A view of electron microscopy from 35,000 feet

Vidya Mangala Prasad
Dr. Kelly K. Lee's lab

MedChem 528
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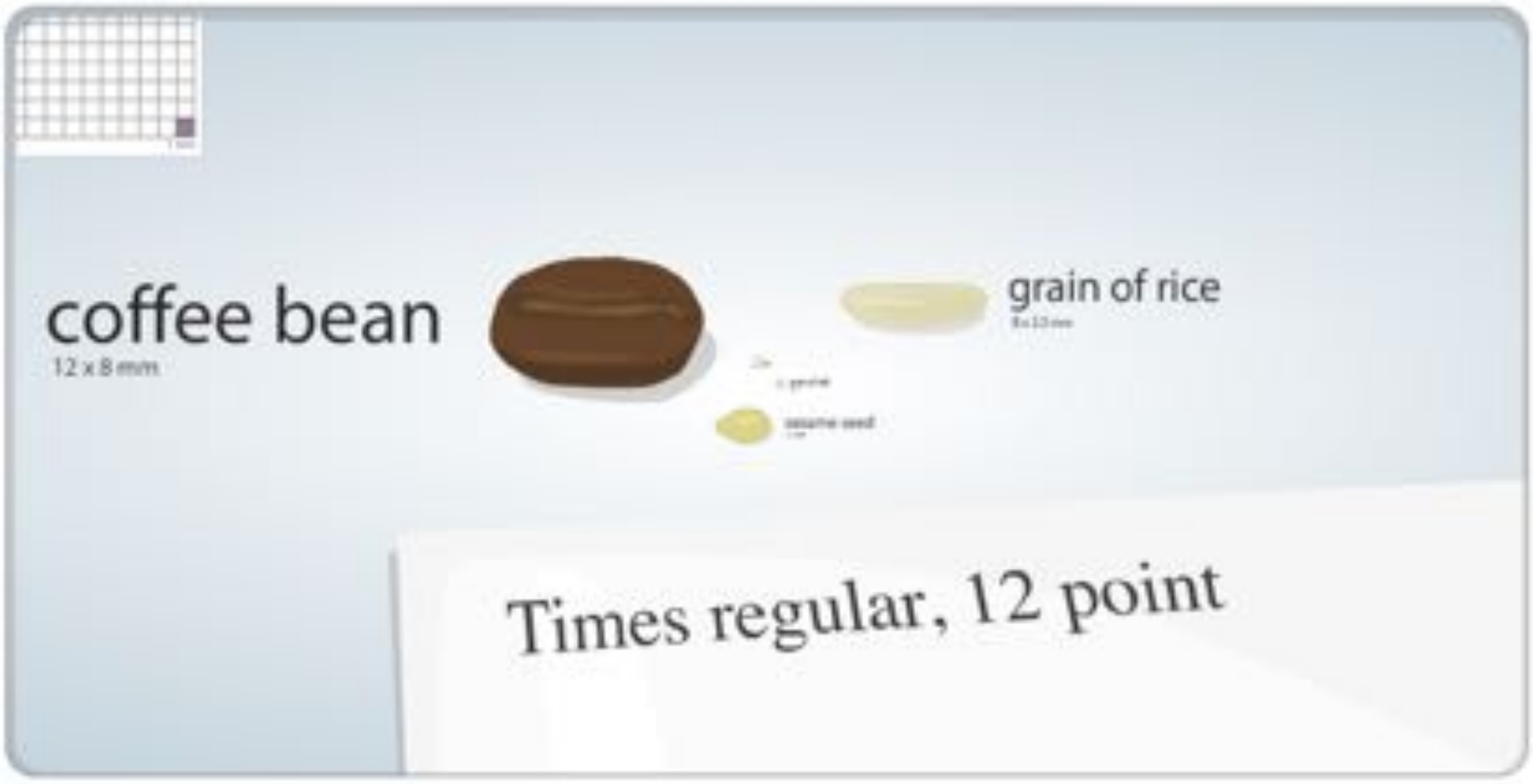
Overview

- What systems can one study? And what information can be obtained?
- A quick glance at the electron microscope
- Negative stain EM
- Cryo-EM single-particle analysis
- Cryo-electron tomography
- Some recent technical advances

Size scales in biology



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Size scales in biology

APPROACHING THE ACUITY LIMIT OF THE HUMAN EYE

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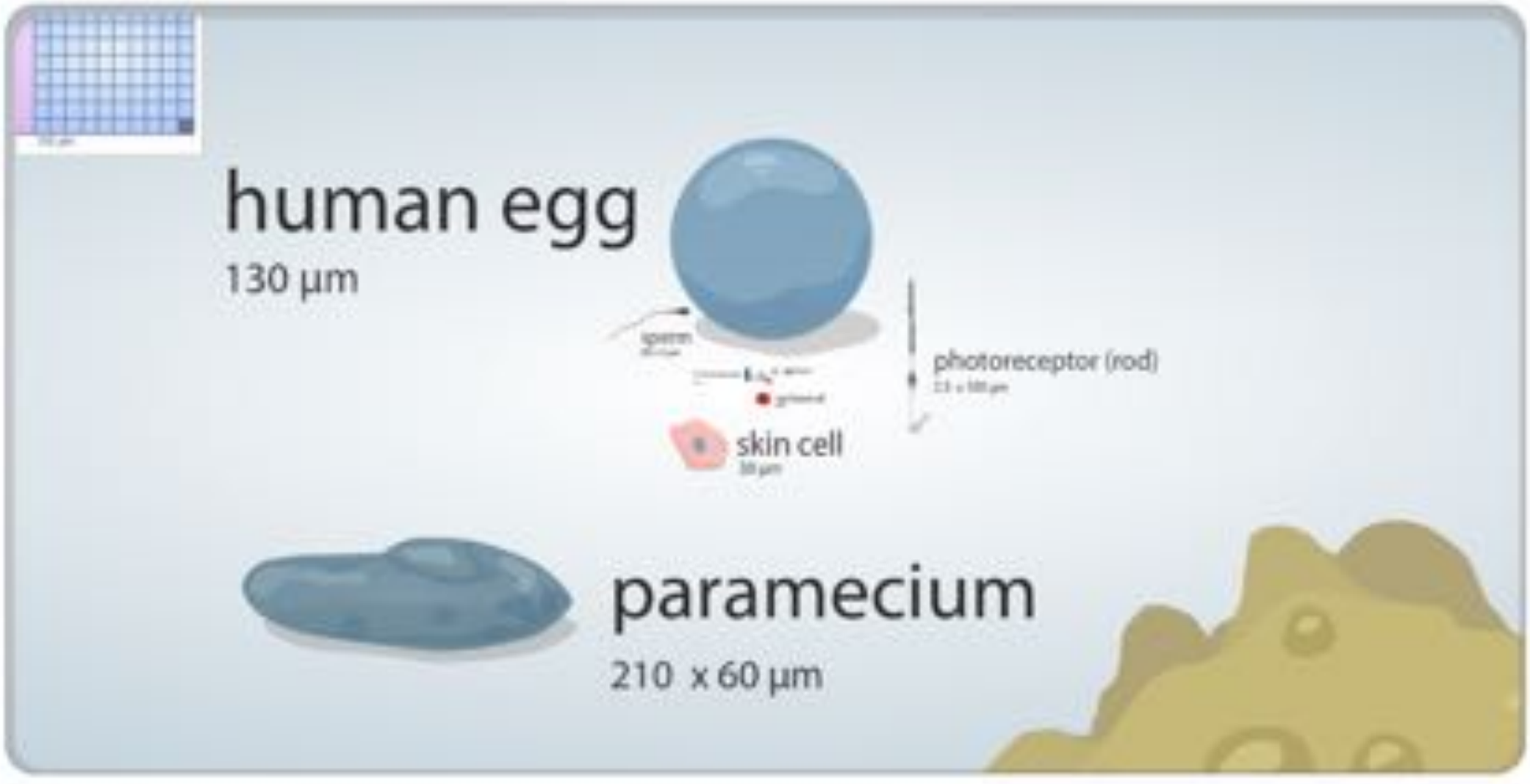
Size scales in biology



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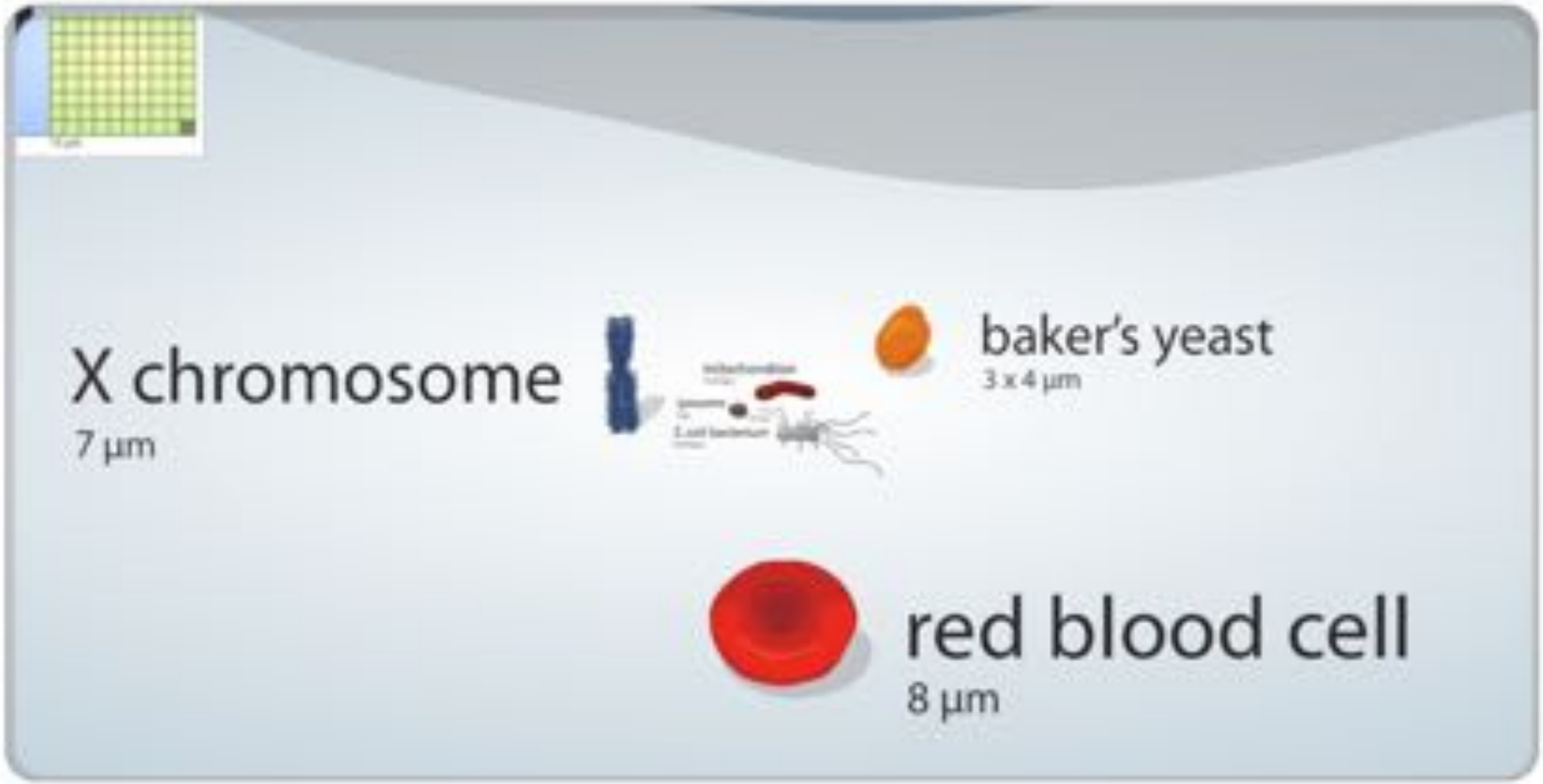
Size scales in biology

APPROACHING THE RESOLUTION LIMIT OF A LIGHT MICROSCOPE



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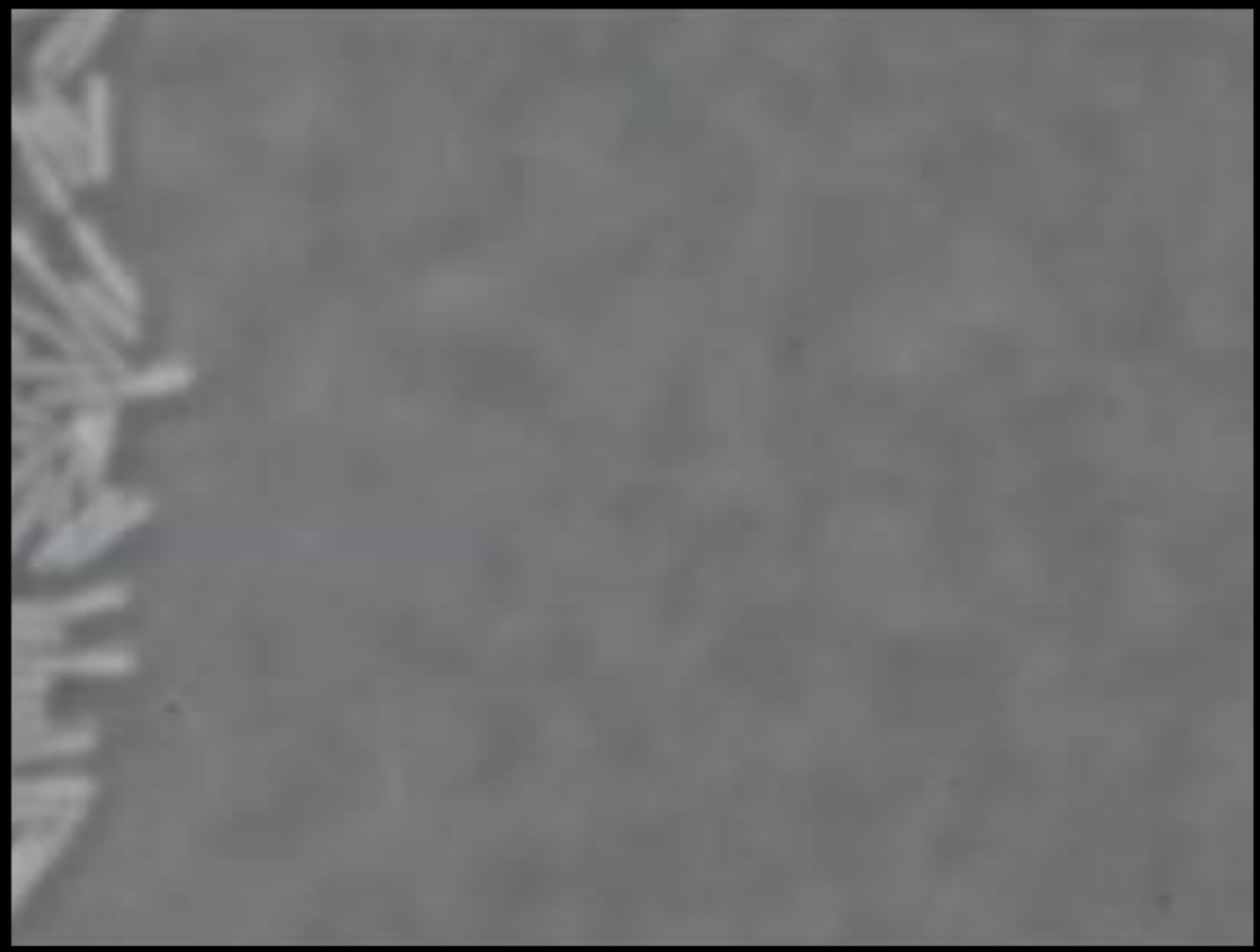
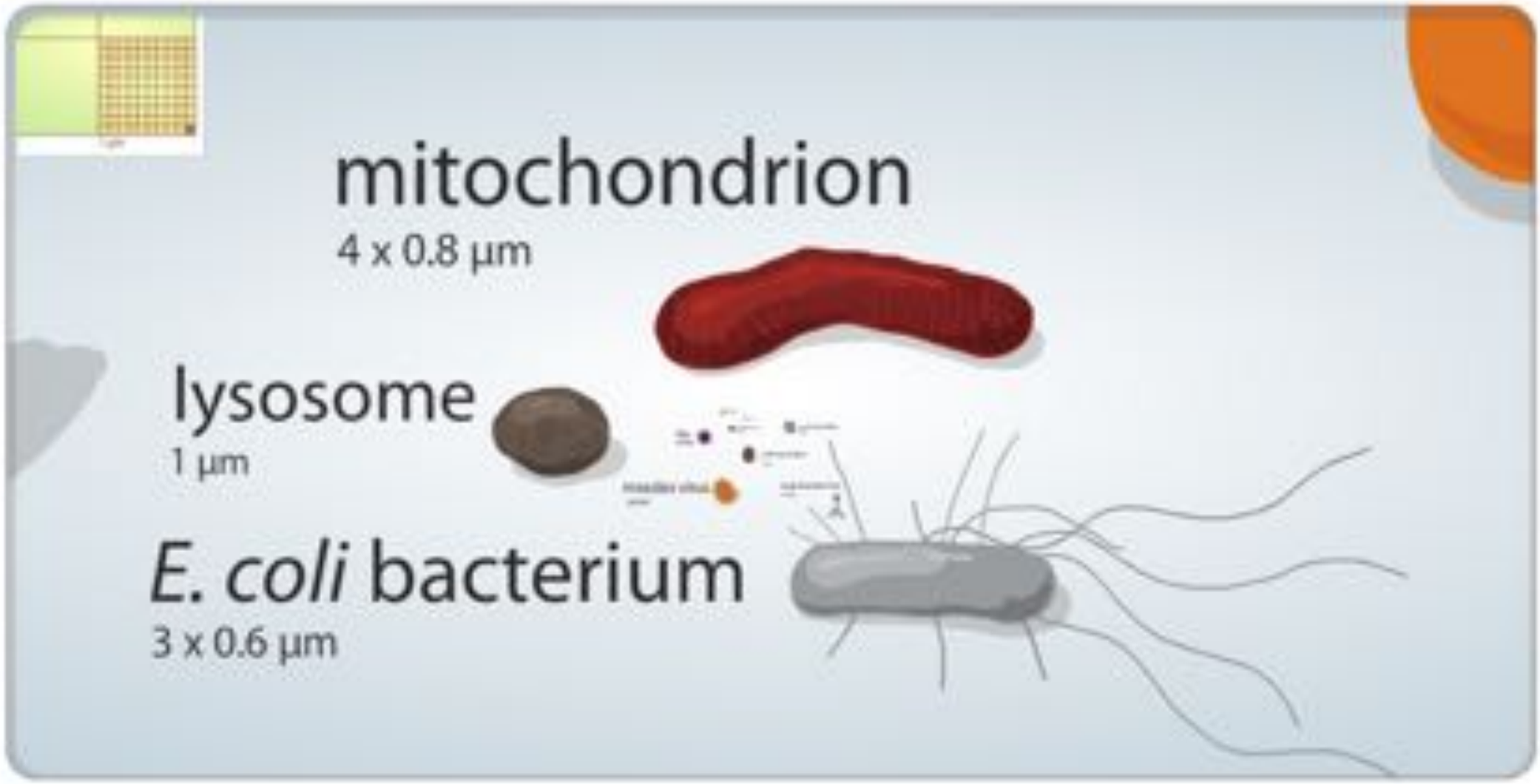


E. coli swarming

Zhang, R., Turner, L. and Berg, H.C. The upper surface of an Escherichia coli swarm is stationary. PNAS **107**: 288–290 (2010)

Size scales in biology

3-D structures of these can be imaged by electron tomography
~3nm resolution limit



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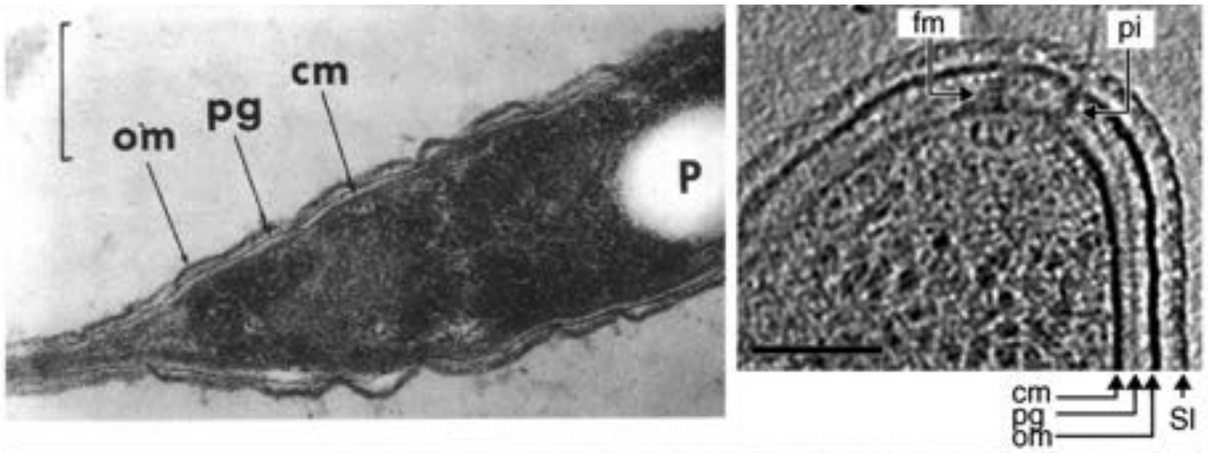
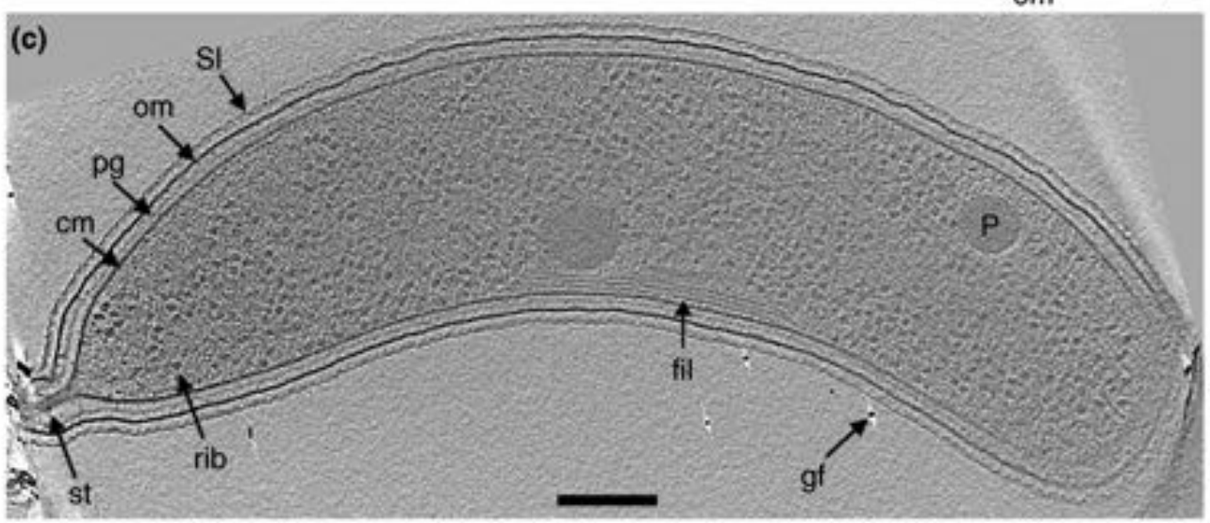
Tomography of whole micro-organisms

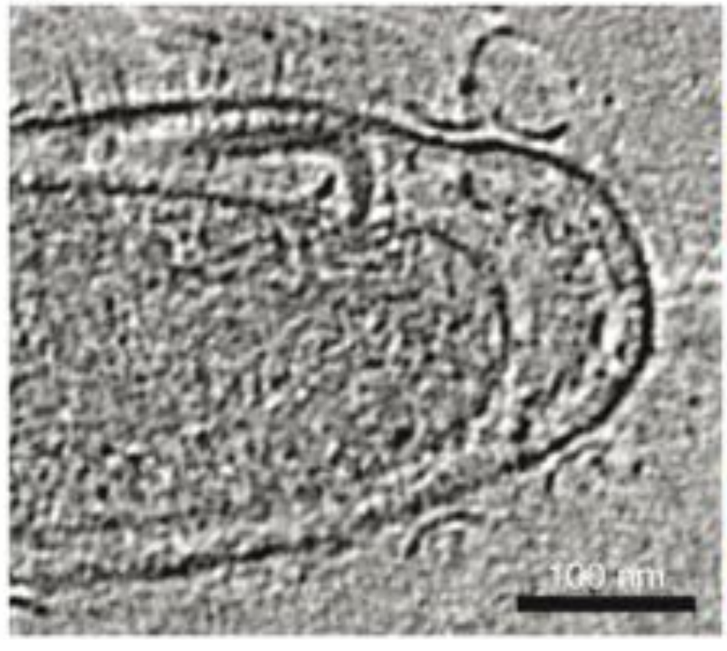


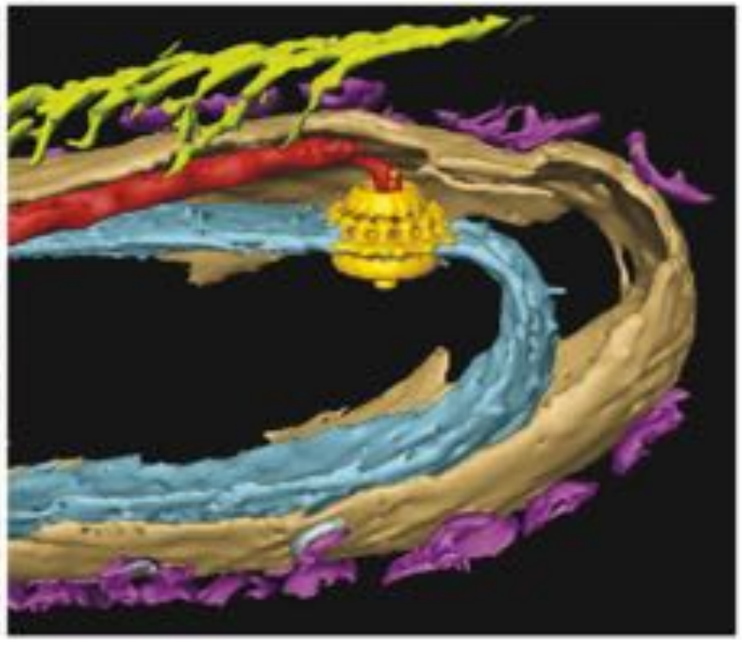


Jensen GJ and Briegel A, Curr Opinion Struct Biol 2007, 17:260–267

Briegel A, Dias DP, Li Z, Jensen RB, Frangakis AS, Jensen GJ, Mol Microbiol 2006, 62:5-14.

Murphy GE, Leadbetter JR, Jensen GJ, Nature 2006, 442:1062-1064.





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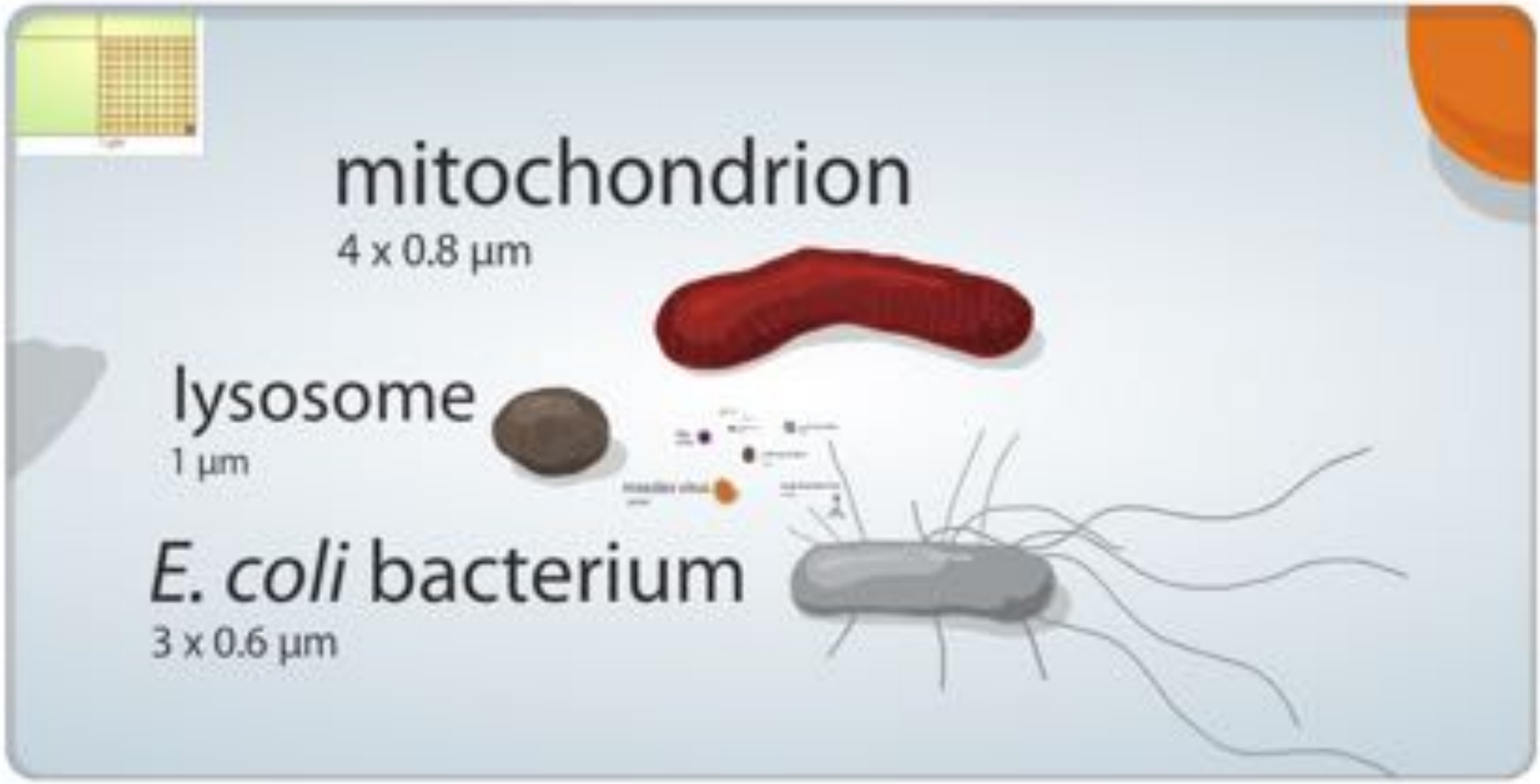

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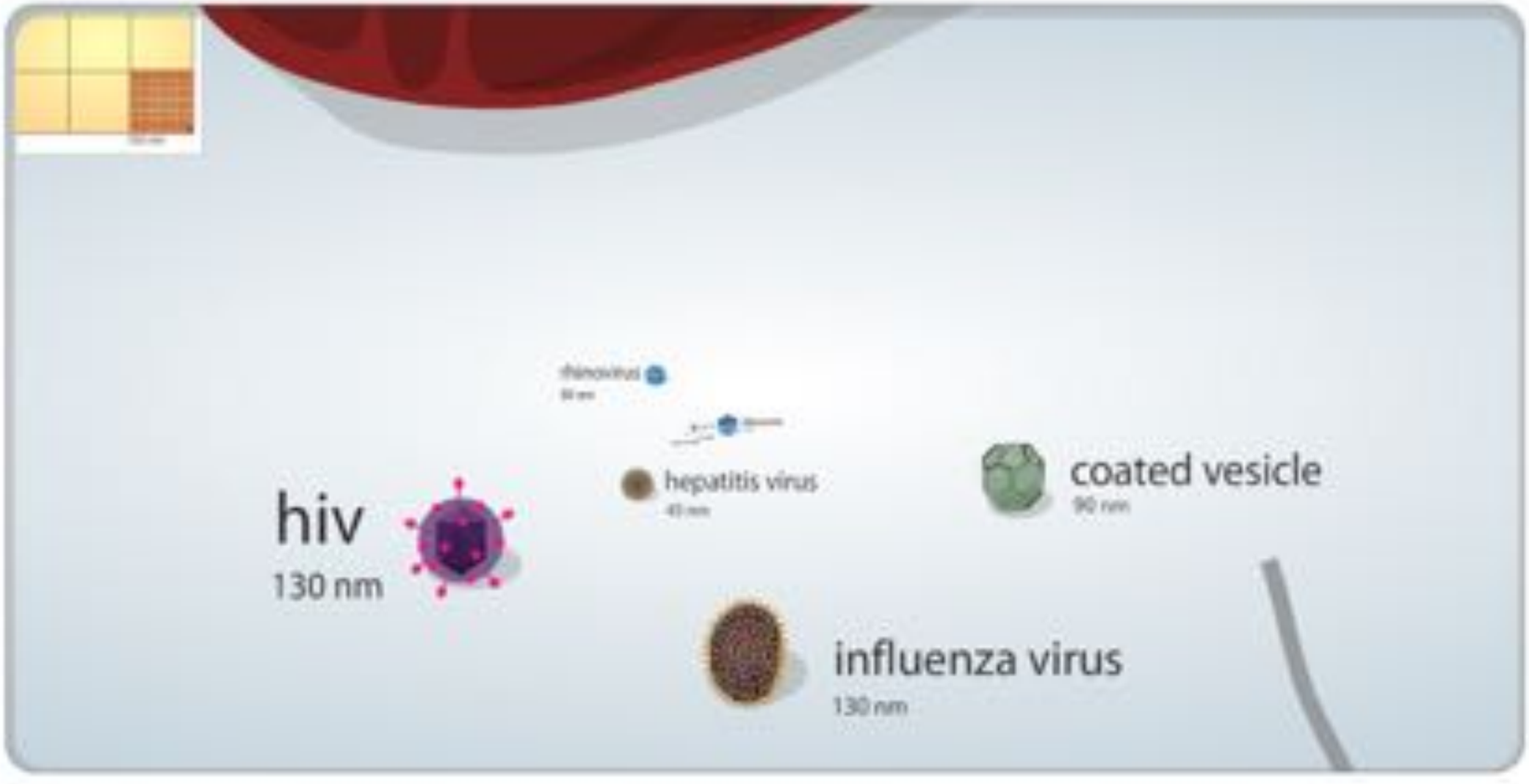

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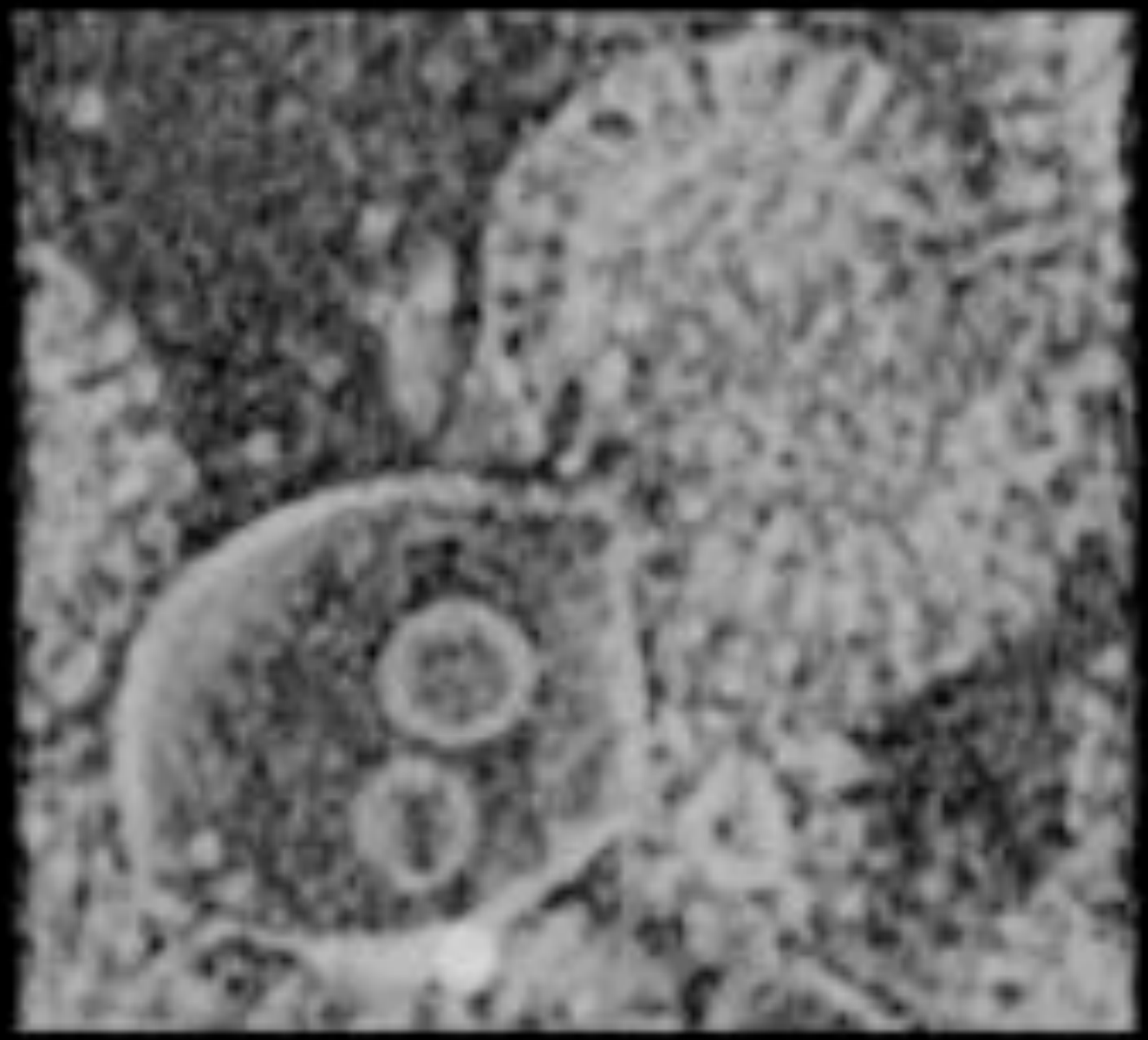
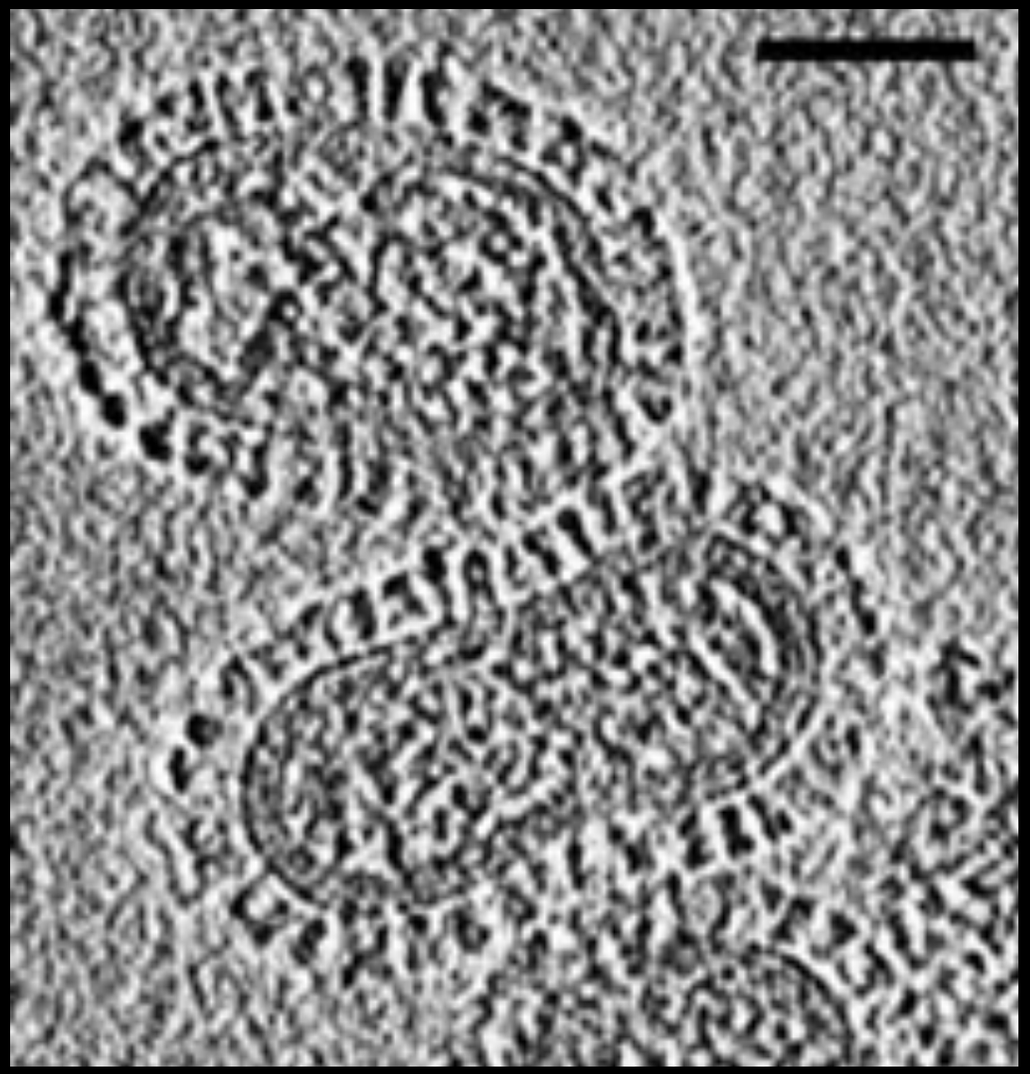
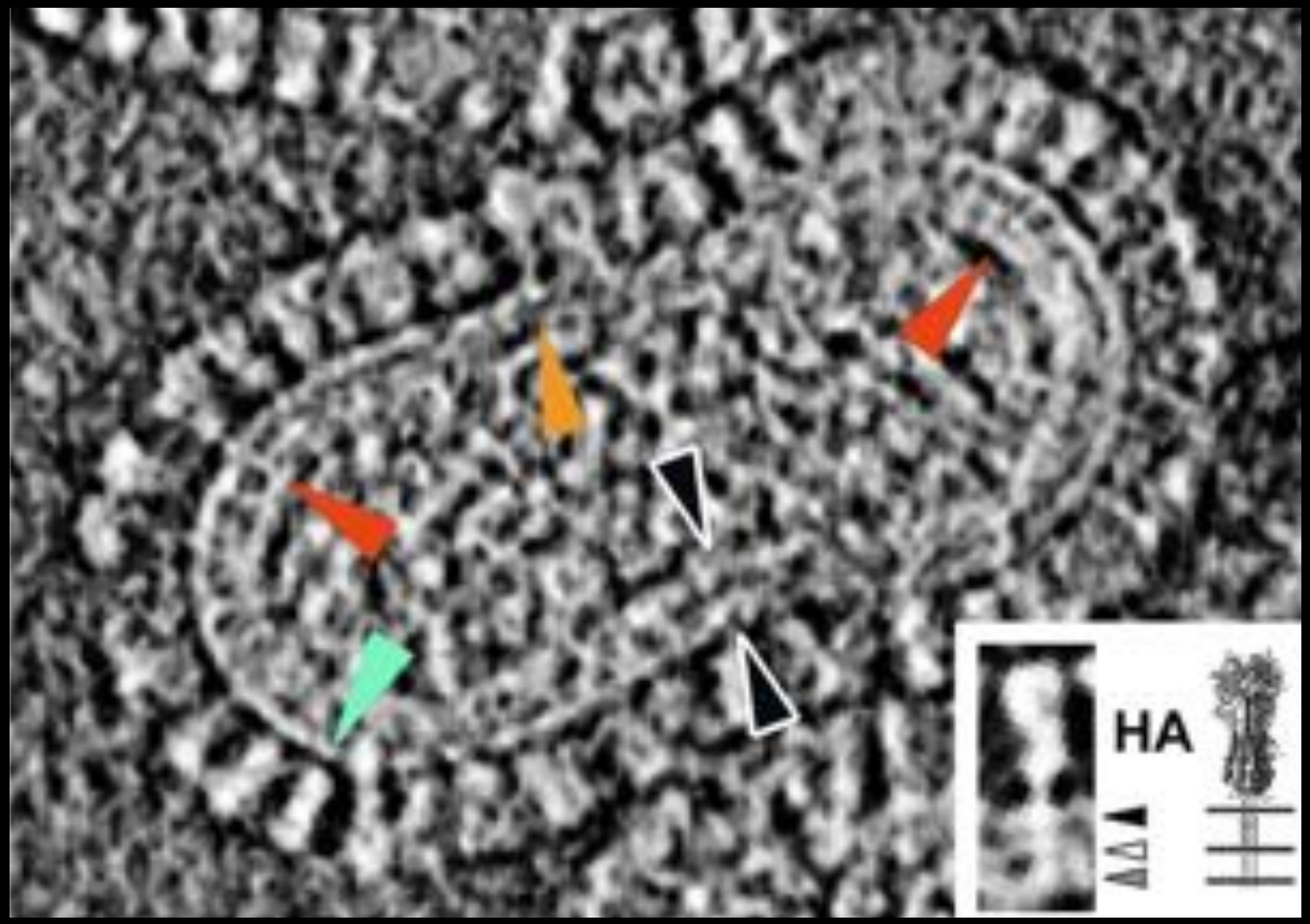
Reconstructed 3-D density of influenza virus bound to a liposome



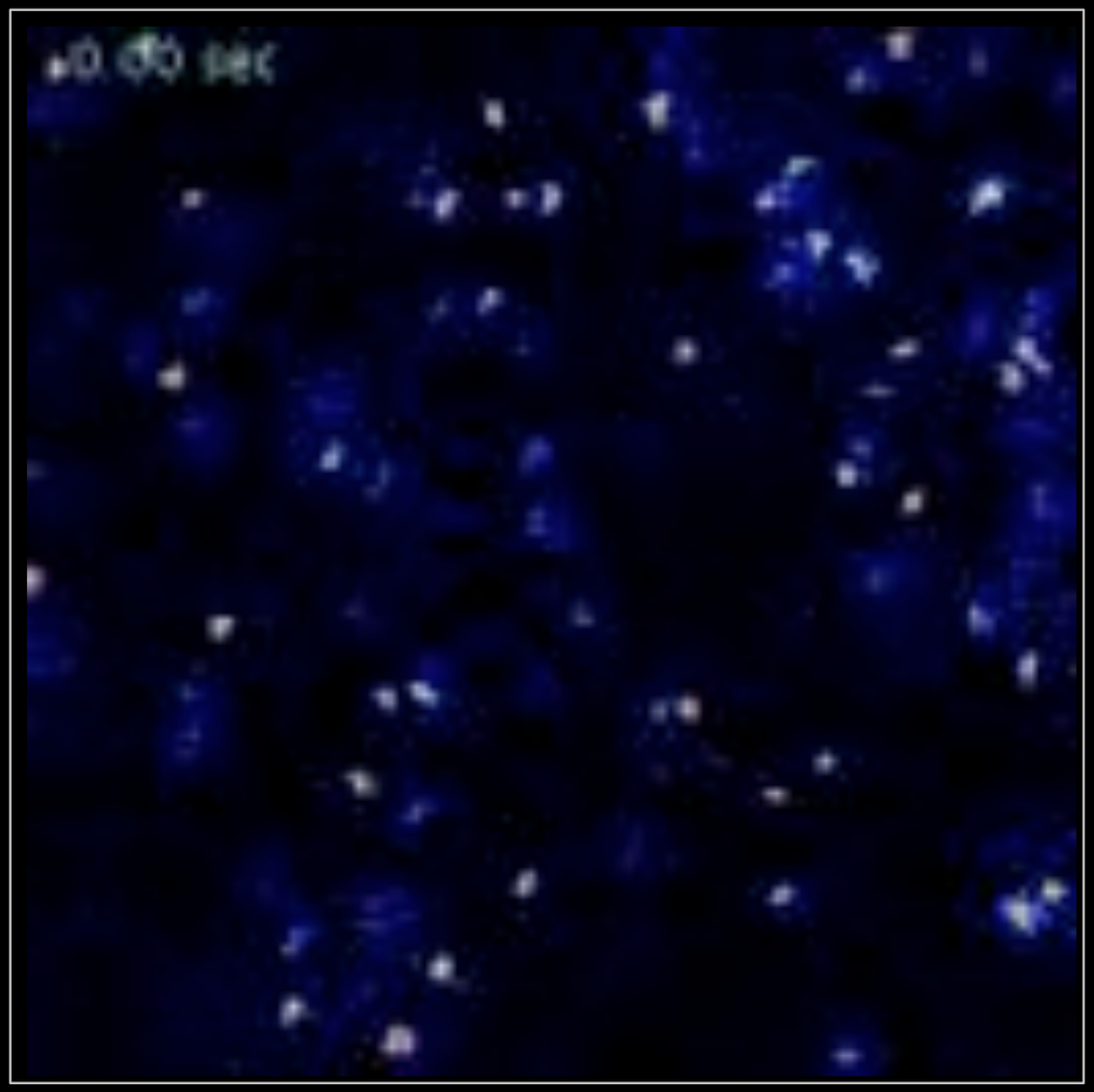
Section through reconstructed electron tomogram





- Hemagglutinin
- Neuraminidase
- Transmembrane anchor
- Envelope

By comparison... this is what you might see in a light microscope
(confocal, fluorescence)



Cy3-labeled 55nm diameter HK97 Prohead-II (PEGylated)
~150pM in SlowFade Gold (~65% glycerol)

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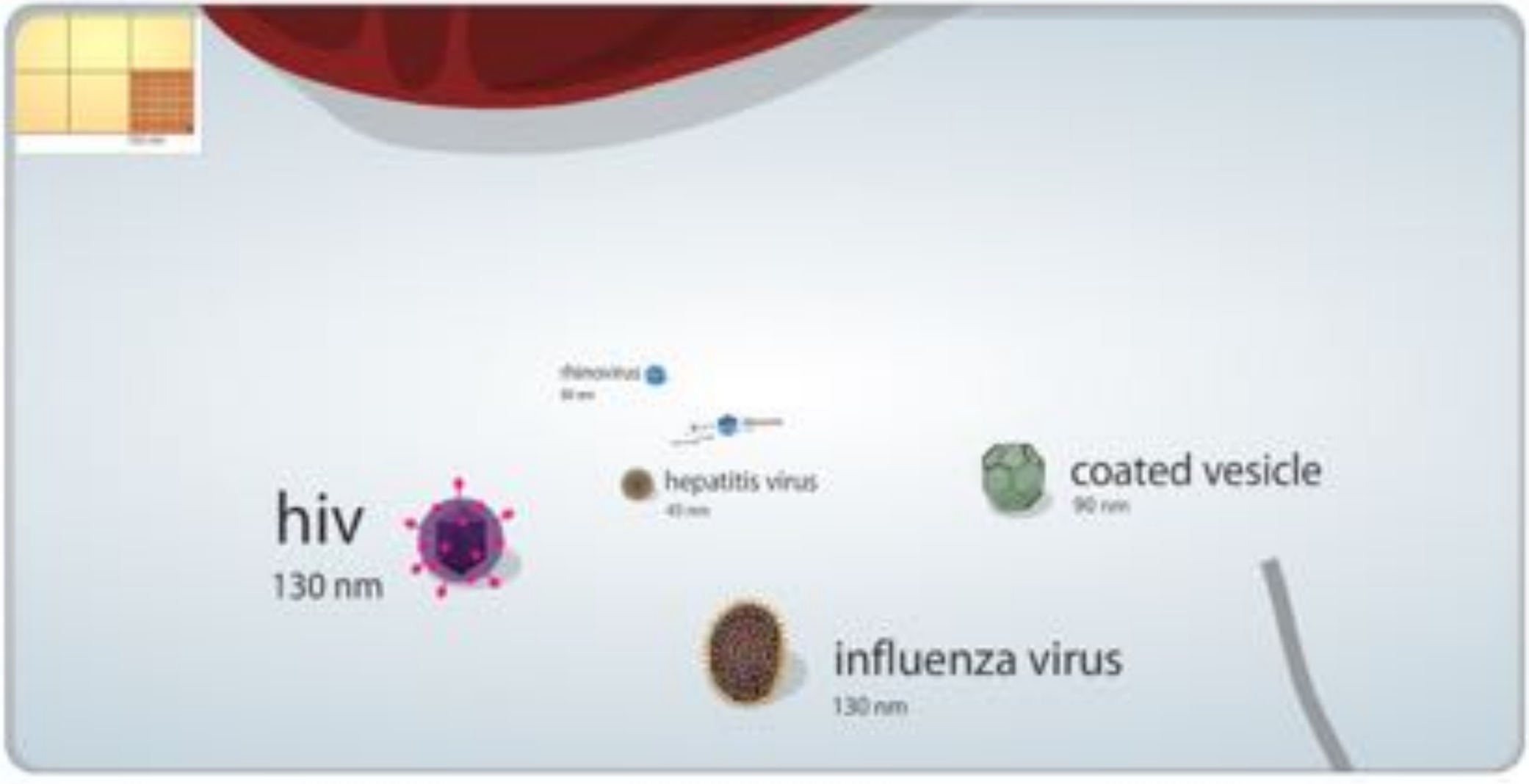

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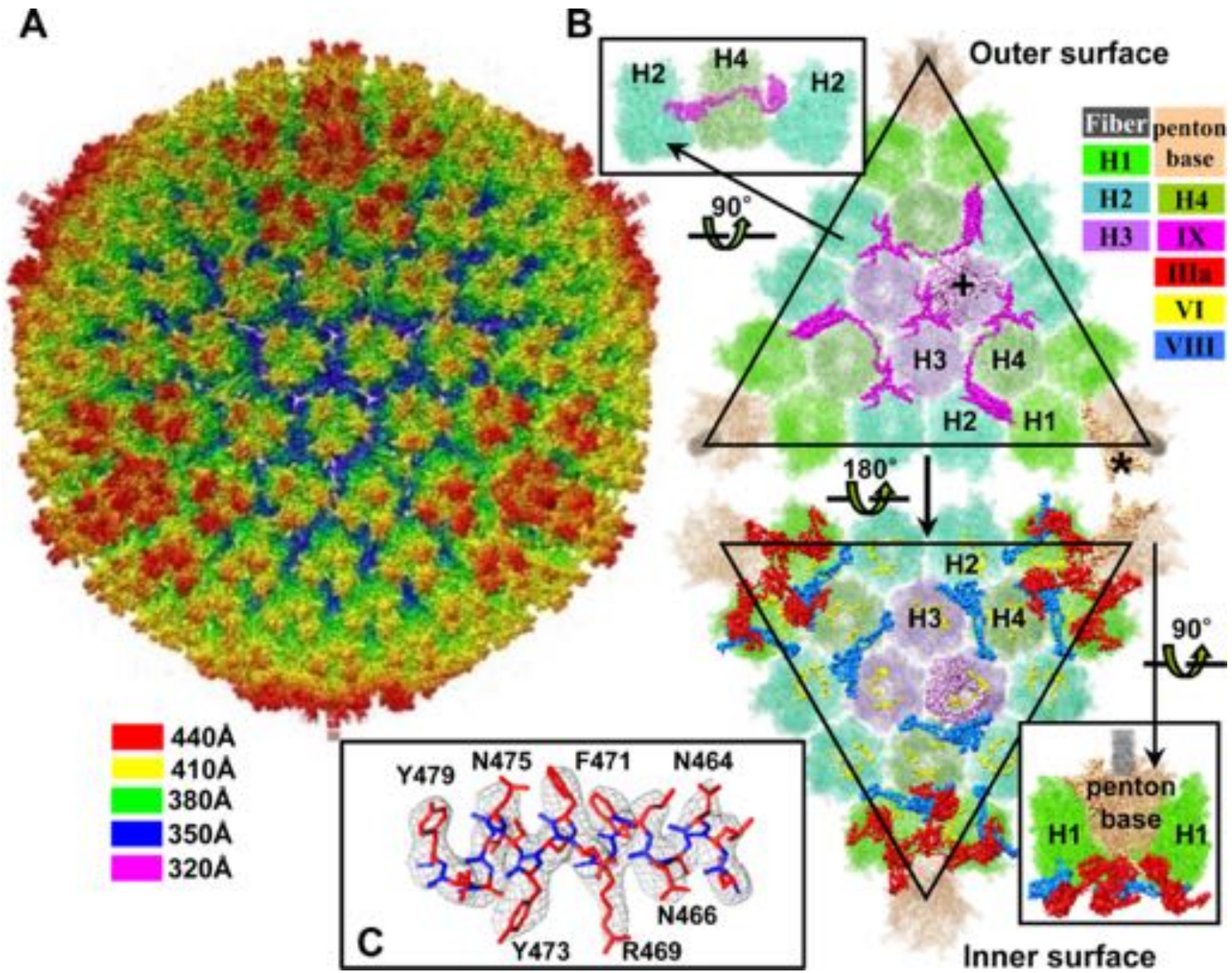
Size scales in biology

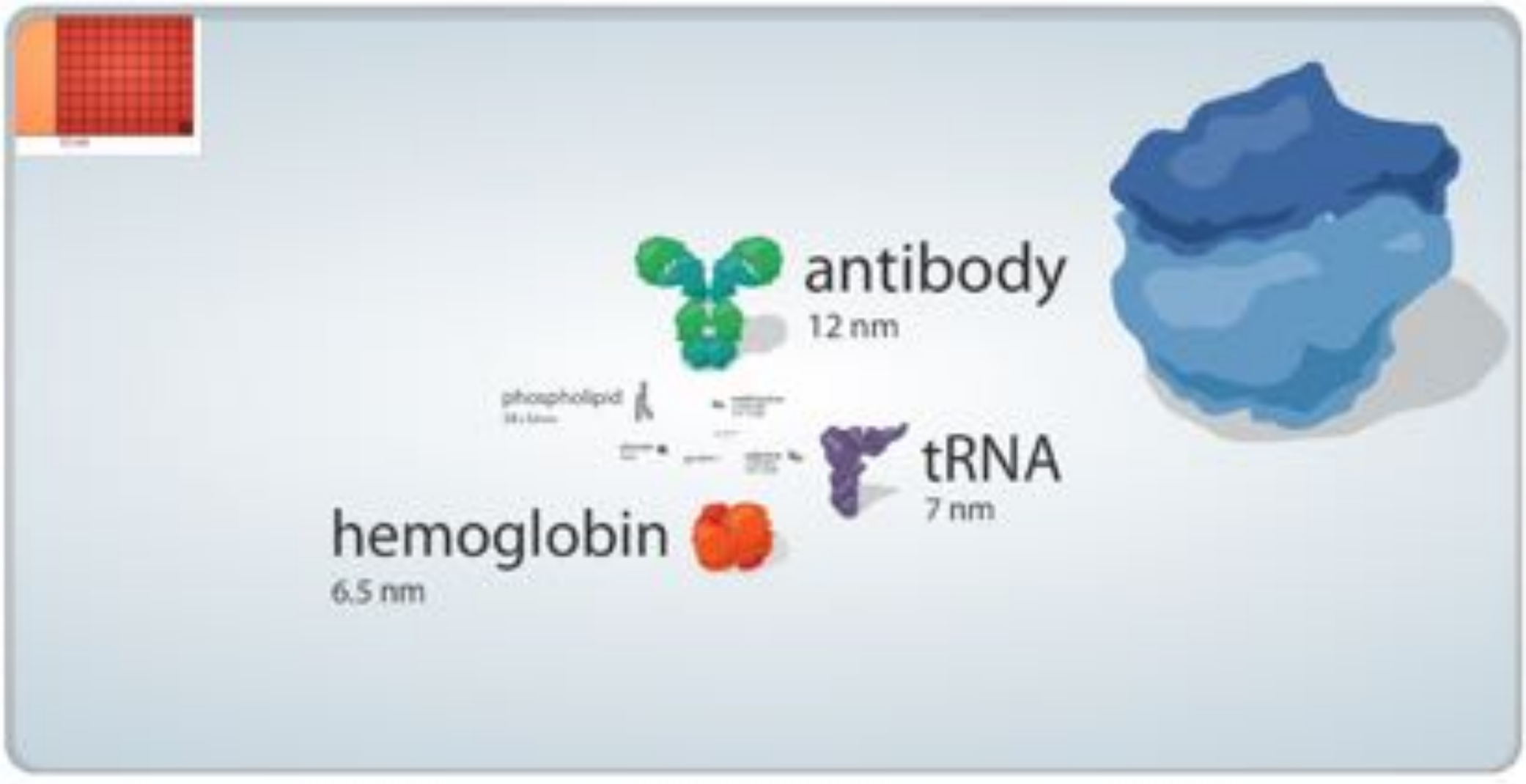
Single-particle cryo-electron microscopy can provide near-atomic resolution of complex macromolecular assemblies (e.g. adenovirus)



Liu et al., *Science* (2010)

Size scales in biology

3-D structures of some of these can be determined by cryo-EM with image reconstruction to ~2-3 Å resolution!



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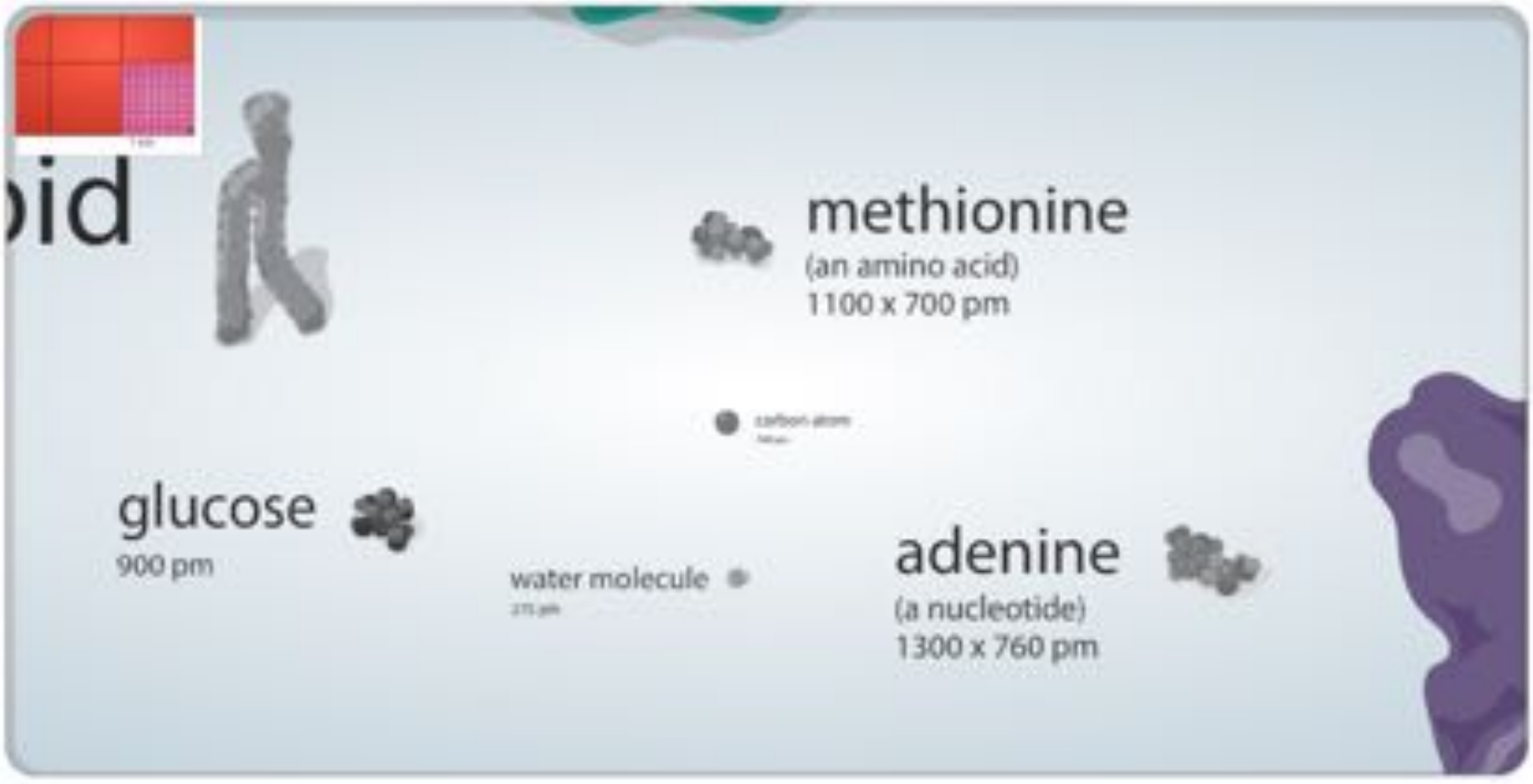

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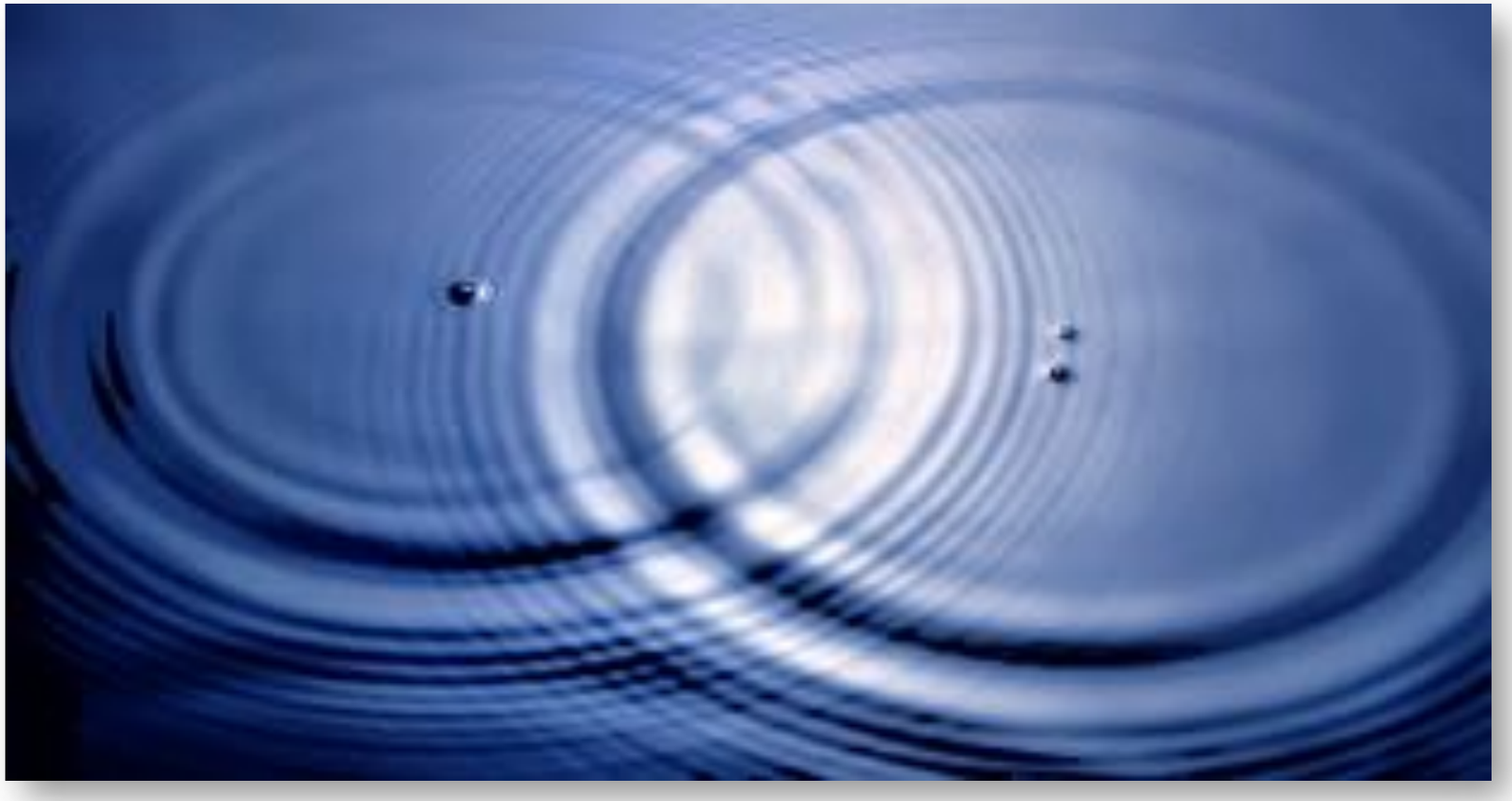
Electrons are waves (and particles)...



...and one can construct an electron microscope that operates analogously to a light microscope, but provides resolution of fine structures to 1 Å instead of ~2000 Å

For a 200kV electron, λ~0.025Å, but electron optics limits practical resolution to ~1Å

Electrons

- Electrons behave as waves
  - amplitude
  - phase
  - undergo diffraction and scattering when they interact with matter

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- The charged nature of electrons make it amenable to bending by electromagnetic lenses.

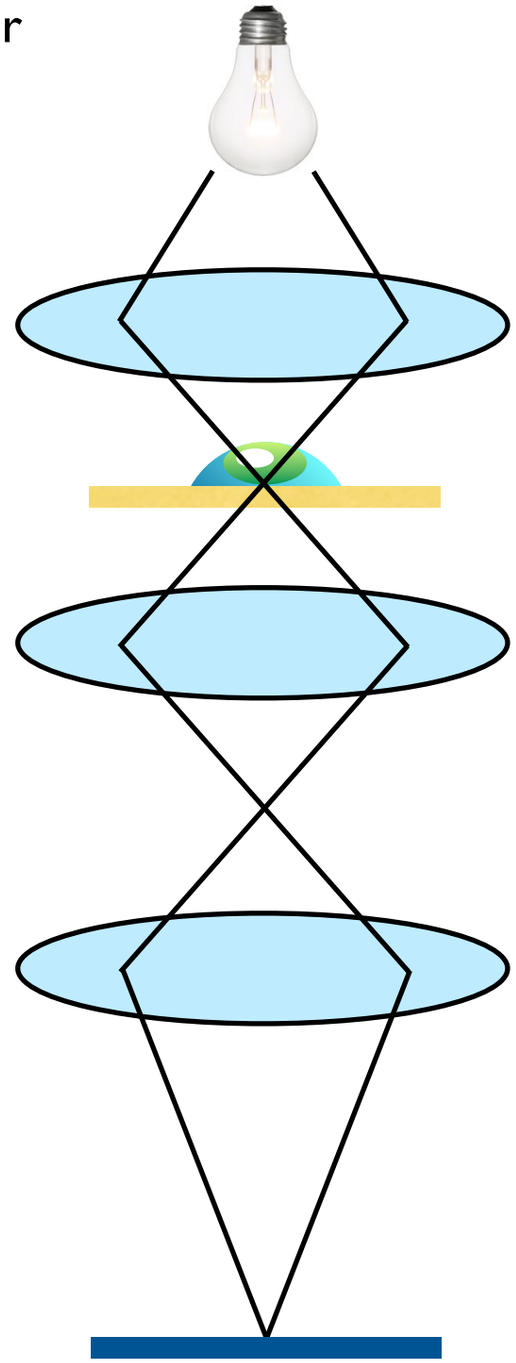
- 100-300kV electrons are also ionizing radiation, rapidly degrades the sample

- Electrons can interact with air molecules as well as the specimen, so the beam and specimen are kept under high vacuum

General similarities of light vs transmission electron microscopes (TEM)

Light source:
Lightbulb
Laser

optical lenses
bend light path



Condensor lens

SPECIMEN

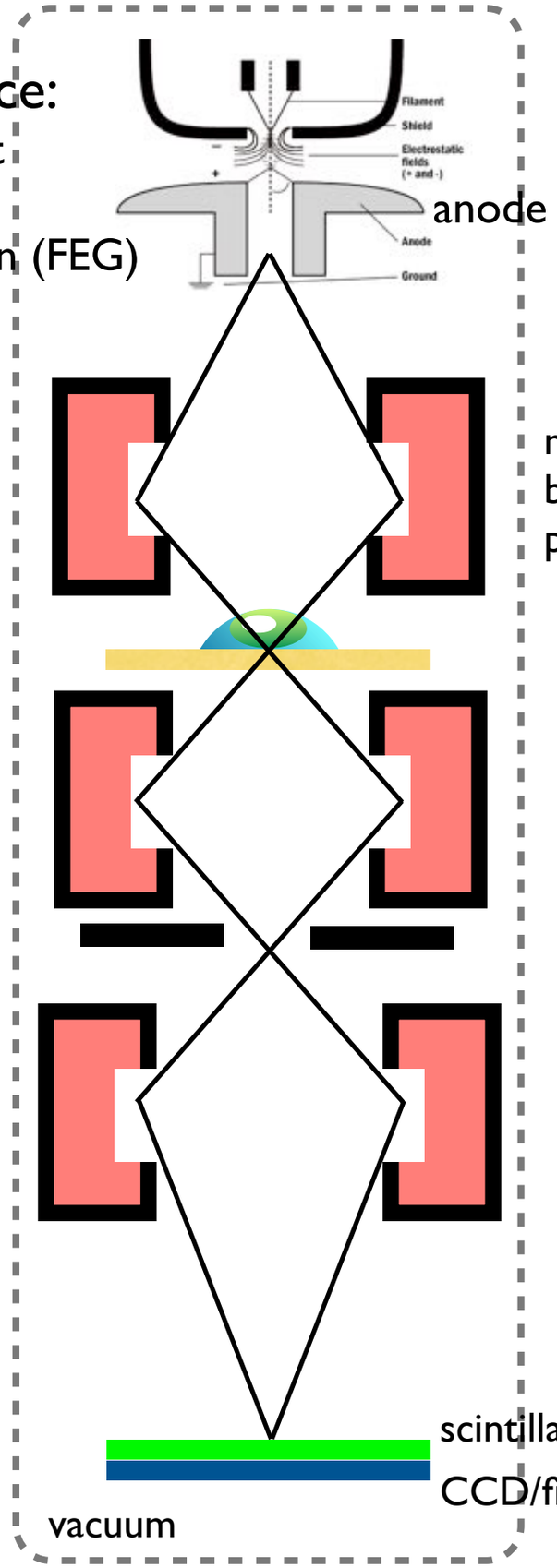
Objective lens

Objective aperture

Projection lens

Detector (CCD, eyeball)

Electron source:
Tungsten filament
LaB_6 filament
Field emission gun (FEG)



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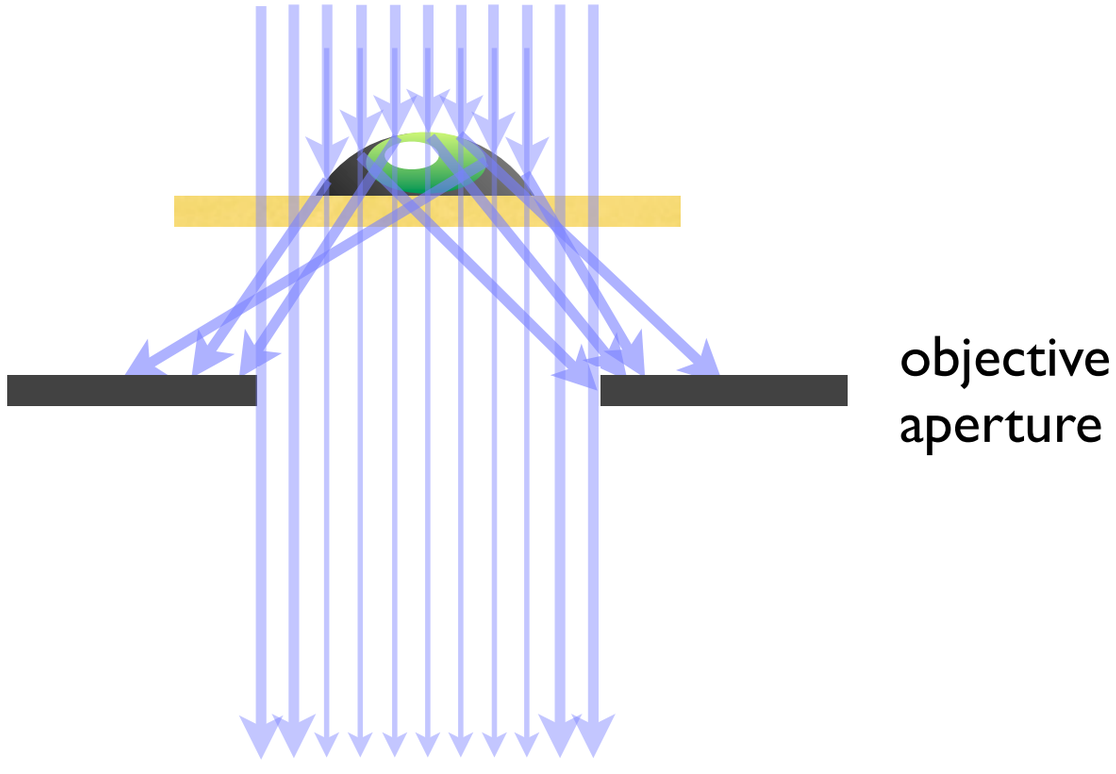
- <u>Amplitude contrast</u>: The difference across the sample in total transmitted (and detected) intensity of the electron waves is imaged.
  - Dominant effect in negative stain-EM. Electrons are scattered by the heavy metals and hence not transmitted along the axial path to the detector; the objective aperture also removes much of these electrons.



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- Phase contrast: Interference between unscattered and elastically scattered electrons that have experienced a phase shift due to interaction with the specimen as well as electron optics.
  - Primary effect in cryo-EM. By imaging under focus, we emphasize phase contrast and can distinguish low frequency (large scale) features more clearly, but we lose high resolution information.

Negative stain TEM

- A specimen is adsorbed to a carbon coated grid and blanketed in a layer of electron dense heavy metal “stain”.

Ohi M et al., *Biol. Proced. Online, 2004*

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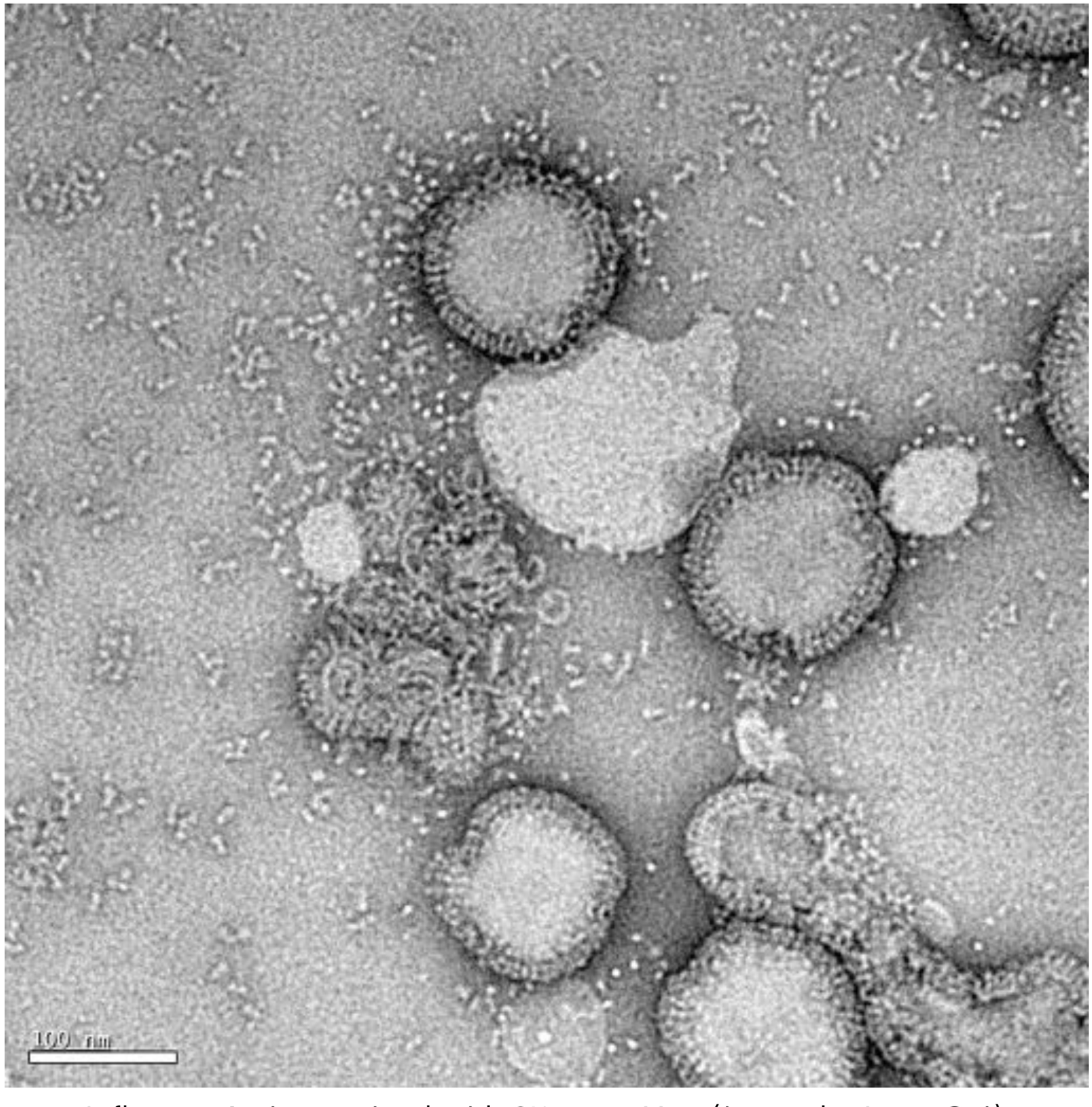
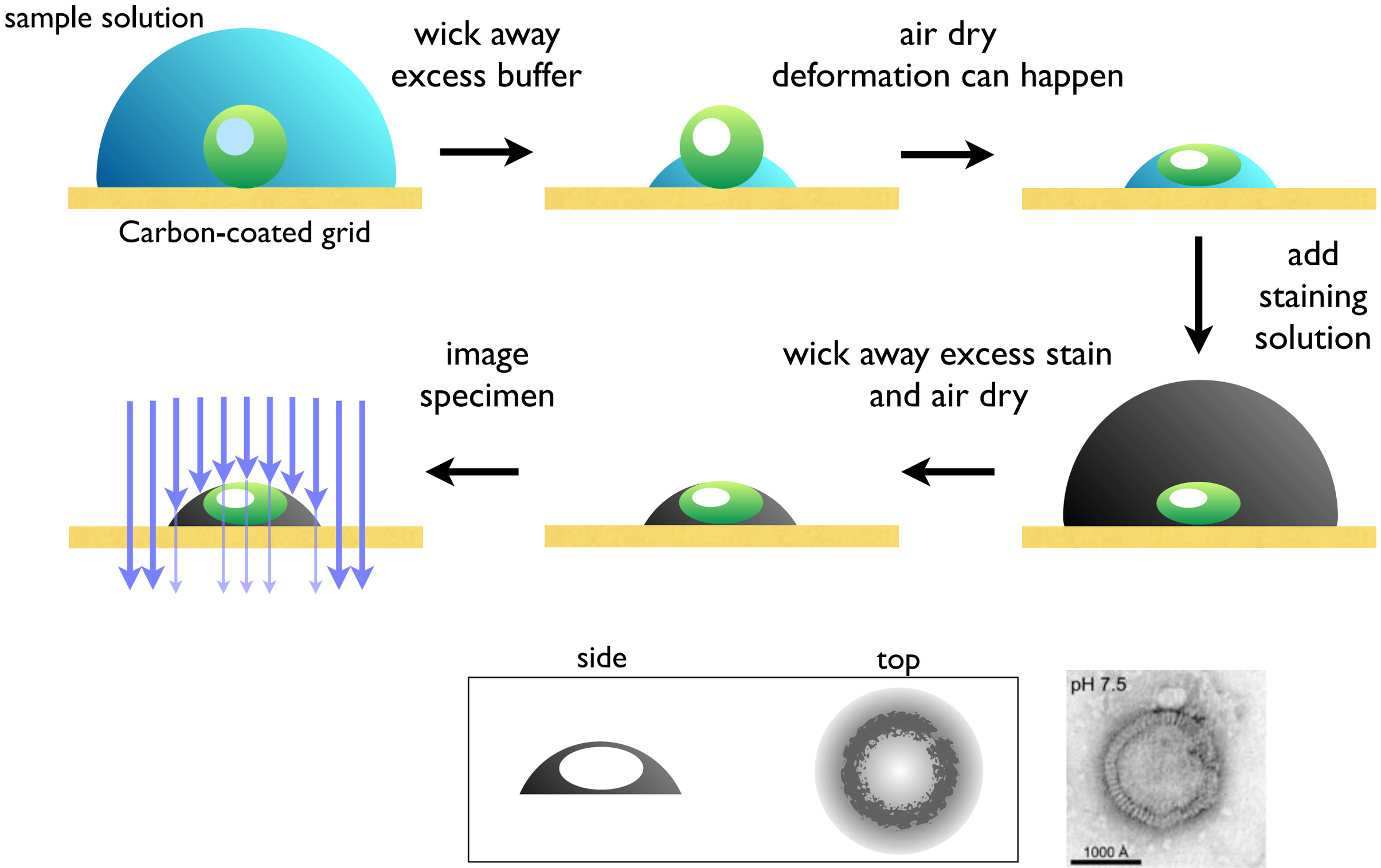


Influenza A virus stained with 2% nano-Van (image by Long Gui)

Overview of negative stain TEM



Sample requirements

- Depending on size of macromolecule, ~3µl of 0.01-1mg/ml to grid.

- Purity not as critical (though it helps). Neg-stain EM is often used to determine how pure or stable prep of a complex or macromolecule may be.

Electron dense heavy metals used in staining

1 H Hydrogen 1.00794																	2 He Helium 4.003
3 Li Lithium 6.941	4 Be Beryllium 9.012182											5 B Boron 10.811	6 C Carbon 12.0107	7 N Nitrogen 14.00674	8 O Oxygen 15.9994	9 F Fluorine 18.9984032	10 Ne Neon 20.1797
11 Na Sodium 22.989770	12 Mg Magnesium 24.3050											13 Al Aluminium 26.981538	14 Si Silicon 28.0855	15 P Phosphorus 30.973761	16 S Sulfur 32.066	17 Cl Chlorine 35.4527	18 Ar Argon 39.948
19 K Potassium 39.0983	20 Ca Calcium 40.078	21 Sc Scandium 44.955910	22 Ti Titanium 47.867	23 V Vanadium 50.9415	24 Cr Chromium 51.9961	25 Mn Manganese 54.938049	26 Fe Iron 55.845	27 Co Cobalt 58.933200	28 Ni Nickel 58.6934	29 Cu Copper 63.546	30 Zn Zinc 65.39	31 Ga Gallium 69.723	32 Ge Germanium 72.61	33 As Arsenic 74.92160	34 Se Selenium 78.96	35 Br Bromine 79.904	36 Kr Krypton 83.80
37 Rb Rubidium 85.4678	38 Sr Strontium 87.62	39 Y Yttrium 88.90585	40 Zr Zirconium 91.224	41 Nb Niobium 92.90638	42 Mo Molybdenum 95.94	43 Tc Technetium (98)	44 Ru Ruthenium 101.07	45 Rh Rhodium 102.90550	46 Pd Palladium 106.42	47 Ag Silver 107.8682	48 Cd Cadmium 112.411	49 In Indium 114.818	50 Sn Tin 118.710	51 Sb Antimony 121.760	52 Te Tellurium 127.60	53 I Iodine 126.90447	54 Xe Xenon 131.29
55 Cs Cesium 132.90545	56 Ba Barium 137.327	57 La Lanthanum 138.9055	72 Hf Hafnium 178.49	73 Ta Tantalum 180.9479	74 W Tungsten 183.84	75 Re Rhenium 186.207	76 Os Osmium 190.23	77 Ir Iridium 192.217	78 Pt Platinum 195.078	79 Au Gold 196.96655	80 Hg Mercury 200.59	81 Tl Thallium 204.3833	82 Pb Lead 207.2	83 Bi Bismuth 208.98038	84 Po Polonium (209)	85 At Astatine (210)	86 Rn Radon (222)
87 Fr Francium (223)	88 Ra Radium (226)	89 Ac Actinium (227)	104 Rf Rutherfordium (261)	105 Db Dubnium (262)	106 Sg Seaborgium (263)	107 Bh Bohrium (262)	108 Hs Hassium (265)	109 Mt Meitnerium (266)	110 (269)	111 (272)	112 (277)	113	114				

58 Ce Cerium 140.116	59 Pr Praseodymium 140.90765	60 Nd Neodymium 144.24	61 Pm Promethium (145)	62 Sm Samarium 150.36	63 Eu Europium 151.964	64 Gd Gadolinium 157.25	65 Tb Terbium 158.92534	66 Dy Dysprosium 162.50	67 Ho Holmium 164.93032	68 Er Erbium 167.26	69 Tm Thulium 168.93421	70 Yb Ytterbium 173.04	71 Lu Lutetium 174.965
90 Th Thorium 232.0381	91 Pa Protactinium 231.03588	92 U Uranium 238.0289	93 Np Neptunium (237)	94 Pu Plutonium (244)	95 Am Americium (243)	96 Cm Curium (247)	97 Bk Berkelium (247)	98 Cf Californium (251)	99 Es Einsteinium (252)	100 Fm Fermium (257)	101 Md Mendelevium (258)	102 No Nobelium (259)	103 Lr Lawrencium (262)

Commonly used electron dense negative stains

STAIN	Conc (w/v)	pH range (check w/ pH paper)	Notes
Sodium Phosphotungstate (PTA)	1-3%	5-8	PTA can perturb membranes. Lower contrast.
Uranyl Acetate (UA)	1-3%	4.2-4.5	Good contrast, fine grain. Acidic. Positive staining (affinity for protein, sialic acid, lipid headgroups). Can precipitate with phosphate.
Sodium Silicotungstate (SST)	1-5%	5-8	Compatible with membranes. Good contrast and grain. Make stain fresh before use, check pH.
Ammonium Molybdate	1-2%	5-7	Lower contrast. Good for osmotically sensitive organelles.
Methylamine Tungstate (Nano-W)	2%	~7	Compatible with membranes. Decent contrast, good grain.

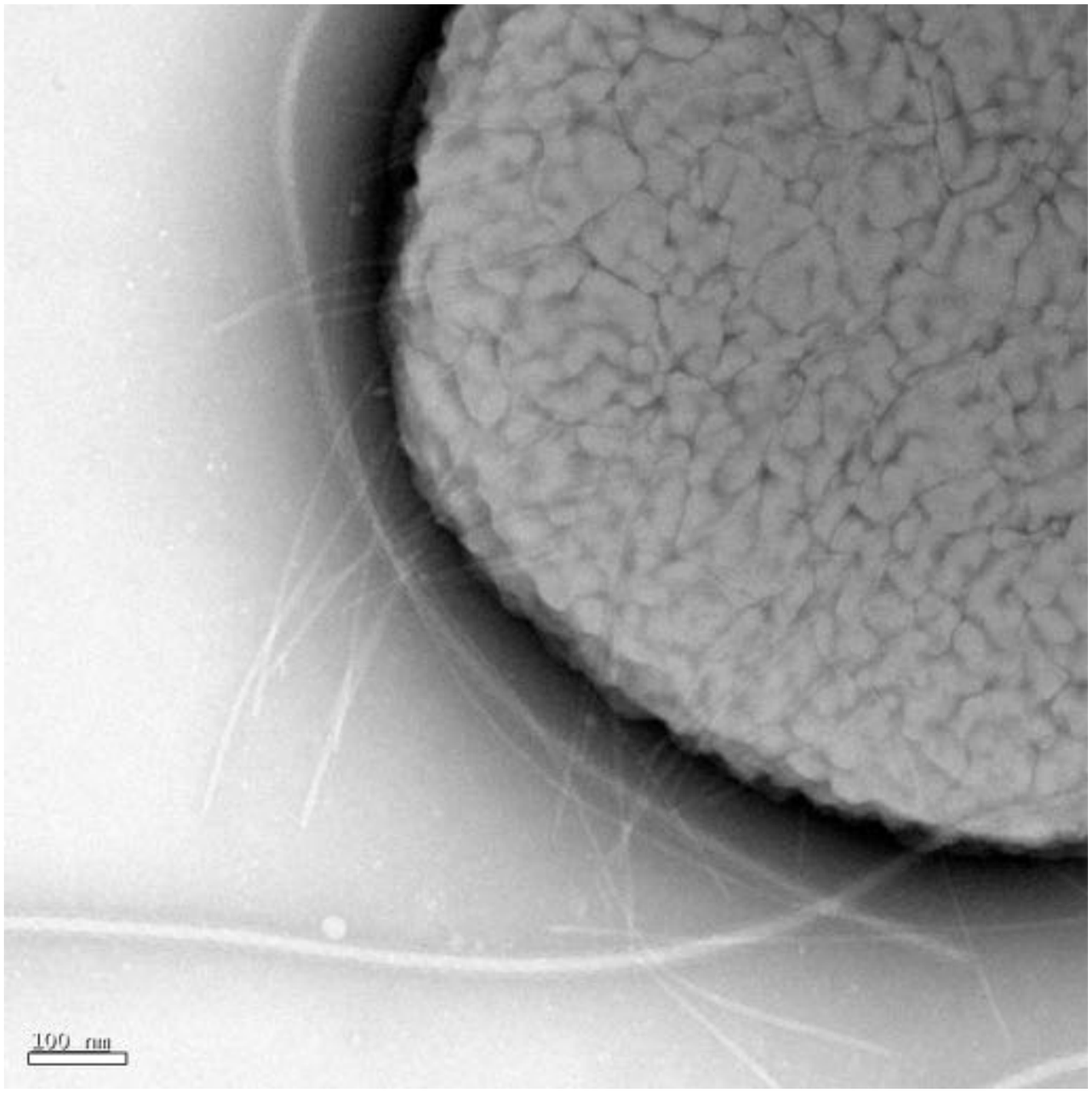
Other stains you may encounter:

- Uranyl formate (pH~4), uranyl oxalate (pH 5-7)
- Methylamine vanadate (pH 8)

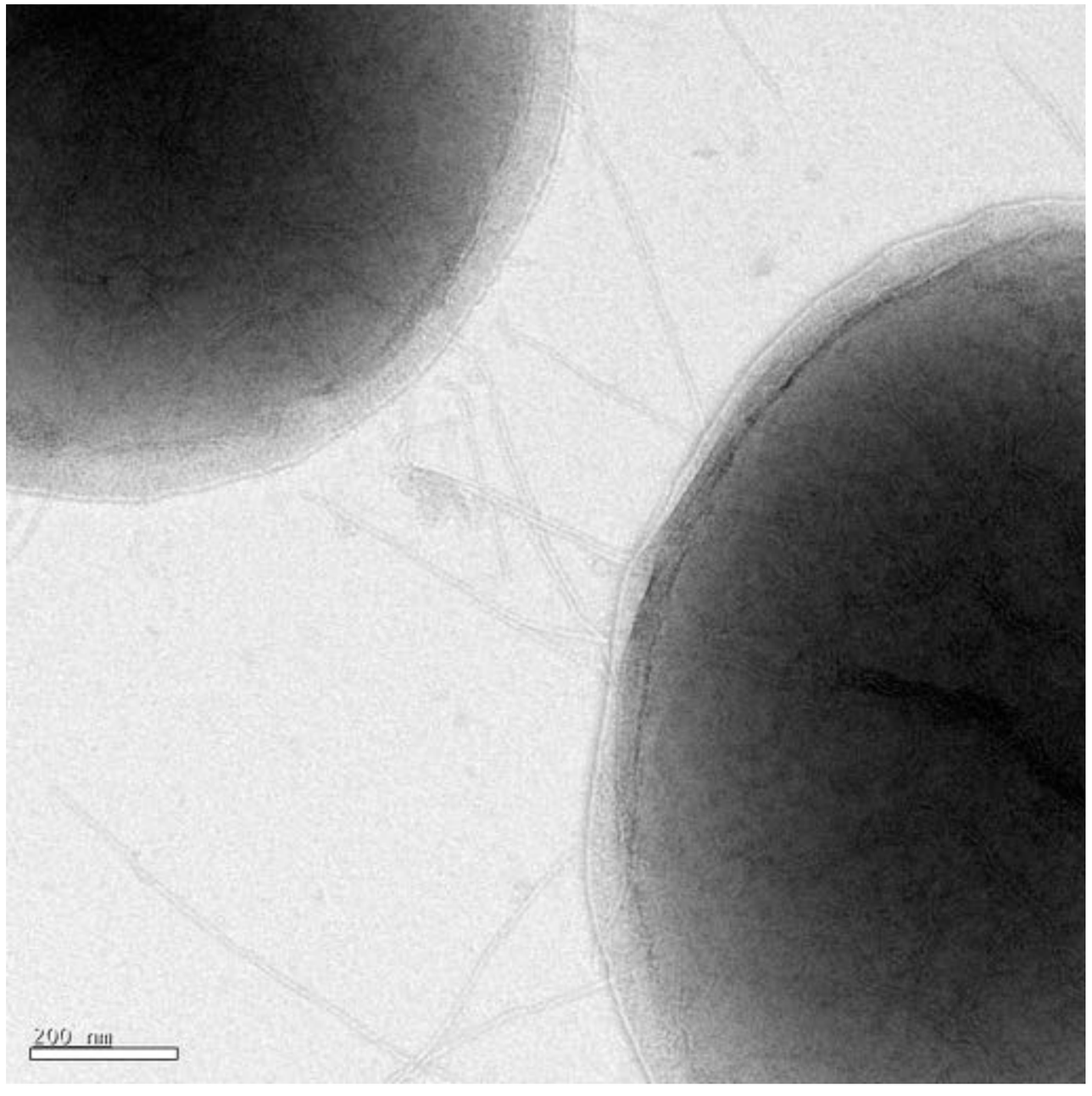
http://web.path.ox.ac.uk/~bioimaging/bitm/instructions_and_information/EM/neg_stain.pdf

The difference a stain can make

E. coli with nano-W



E. coli with nano-Van



- Nano-W (atomic weight of W = 183.8) cannot penetrate into periplasmic space whereas Nano-Van (atomic weight of V = 59.9) appears to be able to go pass through the outer membrane..

Images by Long Gui

Negative stain TEM

- A specimen is adsorbed to a carbon coated grid and blanketed in a layer of electron dense heavy metal "stain".

- Amplitude contrast: The transmitted image shows differential transmission of electrons through varying thicknesses of stain.

- Can be useful for observing complex formation, oligomerization, general shape and global contours, sample "purity"

- Can be used for 3-D reconstruction: limited to nanometer resolutions

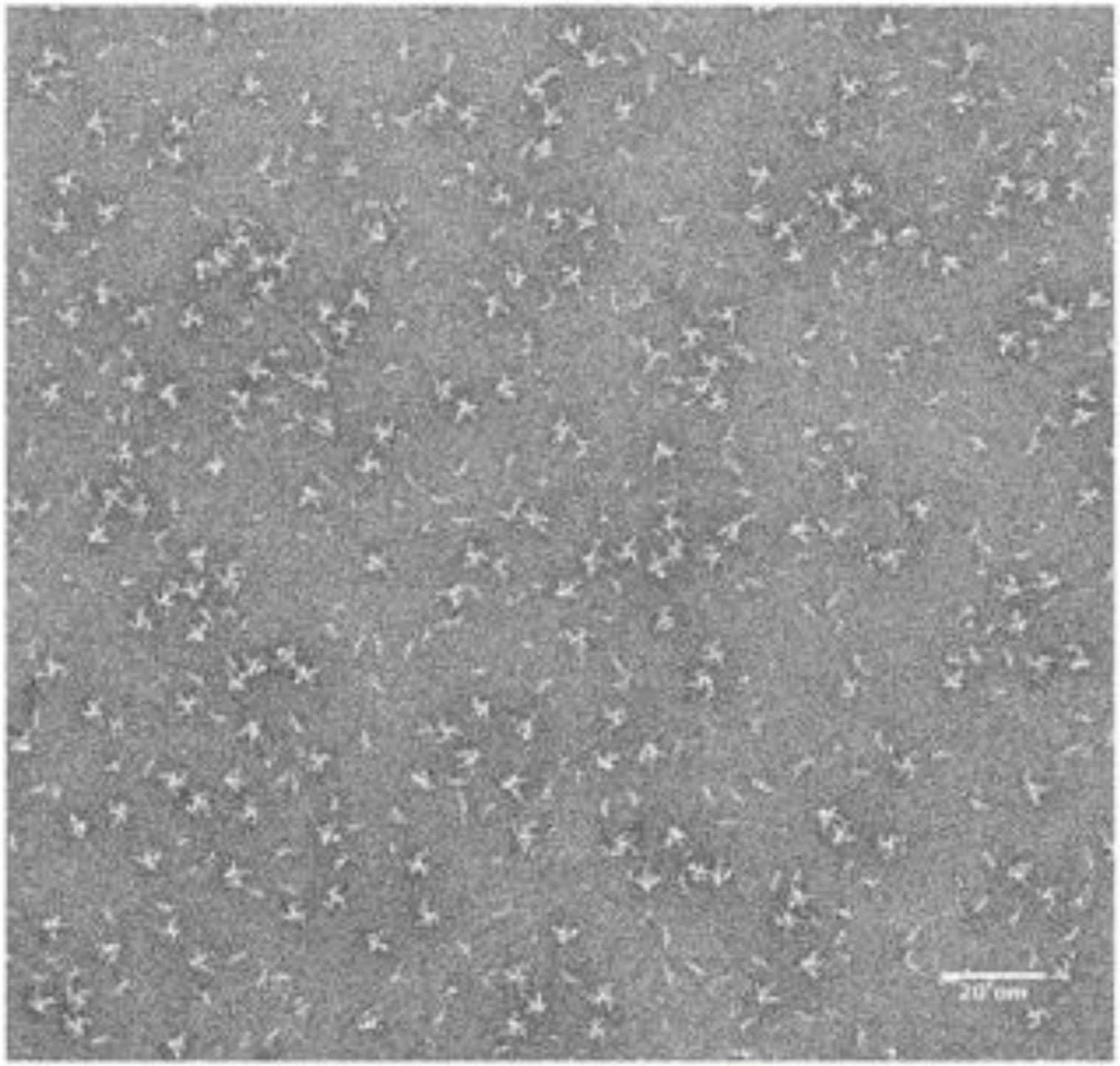
- Samples are dehydrated and frequently end up flattened

- One does not obtain an image of internal organization of the macromolecule

Ohi M et al., *Biol. Proced. Online, 2004*

Example of a negative stain EM reconstruction

Raw negative stain EM micrograph: 3:1 Fab:HIV Env trimer



James Williams and Hans Verkerke

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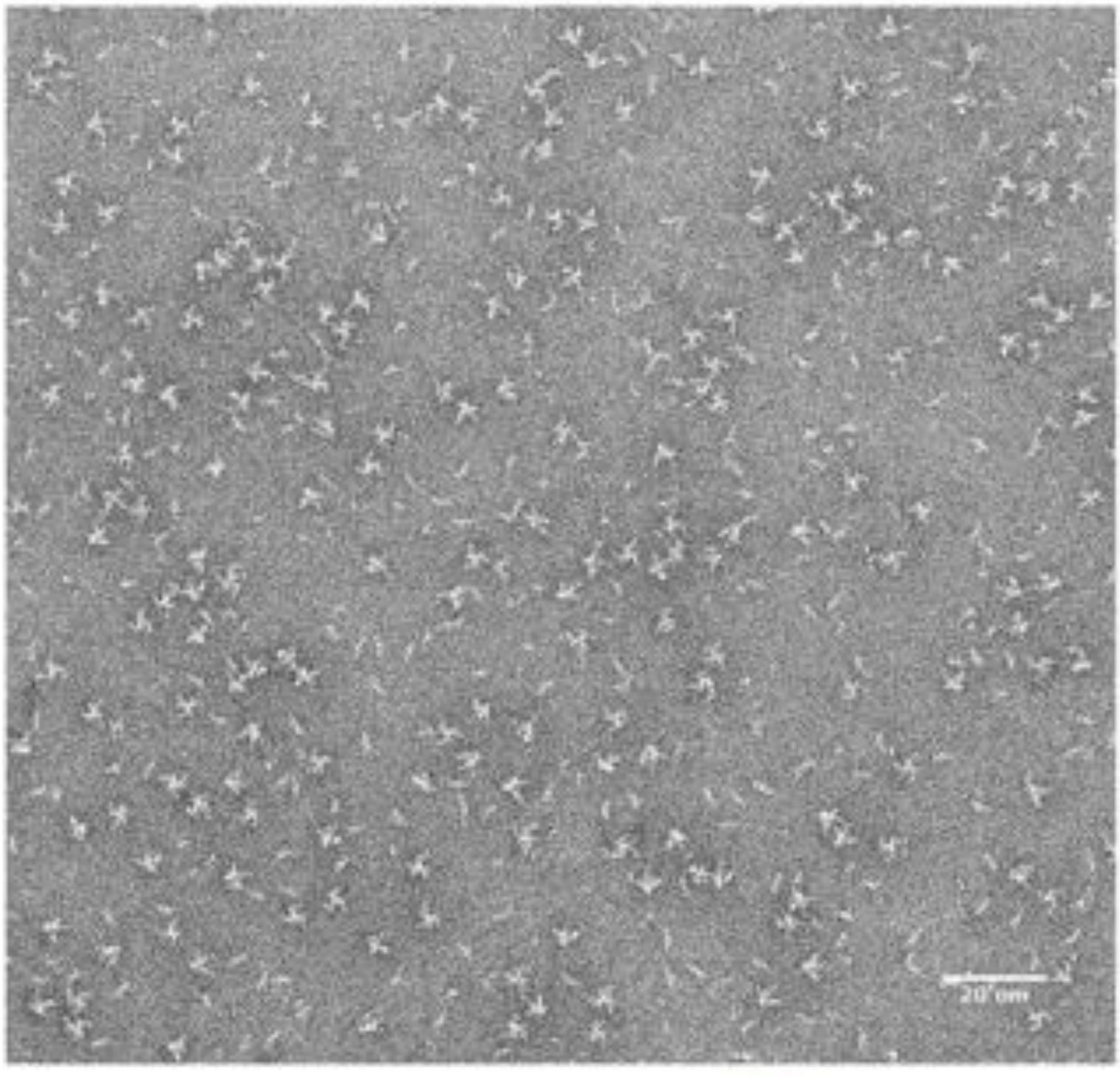
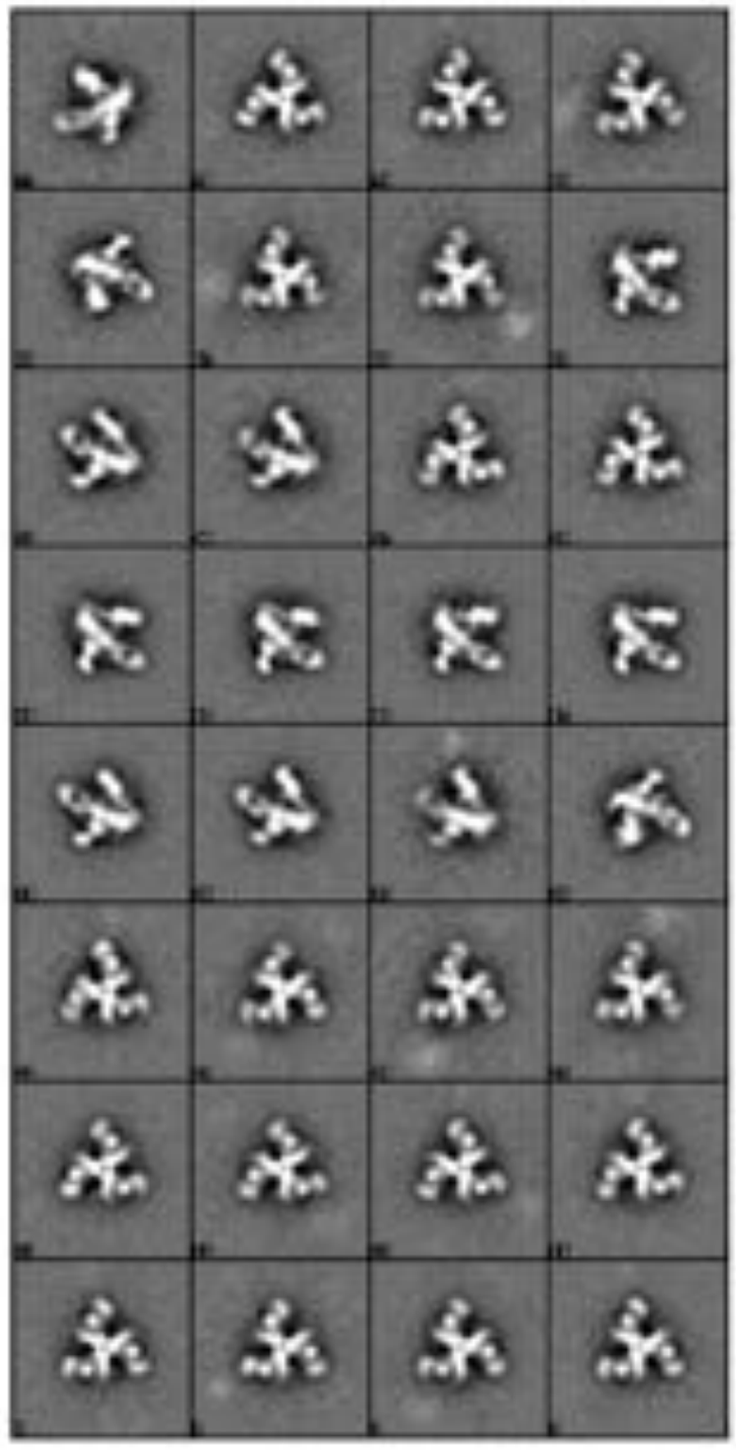


“Class averages”



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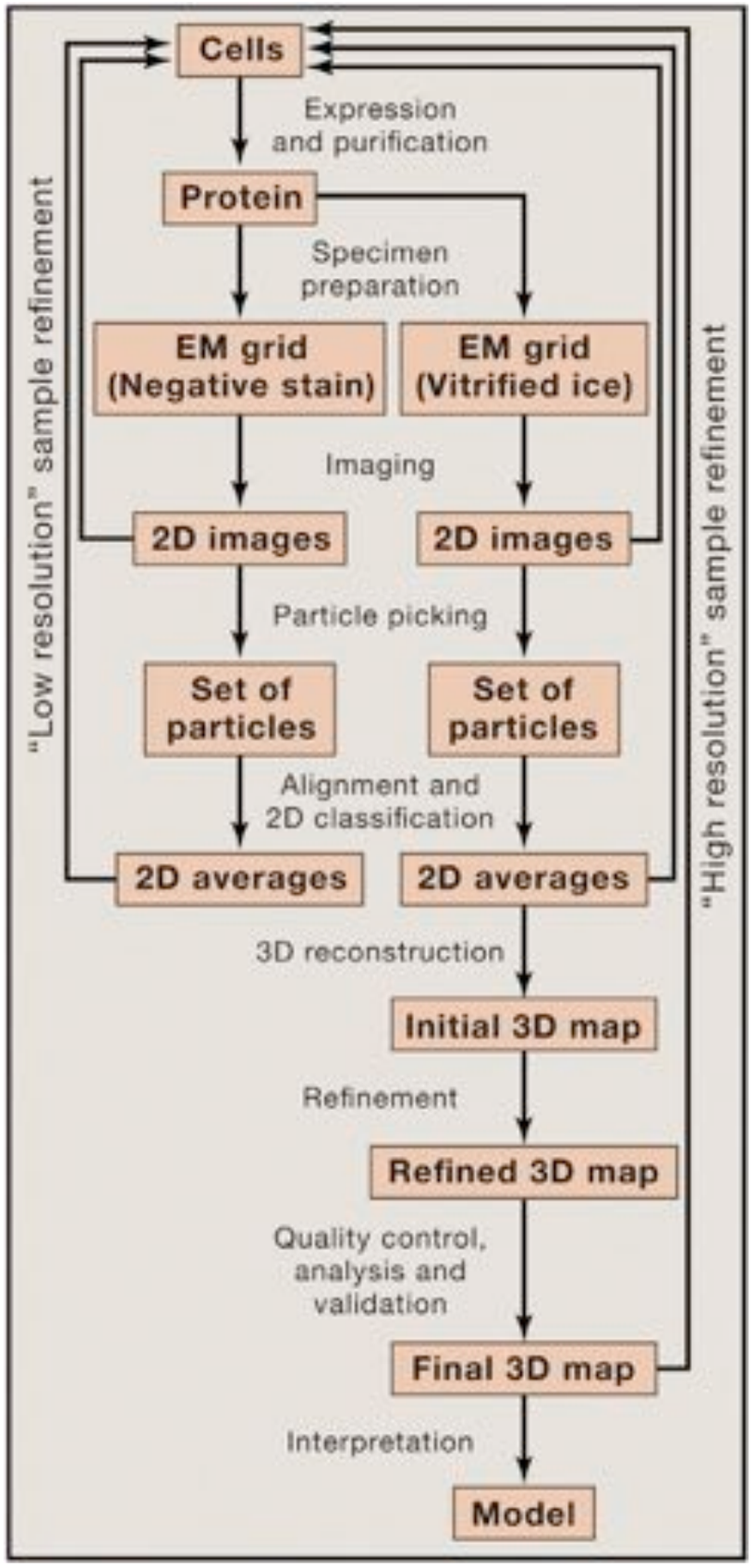
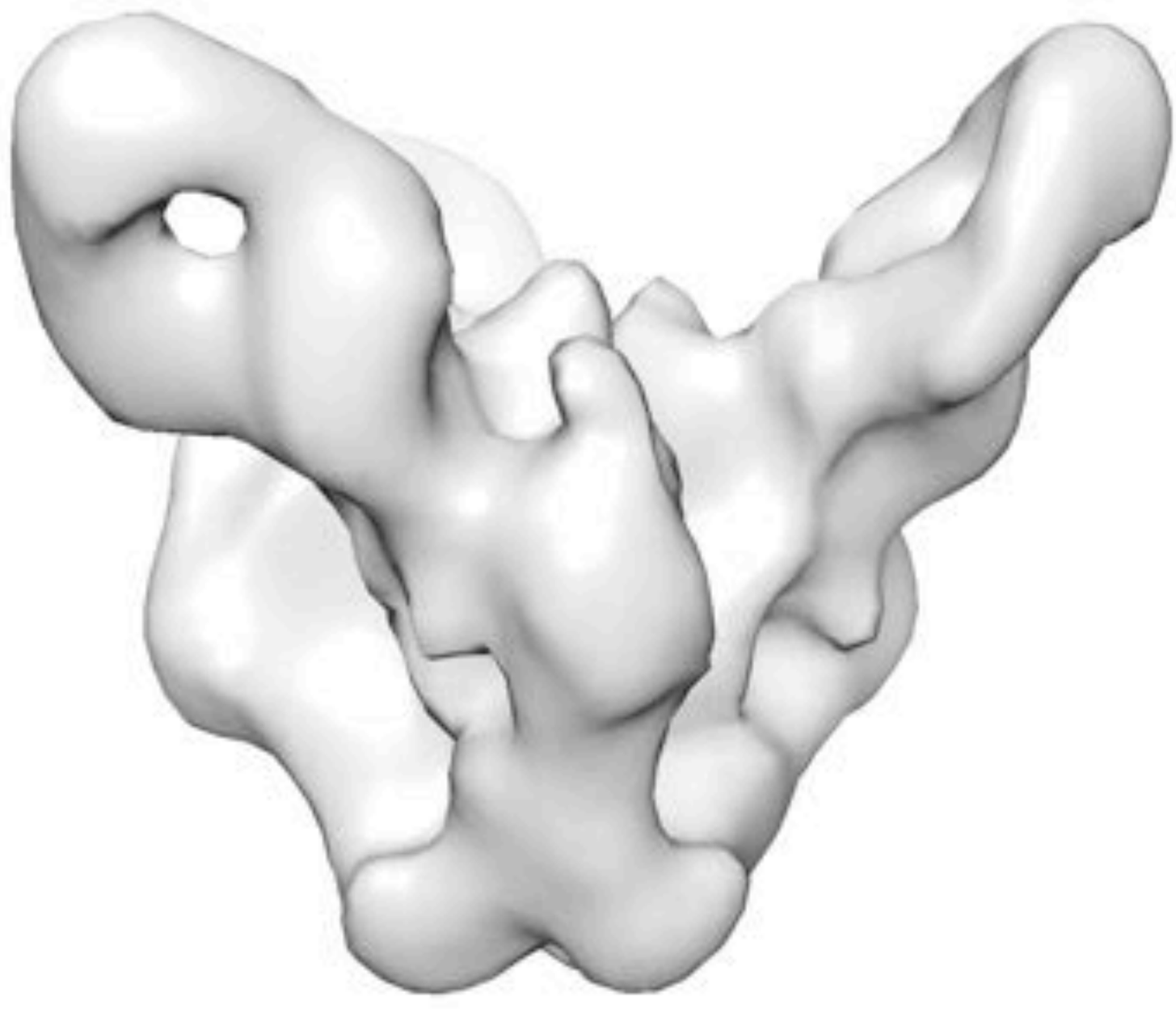


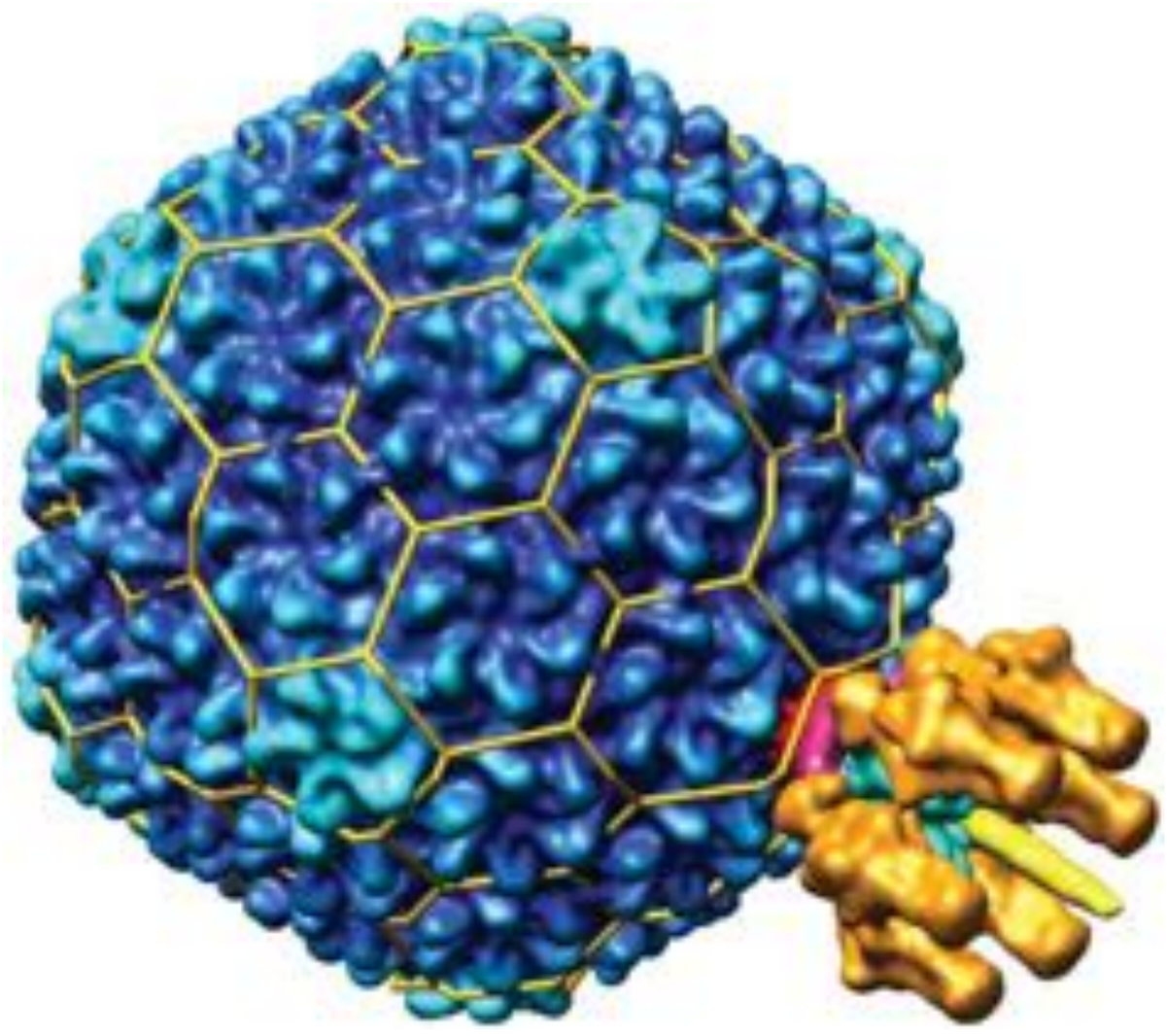
Cheng Y et al., *Cell* (2015)

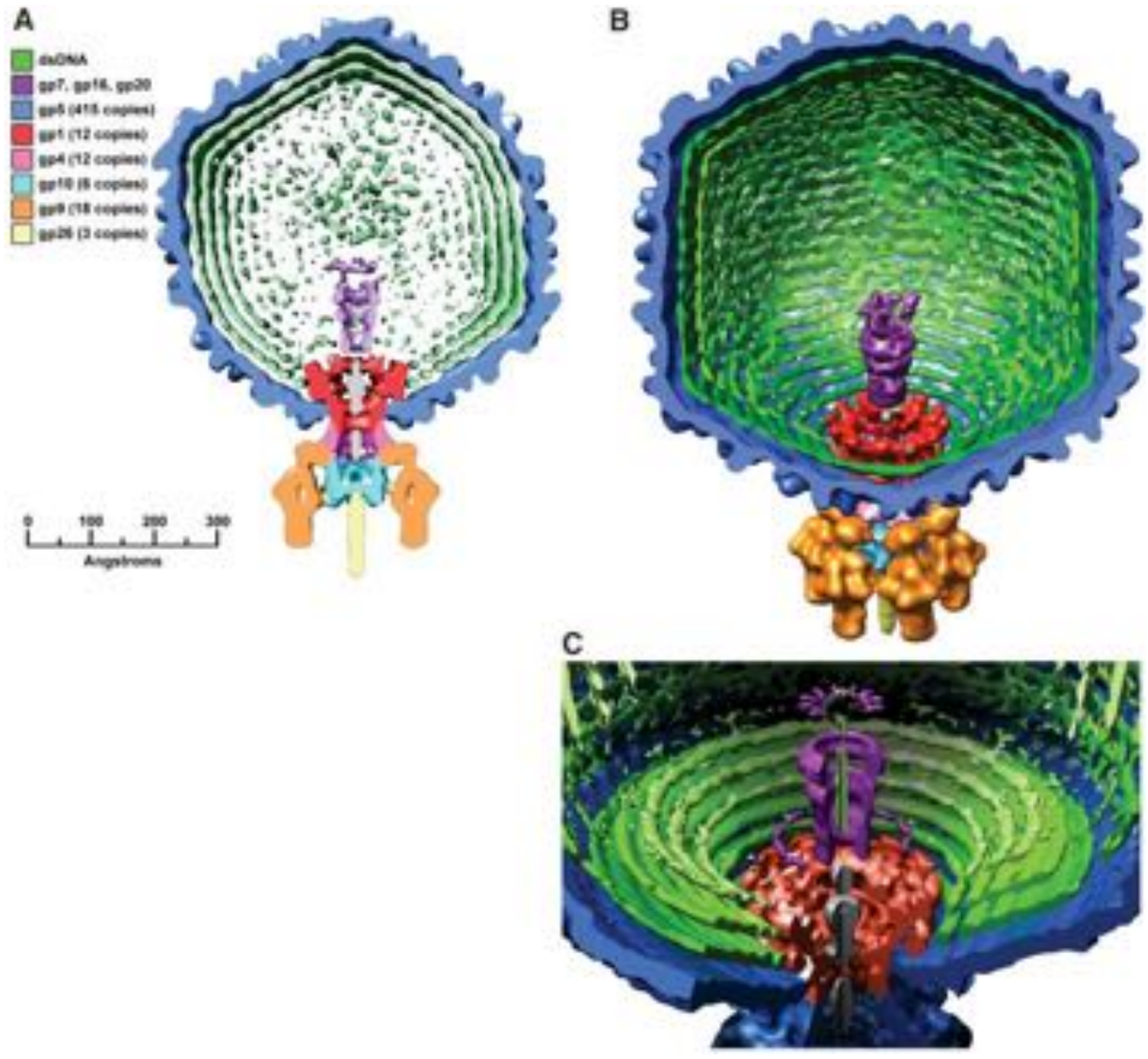
Example of a negative stain EM reconstruction



James Williams and Hans Verkerke

Cryogenic TEM (cryo-EM)





Lander GC et al., Science, 2006

Cryo-EM

- Specimen is embedded in vitreous ice. Samples flash frozen under native conditions (in buffer), no stain

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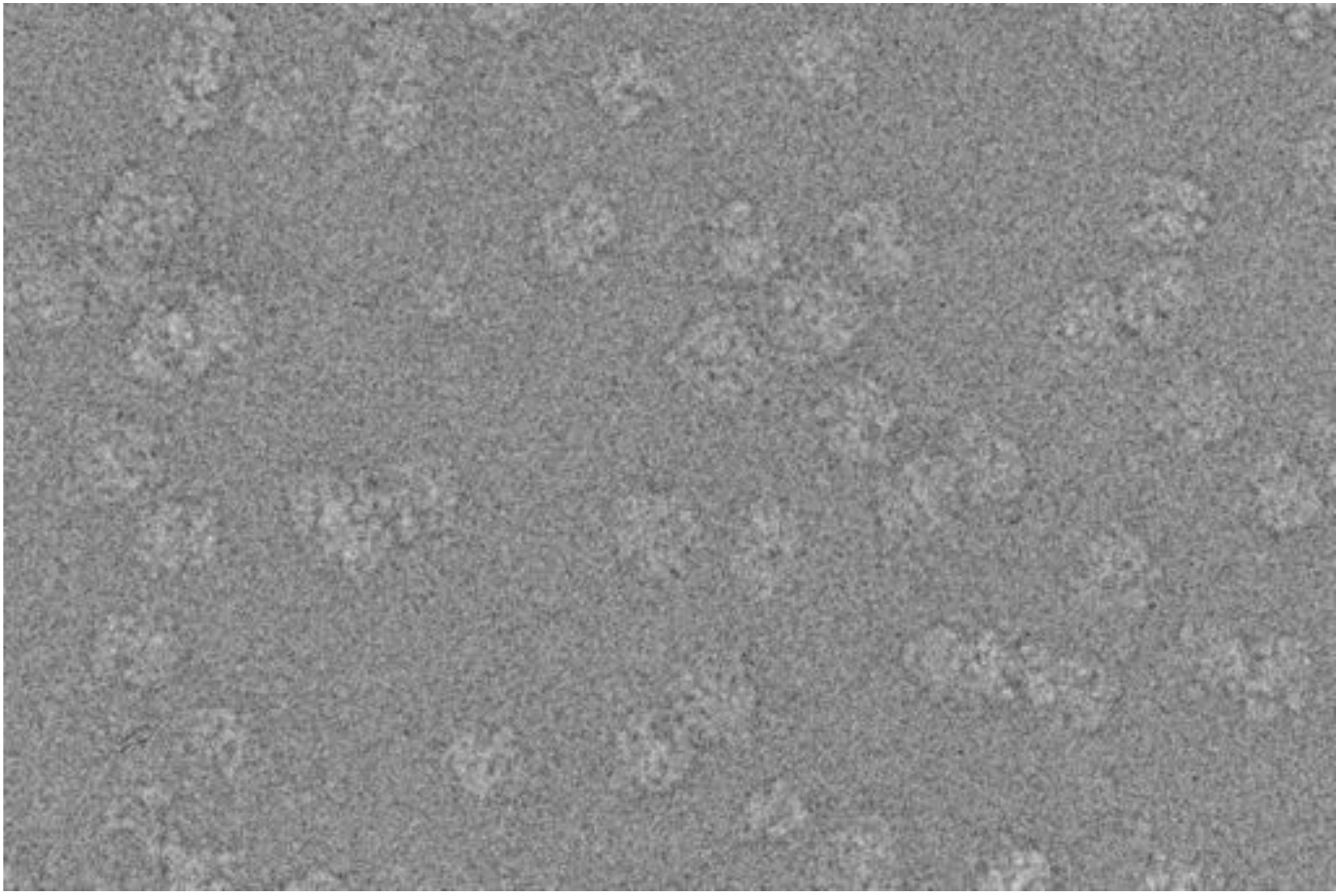
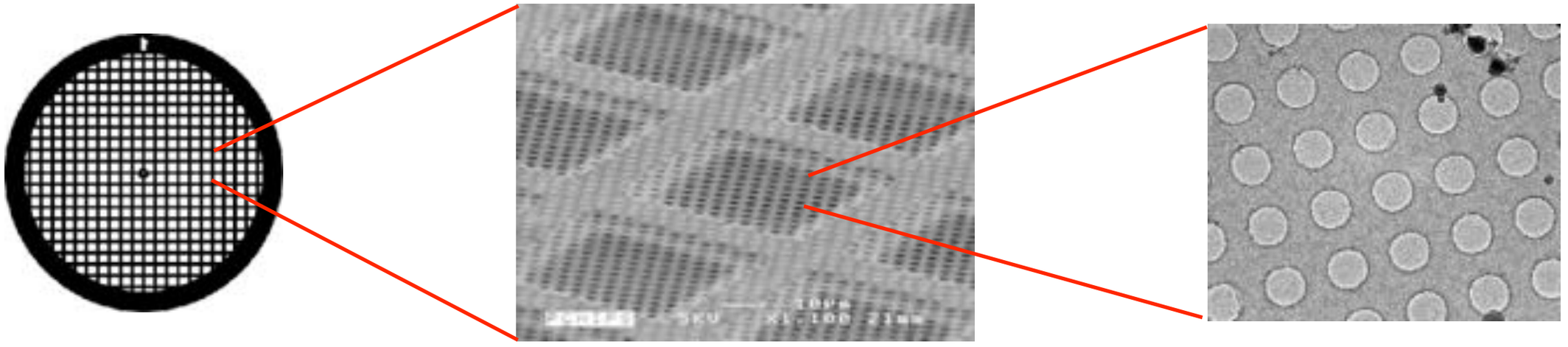
- Extremely low contrast. Signal-noise-ratio <1
- Difficult to identify, orient particles <250 kDa unless they have a very well-defined shape (e.g. a cylinder)

Cryo-EM



Ribosomes imaged by cryo-TEM (image from Joachim Frank)

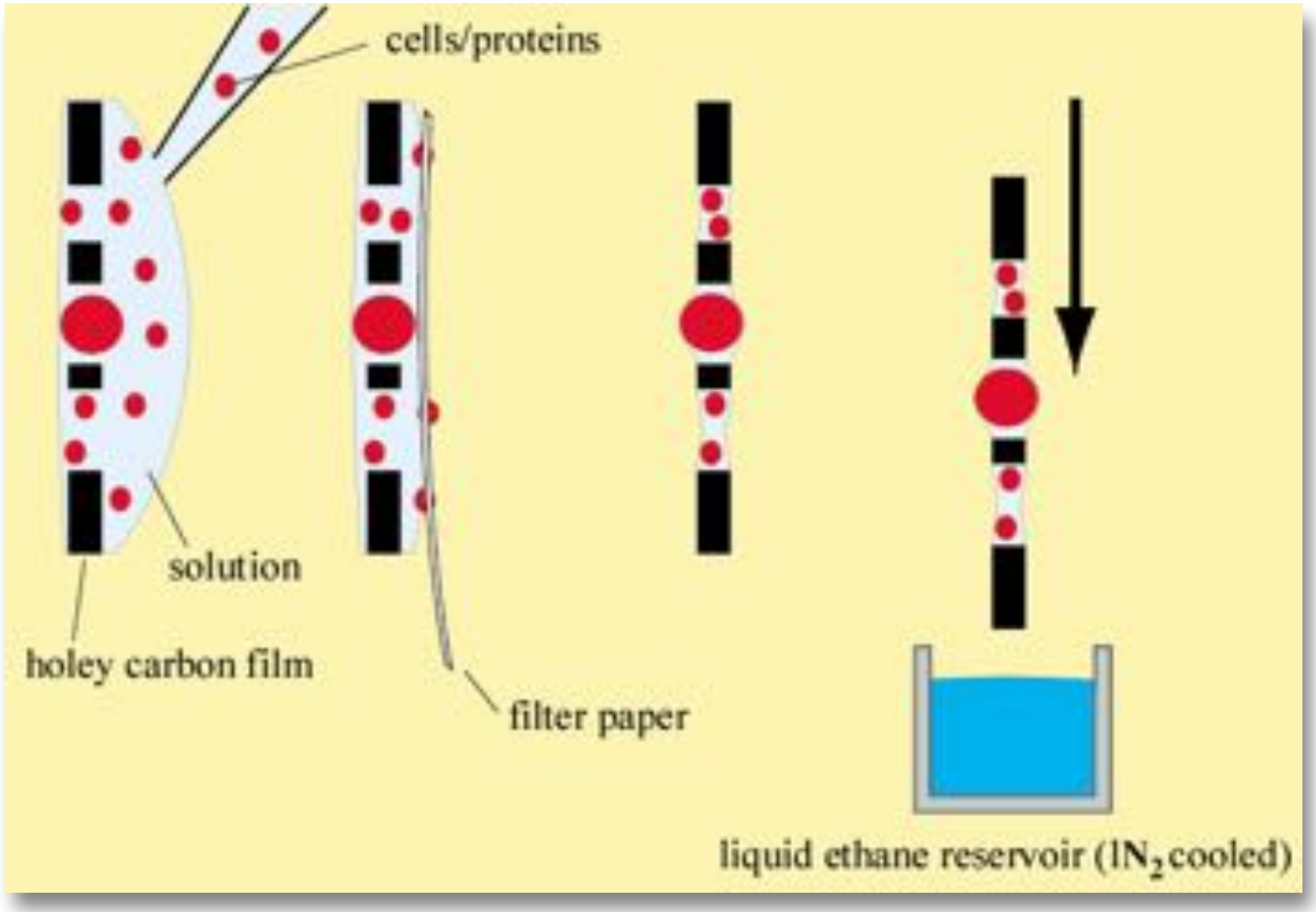
Cryo-EM specimen grids



3mm diameter grid copper mesh carbon-coated	200 300 400 copper mesh	2 or 4µm holes 2 or 4µm separation

Cryo-EM sample grid prep

THIS IS ONE OF THE MOST CRITICAL STEPS, GETTING "GOOD ICE"



Ice layer ~100-200nm thick

Figure by Daniela Nicastro

- Ice thickness impacts contrast, resolution, electron dosage

Plunge freezing

Baker lab, UCSD

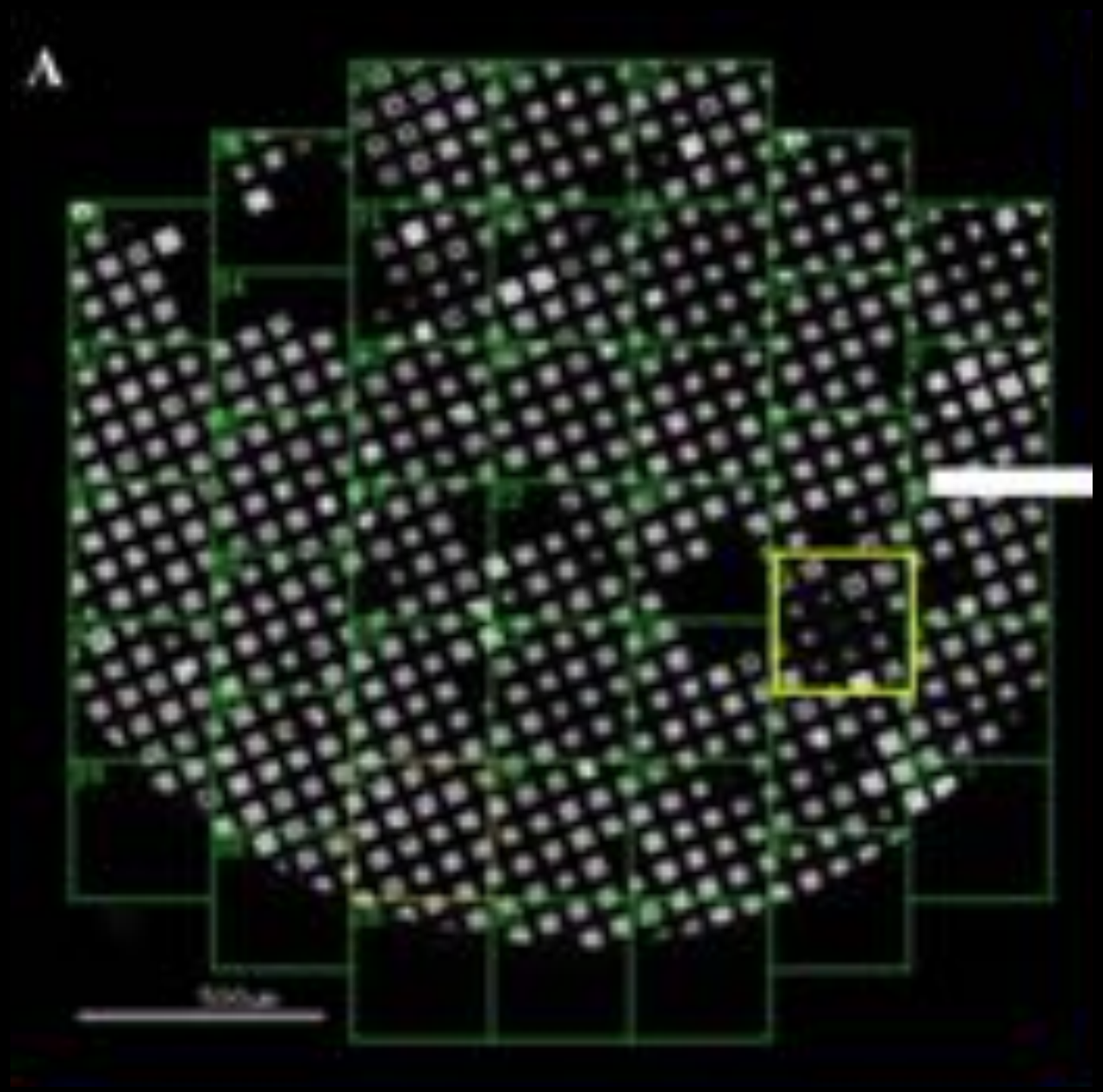
- Plunge the grid into liquid ethane (Tm -182˚C) or propane is equilibrated near liquid nitrogen temperatures

- Rapid freezing (ΔT~10-100,000˚C/s), <ms, can freeze water before crystalline ice can form. Samples are in their hydrated states.

- Despite the vacuum environment of the microscope column, the vapor pressure of vitrified ice held near liquid nitrogen temp (sample temp -195-180˚C) is low enough that the it doesn't sublimate.

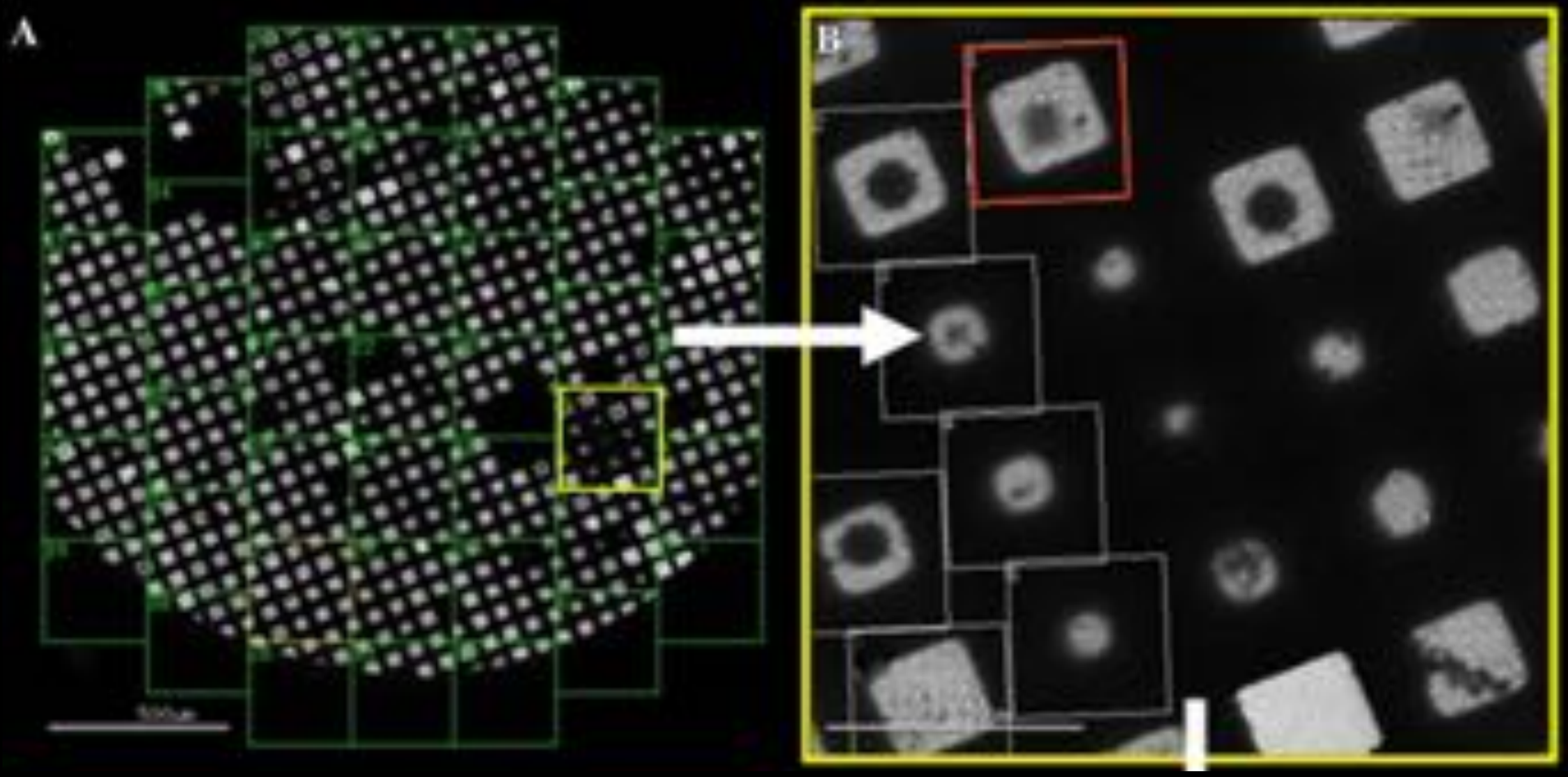
Manual or “robot”

Cryo-EM



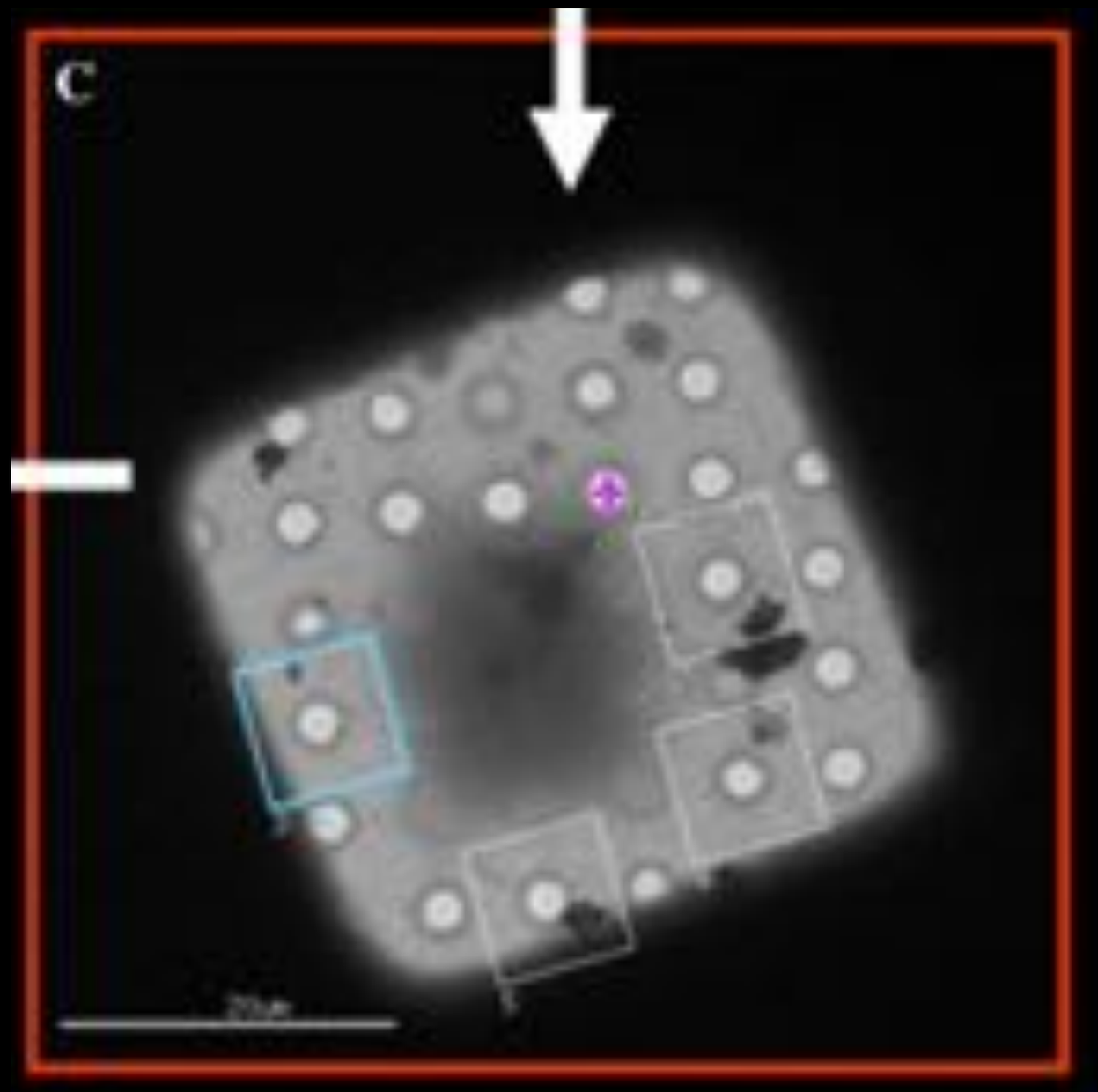
GRID

Cryo-EM



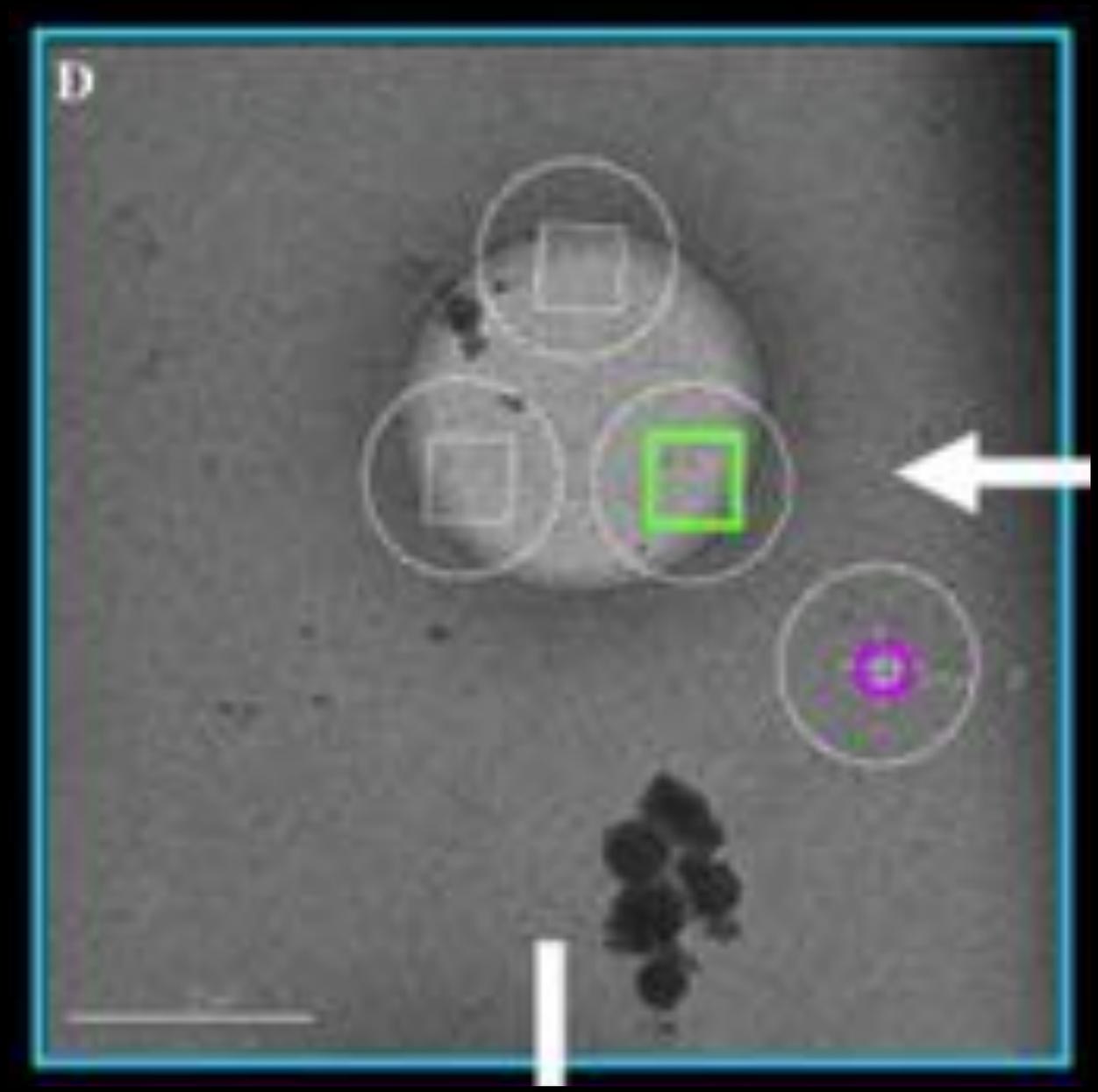
target SQUARE

Cryo-EM



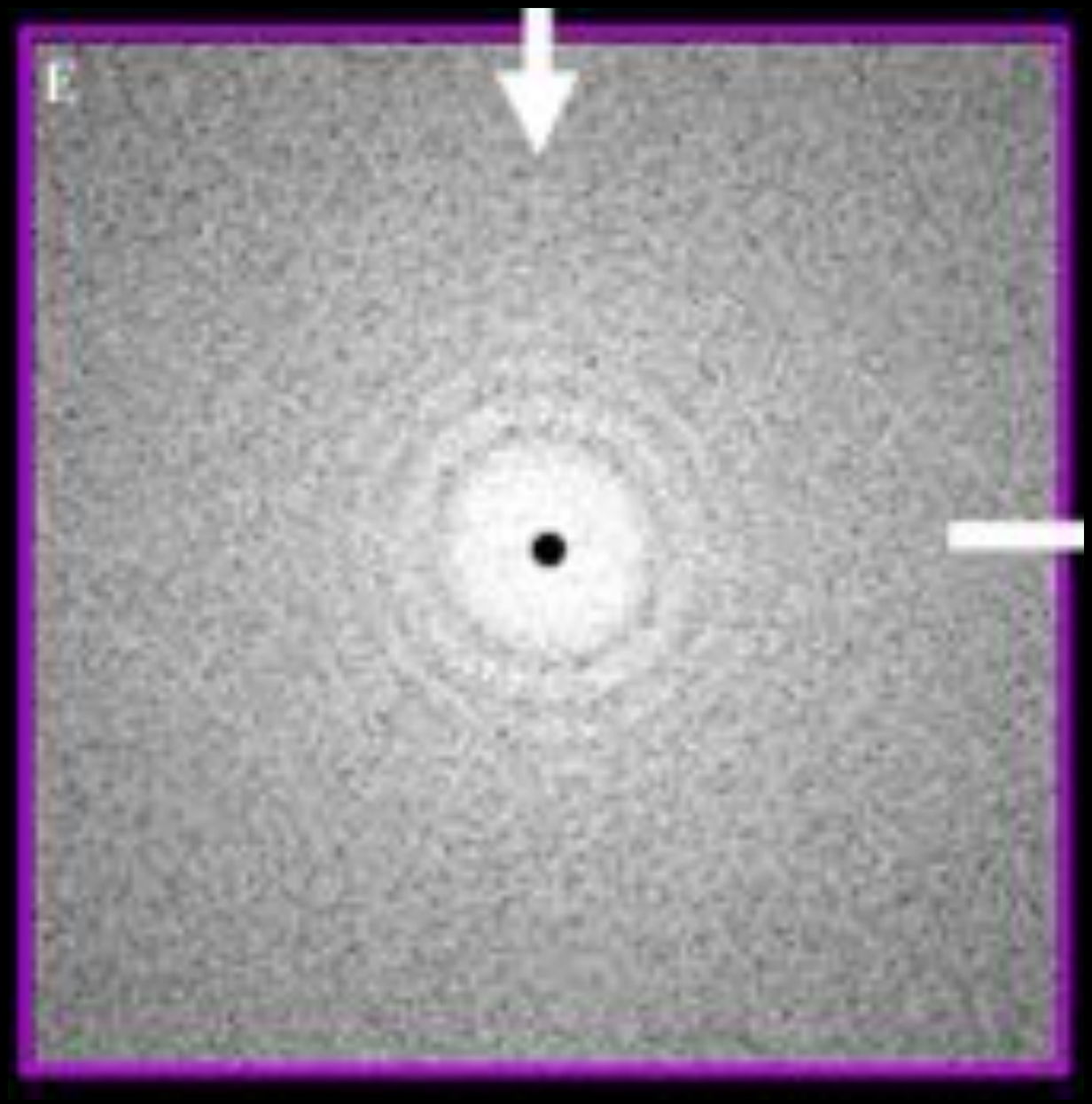
target HOLE

Cryo-EM



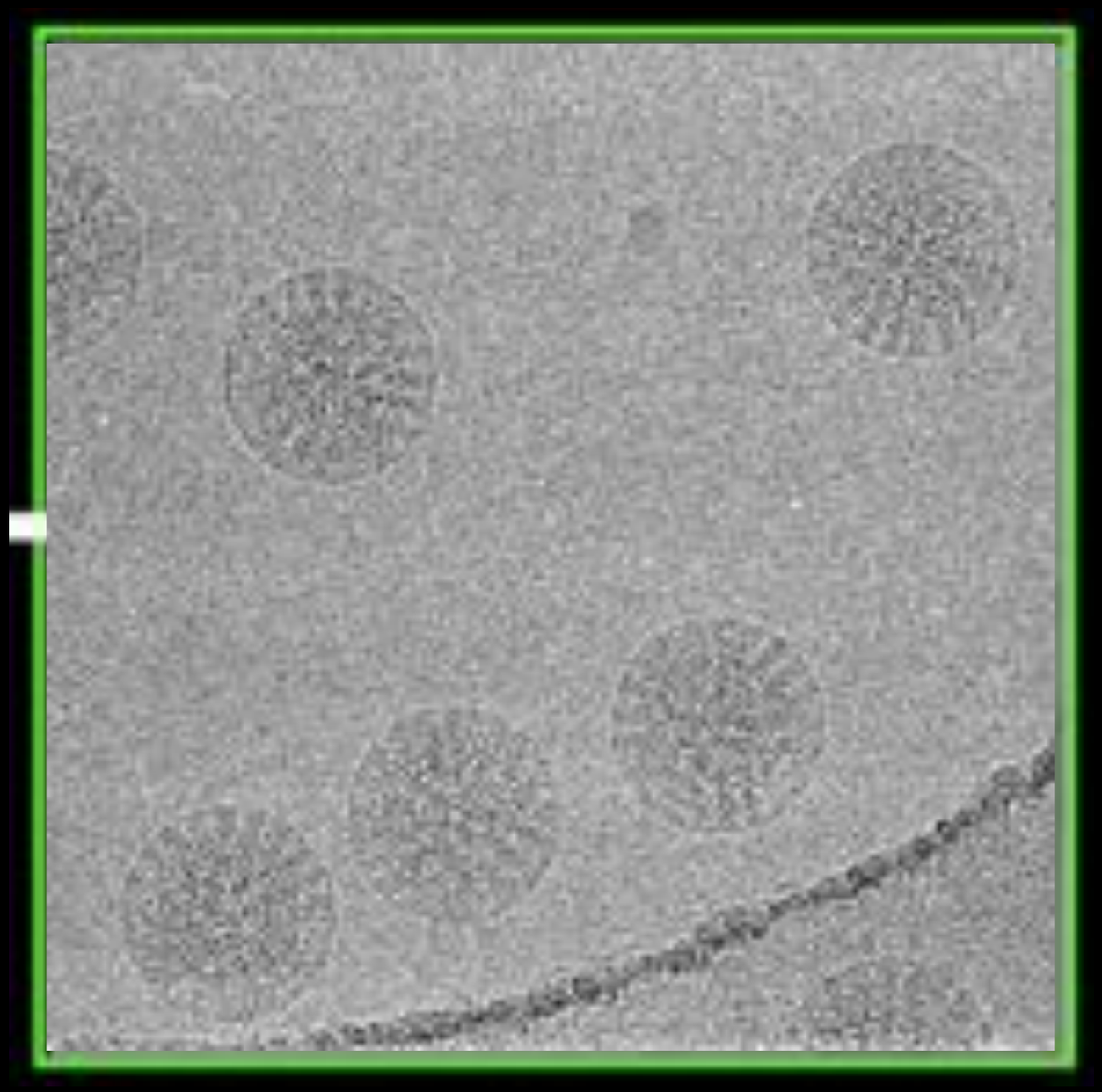
target REGION in hole

Cryo-EM



FOCUS

Cryo-EM



COLLECT DATA

To obtain 3-D information, need to sample different views of the object

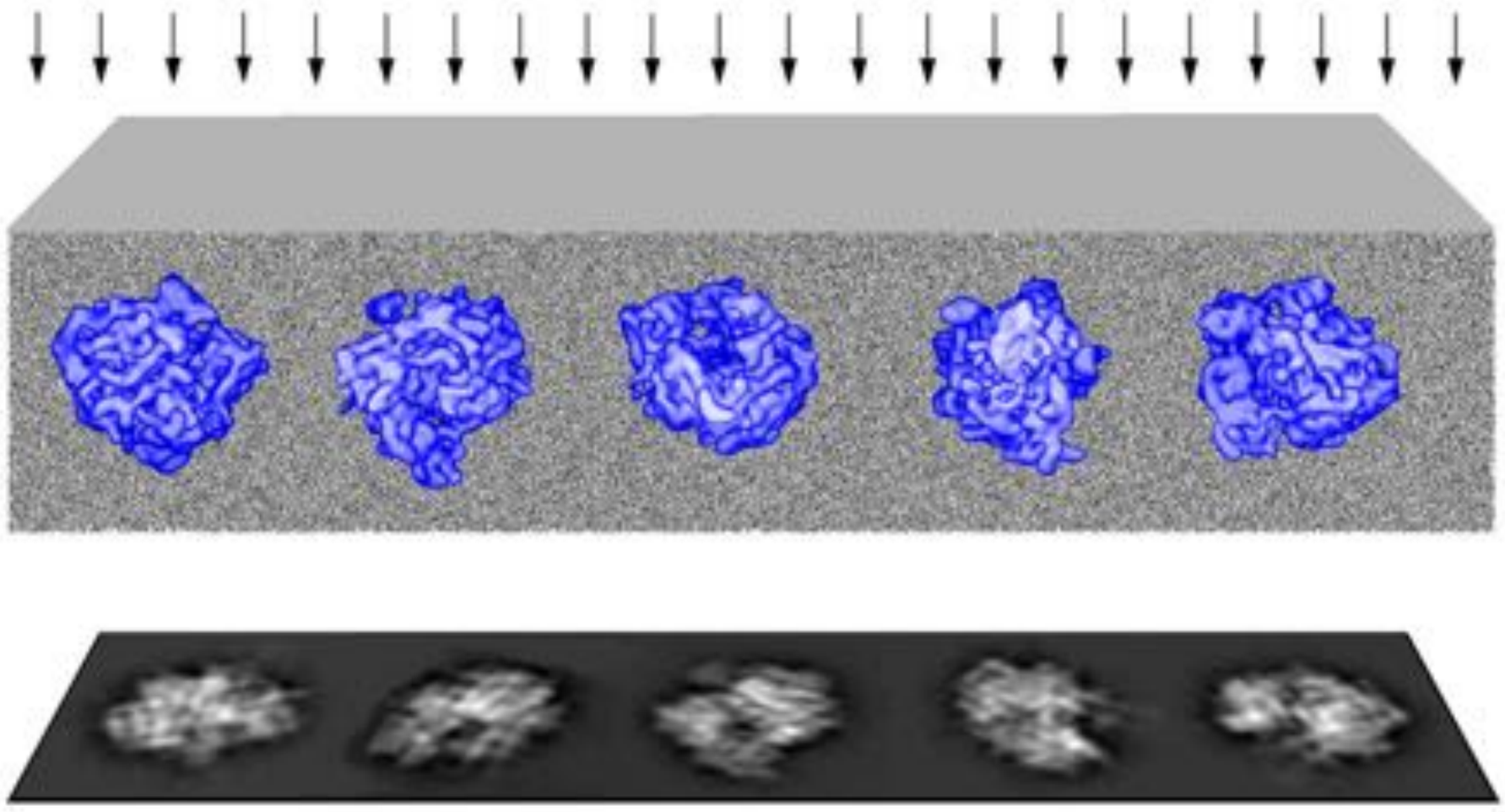


Figure by Joachim Frank

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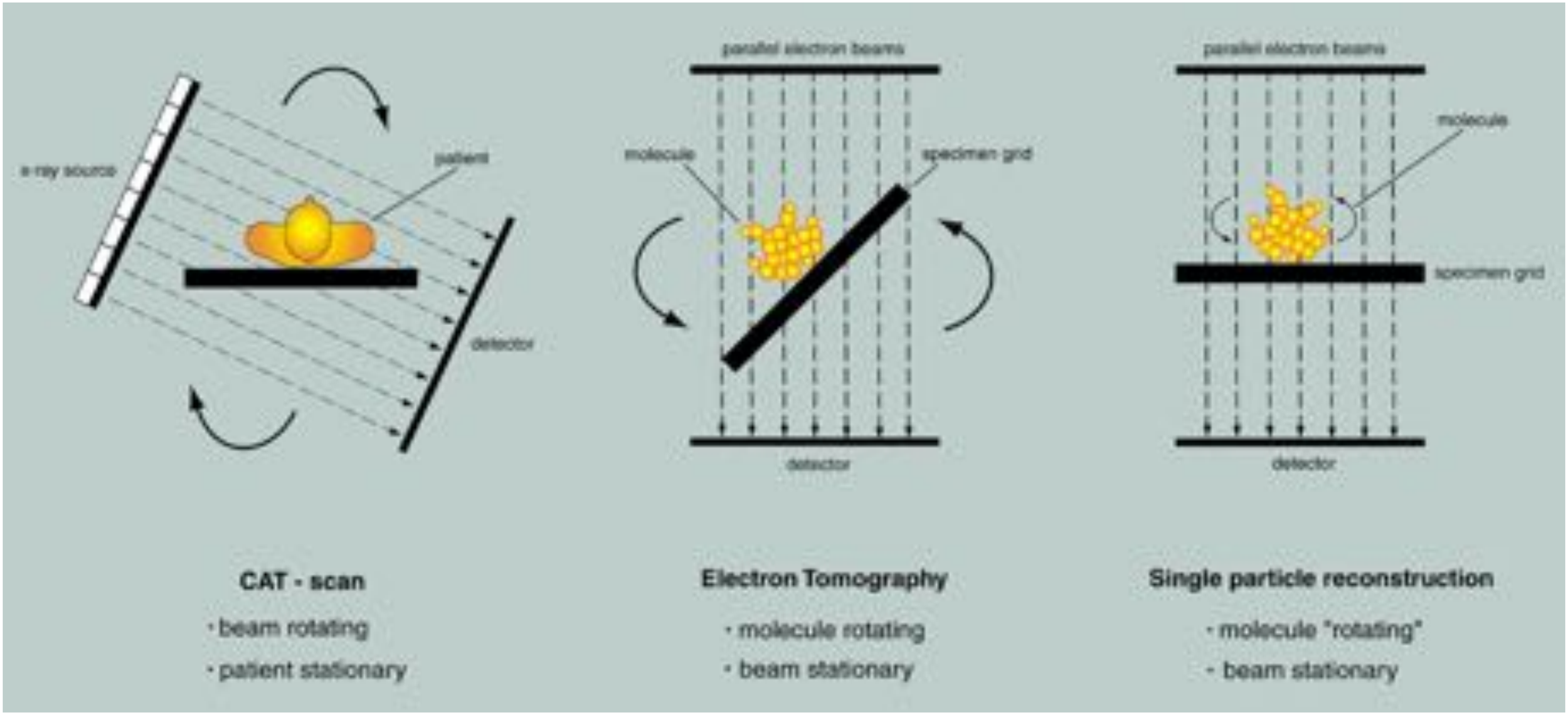
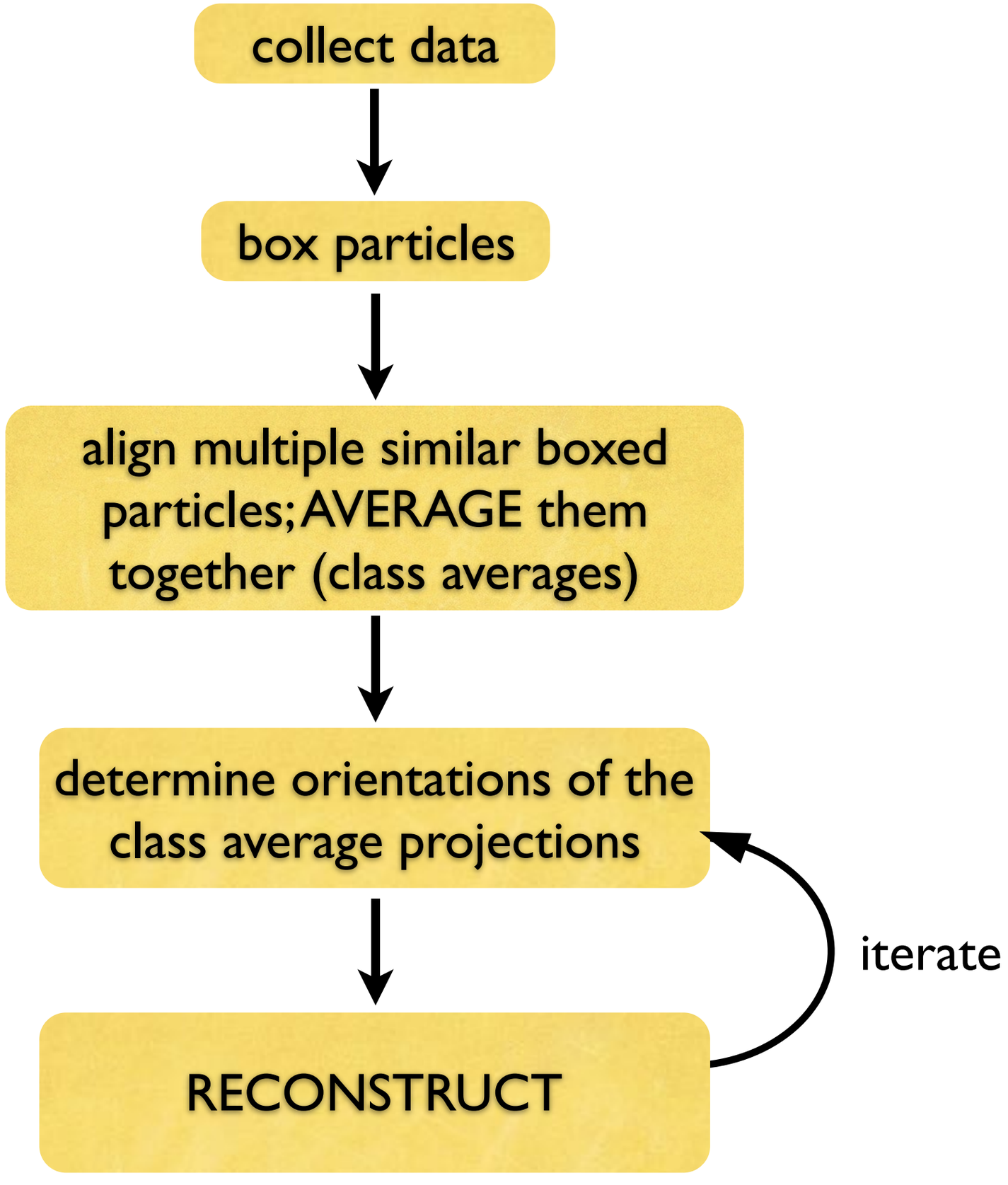


Figure by Joachim Frank

Single-particle analysis



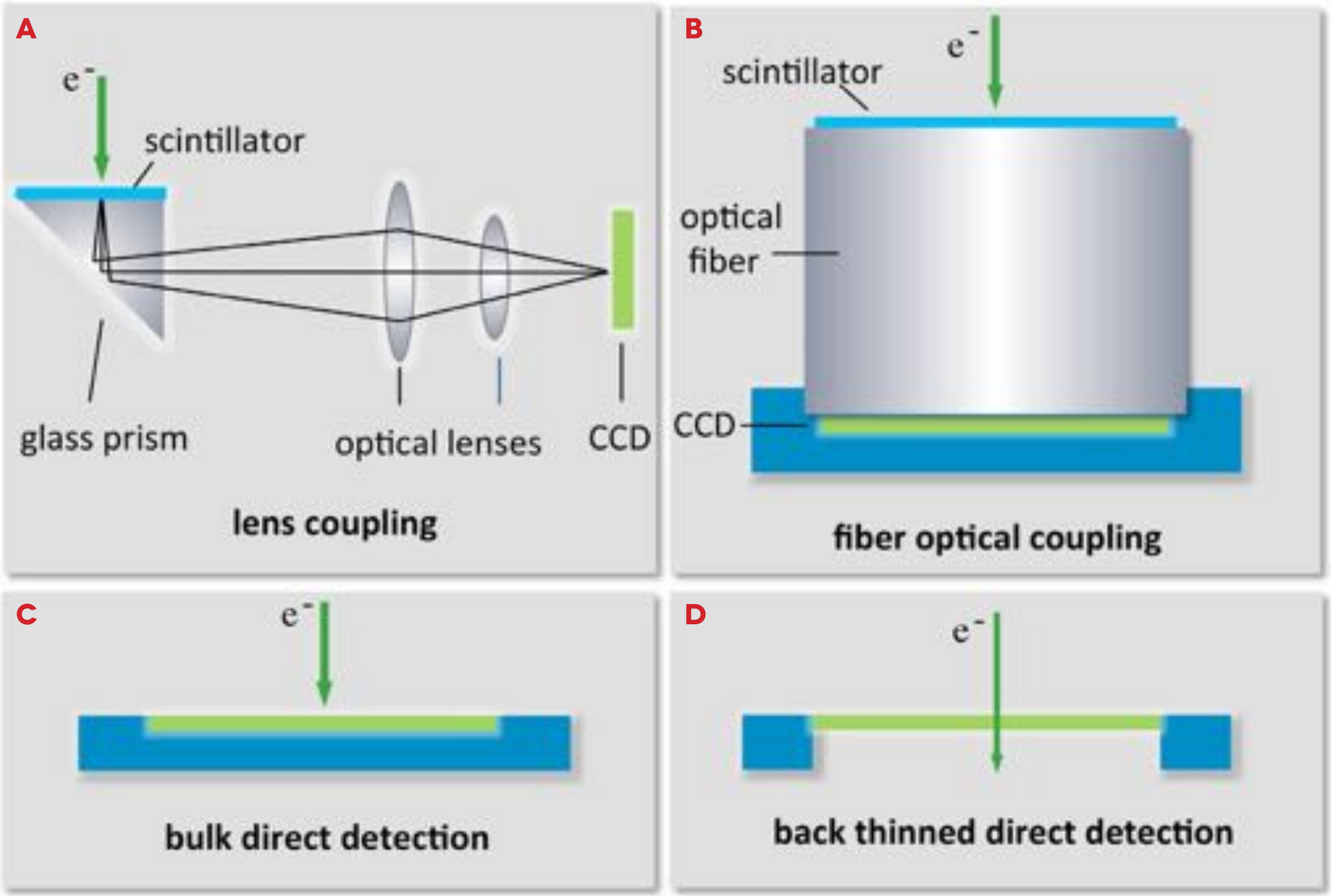
Factors that affect resolution of cryo-EM

- Conformational heterogeneity
- Number of particles used in reconstruction; averaging to improve signal-to-noise
- Orientation determination
- Radiation damage
- Beam-induced movement of particles and grid charging
- Imaging conditions

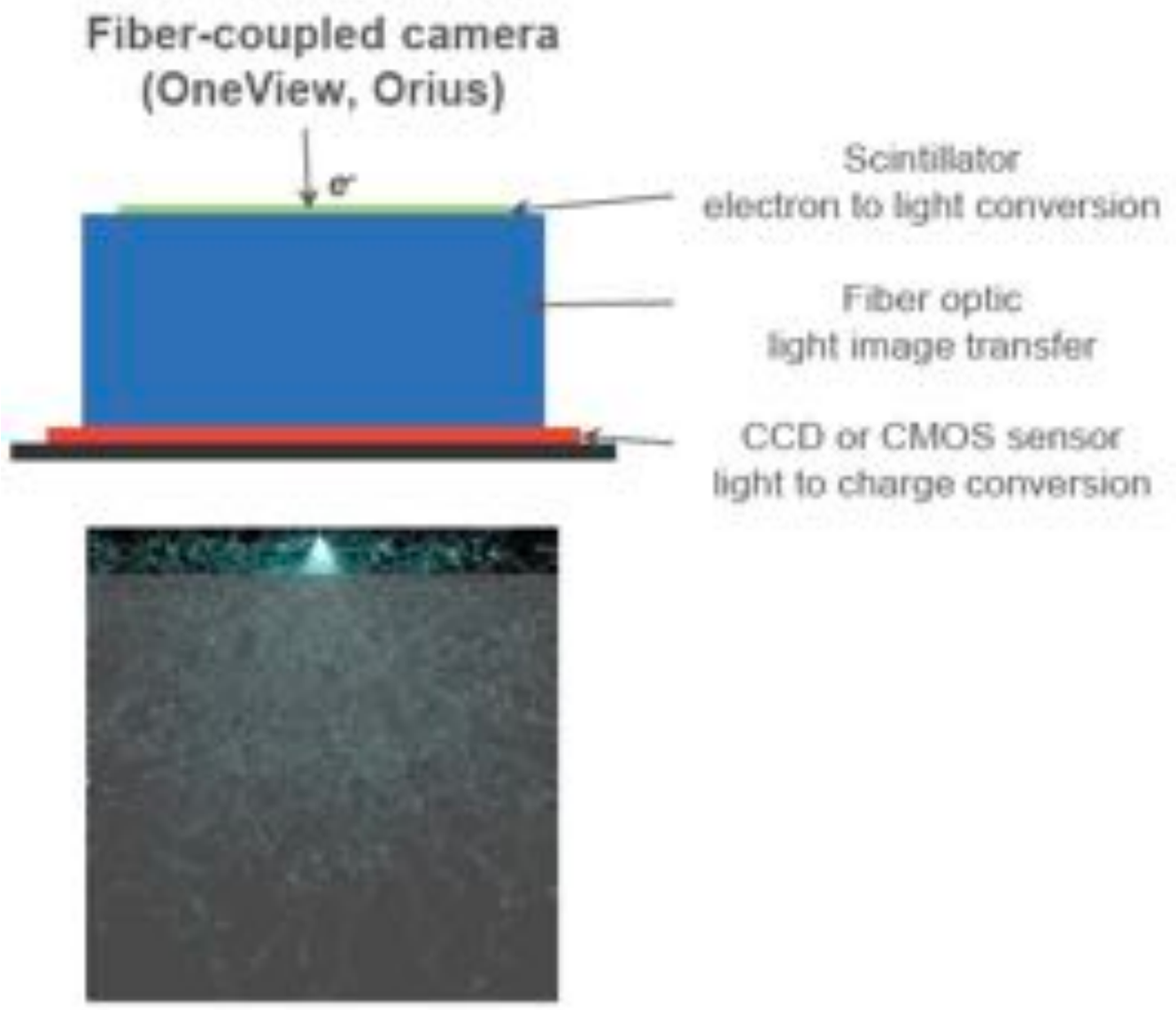
A new type of detector: Direct detection of electrons

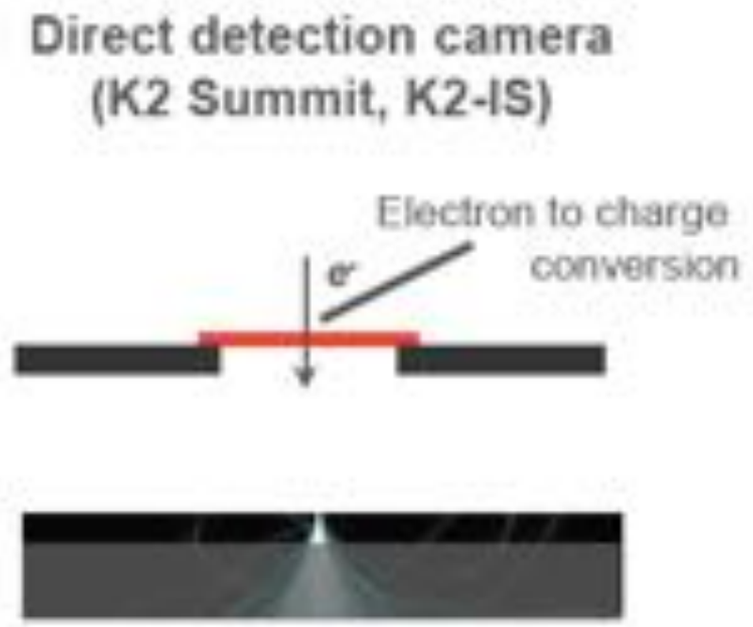
Conversion e- to γ to e-

Direct



A new type of detector: Direct detection of electrons

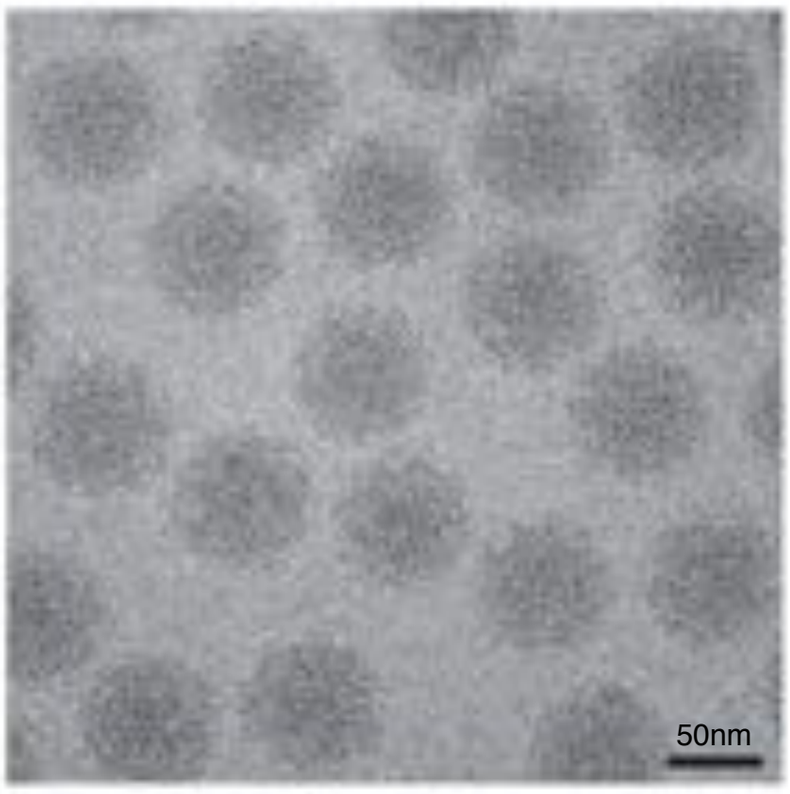




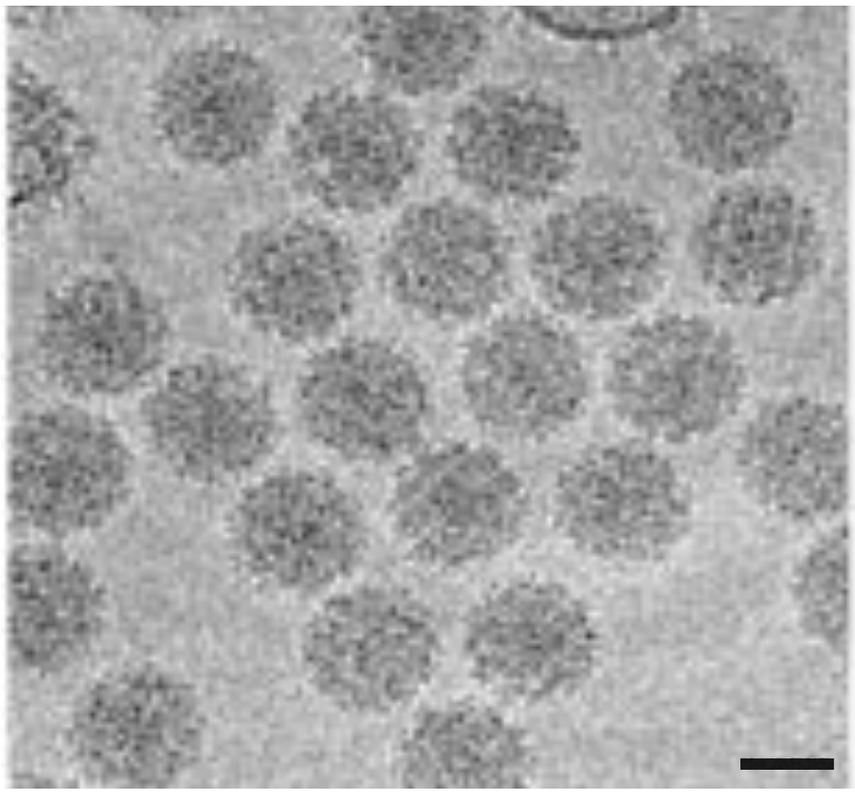
Gatan-workshop (http://workshop.iisertvm.ac.in)

A new type of detector: Direct detection of electrons

- Cryo-EM images of alphaviruses



CCD detector



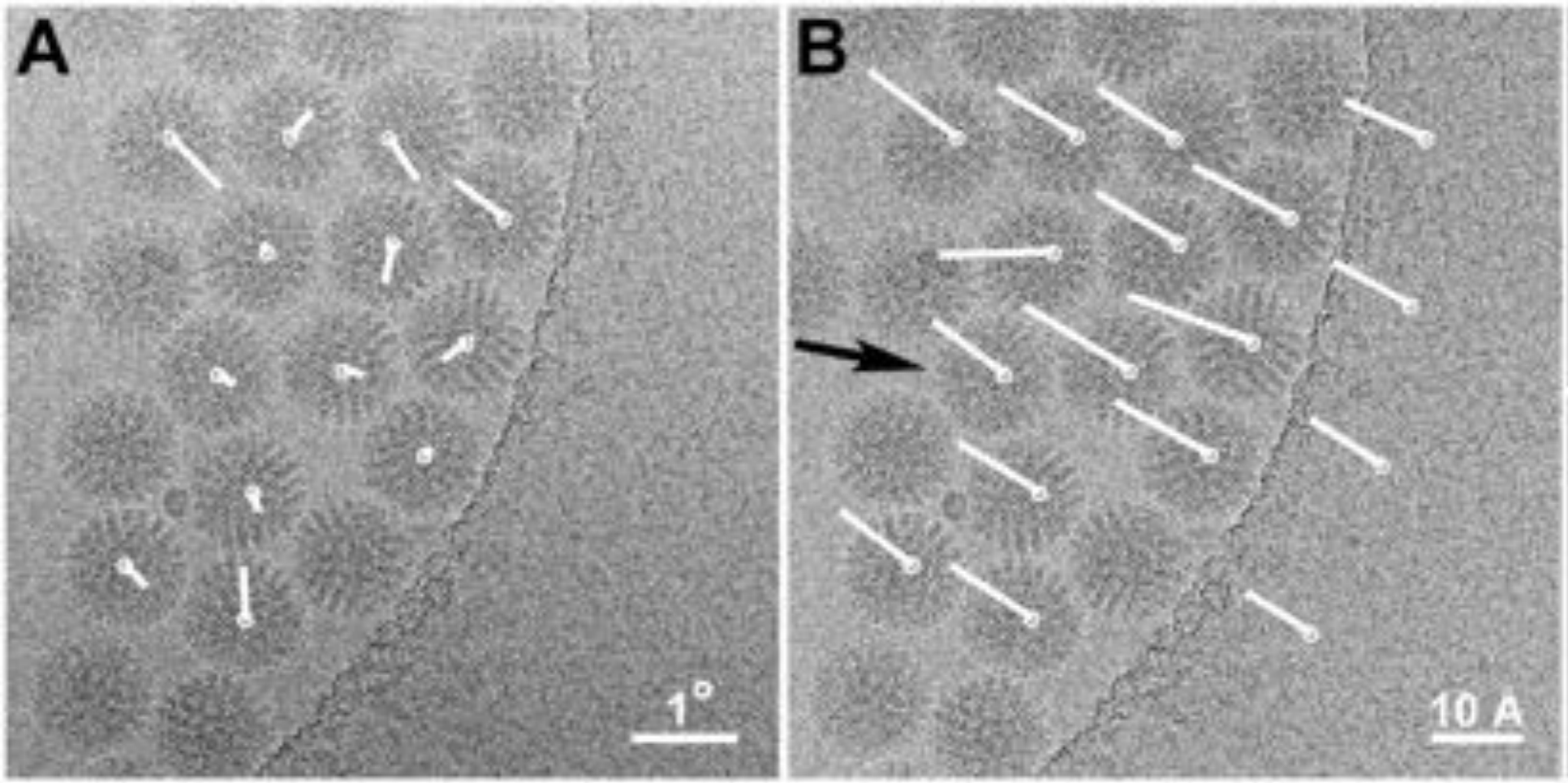
Direct electron detector

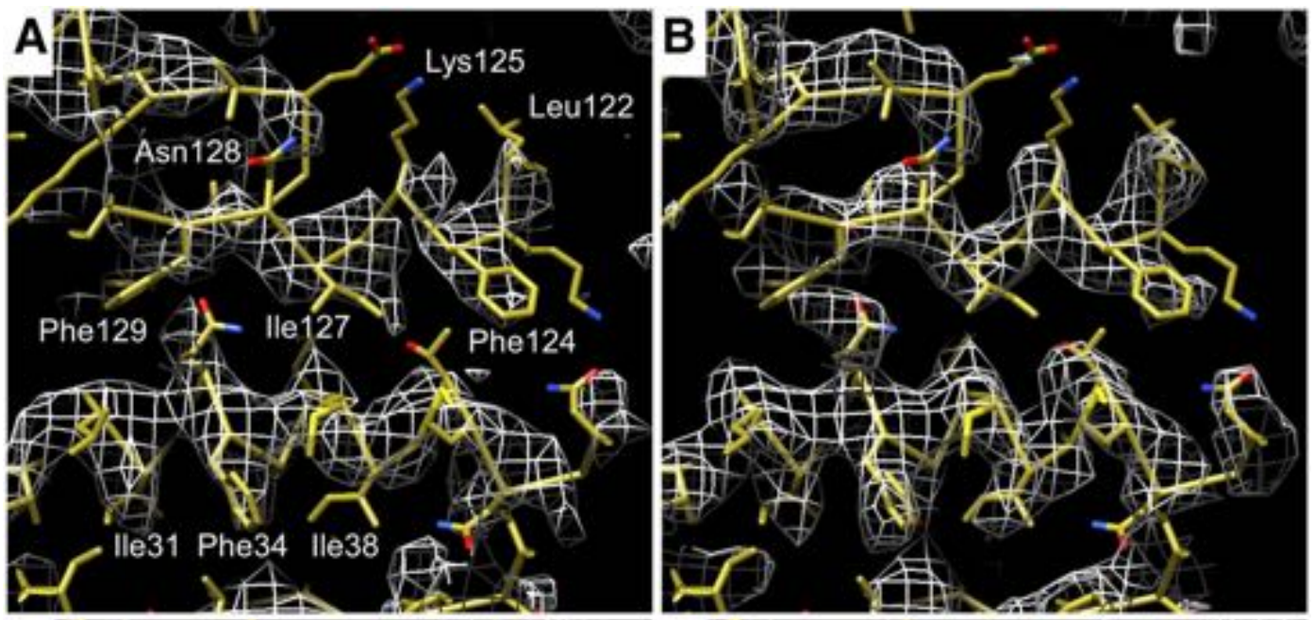
Left: Zhang et.al., EMBO, 2011
Right: IU Bloomington

A new type of detector: Direct detection of electrons

- No intermediary scintillator
  - More efficient detection: greater sensitivity
  - Electron counting
- Higher resolution
- Work closer to true focus (better preservation of high res info)
- Can use lower dosage = less damage to sample
- Very fast readout of frames (20-400 fps), making it possible to correct for mechanical or beam-charging induced specimen drift (sharper image, less motion blurring)
- Dose fractionation/weighting possible due to capturing of images as frame stacks

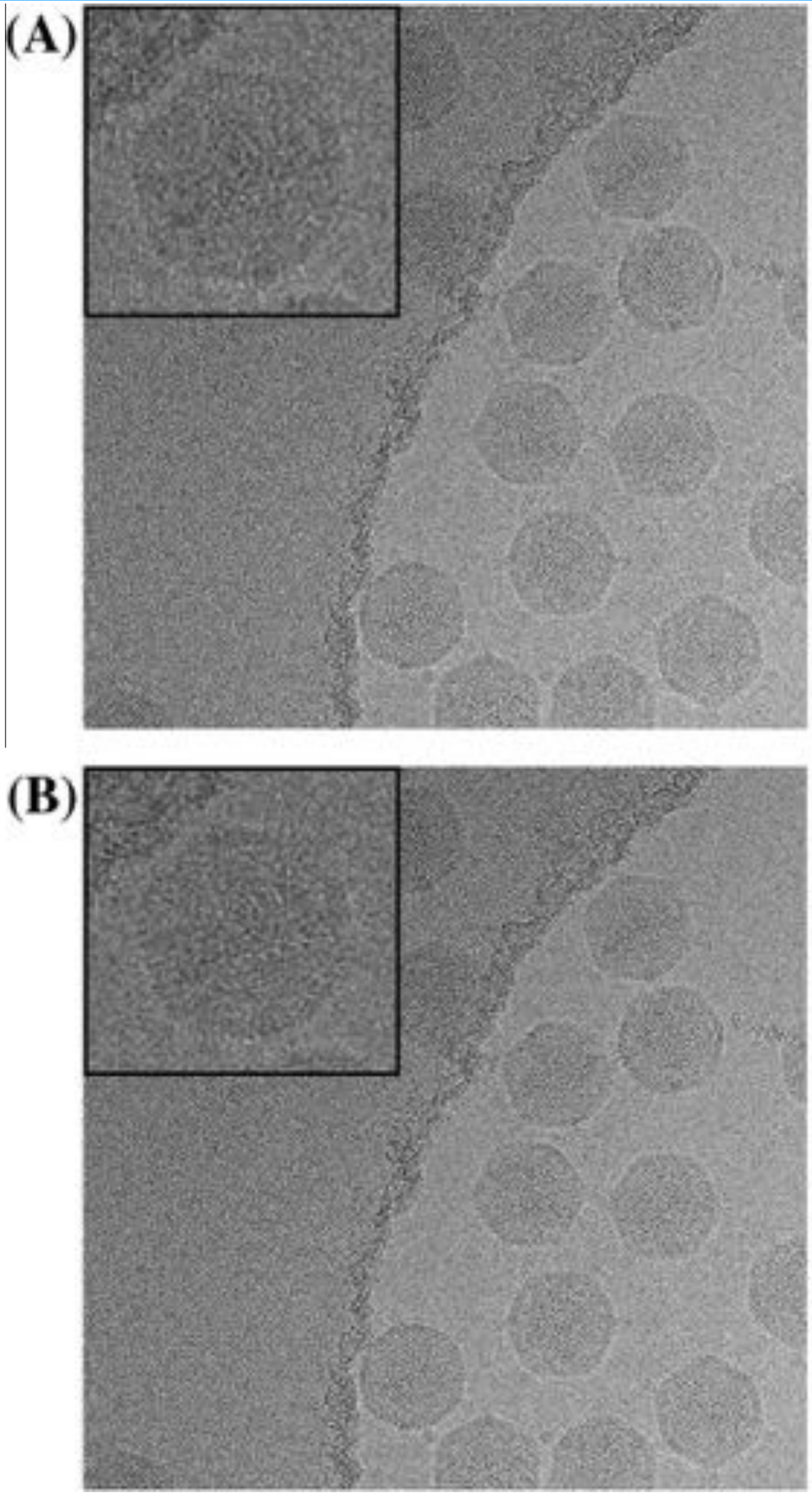
Drift/motion correction (rotational, translational) increases resolution

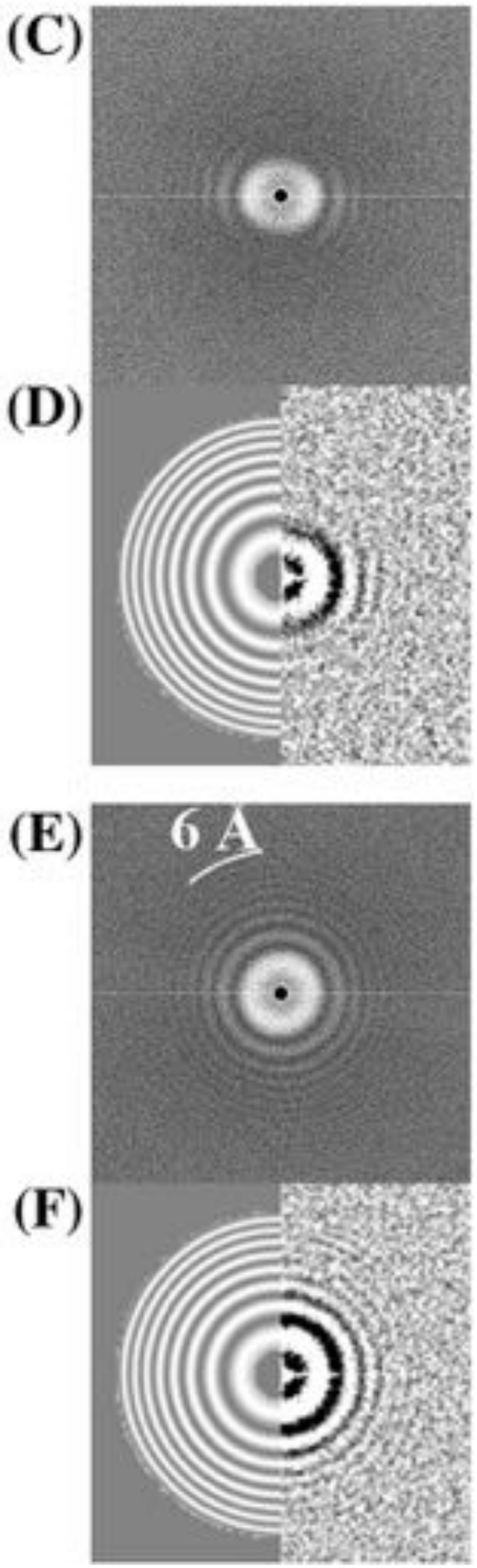


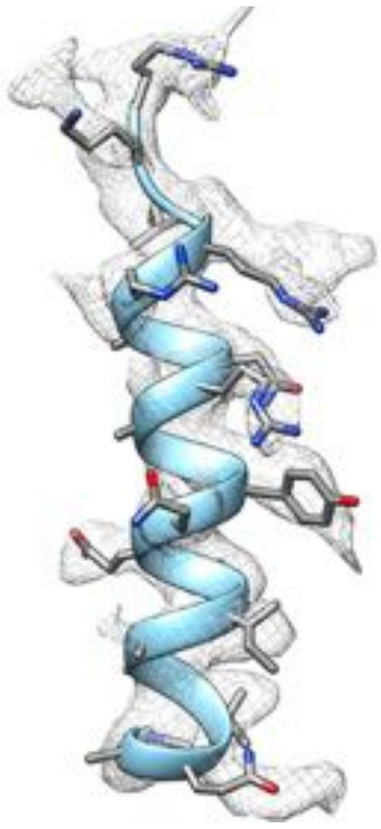


Campbell et al., Structure 2012

Drift correction increases resolution

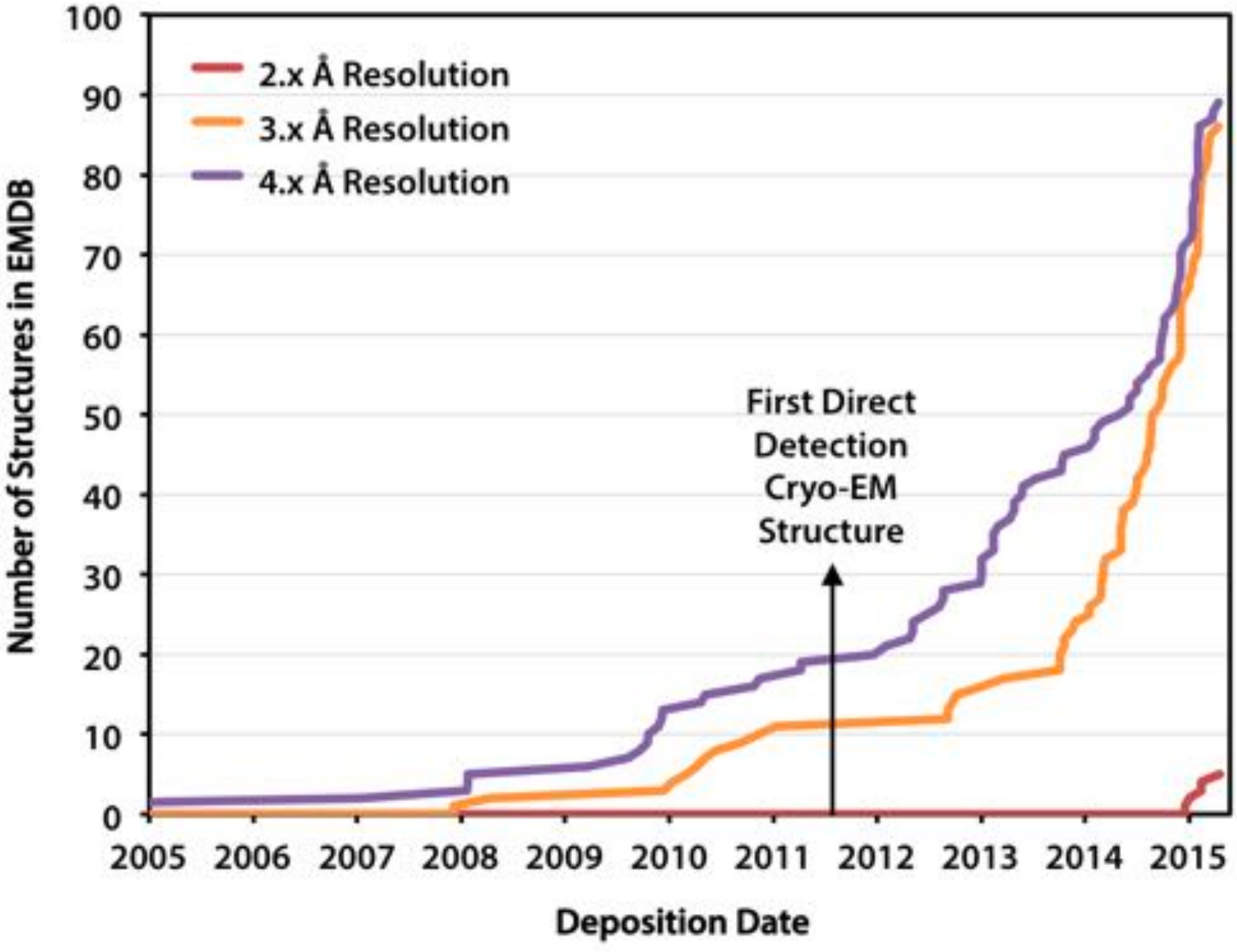






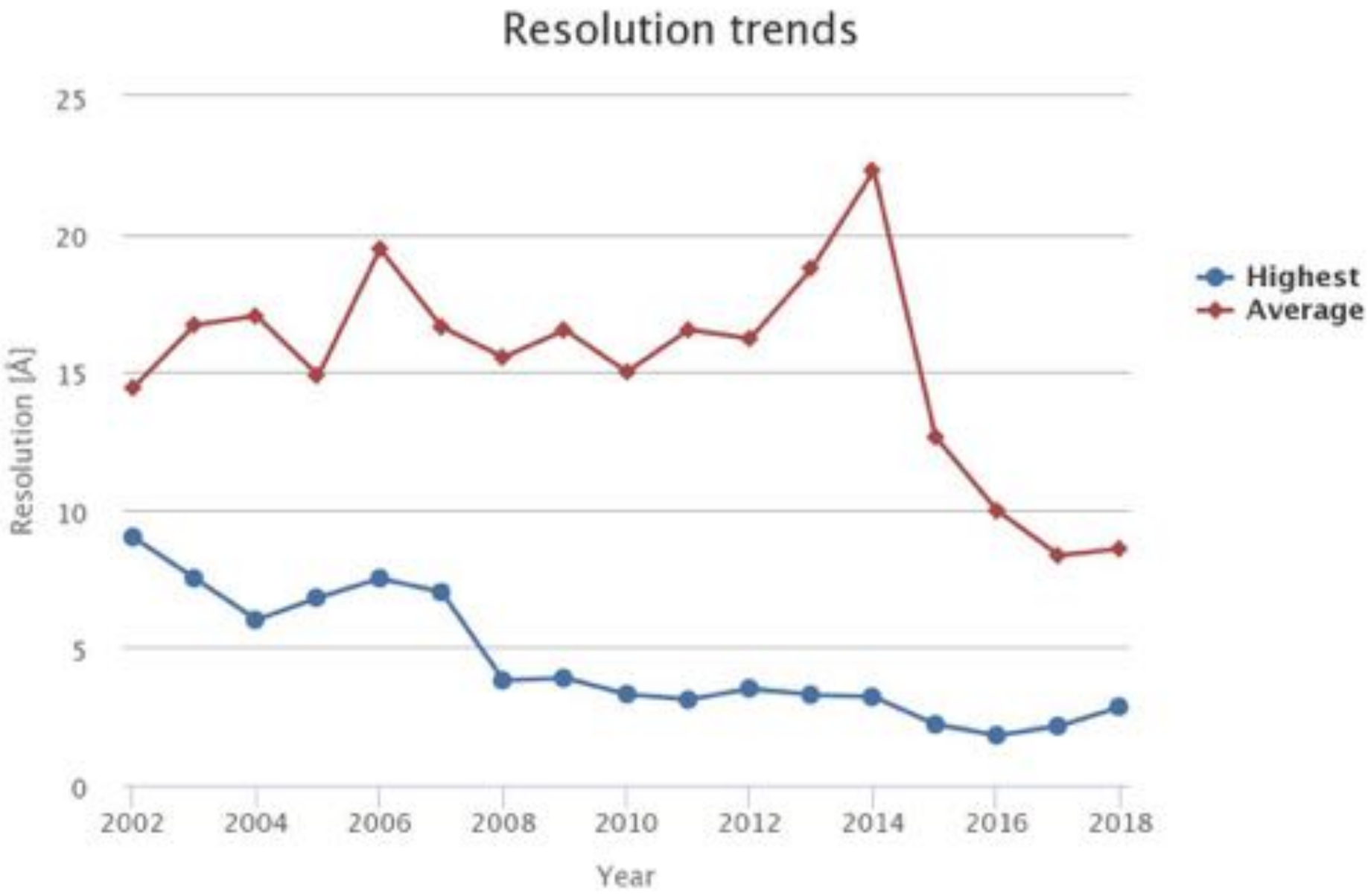
Veesler et al., JSB 2013

Increase in resolution of deposited cryo-EM structures



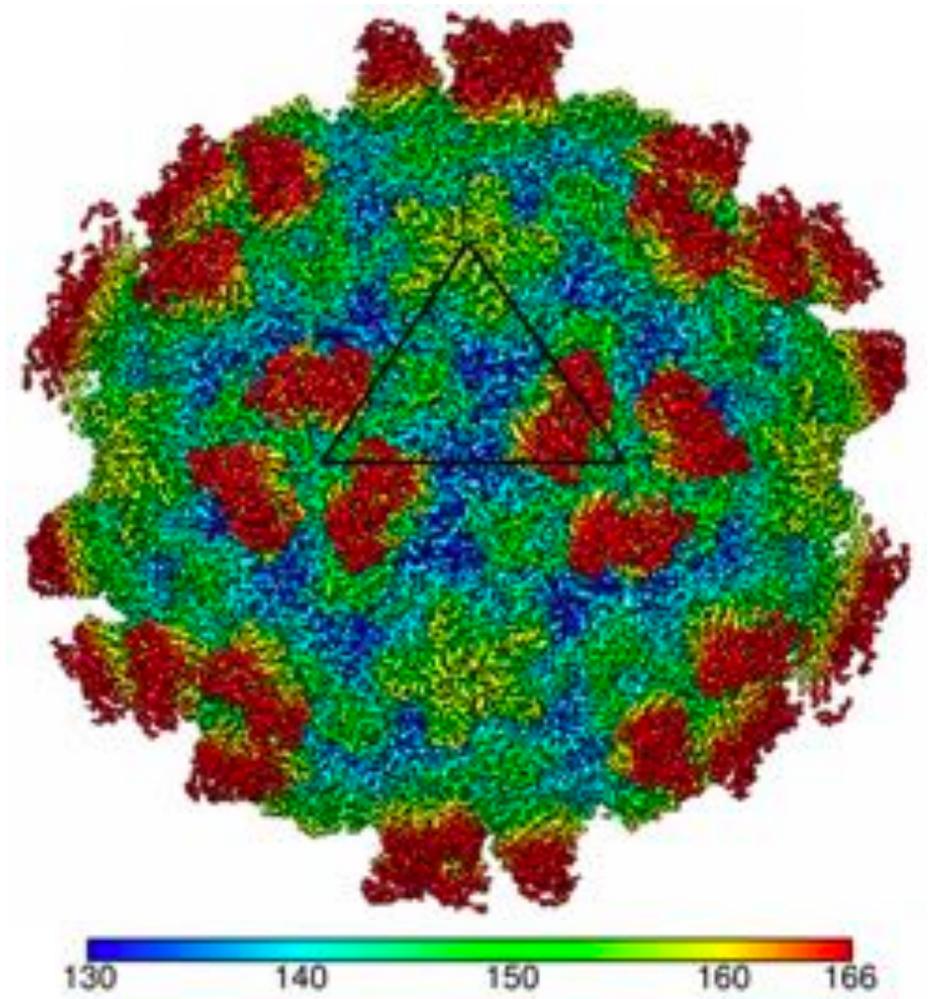
EM DataBank

Trends in Cryo-EM data deposition



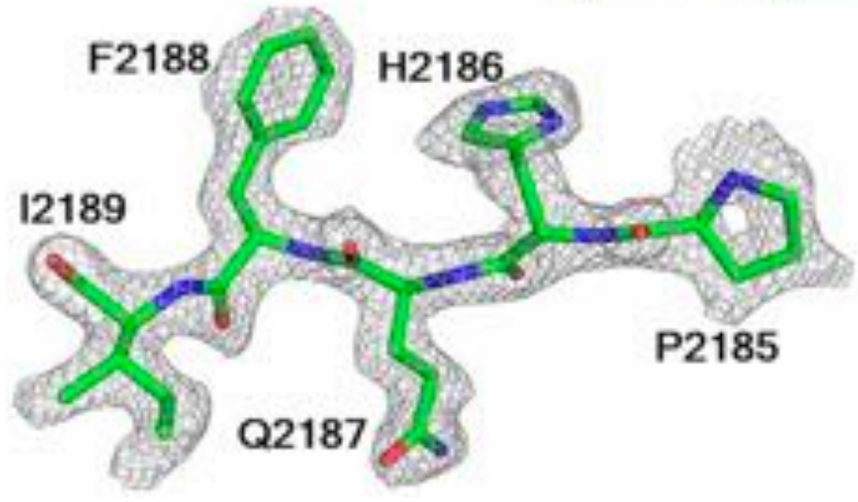
EM DataBank

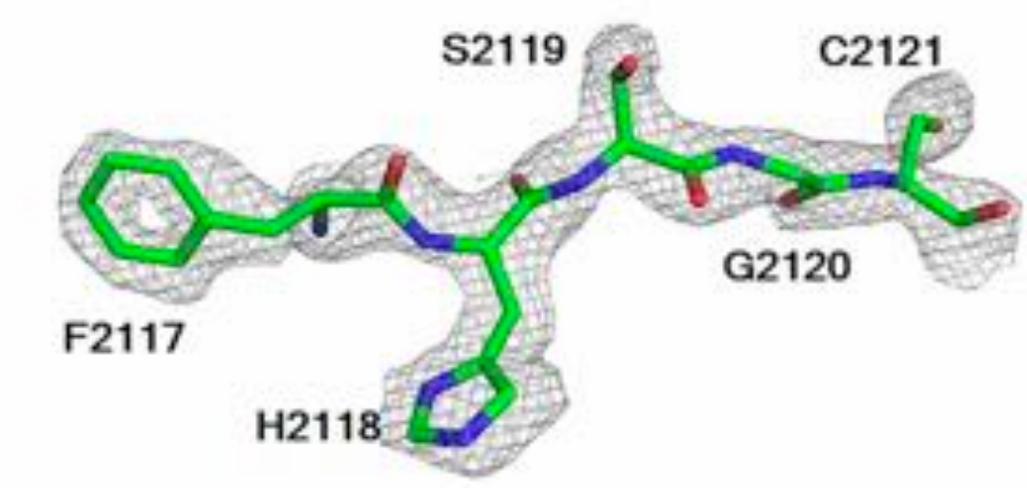
Major advances in EM imaging are starting to challenge crystallography



Rhinovirus B14 - common cold virus
Non-enveloped virus
Resolution: 2.3 Å

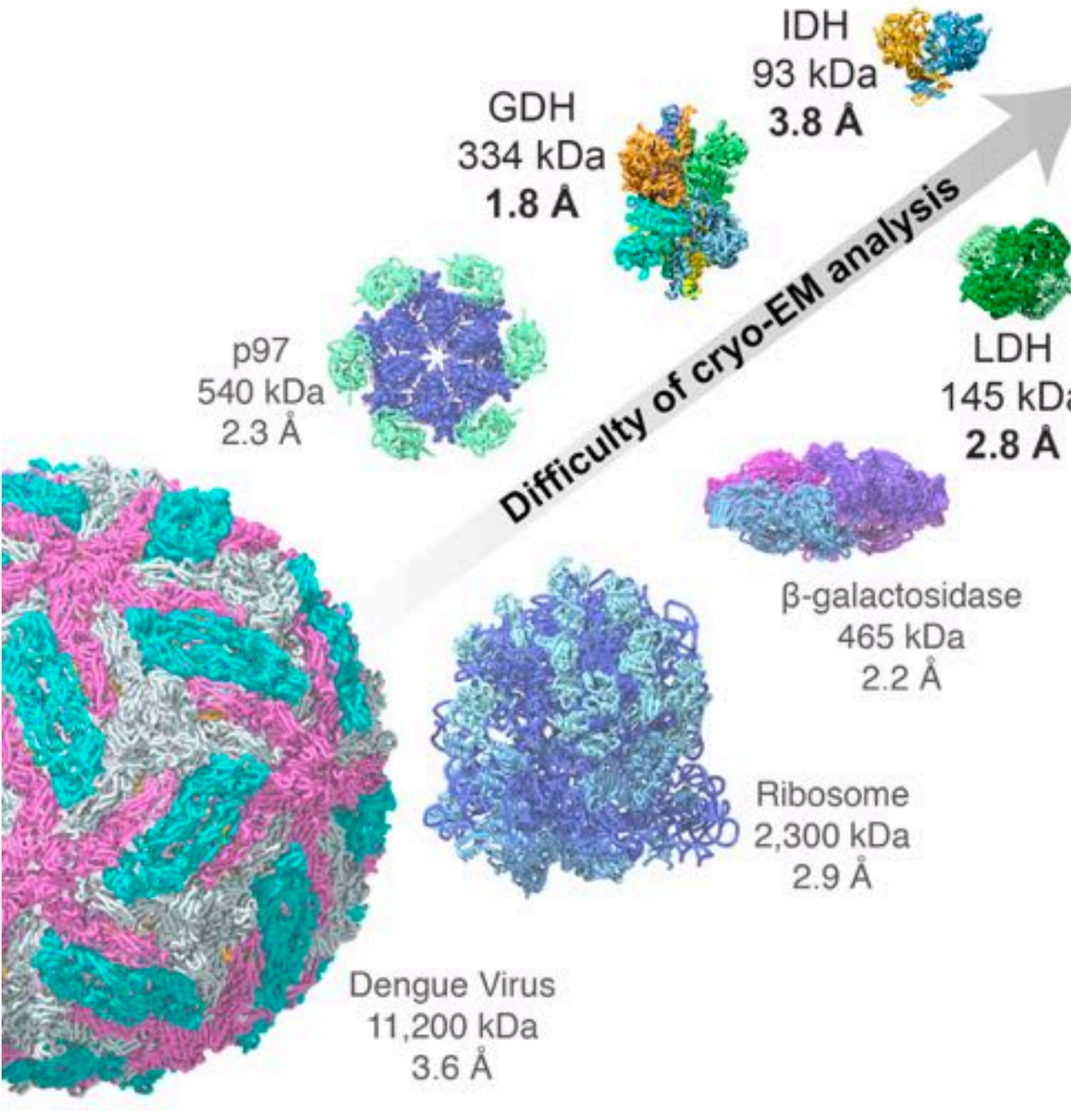
Individual amino acids and side chains can be resolved

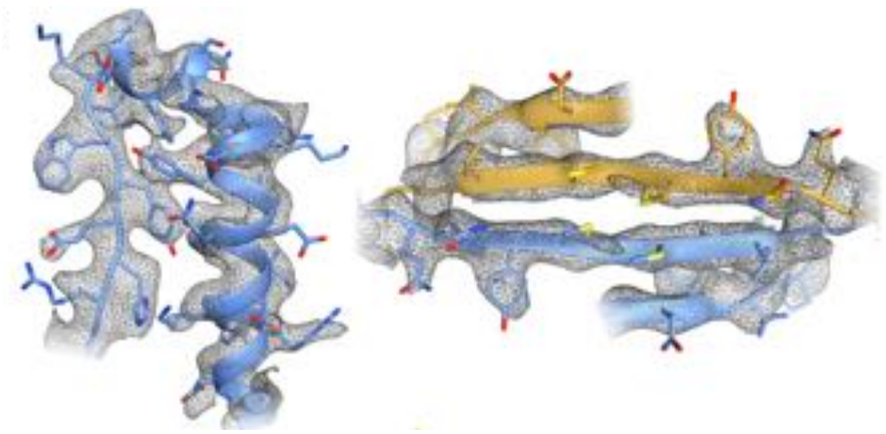


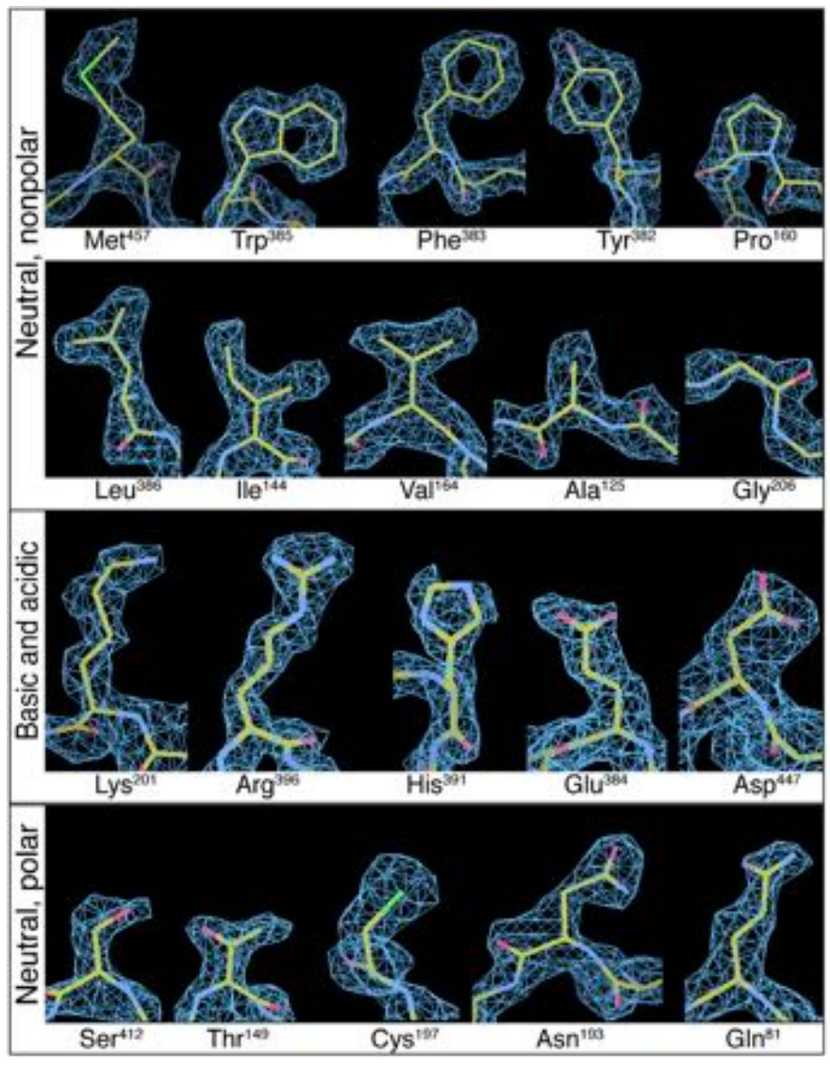


Dong et.al., PNAS, 2017

With near atomic resolutions now achievable for lower symmetry macromolecules also..

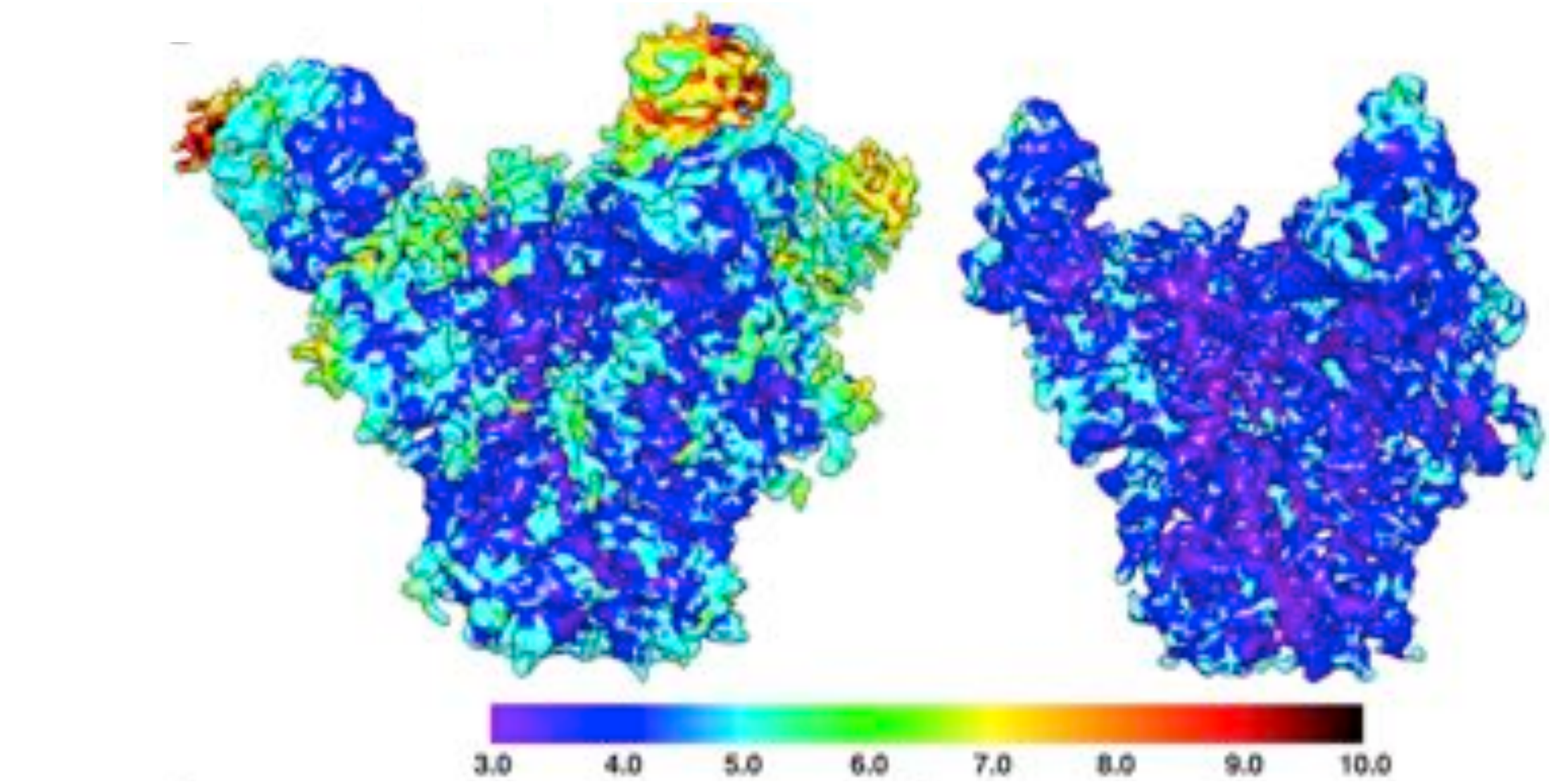


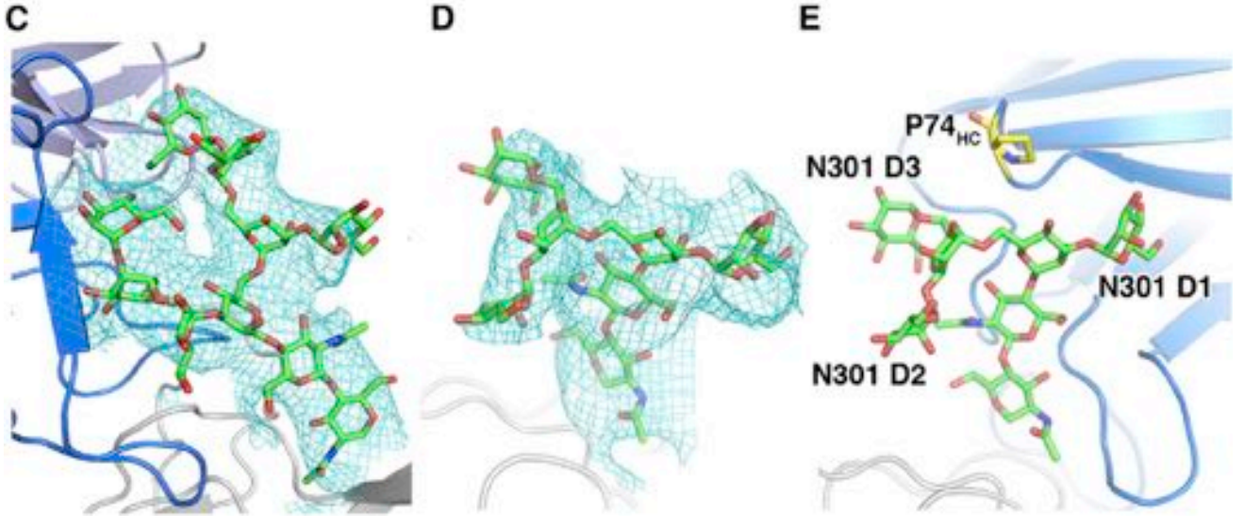




Merck et.al., Cell, 2016

Even for challenging objects like HIV Env glycoprotein (4.4 Å)

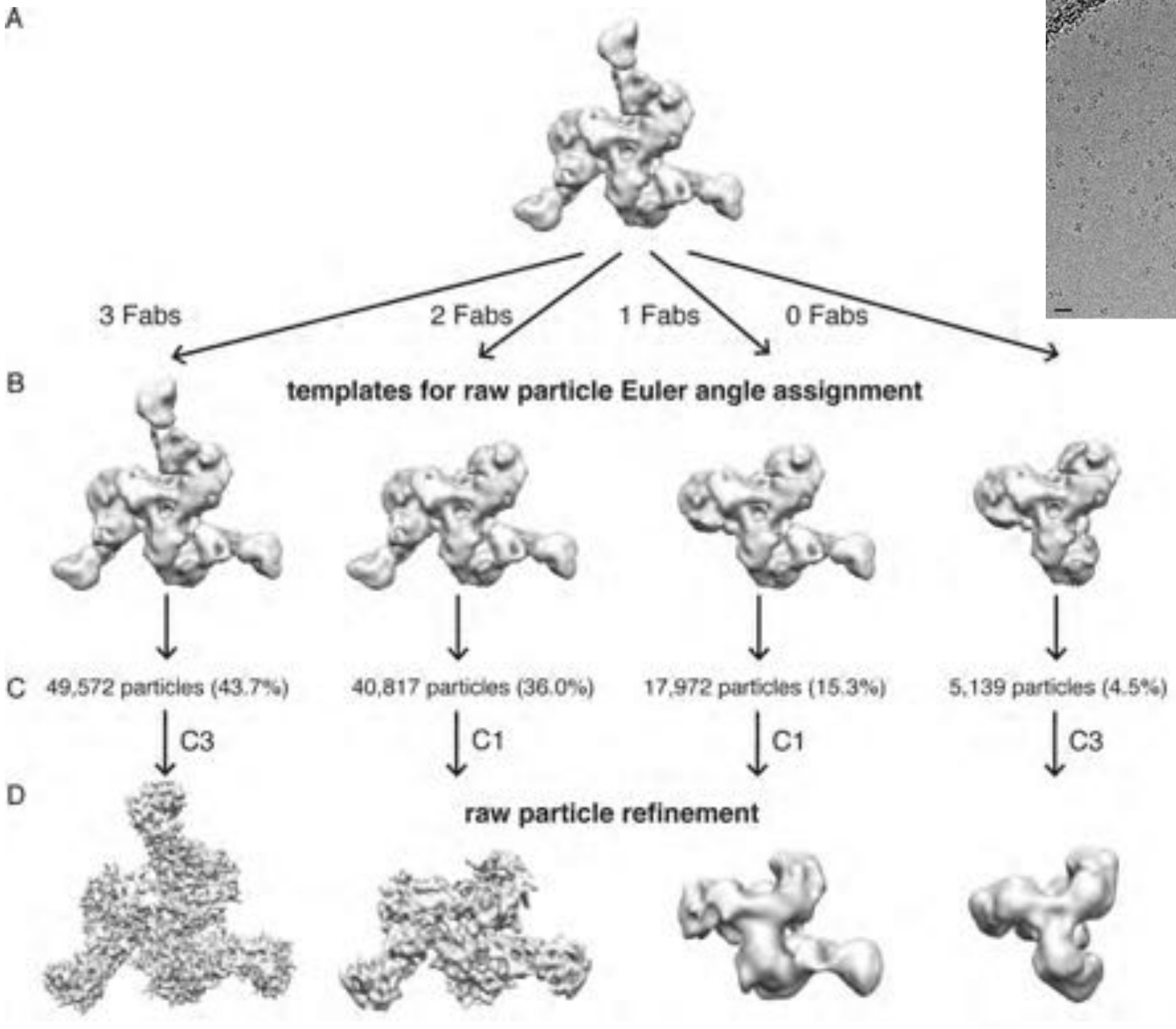


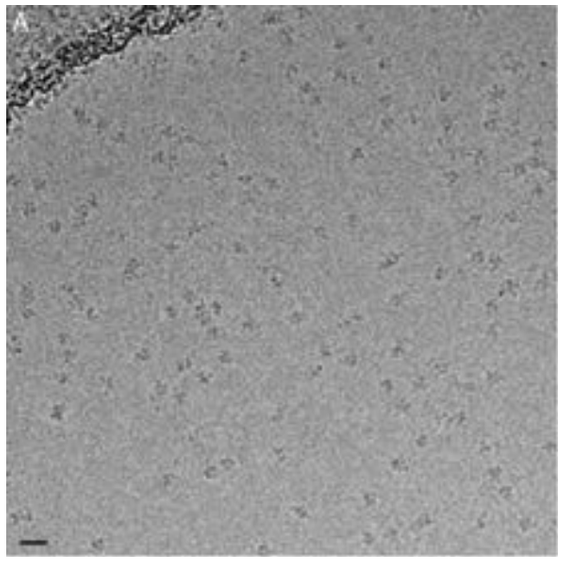


N-linked glycan chains can be resolved

Lee, J.H. et al., *Structure* (2015)

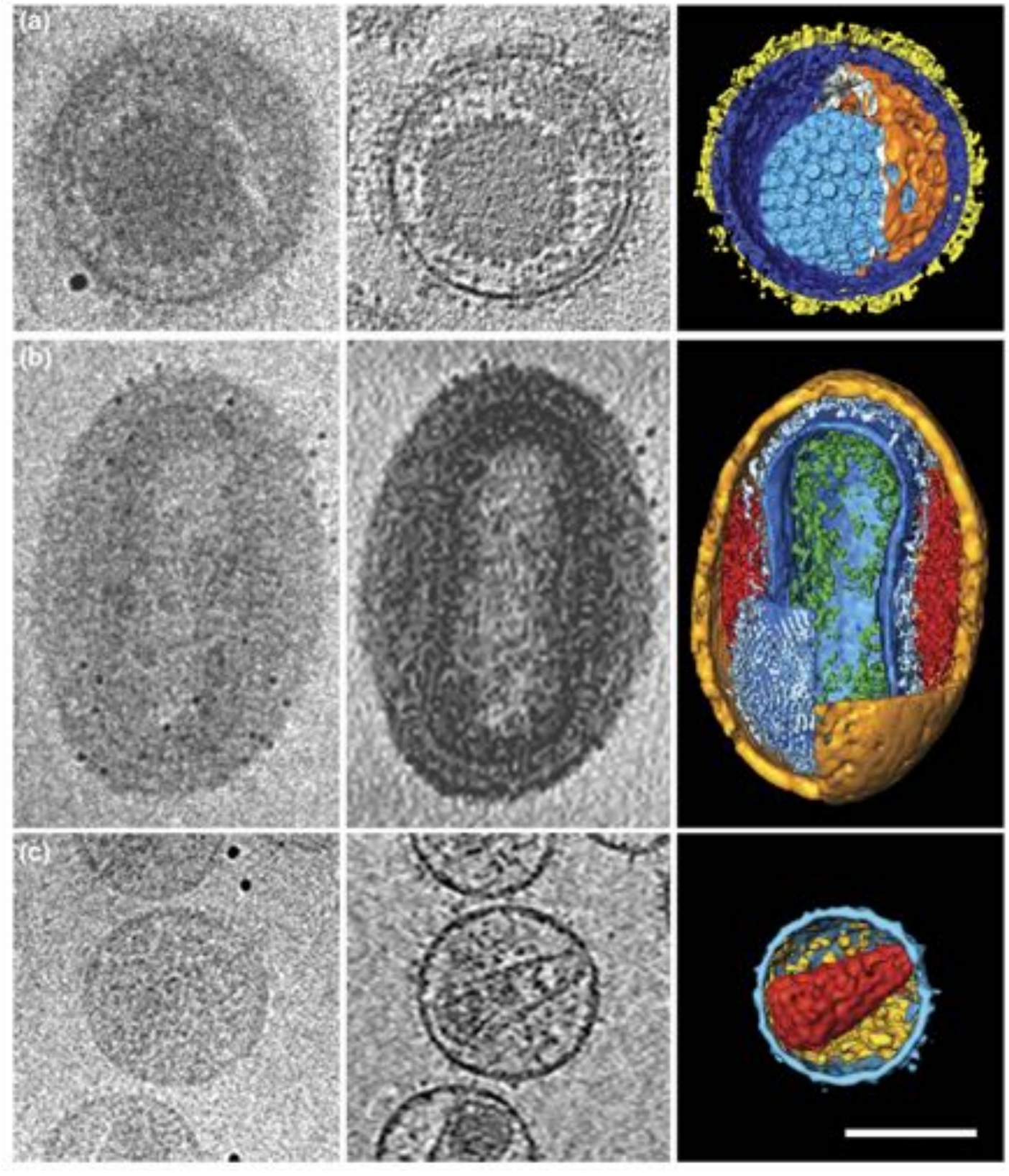
New algorithms enable sub-classification of heterogeneous samples





Lyumkis et al., Science, 2013

Electron Tomography



Grunewald K and Cyrklaff M, (2006) Curr. Op. Microbiol. 9: 437

Electron Tomography

- Obtain 3-D structural information to ~20 Å resolution for non-symmetrical, highly variable structures such as enveloped viruses, bacteria, organelles (e.g. mitochondria)

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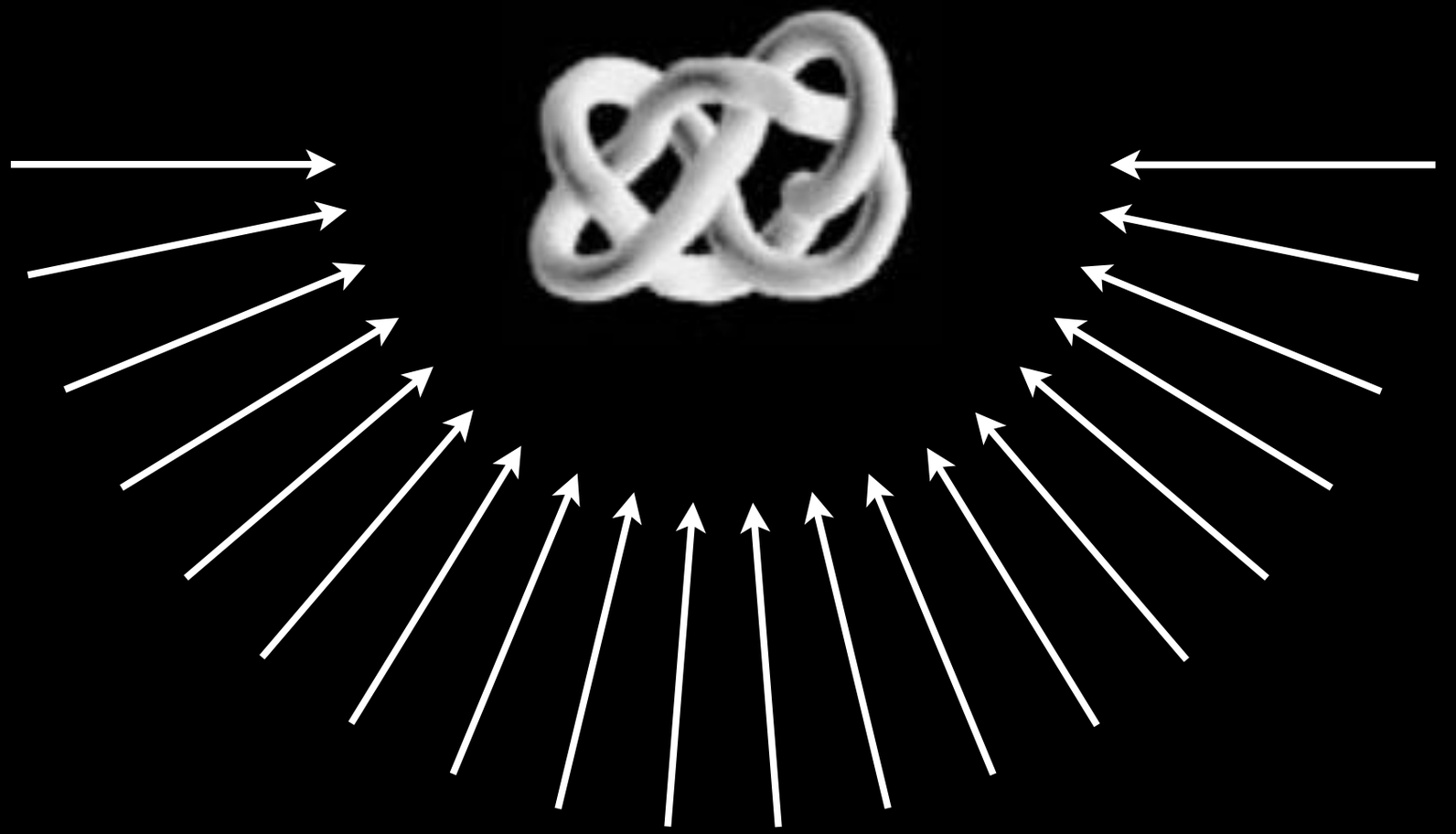
- Incomplete information due to sample geometry (±70°)

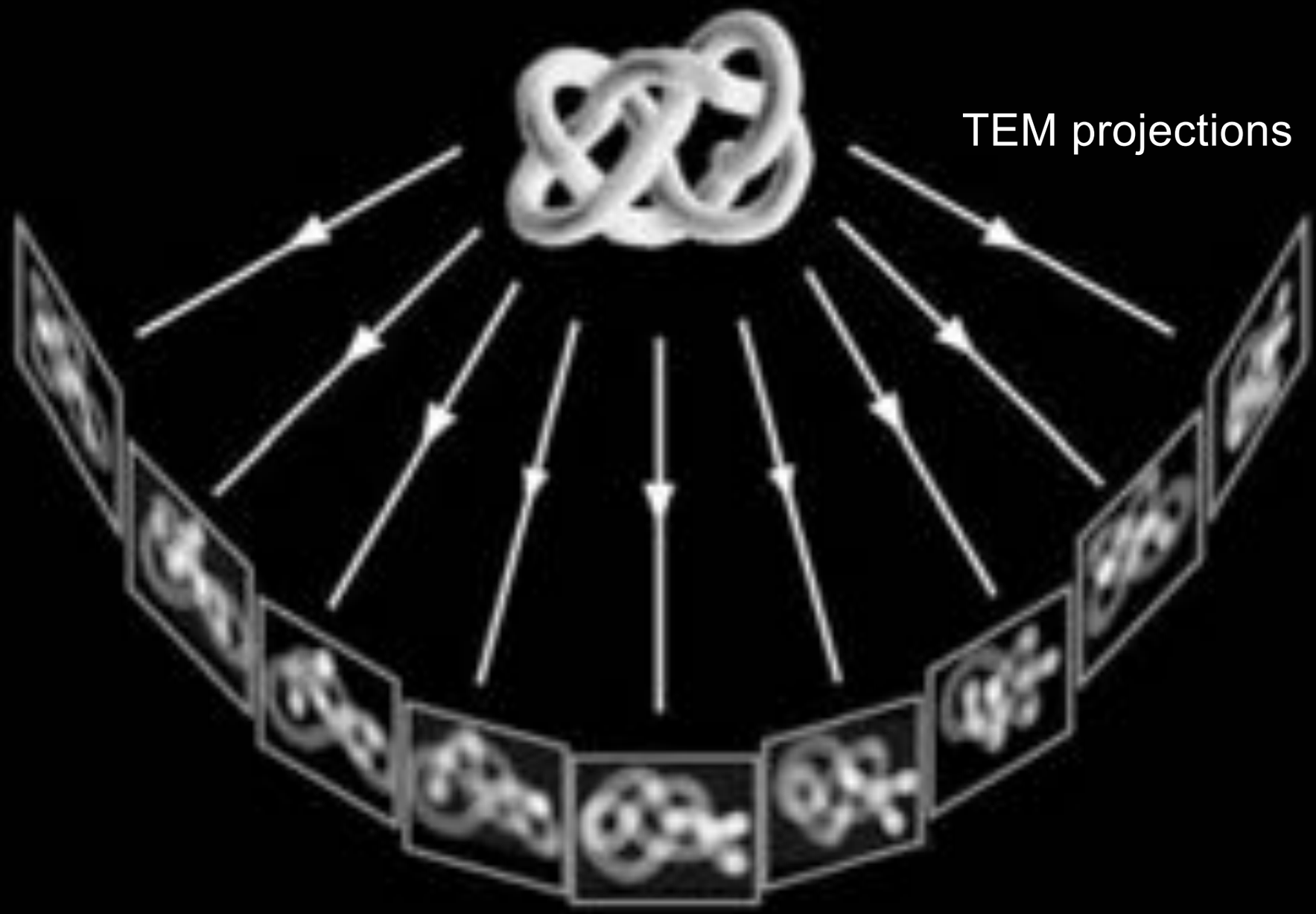
Electron cryo-tomography to determine
3-D architecture of biological nano-scale objects



Ideally would like to gather -90˚ to +90˚ views

Figure adapted from Lucic V, et al., Ann. Rev Biochem, (2005)

Gather projections over as wide an angular range as is possible



Can gather -70˚ to +70˚ ⟶ missing “wedge” of information

Figure adapted from Lucic V, et al., Ann. Rev Biochem, (2005)

The weighted back-projection method is one way to reconstruct the 3-D density

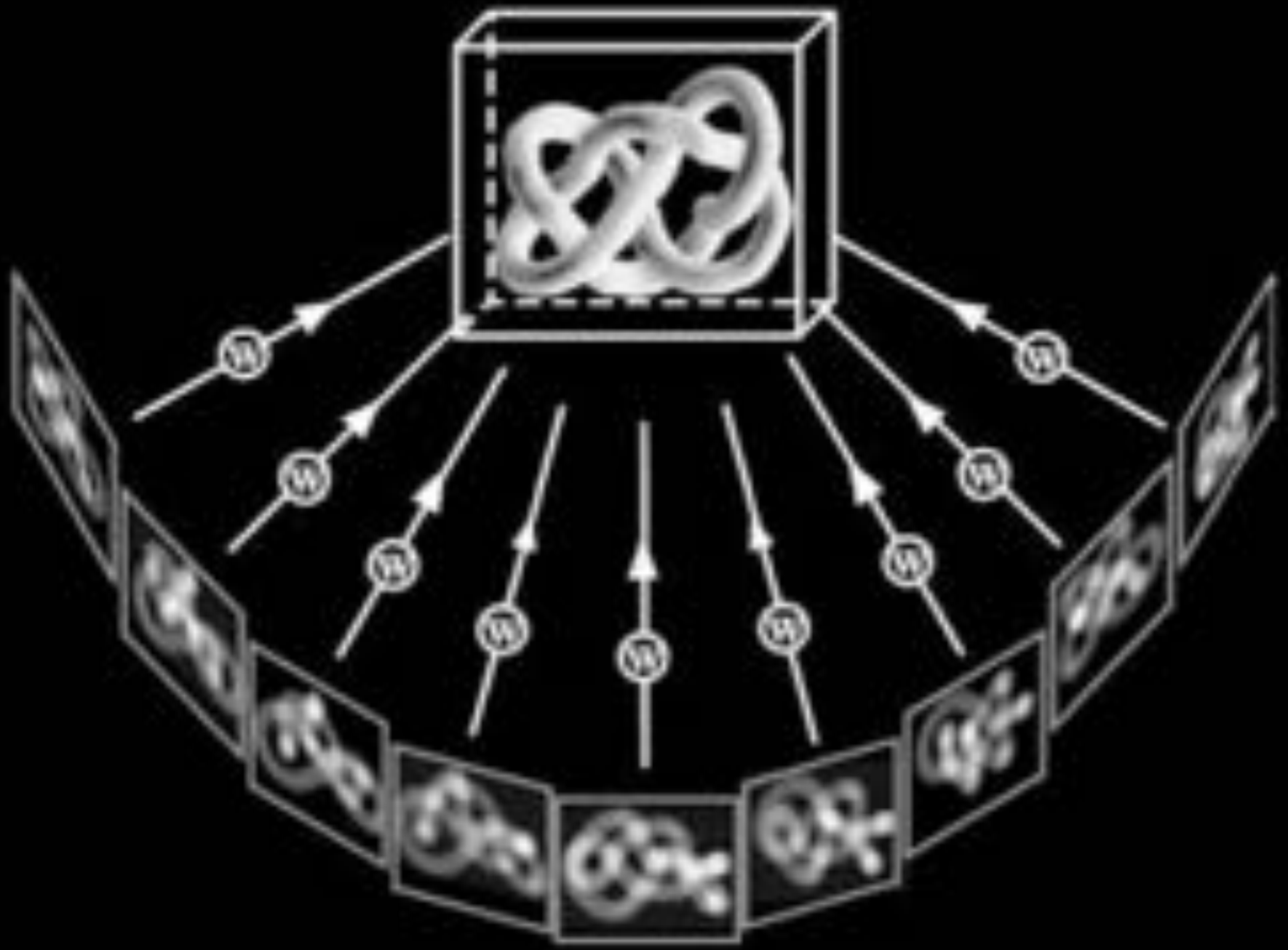
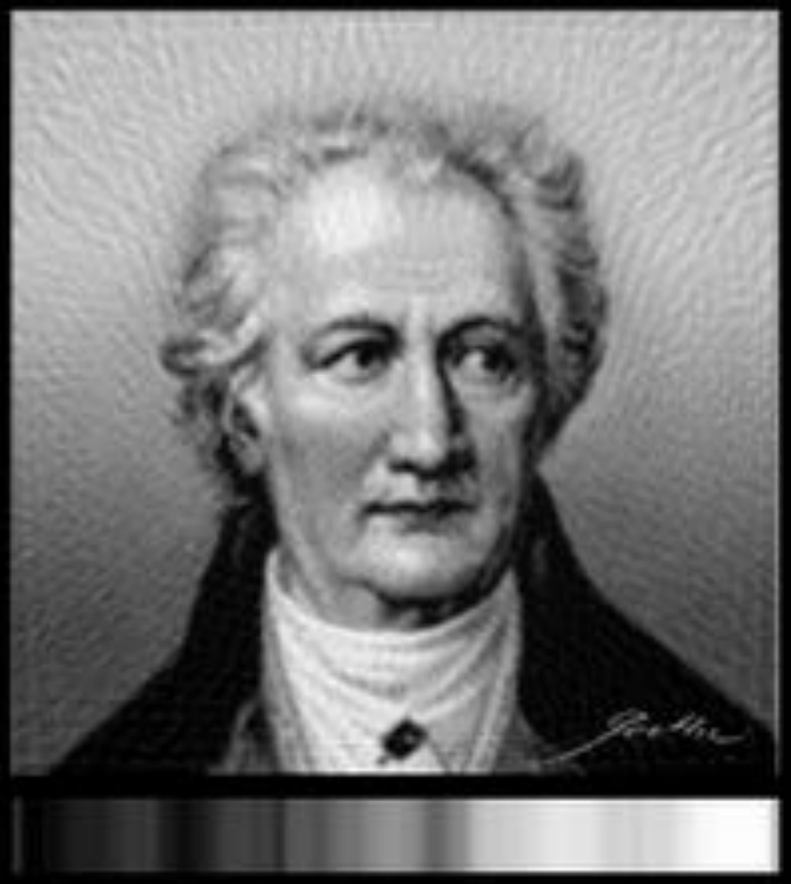


Figure adapted from Lucic V, et al., Ann. Rev Biochem, (2005)

Missing wedge

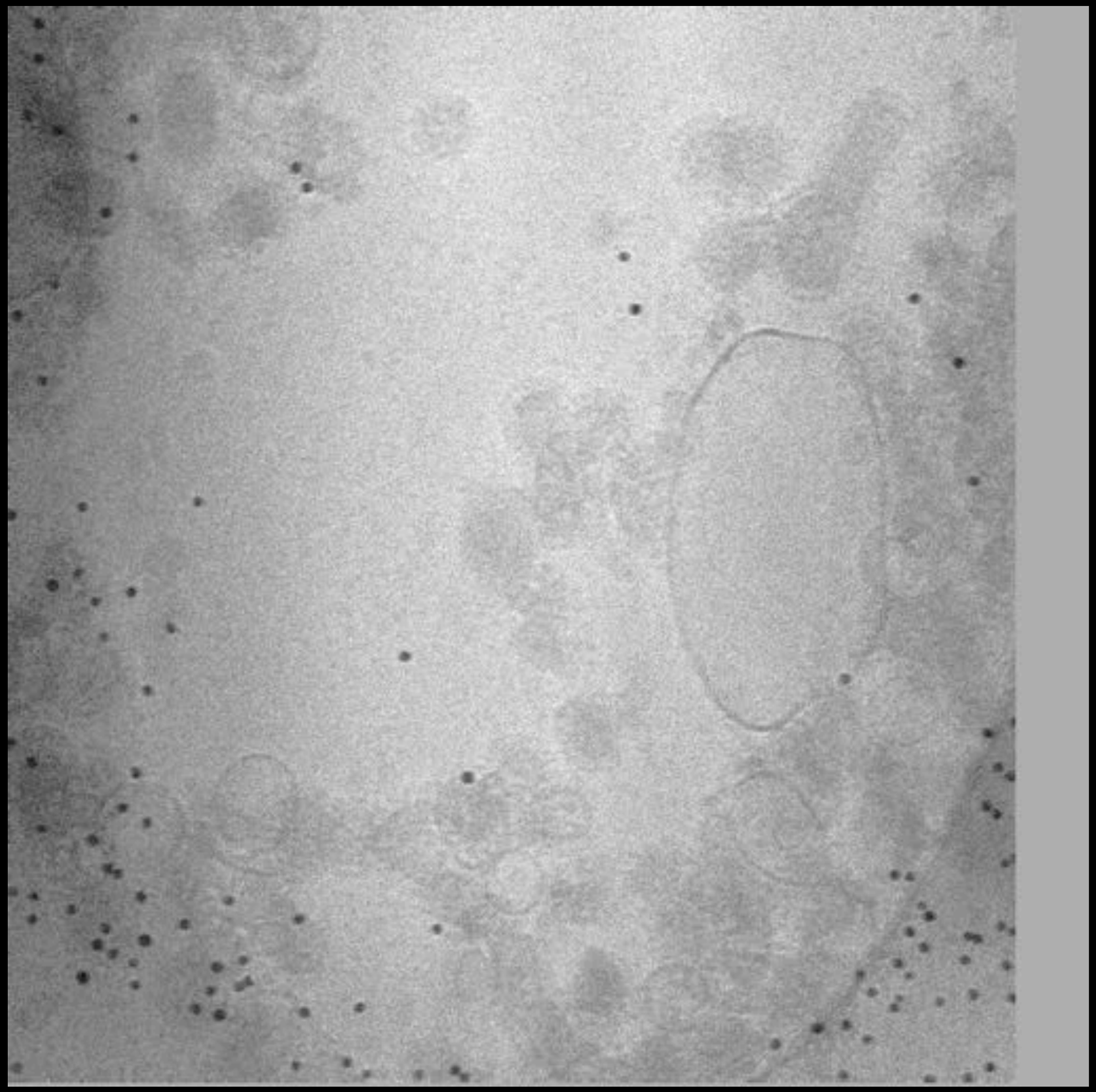
reconstruction of series with
± 90° tilt angle range



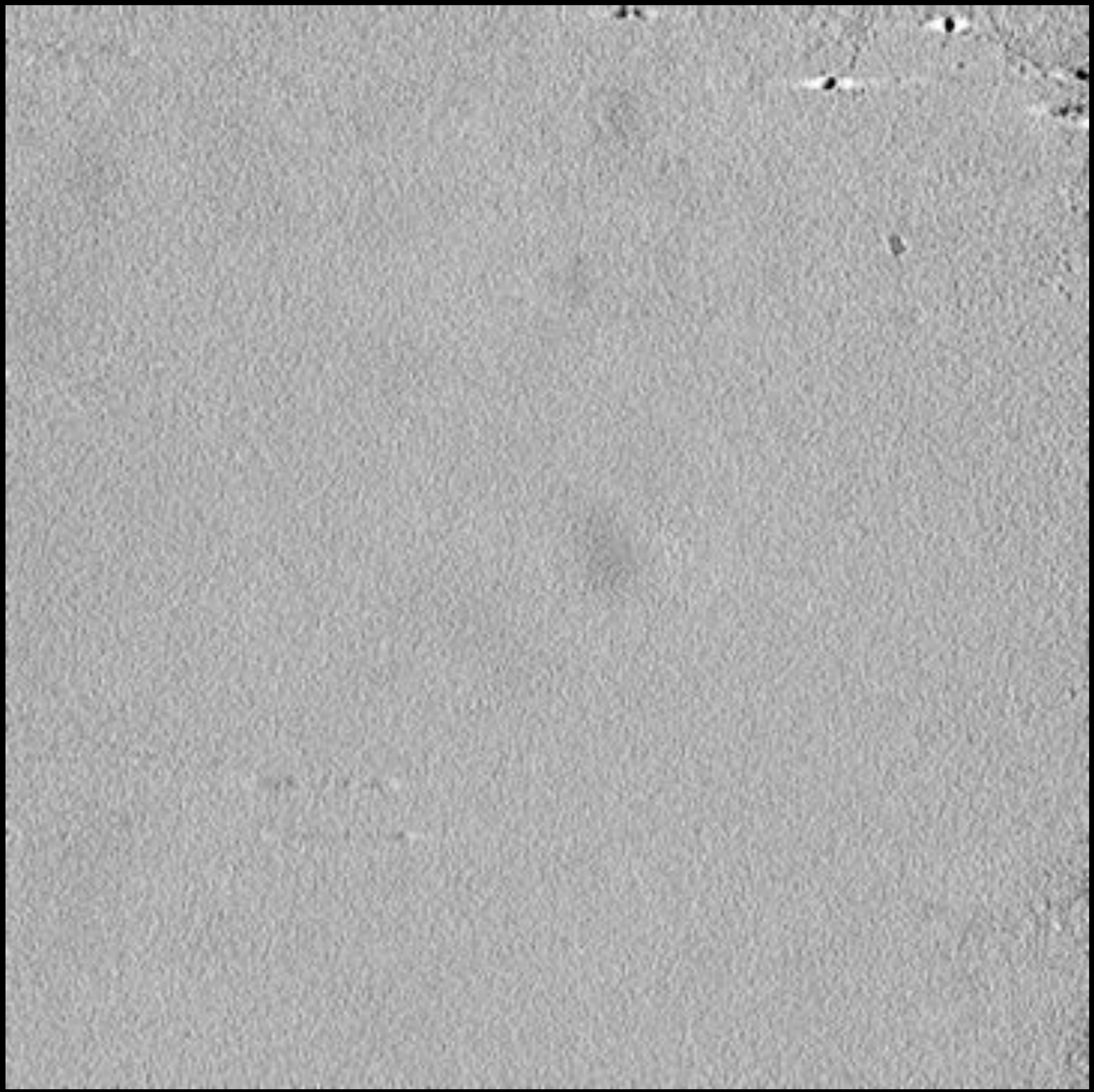
reconstruction of series with
± 60° tilt angle range

Figure by Daniela Nicastro

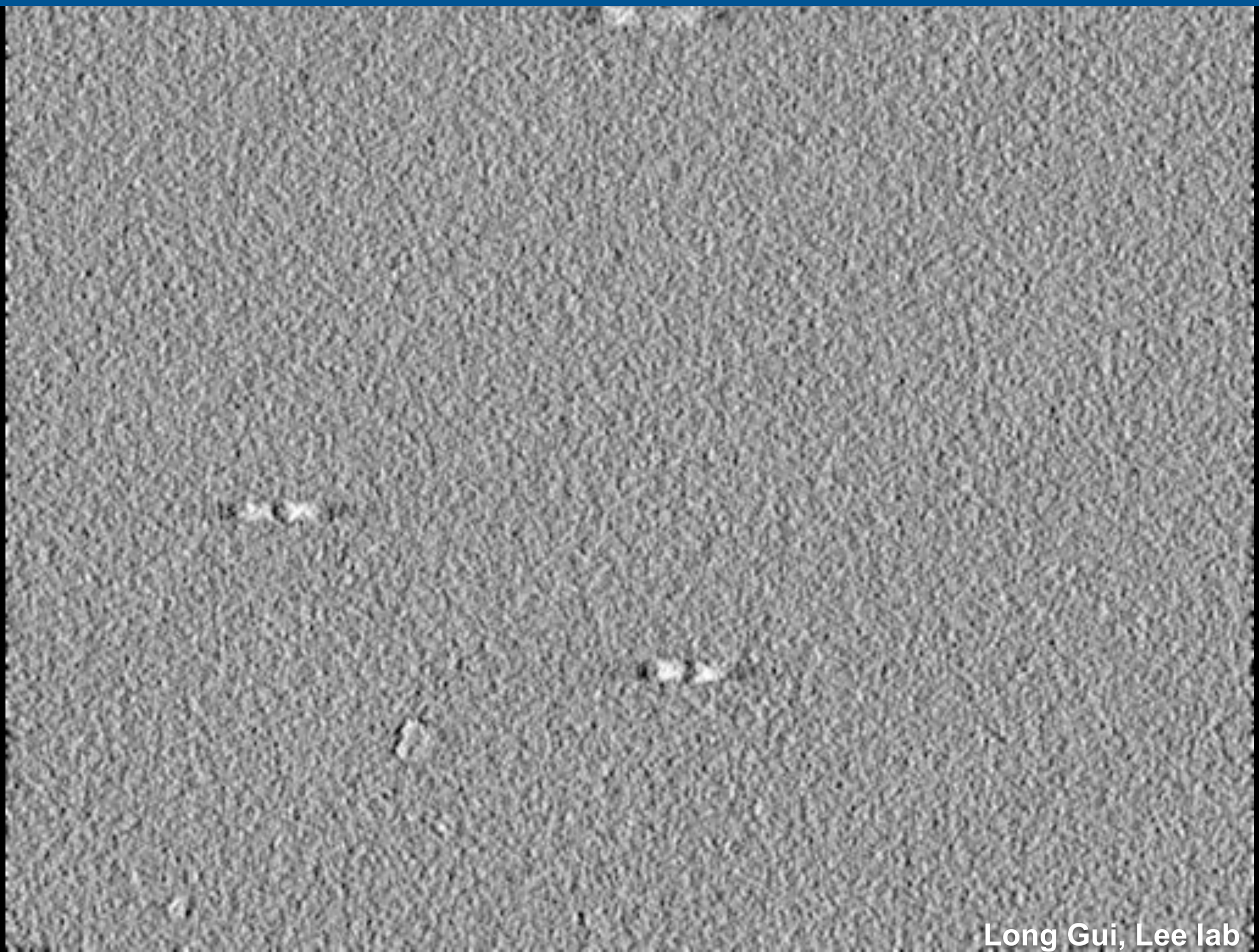
Aligned tilt series showing a field of X31 flu virions and DOPC lipsomes at pH 5.5



120kV FEI T12 microscope; automated data aquisition using Leginon’s Tomography suite

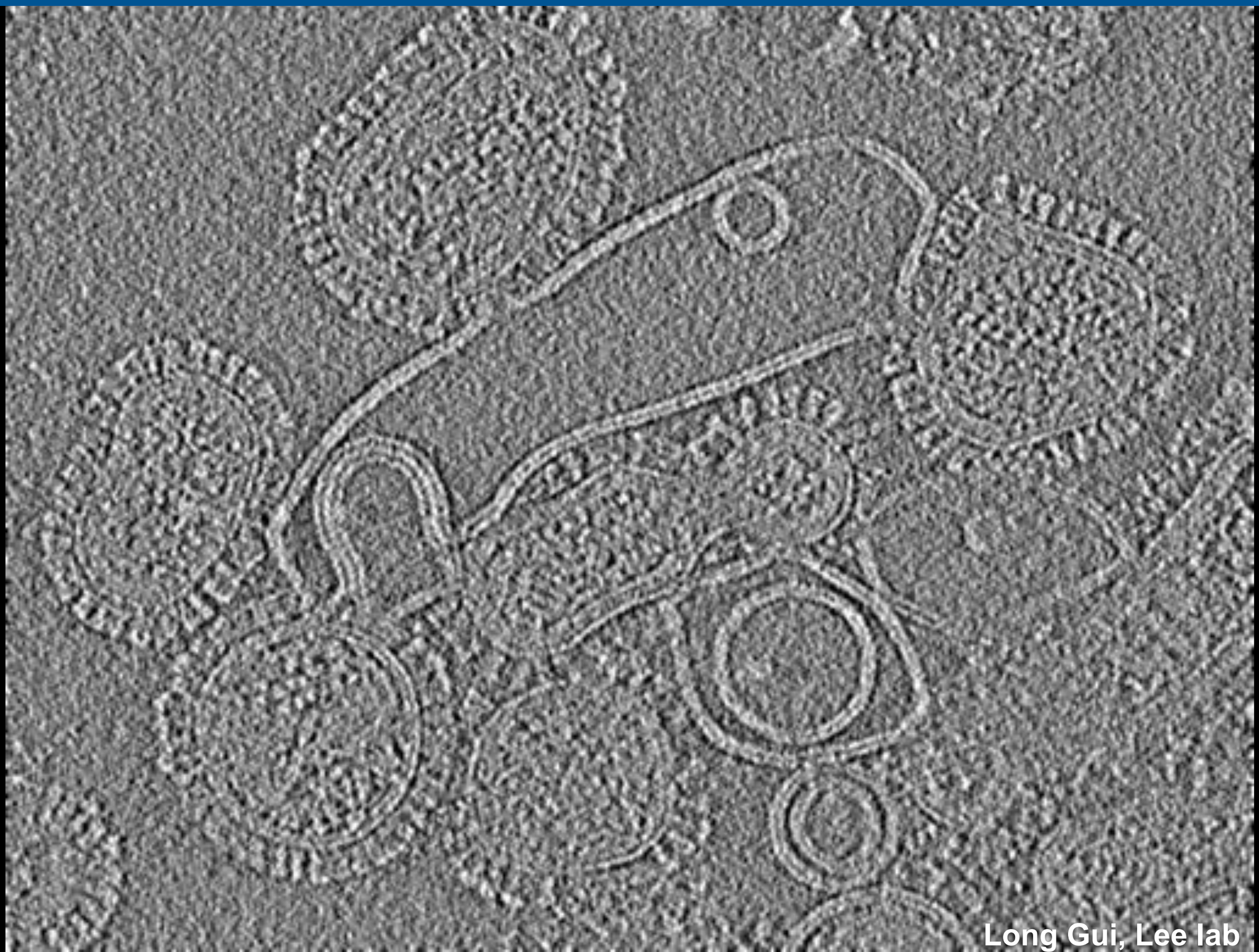


Fusion glycoproteins: Virus machinery for cell invasion



Long Gui, Lee lab

Fusion glycoproteins: Virus machinery for cell invasion



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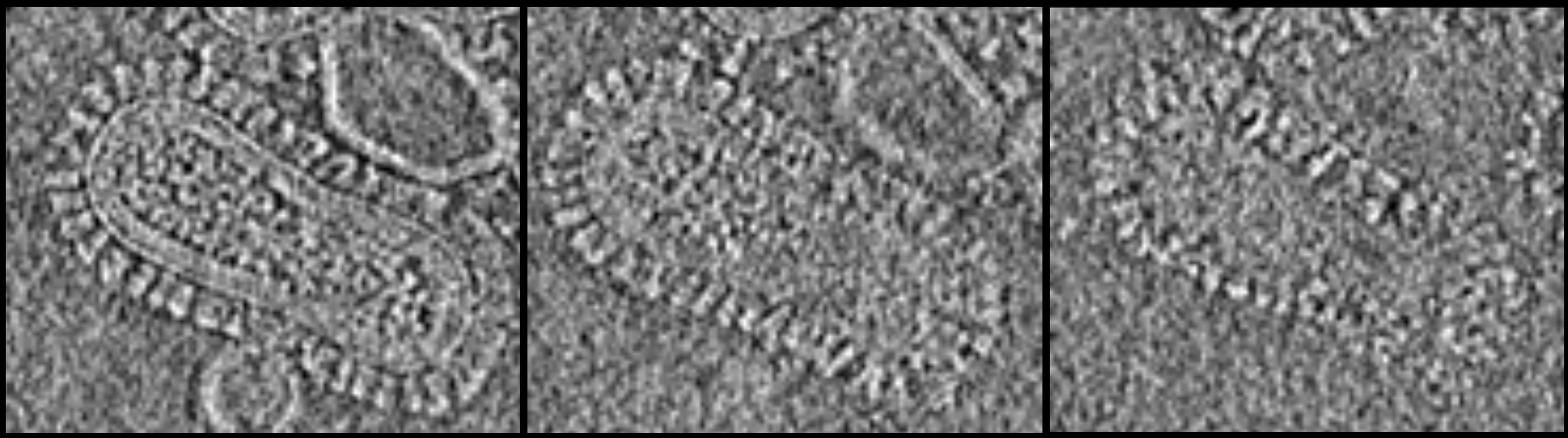
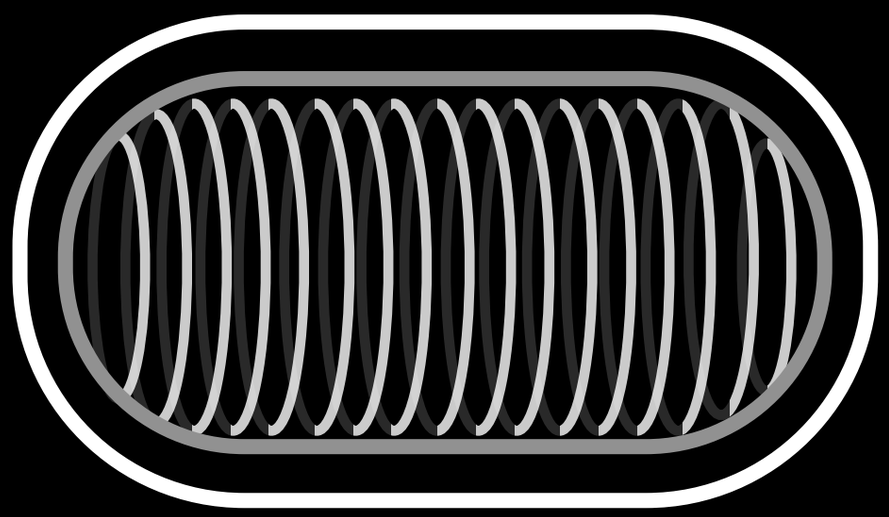
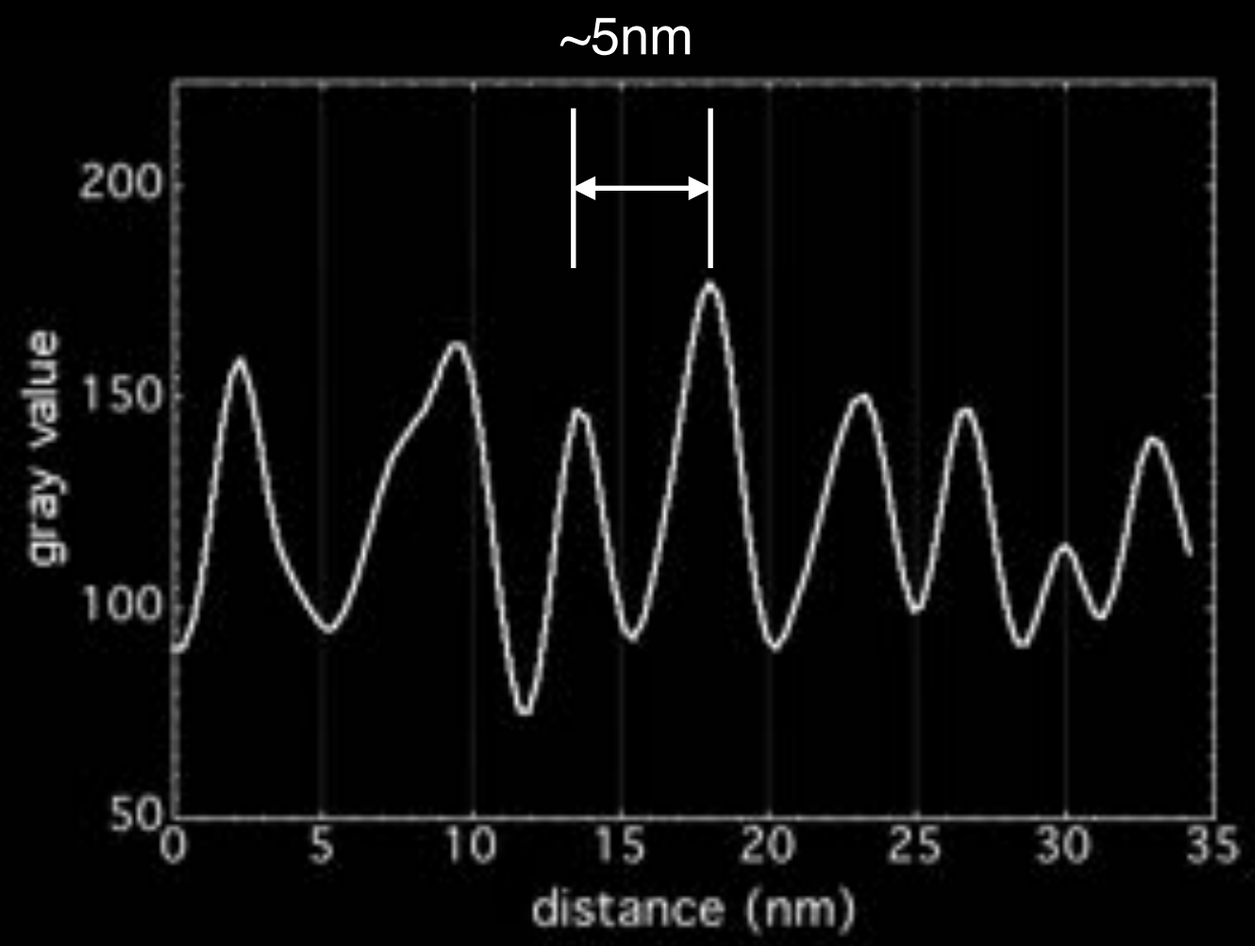
Stacked M1 ribs line the inner surface of the viral envelope





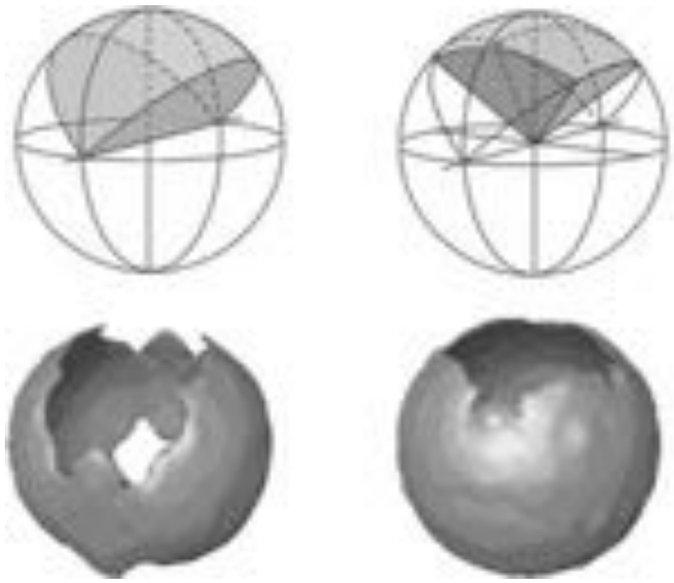


Long Gui, Lee lab

LIMITATIONS

- Missing wedge effect. Incomplete information.
- Radiation damage. Loss of information. Sample changes with imaging.
- Anisotropic resolution
  - Worst in the z-direction
  - Even when good, limited to about 2 nm (20 Å)

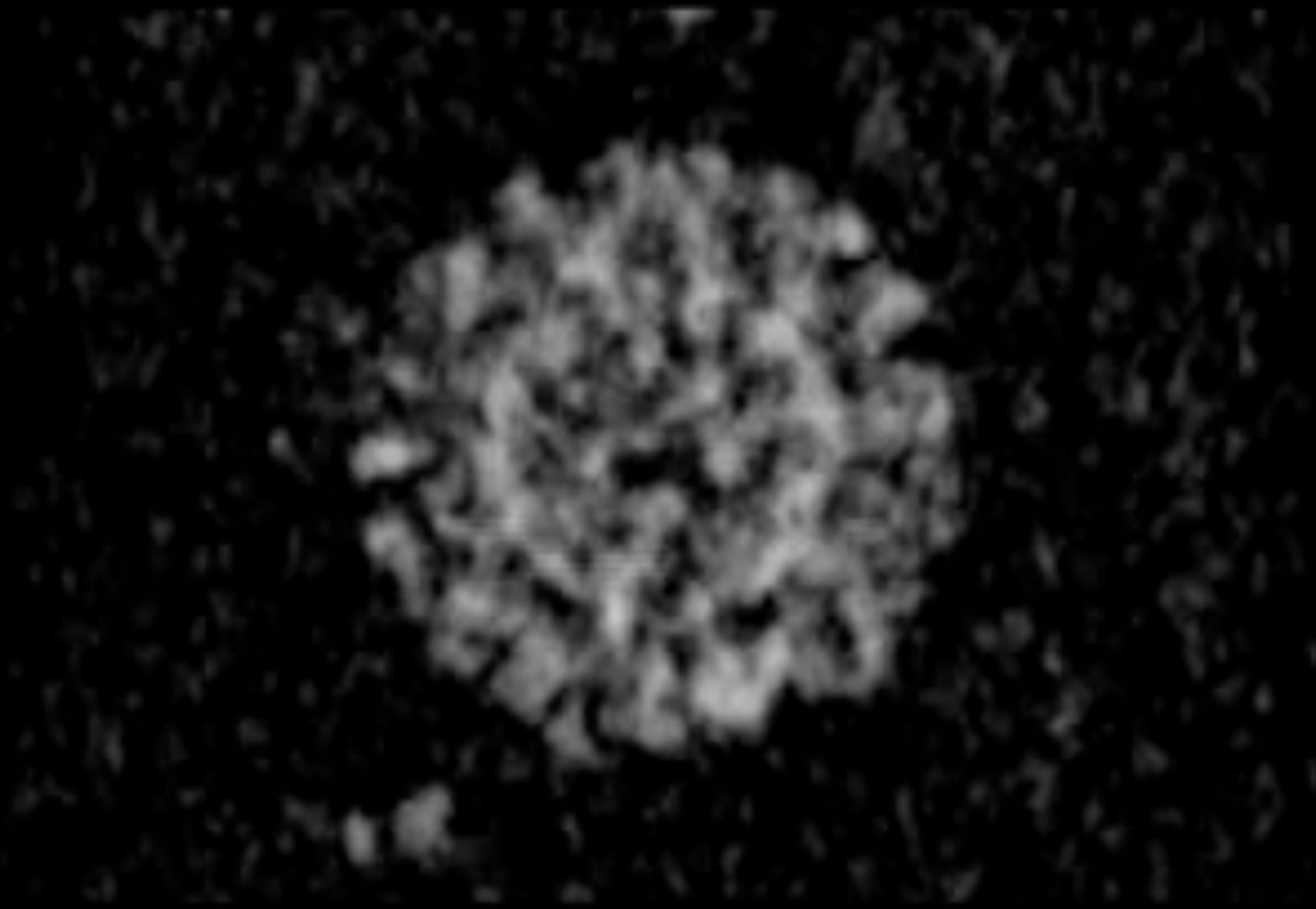
Missing wedge/cone



Tilt range	Single	Double
±70°	78%	93%
±60°	67%	84%
±45°	50%	67%

Figure from Lucic V, et al., Annu Rev Biochem, (2005), 74:833

Anisotropic resolution

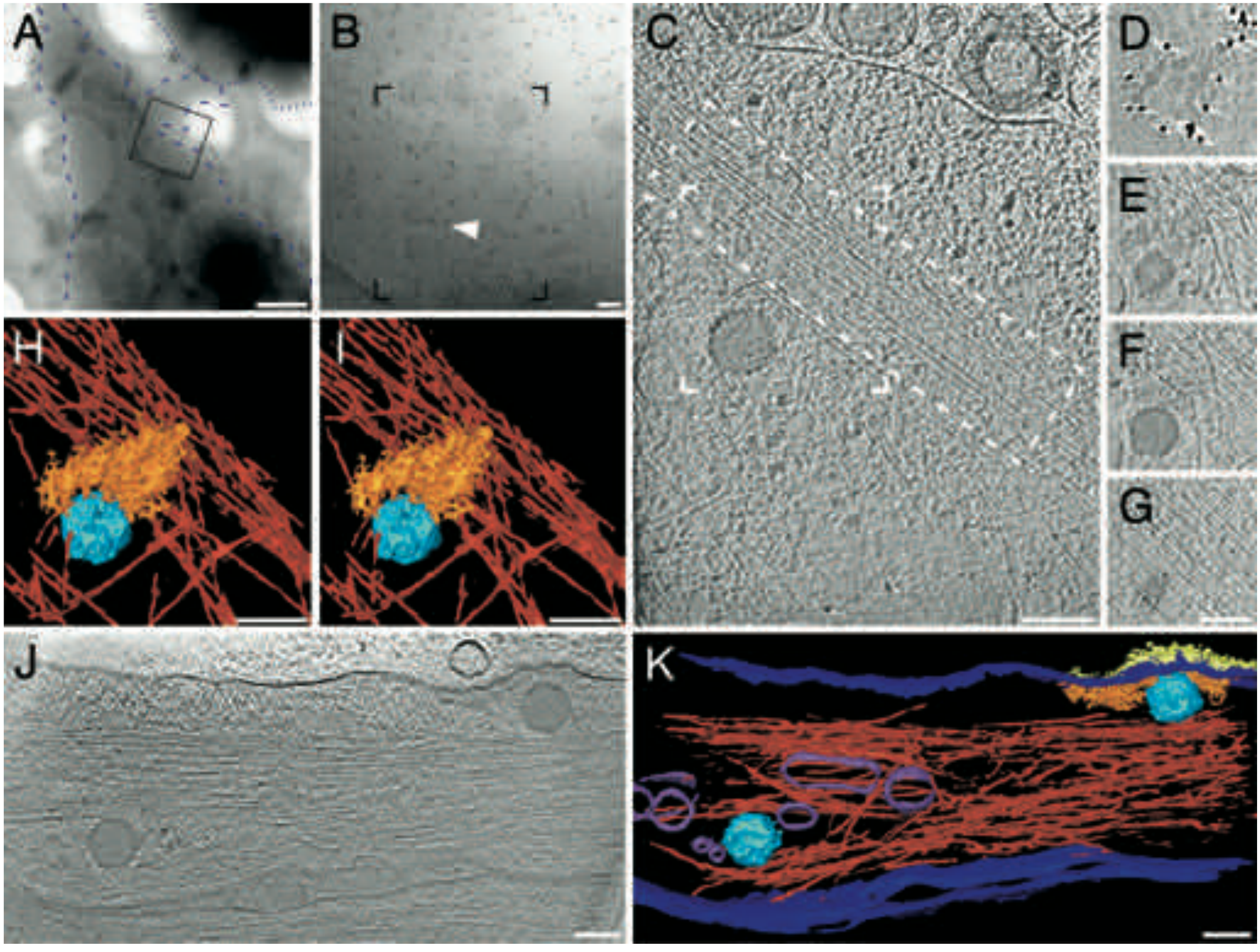


Despite the limitations...

- For some biological questions, there simply is no other way of obtaining structural information

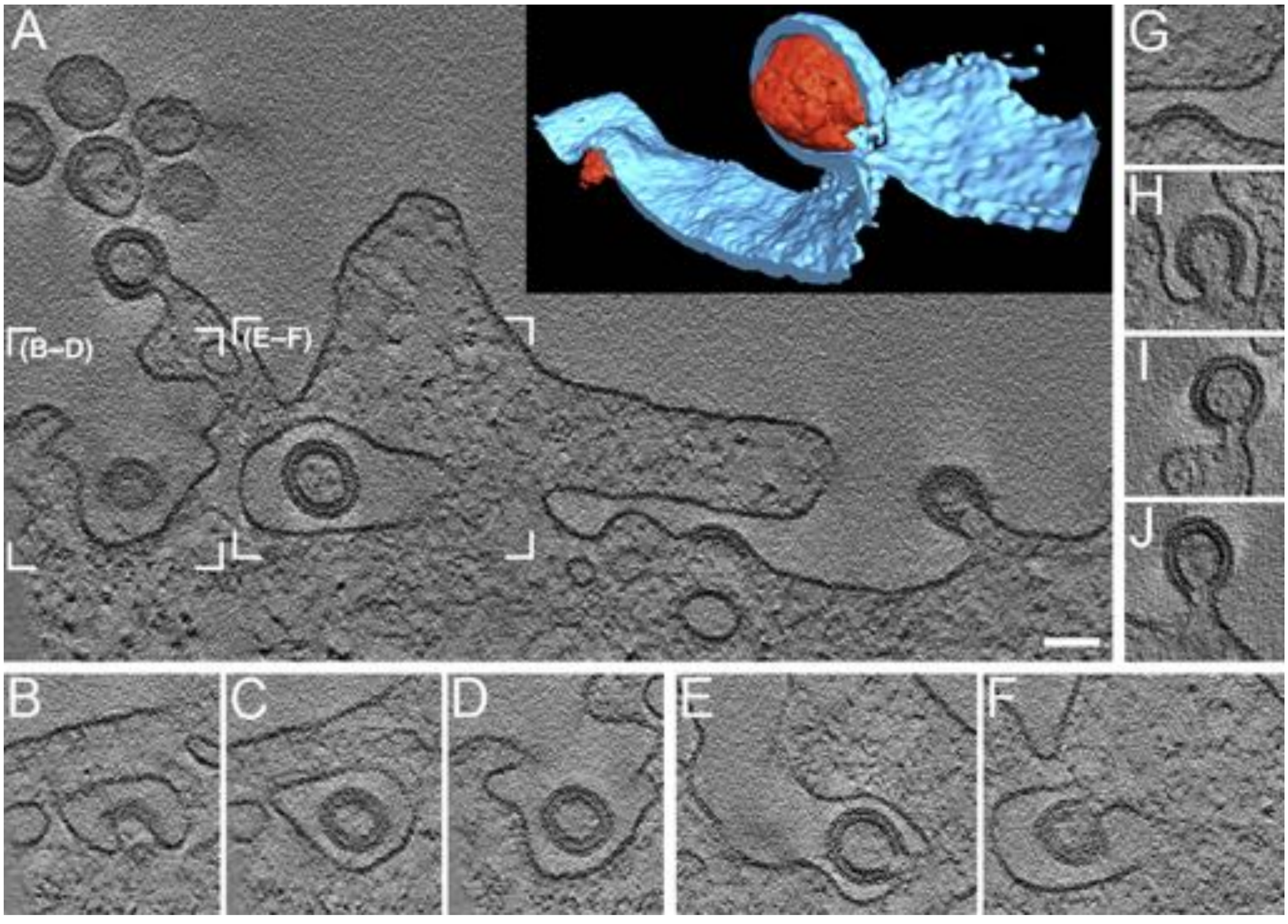
- Good for imaging the architecture, organization of complex systems

Other examples of tomography applications: whole cell imaging



Herpes Simplex Virus-1 cell entry Maurer UE et al. (2008) PNAS 105:10559

Other examples of tomography applications: whole cell imaging



HIV virus budding Carlson L-A et al. (2008) Cell Host Microbe 4:592

Electron Tomography

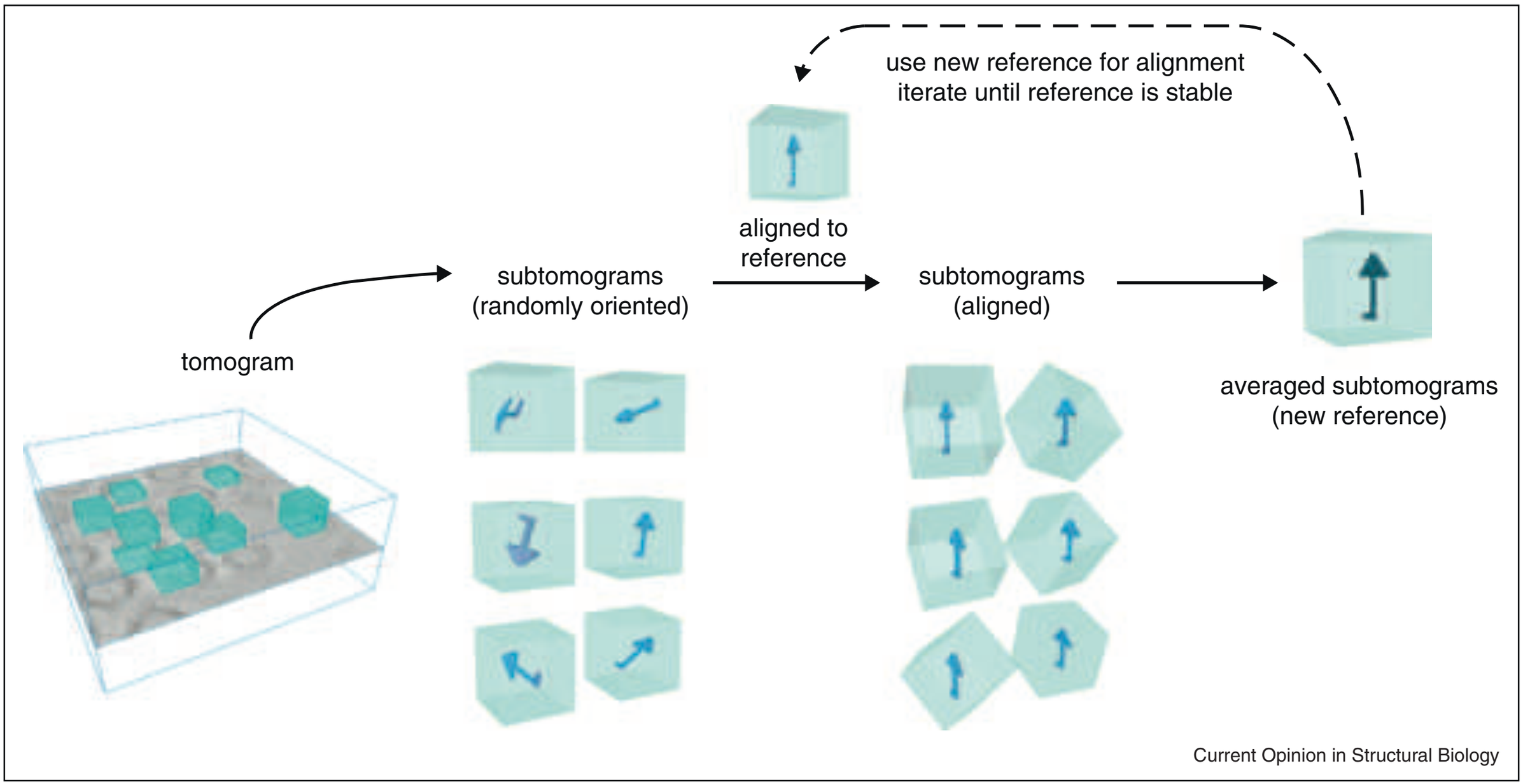
- Obtain 3-D structural information to ~20-30 Å resolution for non-symmetrical, highly variable structures such as enveloped viruses, bacteria, organelles (e.g. mitochondria), etc.

- Thin-section and high-pressure frozen, freeze-substituted specimens can also be examined by tomography allowing whole cells/tissues to be studied

- Feasible with negative stain or cryo samples

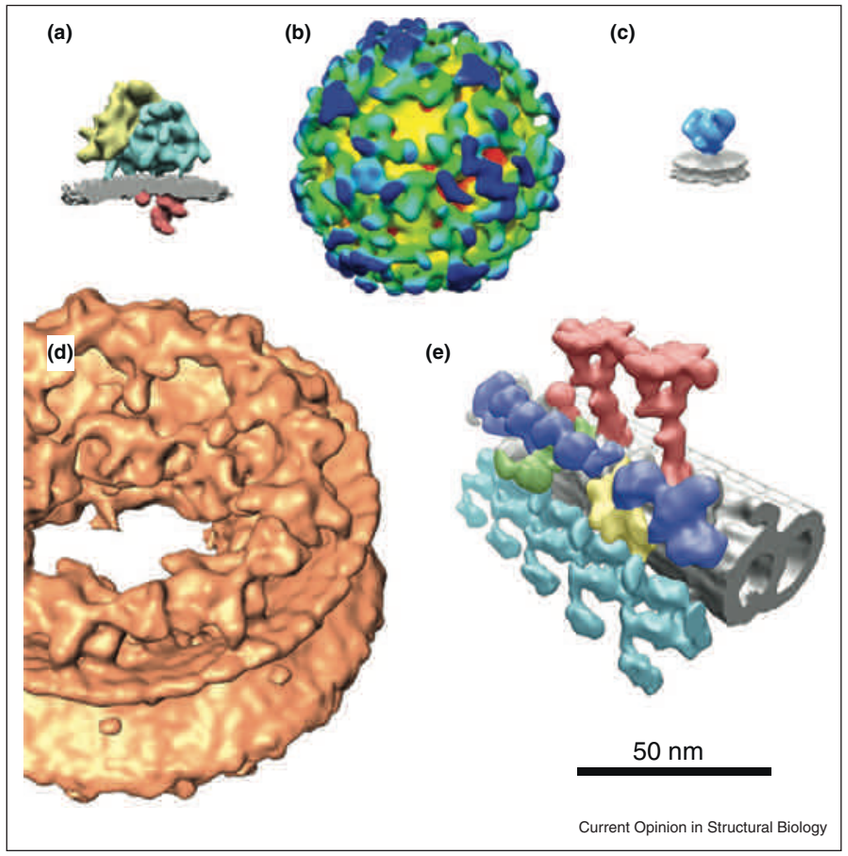
- Incomplete information due to missing wedge

Electron Tomography: sub-tomogram averaging

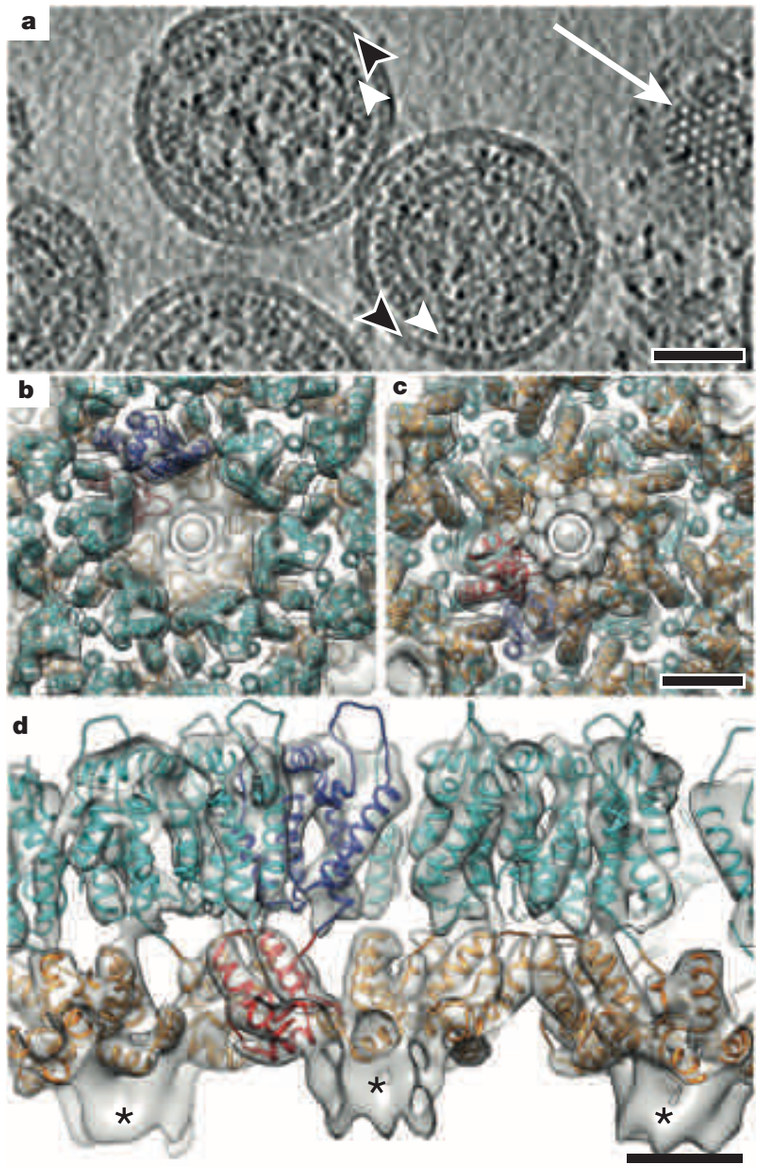


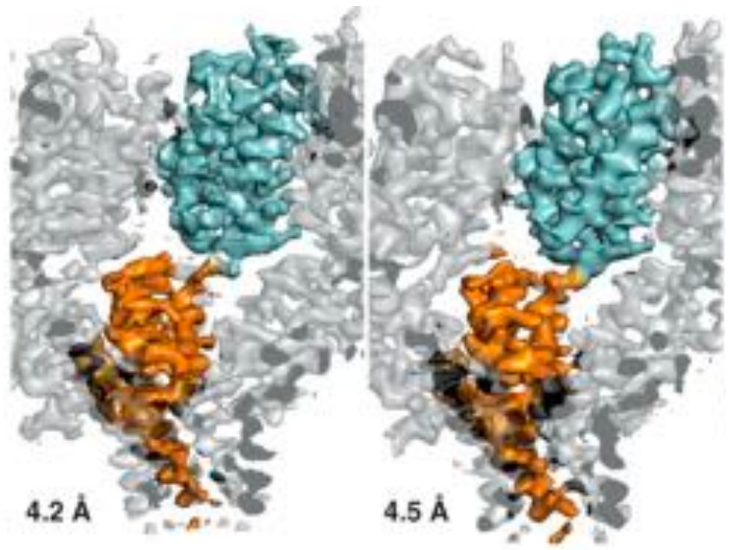
Briggs, JAG, *Current Opinion in Structural Biology*, 2013

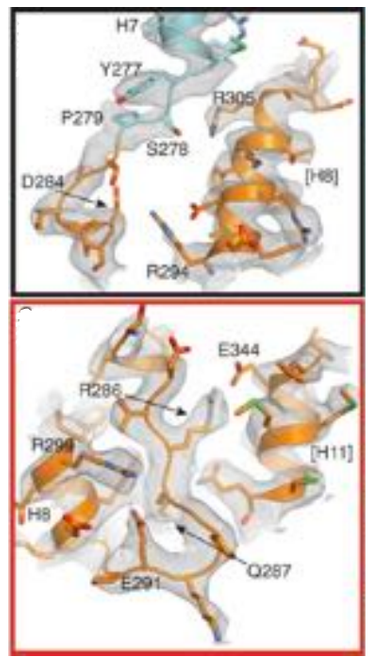
Electron Tomography: sub-tomogram averaging



HIV Gag lattice in intact immature particles





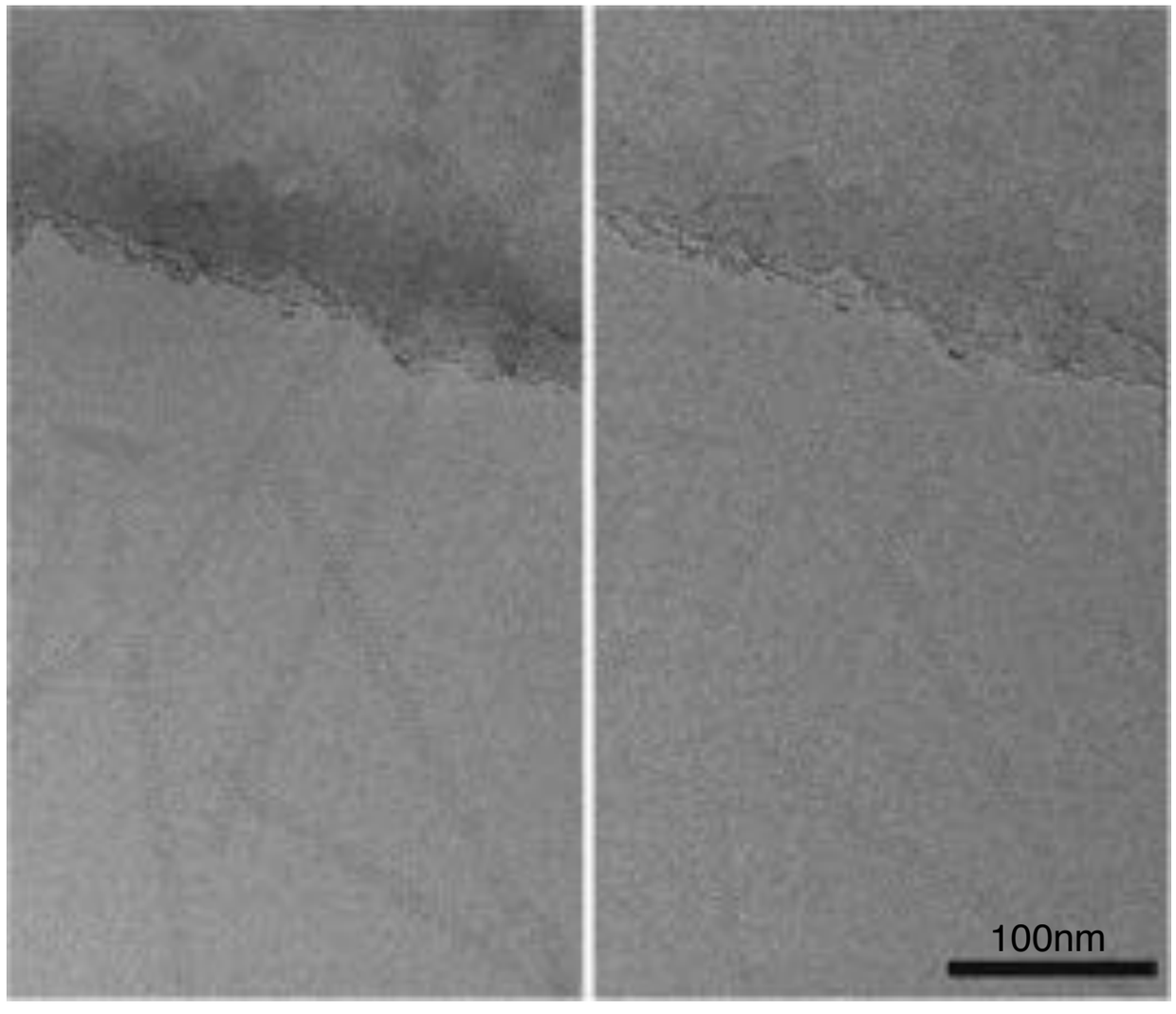


Briggs, JAG, Curr. Opin. Struct. Bio., 2013
Schur et.al., Nature, 2015;
Schur et.al., Science, 2016

TECHNICAL ADVANCES

Energy filter

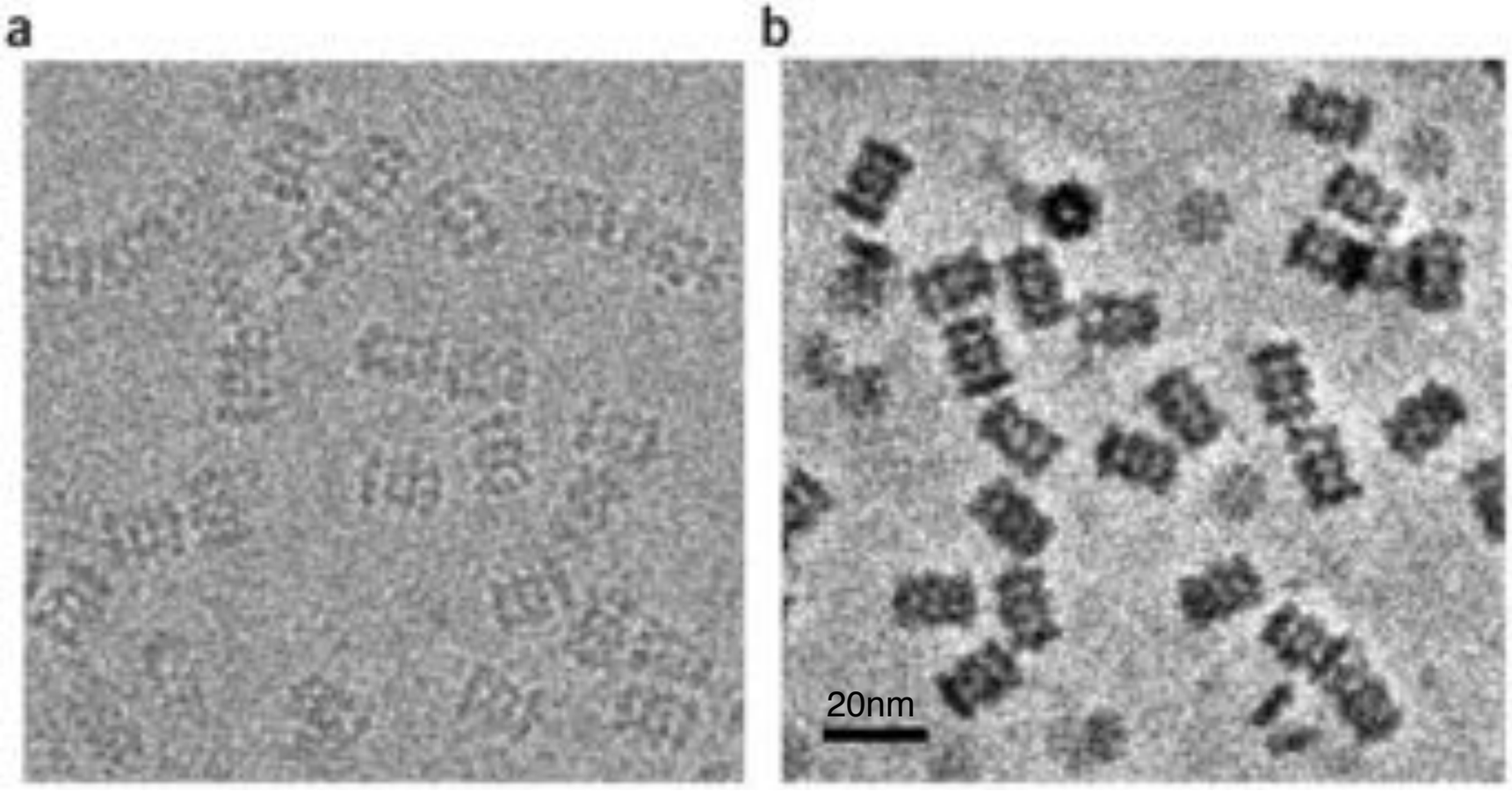
- Enhances amplitude contrast in cryo-EM images by selectively filtering out scattered electrons
- Energy filters used more diligently for cryo-ET due to it's very low signal to noise ratio, but increasingly being used for single particle cryo-EM also



Yonekura et.al., J. Struct. Bio., 2006

Phase plate

- Shifts phase of scattered electron beam to create contrast
- Can achieve near-focus phase-contrast
- Especially advantageous for smaller protein complexes
- Implementation is still tricky, but shows promise



Cryo-EM image of 20S proteasome at ~1.7μm defocus.

Near focus cryo-EM image using Volta phase plate

Max Planck Institute for biochemistry

The case for automation

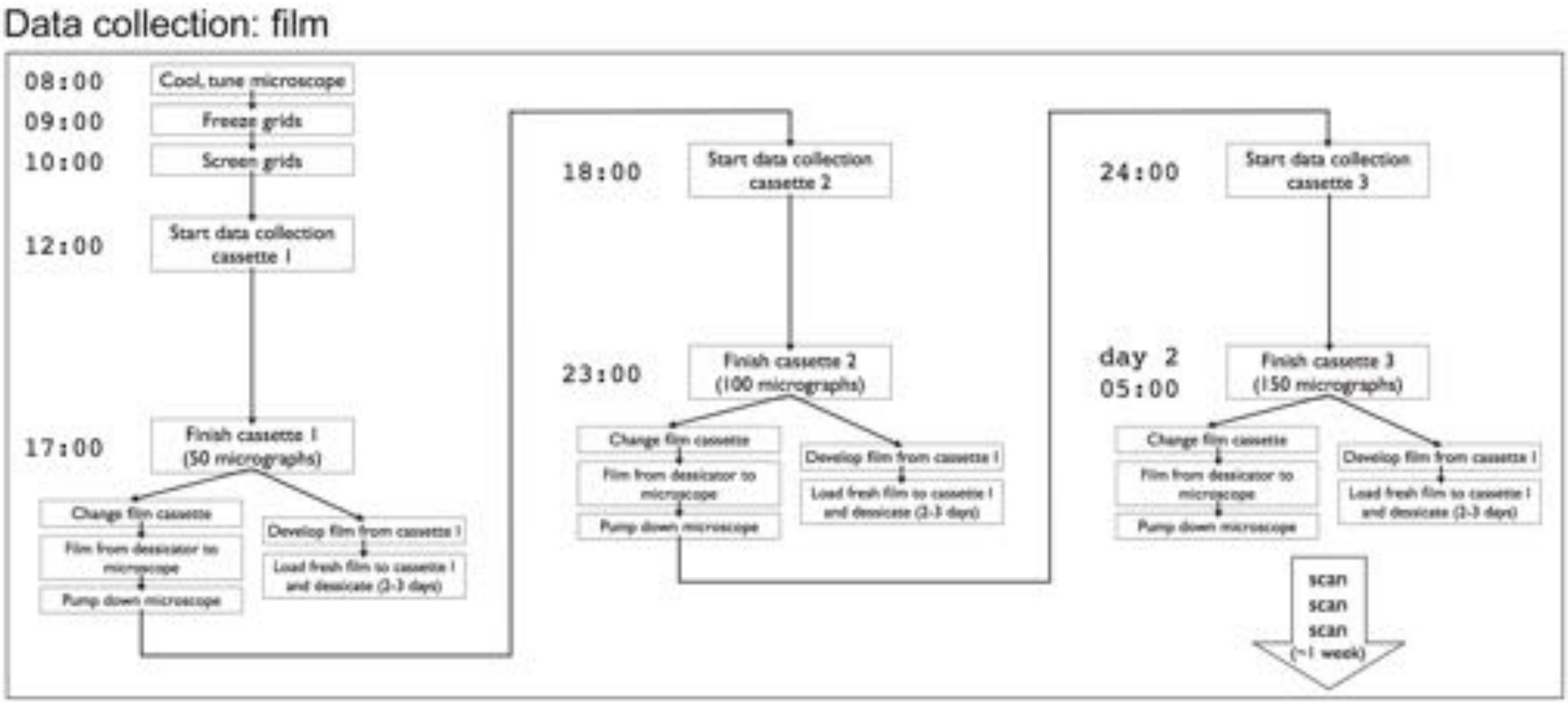
- Can increase the particle sampling by 10-50x over manual data collection (up from 10,000 particles per reconstruction to 500,000)

- Standardization and greater uniformity

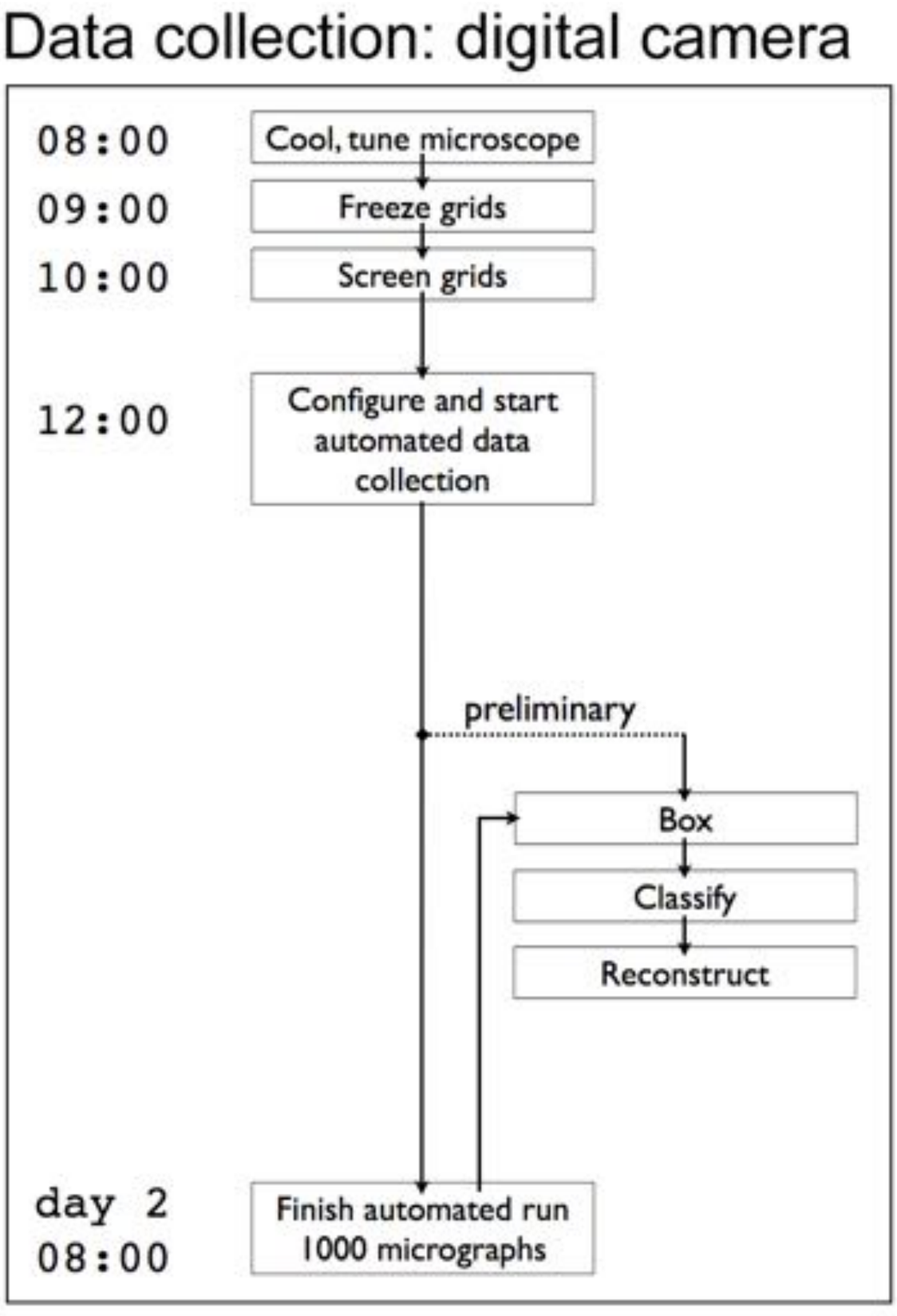
- NRAMM: National Resource for Automated Molecular Microscopy.
  - Developed the "Leginon" software package that controls data collection over an entire grid.

More particles included in reconstruction can improve averaging and signal-to-noise (but if there is heterogeneity among particles in a population, need to sort that out)

The case for automation



The case for automation



SUMMARY

- TEM is a versatile imaging technique that can be used to image a variety of samples, from cells/tissues to high resolution protein structure
- Tomography
- Single particle analysis
- Negative stained or cryo
- What it is NOT good for, though people frequently use it for this:
  - Quantifying populations and *interpreting that as representing populations in solution*: adsorption/grid effects can be dominant.
- Still more advances in methodology ahead… especially in software development

Electron Microscopy REFERENCES

1. Three-dimensional Electron Microscopy of Macromolecular Assemblies. Joachim Frank (2006) Oxford University Press.

2. http://www.rodenburg.org/guide/index.html Describes process of TEM microscope alignment and some of the physics.

3. Ohi M et al., "Negative staining and image classification- powerful tools in modern electron microscopy" (2004) Biol. Proced. Online 6:23.

4. Electron Tomography. Joachim Frank, editor (2005) Springer.

5. Lucic V, et al., "Structural studies by electron tomography" (2005) Annu Rev Biochem 74:833.

6. Numerous reviews in Current Opinion in Structural Biology. Good starting point to get a summary of a field and links to primary references.

7. http://nramm.scripps.edu/seminars/ Some lectures (with slides and audio) from recent workshops on cryo-EM and structure determination held at the National Resource for Automated Molecular Microscopy (NRAMM) in La Jolla, CA.

8. Cryo-electron microscopy of biological nanostructures. Robert M. Glaeser (2008) Physics Today, January: 48.

More recent good reviews..

1. "Cryo-electron tomography and Sub-Tomogram averaging", W. Wan and J.A.G. Briggs. *Methods Enzymol.* (2016) 579:329:67.

2. "A Primer to Single-particle Cryo-electron Microscopy", Y. Cheng, N. Grigorieff, P.A. Penczek, T. Walz. *Cell* (2015)161:438.

3. "Cryo-electron microscopy for structural analysis of dynamic biological macromolecules", K. Murata and M. Wolf. Biochimica et Biophysica Acta (BBA) - General Subjects (2018) 1862(2): 324:334

4. "Cryo-electron Tomography: The Challenge of Doing Structural Biology *in situ*", V. Lucic, A. Rigort, W. Baumeister. *J. Cell Biology* (2013) 202:407.

5. "Cryo-EM: A Unique Tool for the Visualization of Macromolecular Complexity", *Molecular Cell* (2015) 58:677.

6. "How Cryo-EM is Revolutionizing Structural Biology", *Trends in Biological Sciences* (2015) 40:49.

7. "Structural Biology *in situ*- the Potential of Subtomogram Averaging", *Current Opinion in Structural Biology* (2013) 23:261.

8. Grant Jensen's fantastic on-line tutorial about cryo-EM: https://www.youtube.com/watch?v=gDgFbAqdM_c&list=PL8_xPU5epJdctoHdQjpfHmd_z9WvGxK8-

Contrast Transfer Function correction is a step in the processing that is critical for accounting for high resolution information, but beyond the scope of this introduction (see references).

Three-dimensional Electron Microscopy of Macromolecular Assemblies.
Joachim Frank (2006) Oxford University Press