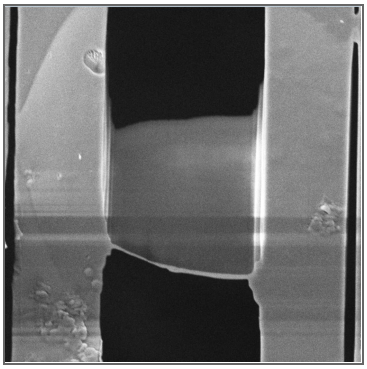

AUG 07, 2023

OPEN ACCESS

Protocol Citation: Josh Hutchings, Tamar Basiashvili, Elizabeth Villa 2023. Cryo-FIB Milling protocol for mammalian cells. **protocols.io** https://protocols.io/view/cryo-fib-milling-protocol-for-mammalian-cells-cxtyxnpw

Protocol status: Working
We use this protocol and it's working

Created: Jul 27, 2023

Last Modified: Aug 07, 2023

PROTOCOL integer ID: 85592

Cryo-FIB Milling protocol for mammalian cells

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ABSTRACT

Cryo-Electron Tomography (Cryo-ET) and Cryo-Focused Ion Beam (Cryo-FIB) milling provides insight into the structure and architecture of various proteins and organelles in cells. Mammalian cells require to be thinned into sections of ~150-200nm to gain high-resolution information about the contents of the cell. Cryo-FIB milling allows us to ablate regions of cells leaving behind a thin section of cellular material called Lamella. In this protocol, we describe how to manually prepare lamellae using a dual-beam FIB/SEM microscope (Aquilos 2+ system).

MATERIALS

1. Aquilos Cryo-FIB/SEM dual beam microscope
2. Liquid Nitrogen
3. Cellular sample on an autogrid designated for milling

Keywords: Cryo-FIB milling, Aquilos , Cryo-EM, In situ structural biology

1. Before you start

1h

1 Check the status of the high-pressure LN2 tank. If empty change it. Set the regulator valve to 80psi.

2 Fill 4L dewer with clean liquid nitrogen (LN2) for sample loading.

3 Fill the heat exchanger tank with liquid nitrogen.

3.1 Once filled place a heat exchanger rod into the tank. Set the flow rate to 190-210 and start cooling down the stage.

4 Retrieve the grids intended for milling.

5 Create a directory on your computer to save the images from the session.

2. Sample loading

30m

6 Prepare the loading station. Slowly add clean liquid nitrogen to the basin and wait till the LN2 equilibrates.

6.1 Load the grids of interest into the sample shuttle. Have grids oriented so that the milling

notch is facing upwards.

7 Once loaded, top-up LN2 in the loading station and maintain to keep the open grid box cold. Leave the base lid on.

7.1 Close the shuttle and place it in the loading position.

8 Insert the shuttle into the microscope using the sample transfer rod.

3. SEM and FIB Overview images

30m

9 On the microscope set the scan rotation to 180^{o} for both SEM and FIB view.

10 Move the stage to the Mapping position and drop SEM magnification to the lowest possible magnification. Check the focus and take an overview image and save it with a desired name. Assess the distribution of cells in SEM overview and do a rough count of mill-able cells.

11 Check hydration of cells in FIB view. Move the stage to the Milling position. Set the current to 10pA, and set it to a magnification that includes most of the center of the grid (or the majority of cells). Check focus and take snapshots and save it in a designated folder.

12 Repeat for the second loaded grid if applicable. If you are happy with the grids and cell distribution, put away open grid boxes in the loading station.

4. Setting up MAPS project

20m

13 For chosen grid, find focus and link focus to the stage. In the mapping position increase the magnification to 250 in the SEM view. Find a focus with the - Reduced area. Link the focus position to the stage.

14 In the SEM lower the magnification to 59x. Open the latest version of MAPS.

15 Start a new project in MAPS.

16 Take a quick SEM snapshot with MAPS.

17 Add a 4x4 tileset and position it over the middle of the snapshot. Set the focus to Fixed and click RUN. 5m

5. Selecting Lamellae sites 10m

18 In MAPS select cells that you would like to mill. Add lamellae sites and rank them in order of preference.

19 Find the eucentric height for each lamellae site.

19.1 Drive to the eucentric position and select calculate.

19.2 Check SEM centering at medium magnification and focus accordingly

19.3 Tilt step of 10^{o}, click the LEFT arrow, update SEM and recenter by double-clicking on the desired feature. Repeat until stage angle = 5^{o}.

19.4 Once centered at 5^{o} stage tilt, click to calculate eucentric position.

19.5 Repeat the above steps for each lamella site.

6. Pre-Milling preparation

15m

20 Sputter coat the grids.

20.1 Click on Prepare for the sputtering button. Choose conditions you would like to use for sputtering (i.e. 30mA, 0.1mbar, 30 sec). Press Run and wait. Once done recover the system.

21 Wake up the beam and move back to the Mapping position. Take a new lowest magnification overview, save the image, and note the contrast change.

22 Deposit PT on the grids.

22.1 Move to the deposition position for the chosen grid. Click on the Cryo-TEM preparation tab in xTUI. Under the Cryo-GIS deposition tab, enter the grid number and set the desired time for the deposition. (e.g. 60 seconds). Click Start to start deposition.

23 Move back to the mapping position, take an overview, save the image, and note the contrast change.

7. Manual Cryo-FIB Milling

45m

24 Go to the Milling position in xTUI and set the desired milling angle. For mammalian cells use 10-11'. Make a note of this for cryo-TEM data collection.

25 In MAPS, drive to eucentric position. Check focus and centering in SEM. (~1200x, 2kV)

26 With FIB at ~2000x at 10pA, center the same cell in FIB view with beam shift knobs and check focus. Increase the magnification to fill FOV with most of the cell.

27 Load a rectangle pattern preset and position at 10pA.

Note

Preset parameters used:
Width 12mm - length - 5mm
The separation between the two patterns - 3-4mm
Stress relief cuts - 500nm

28 Approximate milling routine:

Note

Use scan rotation to make the milling pattern and lamella parallel for more even/smoother milling.

28.1 0.5 nA, 12 um X-width, 3um separation

28.2 0.3 nA, 11.5 um X-width, 1.5 um separation

28.3 0.1 nA, 11 um X-width, 750 nm separation

28.4 50 pA, 10.5 um X-width, 400 nm separation

28.5 30 pA, 10 um X-width, 200 nm separation

28.6 30 pA, 0.5’ under-tilt, 10 um X-width

28.7 Mill under-side from lamella back. 30 pA, 0.5’ over-tilt, 10 um X-width, mill top-side from lamella back.

28.8 Repeat the above steps for all desired lamella.

29 Things to monitor during milling.
GIS layer at the front of the lamella. When this breaks through, becomes very difficult to mill.
Observed transparency in SEM at 2 kV. Becomes homogenous and dark at thickness < 200 nm.

30 Polishing: Return to each lamella and aim for <10 mins of polishing for each.

10 pA top-side only, X-width 10 um, no over/under-tilt.

7. Finishing up

15m

31 Retrieve grids from the microscope and store them.

32 Set the flow of the heat exchanger to 10mg/s and remove the heat exchanger from the dewer.

33 Update your notes and sample information. Update personal spreadsheets/logs with information about the session.