

F30/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. 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 account (for the F20 on both the top and bottom computers). Open software in following order for:
 - a. If using the F20:
 - i. Technai software (peoui.exe; top computer)
 - ii. 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
 - iii. SerialEM (if using; bottom computer)
 - iv. *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).*
 - b. If using the F30:
 - a. peoui.exe (microscope interface; top computer)
 - b. Gatan Filter control (top computer)
 - c. Digital Micrograph (top computer)
 - d. TIA (for communicating the lower computer; top computer)
 - e. SerialEM (lower computer)
 - ii. *If the microscope interphase fails to connect to the microscope check the following on the bottom right bar: Local Area Connection 7 (connected), TEM Server in partial SIM mode (pink 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 and turn on if necessary (go up to 200KV, then in the F30 check FreeHT and increase by 10KV at a time, waiting for emission to stabilize at every step). **THIS IS IMPORTANT IN ORDER TO PREVENT A POTENTIAL ARC AND DAMAGE TO THE TIP***
 - 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, then in the F30 check freeHT and increase by 10KV at a time, waiting for emission to stabilize at every step). THIS IS IMPORTANT IN ORDER TO PREVENT A POTENTIAL ARC AND DAMAGE TO THE TIP. 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. On the F30 you will not be able to see a beam unless the holder is inserted.
 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 4200X. 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.
 - a. On the F30 you can also autofocus in SerialEM with the CCD camera. Do this at a magnification of at least 20kx.
 - i. In Camera -> CCD/TV select CCD. You should hear a noise of it being inserted if it was not selected. If not, you may need to switch back and forth with the Falcon for the system to recognize the switch.
 - ii. If the CCD is not available as an option in the Technai software, check the 'EFTEM' checkbox in the 'Filter Control Panel' in SerialEM to use the CCD.
 - iii. In the Camera menu or SerialEM, click *Setup* and select GIF as the camera.
 - iv. Click *View* to take an image. Make sure that there is an object in your field of view.
 - v. If you are close to focus, use the autofocus feature.

- vi. Your objective lens strength should now be in the range of 88.7-88.9. If it isn't, that means either the focus didn't work properly, or your grid is bent.
 - b. On the F20, you can use the GMS3 software for the initial focusing. Insert the OneView and then click 'View' to obtain an image (you may need to blank 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.
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 cross over (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. If using the F30, perform Falcon protection calibration. (For the F20 skip to step 20). The Falcon detector is more sensitive to electron-beam damage than scintillated CCD and therefore should not be overexposed with an excessive amount of electron dose. A dose protection system has been implemented in the software which protects the Falcon detector from potentially damaging situations. In addition, it protects the camera in unsupported modes.
- a. In Falcon Calibration open flap and choose the file that has OK next to it. If none has an OK it could be due to one of two reasons: The bi-annual falcon calibration has to be done, in which case you should contact Berith. Or the extraction of the microscope isn't set to the extraction for which the bi-annual calibration was done. Check in the FEG panel that the extraction is at that same number, if not, click and unclick *Operate*, to bring it back to the correct extraction (usually 4500).
 - b. In the Falcon Calibration panel press *Beam current* and follow the prompts.
19. Collect a Gain Reference for the Falcon (make sure there is no sample in the beam path)
- a. Go to Low Dose mode on the camera software. Make sure the beam is centered, the intensity looks good, and the magnification for Record is around what you will be using. Update Low Dose settings (Click and Unclick Continuous update), then unclick Low Dose.
 - b. Record an image in Serial EM with the Falcon. Make sure that this looks reasonable (has counts ~3000 for negative stain and 10,000 for cryo)
 - c. In the CCD/TV Camera Open the flap and select *Falcon Reference Image Set* the conditions to acquire 160 frames with 10 images to average and check the 'Remove noise from image' box
 - i. Select *Acquire a single image*
 - ii. If intensity meter at 0% then Unblank (should see following colors on buttons: Insert is Yellow; Blank is Grey; Acquire is White)
 - iii. Adjust the intensity until the Intensity indicator is in the green region
 - iv. Click *Acquire Full Set*
 - v. After it is done select the reference image and click the *Load to TIA buttons*.
 - d. Check that Record in Serial EM is giving a good even image.
20. On the F20, using 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!!!**
21. Close the column valves.
22. Reinsert your sample.
23. Now is a good opportunity to check the centering of the objective aperture (at ~68kx) 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.
- On the F30, take a Record image using the Falcon in low dose mode (see Low Dose Setup in the section below if needed) then under the Process menu, click FFT. On the F20, 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 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 GIF/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 GIF/OneView.
6. Use Falcon/OneView to record high res image. Lattice fringes are visible at 310-470kx on the F30 and at 380-490kx on the F20.

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.
 - b. If baking out holders during your session, the blanking plug can be used instead.
3. Attach the vacuum line and the electrical line to the holder.
4. Start the warm up cycle on the Cold Stage Controller.
 - a. Push the circular button on the left and rotate the dial till "Warm Up" is selected.
 - b. Push the circular button to confirm your choice, then click the button that says 'Yes'.
5. Once the cryo holders have warmed, turn on the power on the pump:
 - a. Note: holder temperature must be above 20°C to be able to start the bakeout process
 - b. F30 room station: a single power on switch is located at the rear right side of the station.
 - c. F20 room station: first, turn on the external diaphragm pump using the green switch on the top left side, then the green power switch on the front right side of the station. **DO not turn on the pumping station without turning on the external diaphragm pump. This will break the turbo pump in the pumping station and is very expensive to repair.**
6. The vacuum reading should quickly decrease to the 10^{-5} Torr range. Once it begins to decrease, open the valve on the cryo holder assembly (where the vacuum tubing is connected).
7. After the vacuum reading begins to decrease again, then the valve(s) on the cryo holder dewar(s).
8. Once the vacuum reading begins, start the zeolite cycle on the Cold Stage Controller and set it to 99 hours.
 - a. Push the circular button on the left and rotate the dial till "Zeolite Cycle" is selected.
 - b. Push the circular button, then click 'Yes', which will bring you to the settings.
 - c. Push the circular button on the number of hours, rotate the dial to read '99', then push the button again to set it.
 - d. Push the button for 'Yes' to start the cycle.
9. Sign the log book.
10. Cryo holders should be allowed to bake out for at least 2 hours, but preferably overnight.
 - a. When turning off current should be below 0.4 A.
11. Once the bakeout is complete, record the current value in the logbook.

12. Close the valve(s) on the holder(s) and the assembly.
13. Stop the zeolite cycle on the Cold Stage Controller.
 - a. Push the circular button.
 - b. It will ask if you want to stop the cycle and click the button for 'Yes'.
 - c. Push any button to return to the main screen.
14. Turn off the vacuum pump.
 - a. On the F20 station, turn off the main power switch on the front, then turn off the diaphragm pump.
15. If you are removing a holder to start cryo work, insert a blanking plug in its place.

Shutdown Procedure

1. Close the column valves and remove your sample.
 - a. Pull back on sample till resistance.
 - b. Turn CW while pulling back. Don't do this too slowly or you may crash the vacuum.
2. Close down software (reverse order from the start up procedure).
 - a. F30:
 - i. Serial EM
 - ii. TIA
 - iii. Digital Micrograph
 - iv. Filter Control
 - v. Technai Software
 - b. F20:
 - i. SerialEM
 - ii. Retract the camera then close the GMS3 software. When closing GMS3, it will ask about the camera temperature. If you are done for the day, warm it to 20°C.
 - iii. Technai software.
3. Log out from both the top and bottom computers on the F20.
4. Initiate the Cryo Cycle:
 - a. Log in to the top computer with 'emuser'.
 - b. Take the LN2 cup off the copper wires and place a pitcher underneath.
 - c. Go to Vacuum tab, click the > arrow, click the Cryo Cycle button. It should start a timer of roughly 3 hours.

If you did not place the copper wires of the sample stage in contact with LN2 you do not need to do a cryo cycle. This will be a rare occasion, but if you are practicing sample insertion or checking the alignment of the scope without doing real imaging work you don't need to cool the sample stage.

Miscellaneous

- On the F20 you need to initially use the black button near the keyboard to manually switch between the two computers so you can log in. Once you've done so, the mouse should move between the two without the use of the button.
- Pixels size for each magnification is provided in the tables below for both scopes. **These will be different for the F20 and F30.**

F20 OneView Pixel Spacings by Magnification

Prepared March 22, 2023

Mag (Screen down)	Mag (Screen up)	Pixel Spacing (Å)
LM 18.5x	LM 21x	5275.66
25x	28x	3956.75
34x	38x	2915.5
50x	56x	1978.37
66x	75x	1477.19
86x	97x	1142.15
105x	120x	925.8
150x	170x	672
195x	220x	502.7
290x	330x	331.5
370x	420x	259
490x	550x	199.3
710x	800x	137.5
970x	1100x	102.9
1350x	1500x	73.78
1850x	2100x	53.14
M 1500x	M 1700x	64.26
2200x	2500x	43.28
3100x	3500x	31.61
SA 4400x	SA 5000x	21.69
5800x	6500x	16.9
6900x	7800x	14.06
8500x	9600x	11.33
10kx	11.5kx	9.605
13kx	14.5kx	7.501
17kx	19kx	5.63
22kx	25kx	4.481
25.5kx	29kx	3.757
44kx	50kx	2.231
55kx	62kx	1.804
71kx	80kx	1.395
89kx	100kx	1.122
135kx	150kx	0.7473
175kx	200kx	0.55929
210kx	240kx	0.466075
250kx	280kx	0.399493
Mh 350kx	Mh 390kx	0.286815
430kx	490kx	0.228282
620kx	700kx	0.159797

F30 Pixel Spacings by Magnification and Camera

Prepared March 23, 2023

Mag (Screen down)	Mag (Screen up)	Pixel Spacing (Å)
		<i>Falcon</i>
M 2050x	M 2300	46.78
2750x	3100	35.11
3500x	3900	28.01
SA 4200x	4700	23.73
8300x	9400	12.04
10.5kx	12kx	9.538
13.5kx	15.5kx	7.277
17.5kx	20kx	5.729
20.5kx	23kx	4.82
27.5kx	31kx	3.621
35kx	39kx	2.873
52kx	59kx	1.89
69kx	78kx	1.461
82kx	93kx	1.21
100kx	115kx	0.9849
135kx	155kx	0.8161
175kx	200kx	0.62683
205kx	230kx	0.527794
Mh 275kx	Mh 310kx	0.385537
350kx	390kx	0.306452
420kx	470kx	0.25429
550kx	620kx	0.192768
710kx	800kx	0.149395

Mag (Screen down)	Mag (Screen up)	Pixel Spacing (Å)
		<i>GIF</i>
LM 51x	62x	1880.5
71x	81x	1534.09
98x	100x	1165.91
120x	125x	929.483
180x	175x	671.811
250x	220x	542.216
310x	280x	412.592
390x	360x	318.079
490x	480x	264.4
590x	650x	186.2
800x	790x	153.6
980x	990x	124.8
1150x	1200x	103.7
1500x	1800x	70.06
1700x	2300x	54.62

1900x	2950x	42.67
M 3000x	M2050x	45.03