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Practical workflow for cryo focused-ion-beam milling of tissues and cells for cryo-TEM tomography

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Abstract

Vitreous freezing offers a way to study cells and tissue in a near-native state by cryo-transmission electron microscopy (cryo-TEM), which is important when structural information at the macromolecular level is required. Many cells -- especially those in tissue -- are too thick to study intact in the cryo-TEM. Cryo focused-ion-beam (cryo-FIB) milling is being used in a few laboratories to thin vitreously frozen specimens, thus avoiding the artifacts and difficulties of cryo-ultramicrotomy. However, the technique is challenging because of the need to avoid devitrification and frost accumulation during the entire process, from the initial step of freezing to the final step of loading the specimen into the cryo-TEM. We present a robust workflow that makes use of custom fixtures and devices that can be used for high-pressure-frozen bulk tissue samples as well as for samples frozen on TEM grids.

Keywords

Cryo-FIB; cryo-SEM; cryo-TEM; vitreous sections; cryo-tomography

1.0 Introduction

Currently, the most faithful electron tomographic reconstructions of eukaryotic cells and tissue are usually carried out on material that has been high-pressure frozen, freeze-substituted, plastic-embedded, sectioned, and imaged at room temperature in the TEM. Since the technique is reliable, not difficult, and does not require specialized equipment (beyond the freezing instrument), it is widely used.

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However, for almost two decades, cryo-electron tomography of vitrified specimens has been the premier method for the study of cellular ultrastructure in a near-native state, but technical challenges have prevented it from being adopted more widely for eukaryotic cells and tissue. More recently, sub-tomogram averaging has emerged as a way to increase resolution in determining macromolecular structure *in-situ* (e.g. Zhu et al., 2006; Frank, 2006; Förster et al., 2008; Wu et al., 2009; Winkler et al., 2009; Amat et al., 2010; Yu et al., 2011; Heumann et al., 2011; Kuybeda et al., 2013). This facilitates study of macromolecular interaction with neighboring cellular components, while yielding sufficient resolution to compare their *in-situ* structures with examples of high-resolution structural maps obtained from isolated macromolecules using the single-particle method or other techniques, most commonly X-ray crystallography.

Tomographic resolution decreases with increasing sample thickness, unless the number of tilt images can be increased. However, increasing the number of tilt images normally increases the electron dose, thus potentially limiting resolution because of specimen damage. In addition, electron-optical image quality decreases with thicker samples, especially when the specimen is tilted to high angles and the electron path length doubles or triples. Thus, samples generally need to be in the thickness range of 150-300 nm for good results in sub-tomogram averaging. While samples just thicker than the macromolecule of interest give the best resolution, the 3-D relationships within the cell may be lost if the sample is too thin.

Although small cells such as bacteria, or the periphery of larger cells, fall into the suitable thickness range for cryo-TEM tomography, most cells, and certainly bulk tissue, need to be thinned. Cryo-ultramicrotomy of vitreously frozen specimens is an effective method of thinning the sample, and the technology has been steadily improving (e.g. Al-Amoudi et al., 2004, 2005; Pierson et al., 2010). The sample area on a grid that is suitable for tilt-series acquisition can be wide, allowing a good choice of targets for reconstruction. Nevertheless, artifacts due to the mechanical cutting action have not yet been completely overcome, and the wrinkled topology of the sections, along with their poor attachment to the TEM grid, can make them difficult to use for cryo-tomography (Hsieh et al., 2006; Marko et al. 2006a).

To avoid the drawbacks of vitreous cryo-sectioning, we proposed (Marko et al., 2005, 2006b), and then demonstrated (Marko et al., 2007) that cells could be thinned for cryo-TEM by means of focused-ion-beam (FIB) milling. We confirmed that samples remained vitreously frozen when observed in the TEM, and that they lacked the artifacts seen after cryo-ultramicrotomy. Since then, development of the technique has been taken up intensively at the Baumeister laboratory (Plitzko et al., 2009; Rigort et al., 2010, 2012a, 2012b), and also in the Zhang laboratory (Strunk et al., 2012; Wang et al., 2012), a group in The Netherlands (Hayles et al, 2010; de Winter et al., 2013), and others (Edwards et al., 2009; Rubino et al., 2012).

However, cryo-FIB preparation remains a challenging technique, often with very low “throughput”. The challenges are (1) handling a small, fragile specimen, (2) keeping the specimen below the devitrification temperature (about -140°C) at all times and (3) avoiding frost accumulation. Each lab that has worked with the cryo-FIB method has approached these problems by specially designed fixtures and devices, and here we show our versions. Unlike any of the other protocols so far published, we emphasize the use of bulk tissue prepared by high-pressure-freezing (HPF), while also ensuring that our workflow can accommodate cells plunge-frozen on TEM grids.

2.0 Methods and Results

2.1 High-pressure freezing

We use an HPF carrier (also known as a platelet, planchet or hat) that has a narrow slot (0.3 mm wide and 2 mm long) rather than a large circular depression. This is desirable so that when the tissue is exposed in the trimming step (Section 2.3 below), the walls of the slot will support the tissue and provide a reasonably short path to ground for charge reduction during FIB-milling. Although the “sectioning quality” of FIB milling is less sensitive to freezing quality than is cryo-ultramicrotomy (Hsieh et al., 2006; Marko et al., 2006), it is best to heed the advice of Studer et al. (1989, 1995) and optimize heat-transfer conditions by using a platelet with the smallest possible volume of tissue and the thinnest possible freezing windows.

For the Bal-Tec HPM 010 (now produced by ABRA Fluid AG, Widnau, Switzerland), the Leica HPM 100 (Leica Microsystems, Vienna, Austria) or the Wohlwend HPF Compact 02 (TechnoTrade International, Manchester, NH), the best platelet choice is the Wohlwend slot type, part number 446. For the Leica EM PACT or EM PACT2 instruments, the best choice is the “Biopsy Carrier”, part number 16706896.

2.2 Intermediate sample holder

The trimmed-down HPF samples are small and difficult to handle under liquid nitrogen, and the FIB-milled TEM lamellae are especially fragile. In order to limit the need to handle the specimen (HPF carrier or TEM grid) directly, we mount the specimen in an intermediate specimen holder (ISH), as shown in Figure 1. Working in the chamber of a cryo-microtome, we use a loading block (Fig. 2) to spread the jaws of the ISH to accommodate the sample. The ISH (available from TGS technologies) is made of annealed and hardened beryllium-copper, and has ample spring tension to hold the sample firmly during all cryo-transfers. For pre-trimming (see below), the ISH is held in the standard vice-type chuck of our cryo-ultramicrotome (Leica UCT/EM-FCS).

2.3 Pre-trimming frozen tissue

When working with tissue, we employ the “H-bar” geometry for FIB-milling. This requires that a free edge of the sample be exposed. This is accomplished by cutting away a substantial portion of the carrier using a diamond trimming knife (Diatome, Biel, Switzerland) in the cryo-microtome. In order to reduce FIB-milling time, we further trim the sample, using the 90-degree diamond trimming knife, down to a 20- μ m thickness. The pre-trimmed region is made in a position that will be within the adjustable eucentric-height range of the goniometer in the cryo-TEM. The trimming geometry is shown in Figure 3. After trimming, the sample--in the ISH--is mounted in the cryo-FIB specimen block (see below), while still in the cryo-microtome chamber. After trimming, the entire assembly can be stored under liquid nitrogen for later FIB-milling.

When working with cells frozen on TEM grids, we sometimes cut part of the grid away (Marko et al., 2007), but we also can use the intact grid, employing the method of Rigort et al. (2010,2012a, 2012b)

2.4 Cryo-transfer at the FIB-SEM

We use a Leica VCT-100 cryo-transfer system and cryo-substage with our Zeiss (Oberkochen, Germany) Neon 40 EsB FIB-SEM and a Leica MED 020 vacuum evaporator equipped with a VCT-100 interface. A frozen and pre-trimmed specimen, mounted in an ISH, is loaded into a pre-tilted, cover-equipped specimen block (Leica part number 16770266, Fig. 4), and the protective cover is closed. This is normally done in the cryo-

microtome chamber, and then the specimen block is brought to the VCT-100 loading box (Figs 5A,B) under liquid nitrogen. The Leica specimen block is intended for standard TEM grids, so we machined wider jaws to accommodate the ISH.

The VCT-100 shuttle (Figs 5 C,D) is pumped to high vacuum in the MED 020, while its internal anticontaminator is cooled with liquid nitrogen. The shuttle is then vented while mounted on the MED 020, but the gate valve is kept closed. It is important that the vent gas is free of water vapor. We found that the only suitable vent gas is the boil-off from a large, lightly pressurized liquid-nitrogen Dewar; the gas is then cooled by flowing through a copper coil submerged in a Dewar of liquid nitrogen (Fig. 5E).

The shuttle is then transferred to the VCT-100 loading box, the gate valve is opened, and the transfer rod is extended under liquid nitrogen, picking up the pre-loaded specimen block. The block is immediately retracted into the shuttle and the gate valve closed. The shuttle is then pumped to high vacuum on the MED 020.

Finally, the shuttle is docked onto the VCT-100 interface on the FIB-SEM. After pumping out the space between them, the two gate valves are opened and the specimen block is inserted into the pre-cooled cyro substage in the FIB-SEM chamber. Removal of the insertion rod opens the cover of the specimen block, exposing the sample inside the FIB-SEM chamber.

After FIB-milling, the specimen block is transferred back to the VCT-100 shuttle. The shuttle is first prepared by evacuation and pre-cooling on the MED 020, so that only the space between the two gate valves remains to be evacuated when attaching the shuttle to the dock on the FIB-SEM. After the gate valves open, the transfer rod is introduced into the specimen block, which automatically closes the cover. The specimen block is then retracted into the shuttle and the gate valves are closed. Thus, the specimen block remains under vacuum at all times, and it is surrounded by the internal anticontaminator when it is in the shuttle.

Next, the shuttle is attached to the dock on the MED 020 and re-pumped to high vacuum before venting (as described above), leaving the gate valve closed after venting. Finally, the shuttle is attached to the VCT-100 loading box, the gate valve is opened, and the specimen block (with the cover still closed) is quickly plunged under liquid nitrogen. The specimen block is stored under liquid nitrogen with the cover closed until the time that cryo-EM work will be done; then it is moved it to the workstation of the TEM cryo-transfer holder.

2.5 Frost analysis

A certain amount of frost is sometimes found on the specimen when first observed in the cryo-FIB/SEM. This may have been picked up in the microtome chamber or in the VCT-100 loading box. We find that this frost can be removed from the specimen surface by brief rastering with the ion beam. We move a rectangular raster (usually at TV rate and 1 nA beam current) across the surface while observing the secondary-electron image generated by the ion beam. The frost particles shrink and disappear, leaving the surface smooth. We observe that the frost particles (hexagonal ice) are much more sensitive to ion-beam irradiation than the vitrified specimen.

We spent considerable time identifying sources of frost. Using a clean Quantifoil grid (Quantifoil Microtools, Jena, Germany), we transferred the grid in and out of the MED 020 evaporator, the VCT-100 loading box, and the FIB-SEM, such that we could observe frost accumulation independently at each step. Figure 6 shows the types of frost that can be observed in the SEM. If the vent gas is not free of moisture, small frost particles (about 50

nm in diameter) are observed). Much larger frost particles (larger than about 0.5 μm in diameter) may accumulate while the sample is transferred through the VCT-100 loading box.

2.6 Cryo-FIB milling

The pre-trimming step in the cryo-microtome is key to excellent results with respect to smooth lamellae in the TEM. Streaks in the milling direction (“curtaining”) are often observed in many types of FIB-milled specimens. We find that curtaining arises from roughness on the surface normal to the ion beam. Since our samples are faced with a diamond knife, this surface is very smooth, so curtaining is minimal. Thus, we can dispense with the protective metal layer (usually Pt) that is sometimes used to protect the specimen (Hayles et al., 2007).

After pre-trimming the HPF carriers in the cryo-microtome, the exposed area of the specimen is typically 300 μm wide and 20-30 μm thick. We mill the specimen in three steps, as shown in Figure 7. At each step a trapezoid-shaped area (as seen in end view in Fig. 7B) is milled on both sides of the sample. The beveled edges of the trapezoid area are at an approximate 30-degree angle, so that the specimen may be tilted to 60 degrees in the TEM without occlusion by the unmilled edge of the sample.

Step 1: Reduce thickness to 10 μm in an area 200 to 250 μm wide by milling on both sides of the lamella, at 5 nA for a total of about 15 min. This makes a lamella about 60 μm deep (as measured down from the free edge of the sample).

Step 2: Reduce thickness to 3 μm in two 80- μm -wide areas, about 40 μm deep, by milling on both sides at 1 nA for a total of about 10 min for each area.

Step 3: Final milling to 100-400 nm thickness in four 10- μm -wide areas within each of the above two areas by milling at 100 pA for a total of about 3 min on both sides for each area. The final TEM lamellae are about 10 μm wide and 20 μm deep.

For recording images with secondary electrons from the ion beam, we use 30 keV and 10 pA, with frame integration. We freeze the scan as soon as an acceptable image is formed. We use the “SESI” (Everhart-Thornley type) detector. For recording electron-beam images, we use 3 keV and about 50 pA beam current, with the in-lens secondary-electron detector.

2.7 Cryo-transfer to the TEM

Once the specimen block is released from the shuttle, under liquid nitrogen in the loading box (Fig 5A; section 2.4, above), it is stored under liquid nitrogen with the handling rod attached (Fig. 5B), with the cover closed. When ready to perform cryo-TEM, the specimen block is placed under liquid nitrogen in the workstation of TEM cryo-transfer holder. The shutter is then opened, and the ISH is removed from the FIB/SEM specimen block and placed in the TEM cryo-transfer holder.

We use a Gatan model 626 TEM cryo-transfer holder (Fig. 8) fitted with our modified tip that accepts the ISH. The ISH is mounted under a hinged cover, which secured by a screw when closed. Because the sample is held only along one edge, about 1.5 mm of the specimen (or TEM grid) is exposed to provide access to the FIB-thinned areas for tilt-series collection. The modified tip can be made for any make of cryo-transfer holder, and there is no need to modify the holder workstation.

2.8 Cryo-TEM imaging

Figure 9 shows cryo-TEM of a section of high-pressure-frozen muscle tissue (from toadfish bladder) prepared by cryo-FIB milling. Our interest is in the association of membranes, the sarcoplasmic reticulum (SR) and transverse tubule (TT) forming the triad junction that is responsible for excitation-contraction coupling, one of which is labeled in Figure 9A. Ryanodine receptors (RyR) are visible in the space between the SR and TT membranes in the cryo-TEM section (arrows in Fig. 9A). Our research involves investigation of structures associated *in-situ* with this molecule, which will involve sub-tomogram averaging for comparison with the same molecule as seen by single-particle reconstruction (Renken et al., 2009). For this work, the sample thickness should not be greater than about 150 nm (Figs. 9A, E-G), to provide good tomographic resolution; the ryanodine receptor is about 30 nm along an edge at the base, so truncation would not be a significant problem.

The overall 3-D arrangement of RyRs in muscle tissue can be appreciated from tomograms of thick sections. Figure 9D shows a projection cryo-TEM image of a 300-nm-thick cryo-FIB-milled lamella. Subsequently, a tomographic tilt series of this region was recorded. Although the projection image shows the presence of RyRs, tomographic reconstruction of the volume (not shown) can reveal the layers of RyRs at each triad junction, which can form a 2-rowed, ordered array in which the two rows extend along the viewing direction in the orientation shown here (Wagenknecht et al., 2002).

Figure 9E is a projection image of a ~100-nm-thick sample of muscle tissue, recorded with $2 \text{ e}^- \text{ \AA}^2$ incident dose at 400 keV with zero-loss energy filtering. Figures 9 F and G show 1-nm-thick x-y slices from the tomogram, recorded at 1-nm pixel size through a tilt range of 120° with a total electron dose of about $80 \text{ e}^- \text{ \AA}^2$. The tilt series was aligned (without gold fiducial markers), and the volume was reconstructed using IMOD (Kremer et al., 1996).

3.0 Summary of steps in the workflow

1. High-pressure freeze a piece of tissue in slot-type HPF carrier or prepare a specimen on a TEM grid by plunge-freezing or high-pressure freezing.
2. In the cryo-microtome chamber, load a sample into an ISH (Fig. 1), using the ISH loading block (Fig. 2) and mount the ISH in the microtome chuck.
3. If using tissue in an HPF carrier, use a diamond trimming knife to expose the tissue, then continue to trim the sample (as in Fig. 3) for efficient FIB-milling.
4. Working in the chamber of the cryo-microtome, load the ISH into the specimen block (Fig. 4).
5. Attach the specimen block to the handling rod (Fig. 5B), closing the cover.
6. Store the specimen block, with the cover closed and the handling rod attached, in a 50-ml plastic tube of liquid nitrogen, kept in a Dewar.
7. When ready to do FIB work, attach the VCT-100 shuttle to the MED 020 evaporator (Fig. 5C) and evacuate it, then cool the internal anticontaminator with liquid nitrogen.
8. Purge the nitrogen lines (Fig 5 F), then vent the shuttle on the MED 020 without opening the gate valve.
9. Attach the shuttle to the loading box (Fig. 5A).
10. Transfer the specimen block from the storage tube to under the liquid nitrogen in the VCT-100 loading box (Fig 5B).

11. Open the gate valve and extend transfer rod in the VCT-100 shuttle to pick up the specimen (Fig. 5A), then close the gate valve.
12. Remove the shuttle from the loading box and attach it to the MED 020; evacuate the shuttle.
13. Move the shuttle to the docking port on the FIB-SEM (Fig. 5 D), attach it and open the gate valves.
14. Extend the transfer rod in the shuttle to move the specimen block to the FIB-SEM cryo sub-stage (thus opening the cover of the specimen block), then retract the transfer rod inside the shuttle, close the gate valves, detach the shuttle, and move it back to the MED 020 evaporator to keep it under vacuum until needed.
15. Carry out the cryo-FIB milling as described in Section 2.6, above.
16. Re-attach the evacuated and cooled shuttle to the FIB-SEM, open the gate valves, and extend the transfer rod to pick up the specimen (thus closing the specimen block cover).
17. Close the gate valves and detach the shuttle from the FIB-SEM.
18. Return the shuttle to the MED 020, attach it and briefly pump to improve the vacuum, then vent the shuttle (Fig 5F) without opening the gate valve.
19. Move the shuttle to the loading box, open the gate valve, and extend the transfer rod to place the specimen block under liquid nitrogen.
20. Pick up the specimen block with cover closed and the handling rod attached (Fig. 5B), and place it in a plastic tube of liquid nitrogen with the handling rod still attached. Store the tube in a Dewar until ready to do TEM.
21. When ready to do TEM, transfer the specimen block from the tube of liquid nitrogen to under the liquid nitrogen in the TEM cryo-transfer workstation, and open the cover to access the specimen in the ISH (Fig. 8A).
22. Move the ISH to the tip of the cryo-transfer holder, close the cover and secure it with the screw, then close the shutter (Fig. 8B-E).
23. Perform the TEM cryo-transfer and observe the specimen in the TEM.

4.0 Discussion and conclusions

The success rate with respect to being able to record a TEM tomogram somewhere in a lamella that was milled in the cryo-FIB is more than 50%, even though some lamellae are partly damaged during cryo-transfer. There is no danger of devitrification, but handling of the sample – even with the aid of the ISH – can cause some lamellae to become damaged, as seen, for example, in Figure 7E. In the case of tissue samples, after TEM imaging we often “reverse-transfer” the ISH back into the cryo-microtome chamber, trim it down, and cycle it again through the cryo-FIB to produce more lamellae. We can repeat this procedure up to three or four times.

The thickness and width can be chosen within limits set by the mechanical stability of the resulting lamellae (which we have not exhaustively explored). Assuming proper set-up of alignment and focus at all of the ion-beam probe currents to be used, the dimensional accuracy of the lamellae depends on the magnification calibration of the FIB-SEM, as well as on charging effects, which are often observed. We can generally obtain lamellae within about 20% of the target thickness. Lamellae are often slightly thinner at the free edge than they are near the base of the lamella. The dimensions of the lamellae are finally confirmed

with respect to width by the TEM image and in thickness by TEM electron energy-loss imaging and by the tomographic reconstruction.

Our practice is to do each of the four procedures on a different day (high-pressure freezing; pretrimming in the cryo-microtome; cryo-FIB milling; TEM tomography). We typically do no more than two samples (i.e. use no more than two ISHs) in a day, although each ISH may accommodate a sample with four to ten lamellae. Others might prefer to link various combinations of these operations in a single day, keeping in mind the time required to cool down and prepare the cryo-FIB and the cryo-TEM before use.

We chose the VCT-100 cryo-(FIB)/SEM system because the transfer shuttle has an actively cooled anticontaminator and because Leica offered a pre-tilted specimen block with automatically operated cover for frost protection. We also considered that conduction cooling, as used on the VCT-100 system, avoids any stage vibration due to gas flow (however, most of the current generation of SEM cryo-stages that are cooled by gas flow reportedly no longer have stage-vibration problems). While conduction-cooled stages take longer to cool, and previously did not cool to very low temperatures, our model has additional cooling bands and can reach -168°C. The full tilt range of the FIB/SEM is available, and at least 90 degrees of rotation is possible, although with the pre-tilted specimen block, only $\pm 10^\circ$ tilt is needed, and our specimens block requires only about 30° stage rotation between the cryo-transfer and milling positions. The VCT-100 cryotransfer system is available for retrofit on any modern FIB-SEM instrument.

For tissue, appropriate HPF carriers are standard and available. When using TEM grids, thin specimens can be plunge-frozen, while thicker ones, including eukaryotic cells cultured in the “wells” formed by the grid bars, can be high-pressure frozen on the grid. Our intermediate specimen holder (ISH) can be used with any make of cryo-(FIB)/SEM cryo-transfer system. Frost control, most important during the post-FIB-milling cryo-transfer steps, will differ with the type of cryo-transfer system. A shuttle to transfer the specimen under vacuum, and a specimen holder with a cover, are both essential. Special care needs to be taken that the gas for venting the shuttle is free of moisture and that the chamber in which the specimen is handled is cold and free of suspended frost particles.

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Figure 1.

Intermediate Specimen Holder (ISH). Two types are shown. On the left in (A) is an ISH designed for holding a standard HPF carrier, which is cut down (nearly in half in this case, see Fig. 3). to expose the tissue. On the right in (A) is an ISH designed for a TEM grid. A. Top and bottom views: Channels on the bottom (arrowheads) fit into the TEM cryo-transfer holder (Fig. 8). The central hole (short arrow) accepts the locating pins in both the loading block (Fig. 2) and in the TEM cryo-transfer holder (Fig. 8). The side holes (long arrows) allow the ISH to be easily manipulated by forceps. B. Side views of both types of ISH (grid-type on the left and HPF-carrier type on the right): The long arrows show the holes where the loading-box retractable pin inserts to spread the jaws. The lower hole forms the hinge. Bars = 1 mm.

Figure 2. ISH loading block. A. The knob on the left spreads the jaws of the ISH by inserting the pin, shown by the arrow in (C). B. View of an ISH in the loading block. The countersunk holes in the loading block are for re-positioning the ISH, if necessary. C. Side view of the empty loading block showing the locating pin (arrowhead) and the spreading pin (arrow). Bars: A = 3 mm; B,C = 1 mm.

Figure 3.

Pre-trimming geometry. A. An HPF carrier, held in an ISH. Nearly half of the carrier has already been trimmed away. The two arrows show the 0.7-mm-wide region (with the tissue in the center) to be further trimmed, as shown in (B) and (C). B. Tissue (*) in a 300- μ m-wide slot of the aluminum HPF carrier, before final trimming. At this stage, a layer of aluminum is still beneath the tissue. C. Final trimming step. The edge of the tissue is trimmed to 20 μ m thickness, supported by aluminum only at the sides. Bars: A = 1 mm; B,C = 100 μ m.

Figure 4.

Leica specimen block. A. Shown with the cover open. Note the thick, curved structures on both ends; these act as additional anti-contaminators, and they also restrict the flow of gas into the ends of the block. In the inset, two ISHs are shown mounted in the block, one with a TEM grid (arrow) and one with a partially-trimmed HPF carrier (arrowhead). B. Shown with the shutter closed. The shutter automatically opens when the block is released in the FIB/SEM coldstage, and it automatically closes when the block is retracted. Bars = 1 cm (3 mm in inset).

Figure 5.

VCT-100 cryo-transfer system and vent gas arrangement. A. The shuttle (*) is attached to the loading box and the gate valve (arrowhead) is opened. The specimen block (Fig. 4) is placed under liquid nitrogen by the transfer rod inside the shuttle. This operation is done both when picking up the specimen block before FIB-milling and retrieving it afterwards. B. The specimen block (black arrow) is introduced into, or removed from, the loading box by means of a handling rod (white arrow). After transfer from the microtome chamber, or before transfer to the TEM cryo-holder workstation, the specimen block, attached to the rod, is stored in a 50-ml plastic tube (black arrowhead) of liquid nitrogen that is kept in a large Dewar. C. The shuttle (*) is shown attached to the MED 020 evaporator with the shuttle gate

valve (arrowhead) and the dock gate valve both open. The shuttle is vented on the MED 020 coating unit, and the vent gas needs to be free of any water vapor. D. The shuttle (*) is attached to the FIB-SEM with both gate valves initially closed. E. A copper coil submerged in a Dewar of liquid nitrogen cools lightly pressurized (8-10 psi) nitrogen gas that is boiled off in a 50-liter Dewar (not shown). F. The anticontaminator in the VCT-100 shuttle is cooled with liquid nitrogen, which can be seen in the reservoir, indicated by the black arrow. Prior to venting, the lines are purged using the added purge valve, shown by the white arrow.

Figure 6.

Types of frost, as deposited on Quantifoil test films with 2-μm-diameter holes. A. If there is water vapor in the vent gas, very small (ca. 50 nm) frost particles are seen. B. The frost is almost eliminated using the arrangement in Fig. 5. C. Larger frost particles may be deposited on the specimen while the specimen block is in the loading box, and can also be deposited while mounting the specimen in the ISH in the cryo-microtome chamber. Bars = 2 μm.

Figure 7.

FIB-milling steps. A. An end view (“ion-beam image”) showing the full 20 μm tissue thickness (double arrowheads) at the far left and far right, milled in the center to about 60 μm deep and 10 μm thick (Step 1, 5 nA). B. Two regions are further milled to about 40 μm deep and 3 μm thick (Step 2, 1 nA). The shape of the trapezoid-shaped milling pattern is outlined at the left region. C. The right-side region from (B) was further milled to about 150-250 nm thick in four sub-areas (Step 3, 100 pA). The arrow indicates the direction of view in (D) and (E). D. An SEM side-view image--perpendicular to the view in (C)--of the final four TEM lamellae. E. A low-mag TEM view of the four lamellae shown in (D), each

about 20 μm deep and 10 wide. The two lamellae on the left were damaged during transfer.
Bars: A,B = 20 μm ; C,D,E = 10 μm .

Figure 8.

TEM cryo-transfer holder in its workstation. A. The FIB-SEM specimen block is opened under liquid nitrogen (but shown here dry), exposing an ISH to be transferred. Both TEM-grid (arrow) and HPF-carrier (arrowhead) samples are shown, but normally only one ISH is used at a time. B. The recess for the ISH is revealed with the cover (black arrow) open. The white arrow shows the locating pin for the ISH. The fastening screw is shown with arrowheads in (B) and (D). C. An ISH with a TEM grid is inserted into the recess, but the cover is still open. D. The cover is closed and secured with a captive screw (arrowhead). The window revealing the TEM grid is apparent. E. The cryo-transfer shutter closed, ready for transfer to the TEM. Bars: A = 1 cm; B-E = 5 mm.

Figure 9.

Example of results -- FIB sections of vitreously frozen muscle tissue (from toadfish). A. Section approximately 150 nm thick, recorded at 400 keV with zero-loss energy filtering and $2 \text{ e}^-/\text{\AA}^2$ incident dose. The sarcoplasmic reticulum vesicles (SR) and t-tubule cross-sections (TT) are surrounded by muscle fibers. Profiles of ryanodine receptors (RyR) are shown at the arrows; in this view, two RyRs are arranged next to each other on both sides of the TT. In this "side view" of the RyRs, the RyR's transmembrane domain (illustrated in the inset as the structure projecting to the right of the RyR's main mass), is inserted in the SR membrane so that only the larger, cytoplasmic region appears in the gap between SR and TT. B. Electron diffraction pattern indicating vitreous ice. C. Cryo-SEM image of the very

thin lamella (*) corresponding to the TEM image in (A). D. Projection image of a 300-nm-thick section, recorded as above, showing a row of five triad junctions. E. Projection image of a ~100-nm-thick slice recorded as in (A). F, G. 1-nm-thick tomographic slices of the triad junctions in (E), left and right respectively; pixel size 1 nm, total dose for the tilt series $100 \text{ e}^-/\text{\AA}^2$. Bars: A,D = 100 nm; E,F,G = 50 nm.