



SIMONS ELECTRON
MICROSCOPY CENTER

NEW YORK STRUCTURAL BIOLOGY CENTER



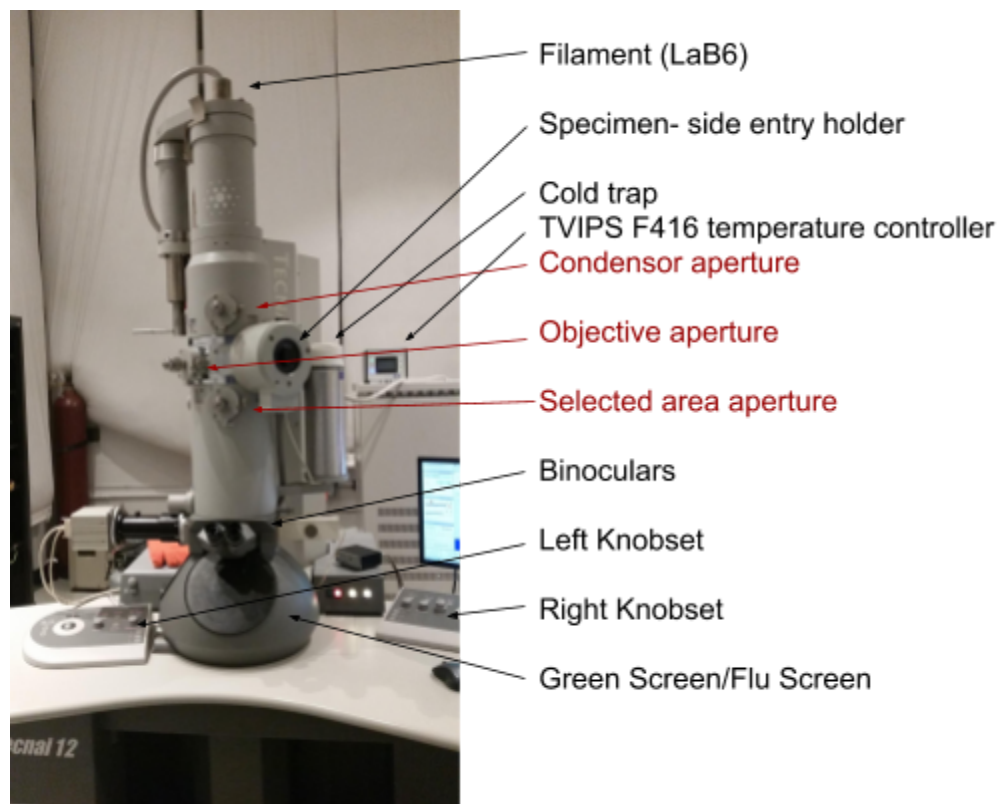
T12 User Manual

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

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T12 Information Sheet



- Aperture sizes

	1	2	3	4
objective	70 μm	100 μm	100 μm	150 μm
condenser	100 μm	100 μm	100 μm	200 μm

- Objective lens

- Cs aberration of objective lens = 6.3 mm

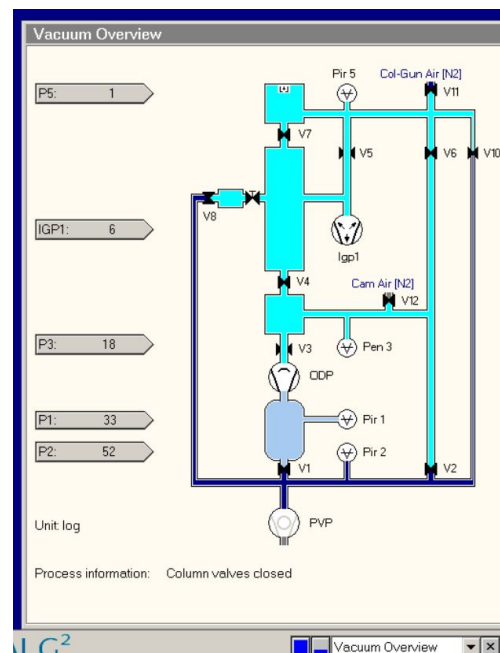
- Min focus step = 9.0 nm
 - Max eucentric tilt = +/- 80°
 - Magnification range 22x - 300,000x

- Camera

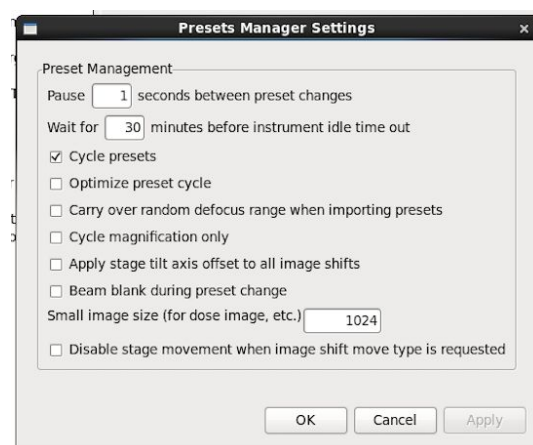
- The TVIPS is a CMOS (complementary metal-oxide-semiconductor) 16 megapixel camera with a 50 frame/sec readout (4k x 4k). It sits below the column of the microscope. It is a robust camera that has good signal-to-noise ratio (SNR) that is used primarily for screening grids. It is housed in a vacuum housing 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.

T12 User Guide (starting your session)

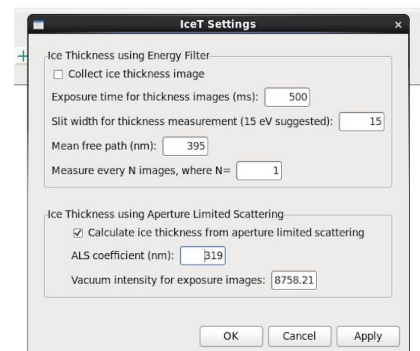
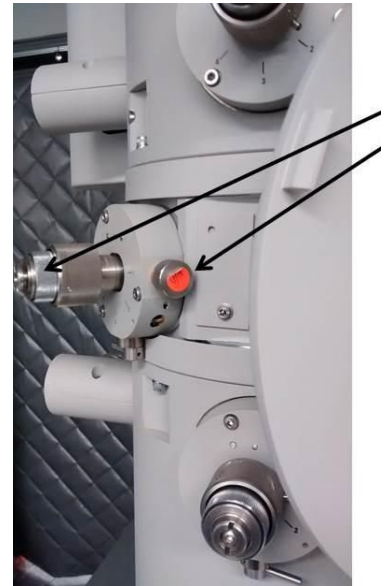
1. Make sure microscope is in stable condition:
 - a. High tension (HT) should be ON (yellow)
 - b. Filament is OFF (grey)
 - c. Column valves are closed (yellow)
 - d. EM column vacuum is stable (IGP1 = 6)
 - e. Stage is neutral (under “Search” tab, click “Holder” button to zero out the X, Y, Z & α coordinates of the stage)
2. Fill cold trap with LN₂ (can last for 6 - 8 hours); refill every 1 - 2 hours
 - a. Fill cold trap with LN₂ from the “wet nitrogen” dewar (LD10); this needs to be done at least 1 hour before your cryo session
3. Grab a tank of fresh LN₂ for your sample
4. Remove cryo holder from the dry pumping station
 - a. See “Dry Pumping Station User Guide (for cryo holders)” for detailed instructions
5. Transfer cryo sample into cryo holder
6. Insert cryo holder into EM
 - a. See “Cryo Holder Insertion/Retraction Guide” for detailed instructions
 - b. CHECKLIST: Make sure that stage is centered, goniometer set to -60°, flu screen of microscope is down, objective aperture is out, shield of cryo holder is closed, airlock pump time is >120 sec
7. After insertion...
 - a. If insertion was successful and the column vacuum DID NOT crash (HT will still be on), then go to step 8
 - b. If insertion was unsuccessful and the column vacuum DID crash (HT will be off); notify staff
 - i. Wait for IGP < 30; Turn HT back on by clicking High Tension button
8. Turn on filament when IGP1 < 30
 - a. Emission = 2 - 10 uA (is > 30 uA then notify staff)
 - b. Emission step = 1 (NOT 3)
9. Login to Leginon computer with your LDAP account
10. Open Leginon client on camera & microscope computer
11. Open Leginon program on Leginon computer
 - a. Open terminal and type betaleginon
12. Setup Leginon session settings:
 - a. Select “create new session” → next
 - b. Select holder (T12-cryo holder)



- i. In description, type information about sample (ex: T12- glutamate dehydrogenase) → next
 - c. Select project → next
 - d. Image directory (use default: /gpfs/legion/username)
 - e. Connect to clients= t12-d297 and t12-f416 → next
 - f. Condenser size = 100 um → finish
13. Once Legion has started up, open the application
 - a. Application → Run → MSI-T2 (3.3)
 - i. Scope = t12-d297
 - ii. Camera = t12-f416
 - iii. Main = biotwin01legion → run
14. Upload your presets
 - a. Preset manager → “import presets from another session” (* icon)
 - i. TEM = tecnai; Digital Camera = Tietz F416
 - b. “Find” presets from past 20 days
 - c. Select presets (usually from the most recent user)
 - d. Highlight all the presets (GR, SQ, HL, FA, FC, EN) and “import” → “done”
15. “Cycle” presets to minimize hysteresis in the beam
 - a. Under Preset Manager node, Settings → “cycle presets” = true (see image)
 - b. Send to EN preset 10X
16. Make sure there is 3 sec pause between exposure images (this ensures there is enough settling time for exposure beam before camera acquires image; otherwise the camera will take exposure images of half the beam)
 - a. Exposure node → settings → wait 3 sec between acquire
17. Open shield of cryo holder when IGP1 < 20; you may see a slight increase in pressure, but should settle within a few seconds
18. Open column valves when IGP1 < 20
19. Center preset beams
 - a. Open column valves; send scope to grid mag (do you see the beam?); move stage until you center square with ice in it (that you don’t mind throwing away)
 - b. Center “square” preset beam
 - i. Send scope to “sq” 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 “hl” 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 FA/FC/EN preset beam
 - i. Send scope to “EN” preset
 - ii. Lower green screen
 - iii. Center beam using left trackball
 - iv. Open the “Edit” box and click “apply beam shift from scope”
 - v. Repeat for FA and FC preset
- 20. Get eucentric height of your grid
 - a. “Z_focus” node→ simulate target (+ icon)
 - b. 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 μm ; this means your grid is fairly flat; if your eucentric height varies much more than that, your grid is probably bent/wrinkled and Leginon will struggle to find focus during your session; may need to work more manually with eucentric height & focus)
- 21. Collect atlas of your grid (this is a series of low mag images that are stitched together to give you a view of your whole grid; ~10 minutes)
 - a. Under the “Grid_targeting” node, select “settings” button (upper left hand side) and input your grid description.
 - i. Radius = 0.0006m (small atlas) or 0.00095m (large atlas)
 - b. Select “calculate atlas”
 - c. Select “play” to collect atlas (this takes ~10 minutes to complete)
- 22. Immediately after atlas is collected, insert and center objective aperture
 - a. Send scope to grid preset
 - b. Center square of non-interest
 - c. Send scope to “EN” preset (make sure beam is centered)
 - d. Go to Diffraction mode (right knobset)
 - i. $D = 680 \text{ mm}$
 - e. Insert objective aperture
 - f. Adjust aperture position to center over diffraction spot (see image on right)
 - i. Note- ALWAYS watch the beam on the flu screen when adjusting the objective position!
 - g. Go out of diffraction mode when done
- 23. Set up Ice Thickness (see image right)
 - a. Go to vacuum area
 - b. Send scope to EN preset (make sure beam is centered)
 - c. Simulate target in exposure node
 - i. Record the mean value

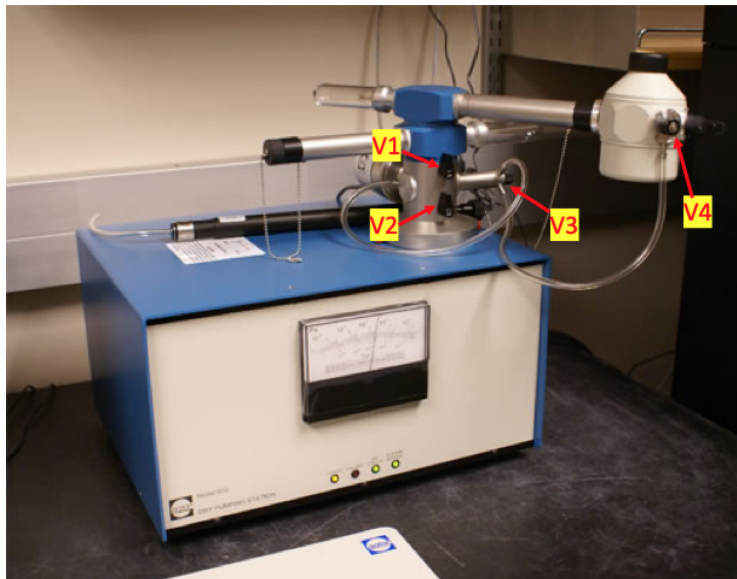


- d. In IceT node → settings → Vacuum intensity for exposure images = value from step above (20c)
 - e. **ALS coefficient = 319 with 100 um objective aperture in**
24. Get the dosage (e^-/A^2) of the EN preset (target = 30 - 60 e^-/A^2)
 25. You are ready to screen now!
 26. In “Square_Targeting” select 1 square of interest
 - a. Using “acquisition” mouse, select the center of square of interest
 - b. Click “Submit target”
 - c. Click “Submit queued targets”
 27. 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
 28. 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”
 29. At this point you have queued up a number of exposure targets for Leginon to acquire
 - a. Go to emgweb.nysbc.org to view images
 30. To collect a new square, repeat steps 22 - 24 until you are done collecting data.
 31. If you are done for the day... read “**T12 User Guide (ending your session)**”

T12 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. Cryo cycle the microscope
 - a. Make sure that EM stage is neutral, column valves closed, flu screen is down, objective aperture is out, and that high mag lenses are left on (send scope to EN/FA/FC preset)
 - b. Adjust time of cryo cycle so that it ends by 8am the next morning
 - c. Click “Cryo Cycle” on FEI interface
3. Remove cold trap from the stand when IGP1 reads 99
 - a. You can keep the remaining LN₂ inside the cold trap (off the coils) for the next user
4. Dump any unused LN₂ into the LD10 tank
5. Bake out the cryo holder
 - a. See “Dry Pumping Station User Guide (for cryo holders)” for detailed instructions
6. Close Leginon client on both “camera” and “microscope” PC
7. Log off the Leginon workstation
8. Cleanup the bench space for the next user
9. Log any issues into the EM notebook or notify SEMC staff directly

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 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

***Cryo holder insertion/retractions on the T12 may only be performed by SEMC staff, or users that have been properly trained with cryo insertions!! NO EXCEPTIONS. ***

Refill holder dewar every 2-3 hours.

Cryo Holder insertion:

1. Make sure IGP1 < 20, filament OFF, High Tension ON, column valves closed, reset holder, objective aperture out, flu screen down.
2. Check that airlock default pump time is >120 sec.
 - a. Setup → settings
3. Set holder alpha value at -60 degrees, goniometer will rotate.
4. Position styrofoam box below goniometer to catch LN₂.
5. Insert holder to first stop with holder top at 3 o'clock position. Stabilize the holder until the airlock pumping time of 2 minutes shows up in the Vacuum Overview window.
6. Select 'Single Tilt 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.
 - a. The stage will rotate back to 0 degrees while the holder top is still at 3 o'clock position.
9. Slowly turn holder counter-clockwise from the 3 o'clock to the 12 o'clock position while keeping a close eye on IGP1 for spikes; if you see a sharp increase in IGP1, then SLOW DOWN
10. 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.
11. Top off cryo holder dewar and cold trap with LN₂ once inserted.
12. After insertion...
 - a. If insertion was successful and the column vacuum DID NOT crash (HT will still be on), then go to step 13
 - b. If insertion was unsuccessful and the column vacuum DID crash (HT will be off); notify staff
 - i. Wait for IGP < 30; Turn HT back on by clicking High Tension button
13. Turn on filament when IGP1 < 30 (takes ~6 min)
 - a. Emission = 2 - 10 uA (is > 30 uA then notify staff)
 - b. Emission step = 1 (NOT 3)

Cryo Holder retraction:

1. Close column valves, reset holder, objective out, flu screen down, filament OFF (wait for countdown to finish), cryo shield of holder closed
2. Gently brace against the column and pull holder back till it stops, then turn clockwise from 12 o'clock to 5 o'clock position, then pull straight out of the column.
3. If vacuum spikes and column pressure crashes, contact an EMG staff member immediately.

T12 Alignments

T12 alignments should be done by EMG staff ONLY!!

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

1. Steps 2-6 should be done over vacuum with specimen out and objective aperture out . You can move the holder out of the path of the beam using a pen.
2. Gun tilt (Tune → Gun → Gun Tilt)
 - a. 1X per day
3. Gun Shift (Tune → Gun → Gun Shift)
 - a. 1X per day
4. Spot size dependent gun shift (Tune → Gun → Spot size-dependent Gun Shift)
 - a. 1X per week
5. Spot Size Intensity Calibration (Tune → beam
HM-TEM → SS intensity calibration)
 - a. 1X per month
6. Condenser Astigmatism
 - a. 1X per week
 - b. ~40K mag
 - c. Tune → Stigmator → Condenser
 - d. Use MF X and Y to make sure beam opens and closes circularly and symmetrically over both sides of crossover
 - e. Click “None” when done
7. Insert sample into column
8. Eucentric Height
 - a. At least 1X per day
 - b. Find a square with some dirt on it
 - c. “Z-focus node” → simulate target
9. Eucentric focus
 - a. At least 1X per day
 - b. “Z-focus node” → “MF”
 - c. Find zero focus; reset focus
10. Beam tilt pivot point X & Y
 - a. At least 1X per day
 - b. Direct alignments → Beam tilt pivot point X
 - c. Beam tilt pivot point Y
11. Rotation center
 - a. At least 1X per day
 - b. Direct alignments → rotation center
12. Beam shift



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

- a. At least 1X per day
 - b. Direct alignments → beam shift
- 13. Objective Aperture Centering
 - a. At least 1X per day
 - b. Send scope to “EN” preset
 - c. Go to Diffraction mode (right knobset)
 - i. $D = 680 \text{ mm}$
 - d. Insert objective aperture
 - e. Adjust aperture position to center over diffraction spot (see image on right)
 - i. Note- ALWAYS watch the beam on the flu screen when adjusting the objective position!
- 14. Objective Astigmatism
 - a. At least 1X per day
 - b. Focus node → “MF” button
 - c. Adjust to $\sim 2 \text{ um}$ defocus (should see 1 - 2 Thon rings)
 - d. Tune → Stigmator → Objective
 - e. Adjust MF Y and X until Thon rings are circular and symmetric
- 15. Gain references
 - a. 1X per month
 - b. For EN, aim for 1 sec exposure time on small screen (SS 3, beam diameter should cover full green screen)
 - i. 2048 x 2048, bin2, 1000ms
 - ii. Dark first
 - iii. Turn Tietz shuttering box off and then back on (should see 2 yellow lights when green screen is up; toggle should be set to “camera”)
 - iv. Bright next
 - v. *DO NOT CLEAR BAD PIXELS*
 - c. For FA/FC/HL/SQ/GR, aim for 0.5 - 1 sec exposure time on small screen (SS3, beam diameter should cover full green screen)
 - i. 1024 x 1024, bin4, 500ms
 - ii. Repeat ii - v
- 16. Preset alignments

Troubleshooting

1. If the column pressure crashes and HT and filament turn off... (This can happen for a number of reasons (bad cryo holder insertion, bad cryo holder removal, etc)
 - a. Notify EM staff
 - b. Wait for TMP rollout to recover (~6 minutes)
 - c. Wait for column pressure to recover
 - d. Manually set HT to 80 kV
 - e. Click “HT” to turn on
 - i. Wait till measured HT is 80 kV
 - f. 80 → 100 kV, 10 kV steps
 - g. 100 → 120 kV, 1 kV steps (need to free high tension and adjust steps)
 - h. Turn filament on
2. If you are targeting in one spot in “Exposure_Targeting” but your actual exposures are ending up somewhere else (this also applies if you are targeting in one spot in “Hole_Targeting” but your “Exposure_Targeting” areas are off)
 - a. Find feature that is visible at low mag (using “navigation” tab, send microscope to “grid” and take picture; using “crosshair tool” and the “clicking tool” center a distinguishable edge of a feature in the center of the stage; refine the centering at “hole” preset”
 - b. In “preset_manager”, highlight “EN” preset and click “align presets to one another”
 - c. Using “stage position”, “crosshair tool” on both left and right images, use “click tool” on image on the right side to center the lower mag image center to match the center of the high mag image on the left (NOTE- always go from high mag (left side) to low mag (right side))
3. Obtain dark and gain reference (OPTIONAL- request staff assistance))
 - a. Note- only needs to be once a month or if camera temperature changes. Use settings that are the same for your high mag “EN” images, like exposure time, camera size and binning.
 - b. For dark - close column valves → “correction” node → “settings” → tecnai & tietz f416 → 4k x 4k bin1, exposure = 1000ms, image to combine = 10, average → “dark” and “both channels” → acquire (you should see black image)
 - c. Bright - open column valves → find empty square → send scope to EN preset → adjust brightness to 0.5sec on small screen → “correction” node → “settings” → tecnai & tietz f416 → 4k x 4k bin1, exposure = 1000ms, image to combine = 10, average → “bright” and “both channels” → acquire (you should see image of the camera sensor)
 - d. Confirm that correction is done → “correction” and “one channel” → acquire; you should see a blank image
 - e. Reset beam blanker after dark reference (unplug USB port from shutter box)
4. Align preset images to one another (OPTIONAL- only necessary if targeting between different mags is inaccurate)

- a. Find feature that is visible at low mag (using “navigation” tab, send microscope to “square” 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 EN mag, and find that feature with the flu screen
- c. Take a picture under EN; center the feature (using stage position)
- 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 in the image)
- i. Go to “preset manager” and apply the image shift to the GR preset “from scope”
- j. You are done!
 - i. Go back to navigation node and reset back to “stage position” for movements