Cryofixation and Freeze Substitution for Ultrastructural Studies of *Neurospora crassa* hyphae

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Introduction

Cryofixation has long been known to provide a much-improved structural and biochemical preservation of cell that is closed to its living state relative to aqueous fixation protocols (Robards and Sleytr, 1985; Giddings, 2003; Gilkey and Staehelin, 1986; Roberson and Chandler, 1998). If ultra-rapid cooling rates, i.e., 10,000 ^{o}C/s, are achieved, intracellular ice crystals formed within the cell upon freezing would be smaller than 3 to 5 nm, thus fulfilling the minimal resolution required for ultrastructural studies of thin-sectioned, e.g., 60 nm thick, resin-embedded biological samples. Not only cytoplasmic features and rapid cellular processes can be visualized with high fidelity but also immuno-reactivity may be retained. Failure to obtain such ultra-fast freezing rates can result in formation of large ice crystals and structural damages in up to 80% or more of cryofixed cells. These artifacts can be apparent ultrastructurally as distorted and fractured in membranes, granular or vacant areas of the cytosol and organelle matrix, collapsed and discontinuous cytoskeletal elements, and fractured cell walls. However, selection of well-preserved cells is done with careful light microscopic screening prior to sectioning (Howard and O'Donnell, 1987), which can eliminate sectioning of poorly preserved cells in many cases.

Five commonly used cryofixation methods are plunge (or quench) freezing, spray freezing, double propane jet freezing, cold metal block freezing (slam or impact freezing), and high-pressure freezing (Gilkey and Staehelin, 1986; Robards and Sleytr, 1985; Roberson and Chandler, 1998). Here we describe manual plunge freezing and freeze substitution protocols used in our lab for ultrastructural studies of germling and hyphal cells of *Neurospora crassa* grown on small membrane supports. These methods are fairly simple, economical, and can successfully freeze monolayer of cells that are no more that 12 to 15 μm in their thinnest dimension.

Precautions

It must be strongly emphasized that using cryogens and chemicals in preparing biological cells and tissues for ultrastructural studies should be handled with extreme care and with full knowledge of the potential hazards. Training from an experienced individual is highly recommended. Handling cryogens can lead to cold burns and to rapid gas expansion, which can be explosive. Fixatives can also 'fix' your tissues just as easily as they can to your specimen's. All workers are advised to read the 'Appendix' on safety by Robards and Sleytr (1985).

Initial Preparation

1. Preparation of Fixatives:

The common fixatives used for standard structural analysis are heavy metals, e.g., uranyl acetate, osmium tetroxide, aldehydes that cross-link proteins, e.g., glutaraldehyde, organic solvents, e.g., acetone, and tannic acid. For immunocytochemical approaches, refer to Skepper (2000), Monaghan et al. (1998) and McDonald (1994).

Prepare freeze substitution (FS) solution prior to cryofixation in appropriate sized vials that can withstand ultra-low temperatures (–85 to –90 °C) and organic solvents. We use simple 25 ml polypropylene scintillation vials. Freeze substitution solutions should be used as soon as possible and long term storage should be avoided. However, if required, freeze substitution solutions can be stored in LN_2 for later use. Great care must be taken when mixing the FS solution. It is mandatory to work in a properly operating fume hood, utilize eye protection, and wear appropriate lab clothing and gloves.

There are many choices of FS solutions. In our lab we often employ a dual stage FS protocol where two solutions are prepared according to Fields et al. (1997). These include a) *Solution One*: 1% tannic acid and 1% glutaraldehyde in anhydrous EM grade acetone (Ted Pella, Inc. Redding, CA) and b) *Solution Two*: 1% osmium tetroxide in anhydrous EM grade acetone (*note: Once osmium and other chemicals are dissolved in acetone, they can easily penetrate the skin upon contact and can quickly disperse into the air; thus, when working with open vials of such solutions, always wear acetone-resistant gloves and work in a properly operating fume hood).* The advantage of using the dual stage FS protocol is that endomembranes are often very well contrasted. Alternatively, a more simple protocol can be used, i.e., single FS solution of 2% osmium tetroxide and 0.05% uranyl acetate in anhydrous EM grade acetone (Hoch 1986). Preparation of the dual stage FS solution for a single sample is described below.

Solution One:

i. In the fume hood, pipette 18 ml of anhydrous acetone into a FS vial. (Note: 100% acetone is maintained and stored in a dissector in screw cap glass containers over molecular sieve)
ii. Add 2 ml of 10% glutaraldehyde (GA) in anhydrous acetone (Electron Microscopy Sciences, Hatfield, PA). The 10% GA/acetone stock solution is purchased in sealed amber ampoules. If you do not have an ampoule breaker, wrap several layers of paper towels around the ampoule before breaking it. Most chemicals supplied in such ampoules come pre-scored and are easy to open. Any excess solution can be stored in appropriate vials in LN_2.
iii. Add 0.1 g of tannic acid. Tannic acid is dissolved into acetone quite easily with brief sonication.

Solution Two:

i. In the fume hood, pipette 18 ml of anhydrous acetone into one FS vial and10 ml to the other and store them in the freezer until chilled (*Note: chilling acetone helps prevent premature oxidation of the osmium tetroxide*).

ii. Break open an ampoule containing 1.0 g of osmium tetroxide crystals and pour the crystals into 10 ml of chilled acetone to make 10 % osmium tetroxide in anhydrous acetone. Osmium tetroxide crystals should dissolve quickly in the chilled acetone.

iii. Add 2 ml of 10 % osmium tetroxide in anhydrous acetone into the FS vial containing 18 ml of chilled anhydrous acetone.

Washing solutions:

As mentioned below, cells need to be thoroughly washed three times with anhydrous acetone at 85 °C prior to the incubation in solution II; thus, at this time pipette 20 ml of anhydrous acetone into three FS vials each and store them at 85 °C.

Place all vials (total of five) in a standard ice bucket or Styrofoam box with the bottom and sides lined with dry ice and store them at –85 °C to –90 °C in an ultra-low freezer for at least one hour prior to the cryofixation. These solutions may be kept at –85 °C for up to one week, however it is strongly advised to use them as soon as possible. We also suggest to either purchase or have your machine shop to make a metal, e.g., aluminum, block holder for these vials. The metal block functions as a holder so that the vials won't tip over thus avoiding the possibility of fixative spilling in your freezer. It also functions as a protection against unwanted temperature increases. *(Note: Since there is always a chance that osmium vapors may leak into the space surrounding the box, it is recommended to have tightly fitting lid on the box. For this reason, the freezer used for FS should not be used for storage of proteins, antibodies or other sensitive materials.)*

2. Growing and handling *N. crassa* cells prior to plunge freezing:

Regardless of the cell type, all samples should be in optimal growth or experimental conditions just prior to cryofixation. Rapid plunge freezing of hyphae and/or germlings into a cryogen can often result in superb results because these cell types can be easily grown as monolayer supported membrane that is permeable to water and nutrients. *(Note: The support membranes should be thin (20 µm) yet rigid enough not to flex while transporting cells from the culture and during freezing. Single-thickness dialysis tubing works well.)*

Support membranes should be prepared by cutting the membrane into ~2 cm lengths and deionized by boiling in deionized water containing 0.1 M EDTA for 1 hour, followed by an additional 7-8 hrs of boiling in water. The water should be changed 6 to 7 times during this period. After complete cleaning, the membranes are placed in beaker containing fresh deionized water and autoclaved for 30 min. The deionized membrane should be stored at 4 °C.

Next, the membrane is placed onto the surface of Vogal's complete medium (VCM) and cut into smaller 5 x 5 mm pieces using a sharp scalpel or razor blade. Some patience is required during this process since the membrane is quite tough and uncooperative to cut sometimes. Now, conidia or small fragments of viable hyphae can be aseptically transferred onto of dialysis membrane. Cryofixation can proceed once conidia have germinated or mature hyphae have produced. Close monitoring of cell growth is required

because one should not allow germlings or hyphae to grow over the edge of the membrane. (*Note: In our hands **N. crassa** is extremely sensitive to any environmental change. Therefore, extreme care should be paid to handle the specimen. Any sign of stress, i.e., alterations in growth rate and cytoplasmic organization will be visible at both the light and electron microscope levels).*

Set Up for Plunge Freezing *(Note: All the procedures must be done in a spark-free fume hood.)*

i. Place a 1 L stainless steel dewar in the fume hood and fill the dewar with LN_2.
ii. Slowly immerse a cryogen reservoir into the dewar. The cryogen reservoir is designed to be built deep so that it provides for the continued decent of specimens through the cryogen bath at the initial entry velocity; moreover, it provides enough room for a propeller of cryogen air-driven stirrer to stir cryogen constantly (Howard and O'Donnell, 1987). Such cryogens reservoirs can easily be made by a university machine shop. Try not to splash LN_2 into the cryogen reservoir.
iii. Cover the dewar with a Plexiglas disc, which has two holes cut in it. One hole is centrally positioned and should be the same size as the outside diameter of the cryogen reservoir. A second hole located at the margin of the cover is used for the addition of LN_2 to the dewar via a polypropylene funnel. The Plexiglas disc should form a secure fit across the dewar and the reservoir to prevent any venting of cold nitrogen.
iv. Because of its low cost, availability, and favorable thermal conductivity, propane is recommended as the cryogen of choice. *(Note: Use extreme caution working with propane. It is strongly recommended that each freezing session does not exceed 30 to 40 min in time and that a gentle, yet constant stream of gaseous nitrogen should be directed over the propane surface; both measures are to lessen the amount of oxygen condensing into the cold liquid propane).* Slowly add propane gas into the reservoir by placing the nozzle on the bottom of the pre-cooled cryogen reservoir until a puddle of condensed liquid propane forms. As the reservoir begins to fill, increase the flow rate until the reservoir is completely full. Maintain the level of LN_2 at or just below the level of propane throughout the filling and freezing procedures.
v. Shut off the valves of propane tank and retract nozzle from the reservoir.
vi. Immerse the propeller of cryogen air-driven stirrer, e.g., Cole-Palmer Instrument, R-4520-30 I, into liquid propane and position it near the bottom of the reservoir. It is important to maintain good circulation of the cryogen throughout during the freezing session.
vii. Submerge some type of specimen basket into the cryogen to store temporary holding of frozen samples, which can be later transferred into the freeze substitution solution. Polypropylene cryovial vials (2 ml; Ted Pella) previously pierced with small holes work well.
viii. Monitor the temperature of liquid propane using a low temperature thermocouple. The temperature should be in the –188 to –192 °C range.

Plunge Freezing

i. Check the hyphae to make sure that they are in optimal growth conditions.

ii. For plunge freezing, grab the membrane with forceps and rapidly plunge it into the propane bath (make sure that no hesitation occurs!). Entry velocity and plunge depth are critical. Plunge the samples so that they enter the fluid slightly off from the vertical entry. Each plunging requires forceps, which are dried completely and at room temperature. For this reason, it is useful to have 5-10 pairs of forceps (curved Dumont stainless steel forceps work well). Place a cryofixed sample in the specimen basket after each plunging.
iii. Remove specimen basket when it gets full; drain any excess propane by shaking once or twice and transfer to either LN_2 (for long time storage) or directly into the FS solution (for dehydration).
iv. Allow each freezing session to last no longer than 30-40 min.
v. At the end of the freezing session, remove the copper pipe reservoir from the LN_2 dewar using a pair of tongs, place it in the metal tray, and leave it in the rear of the hood. In this way, the propane evaporates, and the dilute inflammable vapors can safely escape.

Freeze Substitution and Resin Embedment

If you are not planning to do freeze substitution immediately after the plunge freezing, frozen samples must be kept at colder than -100 °C at all times; therefore, the frozen samples must be kept in LN_2. Carefully but fast when transferring the frozen samples from one container to another since the time that it takes for the thin samples to warm up to about -100 °C is very short. If large numbers of samples are processed on a routine basis, it is advisable to make an investment to purchase a commercial FS unit.

i. Using forceps, pick up the specimen basket containing the frozen samples from the LN_2 bath and quickly transfer it into the Solution One (1%GA, 1% tannic acid in acetone) in dry ice held in an the aluminum block in the ice bucket; this has to be done quickly in the fume hood. Do not screw the lid too tight.
ii. Transfer the ice bucket back to the ultra-low freezer and leave it there for 48 to 72 hrs.
iii. Wash out the GA and tannic acid from the samples by transferring the specimen basket through the series of three cold acetone baths. Make sure to maintain the ultra-low temperatures. Each step of rinsing should last for ~15 min.
iv. Transfer the specimen basket to the Solution Two (1% osmium tetroxide in acetone) and let it incubate for 1 hr at ultra-low temperatures.
v. Allow the sample to warm up slowly to room temperature by first moving the ice bucket to -20 °C freezer. We suggest two options at this time. One option is to leave the sample at -20 °C for 12 to 24 hrs so that the container and its contents slowly warm up to -20 °C as the dry ice gradually evaporates. The other option is to remove the FS vial from the ice bucket and place it into a second ice bucket that has been placed in the -20 °C freezer for at lease 1 hr prior. In this case, allow the sample to warm up to -20 °C over a period of two hrs.
vi. Transfer the sample to a 4 °C refrigerator for 2 hrs.
vii. Transfer the sample to room temperature for 1 hr in the fume hood. By this time, the color of the osmium solution should have turned from yellow to brown.
viii. Wash out the osmium solution through three changes of anhydrous acetone 15 min each.

ix. Procedures of epoxy resin infiltration, embedment, and cell selection have been well described in other reviews. For details of there procedures, please refer to both Hoch (1986) and Howard and O'Donnell (1987).

References

Fields, S.D., Strout, G.W., and Russel, S.D. 1997. Spray-freezing freeze substitution SFFS of cell suspensions for improved preservation of ultrastructure. Micros. Res. Tech. 38: 315-528.

Hoch, H.C. 1986. Freeze-substitution of fungi. In Ultrastructure techniques for microorganisms (ed. H.C. Aldrich and W.J. Todd), pp. 1-16 Plenum Press, New York

Howard, R.J. and O'Donnell, K.L. 1987. Freeze substitution of fungi for cytological analysis. Exp. Mycol. 11:250-269.

Giddings T. H. 2003. Freeze-substitution protocols for improved visualization of membranes in high-pressure frozen samples. J Micro. 212:53-61

Gilkey, J.C. and Staehelin, A. 1986. Advances in ultra-rapid freezing for preservation of cellular ultrastructure. J. Electron Microsc. Tech. 3: 177-210.

McDonald, K.L. 1994. Electron microscopy and EM immunocytochemistry. Methods Cell Biol 44: 411-44

Robards, A.W. and Sleytr, J.B. 1985. Low temperature methods in biological electron microscopy. Pract. Methods Electron Microsc. Vol 10.

Roberson, R.W., Chandler, D.E. 1998. Rapid freezing and deep etching of cells and molecules. *In*: Cell Biology: A Laboratory Handbook. *Eds*. D. Spector, R. Goldman, and L. Leinwand. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. (pp.129.1-129.23).

Skepper, J.N.. 2000. Immunocytochemical strategies for electron microscopy: choice or compromise. J Microsc. 199: 1-36