CryoTEM

Introduction

Version 2 – January 2025

Part II: Preparation

starts at: Procedure preparation
ends with: ready to cryoplunge

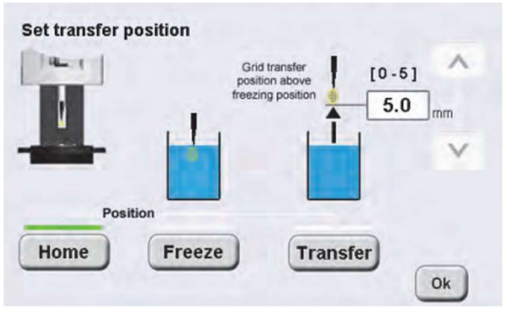

LEICA EM GP

PART II: Preparation

- Plunge parameters

- Automatically plunge: tick this![13]
- If above is ticked, Post-blotting time can be set. Default is 0 sec.[14]
- Skip transfer position (not tocked) and Automatically move to transfer position (ticked)

Explanation and setting:



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

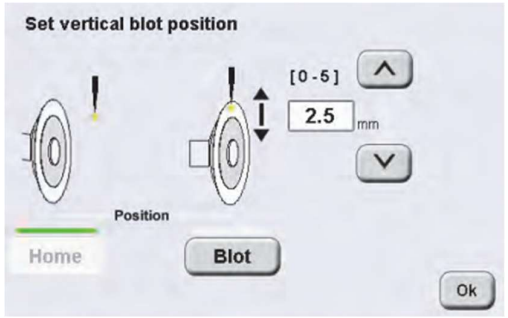
Transfer position: facilitates the transfer of the grid after plunging or to remove excess liquid cryogen.

[13] If "Automatically plunge after blotting" is disabled, the user is required to press the Freeze button on the main screen.

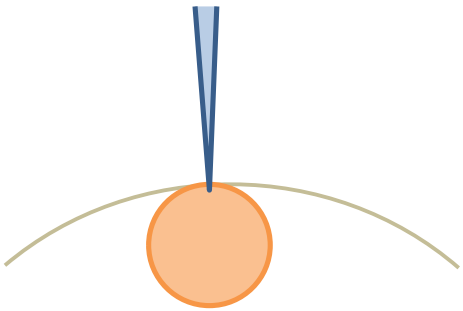
[14] The post-blotting time is desired by some users to allow the water layer even out over the surface of the grid. For default applications, this delay is not required.

Vertical blot position

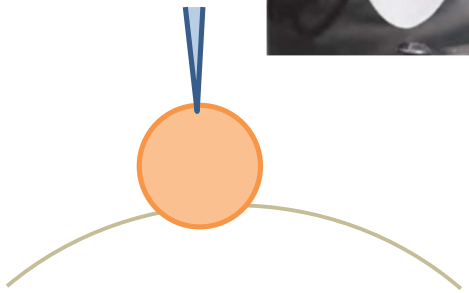
Note: for this step, the chamber must be lowered.



- Press BLOT (filter paper moves to the blotting position)
- With the UP or DOWN arrows, set the mechanism to the desired position[11,12]



Default vertical position



Touch and flow

Running order (in time before plunging)

1 day
- Pump the holder overninght

1 hour
- Check the 25L dewar
- Start a 15 minute bakeout
- Prepare the TEM

45 min
- Fill the bottle of milliQ water
- Fill the 5L dewar with LN2
- Stop the bakeout and install the humidifier

30 min
- Glow discharge of the grids
- Cool down the GP2 system with the primary cryogen

20 min
- Prepare the Gatan station
- Retrieve the Gatan holder and mount it on the Gatan station.
- Cool down both

10 min
- Drying all tools (Heating plate, shoedryer)
- Fill the secondary cryogen

5 min
- Load the grids onto the dedicated tweezers
- Mount the filter paper and prepare the pipettes
- Get your sample

[11] The standard position is where the upper edge of the grid and the upper edge of the filter paper are aligned with eachother.
[12] It is also possible to blot only the lower half of the grid (Called Touch and flow)

Demonstration: Preparation of the cryoholder

WHEN:

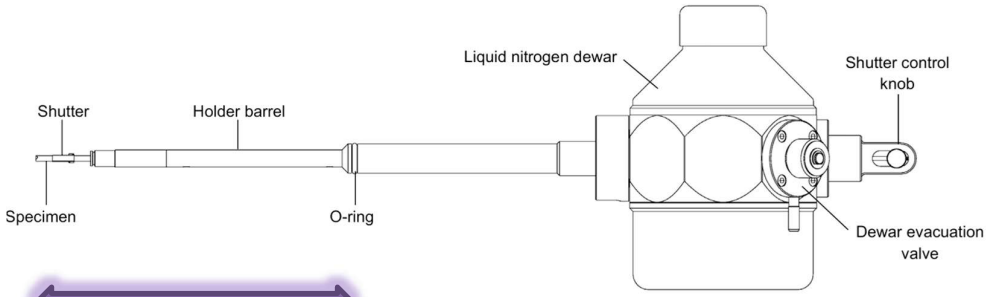
1 day before your cryo-session

ACTION:

Pump the cryoholder

A good vacuum in the cryoholder dewar 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



Never touch the holder in front of the O-ring!!

- The Gatan turbo pump station

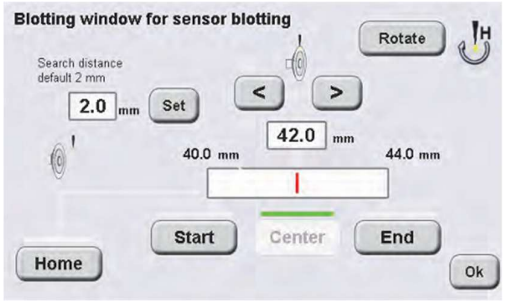
Behind the FIB-SEM on the table, find the Gatan Turbopump station

Experiment: 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!**

Sensor blotting: blotting window

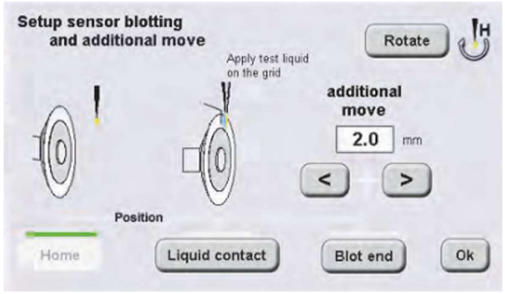


- Insert foreceps with an EM grid
- Press ROTATE to position the grid used for blotting (180°)
- Press CENTER. Now adjust the position of the filter paper with the <, > buttons so the full area of the grid touches the filter paper.[10]

Additional movement

➔ Determines the distance through which the filter paper is moved after the sensor is triggered by the wetting of the paper

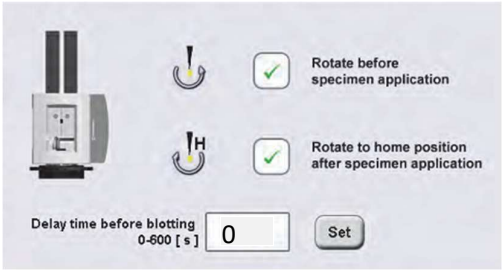
➔ This determines the blotting pressure: using a higher additional movement, more pressure is exerted on the grid while blotting.



- Rotate the grid with ROTATE (the grid side with the film faces the entry port to be used for sample application)
- Apply a sample volume of at least 3 µl (usually 3-5 µl)
- ROTATE the grid (drop now 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 against the grid.

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

- Load specimen parameters



Rotates 180° before sample application (= untick for left handed people)

Rotates 180° back after sample application[8]

Set to 0s (no delay)

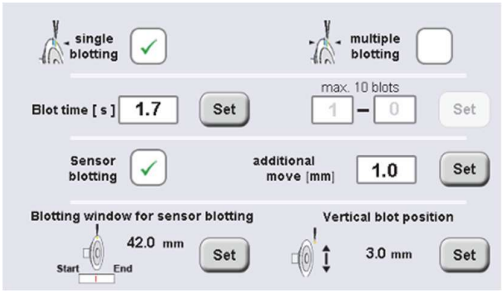
- Blot parameters

Sensor blotting: most automatic setting, highest reproducibility

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

- Warping of the filter paper (caused by humidity)
- Bending of the grid

The sensor is adjusted to 3-5 µl sample volume and Whatman #1



How many times the grid is blotted (default = 1)

The blotting time[9]

Sensor blotting: ticked! Additional move: see below

Sensor blotting and vertical blot positions

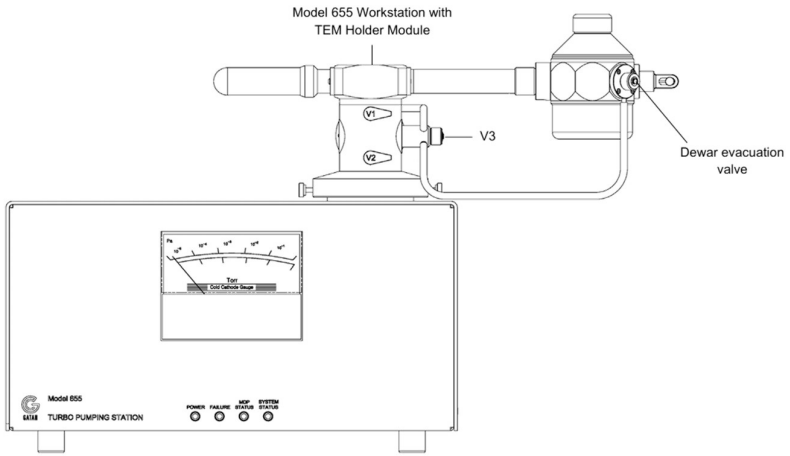
[8] Tick for left handed people of if right handed people want to use the “blot from the back” technique

[9] The actual contact time of the blotting paper with the grid. This parameter is highly sample specific. For 3-5 µl aqueous solution, 3-5 s are usual

2. Remove the black stopper from the lower of the two tubes. Gently insert the holder until the stop (O-ring is inside the tube, with resistance)

3. Connect the plastic tubing to the valve of the cryoholder dewar. Open V1.

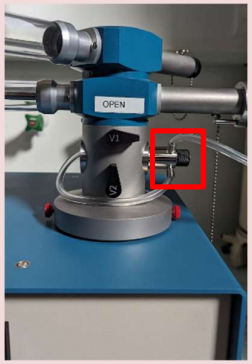
4. Switch on the instrument with the I/O switch in the back.



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

Experiment: evacuation of the cryoholder dewar

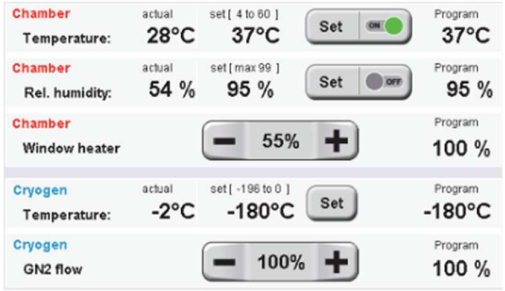
1. On the base of the pump, unscrew the black valve (turn towards you) – not the one on the cryoholder! Do not unscrew farther than **two windings**.



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.

Environment



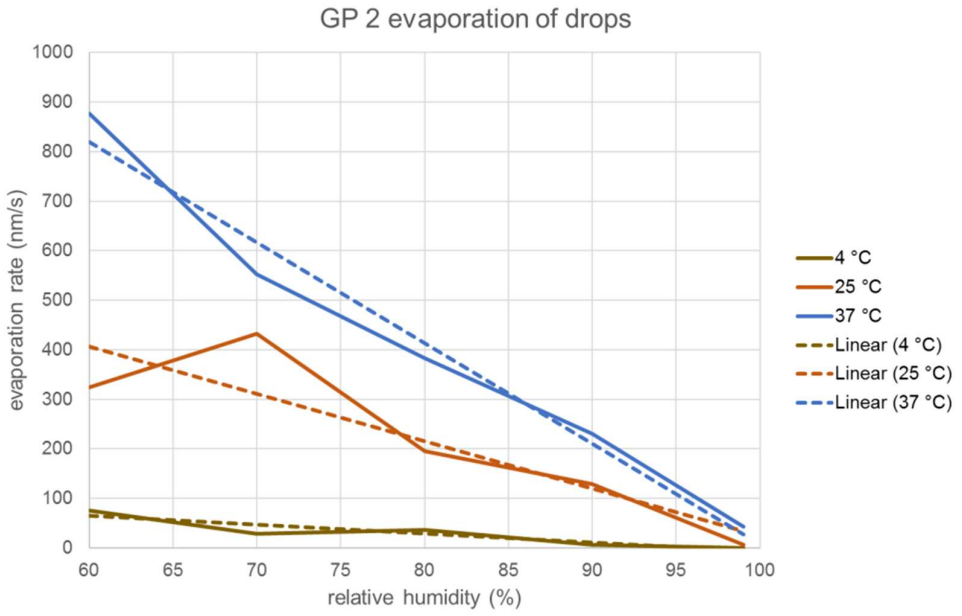
Temperature (e.g. 22°C for liposomes)

Humidity in the chamber[6]

Avoids condensation at chamber door

LN2 temperature: set it to -180°C[7]

For plunging: 100%. Standby: set to 5%



[6] The system cannot lower humidity, only increate it

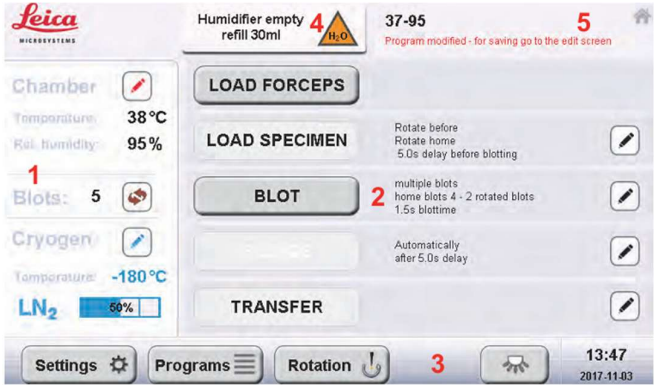
[7] Note: ethane freezes at -182.5 °C. D not set the LN2 temperature below this value

Startup of the system

- Turn on the power with the main switch at the rear of the main unit
- The startup screen appears

- After booting, the following warnings will appear:
  - Replace the filter paper
  - Refill the humidifier tank
  - Cool down the instrument by filling the LN2 container

Just confirm all with the OK button. The main screen appears.



1 The status panel[3]
2 The process panel[4]
3 Functional keys[5]
4 Warnings and error messages.
5 Name and status of the current program.

Demonstration: 1 hour before plunging

WHEN:

1 hour before your cryo-session

ACTION:

Check the LN2 25 L dewar
Start the bake out of the GP2
Get the TEM ready for the cryo-sample

Overlaps with conventional TEM but is easily forgotten

- Check the LN2 25 L dewar

You will need about 10-15 L of LN2 for your session (depending on the number of samples).

Fill the dewar now if it is not at least half-full

- GP2 Bake-out

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

Experiment: short bake-out

1. Open the door of the environmental chamber

2. Start the GP2 (button on the back, right). Let it startup and acknowledge the messages.

3. Once the home screen has loaded, (tap to start) > settings > Bake out. Confirm the three warnings and start the bake out process by pressing start. You cannot set a time limit less than 1 hour.

[3] Info on environment, filter paper and cryogen parameters, alternating periodically between actual and set values.
[4] Where the individual steps of the freezing/plunging workflow can be activated in sequence.
[5] special screens or functions can be called, e.g. rotation of the grid around a vertical axis or chamber illumination on/off.

- Prepare the TEM

Magnification	M 390X	Holder	Inserted. Position 0,0
Camera	out (green LED on)	SAED	Out
Intensity	70%	Vacuum system	Running, Col/Val < 20
obj aperture	Out	Column valves	Closed (V4 & V7)

Experiment: Startup the TEM

1. Login into windows
2. Start the software: Microscope interface, then TIA
3. Fill the cryotrap dewar (1L) with LN_2, if needed
4. Image > CCD/TV camera: select BM-Eagle
5. Reset the goniometer: Image > Stage^2 > flapout > control > reset > holder

Assure the gun/col pressure < 20.

Experiment: Start the filament and retract the holder

1. Start the filament (Setup > Autogun > Light). Wait 120 s.
2. Close the column valves
3. Retract the holder from the goniometer
4. Place the holder in the stand at the loading station. Protect if for dust.
5. Put the stand with the holder somewhere out of the way.
6. On the PC screen: Setup > Autogun > click High tension, then Light
7. On the PC screen: Setup > Autogun > click Align (Gun tilt is shown in the dropdown)
8. Center the beam
9. Finally, set the system to the following settings:
   - Magnification = M 390 X
   - C2 (INT) = 70%
   - Spot size = 2

Note: please switch off the filament now. (Setup > autogun > Light)

- Overview of the system

1 Environmental chamber with humidity and temperature control
2 Humidifier tank
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 (2 pcs.)
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

- Please be informed

LN2 boils at -196.5 °C

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

Gaseous N2 displaces oxygen and can cause fainting. If a person becomes dizzy while working with LN2, move him/her immediately to a well-ventilated area. Do not place those people on the floor!

Demonstration: 45 minutes before plunging

WHEN:

45 minutes before cryoplunging

ACTION:

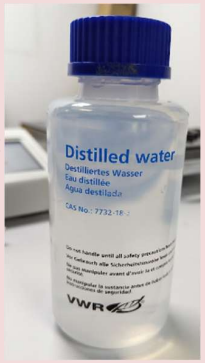
Refill the bottle of milliQ water
Fill the 5L dewar with LN2
Stop the bakeout

Overlaps with conventional TEM but is easily forgotten

- Get the liquid consumables (milliQ water, LN2)

Experiment: get the liquid consumables

MilliQ water
- Only use the provided bottle
- empty the water and **refill to the rim**
- Careful: the blue cap is not closing water tight



Liquid nitrogen
- Fill 5L of LN2 from the 25L dewar
- Make sure there is at least 5L left in the 25L dewar for refills
- Meanwhile, lookm in the camera compartment of the TEM
- Scout for patches that are green (=electron transparent)

- Stop the bake out and install the humidifier

On the GP2, manually stop the bake-out (keep the chamber door open).

- Install the humidifier

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

Mounting the trays

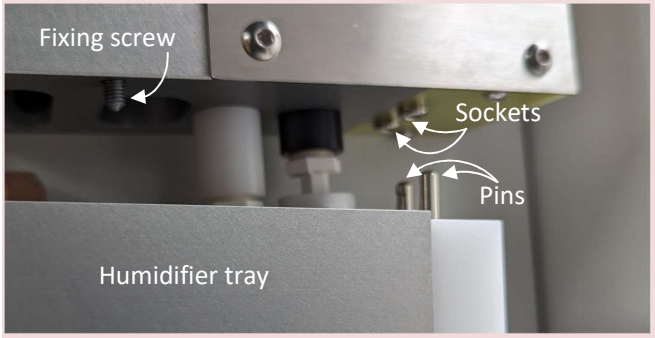
The humidifier tray should be mounted on the right side of the GP2 chamber.

Experiment: mounting the humidifier tray

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

2. Slide the tray in in an upwards movement

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



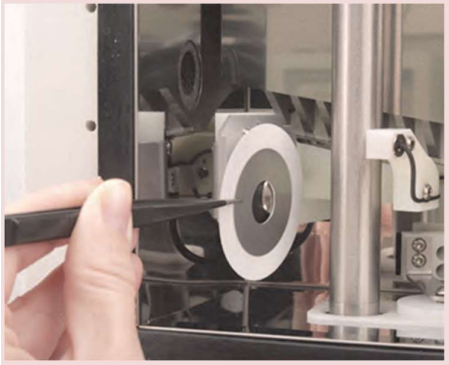
- Mount the filter paper

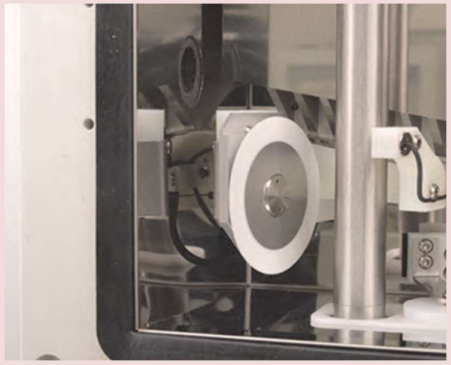
The filter paper starts to wrinkle if it remains longer periods of time in a very humid chamber. Therefore, the mounting is done at the end.

Note: the sensor blotting overcomes most of the wrinking effects.

Experiment: Mounting fresh filter paper

Mount the following stack (in the specific order) on the blotter:
- A Leica contamination ring (in the round black Angstrom box)
- A Whatman #1 filter paper
- A metal ring (also in the Angstrom box)





- If this is not a fresh start, in the main screen, reset the counter[2] 

Get your sample and start plunging!

[2] One filter paper can be used for 10 shots. The counter is automatically reset when the instrument is switched on.

Demonstration: 5 minutes before plunging

WHEN:

5 minutes before cryoplunging

ACTION:

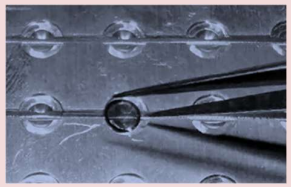
Load the grids onto the dedicated tweezers
Mount the filter paper and prepare the pipettes
Get your sample

Last step before plunging

- Load the grids onto the dedicated tweezers

Pickup a glow discharged TEM grid with the dedicated forceps:

- H shows towards to the front ("home")
- the glow discharged film shows to the left



Prepare all tweezers available (usually 4).

Mounting the dripping tray

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

Experiment: mounting the dripping tray

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

- There are no fixing screws

Connect the demineralized water source

Fresh demineralized water is needed for keeping the humidity in the chamber

Experiment: demineralized water

- Make sure you have topped the provided bottle with fresh demineralized water

- Detach (if needed) and fill the provided plastic syringe with maximum amount of water from the bottle (you need 70 ml to fill the humidifier tray)

- Screw the syringe onto the tube with the blue valve (rotate the syringe, not the tubing)
- Open the blue valve (central arm of the blue part points right or left) and fill the humidifier tray.
- Refill and keep 60 ml of water in the syringe for refilling. Each refilling: 30 ml

- Close the needle valve on the manometer (no not tighten it, just gently close it)

- Close the main valve on the bottle (Zu / ferme / Chiusu)

- Remove the liquefier and place it on the heating plate.

Note: Ethane boils at -88°C and melts at -182.8°C. If you pour liquid nitrogen (e.g. if you have to refill the LN2) into the black ethane container, the ethane will thus freeze. **Do not plunge in ethane ice!** You will break your tweezers and your sample.

Remedy: wait until the GP2 system has warmed up back to -180°C.

Experiment: fill the liquid ethane recipient

- Assure the primary cryogen recipient (black, round, about 1 cm diameter) is installed in the GP2

- Find the liquefier connected to the silicone tubing. It is usually at the back of the GP2 or on the heating plate.

- Place the gas liquefier over the secondary cryogen container.

- Check the temperature on the GP2 display. Wait until the temperature has adjusted (usually to -180°C).

- Open the main valve of the ethane bottle (counterclockwise)

- Adjust the needle valve on the manometer until a white gas flows in to liquefier. (turn towards you will increase the flow, away from you reduce the flow). Be careful with the needle valve!

- After about 10-20 s (depending on the flow), a watery reflection will be visible on the window of the liquefier. This means, the recipient is filled with liquid ethane.

Demonstration: 30 minutes before plunging

WHEN:

30 minutes before cryoplunging

ACTION:

Glow discharge of the grids
Cool down the GP2 system

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

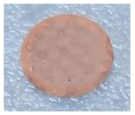
- Glow discharge of the grids

Glow discharge will modify the grid surface, leaving it hydrophilic. This greatly imporves wetting and drop spreading.

The grid block

Find the grid block in the drawer of the cryostation table. **Please bring your own grids.**

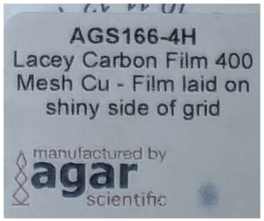
TEM grids have two sides:

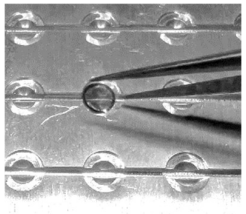


- Shiny side (usually a bit darker)
- Rough side (usually more coppery/bright orange)

Verify on which side is the film

Place the grids with the film up in the grid block (in this case, the shiny side up). When finished, cover the petri dish and move to the glow discharge in the anteroom.





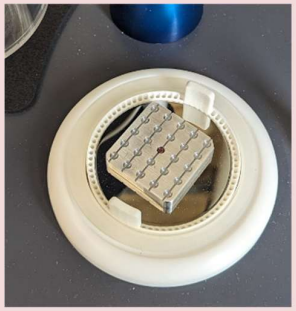
Experiment: Loading samples into the ELMO glow discharge

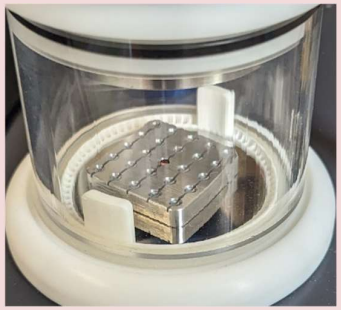
1. Open the right bell (open, then turn)

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 rest completely on the Silicon ring, which assures an air-tight seal





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

Experiment: Step 1. switch on the system

Use the red power switch on the left side of the table.

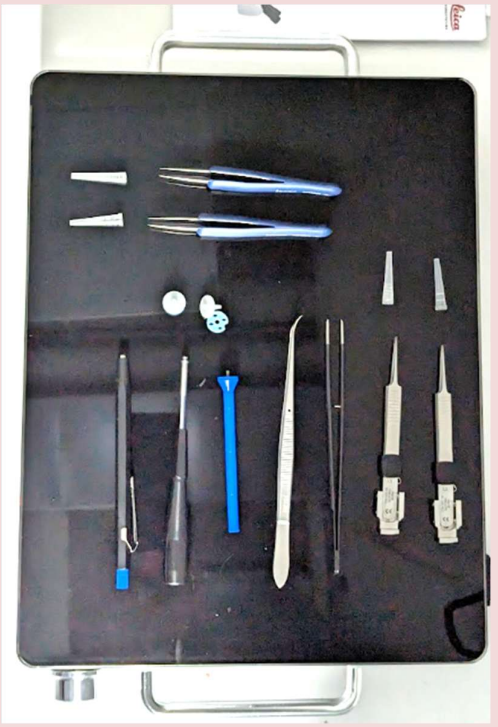
Pressure goal: 2.0 E-1 mbar

Current goal: 2 mA

Time goal: 15 s

Experiment: switch on the heating plate

- On the electrical outlet at the rightmost wall, use the timer to set a 2 hour window



- Fill the secondary cryogen (ethane)

Ethane freezes at -88°C. There is only low Leidenfrost effect, meaning that a drop of liquid ethane on the skin hurts. Much more than a drop of LN2. Be extremely careful with liquid ethane.

<u>Wear safety googles. Also if you are wearing glasses</u>

Demonstration: 10 minutes before plunging

WHEN:

10 minutes before cryoplunging

ACTION:

Drying all tools (Heating plate, shoedryer)
Fill the primary cryogen

It is essential that all tools and recipients are dry

- Drying of the plastic recipients / beakers

Water is your biggest enemy in cryoTEM (and your biggest friend). It is absolutely important that any recipient and object is as dry as possible. Especially the beakers. For this we re-use a home shoe-drying application.

Experiment: assure dry LN2 recipients

A beaker that was filled with LN2 (or still holds LN2) will be prone to ice formation (water from the humidity of the air).

- Place it on the cryotable and put one of the big tubes loosely in it.
Turn on the timer to 15-20 minutes
- Do not use this beaker as long there is water (ice or liquid) in it

- Drying of tools on the heating plate

The heating plate can heat to 100°C and there is no temperature display. It is empirically set to about 50°C. Do not change the temperature settings unless you know what you are doing!

Experiment: Step 2. Setting the dials

- You always use the **right bell.** Therefore the dial selector must point to the right.

- The CURRENT dial points to 3 mA

- The VOLTAGE dial is set to about 60

Experimen: Step 3. Switch on the pump

Flip the pump toggle to I. The pump starts and the pressure begins to drop.

Within 20-30 seconds, pressures below 0.1 mbar (E-1) are reached.

→ If this is not the case then the bell was not properly closed (see page 16).

Caution: the needle valve is extremely delicate and fragile. Proceed with utmost care

Experiment: Step 4. Set a vacuum of 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 further than the slightest of resistance!) until you reach 2.0 E-1 mbar (or there about, +/- 0.5 mbar)

Note: the pump will continue to evacuate the bell and you may need to readjust the vacuum during later steps

Experiment: Step 5. Set the voltage to create a 2 mA current

- Check your pressure. Is it still 2.0 E-1?
- Press the HV button and keep it pressed. A plasma will be instantly created and you can read the current
- Adjust the voltage knob (while holding the HV button pressed) to reach about 2 mA (+/- 0.2 mA).
- Release the HV button

Note: if the CURRENT panel shows SCALE ↓↓ , you have a voltage That is higher than 3 mA. Click the CURRENT dial to 30 mA to get a proper reading and reduce your voltage to reach 2 mA. Note that the voltage is a function of the PRESSURE: the higher the pressure, the more voltage, the more glow discharge.

Note you should recue the pressing of the HV button to a minimum as this will already perform a glow discharge effect.

Experiment: Step 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. E.g. 30 seconds
- Press left or right again to exit the edit mode
- Check vacuum (2.0 E-1)

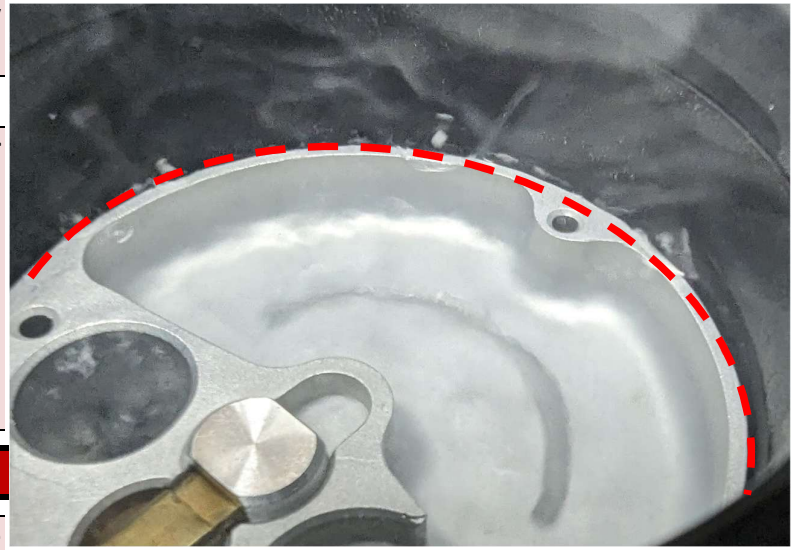
Press OK on the timer to start the glow discharge

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

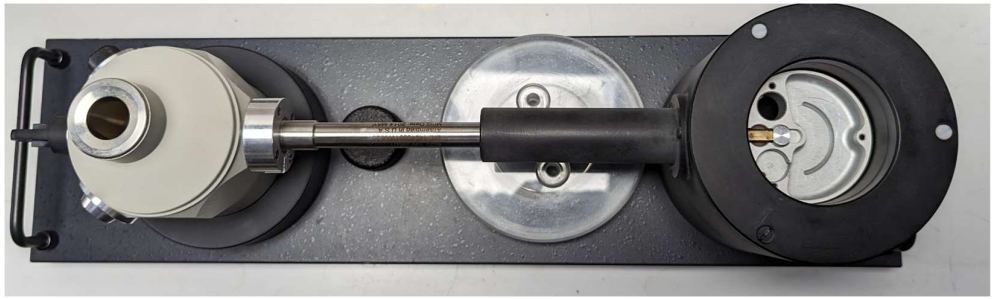
Experiment: Step 7. Switch of the ELMO glow discharge

- Turn off the pump: flip the O/I switch to O. The bell starts to flood with air.
- Open the bell and retrieve the grid block. Close the bell and switch off the entire system.

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



Close with the lid (against ice contamination). Keep an eye on the LN2 level



- Cool down stand and holder

It is important to cool them down at the same time

Experiment: cool down the cryoholder and the stand recipient

1. Pour about 1L of LN2 in a dry plastic beaker

2. Start with the dewar of the holder. Top up the dewar with LN2 (boiling vigorously)

3. Immediately fill the black round recipient of the stand. Do not fill the entire stand: only fill until the rim of the aluminum parts.

Note: you can remove the plexiglass cover, but ideally, you pour through one of the two holes of the cover.

4. Repeat 2-3 until thew LN2 is stable – not boiling vigorously anymore.

- Cool down the GP2 system with the secondary cryogen

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

The positions are counted from the slot notch, clockwise. (the red arrow points to slot 3).

Cryo-gridbox types

Screw caps: do not use

Pin caps: for temporary storage (e.g. max overnight)
They are grabbed, opened and closed with a crayon grabber

Rod closure: best for immediate use

Experiment: prepare for cooldown

Before starting to cool

- Place the cryo transfer container inside the plunging chamber. Remove the lid of the transfer container (the one with the two little holes)

- Place the notch of the cryo-gridbox over the pin in the transfer container

- Mount the warm transfer container to the plunging cylinder using the same pin and notch system.
- Mount the primary cryogen recipient and cover it with the plastic cap.

Cool-Down with the secondary cryogen

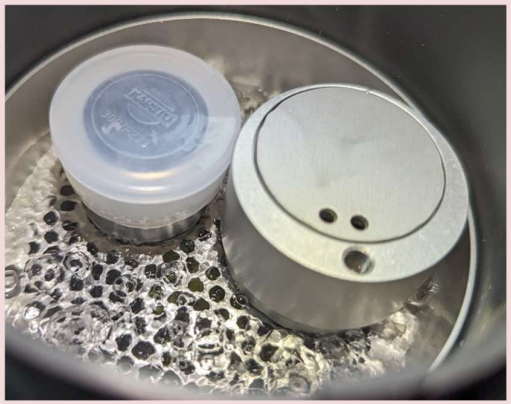
All components in the freezing chamber must be clean and dry

Experiment

The plunging chamber now contains the cryo transfer container (metallic) containing a cryo-gridbox and a black primary cryogen container. Both are covered with their respective lids.

- Fill the 2L beaker with LN2 (you will need about 1.8L)
- Pour until the LN2 starts to bubble through the bottom of the freezing chamber. Then wait a bit.

It can take up to 10 minutes to reach the desired temperature (-180 C)



Demonstration: 20 minutes before plunging

WHEN:

20 minutes before cryoplunging

ACTION:

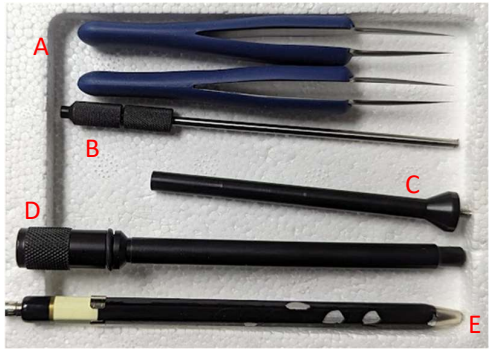
Prepare the Gatan station Retrieve the Gatan holder
Mount it on the Gatan stand. Cool down both

The timing of this step is tricky: doing this too early and you will have to babysit the system and get lots of ice. Do it too late and your system will not be properly cooled.

- Prepare the Gatan station and retrieve the Gatan holder

Assure the stand is ready. Get all the necessary tools at the bionocular of the TEM loading station:

- 2 pair of cryo tweezers (A)
- The Gatan clipring tool (B)
- A cryo-gridbox rod (C)
- The stopper (D)
- If used: a cryo-gridbox pin grabber (E)



Place the Gatan stand under the binocular at the TEM sample loading station

Experiment: retrieve the Gatan Cryoholder

1. Check the tip of the holder while in the pump. Make sure the blanker is closed (grid not visible in the tip)
2. Close the valve at the holder dewar with the black screw. Close V1 (flip to horizonatal).
3. Detach the plastic tubing from the holder dewar inlet and retrieve the holder.

Carefully bring the holder to the Gatan stand and mount it.