Preparation of biological samples for the Transmission Electron Microscope

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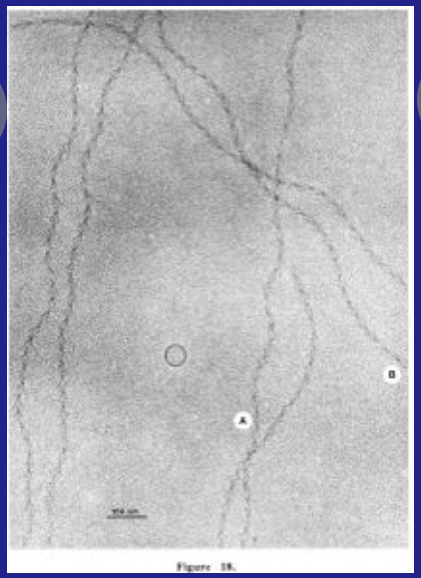
in the group of Bruno Klaholz

ReNaFoBis Ile d'Oléron 5th June 2014

Mon parcours: Master biochemistry (1984 Paris6), phD biophysics (1990 Paris6). I have learned Cryo-electron microscopy 30years ago at EMBL Heidelberg with J. Lepault, J. Dubochet and Marc Adrian. I made static and dynamic studies on actomyosin filaments (fig1). In 1993-1997, I have worked on phasing fragments of fibrinogen crystals (fig2) and tropomysosin sheets at Brandeis University (Waltham, USA). Then, in 1997-2008, I worked on ribosome channel complexes at Boston University with Chris Akey (fig3). I am now in charge of two performant electron microscopes a Polara and a Titan Kryos at IGBMC (Illkirch); I help users collect data in the Frisbi or Instruct network.

1



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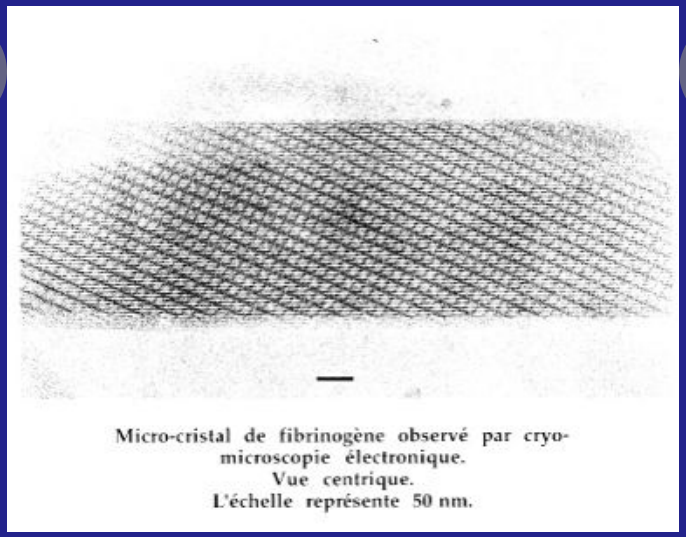
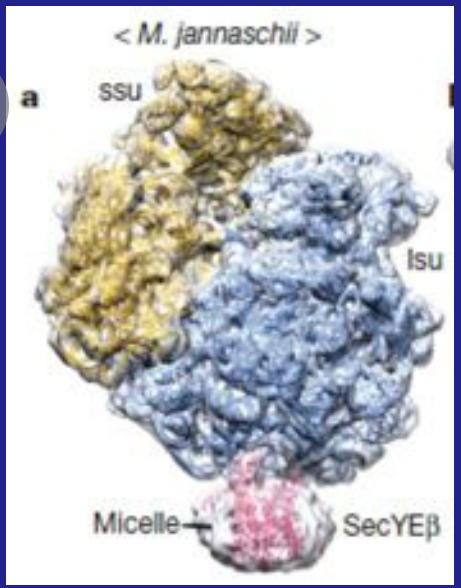


Micro-cristal de fibrinogène observé par cryo-microscopie électronique.
Vue centrique.
L'échelle représente 50 nm.

3



Titan Kryos

It is being installed in IGBMC / CIB / Illkirch

100kV, 120kV and 300kV.
Very stable goniometer.
Very coherent and parallel beam.
12 grids loaded automatically.
Very slow contamination in the column.
Cemos Falcon2 camera

But even the best microscope can be limited by poor grids

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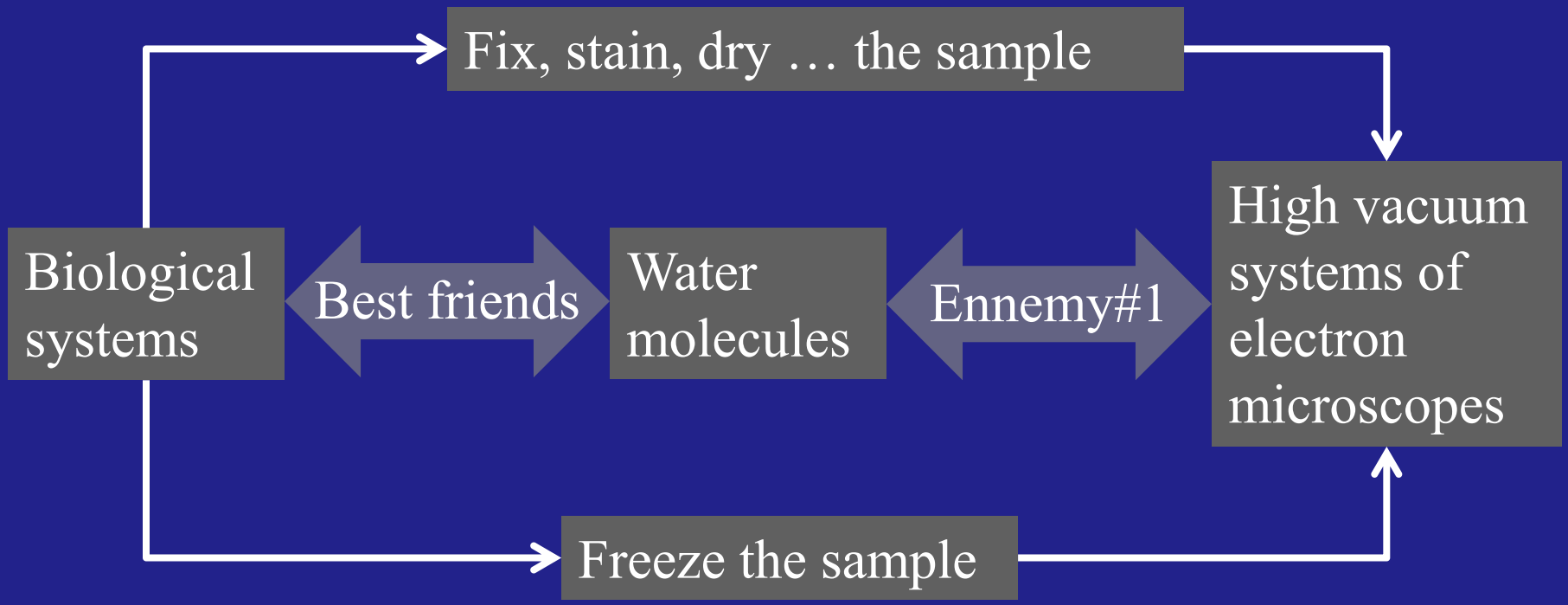
Biological samples and the electron microscopy:



Preparation strategies of biological samples for TEM

Loose the water:

negative staining (Hall 1955)

tissue fixation, dehydration, plastic embedding

Keep the water:

hydration chamber (Parson 1974)

cryo-EM of viruses (Adrian et al. Nature 1984)

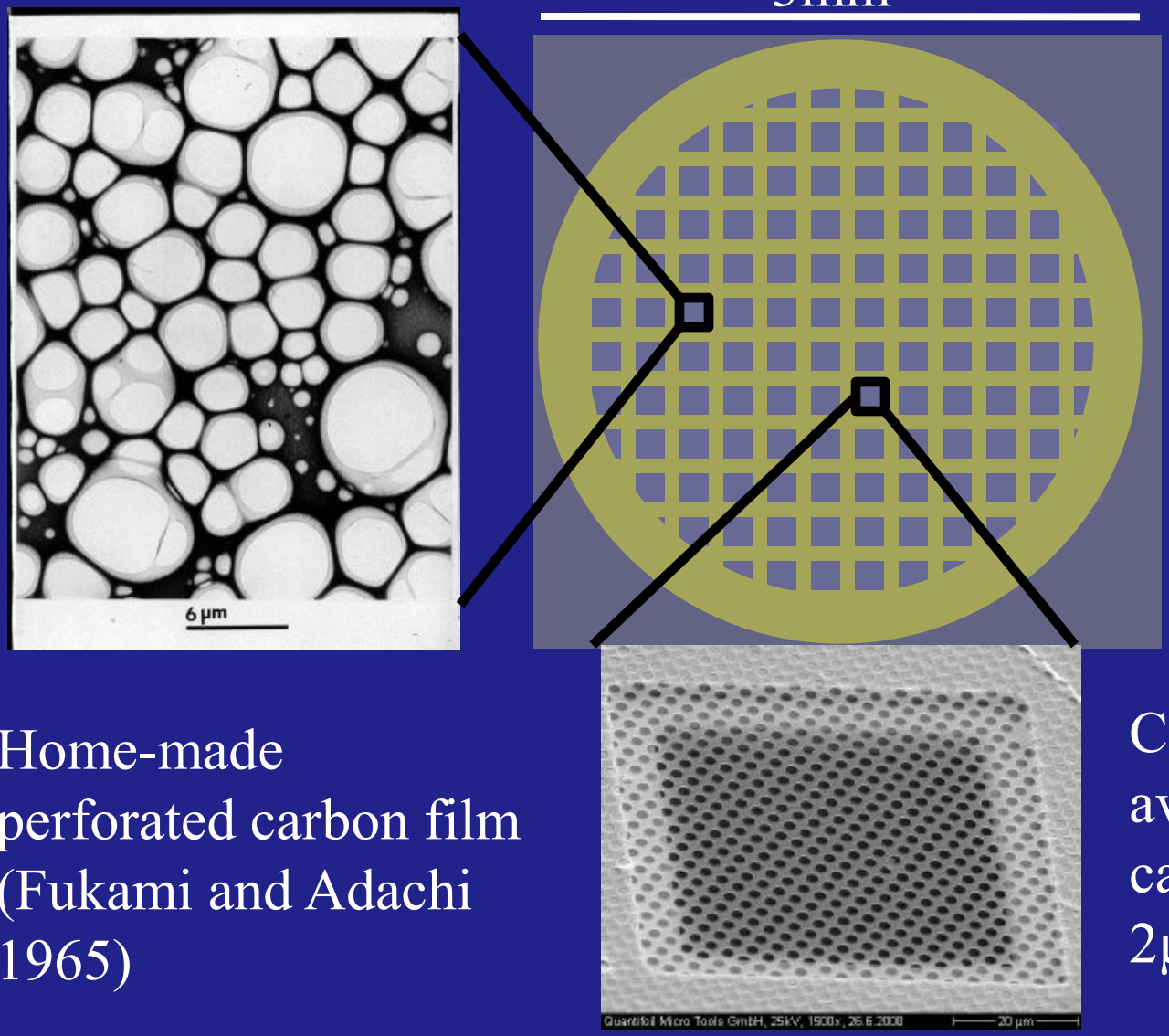
"Water is a beautiful substance"

(quoted from Dubochet et al. 1988)

Sample preparation, related items and applications:

1) Supports used in Electron Microscopy (EM)
2) Shadowing
3) Negative staining
4) Cryo-EM
5) Dynamic studies
6) 2D crystals

1) The grid and the supports



Example 200mesh:
200 squares per inch
(1 inch=25.4 mm)
bar: 28µm
window: 90µm

Grids made of
copper, gold, titanium
or molybdenum …

Home-made
perforated carbon film
(Fukami and Adachi
1965)

Commercially
available perforated
carbon film, 2µm holes
2µm spacing

1) The grids and the supports

Continuous carbon

1) evaporated on a plastic film

2) floated on water from mica; this makes cleaner and more stable carbon film)

Perforated carbon

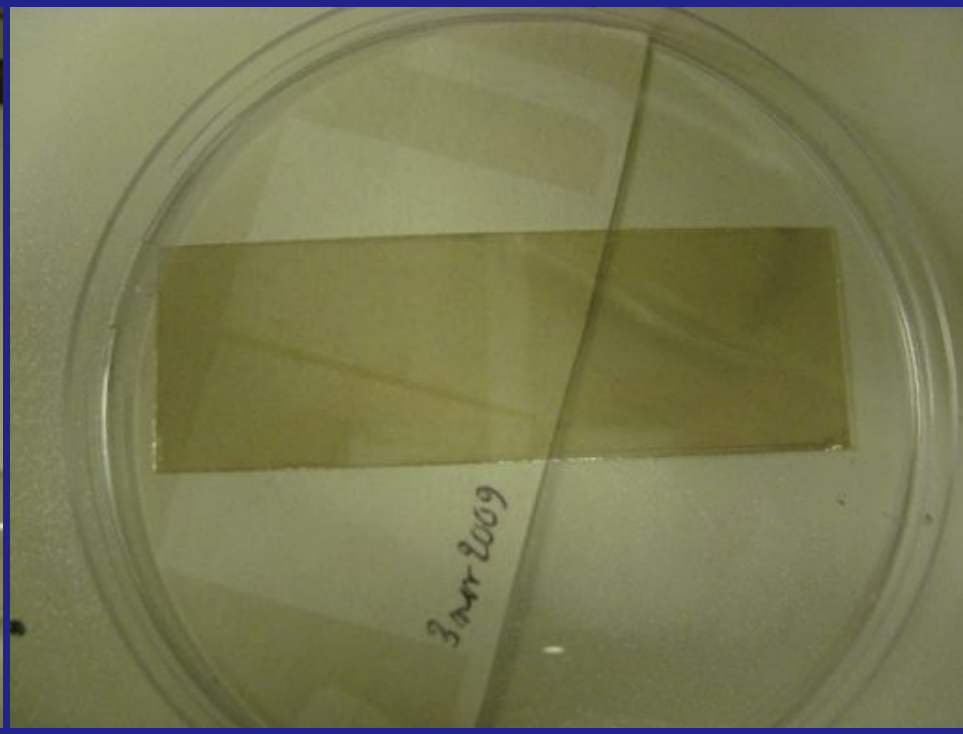
1) home-made plastic film (Fukami and Adachi 1965 …) covered with a thick carbon layer.

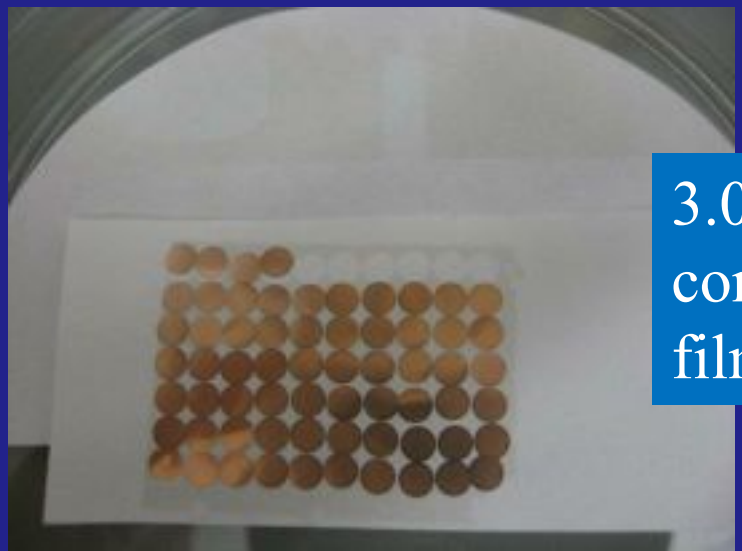
2) commercially bought

Carbon is good to conduct heat and electricity generated by the electron beam

Carbon evaporator (shadower)

1) The grids and the supports





3.05mm grids with continuous carbon film

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1) The grids and the supports

Glow discharge in reduced pressure of:
AIR to make the surface hydrophilic
AMYL AMINE to add negative charges (careful vapors are toxic)
ISOPROPANOL to make the surface hydrophobic

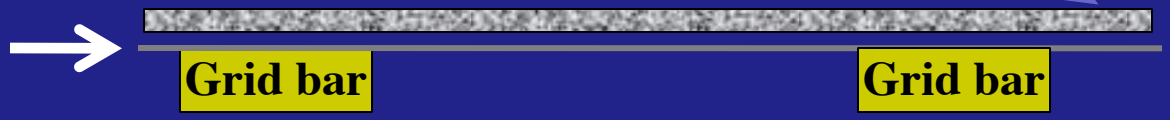
old carbon is hydrophobic but fresh carbon is mildly hydrophilic

THE BEST CARBON is
the one your sample likes

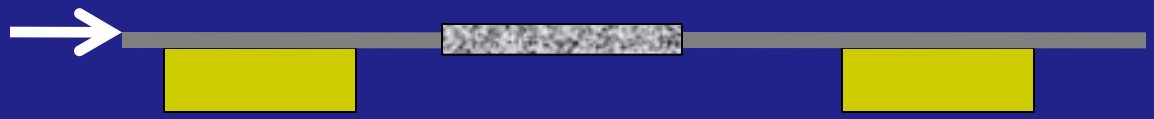
Glow discharge machine

1) The grids and the supports

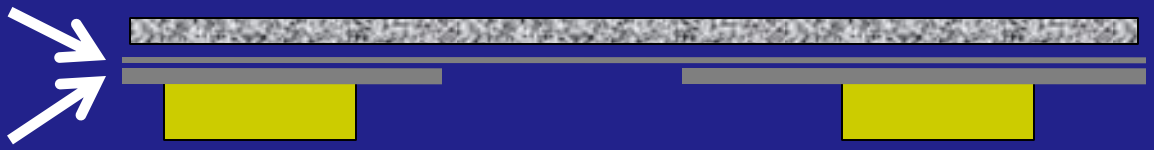
continuous support



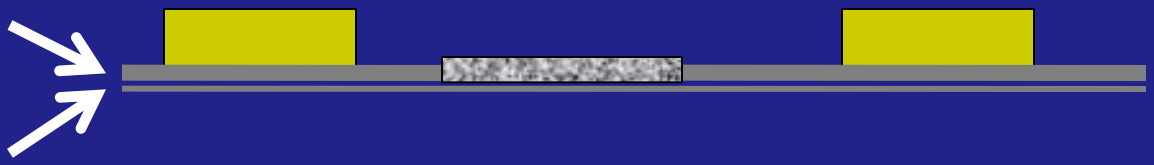
perforated (holey) support



perforated support + continuous thin support



continuous support + perforated support (“basket” configuration)



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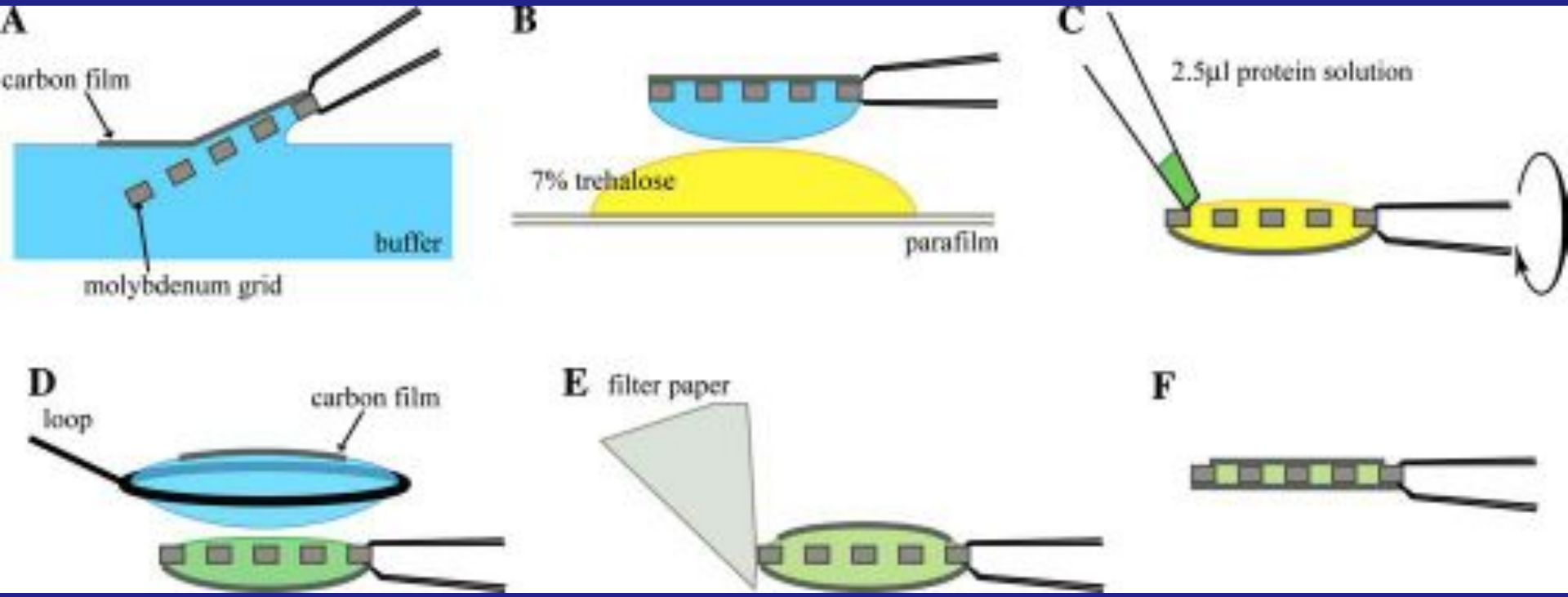
1) The grid and the supports; symmetric carbon sandwich technique



Nobuhiko Gyobu, Kazutoshi Tani, Yoko Hiroaki, Akiko Kamegawa, Kaoru Mitsuoka, and Yoshinori Fujiyoshi *JSB* **146** (2004) 325

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2) Shadowing

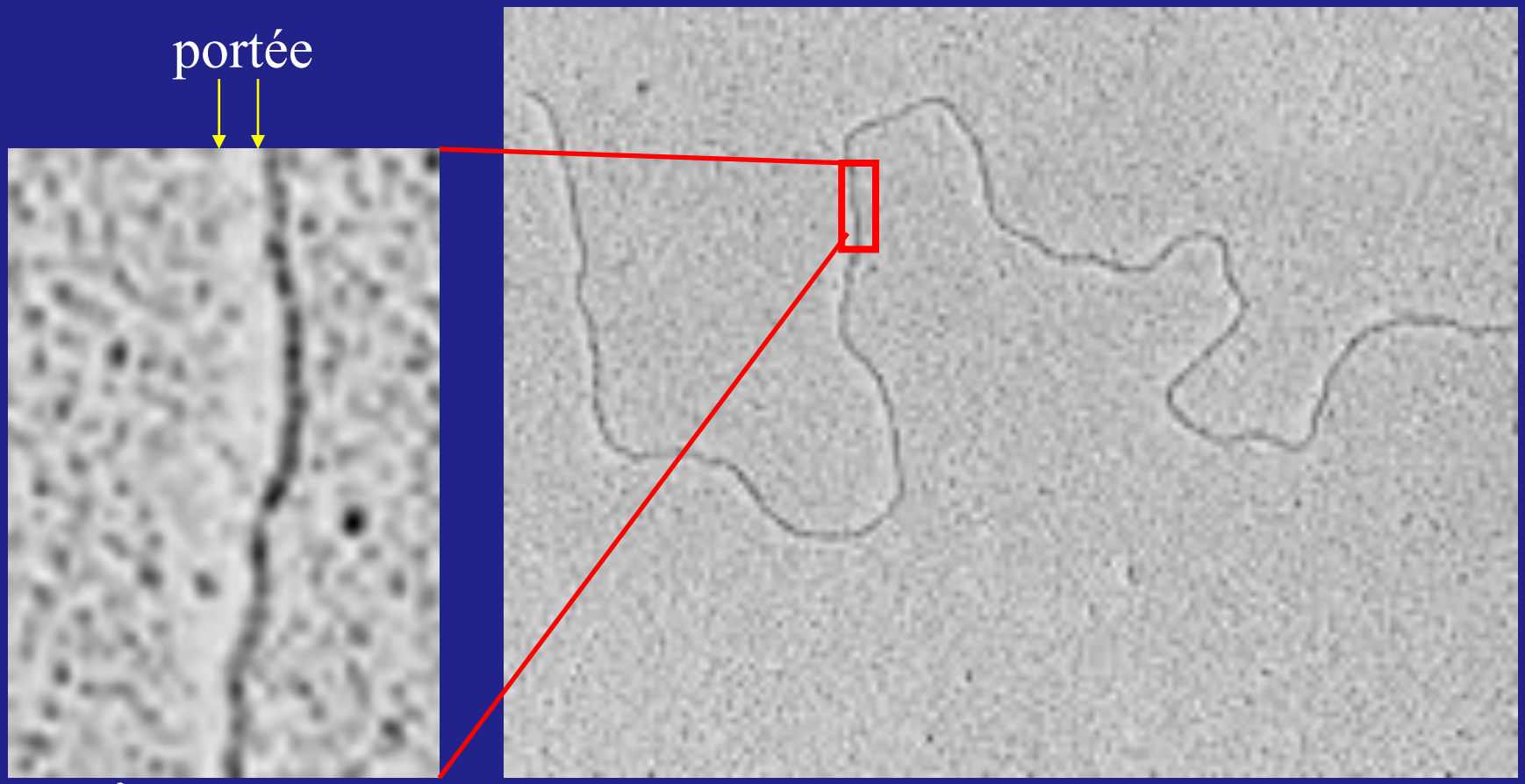
Evaporation of platinum "shadows" the molecules, gives strong contrast but low resolution ~3nm

It can be used to visualize small molecules like:

- DNA or RNA molecules
- DNA-protein or RNA-protein complexes
- single molecule like myosin …

It can be used to determine the handedness of helical objects.

2) Shadowing example: a DNA molecule



~20Å in diameter

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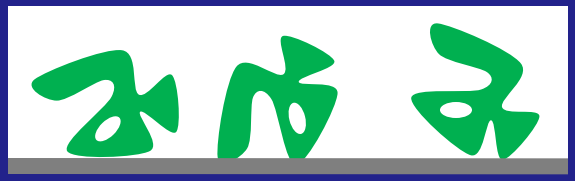
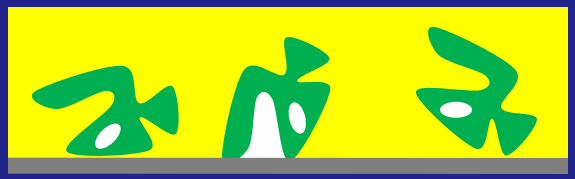
3) Negative staining

1) adsorption



2) wash with 2% uranyl acetate



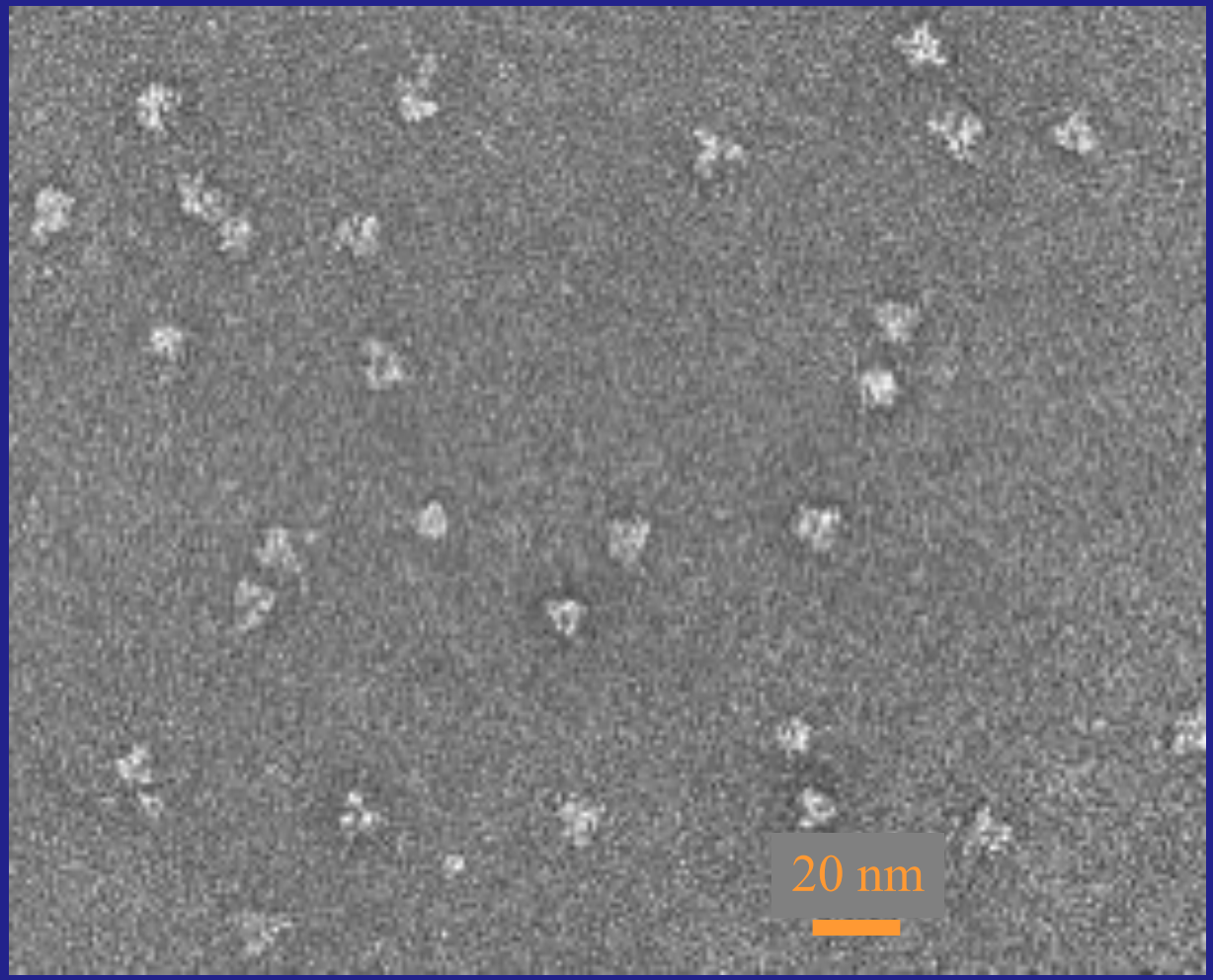
3) air-dry

Heavy metal stains:
ammonium molybdate,
uranyl acetate pH4
uranyl formate pH7
PhosphoTungstic Acid (PTA) pH7
auroglucothionate
and more …

3) Negative staining:

Transcription factors TFIID

Low-dose Image
($20e^{-}/A^{2}$)



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4) Cryo-EM

Cryo-EM is used for 3D reconstruction of asymmetric (best resolution to date: 5.5Å) and symmetric isolated particles (best resolution to date: 3.3Å)

1) No chemical fixation or staining.

2) The structures are preserved in a thin (50 to 70nm) layer of vitreous buffer prepared by rapid freezing.

3) A freezing robot (called VITROBOT) helps keep the sample fully hydrated before it is frozen in liquid ethane.

4) The sample is then kept at liquid nitrogen temperature during its transfer to the TEM and also inside the TEM.

5) The sample is beam sensitive; low-dose (~ $20e^{-}/Å^{2}$) techniques are used to collect data before high-resolution details are being damaged by the electrons.

4) Cryo EM; knowing your sample

Is the sample detergent sensitive ?

detergent can be used to reduce the ice thickness by lowering the surface tension

Do I need to keep sucrose, glycerol in my buffer ?

sucrose and glycerol reduce contrast

Is the sample salt sensitive ?

high limit = damage; subunit dissociation

low limit = aggregation

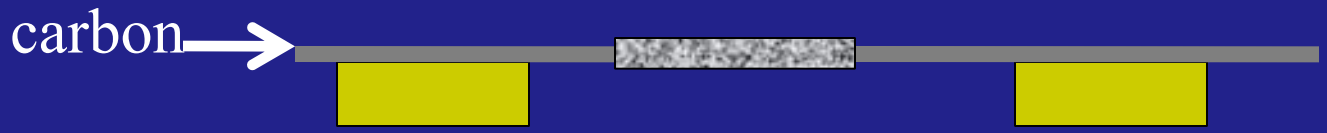
Is the sample temperature sensitive ?

if so, minimize pre-cooling

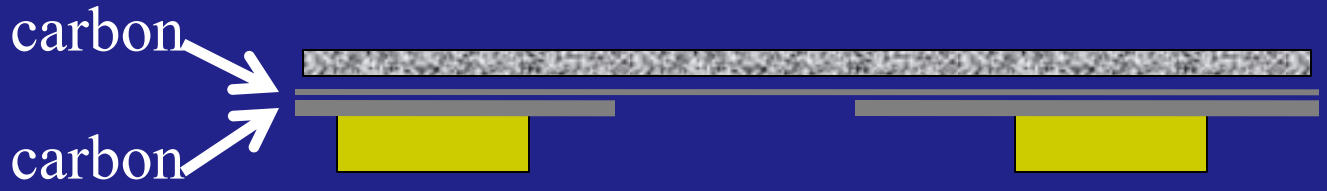
4) The grids for cryo

continuous support "supported cryo"

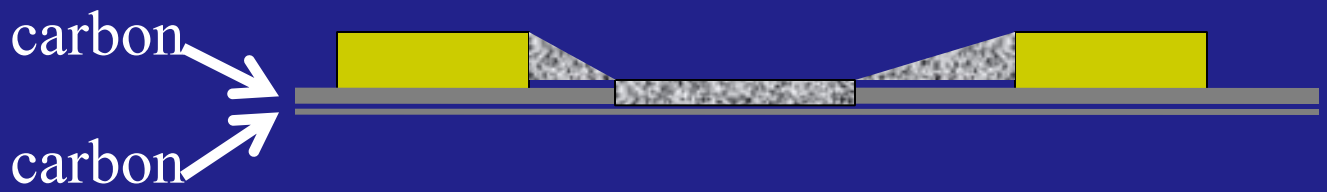
perforated (holey) support "classic cryo" "unsupported" or "self-supported"



perforated support + continuous thin support



continuous support + perforated support ("basket" configuration)



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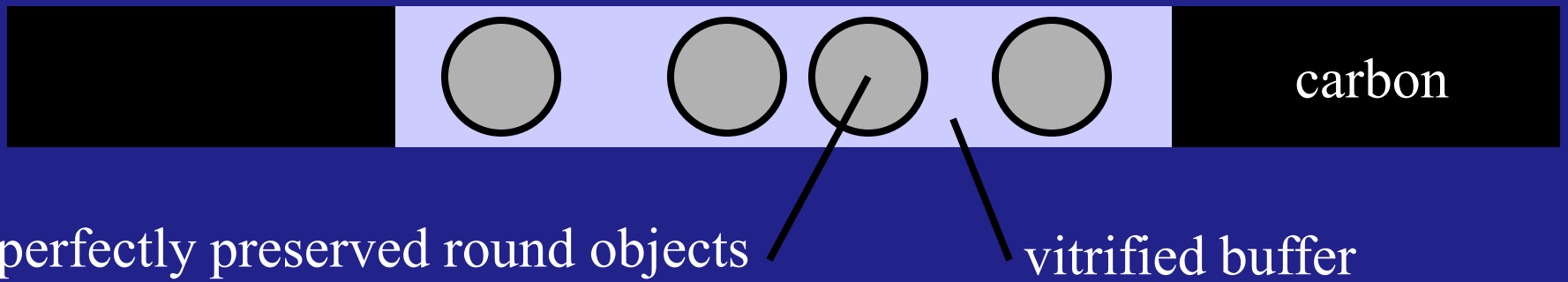
4) “Classic” self-supported Cryo-EM

The specimen is self-supported over holes in perforated carbon film; it is observed in its native state; it is unstained and unfixed.



This is the ideal case

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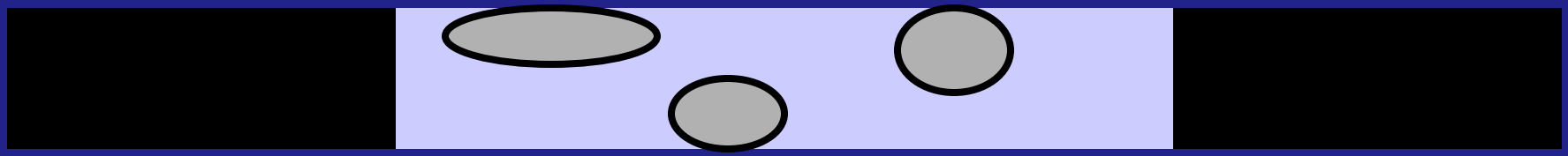
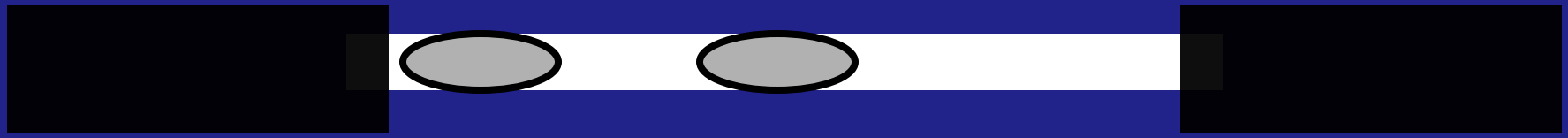
4) Trouble in Cryo-EM

1) interaction with the air-buffer interface can cause damage

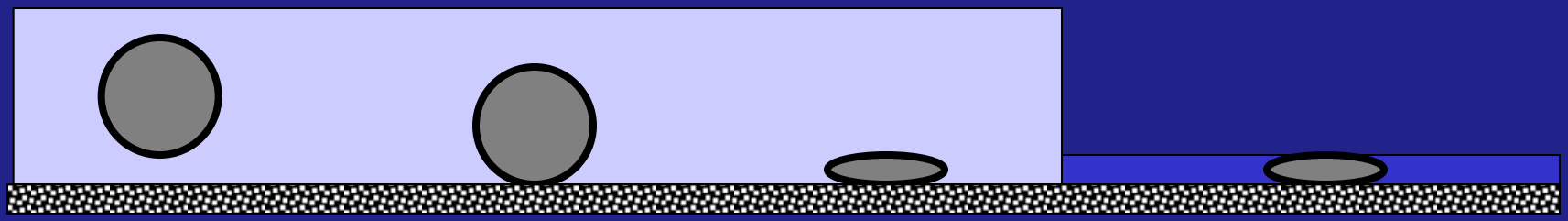
2) drying + flattening



3) ice too thin + flattening



4) Cryo-EM on continuous carbon



"free floating" on "neutral" carbon

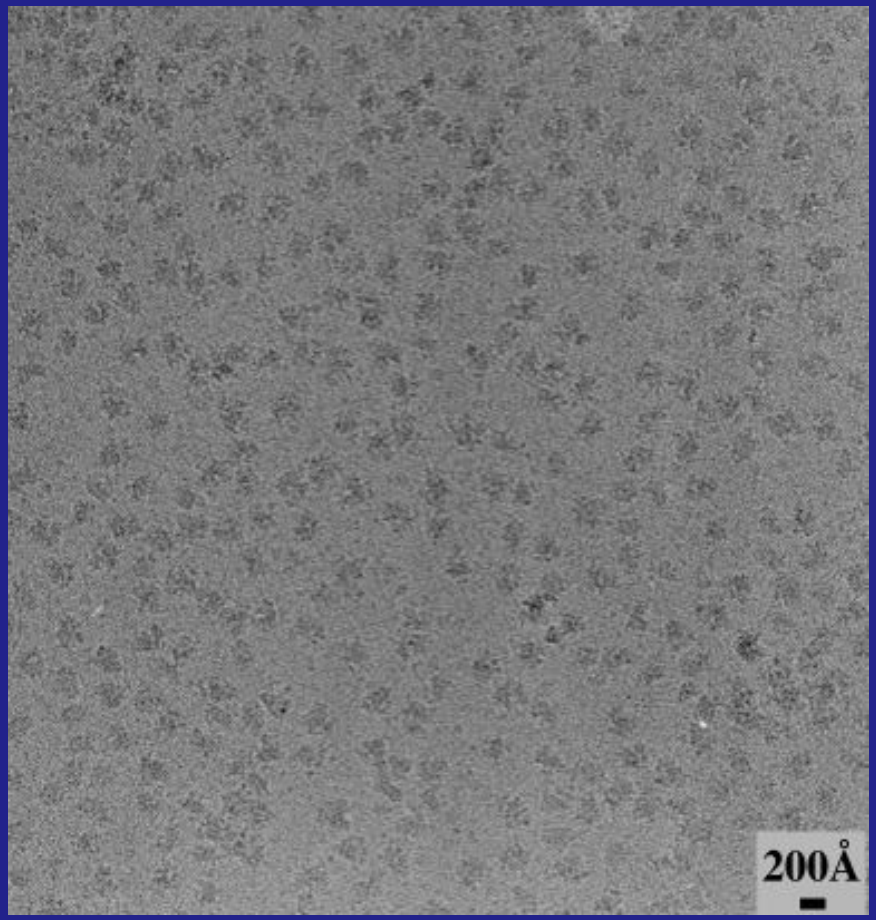
"soft landing" on midly hydrophilic carbon

"crash" on very hydrophilic carbon

drying + flattening

4) Cryo-EM; concentrations

continuous carbon film

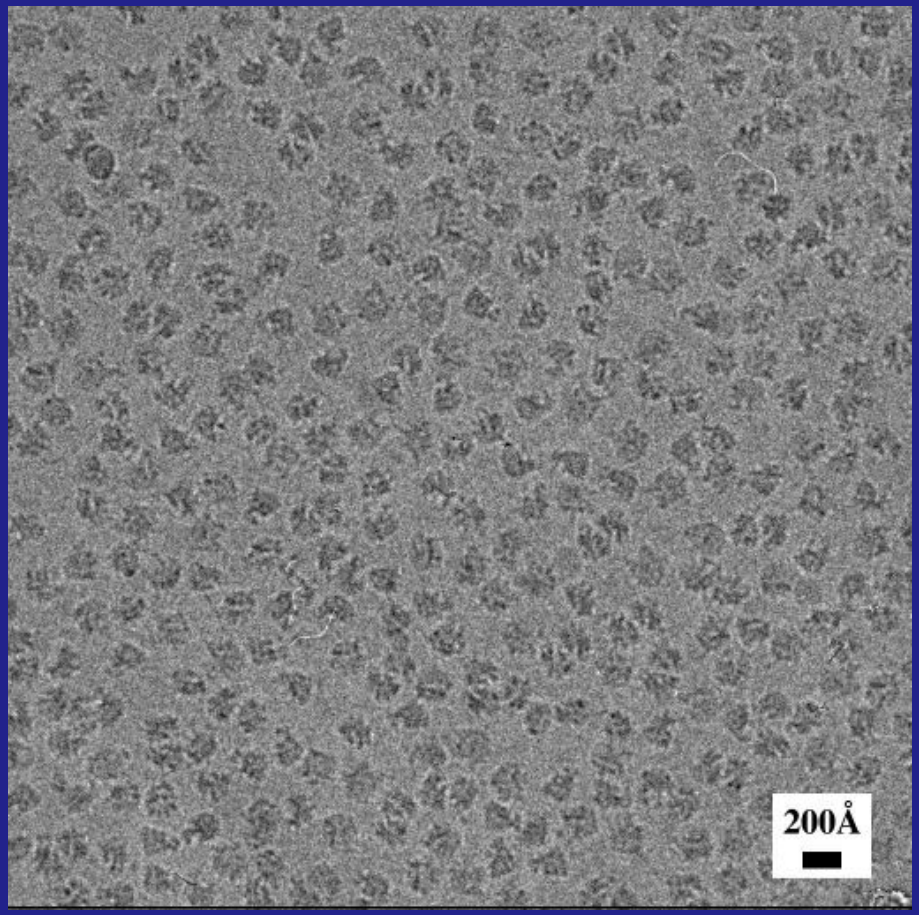


~ 1.4 nanoM

Ribosome channel complexes

~ 1000X

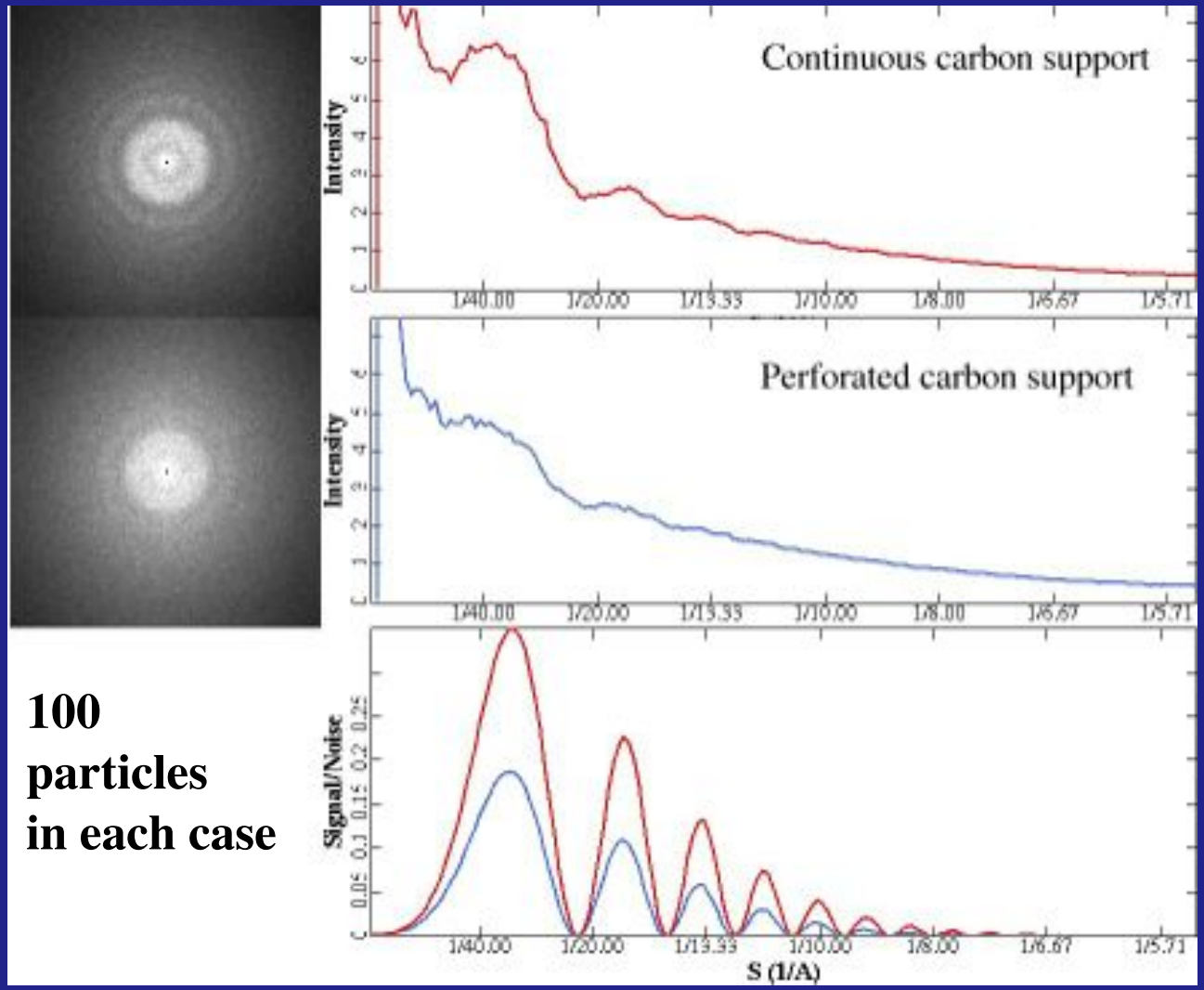
perforated carbon film



~ 1.9 microM

Ecoli ribosomal large subunit

4) Cryo-EM; Contrast Transfer Function

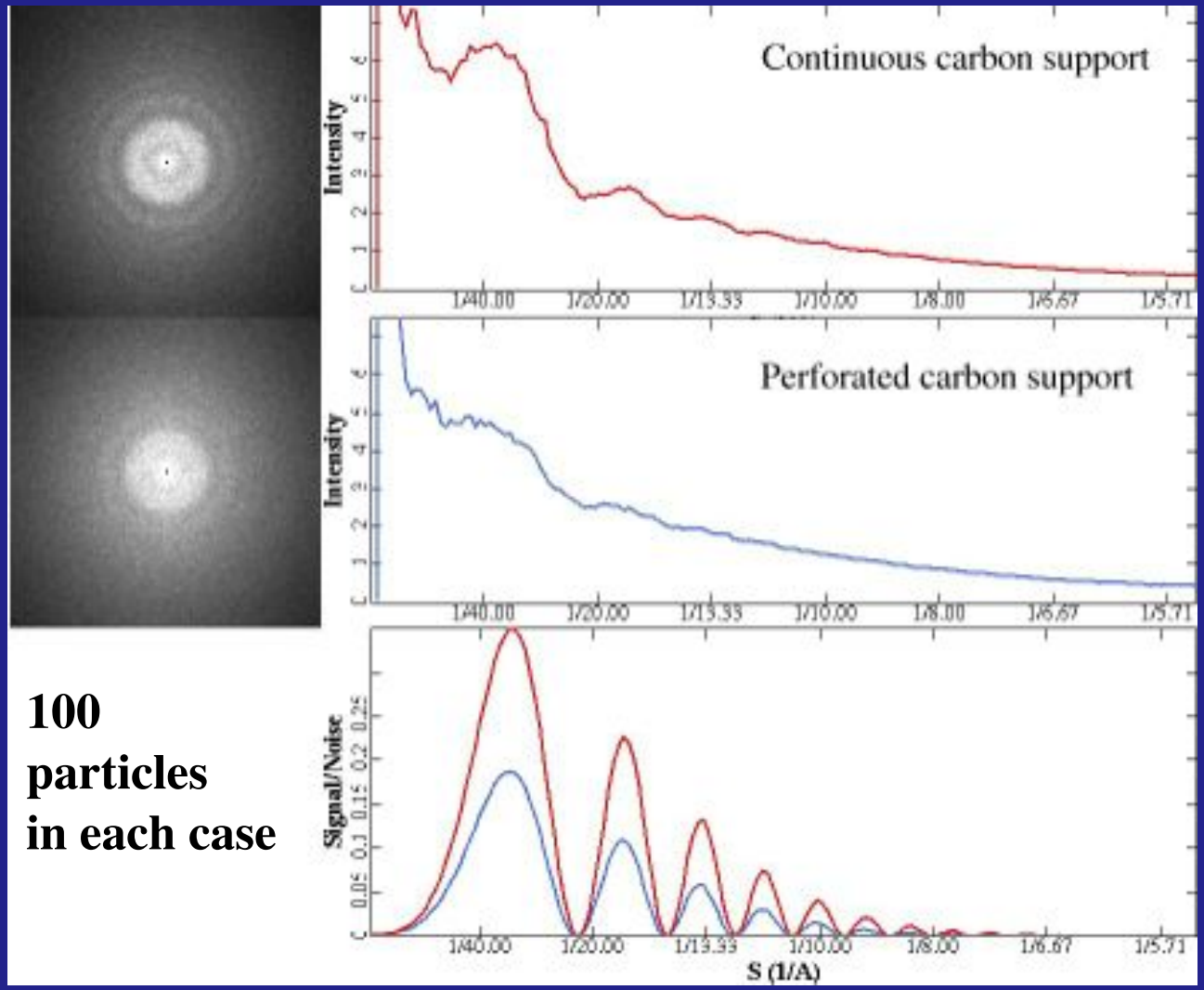


supported

self supported

100 particles in each case

4) Cryo-EM; Contrast Transfer Function



100 particles in each case

Curves include:

CTF,

background noise,

structure factor,

envelope function

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4) cryo-EM; Vitrobot

4) cryo-EM; Vitrobot

Parameters:
1)Humidity
2)Temperature
3)Blotting pressure
4)Blotting time
5)Blotting repeat

4) cryo-EM; Vitrobot

Loading the grid

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4) cryo-EM; Vitrobot

Grid in position before blotting

13 blots per filter paper

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4) cryo-EM; Vitrobot

The grid is being blotted

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4) cryo-EM; Vitrobot

Grid in position before dropping in liquid ethane

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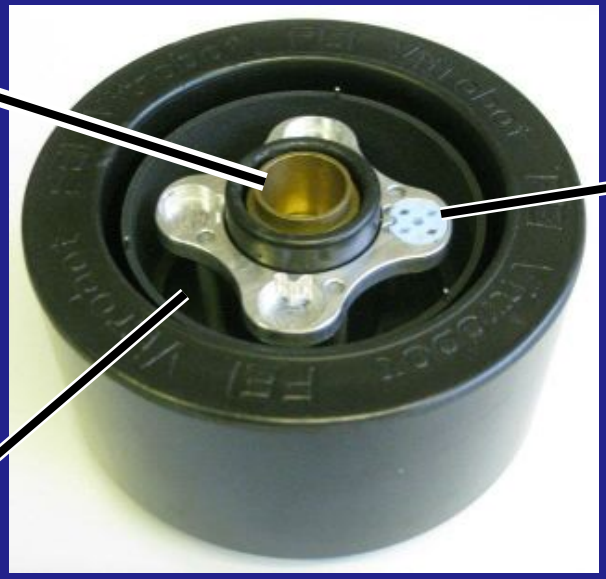
4) cryo-EM; Vitrobot

Liquid ethane
(between -89˚C
and -183˚C)



Grid box

Liquid nitrogen
(between -196˚C
and -210˚C)

Water (and buffer depending on composition)

☹ Hexagonal crystals between 0˚C and -90˚C
☹ Cubic crystals between -90˚C and -140˚C
☺ Amorphous buffer below -140˚C

Rate of cooling: ~10^5˚C/sec

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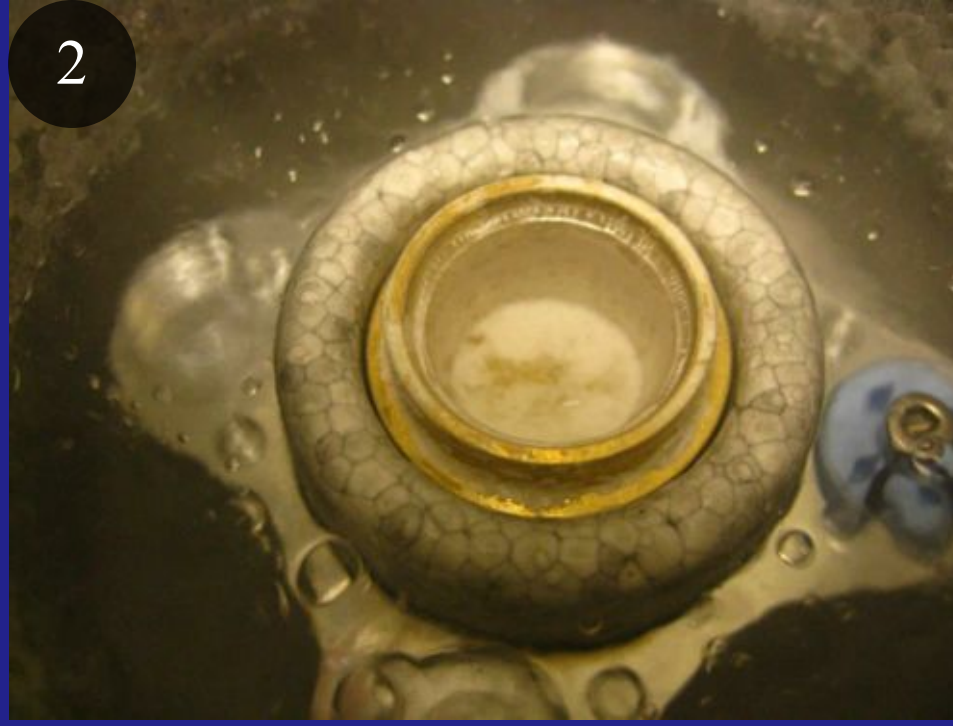
1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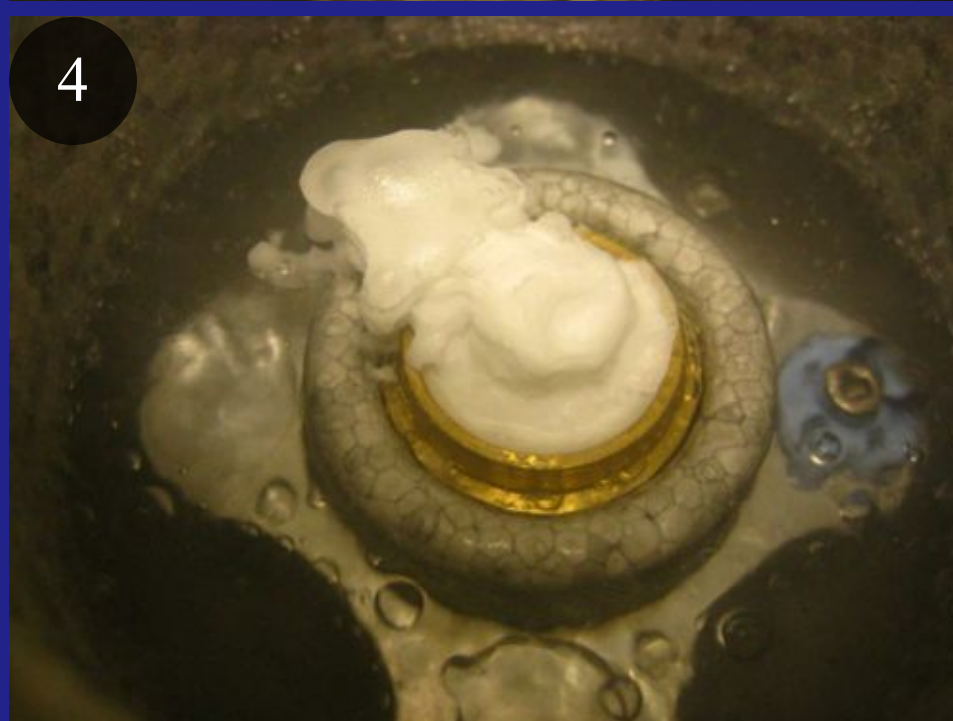

4

4. Freezing the sample

The plunging

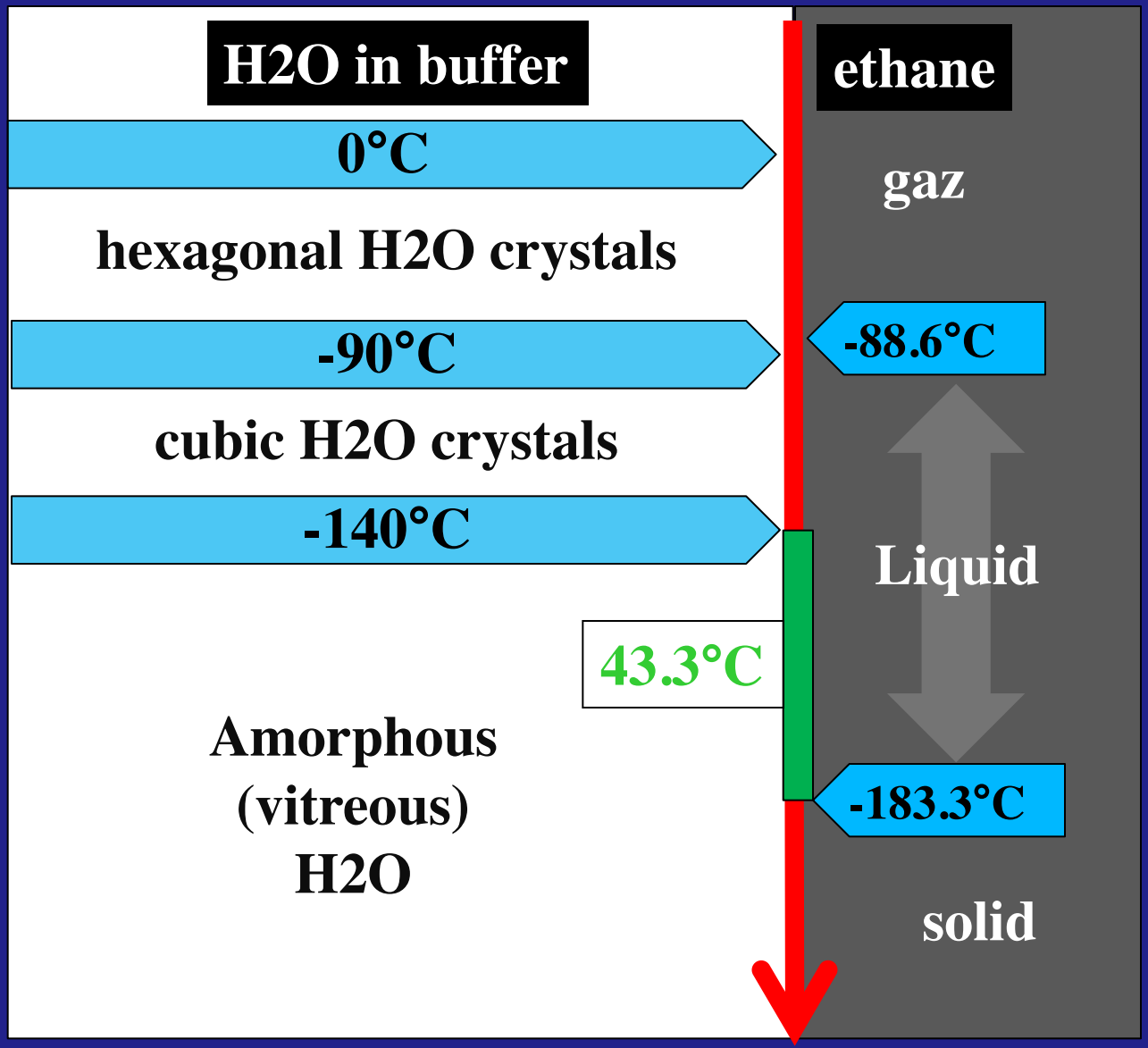
7.8cc = ~7.8g

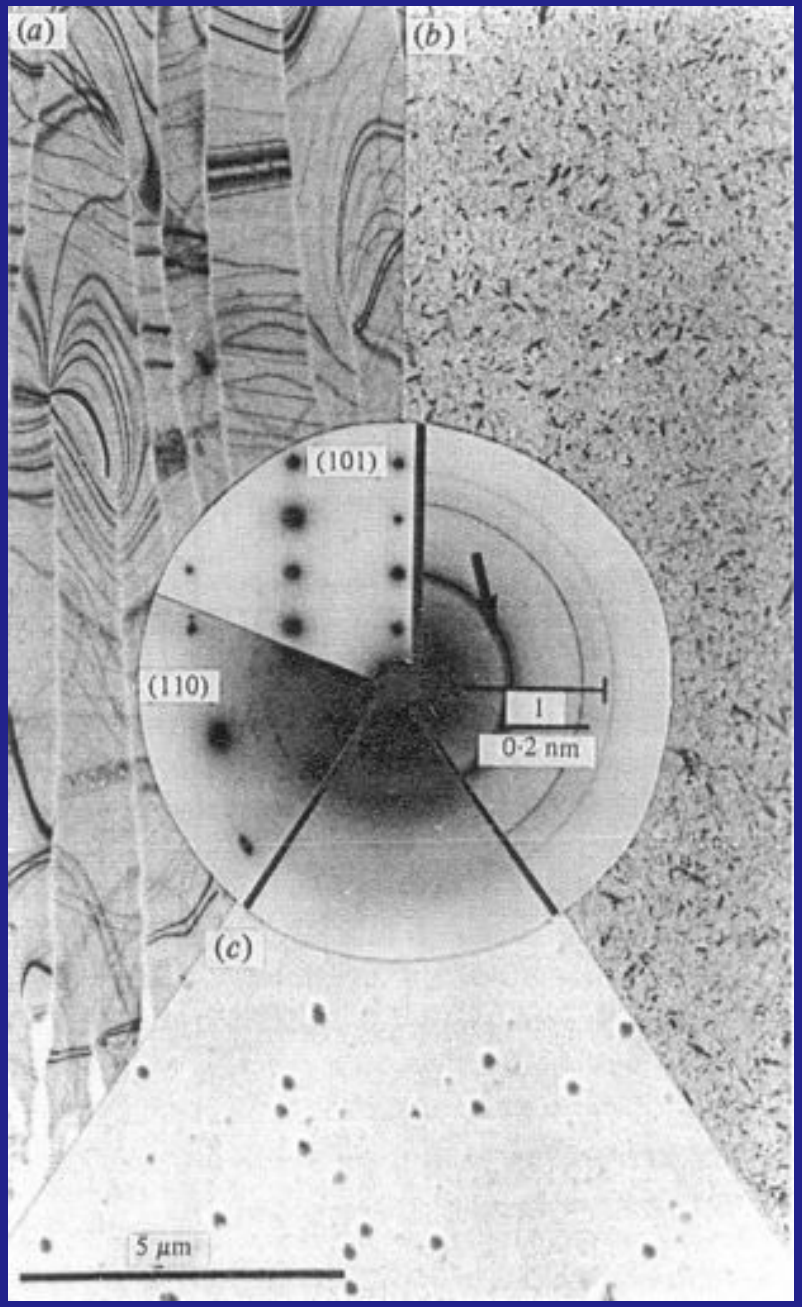
(7.8g) / (30g/mol) = 0.27 mole

0.27mole X 22.42 liter/mole = 6 liters !!!

5. Freezing the sample

5.5. transition temperatures





4) Cryo-EM; states of water seen in image mode and diffraction mode

a) Hexagonal ice
b) Cubic ice
c) Amorphous or vitreous ice

from Dubochet J. et al. Quaterly Review Biophysics (1988) 21 129-228

4) Cryo-EM; contamination

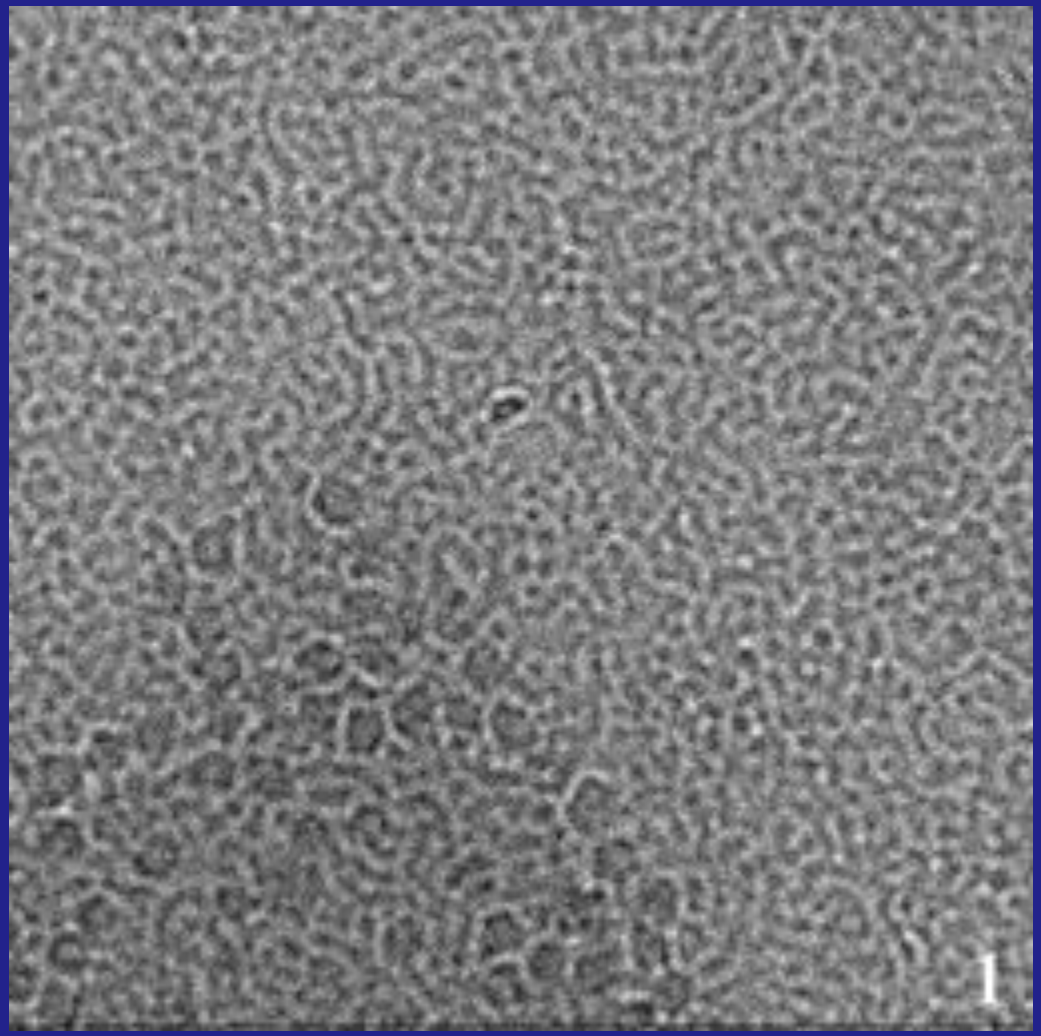
orange skin or
leopard, turtle, gator, snake skin

Contamination deposited on the grid during transfer through the air from liquid ethane to liquid nitrogen.

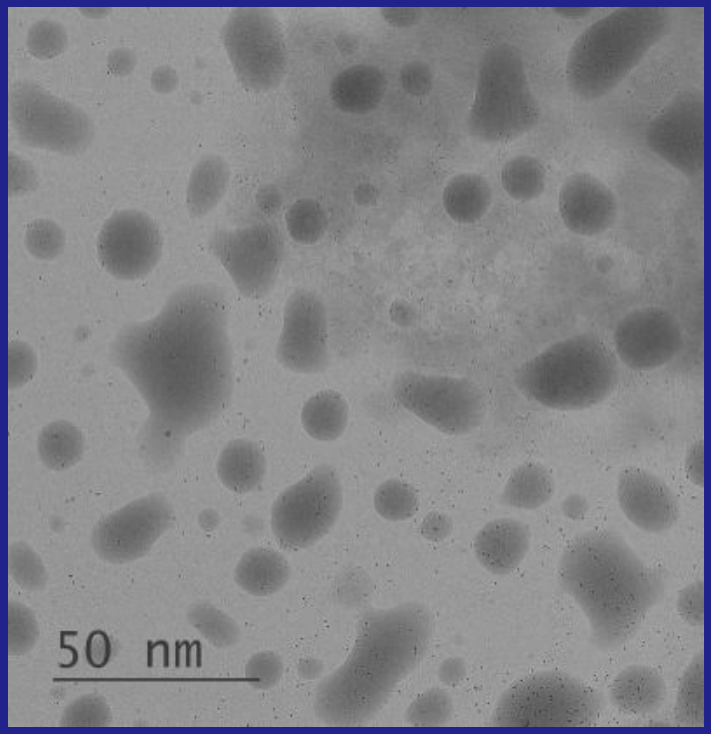
It accumulates also in the microscope with time.

It is sensitive to dose.

from Dr. Yuhang Liu



4) Cryo-EM; contamination

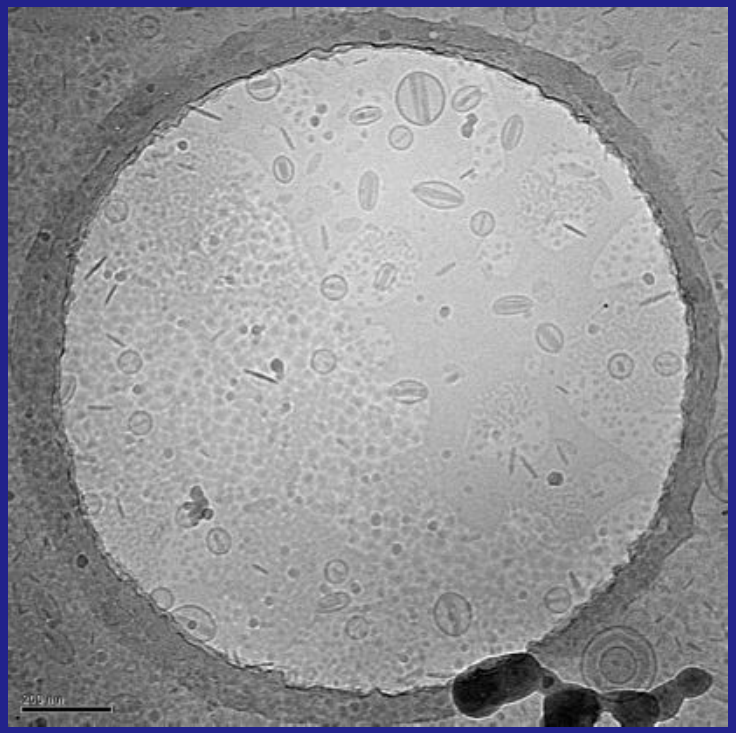


Question on 3DEM mailing list: what's that contamination ?

Analysis: carbon support probably too hydrophobic;

Suggestion: try air glow discharge

4) Cryo-EM; contamination



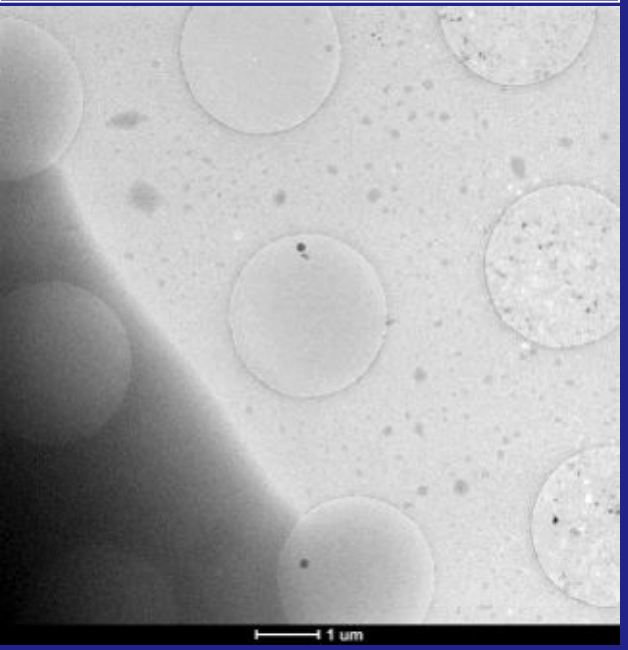
Question on 3DEM mailing list: what's that horrible ice ?

Analysis: "this phenomenon comes and goes" The grid may have been exposed to warm air or surfaces during transfer.

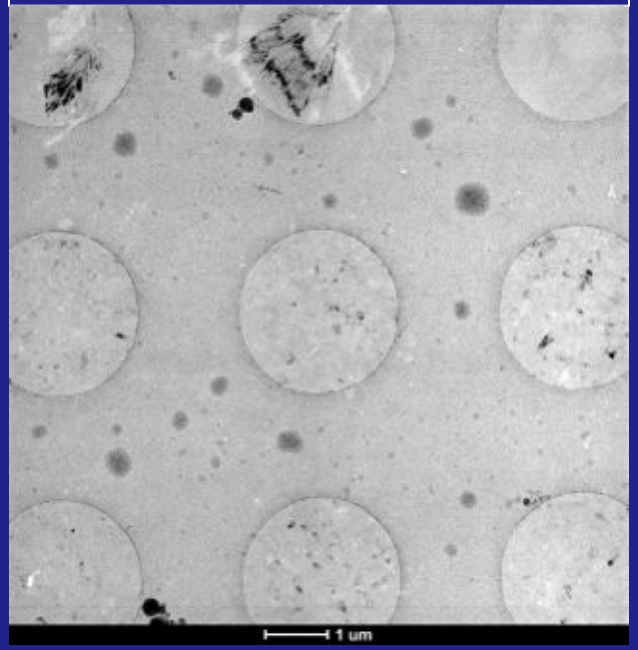
Suggestion: better pre-pumping of the airlock before introduction.

4) Cryo-EM; contamination

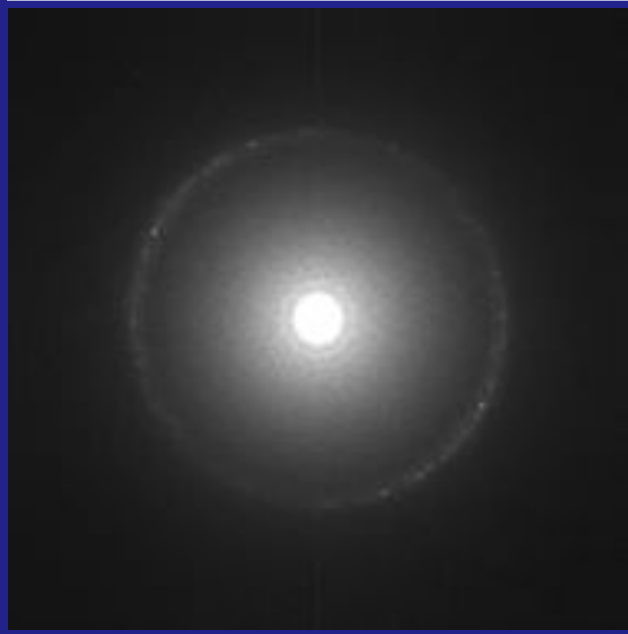
One can find vitreous ice and crystalline ice in a small area



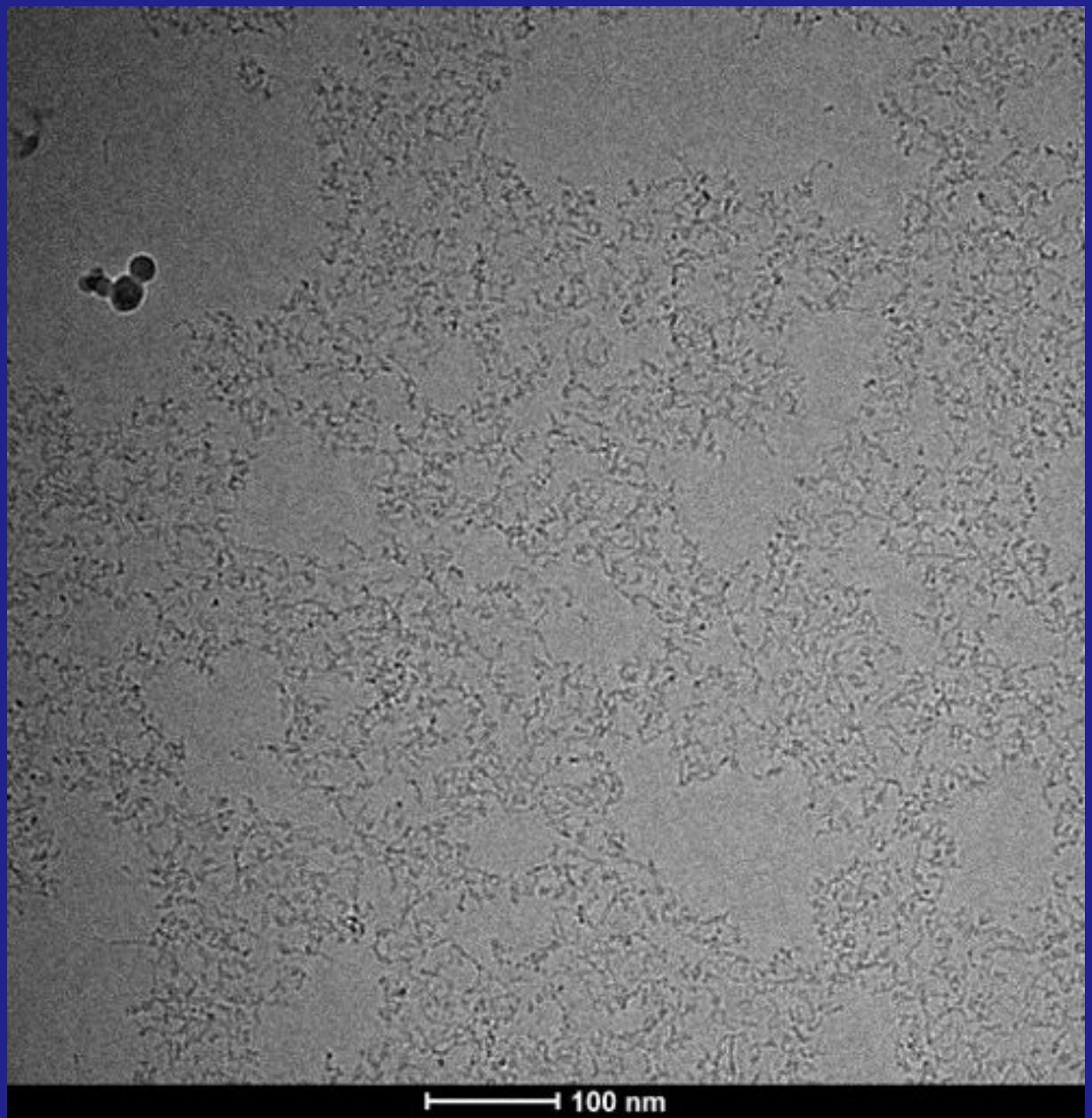
Field of crystalline water



Calculated diffraction showing the presence of water crystals

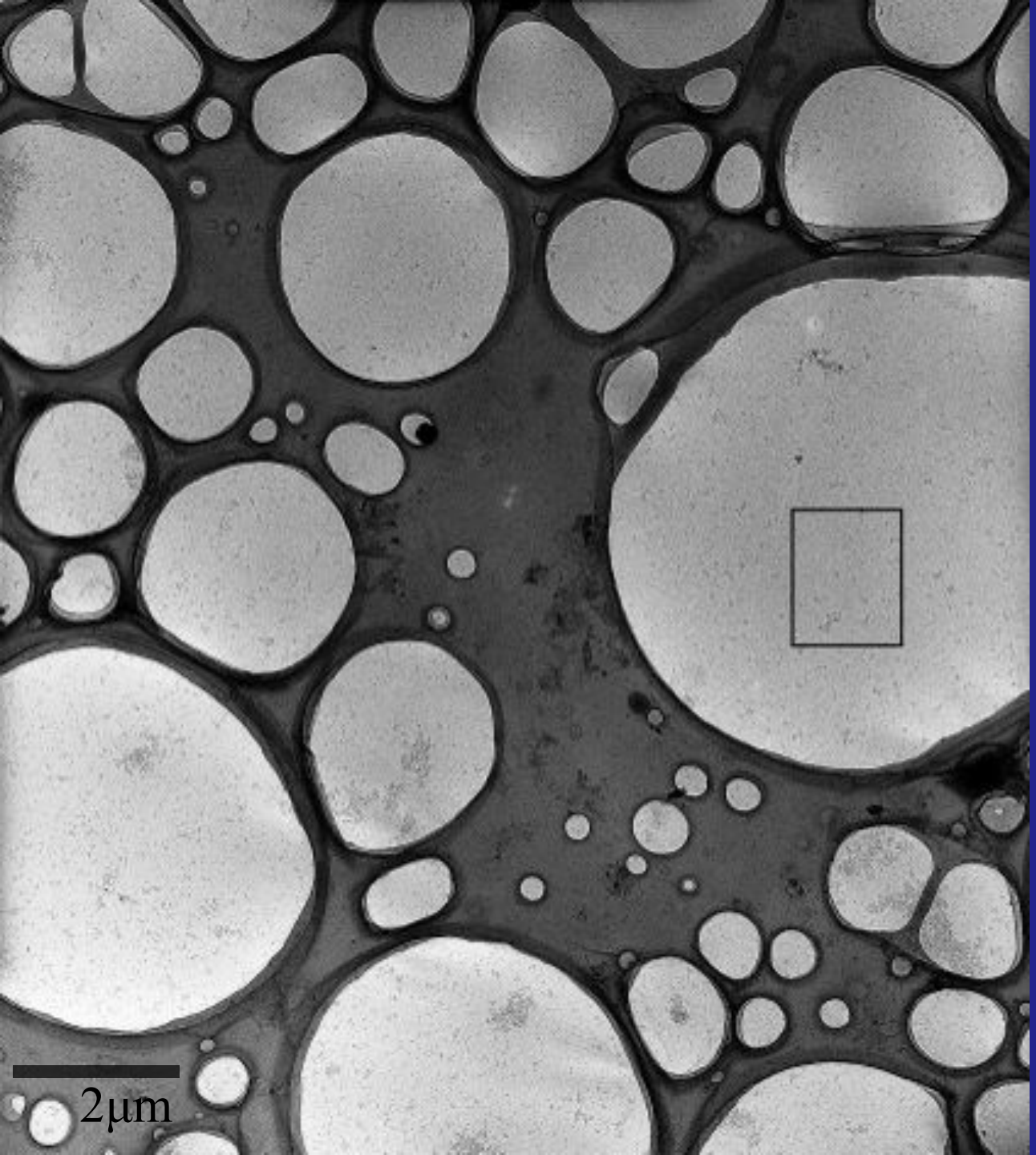


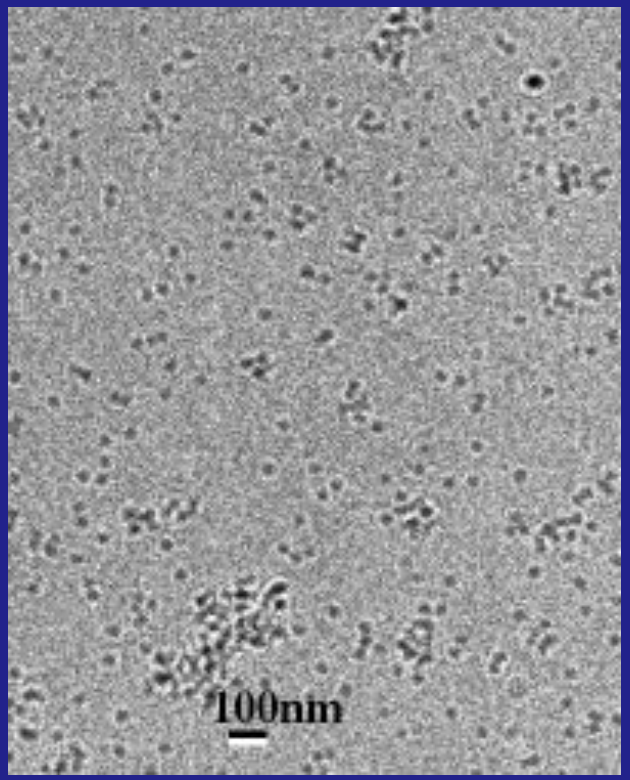
9. Gallerie; the good, the bad and the ugly



Uneven particle distribution

4) cryo-EM; storage

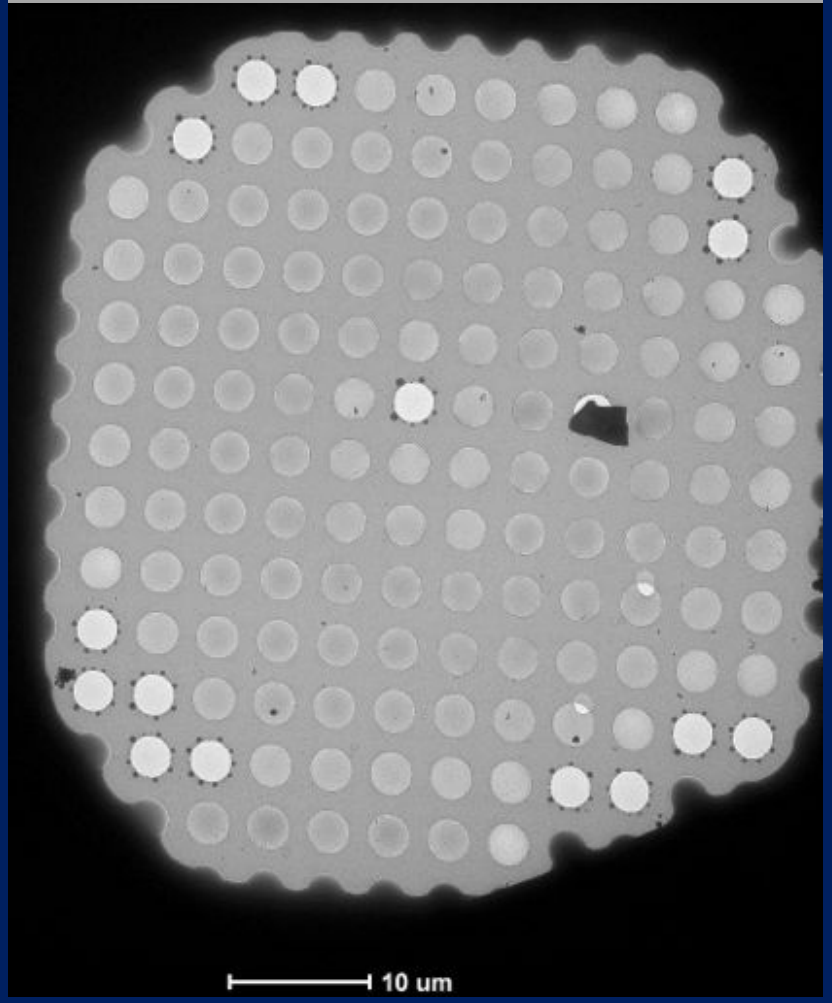




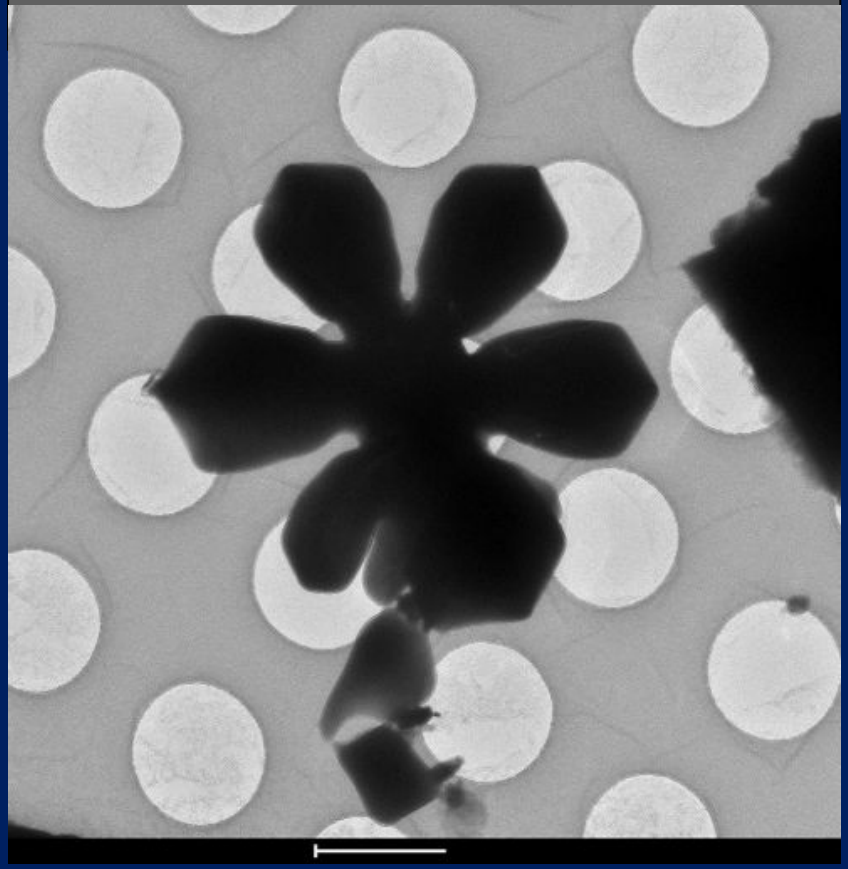
Negative #15893
This grid was stored
for 8 years !!

Gallerie

Looking at the edge of the holes, one can tell if the buffer layer broke before freezing.

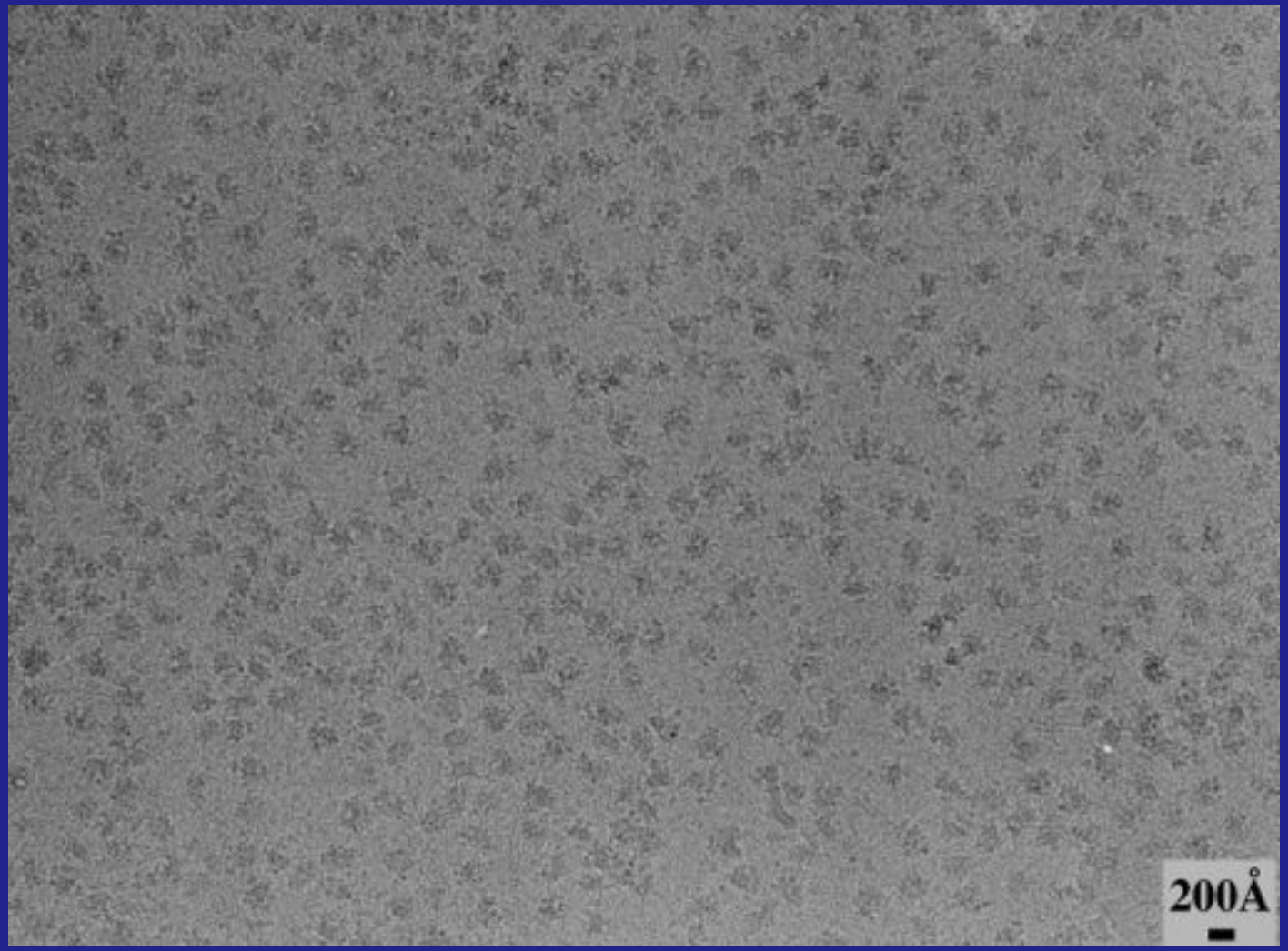


"cryo flower"



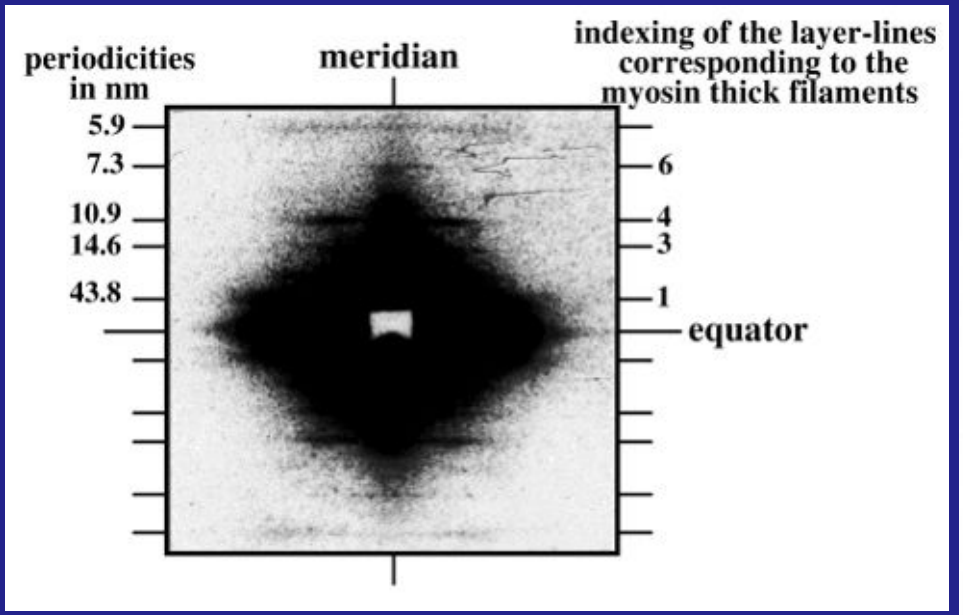
2microns

4) Cryo-electron micrograph of E.Coli ribosome channel complexes on continuous carbon film (~0.005mg/ml)



4) Preservation of fragile samples

Limulus (horse shoe crab) myosin thick filaments



J.S. Wray

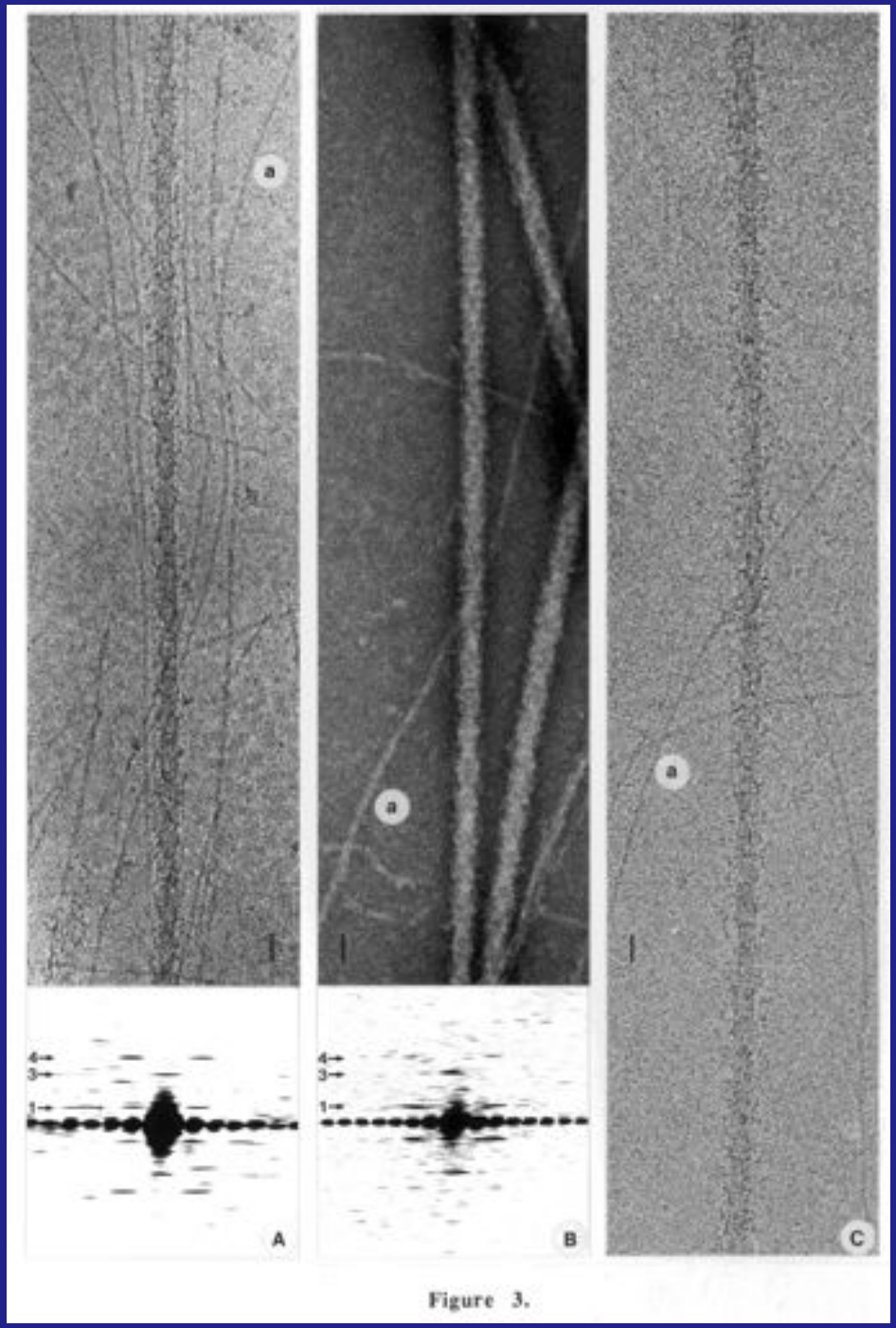


Figure 3.

Scale bar is 14nm

Sample preparation, related items and applications:

1) Supports used in electron microscopy (EM)
2) Shadowing
3) Negative staining
4) Cryo-EM
5) Dynamic studies
6) 2D crystals

5) Dynamic studies in cryo-EM

Photolabile components

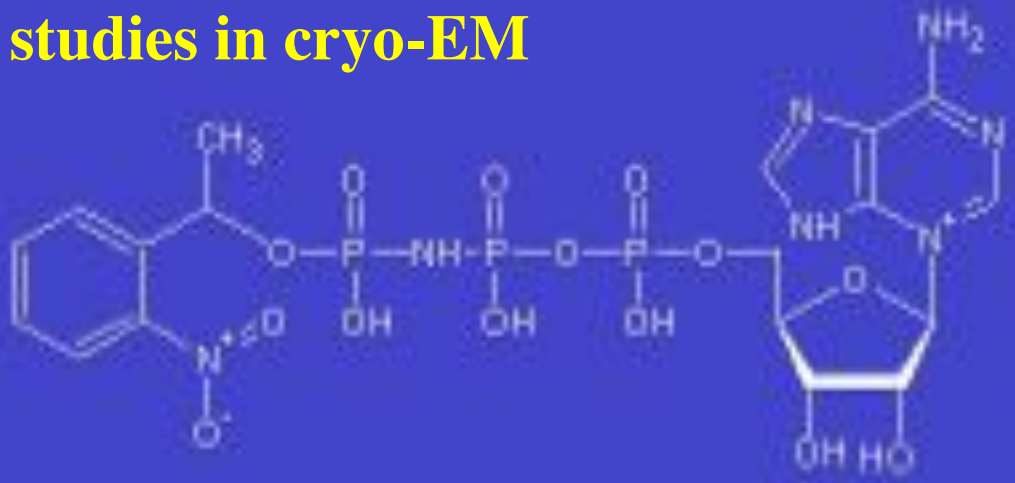
Ménétret JF, Hofmann W, Schröder RR, Rapp G, Goody RS. *J. Mol. Biol.* 1991 219(2):139-44.
Time-resolved cryo-electron microscopic study of the dissociation of actomyosin induced by photolysis of photolabile nucleotides.

Spraying

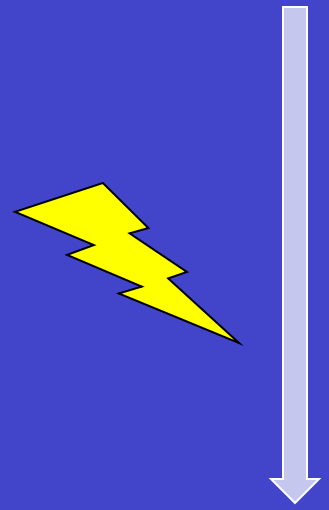
Berriman J. and Unwin N. *Ultramicroscopy*. 1994 56(4):241-52.
Analysis of transient structures by cryo-microscopy combined with rapid mixing of spray droplets.

Unwin N, Miyazawa A, Li J, Fujiyoshi Y. *J. Mol. Biol.* 2002 319(5):1165-76.
Activation of the nicotinic acetylcholine receptor involves a switch in conformation of the alpha subunits.

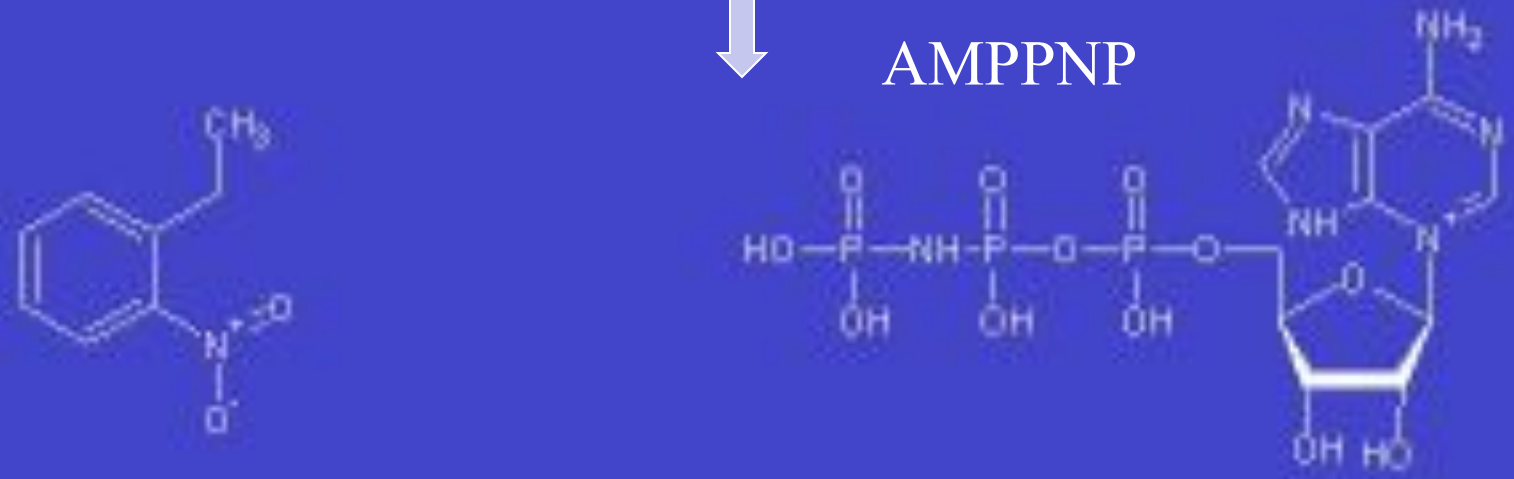
5) Dynamic studies in cryo-EM



Photolabile AMPPNP



AMPPNP



5) Dynamic studies in cryo-EM

The initial state:
Actin filaments decorated with myosin S1

Ménétret JF, Hofmann W, Schröder RR, Rapp G, Goody RS.
J. Mol. Biol. **1991 219(2):139-44.**
Time-resolved cryo-electron microscopic study of the dissociation of actomyosin induced by photolysis of photolabile nucleotides.

Scale bar is 14nm

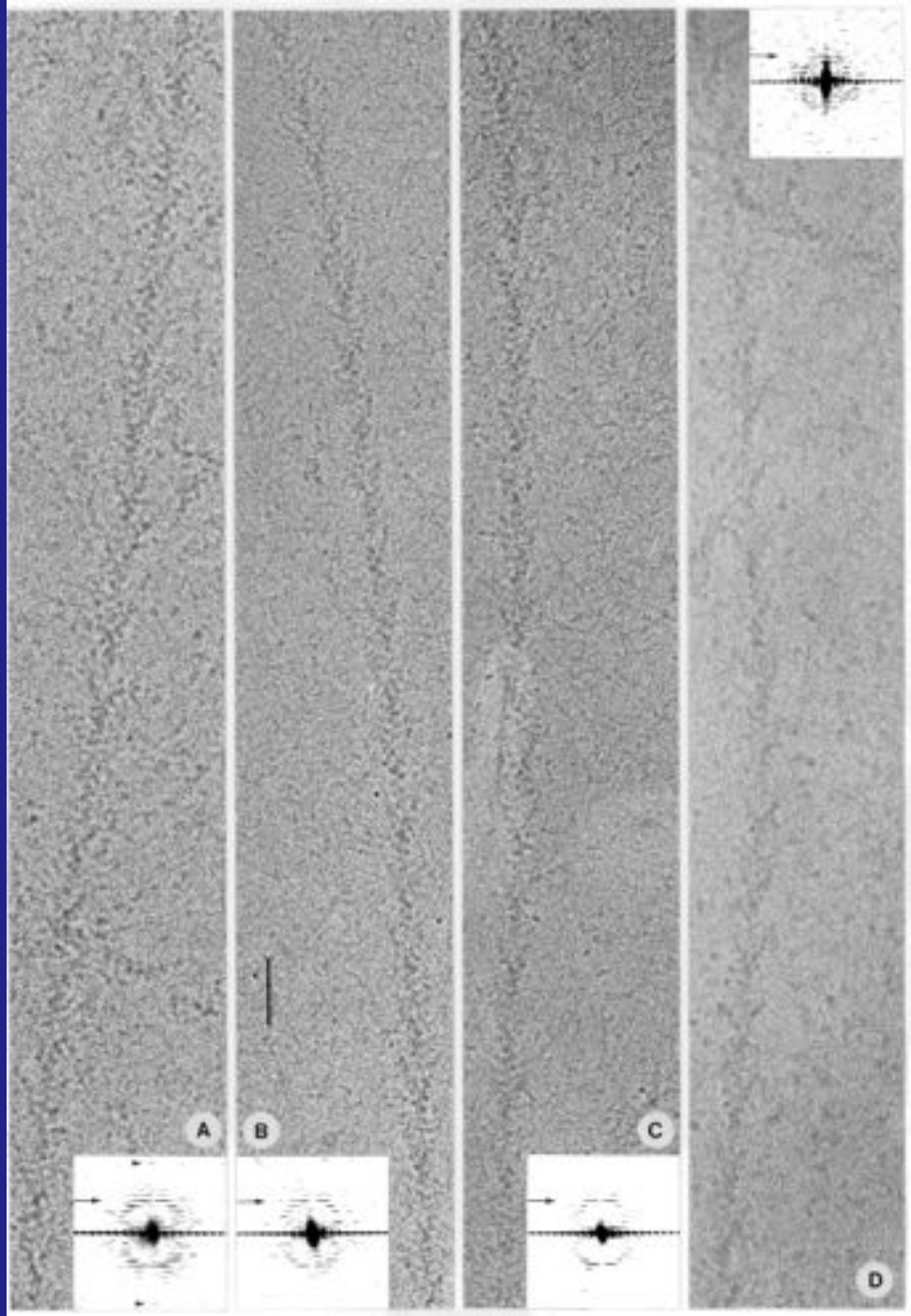
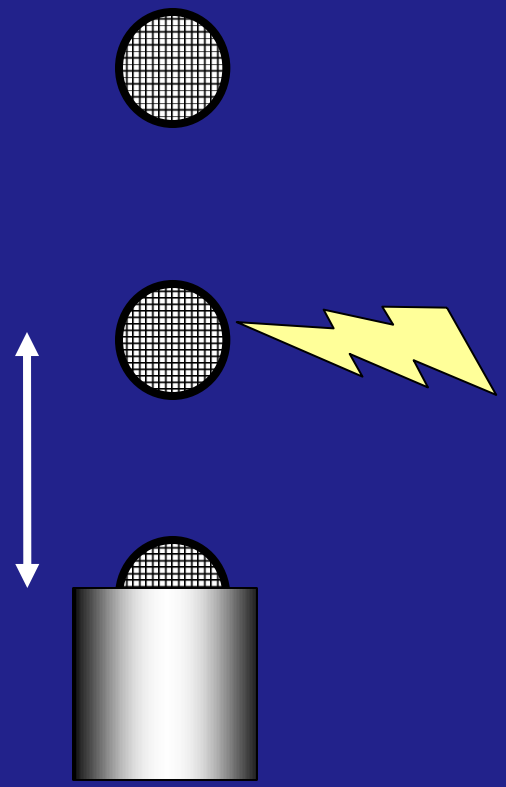
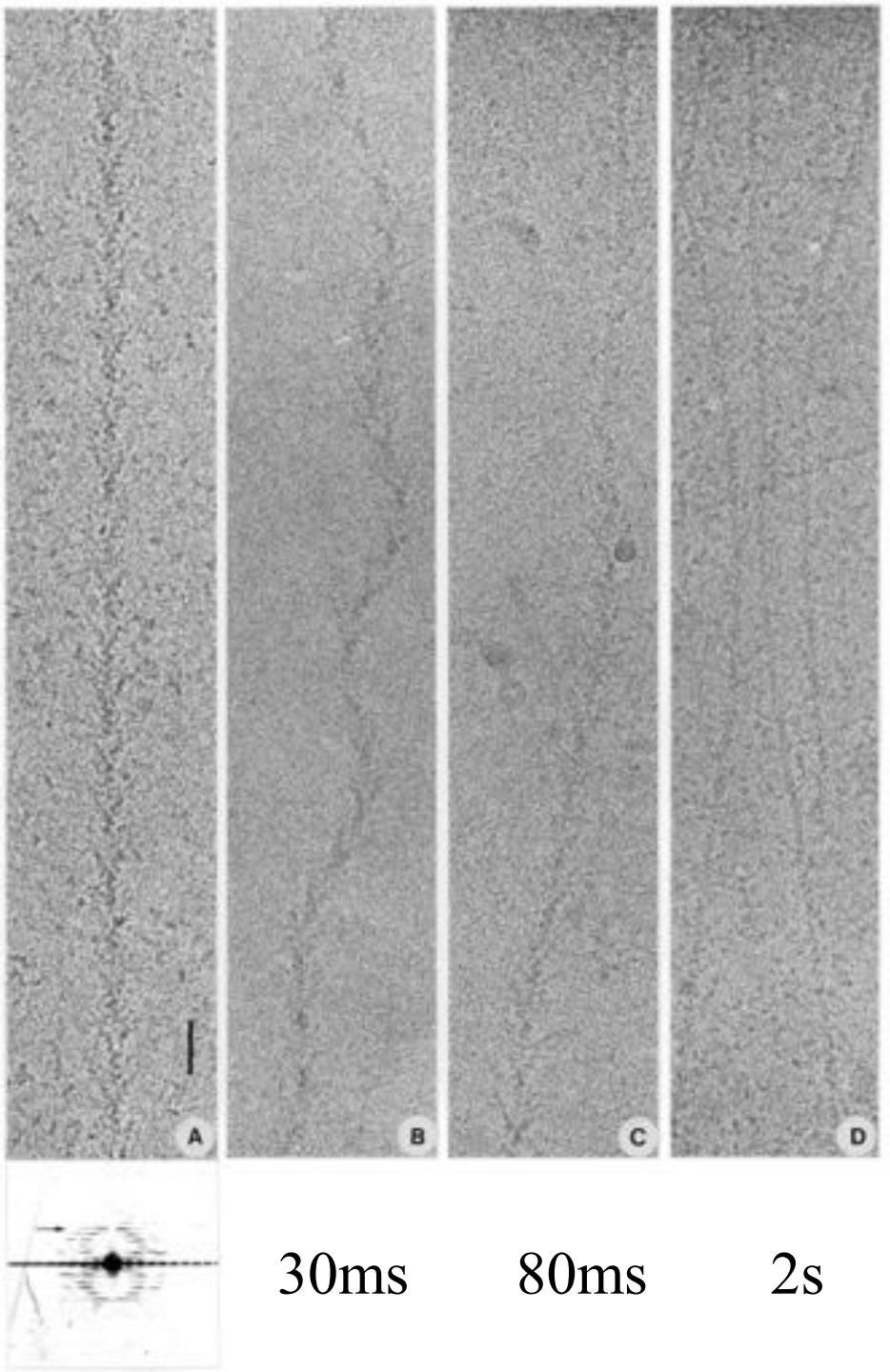


Figure 14.

5) Dynamic studies in cryo-EM

time-resolved dissociation of the myosin heads from the actin filaments





5) Dynamic studies in cryo-EM

Actomyosin
30msec after release of nucleotides by flashing

The released AMPPNP seem to induce a conformational change of each acto-myosin that accumulates along the filaments and promotes their twisting.

Ménétret J-F, Hofmann W, Schröder RR, Rapp G, Goody RS. *J. Mol. Biol.* 1991 219(2):139-44

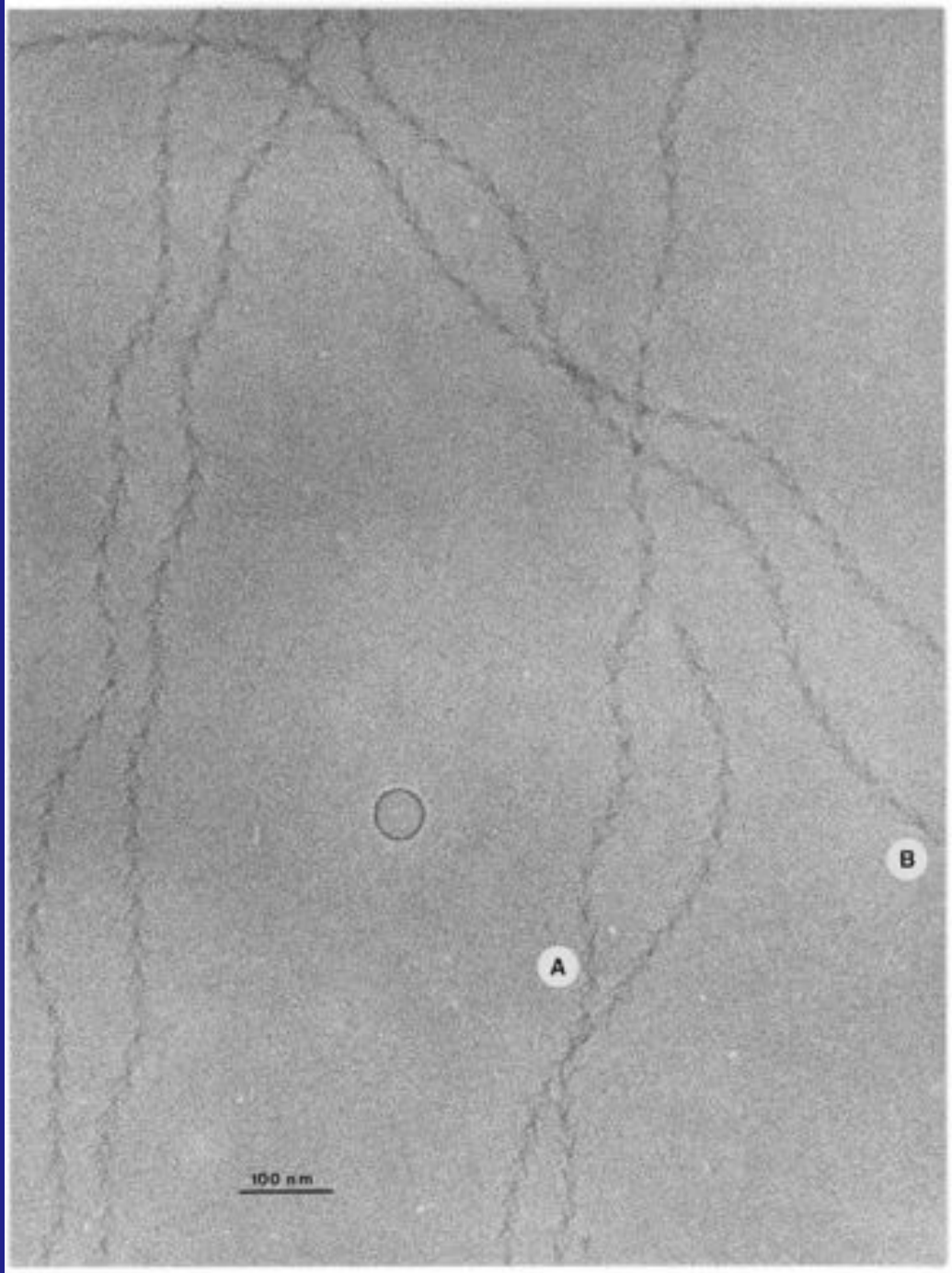


Figure 18.

6) 2D crystals

- One layer-thick crystals are sugar-embedded (trehalose or glucose) and frozen in liquid N2 on a very flat carbon film support.
- The amplitude information is collected using **electron diffraction** and the phase information is calculated from the **images** (illustrated next slide).
- The crystals are tilted to collect 3D information.

6) 2D crystal of soluble proteins on lipid monolayer

What are the advantages of orienting soluble proteins on a lipid monolayer ?

1) to obtain higher resolution information using electron crystallography.

2) to orient the particles in preferred orientations; this could help identify conformational changes.

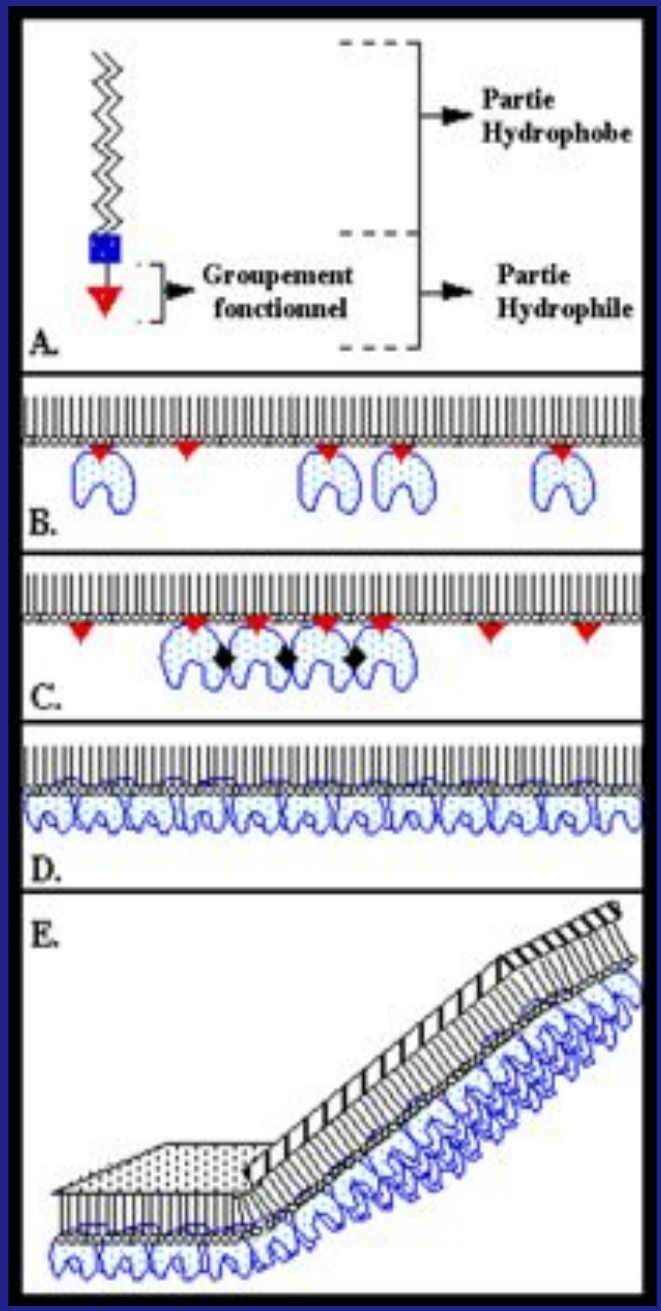
6) 2D crystal of soluble proteins on lipid monolayer

A. adsorption driven by lipid-protein interactions

B. concentration and orientation under the monolayer

C. and D. organisation in 2D crystals

E. picking-up the 2D crystal with a grid



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EM of biological components

	shadowing	Negative staining	Cryo-EM	2D crystal
preservation	bad	good … but	very good	very good
hydration	dry	dry	fully hydrated	hydrated
contrast	very high	high	low	low
aggregation level	single	single	single	crystals
Resolution limit in Å	30	20	6	3
Dynamic studies	no	yes	yes	May be
difficulty	difficult	difficult	very difficult	very difficult

Thank you for your attention