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A new passive system for contamination-free long-distance cryo-transfer of biological tissues

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Abstract. Several new analytical techniques require long-distance cryogenic transfer of samples that need to be kept at stable temperatures for long time periods, but also to be additionally contamination-free. In this study we developed a passive transfer system to fulfil those requirements. With 125mL of liquid nitrogen stored, one cryo-sectioned sample was maintained around 120±1 K and a pressure of about $3x10^{-7}$ mbar for at least 2 hours. With a total transfer weight of 5 Kg this system can be easily handled and carried by any transportation means so that the same sample can be used for different imaging centres located remotely permitting correlative studies.

1. Introduction

Over the past decades, electron-beam imaging techniques, such as Transmission Electron Microscopy (TEM) or Scanning Electron Microscopy (SEM) have become main-stream methods for acquiring ultra-high resolution morphological information of biological systems of interest [1][2]. To minimize volatilization or degassing of the sample in ultra-high vacuum environment of TEM or SEM, classical sample preparation procedures require a chemical fixation of the biological sample and embediment in an epoxy resin matrix before thin section slicing. However, the distribution of soluble compounds (ions, metabolites, drugs, etc.) involved in a multitude of fundamental biological processes are not preserved during the classical sample preparation.

The only certain way to preserve and observe soluble molecular compounds and ions unperturbed in-situ in a biological tissue is to create and maintain highly controlled cryo-conditions throughout the chain of preparative and observational procedures. With the introduction of cryopreservation techniques, cryo-EM imaging techniques have been intensively developed for resolving structural information of the biological samples at the sub-cellular or even atomic level [3][4].

Correlation studies based on cryo-EM and cryo-Fluorescence Microscopy, or cryo-Mass Spectrometry (MS) have showed great potential to study targeted biological process or molecules by

introducing either fluorophore-labeled or stable isotope-labeled biomarker and then overlapping acquired functional images with ultra-high resolution cryo-EM images. Therefore, a reliable vitrified sample transfer system is needed to keep the sample contamination-free during transportation. Currently nearly all commercially-available cryo-transfer system are built only for a specific cryo-imaging or –analysis instrument. Only several customized system has been realized, but they are not designed for long-distance transfer[5][6].

1.1. Operational concept

To conduct a correlation study under cryo-conditions, biological samples are first prepared by labelling biomarkers sensitive to the functional imaging technique. Then those samples will be quickly plunged into liquid nitrogen or high-pressure frozen by cold nitrogen gas so that vitrified state of those samples can be achieved. Before carrying out high-resolution structural imaging, surfaces of the vitrified samples need to be flattened and necessary etching and coating procedures ensure that sample structure of interest exposes under the EM scanning. Critical cryo-transfer at proper temperature and pressure after the EM scanning ensures the same vitrified samples used for mapping their functions of interest without altering samples' vitrified state. Finally, functional images of the vitrified samples are overlapped with structural images so that biological function can be correlated to specific compartment of the studied biological system. A typical operational concept of a correlation study involving cryo-SEM and cryoNanoSIMS can be found in the Figure 1.

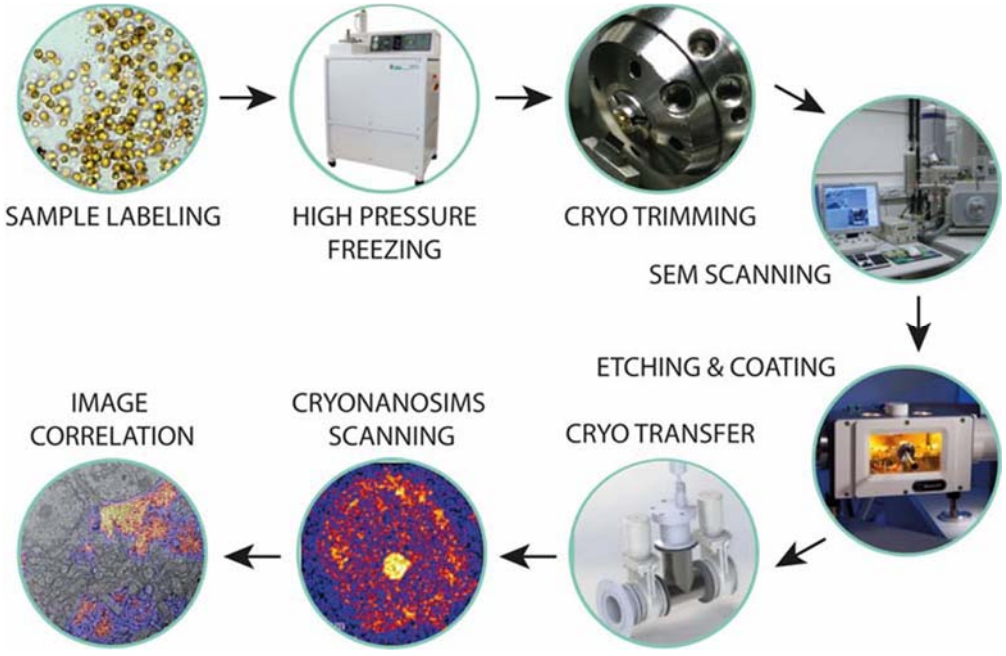

Figure 1. A typical operational concept of a correlation study involving cryo-EM and cryo-MS. A critical cryo-transfer under proper conditions occurs between high-resolution EM imaging and functional MS imaging.

1.2. Requirements

The aim of the present project is to develop a more generally applicable, light-weight transfer system which fulfils the following design requirements for storing and transferring vitrified samples: 1) The system shall provide a contamination-free storage environment for vitrified biological samples with a preferred temperature of 130±5 K for at least two hours; 2) The total transfer weight shall not exceed 5 Kg; 3) The system shall be adapted to different imaging modalities which might be located far away from each other, e.g. hundreds of kilometers in certain cases.

2. Construction of the prototype

The cryo-transfer system was designed with three building blocks: a vitrified sample carrier with a LN2 reservoir in the surrounding vacuum where the sample was stored, an adaptation flange that allowed access to different imaging modalities, and a sample manipulator which could move a sample in translational, rotational and radial directions. An additional mobile LN2 transfer unit was built for cases in which extra coverage of a larger distance would be needed.

2.1 Sample carrier

The sample carrier was based on a high vacuum chamber consisting of a standard stainless steel T-piece (KF50) and a home-made stainless steel extension as main body (1 in Figure 2). To avoid significant thermal convection in the chamber, high vacuum quality was achieved by sealing the vacuum chamber with two mini KF-flanged UHV gate valves (2) on the horizontal axis and a top flange with a VITON O-ring. In the vacuum chamber, an argon-arc welded LN2 reservoir made in 321L type of thin-walled stainless steel (OD 44.45 mm) with a total volume of approximately 125 mL was suspended under the top flange with a SS tube (3) connected and then laser-welded on the top flange for storing LN2.

Considering the conventional vitrified sample loading procedure using the cryo-SEM preparation station (PP3010T, Quorum Technologies, UK), the LN2 filling tube was not located in the middle but close to the rim of the reservoir. The purpose of this specific design was to allow placing the carrier vertically without losing major cooling power of the stored liquid nitrogen. Three short perforated SS tubes (4) were soft-welded around the reservoir in a symmetrical way so that the total weight of the reservoir will not be loaded on the filling tube and at the same time thermal conduction could be minimized.

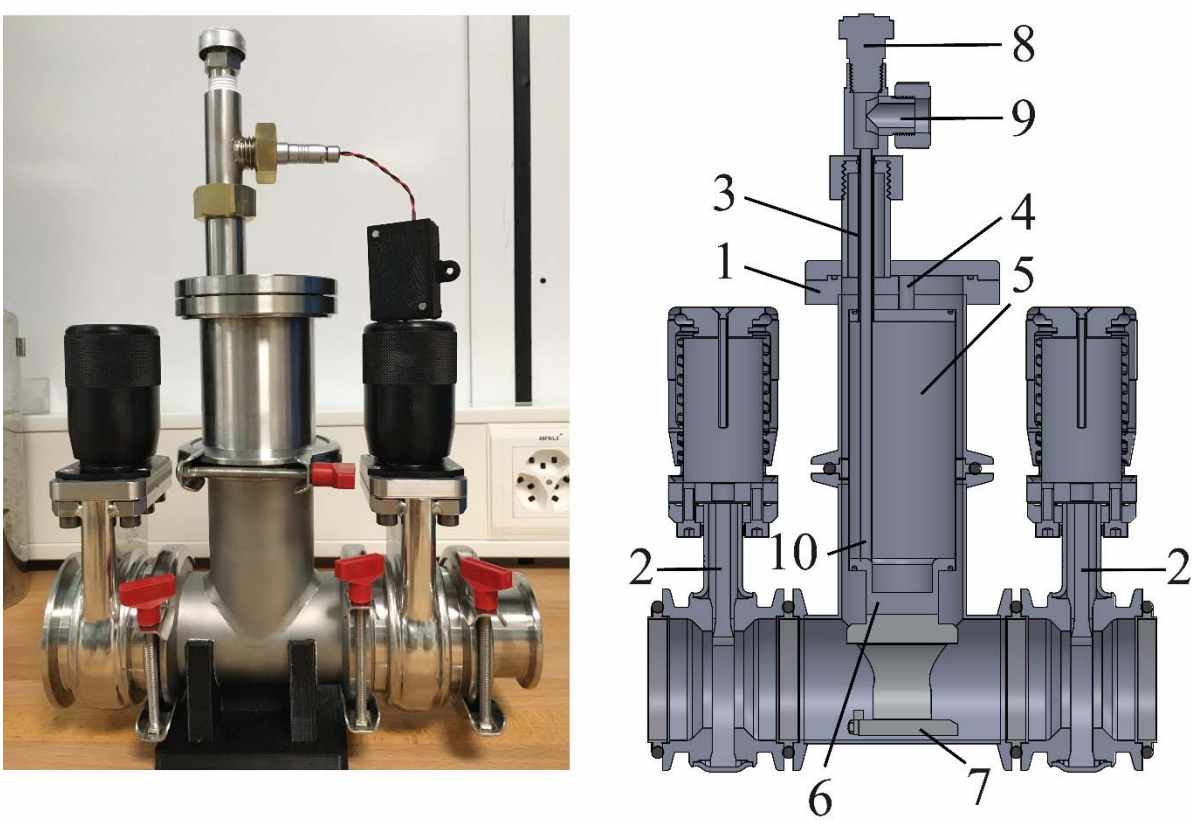

Figure 2. A complete prototype of the vitrified sample carrier (left) with details inside (right): 1. Top flange; 2 Mini gate valves; 3. Filling tube; 4. Supporting tubes; 5. LN2 reservoir; 6. Reservoir bottom; 7. Sample cooling stage; 8. Relief valve; 9. Connection for electronics; 10. Level meter.

Under the LN2 reservoir, a cooling stage (7) made in 6061 type aluminum block was firmly bolted to it to ensure an efficient heat conduction. A large polished body surface of the cooling stage was considered to be both an effective thermal conducting part for cooling the sample and an anti-contaminator for the sample at the same time. Two copper-beryllium (BeCu) springs were made and bolted on the side of the cooling stage and used for reinforcing the contact between standard cryo-SEM sample holder and the cooling stage.

To monitor the LN2 reservoir a level warning insert was built. A chip-based RTD probe (FK422 PT1000B, Heraeus, Germany) was fixed inside the perforated level warning insert (10) at a desired safety level, approx. 20 mm above the bottom of the reservoir in this project (25% of the total volume). Once the LN2 dropped below the safety level, the linear comparator circuit powered by a 3V coin battery would trigger a siren to signal the need for a LN2 refill. Besides, a gas relief valve (8 in Figure 2, D520T1-2M. Circle Seals, USA) was installed on the top of the level warning insert, which was connected to the nitrogen filling port so that nitrogen gas could be safely released to atmosphere once the pressure inside the nitrogen reservoir is higher than the pressure limit (0.1 barG).

2.2 Sample manipulator

Manipulation of vitrified samples was realized by a homemade vacuum-sealed sample manipulator. The manipulator (part C in Figure 3) was connected to one of the VAT gate valves close to the LN2 filling port (see Figure 3). The main challenge of the sample manipulator was to seal the high vacuum and at the same time be able to rotate, translate and wobble the sample. The rotatory and translational movements were ensured by two linear shaft guide made in PTFE and a dynamic seal. To fulfil the requirement of movement in radial direction, a hydroformed bellow (2 in Figure 3, Witzenmann, Germany) was welded to flanges (see Figure 3) so that sample can be tilted with an angle of max. 5° under vacuum. Differ from most of UHV-compatible sample manipulators, this design had a sapphire window (3) enabling not only visual inspection during loading, but also a precise position of the transfer

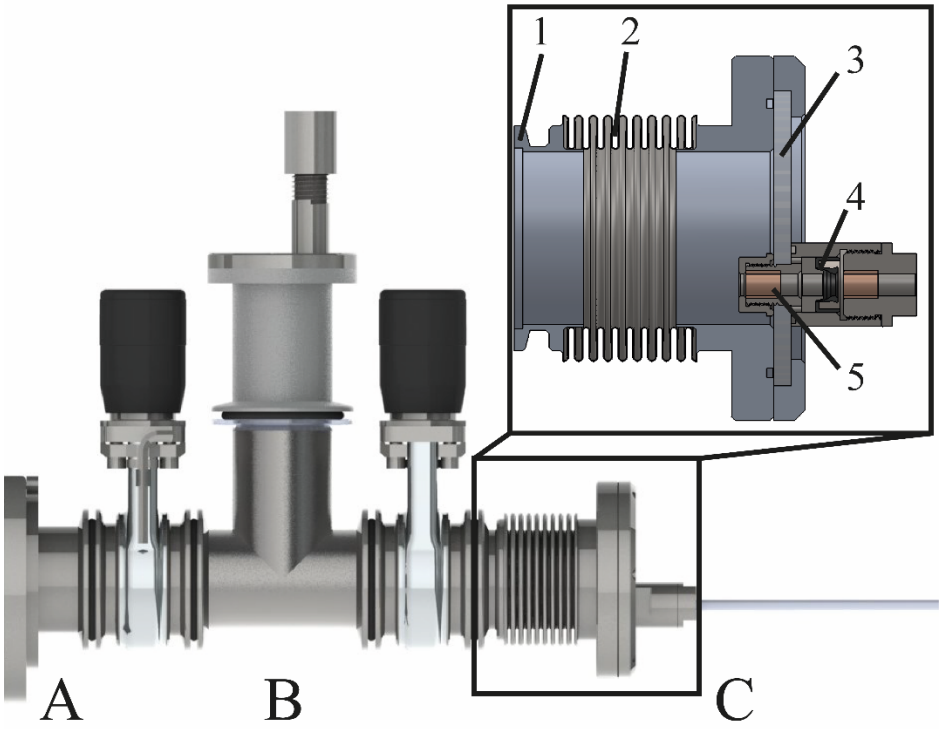

Figure 3. Complete assembly of the transfer system including the adaptation flange (A), the sample manipulator (C) and the sample carrier (B). Cross section of the sample manipulator design is shown at the top-right corner: 1. KF50 flange; 2. Hydroformed bellow; 3. Sapphire window; 4. Dynamic seal; 5. Linear shaft guide bearing.

shaft and its belonging vacuum seal (see Figure 3) so that the loading axes of both cryo-SEM and the sample manipulator were aligned.

2.3 Mobile LN2 filling unit

In addition to the transfer system, a mobile LN2 filling unit with a volume of 1 liter was built. Sufficient overpressure inside the Dewar was built up by manually compressing the evaporated nitrogen gas volume. This forced LN2 to be transferred through a plastic transfer line isolated with foam. The line ended into a sintered outlet to obtain a well-regulated flow into the LN2 reservoir in the vitrified sample carrier and therefore to reduce the total consumption for one refill. Note that the mobile filling unit can be built with any lab grade vacuum LN2 Dewar used for storing LN2. Similar to the principle used for commercially-available product for large volume LN2 filling, evaporating LN2 by heating up a resistor sealed and placed in a LN2 Dewar would generate an overpressure and facilitate the LN2 filling.

3. Experimental results and discussion

To evaluate the performance of the vitrified sample carrier vacuum quality and final temperature measurement on the sample position were carried out. A base vacuum of $5x10^{-5}$ mbar can be achieved at room temperature without continuously pumping on the vacuum chamber. After filling LN2 into the reservoir and stabilizing in a period of 30 minutes, an improved vacuum ~ $3x10^{-6}$ mbar was reached.

Temperature on the position that sample would experience in a real transfer was separately measured by a thermometer clamped on the cryo-SEM sample holder (see Figure 4). After a short cooling down period around 1 hour (Phase I in Figure 4), the actual temperature was stabilized around 120±1 K for a period of 2 hours (Phase II in Figure 4). Note that the filling signal out from the level warning insert would be given around 1.5 hours after cooling down the system from room temperature for the first time. To avoid a temperature increase of the vitrified sample in case of no present of LN2, a mobile LN2 filling unit can be used. Usually 1 L LN2 in the mobile filling unit can fill the reservoir 2-3 times. It was noticed that most of the LN2 consumption was caused by the poorly-isolated transfer line. But even with 2 times refill possibility the sample could be transferred in a much longer time period.

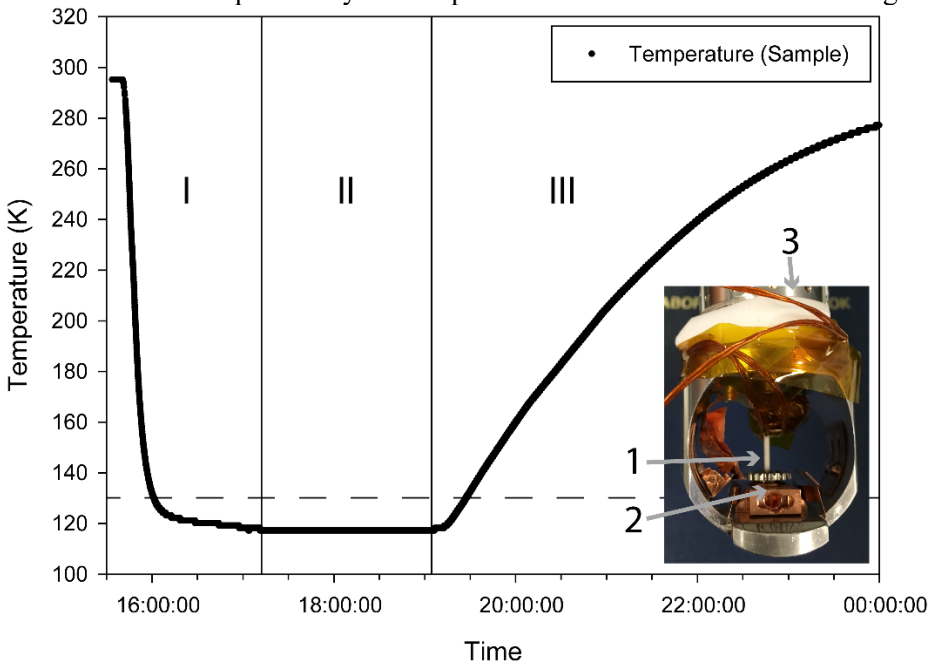

Figure 4. Temperature evolution in one full operational cycle including cooling down (I), transfer (II) and warm up(III) phases. The temperature measurement setup is shown in the image insert at the bottom-right corner. A pt100 thermometer (1) was clamped in the SEM sample holder (2) and the heat dumping of thermometer wiring was done by fixing wires on the bottom of the LN2 container.

This would extend the transfer distance up to hundreds of kilometers through a sample transfer by car.

In the field of cryo-biology research ice contamination often plays an essential role in image quality. Since the measured temperature on sample position was around 120 K, which was 15 K lower than the expected temperature, namely 135 K (reading from the phase-diagram of water at a given pressure of $3x10^{-7}$ mbar), a small amount of ice could be found on the sample after long-distance transfer. To avoid severe ice contamination over long distance transfer, a gas absorber, such as zeolite, was used to improve vacuum quality below $3x10^{-7}$ mbar. In our test transfer no severe ice formation was observed on the SEM images over a long period of 4-5 hours. Another source of contamination was the bad vacuum isolated in the sample manipulator. Once the manipulator was connected after the transfer, this part of blocked air normally could not be pumped out and therefore introduced a potential contamination source for the vitrified sample. In our preliminary test, a bypass bellow (not shown here) connecting both the sample manipulator and the adaptation flange was able to be used for pumping both spaces down to a similar vacuum quality (~ 10^{-7} mbar) as the sample carrier before opening the gate valves to access the vitrified samples. In the future, more considerations will be taken into account regarding to balance the pressure and desired temperature, e.g., thermal simulation of various designs of cooling stage to optimize the final sample temperature.

The versatility of the built transfer system is mainly realized by the widely implemented KF type of quick connection flanges in the present study. Several advantages include that, first, it makes the system easily and quickly adaptable to various imaging modalities. By changing the adaptation flange and its belonging sample manipulator, the entire transfer system can be assembled or disassembled in 10 minutes. Meanwhile, the total weight of the carrier is reduced compared to the case in which other type of standard vacuum flanges, e.g., a CF type, were implemented. Second, since an extra set of adaptation flange and sample manipulator could be stored in the imaging facility in which sample will be transferred, a real cryo-transfer care only needs to be taken for the sample carrier. Therefore, a total transfer weight can be limited to 5 Kg in our case. First series of transfer experiments were carried out by packing the tested vitrified sample carrier in a shoulder bag and carrying a prepared vitrified sample from a cryo-SEM facility to cryoNanoSIMS lab at University of Lausanne. Both labs were located in the same campus in this case, but with a walking distance of 25-30 minutes. The initial temperature of transferred vitrified samples was kept at 125±1 K, which was controlled by the cryo-SEM preparation station. An average total transfer time of 45-50 minutes was able to be achieved and no water condensation outside of the carrier or ice crystal on the surface of the vitrified sample was observed during all transfers (n=6). In the near future, more prepared vitrified sample will be transferred among different cities due to the need for further correlative studies.

Disadvantage of the KF flanges is that ultra-high vacuum is difficult to be achieved or maintained since high temperature baking procedures could not be performed with KF type flanges. Due to the common vacuum quality between 10^{-7} and 10^{-9} mbar in cryo-imaging system, the present transfer system requires a pre-pumping period (dependent on the pre-pumping volume of the imaging modality) to reach the targeted vacuum quality for imaging.

Not to ignore that another advantage of the discussed transfer system is the unnecessariness of any electric power supply, which could become a critical or even limiting factor for vitrified sample transfer. This is because temperature and pressure inside the carrier become very stable during the transfer with the use of gas absorber.

4. Conclusion

In the present study, a contamination-free long-distance cryo-transfer system was realized after solving technical issues such as improving high-vacuum quality in the vitrified sample carrier, approaching the desired temperature on the sample to maintain sample's vitrified state and increasing the system flexibility so that it could be easily handled. It showed a great system stability both with respect to pressure and temperature and was able to be used passively (without sophisticated electronics). Combined with the additional mobile LN2 filling unit, one vitrified sectioned biological sample could be transferred each time over a long distance.

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