Lecture 2

(3 Jul 2016 9:30 am)

# EM preparation methods

## R. Natesh ( kind gift by Carolyn Moores)

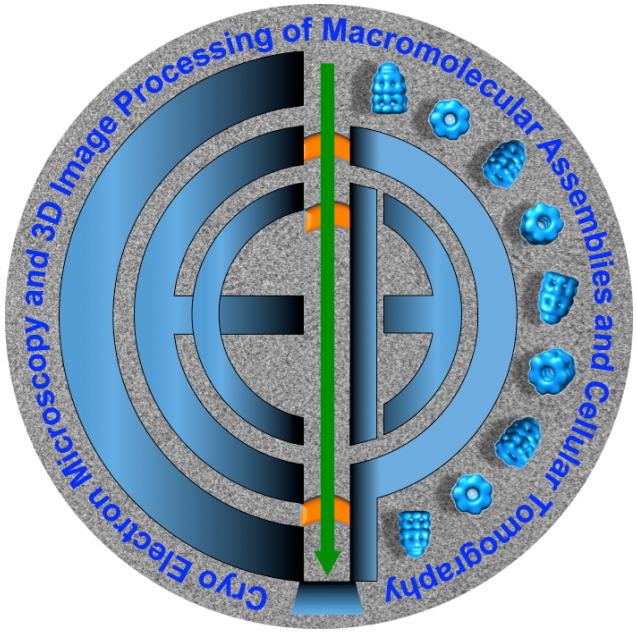



Image Processing for Cryo-EM

2 July - 13 July 2016

IISER Thiruvananthapuram (IISER-TVM), Trivandrum

Cryo Electron Microscopy (analogous to MX)

- ***Sample Preparation***
- ***Specimen preparation***
- ***Data Collection***
- ***Image Processing and 3D Reconstruction***

**Natesh, RESONANCE** | December 2014

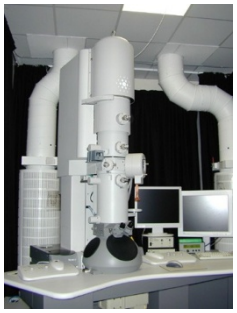
# Outline

Basics
- EM grids + treatment

Molecular EM
- negative stain
- cryo-EM
- sample manipulation

Cellular EM

# EM preparation methods: The challenges

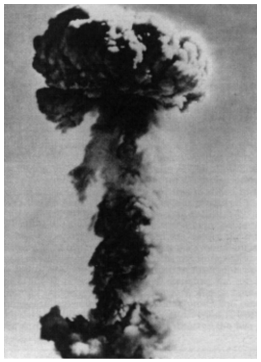


microscope column is a vacuum

minimise artefacts

make sample EM-ready

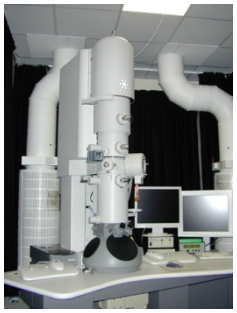
want to see something real



electrons are very damaging

want to actually see something

# EM preparation methods: The challenges



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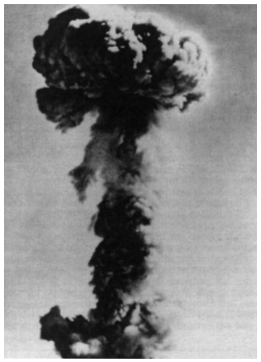

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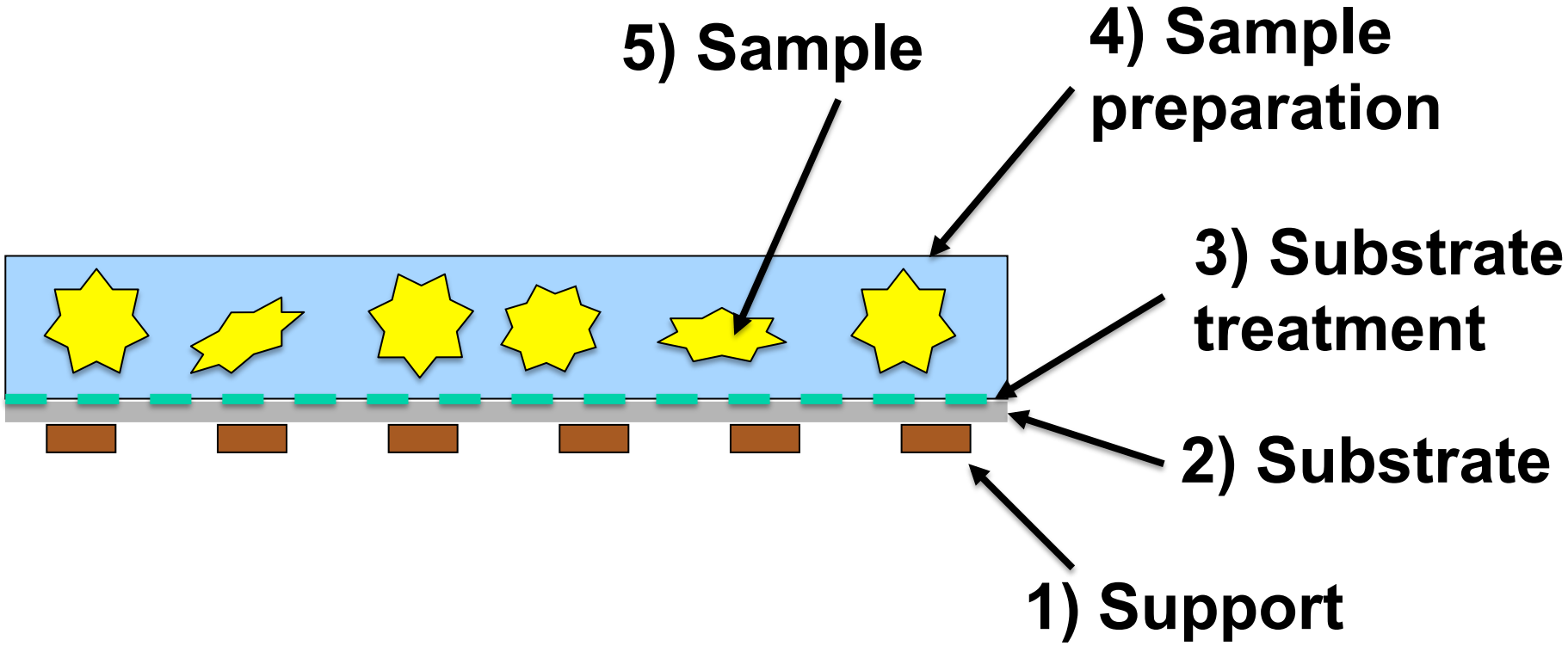
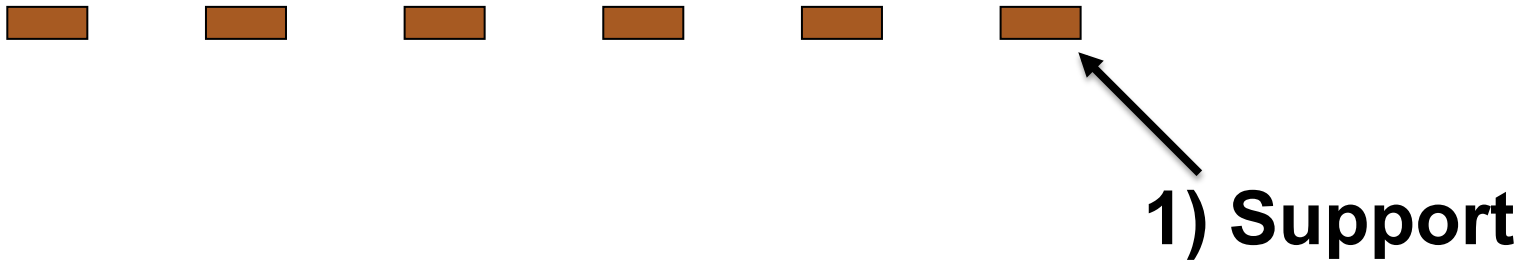
**Samples on the move**

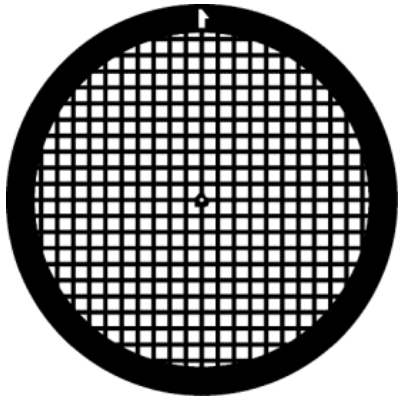
# EM preparation methods - Topics for consideration



# EM preparation methods - Topics for consideration:
# **1) Support**

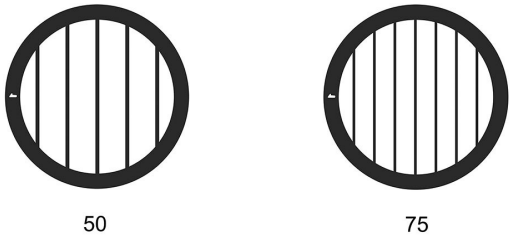


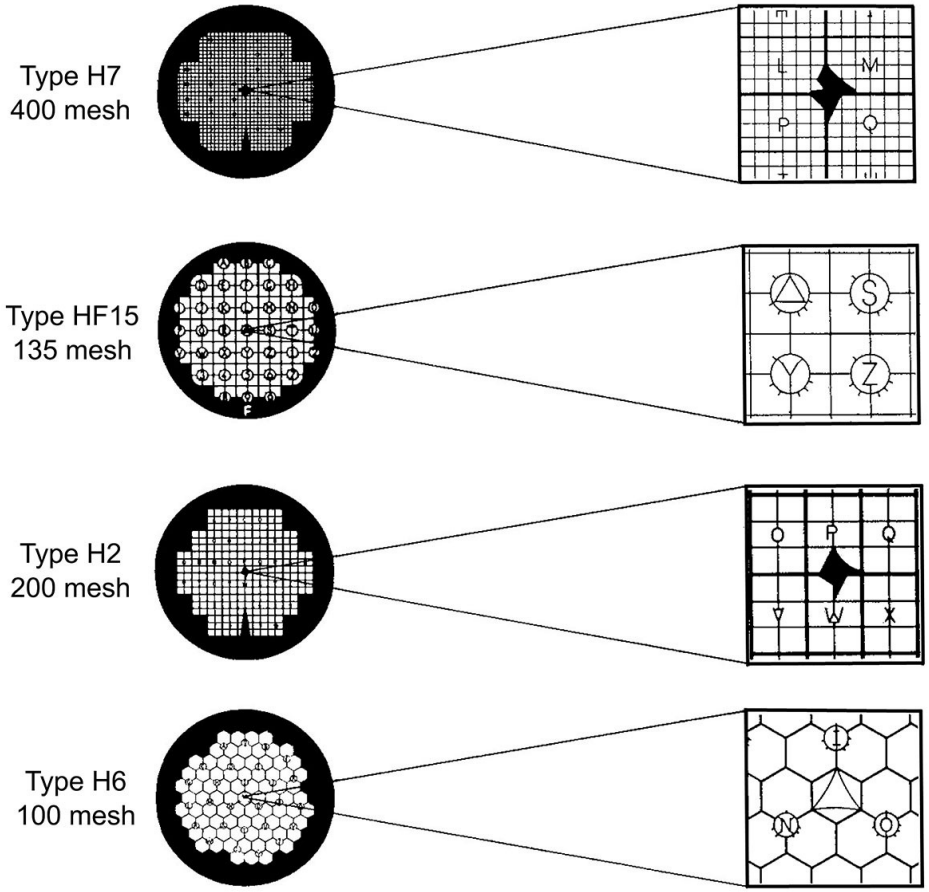
# EM grids

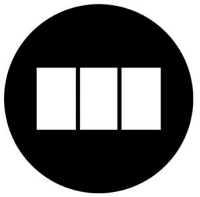


3mm

- metal mesh
- classically copper, or nickel, molybdenum
- gold (inert): e.g. work with cells [more later]

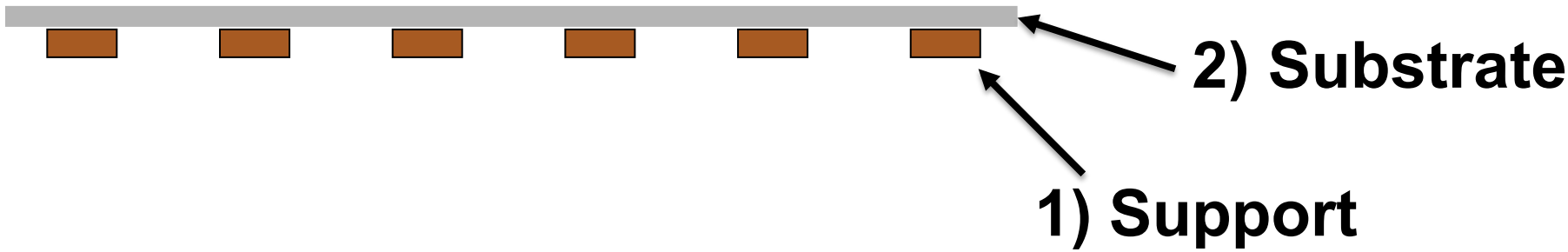


*www.agarscientific.com*

# EM preparation methods - Topics for consideration:
# **2) Substrate**



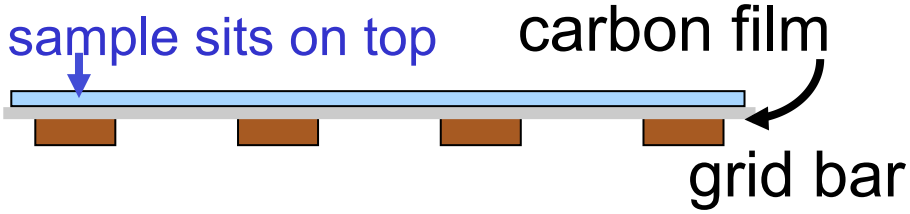
# Carbon film (1)

**Classically: amorphous carbon [am-C]**

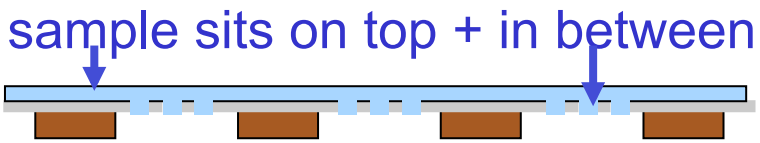
**- inert; strong; electron transparent; electrically (somewhat) and thermally conductive;**

**- prepared cheaply: by evaporation (in-house)**

**- high purity graphite gives better quality film**




- **continuous carbon film**
- thin/thick carbon
- underlying plastic



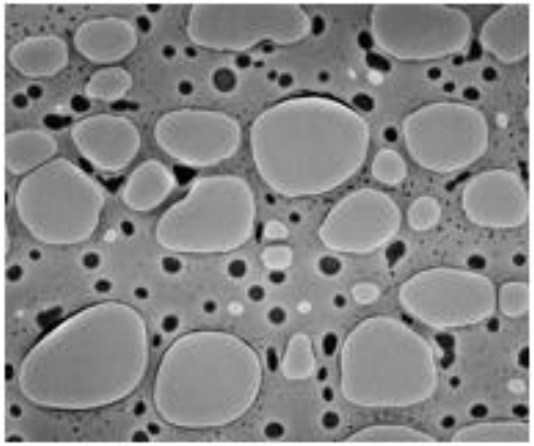

- **holey carbon film**
- thin/thick carbon
- underlying plastic (usually removed)
- size of holes
- regularity of holes

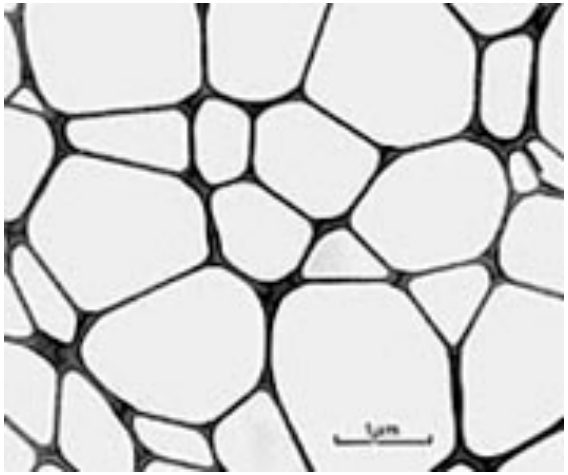
(see Miyazawa et al. 1999)

# Carbon film (2)

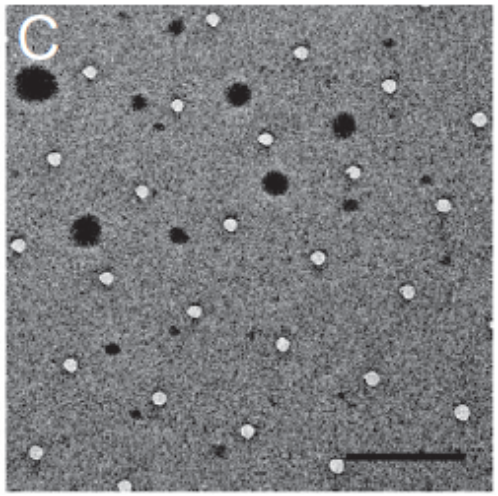
## Types of holey carbon

home-made
(irregular)

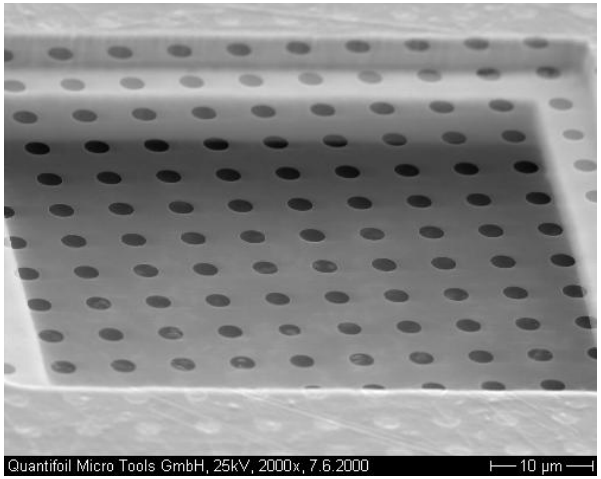




lacey




home-made (regular)
500nm holes



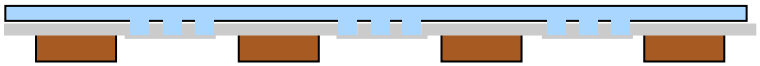

Quantifoil or C-flat

Marr et al ( 2014)

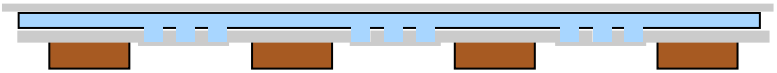
# Carbon film (3)
## Variations (1)

- very little sample
- sample with a particular affinity for carbon
- sample where you want to control views
- for additional sample stability

- a layer of very thin carbon over holey-carbon film



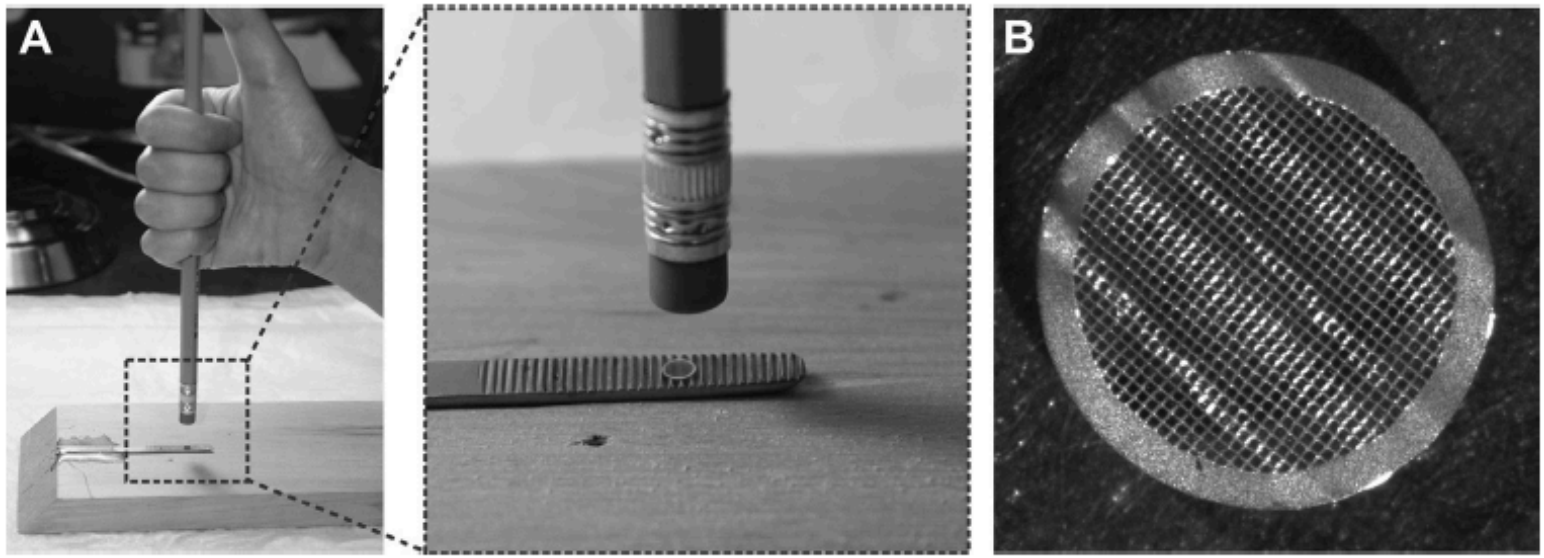
- a thin layer of buffer+sample between 2 carbon layers
- pick up 2nd layer from carbon floating on water/buffer



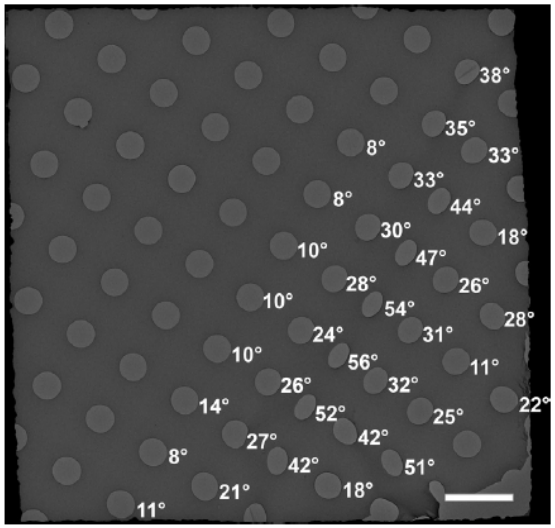
# Carbon film (3)

## Variations (2)

### Carbon: flat or not?



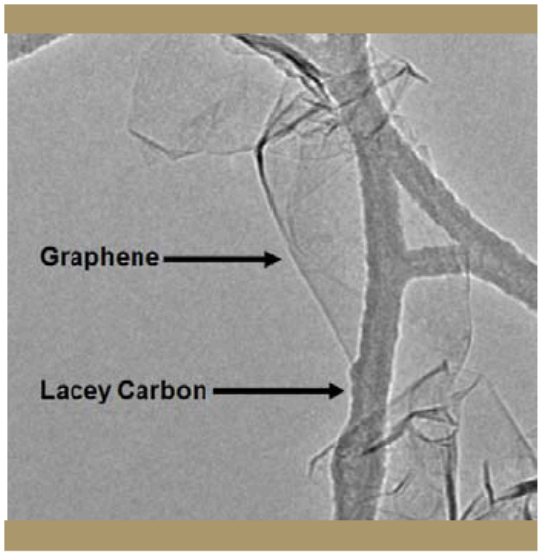

- With preferred orientations, may help to avoid high tilt data collection




Liu et al ( 2013)

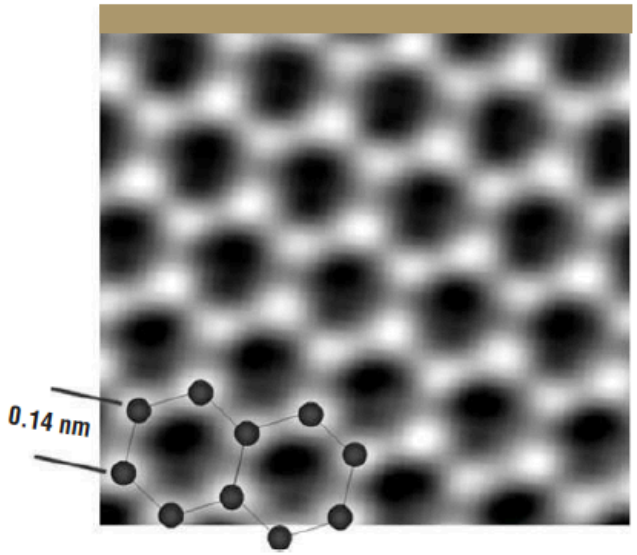
# Carbon film (4)

## Graphene

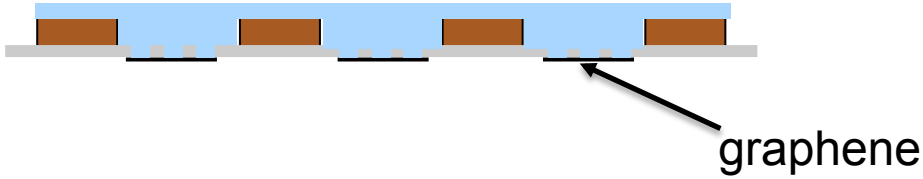



*A typical TEM image of graphene sheets freely suspended on a lacey carbon TEM grid.*

- atomically thin, but strong
- conductivity at all temperatures
- lower background compared to am-C
  - e.g. layer over standard holey grid

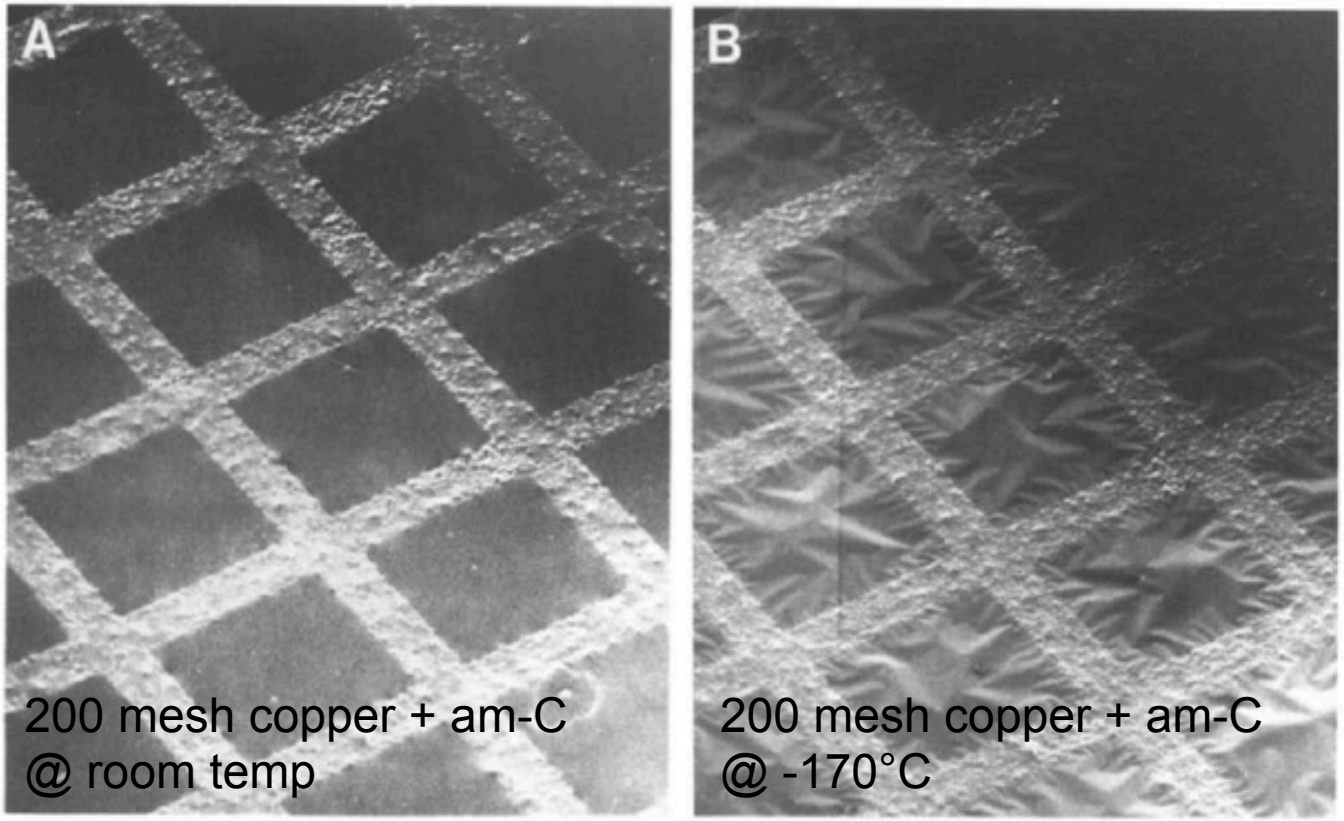



http://www.emsdiasum.com/microscopy/default.aspx




Rhinow et al (2011)
Sader et al ( 2013)
Russo & Passmore (2014a)

# Carbon film (5) + copper
## = potential problem
## “cryo crinkling”

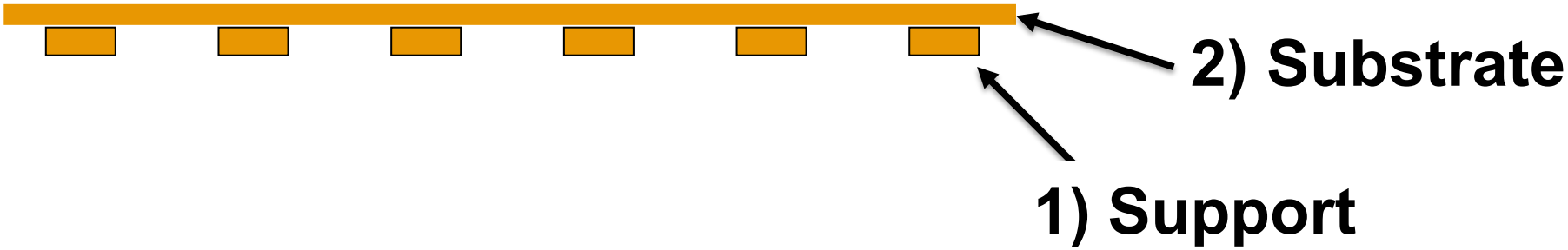



- Differences in thermal expansion coefficient

Booy & Pawley (1993)

# EM preparation methods –
# **1) Support + 2) Substrate**

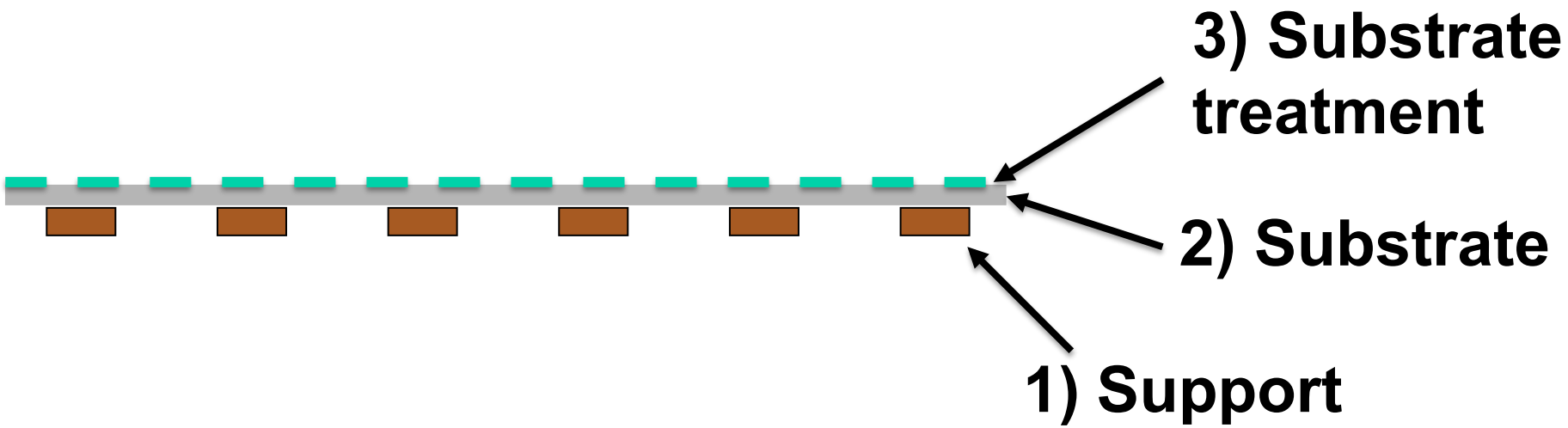
Made of the similar/same material
Improve low temperature stability/conductivity




- **Cryomesh™ (silicon carbide)**
- **Gold**

Yoshioka et al (2010)
Russo & Passmore (2014b)

# EM preparation methods - Topics for consideration:
# **3) Substrate treatment**



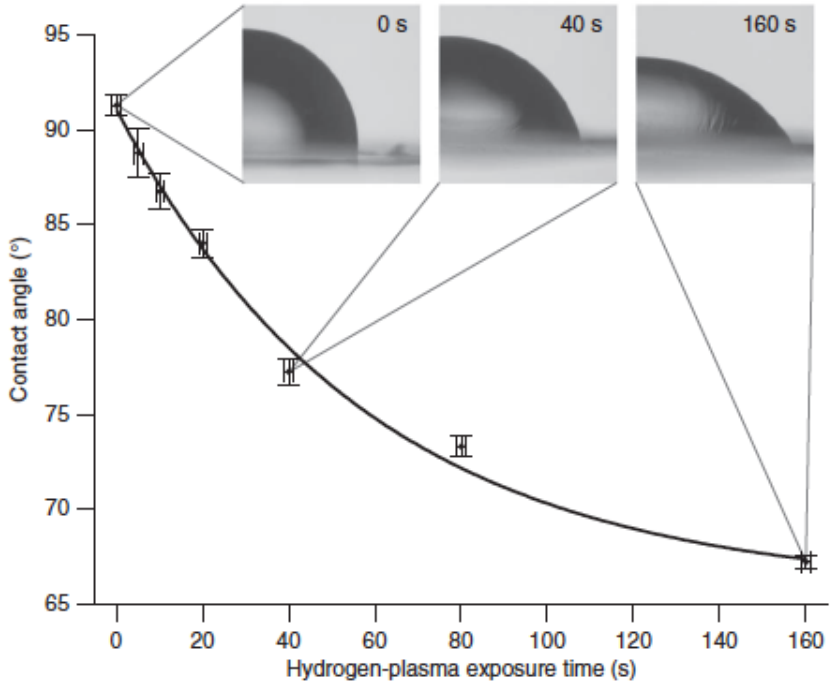
# Substrate treatment

*"details of the surface chemistry.. are poorly understood"*

- **spreading**
- **adsorption/orientation**
- **behaviour of grid on imaging**

- "aging" of the carbon
- plasma cleaning
- glow discharge:

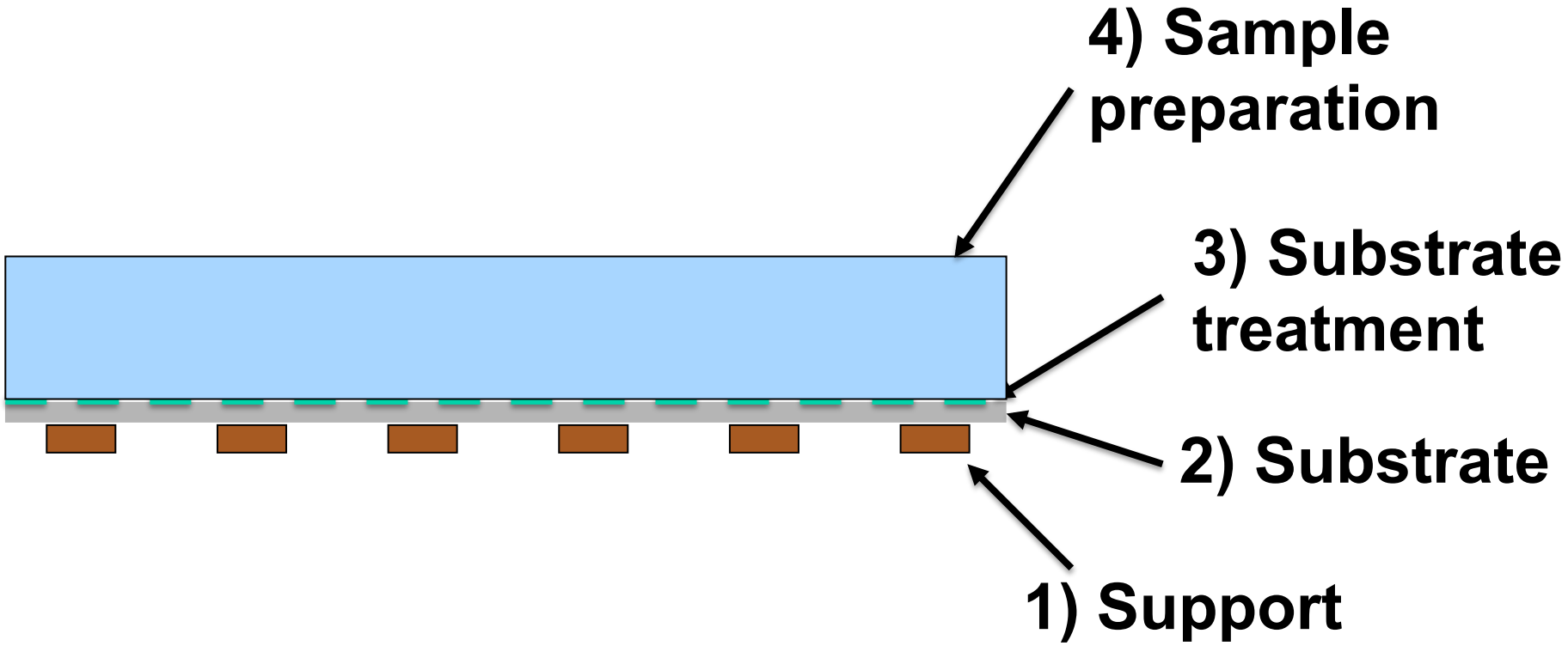
| *Atmosphere* | *Charge* |
|---|---|
| air | - |
| methylamine | + |
| methanol | - |




Russo & Passmore (2014a)

- treatment with UV (Burgess et al., 2004)
- treatment with electrons (Miyazawa et al., 1999)
- additives, eg poly-lysine, detergents, Ni-NTA lipids (Kelly et al 2008)

# EM preparation methods - Topics for consideration: **Sample preparation**



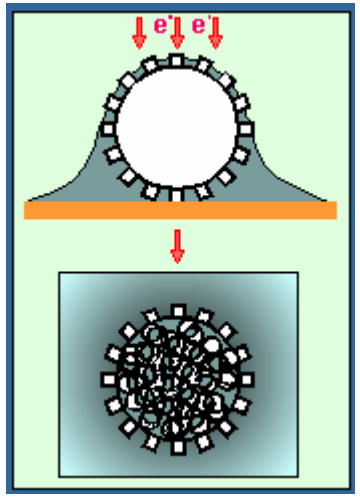
# Negative stain (1)

What is it?

- sample is surrounded by heavy metal solution
- forms a cast around the sample
- “negative” because the stain sits where the protein is NOT

Ideal properties:

- high density
- stability
- solubility
- ability to fix sample
- uniform spreading on support film
- structureless when dry
- chemically inert

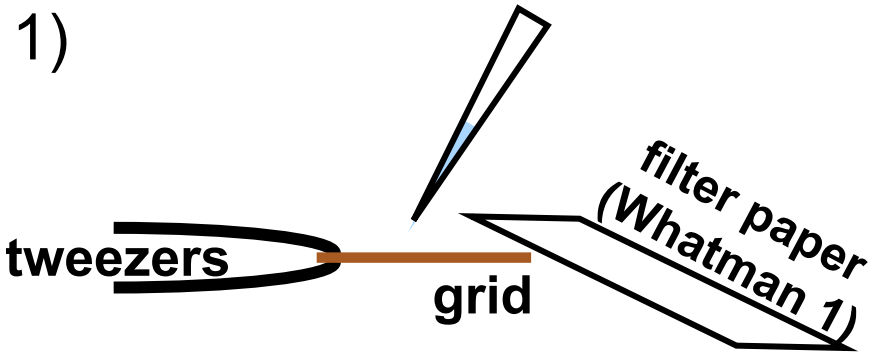


# Negative stain (2)

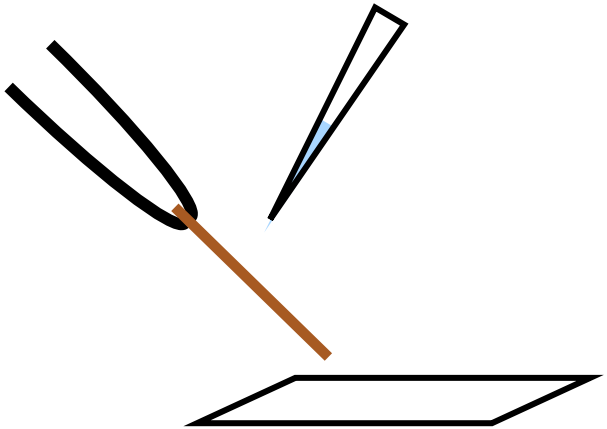
Basic protocol:

- adsorb sample to carbon support film
- apply sample <0.1mg/ml (*must test this*)
- apply heavy metal stain *(maybe multiple times)*
- blot and air dry

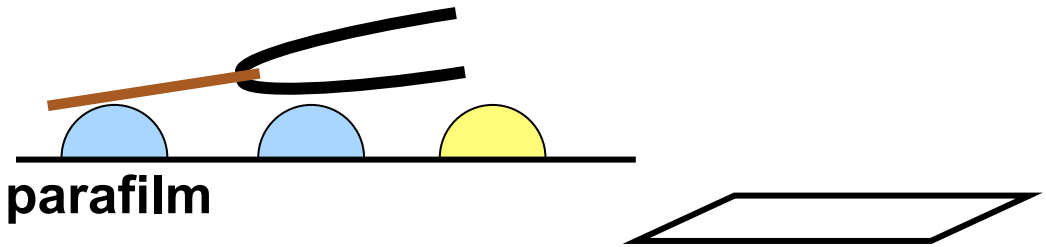
1)




2)



3)



# Negative stain (3)

Pros:

- Quick (~5mins)
- no specialised equipment
- need only a small amount of sample
- good contrast
- sample protection/fixation (time-resolved?)
- can give quite detailed information – including from raw images
- easier to see very small molecules
- reconstruction possible

Cons:

- view a cast of sample, not sample itself
- dehydrates/flattens/distorts sample
- beware of positive staining
- beware of partial staining
- several commonly used stains have extreme pHs
- in best case scenario, resolution still limited by grain size

**Strongly recommended for a new sample**

# Common negative stains

| Stain | Mw | % in soln; working pH |
|---|---|---|
| Phosphotungstic Acid (PTA) | 3315.5 | 1-4%; pH 5-8 |
| Phosphomolybdenic Acid (PMA) | 2041.6 | 1-4%; pH 5-8 |
| Ammonium Molybdate (AM) | 1235.9 | 1-4%; pH 5-7 |
| Uranyl Acetate (UA) | 424.2 | 0.5-4%; pH 4.2 [stable for months] |
| Uranyl Formate (UF) | 414.1 | 0.5-1%; pH 4.5-5.2 [stable for only ~1-2days] |
| Methylamine Vanadate (Nano-Van) | ND | 2%; pH8.0 |
| Methylamine Tungstate (Nano-W) | ND | 2%; pH 6.8 |

0.8-0.9nm grain

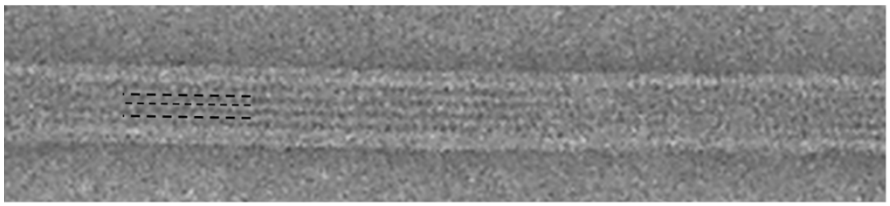
0.4-0.5nm grain

NB Don't forget to filter stain solution through 0.22μm filter

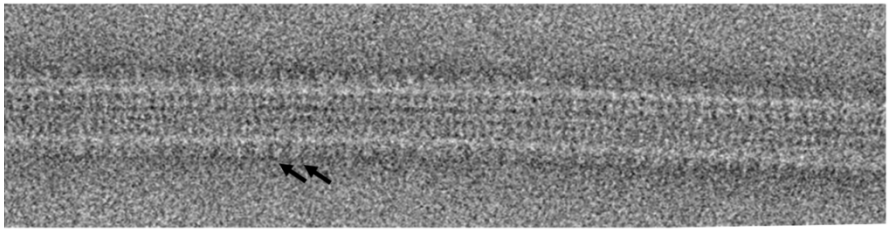
# Getting the most from negative stain (4)

Sample specific

- e.g. can evaluate binding to microtubules in uranyl stains
- squashed but microtubule lattice still intact (good diffraction)
- look awful in Nano-W

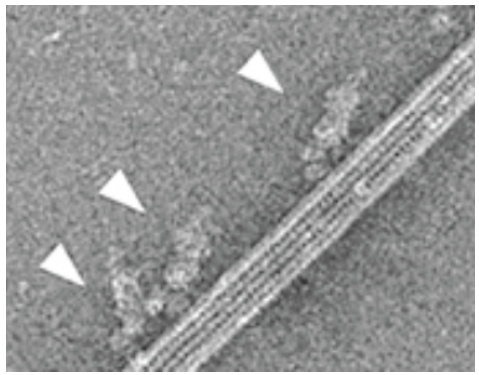


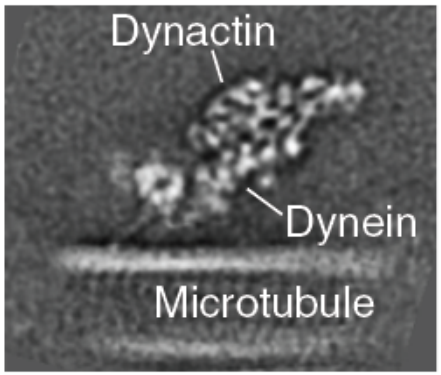
microtubule

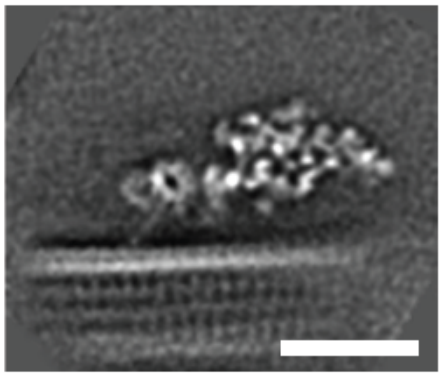


microtubule+kinesin

- benefits for large, dynamic, multi-component complexes





Chowdhury et al (2015)

# Cryo-EM (1)

**What is it?**

• Sample is frozen in a layer of **vitreous** ice

**What is vitreous ice?**

• amorphous – i.e. non-crystalline

• sample preserved in solid solution-like state
(ice crystals destroy samples)

• means it can be placed in vacuum of EM column
(low temperature must be maintained)

• (some) cryo-protection from radiation damage

**How is vitreous ice formed?**

• Quickly (<1ms: estimated cooling rate of 100,000°/s)

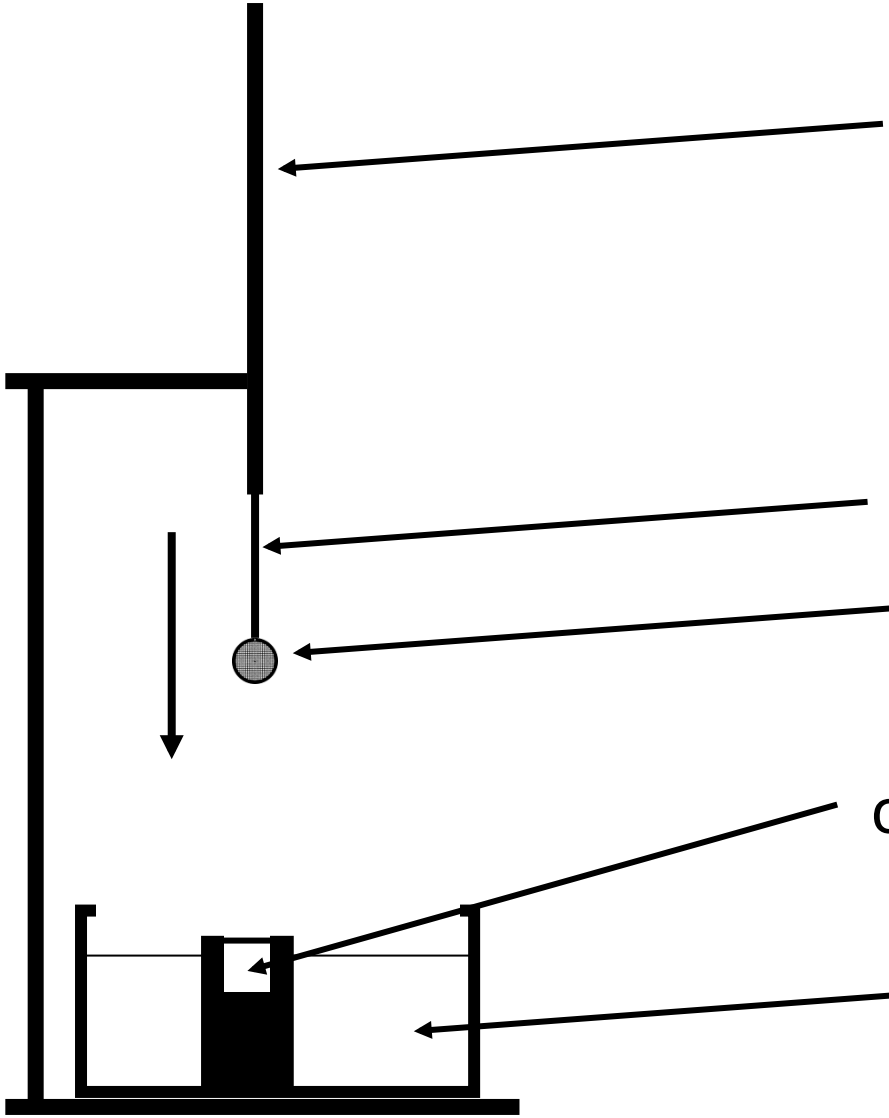
• at atmospheric pressure, forms below ~-160°C

• requires rapid freezing rate to avoid other phase transitions

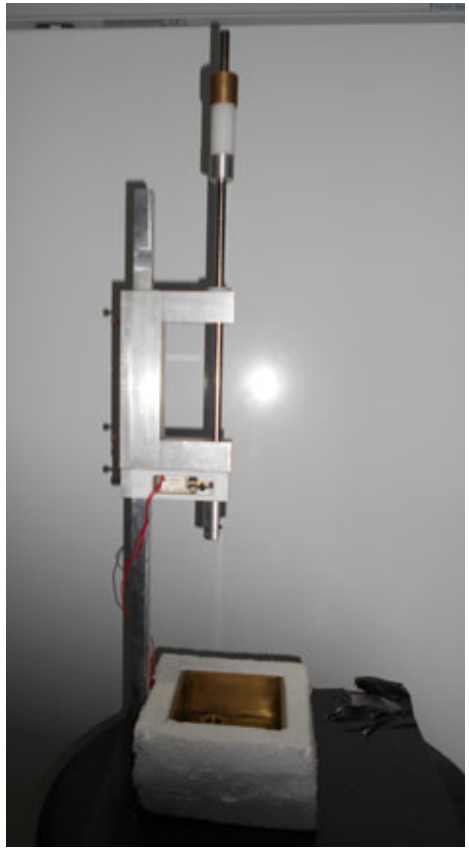
• for rapid freezing, a thin (<10μm) layer of sample is also essential (cf cell sections)

# Cryo-EM (2)

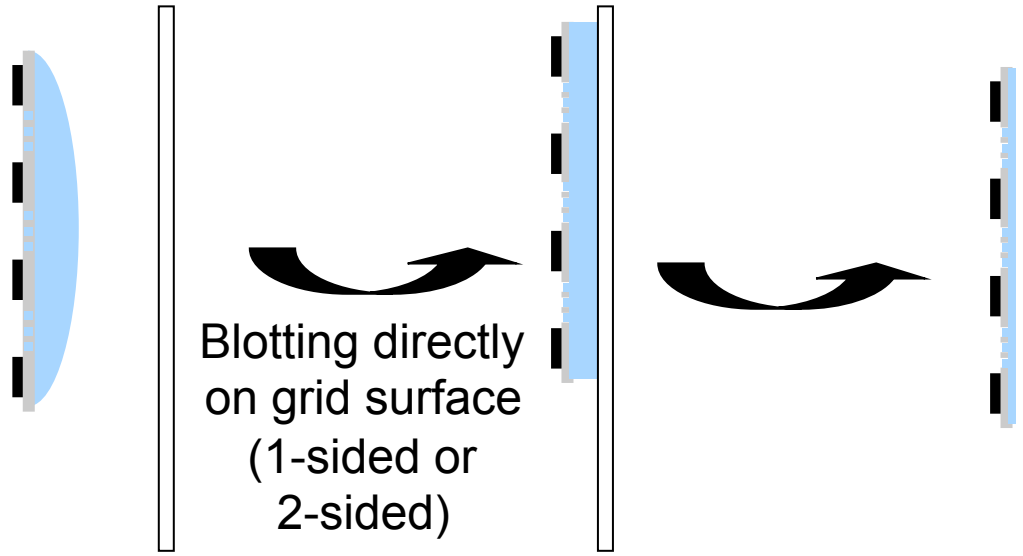
## Basic experimental set-up




Home Made at IISER-TVM



# Cryo-EM (3)
## Grid blotting




**Problem:**

- very thin sample layer means rapid water evaporation; this can
  - increase ionic strength
  - cool sample
- **Solution**: perform freezing in humid atmosphere and/or @4°C

At 99% humidity, rate of evaporation = 5.2Å/s @4°C or 20.4Å/s @25°C
At 60% humidity, rate of evaporation = 207Å/s @4°C or 817Å/s @25°C
(Ken Taylor)

# Cryo-EM (4)
## The cryogen

**Liquid with melting point below ~-160°C;**

Nitrogen Boiling point: -196°C
melting point: -210°C
Boils in thin layer round sample and insulates it (Leidenfrost effect)

Propane Boiling point: -42°C
melting point: -189°C
Flammable

Ethane Boiling point: -88°C
melting point: -183°C
Flammable

# Cryo-EM (5)


The vitrobot!

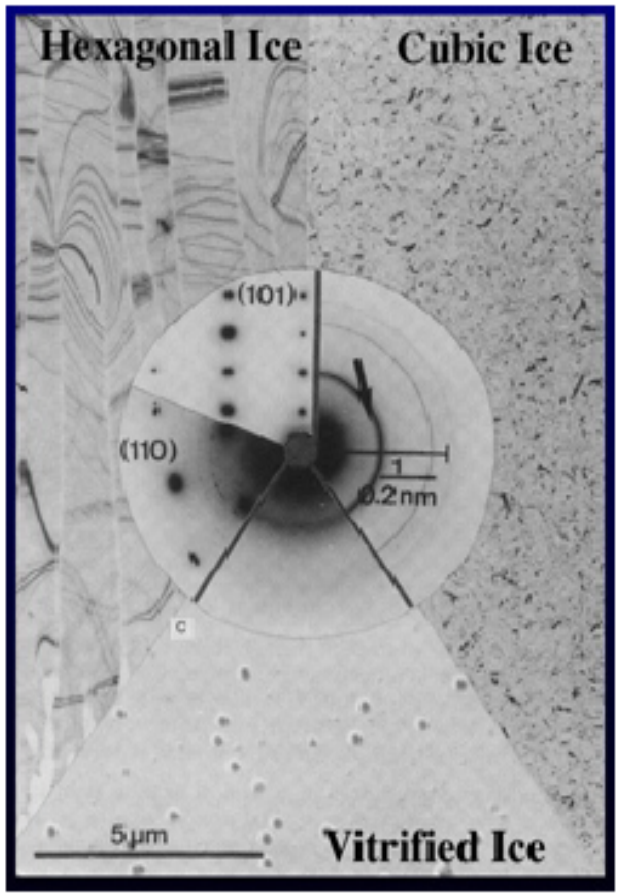

The EM GP!

- helps new users
- improves reproducibility (at least on same machine)

# Cryo-EM (6)

## Preserving vitreous ice

Vitreous ice is metastable and readily converts to other forms of ice so grids must be maintained at <-135°C



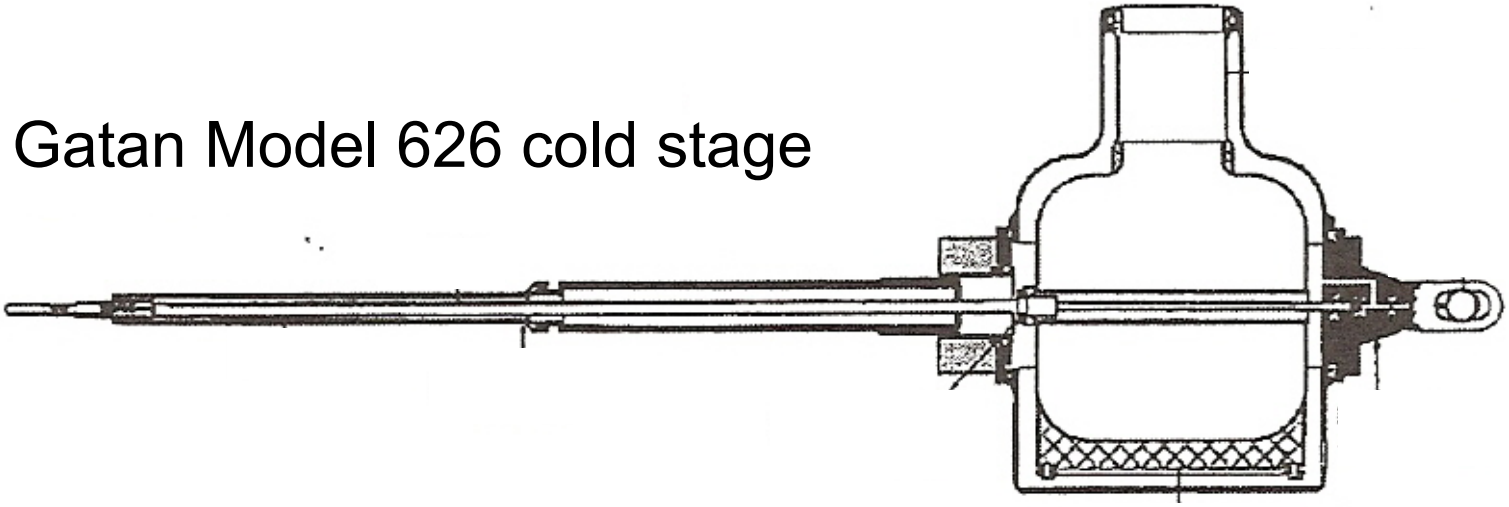

Dubochet et al., 1988

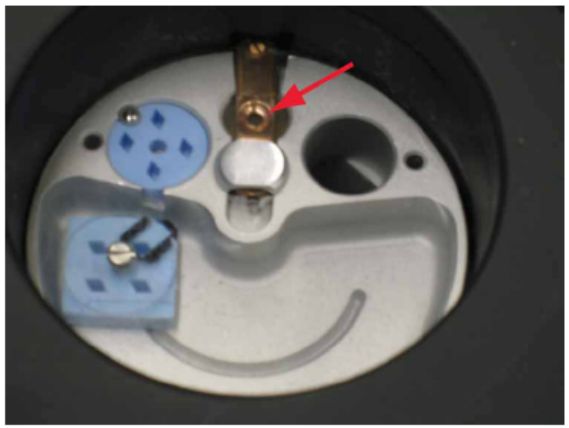
- hexagonal ice
  - crystalline, forms as water cools @ atmospheric pressures
  - likely to form e.g. on cold apparatus
  - (a big problem in a humid atmosphere)
- cubic ice
  - crystalline, formed as vitreous ice warms above ~-135°C
- both crystalline forms of ice are less dense than liquid water; their expansion can damage biological sample as they form

# Cryo-EM (7)

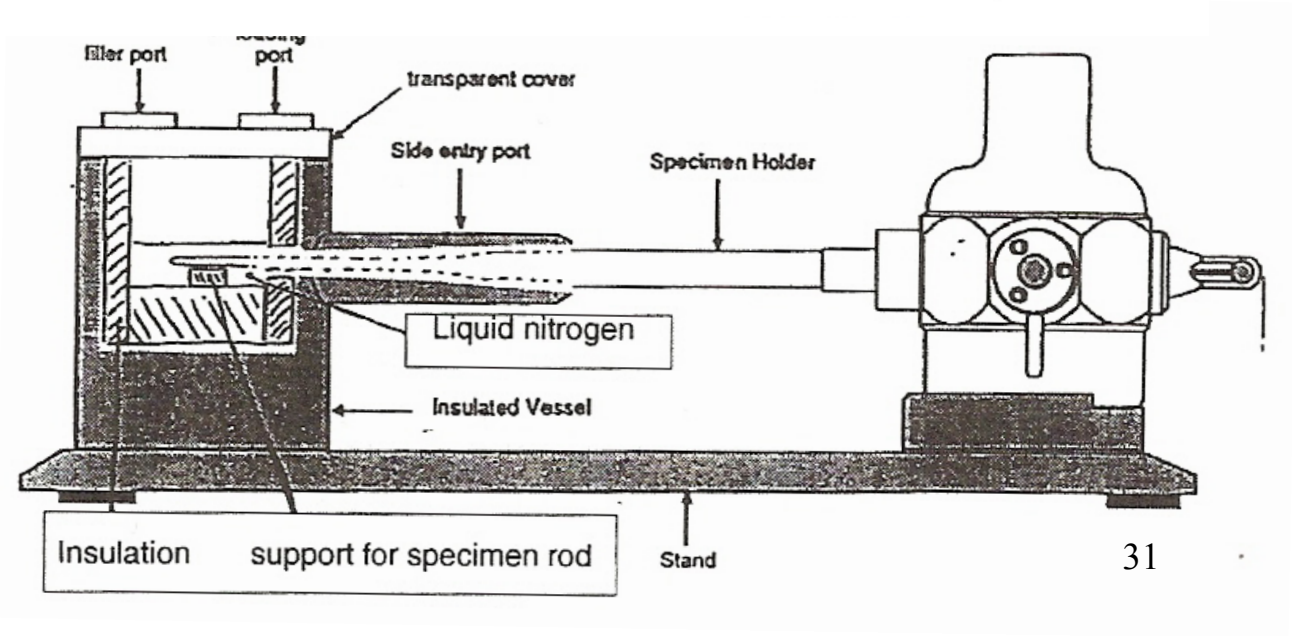
## **Sample holder** (old school)

Gatan Model 626 cold stage



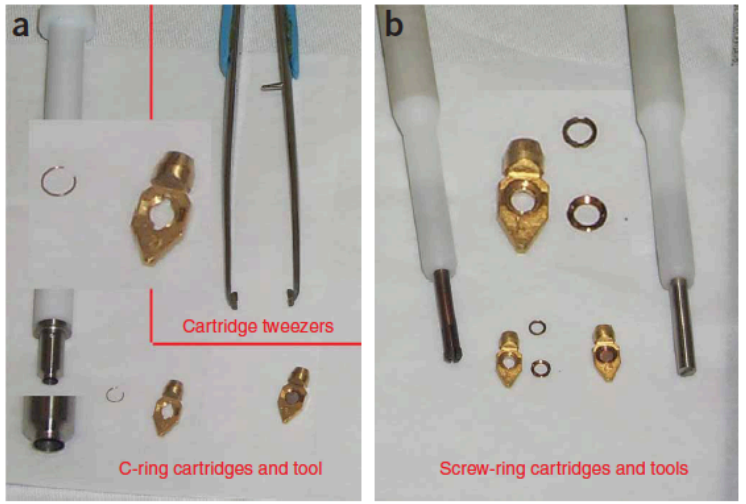


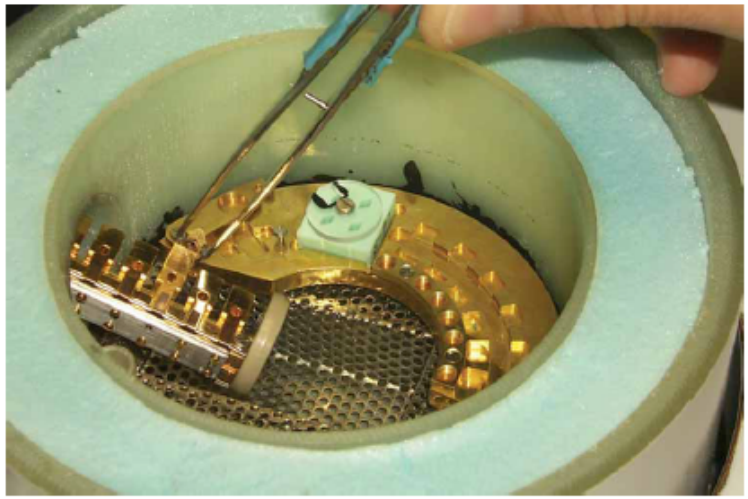
Graussucci et al., 2008



# Cryo-EM (8)

## Cryo-EM cartridge loading system

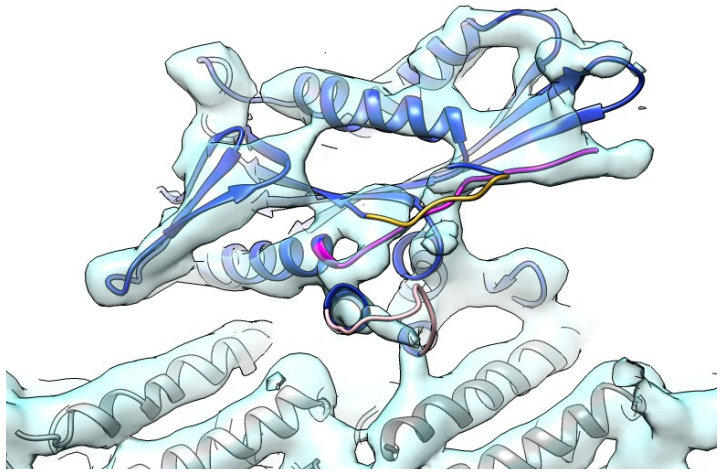



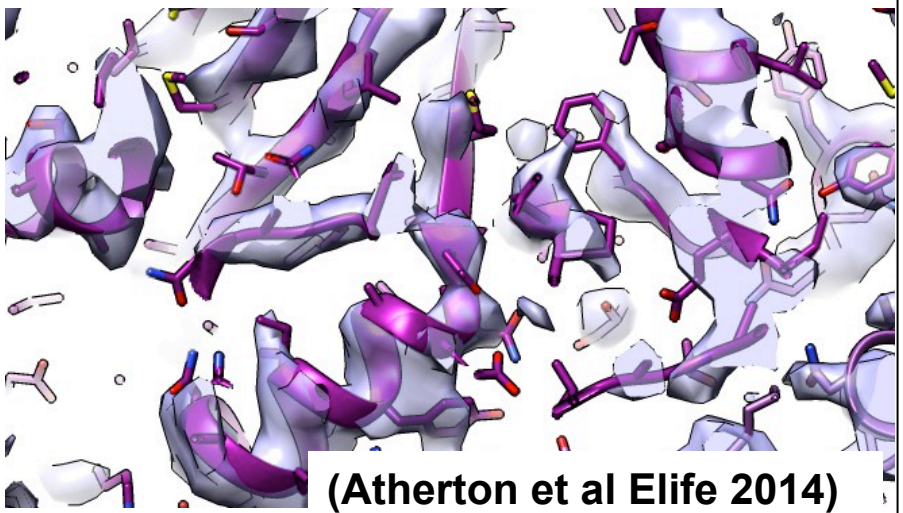
Microscope cooled internally by liquid nitrogen

Graussucci et al (2008)

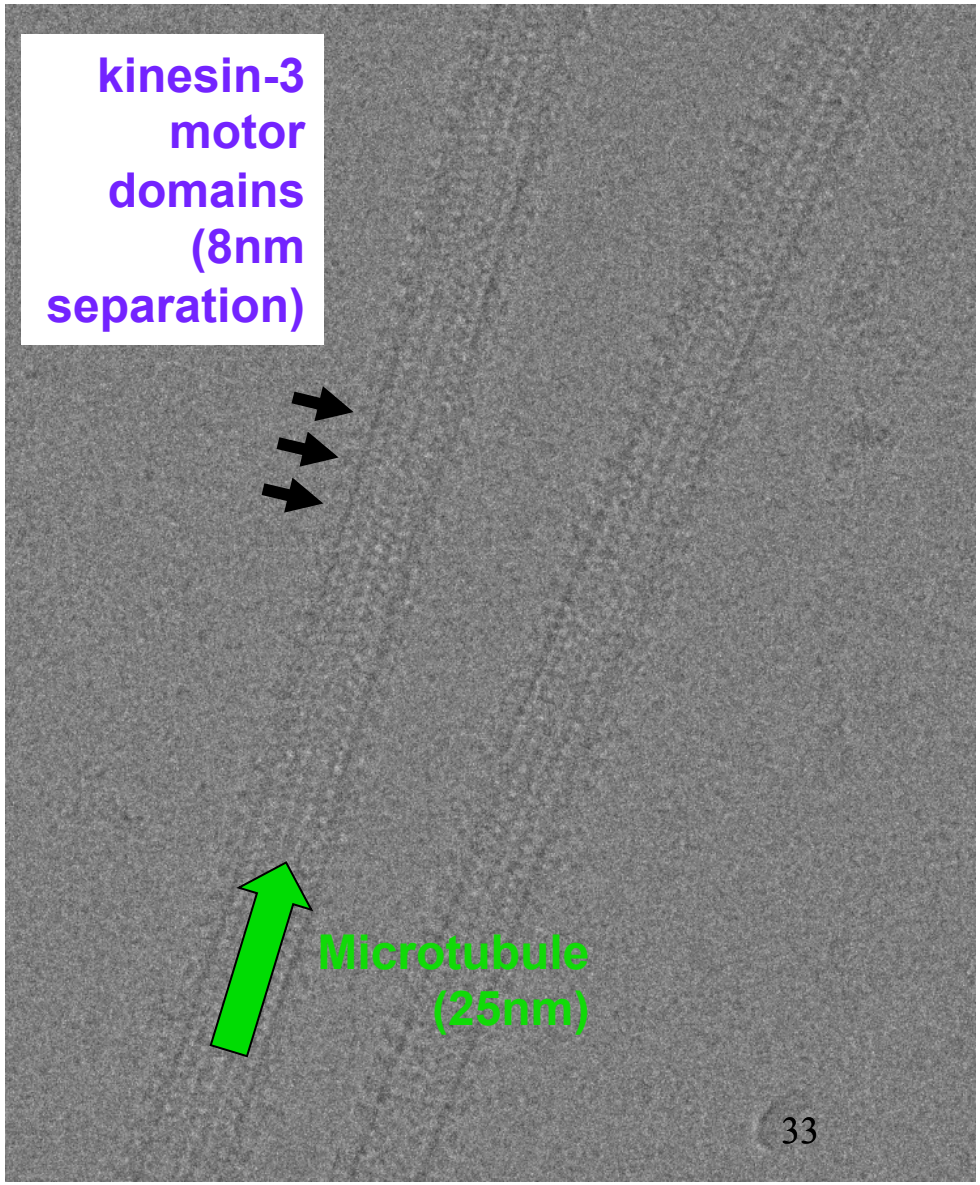
# Kinesin-bound microtubules visualised by cryo-EM

**Sample:**
- **human kinesin-3 motor domain**
- **bound to MT**





(Atherton et al Elife 2014)



# Cryo-EM (8)

**Pros:**

- physiological
- information about sample itself
- information about internal details
- 3D atomic resolution reconstruction theoretically possible
- sample protection
- capturing of transient intermediates

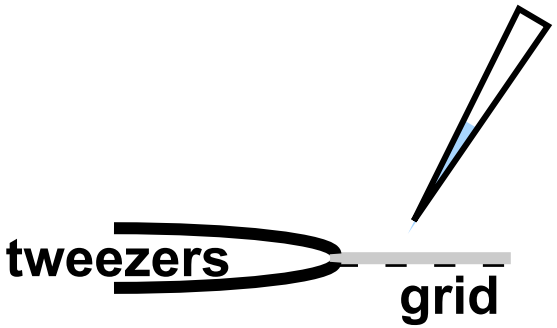
**Cons:**

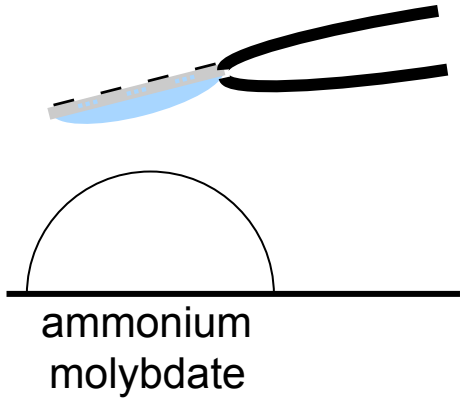
- slower (sample preparation and data collection)
- need more sample than for negative stain
  (~3-5mg/ml though less than eg NMR and X-ray)
- poor contrast
  (~density of vitreous ice (0.9g/cm$^3$) vs protein (1.3g/cm$^3$))
  NB: thinner ice: better contrast but consider surface effects
- requires more specialised equipment
- sample is very electron-sensitive: low-dose imaging essential
  (although low temperature is somewhat protective)

# Cryo-negative staining

- for very small samples
- to improve contrast while retaining sample hydration
- samples less beam-sensitive – can increase dose and therefore further improve contrast
- Limitations: from grain size, stain penetration

Use: 16% ammonium molybdate (adjusted to ~pH7 using NaOH)
[saturated solution: *high ionic strength*]




1) Apply sample to (holey) grid 2) sit grid on drop of stain 3) blot and plunge as usual

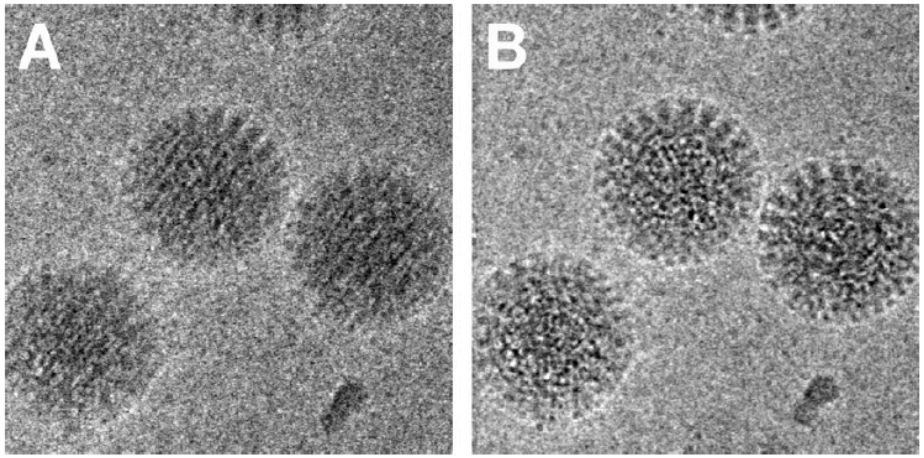
Adrian et al (1998); De Carlo & Stark (2010)

# Cryo-EM (9)

## Samples on the move!

Sample perturbation ~simultaneous with sample imaging:
Beam-induced movement

(Brilot et al 2012)




***BEFORE***
movement correction

***AFTER***
movement correction

Vitreous ice is an insulator - leads to charge accumulation:

- Sample movement due to internal stress
- Changes in carbon film
- Electrostatic effects on beam

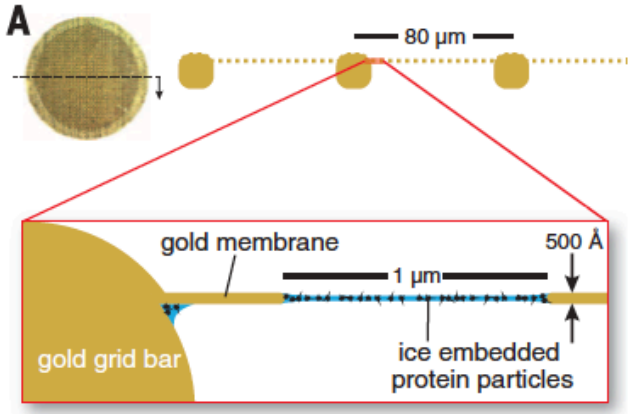
## Solutions: correct (detectors) + minimize (sample)

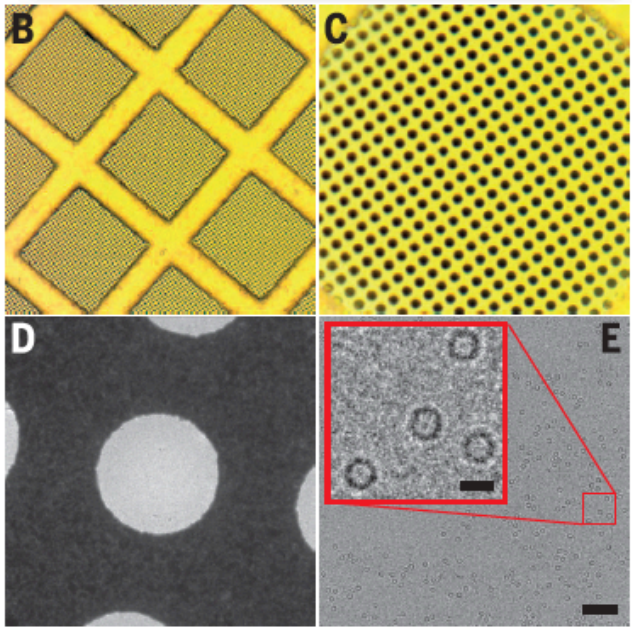
- Imaging protocols (pre-illumination)
- Treatment of carbon/thickness of carbon
- Smaller holes
- Graphene over holes ….. Or…..

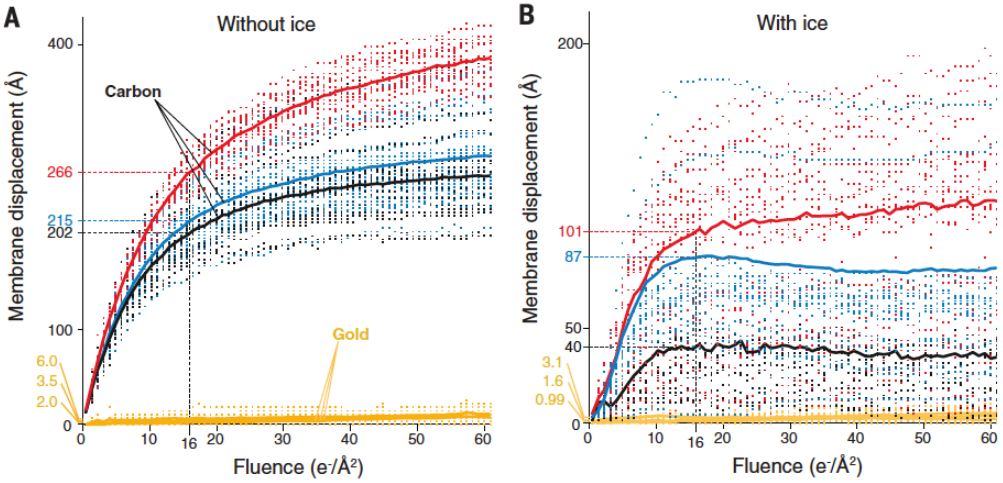
# Ultrastable gold substrates for electron cryomicroscopy

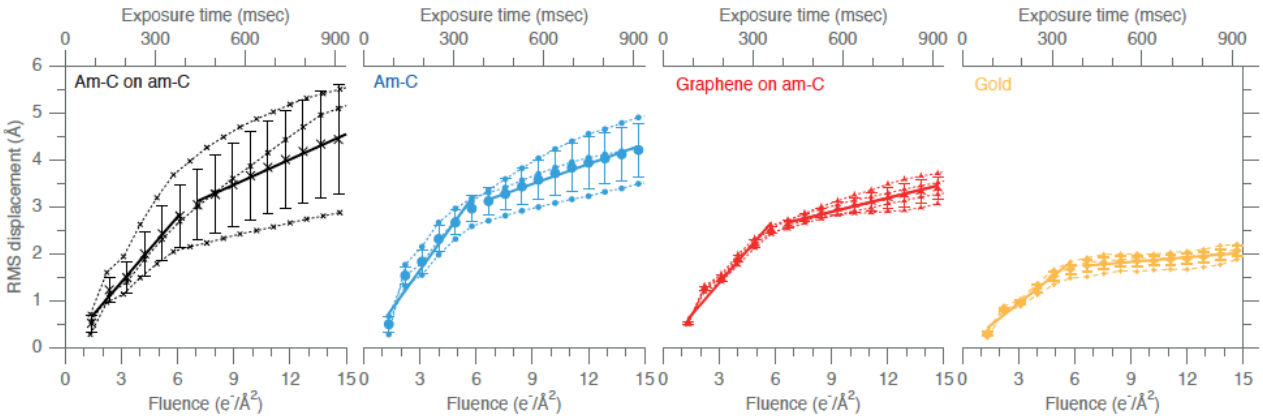
Christopher J. Russo and Lori A. Passmore*

12 DECEMBER 2014 • VOL 346 ISSUE 6215 1377
SCIENCE sciencemag.org



# Ultrastable gold substrates for electron cryomicroscopy

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## Summary

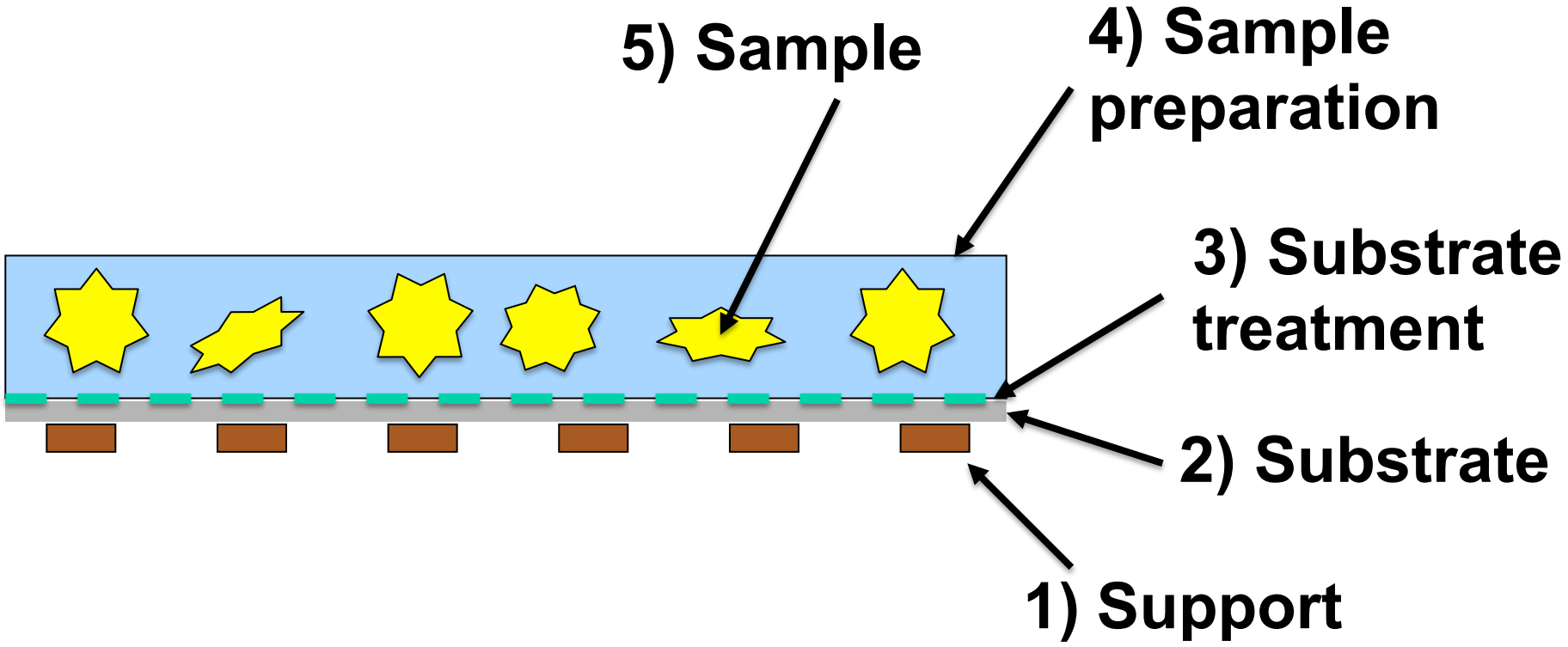
- Nearly eliminates beam-induced movement
- Inert, non-toxic, highly conductive
- Uniform electrical conductivity and thermal contraction

## Considerations

- Optimum thickness: ~500nm – thicker layers make no difference
- Surface treatment/blotting the same
- Measuring defocus: no Thon rings so consider alternatives
- No motion correction needed?
- *COST*?

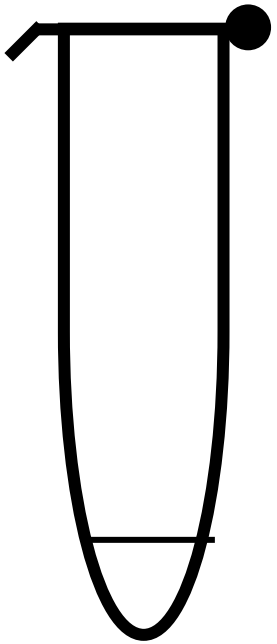
# EM preparation methods - Topics for consideration:

## 5) The sample



# Molecular EM

## Your sample



What do you know about it?

- native/recombinant?
- how purified?
- how pure?
- what buffer is it in?
  - cryo-protectants?
  - phosphate buffer?
  - detergent?
  - high salt?
  - co-factors?
- is it active?
- what shape is it?

# Challenging biological samples require specialised handling

e.g. 1) dynamic, multi-protein complexes:
glycerol gradient centrifugation coupled to chemical cross-linking = “GraFix” Kastner et al (2008)

2) time-resolved sample conformation:
precise timing of mixing/spraying sample+ligand during to grid prep Berriman and Unwin (1994)

3) mixtures of low-abundance complexes absorb samples direct from polyacrylamide gel Knispel et al (2012)

4) Screening sample conditions (e.g. pH, additives) using thermal unfolding = “ProteoPlex” Chari et al (2015)

5) PEGylation of gold: better sample orientation distribution
Meyerson et al (2014)

# More complex samples

- larger dynamic, multi-component complexes
- large organelles/bits of cells
- cell sections
- whole cells

These biological entities are:

- large
- structurally unique and so their structures must be determined using **Electron Tomography (ET)**

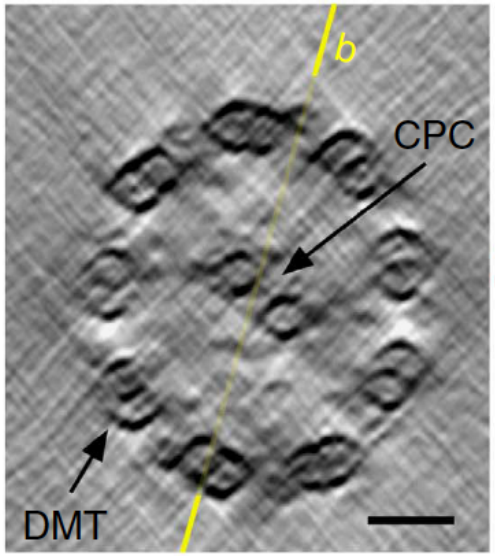
⬇

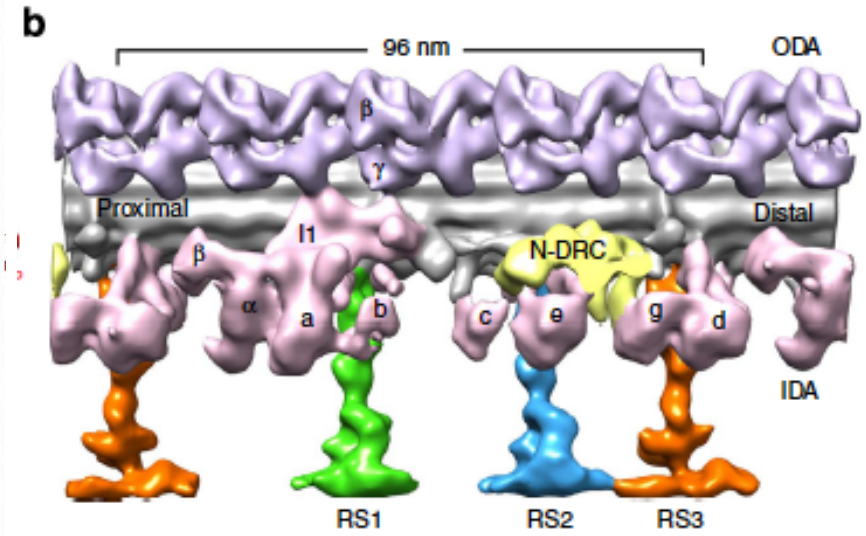
(Prof. Baumeister and Dr. Lu Gan……)

# More complex samples (contd.)

For samples <500nm, use conventional sample preparation techniques (Molecular ET)

- heterogeneous collection of particles – e.g. to get an initial model
- very big, poorly defined complexes
- very small cells (bacteria)




Human epithelial axonemes by cryo-ET
Lin et al (2014)

For ET samples

➔ need to consider whether fiducial markers (~10nm colloidal gold particles) need to be added for subsequent image alignment

➔ benefits of gold grids demonstrated Bharat et al (2015)

# Cellular EM (1)

Most samples will be too thick (>5μm) for conventional freezing to be feasible

1) **Chemical fixation**

Goal: chemical modification of cells to allow sectioning and observation

- Fix cells at room temperature using eg. glutaraldehyde
- Stain cells with eg. osmium tetroxide
- Dehydrate cells using up to 100% EtOH – takes hours/days
- Infiltrate cells with plastic which is subsequently polymerised using eg baking or UV irradiation
- Sections can be cut, ideally serial sections, applied to grids
- Additional staining, labelling, etc

- permeabilises and kills cells, chemically modified/potentially distorted
- cells shrink due to dehydration

# Cellular EM (2)

**2) High-pressure freezing-freeze substitution**

Goal: freeze cells so that intracellular structure is preserved;

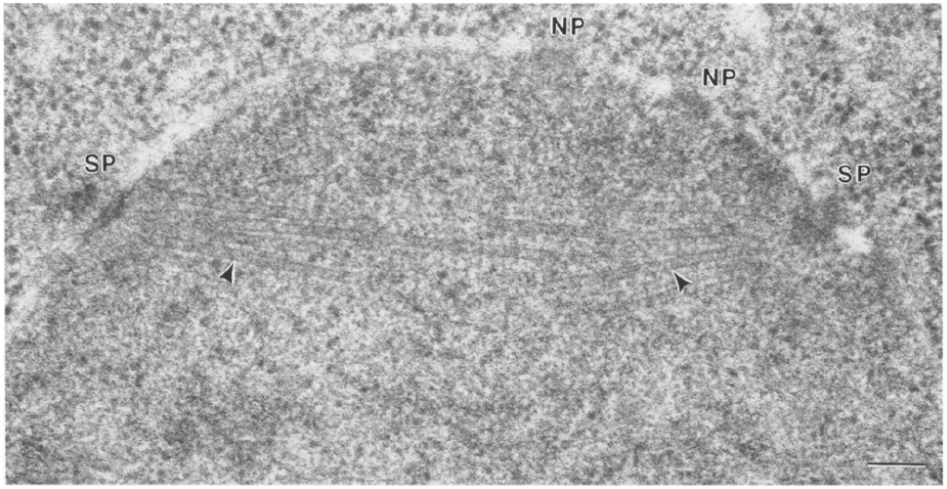
➔ rapid high pressure freezing prevents ice crystal formation

Infiltrate frozen sample for fixation/staining/dehydration for gentler cellular preservation

Resin embedding and sectioning follows

➔ cell shrinkage seen

Samples typically up to 200-300μm




(Winey et al., 1995)

# Cellular EM (3)

**3) High-pressure freezing-cryo-sectioning**

[otherwise known as Cryo-electron microscopy of vitreous sections (CEMOVIS)]

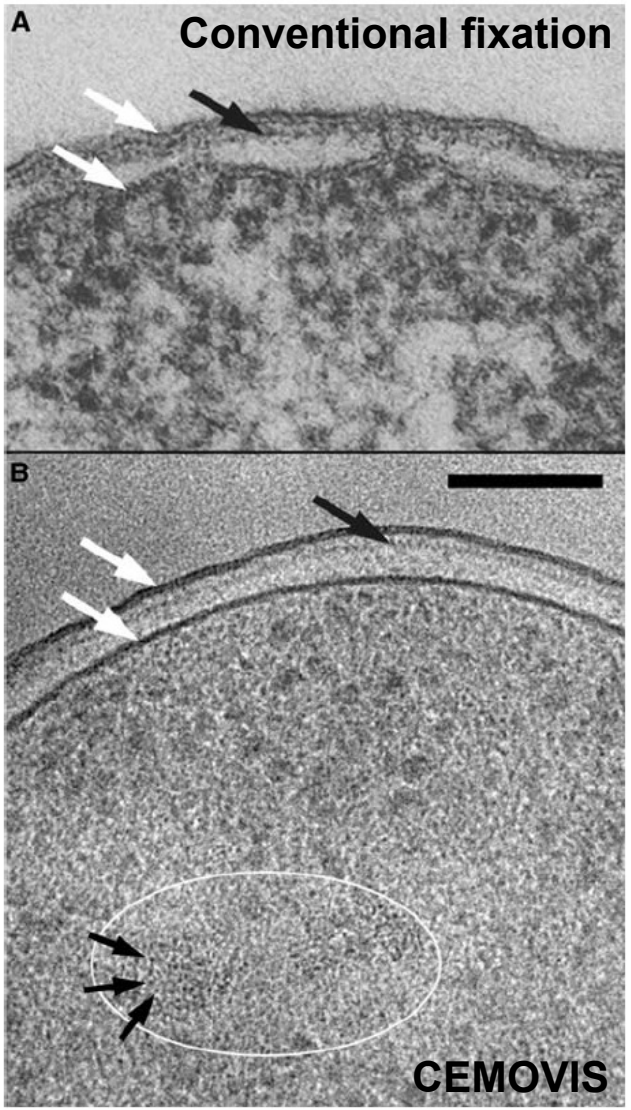
Goals: use no chemical fixation – cut thin sections from frozen-hydrated cells

Considerations:

- bit tricky

  sections must be maintained <-135°C
- artefacts can be introduced by cutting

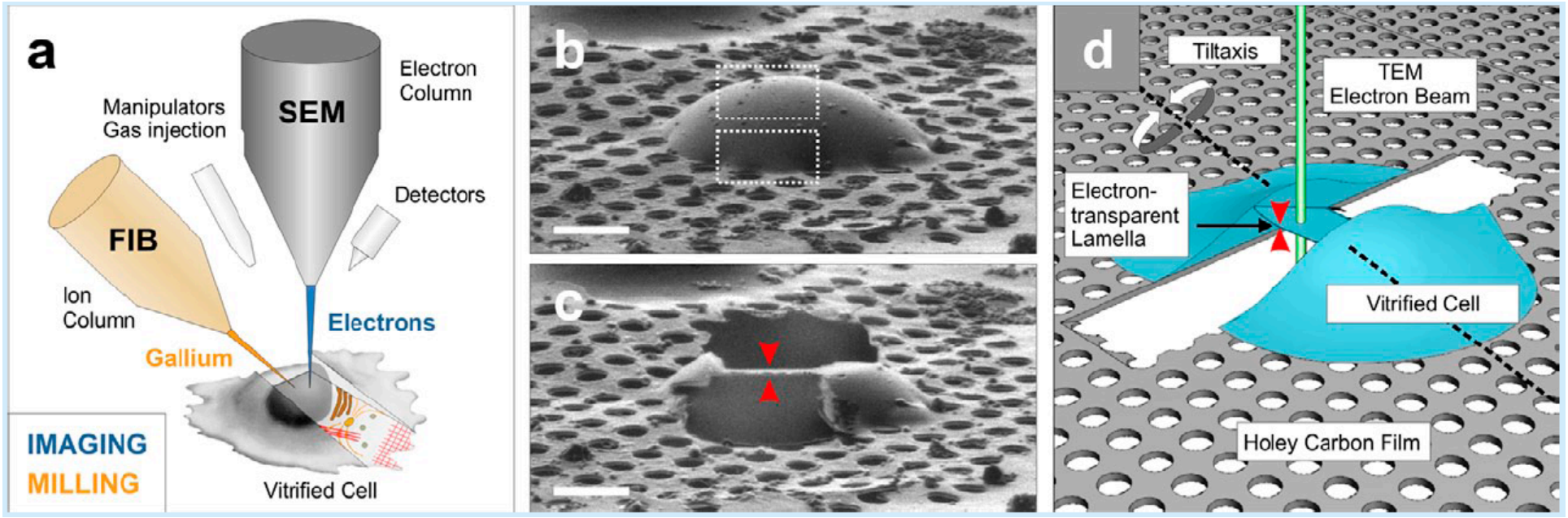
  (especially compression)
- no stain ➔ low contrast




(Al-Amoudi et al. 2004)

# Cellular EM (4)

## Cryo Focused ion beam (FIB) milling



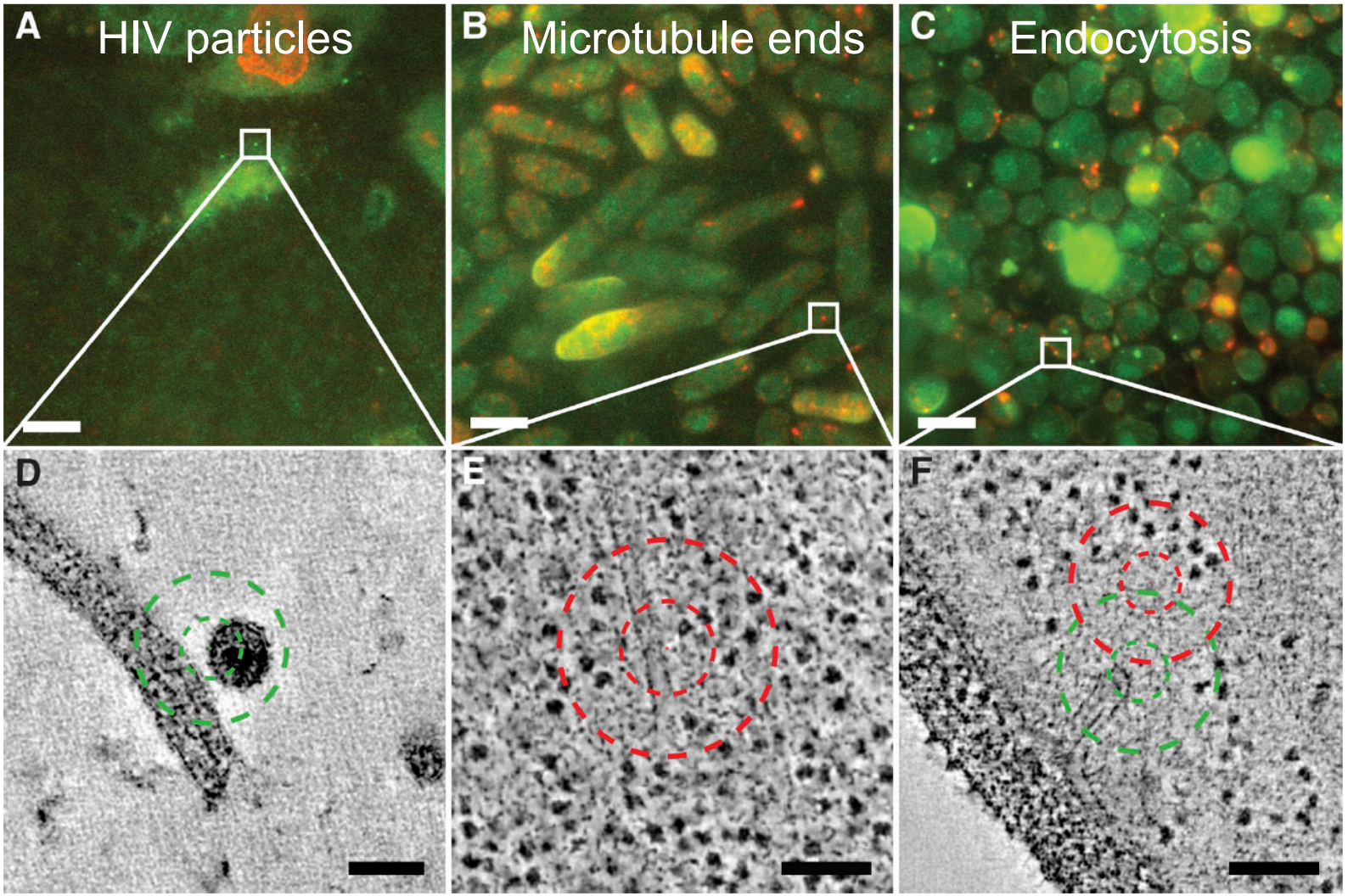

(Lučić et al (2013)

- Sample plunge frozen but too thick visualise
- Subject to cryo-FIB on top and bottom
- Perform cryo-tomography on remaining ~500nm slice

# Cellular EM (5)

## Correlated light and electron microscopy (CLEM)

- emerging approaches using super-resolution methods




( Kukulski et al., 2011)

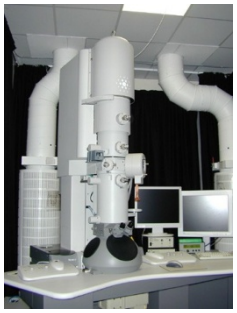
# Sample preparation for EM: The challenge

**Every sample is different**

"Don't waste clean thinking on dirty proteins"

Attributed to Arthur Kornberg

# EM preparation methods: The challenges

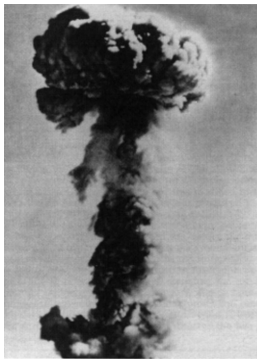


microscope column is a vacuum

minimise artefacts

make sample EM-ready

want to see something real



electrons are very damaging

want to actually see something

**Samples on the move**

# Suggested reading

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# Additional references - contd

Marr et al (2014) Fabrication of carbon films with ~500nm holes for cryo-EM with a direct detector device. J Struct Biol 185: 42-47
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