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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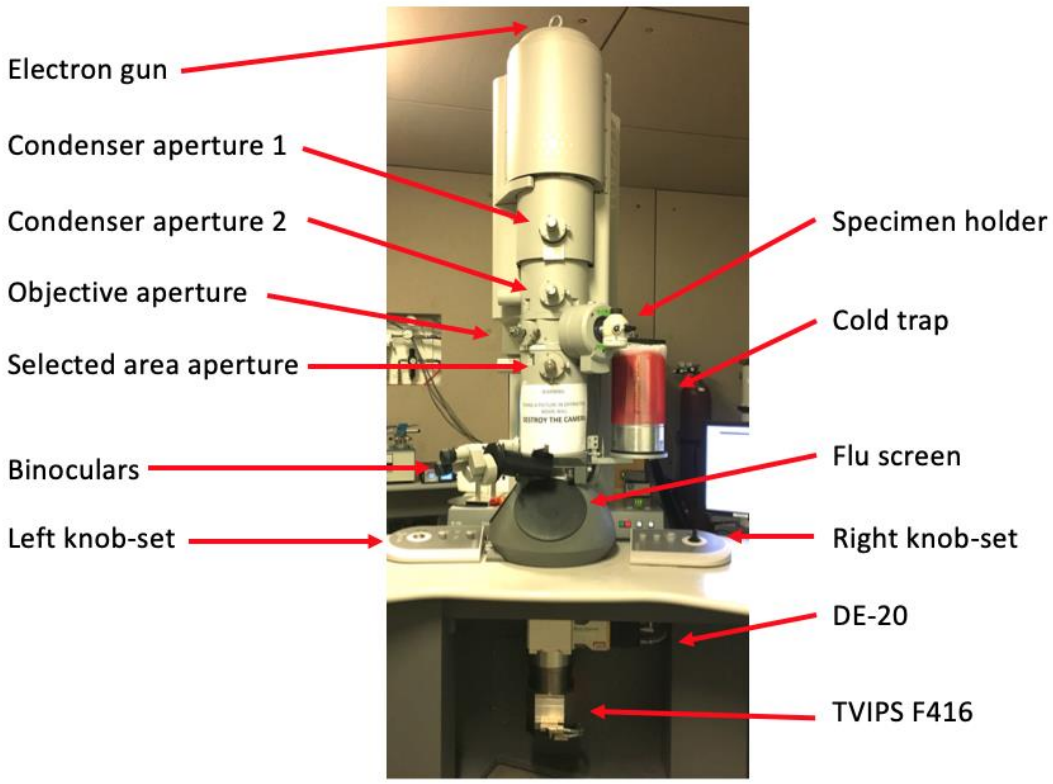
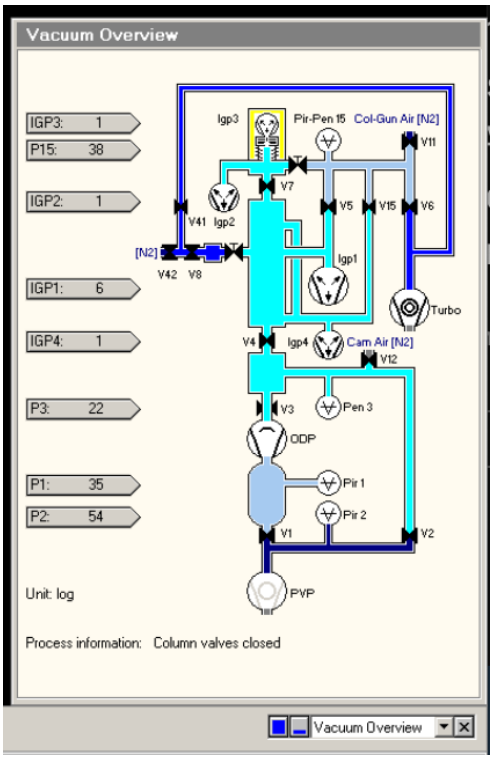
F20 Information Sheet



- 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
- Objective lens Cs = 2.1 mm

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. Setup → "High Tension" and "Operate" are yellow
    b. Column valves are closed
        i. Setup → "Col. Valves Closed" is yellow
    c. EM column vacuum is stable (IGP1 = 6)
    d. Microscope left in SA mag range
    e. Stage neutral
        i. Search → click "Holder" to zero out the X, Y, Z and α coordinates of the stage
    f. Cold trap already filled with LN_2 (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
4. Before insertion,
    a. See Cryo Holder Insertion/Retraction Guide for F20 for detailed instructions
    b. CHECKLIST before insertion:
        1) turbo is on and ready (yellow)
        2) objective aperture is out
        3) flu screen of microscope is down
        4) shield of cryo holder is closed
        5) airlock pump time = 120s
        6) reset holder
        7) alpha = -60°
5. Insert cryo holder into EM.
6. After insertion, wait for IGP1 to reach <10 before opening the column valves
7. Select the camera/acquisition type needed for your session today:
    a. If you plan to screen multiple cryo grids today, use the F416 camera (Cryo Screening with Tietz TVIPS F416)
        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

End of Day Checklist

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. column valves closed
    b. reset holder
    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. 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 tomorrow'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

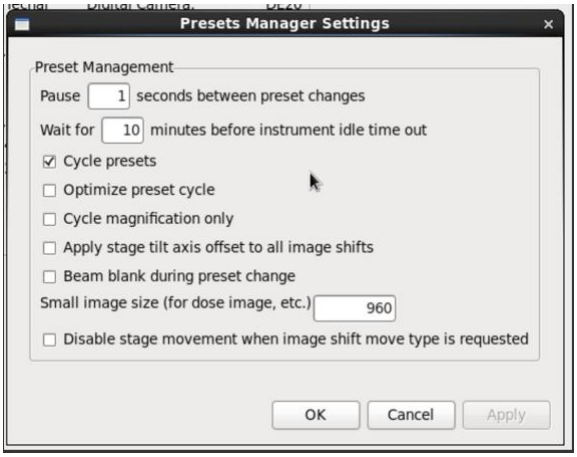
The TVIPS is a CMOS (complementary metal-oxide-semiconductor) 16 megapixel camera with a 50 frame/sec readout (4k x 4k). It sits below the column of the microscope, below the DE20 camera (thus, the DE20 must be retracted to be able to use the TVIPS). It is a robust camera that has good SNR (good contrast) that is used primarily for screening grids. It sits within its own vacuum housing separate from the EM vacuum and is not retractable and always cooled. Because the temperature rarely changes on the camera, 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 EMG or NCCAT Leginon_client (depending on your access type) 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 if using EMG Leginon_client, or nccatleginon if using NCCAT Leginon_client.
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. Main = tf20leginon
        ii. Camera = tf20-f416
        iii. Scope = tf20-d394

    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 = 5,000x; SQ = 1,700x

   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. You may see a slight increase in IGP1, 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 using the flu screen and the stage track ball (square with carbon that you don't mind burning)
    b. Center "grid" preset beam
       i. Send scope to "grid" preset
       ii. Lower flu screen
       iii. Center beam using left trackball
       iv. Open the "Edit" box and click "apply beam shift from scope"
    c. Repeat step b. for square and hole preset
    d. Center and adjust intensity of FA/FC/EN preset beam
       i. Send scope to "EN" preset
       ii. Lower flu screen
       iii. For the EN preset, adjust beam diameter to ~1.5X the size of the second circle and spot size until you achieve target dose of 40 - 70 e^-/A^2 with 1 sec exposure.
       iv. For the FA/FC preset you can make this beam a bit brighter and smaller
       v. Open the "Edit" box and apply intensity "from scope" and beam shift "from scope"
       vi. Repeat for FA and FC preset
11. Get eucentric height and focus
    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.
    d. "Z_focus" node → MF (manual focus)
    e. Click on the upside-down '42' "Toggle show resolution/defocus" and check the defocus at the first zero to see if the set defocus is roughly correct.
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 flu screen and send scope to “DIFFRACTION” mode (right knobset)
      i. Should see bright green small beam on the flu 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 (~3000)
   d. Record the mean value and input into Ice_Thickness node (under ALS)
   e. ALS coefficient with 100 um objective aperture = 392
   f. ALS coefficient with 70 um objective aperture = 302
15. Acquire total dose 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 optical axis (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”
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 End of Day Checklist.

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 5, 3840 x 5120, bin1; HL = 5000x, spot size 5, 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 50,000X this corresponds to ~45 electrons/Å^2 for a 2 second exposure (1.54 Å/pixel).

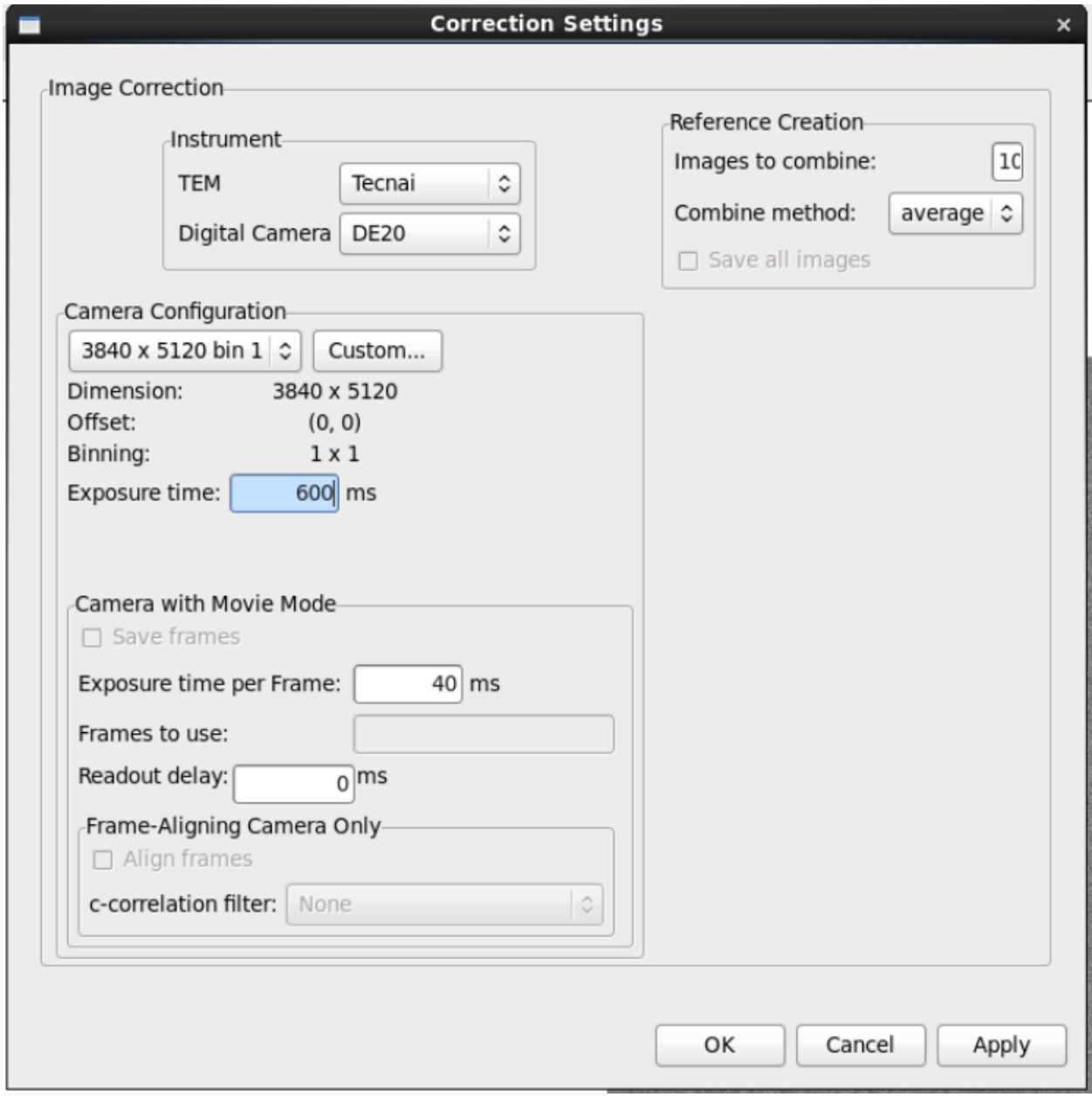
1. After step 6 of F20 User Guide (starting your session)...
2. Open DE20 software to insert and cool down camera (NOTE- can only open MicroManager or Leginon client/server at one time); DE-20 camera control on monitor input DVI-1
    a. Close Leginon client on camera computer
    b. Open "**DE server 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 to -38 Celcius
        i. See Temperature - Detector (Celcius) on the DE Server (Camera Settings)
        ii. Wait for cool down before opening Leginon client (the DE server will stop refreshing and updating temperature reading if Leginon client is open; temperature will not be affected)
    d. "do not autosave" = true
3. Open Leginon client on camera computer (DE20 DVI1), microscope computer (TF20-D394), and main computer (TF20LEGINON)
    a. In terminal, type betaleginon
4. Setup Leginon session settings:
    a. Select "create new session" → "next"
    b. Select holder (TF20-cryo-holder_AMI-2)
    c. In session description, start with F20 and then type info about sample → "next"
    d. Select project → "next"
    e. Image directory (use default… "/gpfs/leginon/username")
    f. Connect to clients= tf20-de20 & tf20-d394 → "next"
    g. C2 size = refer to the white board → "finish"
5. Once Leginon has started up, open the "application"
    a. Run → Application → MSI-T2 (3.3)
        i. Main = TF20Leginon
        ii. Camera = tf20-de20

iii. Scope = tf20-d394→ "run"

6. 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
7. "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
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. 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 grid preset beam
            1. Send scope to grid preset and lower flu screen
            2. Center beam using left trackball
            3. Open the "Edit" box and click "apply beam shift from scope"
        ii. Repeat step i. for square and hole preset
        iii. Center and adjust intensity of FA/FC/EN preset beam
            1. Send scope to "EN" preset and lower flu screen
            2. Center beam using left trackball
            3. Open the "Edit" box and apply beam shift "from scope"
            4. Apply the same beam shift "from scope" for FA and FC preset
11. Get eucentric height and focus
    a. Send scope to square preset and lower flu screen (R1 on knobset)
    b. Using Right Knobset joint stick, move stage until you see a grid square with unique fiducial; center that square on the flu screen
    c. "Z_focus" node→ simulate target (+ icon)
    d. 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
    this means your grid is fairly flat; if your eucentric height varies much more than that, your grid is probably bent/wrinkled)
    e. "Z_focus" node → MF (manual focus
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; ~20 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. Gain references. For gains, it does not matter if objective aperture is in or out. Must be done over vacuum.
    a. EN preset gains
        i. Prepare beam for target dose = 30 - 60 electrons/Å^2 (SS 5, beam diameter ~ 2X bigger than second circle on flu screen)
        ii. Dark reference
            1. Close column valves and flu screen down
            2. In “correction” node → “settings” → **see LEFT image below** → “dark” and “both channels”



3. Acquire (you should see black image)

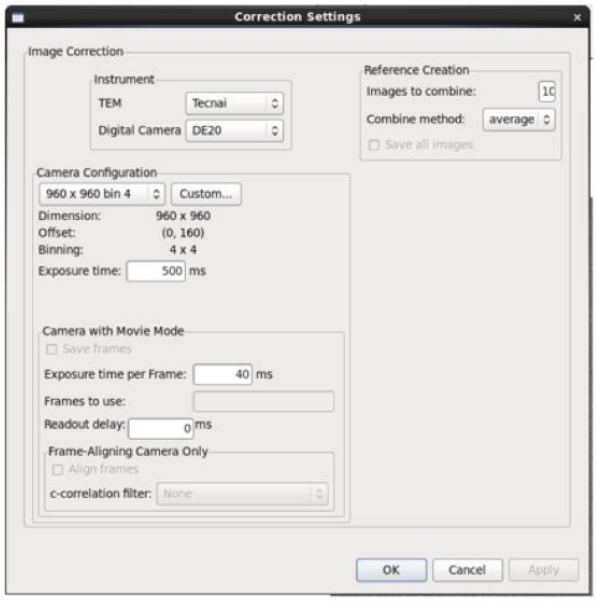
iii. Bright reference
1. Open column valves and flu screen up
2. In “correction” node → “bright” and “both channels”
3. Acquire (you should see image of the camera sensor)

iv. Confirm that correction is done
1. Under “correction” and “both channels”
2. Acquire (you should see a flat fielded image)

v. When done with en preset gains, make sure to apply that same brightness/beam diameter to the en preset in Preset_Manager

b. FA/FC/HL/SQ/GR preset gains (SS 5, beam diameter ~ 2X bigger than second circle on flu screen; target dose = 20 - 30 electrons/Å^2).
i. Repeat step 13a using settings from **RIGHT image below**.

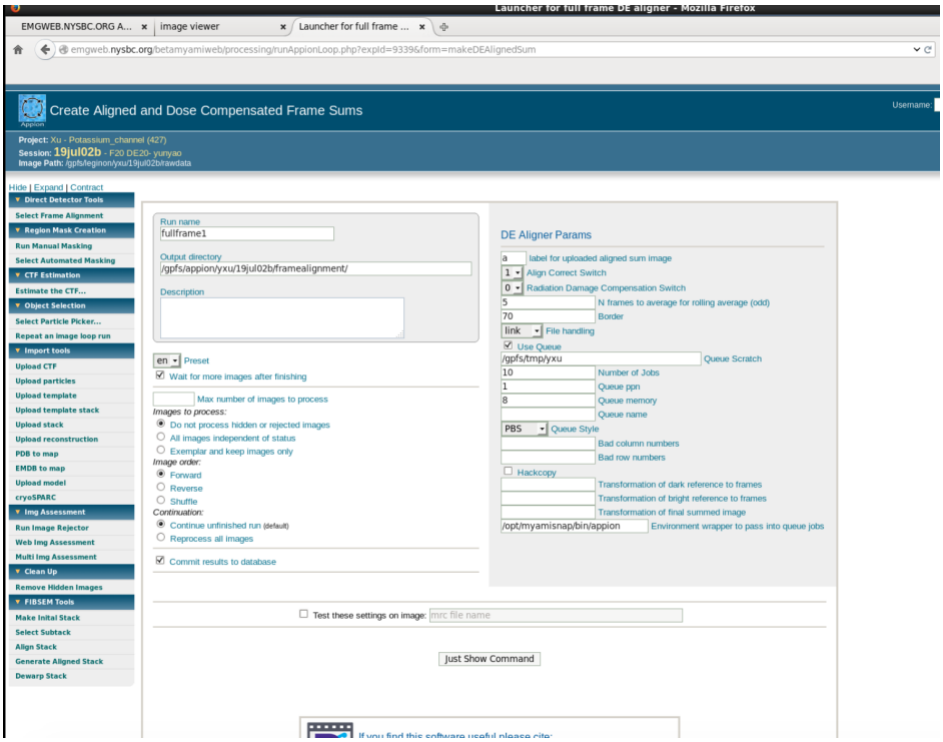
Correction settings for EN preset



Correction settings for FA, FC, HL, SQ, and GR preset

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 box, NOT ZLP)
e. ALS coefficient with 100 um objective aperture = 392
f. ALS coefficient with 70 um objective aperture = 302
15. Get the dosage (e^-/A^2) of the EN preset (target = 30 - 60 e^-/A^2)
16. Insert and center objective aperture
a. Over a junk square, send scope to EN mag
b. Lower flu screen and send scope to “DIFFRACTION” mode (right knobset)

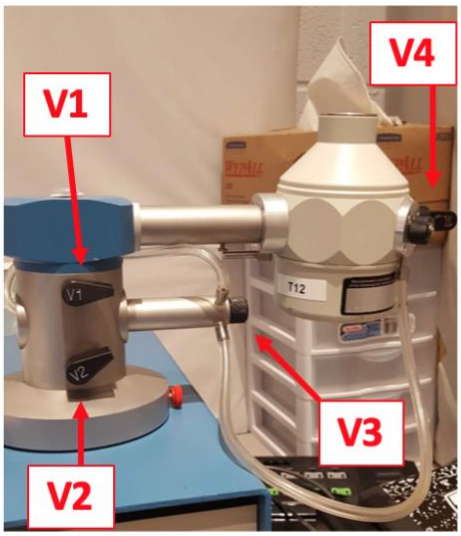
        i. Should see bright green small beam on the flu screen with diffuse carbon diffraction ring around it
    c. Insert objective aperture and center
    d. Exit "DIFFRACTION" mode
    e. If you plan to take a break, send microscope to high mag (FA/FC/EN) to turn on the high mag lenses and close the column valves
17. You are now ready to collect some images!
18. 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"
19. 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>
20. In "Exposure_Targeting" node 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.
21. Under "Hole" node, select "toggle queue timeout" so that column valves will close once exposure queue is done collecting
22. To collect a new square, repeat steps 20 - 22 until you are done collecting data.
23. Start frame alignment
    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.



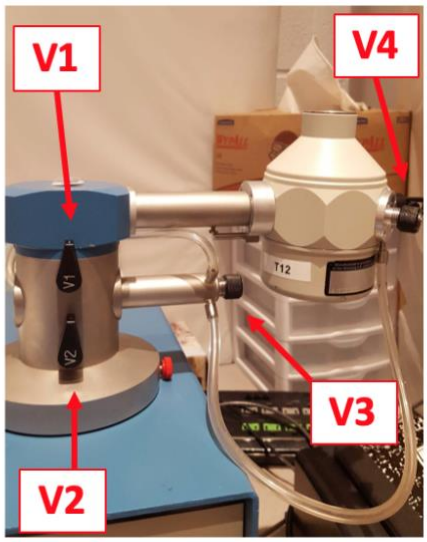
e. Select “Just Show Command”
f. Copy the command line
g. In new screen, type “ssh de20framealignment” or “ssh zeus”
h. Paste the command from step 25e

24. 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)
25. If you are done for the day…refer to End of Day Checklist

Dry Pumping Station User Guide



All valves are closed



All valves are open

Do not overtighten the valves! Finger-tight only!

Normal state at the beginning of the day:

V1 = closed
V2, V3, & V4 = 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?”

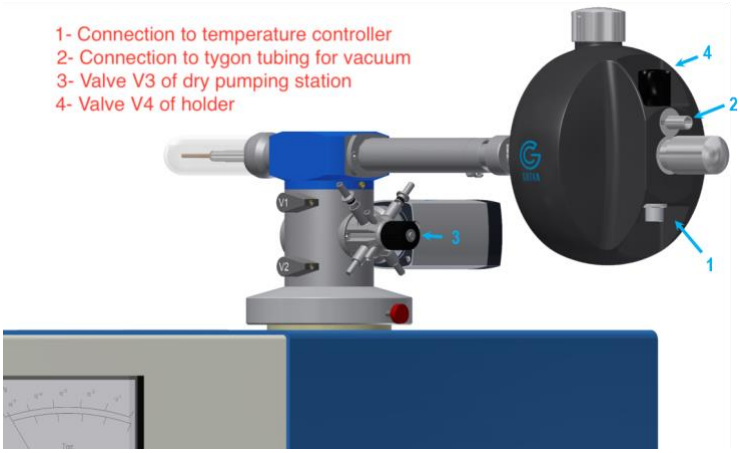
Gatan Elsa cryo holder manual

The Elsa holder is a specialized holder with a large cryogenic dewar that can maintain temperature for ~8 hours.

Elsa holder handling should be done by EMG staff only; this includes loading the sample, insertions, and retractions. Users should only be operating Leginon.

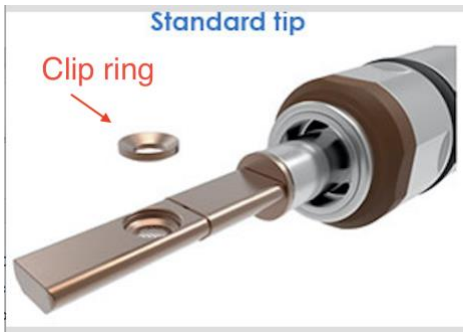
- 1X per week, the holder (V4) should be pumped down
- 1X per month, the holder should be baked out
- Every day the holder is used for cryo, a warm up should be applied at the end of the day

1. Remove Elsa holder from the dry pumping station
    a. V4 and V3 of Elsa will most likely be closed; if not, close these valves first (close V4 and then V3)
    b. If there is a second holder attached to the dry pumping station, close V4 and then V3 of the second holder
    c. Close V1 and then V2
    d. Turn dry pumping station off
    e. Once the "MDP status" and "System Status" turn off, retract holder from dry pumping station
    f. Transfer Elsa holder to the Elsa cryo transfer station



2. Cool down the Elsa holder in "Cryo Transfer" mode (should be done on the pumping station desk)
    a. Attach the Elsa holder to the Elsa temperature controller using the 698 cord (thick grey cord). Make sure to connect red dots to one another.
    b. Switch to "Cryo Transfer" mode and start
    c. Slowly add liquid nitrogen to the tip and dewar sides of the holder until you are at -170°C. This takes ~30 minutes.
3. Transfer sample to the Elsa holder
    a. Open the shield of the holder.
    b. Once the holder is at -170°C you can unplug it from the temperature controller and move the Elsa with its cryo transfer station over to the general bench.
    c. Remove the clip ring from the holder tip. Place the silver end of the tool onto the clip ring and while applying a small amount of vertical pressure on the end of the clip ring tool, slowly turn the tool clockwise 1 - 2 turns until you feel resistance. *Be careful not to turn too far as it will over-tighten the clip ring to the clip ring tool*. Pull up to remove the clip ring.

   d. Transfer cryo grid to the tip of the holder.
   e. Secure the grid with the clip ring. Place clip ring side of the tool onto the grid until you feel a click. While applying a small amount of vertical pressure on the end of the clip ring tool, slowly turn the tool COUNTER- clockwise 1 - 2 turns until the tool moves freely. Pull up to remove the clip ring tool.
   f. Close the shield of the cryo holder.



4. Insert Elsa holder into the microscope
   a. Insertion is the same as the 626 holder.
5. Collect data
6. Retract Elsa holder from the microscope
   a. Retraction is the same as the 626 holder.
7. Warm up the holder
   a. Dump out all excess liquid nitrogen from the dewar of the holder.
   b. Insert holder into the dry pumping station and turn it on.
   c. Once "MDP status" and "System Status" turn green, open V2 and then V1.
   d. Attach the holder to the Elsa temperature controller.
   e. Run the "Warm up" cycle. This takes ~2 hours.
8. On Fridays, pump down the dewar (open V3 and V4).

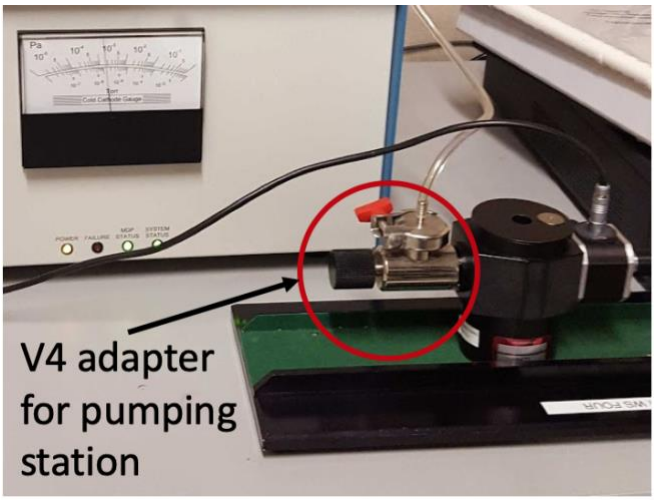
If an overnight collection was done and you want to use the holder the next day, on the next day:

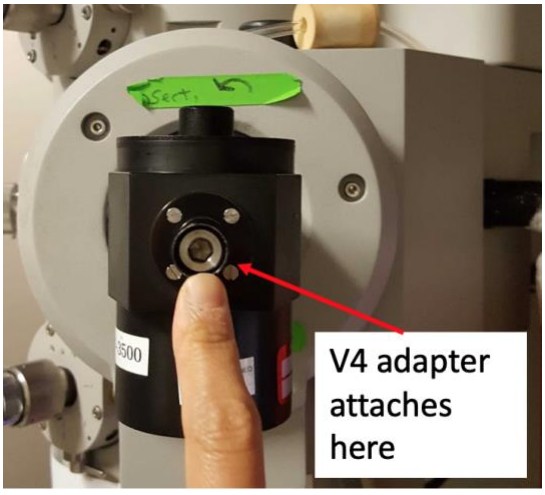
- **<u>If you have time…</u>** retract the holder and run warm up cycle (takes 2 hours); after warm up you can then cool down the holder and use as normal
- **<u>If you don't have time…</u>** cool down the holder in the scope (with the high-resolution cord attached so you can measure the temperature 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)

Gatan CT-3500 (Oxford) holder manual

- This holder takes ~30 min to cool down and should be done while plugged into the temperature controller to monitor cooling.
- It is nearly impossible to recover grids from the CT-3500 holder. Thus, if the grid is good you should plan to collect rather than save the grid.

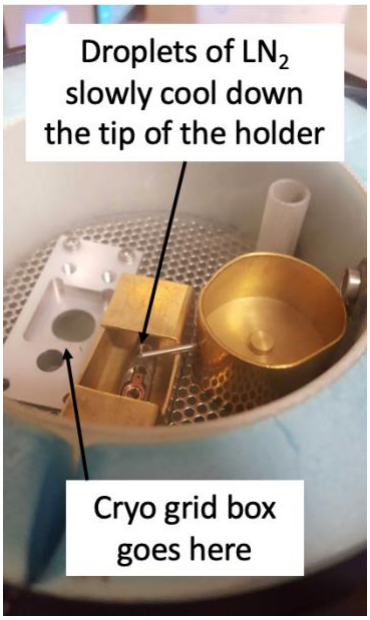
1. Remove holder from dry pumping station.
    a. At the beginning of the day, the temperature control readout should be at 50°C (bake out from the night before). Turn the controller off and then back on so as to stop the heating. Once turned back on you should see the temperature drop from 50°C. Wait until temp drops to at least 30°C before cooling down the holder.
    b. Close V4, V3 and then V1 of the dry pumping station. V2 should already be closed, but if not, then close V2.
    c. Unhook tygon tubing from holder and re-connect to silver stopping plug (near V1 & V2)
    d. Turn the pumping station off and wait for the green "MDP ready" light to turn off
    e. Using one hand to stabilize V1/V2 vacuum area, gently apply force to pull out cryo-holder. Open the holder entry valve on the workstation and insert the holder
    f. Immediately upon removing cryo holder, store in cryo transfer station
    g. Place blanking plug (black) into opening where cryo holder was
    h. Disconnect the silver/black/red vacuum adapter off of the holder. See image.





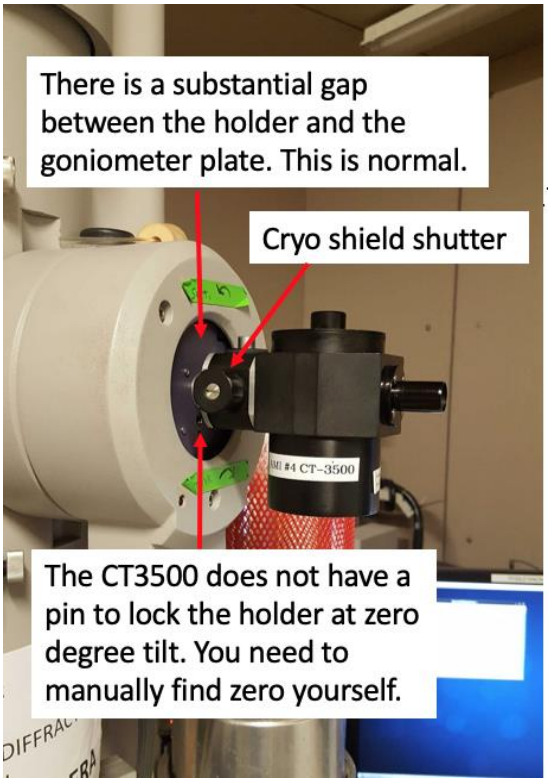
2. Cool down holder to at least -160°C.
    a. Cool down the holder with it connected to the temperature controller module to adequately measure temperature. Cool down the dewar/jacket of the holder using a funnel. SLOWLY, cool down the tip of the holder using the cylindrical filling cup until the temperature reads at least -160°C (takes ~ 30 min). Make sure that while the tip is cooling down, that there is a steady droplet of liquid nitrogen pouring onto the tip of the holder. See image →

b. Care should be taken as LN_2 is added to the filling cup as excess LN_2 will pour out of the back of the transfer station.



3. Transfer cryo grid to the holder.
   a. The gridbox will sit in a small pool of LN_2 on the 9'o clock side of the tip. See image →
   b. All tools are in the drawer labeled "CT3500 only". There are two tools that are used to handle the grid and clip ring: 1) clipping tool and 2) flat head tool. See image below.

   c. The clipping tool is used for loading/unloading clip ring and the flathead tool is used to remove the clip ring if it has been over-tightened. Rotate counterclockwise to remove clip ring off the tip of the holder and clockwise to secure the clip ring in place.
   d. When unscrewing the lid off the gridbox, you will need to use a pair of forceps to hold the box down (station was originally designed for square boxes).

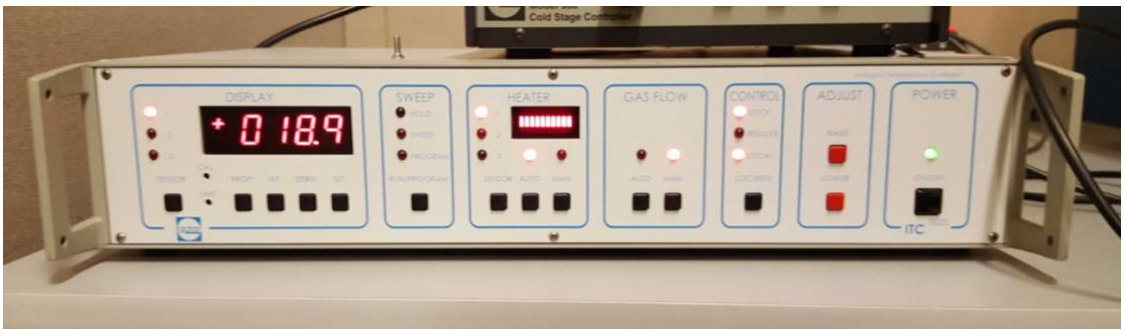


4. Transfer cryo holder into the TEM.
   a. Same insertion mechanism as 626 holder
   b. The CT3500 does NOT have a pin that secures it to the goniometer plate (you need to rotate it to approximately zero degrees by eye)
   c. After insertion, warm up the cryo transfer station (it's not recommended to keep it cold since it gets very frosty)
   d. Open column valves when IGP1 < 20 (will not go < 10).

5. After screening, retract holder from the TEM.

6. Warm up the holder (20°C)
    a. Holder should be warmed up with tip pumped down (open V1 and V2).
    b. Plug in holder to the temp controller. You should see a value in the DISPLAY window (-150°C or so). If not, turn POWER OFF and then back ON. While holding down the SET button (under DISPLAY), hold down the RAISE button (under ADJUST) until the value under DISPLAY reads +20°. Release the SET and RAISE button when done.
    c. Click the AUTO button (under HEATER) to switch out of MAN mode. You should see bars several red bars under the HEATER window which indicates that the holder is actively being heated. This step takes ~30 min.

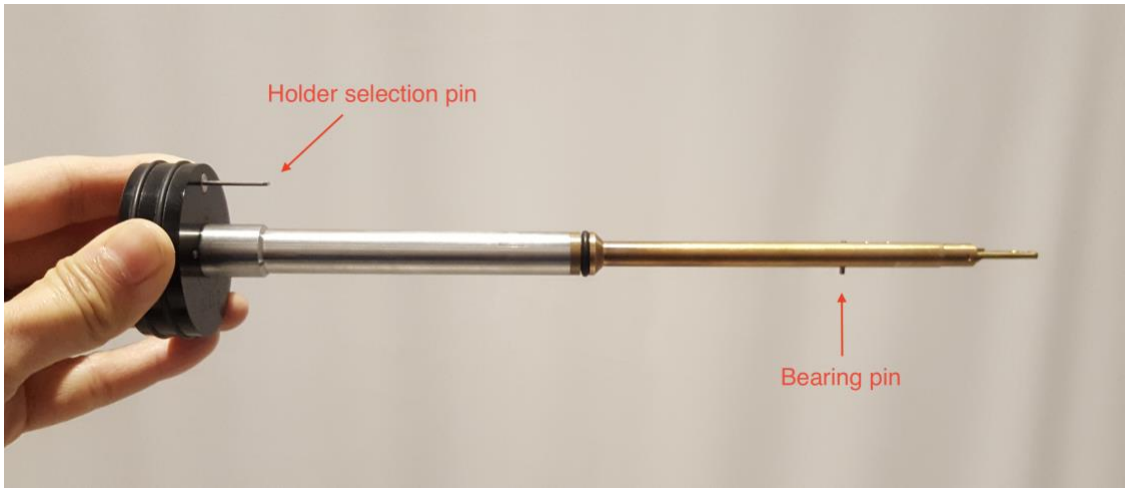
7. Bake out the holder (50°C)
    a. After warm up, the holder should be baked out with dewar/jacket pumped down (close V1, open V2, V3 and V4). You will need to attach the silver/black/red adapter to the end of the holder to open V4.
    b. Connect the holder to the temperature controller cord. See image →
    c. You should see a value in the DISPLAY window (+20°C or so). If not, turn POWER OFF and then back ON. While holding down the SET button (under DISPLAY), hold down the RAISE button (under ADJUST) until the value under DISPLAY reads +50°. Release the SET and RAISE button when done.
    d. Click the AUTO button (under HEATER) to switch out of MAN mode. You should see bars several red bars under the HEATER window which indicates that the holder is actively being heated. This step goes overnight.

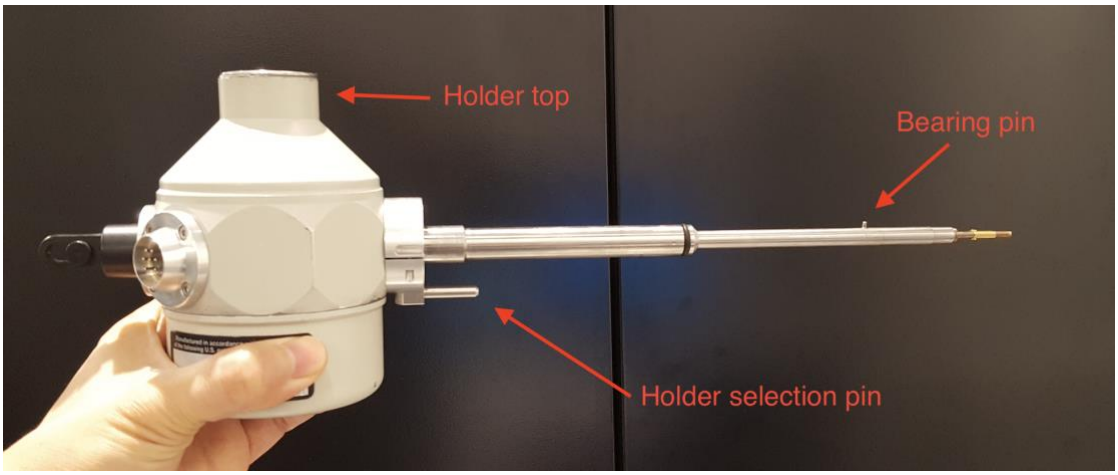


8. You're now done!

Cryo Holder Insertion/Retraction Guide for F20

***Cryo holder insertions on the F20 may only be performed by SEMC staff. NO EXCEPTIONS. ***

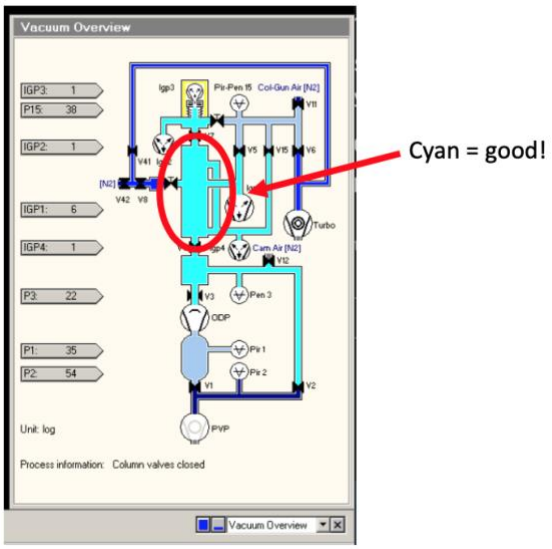




Cryo Holder insertion:

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 holder top 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.

Room Temperature Holder Insertion:

1. Make sure IGP1 < 20, High Tension ON, column valves closed, reset holder, objective aperture out, flu screen down.
2. Check that the airlock default pump time is >120 sec (can go as low as 30 sec).
    a. Setup → settings
3. Insert holder with bearing pin lined up at 5 o'clock position (there is a white line marked on the purple plate). Stabilize the holder until the airlock pumping time of 2 minutes shows up in the Vacuum Overview window.
4. Select 'Single Tilt Holder' as specimen holder.
5. Wait for 2 minute airlock pump time to count down.
6. After airlock pump time is complete, slowly turn holder counter-clockwise so that the holder selection pin moves from the 11 o'clock to the 6 o'clock position. Keep a close eye on IGP1 for spikes; if you see a sharp increase in IGP1, then SLOW DOWN
7. Once at the 12 o'clock position, gently apply resistance on the holder (away from scope) and slowly let the vacuum of the scope pull the holder in.
    a. Give the dewar a slight jiggle to ensure the holder is inserted all the way into the column.

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.

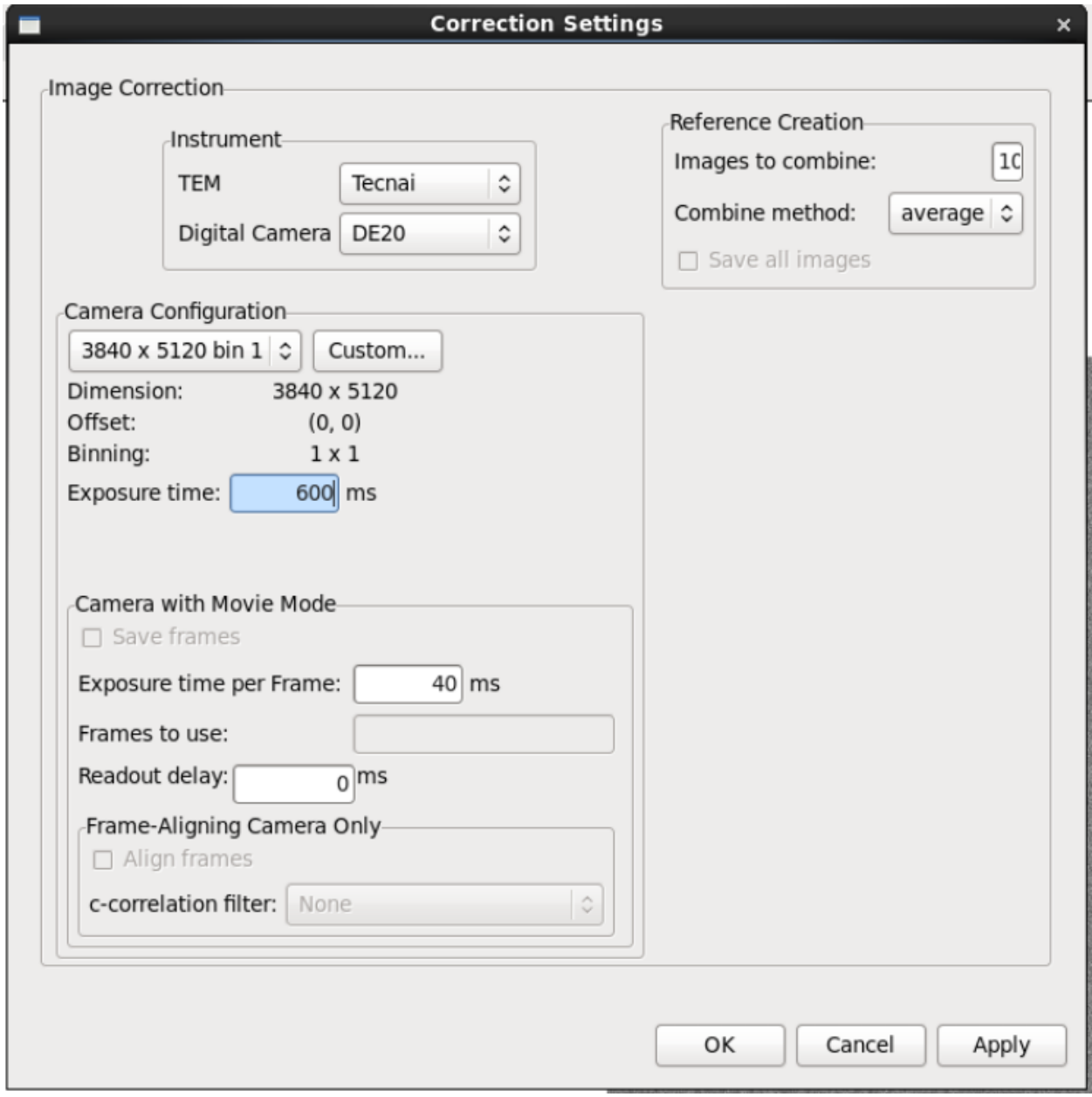
F20 Alignments

- **F20 alignments should be done by EMG staff ONLY!!**
- Alignments can be done with room temperature (RT) holder or cryo holder. Alignments should be done with a grid with carbon support, ideally a cross-grating replica grid.
- Steps 1 - 4 should be done with no specimen in the path of the beam. You can use a ben to pull the holder out partially. If you're confident that gun alignments are good, skip steps 1 - 4.
- Steps 5 - 11 should be done on specimen.

1. Gun tilt ("Tune" → "Gun" → "Gun Tilt")
    a. Focus beam (with intensity knob)
    b. Center beam (beam shift trackball on left knobset)
    c. 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
    d. Condense and center the beam (trackball)
    e. Open beam up to the RIGHT so that it's just past second circle
    f. Center condenser 2 aperture (image →)
    g. 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. NOT tune → alignments → stigmator → condenser stigmator
    b. High mag, ~100kX
    c. Use MF X and Y to make sure beam opens and closes circularly and concentrically over both sides of crossover
    d. 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

   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. Flu screen down. Focus beam to the size of inner diameter circle on flu screen.
   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. 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
9. 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.
10. Coma Free Alignment X and Y
   a. Done via Beam_Tilt_Image node
   b. Click on "Simulate target"
   c. Use click tool to ensure that
11. Preset alignments
   a. Find feature that is visible at medium mag
   b. Center feature on the flu screen using FA preset
   c. Under navigation node, acquire image; 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
12. Gain references. For gains, it does not matter if objective aperture is in or out. Must be done over vacuum.

a. EN preset gains
   i. Prepare beam for target dose = 30 - 60 electrons/Å^2 (SS 5, beam diameter ~ 2X bigger than second circle on flu screen)
   ii. – Check en presets, and in the Correction node settings, choose the camera of interest, and set the appropriate camera configuration, binning, and exposure time.
   iii. Dark reference
      1. Close column valves and flu screen down
      2. In “correction” node → “settings” → **see LEFT image below** → “dark” and “both channels”
      3. Acquire (you should see black image)



iv. Bright reference
    1. Open column valves and flu screen up
    2. In “correction” node → “bright” and “both channels”
    3. Acquire (you should see image of the camera sensor)

v. Confirm that correction is done
    1. Under “correction” and “both channels”
    2. Acquire (you should see a flat fielded image)

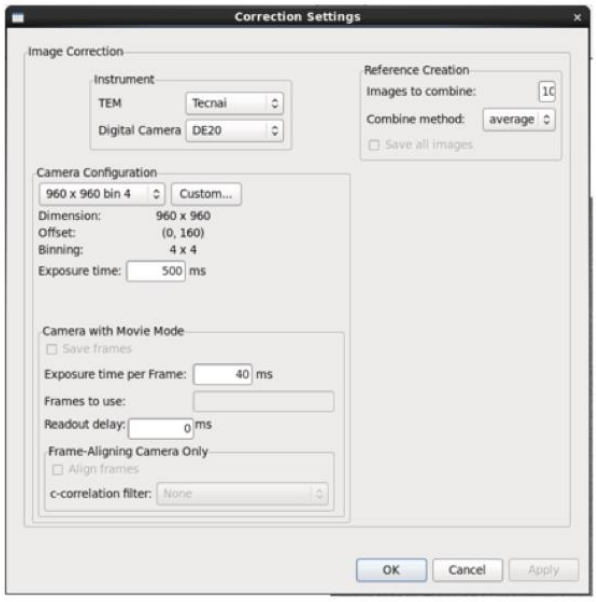
vi. When done with en preset gains, make sure to apply that same brightness/beam diameter to the en preset in Preset_Manager

b. FA/FC/HL/SQ/GR preset gains (SS 5, beam diameter ~ 2X bigger than second circle on flu screen; target dose = 20 - 30 electrons/Å^2).

i. – Check FA preset (especially camera size and binning), and in the Correction node settings, choose the camera of interest, and set the appropriate camera configuration, binning, and exposure time.

ii. Repeat step 13a using settings from **RIGHT image below**.

Correction settings for EN preset



Correction settings for FA, FC, HL, SQ, and GR preset

a.

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)
        i. Turn HT on when IGP2 < 10
    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 ~ 7 uA emission (under HT), FEG = 75 uA
    h. Make sure FEG is operating at 3950 V (may need to select the ↵ button to refresh it)