Protocol for high pressure freezing and cryo-FIB lamella preparation of *Saccharomyces cerevisiae* and *Staphylococcus aureus* culture

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1. Introduction

Cryo-electron tomography (cryo-ET) provides a unique capacity to study macromolecular complexes under near-native conditions *in situ*. However, owing to the strong interaction of an electron with a matter, a suitable specimen has to be no thicker than ~300 nm for transmission electron microscopy (TEM) or ~1000 nm for scanning transmission electron microscopy (STEM), which restricts the direct utilization of cryo-ET only to small cells or peripheral regions of the larger cells. Cryo-focused beam micromachining (cryo-FIBM) has been introduced as a sample preparation method to overcome this limitation. Cryo-FIBM is used to ablate the cellular material and prepare a section (lamella) that is suitable for cryo-ET. Thus, the combination of cryo-FIBM and cryo-ET can be used to obtain high-resolution structural data from any region of the vitrified biological specimen. Plunge freezing vitrifies cellular specimens with a thickness of < 8-10 µm and is usually used for vitrification of bacteria, small to medium-sized eukaryotic cells. However, for the bulkier samples, it is necessary to employ high-pressure freezing (HPF) to assure proper vitrification. HPF is used for vitrification of specimens with a thickness of up to ~200 µm which allows studying not only single cells but also tissue fragments. The Cryo-FIBM protocol requires an additional step in the case of HPF frozen specimens, which comprises the transfer of the volume of interest from the bulk sample to the TEM grid using a micromanipulator. Here, we describe a protocol that we have used to prepare a lamella from the HPF vitrified *Saccharomyces cerevisiae* cell culture for cryo-ET.

2. Protocol

Saccharomyces cerevisiae cell cultivation [1]

High pressure freezing

1. Prepare *S. cerevisiae* cell culture according to protocol in reference *Cultivation of Saccharomyces cerevisiase cell culture in liquid medium* [1] and incubate app. 7h.
2. Measure OD of *S. cerevisiae* cell suspension at 600 nm using UV/VIS spectrophotometer.
3. Gently spin down the cell suspension in centrifuge (100 x g / 5 minutes / ambient temperature) to obtain a compact cell pellet.
4. Discard medium very carefully to preserve the compact cell pellet.
5. Load the cell pellet into HPF planchette hat with diameter 3mm and depth 0.1mm (type A).

6. Cover the HPF planchette hat filled with the cell pellet and cover it with a flat side of the planchette hat (type B) NOTE: Planchette hat (type B) flat side is treated with 2% lecithin dissolved in chloroform to facilitate detachment of both parts of the HPF sandwich after freezing.
7. Freeze the sandwich in the high pressure freezer instrument.
8. Keep the sample in liquid nitrogen conditions for transfer into the cryo-FIB/SEM.

Staphylococcus aureus cultivation

We have used *Staphylococcus aureus* (*S.aureus*) planktonic strain SA 1137 infected with bacteriophage ø812 for the preparation of the HPF specimen.

1. Pre-culture *S. aureus* planktonic strain over night in 20 ml of MIX 6 medium.
2. Seed 50 ml culture at OD_{600} = 0.1 in MIX6 medium.
3. When the *S. aureus* culture reaches $OD_{600} \geq 0.5$, infect with ø812 at MOI 10^1 or 10^2
4. Incubate *S. aureus* with bacteriophage ø812 for 30 min.
5. Spin 3000x g, 5 min, RT.
6. Aspirate media to create dense cell pellet.
7. Load cell pellet into HPF planchette hat with diameter 3 mm and depth 0.1 mm (type A).
8. Cover the HPF planchette hat filled with cell pellet and cover it with flat side of the planchette hat (type B) NOTE: Planchette hat (type B) flat side is treated with 2% lecithin dissolved in chloroform to facilitate dividing of both parts of the HPF sandwich.
9. Freeze sandwich in high pressure freezer.
10. Dessemble sandwich in fresh and clean liquid nitrogen and store planchette hat (type A) with vitrified cell sample in liquid nitrogen conditions for transfer into the cryo FIB SEM.

Materials for HPF:

- 2% lecithin in chloroform
- Liquid nitrogen
- 4L LN2 Dewar
- A high pressure freezer
- A high pressure freezer equipment: HPF planchette hats (Cu/Au) modified for Leica, Type A (3 x 0,52mm, recess 0.1 / 0.2mm), HPF planchette hats (Cu/Au), modified for Leica, Type B (3 x 0,52mm, flat top)
- Cellular sample (*S. cerevisiae* or *S. aureus*) for HPF - at least 10 µL
- Cryo EM and laboratory equipment: tweezers, nitrile or latex gloves, eye protectiol glasses, cryo-gloves, grid boxes, grid box opener, pipettes and pipette tips, ethanol for cleaning.

Loading of the specimen to the cryo-FIB/SEM

1. Draw fresh liquid nitrogen into the dry 4L dewar and microscope cooling dewar.
2. Microscope chamber and preparation chamber (if present) need to be pumped to high vacuum before start of cooling down ($< 4*10^{-4}$ Pa) to prevent contamination growth.

3. Cool down the stage and the anti-contaminator both in the microscope chamber and in the preparation chamber with nitrogen gas (5 L/min) to temperature <-180°C.
4. Cool down the cryo-preparation station with the fresh liquid nitrogen.
5. Insert cryo-shuttle dedicated for HPF sample filler to the left position and half-moon grid to the right position.
6. Cool down tweezers, a gridbox opener and a screwdriver.
7. Insert the box with the samples to the cryo-preparation station and open it with the gridbox opener.
8. Carefully insert HPF specimen into the left slot of the cryo-shuttle with the specimen facing up and half-moon clipped in the AutoGrid cartridge into the right slot with grid pins pointing right.
9. Secure the specimen and the half-moon grid in the shuttle.
10. Flip the shuttle with specimen inside of the preparation chamber to the loading position.
11. Load shuttle with specimen properly to the cooled cryo-FIB/SEM microscope.

Sputter coating and GIS deposition

1. Sputter coating of the specimen surface with conductive metal layer.
   a. Deposit ~10 nm metal layer (Ir,Au,Pt) onto the surface of the HPF sample.

2. Deposition of the protective layer with organometallic platinum using Gas Injection System (GIS).
   a. Set the sample to the eucentric position.
   b. Tilt cryo-stage to 0° and rotate to 110°.
   c. Find center of the grid and set the grid 3 mm below eucentric position.
   d. Set the GIS needle to 26 - 30°C.
   e. Deposit ~300-1000 nm of the organometallic platinum layer on the grid with biological specimen (Corresponds to 30 - 120 s of the GIS deposition)

Milling the trenches for cellular chunk

1. Set scan rotation in both electron and ion beam to 0°
2. Find the suitable region of interest and save the stage position
3. Create upper and lower regular cross section milling patterns (Si-multipass) with following dimensions: X ~ 22 um, Y ~ 25 um and Z = 4 um (Fig. 1).
4. Create right pattern (Fig. 1) to facilitate accessibility of the sample for the micromanipulator needle (X ~ 25-30 um, Y ~ 18-20 um and Z = 4 um).
5. Place right pattern with a slight overlap to both upper and lower pattern and center it closer to the lower pattern.
6. Set lamella (chunk) thickness to 6um.
7. Mill all patterns with current 5-7nA.
8. After rough milling of trenches, create cleaning cross sections with current 0.5nA. Set the lamella (chunk) thickness to 4um (Fig. 2A)

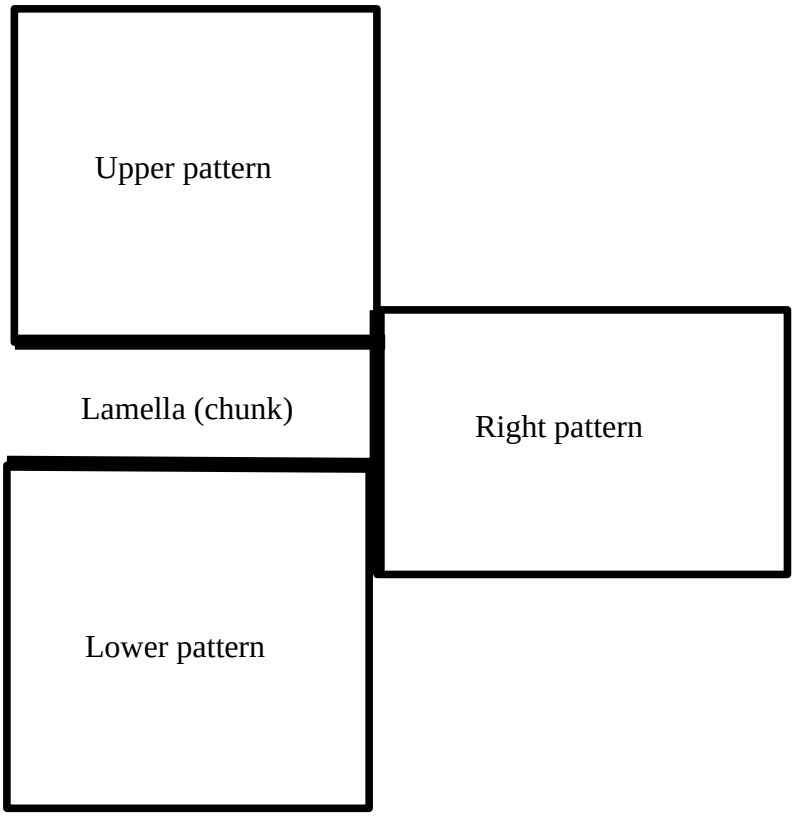

Fig. 1: Schematic depiction of the milling pattern arrangement.

Cryo lift out of the cellular chunk

1. Set the stage rotation to -70° before using the micromanipulator.
2. Set the scan rotation in e-beam and i-beam to 180°
3. Tilt the cryo stage to 30 – 35°.
4. Set milling current to 0.5nA.
5. Create 4 rectangle milling patterns:
   - 2x bottom pattern (with full overlap) with scan direction bottom to top with following dimensions: x= width (x) of the chunk, y = 1.8-2um and z=3-4um.
   - Right milling pattern alongside the right edge of the chunk with z=3-4um (this serves only for cleaning).
   - Left milling pattern is placed to the lower two thirds of the chunk in y dimension.

   NOTE: milling time depends on the chunk thickness. The milling pattern is usually well visible on the side-wall of the upper pattern (trench) once the ions penetrate cut through the chunk. Leave the chunk fixed to the bulk sample by the left side in top third.
6. Navigate the micromanipulator needle in x, y and z direction to the rough proximity of the chunk.
7. Inspect the quality of the micromanipulator needle.
   NOTE: After multiple usage, the needle can be covered with deposited material from the previous runs. In that case, needle needs to be cleaned and sharpened with FIIB using high current (1-3nA and rectangle cross-section). Be careful to clean only deposited material, not the needle material.
8. Navigate the needle carefully to the chunk. Needle should lean on the front side of the chunk and ideally in the top half.
9. Fix the needle to the chunk by biological material deposition. Create regular cross section pattern with following dimensions: x= 5 - 7um, y=1.8 - 2um, z=0.5 - 1um.

Set scan direction was from bottom to top. Multipasses=1, x=2um and z=3-4um (Fig. 2B).

10. Check the proper fixation of the needle to the chunk in electron beam. The layer of the deposited biological material at the contact point of the needle with the chunk (Fig. 2C,D)
11. In case, that the needle is not properly fixed to the chunk, repeat the procedure in the part from the point 9 until the deposition layer on the top of the needle is visible.
12. Create the rectangle pattern and free the chunk from the rest of the sample.
13. Lift the sample chunk slowly up by navigating in all 3 dimensions and when the needle with chunk gets to the safe position, retract the needle.

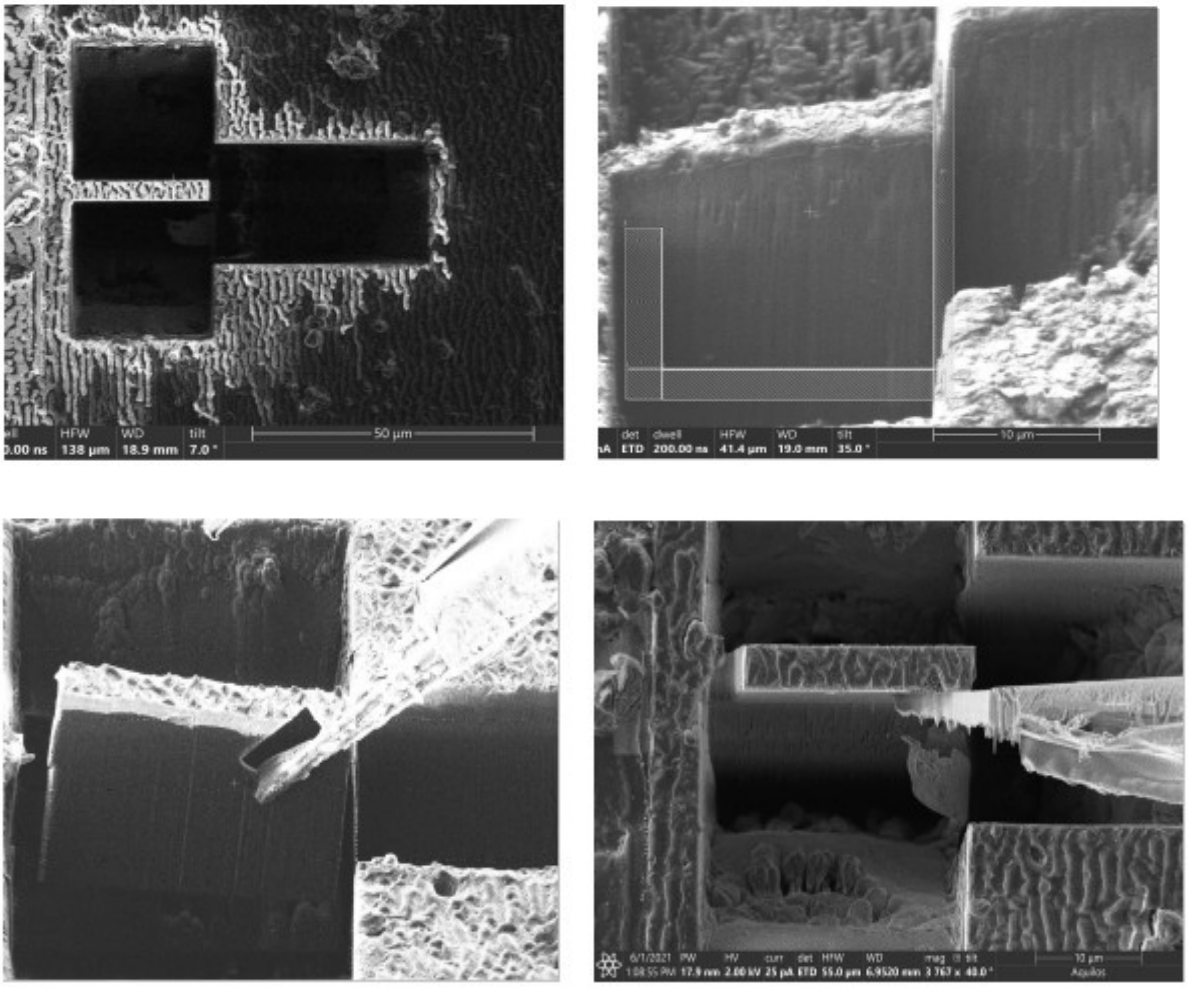

Fig. 2: Cryo-lift out with the micromanipulater. There milling area are created to preparat rough chunk in the area of interest (A). Following the the sample tilt, the chunk is almost released from the bulk specimen at the bottom and on the sides (B). The micromanipulator needle is attached to the chunk by the material deposited from the chunk (C). The chunk is released form the HPF samples and moved away (D).

Transfer and fixation of the chunk to the half-moon grid

1. Rotate the cryo-stage to 110° and tilt to 23°.
2. Set scan rotation in e-beam and i-beam to 0°.
3. Choose a particular pin on the half-moon, where you want to place a sample chunk and set eucentric position for the top of the pin.
4. Insert needle with chunk to a safe distance from the halfmoon grid and slowly navigate in x, y and z directions to the top of the halfmoon pin (Fig. 3A)

NOTE: The chunk needs to be preferably positioned parallel with the half-moon grid pin to simplify the fixation by grid material redeposition.

5. Create three equal regular cross section patterns for chunk attachment with dimensions x=6um, y=2um, z=3um and the scan direction from right to left side. Set milling current to 0.5 nA (1 multipass).
6. The deposited material from the halfmoon pin should attach the chunk to the halfmoon grid. If not, repeat the procedure in point 4 above until a firm connection between cell chunk and half-moon grid is established (Fig. 3B).
7. When the sample chunk is properly attached to the half-moon pin, release the needle from the chunk by needle movement in x and z direction. Finally, retract the needle to the safe position.

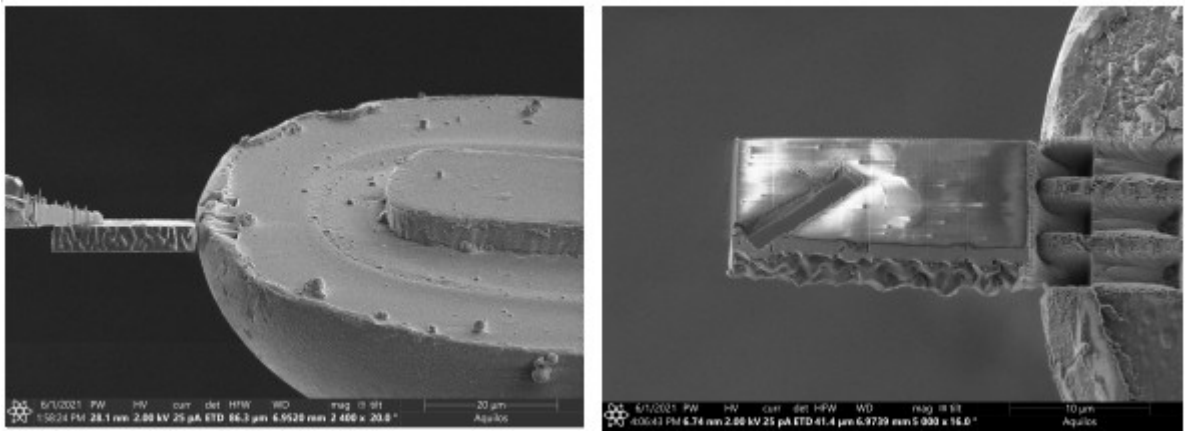

Fig. 3: A micromanipulator with a chunk is positioned close to the half-moon grid pin (A). The lamella is welded by re-deposition of the milled grid pin material (B).

Finalization of the lamella for cryo-TEM

NOTE: Milling of the coarse sample chunk down to the final thickness (200nm) is carried out directly after lift out procedure. Alternatively, the grid with chunks can be stored and re-inserted to the FIB/SEM microscope for the finalization later.

1. Create upper and lower regular cross section pattern.
2. Set parallel milling - upper pattern - box rectangle pattern Top to Bottom scan direction, lower pattern - box rectangle pattern Bottom to Top scan direction.
3. Mill rough lamella in the following milling steps:
   - current = 300 pA / 2um lamella thickness / upper pattern and lower pattern
   - current = 100pA / 1 um lamella thickness / upper and lower box pattern
   - current = 50pA / 500 nm lamella thickness / upper and lower pattern
4. Mill lamella down to the final thickness (< 250nm) in the following milling step:
   - current = 10pA / 0.2um lamella thickness / only upper pattern
5. Unload carefully shuttle with the lamellae from the cryo-FIB/SEM microscope.

Material and equipment for cryo-FIB/SEM work

- a cryo-FIB/SEM microscope equipped with 360° rotation cryo-stage and micromanipulator for lift-out under cryo-conditions
- a cryo FIB/SEM microscope specimen tilted holder (shuttle) suitable for HPF planchette hat and grid cartridge
- a cryo-transfer system with temperature and vacuum control between HPF and cryo-FIB/SEM
- sputter coaer for conductive platinum deposition under cryo-conditions

- cryo-EM and laboratory equipment: precision and flat tweezers, nitrile or latex gloves, eye protection glasses, cryo-gloves, grid boxes, grid cartridge clipping pens and grid box opener.
- half-moon grids clipped into the grid cartridge.

Transfer of the final lamellae into the cryo-TEM

1. Cool down the cryo-TEM loading station with fresh and clean liquid nitrogen.
2. Insert Autoloader casette into the loading station.
3. Cool down flat tweezer and grid box opener.
4. Insert carefully grid box with specimen.
5. Insert half-moon with milled lamellae int the Autoloader casette.
   NOTE: Correct orientation of the lamella with respect to the cryo-TEM stage rotation axis is important. The milling direction of prepared lamellae needs to be perpendicular to cryo-TEM stage tilt axis.
6. Load casette with specimen into the cryo-TEM.

3. References

1. Moravcova J., Pinkas M., Holbova R., Novacek J. (2021). Preparation and cryo-FIB micromachining of *Saccharomyces cerevisiae* for cryo-electron tomography. JoVE 177, e62351.

Acknowledgements

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