CRYO-ELECTRON MICROSCOPY OF BIOLOGICAL TISSUE, CELLS AND ORGANELLES

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Cryo-electron microscopy is a rapidly expanding field that can be divided into two main areas: cryo-preservation for labeling or structural analysis and examination of specimens at low temperatures using a cryo-stage in a transmission electron microscope (TEM). Many types of specimens can be prepared using rapid cooling as the initial fixation step and the specimens can be examined, after processing, at ambient temperature or, without processing, directly at low temperature. Other samples, such as 2D crystalline arrays, can be stabilized with cryoprotectants and slowly cooled for examination at low temperature in a TEM. The choice of which cryo procedure to use is often dictated by the dimensions of the sample. This brief review summarizes the procedures and types of equipment currently available to examine a size range from large samples, such as portions of tissues and organs, to small samples, such as virus particles or cellular components.

The main goals of cryo-preservation with ultrarapid freezing are to stabilize the biological material in its natural state and avoid ice crystal formation and damage. Ultrarapid freezing procedures can be classified based on the physical technique used to extract heat from a specimen. Plunge freezing is the oldest and most commonly used, in part, because of the simplicity of dropping a sample into liquid coolant. The success of plunge freezing, as with other methods, is often based on scale: mm sized tissue fragments in the absence of cryoprotectants cannot usually be frozen without ice crystal damage, some cells in the 20-100 μm range can occasionally be well-preserved, and fragments of cells, including organelles or molecular complexes, can often be perfectly preserved within a matrix of amorphous or vitreous ice. The critical parameter is the thickness of the sample, which should be less than 10 μm. Thus, small cells or fragments sandwiched between thin copper disks can be well preserved by plunging into liquid propane. These frozen samples will probably contain microcrystalline ice that causes little or no damage. To obtain truly amorphous or vitreous ice, the samples must be even thinner, about 100 nm or less. Cell components, single particles and model systems, which can be prepared in a thin aqueous layer suspended over a holes in a support film on an electron microscope grid, can be vitrified by plunging into liquid ethane (a slightly better coolant than propane). This has become the preferred method to consistently prepare biological samples embedded in amorphous ice (the so called, “frozen hydrated” samples) for direct examination in the TEM using a cryotransfer system (to avoid ice crystal deposition on the cold sample) and a cold stage to maintain a stable temperature in the range of -140°C to –170°C (1). Several commercial devices have optimized each critical step in the freezing process, such as blotting the grid to produce a uniformly thin layer (2). These new devices permit a relatively inexperienced user to obtain reproducibly excellent sample preservation in amorphous ice.

An alternative to plunge freezing is to spray a stationary, sandwiched sample with jets of propane from opposite sides. This “propane jet” method gives slightly higher cooling rates compared to the same sample plunged into coolant and thus gives somewhat better preservation of samples 10-20 μm thick (3). A distinct method of bringing sample and coolant into contact forces the flat surface of a sample onto a stationary, liquid-helium-cooled copper block. This is the “quick
freeze” or “slam cooling” method that is often followed by deep etch, rotary shadowing with platinum/carbon (4). The advantage is that cooling is rapid enough to vitrify the surface of the sample without mechanical damage. The limitations are that only a 10 μm thick layer is consistently well preserved and most samples have to be pre-washed in a volatile buffer to allow deep etching. Many sample types have been shown to withstand these treatments and the subsequent images of cellular substructure of often unique and stunning. Large tissue fragments can be slam frozen, as long as a flat region containing the structures of interest can be exposed at the surface that will contact the copper block.

Many samples that do not need to be rapidly cryofixed can be treated with cryoprotectants prior to cooling. The most common cryoprotectants are small molecule solutes that complex water, such as glycerol, sucrose and trehalose (a naturally occurring plant diglucose). When used for example at a concentration of 10-30%, ice crystal formation and growth are sufficiently retarded that relatively slow freezing can be used to give excellent structural preservation. These samples can be frozen by a variety of methods producing slow or modest cooling rates. The test of the extent of preservation is the condition or resolution of the frozen samples, which can be examined in many different ways. For example, thin 2D crystalline arrays of aquaporins embedded in glucose on electron microscope grids were analyzed by cryoelectron crystallography at high resolution (5). In separate procedures large cryoprotected tissue pieces can be cryosectioned, the sections warmed and immunogold labeled for localization analysis at room temperature (6). The cryosections can also be examined at low temperature in a cold stage without stains or artificial contrasting treatments (7).

A different approach to cryoprotection is employed in high pressure freezing (HPF) where the aqueous sample is exposed to a pressure of about 2000 bar just prior to freezing with a coolant, such as a jet of liquid nitrogen (8). The high pressure acts as a cryoprotectant by greatly lowering the temperature of ice crystal nucleation. The main advantage of HPF is that samples typically about 200 μm in the smallest dimension can be preserved throughout in amorphous ice. Such properly frozen macro samples have aqueous regions that are completely clear immediately after freezing. The subsequent processing of the HPF frozen sample generally follows one of three paths: cryosectioning, freeze fracture or freeze substitution. Recent advances in cryosectioning, using a diamond knife connected to an oscillator, have demonstrated that minimally distorted sections can be cut to a thickness of 50 nm and that, when examined using a TEM cold stage, the electron diffraction pattern shows that the ice is amorphous throughout the section (9). This offers the opportunity to study tissues in their natural state and to perform high-resolution cryotomography using only the natural contrast (10). These approaches are still being refined and hold great promise because of the wide applicability to a variety of large structures that loose their biological integrity when dissected or manipulated to conform to other preparation techniques.

After HPF, samples can be freeze fractured conventionally or deep etch, rotary shadowed. In this case the region of interest is exposed at the fracture interface, which is in part dependent on the method of loading the sample into the HPF holder. Access to the entire interior of the HPF frozen sample is available after freeze substitution. Two general approaches to freeze substitution are preservation for structural or labeling analysis. Structural analysis commonly uses osmium tetroxide in anhydrous acetone as the substitution medium for several days at about -90°C. Water molecules are slowly replaced by organic solvent molecules, while the biological structure is immobilized at low temperature. During slow warming, the osmium tetroxide
contributes to the fixation of lipids and proteins, as well as produces stain contrast. After warming, the samples are processed as in conventional TEM including final embedding in an epoxy resin. Additional contrast is obtained by staining thin sections with tannic acid and heavy metals. The second approach commonly employs a non-osmium medium containing mild fixatives, such as uranyl acetate or a small amount of glutaraldehyde. Water soluble resins, such as Lowicryl or LR White for embedding after warming, seem to preserve antigenicity for subsequent labeling of thin sections at room temperature.

In summary, cryo-preservation can be accomplished using a variety of methods appropriate for the specific tissue geometry and the desired structural information. For the highest resolution, thin frozen hydrated samples examined at low temperature are desirable, and special high voltage TEMs are often required to reach the maximum resolution. For cells in culture or naturally thin samples, some form of sandwich holder may be suitable for many applications. For a variety of sample sizes and shapes, HPF offers many advantages as long as the final output is appropriate. If the primary scientific issue is the visualization of the cytoskeleton or extracellular matrix fibers, perhaps only deep etch, rotary shadowing following slam freezing will be suitable. Today there are many options available to find the appropriate sample cryo-EM preparation procedure suitable for most morphological projects.
References.