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Structure Determination by Single Particle Cryo-EM

Christiane Berger-Schaffitzel, 24.06.2022

Resolution Revolution in Cryo-EM

The Nobel Prize in Chemistry 2017
Jacques Dubochet, Joachim Frank, Richard Henderson

The Nobel Prize in Chemistry 2017

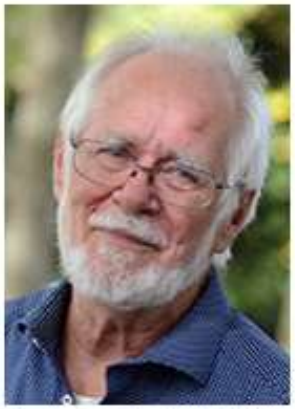


Photo: Félix Imhof © UNIL [CC BY-SA 4.0]
Jacques Dubochet
Prize share: 1/3

Photo: B. Winkowski © Columbia University Medical Center
Joachim Frank
Prize share: 1/3

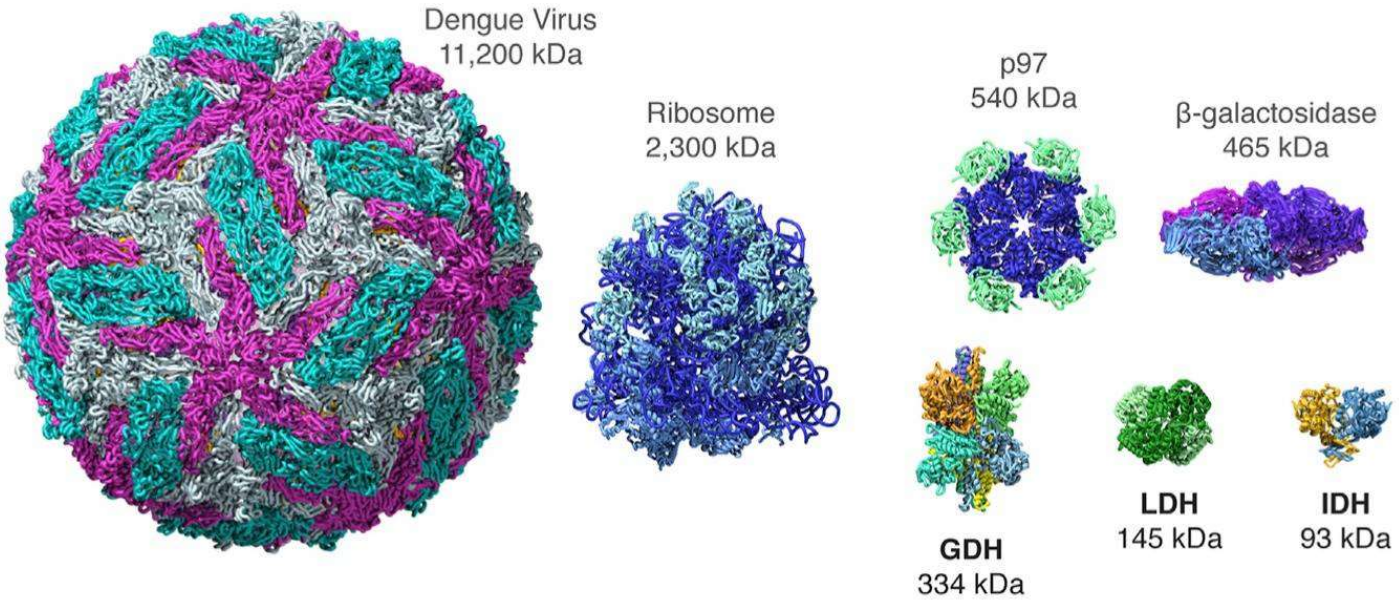
Photo: MRC Laboratory of Molecular Biology
Richard Henderson
Prize share: 1/3

The Nobel Prize in Chemistry 2017 was awarded to Jacques Dubochet, Joachim Frank and Richard Henderson *"for developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution".*

Resolution Revolution in Cryo-EM

Examples of structures solved by cryo-EM to highest resolution (1.22-3 Å)

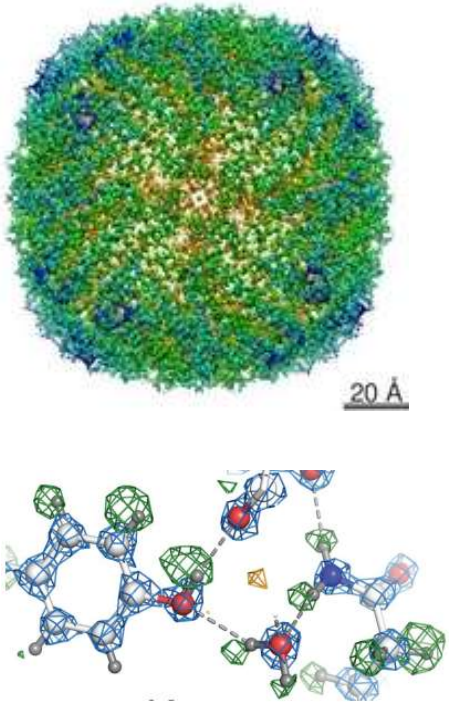
Near-atomic resolution structures (1.8–3 Å)



Molecules shown in relative sizes

Merk *et al., Cell 2016*

Apoferritin, 480 kDa



Single-particle cryo-EM at atomic resolution (1.22 Å)
Nakane et al., *Nature* 2020
Yip et al., *Nature* 2020

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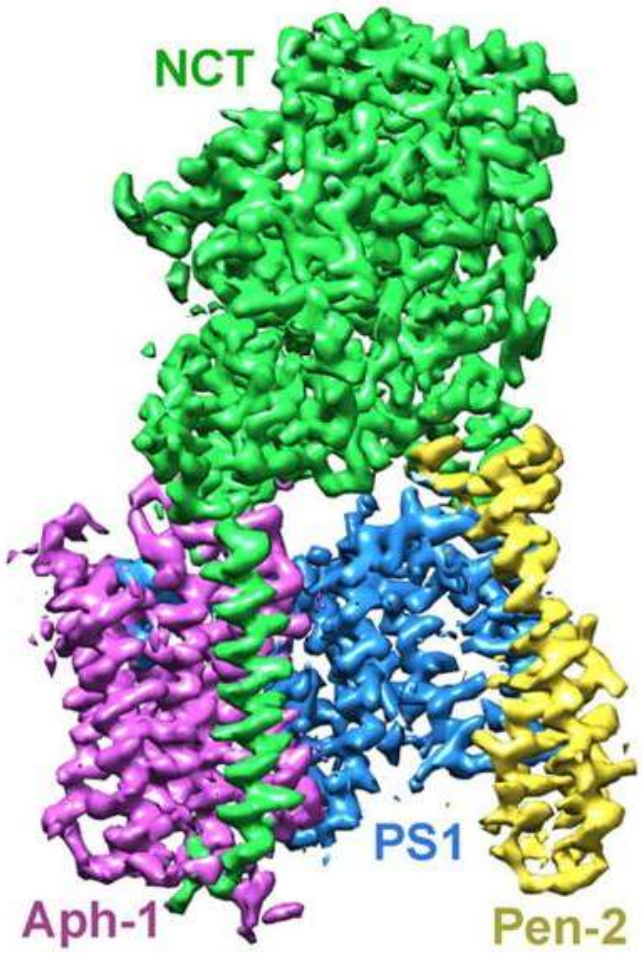
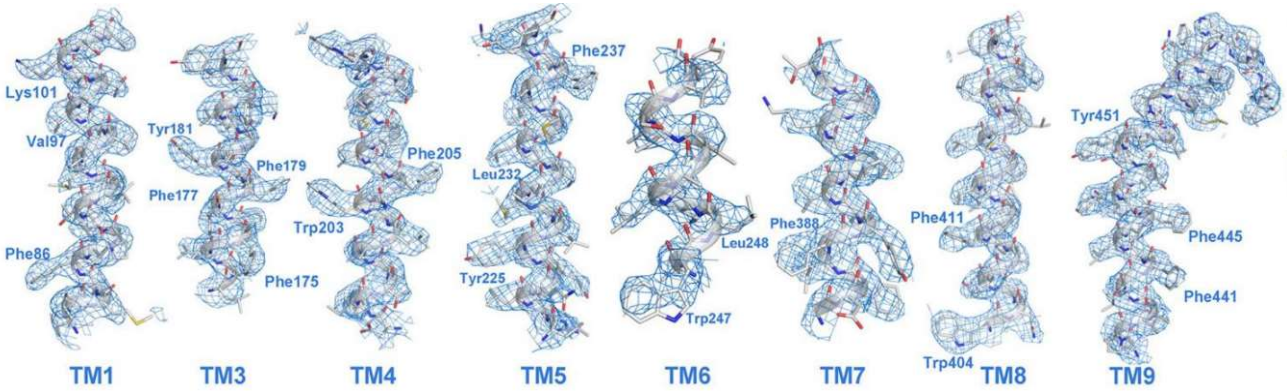
Smaller Asymmetric Membrane Proteins

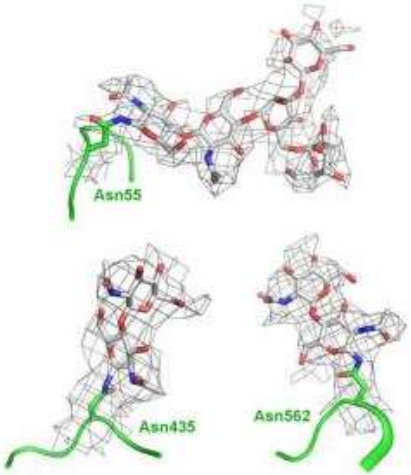
Human γ-secretase complex at 3.4 Å
170 kDa membrane protein complex, glycosylated

Bai *et al., Nature 2015*





density of TM helices of PS1

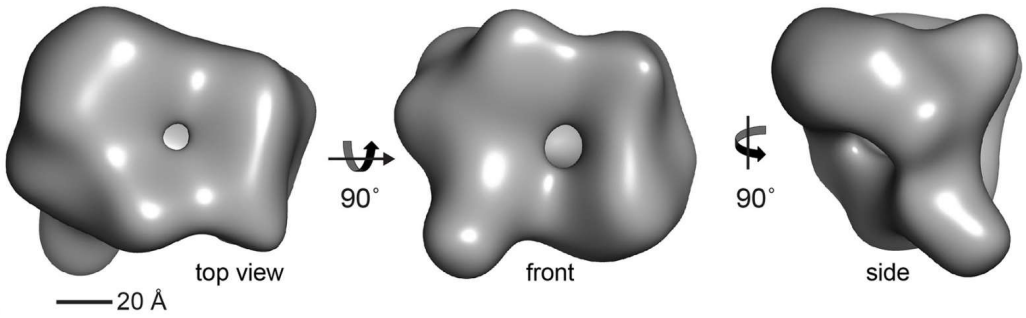


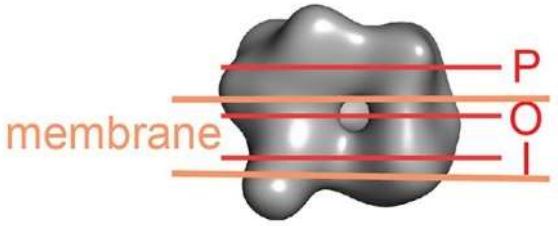
density of glycans of NCT

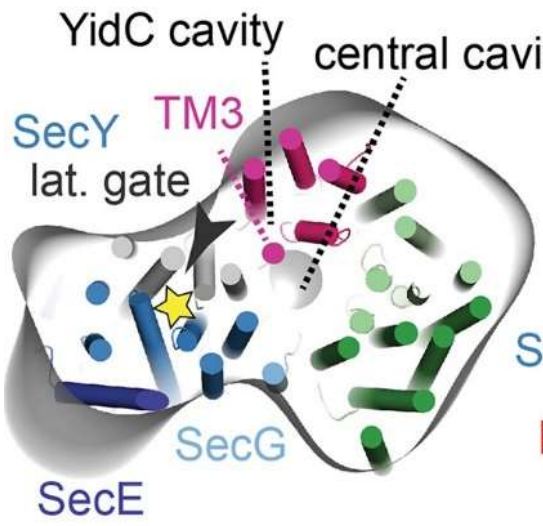
lipid molecules

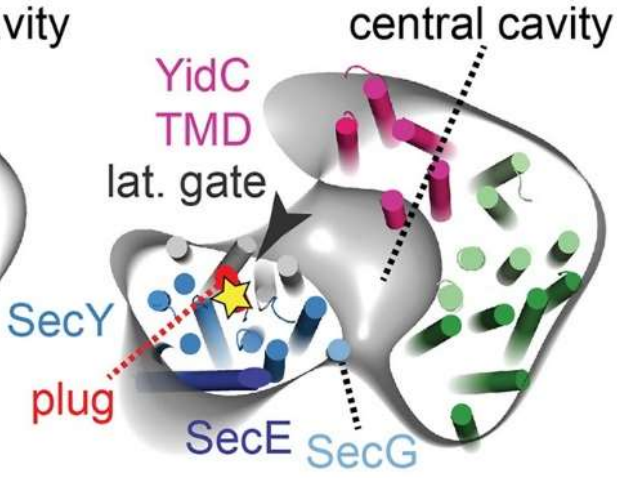
In 2021, ~80% of GPCR structures have been solved by cryo-EM (GPCRpdb, Kooistra *et al.*, NAR 2021).

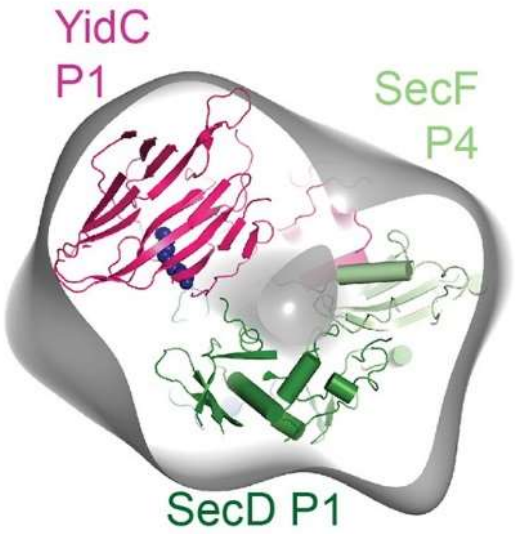
Holotranslocon comprises a Central Lipid-filled Cavity







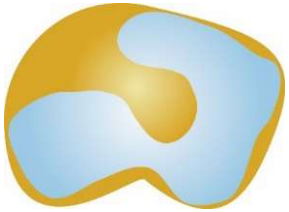




Inner membrane leaflet | Outer membrane leaflet | Periplasmic side

Botte et al. Sci. Reports 2016

Absolute-Scale Small Angle Neutron Scattering Match Point Analysis

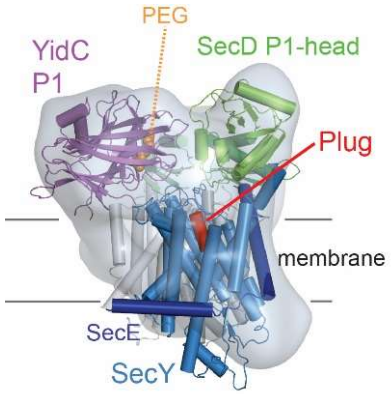


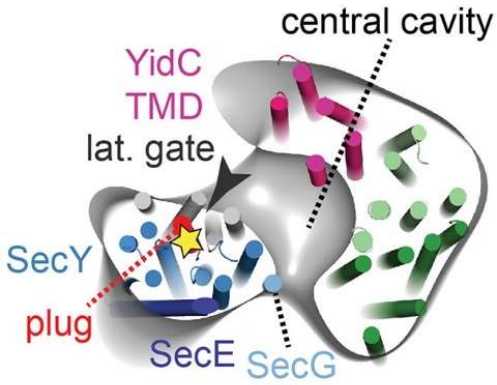
HTL particle is (vol/vol):
~40% protein,
~40% detergent and ~20% lipids.

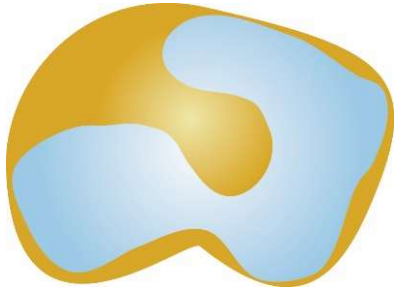
Match point of proteins: 42%
Match point of DDM: 22%
Match point of lipids: 10-14 %

Botte et al. Sci. Reports 2016

SANS Stuhrmann Analysis: HTL has a lipid core surrounded by protein

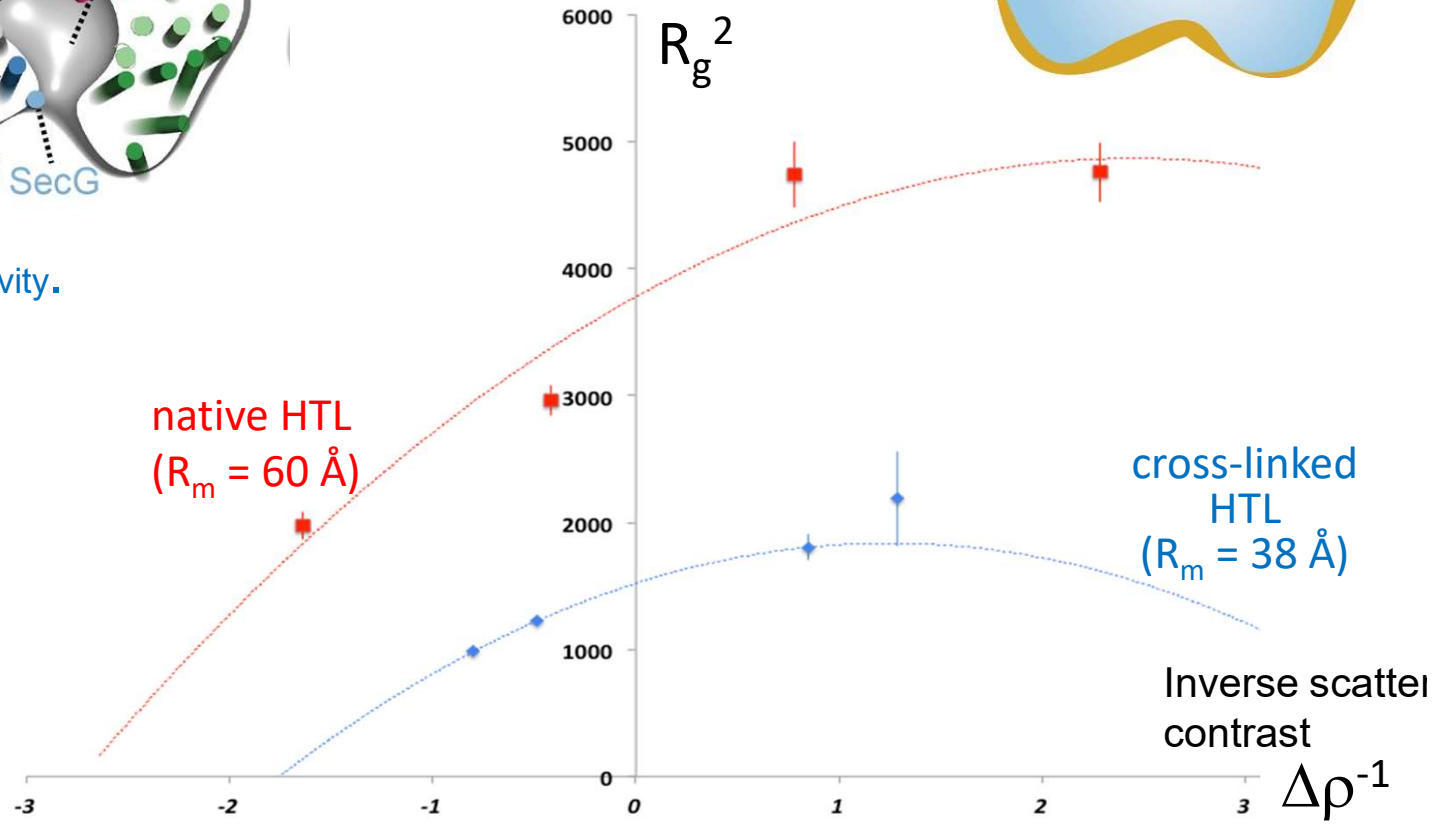






SANS data support a central, lipid-filled cavity.

Positive slope: the high scattering unit (protein) is outside, and the low scattering element (lipid/detergent) is closer to the centre.



Botte et al., Sci Rep 2016

Using deuterated DDM, the volume of the lipid core was determined (1216 Å^3), corresponding to 8-29 *E. coli* lipids.

Martin, Arleth, Collinson et al., Biophys. Journal 2019

Electron Microscopy: The BASICS

1 – The Microscope

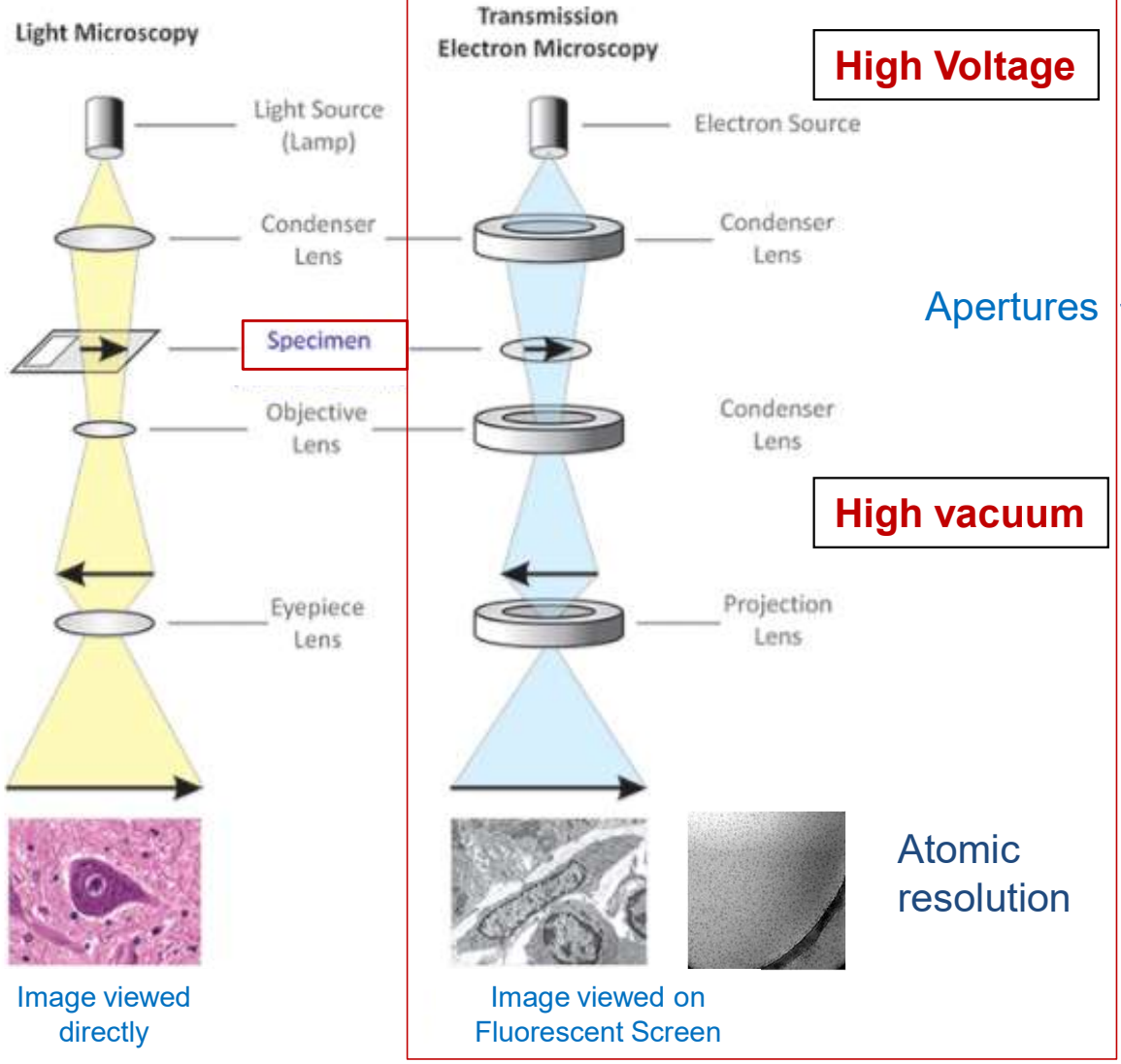
2 – Negative Stain EM and Sample Preparation

3 – The Image & Direct Electron Detectors

4 – Image Processing, the Principle

5 – State-of-the-art Image Processing

Electron Microscope



Electron Microscope T20

Adapted from https://www.pinterest.co.uk

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Differences compared to Light Microscopy

1. It is necessary to analyse samples in a vacuum because airborne contaminants will also scatter electrons. Therefore, samples cannot be visualised in an aqueous solution.

2. High energy electron collisions gives rise to sample damage. Therefore, low electron dosage conditions must be used. This leads to a low signal-to-noise ratio (noisy images).

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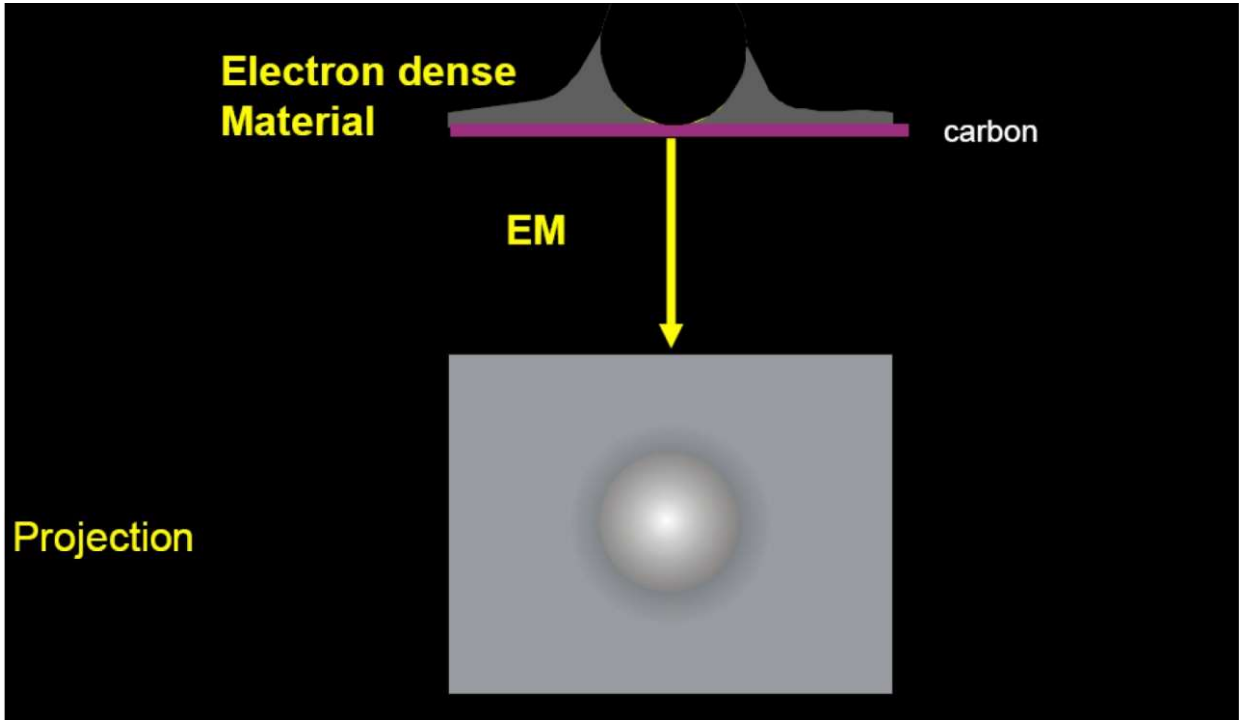
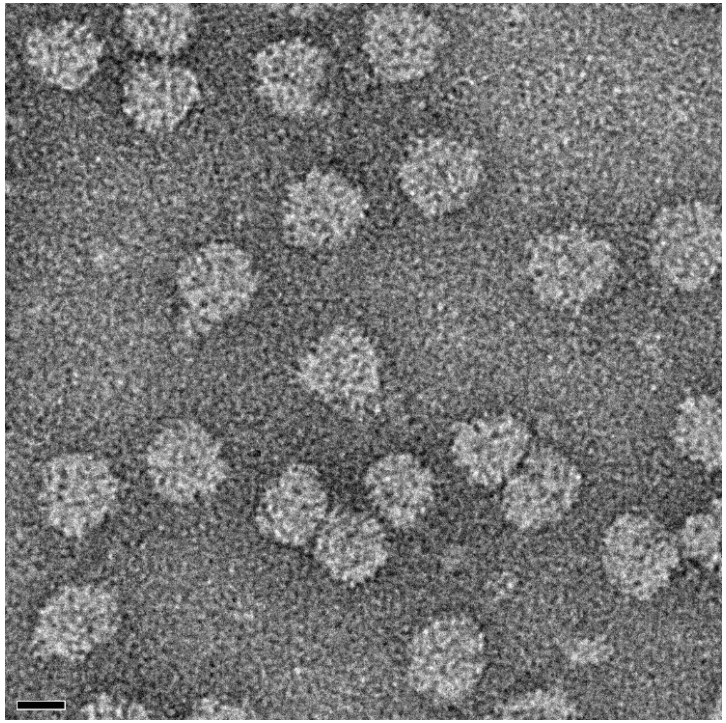
Negative Stain Electron Microscopy





Scale bar : 20 nm

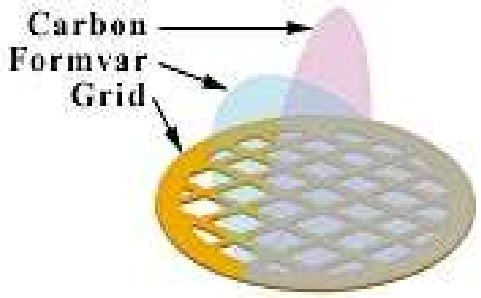
uranyl acetate staining

Advantage: nice contrast of the molecules
can be done at room temperature, fast
great for sample quality control

Disadvantage: the resolution is limited by the stain.
staining artefacts, flattening ,
only the envelope is obtained

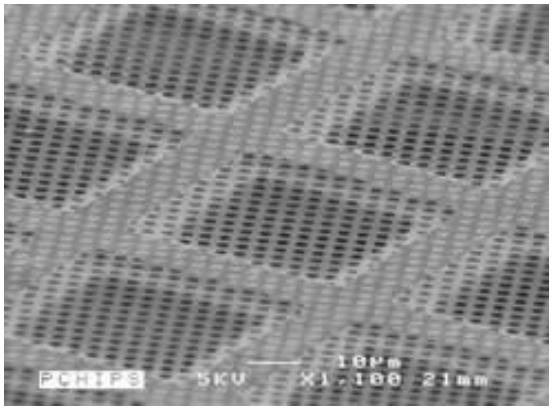
Cryo-EM Sample Preparation

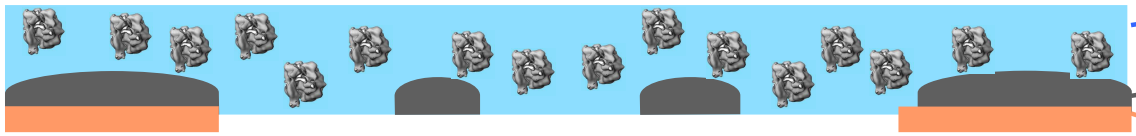
Freezing Grids



3-6 μl of sample (nanomolar concentration) required per grid

Holey carbon film





vitreous ice (200-500 Å)

thick carbon (150 Å)

copper grid

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Vitrification – Cryo-grid preparation: Blotting and Rapid Freezing

- Rapid freezing in liquid ethane leads to formation of vitreous ice.
- Thin ice is required, as the contrast between sample and buffer is low.
- Imaging has to occur at liquid nitrogen temperature to avoid ice contamination.

Video on vitrification: https://www.youtube.com/watch?v=QML_KMQbOMc

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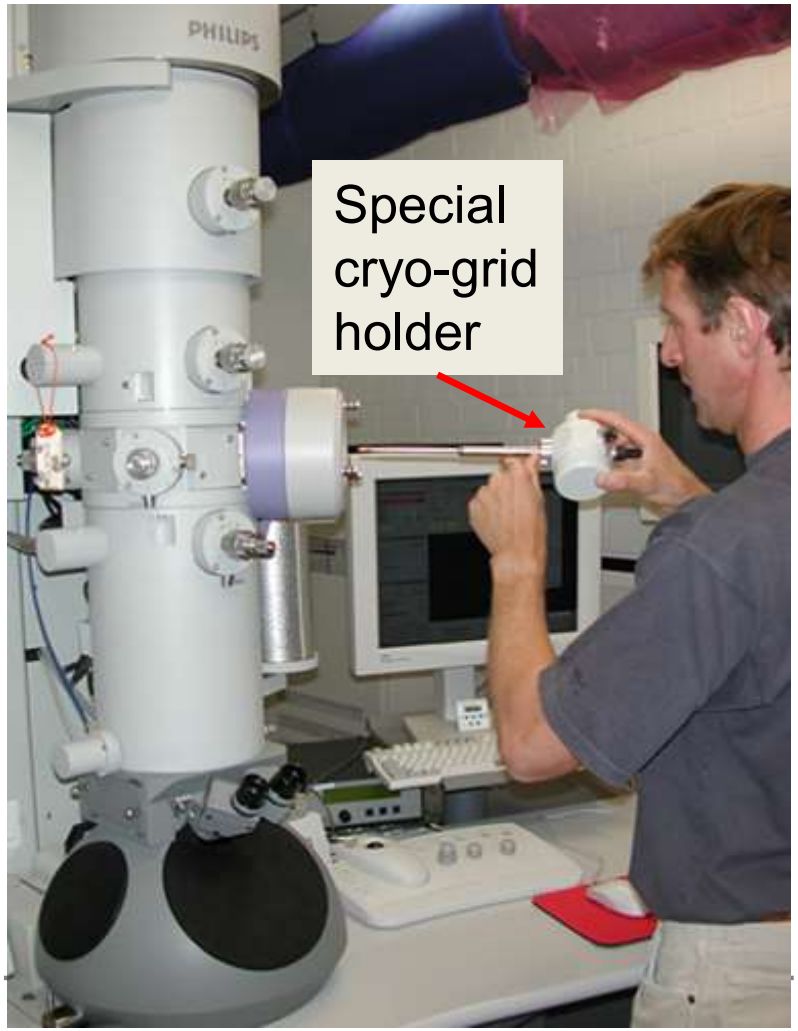
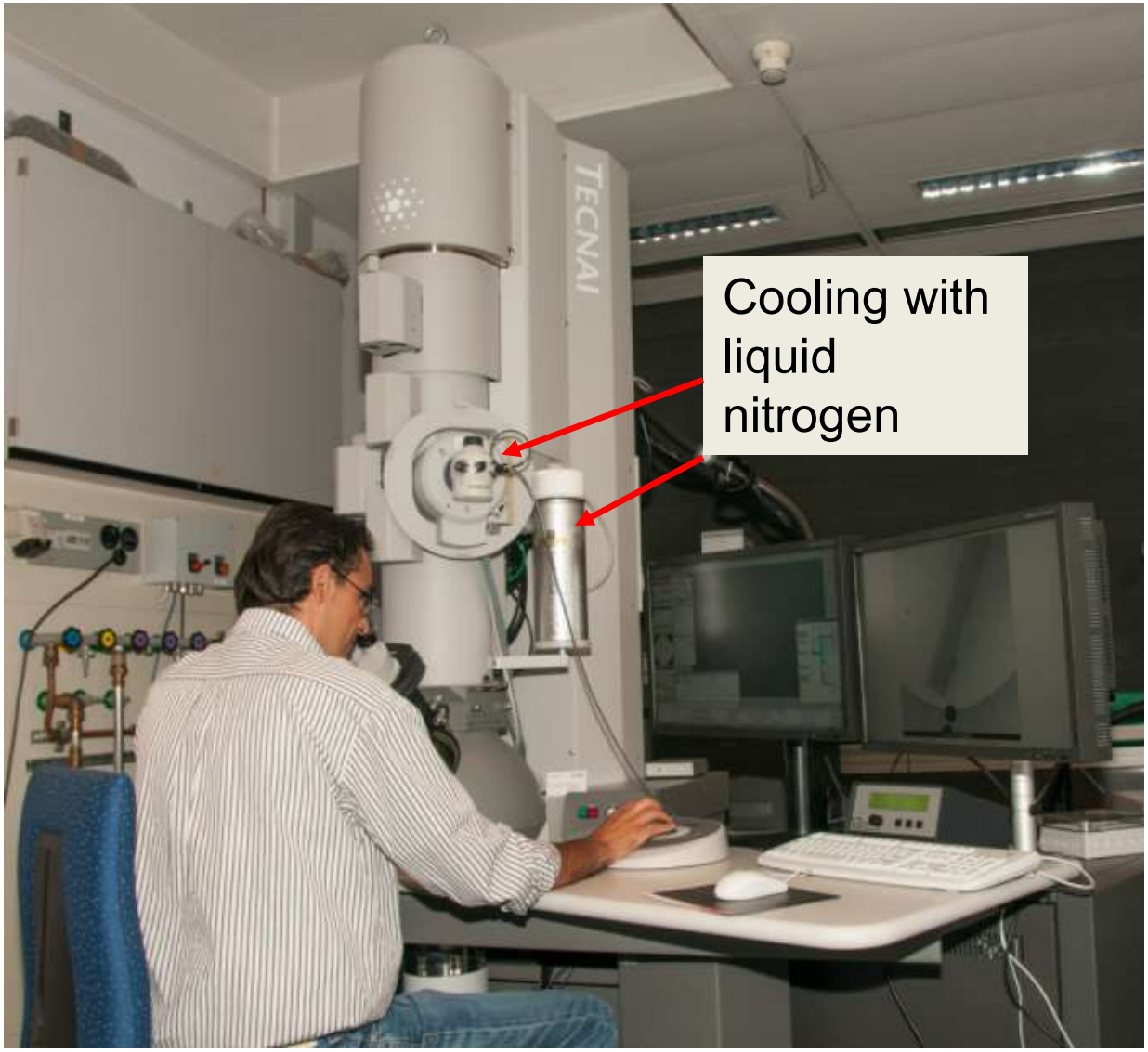
Cryo-Microscopy

Insert specimen holder into microscope





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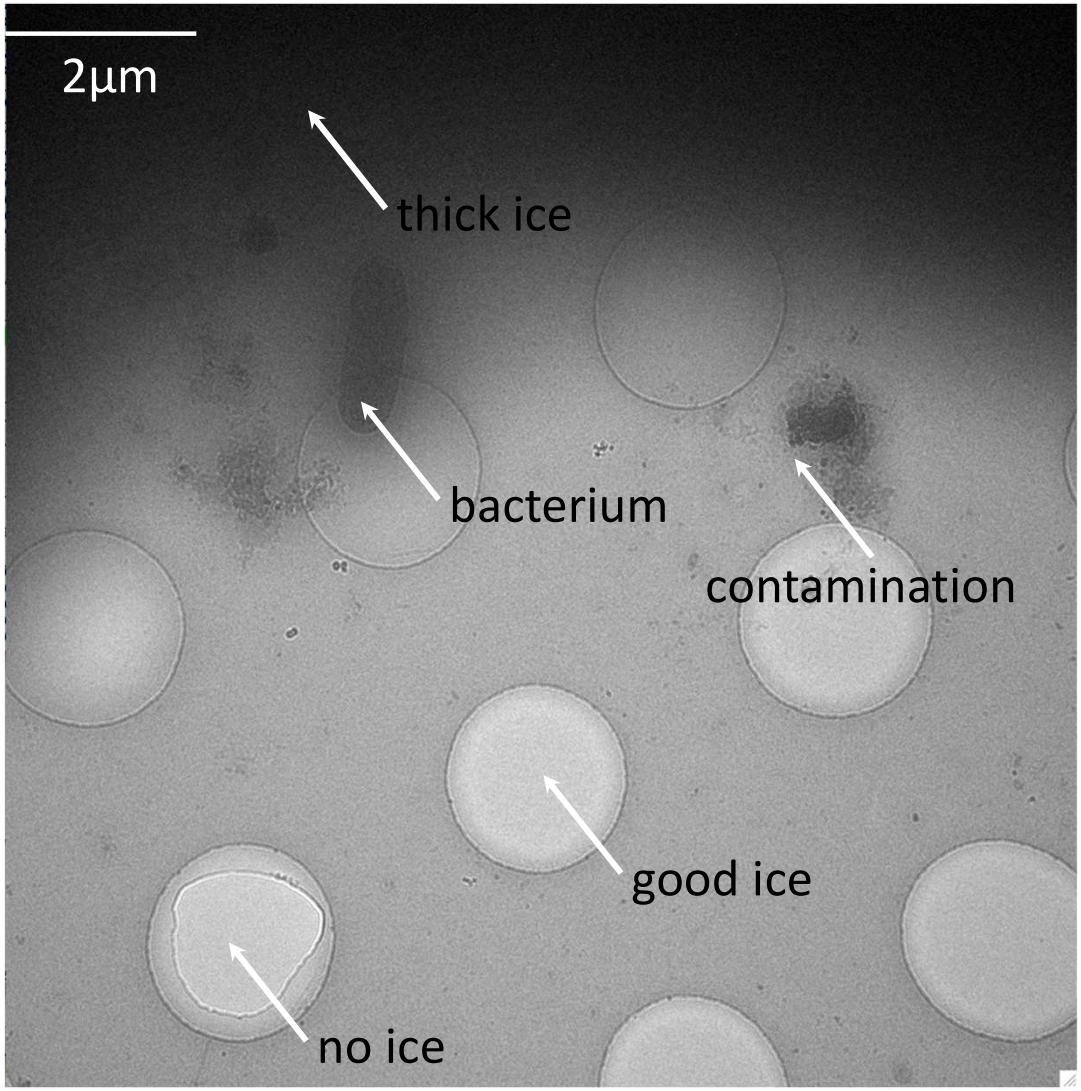
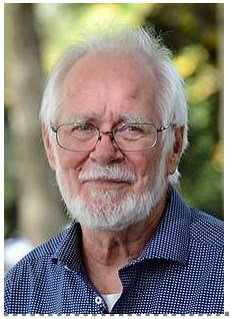
Ice thickness and contamination



Cryo-EM Sample Preparation

- Water, or aqueous sample cannot be imaged in the high vacuum of the electron microscope.
- Sample must be frozen and imaged at cryo-temperatures (cooling with liquid nitrogen, -180˚C).
- Ice crystals are black in the electron microscope, so the freezing step has to be very fast -> flash freezing, avoiding ice crystal formation.
- The ice layer must be thin to be able to see the sample in the ice, optimal contrast is achieved when the ice is just a bit thicker than the sample itself. Larger proteins/ complexes are easier to see.



Jaques Dubuchet, Nobel prize in Chemistry 2017

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Electron Microscopy: The BASICS

1 – The Microscope

2 – Negative Stain EM and Sample Preparation

3 – The Image & Direct Electron Detectors

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Richard Henderson, Nobel prize in Chemistry 2017

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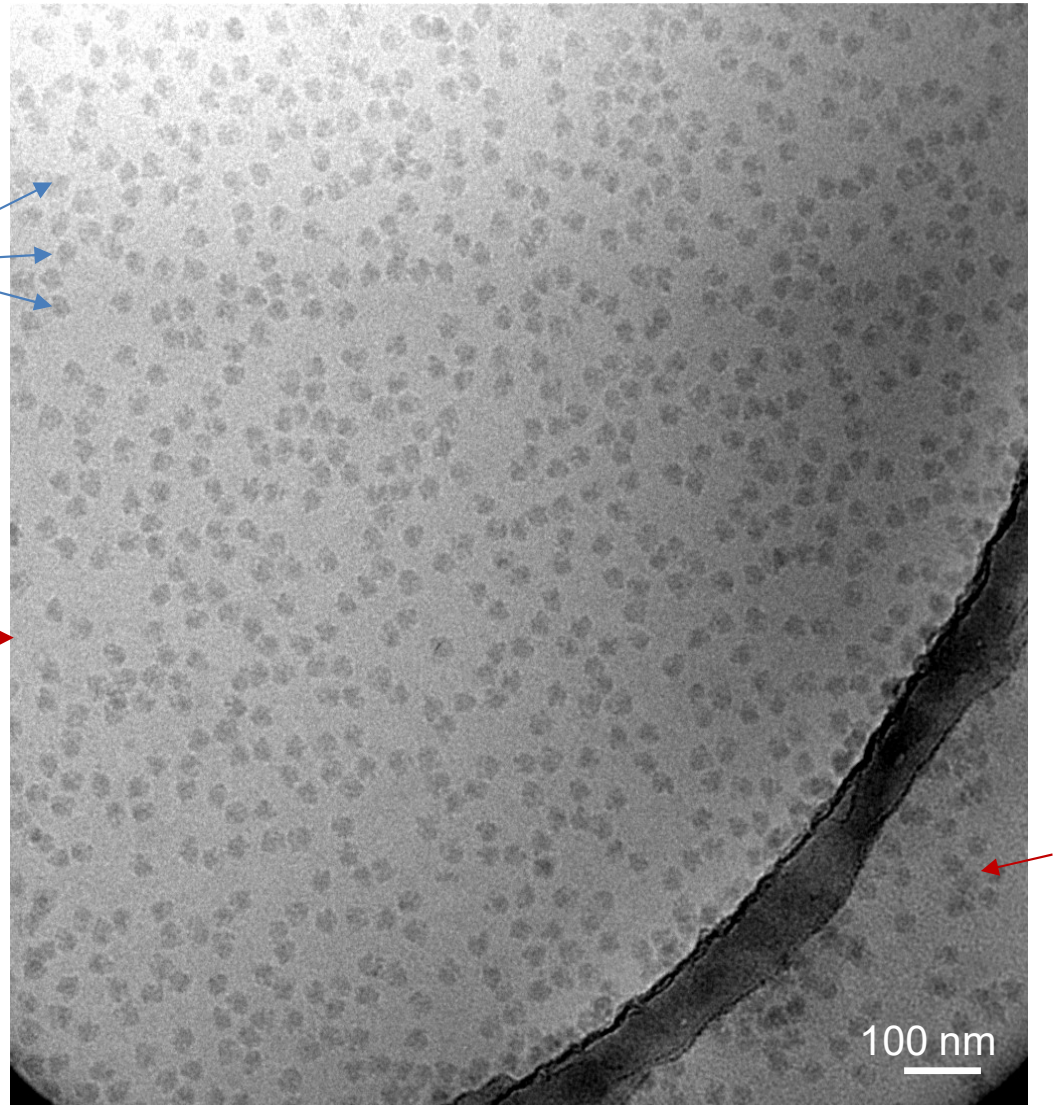
Cryo-Electron Microscopy

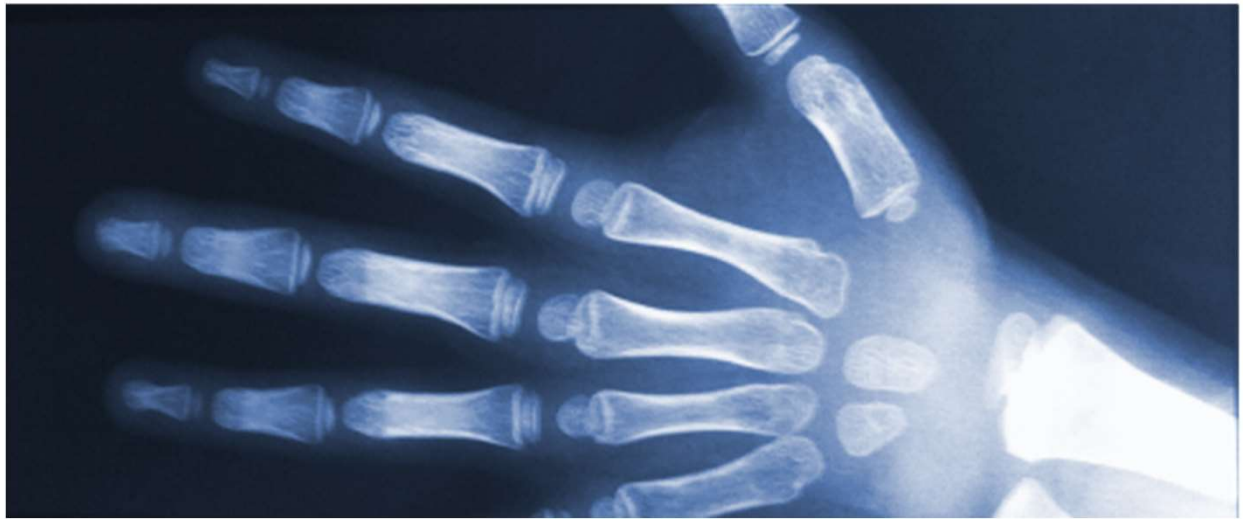


Talos Arctica

University of BRISTOL

What is a Cryo-EM Image?

It is a projection image.

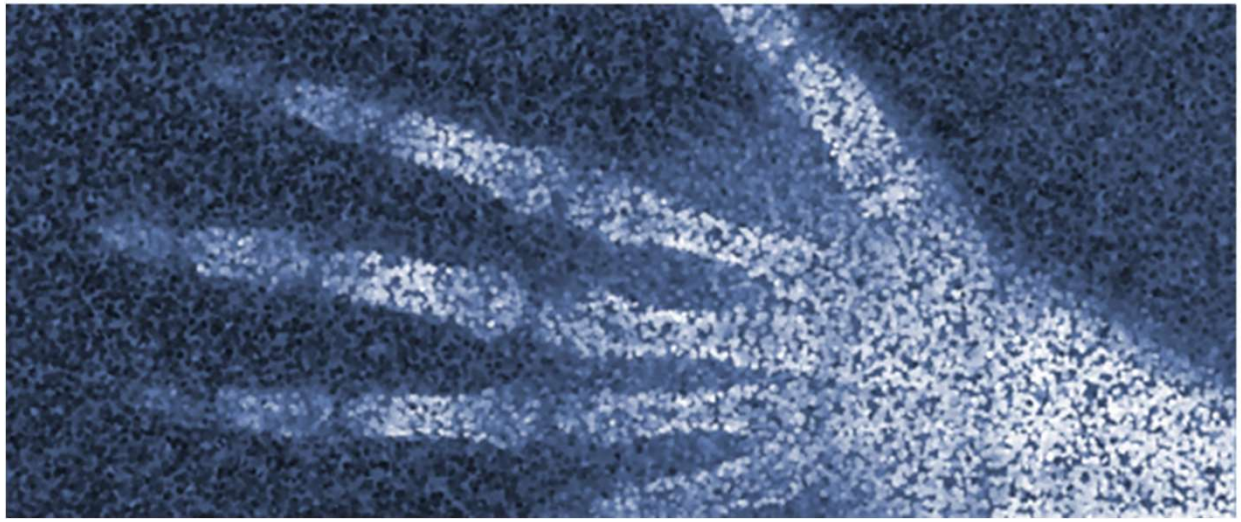


Mark Ian Berger

University of BRISTOL

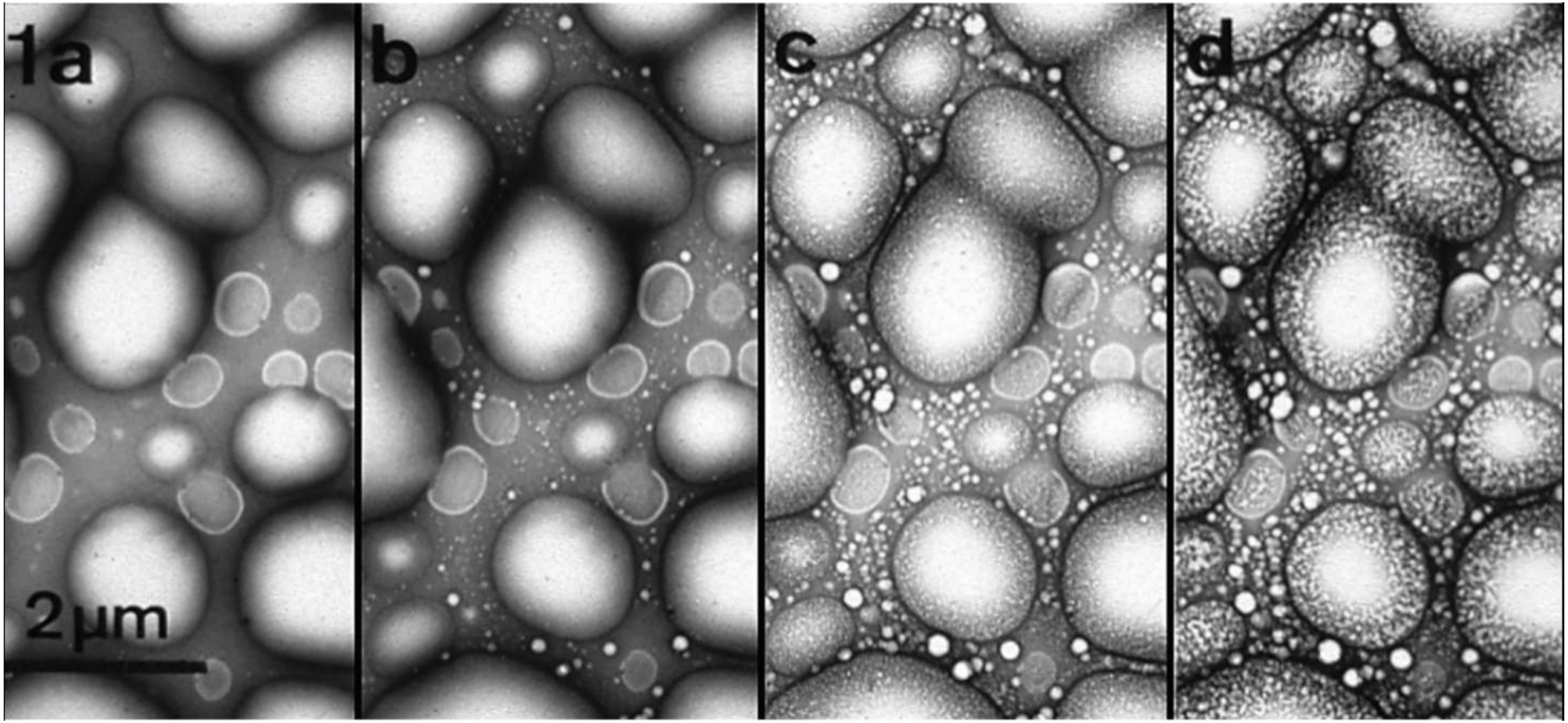
What is a Cryo-EM Image?

It is a very noisy projection image.



Radiation Damage: the Major Factor that Limits the Attainable Resolution of Cryo-EM Structures

It is noisy because of limited electron dose.



Slide: Andy Hoenger

Radiation Damage: the Major Factor that Limits the Attainable Resolution of Cryo-EM Structures

Radiation Damage, another example…

EM Images contain Amplitudes and Phases

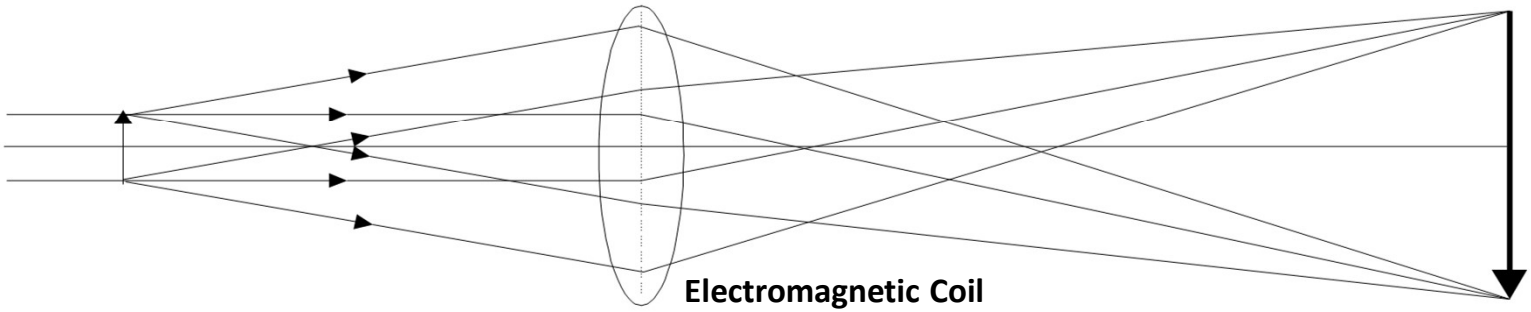
X-RAY DIFFRACTION

Phases are lost.

Object

Diffraction Plane (Amplitudes only)

Image (Amplitudes + Phases)



ELECTRON MICROSCOPY

Electrons can be focussed (unlike X-rays)

Direct Electron Detectors caused the Resolution-Revolution in cryo-EM

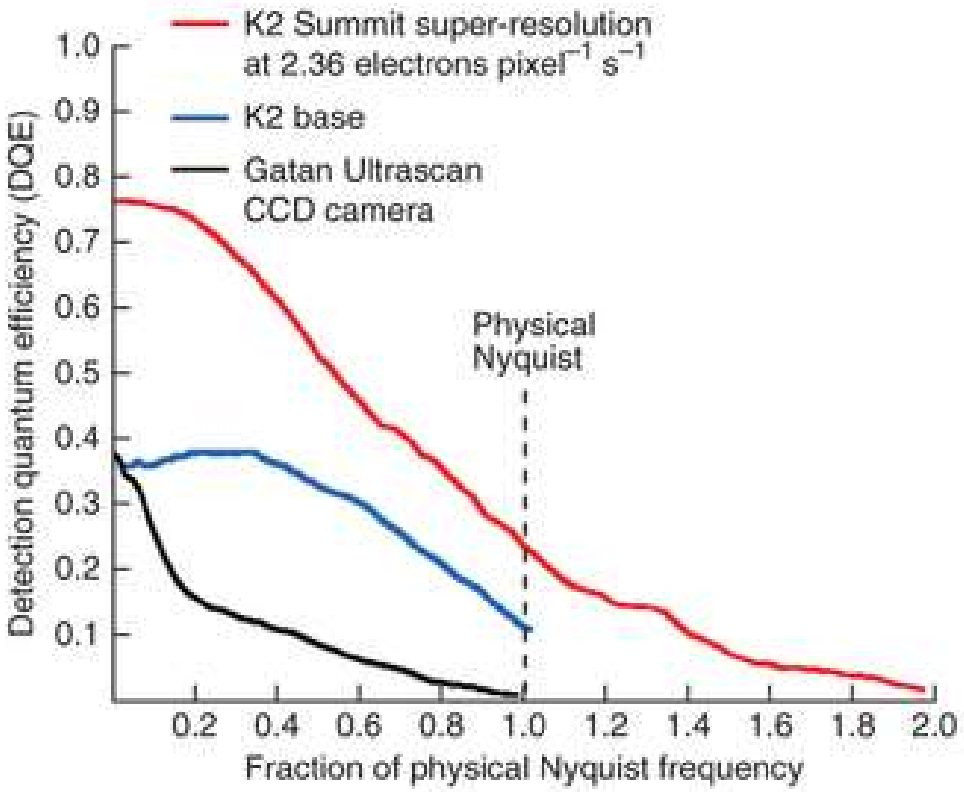
Signal to noise ratio is the major challenge in cryo-EM.

New Direct Electron Detectors contain Complementary Metal Oxide Semiconductor (CMOS) chips.

Advantages :

- More sensitive
- Fast
- Super-resolution imaging

Increased sensitivity:



Li et al., Nat. Methods 2013

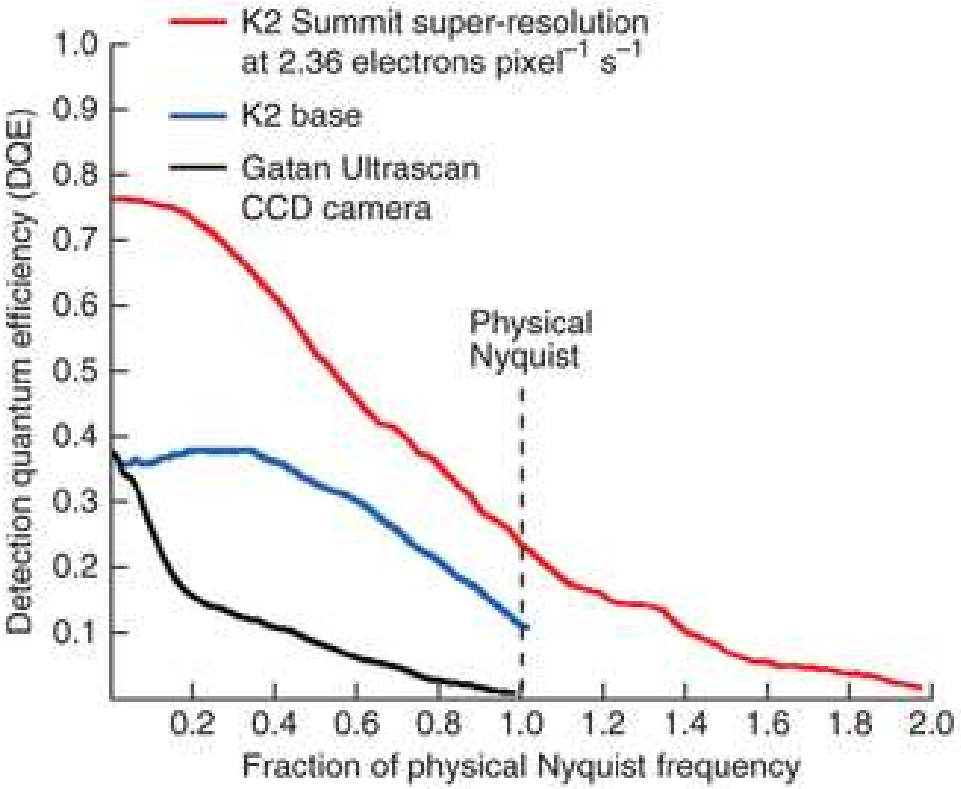
Glossary:

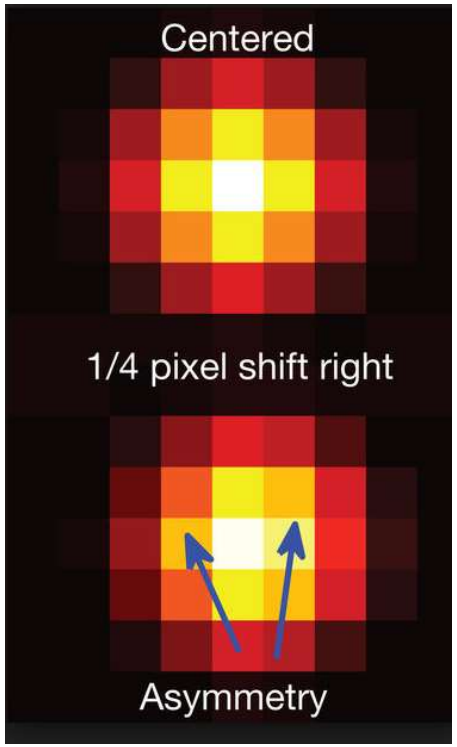
Nyquist frequency: The Nyquist Sampling Theorem states that the sampling frequency (Pixel Size) should be at least twice the highest frequency contained in the signal. Small structures are said to have a high frequency.
I.e. to resolve features of 4Å, a pixel size of at least 2Å/px is required.(protein alpha-helical pitch: 5.4Å)

DQE: frequency-dependent measure for signal to noise ratio performance

Direct Electron Detectors – Super Resolution Imaging

Increased sensitivity:





Why does this work? Because the electron dose is low!

DQE: frequency dependent measure for signal to noise ratio performance

Li et al., Nat. Methods 2013

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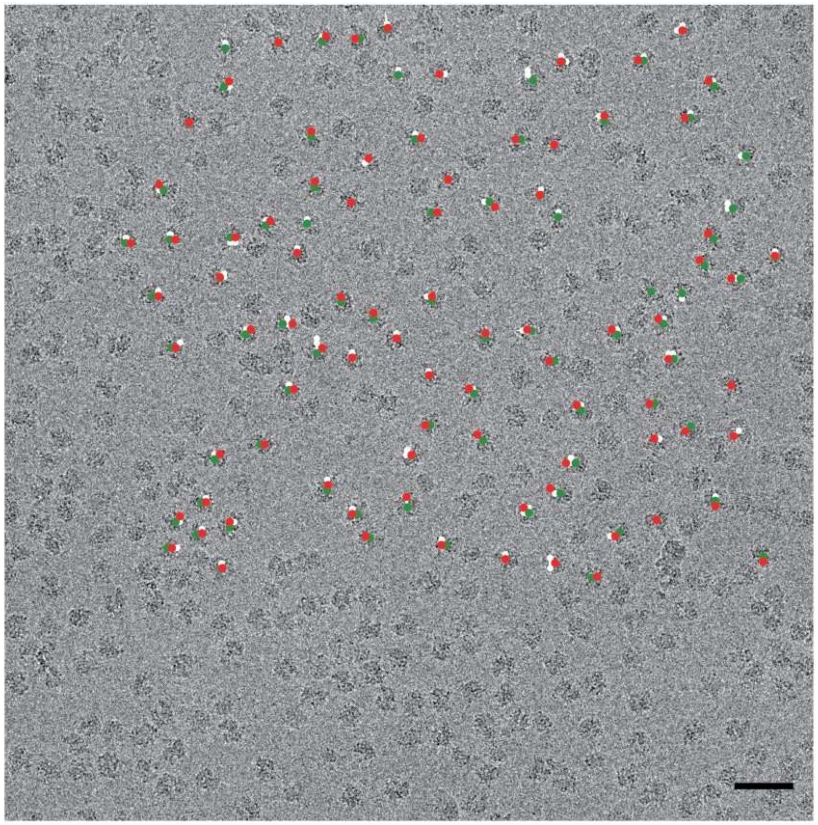
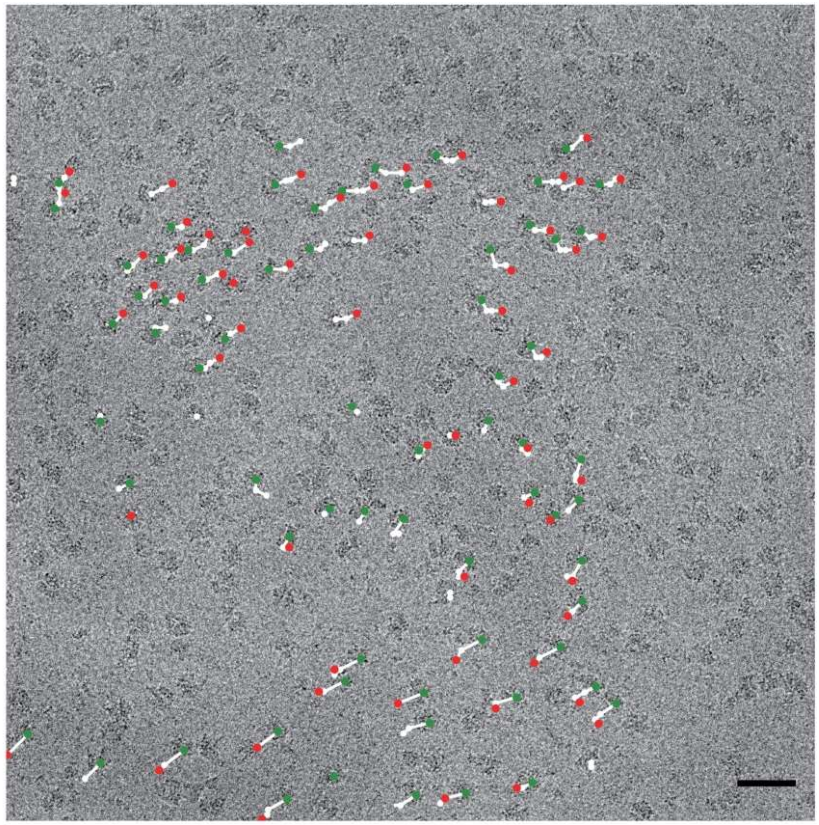
Motion Correction corrects for stage movement and charging effects (particle movement)

Direct electron detectors record movies, e.g the new K3 camera records 1,500 frames/sec (leading to data storage problems!)

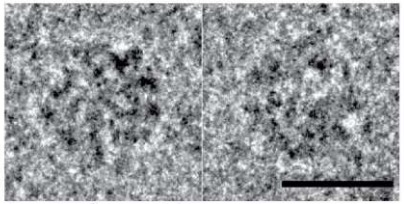




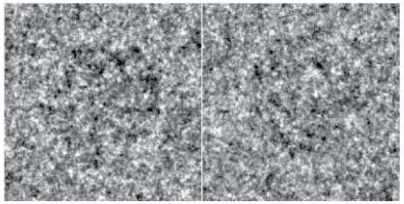
25-fold exaggerated particle movement

10 Å movements!

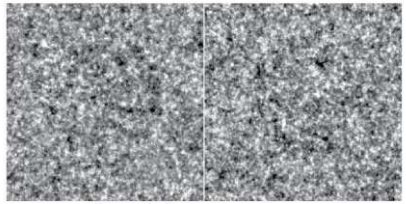
50 nm



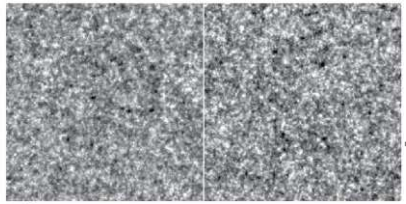
Average from 16 frames



Average from 4 frames



Average from 2 frames



1 frame

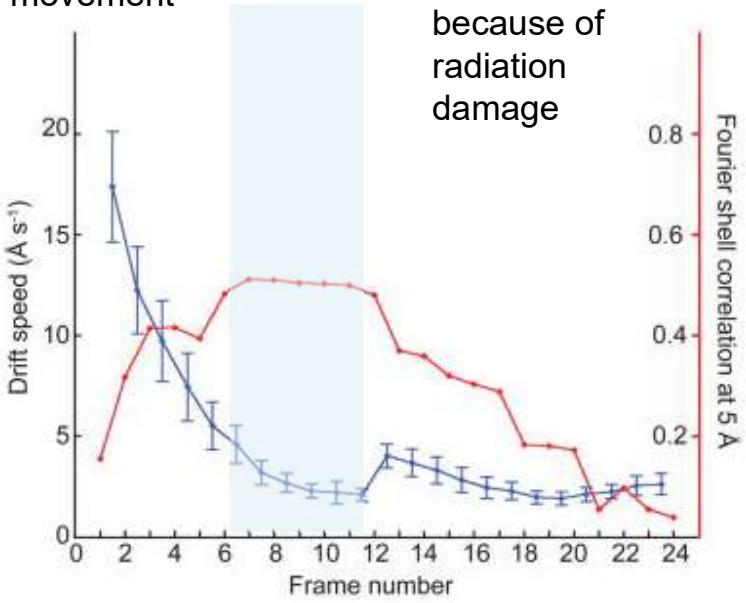
Bai et al., Elife 2013

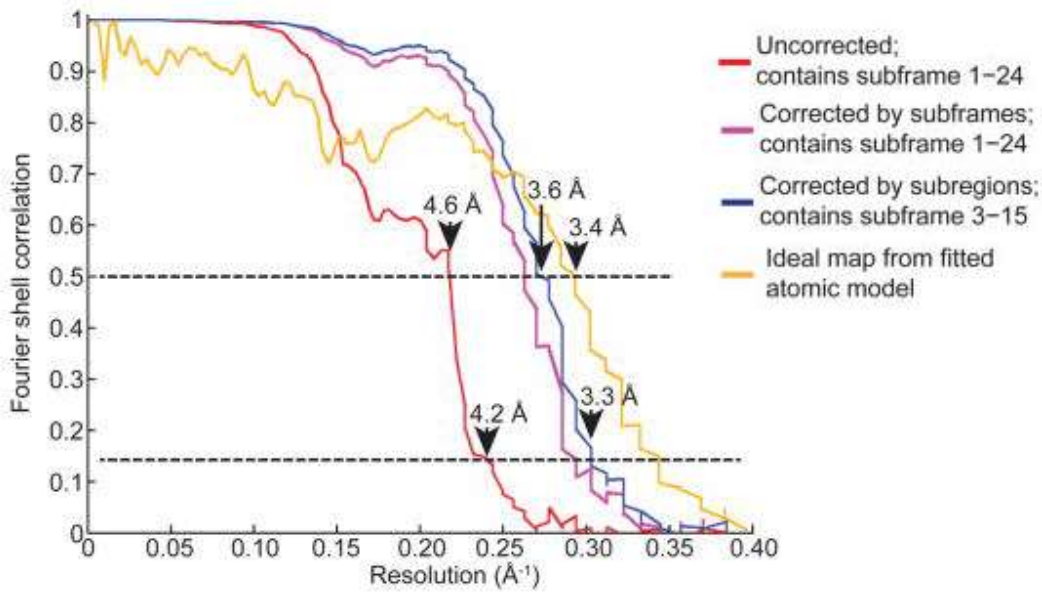
Motion Correction

charge effects, large particle movement

Best frames

Lower resolution because of radiation damage





Motion correction:

- correct for Stage/ Particle movement
- reject frames due to large motion and beam damage

Gain in resolution: 1-2 Å

Li et al., Nat. Methods 2013

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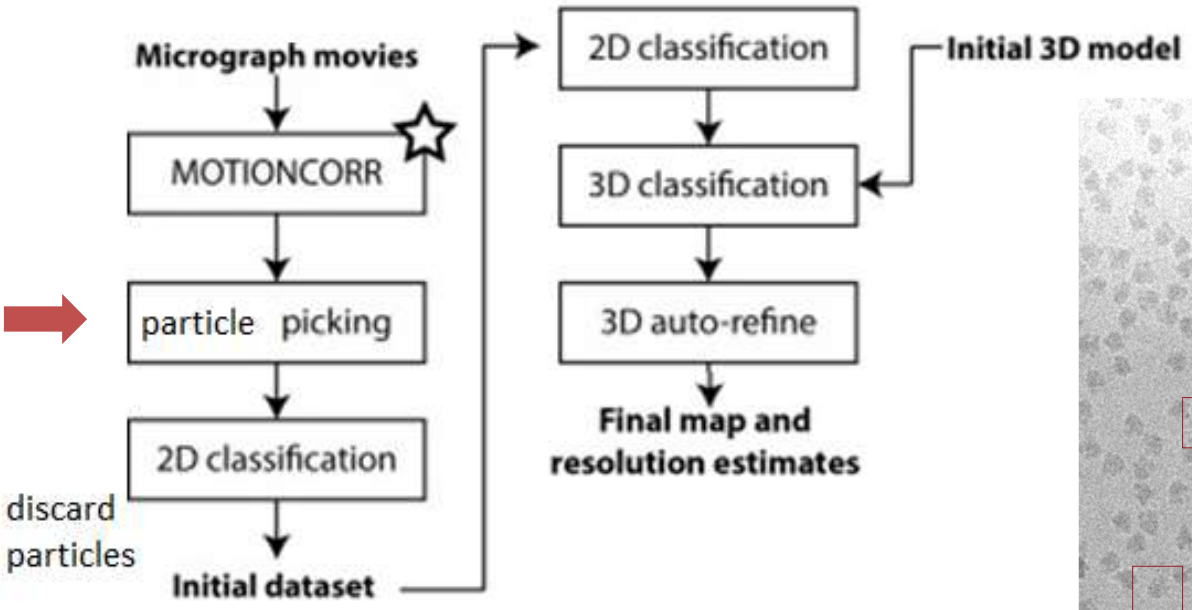
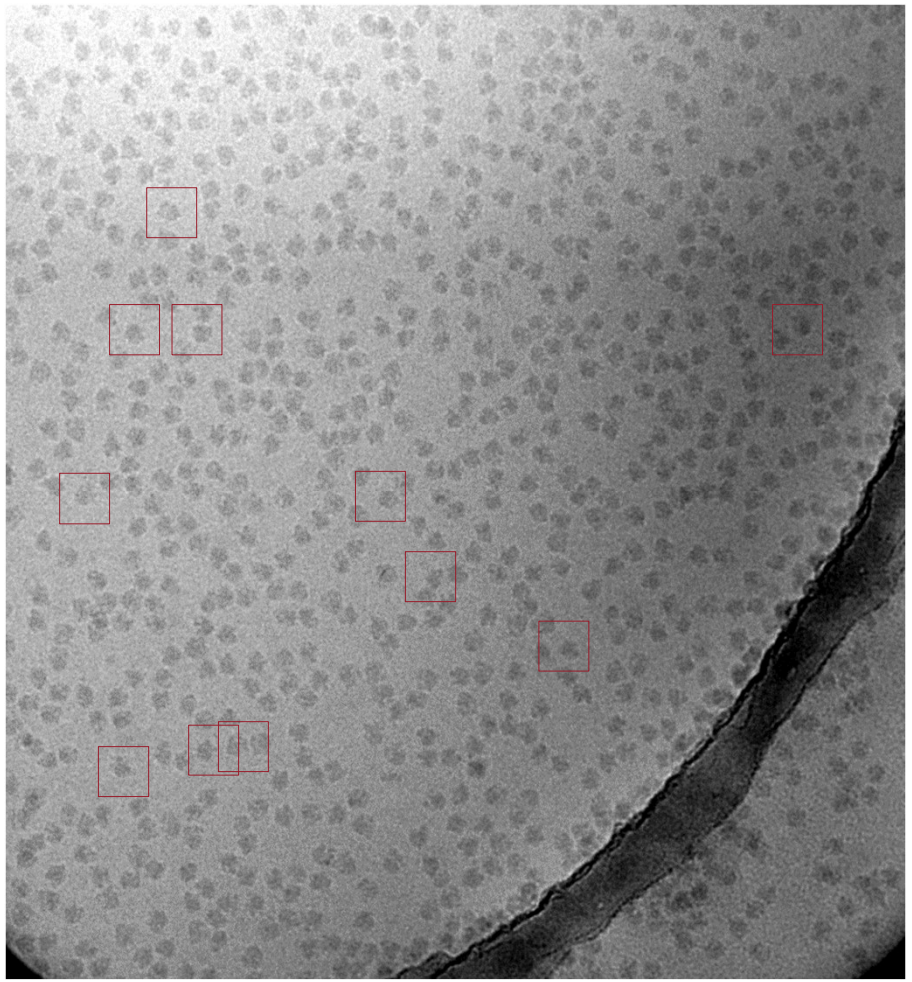
4 – Image Processing, the Principle

5 – State-of-the-art Image Processing

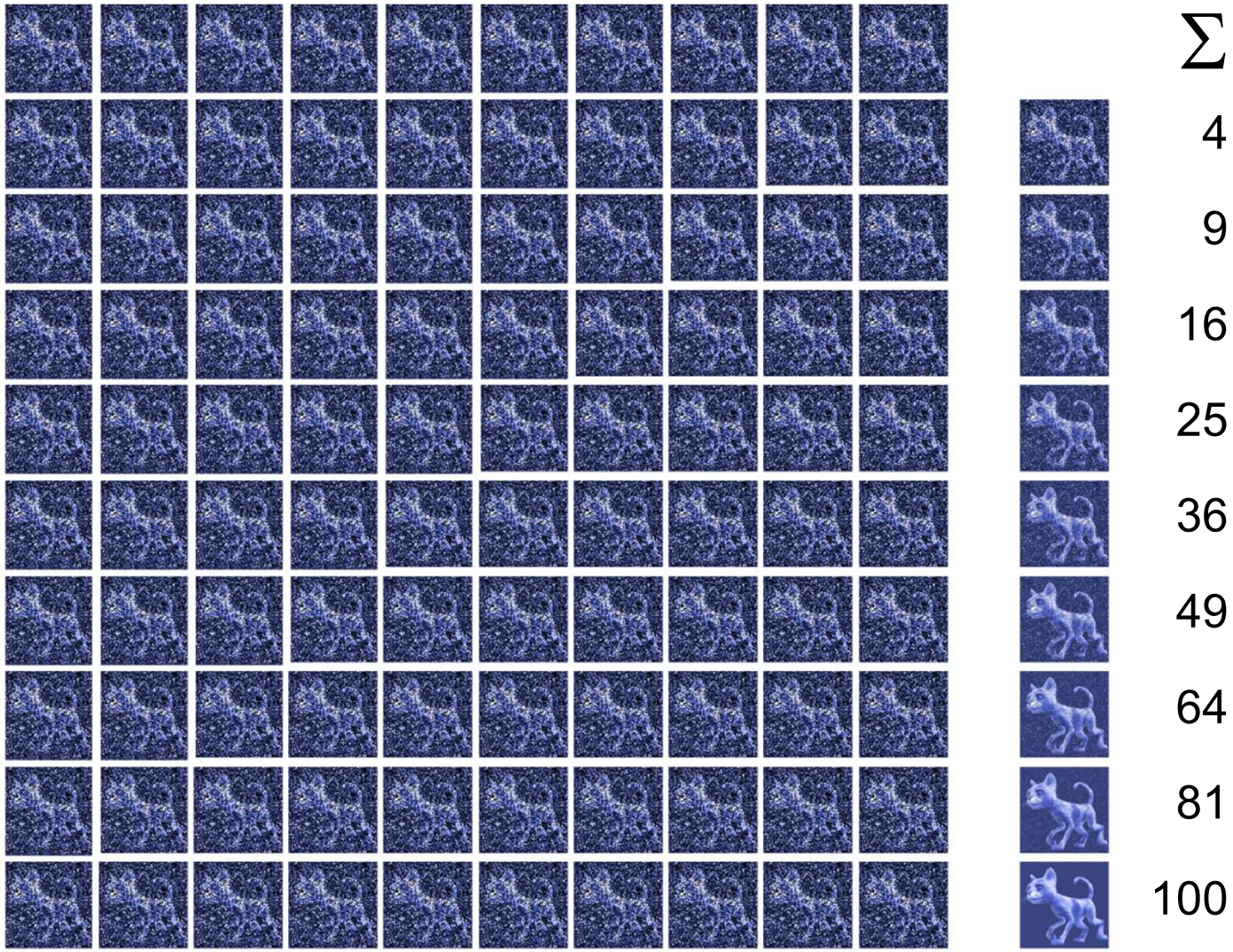
Image Processing Workflow





Particle picking:
selection of particle images from a micrograph, resulting in a large data set each having one particle per image.

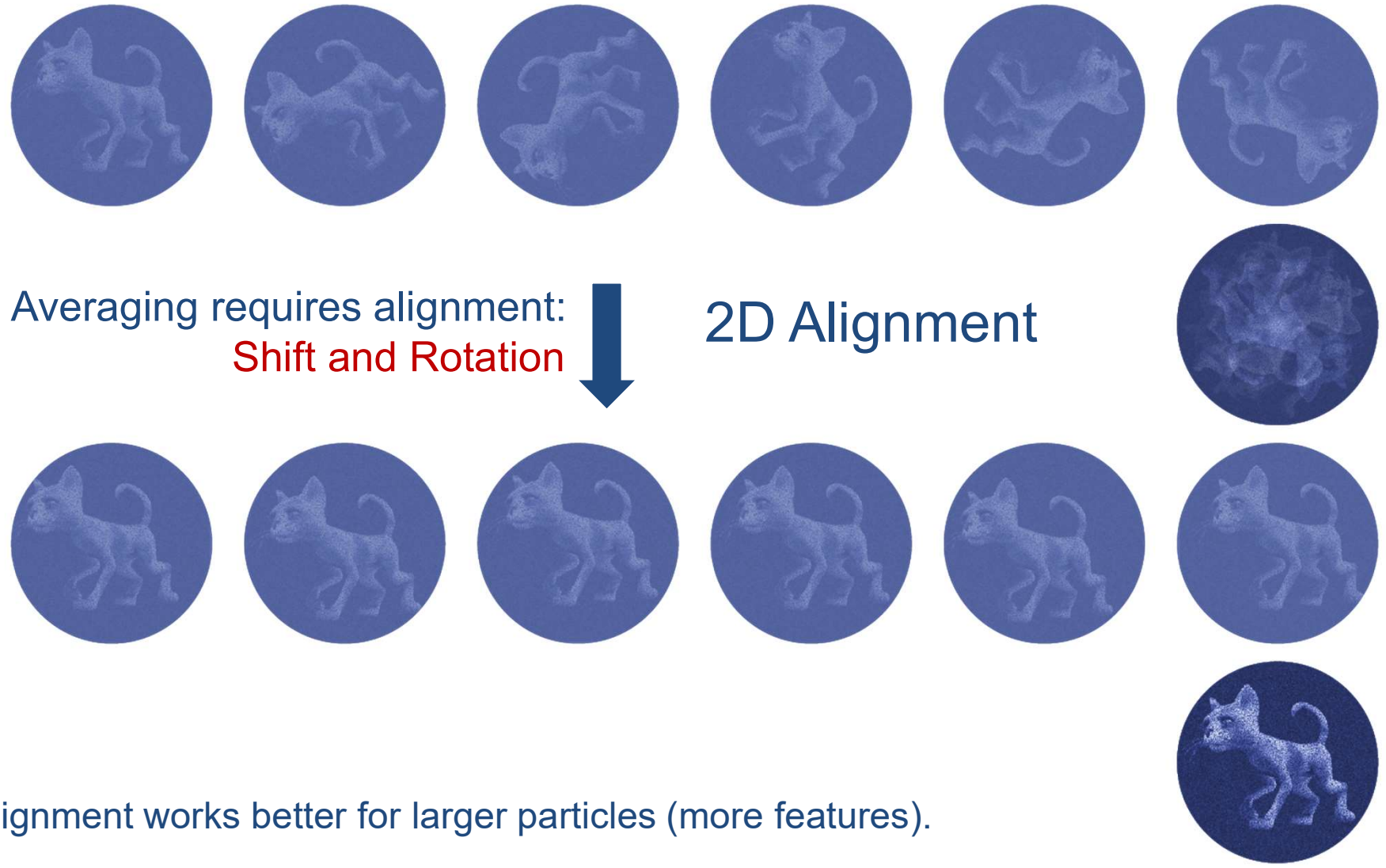
2D Image Processing: Averaging



1. We need to average over many images.
2. We need to obtain identical particles (requires computational sorting).

Signal-to-noise ratio growths with √n

Image Processing: Alignment



Alignment works better for larger particles (more features).

Single Particle EM – Alignment

Alignment - Averaging works only if:

1. All particles are identical.
2. All particles are seen from the same side.

…usually both is not the case.

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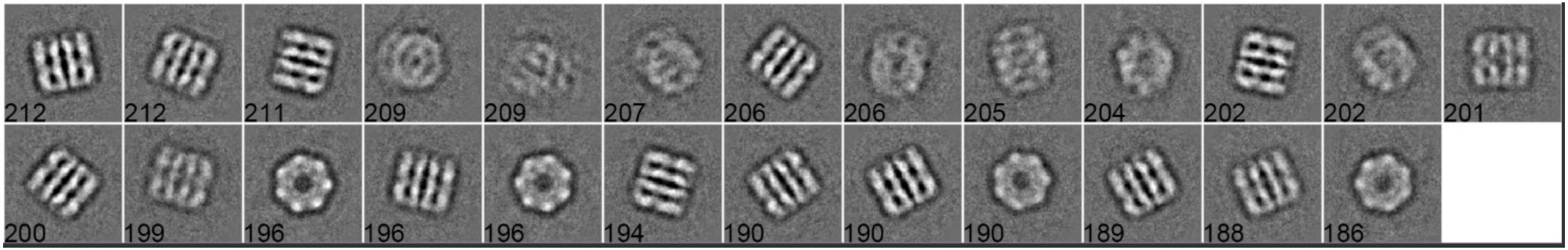
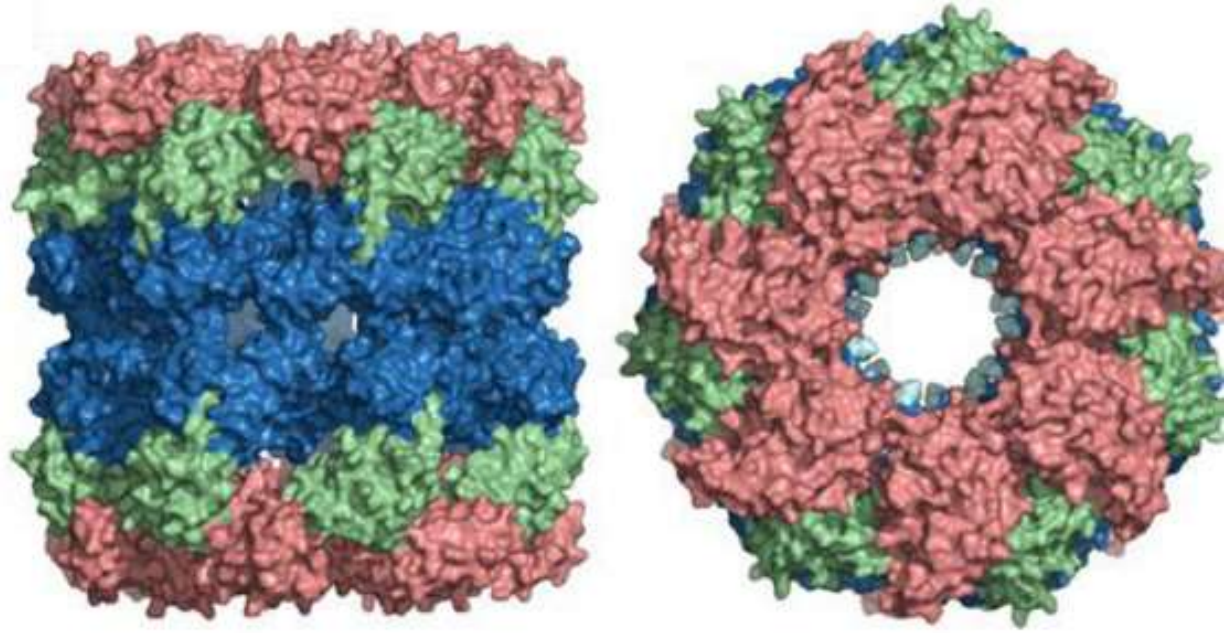
Alignment-Classification-Averaging

GroEL chaperone, 2D class averages



GroEL 3D structure from two views (top & side)

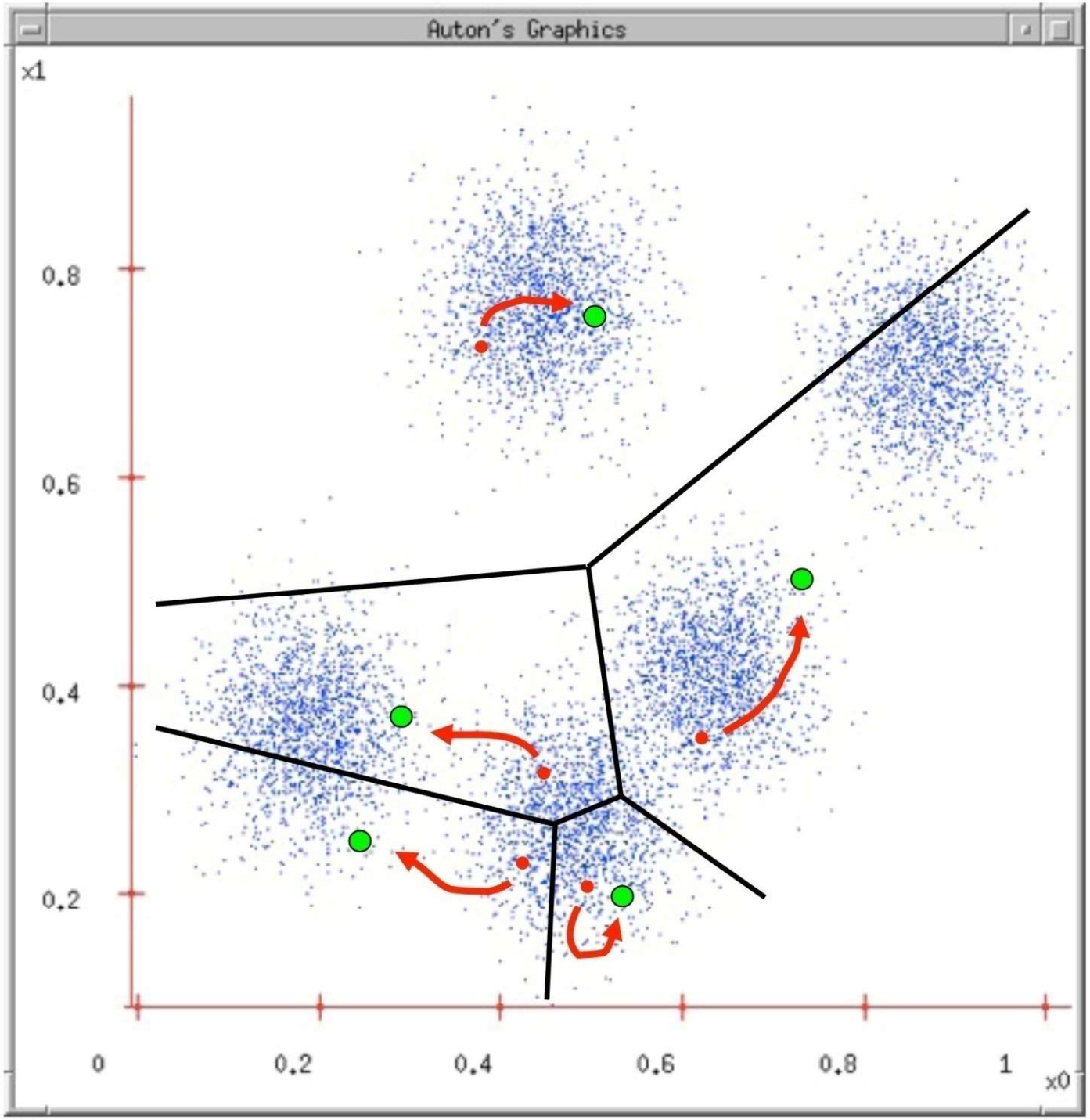


Classification:

- Each image is compared with all other images on a single pixel basis
- Distances between images are related to the similarity between the images
- Clustering identifies similar/close images and follows e.g. k-means algorithms.

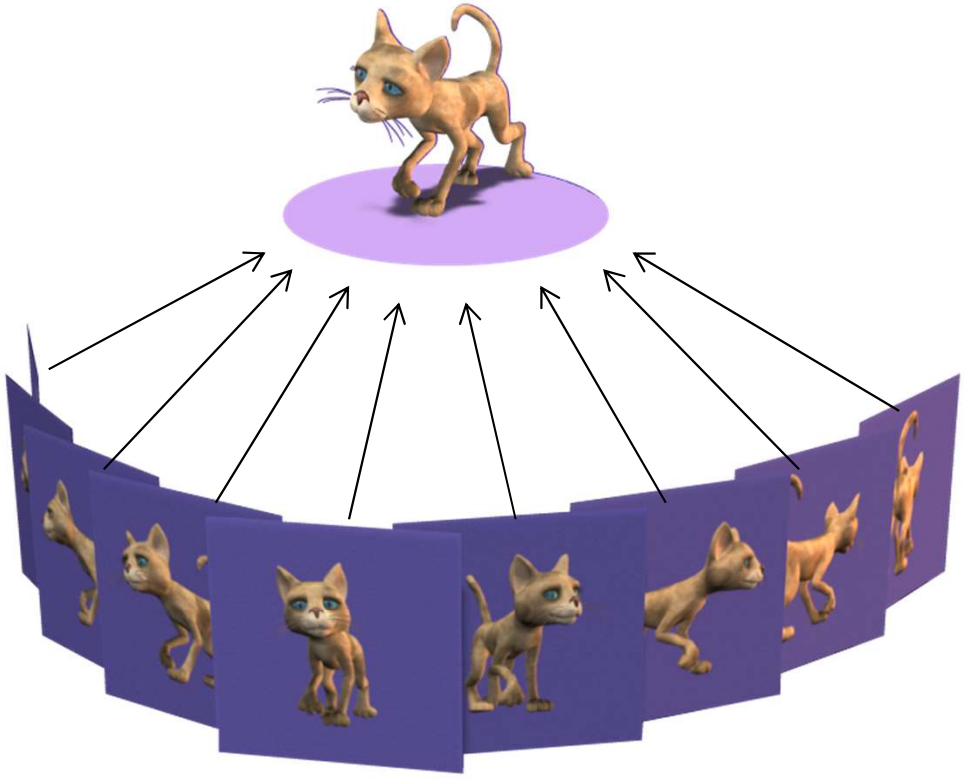
K-means

1. Ask user how many clusters they'd like. (e.g. K=5)
2. Randomly guess k cluster Center locations
3. Each datapoint finds out which Center it's closest to.
4. Each Center finds the centroid of the points it owns



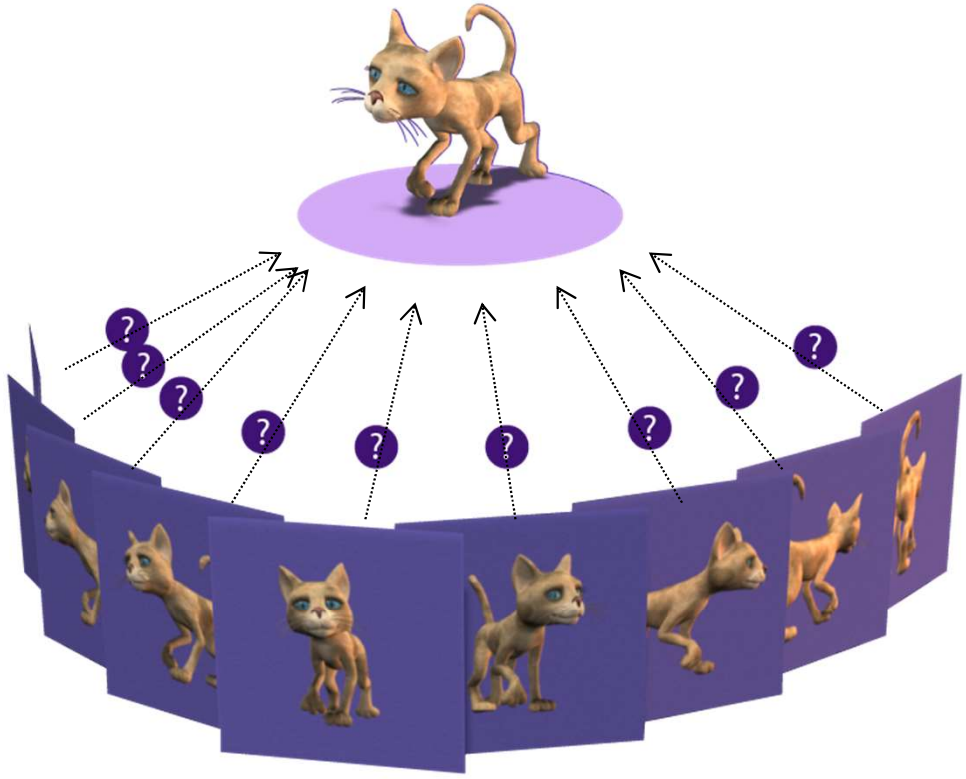
We need an 3D Initial Model for structure calculation – e.g. from Tomography or *ab initio* Calculations

Tomography



Split the electron dose.

Single Particle Analysis



Use the max. electron dose for one image.

Tomography: One object is turned in the electron beam (need to reduce electron dose)
→ many 2D images from one object with known orientation
3D Reconstructions from Tomography have low-resolution and missing information because a 90˚ rotation is not possible.

Many particles with unknown orientation
→ 3D Initial Model required

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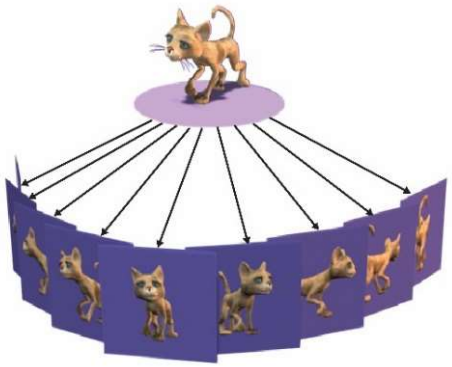
Workflow: 3D Reconstruction

3D Initial Model
e.g. from tomography



3D reconstruction
used as reference for the next round of projection matching

Project

Re-project

Projections
of the starting model
Euler Angles known

Back-project
Use the known angular relationships

Align
rotation, shift

Classify
based on cross-correlation

Average
Generation of 2D class averages

2D classes
Euler angles assigned

Data
Individual Picked Particles

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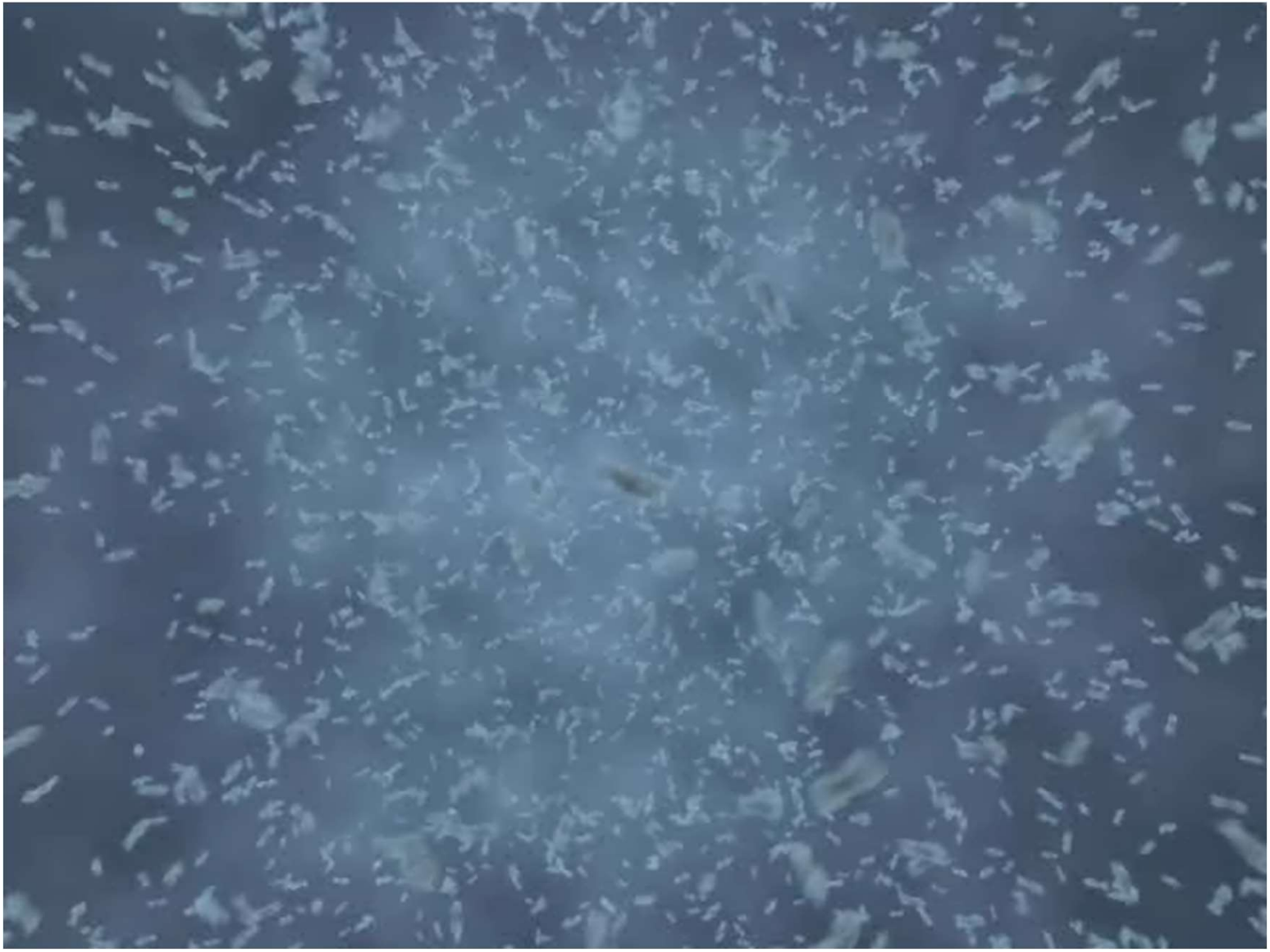
Electron Microscopy



Electron Microscopy: The BASICS

1 – The Microscope

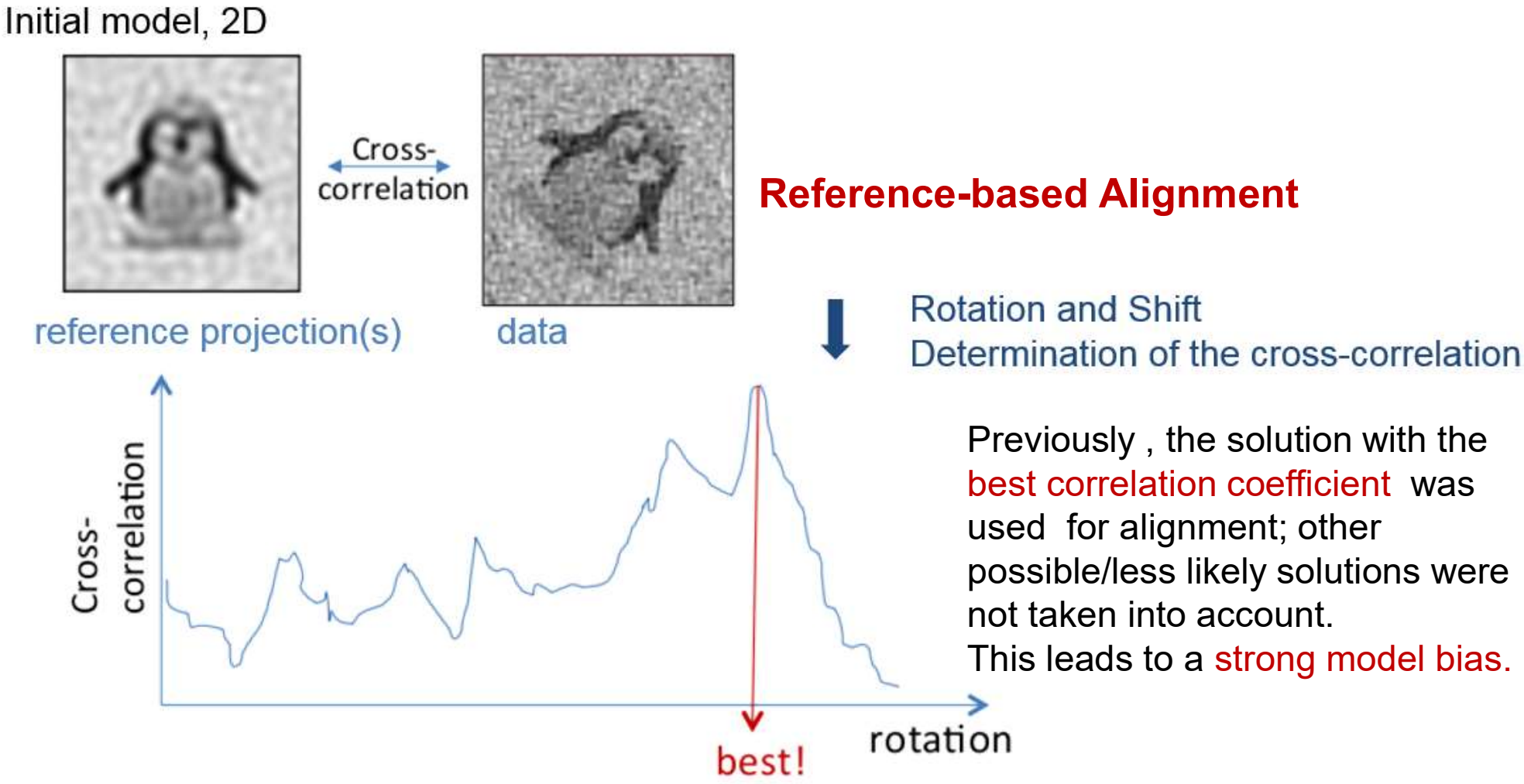
2 – Negative Stain EM and Sample Preparation

3 – The Image & Direct Electron Detectors

4 – Image Processing, the Principle

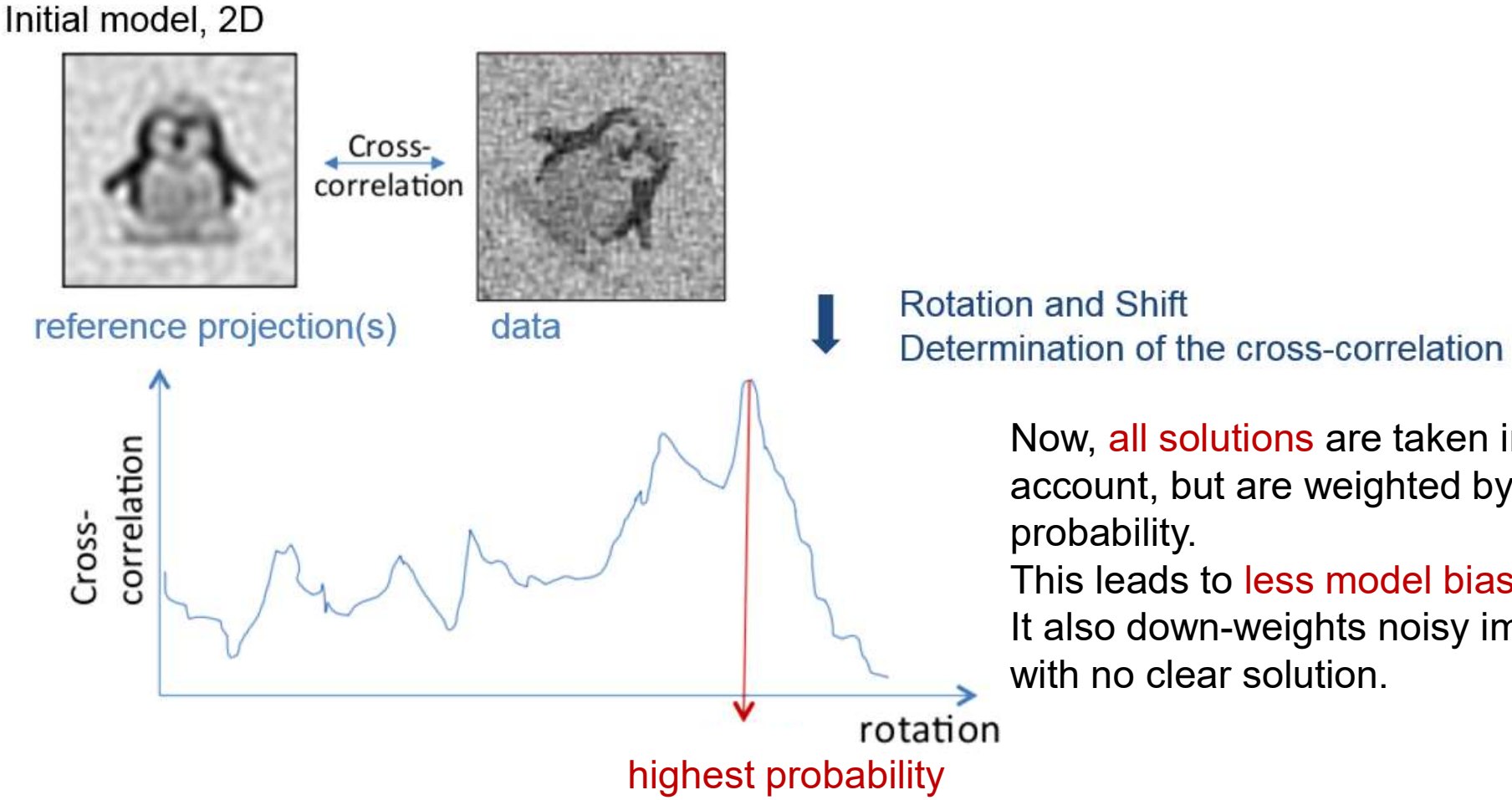
5 – State-of-the-art Image Processing

Increased Computational Power allows to use Maximum Likelihood Algorithms



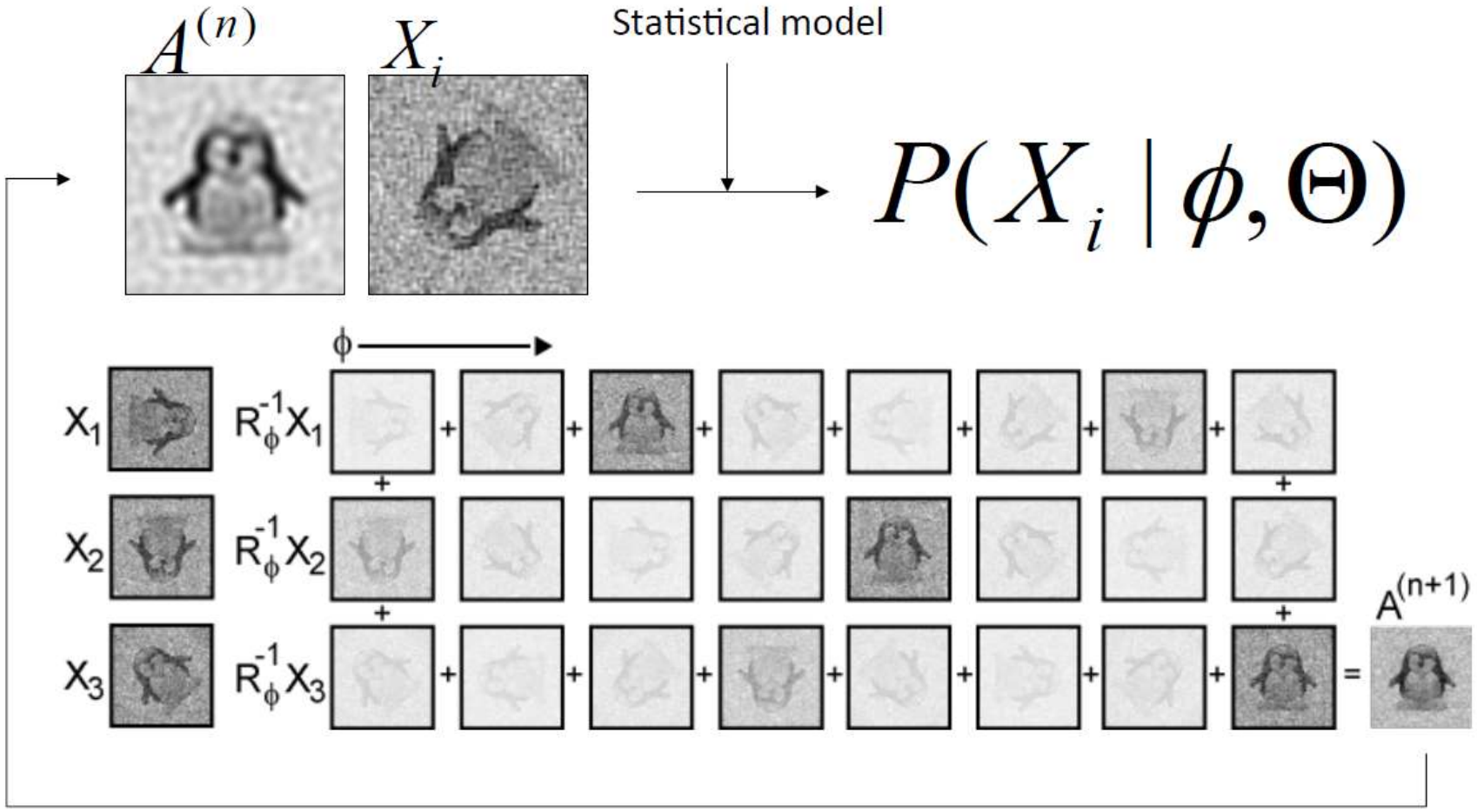
Maximum Likelihood Algorithms are computationally intensive:

… because you go not assign discrete orientations to the data and make hard decisions if the noise in the data does not allow it.



Now, all solutions are taken into account, but are weighted by their probability.
This leads to less model bias.
It also down-weights noisy images with no clear solution.

Maximum Likelihood Algorithms are computationally intensive:



Many iterations required until convergence

Scheres et al., Nat. Protocols 2008

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Workflow: 3D Reconstruction,
Dealing with Heterogeneity

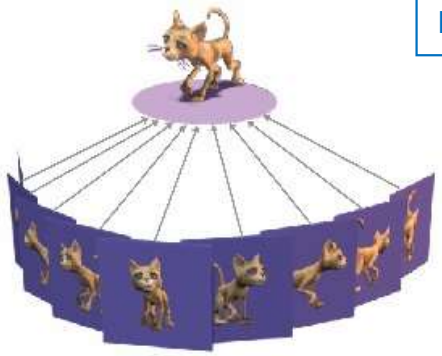
3D Initial Models

Project

Projections
of the starting model
Euler Angles known

Re-project

3D reconstructions
used as references for the next round of projection matching



Back-project
Use the known angular relationships

Data
Individual Picked Particles

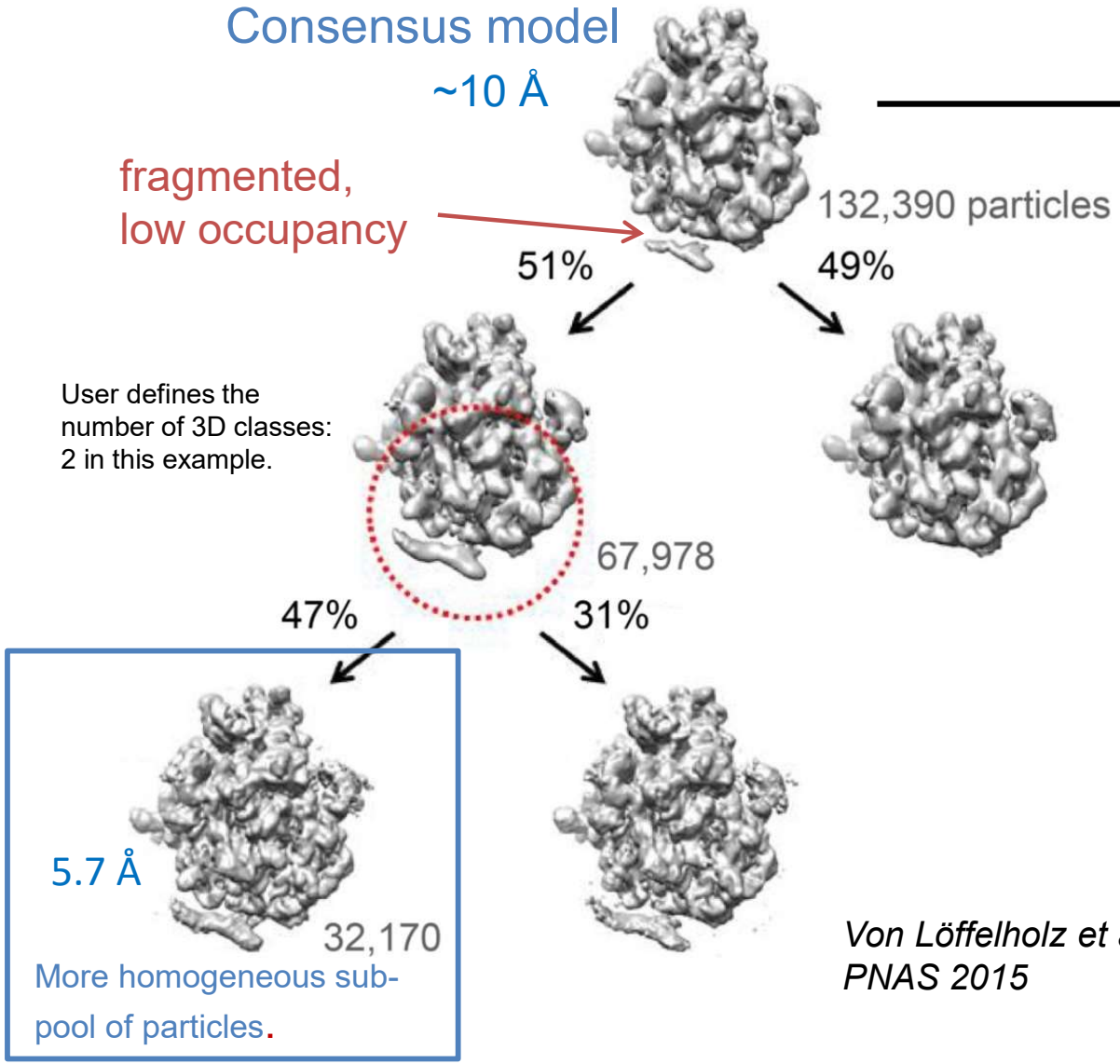
Align
rotation, shift

Classify
based on cross-correlation

Average
Generation of 2D class averages

2D classes
Euler angles assigned

Dealing with Heterogeneity: 3D Classification yields multiple structures

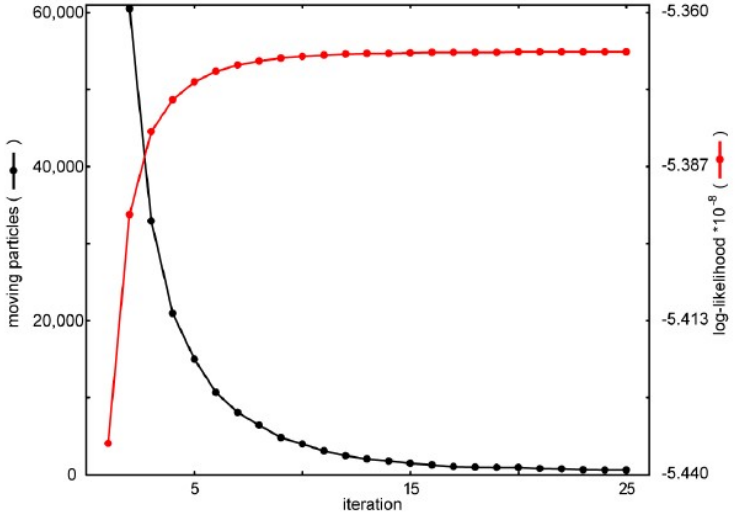


Von Löffelholz et al., PNAS 2015

Filter single consensus model to low resolution (e.g. 80 Å).

User decides on number of 3D classes and programme assigns a random class to each particle in the 1st iteration.

Run many iterations until the maps are stable.



Scheres et al., Nat. Methods 2007

This allows classification without prior knowledge of the differences between the structures present in the data.

3D Refinement, Angular Refinement

3D Initial Model
e.g. from random conical tilt reconstruction

Project

More Projections
More detailed differences
Euler Angles known

Re-project

Use finer angles for the projections:
10˚, 5˚, 4˚,3˚,2˚,1˚…

3D reconstruction
used as reference for the next round of projection matching

Back-project
Use the known angular relationships

Angular Refinement

Data
Individual Picked Particles

Align
rotation, shift

Classify
based on cross-correlation

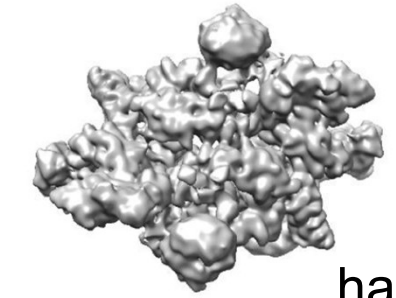
Average
Generation of 2D class averages

2D classes
Euler angles assigned

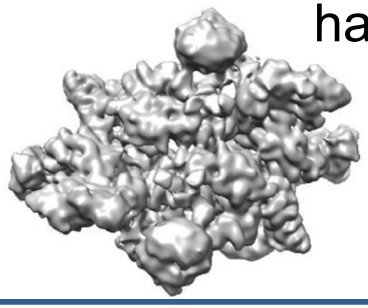
Assessing the Quality of the Structure: Determine the Resolution

Gold standard refinement:

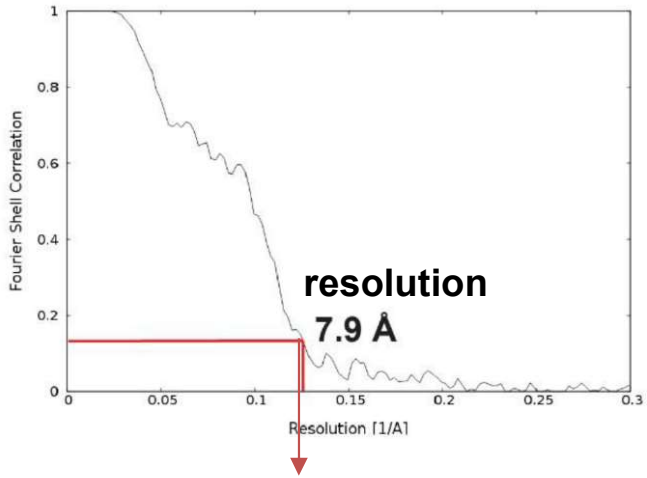
- Split your final particle pool randomly into two.
- Start from the low resolution model and independently refine the two structures.
- Determine (in Fourier space) the correlation at different resolution ranges of the two independent structures = Fourier shell correlation curve.
- A Fourier Shell Correlation Criterion of 0.143 is used to determine the resolution.



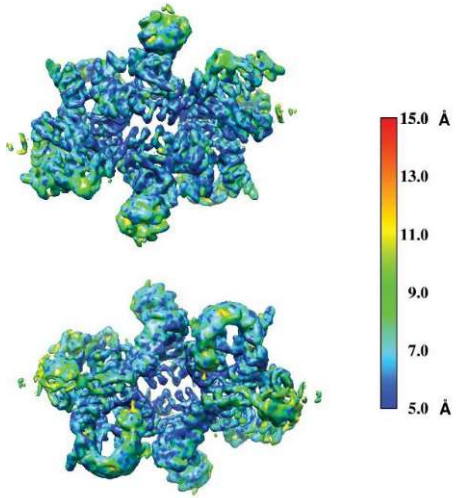
8,085 particles each



half1
half2

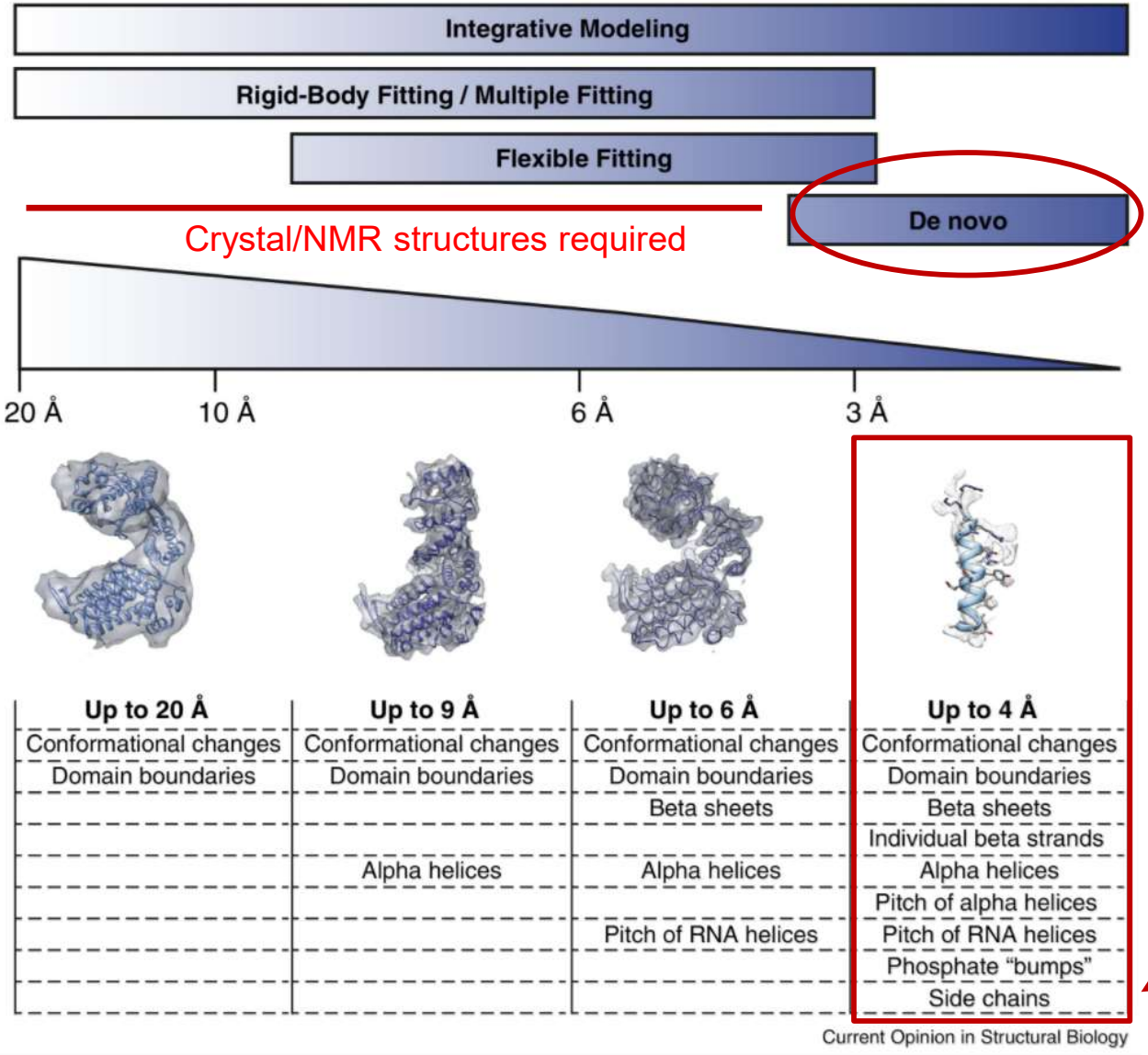


It is important to consider the local resolution: not all parts of a structure are equally well resolved.



Karuppasamy et al., Nature Communications 2017

Interpretation of EM Structures



Ideal case, no further information required.

Model can be built *de novo*

Villa & Lasker , CoSB 2016

Atomic Resolution Electron Microscopy

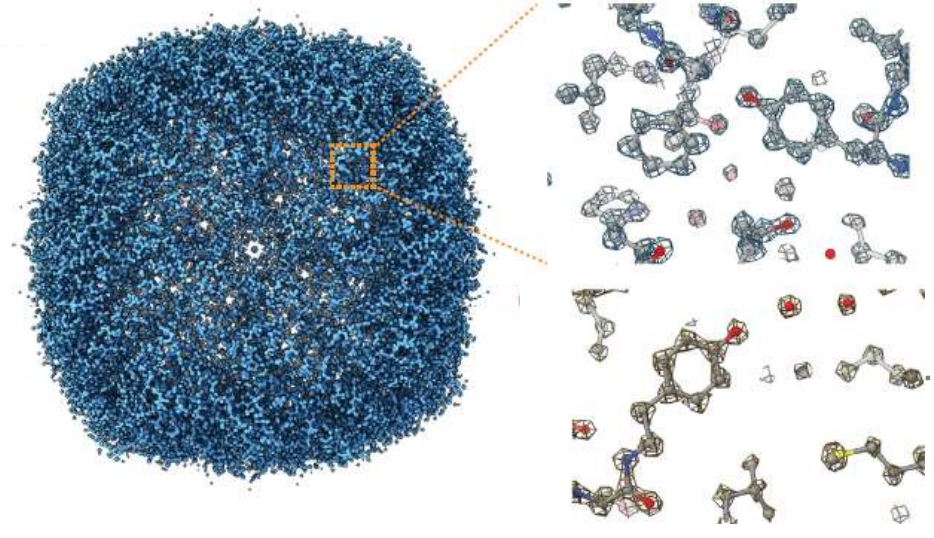
LMB Cambridge team (Nakane et al, *Nature* 2020):

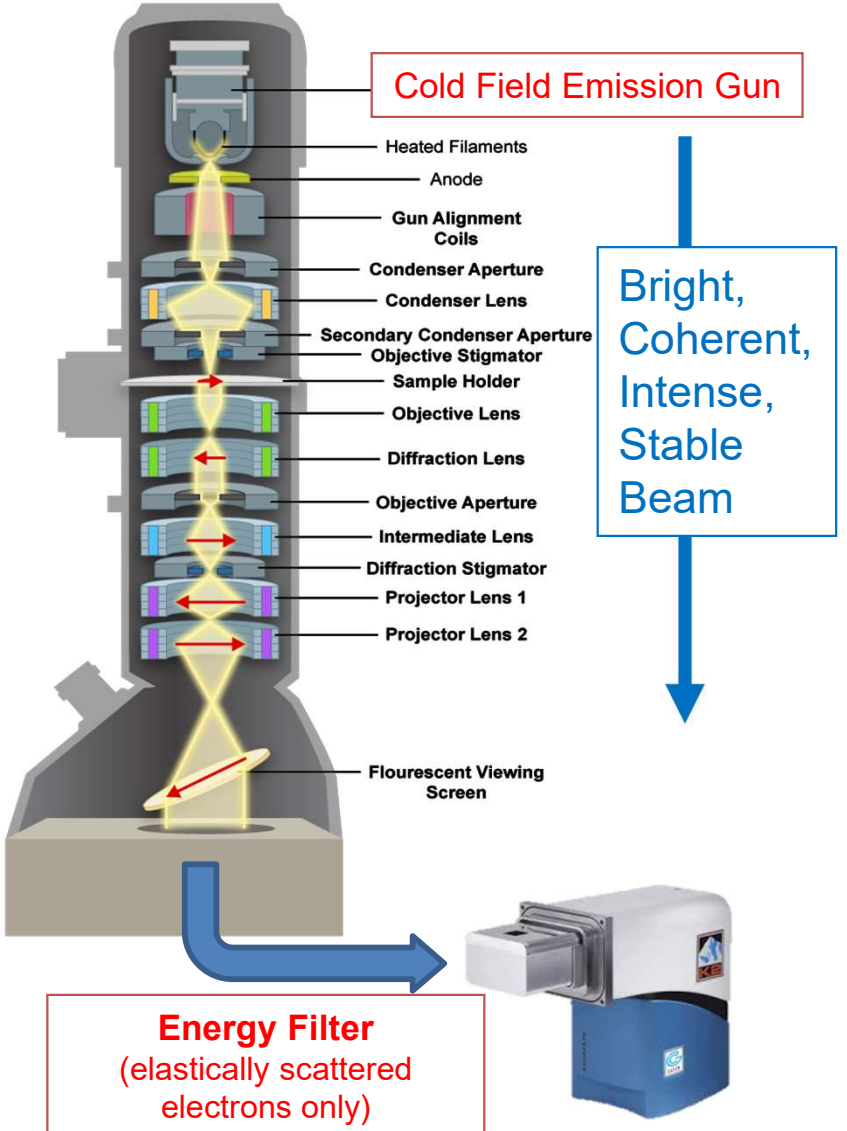
- better electron source (beam with narrow
- energy spread)
- more stable energy filter (removes inelastically scattered electrons more efficiently)
- new generation of DED (faster, more sensitive)
- optical aberration corrections during image processing

MPI team (Yip et al, *Nature* 2020):

- Home-build microscope with advanced electron-optical devices: monochromator and spherical aberration corrector

-> more coherent electron beam and reduce optical aberrations.





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Summary

Advantages:

1. The sample is studied in solution, at near-native conditions
2. Small amount of sample required
3. No crystallisation necessary
4. It is possible to study large macromolecular complexes and membrane proteins, i.e. samples which are difficult to crystallise
5. Computational sorting allows insights into the dynamics of macromolecular machines: more than one structure can be solved from one sample, revealing different functional states.
6. One or several structures can be solved from samples which are not completely 'pure', provided the complex of interest can be identified and computationally purified from the contaminations.

Disadvantages, Limitations and Problems:

1. Low intrinsic contrast of the sample
2. Radiation damage > low signal-to-noise ratio in the images
3. One image per particle – very large data sets required to amplify the signal
4. Determining and dealing with heterogeneity (dynamics, partial complexes, contaminations) is computationally intensive and often limits the resolution of the structure. The basic assumption that the 'sample is homogenous' is usually not correct due to the heterogeneity.
5. Anisotropy resulting from missing views are a common problem, often due to interaction of particles with the air-water interface.

Cryo-EM in Bristol

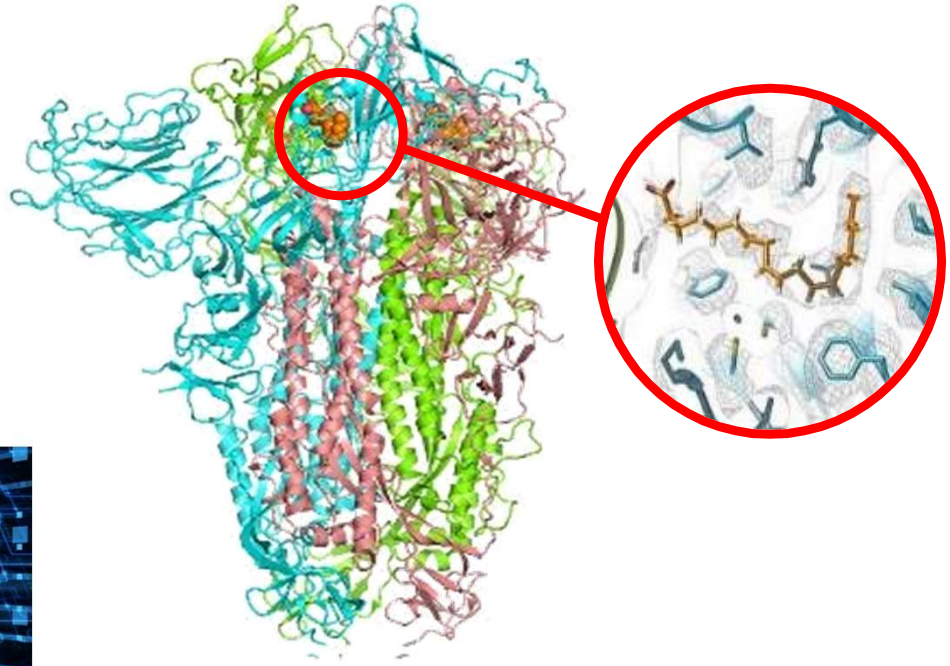
Talos Arctica (200kV)

wellcome trust

BBSRC bioscience for the future

ORACLE

[A Free Fatty Acid Binding Pocket in the SARS-CoV-2 Spike protein]



Toelzer et al., Science 2020;
Gupta et al., Nat. Comm. 2022;
Buchanan et al., Science 2022;
Toelzer et al., BioRxiv 2022

Christiane.berger-schaffitzel@bristol.ac.uk

BrisSynBio
biomolecules to biosystems
from understanding to design

MAX-PLANCK-GESELLSCHAFT

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Learn more about Cryo-EM

Getting started in Cryo-EM , Greg Jensen, Caltech:

https://www.youtube.com/watch?v=gDgFbAqdM_c

LMB Electron Cryo-Microscopy Course:

https://www.youtube.com/playlist?list=PLQbPquAyEw4etKtxyqcvZz4uELPeLDLeF