

SIMONS ELECTRON
MICROSCOPY CENTER

NEW YORK STRUCTURAL BIOLOGY CENTER 

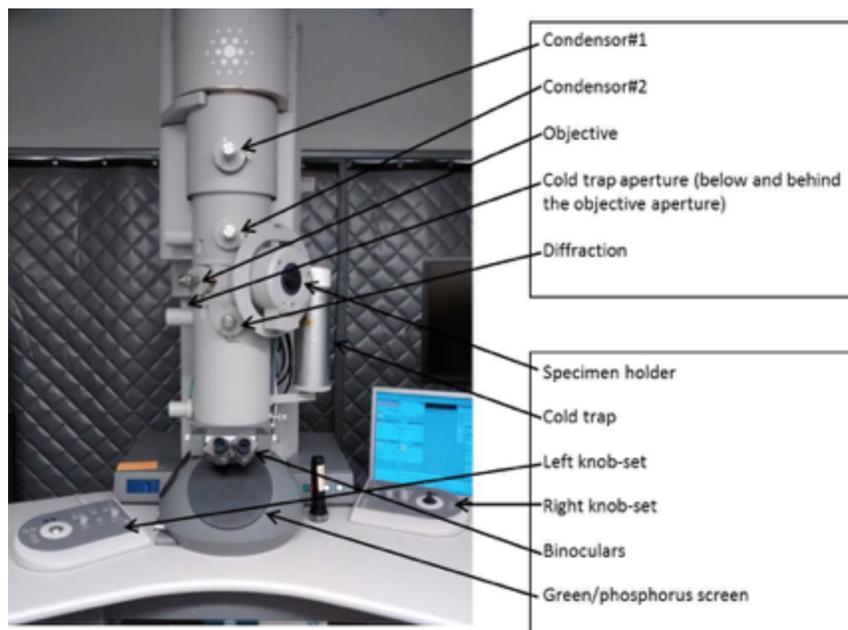
TF20 User Manual

**Simons Electron Microscopy Center
(SEMC) at the New York Structural
Biology Center (NYSBC)**

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F20 Information Sheet

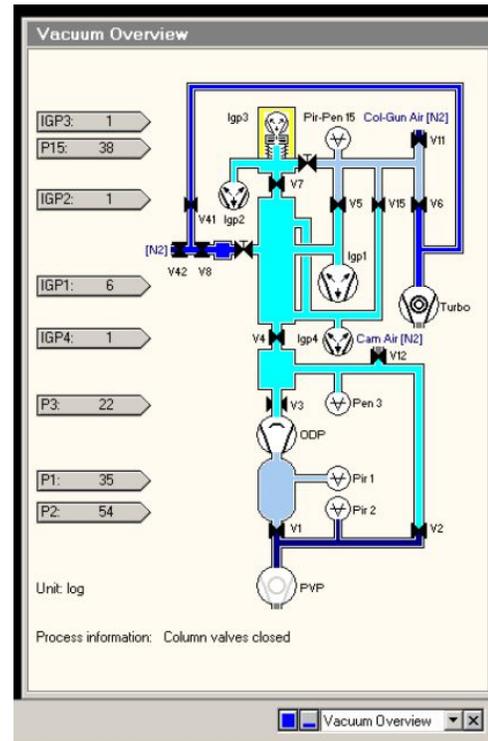


- Wavelengths
 - 80 kV = 0.04176 Å
 - 120 kV = 0.03349 Å
 - 200 kV = 0.02508 Å
 - 300 kV = 0.01969 Å
- Lens & magnification range
 - LM = 21X → 2100X
 - M = 1700X → 3500X
 - SA = 65,000X → 280,000X
 - Mh = 390,000X → 700,000X
- Objective lens Cs = 2.1 mm
- Aperture sizes

<u>Aperture</u>	#1	#2	#3	#4
Condenser	50 um	70 um	100 um	150 um
Objective	40 um	70 um	70 um	100 um
Selected area	40 um	70 um	100 um	200 um

F20 User Guide (starting your session)

1. Make sure microscope is in good condition:
 - a. HT and FEG are on
 - i. Under “Setup” tap, “High Tension” and “Operate” are highlighted yellow
 - b. Column valves are closed
 - i. Under “Setup” tab, “Col. Valves Closed” highlighted yellow
 - c. EM column vacuum is stable (IGP1 = 6)
 - d. Microscope left in SA mag range
 - e. Stage neutral
 - i. Under “Search” tab, click “Holder” button to zero out the X, Y, Z and α coordinates of the stage
 - f. Cold trap already filled with LN2 (can last for 16 hours); refill every 1 - 2 hours
2. Login to leginon workstation with your LDAP account (ex: bcarragher)
3. Transfer cryo holder from the pumping station to transfer station; cool down holder and insert your sample
 - a. See “Dry Pumping Station User Guide (for cryo holders)” for detailed instructions (page 15)
4. Insert cryo holder into EM
 - a. See “Cryo Holder Insertion/Retraction Guide” for detailed instructions
 - b. CHECKLIST:
 - 1) Reset holder
 - 2) Alpha = -60°
 - 3) flu screen of microscope is down
 - 4) objective aperture is out
 - 5) turbo is on and ready (yellow)
 - 6) shield of cryo holder is closed
 - 7) airlock pump time is = 120s
 - 8) pre-pump airlock
5. After insertion, wait for IGP1 to reach <10 before opening the column valves and starting your session
6. Select the camera/acquisition type needed for your session:
 - a. If you are screening multiple cryo grids today, use the F416 camera (“Cryo Screening with TVIPS” guide)
 - i. This camera has a faster readout and yields higher contrast images in a single exposure
 - ii. If your grid looks good, you can switch to the DE20 for data collection
 - b. If you plan to collect data for 2D/3D analysis, use the DE20 camera (“Cryo Data Collection with the DE20” guide)



- i. This camera has a slower readout, but can give you higher resolution details within multiple frames of a movie

F20 User Guide (ending your session)

1. After your screening and/or data collection is done, remove the cryo holder from the microscope
 - a. See “Cryo Holder Insertion/Retraction Guide” for detailed instructions
2. If you used the DE20, make sure the camera is “retracted” and “warmed”
3. Cryo cycle the microscope
 - a. reset = holder
 - b. column valves closed
 - c. flu screen is down
 - d. objective aperture is out
 - e. high mag lenses on (send scope to EN/FA/FC preset; anything in the SA mag range)
 - f. Once turbo is yellow, click “Cryo Cycle” on microscope PC (240 minutes)
4. Remove cold trap from the stand when IGP1 = 99
 - a. You can keep the remaining LN₂ inside the cold trap (off the coils) for the next day’s user
5. Bake out the cryo holder
 - a. See “Dry Pumping Station User Guide (for cryo holders)” for detailed instructions
6. Log off leginon workstation
7. Close Leginon client on both camera and microscope
8. Cleanup the bench space for the next user
9. Log any issues into the EM notebook or notify SEMC staff directly

Cryo Screening with Tietz TVIPS F416

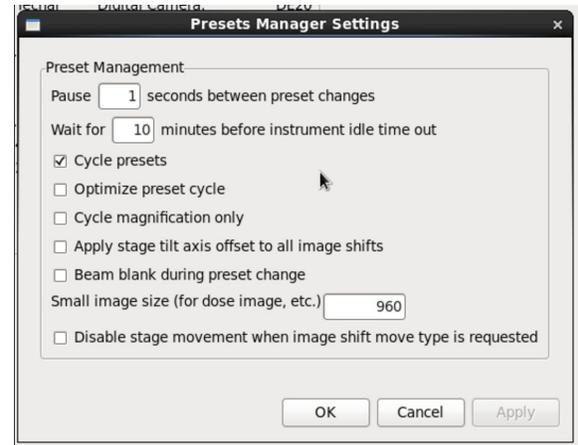
Introduction:

The TVIPS is a CMOS (complementary metal-oxide-semiconductor) 16 megapixel camera with a 50 frame/sec readout (4k x 4k). It sits below the column of the microscope, below the DE20 camera (thus, the DE20 must be retracted to be able to use the TVIPS). It is a robust camera that has good SNR (good contrast) that is used primarily for screening grids. It is housed in a vacuum housing separate from the EM vacuum and is not retractable and always cooled. Dark and gain references are taken infrequently (once per month). It is controlled using “EM-Menu” software, which is found on the “camera” monitor in monitor input “DVI-2”. Suggested mags for EN/FA/FC = 62,000x; HL = 5000x; SQ = 1700x.

MCL server (on microscope computer) must be on in order to turn on EMMENU4 (software for F416) (on camera computer). If F416 computer restarted, login as supervisor.

1. After step 6 of “F20 User Guide”....
2. Open Leginon client on camera & microscope computer
 - a. TVIPS camera control on monitor input DVI-2
3. Open Leginon program on leginon PC
 - a. In terminal, type betaleginon
4. Setup Leginon session settings:
 - a. Select “create new session” → “next”
 - b. Select holder (Elsa or TF20-cryo-holder_AMI-2)
 - c. In session description, type information about sample → “next”
 - i. Make sure the start session name with “F20- ”
 - d. Select project → “next”
 - e. Image directory (use default... “/gpfs/leginon/username”)
 - f. Connect to clients= tf20-f416 & tf20-d394 → “next”
 - g. C2 size = 70 um → “finish”
5. Once Leginon has started up, open the “application”
 - a. Application → Run → MSI-T2 (3.3)
 - i. Scope = tf20-d394
 - ii. Camera = tf20-f416
 - iii. Main = tf20leginon
 - b. “Run”
6. Upload your presets
 - a. Preset manager → “import presets from another session” (* icon)
 - i. TEM = tecnai; Digital Camera = Tietz F416 (if you do not see a camera available, restart both clients on EM and camera computers)
 - b. “Find” presets from past 20 days; select desired preset (usually from the most recent user)
 - c. Highlight all the presets (GR, SQ, HL, FA, FC, EN) and “import” → “done”
 - i. Suggested mags for EN/FA/FC = 62,000x; HL = 5000x; SQ = 1700x
 - d. Close window
7. “Cycle” presets to minimize hysteresis in the beam

- a. Under “preset manager” node, select “settings” button and check the “cycle presets” option (see image)
 - b. Send scope to EN preset 10X
8. Wait until IGP1 = 6
 - a. Open shield of cryo holder when IGP1 <15; you may see a slight increase in pressure, but should settle within a few seconds
9. Center preset beams
 - a. Move to square of non-interest (square with carbon that you don’t mind trashing)
 - b. Center “square” preset beam
 - i. Send scope to “square” preset
 - ii. Lower green screen
 - iii. Center beam using left trackball
 - iv. Open the “Edit” box and click “apply beam shift from scope”
 - c. Center “hole” preset beam
 - i. Send scope to “hole” preset
 - ii. Lower green screen
 - iii. Center beam using left trackball
 - iv. Open the “Edit” box and click “apply beam shift from scope”
 - d. Center and adjust intensity of FA/FC/EN preset beam
 - i. Send scope to “EN” preset
 - ii. Lower green screen
 - iii. For the EN preset, adjust beam intensity to ~0.5 sec (little screen on flu screen), with a beam diameter that is about 1.5X the size of the second circle
 1. For the FA/FC preset you can make this beam a bit brighter and smaller
 - iv. Open the “Edit” box and apply intensity “from scope” and beam shift “from scope”
 - v. Repeat for FA and FC preset
10. Get eucentric height of your grid
 - a. On a square of non-interest...
 - b. “Z_focus” node→ simulate target (+ icon)
 - c. Recommended- record the eucentric height value; it is useful to have an idea of what the eucentric height of your grid is, especially as you navigate around during data collection; (the eucentric height should stay relatively similar, +/- 20 um; this means your grid is fairly flat; if your eucentric height varies much more than that, your grid is probably bent/wrinkled)
11. Collect atlas of your grid (this is a series of low mag images that are stitched together to give you a whole view of your grid; ~10 minutes)
 - a. Under the “Grid_targeting” node, click “settings” button (upper left hand side) and input your grid description
 - i. Radius = 0.0009m = 0.9mm



- b. Click “calculate atlas”
 - c. Click “play” to collect atlas
12. Insert and center objective aperture
- a. Go to square of non-interest
 - b. Send scope to EN mag
 - c. Lower green screen and send scope to “DIFFRACTION” mode (right knobset)
 - i. Should see bright green small beam on the green screen with diffuse carbon diffraction ring around it
 - ii. $D = 460 - 680 \text{ mm}$
 - d. Insert objective aperture and center
 - e. Exit “DIFFRACTION” mode
13. Set up Ice Thickness
- a. Go to vacuum area
 - b. Send scope to EN preset (make sure beam is centered)
 - c. Simulate target in exposure node
 - d. Record the mean value and input into Ice_Thickness node (under ALS)
 - e. ALS coefficient = 319 with 100 um objective aperture in
 - f. ALS coefficient = 247 with 70 um objective aperture in
14. Get the dosage (e^-/A^2) of the EN preset (target = 30 - 60 e^-/A^2)
15. You are now ready to collect some images!
16. In “Square_Targeting” select 1 square of interest
- a. Using “acquisition” mouse, select the center of square of interest
 - b. Click “Submit target”
 - c. Click “Submit queued targets”
17. In “Hole_Targeting” select 1 - 2 regions of holes of interest
- a. Using “acquisition” mouse, select holes of interests (on the carbon, every 2x2 holes away from each other)
 - b. Using “focus” mouse, select area of carbon towards the center of the hole areas you selected in the step above (this is where leginon will acquire the eucentric height of the square)
 - c. Click “Submit target”
 - d. Click “Submit queued targets” when you are done with every hole target
18. In “Exposure_Targeting” select exposure targets
- a. Using “acquisition” mouse, select the center of the holes of interests, on the ice. Select up to 4 holes that are closest to the center of the screen (use “toggle center crosshair” tool to find where center is). This ensures that you do not collect images with a lot of image shift.
 - b. Using “focus” mouse, select area of carbon towards the center of the stage (use “toggle center crosshair” tool to find where center is). This is where leginon will acquire auto-focusing for the exposure targets selected in the step above.
 - c. Click “Submit target”
 - d. Click “Submit queued targets”

- i. Note- at this point you have queued up a number of exposure targets for leginon to acquire. Refer to “Leginon/Appion User Guide” to view micrographs and for further instructions.
19. Under “exposure” node, select “toggle queue timeout” so that column valves will close once exposure queue is done collecting
20. To collect a new square, repeat steps 14 - 16 until you are done collecting data.
21. If you are done for the day... read “**F20 User Guide (ending your session)**”

Cryo Data Collection with the DE20

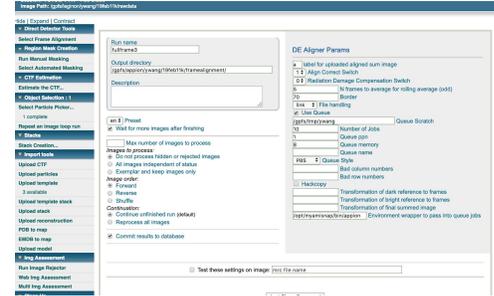
Introduction:

The DE-20 camera system is a direct detection device (DDD) manufactured by Direct Electron. It has high signal-to-noise ratio (SNR) with consistent performance across all spatial frequencies and TEM magnifications. “Movie mode” proves high-speed acquisition of a continuous stream of frames (32 frames per sec max, unbinned full-frame) with nearly 100% duty cycle (no dead time between frames). Increased data quality compared to TVIPS thru drift correction, per-particle motion correction, radiation damage compensation, post-acquisition exposure setting, etc. It has a 5k x 4k field of view (5120 x 3840) with a 6.4um pixel pitch. It is fully retractable and needs to be manually retracted and warmed when not in use, and inserted and cooled when in use. The temperature changes make it so that the camera needs a new dark and gain reference with each new session. The DE20 is sensitive to stray light (operate with window covered and lights off).

Dose should be 2.1 - 2.2 electrons/pixel/frame (no more than 2.5 otherwise clipping will occur). At 29,000X this corresponds to ~ 45 electrons/ \AA^2 for a 2 second exposure (1.54 \AA /pixel).

1. After step 6 of “F20 User Guide”....
2. Open DE20 software to insert and cool down camera (NOTE- can only open MicroManager or Leginon client/server at one time); DE-20 camera control on monitor input DVI-1
 - a. Close Leginon client on “camera” computer
 - b. Open DE server GUI on “camera” computer (this program is laggy; wait 30 sec)
 - c. Open MicroManager on “camera” computer
 - d. Using pull-down flaps, “cool down” camera and “extend” camera (when not in use, DE20 should be retracted and warmed); it takes ~ 5 min to cool down camera
 - e. Make sure that you “do not autosave”
 - f. Close MicroManager (DE server GUI should be left ON); make sure that autosave directory = D://rawframes/DE20
3. Open Leginon client on camera computer
4. Open Leginon client on microscope computer
5. Open Leginon program on main computer (TF20Leginon)
 - a. In terminal, type betaleginon
6. Setup Leginon session settings:
 - a. Select “create new session” \rightarrow “next”
 - b. Select holder (TF20-cryo-holder_AMI-2)
 - c. In session description, type information about sample \rightarrow “next”
 - d. Select project \rightarrow “next”
 - e. Image directory (use default... “/gpfs/legion/username”)
 - f. Connect to clients= tf20-f416 & tf20legion \rightarrow “next”
 - g. C2 size = 70 um \rightarrow “finish”
7. Once Leginon has started up, open the “application”
 - a. Run \rightarrow Application \rightarrow MSI-T2 (3.3)

- i. Scope = tf20-d394
 - ii. Camera = tf20-de20
 - iii. Main = TF20Leginon → “run”
8. Upload your presets
- a. Preset manager → “import presets from another session” (* icon)
 - i. TEM = tecnai; Digital Camera = DE-20
 - b. “Find” presets from past 20 days; select desired preset (usually from the most recent user)
 - c. Highlight all the presets (GR, SQ, HL, FA, FC, EN) and “import” → “done”
 - d. Close window
9. “Cycle” presets to minimize hysteresis in the beam
- a. Under “preset manager” node, select “settings” button and check the “cycle presets” AND “optimize cycling” option
10. Wait until IGP1 = 6
- a. Open shield of cryo holder when IGP1 <15; you may see a slight increase in pressure, but should settle within a few seconds
11. Center preset beams
- a. Send stage to square of non-interest (square with carbon that you don’t mind trashing)
 - i. Under “square_targeting”, select “acquisition” mouse and select square of non-interest; submit target (click green “play” button)
 - b. Once the stage stops moving, check that beam is centered at all presets
 - i. Center “square” preset beam
 1. Send scope to “square” preset
 2. Lower green screen
 3. Center beam using left trackball
 4. Open the “Edit” box and click “apply beam shift from scope”
 - ii. Center “hole” preset beam
 1. Send scope to “hole” preset
 2. Lower green screen
 3. Center beam using left trackball
 4. Open the “Edit” box and click “apply beam shift from scope”
 - iii. Center and adjust intensity of FA/FC/EN preset beam
 1. Send scope to “EN” preset
 2. Lower green screen
 3. For the EN preset, adjust beam intensity to ~0.35 sec (little screen on flu screen), with a beam diameter that is about 1.5X the size of the second circle
 - a. 2.8 electrons/pix/frame, 58 electrons/A², 200 ms @ 29K mag
 - b. 680 ms @ 50K mag = 64 electrons/A²
 - c. For the FA/FC preset you can make this beam a bit brighter and smaller

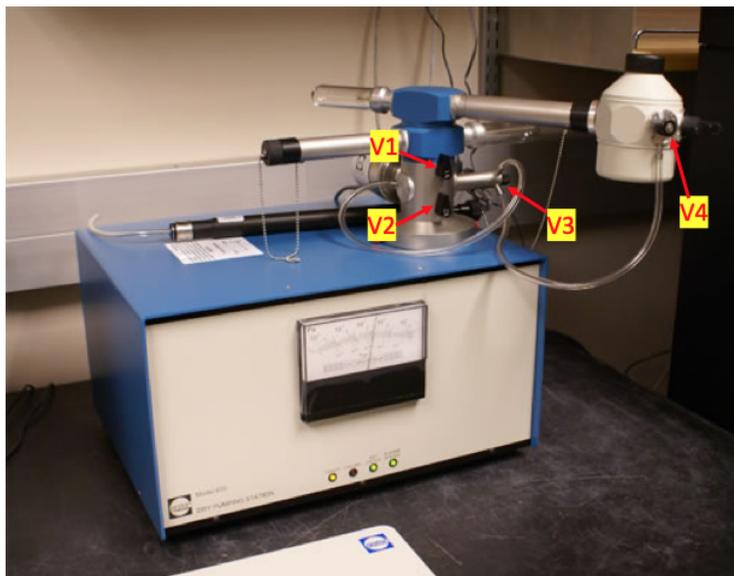


4. Open the “Edit” box and apply intensity “from scope” and beam shift “from scope”
 5. Repeat for FA and FC preset
12. Get eucentric height of your grid
- a. Send scope to sq preset
 - b. Open column valves + lower green screen (R1 on knobset)
 - c. Center beam using Left Knobset trackball
 - d. Using Right Knobset trackball, move stage until you see a grid square with unique fiducial; center that square on the green screen
 - e. “Z_focus” node → simulate target (+ icon)
 - f. Recommended- record the eucentric height value; it is useful to have an idea of what the eucentric height of your grid is, especially as you navigate around during data collection; (the eucentric height should stay relatively similar, +/- 50 um; this means your grid is fairly flat; if your eucentric height varies much more than that, your grid is probably bent/wrinkled)
13. Collect atlas of your grid (this is a series of low mag images that are stitched together to give you a whole view of your grid; ~10 minutes)
- a. Under the “Grid_targeting” node, click “settings” button (upper left hand side) and input your grid description
 - i. Radius = 0.0009m = 0.9mm
 - b. Click “calculate atlas”
 - c. Click “play” to collect atlas
14. Insert and center objective aperture
- a. In “square targeting” node and using green “acquisition” mouse, go to a junk square
 - b. Once at the junk square location, send scope to EN mag
 - c. Lower green screen and send scope to “DIFFRACTION” mode (right knobset)
 - i. Should see bright green small beam on the green screen with diffuse carbon diffraction ring around it
 - d. Insert objective aperture and center
 - e. Exit “DIFFRACTION” mode
 - f. If you plan to take a break, send microscope to high mag (FA/FC/EN) to turn on the high mag lenses and close the column valves
15. Obtain dark and gain reference (needs to be done every time since the camera is warmed up after every use; use same settings as EN preset)
- a. For dark - close column valves → “correction” node → “settings” → tecnai & de-20 → 5120 x 3840 bin1, exposure = 1000ms, frame = 40ms, do not save frames, image to combine = 10, average → “dark” and “both channels” → acquire (you should see black image)
 - b. For bright - open column valves → find empty square → send scope to EN preset → adjust brightness to 0.5sec on small screen → “correction” node → “settings” → tecnai & de-20 → 5120 x 3840 bin1, exposure = 2000ms, frame = 40ms, image to combine = 10, average → “bright” and “both channels” → acquire (you should see image of the camera sensor)

- c. Confirm that correction is done → “correction” and “both channels” → acquire; you should see a blank image
16. Set up Ice Thickness
 - a. Go to vacuum area
 - b. Send scope to EN preset (make sure beam is centered)
 - c. Simulate target in exposure node
 - d. Record the mean value and input into Ice_Thickness node (under ALS)
 - e. ALS coefficient = 319 with 100 um objective aperture in
 - f. ALS coefficient = 247 with 70 um objective aperture in
17. Get the dosage (e^-/A^2) of the EN preset (target = 30 - 60 e^-/A^2)
18. You are now ready to collect some images!
19. In “Square_Targeting” select 1 square of interest
 - a. Using “acquisition” mouse, select the center of square of interest
 - b. Click “Submit target”
 - c. Click “Submit queued targets”
20. In “Hole_Targeting” select 1 - 2 regions of holes of interest
 - a. Using “acquisition” mouse, select holes of interests (on the carbon, every 2x2 holes away from each other)
 - b. Using “focus” mouse, select area of carbon towards the center of the hole areas you selected in the step above (this is where leginon will acquire the eucentric height of the square)
 - c. Click “Submit target”
 - d. Click “Submit queued targets” when you are done with every hole target
21. In “Exposure_Targeting” select exposure targets
 - a. Using “acquisition” mouse, select the center of the holes of interests, on the ice. Select up to 4 holes that are closest to the center of the screen (use “toggle center crosshair” tool to find where center is). This ensures that you do not collect images with a lot of image shift.
 - b. Using “focus” mouse, select area of carbon towards the center of the stage (use “toggle center crosshair” tool to find where center is). This is where leginon will acquire auto-focusing for the exposure targets selected in the step above.
 - c. Click “Submit target”
 - d. Click “Submit queued targets”
 - i. Note- at this point you have queued up a number of exposure targets for leginon to acquire. Refer to “Leginon/Appion User Guide” to view micrographs and for further instructions.
22. Under “exposure” node, select “toggle queue timeout” so that column valves will close once exposure queue is done collecting
23. To collect a new square, repeat steps 16 - 18 until you are done collecting data.
24. Start frame alignments
 - a. In emgweb, select the processing link (upper left side)
 - b. Login with LDAP info (upper right side)

- c. In “Direct Detector Tools” → “Select Frame Alignment” → “Full Frame alignment for Direct Electron Co. Camera”
 - d. Input the following parameters. Everything default except...
 - i. Turn off dose-weighting (radiation damage compensation switch = 0)
 - ii. 5 N frames to average for rolling average (odd)
 - iii. Link file handling
 - iv. /gpfs/tmp/username queue scratch
 - v. 10 number of jobs
 - vi. 1 queue ppn
 - vii. 8 queue memory
 - e. Select “Just Show Command”
 - f. Copy the command line
 - g. Open new terminal and type “screen”
 - h. In new screen, type “ssh de20framealignment” or “ssh zeus”
 - i. Paste the command line from step e
25. Start CTF correction (after first movie is done aligning)
- a. CTF → estimate the CTF → CTFFIND v4
 - b. Default all settings except preset = en-a
 - c. Run on semc-head (or copy/paste into emgbox05)
26. If you are done for the day...refer to “**F20 User Guide (ending your session)**”

Dry Pumping Station User Guide (for cryo holders)



- Normal state (while pumping):
 - V1 = closed
 - V2 = open
 - V3 = open
 - V4 = open
- V1 & V2 open vs closed
 - Open = vertical
 - Closed = horizontal
- Image on left shows V1 & V2 as OPEN
- Do not overtighten the valves!
- Finger-tight only!

Removing holder from dry pumping station:

1. Vacuum should be reading 10^{-4} to 10^{-5} Pa.
2. Unplug cryo holder jacket from temperature controller by pulling straight out (do not twist!)
3. Close V4 (horizontal) on cryo holder, then close V3, and V2 on the pumping station.
 - a. V1 should be closed (horizontal), but if not, then go ahead and close V1 last
4. Unhook tygon tubing from cryo holder and re-connect to silver stopping plug (behind V1 & V2).
5. Turn pumping station off.
 - a. Wait for green “MDP ready” light to go out.
6. Using one hand to stabilize the V1/V2 module, gently apply force to pull out cryo holder.
 - a. Immediately upon removing cryo holder, store in cryo transfer station.
7. Place black blanking plug into opening where cryo holder was.

Inserting holder into dry pumping station (warm up):

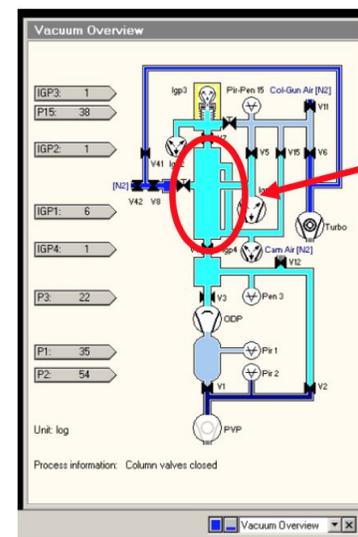
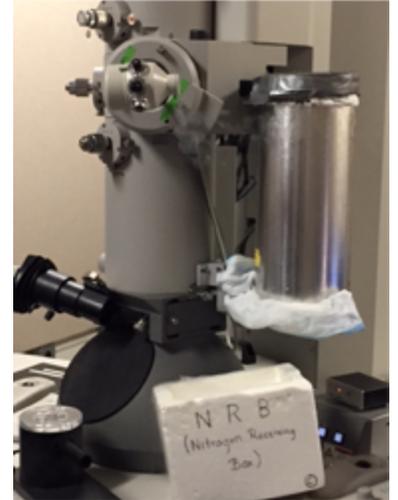
1. Make sure all valves on pumping station are closed.
2. Turn on pumping station.
 - a. Wait for green “MDP ready” light to turn on (~1 minute).
3. Remove blanking plug from pumping station
4. Insert cold cryo holder. Invert holder to facilitate condensation removal.
5. Connect the plastic tygon tubing to V4 on the cryo holder.
6. Open V2, then V1 (vertical) and wait until vacuum stabilizes.
7. Connect cold cryo holder to the temperature controller and run the “warm-up cycle”.
 - a. This takes ~20 minutes
8. Once warm up is done, close V1 (horizontal) and open V3. Wait for vacuum to stabilize.
9. Open V4 on cryo holder.
10. Start the “zeolite cycle” for 2 hours.

Cryo Holder Insertion/Retraction Guide for F20

*****Cryo holder insertions on the F20 may only be performed by SEMC staff. NO EXCEPTIONS. *****
Refill holder dewar every 2-3 hours.

Cryo Holder insertion:

1. Before insertion confirm that IGP1 = 6, column valves closed, holder is reset, objective aperture out, flu screen down, airlock default pump time = 120 sec, turbo on and ready (button will be orange while warming up and yellow when ready).
2. Set holder alpha value at -60 degrees. Goniometer will rotate.
3. "Pre-pump airlock." Takes 4 seconds.
4. Position styrofoam box below goniometer to catch LN₂.
5. Remove holder from the transfer station and rotate the holder so that the "hat" of the holder is at the 3 o'clock position. Immediately insert holder going straight into the goniometer with holder at 3 o'clock position until holder can go no further in.
 - a. You should see the airlock pump time initiate in the Vacuum Overview window
6. Select 'ST Cryo Holder' as specimen holder
7. Wait for 2 minute airlock pump time to count down.
8. After airlock pump time complete, "reset" the alpha value WITH HANDS ON THE DEWAR OF THE HOLDER so that the holder remains stationary
 - a. The stage should rotate back to 0 degrees while the holder top is still at 3 o'clock position.
9. Slowly turn holder counter-clockwise to from the 3 o'clock to the 12 o'clock position while watching IGP1
 - a. If you see a spike in IGP1, stop rotation and wait until the IGP1 value recovers (at least 2 values down OR until the cartoon diagram in vacuum overview is in the cyan region; see image)
 - b. Your goal should be to insert the holder from the 3 to 12 o'clock position within **~1 MINUTE**. The longer the insertion takes, the greater chance you have of crashing the vacuum.
10. Once at the 12 o'clock position, gently apply resistance on the holder (away from the column) and slowly let the vacuum of the scope pull the holder in.
 - a. Give the dewar a slight rotational jiggle to ensure the holder is inserted all the way into the column.
11. Top off cryo holder dewar with LN₂ once inserted.
12. Ensure that "High Tension" and "Operate" are still yellow (did you crash the vacuum? Will be grey if vacuum crashed.)



13. Turn turbo pump off.
14. Do not open the column valves until IGP1/Column < 10. If you are ever in doubt, close the column valves and contact an EMG staff member immediately.

Cryo Holder retraction:

1. Close column valves, reset holder, objective out, flu screen down, cryo shield of holder closed.
2. Gently brace against the purple plate of the goniometer and pull holder back until it stops, then turn holder clockwise so that the “hat” rotates from the 12 o’clock to the 5 o’clock position
3. Pull holder straight out of the column.
4. Transfer holder immediately to the transfer station.
5. If vacuum spikes and column pressure crashes, contact an EMG staff member immediately.
6. Turn turbo on to prepare for next insertion.

F20 Alignments

F20 alignments should be done by EMG staff ONLY!!

Alignments can be done with room temperature (RT) holder or cryo holder, but ideally with a grid with carbon support.

*Note- if you're confident that the alignments on the FEG are good, skip gun alignments (steps 1 & 2)

1. Gun tilt ("Tune" → "Gun" → "Gun Tilt")
 - a. Holder should be out of the path of the beam and objective aperture out
 - b. Focus beam (with intensity knob)
 - c. Center beam (beam shift trackball on left knobset)
 - d. Open beam (intensity knob) CW past crossover (past second circle) and use MF X and Y to get the brightest beam (lowest exposure time)
 - i. Stay away from edge of FEG
 - e. Condense and center the beam (trackball)
 - f. Open beam up to the RIGHT so that it's just past second circle
 - g. Center condenser 2 aperture (image →)
 - h. Click next, repeat steps b-e
2. Gun Shift ("Tune" → "Gun" → "Gun Shift")
 - a. Condense beam, then center beam using MF X and Y
 - b. Repeat for next step
3. Spot size dependent gun shift ("Tune" → "Gun" → "Spot size-dependent Gun Shift")
 - a. Similar to step II, focus beam and center using MF X and Y for all spot sizes
4. Condenser Astigmatism ("Tune" tab → "Stigmator" inset → "Condenser")
 - a. High mag, ~100kX
 - b. Use MF X and Y to make sure beam opens and closes circularly and concentrically over both sides of crossover
 - c. Click "None" when done
5. Eucentric height and focus
 - a. Holder should now be placed back into the path of the beam.
 - b. In legion, "Z-focus" node → simulate target (or do this manually by following steps b - e below)
 - c. Find a piece of dirt that is large (easily visible at low mag, ~5k)
 - d. "Stage/beam" tab → "Stage2" flapout → "Wobbler". Use "Z axis" +/- (right knob-set) to adjust stage z-height until feature on screen stops moving (about -180 um for RT holder; -30 um for cryo holder).
 - e. Rough focus by pressing "Eucentric focus" on right knob-set



This is a side view of the objective aperture, but adjustments to condensor aperture 2 is the same

- f. Focus beam using “Focus” knob on right knob-set and camera/FFT. Reset defocus to 0 (press R3 on right knobset)
6. Beam tilt pivot point X and Y (“Tune” → “Direct Alignments” → “Beam tilt pivot point”)
 - a. At eucentric height and eucentric focus.
 - b. Green screen down. Focus beam to the size of a pencil eraser.
 - c. Click “Beam tilt pivot point x”
 - d. Use MF X and Y to minimize beam shift
 - e. Repeat for pivot point Y
7. Coma free pivot point X & Y (“Coma free pivot point X”)
 - a. Repeat similarly to step 6
8. Coma Free Alignment X and Y (“Tune” → “Direct Alignments” → “Coma free alignment X”)
 - a. Using Live FFT, set the beam to be a little under focused (-250 - 500nm)
 - b. Set the camera so that you can get a quick readout from the camera during the coma free alignment (set exposure to ~0.5s and condense beam to just inside the inner circle)
 - c. Use MF X and Y so that rings to stay the same size (It’s OK if it looks like a “rotation”, but need to correct if the rings are changing in diameter)
9. Objective Aperture Centering (~25 – 50K)
 - a. Focus and center beam to a point
 - b. Click “Diffraction” on right knob-set
 - i. $D = 460 \text{ mm}$
 - c. Insert objective aperture (silver tab pointed left) and center aperture
10. Objective Astigmatism (~25 – 50k)
 - a. At slightly underfocus (2-3 thousand rings) use Live FFT and get rings to be a circle (elliptical = objective astigmatism).
 - b. Use MF X and Y to correct the astigmatism.
11. Preset alignments
 - a. Find feature that is visible at medium mag (using “navigation” tab, send microscope to “hole” and take picture; using “stage position”, “crosshair tool” and the “clicking tool” center a distinguishable edge of a feature in the center of the stage)
 - b. Send scope to FA mag and find that feature with the flu screen
 - c. Take a picture under FA; make sure the feature is centered
 - d. Send scope to “HL” preset and take a picture; using “image shift”, center the feature using the click tool
 - e. Go to “preset manager” and apply the image shift to the HL preset “from scope”
 - f. Under “navigation” node, send scope to “SQ” preset and take a picture; using “image shift”, center the feature using the click tool
 - g. Go to “preset manager” and apply the image shift to the SQ preset “from scope”
 - h. Under “navigation” node, send scope to “GR” preset and take a picture; using “image shift”, center the feature using the click tool (you may need to take out the objective aperture) (from SQ to GR, there is a flip/translation in the image)
 - i. Go to “preset manager” and apply the image shift to the GR preset “from scope”
 - j. You are done! Make sure to reset back to “stage position” for movements

Troubleshooting

HT should be brought up by EMG staff ONLY!!

1. If the column vacuum crashes and HT and FEG turn off...
 - a. Notify EM staff
 - b. Wait for TMP rollout to recover (~7 minutes)
 - c. Wait for column pressure to recover (IGP < 30) (30 - 60 minutes)
 - d. Manually set HT to 80 kV
 - e. Click “HT” to turn on
 - i. Wait till measured HT is 80 kV (~ 2 minutes)
 - f. Click “Operate” to turn FEG on (~ 2 minutes)
 - i. Emission should be < 10 uA; after any increase in HT wait a few minutes for emission to recover and stabilize
 - g. 80 → 200 kV, 10 kV steps
 - i. Free high tension = true
 - ii. 10,000 eV steps
 - iii. Select >
 - iv. At 200 kV, should read ~ 6 uA emission (under HT), FEG = 44 uA
 - h. Make sure FEG is operating at 4050 V (may need to select the ↵ button to refresh it)