## Specimen preparation techniques for high resolution structural study by cryo-EM

Yoshinori Fujiyoshi: Kyoto Univ.

NRAMM Workshop on ATESD

1st G (1986) Kyoto U — 2nd G (1988) Osaka — 3rd G (1994) Kyoto U

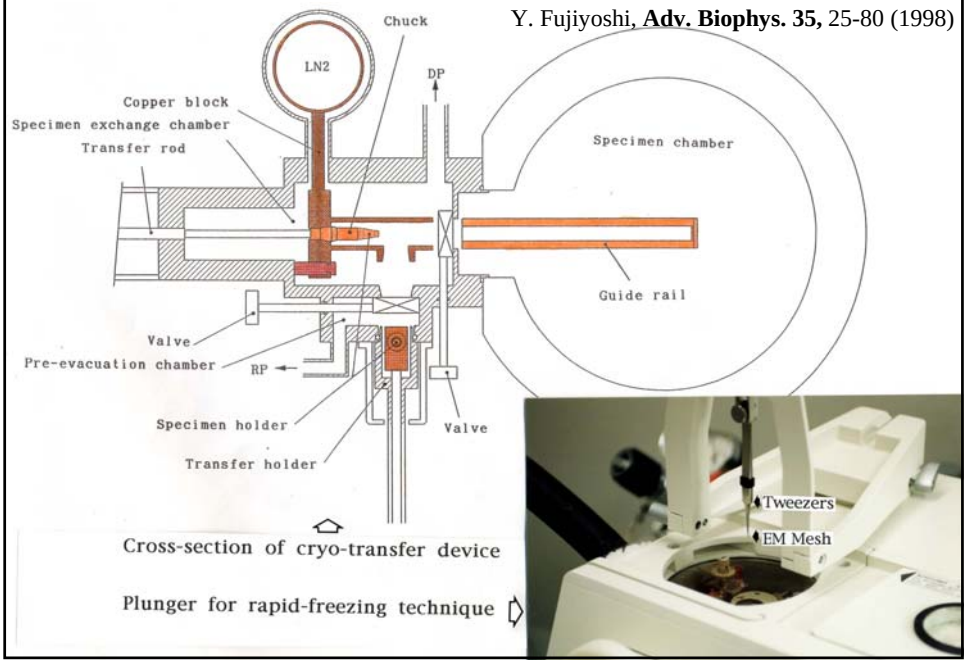
Cryo-EM with helium stage




4th G (2001) Harima, Tokyo — 5th G (2004) Tokyo — 6th G (2006) Kyoto U

## Cryo-transfer system for helium stage

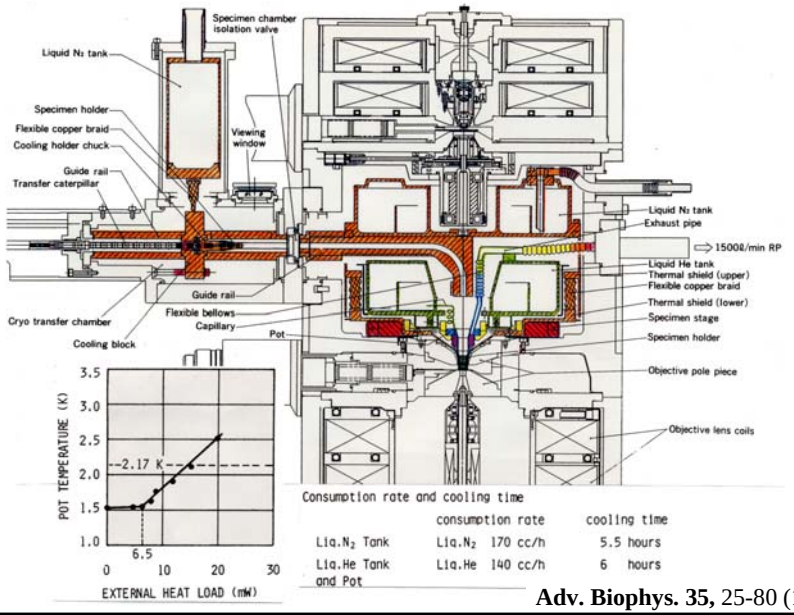
Y. Fujiyoshi, **Adv. Biophys. 35,** 25-80 (1998)




Cross-section of cryo-transfer device

Plunger for rapid-freezing technique

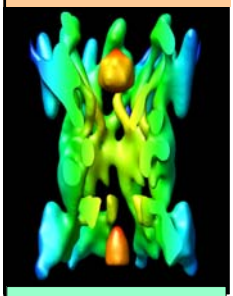
## Quick specimen exchange by our cryo-transfer system helps to optimize specimen preparation techniques




**Adv. Biophys. 35,** 25-80 (1998)

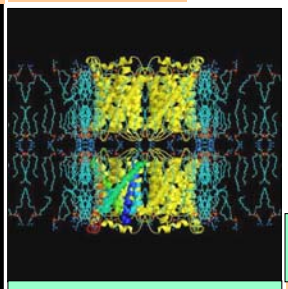
## Structures of membrane proteins analyzed by cryo-EM

**Gap Junction channel**



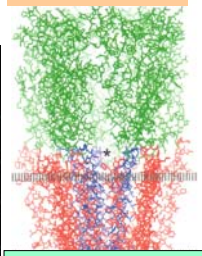
**PNAS, 104,** 10034-10039 (2007)

**Aquaporin-0**



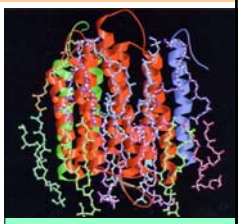
**Nature, 438,** 633-638 (2005)

**AChR**



**Nature, 423,** 949-955 (2003)

**Bacteriorhodopsin**

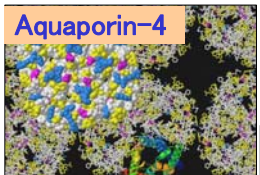


**Nature, 389,** 206-211 (1997)

**MGST-1**

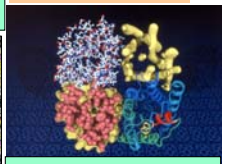
**JMB, 360,** 934-945 (2006)

**Aquaporin-4**



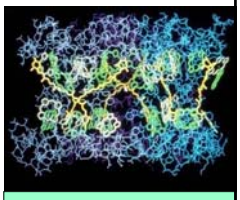
**JMB, 355,** 628-639 (2006)

**Aquaporin-1**



**Nature, 407,** 599-605 (2000)
**Nature, 387**, 624-627 (1997)

**Light-harvesting complex**



**Nature, 367,** 614-621 (1994)

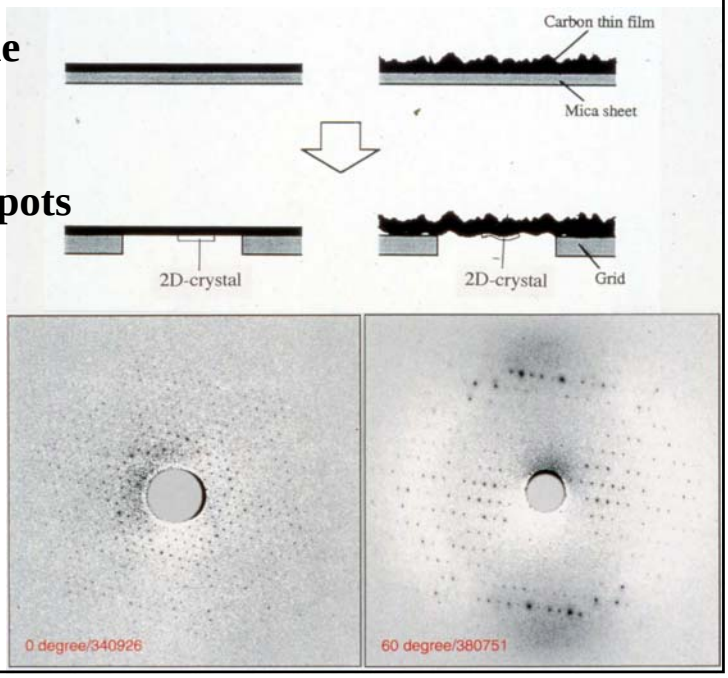
## Requirements for structural study

1) **Flat support**
   - **Atomically flat carbon film**
   - **Smooth Mo grid**
2) **Water evaporation (Dehydration, salt concentration)**
3) **Thinner embedding layer**
4) **Deformation by mechanical interaction**
5) **Suger embedding (Trehalose cushion)**
6) **Image deterioration by beam induced charge**

**How could best EM specimens be prepared?**

## Atomically flat carbon film

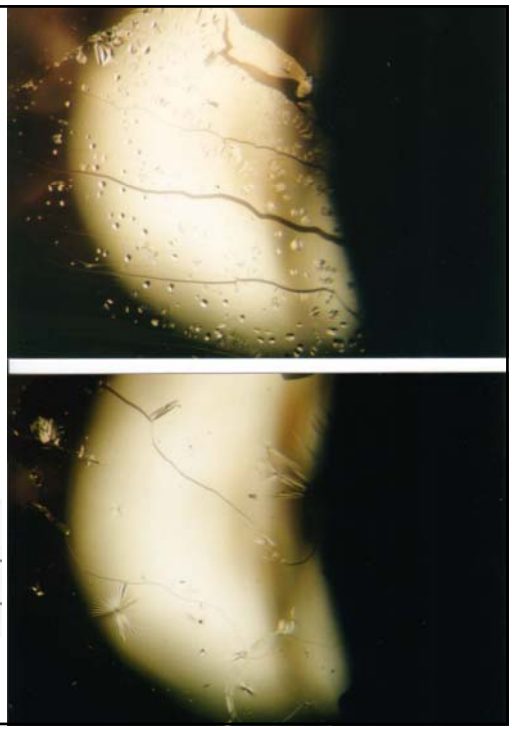
**Reason of the importance: blurring diffraction spots**



## Carbon film with no spark

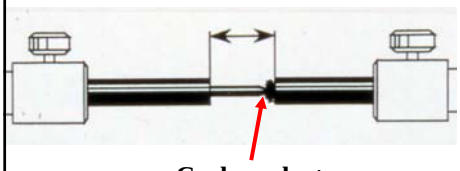
**Atomically smooth carbon film**

**One spark**



**No spark**

**Evaporaton on mica in high vacuum**

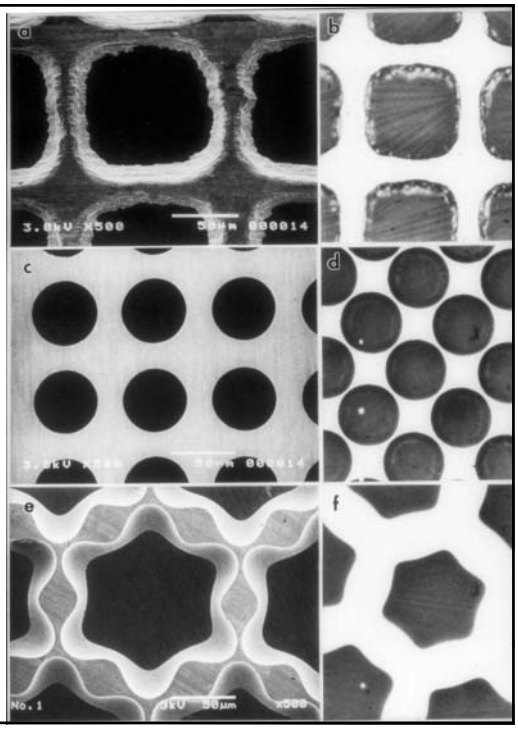


**Carbon cluster**

## Mo grid for minimizing cryo-crinkling

**Commercially avarable Mo grid**

**Very smooth Mo grid**



**Non circular Mo grid**

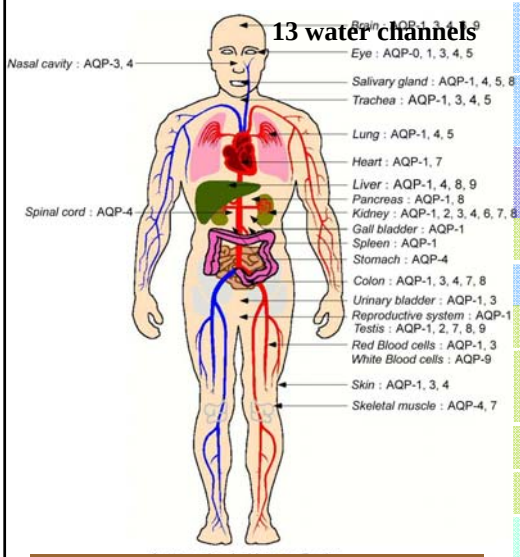
## Requirements for structural study

1) Flat support
   Atomically flat carbon film
   Smooth Mo grid
2) Water evaporation (Dehydration, salt concentration)
3) Thinner embedding layer
4) Deformation by mechanical interaction
5) Suger embedding (Trehalose cushion)
6) Image deterioration by beam induced charge

**How could best EM specimens be prepared?**

## Significance of water channels

Involved in numerous physiological processes



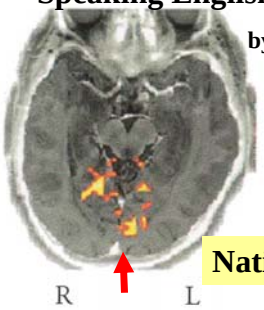

**Aquaporins in Human Body**

- ***AQP0**: cataract, cell adhesion
- ***AQP1**: fast water flow, many organs
- **AQP2**: trafficking according with V2R signal, cardiopathy
- **AQP3**: glycerol, cure incision, beautification
- ***AQP4**: cell adhesion, array, manic-depressive
- **AQP5**: dry eye, salivation
- **AQP6**: permiate not water but anion
- **AQP7**: glycerol, a fat cell, obesity
- **AQP8**:glycerol, alimentary canal, pancreas, acinus, liver
- **AQP9**: glycerol, liver cell
- **AQP10**: glycerol, alimentary canal
- **AQP11**: NPA motif to PNC, nephrogenic diabetes insipidus
- **AQP12**: NPAmotif to NPT

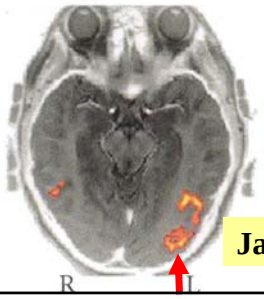
## How is blood flow regulated without smooth muscle

**Speaking English**

**by T. Nakata**



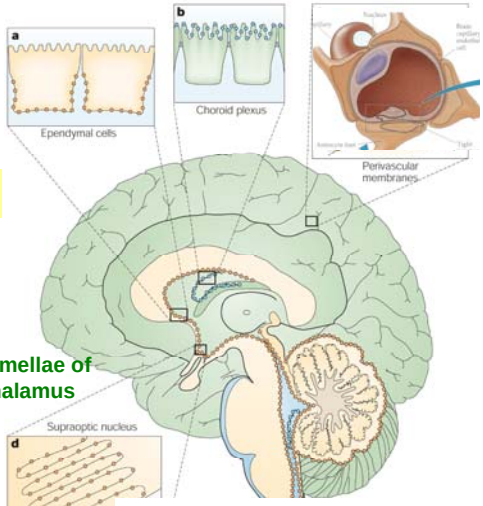

**Native speaker**




**Japanese**

**No vascular smooth muscle in brain**

***endfoot of astrocyte***

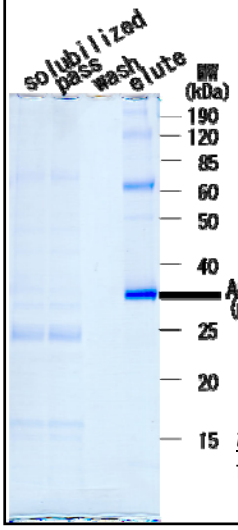



**Glial lamellae of Hypothalamus**

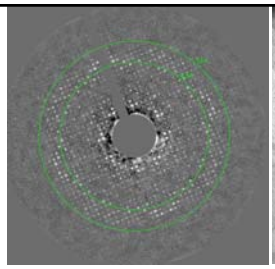
**M.A. Moghaddam, O.P. Ottersen, Nature Rev. Neurosci., 4, 991-1001(2003)**

## Two dimensional crystals of AQP4

***Expression by Sf9 cells & Purification***

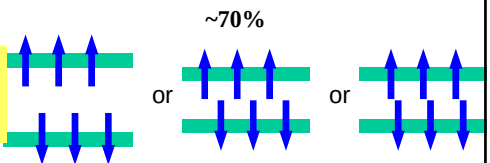



*typical yield:* **~3mg AQP4** from **1-liter of Sf9 cells**

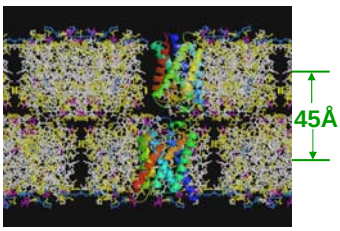


**A typical diffraction pattern from 2D-crystal**

**Yet effective cryo-EM gave us its structure**



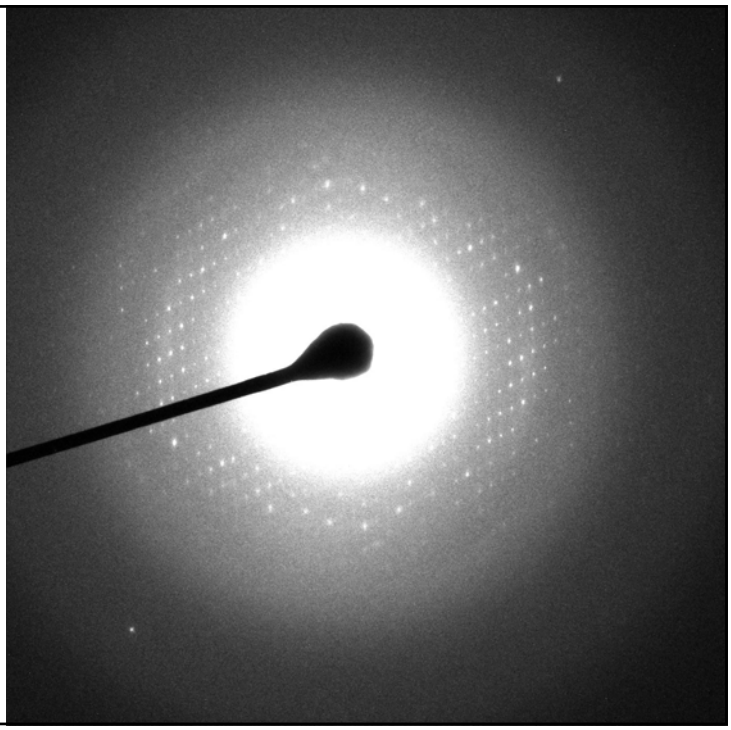

**Double-layered 2D-crystals**




**Molecular arrangement in 2D-crystal**

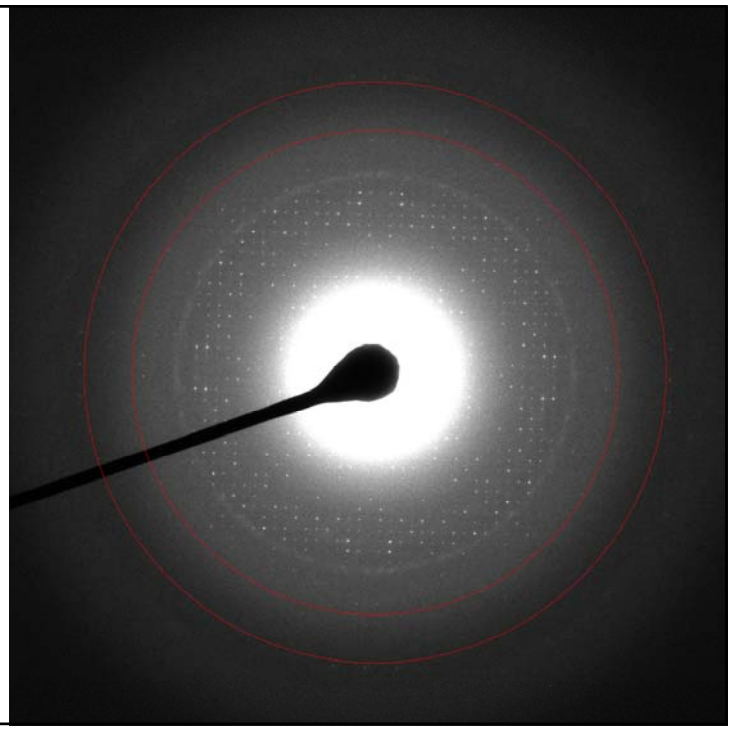
**Diffraction pattern: 60˚tilt**

**Water evaporation causes deterioration of electron diffraction patterns: 60˚tilt**

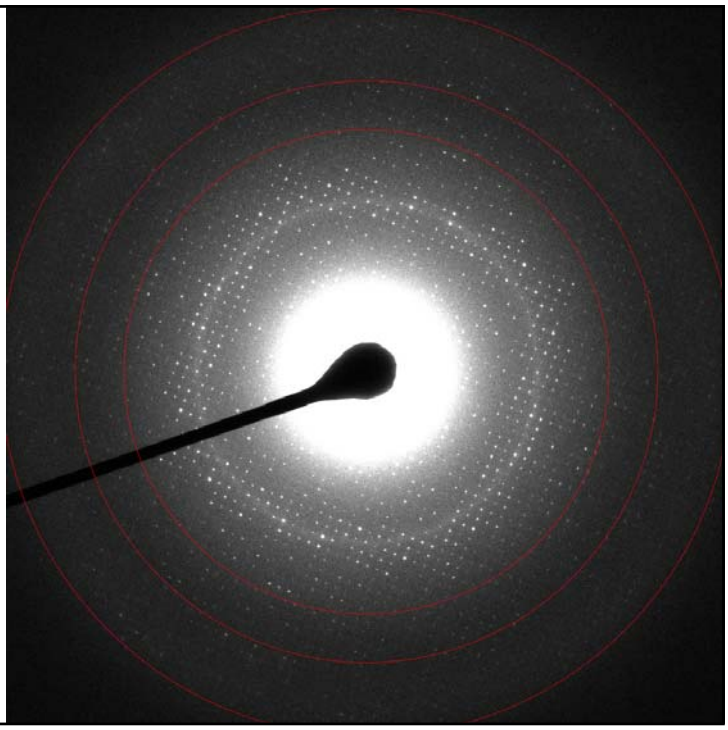


**Dried crystal: untilt**

**Water evaporation causes deterioration of electron diffraction patterns: untilt**

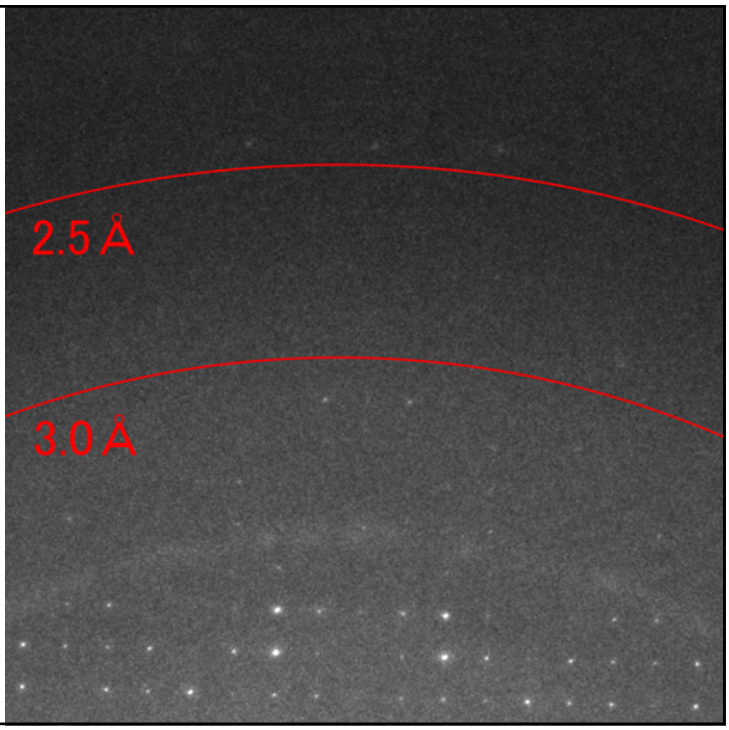


**Good crystal: untilt**

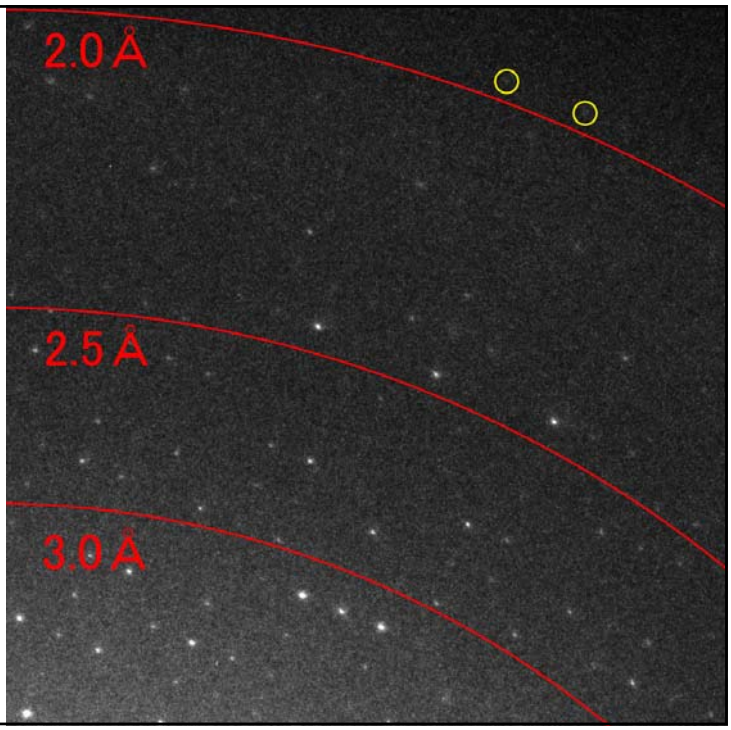


**Dried crystal: untilt**

**Water evaporation causes deterioration of electron diffraction patterns: untilt**

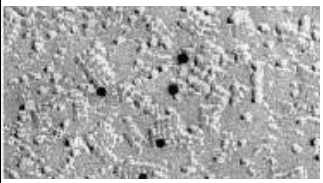


**Good crystal: untilt**

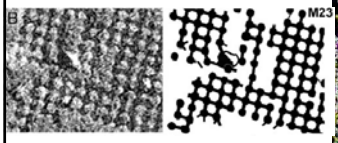



Orthogonal array

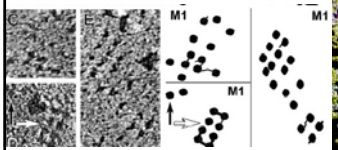
***Size of Orthogonal arrays of AQP4 at endfeet of astrocyte*** by Neely J D et al, Biochemistry (1999) 38: 11156-11163



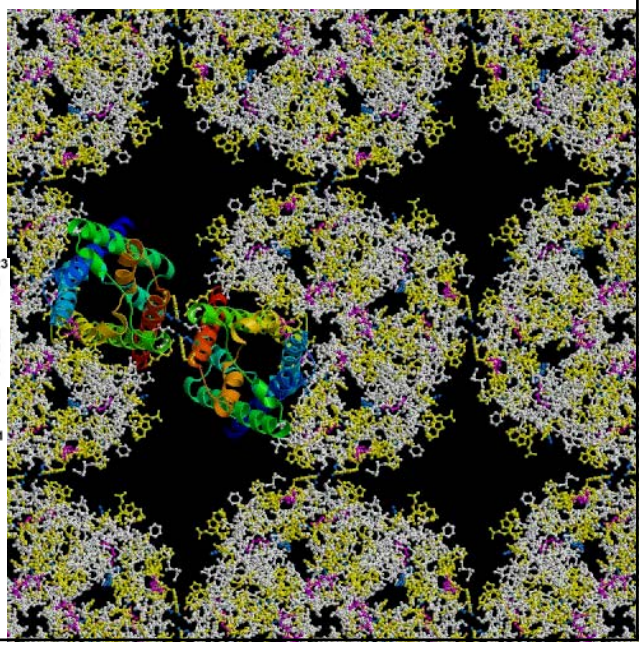
**The orthogonal array structure**




**AQP4M23**




**AQP4M1**

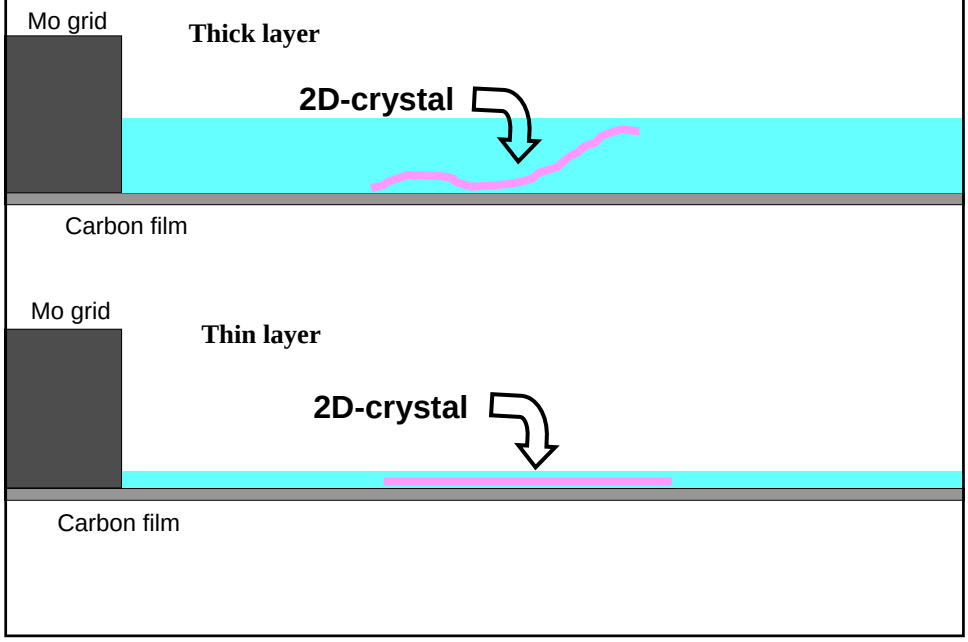


## Requirements for structural study

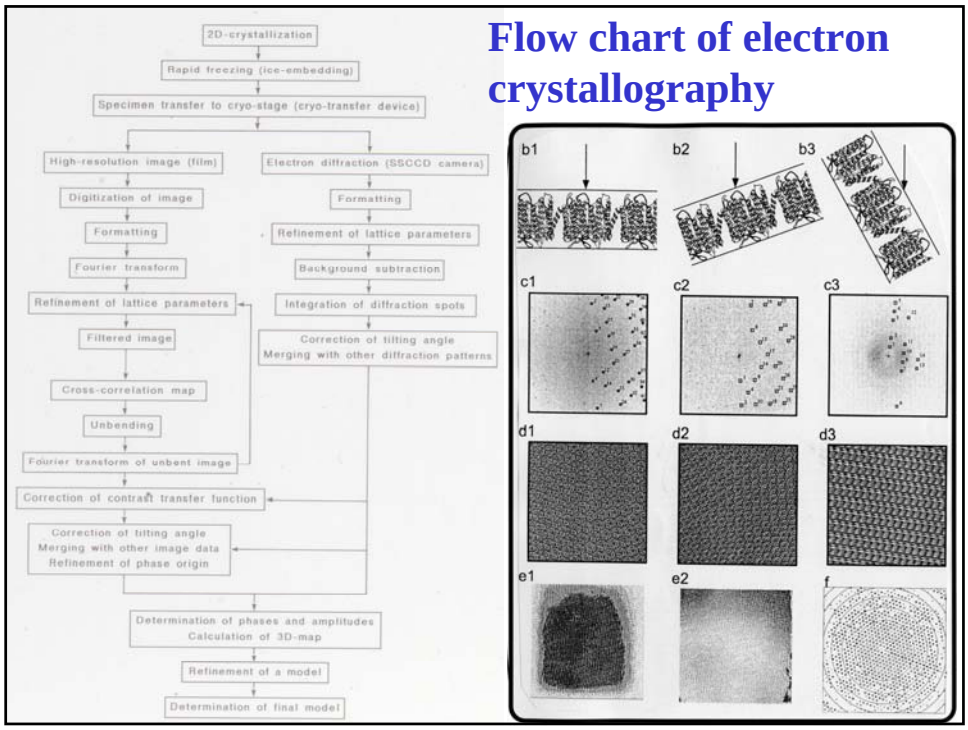
1) Flat support
   Atomically flat carbon film
   Smooth Mo grid
2) Water evaporation (Dehydration, salt concentration)
3) Thinner embedding layer
4) Deformation by mechanical interaction
5) Suger embedding (Trehalose cushion)
6) Image deterioration by beam induced charge

**How could best EM specimens be prepared?**

## Reason why we need thinner embedding layer

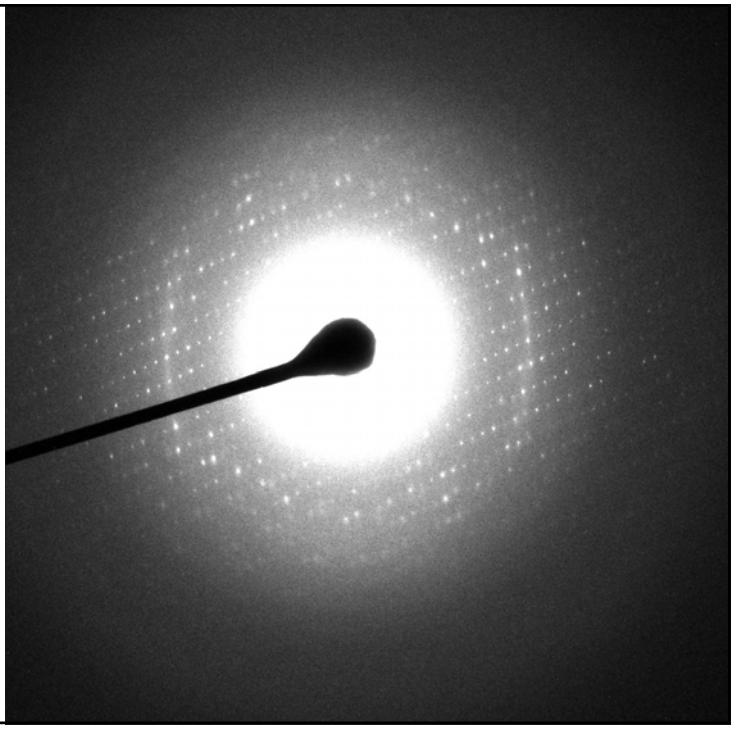


## Flow chart of electron crystallography



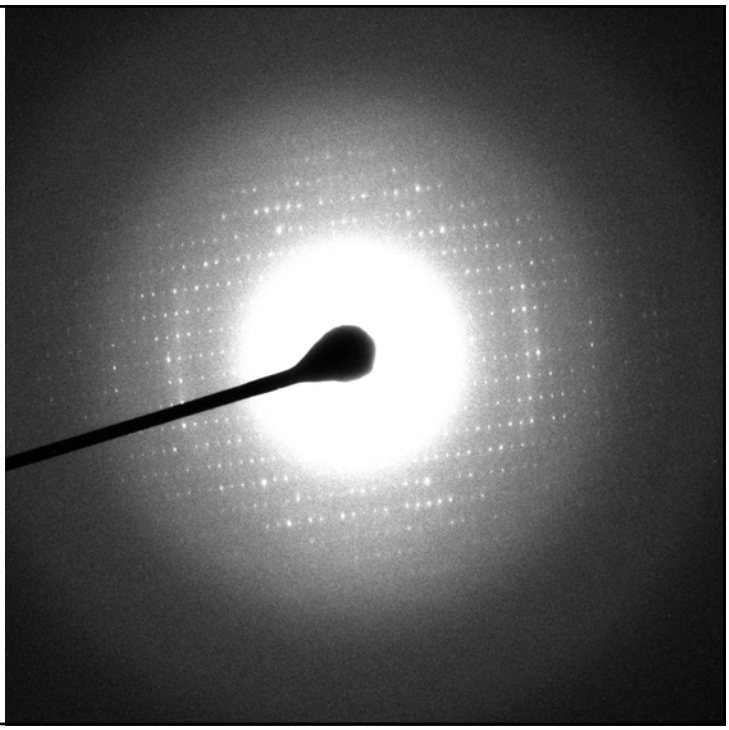

**Bended (undulated) crystal: 60˚tilt**

**Thicker layer makes crystals undulate and less clear diffraction spots in the direction perpendicular to the tilting axis**



**Good crystal: 60°tilt**

**Thinner layer makes crystals less undulate and also gives better S/N ratio**



**Requirements for structural study**

1) Flat support
   Atomically flat carbon film
   Smooth Mo grid
2) Water evaporation (Dehydration, salt concentration)
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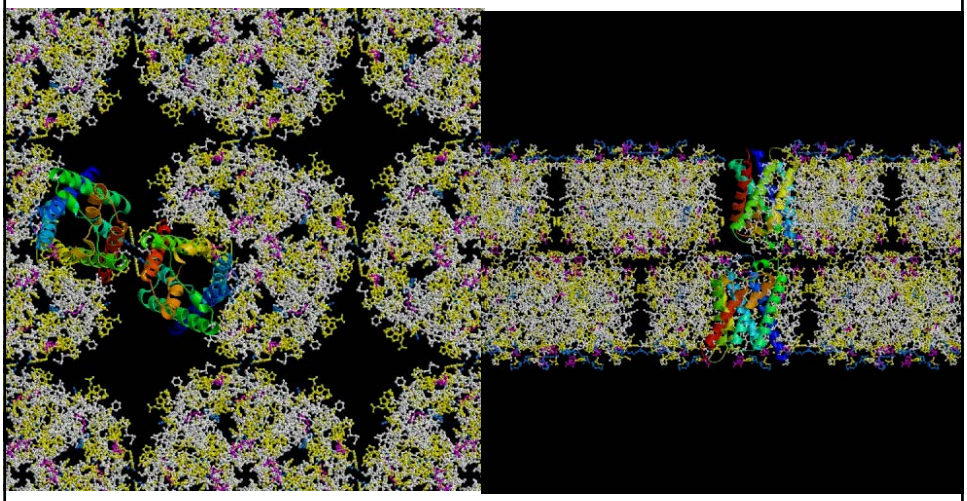
4) Deformation by mechanical interaction
5) Suger embedding (Trehalose cushion)
6) Image deterioration by beam induced charge

**How could best EM specimens be prepared?**

Structure of AQP4



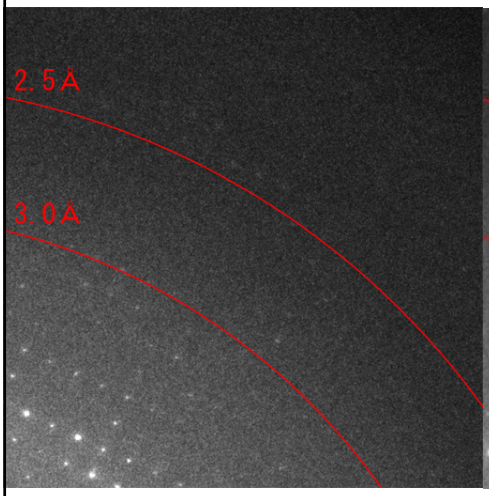
**Requirements for structural study**

**1) Flat support**
**Atomically flat carbon film**
**Smooth Mo grid**
**2) Water evaporation (Dehydration, salt concentration)**
**3) Thinner embedding layer**
**4) Deformation by mechanical interaction**
**5) Suger embedding (Trehalose cushion)**
**6) Image deterioration by beam induced charge**

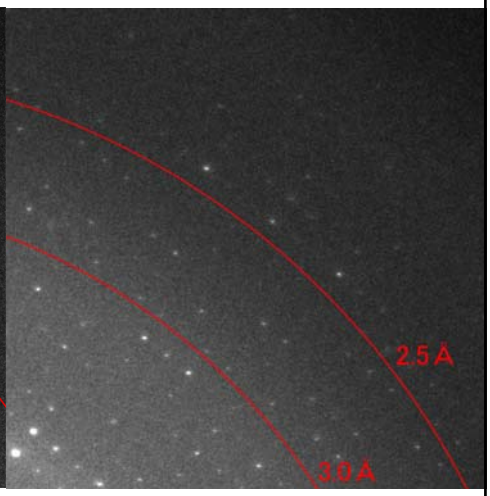
**How could best EM specimens be prepared?**

## Effect of Trehalose cushion

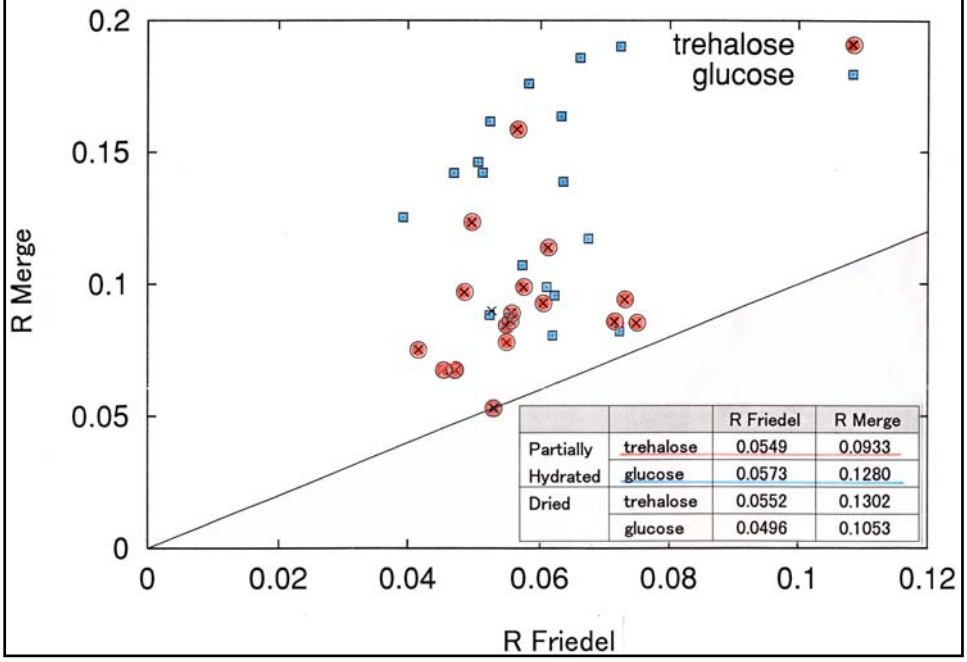
**Low trehalose cushion**




**Higth trehalose cushion**

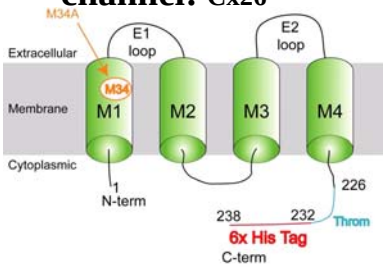


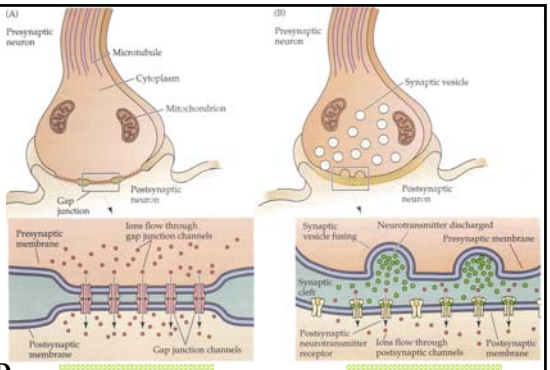

## Trehalose embedding method



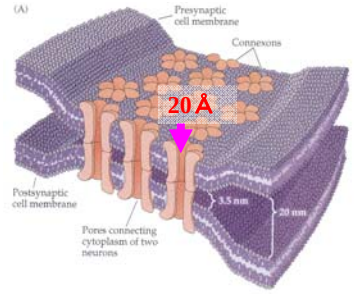

| | | R Friedel | R Merge |
|---|---|---|---|
| Partially Hydrated | trehalose | 0.0549 | 0.0933 |
| | glucose | 0.0573 | 0.1280 |
| Dried | trehalose | 0.0552 | 0.1302 |
| | glucose | 0.0496 | 0.1053 |

## Gap Junction channel: Cx26



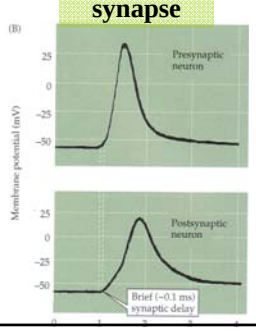




**GJ channels permiate peptides of 1.8kD, gating mechanism for blocking ions?**




**Electrical synapse**

**Chemical synapse**



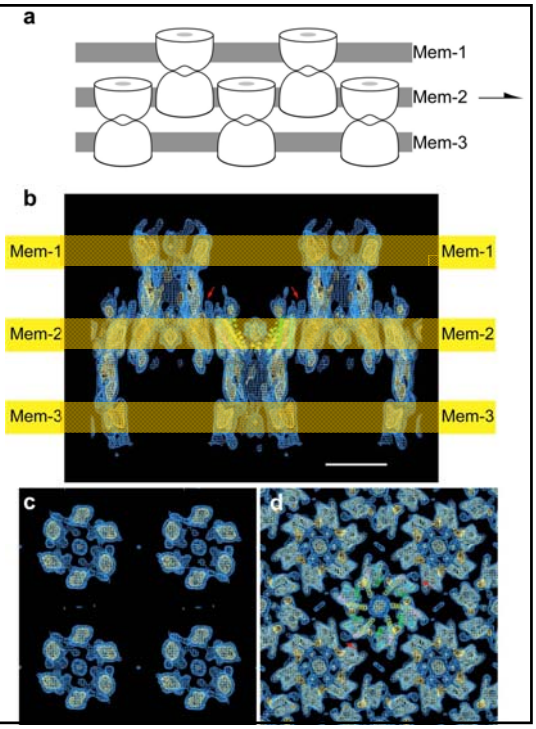

**Long standing questions**

**Multiple gating mechanisms by voltage, calcium ion, phosphorylation, pH**

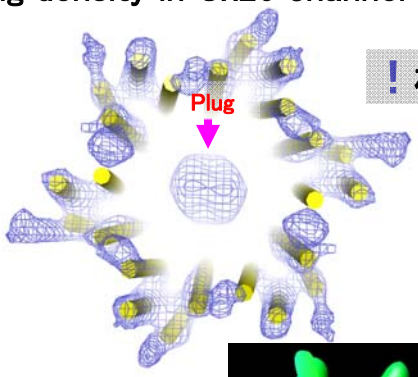
## 2D-crystal of Gap Junction channels

**Surprizingly 3-membrane layers!**

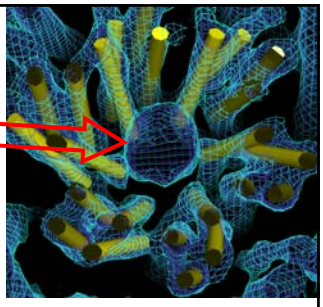
**Crytoplasmic structures at Mem-1 and -3 are easily deformed but these at Mem-2 are protected !**



## Plug density in Cx26 channel

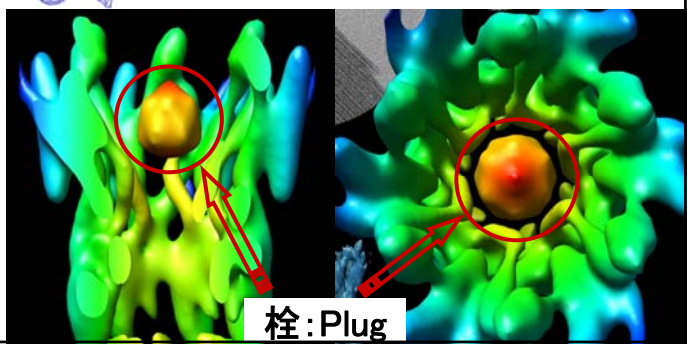



！栓：Plug

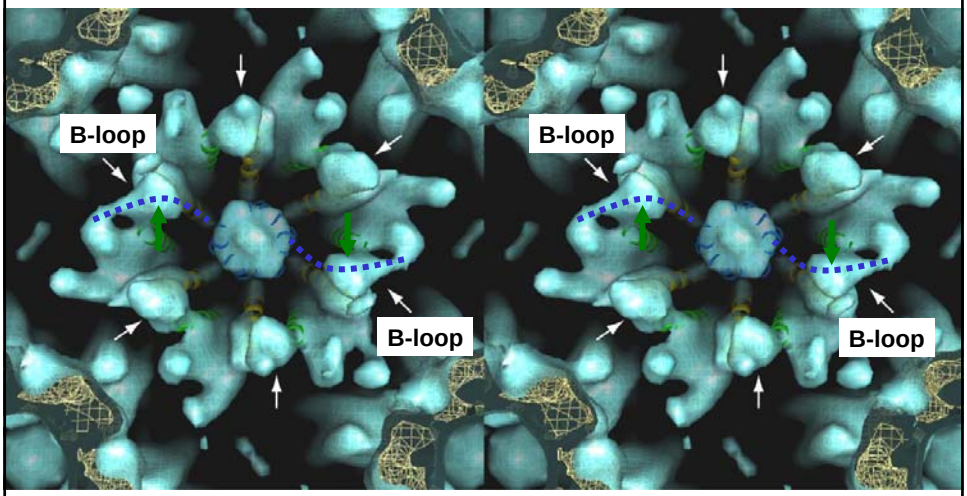


**PNAS, 104**, 10034-10039 (2007)

A New density




## Stereoscopic view at cytoplasmic side

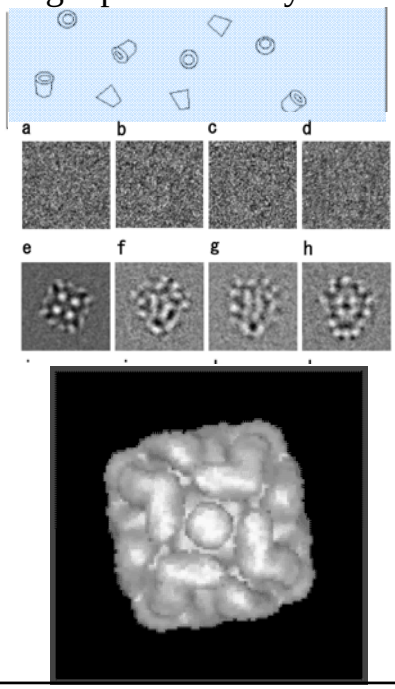


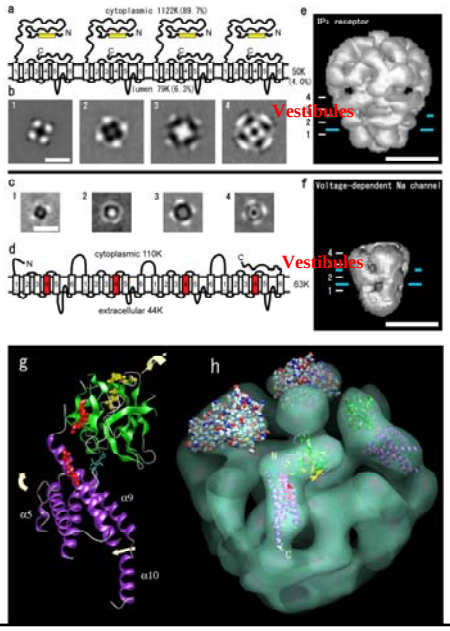

**Structure at cytoplasmic side is related with caracteristic feature of each Cx: White arrows show B loop between Helices2-3 green arrows indicate N-terminal loops interacting with the B loops**

## Single particle analysis

**Na-channel, Nature, 409,** 1047-1051 (2001)

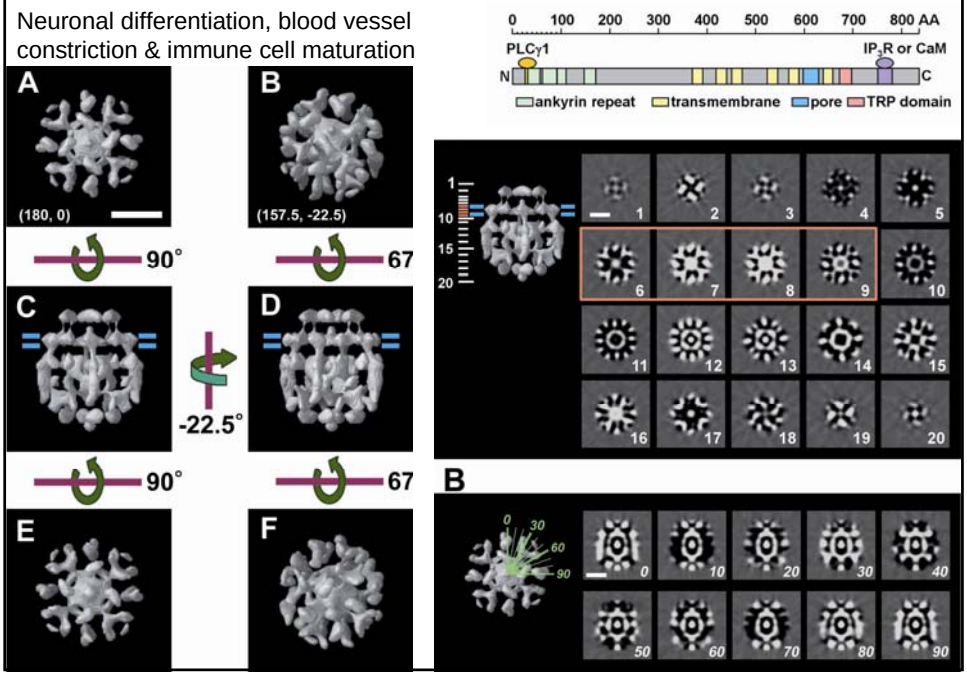
**$IP_3R$., J. Mol Biol., 336,** 155-164 (2004).



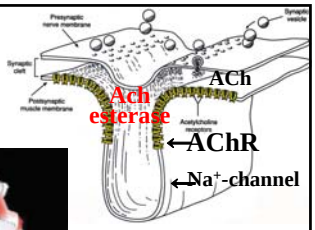

## Single particle analysis of TRPC3

Neuronal differentiation, blood vessel constriction & immune cell maturation

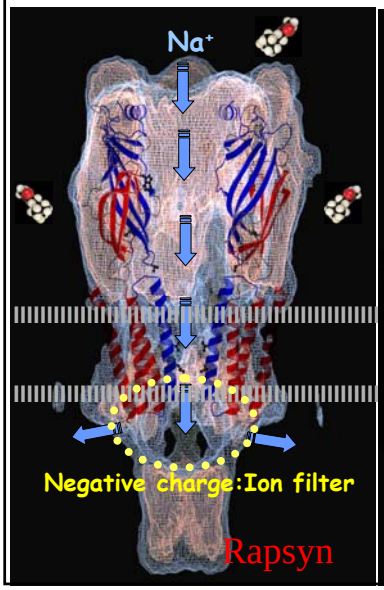


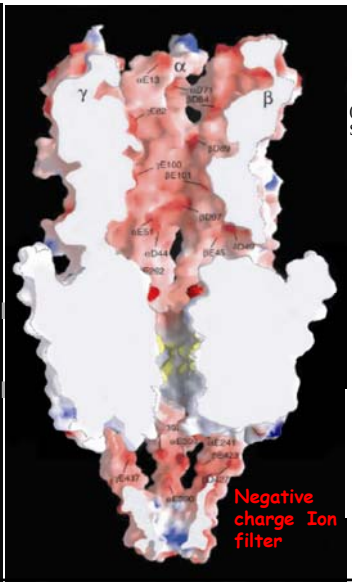
# Neuro-muscular junction

**Nature, 423,** 949-955 (2003)




(B.Hille, 2nd Ed.,SinauerAssociates, Sunderland, MA, 1992)

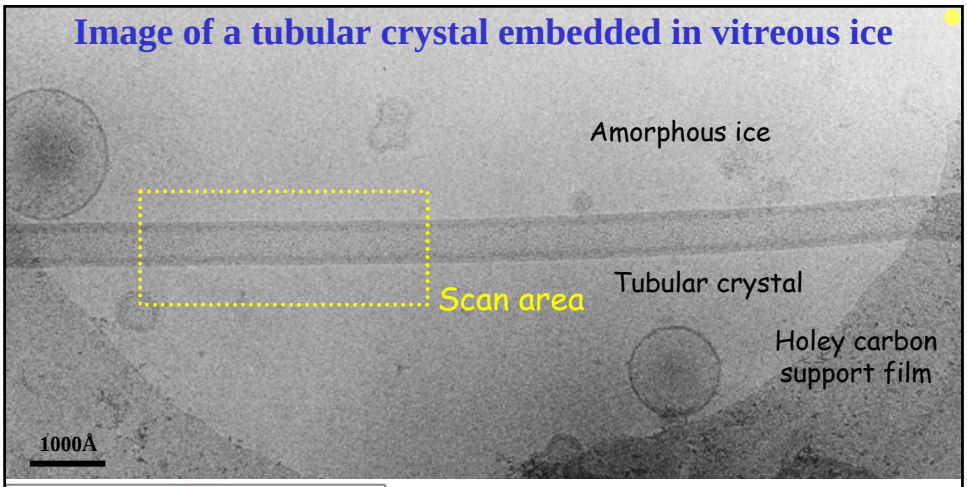


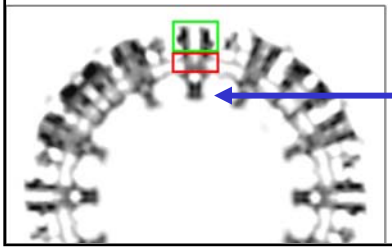




**Ach: Positively charged**

$CH_3CH_2N^+CH_2CH_2OCOCH_3$ (acetylcholine)

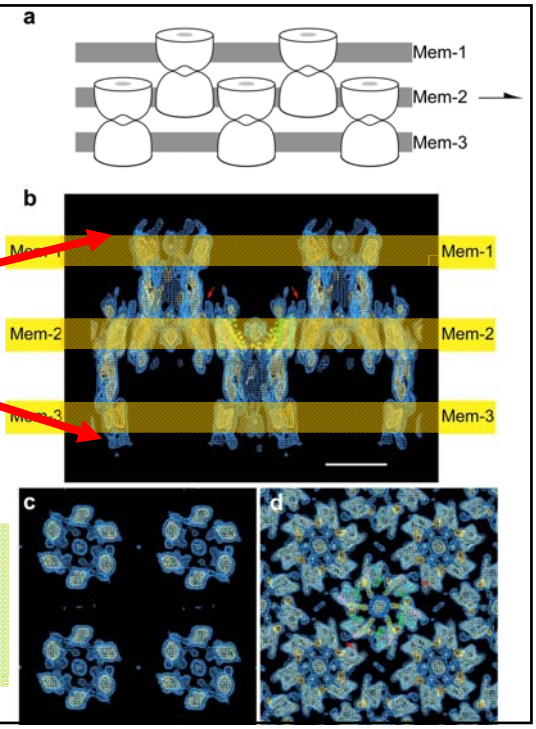
# Image of a tubular crystal embedded in vitreous ice



**No interaction which induces deformation**

**2D-crystal of Gap Junction channels**




**Molecules at outer 1- & 3-membrane layers are deformed but minimized by trehalose cushion**

**Crytoplasmic structures at Mem-1 and -3 are easily deformed but these at Mem-2 are protected by !**

## Requirements for structural study

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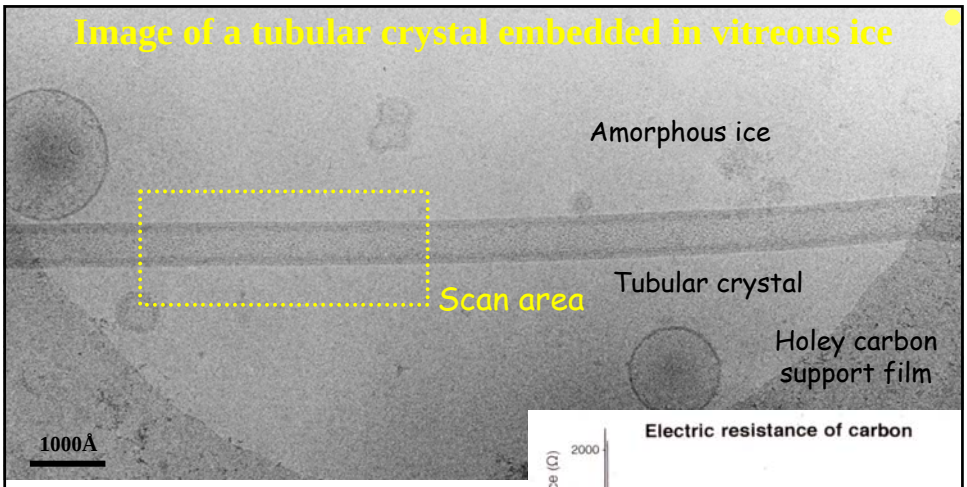
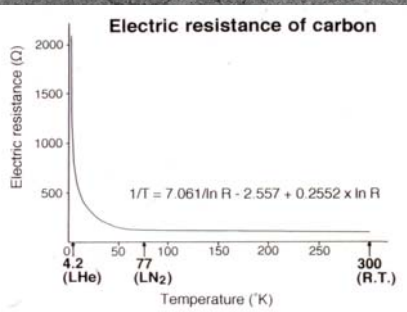
5) Suger embedding (Trehalose cushion)
6) Image deterioration by beam induced charge

**How could best EM specimens be prepared?**

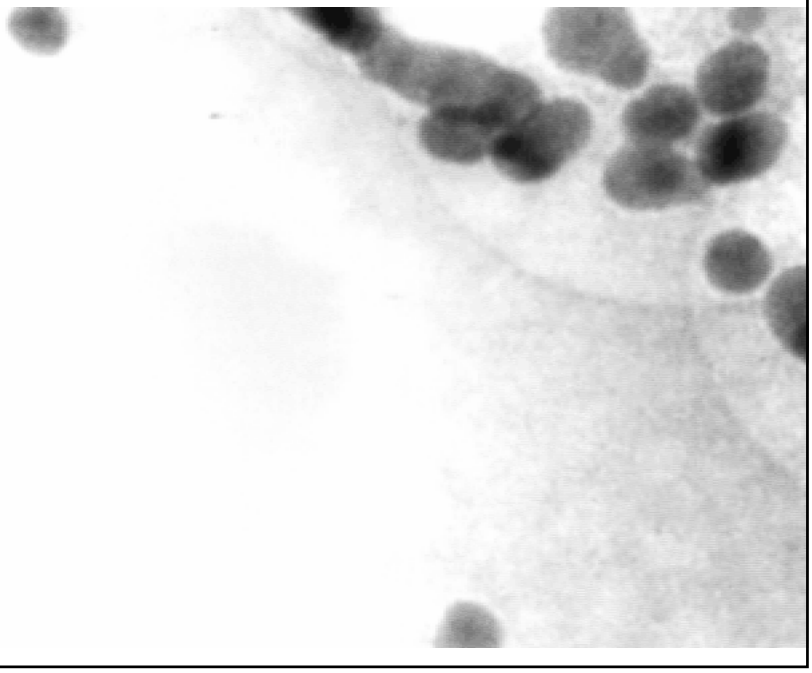
## Image of a tubular crystal embedded in vitreous ice



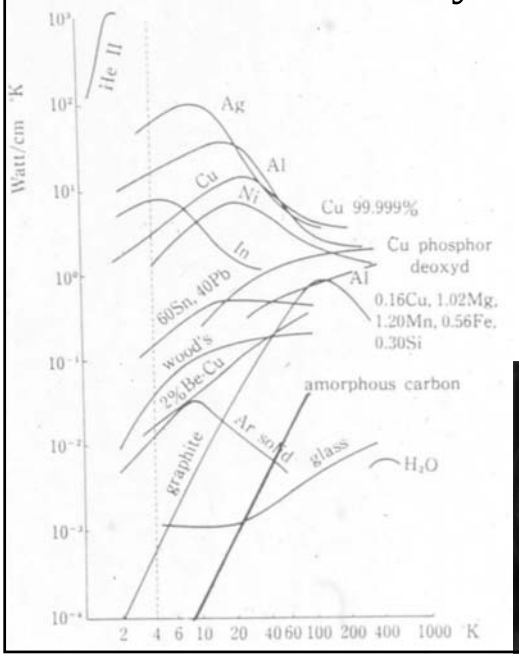

**N. Unwin's image**

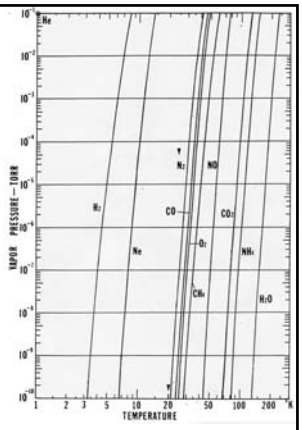


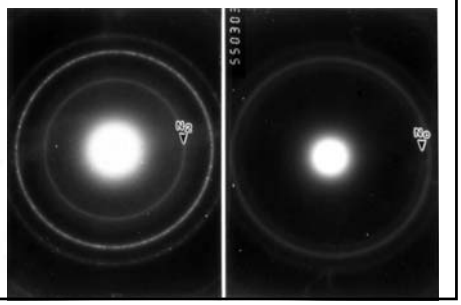

## Gold particles embedded in ice



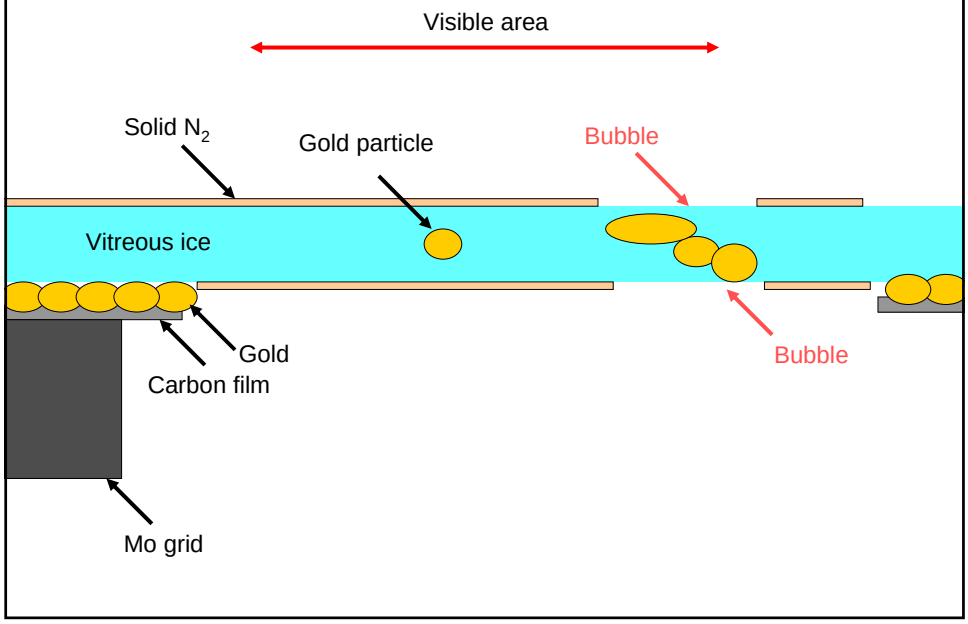
# Thermal conductivity



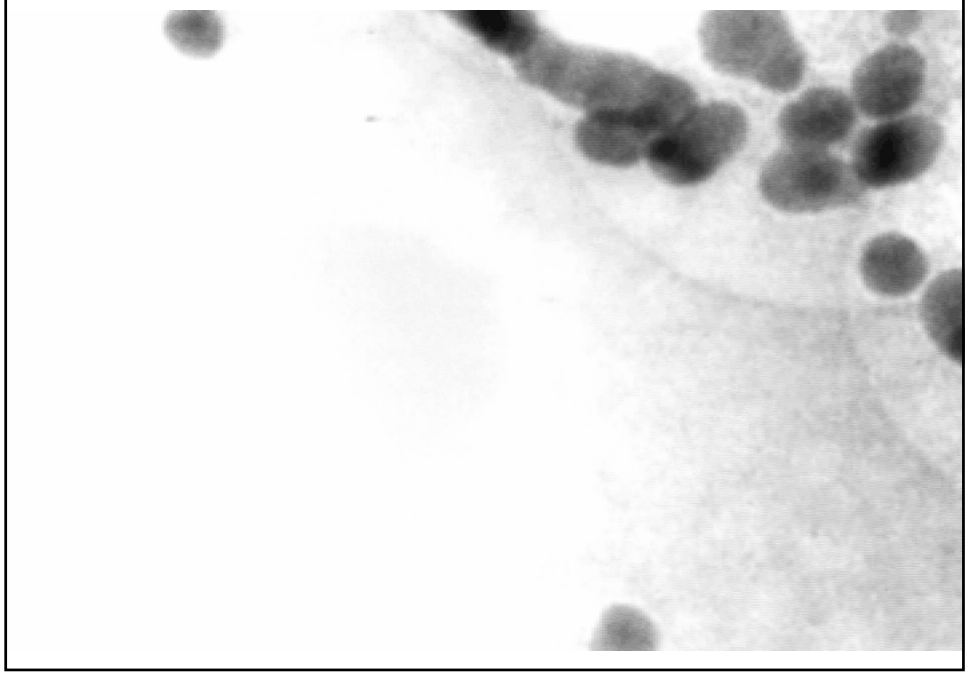



# Gold particles embedded in ice

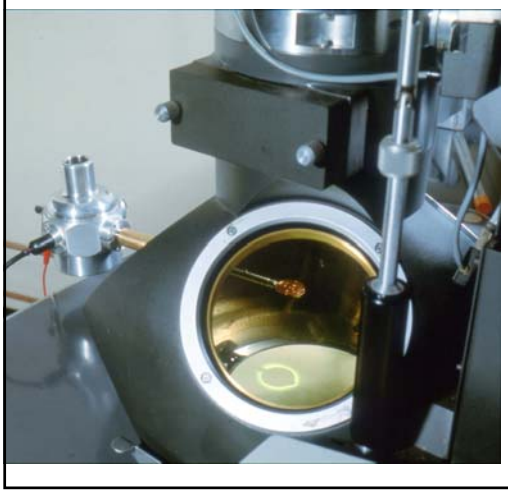


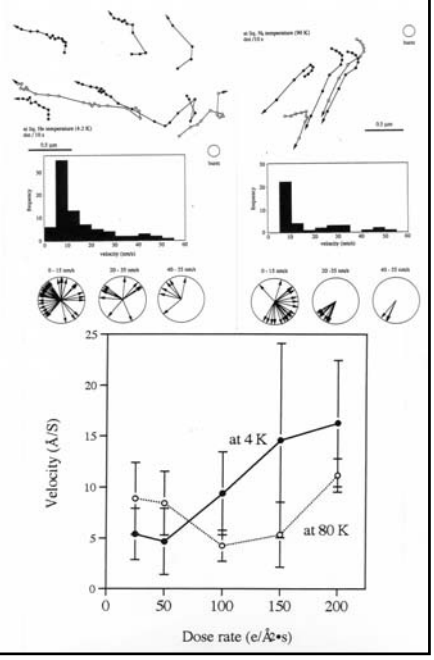
## Gold particles embedded in ice



## Pre-irradiation technique

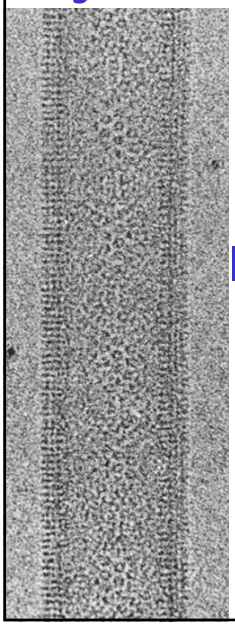
Beam induced movement of a particle embedded in ice





Structure analysis from tubular crystals

Image of tubular crystal

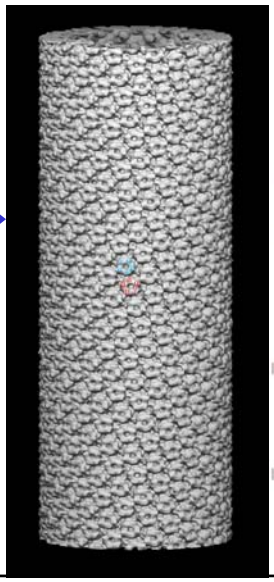


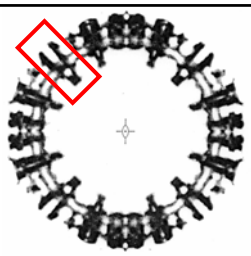
analysis

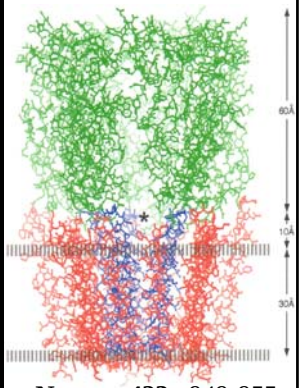
1.unbending

2.averaging

3D-structure







**Nature, 423,** 949-955 (2003)

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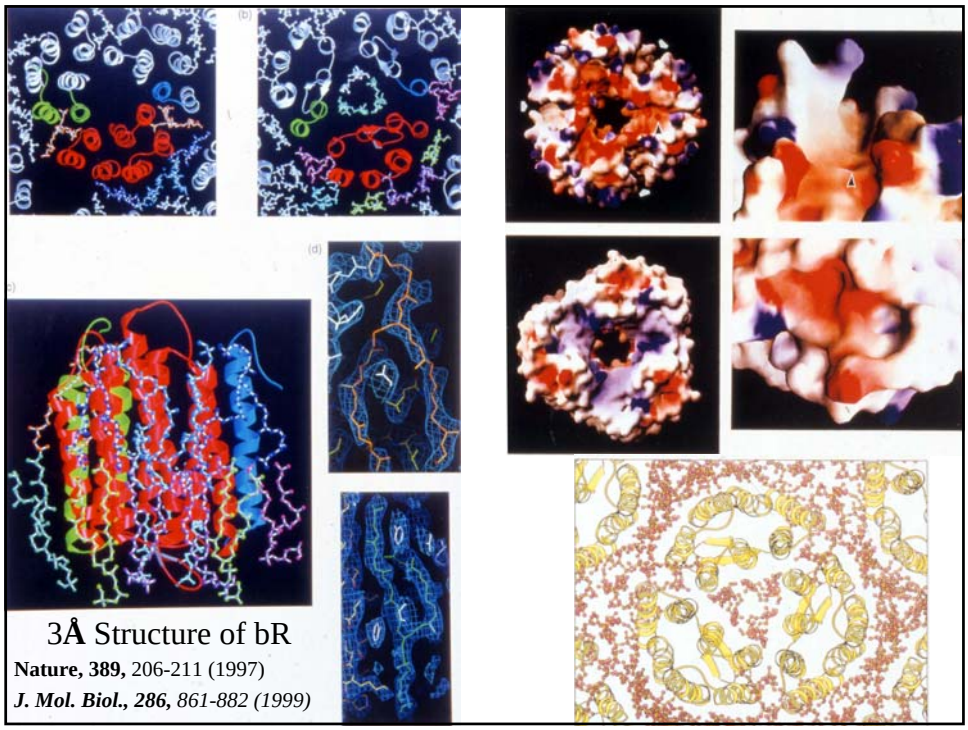
5) **Suger embedding (Trehalose cushion)**
6) **Image deterioration by beam induced charge ⇒ Image shift**

**How could best EM specimens be prepared?**



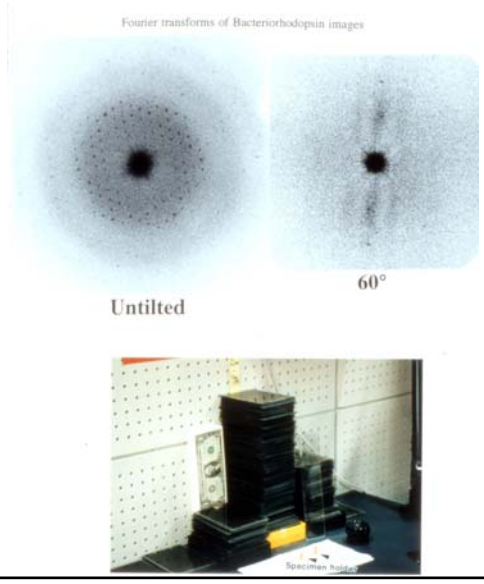
3Å Structure of bR

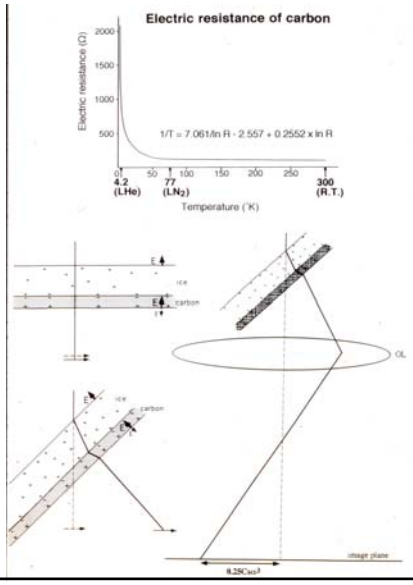
**Nature, 389,** 206-211 (1997)

***J. Mol. Biol., 286,*** *861-882 (1999)*

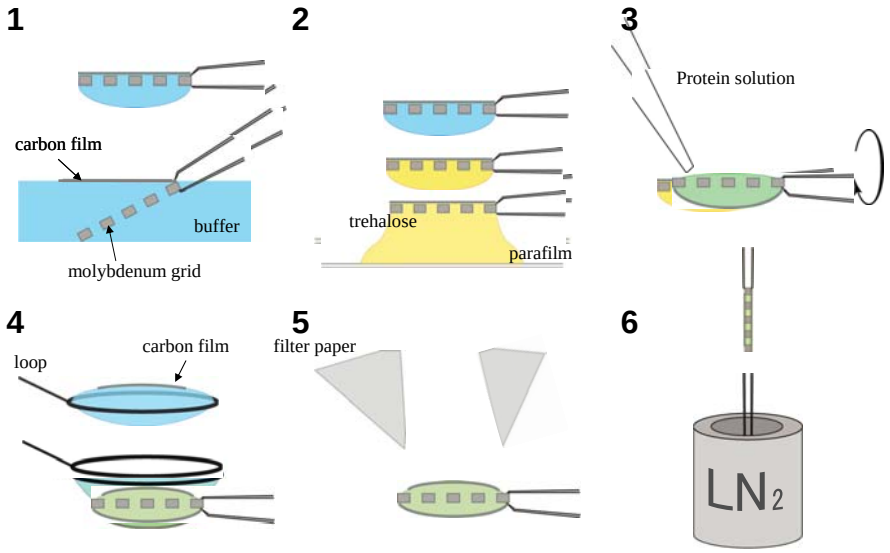
## Image shift caused by charge up

**Very difficult to take good images at tilted conditions**



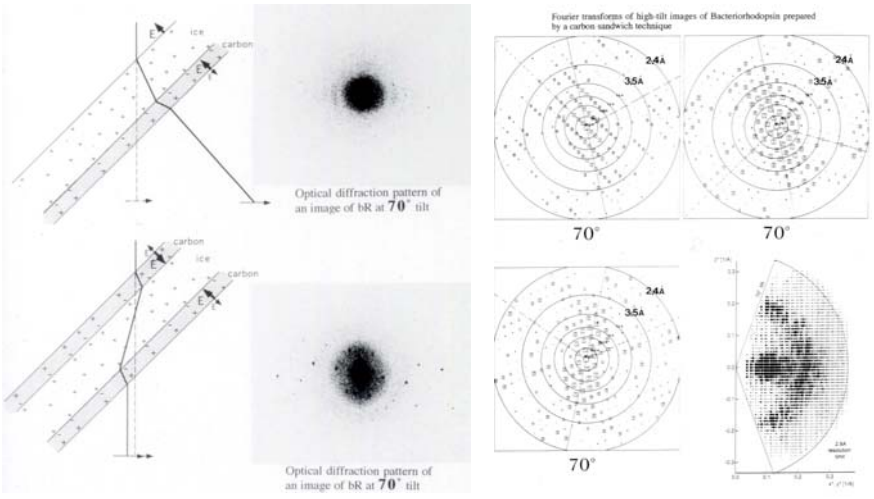
## Carbon Sandwich Specimen Preparation




## Image shift caused by charge up

**Symmetrical specimen＝Carbon sandwich method**

Success ratio from 2% to 95%



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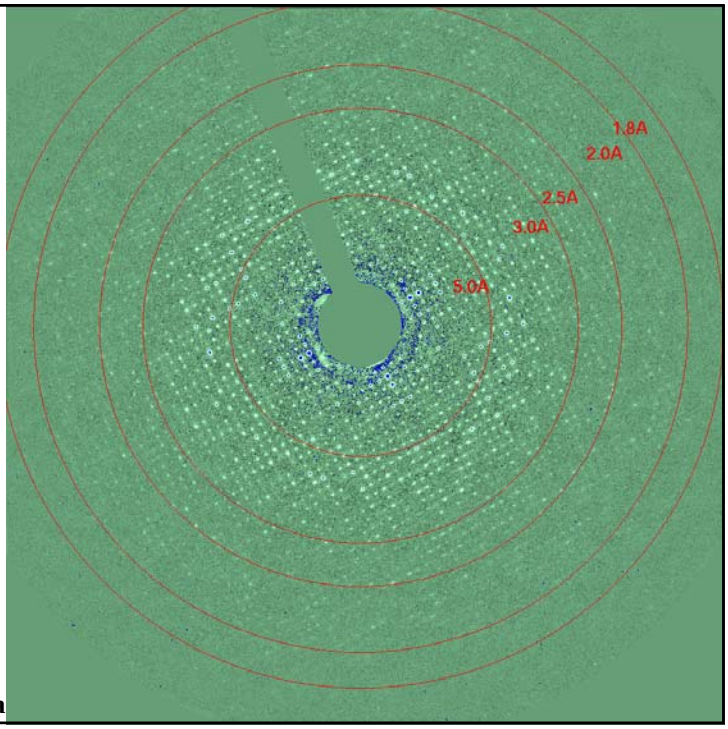
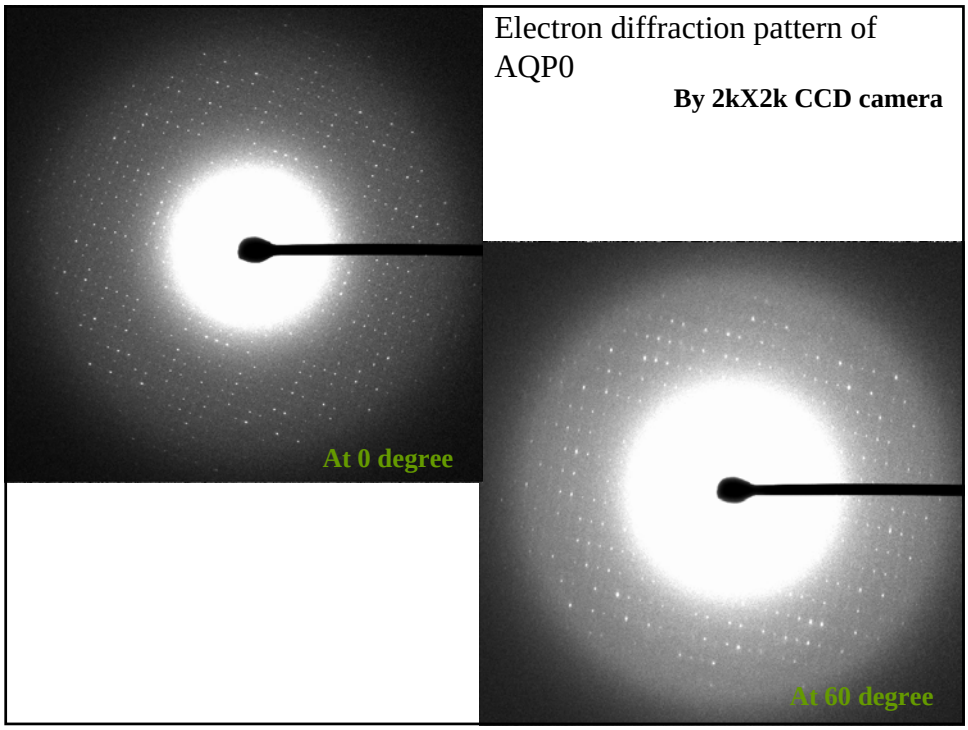
**How could best EM specimens be prepared?**

Electron diffraction pattern of AQP0 at untilted condition



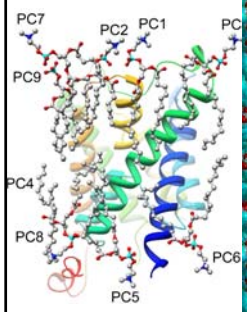

**2kX2k CCD camera**



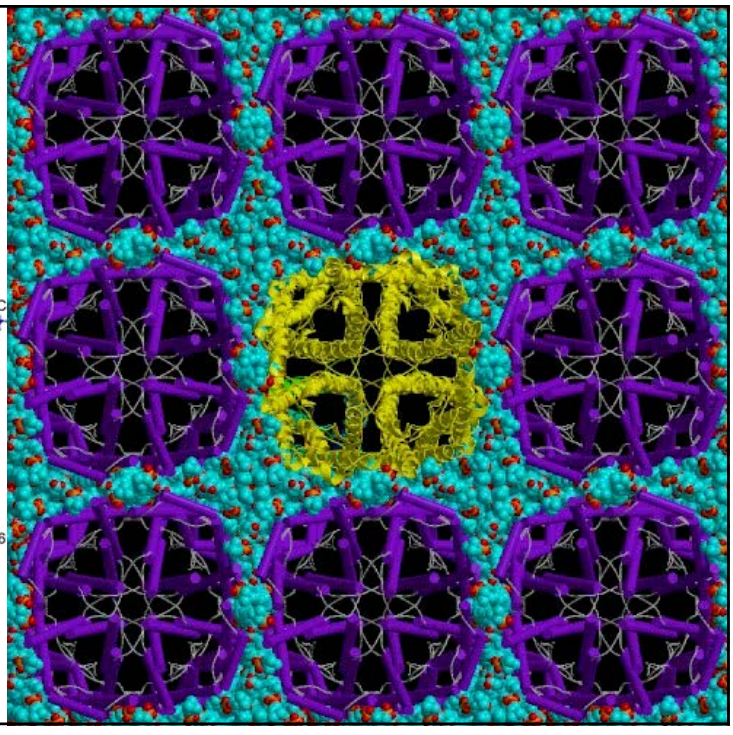

Electron diffraction pattern of AQP0

**By 2kX2k CCD camera**

AQP0 and lipid molecules




**Nature, 438**, 633-638 (2005)



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**6) Image deterioration by beam induced charge**

**Best EM system helps to colect data**


Thank you for having the patience to hear me out!

Collaborators: **Cryo-EM**; Y. Aoki, I. Ishikawa, M. Naruse

**Aquaporin-4;** Y. Hiroaki, K. Tani, A. Kamegawa, T. Mitsuma, N. Gyobu, H. Suzuki, K. Nishikawa, S. Sasaki, K. Mitsuoka

**Aquaporin-0;** T. Gonen, Y. Cheng, T. Walz

**AChR;** N. Unwin, A. Miyazawa

**Gap J**; A. Oshima, K. Tani, Y. Hiroaki, G. Sosinsky

**CCD;** H. Tietz, I. Daberkow , Y. Hiroaki, K. Tani, K. Kobayashi, K. Mitsuoka

**$IP_3R$**; C. Sato, K Mikoshiba

**TRP;** K Mio, T. Ogura, C. Sato, Y. Hiroaki, Y. Tanimura, S. Kiyonaka, Y. Mori


**Helium stage for high resolution electron microscopy**

November, 2007


Yoshinori Fujiyoshi
**Kyoto University**