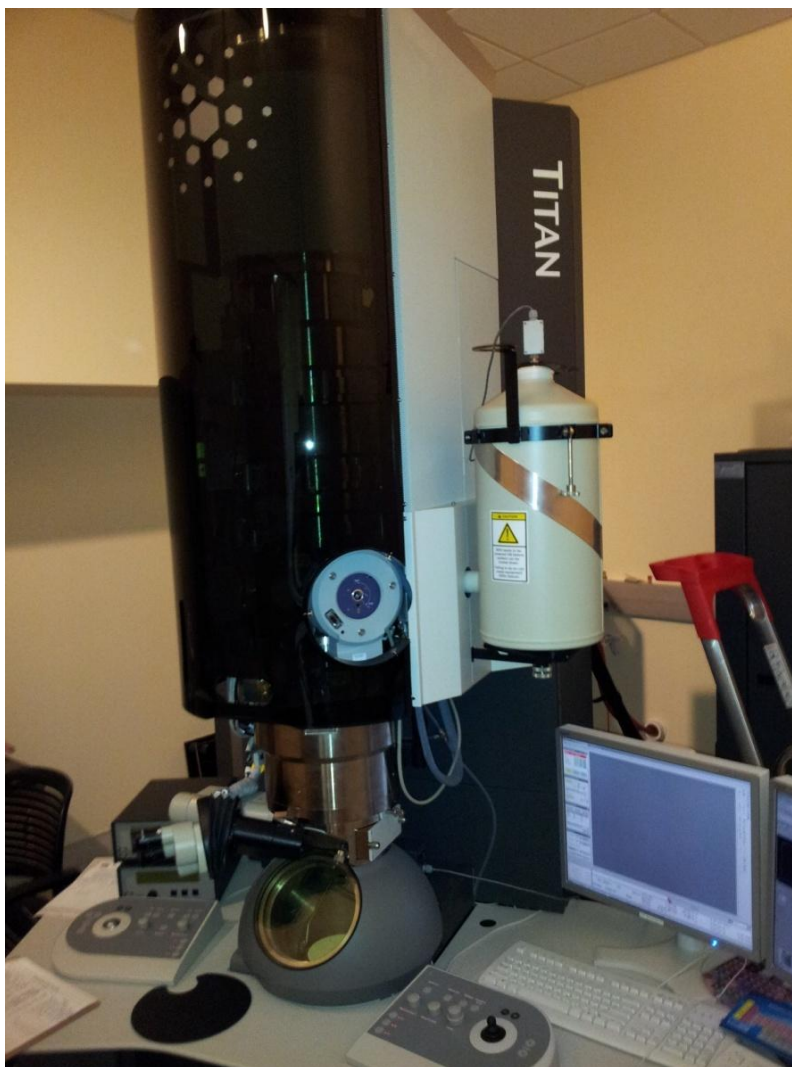




XFEG-Titan Halo 300kV TEM Instructions



THIS MANUAL IS TO BE SERVED AS A DETAILED GUIDELINE FOR GENERAL USE OF THE TITAN HALO 300KV TEM, INCLUDING CRYO-IMAGING. **YOU ARE EXPECTED TO TAKE NOTES DURING TRAINING SESSION** AND KEEP UPDATING THE NOTE BOOK AS YOU PROGRESS THROUGH PRACTISE SESSIONS. **PLEASE ALWAYS BRING THIS MANUAL AND YOUR NOTE BOOK WITH YOU EACH TIME YOU USE TITAN HALO TEM.**

PLEASE ALWAYS REMEMBER TO **ENABLE** AND **DISABLE** TITAN HALO 300KV TEM IN THE BADGER SYSTEM BEFORE AND AFTER YOU USE THE INSTRUMENT.



User Interface Overview

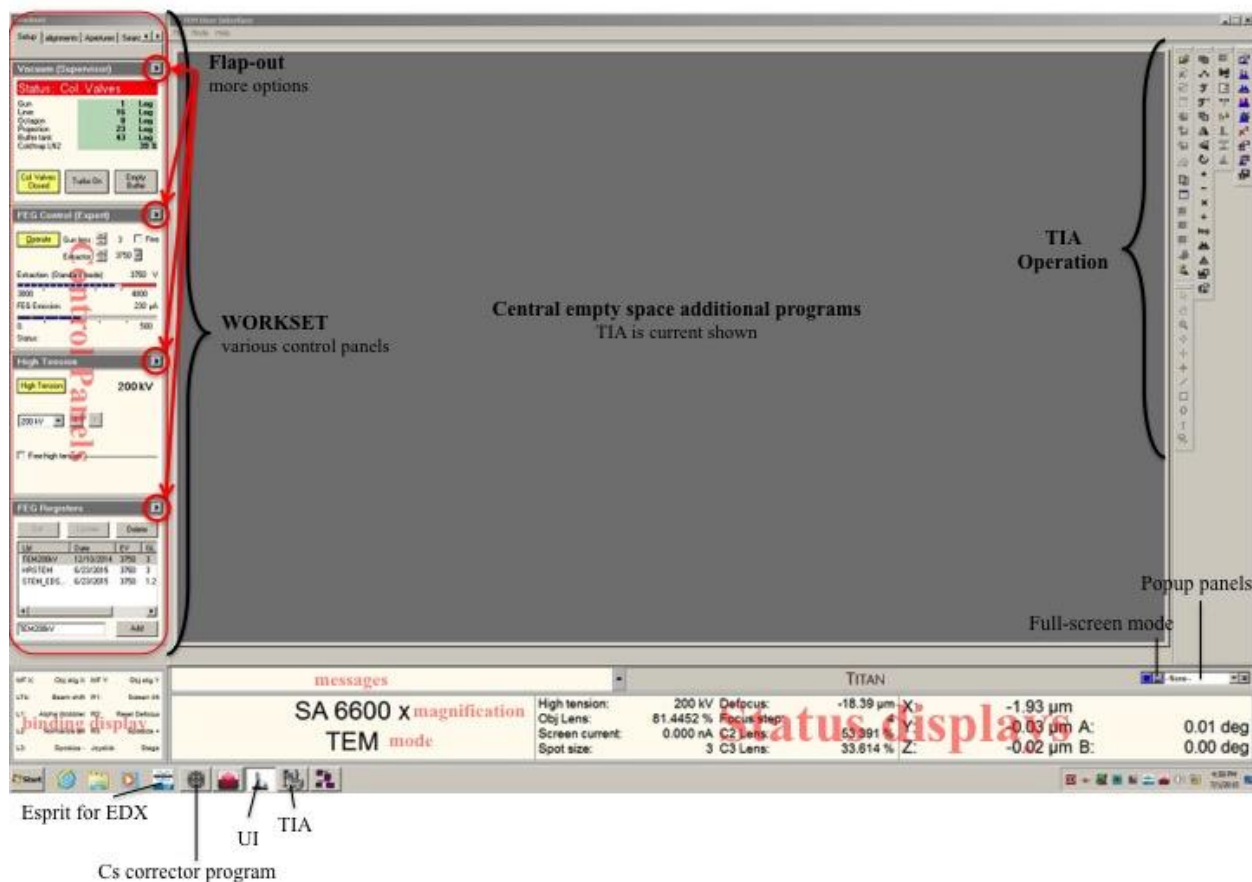


Figure 1

FEI TITAN Halo 300kV TEM is designed for optimal parallel illumination both at room temperature and cryogenic conditions. The interface contains three parts: TEM server (will not covered in this manual), User Interface (UI), and Control pads (left & right). Users interact with TEM server via UI. An overview of UI is shown in Figure 1. UI contains elements that can be divided into three categories:

- Main program (top region): program title and menu bar.
- Control panels (left region): control panels are grouped in worksets that can be selected via a tab at the top-left of the area within control panels.
- Information panels (bottom region): binding, messages, status displays.

The central empty space is reserved for other data display (e.g. TIA, Digital Micrograph).

Notes: Position the mouse cursor over a particular panel and press **F1** on keyboard to invoke the UI online help pages for that particular panel.

Microscope Control Pads Overview

Control pads (left & right) consist of a set of standard TEM controls (Figure 2):



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- Left Pad: Tilt (α & β), User Beam Shift Trackball, Intensity (Brightness), Stigmator, Multifunction X (MF X), programmable buttons (L1, L2, L3).
 - Right Pad: Diffraction, Wobbler, Eucentric Focus, Multifunction Y (MF Y), Magnification, Focus, Focus Step, Joystick, Z height, programmable buttons (R1, R2, R3).
- MF X/Y knobs' default function is "user beam shift X/Y" and can be assigned (bind) to other functions. Programmable buttons are user-defined and can be assigned (bind) to frequently used functions (**please do not re-assign functions to the six programmable buttons**).

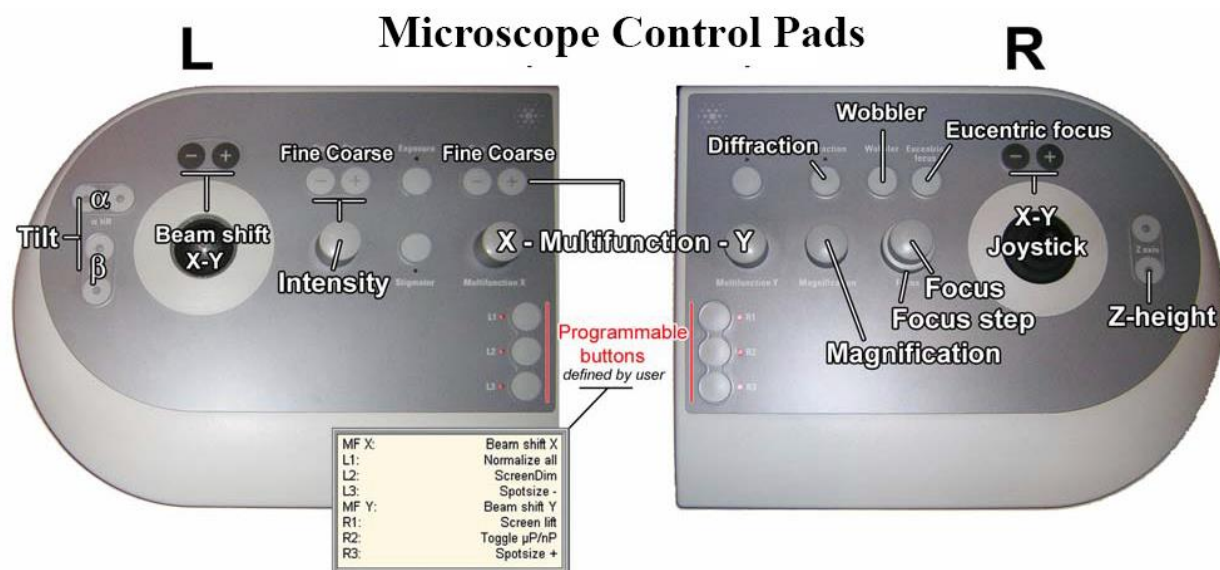


Figure 2

I. Preparation

DO REMEMBER TO SIGN IN BADGER and enable Titan Halo 300kV TEM in Equipment Actions.

1. Please go directly to step 2 if the microscope computer is logged in and TEM user interface (TUI) and TEM Imaging & Analysis (TIA) are already running (Figure 1), Please speak to facility staff if either of the above conditions is not true. The following is only for experienced users: Log in using supervisor account. The microscope server control software (Microscope Software Launcher) should start automatically (Figure 3). Click icon in the windows quick launch panel (Figure 4) if it does not start. Click the play button (green triangle) to start TUI (Figure 3). Launch other software (e.g. TIA) if needed. TUI software launching progress can be monitored by click **window>message** (Figure 3).
2. Check the status of vacuum in **Setup>Vacuum** (Figure 1). It should display **Status: Col. Valves**.



Figure 3



Figure 4



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3. Check the Gun pressure and the liquid nitrogen level (LN₂) in cold trap in **Setup>Vacuum** Figure 1). Please notify Imaging Facility staff immediately if Gun pressure is > 1 log and/or LN₂ is < 15%.
4. Make sure that column valves are closed: **Col Valves Closed** button in **Setup>Vacuum** is yellow (Figure 1).
5. Make sure that camera is retracted: **Insert** button in **Camera>CCD/TV Camera** is grey.
6. Make sure the stage is reset: values for X, Y, Z, α , and β in TEM status window at the lower right corner of TUI are zero or close to zero. If not, click **Holder** in reset panel of **Search>Stage>Control** page to center stage.
7. Make sure the objective lens aperture (obj aperture) is retracted. Objective lens aperture control can be found in **Tune>Apertures**.
8. Make sure **Cryo Box** is retracted (in grey color). Cryo box control can be found in **Setup>Vacuum (flap-out)>Cryo Box**.

II. Grid Loading/Unloading and TEM Holder Insertion/Removal

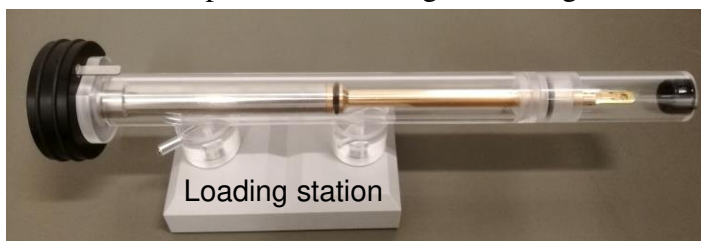
Note:

1. Specimen holder, airlock, and CompuStage are made up of delicate, precisely machined components. You should never have to apply significant force during any step of this procedure. Doing so may result in serious damage to the stage or holder.
2. Loading/unloading TEM grid to single tilt holder or cryo specimen holder should be done in loading station or cryo-transfer station respectively. Please pay attention to facility staff's instruction during the training and take notes. Remember to close specimen shutter after loading grid to cryo specimen holder.
3. Use two hands to move loading station with single tilt holder, one hand on the station and the other on the holder since the holder may slide out of station.

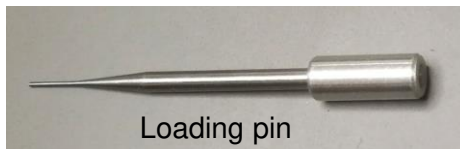
Specimen Loading (single tilt holder):

Note: Never mount magnetic specimen in the clamp holder. The clamp spring is not strong enough to prevent the specimen from attaching to the objective lens pole-piece.

1. Place the sample holder in the grid loading station, remove station cap.



2. Remove the sample-loading pin from station base.



3. Using one hand to hold back end of holder to prevent the holder from slipping out of the station, insert the pin into the pinhole in the grid clamp and gently raise the clamp



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straight up until it stops.



4. Place the grid into the recess at the end of the holder and make sure the grid and the bottom hole are aligned concentrically.



5. Gently lower the clamp straight down with sample-loading pin to hold the grid securely. Return the pin to station base.
6. Retract the holder slightly and turn it $\sim 180^\circ$. Tap the back end several times to check that the grid does not move in the holder.
7. Inspect the holder o-ring and remove any debris using Kim Wipes.

Specimen Loading (cryo-holder):

- 1) Remove cryo holder from pumping station (see pumping station SOP for more details)
- 2) Gently and fully insert it horizontally into the cryo-workstation.



- 3) Keep cryo-holder connected to its controller, fill LN2 to the holder dewar and cryo-workstation, wait for the holder temperature to drop below -180°C (minimum) before loading grid to the holder.

Note: keep surface of LN2 just above the holder tip.

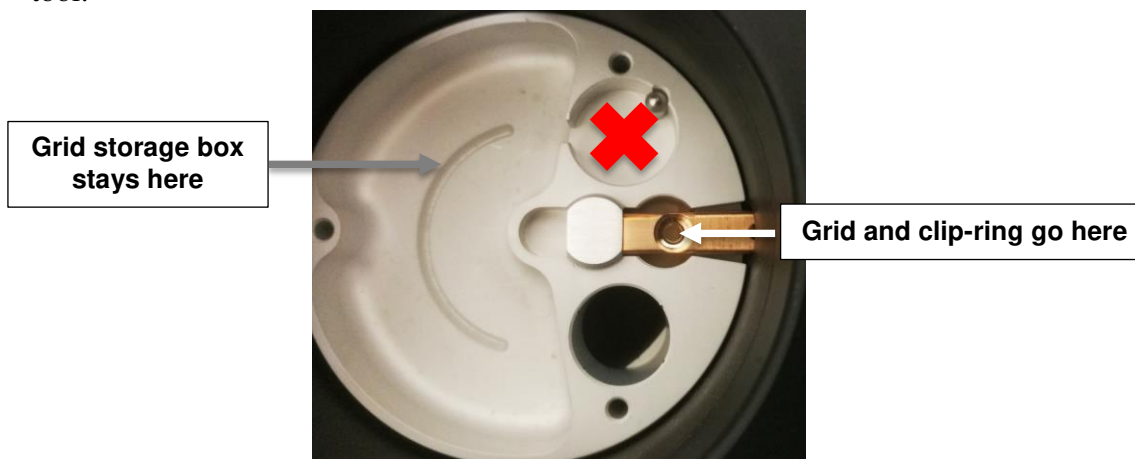
- 4) Quickly transfer cryo grid storage box from storage tube to the cryo-workstation, keep the storage box submerged in LN2 while it is in cryo-workstation. **Do not insert storage box into position marked with Red Cross in Figure below.**

Note: any tools that will touch the storage box, sample grids, and holder tip must be precooled to LN2 temperature.



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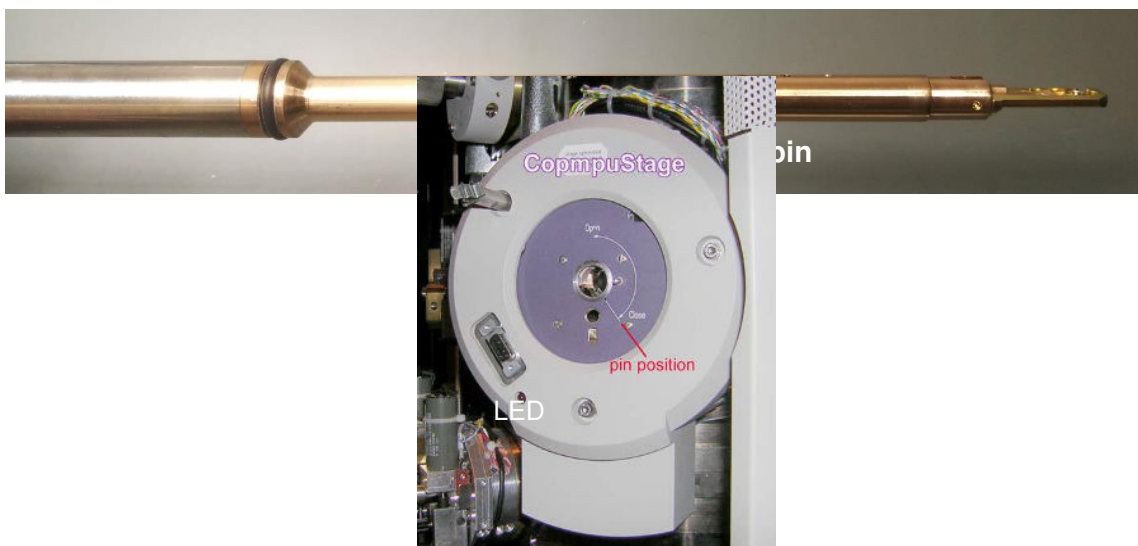
- 5) Unfasten the crew on storage box with precooled screw-driver by rotating it one full turn, gently grip the grid with precooled tweezers and move it from storage box to the holder tip, make sure the grid sit in the center of the holder tip.
- 6) Secure the clip-ring on the clip-ring tool, load clip-ring straight down to holder tip and push down straight to secure clip-ring on the holder tip, release clip-ring from clip-ring tool.



- 7) **Always make sure the clip-ring sits securely on the holder before inserting holder into the TEM column. You can use tweezers to gently touch the edge of clip-ring to check it out.**
- 8) Close specimen shutter on holder, fill LN2 in transfer station to just over holder tip.
Note: specimen shutter must be close all the time during holder insertion into and removal from microscope.

Holder Insertion:

1. Align holder rod well with stage tube and point holder guide pin to 5 o'clock position on the goniometer (**Close** position on the purple surface), gently insert the holder until you feel some resistance. Be careful not to scrape the tip. Push the holder further in for 8 mm with slightly increased force.



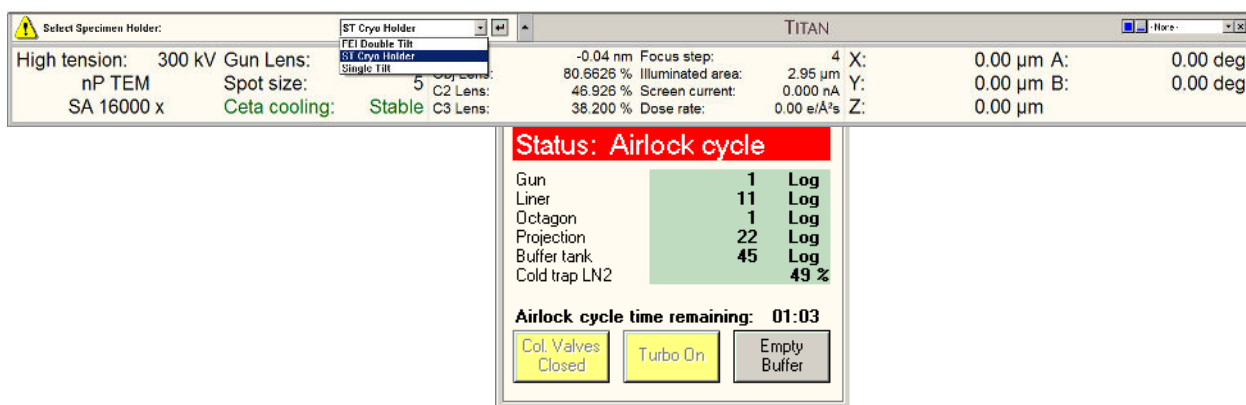
2. The airlock begins pumping and the red light on the CompuStage is on. Do not move the



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holder while the red stage LED is on.

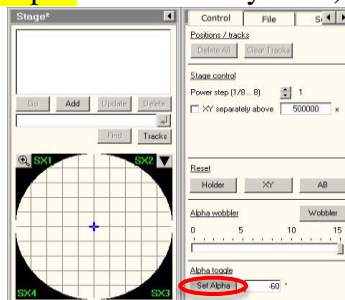
3. The airlock pumping time counting-down is visible in the **Vacuum** control panel.
4. Select the specimen holder type (Single Tilt) from the box in TUI message window. Be sure to click the **✓** button to confirm the selection.
5. When the pumping time counting-down finishes (**Status: COL. VALVES**) and the red stage LED is off, grip the holder securely and slowly turn the holder counterclockwise (~150°). This rotation moves the guide pin approximately from 5 o'clock (Close) to 12 o'clock (Open).
6. Now you feel the column vacuum is pulling the holder in. Continue gripping the holder securely and guide the holder slowly into the microscope column until it stops (**DO NOT let the vacuum pulls the holder in freely**), gently tap the end of the holder to make sure it is securely seated. GUN/Col vacuum may rise to 14-20 log and slowly goes down.



7. Wait for the Octagon to drop <15 log (< 10 log is highly recommended).

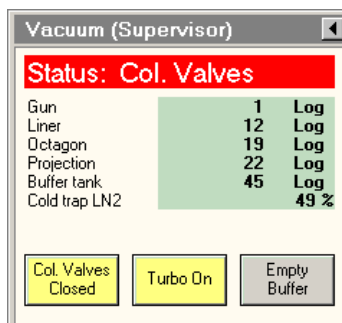
Cryo-holder Insertion:

1. Make sure the column valves are closed. Tilt stage to -60° (α tilt) by click **Set Alpha - 60°** in **Search> Stage** (**Set Alpha** button turns yellow). Now the Close sign is at 3



o'clock position.

2. Start turbo pump by click the **Turbo On** button and wait until the button turn yellow (it takes about 1 min).
3. Remove cryo-holder from cryo-workstation and raise it to the same height as the CompuStage, align holder rod well with stage tube and point holder guide pin to 3



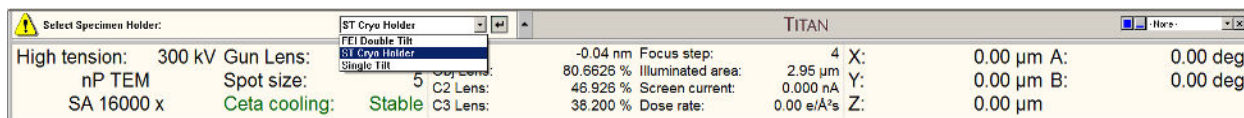


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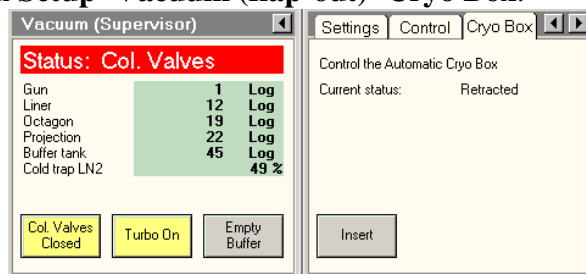
o'clock position on the goniometer (**Close** position on the purple surface), gently insert the holder until you feel some resistance. Be careful not to scrape the tip. Continue without stop to push the holder further in for 8 mm with slightly increased force. Use one hand to stop cryo-holder to rotate counter-clockwise by itself.

Note: Always use a styrofoam box below LN2 dewar of cryo-holder to collect excess LN2 from dewar.

- The airlock begins pumping and the red light on the CompuStage is on. Do not move the holder while the red stage LED is on. The airlock pumping time counting-down is visible in the **Vacuum** control panel.
- Select the specimen holder type (ST cryo holder) from the box in TUI message window. Be sure to click the **✓** button to confirm the selection.



- When the pumping time counting-down finishes (**Status: COL. VALVES**) and the red stage LED is off, grip and turn holder counter-clockwise for 150°. This rotation moves the guide pin to 10 o'clock (Open). Remember the stage is still tilted at -60°.
- Now you feel the column vacuum is pulling the holder in. Continue gripping the holder securely and guide the holder slowly into the microscope column until it stops (**DO NOT let the vacuum pulls the holder in freely**), gently tap the end of the holder to make sure it is securely seated. GUN/Col vacuum may rise to 30-50 log and slowly goes down.
- Tilt stage back to 0° by clicking **Set Alpha -60°** in **Search> Stage** (Set Alpha button turns grey).
- Insert **Cryo Box** in **Setup>Vacuum (flap-out)>Cryo Box**.



- Wait for the Octagon to drop <15 log (< 10 log is highly recommended) before proceeding to next step. This step normally takes 20-30 mins.
- Open specimen shutter on cryo-holder and wait for 1 min before opening column valve.
- Refill LN2 to holder dewar every three hours to guarantee holder temperature to be kept at ~ -180°C.

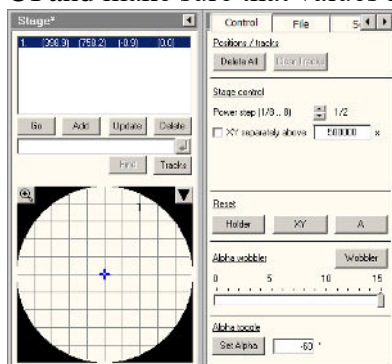
Holder Removal:

- Click **Col Valves Closed** button in **Setup>Vacuum** to close the column valves and make sure the button is in yellow color.



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2. Make sure the stage is centered: click **Holder** in reset panel of **Search> Stage>Control** page to move stage to center, watch the TEM status window at the lower right corner of UI and make sure that values for X, Y, Z, α , and β are zero or close to zero.



X:	-6.07 μm	A:	0.00 deg
Y:	-0.11 μm	B:	0.00 deg
Z:	0.01 μm		

3. Retract obj aperture or Phase Plate if you ever used it.

Note: both obj aperture and stage are motorized; you must wait for them to fully stop before proceeding to next step.

4. Place the viewing screen down; cover the window with the rubber mat.
5. Always keep light pressure on the purple surface of goniometer by left hand, pull the single tilt holder straight back by right hand without rotating until it stops moving (as far as it can go).
6. Turn the holder clockwise gently until it stops, such rotation will move the guide pin approximately from 12 o'clock position to 5 o'clock (150°).
7. While keeping pressure on the purple surface of goniometer, gently pull holder straight back to break airlock vacuum, this step requires small amount of force.
8. Move the holder straight back out of the stage, be careful not to scrape the holder along the inside of stage.

Note: be careful not to touch the holder o-ring and rod with bare hands.

Cryo-holder Removal:

1. If you plan to load another cryo grid, precool the cryo-workstation before removing holder from microscope.
2. Click **Col Valves Closed** button in **Setup>Vacuum** to close the column valves and make sure the button is in yellow color.
3. Make sure the stage is centered: click **Holder** in reset panel of **Search> Stage>Control** page to move stage to center, watch the TEM status window at the lower right corner of UI and make sure that values for X, Y, Z, α , and β are zero or close to zero.
4. Retract obj aperture or Phase Plate if you ever used it.

Note: both obj aperture and stage are motorized, you must wait for them to fully stop before moving on to next step.

5. Place the viewing screen down, cover the window with the rubber mat.
6. Close the shutter on the holder.
7. Retract **Cryo Box** in **Setup>Vacuum (flap-out)>Cryo Box**
8. Tilt stage to -60° (α tilt) by click **Set Alpha -60°** in **Search> Stage** (Set Alpha button turns yellow), follow room temperature operation to remove cryo-holder. In this case clockwise rotation will move the guide pin approximately from 10 o'clock position to 3 o'clock (still 150°).



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9. If you plan to load another grid, insert it into the precooled cryo-workstation, Keep stage α tilt at -60° .
10. If you finished your cryo-imaging session, set stage α tilt back to 0° by clicking **Set Alpha -60°** in **Search> Stage**, empty LN2 from the holder dewar and insert cryo-holder into the pumping station, open valve 1 and 2 on the pumping station, connect the cable, start Warm-up cycle with the old controller or Automastic with new controller (see pumping station SOP for more details).

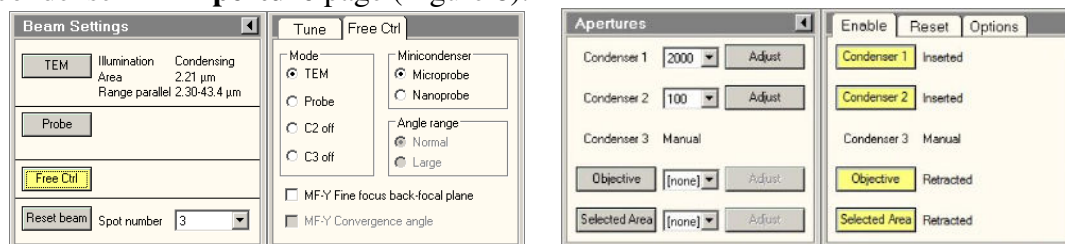
Note 1: Always use a styrofoam box below LN2 dewar of cryo-holder to collect excess LN2 from dewar when inserting/removing cryo-holder from the TEM.

Note 2: You can put both hands on the holder to remove it if you are not able to hold the holder steady by one hand. It is ok that no pressure is applied to the purple surface of the goniometer.

III. Operation and Alignment

Note: Always align the microscope from the top (gun) down. Press F1 on keyboard at any time for online help with an alignment. Begin with the objective apertures removed. Leave a condenser aperture inserted to avoid specimen damage from high beam intensity.

1. Finding the beam
 - 1) Wait for the Octagon to drop <15 log (< 10 log is highly recommended).
 - 2) Click **Col Valves Closed** button to open column valves, **Col Valves Closed** button should change from yellow to grey, find the beam using the fluorescence screen. In case you cannot find the beam, make sure the obj aperture is retracted, reduce the magnification to couple of hundreds in LM and move the stage in X-Y plane using joystick. Find a visible feature on the grids (a black dirt or holey carbon hole would be perfect) and center it in the view.
2. C2 aperture alignment (experienced users)
Set magnification to \sim SA 5000x, turn off C3 lens by check **C3 off** in **Beam Setting>Free Ctrl**. Activate condenser lens 2 (C2) aperture by clicking **Adjust** button for condenser 2 in **Aperture** page (Figure 8):



Method 1 (recommended): Focus the beam using **Intensity** and center the beam using left pad trackball; expand beam on both sides of beam focus and adjust C2 aperture by **MF X/Y** to make sure beam expands and shrinks concentrically.

Method 2: Focus beam and use left pad trackball to center beam; spread the beam to half of the large screen and use **MF X/Y** to center the beam disk. Do this step iteratively until the aperture is centered. In **Beam Setting** control panel (Figure 7), click **TEM** to go back to three-condenser lens system. This is a common mistake, users forget to go back to three-condenser lens system.

Note: It is always a good practice to deactivate button after adjustment (e.g. **Adjust**); make sure **Auto Zoom** in **Tune>Beam Setting** is activated (yellow).



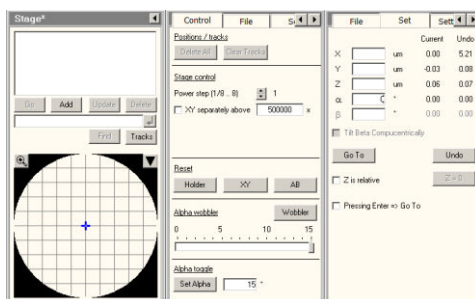
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3. Adjust Eucentric Height at magnification of ~ SA 5000x

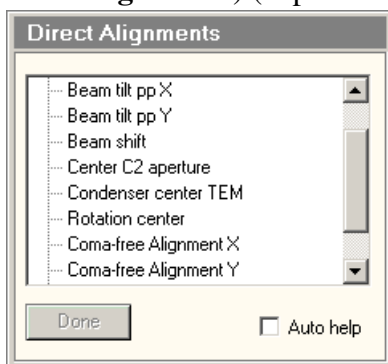
Center a recognizable feather on the grid and bring the sample to Eucentric height by one of the following methods.

Method 1 (recommended): Activate **Alpha Wobbler** in **Search>Stage>Control** (Figure 9), changing Z height by using the Z Axis buttons on right control pad to minimize the movement of image. This is the best way to adjust Eucentric height and recommended, but it needs more time and practice.

Method 2: Center the feature at alpha tilt = 0°, tilt stage to 5° in **Search>Stage>Set** (Figure 9) and adjust Z height to bring the feature back to center. Tilt back to 0° to check if the feature moves. Do this iteratively until the feature is stable while switching between 0° and 5°.



4. Select magnification in SA range (there are LM, SA, and Mh ranges). Note: a range of 30kx to 100kx is most frequently used for negative stained and cryo samples.
5. Direct Alignment (**Tune>Direct Alignments**) (experienced users):

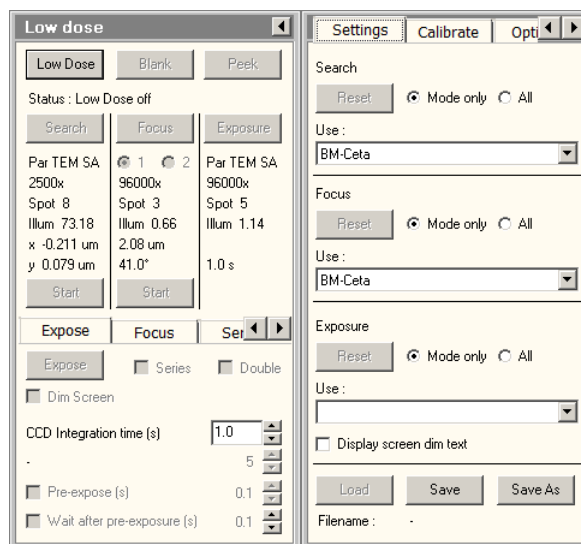


- 1) Click **Eucentric focus** on right control pad.
- 2) X-FEG is quite stable. There is no need to tune **Gun Tilt** and **Gun Shift**.
- 3) Beam tilt pivot point: Focus the beam by **Intensity**, click **Beam tilt pp X** and minimize beam vibration using **MF X/Y** knobs. Click **Done** when finished. Repeat the same procedure for **Beam tilt pp Y**.
- 4) Spread the beam to half of the screen using **Intensity**, move the stage to center a feature on the grid, click **Rotation Center** and minimize image/feature movement using **MF X/Y**. Click **Done** when finished.
- 5) Click **Beam Shift** and center beam by **MF X/Y**. This function is used only in alignment step. Always use left pad trackball for image shift in imaging step.
- 6) Preparation for coma-free alignment (do this alignment only for the high-resolution cryo-EM imaging):
 - Insert CCD camera.
 - Set magnification to 100 kx ~ 150 kx.



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- Set integration time for CCD search mode to 0.25s and activate Live FFT.
 - Monitor counts (mean value) in TIA and keep it to ~500.
 - Set defocus to show multiple FFT rings. Note: always do coma-free adjustment in defocus range.
- 7) Click **coma-free Adjustment X** and you should observe that the image moves between two positions. Watch the first FFT ring (Thon ring) and adjust **MF X** to minimize changing of the first ring. If the first ring switches between two states of larger and smaller you definitely need to adjust **MF X**. Click **Done** when finished.
 - 8) Repeat step g for **coma-free Adjustment Y**.
 - 9) Correct objective lens stigmatism (obj stig) while performing coma-free adjustment. It is good practice to correct obj stig at relatively higher magnification than the final imaging mag.
 - 10) It is not necessary to perform **coma-free pp X/Y** for room temperature imaging. Note: Finding a proper imaging condition to adjust coma-free tilt is difficult and need more patience and practice, remember to click **Done** when finish each alignment.
6. Press **Eucentric focus** button on right control pad and bring the sample to close to focus by gently changing Z height.
 7. Objective Lens Aperture (optional)
 - 1) In **Aperture** panel choose 70 um obj aperture (**Objective** button turns yellow).
 - 2) Press **Diffraction** button on right control pad to enter diffraction mode, set camera length to 500 - 1000 mm by **Magnification** knob and center diffraction spot by adjusting **MF X/Y**.
 - 3) Then click **Adjust** button of **Objective** aperture, use **MF X/Y** to center the aperture. Click **Diffraction** again to return to image mode.



Low Dose Setup for Cryo-EM (carbon film grid only)

1. Click **Low Dose** button in **LowDose** panel in workset to enter low dose mode (LD).
2. Set up individual LD mode. Use CCD camera for all three modes.



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- a. **SEARCH** mode: choose larger spot size 5-8, microprobe beam, low magnification that must be in the same SA range with **FOCUS** and **EXPOSURE** (the lowest magnification in SA range is 2250x), spread beam. Apply large defocus (50-100 um) to achieve good contrast. Set integration time to 0.25s. Note: the principle of setting up **SEARCH** is to keep minimal but enough electron density at sample (count is ~50) to observe sample on CCD camera.
- b. **FOCUS/EXPOSURE** mode: use the same settings for the two modes, choose magnification of 50-100 kx, spot size 3-6, microprobe beam, illumination area 1-2 um.
3. Set up connections between LD modes.
 - a. **EXPOSURE** mode: this is most important of all three modes. Perform **Direct Alignments** and center a feature (a big black dirt, holey carbon holes, or corner of copper bar, it should be something that has good contrast and easy to center).
 - b. Go to **SEARCH**, make sure that **Search Shift** is checked in **Low dose** (flap-out) >**Option**, adjust **MF X/Y** to center the same feature that is centered in **EXPOSURE** mode.
 - c. Go to **FOCUS**: make sure it has the same microscope setting with **EXPOSURE**, define focus distance and angle. Focus distance must be at least 2 times of illumination area; focus angle can be set to 0-360° but only do 0° and 180° for tomography data collection. For Cflat/Quantifoil grid, use focus distance and angle to set focus position in the center of four grid holes.
4. Always suggest to cycle the three mode: **SEARCH>FOCUS>EXPOSURE>SEARCH**. Find area of interest for imaging in **SEARCH**, define defocus in **FOCUS** using Thon ring method (-2.5um for cryo-imaging), and take image in **EXPOSURE**.

Taking Images

1. Focus: move the stage to bring amorphous carbon film in the center of the view. Activate live imaging and live FFT in the CCD camera control panel (**Search**). Focus on carbon film by adjust the **Focus** on the right control pad to make the Thon rings disappear in the live FFT panel, click R2 on right pad to reset defocus and use Focus knob on right pad to set defocus value to -1 ~ -1.5 um for negative stained samples and -2.5 ~ -3.5 um for cryo-samples.
2. Obj stig: Carefully exam Thon rings in live FFT panel. If the Thon rings are not circular, active **Objective** Stigmator to adjust obj stig until the first Thon ring is circular.
3. It is recommended to focus on the sample before taking images.
4. Low Dose: cycle among Search, Focus, and Exposure to find area of interests (AOI), focus area close to AOI and take image of AOI.

Note: data collection using third party software will not be covered in this manual. You should take your notes during training.

Shutdown Procedure

1. Click **Col Valves Closed** button in **Setup>Vacuum** to close the column valves and make sure the button is in yellow color.



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2. Make sure the stage is centered: click **Holder** in reset panel of **Search> Stage>Control** page to move stage to center, watch the TEM status window at the lower right corner of UI and make sure that values for X, Y, Z, α , and β are zero or close to zero.
3. Retract objective lens aperture if you ever used it.
4. Click **Insert** button in **Camera>CCD/TV Camera** to retract CCD (BM-ceta) camera and make sure that the button is in grey color.

Note: Camera, obj aperture and stage are motorized, you must wait for them to fully stop before moving on to next step.

5. Place the viewing screen down, cover the window with the rubber mat.
6. Remove holder from microscope following instructions stated in Holder Removal.

Note: If you are doing cryo-EM, close the shutter on the holder, retract the cryo box, then tilt the stage (α tilt) to -60° , and remove holder from the microscope following instruction in Cryo-holder Removal.

7. Clean up working area, DO NOT leave a mess behind you.

DO REMEMBER TO SIGN IN BADGER again and disable Titan Halo 300kV TEM in Equipment Actions.