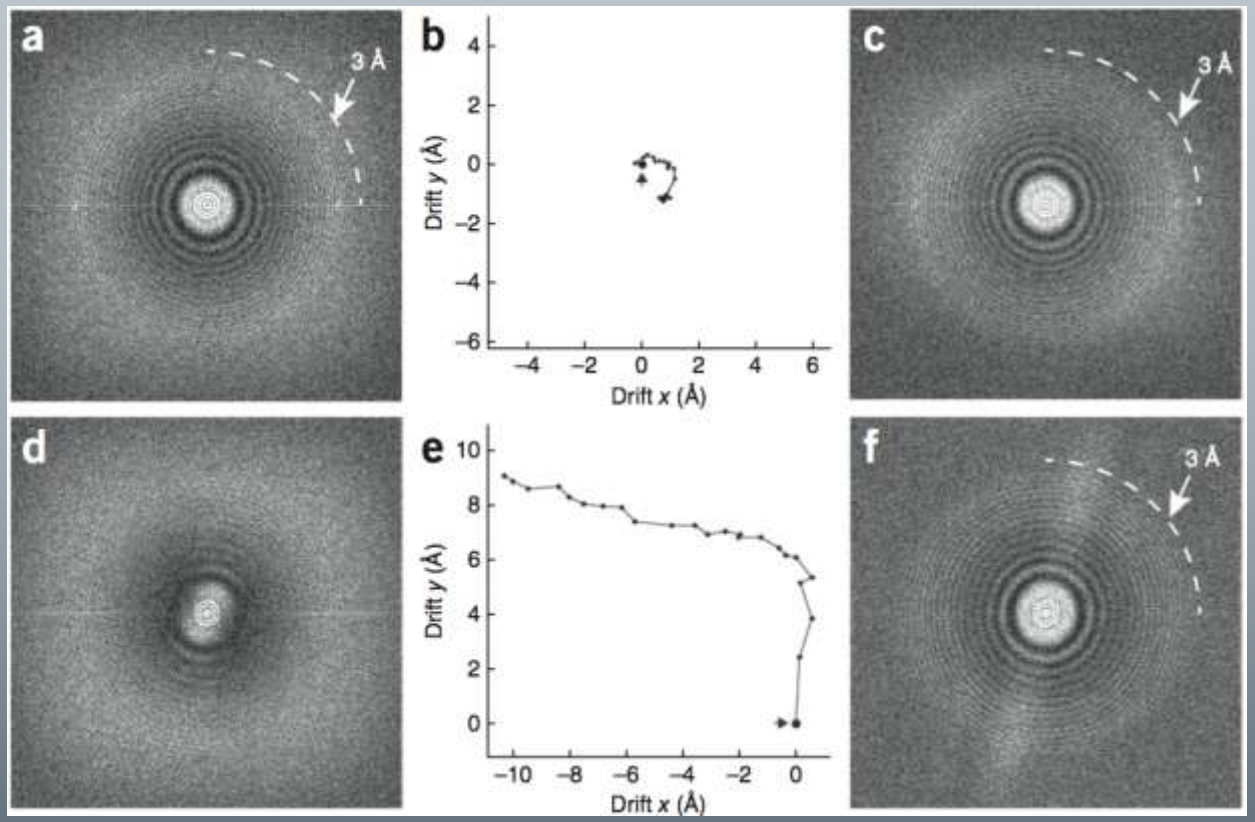
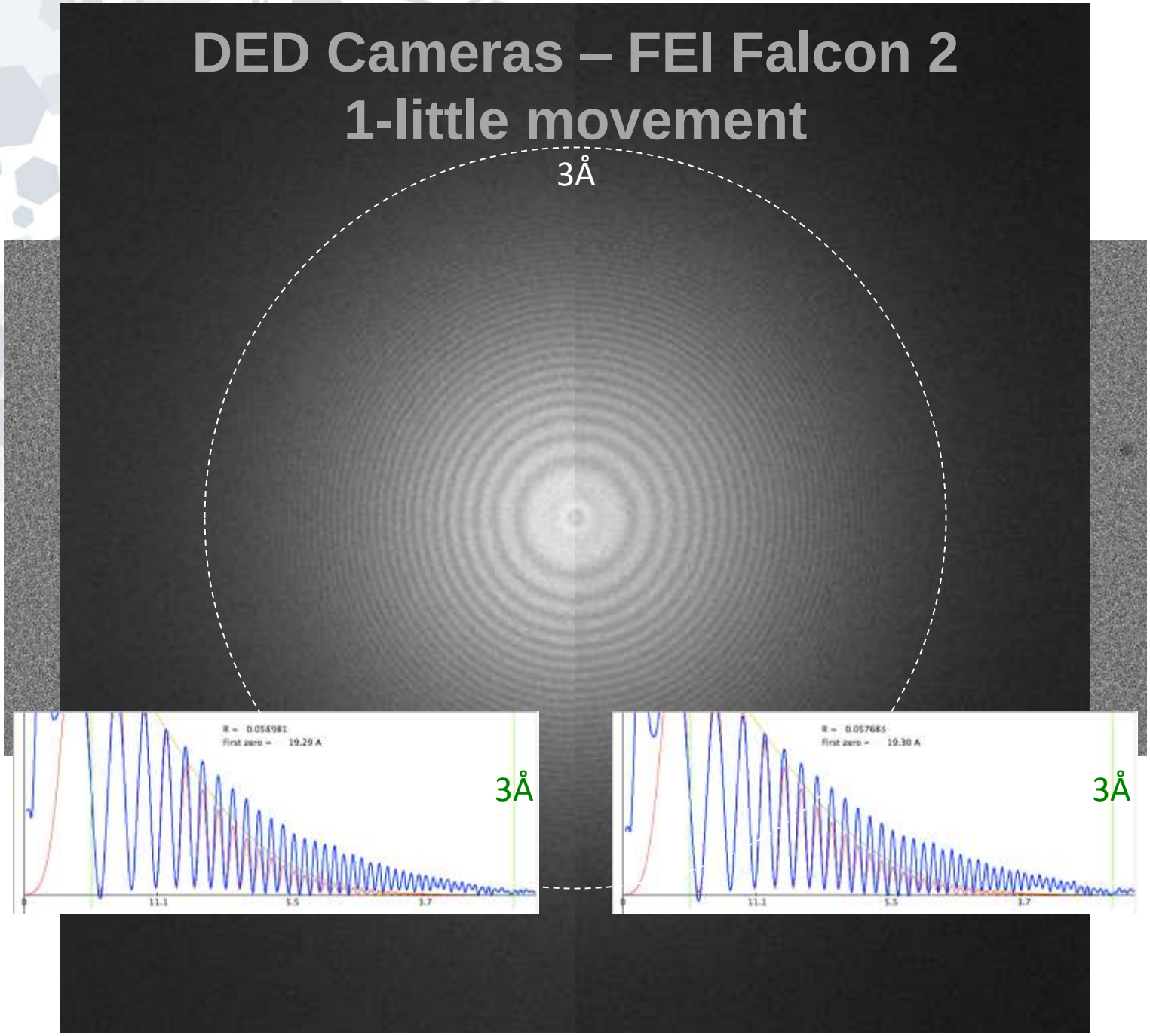


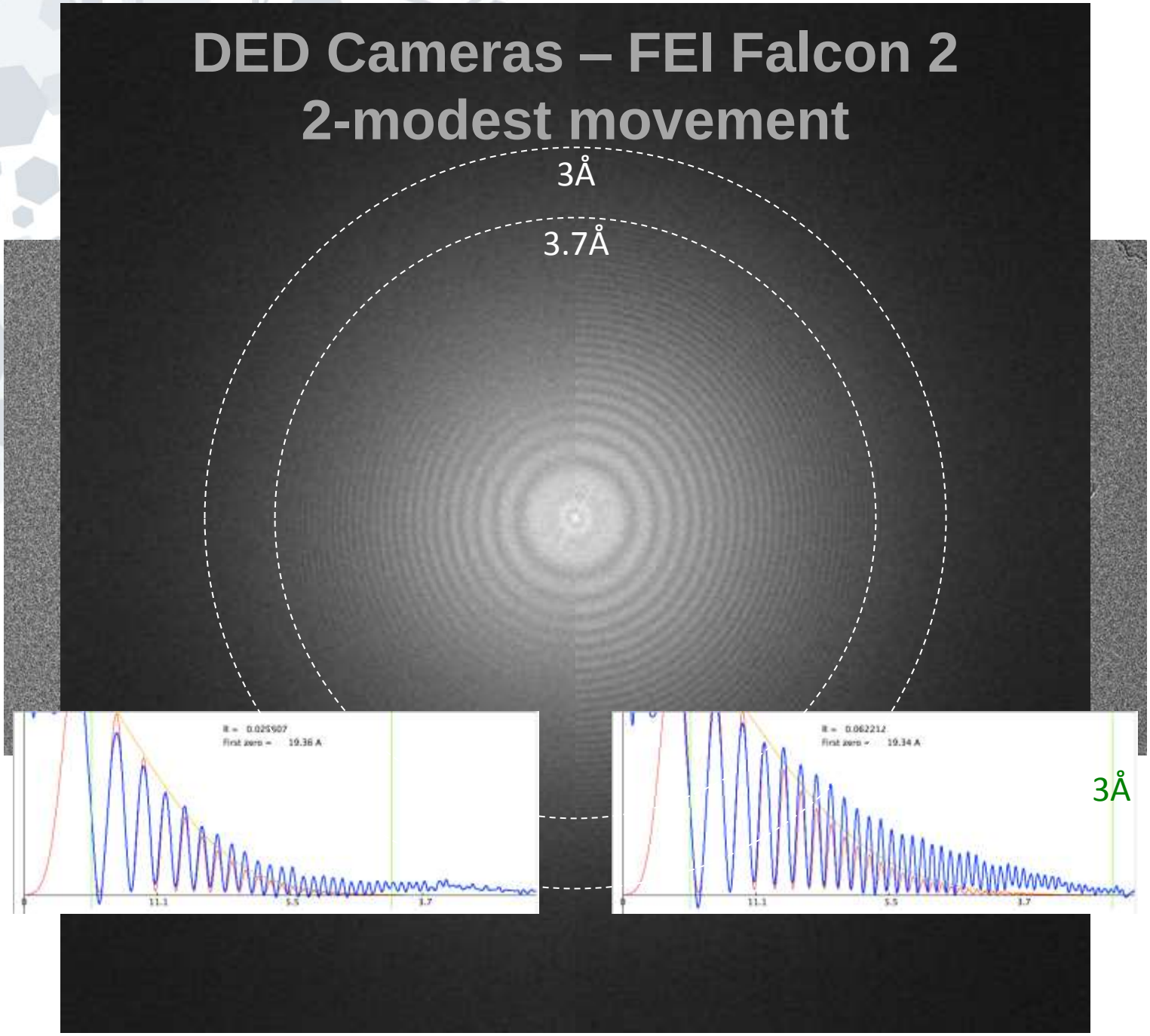
Figure 2. Motion correction restores the lost high-resolution information. (**a**) Fourier transforms of an image of frozen hydrated archaeal 20S proteasomes. Representative 'near-perfect' image in which Thon rings extend to nearly 3 Å. The cross-correlation (CC) between image Thon rings at 5–10 Å and simulated ideal Thon rings over the same resolution range is 0.192. (**b**) The 24 individual subframes used to create **a** were cross-correlated, and relative positional shifts were determined as described in the text and Online Methods. On the basis of these calculations, the path of motion between the first (large black dot with arrow) and last subframes can be determined. (**c**) Fourier transform of **a** after motion correction, where the Thon ring CC is 0.233. (**d**) Fourier transform of a representative imperfect image showing a predominantly unidirectional resolution cutoff at ~20 Å. The Thon ring CC is only 0.092. (**e**) Trace of detected motion between subframes used to create **d**. (**f**) Fourier transform of **d** after motion correction, showing that resolution has been isotropically restored; the Thon ring CC improved to 0.238. The narrow white band was caused by residual fixed-pattern noise in each subframe, which was subsequently eliminated.

Li *et al* (2013) Nature Methods 10, 584-590.

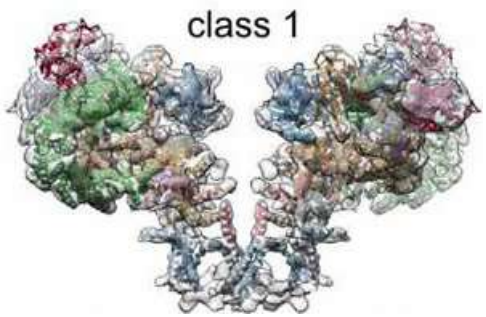
DED Cameras – FEI Falcon 2
1-little movement



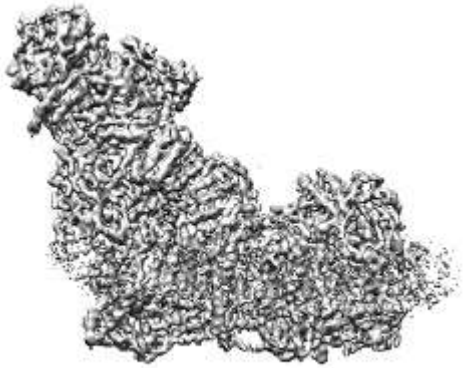
DED Cameras – FEI Falcon 2
2-modest movement



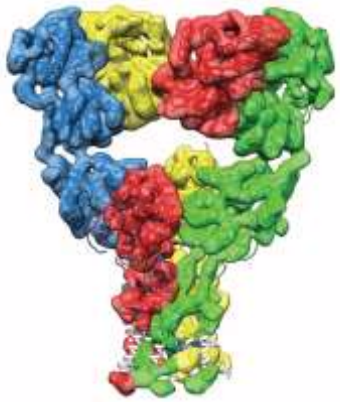
Membrane proteins:



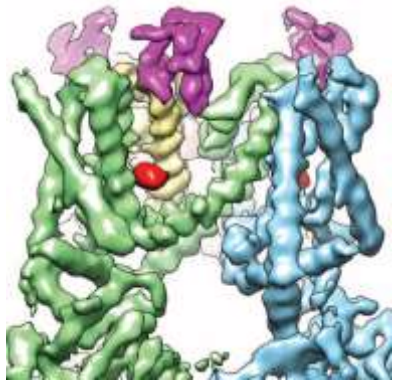
Ryanodine receptor (2.2MD)



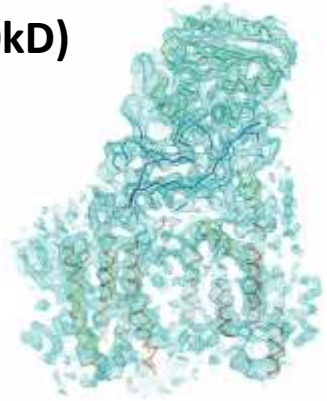
Complex I (1MD)



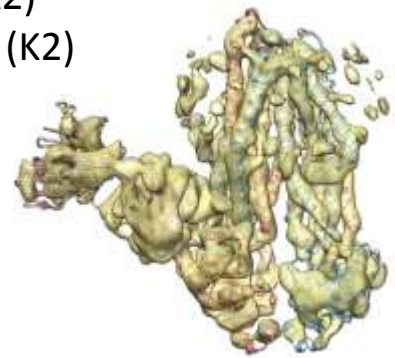
Glutamate receptor (460kD)



TRPV1 (300 kD)



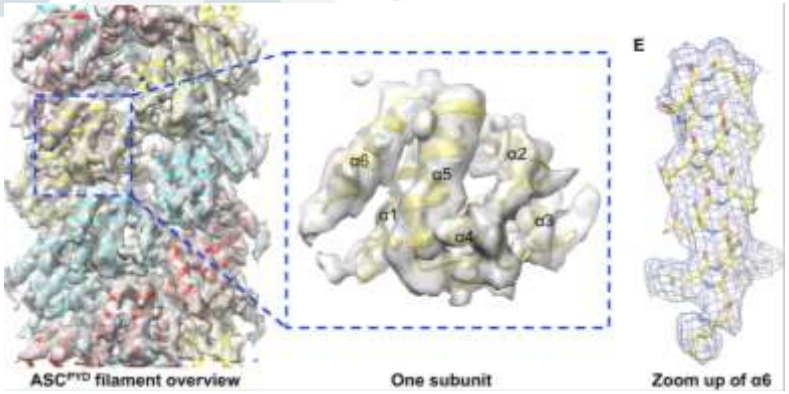
γ-secretase (170 kD)



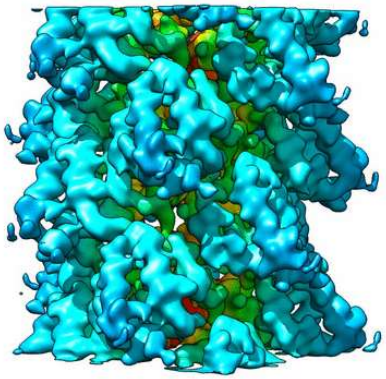
ABC-transporter (135 kD)

Kutti R. Vinothkumar, et al. **Nature** (2014) 515 pp. 80-84 (Falcon)
Erhu Cao, et al. **Nature** (dec 2013) 504 pp. 113-118 (K2)
Maofu Liao, et al. **Nature** (dec 2013) 504 pp. 107-112 (K2)
JungMin Kim, et al. **Nature** (2014) (K2)
Joel R. Meyerson, et al. **Nature** (2014) (Falcon)
Peilong Lu, et al. **Nature** (2014) (K2)
Rouslan G. Efremov, et al. **Nature** (2014) (Falcon)

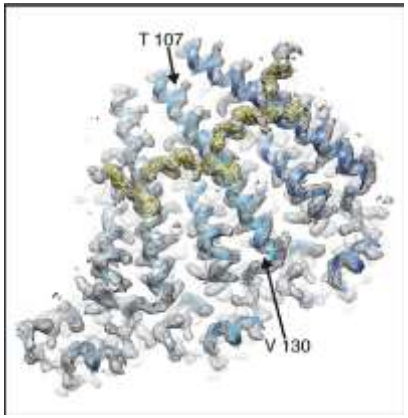
Filaments:



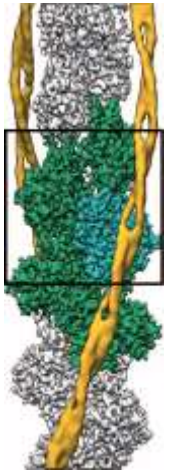
Inflammasomes



MAVS filament



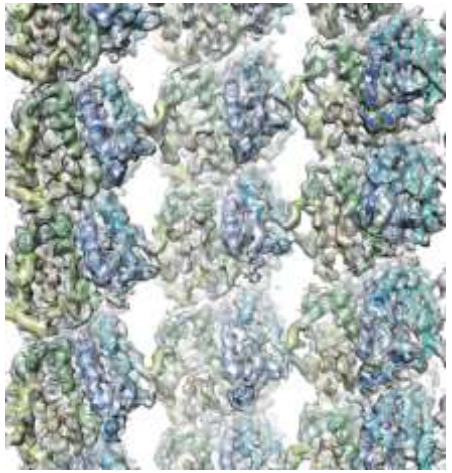
TMV



F-Actin

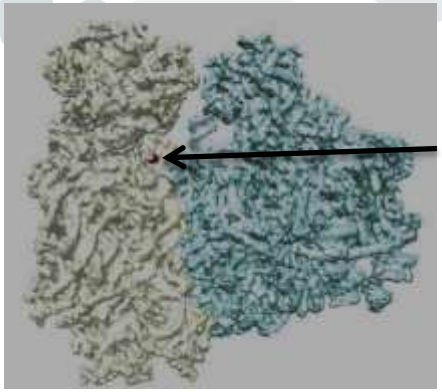
Fromm S. *et al*. **JSB** (2014) (Falcon, K2)
Lu A, *et al*. **Cell** (2014) 156 pp. 1193-1206 (Falcon)
Wu B, *et al*. **Molecular Cell** (2014) 55 pp. 511-523 (K2)
Von der Ecken J, *et al*. **Nature** (2014) (Falcon)
Galkin VE, *et al*. **Structure** (2014) (Falcon)
Alushin GM *et al*. **Cell** (2014) 157 pp. 1117-1129 (film)

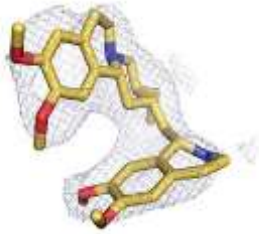
(M)any high resolution structure(s) seems to be a ticket for a high impact publication!



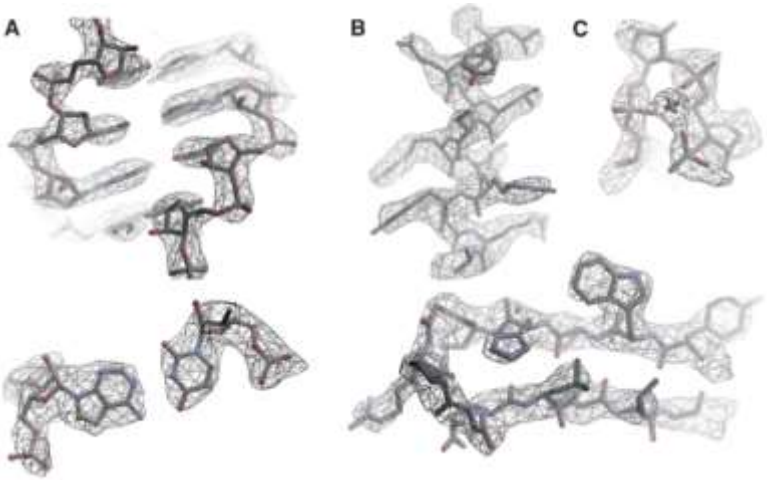
Microtubules

Ribosomes (2014):



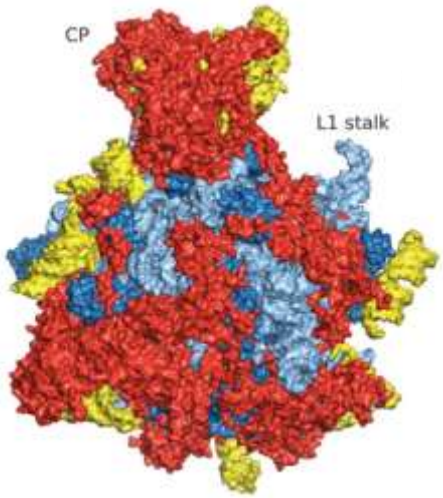


Emetine drug



Hussain T, et al. **Cell** (2014) 159 pp. 597-607 (Falcon)
Bischoff L, et al. **Cell Rep**. (2014) 9 pp. 469-475 (Falcon)
Arenz S, et al. **Molecular Cell** (2014) (Falcon)
Brown A, et al. **Science** (2014) 346 pp. 718-722 (Falcon)
Greber BJ, et. Al. **Nature** (2014) (Falcon)
Shao S, et al. **Molecular Cell** (2014) 55 pp. 880-890 (Falcon)
Voorhees RM, et al. **Cell** (2014) 157 pp. 1632-1643 (Falcon)
Wong W, et al. **eLife** (2014) 3 (Falcon)
Fernandez IS, **Cell** (2014) 157 pp. 823-831 (Falcon)
Amunts A, **Science** (2014) 343 pp. 1485-1489 (Falcon)
Greber BJ, et al. **Nature** (2014) 505 pp. 515-519 (cover) (Falcon)

Full *de novo* model building



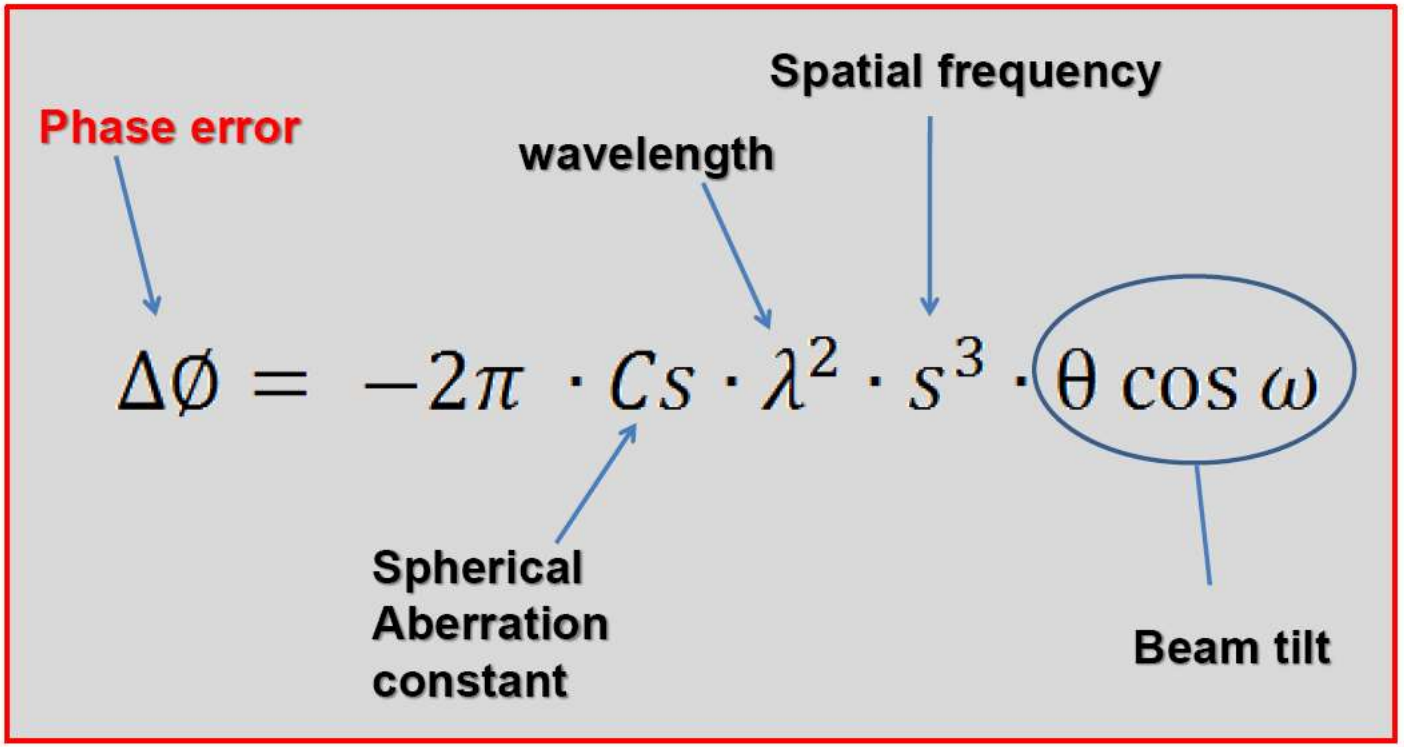
Many high impact publications on ribosomal complexes!

Routinely ≤4Å

Electromagnetic lenses

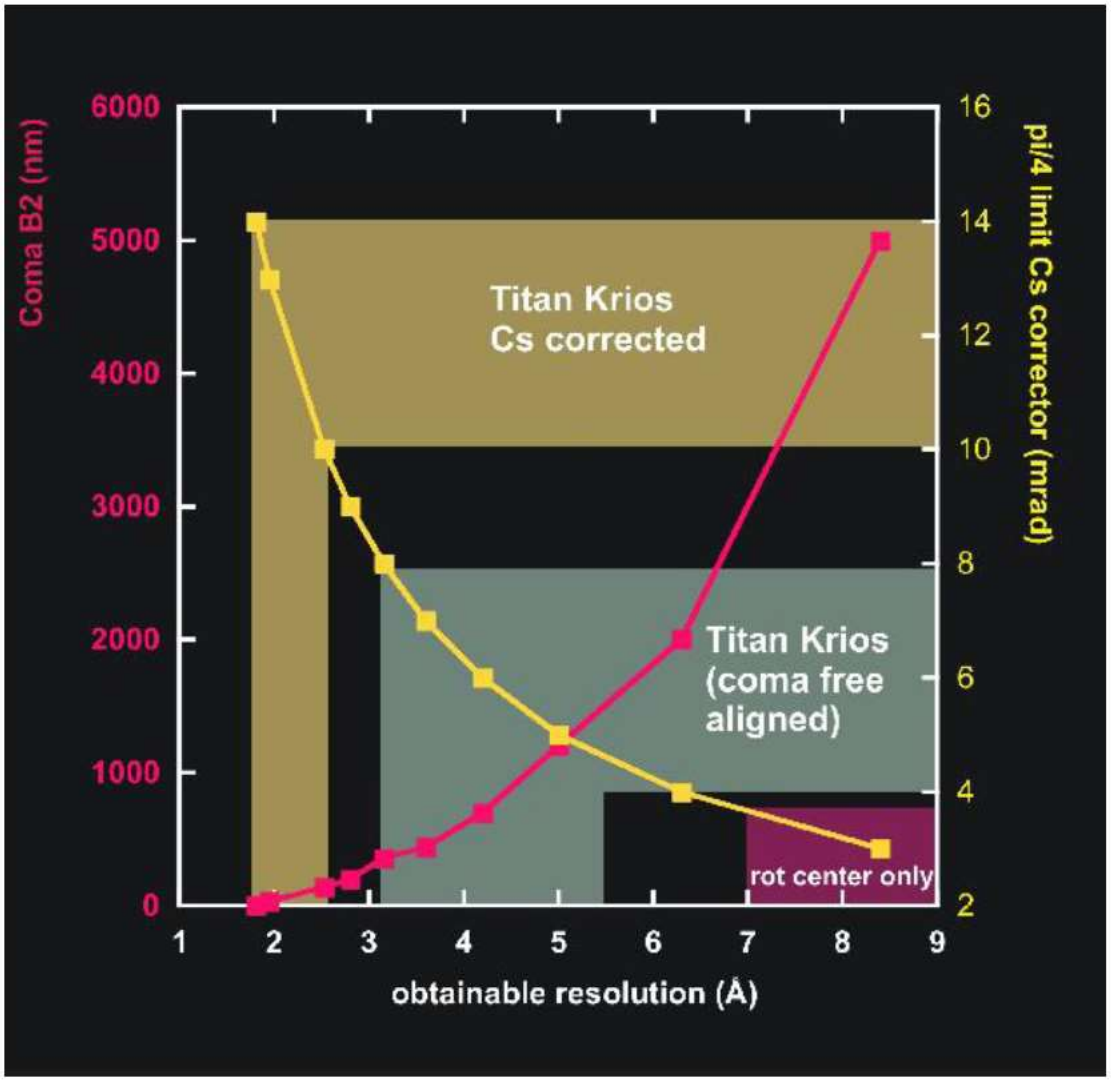
(Spherical aberration)

Round EM lenses -> Cs (aberration)



Beam tilt induces phase errors due to coma
(formula valid for non C_s corrected microscopes only)

Alignment accuracy in a Titan Krios

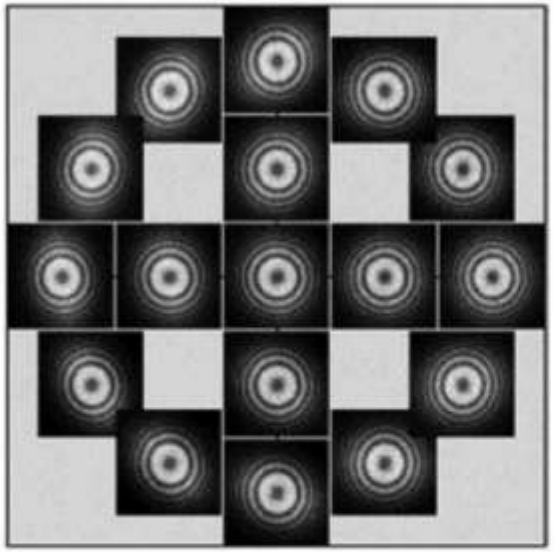


- pi/4 phase error is commonly used as resolution limiting criterion
- a phase error of pi/4 is not a sharp resolution limiting cutoff
- phase errors can be determined by the Zemlin tableau

Aberration-corrected TEM

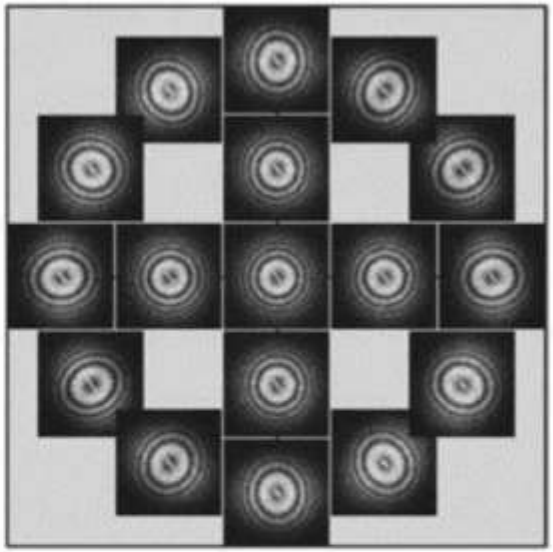
Zemlin Tableau

(a)



Fully corrected

(b)



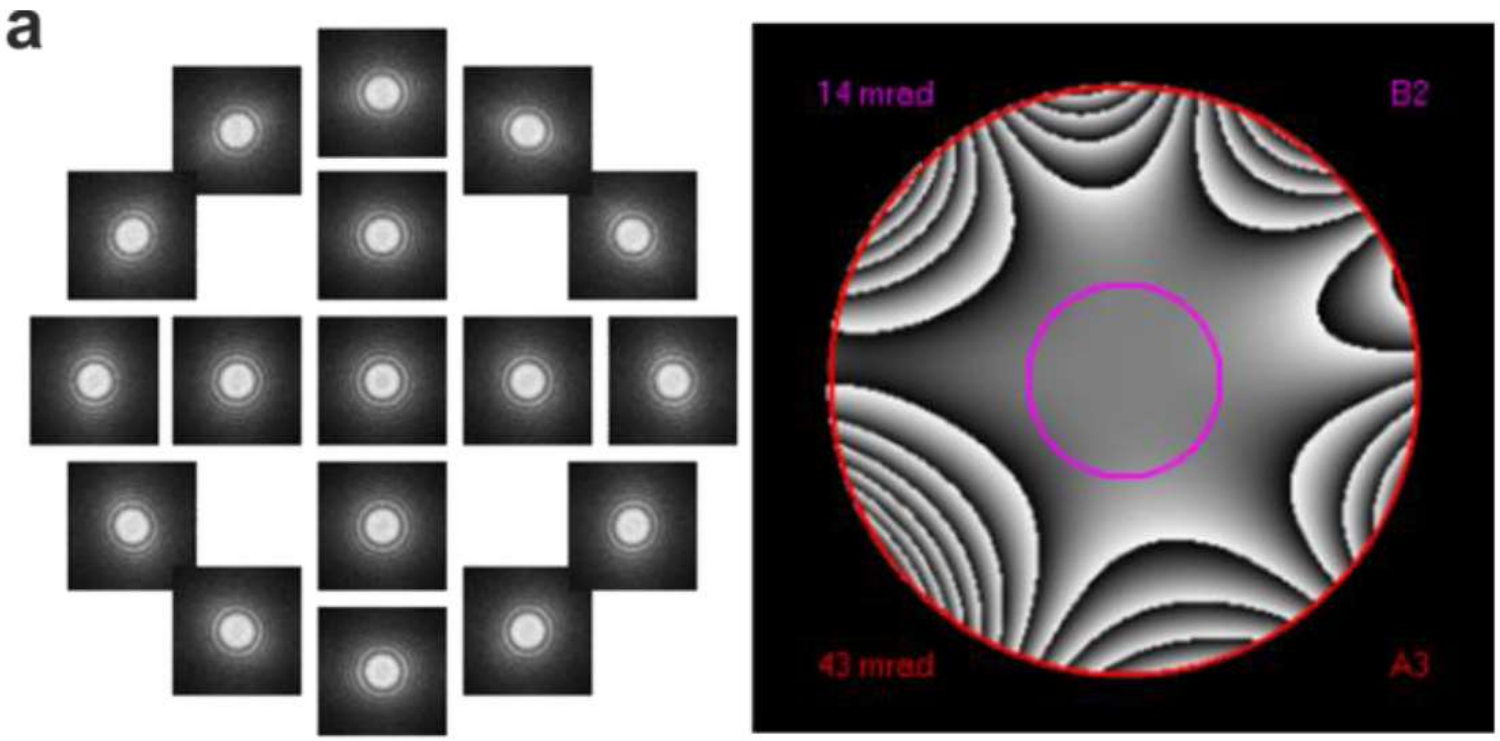
Imposed Cs of 0.1mm (18 mrad)

1. Measure beam-tilt dependent defocus and astigmatism
2. Determine phase errors
3. Correct up to 5th order aberrations

Zemlin et al., Ultramicroscopy 1978

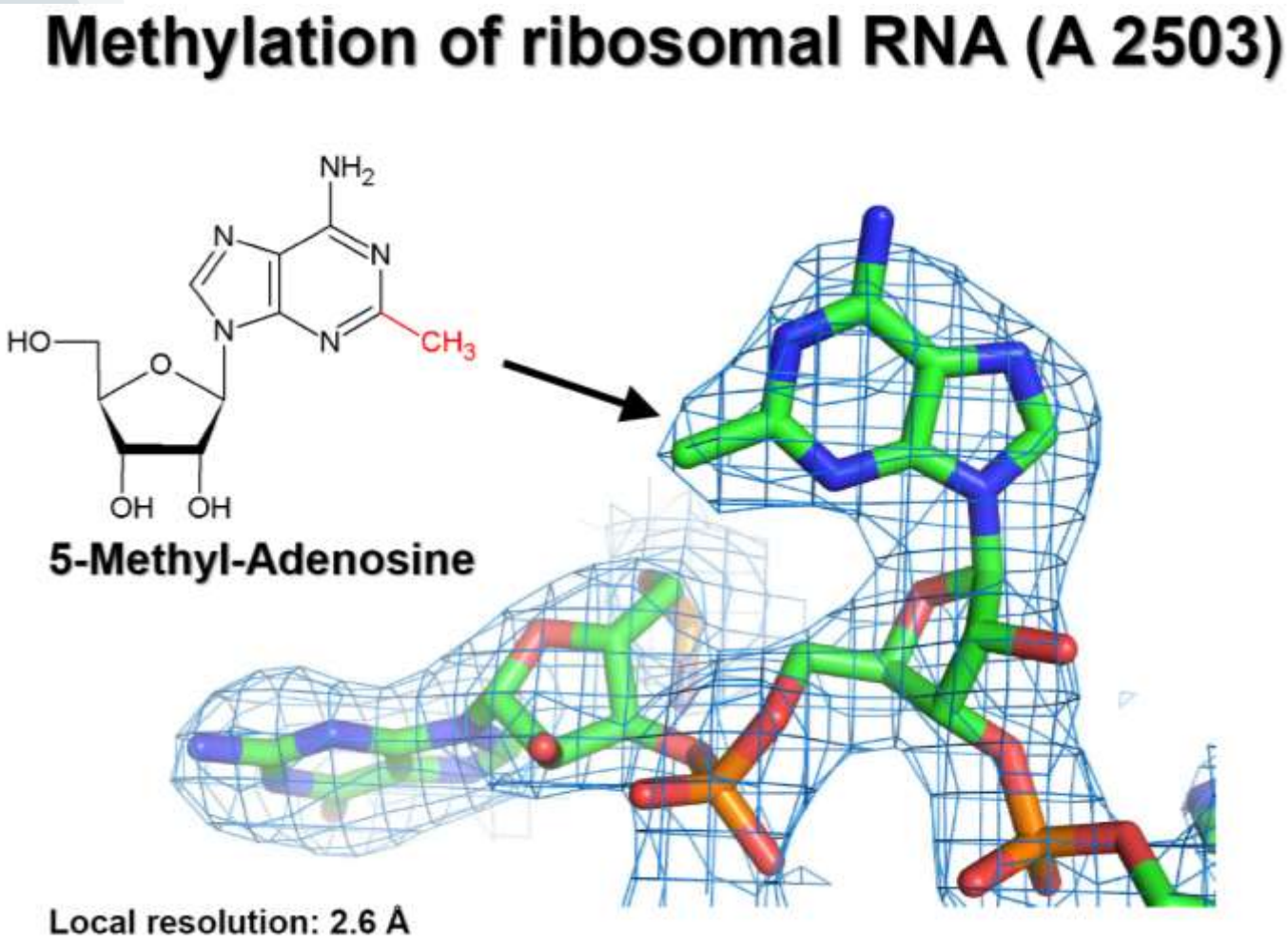
Aberration-corrected TEM

Cs Corrector Alignment



Hexapoles on: phase errors of 45 degrees at scattering angles of 12-15 mrad

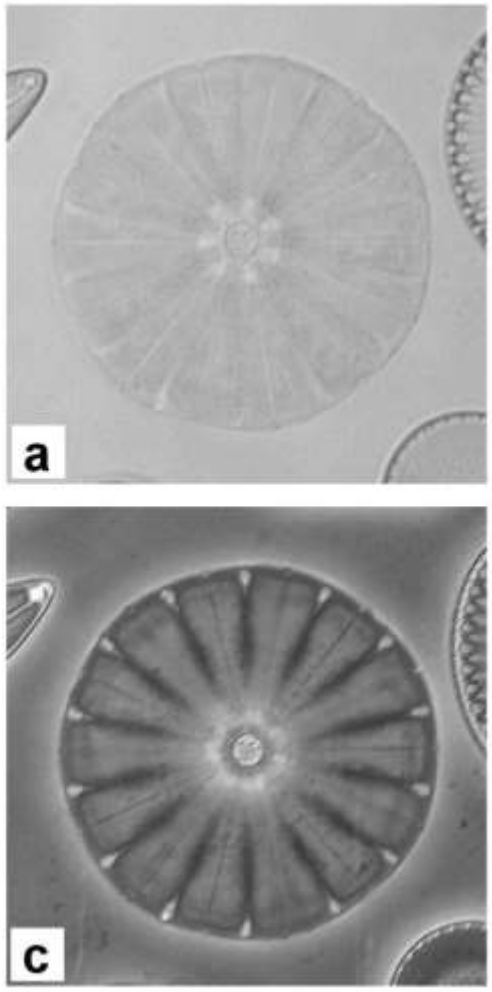
Aberration-corrected cryo-TEM (<3Å)

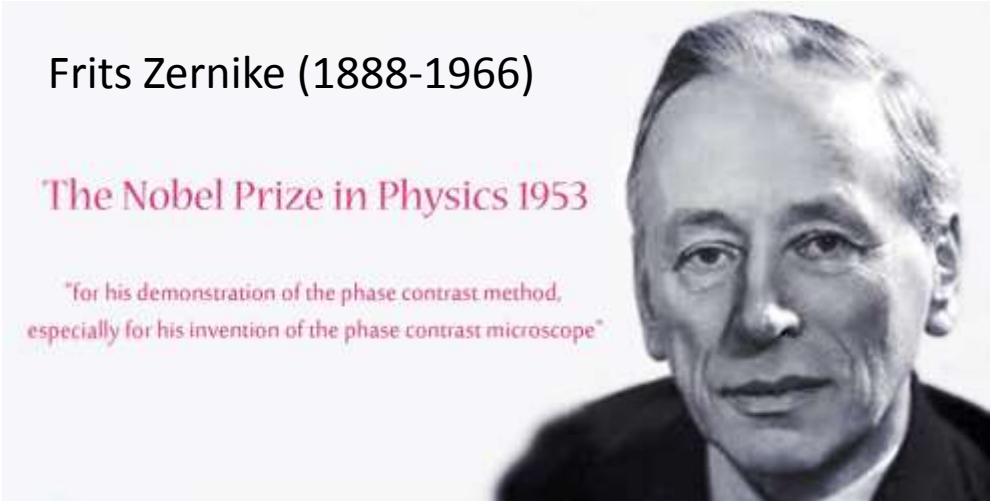


Data (Nature, in press) courtesy of Prof. Holger Stark, MPI Göttingen

Enhancing contrast: Phase Plate

Zernike Phase Contrast

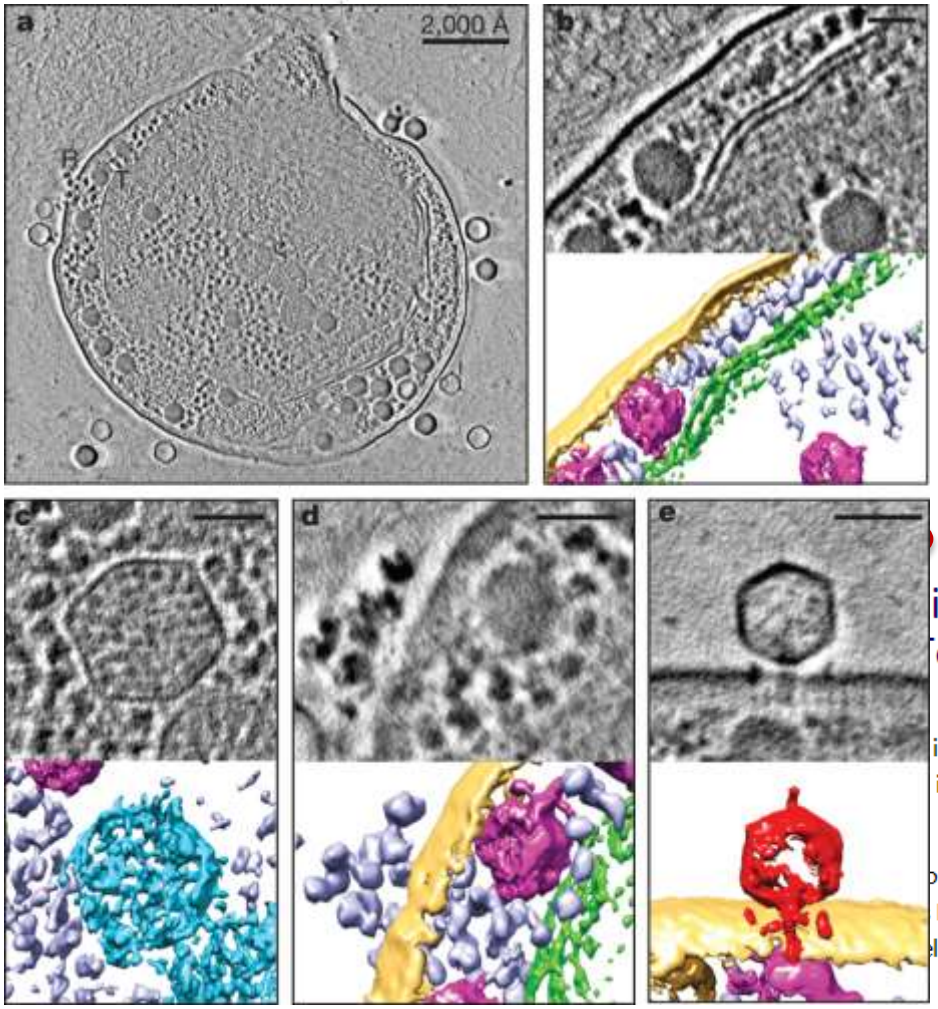




The basic principle to make phase changes visible in phase contrast microscopy is to separate the illuminating background light from the specimen scattered light, which make up the foreground details, and to manipulate these differently

Zernike Phase Plate in TEM

Dai… & Chiu (2014) *Nature Protocols* **9**, 2630–2642



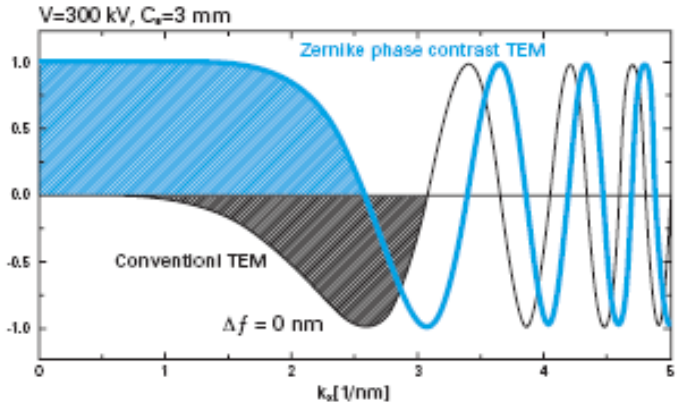


Figure 2

irst Commercially-Available Thin Film echnology for TEM

imaging performance of today's generation of Transmission Electron improved dramatically through the use of a novel technique, the thin film

on microscope supplier to offer commercially-available thin film phase Life Sciences customers, in particular those involved in cryo-electron lectron tomography. The phase contrast imaging capability of a phase-

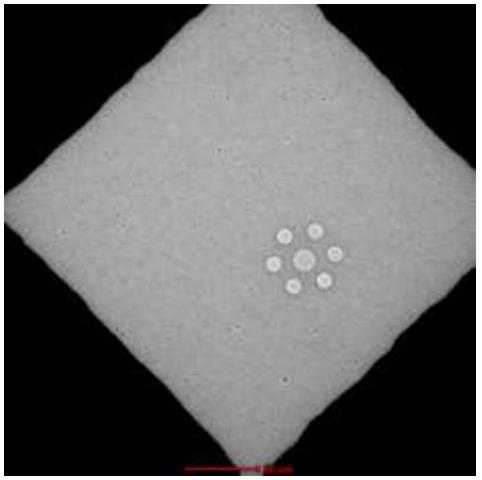
Explore. Discover. Resolve.

FEI™

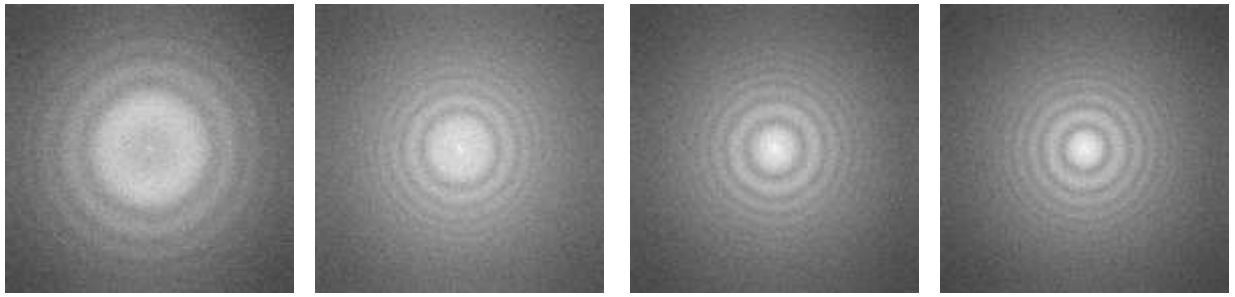
Principle

When an electron beam passes a continuous heated carbon film:

1. The beam leaves a white footprint (underfocus)

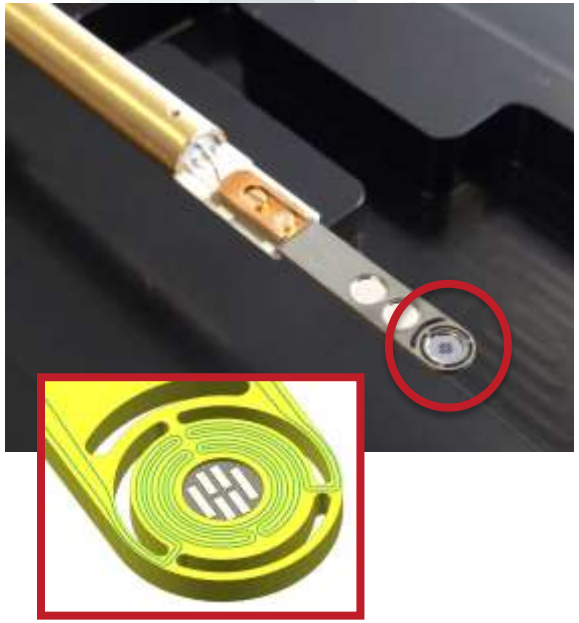


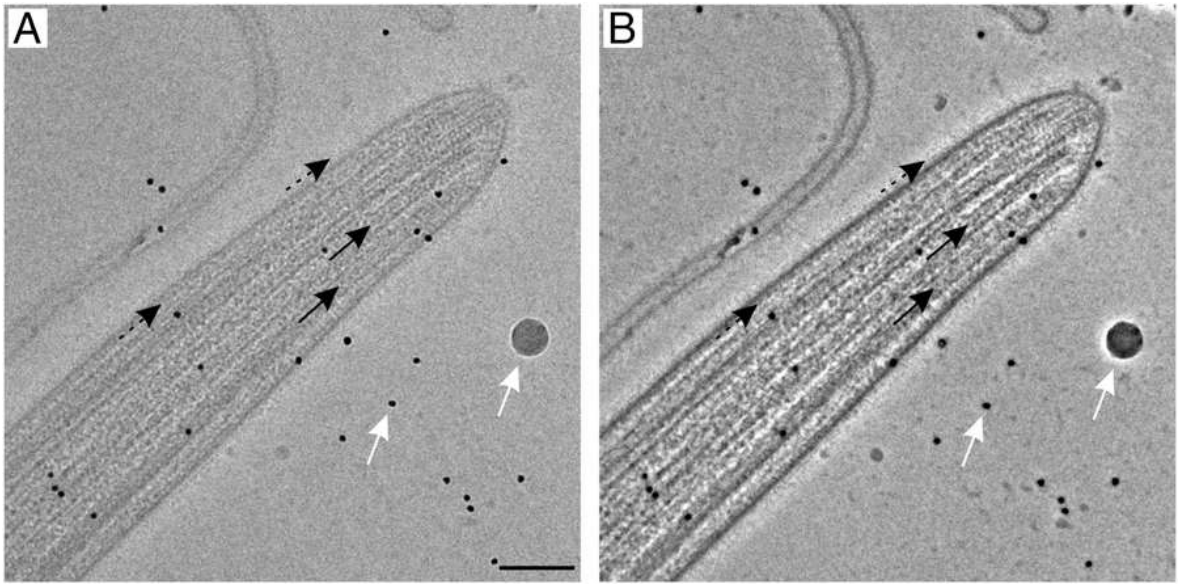
2. A phase shift occurs of the central beam relative to the diffracted beam



π/2

Contrast improvement for tomography

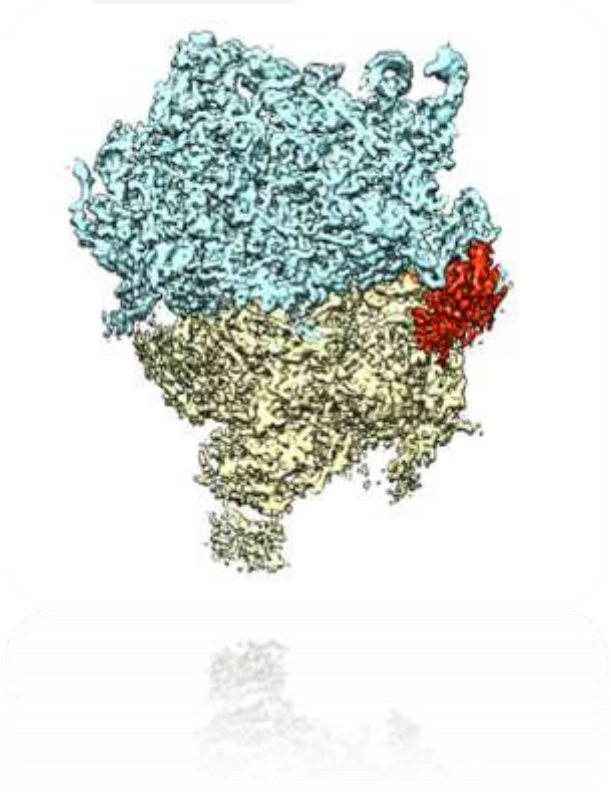


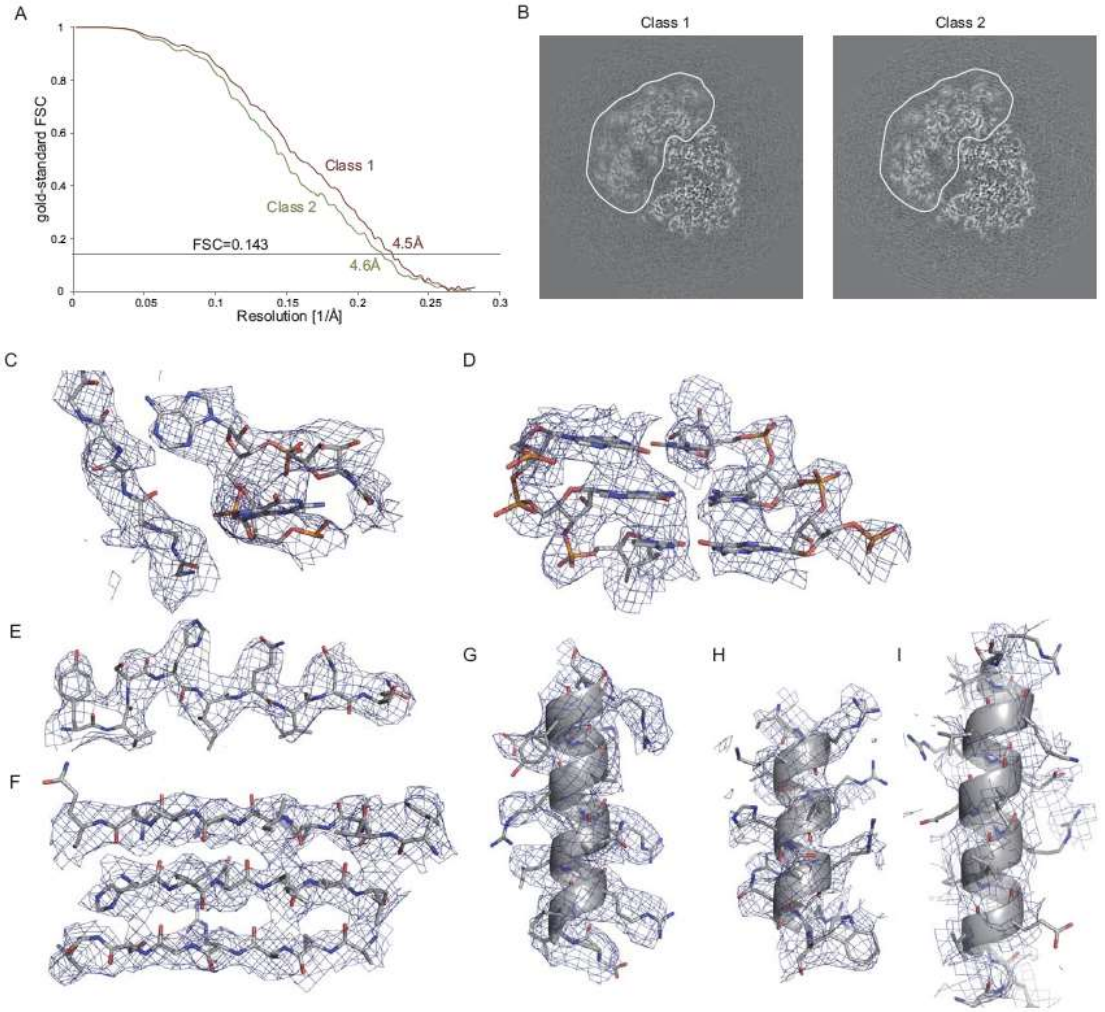


Danev R, *et al*. (2014), **PNAS** 111, p. 15635

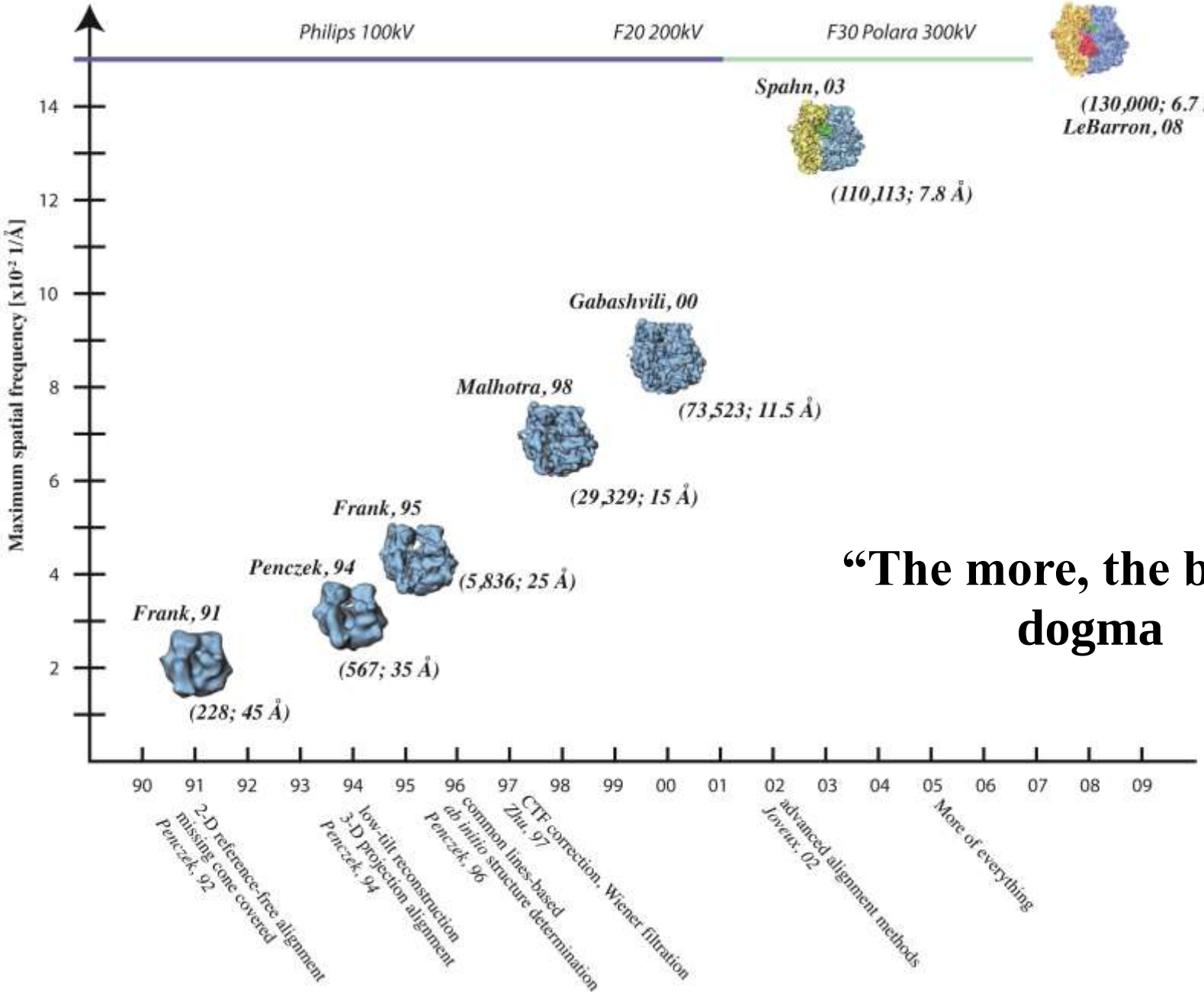
- The FEI Volta Phase Plate provides “high defocus” contrast with in-focus imaging
- The FEI VPP has a long lifetime, regenerates itself, does not require an airlock nor frequent replacements, does not need a centering mechanism and is contamination free
- The FEI VPP is fully automated, easy and simple to use, embedded in the TEM UI and Explore3D tomography software (version 4.1)

With better detectors we maximize output with minimum effort





Bai *et al*. (2013). Ribosome structures to near-atomic resolution from thirty thousand cryo-EM particles. *eLife* **2**



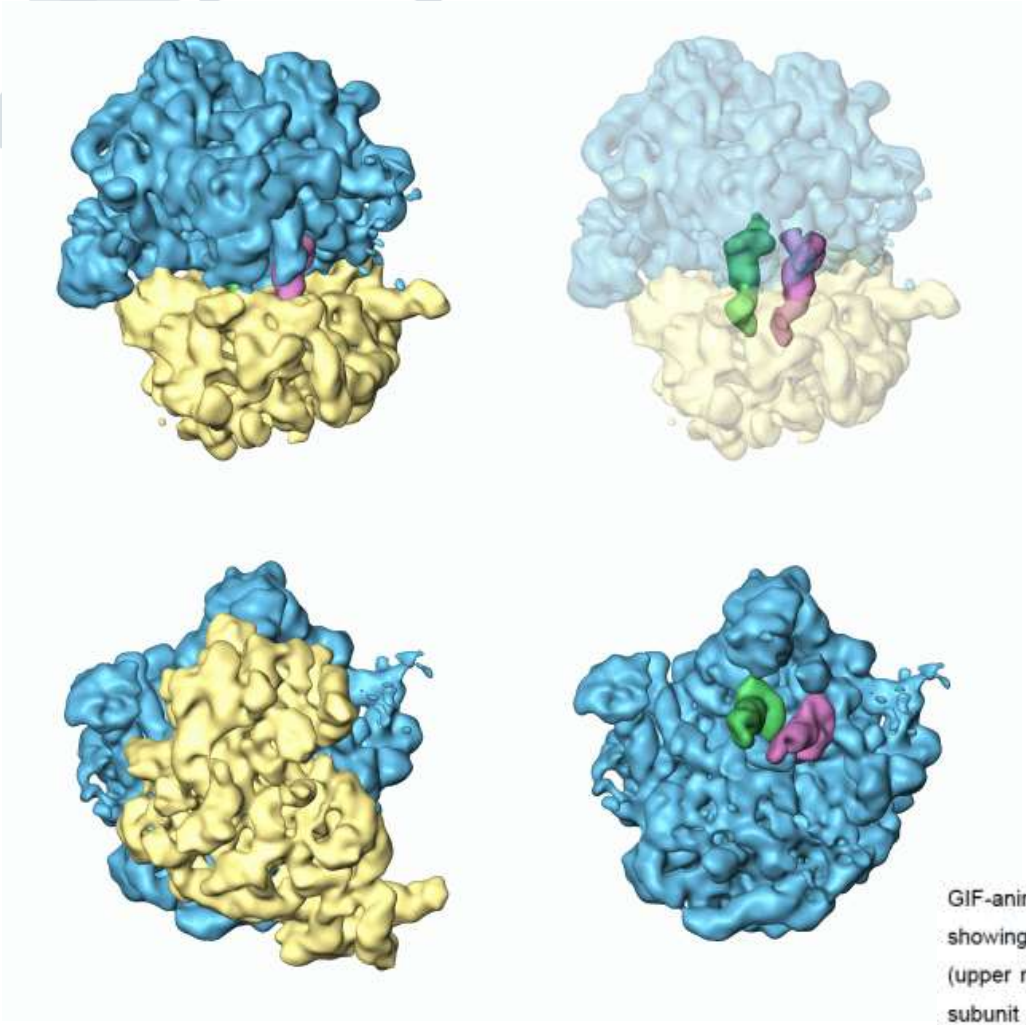
?

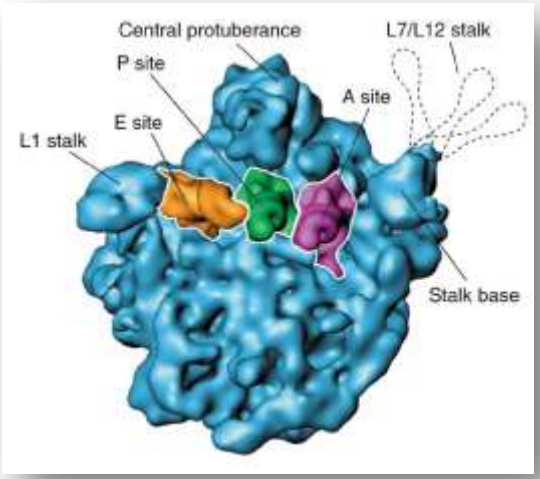
FEI Titan Krios
2,504,547; ?? Å

“The more, the better” dogma

FEI™

“4D” cryo-EM !!





GIF-animation of the same sequence of 20 sub-states as in Supplementary Movie 2, showing for each sub-state a view onto the 70S ribosome cryo-EM maps from the top (upper row), from the solvent side of the 30S subunit (lower left) and onto the 50S subunit and the tRNAs similar as in Supplementary Movie 2 (lower right). Note the coupling between global dynamics of the 30S subunit (yellow; left column), tRNA movement (right column), and local conformational changes of the 50S subunit

Fischer *et al.* (2010) *Nature* **466**, 329-333

Thank You

http://www.fei.com/life-sciences/

FEI™