

F20 Operating Protocol

1. Fill a dewar with LN2. Start cooling the cold finger. As you are working, the LN2 will evaporate, check this roughly every 1.5 hours and top it off. When working with cryo samples, you may want to do this more frequently.
2. Logout of emuser. The top computer will be in this account for cryo cycling. Log out, but don't close the terminal Serial EM connection.
3. Log in to your own accounts on both computers. Open the software in following order:
 - a. Technai software (peoui.exe; top computer)
 - b. GMS3 (Digital Micrograph; bottom computer). Before doing this, make sure that the camera controller's lights are green for CAM PWR, CNTLR, CAMERA, DATA, TEC. If this isn't the case, contact Amanda or Berith
 - c. SerialEM (if using; bottom computer)
 - d. *If the microscope interphase fails to connect to the microscope check the following on the bottom right bar: Support Network (connected), TEM Server in full operation mode (green icon).*
4. In the microscope interface in Setup -> Vacuum, check the vacuum levels. The values should be around 1, 6, 22, 35, 52.
If the camera vacuum is disabled try restarting the vacuum by manually pressing vacuum off and on the panel.
5. FEG, Power, and High Tension (HT) should be yellow.
 - a. *If Pow/FEG grayed out, contact Amanda or Berith.*
 - b. *If HT is gray, check the vacuum. If that is okay, check HT in software, set to 200 kV, and turn on if necessary by clicking the 'High Tension' button.*
 - c. When ready, click 'Operate'.
 - ii. The extraction should be at 4500, Sometimes it's necessary to click twice on operate to set it back to that value.
6. In Search -> Stage open flap and press *Reset XY*
7. Check CryoBox Position. Make sure the CryoBox is at *IN* position. When it is *IN*, the "aperture" indicator points to position "1", close to horizontal direction. This is absolutely needed for a serious cryo session.
8. Insert a room temperature sample.
 - a. **Make sure the Column Valves are closed before inserting!**
 - b. In Setup -> Vacuum, open the flap and press *Prepump Airlock*. This step is crucial during cryo sample insertions.
 - c. Insert sample:
 - i. Insert holder with pin on the barrel aligned to screw (pos 1: 2 O'clock)
 - ii. Turn CW to Position 2 (4 O'clock) and insert a little further while turning. At this point the airlock will be pumped down. There should be a timer for this step. Remember to choose "single tilt" or "cryo" at the bottom of the screen. Otherwise, the red light on the port will not turn off.
 - iii. Once airlock is done pumping (red light on port turned off), turn holder CCW until pin is aligned with "open" position, insert the holder fully and tap.
 - d. *IF THE VACUUM CRASHES: wait for column vacuum to recover.*
When the options become available:
 - i. *In Setup -> HT click on the HT button (increase to 200KV). It should turn yellow.*

- ii. *In Setup -> FEG click on the Operate button. If the extraction starts falling click and unclick the “Operate” button to reset it to 4500.*
9. Wait for the column vacuum (IGP 1) to reach 15.
10. Open the column valves.
11. Make sure you're doing all your work on the clockwise side of the crossover. Beam intensity in SerialEM has only been calibrated for that side.
12. In Align -> Alignments open the flap and load a settings file, either yours or those of a trusted user. **Make sure not to overwrite someone else's setting file when saving them later.**
13. Align eucentricity at mag 4400x. Important for minimizing specimen shift when tilting, for proper alignments later, and for more accurate magnifications. (If you are doing HRTEM, see the alignment procedure in the separate section below.)
 - a. In Search -> Stage click on *Wobbler*. You will need a sample inserted for this step. Zoom in on a grid square, working at a magnification of at least 4400x, with something on it and, using the microscope touch pad, adjust the Z until the sample is not moving side to side.

Proper Z height should be between -100 and 100 um. If that's not the case, the grid could be bent or torn.
14. Manually focus the sample by eye using the small screen in the viewing chamber and the binoculars, or use the GMS3 software for the initial focusing. Insert the OneView and then click 'View' to obtain an image (you may need to unblank the beam). Then click Process -> Live -> FFT.
15. Close the column valves, then retract the holder far enough so that the sample is out of the beam (you can use a pen to hold the holder in place) and take out the objective aperture. Open column valves, once the column vacuum is again below 15.
16. Check the centering of the condenser aperture. The proper alignment of this aperture ensures that it's centered along the optical axis of the microscope and that changing the strength of condenser lens (intensity) does not change beam position. Changing the intensity, check that the beam stays centered while going through crossover (i.e the beam does not “swing”). If the center of the beam moves when going through crossover, the condenser aperture needs to be centered. Do this (only on one side of the crossover) by using the small knob on the aperture and the small knob on the right side of the column. Continue going back and forth with intensity through cross over while doing this.
17. Perform gun and beam alignments. All of these alignments should be performed at magnification of 10kx. (If you are doing HRTEM, see the alignment procedure in the separate section below.)
 - a. In the Align tab do the Gun Shift and Gun Tilt alignments:

This part of the alignment only needs to be done every few sessions. Once you enter this alignment procedure it resets the settings to a default state. If you decide you don't want to continue doing the alignment you will need to reload your latest settings file again.

 - i. Gun tilt: Important for having maximum beam intensity. Adjusts the gun deflection coils so that the electron beam is emitted parallel to the optical axis. This ensures that all the electrons produced can be used for imaging.

Make sure the beam covers the small screen and minimize exposure time with the multifunction buttons

- ii. Gun shift: Adjusts the gun deflection coils such that the electron beam travels straight down the optical axis through the center of the C1 lens. Center the beam with the multifunction buttons.
- iii. Spot size dependent gun shift: ensures that changing spot size will not cause the beam to shift.

The last step of this alignment centers all spot sizes relative to spot 11. In order to do this correctly, first you need to center spot 11 in relation to the column walls. Do this by finding the middle point between the points where the beam disappears (it hits the column walls). Once you have found that, you can shift the beam to the center of the fluorescent screen with the shift x and shift y buttons. Then proceed to move down in spot sizes and center each spot size on the screen using the multifunction buttons.

You can also correct for condenser astigmatism during this process by toggling back and forth with the R2 button on the touchpad. Fixing it for the spot sizes at which you will image ensures that the beam will be circular under your imaging conditions.

- b. Alternatively, you can do a simpler version of the above alignment in the tune tab.
Go through Gun tilt. Then Gun shift at spot size 3 and beam shift at spot size 6. Cycle between the two until the beam stays centered in both.
 - c. In the Tune tab do the beam tilt. Purpose is to decouple the beam tilt and beam shift operations, meaning that beam tilt will not occur when the beam is shifted and vice versa. Important for high resolution imaging in which a beam tilt would result in a change in rotation center. This is also important when using autofocus. Converge the two bright spots using the multifunction knobs.
 - d. In the Tune tab do Rotation center: (you will need to re-insert the sample for this step): make the expanding circle concentric, i.e. it is uniformly expanding from the center.
 - e. You are now done with aligning the scope. Make sure to save the parameters to your Alignment file. Under the Align tab, select your filename and click the Save button. **Be sure to not overwrite anyone else's file!!!**
18. Next, collect a Gain Reference. In the GMS3 software, go to Help -> User Mode -> Power User. This will enable the Camera menu to appear (needed for Gain Reference collection).
- a. Under Camera Monitor, check that the camera temperature has cooled to -5°C.
 - b. Click on the OneView icon to insert it.
 - c. Click TEM imaging.
 - d. Clicking on view will show a live view.
 - i. Make sure the viewport is covered, otherwise a bright glare will be present in the top left corner of the image.
 - e. Do the Gain Reference (under Camera tab). This should be done every session. Follow the prompts on the screen to adjust the intensity accordingly.
 - i. A Dark Reference should be collected ~1x per month.
 - ii. **Do NOT prepare Linearization Reference!!!**
19. Close the column valves.
20. Reinsert your sample.
21. Now is a good opportunity to check the centering of the objective aperture (at ~71kx) on a non-cryo sample:

- a. Insert the objective aperture
 - b. **Make sure the viewing screen is down to prevent damage to the camera,** then click the 'Diffraction' button on the right-hand microscope panel.
 - c. Center the halo around the bright spot manually using the outer knobs on the objective aperture control. Click 'Diffraction' again when finished.
24. Next check the stigmatism of the objective lens. Use the GMS3 software to obtain a View, then click Process -> Live -> FFT.
- a. If the Thon rings are not visible, you may need to increase magnification (to at least 20kx). It's also possible that you need to bring the sample closer to focus and/or Autofocus using serialEM first.
 - b. If you can see the Thon rings, ensure that they are circular. (You can left click on the rings to obtain an overlay of circles in Serial EM.)
 - i. If they are not circular, click on the 'Tune' tab in the scope software and then click 'Objective' under the Stigmators tab. Adjust the values slightly with the Multifunction X,Y knobs. It's a good idea to copy the values (right click) to another column before adjusting them.
 - ii. Take another record image and obtain another FFT.
 - iii. Repeat steps iv and v until the rings are circular, then click none in the Stigmators tab.
25. Remove the objective aperture. Adjust the magnification down to 120x or 180x and spread the beam all the way to prepare for cryo sample insertion and/or full grid montage collection.
- Now you are ready to image your sample. If you are using SerialEM, please see the relevant SerialEM instructions (separate document).

Quick HRTEM Alignment

1. With the sample in, align the eucentric height at 125kx.
2. Close the column valves and retract the sample holder a little, placing a rod between the stage and holder end.
3. Center the C2 aperture and check astigmatism at a spot size of 3.
4. Perform the direct alignments:
 - a. Gun tilt at 10kx
 - b. Gun shift at spot size 3 and beam shift at spot size 9 and magnification 10kx
 - c. Beam tilt pivot point at 125kx
 - d. Close the column valves and reinsert the sample.
 - e. Rotation center.
 - f. Insert the objective aperture, check centering and use the OneView with continuous live imaging and live FFT to correct objective astigmatism.
 - g. Coma free alignment at 350kx using live imaging.
5. With the objective aperture inserted, find focus using the OneView.
6. Use the OneView to record high res image. Lattice fringes are visible at 380 – 490kx.

Baking out Cryo Holders

The facility has two Gatan Turbo Pumping Stations located in the F20 and F30 rooms for baking out holders, which is required after each use. To perform the bakeout:

1. Remove clip rings and grids from the cryo holders that you used during your session.
2. Insert holder into an empty port on the pumping station.
 - a. Note: Holder DH will only fit into the pumping station in the F30 room. The remainder of the holders can be inserted into any station.