CHAPTER THREE

Plunge Freezing for Electron Cryomicroscopy

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Contents

Abstract

Aqueous biological samples must be "preserved" (stabilized) before they can be placed in the high vacuum of an electron microscope. Among the various approaches that have been developed, plunge freezing maintains the sample in the most native state and is therefore the method of choice when possible. Plunge freezing for standard electron cryomicroscopy applications proceeds by spreading the sample into a thin film across an EM grid and then rapidly submerging it in a cryogen (usually liquid ethane), but success depends critically on the properties of the grid and sample, the production of a uniformly thin film, the temperature and nature of the cryogen, and the plunging conditions. This chapter reviews plunge-freezing principles, techniques, instrumentation, common problems, and safety considerations.

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1. Introduction

Because electrons have such high scattering cross-sections, their path through the electron microscope must be kept at extremely high vacuum. Aqueous biological samples must therefore be stabilized or "preserved" before they can be imaged. The first set of methods that were developed to preserve biological samples for EM involved dehydration: protein and viruses were negatively stained; tissues and cells were first chemically fixed, then dehydrated, plastic embedded, sectioned, and then stained. Dehydration perturbs structure, however, so methods were sought to preserve samples in their naturally hydrated state through freezing. The basic problem is, of course, that when frozen gradually, water crystallizes and expands, again denaturing macromolecules and perturbing cellular structures. One approach to solving this problem is to apply high pressures and cryoprotectants ("high pressure freezing"), which inhibit the nucleation and growth of ice crystals (Chapter 8, this Volume).

It was wondered, however, whether water or biological molecules could instead be cooled so rapidly that molecular rearrangements would simply stop before ice crystals had time to form. In the early 1970s, Taylor and Glaeser plunged hydrated catalase crystals into liquid nitrogen and showed that the crystals still diffracted to 3.4 Å (proving that the structure of the proteins had been preserved to at least that resolution; Taylor and Glaeser, 1973, 1974). Then in 1981, the Dubochet group showed that pure water could be frozen in a noncrystalline, liquid-like ("vitreous") state by spreading it into a thin layer across a standard carbon-coated EM grid and plunging it into liquid ethane (Dubochet and McDowall, 1981). At first, this claim was met with skepticism, but the impact the advance would have on structural biology became clear when macromolecular complexes were later added to the water and shown to be preserved in a native, "frozen-hydrated" state (Adrian *et al.*, 1984). The development of dedicated cryo-EM instrumentation (anticontaminators; low-dose kits, and tools to insert and hold frozen grids) and complementary advances in software, computational power, and other aspects of the work have now fully capitalized on this advance, producing reconstructions of specimens in their native states that are interpretable at the atomic level (Chapter 11, this volume; Chapters 9 and 15, Vol. 482).

Today, plunge freezing is being used to study macromolecules, drug delivery vehicles, 2D protein crystals, cell fractionations, vesicle suspensions, filaments, virus particles, thin bacteria, polymers, matrices, colloids, nanoparticulate catalysts, and even emulsion paints (Cerritelli *et al.*, 2009; Finnigan *et al.*, 2006). A variety of plunge freezers and protocols have been optimized for different applications (Grassucci *et al.*, 2007; Iancu *et al.*, 2006). The process of preparing samples for electron cryomicroscopy can be arduous, however, and often requires extensive troubleshooting to

determine the best freezing conditions for each sample. This chapter describes the plunge-freezing protocol in detail and will enable the novice to recognize the potential rewards and challenges that await them.

2. Grids and Supports

The first step in preparing samples for electron cryomicroscopy is to choose the right grid and support film. The grid itself can be made from a variety of metals. Copper is the most common, but if cells are to be grown on or in the presence of the grid, gold is a better choice because it is less toxic. Molybdenum has the advantage that it has a similar coefficient of thermal contraction as carbon, so that when frozen, the grid and the carbon support shrink more similarly, preventing "crinkling" (Booy and Pawley, 1993). Larger mesh sizes (smaller squares between grid bars) provide more support, but the grid bars block more area on the grid, especially at high tilt angles. Specialized "finder" grids are decorated with symbols to help mark particular locations on the grid, which can be critical, for instance, in correlative light and electron microscopy (Chapter 13, this Volume).

Grids for cryo-EM applications are almost invariably coated with a thin carbon film, although just recently, a new silicon ceramic combination called Cryomesh™ has been introduced, which shows promise in providing greater strength and stability (Quispe *et al.*, 2007; Yoshioka *et al.*, 2010). The carbon film can be either "continuous" (no holes) or "holey." Continuous-carbon support films can be better for 2D crystals, for instance, where maintaining a perfectly flat crystal is more important than reducing background noise (Chapter 4, Vol. 482). Holey carbon films allow background noise to be reduced, as samples can be imaged suspended in vitreous ice alone across the holes. While "lacey" grids (prepared either in the lab or purchased) have an irregular array of varying hole sizes (Fig. 3.1A), commercially available Quantifoil® (Fig. 3.1B) and C-flat™ films have a regular pattern of holes to facilitate automatic image acquisition. Typical hole sizes are around 1 μm. Larger holes maximize the sample imaging area, but it is helpful to have at least some carbon film (and maybe the full periphery surrounding a hole) in each image to reveal the defocus more precisely (Chapter 9, Vol. 482) and to reduce charging (Chapter 10, this volume).

Unfortunately, in our experience, the surface properties and integrity of the support film vary from batch to batch. We therefore recommend that a few grids from each batch be tested before use. The integrity of the carbon film can be checked easily in a light microscope, both before and after plasma cleaning (see Section 3). The surface properties can be checked by plunge-freezing pure water on a grid with standard plasma-cleaning and plunge-freezing protocols to make sure that uniformly thin vitreous ice is formed. Commercial suppliers can

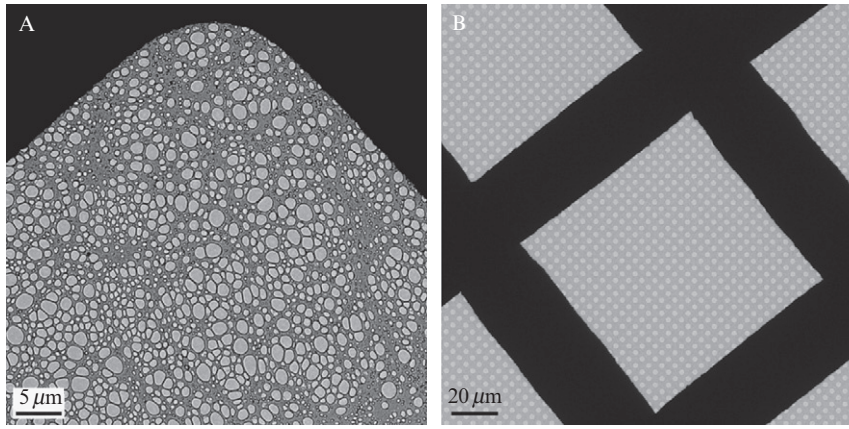

Figure 3.1 EM grid types. (A) Lacey carbon support film with an array of various hole sizes (grid bar 5 μm) and (B) Quantifoil® grid showing regular pattern of holes in carbon (grid bar 20 μm).

customize their support films to particular needs to reduce substrate bubbling, for instance, or improve film stability through the use of extra thick layers.

3. Cleaning the Grids

Freshly prepared carbon films are hydrophilic, but they become progressively more hydrophobic over time. It is therefore usually necessary to restore their hydrophilicity so that the liquid sample will spread evenly over their surface. Before the advent of electron cryomicroscopy, plasma cleaning, also called "glow discharging," was used to modify the adhesive properties of a variety of substrates for room temperature microscopy (Dubochet *et al.*, 1971). The plasma is created from the ionization of a gas, such as air, argon, oxygen, and hydrogen, or combinations thereof, such as argon/oxygen or hydrogen/oxygen, under low vacuum. Radicals within the plasma react with the surface of the substrate. As a result, the surface of the grid typically becomes hydrophilic. When liquid samples are then placed on the grid, they spread evenly across the surface and can be blotted to form films as thin as just tens of nanometers thick (Gan *et al.*, 2008). On a properly cleaned grid, in the chamber of a humidity-controlled plunge freezer, this thin sample film is remarkably stable and can remain suspended across holes in the grid for many seconds. If the plasma cleaning does not make the whole surface of the film uniform, the liquid will not spread over the grid or blot evenly off the grid, causing denser ice in some areas. One result can be, for instance, a bulge of ice in the center of each grid square (Fig. 3.2C).

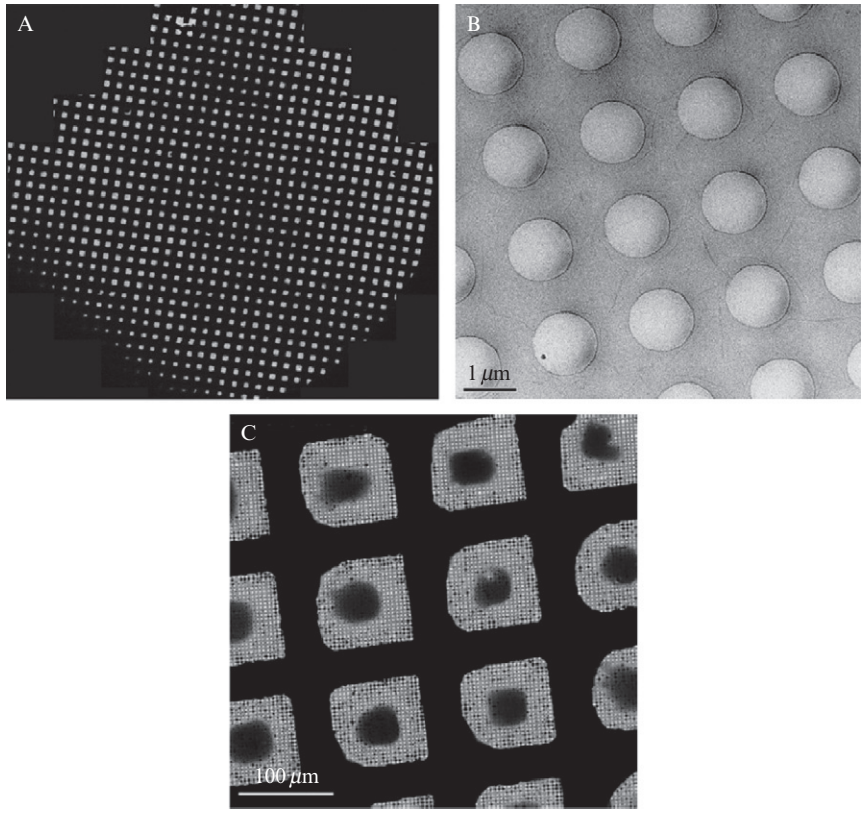

Figure 3.2 The effect of plasma cleaning on the ice. (A) Montaged serial EM atlas of a grid showing uniform ice thickness, image courtesy of Dr. Guenter Resch. (B) A Solarus™ plasma-cleaned Quantifoil® film with thin, uniform ice in the holes, image courtesy of Dr. Chen Xu, Rosentiel Basic Medical Sciences Research Center, Waltham, MA (grid bar 1 μm). (C) When plasma cleaning fails, one result can be a dense core of ice in the center of each grid square (grid bar 100 nm).

Plasma-cleaning parameters, such as the chamber pressure, radio frequency (RF) power, the gas mixture used to form the plasma, and the overall system geometry, should all be explored and optimized. The system settings can vary for each machine and application, but in our lab at Caltech, we use a platform height of 35 mm, a glow time of 60 s and an electrical current of 15 mA. If the fields are too strong or the glow time is too long, bombardment by the highly energetic ions can break the carbon film. The carbon film can also break if the vacuum is vented too quickly. Small organic molecules like amylamine and polylysine can be introduced as vapors during the ion discharge and subsequently affect how purified macromolecules partition in the ice over the carbon film and the holes. Once cleaned, grids can be stored in their

original storage grid box and sealed in an air-tight bag or chamber for later use. There are several diagrams available that show the proper setup for a plasma-cleaning system (Aebi and Pollard, 1987; Kumar *et al.*, 2007).

Building homemade plasma cleaners can be dangerous because of the high currents and voltages used and the specialized gases required to create the plasma. Commercial instruments are widely available. Some are designed solely for plasma cleaning and others also offer carbon coating. The Cressington 208 plasma-cleaning module attached to the Cressington carbon coater has a fixed 40-mm grid platform height and programmable time and power values. The Emitech K100X free standing unit uses a programmable protocol sequence, an adjustable height platform, and options for introducing alternate gases, making this a versatile unit. The smaller Harrick PDC-32 unit has fewer control settings, a fixed glow tube diameter, and may be easily transported to more remote research locations. Further development by Gatan, Inc. has produced the autotuning Solarus™ 950 Advanced Plasma Cleaning System, with a chamber to accommodate grid cleaning as well as two ports for cleaning microscope specimen holders. The Solarus™ 950 is configured to use a hydrogen/oxygen gas mixture that cleans with minimal sputter damage, making it especially suitable for cleaning fragile carbon support substrates. Because of the efficiency of the hydrogen/oxygen plasma, the cleaning time for carbon substrates is very short, typically 15–30 s using a hydrogen/oxygen gas mixture and a RF setting of 50 W. This has produced carbon films that are uniformly hydrophilic and can remain so for several weeks (Melanson, 2009b).

Though plasma cleaning is the preferred method to clean grids, when plasma cleaners are not available, the grids can also be dipped into ethanol, acetone, or chloroform or be recoated with a fresh carbon layer (Quispe *et al.*, 2007). Grids can also be coated with polylysine or other organic molecules to promote the adherence of cells, for instance. We and others have found that more extreme grid treatments (such as overnight "preirradiation" in an EM) can cause certain macromolecular complexes to partition into the holes in the carbon.

4. Preparing the Cryogen

For water to vitrify, the temperature has to drop faster than $\sim 10^5$ K/s (Dubochet and McDowall, 1981). The reason why samples have to be thin is that the heat conductivity of the water in the sample is the limiting factor. The cryogen that the sample is plunged into has to have a high thermal conductivity in order to transfer heat out of the specimen quickly, a freezing point below the temperature needed to vitrify the sample, and both a high boiling point and a large heat capacity to prevent a layer of vapor forming between the sample and the cryogen (Bellare *et al.*, 1999). While the temperature of liquid nitrogen at ambient pressures is very low (77 K), it is readily available, and it is relatively

inexpensive, unfortunately its thermal conductivity is only about 400 K/s and so frequently produces crystalline ice. The most commonly used cryogens are therefore ethane and propane, primarily because their thermal conductivity is 300–400 times higher (in excess of 13–15 kK/s). The freezing point of ethane is 90 K, its boiling point is 184 K, and it has a high heat capacity (68.5 J/mol K at 94 K). Liquid nitrogen is used instead as the primary coolant to first liquefy the ethane or propane and then keep it cold during the procedure.

An inconvenience arises, however, because the freezing points of both ethane and propane are higher than the temperature of the nitrogen, so they slowly solidify during the experiment. Some plunge freezers have therefore been constructed with built-in heating elements or special designs that limit the heat transfer between the nitrogen and ethane/propane cups to maintain the cryogen just above its melting point. Tivol *et al.* (2008) found that a mixture of 37% ethane and 63% propane remains liquid even when in direct contact with liquid nitrogen. This mixture produces consistently thin vitrified layers and facilitates long plunge-freezing sessions without heaters or special cup configurations.

After the grid is plunged into the cryogen and then transferred into liquid nitrogen for storage, excess ethane (or propane) on the grid will freeze, forming a solid crust. Usually, this crust falls off the grid in subsequent handling, but if not, it will sublime rapidly when the grid is inserted into the high vacuum of the microscope. Impurities will remain, however, so it is important to use very pure cryogen. Lower grade ''camping gas propane'' is too full of contaminants to produce clean samples.

4.1. Condensing the cryogen

Cryogens come as compressed gases and therefore need to be liquefied. This is done by releasing the gas slowly into a cup cooled by liquid nitrogen. The flow of the gas can be controlled with a 2-stage regulator fitted with a needle valve on the second stage and narrow-bore Tygon tubing on the nozzle. A pipette tip is usually inserted into the end of the Tygon tubing to further restrict and better direct the gas flow. The following is a typical protocol for condensing the cryogen:

1. Work in a fume hood and wear a lab coat and goggles.
2. Pour liquid nitrogen into the space around the cryogen cup. When the cup has reached at least $-175\ ^{\circ}C$, the liquid nitrogen will stop bubbling violently (the ''Leidenfrost point''). Depending on the plunge freezer design, this can take 5–15 min, and the procedure is usually outlined in the instruction manual specific to the instrument being used.
3. Before starting the condensation process, check to make sure that the cryogen cup is free of any residual liquid nitrogen.
4. With the needle and main tank valves on the 2-stage regulator closed at this point, adjust the gas outlet pressure on the second stage to

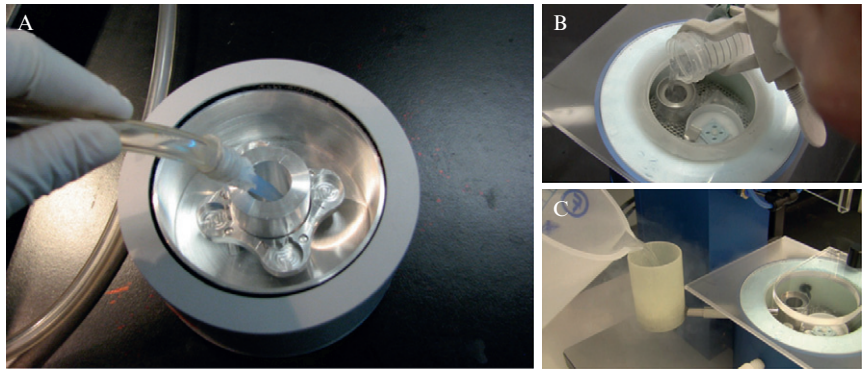

Figure 3.3 (A) Condensing the cryogen by flowing cryogen gas into a precooled cup surrounded by liquid nitrogen. (B) Pouring liquid ethane into the cold cryogen cup after condensing ethane gas in a separate container. (C) Refilling the liquid nitrogen through an external port maintains a clean, cold nitrogen gas environment. (B) and (C) Images courtesy of Gatan, Inc., Pleasanton, CA.

approximately 0.14–0.28 bar. Use low pressure to avoid unnecessary venting of the gas into the fume hood or splashing of condensed cryogen.

5. Place the tubing attached to the gas tank regulator into the bottom of the precooled cryogen cup (as in Fig. 3.3A).
6. Open the main tank and needle valves to allow delivery of the gas at the preset pressure.
7. You will start to notice the liquid filling the cup. When the liquid reaches the top, decrease the flow of gas and slowly pull the tip of the tubing out. Quickly turn the gas off. If you turn the gas off while the tip is still submerged, the liquid will aspirate back into the tubing.
8. Remember to close the main tank valve on the cylinder and bleed the line of any residual gas. Always leave the gas cylinder in a safe configuration as defined by the safety procedures for your laboratory.

Alternatively, the cryogen can be condensed in a separate container cooled by liquid nitrogen and then poured into the precooled cryogen cup (Fig. 3.3B).

4.2. Safety considerations

Before handling cryogens, read about them thoroughly in the latest Materials Safety Data Sheets. Ethane and propane are highly flammable and are even more so when condensed, so do not condense these gases in the presence of an open flame. Only condense the smallest volume necessary to fill the cryogen cup (usually less than 10 ml). Rather than having one large tank of cryogen gas, try to limit the size and keep reserve tanks in flameproof cabinets. Two refillable cylinders containing 67 lb of gas last 2–3 months in a busy laboratory. Ethane

or propane gas cylinders, and their associated 2-stage regulators, should be ordered and installed in consultation with the on-site laboratory safety officer.

The liquid nitrogen that is used to maintain the low temperature of the condensed cryogen will evaporate over time and must be continually refilled during a freezing session. Replenishing the liquid nitrogen also serves to maintain a layer of cold, dry nitrogen gas surrounding the condensed cryogen. This helps to minimize condensation of atmospheric moisture into ice that will contaminate the cryogen and the sample, and provide a protective interface for transferring the frozen specimen grid. However, try not to splash liquid nitrogen into the cryogen. The surface of the cryogen can freeze solid, entrapping an underlying volume of warmer, liquid cryogen that can explode through the frozen layer. The Cryoplunge™ 3 has a shield over the workstation to prevent splashing, as well as an external funnel for refilling the liquid nitrogen (Fig. 3.3C). Physical exposure to these low temperature cryogens can produce severe frostbite. Always wear adequate eye and face protection when working with these cryogens. Also, exercise caution when handling any materials that come in contact with the condensed cryogen, since these surfaces can also freeze skin and underlying tissues.

While small volumes of liquid nitrogen can safely be poured over a large ventilated surface, such as a floor, to dispose of it, it is recommended that propane and ethane be allowed to evaporate in a dedicated fume hood for several reasons. First, they are highly flammable. As the cryogens evaporate, they will expand rapidly by factors in excess of 700 times. Since the cryogens are odorless and colorless, there is also a risk of asphyxiation as atmospheric oxygen is displaced. Even at low concentrations, ethane gas can cause narcotic effects with symptoms of dizziness, headache, nausea, and loss of coordination. The plunge-freezing area should be well ventilated, and labs handling large volumes of cryogens can be equipped with oxygen displacement sensors to warn people when oxygen gets low. In addition to educating the staff on the risks from cryogens, always provide plenty of protective cryo gloves and eye shields and post signs alerting visitors and emergency responders to the location of cryogens.

5. Plunging the Grid

5.1. Basic procedure

The process of plunge freezing generally involves three main steps: a small liquid droplet containing the specimen is applied to the carbon surface of an EM grid, the liquid droplet is blotted with filter paper until only a very thin film of fluid remains, and then the grid is plunged into the cryogen. The grid is then stored in liquid nitrogen in a custom-made grid box until it is finally loaded into the electron cryomicroscope for imaging. Blotting can be done from either one or two sides. Unilateral blotting can be particularly helpful in

reducing the direct contact of fibers in the blotting paper with cells, for instance, growing on the other side (Lepper *et al.*, 2010). The best ice thickness depends on several factors, including the size and shape of the specimen and the accelerating voltage of the electron cryomicroscope that will be used. Thicker ice may provide more stability, but if the fluid sample to be vitrified is too thick, the ice may not vitrify. If too much fluid is blotted away, the cells can become dehydrated. The thinness of the ice will also effect how particles distribute across the holes: large particles may be displaced to deeper regions of the film, such as the edge of a hole. Particles may also be oriented preferentially in very thin layers in part because of surface charges at the air/liquid interface (Glaeser *et al.*, 2007). The temperature, humidity, blotting pressure, and blotting duration should be optimized for each specimen. It has recently been shown that blotting can damage and even kill large cells (Lepper *et al.*, 2010). Such samples should therefore be blotted gently for longer times.

As an example protocol for plunge-freezing protein or bacteria,

1. Suspend the sample in an aqueous medium (e.g., water or low ionic buffer solution to reduce background noise during imaging) at a concentration of 1–3 mg/ml for protein complexes or an OD_{600} of 0.5 for bacteria.
2. Plasma clean EM grids, following the instructions provided in the user manual for your particular machine.
3. Secure an EM grid with the tweezers provided with your plunge freezer and attach the tweezers to the machine.
4. If the plunge freezer has a humidity-controlled chamber, set the humidity to 100%.
5. Apply 3–5 μl of the sample to the carbon side of the grid (see the manufacturer's instructions on the grid box).
6. Blot the EM grid with #1 grade filter paper for 1–3 s to produce an aqueous film less than 1 μm in thickness.
7. Plunge into liquid cryogen to produce a thin glass-like solid.
8. Transfer the grid into a labeled four-grid-slot box in liquid nitrogen, being careful not to expose the grid to atmospheric moisture.
9. Grid boxes are stored within a 50-ml conical tube placed in a large nitrogen cryostorage dewar.

Part of the skill of plunge freezing is knowing when the cryogen is at the right temperature. When gaseous cryogens are first liquefied, they are still warmer than the surrounding liquid nitrogen, and it takes time for them to cool further. The best indication for when the liquid ethane reaches the right temperature for plunge freezing is when the bottom of the cup freezes, but enough liquid remains at the surface for plunging the grid. This state does not last very long before the rest of the volume freezes, however, so unless a mixture of ethane and propane is used or the freezing device somehow keeps the cryogen temperature just above its freezing point (see earlier), the cryogen will have to be melted periodically. This can be done by inserting a warm

metal rod or adding more (room temperature) cryogen gas, but neither strategy is ideal, since rods can introduce contamination and adding more gas can cause the cryogen to overflow the cup. One must also wait again until the cryogen has recooled to its freezing point before the next grid is frozen.

After a grid has been plunged, it should be handled very carefully to avoid damage. The grid should never be bent, because it will then fail to seat securely in the holder, causing drift and instability, so try to avoid touching the grid to any walls of the freezing cup during manipulation. When transferring the frozen grid from the cryogen cup to the storage holder, the grid may need to be lifted out of the cryogen very quickly in a space filled with cold dry nitrogen gas to prevent exposing it to moisture in the air. Floating cylindrical barriers and purpose-built covers are also to be used to trap more dry nitrogen gas and protect the specimen. For more details on the plunge-freezing procedure, see Iancu *et al.* (2006). Training courses are frequently available from vendors and the NIH-funded National Research Resource Centers.

5.2. Controlling humidity

Atmospheric moisture in the cryolab is undesirable. If precautions are not taken, moisture will form ice crystals on liquid nitrogen storage containers and subsequently on the grid sample. In humid regions of the world, cryolabs employ complex ventilation systems and dehumidifiers for reducing relative humidity to less than 25%. Additionally, instruments may be entirely enclosed within a humidity-controlled chamber. (A cautionary tip: low humidity may increase static electricity. Certain floor coverings and clothes can reduce these discharges.) The regulations at some institutions require that plunge freezers be operated in a fume hood. The strong air currents within fume hoods can introduce ice contamination and air-drying artifacts. A shield around the plunging area is recommended. Automated plunge freezers now provide covers that facilitate a dry nitrogen gas flow over the cryogen container to reduce contamination. In addition, all liquid nitrogen dewars must be kept dry between freezing sessions and fitted with a loose lid to reduce water vapor condensation. Always invert portable tanks and dewars to dry, since moisture will collect on their cold surfaces. A large drying incubator at 30 °C is useful to ensure that all components remain moisture free. Workstations with heat blocks set at 50 °C can be used to dry small tools, and a source of low pressure ''lab air'' can be used to dry fixtures.

While atmospheric moisture must be controlled to avoid contamination, a higher relative humidity in the immediate area of the sample grid is preferred for preventing desiccation of the sample prior to freezing. Many commercial plunge-freezing instruments offer an environmental chamber for controlling temperature and humidity. This controlled environment is especially important when blotting, as evaporation and surface air/liquid interfaces play important roles in electrostatic forces and on how macromolecules organize at the

surface. In general, the humidity in the chamber before plunging should be greater than 80%, as demonstrated in the preservation of liposomes. Humidity values in the 40% range will create osmotic imbalances as water evaporates from the film, causing liposome inversions (Frederik and Hubert, 2005). Other effects are seen in the preferential organization of viral capsid complexes as they align because of surface interactions (Dubochet *et al.*, 1985).

5.3. Instrumentation

The designs of plunge freezers used in pioneering experiments contributed to the design of modern-day instruments (Fernandez-Moran, 1960; Handley *et al.*, 1981; McDowall *et al.*, 1984). Early laboratory prototypes for plunge freezers were often a basic construction of makeshift stands and Styrofoam boxes (Dubochet *et al.*, 1983; McDowall *et al.*, 1983; McDowall, 1984). One of the first plunge freezers was a simple pivoting fine-forcep holding a carbon-coated grid. The grid fell in a gravity arc past a vaporized mist sprayed through an aperture slit. The continuous-carbon substrate collected microfine droplets, which were vitrified in a pot of viscous ethane. The first image of vitreous water was prepared in this way (Dubochet and McDowall, 1981). Eventually, the carbon substrate was removed and the ''bare grid'' method was the precursor of the unsupported liquid film (Adrian *et al.*, 1984). The Dubochet group's 1980s plunge freezer design was an elastic-driven rod supporting gold electronic circuitry pins for freezing suspensions and filaments. A water-driven magnetic stir bar kept the ethane fluid and successfully vitrified samples for early cryosectioning experiments (Dubochet *et al.*, 1988). The plunge freezers today are much more sophisticated but owe their design to these early prototypes and years of experience in many laboratories.

A key requirement for obtaining good frozen-hydrated specimens is the ability to produce the uniformly thin vitrified ice layer. In the early days of cryospecimen preparation, many of the ''homemade'' plunge-freezing instruments required manual blotting of the specimen grid. Although, very high-quality results can be obtained in this manner, manual blotting of the liquid from the surface of a fragile EM grid is often variable, and success depends on the skill of the individual. Manual plungers rely on gravity to plunge the EM grid into a cryogen and have therefore been called ''gravity plungers'' (Fig. 3.4A). Because they are not automated they offer more user control over the blotting. For experiments involving cells growing on the EM grid, harsh blotting pressures from both sides in an automated plunger run the risk of ''peeling'' the adherent cells off the grid. In the case of gravity plungers, the user can blot from the back of the grid if the intent is not to disturb the cells. The liquid flows into the filter paper through the holes in the carbon. A typical practice is to blot with filter paper from one side until the liquid stops wicking into the filter paper (Fig. 3.4B), but a variation of blotting times should be tested for each sample. The gravity plungers are fairly mobile and are often used

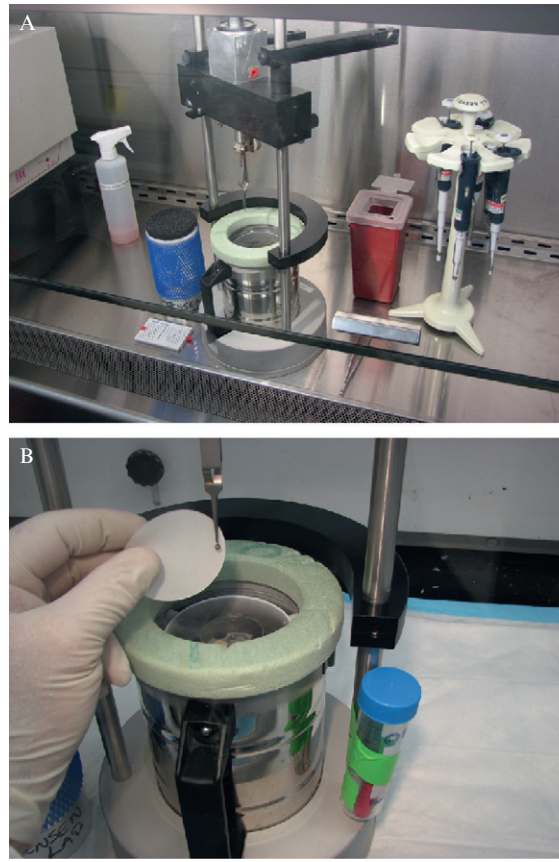

Figure 3.4 (A) Manual plunger (custom-made in the Department of Biochemistry, Max Planck Institute, Martinsried, Germany) in a biosafety cabinet. (B) Manually blotting the liquid from the back of the grid before plunging.

when traveling to laboratories where specimens are to be frozen on-site. However, the lack of an environment-controlled chamber means that the grids are exposed to atmospheric humidity and temperature, and the users are more exposed to the danger of having sharp tweezers and biological samples near their hands. Modern manual plungers have built-in lights to help visualize the blotting process and a foot pedal for dropping the tweezers.

Currently, there are a variety of automated plunge-freezing instruments designed to make the plunge-freezing process efficient and reproducible (Fig. 3.5). Automated plunge freezers provide precise control of several parameters such as humidity, blot pressure, and blot duration in order to eliminate variability in the thickness of the vitrified ice layer. They also provide the means to select, store, and recall a set of parameters. Finally, automated plunge freezers incorporate a variety of safety features for protecting the user.

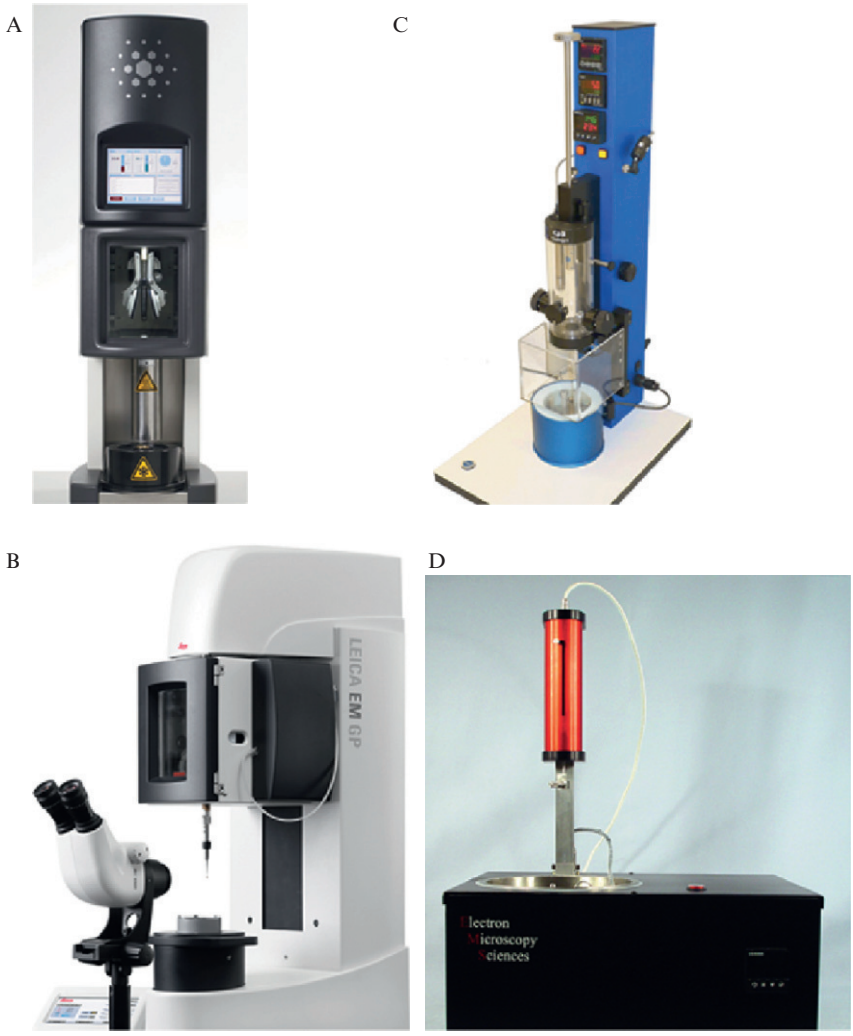

Figure 3.5 Variety of automated plunge freezers. (A) Vitrobot™ Mark IV, image courtesy of FEI, Inc., Hillsboro, OR. USA. (B) Leica EM GP, image courtesy of Leica Microsystems, Inc., Vienna, Austria. (C) Cryoplunge™ 3, image courtesy of Gatan, Inc., Pleasanton, CA. (D) EMS-002 Rapid Immersion Freezer, image courtesy of Electron Microscopy Sciences, Hatfield, PA, USA.

The first fully automated, computer-controlled plunge freezer was developed in the late 1990s by Dr. Peter Frederik and Paul Bomans (Frederik and Hubert, 2005). This machine, the Vitrobot™ Mark I, was the first in a series of "vitrification robots" now commercially offered through the FEI Company. The Vitrobot Mark IV is the latest version, and can dip the grid into a liquid

sample or allow a sample to be pipetted onto the grid from an opening in the side. The Leica EM GP, developed in conjunction with Dr. Gunter Resch, provides one-sided blotting of cell monolayers grown on the specimen grid. An attached stereomicroscope allows the user to view the specimen grid to monitor the process. Cryoplunge™ 3 from Gatan, Inc. is a versatile, semiautomated plunge-freezing instrument that provides timed blotting functions, a removable humidity chamber, and the temperature of the liquid ethane can be held just above the melting point of the cryogen. A shield over the cryogenic workstation provides a protective environment for transferring the frozen-hydrated grid to its storage container to prevent the formation of contaminating ice (Melanson, 2009a). The Rapid Immersion Freezer from Electron Microscopy Sciences requires that the specimen be manually blotted, but it is a portable and economical plunge freezer that provides an environmental chamber, temperature control of the cryogen, and freeze-substitution capabilities.

Consistency of results, control parameters, and overall cost are some of the criteria that can be used to determine which type of plunge freezer best suits the needs of the individual investigator. Some laboratories use several different types of plungers based on the features required to prepare their diverse specimens.

5.4. Safety considerations

Many people with different samples typically share a plunge freezer. A good practice is to decontaminate surfaces and mechanical parts of the machine with 70% ethanol before and after every use. In order to keep track of all the samples that come in contact with the machine, an accurate sample history should be kept on a database and staff should be informed of the daily freezing schedule. Only microliter volumes should be cultured and used in freezing procedures, and aseptic technique should be followed in safety hoods. For biohazardous samples in automated plunge freezers, a heat cycle overnight will help to decontaminate the chamber. In manual plungers, the whole machine can be placed in a biosafety cabinet in an isolated room. When using manual plungers, be aware of the location of the foot pedal to avoid premature release of the tweezers and try to keep fingers from ever going beneath the sharp tweezers. The automated plunge freezers are usually controlled by compressed air and have enclosed chambers so the user is less likely to be injured by the tweezers. Training for new staff and yearly refresher lab safety courses should be offered.

6. Common Problems and Their Diagnoses

Ideally, the ice across a grid will be uniformly thin and vitreous (Fig. 3.2A). If the ice and embedded sample is too thick, or there is too much contamination, no electrons will penetrate through the grid. If the ice is at least thin enough for

electron penetration, electron diffraction can be used to evaluate the quality of the ice. Vitreous ice is the absence of a detectable crystalline structure and most closely resembles the liquid state of water. It is the preferred form of "ice" for electron imaging and is thought to be the least damaging to structures. However, there are many points in the freezing, transfer, and loading procedures that can cause damaging hexagonal or cubic ice crystals to form: slow cooling of the sample because the cryogen was too warm or had a low thermal conductivity, contaminating ice floating in the cryogen or storage liquid nitrogen, or the frozen sample warming to a temperature higher than −135 °C at any time during storage or transfer (Cavalier *et al.*, 2009; Dubochet *et al.*, 1988). Contaminating ice can adhere to the sample during the transfer of the grid from the cryogen to the storage container or during loading into the microscope. Care should be taken at these points to protect the EM grid by always keeping it in liquid nitrogen, limiting exposure to atmospheric moisture, and always cooling tools in liquid nitrogen before using them to manipulate the grid.

Hexagonal ice is the most common ice on earth and is formed when water molecules attach to each other at each point of their tetrahedral structure and extend indefinitely (Dubochet *et al.*, 1988). This proliferation of bonded molecules can severely damage the cellular ultrastructure. Hexagonal ice can form during slow freezing (streaks of crystalline ice, as seen in Fig. 3.6A), or could have condensed after freezing in the form of discrete spherical spots. Cubic ice is very similar to hexagonal ice, except that the bond angles of neighboring water molecules are rotated 180°, making it only stable below −70 °C (Dubochet *et al.*, 1988). The dimensions of cubic ice crystals range from 30 nm to 1 μm and they usually look like fine grain spots (Fig. 3.6B). While cubic ice does not tend to cause extensive structural damage to the specimen, it can cause background noise that will disrupt the image. For reference, the three forms of ice and their diffraction patterns can be found in the landmark article by Dubochet *et al.* (1988).

There are many decisions to be made and conditions to be controlled during the plunge-freezing process in hopes of creating a well-preserved sample in a thin, vitreous ice. However, when the process works, high-resolution structural detail of a sample can be obtained and the reward can be great. Figure 3.7 demonstrates the level of detail that can be achieved in different samples.

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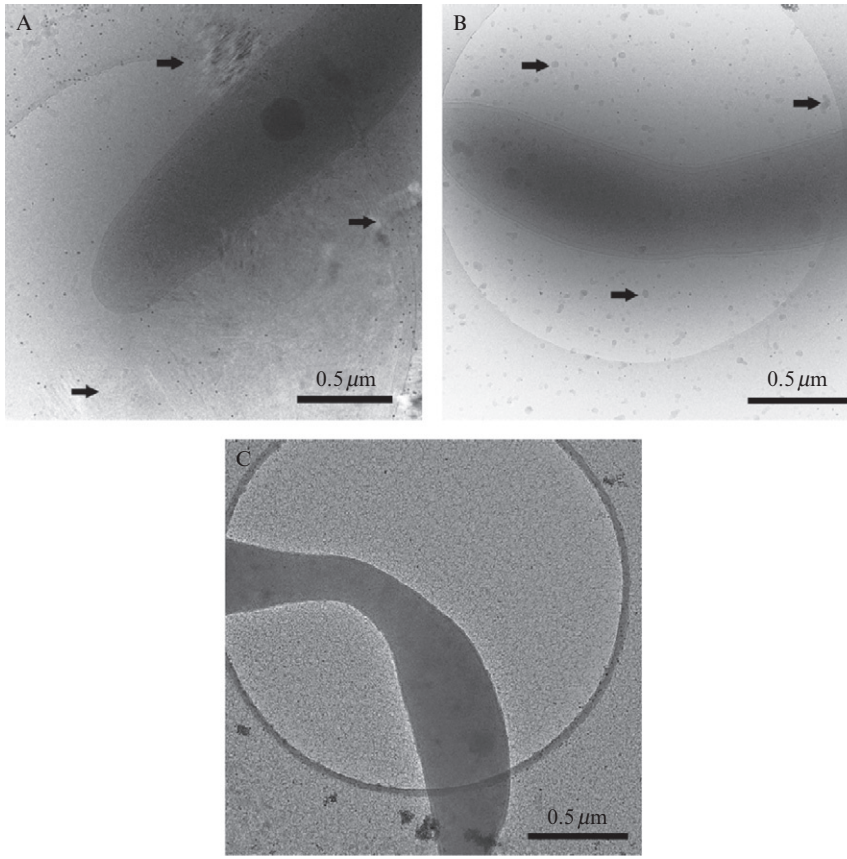

Figure 3.6 Examples of poor ice. (A) Hexagonal ice surrounding the bacterium, indicated by the arrows. (B) Cubic ice contamination, indicated by arrows. (C) The result of extreme rewarming: loss of water and structural detail. Grid bars = 0.5 μm.

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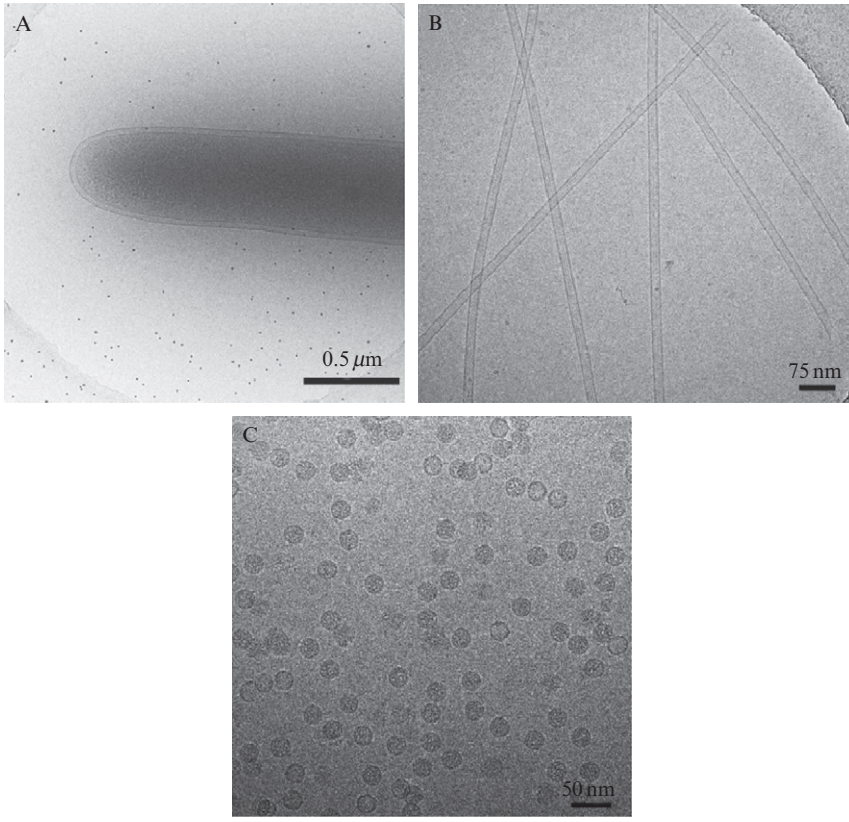

Figure 3.7 **Examples of good ice. (A) A bacterium surrounded by gold fiducials, which are used to align tomographic tilt-series (grid bar 0.5 μm). (B) Microtubules, image courtesy of Dr. Guenter Resch (grid bar = 75 nm). (C) Rhinovirus particles labeled with Fab fragments, image courtesy of Angela Pickl-Herk (grid bar = 50 nm).**

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