New approaches to substrates and specimen preparation

Workshop on Advanced Topics in EM Structure Determination: Challenges and Opportunities.
October 29 - November 3, 2017

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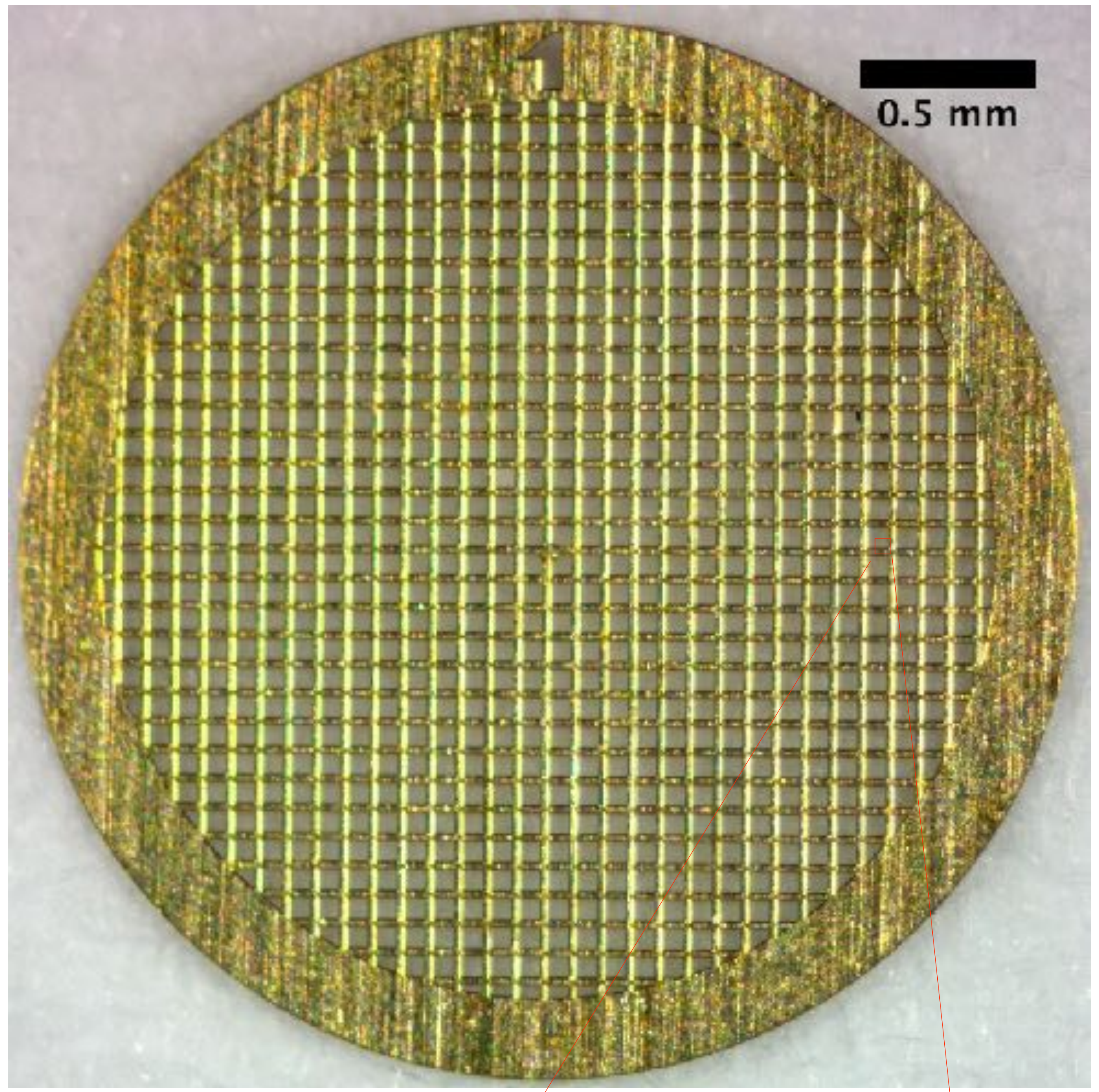
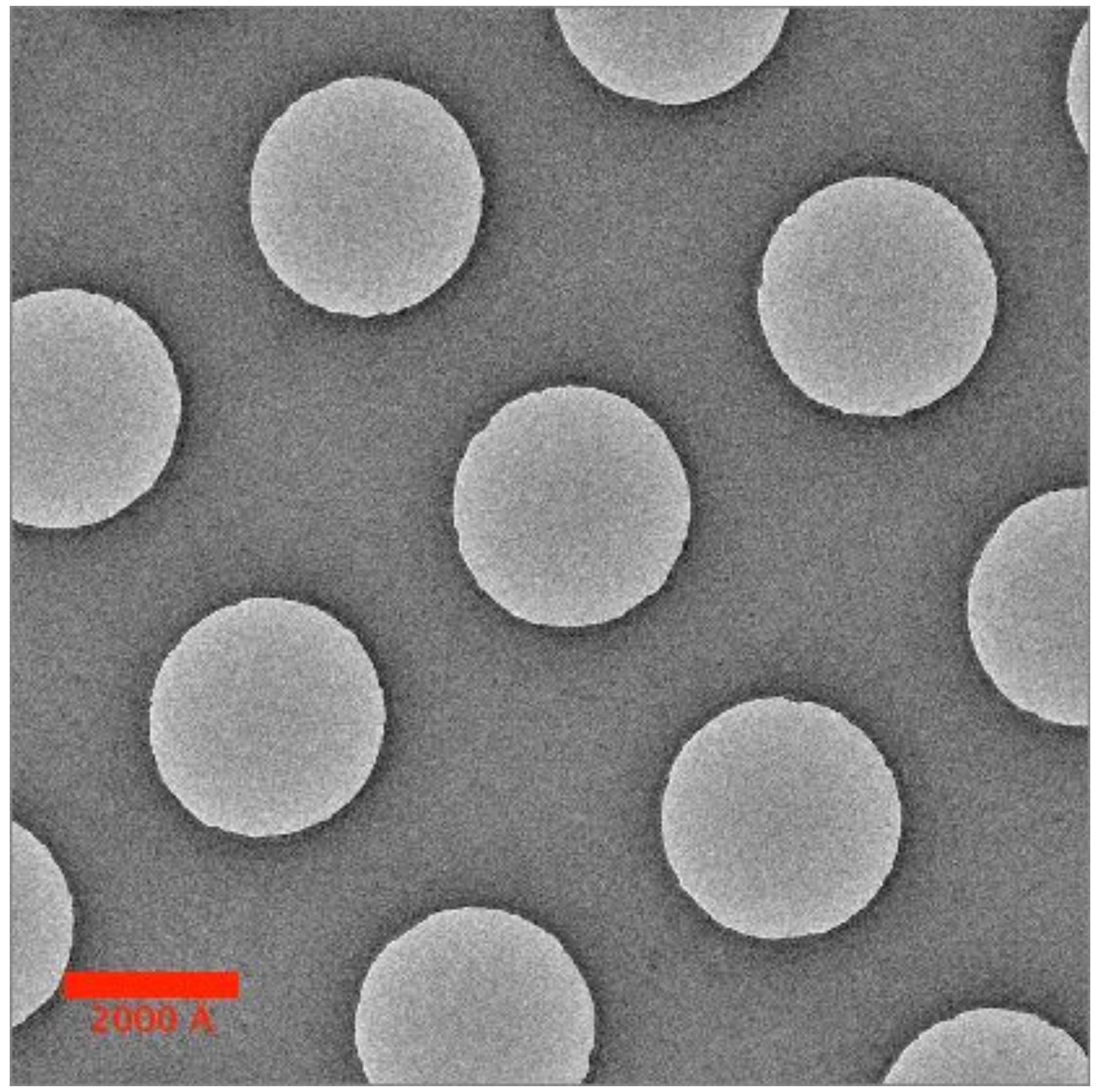
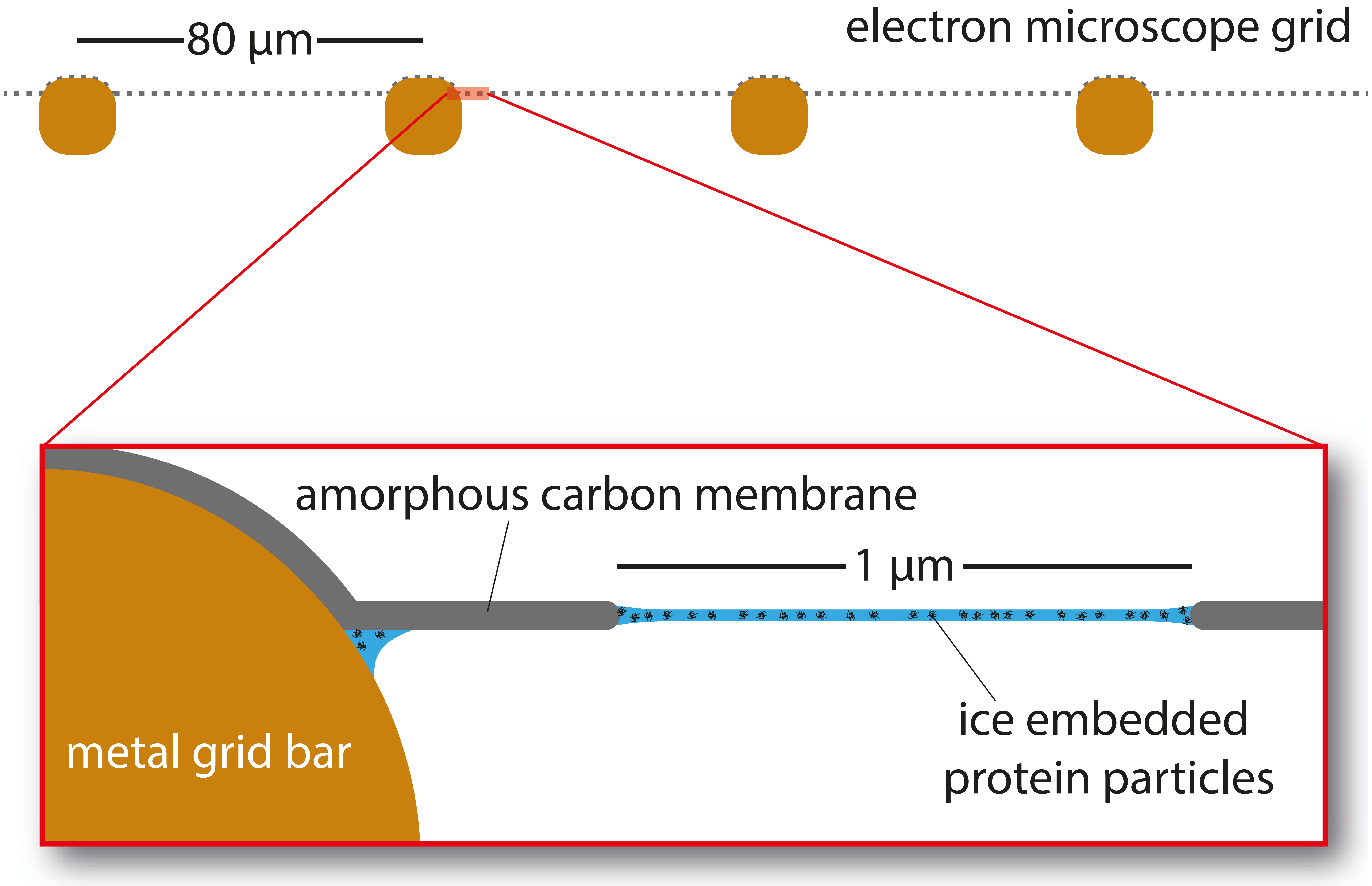
MRC Medical Research Council

• Are there treatments that can be applied to thin carbon that are advantageous?	*yes*
• Should we be using carbon at all?	*<no|yes*>*
• Are there new substrates that offer advantages over the traditional thin carbon?	*yes*
• Can we envision improving on these further using surface treatments?	*yes*

Standard cryo-EM specimen







First Biological EM
Drosera intermedia
on copper "net"

L. Marton
1934

For liquid hydrogen iodide we find a Raman line of a very diffuse character. As in the case[2] of hydrogen chloride and bromide, it has a complex structure. The wave numbers in the liquid and solid state as compared with the gaseous state are given in the accompanying table. The structure of the line in the liquid may be seen from Fig. 1. The points marked on the curve have the following wave numbers: *a*, 2178 cm.$^{-1}$; *b*, 2167 cm.$^{-1}$; *c*, 2165 cm.$^{-1}$; *d*, 2162·5 cm.$^{-1}$; *e*, 2151 cm.$^{-1}$. It may be emphasised that the structure and the wave numbers are not very accurately known.

From these results, it will be seen that although the low temperature apparatus already used is quite adequate for our purpose, a spectrograph of higher dispersion will be necessary if we are to carry this investigation further. We have therefore begun to construct a new type of spectrograph with a liquid prism.

H. EPSTEIN.
W. STEINER.

Physikal. Chem. Institut d. Universität, Berlin.
Laboratory of Physical Chemistry, Cambridge.
May 2.

[1] Grassmann, *Z. Phys.*, **82**, 767; 1933.
[2] E. O. Salant and A. Sandow, *Phys. Rev.*, **37**, 373; 1931. E. O. Salant and D. Callihan, *Phys. Rev.*, **43**, 590; 1933.

Magnetic Moment of the Deuton

IN a previous note[1] we reported, together with Mr. Frisch, on experiments concerning the deflection of a beam of 'ordinary' hydrogen molecules in an inhomogeneous magnetic field. From these experiments, we were able to derive the magnetic moment of the proton. The value obtained was 2·5 nuclear magnetons (not 1, as expected theoretically).

by contact with an extremely thin metal foil which is cooled by conduction).
(2) Impregnating the object with a substance which makes the object less destructible.
(3) Impregnating the object in such a way that a framework of the object is preserved although the object itself is destroyed.
(4) Combining methods (1) and (2), or (1) and (3).

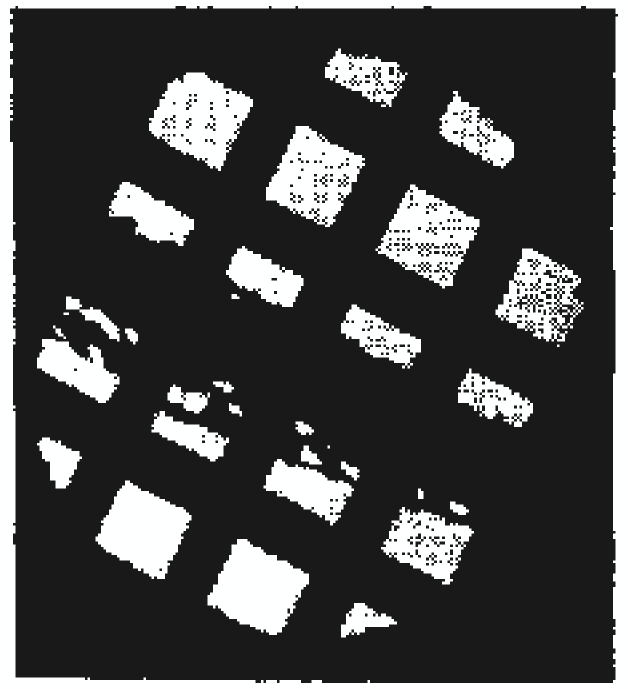
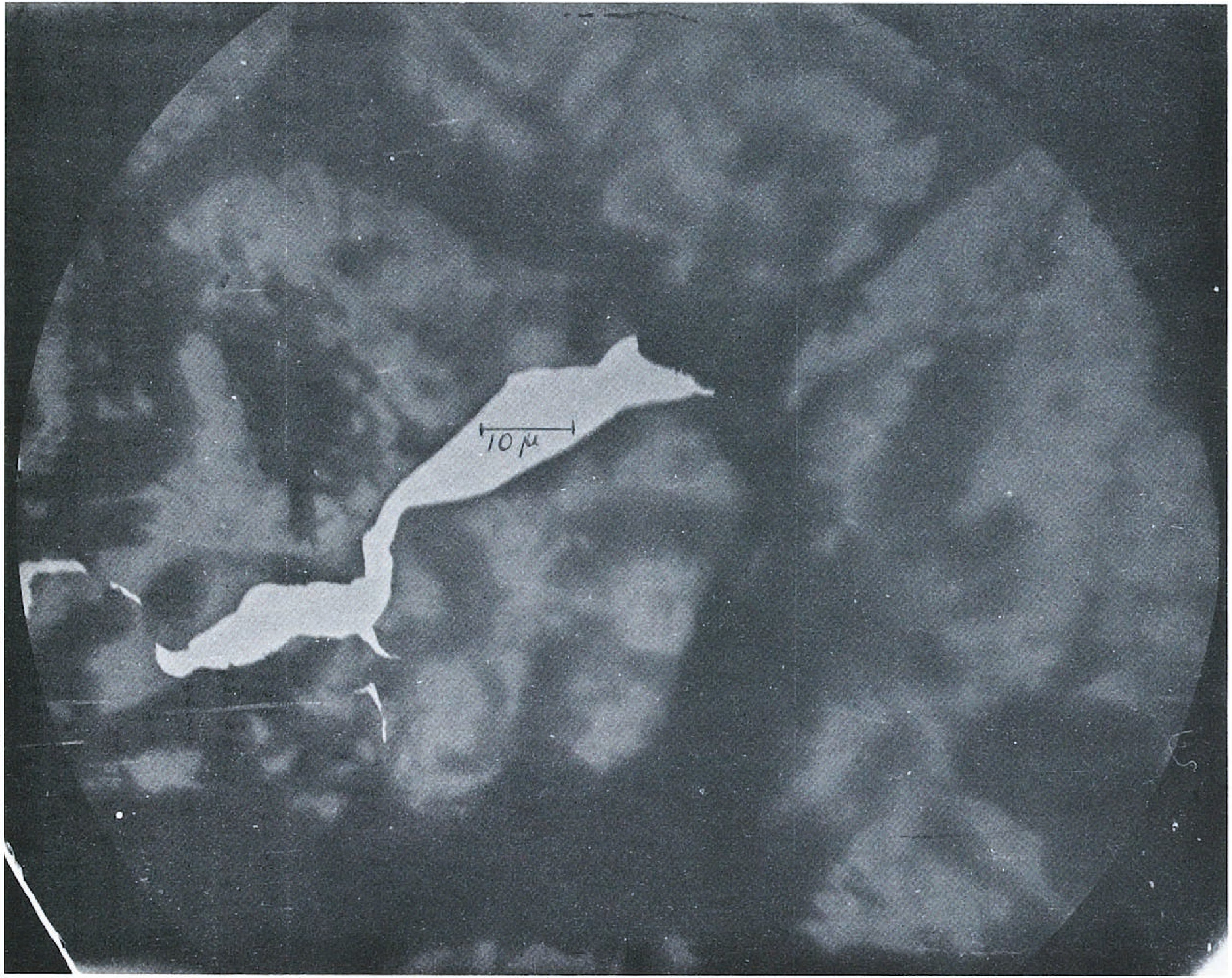


FIG. 1. × 65.

We obtained the best results by using the third method. To arrive at good results by this method the following conditions must be satisfied by the metallic or other framework: It must be (*a*) geometrically similar to the object; (*b*) of high melting point and good thermal conductivity; and (*c*) of high atomic weight.

Root of *Neottia nidus avis*
on torn collodion foil.

L. Marton
22 Jaunary 1936



First amorphous carbon films for EM

D. E. Bradley
1953

Evaporated carbon films for use in electron microscopy

By D. E. BRADLEY, Research Laboratory, Associated Electrical Industries Ltd., Aldermaston, Berks.

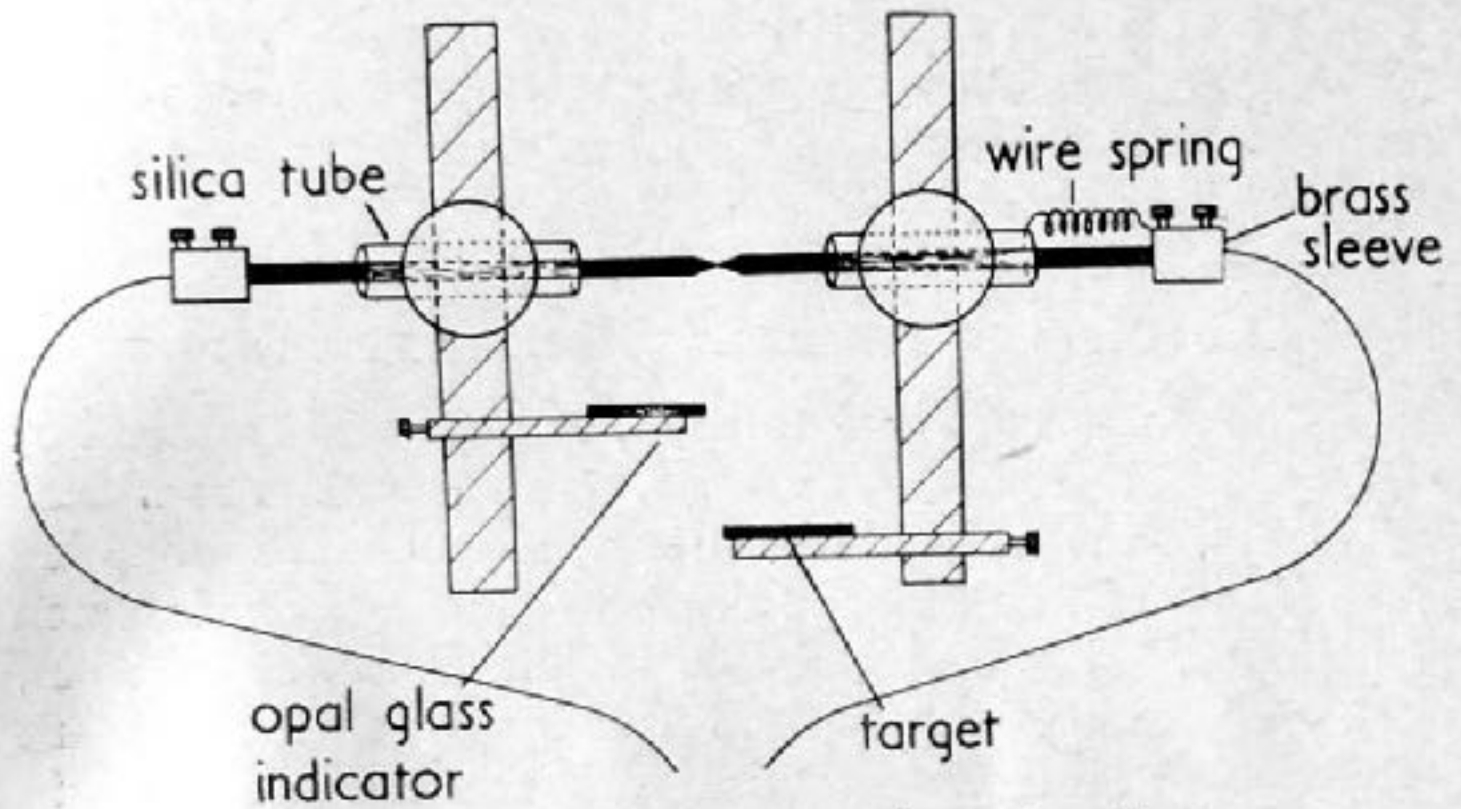
[*Paper received* 5 *August*, 1953]

A method has been developed for producing carbon films suitable for electron microscope specimen supports. Carbon is evaporated on to an extremely soluble substrate, which is dissolved away leaving very thin films.

It has been found possible to evaporate carbon to form uniform amorphous films suitable for electron microscope specimen supports. These films are exceptionally strong and can therefore be made extremely thin, so that, since they have a low atomic number, they are highly transparent to electrons.

The method of evaporation is to pass an alternating current of between 20 and 50 A through two pointed carbon rods in a vacuum chamber, with the points held lightly together so that they are not parted during the process. Intense local heating occurs in the region of contact.

The apparatus used is illustrated in the figure. The 0·5 cm diameter carbon arc rods are supported in 0·6 cm internal diameter silica tubing held in bosses screwed to two upright rods. To maintain a slight pressure between the pointed



Apparatus for the evaporation of carbon

ends, one electrode is fixed, and a small spring made from 0·01 in. tantalum or tungsten wire is fitted between the terminal on the other rod and a small hole blown in the silica

found to be Bedacryl 122 X, supplied in a 40% solution in Xylene or in solid form by Imperial Chemical Industries Ltd. Boron oxide or glycerol can also be used and the film mounted from a water surface, but the thinner films break with these substrates.

To coat the target slide, the Bedacryl is first diluted with redistilled benzene to a strength of 6–8% weight/volume. A quantity is then poured over a clean microscope slide and allowed to drain off. The film of resin dries in a few seconds under a lamp.

After evaporation, the film is scored into small squares and floated on to a water surface. Stripping is made easier by breathing heavily on to the film, as with formvar, and some teasing at the edges may be necessary, otherwise Bedacryl strips very easily.

A specimen grid is now slightly bent in such a way that it lies on a flat topped peg supported at the edges only. Alternatively, a peg with a convex top can be used and the bending can be omitted. The bending of the grid or the use of a round-topped peg prevents contact of the film with the surface of the peg over a large area where the film would otherwise subsequently break.

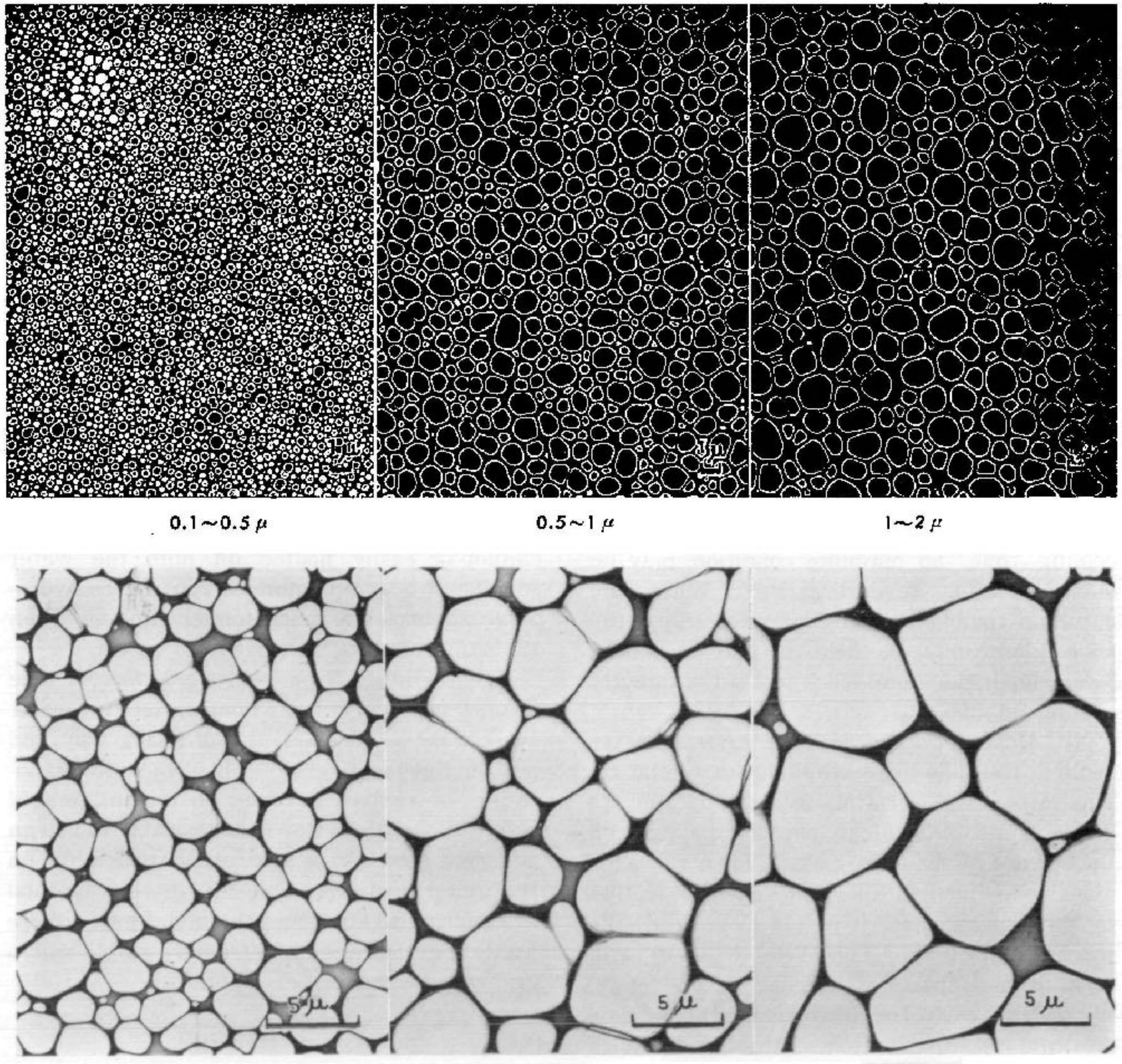
The floating squares are picked up with a lifter consisting of a bent metal strip with a $\frac{3}{16}$ in. diameter hole in it. They are then inverted over the grids mounted on the pegs in the order: grid, carbon, Bedacryl. If the hole in the lifter is too small, the resin film may break.

At this stage, it has been found necessary to bring the carbon into intimate contact with the grid surface, otherwise the thin film will float off in subsequent washing. This is brought about by rendering the Bedacryl tacky, and then drying it. Thus, four or five drops of methylated spirits are allowed to fall on the specimen and surplus liquid is removed

Patterning plastic with holes for EM

Fukami and Adachi 1965

116 Akira FUKAMI and Kôichi ADACHI (34)



0.1~0.5 μ 0.5~1 μ 1~2 μ

2~3 μ 3~6 μ 6~10 μ

Fig. 2. Self-perforated micro grids with various hole sizes.

Published September 1, 1962

'Graphene'
for EM

Beer
1962

A SIMPLE PREPARATION OF GRAPHITE-COATED GRIDS FOR HIGH RESOLUTION ELECTRON MICROSCOPY

MICHAEL BEER and PETER J. HIGHTON. From the Department of Biophysics, Johns Hopkins University, Baltimore, Maryland

High resolution electron microscopy requires that the specimen supports be smooth. With this in mind a procedure was developed for cleaving single graphite crystals into thin flakes suitable as specimen supports. Fernández-Morán (1) was the first to use graphite and mica crystals for specimen supports. He as well as Sprague (2) obtained the thin sheets of mica and graphite required through successive cleavages of single crystals by attaching adhesives or plastics to each side of the crystal and then pulling these apart. We have found the complete removal of the adhesive virtually impossible. In this note we report a method in which no adhesives are used, and smooth, clean, thin graphite films are readily obtained.

Single crystals of graphite are obtained from marble embedded with graphite (2). This is mined in Essex County, New York, and can be purchased through Ward's Natural Science Establishment, Inc., Rochester, New York. Concentrated nitric acid dissolves the marble, leaving the graphite and a small amount of quartz. This residue is washed in tap water. A drop of clean ethanol is put on a clean, glass microscope slide. A good, unfragmented, smooth, graphite crystal is placed on the drop of ethanol. Another slide is placed over this, and the sandwich is lowered into liquid nitrogen and left there until the rapid boiling ceases. The glass-graphite sandwich is then removed and the glass slides are forced apart. It is found that the frozen ethanol acts as an adhesive and the graphite is cleaved, leaving part of the crystal attached to each glass slide. The glass slides are allowed to warm up, the condensed water removed, another drop of ethanol placed on each crystal, and the procedure repeated. Successive splittings are continued until at least a portion of the crystal is sufficiently thin to be light grey. Such flakes are then floated off on water after much of the ethanol has evaporated, but before the condensed water has completely dried off. The graphite flakes then can be picked up on grids coated with polybutene (3). During one afternoon perhaps two dozen graphite-coated grids can be prepared with a little practice. Light grey flakes are thin enough for high resolution microscopy. The adsorption properties appear to be similar to those of evaporated carbon and the graphite grids can be used for the recovery of long DNA molecules in a manner similar to that described previously (4). Quantitative aspects of the improvement in background are now under investigation.

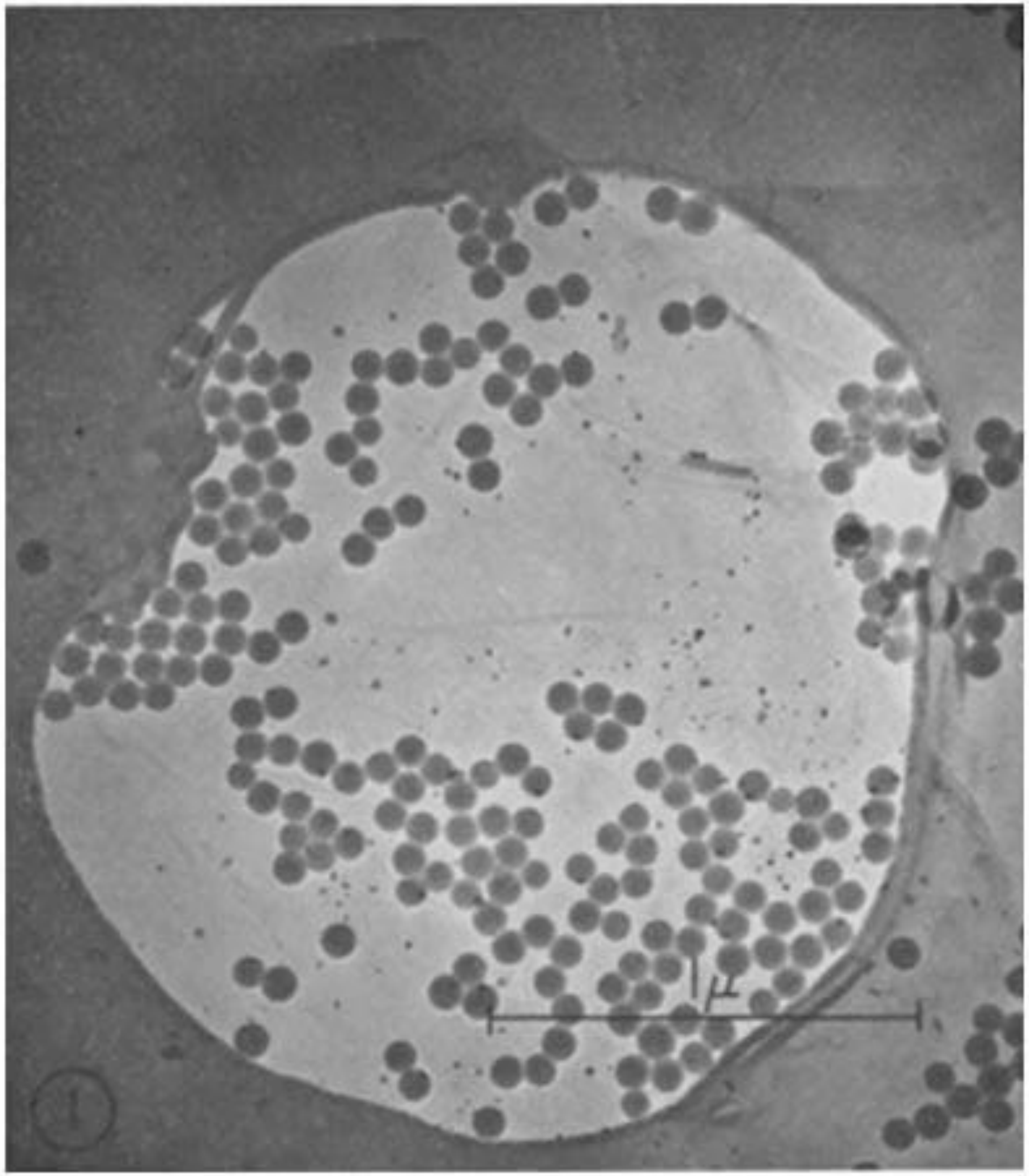
Received for publication, December 5, 1961.

REFERENCES

1. Fernandez-Moran, H., *J. Appl. Physics*, 1960, **31,** 1840.
2. Sprague, R., 1960, personal communication.
3. Barton, A. A., *Stain Technol.*, 1960 **35,** 287.
4. Beer, M., and Zobel, C. R., *J. Mol. Biol.*, 1961, **3,** 717.

Reduced 'graphene oxide'
on lacey carbon

Dobelle & Beer
1968




Jacques Dubochet

32 ARTICLES NATURE VOL. 308 1 MARCH 1984

Cryo-electron microscopy of viruses

Marc Adrian, Jacques Dubochet, Jean Lepault & Alasdair W. McDowall

European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg, FRG

Thin vitrified layers of unfixed, unstained and unsupported virus suspensions can be prepared for observation by cryo-electron microscopy in easily controlled conditions. The viral particles appear free from the kind of damage caused by dehydration, freezing or adsorption to a support that is encountered in preparing biological samples for conventional electron microscopy. Cryo-electron microscopy of vitrified specimens offers possibilities for high resolution observations that compare favourably with any other electron microscopical method.

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

Printed in Great Britain

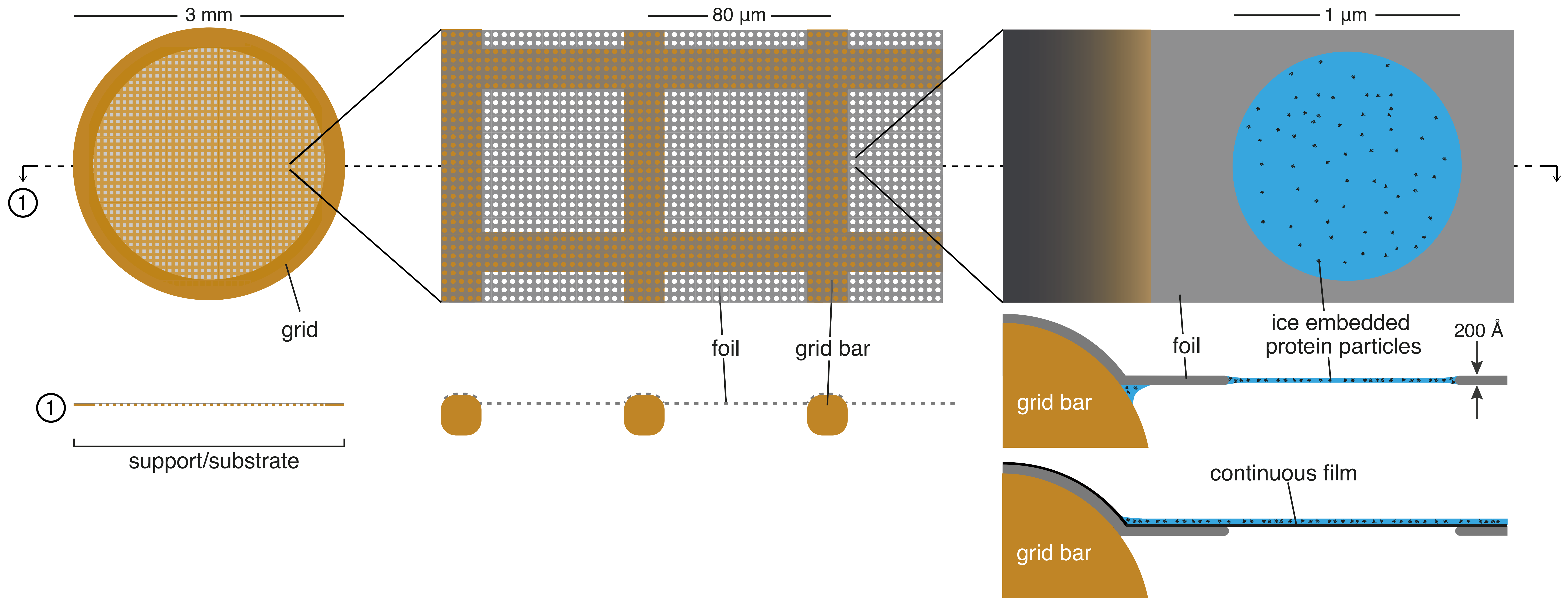
Cryo-electron microscopy of vitrified specimens

Vitreous = glass, amorphous
NOT crystalline

JACQUES DUBOCHET[1], MARC ADRIAN[2], JIIN-JU CHANG[3], JEAN-CLAUDE HOMO[4], JEAN LEPAULT[5], ALASDAIR W. McDOWALL[6] AND PATRICK SCHULTZ[4]

European Molecular Biology Laboratory (EMBL), Postfach 10. 2209, D-6900 Heidelberg, FRG

Types of specimen supports



Grid materials

Copper
Nickel
Titanium
Silicon

Gold
CuRh
Molybdenum
Aluminum
Tungsten

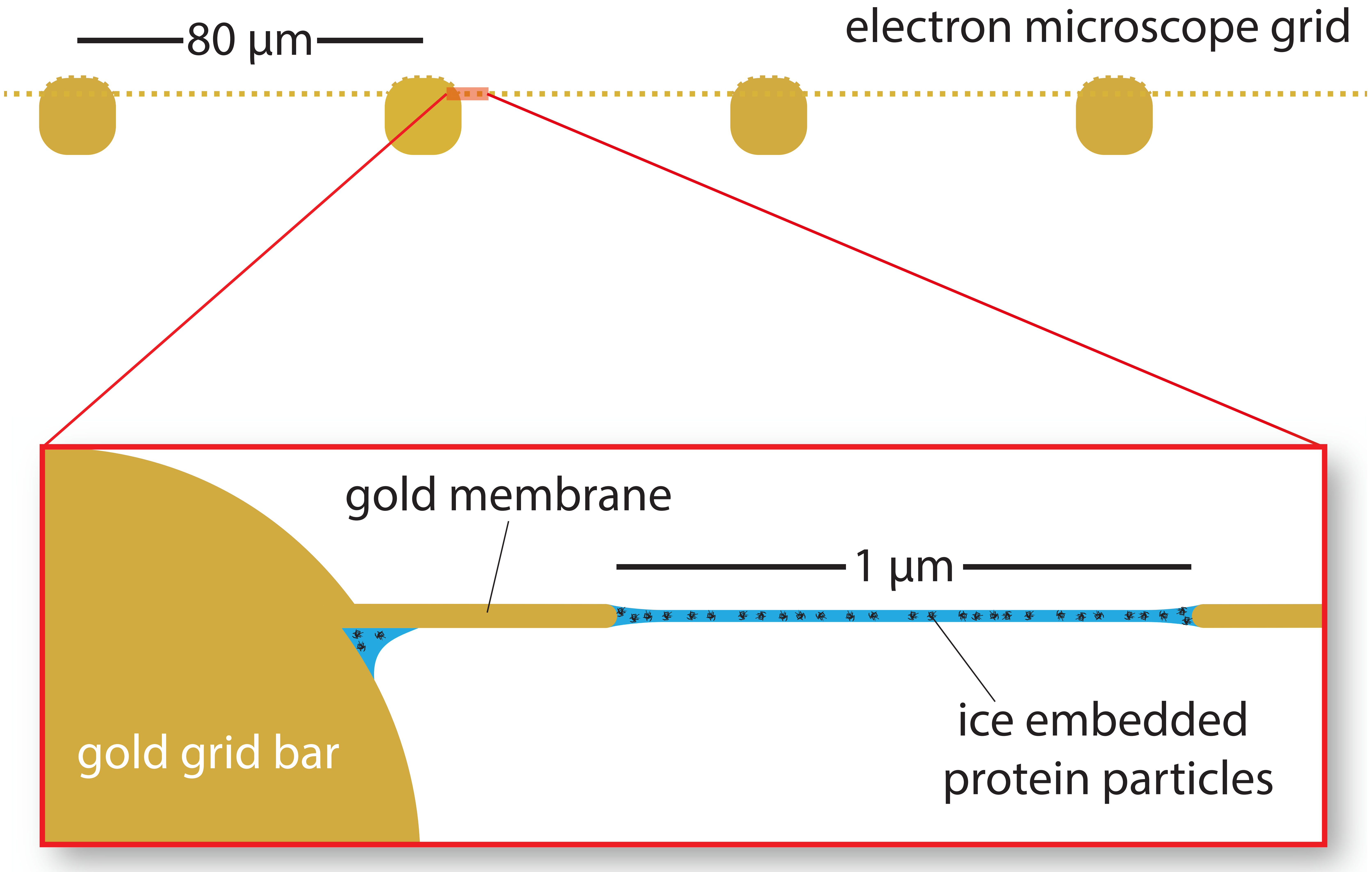
Foil materials

Amorphous carbon
Gold
TiSi SiN
SiO_2 SiC

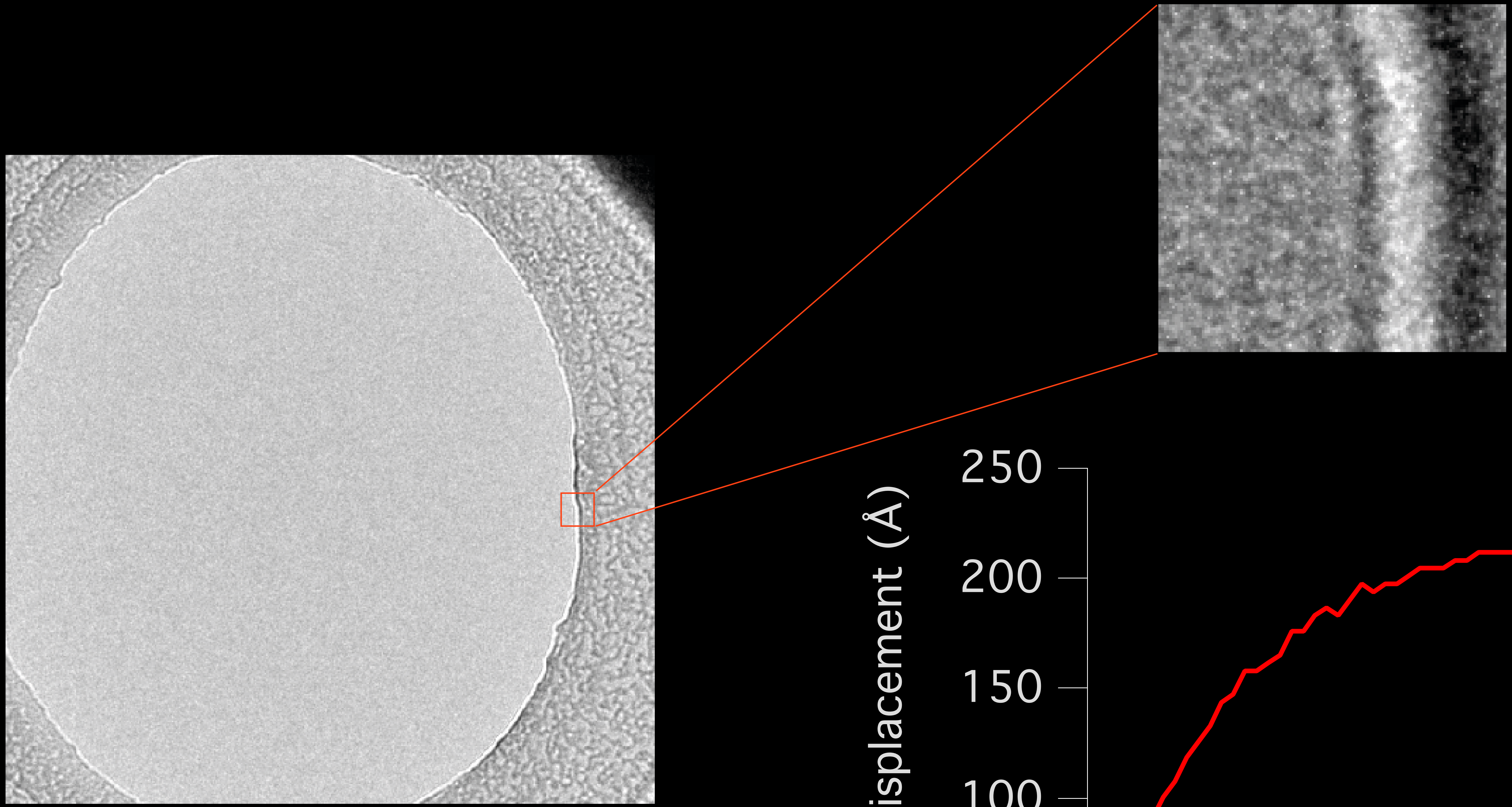
Film materials

Amorphous carbon
Graphene
Graphene oxide
TiSi
SA 2D crystal
& others

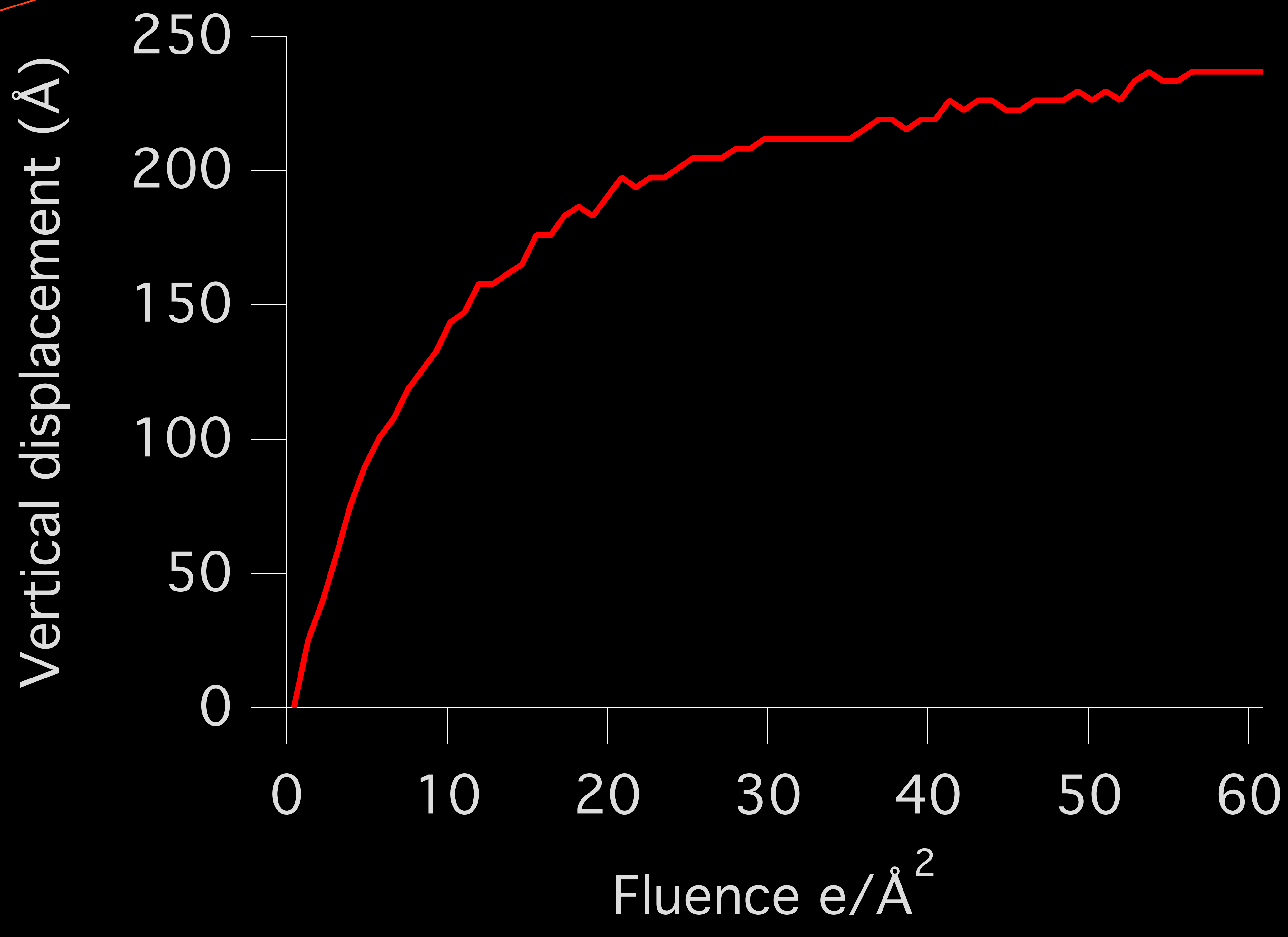
Passmore & Russo MiE 2016


80 µm
electron microscope grid
gold membrane
1 µm
ice embedded
protein particles
gold grid bar

Grid vertical movement



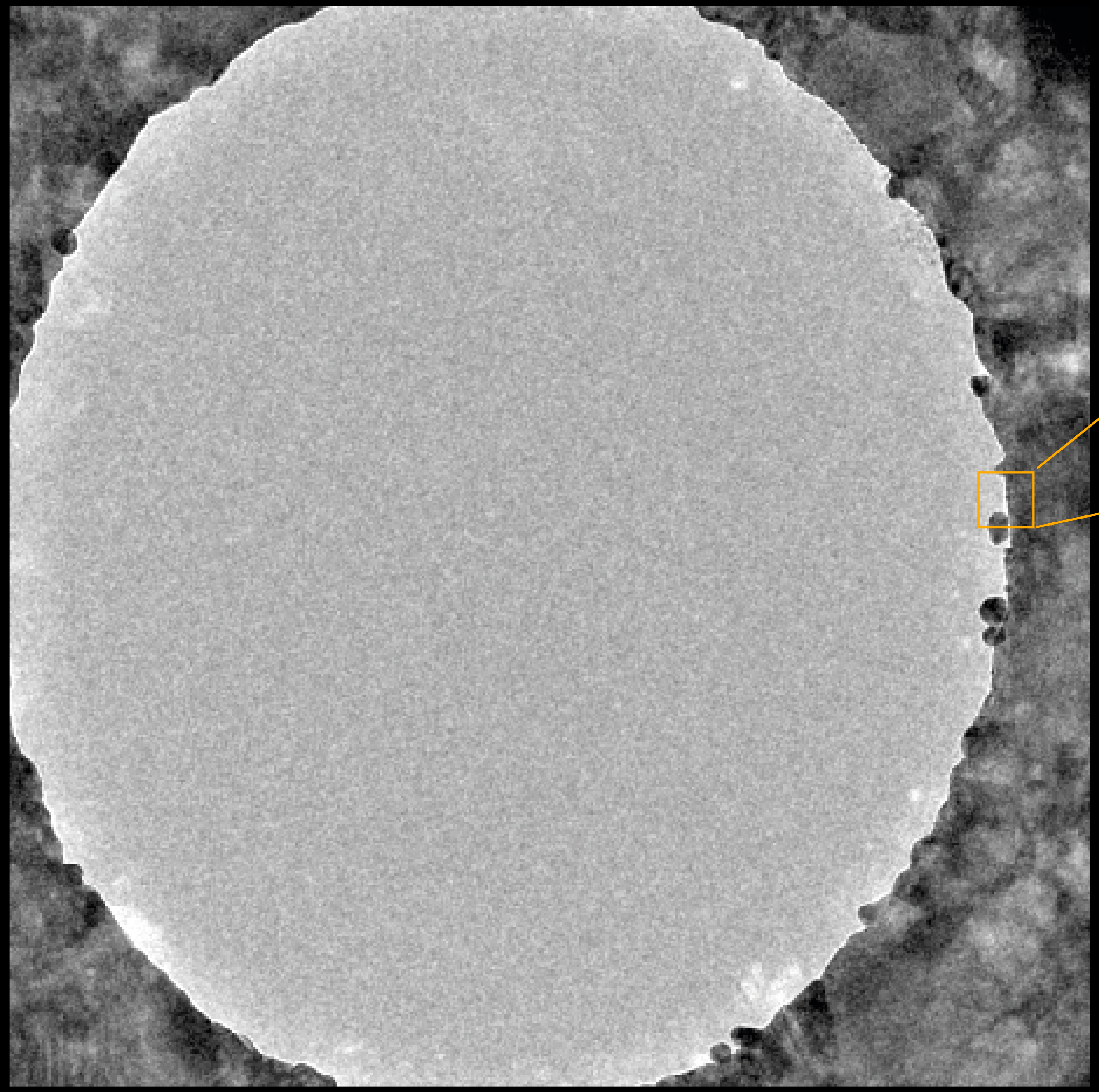
Quantifoil

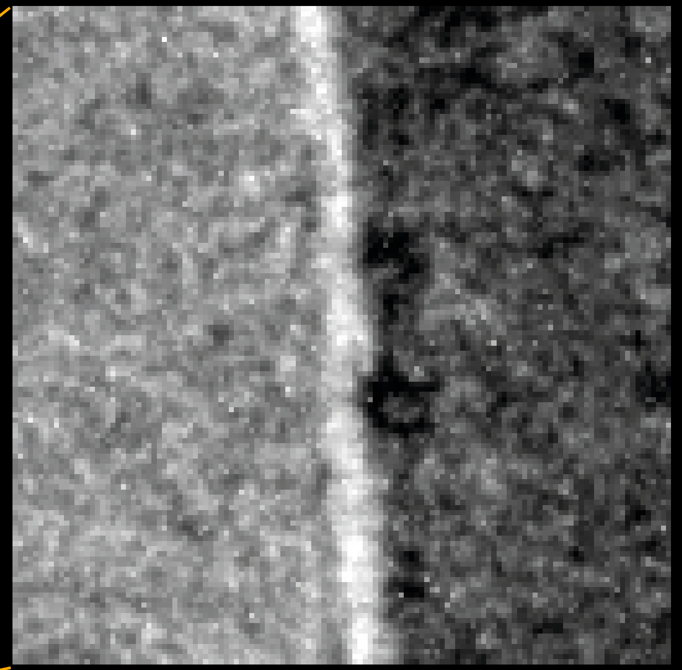


Russo & Passmore *Science* 2014

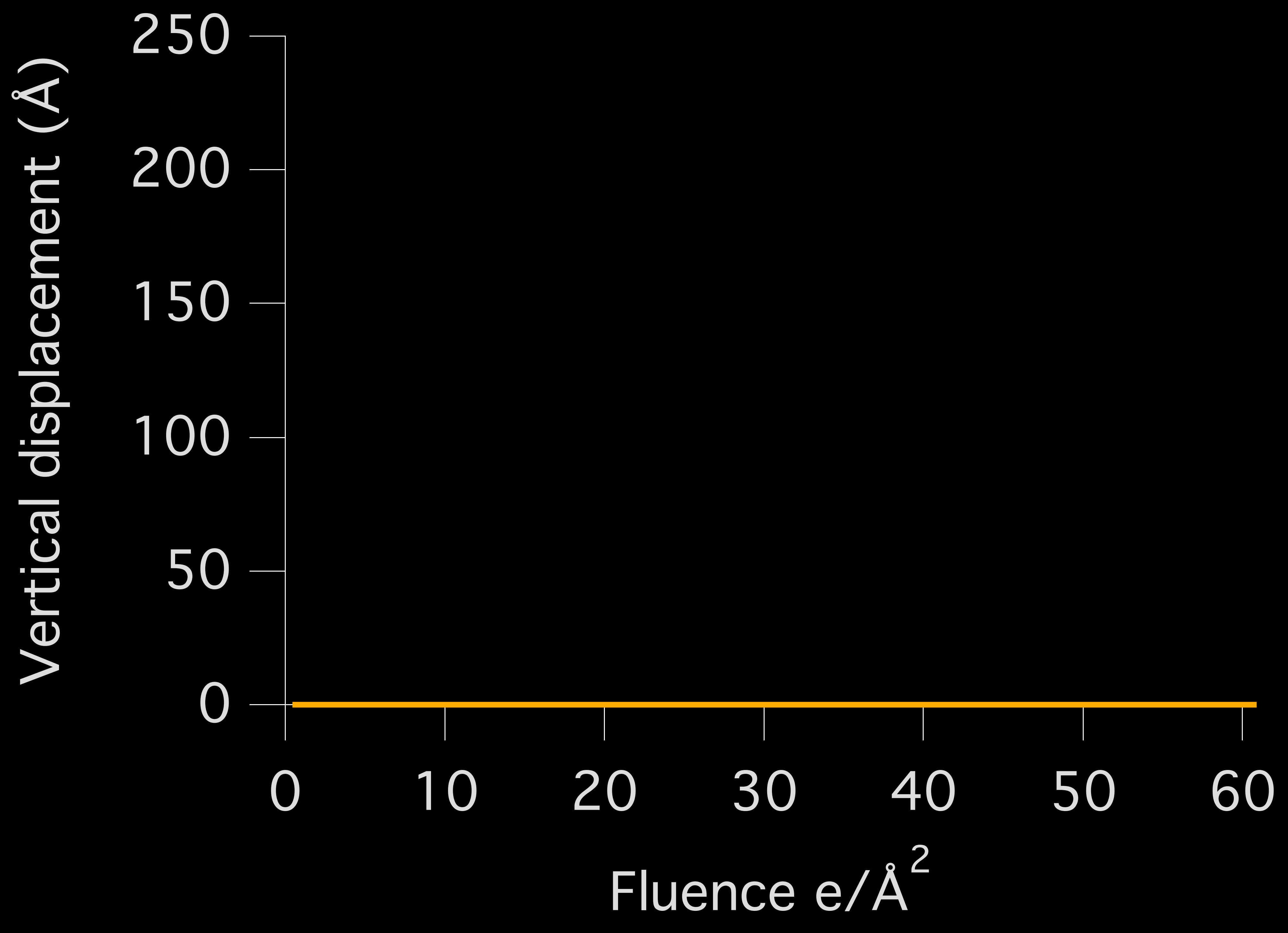
16 e/Å^2/s
30° tilt
real time

Grid vertical movement





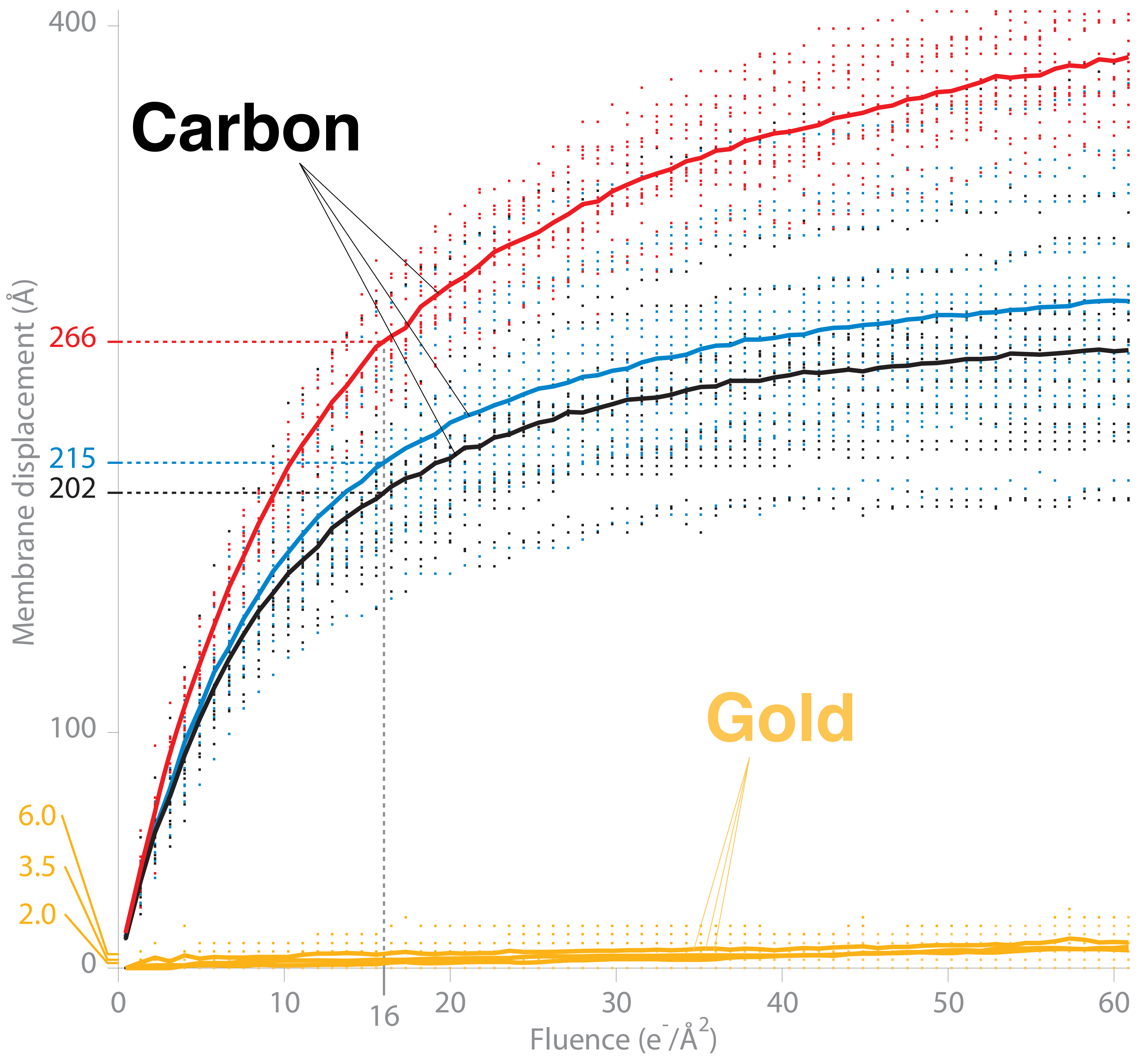
Gold



16 e/Å²/s
30° tilt
real time

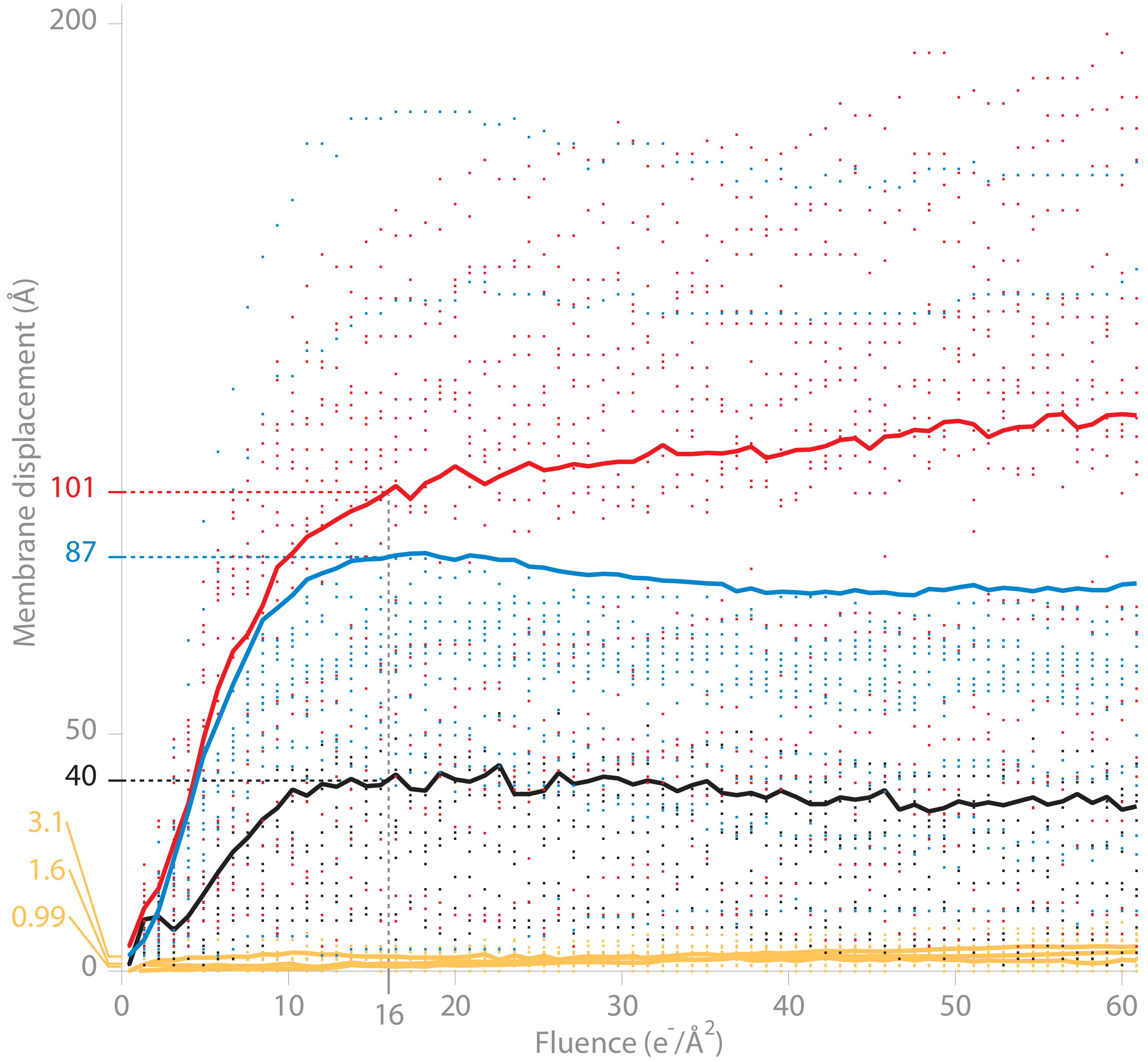
Russo & Passmore *Science* 2014

No ice



228 Å vs. 3.8 Å

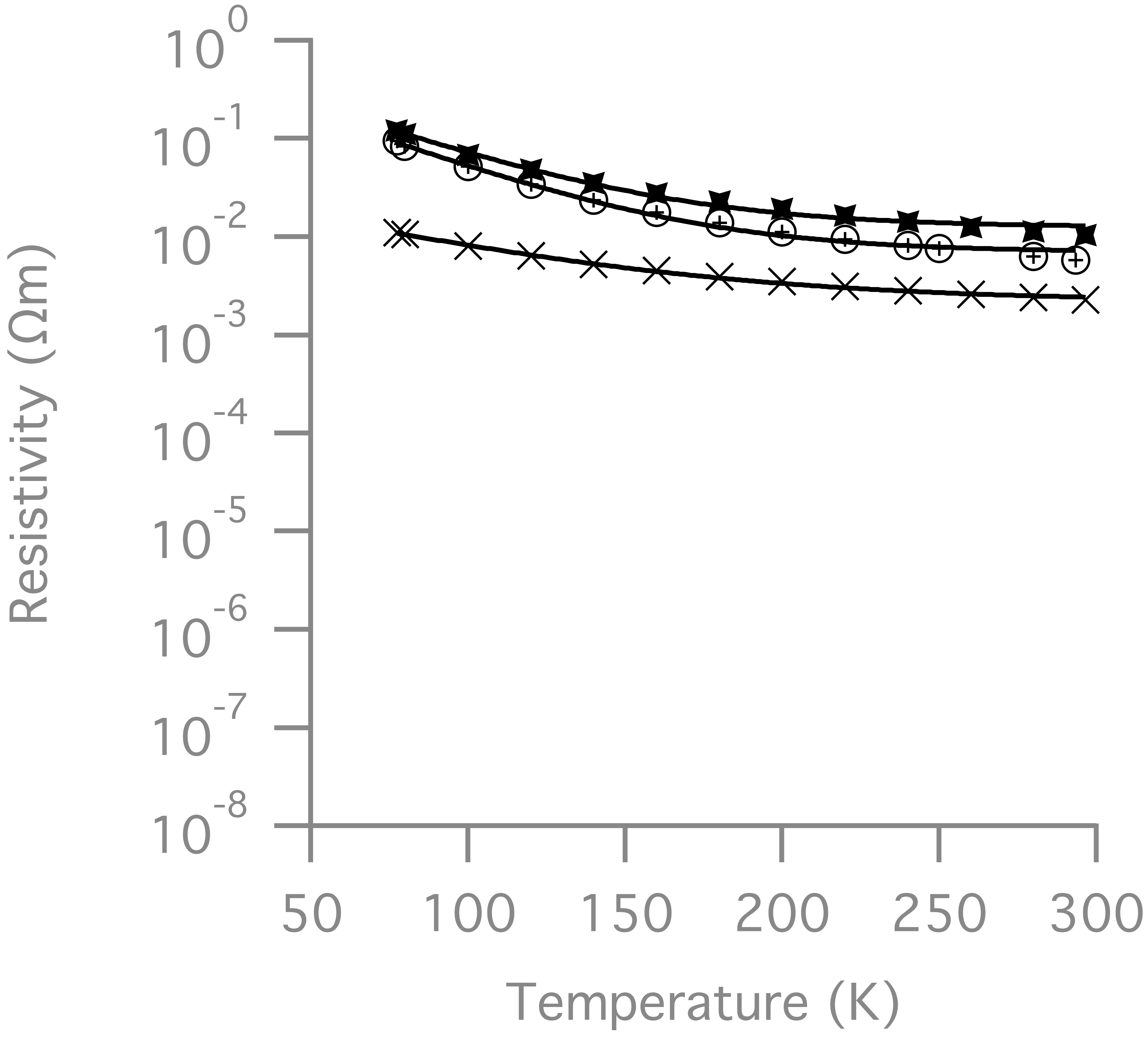
With ice



76 Å vs. 1.9 Å

Russo & Passmore *Science* 2014

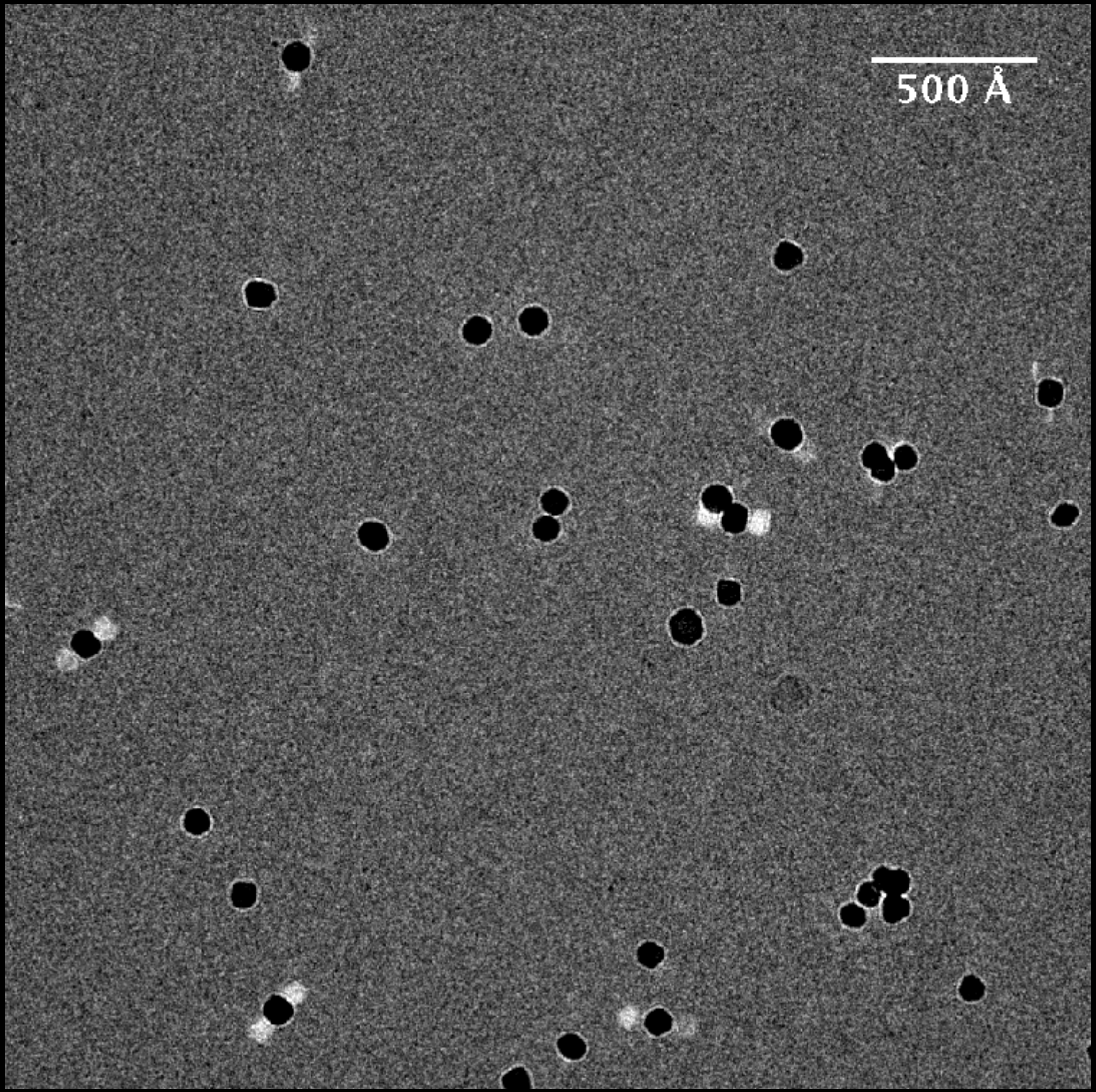
Thin film resistivity measurements



Carbon

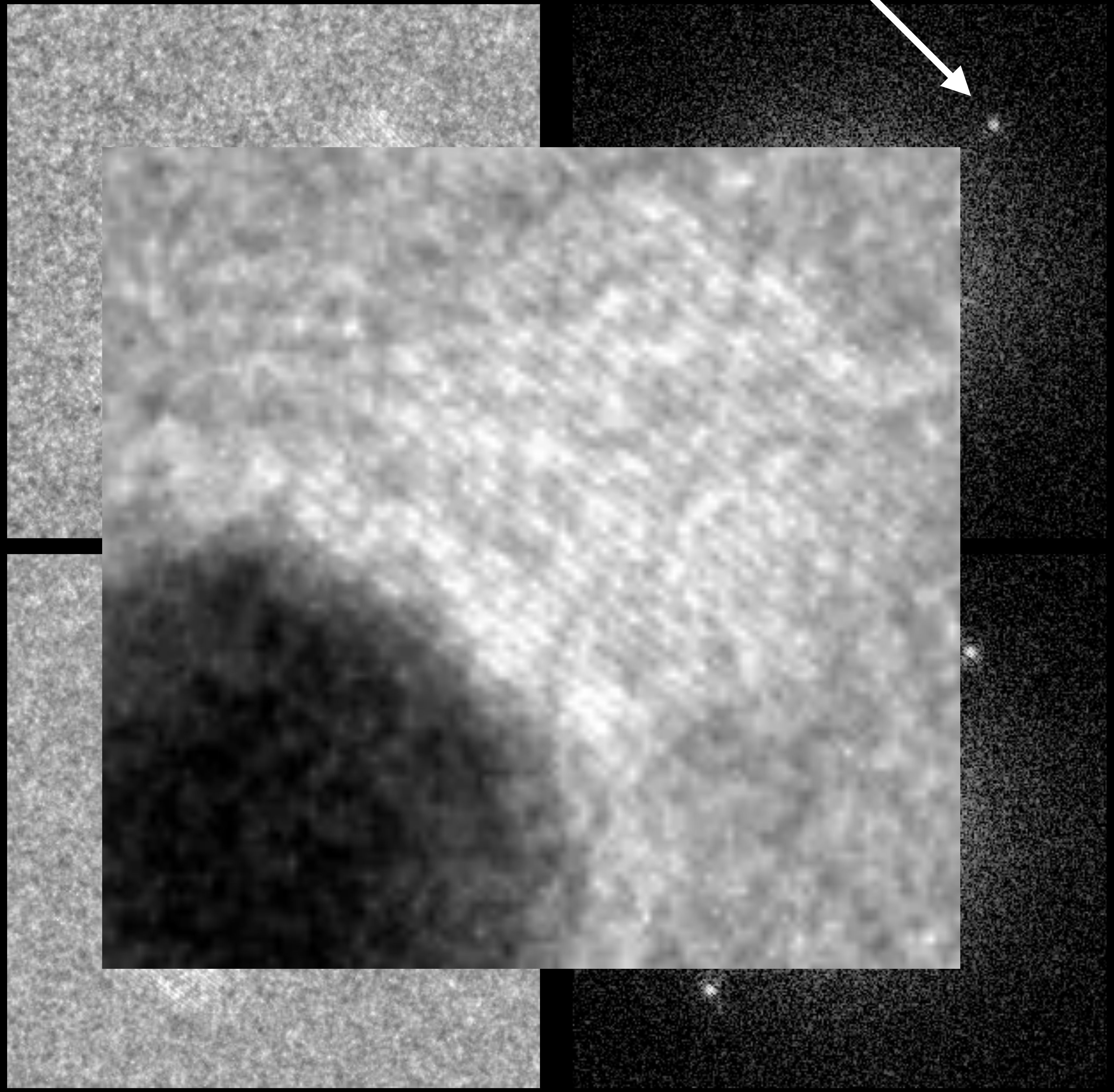
Russo & Passmore 2016a

Seeing atoms in ice



100 Å gold particles in ice

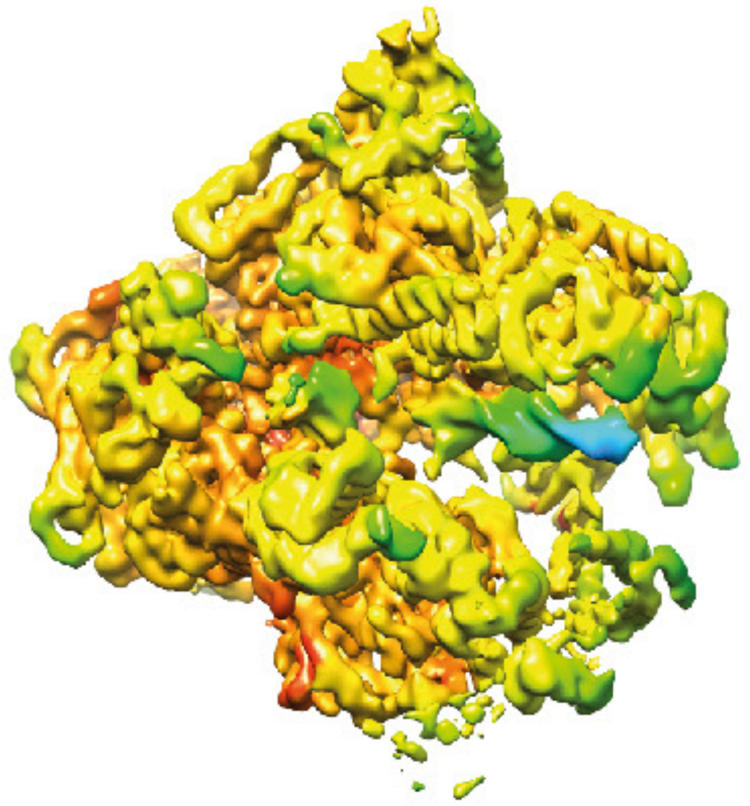
gold 111
2.35 Å



300 keV 20 e⁻/Å² Polara Falcon 3
Raw micrograph no motion correction

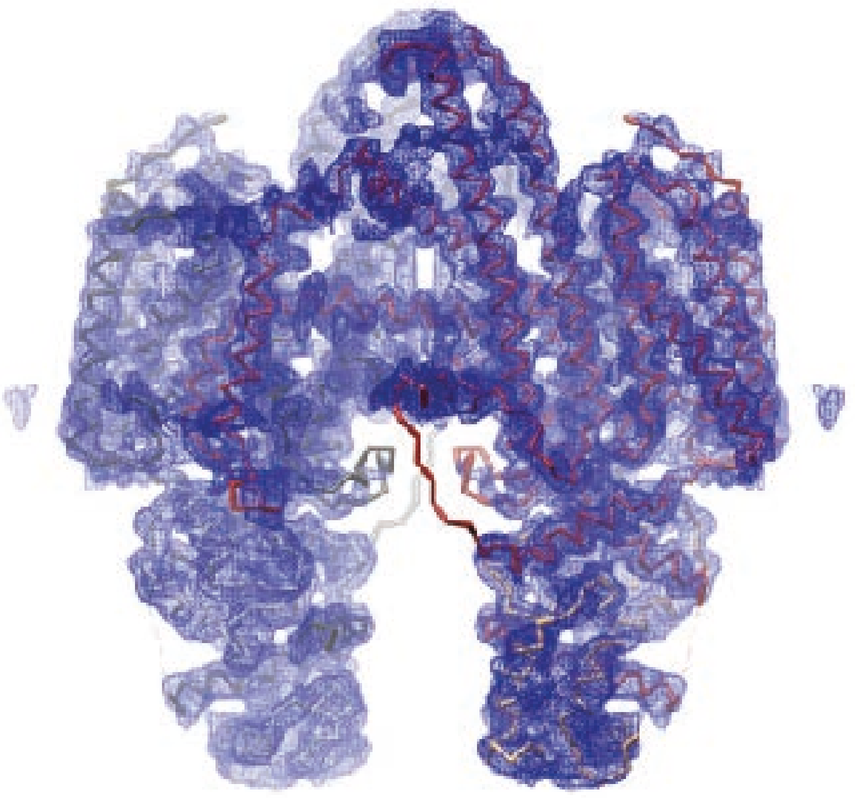
Pol I
3.8 Å

Neyer et al. 2016



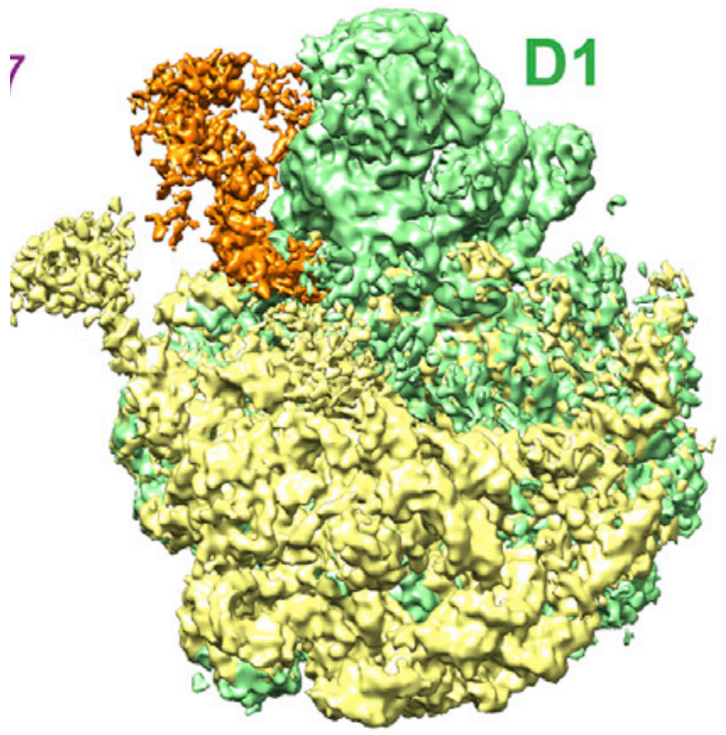
STRA6
3.9 Å

Chen et al. 2016



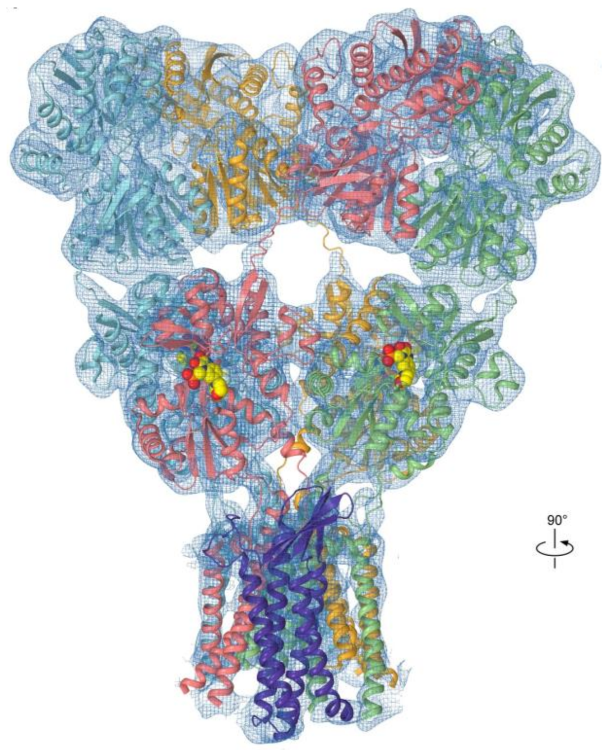
60S ribo
4.4 Å

Davis et al. 2016



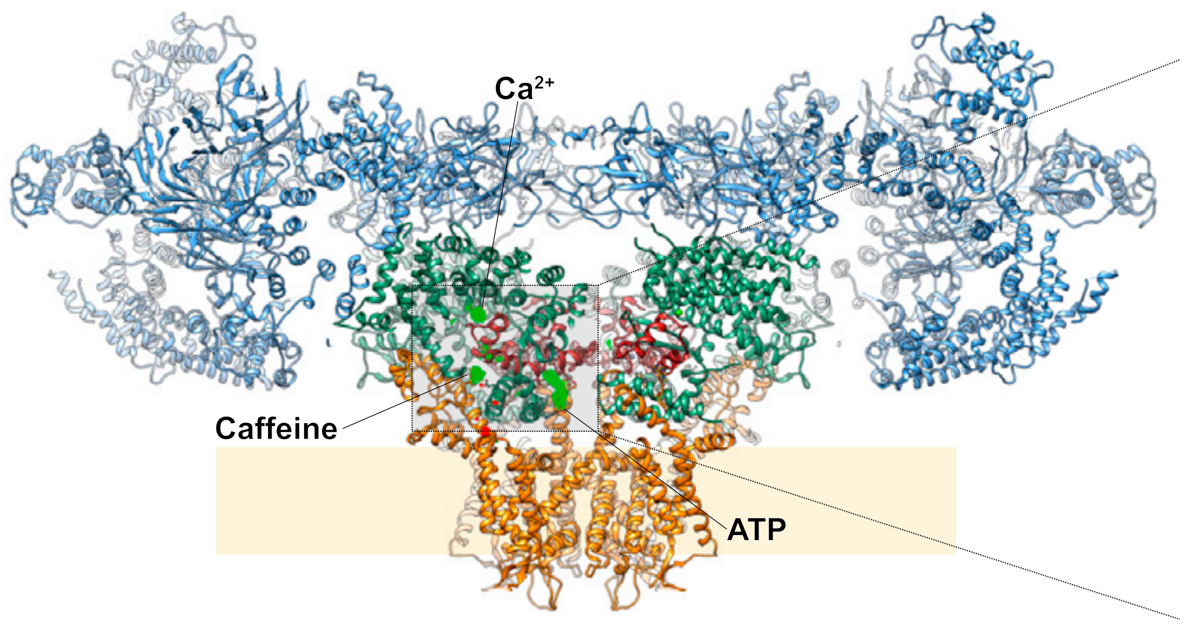
AMPA receptor
5.6 Å

Twomey et al. 2016



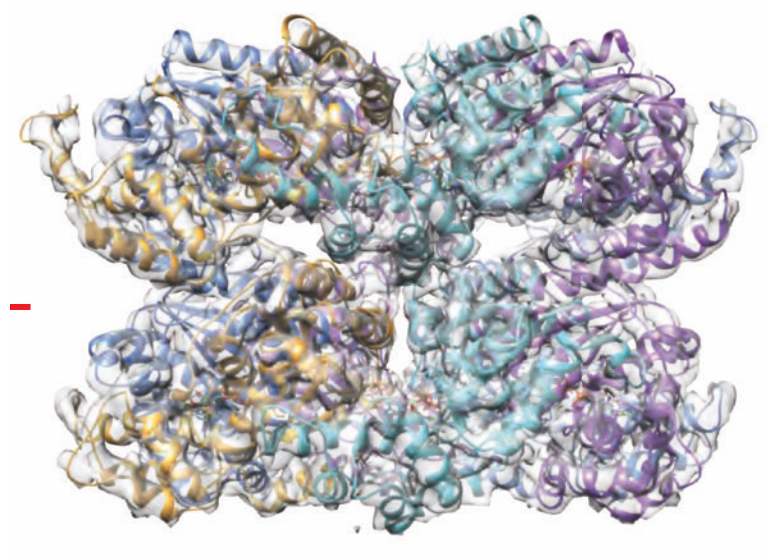
RyR1
3.6 Å

des Georges et al. 2016



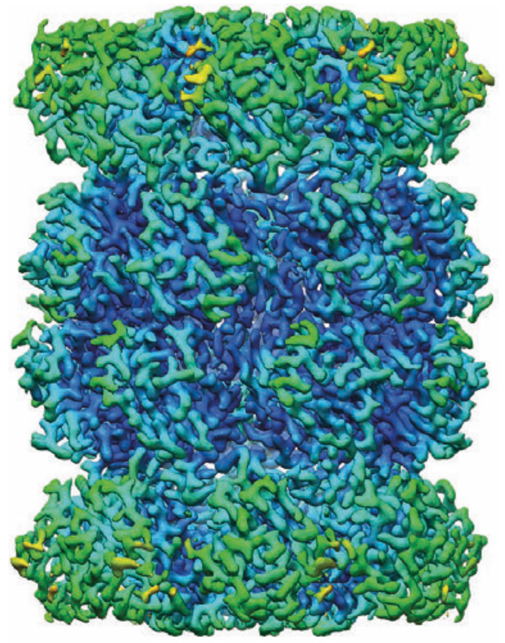
p97 AAAase
3.9 Å

Ripstein et al. 2017



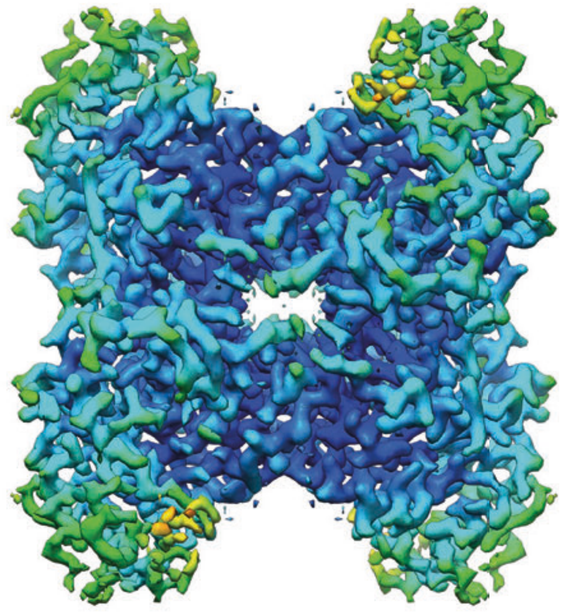
Proteasome
3.1 Å

Herzik et al. 2017



Adolase
2.6 Å

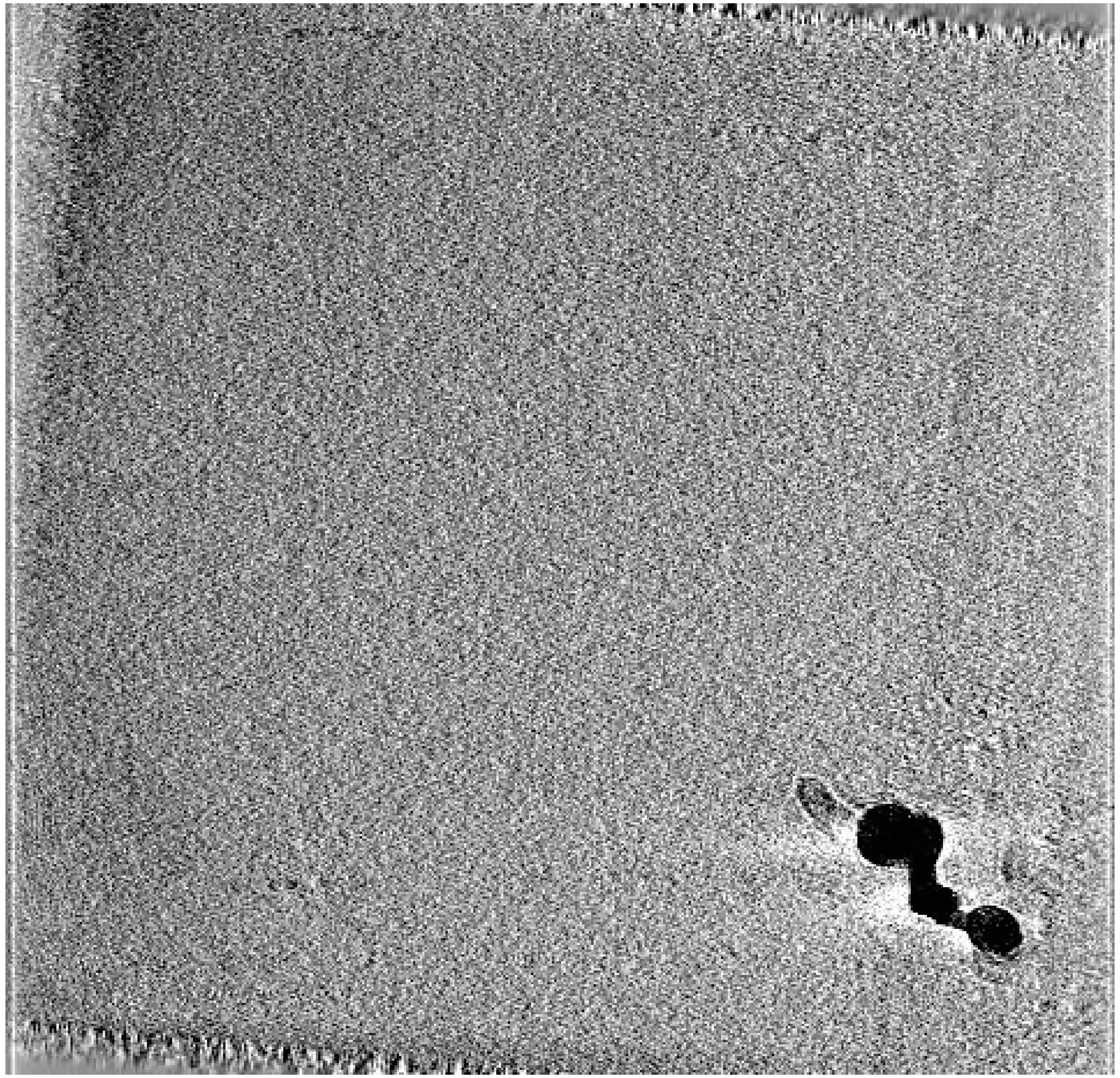
Herzik et al. 2017



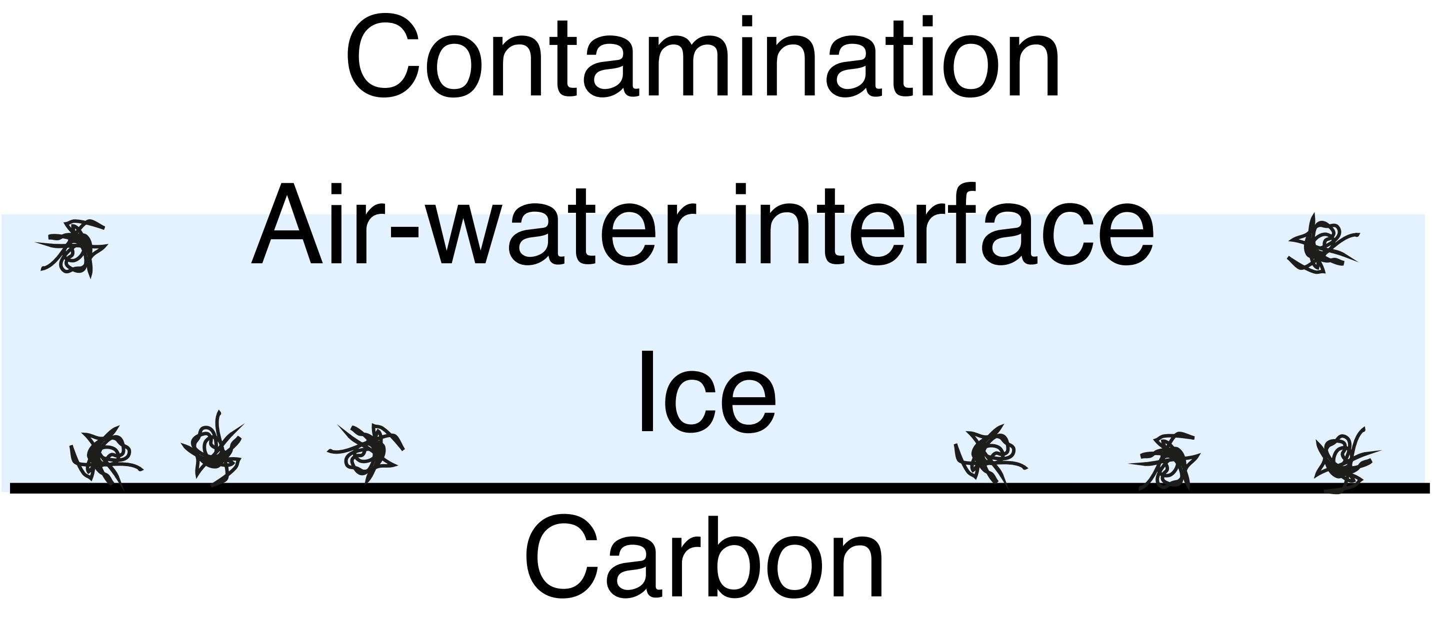
Surfaces:

Why do they matter?

Proteins adhere to surfaces

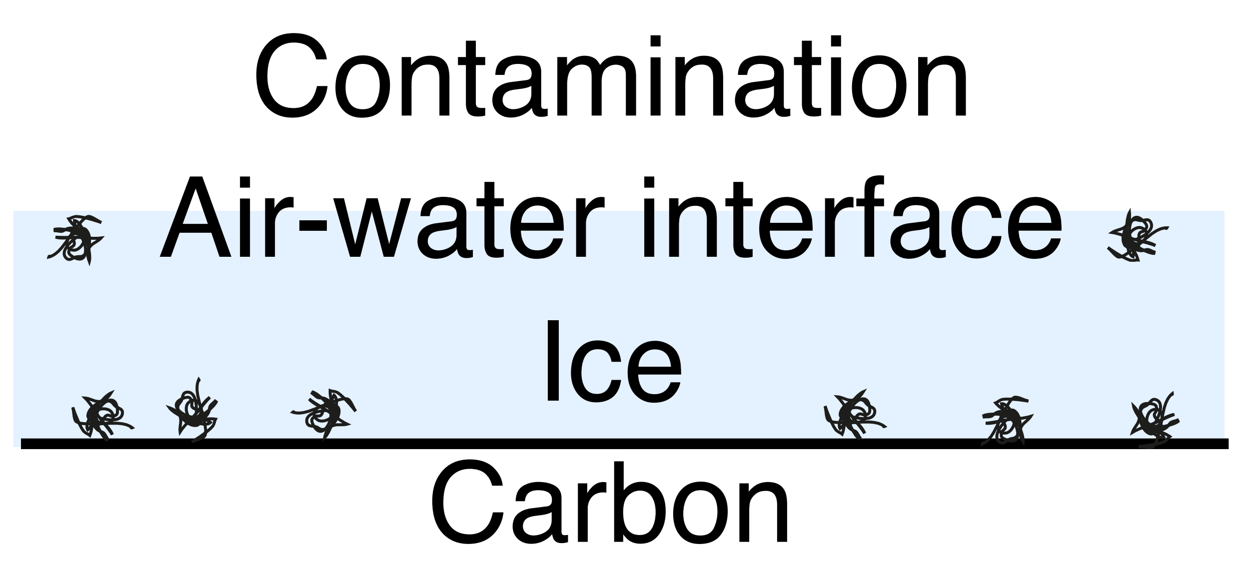


Tomogram of ribosomes in ice on an amorphous carbon film courtesy of Tanmay Bharat



See also Alex Noble

Proteins interact with water surfaces before freezing



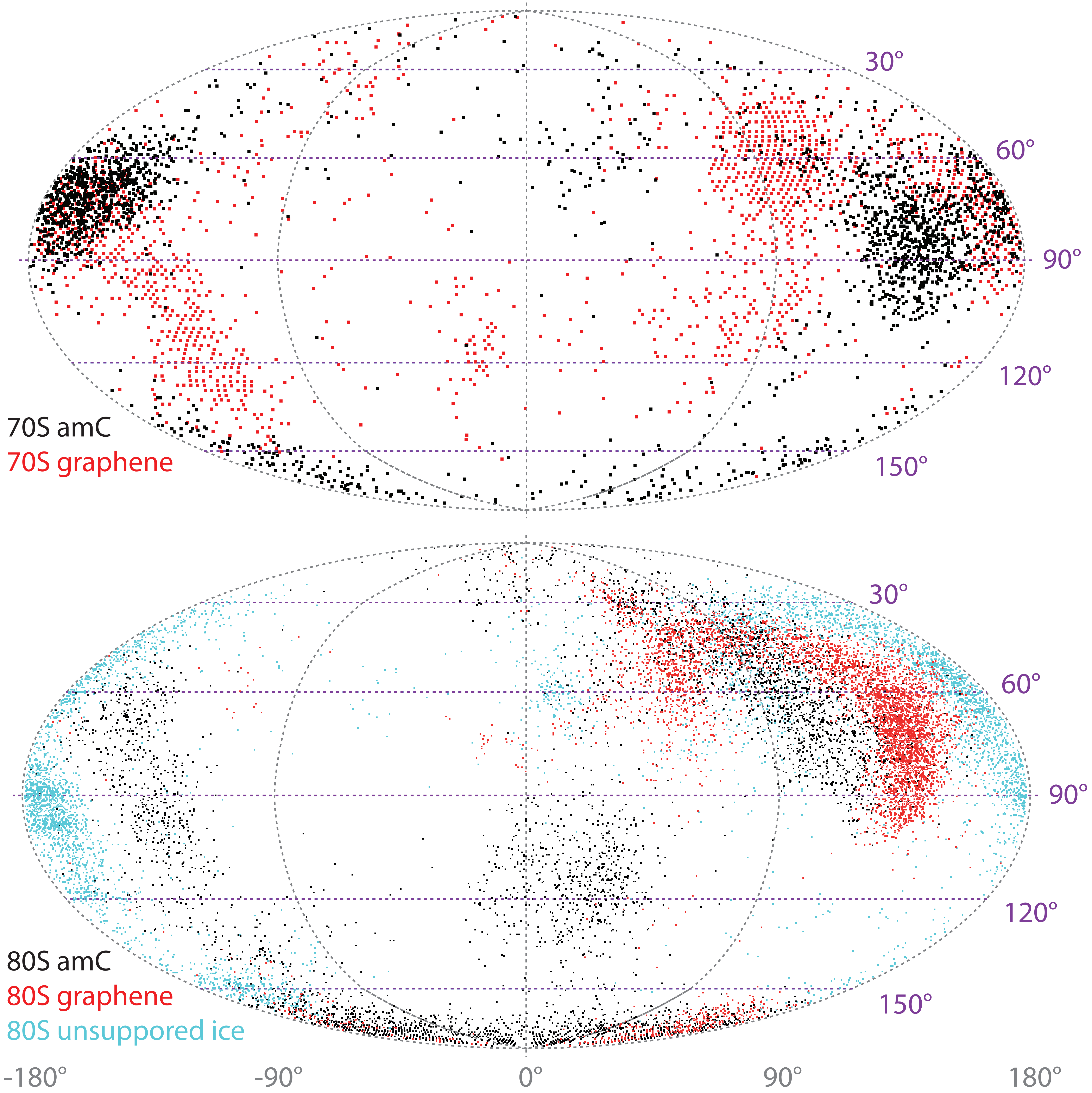
$$\Delta x = \sqrt{2Dt} \qquad D \propto MW^{-1/3}$$

Supplementary Table 1: **Diffusion of molecules in a thin film of water**

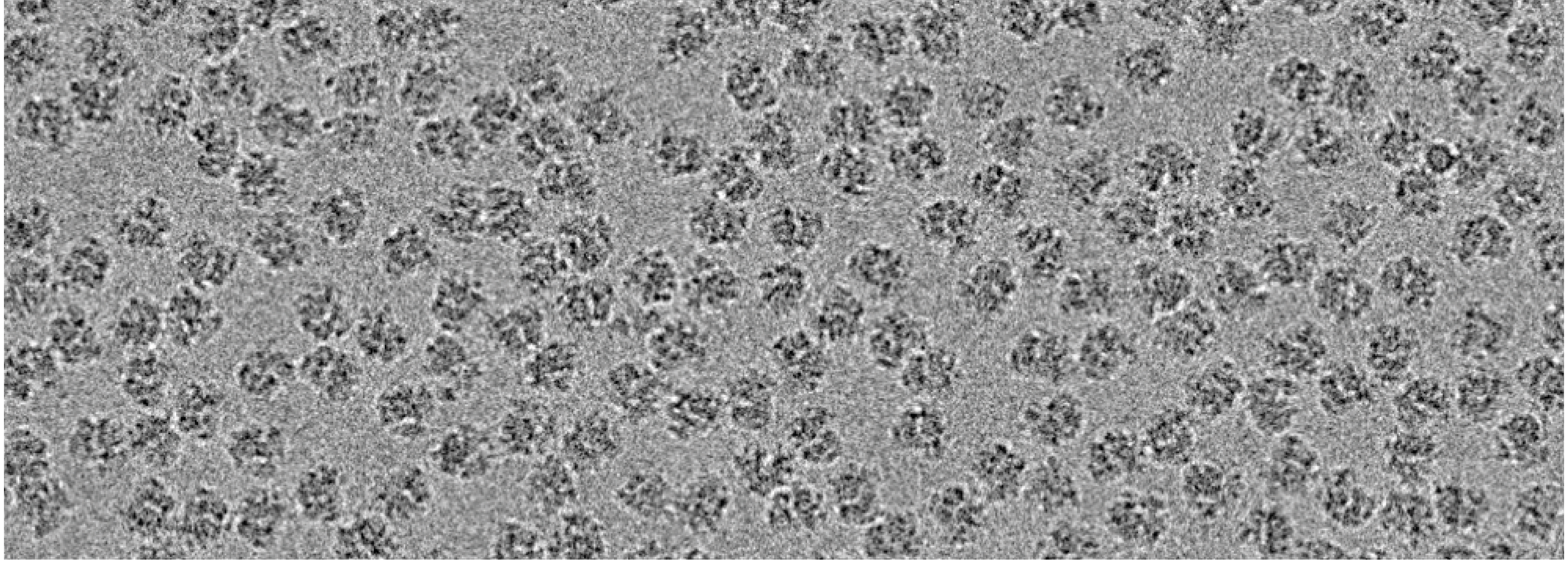
MW (kDa)	D (cm^2s^{-1})	Residence time (s)	d (Å)	Num. short-range interactions	Num. hydrophobic interactions	Num. electrostatic interactions
10	1×10^{-6}	10	500	10^3	10^3	10^3
10^2	5×10^{-7}	10	500	10^3	10^3	10^3
10^3	2×10^{-7}	10	500	10^3	10^3	10^3
10^4	1×10^{-7}	10	2000	10^2	10^2	10^2
10	1×10^{-6}	1	500	10^3	10^3	10^3
10^2	5×10^{-7}	1	500	10^2	10^2	10^3
10^3	2×10^{-7}	1	500	10^2	10^2	10^2
10^4	1×10^{-7}	1	2000	10	10	10^2
10	1×10^{-6}	10^{-3}	500	10	10	10
10^2	5×10^{-7}	10^{-3}	500	10	10	10
10^3	2×10^{-7}	10^{-3}	500	10	10	10
10^4	1×10^{-7}	10^{-3}	2000	1	1	1

Naydenova & Russo, *Nature comm.* 2017

See also Glaser and Han 2016



Russo & Passmore 2014



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

Concentration

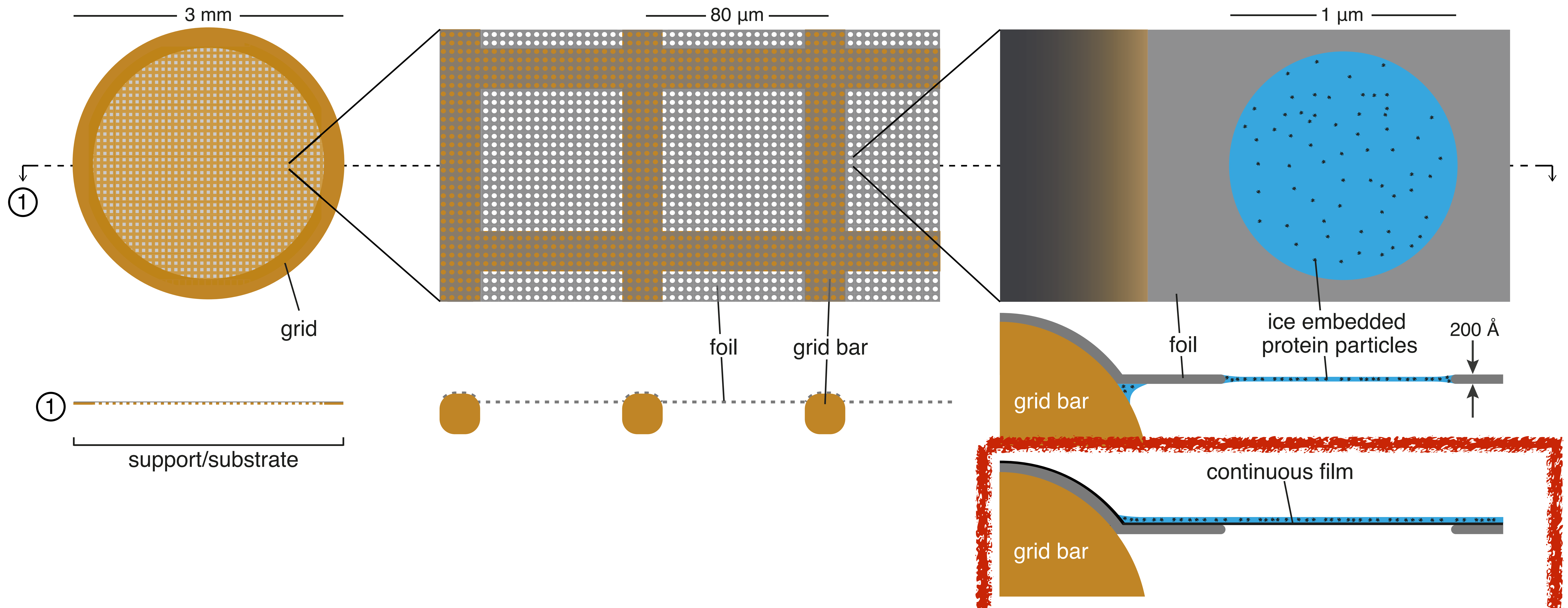
M.W.	10mg/ml	2mg/ml	0.5mg/ml	0.1mg/ml	20μg/ml
10 kD	48000 (45Å)	10000 (100Å)	2500 (200Å)	500 (450 Å)	100 (1000 Å)
50 kD	10000 (100Å)	2000 (220Å)	500 (400Å)	100 (1000Å)	20 (0.2μm)
250kD	2000 (220Å)	400 (500 Å)	100 (1000 Å)	20 (0.2μm)	4 (0.5μm)
1 MD	500 (400Å)	100 (1000Å)	25 (0.2μm)	5 (0.4μm)	1 (1μm)
5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

Vinothkumar & Henderson 2016

Surfaces:

What are the current options?

Types of specimen supports



Grid materials

Copper
Nickel
Titanium
Silicon

Gold
CuRh
Molybdenum
Aluminum
Tungsten

Foil materials

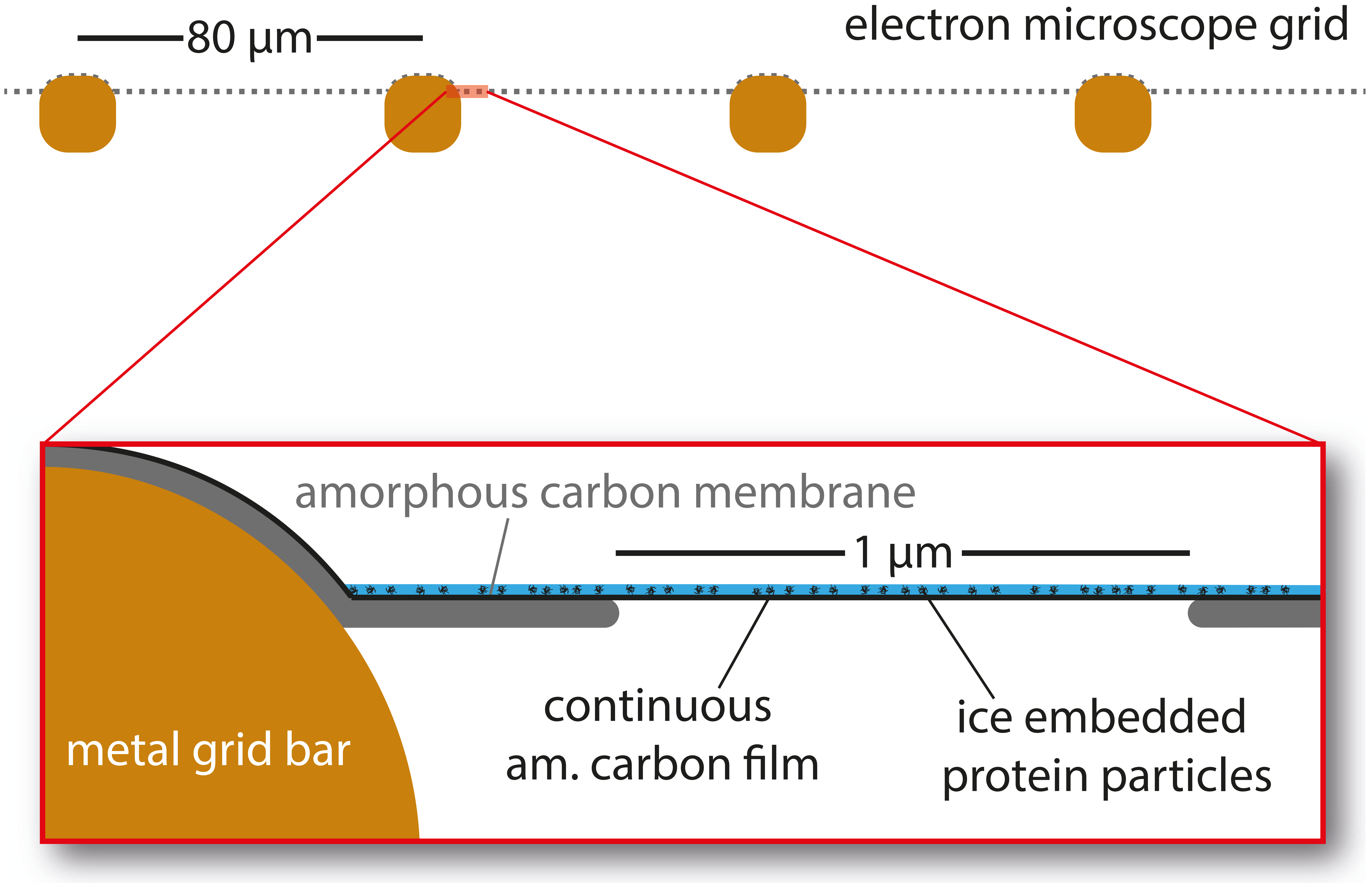
Amorphous carbon
Gold
TiSi SiN
SiO_2 SiC

Film materials

Amorphous carbon
Graphene
Graphene oxide
TiSi
SA 2D crystal
& others

Passmore & Russo MiE 2016

Continuous carbon films



Surfaces:

How do we modify them?

Plasma treatment

- Plasma created by ionisation of a low pressure gas
  - E.g. in air (glow discharge), oxygen, argon, hydrogen
- Ions interact with surface to remove adsorbed contamination and render it hydrophillic
- Other molecules can be introduced to alter the surface, e.g. Amylamine

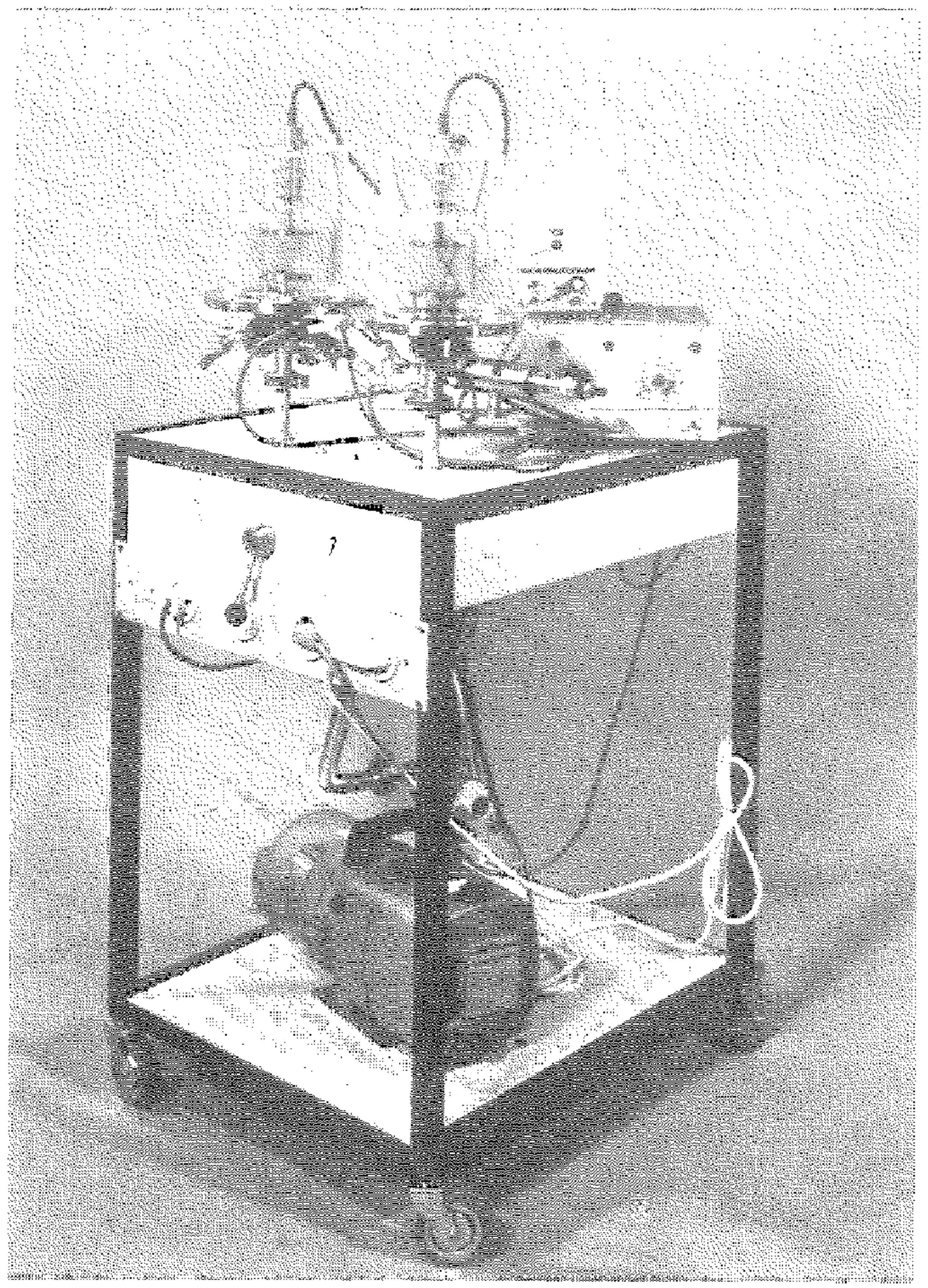
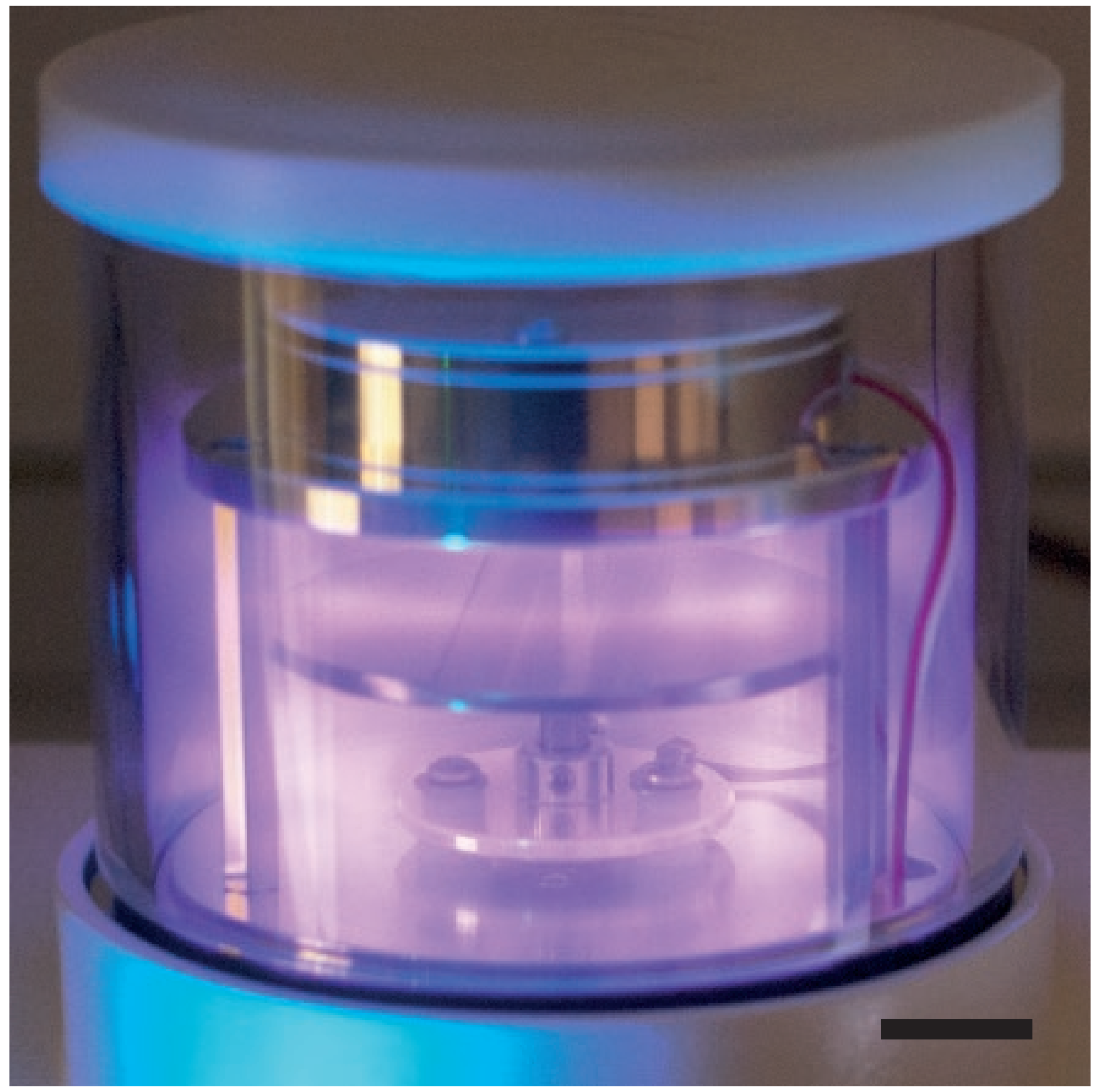
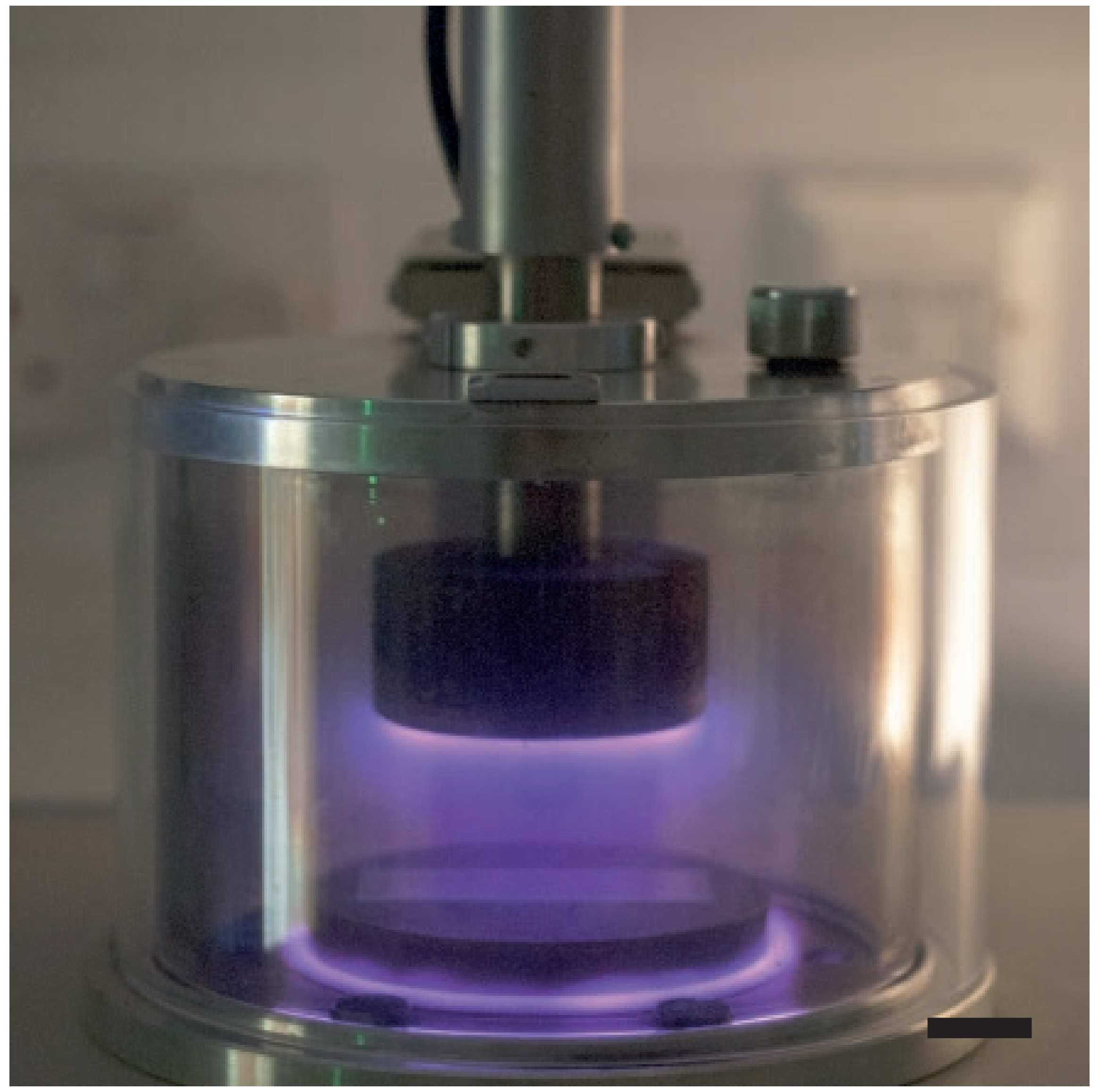


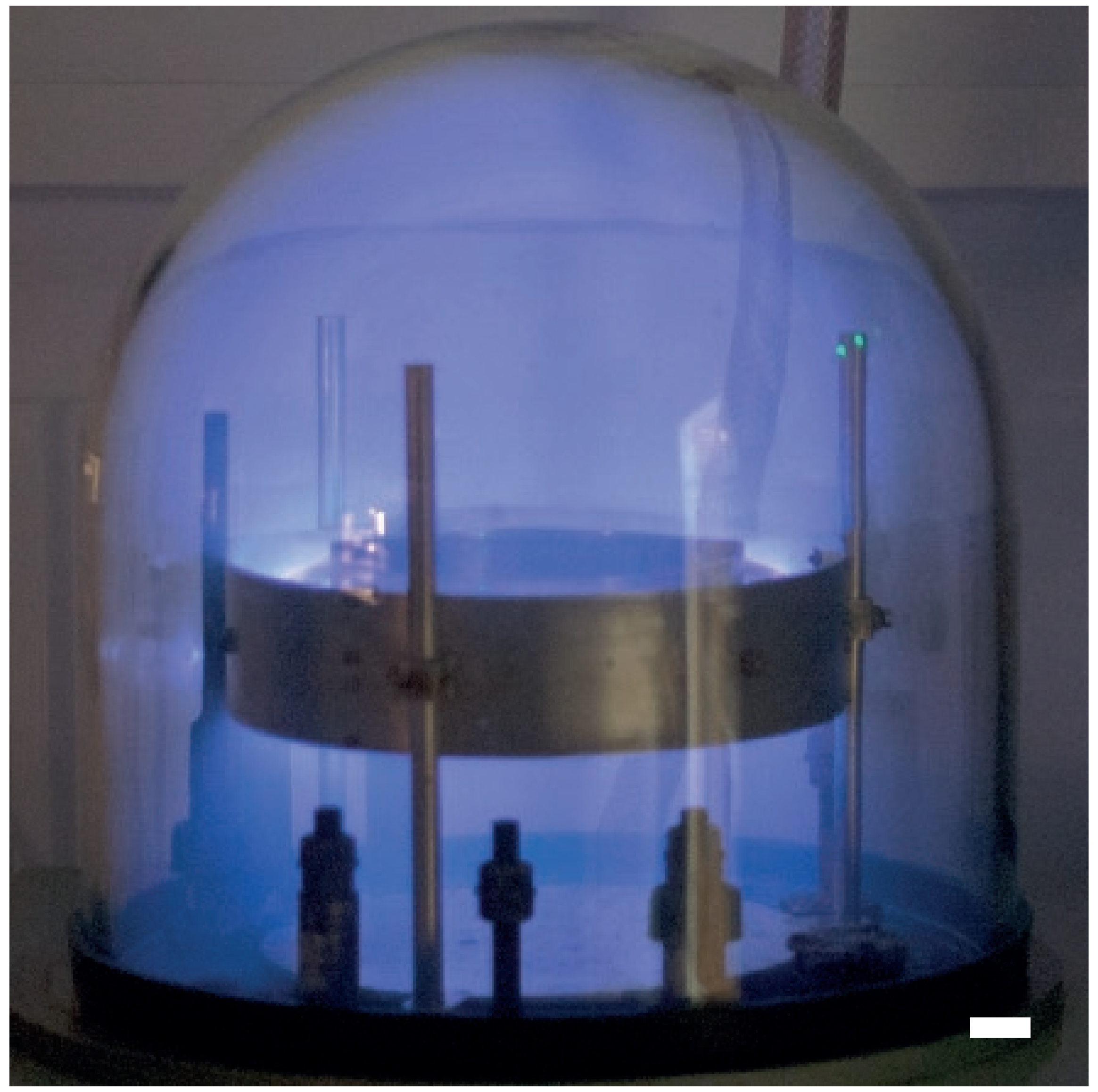
FIG. 6. Photograph of the glow discharge apparatus.

Dubochet et al. 1982

Residual air plasmas: glow discharge


Ted Pella easyGlow (c. 2015)


Edwards S150B (c. 1995)


Edwards 12E6 (c. 1962)

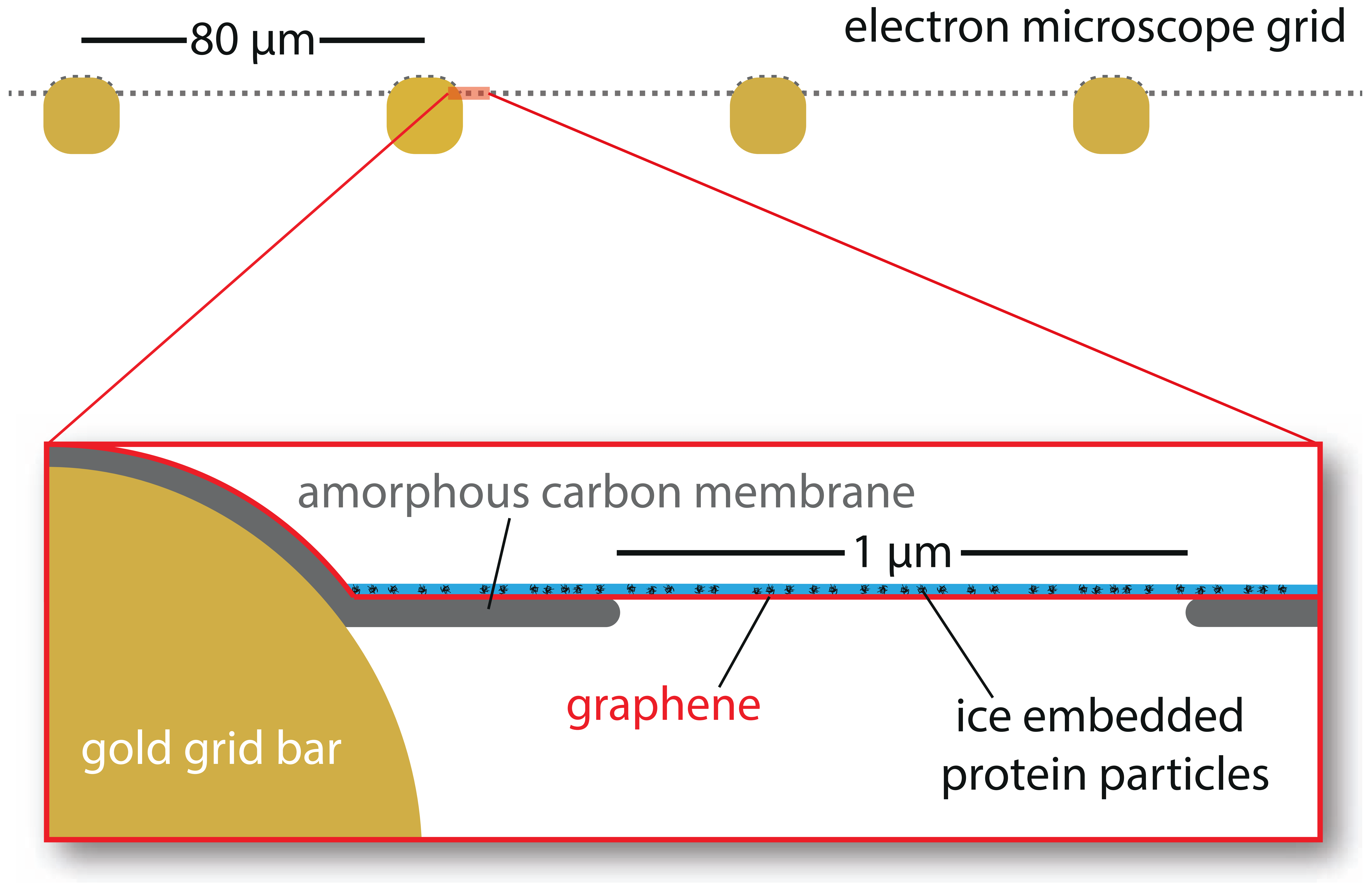
Passmore & Russo MiE 2016

Dedicated plasma systems

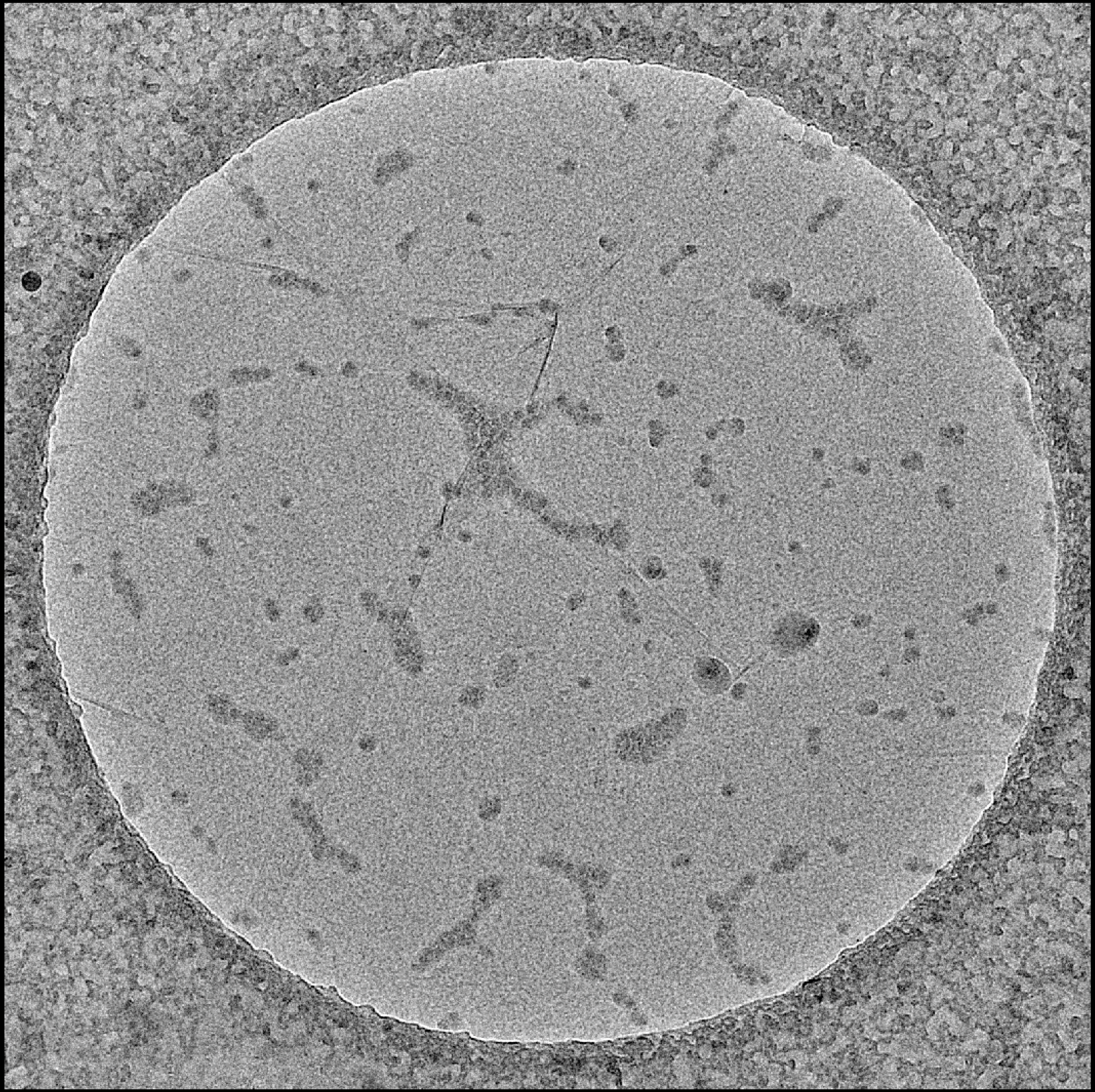
Passmore & Russo MiE 2016, image B. Carragher

Surfaces:

What about something thinner?


80 µm
electron microscope grid
amorphous carbon membrane
1 µm
graphene
ice embedded
protein particles
gold grid bar

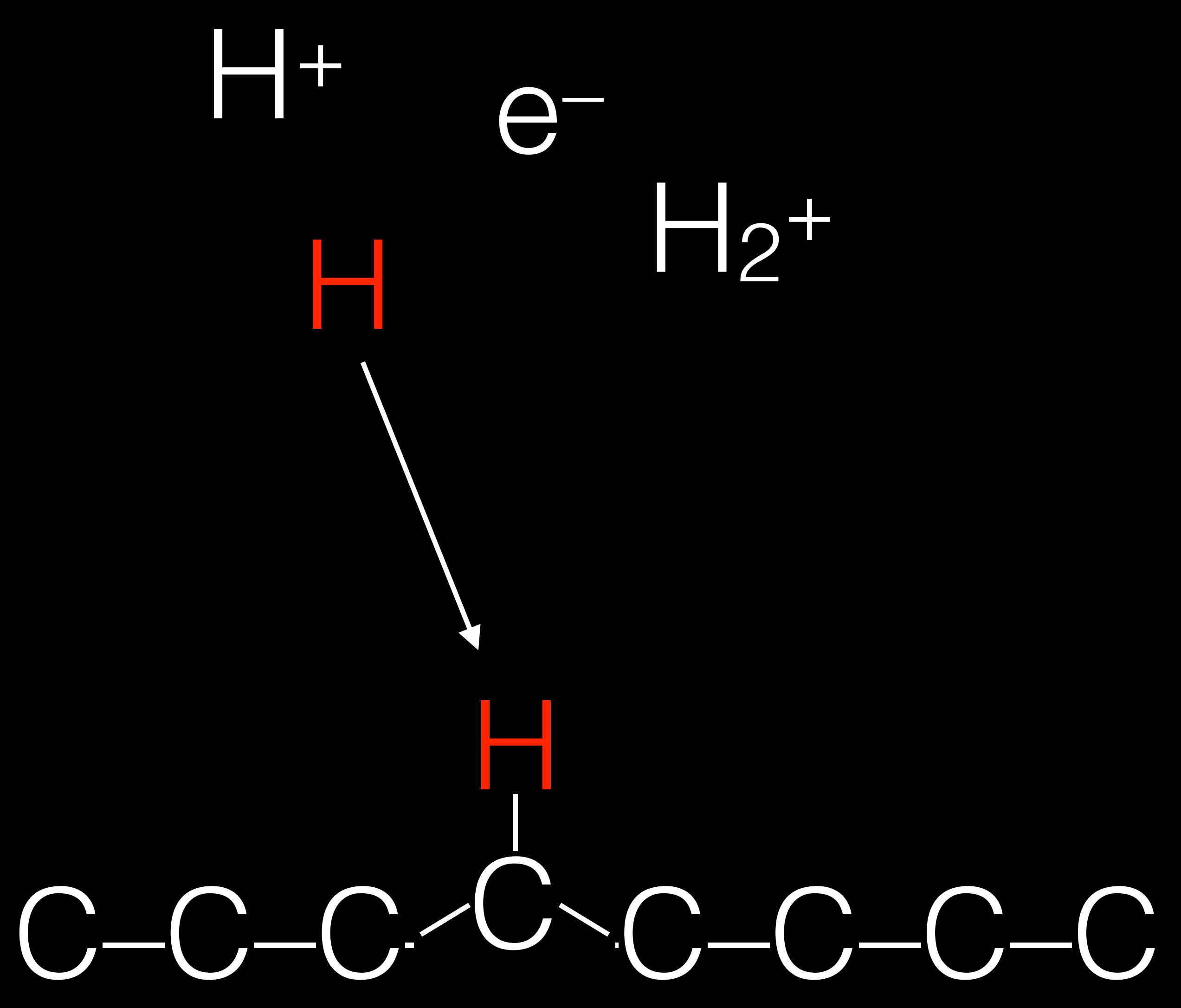

10 Å



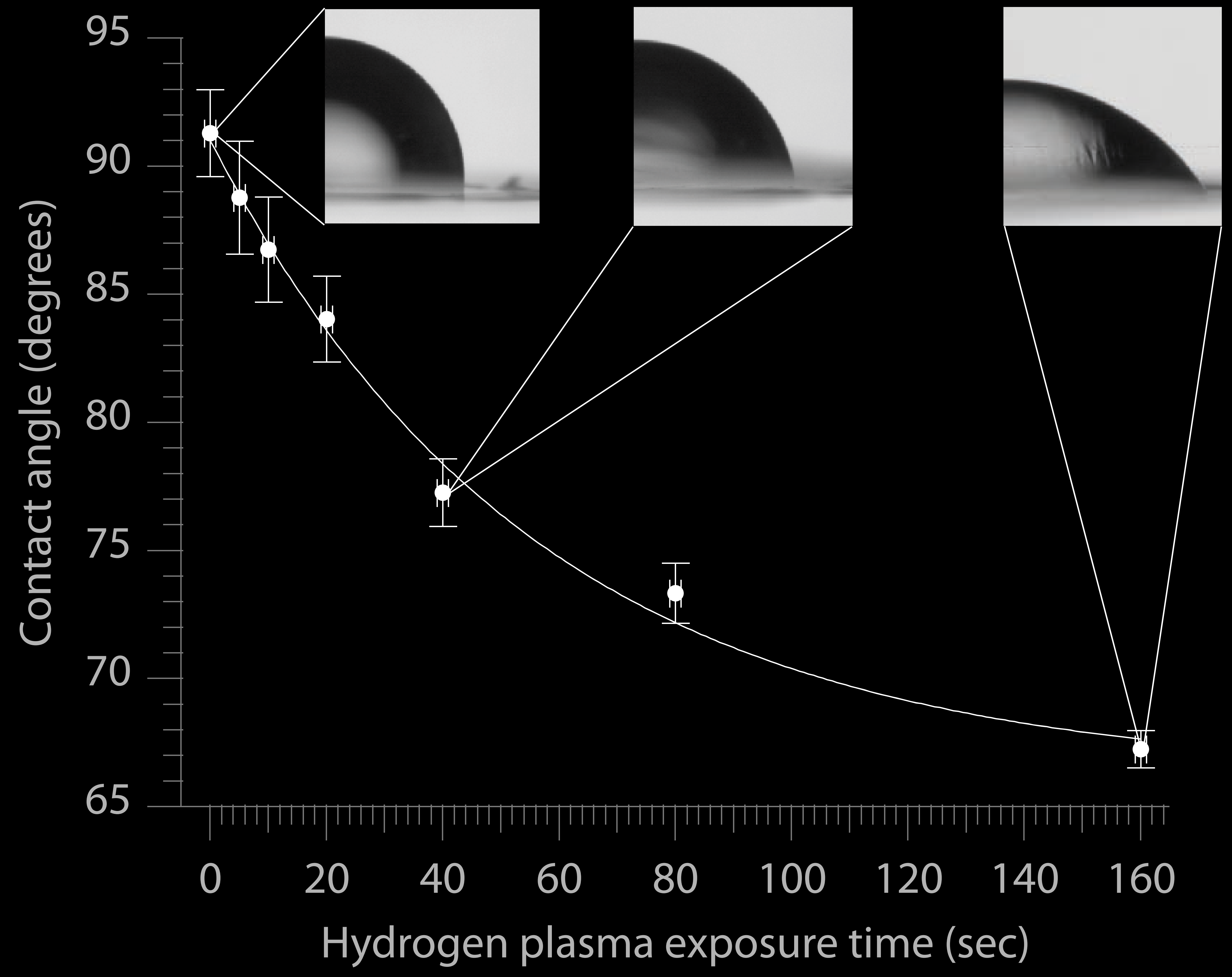
70S Ribosomes
on graphene as
synthesised

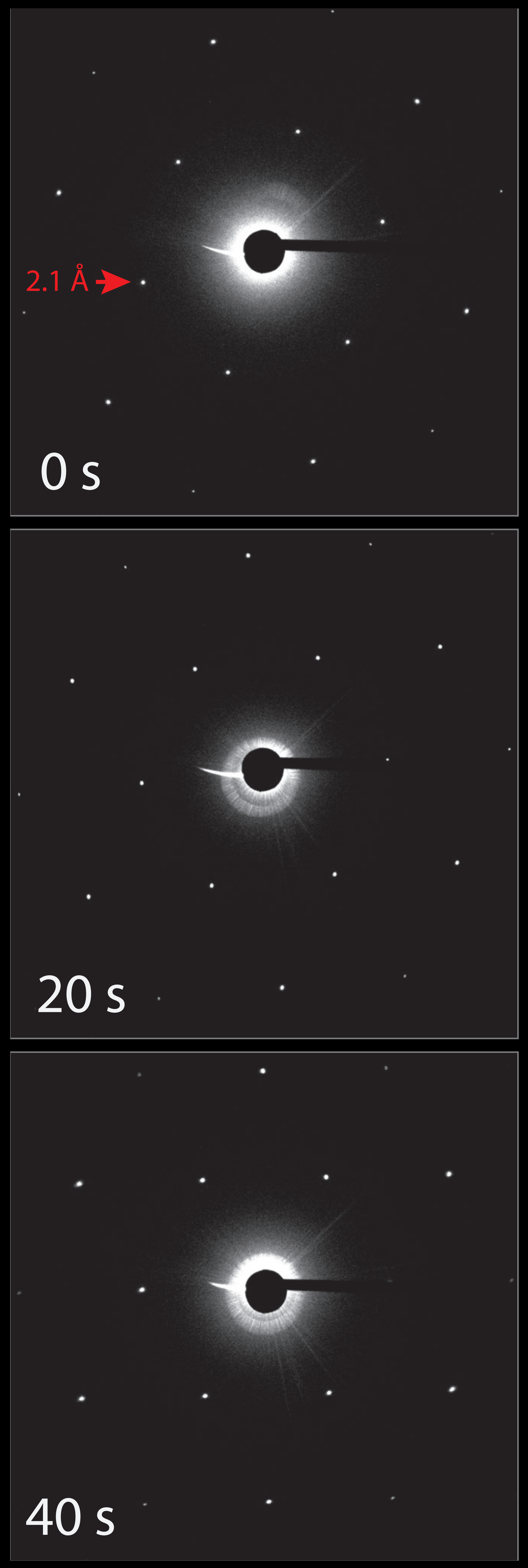
1.2 μm hole

Hydrogen plasma

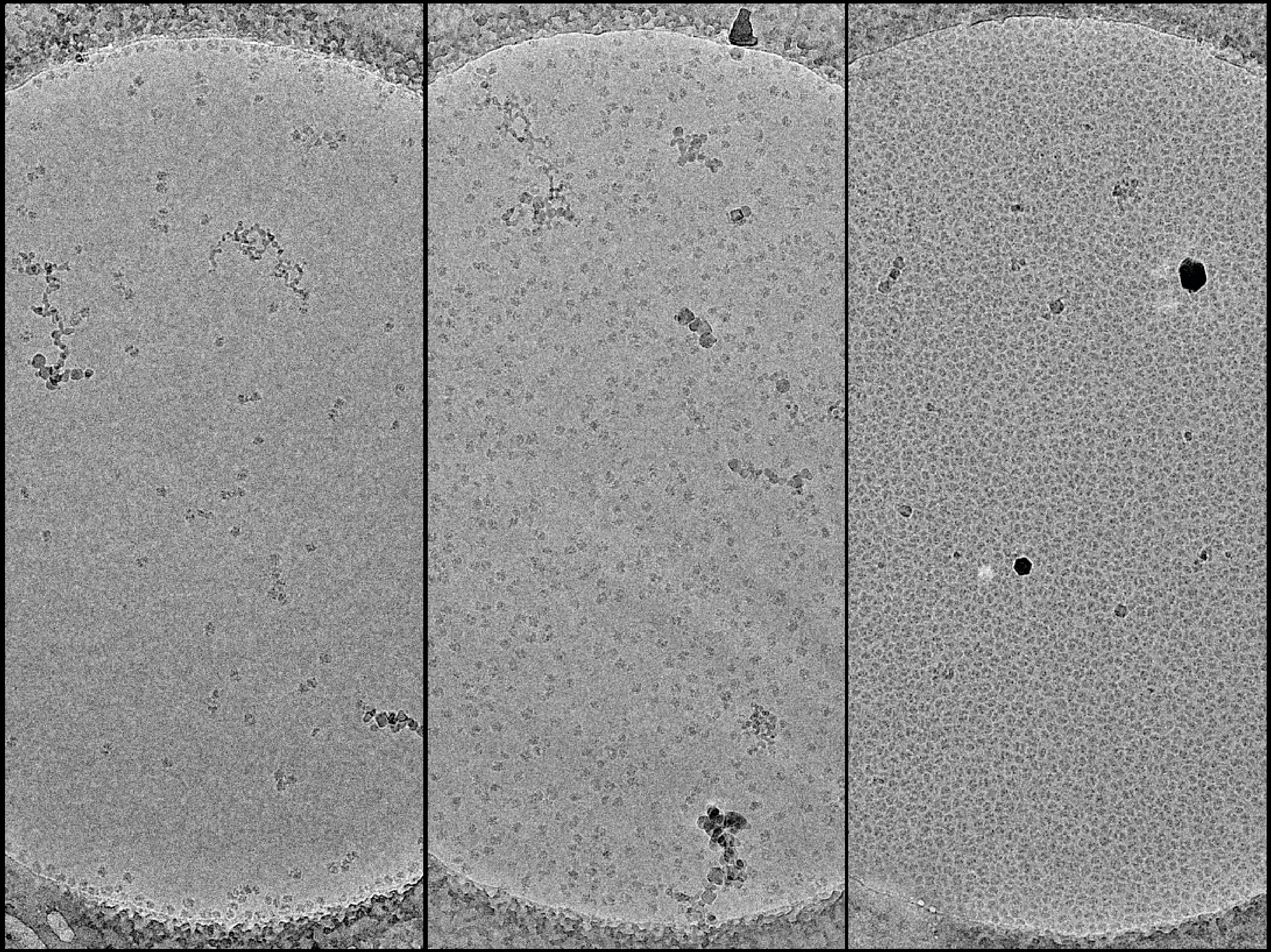


Graphene sp2 bond





Controlled adsorption of proteins to graphene



no graphene

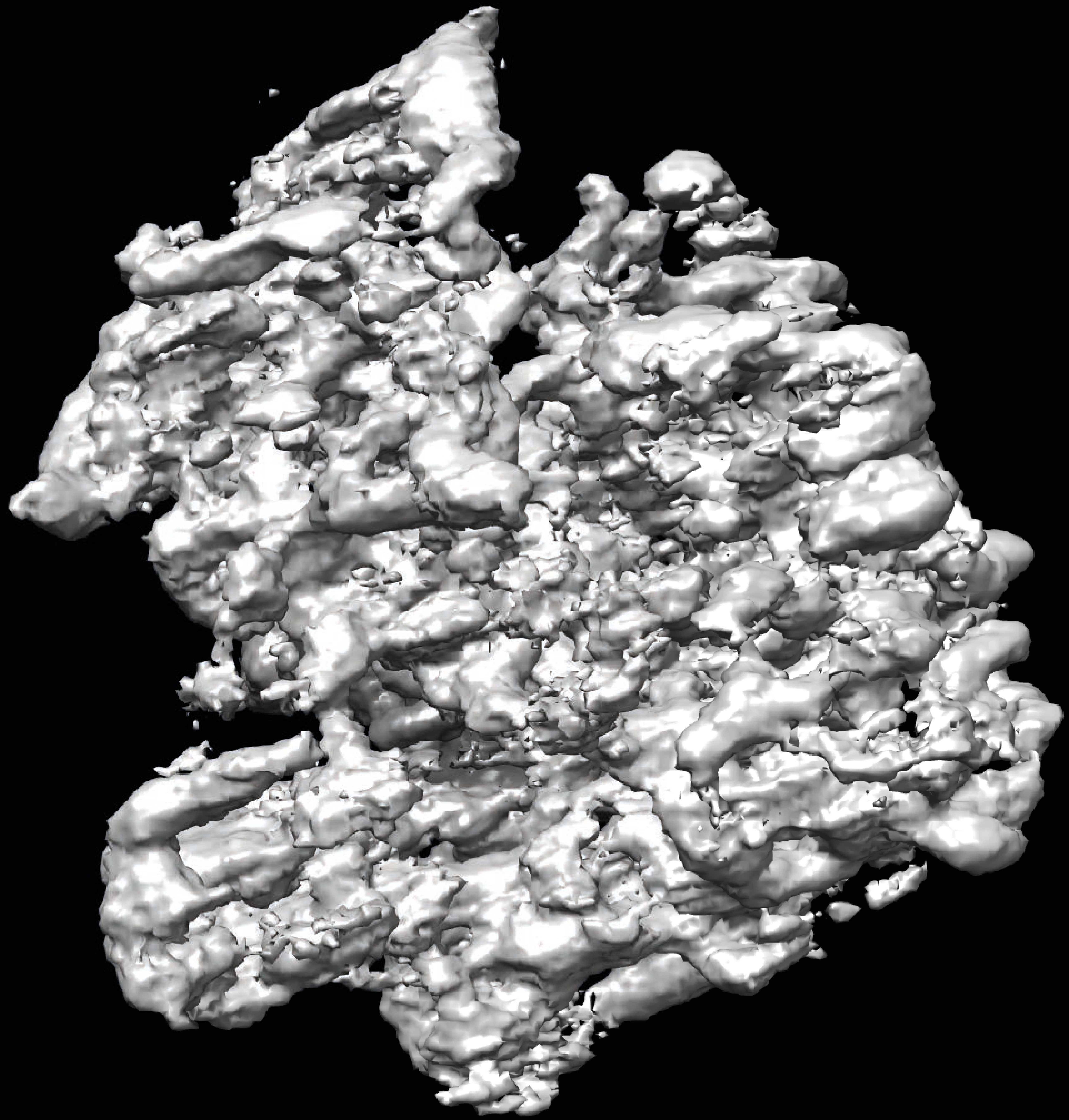
graphene +
20 s hydrogen

graphene +
40 s hydrogen

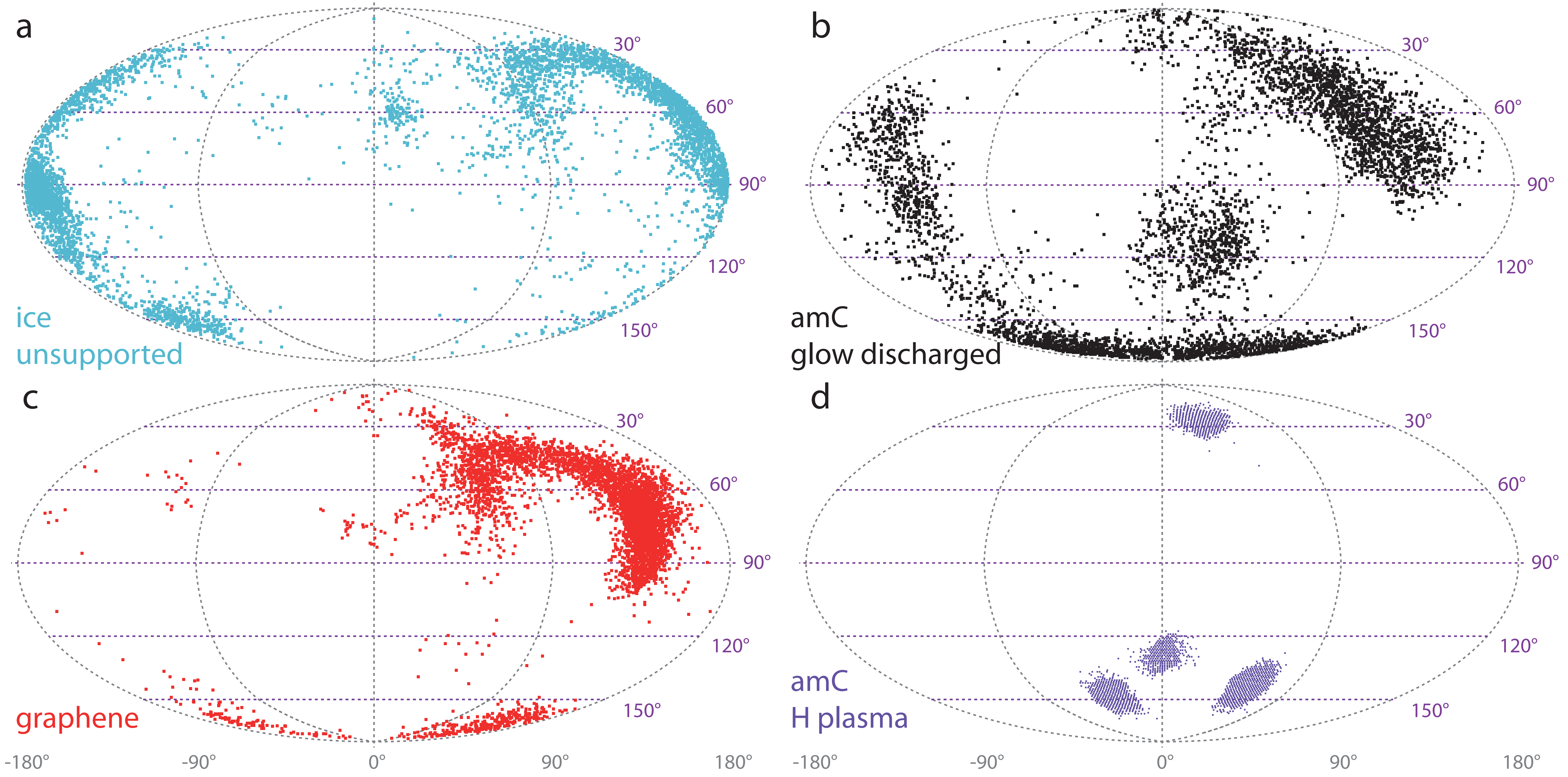
1.2 um holes

Surfaces:

How to tell if one is better than another?



Which is better?



Russo and Passmore. Nature Methods 11.6 (2014): 649-652.

Filling Fourier space with information

Two particles

10000 particles

Completely undetermined Fourier components

Transfer functions

Naydenova & Russo, *Nature Comms* 2017

Fill Fourier space for each orientation plane:

$$C_i(\mathbf{k}) = \sqrt{N_i}\exp(-\frac{B|\mathbf{k}^2|}{4})$$

Sum up contributions from all views:

$$\tau(\mathbf{k}) = \sqrt{\sum C_i^2(\mathbf{k})}$$

Normalize to unit power:

$$\int \tau^2(\mathbf{k})d^3\mathbf{k} = 1$$

Recall the formation of the image in Fourier space:

$$I(\mathbf{k}) = O(\mathbf{k}) \times \tau(\mathbf{k})$$

Take inverse Fourier transform:

$$i(\mathbf{r}) = o(\mathbf{r}) * s(\mathbf{r})$$

3D image

actual object

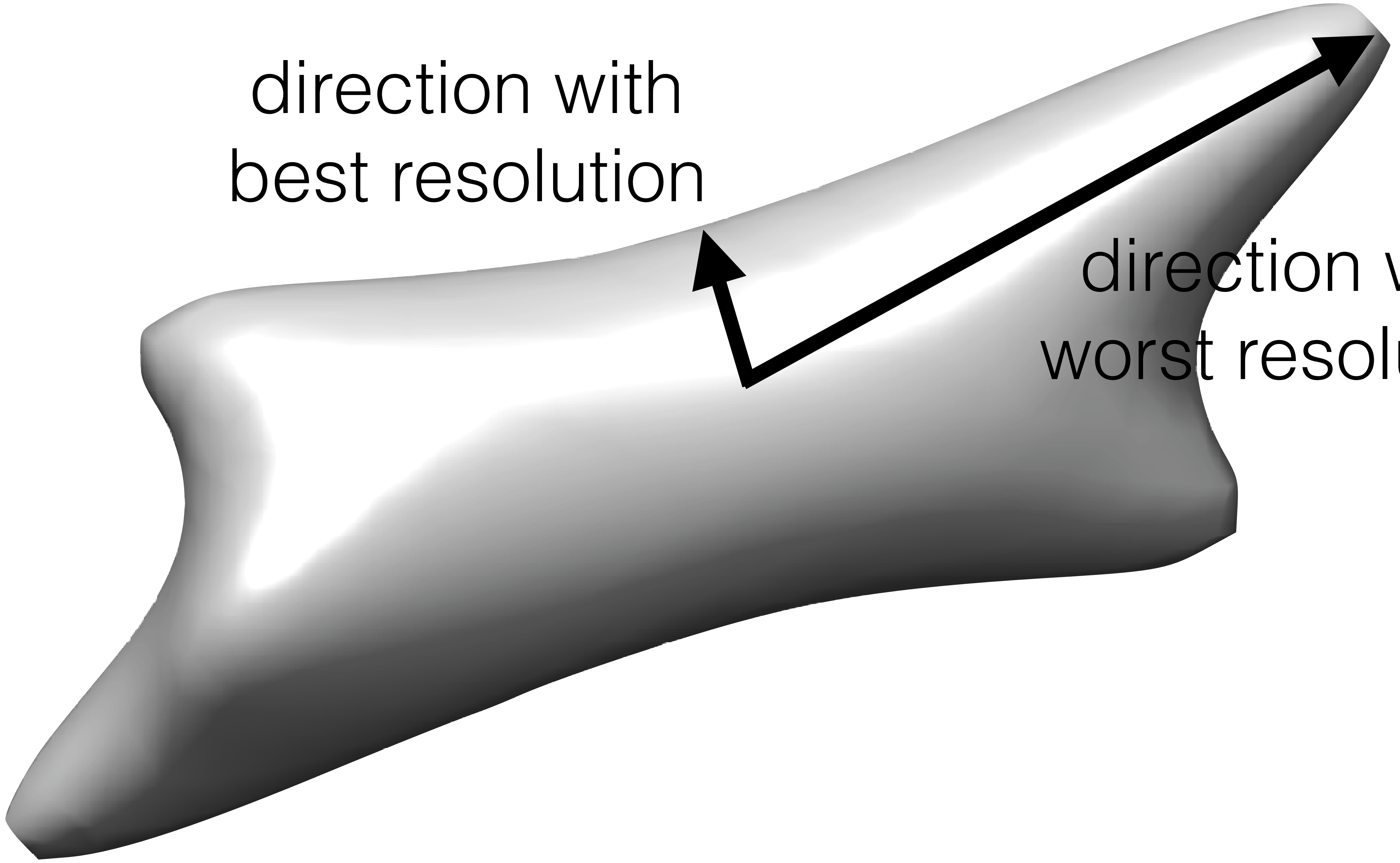
Point spread function (inverse transform of the transfer function)

Shape of the PSF at $1/e^2$ point: anisotropic resolution

The perfect PSF

radius = resolution

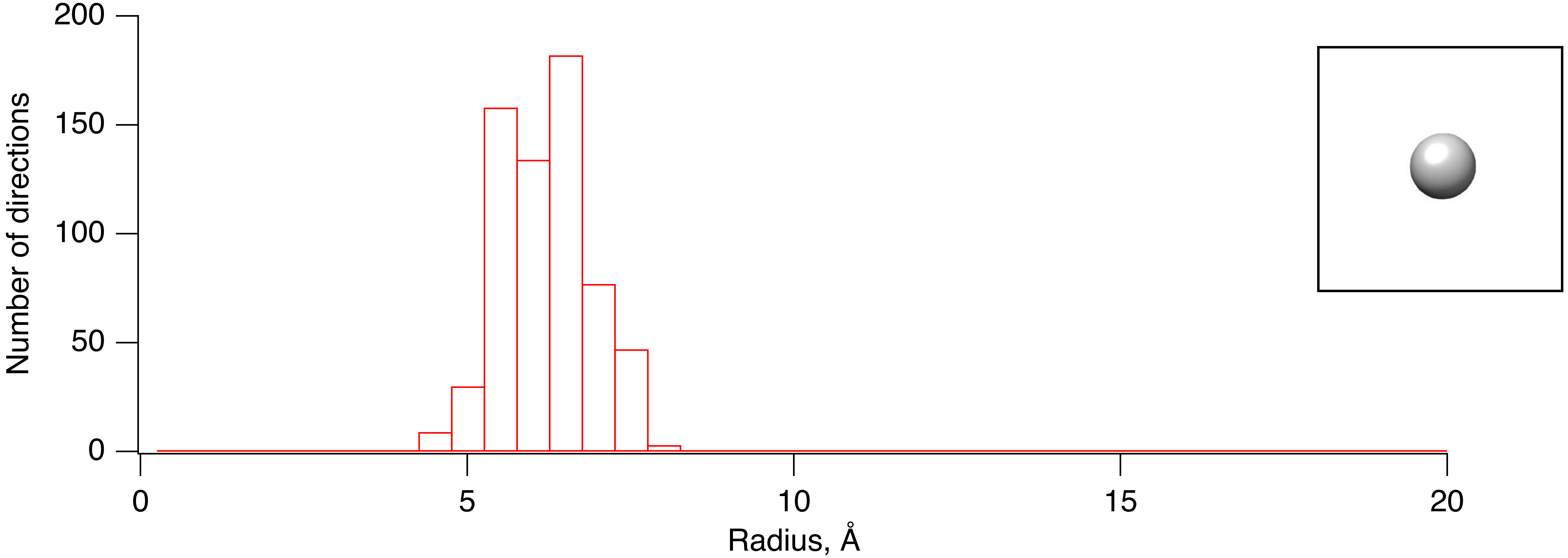
direction with best resolution



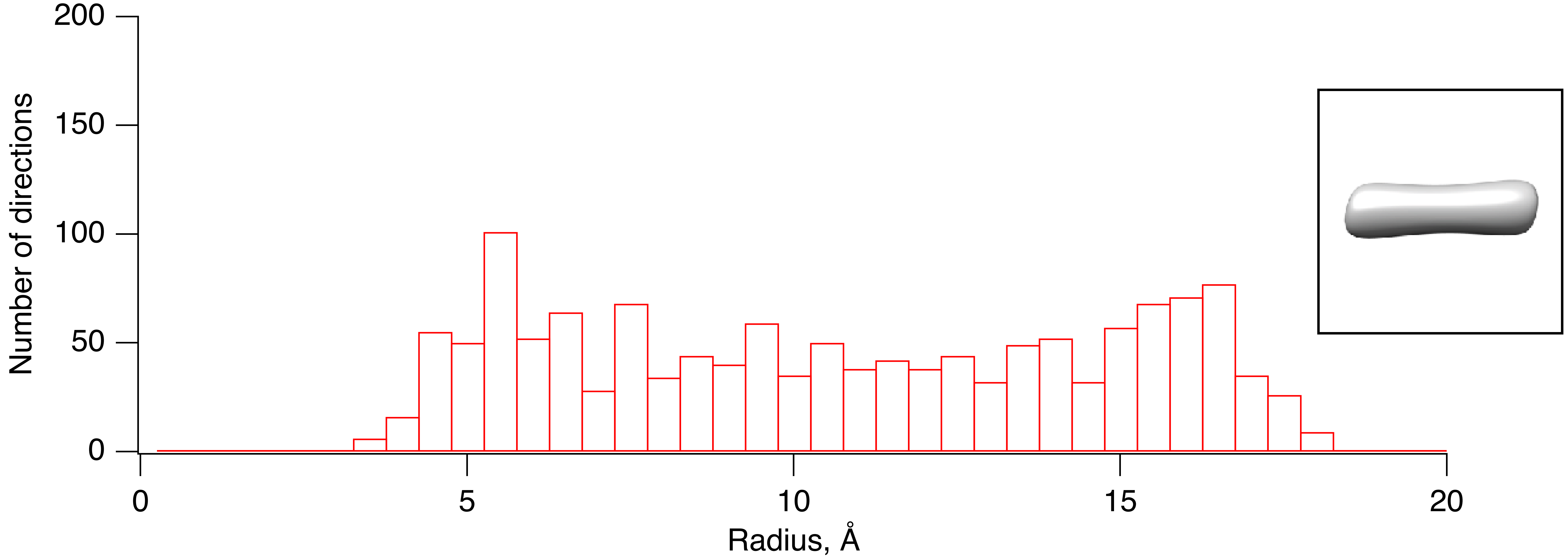
direction with worst resolution

mean resolution = ?
variance in the resolution = ?

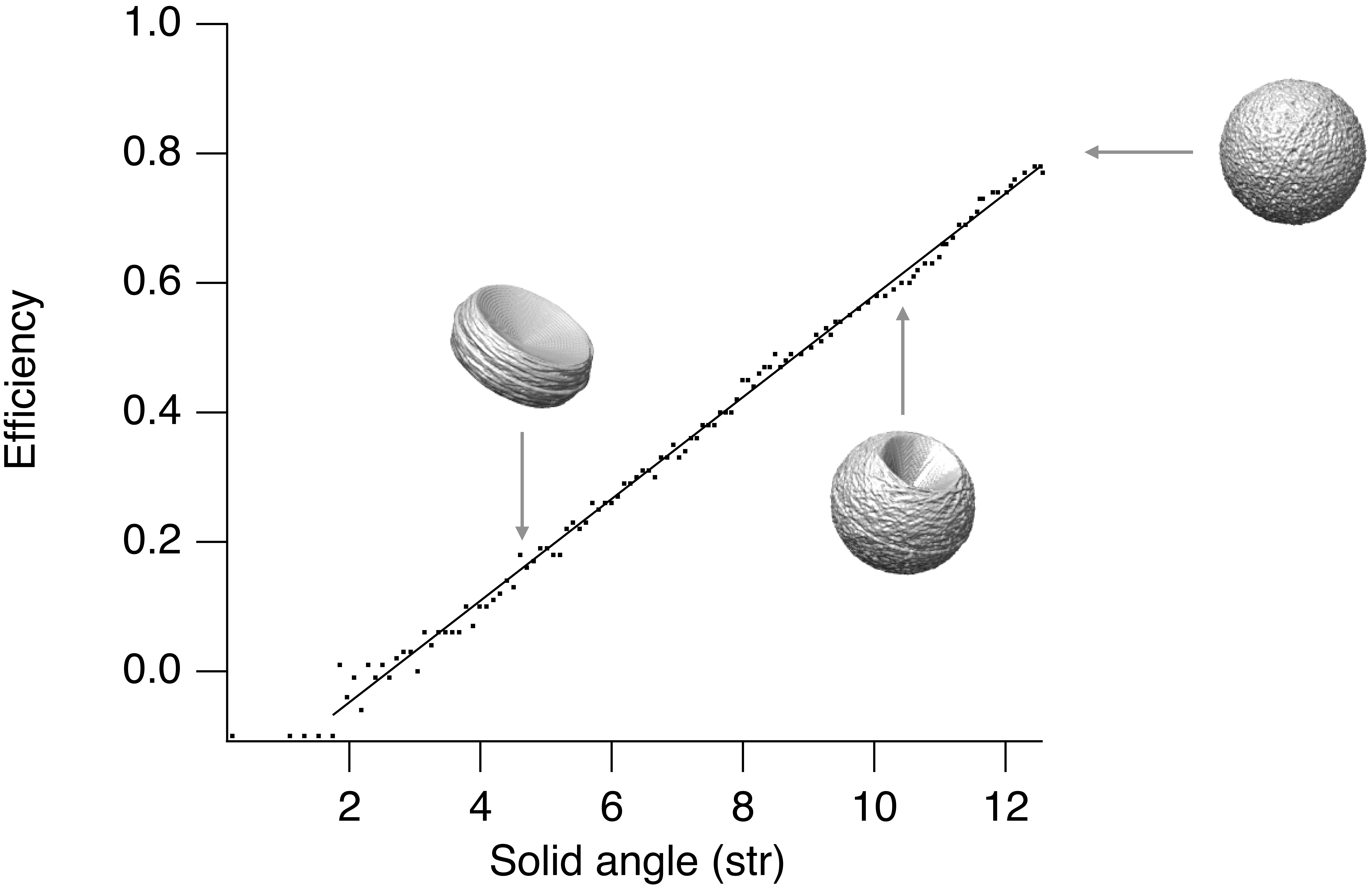
Efficiency = 1 - 2 x StDev / Mean



Efficiency 0.8

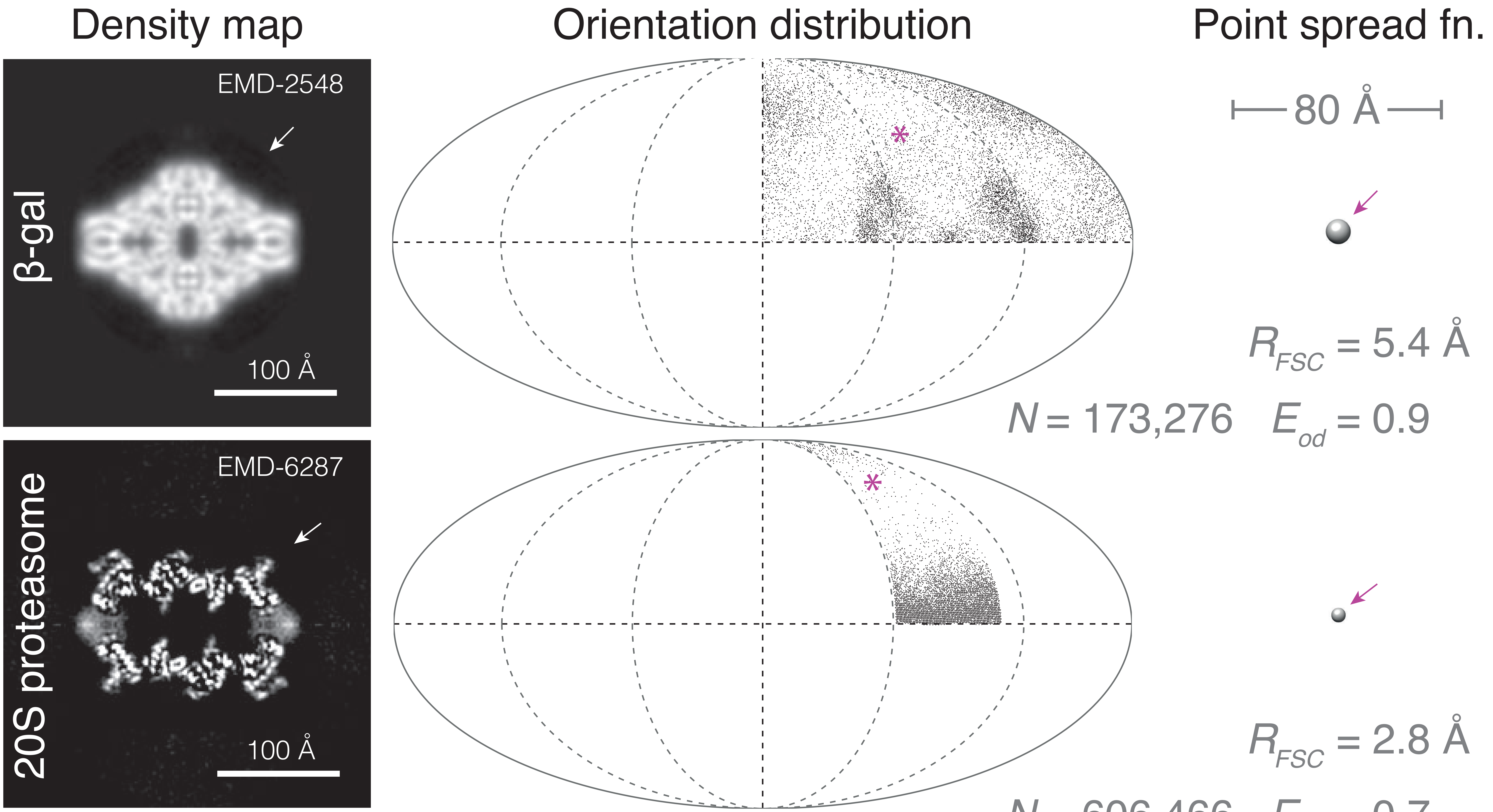


Efficiency 0.2

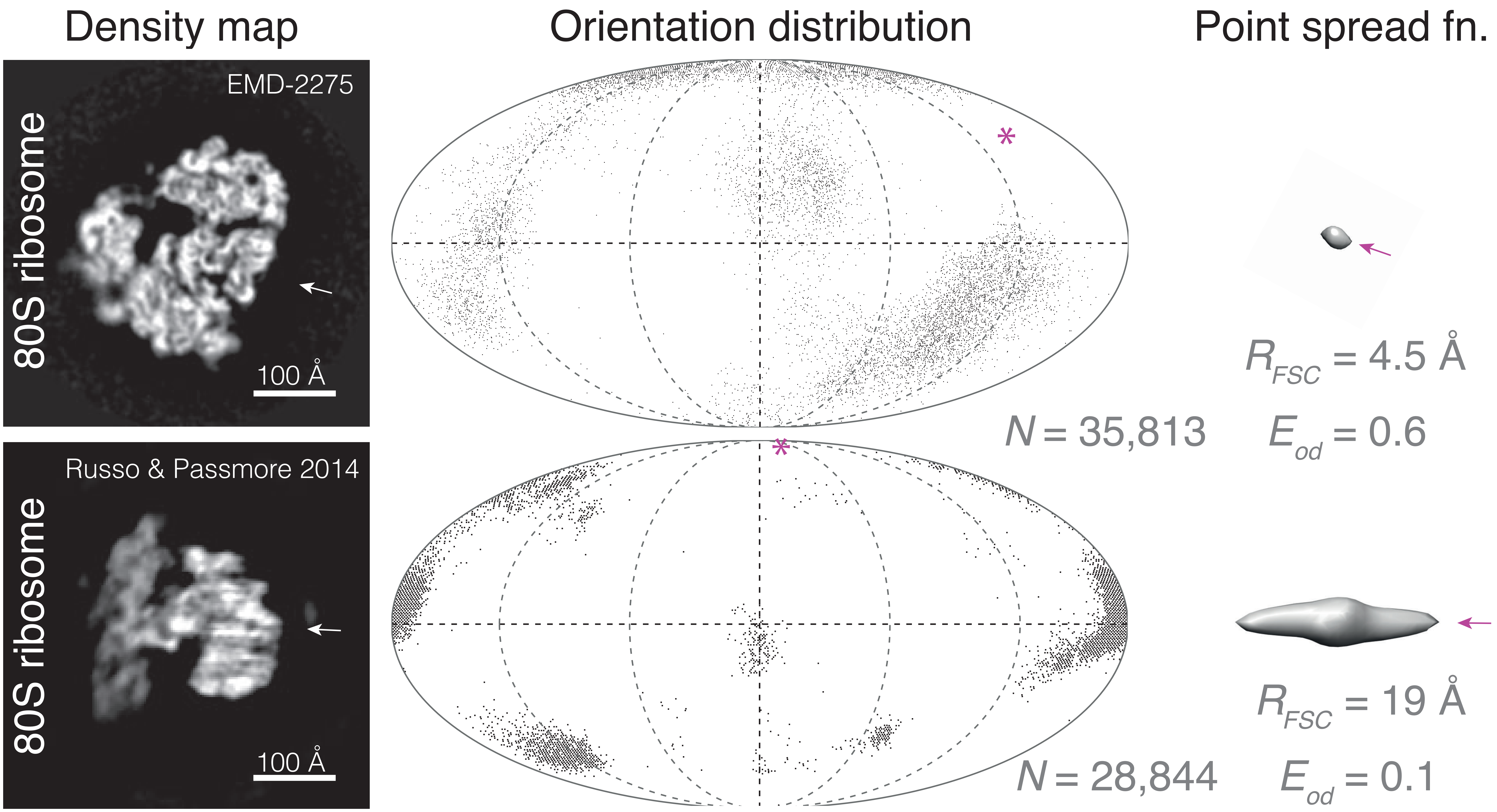


Efficiency
1.0
0.8
0.6
0.4
0.2
0.0
2
4
6
8
10
12
Solid angle (str)

Orientation distribution
Fourier space coverage (projection)
Point spread function
Efficiency
1
0.6
0.2
20 Å

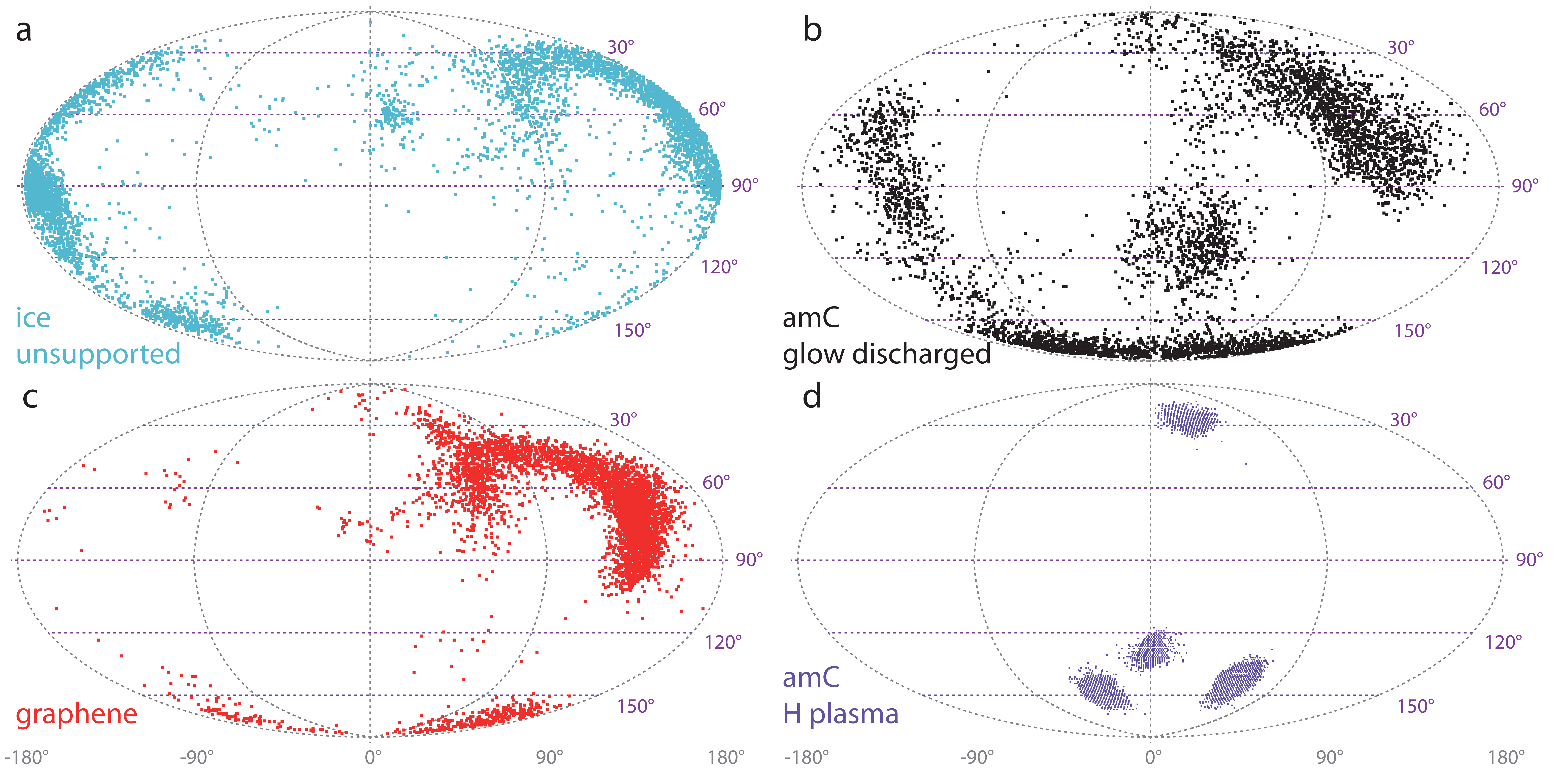


Naydenova & Russo, *Nature Comms* 2017

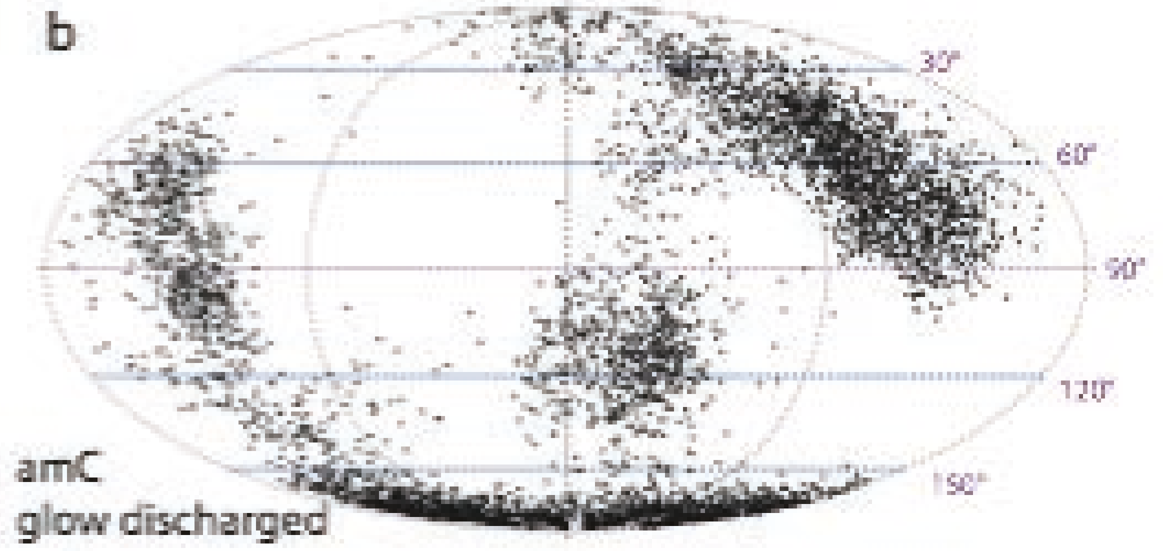
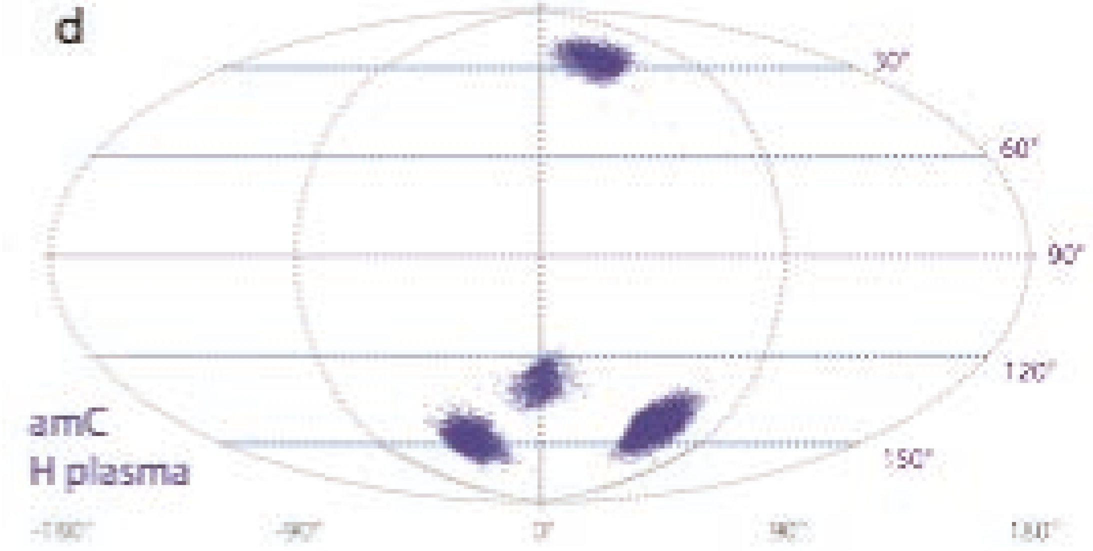
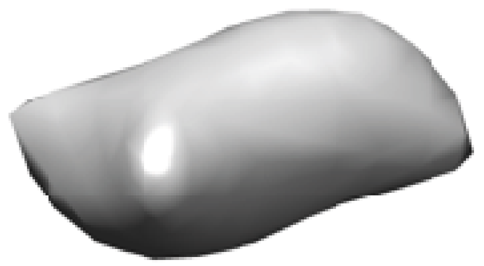
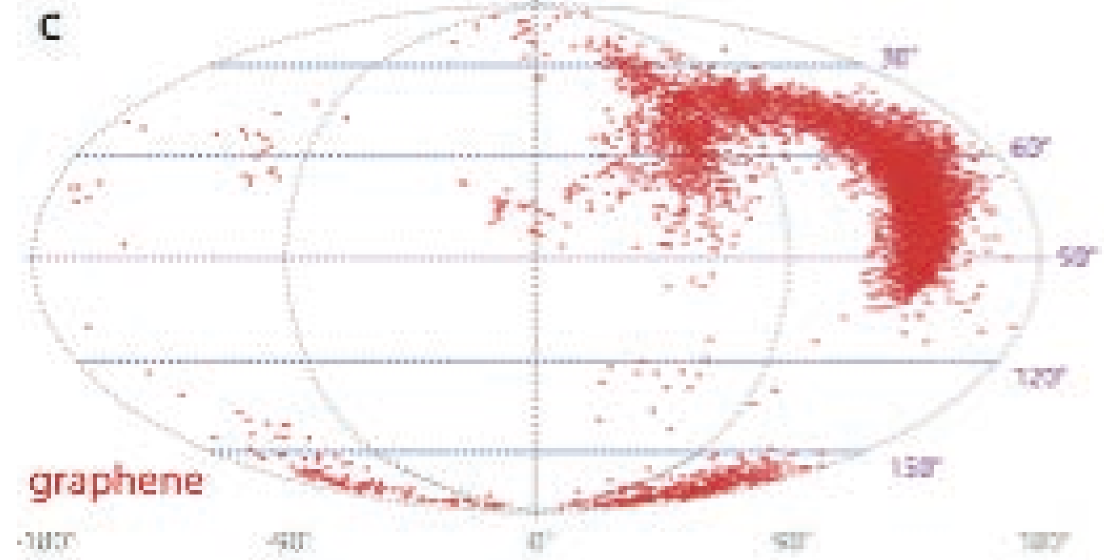
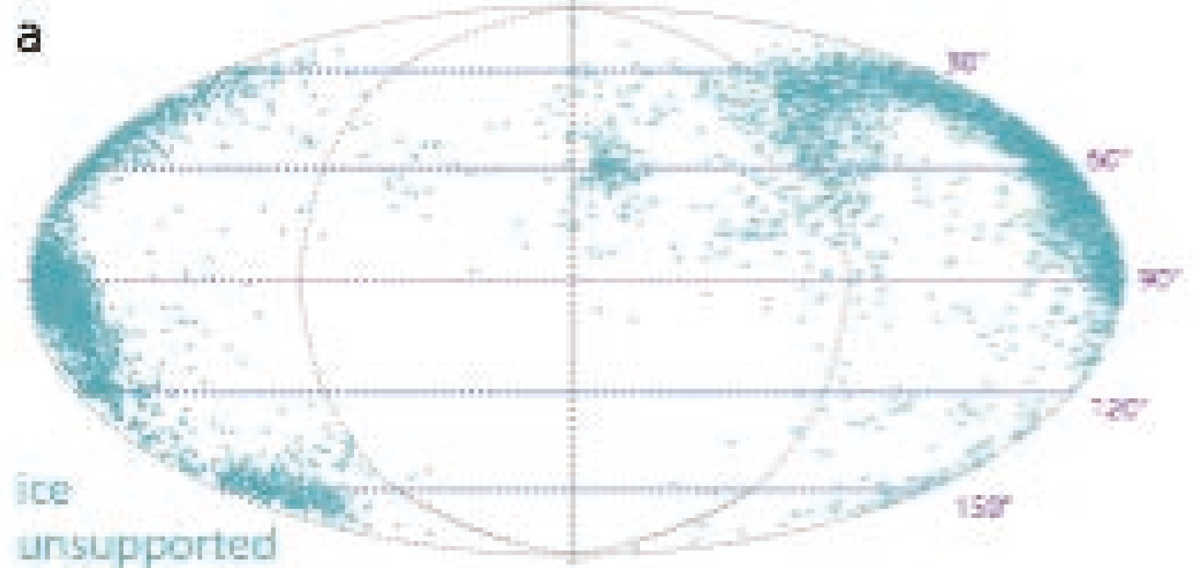
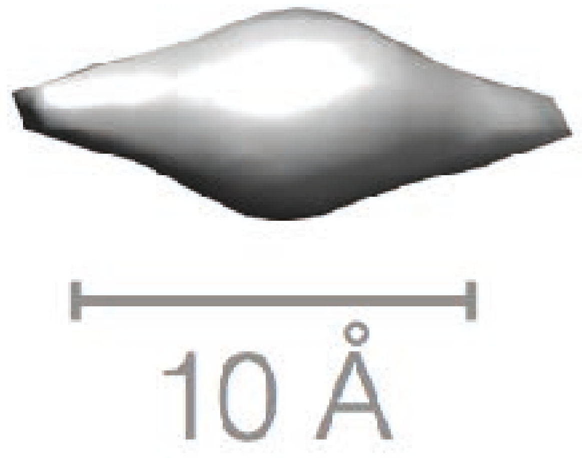


Naydenova & Russo, *Nature Comms* 2017

Which is better?

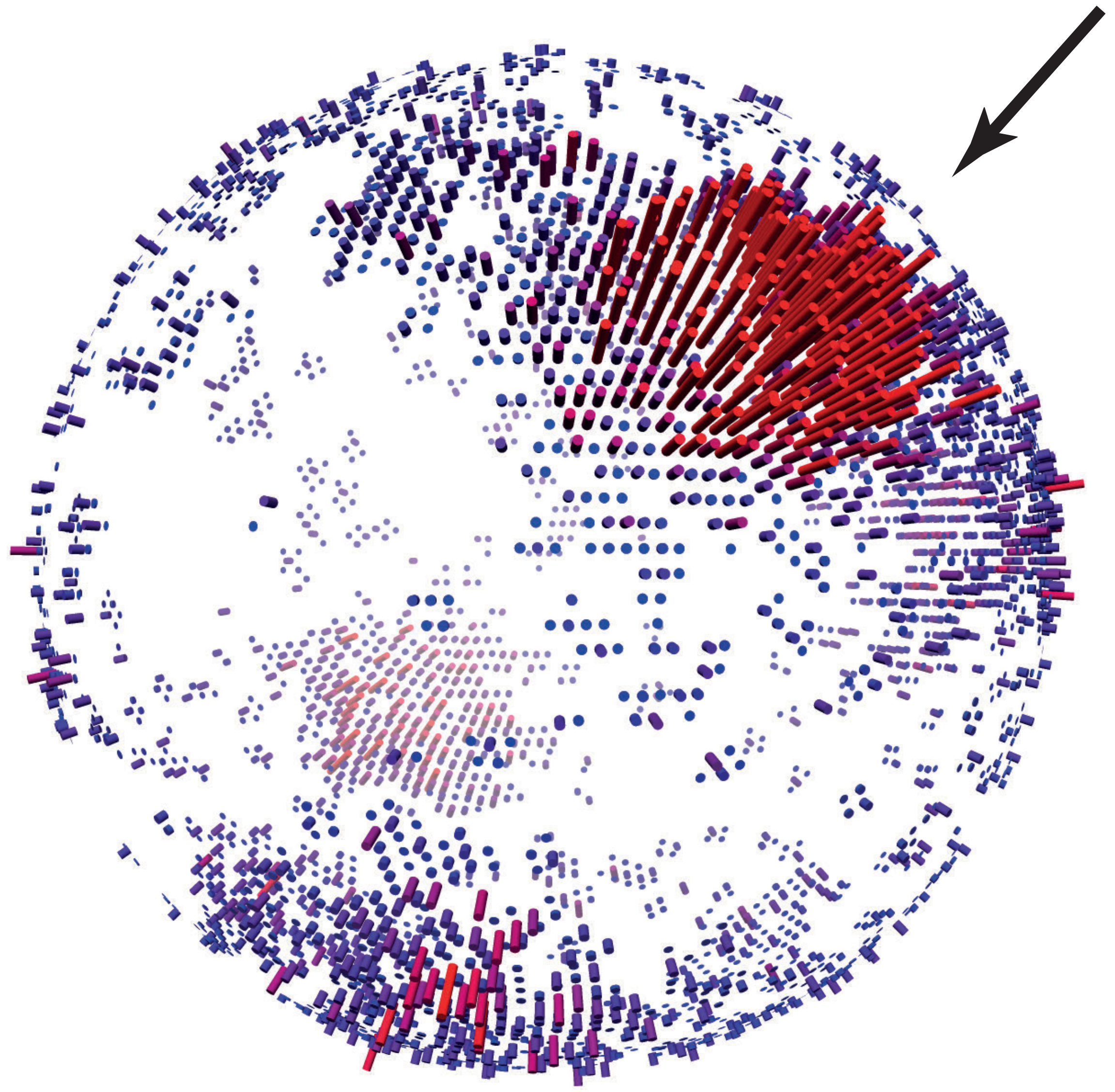


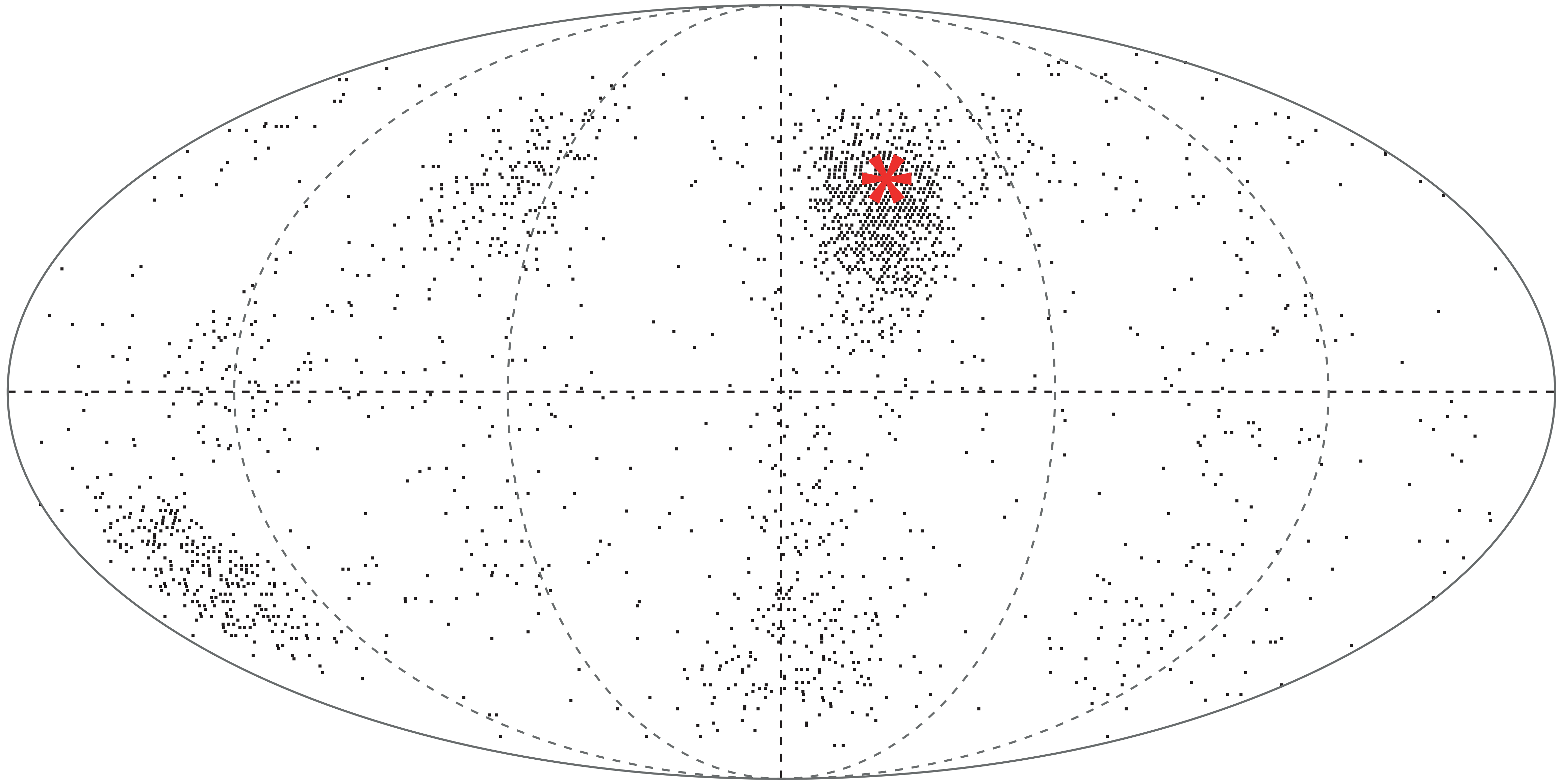
Russo and Passmore. "Controlling Protein Adsorption On Graphene For Cryo-EM Using Low-Energy Hydrogen Plasmas". Nature Methods 11.6 (2014): 649-652.

Substrate	OD	Number of particles	PSF	Efficiency
Amorphous C Glow discharged		10009		0.55
Amorphous C H-treated		4620		0.45
Graphene		6270		0.38
None		10412		0.29

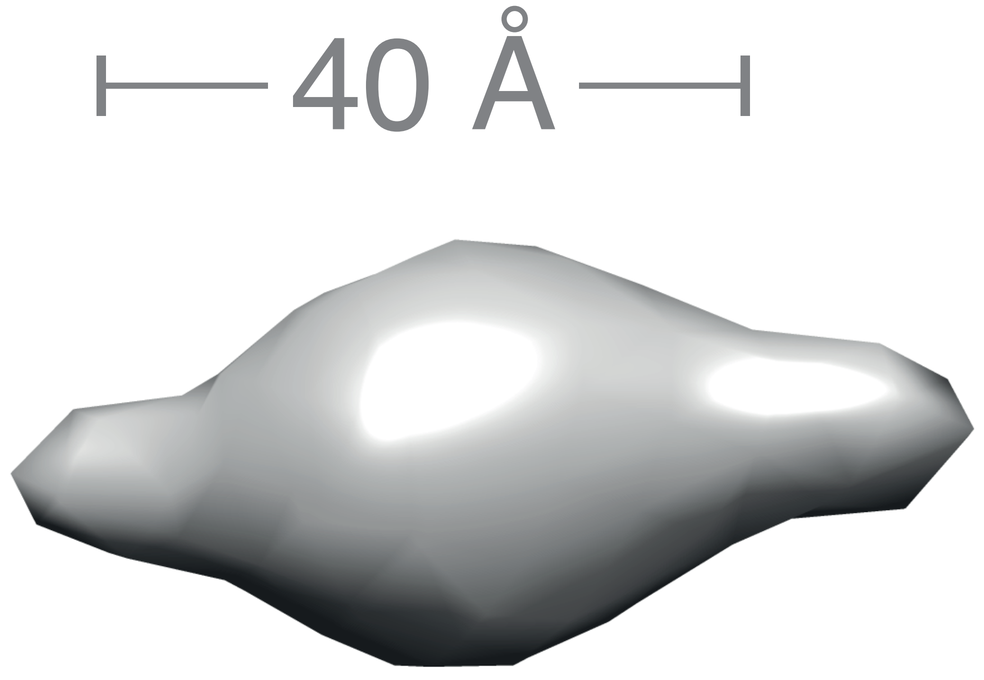
Tilting may improve the efficiency

Orientation distribution


$N = 2444$



Point spread fn.

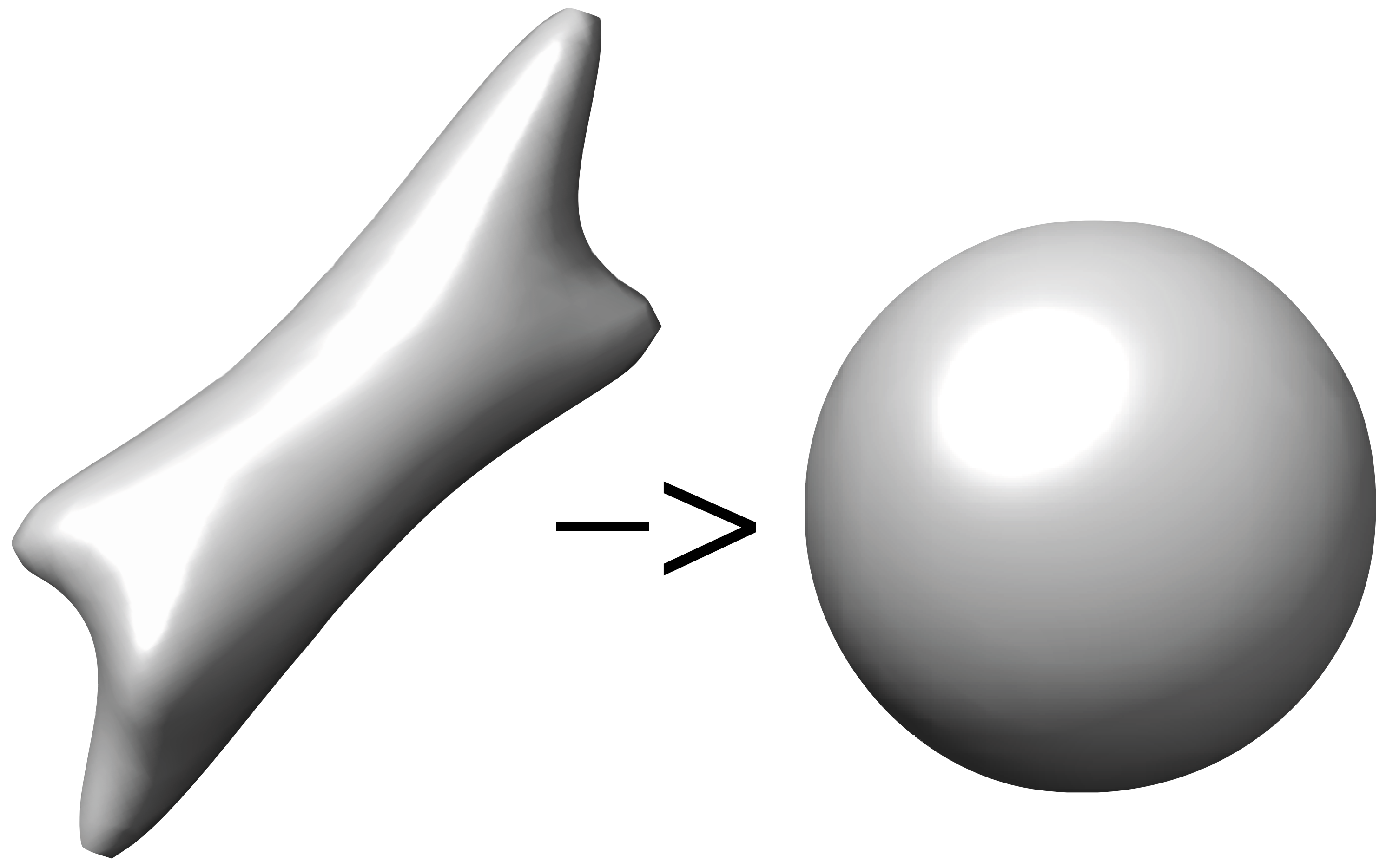


$R_{FSC} = 13.6$ Å
$E_{od} = 0.3$

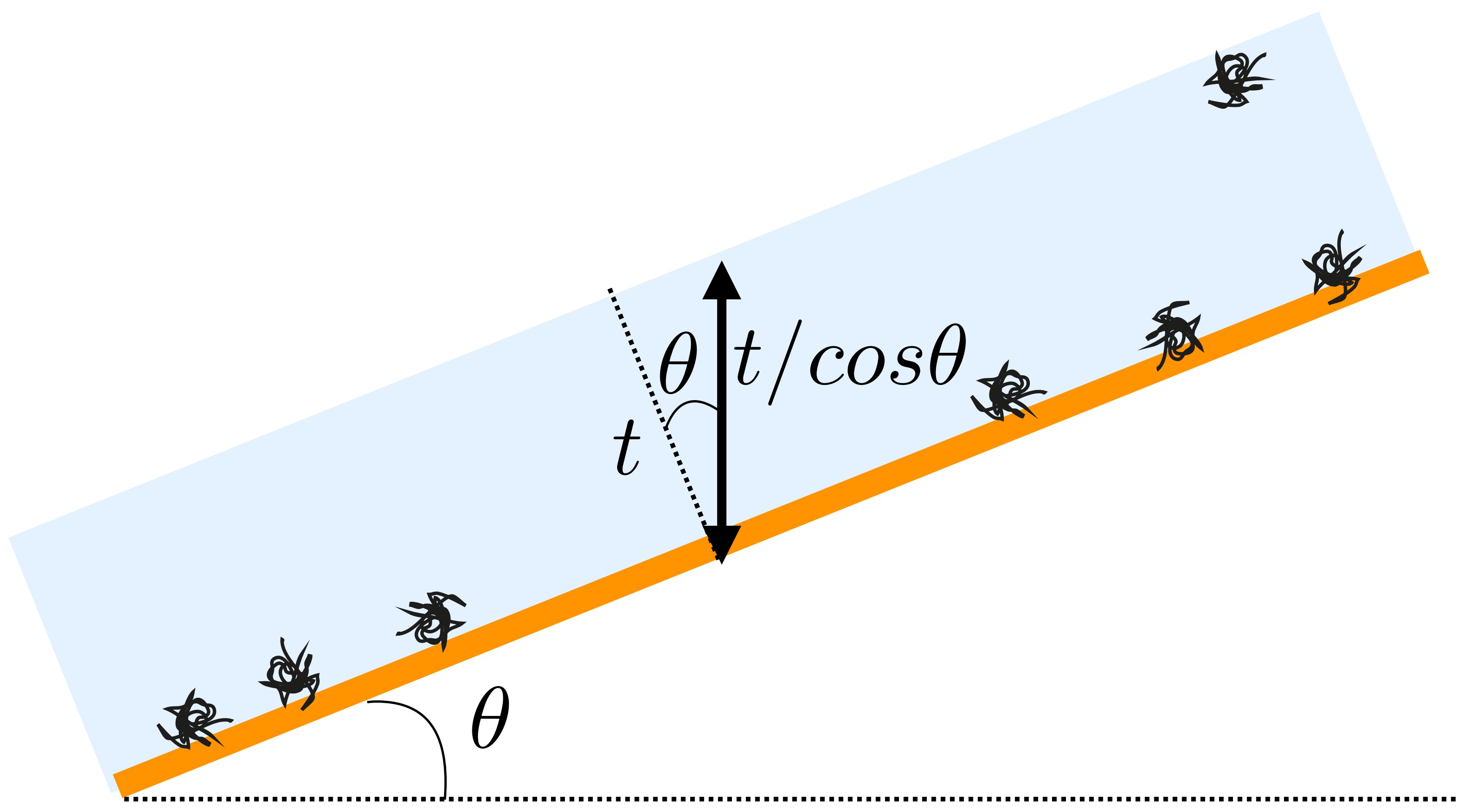
Naydenova & Russo, *Nature Comms* 2017

see also: Tan et al. *Nature Methods* (2017)

Optimal tilt angle determination



vs



filling up missing views

quality loss


Efficiency = 0.42
Combined Efficiency = 0.72
Tilt 29°

Input

Euler angles from 3D reconstruction
(RELION star file, FREALIGN par file, etc)

size of object

box size

FSC resolution

B factor value

symmetry group

CPU time ~ 0.1 - 10 hours

Output

K-space coverage map (.mrc and projection)

PSF (.mrc) and threshold for viewing

Efficiency estimate

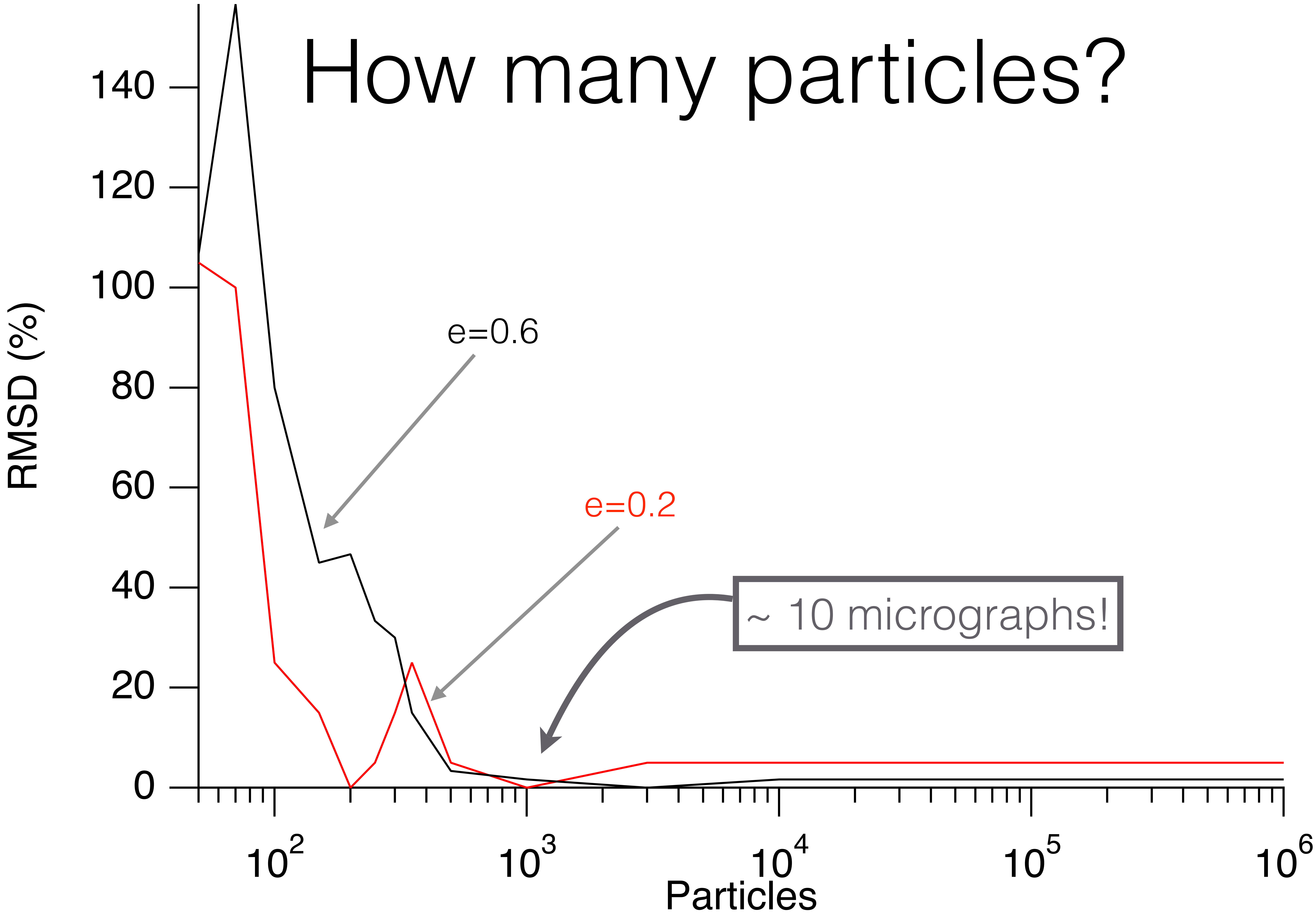
Weakest & strongest directions

Tilt recommendations

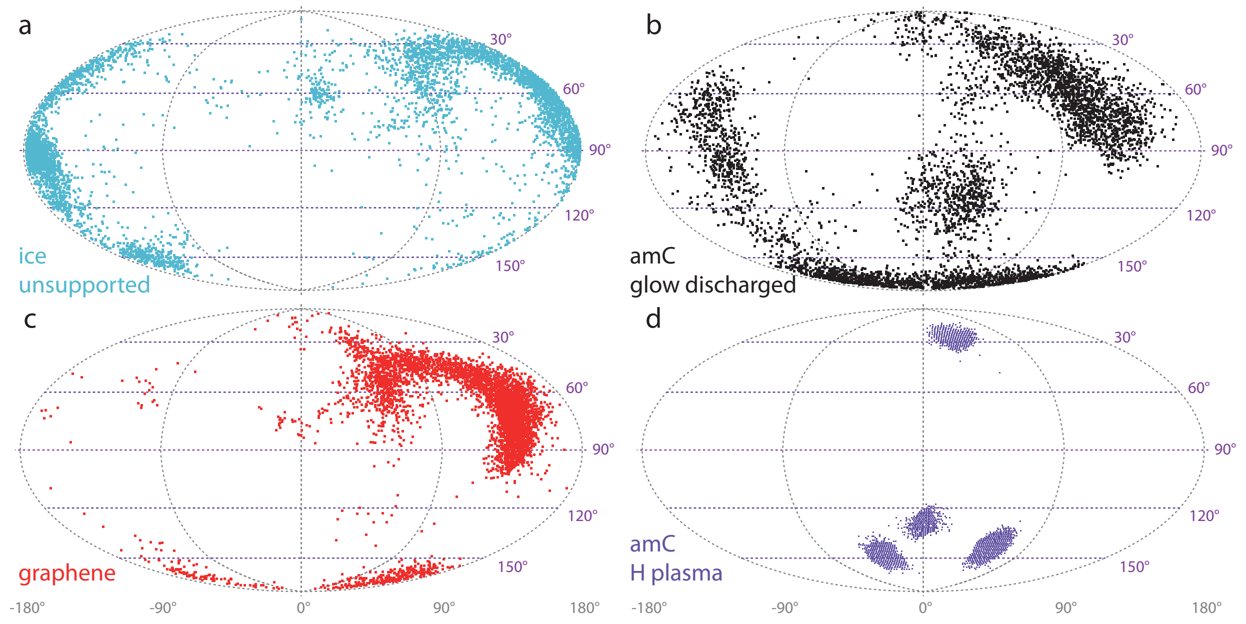
Naydenova & Russo, *Nature Comms* 2017

www.mrc-lmb.cam.ac.uk/crusso/cryoEF

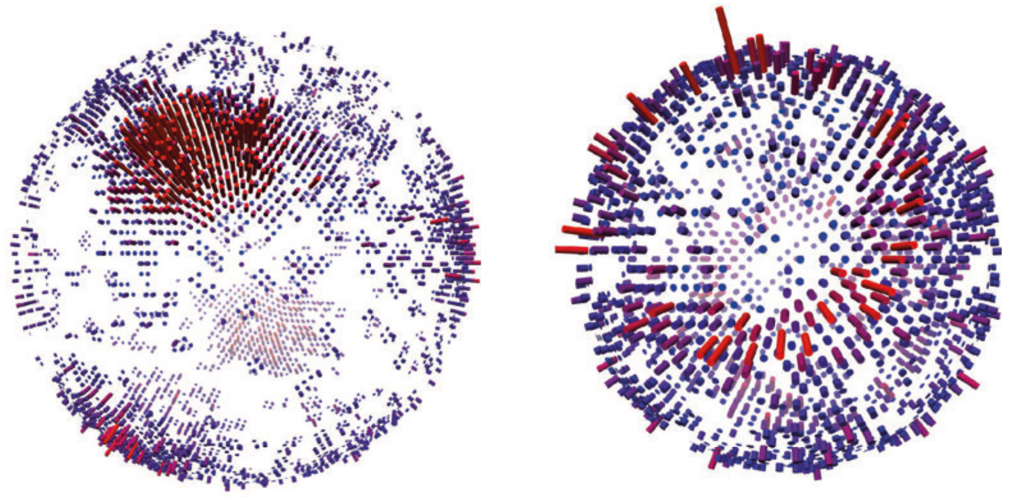
How many particles?



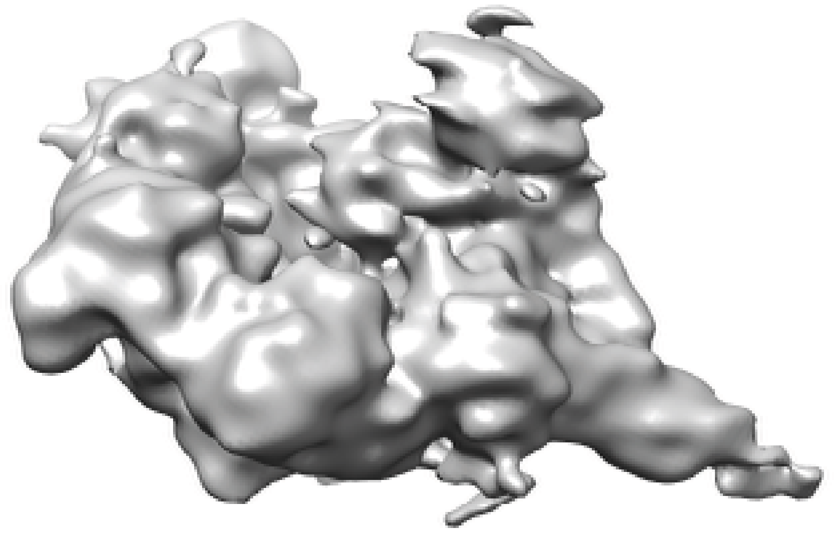
- Rigorously measure quality (efficiency) of the orientation distribution (OD) by analysing the anisotropic PSF.



- Use of the algorithm allows rapid (1000 particles!) assessment of different experimental conditions, support surfaces and their effect on the OD.



- Tilt can partially compensate for inefficient OD and the appropriate angles can be predicted from the PSF.



- Efficiency of the OD is at least as important as amount of data collected in reaching high resolution.

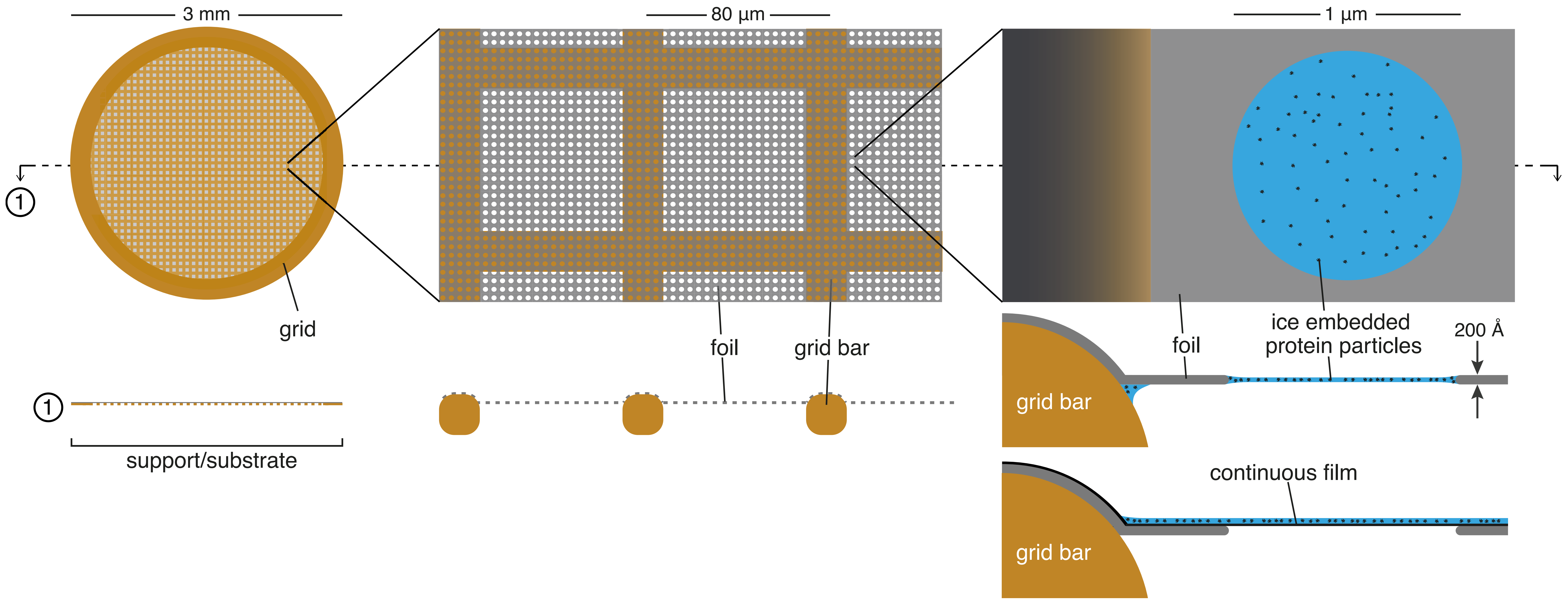
- Are there treatments that can be applied to thin carbon that are advantageous? *yes*

- Should we be using carbon at all? *<no|yes*>*

- Are there new substrates that offer advantages over the traditional thin carbon? *yes*

- Can we envision improving on these further using surface treatments? *yes*

Types of specimen supports



Grid materials

Copper	**Gold**
Nickel	CuRh
Titanium	Molybdenum
Silicon	Aluminum
	Tungsten

Foil materials

Amorphous carbon
Gold

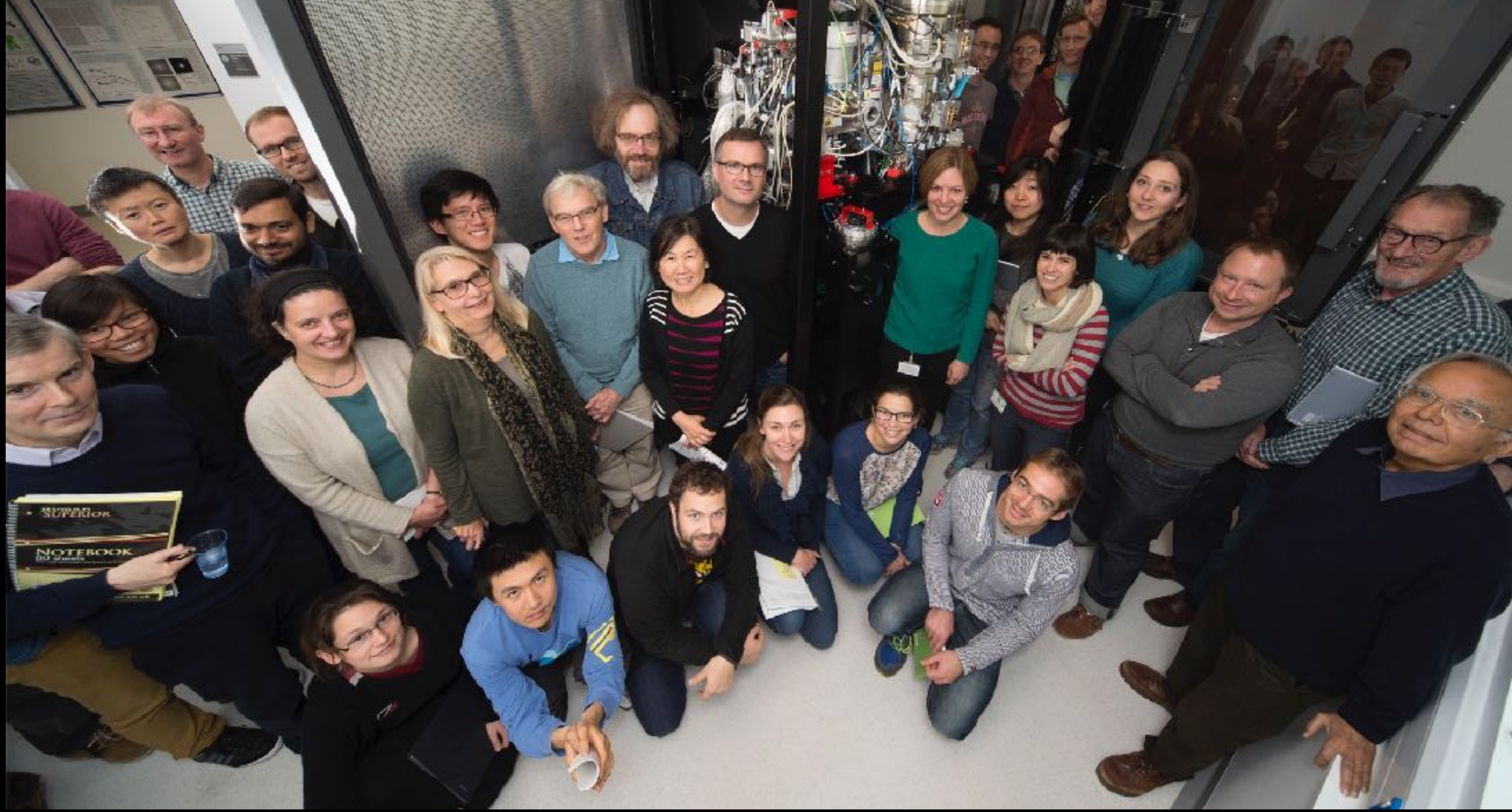
TiSi SiN
SiO_2 SiC

Film materials

Amorphous carbon
Graphene
Graphene oxide
TiSi
SA 2D crystal
& others

Russo & Passmore Current Op. 2016

image from GE Life sciences



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Lori Passmore
Tony Crowther
Greg McMullan

Israel Fernandez
Tanmay Bharat
Jan Löwe
Venki Ramakrishnan

Postdoc and
PhD positions
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