Cryo SEM Protocol (LowVac)

Inserting sample

1. Check electronics are not set to standby.
2. Turn on **rotary pump** and **backing valve**.
3. Check the cryo stage is in the microscope.
4. Open nitrogen regulator (located on wall next to plunging station) to 10psi.
5. Open remote dewer regulator to 5psi.
6. Wait at least 10 minutes. Longer is better.
7. Cool the prep chamber. Fill with liquid nitrogen using a funnel ensuring the vent is pointing away from you whilst filling. The temperature will register on the keypad.
8. Fill the remote dewer with liquid nitrogen using a funnel. Increase the nitrogen flow to 7psi to facilitate faster cooling. Once the temperature has registered as -180^{o}C on the keypad the flow can be adjusted until -140^{o}C to -150˚C is reached. The prep chamber and remote dewer can be filled at the same time on the LowVac,
9. Attach the sample to the stub using 50:50 of tissue tek : graphite. Place stub in cryo sledge and tighten screw to secure it.
10. Fill the polystyrene beaker in the plunging station with clean liquid nitrogen and cover with lid.
11. Press **backing valve** off.
12. Press **slush/vent**. Once slush forms press **slush/vent** again to vent. Turn on lamp to help see when slush forms.
13. Attach cryo sledge to vacuum transfer device (VTD).
14. Place the sample quickly into the nitrogen, once the bubbling has stopped attach the VTD onto the cryo station ensuring the rod does not get immersed. Turn on

slush/vent. While slushing hold sample so the end of the sledge is in the nitrogen but the sample is just above it.

15. As the slush forms then withdraw the sample into the VTD.
16. Close the VTD valve. Press **slush/vent**.
17. Attach the VTD to the gate valve on the prep chamber and press **load pump** while holding in place.
18. Wait around 20 seconds. Slightly open the gate valve. Allow the vacuum to recover then fully open the valve.
19. Advance sample into prep chamber, line up between first two dots.
20. If sample requires freeze fracturing Use the scalpel to slice off the desired section of the sample. Place knife back in holder.
21. Set the **heater** temperature. Turn on and wait for it to reach the desired temperature. Set a timer for etching time.
22. Turn the heater off. Wait for the temperature to reach below -100^{o}C before continuing.
23. Line up the stub with the dots at the end of the cold block.
24. Set the timer for sputter coating using the **raise/lower** buttons while holding down **set timer**.
25. Open the argon cylinder by turning the valve on the cylinder 4 full turns counter clockwise.

26. Press **sputter**, if it doesn't sputter press reset. The current should be 10mA- adjust the voltage dial on the electronics box if needed. If the plasma flickers, open the needle valve until stable. The plasma should be a blue, if consistently pink ask a member of bioimaging staff. May be prep chamber has warmed up or the vacuum needs checking.
27. Press **reset** and close the argon cylinder.

28. Check that the SEM stage is in the correct position for the transfer (x=22, y=2, z=48).
29. Once the light on the ball valve turns green, open the ball valve and advance the sledge into the SEM, watching through the open ball valve.
30. When the sledge is in place in the stage, detach the rod by turning it a quarter turn anticlockwise.
31. Withdraw the rod back into the prep chamber and close the ball valve.
32. The sample is now ready for imaging.

Removing sample

1. Check the HT is off. Move the stage position to x=22, y=2, z=48.
2. Open ball valve and attach VTD to sledge turning 90^{o} clockwise watching through open ball valve.
3. Retract the sledge into the VTD. Close ball valve then close gate valve.
4. Press **load pump** twice to vent.
5. Place VTD into cryo station and open VTD valve. Pull it back into the freezing station then turn to prevent it falling.
6. Remove VTD from cryo station and detach sledge from VTD.