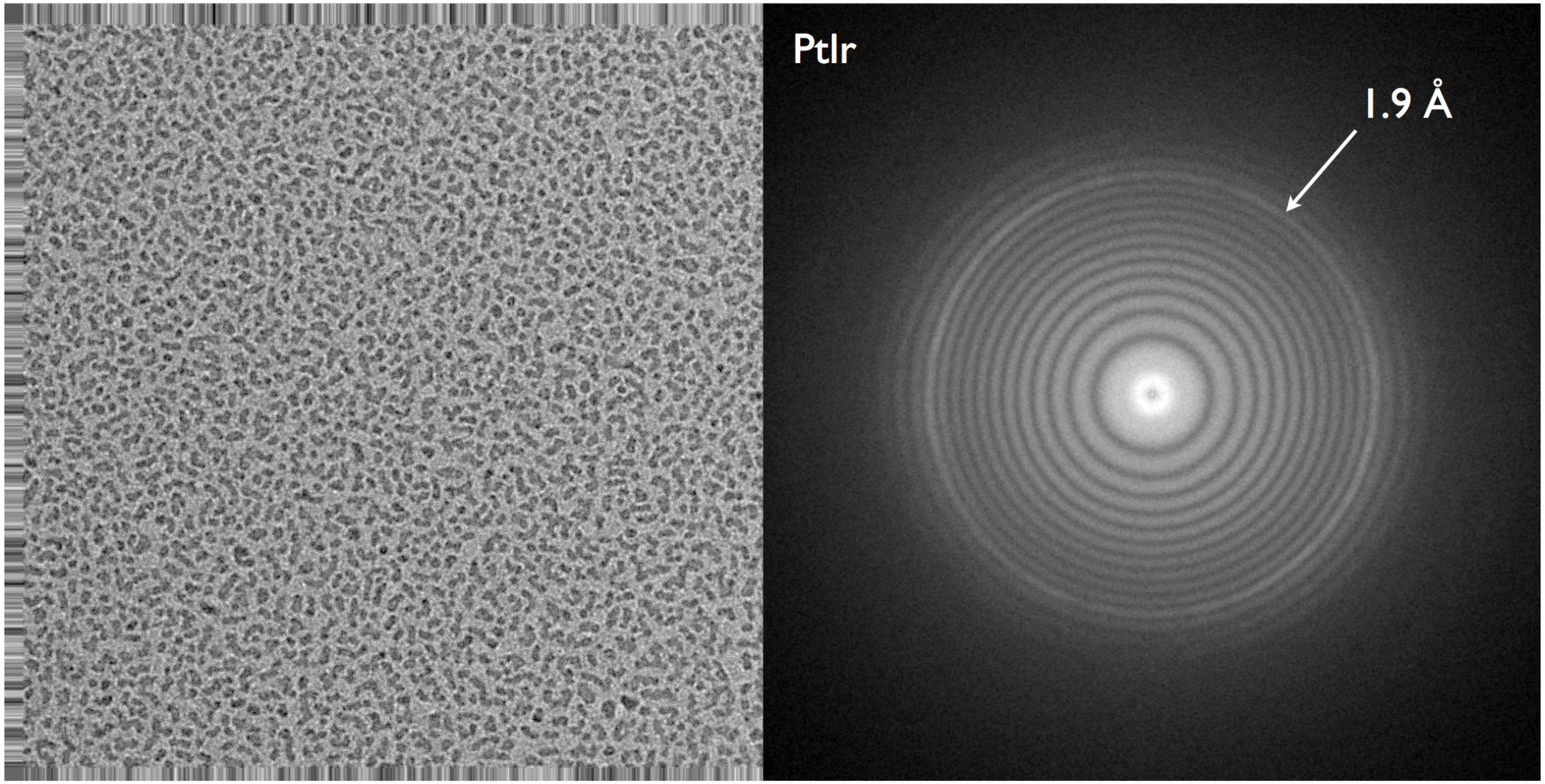
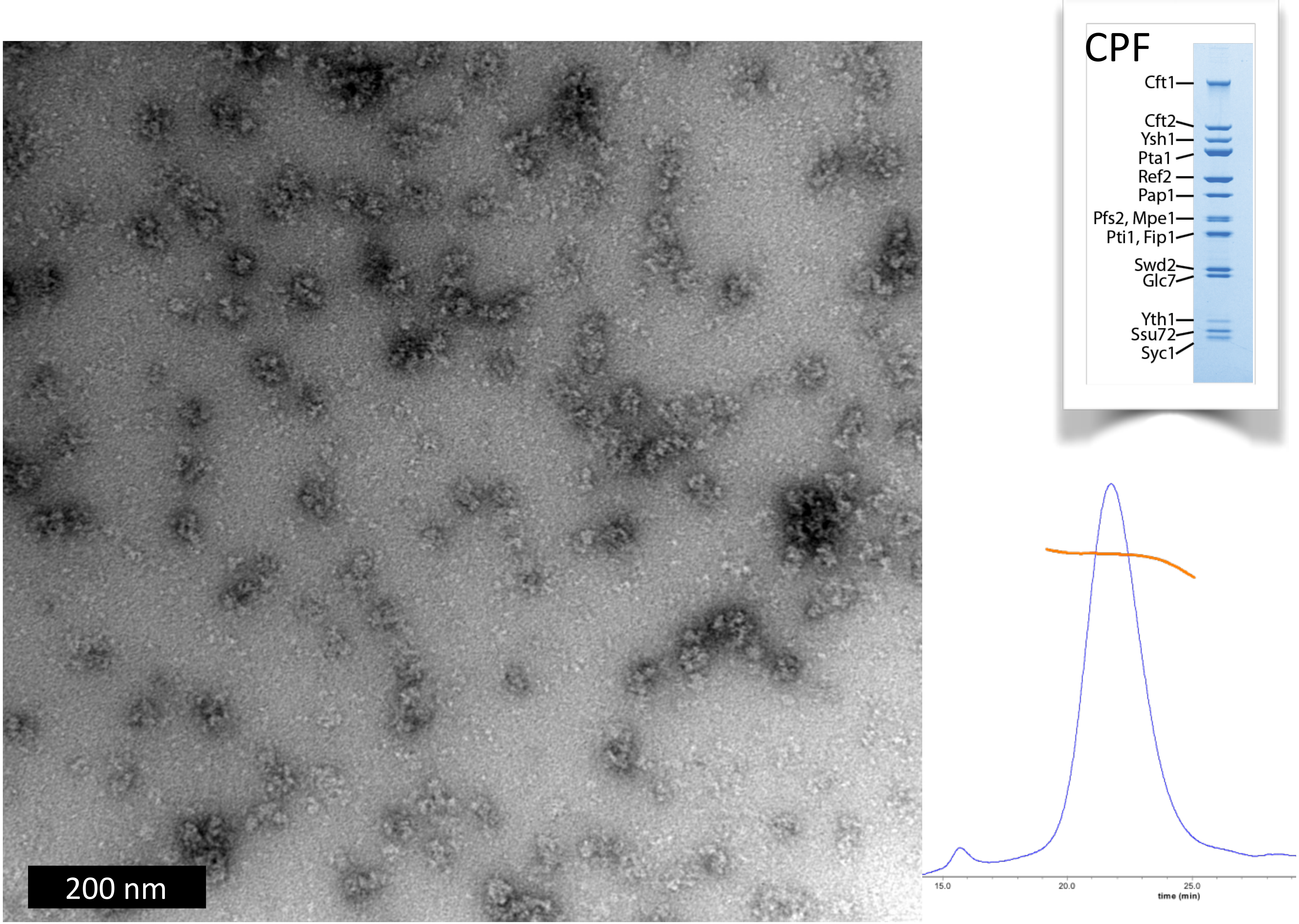
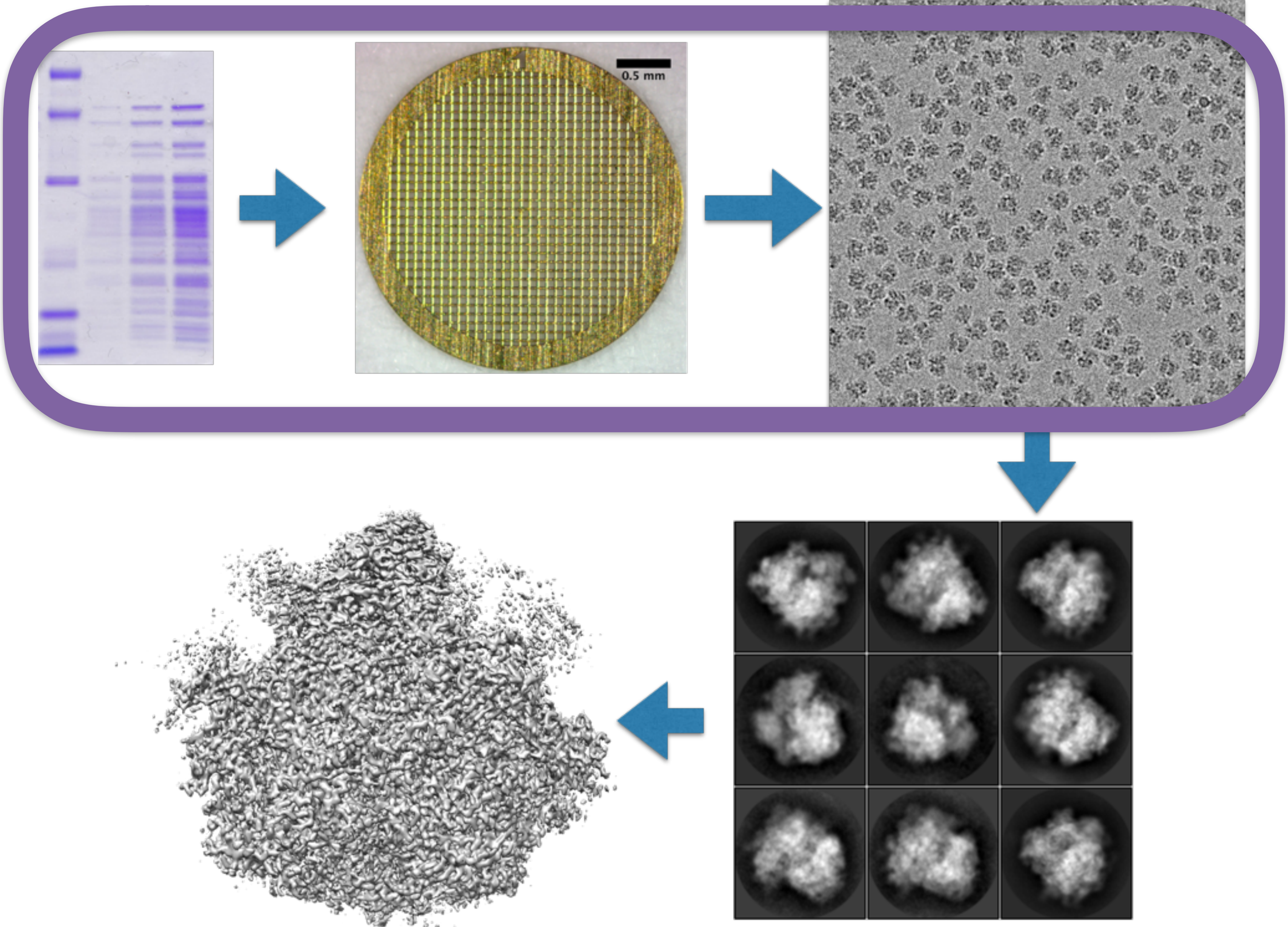
Specimen preparation for cryo-EM

Lori Passmore

What prevents us from routinely achieving atomic resolution in biology?




200 nm
CPF
Cft1
Cft2
Ysh1
Pta1
Ref2
Pap1
Pfs2, Mpe1
Pti1, Fip1
Swd2
Glc7
Yth1
Ssu72
Syc1
15.0
20.0
25.0
time (min)



Passmore & Russo 2016 Methods in Enz

Limitations of electron beam instruments

- **Vacuum**
- Damage Damage **Damage** Damage Damage Damage Damage

  Electron microscopes are used to simulate damage in the core of a nuclear reactor!

- Electron lenses terrible (relative to photon lenses) and hard to make
- Have to record many many **noisy** images, *lots* of data (just ask Jake & Toby!)
- **Charging**: non-conductive samples charge up and act like lenses
- **Samples must be very thin and are quite fragile, move around in the beam and are often difficult to make**
- Expensive (From £300k to £10M) Krios is £3000/day

Chris Russo

Goal of sample preparation

- **Preserve** the biological specimen in a **native state** such that it will give **maximum contrast**

- To obtain a high resolution structure:
  - Best specimen
  - Use optimal magnification
  - Use detector with best DQE possible
  - Obtain best micrographs possible

- Leading to **highest resolution structure** with **least data**

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

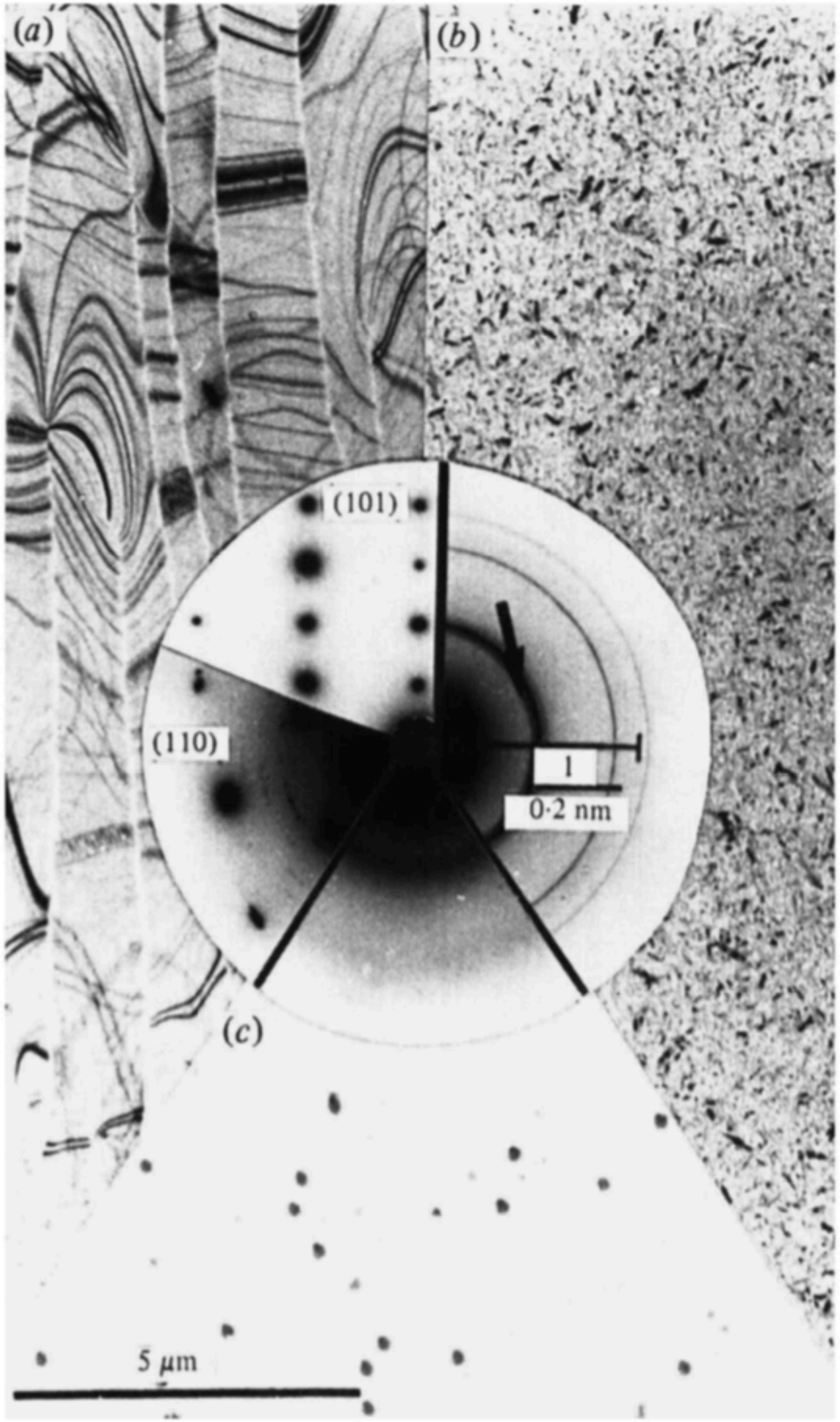
Cryo-electron microscopy of vitrified specimens

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

Hexagonal ice



Cubic ice

Vitreous ice

Dubochet et al QRB 1988

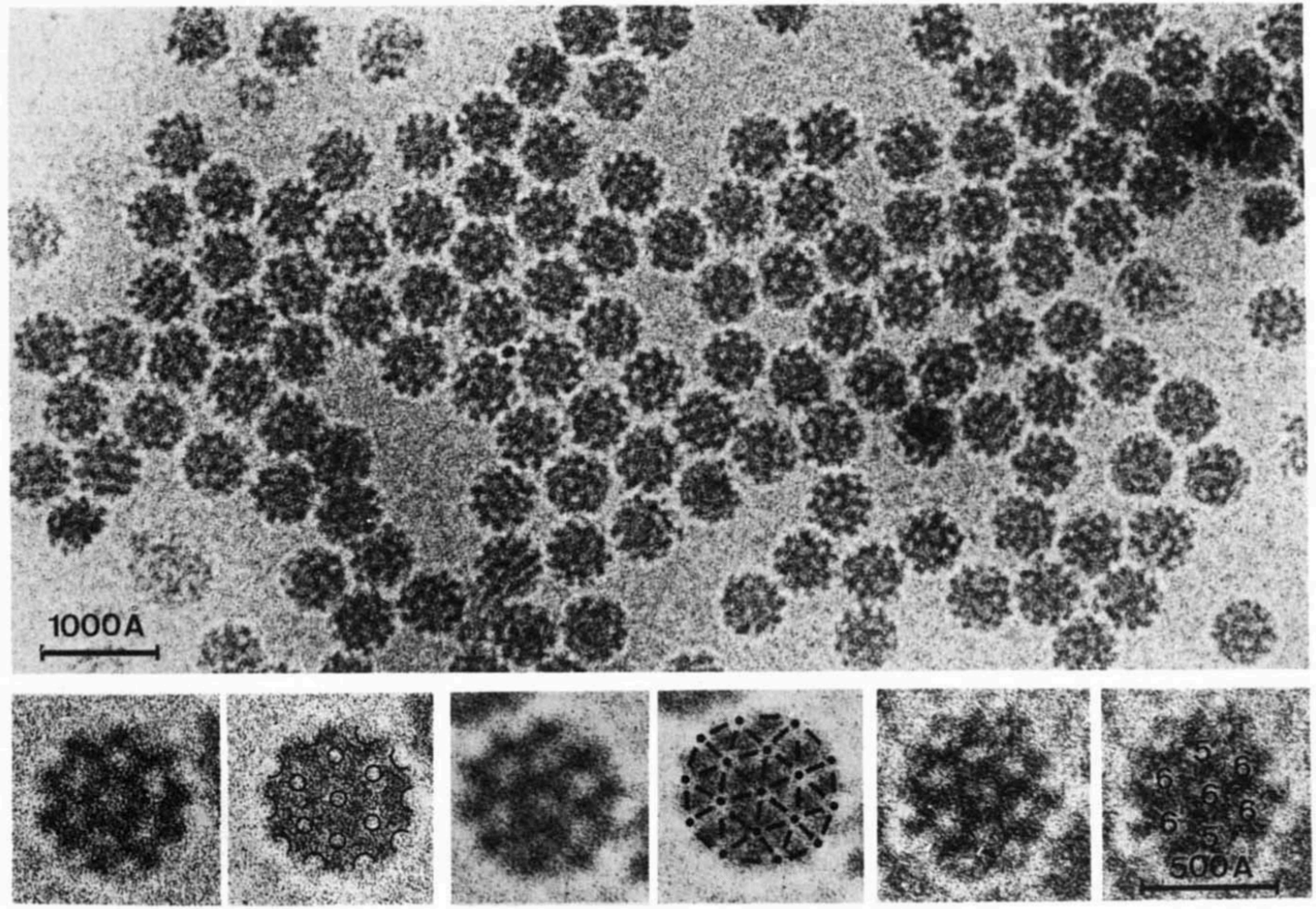
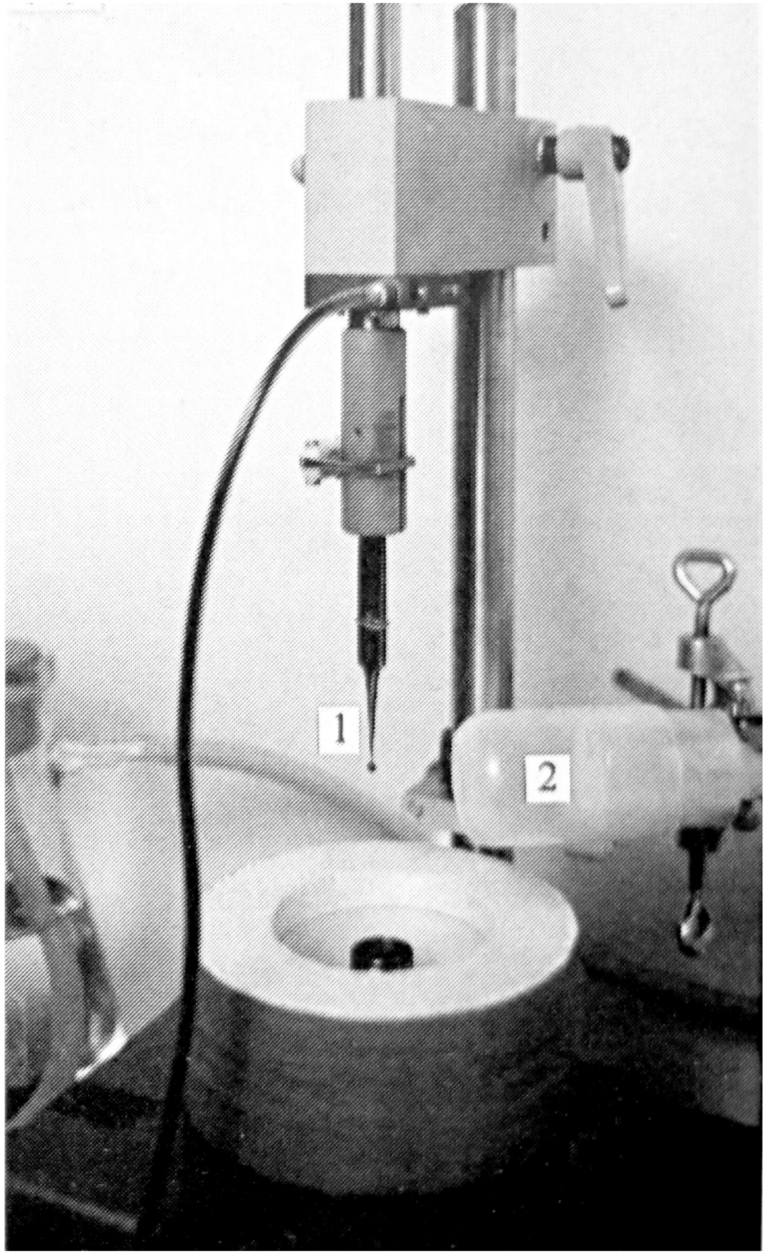


Fig. 4 Semliki Forest viruses prepared by the perforated film method. A suspension containing ~5 mg protein per ml in 50 mM Tris, 100 mM NaCl[32], was prepared as for Fig. 2. Electron optical magnification ×15,000. Underfocusing, 3.5 μm. Three selected images of virions projected along their 2-fold axis are shown at higher magnification. They are underfocused by 6–9 μm. Each image is duplicated and superimposed with the schematic outline of the structure, the triangular network, or a designation of the 5- and 6-fold low density nodes.

©1984 Nature Publishing Group

Adrian et al Nature 1984

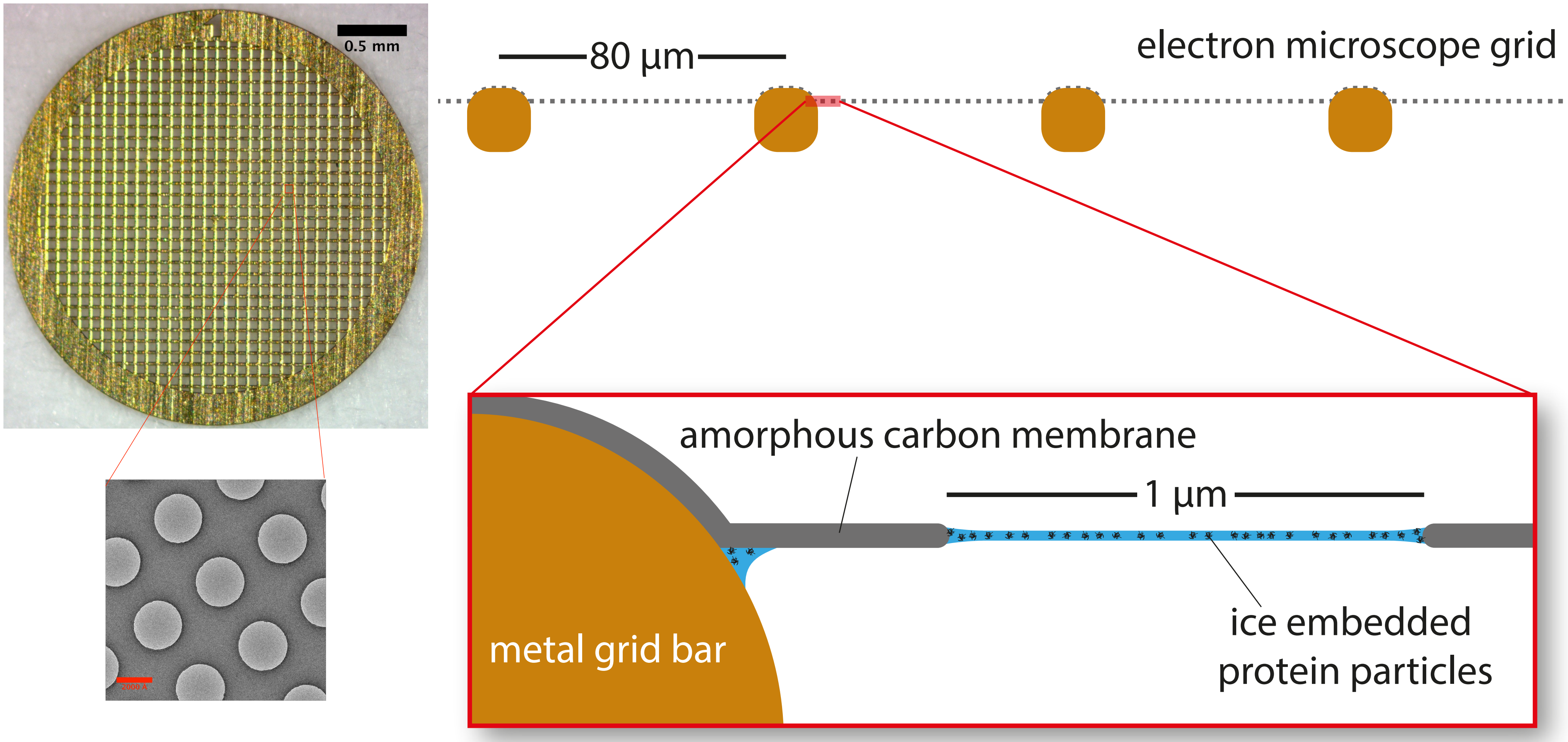
Vitrification

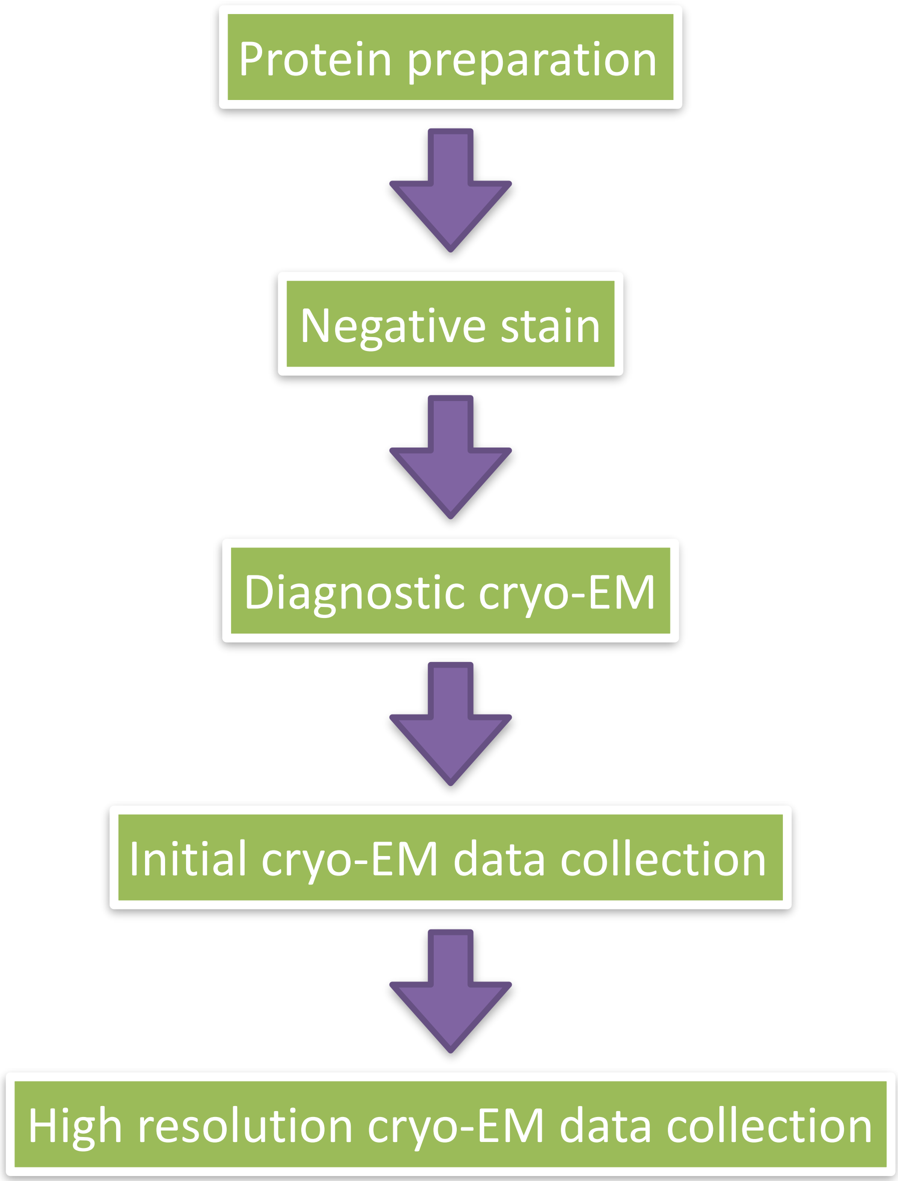


Dubochet and colleagues 1984   EMBL

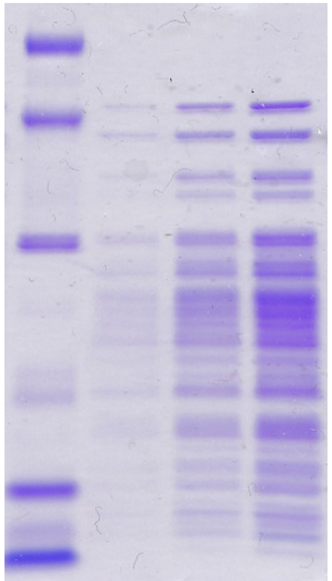
Vitrification

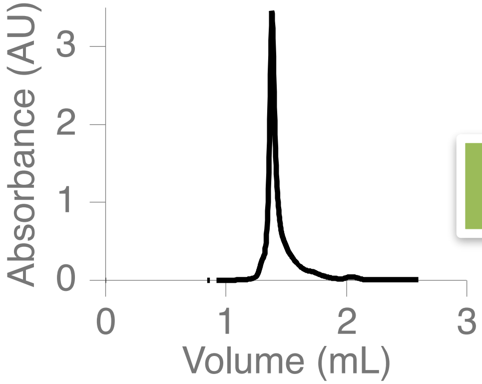
Traditional substrates for cryo-EM





Passmore & Russo MiE 2016



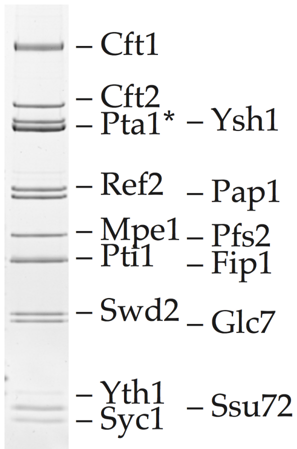


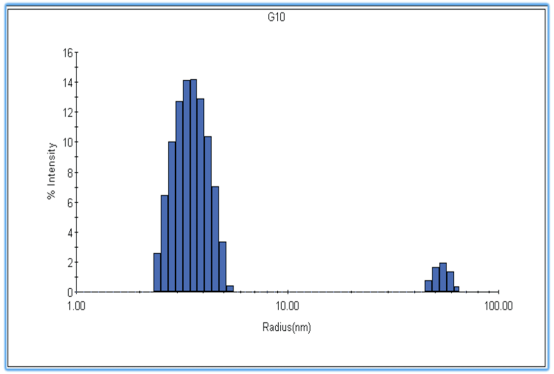
Protein preparation

- Composition
- Purity homogeneity
- Stability
- Biochemical activity

Passmore & Russo MiE 2016

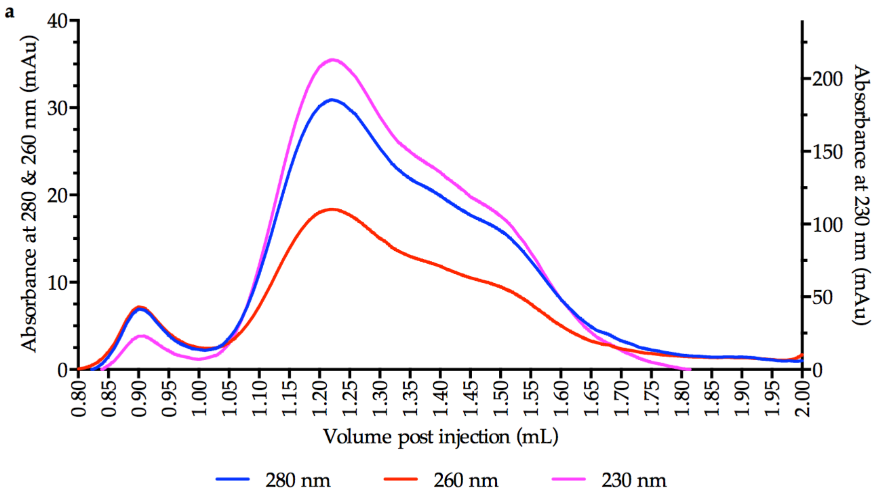
Homogeneity



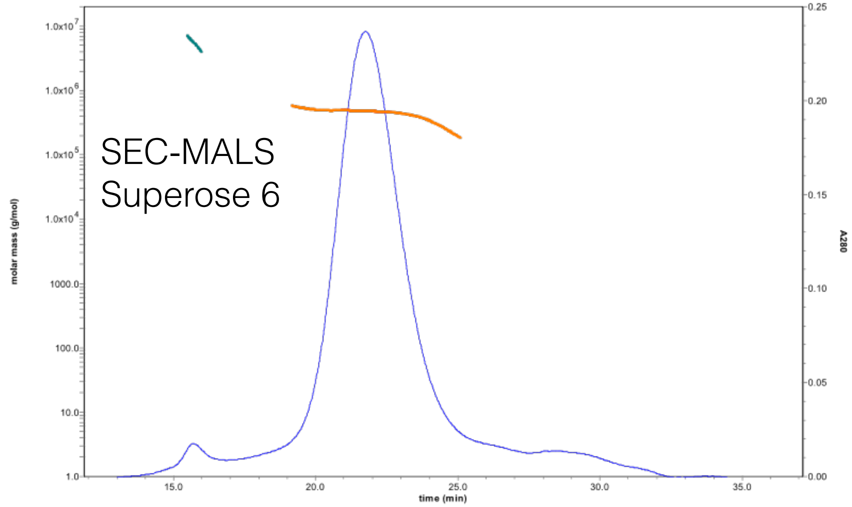


DLS
(Dynamic light scattering)

*Optimise buffer conditions
(salt, pH, detergent, etc)



Size exclusion chromatography



SEC MALS
(Size exclusion chromatography coupled to multi-angle light scattering)

Quantity

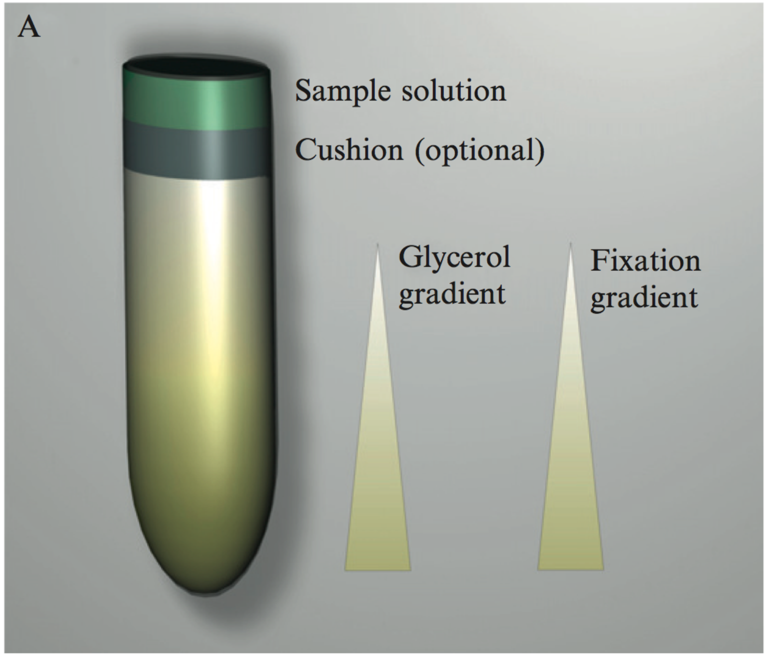
2 – 4 μl / grid

50 nM – 5 μM concentration

- Make sure your complex is stable at these concentrations (e.g. consecutive SEC runs)

What if my complex dissociates at low concentrations?

- Work at higher concentrations, adjust plasma and blotting
- Buffer conditions
- Crosslinking can stablise protein-protein interactions (between subunits or domains)
  - Changes surface properties so can change particle orientations on grid
  - Must minimise or remove aggregates due to intra-complex crosslinks



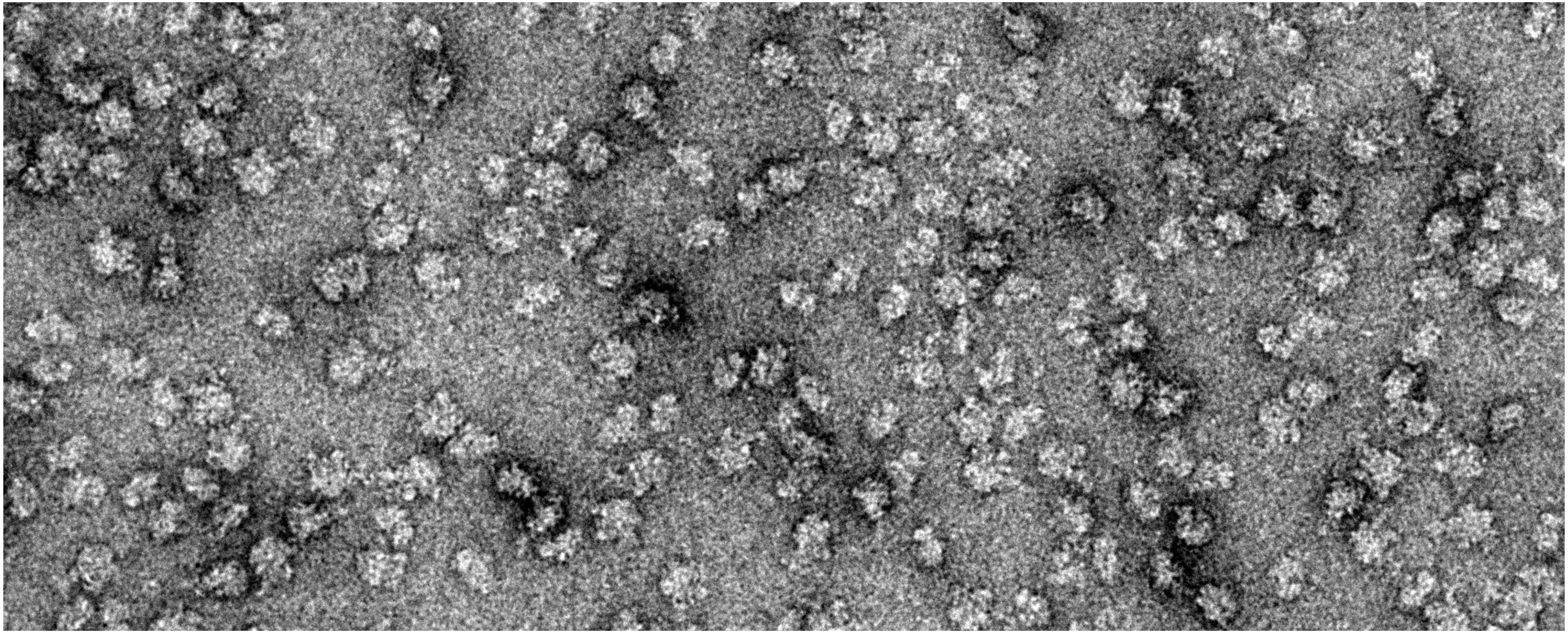
Kastner et al (2008); Stark (2010)

Protein preparation

Negative stain

Entry level
electron microscope

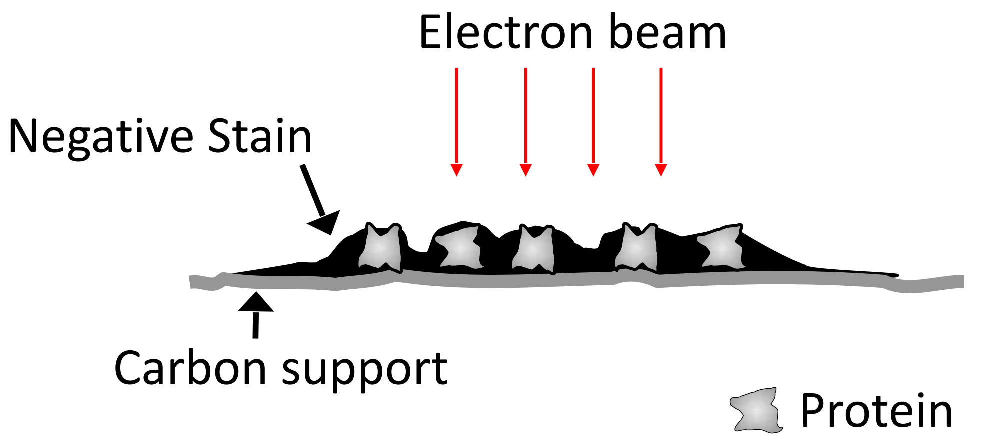
- Discrete particles
- Stability
- Particle size and shape



Passmore & Russo MiE 2016

Negative stain

- Sample is embedded in a layer of heavy metal salts (e.g. uranium, molybdenum, tungsten) which surrounds the protein like a shell
- Reveals the solvent-excluded surface and shape of the molecule



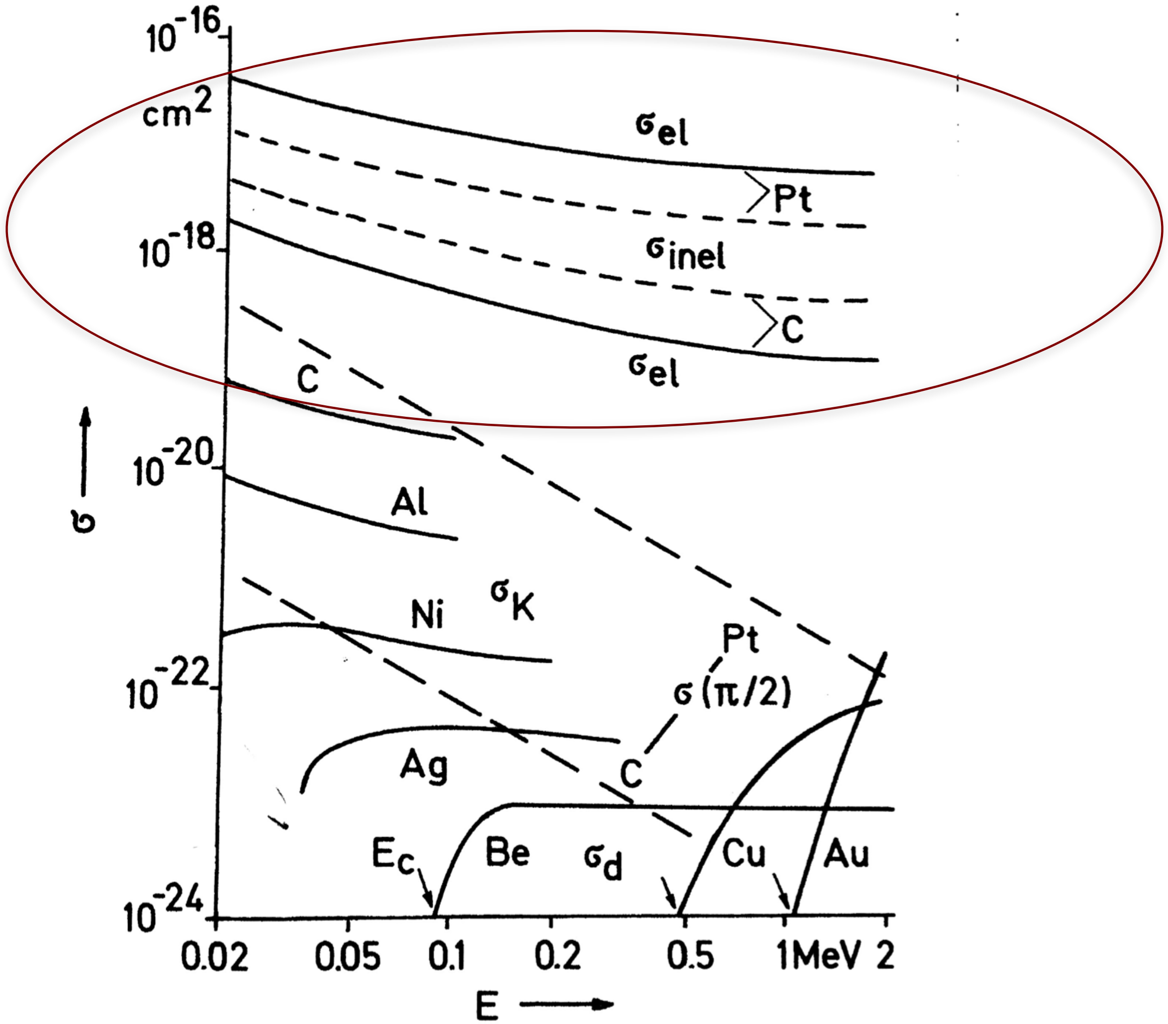
Biochim Biophys Acta. 1959 Jul;34:103-10.

A negative staining method for high resolution electron microscopy of viruses.

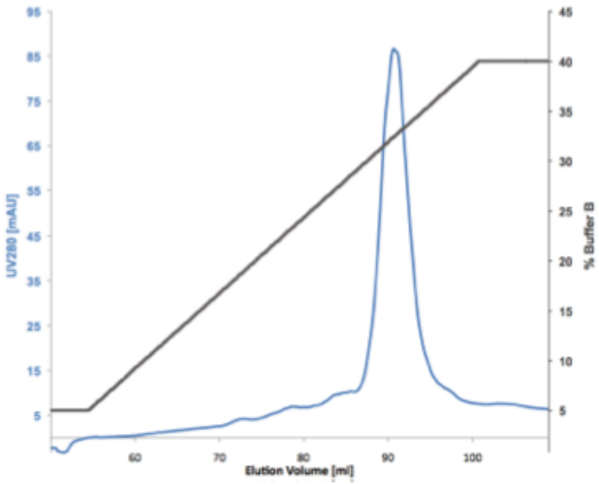
BRENNER S, HORNE RW.

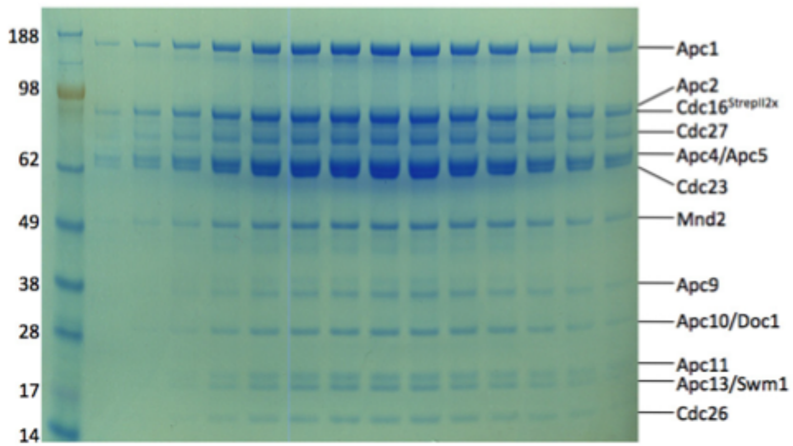
PMID: 13804200 [PubMed - indexed for MEDLINE]

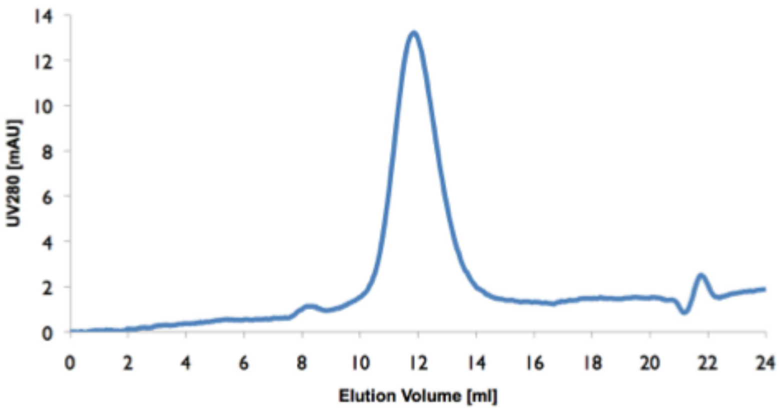
Inelastic vs elastic scattering

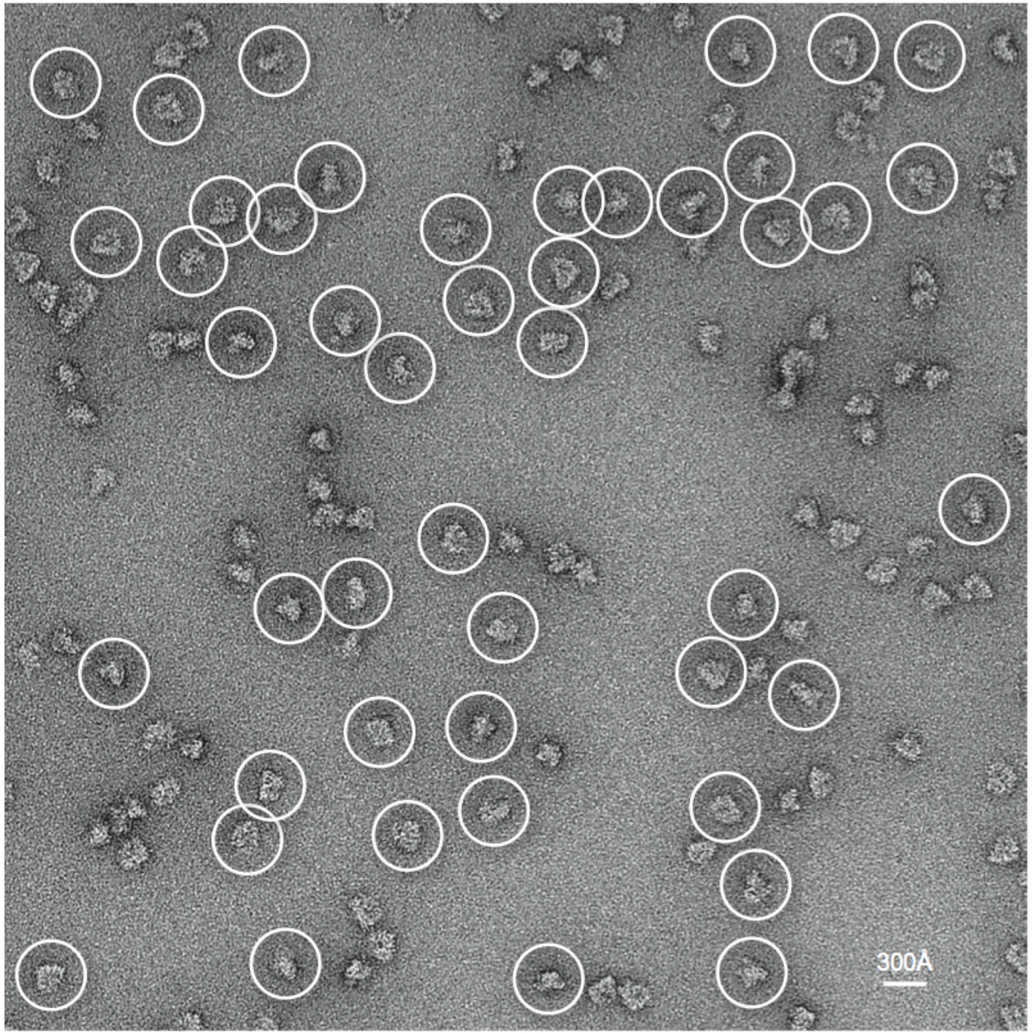


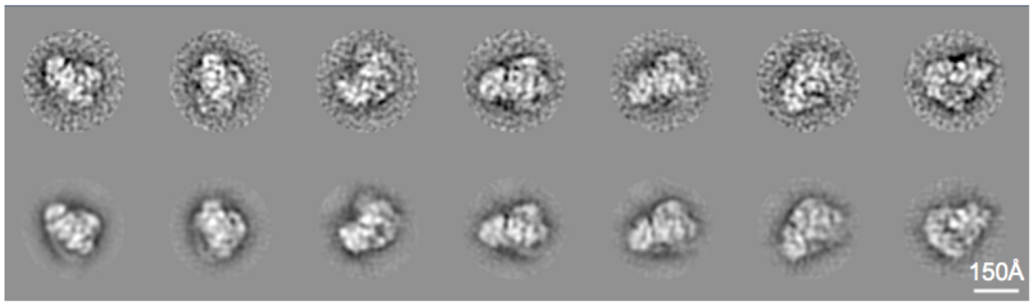
Reimer 2008











Schreiber *et al* (2011) Nature
Da Fonseca *et al* (2011) Nature

Negative stain

- **Advantages**
  - **Quick** to screen lots of conditions (10-20 in a day)
    - Evaluate homogeneity and size, presence of binding partners
  - Very high contrast – small molecules
  - Mildly radiation sensitive
  - Relatively straightforward

- Disadvantages
  - Limited resolution (20 Å)
  - Protein damage or distortion (high salt, low pH, dehydrated)
    - often proteins are flattened
  - Uneven staining leads to difficulties with processing/classification

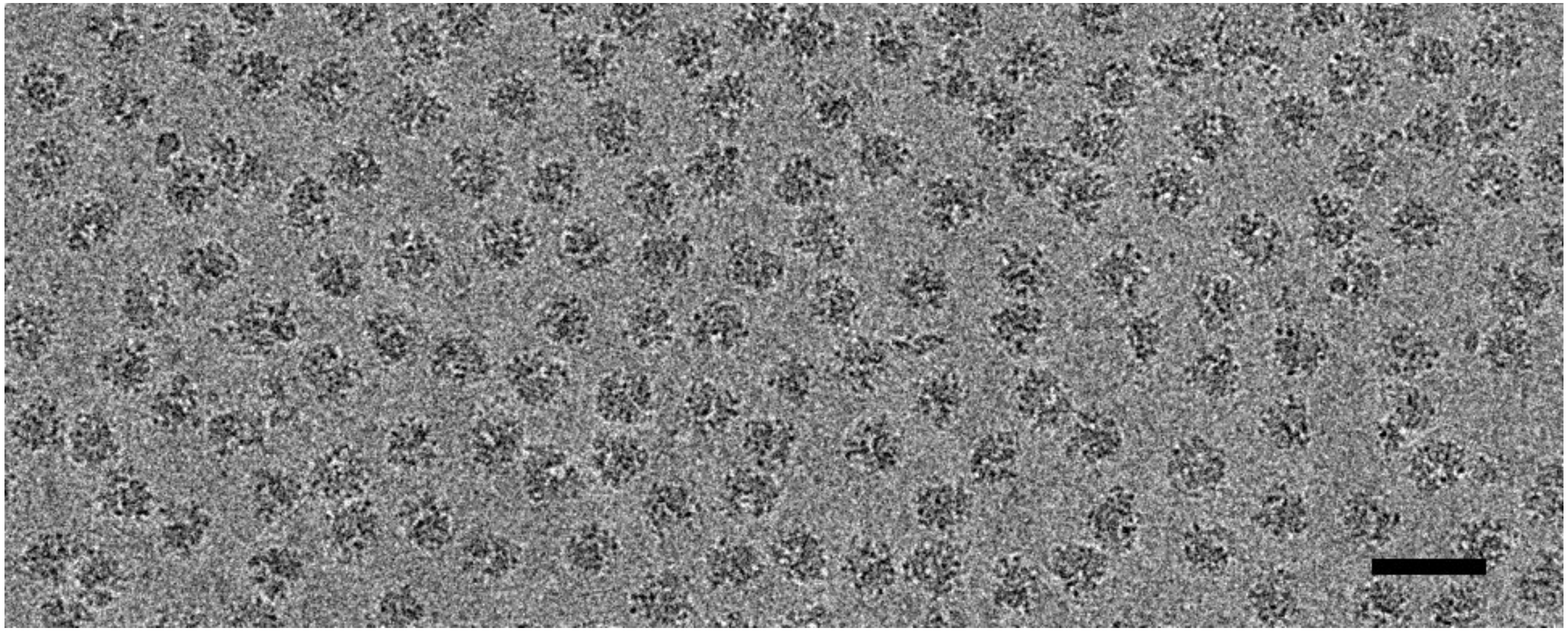
Protein preparation

Negative stain

Diagnostic cryo-EM

Entry level or mid-range electron microscope

- Stability
- Particle size & shape
- Particle distribution vs concentration



Passmore & Russo MiE 2016

Cryo-EM

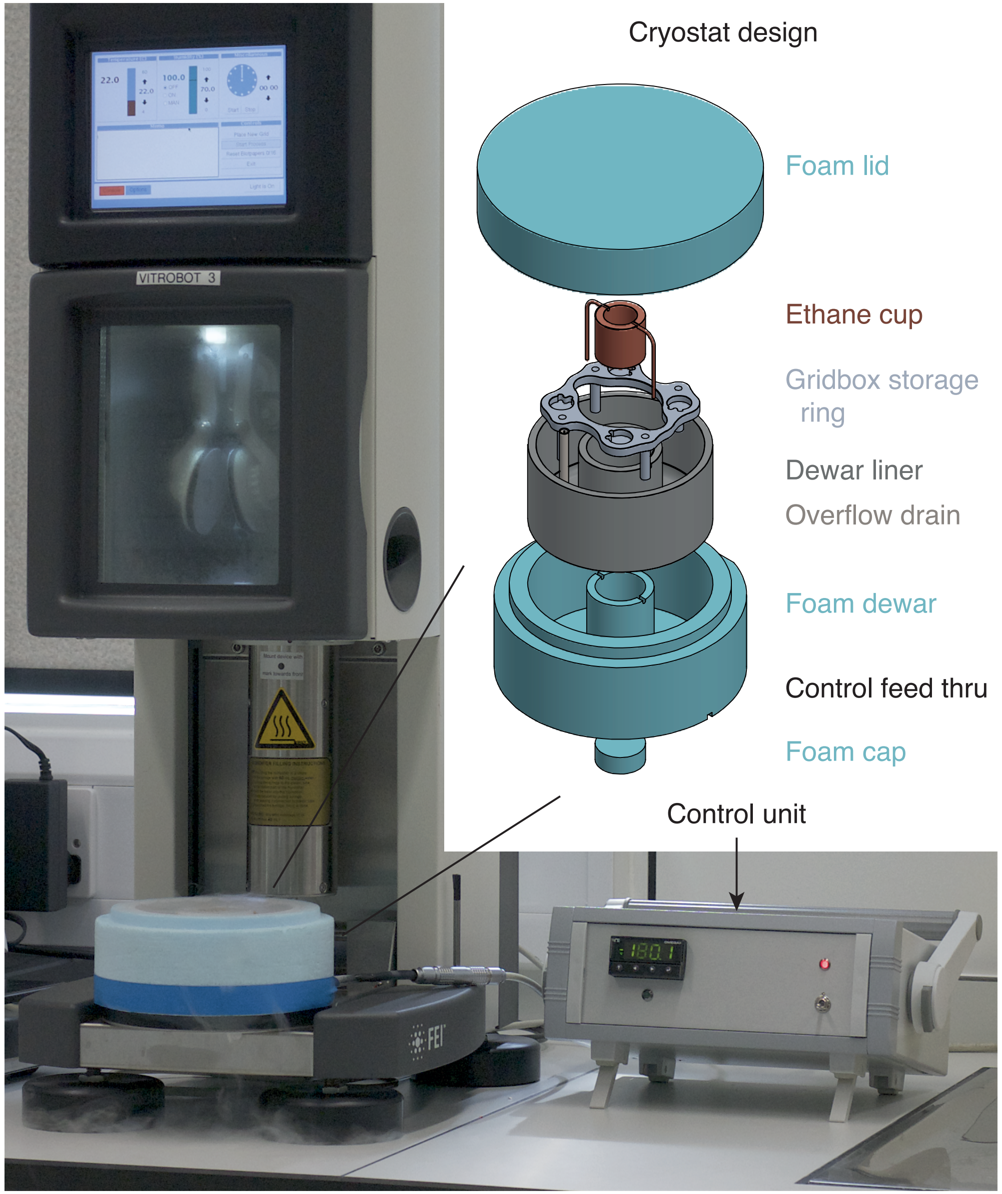
- Sample is <u>rapidly</u> frozen in buffer (vitrification) to prevent formation of crystalline ice
  - Vitrification is fast (10^{-4} s)
  - Protein is preserved in a "hydrated", near-native environment (amorphous ice)

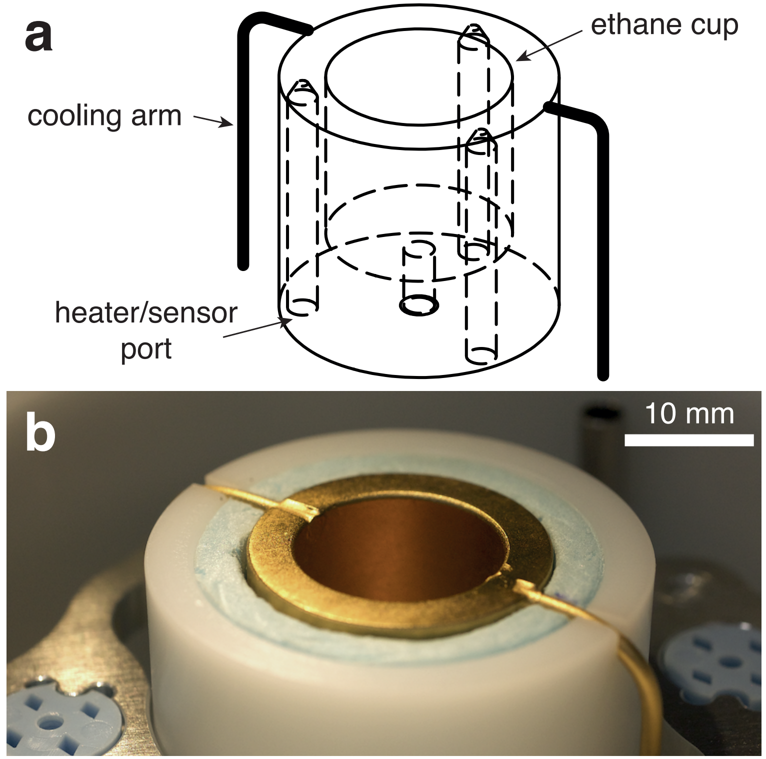
- Contrast is low so need to minimise background signal
  - Protein is 1.36 g/cm^3 (compared to water at 1.0 g/cm^3)

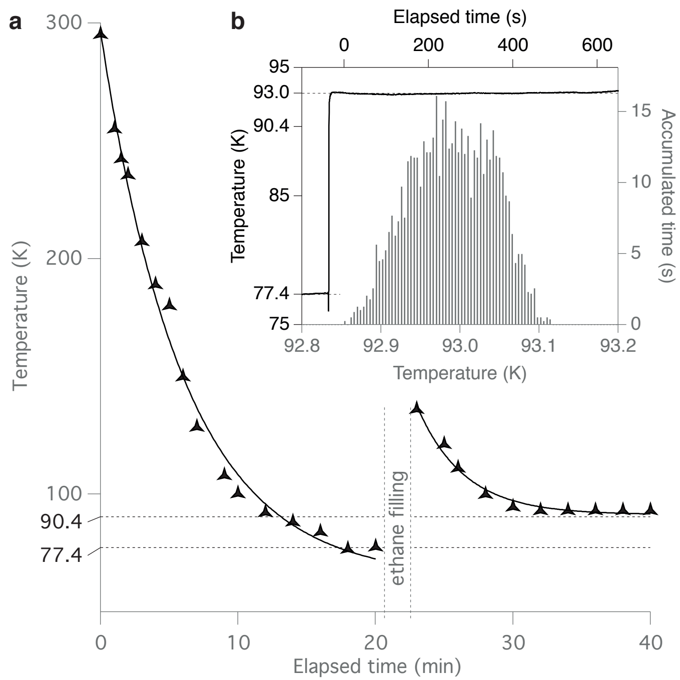
- Layer of water must be thin

- At 4°C and 90% relative humidity, the evaporation velocity is of order 100 Å/s so in the 2 s between the blot and the freeze, a 400 Å film can be concentrated by a factor of two

Ethane temperature

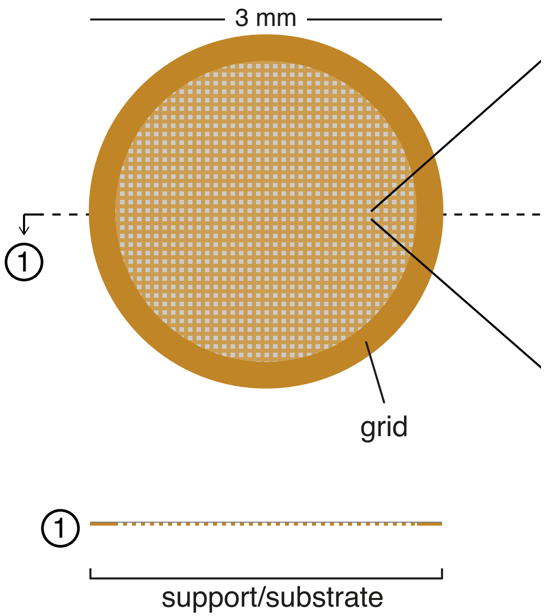






Russo et al. 2016 Rev Sci Instrum

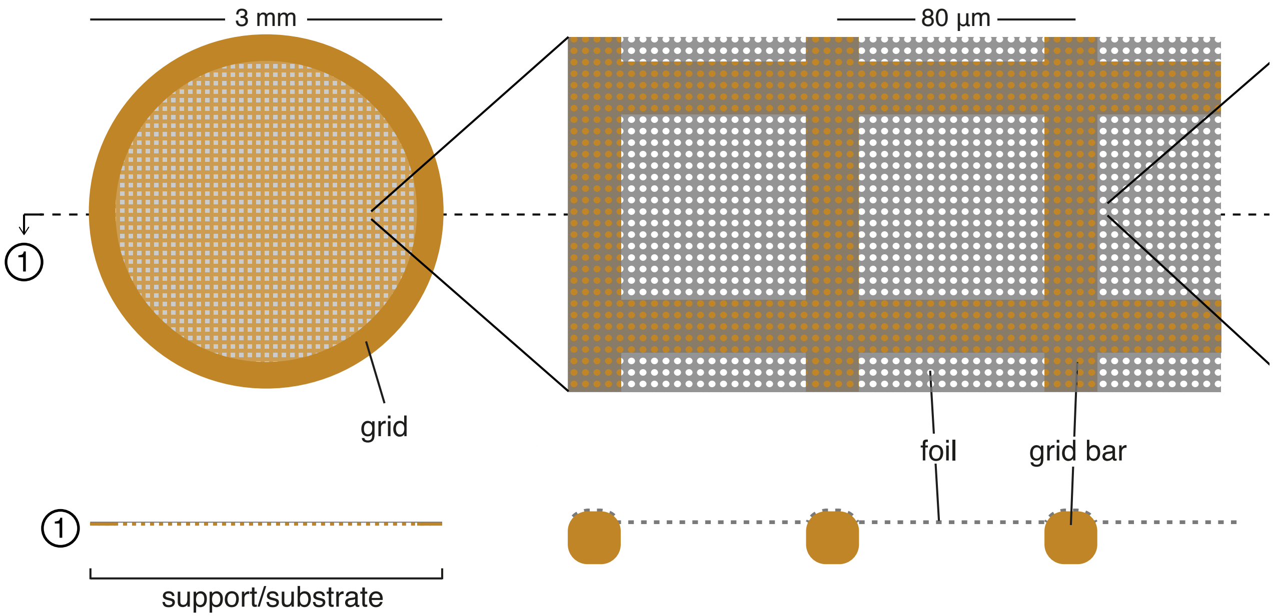
Types of specimen supports

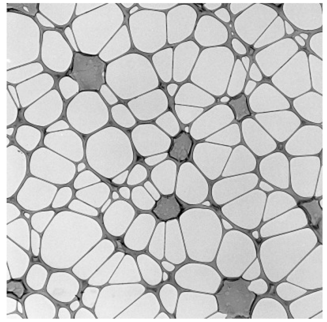


Grid materials	
Copper	**Gold**
Nickel	CuRh
Titanium	Molybdenum
Silicon	Aluminum
	Tungsten

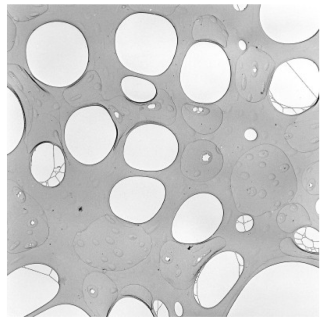
Russo and Passmore, Curr Op Struct Biol, 2016

Types of specimen supports





Lacey carbon



Holey carbon

Grid materials

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

Foil materials

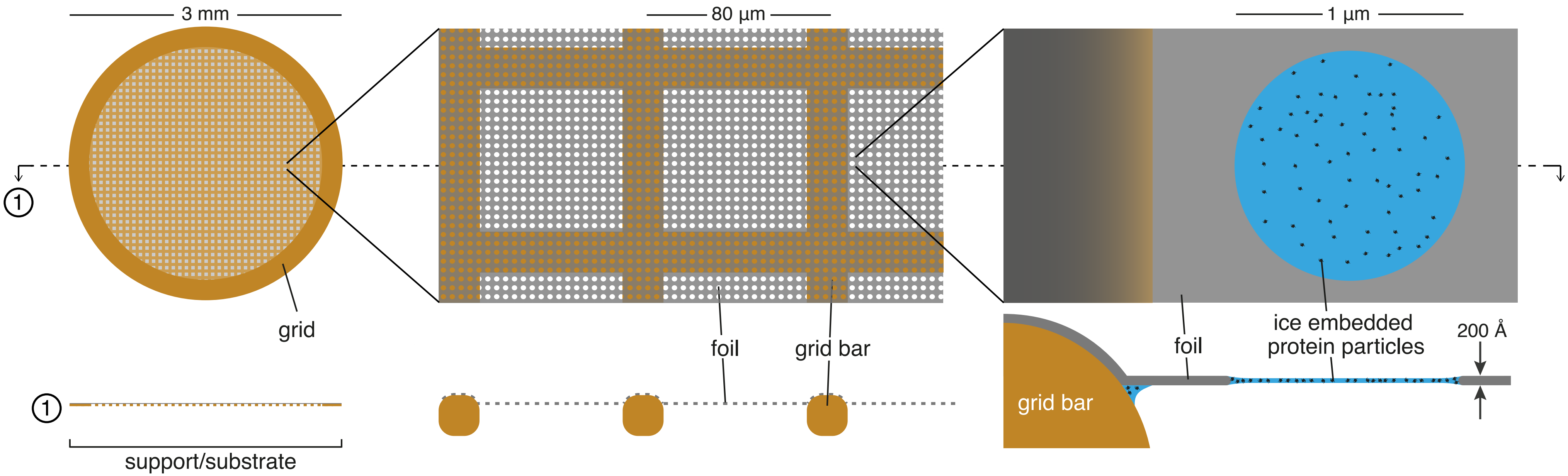
Amorphous carbon

Gold

TiSi	SiN
SiO_2	SiC

Russo and Passmore, Curr Op Struct Biol, 2016

Types of specimen supports



Grid materials

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Titanium	Molybdenum
Silicon	Aluminum
	Tungsten

Foil materials

Amorphous carbon

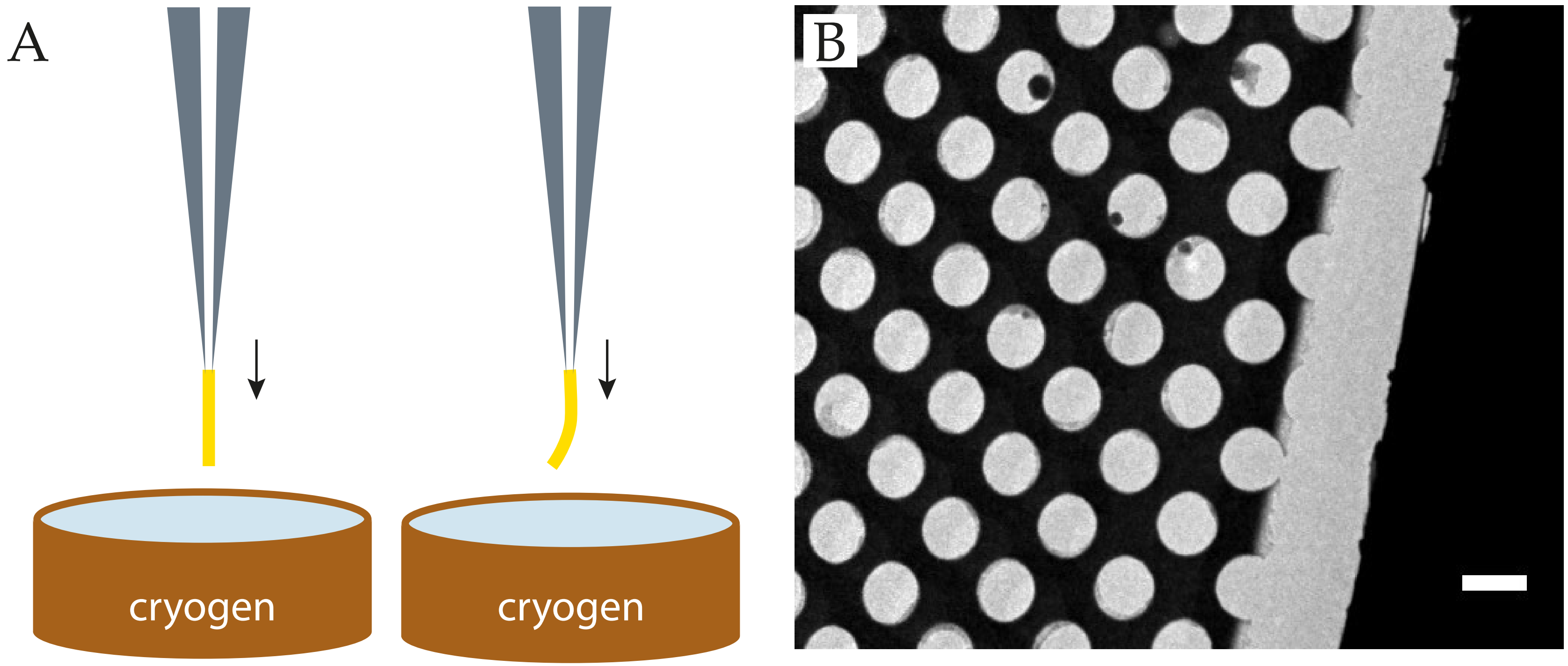
Gold

TiSi SiN

SiO_2 SiC

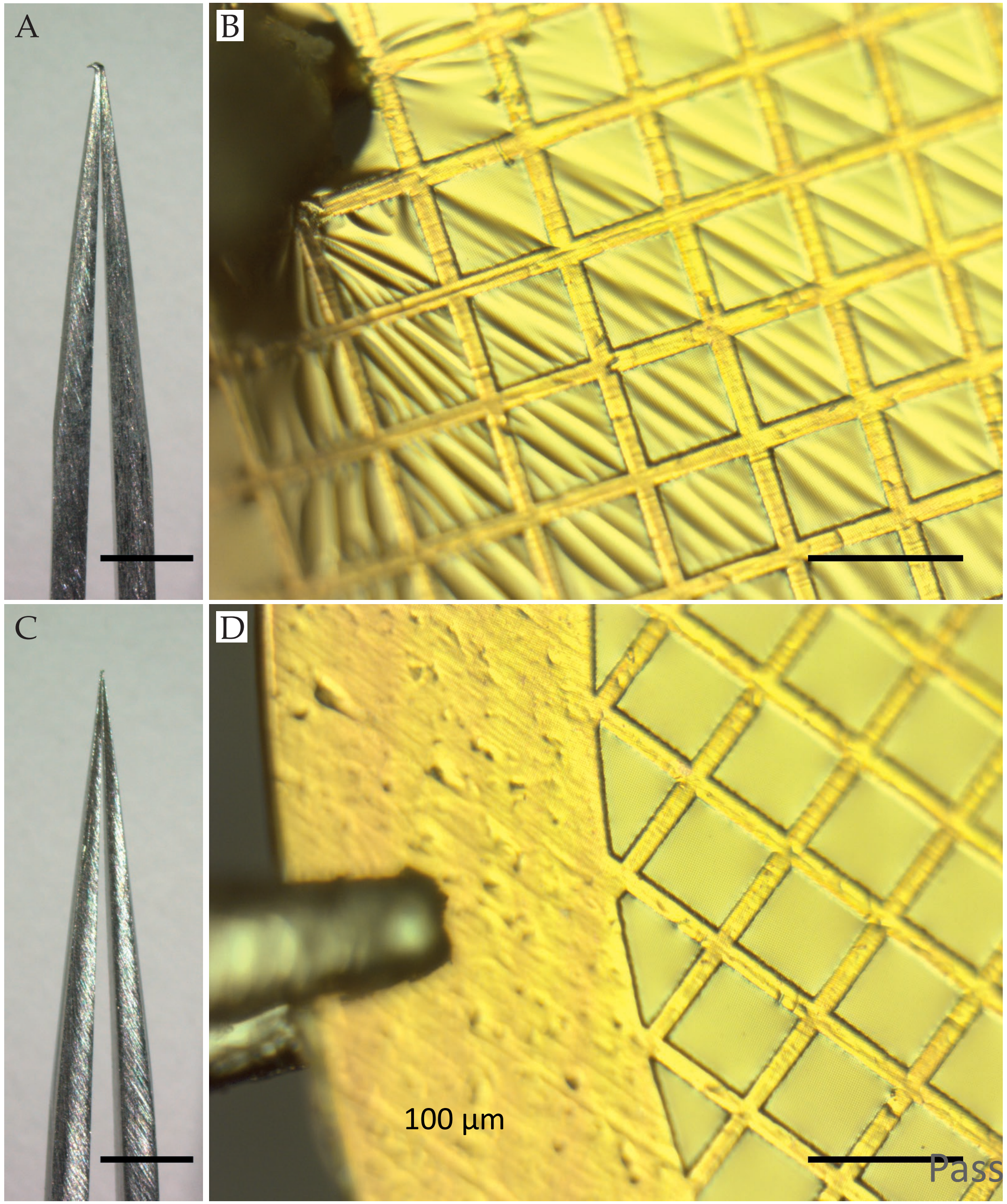
Russo and Passmore, Curr Op Struct Biol, 2016

Handling of supports

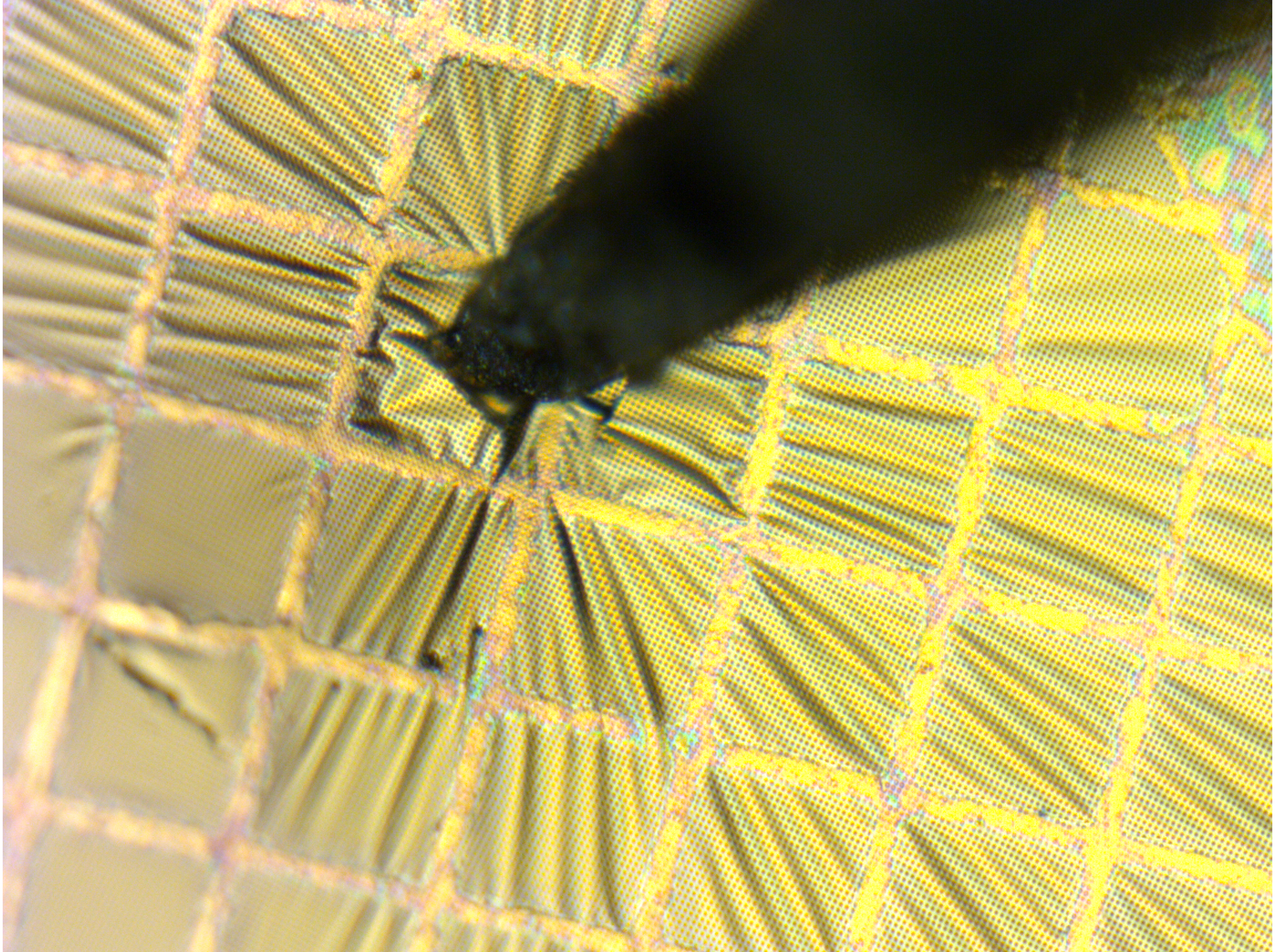


- Always check in optical microscope before use
  - Discard any grids with defects
- Clean tweezers, glass slides and storage dishes

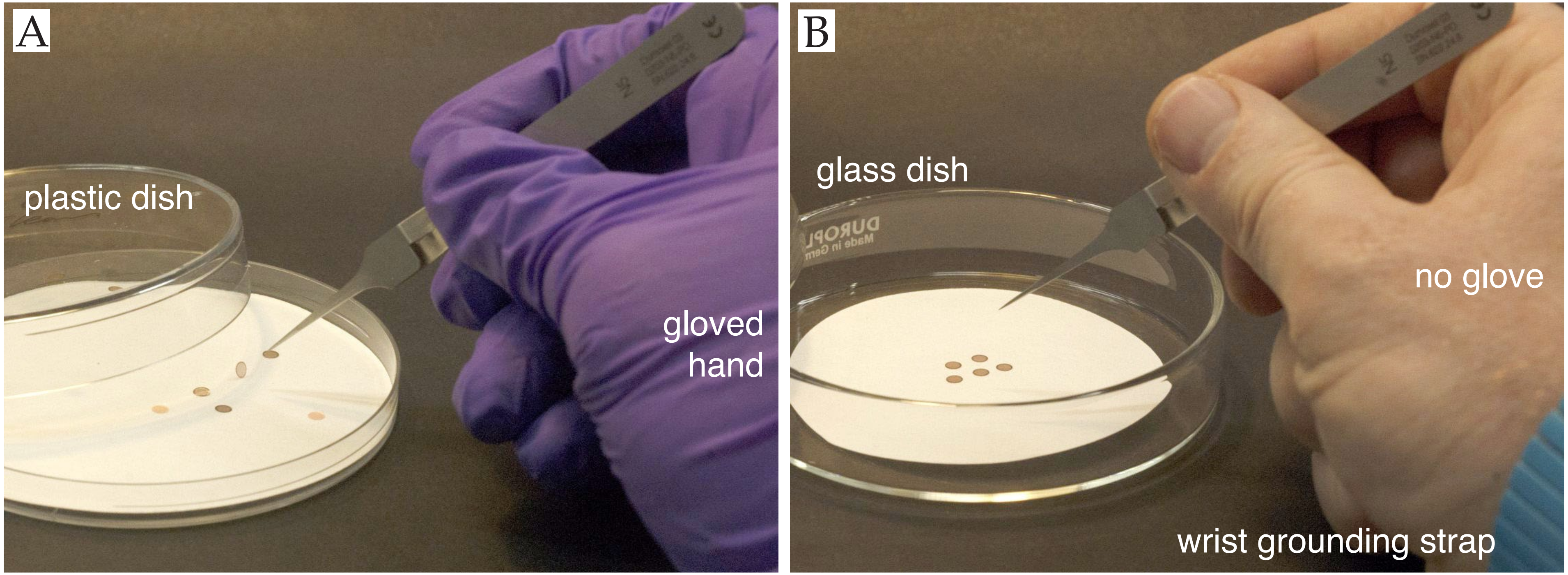
Handling of supports



Passmore & Russo, MiE (2016)



Storing grids



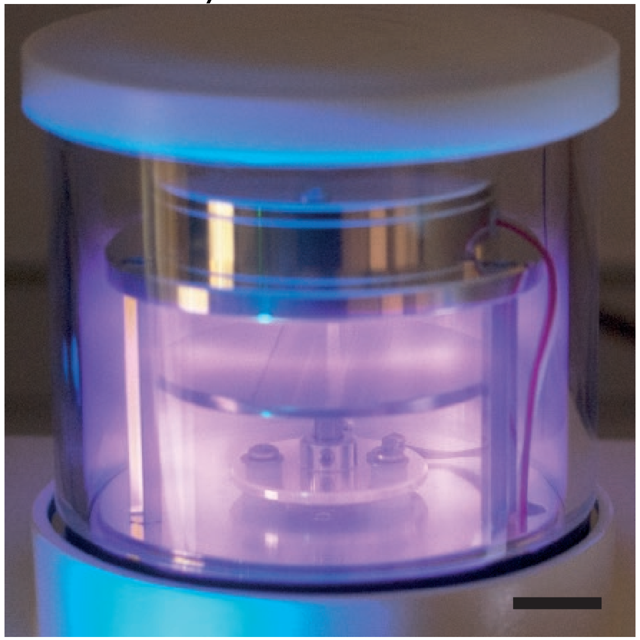
Store in glass dish in desiccator cabinet, ideally in dry nitrogen

Plasma treatment

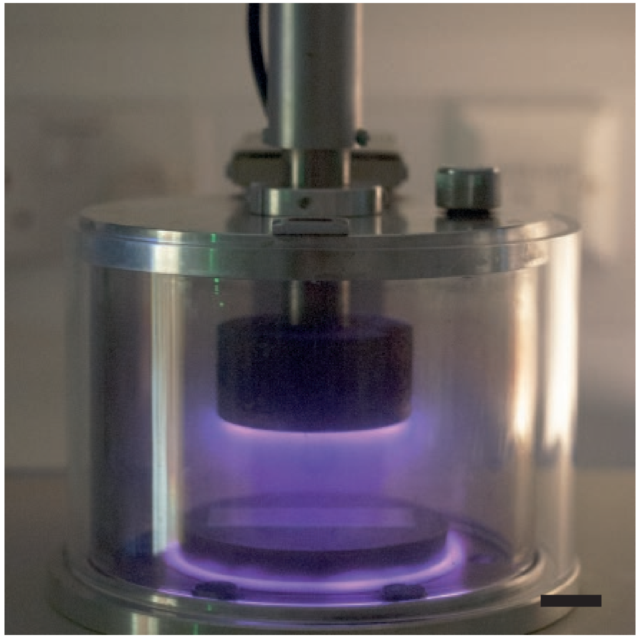
- Plasma created by ionisation of a low pressure gas
  - E.g. in air (glow discharge), oxygen, argon, hydrogen

- Ions interact with surface to remove organic contamination and render it hydrophillic

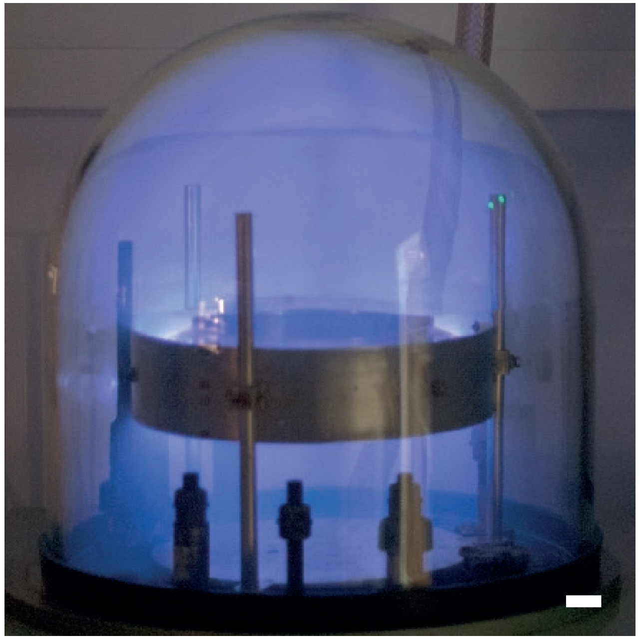
- Other molecules can be introduced to alter the surface, e.g. Amylamine



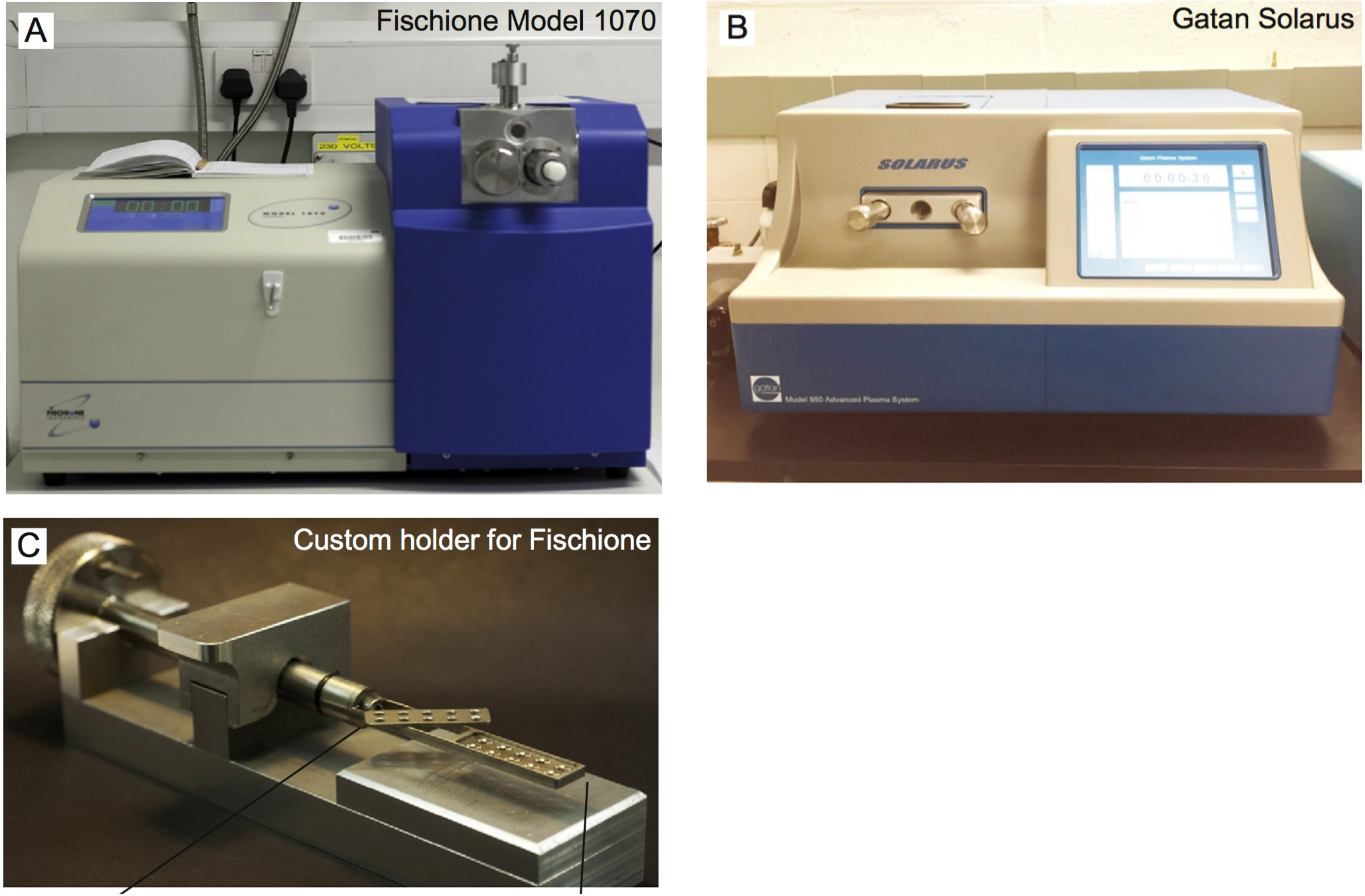
Ted Pella easyGlow (c. 2015)

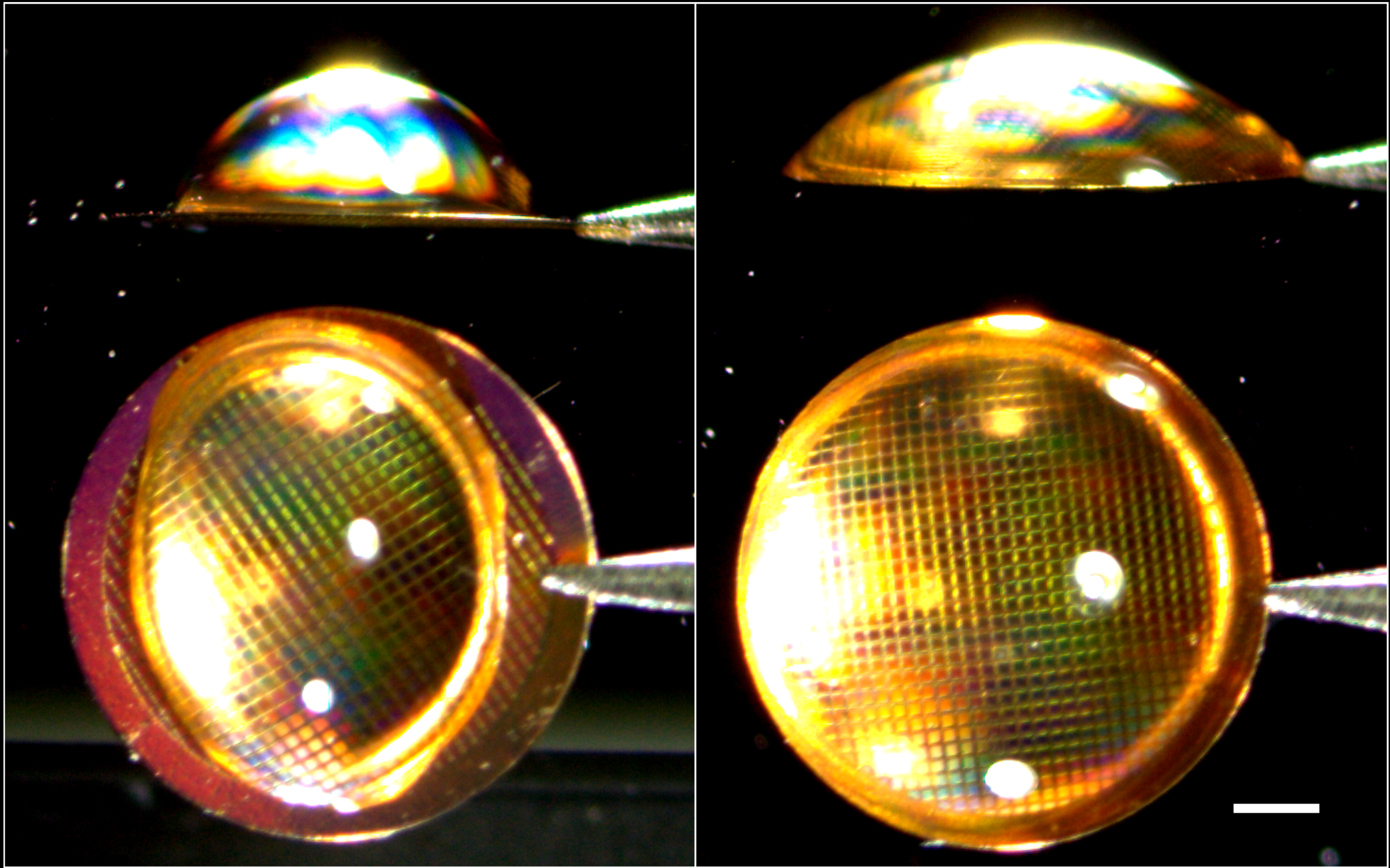


Edwards S150B (c. 1995)



Edwards 12E6 (c. 1962)


A
Fischione Model 1070
B
Gatan Solarus
SOLARUS
Model 950 Advanced Plasma System
C
Custom holder for Fischione



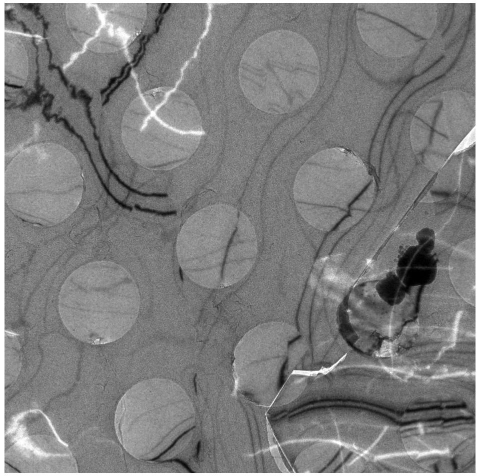
No treatment

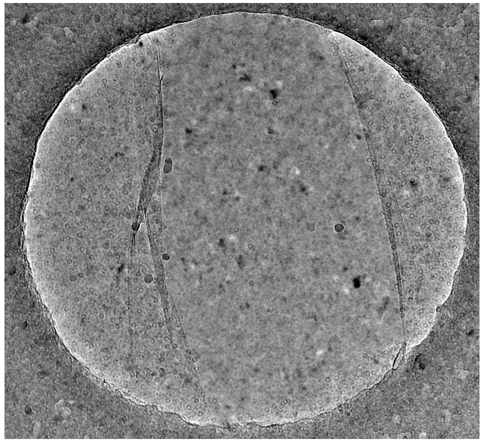
after 20 sec 9:1 Ar:O_2 plasma

Evaluate:

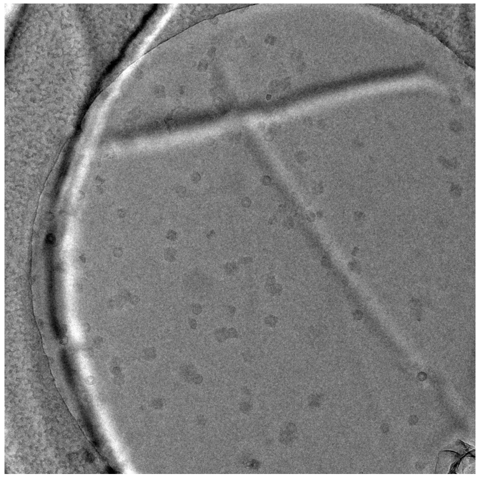
1. Ice thickness and uniformity
2. Phase of the ice
3. Protein concentration and stability

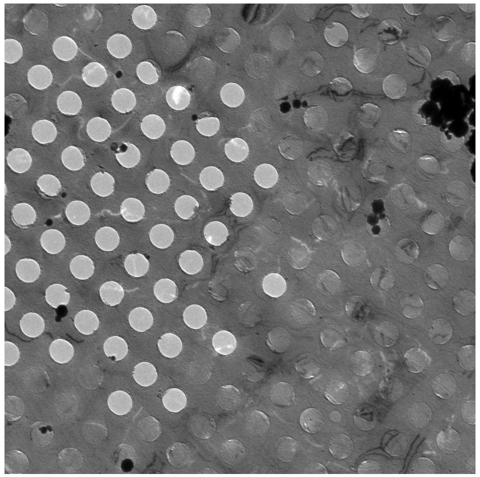
1. Ice quality

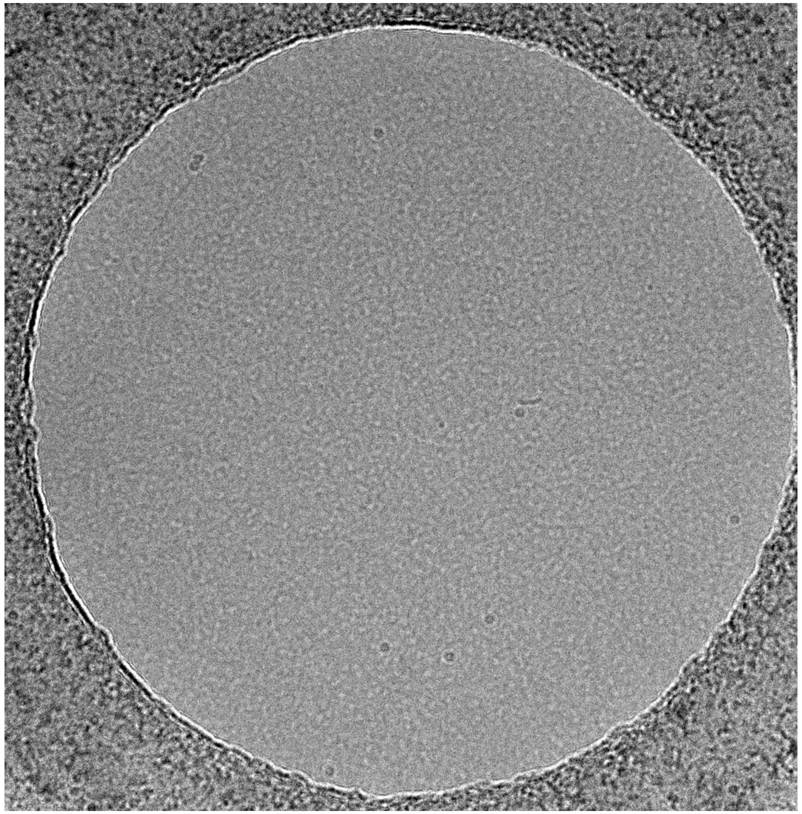




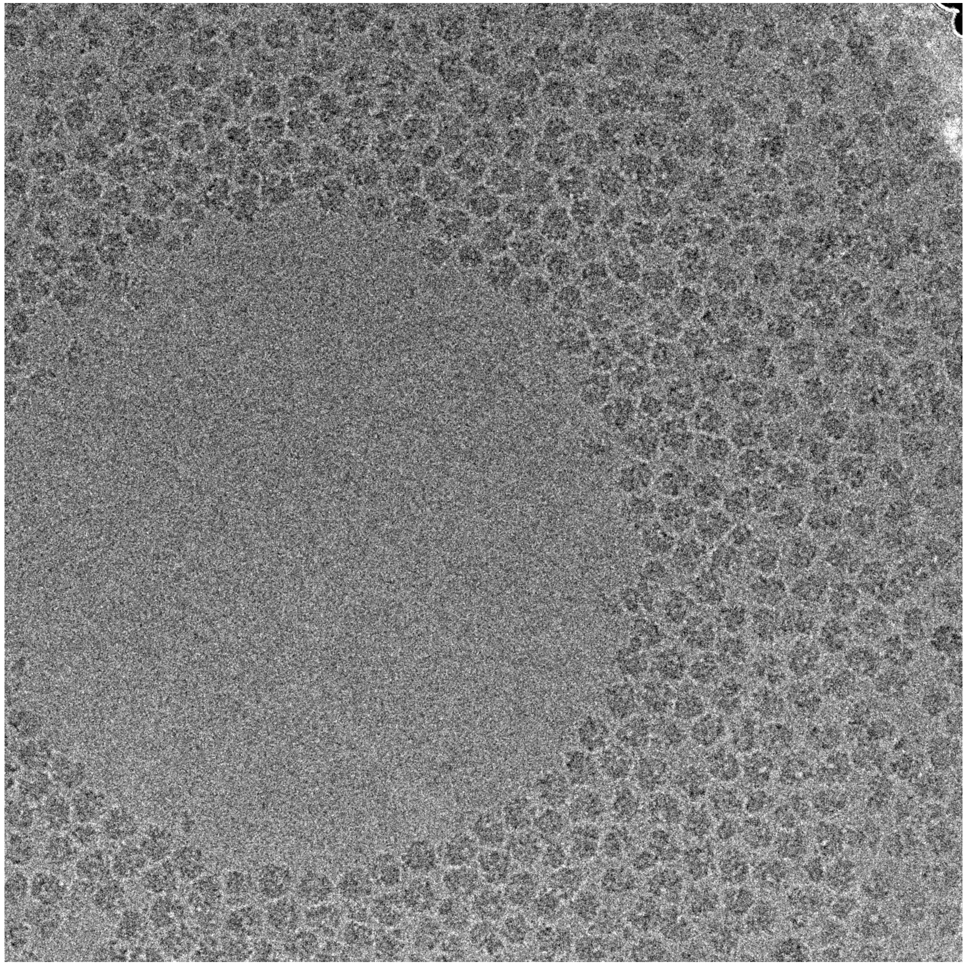
- Vitreous
- Free of contamination



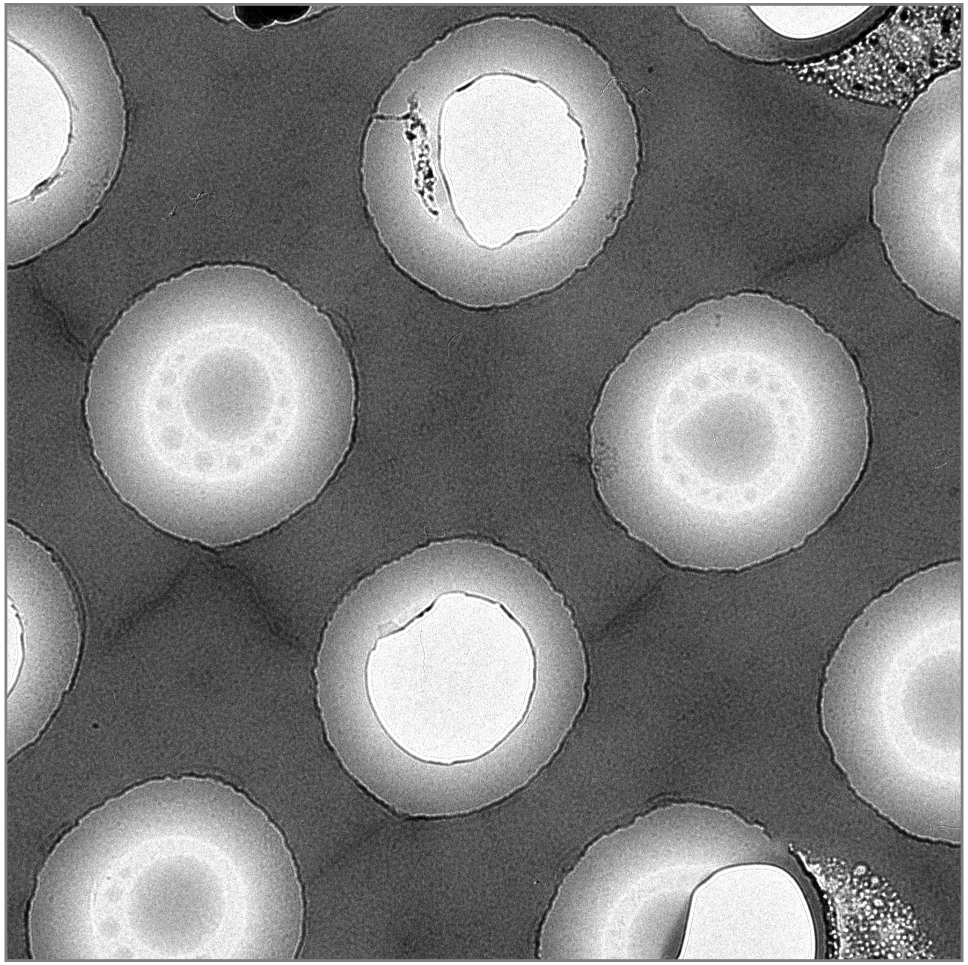




1. Ice thickness and uniformity

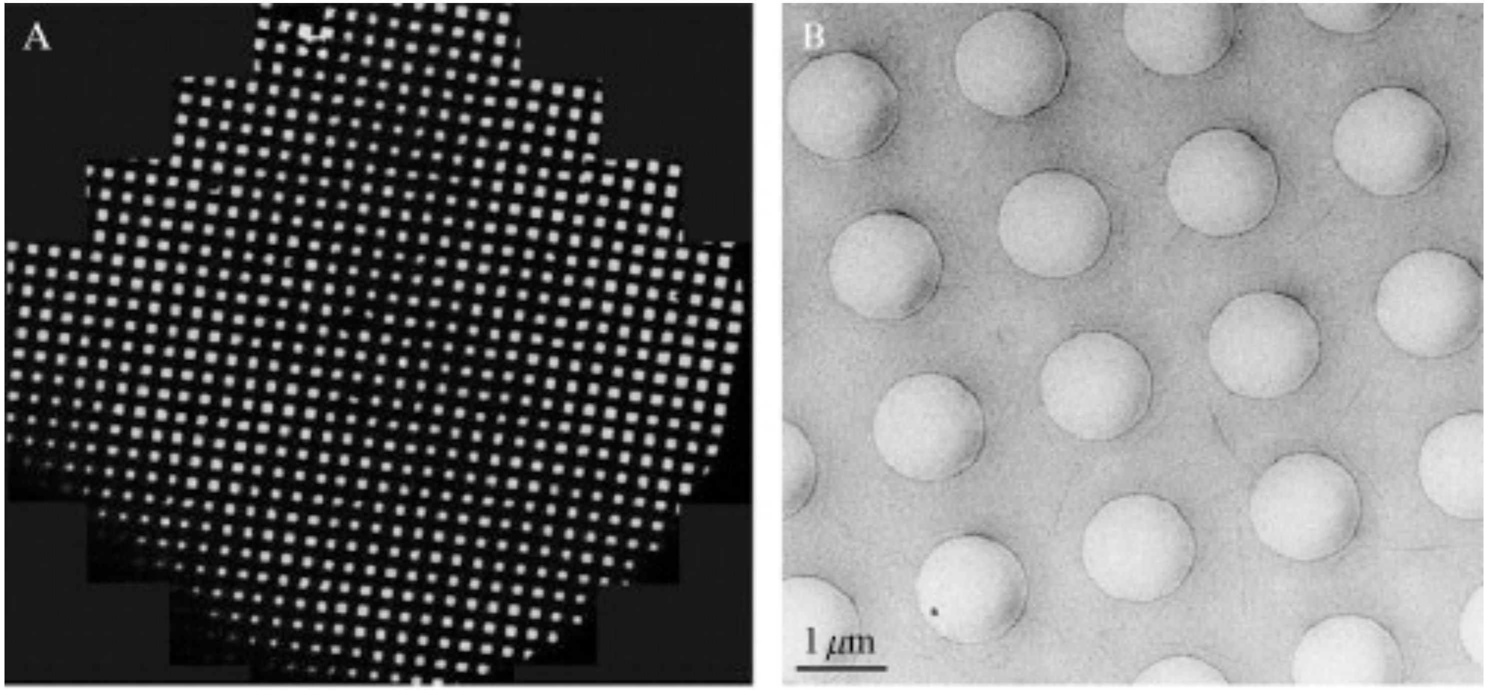


Ribosomes, Chris Russo

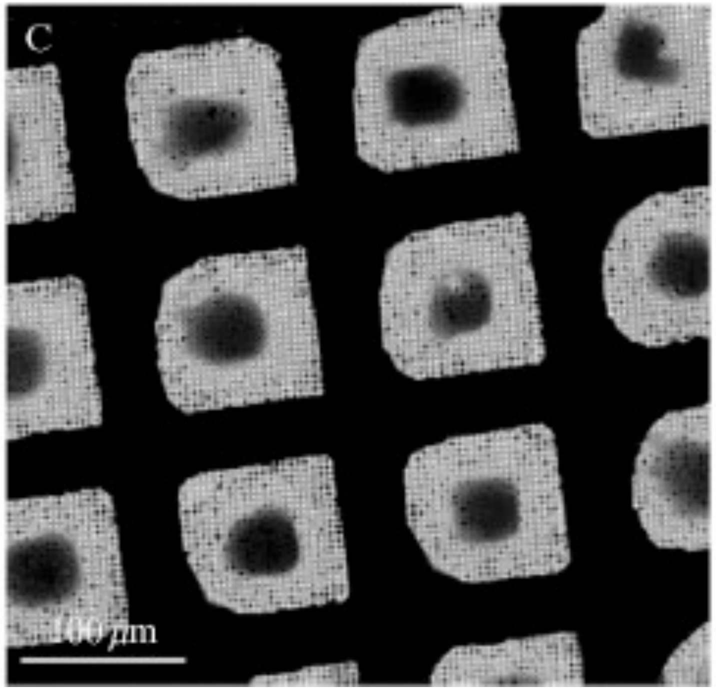


Vinothkumar

Optimise plasma exposure time and blot force.

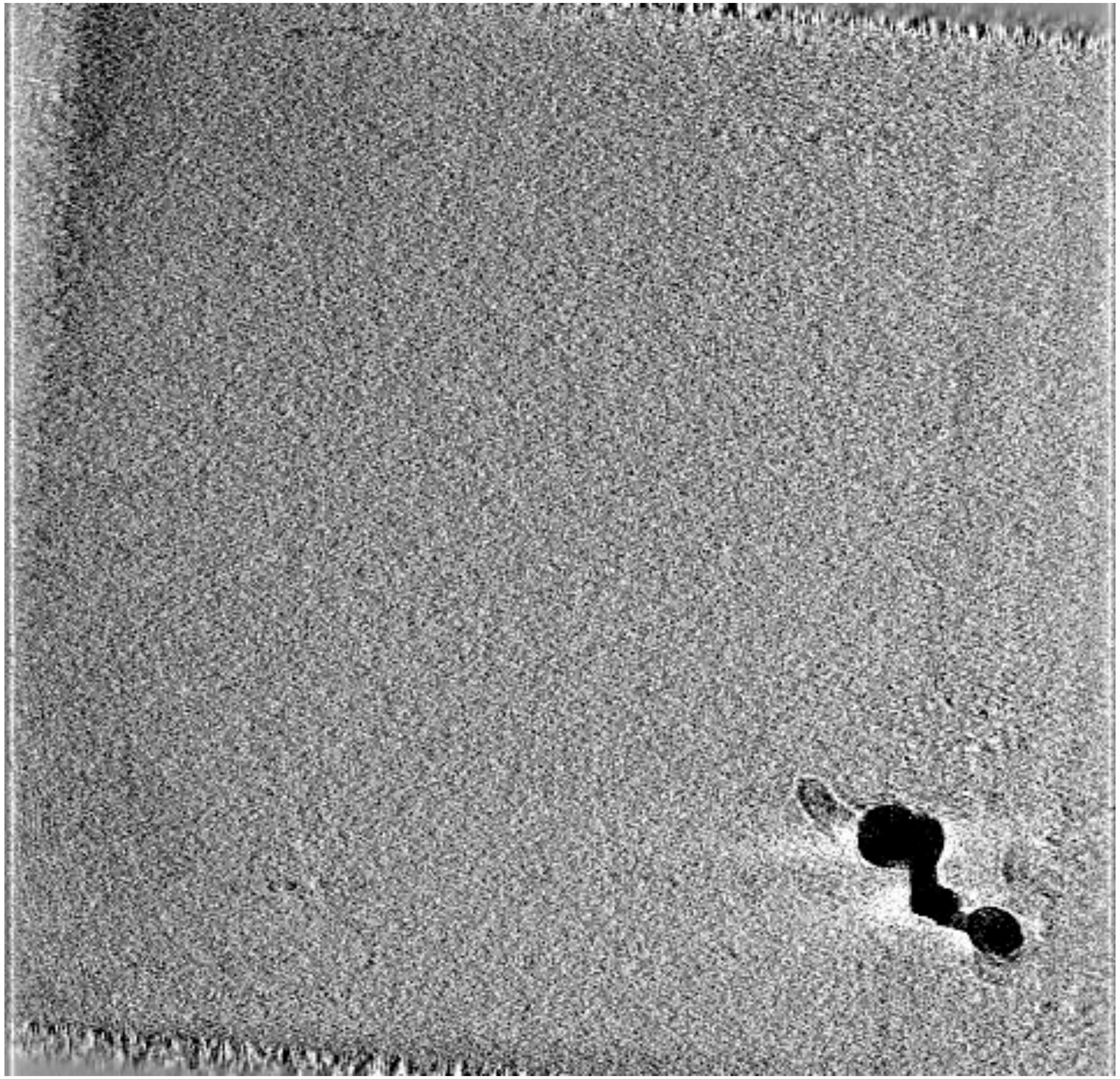


Incomplete
wetting

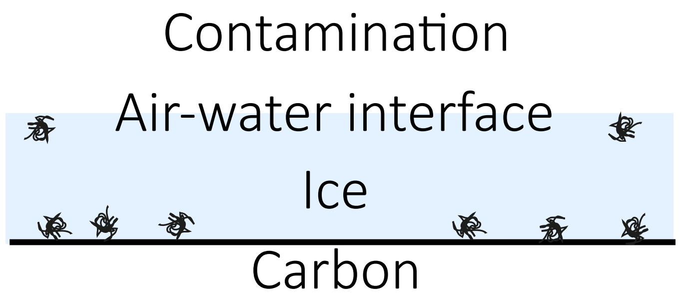


Dobro *et al* (2010)

Protein distribution in ice



Tomogram of ribosomes in ice.
Tanmay Bharat, Jan Löwe MRC LMB



Do the number of particles in the image match what you would expect from the protein concentration in free solution?
e.g. 250 kDa protein at 2 mg/ml
→400 particles/µm²

(Vinothkumar and Henderson 2016)

Expected particle distribution on grids

Given the concentration of the molecule of interest in mg/ml and the molecular weight (MW), how many particles should you see in the image if the frozen specimen has the same concentration of molecules that you expect in free solution?

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)

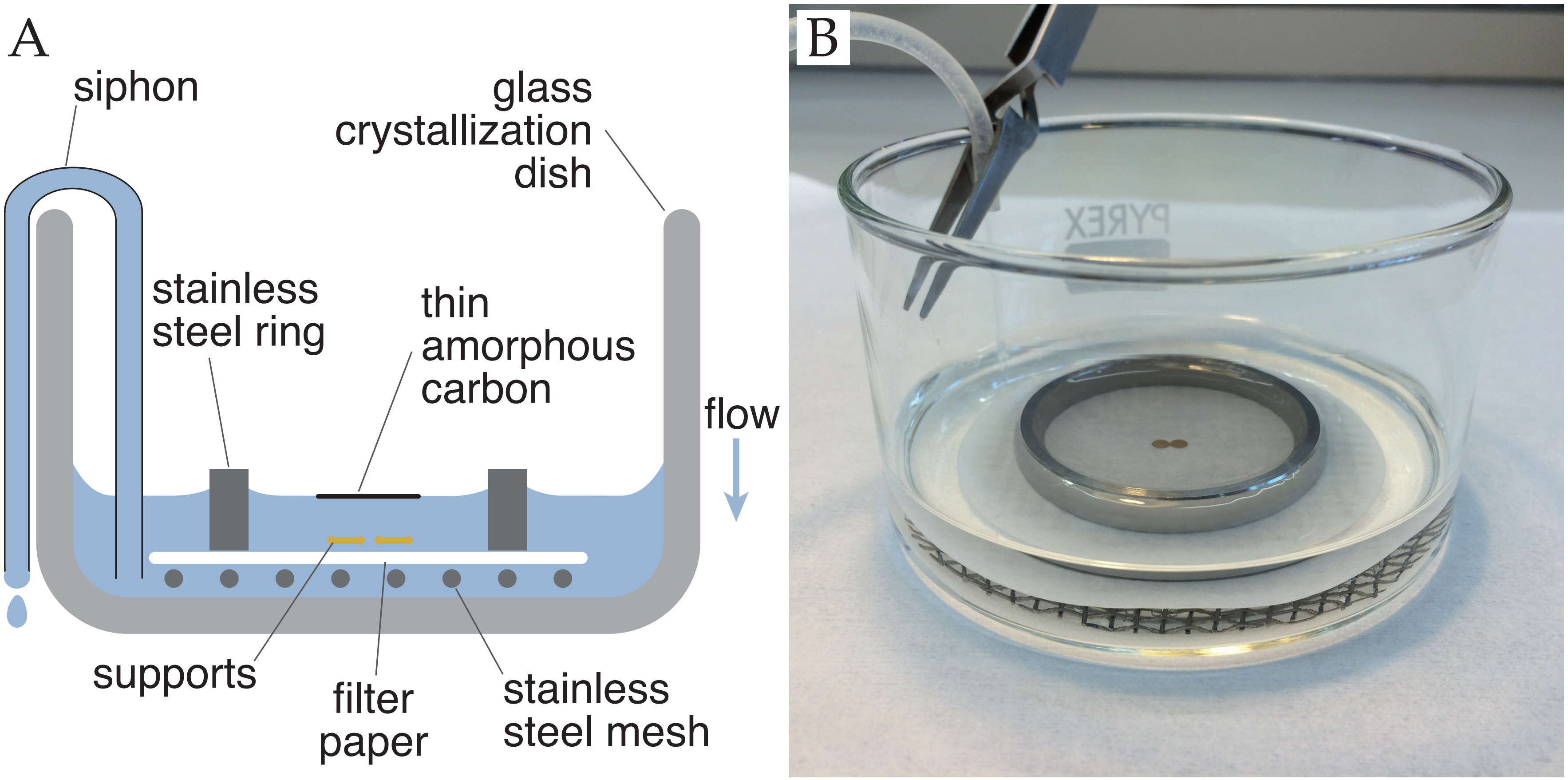
Vinothkumar & Henderson 2016

What if I don't see the expected number of particles?

If it is stable in solution, the following parameters can be optimised:

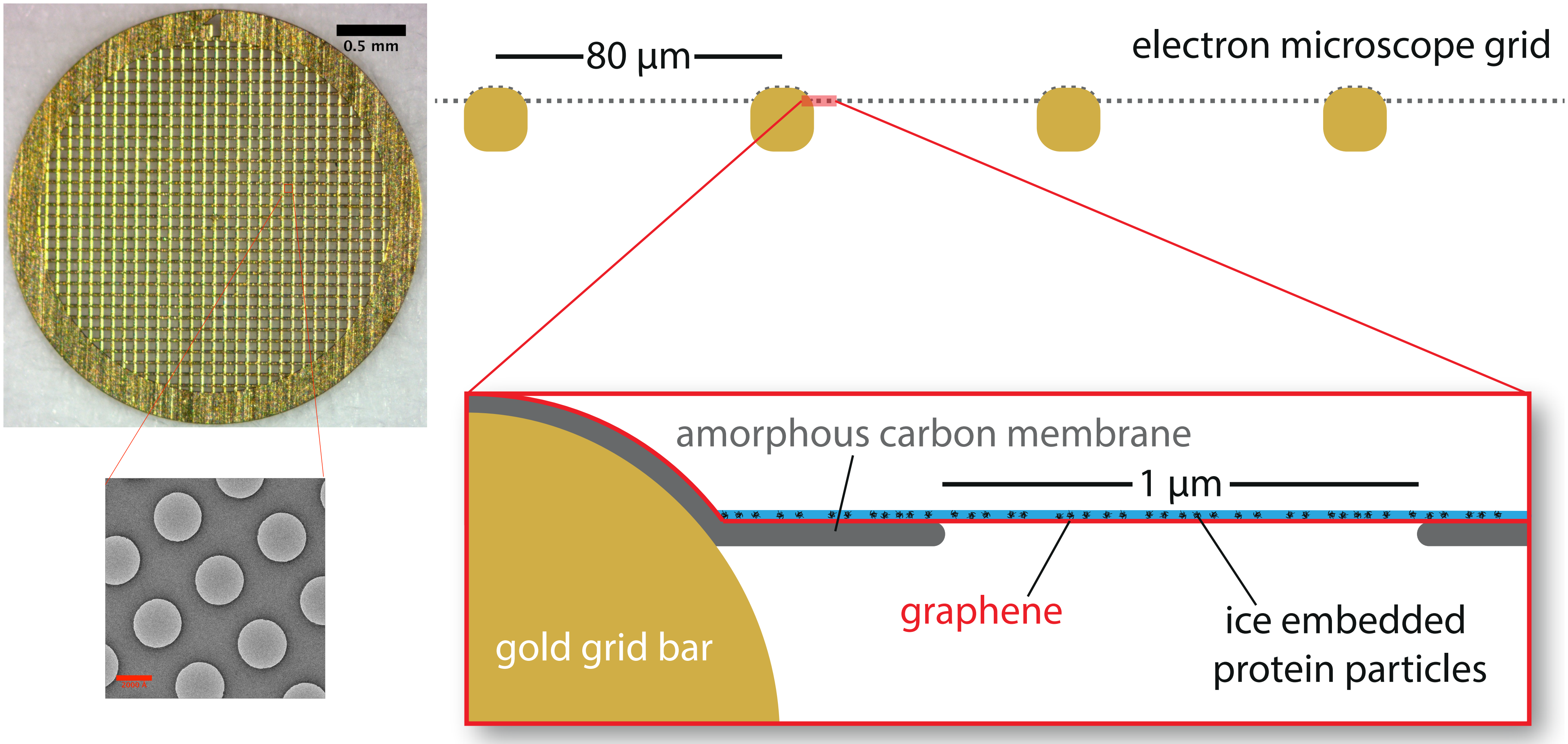
- Buffer optimisation (pH, salt), crosslink
  - change charge on protein
- Addition of small amounts of detergents, lipids
  - Protect protein from air-water interface
- Blotting and plasma conditions
  - Is the ice too thick? Too thin?
- Use of an extra continuous supporting layer
  - Provides another surface for proteins to adsorb to

Depositing thin carbon onto grids

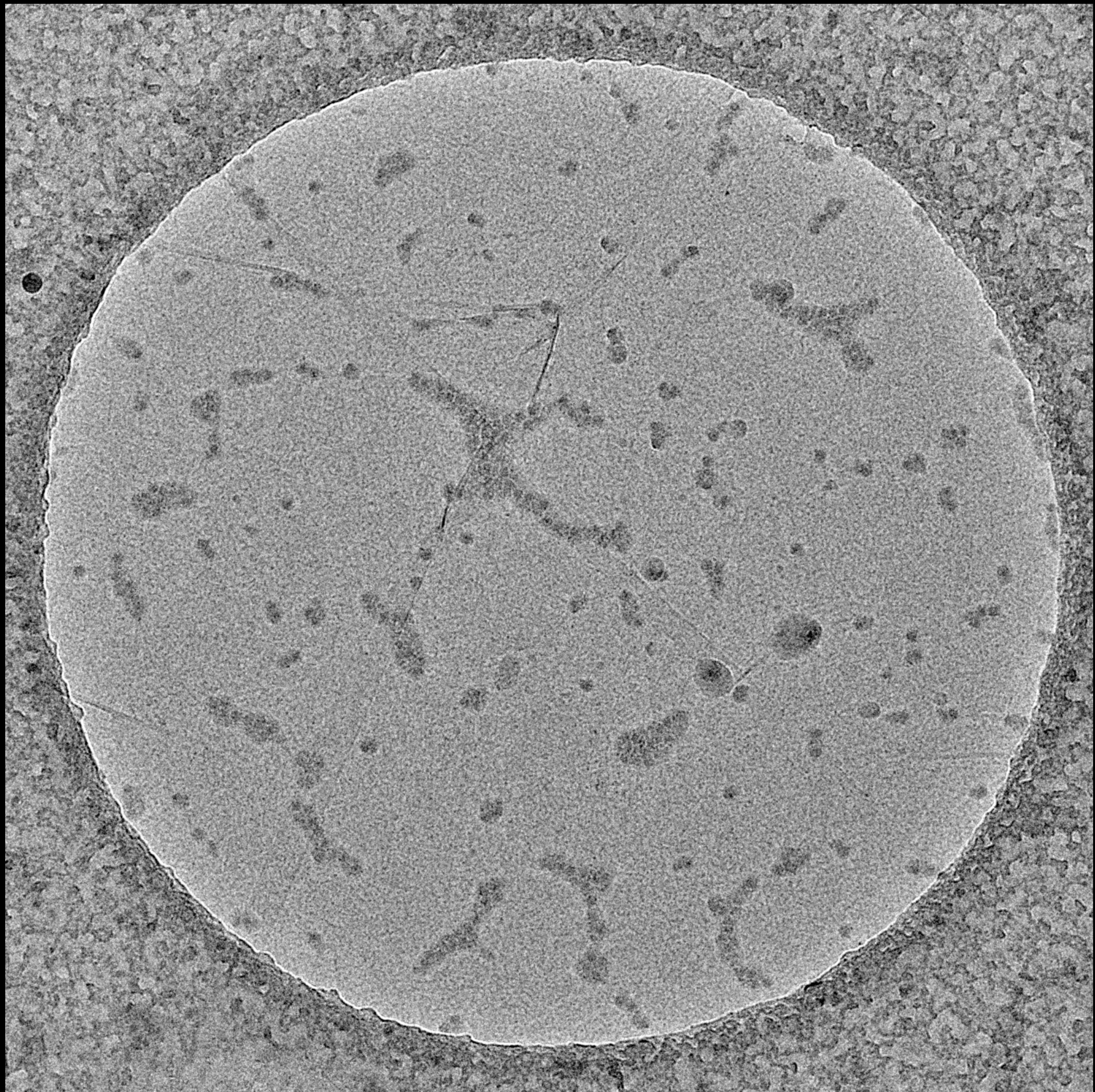


Passmore & Russo MiE 2016

Graphene supports for cryo-EM



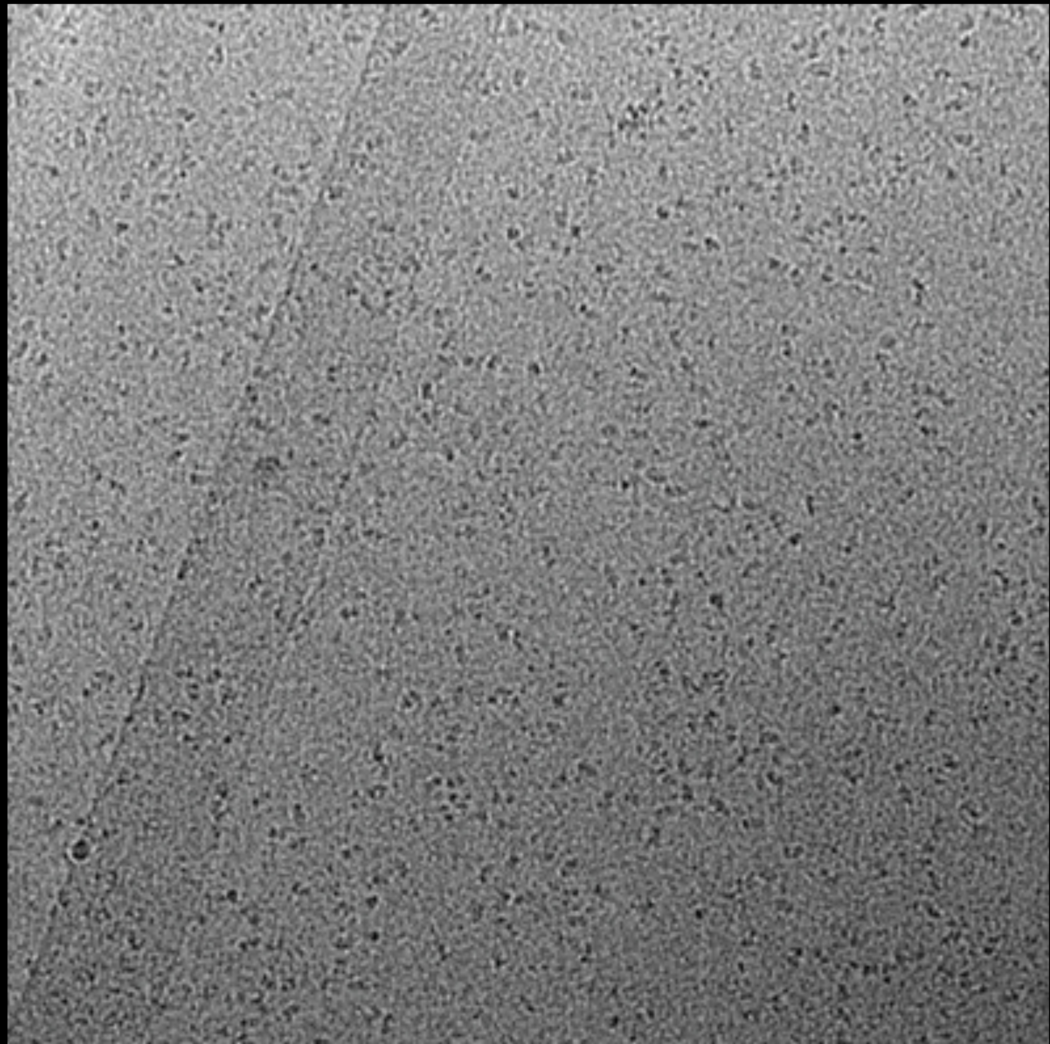
Russo and Passmore (2014) Nature Methods



70S Ribosomes
on graphene as
synthesised

1.2 µm hole

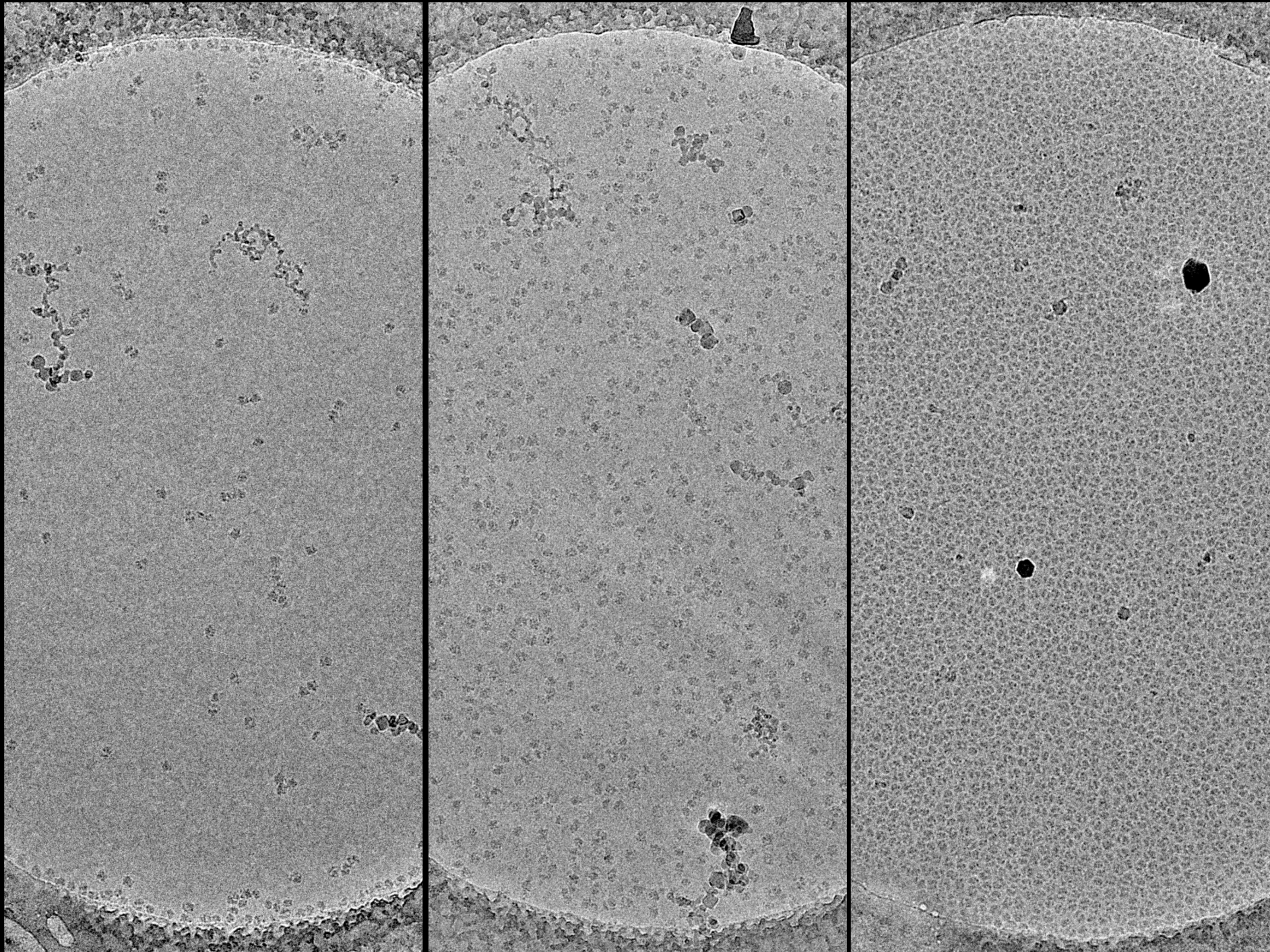
Partial Hydrogenation: Russo & Passmore 2014 Nat Methods
Passmore & Russo 2016 Methods in Enzymology
Graphene oxide: Pantelic et al 2010; Martin/Scheres 2016
https://figshare.com/articles/Graphene_Oxide_Grid_Preparation/3178669



Graphene oxide: Pantelic, Thomas Martin

Separase–securin Boland et al NSMB 2017

https://figshare.com/articles/Graphene_Oxide_Grid_Preparation/3178669

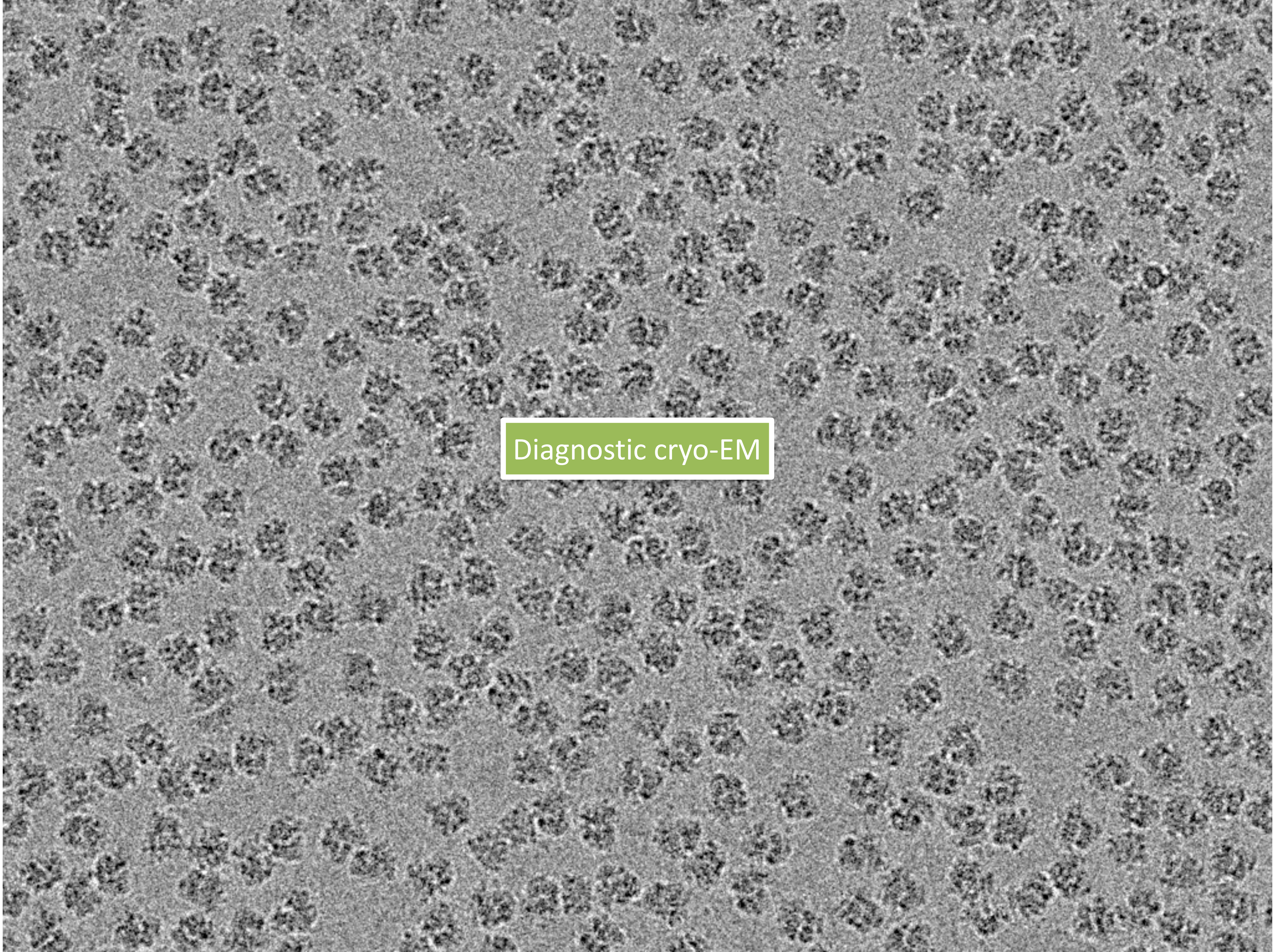


no graphene

graphene +
20 s hydrogen

graphene +
40 s hydrogen

Russo and Passmore (2014) Nature Methods


Diagnostic cryo-EM

Protein preparation

↓

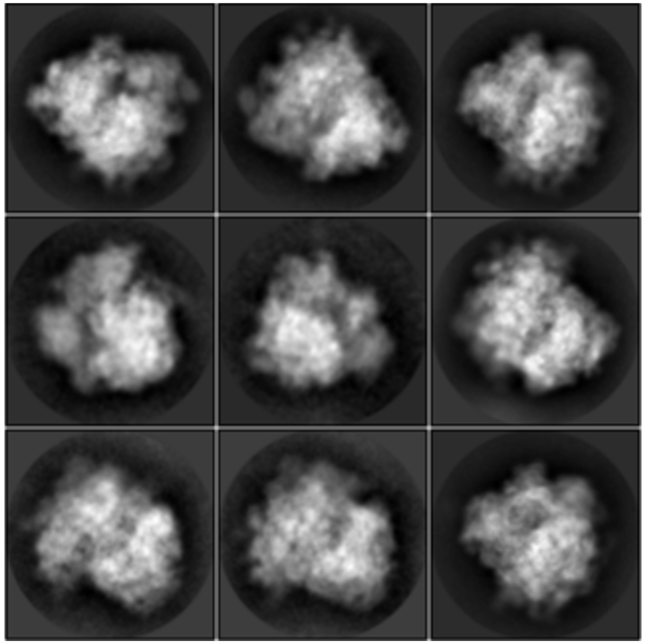
Negative stain

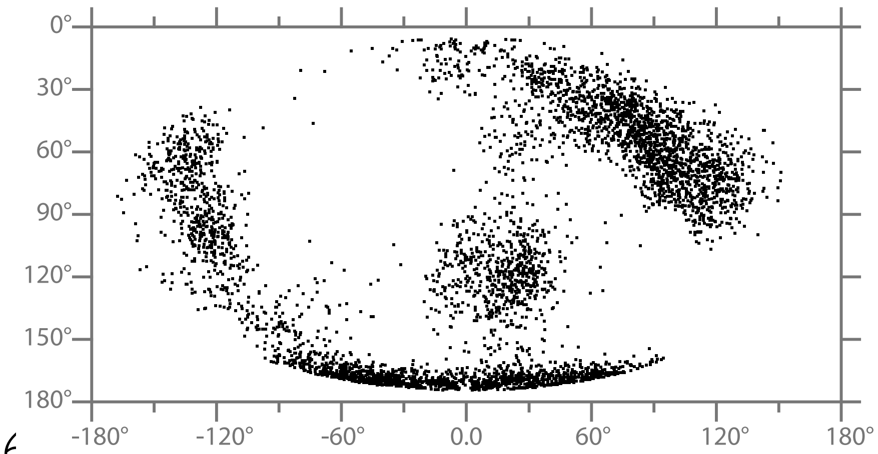
↓

Diagnostic cryo-EM

↓

Initial cryo-EM data collection

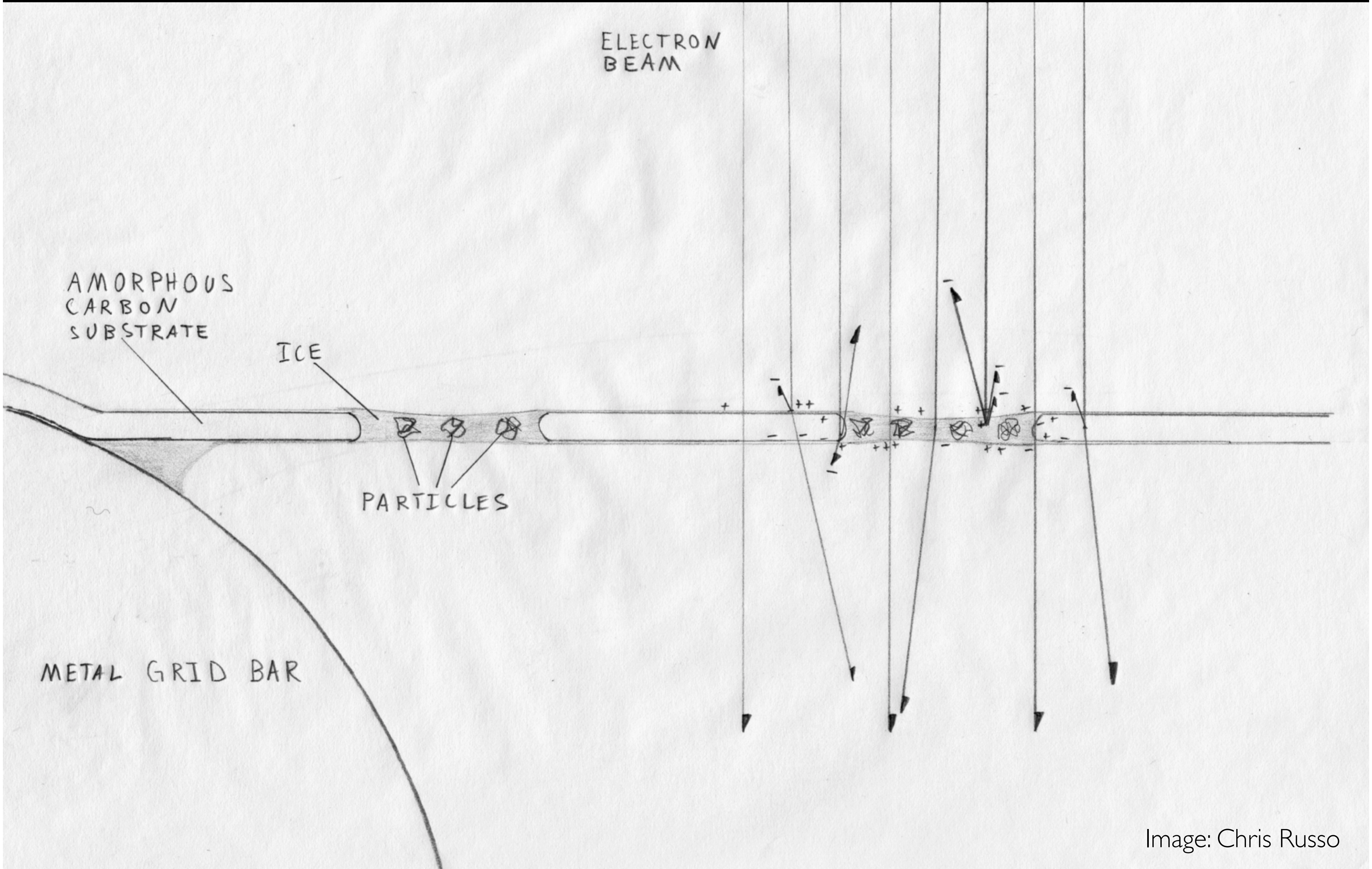




- High resolution 2D classes
- Initial 3D model
- Orientation distribution
- Particle yield

Mid-range or high-end electron microscope

Passmore & Russo MiE 2016



Glaeser, Henderson, Downing, Jensen, Grigorieff, Kühlbrandt, Scheres, Cheng ….

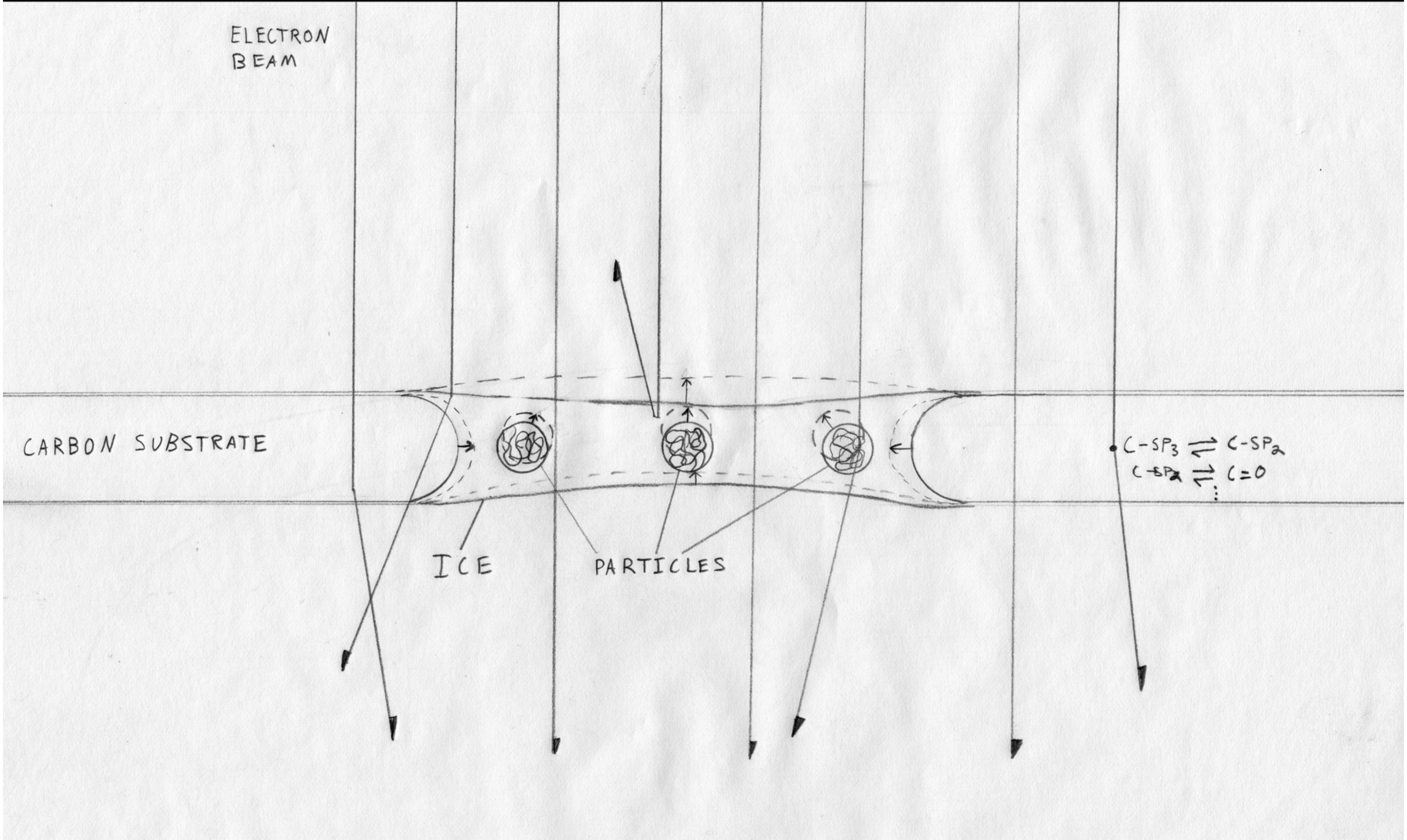
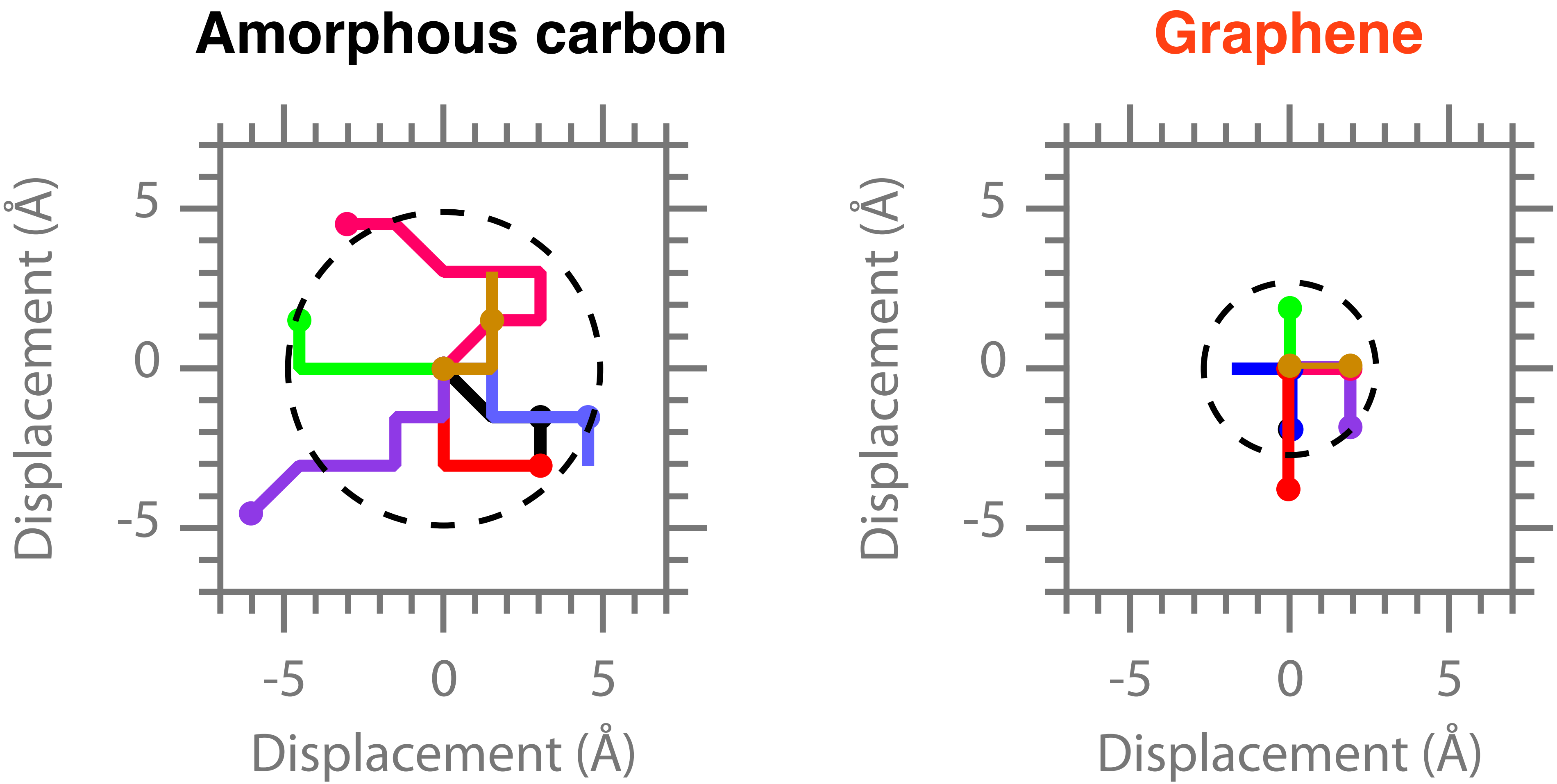
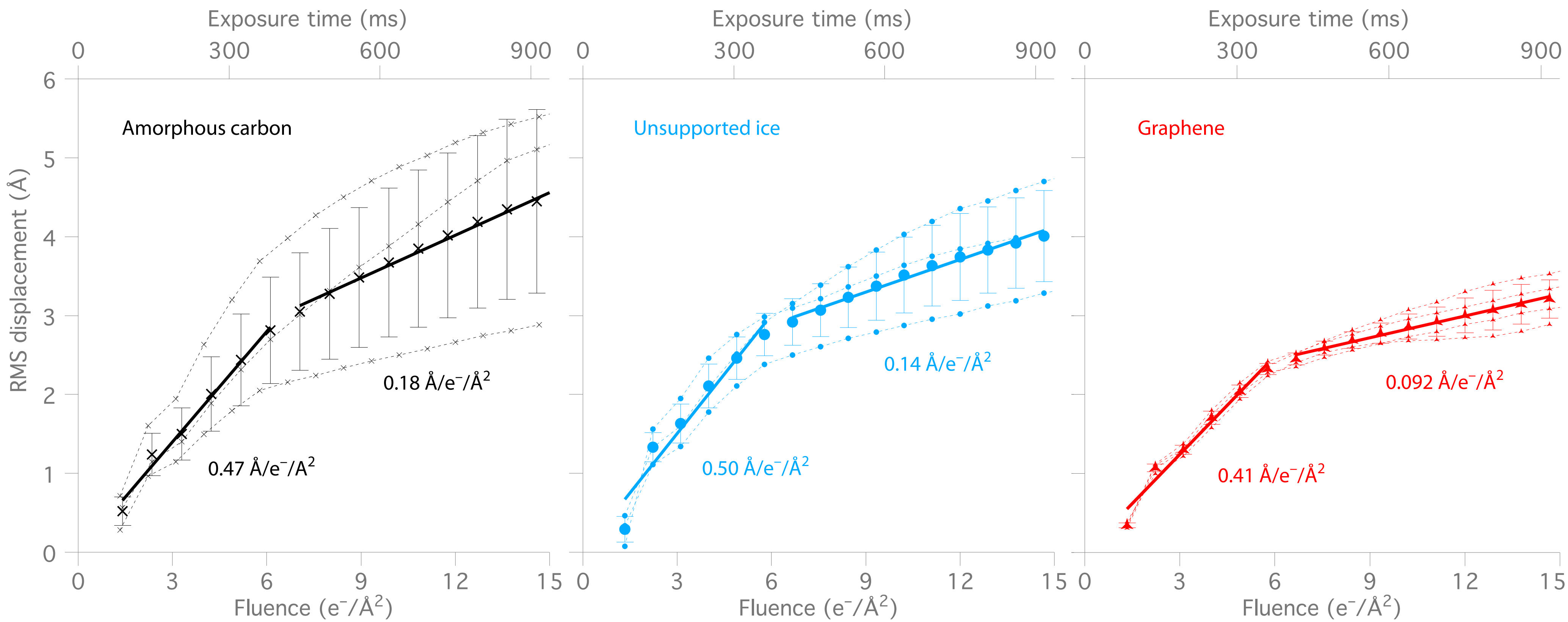


Image: Chris Russo

Glaeser, Henderson, Downing, Jensen, Grigorieff, Kühlbrandt, Scheres, Cheng ….


Amorphous carbon
Graphene
Displacement (Å)
Displacement (Å)
Displacement (Å)
Displacement (Å)
5
0
-5
-5
0
5
5
0
-5
-5
0
5

Ribosome speed plots on different substrates

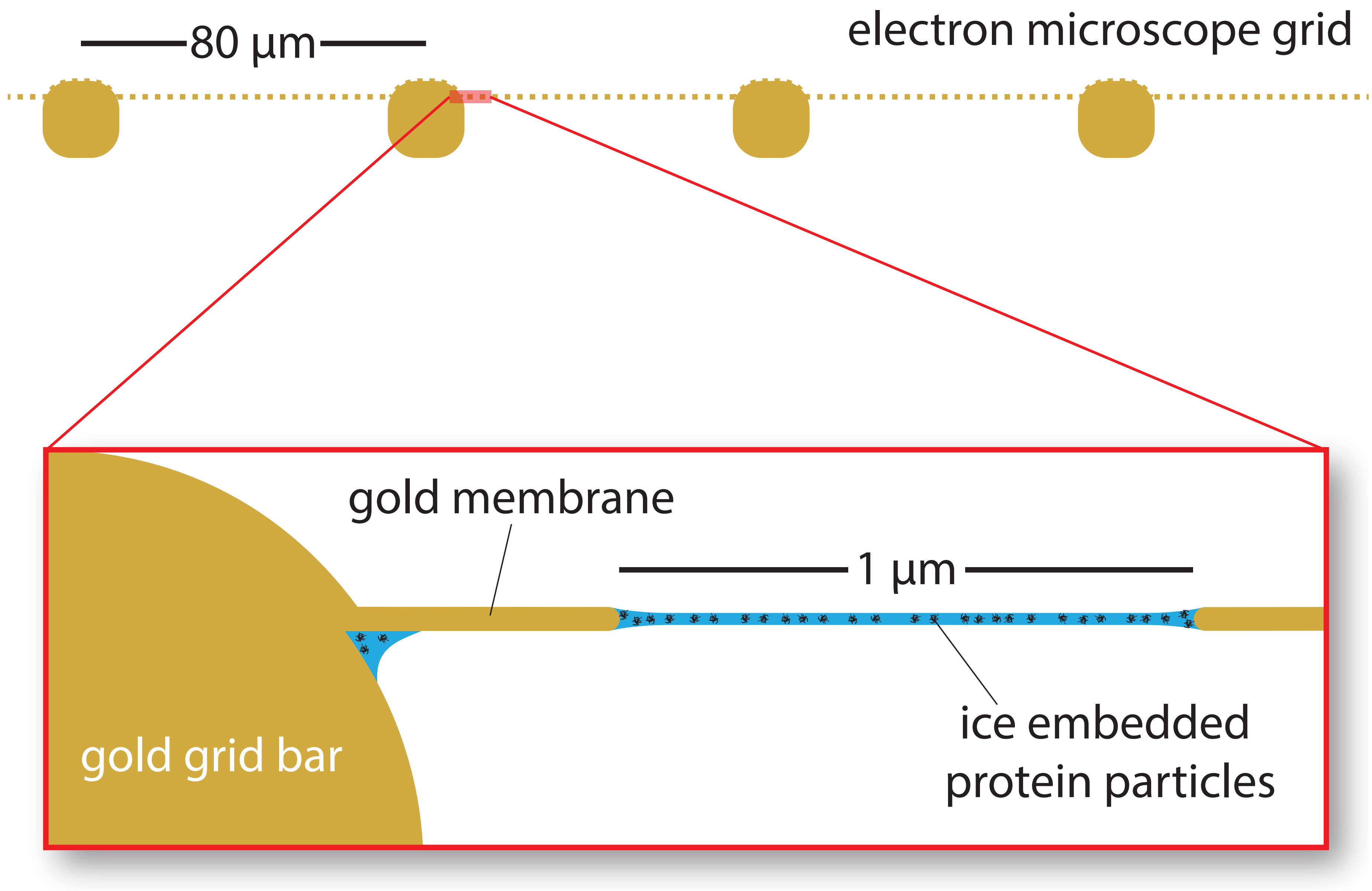


amorphous carbon
on quantifoil

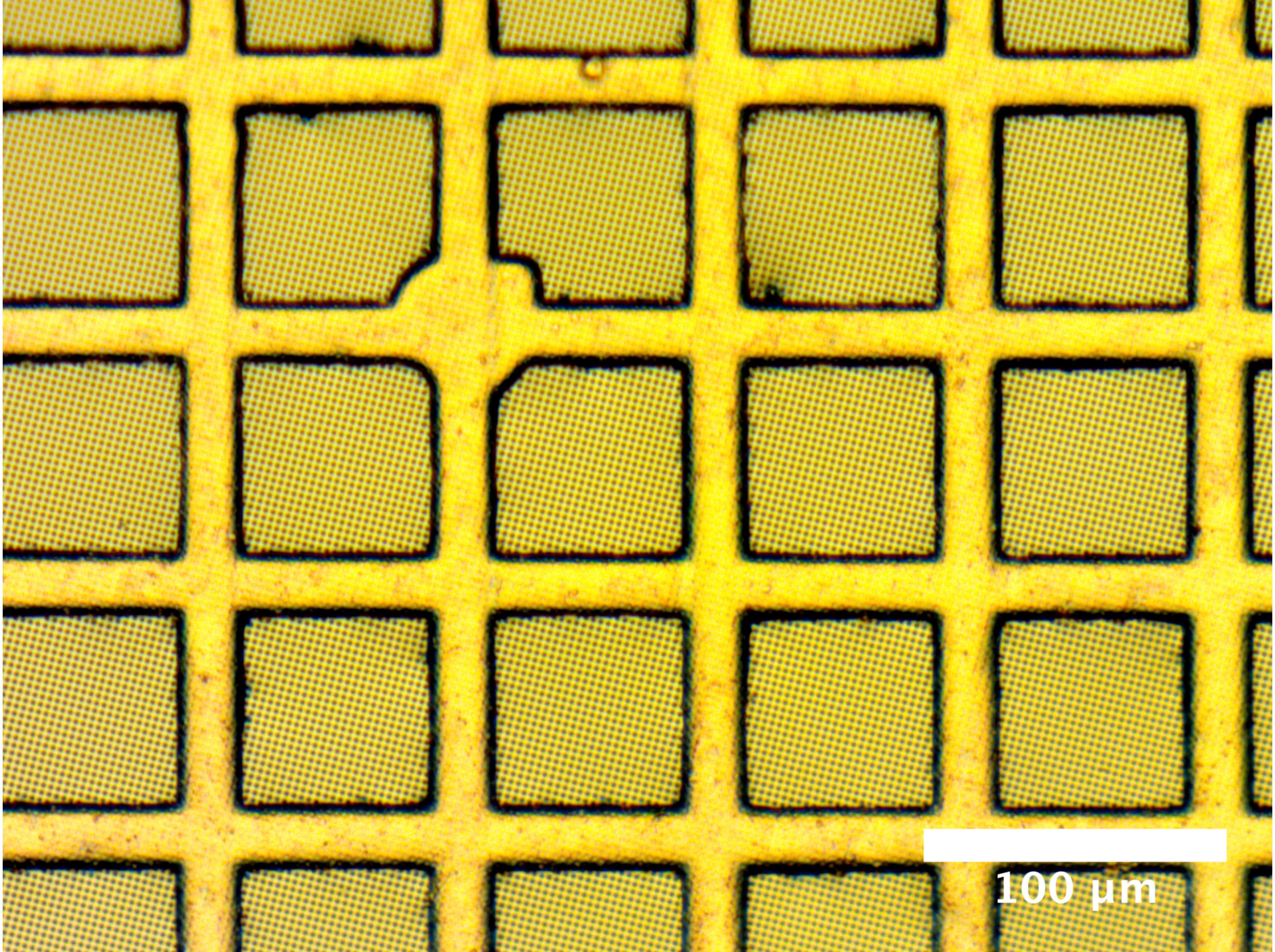
quantifoil

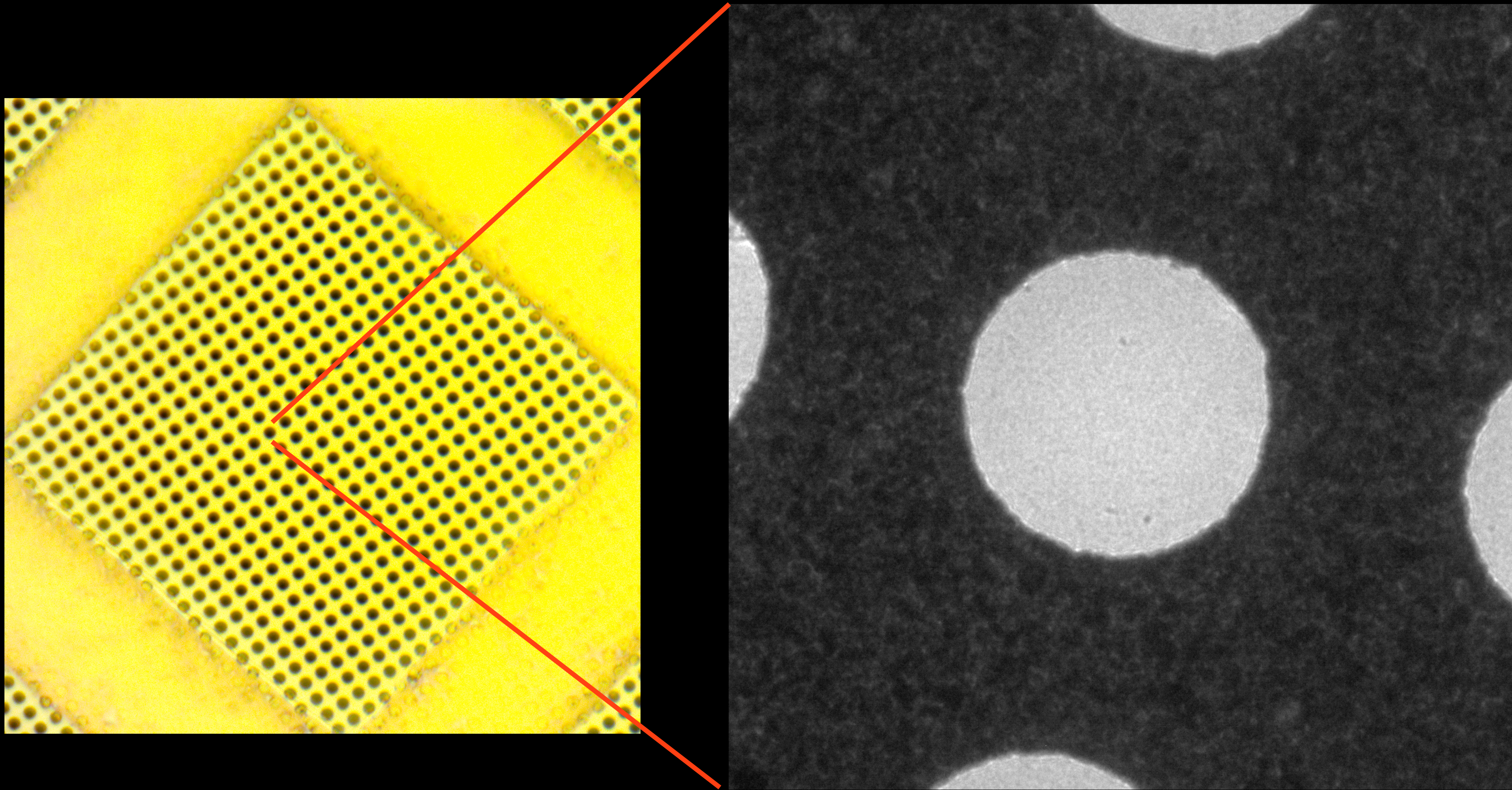
graphene
on quantifoil

UltrAuFoils



Russo and Passmore (2014) Science


100 µm

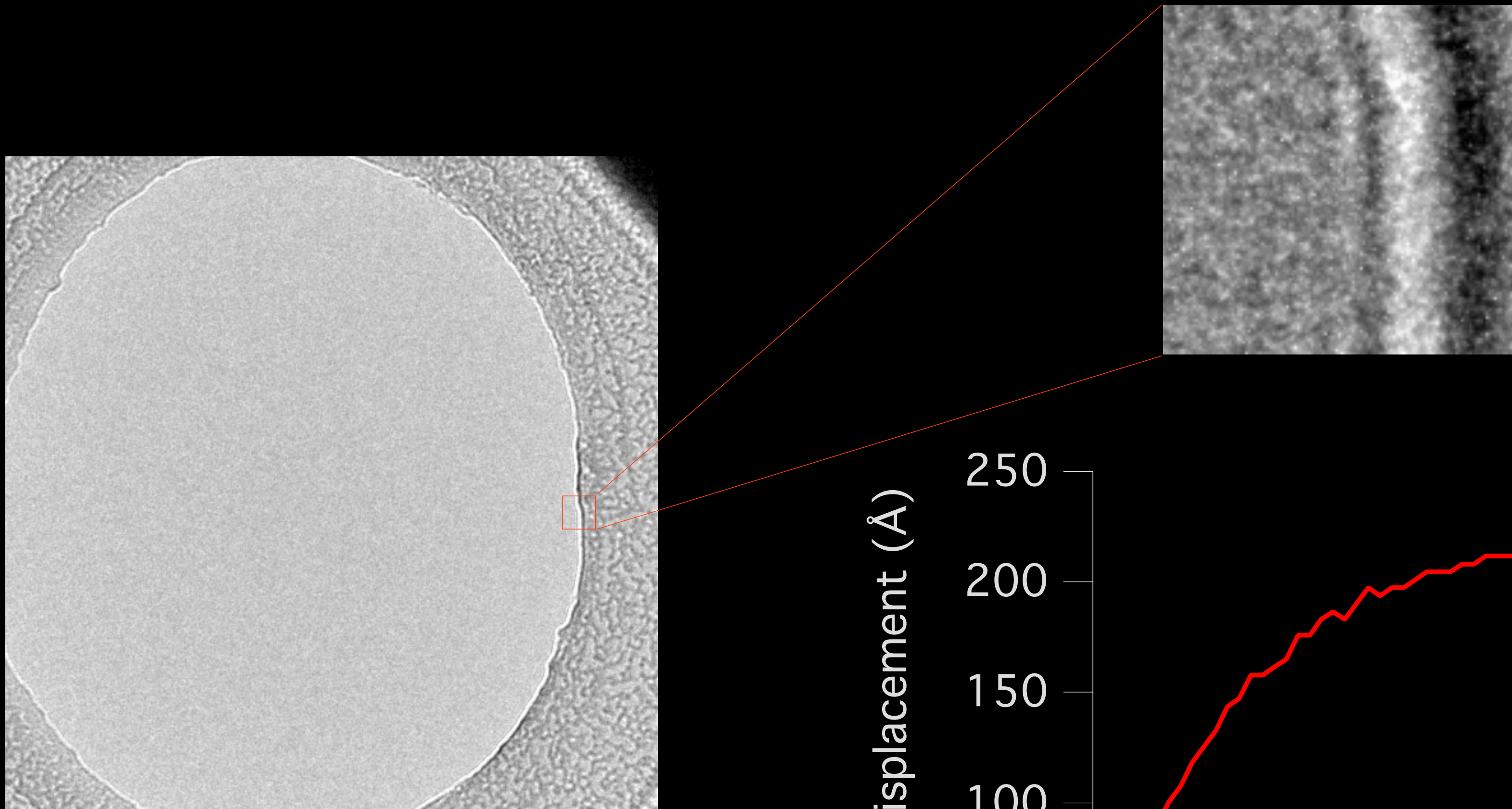


1.2 µm holes

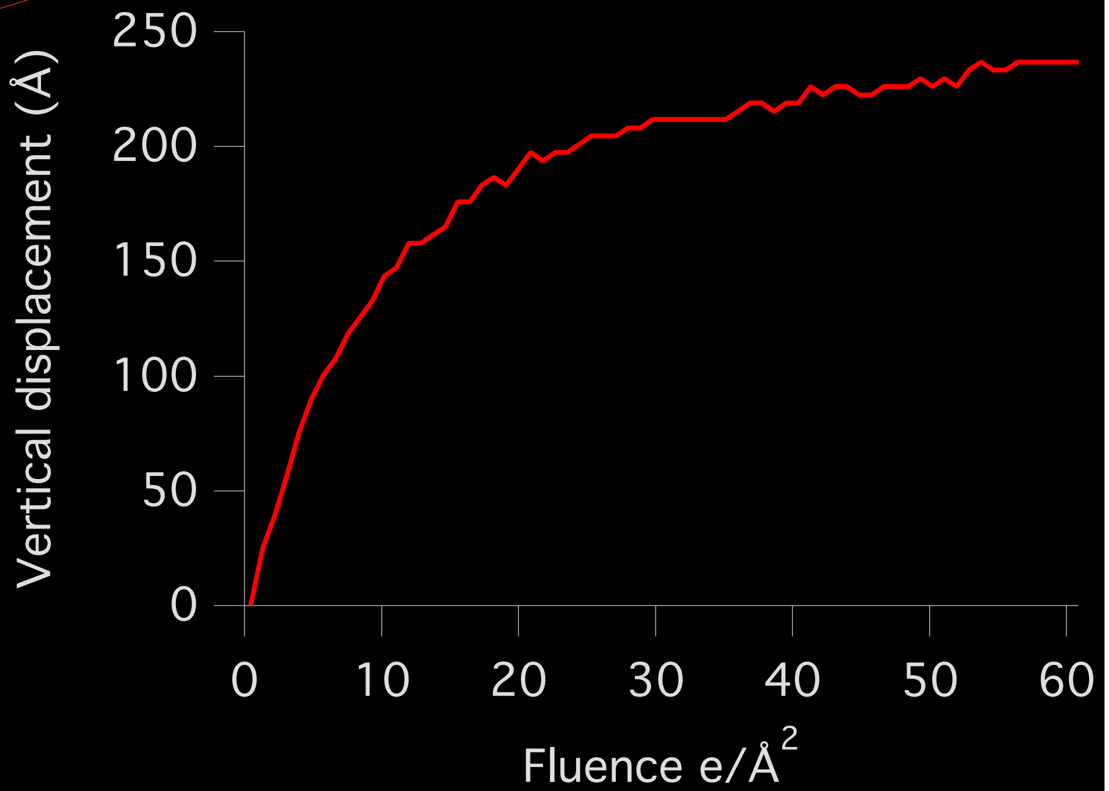
UltrAuFoils™ (Quantifoil)

Grid vertical movement

16 e/Å²/s

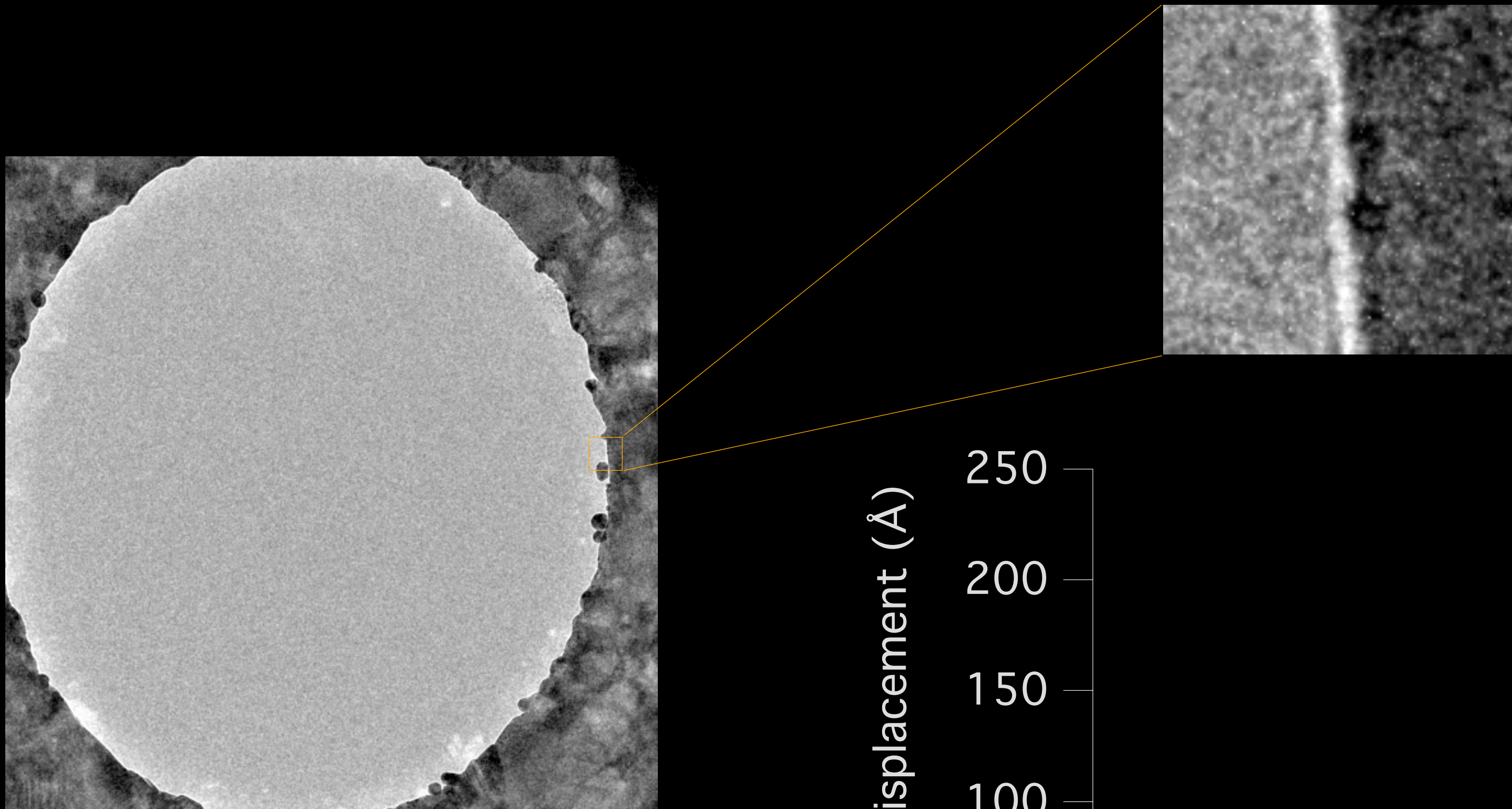


Quantifoil

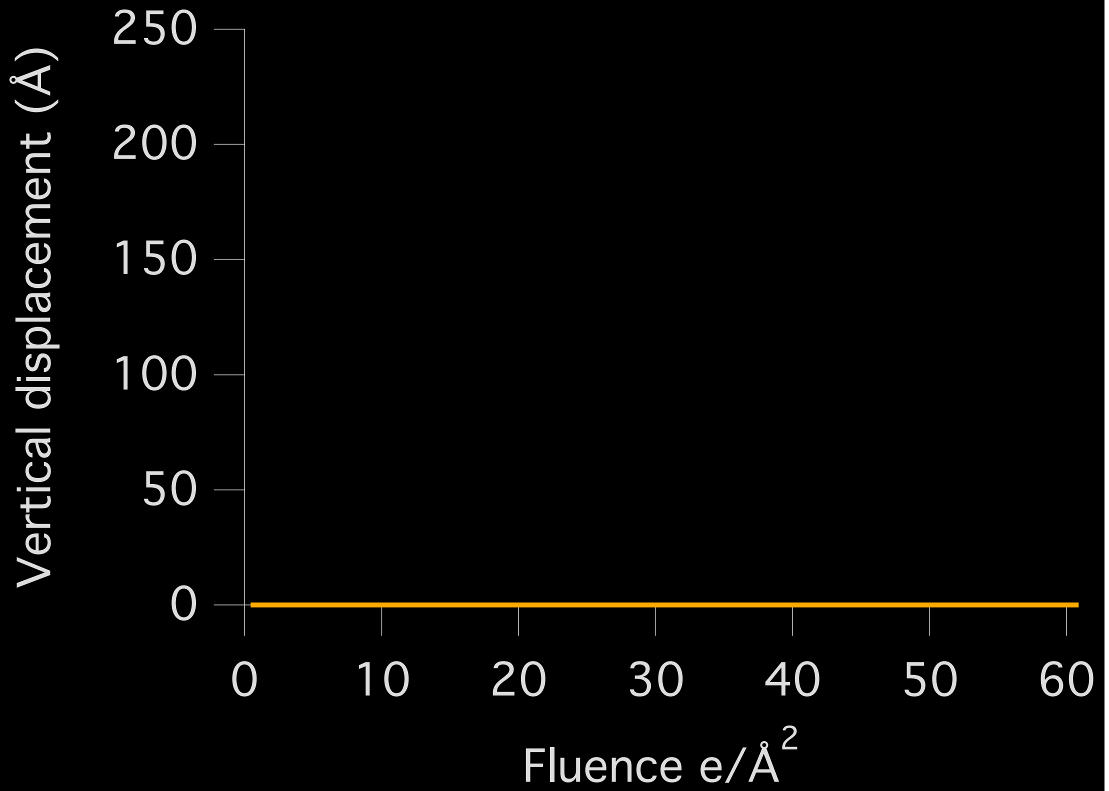


Grid vertical movement

16 e/Å²/s



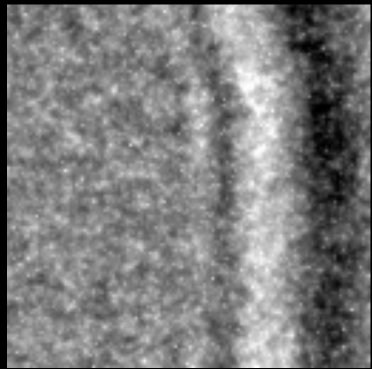
Gold



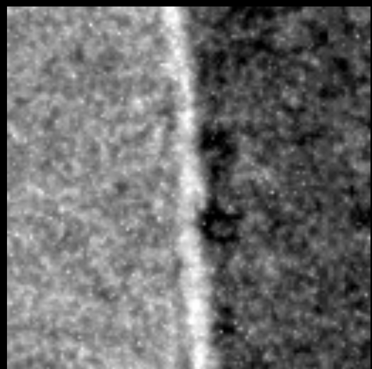
16 e/Å^2/s

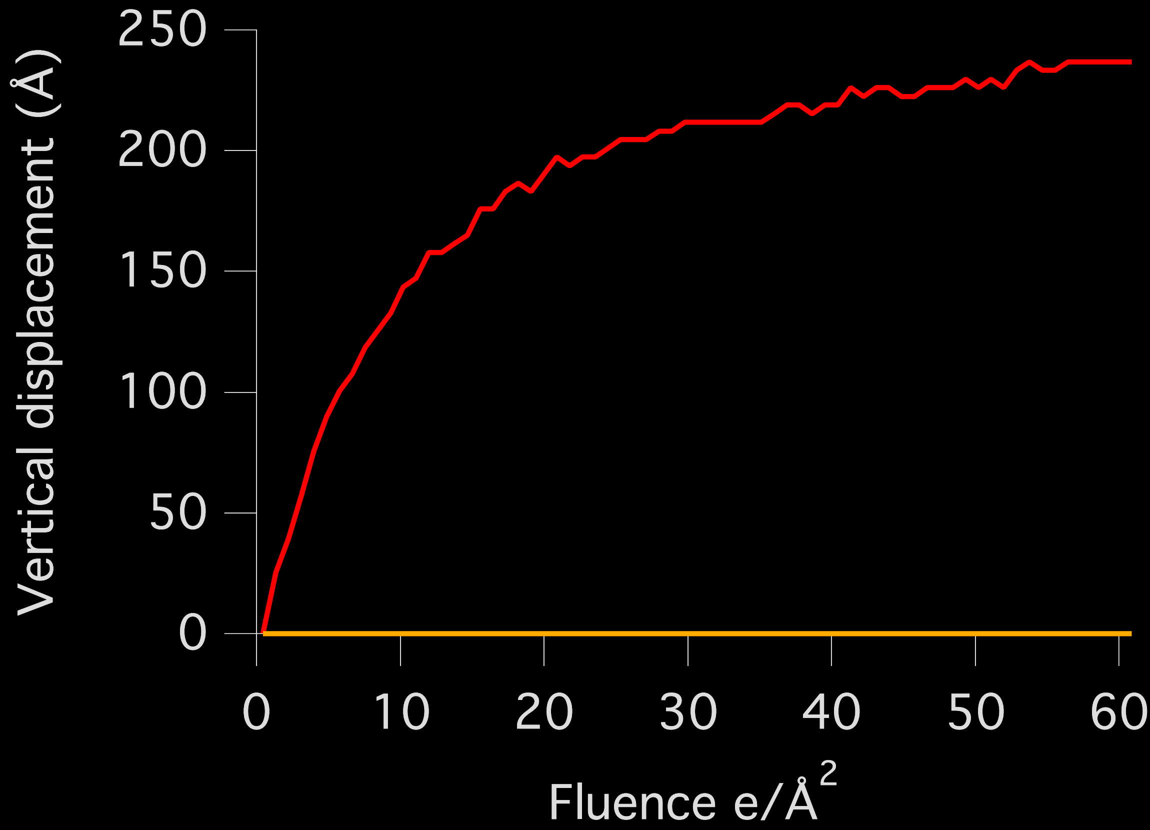
Grid vertical movement

Quantifoil

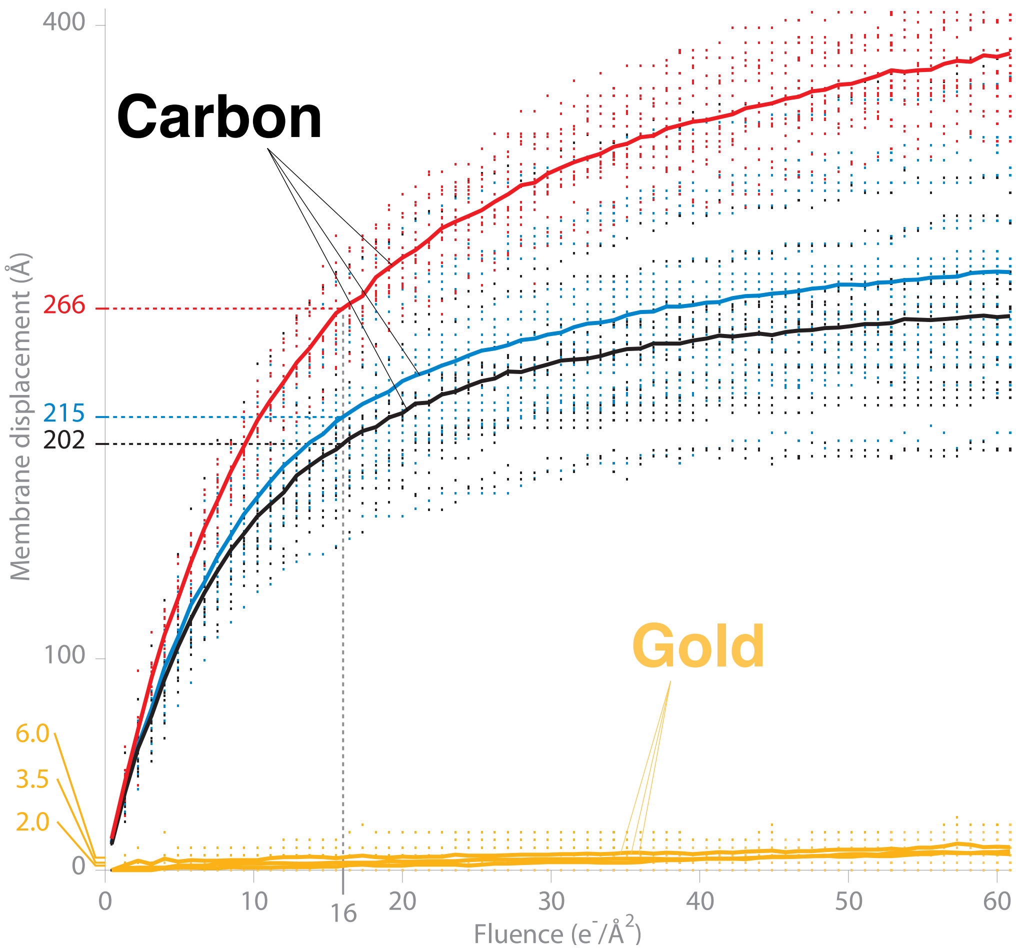


Gold





50x less movement in the z direction

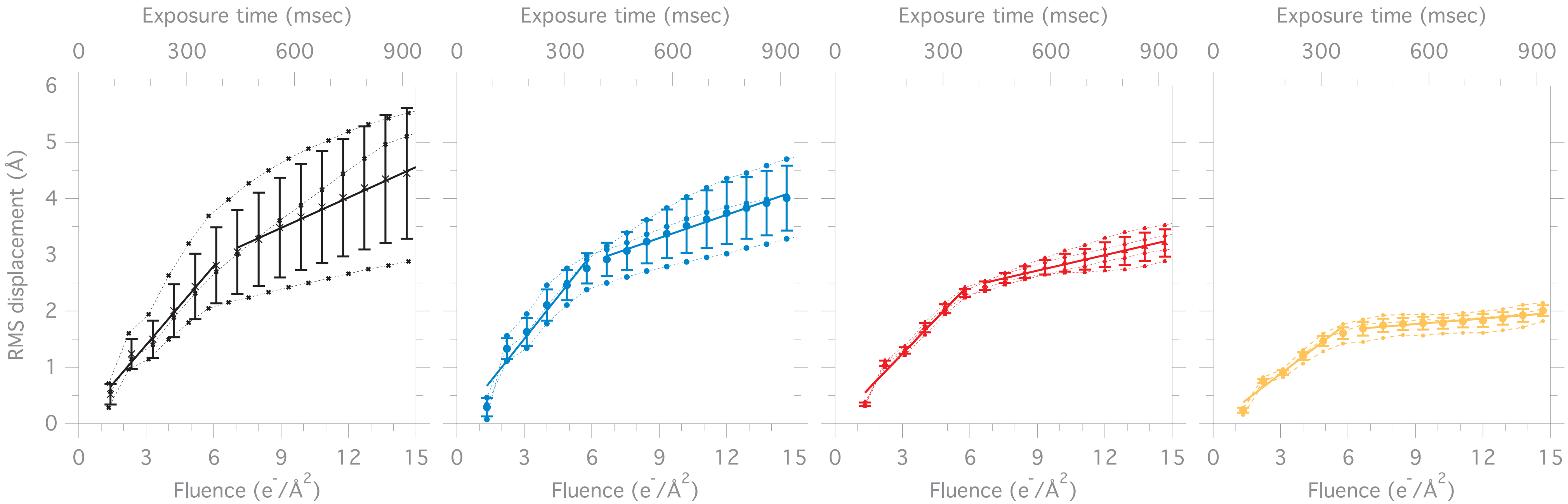


228 Å vs. 3.8 Å

No ice

Russo and Passmore (2014) Science

Ribosome speed plots on different substrates



amorphous carbon on quantifoil

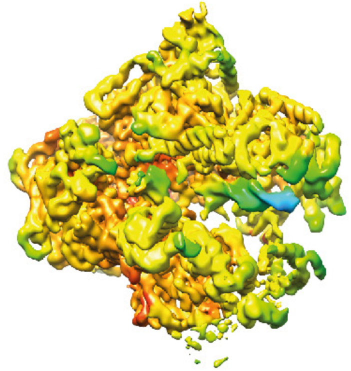
quantifoil

graphene on quantifoil

gold

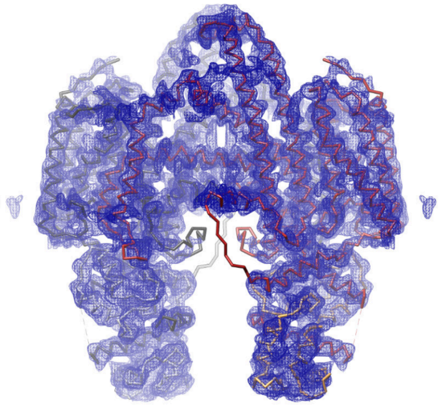
Pol I
3.8 Å

Neyer et al. 2016



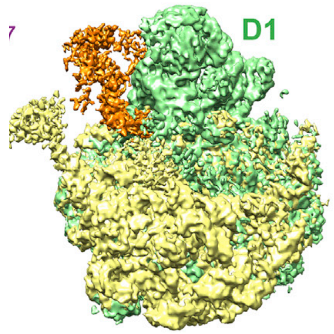
STRA6
3.9 Å

Chen et al. 2016



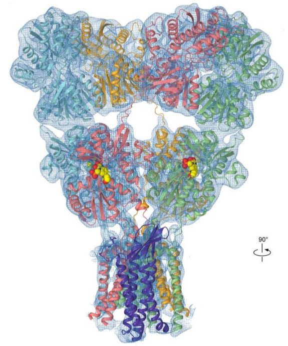
60S ribo
4.4 Å

Davis et al. 2016



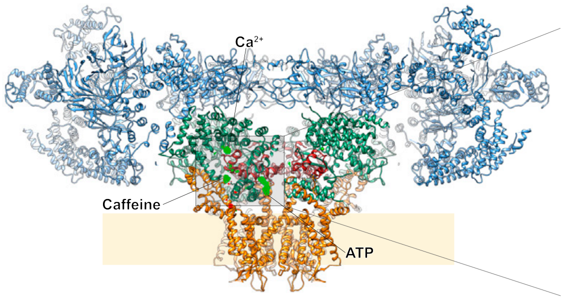
AMPA receptor
5.6 Å

Twomey et al. 2016



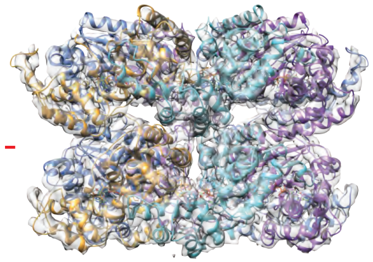
RyR1
3.6 Å

des Georges et al. 2016



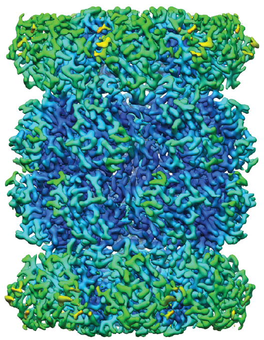
p97 AAAase
3.9 Å

Ripstein et al. 2017



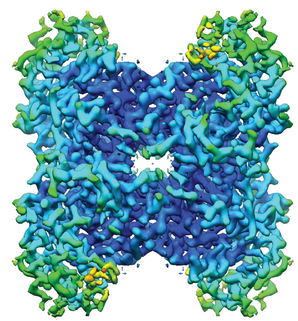
Proteasome
3.1 Å

Herzik et al. 2017



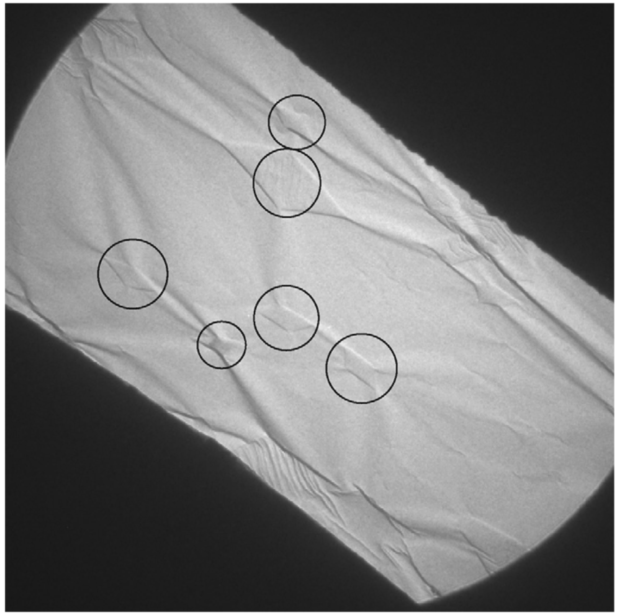
Adolase
2.6 Å

Herzik et al. 2017



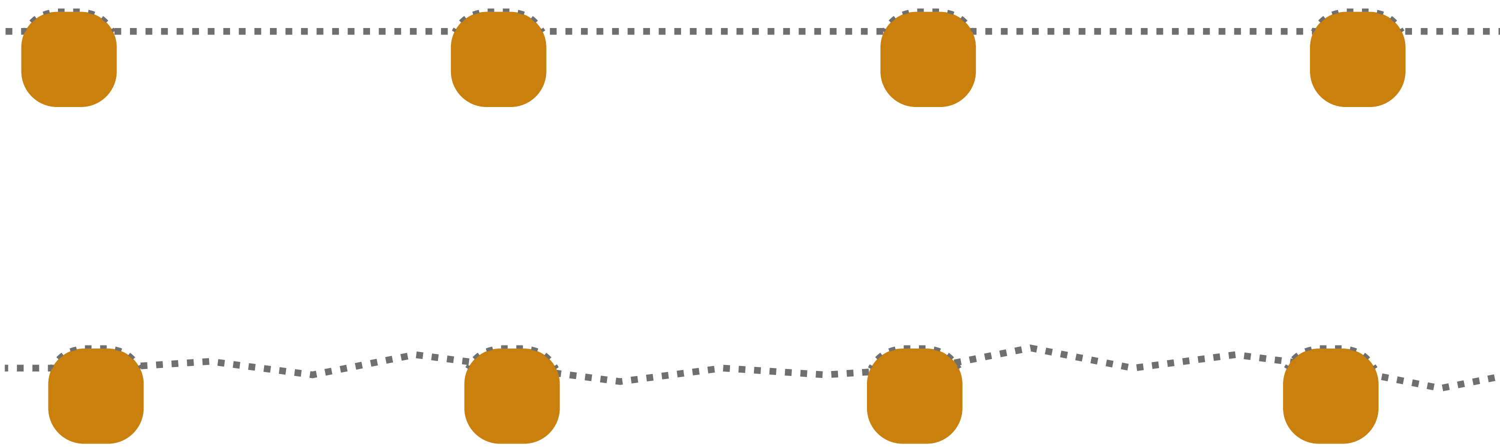
Coefficient of thermal expansion

$$\alpha_L(copper) = 16.6 \times 10^{-6} m/mK$$
$$\alpha_L(carbon) = 2 - 6 \times 10^{-6} m/mK$$



300 K

100K

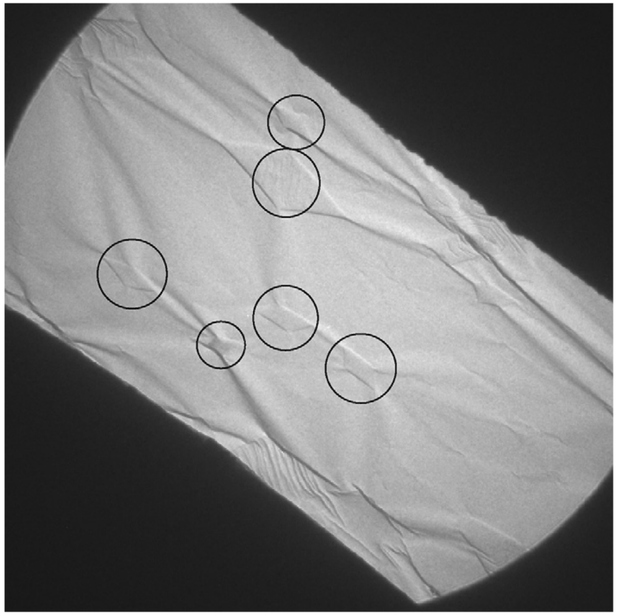


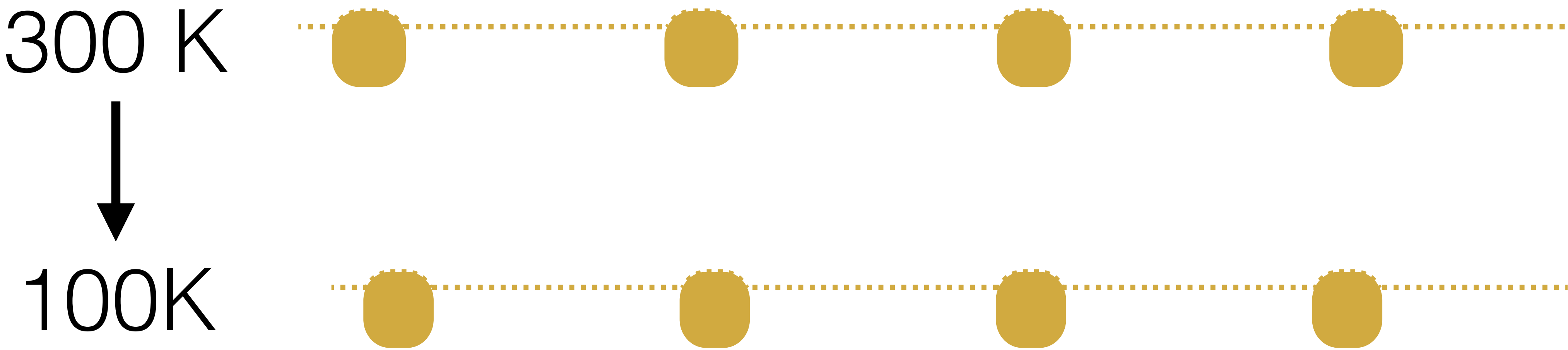
0.1% relative shrinkage

eg. Glaeser..Henderson 2011

Coefficient of thermal expansion

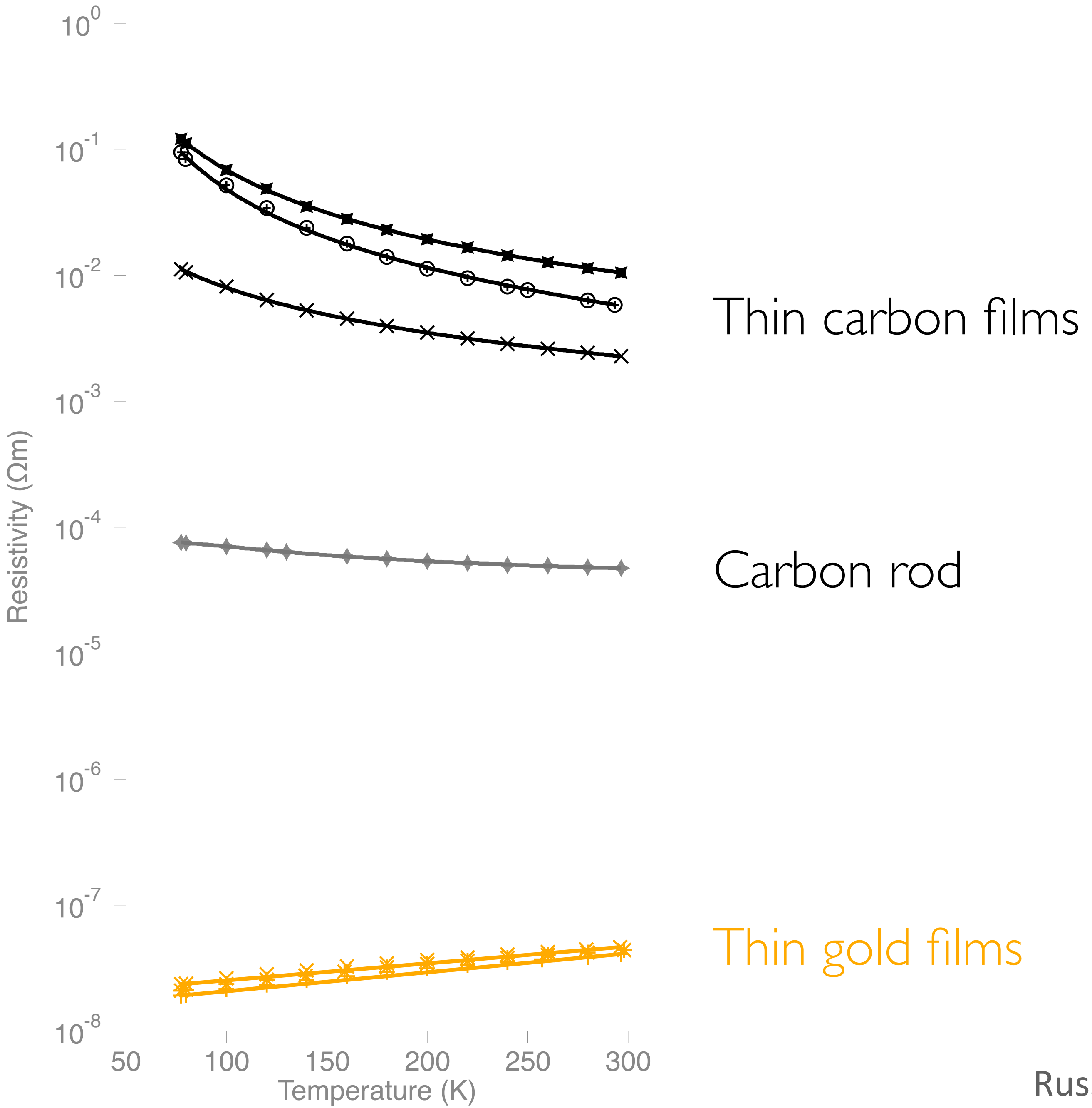
$$\alpha_L(copper) = 16.6 \times 10^{-6} m/mK$$
$$\alpha_L(carbon) = 2 - 6 \times 10^{-6} m/mK$$



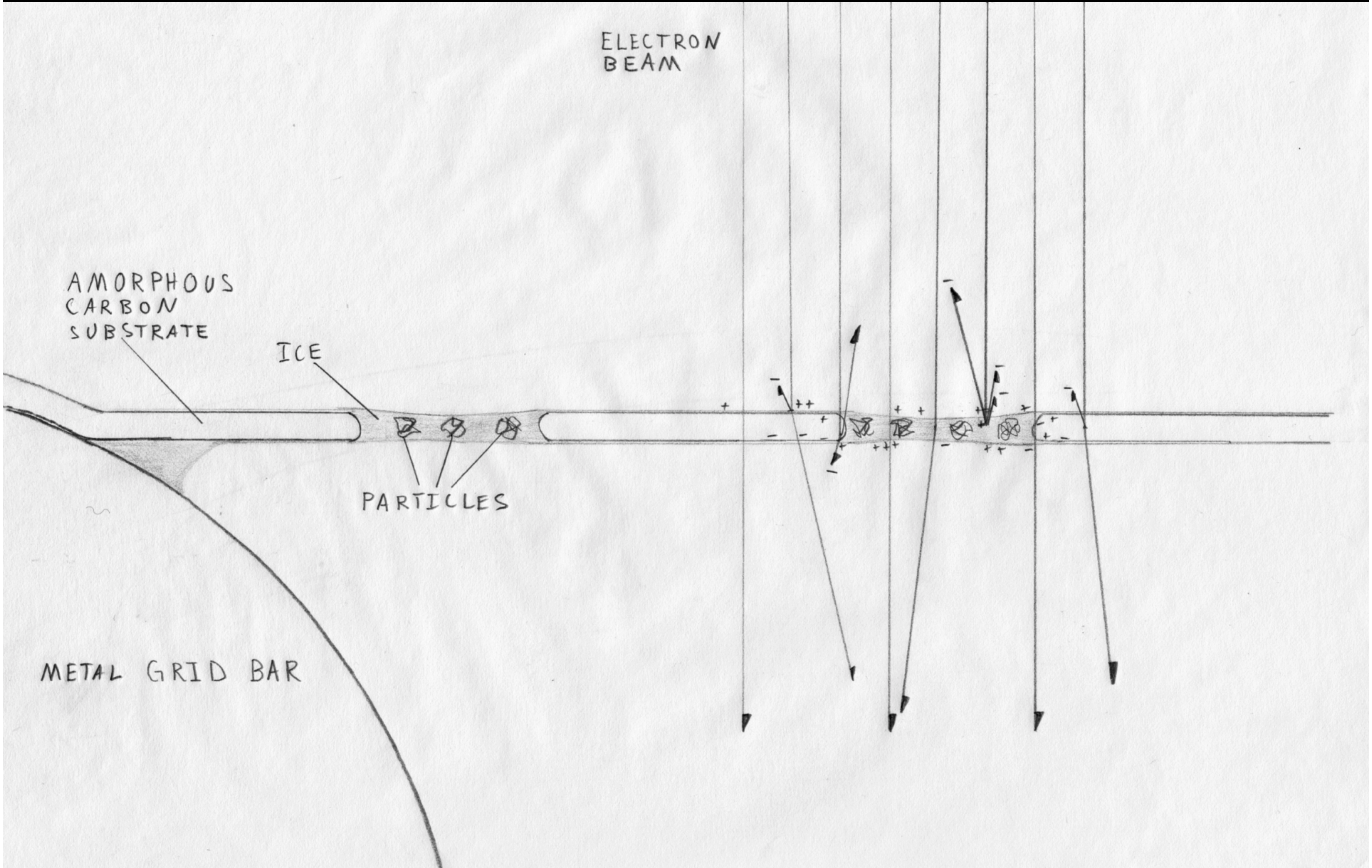


eg. Glaeser..Henderson 2011

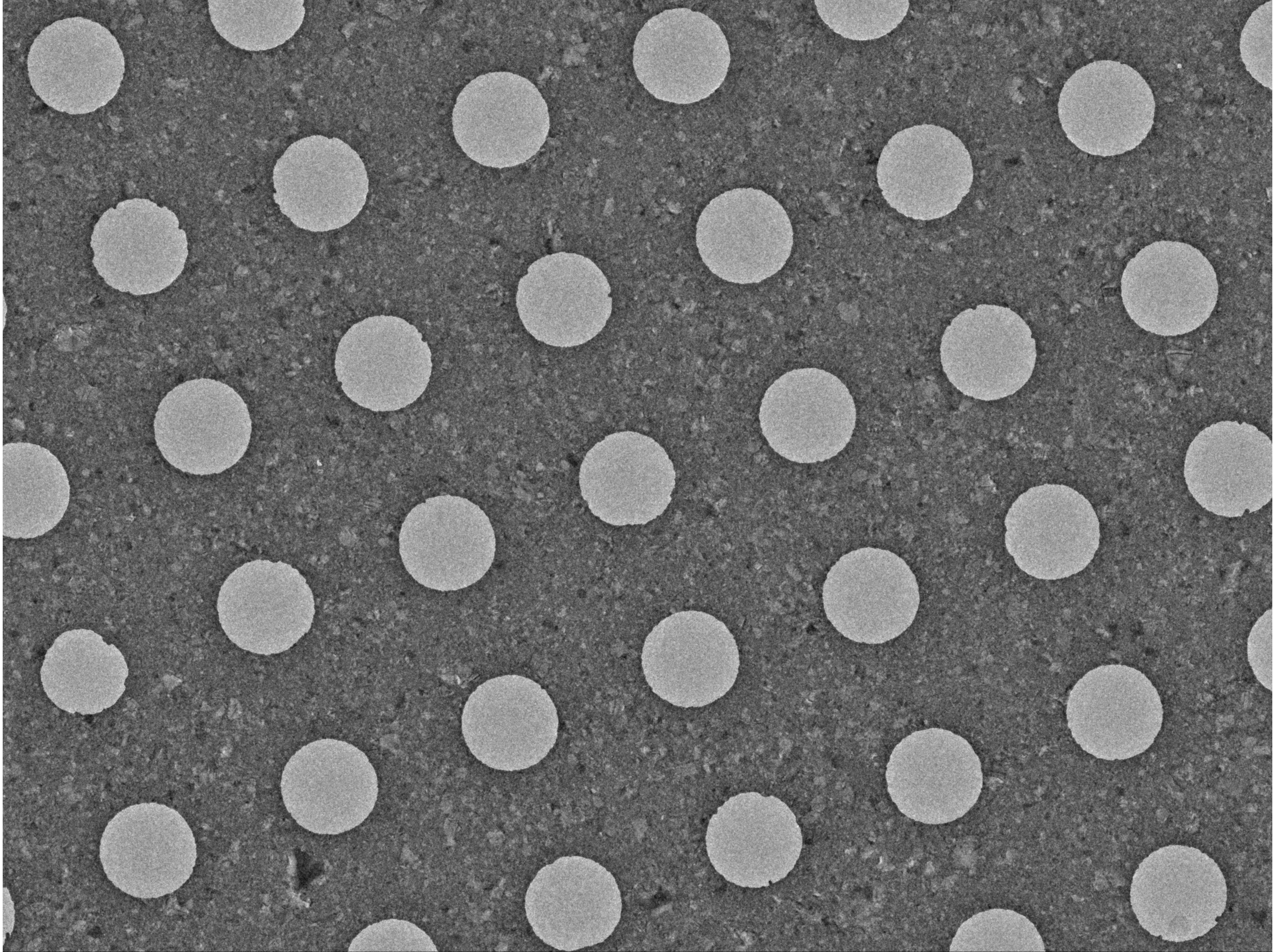
Conductivity measurements

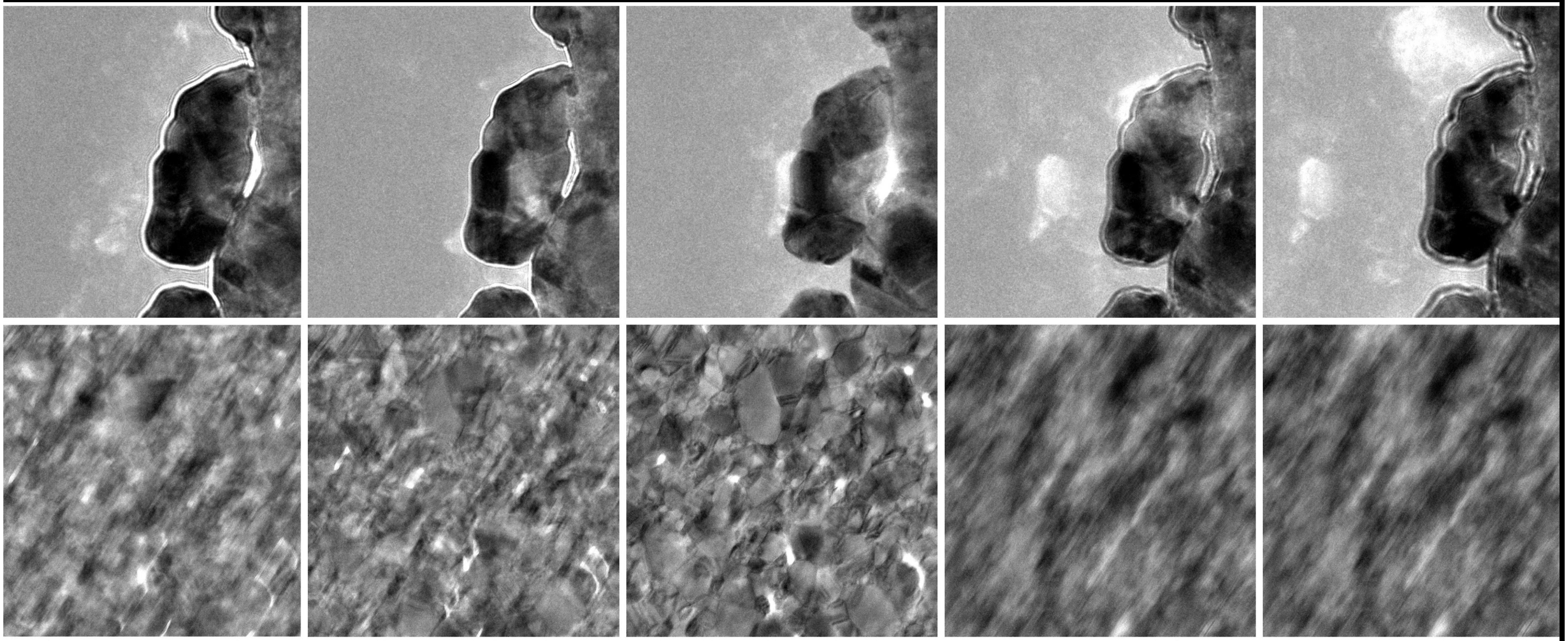


Russo and Passmore (2016)


ELECTRON
BEAM
AMORPHOUS
CARBON
SUBSTRATE
ICE
PARTICLES
METAL GRID BAR

How do I focus without carbon?

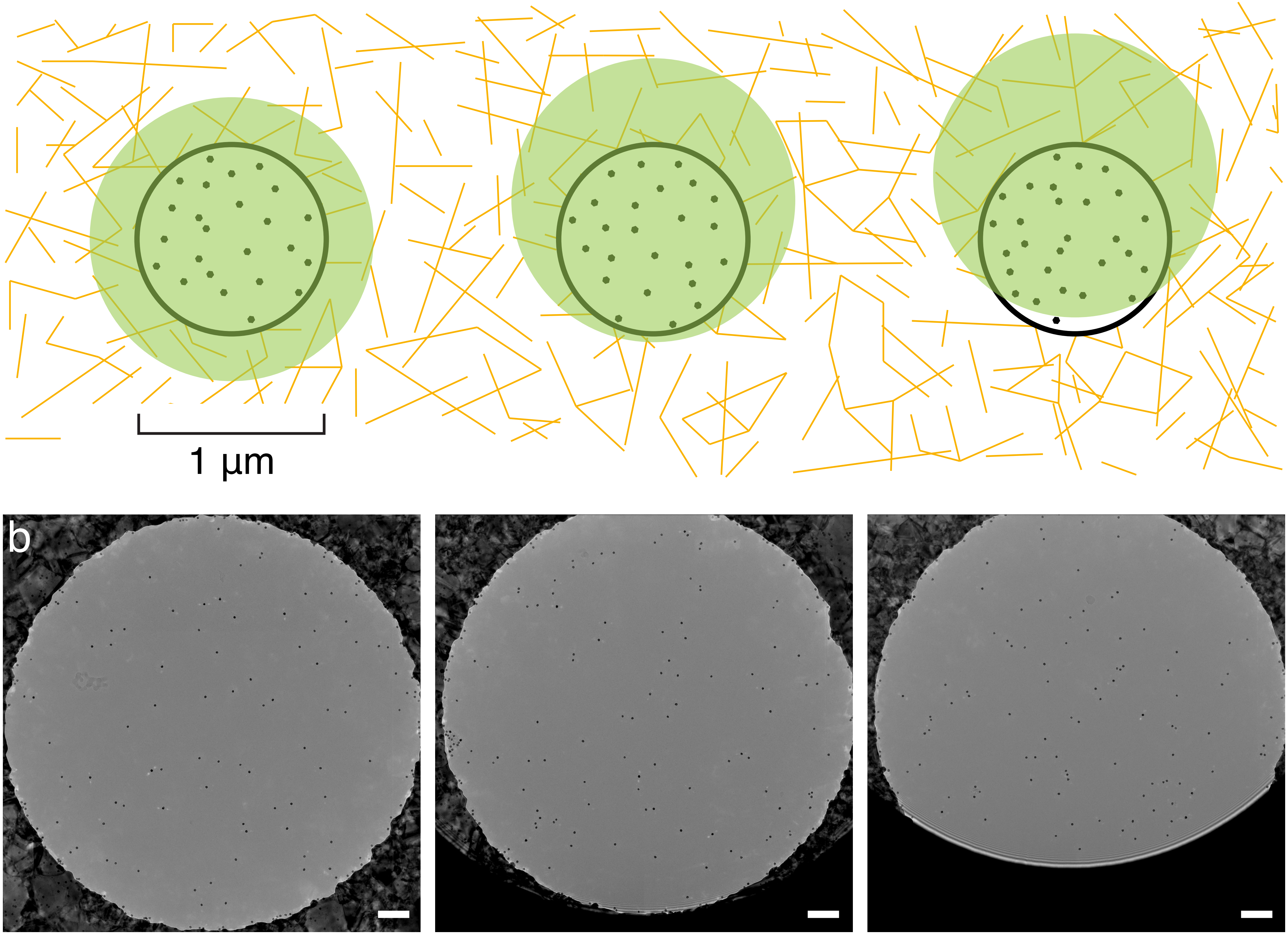


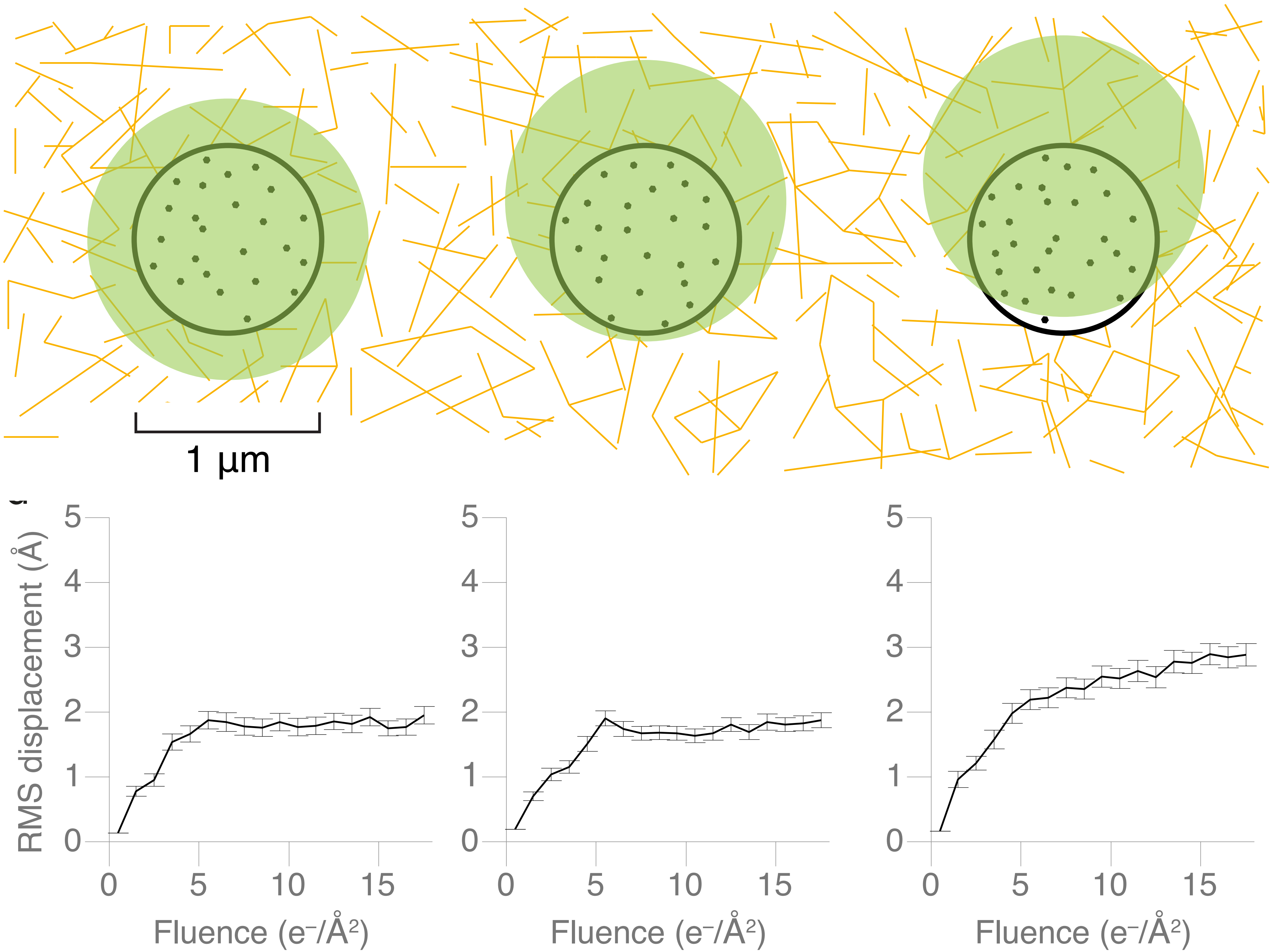


–4 µm –2 µm 0 µm +2 µm +4 µm

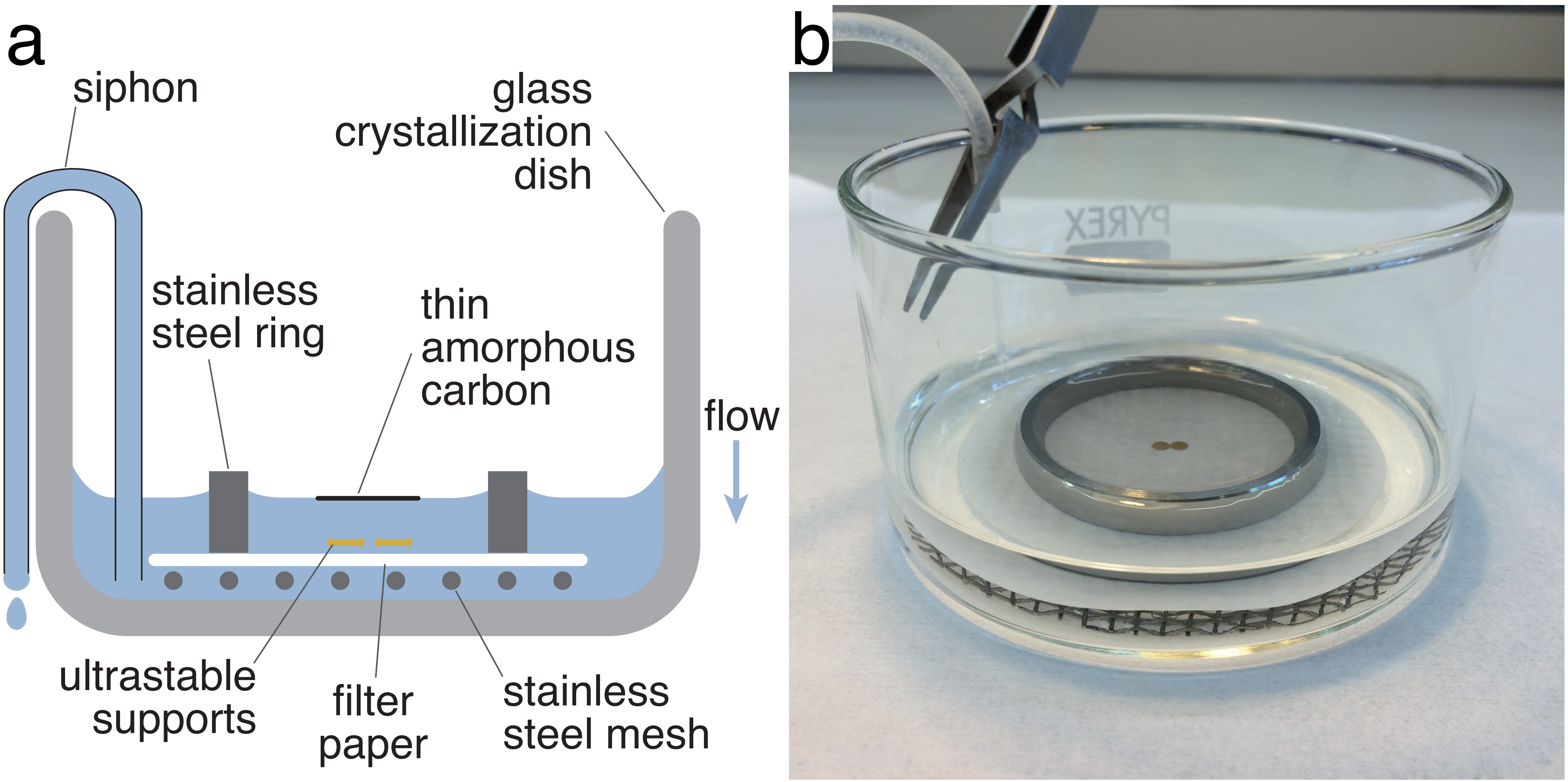
Defocus series

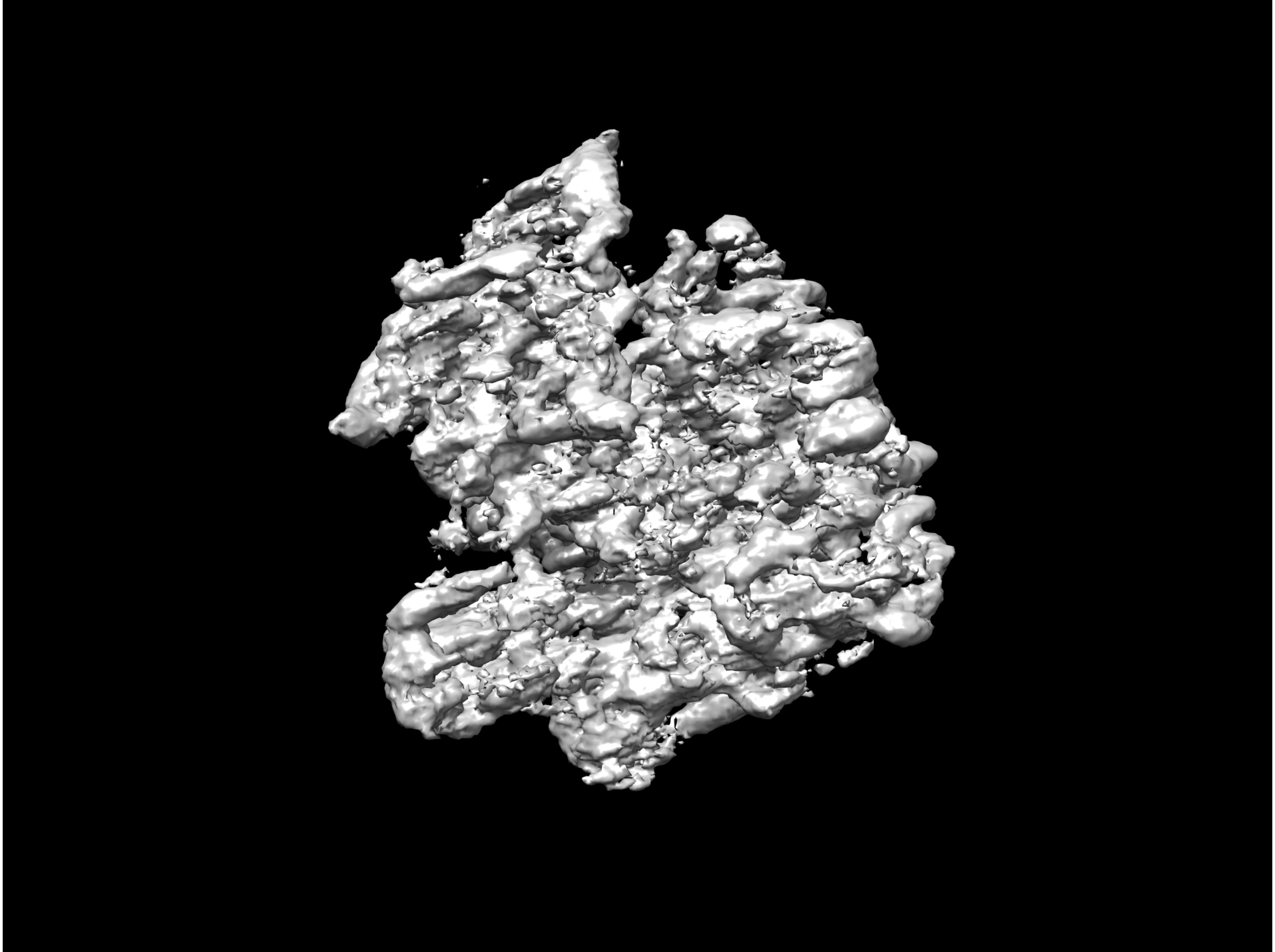
How should I set up the electron beam?


1 µm
b

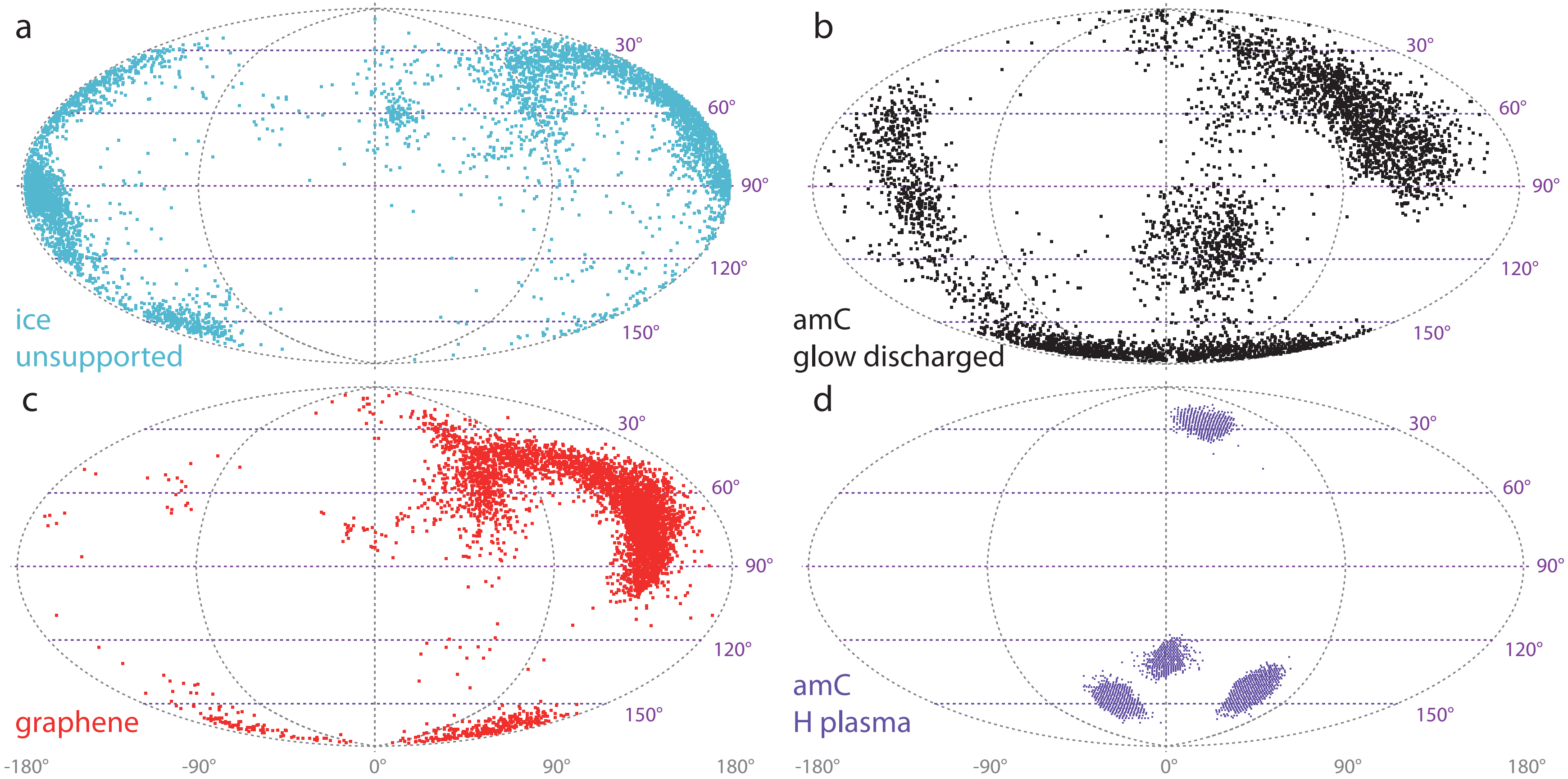


1 μm
RMS displacement (Å)
0
1
2
3
4
5
0
5
10
15
Fluence (e⁻/Å²)
Fluence (e⁻/Å²)
Fluence (e⁻/Å²)


a
siphon
glass
crystallization
dish
stainless
steel ring
thin
amorphous
carbon
flow
ultrastable
supports
filter
paper
stainless
steel mesh
b
PYREX



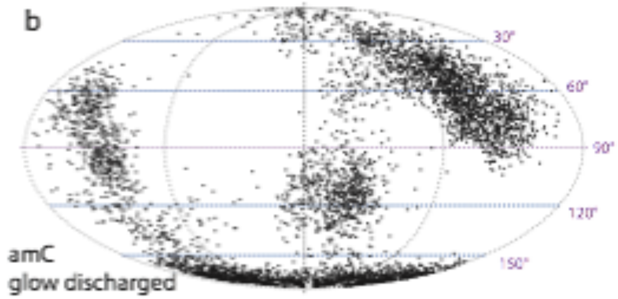
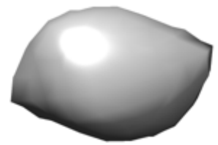
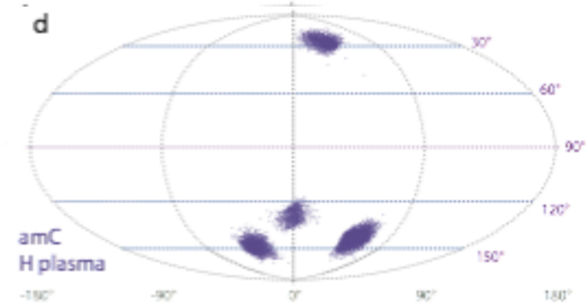
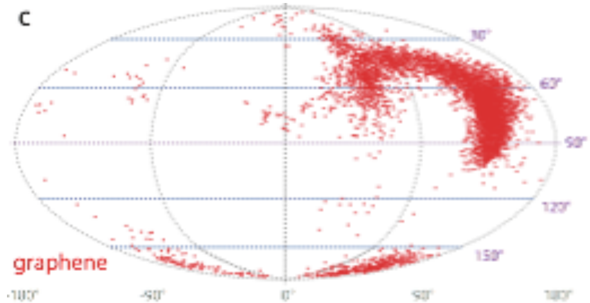
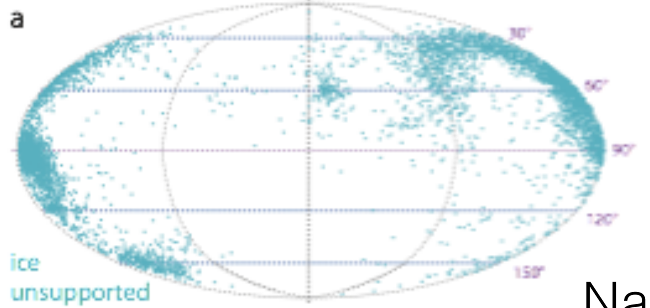
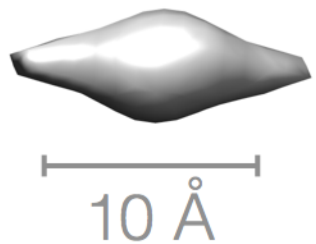
Efficient EM: Measuring the effect of orientation distribution on 3D reconstruction



Which is better?

Katerina Naydenova, Chris Russo

Measuring the quality of an orientation distribution

Surface	Orientation distribution	PSF	Efficiency
Amorphous C Glow discharged			0.55
Amorphous C H-treated			0.45
Graphene H - treated			0.38
Air-water			0.29

Naydenova & Russo *in review 2017*

Protein preparation

↓

Negative stain

↓

Diagnostic cryo-EM

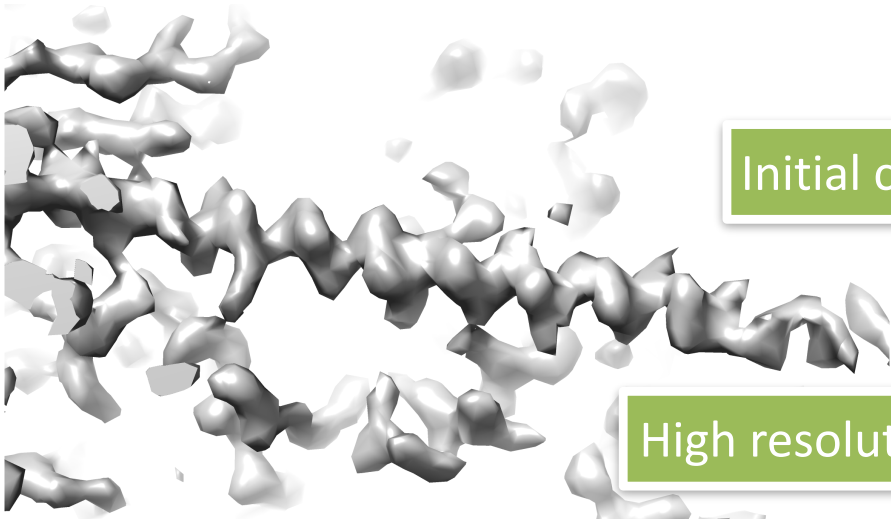
↓

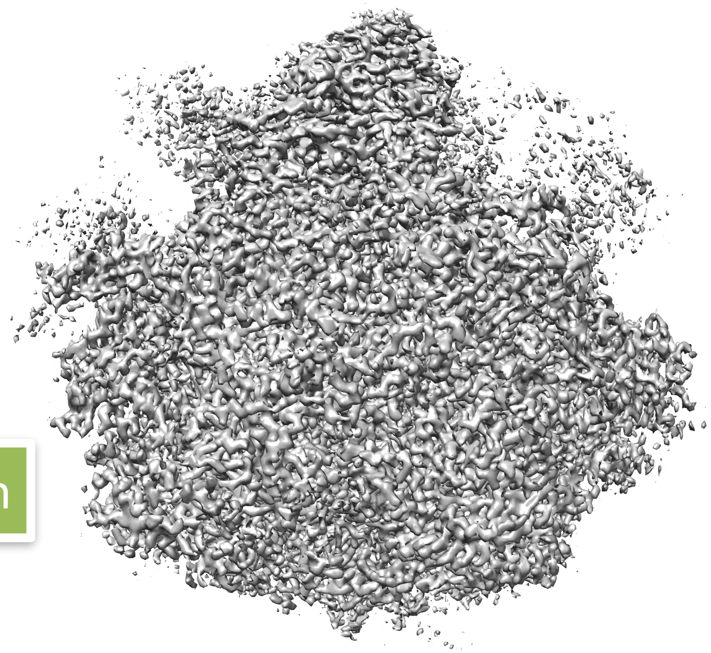
Initial cryo-EM data collection

↓

High resolution cryo-EM data collection

High-end
electron microscope



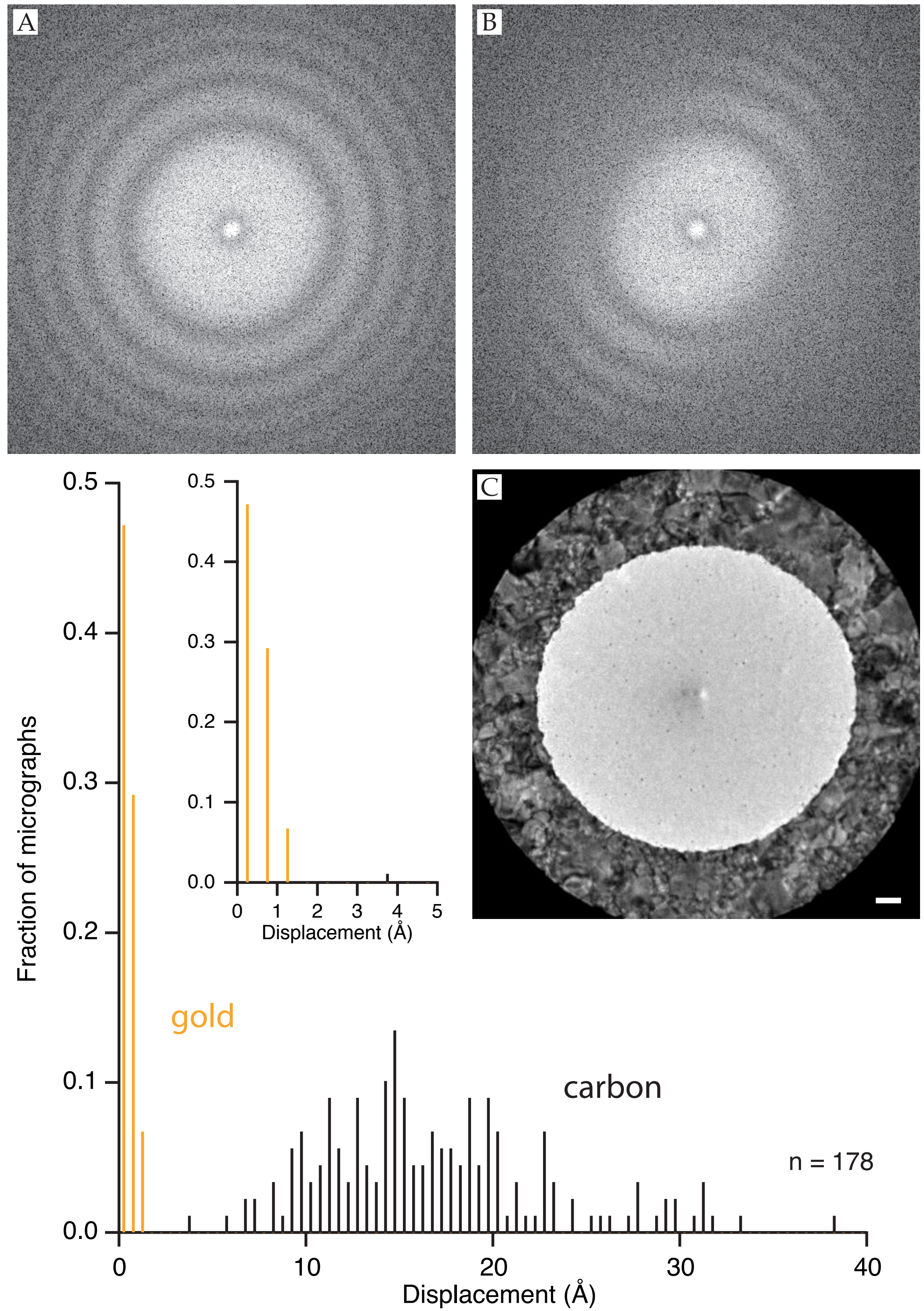


- Validation
- Motion statistics
- Angular accuracy
- Local/overall resolution
- Conformational states

Passmore & Russo MiE 2016

Motion tracking software:

Can now use to detect problems
with microscope
and incorrect beam alignments



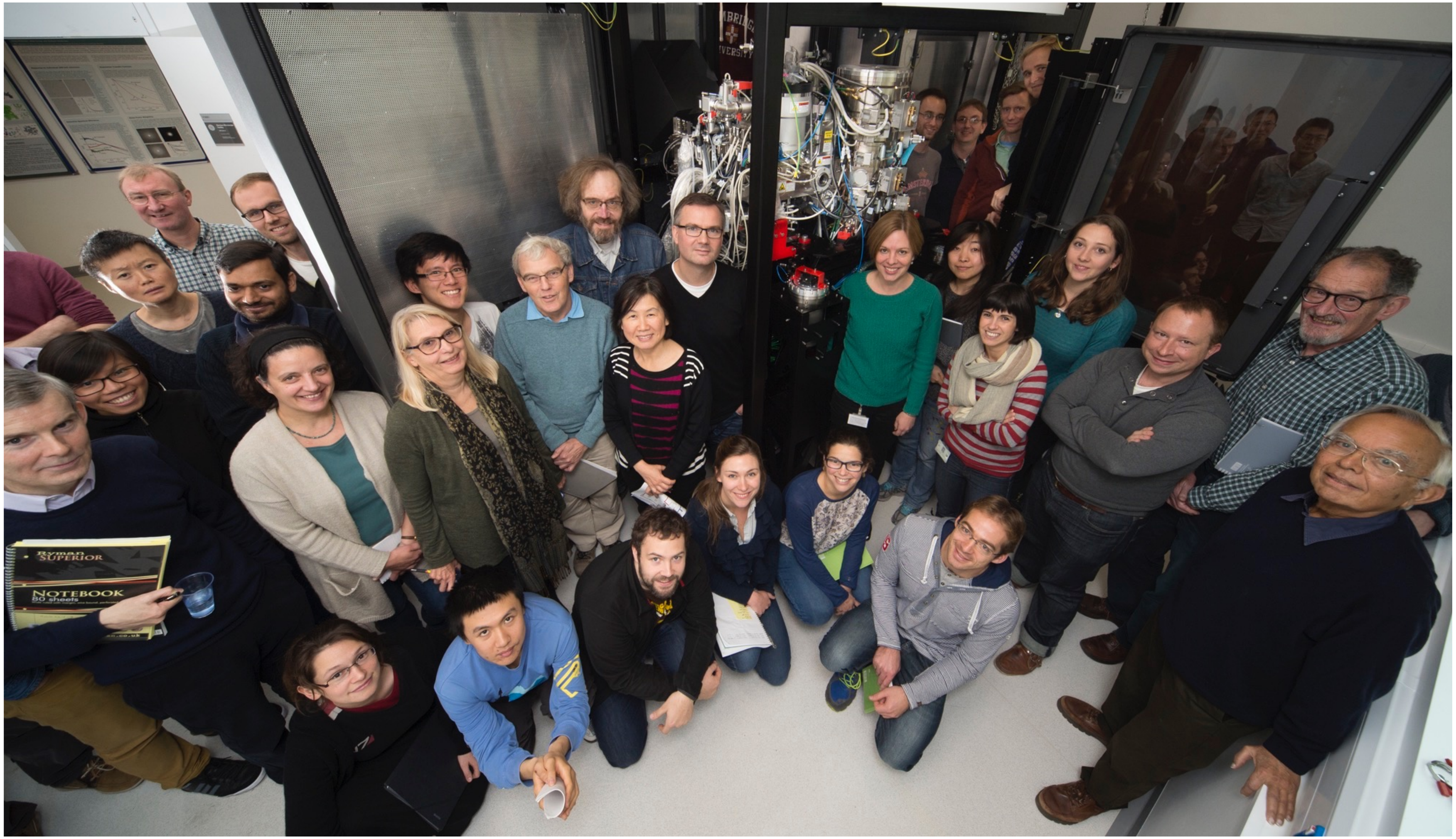
Future Challenges

Moving from a **trial-and-error** process to a **controlled** and **reproducible** method

This will need to address

- better vitrification methods
- rapid screening methods
- radiation induced motion, charging
- tunable interaction with surfaces

Acknowledgements



Chris Russo

Sjors Scheres
Greg McMullan
Shaoxia Chen, Christos Savva
Richard Henderson

LMB computation
LMB workshops

MRC Laboratory of Molecular Biology

EMBO Young Investigator

erc