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# A new HPF specimen carrier adapter for the use of high-pressure freezing with cryoscanning electron microscope: two applications: stearic acid organization in a hydroxypropyl methylcellulose matrix and mice myocardium

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**Key words.** Cryogenic scanning electron microscopy, Cryogenic fracture, Cryogenic freezing, High-pressure freezing, HPF specimen carrier adapter.

## Summary

Cryogenic transmission electron microscopy of high-pressure freezing (HPF) samples is a well-established technique for the analysis of liquid containing specimens. This technique enables observation without removing water or other volatile components. The HPF technique is less used in scanning electron microscopy (SEM) due to the lack of a suitable HPF specimen carrier adapter. The traditional SEM cryotransfer system (PP3000T Quorum Laughton, East Sussex, UK; Alto Gatan, Pleasanton, CA, USA) usually uses nitrogen slush. Unfortunately, and unlike HPF, nitrogen slush produces water crystal artefacts.

So, we propose a new HPF specimen carrier adapter for sample transfer from HPF system to cryogenic-scanning electronic microscope (Cryo-SEM). The new transfer system is validated using technical two applications, a stearic acid in hydroxypropyl methylcellulose solution and mice myocardium. Preservation of samples is suitable in both cases. Cryo-SEM examination of HPF samples enables a good correlation between acid stearic

liquid concentration and acid stearic occupation surface (only for homogeneous solution). For biological samples as myocardium, cytoplasmic structures of cardiomyocyte are easily recognized with adequate preservation of organelle contacts and inner cell organization. We expect this new HPF specimen carrier adapter would enable more SEM-studies using HPF.

## Introduction

Cryoscanning electron microscope (cryo-SEM) examination of fully hydrated cryofixed chemical and biological specimens for morphological studies has been extensively used (Wergin *et al.*, 1993; Nakatomi *et al.*, 2005; Nguyen *et al.*, 2015; Engström *et al.*, 2016).

For the past decade, cryo-SEM observation has experienced a wonderful development thanks to the improvement of cryo-observation modules. However, cryopreparation of samples is key to a cryo-observation of quality and can be very challenging.

Tissue which has been cryofixed remains hydrated and specimen components are rapidly immobilized, which is not the case using critical point drying by carbon dioxide (Polliack *et al.*, 1973) or hexamethyldisilazane (HMDS) (Hazrin-Chong & Manefield, 2012). Cryofixation involves immersing fresh

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and hydrated samples into a liquid cryogen. This technique is commonly followed by freeze fracture and freeze drying (Pieniasek & Messinat, 2016).

The technique of nitrogen slush cryofixation is also based on rapid freezing of the sample. However, the nitrogen liquid calefaction phenomenon resulting from this technique induces slow cooling by freezing and results in water crystals formation due to nucleation and ice crystal growth (Studer *et al.*, 1989). Usually, nitrogen slush fixation is used in SEM systems fitted with cryopreparation chamber.

Many cryofreezing techniques are proposed as nitrogen slush alternative. Studies have performed for freon (Lyer *et al.*, 2005), ethane (Issman & Talmon, 2012), propane (Inoué & Koike, 1989), propane jet (Walther, 2003), and high-pressure freezing (HPF) (Walther, 2003; Fujita *et al.*, 2012) cryofixation.

Unlike the techniques above, HPF enables the freezing of specimen up to a thickness of about 200  $\mu\text{m}$  without the use of cryoprotectant (Walther, 2003) (although preserved frozen sample thickness is dependent of sample kind (Studer *et al.*, 1995)). The absence of cryoprotectant is essential for sublimation after cryofracture, because cryoprotectant slows down sublimation.

HPF uses high pressure and very low temperatures to achieve high cooling rates (Bald & Robard, 1978). HPF is a technique to produce vitreous ice which preserves hydrated samples (Dubochet *et al.*, 1983; Moor, 1987; Studer *et al.*, 1992). This technique prevents cryofixation artefacts and has been recommended for better preservation of hydrated samples (Dubochet *et al.*, 1988; Vanhecke *et al.*, 2008).

After HPF, sample cryofracture and freeze etching can be done before SEM examination. This freeze etching corresponds to a sublimation of frozen water (ice) at low temperature and high vacuum. Sublimation of surface ice under vacuum reveals details of the fractured face that were originally hidden (Echlin, 2009). After deep etching, structures protruding from the remaining ice are generally not collapsed. This is probably a result of the retention of unfreezable water (also known as 'hydration' or 'bound' water) (Given, 1991).

Each step of cryopreparation can be challenging and is key to a suitable examination of samples. That is why many parts of cryopreparation system have been designed specifically to answer specific needs (Inoué & Koike, 1989; Lyer *et al.*, 2005; Issman & Talmon, 2012)

One of the limits for the use of HPF for SEM observation is linked to the lack of well adapted modules for transfer of HPF specimen carrier from HPF systems (EM-ICE, Leica, Nanterre, France, or, HPM100, Leica, Nanterre, France) to the SEM cryotransfer systems (Alto, Gatan, Pleasanton, CA, United States or PP300 and PP3010T Quorum, Laughton, East Sussex, UK). In order to allow this HPF specimen carrier transfer it is necessary to implement an adapter. However, this adapter has to be developed within specific constraints. Firstly, it is

essential that the HPF specimen carrier sandwich is firmly fixated to the adapter, as it will be necessary to apply pressure on the sandwich to open it and to create a fracture using the scalpel. Secondly, the HPF carrier sample fixation has to be done in liquid nitrogen. Finally, the HPF specimen carrier adapter has to be locked to the specimen shuttle (this specimen shuttle is designed by Quorum and Gatan cryopreparation chamber).

Taking all these constraints into account, we designed an HPF specimen carrier adapter. To validate its use we tested two specimens: a chemical one [hydroxypropyl methylcellulose (HPMC) with stearic acid (SA)] and a biological one (mice myocardium).

Cellulose derivatives, such as HPMC, are often used in granulation processes as a protective colloid to coat hydrophobic particles such as SA (Mahato & Narang, 2011). When dispersed in water, hydrophobic SA particles tend to minimize their interaction with the water and thus form large aggregates, whereas HPMC can be dissolved in water. HPMC polymer is able to form a gel network when mixed with water. Upon dispersing SA in the HPMC-water blend, the HPMC gel will entrap SA particles and prevent their aggregation, thus producing stable SA particles (Jarray *et al.*, 2015). Particle dispersion is known to be stable when the particles remain dispersed over a long time (i.e. the total number of particles remains constant over time). As, HPMC-SA is hydrated, it is a good model to test cryofixation quality. It can be verified if, by examination, the HPMC-SA correlated to reality. To this purpose, we have calculated SA-surface in total surface and compared it to real SA concentration.

For biological sample, we chose mice myocardium, because cardiomyocyte morphology and inner cell structure organization are well established. It is therefore possible to evaluate technical quality of the technique thanks to the examination of the SEM-myocardial ultrastructure.

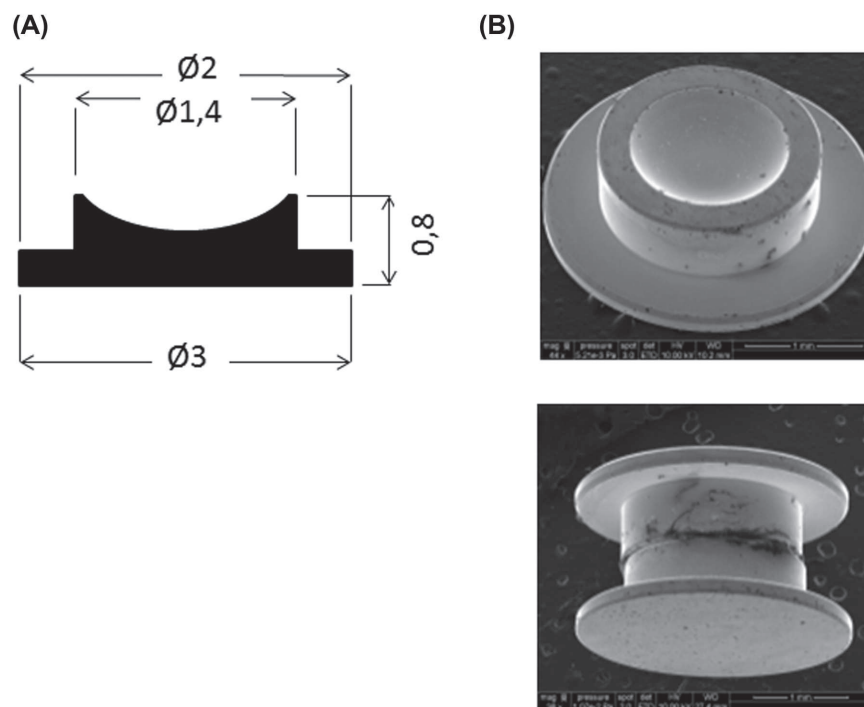
## Materials and methods

### HPMC-SA sample preparation

Aqueous polymeric solutions of HPMC-SA are prepared by dispersing SA in a solution of HPMC and water at 85°C, under agitation for 30 to 60 mins with a rotor-stator homogenizer (Ultraturrax T25, Janke and Kunkel, Staufen, Germany). The solution is then crystallized by cooling the suspension to room temperature. Solutions are thereafter degassed at 50 mbar for 2 h and stored at 5°C for at least 24 h.

### Mice myocardial preparation

Studies were performed on 2-month-old male C57BL/6J0laHsd mice (purchased from Envigo). Experimental animal protocols were carried out in accordance with the European and French regulation guidelines for animal experimentation



**Fig. 1.** HPF specimen carrier. In HPF (HPM100 or ICE, Leica, Nanterre, France) samples are frozen in HPF specimen carrier. (A) manufacturer diagram of this carrier with size of each component (in mms). (B) shows the cavity where the sample is inserted and the (C) shows the sandwich which allows to freeze the sample between two HPF specimen carrier. This technique allows a fracture when separating the two carriers and a sample protection from any water contamination.

and were approved by the local ethical committee. Mice were euthanized through intraperitoneal injection of pentobarbital sodium (160 mg/kg).

Immediately after removal, the heart is plunged in cold PBS during a few seconds thus stopping immediately heart beating. Then, the heart was cautiously sliced with razor blade (few mm thick) on ice bed without any torsions or pressures.

#### *HPF-Cryofixation of HPMC or HPMC-SA or mice myocardium*

The HPMC or HPMC-SA is inserted between two HPF specimen carriers dedicated for cryofracture (Leica) to create a 'sandwich' (Fig. 1), and it is loaded immediately into a HPM100 HPF machine (Leica). The sample is fixated and frozen within 5 ms at 2050 bar and samples are then transferred to cryovials under liquid nitrogen.

For mice myocardium, each myocardial piece is briefly immersed in hexadecane solution, and then inserted between two HPF specimen carriers, loaded into a HPM100 HPF machine (Leica) and fixated following the same procedure as HPMC.

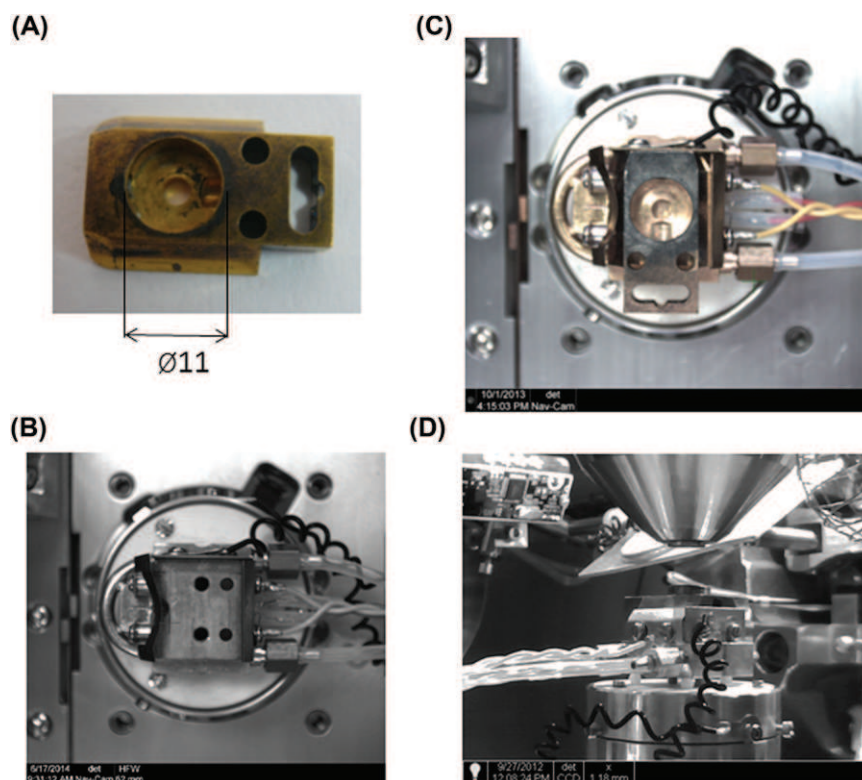
#### *New HPF specimen carrier adapter*

The HPF specimen carrier adapter, made in aluminum, is designed to receive the HPF specimen carrier sandwich (Fig.1

and Figs. 3 and 4) and settle down on the specimen shuttle (AL200077B, Quorum) which is slipped into the cryostage of cryochamber preparation or of SEM cryochamber (Fig. 2). The technical diagram, the picture and the 3D graphic of the new HPF specimen carrier adapter are shown in Fig. 3. The specimen shuttle, HPF specimen carrier adapter, and HPF specimen carrier constitute a combined unit (Fig. 4A). The HPF specimen carrier adapter is organized into two areas, a deposit area and a fixative area (Fig. 4B). Fixation of the HPF sandwich specimen carrier was achieved by slipping it onto slide channels 0.26 mm thick (Fig. 4B, C). This easy fixative method can be used in nitrogen liquid. Moreover, HPF sample fixative area is designed with same principle to accept two specimens reducing the whole procedure duration (Fig. S2).

#### *Transfer of the HPF prepared sample*

HPF specimen carrier adapter (Fig. 3), is first fixated by a screw to the specimen shuttle (Quorum; Figs. 2 and 4) and dried carefully. The transfer occurs in a liquid nitrogen dewar. A large slusher pot full of liquid nitrogen is used to place the HPF specimen carrier (Leica) on the new HPF specimen carrier adapter. The following steps to introduce the sample in the cryopreparation chamber are well established and follow user manual steps (PP3000T, Quorum).



**Fig. 2.** Specimen shuttle. This shuttle is used for cryopreparation chamber (ref AL200077B, Quorum PP3000T, Laughton, East Sussex, UK). It has a hole of 11 mm (A) in its centre and a screw that allows stubs binding suitable for various samples. It uses to perform samples freezing in nitrogen slush. Specimen shuttle is adapted for SEM cold stage, picture B shows the top view without the specimen shuttle and C with it. (D) overview of SEM chamber with the implement of the specimen shuttle.

### *Sample cryofracture*

The fracture of the sandwich specimen carrier HPF is produced in a PP3000T cryochamber by contact with scalpel blades (Fig. 5A); the whole surface of the sample is fractured (Fig. 5B). If the separation of the sandwich specimen carrier occurs without fracture, it is possible to perform the fracture directly by contact of the sample with a scalpel blade, which will induce a small fracture area (Fig. 5C).

### *Freeze drying of the surface of the sample*

Ice sublimation is necessary for surface sample observation. Ice is directly transformed into water vapor at  $-95^{\circ}\text{C}$  in 30 mins. Sublimation is performed in the cryostage of cryopreparation chamber (Quorum).

### *Examination with scanning electron microscope*

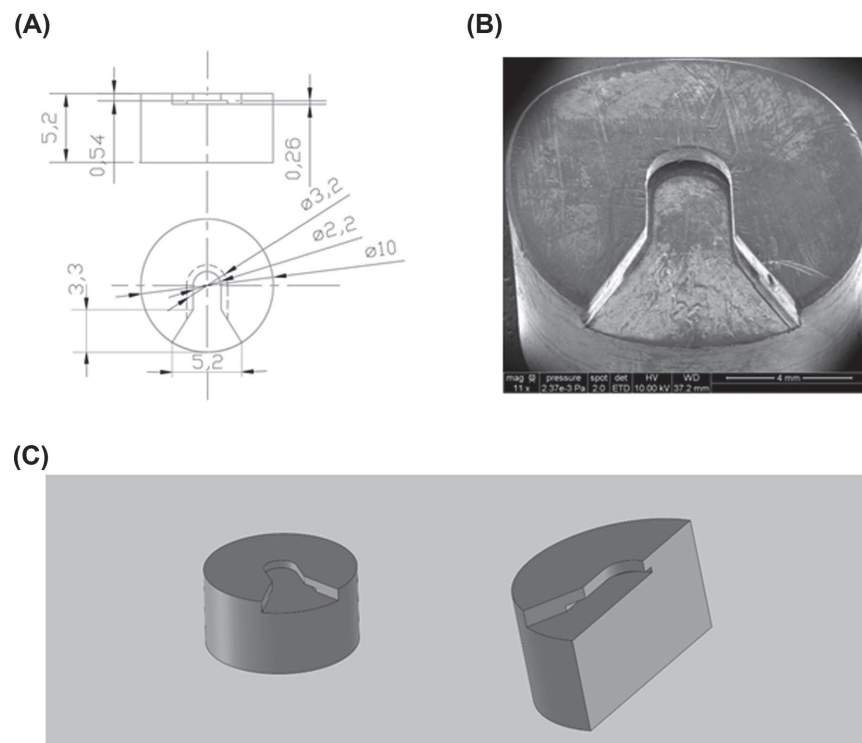
Samples are metalized in cryostage of Quorum cryochamber preparation with Platinum for 60 s at 10 mA. The platinum layer is thinner than 1 nm but allows examination at high voltage with better resolution. Sample was introduced into SEM chamber Quanta 250 FEG FEI SEM (Thermo Fisher, Hillsboro,

OR, USA) and was observed at 1–5 kV with spot size 2 and 0.9–1.1 cm working distance. We used secondary electrons detector located in SEM chamber, so the optimal working distance recommended is around 1 cm. The temperature was kept below  $-140^{\circ}\text{C}$  during SEM imaging.

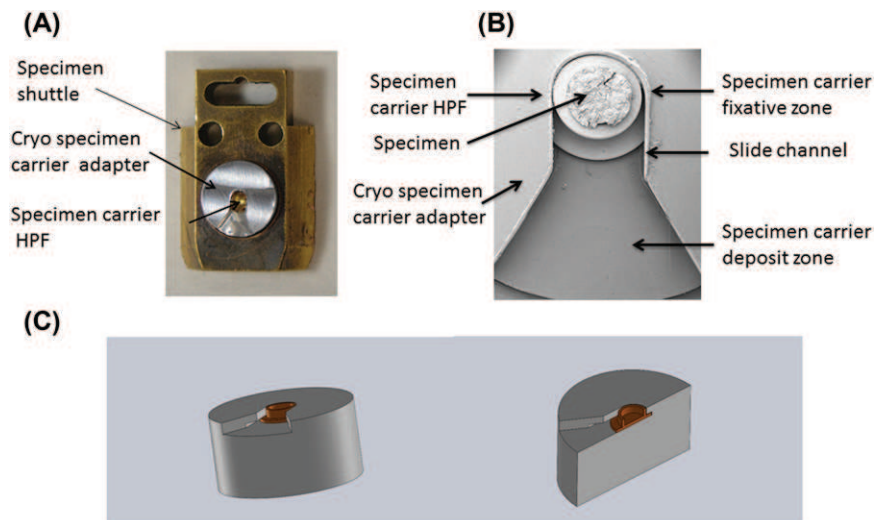
### *SA counting by image analysis*

The density of the SA in the HPMC mixture is measured with the ImageJ image processing software (Rasband, 1997). We chose this software because previous studies validated it in a similar context: Hermana & Walz (2015) to determine the size distribution of adsorbed latex nanoparticles in colloid dispersion; Ellison and collaborators (2014) for the calculation of the mean particle diameter of coated silver nanoparticles; and Baghbanzadeh *et al.* (2015) to measure the surface roughness of membranes.

In ImageJ, in order to remove smooth continuous backgrounds from SEM images of HPMC-SA dispersions, we applied a 'rolling ball' of 30 pixels. To improve the counting and selection of high intensity objects, median (radius 2 pixels) and high pass (radius 0.7 pixel) filters are applied. These filters remove the background noise in the images and increase the intensities of the particle signals in the dispersions. A



**Fig. 3.** HPF specimen carrier adapter. This adapter is designed to be locked in the specimen shuttle and to fasten HPF the specimen carrier. (A) HPF specimen carrier adapter manufacturer technical diagram. (B) SEM picture of this adapter (C) 3D overview diagram and cutting diagram.



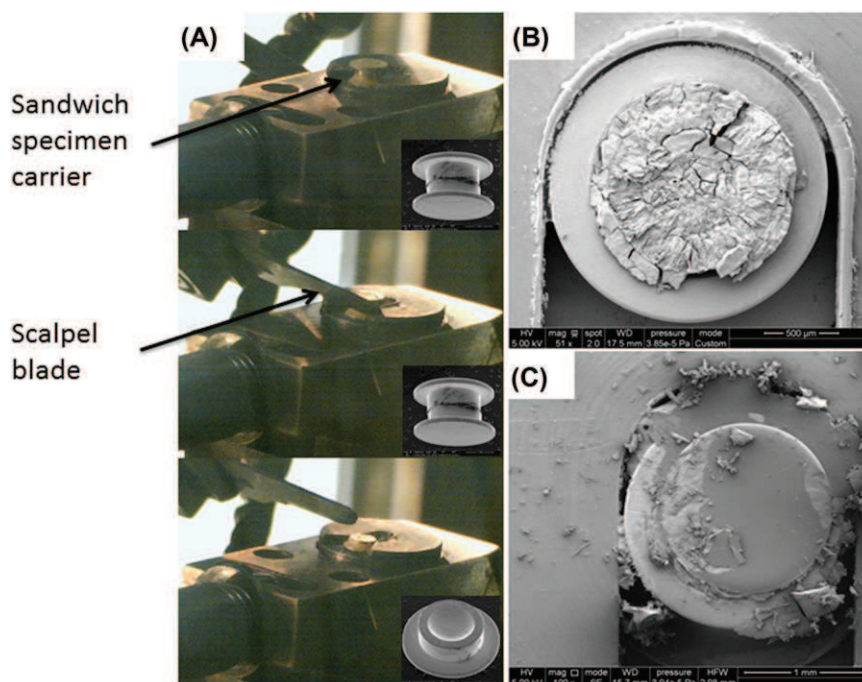
**Fig. 4.** Assembly of technical parts (specimen shuttle, HPF specimen carrier adapter, HPF specimen carrier). (A) overview of every parts assembled. HPF specimen carrier is fastened in HPF specimen carrier adapter, itself placed in the specimen shuttle. (B) SEM picture of HPF specimen carrier adapter areas. Deposit area is used to easily place the HPF specimen carrier. Slide channels enable to slip HPF specimen carrier to the fixative area. (C) 3D-overview and cutting diagrams depict HPF specimen carrier adaptor with HPF specimen carrier in fixative area.

threshold is applied to the images to select objects to be counted to those with intensity between 90 and 255 in grey levels (8 bit images), with a save of the count mask of the outlines of the counted objects. A second count mask is applied to select the counting area on the flat regions.

## Results

### *Sample transfer with our HPF specimen carrier adapter*

Sample transfer is easy but some precautions need to be taken. A screw must be used to fixate the HPF specimen carrier



**Fig. 5.** Specimen cryofracture by removing HPF specimen carrier sandwich top in cryochamber preparation with scalpel blades. (A) (top picture) sandwich HPF specimen carrier in HPF specimen carrier adaptor in the cryochamber PP3000T. (middle picture) scalpel blade pushing sandwich HPF specimen. (bottom picture): HPF specimen carrier bottom remaining after sample fracture and after removing the top of the HPF specimen carrier. (B) sample cryofractured due to HPF specimen carrier dissociation. (C) without sample cryofracture after HPF specimen carrier dissociation the cryofracture is produced manually with scalpel blade.

adapter on the specimen shuttle. It is very important to dry the specimen carrier adapter before nitrogen liquid immersion to prevent water crystals in the slide channel. Ten minutes are necessary for the system to reach liquid nitrogen temperature; this is characterized by boiling around the end of the piece. The HPF Specimen carrier transfer from the HPF (or cryotube storage) to the HPF specimen carrier adapter, occurs in an adapted box containing liquid nitrogen. Cryoelectron microscopy tweezers at liquid nitrogen temperature are used to put HPF specimen carrier on the deposit area of the HPF specimen carrier adapter and to push it on the slide channels to the fixative area. This is technically very simple, however the sliding of the carrier could be blocked by water crystals if this system isn't well dried or if the sample overflows from the HPF specimen carrier during HPF. Under these conditions, the specimen carrier can slide if two tweezers are used for a strong push. Increasing the size of the slide channel would be a technical solution to avoid crystal blocking but this solution increases the risk to drop the HPF specimen carrier during transfer.

The use of two HPF specimen carriers during HPF provides a specimen carrier sandwich. Specimen carrier sandwich separation on the SEM cryotransfer chamber is very easy with the scalpel blade. Therefore, the whole surface of the sample is cryofractured without contamination by water crystals. Should

the fracture not occur, it has to be done manually in SEM cryotransfer chamber. However, extraction of the plastic support for HPF can sometime result in a premature cryofracture, which can be responsible for pollution of the specimen by the emergence of water crystals. This pollution can be removed in the SEM cryotransfer chamber by directly fracturing the specimen again with the scalpel blade or by sublimation.

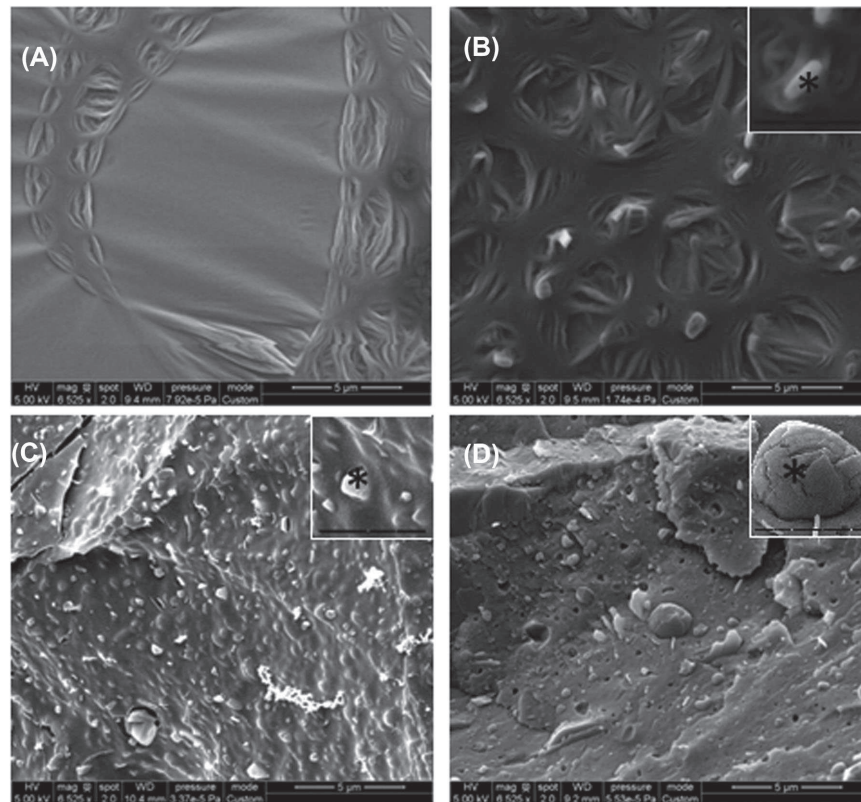
#### *HPMC and HPMC-SA examination*

HPMC does not contain any perforations and has a smooth surface, meaning there is no water crystal artefacts. Some pleating can be observed (Fig. 6A). This data confirms the suitability of cryofreezing by HPF, compared to nitrogen slush which induces holes 'honeycomb-like structure' (Fig. S1).

When HPMC is mixed with SA, white crystals appear. Those crystals have been previously described as SA crystals (Jarray, 2015, 2016) and never occur in 'pure HPMC-water' (Fig. 6).

The general appearance of HPMC-SA mixtures in water at SA 2% (Fig. 6B) or at SA 10% (Fig. 6C) is quite same, even if the number of SA crystals increase significantly for the SA 10% mixture. White SA crystals are distributed in the HPMC-water with a size below 1  $\mu\text{m}$ . Some of the SA crystals are outside (or partially outside) the HPMC-amorphous phase, whereas others are covered with the HPMC-water phase but are still





**Fig. 6.** SEM examination fractured surface of various SA concentration in HPMC. (A) HPMC in water [10% (w/w)] exhibits a smooth, sometime pleating surface. HPMC-SA in water under different SA concentrations; (B) HPMC-SA 2%, SA is spread in HPMC, inset: small SA crystal (\*). (C) HPMC-SA 10%, SA crystals are numerous but still scattered, inset: small SA crystals (\*). (D) HPMC-SA 20%, HPMC cannot keep isolated SA crystals and SA agglomerates appear. Inset: high magnification of an SA agglomerate (\*). HPMC: hydroxypropyl-methylcellulose; SA: stearic acid. (Scale bar 5  $\mu\text{m}$ ).

distinguishable from the swollen HPMC–water surface. There are no big agglomerates.

At SA 20% (Fig. 6D) the structure of the HPMC-SA mixture changes; the SA crystal distribution becomes polydispersed and it becomes difficult to distinguish SA crystals in the aggregates. There is also formation of big agglomerates of SA with different morphologies (Fig. 6D).

#### Image analysis

Quantification of total HPMC–water surface covered by SA crystals (compared to total surface and expressed in percentage), with the scientific image analysis program ImageJ (Rasband, 1997) demonstrates an increase of this SA surface correlated with SA concentration. SA crystals surface calculated is well correlated with SA concentration until SA 10% (Fig. 7). At SA 20%, the surface calculated is underestimated because of emergence of aggregates.

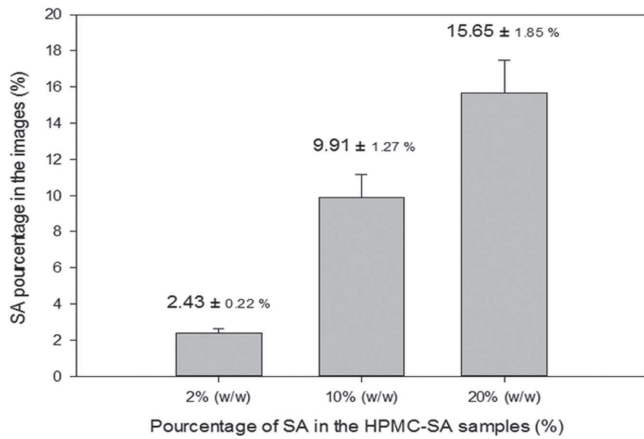
#### Mice myocardium examination

SEM frozen tissue examination can be sensitive because this technique needs to optimize freezing without water crystals

artefacts and enough etching to distinguish cell inner structures details. As expected, HPF-myocardium is well preserved and cryofracture enables a near 3D-examination of cardiomyocytes organization in myocardium (Fig. 8A). Cardiomyocyte cytoplasm is more homogeneous with HPF than with nitrogen slush freezing, with fewer water crystals artefacts (Fig. S1). Contractile apparatus of the inner cardiomyocyte is discerned. Specific organization of interfibrillar mitochondria along contractile apparatus can be observed (Fig. 8B). At high magnification, Z lines appear and can be measured (Fig. 8C), inner mitochondrial structures are well seen and cristae can be detected (Fig. 8D).

#### Discussion

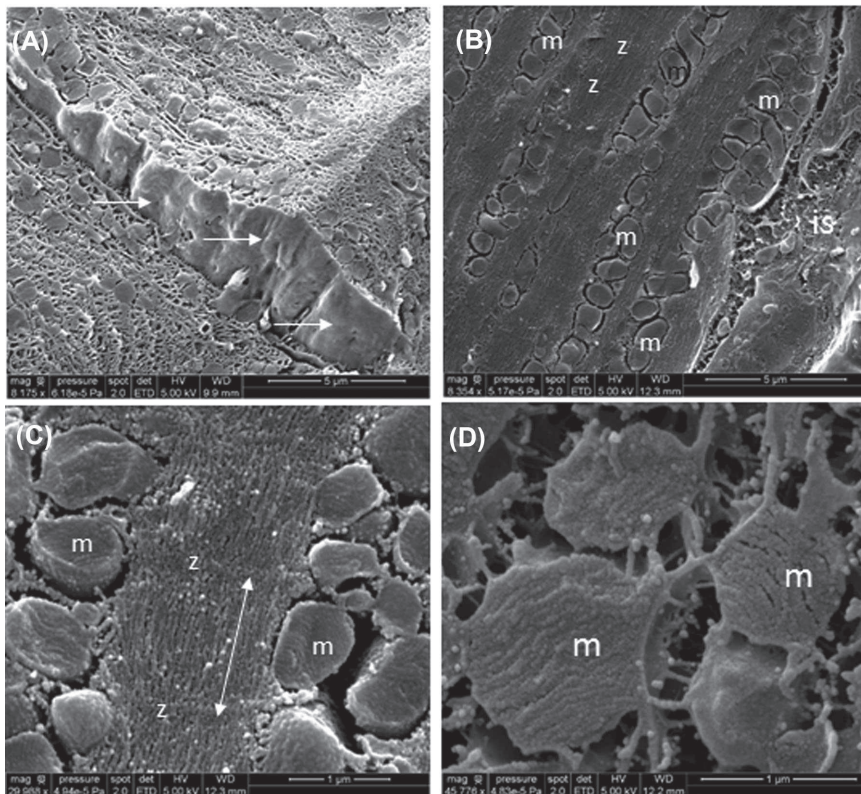
Cryo-SEM examination practices increased tremendously over the last decade. The crucial step for cryo-SEM observation of hydrated samples is cryopreparation. There are various techniques of cryofixation available. HPF is a cryofixation validated and adequate technique for hydrated sample particularly, samples size are preserved on around 200  $\mu\text{m}$  of thickness. Furthermore, this technique does not entail the use



**Fig. 7.** Measure of area covered by SA in HPMC at various SA concentrations. SA surface is measured from SEM pictures by imageJ and expressed as percentage of SA area compared to total area. There is a good correlation at SA 2% and SA 10% between SA concentration in HPMC mixture and the SA area measured. At SA 20%, SA area measure underestimated compared to the real SA concentration.

of any cryoprotectant. Cryoprotectants are known to prevent a correct examination of the sample after cryofracture and after a long sublimation. Indeed, cryofracture allows observation of the cell surface and inside the inner cell, a near 3D-estimation of tissue level organization (depending of the random fracture) and is acclaimed for morphological examination. However, the transfer of cryosample in cryo-SEM can be challenging because of the lack of suitable HPF-cryo-transfer system. In this study, we designed a new HPF specimen carrier adapter for HPF transfer sample.

Various technical constraints have been considered in the design of the carrier, linked to the steps of the cryopreparation and linked to the design of the existing specimen carrier or specimen shuttle currently produced by manufacturers. First, the HPF specimen carrier adapter should be locked to the specimen shuttle and must not show significant expansion under the action of low temperature. That is why we have chosen aluminum material because its shape doesn't change at low temperature. Its diameter is 10 mm to be adapted to 11 mm of specimen shuttle hole. Then the sample is frozen between two HPF specimen carriers. This 'sandwich' should



**Fig. 8.** SEM examination fractured of ultrastructure mice myocardium. (A) At low magnification, cardiomyocytes can be examined in the myocardium with a 3D view (sarcolemma is apparent: arrow). (B) intracellular structures of cardiomyocyte are easily recognized: contractile apparatus (Z) and inter-fibrillar mitochondria (m) are well distinguished. Inter-fibrillar space (is) is noticeable. (C) at high magnification, mitochondria (m) are well visualized and also their organization in the cardiomyocyte, between contractile apparatus. Z-line can be measured (Z; double arrow). (D) mitochondria are suitable cryofixed and cristae organization can be observed.

be fixated firmly to the HPF specimen carrier adapter and this fixation must be performed in liquid nitrogen. The two parts of sandwich should be separated creating a sample-fracture. This operation requires some manual pressure on the system and therefore implies a stress on the fixation system. That is why we have chosen a fixation system in slides which allows fixation in immersion in the liquid nitrogen and which also firmly fixates the HPF support carrier on the HPF support carrier adapter.

Use of our HPF specimen carrier adapter is easy without any damage or any disadvantage requiring redesigning the adapter. A few small difficulties, such as the separation of the two parts of the sandwich without generating fracture, are easily resolved by fracturing manually with the scalpel blade.

To validate our HPF specimen carrier adapter the author tested it in 'real' condition using two samples: a chemical and aqueous sample (HPMC) and a biological sample (mice myocardium). HPMC is a nonionic cellulose ether, which is soluble in water and has thermal gelation properties. Because of its mechanical properties, HPMC is largely used in pharmaceutical industries. It forms a film that can be used as a matrix containing various substances (e.g. nanoparticles or SA) (De Moura *et al.*, 2008). Its aqueous nature makes it a good candidate for cryotests. For biological sample, we chose myocardium because its morphology is well established and inner cardiomyocytes structures are well described. After cryofracture, ice etching is performed at  $-95^{\circ}\text{C}$  during 30 min. At this temperature, amorphous ice does not present major modification due to recrystallization because artefactual modifications are not apparent at the magnification the samples are examined. According to DJ Prior data (Prior *et al.*, 2015), we can suppose that if ice grains are present, their size is smaller than 1 nm, and they are therefore not detectable. Anyway, these data concern water and dirty water freezing. For biological samples, the removal of water by etching leads artefactual morphological modifications due to the shrinking and collapsing structures (Walther, 2003) which hide recrystallization artefacts.

The superior quality freezing with HPF compared to nitrogen slush freezing needn't be demonstrated (Yong *et al.*, 2014), and as expected we observed a suitable quality of cryofixation with HPF. After HPF, HPMC is homogenous and white crystals of SA are easily distinguishable. At high pressure, water is more viscous than at atmospheric pressure, which drastically reduces the nucleation and thus the growth rate of water crystals. Therefore our HPF test permits observation of HPMC structural details similar to its native state. This first step validates our adapter confirming no disturbance in transfer of the HPF cryosample.

Thereafter we have tested the stability of HPMC freezing by mixing HPMC with SA at different concentrations. As expected, at low SA concentrations in the HPMC-SA mixture, SA crystals are stable with good preservation of the morphology of the blend. But at SA 20%, we observed big agglomerates of

SA with different morphologies, meaning that HPMC becomes unable to stabilize SA. Our data are in accordance with literature (Jarray *et al.*, 2016). Overall, in our study, there is no water crystal perforation and the structure of the SA crystals in the HPMC polymer gel is well preserved. To confirm our observations, we counted SA crystals with ImageJ. Our data indicates that SA crystals are well dispersed in the HPMC-water mixture. They also show that the HPF technique is able to preserve the structure of dispersed particles. At SA 20% (w/w), we obtain  $15.65\% \pm 1.85$  of SA surface on the sample total surface. This value, lower than the real SA concentration in HPMC-SA (i.e. 20% (w/w)), can be explained by a polydispersity in the mixture; at high polydispersity the number of chosen images treated by ImageJ become less representative of the distribution of the particles, but also by SA-aggregates. That's why a high calculation error may be obtained.

Heart is a biological sample SEM-examined for more than 30 years and cardiomyocyte inner structures are well known. However, studies of 80's with various freezing techniques and more recent (Ashraf M, 1982; Dalen H *et al.*, 1983; Mühlfeld *et al.*, 2006), demonstrated that it can be difficult to observe myocardium without water crystals artefacts. Therefore testing the new HPF specimen carrier adapter with mice myocardium seemed challenging enough. Despite the challenge the result is that the HPF-myocardium is adequately preserved, without any water crystals artefacts, allowing a good examination of sarcolemma, contractile apparatus, interfibrillar mitochondria with cristae details and also allows if necessary to measure sarcomeres length.

## Conclusion

The HPF specimen carrier adapter we designed, is available for transfer of HPF specimen carrier. It can be used with cryotransfer system (Gatan et Quorum) and therefore can be used with cryo-FIB provided with these system.

The tests performed with this adapter, with HPF cryofreezed HPMC without and with SA, confirm that aqueous samples can be frozen and transferred adequately to allow examination but also quantitative analysis. Same quality is obtained with myocardium extending the adapter practice to biological samples. It has to be however noted that every tissues being different the protocol might not be relevant for other organs. We, however, hope that the HPF specimen carrier adapter will allow future biological tests.

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### Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Supplementary method: Cryofixation of HPMC, HPMC-SA or mice myocardium by pasty nitrogen.** The sample is immersed into pasty nitrogen slusher pot of workstation. Under vacuum conditions, (primary pump) liquid nitrogen ( $-196^{\circ}\text{C}$ ) is transformed into pasty nitrogen ( $-210^{\circ}\text{C}$  approximately). Sample transfer to column mounted preparation chamber PP3000T Quorum Technologies is performed by a cryotransfer-rod. Cryofracture is produced by scalpel blades in the cryochamber. Sublimation is performed until  $-95^{\circ}\text{C}$ .

### Fig. S1. SEM micrographs of HPMC-SA in water (10%–10% (w/w) and myocardium using nitrogen slush.

A: cryofixation using nitrogen slush is a slow freezing process that generates ice crystals, consequently, HPMC exhibits large pores after sublimation (arrow). SA crystal can indeed be observed (star). Bar scale:  $2\ \mu\text{m}$ .

B: examination of cardiomyocyte nitrogen slush freezing is possible and enables to observe endoplasmic reticulum and mitochondria tight contacts. Contractile apparatus is noticeable. Anyway, some crystallization artefacts and some distortion of structures can be distinguished (arrow). Bar scale:  $2\ \mu\text{m}$ .

**Fig. S2. HPF specimen carrier adapter for two HPF specimens carrier.** The adapter shown in this manuscript, is designed with the same technical principle to accept two specimens. A: HPF specimen carrier adapter manufacturer technical diagram. B: SEM picture of this HPF specimen carrier adapter with two HPF specimens carrier.