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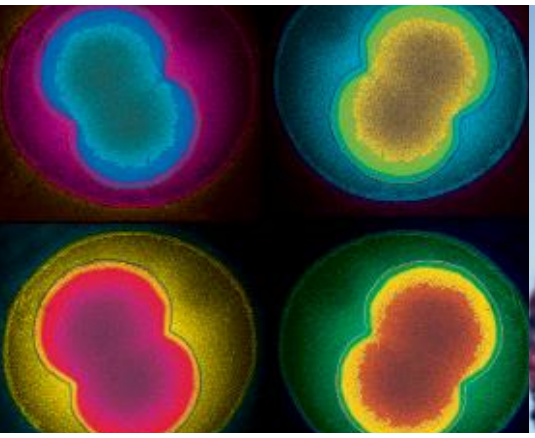
Good Practice Guide

Cryogenic Sample Preparation for Vacuum Based Metrology Instruments



November 2018

EMPIR
EURAMET
The EMPIR initiative is co-funded by the European Union's Horizon 2020 research and innovation programme and the EMPIR Participating States

MetVBadBugs

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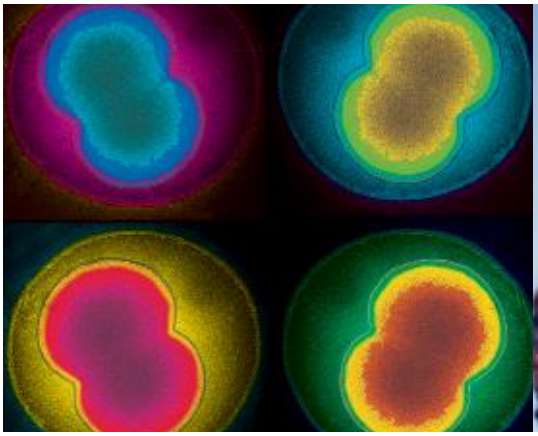

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MetVBadBugs

This information is also available on the http://empir.npl.co.uk/metvbadbugs/ website

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Alternative format versions of this report are available on request from [Kirsty.MacLellan-Gibson@nibsc.org].

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Forward

Water is an essential component of life; our bodies are approximately 65% water . The numerous cellular and molecular processes that are life are conducted in the liquid water of the body. [1]. However, this liquid water is not directly compatible with many of the analytical and structural methodologies of today. Over the last 60 years advances in the field of electron microscopy have addressed this issue and it is now possible to routinely observe fully hydrated biological samples in the high vacuums required by electron microscopes. This good practice guide aims to introduce the main concepts of how to prepare biological samples for metrology without greatly affecting their ultrastructure through the disruption caused by the removal of water from the sample.

Scope of this Guide

The scope of this guide is to provide information about cryo-fixation for vacuum-based metrology methods. These cryogenic sample preparation methods are essential to enable ‘liquid’ (vitrified) water to be present in the vacuum of high performance metrology instruments, without ultrastructural biological sample reorganisation and translocation of exo/endogenous molecules. Within this guide we will discuss both the theory behind and practical implementation of getting stable ‘liquid’ water into a vacuum. The information contained within this guide is aimed at allowing individuals to be able to produce reliable results, which are comparable across different laboratories and to ensure that the method is correctly applied.

The guide does not aim to cover every possible permutation of cryogenic sample preparation but aims to act as a source of information for the individual. Out of scope techniques for this guide are anywhere the sample enters the vacuum of the metrology instrument at a temperature greater than 136K (-137°C), or any sample which has had the liquid water removed prior to entry into the vacuum of the metrology instrument. This guide will not cover the methods of slam freezing, self-pressurised freezing or progressive lowering of temperature. The guide will, however, cover cryo-fixation by high pressure freezing and plunge freezing.

Definitions

Cryo-fixation – a technique to fix or stabilise biological components within a sample prior to analysis or further processing.

Vacuum based metrology – any metrology method where the sample is analysed in a vacuum, i.e. electron microscopy or NanoSIMS.

High pressure freezing – rapid cryo-fixation of specimens at ~2000 bar pressure and at liquid nitrogen temperatures (-196 °C).

Plunge freezing - rapid cryo-fixation of specimens by plunging into a liquid cryogen at temperatures lower than -137 °C.

Amorphous water ices – referring to any of the non-crystaline amorphous states of water; hyper-quenched glassy water (HGW); amorphous solid water (ASW); high density glassy water (HDG); low density (LDA), intermediate density (IDA), high density (HDA) and very-high density amorphous ices (VHDA).

LDA – low density amorphous ice with a density of 0.94 g x cm^{-3}

HDA - high density amorphous ice with a density of 1.17 g x cm^{-3}

Vitreous water – non-crystalline amorphous solid form of water, usually referring to LDA and HDA forms.

Cryoprotectant – any substance that protects the sample from freezing damage caused by ice crystals or that can direct the sample towards vitrification, rather than crystallisation.

Introduction

In an ideal world analysis of a biological specimen should be performed on a sample which is identical to that found in nature; in practice however, biological samples need to be prepared to ensure that the analysis can be performed reproducibly under conditions not usually conductive to life. Inevitable this means some form of fixation or immobilisation of the sample is required. The ideal sample preparation will result in preservation of the specimen with no damage caused by the preservation methodology. Historically the main problem facing preparation of biological specimens for high resolution metrology is the paradox that water contained within and around biological specimens is not miscible with the vacuum required for high resolution metrology.

Leading the field in the area of biological sample preparation for vacuum-based methodologies is sample preparation for electron microscopy, initially with dehydration of biological samples [2] and then substitution of the liquid water for a curable resin [3, 4]. In the 1960's Fernandez-Moran proposed that 'significant advances in the study of life processes under the conditions of minimum perturbation will depend on a large extent on the further development of the unique potential inherent in the low temperature domain' [5]. By the 1980's two groups had managed to successfully immobilise water in a liquid state and analyse it in high vacuum metrology systems: X-ray diffraction and differential thermal analysis or electron microscopy [6, 7]. This leap forward in sample preparation paved the way for the preparation of fully hydrated biological specimens [8], which are still employed today. Below is a brief introduction to the water molecule and its properties that allowed these ground-breaking methodologies to be developed.

Pure water

There are many resources that explain in great the many remarkable properties of water. This guide is not such a resource, but it will provide a brief description of water and the some of the important properties it has. It is worth noting that pure water and water within a biological system will behave in a different manner and that sample preparation techniques for one will not necessarily work for the other.

Pure water is a simple molecule comprised of one oxygen atom and two hydrogen atoms (Figure 1a). There are 10 electrons within the water molecule arranged as five electron pairs; of these, one pair stay with the oxygen molecule; two pairs make the OH bonds and there are two lone pairs, which are free to form hydrogen bonds. These electron pairs are spaced equally about the nucleus in a tetrahedral arrangement. All 10 electrons are drawn towards the oxygen nucleus leaving the hydrogen nucleus partially denuded of electrons giving it a positive charge and the oxygen a slight negative charge. The presence of these charges on the oxygen and hydrogen atoms causes different water molecules to attract each other forming a hydrogen bond (Figure 1 Diagram of Water MoleculeFigure 1b). This tetrahedral structure in liquid water is transient with hydrogen bonds lasting in the order of milliseconds [9]. The tetrahedral clusters are found with decreasing frequency with increasing temperature and not within the vapour form; they are however found extensively within the crystalline forms.

Figure 1 Diagram of Water Molecules

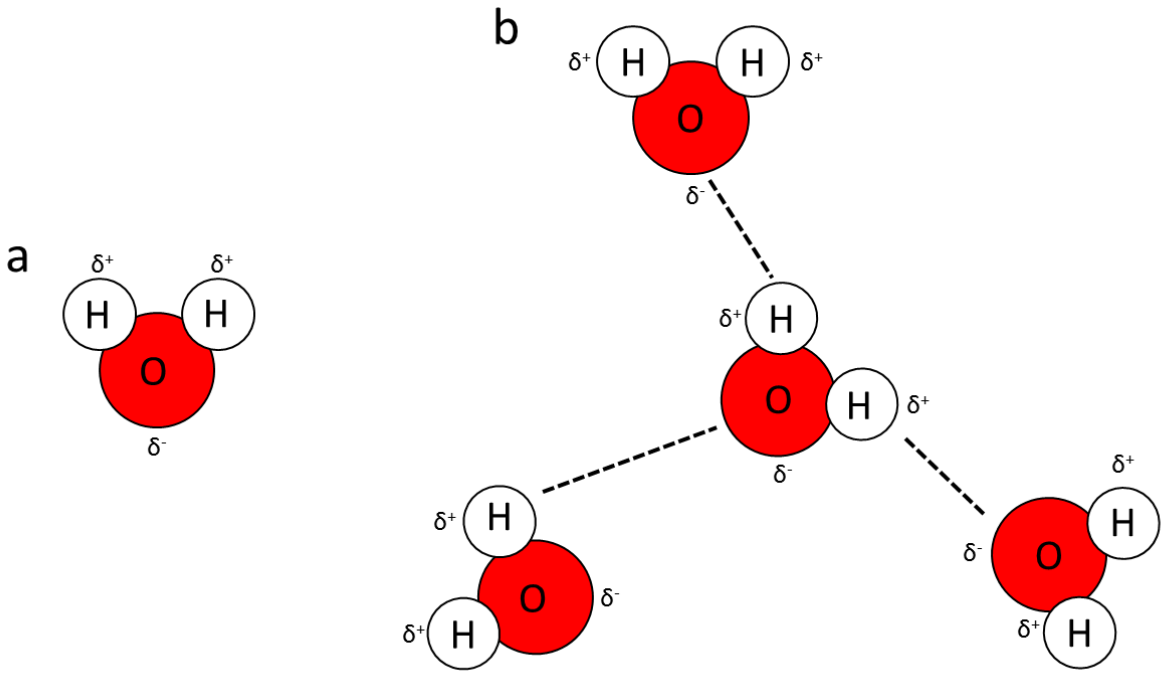

Figure 1a, representation of the v-shaped water molecule comprising of one oxygen atom (red) and two hydrogen atoms (white) showing negative charge (δ-) for the oxygen atom and the positive charges for the hydrogen atoms (δ+); b, arrangement of water molecules in the liquid phase, dotted lines represent hydrogen bonds between the negatively charged oxygen atoms and the positively charged hydrogen atoms.

As well as the vapour and liquid phases of water there are approximately 18 crystalline numerous non-crystalline glasses have been described: hyper-quenched glassy water (HGW); amorphous solid water (ASW); high density glassy water (HDG); low density, intermediate density, high density and very-high density amorphous ices (LDA, IDA, HDA and VHDA respectively). The amorphous ices LDA (0.94 g x cm^{-3}) and HDA (1.17 g x cm^{-3}) are of particular interest to us as they are similar in density to liquid water [10].

Low density amorphous ice is formed by very rapid cooling of liquid water into a glassy state at atmospheric pressure; the cooling rate needs to be greater than 10^5 K s^{-1} [11] it can also be created by warming HDA or high pressure Ice VIII to ~-150 °C under atmospheric pressure. LDA is readily achieved in the lab using either plunge freezing or high pressure freezing. Melting of LDA into crystalline ices occurs at approximately -137 to -117 °C [12-15].

High density amorphous ice is formed by submitting low density ices (hexagonal, cubic and XI) or LDA to high pressure and is stable at -196 °C under atmospheric pressure. This form may be created by high pressure freezing but rapidly transforms to LDA upon slight warming, such as removal transfer through vapour phase from high pressure freezer to liquid nitrogen.

Aqueous systems

Whilst biological specimens are largely comprised of liquid water, they contain many different dissolved components, which alter the freezing properties of the cell. Research carried out at EMBL in the 1980's lead to the development of a method to vitrify pure liquid water and to transfer

it to the vacuum of an electron microscope [7]. This then rapidly lead to the application of this methodology to aqueous solutions and biological samples [8, 16].

Complete vitrification of the water within a biological specimen is essential as the formation of ice crystals within a cell can cause disastrous effects on ultrastructure [17]. Freezing cells using low cooling rates (such as in a freezer or dry ice) can cause in phase separation between water and the solutes within the cell (resulting in increasing concentrations of organic matters and salts as the forming ice crystals remove water) it may even lead to ice crystals poking through cell membranes or destroying organelles [18]. These tissues when thawed are nothing but mush. Biological material frozen in the presence of anti-freeze solutions (DMSO, sucrose, glycerol etc…) readily survive slow freezing, however there are often ultrastructure changes induced by the anti-freeze used [18]. In order to prevent these issues, the water within the biological sample needs to be immobilised even without cryoprotectants [19]. The section below describes the main ways to achieve this goal of creating vitreous water within biological samples.

Vitrification of biological samples

The likelihood of successfully vitrifying a sample is greatly enhanced by the addition of cryoprotectants, increasing pressure, reduction of sample size and by increasing cooling rate. Below we will consider each one in turn and how to practically achieve this in the lab.

Cryoprotection

Cryoprotection is a way in which the sample can be directed towards vitrification, rather than crystallisation. Vitrification can be further enhanced when the free water is diluted by the addition of another component, as this can depress the free energy and promote vitrification; however, these components are not always compatible with biological materials, ones that are, are referred to as cryoprotectants. [20]. Cryoprotection of the sample can increase depth of freezing within thicker samples and offer a supporting media for suspension samples. Careful choice of a cryoprotectant will allow you to perform your chosen metrology on your prepared samples with no impact from the cryoprotectant, either through damage to the sample or masking of signal by the cryoprotectant itself. Cryoprotectants are divided into two classes, extracellular or intracellular. Extracellular cryoprotectants, ones which do not penetrate the biological sample, are often comprised of sugars: sucrose, dextrans and Ficoll; biological components, cold water fish gelatines, yeast pastes or serum albumins; or carbon rich compounds such as 1-hexadecene. Intracellular cryoprotectants include: Glycerol, methanol, ethanol, dimethyl sulfoxide and ethelene glycol. The idea cryoprotectant should be:

- Effective at heat transfer. Water tends to turn to crystalline ice when frozen, the process of crystallisation produces heat (latent heat of fusion), which slows cooling of surrounding sample. Additionally, ice is a poor conductor of heat and any present within the sample will reduce cooling rates, which in turn will increase the likelihood for further ice crystal formation.
- Should not affect the biological sample, nor cause structural alterations to the sample.
- Should be physiologically compatible with the sample, i.e. no osmotic effects.
- Should have cryoprotective properties, i.e. prevent water from forming into ices.
- Should be compatible with the metrology method the samples are to be analysed with.

Addition of cryoprotectant can extend freezing to 400 µm in the case of high pressure freezing or can surround suspensions of biological material for plunge freezing [21].

Increasing pressure

The application of high pressure, 210 MPa (2048 bar) to liquid water reduces the cooling rate required to vitrify water from 10^4 K s^{-1} to 10^2 K s^{-1} [22-24]. Commercially available high-pressure freezers operate at this pressure and are suitable for vitrifying small samples a few hundreds of micrometres thick. Thicker samples are more difficult to vitrify as the heat does not dissipate from the centre of the sample quickly enough [25], so a combination of approaches is often needed i.e. high pressure freezing in the presence of a cryoprotectant.

Decreasing sample size

Sample size, specifically sample depth greatly effects the success of vitrification. The thinner the sample the better the vitrification, maximal cooling rates of 10^8 K s^{-1} can be achieved by plunge freezing into an appropriate cryogen (liquid ethane or propane). However, vitrification depth is usually only a few micrometers from the surface [17, 26]. The shallow depth of vitrification achieved by this method is due to water being a poor conductor of heat, resulting in much slower cooling of the sample, which in turn results in the formation of ice crystals [19, 27, 28].

Increasing cooling rate

For full vitrification of the sample at ambient pressures a cooling rate of 10^5 K s^{-1} is required [25, 29]. By increasing the cooling rate, close to that of the theoretical maximum (10^8 K s^{-1}) improved vitrification of the sample can be observed.

Techniques

Plunge freezing

Plunge freezing is the ideal technique for liquid suspensions of material i.e. bacteria, viruses or small proteins in solution; small cells and organisms can also be frozen with this technique but samples that are too thick (<1um) may not fully vitrify. The plunge freezing techniques used to vitrify thin films of samples. This requires a careful selection of liquid cryogen: specifically one that is liquid at a temperature below -137°C; that does not cause a Leidenfrost effect (such as liquid nitrogen, which boils so rapidly an insulating layer of gas is trapped between the sample and the cryogen, thereby reducing the cooling rate and preventing required cooling rates to be achieved) and that has a great difference between melting and boiling points, allowing a large range of plunging `temperatures to be achieved. Below is a list of suitable cryogens, the top three cryogens, based on the points above are preferable.

Table 1 Potential Cryogens

Cryogen	Melting point °C (K)	Boiling point °C (K)	Temperature difference (melting – boiling points) °C/K
Ethane	-183.45 (89.7)	-88.75 (184.4)	-94.7
Propane	-189.85 (83.3)	-42.25 (230.9)	-147.6
Isopentane	-161 (112)	27.8 (300.9)	-188.8
Helium	-270.95 (2.2)	-268.95 (4.2)	-2
Hydrogen	-259.05 (14.1)	-252.75 (20.4)	-6.3
Methane	-182.65 (90.5)	-164.15 (109)	-18.4
Nitrogen	-210.05 (63.1)	-195.85 (77.3)	-14.2

Table 1 list of potential cryogenic liquids for use in plunge freezing, light blue highlighting indicated commonly used cryogens.

In order to produce a vitreous film, the sample needs to be rapidly cooled in the liquid cryogen. Using liquid ethane as an example plunge freezing can be achieved by. Condensing ethane gas in a liquid nitrogen cooled (-196 °C) container, which is then held at ~-170 °C. A thin film of the sample is rapidly plunged, usually by a guillotine, into the liquid ethane to vitrify the sample, achieving a cooling rate of 10^5 °C/s.

High pressure freezing

High pressure freezing is used when samples are too thick for plunge freezing, such as whole cells, tissue biopsies and small organisms [30]. Larger volumes of liquid suspensions can also be frozen, as can combinations such as bacterial biofilms and planktonic bacteria. Commercially available high pressure freezers enable the vitrification of samples up to 500µm thick (though these often require the addition of cryoprotectants to achieve this). The high pressure freezers on the market today achieve the greater depth of vitrification by increasing the pressure of the sample to greater than 2000 bar, whilst lowering the temperature to that of liquid nitrogen. This all occurs in a few milliseconds [31].

Verification of vitreous states

In an ideal world each sample that you prepare will be fully vitrified, however this is rarely the case and achieving 100% successful vitrification of every sample is unrealistic. Therefore, before using a sample in an experiment verification of vitrification is recommended. The methods below are not guarantees that the sample is fully vitrified all the way through, as it is possible to have localised regions of poor or incomplete vitrification; it is also possible to warm the sample during transfers between machines, causing a previously good sample to be destroyed.

Visual inspection of sample

The simplest method of checking the vitreous states, is a visual inspection with the naked eye. This method will provide a rapid check to see if there are any major problems with the sample but will not allow accurate indications of the vitreous state of the material. Samples with good vitrification should appear glassy and either black (if in a well) or transparent if alone (Figure 2a), samples that contain crystalline material appear cloudy, cracked and or crystalline with either small or large ice crystals (Figure 2 b, small, and c, large). In this example only, the sample in Figure 2a would be suitable. This can be confirmed by inspecting in a cryo SEM and looking for crystalline material. Plunge frozen material is more difficult to assess by eyeballing and will require visual inspection in a cryo-electron microscope to identify any areas of crystalline material (Figure 2d). However, if it is not possible to perform electron microscopy to verify the state of the material samples may need to be used without any verification of their vitreous states.

Figure 2 Visual verification of high pressure frozen material

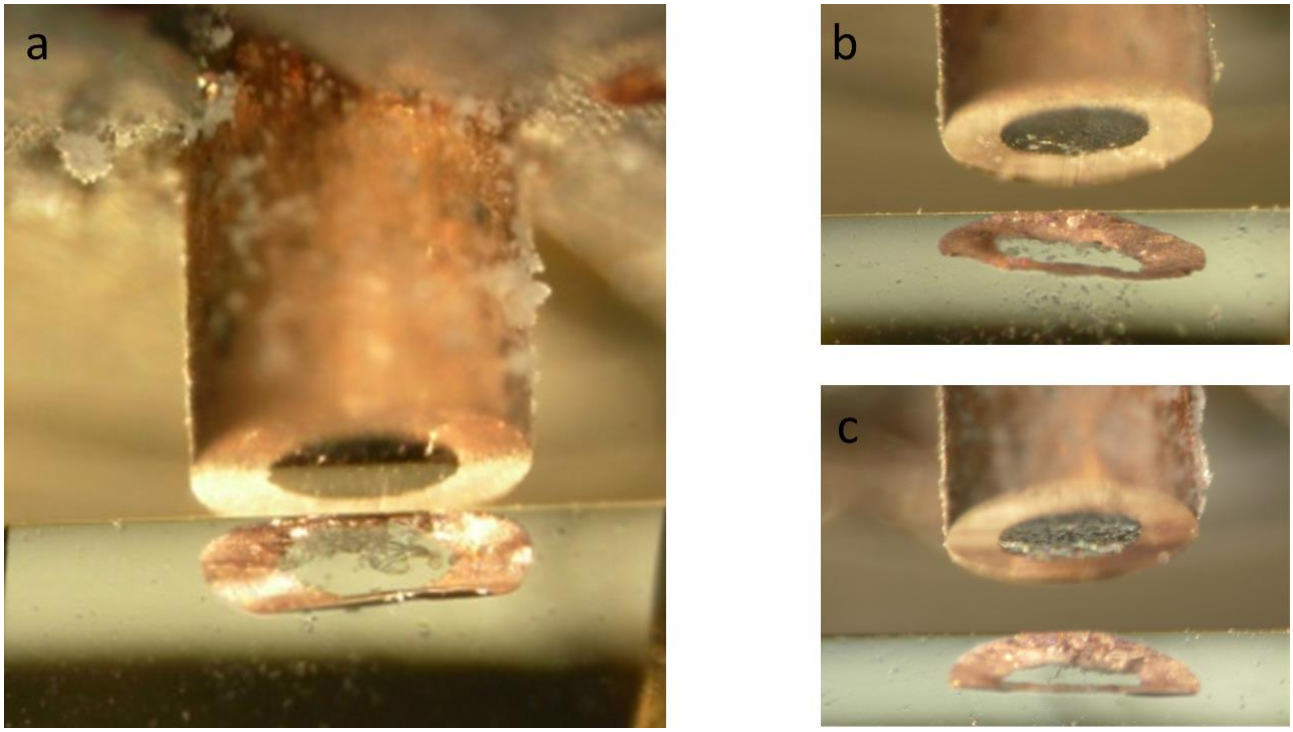

Figure 2a, high pressure frozen dextran solution in a copper tube showing smooth glassy black surface indicative of vitreous material (arrows); b, high pressure frozen dextran solution in a copper tube showing textured black surface indicating the presence of small ice crystals; c, high pressure frozen dextran solution in a copper tube showing large ice crystals throughout. The high pressure frozen dextran solutions in b and c are not vitreous.

Electron diffraction

A more thorough method of identifying well vitrified samples is to perform electron diffraction (x-ray diffraction may also be performed if it is available). High pressure frozen samples will need to be prepared to allow imaging in a cryo-transmission electron microscope to allow confirmation of vitrification through the depth of the sample, or for surface verification in a cryo-scanning electron microscope. Examples of vitreous and crystalline material and their diffraction patterns are shown in Figure 3. Vitreous material (Figure 3a) has a distinct diffraction pattern with diffuse rings of diameter 0.37 and 0.21 nm [16]. Cubic ice has a set of sharp rings (with the strongest at 0.366nm diameter) indicative of a powder diffraction (Figure 3b and c). Hexagonal ice (Figure 3d), by contrast, appears as a set of punctate dots corresponding to the crystal lattice.[16]. Material which shows a crystalline diffraction pattern should be discarded; it is recommended to look at a couple of areas to confirm vitrification as sometimes there are localised regions of crystallisation in a sample such as around vacuoles or air bubbles.

Figure 3 Electron diffraction of vitreous and crystalline material

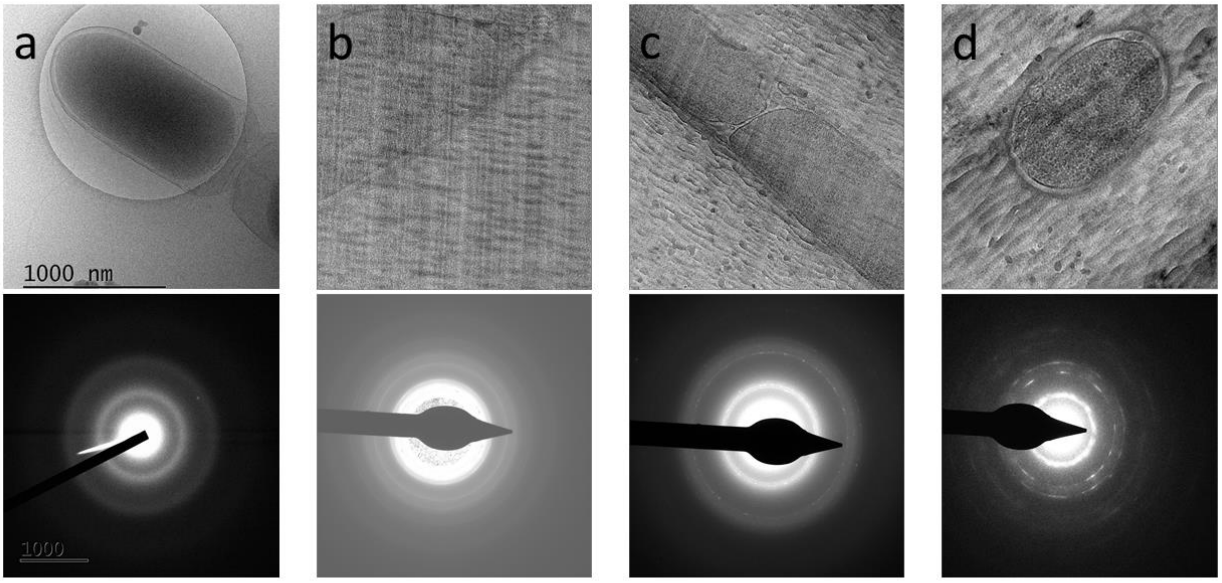

Figure 3a, Cryo-electron micrograph of a vitreous plunge frozen bacteria and its corresponding electron diffraction pattern; b, Cryo-electron micrograph of non-vitreous CEMOVIS section of bacteria and its corresponding electron diffraction pattern, showing a powder diffraction profile; c, Cryo-electron micrograph of non-vitreous CEMOVIS section of bacteria and its corresponding electron diffraction pattern, indicating the sample contains many small crystals; d, Cryo-electron micrograph of non-vitreous CEMOVIS section of bacteria and its corresponding electron diffraction pattern, indicating the sample contains many large crystals.

Protocols

Correct sample preparation is key to getting the best results from sample vitrification, the all too familiar adage of *rubbish in, rubbish out* is high applicable for sample vitrification. There are a few key rules to getting the best out of your sample.

General Rules

Use the best sample you can

The samples used must be in optimal conditions to minimise damage and the formation of artefacts, as well as to get the best freezing and ultrastructural preservation form them. Stresses on the sample may result in poor freezing and non-reproducible results. Ideally tissues should be freshly harvested and sitting in a physiological buffer/media at the correct temperature. Cultured cells in suspension should be taken at log phase growth (unless the experimental design dictates otherwise) and kept at an appropriate temperature in a physiological buffer/media, if cells are concentrated care should be taken not to damage them during this process (see below). Small live organisms, such as worms, embryos, larvae, eggs etc… should be kept at optimal conditions until the last minute.

Work quickly

Working quickly enables your sample to be preserved in as near to native state as possible. Concentration of the sample and mixing with cryoprotectants may introduces stresses to the biological material, which may cause artefacts with in the sample. As stated by McDonald ‘A well-frozen dead or sick cell is not worth of study’ [21].

Do not let samples dry out

In general sample volumes for both high pressure freezing, and plunge freezing are less than one microliter. Under mid to low humidity conditions these small volumes will rapidly dry out it may be necessary if the sample loading is particularly onerous working in a humidified environment (moist chamber or regularly moistening your sample) will minimise any drying effect.

Avoid water

Water and other aqueous medias can crystallise and slow down or stop the transfer of heat from the centre of the sample. The addition of a suitable cryoprotectant can minimise the presence of free water surrounding the sample and negate this effect.

Avoid damage

Mechanical damage caused by poor cutting techniques, blunt tools or improper dissection techniques will damage the cells. Always use the sharpest tools for sample cutting; disposable biopsy needles or punches are useful, if available. Damage may occur through overfilling on specimen holder resulting in crushing of the sample, it is better to have a smaller sample surrounded than cryoprotectant than a crushed specimen. For suspension cultures damage may occur through over zealous centrifugation, preferable a maximum speed of 3000rpm should be used or a gentle vacuum filtration may be used. Shear stresses form pipetting the sample will also cause mechanical damage so good pipetting techniques should be employed.

Avoid air bubbles

Air acts as an insulator during freezing, thus preventing the rapid transfer of heat from the sample that is essential to optimum vitrification. Air bubbles are likely to collapse under pressure, which can cause shear stresses on the sample and negatively affect sample vitrification.

Use small volumes

The thinner the sample the better the chance of complete vitrification. Samples of over 400µm are difficult to vitrify even in the presence of a cryoprotectant.

Use appropriate specimen support

Using a specimen support or sample holder that is not optimised for rapid heat transfer will result in sub-optimal freezing of the sample. Most supports are made up of copper, aluminium, sapphire or gold to allow rapid transfer of heat from the sample into the cryogen. Specimen supports made from an insulating material i.e. plastics, ceramics or glass will retard the heat transfer and prevent successful vitrification.

Keep samples and tools below devitrification temperature

It goes without saying that if you have managed to produce a vitreous sample that you want to keep it that way. Ensuring that the sample is correctly transferred from the freezing apparatus and stored until required and that all tools which it comes into contact with are adequately cooled will prevent the sample devitrifying. In general, keeping the sample under liquid nitrogen, or at temperatures lower than -137°C will be sufficient to prevent this.

High pressure freezing

Sample preparation for suspension cultures (planchettes)

Clean type A or B planchettes in a small beaker with a few millilitres of 100% ethanol in a sonicating water bath, remove the planchettes to dry on clean filter paper. Commercially available planchettes have well depths of 100 µm and 200 µm (type A) or 300 µm (type B). Gently pellet cells by centrifugation (3,000 rpm maximum) or if growing on agar plates or in monolayer's by scraping (this can be done in the presence of a small volume of cryoprotectant or PBS). Motile cells and small organisms can be concentrated by filtration in a sterile vacuum filter unit and scraping the concentrated cells from the filtration membrane. Pipette ~1µl of the cell suspension into a well of the planchette and assemble as a sandwich (Figure 4). Freeze according to high pressure freezer protocols for your high-pressure freezer.

Figure 4 Sample loading for HPF of cell suspension

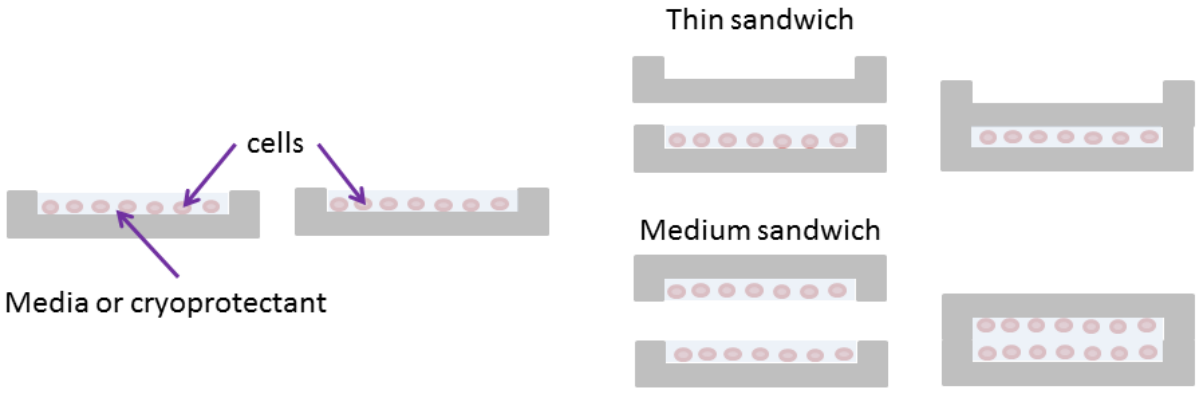

Figure 4 schematic to show how to prepare planchette sandwiches of cell suspensions for high pressure freezing.

Sample preparation for suspension cultures (tubes)

Tube systems can be used for some vacuum based metrologies, where sections of the material are required. It is important to note the internal diameters of the tubes as not all cells will fit inside them. Clean copper or gold tubes in a small beaker with a few millilitres of 100% ethanol in a sonicating water bath, remove the planchettes to dry on clean filter paper. Commercially available tubes have internal diameters of 350 µm (copper) or <200 µm (gold or cellulose). Prepare samples as above. Pipette ~10µl of the cell suspension onto a clean surface and submerge one end of the tube into the cell suspension. Place a piston (narrow gauge wire) into the tube and suck the suspension into the tube, remove tube and piston and repeat for the opposite end to ensure that no air bubbles are trapped (Figure 5). Note that if cellulose capillary tubes are used they will need to be frozen in a planchette surrounded by a cryoprotectant (such as 1-hexadecene). Freeze according to high pressure freezer protocols for your high-pressure freezer.

Figure 5 Sample loading for HPF of cell suspension

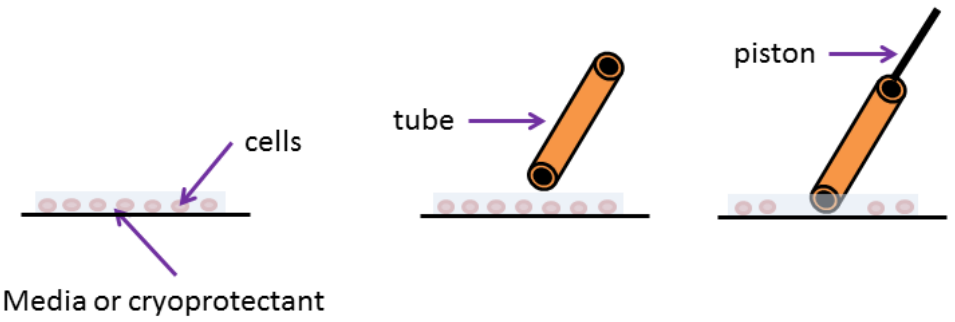

Figure 5 schematic to show how to prepare copper tubes filled with cell suspensions for high pressure freezing.

Sample preparation for adherent cells

Clean type A and B planchettes in a small beaker with a few millilitres of 100% ethanol in a sonicating water bath, remove the planchettes to dry on clean filter paper. Gently remove the growth media from the adherent cells by aspiration (Note: it is easiest to use a 1ml automatic pipette with the tip cut, reduce shear stress). Wash the cells gently with media twice then twice with cryoprotectant or filler of choice and remove the sapphire for assembly into a sandwich for high pressure freezing (Figure 6). Place a cleaned type A planchette 100 µm side up and fill it with cryoprotectant or filler of choice, place the sapphire biofilm side down onto the cryoprotectant or filler of choice and place the flat face of a type B planchette on top of the sapphire. Note some people might find it easier to place the sapphire on the flat planchette first, cells face up, then transfer the sapphire and the planchette together. Freeze according to high pressure freezer protocols for your high-pressure freezer.

Figure 6 Sample loading for HPF of adherent cells

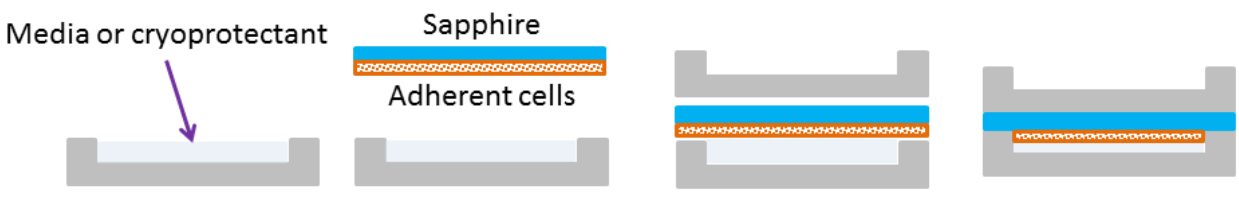

Figure 6 schematic to show how to prepare planchette sandwiches of adherent cells for high pressure freezing.

Sample preparation for Biopsies or tissue samples

Clean type B planchettes in a small beaker with a few millilitres of 100% ethanol in a sonicating water bath, remove the planchettes to dry on clean filter paper. Fill the well of the type B planchette with the selected cryoprotectant ensuring no air bubbles are present. Gently remove the biopsy or tissue sample from the storage media and place into the 300 µm well of the planchette. Place a second type B planchette flat side down on top of the well containing the sample (Figure 7). Freeze according to high pressure freezer protocols for your high-pressure freezer.

Figure 7 Sample loading for HPF of biopsies or tissue samples

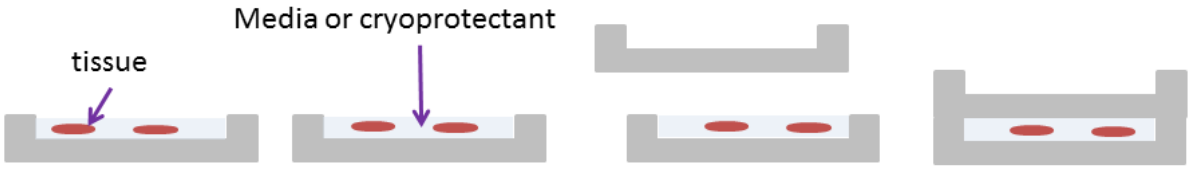

Figure 7 schematic to show how to prepare planchette sandwiches of biopsies or tissue samples for high pressure freezing.

Plunge freezing

Preparation of grids

Samples need to be suspended in a thin film ~ 50-300 nm. In order to do this a thin film of liquid is created by blotting away most of a droplet of the suspension from a hydrophobic support film. The support film may be a continuous carbon film suspended on a metallic 3.05mm TEM grid or one of the various holey carbon films (Quantifoil, C-flat, holey or lacey carbon). To create the hydrophobic support films the carbon coated grids are glow discharged or plasma cleaned (in a 25% oxygen/argon) mixture immediately before plunge freezing. This step allows even spreading of the sample across the grid.

Sample preparation plunge freezing

Various sample types can be plunge frozen; here are a few guidelines to getting the best out of your sample: Do not plunge freeze samples containing large amounts of sugars, as they may interfere with some measurements; Thicker solutions, (lipids, emulsions and gels) need longer blotting times; When working in high humidity environments or on a humid day blotting time may need to be increased, the inverse is also true.

Plunge freezing suspensions

Grip the grid at the edge without the forceps touching into the fragile grid bars. Tighten the forceps and gently tap the edge of the forceps with your finger to make sure that the grid does not move or drop out. Load the forceps into the plunge freezing apparatus or guillotine and secure. There are multiple suppliers of automated or semiautomated grid plungers (Thermo scientific, Gatan, Leica, Electron Microscopy Sciences). These devices allow the liquid ethane to be maintained at optimal temperature and often perform the plunge freezing in a humidity and or temperature-controlled environment. Cool down the plunge freezing apparatus and position the ethane container below the forceps. Once it has reached the desired temperature, either according to manufactures instructions or if using a homemade guillotine (Figure 8), when the liquid nitrogen has stopped

boiling and is still make the liquid cryogen. There are numerous possibilities for cryogen the main requirement is that it is a liquid at temperatures below -137 °C (136 K) see Table 1.

Figure 8 plunge freezing apparatus

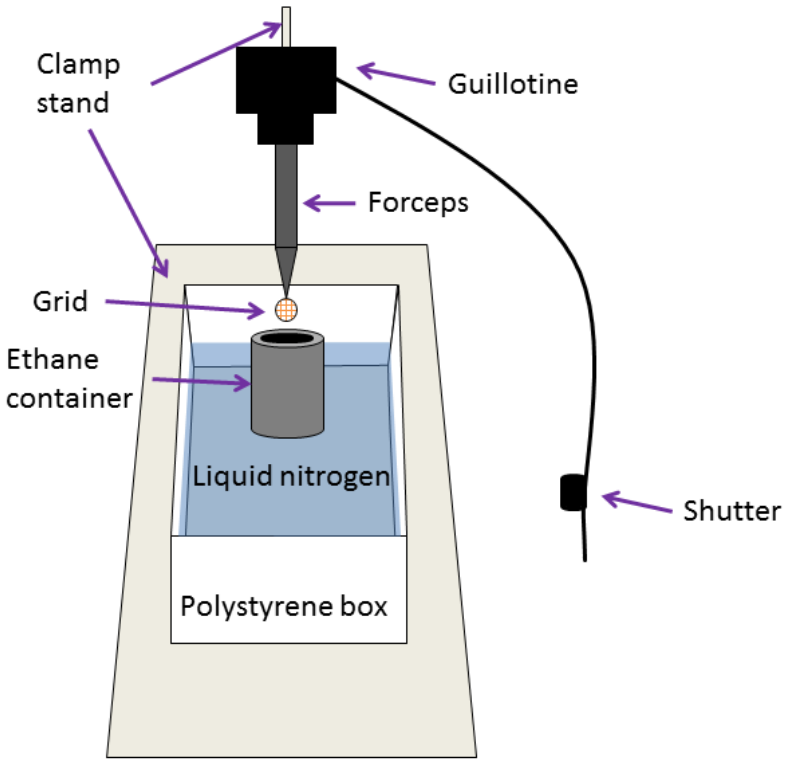

Figure 8 schematic of manual plunge freezing apparatus.

If using the manual plunge freezing apparatus thaw the ethane by adding fresh liquid ethane to the ethane container, the ideal consistency is for the ethane to be slightly slushy. Mix the sample gently by flicking the tube containing the sample and remove 4µl of sample using a pipette. Holding the pipette, as near to horizontal as possible and load the sample gently onto the grid. Remove excess liquid from the grid by blotting with filter paper, for approximately 4 seconds, until the paper comes away from the grid (this is easier to see with back lighting). Blotting times will depend on sample concentration, composition and relative humidity and a few attempts may be needed on new samples. Once the excess liquid has been removed immediately plunge the grid into the liquid ethane. After plunging remove the forceps from the guillotine and rapidly transfer the grid into liquid nitrogen (often there can be a large droplet of ethane on the grid, this can often be removed by gently touching the grid to the side of the ethane container, on the way out of the container. Place the grid into a grid storage box by moving it under the liquid nitrogen, or well below the vapour phase if using a commercially available plunge freezer, ready for transfer into the microscope.

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