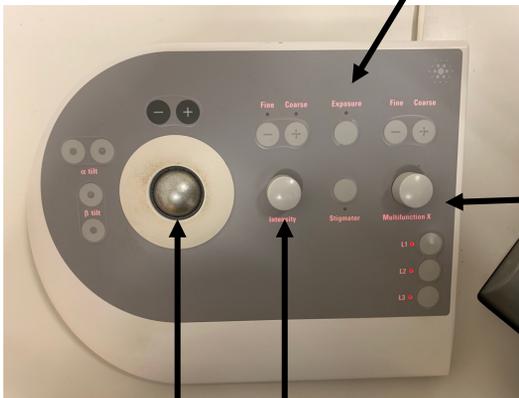
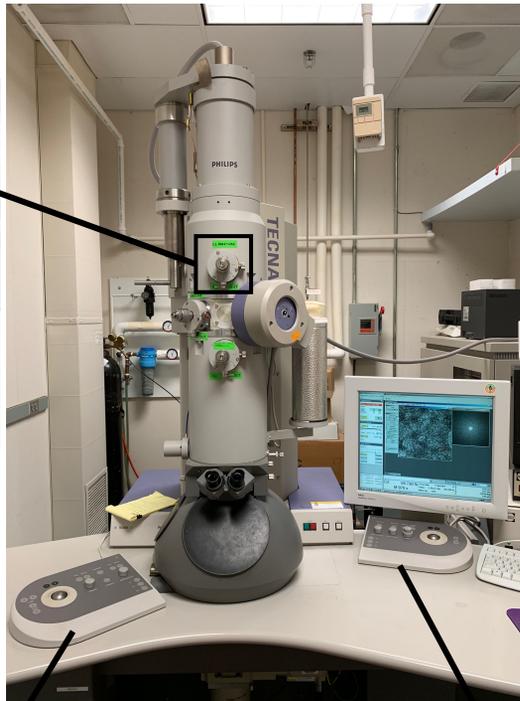


T12 Standard Operating Procedures (Negative Stain)

The Tecnai T12 is generally used for screening freezing conditions and sample distribution on your grid. It can also be used to perform some initial image processing to get an idea of how well your sample is behaving in cryo. The T12 is also used to screen and collect data from negative stain grids. The negative stain images can be processed to yield more information about your sample. Below are the steps to guide you to get the best data out of your session at microscope.

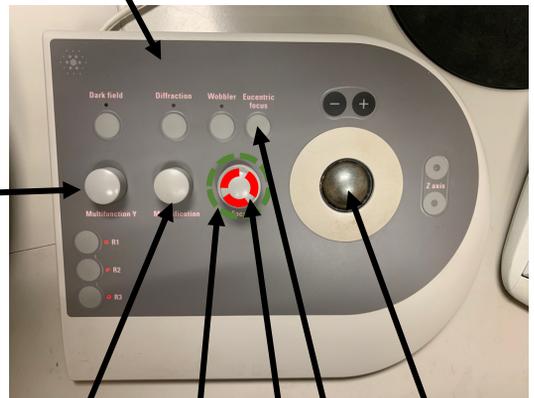
T12 Anatomy



Trackball to align beam

Intensity

Multifunction X/Y



Magnification

Focus Step

Focus

Stage Trackball

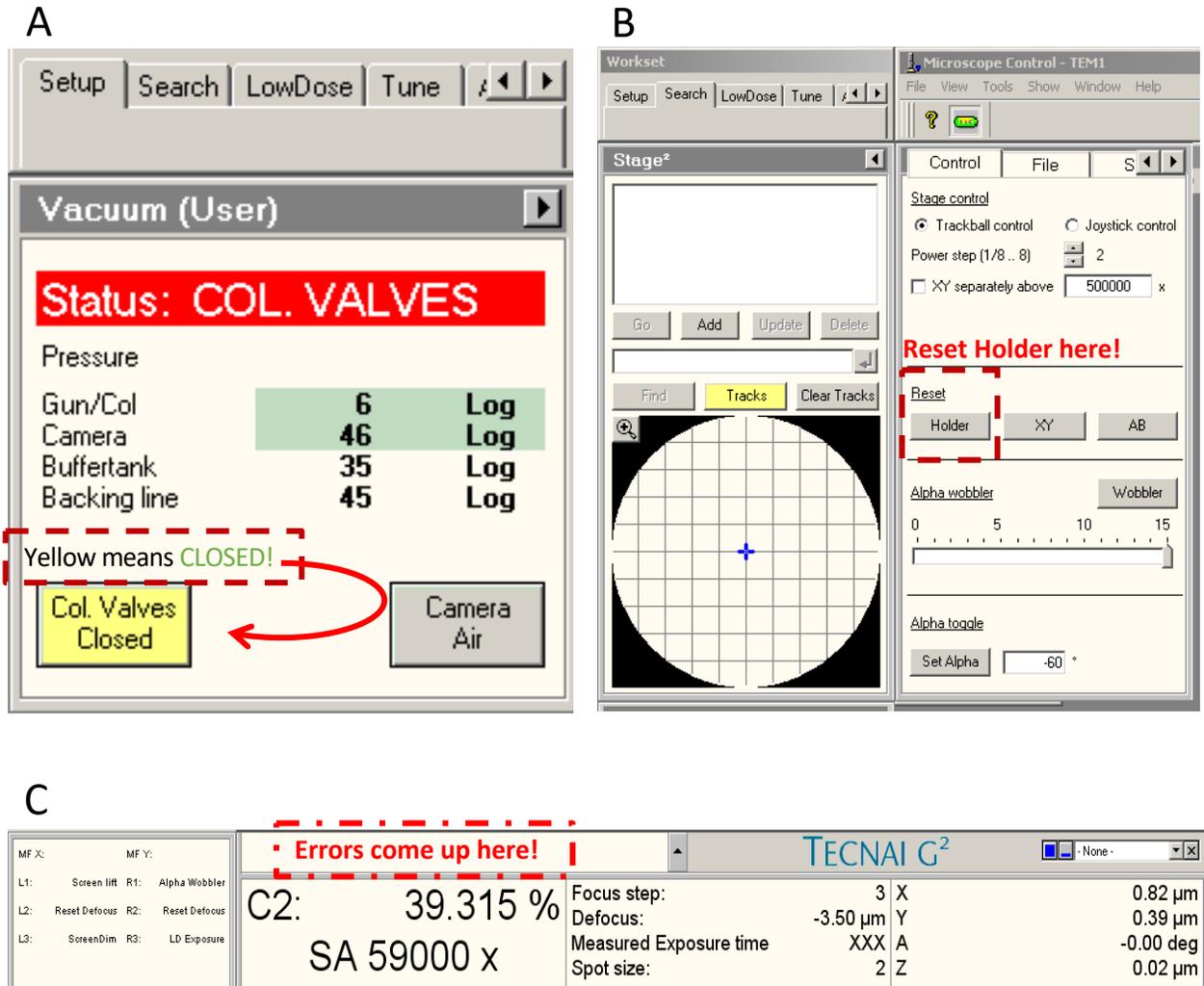
Eucentric Focus

I. Starting your session

Before you start your session, make sure that:

1. You check the last user made an entry in the Log book. *Were there problems?*
2. Note any Error messages on the Tecnai interface (Figure 1C).
3. The vacuum status, mainly the Gun/Col value. The values should be close to those shown in Figure 1A.
4. The Column Valves are closed (should be yellow, see Figure 1A).
5. The stage is centered (Figure 1B). (Search > Stage² > Reset Holder)

Figure 1: The status of the microscope at the start of your session



After confirming the above points:

1. Check the **High Tension** status. If you are the first user, you will have to turn it on from the Microscope user interface, see Figure 2. After turning it on you will hear a high-pitched sound and the emission value will spike and then should return below $2 \mu\text{A}$ (red arrow oval Figure 2).
2. Cool the cold trap on the microscope with liquid nitrogen. Allow for 15-20 minutes before you insert the specimen holder. You can align the beam during this time.
3. If the the emission value has fallen below $2 \mu\text{A}$, turn on the **Filament** (yellow means on).

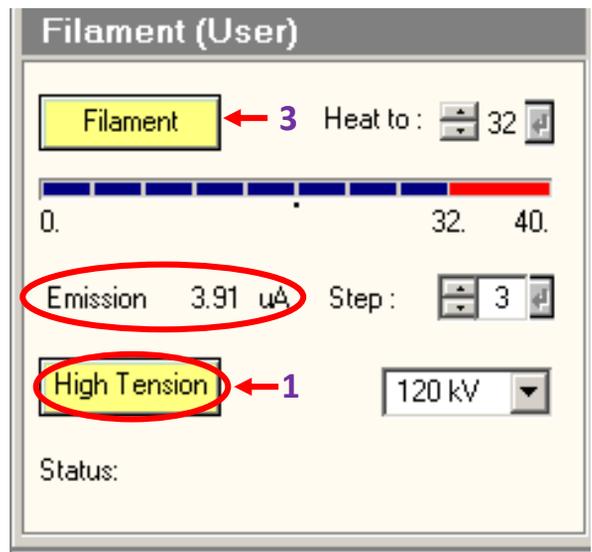


Figure 2: High Tension and Filament

II. Find beam and perform direct alignments.

One has to correct any defects in the way the electron beam is incident on your sample. Not aligning the microscope well will result in poor image quality and difficulty in processing and interpreting data. Therefore, this is the most important thing you need to check before you take your first image. As such on a day to day basis, the alignments should not vary a lot from user to user.

NOTE: All imaging (even negative stain images) is done in Low Dose mode.

The alignments are carried out by going into the TUI window (Figure 3 left) and selecting Low Dose > Exposure Mode (Figure 3 right) and then switch to Tune > Direct alignments.

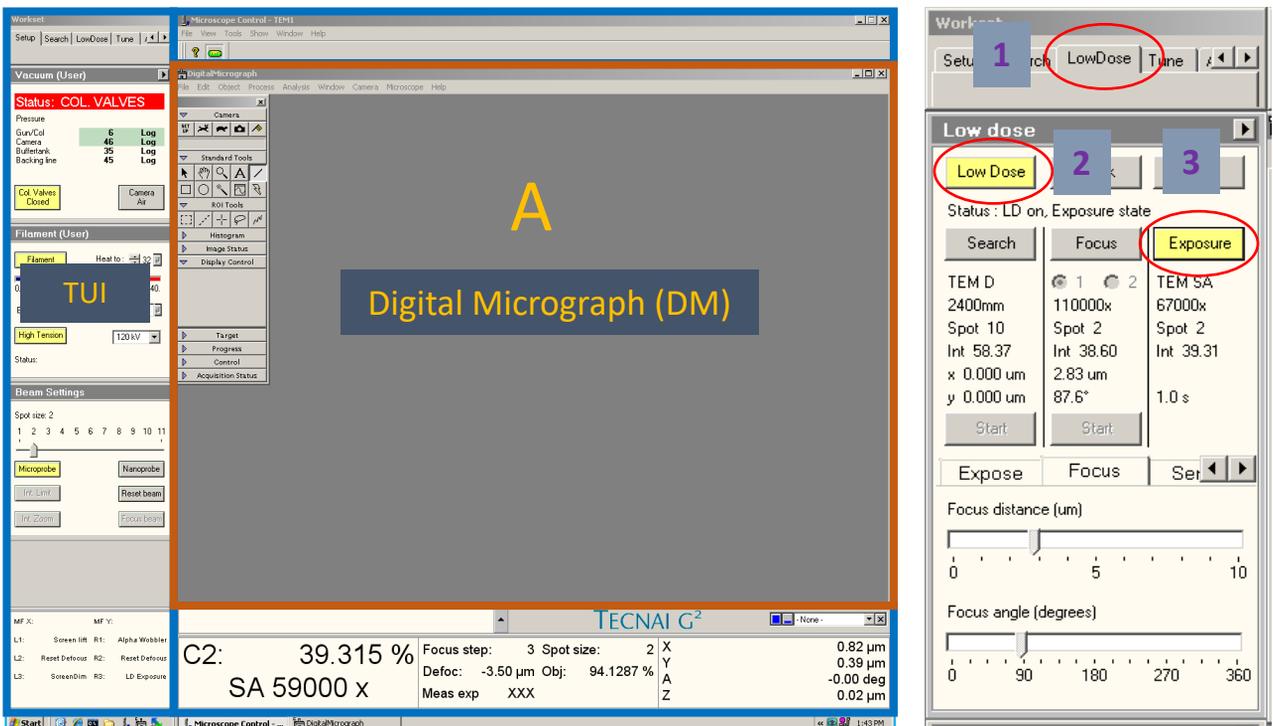


Figure 3: Microscope control windows (left) and low dose panel (right)

Follow the steps below for guidance through alignment of the T12 before inserting a sample.

What is being aligned	How	What you are looking for
Gun Tilt (in Direct Alignments)	Lower the fluscreen (usually L1) and raise the small screen. Spread the beam to imaging size i.e. close to the hash marks. Click Gun Tilt . Alternate between Multi Function (MF) X and Y to achieve the lowest Measured Exposure time (Figure 1C). Remove the small screen when finished.	Lowest Meas. Exp. time which is also the brightest beam.
Gun Shift (in Direct Alignments)	a. Click Gun Shift . Go to spot size 3 (~37% C2) and condense the beam using the Intensity knob. Center the beam with MF X/Y. b. Drag the slider to spot size 9 (~34.8% C2), condense the beam with the Intensity knob, and center the beam with the left trackball. c. Slide the slider back to spot size to 3 and check if the beam is centered. If not, use MF X/Y. d. Repeat points b and c until the beam remains centered. e. Click DONE f. Go back to the working spot size when satisfied.	The beam stays in the center at the two spot sizes.
C2 Aperture (manual alignment)	a. Using the Intensity knob, go to the correct side of the crossover i.e. Beam should get bigger when the Intensity knob is turned clockwise. b. Center the beam with the left trackball. Go to the wrong side of the crossover, if the beam has moved, then use the C2 aperture knobs to bring the beam half-way back to the center (see the T12 Anatomy). c. Iterate until beam stays in the center.	The beam should symmetrically expand and contract when going through crossover from correct to incorrect side.
Beam tilt Pivot Point X and Y (in Direct Alignments)	Condense the beam to as small as possible using the Intensity knob. Click Beam tilt PP X . Use MF X/Y reduce the amount of beam jitter, until the beam stops moving. Click Beam tilt PP Y and repeat.	You are looking for a single beam instead of two and minimizing the tilt of the beam.
Beam Shift (in Direct Alignments)	Adjust the intensity of the beam until it is up to hash marks on the fluscreen (imaging size). Click Beam Shift . If the beam moves to a side, use MF X/Y to bring it back to the center of the fluscreen.	The beam should be in the middle of the small circle on the fluscreen.

CLICK **DONE** WHEN COMPLETE!

After you are satisfied with the alignments, cycle through the Low Dose presets to check that the beam remains centered. If the beam is off-center in either Search, Focus or Exposure modes, use the **trackball** to bring it back to the center.

Before inserting the specimen holder double check these **4 important steps**:

- The Column valves are closed.
- The stage is reset to all zero positions (Search > Stage² > Reset Holder).
- The camera is retracted (in the DM window Camera > Retract Camera).
- The fluscreen is lowered.

III. Inserting your **NEGATIVE STAIN** grid in the microscope

Once you have successfully placed your grid in the holder:

- Open the **Vacuum Overview** from the bottom right of the TUI.
- With one hand on the end of the sample holder and the other stabilizing the shaft of the holder (**WARNING: Do NOT touch the sample holder beyond the O ring!**) orient the sample holder so that the small pin (closer to grid) is in the 3 o'clock position and insert the holder into the stage until it stops. Immediately, rotate the sample holder to the **Close** position and push the holder in about 2 cm. If this step is done correctly, then the vacuum cycle is initiated, and you will hear the pump running. At the same time, a countdown from 1min 30s will start in the vacuum overview tab. The system will prompt you to choose a holder. Select **Single Tilt** holder.
- After the countdown has ended, rotate the sample holder counter-clockwise from **Close** to **Open**, and gently guide the holder into the stage (Do NOT let go!). The large metal tab on the holder lines up with the hole at the 6 o'clock position (see Figure 4). The holder should be 1-2 mm from the purple plate if fully inserted.
- Check that the IGP value (column pressure) is below 20 log and if so, you can open the **Column Valves**.

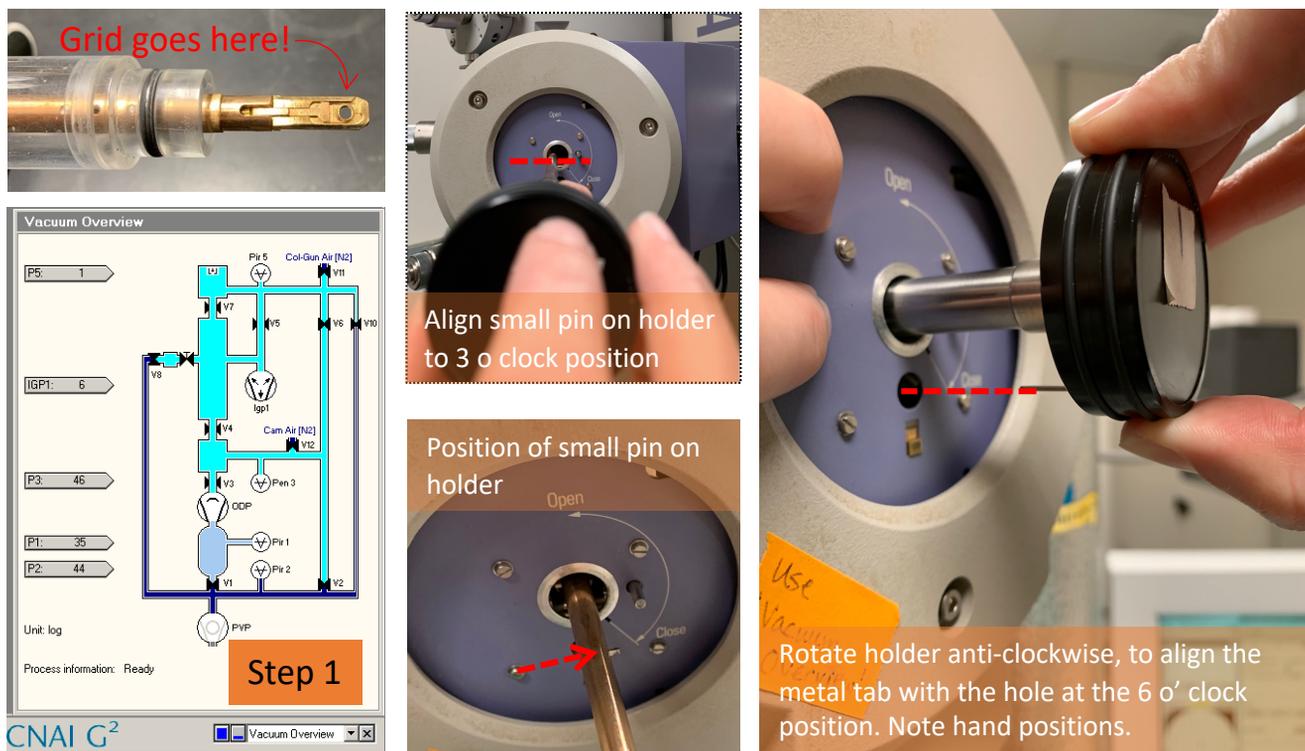
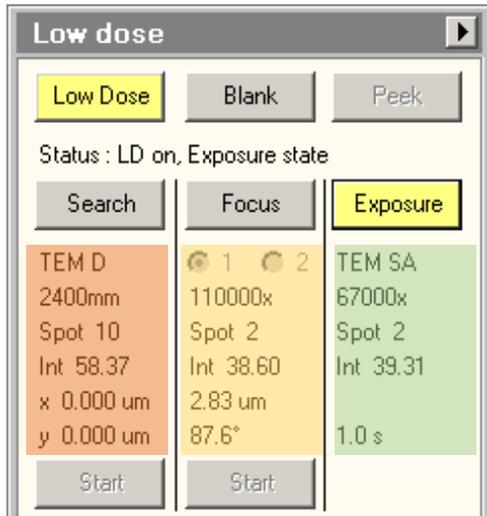


Figure 4: Visual guides for sample holder insertion

IV. Imaging your grid

Imaging is always performed in **Low Dose Mode** mode, even if you are imaging negative stain grids. Always use the following sequence when cycling through the presets/modes: **Search > Focus > Exposure**. Example imaging parameters are listed in the following figure. The current values are set by facility staff and you can find the values in the log book or posted on the microscope.



Overview:

1. In the Search preset:
 - a. Move to a square, find a piece of junk
 - b. Use continuous imaging in DM
 - c. Use alpha wobbler to find the eucentric height (Z height)
2. In the Focus preset:
 - a. Press Eucentric Focus
 - b. Use continuous imaging in DM
 - c. Start the Live FFT to view the Thon rings
 - d. Adjust the focus to true (zero) focus and reset the Defocus to 0 μm
 - e. Defocus to approx. -1.5 μm
3. In the Exposure preset:
 - a. Record the image with the camera button in DM
 - b. Use the **Blank** button to shutter the beam to protect your sample

V. Adjusting Eucentric Height of the stage (rough focusing)

1. In **Low Dose > Search Mode** and start **Continuous Imaging**, from the Digital Microscope window (🖥️).
2. In the resulting **View** window, using the **Line tool**, draw an X across the screen.
3. Use the stage trackball to move some junk or a recognizable feature into the center of the cross that you put on the **View** window.
4. Activate the α -wobbler from the right-hand control pad (usually R1), this will swing the stage from +15° to -15°. (**NOTE:** α -wobbler can also be started/stopped from **Search > Stage² > Wobbler** (see Figure 1)).
5. Adjust the Z-height (right-hand control pad, Z axis buttons (Figure 6)). As a rule of thumb, the value of the stage for the single-tilt holder is approx. -100 μm . However, be prepared to increase or decrease this value based on which part of the grid you are imaging. Sometimes, your feature of interest might move out from view. In that case, stop the α -wobbler by pressing R1 again, move the feature back to the field of view using the track ball, and restart the process.
6. As you approach the Eucentric Height of the sample, the feature should translate less. Try to reduce the feature's movement as much as possible. When you are satisfied, stop the α -wobbler by pressing Alpha Wobbler (usually R1).

Eucentric height (Z height) is now adjusted.

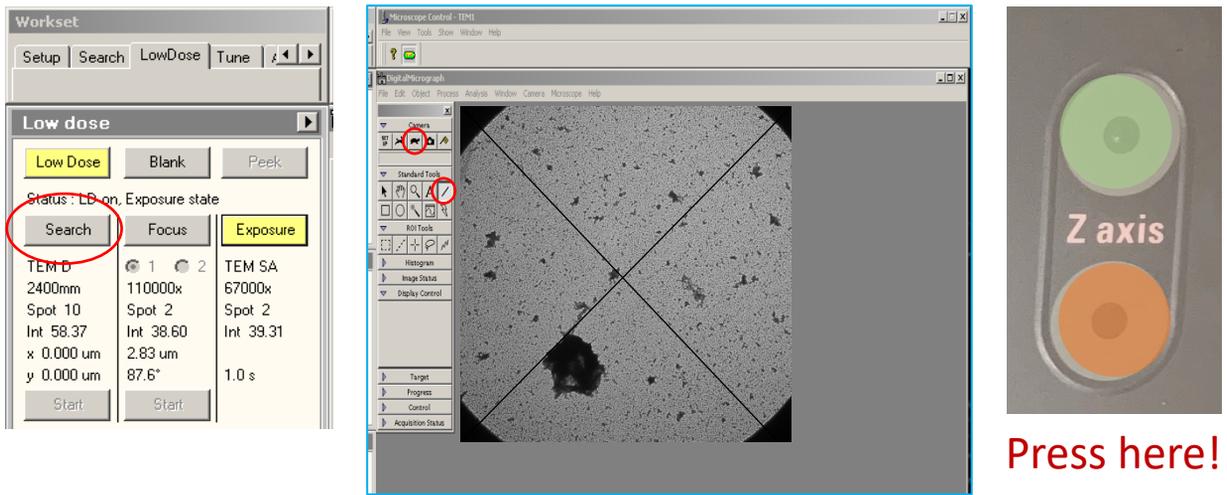


Figure 6: Setting eucentric height - Use the Search Preset

VI. Adjust Focus and Take an Image

1. Switch to **Low Dose > Focus Mode** and press the **Eucentric Focus** button only once on the right-hand control pad to set the objective lens to the correct pre-calibrated value. Should only need to press Eucentric Focus once per grid or if the focus is far off.
2. In **Low Dose > Focus** check if the beam is centered on the fluscreen. If it has moved, bring it back into the center using the trackball.
3. Raise the fluscreen, and from the Camera menu in the DM window, activate continuous FFT (**Process > Live > FFT**)
4. Using the Focus Knob on the right-hand control pad, find true focus. You know you are at true focus when the FFT resembles the one shown in Figure 7.
5. Once you are true focus, press the 'Reset defocus' button (usually R2). This will change the relative defocus value to 0.00 μ m.
6. Using the focus knob, dial down to -1.5 μ m.

➤ Switch to **Low Dose > Exposure**, click the camera button to take a single picture!

Check the FFT of the image **Process > FFT**

If the beam has astigmatism then follow these steps to correct it (Fig. 7).

1. In the TUI window, go to **Tune > Stigmator > Objective**
2. Use MF X/Y to correct the astigmatism of the beam.
Examples of astigmatic and corrected beams are shown below.
3. Click NONE, when done.

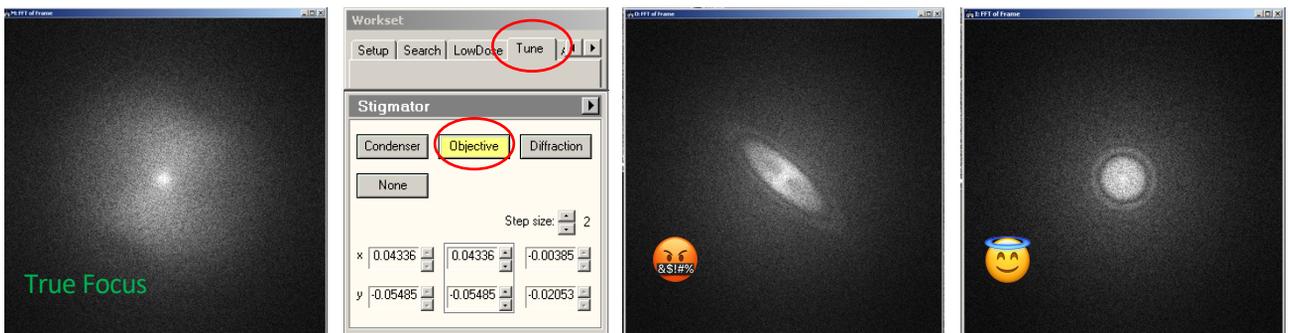


Figure 7: (From L-R), image taken at focus, the objective Stigmator window, an astigmatic FFT, a corrected FFT.

Once your sample is loaded with the correct Z height and focus set, you can more accurately perform the following alignments. You should be in the Exposure preset/mode to perform both alignments.

VII. Align the objective aperture (manual alignment)

1. Lower the fluscreen → *very important step to protect the camera!*
2. Press the Diffraction button on the right-hand control pad.
3. You will see a small bright beam surrounded by a dim halo.
4. Check if the halo and the bright beam are centered relative to each other.
5. If not, using the objective aperture knobs on the microscope, center the halo relative to the bright beam. **Note:** you are NOT trying to center the bright beam to the center of the fluscreen.
6. When satisfied, press the Diffraction button again to exit diffraction mode.

VIII. Adjust rotation center (in Direct Alignments)

1. Find a distinct piece of junk on the grid and use the eyepiece to view the image on the small fluscreen.
2. Go to Tune > Direct Alignments > Rotation center.
3. Using MF X/Y try to reduce the image translation. This is a somewhat hard alignment to perform.
4. If you have adjusted the parameter right, you will find that the image does not “move” left/right/up/down or moves very little. Rather it might seem to “pulse” in place. Once you are satisfied, click Done.

Once the beam is aligned down the microscope, you need to align the Low Dose modes/presets relative to the Exposure mode.

IX. Align Search preset to the Exposure preset using Image Shift

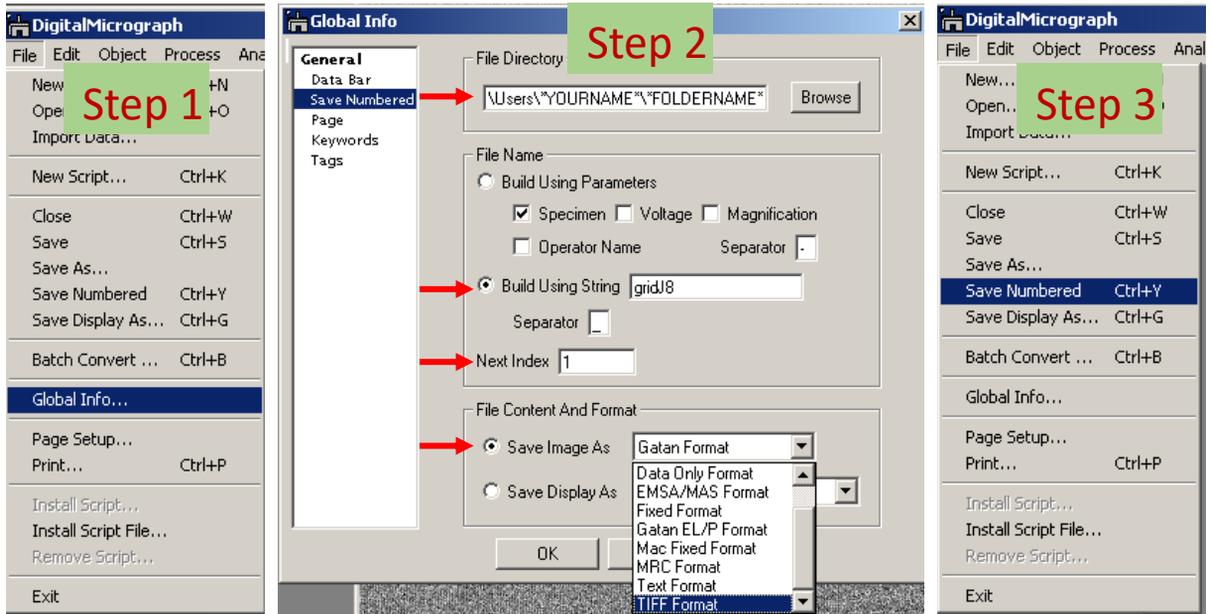
1. In Low Dose > Exposure, find some junk or a recognizable feature with the fluscreen down, ideally in the middle of a grid square. Center the feature with the stage track ball to the middle of the fluscreen. Alternatively, you can lower the fluscreen for at least 30s anywhere on your grid to create a burn mark.
2. Switch to Search mode, click the continuous imaging button, and find the same feature or your burn mark. Use MF X/Y (Image Shift) to move the feature/burn mark to the previously drawn 'X' in the center of the View window. Now the spot you select to image in Search mode will be the same spot you image in Exposure mode.

X. Adjust the Focus preset position to be adjacent to the Exposure preset

1. With a known stage position in the Exposure mode, switch to Focus mode. Because the magnification is higher, it is easily to create a burn mark. Lower the fluscreen for at least 30s.
2. Without moving the stage, switch from Focus > Exposure > Search mode and click the continuous imaging button. Verify the Focus position does **not** overlap with your Exposure position. If it is far enough away from your Exposure spot (approx. 1.6 μ m – 2 μ m, depending on your beam size), you are ready to continue imaging. If not, proceed to the next step.
3. In Low Dose > Focus, put the fluscreen down and lower the magnification a couple clicks and use the Intensity knob to spread out the beam. Use MF X/Y (Focus angle & Focus distance) to move the **image** away from the Exposure position. As you adjust MF X/Y, you'll need to re-center the beam with the trackball.

If these two Low Dose alignments are performed correctly, you should see 2 distinct burn marks when in Search mode: 1 large mark from Exposure and 1 small mark from Focus.

XI. Saving and transferring your image files



Before collecting your data, you can set up an automated naming scheme for your saved images, to streamline your data collection process. In order to set that up, follow these steps. From the DM window:

1. File > Global Info

2. In the resulting window,

a. go to **Save Numbered** page and set the destination path for your images

(e.g. E:\Users\yourfoldername\).

b. In the build using string box enter a unique name (e.g. samplename)

c. Set **Next Index** to 1

d. Finally, in the Save Image As field, save as Gatan format (.dm3) and convert to your desired format later in Digital Micrograph or ImageJ.

3. Save images from the DM window (File > Save Numbered), or use the keyboard shortcut **Ctrl+Y** to speed up the process. All images will now be stored in your destination folder with the appropriate tag.

Alternatively, you can save images in your folder by clicking File > Save As... and typing a file name into the dialogue box. It is recommended that you save your files as .dm3 format and convert to your desired format afterward.

Transferring your images:

• New Users

Move your images to your folder on the M: drive. Contact SBGrid at help@sbgrid.org. SBGrid will provide you a rsync command to copy the data from your M drive folder to your destination storage.

• Established Users (those who already have an SBGrid account set up)

Use WinSCP (blue-green, double arrow on the bottom left of the screen). Log into your xtal account (yoursbgridname@xtal200.harvard.edu) and drag the files from your folder in E:/Users/youruserfolder to destination storage.



XII. Removing the sample holder:

As your files are getting copied over to the desired location, use the time to extract the holder from the microscope and place it in its protective case. To remove the sample holder follow the same 4 important steps as listed before:

- a. The column valves are closed.
- b. Holder is reset to 0,0 position (Search > Stage² > Reset Holder).
- c. Camera is retracted (in the DM window Camera > Retract Camera).
- d. The fluscreen is lowered.

Having made sure of the above four steps, you are now ready to remove the sample holder. Below are the steps to follow.

1. Brace the purple plate of the stage with one hand (use any position you are comfortable with) – the microscope should not move.
2. With your other hand, pull the sample holder straight out, no turning until it stops. **NOTE:** This step will take higher than expected strength on your part as the vacuum in the column will be pulling at the other end.
3. Then, turn the holder clockwise such that the small pin at the sample end of the holder lines up with the CLOSE position on the stage (see Figure 4). **NOTE:** the holder will not go further than that - Do not force the holder.
4. Next, grasp the metal part of the holder with one hand and the black part of the holder with the other. In a single fluid motion, extract the holder from the stage. Try not to rattle the holder on its way out as this will trip the Vacuum system on the scope and lead to an error message on the TUI window.
5. Once the holder is out, remove your grid, and store the holder in its protective case and return it to the cabinet at the end of your session.

XIII. Ending your session

At the end of your session, you must leave the microscope in a usable state for the next user or get a staff member to help you fix any problems. Perform the following actions before leaving the room:

1. Assure the Column Valves are closed, the stage is centered, and the camera is retracted.
2. Confirm that the Low Dose settings for each preset are correct.
3. Confirm that the Camera settings for each preset are correct.
4. Check the schedule in PPMS: if someone is using the microscope after you, turn off the filament and top off the LN2 dewar. Leave the High Tension ON.
5. If you are the last user of the day, initiate the cryo-cycling protocol (remove the cold trap and press the Cryo Cycle button (Vacuum (User) > Cryo Cycle).
6. Most importantly, sign the Log Book, noting any difficulties or errors. This is our only way to keep track of what happens to the microscope, so please write down any issues like vacuum crashes or errors.