TEM tomography and single particle reconstruction

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Motivation

TEM-image represents a projection image of the specimen.

->Features at different depths in the structure are all superimposed.

->Analysis of 2D projections can lead to misinterpretations.

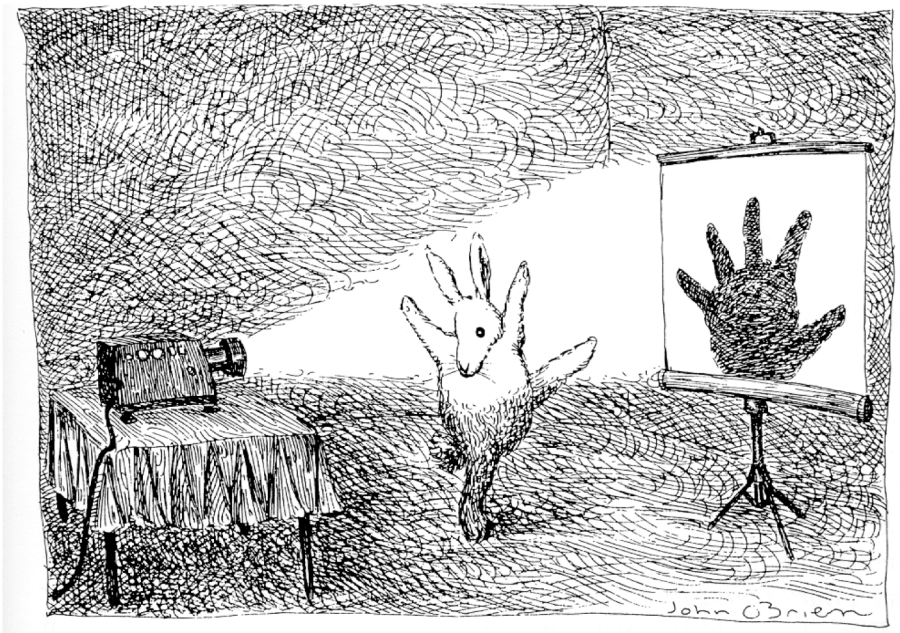


Fig. 5.1. A single projection image is plainly insufficient to infer the structure of an object. Drawing by John O'Brien; © 1991 The New Yorker Magazine.

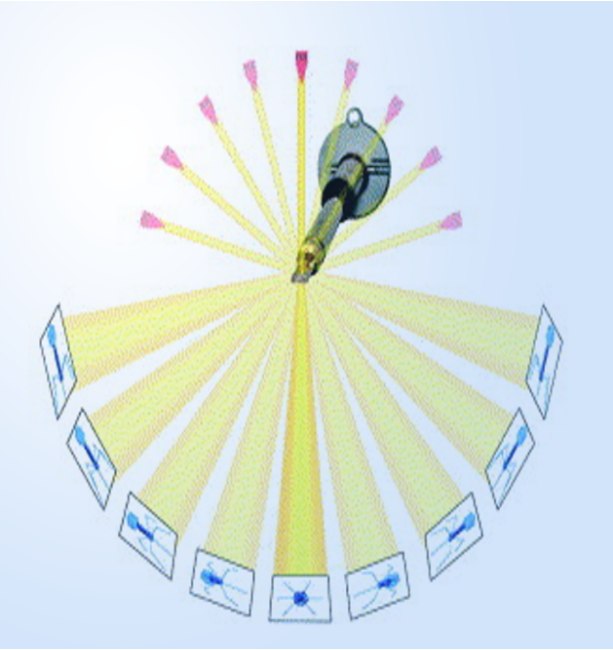
The New Yorker

Solution: combine projections taken at different angles

Tomography as solution

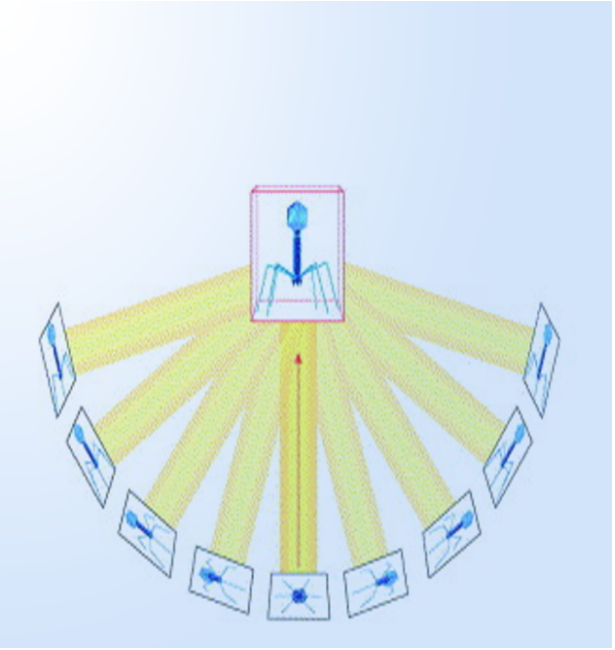
Principle of electron tomography:

DATA COLLECTION



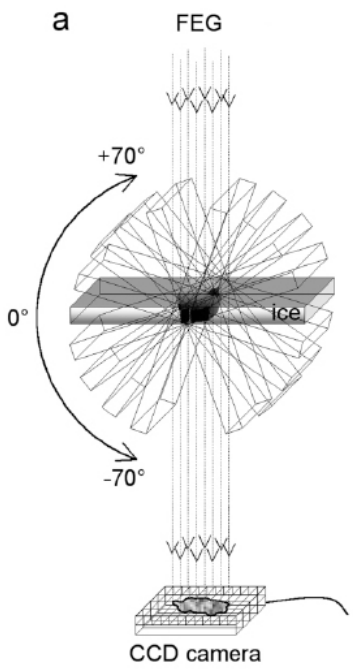
3D object => 2D-projections

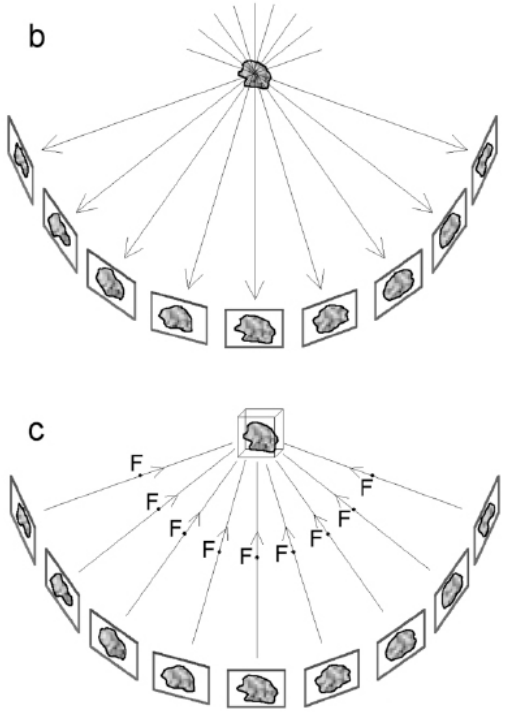
RECONSTRUCTION

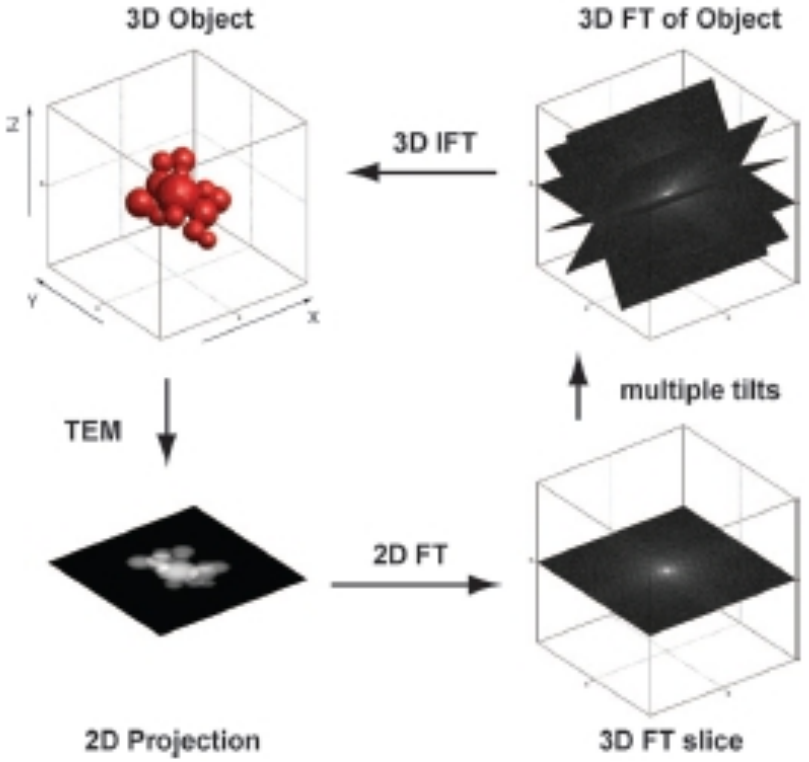


2D-projections => 3D-reconstruction

Process in a nutshell

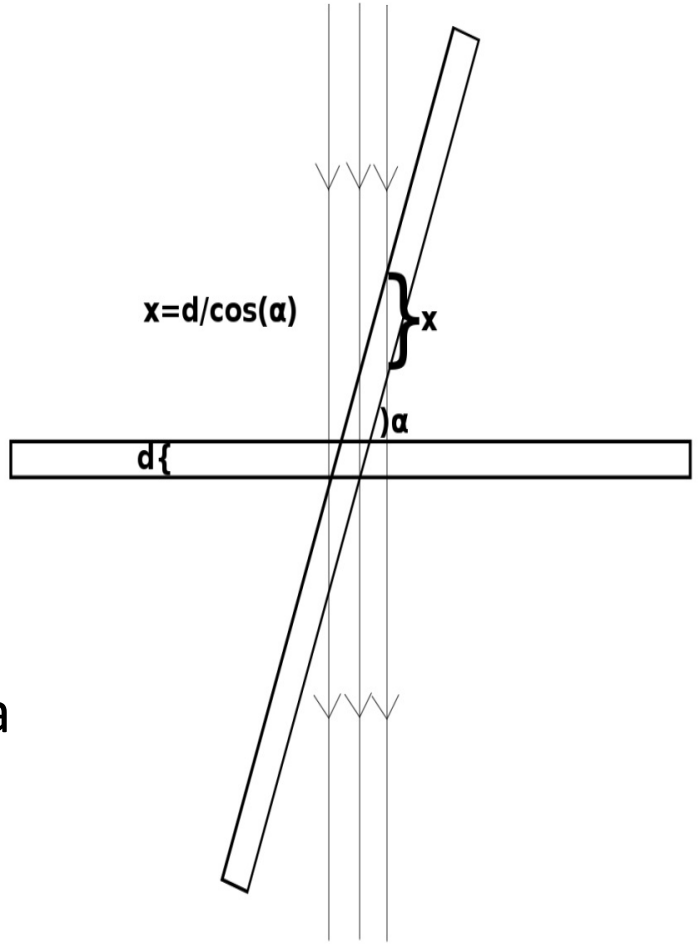






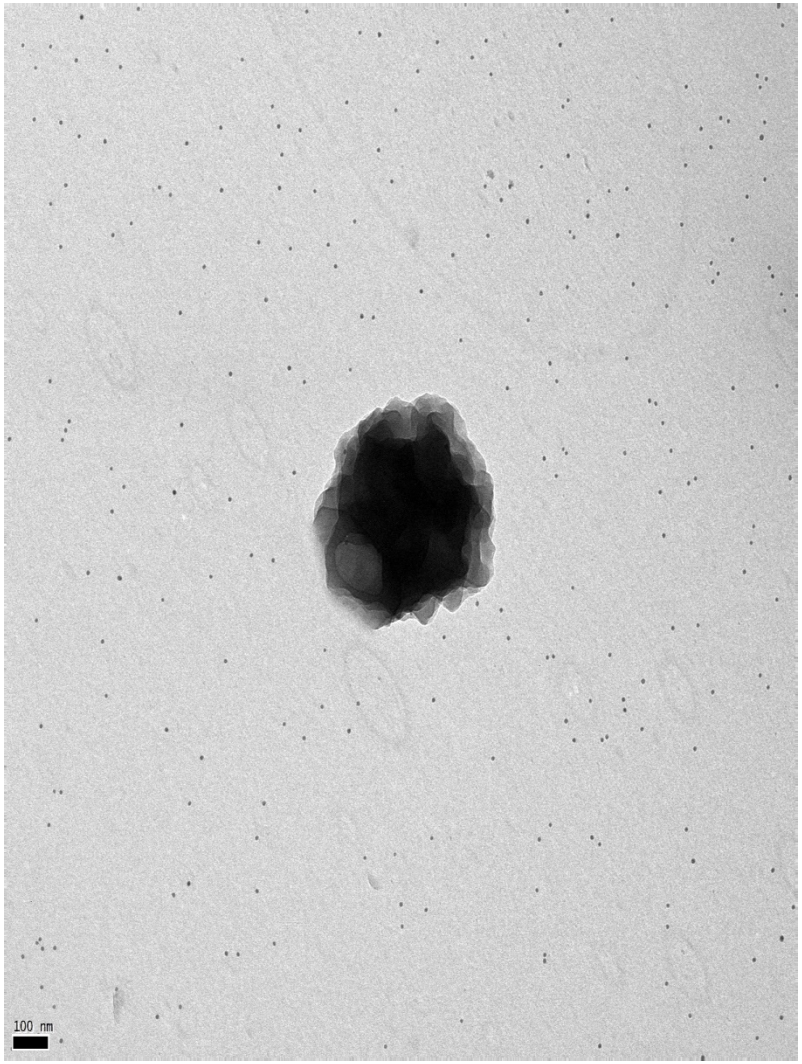
Perceived sample thickness and tilt angle

- The distance the electron beam must travel through the sample increases as the sample is tilted
  - At 60° tilt the distance is doubled
  - At 70° tilt the distance is nearly tripled
- The effect of perceived thickness can be somewhat compensated for by adjusting imaging conditions as a function of tilt angle
- Thin sample is preferable (<200nm)
- Applies to large samples (sections, cryo)

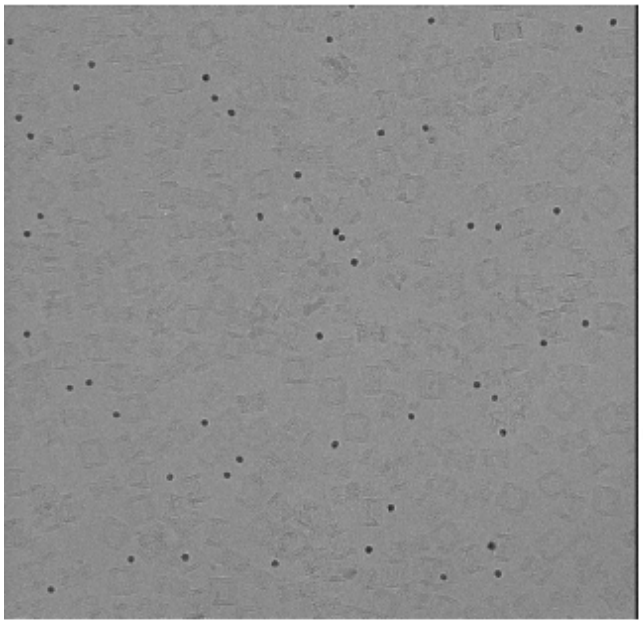


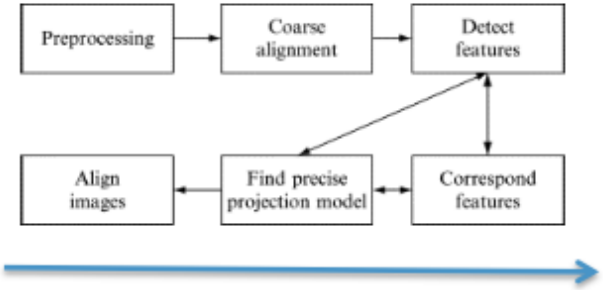
Tracking with fiducial markers

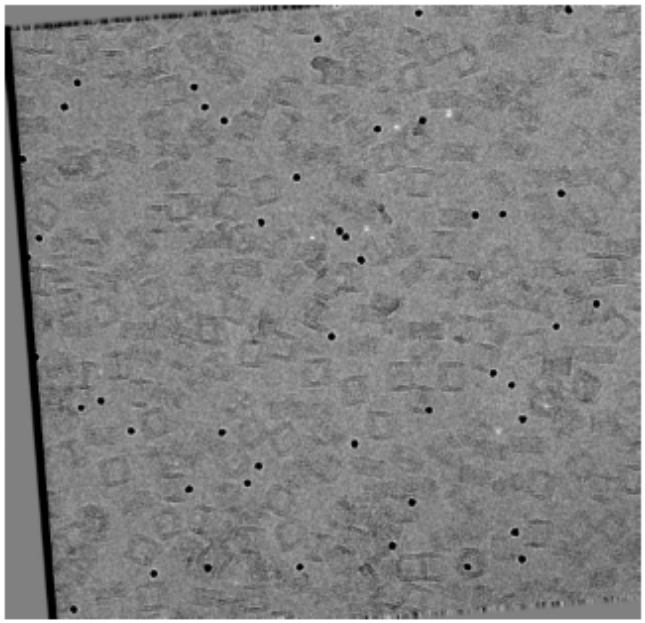
- Fiducial markers are typically gold particles of controlled size
- Size of fiducials should be selected with the intended magnification in mind
- Fiducials are added during sample preparation to aid image alignment later on
- For samples prepared on ”solid” support, fiducials can be added prior to sample application



Stack alignment



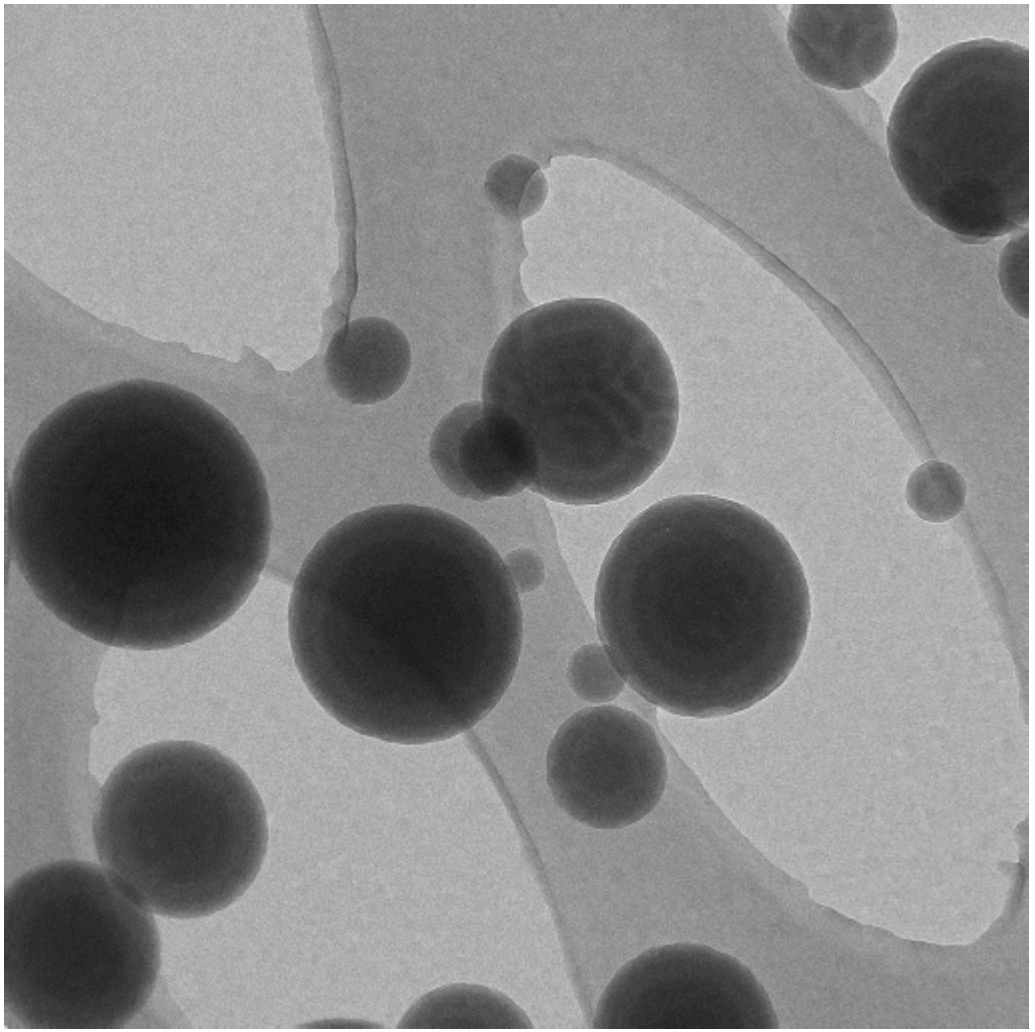




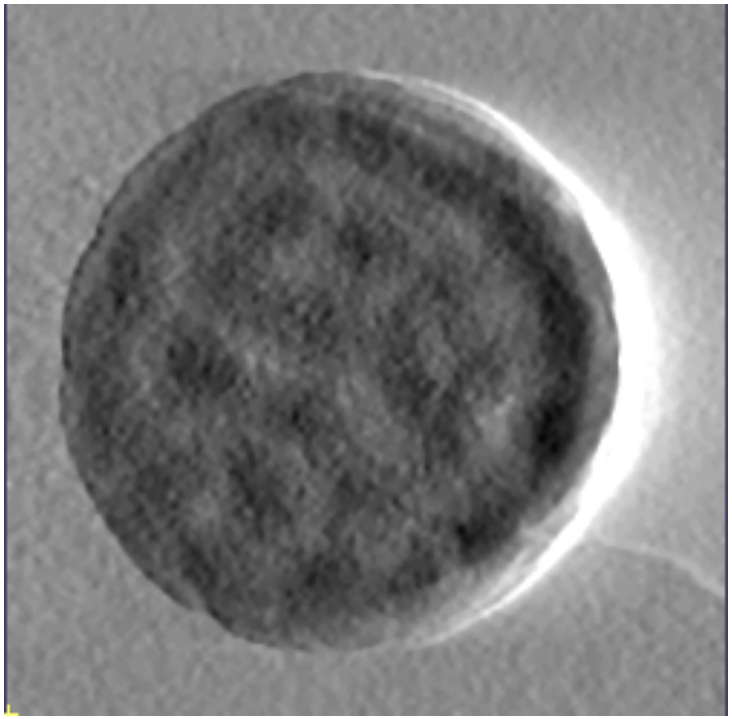
Original tilt series

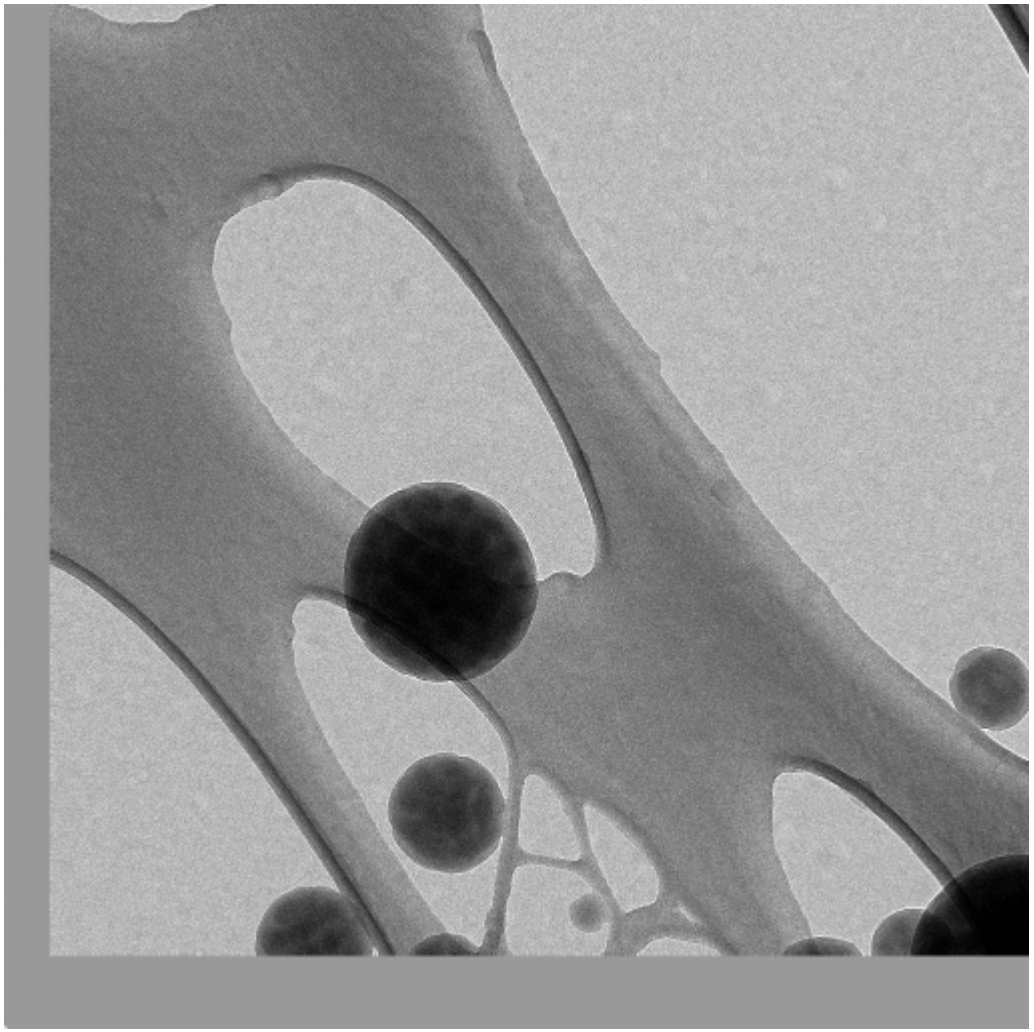
Aligned tilt series

Unaligned stack

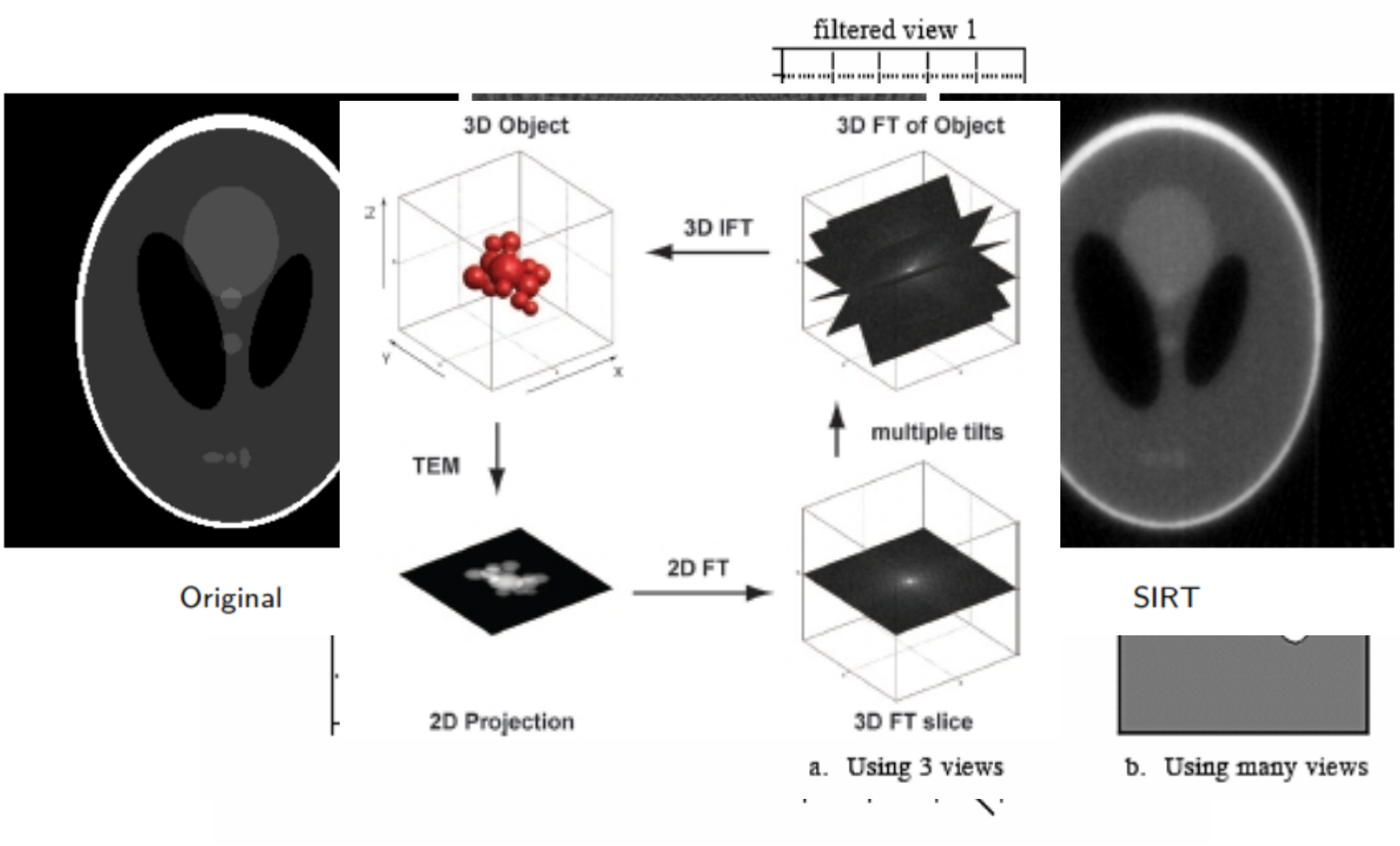


Aligned stack (coarse only)



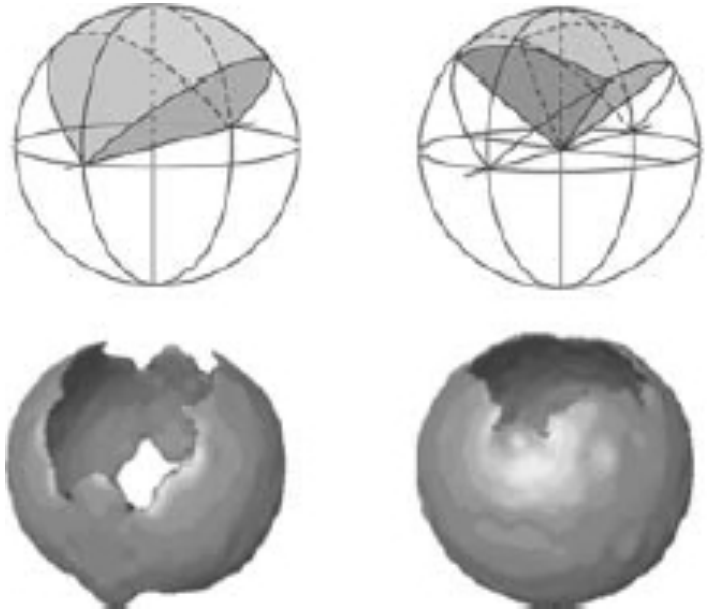


From stack to volume



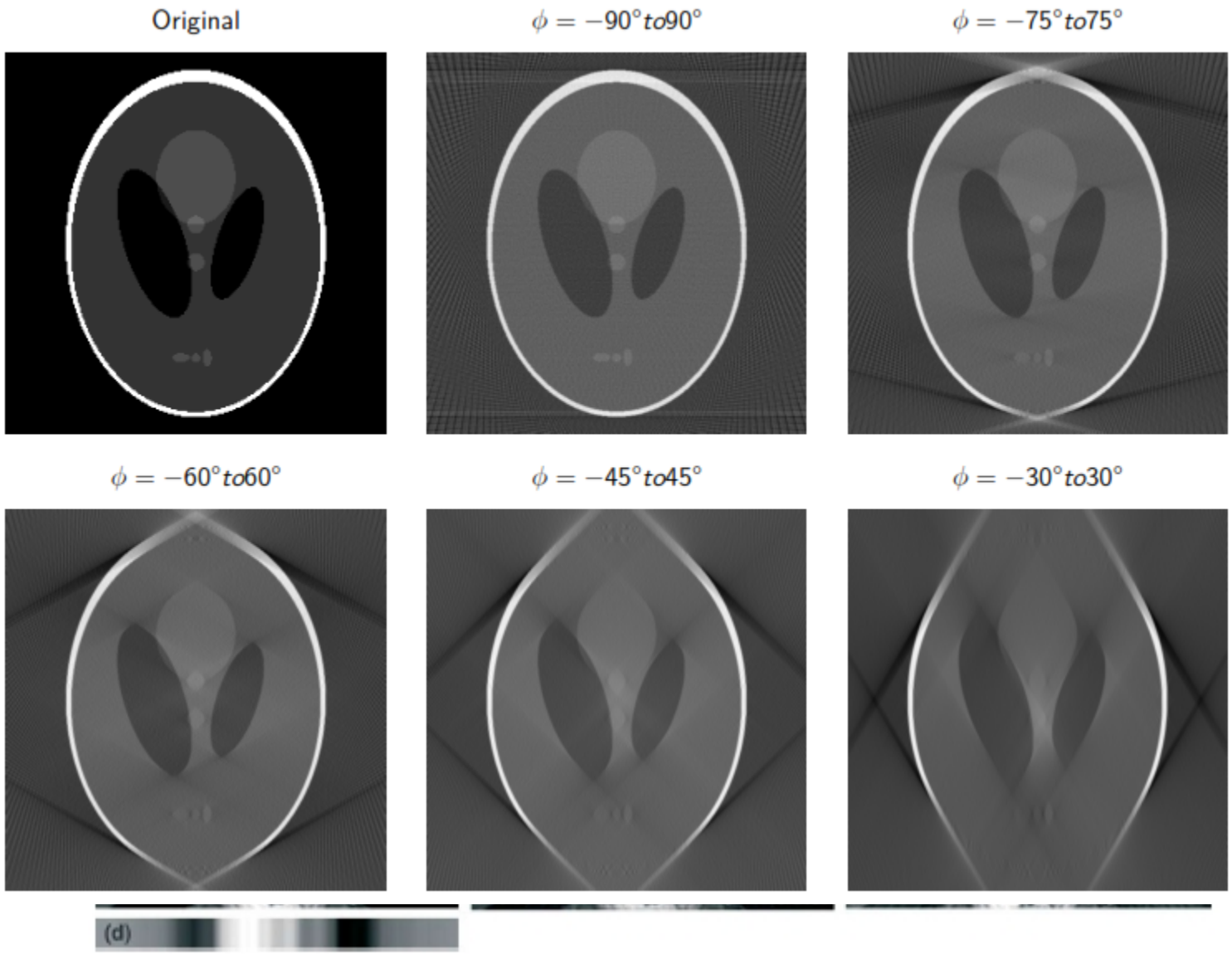
Missing wedge/cone

- Due to limited tilt range all the projections can not be collected
- With a single tilt axis the missing projections form a wedge
- More information can be collected by tilting the sample around two axis

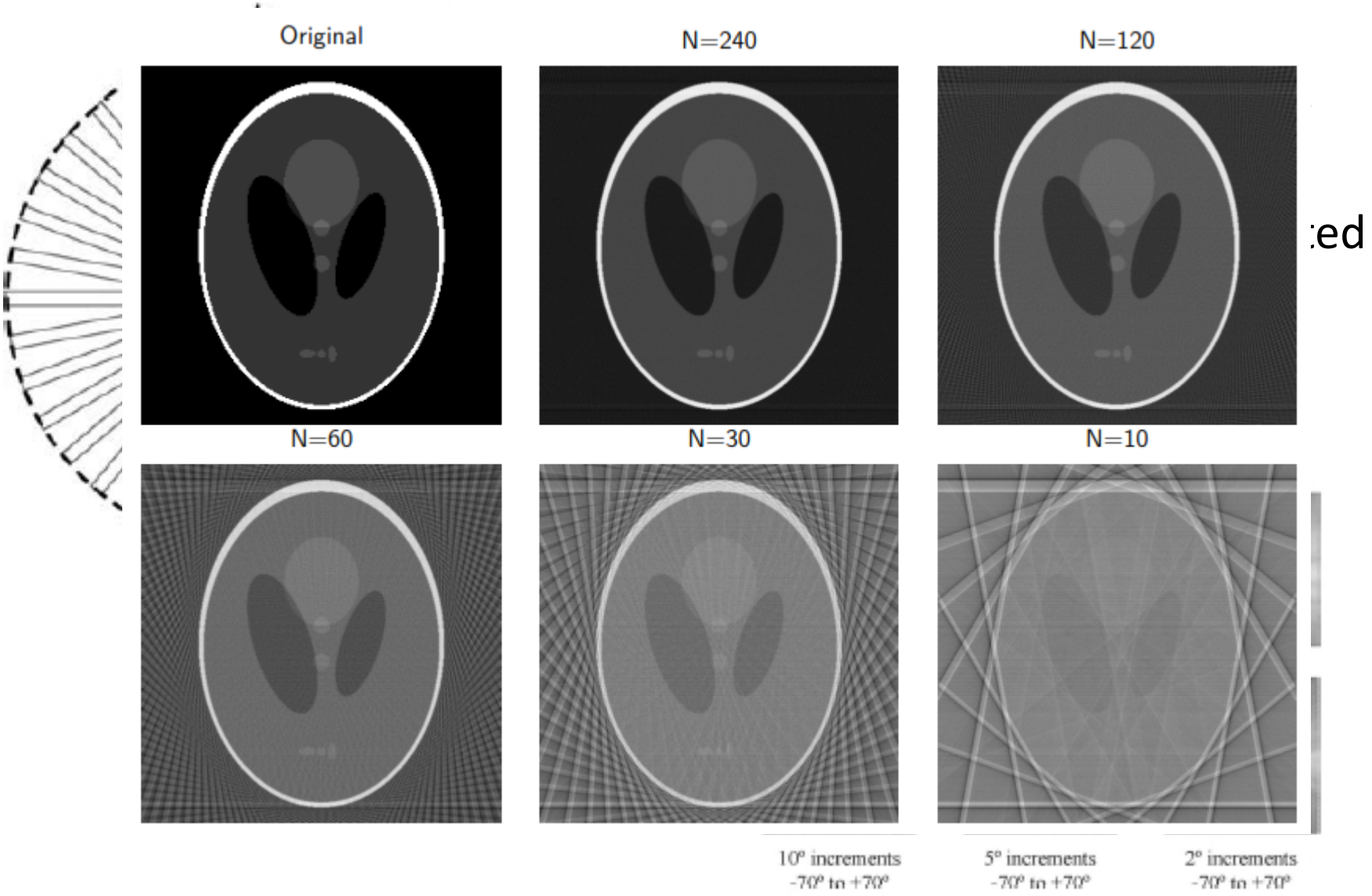


Tilt range	Single	Double
±70°	78%	93%
±60°	67%	84%
±45°	50%	67%

Effect of missing wedge

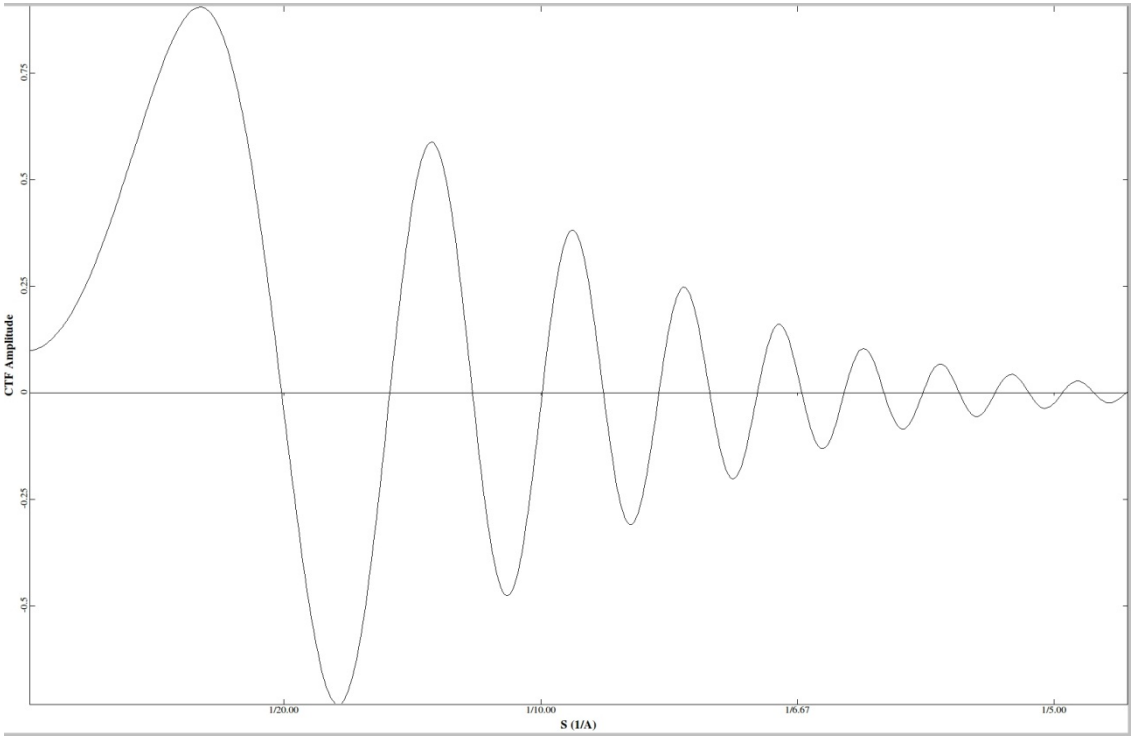


Sampling & resolution



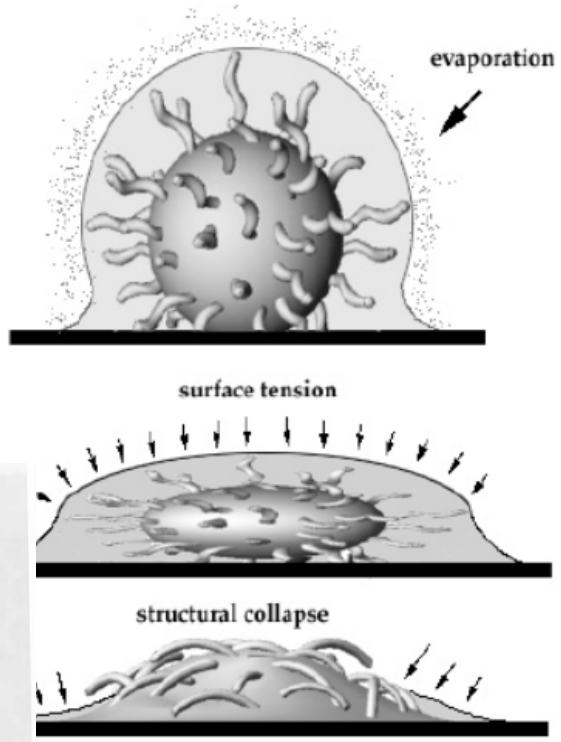
CTF correction

- The height variation leads to differences in defocus → opposite edges can have a difference of a few micrometers in defocus
- Typical method for correction is to truncate at the first node

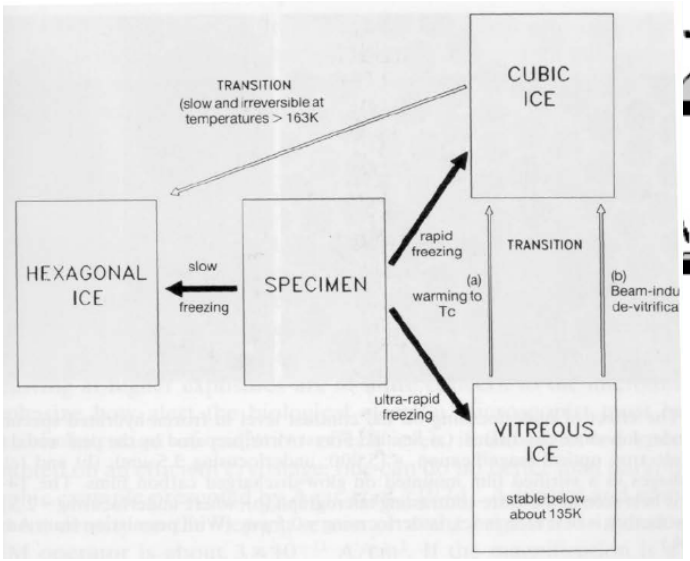


Towards vitrified samples

- Vitrification encloses the sample in a layer of vitrified water
- This allows preservation of native structure



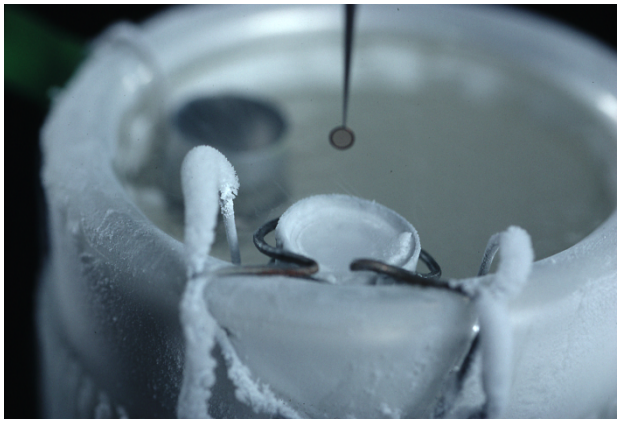
- Water is suspended in a metastable state → careful handling required

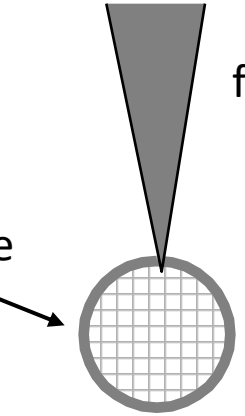


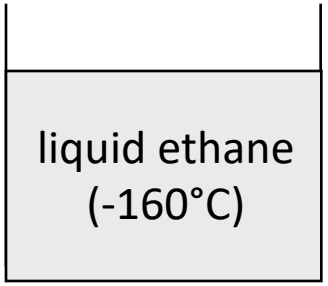
Preparation of vitrified specimens

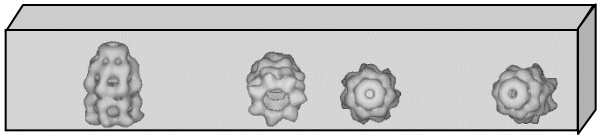
- Samples are embedded in super-cooled liquid, called vitreous ice (that's why it's *cryo*-EM...)
- Preparation:
  - Rapid cooling (10 000 K/s; -160 ^{o}C) ->
    vitrified metastable state, no ice crystal formation
  - No staining used
- Imaging:
  - Structureless, hydrated medium preserved despite the vacuum of the microscope

Sample preparation for cryo-ET

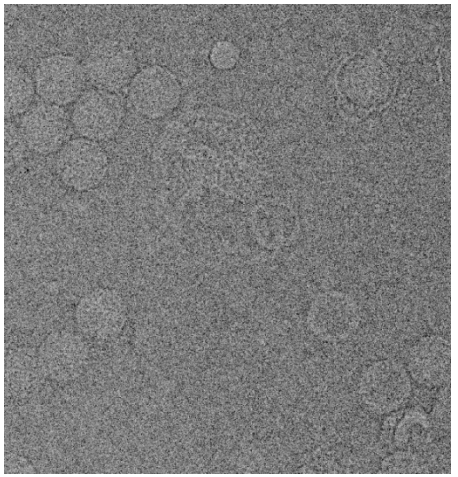








Edge-on view of an unsupported part of the water layer



image

Sample is imaged in native state

Preparation of vitrified specimens

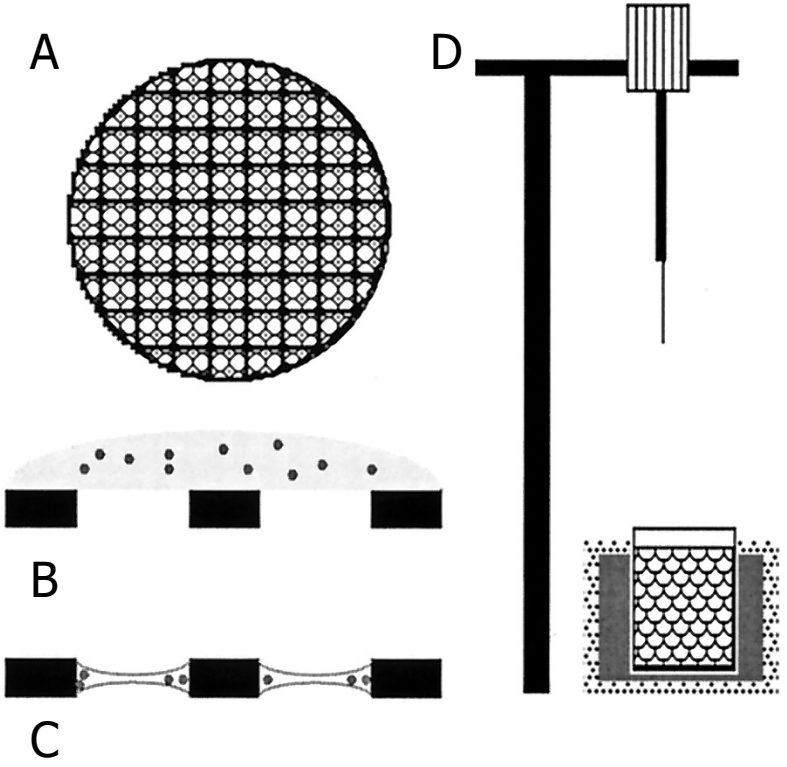
Vitrification

A. EM grid covered with a “holey” layer of carbon

B. Sample (3μl) in solution is pipetted over the grid

C. The grid is blotted to remove excess of solution

D. Guillotine holding tweezers is released to plunge the grid into liquid ethane bath cooled by liquid nitrogen



Preparation of EM specimens

Negative-stained specimens

- Pros:
  - High image contrast
  - Low sensitivity to electron beam
- Cons:
  - Only surface details visible, not the density of the object
  - Resolution is limited (incomplete stain penetration, stain movement during imaging, variable flattening of the structure)

Preparation of EM specimens

Vitrified, unstained specimens

- Pros:
  - Internal details accessible
  - Object is in a native-like state
  - Resolution is not limited by the sample preparation
- Cons:
  - Limited sample thickness
  - Tedious sample preparation and handling
  - Special equipment required

Various states of water

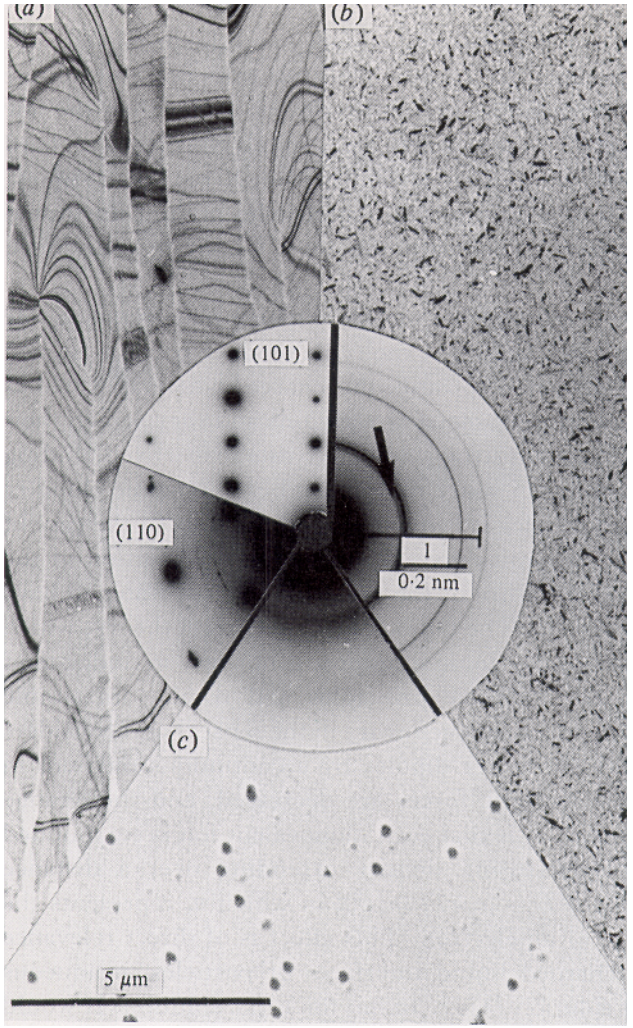
Plastic beads (~100 nm) in three forms of solid water:

(a) hexagonal ice

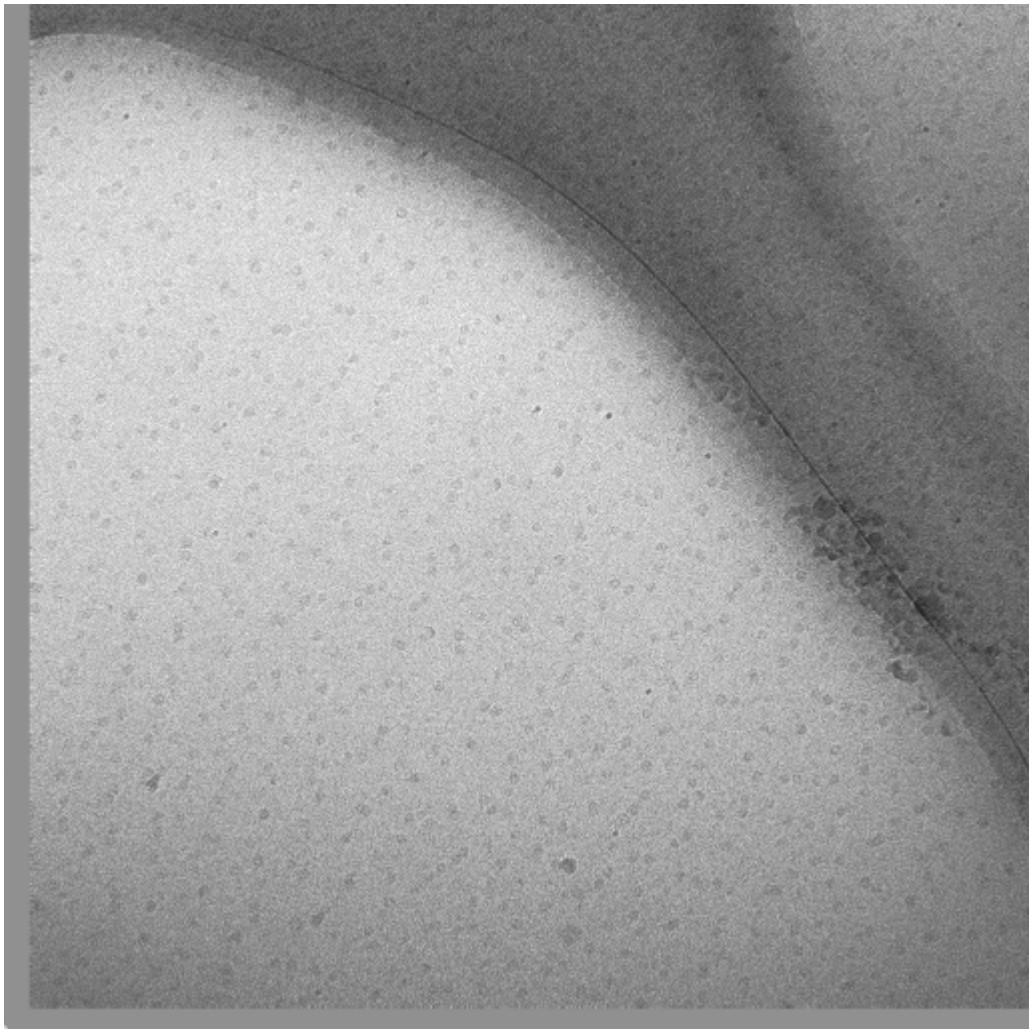
(b) cubic ice

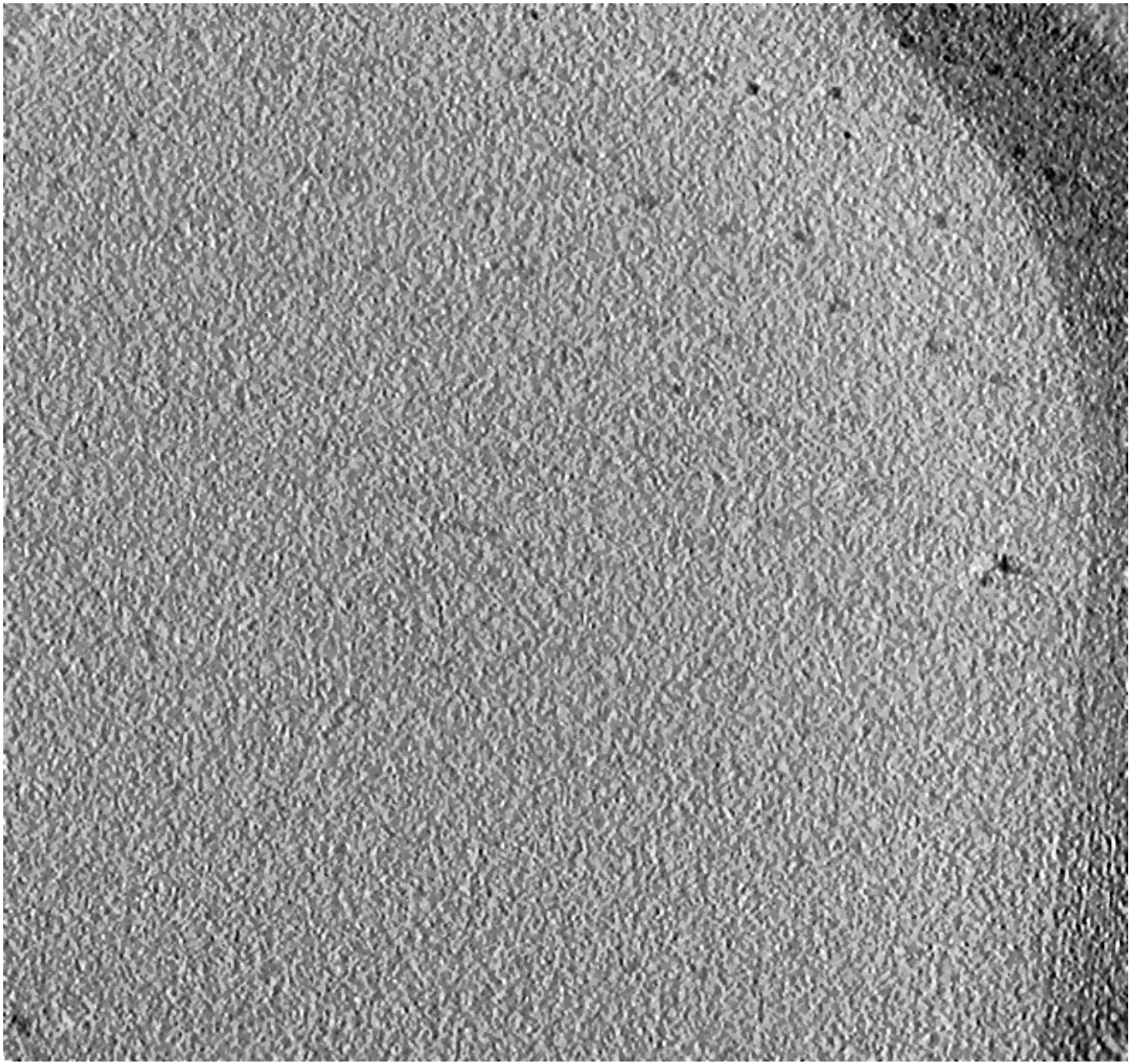
(c) vitreous ice

Characteristic diffraction patterns



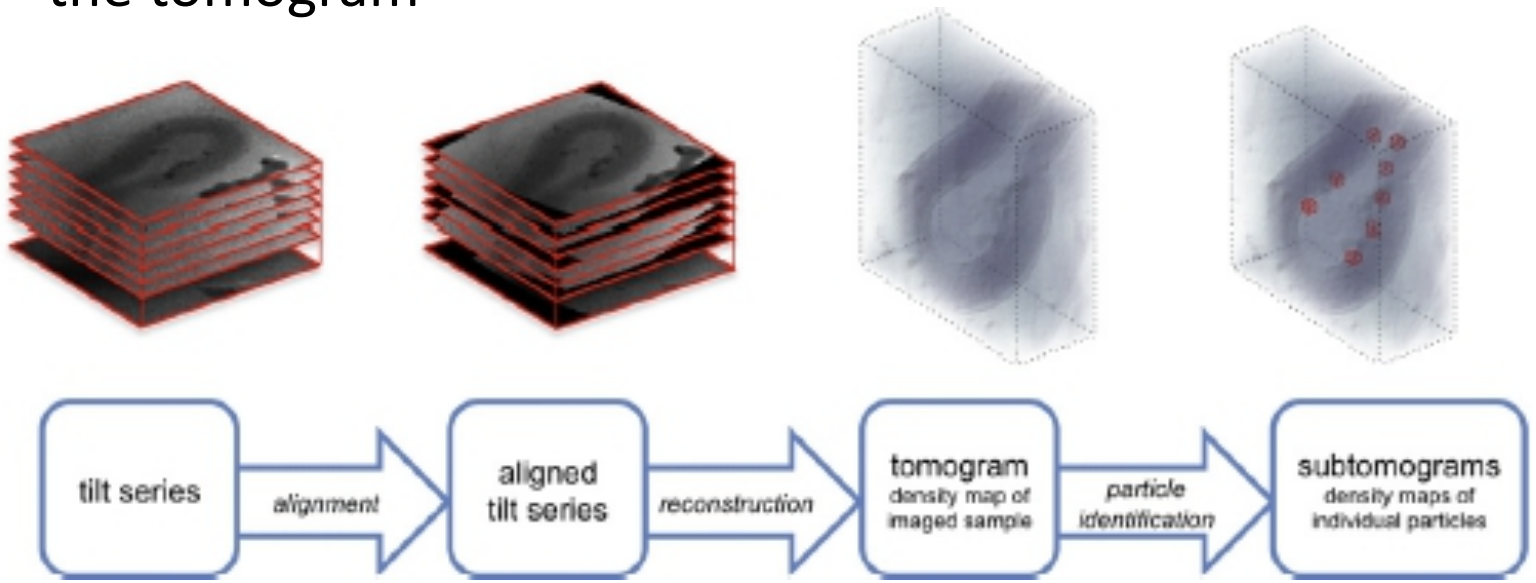
Example of cryoET





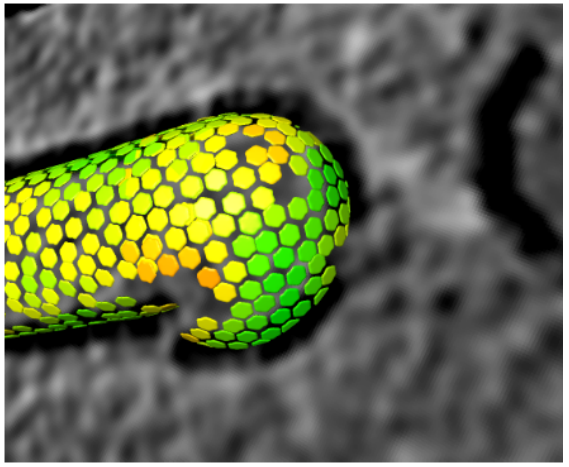
Subtomogram averaging (1)

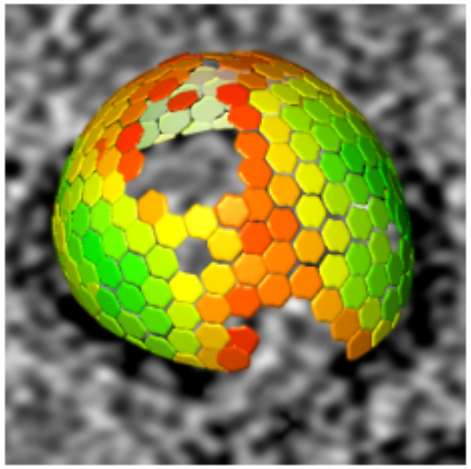
- “Raw” data are small volumes extracted from tomograms
- Idea is similar to single particle, but the “raw” data is already in 3D → additional degrees of freedom
- Requires significant computational resources
- Target molecule must be (relatively) easily recognizable in the tomogram

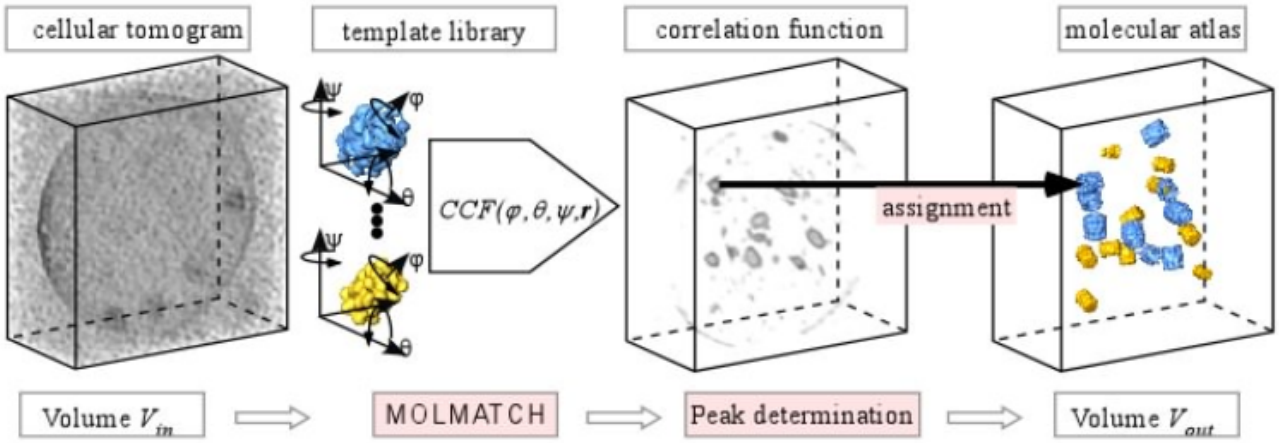


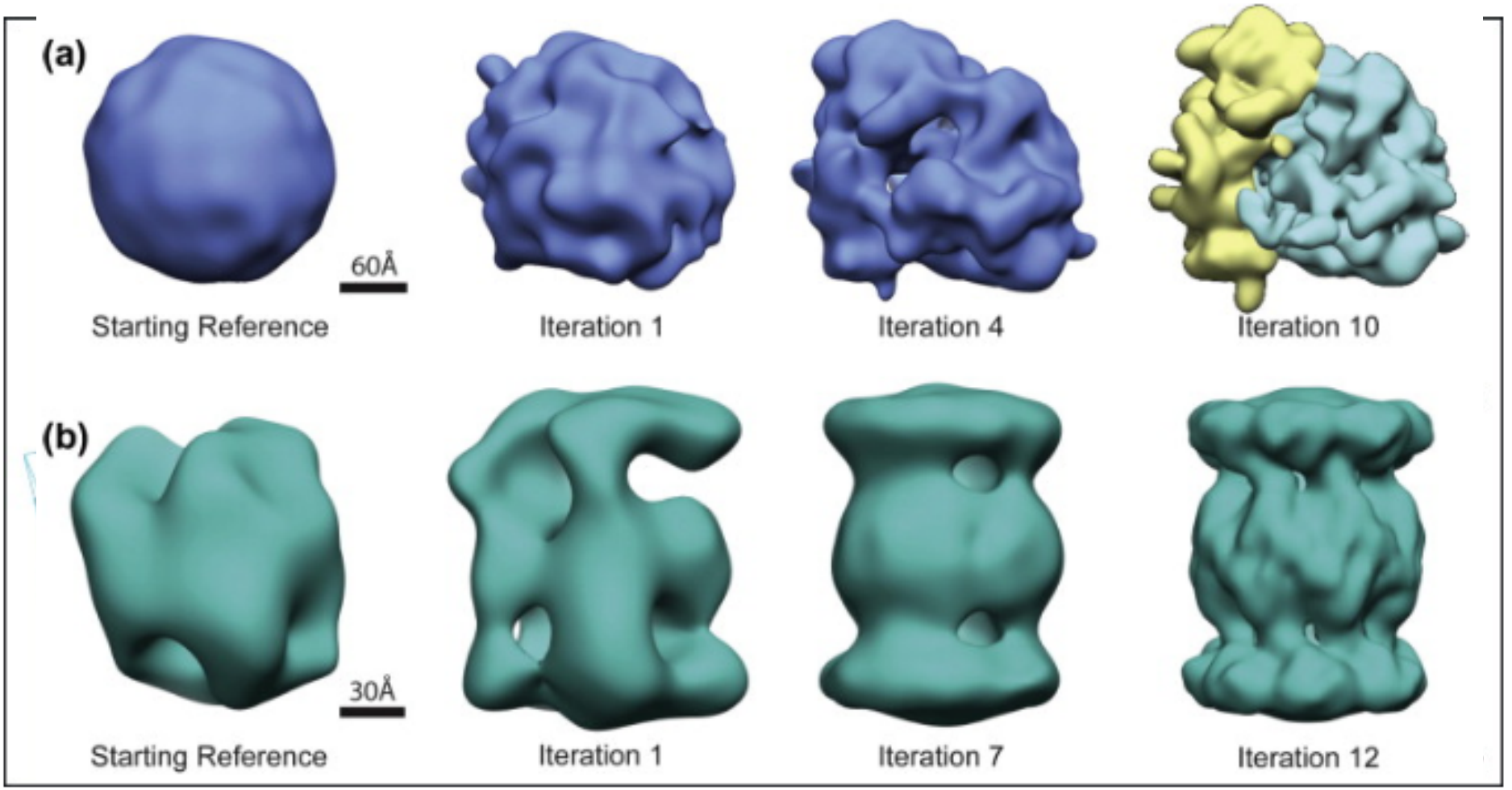
Subtomogram extraction

- At least three options for selecting subtomograms:
  - Manual (tedious)
  - Geometric
  - Template matching
- Can reach high resolution → CTF correction becomes important



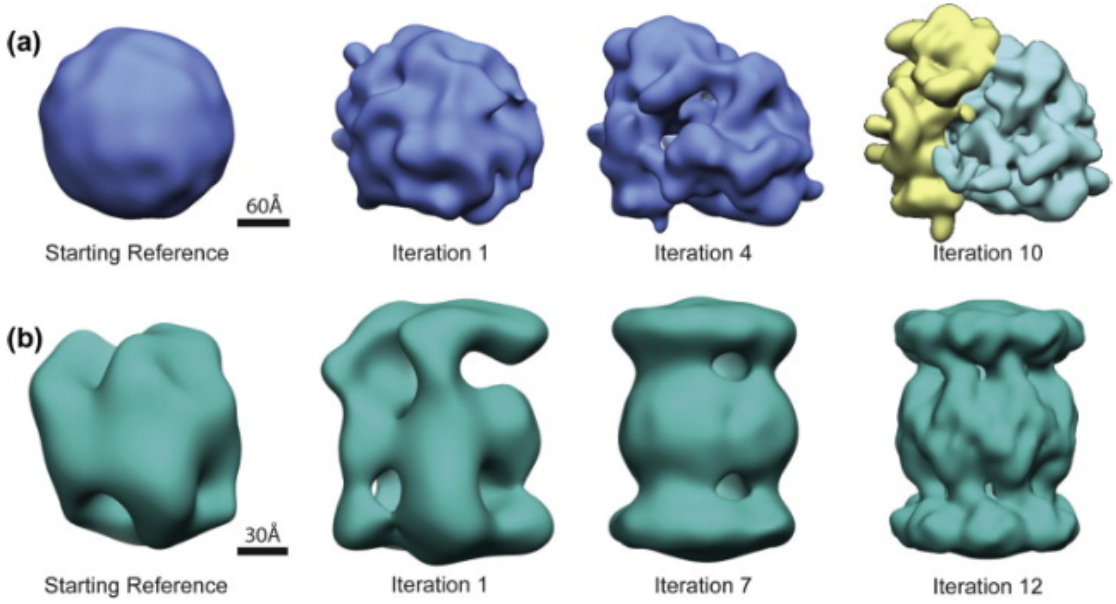





(a)
60Å
Starting Reference
Iteration 1
Iteration 4
Iteration 10
(b)
30Å
Starting Reference
Iteration 1
Iteration 7
Iteration 12

Conclusions for tomography

- For pleomorphic/unique structures tomography is one of the few 3D imaging options
- Identical subvolumes can be cut from the tomogram and averaged similarly to single particle reconstructions
- Tomogram resolutions around 15Å according to databases
- Subtomogram averages around 4Å (DBs)



Contents for single particle

1. Goals and requirements
2. Sample preparation, imaging & image quality
3. Image processing & reconstruction
4. New detectors & current state of the art

Goals for successful SPR

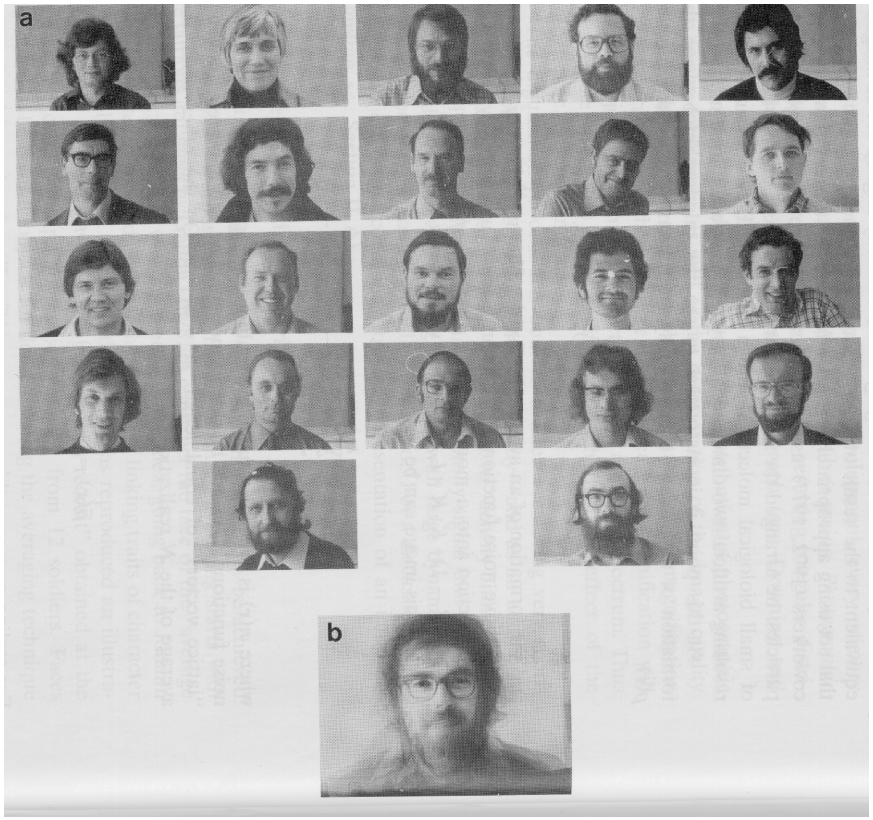
- Eliminate background AND alter your sample as little as possible during preparation
- Record as much detail as possible during imaging (minimize damage to fine structure)
- Obtain 3D model and enhance fine structure via averaging

MOST IMPORTANT STEP?

Garbage in ------> garbage out

”Tim Baker”

Requirements on the object of interest



- Size (depending on detector and material) min. 100-200kDA
- Structure between molecules must be **IDENTICAL**

A random sample of complexes may have:

*differences in mobile subunits
*minorities
*cofactors
*damaged complexes

<-- 2D average of 22 complexes

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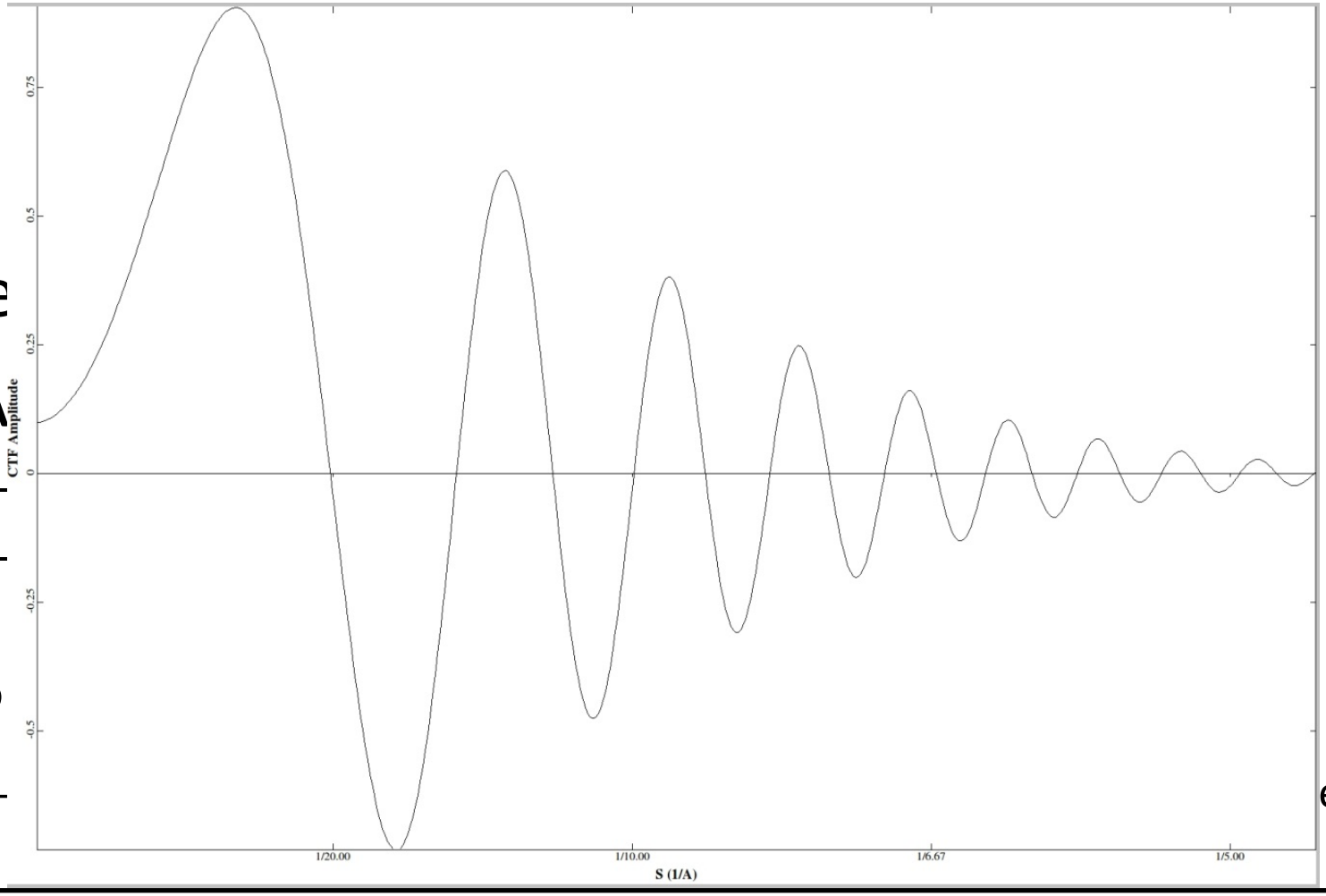
The

- A
  -
  -
- P
  -



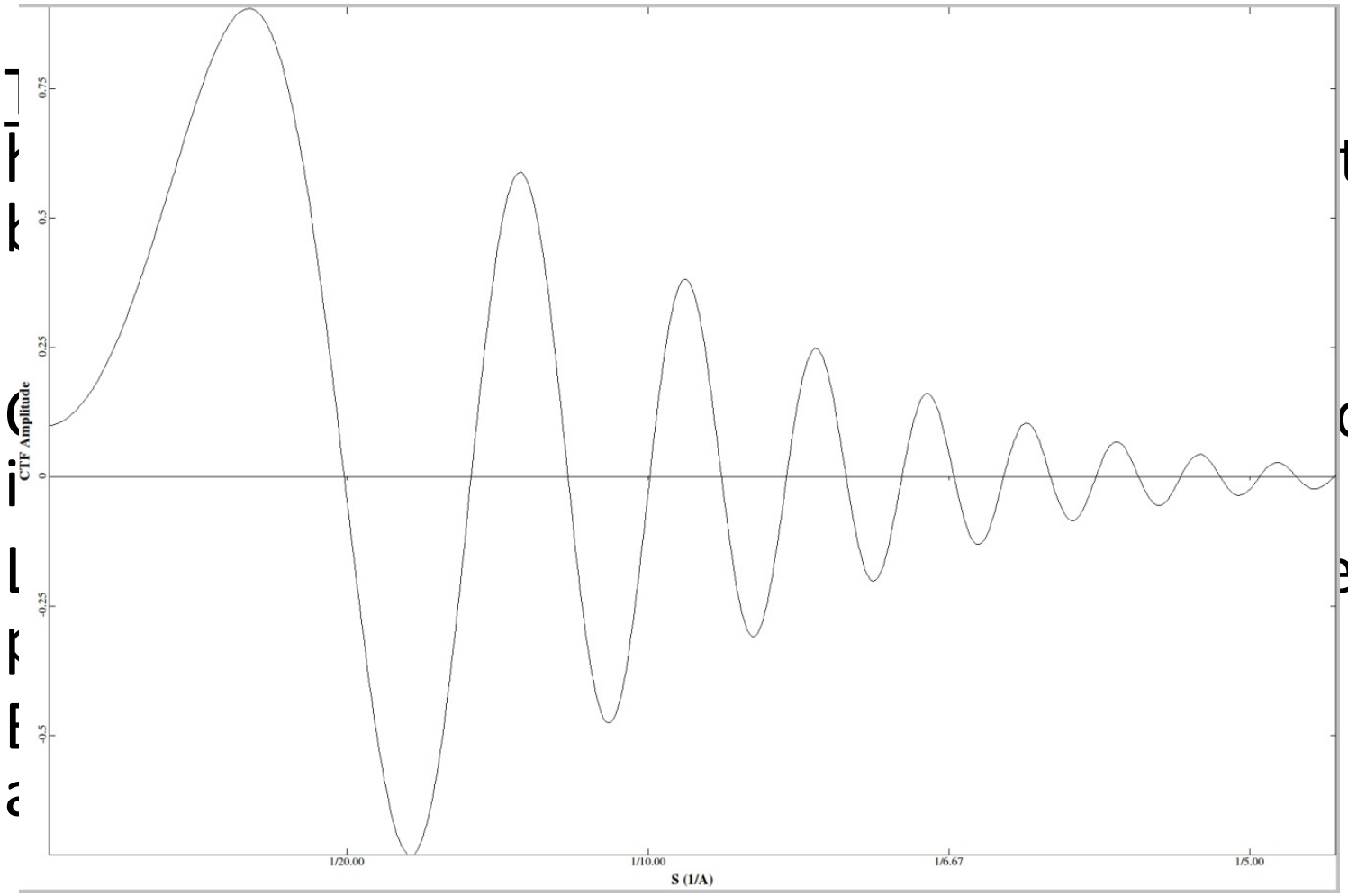
e

$$CTF(\nu) = -\{(1 - F^2_{amp})^{1/2} \cdot \sin(\chi(\nu)) + F_{amp} \cdot \cos(\chi(\nu))\} \cdot e^{-(\delta\nu)2}$$

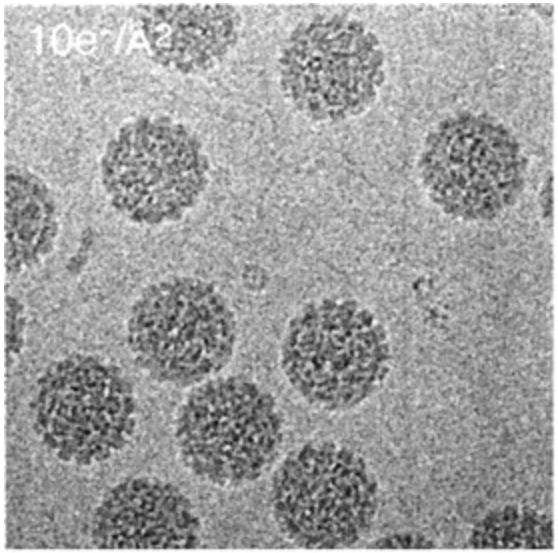
where: $\chi(\nu) = \pi \cdot \lambda \cdot \nu^2(\Delta f - 0.5 \cdot C_s \cdot \lambda^2 \cdot \nu^2)$

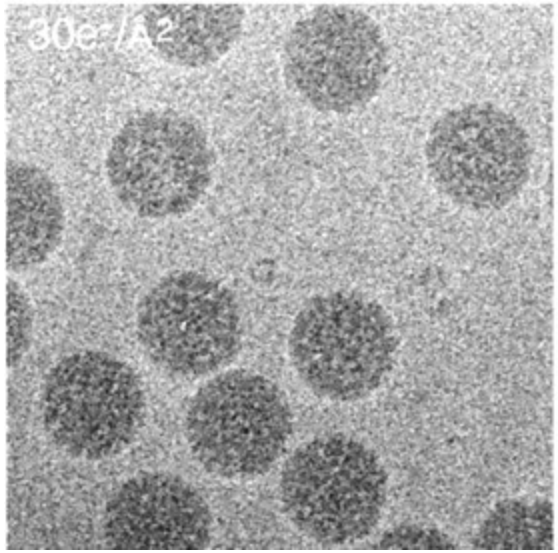
Imaging of vitrified specimens

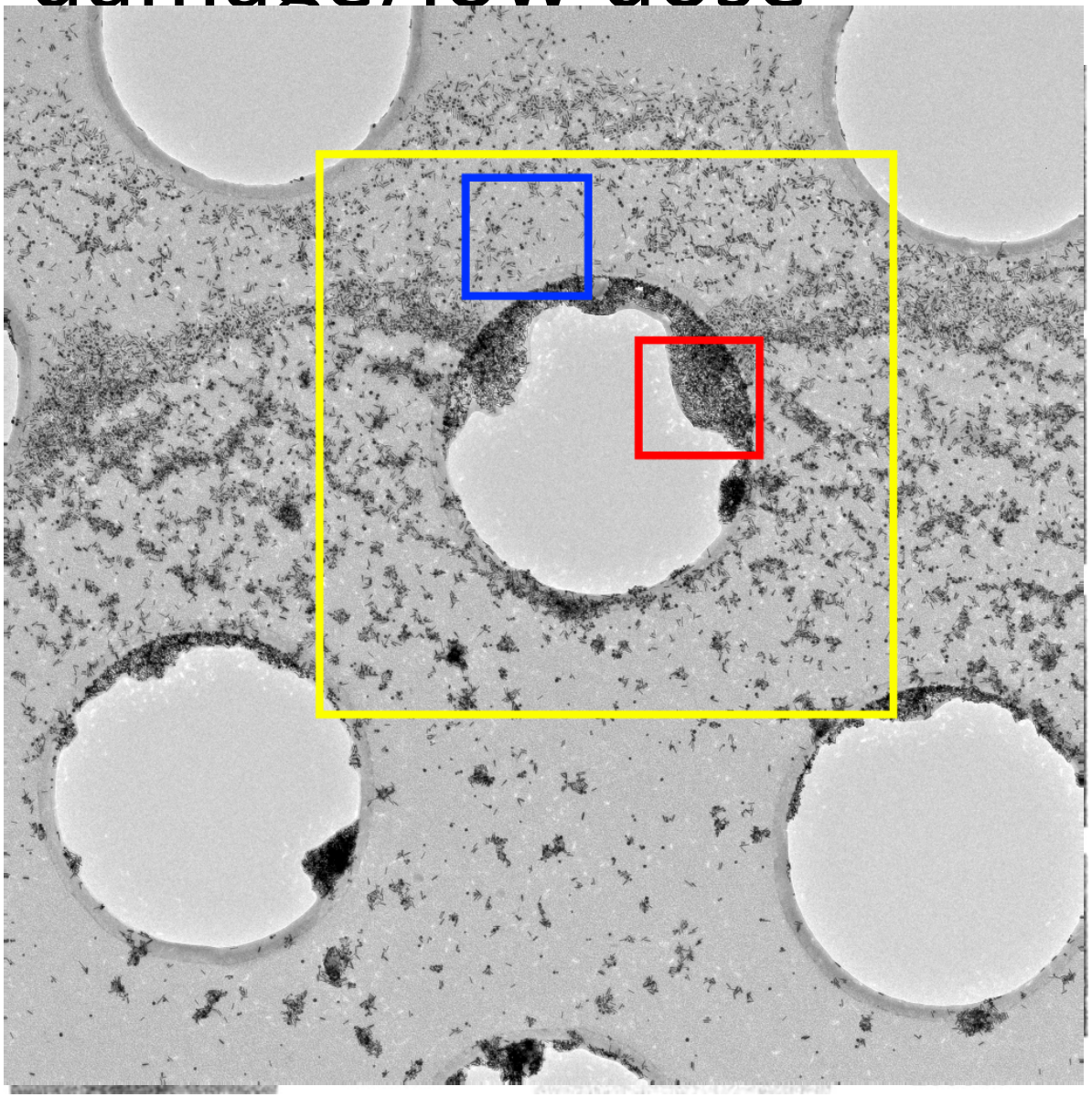
- [illegible]ted
- [illegible]o
- [illegible]ed
- [illegible]



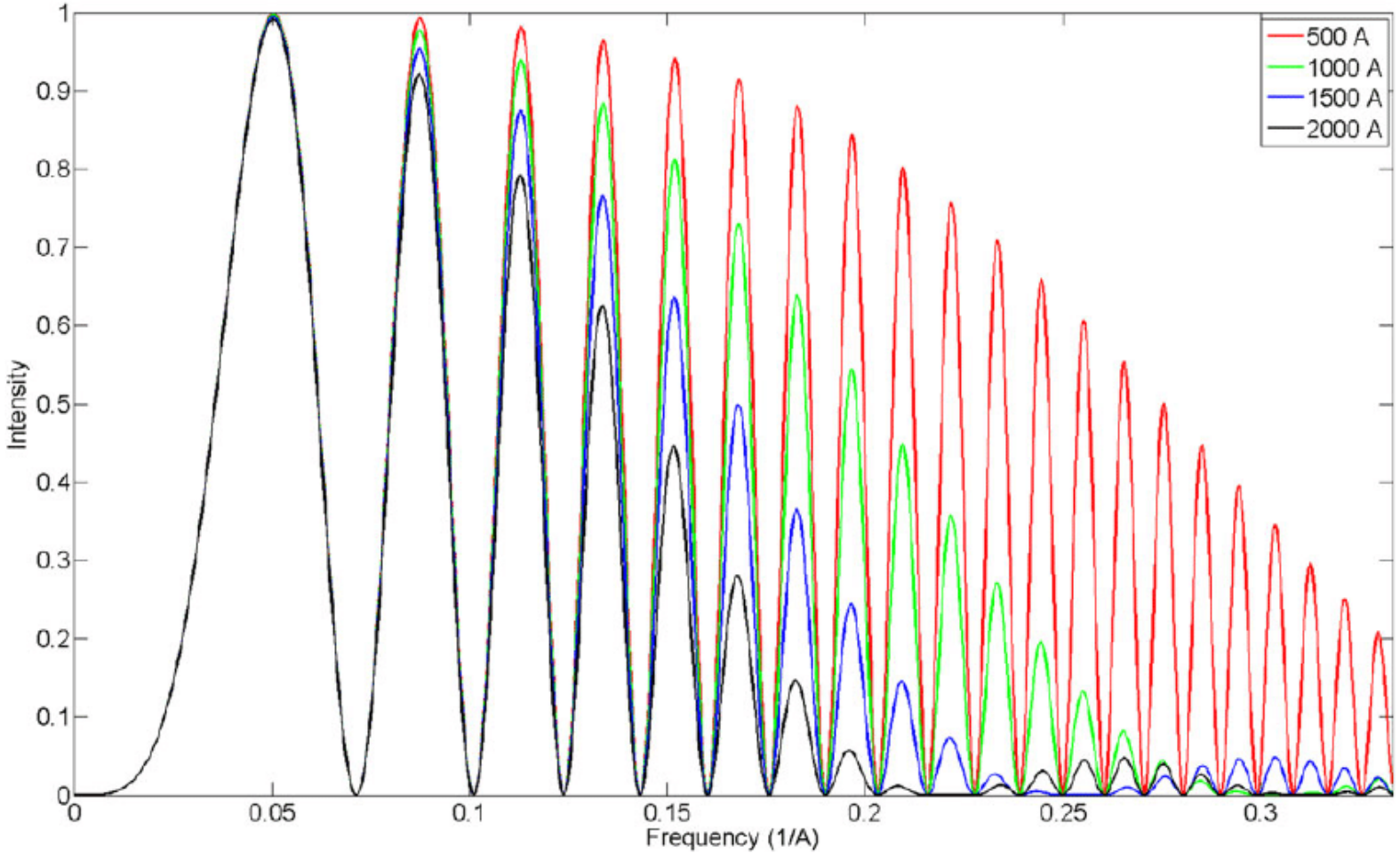
Beam damage/low dose

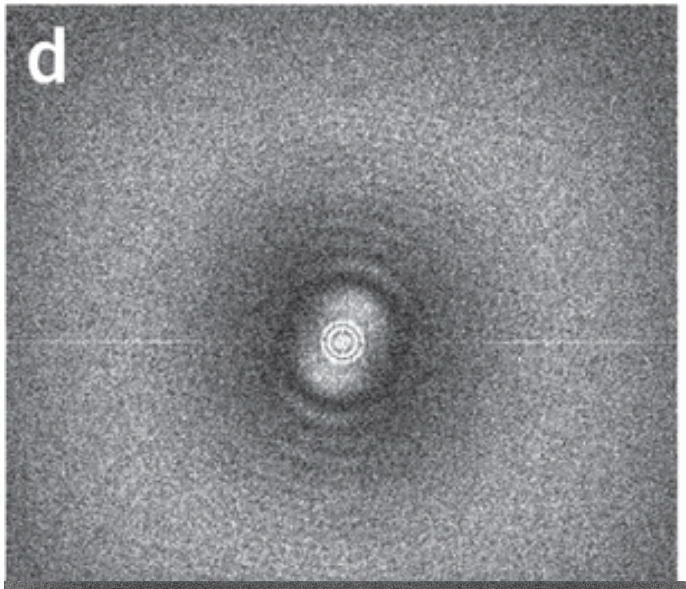






Tungsten filament (80kV)

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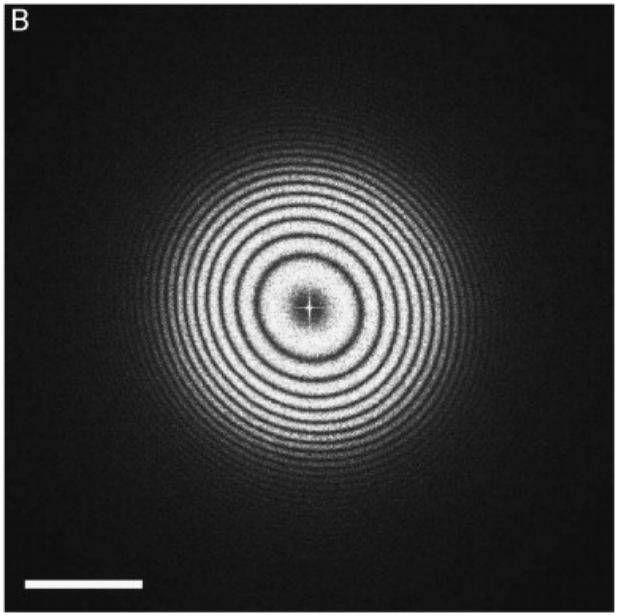
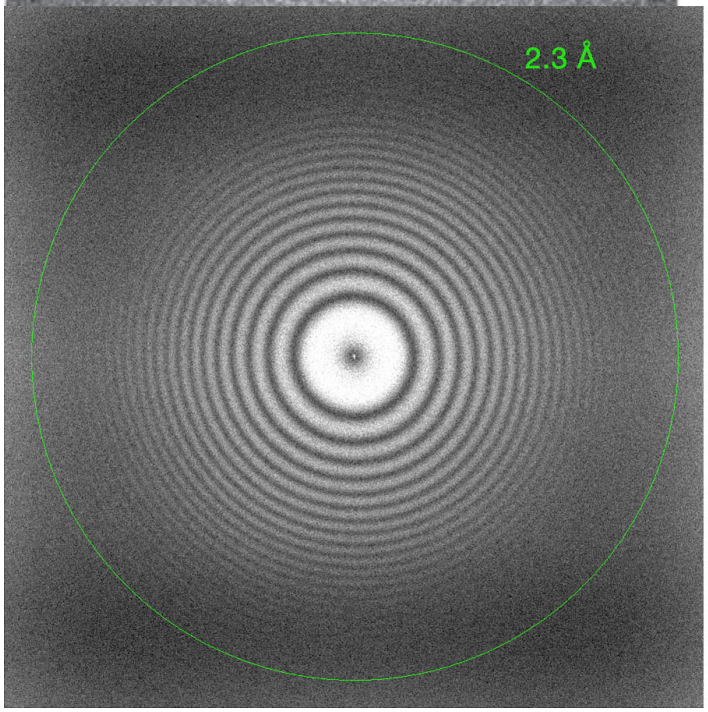
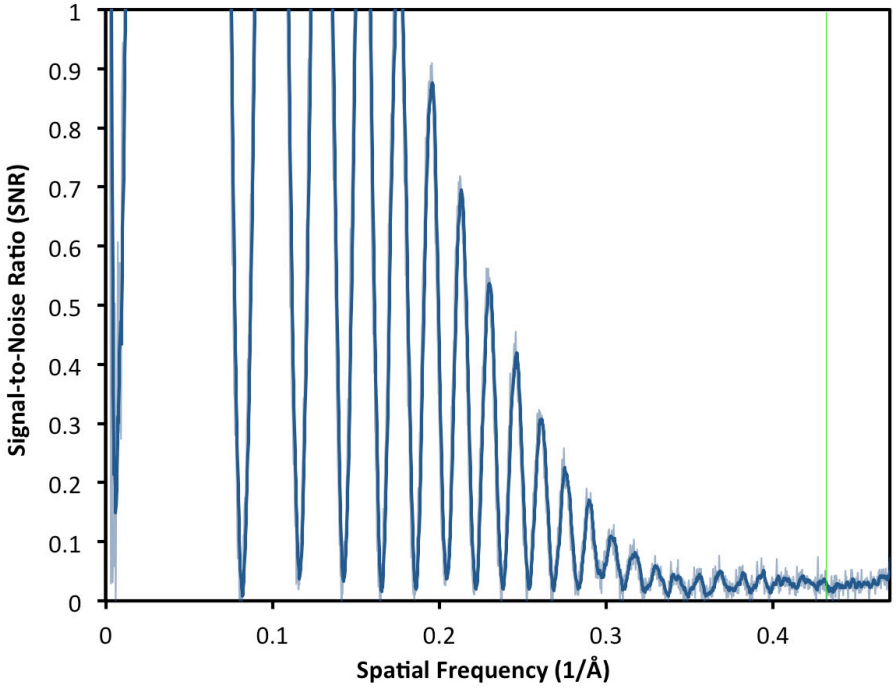

B


2.3 Å


Signal-to-Noise Ratio (SNR)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.1
0.2
0.3
0.4
Spatial Frequency (1/Å)

Contents

1. Goals and requirements
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3. Image processing & reconstruction
4. New detectors & current state of the art

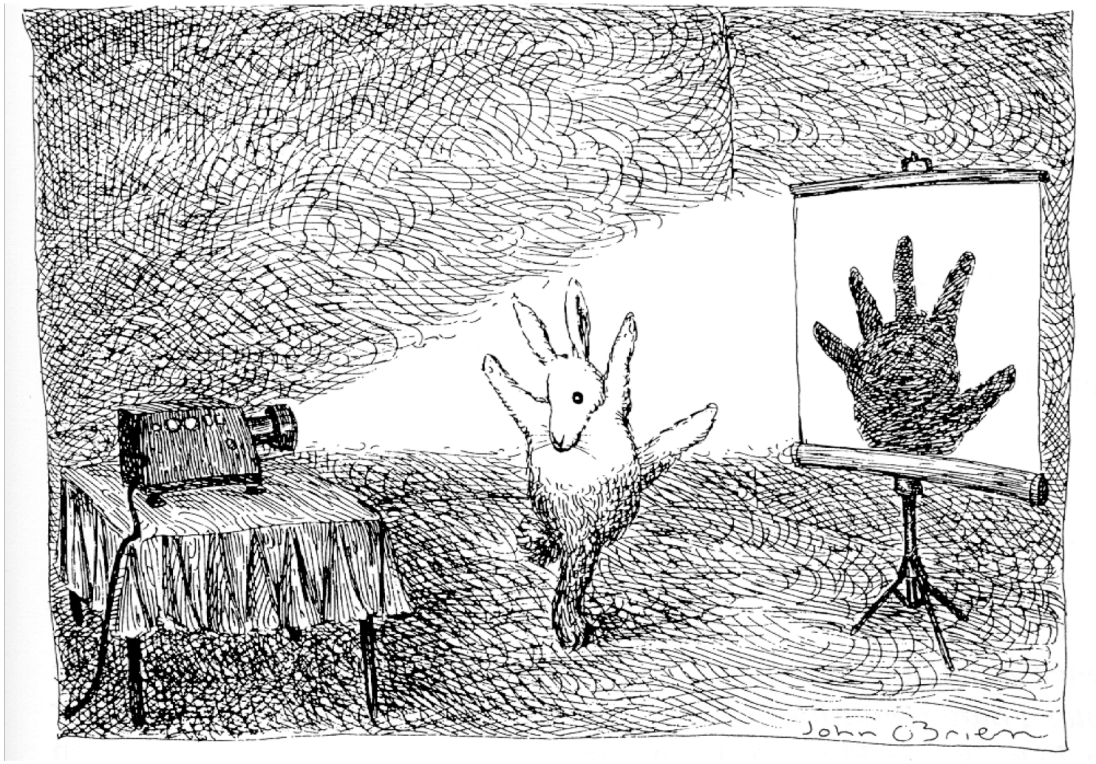
3D Image reconstruction

Goal: Reconstruct a 3D model from 2D TEM-images

TEM-image represents a projection image of the specimen.

->Features at different depths in the structure are all superimposed.

->Cannot generate a 3D structure from a single 2D projection.



Solution: combine projections taken at different angles

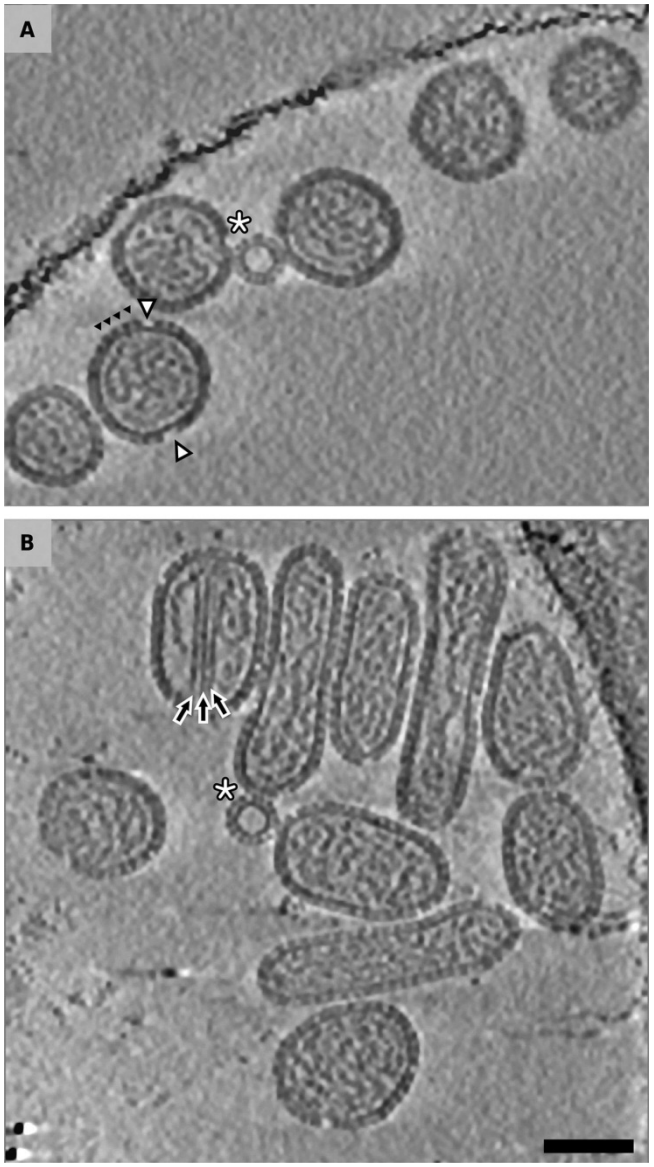
3D Image reconstruction

Method choice depending on the sample:

- Single particle analysis
  - identical unordered copies of the object (mass > 200 kDa (or: around 100kDa, DDs), e.g. ribosomes, viruses)
  - Symmetry can be exploited, helical can be a special case
- 2-D crystals
  - identical copies of the object (e.g. a membrane protein) forming an ordered 2D-crystal
- Tomography
  - one unique object (e.g. mitochondrion, cell section)

Single particle approach

- Requires tons of images of individual, but mutually identical objects, in different (random) orientations
- Works with symmetric and non-symmetric objects
- Most critical is the determination of orientation of objects in respect to each other
- Images at different defoci are used for CTF compensation
- Routinely reaches sub-nanometer resolutions (record is around 2Å)

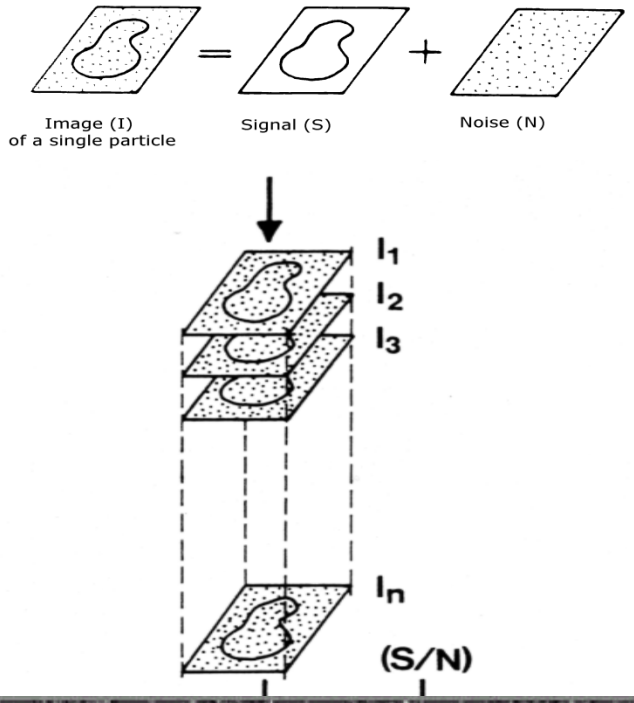


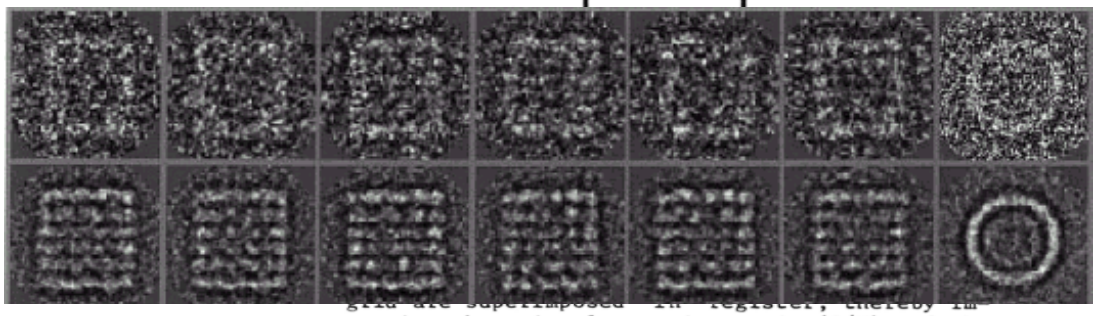
Single particle analysis

- Images are projections of different (identical) particles in random orientations
- Five parameters must be defined before the 3D reconstruction can be calculated from the 2D projections:
  - Centre of the particle (2 coordinates: x, y)
  - Orientation of the particle (3 eulerian angles θ, φ, ω)

Single particle analysis

- A large number of images (typically 1 000-10 000) is needed to increase the signal (S) and decrease noise (N)
- Images in the same orientation can be averaged

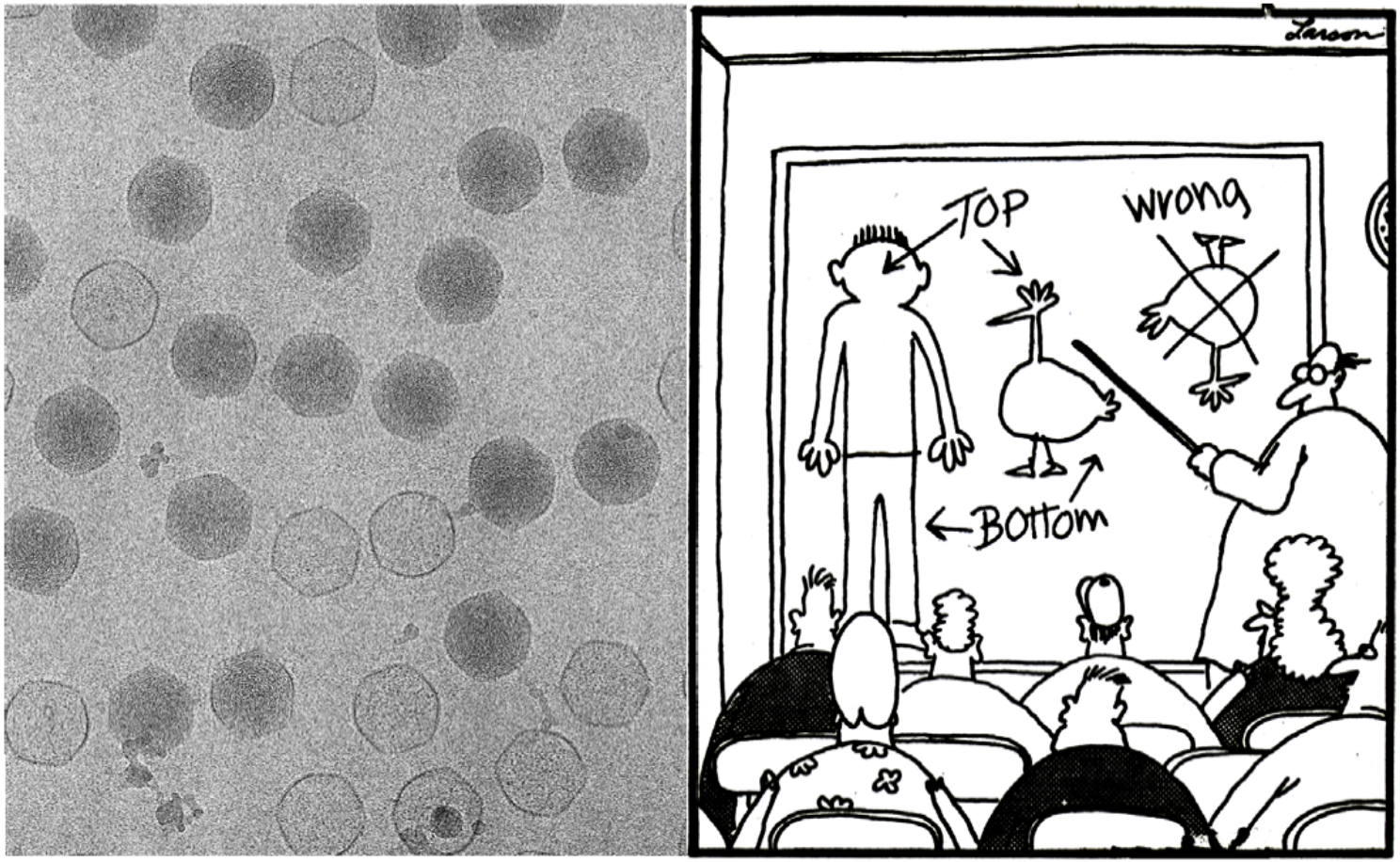




proving the signal-to-noise ratio (S/N) of the common structural features by a factor of $n^{1/2}$.

Icosahedral Virus 3D Reconstruction Scheme

Determine Origin and Orientation (θ,ϕ,ω,x,y)



People who don't know which end is up

Slide courtesy of Tim Baker, UCSD, and Larson, The Far Side

How do we determine the (θ, ϕ, ω, x, y) parameters?

Two methods:

1. *Ab initio* (*e.g.* Common lines)

   New or unknown structure

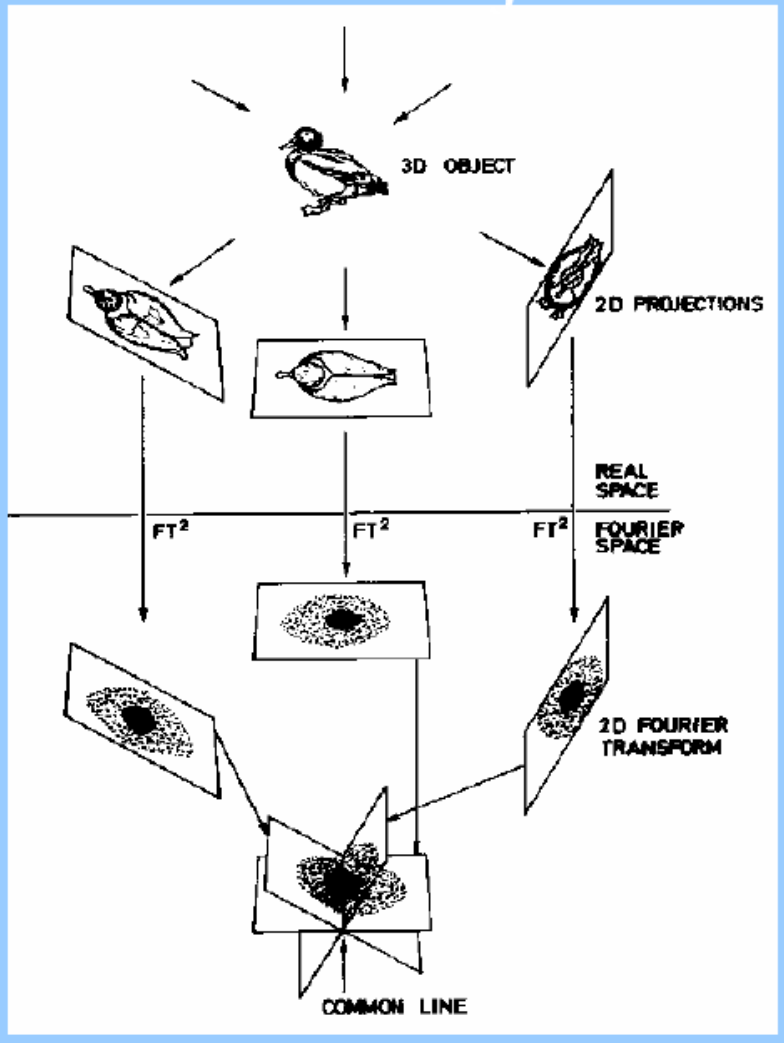
2. Model-based (template) matching

   General features of structure are known or a crude model can be generated (...or, sometimes, even a lousy model will work)

Slide courtesy of Tim Baker, UCSD

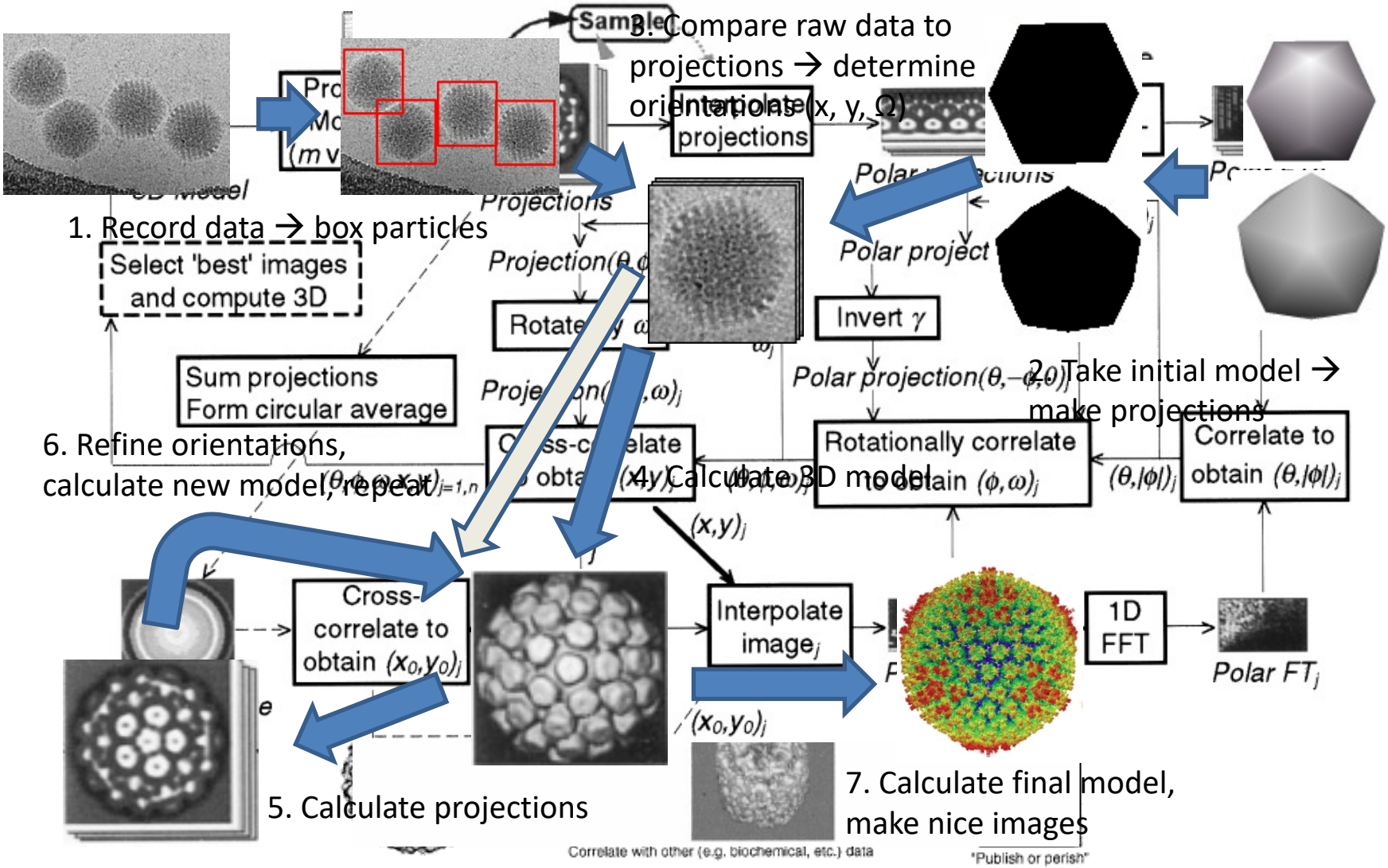
it's the common line!

Projection Theorem



The two-dimensional Fourier transform of the projection of a three-dimensional density is a central section of three-dimensional Fourier transform of the density perpendicular to the direction of projection.

The model based method



1. Record data → box particles

2. Take initial model → make projections

3. Compare raw data to projections → determine orientations (x, y, Ω)

4. Calculate 3D model

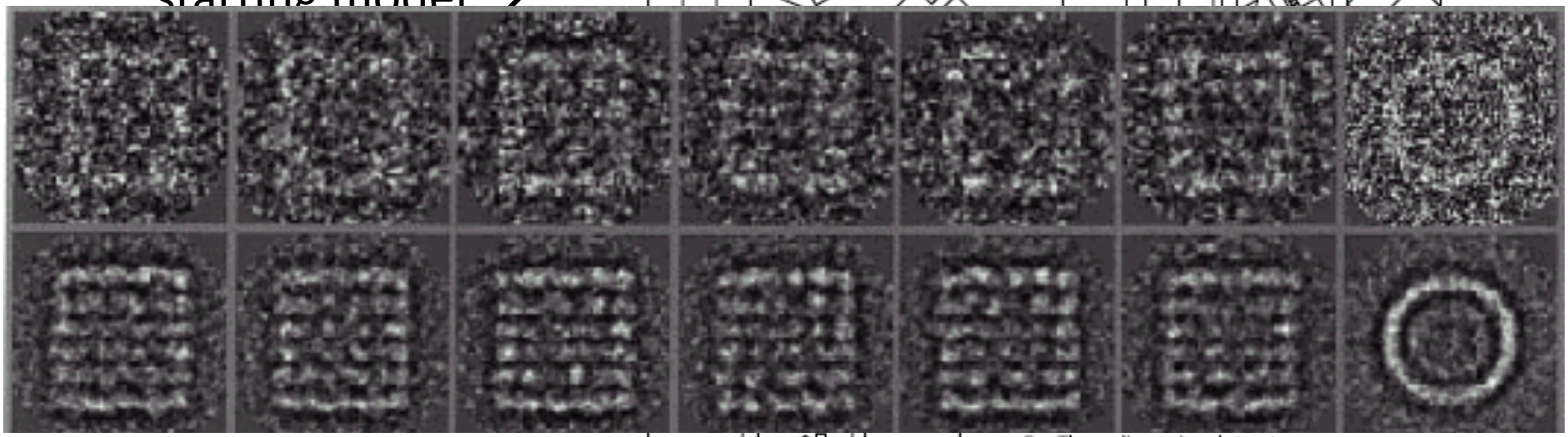
5. Calculate projections

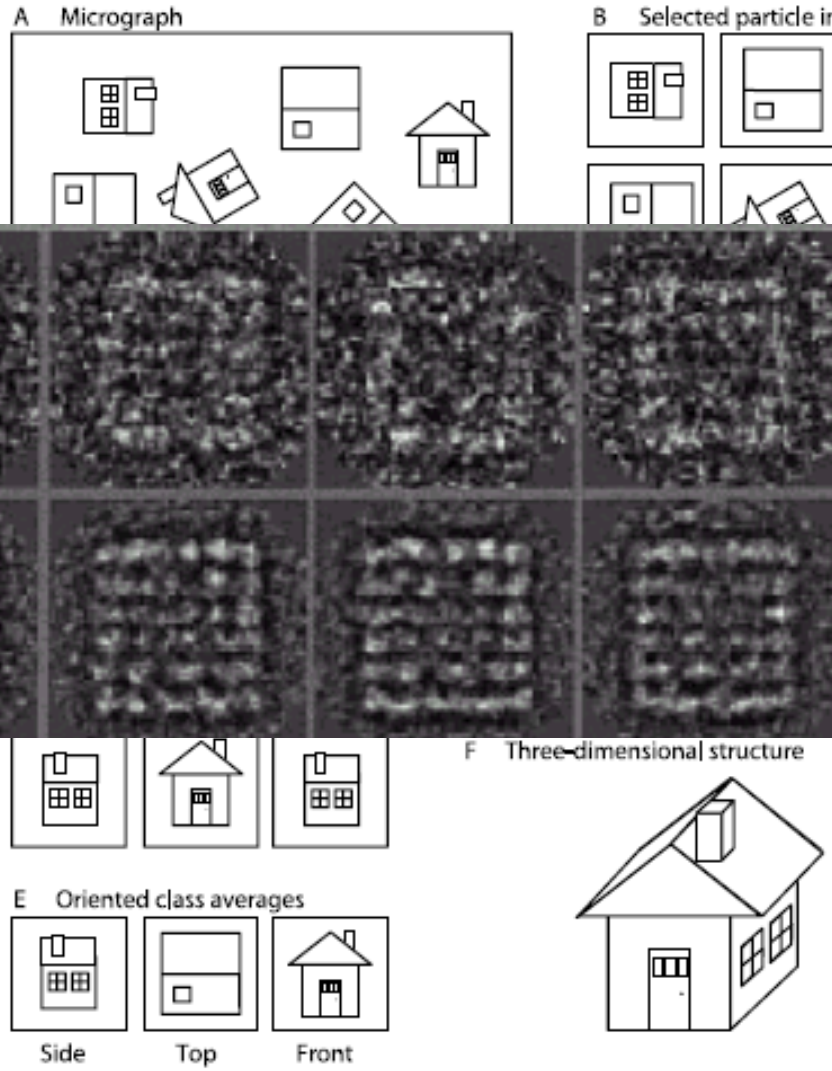
6. Refine orientations, calculate new model, repeat

7. Calculate final model, make nice images

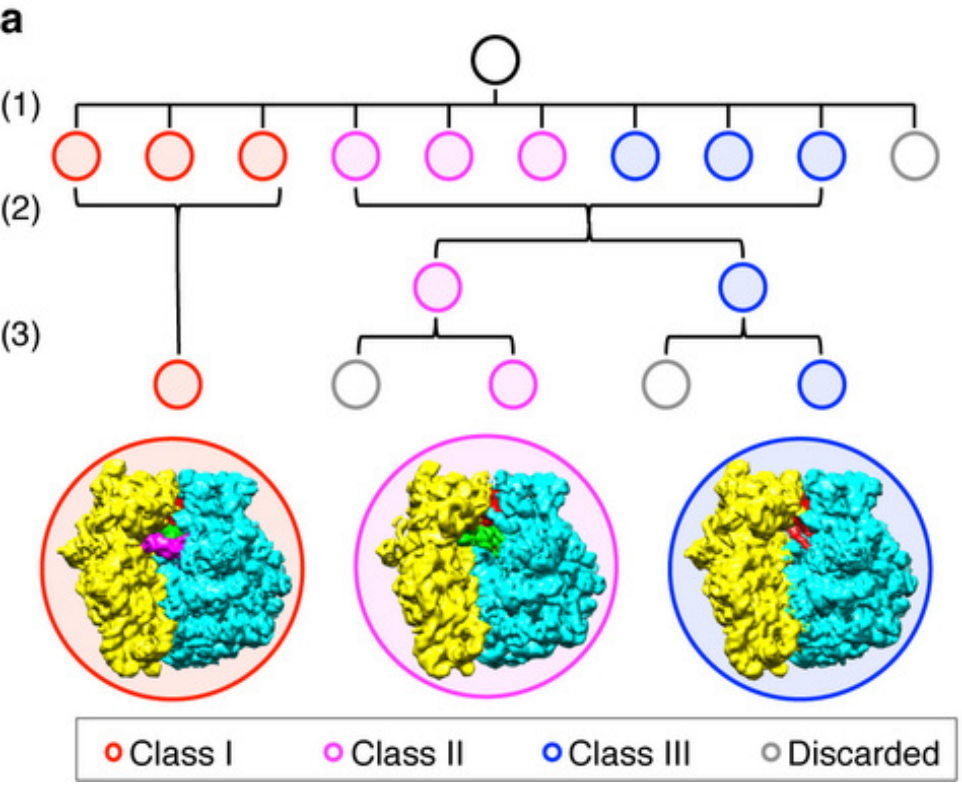
The classification method

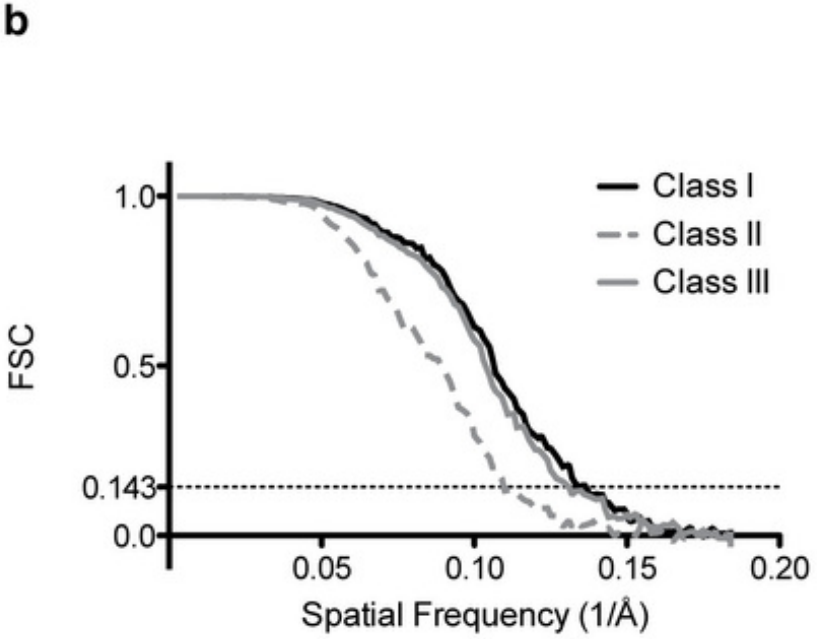
- No need for a starting model →



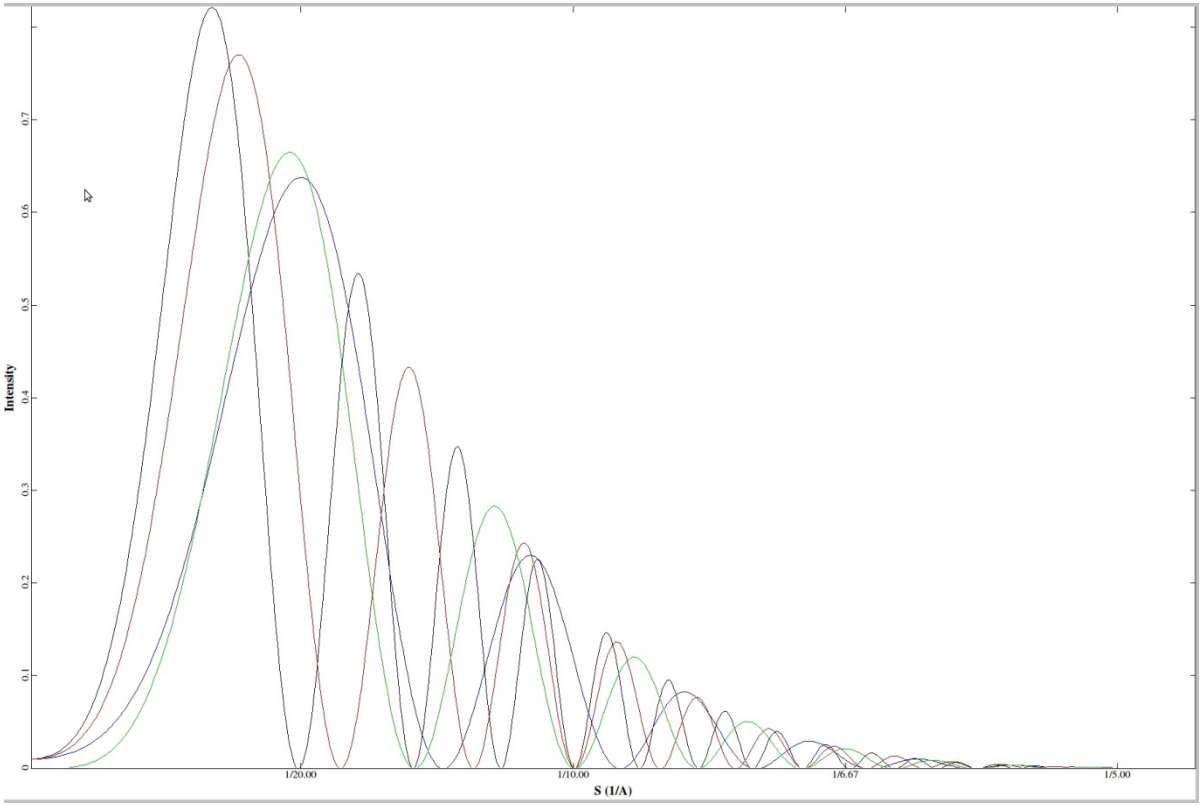


3D classification





CTF correction



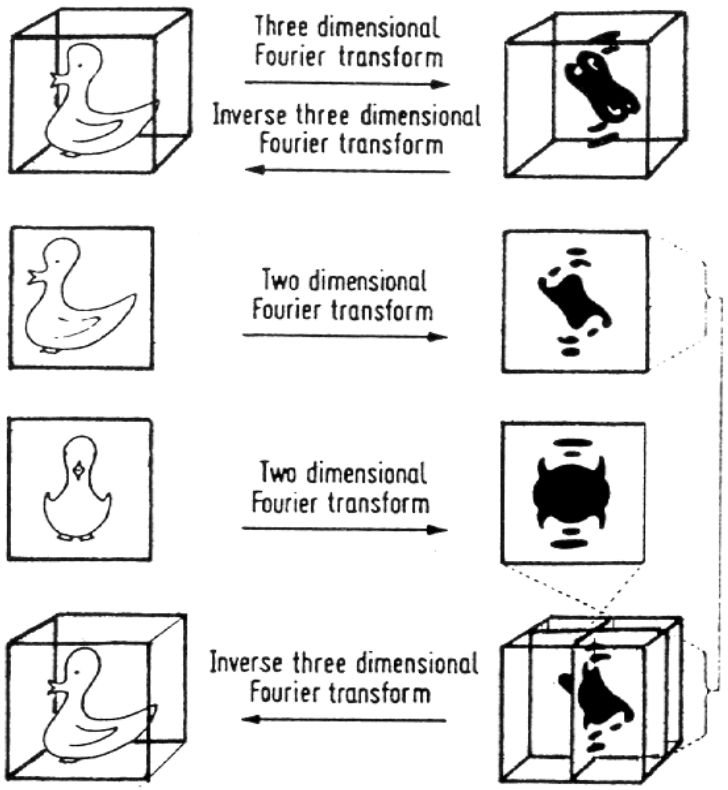
Compensation: 1) phase flips

2) decay at high res

3) zeros

Single particle analysis

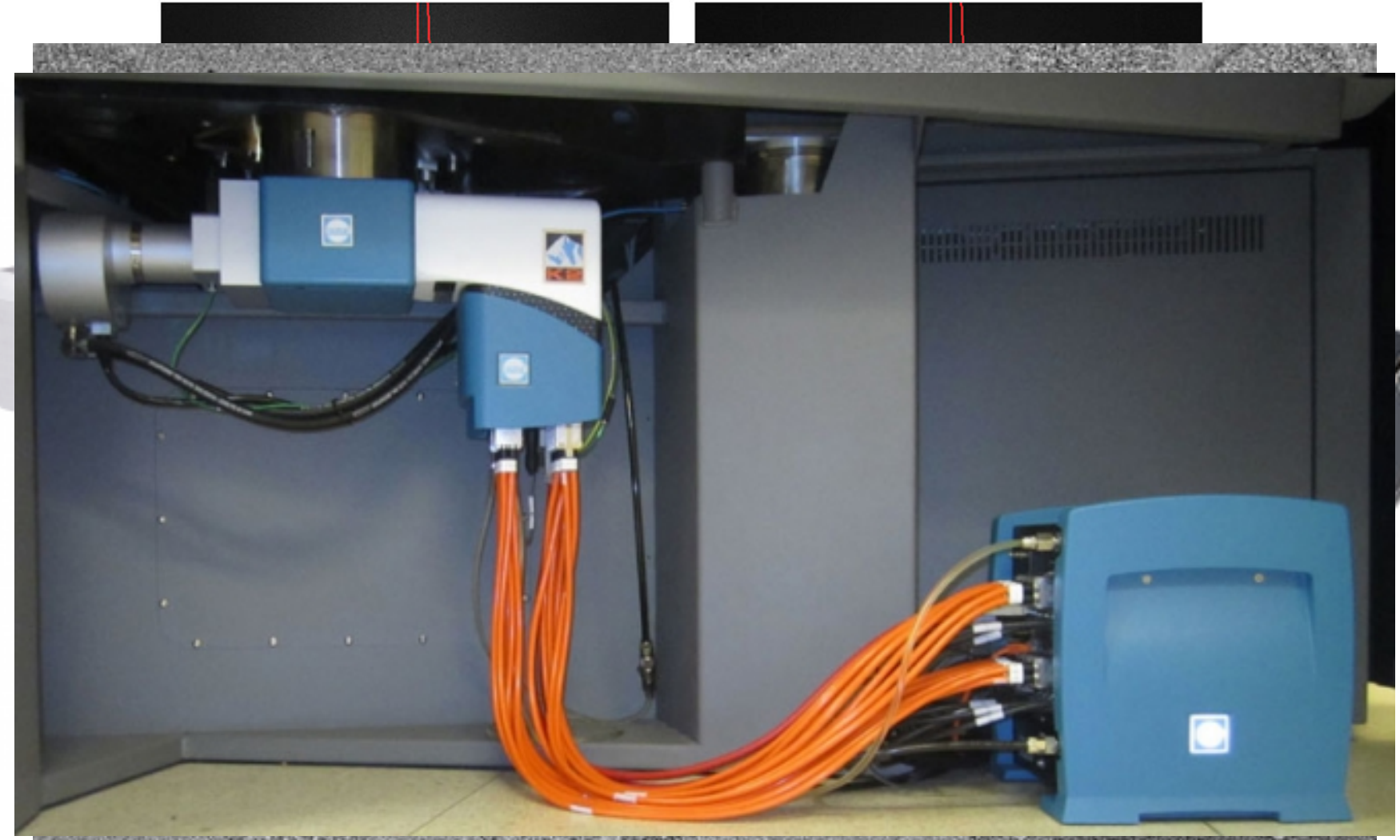
- 2D Fourier transform (FT) is calculated for every projection
- These correspond to central sections in 3D FT of the object
- Reconstruction can be done by “filling” the 3D Fourier space with these sections



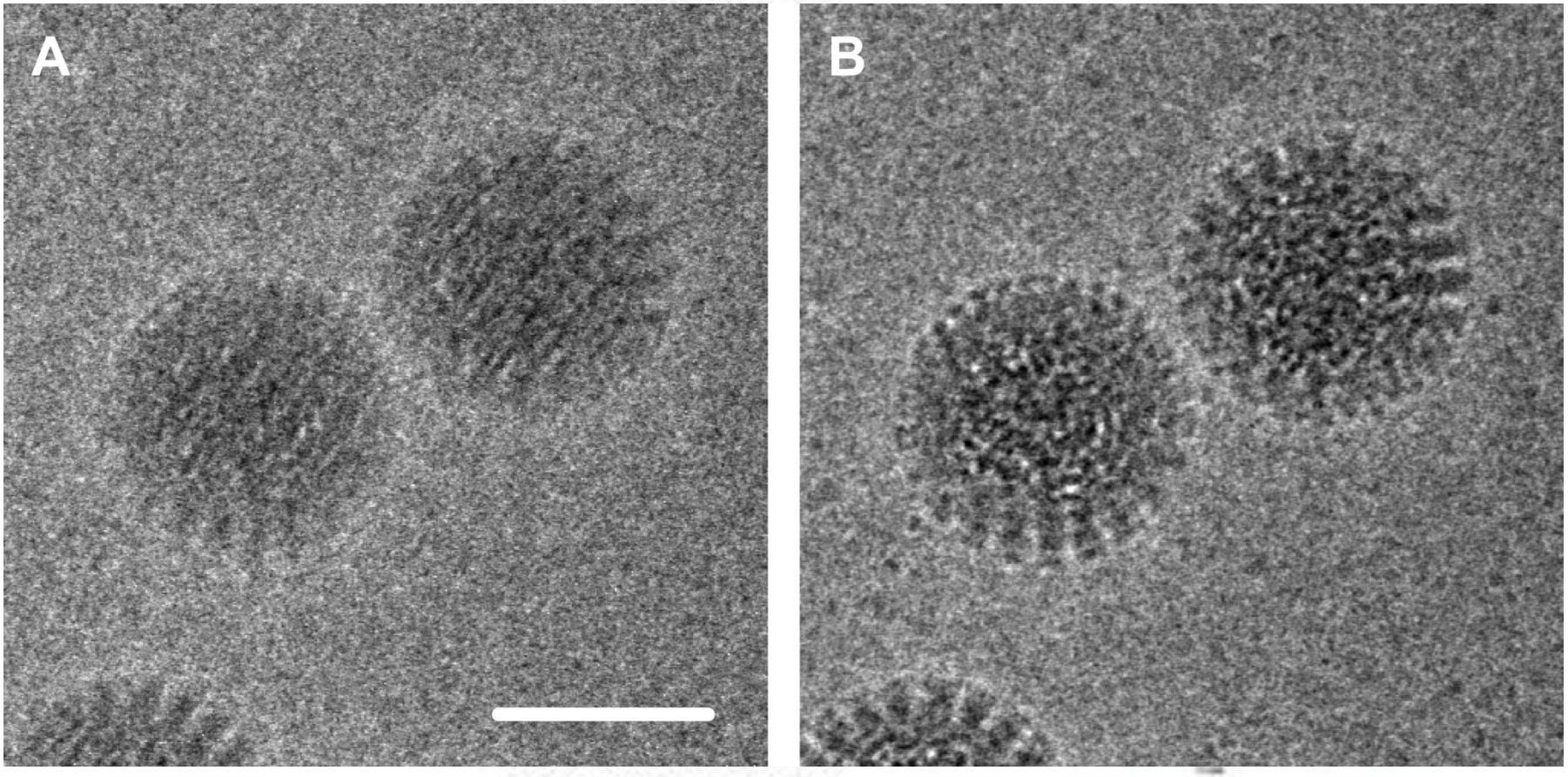
Contents

1. Goals and requirements
2. Sample preparation, imaging & image quality
3. Image processing & reconstruction
4. New detectors & current state of the art

Direct electron detectors



Beyond sensitivity



H)

1. EM
2. EM bectively
3. EM

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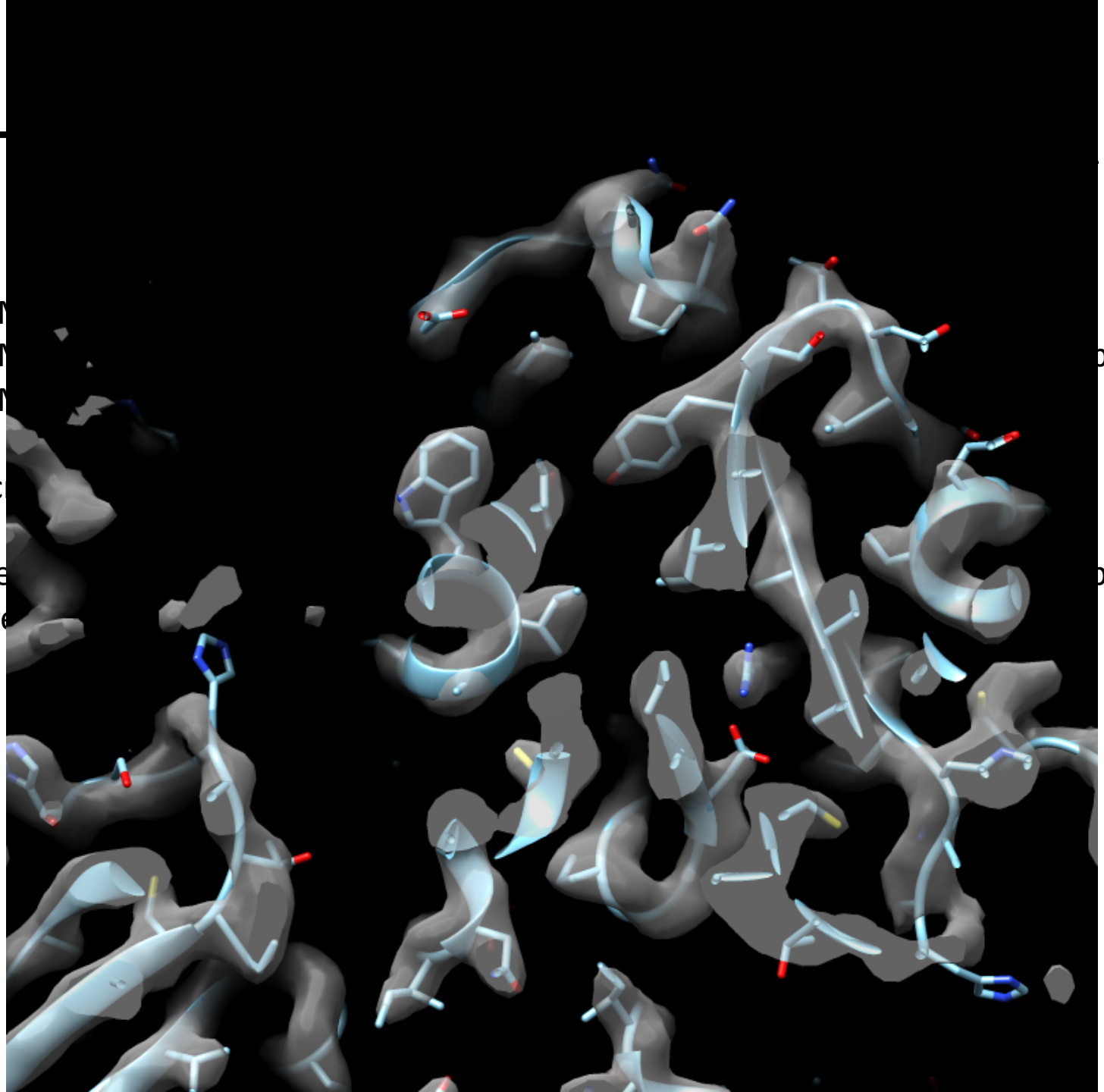


2017 NOBEL PRIZE IN CHEMISTRY

The Nobel Prize in Chemistry 2017 was awarded to Jacques Dubochet, Joachim Frank, and Richard Henderson for the development of cryo-electron microscopy for determining biomolecule structures.

X-RAY CRYSTALLOGRAPHY

Structures of proteins that form crystals

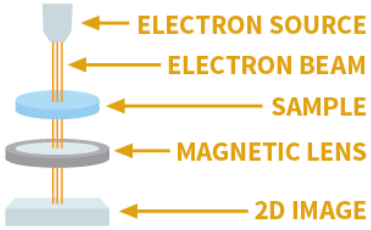
NMR SPECTROSCOPY

Structures of small proteins in solution

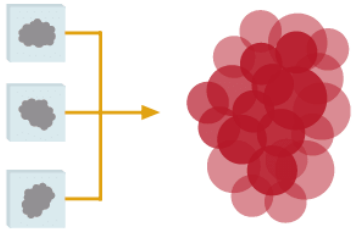
CRYO-EM

Structures of large, non-crystalline proteins

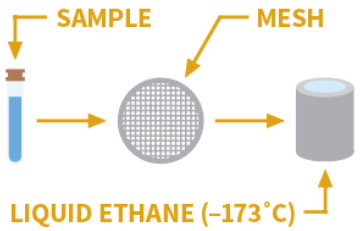
Cryo-electron microscopy (cryo-EM) is a technique that makes it possible to produce 3D images of biomolecules at atomic resolution. It can be used to capture images of biomolecules which could not be visualised with previously existing techniques.



Henderson pioneered the use of electron microscopy (EM) to visualise proteins. Using it, he produced the first atomic resolution image of a protein, bacteriorhodopsin, in 1990.



Frank developed an image analysis method that allowed computers to assemble a high resolution 3D image from many 2D EM images, improving the quality of biomolecule images.



Biological samples dry out and are damaged when in vacuum during EM. **Dubochet** solved this by rapidly freezing samples in water at –173˚C to form an icy glass instead of crystals.

WHY DOES THIS RESEARCH MATTER?

Cryo-EM allows scientists to reveal how proteins move and interact with other molecules, freezing and observing them mid-process. It could improve our understanding of drug targets and biological processes.

Nobel Prize in Chemistry Press release: https://www.nobelprize.org/nobel_prizes/chemistry/laureates/2017/press.html

COMPOUND iNTEREST

Conclusions

- SPR can produce atomic resolution models of macromolecules with relatively easy sample preparation
- Strict requirement for identical particles → however, 2D- & 3D-classification can work with mixed populations (very laborious)
- New detectors (and computational methods) have changed the process significantly