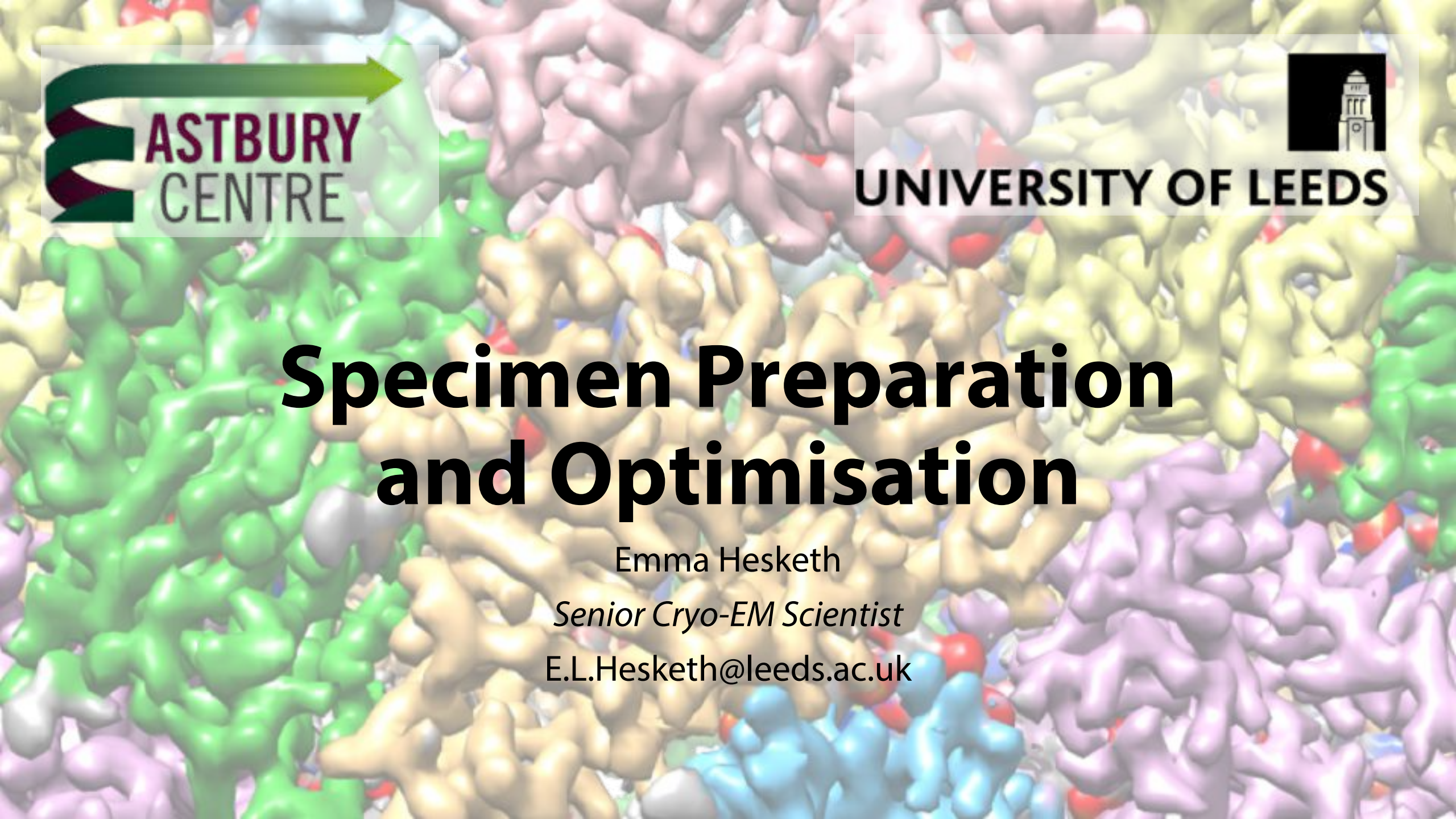


ASTBURY CENTRE

UNIVERSITY OF LEEDS

Specimen Preparation and Optimisation

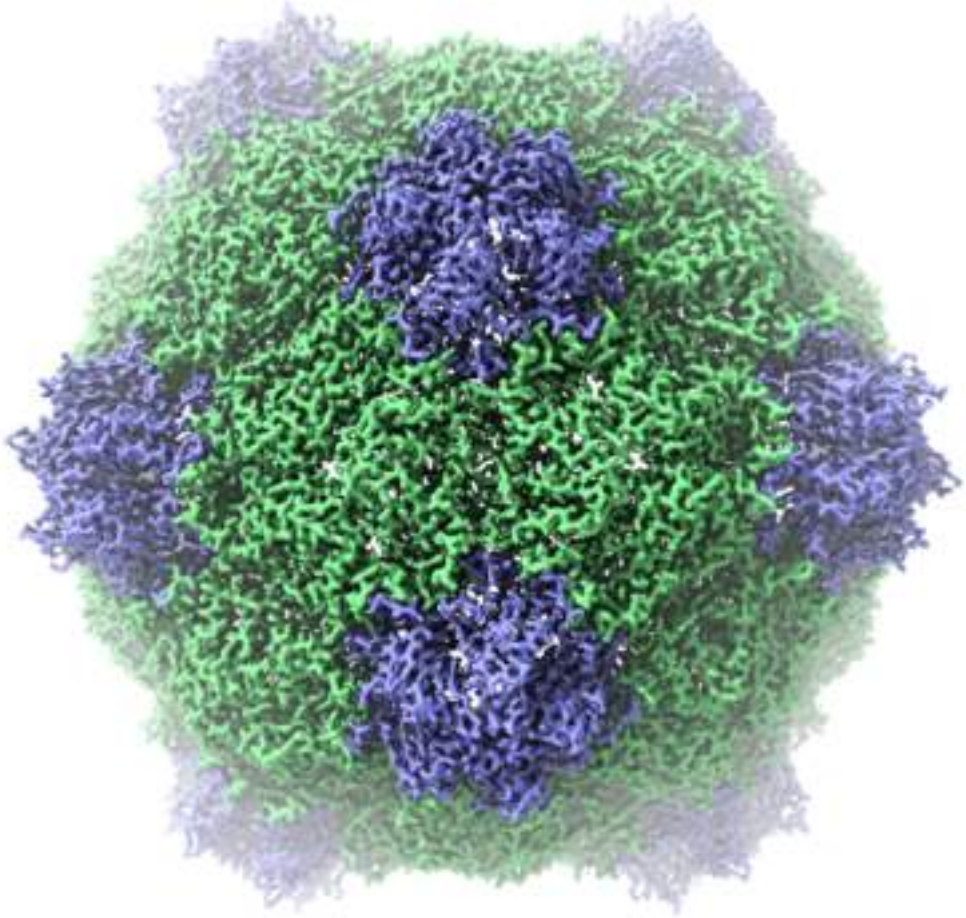
Emma Hesketh

Senior Cryo-EM Scientist

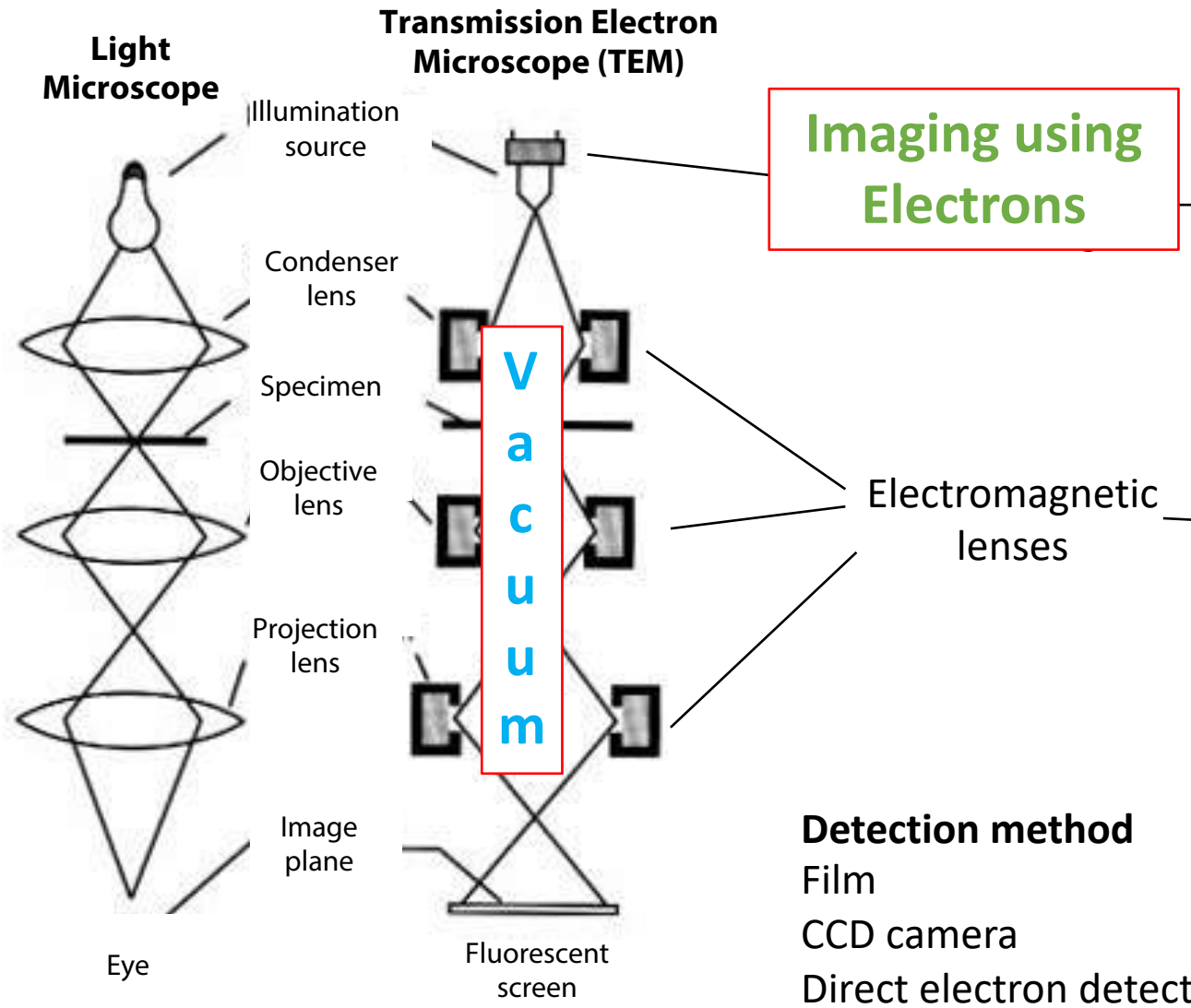
E.L.Hesketh@leeds.ac.uk

Overview of todays talk

- Brief introduction to electron microscopy
- Why use cryoEM?
- What specimens are suitable for cryoEM?
- How to routinely prepare samples for cryoEM?
- Alternative methods for cryoEM grid preparation



Electron microscope anatomy



Imaging using Electrons

Electromagnetic lenses

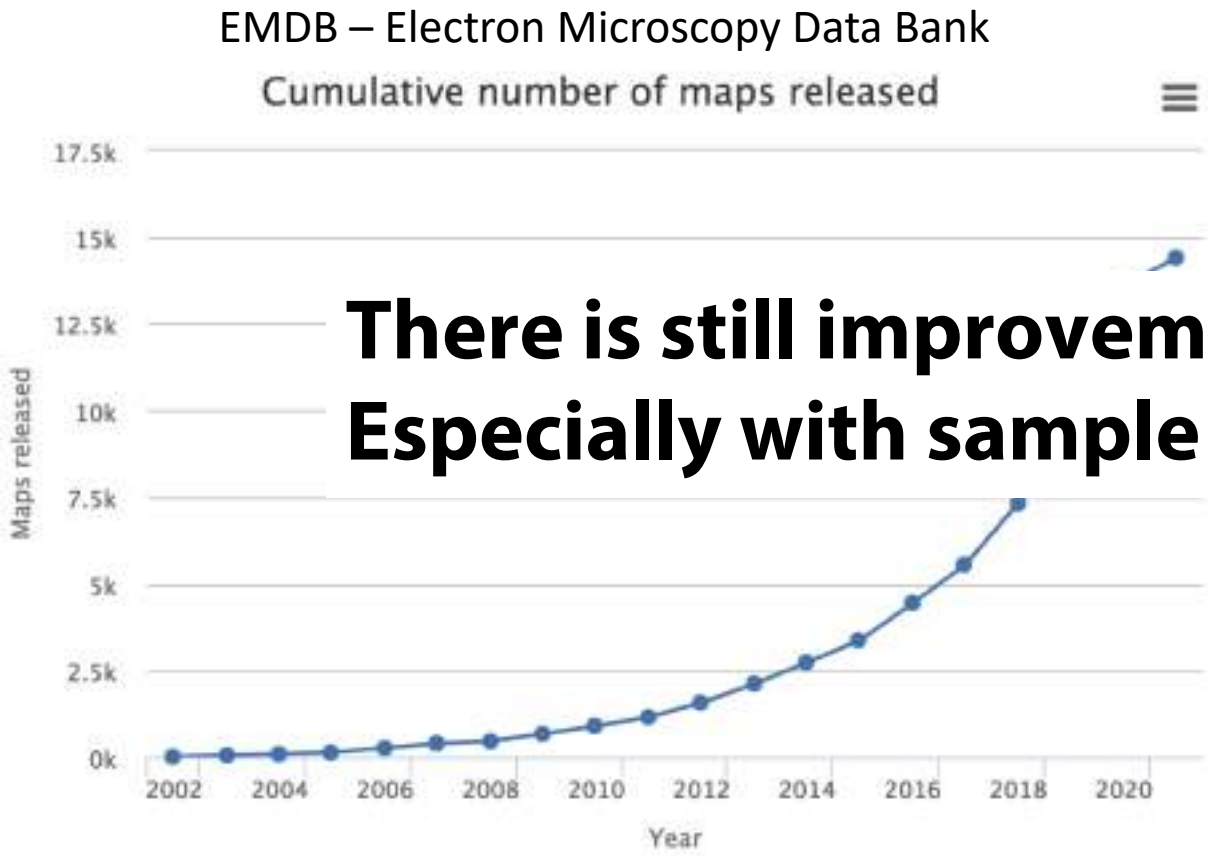
Detection method

Film

CCD camera

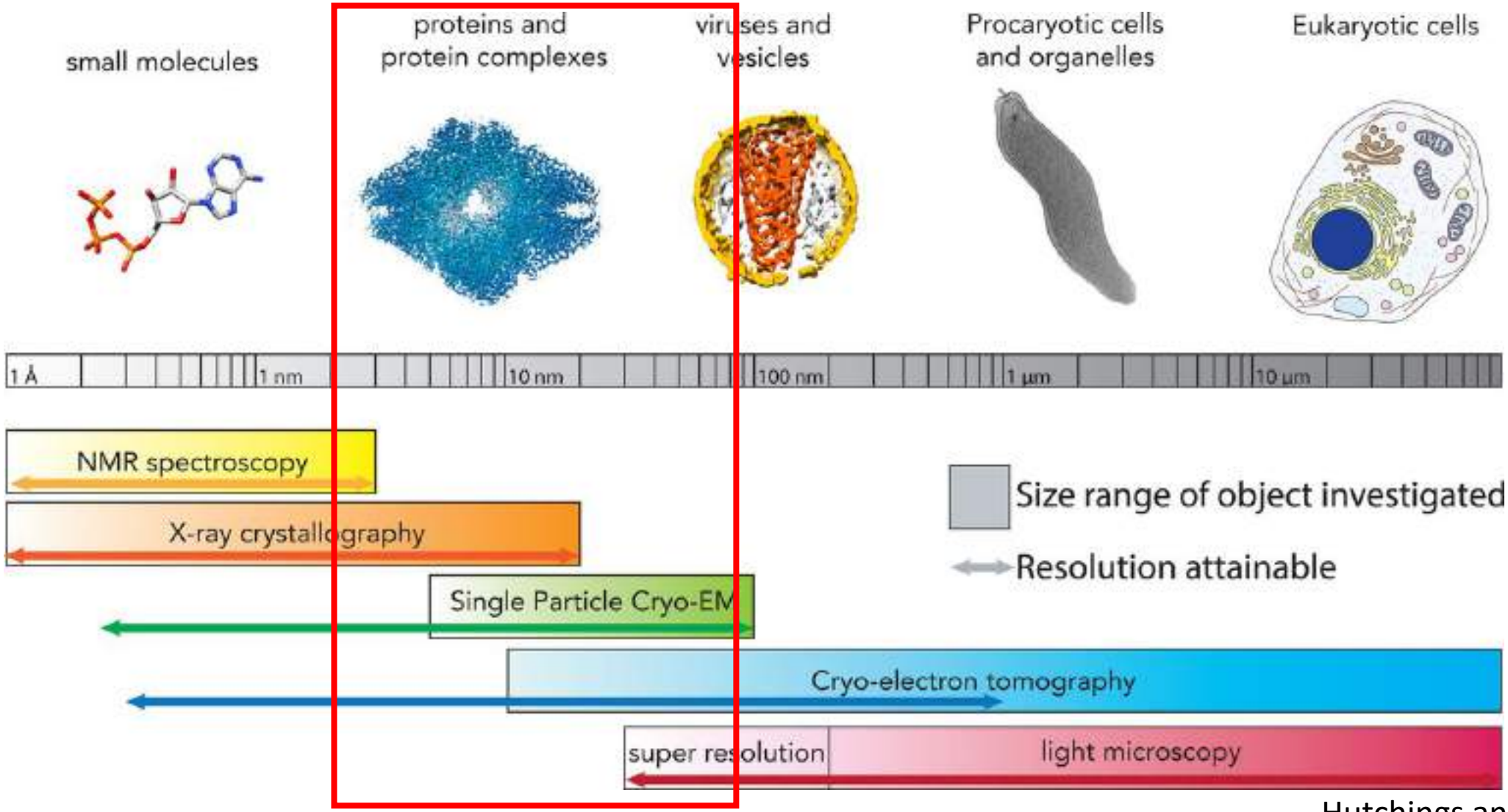
Direct electron detector

Electron microscopy



There is still improvement to be made.
Especially with sample preparation

Structural biology techniques



Hutchings and Zanetti 2018

Why use cryoEM?

Between 50 nM and 5 µM

Avoid organic molecules such as Glycerol, High salt, Caesium

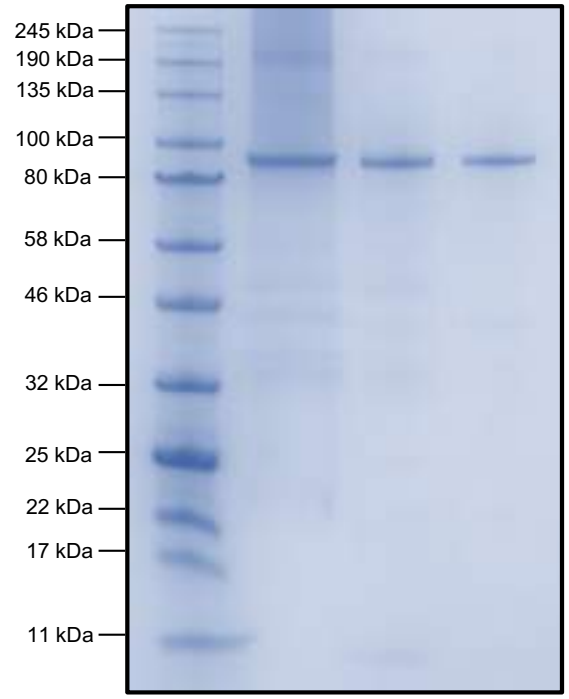
The higher the purity/quality of your sample, the better data achievable.

Theoretically <2 Å achievable, in reality HIGHLY dependent on your sample <4 Å fairly routine

< 100KDa has now been achieved but it is not yet routine

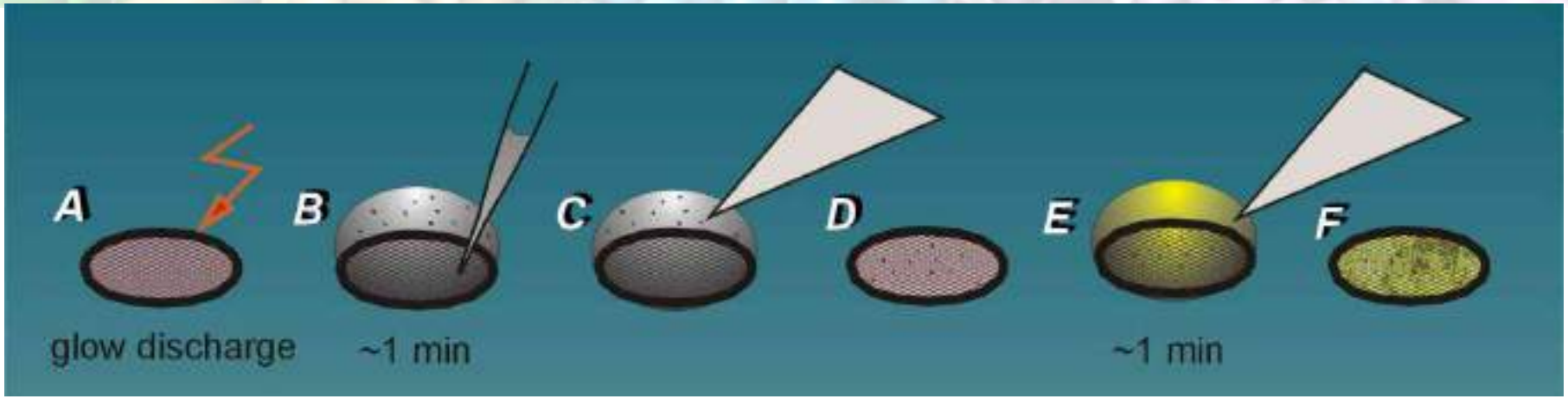
Rubbish in is rubbish out

- Before going to cryo-EM make sure you have the best preparation available
- Make sure there is a band on SDS – PAGE …… BUT a single band on an SDS gel does not mean the protein is ”good”
- Use a range of Biochemical and Biophysical techniques to check your sample before cryoEM.
  - For example, SEC-MALLS, circular-dichroism, thermal stability assays, binding/activity assays, negative stain EM



Don’t waste clean thinking on dirty proteins (Arthur Kornberg)

Why negative stain EM?



Quickly assess for:

Purity

Heterogeneity

Aggregation

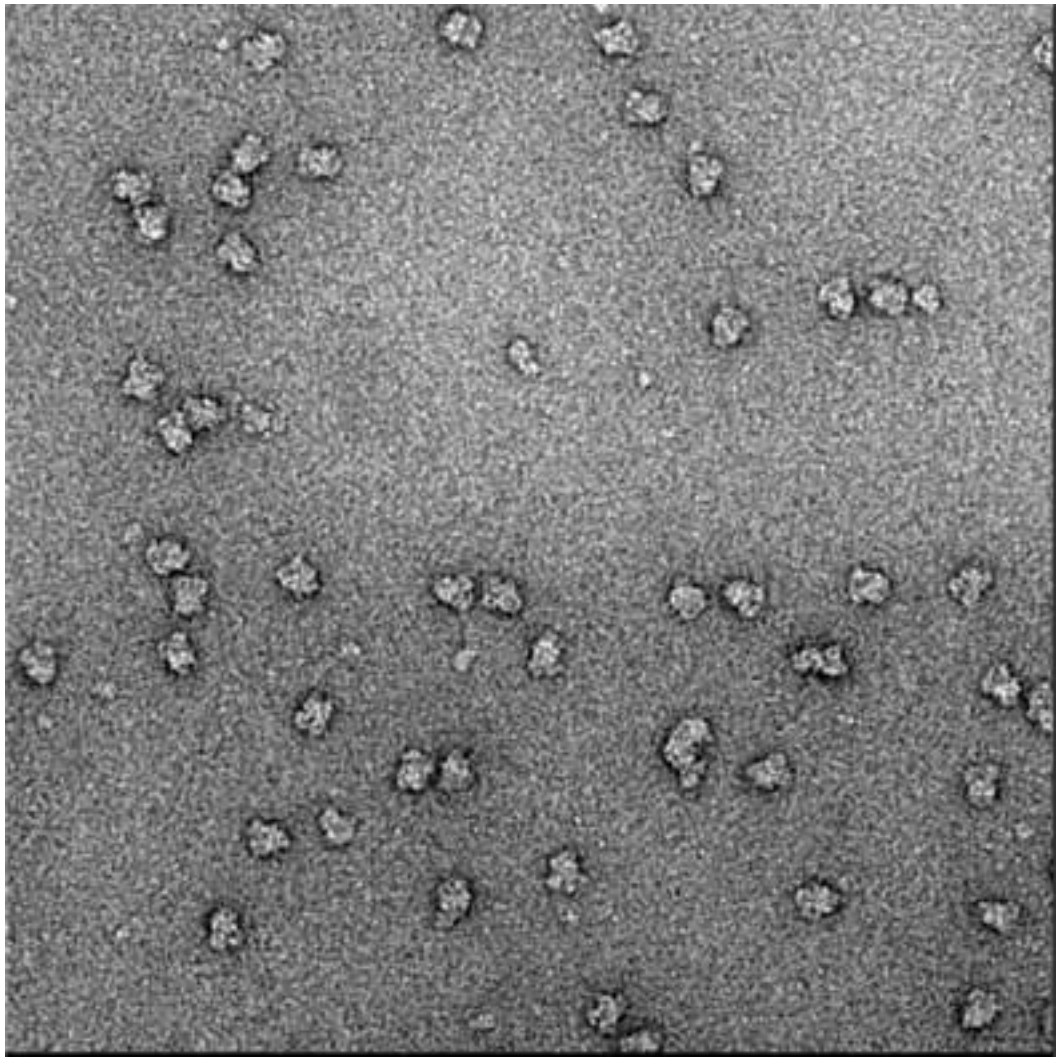
Degradation

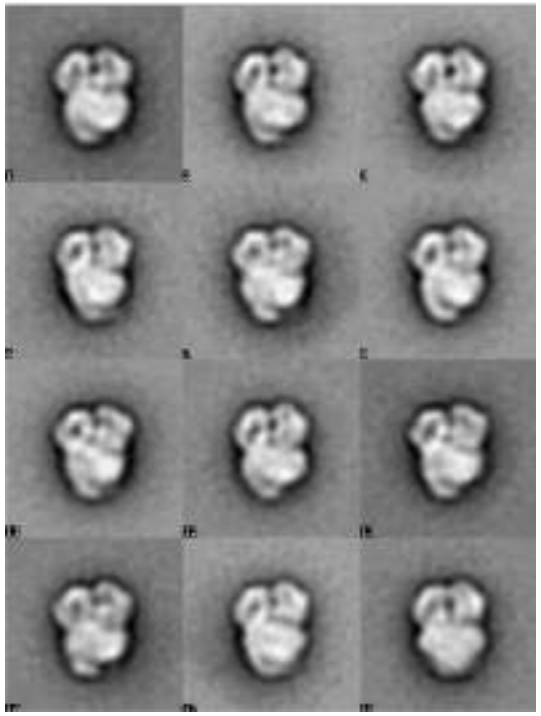
***Bit more time (weeks/months)*:**

Flexibility

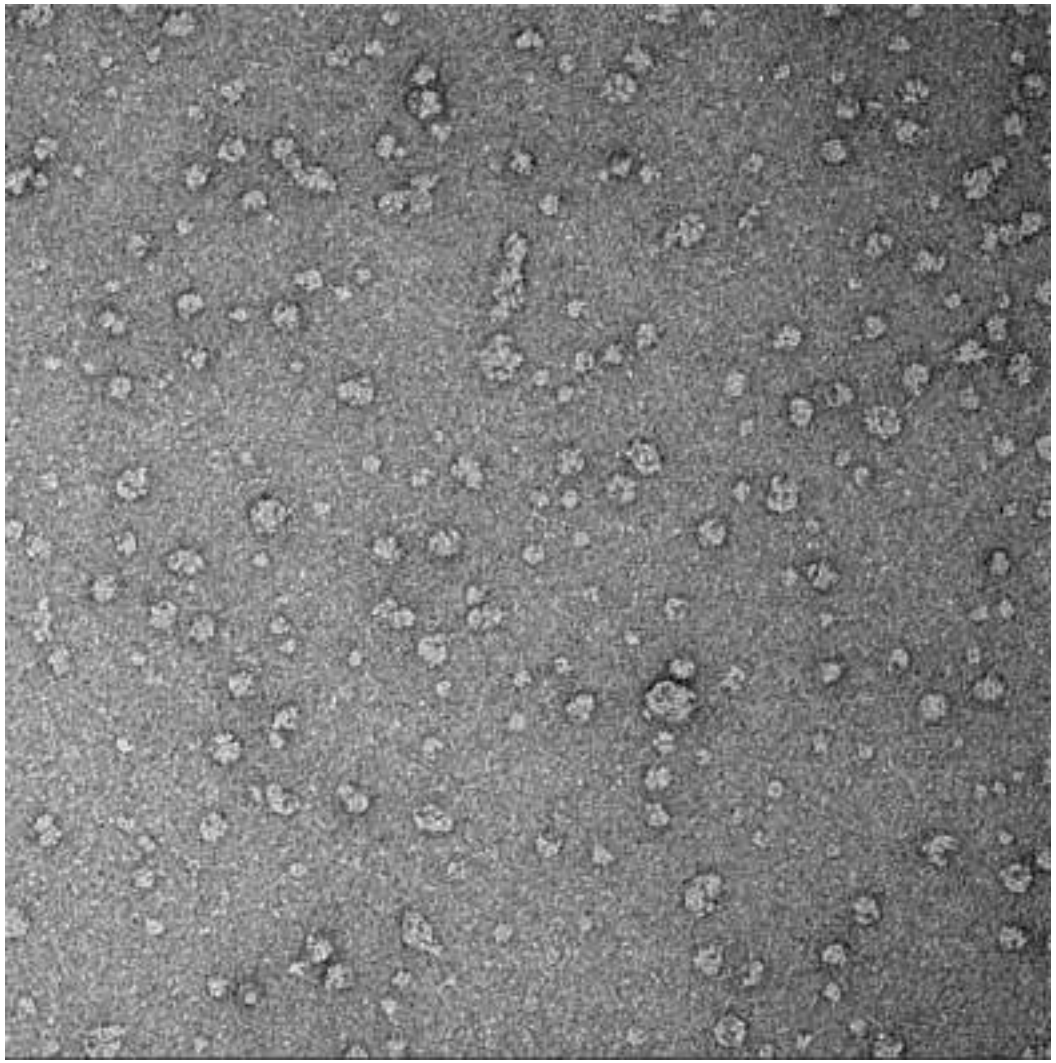
Stoichiometry

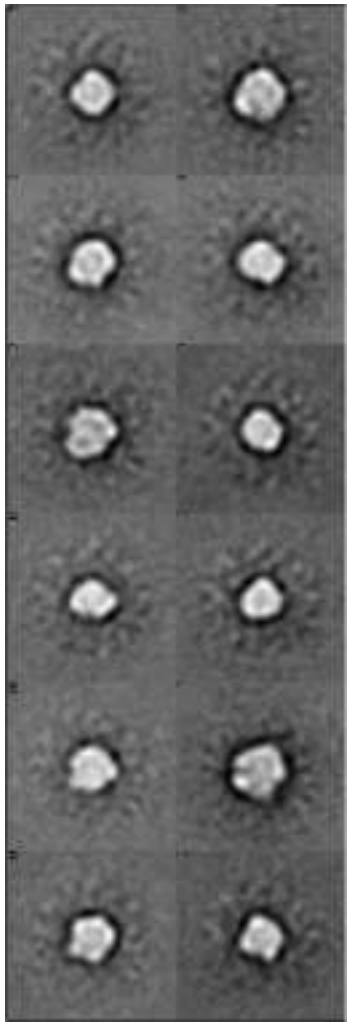
Is it bad or good?





Is it bad or good?





Single Particle CryoEM Standard workflow

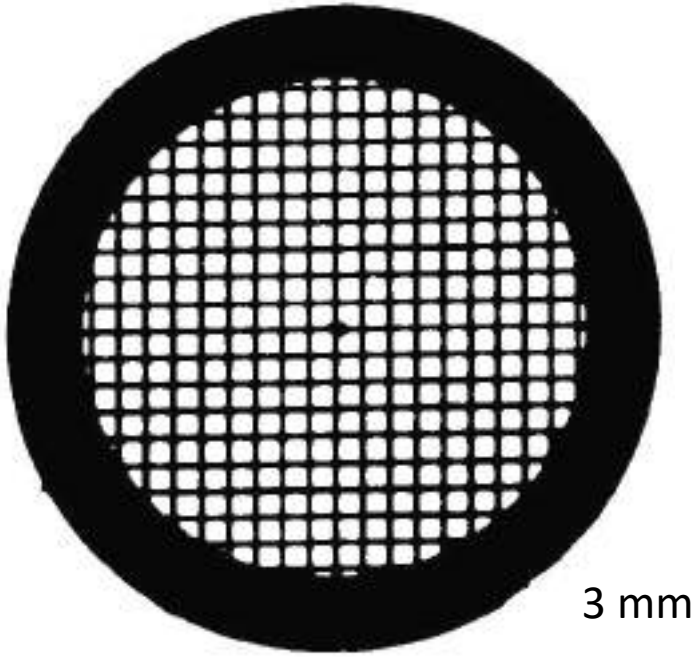
1. Selecting an EM grid and support film

2. Rendering that support film hydrophilic

3. Apply the sample to the EM grid and forming a thin film, within which the sample is suspended, by blotting away excess liquid

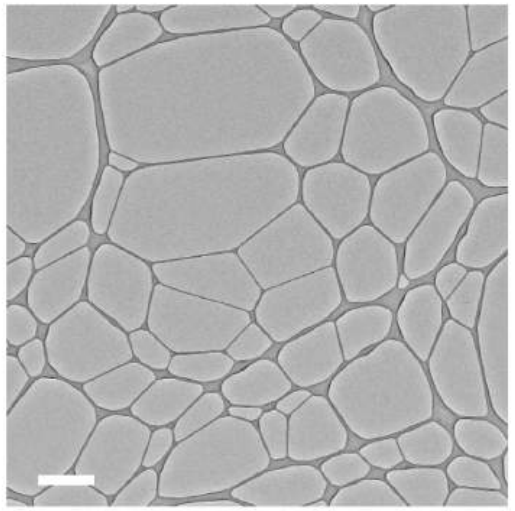
4. Vitrifying the sample by plunge freezing into a cryogen such as liquid ethane.

1. Selecting an EM grid and support film

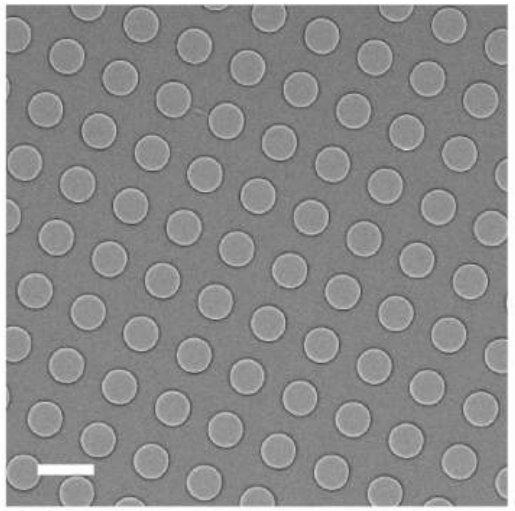


3 mm

Irregular mesh “Lacy”



Regular mesh



Grids most commonly purchased from Quantifoil, Agar Scientific or C-flats but many can be made “in-house”

Lots of options available AND worth trying if you have problems

1. Selecting an EM grid and support film

Support films

2nm/3nm Carbon coated (Quantifoil/**Agar Scientific**)

Graphene oxide – commercially available or home made

2. Rendering that support film hydrophilic

- Glow discharge in air

- Glow discharge in vapour
  - Amylamine

- Plasma cleaning in defined gas mixes

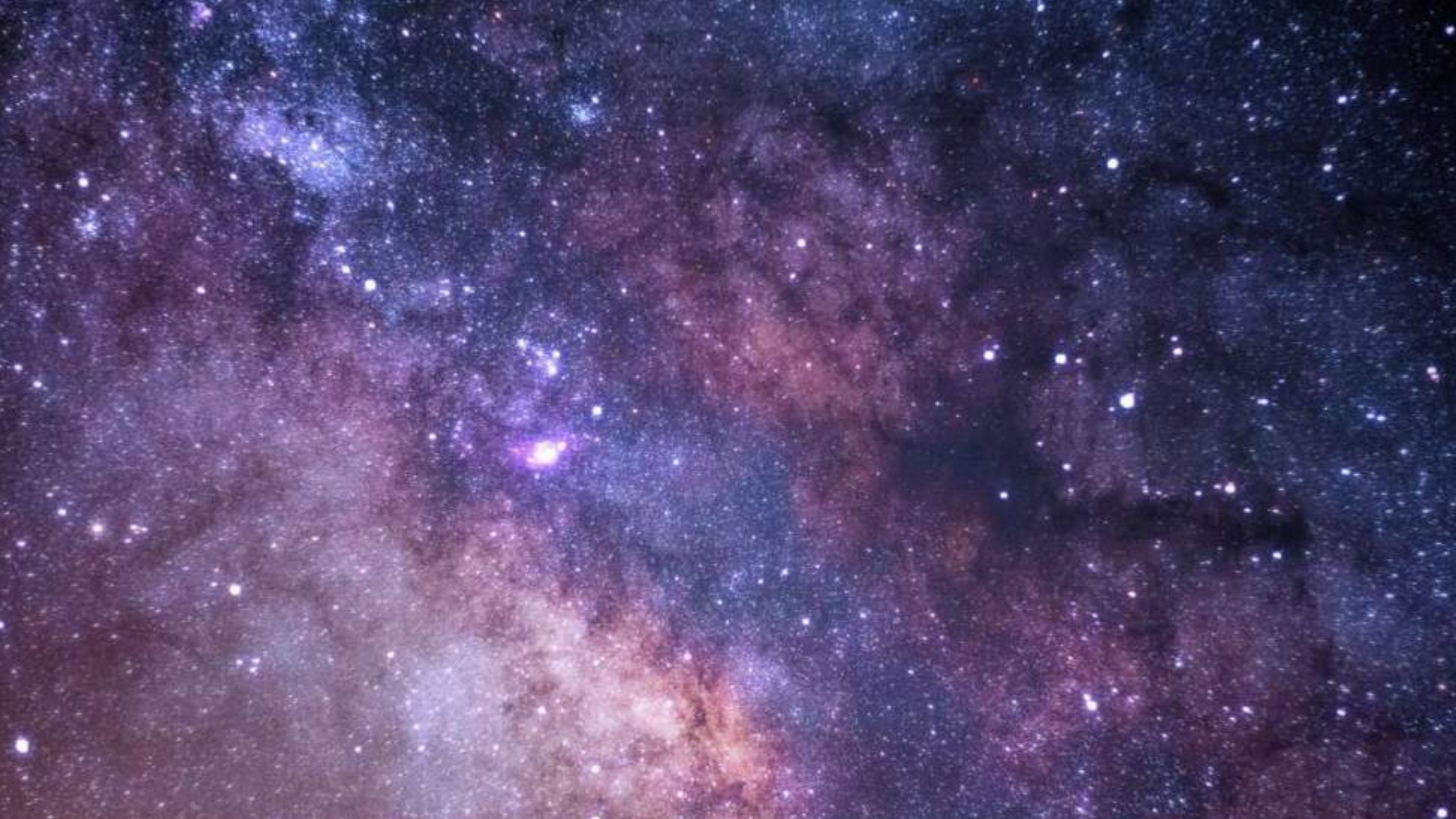
Single Particle CryoEM Standard workflow

1. Selecting an EM grid and support film

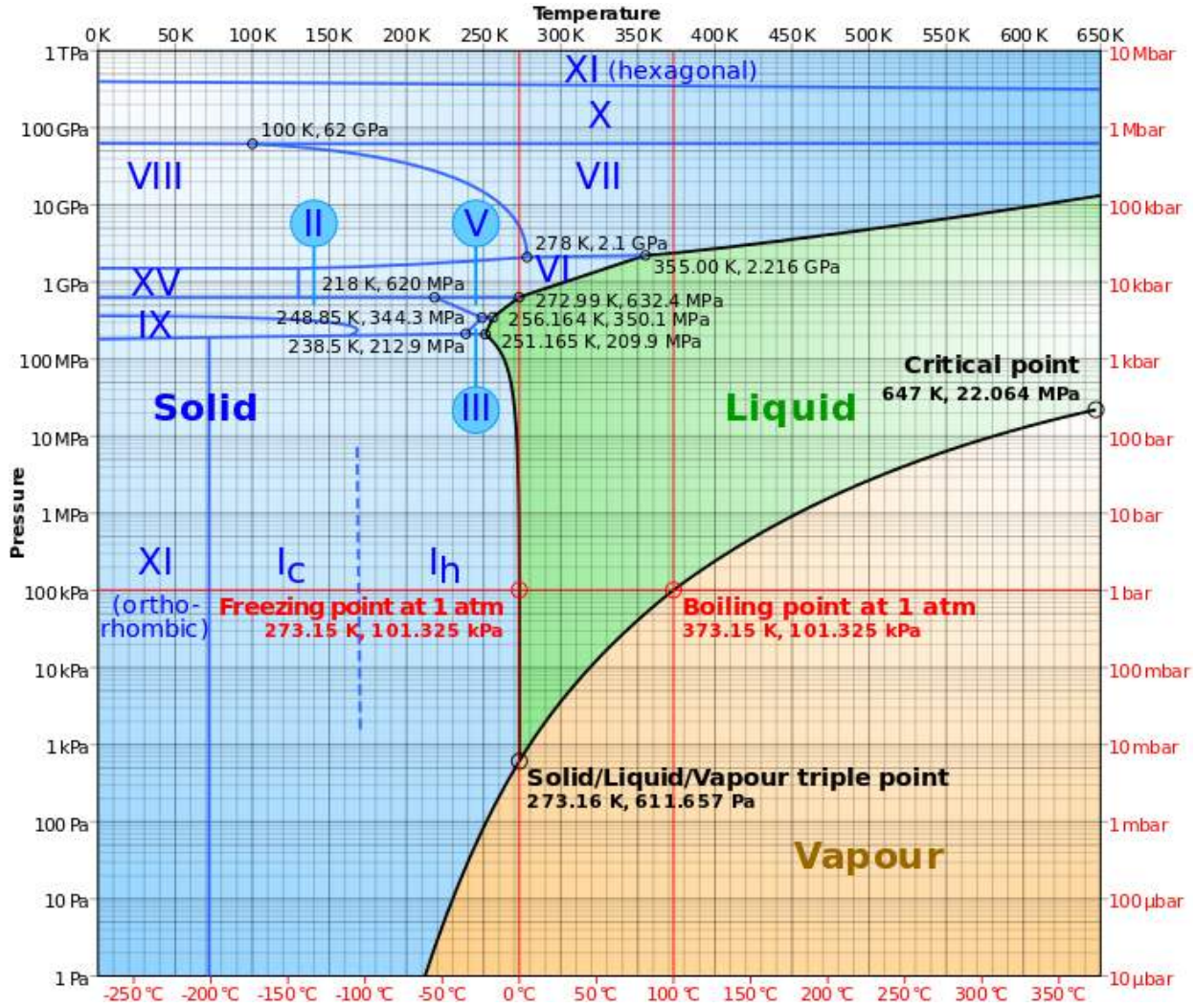
2. Rendering that support film hydrophilic

3. Apply the sample to the EM grid and forming a thin film, within which the sample is suspended, by blotting away excess liquid

4. Vitrifying the sample by plunge freezing into a cryogen such as liquid ethane.



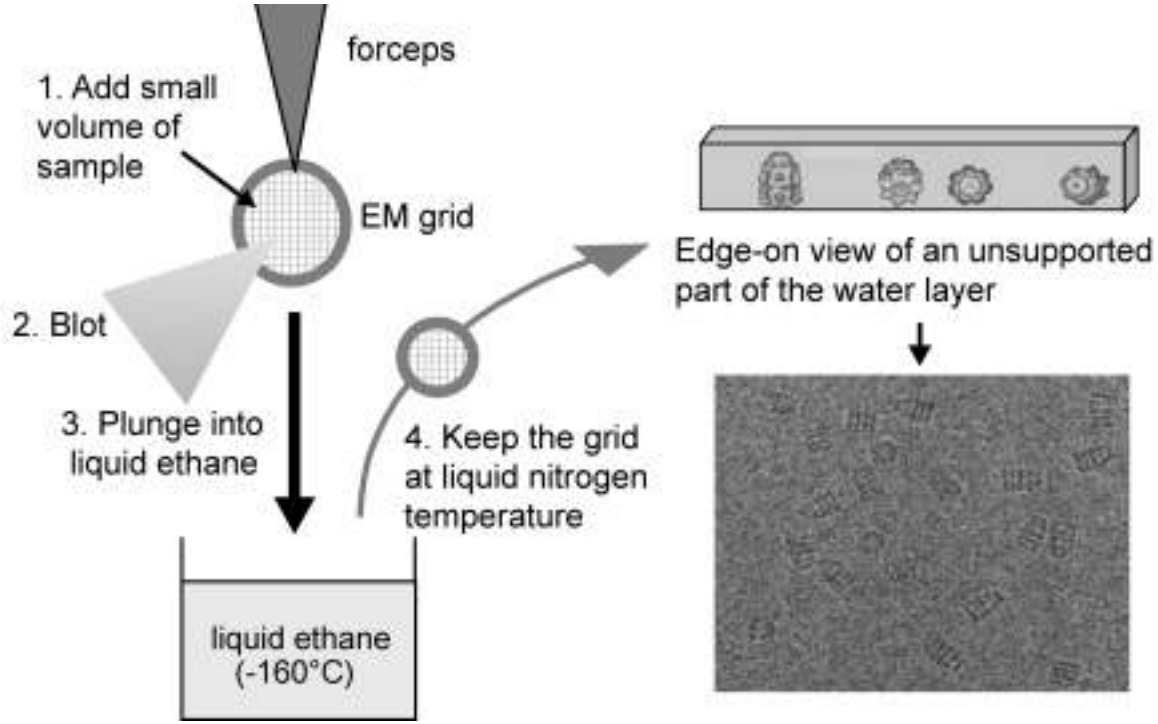
Vitrification of water

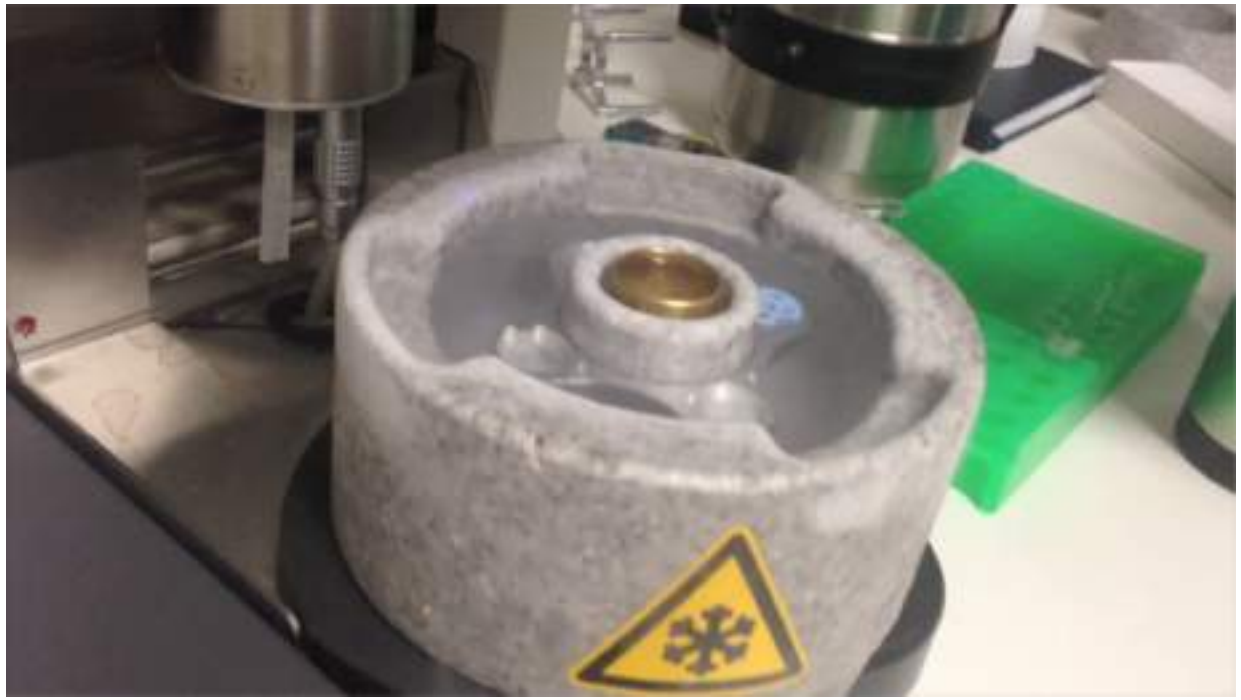


- Rapid freezing
- Water in, and surrounding the specimen is fixed in a vitreous state.

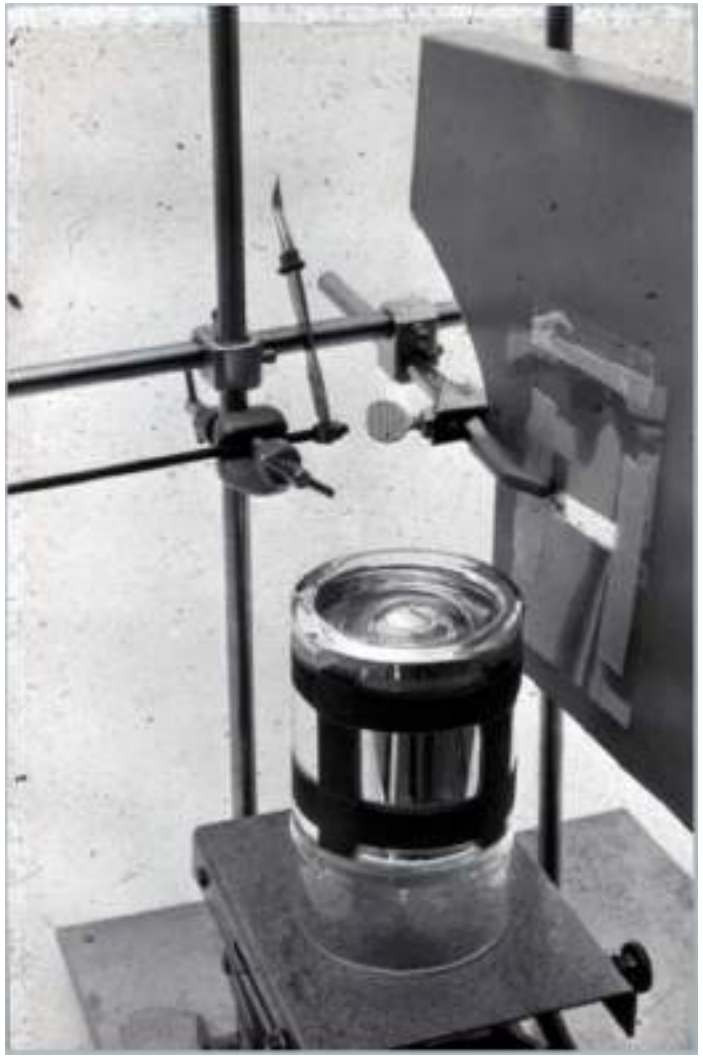
- If freezing occurs too slowly, or the specimen is subsequently warmed above –137 °C, the temperature at which water devitrifies, crystalline ice is formed

Sample Preparation – Cryo-EM





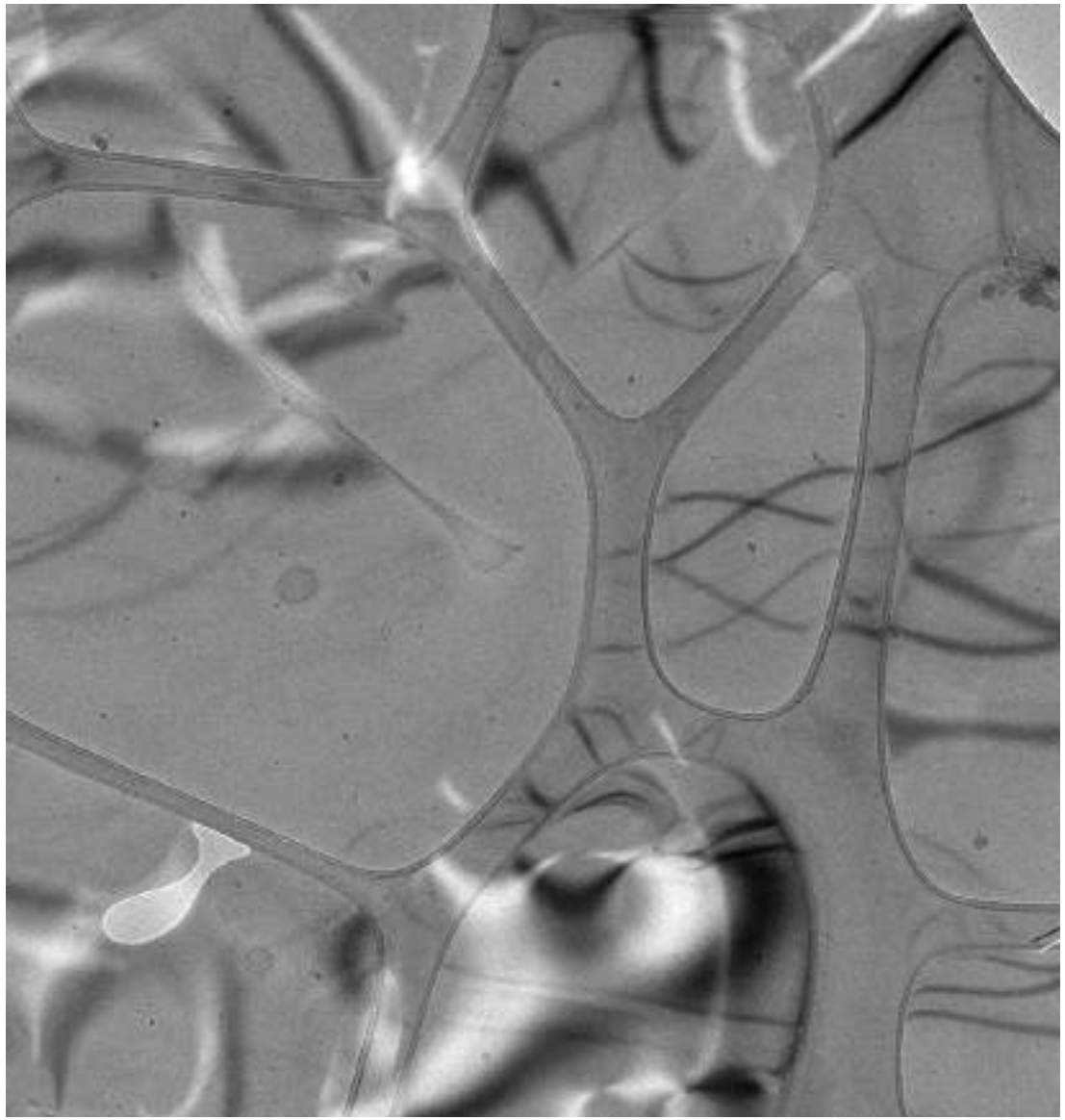
Sample Preparation – Cryo-EM



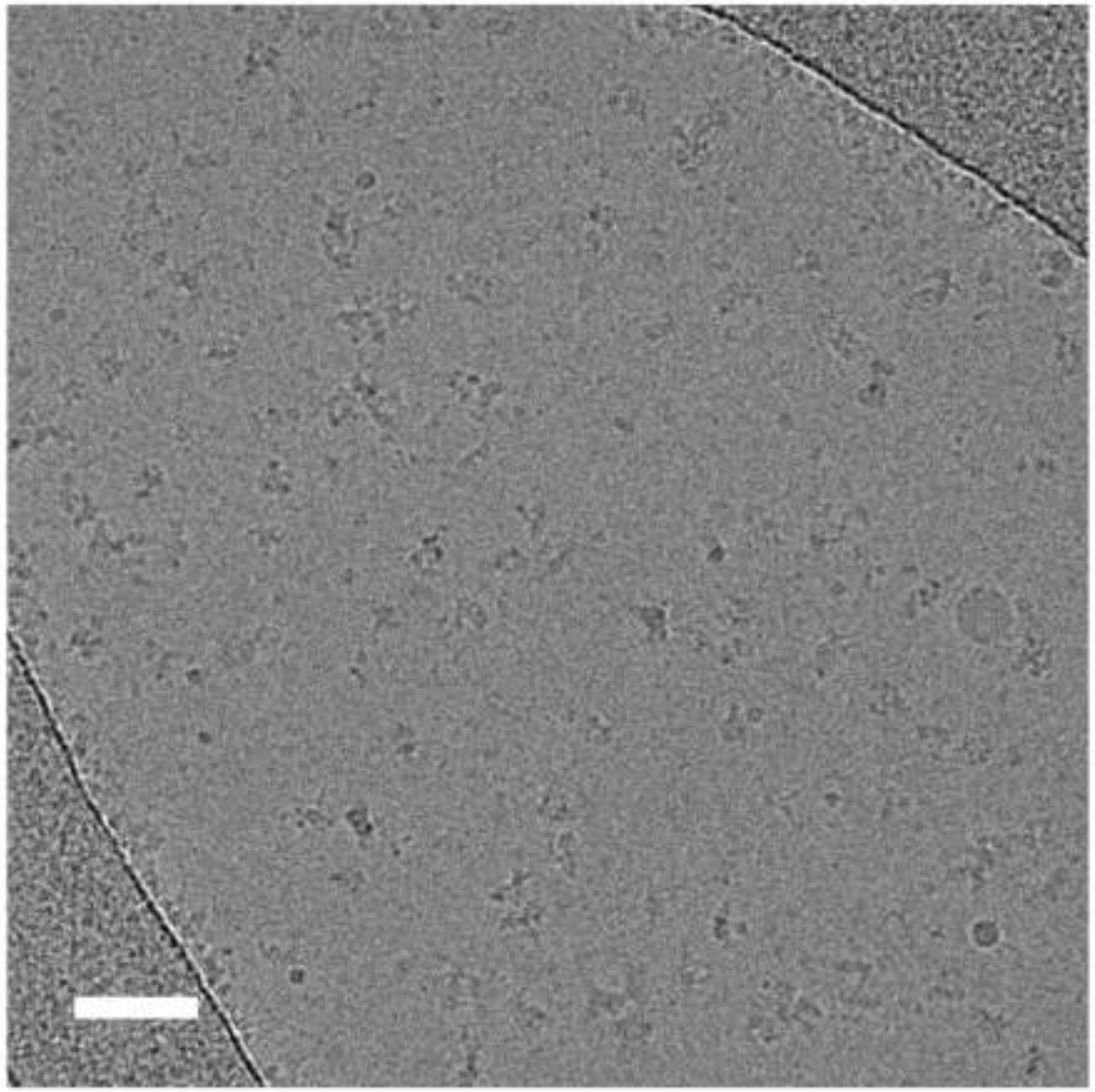
1980 Dubochet

FEI Vitrobot

Leica EM GP

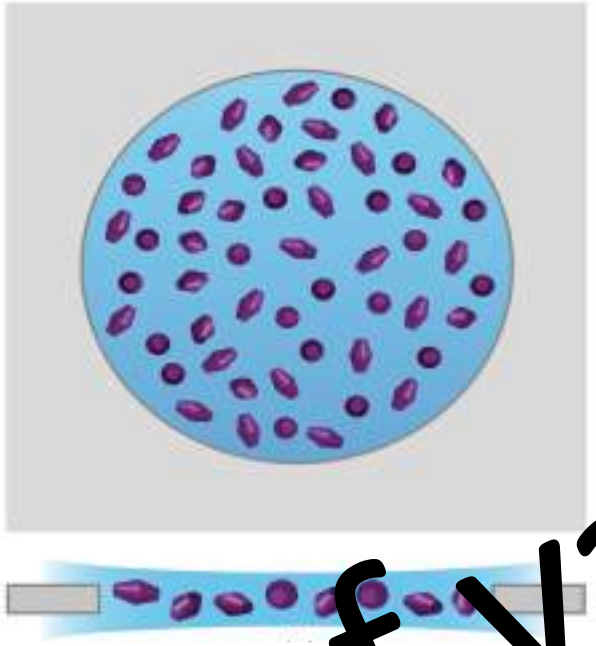


Non-vitreous ice

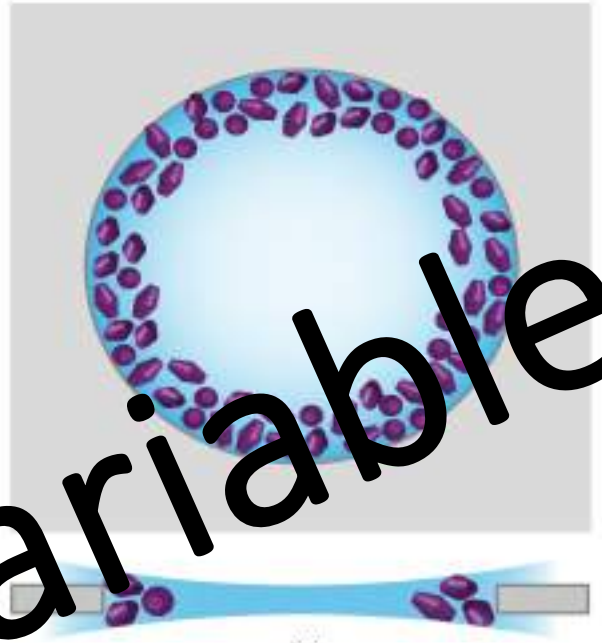


Vitreous ice with particles

Well distributed

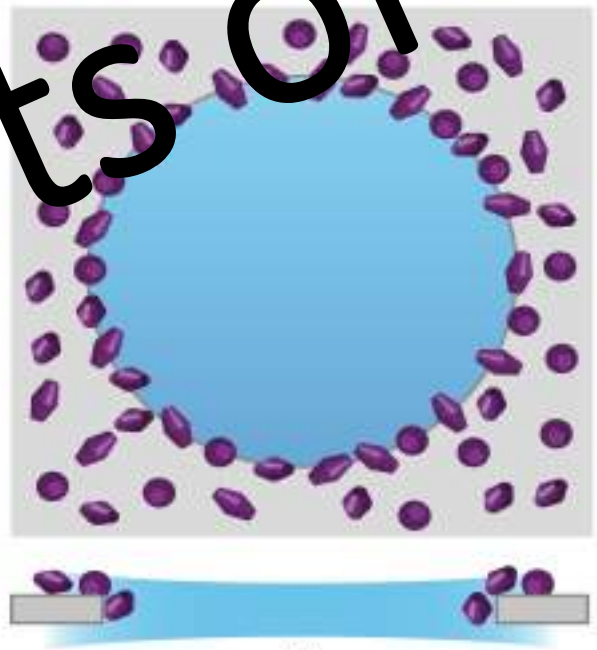


Thin ice in center of hole pushing particles to the hole edge

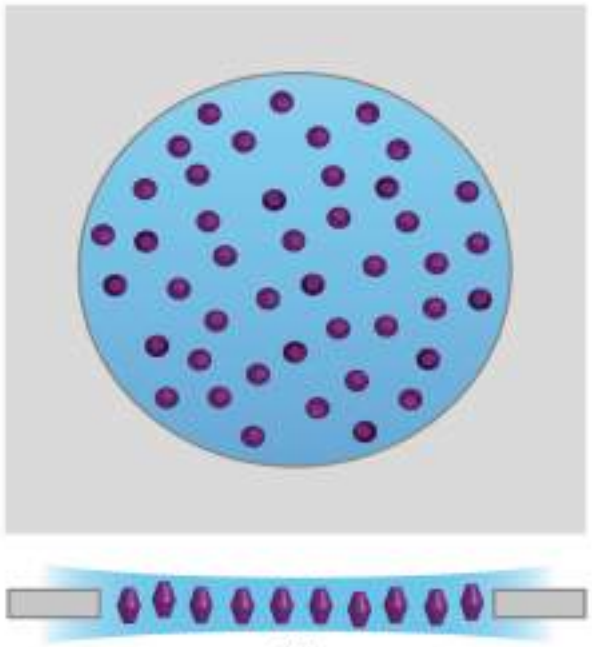


Lots of variables!

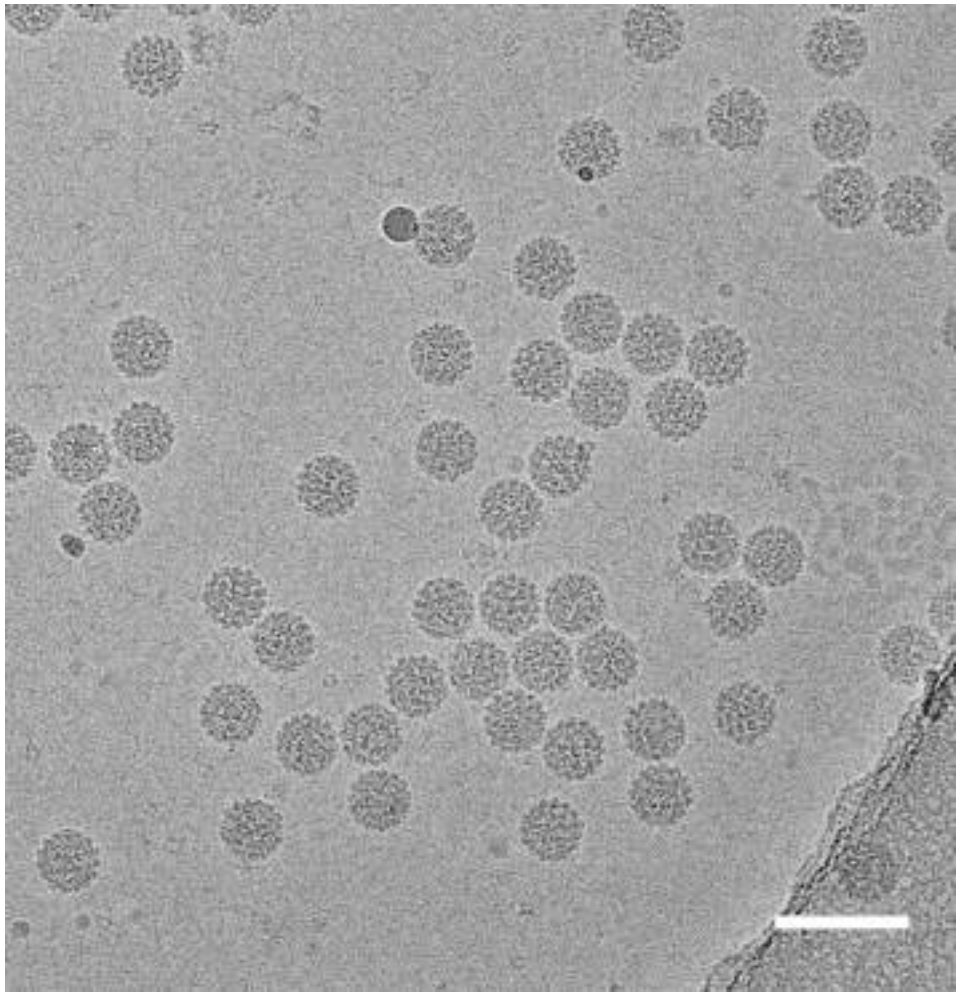
Affinity for the carbon support



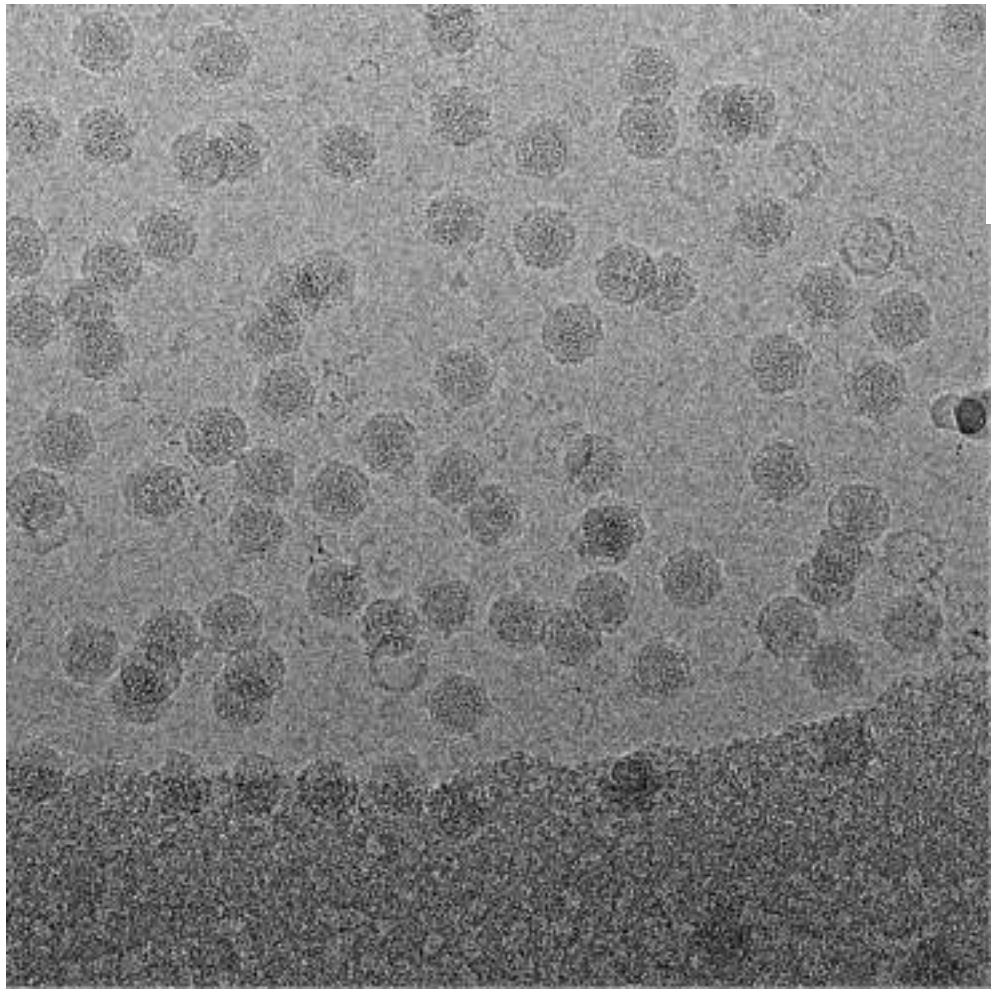
Preferred orientation at air water interface



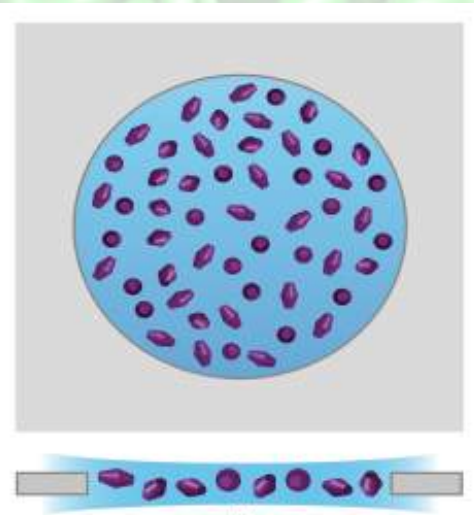
Example electron micrographs



A human Polyomavirus, Dan Hurdiss

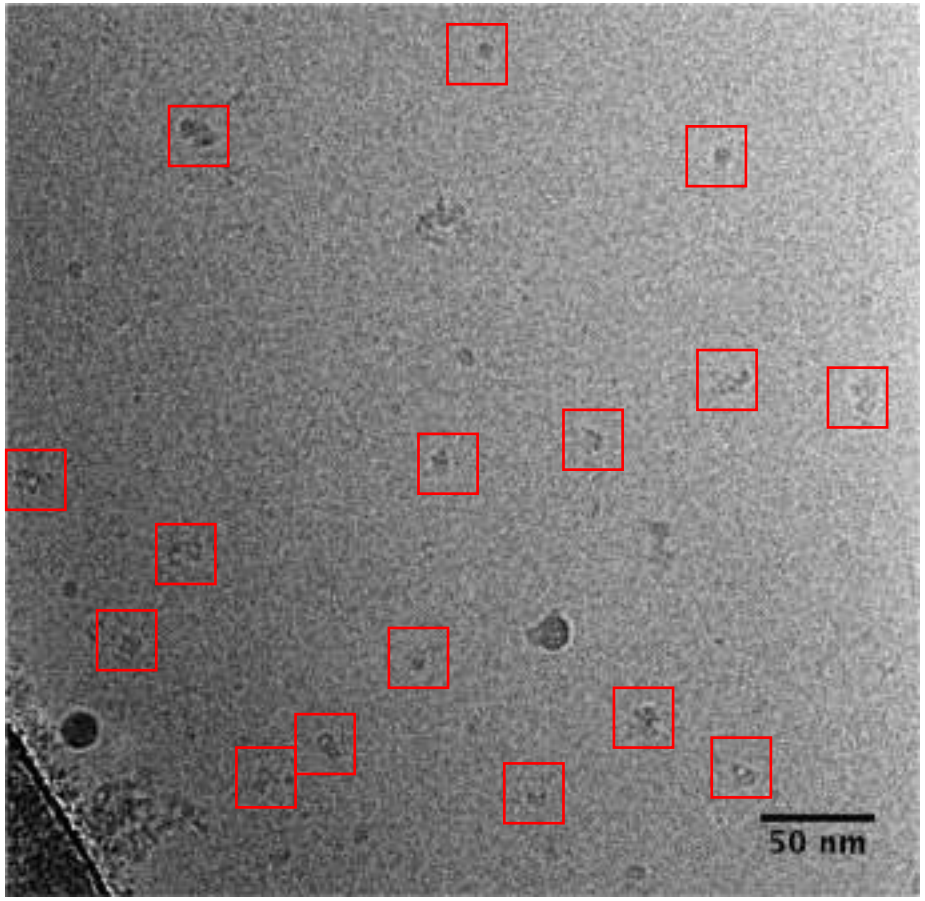


Plant virus, Emma Hesketh

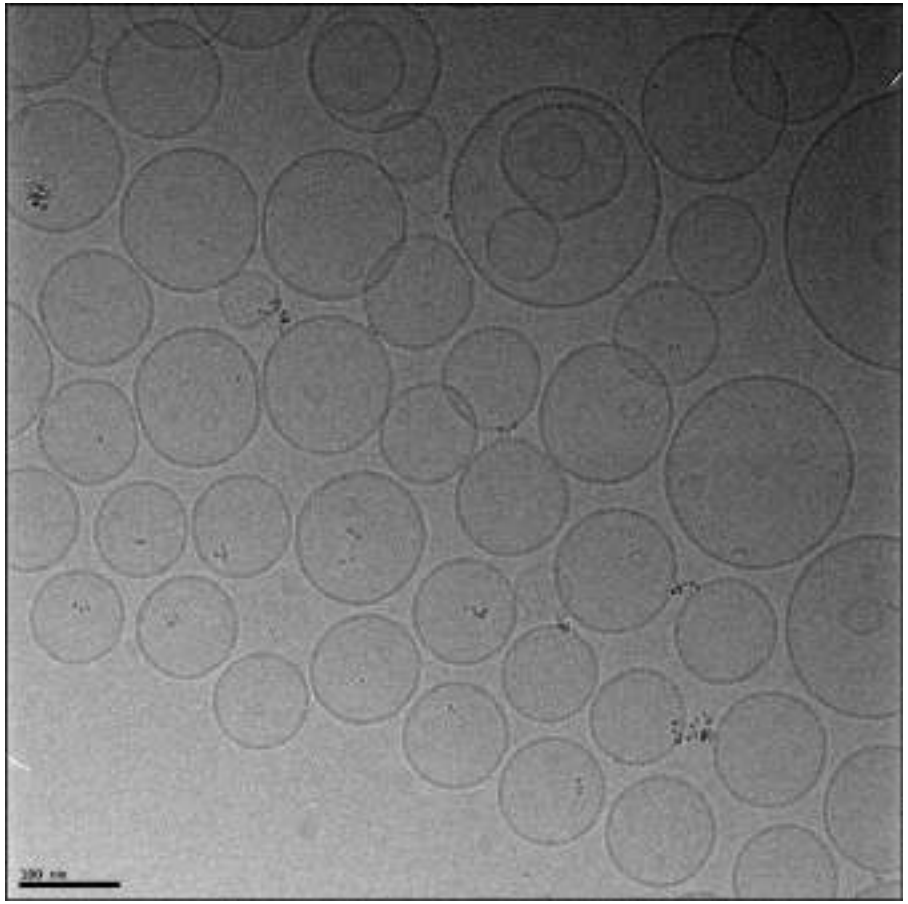


Well distributed

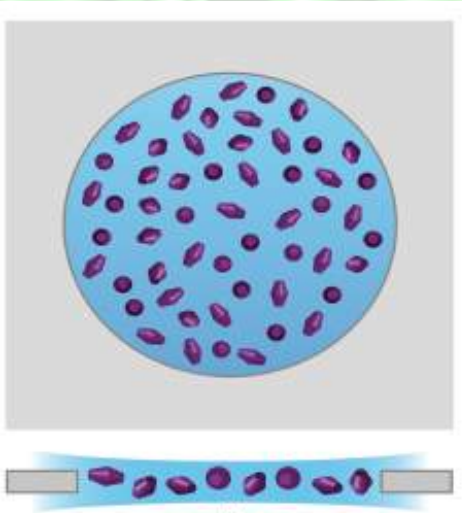
Example electron micrographs



Membrane protein

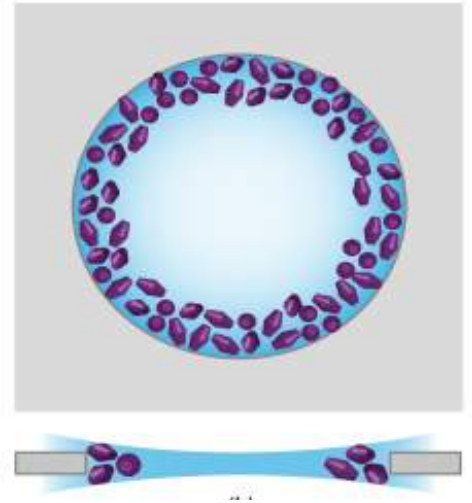


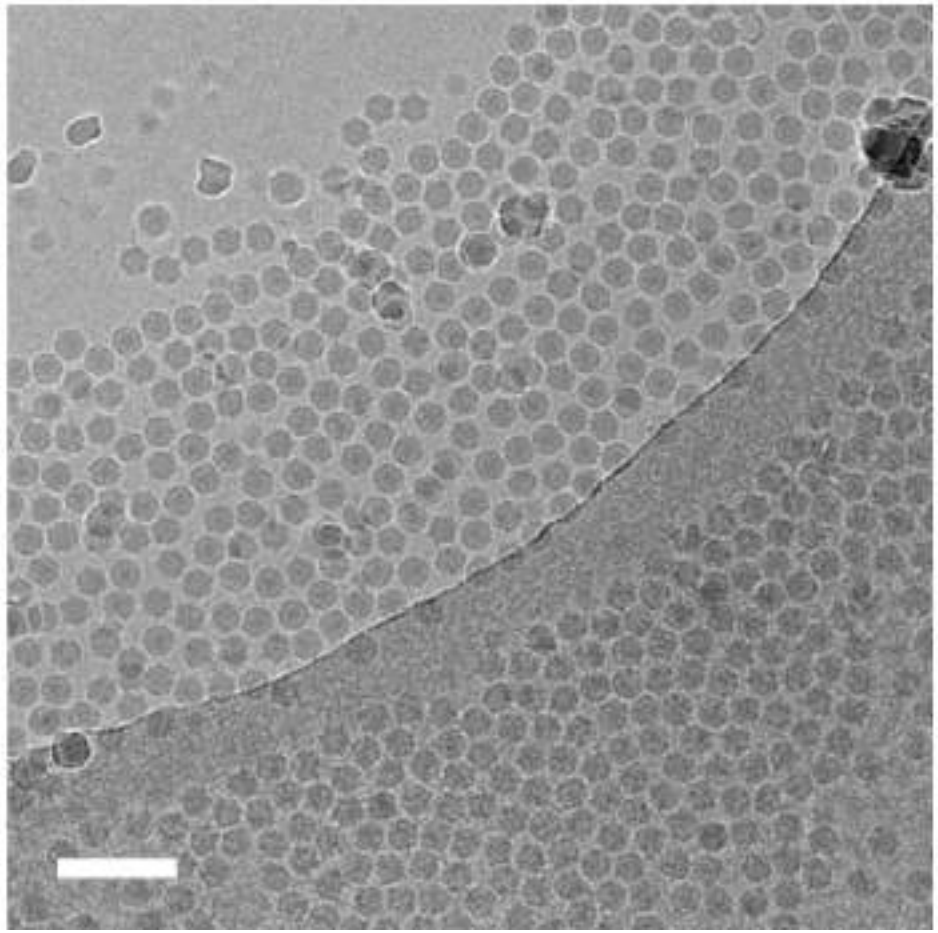
Liposomes

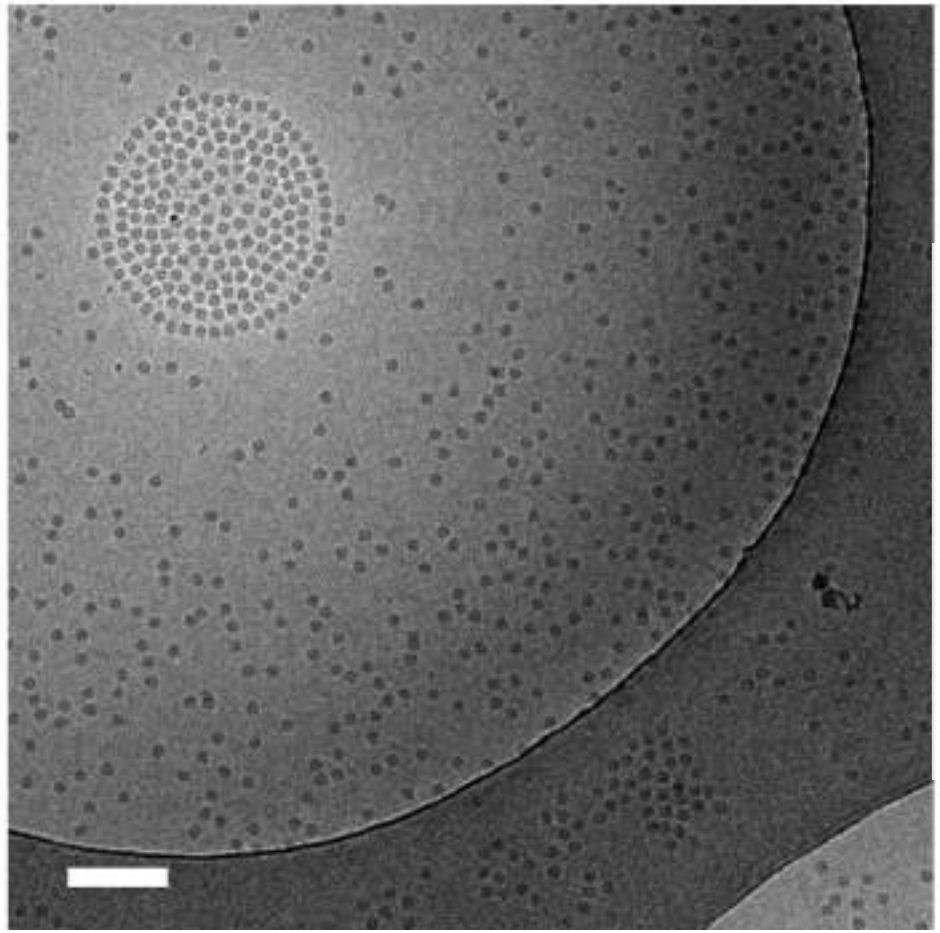


Well distributed

Example electron micrographs





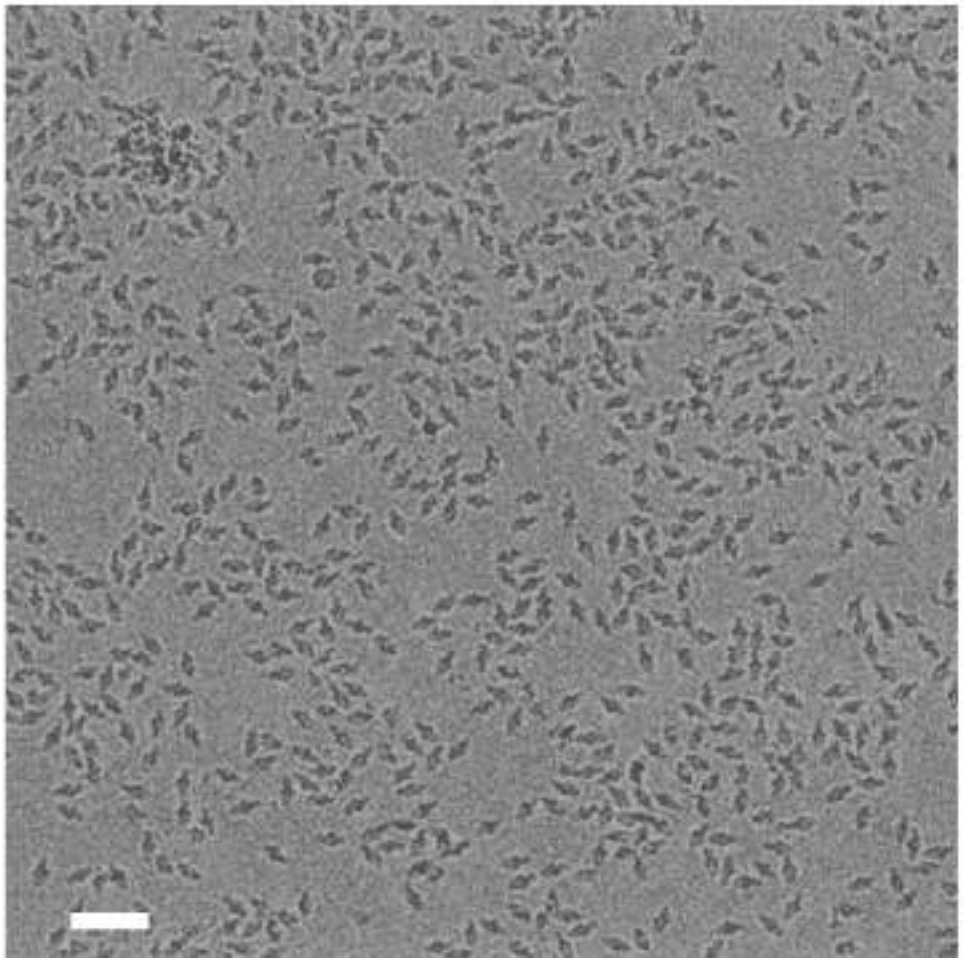


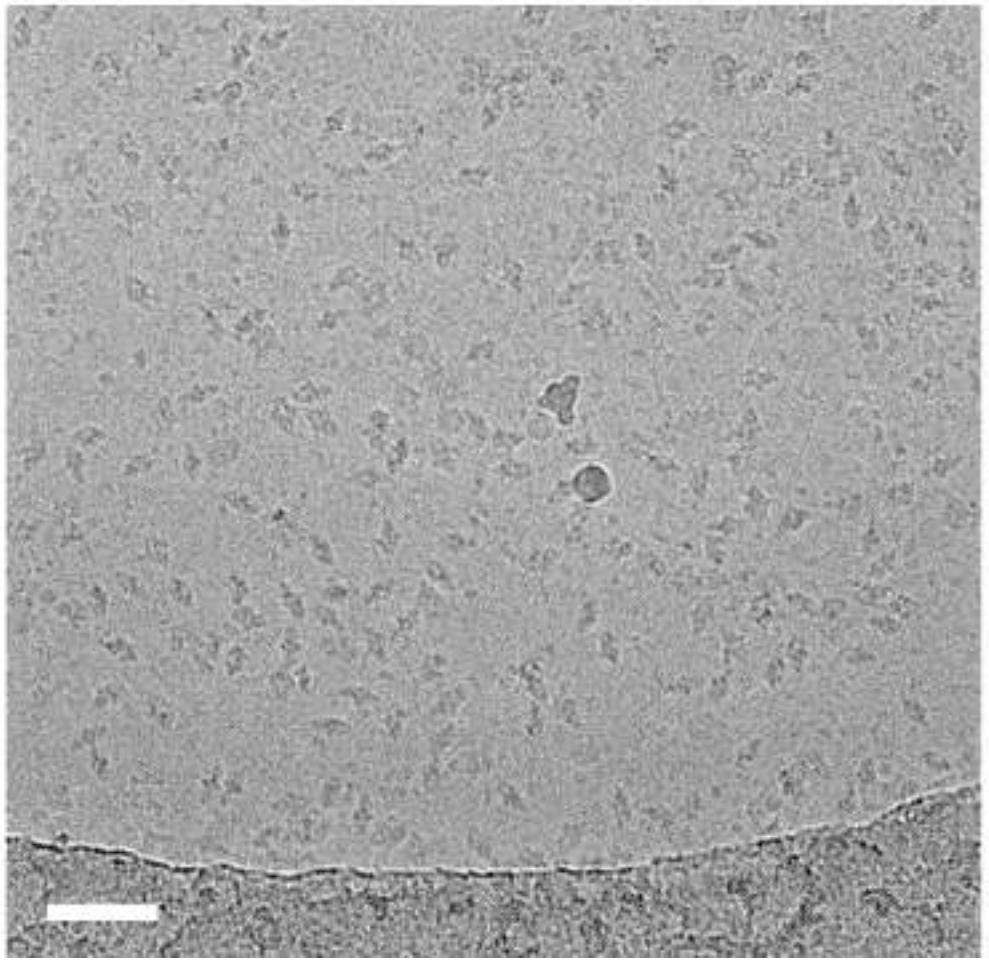
Thin ice excluding particles from the centre of the holes

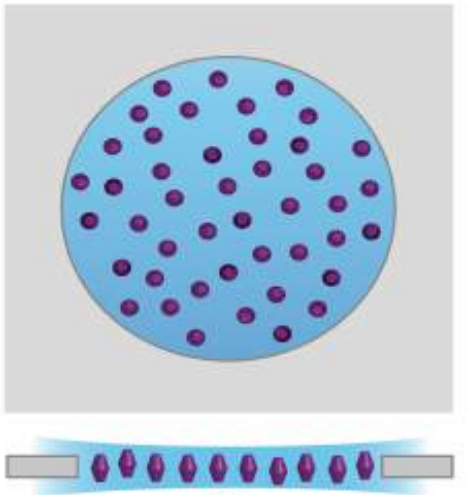
Ice thickness

Drulyte *et al* 2018

Example electron micrographs



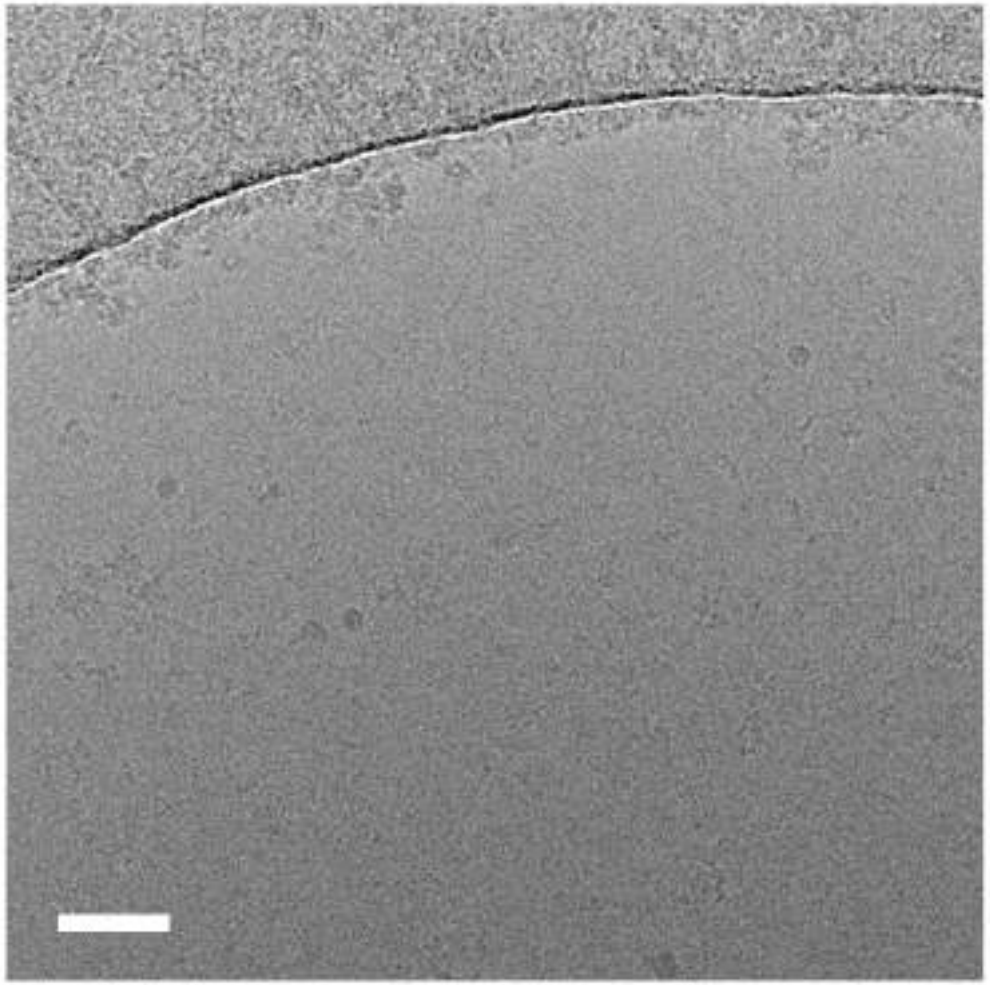


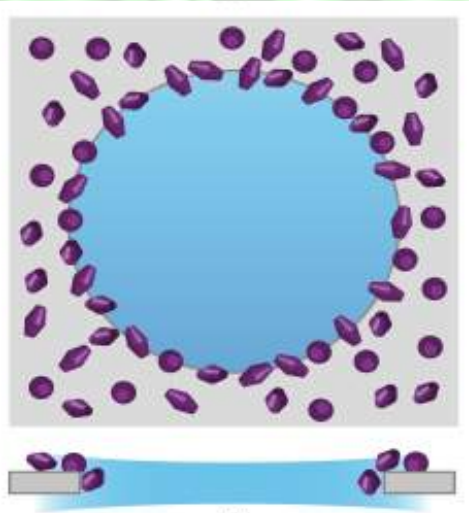


Preferred orientation

Drulyte *et al* 2018

Example electron micrographs



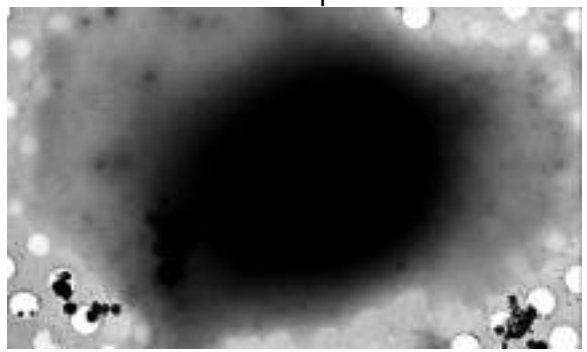


Affinity for the carbon support

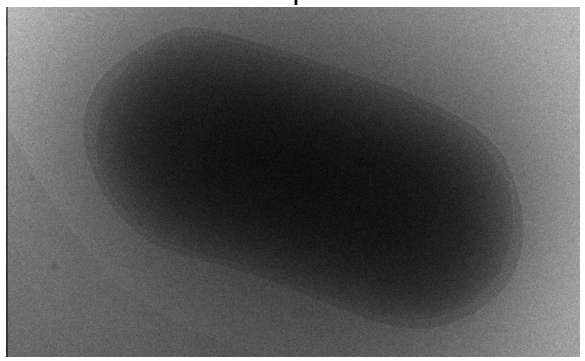
Drulyte *et al* 2018

Plunge freezing can be used for a wide range of specimens

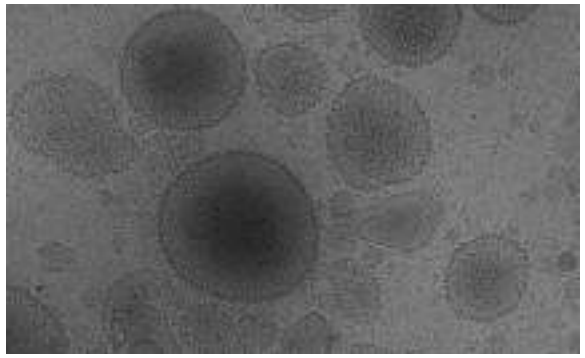
Eukaryotic cells
10-100 µm



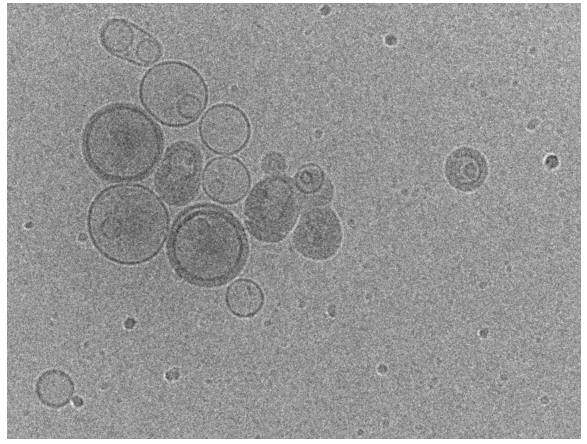
Prokaryotic cells
0.1-5 µm



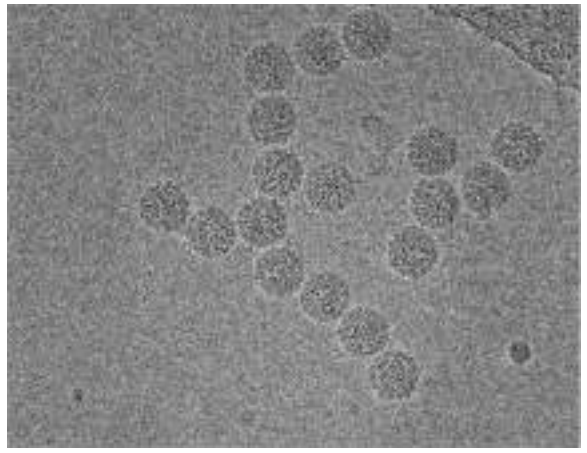
Isolated organelles
100 nm-2 µm



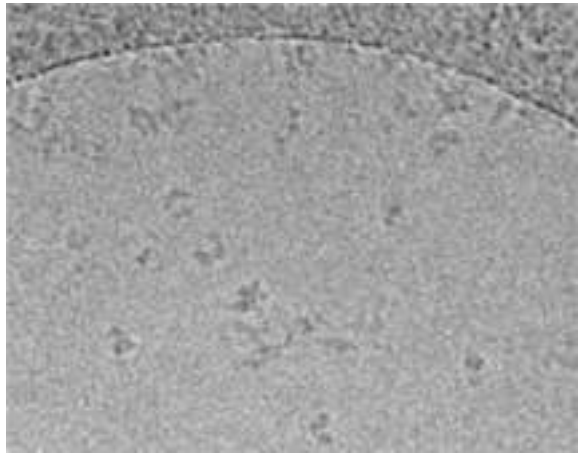
Synthetic liposomes
20 nm-500 nm



Viruses
20-400 nm



Macromolecular complexes



Disadvantages of plunge freezing techniques

1. Large amounts of wasted sample
2. Proteins dissociate at air water interface
3. Blotting paper contains impurities i.e. divalent metal ions.
4. **Low reproducibility**

Alternative methods to prepare SPA cryoEM grids

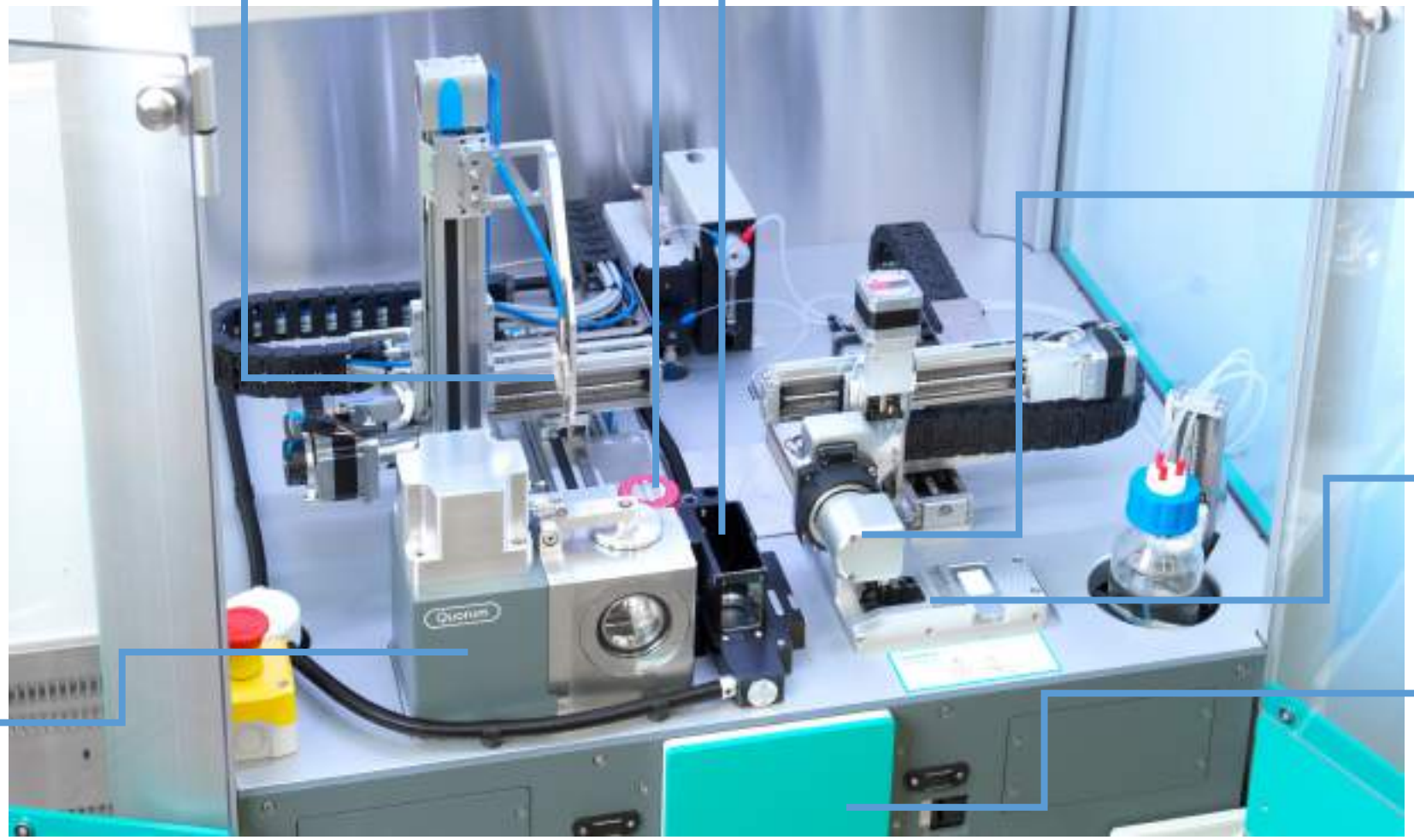
- **Spotiton** – commercialized as **Chameleon** (Noble et al., 2018).
  - Piezo electric inkjet dispensing system … and more
- **cryoWriter** – microcapillary action (Arnold et al., 2017).
- **Time resolved methodologies** – spray micro droplets onto grids and immediately plunge freezing (Kontiziampasis et al., 2019).
- **Vitrojet** - pin-printing and jet vitrification (Ravelli et al., 2020).
- **Shake-it-off** – ultrasonic humidifier using parts from a 3D printer! (Rubinstein et al., 2019)

Alternative methods to prepare SPA cryoEM grids

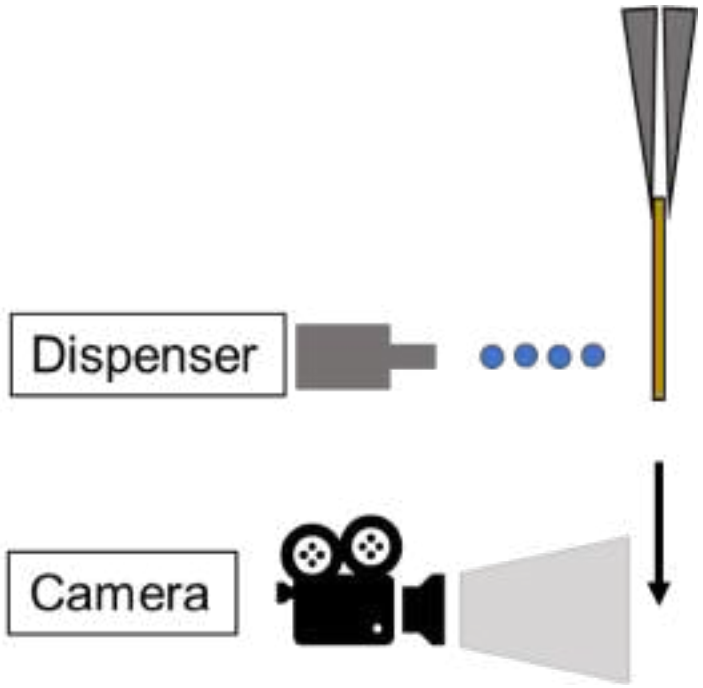
- **Spotiton** – commercialized as **Chameleon** (Noble et al., 2018).
  - Piezo electric inkjet dispensing system … and more

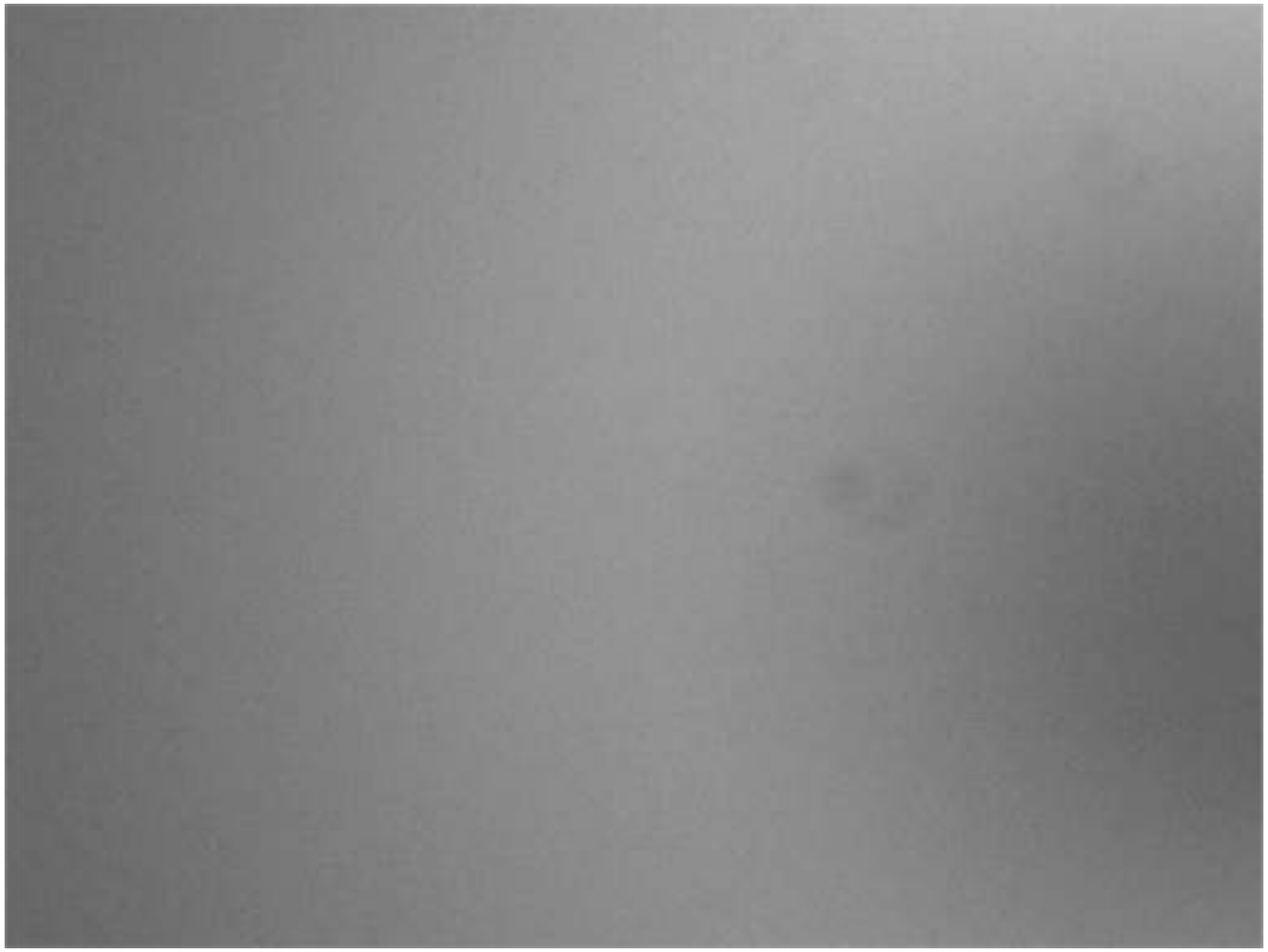
Michele Darrow - Michele.Darrow@sptlabtech.com

chameleon


Grid Holder
Humidity Shroud
Robotic Tweezers
Dispenser
Sample Block
Glow Discharger
Cryogen Drawer

Capturing the wicking process





Chameleon videos:

An animation showing the use and concepts of chameleon can be viewed here:

https://www.sptlabtech.com/products/sample-preparation/chameleon/

An video of the actual instrument workflow in use can be viewed here:

https://www.sptlabtech.com/resources/videos/a-closer-look-at-chameleon-providing-rapid-and-efficient-path-to-quality-frozen-grids-for-cryo-EM/

An link to last years webinar can be viewed here (bit out of date now):

https://discovery.sptlabtech.com/chameleon-delivering-automated-sample-prep-for-next-generation-cryo-em-webinar

chameleon – published/open structures (12 Oct 2020)

Horse spleen apoferritin
2.1 A: 6PXM

GroEL
3.8 A

β-galactosidase
3.5 A

30S ribosome
6.8 A

HSPD1
8.0 A

FACT subnucleosome complex 2
7.4 A; 6UPL

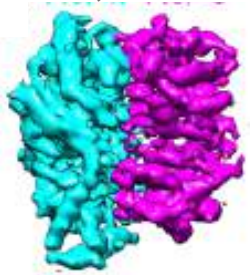
FACT subnucleosome complex 1
4.9 A; 6UPL

DNA polymerase Zeta/DNA/dNTP Ternary Complex
3.10 A; 6V93

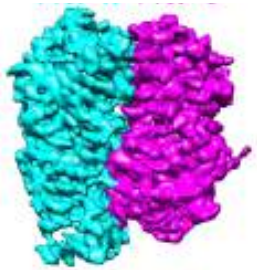
70S ribosome
4.9 A

50S ribosome
4.1 A

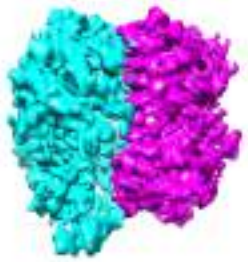
H11-H4 nanobody bound to Spike
3.7 A; 6ZHD



PreP – Aβ partial Closed
3.3 A



PreP – CS presequence partial Closed
4.6 A



Apo-PreP (partial Open 2)
3.9 A

Apo-PreP (partial Open 1)
3.7 A

Apo-PreP (Open)
4.0 A

Alternative methods to prepare SPA cryoEM grids

- **Time resolved methodologies** – spray micro droplets onto grids and immediately plunge freezing (Kontiziampasis et al., 2019, Klebl et al., 2020).

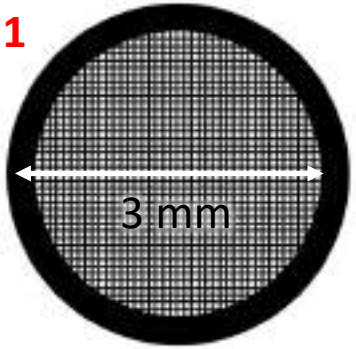
University of Leeds

Steven Muench

David Klebl

Fast grid preparation setup

1



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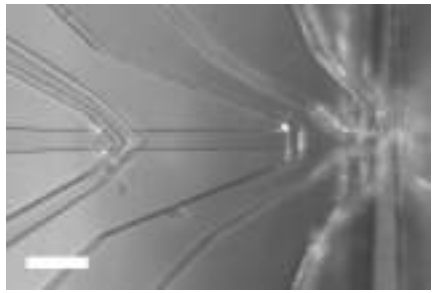
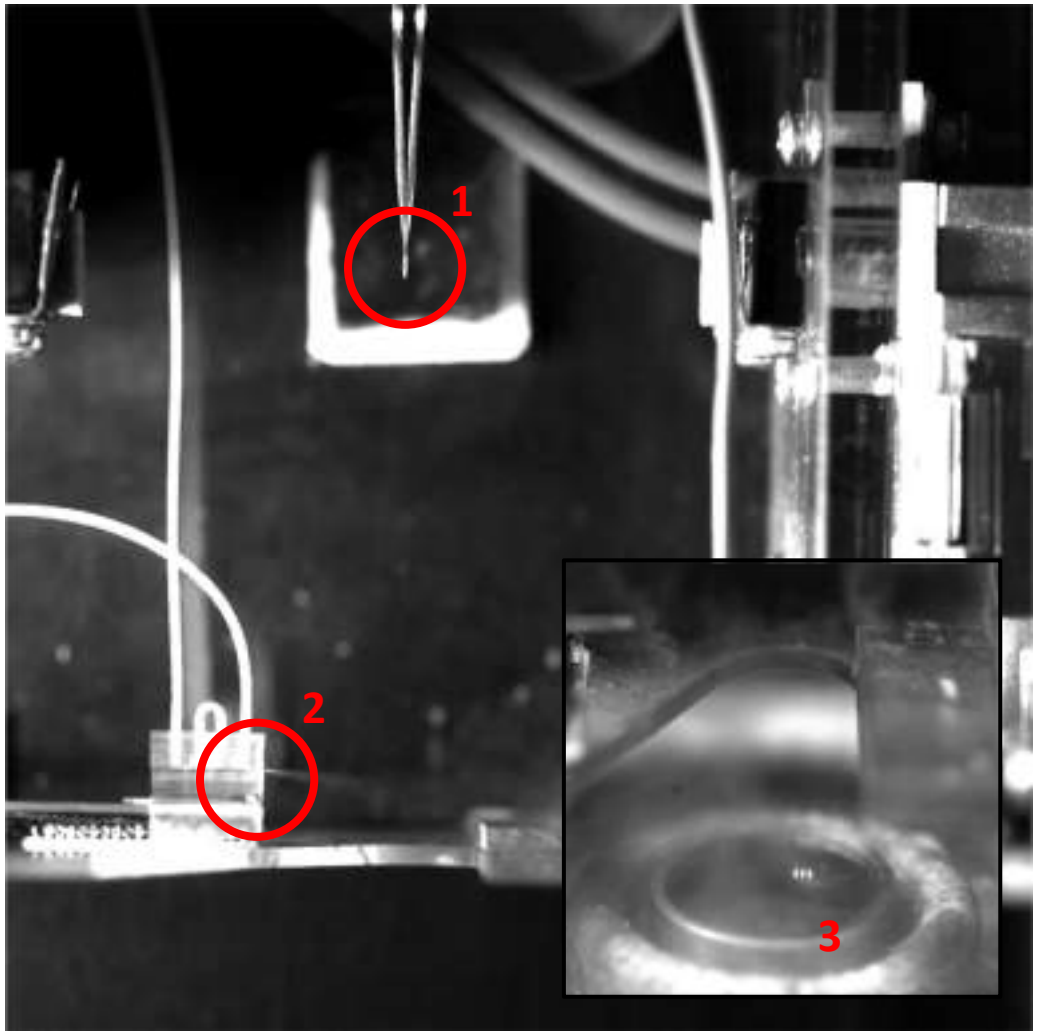
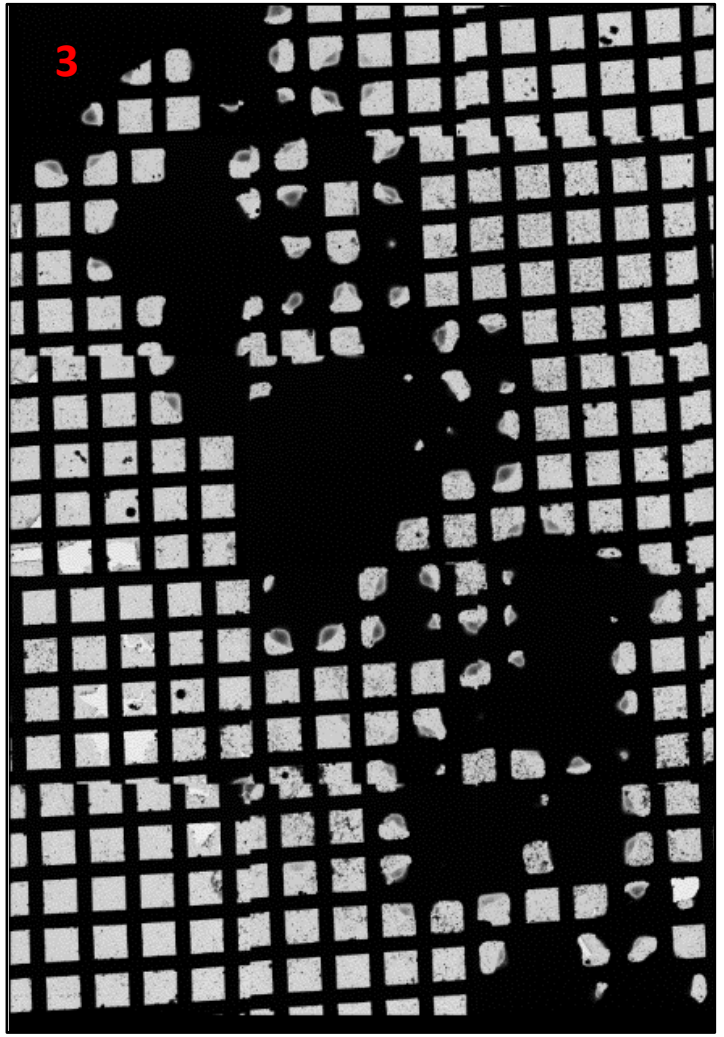
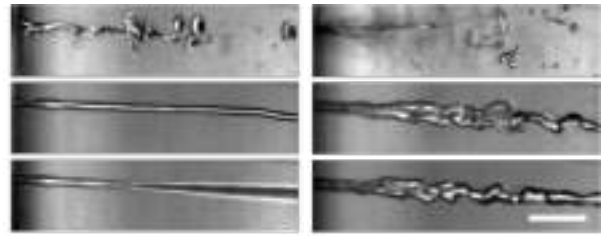






Fast grid preparation setup



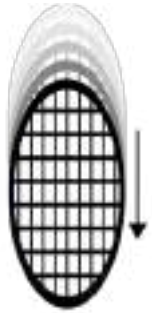
droplets ~ 20 – 40 µm
work well

sample A

N_2 gas

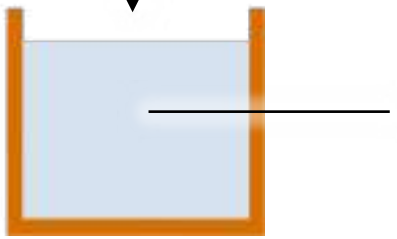
microfluidic mixer/
spray nozzle

nozzle/grid distance
~ 1 cm



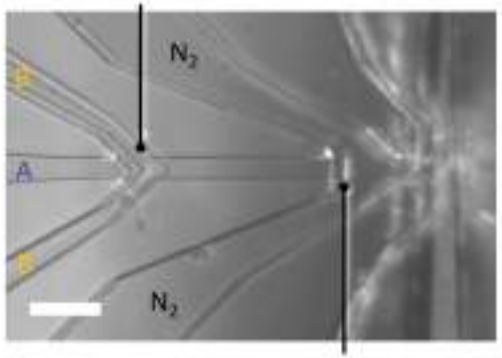
grid speed: 0.5 - 3 m/s

spray/ethane distance ~1-5 cm



at the time of freezing,
sample must be ~ 100 nm thin
freezing time ≤ 1 ms

flow focussing/mixing

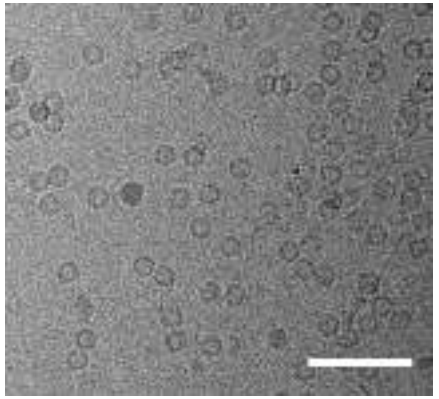


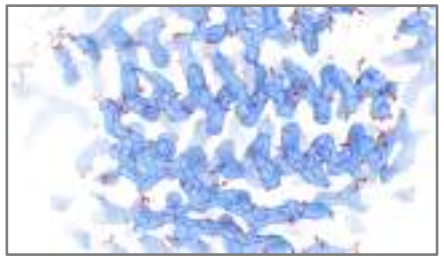
gas focussing/atomization

~ 30 nm

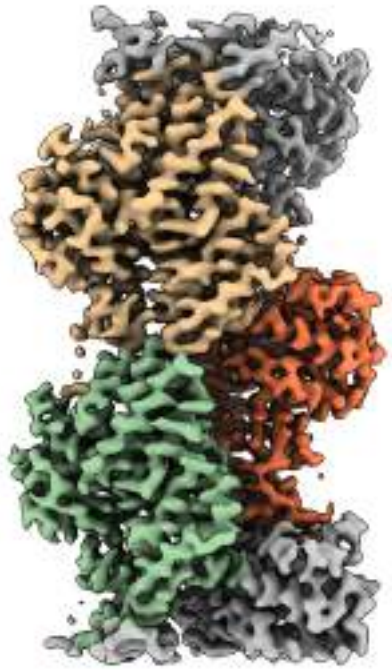
Results from fast grid preparation

High-resolution structures for single particles and helical assemblies:



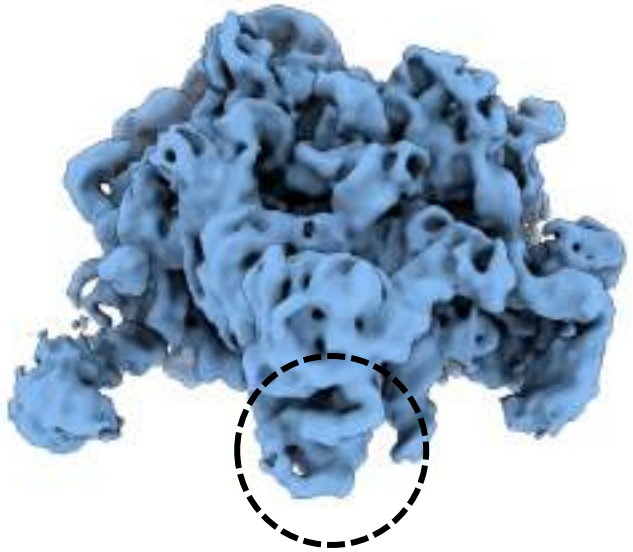


Apoferritin, 3.5 Å



F-actin, 3.4 Å

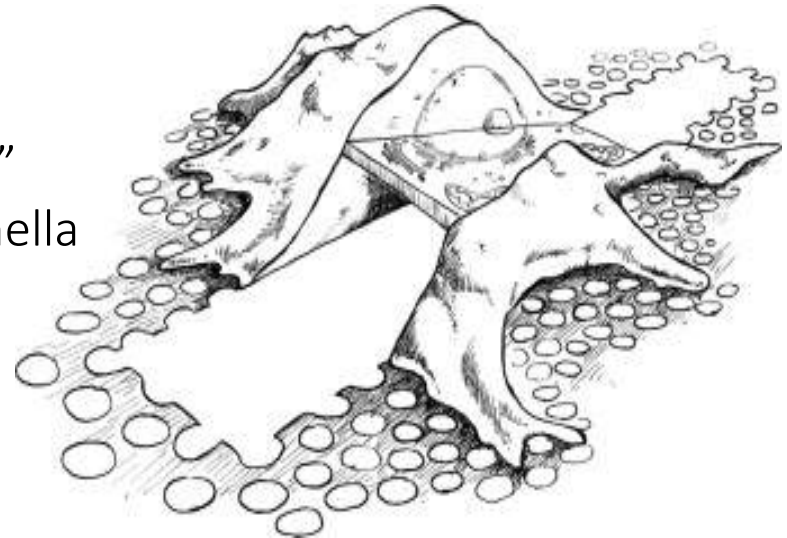
Fast grid preparation can improve particle orientations and prevent protein denaturation



50S ribosome at 13, 54, 200 and 6 000 ms

Sample preparation of cells, tissues and organisms

- **High pressure freezer-** for vitrification of cells, tissues and organisms like *C. Elegans* - **Leica EM ICE**
- **Freeze substitution** of high pressure frozen specimens **Leica AFS2**
- **Ultramicrotome for sectioni**ng of ambient/Tokyasu/vitreous (CEMOVIS) samples - **Leica UC7 cryo**
- **Cryo FIB** – uses ion beam to “mill” away sample so left with thin lamella for imaging.



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BBSRC
bioscience for the future

UNIVERSITY OF LEEDS


Any questions?