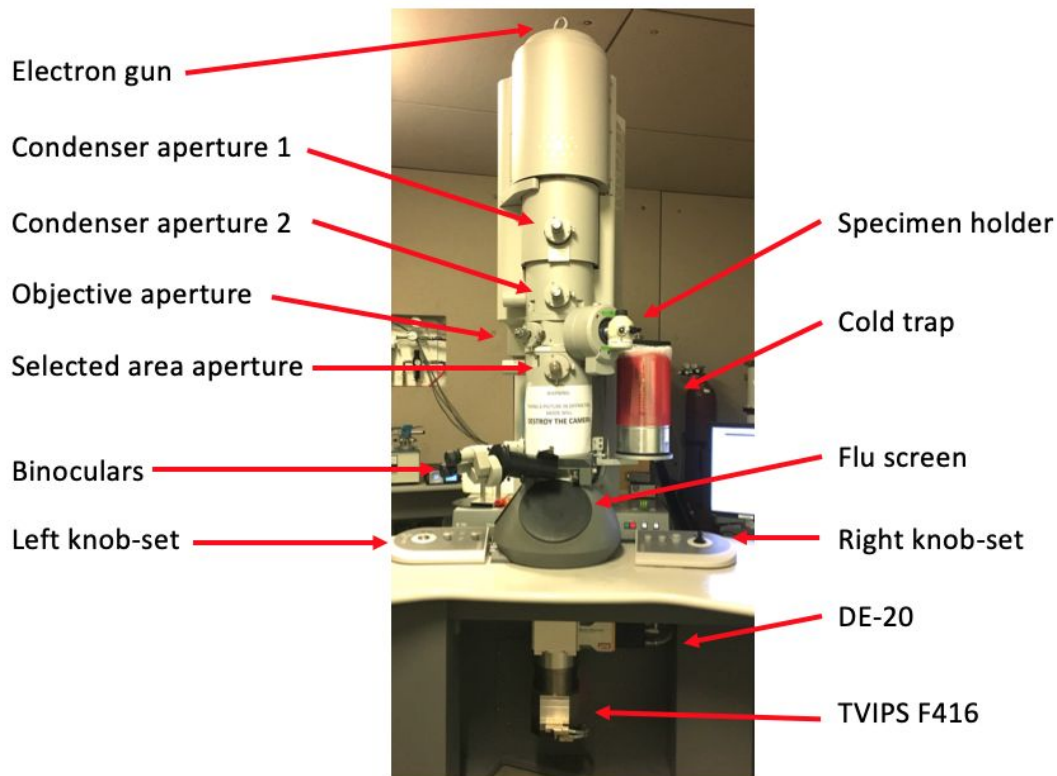# TF20 User Manual

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

Table of Contents

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
- Objective lens Cs = 2.1 mm
- Aperture sizes

Aperture	**#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 yellow
   b. Column valves are closed
      i. Under "Setup" tab, "Col. Valves Closed" is 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
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 F20" 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 = 120s
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 faster throughput 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)
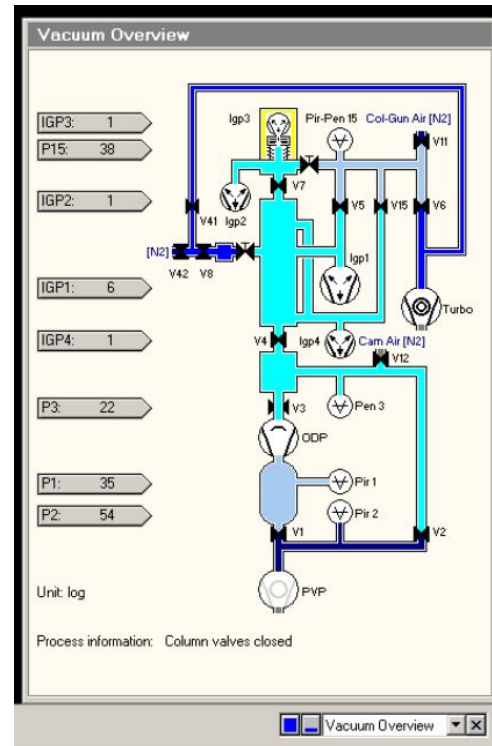
i. This camera has slower throughput, but can give you higher resolution details within multiple frames of a movie

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 F20" for detailed instructions
2. If you used the DE20, make sure the camera is "retracted" and "warm up"
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 LN_2 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

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 = refer to the white board → "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. Open shield of cryo holder when IGP1 <15; you may see a slight increase in pressure, but should settle within a few seconds

9. Open column valves when IGP <10

10. Center preset beams

    a. Move to square of non-interest (square with carbon that you don't mind burning)

    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

11. 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, +/- 50 um. This means your grid is fairly flat. If your eucentric height varies much more than that, your grid is bent/wrinkled.

12. 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"
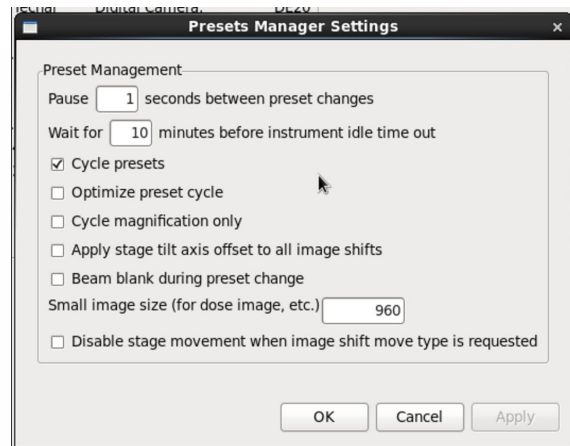
      c.   Click "play" to collect atlas

13. 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 mm
      d.   Insert objective aperture and center
      e.   Exit "DIFFRACTION" mode

14. 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

15. Get the dosage (e^-/A^2) of the EN preset (target = 30 - 60 e^-/A^2)

16. You are now ready to collect some images!

17. 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"

18. 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" <u>when you are done with every hole target</u>

19. 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 analyze data.

20. Under "Hole" node, select "toggle queue timeout" so that column valves will close once exposure queue is done collecting

21. To collect a new square, repeat steps 17 - 19 until you are done collecting data.

22. Once you are done for the day, read "**F20 User Guide (ending your session)"**

Cryo Data Collection with the DE20

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). Suggested mags for EN/FA/FC = 50,000x, spot size 3, 3840 x 5120, bin1; HL = 5000x, spot size 3, 960 x 960, bin 4; SQ = 550x, spot size 5, 960 x 960 , bin 4; GR = 97x, spot size 5, 960 x 960 bin 4.

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/$Å^2$ for a 2 second exposure (1.54 Å/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 (OLD)**" on camera computer (this program is laggy; wait 30 sec)
   c. "Cool down" and "extend" camera (when not in use, DE20 should be retracted and warmed); it takes ~ 5 min to cool down camera
   d. Make sure that you "do not autosave"
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" → "next"
   b. Select holder (TF20-cryo-holder_AMI-2)
   c. In session description, type information about sample → "next"
   d. Select project → "next"
   e. Image directory (use default… "/gpfs/leginon/username")
   f. Connect to clients= tf20-f416 & tf20leginon → "next"
   g. C2 size = refer to the white board → "finish"
7. Once Leginon has started up, open the "application"
   a. Run → Application → MSI-T2 (3.3)
      i. Scope = tf20-d394
      ii. Camera = tf20-de20
      iii. Main = TF20Leginon → "run"
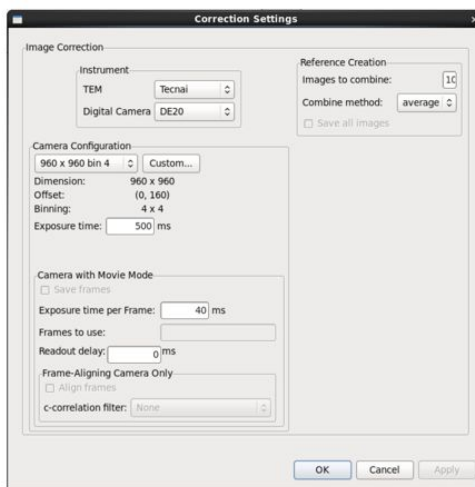
8. Upload your presets
    a. Preset manager → "import presets from another session" (* icon)
        i. TEM = tecnai; Digital Camera = DE-20
    b. "Find" presets from past 20 days; select desired preset (usually from the most recent user)
    c. Highlight all the presets (GR, SQ, HL, FA, FC, EN) and "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. Open shield of cryo holder when IGP1 <15; you may see a slight increase in pressure, but should settle within a few seconds
11. Open column valves when IGP < 10.
12. 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. Center beam using left trackball
            4. Open the "Edit" box and apply beam shift "from scope"
            5. Apply the same beam shift "from scope" for FA and FC preset
13. Get eucentric height of your grid
    a. Send scope to sq preset
    b. Lower green screen (R1 on knobset)
    c. Using Right Knobset trackball, move stage until you see a grid square with unique fiducial; center that square on the green screen
    d. "Z_focus" node→ simulate target (+ icon)
    e. 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)

14. 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

15. Acquire new gain references for the EN preset (over vacuum). For gains, it does not matter if objective aperture is in or out.
    a. For dark - close column valves → "correction" node → "settings" → see image below → "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 0.5 sec on small screen → see image below → "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 flat image
    d. Repeat for FA, FC, HL, SQ and GR preset (see image below to camera settings)
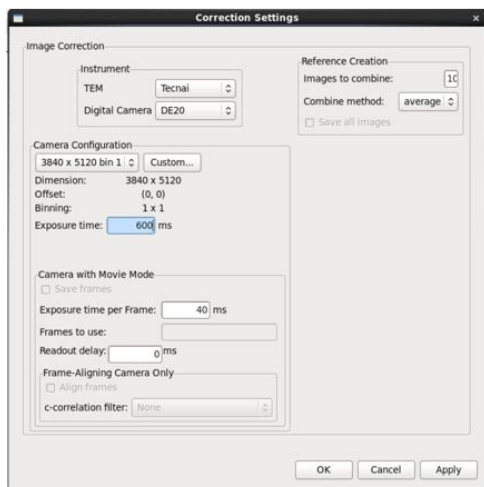
Correction settings for EN preset   Correction settings for FA, FC, HL, SQ, and GR preset

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. Insert and center objective aperture
    a. Over a junk square, send scope to EN mag
    b. 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
    c. Insert objective aperture and center
    d. Exit "DIFFRACTION" mode
    e. 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
19. You are now ready to collect some images!
20. 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"
21. 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" <u>when you are done with every hole target</u>
22. 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.
23. Under "Hole" node, select "toggle queue timeout" so that column valves will close once exposure queue is done collecting
24. To collect a new square, repeat steps 20 - 22 until you are done collecting data.
25. 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.

e. Select "Just Show Command"
f. Copy the command line
g. In new screen, type "ssh de20framealignment" or "ssh zeus"
h. Paste the command from step 25e

26. Start CTF correction (after first movie is done aligning)
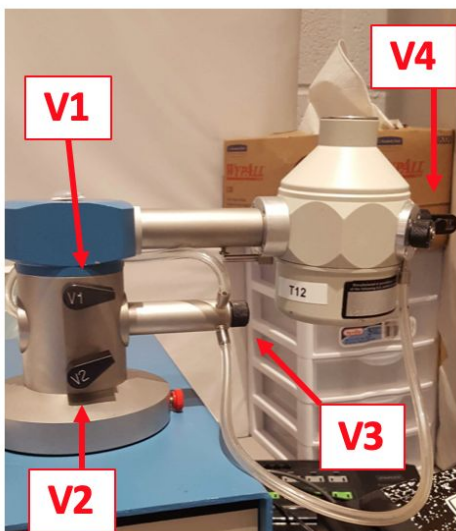   a. CTF → estimate the CTF → CTFFIND v4
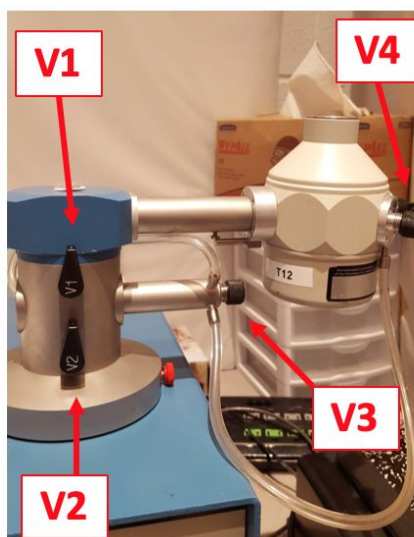   b. Default all settings except preset = en-a
    c. Run on semc-head (or copy/paste into emgbox05)

27. If you are done for the day…refer to "**F20 User Guide (ending your session)**"

Dry Pumping Station User Guide

V4
V1
V4
V1

Do not overtighten the valves! Finger-tight only!

T12
T12

Normal state at the beginning of the day:

V1 = closed
V2, V3, & V4 = open

V3
V2
V3
V2

All valves are closed   All valves are open

Removing holder from dry pumping station:
1. Vacuum should be reading 10^{-3} to 10^{-5} Pa.
2. Remove temperature control cord by pulling straight out from base of plug from the cryo holder jacket. Do not twist or pull the wire!
3. Close V4, then close V3. If there is a second holder attached, close V4 and then V3 on that holder.
4. Close V2. V1 should be closed already, but if not then close V1 last.
5. Unhook tygon tubing from cryo holder and re-connect to silver stopping plug (behind V1 & V2).
6. Turn pumping station off. Wait for green "MDP status" and "System Status" lights to turn off.
7. Using one hand to stabilize the V1/V2 module, gently apply force to pull out cryo holder.
   a. Transfer cryo holder from pumping station into cryo transfer station.
8. Place black blanking plug into opening where cryo holder was.

Inserting holder into dry pumping station and warming it up:
1. Make sure all valves on pumping station are closed. If there are 2 holders, make sure the second holder has V4 and V3 closed (in that order).
2. Remove blanking plug from pumping station
3. Insert cold cryo holder. Invert holder to facilitate condensation removal.
4. Connect the plastic tygon tubing to V4 on the cryo holder.
5. Turn on pumping station. Wait for green "MDP status" and "System Status" light to turn on (~1 min).
6. Open V2, then V1 and wait until vacuum stabilizes.
7. Connect cryo holder to the temperature controller and run the "WARMUP CYCLE" (~20 min)
   a. Warm up on the Elsa holder takes ~ 2 hr. Because of the long time to warm up, only keep V1 and V2 open (V3 and V4 of BOTH holders CLOSED) during the warmup cycle.

Make sure that the 626 holder has a good vacuum. Finally, send message in slack in #EMG channel: "Elsa is warming up now. V1 and V2 open, V3 and V4 of both Elsa and 626 are closed. 626 has a good vacuum and should be ready to go first thing in the morning."

   b. You are done after this step.

   c. If warming up the 626 holder, proceed to step 8.

8. Once warm up is done, close V1.
9. Open V3, then open V4 of holder #1.
10. Open V3, then open V4 of holder #2.
11. On Fridays, start the "ZEOLITE CYCLE" for 2 hours.

==*****If for any reason you cannot finish warming up the 626 and/or Elsa holder and open V3 and V4 to pump down the jacket of the holder by the end of your session, please send an email to emg@nysbc.org OR slack in #EMG channel:**==

==**"626 and/or Elsa is still warming up right now. Only V1 and V2 are open, pumping down the tip only. Can someone from EMG please close V1 and then open V3 and V4 first thing in the morning to pump down the jacket of the holders?"**==

Elsa cryo holder manual (for EMG staff only)

ONLY EMG STAFF should be handling the Elsa holder. This includes loading the sample, insertions, and retractions. Users should only be operating Leginon

- High-resolution cable only needs to be attached during warm up of holder. Warm up cycle takes ~2 hours.
- High-resolution cable should only be used AT THE PUMPING STATION. Do not extend the cable and use it at the general bench (it becomes a trip hazard).
- 1X per month, the holder should be baked out (must use the thicker cable to do this).

After an overnight collection, if you need to use the Elsa holder again on day 2:
- **If you have time…** retract the holder and run warm up cycle (takes 2 hours); after warm up you can then use as normal
- **If you don't have time…** cool down the holder in the scope (with the high-resolution cord attached, in cryo transfer mode, so you can measure the temperature of the tip); after the tip reaches ~ -170C, you can retract the holder into a cold transfer station and switch to the grid of your choice (you will discard the current grid since it has warmed up in the column and is unusable now)
- **Otherwise...** Warm up the Elsa and use the Gatan 626 holder instead.

Cryo Holder Insertion/Retraction Guide for F20

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 LN$_2$.
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 LN$_2$ 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.
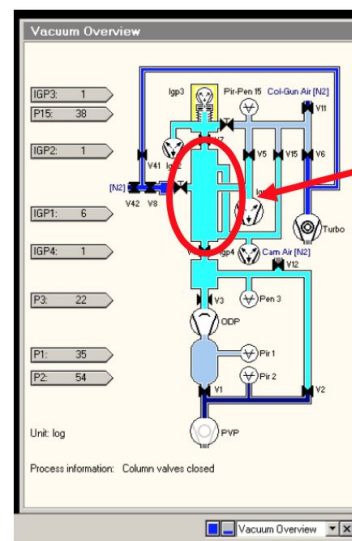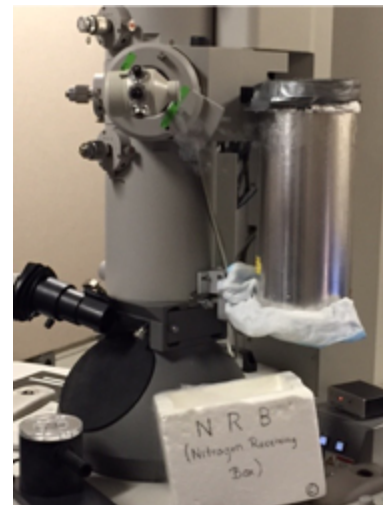
14. Do not open the column valves until IGP1/Column < 10. If you are ever in doubt, close the column valves and contact an EMG staff member immediately.

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

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 leginon, "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 an 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 mm
     c.   Insert objective aperture (silver tab pointed left) and center aperture

10. Objective Astigmatism (~25 – 50k)
     a.   At slightly underfocus (2-3 thon 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

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)