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CryoTEM

Introduction

Version 1 – May 2024

Part IV: Grid transfer

starts at: samples ready to transfer to cryoholder
ends with: grid inserted in TEM, ready to image


LEICA EM GP

PART IV: Grid transfer

- Warm up the workstation

Experiment: heating up and drying of the workstation after use

- Remove all loose parts from the workstation (e.g. lid and blanking rod)

- Tilt the workstation slightly (not upside down!) and let the LN2 pout out (e.g. in a dirty LN2 container)

- Place the workstation on the small by-table and mount a heating tube pointing directly into the workstation dewar and another one fitted over the side entry port. The blanking rod must be removed. Activate a 15-20 minutes timer on the heater.

- Meanwhile: address your sample in the TEM

NEVER (never!) turn the workstation upside down. The entire aluminum block may fall out!

- Keep the workstation cold

In this state, a new grid can be plunged and inserted.

Experiment: keep the workstation cool

- Place the plastic cover on the workstation and insert the plug (make sure it is dry![1]).

- Fill the workstation with LN2, not more than 1cm above the rim

- Make sure to refill it regularly.

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eze
and you will not be able to take out the blanking rod. The only option is to warm it up and dry the entire workstation. (believe me, I was in this situation on several occasions).

Demonstration: first glimpse of the sample and how to deal with the workstation

PREREQUISITES:

- A TEM gird is inserted
- The workstation is still cooled and filled with LN2

How to proceed after you loaded you sample

Do not yet open the column valves

When the vacuum is OK (< gun/col 20), retract the shutter with the shutter know at the end of the cryoholder dewar. This usually causes a loss of vacuum of 1-2 units.

Experiment: Low mag scan the grid for good quadrants

- With the vacuum under 20 and the shutter retracted, open the column valves

- The magnification was set to M 390 X.

- Use the Stage2 window (Image > Stage2) to search the entire grid. Use the track to help you keeping track which part you searched.

- Goal: finding quadrants that are transparent for the electron beam. Ideally, they will have some darker parts near the edge of the quadrant.

- When found, insert the objective aperture. If the quadrant is still transparent for the beam, you have found good ice.

Depending on the ice you have found, you can now decide to warm up the workstation (good ice found) or keep it cooled and plunge another grid.

Demonstration: Prepare the TEM

WHEN:

Setup the TEM to accept a cryogrid

You are ready to load the grid in the cryoholder and observe it in the TEM

Prepare the TEM

Select the Veleta camera

Image > CCD/TV: select WA-Veleta camera

Reset the goniometer

Image > stage^2 > flapout > control > Reset > Holder

(the goniometer is centered to 0,0,0 (x,y,z)

Take out the objective aperture

On the column: Check if the objective aperture is out, if not, flip mechanism to OUT

Remove the single tilt holder

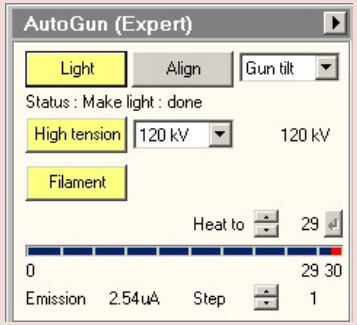
Place it in its stand near the binocular

Check the vacuum and start the filament

GUN/COL < 20 / 120 kV

Align the gun tilt

In the tab Setup > Autogun > Click “Align”.



Finally: Set to the following settings:

- Magnification: M 390 X
- Spotsize: 2
- C2: 100%

Demonstration: Transfer to the Gatan TEM cryoholder

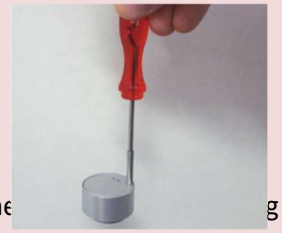
WHEN:

Sample is vitrified and in the cryo-gridbox and the Gatan transfer tool is cooled down

You are ready to load the grid in the cryoholder and observe it in the TEM

Transfer the gridbox

- close lid of the cryo-gridbox with the gripping tool (or use a handling rod)



- Move the gridbox to the cooled blue sytrofoam shuttle Container. Alterantively, attach the pre-cooled M4 cryotool (#5, page **Error! Bookmark not defined.**) and lift the containe g chamber
into the Gatan transfer tool.

Note: try not the breath towards the cooled tools you are using. This causes severe water contamination

Some remarks

Sweating

Check the cryoholder dewar: it must be dry. Wet or condensated holders are a very strong sign that the vacuum in the holder is poor and that the holder pumping (page **Error! Bookmark not defined.**) was not done correctly.

Settling

After insertion and refilling the cryoholder dewar, temperatures will drop. Usually, it will settle around -175 to -180°C. This takes about 10-15 minutes. During this time, high resolution imaging is not possible (due to drift), but low magnification is possible, e.g. to scan the quadrant for proper ice thickness.

Upper temperature

After insertion and filling the dewar, the highest temperature that should be reached is somewhere around -140°C (the recrystallization temperature, Tm, see page **Error! Bookmark not defined.**). If you are close to this temperature, the cryoholder and workstation were cooled down too late or warmed up after cooling. Ideally, the temperature never drops below -160°.

Cryodewar bubbling

The cooking of nitrogen in a fully filled cryoholder dewar can cause instability (the N2 bubbles bit the slanted walls at the top of the Dewar). To remedy: remove the black cap and insert your little finger about 1 cm. Some gas and LN2 will spray out.

Clean LN2 vs dirty LN2

You will notice that small ice crystals for in containers containing LN2 (the workstation, the plastic containers, …). Such volumes of LN2 are called ‘dirty’. Never pour dirty LN2 into a compartment that contains a sample. Use the heating tubes to clean (i.e. dry) any dirty container.

- Tilting the goniometer

An alternatively method for transfer is possible. Using this method, some liquid nitrogen is kept in the dewar. The setup of the station is the same.

Experiment: insert the cryoholder (with pretilting)

- Using the software (Image > Stage2 > Flapout > settings), pre-tilt the goniometer around to -60°. This way, the holder can be inserted with the dewar opening pointing upwards.

- Insert the holder, and slide the pin into the groove. Pumping starts. Minimal to no loss of LN2 should occur.

- When the airlock is pumped, set the pre-tilt to 0° (using the software) while simultaneously holding the dewar. This brings the goniometer tilt back to zero degrees while maintaining the holder dewar in its upright position.

- Now the holder can be fully inserted into the microscope.

Note that this method leads to a much higher risk of airleaks.

With the holder successfully inserted, attach the temperature controller

Experiment: Connecting the controller

- Connect the control unit to the dewar. Switch the controller on (power switch in the back).

- The temperature reading at the tip of the specimen rod should now be displayed.

- Place the black plastic cap over the opening of the dewar to reduce frosting on the dewar. A full cryoholder dewar should last about 2 hours.

- Allow the vacuum in the TEM specimen chamber to fully recover before withdrawing the cryoshield.

Demonstration: Standby, shutting down and bakeout

WHEN:

Sample is vitrified and in the cryo-gridbox and the Gatan transfer tool is cooled down

You have good ice in the TEM

Standby

If you need 15-30 minutes at the TEM to check for good ice, but do not want to warm up the system yet.

1. Cover the freezing chamber with the foam cover (#2, page **Error! Bookmark not defined.**)
2. Set the GN2 flow (Environment settings, page **Error! Bookmark not defined.**) to 5%

Shutting Down and Bake Out

1. transfer the secondary cryogen container (#11, page **Error! Bookmark not defined.**) using insulated forceps to the small Styrofoam container provided. Place the container in the fume hood of room A021 to evaporate. Retrieve the container 15 minutes later.

2. Remove any remaining water from the humidifier tank (use the syringe).

3. Remove the humidifier tank and the drip collector. Put them upside down on paper.

4. Remove any used blotting paper in a proper way (solid NP waste!)

5. Leave the door of the environmental chamber open.

5. Go to the **SETTINGS** screen and press **BAKE OUT**. Confirm the warnings, start the process by pressing START (typically 1 hour, or 15 minutes at the start of your session).

Demonstration: loading a sample into the Gatan cryoholder

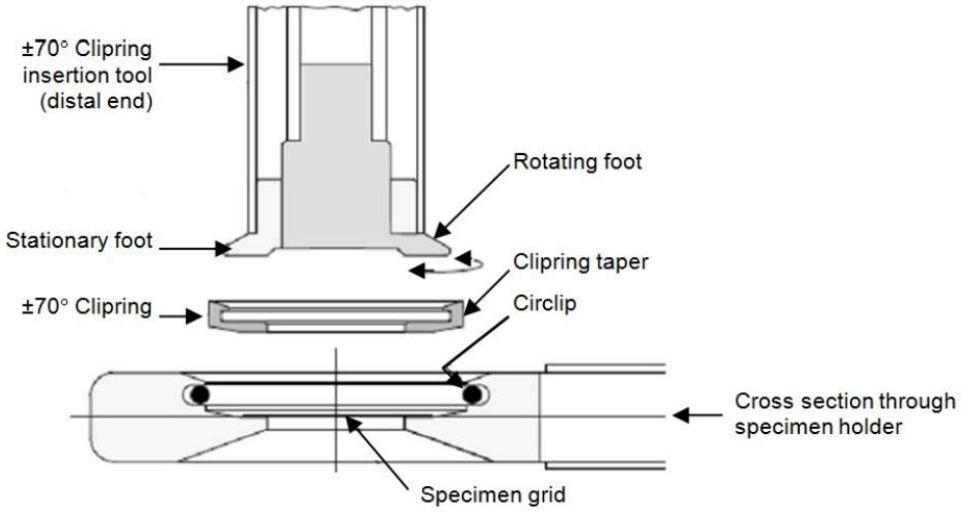
PREREQUISITES:

- A TEM grid is plunged and ready to be transferred
- The cryoholder and the workstation are cooled down and under LN2 (for > 15')

Ready to load

It is helpful to practice inserting and removing the Clipring at room temperature to develop the skills required to handle these small tools

Always precool the tweezers, the clipring and the Clipring insertion tool in liquid nitrogen prior to securing the frozen hydrated specimen grid.



- Emptying the cryoholder dewar

This is the easiest and most straightforward way of inserting the cryoholder.

Experiment: insert the cryoholder (no pretilting)

- Prepare a plastic container (1L) and place it on the desk, at the lower left of the right panel. Remove the small black cap from the holder dewar.

- Pull out the holder and insert it straight into the microscope (do not care about the 4 O'clock tilt). At some point you will hit a metallic stop.

- If you have a second pair of hands – or are very quickly – place the blanking plug into the workstation to avoid further LN2 loss.

- Now rotate the holder away from you until the holder pin finds the groove (similar to a standard holder insertion angle). Most of the LN2 from the holder dewar will pour into out. Use the plastic container to catch (most of) the LN2.

- The holder should now be in the TEM airlock and the usual 1 minute countdown starts.

- After pumping, rotate the holder towards you (120 degrees, as per usual holder insertion). When the dewar is in its upright position again, you can insert the holder further into the goniometer, thereby finishing the insertion.

- The dewar must now be refilled with liquid nitrogen as soon as possible (use the LN2 you caught in the plastic container). Note: the thermal capacity of the dewar is enough, even when empty, to maintain a low specimen temperature for this short period of time.

Demonstration: inserting the Gatan cryoholder into the TEM

PREREQUISITES:

- A TEM grid is transferred to the cryoholder
- The cryoholder and the workstation are cooled down and under LN2 (for > 15')

Ready to load your grid into the TEM

Prior to transfer, setup the TEM

Experiment: setup the TEM

- Login, take out the objective aperture and center the goniometer. Take out the camera.

- Start the HT and click "light"

- run the gun alignment. Assure you have a good beam.

- Use spotsize 1-3

- Go to magnification M 390 and open C2/INT until most of the screen is filled with green light.

- Close the column valves

- Pickup the Gatan temperature controller and place it on top of the cooling cabinet. Connect it to a AC power outlet. Place the temperature connection cable over the screen.

Transfer the station to the TEM desk, the holder pointing towards the TEM.
Place an object of 2 cm high and 10 cm wide under the dewar side of the workstation to tilt the station slightly.

Place the lid on the workstation

Two methods were developed to deal with the LN2 in the TEM holder.

Experiment: step 1

- Check the liquid nitrogen in the workstation. Ideally, the reservoir is filled, but the cryobox space is not submerged. Also the tip of the holder should be just above the level of liquid nitrogen.

- Remove the shutter at the holder tip (pull on the shutter control knob at the rear of the dewar)

- Take the cryo-gridbox from the blue Styrofoam shuttle container and in one swift movement, place it into the gridbox reservoir.

- remove the pin cap or handling rod.

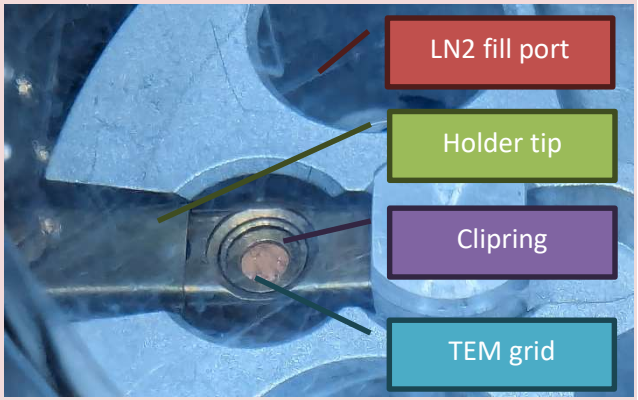
Important notice: static electricity sometime causes the grids to stick to the plastic pin cap or rod. When removing the cap or rod, first move it to the liquid nitrogen reservoir of the workstation and check if this is the case.

Now things must go quick.

Experiment: step 2

- Hold the clipring insertion tool perpendicular to the Clipring while opening the feet by turning the top part. Rotate 180°.

- The clipring can now be lifted from the holder tip and stored in the liquid nitrogen reservoir of the workstation, while still being attached to the clipring insertion tool.



Adjust the level of liquid nitrogen again if needed.

Experiment: Step 3

Dip the tips of the tweezers in liquid nitrogen reservoir for about one minute to cool or until the liquid nitrogen stops bubbling

- Pick out a grid from the grid box and transfer it into the recess in the holder tip.
    - Which side is up is not relevant
    - Try to touch the grid only on the rim
    - Try not to bend the grid
    - check for obvious ethane ice spot (usually white small hills on the grid). Discard these grids.

- Check if the grid is properly centered. Remove any visible ice crystals from the grid area.

The last step, step 4, is the most tricky one…

Experiment: Step 4

- Take the transfer tool with the clip ring still attached. Lower it perpendicular onto the holder tip with the grid.

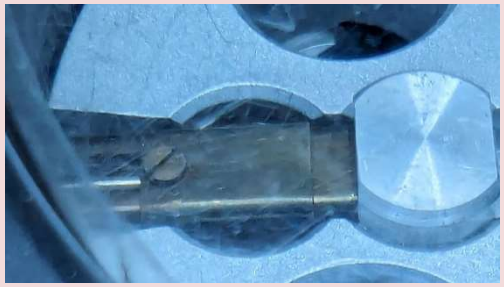
- Press the Clipring™ down onto it with a force of about what you need if you press a code in a keypad.

- You might hear the clipring clicking in, but this is not always the case.

- Gently rock the clipring tool slightly back and forth and left and right (about 5-10°)

- The Clipring insertion tool can now be released: very gently rotate the tip of the tool without changing its position.

- very gently move the clipring insertion tool upwards. Make sure you do not hook the feet around the clipring. Finally, remove the clipring insertion tool but keep it in the workstation (see below).



- Check with tweezers that the Clipring is properly sealed: tap it ever so gently: it should not move when probed with the cold tweezers.

- If the Clipring is loose it must be picked up again by the transfer tool and the procedure repeated.

- Finally: close the shutter with the shutter knob at the back of the cryoholder dewar. Add LN2 to the workstation to assure it is cooled for the next step in the procedure.

During the procedure of loading, the holder tip should never show any sign of frosting. If this is the case, add more liquid nitrogen.