Cryo-electron microscopy,
a key player in integrative structural biology

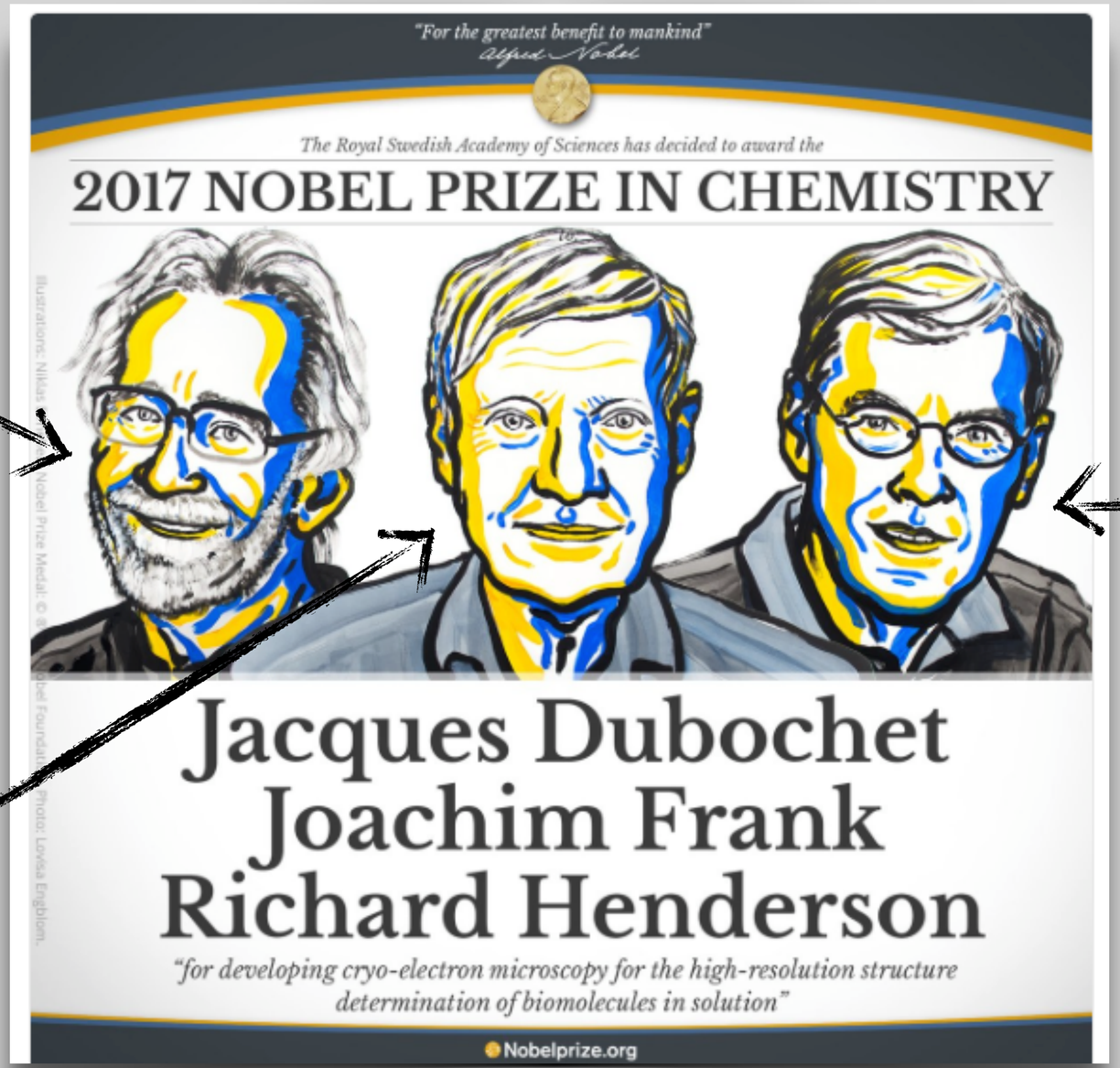
Adeline GOULET

RéNaFoBiS

Oléron 2018

Sample vitrification

Image analysis

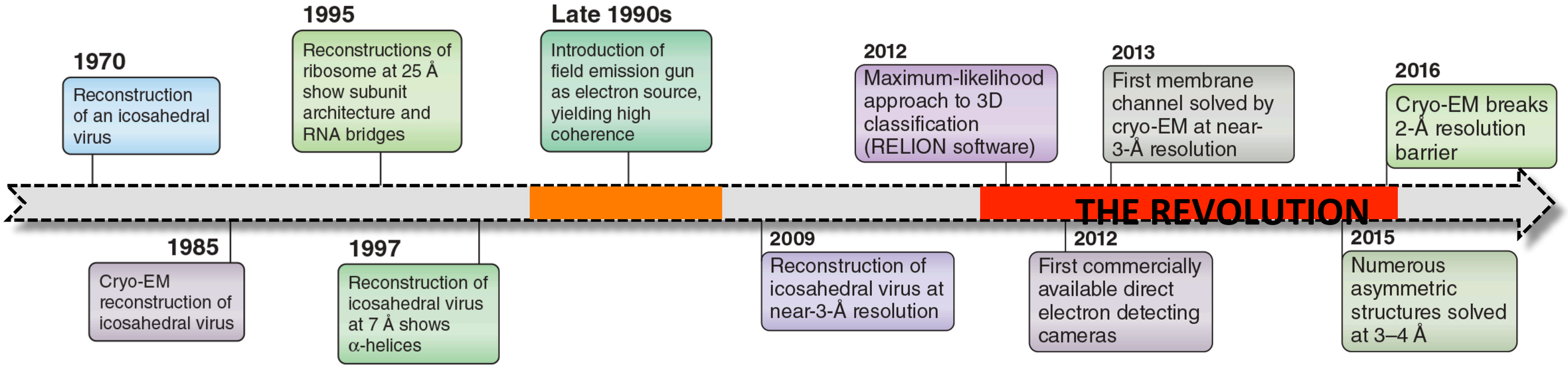


First atomic structure

« It's opened up a previously unapprochable area of structural biology » R. Henderson

A ‘revolution in resolution’

Joachim Frank, Nature protocols, 2017



Acquisition

Detection

Computation

A ‘revolution in resolution’

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.

How Cryo-EM Became so Hot

Yifan Cheng,[1] Robert M. Glaeser,[2,*] and Eva Nogales[2,3]
[1]Department of Biochemistry and Biophysics, and Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, CA, USA
[2]Lawrence Berkeley National Laboratory, Berkeley, CA, USA
[3]Department of Molecular and Cell Biology, and Howard Hughes Medical Institute, University of California, Berkeley, Berkeley, CA, USA
*Correspondence: rmglaeser@lbl.gov
https://doi.org/10.1016/j.cell.2017.11.016

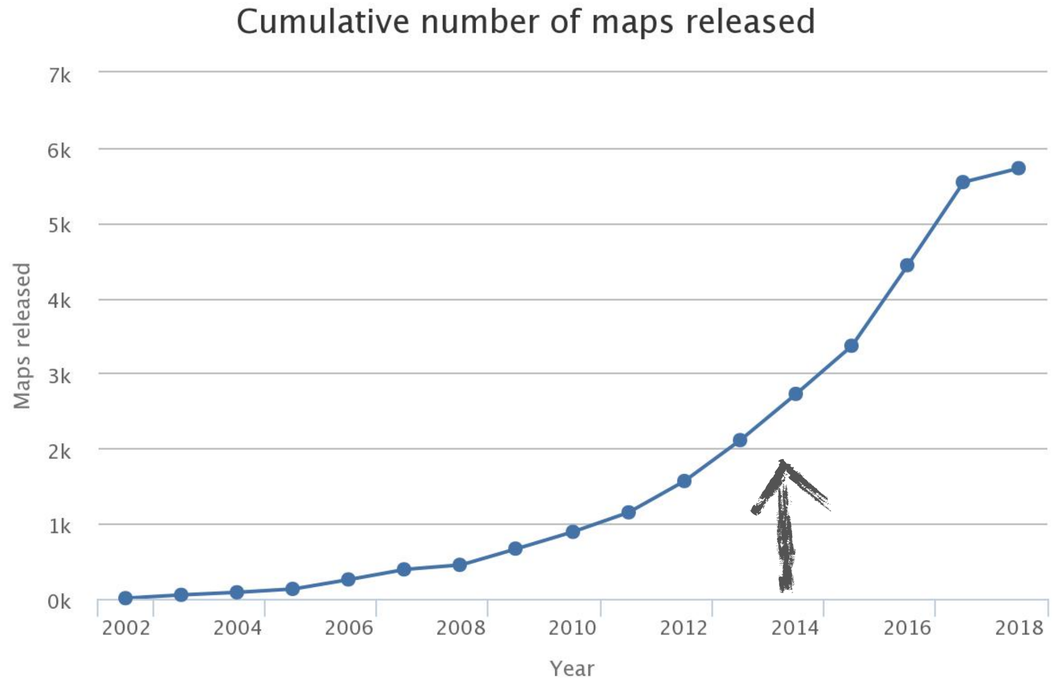
Review

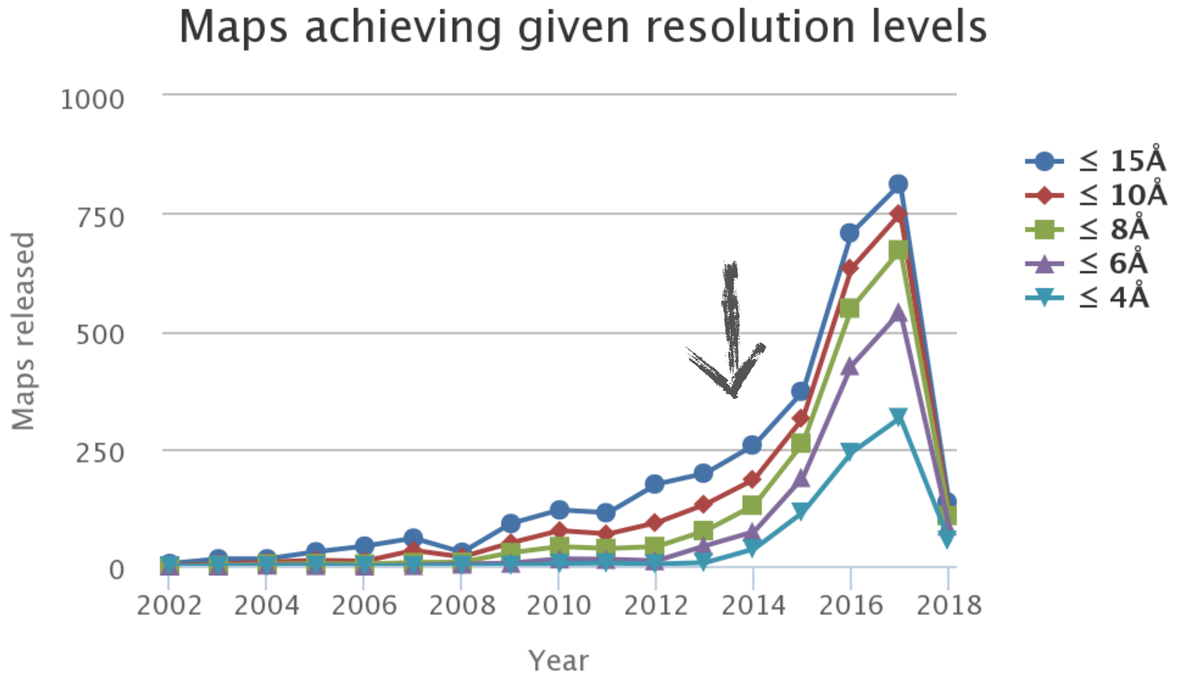
CellPress

How cryo-EM is revolutionizing structural biology

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

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





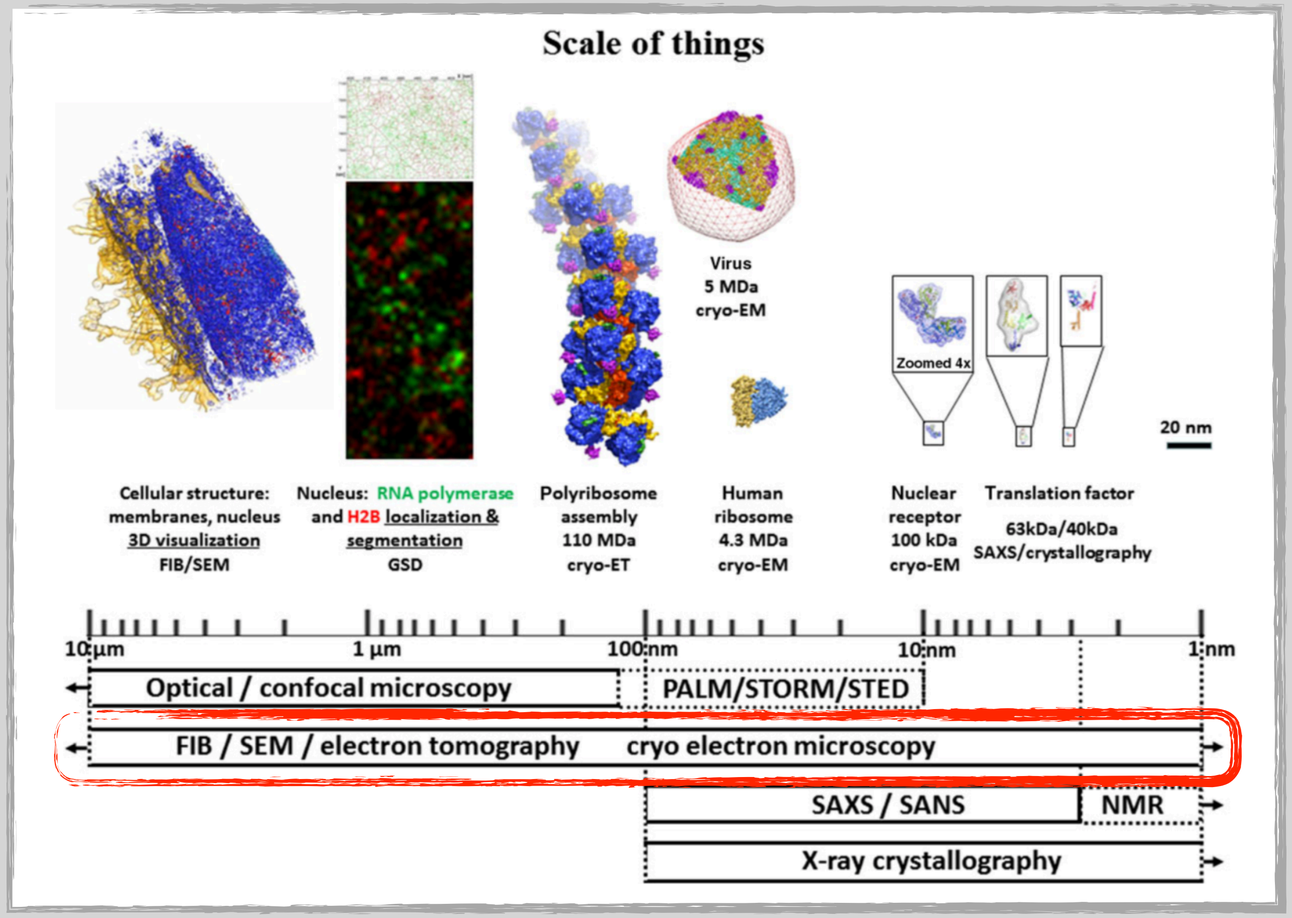
Biol. Cell (2017) **109,** 81–93 DOI: 10.1111/boc.201600042

Review

The integrative role of cryo electron microscopy in molecular and cellular structural biology

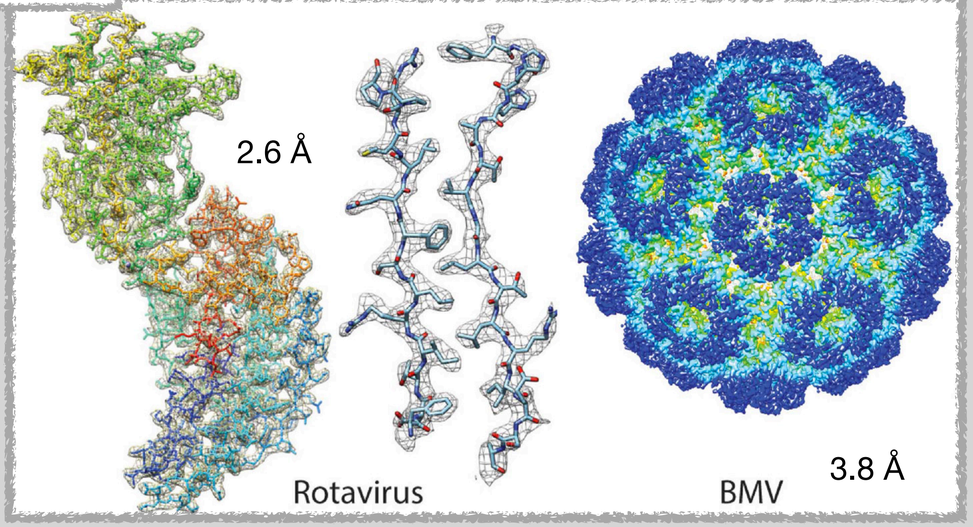
Igor Orlov*†‡§, Alexander G. Myasnikov*†‡§, Leonid Andronov*†‡§, S. Kundhavai Natchiar*†‡§, Heena Khatter*†‡§[2], Brice Beinsteiner*†‡§, Jean-François Ménétret*†‡§, Isabelle Hazemann*†‡§, Kareem Mohideen*†‡§, Karima Tazibt*†‡§, Rachel Tabaroni*†‡§, Hanna Kratzat*†‡§, Nadia Djabeur*†‡§, Tatiana Bruxelles*†‡§, Finaritra Raivoniaina*†‡§, Lorenza di Pompeo*†‡§, Morgan Torchy*†‡§, Isabelle Billas*†‡§, Alexandre Urzhumtsev*†‡§ and Bruno P. Klaholz*†‡§[1]

*Centre for Integrative Biology (CBI), Department of Integrated Structural Biology, IGBMC (Institute of Genetics and of Molecular and Cellular Biology), Illkirch, France, †Centre National de la Recherche Scientifique (CNRS) UMR 7104, Illkirch, France, ‡Institut National de la Santé et de la Recherche Médicale (INSERM) U964, Illkirch, France, and §Université de Strasbourg, Strasbourg, France

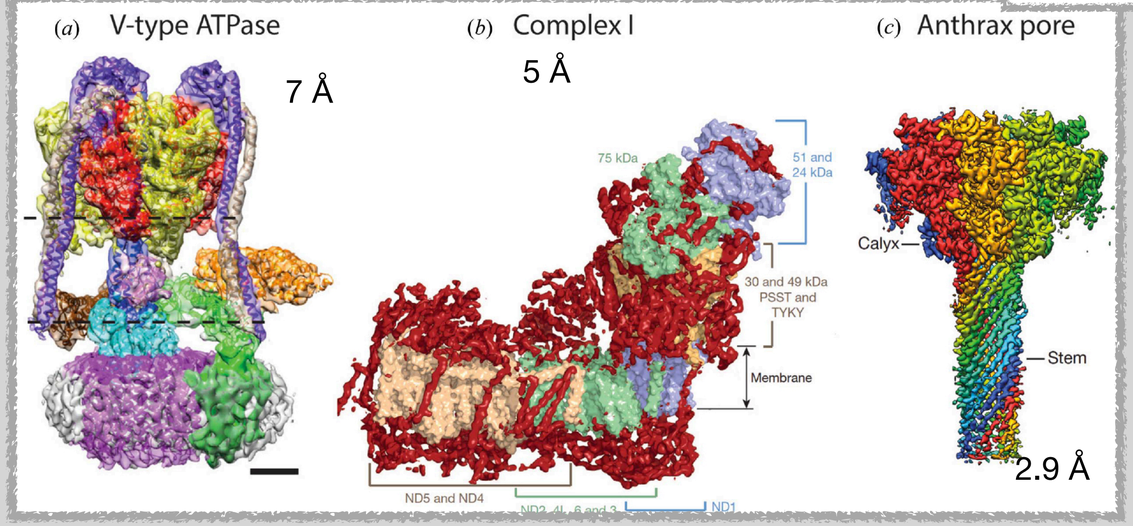


Molecular cryo-EM

Large

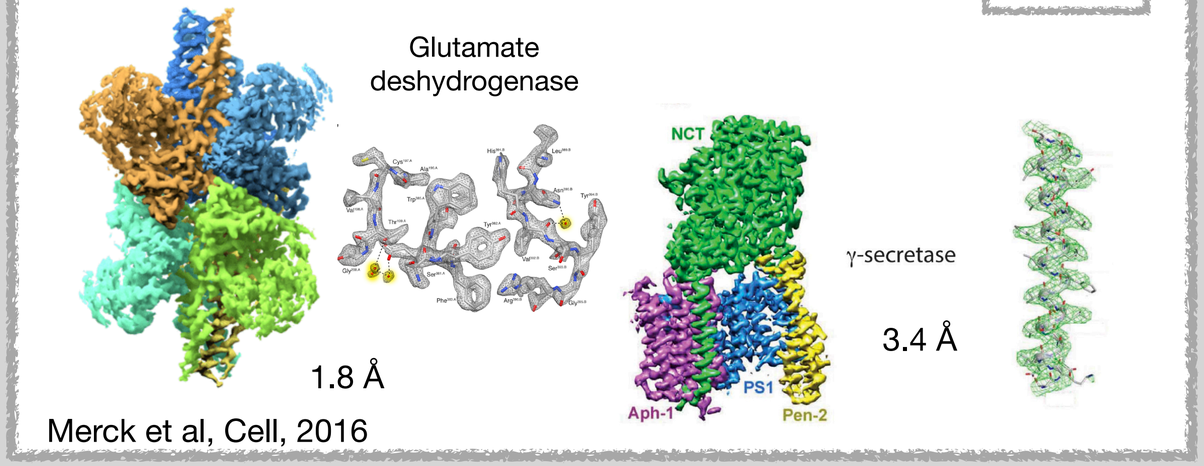


Medium



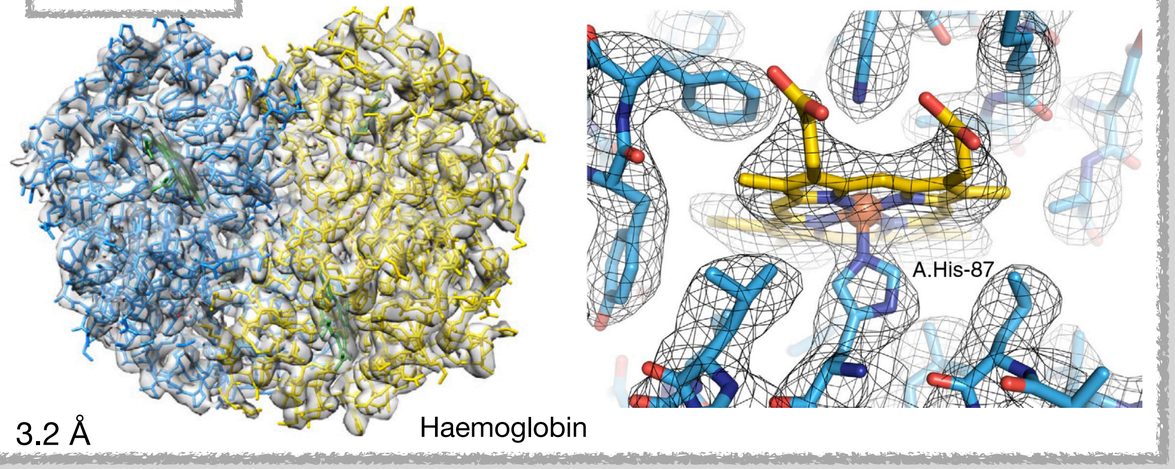
Subramanian et al, IUCRJ, 2016

Small



Merck et al, Cell, 2016

X-small



Haemoglobin

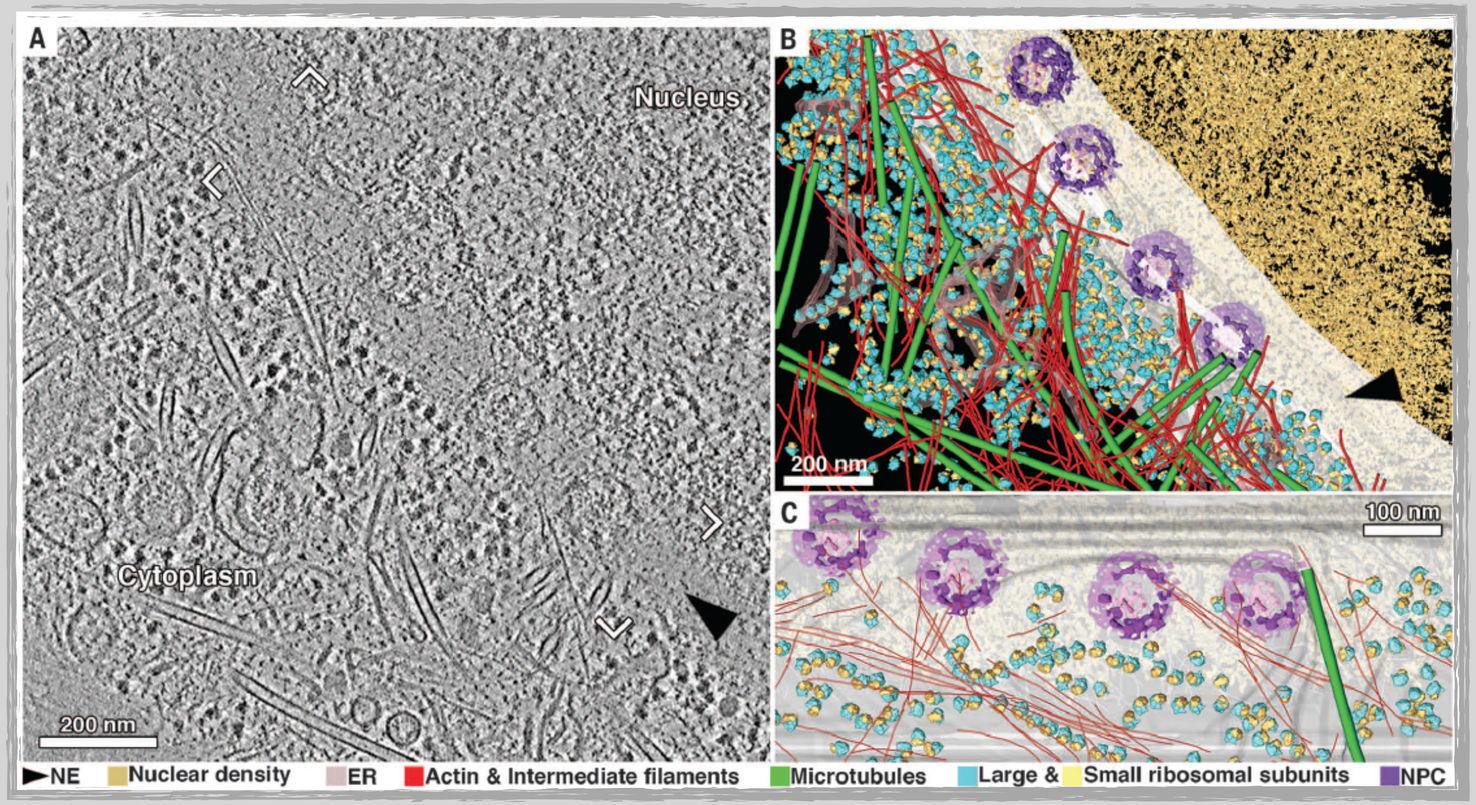
Cellular cryo-EM

CELL NUCLEUS

SCIENCE

Visualizing the molecular sociology at the HeLa cell nuclear periphery

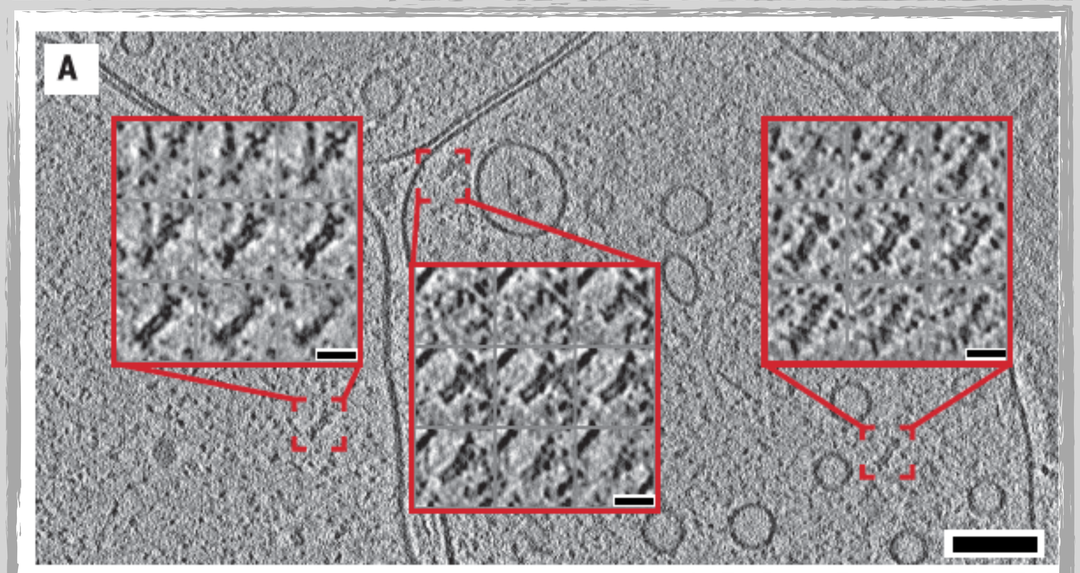
Julia Mahamid,[1]* Stefan Pfeffer,[1] Miroslava Schaffer,[1] Elizabeth Villa,[1,2] Radostin Danev,[1] Luis Kuhn Cuellar,[1] Friedrich Förster,[1] Anthony A. Hyman,[3] Jürgen M. Plitzko,[1] Wolfgang Baumeister[1]*



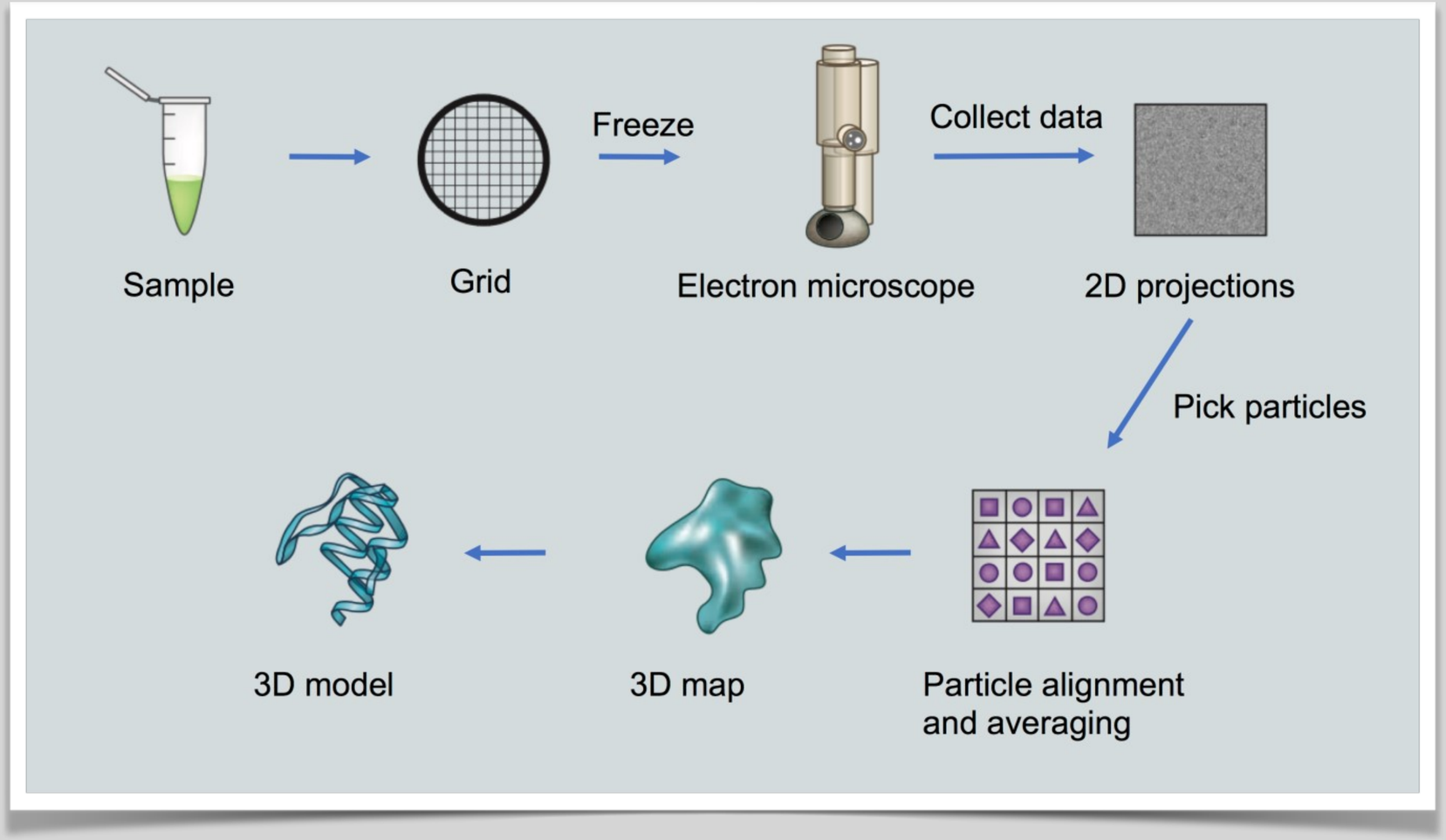
SCIENCE sciencemag.org 23 JANUARY 2015 • VOL 347 ISSUE 6220

A molecular census of 26*S* proteasomes in intact neurons

Shoh Asano,* Yoshiyuki Fukuda,* Florian Beck, Antje Aufderheide, Friedrich Förster, Radostin Danev, Wolfgang Baumeister†



HOW TO DETERMINE THE STRUCTURE OF YOUR FAVORITE BIOLOGICAL SAMPLE USING CRYO-EM AND SINGLE PARTICLE ANALYSIS?



INTRODUCTION TO SAMPLE PREPARATION AND IMAGE FORMATION/DETECTION IN TRANSMISSION ELECTRON MICROSCOPY

Adeline GOULET

Oléron 2018

1) Introduction to image formation

- basic anatomy of a microscope
- amplitude vs phase contrast

2) Overview of sample preparation for SP EM

- Negative staining
- Flash Freezing
- EM grids

Why electrons?

1924

Louis de Broglie

- **Wave-particle duality (Louis de Broglie) :**

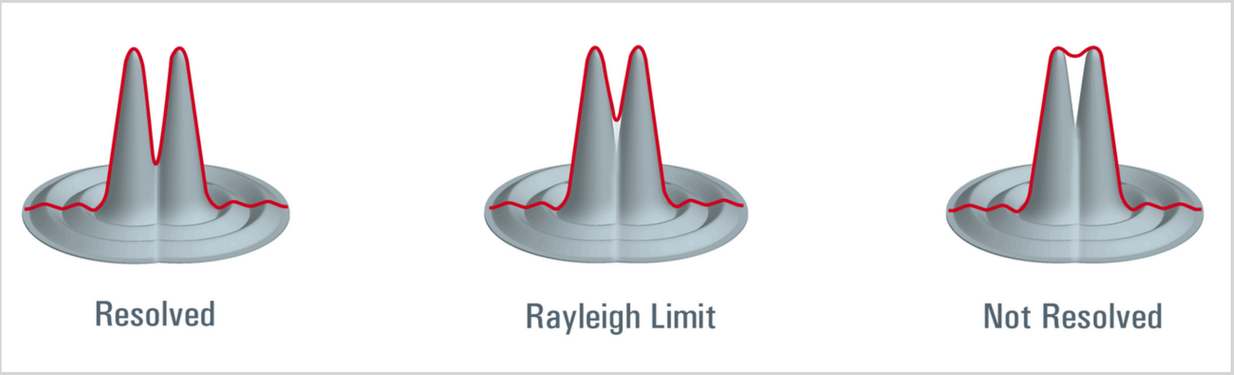
  A particle with a mass m and a speed v is characterized by a wave length λ

  $\lambda = h/(m.v)$ **(h=Planck constant)**

- **Rayleigh criterion:** **d (resolution) ~ 0.61 x λ**

Tension (kV)	Wavelength (Å)	Resolution (Å)
100	0,037	0,023
300	0,0197	0,012

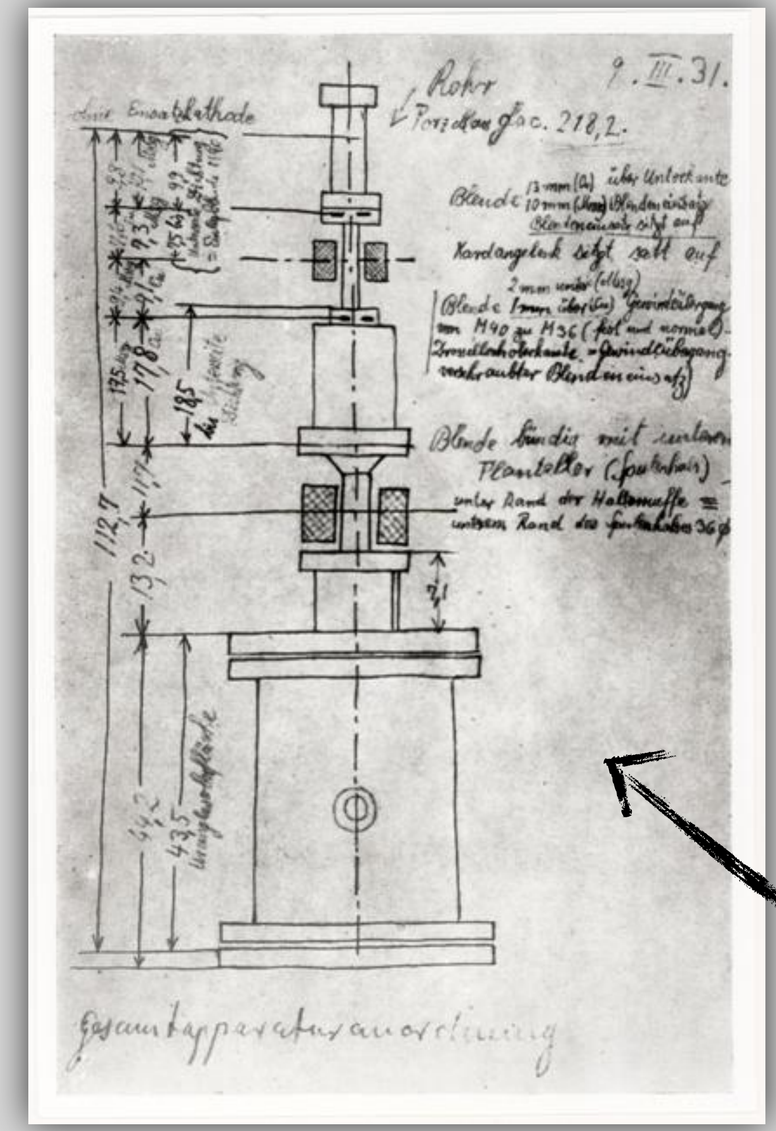
average atom radius ~ 1 Å // C-C ~ 1.54 Å



The electron beam wavelength is NOT a limiting parameter in getting atomic structures

Why electrons?

1931



Szilard (physicist) : « *Busch has shown that one can make electron lenses, de Broglie has shown that they have sub-Angstrom wave lights. Why don't you make an electron microscope, one could see atoms with it!* »

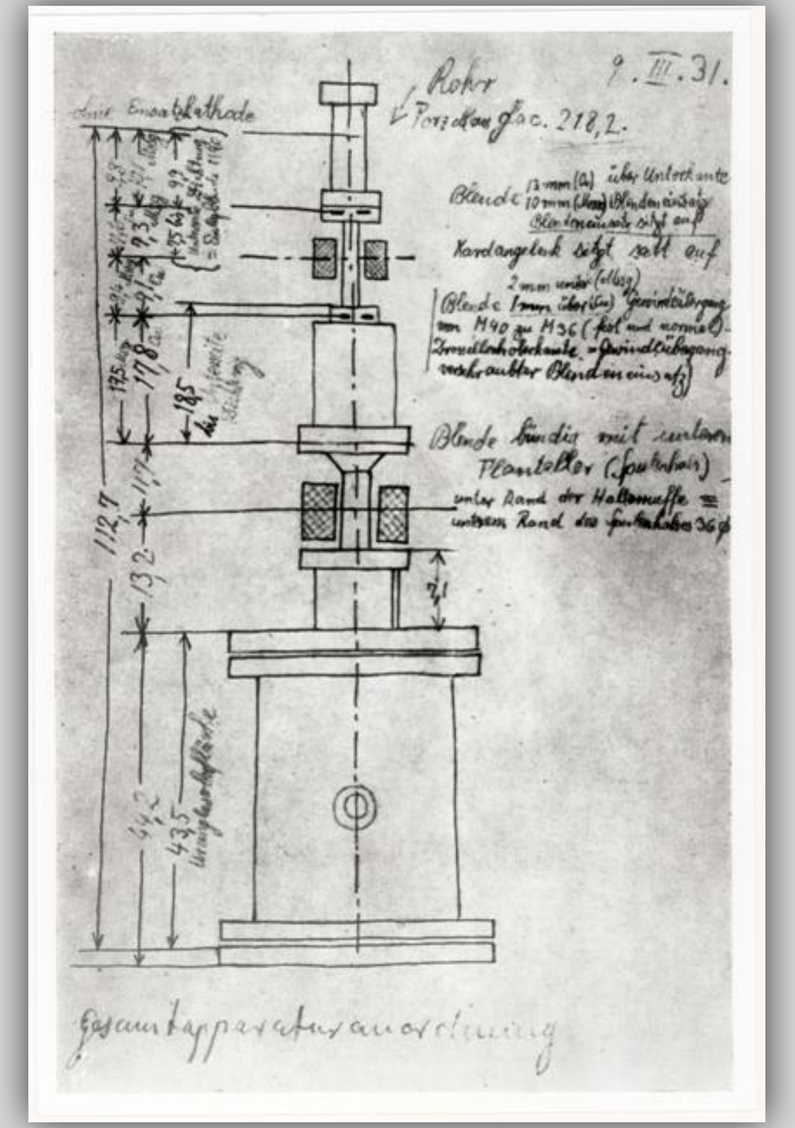
Gabor (physicist): « *Yes, I know. But one cannot put living matter into a vacuum and everything will burn anyway to a cinder under an electron beam* »

Ernst Ruska built the first TEM (mag 17.4)

Noble Prize in 1986

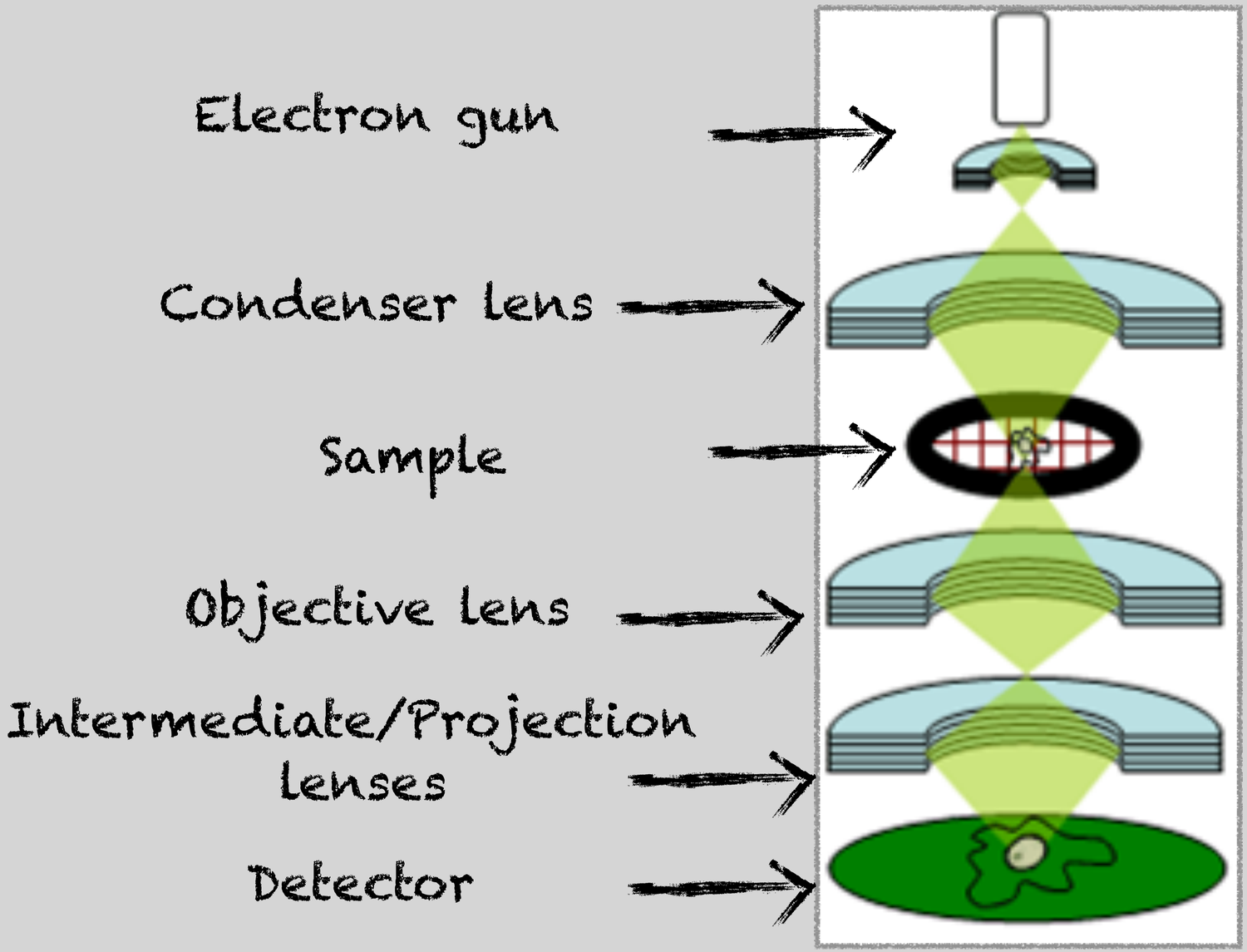
Why electrons?

1931



2005 TITAN Krios Thermo Scientific™

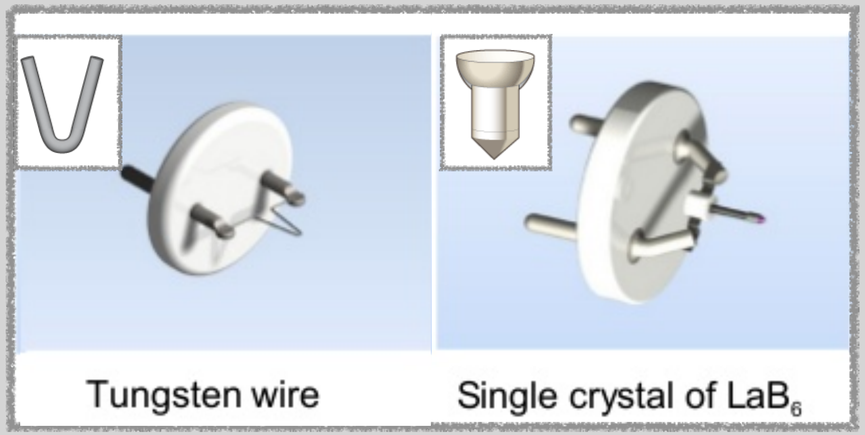
Basic anatomy of an EM



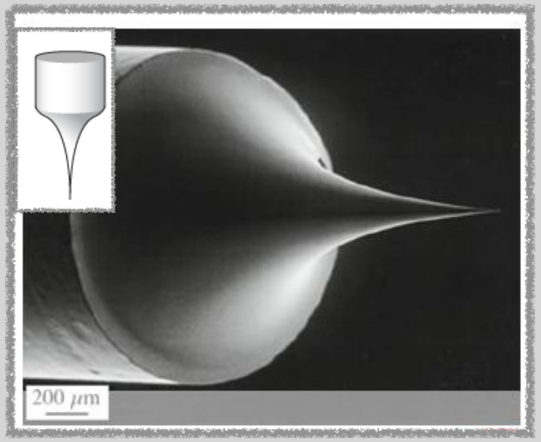
High vacuum ~ 10^{-6} Pa

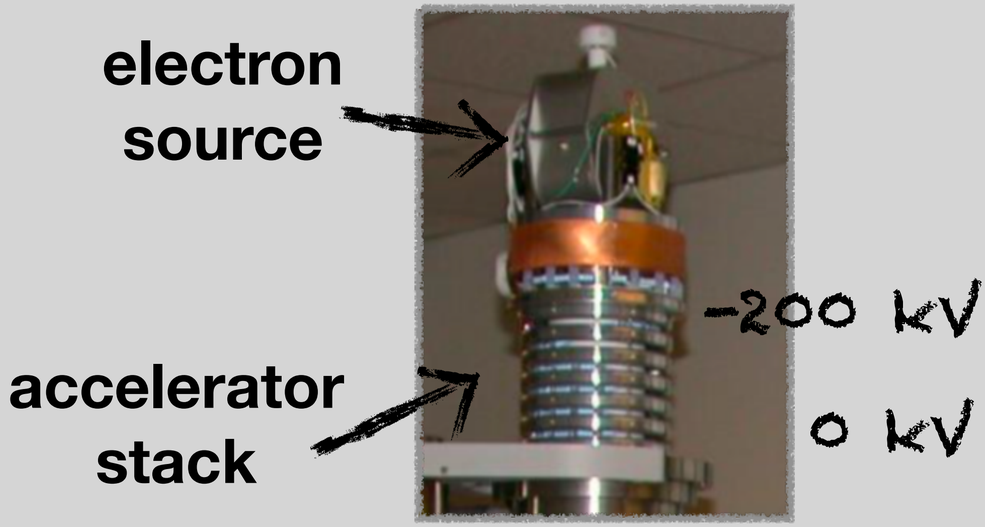
Electron sources

Thermionic emission



Field Emission Gun

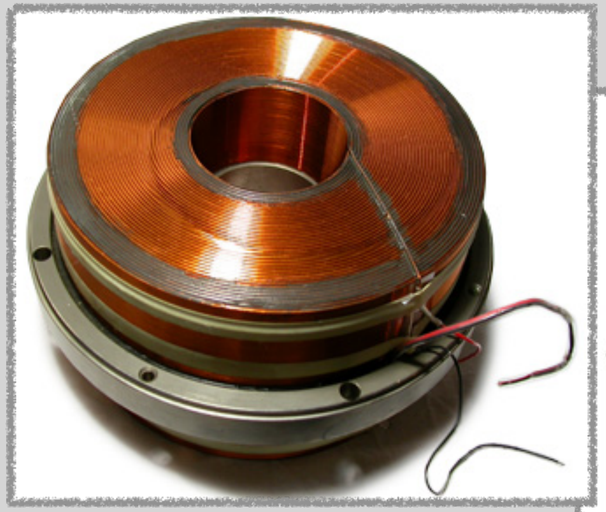


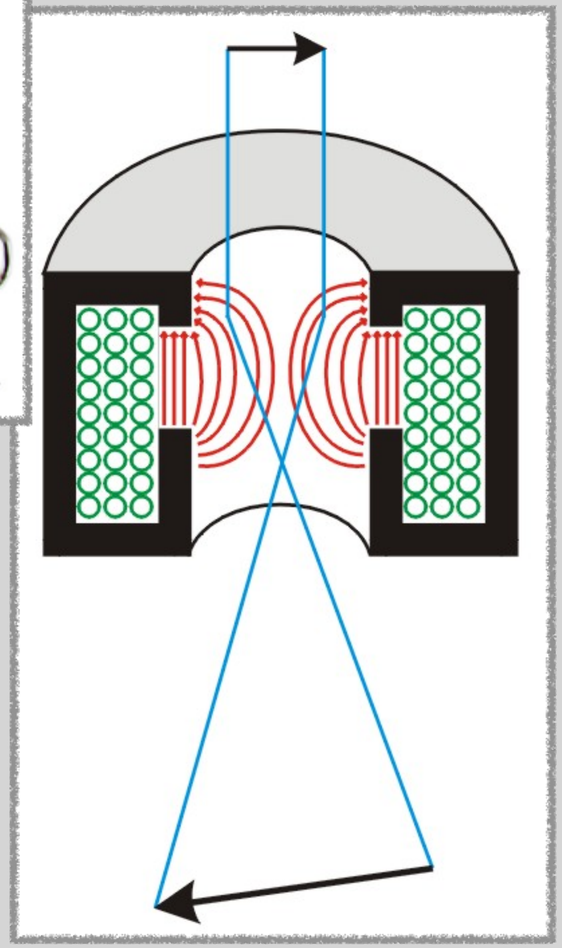


Coherence & brightness

- Brightness or spatial coherence (~ electrons density)
- Temporal coherence (same wavelength)

Electromagnetic lenses





object

magnetic field

copper wire (lens)

electron trajectories

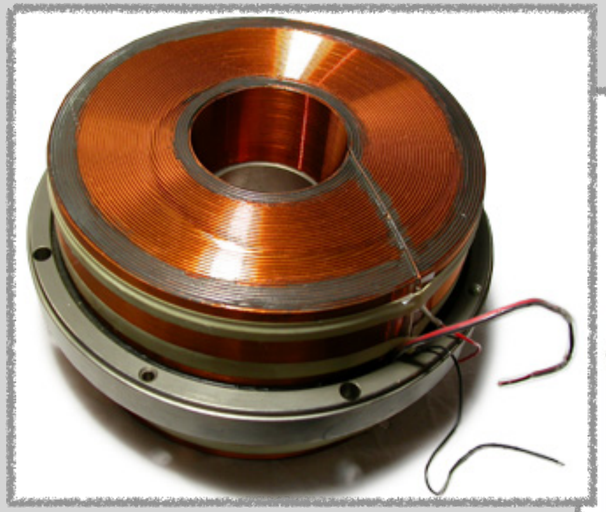
magnified image

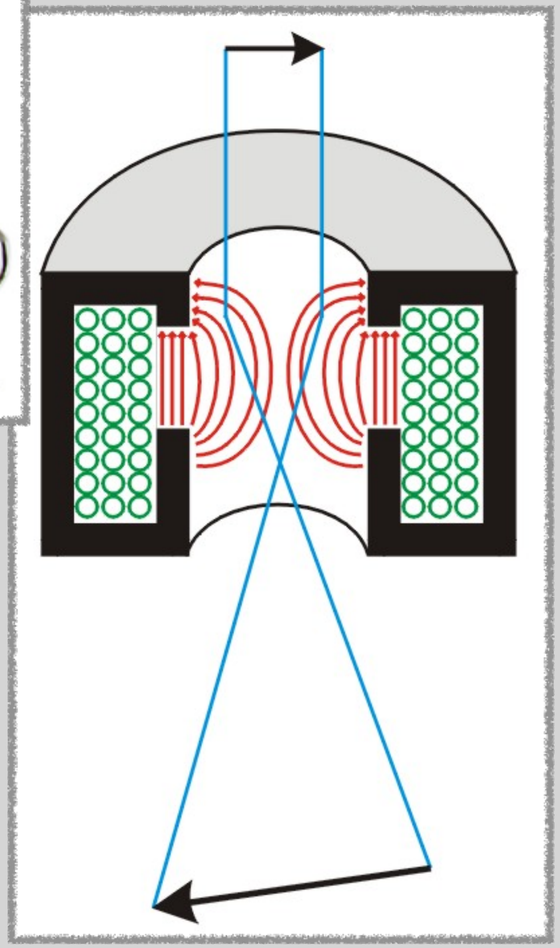
Lorentz force

$$\vec{F}=-e(\vec{v}\wedge\vec{B})$$

Electromagnetic lenses deviate and focalise electrons

Electromagnetic lenses





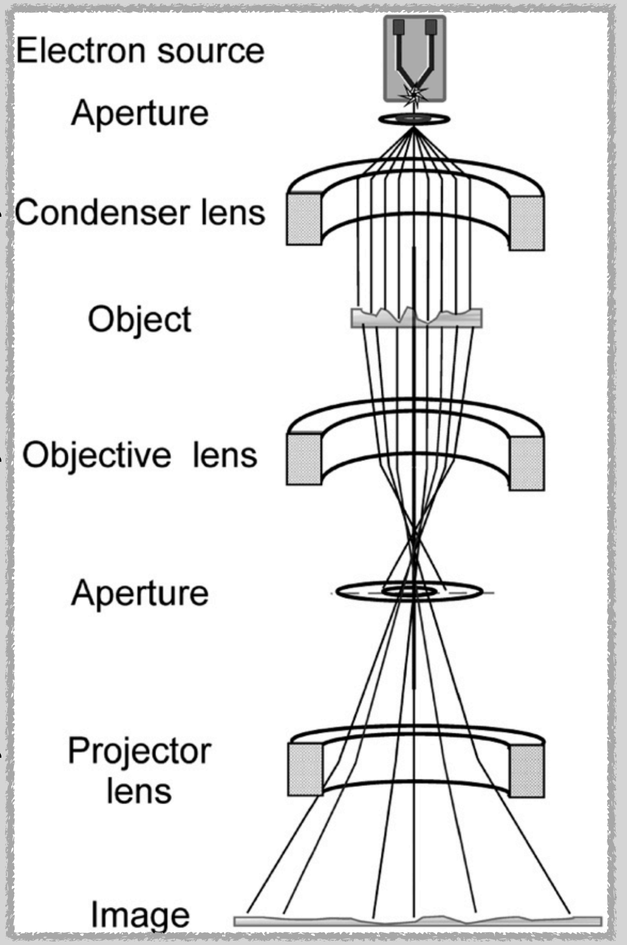
Lorentz force

$$\vec{F}=-e(\vec{v}\wedge\vec{B})$$

beam convergence → spot size

Image formation →

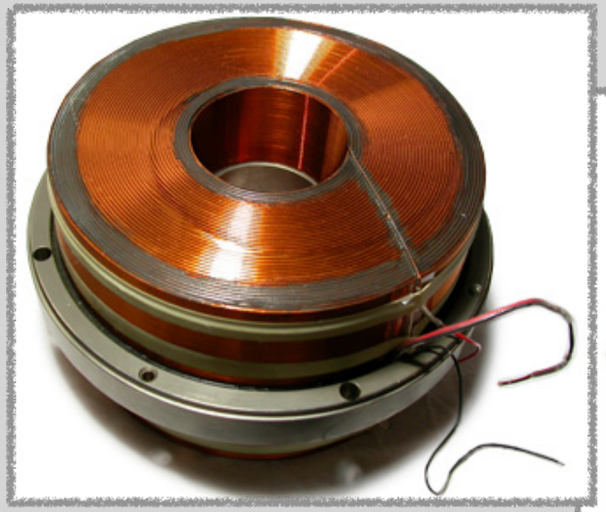
Image magnification →

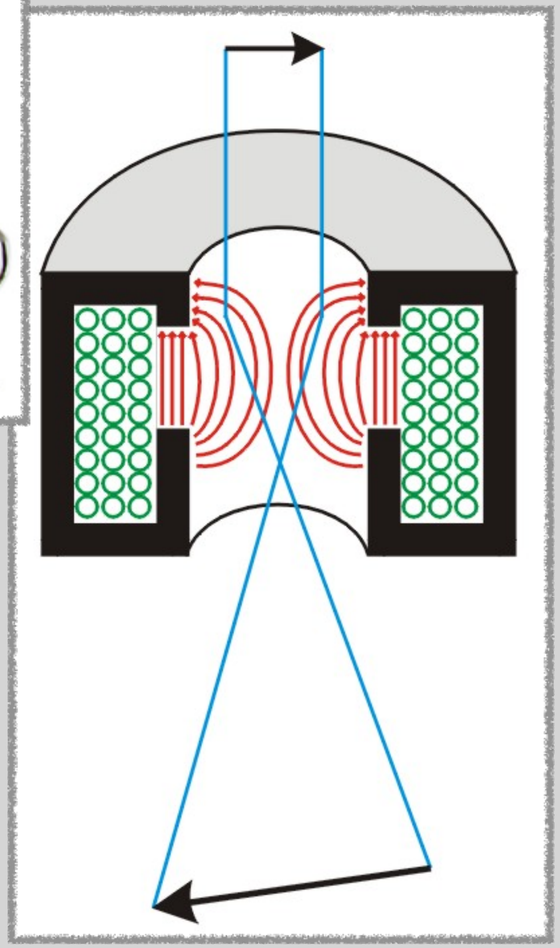


EV Orlova & HR Saibil, Chemical Reviews, 2011

Electromagnetic lenses deviate and focalise electrons

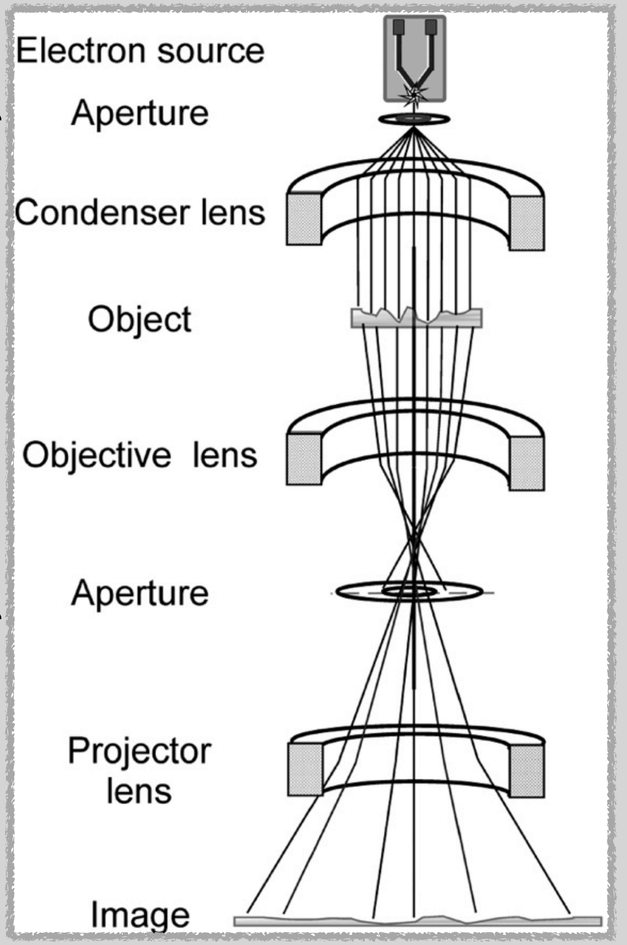
Electromagnetic lenses





selects electrons that make the beam

selects electrons that form the image



EV Orlova & HR Saibil, Chemical Reviews, 2011

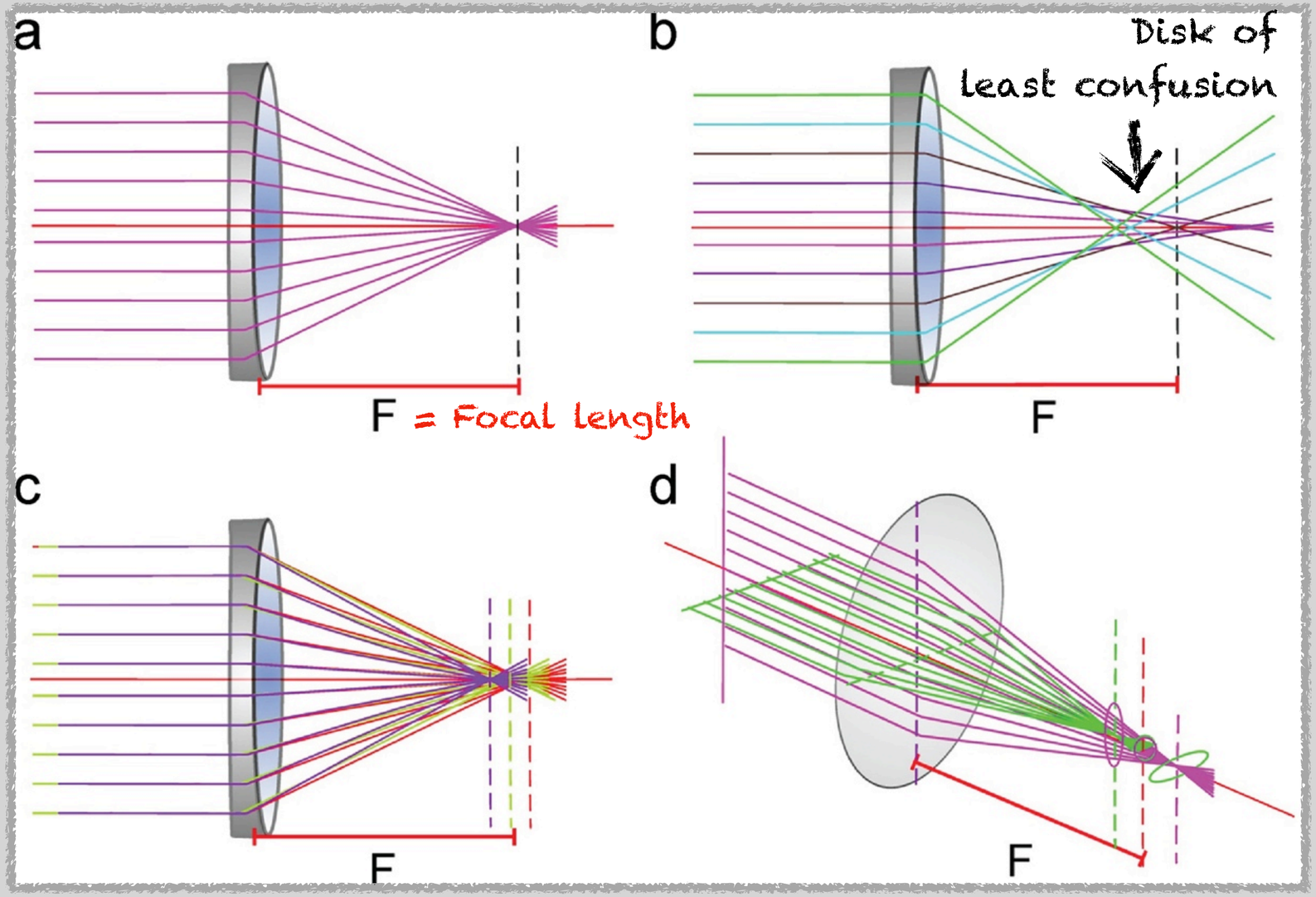
Lorentz force

$$\vec{F}=-e(\vec{v}\wedge\vec{B})$$

Electromagnetic lenses deviate and focalise electrons

Electromagnetic lenses

Perfect lens



Chromatic aberration

$ds = 0.5 \times Cs \times \alpha^3$

Cs: spherical aberration coefficient
α: angular aperture

Spherical aberration

****Cs correction****

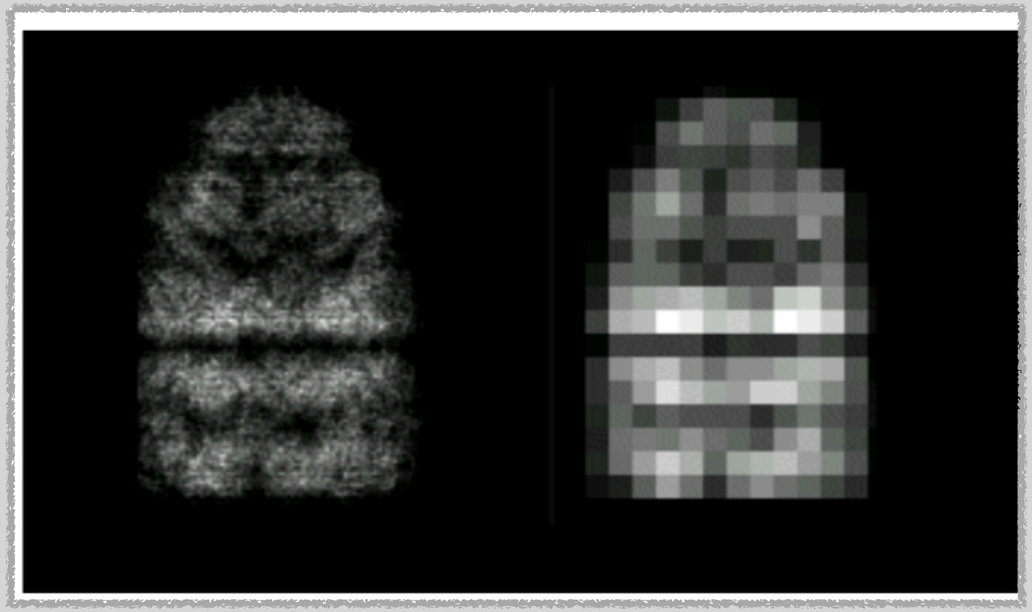
Astigmatism

(corrected with stigmators)

EV Orlova & HR Saibil, Chemical Reviews, 2011

Objective lens aberrations (focalisation defects) limit the resolution

Detectors

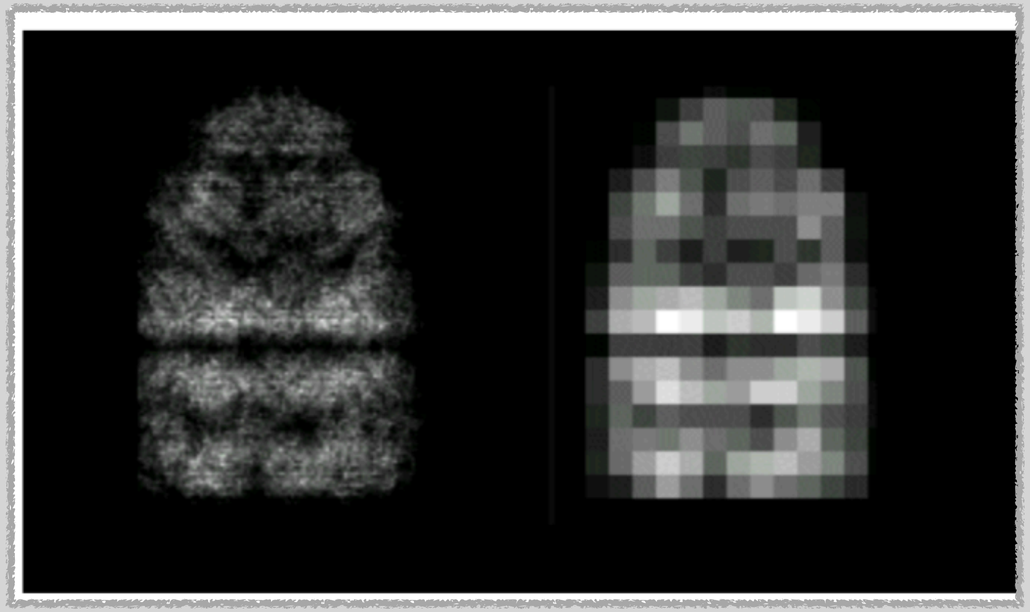


The image is divided up into pixels with a given sampling that determines the highest resolution to analyse.

Sampling (Å/pix)	Resolution (Å)
2	4
5	10

Nyquist limit = 2x(pixel size)

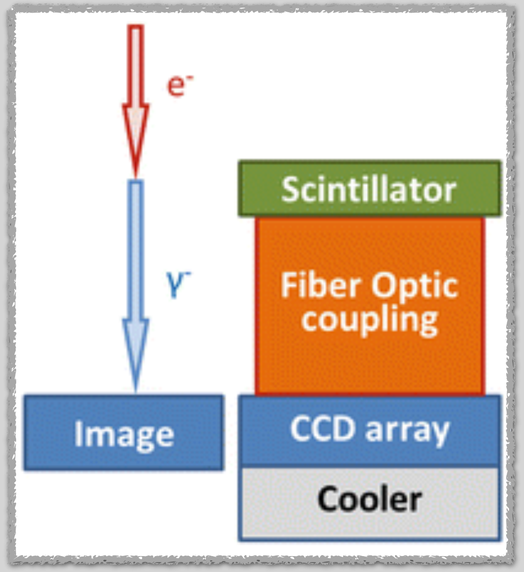
Detectors



Photographic film

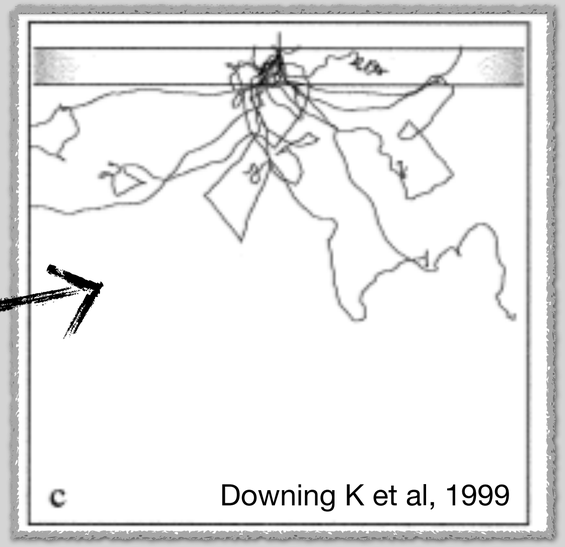
- Digitization (1 pixel= 8 µm)
- 1s/frame

CCD camera



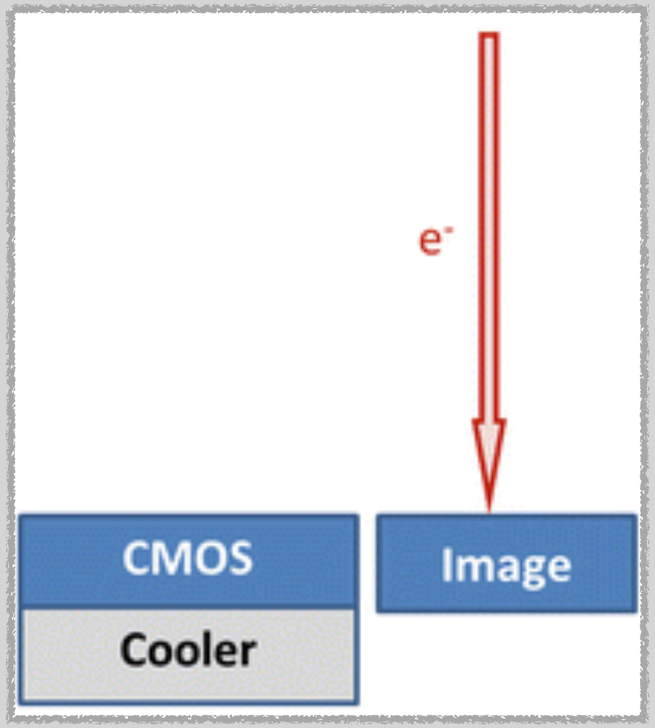
- Digital image (1 pixel= 14 µm)
- 1s/frame

Noise affecting resolution



Detectors

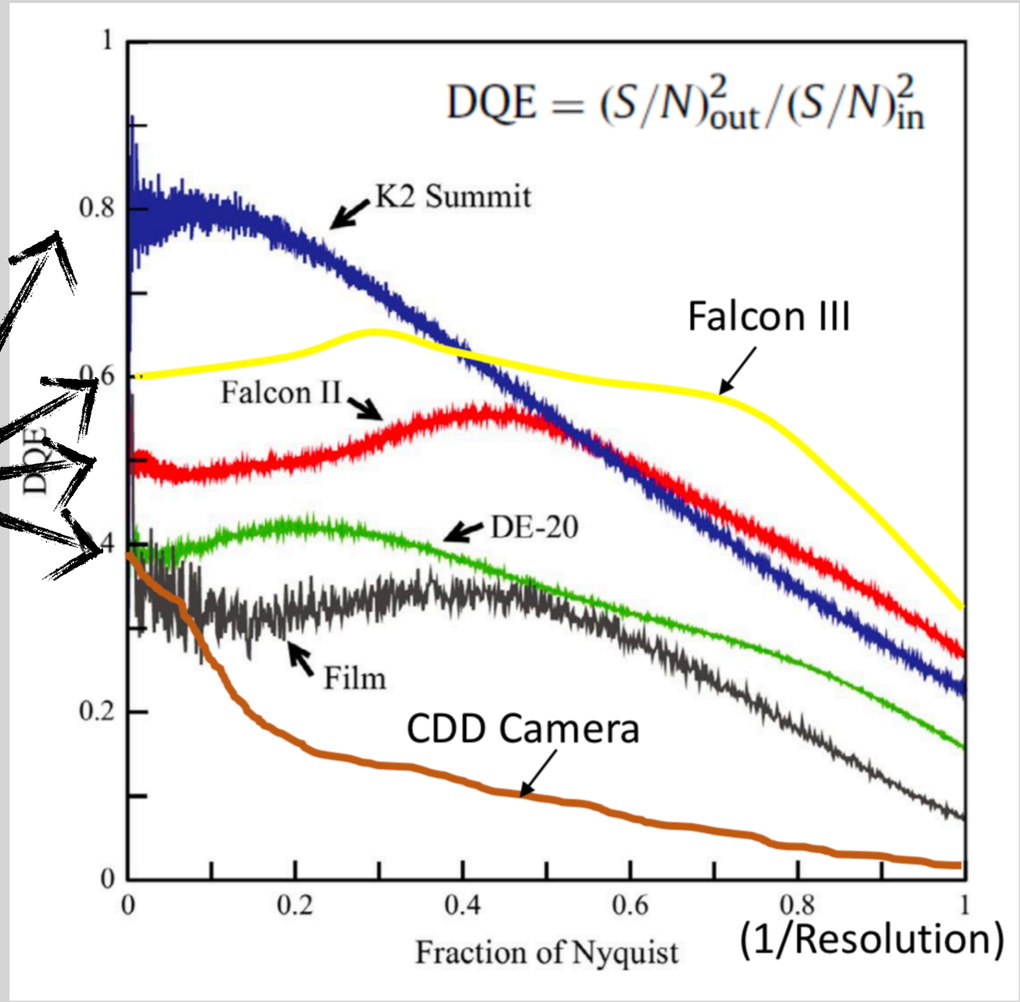
Direct electron detector



Higher DQE for DED

- Digital image (1 pixel= 5 µm)
- high speed read-out —> 400 images/sec

DQE (Detective Quantum Efficiency) ~ Sensitivity



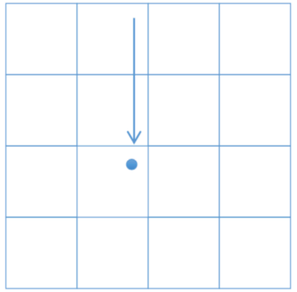
Courtesy of O. Lambert

Detectors

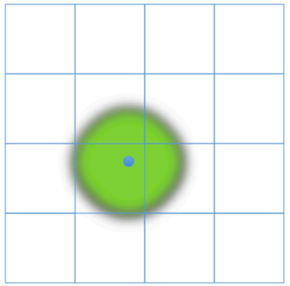
DED → Rolling read-out reduces noise in images

© Gatan

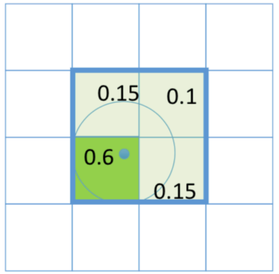
1. Electron enters detector



2. Signal is scattered

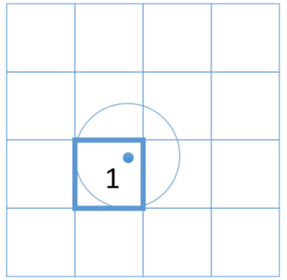


3. Charge collects in each pixel



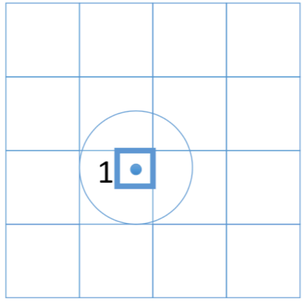
Integration mode

4. Events reduced to the highest charge pixels

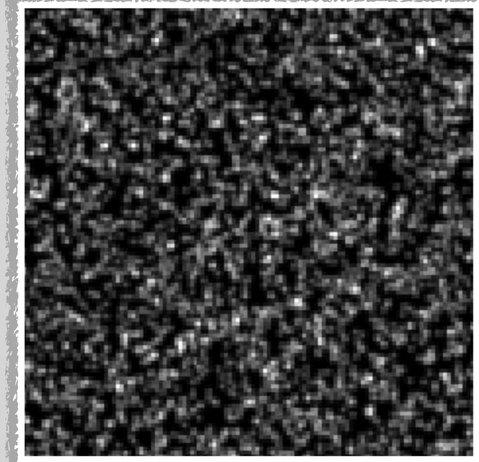


Counting mode
Improved DQE

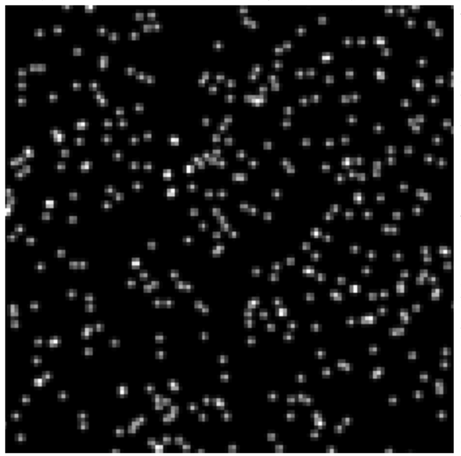
4b. Events localized with sub-pixel accuracy



Super-Resolution counting mode
Improved DQE **7680 x 7424** pixels



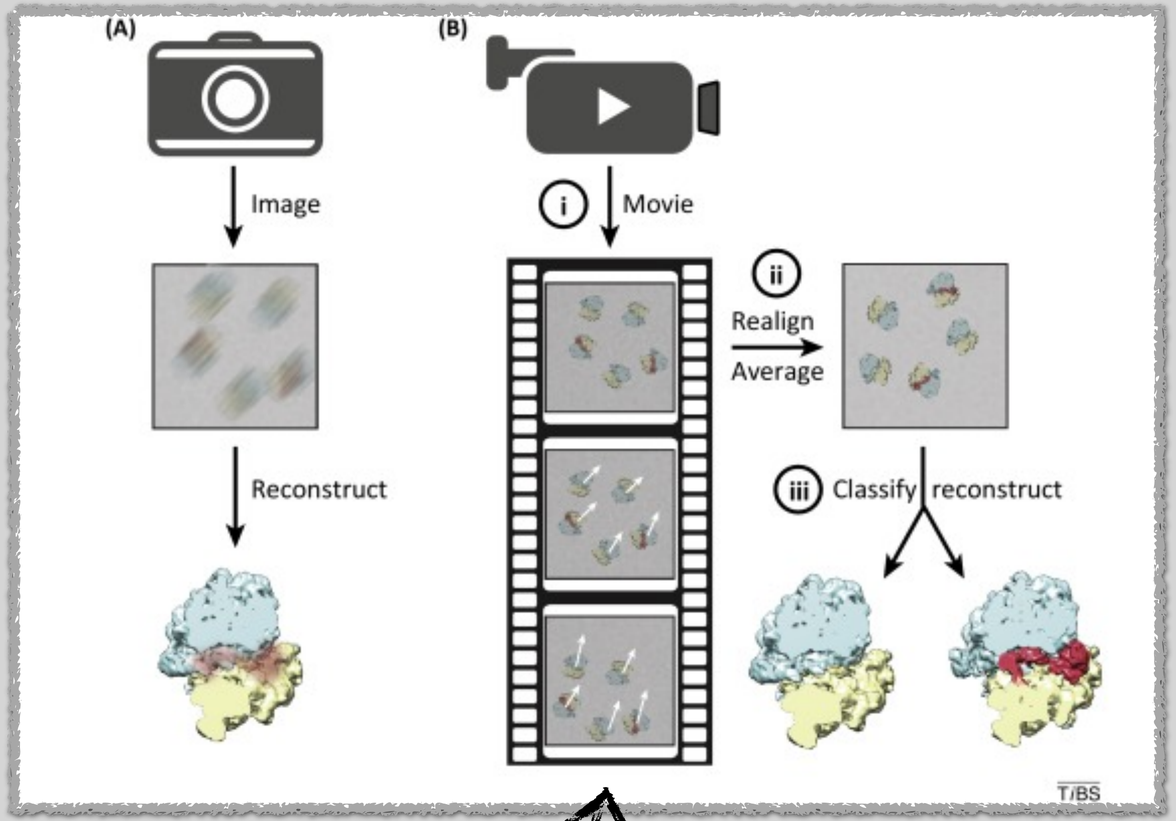
40 frames per second: events overlap and cannot be resolved.



400 frames per second: events are resolved.

Detectors

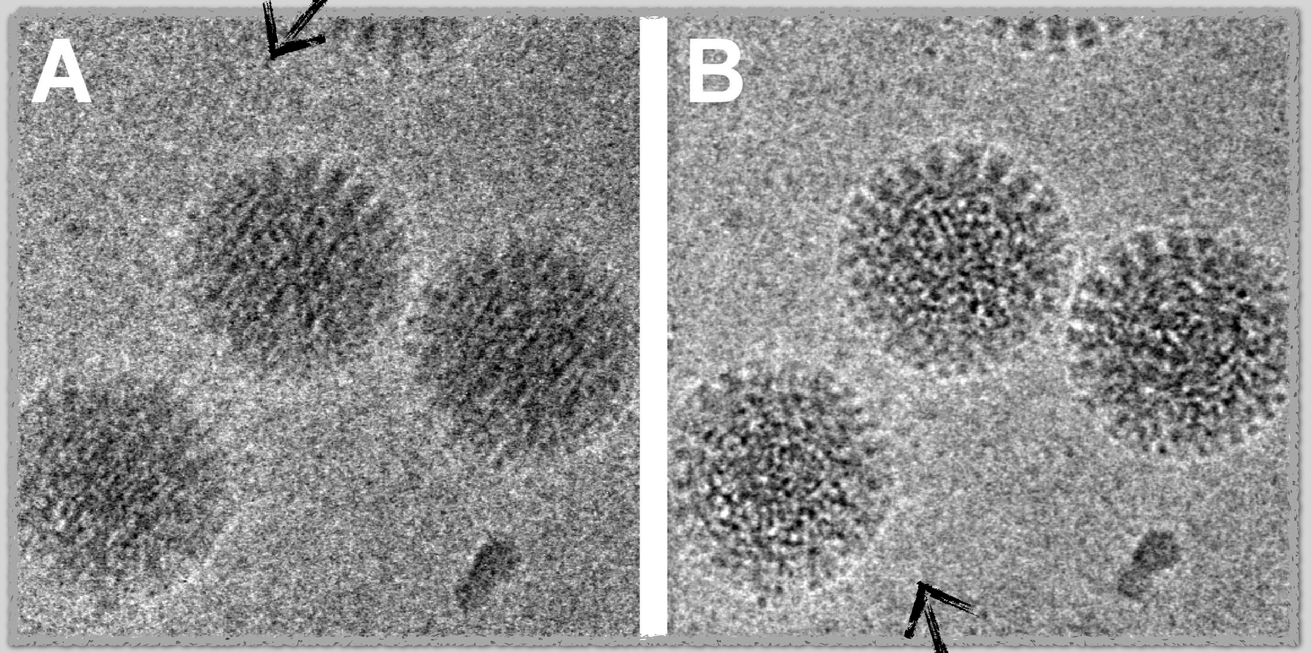
DED → Rolling read-out enables motion correction



Bai X et al, TiBS 2015

dose fractionation
40 e^-/A^2 total
1 $e^-/A^2/0.4s$ —> 40 frames

Average of frames that experienced translations



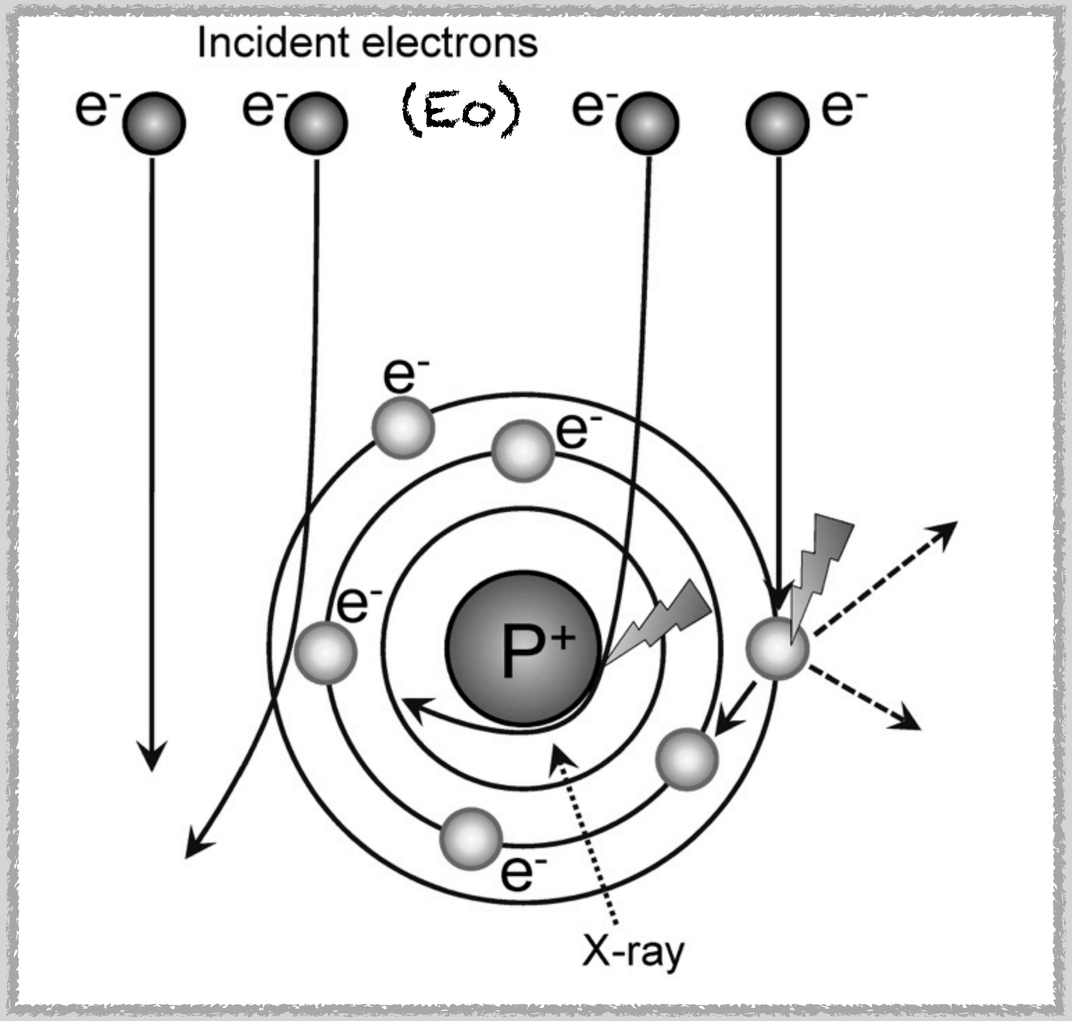
Brilot A et al, JSB 2012

Average after translational alignment of individual frames

Image formation

Unscattered electrons
(Eu=E0)

Elastic scattering
(Ee=E0)



EV Orlova & HR Saibil, Chemical Reviews, 2011

Inelastic scattering
(low angle, Ei<E0)

RADIATION DAMAGE

= Fundamental limit for resolution

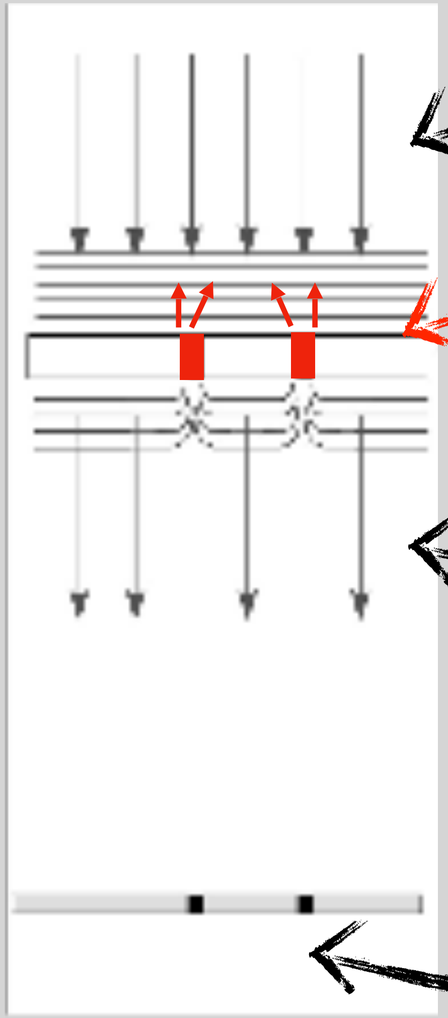
Image formation

Amplitude contrast (electrons as particules)

Amplitude contrast

=

lose of electrons



Incident electrons

Backscattered electrons

dense object, heavy atoms

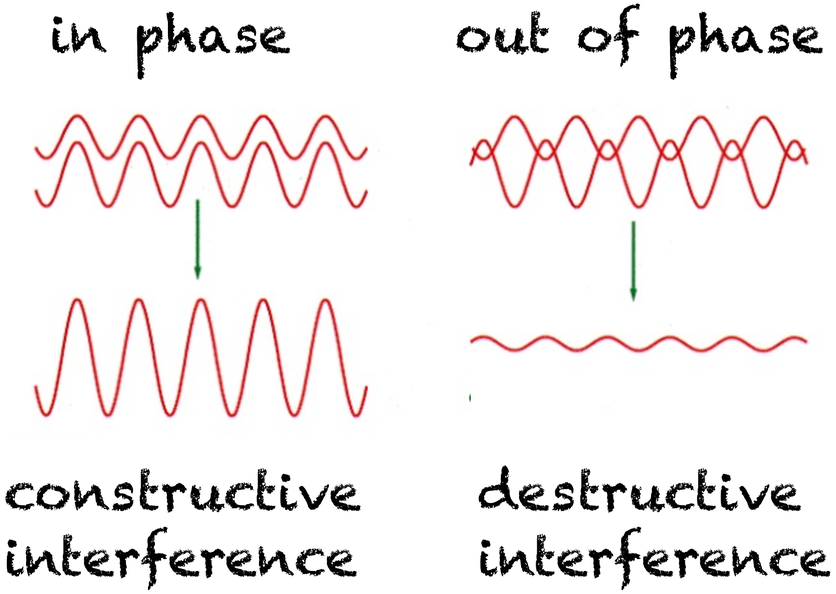
Transmitted electrons

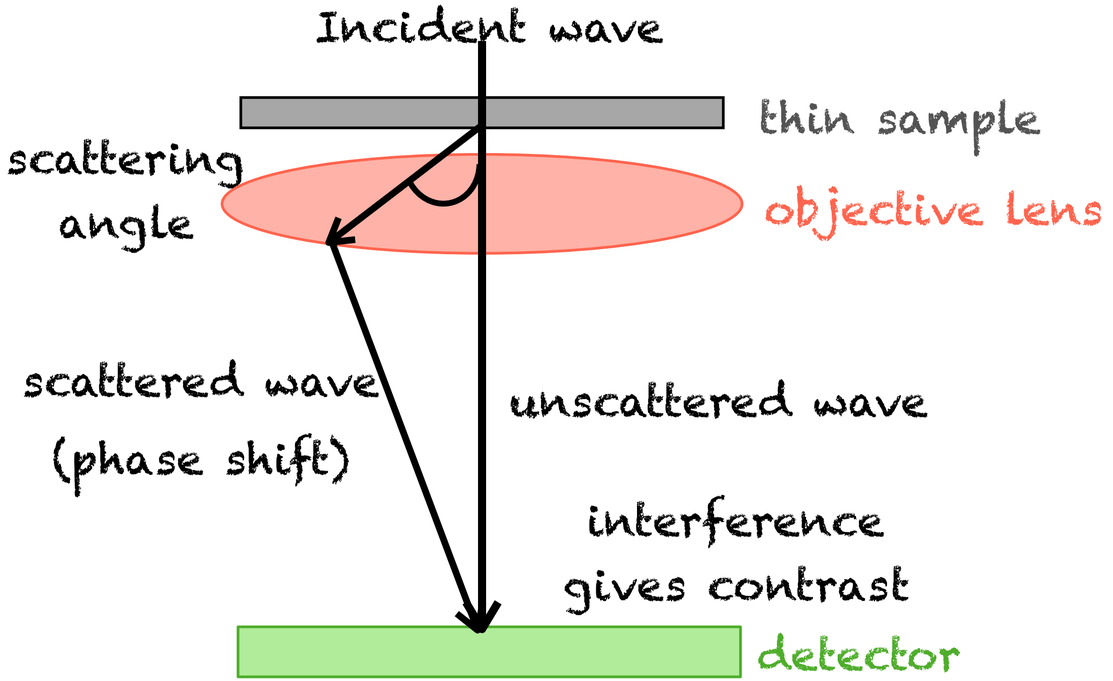
Backscattered vs transmitted electrons create contrast

EV Orlova & HR Saibil, Chemical Reviews, 2011

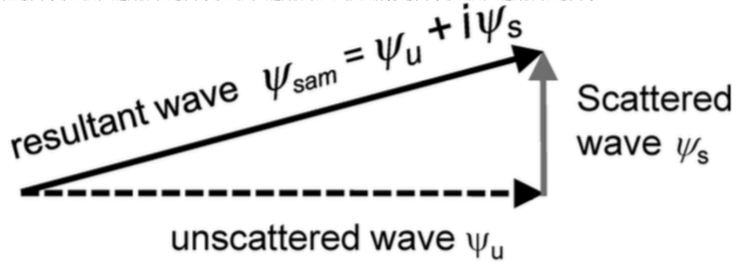
Image formation

Phase contrast (electrons as waves)





$$\Psi_{sam}(\vec{r}) \approx \Psi_0(1 + i\sigma\varphi_{pr}(\vec{r}))$$



- 2D projection of the sample's Coulomb potential
- biological samples are weak phase objects
- small phase shift —> small amplitude variation

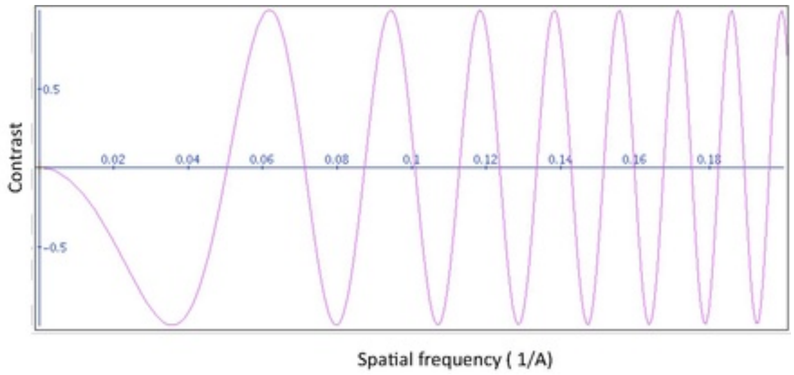
Image formation

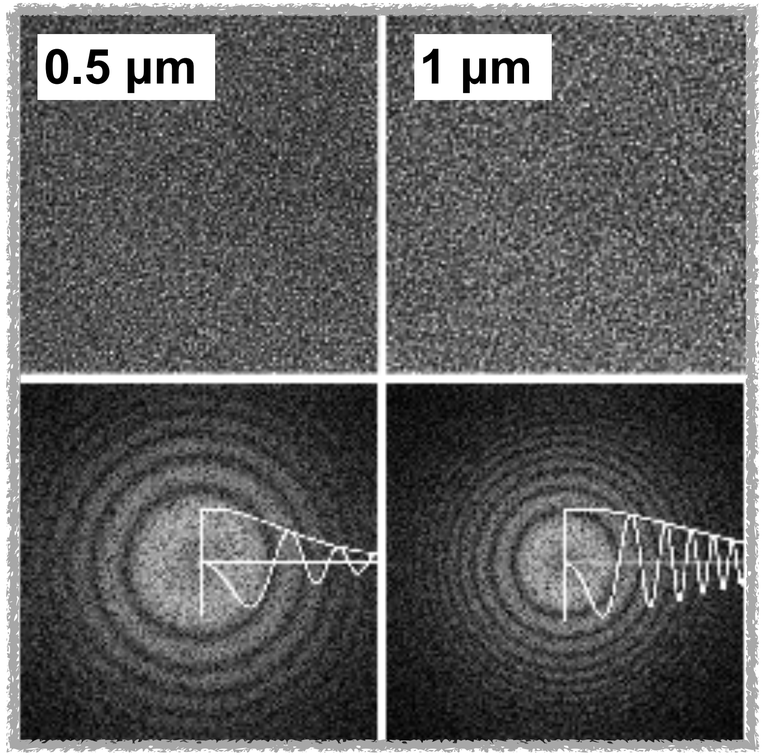
Contrast is enhanced by taking under-focus image

Contrast transfer function

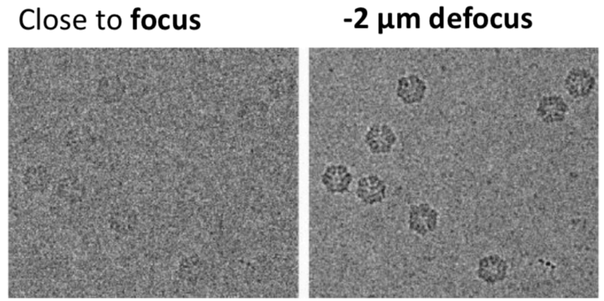
$$\gamma(\vec{R}) = -2\pi\left(\frac{1}{2}\Delta\lambda\vec{R}^2 - \frac{1}{4}C_s\lambda^3\vec{R}^4\right)$$

Defocus

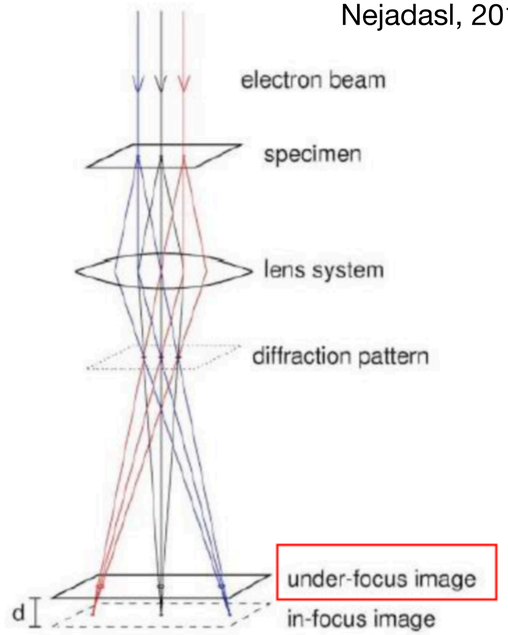




EV Orlova & HR Saibil, Chemical Reviews, 2011



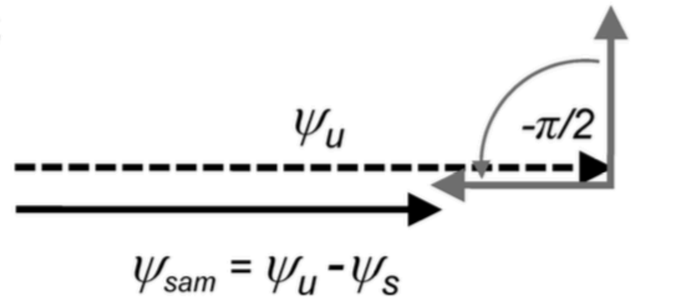
Nejadasl, 2011

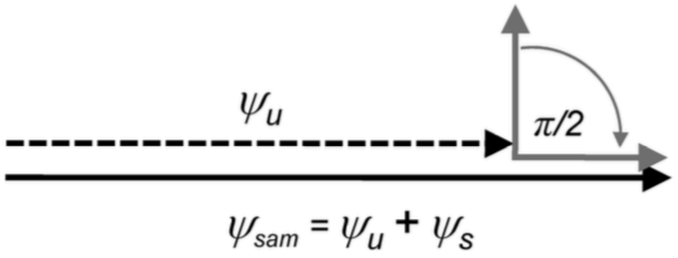


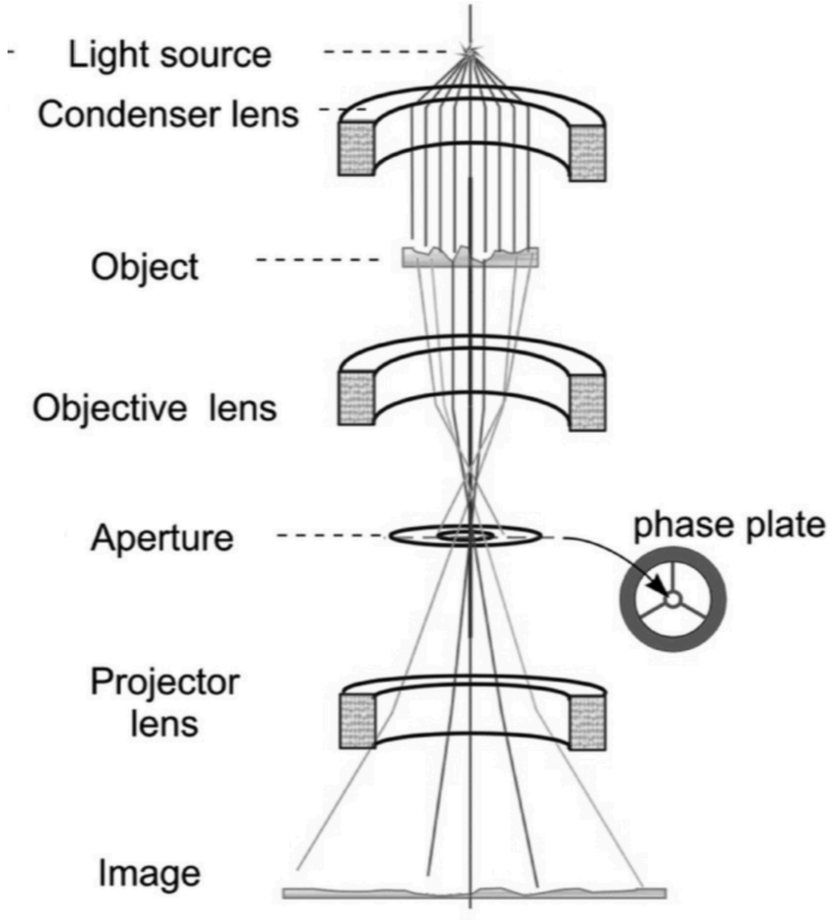
Volkmann & Hanein, 2002

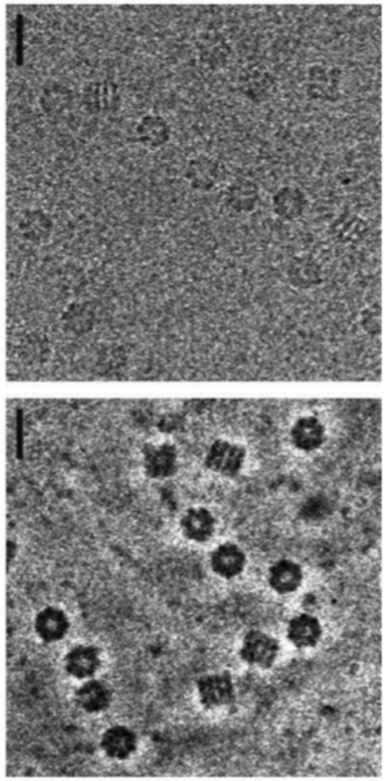
Image formation

*** Phase plate *** introduces contrast over a large resolution range









EV Orlova & HR Saibil, Chemical Reviews, 2011

Image formation

*** Phase plate *** introduces contrast over a large resolution range

nature COMMUNICATIONS

ARTICLE

Received 27 Apr 2017 | Accepted 26 May 2017 | Published 30 Jun 2017

DOI: 10.1038/ncomms16099 OPEN

Cryo-EM structure of haemoglobin at 3.2 Å determined with the Volta phase plate

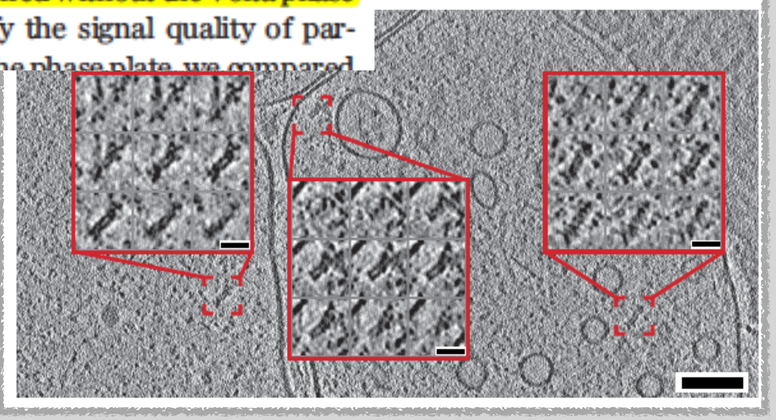
Maryam Khoshouei[1], Mazdak Radjainia[2,†], Wolfgang Baumeister[1] & Radostin Danev[1]

SCIENCE sciencemag.org 23 JANUARY 2015 • VOL 347 ISSUE 6220

A molecular census of 26S proteasomes in intact neurons

Shoh Asano,* Yoshiyuki Fukuda,* Florian Beck, Antje Aufderheide, Friedrich Förster, Radostin Danev, Wolfgang Baumeister†

all (>3σ) 26S proteasome particles present in the tomograms were detected. In contrast, we could not identify 26S proteasome particles reliably in cryotomograms acquired without the Volta phase plate (*9*). To quantify the signal quality of par-



CELL NUCLEUS SCIENCE

Visualizing the molecular sociology at the HeLa cell nuclear periphery

Julia Mahamid,[1]* Stefan Pfeffer,[1] Miroslava Schaffer,[1] Elizabeth Villa,[1,2] Radostin Danev,[1] Luis Kuhn Cuellar,[1] Friedrich Förster,[1] Anthony A. Hyman,[3] Jürgen M. Plitzko,[1] Wolfgang Baumeister[1]*

in the TEM (*8*, *9*). We resolved this by sputtering a fine metal coating onto the FIB-lamellas, as routinely applied for biological cryo–scanning electron microscopy (SEM) imaging (*10*). (ii) Direct detectors improve the quality of cryo-TEM images substantially (*11*), and (iii) the recently developed Volta phase plate (VPP) enhances (phase) contrast especially for lower spatial frequencies without the need of defocusing, rendering the tomograms directly interpretable (*9*, *12*, *13*). Here, we combined these developments to produce in situ high-resolution 3D snapshots of the HeLa cell nuclear periphery.

1) Introduction to image formation

- basic anatomy of a microscope
- amplitude vs phase contrast

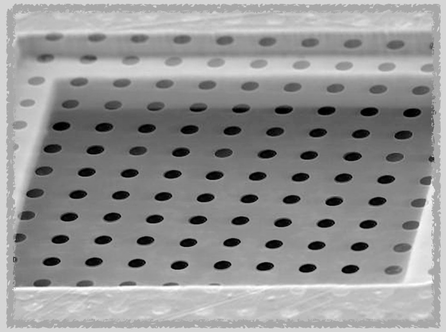
2) Overview of sample preparation for SP EM

- Negative staining
- Flash Freezing
- EM grids

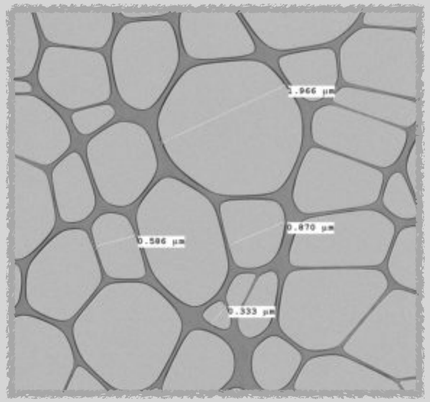
Sample preparation

- continuous

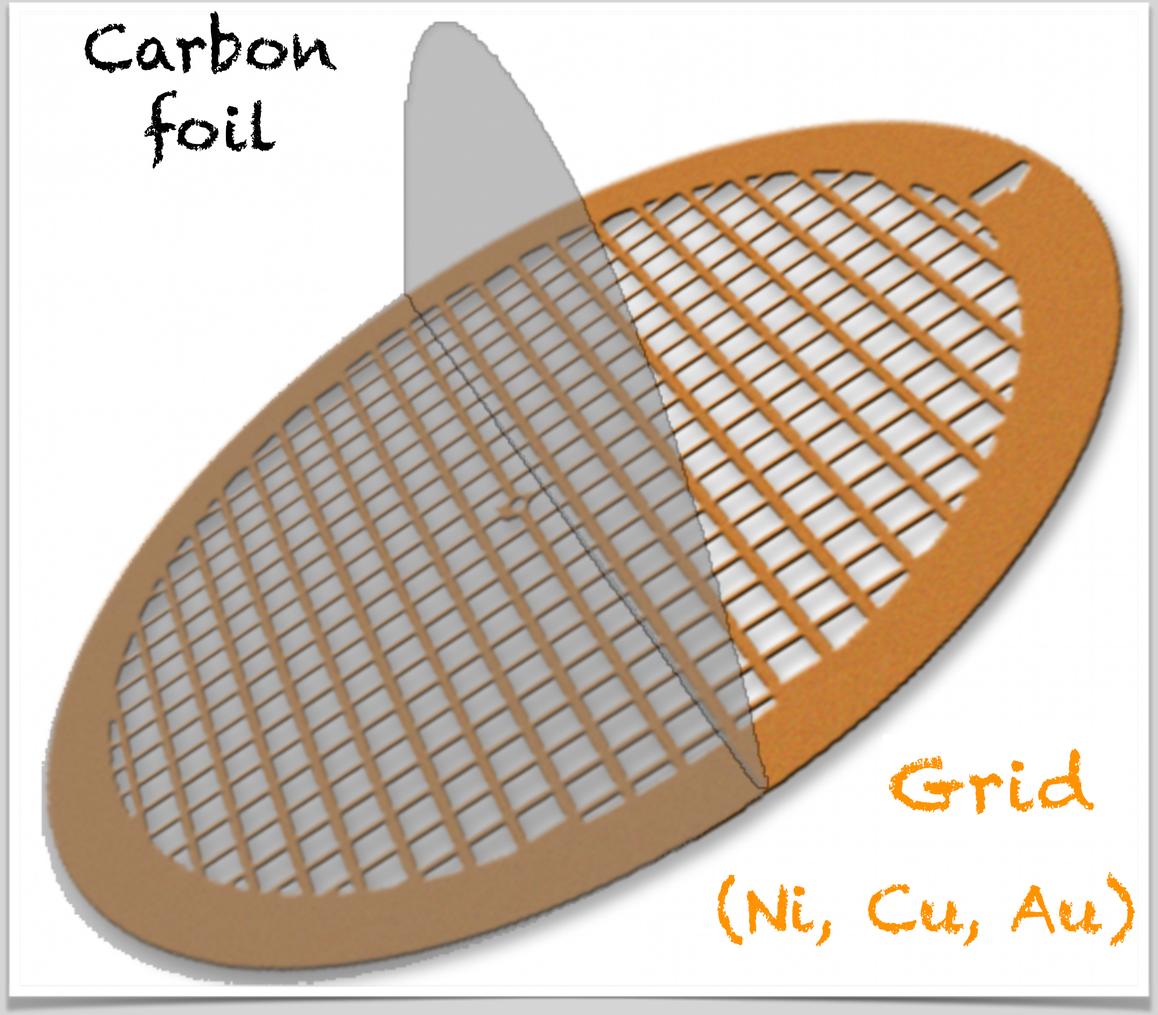
- holey



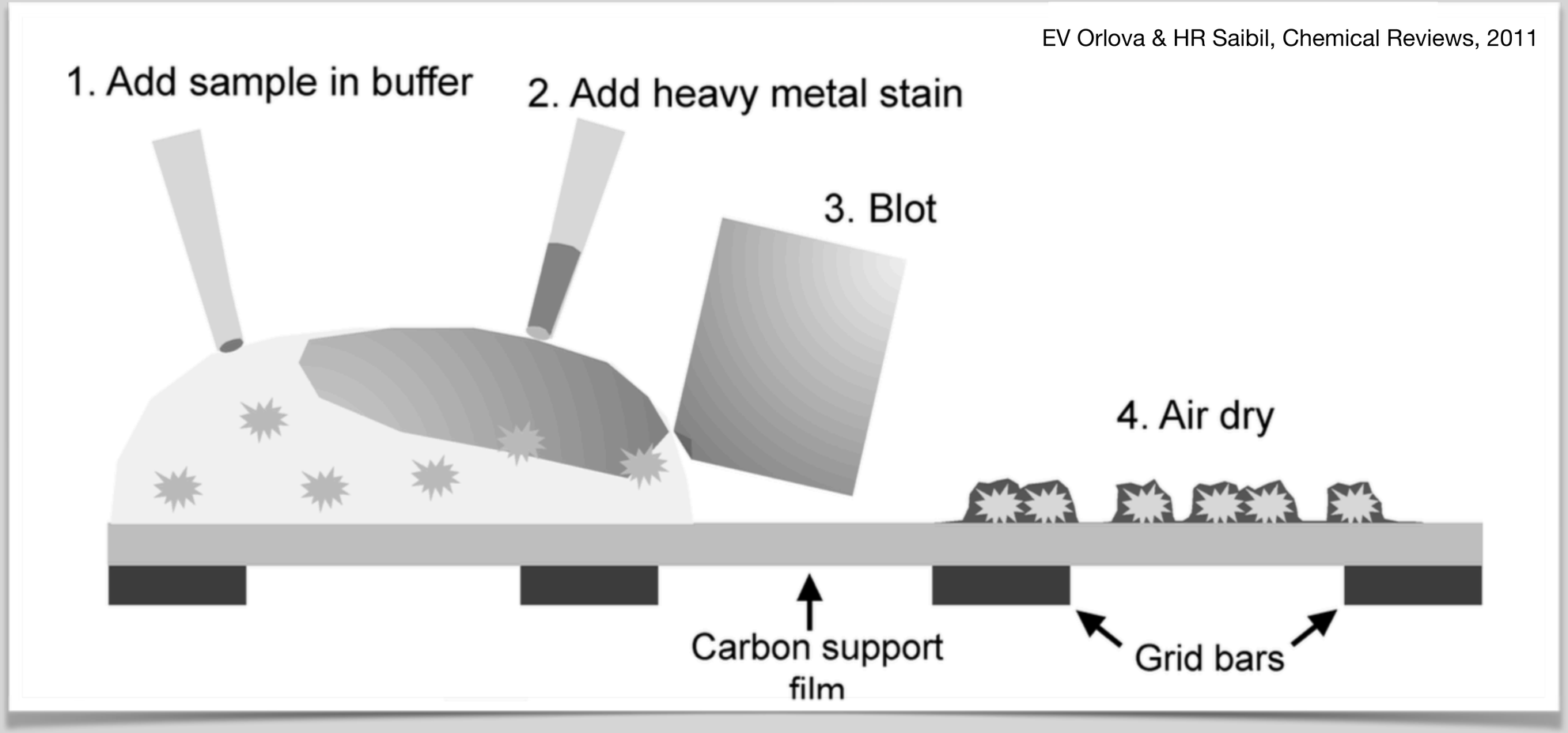
- lacey



- functionalized



Negative staining



The macromolecular shape is seen by exclusion rather than binding of stain (negative staining)

Negative staining

Advantages

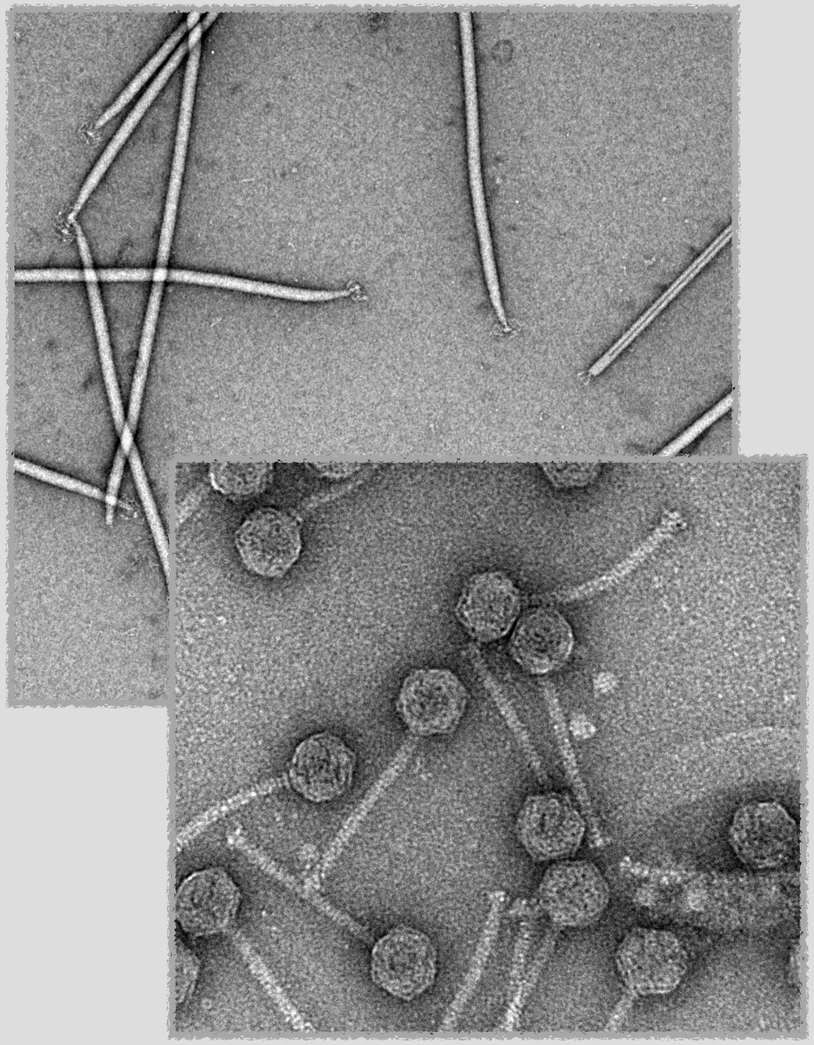
- **quick & easy**
- **Few amount of sample (~15-50 µg/mL)**
- **high contrast**
  (uranyl acetate, phosphotungstic acid, molybdate, …)
- **Evaluate sample homogeneity, size, shape, symmetry, complex formation**

Disadvantages

- **Limited resolution (20Å)**
- **Sample damage (dehydration, pH, particles often squashed)**
- **Uneven staining makes difficult data processing and analysis**

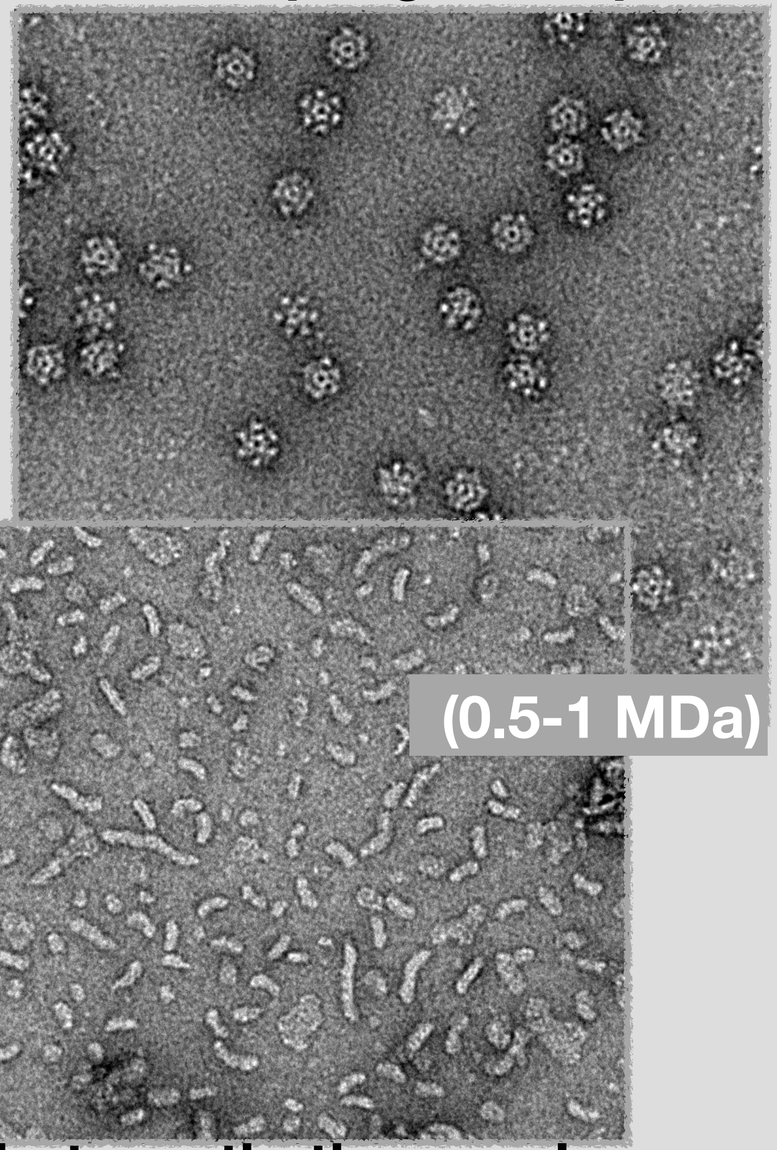
Negative staining

Archaeal virus



Bacteriophage

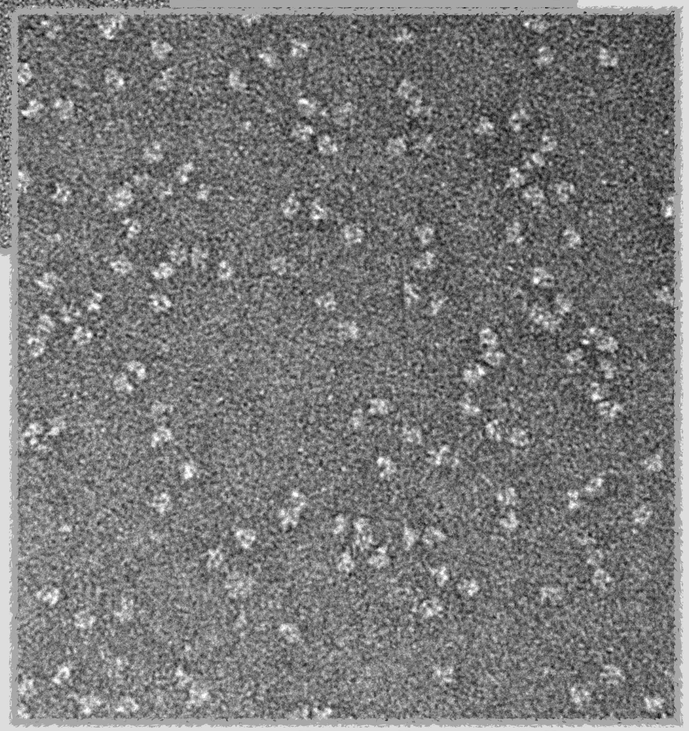
Bacteriophage baseplate



photosynthetic complex

PKS module

Anti-CRISPR/Cas9



Flash-freezing

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 fa

Quarterly Review of Biophysics **21**, 2 (1988), pp. 129–228 129

Printed in Great Britain

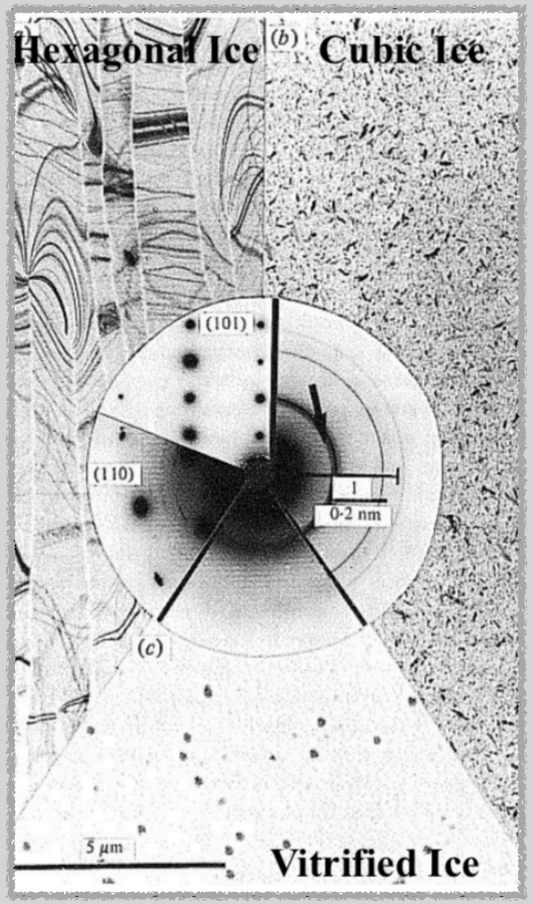
Cryo-electron microscopy of vitrified specimens

Vitreous = glass, amorphc
NOT crystalline

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

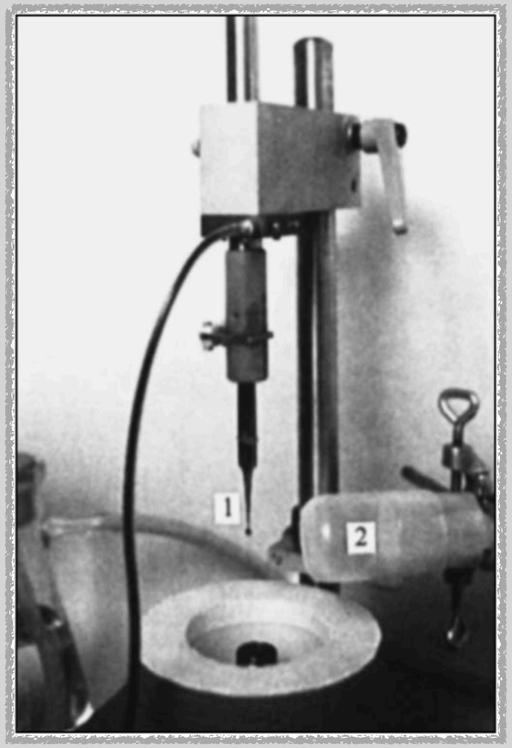
Vitrification requires a drop of temperature faster than 10^5-10^6K/s



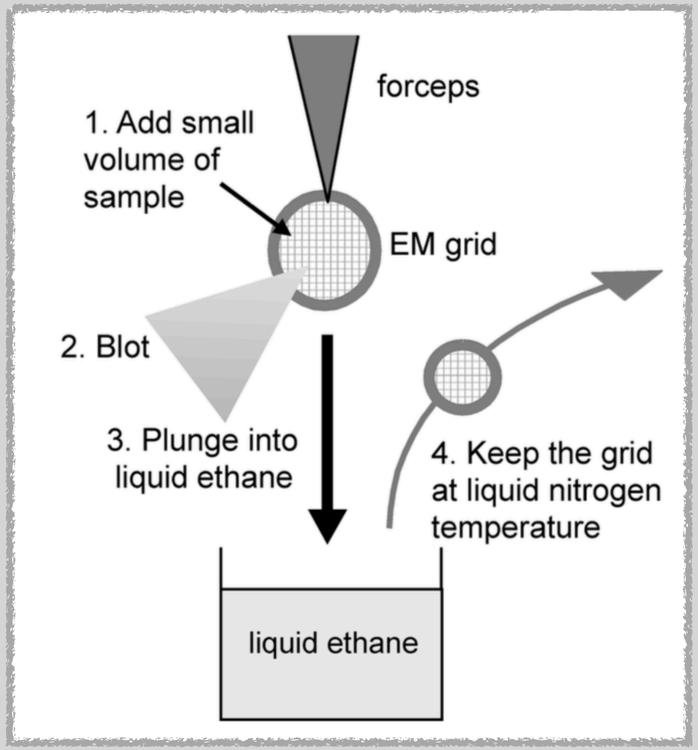
Flash-freezing traps biological samples in a vitreous, amorphous ice close to their native state

Flash-freezing

Guillotine

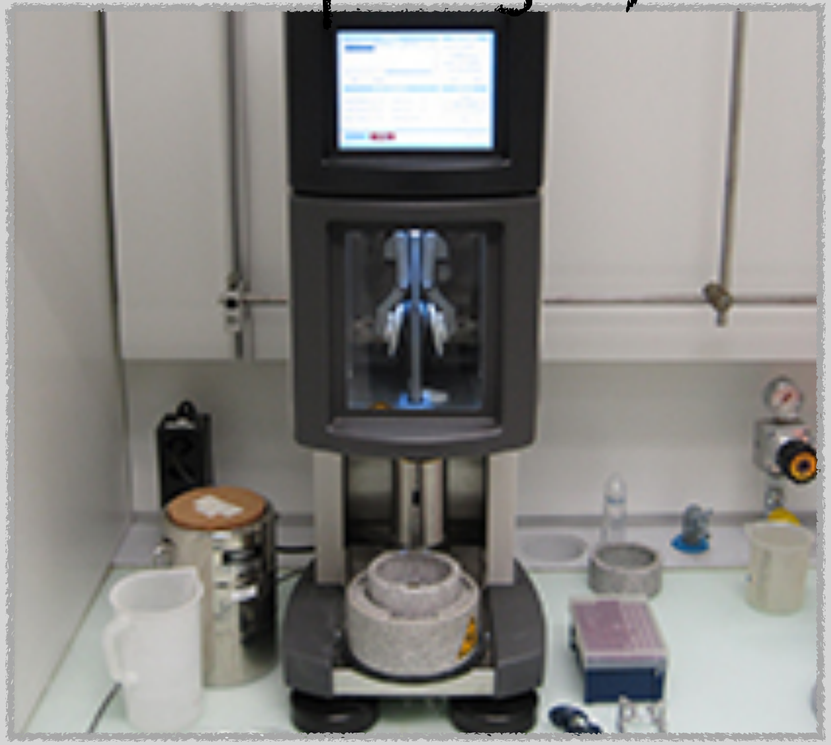


Cheng et al, Cell, 2017



EV Orlova & HR Saibil, Chemical Reviews, 2011

Automated plunge freezer



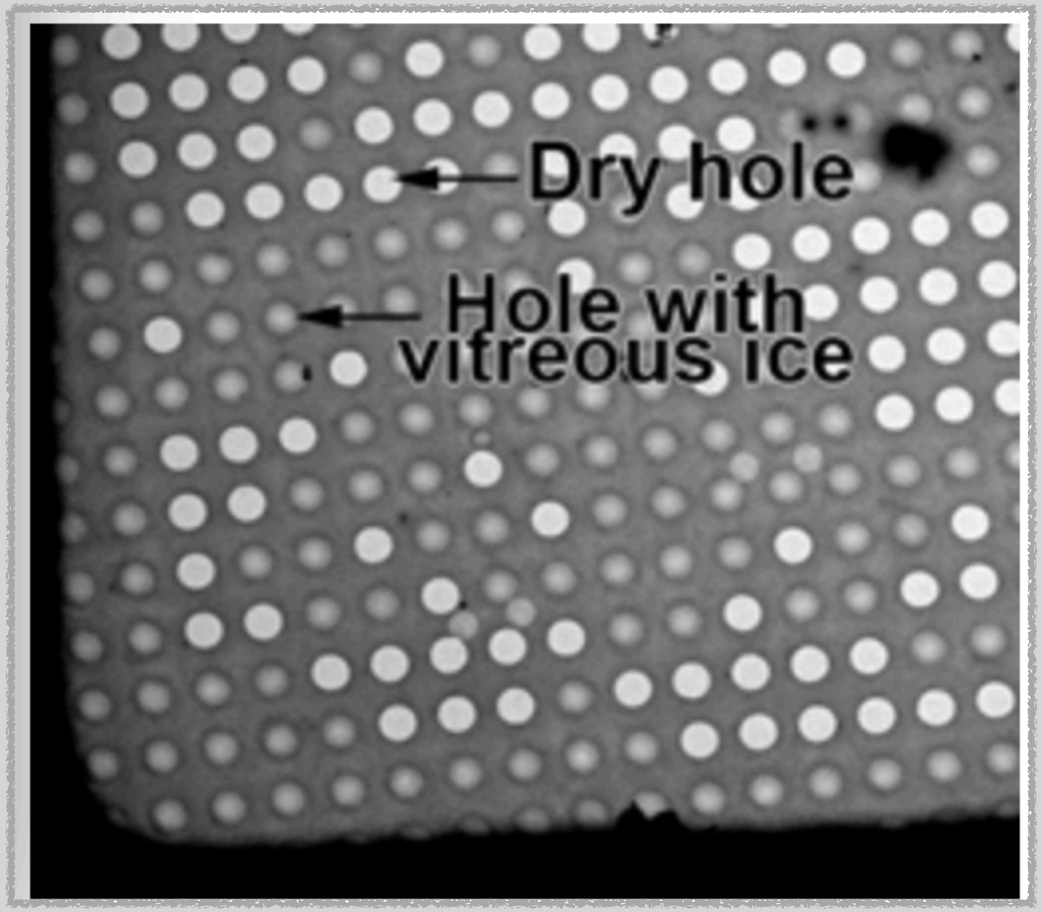
- **sample < 3 µm**
- **liquid ethane** (melting point ~ -183°C vs LN2 boiling point ~ -196°C)
- **plunge at > 1m/s**

Controls:

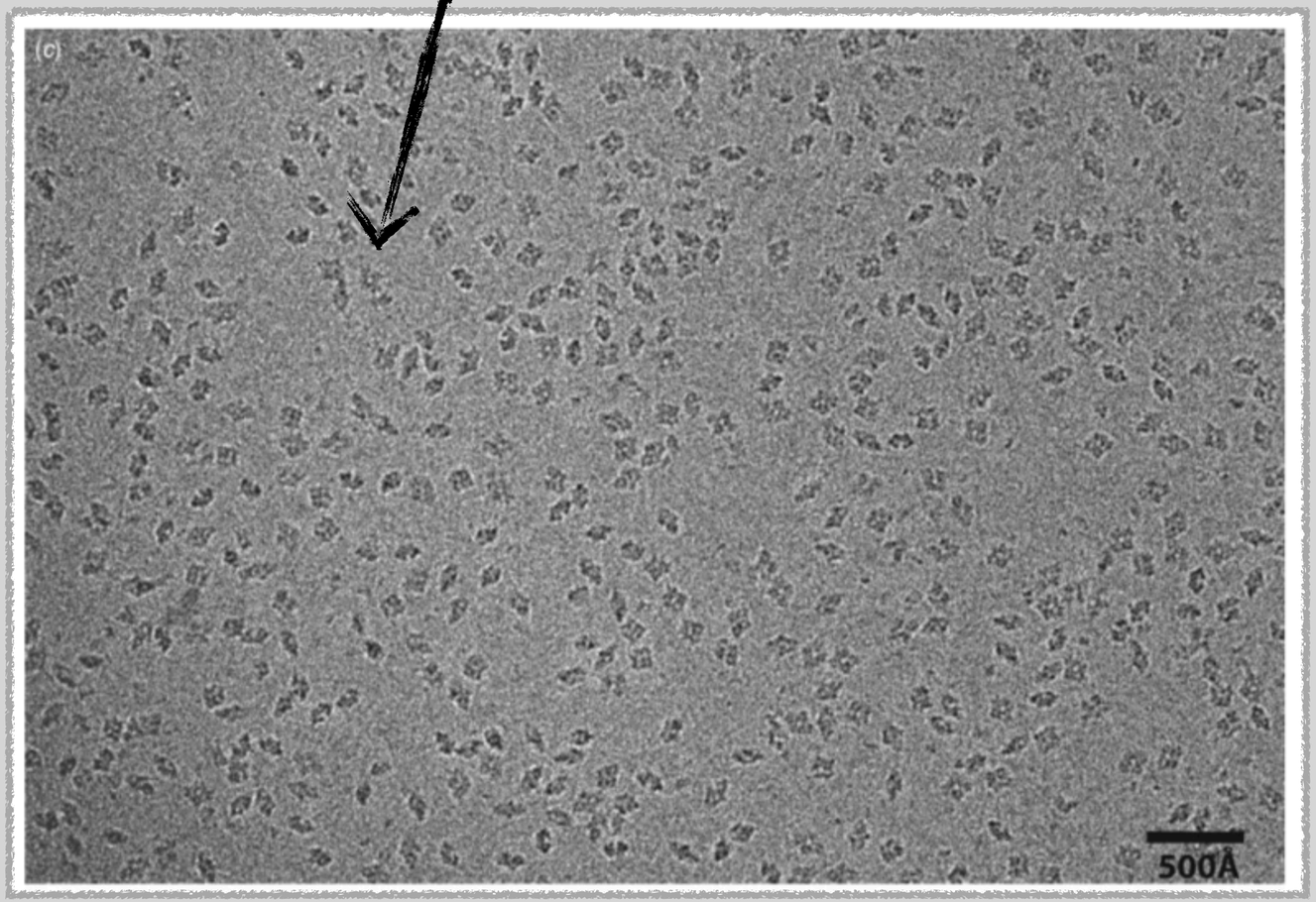
- **T° & humidity**
- **blot force/time, single/double side**
- **liquid ethane temp**

Flash-freezing

Low mag view of a grid square



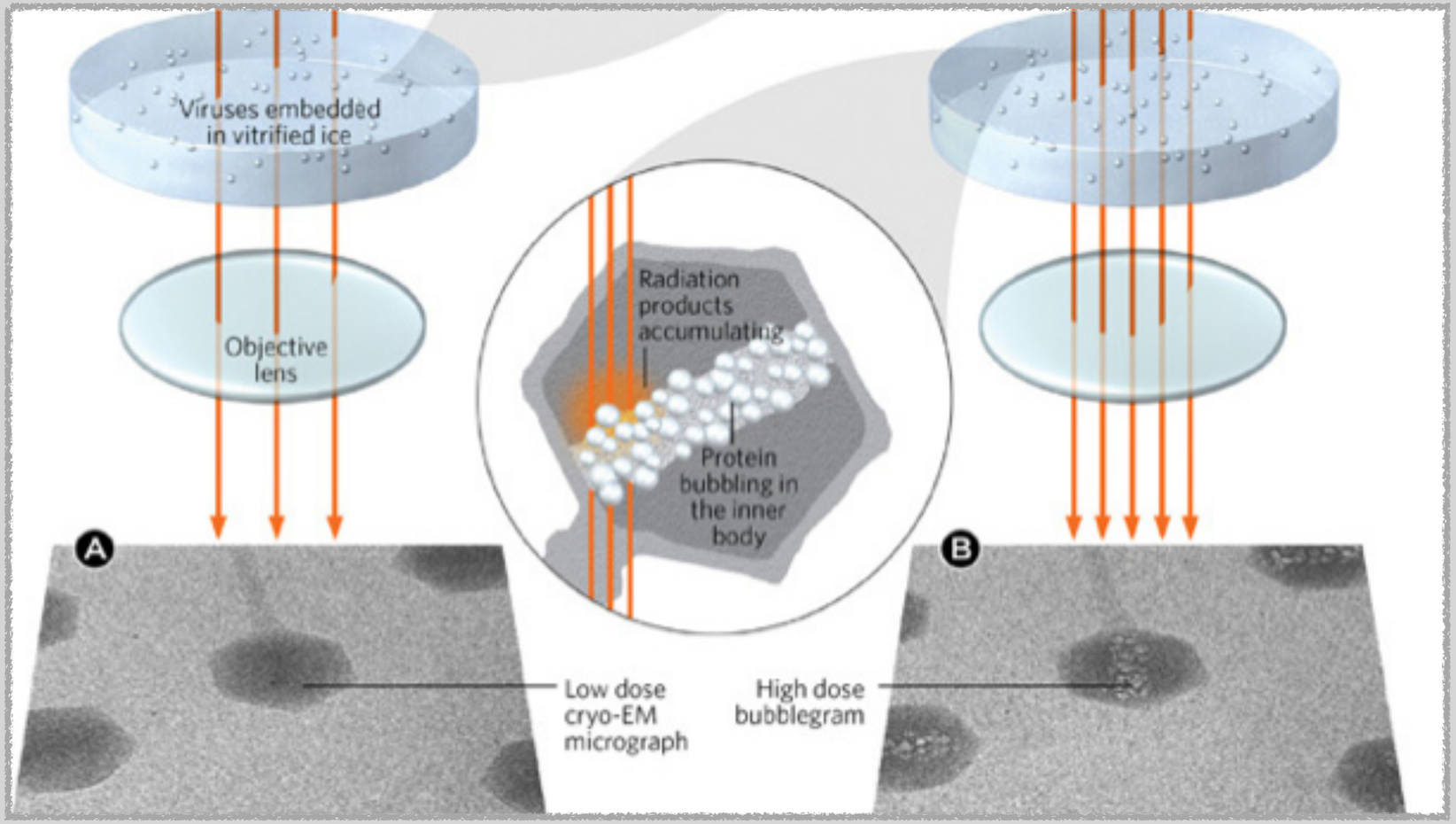
https://cryoem.ucsd.edu



Vinothkumar & Henderson, QRV, 2016

K2 DED, 300 kV, 3-4 μm defocus, 17 $e^-/A^2/s$, 4s exp

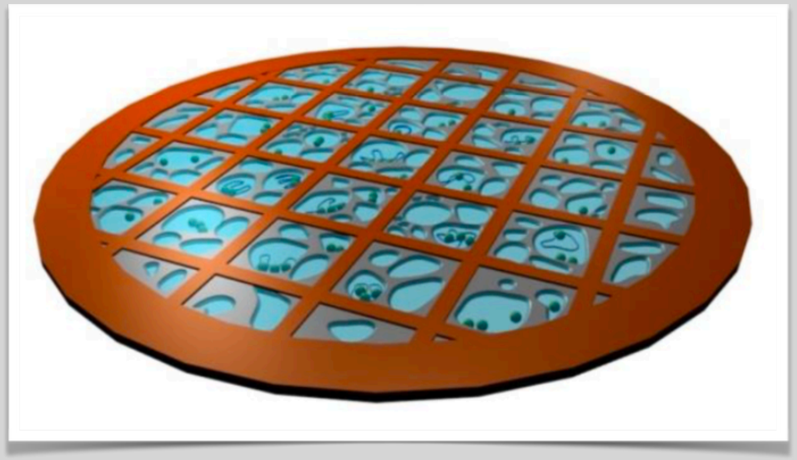
Flash-freezing



Frozen samples suffer from radiation damage

Flash-freezing

Sample



Concentration

Number of particles in projection/μm^2 in 800 Å thick ice film (separation)

	Concentration				
M.W.	10mg/ml	2mg/ml	0.5mg/ml	0.1mg/ml	20µg/ml
10 kD	48000 (45Å)	10000 (100Å)	2500 (200Å)	500 (450 Å)	100 (1000 Å)
50 kD	10000 (100Å)	2000 (220Å)	500 (400Å)	100 (1000Å)	20 (0.2µm)
250kD	2000 (220Å)	400 (500 Å)	100 (1000 Å)	20 (0.2µm)	4 (0.5µm)
1 MD	500 (400Å)	100 (1000Å)	25 (0.2µm)	5 (0.4µm)	1 (1µm)
5 MD	100 (1000Å)	20 (0.2µm)	5 (0.4µm)	1 (1µm)	0.2 (2.2µm)
25 MD	20 (0.2µm)	4 (0.5µm)	1 (1µm)	0.2 (2.2µm)	0.04 (5µm)

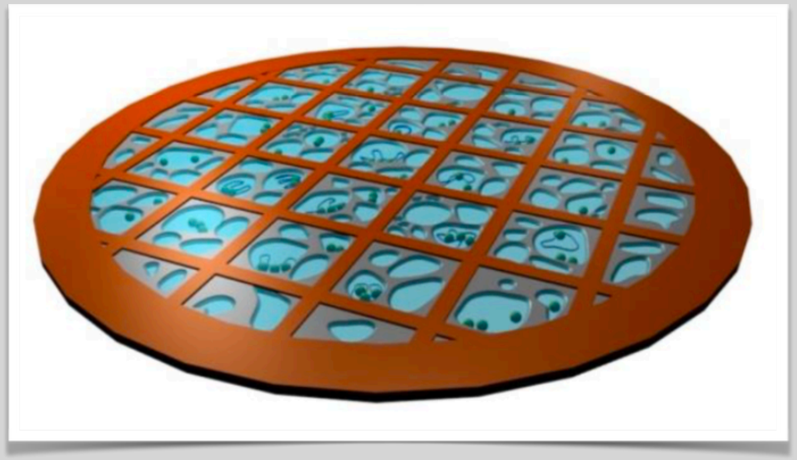
Vinothkumar & Henderson, QRV, 2016

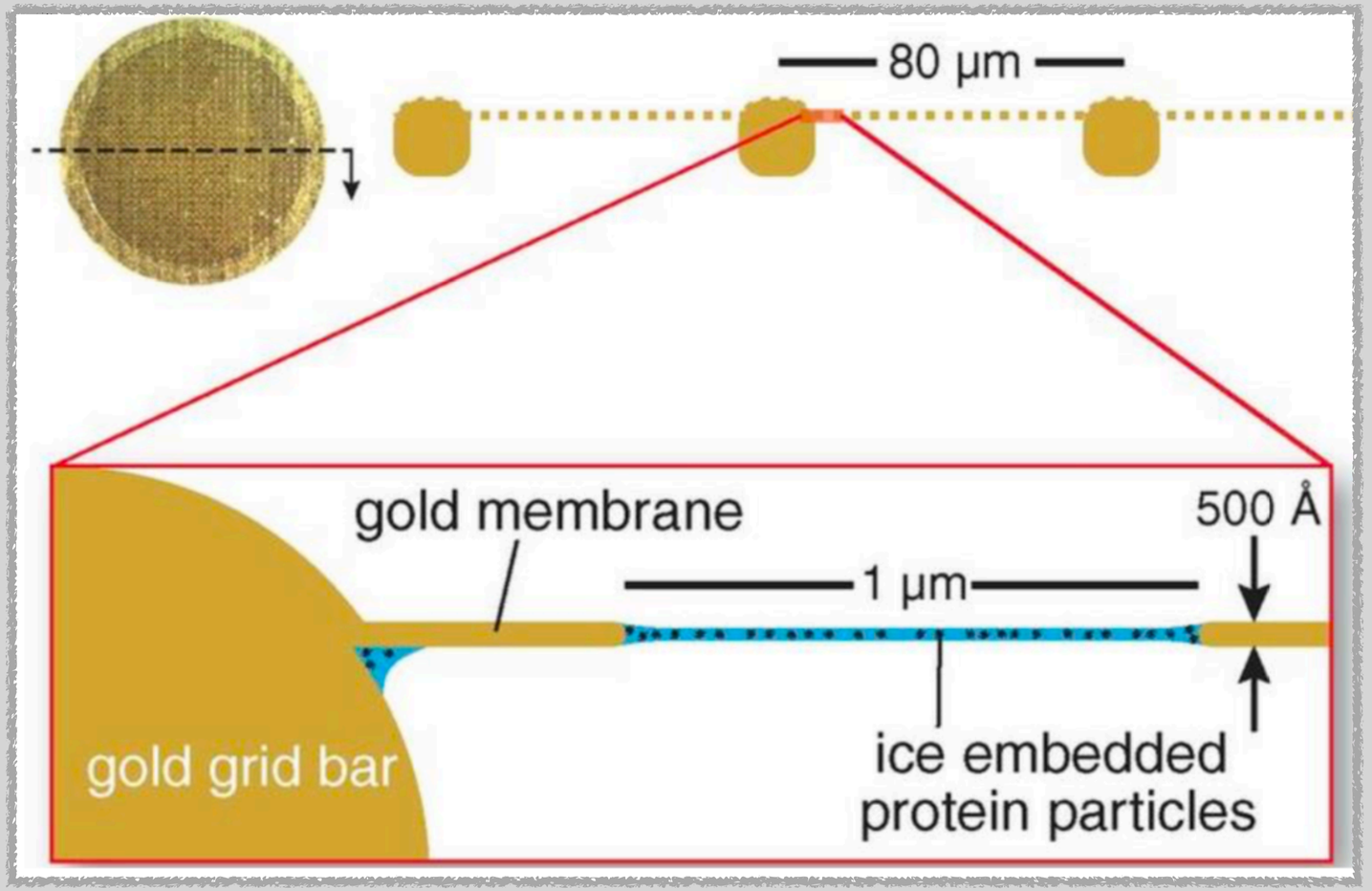
Buffer composition

- low salt (< 150 mM NaCl)
- < 5% glycerol, sucrose, …
- Low conc. detergent

Flash-freezing

Gold grid



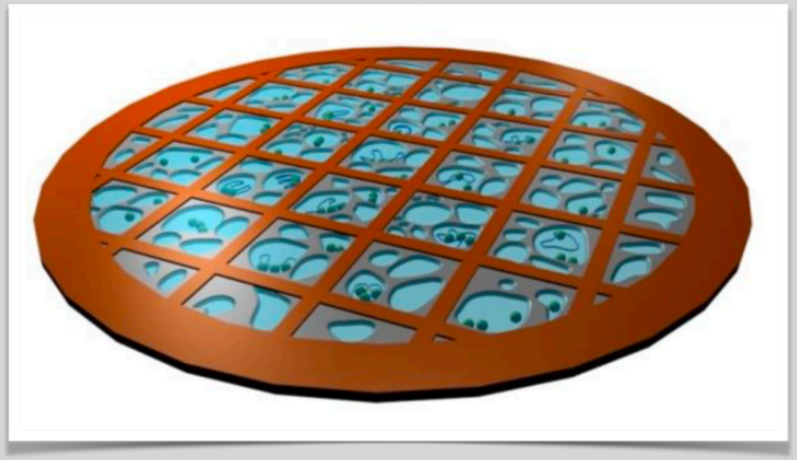


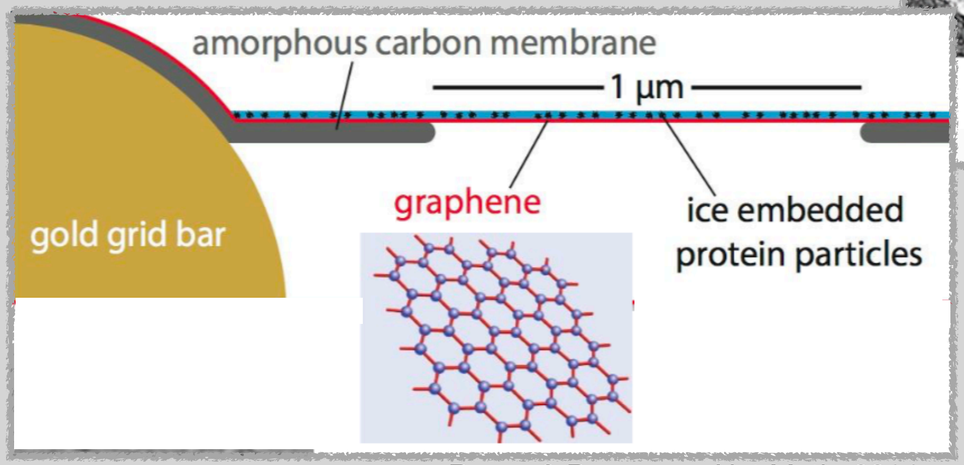
Russo & Passmore, Science, 2014

A gold specimen support nearly eliminates substrate motion during irradiation

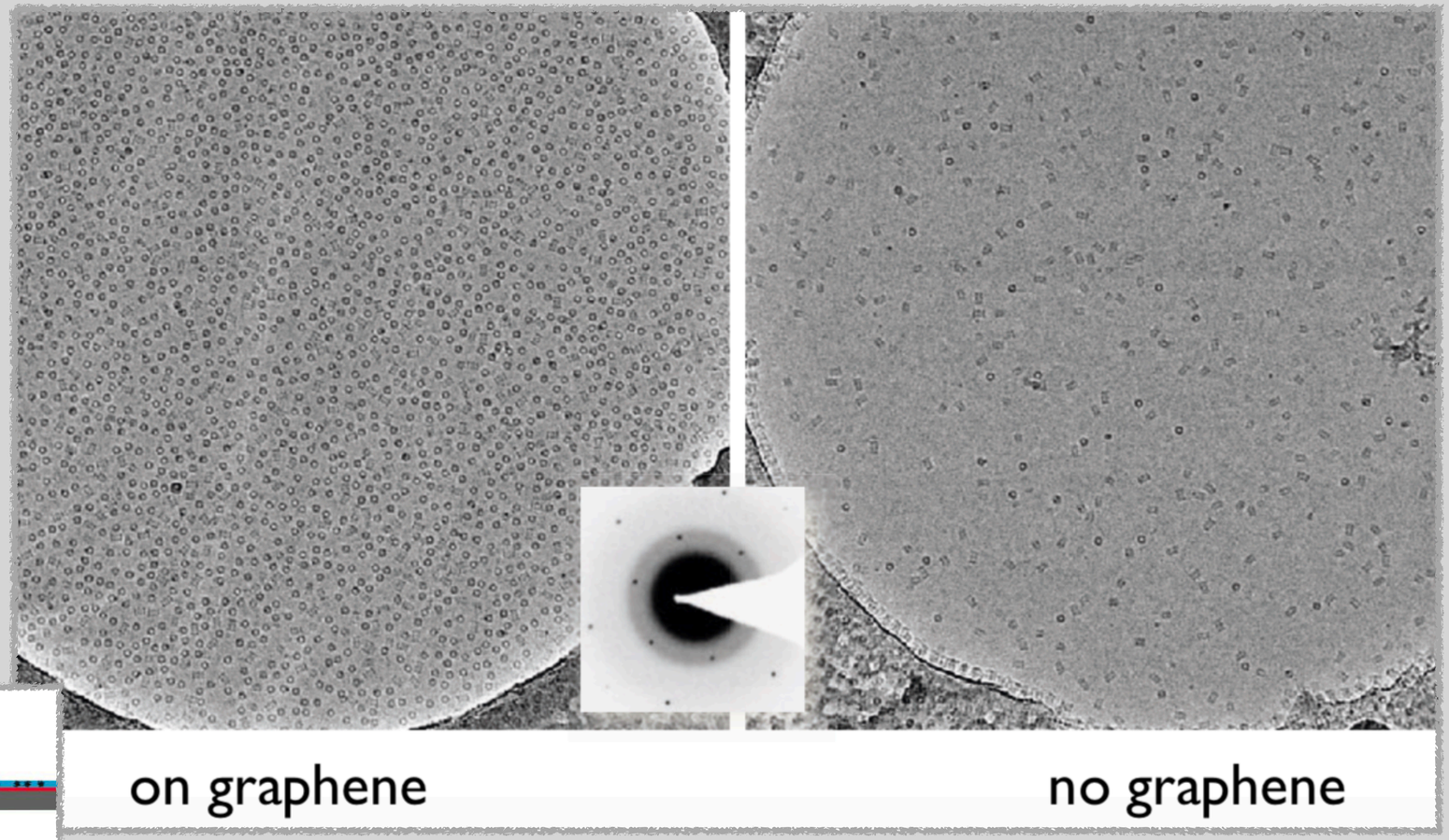
Flash-freezing

Graphene





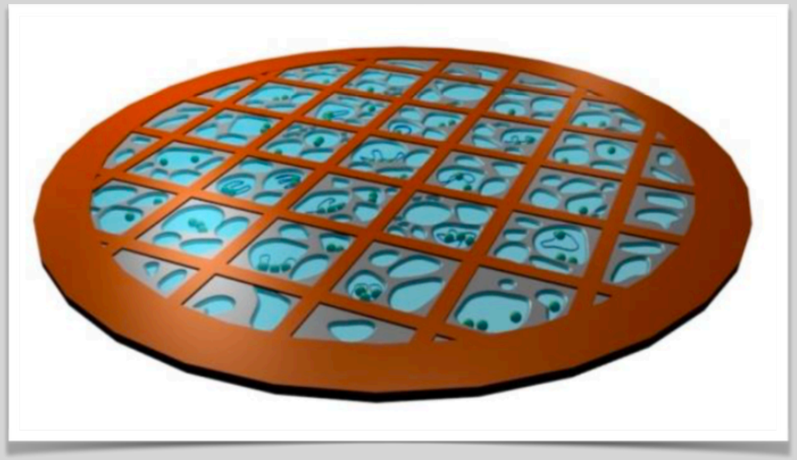
Russo & Passmore, Nat Meth, 2014

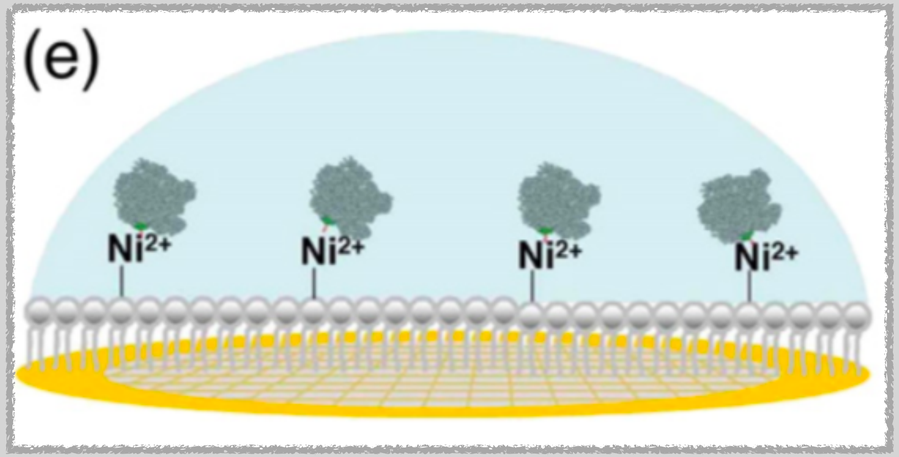


- **Single layer of graphene**
- **to control sample distribution**

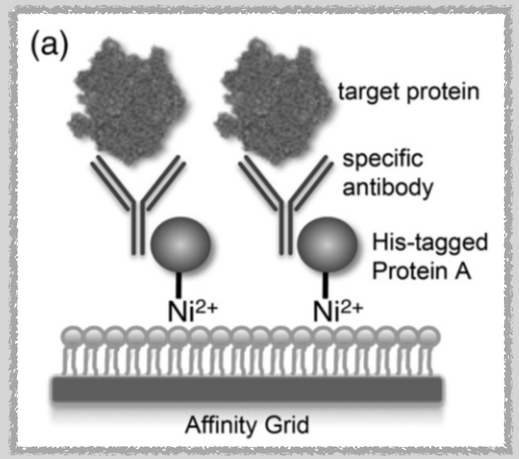
Flash-freezing

Affinity grids





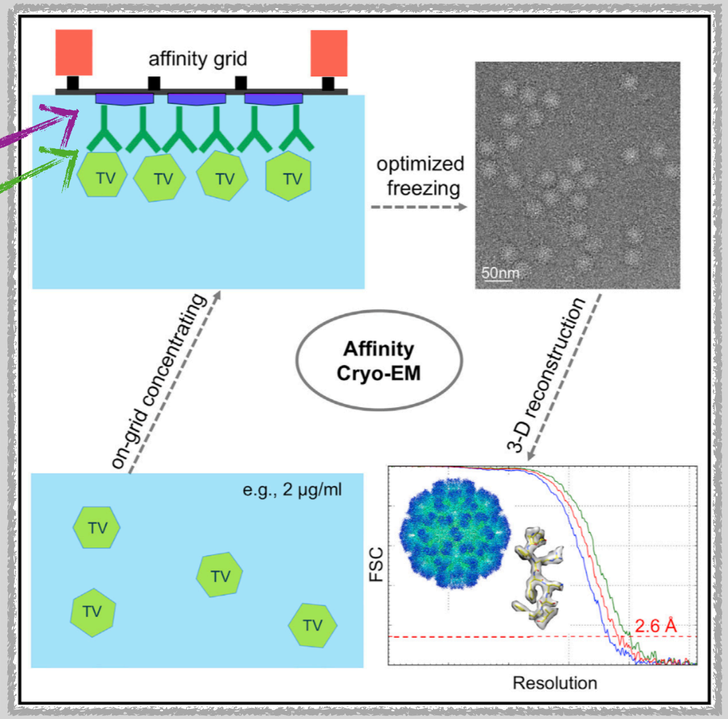
Kelly D, JMB, 2008



Kelly D, JMB,2010

protein A

IgG



Article

Structure

Antibody-Based Affinity Cryoelectron Microscopy at 2.6-Å Resolution

Yu et al., 2016, Structure *24*, 1984–1990
November 1, 2016 © 2016 Elsevier Ltd.
http://dx.doi.org/10.1016/j.str.2016.09.008

CrossMark

CellPress

HOW TO DETERMINE THE STRUCTURE OF YOUR FAVORITE BIOLOGICAL SAMPLE USING CRYO-EM AND SINGLE PARTICLE ANALYSIS?

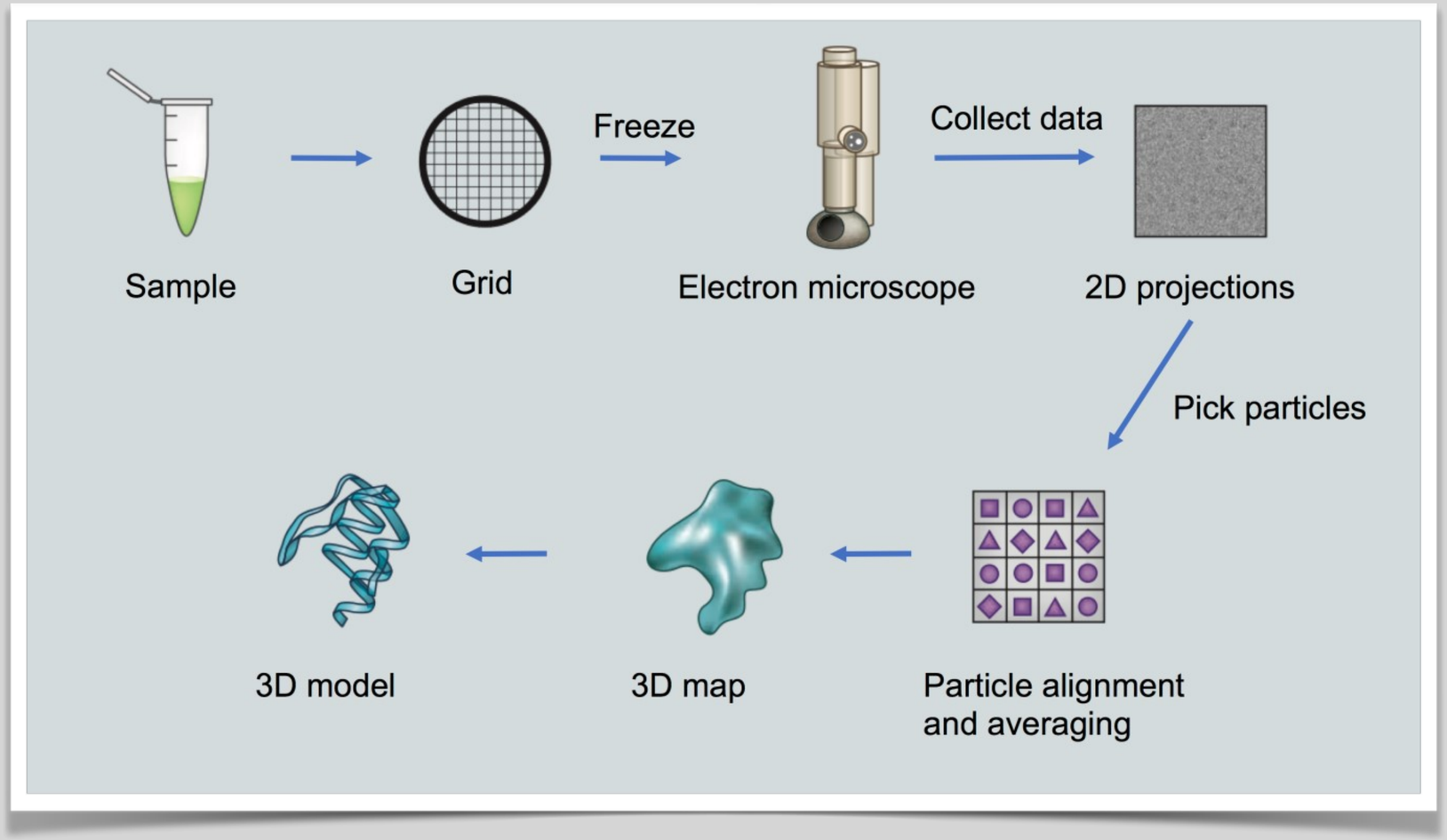


Image analysis,

3D reconstruction validation & interpretation

Leandro Estrozi
Ottilie Loeffelholz von Colberg
Célia Plisson