African Journal of Biotechnology Vol. 10(68), pp. 15426-15429, 2 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1175
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Applicable technical method for lower temperature freeze-substitution of cryo-fixed cells

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Accepted 13 July, 2011

Cryo-fixation and freeze substitution followed by microscopy are commonly used sample preparation methods for visualizing the morphology of intracellular organelles. Freeze substitution is an especially important preparative step because it enables the preservation of intracellular structures in cryo-fixed cells close to the living states for visualization. In this study, we describe a novel freeze substitution method and device that uses both dry ice and liquid nitrogen as coolants rather than dry ice alone. We found that our approach achieved lower temperatures for longer periods of time than conventional devices and therefore allows major improvements in preservation quality, ultimately facilitating high contrast imaging of intracellular organelles. The technical approaches that are realized by this valuable approach are simple, rapid, and applicable for visualizing cellular events of interest.

Key words: Cryo-fixation, high pressure freezing, freeze substitution, electron microscopy.

INTRODUCTION

A variety of sample preparation methods including conventional chemical fixation (Hayat, 1989) and cryo-fixation techniques (Quintana, 1994) are available for the microscopic visualization of intracellular organelles. Cryo-fixation, plunge freezing, propane jet freezing, cold metal block freezing, and high pressure freezing provide advantages over chemical fixation because they offer good preservation of ultrastructures within cells (Porta and López- Iglesias, 1998; Ding et al., 1991; Kiss et al., 1990). Among these methods, combinations of high pressure freezing (HPF) and freeze substitution (FS) are excellent commonly used cryofixation methods (McDonald and Morphew, 1993; Parthasarathy et al., 1995). High pressure frozen cells can be prepared as frozen hydrated specimens by FS which is the process of dissolving ice within frozen samples by removing water with organic solvents at low, temperatures and reacting fixatives with cellular components while raising the temperature (Giddings, 2003). Optimized transfer temperature during preparation is a crucial factor in order to obtain high yields of adequately frozen specimens, because intracellular organelles are surrounded by vitreous ice and their ultrastructures can be seriously damaged by growing ice crystals (Steinbrecht, 1993).

Vitreous ice is produced by the rapid freezing of liquid solution and is converted to cubic ice at temperatures higher than -135°C, which in turn starts to change into hexagonal ice at -80°C (Cavalier et al., 2009). To avoid damage to the ultrastructures of intracellular organelles by the formation of hexagonal ice crystals, the dehydration steps of FS should be perform below -80°C for several hours (Robards and Sleytr, 1985).

Here, we describe a new device and method for achieving and retaining optimal dehydration temperature that uses both dry ice and liquid nitrogen in an insulated box. In this coolant system, dehydration may be maintained below -80°C for 22 h; enough time to facilitate good ultrastructural preservation of frozen cells. Although commercial FS systems equipped with liquid nitrogen cooling are available, these often incur high expenses in

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Abbreviations: HPF, High pressure freezing; **FS,** freeze substitution; **TEM,** transmission electron microscopy.

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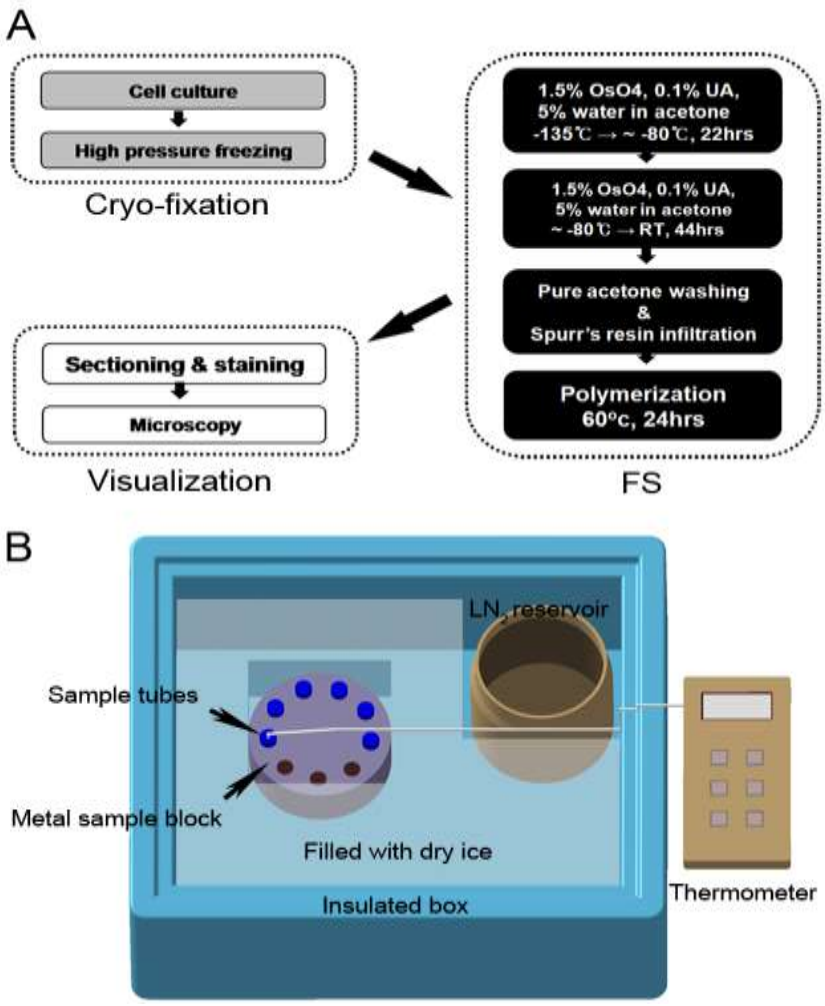

Figure 1. Overview of sample preparation steps (A) and the home-made FS device (B) used in this study. The detailed methods summarized in (A) are described in the materials and methods.

purchase and maintenance. Other home-made devices using only dry ice as a coolant are also often used, but most achieve a low temperature for dehydration starting at only -78°C and are characterized by low reproducibility of adequately frozen specimens. Our technical approach, which uses both dry ice and liquid nitrogen as coolants, is characterized by a high success rate and is therefore suitable for the applications in which consistently excellent preservation of intracellular ultrastructures is required.

MATERIALS AND METHODS

Sample preparations using HPF and FS

MRC 5 cells were cultured as previously described (Jayarama et al., 2006). For high pressure freezing, cells were grown while attached to 3.0 mm sapphire discs (Technotrade International, Inc., USA). The bottom of each sapphire disc was placed on the flat side of a 0.3 mm aluminum platelet, and the cell side of the disc was covered with a 0.1 mm aluminum platelet. The space between the sapphire disc and the 0.1 mm aluminum platelet was filled with 20% bovine serum albumin (Sigma-Aldrich, USA) in phosphate-buffered saline. The discs and platelets were frozen using HPM 010 (Bal-Tec, Liechtenstein) and stored in liquid nitrogen prior to FS. 2 ml of the substitution medium in 3 ml screw cap tubes were frozen in liquid nitrogen. The frozen discs were placed on top of the frozen medium. For FS, the tubes were transferred to a metal sample block that was pre-cooled to -135°C with 12 kg of dry ice and 700 ml of liquid nitrogen in an insulated box (35 × 25 × 25 cm; Figure 1B). The liquid nitrogen chamber was filled with 1, 200 ml of liquid nitrogen. Samples were freeze-substituted in acetone containing 1.5% OsO_4, 0.1% uranyl acetate, and 5% water for 22 h. After placing 3 kg of dry ice under and around the metal sample block, most of the dry ice was removed and the temperature was raised to 20°C for 44 h. The samples were washed three times in anhydrous acetone for 10 min each and then infiltrated according to the following steps: 2 parts anhydrous acetone + 1 part Spurr's resin (Electron Microscopy Science, USA) for 2 h; 1 part anhydrous acetone + 1 part Spurr's resin for 2 h; 1 part anhydrous acetone + 2 parts Spurr's resin for 4 h; 100% Spurr's resin for 4 h (twice); and Spurr's

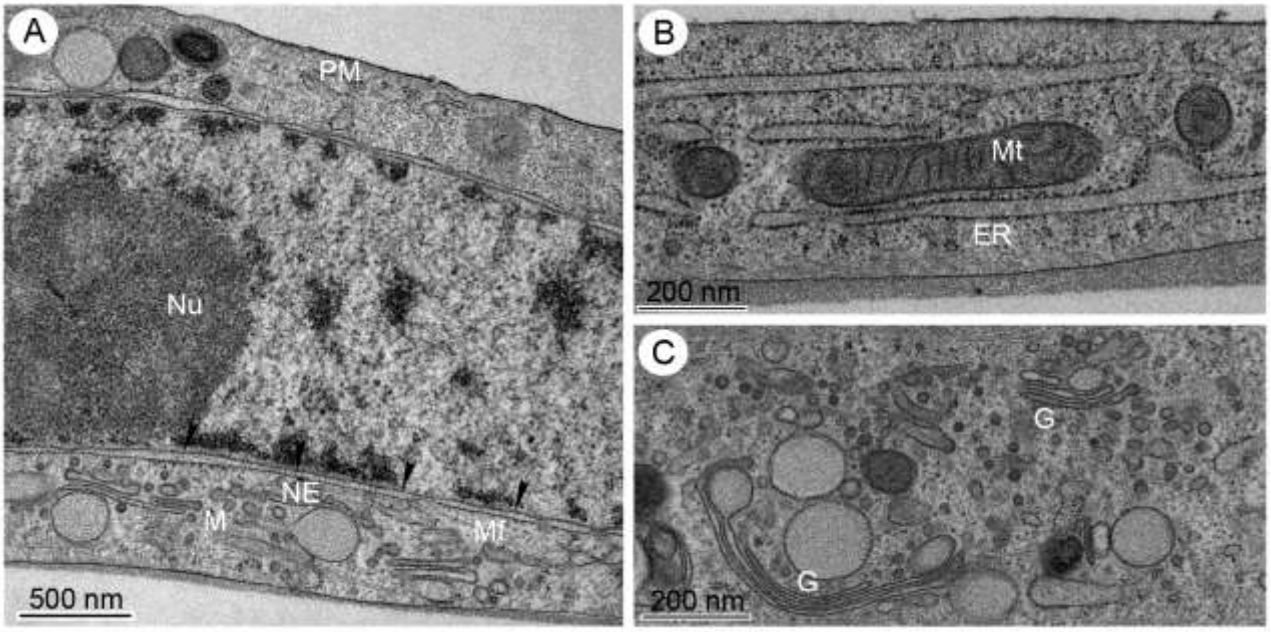

Figure 2. Intracellular ultrastructures observed during cryo-fixation using our novel FS technique followed by HPF. (A-C) Micrographic images of MRC 5 cells showing well defined structures of each organelle: plasma membrane (PM), nucleus (Nu), nuclear envelope (NE), microtubules (M), microfilaments (Mf), mitochondria (Mt), endoplasmic reticulum (ER), and Golgi apparatus (G). The arrow heads in (A) indicate a widened perinuclear envelope, which may be induced by adding 5% water to the substitution media (Zechmann et al., 2007), and a well-preserved ER with attached ribosomes is shown in (B). Scale bars represent 500 nm and 200 nm in (A) and (B and C), respectively.

resin overnight. The sapphire discs were embedded in Spurr's resin and polymerized at 60°C for 24 h.

Electron microscopy

The sapphire discs were removed by immersion in liquid nitrogen. Ultrathin sections (70 nm thin-sections) were cut on a Ultracut-S microtome (Leica, Austria) using ultra 35° diamond knife (DiATOME, Switzerland) and collected on formvar coated copper grids. It was followed by staining with aqueous solution of 2% uranyl acetate for 10 min and Reynolds' lead citrate (Reynolds, 1963) for 5 min. They were examined in Tecnai G^2 Spirit Twin transmission electron microscopy (FEI, US) operated at 120 kV. Images were recorded on 4Kx4K Ultrascan 895 CCD (Gatan, U.S).

RESULTS AND DISCUSSION

We devised and tested a simple home-made FS device (Figure 1) for the preservation of ultrastructures in high pressure frozen cells. Our device uses a mixture of dry ice and liquid nitrogen as coolants, while conventional devices generally use only dry ice alone, to maintain temperatures below -80°C. As the coolant is exhausted, the temperature slowly increases and dehydration occurs in the blocks of frozen cells. Under our coolant system, dehydration begins at -135°C, when the liquid nitrogen chamber is fully filled. In practice, frozen cells were dehydrated for 22 h; the temperature was raised from -135 to -90°C rapidly and then slowly raised to -80°C and held there for 22 h. Images of the resulting specimen preparations are shown in Figure 2. The plasma membrane (PM) and nuclear envelope (NE) appear as continuous and smoothly outlined characteristics that are known advantages of cryo-fixed samples (Figure 2A). Our images of darkly stained mitochondria (Mt; Figure 2B) and well-defined Golgi apparatus with vesicles (G; Figure 2C) in well-frozen areas demonstrate that intracellular organelles were highly preserved without extraction or ice damage by our device and method.

The aim of this study was to develop a FS device that uses a double coolant system (Figure 1). Our technical approach allows cryo-fixed biological samples to be held longer at -80°C, ultimately resulting in the visualization of well-preserved intracellular organelles by preventing ice crystallization during dehydration (Figure 2). Our FS technique is an effective sample preparation method with an excellent success rate (~80%) for preparing adequate cryo-fixed samples at low cost. It is therefore suitable for preparing samples intended to be used in structural characterizations of intracellular organelles and the localization of nano-materials within cells.

ACKNOWLEGEMENTS

This work was supported by Korea Basic Science

Institute grant (T3121A) to HS Jung.

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