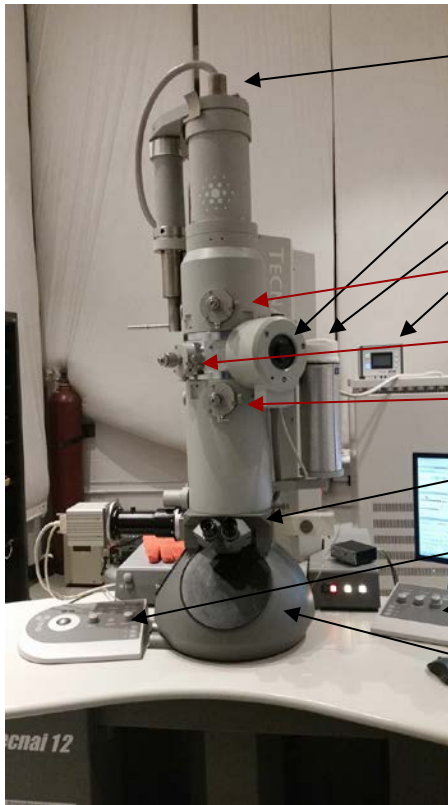


T12 User Manual at the
Simons Electron Microscopy Center

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T12 Information Sheet

Filament (W or LaB6)

Specimen- side entry holder

Cold trap

TVIPS F416 temperature controller

Condensor aperture

Objective aperture

Selected area aperture

Binoculars

Left Knobset

Right Knobset

- Aperture sizes

	1	2	3	4
objective	70um	100um	150um	150um
condenser	30um	50um	100um	200um

- Objective lens

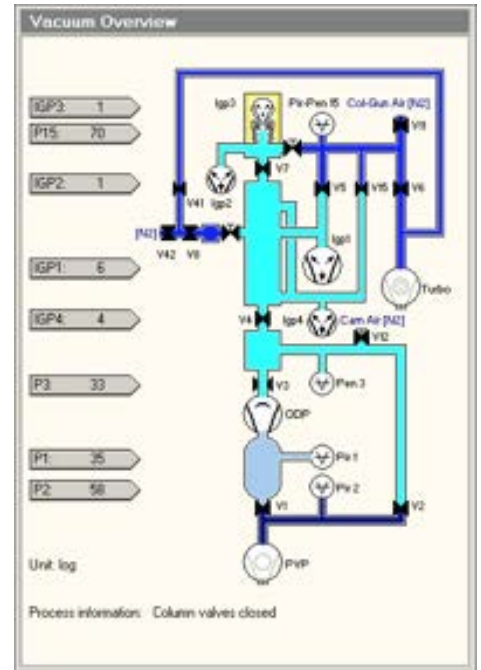
- Line resolution = 0.34 nm
- Cs objective = 6.3 mm
- Cc objective = 5.0 mm
- Focal length = 6.1 mm
- Min focus step = 9.0 nm
- Max eucentric tilt = +/- 80°
- Mag 22 - 300,000

- Camera

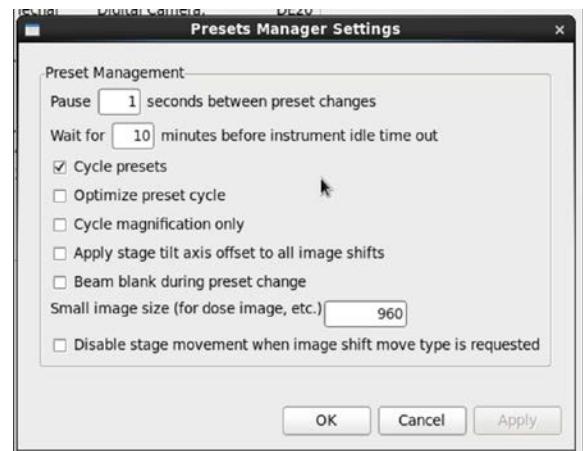
- The TVIPS is a CMOS (complementary metal-oxide-semiconductor) 16 megapixel camera with a 50 frame/sec readout (4k x 4k). It sits below the column of the microscope. It is a robust camera that has good SNR (good contrast) that is used primarily for screening grids. It is housed in a vacuum housing separate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

T12 User Guide (starting your session)

1. Make sure microscope is in stable condition:
 - a. Turn HT is 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 “dry” nitrogen 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 (page 8)
5. Transfer cryo sample into cryo holder
6. Insert cryo holder into EM
 - a. See “Cryo Holder Insertion/Retraction Guide” for detailed instructions (page 9)
 - 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 $>120s$
7. After insertion...
 - a. If insertion was successful and you did not crash the column vacuum, HT will still be ON (yellow)
 - b. If insertion was unsuccessful and you did crash the column vacuum, HT will be OFF (grey); click HT to turn back on; wait 5 minutes for vacuum to recover
8. Turn on filament when IGP1 < 25
6. Login to leginon computer with your LDAP account (first initial, last name)
 - a. You received this on a small sheet of paper at the new user orientation
7. Login to Leginon/Appion webviewer
 - a. Same username and password as LDAP
8. Open Leginon client on “camera” & “microscope” computer
9. Open Leginon program on leginon computer
 - a. In terminal, type `betaleginon`
10. Setup Leginon session settings:
 - a. Select “create new session” → next
 - b. Select holder (T12-cryo holder)
 - i. In description, type information about sample (ex: glutamate dehydrogenase (T12 F416)) → next
 - c. Select project → next
 - d. Image directory (use default: `/gpf/leginon/username`)



- e. Connect to clients= t12-d297 and t12-F416 → next
 - f. Condenser size = 100 um → finish
11. Once Leginon has started up, open the application
- a. Application → Run → MSI-T2 (3.3)
 - i. Scope = t12-d297
 - ii. Camera = t12-f416
 - iii. Main = biotwinleginon01 → run
12. 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, shut down leginon and restart both clients on EM and camera computers)
 - b. “Find” presets from past 20 days
 - c. Select desired presets (usually from the most recent user)
 - d. Highlight all the presets (GR, SQ, HL, FA, FC, EN) and “import” → “done”
 - i. Suggested mags for EN/FA/FC = 49,000x; HL = 4,800x; SQ = 890x; GR = 100x.
We recommend the use of condenser aperture #4 = 200um with beam spot size 5 for EN preset
 - e. Close window
 - f. “Cycle” presets to minimize hysteresis in the beam
 - g. Under “preset manager” node, select “settings” button and check the “cycle presets” option (see image)
13. 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
14. Wait until IGP1 = 6 (or < 20)
- a. Open shield of cryo holder when IGP1 <20; you may see a slight increase in pressure, but should settle within a few seconds
15. Center preset beams
- a. Send stage to square of non-interest (square with carbon that you don't mind trashing)
 - i. Under “square_targeting”, select “acquisition” mouse and select square of non-interest; submit target (click green “play” button)
 - b. Once the stage stops moving, check that beam is centered at all presets
 - i. Center “square” preset beam
 1. Send scope to “square” preset
 2. Lower green screen
 3. Center beam using left trackball
 4. Open the “Edit” box and click “apply beam shift from scope”
 - ii. Center “hole” preset beam



1. Send scope to "hole" preset
2. Lower green screen
3. Center beam using left trackball
4. Open the "Edit" box and click "apply beam shift from scope"
- iii. Center and adjust intensity of FA/FC/EN preset beam
 1. Send scope to "EN" preset
 2. Lower green screen
 3. For the EN preset, adjust beam intensity to ~0.5 sec (little screen on flu screen), with a beam diameter that is about 1.5X the size of the second circle
 - a. For the FA/FC preset you can make this beam a bit brighter and smaller
 4. Open the "Edit" box and apply intensity "from scope" and beam shift "from scope"
 5. Repeat for FA and FC preset
16. Get eucentric height of your grid
 - a. Send sq preset "to scope"
 - b. Open column valves + lower green screen (R1 on knobset)
 - c. Using right knobset trackball, move stage until you see an intact grid square with visible ice & carbon; center that square on the green screen; NOTE- work quickly as not to overexpose your grid to the beam
 - 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, +/- 20 um; 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)
17. 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)
18. Immediately after atlas is collected, insert and center objective aperture
 - a. Under “square targeting”, select square of non-interest
 - b. Using flu screen and right knobset trackball, center in the middle of 4 squares on the grid bar (this way you can see the edge of the beam on 4 squares)
 - c. While watching the beam on the flu screen, insert the objective aperture
 - d. If you see the same beam as pre-aperture, no centering needs to be done
 - e. If a portion of the pre-aperture beam is blocked out, adjust objective aperture centering using objective adjustment knobs (see picture on the right →)
 - f. Note- ALWAYS watch the beam on the flu screen when adjusting the objective position!
19. In “Square_Targeting” select 1 square of interest
 - a. Using “acquisition” mouse, select the center of square of interest
 - b. Click “Submit target”
 - c. Click “Submit queued targets”
20. In “Hole_Targeting” select 1 - 2 regions of holes of interest
 - a. Using “acquisition” mouse, select holes of interests (on the carbon, every 2x2 holes away from each other)
 - b. Using “focus” mouse, select area of carbon towards the center of the hole areas you selected in the step above (this is where leginon will acquire the eucentric height of the square)
 - c. Click “Submit target”
 - d. Click “Submit queued targets” when you are done with every hole target
21. In “Exposure_Targeting” select exposure targets
 - a. Using “acquisition” mouse, select the center of the holes of interests, on the ice. Select up to 4 holes that are closest to the center of the screen (use “toggle center crosshair” tool to find where center is). This ensures that you do not collect images with a lot of image shift.
 - b. Using “focus” mouse, select area of carbon towards the center of the stage (use “toggle center crosshair” tool to find where center is). This is where leginon will acquire auto-focusing for the exposure targets selected in the step above.
 - c. Click “Submit target”
 - d. Click “Submit queued targets”
 - i. Note- at this point you have queued up a number of exposure targets for leginon to acquire. Refer to “Leginon/Appion User Guide” to view micrographs and for further instructions.
22. Under “exposure” node, select “toggle queue timeout” so that column valves will close once exposure queue is done collecting



23. To collect a new square, repeat steps 19 - 21 until you are done collecting data.
24. 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 and IGP4 read 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 “wet nitrogen” tank
5. Bake out the cryo holder
 - a. See “Dry Pumping Station User Guide (for cryo holders)” for detailed instructions
6. Close Legimon client on both “camera” and “microscope” PC and log off the Legimon workstation
7. Cleanup the bench space for the next user
8. Log any issues into the EM notebook or notify SEMC staff directly

Dry Pumping Station User Guide (for cryo holders)



Normal state:

- V1 – closed
- V2 – open
- V3 – open
- V4 – open

Warm up holder

~30min cycle

Removing holder from dry pumping station:

1. Unplug holder jacket from temperature controller
2. Close V4, V3, V1, then 2 of holder
3. Unhook tygon tubing from holder and re-connect to silver stopping plug (near V1 & V2)
4. Turn pumping station off
 - a. Wait for green “MDP ready” to turn off
5. Using one hand to stabilize V1/V2 vacuum area, gently apply force to pull out cryo holder
 - a. Immediately upon removing cryo holder, store in cryo transfer station
6. Place blanking plug (black) into opening where cryo holder was

Inserting holder into dry pumping station (warm up):

1. Connect cold holder to the temperature controller and run the “warm up cycle”
2. Turn on pumping station and wait for green “MDP ready” state (~1 minute).
3. Make sure all valves are closed.
4. Remove the blanking plug and insert cryo holder and invert it to facilitate condensation removal.
5. Connect the plastic tygon tubing to V4 of the cold cryo holder
6. Open V2, then V1 and wait until vacuum stabilizes
7. Once warm up is done, open V3 and V4
8. Start the “zeolite cycle” for 2 hours

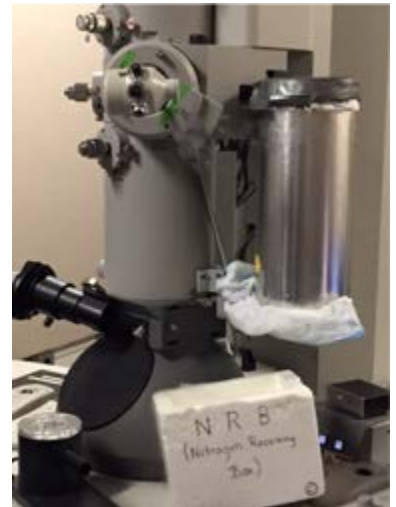
Cryo Holder Insertion/Retraction Guide

*****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, HT ON, column valves closed, reset holder, objective out, flu screen down.
2. Check that airlock default pump time is >120sec.
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 shows up in the Vacuum Overview window.
6. Select 'ST Holder' as specimen holder.
7. With hand on the holder's dewar, reset alpha value. The stage will rotate back to 0 degrees.
8. Turn holder counter-clockwise to second stop and apply slight resistance to slowly let the vacuum pull the holder in.
 - a. Top off holder dewar with LN2 once inserted
9. Watch IGP1/Column vacuum for spike and wait for it to get below 10. If vacuum spikes and column pressure crashes, contact an EMG staff member immediately.
10. 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.
11. Turn filament ON.



Cryo Holder retraction:

1. Close column valves, reset holder, objective out, flu screen down, filament OFF, cryo shield of holder closed
2. Gently brace against the column and pull holder back till it stops, then turn clockwise to 5 o'clock and pull straight out of the column.
3. Watch IGP1/Column vacuum for spike. 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.

1. Gun tilt
 - a. Remove objective aperture (silver tab pointed right)
 - b. Center the stage (with stage shift trackball/joystick on right knobset) over a square with intact carbon and some large features that are visible at low mag.
 - c. With microscope computer, under "Tune" tab → "Gun" folder → "Gun Tilt"
 - d. Focus beam (with intensity knob)
 - e. Center beam (beam shift trackball on left knobset)
 - f. 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 filament
 - g. Condense and center the beam (trackball)
 - h. Open beam up to the RIGHT so that it's just past second circle
 - i. Center using condenser 2 aperture
 - j. 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. Eucentric Height
 - a. Find a square with some dirt on it
 - b. "Z-focus node" → simulate target
4. Eucentric focus
 - a. "Z-focus node" → "MF"
 - b. Find zero focus; reset focus
5. 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
6. Condenser Astigmatism (high mag, ~135k)
 - a. "STIG/FEG" tab → "Condenser"
 - 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
7. Objective Aperture Centering (~25 – 50K)
 - a. Focus and center beam to a point
 - b. Click "Diffraction" on right knob-set
 - c. Center beam using MF X and Y
 - d. Insert objective aperture (silver tab pointed left) and center aperture
8. Objective Astigmatism (~25 – 50k)



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

- a. At slightly underfocus (2-3 thin rings) use Live FFT and get rings to be a circle (elliptical = objective astigmatism). Use MF X and Y to correct the astigmatism.
9. Get eucentric height & focus again
10. Pivot point alignment
 - a. Direct alignment → pivot point x
 - b. Use MF-X and MF-Y to minimize beam movement
 - c. Click “done”
 - d. Repeat for pivot point y
11. Beam shift alignment
 - a. Direct alignment → beam shift
 - b. Use MF-X & MF-Y to center beam
 - c. Click “done”

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