Cryo EM sample preparation

13th November, 2019

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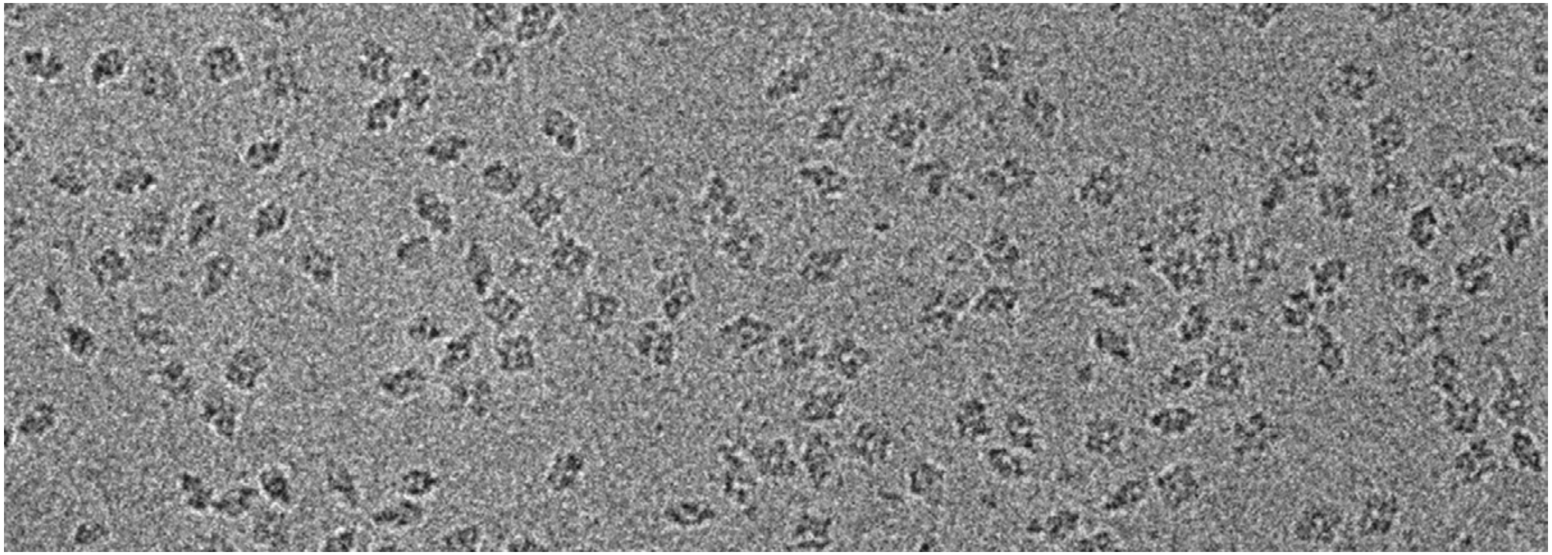
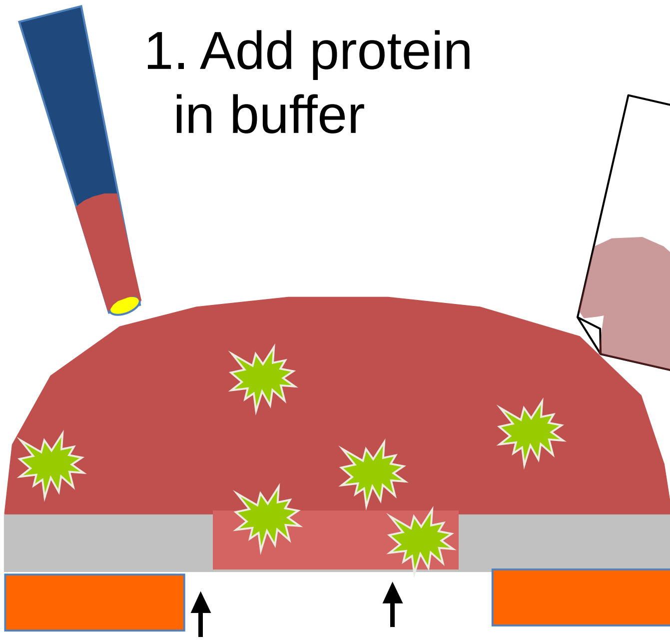
Cryo EM sample preparation: goals

- Preservation/stabilisation of native structure in aqueous solution
- Maximize contrast for best results

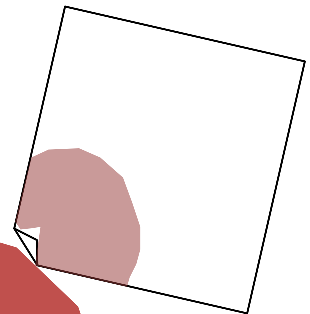


Traditional cryo EM sample preparation

1. Add protein in buffer



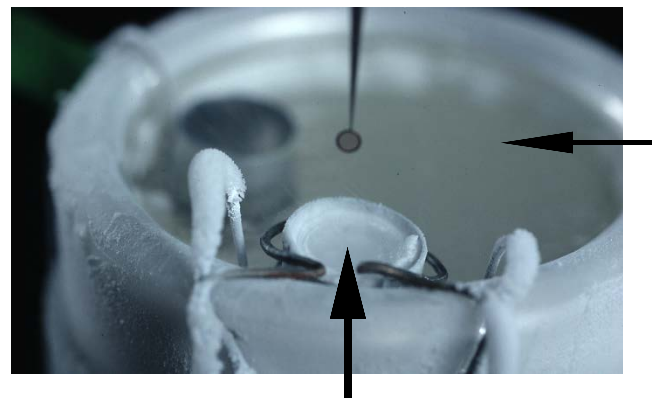
2. Blot



Carbon support film

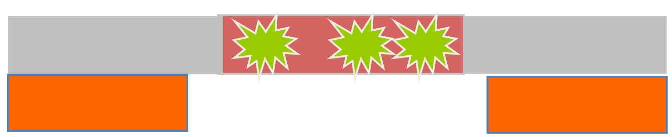
Holes in carbon support

3. Fast - 10^5-10^7 °C/sec – freezing result in vitreous ice. Could be achieved by plunging into liquid ethane



Liquid nitrogen - 196°C

Liquid ethane -172°C



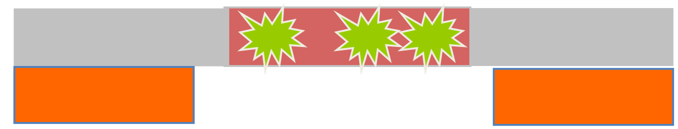
Grid bars

Vitrification of cryo EM sample

- Use of liquid ethane **just above the freezing point** of (−183 °C) allows the thin film of protein suspension (mostly water) to supercool sufficiently fast that ice crystals do not have time to nucleate and grow, resulting in low-density amorphous ice.
- **Regions near the metal grid bars** of the EM grid cool down more slowly due to the thermal inertia of the metal, resulting in a 3–5 µm wide region near each grid bar where the ice is frequently crystalline.
- As the temperature drops **metal grid bars shrink** more than carbon film (0.2% for copper vs ~0.02% for carbon), that thus develops wrinkles as it cools (cryo-crinkling).
- While metal and carbon shrinks, thin film of **buffer expands** as it is transformed into amorphous ice by ~2.5%. These differential changes in volume create stresses in the frozen film of amorphous ice, which are locked into the specimen at least until the electron beam is switched on.

Ideal single particle cryo EM sample

Ideal sample:



the majority of areas in holes maximally occupied by free-floating, non-interacting particles **10-20 nm from the air-water interfaces**; particles oriented randomly, no overlap in the beam direction.

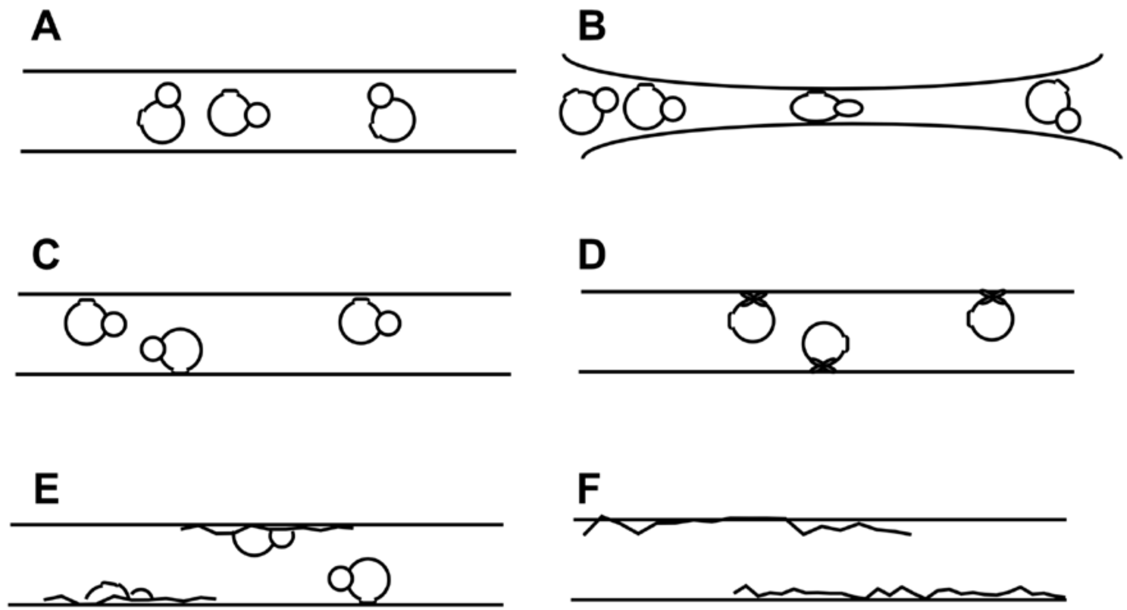
Concentration

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)

Expected particle distribution on holey grids: number per μm^2 and the expected particle separation for 800 Å thick ice (if the frozen specimen has the same concentration of molecules that you expect in free solution).

Vinothkumar & Henderson, 2016

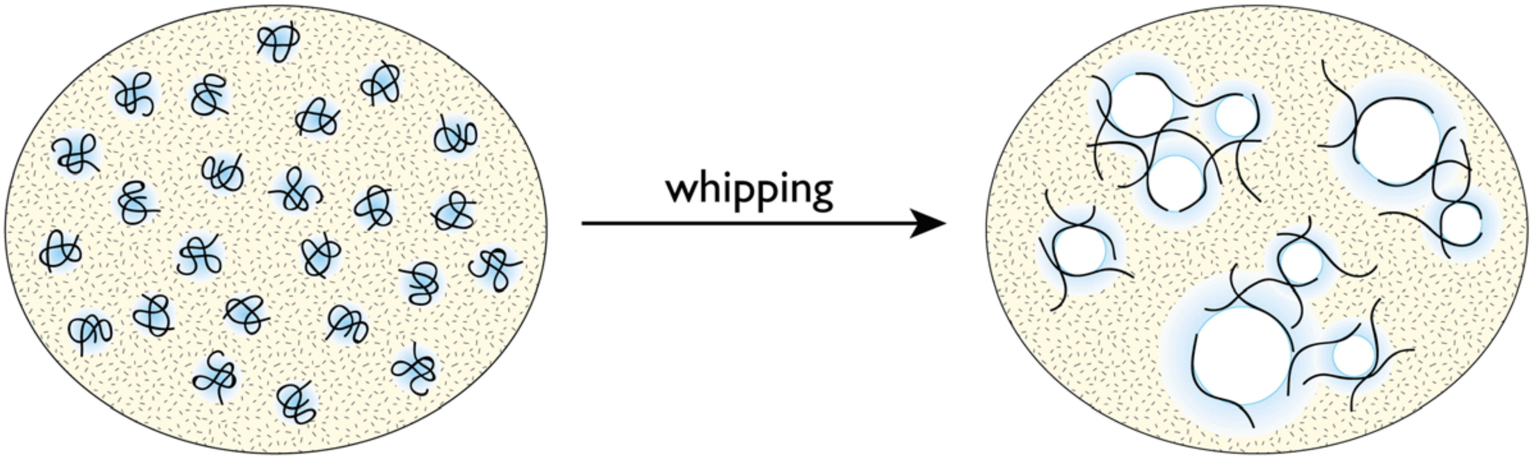
Cartoon-representations of a cryo EM preparation



A, ideal; **B**, particles fully embedded but some may be partially "flattened"; **C**, particles are adsorbed to the air-water interface with one or more preferred orientations; **D**, particles are adsorbed to the air-water interface and partially or completely denatured; **E**, some particles denature and spread at the air-water interface; for others "half" of structure may remain intact in the aqueous subphase; **F**, no particles are seen even though an appropriate concentration of protein was applied

Taylor & Glaeser, 2008

Air-water interface



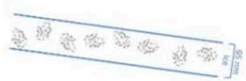
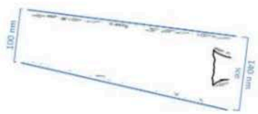
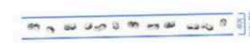
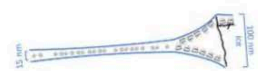
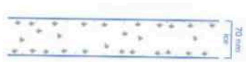
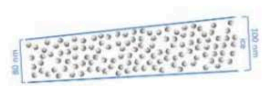
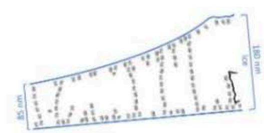
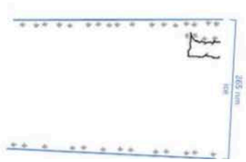
Air-water interface and cryo EM sample preparation

- Various proteins (10–1,000 kDa and at ≲1 mg/mL) in buffer commonly adsorb to the air-water interface and form <10 nm thick denatured viscoelastic protein network films.
- Adsorption due to bulk diffusion requires ~0.1-1 ms to begin, depending on the protein, i.e., starts immediately after sample deposition on the grid.
- For a protein that denatures at the air-water interface (surface diffusion), tens of milliseconds are required depending on protein and concentration, surface hydrophobicity, amount of disordered structure, secondary structure, concentration of intramolecular disulfide bonds, buffer, and temperature

Taylor & Glaeser, 2008; Vinothkumar & Henderson, 2016

Single particle cryo EM sample and grid characterization by tomography

The primary result gleaned from over 1,000 tomograms of over 50 different single particle grid/sample preparations is that the vast majority of all particles (approximately 90%) are local to an air-water interface.

Sample # Name	Example cross-sectional schematic diagram	Sample # Name	Example cross-sectional schematic diagram	Sample # Name	Example cross-sectional schematic diagram
1* 32 kDa Kinase		**14*** Neural Receptor		**27*** IDE	
4*† Hemagglutinin		**17*** Protein with Bound Lipids (deglycosylated)		**30*†** GDH	
5* HIV-1 Trimer Complex 1		**18** Protein with Bound Lipids (glycosylated)		**31*†** GDH	
6* HIV-1 Trimer Complex 1		**19*** Lipo-protein		**32*†** GDH + 0.001% DDM (2.5 mg/mL)	
7* HIV-1 Trimer Complex 2		**20** GPCR		**33*†** DNAB Helicase-helicase Loader	

Noble *et al.*, 2018

Single particle cryo EM sample and grid characterization by tomography

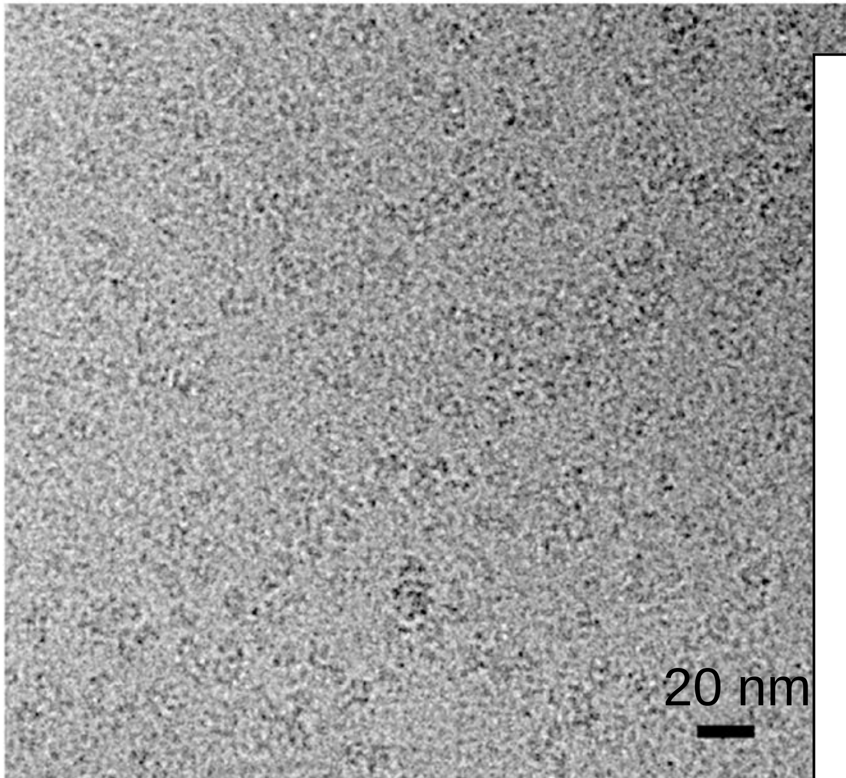
- Only two samples, protein in nanodisc and protein on streptavidin, exhibit ideal characteristics – less than 100 nm ice thickness, no overlapping particles, little or no preferred orientation, and no particle-air-water interface interaction.
- Several samples show an asymmetry between particle saturation at the top and bottom air-water interfaces.
- A large fraction of the samples contain imaging areas in holes (sometimes limited to near the edges of holes, where in addition to a single layer of particles at one air-water interface, there are free floating particles and/or a second layer of particles at the apposed air-water interface).
- Most of the air-water interfaces are tilted between 0° and 16° relative to the electron beam when at a nominal stage tilt of 0°.

Noble *et al.*, 2018

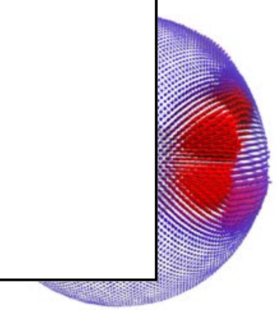
Controlling protein adsorption: continuous support

- 3-4 nm amorphous carbon or graphite (for large protein/complexes only because of the background)
- Graphene: an atomically thin, mechanically robust conductor; hydrophobic and is susceptible to surface contamination during manufacturing, handling and storage
- Graphene oxide: it contributes nearly as much background signal as thin graphite, it is often an insulator; it has decreased mechanical strength, making it less stable than graphene

Controlling protein adsorption: continuous carbon support



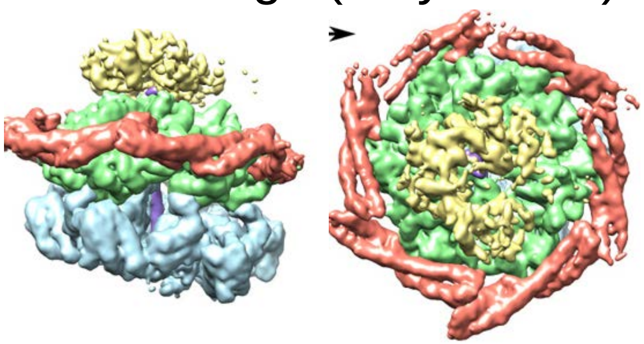
Cryo EM structure of Tra1 at

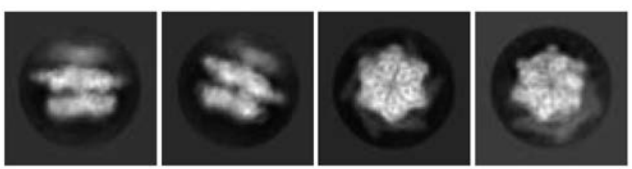


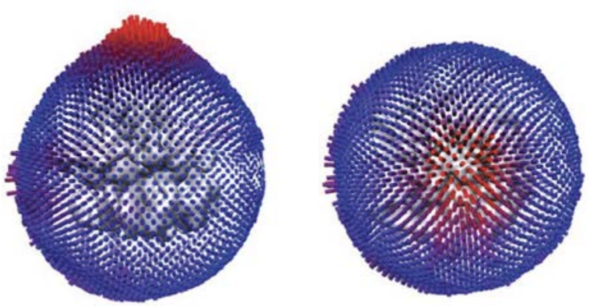
Díaz-Santín *et al.*, 2017

Cryo EM of *E. coli* disaggregase ClpB

2-3 nm carbon over holes, positive glow discharge (amylamine)

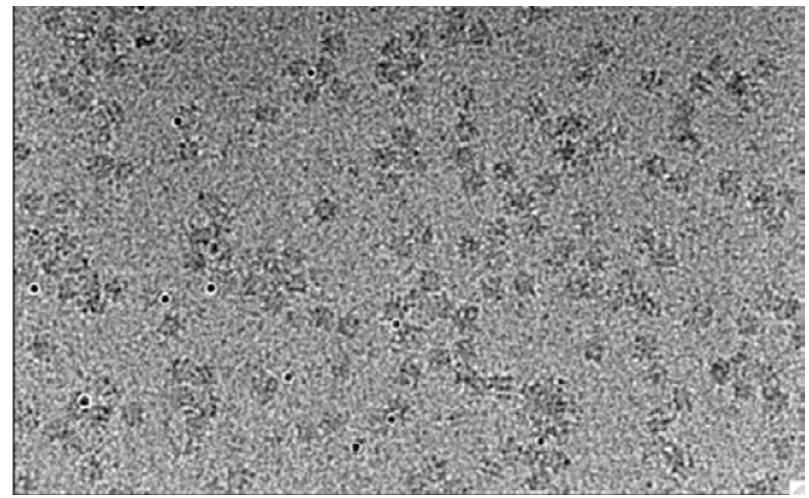


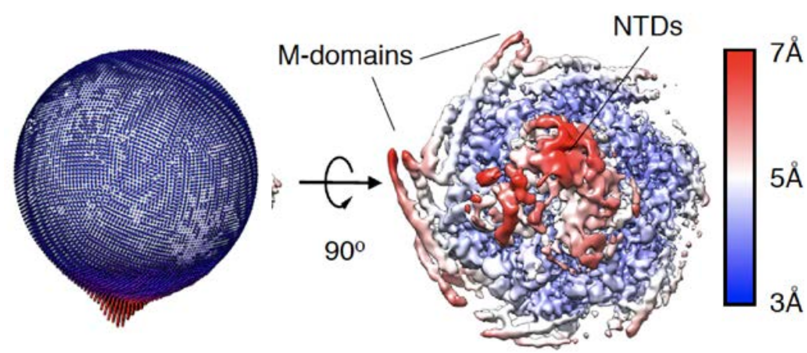




Deville *et al.*, 2017

graphene oxide over holes

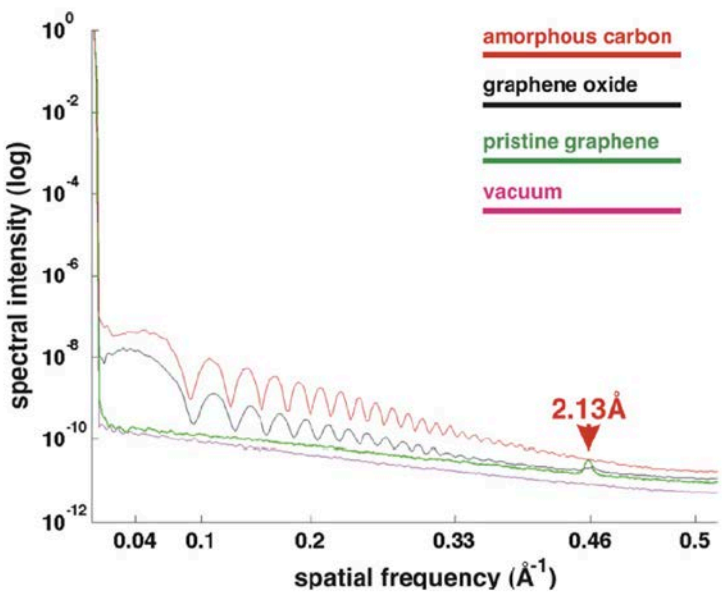




Deville *et al.*, 2019

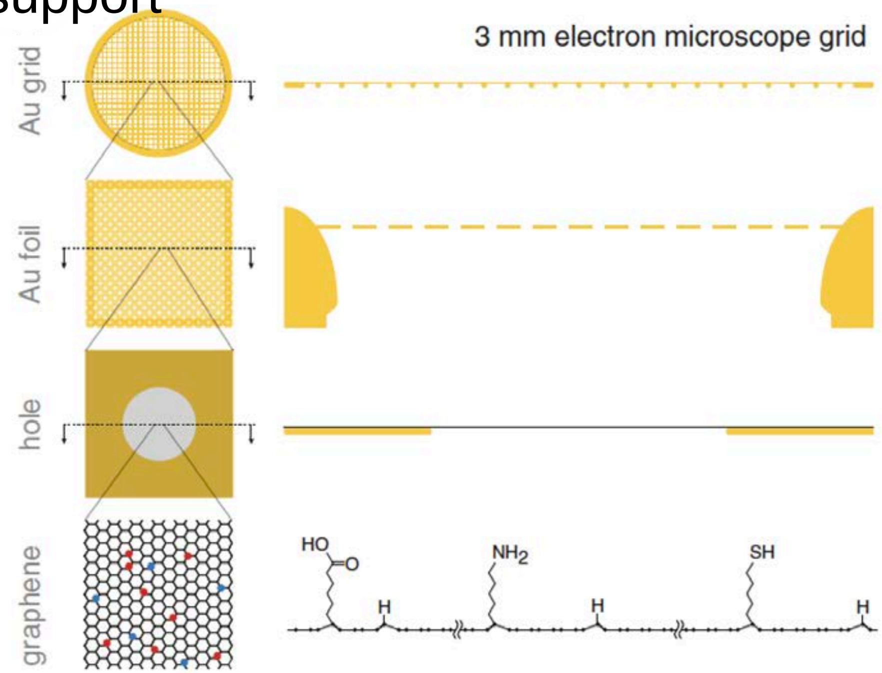
Graphene supported ice

Comparison of introduced substrate signal by power spectra



Pantelic *et al.*, 2011

Multifunctional and ultrastable graphene support



Graphene can be covalently functionalised by exposure to a low-energy helium plasma containing precursor molecules (shown for amylamine, hexanoic acid, 1-pentanethiol, and 4-pentylphenol).

Naydenova, Peet, & Russo, 2019