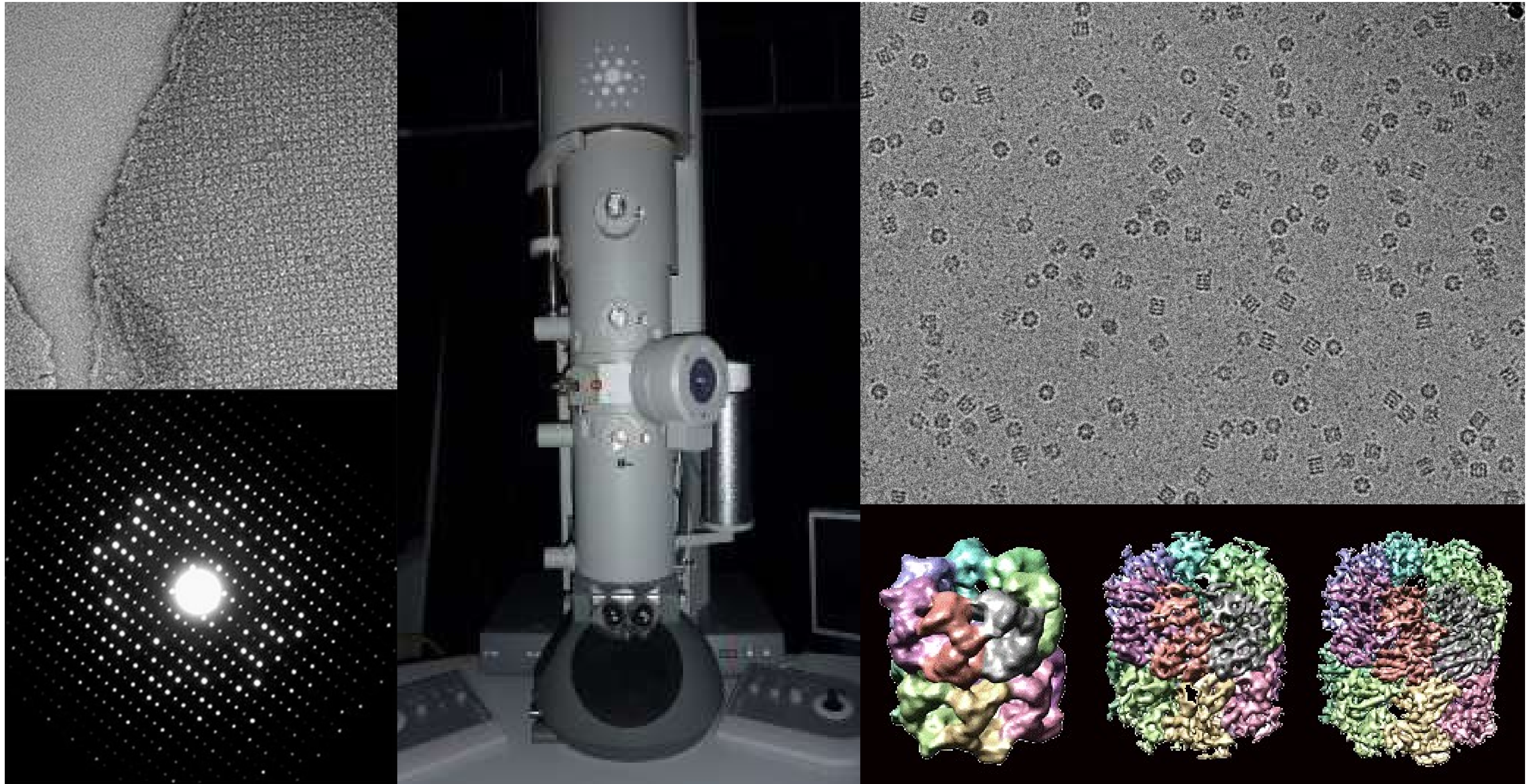
Electron Microscopy techniques for Structural Biology



Corso di Biocristallografia e Microscopia Elettronica

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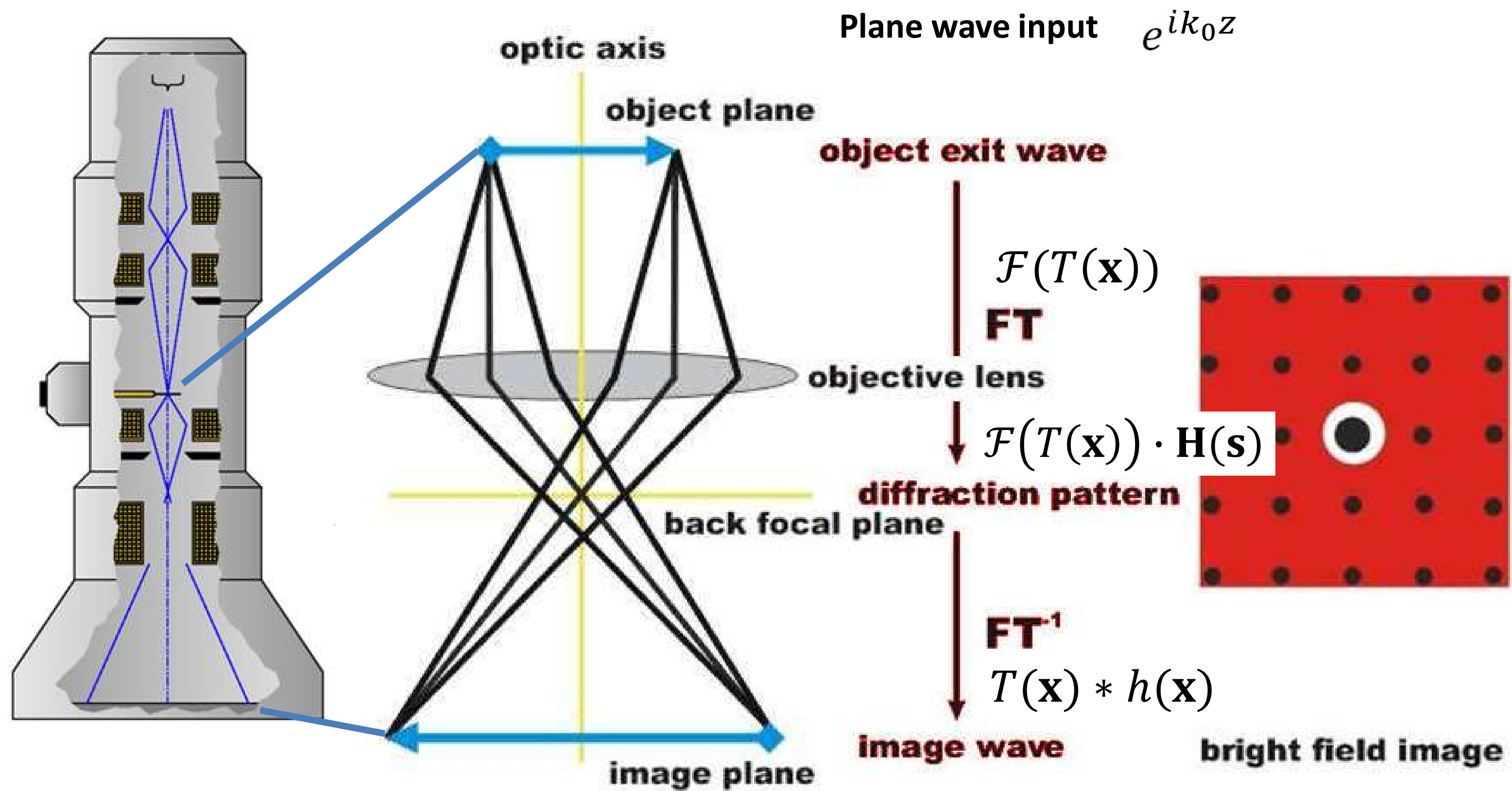
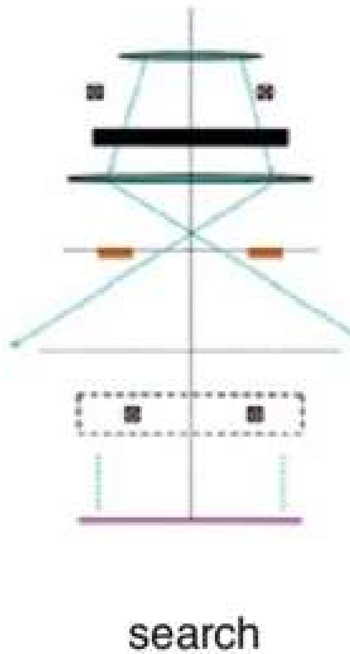
Electron microscopy techniques for high-resolution structural biology

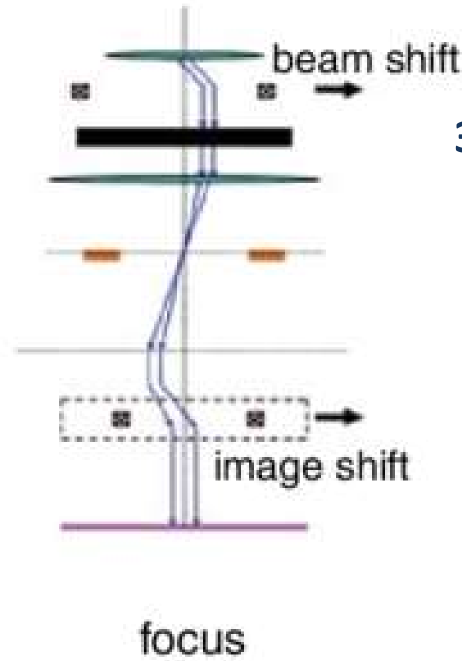
	ELECTRON CRYSTALLOGRAPHY	SINGLE-PARTICLE ELECTRON MICROSCOPY
Sample	2D protein crystals (usually membrane proteins…)	Protein solution (concentration < 1 mg/mL)
Data collection	Diffraction + imaging	Imaging
Resolution	Up to 2 Å	Up to 3-4 Å (with DDD camera!)
Implementation	Manual and time consuming	Increasingly automated (for both sample preparation and data collection)
Data analysis	Methods adapted from X-ray crystallography	2D classification and 3D reconstruction

Image plane and back focal plane

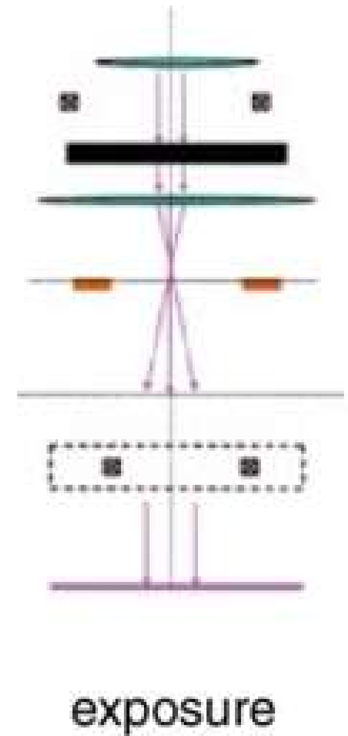




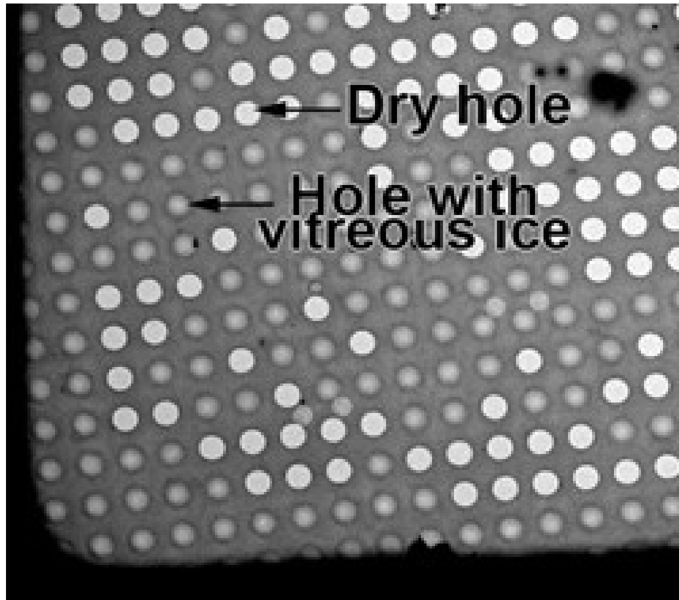
1. **Search mode**: scan a large area with low magnification and low dose settings, intended to minimize electron beam induced damage of the sample.

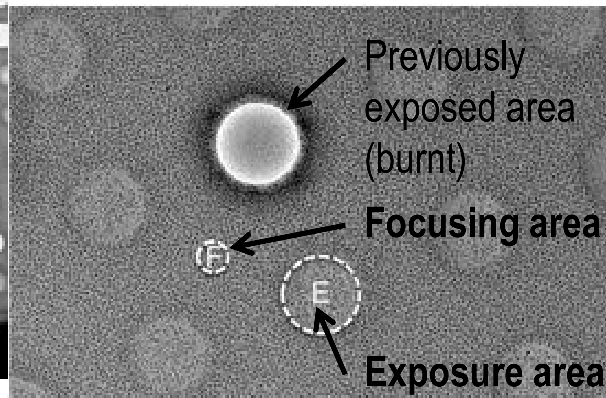


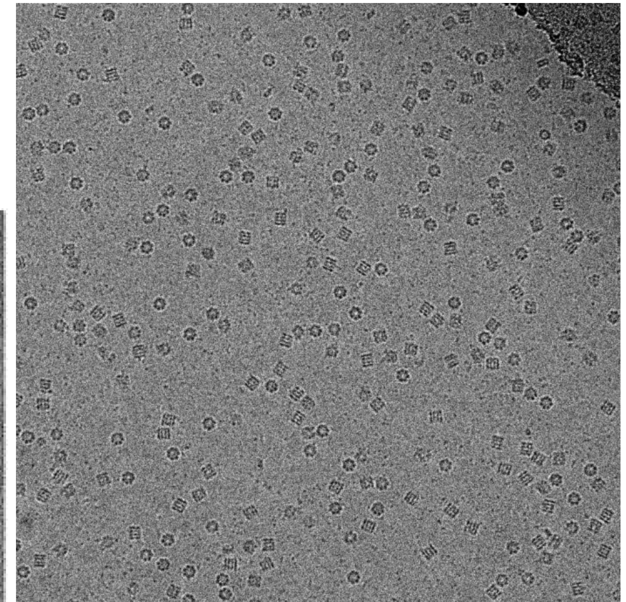
3. **Exposure mode**: the beam is shifted back on the sample and the area is exposed at high doses for data collection.



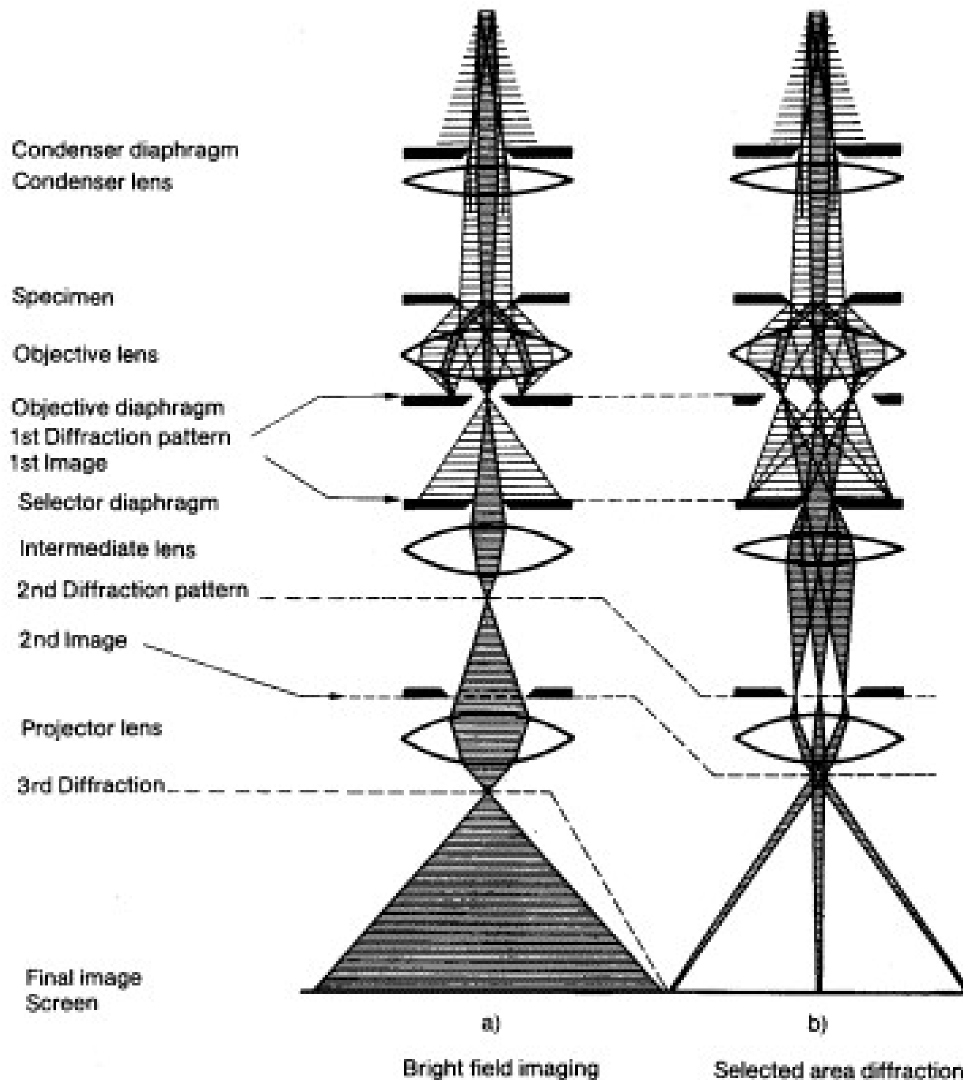
2. **Focus mode**: the beam is shifted off the area of interest and focused at high magnification.







Diffraction vs Imaging



For diffraction:

- **flatness** of the sample is crucial!!
- **tilting** of the sample is required for 3D structure; tilting can cause **charging** of the sample that, in turn, results in **drifting**
- diffraction is not sensitive to drifting

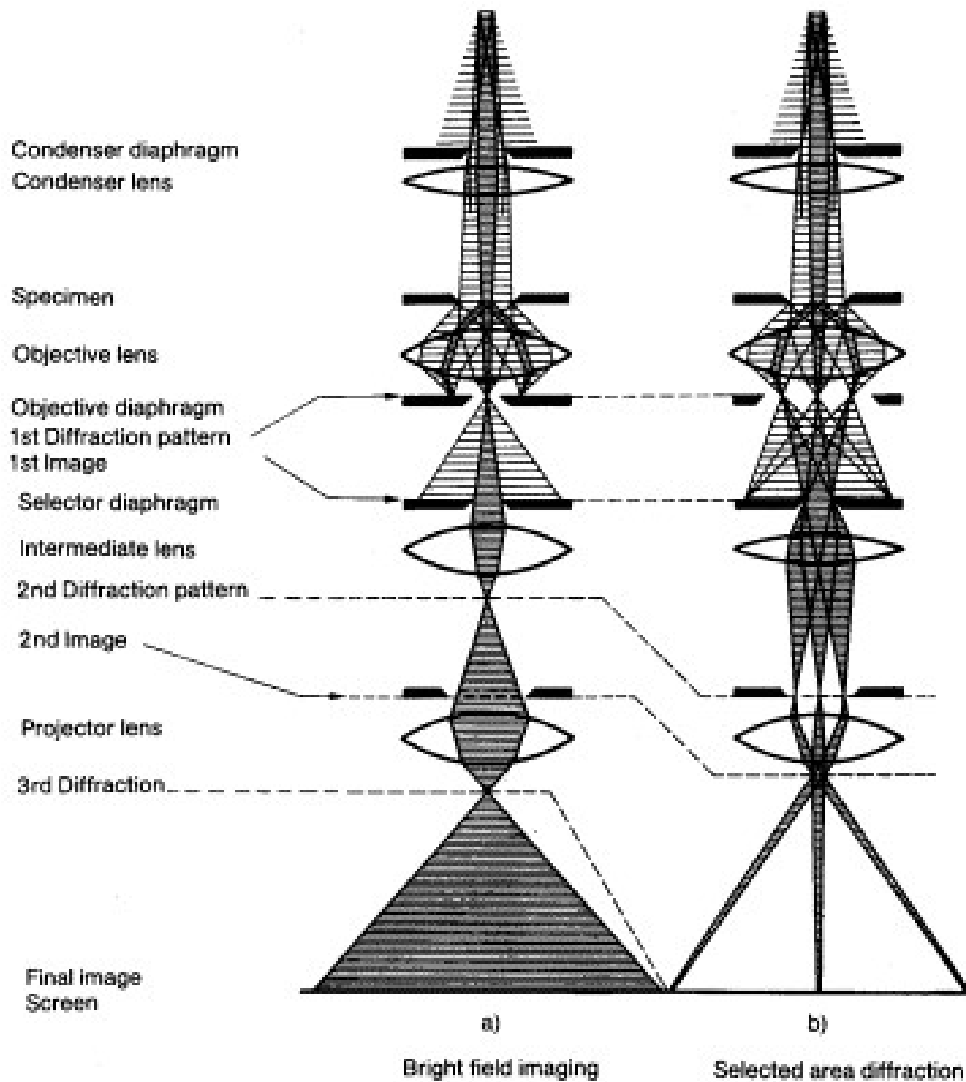
For imaging:

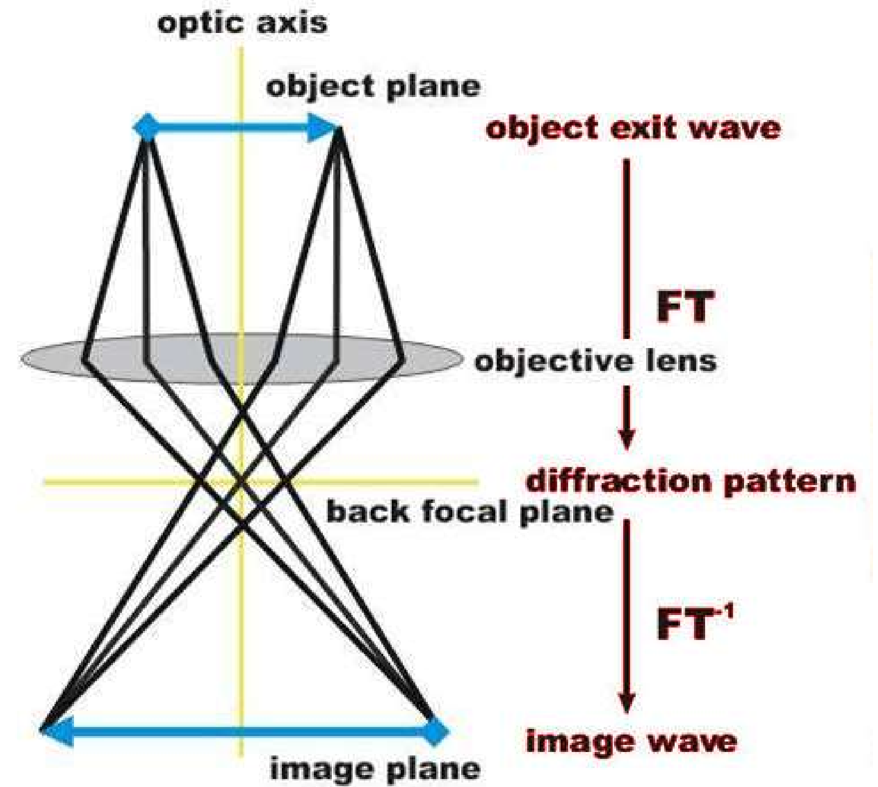
- imaging is very **sensitive to drifting**, due to **vibration** or **charging**
- **stability** of the stage is a requirement

DDD CAMERA!!

Can correct small drift and improve S/N

Diffraction vs Imaging





Electron crystallography

Ideal for membrane proteins

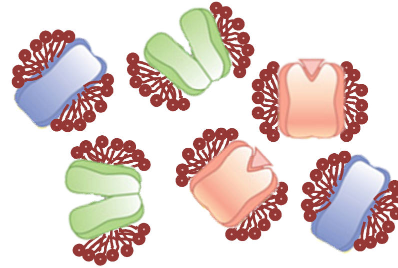
Less sample than MX

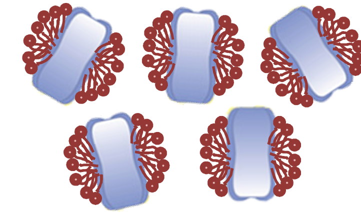
Resolution up to ~2 Å

Structure can be solved at low res

Bottleneck of crystallization

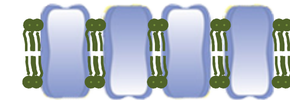
Membrane solubilization

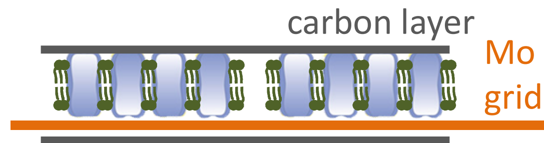




Protein purification

2D crystallization

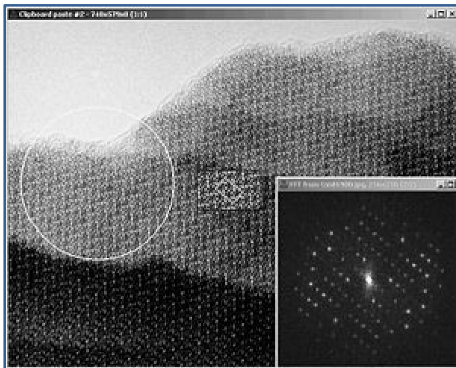




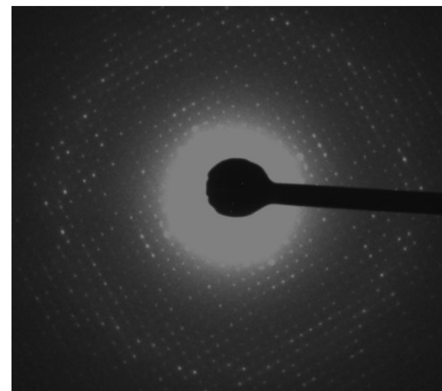
Mo grid

Sample preparation

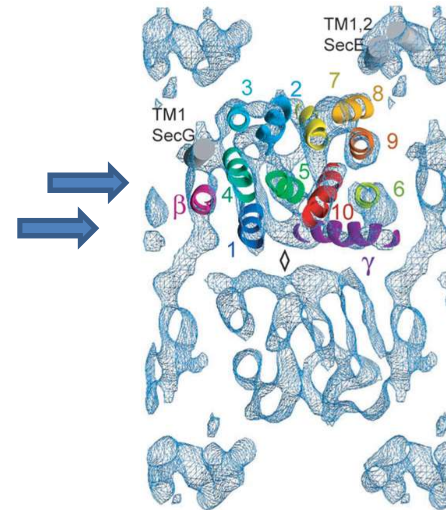
Electron microscopy



Imaging & tilting
(Phase information)



Diffraction & tilting
(Amplitude information)



3D reconstruction & atomic model

2D crystallization

Protein crystals ordered in 2D.

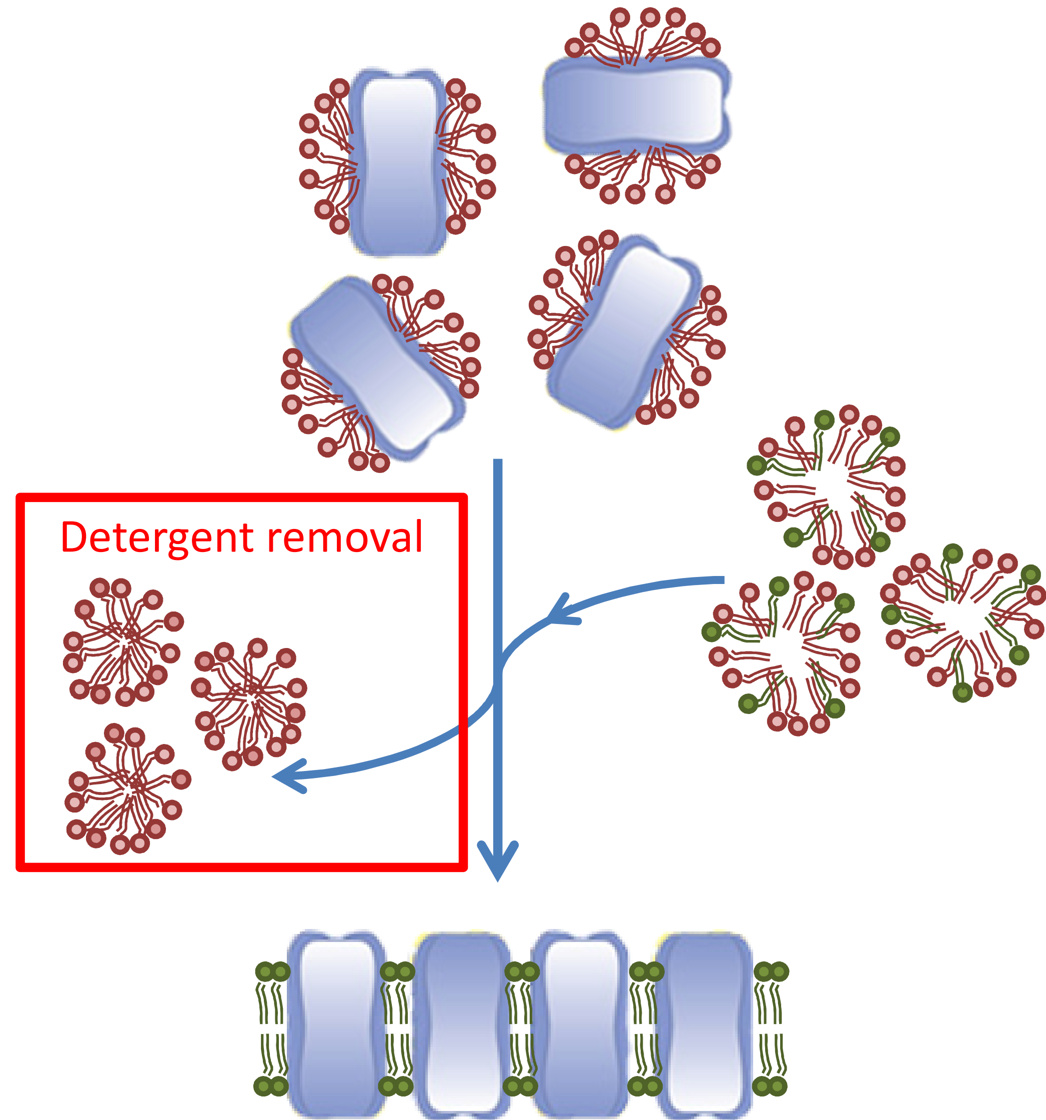
Environment is similar to the one in the biological membrane (native environment of membrane proteins).

Some proteins form natural crystals in cell membranes (Bacteriorhodopsine in the 'purple membrane').

<u>Sample requirements</u>

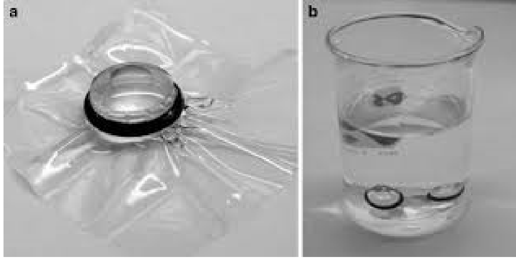
Protein: high purity, stability in detergent, amount < 1 mg, concentration ~ 0.5-2 mg/mL.

Lipid: detergent-solubilized, concentration ~2-5 mg/mL, freshly prepared to avoid oxydation.



Dialysis

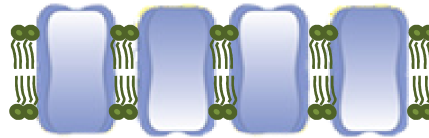
For high c.m.c. detergents, rate of detergent removal difficult to control, slow



Dilution

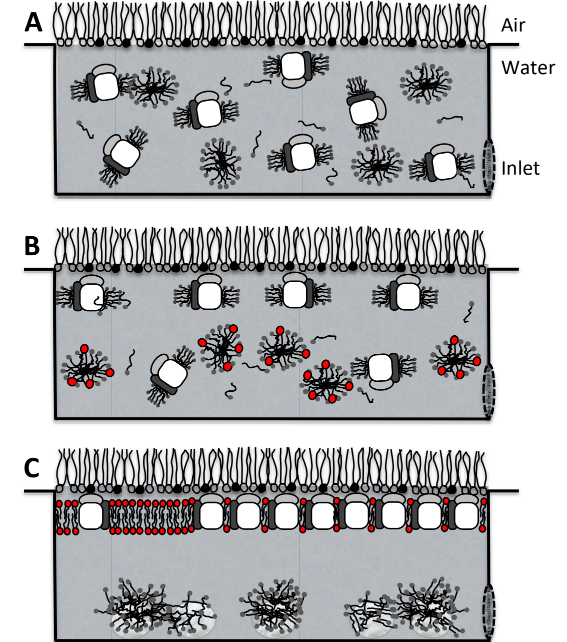
Control of rate of detergent removal, needs high protein concentration

2D crystallization



Detergent removal

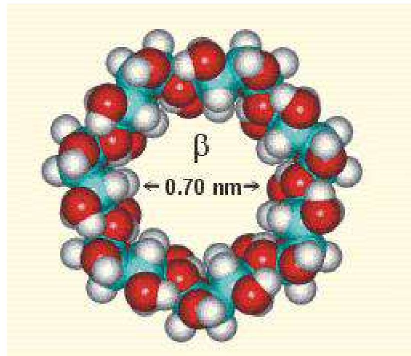
Monolayer technique



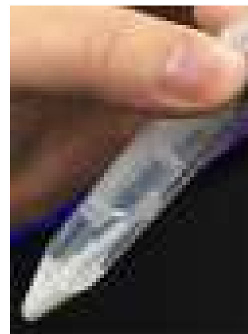
Low protein amount, low protein concentration

Cyclodextrin soln

Binding to detergent with precise stoichiometry, compatible with automation



Biobeads



Polystyrene beads that remove detergent but not lipids, good for low c.m.c. detergents

Negative staining vs Cryo

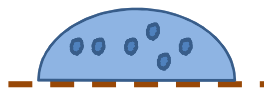
	NEGATIVE STAINING	CRYO
Sample preparation	Staining with heavy atom (usually U)	Freezing at 77 K (and data collection at 77 K)
Contrast	Good	Poor
Resolution	Up to 20-15 Å	Atomic! Up to 1.5 Å
Implementation	Easy and fast	Tricky and time consuming
Radiation damage	Very low	Very high
Equipment	High voltage not required	200-300 kV
Used for	Low resolution information, domain assignment, protein-protein interactions	High resolution information

Negative staining EM – Sample preparation

Sample is embedded in a layer of dried heavy metal salt (salts of Molybdenum, Uranium or Tungsten)
Heavy metal generates more contrast on the images; proteins are imaged as white objects

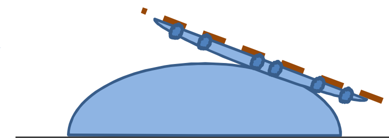
For 2D crystals (electron crystallography) or protein solutions (single-particle EM)

1. Crystal suspension/ protein solution on hydrophilic Cu grid with carbon layer – Incubate ~20 sec

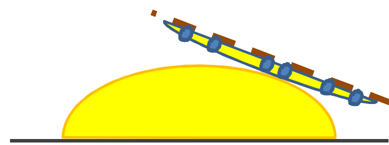


2. Blot on filter paper from the edge

3. Wash quickly on water drop, blot quickly on filter paper (twice)



4. Stain with Uranyl Acetate 0.5-2% and blot (twice)



Problems of NS embedding:

- Distortions due to air drying of grid
- Incomplete staining for large particles
- Limited resolution due to dimension of staining grains (15-20 Å)
- For single-particle EM: Usually orientation of the protein not random (preferred orientation)
- For single-particle EM: Possible effects of staining on protein structure (e.g. flattening)

Cryo Electron Microscopy

Problems:
- Preserve the hydrated sample in the vacuum condition of the microscope
- Avoid specimen flattening due to stain
- Reduce occurrence of partial staining
- Reduce radiation damage

Cryo-electron microscopy: sample kept at low temperature (usually liquid N_2 temperature)

Problem:
Mother liquid crystallization and increased thickness of the sample

Use of cryoprotectant

Problem:
Cryoprotectant scattering power (reduces contrast of background compared to protein)

Sugar (glucose, threalose) or tannic acid embedment

Sugar embedment replaces the mother solution with a ~ 1% sugar solution

Works for 2D crystals, but not for single-particles (contrast is too low!!!)

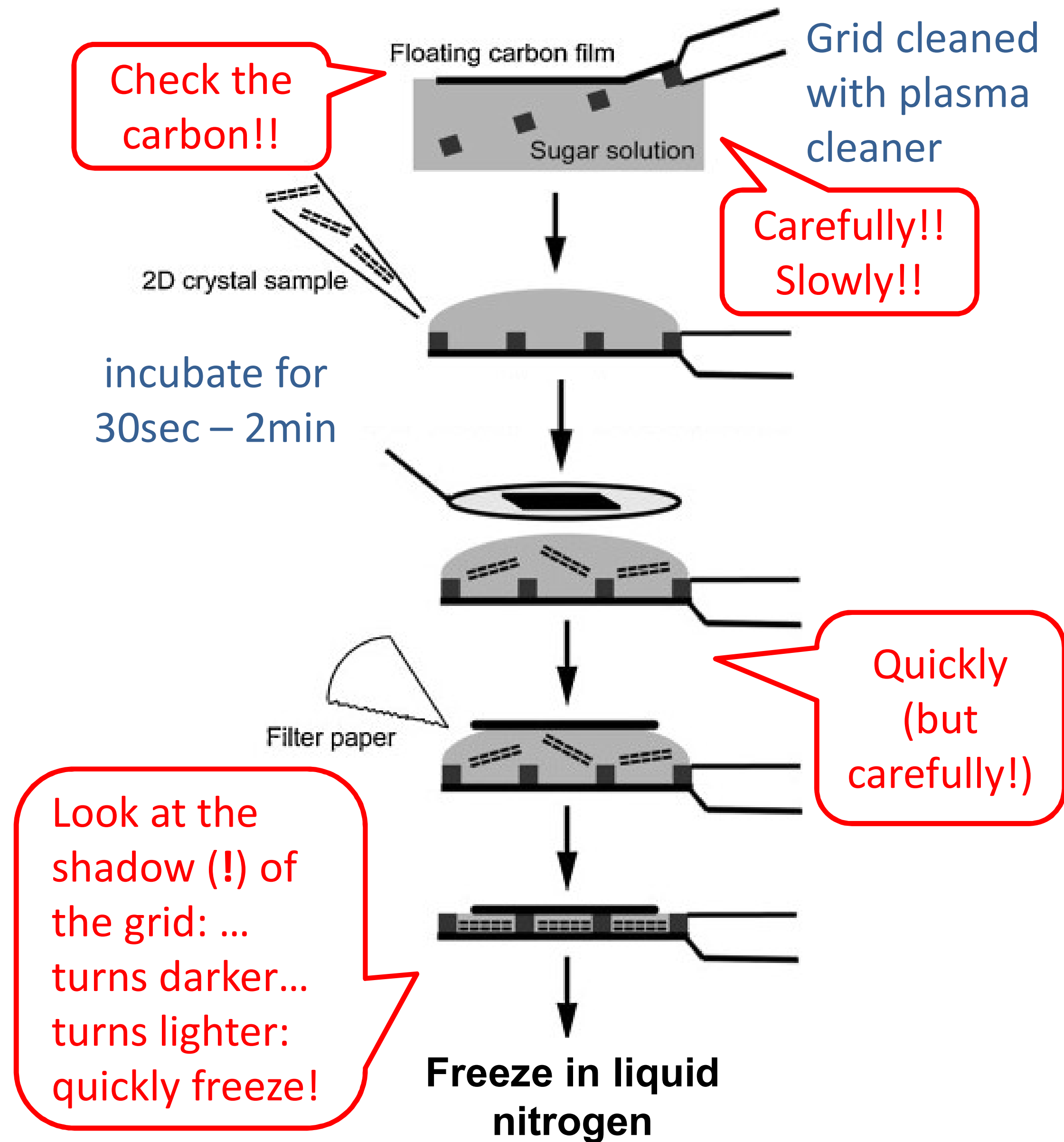
Cryo Electron Crystallography - Sample preparation

Flatness of the sample!!
Important for diffraction (and imaging)

Cryo preservation
Sugar embedding, to preserve crystal and avoid crystallization of water

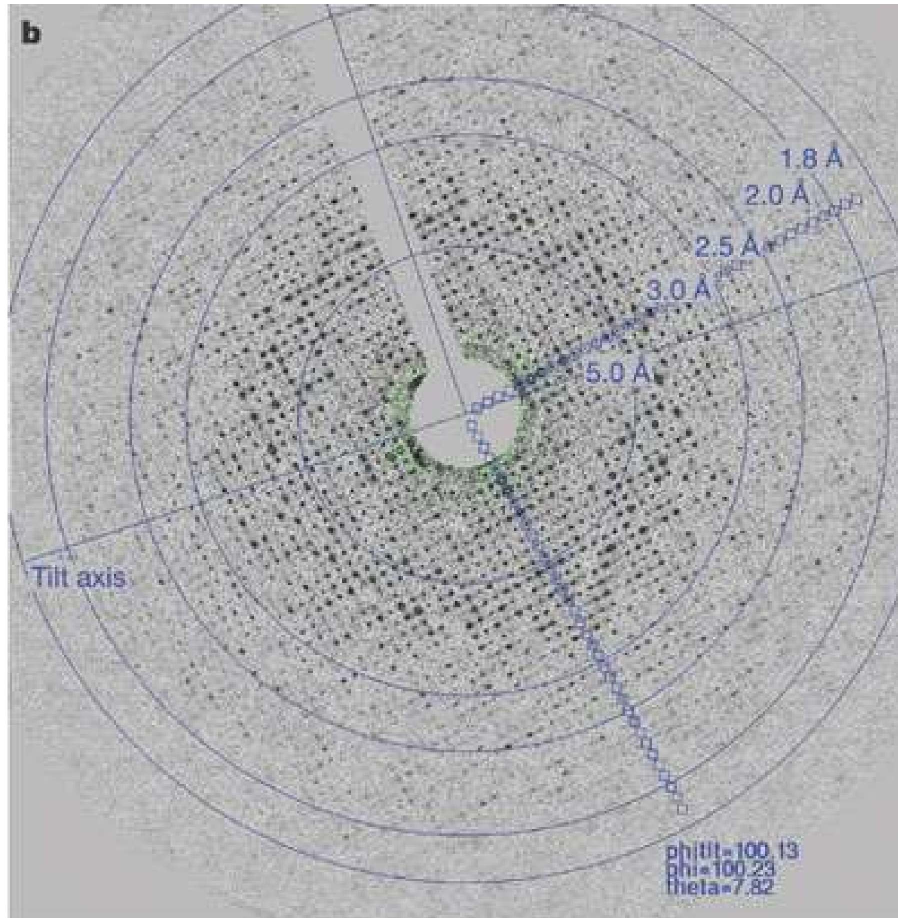
Back injection method
Carbon layer floated on sugar solution, then picked up with grid; crystal suspension on back of grid; blot.

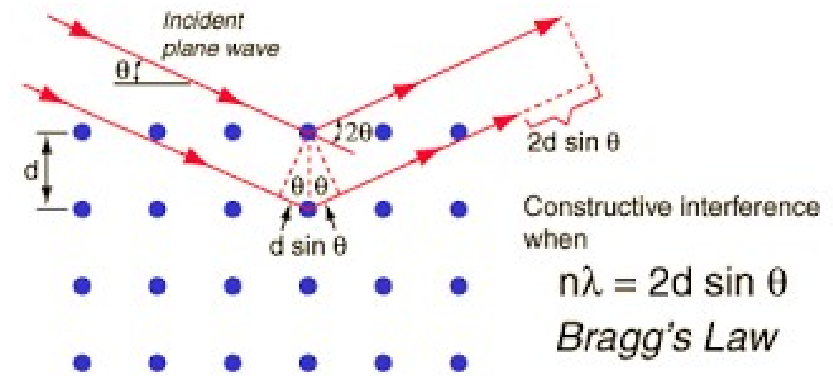
Sandwich method
Crystals between 2 layers of carbon, optimal preservation!!

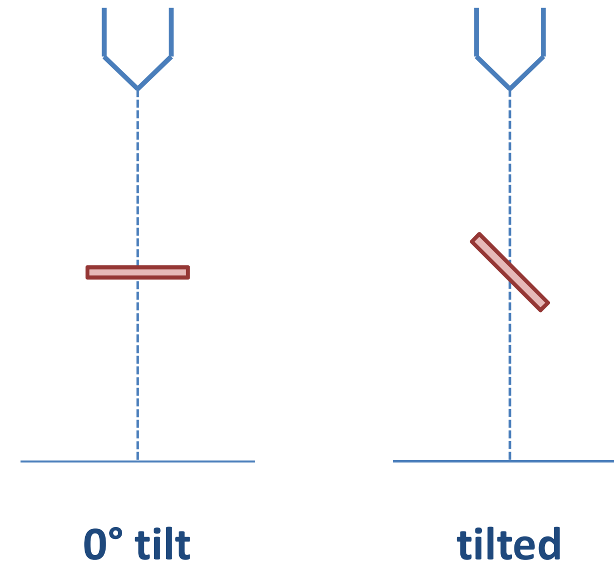


Electron diffraction – data collection

Diffraction mode of microscope:
lens settings to obtain diffraction pattern on screen

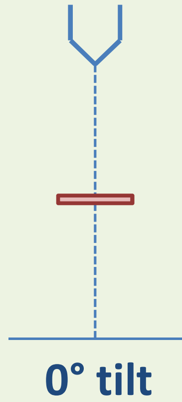


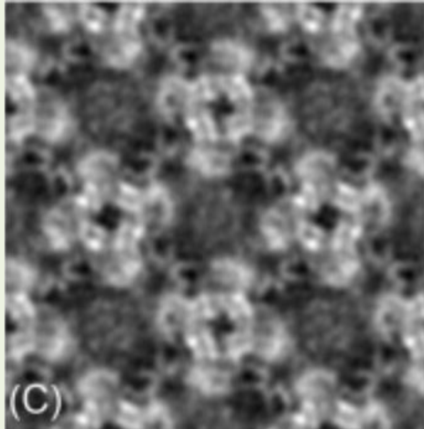




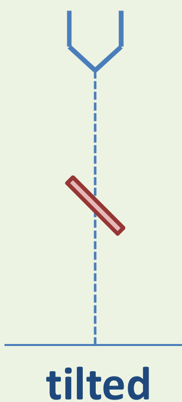
Reciprocal space of 2D crystals

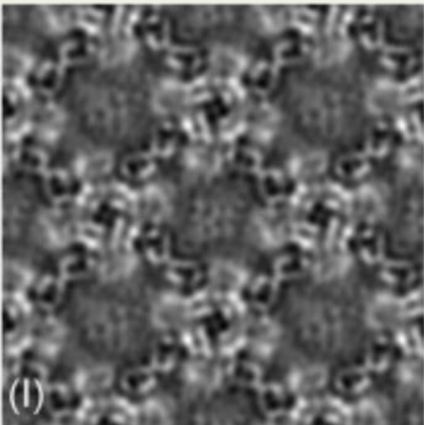
Real space

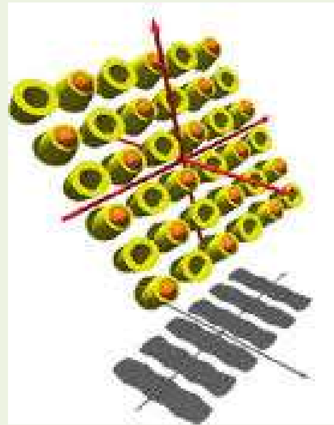




Ordered 2D array of protein molecules (no order in the 3rd direction)



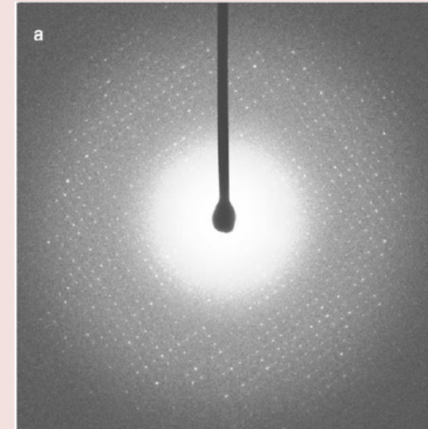


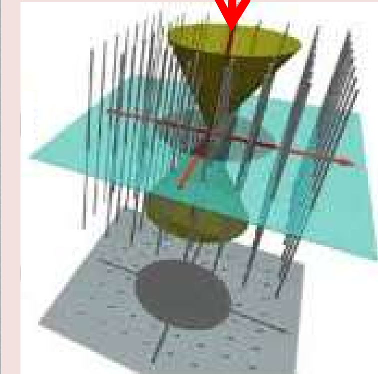


Up to 65-70°

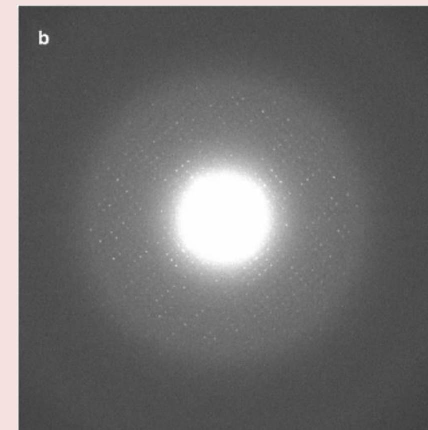
Reciprocal space

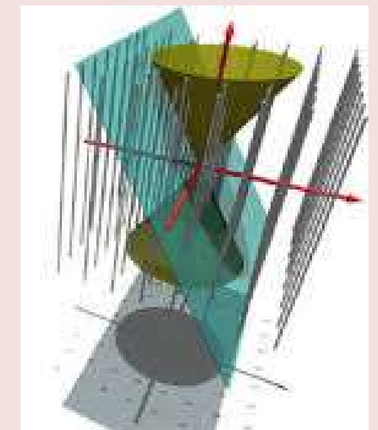
Missing cone





Discretized function in 2 directions, but continuous function in the 3rd direction





Sampling of lattice lines

Electron diffraction – data analysis

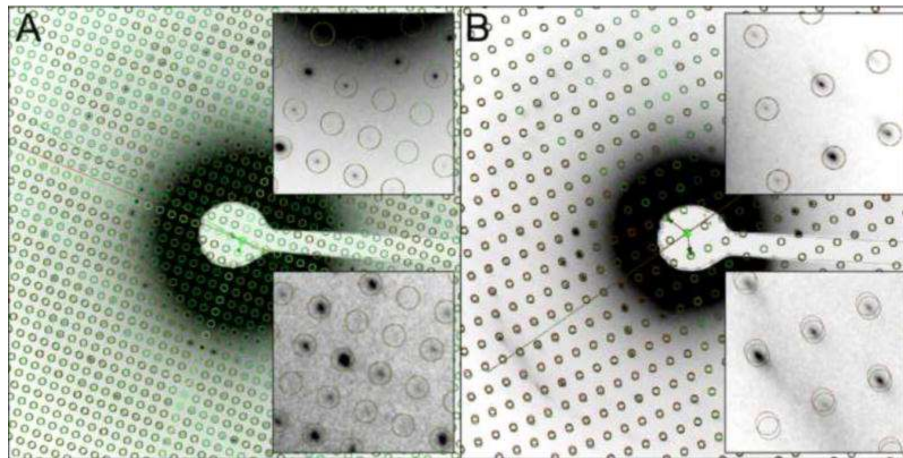
0° tilt
Indexing
Integration

20° tilt
Indexing
Integration

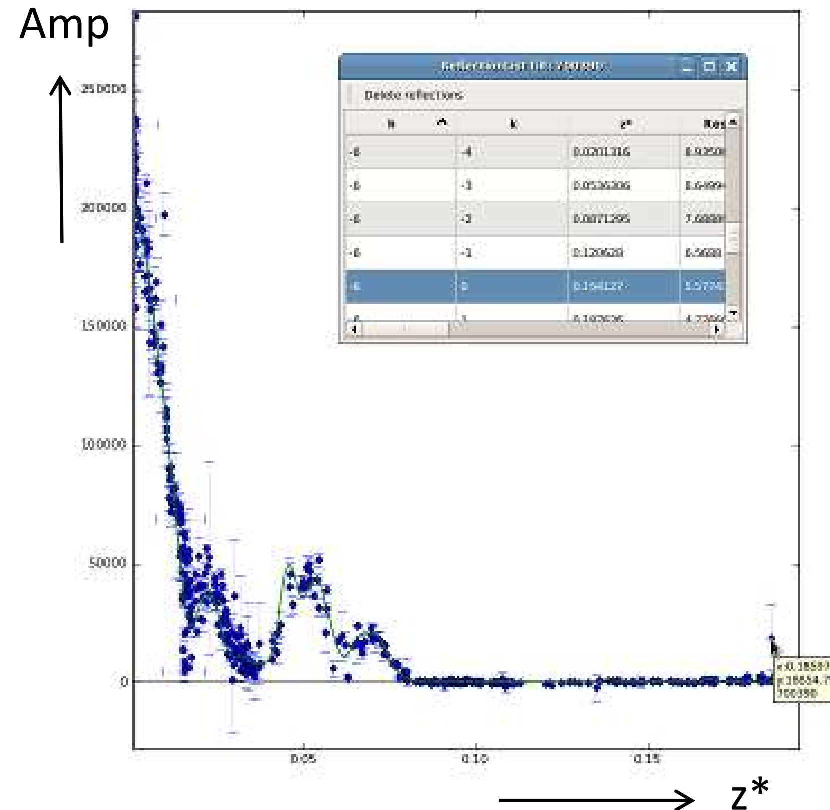
45° tilt
Indexing
Integration

60° tilt
Indexing
Integration

INDEXING & INTEGRATION
(h, k) + tilt axis and angle refinement



SCALING & MERGING
Plot of lattice lines and z* refinement



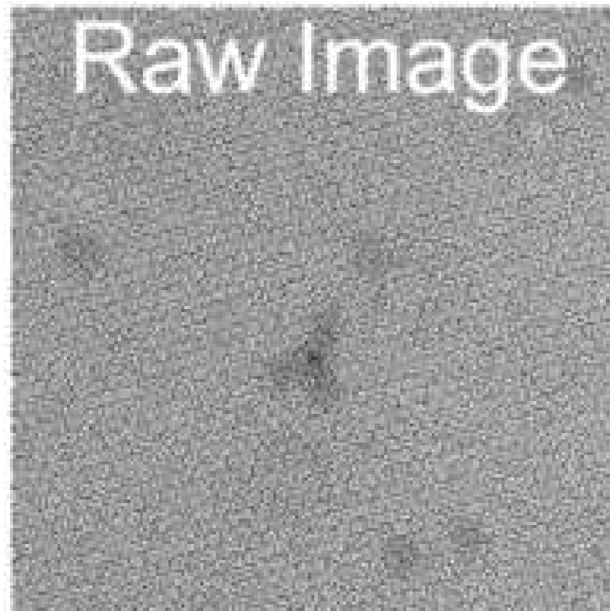
PHASING: Phase information from Molecular Replacement or Images

REFINEMENT (as for X-ray crystallography)

Structure!!

Image analysis

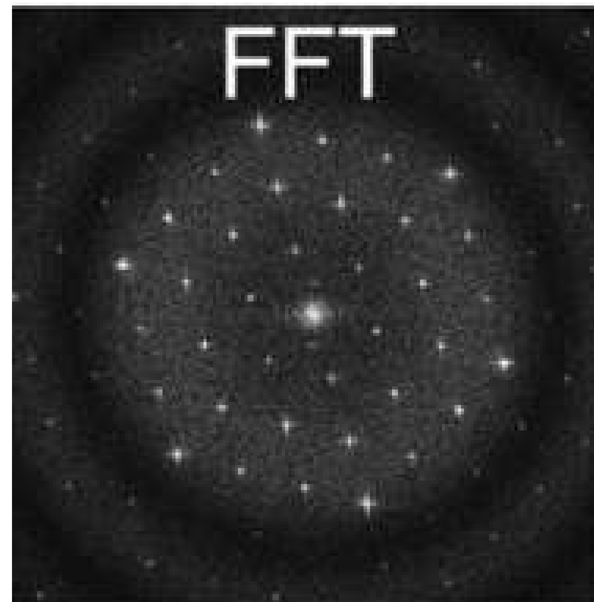
Electron microscopy image of the crystal **in real space**



Noisy! Due to presence of defects in the crystal, inelastic scattering, image acquisition…

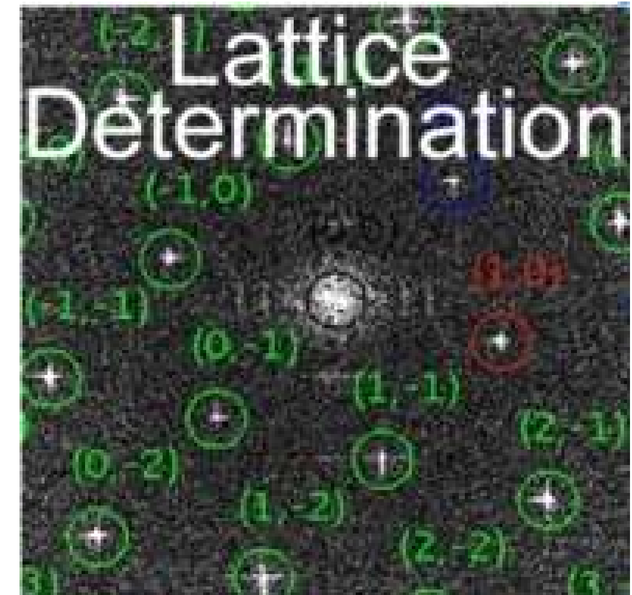
1 Data analysis conveniently performed in reciprocal space!

Power spectrum of the image



Due to order of proteins in the crystal: discretized signal in R*

2 To analyze image, its power spectrum is indexed

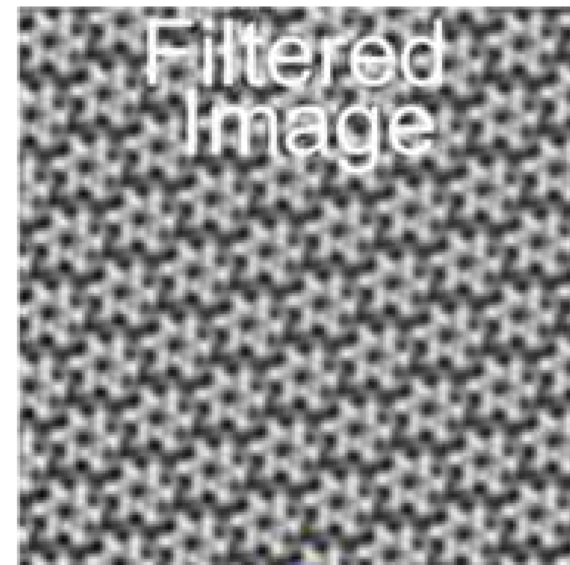


Considering the discrete signal of the crystal in the reciprocal space, part of the noise of the image can be **filtered out**.

3 To filter noise:
In reciprocal space, generate a **mask** considering lattice parameters previously determined and superimpose it to the power spectrum

By masking areas surrounding the spots, a large part of the noise is removed

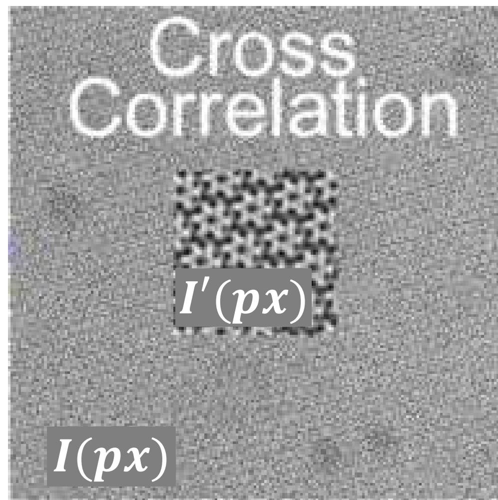
4 An inverse Fourier transform using the signal of the masked FFT generates a **filtered image** of the crystal



The filtered image does not contain details, but can be used for **unbending**

In real space, **UNBENDING**: correction of the noise generated by crystal defects, i.e. the (small) shift of different domains within the crystal

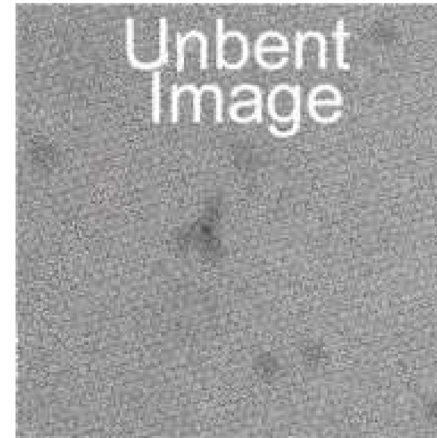
5 The filtered image is used as model, to calculate shift vectors for small domains of the full image by cross-correlation



Cross-correlation function:

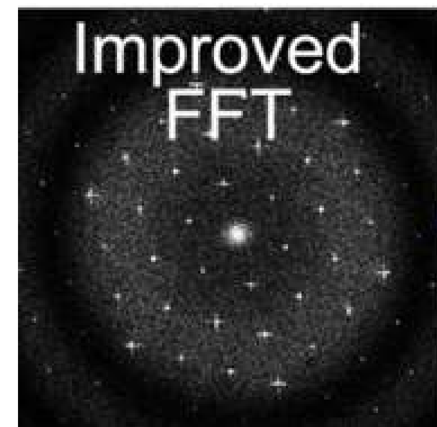
$$CC(\boldsymbol{t}) = \sum_{i}^{domain} I'(\boldsymbol{px}_i) I(\boldsymbol{px}_i + \boldsymbol{t})$$

6 Shift vectors maximizing cross-correlation value are used on each small domain



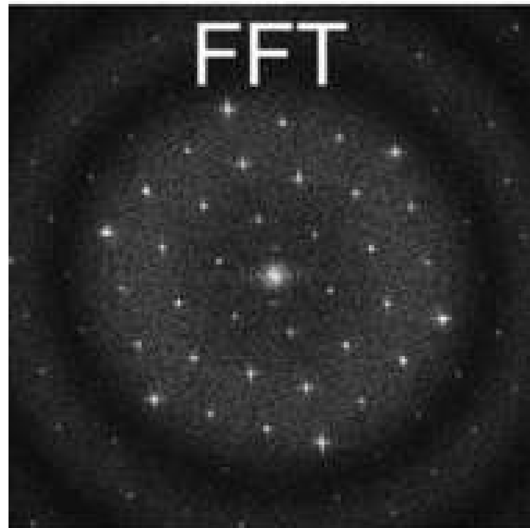
Unbent image (corrected)

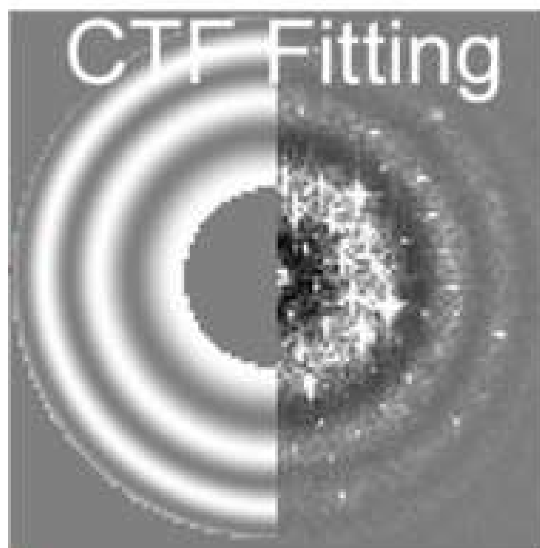
7 Fourier Transform of the unbent image yields an improved power spectrum



CTF correction:

8 From initial power spectrum, CTF is calculated



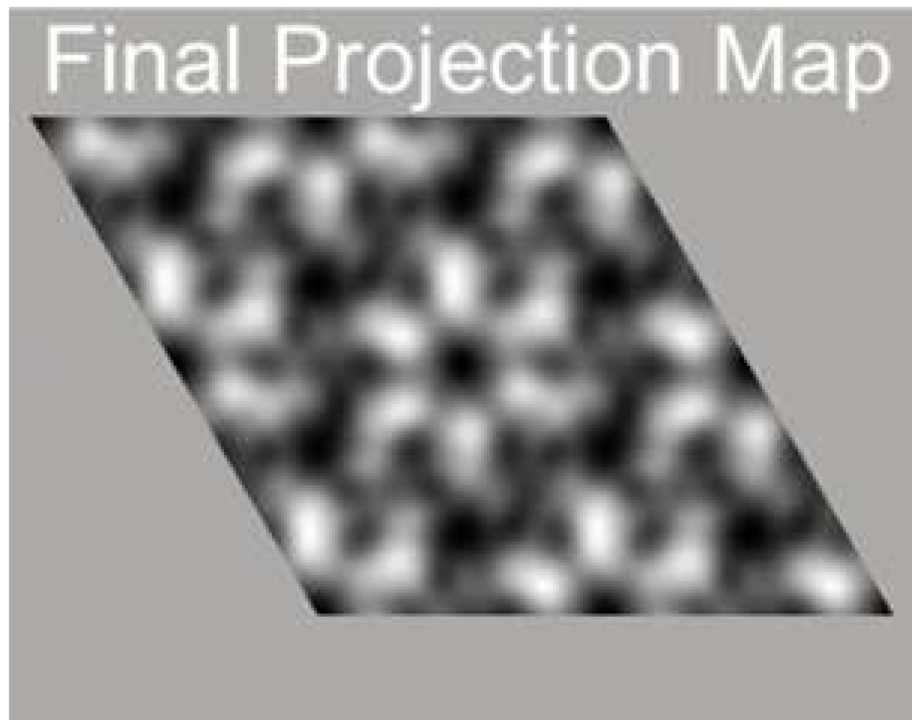


9 CTF correction (phase flipping, Wiener filter…) is applied to the improved power spectrum…

…and values of both amplitudes and phases are extracted

10 In reciprocal space:
merging of many crystals (>10)

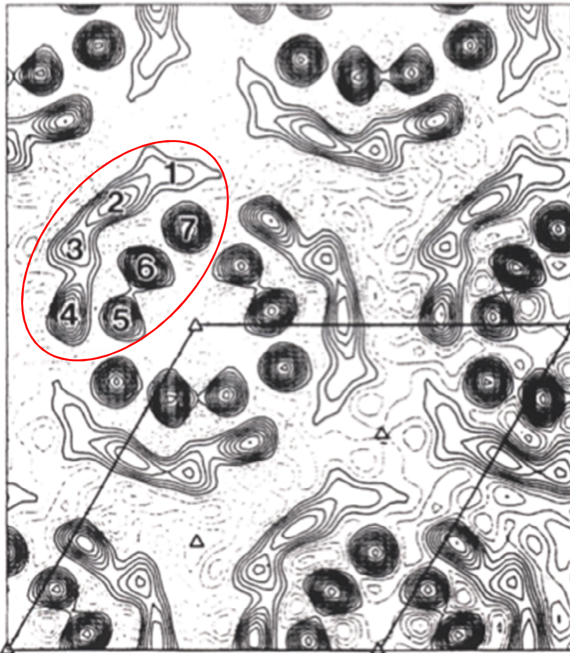
...and the final projection map is calculated by inverse Fourier Transform

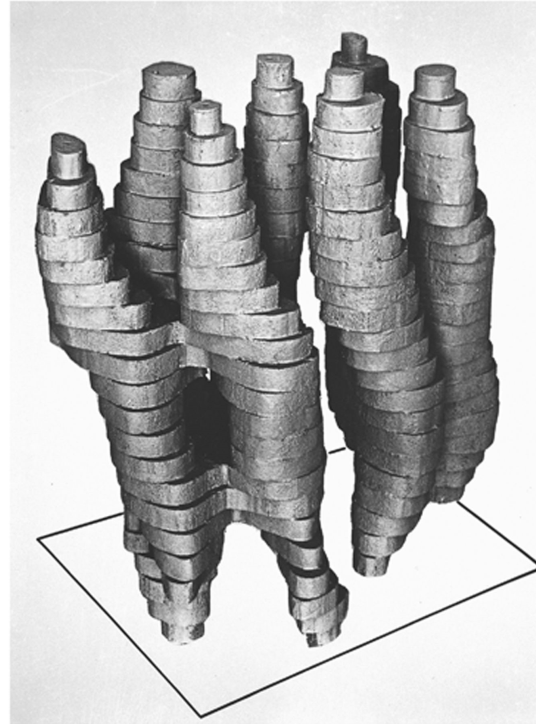


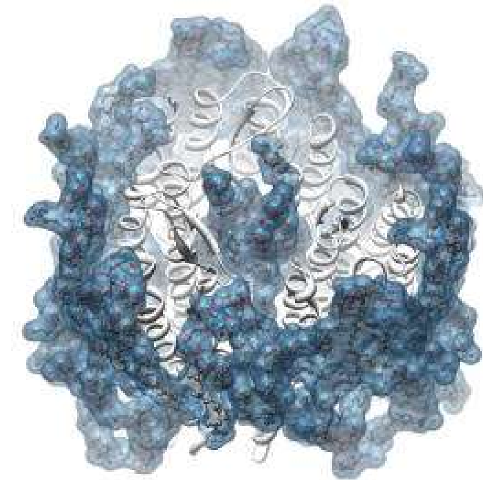
- From the projection map: position and number of helical elements of helical membrane proteins
- If tilted images are available: complete reconstruction of the model (up to 4-5 Å resolution)
- If diffraction patterns are available: combine phases from images and amplitudes from diffraction data to obtain better reconstruction (amplitudes from images have large errors)

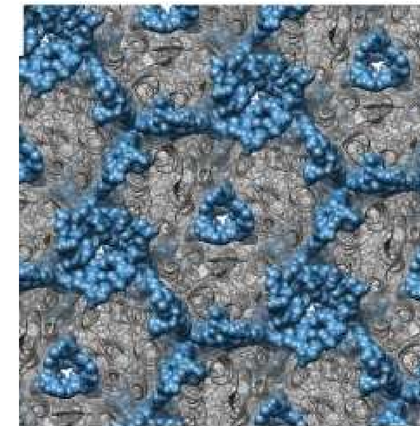
Bacteriorhodopsin

Unwin & Henderson
(MRC - Cambridge)
1975



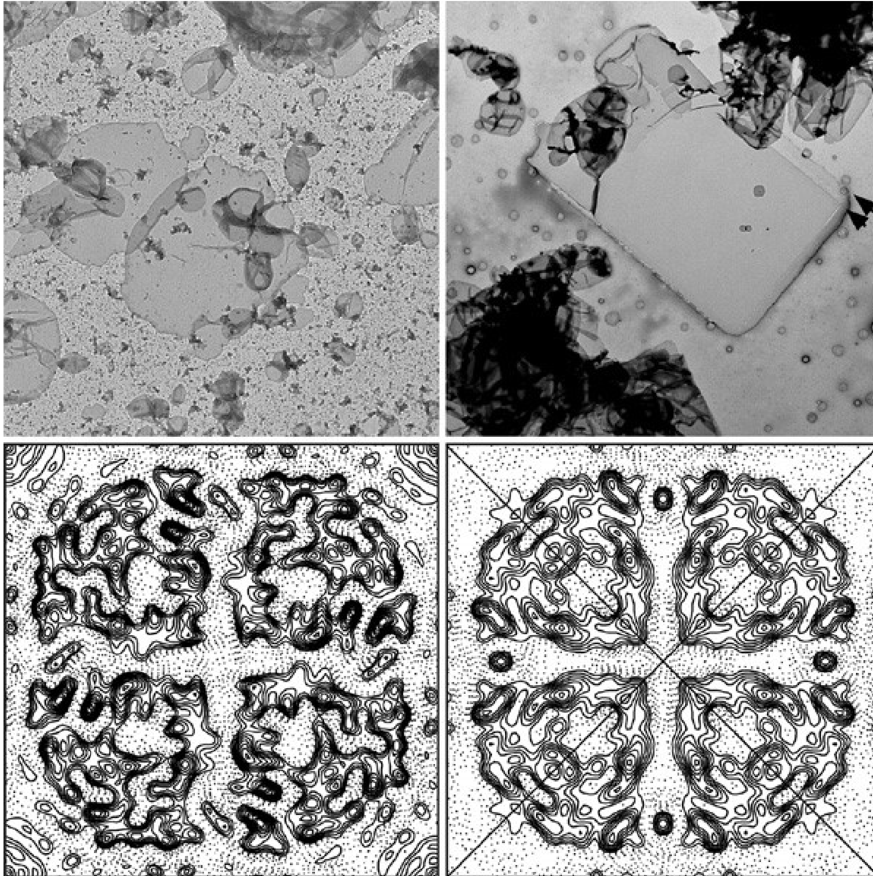




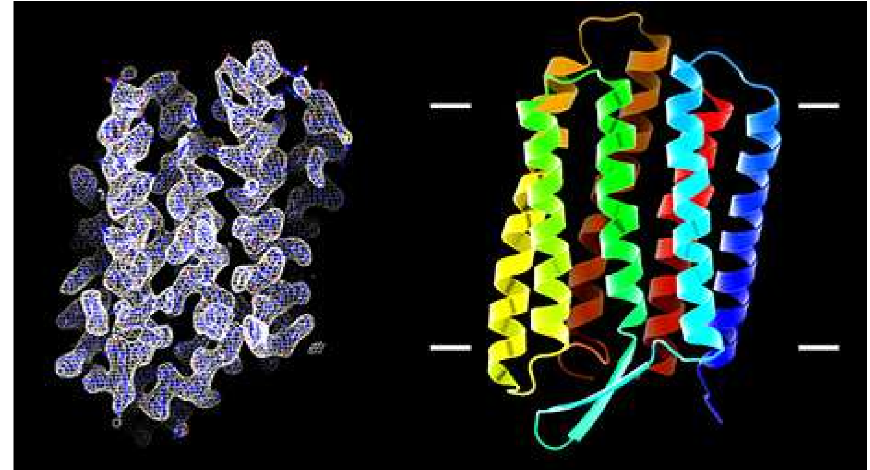


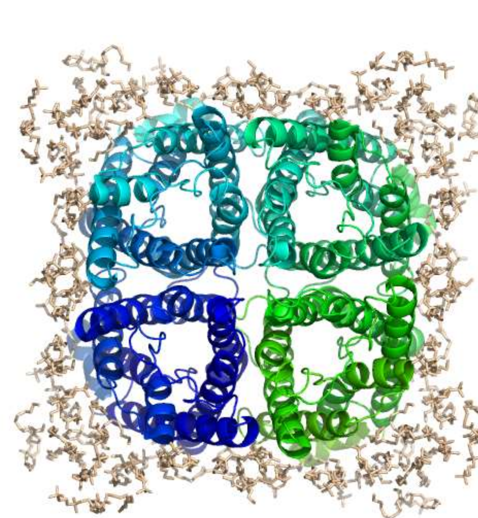
Fujiyoshi's group
(Kyoto University)
1999 – 3 Å

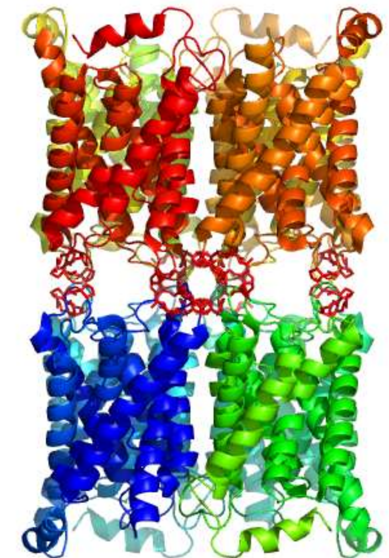
Aquaporin-0



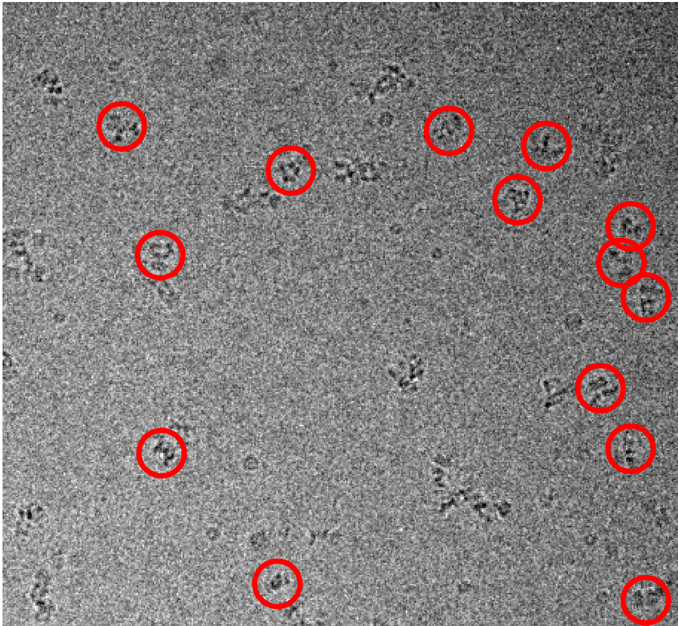
Walz group
(HMS - Boston)
2005 – 1.9 Å





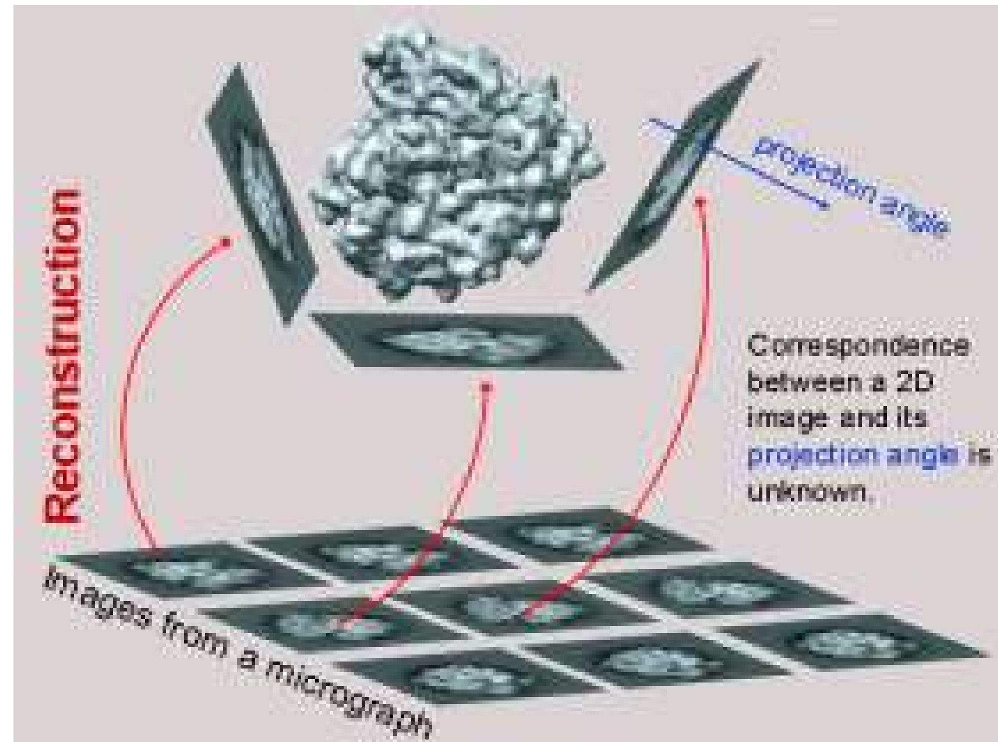


Single particle EM



Different views of the same particle allow 3D reconstruction, but high SNR requires averaging of many particle images in the same orientation

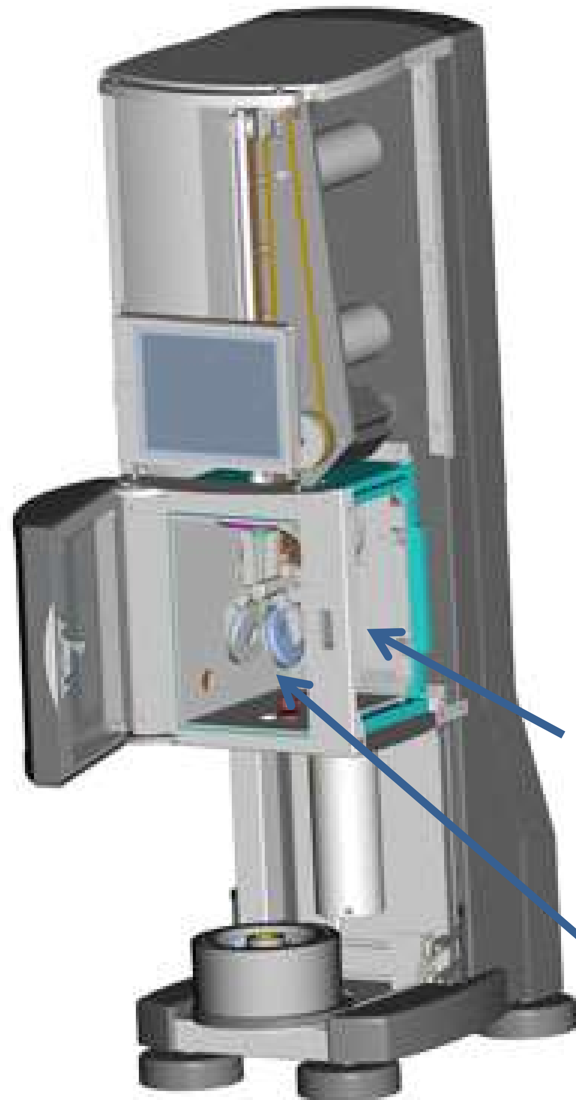
Euler angles (projection angles) allow 3D reconstruction



For particle picking & determination of Euler angles, particles have to show features

Size limitation: proteins not smaller than ~150 kDa

Cryo EM – Sample Preparation



- No cryoprotectants! Increased background!
- Freezing must be very fast to avoid formation of cubic (crystalline) ice that has strong contrast
- Heat capacity of nitrogen is not enough to avoid formation of cubic ice

Plunge-freezing in ethane (high heat capacity) to vitrify water and preserve structural information of protein

Chamber conditions tightly controlled to increase reproducibility

Grid with sample

Grid with sample

Ethane

Nitrogen (to cool ethane)

References

- 2D crystallization: Schmidt-Krey I., "Electron crystallography of membrane proteins: two-dimensional crystallization and screening by electron microscopy.", **Methods. 2007**, *41(4)*:417-26; Schmidt-Krey I. & Rubinstein J.L., "Electron cryomicroscopy of membrane proteins: specimen preparation for two-dimensional crystals and single particles.", **Micron. 2011**, *42(2)*:107-16

- Electron crystallography: Walz T. & Grigorieff N., "Electron Crystallography of Two-Dimensional Crystals of Membrane Proteins.", **J Struct Biol. 1998**, *121(2)*:142-61; Ubarretxena-Belandia I. & Stokes D.L., "Membrane protein structure determination by electron crystallography.", **Curr Opin Struct Biol. 2012**, *22(4)*:520-8.