1 Day
- Pump the holder overnight

1 hour
- Start a 15 minute bakeout of the GP2
- Fill and mount the 1L TEM LN2 dewar.
- Login onto the TEM, start the TEM software and take out the single tilt holder

45 min
- Fill the bottle of milliQ water
- Fill the 5L dewar with LN2

30 min
- Prepare and glow discharge the grids
- Prepare the GP2. Cool down the system

20 min
- Retrieve Gatan holder from the pumping station
- Mount the holder on the workstation and cool down both

10 min
- Switch on the heating plate and set to 50°C.
- Place all tools (tweezers, grips, screwdrivers) on the heating plate

5 min
- Prepare the pipette and the pipette tips
- Get your sample

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CryoTEM

Introduction

Version 1 – May 2024

Part II: Preparation

starts at: Procedure preparation
ends with: ready to cryoplunge


LEICA EM GP

PART II: Preparation

- Prerequisites: the evening before

1. Insert the holder into the appropriate adapter on the Model 655 Dry Pumping Station and evacuate the cryoholder dewar.
2. (optional) heat the cryoholder dewar to at least 60°C for more than two hours according to the procedure in Section 5 of the cryoholder manual.

- Cooling down the workstation and the holder

At the start of any experiment the transparent cover of the workstation should be in place together with the two plastic caps covering the access ports.

Experiment: cool down the cryoholder

1. Remove the blanking plug. Mount the cryoholder in the workstation.

2. Remove the plastic lid of the workstation and the black cap of the cryoholder.

3. Alternatively fill the workstation Dewar (use the fill port) and the cryoholder with LN2.

4. Repeat until the liquid nitrogen eruption passes and the liquid becomes less chaotic. Close off the workstation with the lid and the cryoholder with the cap.

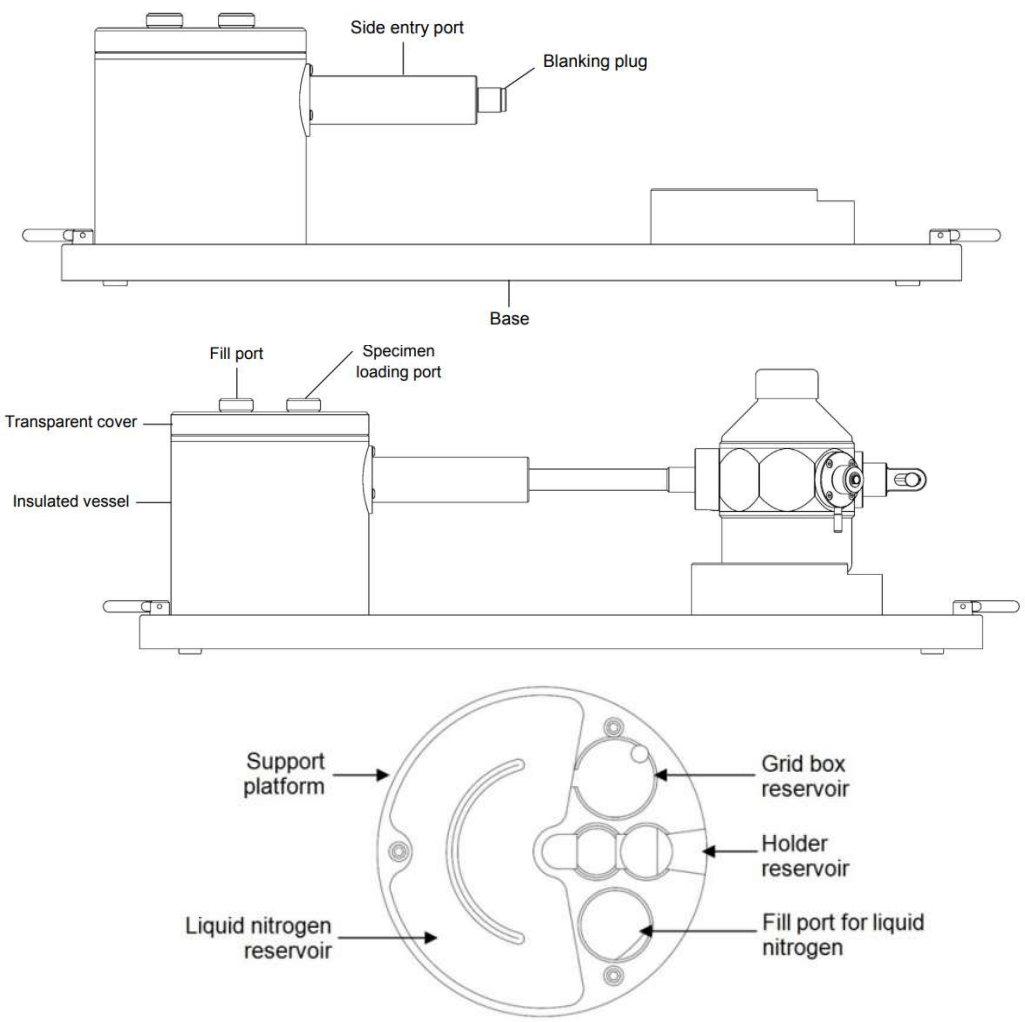
Keep an eye on the workstation: it should not warm up. The liquid nitrogen reservoir should never fall without liquid nitrogen!

Demonstration: preparing the Gatan cryoholder

WHEN:

Before you start plunging

The cryoholder is the technology that allows cryoTEM



Running order (in time before plunging starts)

1 Day
- Pump the holder overnight

1 hour
- Start a 15 minute bakeout of the GP2
- Fill and mount the 1L TEM LN2 dewar.
- Login onto the TEM, start the TEM software and take out the single tilt holder

45 min
- Fill the bottle of milliQ water
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5 min
- Prepare the pipette and the pipette tips
- Get your sample

Demonstration: Preparation of the cryoholder

WHEN:

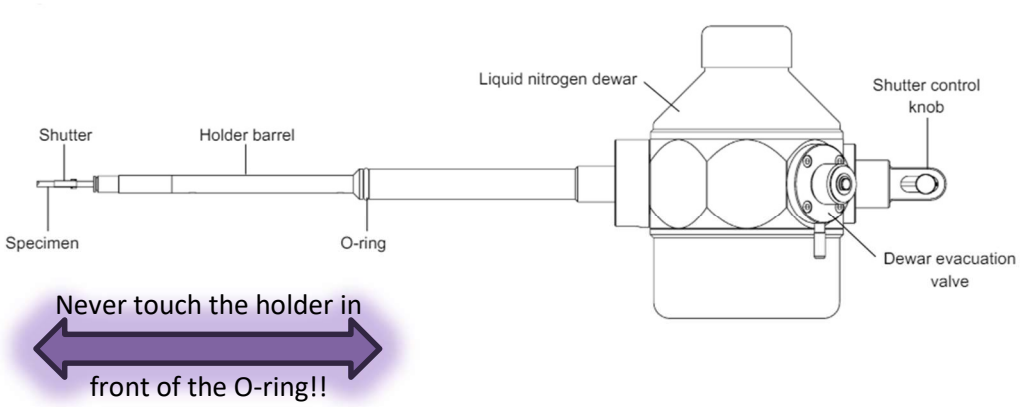
1 Day before cryoTEM

ACTION:

Pump the cryoholder

A good vacuum in the cryoholder is absolutely crucial. Do not start your session without assuring at least 10^{-3} Pa (=10^{-5} mbar) in the holder (or better)

- A short intro to the cryoholder



- The Gatan Turbo pump station

Behind the Instrument, find the Gatan Turbo pump station.

Mount the cryoholder for pumping

1. Close V1 (tipped to the right) and open V2 (tipped upwards)[1]

[1] V2 should always be open. **Always!**

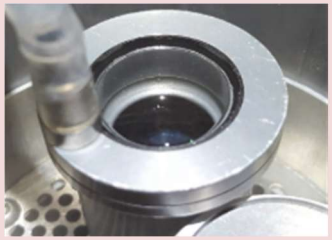
cryogen container drops to or close to the set value (usually around -180°C)

- Open the main ethane bottle valve. Turn it open completely

- Adjust the needle valve on the manometer until a white gas flows out to the liquefier[17].

- Monitor the temperature of the secondary cryogen container (left image).

- Check the cryogen level continuously (watery reflection of the surface, right image) at a flat angle until the container is filled to the brim.



- Close the main ethane bottle vale and the manometer valve

- Remove the liquefier and place on the heating plate. Avoid contact!

Note:

Ethane freezes at around -182.8°C. If you pour liquid nitrogen (-196°C) into the black ethane container, the ethane will solidify. **Do not plunge in solid ethane**: you will damage the tweezers and your sample.

Remedy: wait until the system has settled back at -180°C (see "Environment, page 25)

[17] Often, after a few minutes, condensation of cryogen will form at the inner lip of the liquefier

- Freezing Chamber and Cool-Down

All components of the freezing chamber must be clean and completely dry before starting to work

→ Run a bake-out (at least 15 minutes) prior to operating the plunge freezer

Prepare the primary cryogen (LN2)

1. Load a cryo-grid box
2. Position the black secondary cryogen container (#8 on page 23). Cover the container[16] with the plastic cap (or a plastic bottle cap)
3. Position the container (#11 page 23) on its platform at the front right in the freezing chamber.

→ Pour LN2 directly into the EM GP2 Dewar
→ It takes around 1.8 litres of LN2 to cool the Dewar and fill it to 100%

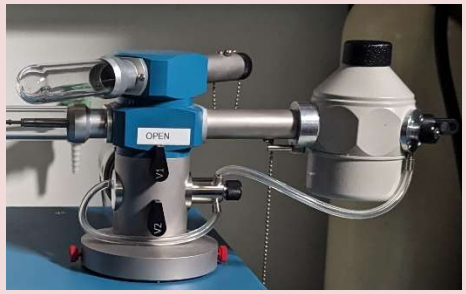
Wait until the desired temperature and humidity is reached (usually -180°C, 95%). Due to the 15 minutes bakeout, this can take up to 10 minutes

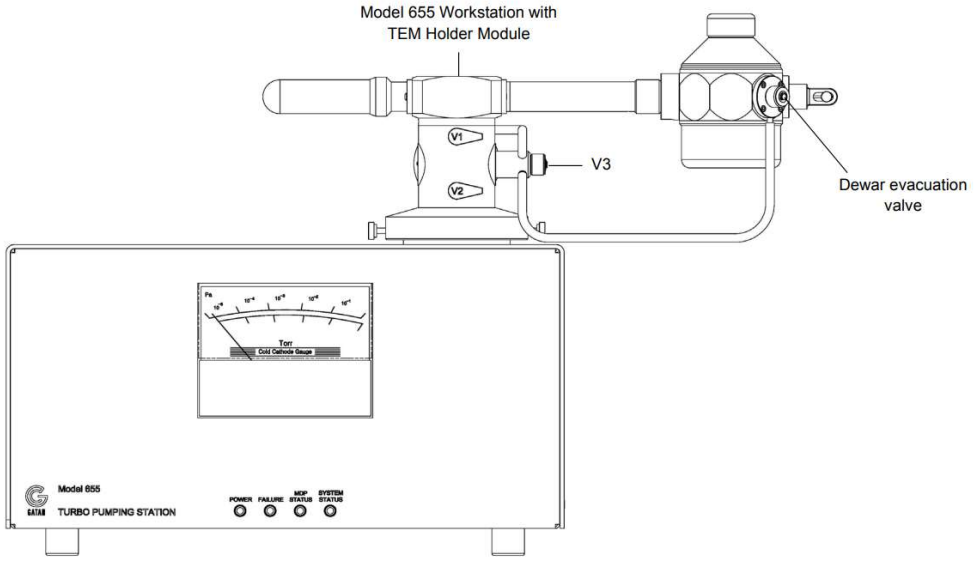
Prepare the secondary cryogen (ethane)

- Connect the liquefier (#10 page 23) to the silicone tubing of the ethane manometer
- Insert the gas liquefier in the chamber over the secondary cryogen container
- wait until the temperature of the

[16] Do not spill LN2 over the surface of the secondary cryogen (when present): it might freeze over, leading to pressure build-up and explosion.

2. Remove the black stopper from the lower of the two tubes. Gently insert the holder until the O-ring is inside the tube (bit of resistance).
3. Connect the plastic tubing onto the valve of the cryoholder dewar. Open V1.
4. Switch on the instrument with the 1/0 switch in the back





The pump will start and after a few minutes you will see the pressure dropping (needle to the left)

Evacuation of the cryoholder dewar

1. On the base of the pump, unscrew the black valve (turn towards you) – not the one on the cryoholder! Do not unscrew further than **two windings.**



As soon as the valve allows flow, the needle will drop to atmospheric pressures, but should recover fast.

2. Once a good vacuum is achieved (10^{-2}-10^{-3} Pa), open the black screw valve on the dewar. Pump the holder for several hours, preferably **overnight**. You want to reach a pressure (well) **below 10^{-3} Pa**.


- Cryo-gridbox

The cryo-gridbox is the shuttle between the GP2 and the cryoholder. The cryo-gridbox provides space for 4 grids.

The positions are counted from the slot, counterclockwise. (hence, the grid on the left is positioned in #3).

Do not use the gridboxes with a screw: too tedious to handle. Use the pin lids and the accompanying gripping tool or the handling rod (pin and tool in one)

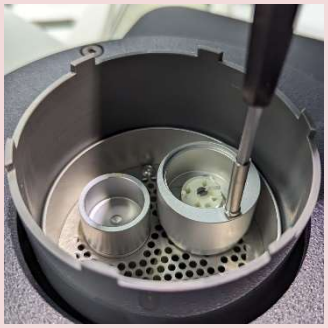
The cryotransfer container

Do this before you cool the dewar with LN2

- Remove the lid of the grid box cryo transfer container (#11 on page 23) using cryo foreceps. Leave the lid inside the LN2 Dewar to avoid contamination.

- Place the opening of the cryo-gridbox over the pin in the transfer container.





- Filter paper

Mounting a fresh filter paper

Mount the following stack (in this order) on the blotter:
- A leica contamination ring (Angstrom)
- A whatman #1 filter paper
- A metal ring

- If this is not a fresh start, In the main screen, press the reset counter 
- 1 Filter paper = 10 shots[15] (then it needs to be replaced)
- For reproducibility, allow a fresh filter paper to equilibrate in the humid chamber (5 minutes) before being used for blotting.

[15] The counter is automatically reset to zero when the instrument is switched on

Demonstration: Prepare the TEM

WHEN:

About 1 hour before plunging

ACTION:

Get the TEM ready for the vitreous sample

Overlaps with conventional TEM but is easily forgotten, leading to stres

- TEM

Vacuum system running: Gun/Col Log < 20
Holder in the goniometer, in position 0,0
No sample in the holder
The Veleta camera is out
The column valves (V4 and V7) closed
The objective aperture is out
Magnification 390 M
Fluorescent screen is down (R1)

Experiment: startup the TEM system

1. Windows Login
2. Software startup: Microscope Interface, then TIA.
3. Fill the cryotrap dewar with LN_2.
4. Image > CCD/TV: select BM-Eagle camera
5. Reset the goniometer: Image > stage[2] > flapout > control > Reset > Holder
6. Take out the objective aperture

Make sure the TEM is ready. Then start, the filament

Experiment: Retract the single tilt holder

1. Remove the holder
2. Place is, dust protected, on the stand at the sample loading station (binocular)
3. Place the stand out of the way, so it cannot get damaged when the cryo loading stage is used.

Experiment: startup the filament

1. On the PC screen: tab Setup > Autogun[2] > High Tension, then Light
2. On the PC screen: tab Setup > Autogun > Align (Gun tilt is shown)
3. Run condenser astigmatism alignment
4. Finally, set the system to:
    - Mag: 390 X
    - C2 = 100%
    - Spot size = 2

Note: you may switch off the filament to save filament lifetime (press “Setup > Autogun > Light”).

- GP2

Start a 15 minutes bake out of the GP2 system to assure a water free system.

Experiment: Bake Out

1. Leave the door of the environmental chamber open.

2. Start the GP2 (button on the bottom back), let it startup.

2. Once the home screen loads, go to the **SETTINGS** screen and press **BAKE OUT**. Confirm the warnings, start the process by pressing START. Set the time to 15 minutes.

[2] If the High Tension is off, (not yellow): Assure that 120 kV is selected from the dropdown menu (Figure B) and click the High Tension button. If the button “High Tension” is greyed out and the Status reads: High tension not enabled, press the physical button “HT” on the right of the microscope column.

Connect the demineralized water source

Demineralized water

- Fill the provided bottle to the top with fresh demineralized water. Keep closed if not used.

- Fill the provided plastic syringe. Mount the syringe onto the tube.

- Open the blue valve (central arm of the blue part points up) and fill the humidifier tray with 70 ml of water (yes, you need two fillings).

- Keep at 50 ml in the syringe for refillings. At each refill: add 30 ml.

Bottle for demineralized water

No syringe attached, valve closed

Syringe attached, valve open

3. Tighten the black screw on top of the humidifier assembly to keep the tray in place (you might have to wiggle the tray a bit to find purchase.

Mounting the dripping tray

Experiment: mounting the dripping tray

Below the chamber, find the mounting mechanism for the dripping tray.

- Just slide in the dripping tray in a horizontal motion.

- There is no fixing screw on the dripping tray

Demonstration: Preparing humidity and cooling resources

WHEN:

About 45 minutes before plunging (the effect wears off!)

ACTION:

Glow discharge treatment of the TEM grids

Make sure you have all you need before you start the procedure

You need distilled water (MilliQ), about 200 mL and liquid nitrogen, about 5 L.

Experiment: get resources

MilliQ water

- Only use the provided bottle
- Fill it to the rim
- Careful: the blue cap is not closing it watertight

Liquid nitrogen

- Fill 5L of LN2 from the 25L dewar
- If needed refill the 25L dewar

Demonstration: surface treatment of the TEM grids

WHEN:

Not more than 30 minutes before plunging (the effect wears off!)

ACTION:

Glow discharge treatment of the TEM grids

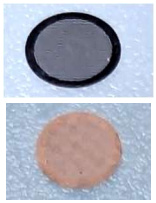
Improved wetting by glow discharge helps to get a thin layer of liquid on the grid

Glow discharge will modify the grid surface, leaving them hydrophilic and negatively charged. This greatly improves the wetting.

- The grid block

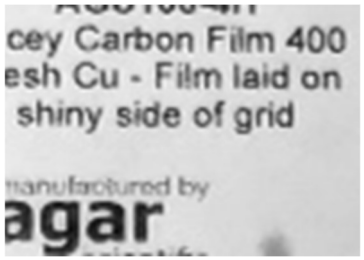
Find the grid block in the drawer of the cryostation table. Please bring your own grids. TEM grids have two sides:

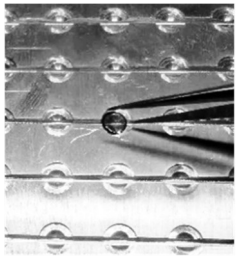
- Shiny side (usually a bit darker, and shiny)
- Rough side (usually more copper/ bright orange)



- Verify on which side is the film

Place the grids with the film up in the gridblock (in this case, the shiney side up). When finished, cover the petridish and move to the glow discharge in the anteroom of A017.





Demonstration: preparing the Leica GP2 plunge freezer

WHEN:

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

Setup the GP2 for plunge freezing

- Humidifier

Find the humidifier tray (below, left) and the dripping tray (below, right). They are usually on the heating plate.

Mounting the trays

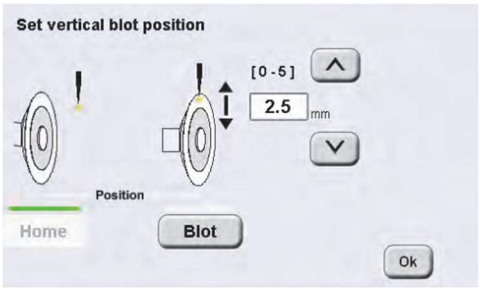
- The humidifier tray should be mounted on the right side of the GP2 chamber.
- The black cover is never mounted.

Experiment: mounting the humidifier tray

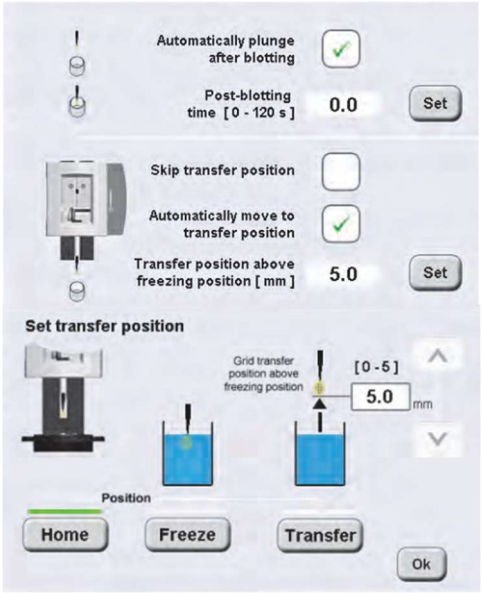
1. Align the two pins of the tray with the two sockets on the right side (outside) the chamber.

2. Slide in the tray in an upwards movement

Note: for this step, the chamber must be lowered.
- Press BLOT (filter paper moves to the blotting position)
- Adjust the mechanism to the desired position.[11,12]



- Plunge / Transfer Parameters



Automatically plunge: tick![13]

If above it ticked, this can be set. Default: 0[14]

Do move to transfer position, and do this automatically (unticked / ticked).

Explanation and setting:

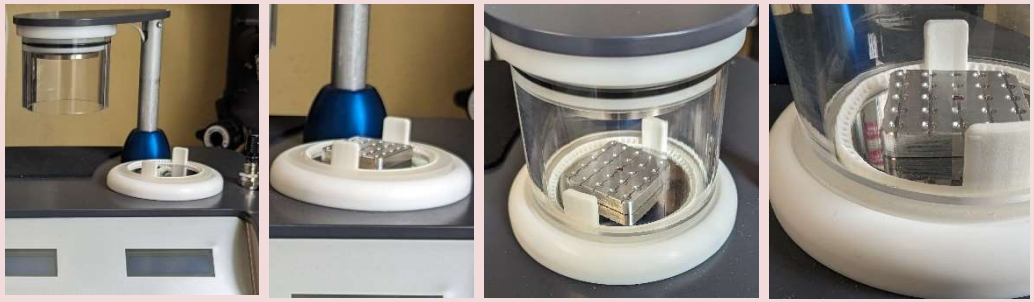
The freezing position is fixed to 4 mm from the base of the secondary cryogen container.

Transfer position: facilitates the transfer of the grid after plunging or to remove excess liquid secondary cryogen.

[11] The recommended position is where the upper edge of the grid and the upper edge of the filter paper are aligned with each other
[12] It is also possible, however, to blot only half the grid (Touch and Flow)
[13] If "Automatically plunge after blotting" is disabled, the user is required to press the "Plunge" button on the main screen/workflow. This "Plunge" button is di when ticked.
[14] The post-blotting time is desired by some users to allow the water layer even out over the surface of the grid. For typical applications, this delay is not required.

Mounting samples in the ELMO glow discharge

1. Open the right bell (pull, then turn)
2. Place the grid block – without the plastic petri Dish - with your grids on the Electrode (=the mirror-like bottom of the bell)
3. Close the bell
4. Make sure the bell rests completely on the Silicon ring, which assures an air-tight seal



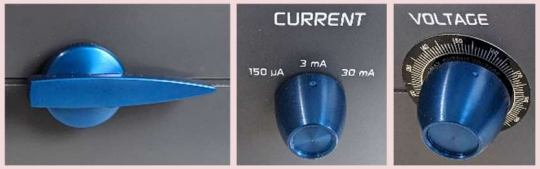
<u>If the vacuum detailed below cannot be reached, then the bell was not properly closed / does not properly rest on the silicon seal ring.</u>

1. Switch on the system

Use the red power switch on the left side of the table. The goal is to glow discharge:
- at 2.10^{-2} mbar
- at 2 mA
- for 15-60 seconds

2. Setting the dials

- the bell selector pointing to the right
- The CURRENT dial point to 3 mA
- The VOLTAGE dial is set to about 60



3. Turn on the pump

flip the pump toggle to 1. The pump starts and the pressure starts dropping. Within about 20-30 seconds, pressures below 0.1 mbar (E-1) are reached.

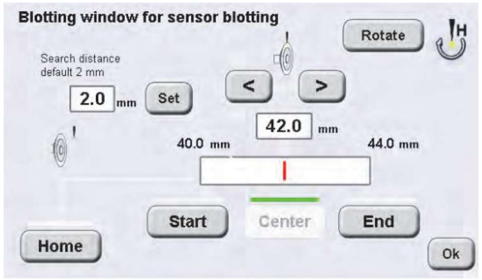
→ If this is not the case: the bell was not properly closed

Caution: the needle valve is extremely breakable. Proceed with utmost care

4. Set the vacuum to 2.0 E-1 mbar using the needle valve

- Find the needle valve on the right of the right bell.
- Adjust it (never tighten it!!) until you reach 2.0 E-1 mbar, or there about.

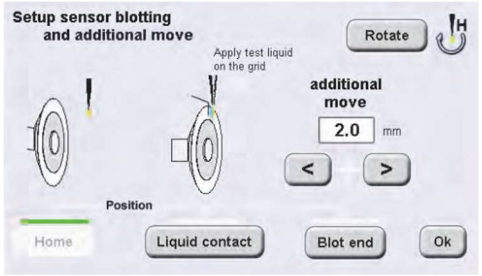
Note: the pump will continue to evacuate the bell: you may need to readjust the vacuum.



- Insert forceps with an EM grid
- press ROTATE to position the grid used for blotting (180°)
- press CENTER position. Adjust the position of the filter paper with the left/right arrow buttons to touch the full area of the grid[10]

Additional movement

- ➔ determines the distance through which the filter paper is moved after the sensor is triggered by the wetting of the filter paper
- ➔ By this, determines the blotting pressure: using a higher additional movement, more pressure on the grid will exert while blotting.



- Rotate the grid with ROTATE (the grid side with the film faces the entry port to be used for sample application).
- Apply a test sample volume of at least 3 µl.
- ROTATE the grid (drop faces the filter paper)
- Press LIQUID CONTACT. This will advance the filter paper until the blotting sensor has been triggered.
- Press BLOT END. Set the move to achieve the desired pressure of filter paper vs grid.

Vertical blot position

[10] The default size of the blotting window (START/END) does not require adjustment.

- H (home) faces front: film is facing left (towards the blotter)

- Right-handed users: Tick both boxes (to apply sample from the right): the right port is used for specimen application, the grid rotates back 180° and is blotted from the left side.

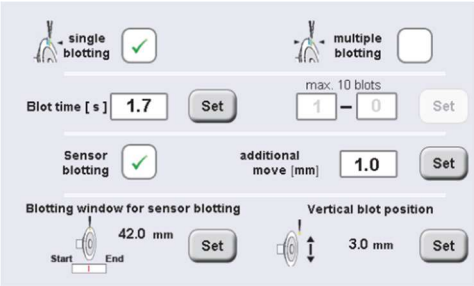
- Blot Parameters (single blotting with sensor)

Sensor blotting = most automatic setting, highest reproducibility

Action: a sensor will continuously check paper wetting, irrespective of:

- warping of the filter paper (caused by high humidity in the chamber)
- bending of the grid

Note: the sensor is adjusted for Whatman #1 and 3 µl sample volume



How many times the grid is blotted

The blotting time[9]

Sensor blotting: ticked. Additional move: see below

Sets up the sensor blotting parameters and the vertical blot position (see below)

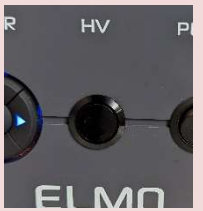
Sensor blotting requires two parameters to be set up:

- the “blotting window” for the sensor
- the additional movement after the sensor signal has been triggered.

Blotting window

[9] The actual contact of filter paper with grid. This parameter is highly specimen specific. For 3 to 5 µl aqueous solution, 0.5 to 3.0 s blotting are usual.

5. Set the voltage to create 2 mA current



- Press the HV button and keep it pressed. A plasma will be created instantly and you can read its current.
- Adjust the voltage knob to reach about 2 mA.
- look closely, you can see a slight purplish glow
- Do this quickly! Then release the HV button.

Note: if you see ↑↑↑ SCALE or ↓↓↓ SCALE instead of a reading in the CURRENT panel, your current voltage produces a current that is off the current scale.

- Change the CURRENT dial to find your value
- Adjust the VOLTAGE dial to reach 2.0 mA

6. Set the timer

- On the TIMER dial, press left or right to enter the edit mode
- Use up and down on the TIMER dial to set your time, eg. 30 seconds.
- On the TIMER dial, press left or right to exit the edit mode
- Finally, press the OK button on the TIMER dial to start the glow discharge.

Be ready to fine-tune your vacuum (needle valve) and the current!

7. Switch off

- Turn off the pump with the 0/1 flip switch. The bell starts to flood with air.
- When atmospheric pressure is reached, open the bell and retrieve you grid block. Place it in the petri dish and close it for transport to the GP2.
- Close the bell
- Finally, switch off the ELMO system with the red button on the left of the table.

Demonstration: Retrieve the cryoholder and cool down

WHEN:

About 15-20 minutes before plunging

Don't do this too early (ice crystal contamination), but don't do this too late (the system needs time to properly cool down)

Assure the stand is ready to receive the holder:

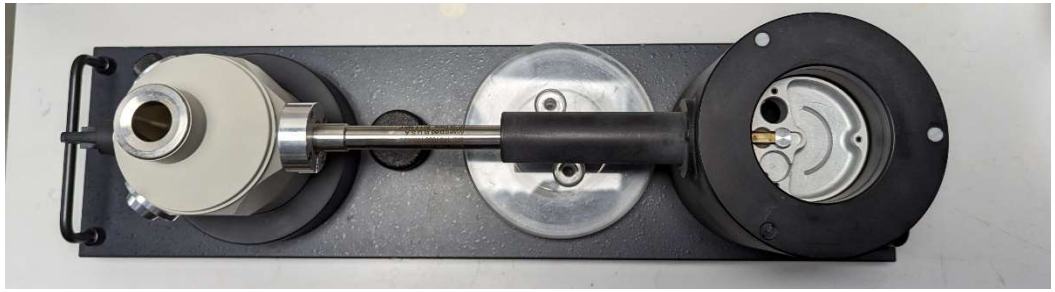
- Placed on the cryotools table, or directly under the binocular of the TEM specimen loading station.
- Remove the blanker

- Retrieving the cryoholder from the Gatan Turbo pump station

Retrieving the holder

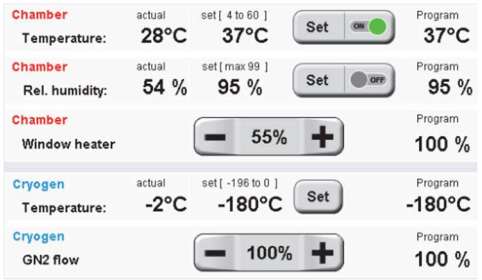
1. Close the black screw valve on the cryoholder and the black screw valve on the pump base. Close V1 (flip horizontally)
2. Detach the plastic tube from the dewar inlet
3. Gently pull back the holder from the glass tube and replace it by the dummy. Turn off the pump with the switch in the back. The holder is now ready to use for cryoTEM.

Carefully bring the holder to the stand and mount it



2. The process panel[4]
3. Functional keys, with which special screens or functions can be called
4. Warnings and error messages.
5. Name and status of the current program.

- Environment



Temperature (e.g. 22°C for liposomes)

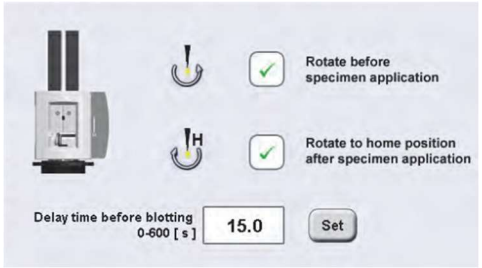
Humidity in the chamber[5,6]

Avoid condens. at the chamber door window

LN2 temperature: set it to -180°C[7]

For plunging: 100%, standby: set to 5%

- Load Specimen Parameters



Rotates 180° before sample application.

Rotates 180° back after sample application[8]

Set to 0 s (no delay)

[4] Here, the individual steps of the freezing/plunging workflow can be activated in sequence.
[5] The humidity should be chosen as high as possible, particularly at room temperature and above, to avoid evaporation of the thin water layer left after blotting (and subsequent changes in concentration of solutes in the sample). Generally, low humidity to promote evaporation yielding thinner ice is not recommended.
[6] The system cannot lower humidity, only increase it.
[7] Note: ethane **freezes** at -183°C. Don't set it the LN2 temperature below this value.
[8] Except if you want to "Blot from the back". Could be useful for NPs

Demonstration: parameters of the Leica GP2 plunge freezer

WHEN:

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

Setup the GP2 for plunge freezing

Startup of the system

- Turn on the power with the main switch at the rear of the main unit.
- The following screen will appear:

- After booting, the following warnings appear:

a) replace filter paper

b) refill the humidifier tank and remove water from the drip tray

c) to cool down the instrument by filling the LN2 container.

Just confirm them with "OK". The main screen appears.

1. The status panel[3]

[3] Info on environment, filter paper, and cryogen parameters, alternating periodically between actual and set values.

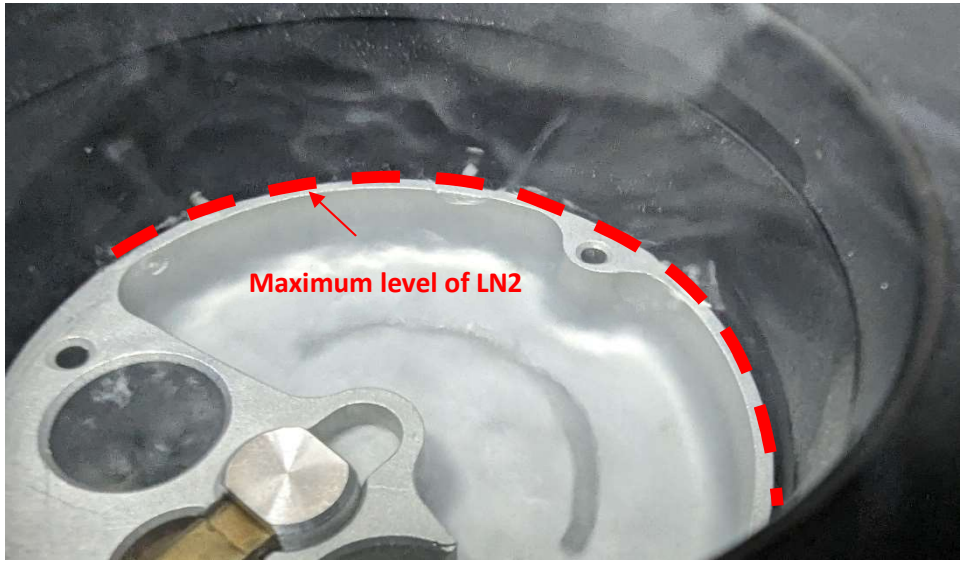
- Cooling down station and holder

It is important to cool them down at the same time

Cooling down the cryoholder and station

1. Pour about 1 L of LN2 in a plastic beaker
2. Top up the cryoholder dewar, about 100 mL (remove the round black cap)
3. Immediately fill 200 mL in the fill port of the stand. Do not fill the entire stand recipient! You can remove the transparent cover, but better is to keep it and pour though ne of the two holes, directly in the filling port.
3. Repeat until the LN2 remains – is not vigorously boiling anymore - stable in both recipients
4. Place the round black cap on the dewar, and the plastic cover on the stand.

Do not overfill the stand recipient! LN2 should not have a lever higher than the rim of the metal



Keep an eye on the LN2 level. Refill roughly every 10 minutes

- Overview of the system

1. Environmental chamber with humidity and temperature control
2. Humidifier tank (panel removed)
3. Drip tray
4. Magnetic holder for filter paper
5. Forceps with forceps adapter
7. Moveable ring for contamination protection
8. Freezing chamber with secondary *cryogen and cryo transfer container
9. Secondary cryogen liquefier
10. Cryo transfer container
12. Touch panel

1. Special forceps with insulation coating
2. Foam cover
3. Styrofoam box and magnetic rings (2 pcs.)
4. Punched filter paper
5. M4 Cryotool
6. Forceps with forceps adapter
7. Cryo-grid box
8. Secondary cryogen container
9. Secondary cryogen container cover
10. Secondary cryogen gas liquefier
11. Cryotransfer container

Demonstration: The Leica GP2 plunge freezer settings

WHEN:

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

Humidity and temperature assure reproducibility of the plunge freezing process

- Please be informed:

When LN2 evaporates, it expands at a ratio of 1:700. Never place LN2 in a closed vessel!

GN2 displaces oxygen and can cause fainting. If a person becomes dizzy while working with LN2, move him/her to a well-ventilated area immediately.

If your settings are already set, skip to the next hands-on chapter, on page 29.

- Keeping the beakers water-free

Drying the plastic beakers

Water is the biggist issue in cryoTEM. It is important the any recipient that is used to pour LN2 is absolutely dry. For this we re-use a home shoedrying application.

- After use of a beaker, you will see ice forming on its surfaces
- place it on the cryotable and put one (or more) big tubes in it.
- Turn the timer to about 20 minutes.

This beaker is not available for at least 20 minutes

Demonstration: The heating plate, pipettes and samples

WHEN:

About 10 minutes before plunging

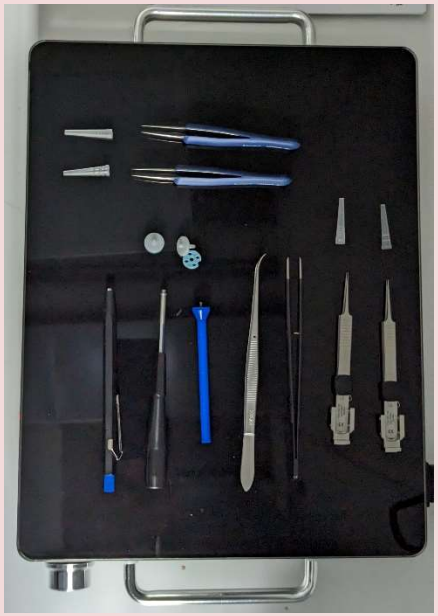
All tools must we as dry as possible

The heating plate can heat to 100°C. There is no temperature display.

- The heating plate

Setting the heating plate to about 50°C

- On the electrical outlet, use the timer to set a 2 hour window
- If needed, turn the heating plat knob into the Range marked in red.
- Place all needed tools on the heating plate



- The pipette

Prepare the pipette: 3-4 ul

Do not place the pipette or the tips on the heating plate

- Sample

Get your sample