Lecture 1

Carolyn Moores

EM preparation methods

EM sample preparation Outline

1) Cryo-EM macromolecule sample prep: fundamentals

2) Practicalities and challenges
   - Typical work flow
   - Trouble-shooting

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

microscope column is a vacuum

minimise artefacts

make sample EM-ready

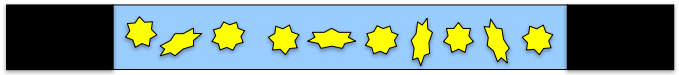
want to see something real

electrons are very damaging

Samples on the move

want to actually see something

Cryo-EM of macromolecules
The ideal



- **Thin layer of vitreous ice**
- **Some kind of support**
- **Single particles, all orientations**
- **Single particles, well distributed**

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Vitreous ice: a very brief history

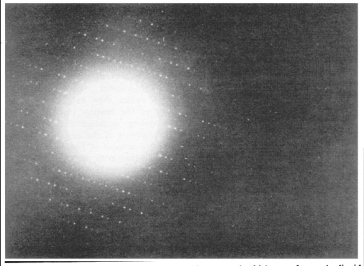

Fig. 1. Electron diffraction pattern of a catalase crystal which was frozen in liquid nitrogen and observed on a specimen stage cooled with liquid nitrogen. The resolution of the photographic reproduction is 4.5 Å, although that of the diffraction pattern on the original plate was 3.4 Å.

Taylor & Glaeser (1974)
Science 186, 1036

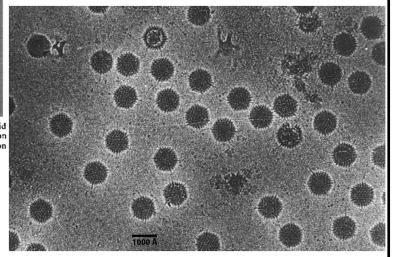

Fig. 1 Thin layer of vitrified suspension of unstained and unfixed adenoviruses type 2, pre-

Adrian et al (1984) Nature 308,32

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Samples for cryo-EM

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 10^5 - 10^6 K/s
Dubochet et al (1988) Quart Rev Biophys 21, 129
• Forms below ~-160° C at atmospheric pressure
• Thin (<3μm) sample is required for rapid freezing:
ice is a poor thermal conductor

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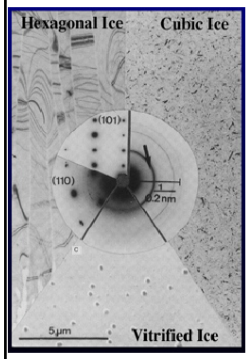
Cryo-EM
Preserving vitreous ice

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



• 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

Dubochet et al (1988) Quart Rev Biophys 21, 129

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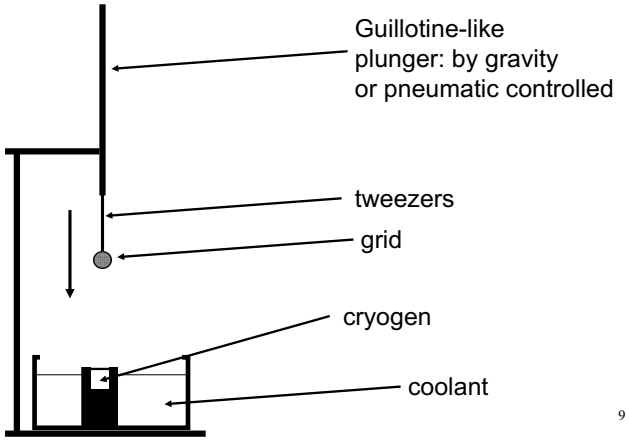
Cryo-EM
Basic vitrification set-up



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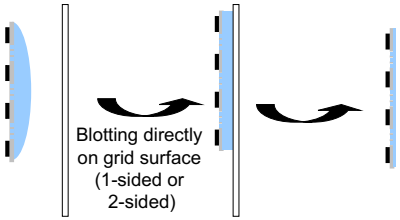
Cryo-EM
Single sided grid blotting



Aim:
• Obtain very thin layer of liquid slightly thicker than sample
Problem (1):
• very thin sample layer means rapid water evaporation
• @4° C 90% humidity, evaporation rate ~100Å/s ➔
- increase ionic strength
- cool sample
Solution:
• perform freezing in ~100% humidity @4° C

Passmore & Russo (2016) Meth Enzymol 579, 51

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Cryo-EM
The cryogen

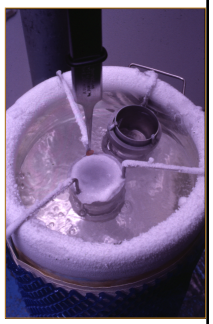
Liquid with melting point below ~-160° C;

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

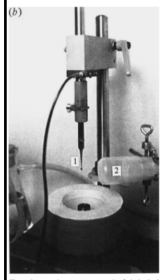
Ethane Boiling point: -88° C
melting point: -183° C

Propane/Ethane (67/33) Boiling point: -42° C
melting point: -196° C



Tivol et al (2008) Micro Microanal 14, 375

Plunging devices



Dubochet et al (1988)
Quart Rev Biophys
21, 129

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

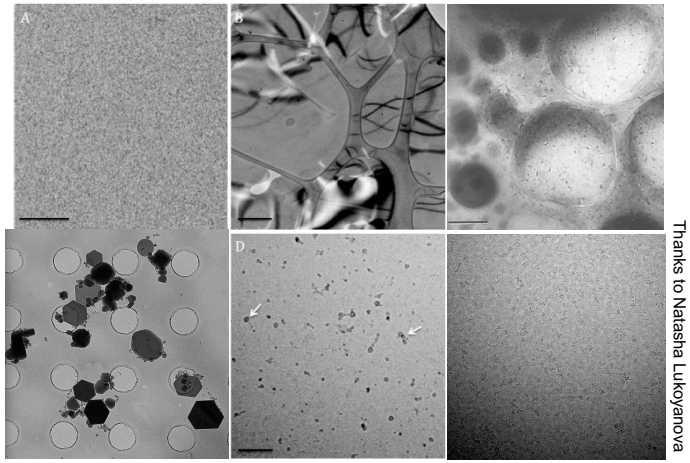
Table 2
Comparison of commercially available plunge-freezing devices.

Model	Humidity control in chamber	Temperature control in chamber	Automatic control of ethane temp	Blotting modality	Additional information
Leica EM GP	✓	✓	✓	Single sided	Optional stereomicroscope to monitor blotting
Gatan Cryo-plunge 3	× (Chamber set at 98%)	× (Operates at ambient temperature)	✓	Single and double sided	Removable humidity chamber
FEI Vitrobot	✓	✓	×	Double sided	Currently the most common plunge freezing device.

Thompson et al (2016) Methods 100, 3

Problems with ice

• Cubic • Hexagonal • Leopard
• Hexagonal • Vitreous • Ethane



Thanks to Natasha Lukoyanova

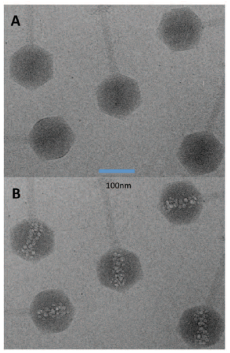
Thompson et al (2016) Methods 100, 3

Cyrklaff & Kuhlbrandt (1994) Ultramicros 55, 141

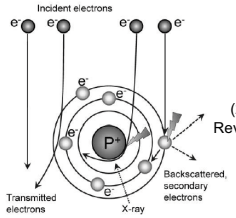
Cryo-EM

Vitreous ice vs electrons

Bubblegrams Reveal the Inner Body of Bacteriophage ϕKZ



Wu et al (2012) Science 335, 182



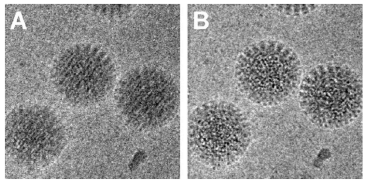
Orlova & Saibil (2011) Chemical Reviews 111, 7710

• Inelastic scattering deposits energy (radiolysis in ice and specimen)
➔ release of H2 gas

• Specimen motion
1) Charging
2) Beam-induced sample movement

Cryo-EM

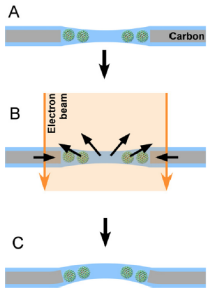
Sample perturbation ~simultaneous with sample imaging:
Beam-induced movement



BEFORE movement correction

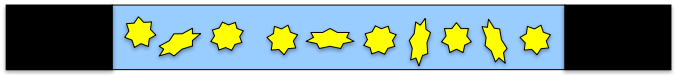
AFTER movement correction

Brilot et al (2012) JSB 177 630



- Electrostatic effects of beam
- Vitreous ice is an insulator - leads to charge accumulation
- Sample movement due to relief of internal stress: Differential contraction of ice vs support vs grid on vitrification

Cryo-EM of macromolecules
The ideal



- **Thin layer – as close as possible to dimensions of particle**
- **Cryo temperatures offer (a little) protection from radiation damage**
- **Sample moves**

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Cryo-EM samples: Possible problems

Preferred orientations

Hole edges preferred – possibly linked to ice thickness

Holes disfavoured

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Cryo-EM samples: Effects at the air:water interface

Cryo-electron microscopy of helical particles TMV and T4 polyheads

J Microscopy 140, 73 (1985)

by J. LEPAULT, *European Molecular Biology Laboratory,*

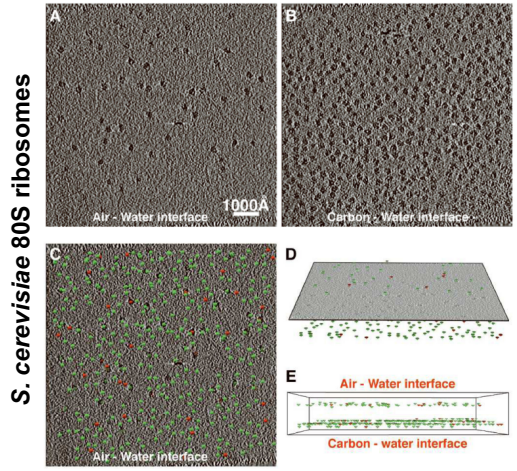
J Microscopy 161, 21 (1991)

Interfacial energies and surface-tension forces involved in the preparation of thin, flat crystals of biological macromolecules for high-resolution electron microscopy

by R. M. GLAESER*, A. ZILKER†, M. RADERMACHER†, H. E. GAUB†, T. HARTMANN‡ *and* W. BAUMEISTER‡, **Division of Biophysics and Cell*

- There is time for all particles to move to air water interface during grid prep
- Is the blotting paper itself part of the problem?
- May be indicated by preferred orientations

Effects from the air:water/substrate/water interface



Bharat & Scheres (2016) Nat Protoc 11, 2054; Noble et al (2018) eLife 7, e3457; D'Imprima et al (2019) eLife 8, e42747

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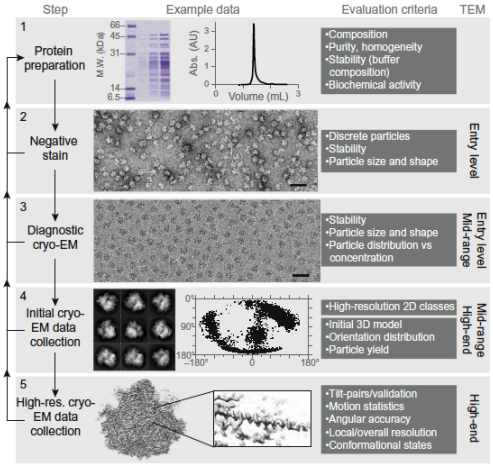
Practicalities & challenges



AIMS

- Good particle distribution
- Good range of particle orientations
- Low background
- Minimise sample movement
- Multiple iterations will be needed

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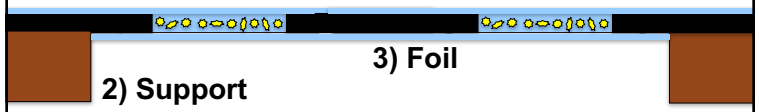
Passmore & Russo (2016) Meth Enzymol 579, 51

Practicalities & challenges



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Sample

- establish conditions for sample stability before cryo-EM
- 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
- need more sample than for negative stain (~3-5mg/ml)

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) Quart Rev Biophys 49, e13

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Challenging biological samples require specialised handling

e.g. 1) Screening sample conditions (e.g. pH, additives) using thermal unfolding = "ProteoPlex" Chari et al (2015) Nat Meth 12, 859

2) dynamic, multi-protein complexes:
glycerol gradient centrifugation coupled to chemical cross-linking = "GraFix" Kastner et al (2008) Nat Meth 5, 53

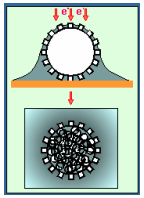
3) membrane protein stabilisation – can include detergents, amphipols, lipid nanodiscs, SMALPs e.g. Sgro and Costa (2018) Front Mol Biosci 5, 74

4) time-resolved sample conformation (see also later):
precise timing of mixing/spraying sample+ligand during to grid prep Berriman and Unwin (1994) Ultramicro 56, 241

Negative stain (1)

What is it?
- sample is adsorbed to continuous carbon support film
- sample is surrounded by heavy metal solution, which is air-dried
- 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



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Ohi et al (2004) Biol Proced Online 6,23

Negative stain (2)

Pros:
- Quick (~5mins)
- need only a small amount of sample ~<0.1mg/ml
- good contrast
- helpful for initial info re sample homogeneity
- 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
- continuous carbon can distort sample

Strongly recommended for a new sample

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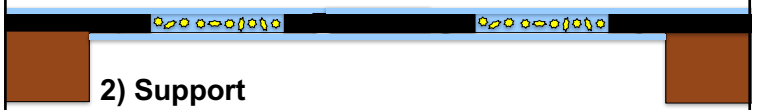
Practicalities & challenges

1) Sample



2) Support

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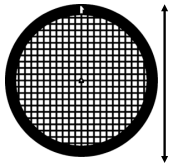
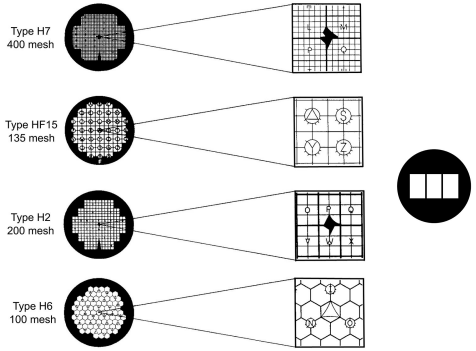
EM grids



3mm

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



www.agarscientific.com

Practicalities & challenges

1) Sample

3) Foil

2) Support

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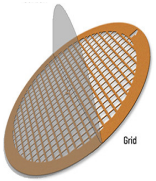
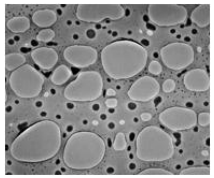
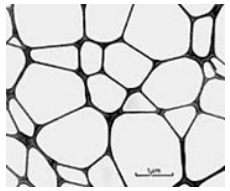
Carbon film



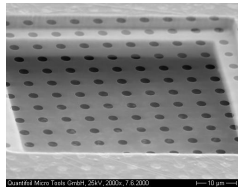
home-made (irregular)



- **Inert, strong, electron transparent**
- **(Somewhat) electrically and thermally conductive**
- **Cheap**



lacey

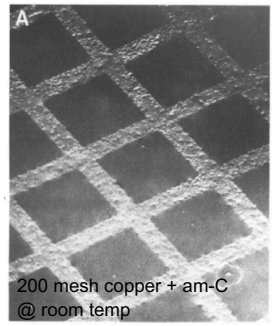


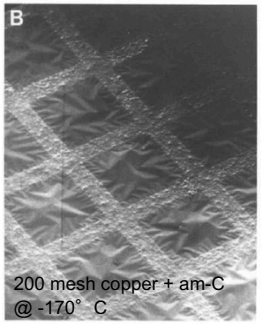
Quantifoil or C-flat

EM automation

Carbon film + copper

= potential problem: "cryo crinkling"

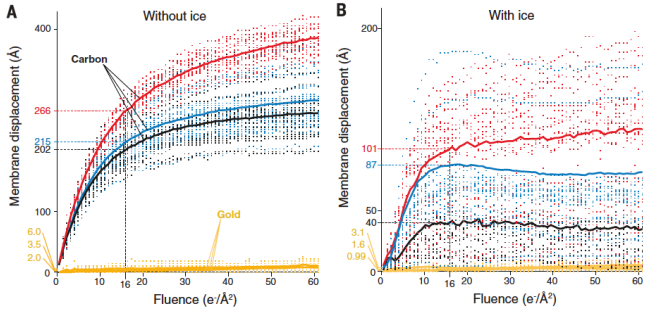




- Differences in thermal expansion coefficient
- Potential source of support movement on illumination

Booy & Pawley (1993) Ultramicro 48, 273

Carbon moves under the electron beam



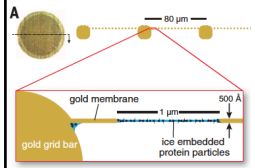
Russo & Passmore (2014) Science 346, 1377

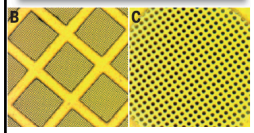
- Carbon becomes a semi-conductor at LN2 temperatures

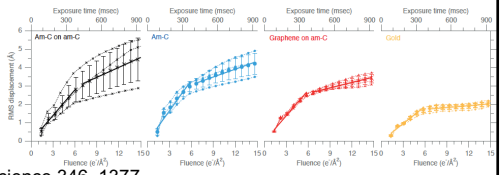
➔ charge accumulation and instability on illumination

Ultrastable gold substrates for electron cryomicroscopy

Christopher J. Russo and Lori A. Passmore*
12 DECEMBER 2014 • VOL 346 ISSUE 6215 1377
SCIENCE sciencemag.org







- **Uniform electrical conductivity and thermal contraction**
- **Minimal beam-induced movement**
- **Inert, non-toxic**

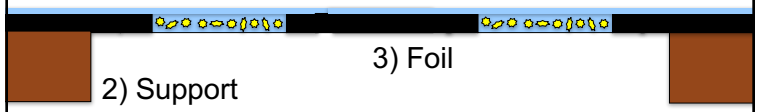
>> consider sample spreading
>> can't use with a phase plate

Russo & Passmore (2014) Science 346, 1377
Russo & Passmore (2016) JSB 193, 33
Russo & Passmore (2016) COSB 37, 81
Passmore & Russo (2016) Meth Enzymol 579, 51

Practicalities & challenges

4) Foil treatment

1) Sample



3) Foil

2) Support

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Foil treatment (1)

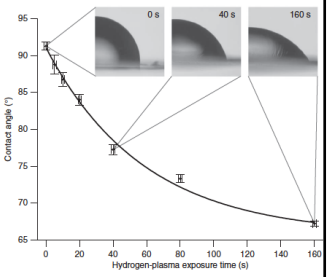
"details of the surface chemistry.. are poorly understood"

- **spreading**
- **adsorption/orientation**

• plasma cleaning (oxygen, hydrogen, argon)

• glow discharge (*consider having 2*)

Atmosphere	*Charge*
air	-
methylamine	+
methanol	-

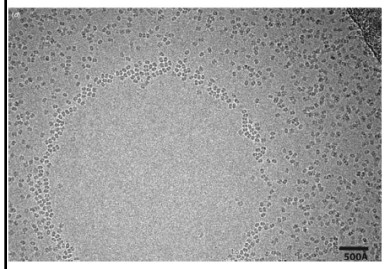


Russo & Passmore (2014) Nat Meth 11,649

• treatment with UV Burgess et al (2004) J Mol Biol 147, 247

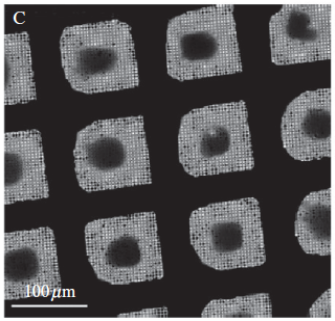
• treatment with electrons Miyazawa et al (1999) J Mol Biol 288, 765

Cryo-EM samples: Possible problems: examples



Human erythrocyte catalase (240kD)
Ice thickness affects sample orientation

Vinothkumar & Henderson (2016) Quart Rev Biophys 49, e13
See also Drulyte et al (2018) Acta Cryst D74, 560

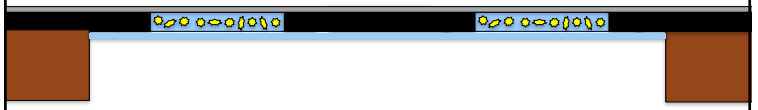


When plasma cleaning fails… Dense ice in grid square centre

Dobro et al (2010) Meth Enzym 481, 63

Foil treatment (2)

Additional films may help with
a) low sample concentrations;
b) particle distribution
c) orientation
d) air water interface?



- **3-4 nm additional amorphous carbon/graphite**
  - Adds background to images
  - Grid properties may vary during carbon preparation: suggested to make large batches for reproducibility
  - Carbon is a semi-conductor at LN2 temperatures

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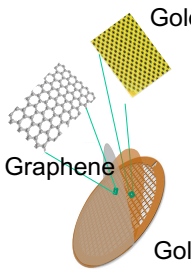
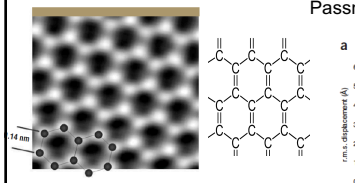
Foil treatment (3): Graphene



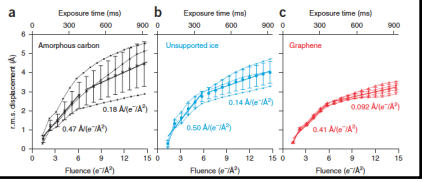
- atomically thin (0.34nm)
- high mechanical strength
- high conductivity at all temperatures
- featureless down to resolution of ~0.2nm
- hydrophobic
- low-energy hydrogen plasma treatment

➔hydrophilic

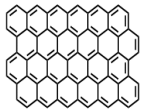
Pantelic et al (2011) JSB 174, 234
Sader et al (2013) JSB 183, 531
Russo & Passmore (2014) Nat Meth 11, 649
Passmore & Russo (2016) Meth Enzym 579, 51



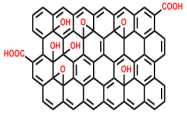
http://www.emsdiasum.com/microscopy/default.asp



Graphene vs Graphene Oxide


Graphene

oxidation
H_2SO_4,
H_2O_2
$KMnO_4$



Graphene oxide

Graphene	Graphene Oxide
Hydrophobic	Hydrophilic
High conductivity	Low conductivity
0.34 nm thickness	~ 1 nm thickness
Extremely low background (> 2.13 Å)	Lower background than am-C

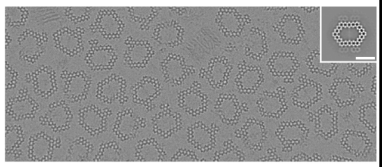
Pantelic et al (2010) JSB 170, 152
Bokori-Brown et al (2016) Nat Commun 7, 11293
Palovcak et al (2018) JSB 204, 80
https://figshare.com/articles/Graphene_Oxide_Grid_Preparation/3178669 (Thomas Martin @LMB)

Summary of other approaches

- Detergents (below CMC): protein specific: reported to help with distribution and orientation (e.g. 0.085mM dodecyl maltoside; Lyumkis et al (2013) Science 342, 1484); digitonin, 0.05% (v/v) Tween 20: Fernandez-Leiro et al (2015) eLife 4, e11134
- Ni-NTA lipids Kelly et al (2008) JMB 382, 423
- Use affinity grids or antibodies reviewed in Earl et al (2017) COSB 46, 71
- Functionalised graphene oxide Wang et al (2019) bioRxiv http://dx.doi.org/10.1101/657411doi:
- PEGylation of gold: better sample orientation distribution Meyerson et al (2014) Sci Rep 4, 7084

• make a DNA cage to put protein in middle Martin et al (2016) PNAS113, E7456-E7463

• If you're stuck with preferred orientations, tilt Tan et al (2017) Nat Meth 14, 793

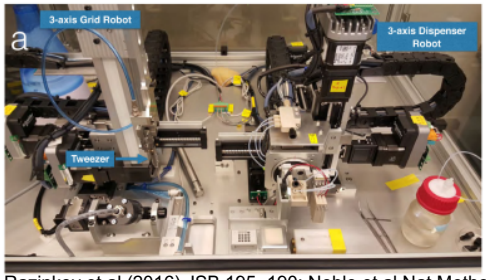


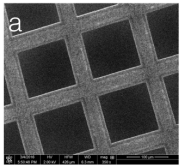
Effects at the air:water interface

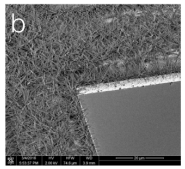
Potential solutions: freeze faster

E.g. 1: Self-blotting grids + ink jet robot (Spotiton)

- dispenses pl sample volumes (less wasteful)
- multiple samples per grid

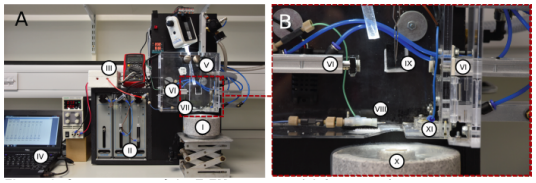




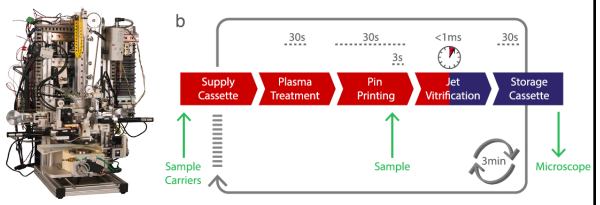


Razinkov et al (2016) JSB 195, 190; Noble et al Nat Methods (2018) 15, 793

Potential solutions: freeze faster



E.g. 2: Stopped-flow-based rapid mixing/spraying/vitrification
Kontziampasis et al (2019) bioRxiv doi: http://dx.doi.org/10.1101/563254.



E.g. 3: Deposit sub-nanoliter sample volume, vitrify with ethane jet
Ravelli et al (2019) bioRxiv doi: http://dx.doi.org/10.1101/651208.

EM sample preparation

1) Cryo-EM macromolecule sample prep: fundamentals
2) Practicalities and challenges
   - Typical work flow
   - Trouble-shooting

The challenge:

Every sample is different

More complex samples for cryo-ET

➡ Kay Grunewald, Julia Mahammid

Suggested reading

Dubochet, Adrian, Chang, Homo, Lepault, McDowall, Shultz (1988) Cryo-electron Microscopy of vitrified specimens. Q. Rev. Biophys. 21: 129-228
Grassucci, Taylor, Frank (2007) Preparation of macromolecular complexes for cryo-electron microscopy. Nat Protoc 2: 3239-3246

Methods in Enzymology Volume 481, Pages 2-410 (2010)
Cryo-EM Part A Sample Preparation and Data Collection
Edited by Grant J. Jensen
ISBN: 978-0-12-374906-2

Methods in Enzymology Volume 579, Pages 2-445 (2016)
The Resolution Revolution: Recent Advances In cryoEM
Edited by R.A. Crowther
ISBN: 978-0-12-805382-9

Common negative stains

Stain	Mw		% in soln; working pH
Phosphotungstic Acid (PTA)	3315.5	0.8-0.9nm grain	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	0.4-0.5nm grain	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

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

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