Modification of Leica EM PACT2 and AFS Equipment for More Efficient High-Pressure Freezing and Freeze-Substitution Sample Preparation

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Introduction

Preparation of biological specimens using high-pressure freezing-freeze-substitution (HPF-FS) gained popularity, owing to a high level of sample preparation quality. Different preparation procedures were developed and successfully applied to various samples and research questions [1]. Yet, the preparation of biological samples for HPF-FS is a time-consuming procedure that requires careful manipulation during the freezing step. Higher efficiency gained during any preparation step should speed up sample treatment while reducing the cost of preparation and equipment use. An example of equipment development for higher efficiency is the rapid loader [2].

This article describes modifications to commercial EM (electron microscopy) equipment: Leica EM PACT2 and AFS machines (Leica GMB, Austria) extensively used in many EM facilities. For EM PACT2, I propose a more convenient unloading station variant for five pods, as well as a new sample carrier storage box that facilitates transfer and storage. For more precise carrier transfer from the box to the AFS reagents basins, I propose carrier sliders. Finally, to improve each run of the AFS, I describe specimen holders that will double the capacity of each run. These modifications should help to make the entire HPF-FS procedure more effective and efficient.

Materials and Methods

The proposed modified parts are not commercially available, yet they can be reproduced in university workshops. All equipment modifications described here were performed in the mechanical workshop of the University of Rennes, France. Measurements

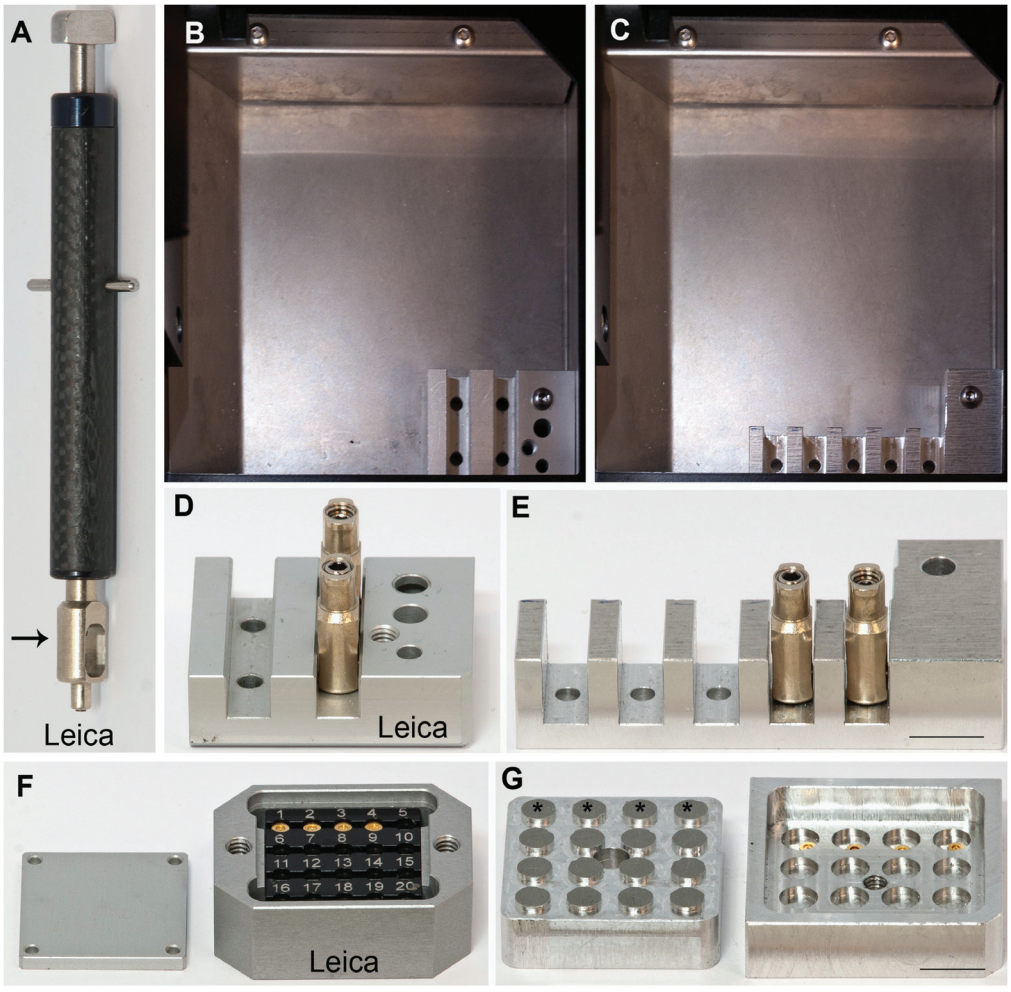

Figure 1: Custom-designed mechanical parts for the Leica EM PACT2. (A) Leica EM PACT2 bayonet of rapid loader with an attached pod (arrowhead). **(B)** Leica EM PACT2 freezing basin with the original unloading station. **(C)** Leica EM PACT2 freezing basin with the custom-modified unloading station. **(D)** EM PACT2 bayonet uploading station (originally provided by Leica) with two pods. Scale bar = 1 cm. **(E)** Linearized, custom-made version of the EM PACT2 bayonet uploading station. In addition to the altered shape, it contains five, instead of four, bayonet sockets. Scale bar = 1 cm. **(F)** Original Leica grid storage box with cover (at left). **(G)** Modified storage box with cover (at left). The sockets for sample storage are wider and can receive the sample while it is being held by tweezers. The lid is carved to fit the sockets (asterisks on the first row) and to hold the carriers precisely in place. Scale bar = 1 cm.

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and manufacturing details are available upon request from the Rennes imaging facility.

Leica EM PACT2 Modifications

Unloading station for bayonet pods. Freezing of tissue from a single sample is usually done with several repeats, preferably with minimal delay. HPF carriers are introduced to the machine by a bayonet pod (Figure 1A). After freezing, samples are transferred to an unloading station situated inside the HPF liquid nitrogen basin (Figure 1B). The original unloading station could accommodate only four pods simultaneously, two in each row (Figure 1D). Such arrangement can cause sequence confusion when multiple samples (for example, control samples plus experiment samples) are treated in parallel. Linear arrangement permits easier tracking of sample placement during experiments, lessening confusion (Figure 1C). The dimensions of the inner parts (pods recipient) were kept identical to the original piece (Figure 1D).

Cryo-transfer box for carriers. The bayonet, detached from the handle and the sample carriers (abbreviated as "carriers" hereafter) is released and stored in the EM PACT2 sample box, fit to host twenty standard carriers (Figure 1F). The socket diameter has been designed to precisely encompass the carrier, leaving little room for manipulation. The minute size of the carrier (1.5 mm in diameter, 500 nm thick) renders the grip with tweezers unstable, sometimes resulting in confusion about the sample identity.

The modified carrier box design facilitates manipulation during sample freezing. The enlarged sockets (2.5 mm in diameter) permit direct carrier installation using tweezers, and the concave lid fits precisely into the carrier slots, securing samples in place during transfer (Figure 1E). The depth of the box and the thick concave lid are sufficient to transfer the samples without the risk of heating. The lid and the box itself can be secured with the M4-thread screwdriver provided with the EM PACT2, and carriers can be safely stored in the box under liquid nitrogen until the substitution step.

Sample transfer supports. It is crucial to keep the samples under the temperature of ice crystallization during the transfer of the carriers from the freezing to the substitution step. Usually, the samples are transferred under liquid nitrogen to avoid sample heating from insufficiently chilled manipulation tools. As with freezing, the difficulty of the transfer under liquid nitrogen is caused by limited visibility. The AFS2 unit is equipped with reagent basins for several kinds of sample separator inserts (Figure 2A). Transfer supports were custom-designed to facilitate the transfer of the carriers from the storage box to the reagents basin or to cryo-vials.

Transfer supports have a platform adapted to contain the sample carrier box, and a slider, with different angles of sliders for each kind of spacer and adaptation for standard and modified carrier boxes (Figures 2B, 2C, 2D). To transfer the samples, the chemical basins and the carrier storage box are transferred into liquid nitrogen with the slider aligned to a precise spacer position inside the basins (Figures 2E and 2F). The carrier is transferred on a slider, and, after it reaches the basin bottom, its landing position is verified. The chemical basin is transferred to the AFS with the liquid nitrogen trapped inside, reducing the possibility of sample mishandling and confusion during manipulation. The slider can be used for EM PACT2 or HPF100 carrier transfer owing to a double-notch system inside the slider (with external diameter, 3 cm; internal diameter, 1.5 cm).

Leica AFS1, AFS2

AFS manipulation holder. Leica AFS machines are frequently used in EM labs for the substitution step. The main mechanical difference between the AFS and the AFS2 is that the AFS liquid nitrogen feeding tube is inside the manipulation chamber, whereas it is outside that of the AFS2. The manipulations during substitution are performed inside this chamber, while liquid nitrogen from the tank together with

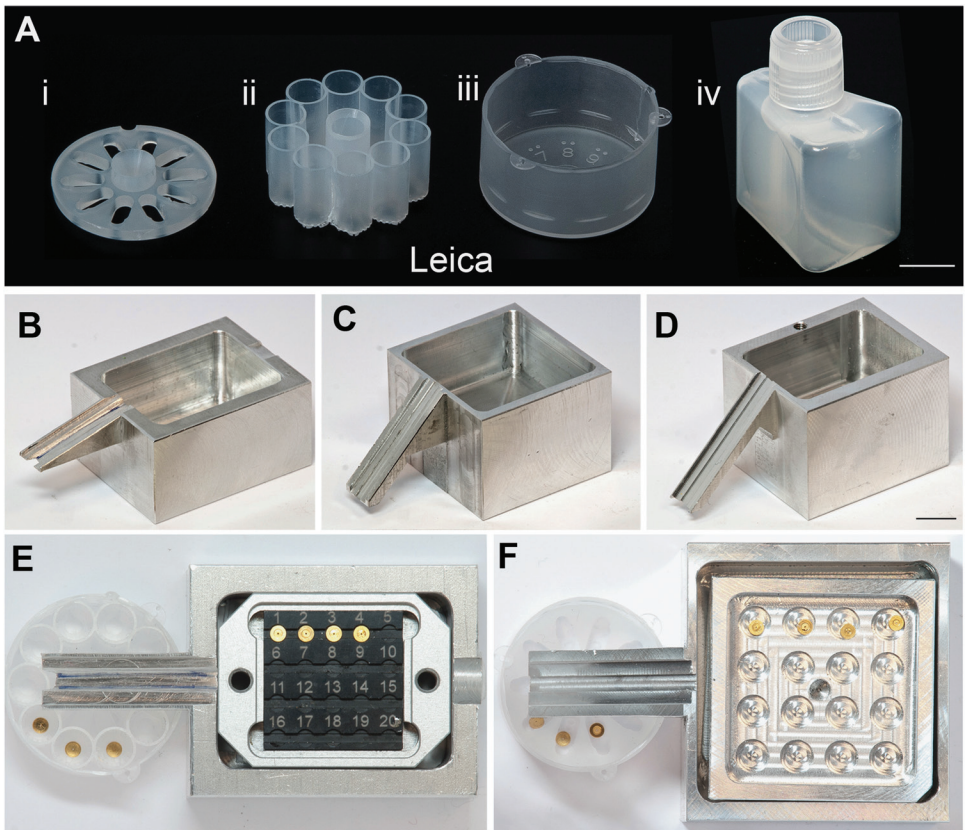

Figure 2: Carrier transfer supports. **(A)** Commercial consumables for automatic freeze-substitution. i. Reagents bath. ii. Reagents bath with flat embedding inserts. iii. Carriers for tubes. iv. Reagents container. **(B–D)** Custom-designed sample transfer supports adapted for the original Leica carrier transfer box and for the modified carrier box. The slider angle will fit cryo-tubes (B), a barrel-like spacer (C), or a flat embedding spacer (D). Original Leica **(E)** and modified **(F)** grid storage boxes with sliders and reagent bath in working state. Grid transfer beneath the slider.

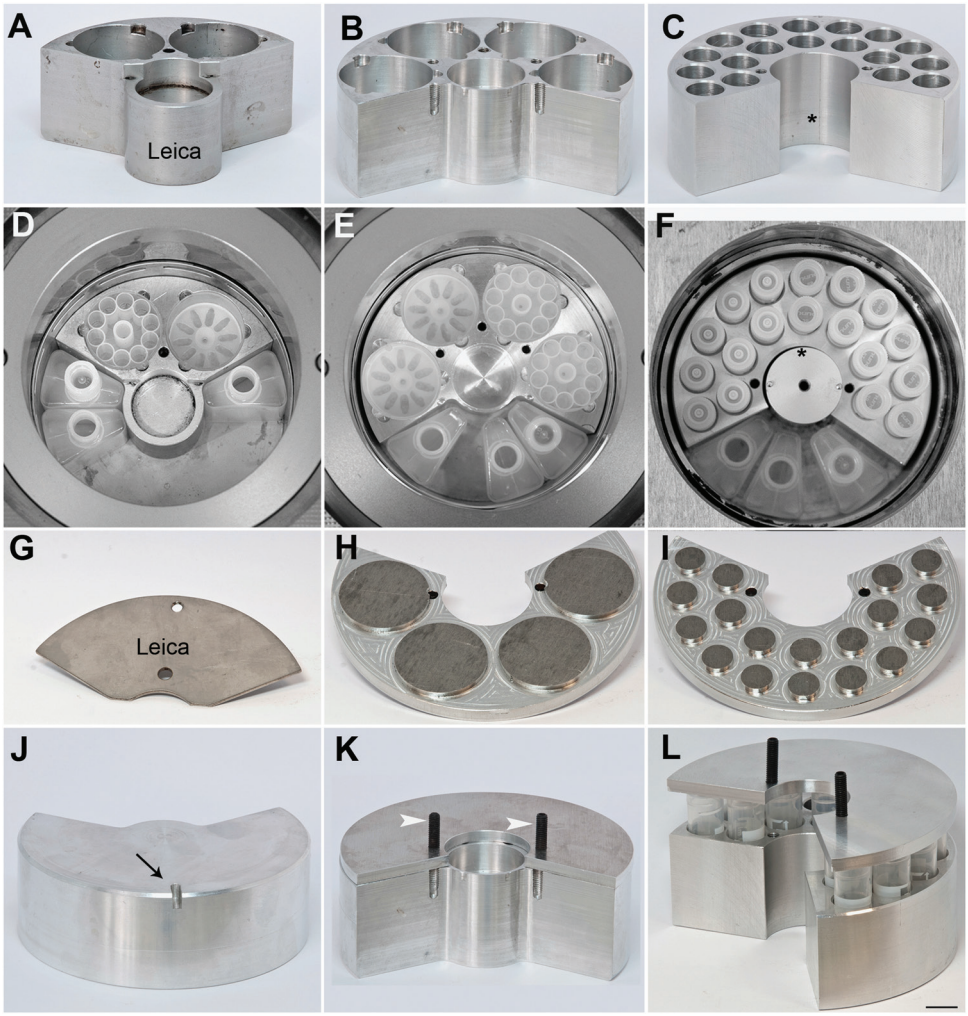

Figure 3: Modified custom-designed holder, adapted for Leica AFS 1 equipment. **(A)** Processing holder for Leica AFS2 chamber. Suitable for automatic freeze-substitution with two round supports. **(B)** Custom-designed processing holder adapted for four round containers. This version is adapted for the Leica AFS2 with a working table in the middle (asterisk). **(C)** Custom-designed processing holder adapted for twenty cryo-tubes. This version is adapted for the Leica AFS1 with an empty space in the middle to fit a liquid nitrogen tube (asterisk). **(D)** AFS2 manipulation chamber in working position with a standard processing holder. **(E)** AFS2 manipulation chamber in working position with a custom-designed holder for the round containers. **(F)** AFS manipulation chamber in working position with a custom-designed holder for the round containers (asterisk on liquid nitrogen tube). **(G)** Leica AFS holder lid. **(H)** Lid for the modified holder, required when longer sample incubations are expected. **(I)** Lid for the modified holder, for cryo-tubes. It eliminates the need to close each tube individually. **(J)** Reverse view of the modified container with the notch required for proper installation inside the AFS2. **(K)** Custom-designed specimen holder for the AFS2, covered with the lid. Arrowheads point to the handles that facilitate placement of the lid. **(L)** Custom-designed tube holder for the AFS, covered with the lid. Scale bar is 1 cm for all images.

digital temperature control assures the required temperature. Usually, samples are cryo-substituted inside the freezing vials directly placed inside AFS within an aluminum cup filled with ethanol, a solution that is inconvenient because of lack of stability. The AFS2 version can be equipped with a holder that fits the AFS chamber, necessary for automatic operation with the FSP module (Leica; Figures 3A, 3D). It offers capacity for twenty samples in two experimental conditions for each preparation run.

The limited number of samples/conditions for each run is a drawback. In addition, when multiple types of samples are treated simultaneously, they are equally prone to manipulation mix-ups. To increase the efficiency of each manual operation AFS machine run, I designed special holders adapted for AFS machines (Figure 3B). The round-basin design allows for the preparation of up to 40 samples or 4 experimental conditions on a single run. In some experiments, cryo-vials are preferred during the substitution step. The alternative design permits stable handling of 20 cryo-vials per run (Figure 3C). These modifications were done with respect to the differences between the AFS models (room for the liquid nitrogen tube inside AFS, Figures 3B, 3C, 3E, 3F, asterisk). Each of the designs contains the notch on the reverse side, necessary to fit the AFS2 chamber (Figure 3J). Each version can support AFS chemical flasks: two flasks contain the pre-chilled substitution mixes, and the remaining flask is a waste container (Figures 3E, 3F).

The original lid for a standard holder was aimed at preventing evaporation of the substitution mixture during the heating phase (Figure 3G). The custom-made lids were designed with convex shapes carved in the lid to enable tight closure of the containers, and the handles permit easier lid placing (Figures 3H, 3I, 3K). For the cryo-preservation vials holder, the lid is highly beneficial, compared to the alternative of removing and replacing the vial caps (Figure 3L). Cryovials can be secured inside the holder to prevent their displacement during agitation. The lid can be fastened to the holder by wrenching in the handles. In this arrangement, the holder can also serve as a fast substitution procedure that does not require the AFS machine [10].

Discussion

Modern technological advancements are updating the field of EM. Focused ion beam and serial block-face scanning electron microscopy permit the acquisition of large volumes of tissue serial sections [3, 4]. Tomography enables acquisition of high-resolution ultrastructural data from thick sections [5]. Correlative microscopy facilitates the correlation between dynamic light microscopy and resolving electron microscopy [6–8]. Successful sample preparation remains a prerequisite for any of these methods. Attempts are constantly made to improve preparation protocols [9, 10]; the same is true for the addition of technological modifications [11, 12].

In this work, I describe a number of upgrades to commercial equipment (Leica EM PACT2, AFS) for more efficient sample preparation, summarizing my attempts to make the entire procedure more ergonomic and precise. The totality of illustrated technical developments permits more efficient exploitation of Leica HPF-FS equipment with reduced risk of sample confusion during the preparation step. These upgrades can be used all together for one experiment, or separately, as the need arises. Given the difficulty of the acquisition of transmission electron microscopy (TEM) results, and the time and labor required for sample preparation and data analysis, these proposed changes could advance the use of EM in certain applications: EM could provide answers to organismal biological questions and a better understanding of cellular ultra-structure.

Conclusion

This article describes modifications and new developments aimed to improve the efficiency of Leica HPF-FS equipment (EM PACT2 and AFS). These developments together or separately can benefit TEM facilities that use this equipment for sample preparation.

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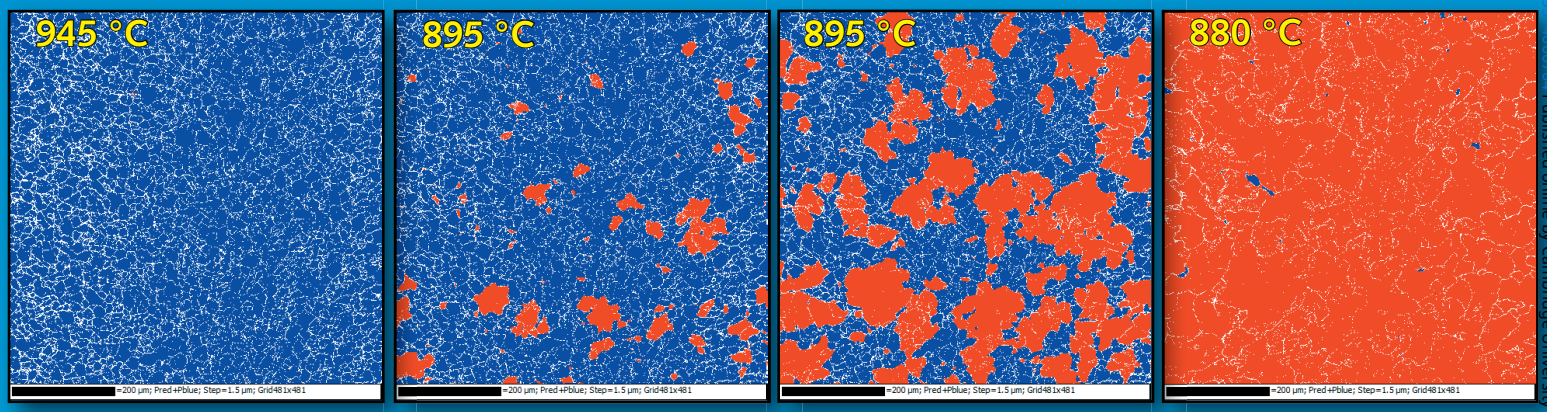

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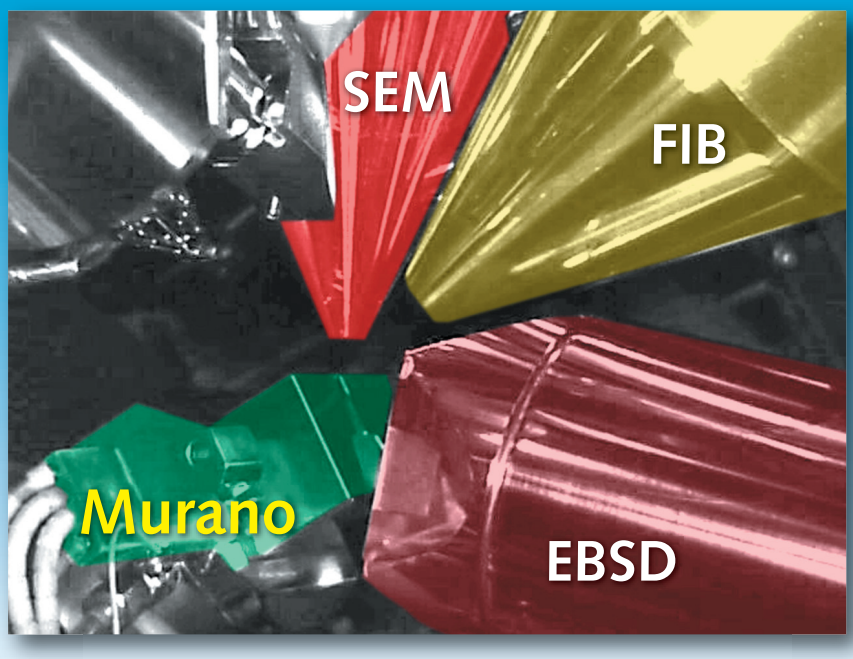

Top Images: EBSD phase maps showing austenite to ferrite transformation from 945 °C to 880 °C, austenite is blue (dark) and ferrite is red (light). Measurements were taken using a single low carbon steel specimen heated to 945 °C then cooled at 1 °C per minute until the start of phase transformation was observed. Once transformation started, temperature was held to observe development of the phase change in individual grains before cooling resumed. Data courtesy of Dr. Singh Ubhi from Oxford Instruments. Bottom Image: SEM chamber view with EBSD, FIB, SED.

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