Cryo-Electron Microscopy

Sarah Butcher, Helsinki University

Principles of cryo-em

One of the cherished goals of biological electron microscopy is to be able to examine artefact-free preparations, in which the morphology and chemistry of the observed specimen bears a close resemblance to its in vivo state. Unfortunately, during most of the history of electron microscopy this idea has not been achieved: in the case of specimens suspended in liquids for example, damage is caused during 'conventional ' preparation procedures by drying, adsorption onto the support film and by contrasting, staining media. The structural consequences of dehydration alone are potentially catastrophic.

Dramatic developments in the field of cryo-electron microscopy during recent years has changed this perspective. An early indication of the potential value of cryopreparations in conjunction with high resolution imaging was provided by (Taylor and Glaeser, 1974) who demonstrated by electron diffraction that 0.3 nm resolution could be preserved in frozen-hydrated catalase crystals. A further crucial development was the discovery that liquid water could, under certain circumstances, be cooled in to a vitreous (i.e. glass-like) solid without the formation of structurally disruptive crystals. Hence the path was paved for the examination of favourable specimens embedded in the most natural substance of all: non-crystalline water. This talk will concentrate on the methodology and high resolution transmission electron microscopy of 'vitrified thin film' specimens.
The key details of this technique are

1. In non-cryoprotected specimens, successful vitrification can only be achieved in thin aqueous films, preferably < 1μm thick. Specimen preparation is however rapid and easy.
2. The advantages of cryoEM are already apparent at 110 K, so that liquid nitrogen can be used as a coolant for the microscope parts. However, liquid helium is used in some microscopes.
3. All the necessary hardware (cold stages and cold-transfer devices) is commercially available.
4. Vitreous ice and amorphous ice are not synonymous. In amorphous ice, minute ice crystals not resolvable by direct imaging can be juxtaposed. The vitrified state is defined by the nature of the electron diffraction pattern.

Electron microscopy of thin vitrified films can be divided into 4 major procedural steps:

1. forming a thin layer of pure water, solute solution or materials in aqueous suspension;
2. rapidly cooling the films to the vitreous state;
3. transferring to the EM without re-warming above the devitrification temperature (140 K);
4. observing the specimen, in which the state of the water has been characterised by electron dffraction, below 140 K with an electron dose low enough to preserve vitrified water.

Preparation of vitrified thin aqueous films

Vitrification of aqueous solutions and suspensions (of, for example, viruses and fibrous proteins) can be achieved in practice if the specimen size is reduced sufficiently to facilitate high cooling rates. Specimens which are small enough to allow vitrification are fortunately most suitable for EM. In forming a thin aqueous film it is necessary to overcome the tendency of water to minimize the surface-to-volume ratio (a consequence of its high surface tension). Maintaining the fragile thin fluid layer long enough for it to be solidified by freezing is also a difficulty.

In practice the most common method used is the following:
Thin films (50-300 nm, preferably < 200nm) are produced on thin carbon films suspended over electron microscope grids (200-400 mesh). Depending on the sample, the support film may be perforated ((Fukami and Adachi, 1965). The key factor is the surface property of the support film. Carbon support films tend to be hydrophobic and must be rendered strongly hydrophilic. This can be done by glow discharging for 10 seconds in an alkylamine atmosphere at a pressure of 100 Pa, or in air.

After glow discharging, a drop of aqueous solution (~ 3 μL) is pipetted onto the hydrophilic film, with the grid held in a pair of forceps mounted on a guillotine suspended over a liquid cryogen (liquid nitrogen-cooled ethane or propane) bath. Most of the liquid is removed from the drop by blotting

briefly with a piece of dry filter paper. The grid is then plunged into a cryogen and transferred to liquid nitrogen using pre-cooled forceps. The choice of liquid cryogen should ensure that gas formation at the specimen-coolant interface (film boiling) should be minimal and heat transfer maximal. So liquid cryogens are selected for their low melting points and high boiling points. The formation of a stable gas layer around the specimen tends to insulate it from the coolant; for this reason liquid nitrogen is not an efficient primary cryogen for high resolution studies. In addition, a good coolant should have good heat conduction, high heat capacity, high fluidity at the low temperature required, high density, be safe to use and inexpensive. Apart from their explosive nature, ethane and propane fulfill these requirements very well. One should avoid using liquid nitrogen that has been exposed to the air since this will cause heavy ice contamination.

The vitrified specimen may be stored under liquid nitrogen or transferred carefully to a TEM cold stage. The cold stage has a nitrogen dewar which keeps the specimen stable at liquid nitrogen temperatures during imaging. One disadvantage of placing a cold stage into the high vacuum of the microscope column is that any free moisture will accumulate onto this cold surface. Hence over a period of a few hours a thick layer of contaminating ice will accumulate on the specimen, making it gradually unusable. To reduce this problem, a second cold surface is present in the column – an anticontaminator, that is also cooled by liquid nitrogen, enclosing most of the tip of the cold stage, and trapping free molecules. One common design is that of the double-bladed anticontaminator (Homo *et al.*, 1984)

Structure of ice

The structure of ice at normal or low pressure can take three forms. Hexagonal ice (I_h) consists of large (μm) crystals. Cubic ice (I_c) appears as a powdery mosaic of small crystals with average dimensions ~0.1 μm that cannot be detected by electron diffraction when < 30 nm. Vitreous or amorphous ice (I_v) is smooth and featureless, but possesses a number of diffuse diffraction rings with well-defined radii and widths. These three phases can be distinguished by their general appearance and more definitely by electron diffraction. A material is said to be in the vitrified state if first, it is amorphous, and secondly, if it suffers a phase transition leading to a crystalline state when warmed above the devitrification temperature. The form and size of the ice within a specimen depends on the rate of cooling experiences by the specimen. It is possible to compromise the specimen severely after freezing because under certain circumstances, phase transitions occur from I_v to I_c and from I_c to I_h.

Imaging frozen hydrated specimens

Imaging frozen hydrated specimens is difficult. The main problem is that frozen hydrated specimens are low in contrast. The density of the specimen is e.g. 1350 kg m^{-3} for protein and that of vitreous ice is 930 kg m^{-3}. However, the signal-to-noise ratio is good because there is no stain present to contribute to the noise, there may be no support film and the specimen is relatively undamaged. Contrast in the electron microscope is a mixture of amplitude contrast and phase contrast (Lecture B). Amplitude contrast dominates in the case of large objects, and phase contrast becomes increasingly important for smaller objects until it becomes almost the only source of contrast for small organic objects less than about 6nm in diameter. To make the very best use of phase contrast, micrographs of frozen-hydrated specimens must be recorded with large defocusing values, because at Gaussian focus the image contrast is at a minimum. Enhancing phase contrast by underfocusing the objective lens results in a loss of resolution for certain spatial frequencies (depending on the degree of defocus). Accurately defocusing by a predetermined amount is a prerequisite for good imaging. The TEM is thus calibrated so that defocus values are converted into decrements of objective lens current.

Beam damage

Once a specimen has been vitrified, what is the influence of electron radiation on the specimen? Can it withstand irradiation under conditions that permit high resolution imaging? Does the presence of water make the organic specimen more or less sensitive to beam damage? Beam damage is reduced around liquid nitrogen temperatures, liquid helium temperatures (10 K) increase radiation resistance about 2 fold compared to liquid nitrogen.

Low dose imaging

All organic specimens are potentially subject to damage during irradiation in the EM. The damage can be subtle structural changes induced by relatively mild electron exposures that produce significant artefacts in the context of high resolution structural studies. Or at higher doses may result in significant mass loss. Hence radiation damage limits resolution. The electron dose required to produce a high resolution image is in the order of 100 $e^- nm^{-2}$. A subminimal dose or 'safe' dose in EM is considered to be near the lower end of the 5-100 $e^- nm^{-2}$ range (where 6.25 $e^- nm^{-2} = 1 C m^{-2}$ and the usual dose for aligning a column is about 62000 $e^- nm^{-2} \equiv 4 \times 10^5$ Mrad). Three points should be noted,

1. Every doubling of the image magnification results in a 4 x increase in the exposure dose at the specimen plane.
2. Radiation damage is proportional to total dose (i.e. the total number of electrons delivered per unit area of a specimen) and not to the rate at which it is delivered. Thus a short exposure to a high intensity is as destructive as a prolonged exposure to a milder intensity.
3. Radiation damage is inversely proportional to the accelerating voltage.

In practice, low dose imaging requires one to restrict the electron dose suffered by a specimen in the region of interest exclusively to that needed to expose the photographic plate or CCD, and therefore to eliminate dosing the chosen region during field selection, focusing and other optical manoeuvres. The most common method to achieve this, is using beam tilting. It does not require the specimen stage to be moved to bring the region to be photographed into the beam axis, and it permits focusing and astigmatism correction to be performed at high magnifications on an off-axis region of the specimen which nevertheless lies immediately adjacent to the region of interest. For effective low dose imaging, firstly the microscope must be accurately aligned. Secondly, in search mode, the region of the specimen to be photographed and a similar, adjacent area to be used for focusing are selected at low magnification (x 2000). Once the area of interest is selected, the beam is shuttered to prevent further damage to the specimen. Thirdly, the focus area is illuminated at high magnification (x 100000) for critical focusing using the extinction of background granularity. The beam is again shuttered. One then defocuses by a known amount to provide optimal phase contrast. Fourthly the magnification is reduced (x 20000 to x 50000) for photography on the region of interest. Photography should be performed at the lowest tolerable magnification to ensure the lowest possible electron dose. All modern electron microscopes are now routinely equipped with software for carrying out lose dose imaging.

An approach used to considerable effect for the high resolution imaging of radiation sensitive objects is signal averaging. This is applied most effectively to periodic structures and entails acquiring noisy images at low exposures well below the critical damage level and then combining them in a computer to yield an acceptable image (image processing lectures).

Uses

Even within the resolution range that is commonly now achieved with single particle methods (1/40 to 1/15 $Å^{-1}$) reconstructions of macromolecular assemblies allow very detailed models to be designed which serve as a framework for functional interpretations. Improvements in methodology are bringing that range repeatedly now to 7Å where secondary structure begins to be recognised. For the case of two-dimensional crystals, this is now the accepted norm.

The vitrified thin film method has been used to obtain new morphological descriptions of the high resolution structures of viruses, membranes, ribosomes, actin, membrane channels and various other organic macromolecules. The technique has been extended to work with thicker specimens by the advent of high pressure freezing, and adapted by the introduction of cryoprotectants to increase the preservation of antigens and ultrastructure during the preparation of tissues and cells for sectioning.

The rapid preparation makes this a suitable technique for time- and temperature-resolved studies on the millisecond time scale, where it has been used to study microtubule dynamics (Chretien *et al.*, 1992), the photocycle in bacteriorhodpsin (Subramaniam *et al.*, 1993), low pH induced changes in viruses (Fuller *et al.*, 1995), and the conformational changes involved in protein folding by chaperonins (Rye *et al.*, 1999) to name but a few. Reactions maybe carried out under controlled conditions on the grid, or even activated during the fall of the grid into the cryogen by a flash of light, or the spraying of a chemical onto the sample.

Besides yielding information on quaternary structure, low-resolution maps of large macromolecular assemblies have provided powerful constraints for the phasing of X-ray data. Examples include the

ribosome and several icosahedral viruses. Quantitative comparison of data from cryo-electron microscopy and image reconstruction with X-ray crystallography have shown excellent agreement throughout the molecule. If one combines the two techniques then it is possible to resolve very large structures to atomic resolution. Often components of a macromolecular assembly are suitable for X-ray crystallography, but the entire assembly may be too large, flexible or variable. If its image is obtained at low resolution by EM, the X-ray can be fitted into the EM with high positional accuracy, giving a quasi-atomic resolution model.

A 55-gold atom cluster (Nanogold) bound to a single maleimide site had been used to specifically link to exposed cysteine residues. The scattering density of this gold cluster is high enough to outweigh the contribution to the EM image of a projected thick (200-300 Å) protein mass. Hence it has been successfully used in cryo-EM reconstructions to for instance map the substrate protein to the cavity of GroEL. This is an example of a general technique called difference imaging, where two reconstructions are compared to see if a feature present in one, but not in the other, can be identified. Other examples include the use of antibody labelling, omitting a particular protein through the use of mutants, or by biochemical treatment of an assembly to remove certain components. In addition cofactors and ligands may be added to see if they cause conformational changes, or can actually be localised.

Further reading, information sources

Cryo electron microscopy:

1 Chretien, D., Metoz, F., Verde, F. and Karsenti, E. (1992) Lattice defects in microtubules: protofilament numbers vary within individual microtubules. *Journal of Cell Biology*, **117**, 1031-1040.

2 **Dubochet, J., Adrian, M., Chang, J.J., Homo, J.C., Lepault, J., McDowall, A.W. and Schultz, P. (1988) Cryo-electron microscopy of vitrified specimens. *Quart. Rev. Biophys.*, **21**, 129-228.

3 Fukami, A. and Adachi, K. (1965) A new method of preparation of a self-perforated micro-plastic grid and its application. *J. Elec. Microsc. (Japan)*, **14**, 112-118.

4 Fuller, S.D., Berriman, J., Butcher, S.J. and Gowen, B.E. (1995) Low pH induces swiveling of the glycoprotein heterodimers in the Semliki Forest virus spike complex. *Cell*, **81**, 715-725.

5 Homo, J.-C., Booy, F., Labouesse, P., Lepault, J. and Dubochet, J. (1984) Improved anticontaminator for cryo-electron microscopy with a Philips EM 400. *J. Microscopy*, **136**, 337-340.

6 **Roos, N and Morgan, A. J. 1990. Cryopreparation of thin biological specimens for electron microscopy: methods and applications. Royal Microscopical Society Microscopy Handbooks 21, Oxford University Press. (have in library)

7 Rye, H.S., Roseman, A.M., Chen, S., Furtak, K., Fenton, W.A., Saibil, H.R. and Horwich, A.L. (1999) GroEL-GroES cycling: ATP and nonnative polypeptide direct alternation of folding-active rings. *Cell*, **97**, 325-38.

8 Subramaniam, S., Gerstein, M., Oesterhelt, D. and Henderson, R. (1993) Electron diffraction analysis of structural changes in the photocycle of bacteriorhodopsin. *EMBO J*, **12**, 1-8.

9 Taylor, K.A. and Glaeser, R.M. (1974) Electron diffraction of frozen, hydrated protein crystals. *Science*, 186, 1036-1037.

Principles of electron microscopy and image processing:

http://em-outreach.sdsc.edu/web-course/toc.html

10 Frank, J. (1996). Three-dimensional electron microscopy of macromolecular assemblies. Academic Press (have in library)

** Excellent source of information