Video Article

Tandem High-pressure Freezing and Quick Freeze Substitution of Plant Tissues for Transmission Electron Microscopy

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Abstract

Since the 1940s transmission electron microscopy (TEM) has been providing biologists with ultra-high resolution images of biological materials. Yet, because of laborious and time-consuming protocols that also demand experience in preparation of artifact-free samples, TEM is not considered a user-friendly technique. Traditional sample preparation for TEM used chemical fixatives to preserve cellular structures. High-pressure freezing is the cryofixation of biological samples under high pressures to produce very fast cooling rates, thereby restricting ice formation, which is detrimental to the integrity of cellular ultrastructure. High-pressure freezing and freeze substitution are currently the methods of choice for producing the highest quality morphology in resin sections for TEM. These methods minimize the artifacts normally associated with conventional processing for TEM of thin sections. After cryofixation the frozen water in the sample is replaced with liquid organic solvent at low temperatures, a process called freeze substitution. Freeze substitution is typically carried out over several days in dedicated, costly equipment. A recent innovation allows the process to be completed in three hours, instead of the usual two days. This is typically followed by several more days of sample preparation that includes infiltration and embedding in epoxy resins before sectioning. Here we present a protocol combining high-pressure freezing and quick freeze substitution that enables plant sample fixation to be accomplished within hours. The protocol can readily be adapted for working with other tissues or organisms. Plant tissues are of special concern because of the presence of aerated spaces and water-filled vacuoles that impede ice-free freezing of water. In addition, the process of chemical fixation is especially long in plants due to cell walls impeding the penetration of the chemicals to deep within the tissues. Plant tissues are therefore particularly challenging, but this protocol is reliable and produces samples of the highest quality.

Video Link

The video component of this article can be found at https://www.jove.com/video/51844/

Introduction

Our knowledge of cell ultrastructure comes mainly from electron microscopy, which can resolve details in the range of a few nanometers [1]. Despite being so powerful in resolution TEM is not considered user-friendly, as sample preparation requires time-consuming and laborious protocols, and demands some expertise from the practitioner. Traditional fixation of samples has combined the use of aldehydes and osmium tetroxide before further processing that includes dehydration, embedding in resin and then sectioning to produce ultra-thin sections that are then stained with heavy metals. However, it is known that chemical fixation can produce artifacts including protein aggregation and loss of lipids [1], and changes to membranes that ultimately affect several cellular compartments [2]. These artifacts are largely attributed to the slow rate of fixation and dehydration at room temperature [3,4,5].

Cryofixation by high pressure freezing (HPF) avoids most of the artifacts caused by chemical fixation. The principle of cryofixation is that it lowers the freezing point of water by 20 degrees, slows down the nucleation and growth of ice crystals and increases the viscosity of water in a biological sample so that cellular constituents are essentially immobilized [6, 7]. HPF decreases a sample's temperature to that of liquid nitrogen, under very high pressure (210 MPa or 2,100 bar) in milliseconds. When done properly HPF prevents formation of large ice crystals that can cause major damage to cell ultrastructure. HPF can be used to fix samples of 100-200 µm thickness at typical concentrations of biological solutes [7]. There are numerous reviews on the physics and principles underlying HPF, *e.g.*[1,7,8].

After HPF, samples are incubated at low temperature (-78.5 °C to -90 °C) in the presence of liquid organic solvent containing chemical fixatives like osmium tetroxide, generally for a few days. At this low temperature, the water in the sample is replaced by the organic solvent, typically acetone or methanol [1,9]. Thus, this process is called freeze substitution (FS). The sample is then gradually warmed and during this time is fixed, usually with osmium tetroxide and uranyl acetate [9]. Crosslinking at low temperatures has the advantage of fixing molecules that are immobilized [1]. FS therefore produces samples of superior quality compared to those fixed by conventional chemical fixation at room temperature, in particular it results in improved ultrastructural preservation, better preservation of antigenicity and reduced loss of unbound cellular components [10,11].

Most FS is carried out over long time periods, typically up to several days. This is particularly true for plants samples [12,13,14]. A recent protocol developed by McDonald and Webb greatly reduces the time for FS from several days to a few hours [15]. In their quick freeze substitution (QFS) procedure, FS is carried out over 3 hours, while in the super quick FS (SQFS) samples are processed in 90 minutes. The quality of samples produced by these methods is comparable to those yielded by traditional FS protocols. We have adopted the QFS protocol for downstream processing of plant samples after HPF. This has proven to save not only time but also money, as QFS and SQFS use common lab equipment instead of the costly commercially available FS machines.

Plant tissues are often very challenging to prepare for TEM. On average, plant cells are bigger than either bacterial or animal cells. The presence of hydrophobic waxy cuticle, thick cell walls, large water-filled vacuoles containing organic acids, hydrolases and phenolic compounds that may occupy up to 90% of the total cell volume [16], and the presence of aerated spaces severely decreases heat conductivity of the system [17]. Further, in the case of plants, the sample thickness almost always exceeds 20 µm, the limit for use of chemical fixation. At these thicknesses, the low heat conductivity of water prevents a freezing rate more than –10,000 °C/sec in the center of the sample. That rate is required to avoid damaging hexagonal ice formation (ice crystals with a lower density and bigger than 10 to 15 nm) [8]. Together, these present challenges to both proper freezing of the sample and subsequent FS. Nonetheless, cryofixation is the best method for fixing plant samples. Here a protocol for HPF-QFS of plant tissue samples is presented. It focuses on the model species *Arabidopsis thaliana*, but has also been used with *Nicotiana benthamiana*. The typical results demonstrate that HPF-QFS produces samples of comparable quality to traditional HPF-FS in a fraction of the time. With proper adjustments, this protocol may also be used for other relatively thick biological samples.

Protocol

NOTE: The QFS procedure requires extreme care and caution by the user and we highlight these safety precautions here as Cautions and Notes where applicable.

1.Preparation for HPF Run

1. Before beginning sample preparation, turn on the high-pressure freezer following manufacturer's instructions.
   NOTE: The HPF unit used in this protocol is a Wohlwend Compact 02 unit (**Figure 1A**), and it takes approximately one to one-and-a-half hours of start-up procedure before HPF runs can commence.
   1. Switching on the Wohlwend Compact 02 High Pressure Freezer.
      1. Switch on the instrument by turning the main switch from "**0**" to "**1**".
      2. Release compressed air to the machine.
      3. Release liquid nitrogen to the machine.
      4. Press the "SYSTEM DRY UP" button.
      5. After 30 min, press the "SYSTEM DRY UP" button again.
      6. Press the "ON/OFF" button to switch on the instrument.
      7. Press the "NITROGEN" button.
      8. Allow the machine to cool down for 20 min even if the "DRIVE IN" button has already lit up.
      9. The "DRIVE IN" button lights up.
      10. Press the "DRIVE IN" button.
      11. Press the "AUTO" button.
      12. The "SYSTEM READY" button lights up.
      13. Place the temperature-and-pressure probe into the pressure chamber and lock it with the pin.
      14. Press the "JET AUTO" button. This step checks that the pressure increase and temperature decrease are as desired; essentially a trial run. Sharp drops in temperature and sharp increases in pressure are expected (**Figure 1B**).
      15. Carry out two more JET cycles.

2. Preparation for Receiving Frozen Samples

1. Fill an insulated box containing cryovial holders with liquid nitrogen (see Safety Warnings), so that the holders are completely covered (**Figure 1C**).
   NOTE: Use PPE including cryogloves and goggles when handling liquid nitrogen.
2. Place the appropriate number of vials containing the FS medium into the aluminum tube holders in the liquid nitrogen (**Figure 1C**). With this protocol up to four discs each containing a single sample can be placed in a single cryovial.
   NOTE: The vials should be labeled with a sharp instrument like a diamond-tipped scribe and very soft pencil.
   1. For fixative during QFS use 1% OsO_4 and 0.1% uranyl acetate in acetone [9]. Prepare the solution in large volume, dispense aliquots of 1.5 ml into cryovials and store frozen in liquid nitrogen.
      NOTE: Follow Safety Warnings for handling OsO_4 and uranyl acetate.
      NOTE: OsO_4 and uranyl acetate are dangerous chemicals and should be handled in the fume hood while wearing appropriate personal protective equipment (PPE) including closed-toed shoes, lab coat and gloves.
      NOTE: The cryovials used to hold samples for QFS should have a hard O-ring to ensure that the tubes remain sealed during QFS to avoid leakage of OsO_4.

3. Prepare yeast paste by mixing baker's yeast with a roughly equal volume of 10% methanol with a toothpick or other such instrument until the paste is smooth. The yeast paste acts as an extracellular cryoprotectant and is used to fill the space around the sample in the specimen

carrier. The amount of paste depends on the number of samples; for 10 samples or fewer the paste (1 ml) can be mixed in a microcentrifuge tube.

3. High-pressure Freezing of Samples

1. Remove a leaf (or other tissue) of interest from the plant and gently place it on a piece of dental wax or other cutting surface. Use a punch of 2.0 mm or desired size to cut a sample out of the leaf. Handle the sample gently with a pair of forceps and work as quickly as possible.
2. Working under a dissecting microscope, place the leaf disc in the 0.2 mm side of the Type A specimen carrier and cover completely in yeast paste. Ensure that the disc is completely filled and the paste is level with the rim of the holder by smoothing out the paste with a fine paintbrush (**Figure 2**).
3. Place the carrier in the specimen holder. Cover the sample with the Type B specimen carrier, flat surface down.
   NOTE: The specimen holder should be dry and at room temperature.
4. Take sample in specimen holder, insert it into machine and initiate a freezing cycle by pressing the “JET AUTO” button, which should complete in a second or two.
5. Working as quickly as possible, remove the holder from the machine and place the tip holding the sample into liquid nitrogen in the insulated box on top of the HPF machine. Immerse the tips of two pairs of forceps in the liquid nitrogen to chill them.
   NOTE: After freezing, the carriers should only be handled with liquid nitrogen-cooled forceps. Warm (room temperature) forceps are often the cause of failure in cryotechniques.
6. Open a cryovial containing the FS media, and place the lid on the side of the box. With the aid of a pair of liquid nitrogen-cooled forceps, gently remove the disc from the specimen holder, ensuring that the disc is always in the liquid nitrogen or vapor. Working in the liquid nitrogen vapor, hold the FS tube with one pre-cooled forceps and use the other to place the holder in the FS vial. Screw the lid of the FS vial back on. Do not trap any liquid nitrogen in the vial.
   NOTE: When transferring samples to cryovials for QFS, ensure that no liquid nitrogen is trapped in the vials. Liquid nitrogen expands 700-fold during warming and any trapped liquid nitrogen can cause explosions during this time.
7. Repeat steps 3.1 to 3.7 until all desired samples are frozen. Multiple leaf discs containing the same type of sample can be placed in the same vial. Note that the specimen holder needs to be dried and brought to room temperature between freezing runs. To do this quickly, heat it with a blow dryer, and monitor its temperature by touch.

4. Preparation for Freeze Substitution

1. Completely immerse the aluminum heater block in liquid nitrogen for 10 min or until nucleate boiling stops. While this is going on, place a layer of dry ice (1-2 cm) in the bottom of the container used as the FS chamber (**Figure 3**). A styrofoam container or ice bucket can be used for FS, along with crushed dry ice or pellets.
2. Quickly insert the cryovials containing the samples along with the temperature probe into the middle rows of the heater block. Be sure that the lids on the vials are tightly screwed on so that the FS media does not leak out during FS. Begin recording the temperature.
   1. Make the temperature probe by placing a thermocouple through the top of a cryovial so that it reaches the bottom of the tube. Seal the lid of the tube with resin so that no liquid leaks out. Fill the tube with 1.5 ml acetone at the beginning of each QFS run.
3. Using insulated cryogloves or large forceps, pour out all the liquid nitrogen from the heater block. Take care not to pour out the cryovials.
   NOTE: Use PPE including cryogloves and goggles when handling liquid nitrogen.
4. Place the block containing the vials onto the layer of dry ice in the QFS chamber. Make sure the block is placed so that the tubes are lying horizontally but with a slight upward tilt. There should be no leakage if the correct cryovials are used.
5. Pack the QFS chamber with dry ice so that the FS tubes are covered, although it is not necessary to cover the top of the block. Place the lid on the chamber.

5. Quick FS

NOTE: Perform the QFS run in a fume hood in the event that any leakage of OsO_4 inadvertently occurs despite other precautions.

1. Place QFS chamber on a platform rotary shaker in a fume hood and rotate at 125 rpm for 120 min. During this time the temperature of the block should increase gradually to about -80 °C. The agitation ensures mixing of the components for better FS.
   NOTE: The placement of the shaker in the fume hood and the position of the fume hood door should be the same throughout the QFS procedure and for every QFS run to ensure consistent warming of samples.
2. Remove the dry ice from the chamber and continue shaking for another hour.
   NOTE: the temperature should increase to about -15 °C to -20 °C.
3. Remove the samples and temperature probe from the QFS chamber and place on the shaker at room temperature for another 10-15 min, until they reach room temperature. Stop recording the temperature.
4. During FS, turn off the HPF machine.
   1. Close the tap of the liquid nitrogen tank while the HPF machine is being filled with nitrogen.
   2. Press the “NITROGEN” button.
   3. Wait until the red “PISTON DOWN” light extinguishes.
   4. Press “ON/OFF” button.
   5. Press the “SYSTEM DRY UP” button.
   6. Cut off the supply of compressed air to the machine.
   7. After a minimum of 12 hr (to ensure drying of any moisture), switch off the machine by turning the main switch from “1” to “0”.

6. Post FS Processing

1. Carefully remove the FS media with plastic transfer pipettes and place in the appropriate container for toxic waste.
   NOTE: OsO_4 and uranyl acetate are dangerous chemicals and should be handled in the fume hood while wearing appropriate personal protective equipment (PPE) including closed-toed shoes, lab coat and gloves.
2. Wash samples four times with 100% acetone. Collect the first two washes and place in the toxic waste container.
3. Using fine forceps, remove the tissue samples from the holders. Keep samples wet with acetone as this is done, and work very gently to avoid breaking samples.
   NOTE: It is not unusual and it may actually be useful to have the yeast paste fall away from the samples at this point. The yeast paste is usually very dark brown while the leaf tissue is still green.
4. Collect the samples in cryovials containing acetone. Proceed with sample preparation (infiltration and embedding) for TEM according to usual protocols.

Representative Results

Results presented below have been obtained using a Wohlwend Compact 02 for HPF (**Figure 1A**). One major advantage of this instrument is the ease of use of the specimen carriers and its holders. When using other instruments, McDonald recommends that two users should carry out the sample preparation and HPF, one preparing the samples while the other does the freezing and transfer to the FS cryovials [9]. However, the Wohlwend specimen carriers and holder are easy enough for a single user to manipulate independently (**Figure 2A, B, E, F and G**). It should be mentioned however that one should perform a few trial runs to become familiar with the HPF instrument before working with valuable samples. An experienced user should be able to fix several (10 or more) samples in an hour. However, the principles presented in this protocol can be used with any of the commercially available HPF machines.

Perhaps the most critical part of the HPF-QFS procedure is the sample preparation for loading into the specimen carrier (**Figure 2C**). Although this may not be intuitive, it is the step in the protocol over which the researcher has the most control. The HPF machine is robust, and with proper use and maintenance should produce the expected changes in pressure and temperature with little variation between samples. If a sample is poorly handled during preparation then no other step will redeem the damage so caused. Plant tissues are fairly easy to manipulate during this stage of the process as they are not cultured in medium and their cells walls give the cells good strength. It is nonetheless important that samples are handled quickly to avoid ultrastructural changes that result from removal from the parent plant and to limit stress and wounding responses. The smallest specimen carrier that can accommodate a sample should be used to ensure efficient and consistent HPF (**Figure 2C**). Finally, the specimen carrier should be filled but not overflowing (**Figure 2D**). The filled specimen carrier is easily inserted into the HPF machine for freezing (**Figure 2 H and I**).

The first steps in the QFS protocol can be challenging for a beginner, so it is recommended that two people should work together until users are comfortable with the procedure (**Figure 3A-E**). Care should be taken at all times when handling liquid nitrogen and the fixatives osmium tetroxide and uranyl acetate. A typical curve for temperature changes during QFS is shown in **Figure 2J**. The temperature increases rapidly from -196 °C, the temperature of liquid nitrogen, to about -80 °C. It is at temperatures of around -78 °C to -90 °C that freeze substitution is believed to occur [9]. One challenging step in the QFS protocol is the removal of dry ice after 2 hours of FS. At this step, mishandling of samples can produce a spike in temperatures. For this, the heater block should be swiftly lifted out of the QFS chamber using a cryoglove and the dry ice quickly poured out into a secondary container.

HPF has been used to fix various plant tissues including *Nicotiana benthamiana* leaves and Arabidopsis leaves and embryos. It is challenging to fix samples from mature leaves due to the large central vacuoles of most cells. Younger leaves contain smaller vacuoles but the trichomes are usually quite densely packed. The presence of the trichomes can make it difficult to pack the samples with yeast paste but care must be taken to ensure that this is properly done to minimize the amount of air trapped between the leaf surface and the paste. The trapped air will impede heat transfer during HPF and reduce the quality of fixation. This is true for any sample.

After HPF and QFS samples may be prepared for viewing under the TEM by infiltrating and embedding with resin. Thin sections of 65-100 nm may then be prepared by sectioning. Typical results are shown in **Figure 4**. The images shown are all from Arabidopsis leaf samples. Plasma membranes are typically smooth and pressed against the cell wall, a sign of good fixation (**Figure 4A, C and E**). Other organelles including chloroplasts (**Figure 4A, D, F and H**) and thylakoids (**Figure 4B**), mitochondria (**Figure 4D and F**), Golgi (**Figure 4 G**), microtubules (**Figure 4E**) and ribosomes (especially **Figure 4C**) are also clearly visible and the large central vacuoles remain intact (**Figure 4A**). Poor handling during HPF-QFS results in artifacts including ice crystal-induced damage (**Figure 4D**) and plasmolysis (**Figure 4H**). Lead precipitate may also form during staining of sections (**Figure 4F**).

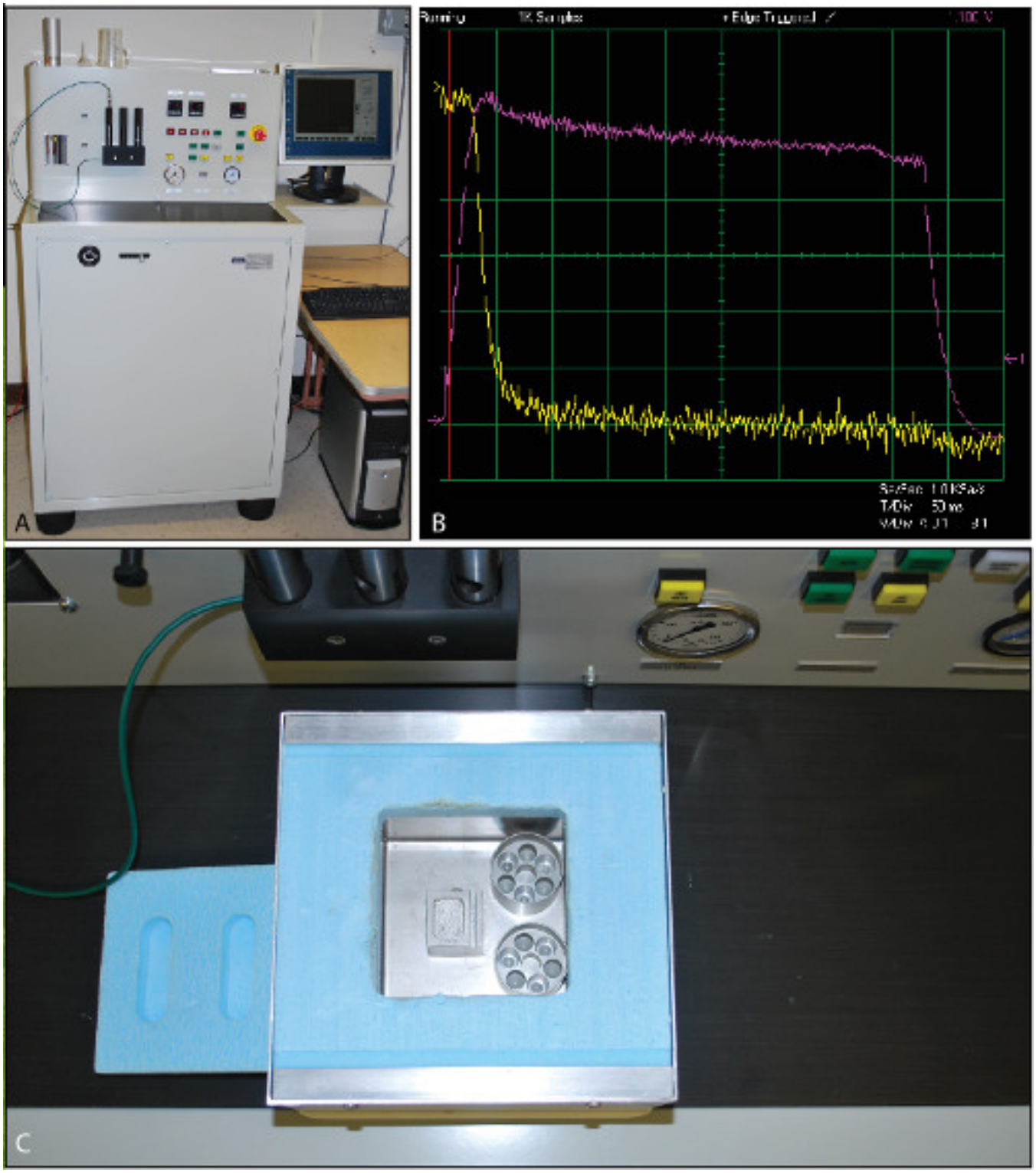


Figure 1: The Wohlwend Compact 02 High-Pressure Freezer. (A) The Wohlwend Compact 02 HPF machine used for cryofixation with the attached computer terminal. Samples are inserted into the front of the machine (small circle) for freezing. A temperature curve can be generated on the computer screen for each run, as desired by the user. **(B)** A typical temperature- pressure curve for a HPF run. The yellow and purple lines represent the temperature and pressure, respectively. Note the steep slopes of both curves. The high pressure is maintained for about 400 msec. Each interval on the x-axis represents 50 msec. Data was collected with EasyScopeII for DSIM12 software. **(C)** The insulated box and cover used for storage of frozen samples immediately after freezing can be conveniently placed on top the HPF machine. It is filled with liquid nitrogen. The round aluminum containers hold the cryovials.

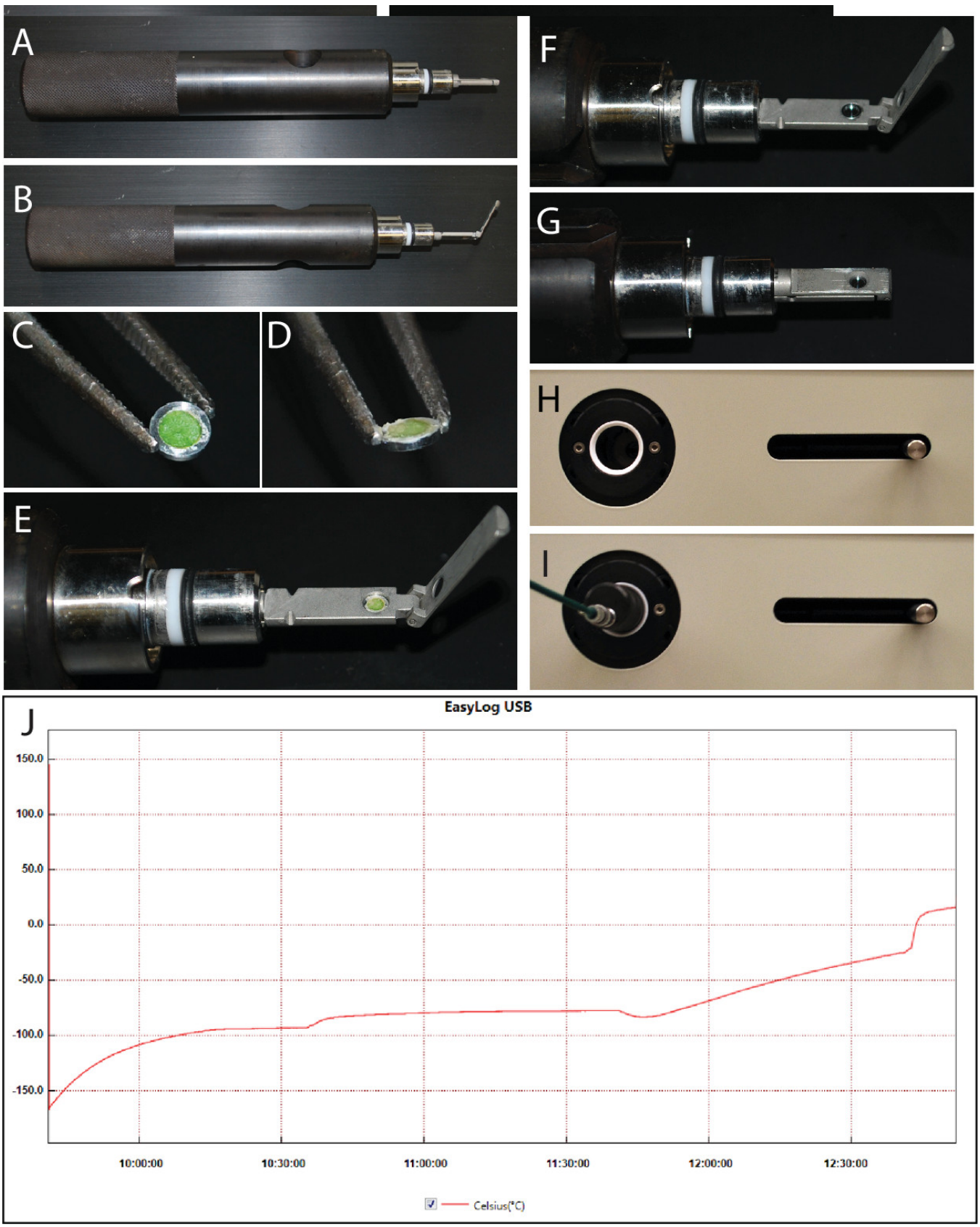


Figure 2: Preparing tissue sample for HPF. (A) The specimen holder for the Compact 02 HPF machine in its closed configuration. **(B)** The specimen holder is open. **(C)** A leaf sample in the 0.2 mm well of a Type A specimen carrier. The other side of this carrier is 0.1 mm deep. **(D)** The leaf sample covered in yeast paste. Note that the carrier is full but not overflowing. **(E)** The specimen carrier in the specimen holder. **(F)** The sample is covered with the Type B carrier. This carrier has one flat surface and on the other side a well that is 0.3 mm deep. Here the flat surface is used to sandwich the sample. **(G)** The sample holder is closed and ready for insertion into the HPF machine. **(H)** The orifice on the front of the HPF instrument into which the specimen holder will be inserted for freezing. **(I)** The temperature and pressure probe/holder inserted into the HPF machine. **(J)** A typical temperature curve for a QFS run (data collected with EasyLog software). Temperature was recorded each second. The spike at the beginning of the run is due to the electronics of the probe used and does not reflect a real temperature measurement.

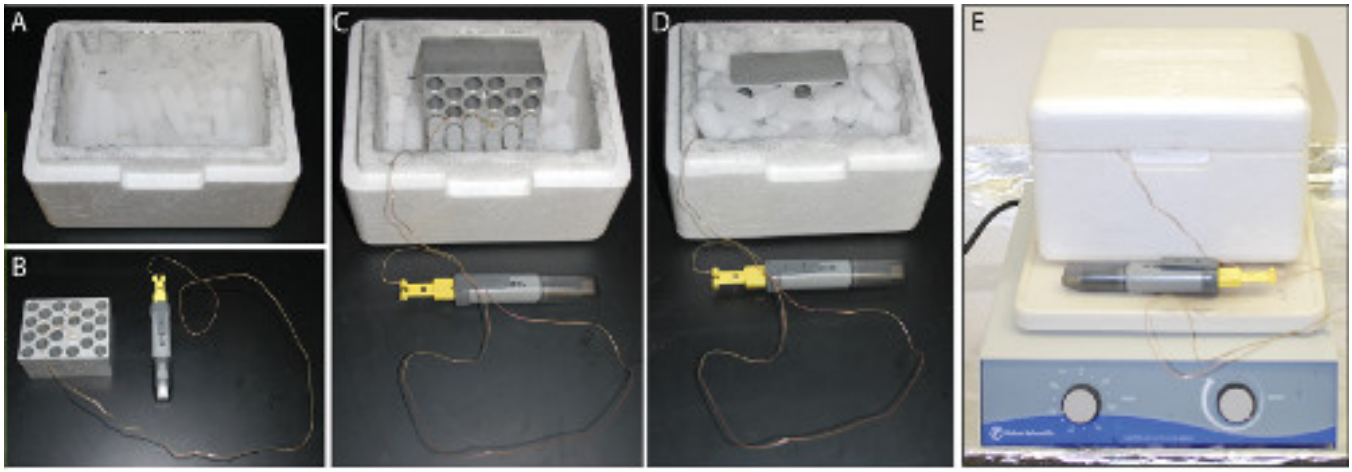
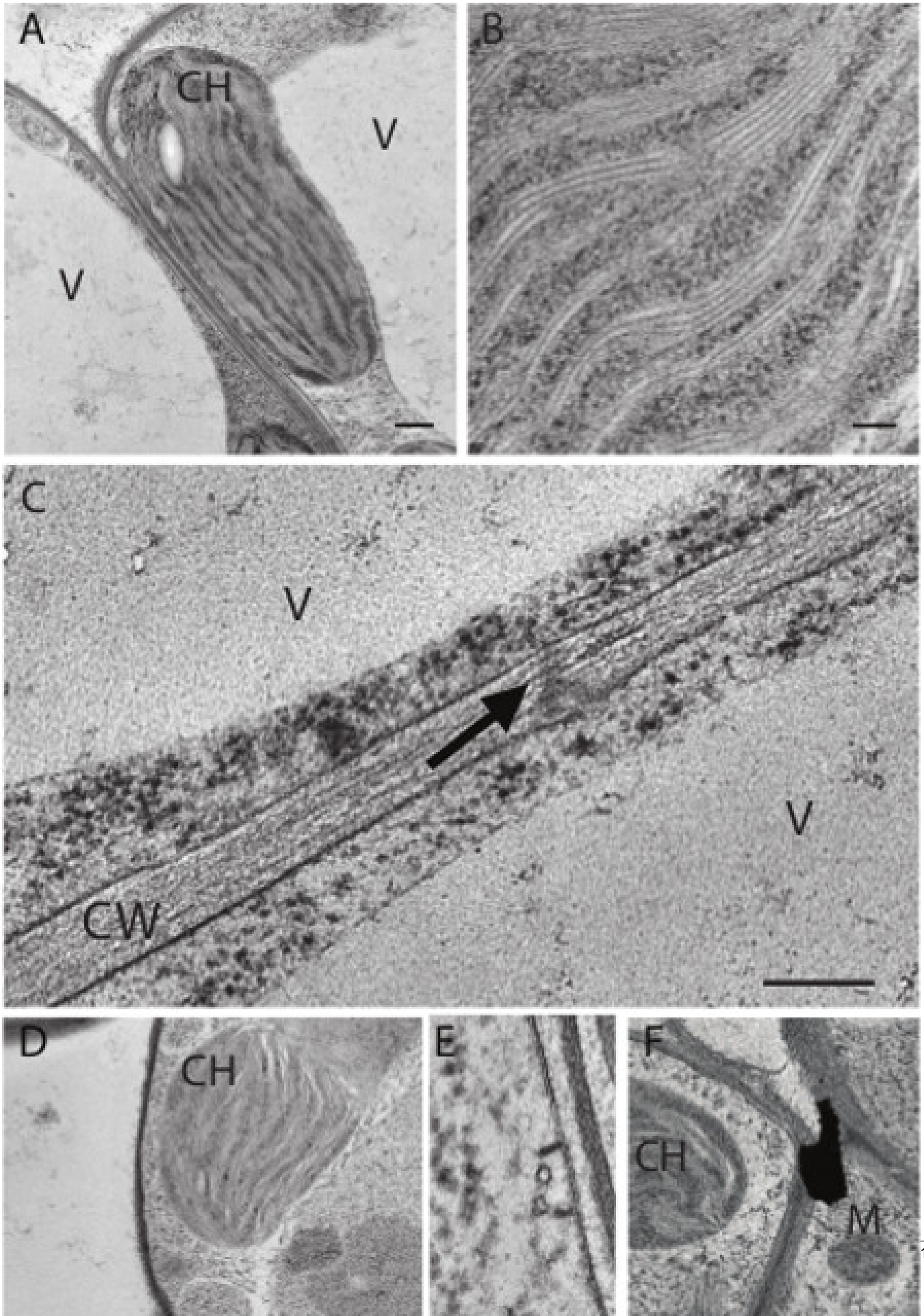


Figure 3: Equipment used in QFS. QFS can be carried out in any insulated container such as a Styrofoam box or an ice bucket. **(A)** A layer of dry ice 1-2 cm deep covers the bottom of the QFS chamber, here a styrofoam box. **(B)** A heater block with 13 mm holes is used to house samples during QFS. The temperature probe with digital data logger is placed in the heater block. **(C)** After cooling the heater block in liquid nitrogen the samples are placed in the block, the liquid nitrogen is poured out, and the entire assembly is placed in the QFS chamber with the dry ice. **(D)** The QFS chamber is filled with dry ice so that the samples are covered; there is no disadvantage to covering the entire heater block with dry ice. **(E)** The box is covered and then moved onto the rotary shaker in a fume hood where the samples are agitated throughout the QFS procedure.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Discussion

The success of the protocol presented here depends heavily on the user. First, advanced preparation is required to ensure that all necessary materials are readily available and in sufficient quantity to complete an entire HPF-QFS run. Second, the user must work quickly, moving from step-to-step in an efficient manner that minimizes sample handling, thus minimizing changes to the native state of the tissue. Once samples are frozen and before they are dehydrated it is imperative that they be kept cold, so care must be taken to avoid any handling that can inadvertently heat up the sample, for example, handling with a gloved hand instead of pre-cooled forceps. Speed and efficiency increase with familiarity with the protocol, so practice runs are recommended before attempting to fix one's samples of interest. Third, the user must remain aware of the dangers of the chemicals used in the FS medium as well as the dangers of working with liquid nitrogen and dry ice. A fume hood is necessary for the preparation of the FS medium and for the QFS procedure. For all other steps, the necessary personal protective equipment including gloves, lab coat, closed-toe shoes and goggles are recommended.

The advantages of using cryofixation instead of chemical fixatives at room temperature for preparing samples for TEM, including plants, have long been known [10]. One study comparing the ultrastructure of root tips of *Nicotiana* and *Arabidopsis* fixed by HPF or chemical fixatives found that the HPF-FS method gave far superior results [5]. In tissue prepared by HPF-FS the plasmalemma and other cellular membranes were smoother, the plasmalemma was flush against the cell wall, and generally organelles including microtubules were better preserved than when samples were fixed by conventional methods. Another study of soybean root nodules concluded that "Chemical fixation by buffered glutaraldehyde does not preserve the ultrastructure of soybean root nodules in a state that permits the correlation of structure and function." And it continues to say that the only available alternative is HPF-FS [2]. Plasmodesmata are membrane-bound channels that connect plant cells to their neighbors, but whose substructure is difficult to resolve even under TEM. Cryofixation by HPF or with a propane jet freezer followed by FS was chosen over chemical fixation in studies aimed at elucidating the fine details of plasmodesmal structure [18]. Despite these early findings, TEM studies on conventionally fixed tissues are still the norm in plant sciences. This may be due to a simple adherence to traditional methods or to the seemingly prohibitive cost of HPF-FS equipment coupled to the long preparative time for currently used FS protocols.

The procedure for QFS is a recent innovation in speeding up sample preparation for TEM [15]. QFS has been used to prepare whole *C. elegans* and *N. benthamiana* leaves after cryofixation [15]. These are relatively thick samples to be fixed by HPF, and preparation of *Nicotiana* samples by HPF-FS has traditionally used very long FS times [12,13,14]. For this protocol we have used leaves from the model plant Arabidopsis. The rationale for long FS times for Nicotiana and other plant samples is that the exchange of solvents and water would likely be slowed by the large vacuoles of the cells. However, it is expected that devitrification of a well-frozen sample should result in minimal damage to the cells ([15] and refs. therein). If ice damage occurs it is most likely caused by the freezing itself and not the QFS. An even shorter protocol for FS has been reported [15]. This super quick FS (SQFS) uses only 90 min for FS. In this protocol, the samples in the heater block are rotated at 100 rpm in the absence of dry ice without covering the SQFS chamber. This allows the samples to reach -80 °C rapidly. The samples are then removed from the heater block and allowed to reach room temperature on the rocker. SQFS has been successfully used to dehydrate cells grown in culture and *E. coli* cells. We have not yet attempted to use SQFS with plant samples due to the thickness of the plant tissues.

The protocol for cryofixing and then dehydrating plant samples by tandem HPF and QFS represents a significant improvement over current protocols. The time for sample preparation before resin infiltration is now reduced to a few hours instead of several days. In addition to developing the QFS method [15], McDonald has also recently published protocols for rapid infiltration of plant tissues with resin following SQFS without dry ice [19]. Plant tissues are usually slowly infiltrated with resin by several long incubations, including overnight. These new protocols result in even shorter sample processing times: 6 hr from freezing to sectioning. Thus in the future, the combination of HPF-QFS and rapid infiltration should replace current protocols for plant-sample preparation for TEM. In addition, the QFS procedure uses common lab equipment that is inexpensive and can be used for multiple purposes, although a commercial QFS Kit is currently available through Electron Microscopy Sciences (http://www.emsdiasum.com). Either option represents a tremendous savings over the cost of purchasing a dedicated freeze substitution unit that can cost well over $50,000.

It should be noted that the applications of samples prepared by HPF and FS extend beyond routine TEM analysis. Samples prepared in this manner retain their antigenicity and can therefore be used in antibody-based approaches, including immunogold labeling [17,19]. These samples may also be used for advanced three-dimensional structural analyses via electron tomography [20]. HPF-FS samples can even be used with correlative light and EM [21,22]. Thus, continued improvements in the procedures for cryofixing and then dehydrating samples will be beneficial to a wide variety of investigators working in diverse systems.

Disclosures

The authors have nothing to disclose.

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