- 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 cool

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![6]).
- Fill the workstation with LN2, not more than 1cm above the rim
- Make sure to refill it regularly.

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[6] If you insert a wet blanking rod in the side port of a cooled workstation, the water will freeze 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).

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CryoTEM

Introduction

Version 1 – May 2024

Part III: Cryoplunging

starts at: plunging grids
ends with: samples ready to transfer to cryoholder

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

WHEN:

A TEM grid is transferred to the cryoholder
The cryoholder is inserted into the TEM

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 390 M.

- 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 tranparant 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.

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.

PART III: cryoplunging

Demonstration: Plunging a grid

WHEN:

Cryoholder is well pumped, TEM grids are surface treated, GP2 is ready

Vitrify your sample!

- **Only work with clean and dry tools (use the heating plate)**
- **Only approach frozen specimen with pre-cooled tools (no boiling in LN2).**

Check:
- if the environmental chamber has equilibrated at the set humidity and temperature
- assure that the secondary cryogen has reached the set temperature

STEP 1: Attach the Forceps/Grid

1. Pickup a TEM grid with the dedicate forceps:
   - H shows towards to the front (“home”)
   - the glow discharged film shows to the left

2. Press LOAD FORECEPS to bring the chamber in the correct position

3. Lock the forceps by pushing the black locking sleeve to the first groove, then slip the forceps into position on the gantry of the EM GP2.

- 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.

- 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.

STEP 2: Lower the chamber

1. Press LOAD SPECIMEN on the main screen
The chamber will lower[1] the forceps will rotate 180° (if setup for right-handed users, page **Error! B**

2. Adjust parameters or load a program[3]

STEP 3: Apply the specimen

Either port, located to the left and right, can be used, depending on the user preference. It is important that the forceps orientation (home or 180°) is set correctly for the port through which the sample shall be applied.

- Open the side port of the environmental chamber

- dispense a 3 - 5 µl fluid droplet onto the grid.

- Close the side port immediately to maintain a stable atmosphere.

STEP 4: Blot

Press BLOT on the main screen (the forceps will possibly rotate to "home" first).[4]

[1] During movement of the environmental chamber, a "STOP" button will appear on the touch panel. Pressing this button will freeze the movement. To continue, the workflow must be reset via the LOAD FORCEPS button.
[2] When the environmental chamber is in the lower position, the shutter closing the feedthrough to the cryogen will wiggle in regular intervals to prevent freezing to the bottom of the chamber.
[3] For a first run with a new specimen, prepare a sequence of different blot times to achieve the desired film thickness, e.g. 0.5, 1.0, 2, and 4 seconds.
[4] If a pre-blot is programmed, the screen will show a progress bar. Press BLOT STOP to stop the process.

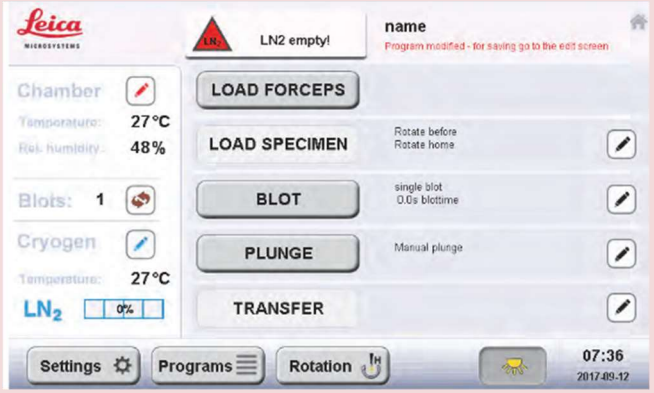
STEP 5: Freezing

- If “automatically plunge after blotting” is selected in the parameters (page **Error! Bookmark not defined.**) the grid will plunge automatically into the secondary cryogen after blotting

- If this option is not enabled, the user must press the PLUNGE button on the main screen.

After plunging, the environmental chamber will rise, allowing access to the frozen grid. Then, the grid will be automatically lifted to the “transfer” position a few mm (depending on the setting, page **Error! Bookmark not defined.**).

- press TRANSFER if the transfer position is not reached automatically



Demonstration: inserting the Gatan cryoholder into the TEM

WHEN:

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.

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.

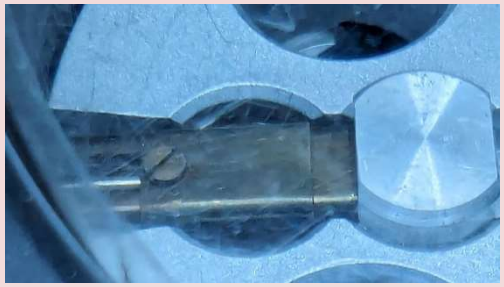
- Press the Clipring™ down onto it with a force of about what you need if you press a code in a keypad.

- 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.

Demonstration: Storing a grid in the cryo-gridbox

WHEN:

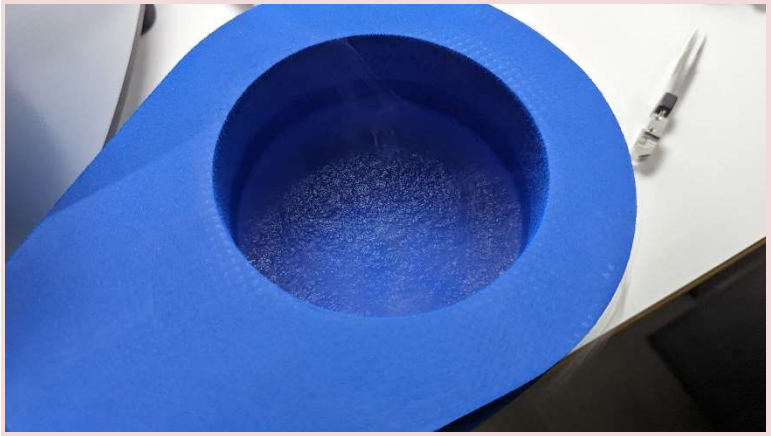
Sample is vitrified

The cryo-gridbox is your shuttle between the plunger and the TEM cryoholder

This step is not trivial and might need some exercise to master

Shuttle the plunged grid from the tweezers to the cryo-gridbox

- fill about 250 ml of liquid nitrogen in a a blue Styrofoam shuttle container.



Lift the lid of the cryotransfer container and place it in the liquid nitrogen container. Assure the lid of the cryo box is open (place the transfer tool with the lid or the handling rod submerged in the blue styrofoam shuttle. Cover with the Styrofoam lid as much as possible.

- Remove the forceps from the gantry by tilting it out forwards. Use the one-handed release if you master it. Assure that the position of the tip remains the same. At no point the grid should leave the liquid ethane.

Do not touch the wall of the container with the frozen grid

- Keep the tweezers actively closed while sliding black tweezer ring upwards. Caution: this will open the tweezers and drop the grid unless you actively keep them closed. The grid is still in submerged in liquid ethane.

- With one quick movement, move the grid from the ethane to just above an empty position of the cryo-gridbox in the cryo-transfer container.

- very gently slide the grid in a slot in the Gridbox and release the tweezers.

Be careful: sometimes the grid sticks to one Of the tweezer arms (is frozen to it).[5]

To commence another run, press LOAD FORECEPS in the workflow.
Alternatively, proceed to transfer the grid to the Gatan cryoholder.

[5] In this case: keep the grid inside the cryo transfer box. Release (open) the tweezers, the grid will stick to one of the tweezer arms. With the tweezers opened, lower the grid into a position in the gridbox and move the tweezer arm in a horizontal way, away from the grid.

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.

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.

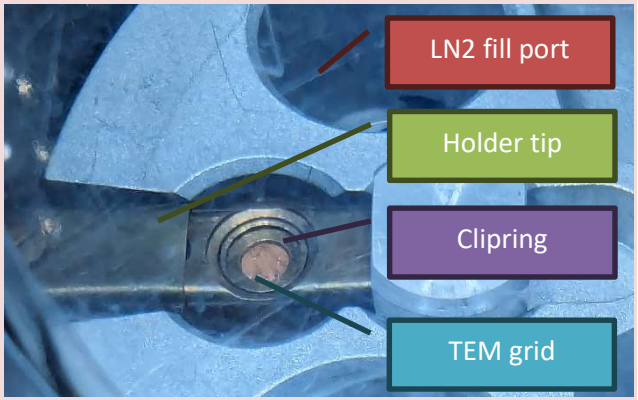
Important notice: static electricity sometime causes the grids to stick to the plastic pin cap or rod. When removing the cap or rod, first move it to the liquid nitrogen reservoir of the workstation and check if this is the case.

Now things must be done swiftly.

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.



Demonstration: Transfer to the Gatan TEM cryoholder

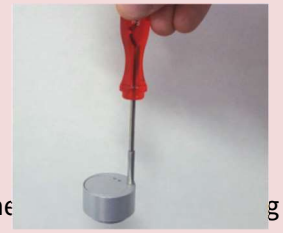
WHEN:

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

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

WHEN:

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.