Sample Preparation

Electron Crystallography Workshop

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University of Basel

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Overview

- Negative staining

- Cryo-EM sample preparation
  - Preparing flat specimens
  - Sugar embedding
  - Back injection method
  - Carbon sandwich method

Negative staining

- Embedding a specimen in a layer of heavy metal salts, such as uranyl acetate, phosphotungstic acid, and ammonium molybdate.

- Provides high contrast for imaging

- Very quick and easy procedure

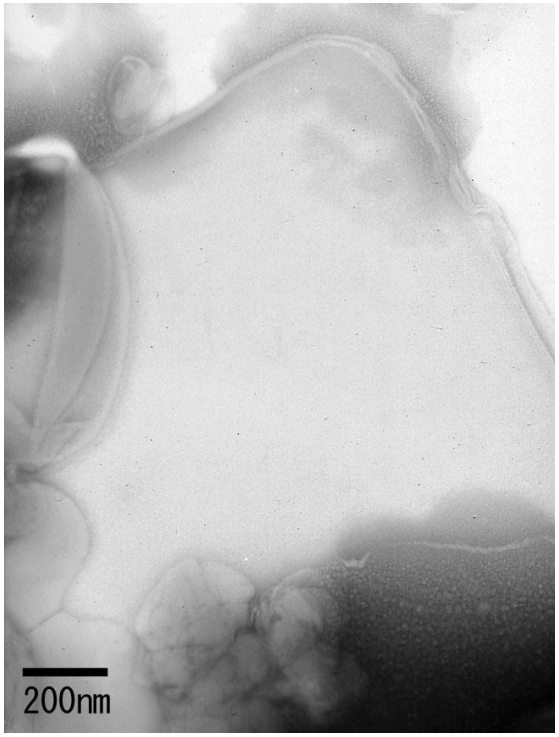
① 2.5μl sample solution is adsorbed to a carbon-coated grid (made hydrophilic by a glow discharge)

② blot the grid with filter paper

(③ wash with several drops of water)

④ stain with two drops of stain

⑤ blot the grid with filter paper and completely dry

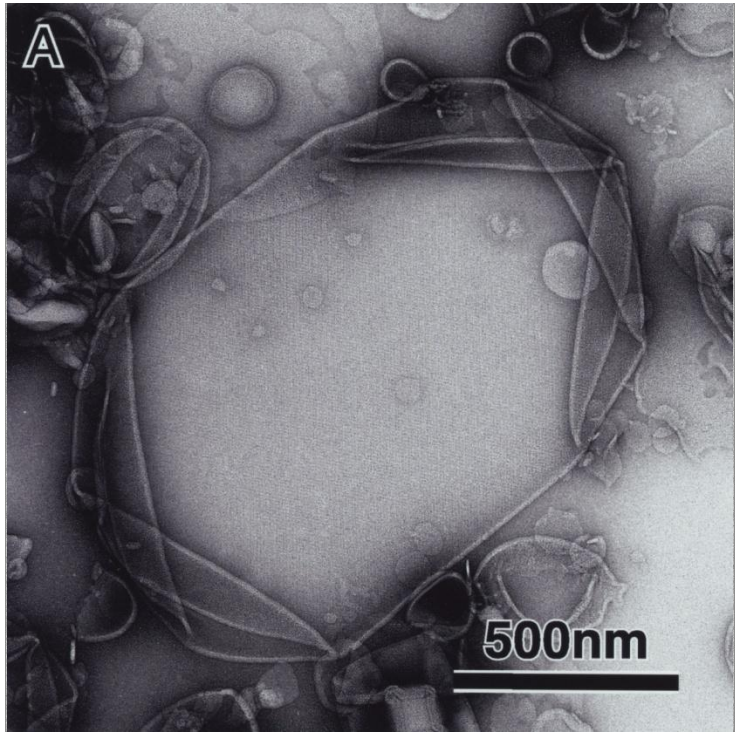


Negative staining

- Screening of crystallization conditions. The information on the morphology and quality of the specimen. Detection of crystalline arrays.

- Crystallographic study at 2-3 nm resolution. Rough estimate of the molecular surface, shape and the packing arrangement.

- Staining and drying results in distortions of the molecules. Incomplete stain embedding gives artifacts.



Cryo-electron microscopy

- Specimens are unstained. They are nearly in a native environment and artifactfree.

- Provides low contrast. The information of negative staining of the same specimen (crystal shape, lattice locallization, size of crystals, …) helps when taking cryo-EM data.

- Crystallographic study at a middle resolution to an atomic resolution.

Atomic models of biological macromolecules by cryo-electron microscopy

Protein	Year	Sample Preparation	Embedding Medium
Bacteriorhodopsin	**1990**	**2D crystals**	**Glucose**
Plant light-harvesting complex (LHC-II)	**1994**	**2D crystals**	**Tannin**
α, β - tubulin	**1998**	**2D crystals**	**Tannin-glucose**
Aquaporin-1	**2000**	**2D crystals**	**Trehalose**
Acetylcholine receptor	**2003**	**helical crystals**	**Ice**
Bacterial flagellar filament	**2003**	**helical crystals**	**Ice**
Aquaporin-0	**2004**	**2D crystals**	**Glucose, Trehalose**
Aquaporin-4	**2005**	**2D crystals**	**Trehalose**
Microsomal Glutathione transferase 1 (MGST1)	**2006**	**2D crystals**	**Trehalose**
Microsomal prostaglandin E synthase 1 (MPGES1)	**2008**	**2D crystals**	**Trehalose**

Preparing flat specimens

Lack of specimen flatness is caused by:

・the roughness of the carbon support film

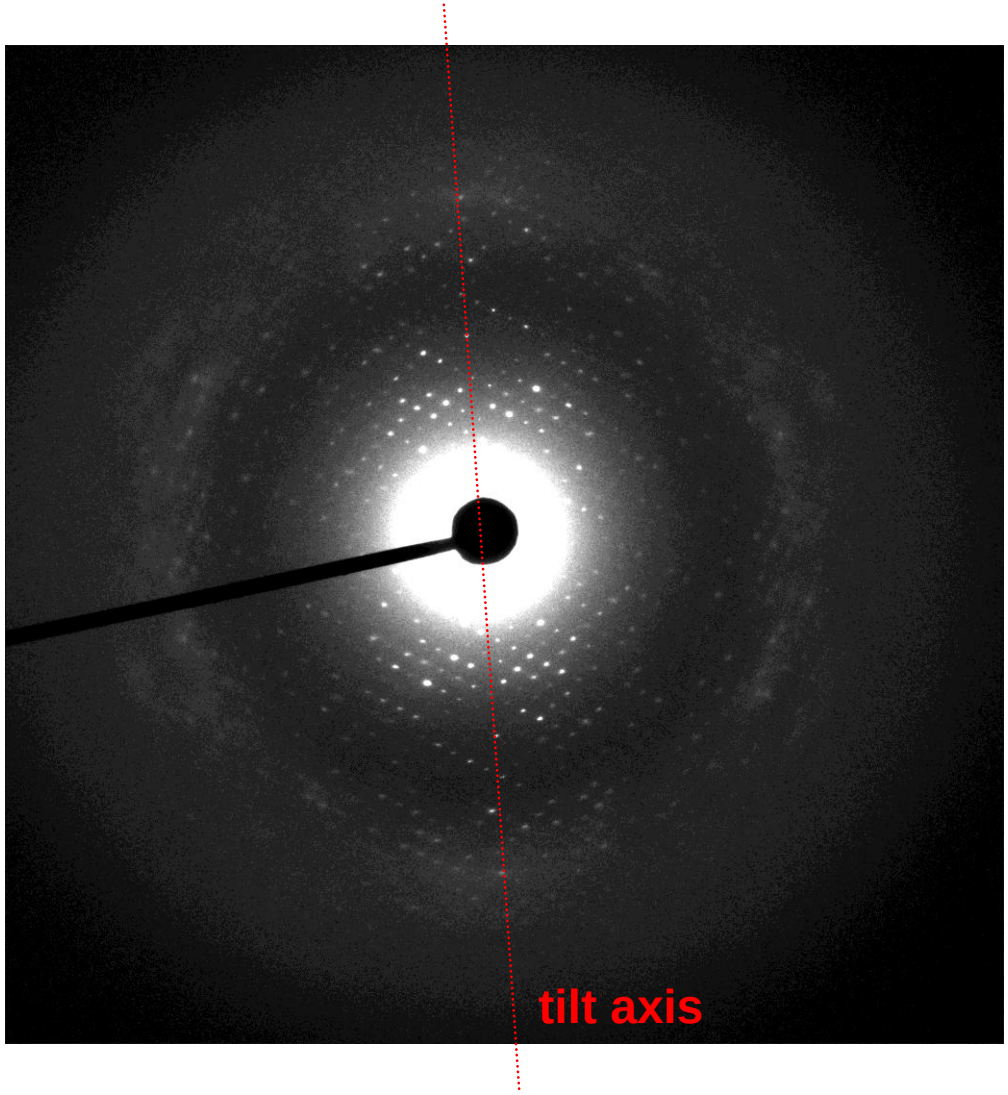
・the wrinkling of carbon film supported by EM grid upon cooling (cryo-crinkling)

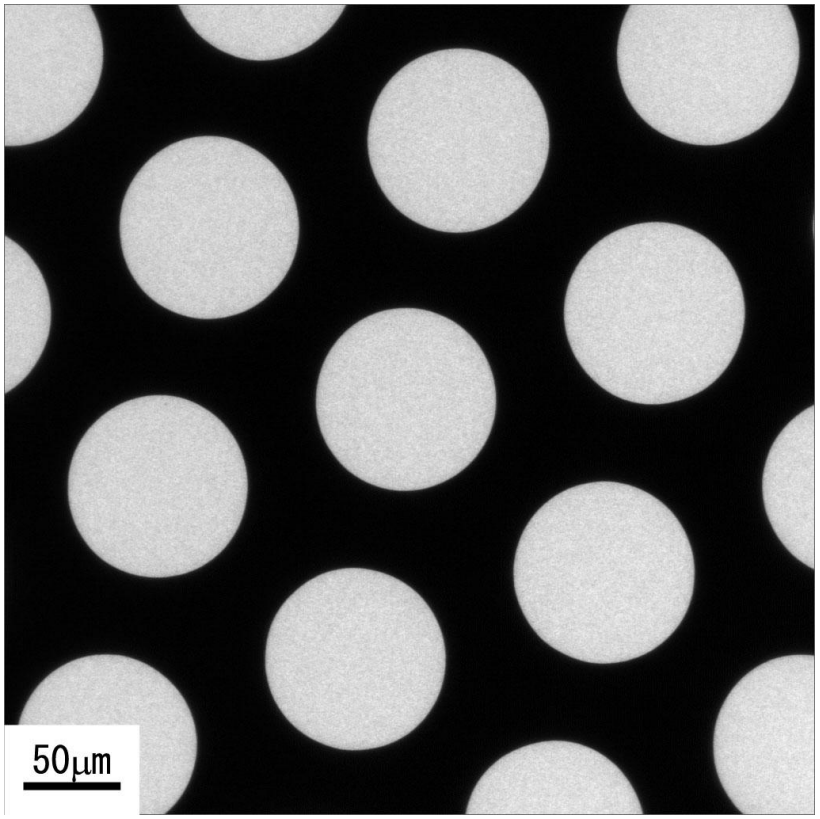
Solutions:

・Use of molybdenum grids (the thermal expansion coefficient is similar to that of carbon)

・Use of flat carbon support films by spark-less evaporation

Electron diffraction pattern of a tilted specimen that shows imperfect specimen flatness



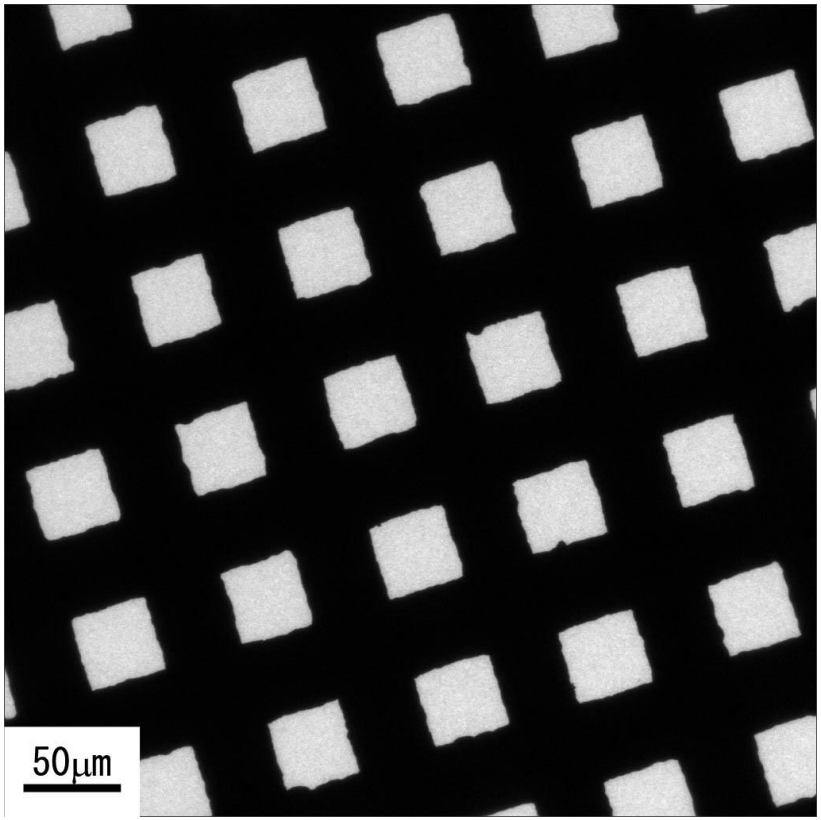


Special Mo grid from JEOL Ltd.

・smooth surface

・prevents carbon films from wrinkling

・larger holes; larger visible area upon taking tilted data



Mo grid in common use

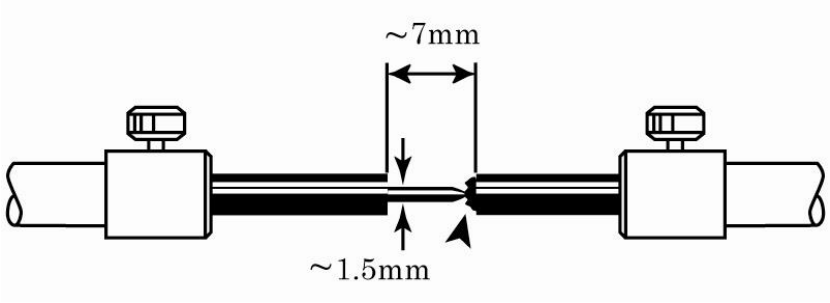
・rough surface

・causes wrinkling of carbon films

How to prepare a high-quality flat carbon film by vacuum evaporation

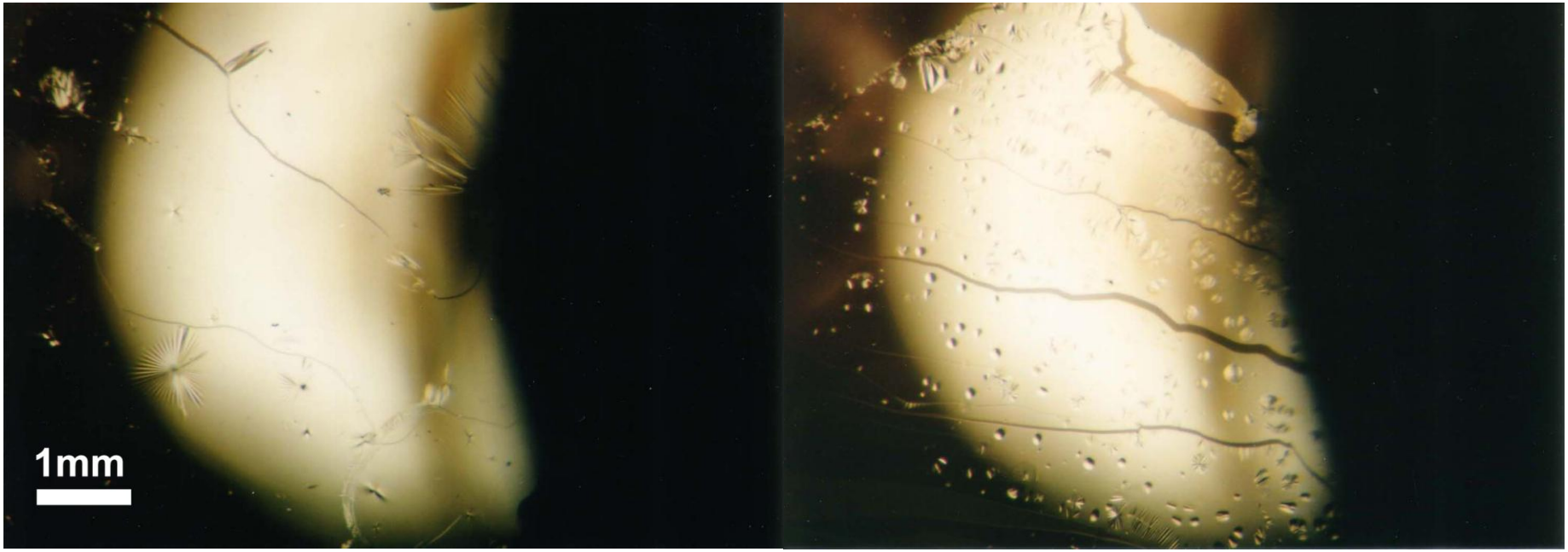
- Use of pure carbon rods with a purity of 99.9999% and good-quality mica plates
- Evacuation for more than one day before evaporation
- Pre-evaporation of the carbon rod
- Evaporation on freshly-cleaved mica plates to a thickness of 5–10 nm
- The vacuum: better than 2 x 10^{-6} Torr (= 2.66 x 10^{-6} mbar)

Vacuum evaporator JEOL JEE-420



the setting of the carbon rods

High-quality carbon support film



Spark-less evaporation

Evaporation with sparkling

Sugar embedding

The specimen has to be in vacuum in the electron microscope

→ dehydration of the specimen
flattening and collapse of the protein structure

Solutions:

・Sugar embedding
Water surrounding the specimen is replaced by a less volatile sugar which mimics the native environment.

Atomic models by cryo-electron microscopy and embedding medium used

Protein	Year	Embedding Medium
Bacteriorhodopsin	**1990**	**Glucose**
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α, β - tubulin	**1998**	**Tannin-glucose**
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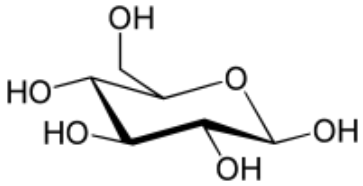
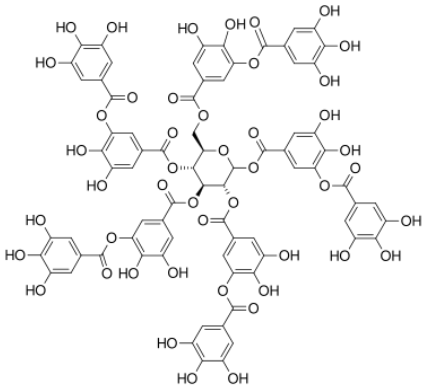
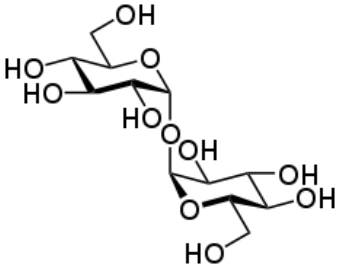
Aquaporin-4	**2005**	**Trehalose**
Microsomal Glutathione transferase 1 (MGST1)	**2006**	**Trehalose**
Microsomal prostaglandin E synthase 1 (MPGES1)	**2008**	**Trehalose**



glucose



tannic acid



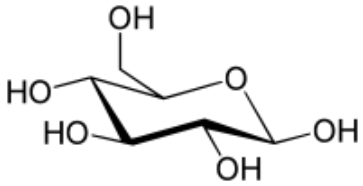
trehalose

Sugar embedding

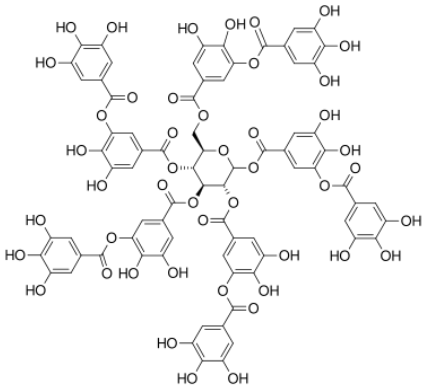
・Sugar molecules fill in the crevices and grooves within proteins, preventing the flattening and collapse.

・Sugar molecules are substituted for water molecules on the protein surface and form interactions with proteins to preserve a native, hydrated state.

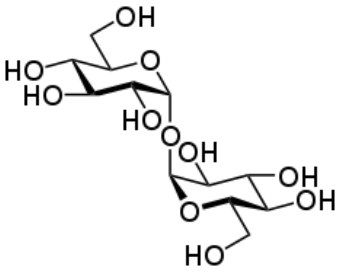
・Trehalose prevents ice crystal formation.



glucose



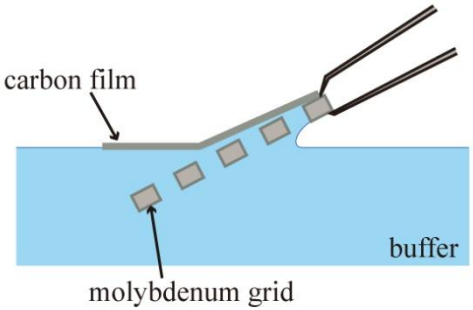
tannic acid



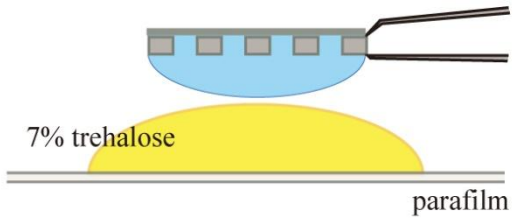
trehalose

Back injection method

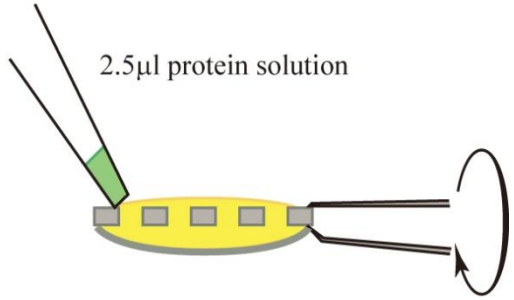
(a)



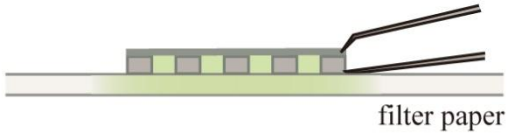
(b)



(c)



(d)



(e)

Air drying

Carbon sandwich method

The beam-induced image shift

→ very low yield of good-quality images when taking tilted images using a conventional back injection method

e.c. The success rate of taking good images of AQP4 crystals at 45 degree tilt is 30% for back injection method. That of bacteriorhodopsin at more than 60 degree tilt is several %. That of H^+, K^+-ATPase is 5% at 20 degree and zero at more than 45 degree.

Solutions:

- Carbon sandwich method

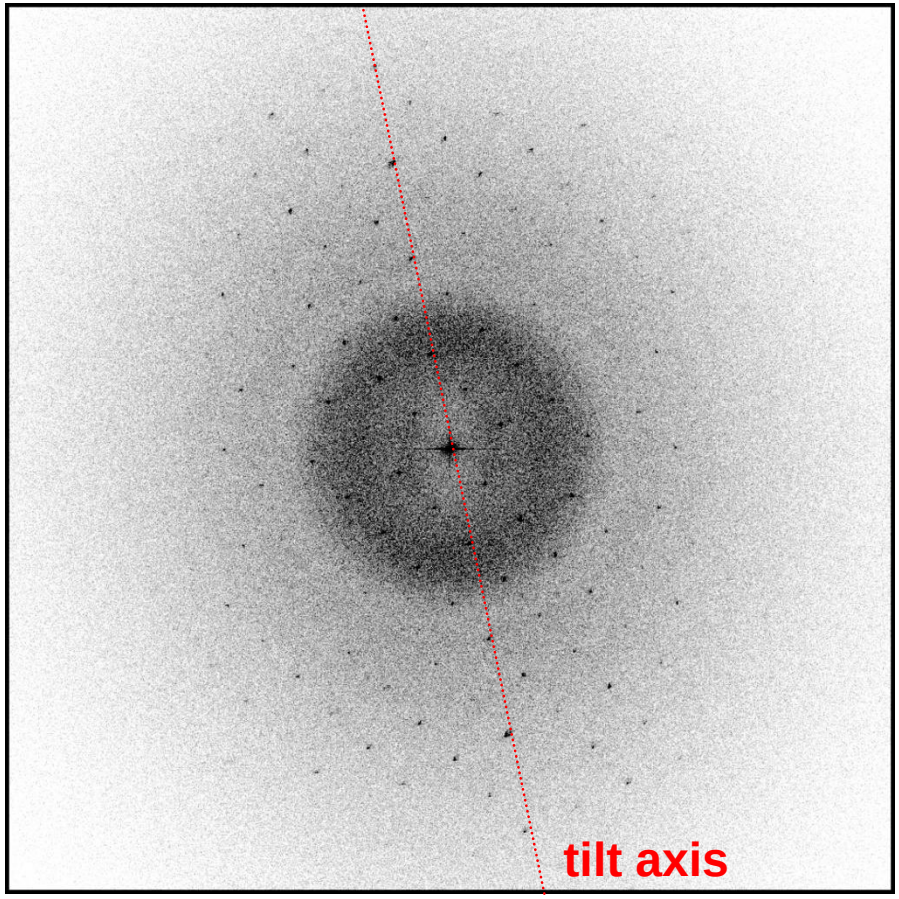
  The specimen is symmetrically sandwiched between two carbon films

  Extremely high success rate of taking good-quality tilted images

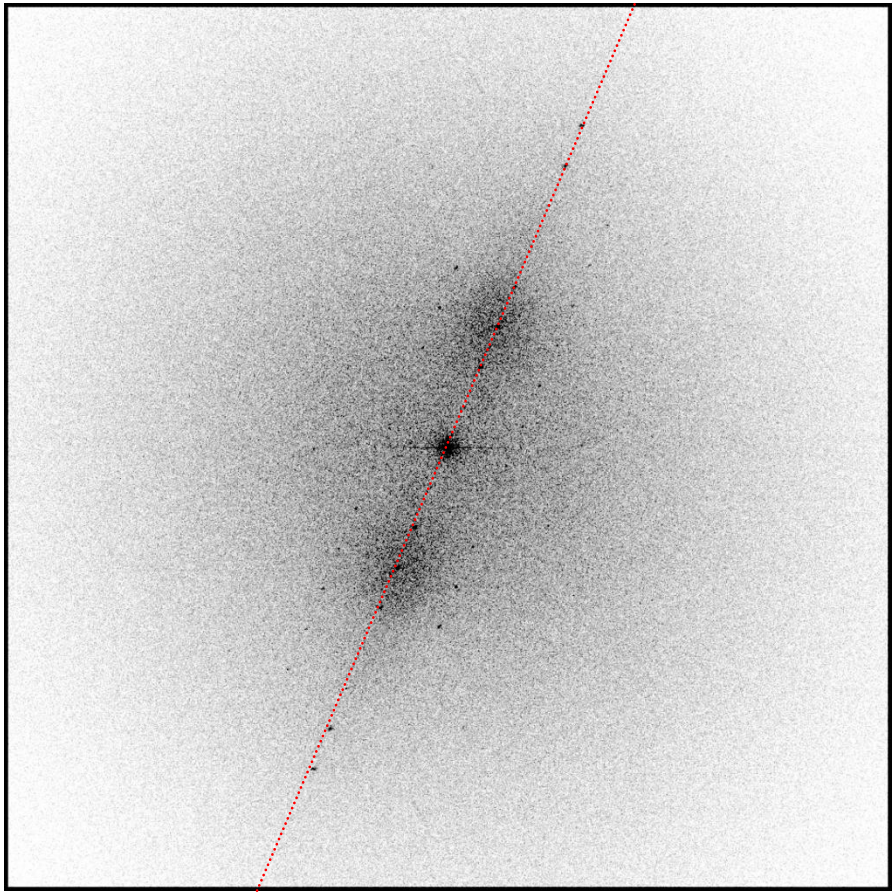
e.c. The success rate of taking good images of AQP4 crystals at 45 degree tilt is 90% for carbon sandwich method. That of bacteriorhodopsin at more than 60 degree tilt is 90%. Images up to 70 degree tilt has been collected for H^+, K^+-ATPase.

Fourier transforms of AQP4 crystals at 45 degree tilt

Without beam-induced image shift

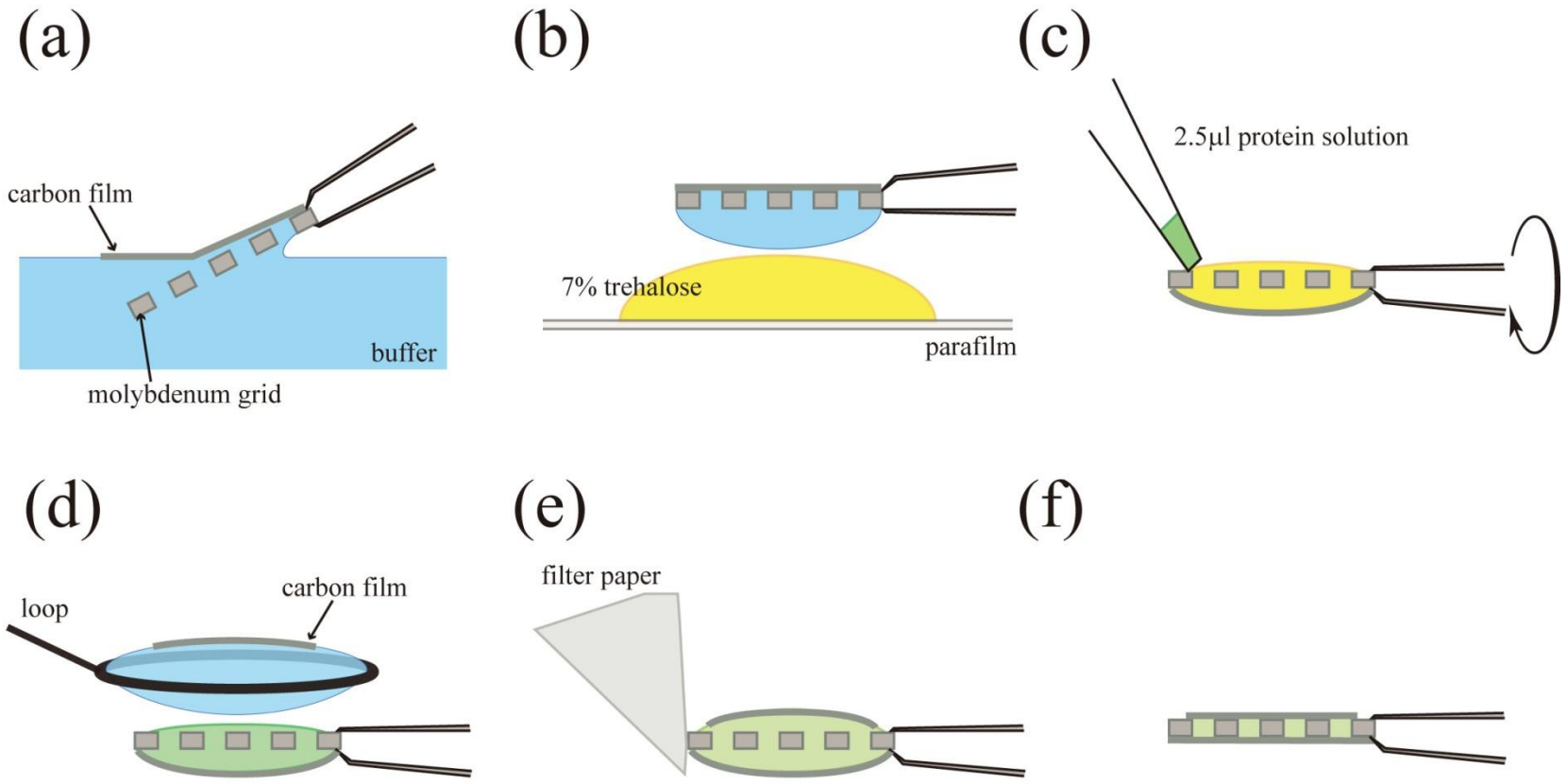


With beam-induced image shift



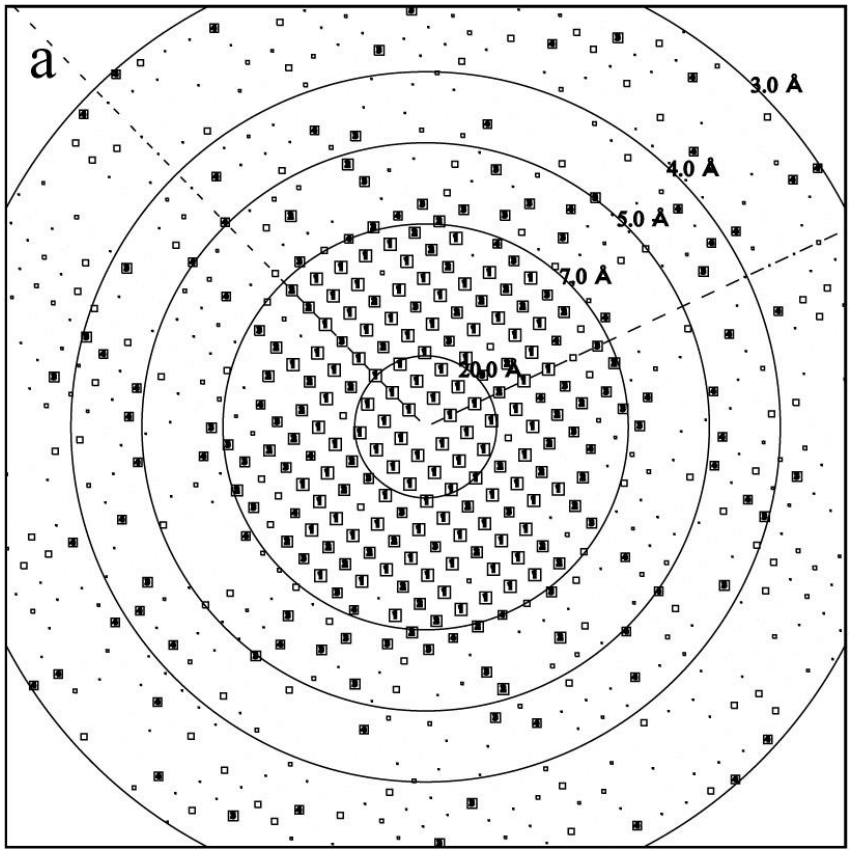
Carbon sandwich method

Gyobu et al. *J. Struct. Biol.* (2004) 146, 325

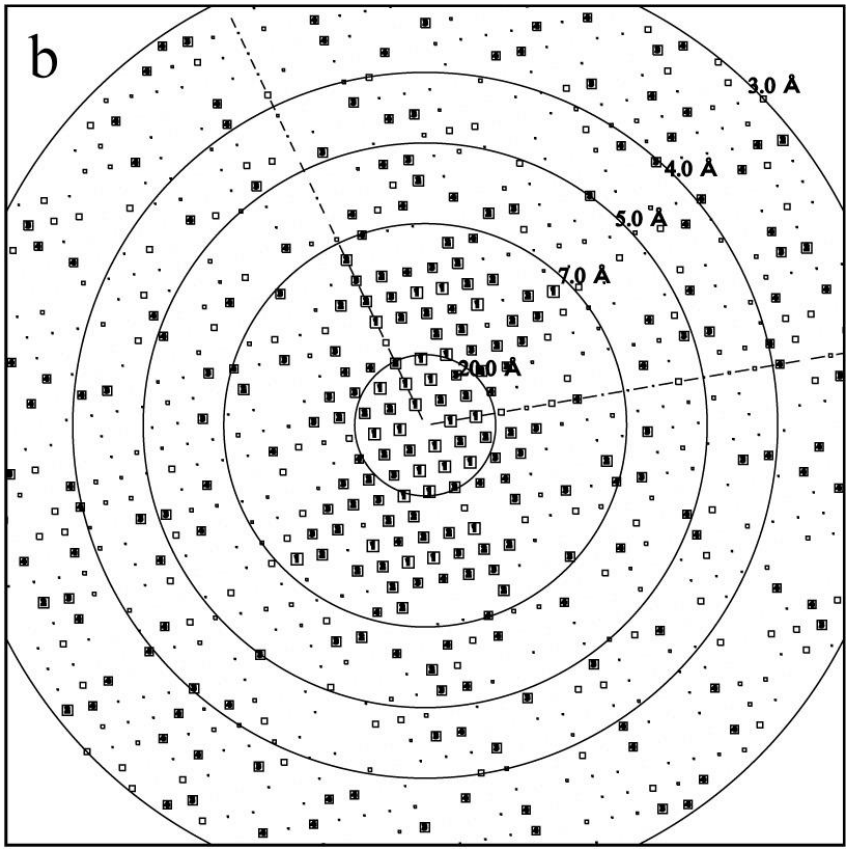


Carbon sandwich method produces images of better quality

Fourier components of good images of AQP4 crystals tilted by 45 degree

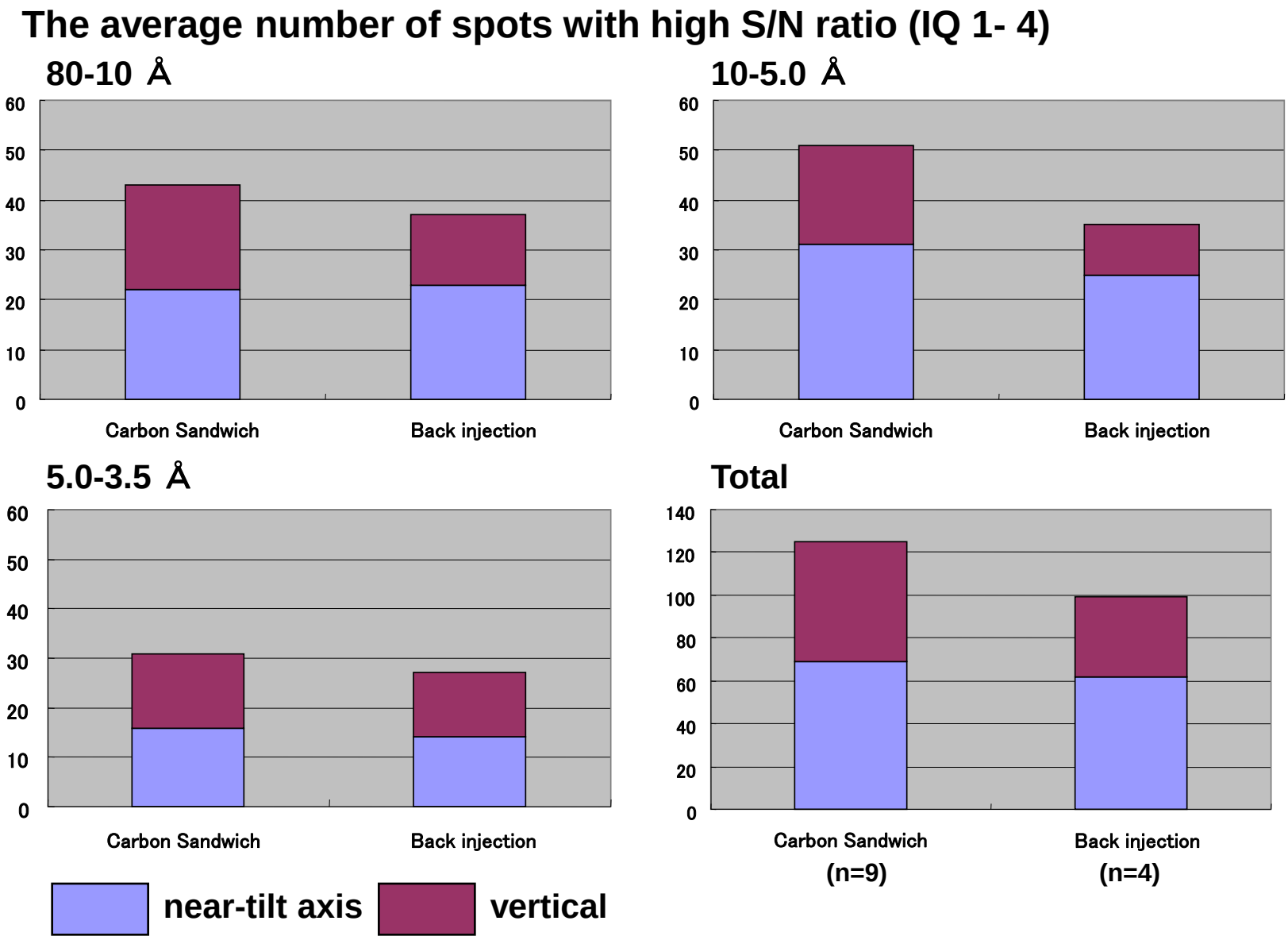


Carbon sandwich



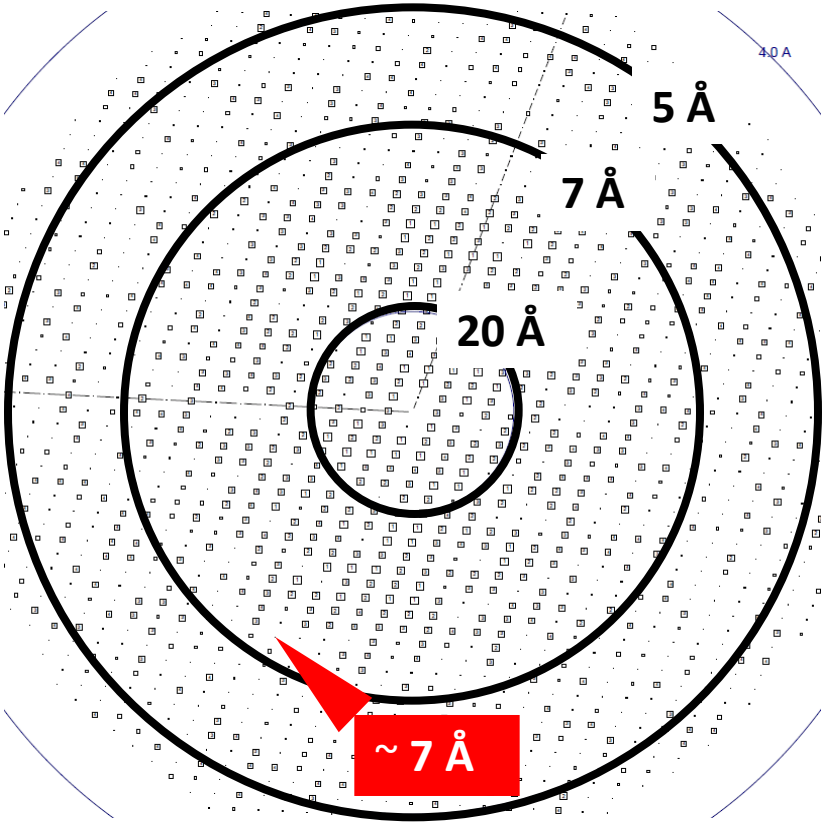
Back injection

Carbon sandwich method produces images of better quality

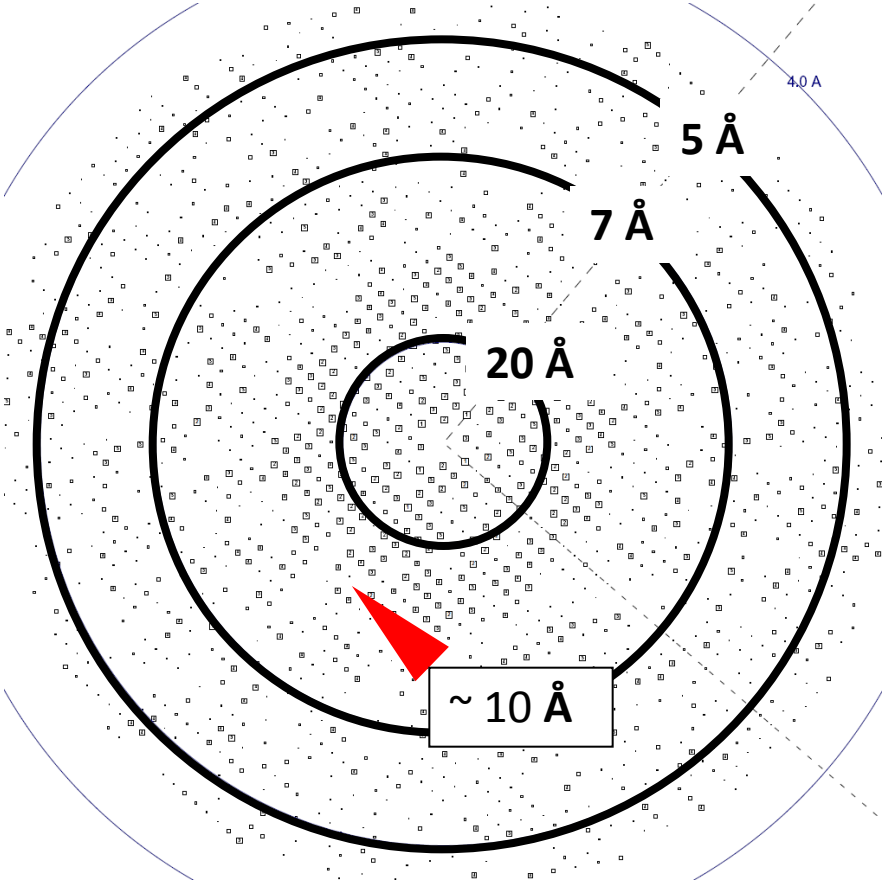


Improvement in resolution using H^+, K^+-ATPase 2D crystals

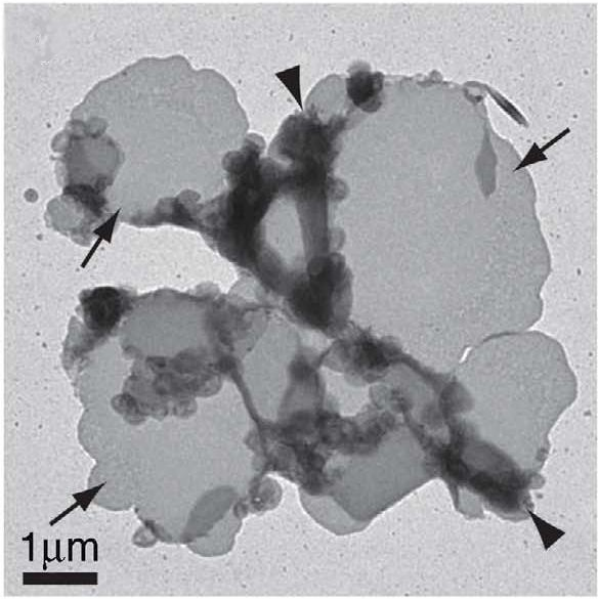
Carbon sandwich method

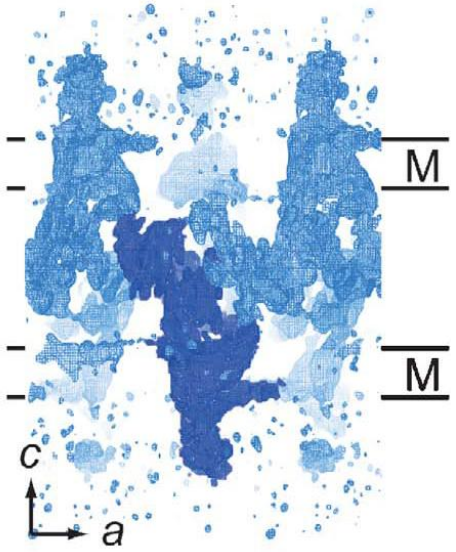


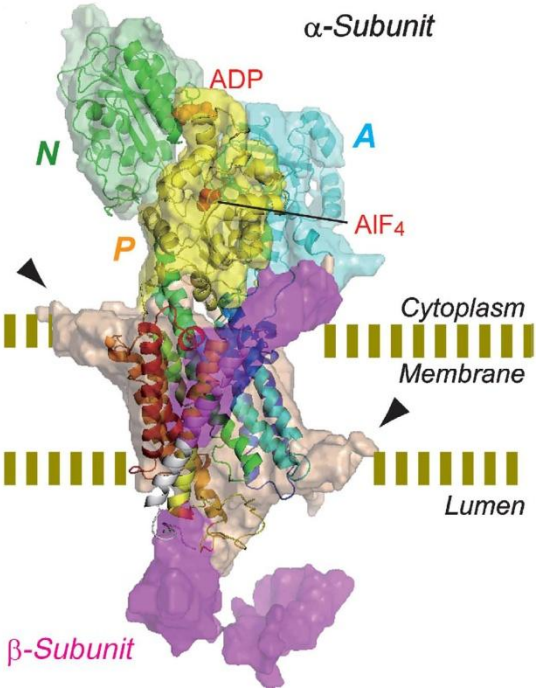
Back injection method



Cryo-EM structure of H^+, K^+-ATPase at 6.5 Å resolution



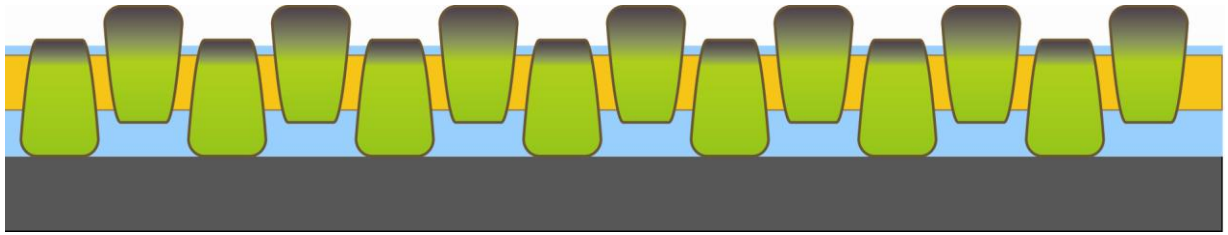




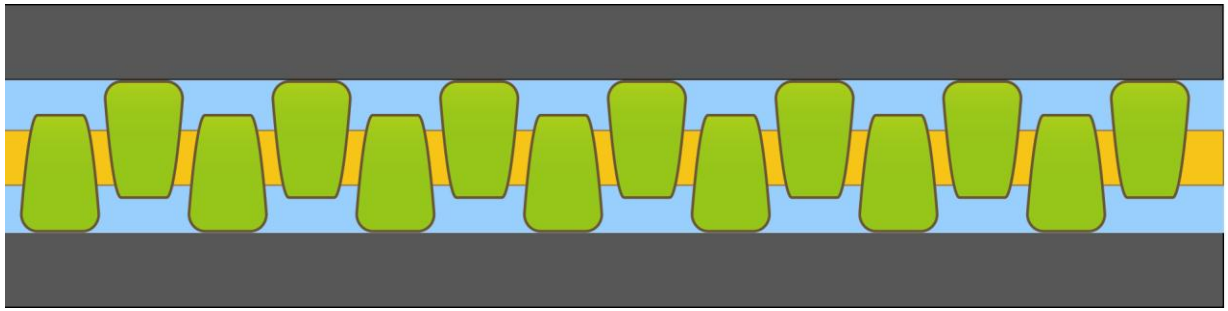
Abe K. *et al.* (2009) *EMBO J.*

Proteins are completely kept hydrated in carbon sandwich method

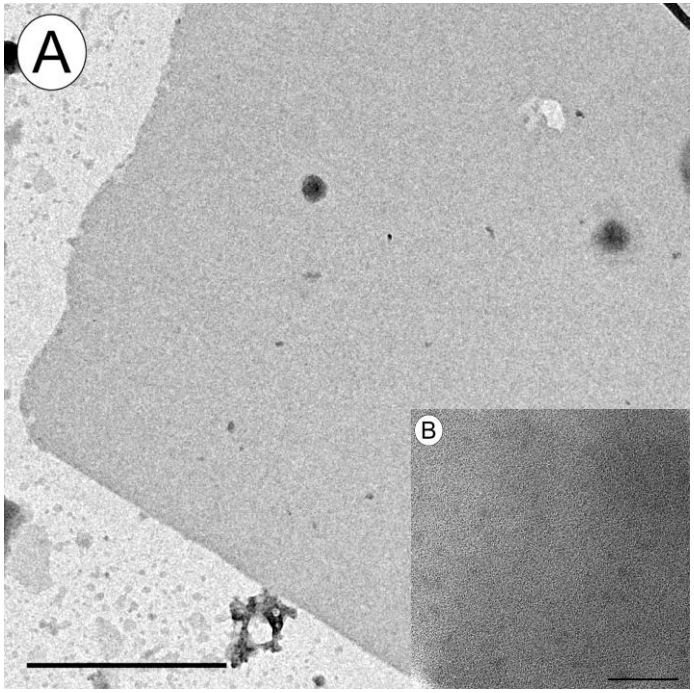
Back injection method

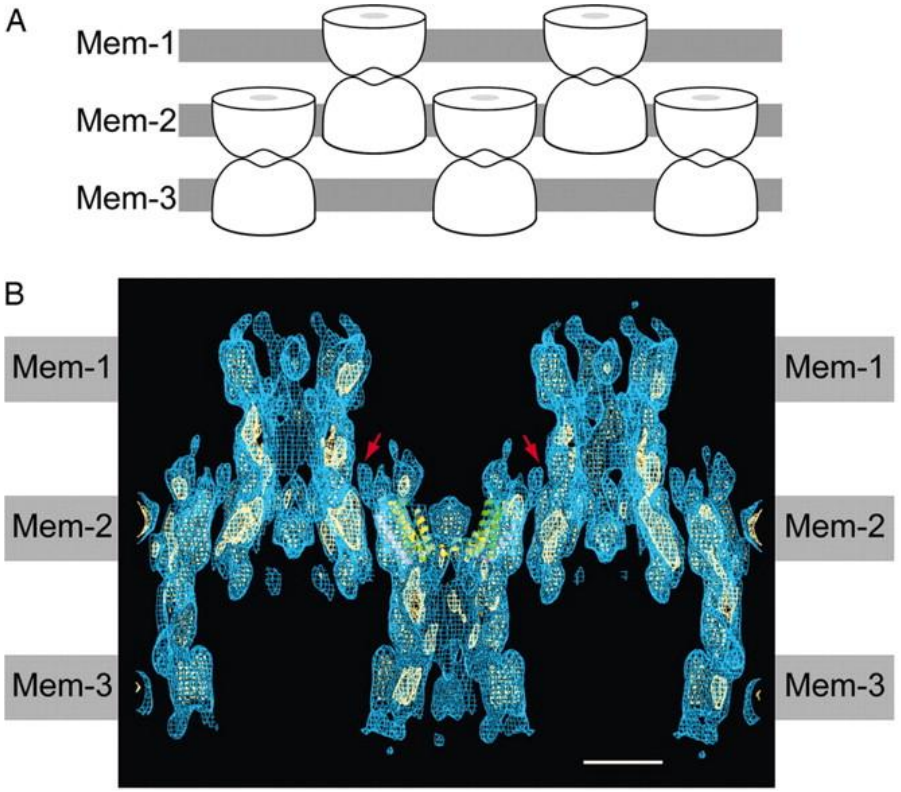


Carbon sandwich method



Cryo-EM structure of connexin26 gap junction channel





Oshima A. *et al.* (2007) *PNAS*

Parameters of sample preparation

- embedding medium: type, concentration

1-20% glucose, 1-3% tannic acid, 1-40% trehalose

- room temperature or 4℃

- how to sample to spread larger number of crystals onto the carbon film
  - thorough agitation before sampling from the storage tube or sampling from the bottom without serious agitation
  - incubation after injecting a sample may induce crystals to adhere to the carbon film
  - purifying crystals from a mixture of crystals, vesicles and aggregates on a sucrose gradient

Parameters of sample preparation

- length of blotting and air-drying

- high concentration of solutes in the crystallization buffer may hinder

  salts, glycerol, …

- back injection or carbon sandwich

  back injection: 1st choice. specimens tough against drying.

  carbon sandwich: dehydration-sensitive specimens. highly tilted data, higher resolution.