Freeze fixation of cells and tissues provides a method of avoiding chemical fixation artifacts (9), and it often improves immunolocalization (1,4,9). Various devices and techniques have been devised for freeze fixation of different microbial, plant and animal materials (2,5,6,9,10). One of the simplest methods is plunge freezing, in which samples for microscopy are quickly plunged into a suitable cryogen and held there until completely frozen. Often, liquid propane cooled by liquid nitrogen is used as the cryogen because it has good freezing properties and is relatively inexpensive. High rates of heat transfer are desirable to minimize or avoid crystalline ice formation that damages cellular structure, so copper is a common choice for parts of freezing devices. Commercial or custom-made, machine-tooled devices using expensive high-grade, highly polishes copper parts can be used, but we report excellent freezing results using a simple, inexpensive and quickly assembled device that is composed in part of commonly available copper plumbing supplies.

For freezing, tobacco seeds [Nicotiana tabacum (L.) cv Xanthi] were germinated on wet filter paper in a humidified chamber for 3 days until the primary root had just broken through the seed coat. Then the seedlings, including the embryos, endosperms and seed coats, were cryoprotected by immersion in 20 mM 2-[N-morpholino]ethanesulfonic acid (MES), pH 5.5, 2 mM MgCl₂, 2 mM CaCl₂, 2 mM KCl, 0.2 M sucrose for 0.5–1 h (9), cut into 0.5-mm pieces, loaded onto Formvar™ (Toki, New York, NY, USA) coated 0.3-cm nickel loops and plunge-frozen in liquid propane cooled by liquid nitrogen while held using insulated forceps using the device shown in Figure 1. The liquid propane had been produced by blowing a gentle stream of gaseous propane from a commercial blowtorch cylinder into the liquid nitrogen-cooled copper cup (Figure 1). This cup is very smooth to the touch and was easily polished to a high shine using commonly available metal polish.

Seedlings were substituted for 3 days at -85°C in either acetone (for electron microscopy) or ethanol (for light microscopy). The samples were then placed in different temperature compartments to gradually warm the samples to room temperature in a step-wise manner: to -20°C overnight, to 4°C over 4 h and to 25°C over 1 h, followed by washes in fresh solvent.

Figure 1. Diagram of the freezing device. For freezing specimens in liquid nitrogen-cooled liquid propane, a used styrofoam box [1] approximately 25 cm³ was used. A piece of styrofoam [2] was placed in the bottom of the box to allow insertion of a 15-cm length of common plumbing-grade 0.5-in diameter copper pipe [4] into the styrofoam without puncturing the box. The pipe had a homemade wire basket [5] looped over its top to hold a 0.5- or 0.75-in diameter cup-shaped copper tube cap (Elkhart Products, Elkhart, IN, USA) [6], normally used for the end of copper plumbing pipes. This copper cap was filled with propane by blowing a gentle stream of gaseous propane into the cup after the space between the cup and the styrofoam box [3] had been filled with liquid nitrogen. This caused the propane to condense in the cup.

Figure 2. Light and electron micrographs of thin sections of freeze-substituted tobacco seedlings. (A) Toluidine blue-stained 0.5-μm section of a well-frozen freeze-substituted tobacco seedling. The seed coat (arrow), one cotyledon (ct) and the endosperm (e) are indicated. Bar = 100 μm. Good freezing occurs for several cell layers into the endosperm. (B) High-magnification view of endosperm cell cytoplasm showing good freezing representative of the freezing seen throughout Panels C and D. Ribosomes are well-preserved (e.g., arrow), as are the rest of the cytoplasm (c) and the vesicles (v). Bar = 100 nm. (C and D) Electron micrographs of a well-frozen tobacco endosperm cell displaying vesicles and well-preserved cytoplasmic strands (medium arrow) and ribosomes (small arrow). This preservation is seen even where several thin cytoplasmic strands come together (large arrow). Note the tight adherence of the plasma membrane (open arrow) to the cell wall (w), indicating excellent freezing. Panel C shows good freezing along 5–6 μm of this cell, depending on whether the long edge or the diagonal of the micrograph is measured, while Panel D shows 4–5 μm from a different cell. Bars = 0.5 μm.
Samples were then gradually infiltrated in a stepwise manner with increasing concentrations of either L.R. White resin (Electron Microscopy Sciences, Fort Washington, PA, USA; 1 µL of accelerator per 10 mL resin in final step to reduce rapid heating due to polymerization) or 1:4 methyl/butyl methacrylate (light microscopy; Electron Microscopy Sciences; 1% benzoyl peroxide in final step as a polymerizing agent; References 1, 3, 7 and 8). Infiltrated samples were embedded in dried gelatin capsules and polymerized by heating overnight to 60°C.

Samples were then processed for light microscopy by cutting into 0.5–1-µm sections using a Reichert OM-U2 Ultramicrotome (Reichert Ophthalmic Instruments, Buffalo, NY, USA) or for electron microscopy by cutting into 80–100-nm sections using a Reichert Ultracut E ultramicrotome. Light microscopy sections were dried onto Fisherbrand® Superfrost® Plus slides (Fisher Scientific, Pittsburgh, PA, USA), stained with 0.1% toluidine blue in 0.1 M sodium phosphate (pH 8.0) and mounted in Permount® (Fisher Scientific). Light microscopic samples were photographed using an Olympus® AD photosystem (Olympus, Lake Success, NY, USA) equipped with a home-made orange filter on Kodak Plus-X Pan film (Scientific Imaging Systems [Eastman Kodak], New Haven, CT, USA). Samples for electron microscopy were picked up on Formvar-coated nickel grids and stained for 8–10 min with 2% uranyl acetate in 1:1 ethanol/water and for 3–5 min in Reynold's lead citrate. Specimens were then viewed on a Model H-600 transmission electron microscope (Hitachi Scientific Instruments, Mountain View, CA, USA) and photographed on Kodak 4489 EM film. All prints were made using Kodak Polyclar III RC paper and Multigrade® filters (Ilford, Paramus, NJ, USA).

From Figure 2, this method clearly gives good freezing at both the light (Figure 2A) and electron microscopic (Figure 2, B and C) levels. This can be seen at both intermediate (Figure 2, C and D) and high magnification (Figure 2B) on the electron microscope. Based on experience using commercial or much more expensive devices, our device gives similar depth and freezing quality when plunge-freezing. At the electron microscopic level, good freezing has been achieved to a depth of 5–10 µm; i.e., one cell deep and sometimes into a second cell. At the light microscopic level, the appearance of good freezing occurs several cell layers deep into the frozen explant. Ribosomes, cytoplasmic strands and similar features are well-preserved (Figure 2, B–D, especially 2B).

Because one purpose of this work was to show that specimens can be prepared for immunolocalizations and because epitopes can be sensitive to the aldehyde fixatives and the OsO₄ sometimes used to improve ultrastructural preservation, these fixatives were omitted from this work. Membrane bilayers are not clearly seen in our micrographs because no OsO₄ was used to fix the lipids, though vesicle shape and structure are well-preserved (Figure 2, B–D). These additional fixatives, beyond the coagulative effect of the organic solvents used here, could simply be added to the organic solvents used for substitution.

Given the following considerations:
(i) the inexpensive parts needed to construct this device (at less than $5.00 [US]), (ii) the rapidity and simplicity with which it can be assembled (in about 30 min) and (iii) the ready availability of liquid nitrogen and gaseous propane, this device could allow a laboratory that infrequently needs to cryofix material to do so without great expense of time or money and with good results. This is especially valuable in a time when more molecular biology work includes microscopic examination of experimental material.

REFERENCES

Simultaneous Purification of RNA and DNA from Liver Using Sodium Acetate Precipitation

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Several methods for the isolation of RNA use guanidinium solutions for cell lysis to provide optimal protection from ribonucleases (2,4). It is sometimes necessary, however, that both RNA and DNA be harvested from the same tissue sample. Separation of RNA and DNA from guanidinium isothiocyanate (GITC) lysates has been achieved by cesium chloride ultracentrifugation (2,7) or by acidic phenol extraction followed by recovery of DNA from the phenol phase (1,3,6). We present an alternative method using sodium acetate precipitation. Selective precipitation of RNA using sodium acetate or lithium chloride has been previously used for RNA isolation (5,8), but we demonstrate that high-quality DNA can be obtained simultaneously.

Mouse liver tissue samples were homogenized on ice in GITC solution (4 M GITC, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% sodium lauryl sarcosinate, 1% β-mercaptoethanol). The homogenization volume was 2–4 mL/100 mg tissue. A motor-driven Teflon® pestle with a tight-fitting glass vessel was used at low speed (≤300 rpm) to homogenize the tissue with minimal shearing of the DNA. The homogenate was extracted twice with an equal volume of buffered phenol/chloroform/isooamyl alcohol (25:24:1; buffer with Tris-HCl to pH 8.2; Life Technologies, Gaithersburg, MD, USA) and once with an equal volume of chloroform/isoamyl alcohol (24:1), mixing gently by inversion. After the final extraction, 3 vol of 4 M sodium acetate (pH 7.0) were added to the aqueous layer. The sample was mixed gently, stored on ice at 4°C overnight and centrifuged at 30 min at 5000×g. The supernatant (containing DNA) was decanted into a polypropylene tube and set aside while the RNA pellet was processed, and GITC solution was added immediately to the RNA pellet (2 mL/100 mg tissue).

In samples with very little RNA, components of the GITC tissue homogenate can interfere with salt precipitation of the RNA (5). Therefore, for tissue samples <50 mg, the method was modified to include an ethanol precipitation just prior to the sodium acetate precipitation step: 0.1 vol of 3 M sodium acetate and 2 vol of ethanol were added, the sample was centrifuged for 5

Figure 1. (A) Agarose gel analysis of total RNA. Total RNA was purified by the sodium acetate precipitation method, and 10-µg aliquots were run on 1.4% agarose/6% formaldehyde gels and stained with ethidium bromide. Normal liver tissue (N) and 2 liver tumors (T1 and T2) from each of 2 mice (A and B) are shown. (B) Agarose gel analysis of genomic DNA. DNA was purified from 6 mouse liver samples (lanes 1–6) by the sodium acetate precipitation method, and 1-µg aliquots were run on a 0.8% agarose gel and stained with ethidium bromide. HindIII-cleaved λDNA (M) was used as a size marker.