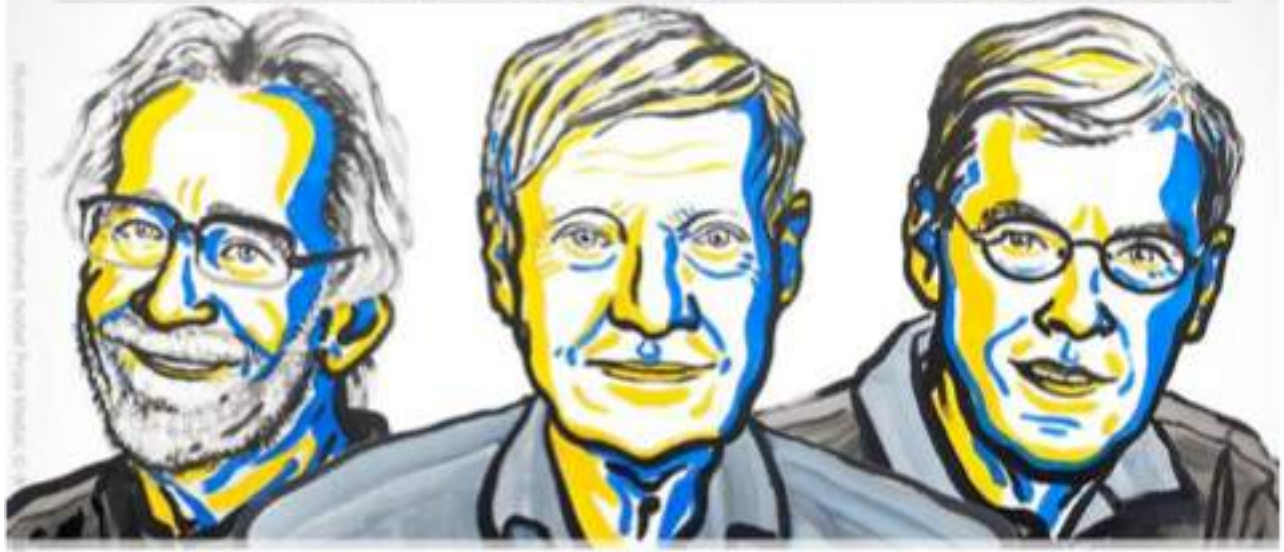
Introduction to single particle electron microscopy

Hélène Malet
Electron microscopy group, IBS

The Royal Swedish Academy of Sciences has decided to award the

2017 NOBEL PRIZE IN CHEMISTRY



Jacques Dubochet
Joachim Frank
Richard Henderson

"for developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution"

Science
AAAS

BIOCHEMISTRY

The Resolution Revolution

Werner Kühlbrandt

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

Review

CellPress

How cryo-EM is revolutionizing structural biology

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

MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge Biomedical Campus, Cambridge

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NATURE | NEWS FEATURE

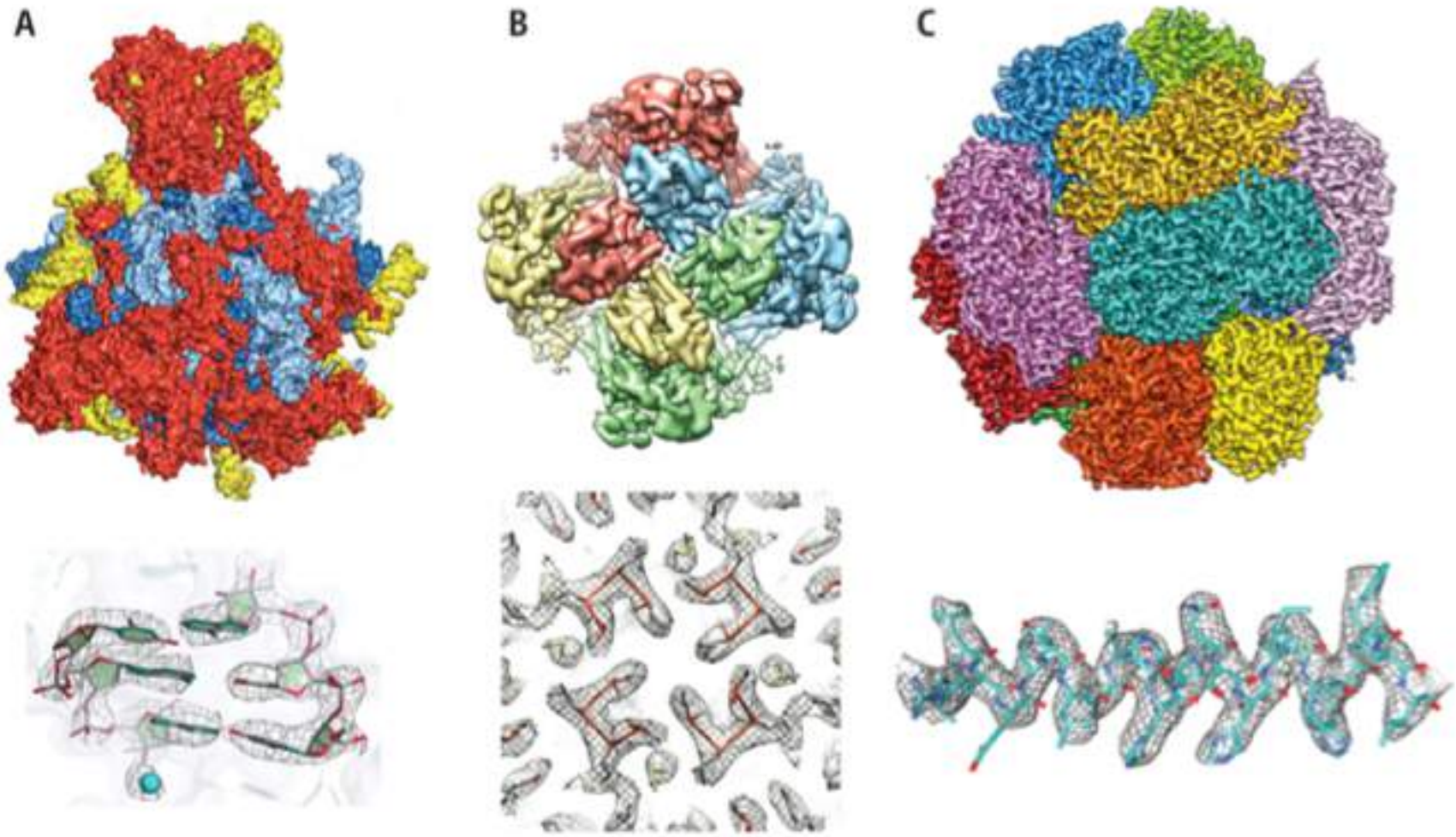
The revolution will not be crystallized: a new method sweeps through structural biology

Move over X-ray crystallography. Cryo-electron microscopy is kicking up a storm by revealing the hidden machinery of the cell.

Method of the Year 2015

The end of 'blob-ology': single-particle cryo-electron microscopy (cryo-EM) is now being used to solve macromolecular structures at high resolution.

The resolution revolution

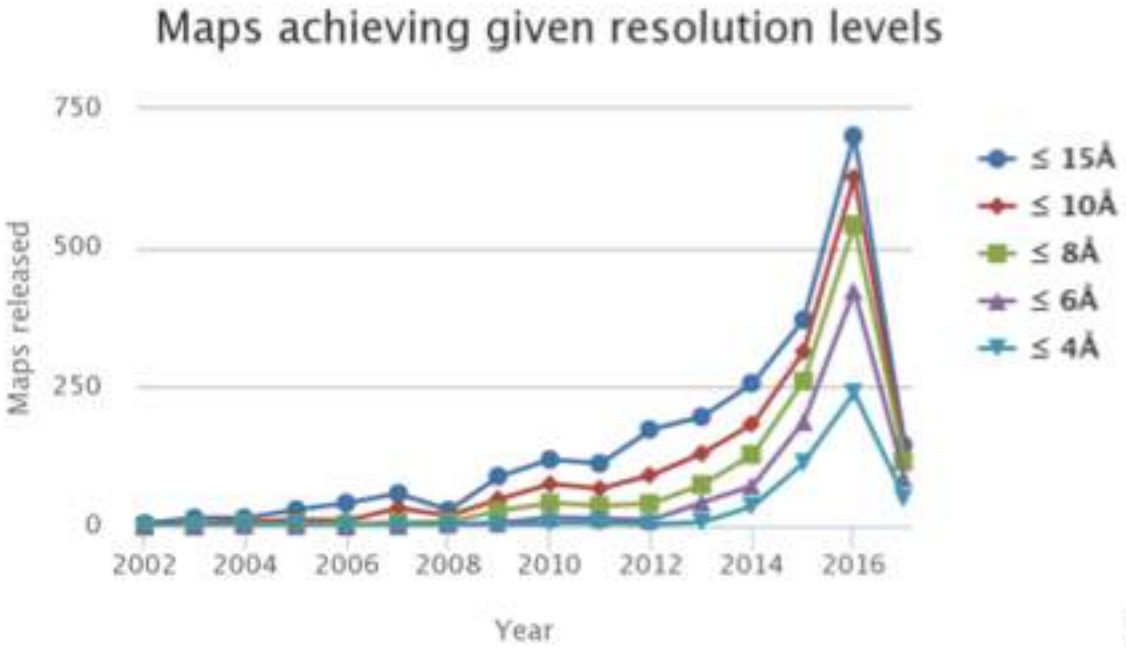


Mitoribosome
Amunts et al (2014)

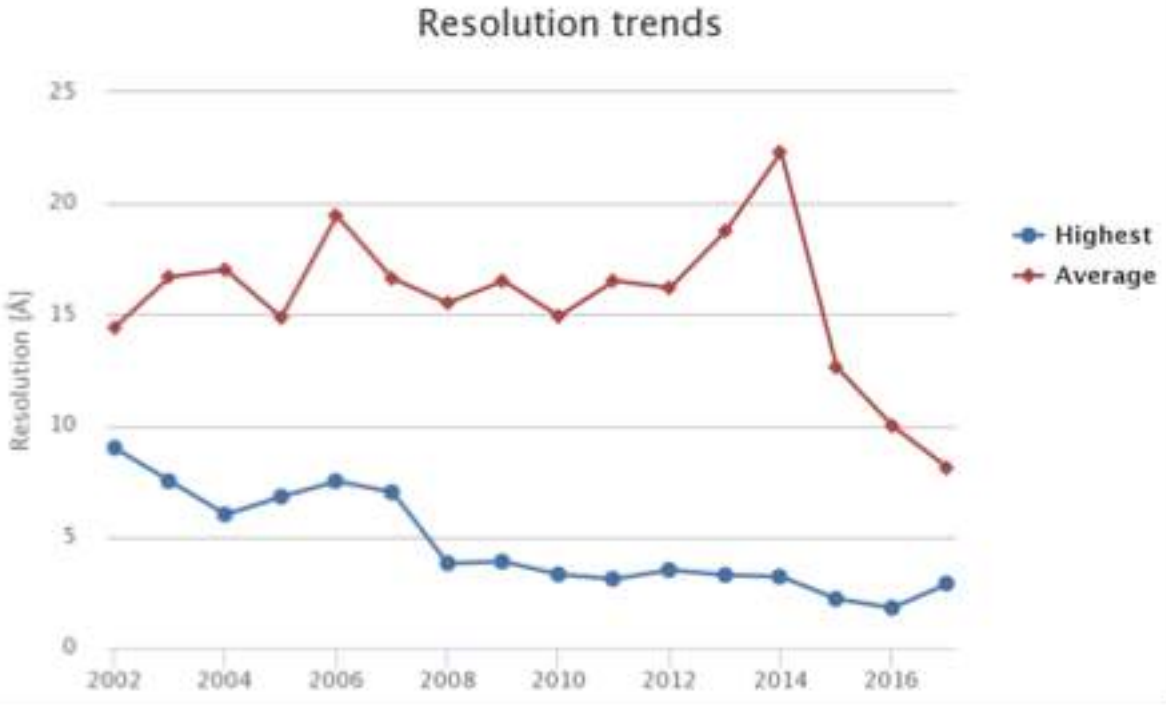
TRPV1 ion channel
Liao et al (2013)

NiFe hydrogenase
Allegretti et al (2014)

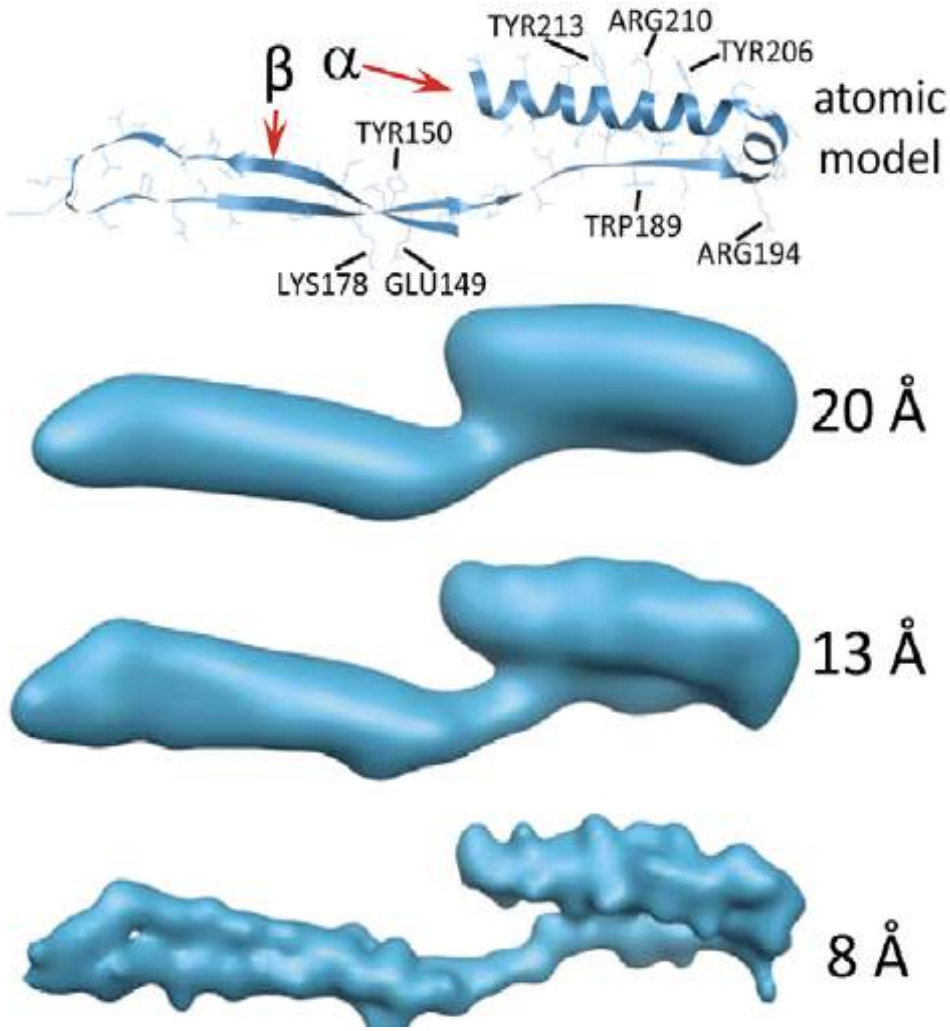
The resolution revolution

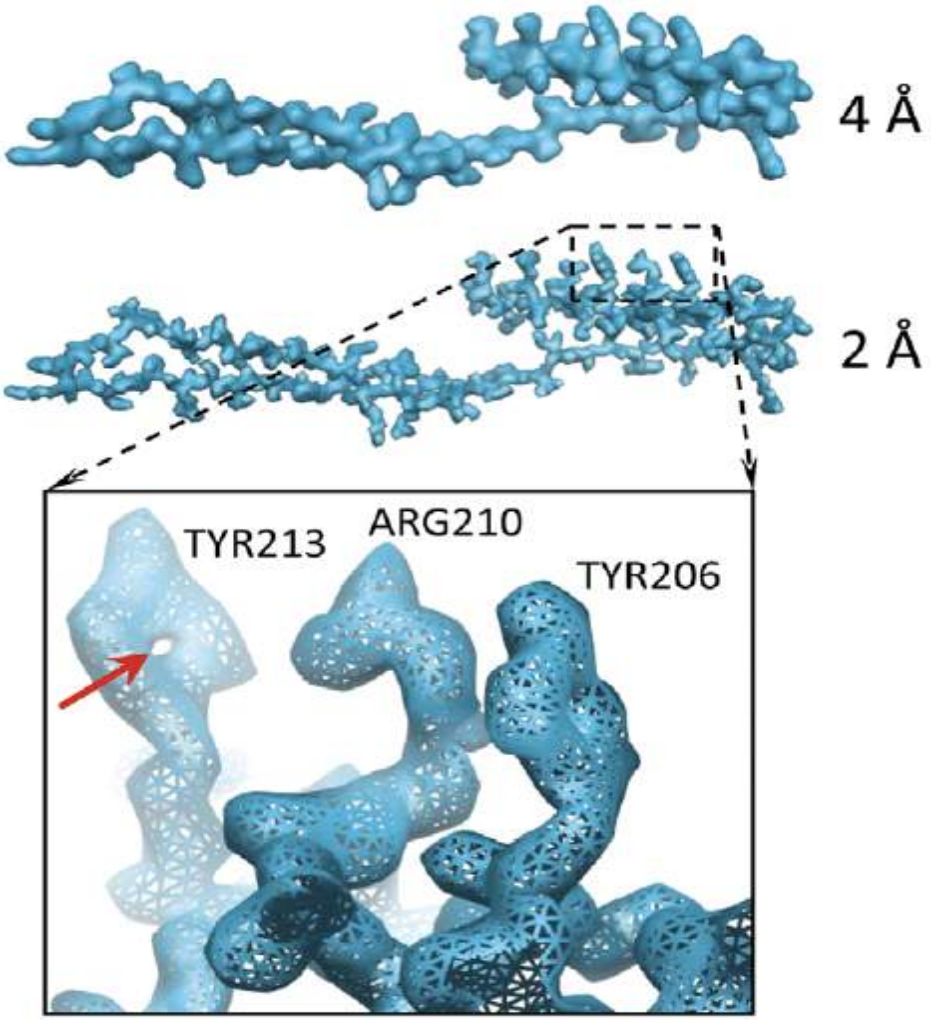


EM Databank Statistics
(March 2017)



Resolution: what do we see ?





Lecture outline

Introduction

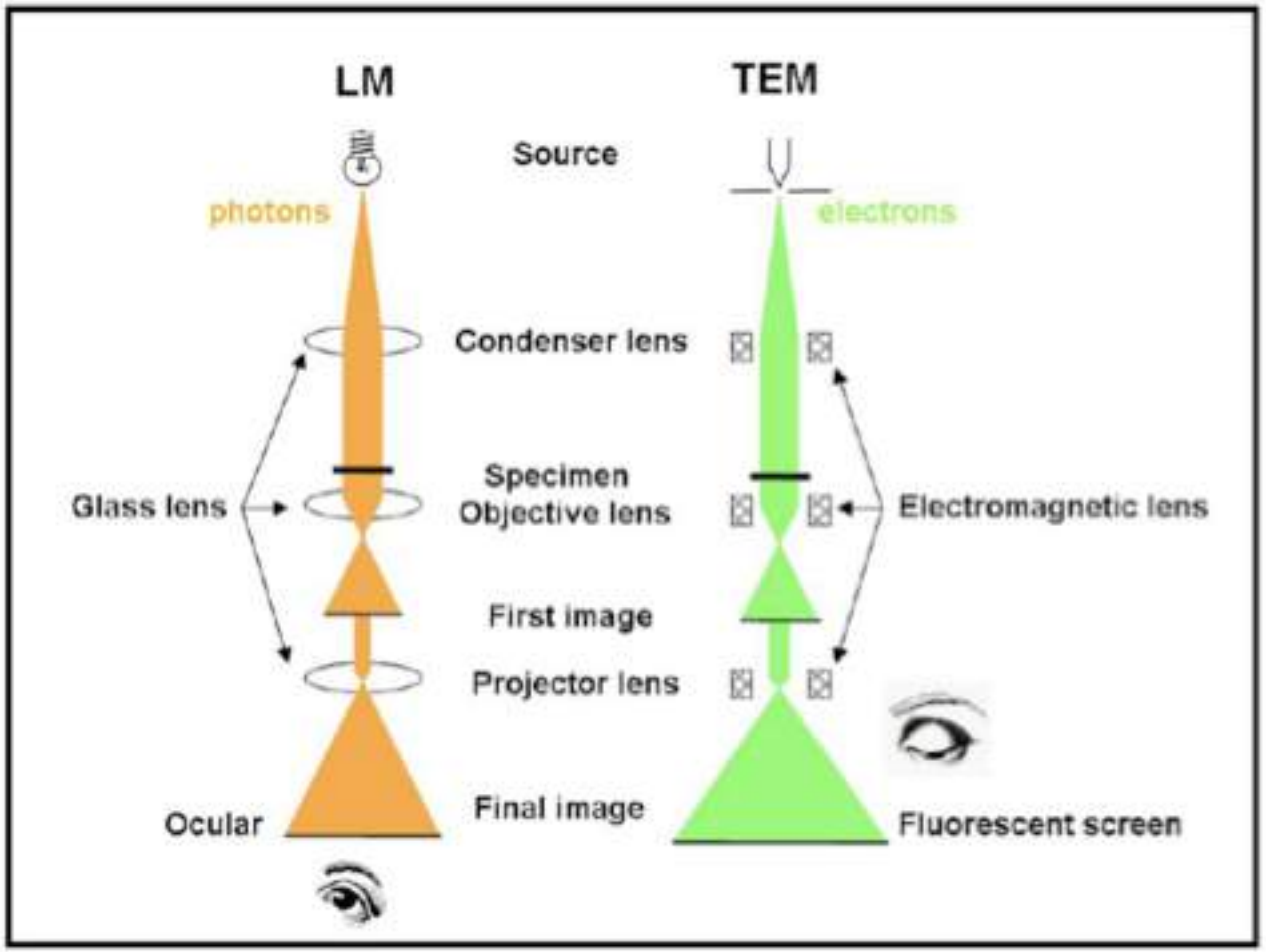
The electron microscope

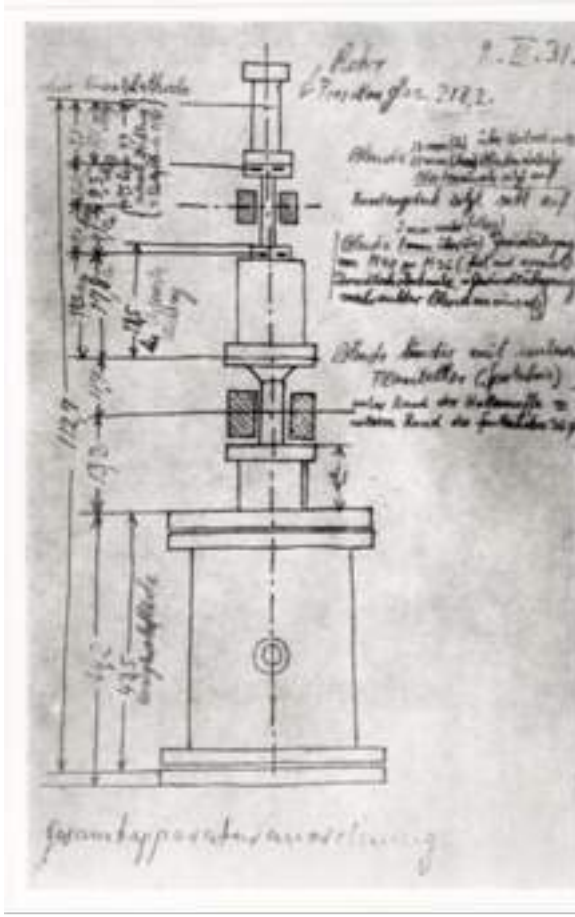
Sample preparation for EM

Data collection – Direct detectors

Image processing & applications

Light microscope *vs* Electron microsope





First EM: Ernst **Ruska**, Berlin, 1931 (Nobel in 1986)

Electron gun

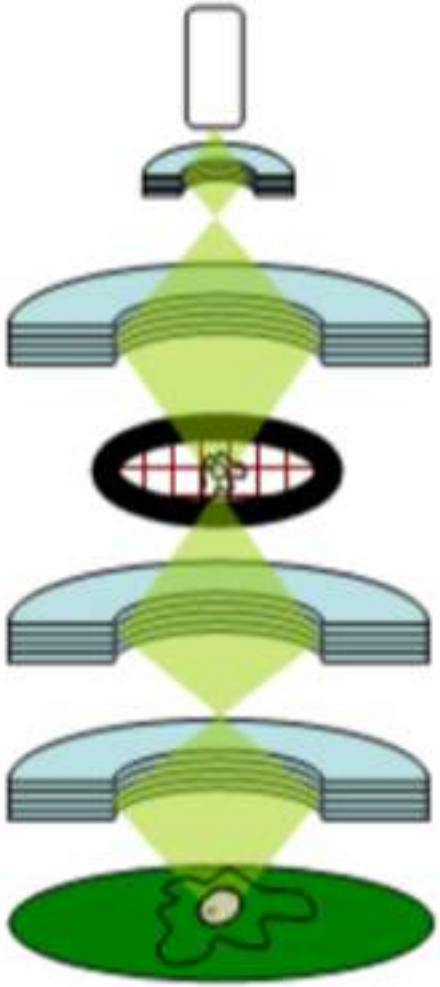
Condenser lens

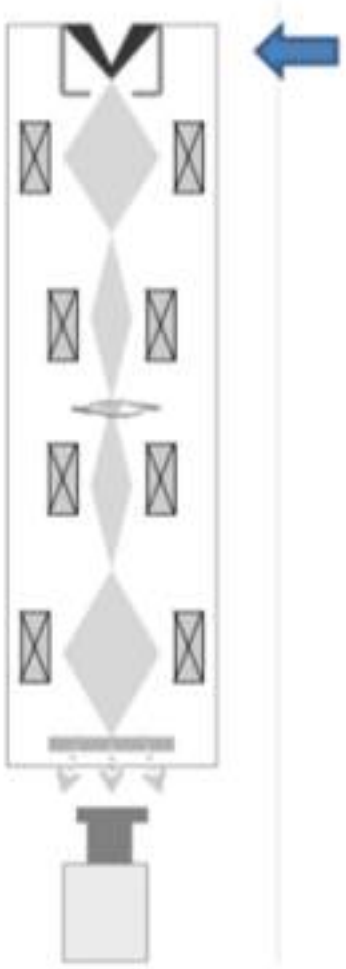
Sample grid

Objective lens

Projection lens

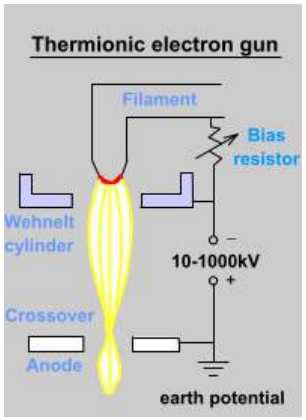
Phosphor screen

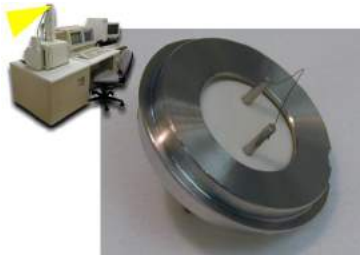




Electron source

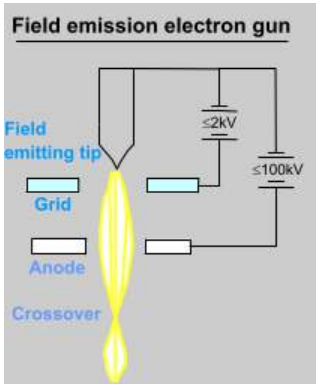
- Purpose: generation of electrons that can be accelerated by high tension to obtain the illuminating electron beam

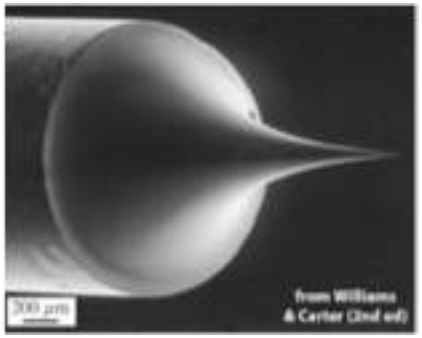




Thermionic gun: W or LaB6

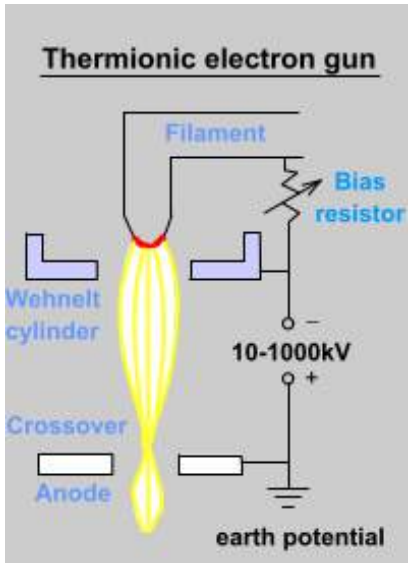
Electrons come out when the emitter is heated



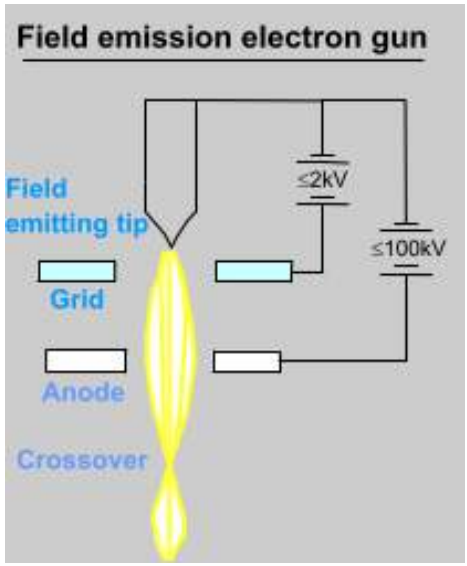


Field emission gun

Central development for high resolution EM !


Thermionic electron gun
Filament
Bias resistor
Wehnelt cylinder
10-1000kV
Crossover
Anode
earth potential

VS


Field emission electron gun
Field emitting tip
≤2kV
≤100kV
Grid
Anode
Crossover

VS

Electron gun

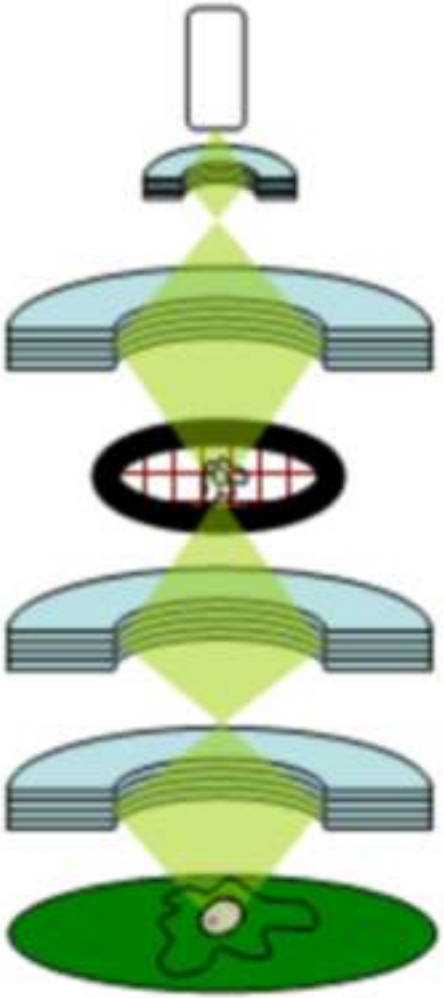
Condenser lens

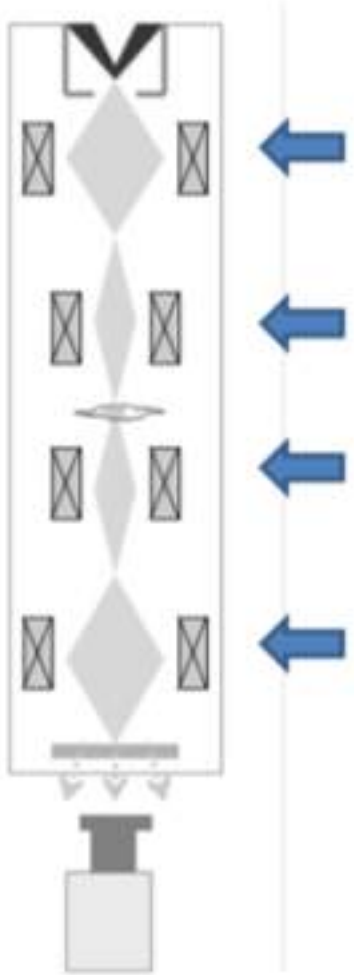
Sample grid

Objective lens

Projection lens

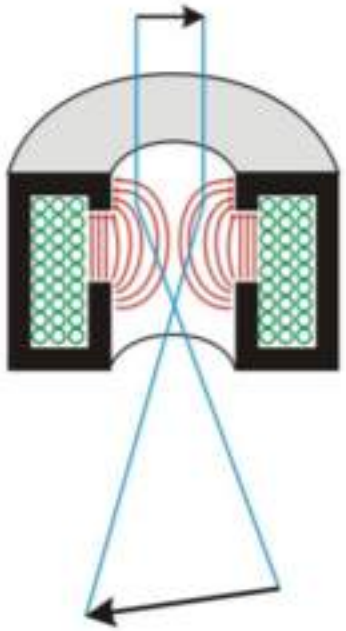
Phosphor screen

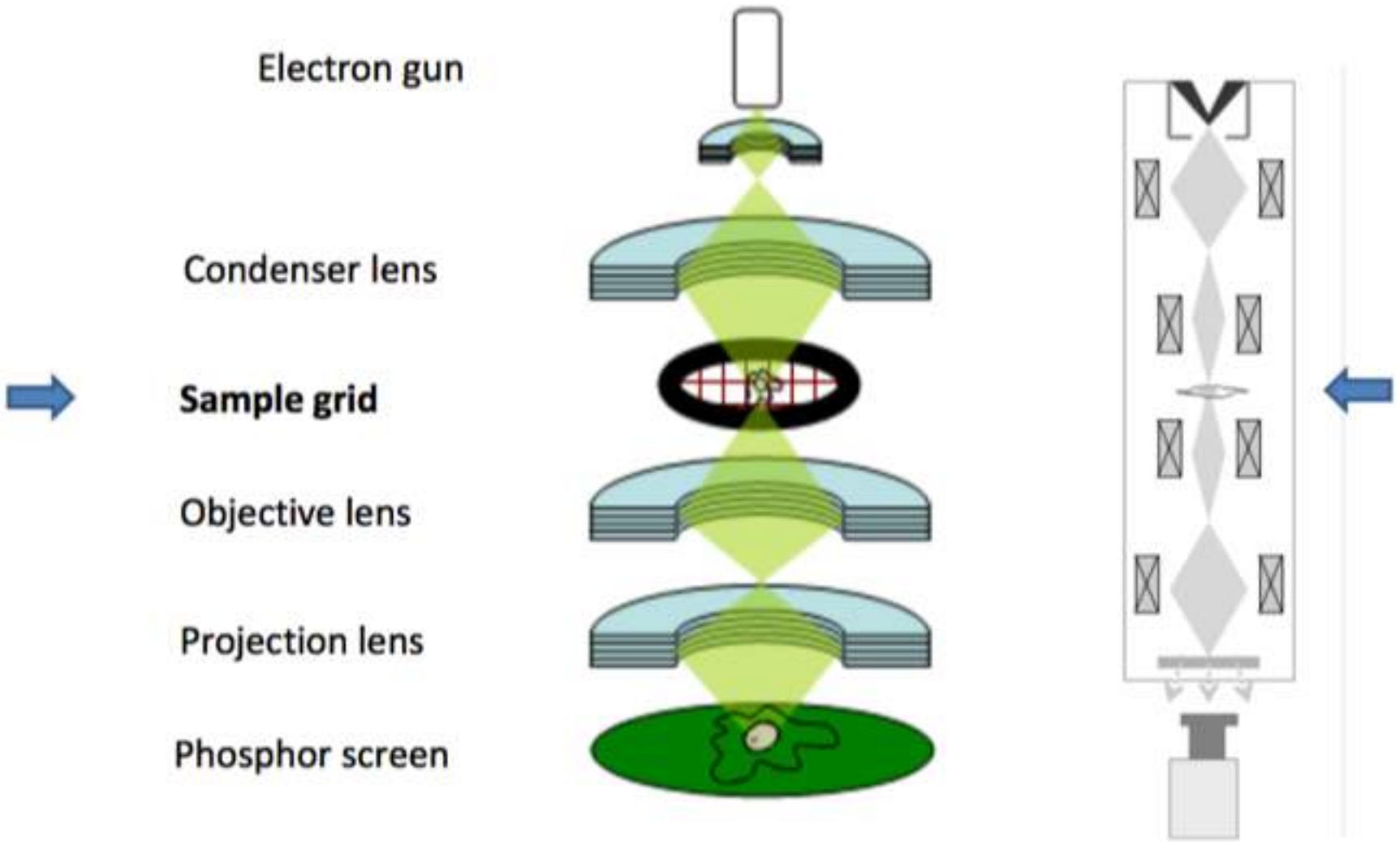


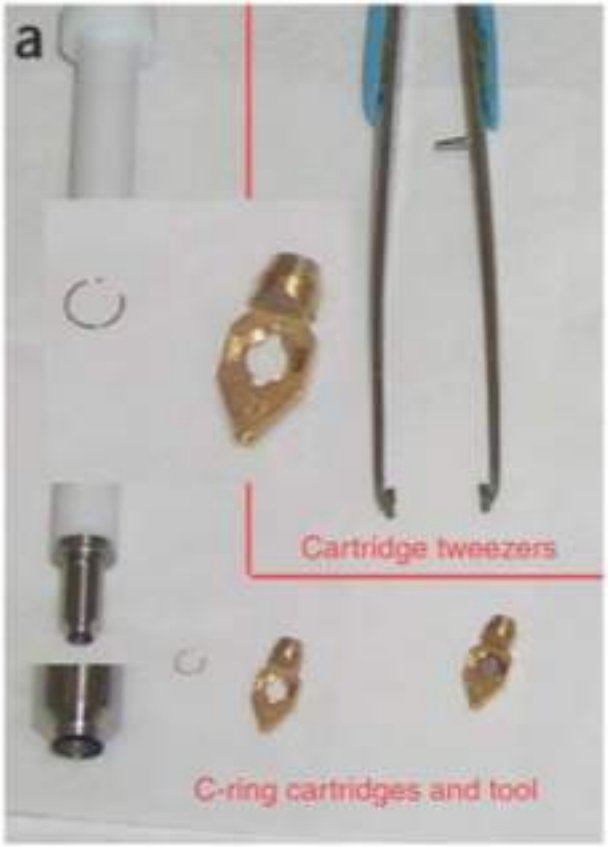


What is an electromagnetic lens ?

- Purpose of lenses: Generation of an electron beam that can be adapted in size and dose to the specimen




Electron gun
Condenser lens
Sample grid
Objective lens
Projection lens
Phosphor screen



Central development for high resolution EM !

Electron gun

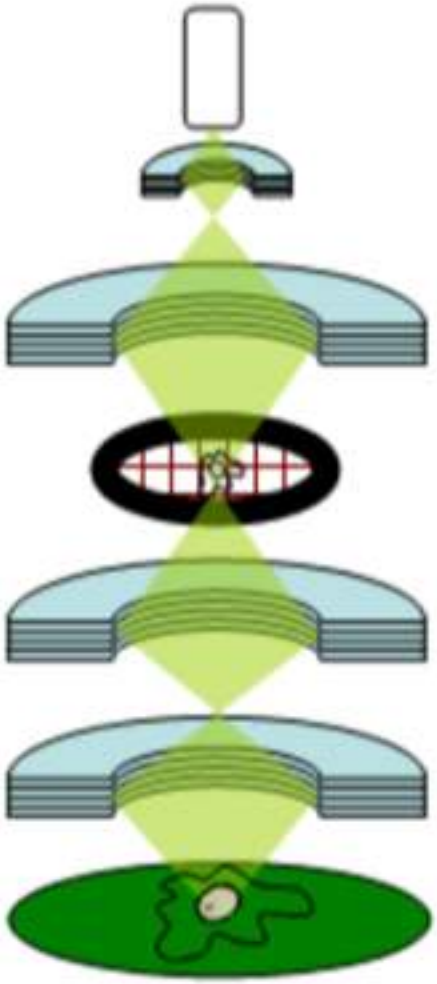
Condenser lens

Sample grid

Objective lens

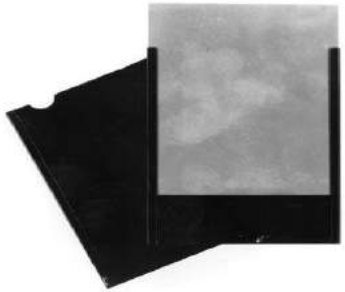
Projection lens

Detector

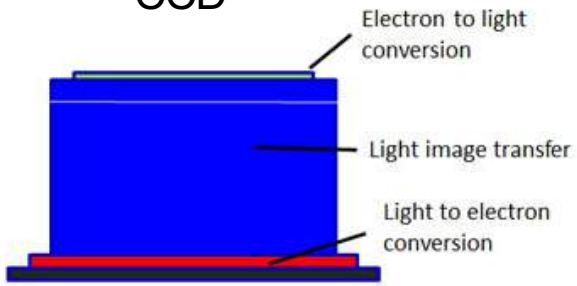


Detectors

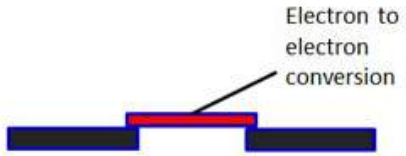
Photographic film

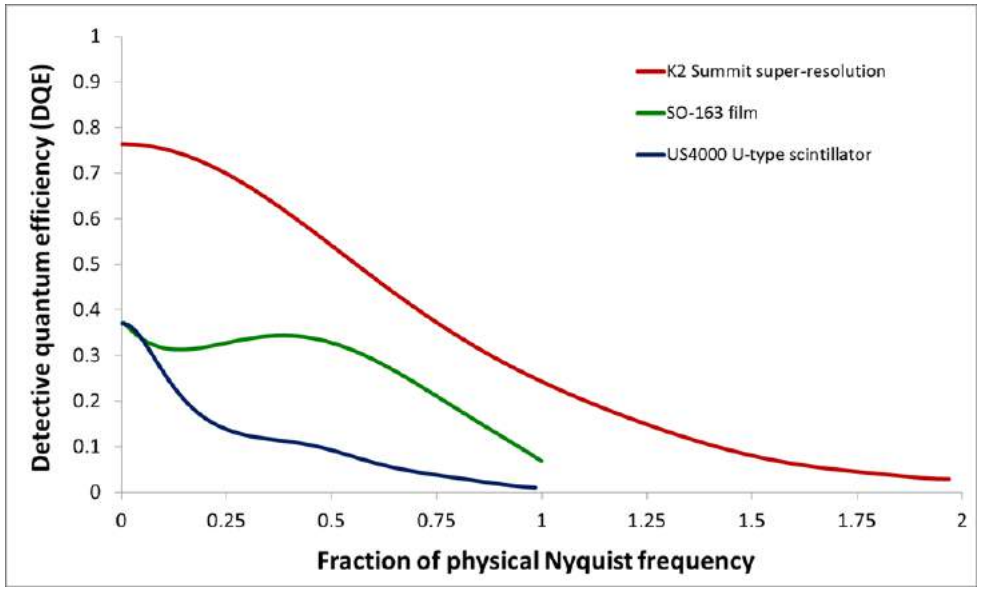


CCD



Direct electron detector





Direct electron detector: Central development for high resolution EM !

Lecture outline

Introduction

The electron microscope & image formation

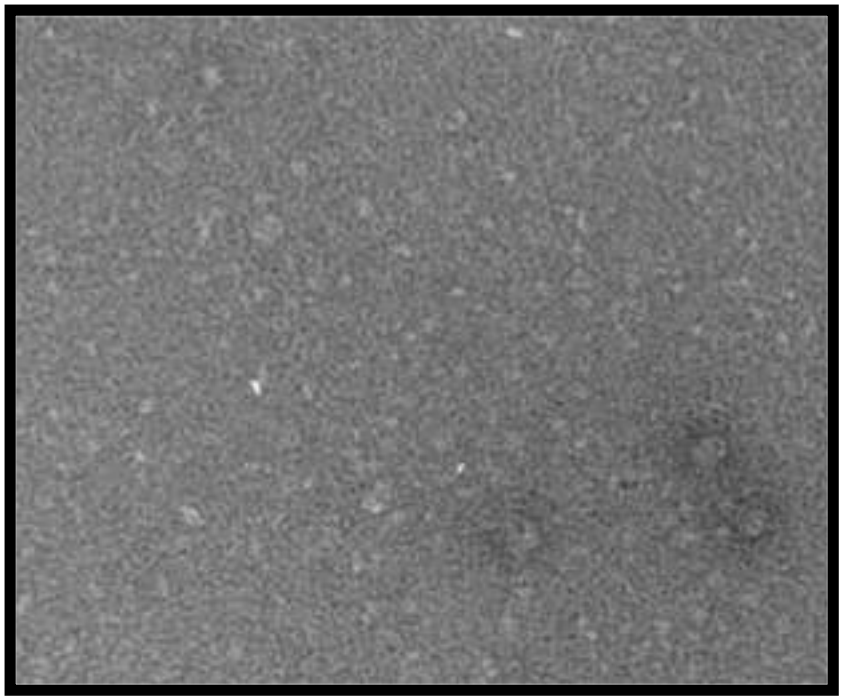
Sample preparation for EM

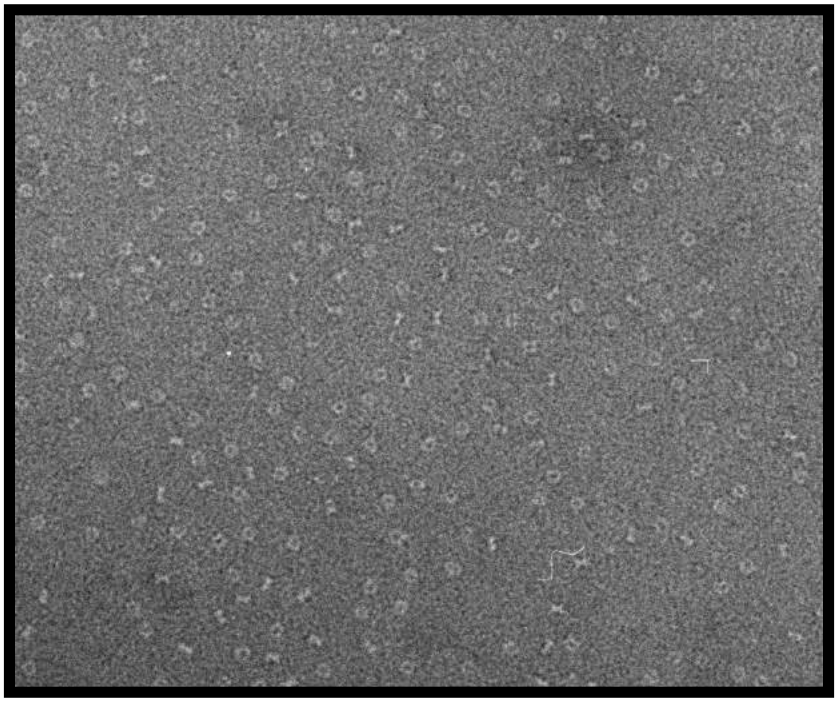
Data collection – Direct detectors

Image processing & applications

First thing: you need to obtain a good sample

➔ Clone, express, purify a sample to homogeneity





Heterogeneous

150 kDa

Homogeneous (quite)

Rubbish in

Whatever expensive microscope

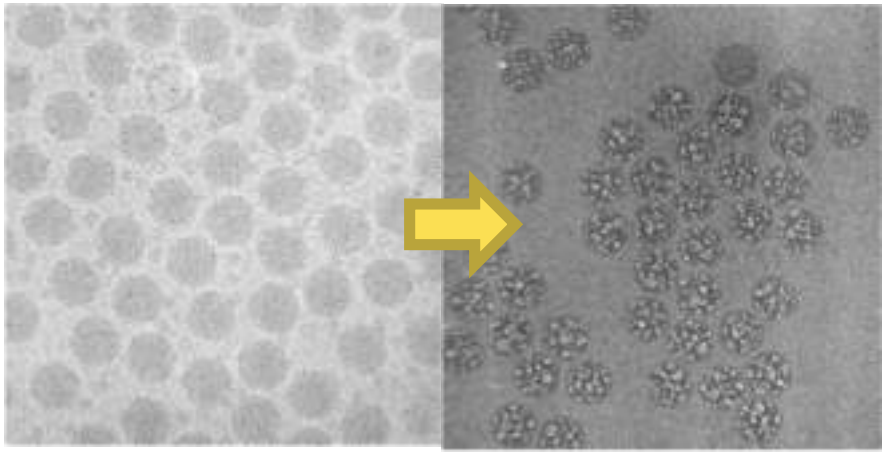
Rubbish in

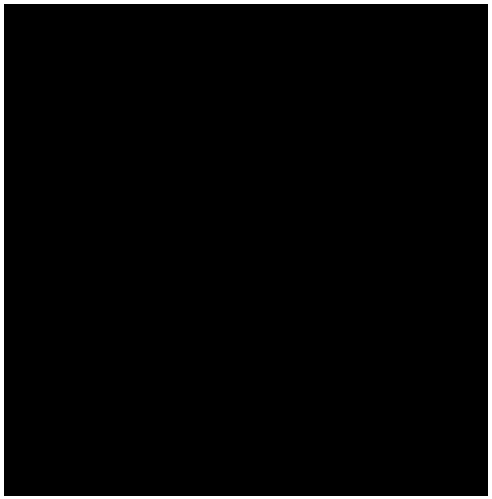
Whatever expensive microscope

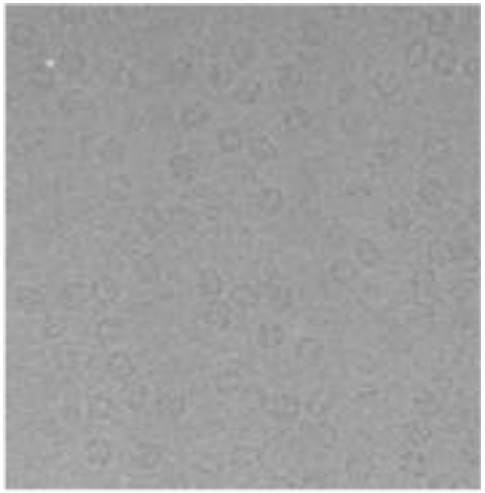
Rubbish out

It is worth spending time getting a good sample !!!

Sample requirements to be imaged in an EM

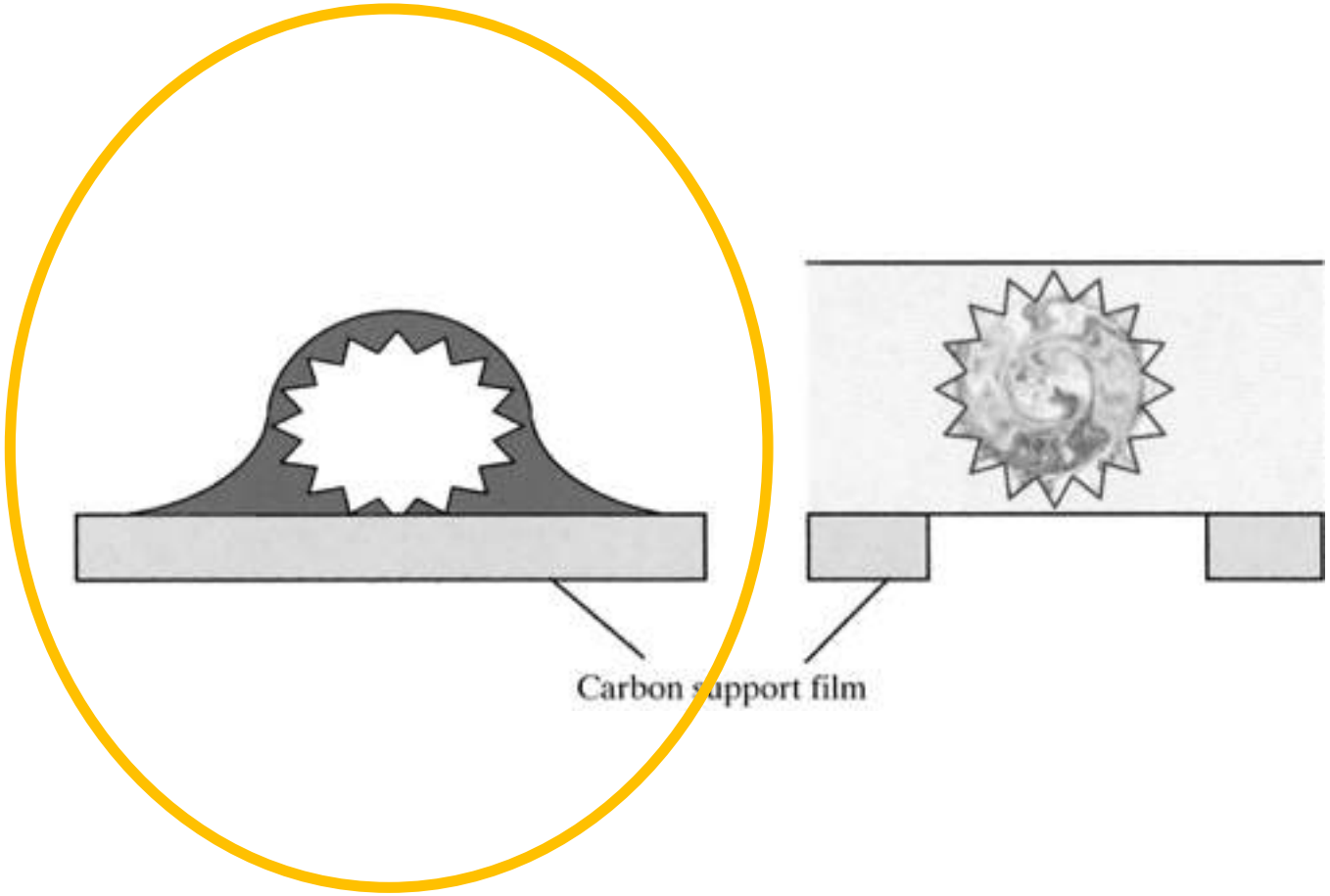






- Resist to the vacuum = be solid
- Resist to the electrons
- Being thin enough
- Have enough contrast

Two main methods

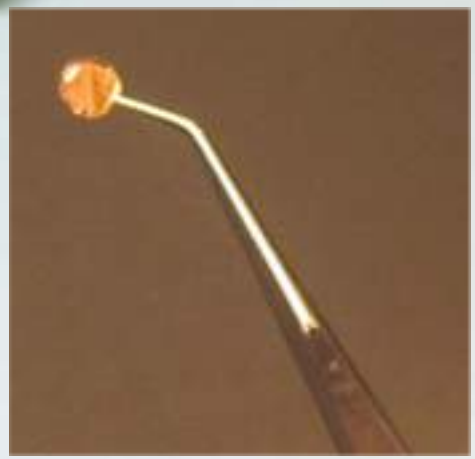


Negative stain
High contrast

Cryo
Native state

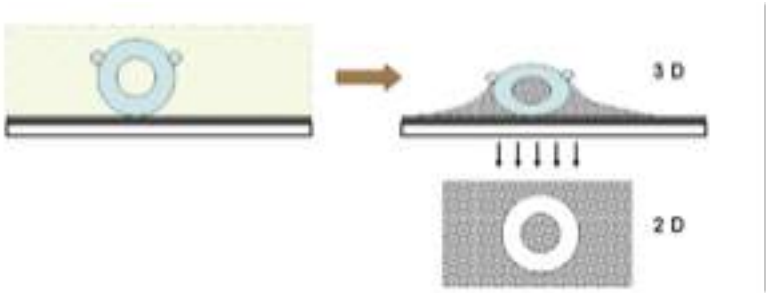
What is negative stain electron microscopy ?

Sample preparation



Advantages/drawbacks

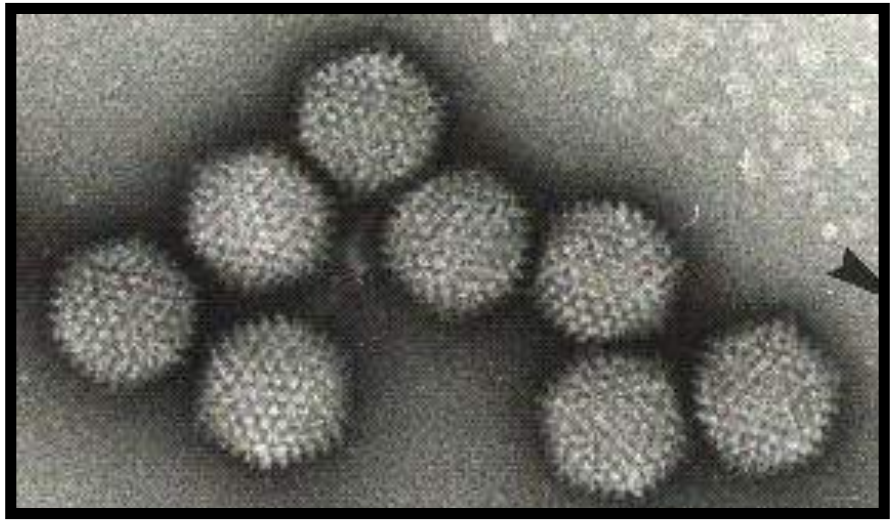
- Advantages:
  - Fast
  - Small amount of protein (concentration around 0.01-0.1mg/ml, few ul)
  - Small proteins visible (>50-100kDa)
  - High contrast

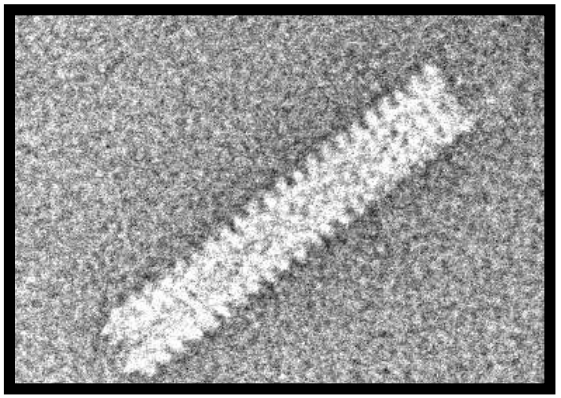


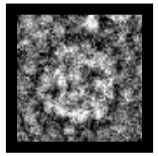
- Drawbacks:
  - Flattening and drying of the sample.
  - Artifacts due to the stain.

What we see in negative stain

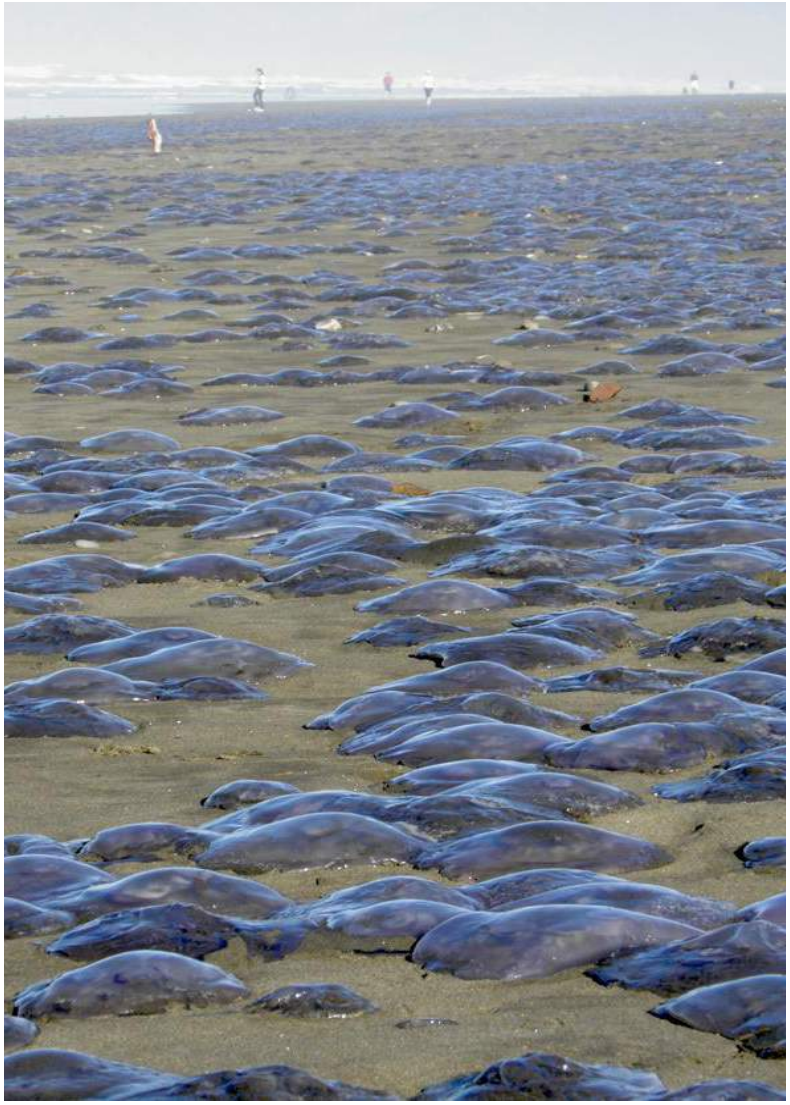
Single particles visualized by negative stain EM: examples





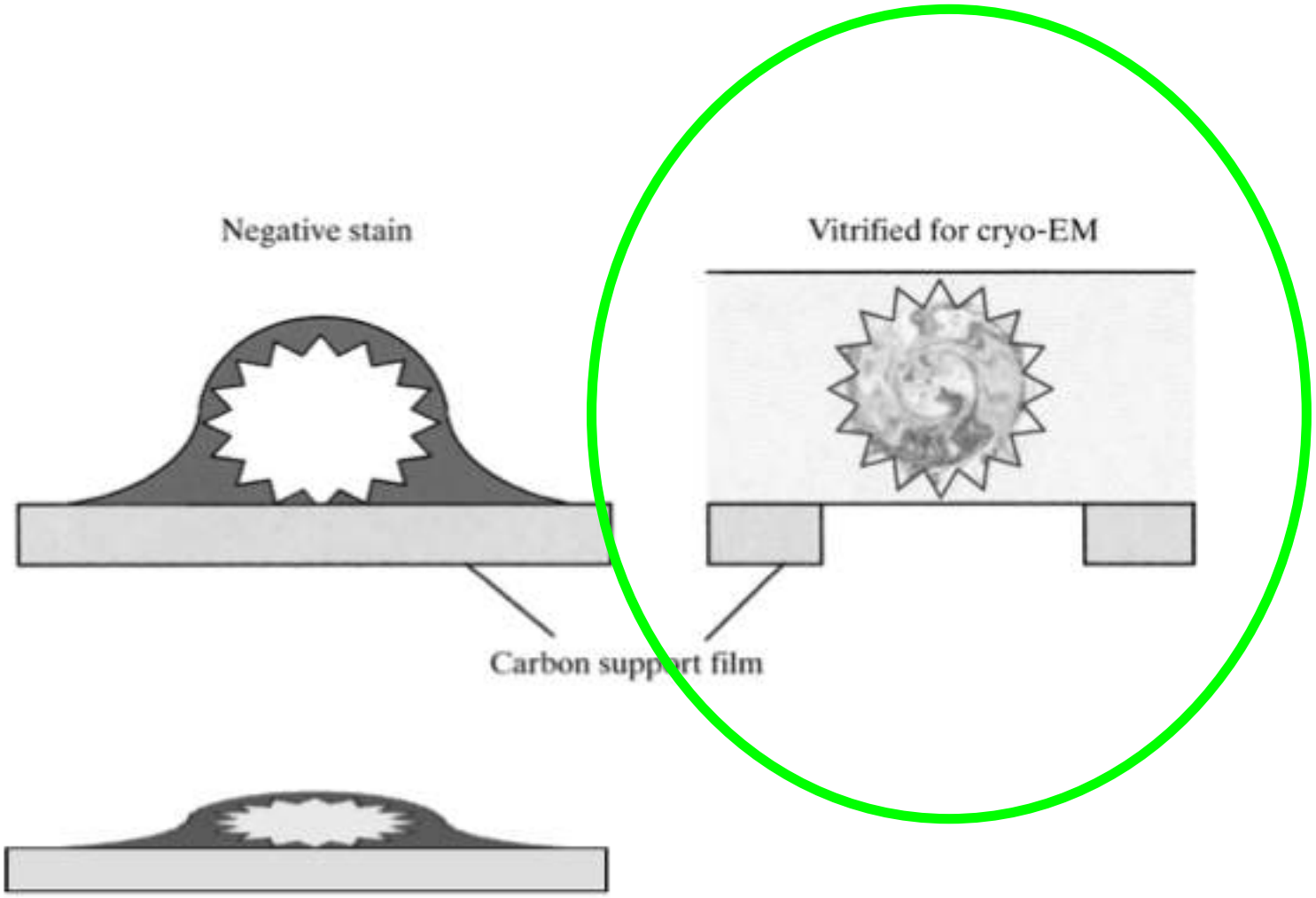


Object seen in negative stain



Real object to be visualized

To solve this problem: preparation of cryo grids



1984, EMBL Heidelberg

Cryo-electron microscopy of viruses

Marc Adrian, Jacques Dubochet, Jean Lepault & Alasdair W. McDowall

European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg, FRG

Thin vitrified layers of unfixed, unstained and unsupported virus suspensions can be prepared for observation by cryo-electron microscopy in easily controlled conditions. The viral particles appear free from the kind of damage caused by dehydration, freezing or adsorption to a support that is encountered in preparing biological samples for conventional electron microscopy. Cryo-electron microscopy of vitrified specimens offers possibilities for high resolution observations that compare favourably with any other electron microscopical method.

Jacques Dubochet

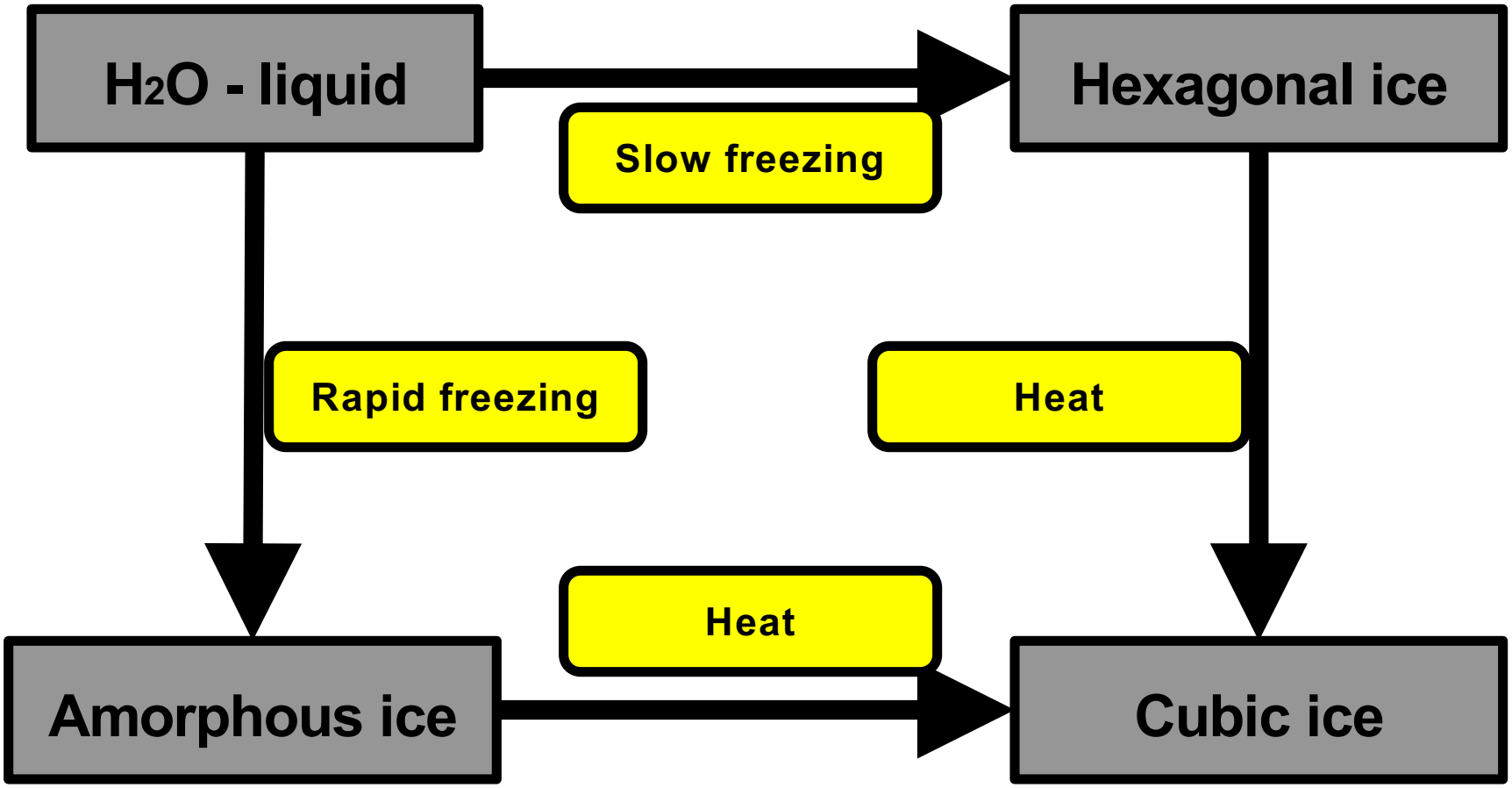
Quarterly Review of Biophysics 21, 2 (1988), pp. 129–228 129
Printed in Great Britain

Cryo-electron microscopy of vitrified specimens

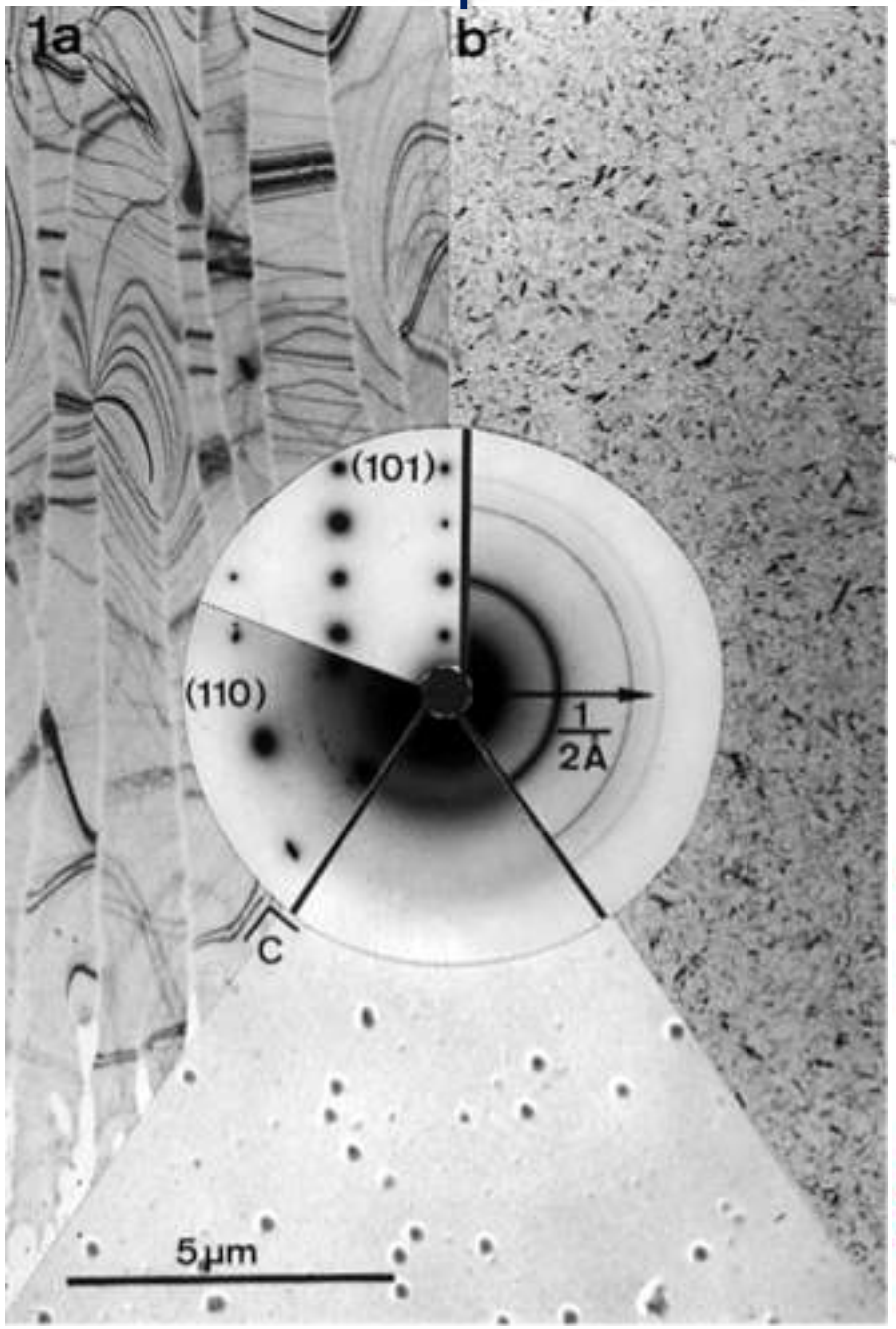
JACQUES DUBOCHET[1], MARC ADRIAN[2], JIIN-JU CHANG[3], JEAN-CLAUDE HOMO[4], JEAN LEPAULT[5], ALASDAIR W. McDOWALL[6] AND PATRICK SCHULTZ[4]

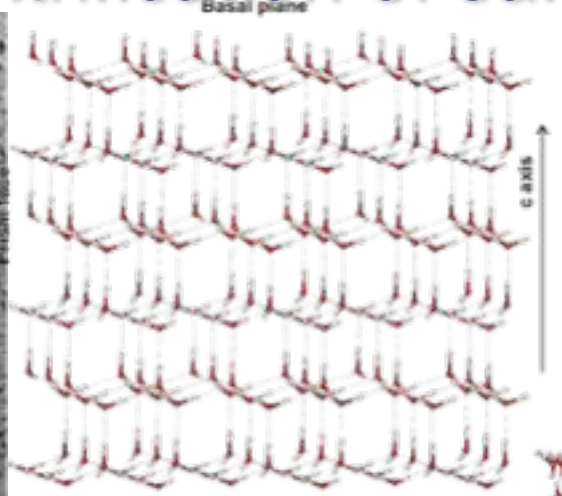
European Molecular Biology Laboratory (EMBL), Postfach 10.2209, D-6900 Heidelberg, FRG

Principle behind vitrification of sample



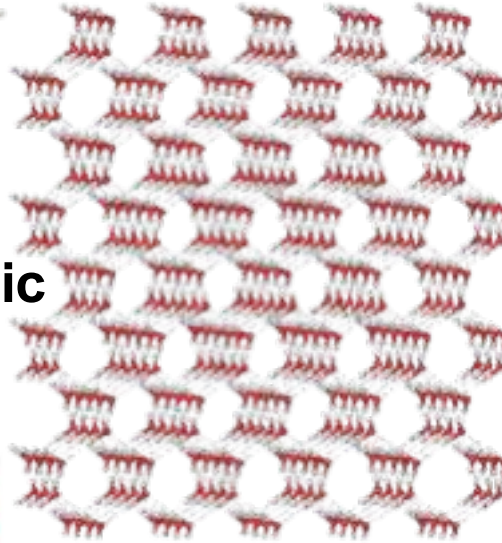
Principle behind vitrification of sample





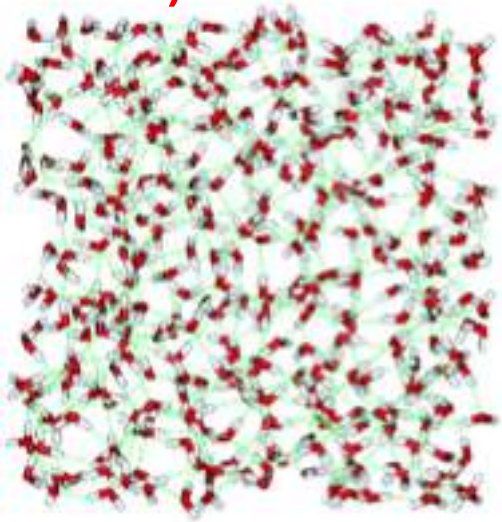
Dubochet et al., (1982)

a) hexagonal



b) cubic

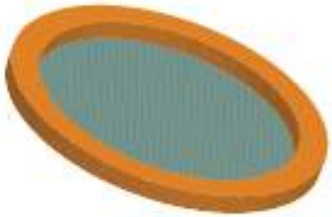
c) vitrified



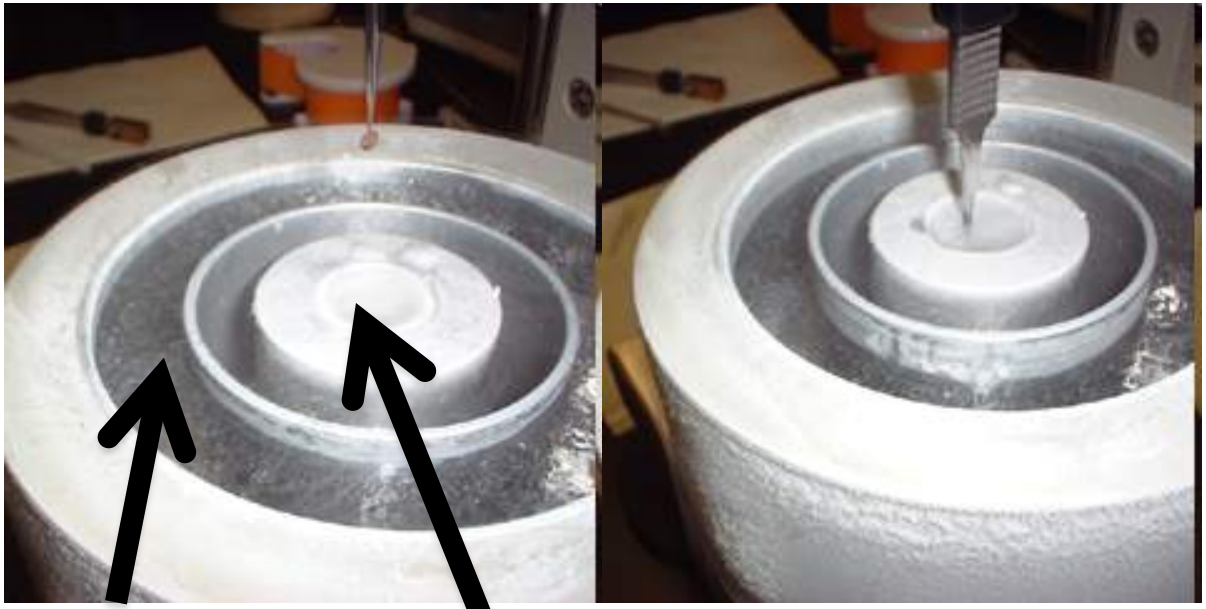
Vitrification of sample : plunge-freezing

EM grid

Apply sample



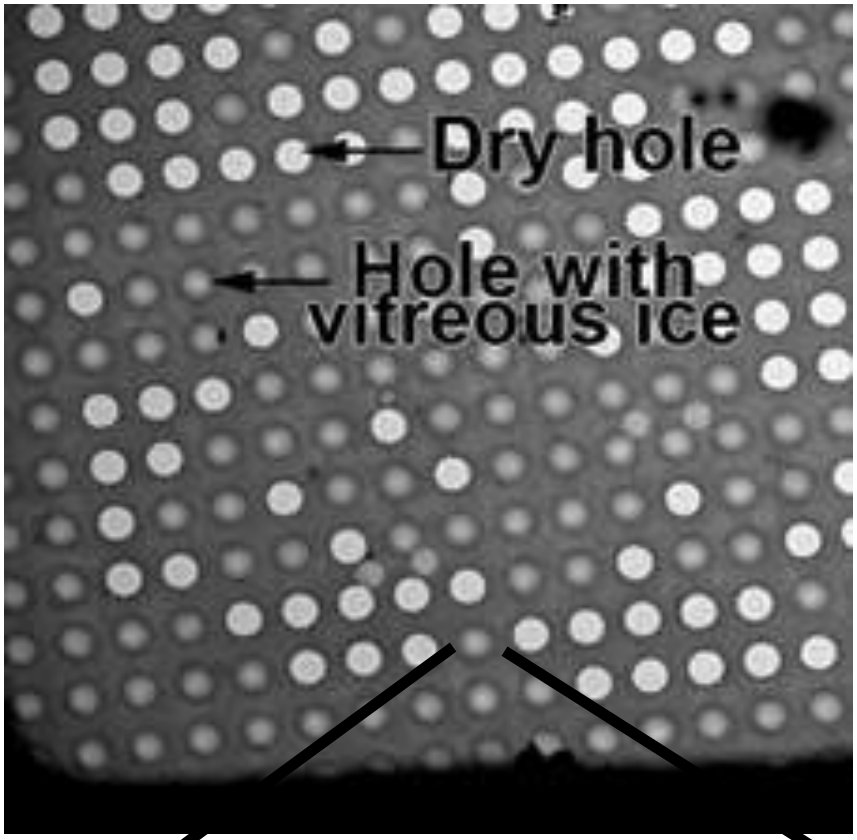
Blot excess buffer before plunging

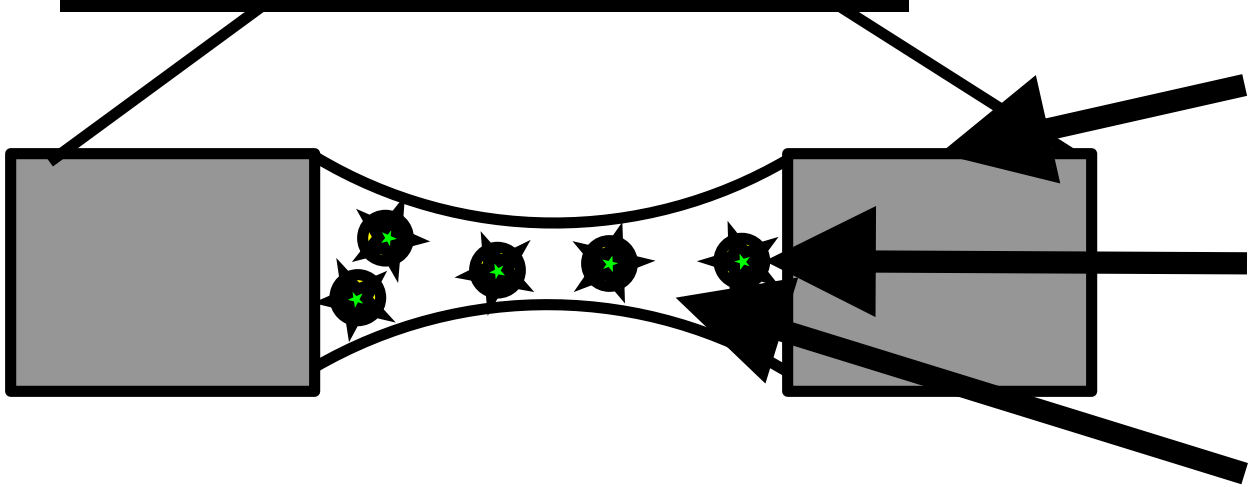


Liquid Nitrogen

Liquid Ethane

Cryo grid under the EM



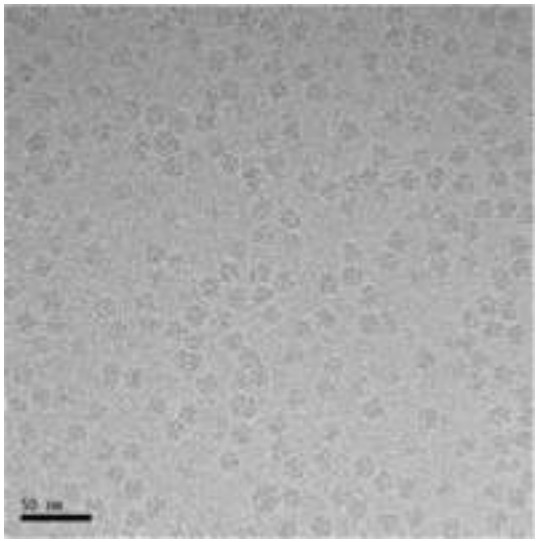


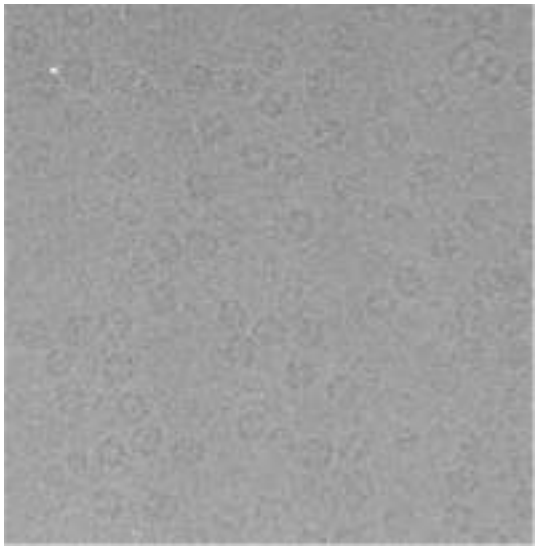
Carbon support

Particles in random orientations

Vitreous Ice (Thin !)

Single particles: cryo preparation





- Advantages:
  - Native state
  - Higher resolution
  - Small amount of protein (1 grid = 4 ul at 0.1-1mg/ml)
- Disadvantages:
  - Low contrast
  - Highly sensitive to radiations
  - Minimal size limit: 150 kDa
  - Difficult

Lecture outline

Introduction

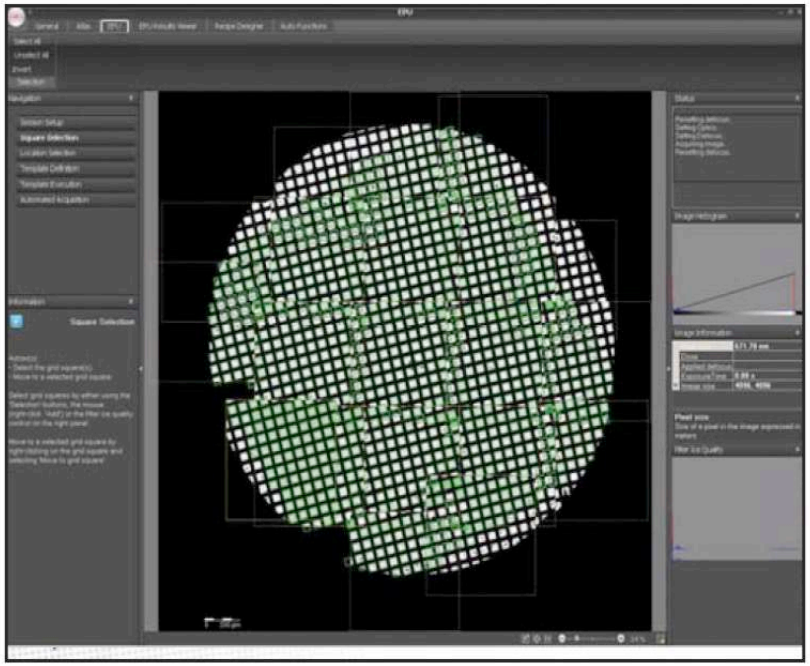
The electron microscope

Sample preparation for EM

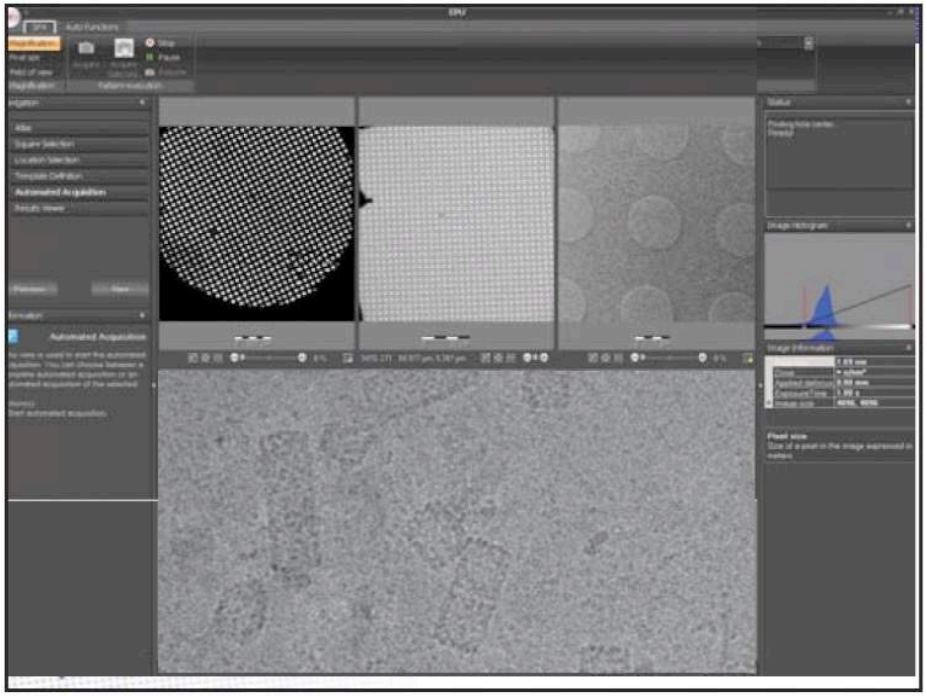
Data collection – Direct detectors

Image processing & applications

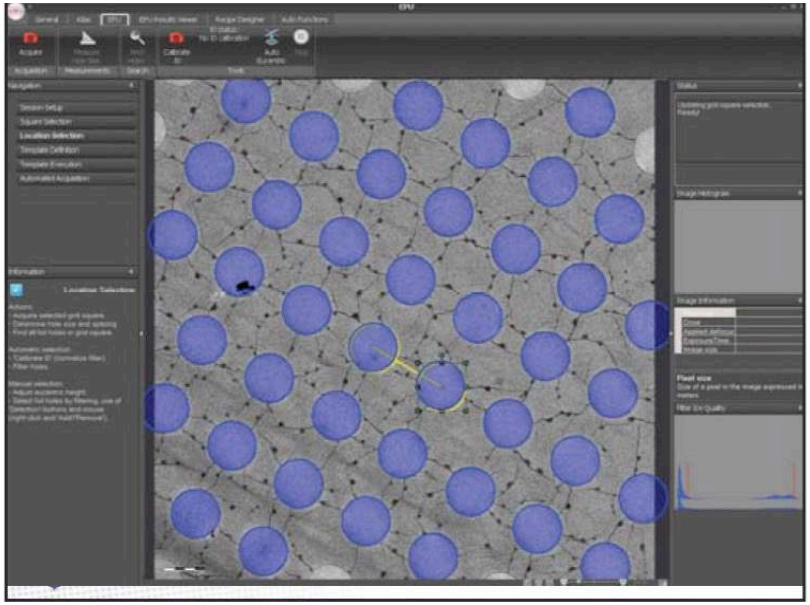
Semi-Automated data collection



1/ Grid square selection

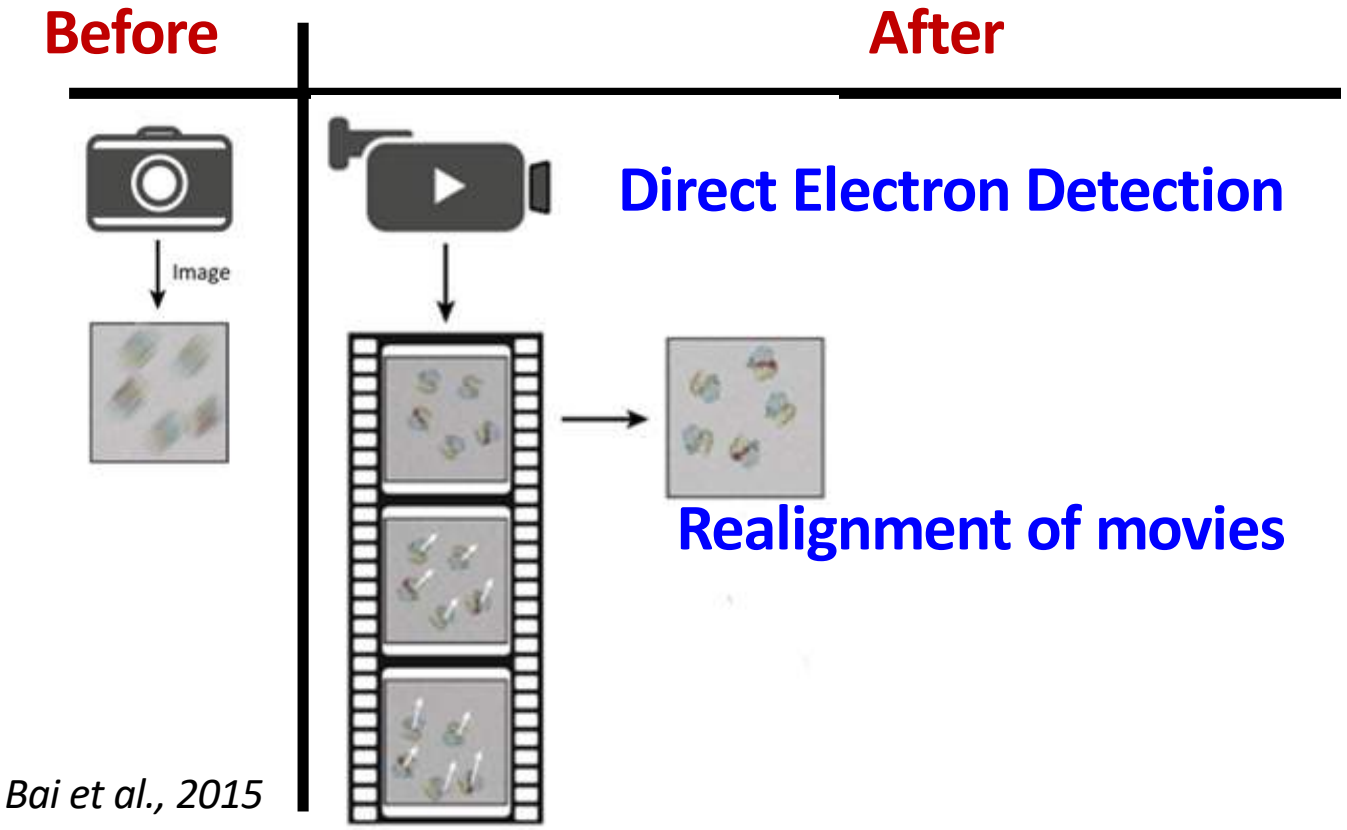


3/ Automated acquisition (autofocus, …)



2/ Assisted hole selection

With direct electron detector, we can now correct for the drift that happened during the data collection



Lecture outline

Introduction

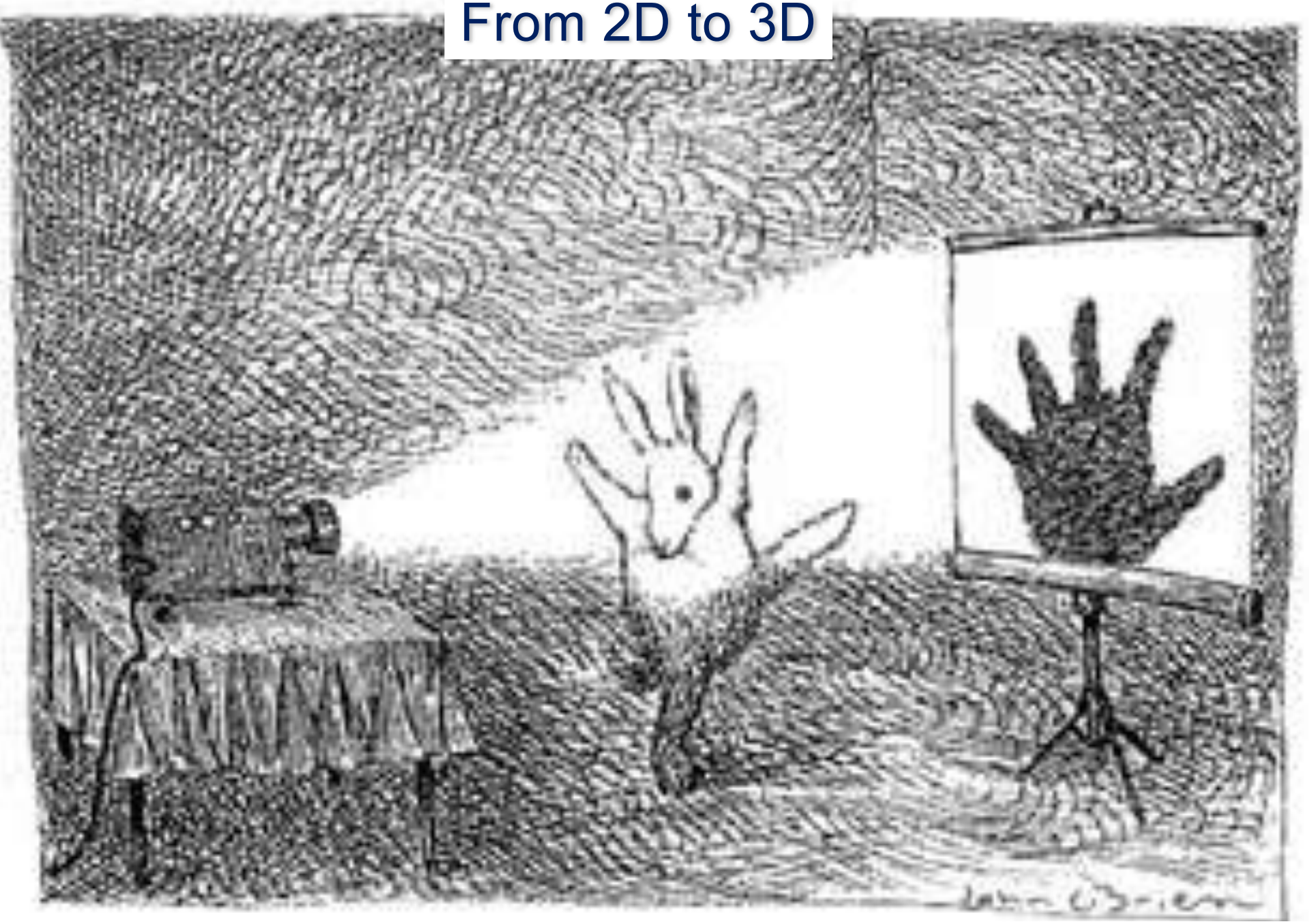
The electron microscope

Sample preparation for EM

Data collection – Direct detectors

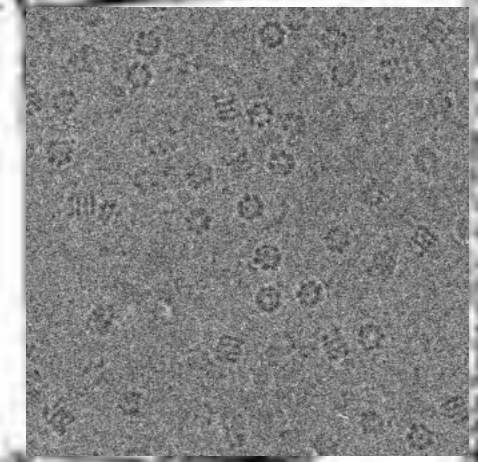
Image processing & applications

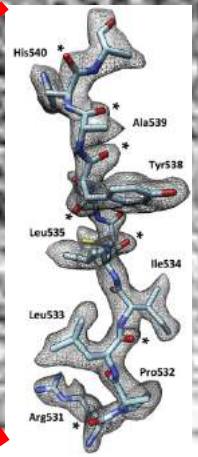
From 2D to 3D



From 2D to 3D

Electron microscope



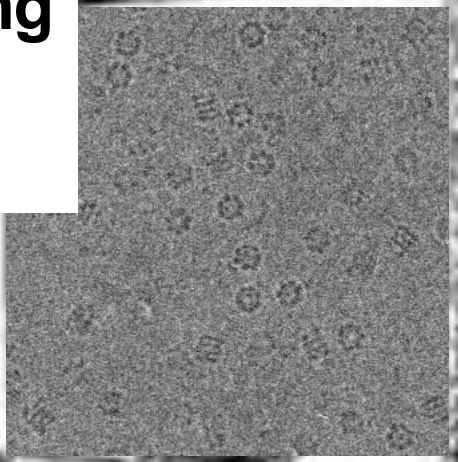


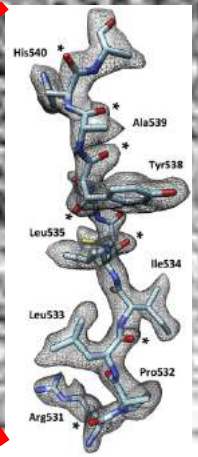
From 2D to 3D

We need to combine many different views of the object of interest

Single-particle image processing
=~ determining and combining orientations

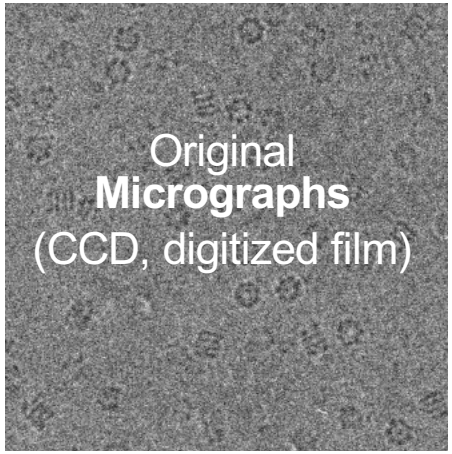
Electron microscope





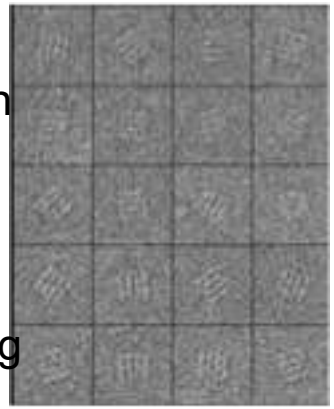
How do we determine and combine orientations ?

How do we determine and combine orientations ?

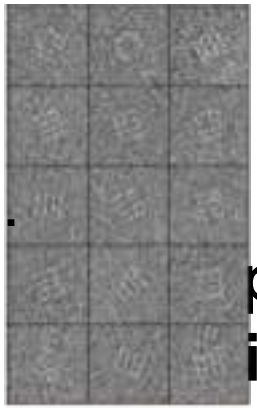


Micrograph selection
Particle picking

CTF determination
CTF correction
Image pre-processing

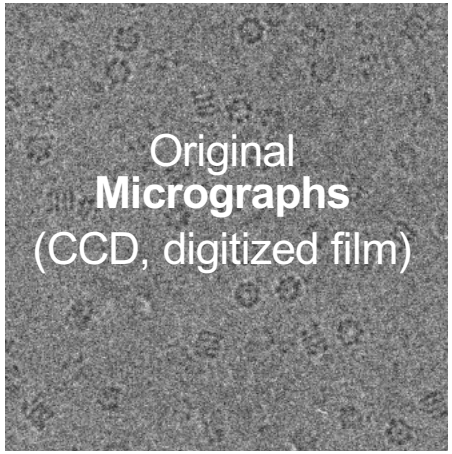


...



Stack of particle images

How do we determine and combine orientations ?



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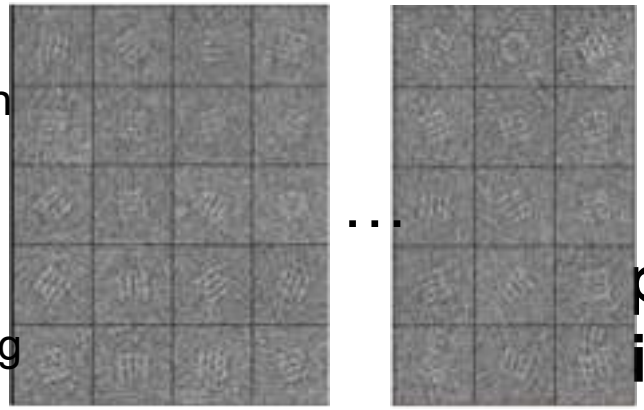
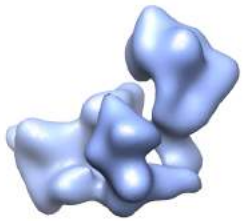
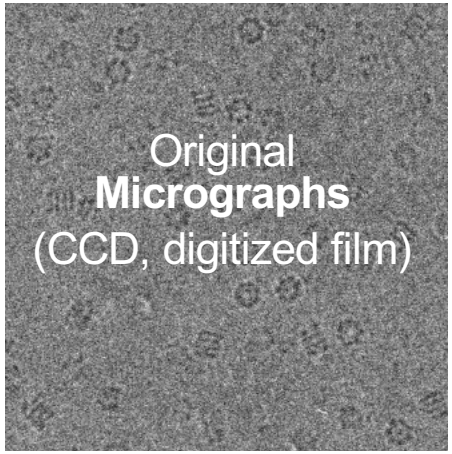


Stack of particle images



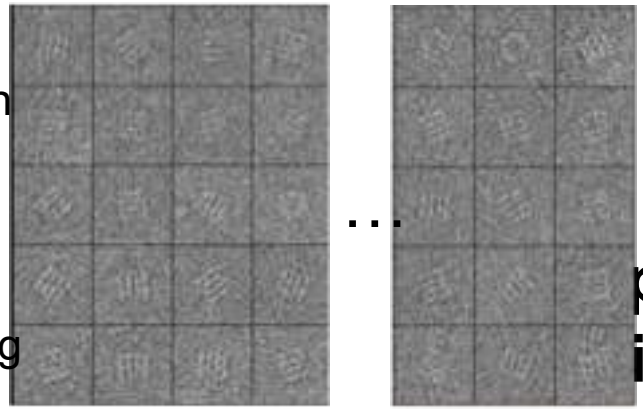
Initial model

How do we determine and combine orientations ?



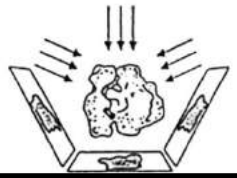
Micrograph selection
Particle picking

CTF determination
CTF correction
Image pre-processing

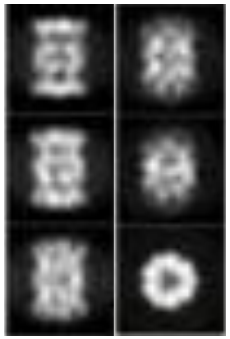


Stack of particle images

Initial model

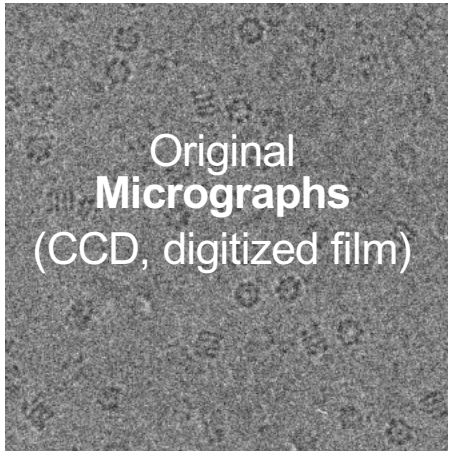


Calculate reprojections in all orientations



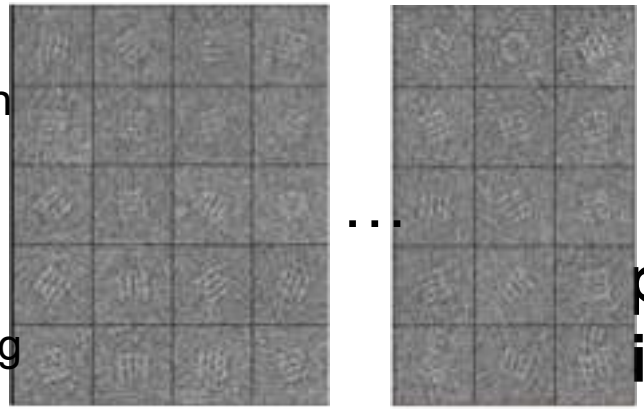
2D template projections

How do we determine and combine orientations ?



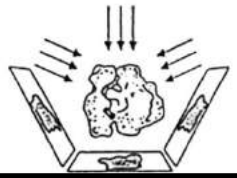
Micrograph selection
Particle picking

CTF determination
CTF correction
Image pre-processing

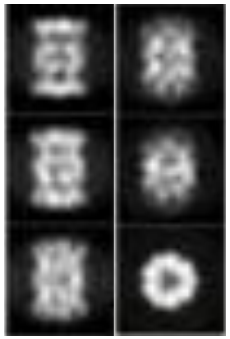


Stack of particle images

Initial model



Calculate reprojections in all orientations



2D template projections

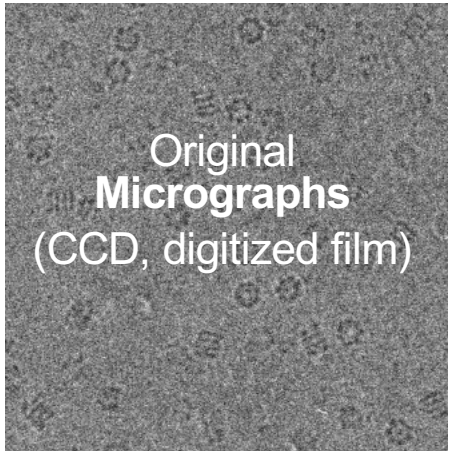
Calculation of 2D ccfs (in-plane rotation, x and y shift)

ccf1
ccf2
ccf3
ccf4
ccf5
ccf...

maxk
ccfk

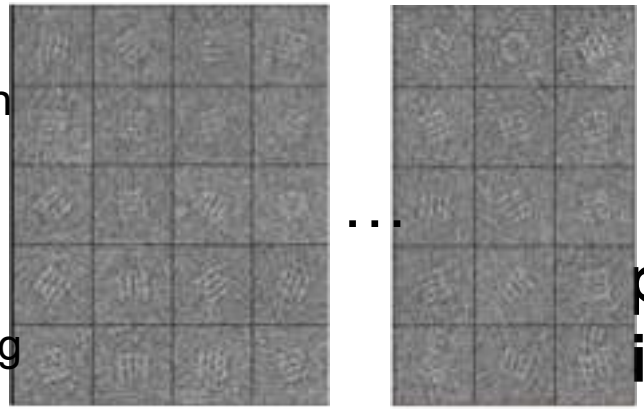
Orientation parameters

How do we determine and combine orientations ?

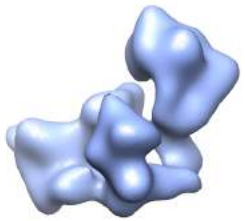


Micrograph selection
Particle picking

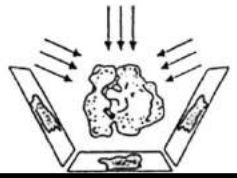
CTF determination
CTF correction
Image pre-processing



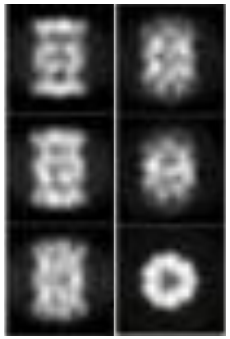
Stack of particle images



Initial model



Calculate reprojections in all orientations



2D template projections

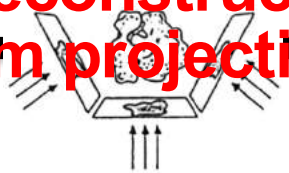
Calculation of 2D ccfs (in-plane rotation, x and y shift)

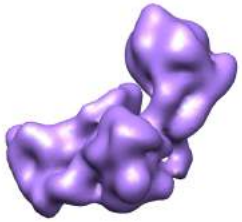
ccf1
ccf2
ccf3
ccf4
ccf5
ccf...

maxk
ccfk

Orientation parameters

3D reconstructions from projections





3D map

Lecture outline

Introduction

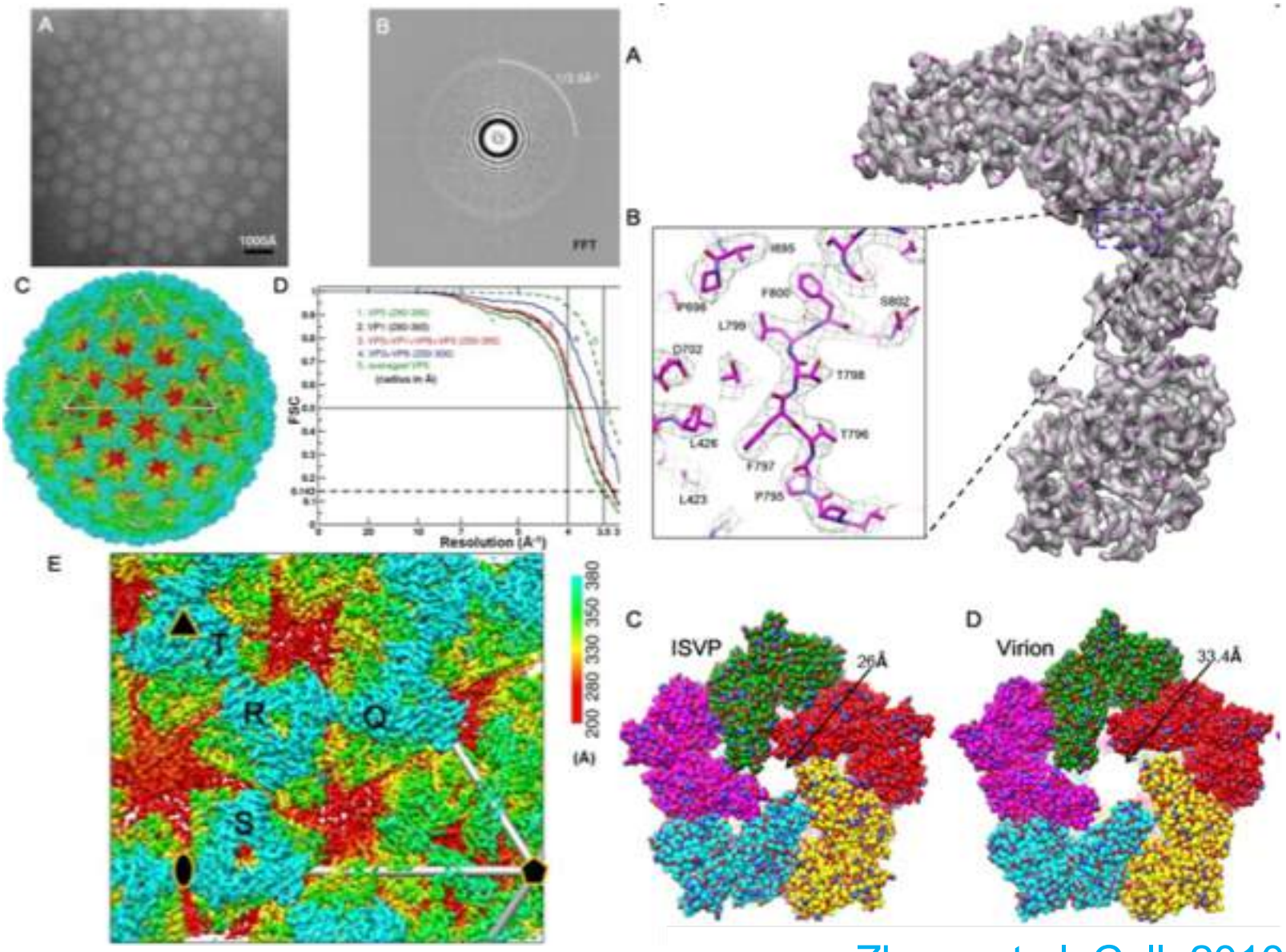
The electron microscope

Sample preparation for EM

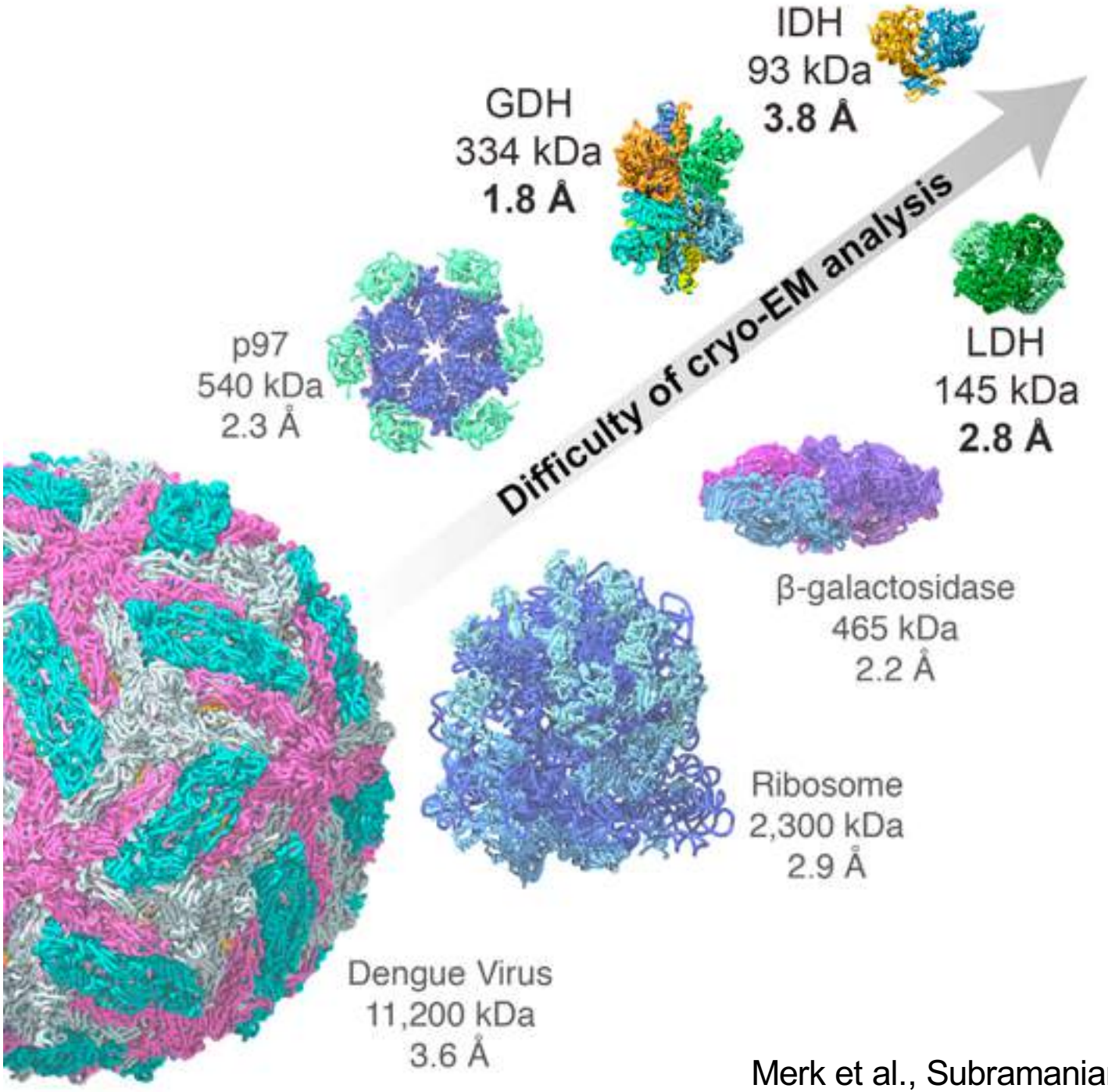
Data collection – Direct detectors

Image processing & applications

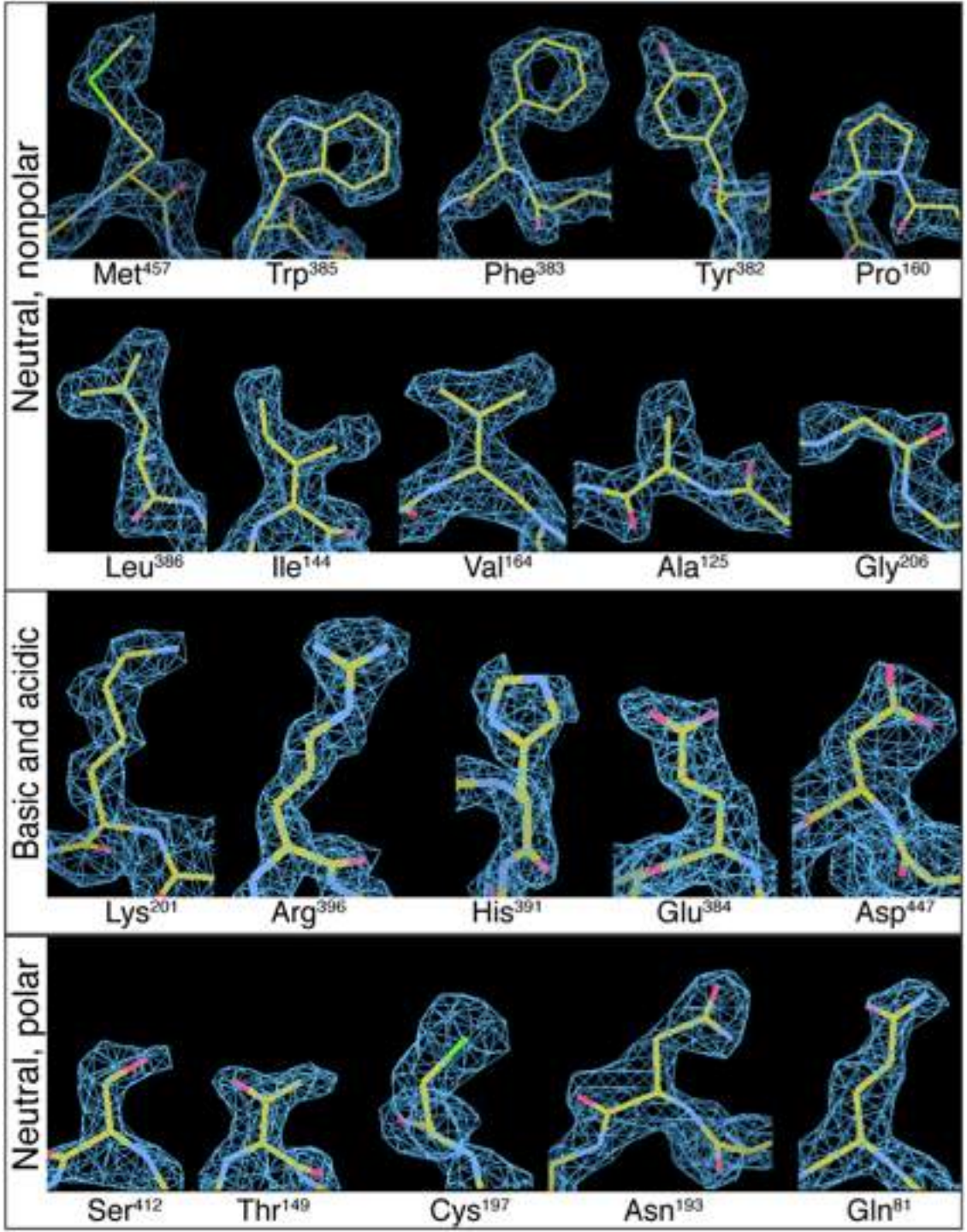
If samples are homogeneous: high resolution EM



Zhang et al, Cell, 2010

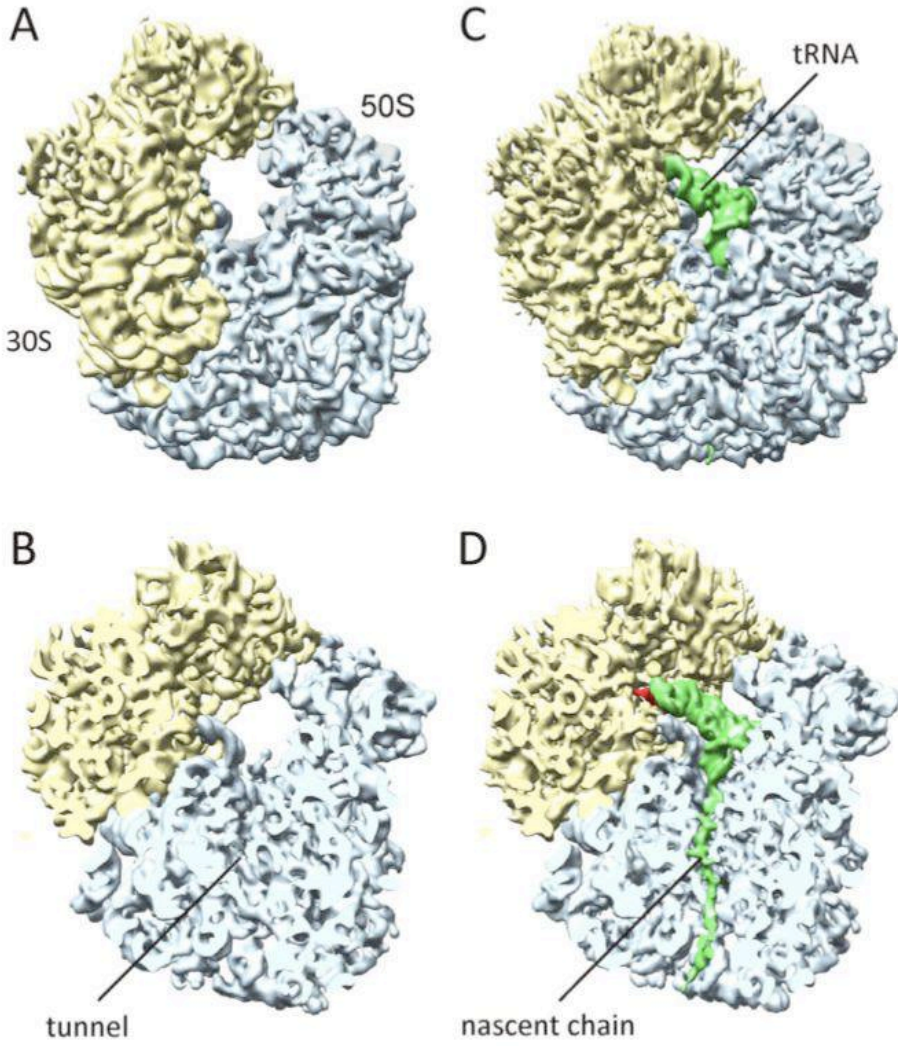


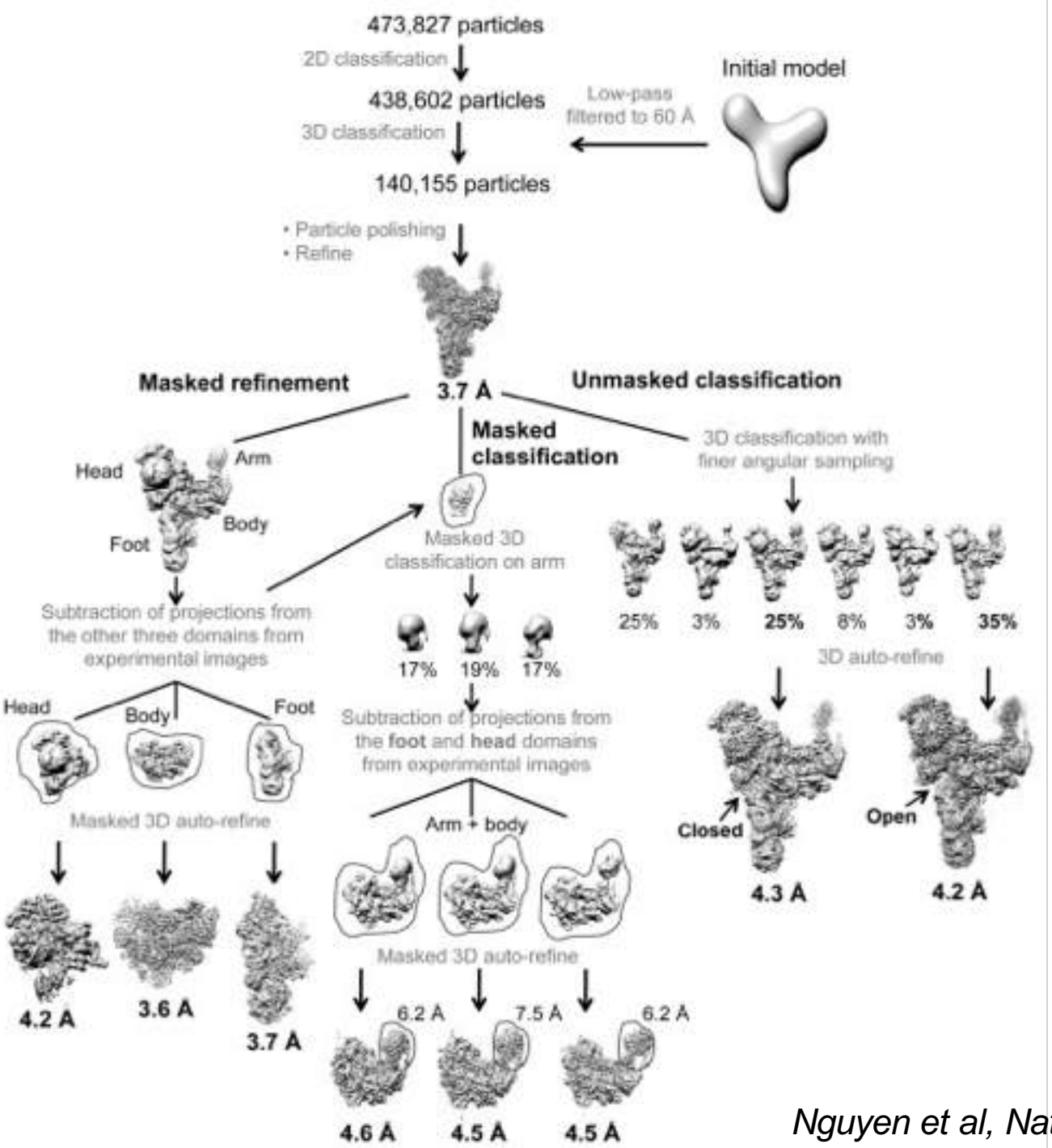
Merk et al., Subramaniam, Cell 2016


Neutral, nonpolar
Met457
Trp385
Phe383
Tyr382
Pro160
Leu386
Ile144
Val164
Ala125
Gly206
Basic and acidic
Lys201
Arg396
His391
Glu384
Asp447
Neutral, polar
Ser412
Thr149
Cys197
Asn193
Gln81

If complexes are heterogeneous: separation of different populations within the same sample

- Different 3D volumes with particles chosen randomly.
- 3D classification to determine to which structure each particle belongs





Nguyen et al, Nature, 2016

What about glycosylated proteins?

Structure
Article

Model Building and Refinement of a Natively Glycosylated HIV-1 Env Protein by High-Resolution Cryoelectron Microscopy

Jeong Hyun Lee,[1,2] Natalia de Val,[1,2] Dmitry Lyumkis,[3] and Andrew B. Ward[1,2,*]
[1]Department of Integrative Structural and Computational Biology, Center for HIV/AIDS Vaccine Immunology and Immunogen Discovery, The Scripps Research Institute, La Jolla, CA 92037, USA
[2]International AIDS Vaccine Initiative (IAVI) Neutralizing Antibody Center and Collaboration for AIDS Vaccine Discovery (CAVD), The Scripps Research Institute, La Jolla, CA 92037, USA
[3]Laboratory of Genetics and Helmsley Center for Genomic Medicine, The Salk Institute for Biological Studies, La Jolla, CA 92037, USA
*Correspondence: abward@scripps.edu
http://dx.doi.org/10.1016/j.str.2015.07.020

SUMMARY

Secretory and membrane proteins from mammalian cells undergo post-translational modifications, including N-linked glycosylation, which can result in a large number of possible glycoforms. This sample heterogeneity can be problematic for structural studies, particularly X-ray crystallography. Thus, crystal structures of heavily glycosylated proteins such as the HIV-1 Env viral spike protein have been determined by removing the majority of glycans. This step is most frequently carried out using Endoglycosidase H (EndoH) and requires that all expressed glycans be in the high-mannose form, which is often not the native glycoform. With significantly improved technologies in single-particle cryoelectron microscopy, we demonstrate that it is now possible to refine and build natively glycosylated HIV-1 Env structures in solution to 4.36 Å resolution. At this resolution we can now analyze the complete epitope of a broadly neutralizing antibody (bnAb), PGT128, in the context of the trimer expressed with native glycans.

ation pathway. For example, GnTI-deficient HEK293S cells produce proteins with all high-mannose glycans that can be deglycosylated with Endoglycosidase H (EndoH), leaving only the N-linked core N-acetylglucosamine (N-GlcNAc) (Depetris et al., 2012; Julien et al., 2013a; Lee et al., 2008; Pancera et al., 2014). However, the major caveat of this approach is that the protein cannot be crystallized in its native form, which is especially disadvantageous when attempting to solve structures of glycoprotein complexes in which the binding partner recognizes complex glycans.

HIV-1 envelope glycoprotein (Env), the fusion machine on the surface of HIV-1, is metastable and one of the most highly glycosylated protein complexes known, and has been subject to all of the above challenges. A soluble Env trimer construct termed BG505 SOSIP.664, derived from the sequence of clade A virus BG505, with an introduced gp120-gp41 heterodimer linking disulfide bond (SOS) (Binley et al., 2000), and pre-fusion conformation stabilizing I559P mutation (IP) (Sanders et al., 2002) was used to overcome the lack of trimer stability for multiple structural studies (Garces et al., 2014; Huang et al., 2014; Julien et al., 2013a, 2013b, 2013c; Khayat et al., 2013; Kong et al., 2013; Lyumkis et al., 2013; Pancera et al., 2014). Although the ability to generate a stable native-like soluble Env trimer was a major breakthrough, structural characterization of Env glycans remains an arduous task. Despite the fact that the outer domain of Env is heavily packed with glycans they are highly flexible, and

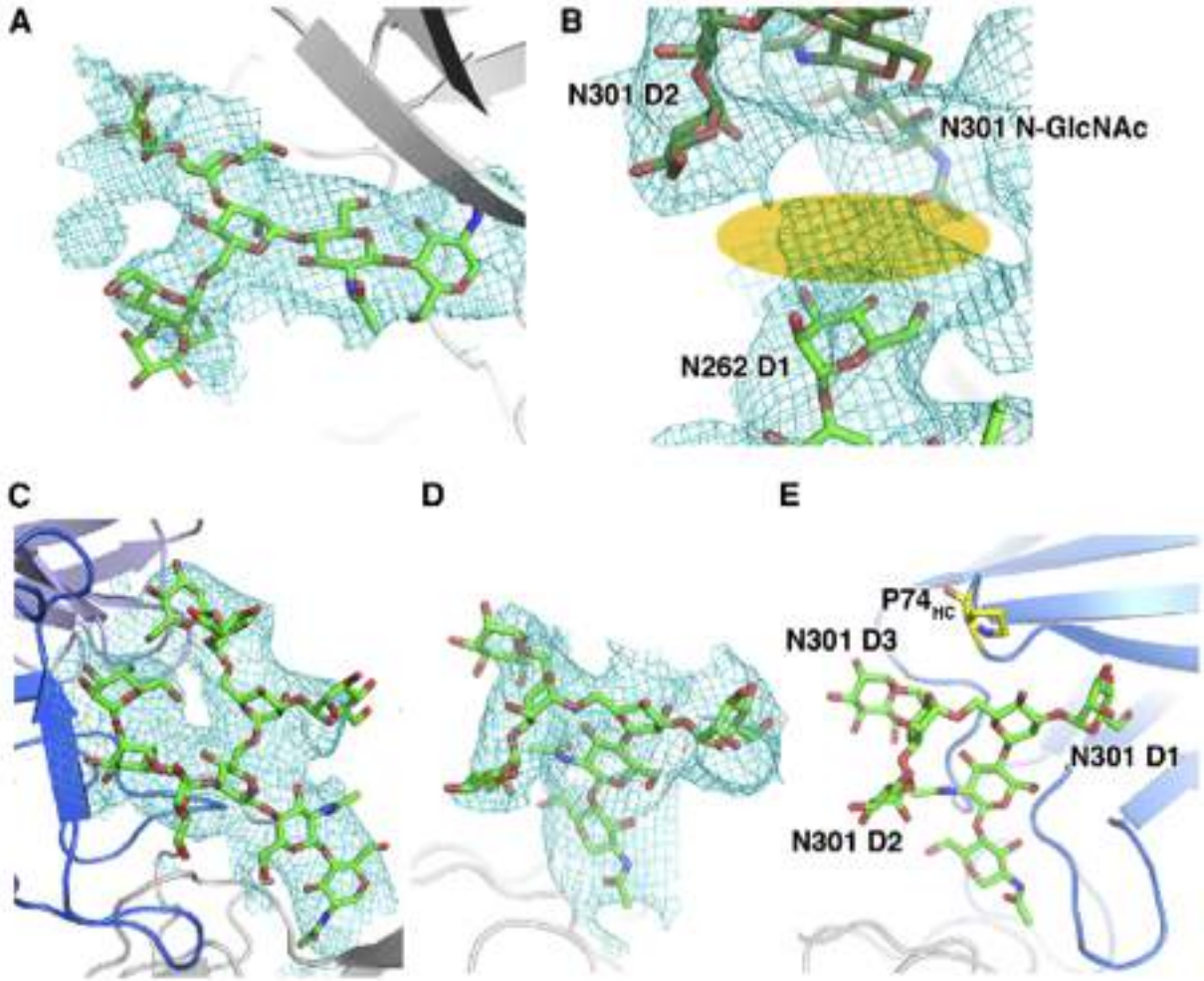


Figure 4. Glycan Interactions Resolved in the Cryo-EM Structure

(A) Clear density for a Man_5 glycan at N262 can be seen in the EM map.

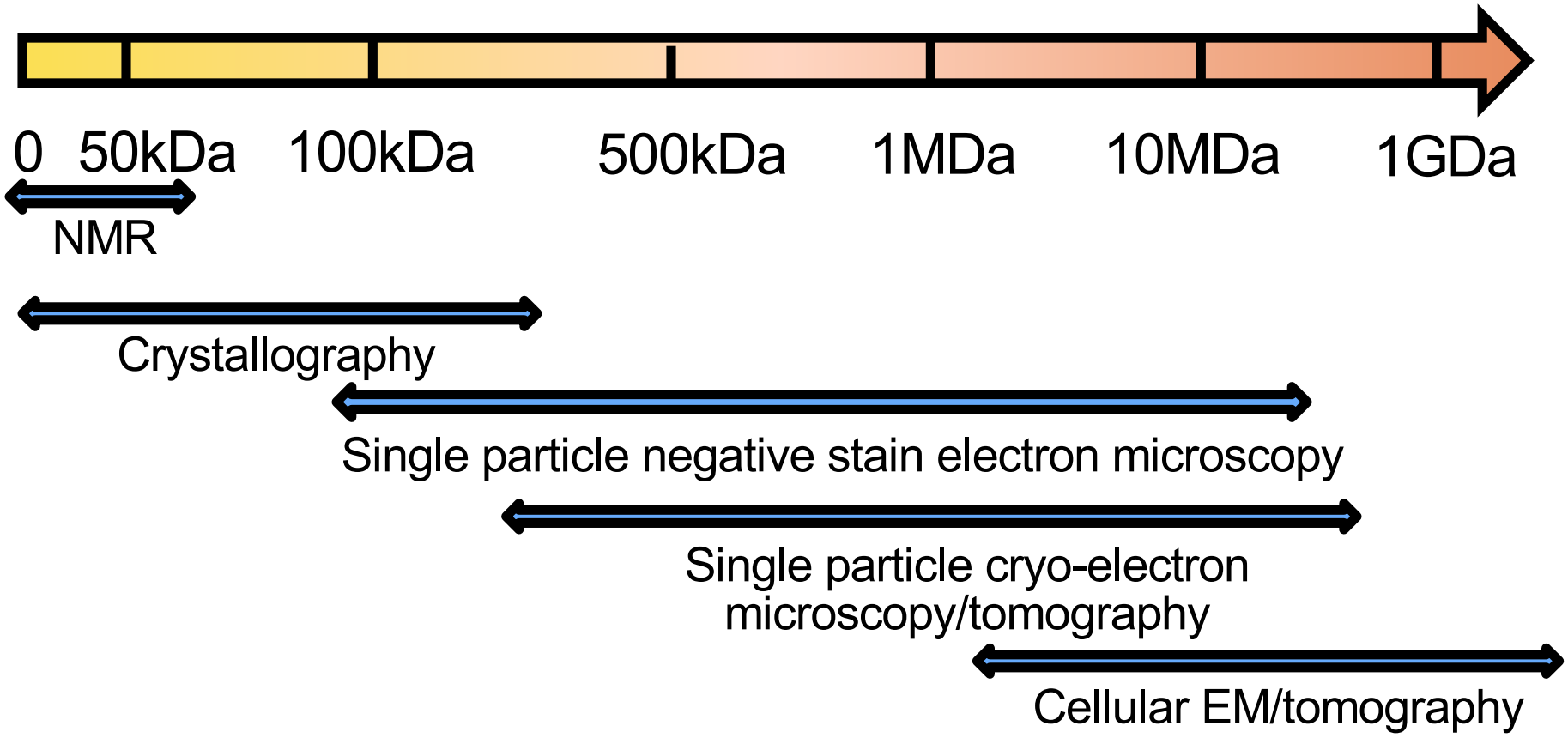
(B) While the last glycan in the D1 arm has not been built, we see a small amount of density in this region (orange) connecting the N262 D1 arm glycan to the base and D2 arm of the N301 glycan.

(C) The N332 glycan from 3TYG structure fits tightly into the EM density. In blue and lavender are PGT128 Fab HC and LC, respectively.

(D) The N301 glycan and its density.

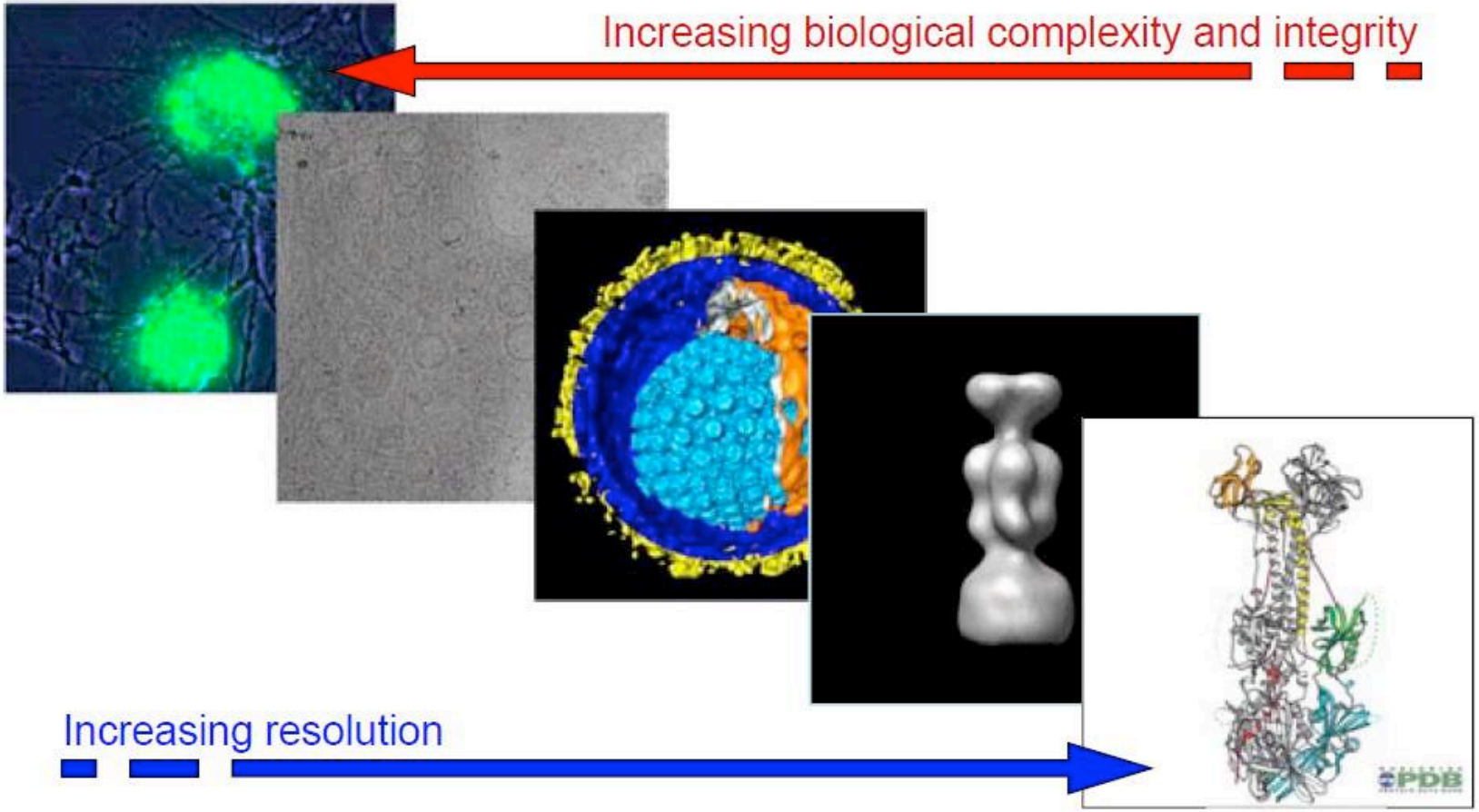
(E) The D3 arm of N301 does not interact with P74 (yellow) in PGT128 (HC shown in blue) as was suggested by the X-ray structure.

Electron microscopy in structural biology: Which objects can be analysed



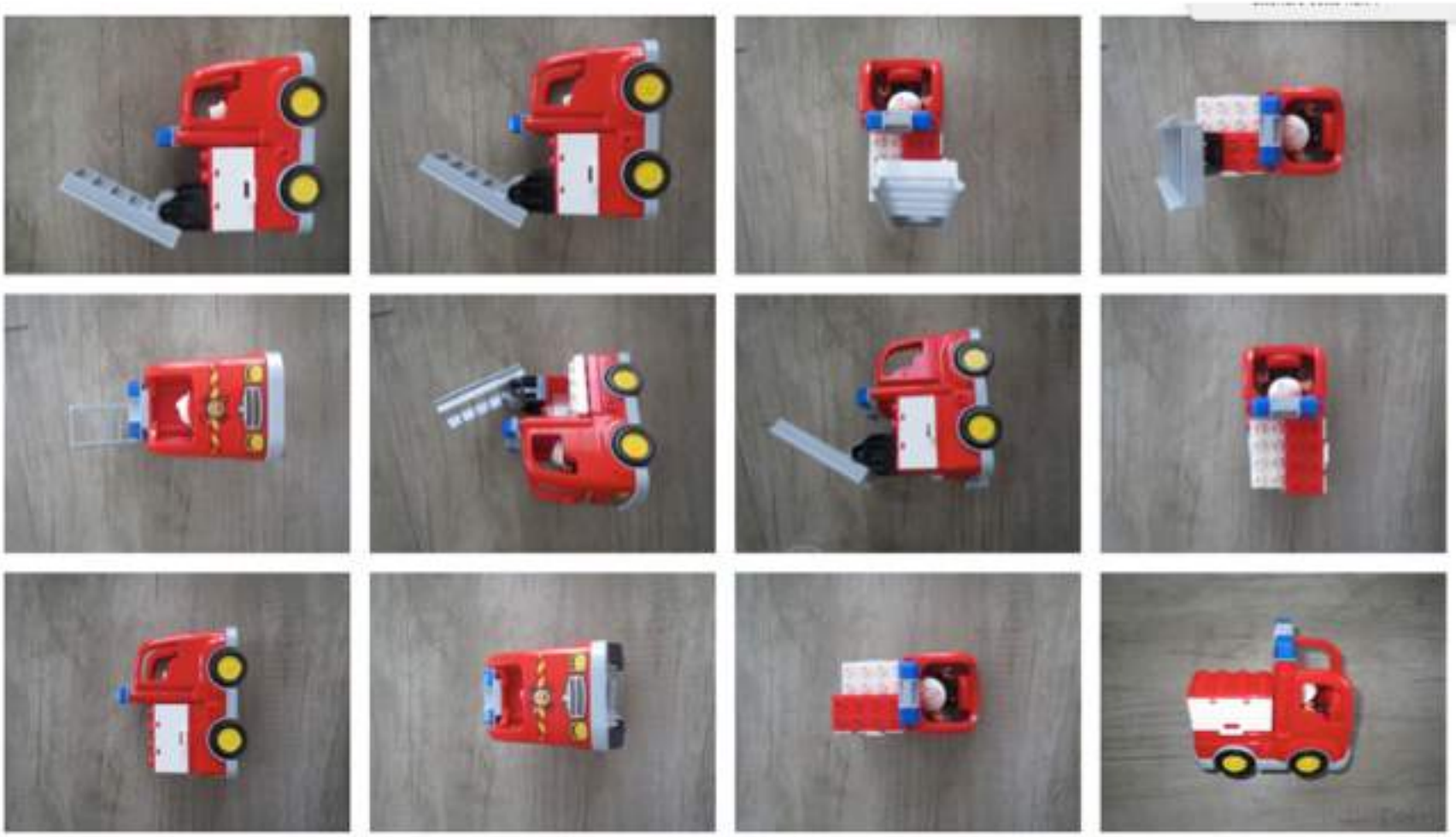
Method combinaison to study a biological process

And a dream begins to becomes true: understanding a system from the cellular context to the atomic level



How to deal with structural heterogeneity ?

- Most datasets are, to some extent, heterogeneous.
- For example, you can have a dataset with your complex in both active and inactive states.



- Cryo-EM image analysis allows to classify these 3D heterogeneities
- Generation of X initial reconstructions (can be obtained from random subsets of images with assigned euler angles)
- « Competitive » projection matching.