

Aquilos 2 Protocol 1 – FIB Milling using AutoTEM

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Aquilos2 and Prep Pot Preparation

Purge the sputter vacuum and GIS (**Performed by staff on Monday** before cooling the machine)

- a. In the FlowView software > flow rate to 200 mg/s to dry out the instrument.
- b. Click the double-arrow button on the top of the xT UI to the check vacuum the entire instrument. Everything should be green (some even olive green).
- c. Go to Beam control (atom symbol) on the right pane of xt server. Vacuum = Sputter Vacuum (chamber pressure to ~10 Pa). After chamber pressure stabilizes around 10 Pa, a purge button will be available. Purge for 5 cycles. Once done, immediately recover. Click High Vacuum again.
- d. Go to Cryo TEM preparation (snowflake symbol) on the right pane of XT server. Enter 2 mins for Flow deposition under Cryo GIS Deposition and click purge. (If chamber pressure (bottom left) changes by an order of magnitude (10^{-4} to 10^{-3} Pa), repeat the purge!)

Cooling the Aquilos 2 (**Performed by staff on Monday**)

Slowly, carefully drop the Heat-exchanger cane into the sub-angstrom dewar pre-filled to the top. (Try not to bump it around. Perfect in-and-out ideally, watch out the lines connected to the heat-exchanger)

- a. In the FlowView software > flow rate to 200 mg/s.
- b. Monitor the FIB temperatures and pressures with time.
 - i. Temperatures (bottom right-center): The stage shield and stage will cool to -194°C or lower; the instrument records temperatures after it reaches $< 10^{\circ}\text{C}$.
 - ii. Chamber Pressure (bottom left): The chamber pressure should drop to $< 5 \times 10^{-5}$ over time. If the pressure is sinusoidal or wobbly, there's an issue.

Purging PrepPot

Fully assemble the preparation pot (prep pot) and purge for at least 30 minutes.

- a. Grab the black prep pot and move it onto the purging platform.
- b. Ensure the vacuum lid is closed up and placed on top of the pot.

- c. Hit the purge button and listen for airflow.
- d. Turn on the heating plate to start warming up tools.

Loading the samples

Cooling the prep pot.

- a. Hit the close button, ensure there is no airflow (NOT purging anymore).
- b. Replace the vacuum chamber lid with the movable two-piece lid.
- c. Move the entire prep pot lid to the side, exposing one of the holes in the black peripheral ring.
- d. Use a plastic funnel to fill the ring with LN2 (you can use an N95 mask to filter ice from any LN2 you pour into the funnel).
- e. When you see LN2 bubbling out of the other peripheral ring hole (the one covered by the lid), stop filling and allow to equilibrate.
- f. Shift the lid back into place. Open the middle lid.
- g. Pour LN2 directly into the silver metallic cup, up to the bottom of the black notch. Place the middle lid back on while equilibrating.

Move your samples into the prep pot.

- a. Move your sample from a storage dewar to the transfer dewar. With the help of pre-cooled large tweezers and box-lid pen and move and secure your grid box/es in the groove(s).
- b. Use pre-cooled tweezers to flip the shuttle into an orientation where you can see the slots to load your grids.
- c. Lock the shuttle into place with the white plastic lever.
- d. Cool down autogrid tweezers and a small screwdriver to open/close the shuttle screw.
- e. Ensure that the shuttle is in the “open” position (screw touching the bottom edge). You can align the grid to the tweezer in the inspection area in the loading station before moving to slot. Place the grids in the position 1 and 2 slots of the shuttle using the autogrid tweezers. Cell/tissue side facing you and while the milling notch on the top (12 O’clock position). Tighten the screw in the “close” position (screw touching the top edge). I recommend marking the clip rings prior to clipping to get better sense of loading the grids for milling or TEM.
- f. Unlatch the white plastic lever. Tilt the shuttle away from you with a pin hole facing up.

Make sure you can see inside the FIB chamber.

- a. Click the double-arrow (sample exchange) button at the top left of the xT UI.
- b. Change the tab on that window to show the live chamber camera.
- c. Tilt right most computer screen towards the sample arm, just so you can see the live view of the chamber while loading.

Pre-check before loading

- a. Remove the normal lid from the prep pot and replace it with the vacuum chamber lid. Ensure that the silver door between the lid and prep pot is closed.
- b. Confirm that the transfer arm is closed and under vacuum. On the side of the aquilos with the transfer arm:
 - i. Check whether the transfer arm door is closed or open.
 - ii. If closed, we can assume it's under vacuum. Press "P" to pump. Open the transfer arm door. Press "P" to pump. Close the transfer arm door.
 - iii. If open, press "P" to pump. Close the transfer arm door.

Loading the sample shuttle using the loading arm

- a. Press "V" to vent the intermediate chamber on the aquilos.
- b. Unhook the transfer arm and hook it to the vacuum lid on the prep pot. At the prep pot station, press "Pump."
- c. (Optional) While that's pumping, cover the hole on the intermediate chamber with the placeholder block and press "P" to pump (saves gas).
- d. Open the transfer arm door. Press "Vent" to vent the intermediate chamber on the vacuum chamber lid. As soon as the arm and chambers are vented, open the door to the prep pot.
- e. Unlock the transfer rod by twisting the black cap at the end of the transfer arm to the unlocked position. Unlock the transfer rod "thumb" by twisting the cap of the black lever that determines the position of the rod.
- f. Carefully slide down the transfer rod into the prep pot using the black lever, looking through the plastic lid to ensure that the rod comes into contact with the shuttle.
- g. When the rod successfully latches into the shuttle, twist the lever cap to lock the shuttle in place (close the "thumb").
- h. Carefully raise the transfer rod using the black lever, taking the shuttle with you.

- i. When fully extracted from the pot, twist the black cap at the top of the transfer arm to lock the rod into place.
- j. Close the silver door between the prep pot and vacuum lid. Press “Pump” to put the transfer arm and intermediate chamber of the prep pot lid under vacuum.
- k. (If you performed step “c”) On the FIB, press “V” to vent and remove the placeholder block.
- l. Close the transfer arm door. Press “Vent” to release the pumped transfer arm from the prep pot lid.
- m. Unhook the transfer rod and hook it to the intermediate chamber on the aquilos.
- n. Press “P” to pump. If you listen carefully, you should hear a faint click. This click is the signal that the door between the intermediate chamber and main chamber on aquilos is unlocked, which will only remain unlocked for a short amount of time.
- o. In quick succession, open the door between the intermediate chamber and main chamber, then open the transfer arm door.
- p. Unlock the transfer rod by twisting the black cap at the back of the arm. The vacuum will naturally pull the transfer rod into the chamber; use your hand to gently guide the rod into the chamber at a controlled speed.
- q. Watch the shuttle dock into the stage on the monitor.
- r. You should feel the rod stop; apply light pressure to confirm the shuttle is docked. Once the shuttle is fully docked, twist the cap of the black lever to unlock the transfer rod “thumb.”
- s. Carefully retract the transfer rod. The shuttle should stay in place. Once fully retracted, lock the rod in place with the cap at the back of the arm.
- t. Close the door between the main chamber and intermediate chamber.

Clean up around you

- a. Put the placeholder block onto vacuum lid and press the “Close” button.
- b. Any tools in the prep pot should be placed on the heat block.
- c. Save rest of your samples from the Prep pot if you are not exchanging the already loaded samples.

FIB-SEM session preparation

Home Stage (check with staff if required)

If the stage is funky - out of place, somewhat unstable, etc. – Home the Stage. Top ribbon; Stage > Home Stage (Shift + F3).

Take a NavCam photo.

Top; Stage > Take Nav-Cam Photo (Ctrl + Shift + Z). Side; Detectors Tab (eye symbol) > Digital Zoom. Click on the bottom left window, where the NavCam photo is, then adjust digital zoom so that you can see your grids. Sometimes this is important for checking grid quality and loading alignment.

Wake up the beams.

Side; Beam Control Tab (atom symbol) > System > Wake Up . Takes a second. Often the liquid metal ion source (LMIS) of the FIB will need some time to warm up.

Confirm scan rotation is at 180 degrees.

Click on the SEM quadrant, then Shift + F12. Select 180.

Click on the FIB quadrant and select 180. Then Shift + F12 to exit the scan rotation option.

SEM preparation

- a. Move to mapping position on Grid 1. Side; Cryo TEM operation (snowflake symbol) Tab > Shuttle Controls > Mapping Position (Grid 1 selected)
- b. Check the SEM column is at reasonable settings (e.g., 2 kV, 13 pA).
- c. Move to a part of the grid where lamellae won't be made (blown out foil, ice patch) but there is still a distinguishable feature (such as grid holes, ice), ideally near the center of the grid. Zoom in to 5000x or higher magnification.
- d. Adjust your focus.
 - i. Press F7, reduced area. Place over a well-defined feature.
 - ii. Use the knobs on the control panel to adjust your focus. Try moving fully in and out of focus before settling down. There is also Autofocus button (fainted half moon) on top ribbon if you want to use that.
 - iii. Link the Z-Height to Working Distance (oval and beam button, top leftish).
- e. Adjust your stigmators.
 - i. Press F7, reduced area. Place over a well-defined feature (can be same as above).

- ii. Use the knobs on the control panel to adjust your stigs.
 - iii. It's very similar to focus; the image will blur in and out.
 - iv. There's also Top; Tools > AutoStigmator if you want to use that.
 - f. Repeat step 4 after stig correction.
 - g. Check the beam crossover.
 - i. Side; Direct Adjustments Tab (box w/ crosshair) > Beam > Crossover
 - ii. The SEM view should change to a small circle in a black background with a crosshair over it (this is the electron beam in the column). If it's circular and the crosshairs are mostly centered, you're good. Else, hit "Auto."
 - h. Check the beam wobble (Lens Alignment).
 - i. Press F7, reduced area. Place over a well-defined feature (can be same as above).
 - ii. Press the Lens Alignment button (Shift + F4; target button, top leftish). The image will begin bouncing in and out of focus. Move the green crosshair around on screen until that bouncing causes no translation (slight rotation is fine). Press the Lens Alignment button again.
- Or
- Side; Direct Adjustments Tab (box w/ crosshair) > Beam > HV modulator change modulation using the slider to stop the translation.
 - i. Find your focus and stigs again (fully repeat steps 4, 5, and 6).
 - j. Return to the lowest SEM magnification and return to the mapping position on Grid.

MAPS

Take a pre-sputtering atlas of Grid 1.

- a. Top; ThermoScientific > Maps 3.28
- b. The program will open. Create a new project.
YYYYMMDD_Details_Initials_Maps . Save to your folder in the data drive/server.
- c. It will usually ask you to ensure that the grid is in the mapping position. In the main UI. Side; Snowflake Tab > Shuttle Controls > Mapping Position.
- d. Once open, take a snapshot. In Maps, Top Right Corner; "Take Snapshot." (Right-click the layer in the left tab to save image to file.)
- e. Select your atlas tile set template. In Maps, Top Left Corner; "TEMPLATE" drop-down menu > Aquilos_template (if at cEMc).

- f. Add the template tile set. In Maps, Top Left Corner (even further left); “Add tile set” button. A tile set will appear on top of your most recent electron snapshot.
- g. Adjust the tile set as desired by clicking and dragging the edges of the tile set such that the majority of tiles are in the center(ish) of your grid.
- h. In the bottom left, there are several options one can pick and change as preferred. In the bottom left-center of the screen, hit “RUN.”

Take a pre-sputtering atlas of Grid 2.

- a. Side; Cryo Tab > Shuttle Controls > Mapping Position (Grid 2 selected).
- b. Adjust your focus at a location where lamella won’t be made, near center of grid (you can skip this if using “aquilos_template” Tile template as it will autofocus and autobrightness-contrast in the center tile once and spiral out to take map).
- c. Return to the lowest SEM magnification and mapping position on Grid 2.
- d. In Maps, Top Right; “Take Snapshot.”
- e. In Maps, Top Left Corner; “TEMPLATE” drop-down menu > Aquilos_template (if at cEMc). Switch off autofocus and autobrightness contrast in left panel template settings if already performed.
- f. In Maps, Bottom Left-Center; “RUN.”

Finding SEM and FIB coincidence

- a. Side; Cryo Tab > Grid 1 or 2 Milling Position
- b. In the SEM, find a feature on your grid that you’d like to focus on (ice).
- c. Move the stage such that the crosshair is right on top of the object.
- d. Click on the FIB quadrant. Top; Stage > Z-Tracking (Ctrl + F12). An arrow should come up on the right side of the FIB quadrant.
- e. In the live view of the FIB, click and drag on the arrows to move the stage up or down such that the crosshair approaches your feature. You need not move the crosshair directly on top of the feature for now.
- f. Check in the SEM whether your feature has moved. Move the stage such that the crosshair is centered on your feature in SEM quadrant.
- g. Move back to the FIB view. In the live view, click and drag the arrows to once again move the stage such that the crosshair approaches your feature. This time, you can move even closer.
- h. Check the SEM view again. Move the stage such that the crosshair is centered on your feature.
- i. Repeat steps g and h until the crosshairs of the SEM and FIB are over the feature.
- j. Turn off Z-tracking (Ctrl + F12).

WORK UNDER CONSTRUCTION (BELOW) 😊

Prepare for Milling

The sputter coating primarily dissipates charging across the sample. The organometallic platinum forms a strong layer that protects the leading edge of your lamellae from being degraded by the FIB.

Platinum Rough Sputtering, Round 1:

- a. Open the sample exchange window (two arrows at top left of xTUI) and monitor both the chamber pressure (color) and the valves to the Argon line in particular!
- b. In FlowView, change the flow to 210-220 mg/s.
- c. Side; Cryo Tab > Sputter Coating > Prepare for Sputtering
 - i. The chamber should turn blue in color, and the Argon line valves should be green (open). Chamber pressure should be around 10 Pa.
 - ii. In the live chamber camera view, a metal shield should come out and nearly cover your grids.
- d. Side; Cryo Tab > Sputter Coating > Preset 1, then Run
 - i. Your grids should move towards the metal shield; watch closely to see if a light sort of “illuminates” your grids.
 - ii. If you don’t see a light, repeat the sputter.
- e. Side; Cryo Tab > Sputter Coating > Recover from Sputtering
 - i. Your grids should return to their original positions and the chamber pressure should be reestablished around 10^{-5} Pa.
 - ii. If your chamber pressure doesn’t recover to where it was previously, check to make sure both valves to the Argon line are closed (red in color). If they are not closed, try to switch to sputter vacuum and back to high vacuum.

GIS Coating (organometallic platinum):

- a. Side; Cryo Tab > Cryo GIS Deposition > Check Boxes, Select Time, Run
 - iii. Note 1: GIS Deposition Time will depend on sample; the more time, the thicker the GIS layer. 15 sec is a good starting point.
 - iv. Note 2: Selecting Grid 1 or Grid 2 only will still end up depositing GIS on both grids, just a lot more on one grid.

Platinum Rough Sputtering, Round 2:

Same as Round 1

MAPS – sputter coated maps

- a. In Maps, take new snapshots and atlases (see above).
- b. Wake Up the beams.
- c. In the cryo tabs, hit Grid 1 or 2 mapping position, ensure you're in focus and then zoom to lowest magnification.
- d. In Maps, in the top right corner hit "Take Snapshot"
- e. Then take atlases (see steps above).
- f. Align the OLD to your NEW stuff:
 - i. Ctrl Click old snapshot and atlas for one grid, Right Click, Alignment.
 - ii. Press the bottom left button on the menu twice and follow prompts to align the old stuff to new stuff using 3 point method to triangulate.

Define Lamellae sites

Using the older or newer images, search for lamella locations:

- a. Zoom around in maps, find cellular positions that are suitable for lamella (mostly sitting in the middle of the grid squares, but not necessary).
- b. Middle "9x9" squares is best for milling using regular Clip rings. You can try milling using non-grazing angles and AUTOFIB/Nanosoft Clip rings in the squares outside this region.
- f. At the region of interest, in Maps, right-click and "Add Lamella Site"
- g. Continue adding sites until satisfied. When finished, re-evaluate the sites that are best/necessary

AutoTEM Milling

Once a workflow is optimized for a sample, users can successfully get 120-150 nm lamella from AutoTEM alone. However, it is as per user preference to perform Milling steps (Rough, Medium, Fine and finer Milling) using AutoTEM and Thinning manually.

Initiate AutoTEM.

- a. Top; ThermoScientific > AutoTEM Cryo 2.4
- b. The program will open. Create a new project by importing your Maps project.

- i. A window will pop up showing various AutoTEM projects. Your Maps project should appear with a green “online” symbol as its picture. Click on it.
 - ii. YYYYMMDD_Details_Initials_AutoTEM
 - iii. Save to your folder in the I drive in your respective folder.
- c. Once open, AutoTEM should import the lamella sites you’ve made in the maps project, as well as their tiles on your most recent atlas.

Prepare your favorite template.

- d. In the top right, click the “Templates” tab.
- e. Select or create a template for your sample.
 - i. On the left side of the screen, you will see various templates for milling.
 - ii. However, if no program suits your needs, you can right-click on templates to “Clone” and “Rename” them, making them your own.
 - 1. Modify new template but DO NOT change other users’ templates!
 - 2. Only maintain a few (1-3) templates under your name; creating too many templates clutters up the menu for other users.
- f. There are simply too many parameters in the AutoTEM workflow to cover each of them here in full detail. However, when modifying your own template, here are some important considerations:
 - i. Preparation: Milling Angle Tab > Target Milling Angle
 - 1. Smaller/shallower angles are *usually* better to get longer lamella.
 - ii. Milling: Lamella Size (at the top)
 - 1. 10 µm x 5-10 µm is typical, but can vary widely based on sample.
 - iii. Milling: Rough > Medium > Fine > Finer Milling Tabs
 - 1. Typical Milling Currents might proceed like so:
1 nA > 0.3 nA > 0.1 nA > 50 pA
 - 2. Typical Pattern Offsets might proceed like so:
1 µm > 600 nm > 350 nm > 250 nm
 - 3. Rough Milling is typically performed with a “Rectangle” Pattern Type. Fine and Finer Milling is typically performed with a “CleaningCrossSection” Pattern Type. Medium Milling is up to user preference (though most prefer “CleaningCrossSection”).
 - 4. Depth Correction typically varies between 140-160%
 - 5. Pattern Overlap typically stays around 200% or above
 - iv. Polishing: Polishing 1 Tab
 - 1. If you’re allowing AutoTEM to polish your lamella, the typical Pattern Offset is 150 nm and the typical Milling Current is 30 pA.

- v. REMINDER: None of these “typical” parameters are hard and fast rules! Everything varies by sample and the user should try to optimize milling for their purposes.

Apply template(s) to your lamella.

- g. In the top right, click on the tab titled after your project.
- h. On the left side of the screen, click on a lamella. On the right side, there should be a list of templates. Select your favorite template and hit “Apply.”
 - i. You can also right-click the template and hit “Apply to all”
- i. Apply the appropriate template to each lamella.

Run the Preparation step.

- j. In the bottom left, click on the “RUN” button. A window should appear.
- k. Uncheck the boxes under “Milling” and “Thinning”
- l. Click “PREPARE.” AutoTEM will now go through all your lamella, finding the eucentric height and milling angle at each lamella site (so long these options were active on your template). Takes about 5-6 min per lamella site.
- m. When complete, you will place the milling patterns manually.
 - i. If you can’t see your cell or feature in the image, you can use the xTUI to zoom out, then press the “Update” button at the top right of AutoTEM.
- n. Assess the quality of your lamella sites, unchecking the ones that look the least promising.

Run the Milling (& Thinning) step(s).

- o. In the bottom left, click on the “RUN” button. The same window as previously seen should appear.
- p. To your preference, check the boxes under milling and thinning.
- q. In the bottom left, ensure that execution mode is set to “Step-wise.” (“Site-wise” is fine for a few lamella, but an overnight site-wise run will lead to ice contamination on your earliest prepared lamella.)
- r. Click “RUN.”

FOR OVERNIGHT RUNS:

- s. Select 15-20 lamellae and let it run overnight. Generally all steps are done by 8 am based on the template settings.