



Cryo Electron Microscopy Research Center

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF WISCONSIN-MADISON



Talos L120C User Manual
Serial # 9964195

L120C OPERATIONS: BEFORE YOU BEGIN

IMPORTANT! DO NOT OPERATE THE L120C ON YOUR OWN UNTIL YOU HAVE BEEN CERTIFIED BY CEMRC STAFF FOR INDEPENDENT OPERATION!

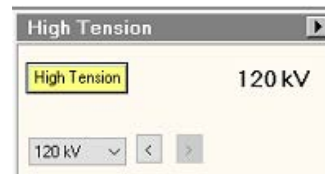
Make sure all the following are true. See the images below for details.

- 1) High Tension is on 120kV (Button is yellow)
- 2) Column valves are closed (Button is yellow)
- 3) All vacuum levels OK (All highlighted in green,
- 4) Accelerator = 1, Column = 15 and under)
- 5) Liquid nitrogen > 30%
- 6) No errors or warnings showing (see picture)
- 7) Filament is off (Button is grey)
- 8) The red light on the stage is not on
- 9) The single-tilt holder is inserted
- 10) Fluscreen is down
- 11) The camera is retracted
- 12) The microscope is in SA mode (magnification is 8500x)
- 13) Objective & Selected Area apertures are retracted

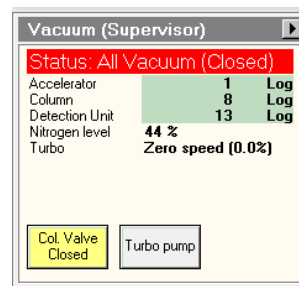
Alert staff and **DO NOT PROCEED** if any of the items 1-7 are incorrect.
Alert staff and make a note in the logbook about any issues.

See below for details on how to check each item.

- 1) High Tension is on 120kV (Button is yellow)
Box is located on the Gun tab of TEM UI



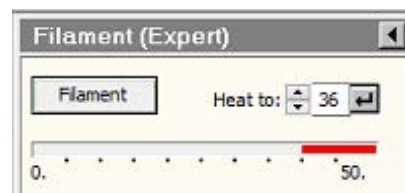
- 2) All vacuum levels OK
Box is located on the Gun tab of TEM UI
All highlighted in green,
Accelerator = 1, Column = 15 or less



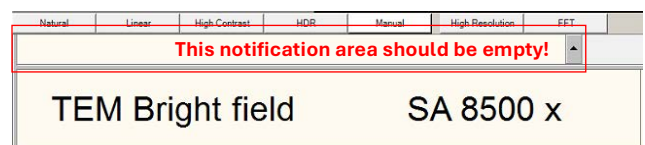
- 3) Column valves are closed (Button is yellow)

- 4) Nitrogen level greater than 30%

- 5) No errors or warnings showing
Box is located near bottom left corner TEM UI



- 6) Filament is off (Button is grey)
Box is on the Gun tab of TEM UI



7) Red light on the stage is not ON

Red LED is located on the microscope
as indicated in the photo



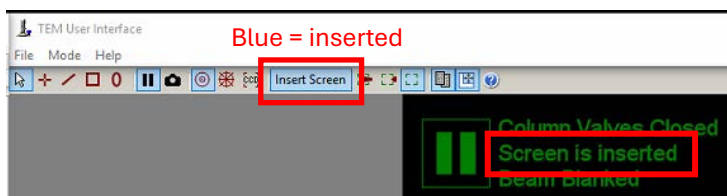
8) Single-tilt holder is inserted

Check the microscope for the holder

**Holder is
inserted**

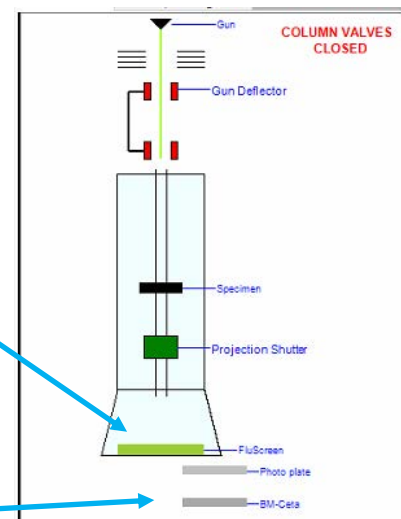
9) Fluscreen is down

Can be checked near the top of Flucam window
or in blanker/shutter monitor window



**Fluscreen
is down**

**Camera is
retracted**



10) Camera is retracted

Can be checked from the blanker/shutter monitor

OR from the Camera tab of TEM UI (Insert button is grey)



11) Microscope is in SA mode (magnification is 8500x)

Box is located near the bottom left corner TEM UI

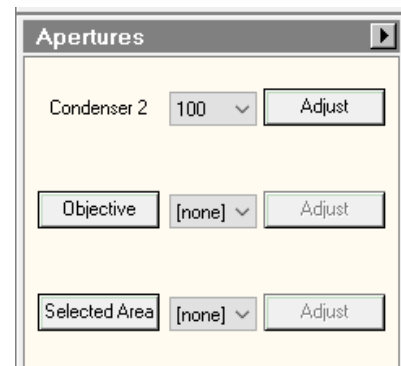


12) Objective aperture and Selected Area apertures are retracted

Box is on the Gun tab of TEM UI

Objective button is grey, [none] is displayed

Selected Area button is grey, [none] is displayed



**ALERT STAFF AND MAKE A NOTE IN THE LOGBOOK ABOUT
ANY ISSUES.**

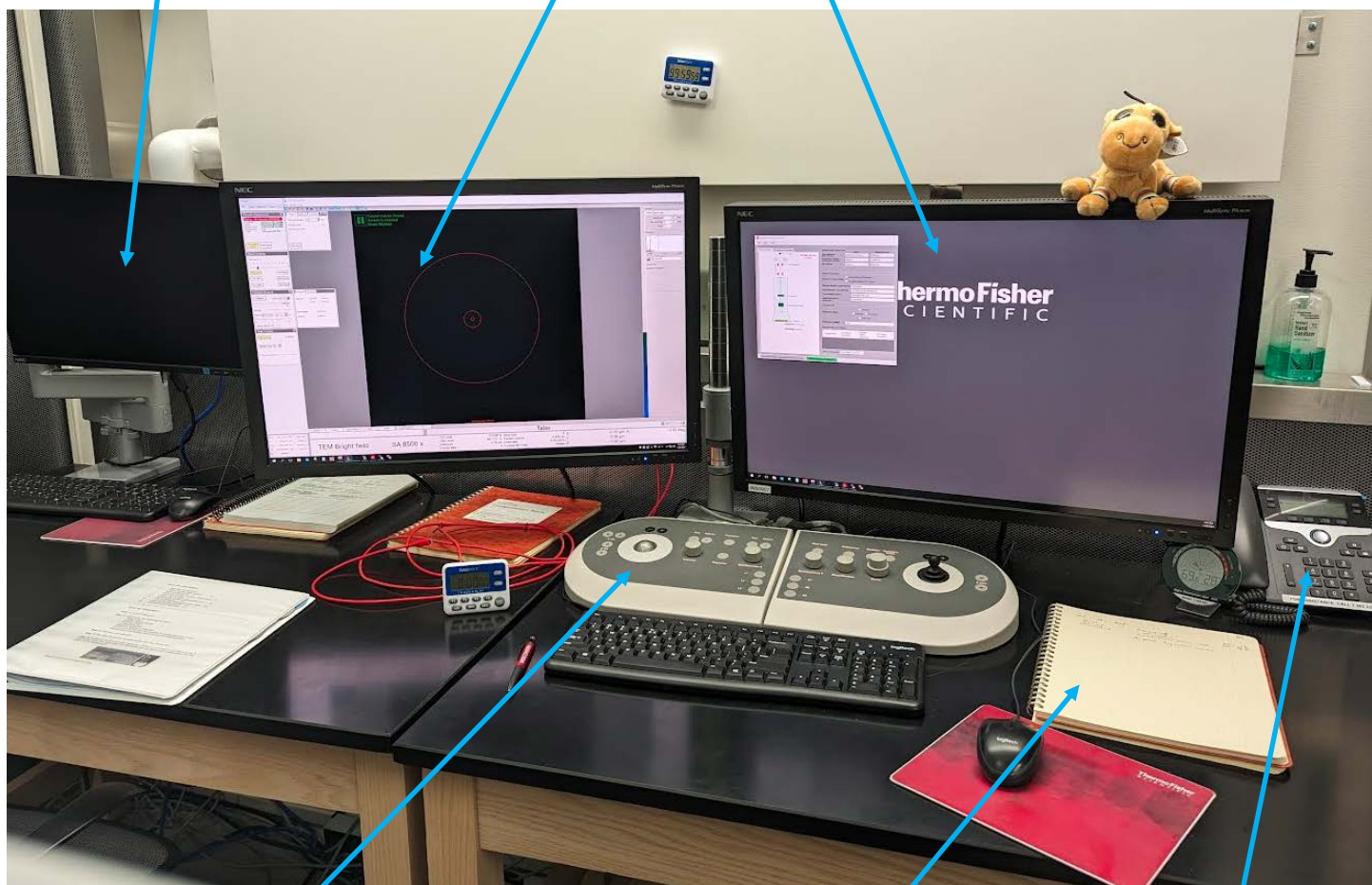
Talos L120C 120kV TEM B1414A DeLuca Biochemical Sciences Building

Microscope PC monitors

TFS support PC

TEM UI/Flucam

SerialEM/TIA/EPU etc.

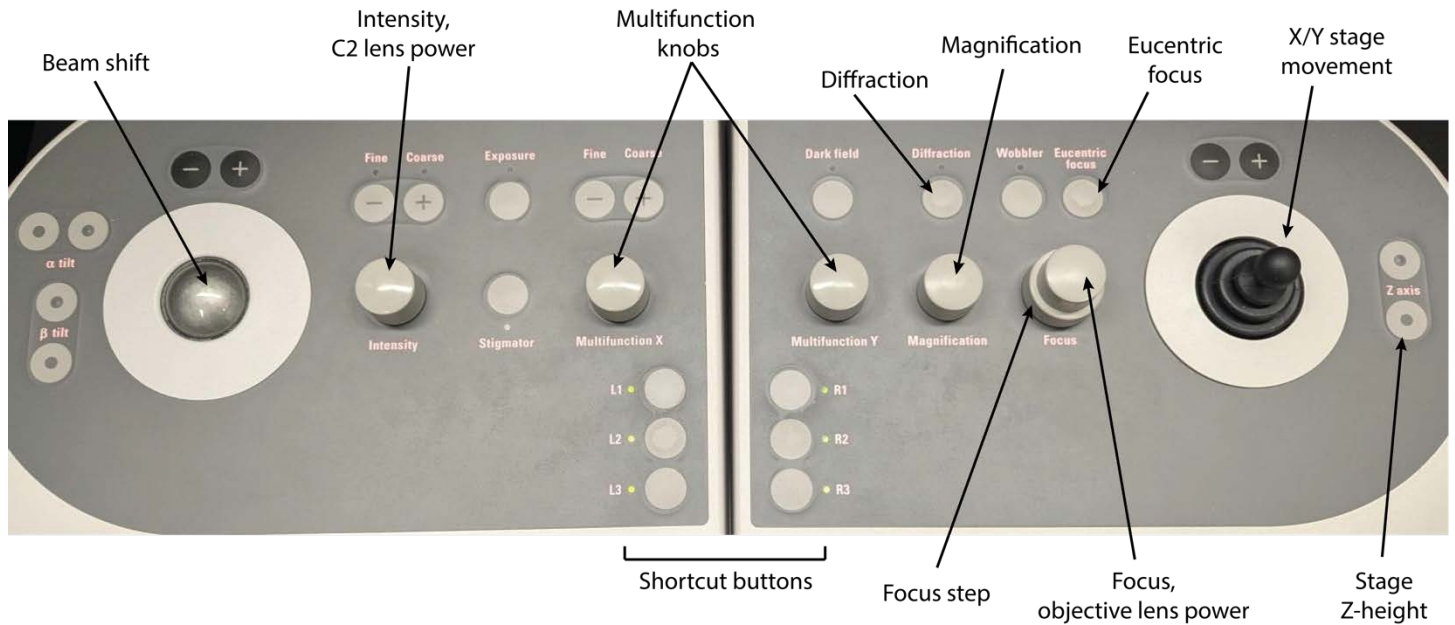


User hand panels
that control the TEM.

Log notebook
you will fill out.

Phone to use
for support.

Overview of L120C Hand Panels



Shortcut Buttons

MF X:	Beam shift X
L1:	Screen lift
L2:	Normalize all
L3:	Spotsize -
MF Y:	Beam shift Y
R1:	Screen lift
R2:	Reset Defocus
R3:	Spotsize +

MF X	Beam Shift X
L1	Screen lift
L2	Normalize all
L3	Spotsize -
MF Y	Beam shift Y
R1	Screen lift
R2	Reset Defocus
R3	Spotsize +

Filling Out the CEMRC Talos L120C Logbook

Fill out the logbook. **THIS IS NOT OPTIONAL**, and you will lose use privileges if you fail to do this (or do so in a way that is not interpretable to facility staff). You need to capture the following details in your logbook entry:

Date – Your name (and any other users present)

Accelerator (Gun) vacuum	Filament heat
Column vacuum	Emission*
Detection (Camera) vacuum	Bias*
Liquid nitrogen level	

*These values are recorded after calculating your emission dose for data collection.

- ☐ Empty holder inserted?
- ☐ Left in SA mode?
- ☐ Camera gains acquired?

Sample description

Software used (SerialEM or EPU)

Imaging conditions (spot size, magnification, objective aperture, exposure time, dose rate...)

Notes / Issues - **CONTACT STAFF ABOUT ANY ISSUES IMMEDIATELY!**

This is what a good entry would look like:

2025-07-11	Preeti Verma			
Accelerator	1	Filament	32	cryo-cycle - No Leave in SA - Yes Leave empty holder - Yes (inserted)
Column	8	Emission	5µA	
Detector	13	Bias	400.633V	
Nitrogen level	39%			
prepare new gains - yes Imaging conditions → Apoferritin negative stain (12 µg/ml) (2).42.627%, spot size 5, 45000x, OA in 100µm, exposure time 1s beam size: 3.14µm				
* No issues				

IMPORTANT: IF THERE ARE NO ISSUES, PLEASE WRITE "NO ISSUES."

Beginning of the Imaging Session

Step 1: Ensure microscope is ready to go by checking the following items **BEFORE YOU BEGIN**. Ensure that your time has been properly booked on iLab.

High Tension is on 120kV	Column valves are closed	All vacuum levels good
No errors or warnings showing	Filament is off	Red light on stage is off
Liquid nitrogen level above 30%		

If the liquid nitrogen level runs below 30%, please contact CEMRC staff.

Step 2: Make sure all the necessary control software is running. You need the following to be visible:

- 1) TEM User Interface
- 2) FluCam viewer
- 3) TIA (for viewing/saving images)
- 4) BlankerShutter Monitor

If the TEM User Interface is not running, please contact CEMRC staff. The rest can be started from the taskbar or from the desktop icons.



Step 3: Make sure the last user left the microscope at SA-level magnification. If not, change to an SA-level magnification now, as it can take a while for the projection system to equilibrate if it was left at an LM-level magnification for a prolonged period.

Step 4: Fill out the logbook. **THIS IS NOT OPTIONAL**, and you will lose use privileges if you fail to do this (or do so in a way that is not interpretable to facility staff). See above for more details.

OVERVIEW - Dry Sample Insertion and Removal

This is a quick, at-a-glance, version of the protocol (more details on the following pages):

Step 1: Make sure an empty holder was left inserted in the microscope.

Step 2: Make sure the following are done prior to removing the holder from the microscope:

- 1) Column valves closed
- 2) All vacuum levels are OK (nothing highlighted grey or red)
- 3) Objective and Selected Area apertures are retracted
- 4) Reset (“zero”) stage
- 5) Fluscreen is down/inserted (Fluscreen can be inserted using **R1** button on the hand panel)
- 6) Camera retracted

Step 3: Begin removing the sample holder by slowly and gently pulling it straight out about 3 inches until you feel a hard stop (but don’t pull hard against that stop!).

Step 4: Now rotate the sample holder clockwise by ~120-degrees. Do this slowly.

Step 5: Complete the removal of the holder from the microscope and transfer to the stand.

Step 6: Raise the grid shield and insert the new grid in the holder.

Step 7: Close the grid shield and rotate the holder to make sure the grid does not fall out.

Step 8: Inspect the holder O-ring to ensure there are no fibers or dust on it.

Step 9: Make sure the microscope is still in the same state as in step 2.

Step 10: Insert the sample with the pin at ~120-degree tilt.

Step 11: Select holder type and pump out airlock. The airlock does not start pumping until **AFTER** the turbo is at full speed, so there are two delays to keep an eye on here: 1) the turbo power-up and 2) the pumping time.

Step 12: Watch the countdown timer.

Step 13: Remind yourself to keep the holder as level as possible in the following steps.

Step 14: Immediately after the countdown is over, slowly turn the holder counterclockwise by ~120-degrees.

Step 15: Complete insertion at the end of the ~120-degree rotation. Don’t let the column vacuum draw the holder in freely.

Step 16: Turn off the turbo pump when the status in the Vacuum tab reads “All Vacuums (Closed) / All Vacuums Ready”. The Turbo pump cannot be turned off when the status is “Busy”.

Detailed Dry Sample Insertion and Removal Protocol

Step 1: At the beginning of your session, you should find an **EMPTY** sample holder inserted into the microscope. We leave the sample holder inside the microscope to prevent dust from accumulating inside the goniometer and causing problems with the vacuum seals.

Step 2: Make sure the following are done prior to removing the holder from the microscope.

- ☐ Column valves closed
- ☐ All vacuum levels are OK (nothing highlighted grey or red)
- ☐ Objective and Selected Area apertures are retracted
- ☐ Reset ("zero") stage
- ☐ Fluscreen down (Can be inserted using R1 button on the hand panel)
- ☐ Camera retracted

The following screenshots illustrate the required states for the microscope components, with red arrows and text explaining the meaning of colors and values:

- Vacuum (Supervisor) window:**
 - Status: All Vacuum (Closed) - **good vacuum (all green)**
 - Accelerator: 1 Log
 - Column: 8 Log
 - Detection Unit: 13 Log
 - Nitrogen level: 44 % - **liquid nitrogen level**
 - Turbo: Zero speed (0.0%) - **yellow means closed**
 - Col. Valve Closed (Yellow button)
 - Turbo pump (Grey button)
- Apertures window:**
 - Condenser 2: 100 Adjust
 - Objective: [none] Adjust - **grey means**
 - Selected Area: [none] Adjust - **grey means**
- CCD/TV Camera window:**
 - Camera: BM-Ceta
 - Integration time [s]: 0.25
 - Sampling: 2
 - Readout area: Full
 - Blank, Search, Preview, Acquire, Insert, Auto Focus, Live FFT buttons
 - Insert button - **grey means retracted**
- Stage* window:**
 - Reset stage (Red text)
 - Reset Holder (Grey button)
 - Alpha wobbler: 0 to 15
 - Alpha toggle: 0° tilt - **grey is**
- Control window:**
 - Positions / tracks: Delete All, Clear Tracks
 - Stage control: Power step (1/8 .. 8) 1/2
 - XY separately above 500000 x
 - Reset Holder, XY, A buttons
 - Alpha wobbler: 0 to 15
 - Alpha toggle: 0° tilt - **grey is**
- Bottom status bar:**
 - X: 0.00 μm A: 0.00 deg
 - Y: 0.00 μm B: 0.00 deg
 - Z: -0.02 μm Cold trap LN2: 43 % - **liquid nitrogen level**
- Diagram of the microscope column:**
 - Gun, Gun Deflector, Specimen, Projection Shutter, Fluscreen, Photo plate, BM-Ceta
 - Fluscreen is down (Red text)
 - camera is retracted (Red text)
 - liquid nitrogen level (Red text)
 - reset stage (Red text)
 - Fluscreen is down (Red text)
 - camera is retracted (Red text)
 - liquid nitrogen level (Red text)

Here's the coloring for control software buttons in the ThermoFisher world:

Grey = click me to do that

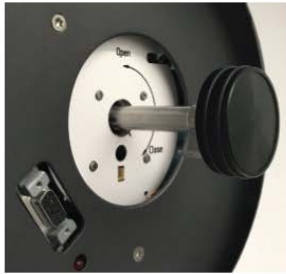
Yellow = this is being done already

Orange = I'm working on this, leave me alone for a bit

Grey text inside a box means that the microscope won't let you do that now, so clicking the corresponding box will do nothing.

Holder removal

Step 3: Begin removing the sample holder by **SLOWLY** and **GENTLY** pulling it straight out about 3 inches until you feel a hard stop. The stop that you feel is a small metal pin hitting a stopping point. If you pull too hard against the stop, you will break this pin and cause a catastrophic problem with the goniometer and column vacuum.



you will feel a hard stop around here



PLEASE BE GENTLE ON THE PIN!

For more information refer to this Bucky Cryo's video link on Dry Holder Removal here: <https://youtu.be/ltkzMPyTy8Y>



Step 4: Now rotate the sample holder clockwise ~120-degrees. **DO THIS SLOWLY.** This turning motion closes a valve that isolates the airlock from the column interior. Remember that fragile metal pin? You can break it here also if you turn too hard. At the end of the turn, the pin becomes aligned with an exit channel that allows further withdrawal of the holder.

Step 5: Once you have finished rotating the sample holder, you are ready to remove it from the microscope. You want to do this as a single continuous motion; you don't want the sample holder tip to bounce around inside the goniometer on its way out. Here is an image of how you should hold it while withdrawing the holder.



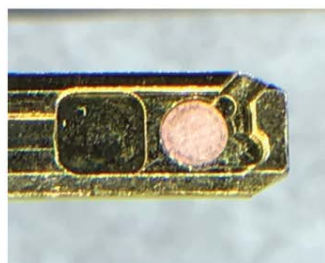
IF YOU BLOW THE VACUUM, YOU SHOULD FINISH THE UNLOADING PROTOCOL ANYWAY, THEN CONTACT FACILITY STAFF.

Sample loading

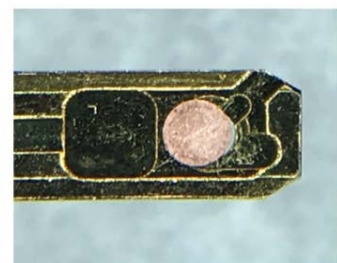
Step 6: Use a magnifying lens to view the tip of the holder as you insert your new grid. To open the holder, use the small metal tool provided to lift the grid shield. It's really important to keep your grids as flat as possible. This isn't easy, and it's almost impossible without a magnifying lens to see what you are doing.



raising shield



good grid placement



not good
(not centered)

Step 7: Close the grid shield and rotate the holder to make sure the grid does not fall out. If there's an issue with the grid being secured, you want to see it here, not inside the scope!

Step 8: Inspect the O-ring to ensure there are no fibers or dust on it. If so, they will prevent the formation of a good seal, and you will end up blowing the column vacuum during sample insertion. If you see anything, gently wipe the O-ring with a gloved hand or use a can of compressed air to clean off the O-ring. Talk to facility staff if that is not sufficient to clean the O-ring. **DO NOT PUT GREASE ON THE O-RING WITHOUT STAFF PERMISSION.**

Holder insertion

Step 9: Make sure the microscope is still in the same state as in Step 2. Column valves closed; all vacuum levels are good; Objective and Selected Area apertures retracted; stage reset; Fluscreen down; camera retracted.

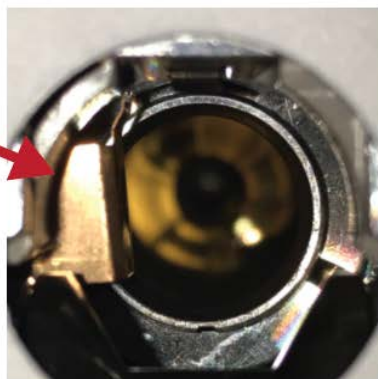
Step 10: You will first insert the sample with the pin at around ~120-degree tilt. This is the same angle at which you finished removing the holder earlier. Remember that delicate pin? You will insert the holder until the pin hits a hard stop, after which point you will gently rotate the holder (only +/- a few degrees!) to make sure the pin is fully seated and can no longer freely rotate in the goniometer. Once you have done this, the turbo will begin to power on so it can pump out the airlock (so you don't introduce too much atmosphere and water into the column later).

For more information refer to this Bucky Cryo's video link on Dry Holder Insertion here:

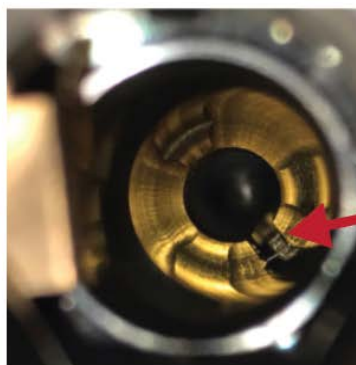
<https://youtu.be/J83G98UrJKE>



sensor
for
turbo
pump



line pin
up with
this
mark

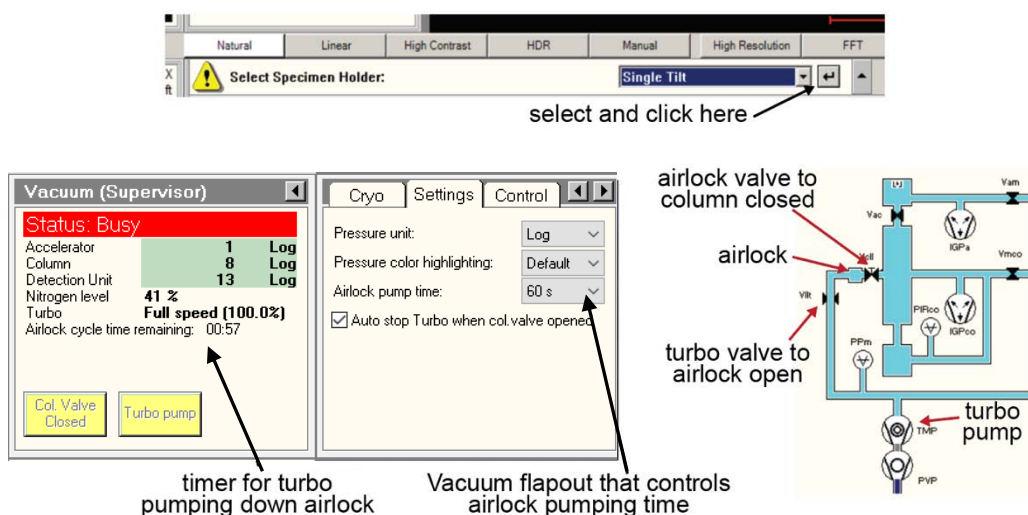


pin needs
to be
completely
seated in
this slot

During this first insertion step, the sample holder physically trips a sensor that waits 2 seconds and then opens the airlock to the turbo pump. But it takes the turbo a few minutes to get up to speed before it's ready to actually pump.

Step 11: You are now at a waiting step, where the turbo is pulling atmospheric and water vapor out of the airlock. This is set to 1 minute by default for the dry holder, and only facility staff can adjust this. During this waiting period, you need to tell the microscope which holder you just inserted (in this case, the single-tilt holder). Watch the countdown and move to the next step as soon as it is finished.

NOTE: The vacuum in the airlock will rapidly begin to decay after the end of the countdown. The O-ring on the holder doesn't seal very well until the holder is fully inserted into the microscope.



You can define the holder type after the insertion is complete if that is more convenient.

Step 13: Remind yourself to keep the holder as level as possible in the following steps.

Step 14: Immediately after the countdown is over, make sure the red light by the stage is off. You will slowly turn the holder counterclockwise ~120-degrees. This turning motion opens a valve that previously separated the airlock from the column interior. The actual opening point is around halfway through the ~120-degree turning motion. You will see the column vacuum spike as the valve is being opened. And you'll probably hear the column Ion Getter Pump (IGP) temporarily peak at full power to clear all the atmosphere and water that was still in the airlock. Good thing you had those column valves closed to protect the gun! If the IGP is not capable of clearing this spike in vacuum, it will shut down and undergo a recovery process. If this happens, talk to facility staff so we can get the scope right again.

Remember that fragile metal pin? Don't rotate too hard, or you will break it here.

! IF YOU LOSE THE VACUUM, YOU SHOULD COMPLETE THE INSERTION PROTOCOL ANYWAY AND THEN CONTACT FACILITY STAFF.

Step 15: At the end of the ~120-degree rotation, you will feel the column's vacuum pulling the sample holder in. With two hands, you should do your best to keep the holder level and reduce the insertion rate. A good rule of thumb is 10 seconds to complete insertion. Once fully inserted, you can gently rotate the sample holder to make sure it's fully seated inside the goniometer.

Step 16: Turn off the turbo pump when the status in the Vacuum tab reads "All Vacuums (Closed) / All Vacuums Ready". The Turbo pump cannot be turned off when the status is "Busy".

OVERVIEW - Microscope Alignment

This is a quick overview, at a glance, version of the protocol (more details on the following pages):

Step 1: Insert a standard **sample**. Ideally, a cross-grating with a mixture of intact and empty grid squares, since some alignments will require imaging on foil and others over vacuum.

Step 2: Make sure the **HT** is active and set to 120 kV. You should never turn this off or alter it.

Step 3: Filament heat should be set to **34**. Turn on filament. As of February 2026, this will yield a slightly undersaturated beam, which you will use for gun tilt alignment. It takes about 5 minutes for the filament to reach full heating. Press the eucentric focus button here too.

Step 4: Open **column valves**. Go to **155x** and in the Stage tab record stage positions for foil-covered and empty (over vacuum) positions on the cross-grating grid. You'll need to come back to these later.

Step 5: Move the stage to an empty position or **hole**. Set **magnification to 13,500x**. Go to **spot size 3**. Center the beam at cross-over using beam shift.

Step 6: Gun Tilt under Direct Alignments. With the beam condensed to a cross-over, center the beam using the multifunction X and Y knobs.

Step 7: Gun Shift under Direct Alignments. Likewise, center the beam using the multifunction X and Y knobs.

Step 8: Go through one more iteration of **Steps 6-7** before moving on to **Step 9**.

Step 9: Center C2 aperture. (**100 μm**) by expanding the beam to the outer red circle and making sure the beam doesn't shift when going to the crossover.

Step 10: Carefully adjust the **stigmation of the C2** lens at each spot size you intend to use today.

Step 11: Make sure filament heat is at 34. Don't go above heat 34 without talking to facility staff first. If a fully saturated beam is required, reach out to staff for assistance.

Step 12: Prepare a **new gain reference** for the Ceta camera if needed. See **Page 24**.

Step 13: Move the stage onto the saved **foil** position.

Step 14: Press the "**Eucentric Focus**" button on the control panels. Also, "**Reset Defocus**" on the control panels (it's linked to the R2 button).

Step 15: Adjust stage **Z to the eucentric height**, then update the Z in the stage panel.

Step 16: Insert a **100 μm objective aperture**.

Step 17: Center the objective aperture in diffraction mode.

Step 18: Go to **120,000x** and **adjust the stigmatism** of the objective lens.

Step 19: Find focus (minimal contrast in real space or no Thon rings in reciprocal space).

Step 20: Check the **pivot point alignments**. Go to **45,000x and adjust intensity to crossover**. Center the beam using beam shift, then select “**Beam tilt pp X**” under direct alignments. Adjust multifunction knobs to minimize translation. Repeat with “**Beam tilt pp Y**”.

Step 21: Go to **120,000x** and expand the beam to flood the Fluscreen. Perform “**Rotation center**” alignments from the direct alignments tab.

Step 22: You are now ready to image your sample(s). But since you will remove the objective aperture during sample exchange, be sure to repeat **Steps 16-17** after inserting your sample. This will burn a small region of your sample, but that's OK.

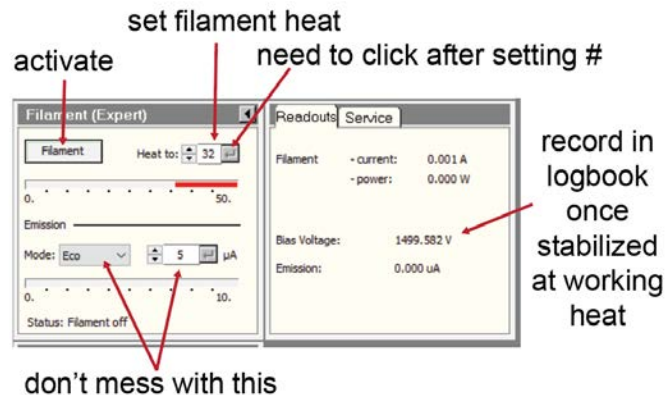
Complete Detailed Microscope Alignment

In general, most of the gun alignments should be quite stable. So, as a rule of thumb, you should not have to make significant changes to anything in **Steps 5-8**. Please notify facility staff if you find that anything was significantly off its optimal setting, because that may indicate an imminent equipment failure.

Step 1: Insert a standard sample. Ideally, a cross grating with a mixture of intact and empty grid squares, since some alignments will require imaging on foil and others over vacuum.

Step 2: Make sure the HT is active and set to 120 kV. You should never turn this off or alter it.

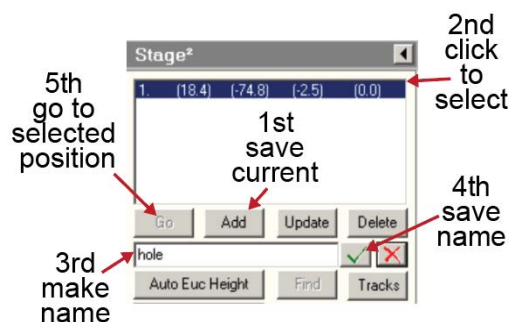
Step 3: Filament heat should be set to **34**. Turn on filament. As of February 2026, this will yield a slightly undersaturated beam, which you will use for gun tilt alignment. It takes about 5 minutes for the filament to reach full heating. Press the eucentric focus button here too.



Step 4: Open column valves. Go to LM 155x and record stage positions for foil-covered and empty hole (vacuum) positions on the cross-grating grid.

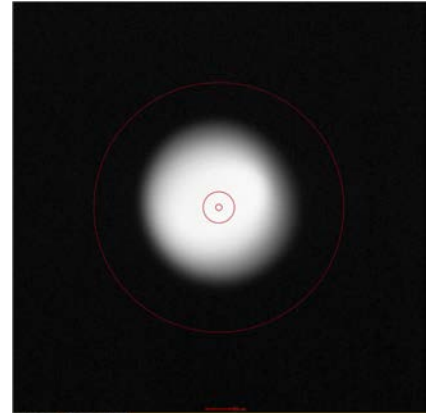
- Go to **155x** magnification.
- Use **spot size 7** and the intensity knob on the hand panels to adjust C2 intensity to 100% (if it is not already).
- You can adjust contrast if needed by clicking on Natural near the bottom of the Fluscreen viewer. Scrolling with the middle mouse button automatically switches the mode to manual and adjusts contrast as you scroll.
- Use the joystick to move to a position over a **hole** and add it to the Stage panel. Do the same for a position over **foil**.

You'll need to come back to these later, and it's a waste of time to search for them over and over.



Step 5: Prepare for gun tilt alignments.

- Move the stage to a hole
- Ensure Fluscreen is down
- Step the magnification up to SA **13,500x**
- Go to **spot size 3**
- Press Eucentric Focus on the control panels
- Under Direct Alignments select Beam Shift to center beam using multifunction X and Y.
- Adjust the beam with the intensity knob so the illuminated area extends approximately halfway between the outer two red rings of the Fluscreen.

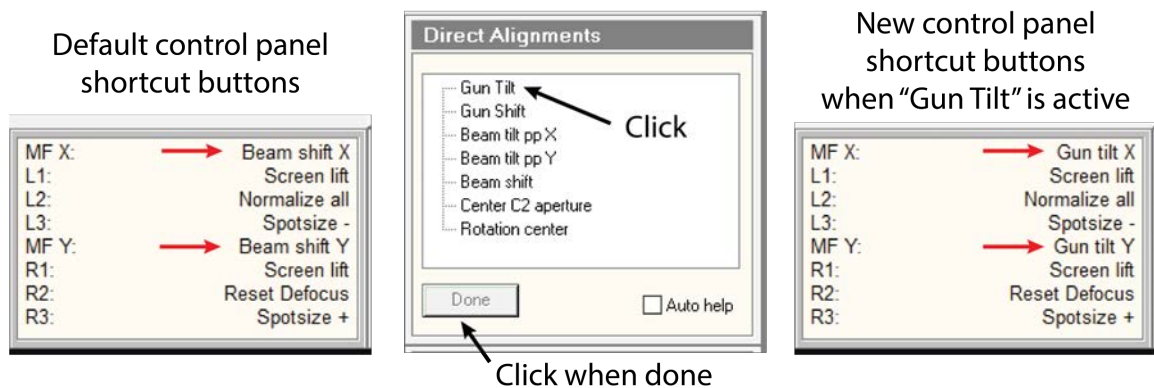


Step 6: Gun tilt alignment.

Method A – Maximize screen current

- Use the optical parameters described in **Step 5**
- Go to the Direct Alignments (under the alignments tab) and select “Gun Tilt”. Doing this will change the behavior of the multi-function knobs on the control panels.
- Adjust multifunction X to maximize screen current. Then adjust multifunction Y to maximize screen current. Iterate between X and Y until you can no longer increase the screen current. If the beam shifts outside the outer red circle, stop the gun tilt, use the beam shift direct alignment to recenter the beam, then resume the gun tilt direct alignment.
- The screen current should be between 9-10 nA using 34 heating index.

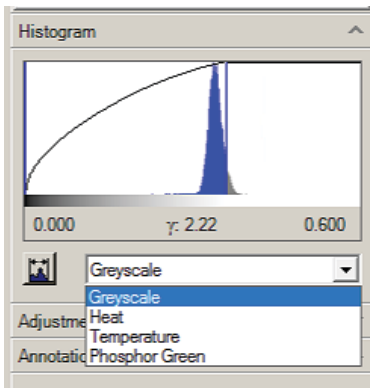
Make sure you click the “Done” box once you are done here; otherwise, you will accidentally change gun tilt when using the multifunction knobs.



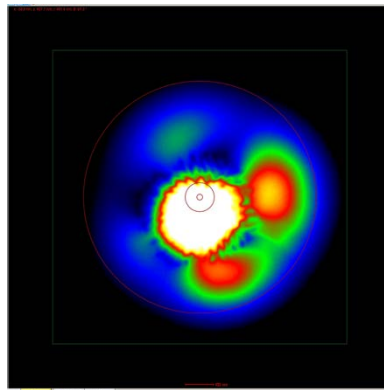
Method B – Optimize filament shape

- Step up the magnification to **57,000x**, condense beam to **cross-over** using the intensity knob
- Drop heating index from 34 to **32**.
- You should be able to see the structure of the filament under these conditions.
- Go to the Direct Alignments (under the alignments tab) and select “Gun Tilt”. Doing this will change the behavior of the multi-function knobs on the control panels.
- Adjust multifunction X and Y until the image of the undersaturated LaB6 filament looks as symmetric as possible. If the beam shifts out of the outer red circle, stop gun tilt, use the beam shift direct alignment to recenter the beam, and resume the gun tilt direct alignment. Make sure you click the “Done” box once you are done here, otherwise you will be accidentally changing gun tilt when using the multifunction knobs.

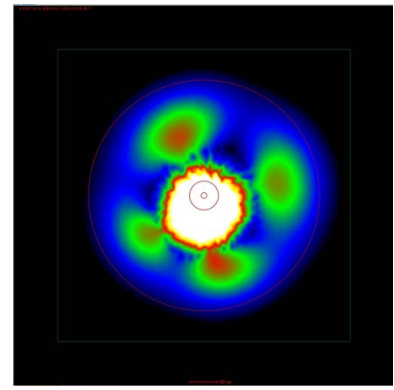
- Return heating index to 34 before proceeding.



Switching Fluscreen to temperature can help visualize symmetry



Asymmetric
Use multifunction X/Y to improve



Aligned filament image
Click done to complete alignment

Step 7: Gun shift alignment.

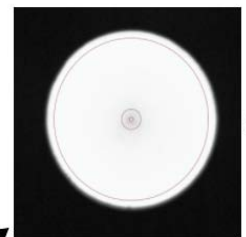
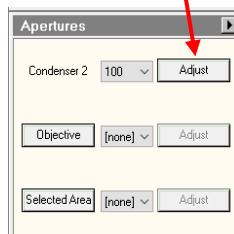
- Go to magnification **13,500x**, **spot size 9** and adjust the intensity knob until you are at crossover.
- Go to the Direct Alignments and select "Beam shift". Center the beam with the multifunction knobs and click "Done".
- Now go to **spot size 3** and adjust the intensity knob until you are at crossover.
- Go to the Direct Alignments and select "Gun Shift" and center the beam with the multifunction knobs and click "Done".
- Now go back to **spot size 9** and crossover. In the Direct Alignments, select "Beam shift" and center the beam. Click "Done" and go to spot size 3 to re-center with "Gun shift".
- Iterate this process until the beam is centered at spot size 3 and 9 without having to adjust "Gun Shift (use at spot 3)" or "Beam shift (use at spot 9)". Remember to select "Done", otherwise you'll be messing things up later.

Step 8: Go through one more iteration of **Steps 6-7** before moving on to **Step 9**.

Step 9: Align Condenser Aperture.

- Reduce magnification to **2,600x**, go to **spot size 3**.
- Center the beam at crossover using Beam Shift under direct alignments and then expand the beam by rotating the intensity knob clockwise, until the beam is the size of the outer red circle.
- In the Apertures box on the Alignments tab select "Adjust" for Condenser 2 (this should always be set to 100 μm size).
- This again changes the behavior of the multifunction knobs on the control panel.
- Adjust those so the spread beam is centered on the screen. Click the "Adjust" box to return the multifunction panel to their native state of controlling beam shift.
- Once the C2 aperture is aligned, you should see very little translation in the beam center as you expand/contract the beam from/to crossover with the intensity knob.

click to adjust C2 aperture



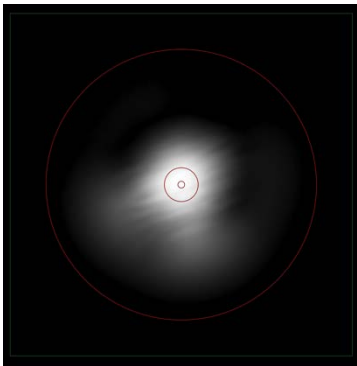
expand beam



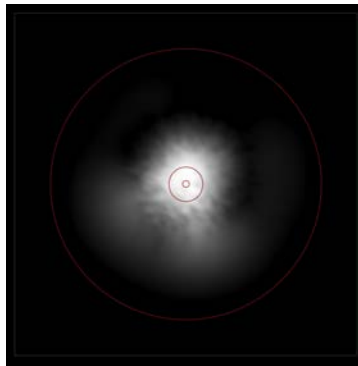
Step 10: Correct condenser lens stigmatism.

- Go to **spot size 4 and 57,000x**, and adjust the beam to crossover with the intensity knob. You should be able to see some fine structure in the filament at crossover. Fine tune intensity to make the features as sharp as possible.
- Go to the Stigmator (on Camera tab) and select “Condenser”. This again changes the behavior of the multifunction knobs.
- Adjust the stigmatism with multifunction X to minimize smearing and show as much fine structure as possible in the filament.
- Adjust the stigmatism with multifunction Y to minimize smearing and show as much fine structure as possible in the filament.
- Iterate adjustment of intensity, multifunction X, and Y to make the image as sharp as possible.
- Click None in the Stigmator when finished.

You can do better



Good



This needs to be done for every spot size you intend to use for imaging.

Image provided as an example. The features of the filament may not look exactly as pictured due to changes over time.

Step 11: Saturate filament. Set filament heat to **34**. As of February 2026, this will yield a close to saturation beam suitable for imaging. Please contact the center staff if you feel the beam is too undersaturated at heat 34. Recheck the gun tilt alignment to ensure the brightest beam.

Step 12: Prepare a new gain reference for the Ceta camera if needed. See **Page 24**.

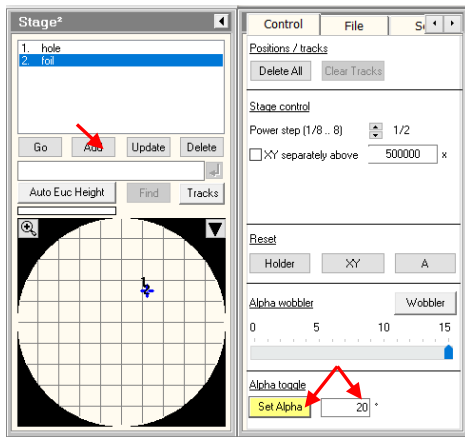
Step 13: Move the stage onto the saved foil position.

Step 14: Press the “Eucentric Focus” button on the control panels if it hasn't been done already. Also, “Reset Defocus” on the control panels (it's currently linked as button R2). Go to **11,000x**, set the **spot size to 4**, center the beam using beam shift, and expand the beam to evenly illuminate the Fluscreen.

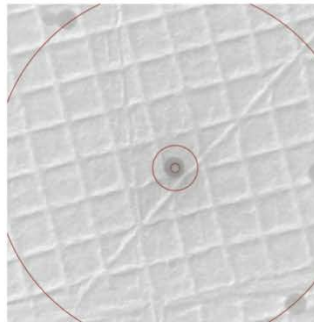
Step 15: Find the eucentric height.

- Use the joystick to center on an easily discernible landmark.
- Type in 10 into the “Set Alpha” field at the bottom of the Stage flapout and click the box (button should turn yellow).
- The field of view will move laterally as the holder rotates because the sample is probably not at eucentric height (if it moves so far that you can't tell what's going on, you can decrease magnification, go back to 11,000x for the following iterations).
- Use the “Z axis” buttons on the control panels to re-center that landmark. **DON'T USE THE STAGE MOVEMENT JOYSTICK** for this step. Buttons are pressure-sensitive.
- Press “Set Alpha” to return the stage to 0° tilt (button should turn grey), and the landmark should be close to the center.
- Center the feature again using the joystick if necessary.
- Type 20 into “Set Alpha”, press the button to tilt the stage to 20°. Center the object using the “Z axis” buttons on the control panel.

- Press “Set Alpha” to return the stage to 0° and center the object with the joystick.
- Type 30 into “Set Alpha”, press the button, and center the object using the “Z axis” buttons on the control panel.
- Press “Set Alpha” to return the stage to 0°, and the object should remain centered. Repeat centering the object using the joystick at 0° and the z-axis buttons at 30° until the object remains within the second red circle on the Fluscreen at both 0° and 30°.
- Once the object remains centered in the second red circle at both 0° and 30°, update the Z-height to the foil area in the Stage panel.

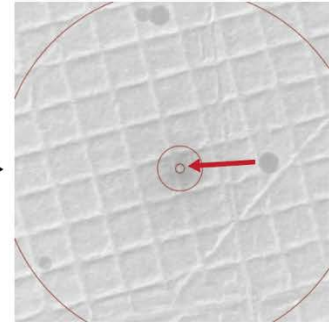


center on landmark
with joystick



tilt stage
(adjust α)

recenter with Z
height adjustment



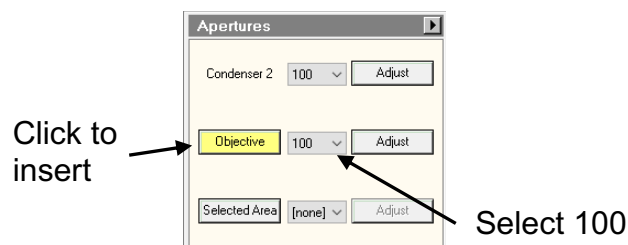
NOTE: When you change the alpha to 10°, 20°, or 30°, pay attention to the tilt axis value in the TEM UI—it should update accordingly to 10°, 20°, or 30°, and return to 0° afterwards. See the screenshot below for reference (an example is shown for 20°).

Pay attention to this tilt axis value					
X:	250.13 μm	A:	19.99 deg	X:	250.17 μm
Y:	309.96 μm	B:	0.00 deg	Y:	309.65 μm
Z:	-58.93 μm	Cold trap LN2:	42 %	Z:	-58.93 μm
					Cold trap LN2: 42 %

Step 16: Insert the objective aperture.

Increase the magnification to **45,000x** and set the **spot size to 4** (or to your data collection magnification). Adjust C2 lens power (aka “Intensity”) so that the screen current is around 0.5 nA with the beam evenly illuminating the screen. Make sure you are on the correct (right) side of crossover (beam expands as intensity knob is turned clockwise).

- Insert 100- μm objective aperture. Make sure you are using the 100- μm aperture, or you can’t do the next step.

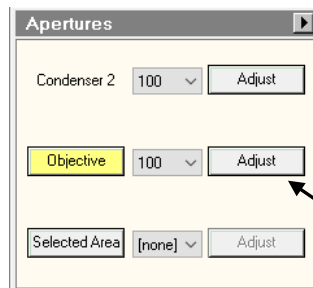


Step 17: Center the objective aperture.



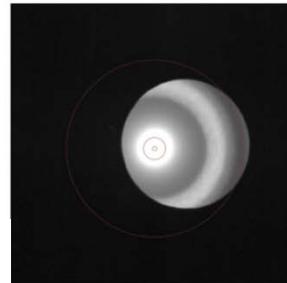
Ensure the Fluscreen is down before entering diffraction mode!

- Go to diffraction mode by pressing the diffraction button on the control panels.
- Adjust the contrast on the Fluscreen by rolling the mouse wheel until you see a halo of rings surrounding the central beam. Click the “Adjust” button beside the “Objective” aperture to change the behavior of multifunction knobs. Move the aperture so its shadow is concentric with the rings. You can adjust “camera length” with the magnification knob to expand or contract the image here.
- After making the necessary adjustment, click “Adjust” again to stop adjusting and exit diffraction mode once done.

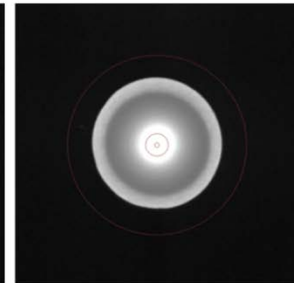


Click to adjust

you can do better



good



- After centering the Objective aperture, click Objective to take it out.

Step 18: Find focus.

- Go to **120,000x** and press the “Eucentric focus” panel button (if it hasn't been done already). Click Natural Contrast for the Fluscreen if you adjusted it in the previous step.
- Adjust C2 so that the beam evenly illuminates the screen, and the screen current is around 0.5 nA. You may need to change the spot size to accomplish this. Currently, **spot size 4** works.

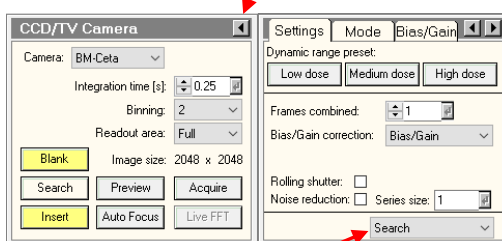


IMPORTANT: Do not expose the Ceta to screen currents > 1.5 nA.

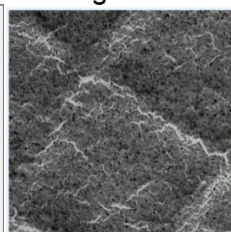
- Insert the Ceta camera, and open the flap in the CCD/TV camera tab. In the Settings tab, make sure “Search” is selected in the dropdown menu at the bottom of the flap-out (the Search button will be white).
- Set sampling (aka binning) to 2 and integration time to 0.25 seconds.
- Press “Search”
- Raise the Fluscreen.

You should now be able to see the camera output in real time in TIA. Click on FFT to show the Fourier transform of the image in parallel with the real space image in TIA.

- Use the bottom, larger focus knob to set the Focus step to 3
- Use the smaller, top focus knob to adjust focus so that the center spot is as large as possible, no Thon rings should be visible.
- Press “Search” again to stop the camera when done.



real space image in TIA



reciprocal space image in TIA

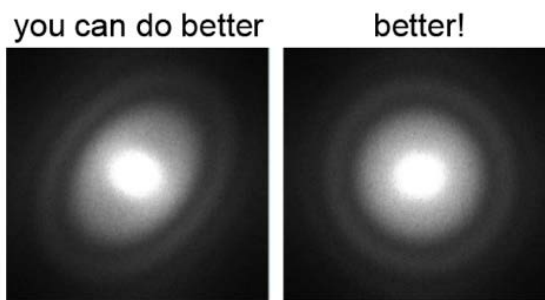


The beam is stigmatized and underfocus in this image; we will correct the stigmatism in the next step.

Thon rings!

Step 19: Correct objective lens stigmatism.

- Restart Search with the FFT showing in TIA.
- Turn the focus knob counterclockwise to defocus one to two μm , using the Defocus readout in the TEM UI as a guide.
- Go to the Stigmator (on Camera tab) and select “Objective”.
- Adjust the stigmatism with multifunction X to make the Thon rings as round as possible.
- Adjust the stigmatism with multifunction Y to make the Thon rings as round as possible.
- Expand the Thon rings a bit by adjusting the focus knob closer to the lens (astigmatism is easier to see when you get close to focus).
- Iterate adjustment of multifunction X and Y to make the Thon rings as round as possible.
- Click None in the Stigmator when finished.
- Use the focus knob to return to focus (central ring fully expanded) for the next steps.
- Press “Search” again to stop the camera when done.
- If astigmatism is bad, start at a defocus of $-2\text{ }\mu\text{m}$, adjust the Stigmator using Mx and My, then go to defocus of $-1\text{ }\mu\text{m}$ and do a final adjustment.



Step 20: Adjust pivot points.

- Lower Fluscreen (if necessary)
- Adjust the mag to 45,000x, the spot size to 4, and the intensity to crossover.
- Center the beam at the crossover using Beam Shift under direct alignments.
- Select “Beam tilt pp X” under direct alignments. This is known as the “pivot points” alignment. The beam will start wobbling between two tilt settings.
- Adjust the multifunction X knob to minimize translation (maximize overlap) of the beam between the positions and adjust the multifunction Y knob to reduce translation of the beam in the perpendicular direction. (You can decrease mag if the beam is translating off the screen.)
- Click done.
- Repeat this alignment for “Beam tilt pp Y”. Click done.

For more information refer to this Eric Montemayor's video link on Pivot Point Alignment here:

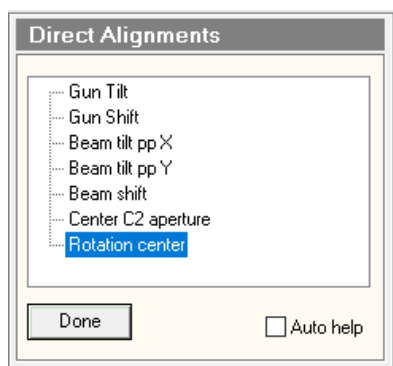
<https://youtu.be/MFxFbnOsq68>



Step 21: Rotation center alignment.

- Go to **120,000x** and expand the beam to flood the Fluscreen.
- Select “Rotation center” in direct alignments. The image will appear to pulse and may have lateral translation as well. The rotation center is well aligned when the image expands and contracts without any concurrent translation.
- Watch a small area of the image (such as something near one of the red circles) and adjust multifunction X to minimize image translation. If the translation appears to change direction, switch to multifunction Y.
- Adjust the multifunction Y to minimize image translation. If the translation appears to change direction, switch back to multifunction X. You may need to iterate between X and Y several times.

For more information refer to this Eric Montemayor's video link on Rotation Center Alignment here:
<https://youtu.be/EPuryhFiD2w>



Step 22: You are now ready to image your sample(s). But since you are going to remove the objective aperture during sample exchange, be sure to repeat **Steps 16-17** after inserting your sample. This will burn a small region of your sample, but that's OK.

BEFORE UNLOADING

Make sure all the following are true. See the images below for details.

- 1) Column valves are closed (Button is yellow)
- 2) Stage reset to 0 (X, Y, Z, and A; reset holder)
- 3) Objective and Selected Area apertures are retracted
- 4) All vacuum levels OK (All highlighted in green, Accelerator = 1)
- 5) The red light on the stage is not on
- 6) Fluscreen is down
- 7) Camera is retracted

Keep an eye on the liquid nitrogen level; it should remain above 30% while you are working.

The screenshots and their annotations are as follows:

- Vacuum (Supervisor) Screenshot:** Shows a table of vacuum levels. The status bar at the top says "Status: All Vacuum (Closed)". Annotations include:
 - "good vacuum (all green)" pointing to the green status bar.
 - "yellow means closed" pointing to the "Col. Valve Closed" button.
 - "liquid nitrogen level" pointing to the "44 %" value.
- Apertures Screenshot:** Shows settings for "Condenser 2" (100) and "Objective" ([none]). Annotations include:
 - "grey means retracted" pointing to the "Objective" dropdown menu.
- CCD/TV Camera Screenshot:** Shows camera settings like "Camera: BM-Ceta" and "Integration time [s]: 0.25". Annotations include:
 - "grey means retracted" pointing to the "Auto Focus" button.
- Condenser 2 Screenshot:** Shows the "Condenser 2" value set to 100. Annotations include:
 - "Make sure OA is retracted" pointing to the "Objective" dropdown.
 - "Make sure SA is retracted" pointing to the "Selected Area" dropdown.
- Reset Screenshot:** Shows a "Reset" button and a "Holder" button. Annotations include:
 - "Make sure holder is reset" pointing to the "Holder" button.
- Diagram Screenshot:** A schematic diagram of the instrument's internal components. Annotations include:
 - "COLUMN VALVES CLOSED" pointing to the top section.
 - "Fluscreen is down" pointing to the "FluScreen" component.
 - "camera is retracted" pointing to the "BM-Ceta" camera at the bottom.

Preparing the Ceta Camera for Data Collection

The Ceta gain references are acquired from within the microscope control software. References should be taken **at least** once a week. Always check by taking an image over the vacuum, then look for gain artifacts before deciding whether a new gain reference is needed.

Step 1: At your data collection magnification, make sure your illumination system is aligned to provide a uniform beam.

Step 2: Move the stage so that you are imaging over a vacuum (nothing in the field of view).

Step 3: Insert the Ceta camera. Keep Fluscreen down for now.



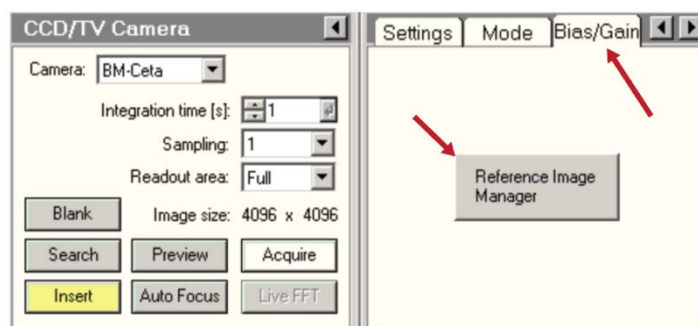
WARNING: Never change magnification, spot size, or C2 lens power (intensity) with the screen lifted

Step 4: Go to **spot size 4** and **45,000x** (or your data collection mag and spot size if known). Adjust C2 lens power (aka “Intensity”) so that the screen current is around 0.3 to 0.65 nA. This should provide a **uniform beam that floods (completely covers) the Fluscreen**. Adjust the beam shift to center the beam if necessary.

For optimal gain correction, you need to match the dose rate of your imaging state as closely as possible. This means you may want to optimize your imaging state in SerialEM, EPU, or other software before collecting your gain reference. You may also choose to collect a complete set now by pressing “Acquire Full set” in the Ceta 2 Reference Image Manager and collecting a fresh “Acquire Single Gain Reference” after setting up your imaging state.

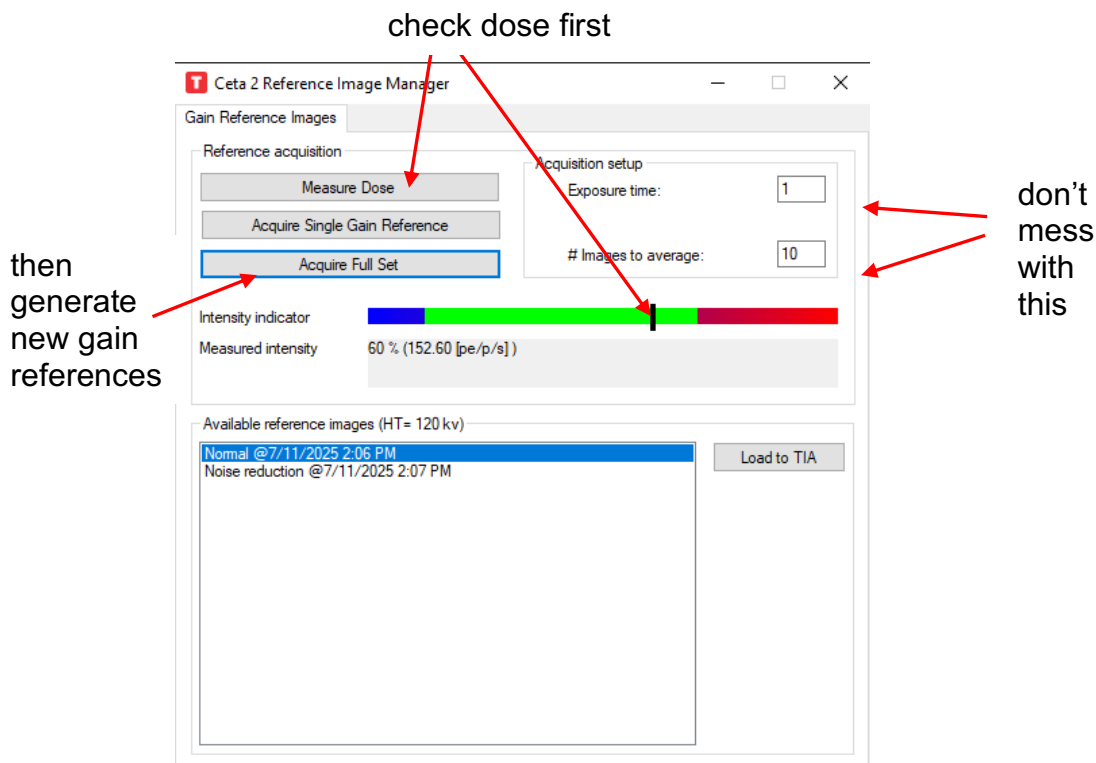
You may need to manually raise the Fluscreen to record an image on the Ceta.

Step 5: Go back to the CCD/TV Camera under the Camera tab. Activate the flap-out, then go to the Bias/Gain tab. Click “Reference Image Manager”

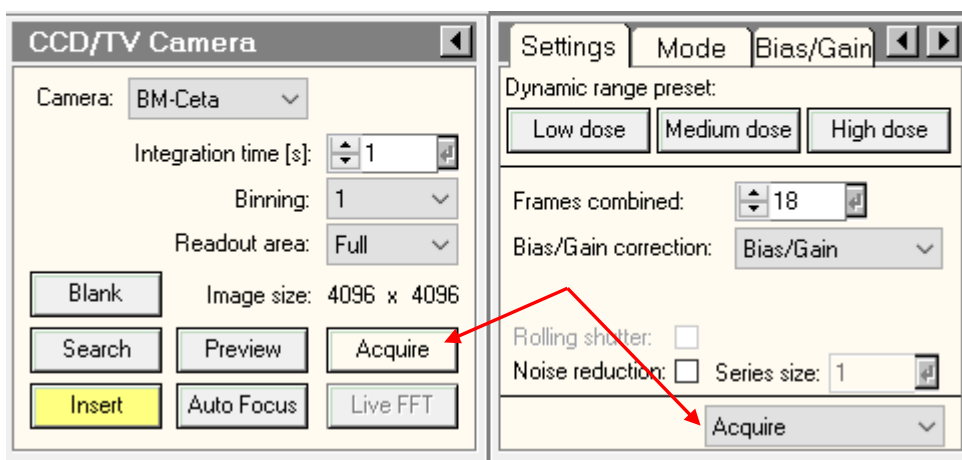


Step 6: With the Fluscreen raised, select “Measure Dose”. This should yield a dose within the camera's dynamic range (along the green slider). Expand/contract the beam or change the spot size if needed to adjust the dose. If you contract the beam, make sure it is still uniformly covering your field of view.

Step 7: When the dose is in the ideal range, select “Acquire Full Set”. This process will run automatically and will take approximately 1-2 minutes.



Step 8: Once done, take an image using the “Acquire” button while still over the vacuum, and ensure both the image and its Fourier transform are uniform. Make sure “Acquire” is selected in the dropdown menu at the bottom of the flap-out. For acquire, binning should be 1 and integration time 1 second.

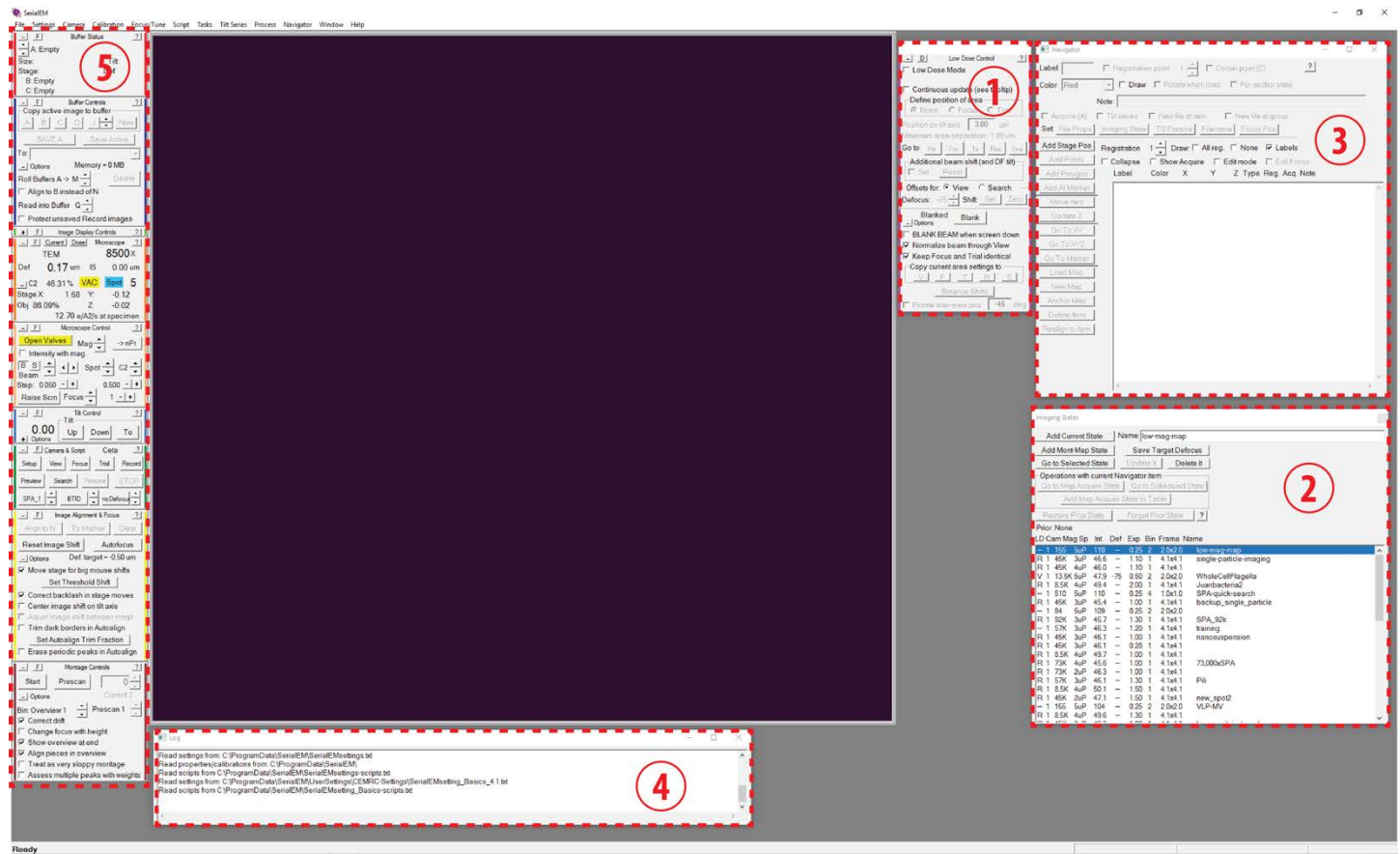


Step 9: Be sure to retract the camera when no longer in use.

If the gains have been acquired recently and you do not see any artifacts in your images, you may skip the gain reference collection step.

Single Particle Data Collection with SerialEM

An overview of the SerialEM GUI is shown in the image below. Key dialog boxes are identified by red circled numbers.



- 1. Low Dose Control:** The Low Dose Control panel is used to configure and verify the low-dose image states. This panel provides a workflow for establishing, verifying, and executing the low-dose collection mechanism.
- 2. Imaging States:** This Image States dialog allows you to store a set of imaging settings and then easily return the microscope to a set of microscope settings and Record parameters.
- 3. Navigator:** The Navigator panel and its associated menu entries provide the capability to save stage positions and return to them; to make and save a map of areas of interest; to mark positions on these maps; to go to the marked positions; to accurately return to positions on maps, and to transform positions after moving the grid in the specimen holder.
- 4. Log:** The Log panel records messages (eucentric height, electron dose calibration, focus, etc.).
- 5. Control panels:** Many controls and status indicators are arranged in a set of control panels. Each control panel has a colored vertical border. Throughout the workflow, you will be asked to navigate these control panels in setting up your automated collection process.

SerialEM Control Panels. Detailed descriptions of panels from (5) above.

Buffer Control panel. This panel allows you to save images in the active buffer or a previously recorded image if it is still stored in a buffer.

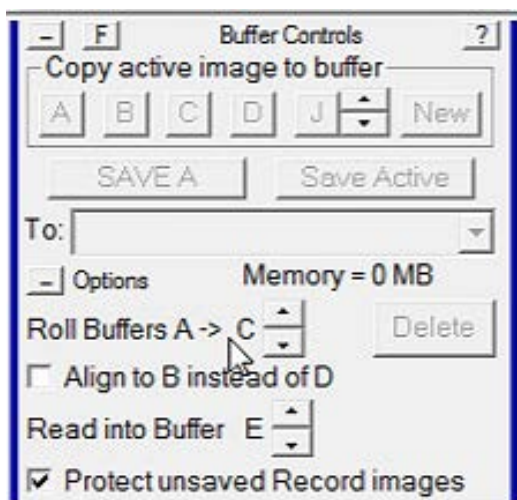
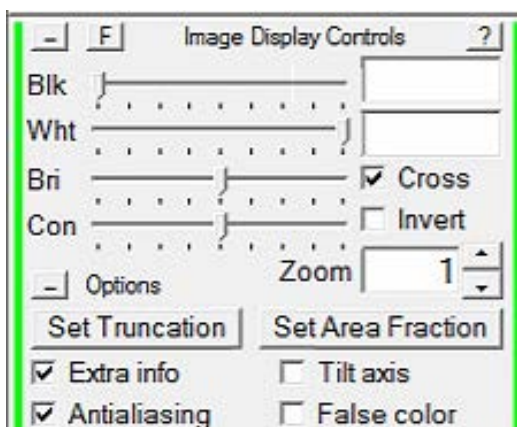
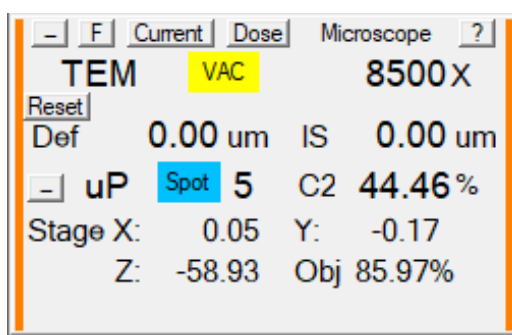


Image Display Control Panel. This panel controls the image, specifically the black-and-white levels. The software analyzes pixels in a central area and truncates to a certain fraction. You can manually change the black and white values to adjust the image contrast.



Microscope status panel. The dose button creates a floating dose meter. This control panel also displays the defocus, magnification, spot size, and other useful microscope information.



Camera and macro controls. Controls camera setup and camera acquisition modes. Used to acquire images using the stored imaging states.

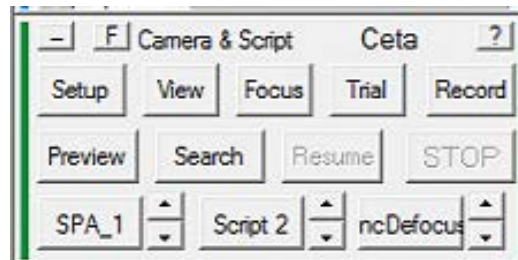
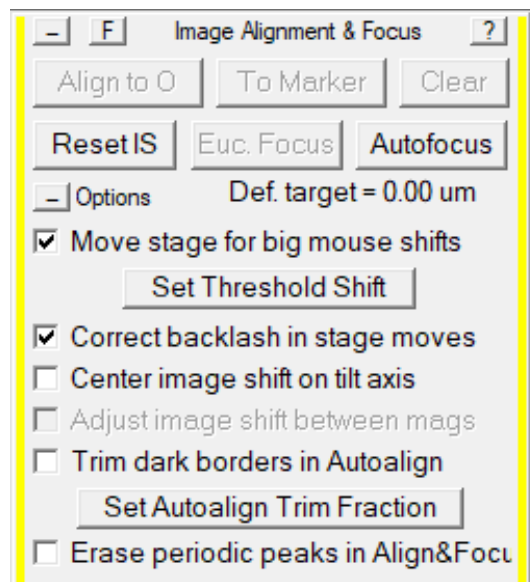


Image alignment and focus.



Reset IS image shift [button]. Clears any currently applied image shift and moves the stage to keep the current target centered.

Move stage for big mouse shifts [checkbox]. Always moves the stage for substantial shifts rather than image shifts.

Section 1: Acquiring a Low Mag Map (LMM Or Grid Atlas)

- 1) Make sure the beam is blanked and the column valves are closed to avoid exposing your sample during setup.
- 2) Open SerialEM. From the menu bar, open Settings>C:\ProgramData\SerialEM\UserSettings\CEMRC\SerialEMsettings.txt
Minimize and expand SerialEM if the log (or similar) windows are not visible when they should be visible.
- 3) From the menu bar, open Navigator (**Navigator -> Open**). If you do not see the Navigator and Imaging States windows, they may be minimized or hidden behind another window. The Imaging state window can be opened from the Navigator menu.
- 4) Select the appropriate imaging state by double-clicking on the state in the Imaging States window. Make sure you are not in low-dose mode (the box near the top of the low-dose panel should be unchecked), and that the microscope is set to **LM 155x, spot size 7, and C2 100%**.
- 5) Click Setup in the "Camera & Script" panel. This is on the left-hand side of the UI. It can also be accessed by the menu running along the top of the UI. **Camera -> Setup**.
- 6) Select the Record radio button at the top of the window.

Set the Record camera parameters as follows:

Acquisition: Single Image

Processing: Gain Normalized

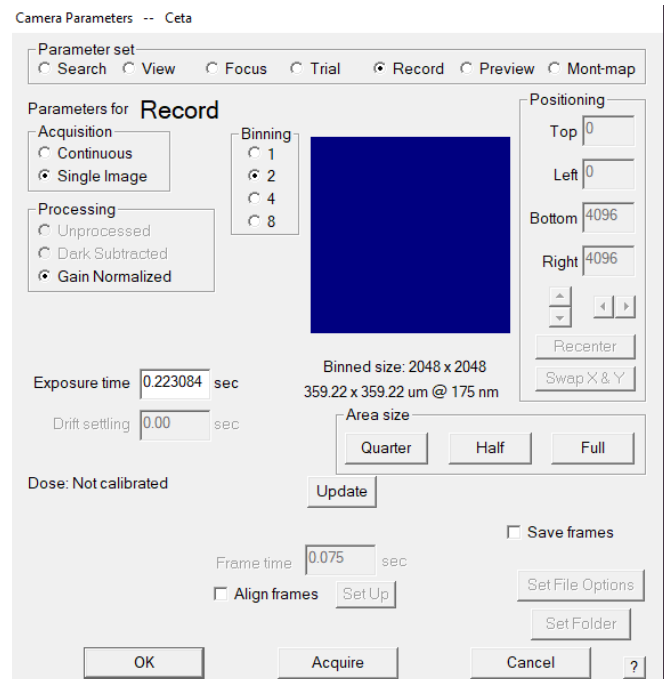
Exposure time: 0.22s

Drift settling: 0s

Binning: 2

Area size: Full

Save frames: Unchecked



- With the screen down, open the column valves and unblank the beam (if necessary). Check that the beam is spread and centered. You can see your grid; there are no apertures in the way (Objective and Selected Area apertures should be retracted), etc.
- Press the Acquire button in the Camera Parameters panel (or Record) to acquire a single Record image in SerialEM and check that the images match.

9) From the menu bar, select **Navigator>Montaging & Grids> Setup Full Montage:**

Select the following settings:

Magnification 155x

Binning: 2

Number of pieces X and Y: 4x4

Minimum overlap 10% and 0.5 μm

Select checkboxes:

‘Move the stage instead of shifting image’

‘Make a map from each montage if Navigator open’

10) When done, click ‘OK’.

Montage Setup

FITTING TO NAVIGATOR AREA: Change mag to adjust number of pieces. Changing mag, binning, overlap, or "Move stage" will refit to area

Magnification: 155 Binning: 2

Pixel size: 175 nm

Number of pieces in X: 4 Y: 4

Piece size in X: 2048 Y: 2048

Overlap in X: 306 Y: 306 Reset

Minimum overlap: 10% and 0.5 micron

Total Area: 7274 x 7274 pixels 1276 x 1276 microns Update

☒ Move stage instead of shifting image

☐ Image shift in blocks, IS up to 35 microns

☐ Skip pieces outside Navigator item 0

☐ Do full rectangle; ignore list of pieces to skip

☒ Make map from each montage if Navigator open

☐ Close file when montage is done

☐ Use Montage Mapping, not Record parameters

☐ Acquire with Multiple Record routine

☒ Use continuous mode with settling factor 0.5

☒ Turn off Drift Correction for stage montage

☐ Use settings for high-quality stage montage

OK Cancel ?

11) A file properties window will appear, select the following settings:

Save data as: ‘Integers’

Save in extended header: Check all boxes

Select ‘Save extra information in a ‘.mdoc’ metadata file’

Save images to MRC stack file

Click ‘OK’

File Properties

File type

Save images to

☒ MRC stack file

☐ HDF stack file

☐ TIFF file (one image per file)

☐ Series of TIFF files listed in an Autodoc file

☐ JPEG file (one image per file)

Type of compression in TIFF or HDF file

☒ None ☐ ZIP ☐ LZW ☐ JPEG

Image data treatment

Save non-float data as

☐ Bytes

☒ Integers

When saving 16 bit data

☒ Truncate above 32767

☐ Divide by 2

☐ Subtract 32768

Percent of pixels to truncate converting to bytes

As black (0): 0.2 As white (255): 0.2

Metadata

Save in extended header

☒ Tilt angle

☒ Intensity

☒ Stage position

☒ Magnification

☒ Exposure dose

Maximum number of montages: 360 (Be generous)

☒ Save extra information in a .mdoc metadata file

☐ Skip this dialog in future (re-enable in File menu)

OK Cancel ?

12) Choose a destination and name for your file. Make sure to follow facility data standards. These standards ensure you can access your data after the collection is complete.

OffloadData:/Temscribing/BM-Ceta/ /YYYY_MM_DD_netid/ flexible

Recommended:

OffloadData:/Temscribing/BM-Ceta/
/YYYY_MM_DD_netid/sample_name/grid_box/grid/

13) The montage control panel should now be visible on the left side of SerialEM. Select the following settings:

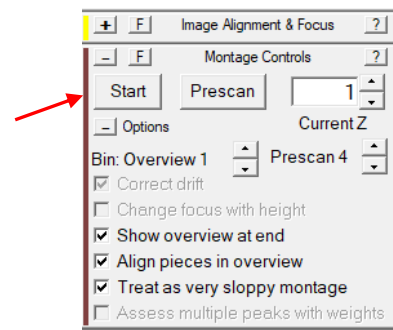
Bin: Overview = 1, Prescan = 1

Check boxes for:

‘Show overview at end’

‘Align pieces in overview’

‘Treat as very sloppy montage’



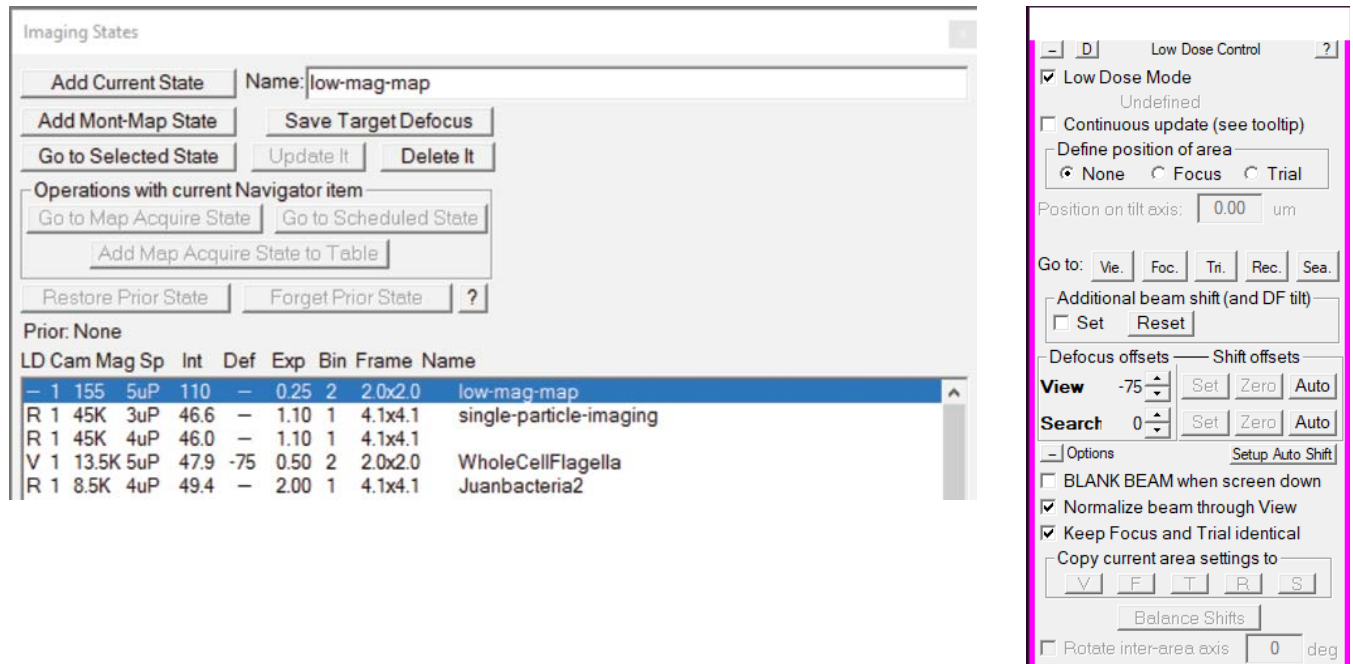
14) Click the **Start** button in this panel to begin acquiring the montage. It is best not to touch anything during this time. The progress is visible in the bottom right corner.

15) When it is finished, say yes and that you would like to make a map. This map will now be item 1 in the navigator window. From this point forward, this map will be referred to as a low magnification montage (LMM).

16) You can reload the map by double-clicking on the Navigator item at any time. To always keep a copy of the map open (optional), go to **Window > New Window** on the menu bar.

Section 2: Registering Your Map and Setting Up Low Dose Mode

- 1) From the Imaging States window, select an appropriate View imaging state (will have a V in the LD column) and an appropriate Record imaging state (R in the LD column) by double-clicking on them. A small † will appear before the Name of any loaded state.



- 2) The checkbox by Low Dose Mode in the Low Dose Control Panel should be checked once you select the imaging state.

VIEW (and map registration)

- 3) Press Go to: View In low dose control panel.
- 4) The microscope settings should update to settings for acquiring square maps.
- 5) Check the microscope Mag, Spot Size, and C2 to ensure they are what you expect.
- 6) To change the mag, spot size, or C2 settings, check the Continuous Update box in the Low Dose control panel. With the screen down, use the hand panels to set your desired settings, and make sure to uncheck Continuous Update.
- 7) Make sure that Offsets for Defocus: View in the low dose control panel is set to -75 µm.
- 8) Move the stage over vacuum (preferred) or an area of the grid that you don't plan on collecting from. To do this, left-click on the map to add a green cross over the location you would like to move to and select "Go to Marker" on the left-hand side of the Navigator menu. Since the map is not yet registered, you may be ~20 µm off after moving. Lower the Fluscreen and use the joystick to move to the desired location.
- 9) Lower the screen and make sure the beam covers the screen, and that the screen current is around 0.5 nA.
- 10) Click Setup in the Camera & Script panel.

- 11) Select the View radio button at the top of the window.
- 12) Make sure the View camera parameters are as follows:
 - Acquisition: Single Image
 - Processing: Gain Normalized
 - Exposure time: 0.5s
 - Binning: 2
 - Area size: Full
 - Save frames: Unchecked
- 13) Add one point (left-click) at an easily identifiable object (asymmetric tear or debris) on the low mag map.
- 14) Click Add Marker on the left panel of the Navigator window. This point will now be item 2.
- 15) With item 2 selected, click 'Go to XY' on the left side of the navigator window.
- 16) Your object (Item 2) should be visible and labeled in the LMM map.
- 17) In the menu bar, select **Tasks>Eucentric - Rough**.
- 18) SerialEM will now run an automated series of tilts and acquisitions to determine the eucentric height. The new height will be reported in the log window when it is done.
- 19) Select the map item (LMM) in Navigator and select "Update Z".
- 20) After it is done, take another View image by pressing View in the Camera & Scripts panel.
- 21) This View image is rotated relative to your LMM, so make sure your reference image is an asymmetrical object, not just a round dot.
 - a. If the object you had selected is visible in the View image you just took, left-click at the exact position of Item 2 that you had previously selected in your low-mag map (LMM). This will place a green cross in the same position on the View image you just took.
 - b. If it is no longer in the image, that means your image shift will have to be greater than the image the camera can capture. In this case, lower the Fluscreen and re-center the item before taking another View. Once the object is visible in the new View image, left-click at the exact position of Item 2 that you had previously selected in your low mag map (LMM).
- 22) In the SerialEM interface, go to the '**Navigator**' menu in the menu bar and select **Shift to Marker**, (**Navigator>Shift to marker**) and click yes.
- 23) If your shift worked, the label for Point 2 will now appear as orange in the View image you were just in.
- 24) Before applying the shift, you will be prompted for confirmation.
- 25) Make sure the shift is reasonable (not 100- μ m).
- 26) Close the map file by selecting **File>Close** in the menu bar.
- 27) Save the Navigator file to your directory from the menu bar: **Navigator > Save As**.
- 28) In the Low Dose control panel, make sure the options 'Normalize beam through view' and 'Keep Focus and Trial Identical' are selected.

NOTE: It is a good idea to save the Navigator file periodically as you add items.

RECORD

- 1) In the Low Dose Mode panel, press the Go to: Rec. button, and press the Eucentric focus on the hand panel.
- 2) Insert and center the objective aperture if you haven't done so
- 3) Check the microscope Mag, Spot Size, and C2 to ensure they are what you expect.
- 4) To change the mag, spot size, or C2 settings, check the Continuous Update box in the Low Dose control panel. With the screen down, use the hand panels to set your desired settings, and make sure to uncheck Continuous Update. Adjust C2 or the spot size to achieve approximately 0.5 nA screen current with an evenly illuminated screen.
- 5) With the screen still down, check the beam position. Adjust the intensity/C2 to near crossover to see if the beam is centered (Make sure Continuous update is not checked). Use the Beam Shift alignment in the Direct Alignments to center the beam. In the Low Dose Mode panel, press the Go to: Rec. button to reset the beam to the initial intensity.
- 6) Verify that the defocus offset for View in the TEM user interface is **-75 μm** , as set earlier.
- 7) Move over to vacuum and take an image with your record settings.

Acquisition: Single Image

Processing: Gain Normalized

Exposure time: 1.0s

Binning: 1

Area size: Full

Save frames: Unchecked

If you can see a gradient, acquire a new gain (Acquire a single gain reference is sufficient). If the gradient persists, you may need to reduce your dose rate and increase your exposure time.

For the Talos L120, a gradient will always be visible; however, we aim to minimize the intensity of these lines.

- 8) Once you have an even image over vacuum, select **Tasks → Calibrate electron dose** from the menu bar. It will ask you to confirm that the current image is over vacuum, and the calibrated electron dose will be shown in the log window. The total dose for a Record image will now be shown in the top right of the low-dose control panel.
- 9) **Make note of the dose rate in the logbook.**
- 10) To adjust the dose, check Continuous Update, then change the spot size or C2% as necessary. Uncheck Continuous Update when done.
- 11) Near the bottom of the Low Dose control panel, press Copy current area settings to F and T and confirm that you want to do this.
- 12) Measure the beam diameter. Use the default display mode (Natural) on the Fluscreen while measuring.
 - a. Make sure the "Continuous update" option is not checked.
 - b. Make sure Int. Zoom is not on in the Beam Settings panel in the TEM User Interface.
 - c. With the screen still down, decrease the magnification until the entire beam is visible on the screen, and use the scale bar to approximate the diameter.

- d. You will need to know this to define the Focus offset distance when setting up the Focus/Trial settings below.
- e. Return the mag to its starting value before lifting the screen. **DO NOT** expose the Ceta camera to the tight beam.



FOCUS

- 1) Take a View image if one is not on the screen.
- 2) In the Low Dose Mode panel, press the Go to: Foc. Button, lower the screen, and check that the beam is spread and evenly illuminating the screen. Raise the screen after checking.
- 3) Select Focus in the Define position of the area in the Low Dose Mode panel.
- 4) Two boxes will appear on the View image: green indicates the position of the Record, and yellow indicates the Focus/Trial area.
- 5) The Focus area will be set along the tilt axis, above or below your record area.
- 6) Enter an appropriate value in the Position on tilt axis box so that the Focus beam will not overlap your record area. In general, the focus shift should be as large as (or slightly larger than) the Record beam size you measured earlier. The value can be + or –, depending on which side of the object you want to focus on.
- 7) Select None in the Define position of area.
- 8) If the beam is not centered, check “Set” under Additional Beam Shift (and DF tilt) in the low dose control panel and center the beam with multifunction X and Y. This should not be off by very much. If it is way off, talk to facility staff, as that may indicate a larger problem with the microscope’s base image shift alignment (something only the TFS engineers can adjust).
- 9) Uncheck Set.
- 10) In the menu bar, select **Focus/Tune>Set target**.
- 11) Enter the defocus value that you would like autofocus to apply for screening (generally in the range of -0.5 to -1.5 μm). This will be superseded by the value in the script later, once you start automated collection of a dataset.

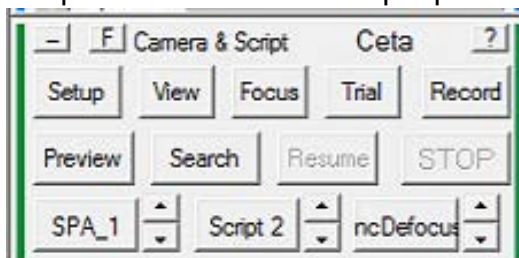
TRIAL

- 1) In the Low Dose Mode panel, press the Go to: Tri. Button.
- 2) The beam should be identical to Focus; if not, make sure ‘Keep Focus and Trial Identical’ is selected.
- 3) In the Low Dose Mode panel, press the Go to: Rec. Button and check the beam.
- 4) If necessary, repeat the steps above for Record.

With the Fluscreen down, go through Rec.>Vie.>Foc.>Tri.>Rec. and make sure all of the settings match what you expect, and that the beam shift is centered for Record. Correct Beam Shift for Record now if necessary.

CAMERA PARAMETERS

- 1) Press Setup in the Camera & Scripts panel.



- 2) Select the View radio button at the top of the window.
- 3) This should remain as you set it previously:

Acquisition: Single Image

Processing: Gain Normalized

Exposure time: 0.5s

Binning: 2

Area size: Full

Save frames: Unchecked

- 4) Select the Focus radio button and set as:

Acquisition: Single Image

Processing: Gain Normalized

Exposure time: 0.5s

Binning: 2

Area size: Full

Save frames: Unchecked

- 5) Select the Trial radio button and set it to match Focus.

- 6) Select the Record radio button and set it as:

Acquisition: Single Image

Processing: Gain Normalized

Exposure time: 1.0s (depending on desired dose, ideally ~1 sec, not more than 2 sec)

Binning: 1

Area size: Full

Save frames: Unchecked

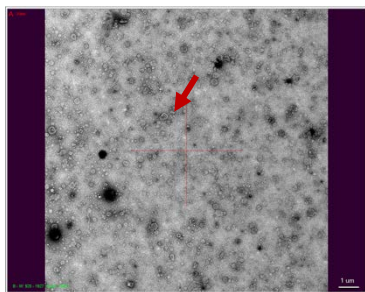
Align View and Record (Center the image).

NOTE: If you change spot size or mag for View or Record after this alignment, you will need to redo it.

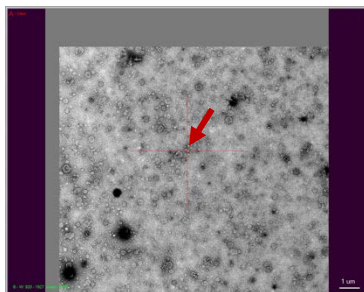
- 1) From the Camera & Script panel, press View to acquire a View image.
- 2) Find an identifiable object within the image.
- 3) By clicking and holding down the right mouse button, drag the image so that the object is centered on the screen.
- 4) Take another View image and ensure the item is centered in the image; if not, drag the image to center the item again.
- 5) Once the item is centered in view, do Autofocus and take a Record image.
- 6) If the item is still centered, you are done.
- 7) If the item is not centered in the Record image, drag the image to center the item.
- 8) Take another record image and confirm that the item is centered.
- 9) Take a View image.
- 10) Drag the View image so that the item is centered, do not take another view.
- 11) In the low dose control panel, under Offsets for View: Shift: press Set.
- 12) SerialEM will acquire another View image.
- 13) If the item is not centered, drag the image again and press Set once more.
- 14) Once the item is centered in view, take a Record image.
- 15) If the item is not centered in the Record image, you will need to iterate this process, starting with taking a Record with the item centered.

The alignment will likely never be perfect; for many purposes, getting the alignment within <250 nm will be sufficient. If the item isn't centered in View and Record after two or three iterations, there may be a problem with the low-dose setup or microscope alignments.

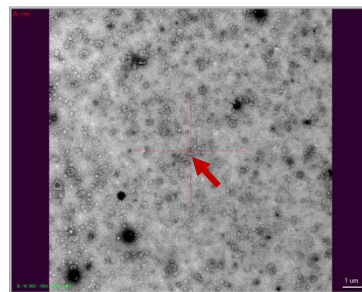
Align View and Record (Center the image)



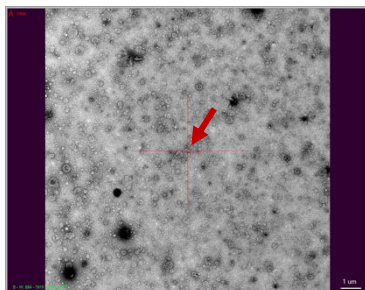
Find an identifiable object.



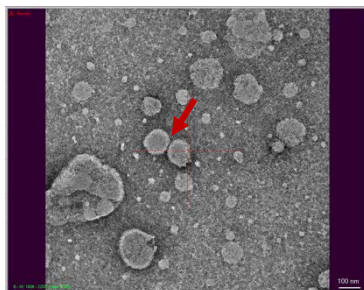
Click and hold down the right mouse button, center the object.



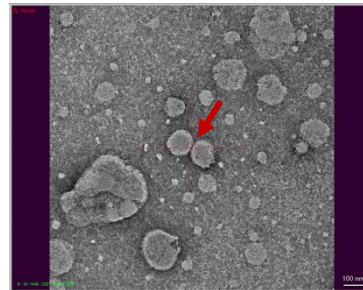
Take a View image. Drag and center the item if it is not centered.



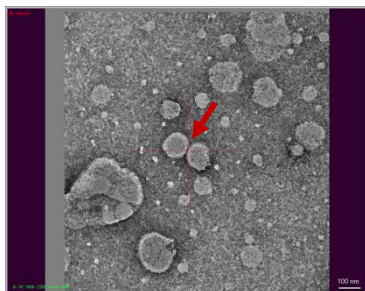
Take another View image and ensure the item is centered.



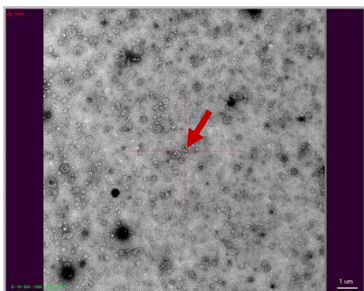
Take a Record image.



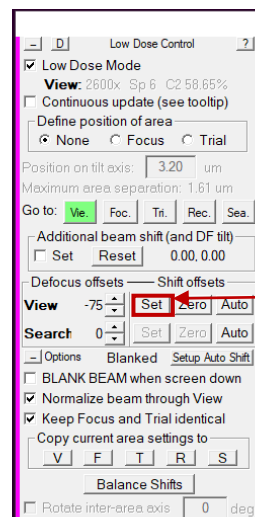
Click and hold down the right mouse button, center the object.



Take another Record image and ensure the item is centered.



Take a View image, center the item, do not take another View image.



Press

Section 3: Setting Up Grid Screening in SerialEM

Checking for Good Squares

- 1) Double-click your low-mag map navigator item to load the map.
- 2) If you can already tell which squares you want to collect from, you can proceed to setting up square map montages.
- 3) Left-click on a square you want to image and press Add Marker in the Navigator Window.
- 4) This will add the point to your map and in Navigator so that you will know where you have already imaged when setting up points for data collection.
- 5) Press Go to XY in the Navigator window.
- 6) To autofocus at this position, press Autofocus in the Image Alignment & Focus panel.
- 7) When it is done, the measured starting defocus and adjustment will be reported in the log window.
- 8) You can now take a View or Record image at this position using the buttons in the Camera & Scripting panel.

If the measured defocus is 0.0, autofocus probably failed. This may occur if the position is far from the eucentric focus. If Autofocus fails, you will need to find the eucentric height and try again. It is not necessary to find the eucentric height for each square at this point if Autofocus is working.

To find the eucentric height for the stage position, in the menu bar, select **Tasks>Eucentric-Rough**. SerialEM will run an automated series of tilts and acquisitions to determine the eucentric height. The stage will be adjusted, and the new height will be reported in the log window when it is done.

To move the stage to a specific location within a View image, you can drag the View image with the right mouse button so that the position is centered, or you can left-click on the image and select Go to Marker from the navigator window.

Once you have an idea of what a good square looks like, you can set up square maps in Navigator.

Saving Images

- 1) You will want to save some of the images you acquire while screening squares. While the image is on the screen, go to File>Open New.
- 2) A file properties window will appear, select the following settings:
 - Save data as: 'Integers'
 - Save in extended header: Check all boxes
 - Select 'Save extra information in an '.mdoc' metadata file'
 - Save images to MRC stack file
 - Click 'OK'
- 3) Choose a destination and name for your file. Make sure to follow facility data standards:
OffloadData:/Temscripting/BM-Ceta/ /YYYY_MM_DD_netid/ /**flexible**
You will need to type the *.mrc extension at the end of the name.
Everything needs to be lower case, for dates use "2025_07_01" rather than "2025_7_1".

- 4) After the file is created go to File>Save Active in the menu bar
- 5) Once the image is saved go to File>Close in the menu bar.

You can save multiple images of the same type (i.e., View or Record) in a single file as an *.mrc stack. If you want to save more images of the same type, you can leave the file open and go to **File>Save Active** each time you want to save a new image. Make sure to close the file when you are done or when you want to save a different image type.

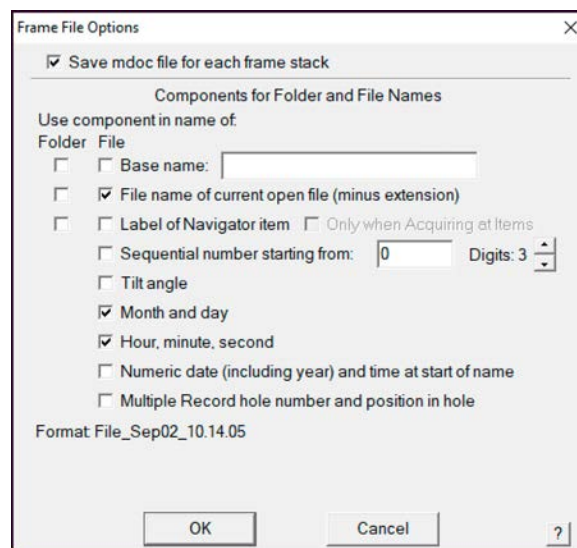
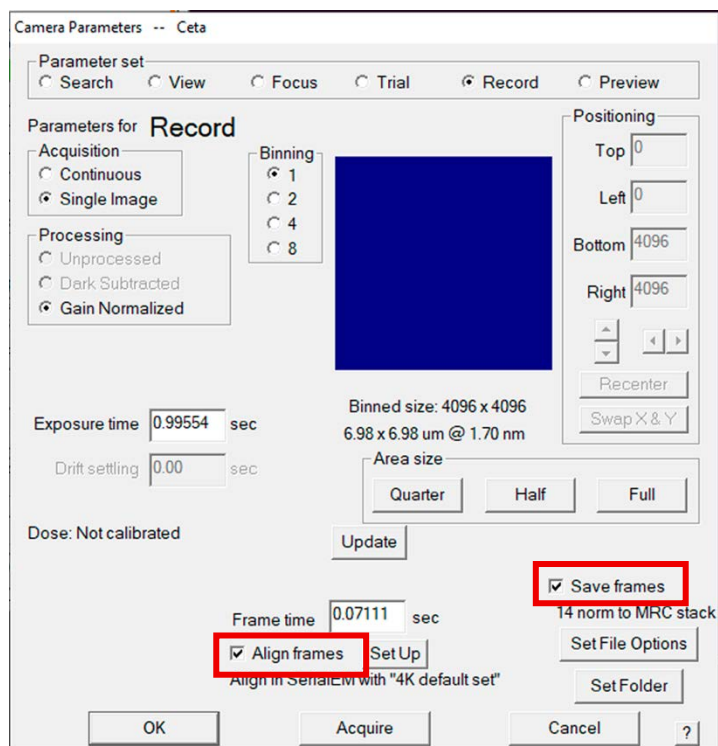
If you want to save an image in a previously closed file, you can go to File>Open old and open the file you wish to save the image to and then go to File>Save Active. Make sure to close the file again when you are done.

Advanced users may choose to leave multiple files open (i.e., one for View and one for Record) and switch between them using the To file: option in the buffer control panel. All currently open files and their numbers are listed in the SerialEM title bar. Make sure to close the files when you are done.

Saving Frames (optional)

The Ceta-F camera can save movie frames at ~14 fps, in addition to integrated single images. You can enable this by checking the “Save frames” box in the Record camera parameters.

If you select “Save frames,” you MUST also check the “Align frames” box. With only “Save frames” checked, SerialEM will save the frames but will not save an integrated image or display the recorded image on the screen.



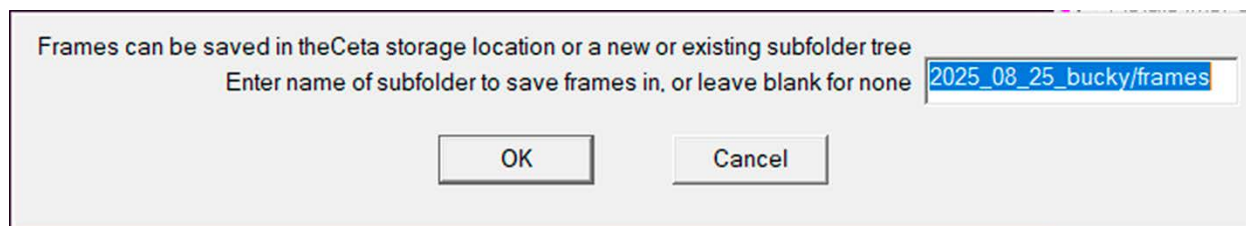
After checking “Save frames,” two additional buttons, “Set File Options” and “Set Folder,” become clickable in the camera parameters window. Set File Options is used to set the filename for saved frame stacks. We recommend checking:

- Save mdoc file for each frame stack
- File name of current open file (minus extension)
- Month and day
- Hour, minute, second

Set folder is used to set where the frames are saved. Using the Windows file explorer, create a folder named frames inside of your folder for the day’s session.

E.g., OffloadData:/TemScripting/BM-Ceta/YYYY_MM_DD_netid/**frames**

For Set Folder, type the name of the last two folders: YYYY_MM_DD_netid/frames



Setting Up Square Map Montages for Screening Collection

- 1) Double-click your low-mag map Navigator item to open the map.
- 2) Left-click at the center of each square you would like to collect from and press Add Marker in the Navigator window to create a Navigator item at this point.
- 3) Once you have all the points added, highlight the first point (single click) and check the Acquire checkbox near the top left of the Navigator window. You can also hit “A” on the keyboard while the point is highlighted. This will add an “A” to the end of the item in the item list.
- 4) Do this for each point, defining a square.
- 5) In the Low Dose control panel, select Go to: Vie.
- 6) Make sure no files are currently open in SerialEM (open files are listed and numbered in the title bar).
- 7) From the menu bar go to **File>New Montage**. SerialEM will collect a new montage for each selected square.
- 8) Select the follow settings:

Select checkboxes: ‘Move stage instead of shifting image’, ‘Ask about making map after montage’, and ‘Use View parameters in Low Dose mode.’

Magnification 2600 x

Binning: 2

Number of pieces X and Y: 4x4

Minimum overlap 10% and 0.5 μm

NOTE: A 4x4 montage at 2600x will cover an entire 200 mesh grid square; you can adjust the number of pieces if necessary.

9) When done, click 'OK'. You may need to adjust number of pieces depending on your grid mesh size.

10) A file properties window will appear. Select the following settings:

Save data as: 'Integers'

Save in extended header: Check all boxes

Select 'Save extra information in an '.mdoc' metadata file'

Save images to MRC stack file

Click 'OK'

Make sure to save your file in the appropriate directory and type the .mrc extension at the end of your file name.

11) From the file menu, go to **Navigator>Acquire at Items**:

12) Select the following settings (Image on next page):

Primary Action: Acquire and save an image or montage

Check the box for "Make Navigator map"

Primary Action-related: Acquire non-montage images in Low Dose with "View"

Tasks before or after Primary: Rough Eucentricity

General options: Close column valves at the end (make sure to reopen them before proceeding if you choose this)

13) Press GO. SerialEM will now go to each Navigator item you checked "Acquire" for. It will determine the eucentric height at each position, collect a 4x4 montage using your View parameters, and save the montage to the file you created. It will create a new Navigator Item for each point; the new items will be identified as a map in the type column. The eucentric height of each map will be updated in the Z column.

14) Go to **File>Close** in the menu bar to close the file.

Section 4: Targeting & Collecting Data with SerialEM

After collecting your grid square maps, you can manually add points at positions within each map for data collection, or you can use SerialEM's Add Grid of Points feature. This protocol will only cover the most basic use of the Add Grid of Points feature for negative-stain EM. SerialEM has advanced features for defining and setting up collection within holes of a cryo-grid, which is not covered here.

Option 1. Manually Adding Points for Collection

- 1) Load your first square map by double-clicking the map item in the Navigator window.
- 2) With the map item still highlighted in the Navigator window, click Go to XYZ – this ensures that the points you add will have the correct eucentric height from the map.
- 3) In the Navigator window, click Add Points.
- 4) Click on each point within the map that you would like to acquire a Record image. The points will be acquired in the order that you add them. Keep your beam diameter and the location of your focus position in mind while you add points. You do not want to collect over an area that has already been exposed to high dose.
- 5) When you are done click Stop adding in the Navigator window.
- 6) Double-click your following square map item to load it and add points as desired. Repeat this until you have points on all of your maps.
- 7) Once all your points are selected, highlight them in the Navigator window and check the “Acquire” box for each as you did when defining squares previously.

Option 2. Add Evenly Distributed Points Using Add Grid of Points

- 1) Load your first square map by double-clicking the map item in the Navigator window.
- 2) With the map item still highlighted in the Navigator window, click Go to XYZ – this ensures that the points you add will have the correct eucentric height from the map.
- 3) Go to **Navigator>Montaging & Grids>Add Grid of Points** in the menu bar.
- 4) SerialEM will prompt you to enter the desired spacing between points. Keep your beam diameter and the Focus position in mind. If you have a 5 μ m beam diameter and a 4 μ m Focus position offset, the points will need to be 9 μ m apart to avoid overlap.
- 5) SerialEM will prompt you to choose whether to move in a Zig-Zag pattern or away from the focus position. Select the Zig-Zag pattern, which minimizes stage movement and allows faster acquisition. Selecting to move away from the focus position would allow you to arrange the points closer together (not covered here).
- 6) SerialEM will ask if you want to flag the points for acquisition; select yes.
- 7) SerialEM will now seed a grid of points and add them to your Navigator window. You can eliminate any bad points by deleting them from the Navigator (Highlight and press Delete Item).
- 8) Repeat this process for each square map.

Collect Images

At this point you should have several points defined in Navigator and flagged as 'Acquire'.

- 1) Make sure no files are currently open in SerialEM (open files are listed and numbered in the title bar).
- 2) Save your Navigator file from the menu bar Navigator>Save or Navigator>Save As.
- 3) From the menu bar, go to **File>Open New**.
- 4) A file properties window will appear. Select the following settings:

Save images to Series of TIFF files listed in an Autodoc file (will save single TIFF per image and an .idoc metadata file)

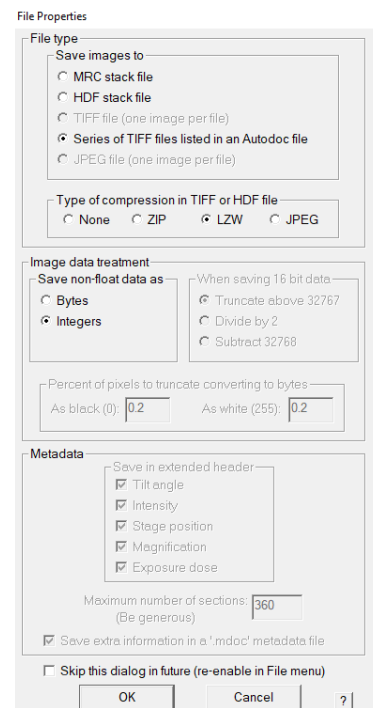
– OR –

MRC stack file (will generate a single MRC file with all images and an .mdoc metadata file).

Type of compression in TIFF file: LZW

Save non-float data as: 'Integers'

Click OK



- 5) Make sure to save your file in the appropriate directory.
- 6) In the menu bar, go to **Navigator>Acquire at Items**.
- 7) Select the following settings:

Primary Action: Acquire and save an image or montage

Tasks before Primary: Run Script before Action

Script to run: IncDefocusEucentricity

(Optional) Realign to Item; select this option if you manually selected points, and it is important that the record image is well centered over your point. SerialEM will take a view image at each point and realign to the map to correct for any errors in stage movement.

General options: Close column valves at the end (make sure to reopen them before proceeding if you choose this).

The script will run Autofocus and rerun rough eucentricity if Autofocus fails. It will also iterate defocus from $-0.5\ \mu\text{m}$ to $-2.0\ \mu\text{m}$. If you do not run this script, you will need to set your target defocus (Focus/tune>Set Target) to the appropriate value and check Autofocus before Primary instead of Run script before Action.

- 8) Make sure the column valves are open before starting.
- 9) Press GO. The IncDefocusEucentricity script will adjust the target defocus for each image to acquire a range of defocus from $-0.5\ \mu\text{m}$ to $-2.0\ \mu\text{m}$, using a $0.25\ \mu\text{m}$ increment. SerialEM will now go to each point, Autofocus at the focus position, adjust to the set defocus, take a Record image at the point, and save it to the file. This may take a while, depending on the number of points added.
- 10) After the collection is done, save your Navigator file from the menu bar Navigator>Save or Navigator>Save As if you didn't save it previously.
- 11) Save your log file from the menu bar, File>Save log file as...
- 12) Close the image file from the menu bar File>Close.

You have now collected a dataset in SerialEM! If you are going to collect on a second grid, do not close SerialEM, but do EXIT Low Dose Mode by unchecking the box in the low dose control panel while you are not working in SerialEM.

BEFORE UNLOADING

Make sure all the following are true. See the images below for details.

- 1) Column valves are closed (Button is yellow)
- 2) Stage reset to 0 (X, Y, Z, and A; reset holder)
- 3) Objective and Selected Area apertures are retracted
- 4) All vacuum levels OK (All highlighted in green, Accelerator = 1)
- 5) The red light on the stage is not on
- 6) Fluscreen is down
- 7) Camera is retracted

Keep an eye on the liquid nitrogen level; it should remain above 30% while you are working.

All your camera parameters, low dose settings, dose calibration, and view-record alignment should be ready for the second grid. It is a good idea to go through the low-dose setup and ensure the beam remains centered across all settings.

The image displays four screenshots of microscope control software interfaces with red annotations indicating required states before unloading:

- Vacuum (Supervisor) window:**
 - Annotation: "good vacuum (all green)" points to the green status bar.
 - Annotation: "yellow means closed" points to the yellow "Col. Valve Closed" button.
 - Annotation: "liquid nitrogen level" points to the "44 % Zero speed (0.0%) Turbo" text.
- Apertures window:**
 - Annotation: "grey means retracted" points to the greyed-out "Objective" and "Selected Area" buttons.
- CCD/TV Camera window:**
 - Annotation: "grey means retracted" points to the greyed-out "Insert" button.
- Stage* window:**
 - Annotation: "reset stage" points to the "Reset" button.
 - Annotation: "grey is 0° tilt" points to the greyed-out "Alpha toggle" button.
 - Annotation: "all zero" points to the X, Y, and Z coordinates (0.00 μm, 0.00 μm, -0.02 μm).
 - Annotation: "all zero" points to the A and B angles (0.00 deg, 0.00 deg).
 - Annotation: "liquid nitrogen level" points to the "Cold trap LN2: 43 %" value.
- Diagram window:**
 - Annotation: "Fluscreen is down" points to the "Fluscreen" component.
 - Annotation: "camera is retracted" points to the "BM-Ceta" camera.
 - Annotation: "liquid nitrogen level" points to the "Cold trap LN2" level.

Section 5: End of the Imaging Session

Step 1: Close column valves and turn off filament

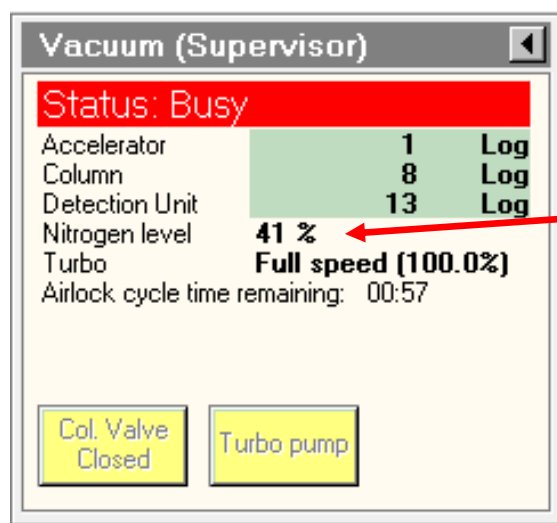
Step 2: Make sure the following are done:

- ☐ Column valves closed
- ☐ Reset stage (holder)
- ☐ Objective and Selected Area apertures are retracted
- ☐ All vacuum levels are OK (nothing is highlighted grey or red)
- ☐ Fluscreen down
- ☐ Camera retracted
- ☐ Close SerialEM (leave all other software open)
- ☐ The microscope is at 8,500x (must be an SA magnification)

Step 3: Remove your sample

Step 4: Re-insert an empty sample holder into the microscope

Step 5: Record the time and liquid nitrogen level in the logbook. **If the liquid nitrogen level is less than 30%, please inform CEMRC staff. Please do not cryocycle.**



Note down Liquid
nitrogen level in
logbook

Step 6: Finish your logbook entry and record the emission and bias values before turning off the filament.

Step 7: Enter session data into the CryoEM Research Center GUI (UW Madison logo on taskbar)

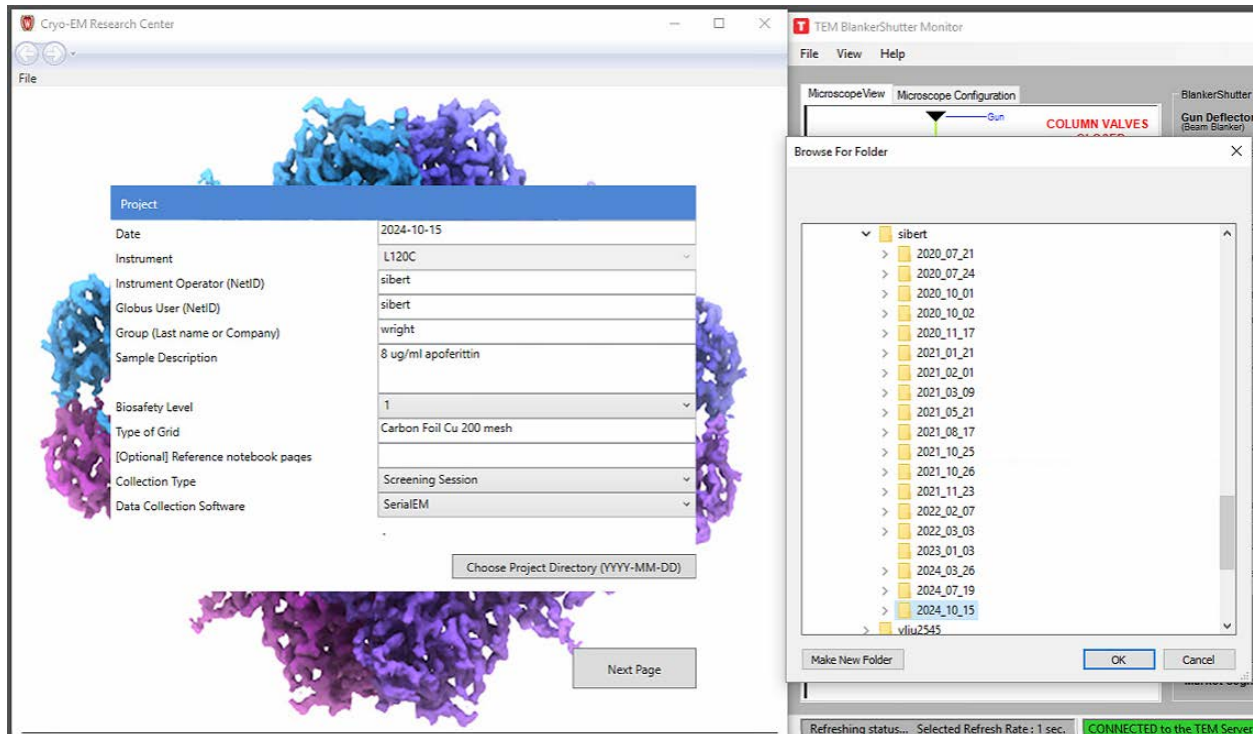
Step 8: Confirm that the filament is off, column valves are closed, and all vacuum levels are good and there are no errors displayed in the TEM user interface.

Section 6: Record Imaging Session Information

Click the UW-Madison logo to open the project information GUI.

Fill out all fields to the best of your ability.

You must select the YYYY_MM_DD directory containing your data in the “Choose Project Directory” field. After you complete the steps in the GUI, a text file will be generated in the directory.



Data Recording Policy

You must follow the protocols outlined in the microscope operations manual. Movement of data from the microscopes to Globus is controlled by the file paths that you generate, and if you do it incorrectly, you will not be able to access your data.

Z:/TemScripting/BM-Ceta/YYYY_MM_DD_netid/**flexible**

You will not be able to access your data unless you strictly follow the:

Z:/TemScripting/BM-Ceta/YYYY_MM_DD_netid/**flexible** part of this policy. Here are a few examples of how this could be done:

Z:/TemScripting/BM-Ceta/2020_07_02_emontemayor/betagal/1/W1804/1/data/Micrographs/*.tif

Appendix 1: L120C Ceta F Magnification Table

Magnification (x)	Mode	Field of View (μm)	Pixel size (Å)
62	LM	876.54	2140.00
84	LM	651.67	1591.00
115	LM	484.15	1182.00
155	LM	359.22	877.00
210	LM	269.23	657.30
280	LM	200.70	490.00
380	LM	149.46	364.90
510	LM	111.74	272.80
700	LM	81.59	199.20
940	LM	61.28	149.60
1300	LM	45.18	110.30
1700	LM	33.50	81.78
2300	LM	24.42	59.63
1250	M	46.86	114.40
1600	M	36.45	88.99
2000	M	28.39	69.30
2600	SA	22.28	54.39
3400	SA	17.26	42.13
4300	SA	13.47	32.89
5300	SA	10.93	26.69
6700	SA	8.86	21.62
8500	SA	6.98	17.05
11000	SA	5.51	13.44
13500	SA	4.33	10.57
17500	SA	3.39	8.28
22000	SA	2.65	6.48
28000	SA	2.05	5.01
36000	SA	1.69	4.12
45000	SA	1.32	3.22
57000	SA	1.04	2.54
73000	SA	0.81	1.98
92000	SA	0.64	1.57
120000	SA	0.50	1.23
150000	SA	0.39	0.96
190000	SA	0.31	0.75
240000	SA	0.24	0.58

Appendix 2: Quick Reference Protocol

1. PURPOSE OF THIS WORKFLOW

The short version protocol for the L120C is designed to serve as a rapid reference guide for experienced or trained users when operating the microscope to screen and acquire images in SerialEM.

2. BEFORE YOU BEGIN

- ☐ **Make sure that your time has been properly booked on iLab**
- ☐ **High Tension is on 120kV**
- ☐ **Column valves are closed**
- ☐ **All vacuum levels are OK (nothing highlighted grey or red)**
- ☐ **Liquid nitrogen > 30%**
- ☐ **No warning/errors**
- ☐ **Filament is off**
- ☐ **The red light on the stage is not on**
- ☐ The single tilt holder is inserted
- ☐ Fluscreen down (using R1 button)
- ☐ Camera retracted
- ☐ The microscope is in SA mode at 8500x
- ☐ Objective & Selected apertures are retracted
- ☐ Reset ("zero") stage



ALERT STAFF AND DO NOT PROCEED IF ANY OF THE ITEMS IN BOLD ARE NOT CORRECT.

3. DRY SAMPLE INSERTION AND REMOVAL

Step 1: Ensure the Following Are Done Prior to Removing the Holder

- ☐ Column valves closed
- ☐ All vacuum levels are OK (nothing highlighted grey or red)
- ☐ Objective and Selected Area apertures are retracted
- ☐ Reset ("zero") stage
- ☐ Fluscreen down
- ☐ Camera retracted

Step 2: Sample Holder Removal and Insertion Procedure

- ☐ Begin removing the sample holder by gently pulling it straight out about 3 inches until you feel a hard stop (but don't pull hard against that stop!).
- ☐ Slowly rotate the sample holder clockwise by ~120-degrees until you feel a stop.
- ☐ Gently pull to remove the sample holder from the microscope completely. **IMPORTANT! If you blow the vacuum, you should finish the unloading protocol anyway, then contact facility staff.**
- ☐ Load a new grid into the holder using the holder station.
- ☐ Inspect the holder O-ring to ensure there are no fibers or dust on it.
- ☐ Make sure the microscope is still in the same state as in **Step 1**.
- ☐ Insert the sample holder with the pin at ~120-degree tilt.

- ☐ Select the holder type and pump out the airlock. The airlock does not start pumping until **AFTER** the turbo is at full speed, so there are two delays to keep an eye on here: 1) the turbo power-up and 2) the pumping time.
- ☐ Watch the countdown timer.
- ☐ Remind yourself to keep the holder as level as possible in the following steps.
- ☐ Immediately after the countdown is over, slowly turn the holder counterclockwise by ~120-degrees.
- ☐ Complete insertion at the end of the ~120-degree rotation. **IMPORTANT! Don't let the column vacuum freely pull in the holder.**
- ☐ **IMPORTANT! If you blow the vacuum, you should finish the insertion protocol anyway, then contact facility staff.**
- ☐ Turn off the turbo pump.

4. MICROSCOPE ALIGNMENT AND CETA CAMERA PREPARATION

Step 1: Insert a Standard Sample

- ☐ Insert a cross-grating grid with a mixture of intact and empty grid squares.

Step 2: HT and Filament Settings

- ☐ Make sure the HT is active and set to 120 kV. **IMPORTANT! You should never turn this off or alter it.**
- ☐ Filament heat should be set to 34. Turn on filament. As of February 2026, this will yield a slightly undersaturated beam, which you will use for gun tilt alignment. It takes about 5 minutes for the filament to reach full heat. Press Eucentric focus button here too.

Step 3: Direct Alignments

- ☐ Open column valves. Go to 155x and record stage positions for foil-covered and empty (vacuum) positions on the cross-grating grid.
- ☐ Move the stage to an empty position or hole. Adjust the intensity knob to the crossover setting and set the magnification to 13,500x. Go to spot size 3.
- ☐ Gun Tilt under Direct Alignments can be achieved by maximizing screen current or optimizing filament shape.
- ☐ Gun Shift under Direct Alignments by centering the beam at spot sizes 3 and 9 without having to adjust "Gun Shift (use at spot 3)" or "Beam shift (use at spot 9)".
- ☐ Go through one more iteration of Gun Tilt and Gun Shift.
- ☐ Center 100 μ m C2 aperture at magnification 2,600x.
- ☐ Go to magnification 57,000x and spot size 4. Adjust the stigmation of the C2 lens by making the features on the filament at crossover as sharp as possible. **IMPORTANT! Do this at each spot size you intend to use today.**
- ☐ Ensure filament is still set to 34 heating index. **IMPORTANT! Don't go above heat 34 without talking to facility staff first.**
- ☐ Once the filament heat is stable, note down the Emission and Bias values in the logbook.
- ☐ If gun tilt was set using geometry shape it is recommended to recheck the gun tilt alignment at 34 to ensure the brightest beam.
- ☐ Move the stage onto the saved foil position.
- ☐ Press the "Eucentric Focus" button on the control panels. Also, "Reset Defocus" on the control panels.
- ☐ Set the magnification to 11,000x, adjust the stage Z to the eucentric height, and update it in the Stage panel.

- ☐ Insert a 100 μm objective aperture and set to diffraction mode. **IMPORTANT!** Make sure the screen is down before entering diffraction mode.
- ☐ Center the objective aperture in diffraction mode. **IMPORTANT!** Leave diffraction mode as soon as you are done.
- ☐ Adjust the screen current intensity to around 0.5 nA. **IMPORTANT!** Never expose the Ceta camera to a screen current beam exceeding 1.5 nA.
- ☐ Insert the Ceta camera and set the camera settings to binning 2 and integration time 0.25 seconds for “Search”, and binning 1 and integration time 1 second for “Acquire”.
- ☐ Go to 120,000x and stigmatize the objective lens.
- ☐ Find focus (minimal contrast in real space or no Thon rings in reciprocal space).
- ☐ Go to 45,000x and adjust intensity to crossover. Center the beam using beam shift and select “Beam tilt pp X” under direct alignments. Adjust multifunction knobs to minimize translation. Repeat with “Beam tilt pp Y”.
- ☐ Go to 120,000x and expand the beam to cover the Fluscreen. Align “Rotation center” in direct alignments.

Step 4: Ceta Camera Preparation for data Collection

- ☐ Make sure the Fluscreen is down. **IMPORTANT!** Never change magnification, spot size, or intensity with the screen lifted.
- ☐ Move the stage over vacuum.
- ☐ Go to your data collection magnification. **IMPORTANT!** Make sure the beam is uniform and completely covers the Fluscreen.
- ☐ Adjust the screen current intensity to around 0.5 nA.
- ☐ With Fluscreen raised, “Measure Dose” in Reference Image Manager. **IMPORTANT!** The dose should be within the camera's dynamic range (along the green slider).
- ☐ Select “Acquire Full Set”.
- ☐ Acquire an image and ensure that the image and its Fourier transform are uniform.

5. IMAGING IN SERIALEM

Step 1: LMM Collection

- ☐ Open SerialEM and load the correct setting file
Settings>C:\ProgramData\SerialEM\UserSettings\CEMRC\SerialEMsettings.txt
- ☐ Go to **Navigator > Open Navigator** and verify that the imaging states are open.
- ☐ Make sure the column valves are closed so your sample is not exposed during setup.
- ☐ Make sure that the objective aperture is retracted.
- ☐ Select the appropriate LMM imaging state and cross-check the Record parameters on the microscope are set to LM 155x, spot size 7, intensity 100%.
- ☐ Set up the Record camera parameters. Settings are: Acquisition: Single Image, Processing: Gain Normalized, Exposure time: 0.22 s, Binning: 2, Area size: Full.
- ☐ Open column valves and check that the beam is well spread and centered. You can see your grid, and no apertures are in the way.
- ☐ Acquire a Record image and ensure the image matches.
- ☐ Set up LMM montage in **Navigator>Montaging & Grids> Setup Full Montage**
- ☐ Make sure the settings are: Magnification 155x, Binning: 2, Number of pieces X and Y: 4x4, Minimum overlap 10% and 0.5 μm . Select checkboxes: ‘Move stage instead of shifting image’, ‘Ask about making map after montage’, and Save images to MRC stack file.
- ☐ Make sure to save your file in the appropriate directory.
- ☐ Start the acquisition of LMM.
- ☐ Ensure that a Navigator map item is generated in the Navigator once the LMM collection is complete.

Step 2: Registering LMM and Setting Up Low Dose Mode

- ☐ Select appropriate View and Record imaging states in the imaging state window.
- ☐ Make sure that the Low Dose Mode is checked in the Low Dose Control panel.
- ☐ Move to vacuum or a non-collectable area.
- ☐ Press Go to: Vie. in the Low Dose Control panel and make sure the microscope magnification, spot size, and intensity are what you expect.
- ☐ Check the Continuous Update box in the Low Dose control panel while changing the magnification, spot size, or intensity. Once done, uncheck it.
- ☐ Make sure that the defocus offset for View is set to -75 μm .
- ☐ Make sure the beam covers the screen, and the screen current is around 0.5 nA.
- ☐ Set up the View camera parameters. Settings are: Acquisition: Single Image, Processing: Gain Normalized, Exposure time: 0.5 s, Binning: 2, Area size: Full.
- ☐ Add a point in the Navigator at an easily identifiable object on the LMM.
- ☐ Go to this point, do eucentric height by selecting **Tasks>Eucentric-Rough** and then "Update Z" for this item in the Navigator.
- ☐ Acquire a View image in which the identifiable object is visible.
- ☐ Click on this identifiable object, and with the corresponding Navigator item selected, go to **Navigator>Shift to marker** to register the LMM.
- ☐ Move to vacuum or a non-collectable area.
- ☐ Press Go to: Rec. and make sure the microscope magnification, spot size, and intensity match your expectations.
- ☐ Check the Continuous Update box in the Low Dose control panel while changing the magnification, spot size, or intensity. Once done, uncheck it.
- ☐ Make sure the beam covers the screen and the screen current is around 0.5 nA.
- ☐ Insert the objective aperture if you haven't done so.
- ☐ Measure the beam diameter.
- ☐ Set up the Record camera parameters. Settings are: Acquisition: Single Image, Processing: Gain Normalized, Exposure time (depending on dose): 1-2 s, Binning: 1, Area size: Full.
- ☐ Acquire a record image over vacuum and calibrate the electron dose at **Task>Calibrate electron dose**
- ☐ Copy the Record parameters to Focus and Trial in the Low Dose Control panel.
- ☐ Set up the Focus and Trial camera parameters. Settings are: Acquisition: Single Image, Processing: Gain Normalized, Exposure time: 0.5 s, Binning: 2, Area size: Full.
- ☐ With the screen inserted, make sure the microscope's View, Focus, Trial, and Record settings are as expected, and that the beam uniformly covers the entire screen.

Step 3: Align View and Record

- ☐ Acquire a View image, find an identifiable object within the image, and center it.
- ☐ Acquire another View image and make sure the item is centered.
- ☐ Acquire a Record image, drag the image, and make sure the item is centered by acquiring another Record image.
- ☐ Acquire a View image, drag the image so that the item is centered, do not take another View.
- ☐ In the low dose control panel, under Offsets for View: Shift: press Set.
- ☐ SerialEM will acquire another view image.
- ☐ If the item is not centered, then repeat the above steps.

Step 4: MMM Collection

- ☐ Load the LMM.
- ☐ Create a Navigator item by adding points in the center of each square you want to collect.
- ☐ Check the Acquire checkbox for each of these items in the Navigator window.
- ☐ Make sure no files are currently open in SerialEM.
- ☐ Go to **File>New Montage** to set up MMM collection for each selected square.
- ☐ Make sure the settings are: 'Move stage instead of shifting image', 'Ask about making map after montage', and 'Use View parameters in Low Dose mode'. Magnification 2,600x, Binning: 2, Number of pieces X and Y: 4x4, Minimum overlap 15% and 0.5 μm , and Save images to MRC stack file.
- ☐ Make sure to save your file in the appropriate directory.
- ☐ Go to **Navigator>Acquire at Items** to acquire MMM montages.
- ☐ Make sure the settings are: Primary Action: Acquire and save image or montage, Check the box for "Make Navigator map", Primary Action-related: Acquire non-montage images in Low Dose with "View", Tasks before or after Primary: Rough eucentricity and Close column valves at end.

Step 5: Data Collection

- ☐ Open column valves.
- ☐ Load the first MMM.
- ☐ Click Go to XYZ of the map item in the Navigator window.

Option 1. Manually Adding Points for Collection

- ☐ Add points within the MMM that you would like to acquire a Record image. **IMPORTANT!** Keep your beam diameter and the location of your focus position in mind while you add points.
- ☐ Click Stop in the Navigator window when you are done adding points.
- ☐ Check the Acquire checkbox for each of these items in the Navigator window.
- ☐ Load the second MMM and repeat the steps.

Option 2. Add Evenly Distributed Points Using Add Grid of Points

- ☐ Go to **Navigator>Montaging & Grids>Add Grid of Points**
- ☐ **IMPORTANT!** Keep your beam diameter and the location of your focus position in mind while entering the desired spacing between points.
- ☐ Select to move in a Zig-Zag pattern and flag the points for collection.
- ☐ Load the second MMM and repeat the steps.
- ☐ Make sure no files are currently open in SerialEM.
- ☐ Go to **File>Open New**
- ☐ Make sure the file properties settings are: Save data as: 'Integers', Save images to Series of TIFF files with LZW compression or MRC stack files.
- ☐ Make sure to save your file in the appropriate directory.
- ☐ Go to **Navigator>Acquire at Items** to acquire images at these points
- ☐ Make sure the settings are: Primary Action: Acquire and save image or montage, Tasks before Primary: Run Script before Action with Script to run: IncDefocusEucentricity, and Close column valves at end.

6. END OF SESSION

Step 1: Make Sure Following Are Done

- ☐ Column valves closed
- ☐ Filament is off
- ☐ Reset stage (holder)
- ☐ Objective and Selected apertures are retracted
- ☐ All vacuum levels are OK (nothing is highlighted grey or red)
- ☐ Fluscreen down
- ☐ Camera retracted
- ☐ Close SerialEM (leave all other software open)
- ☐ The microscope is at 8,500x (must be an SA magnification)
- ☐ No warning/errors

Step 2: Remove Your Sample From the Microscope

Step 3: Re-insert Empty Holder Into the Microscope

Step 4: Record the liquid nitrogen level in the logbook. If the liquid nitrogen level is less than 30%, please inform CEMRC staff. **IMPORTANT! Do not start a cryo-cycle.**

Step 5: Finish your logbook entry and record the emission and bias values before turning off the filament. **IMPORTANT! Make sure any issues are reported immediately to staff and recorded in the logbook.**

Step 6: Enter Session Data Into the CryoEM Research Center GUI

Step 7: Make Sure That the Points in Step 1 Are Still Correct

Appendix 3: Glossary

Aberration: Aberration is an imperfection in the way a lens focuses light, causing the rays not to converge at a single point, which reduces resolution. Astigmatism, chromatic aberration, spherical aberration, and coma are examples of lens aberration.

Alpha: Alpha (α) is the stage tilt angle along the alpha axis (alpha tilt). The set alpha function provides a rapid toggle of stage tilt along the alpha axis between an angle and back to 0°.

Aperture: An aperture is an opening in the electron beam pathway that controls the illumination. An aperture can be of different sizes depending on the beam size and the illumination required for imaging. Larger apertures permit most of the electrons to pass through, whereas smaller apertures cut out more peripheral electrons.

Astigmatism: Astigmatism is a lens aberration that results when a lens field is not symmetrical in strength, being stronger in one plane and weaker in another, so that only part of the image is in focus at any given time. Condenser astigmatism is visible as elliptical illumination on the flucam viewer, and objective astigmatism is visible as elliptical Thon rings of the Fourier transform of the image on the TIA.

Back Focal Plane: The Back focal plane is an imaginary plane behind the objective lens, where the diffraction image of the specimen forms. It is the focal plane of the objective lens where parallel electron rays converge. It is the location of the objective aperture and the phase plate.

Beam Shift: Beam shift is the shifting of the electron beam over the specimen by adjusting the double deflection coils below the second/third condenser lens. It is done through the multifunction X and Y knobs or the trackball on the hand panel.

Bias (Emission): Bias is the voltage difference between the Wehnelt cylinder and the filament. It generally controls the percent of electrons coming out of the filament tip and is passed through the Wehnelt cylinder towards the accelerator stack.

*For more information refer to this Grant Jensen's lecture on Detectors here:
<https://www.youtube.com/watch?v=-0bjbWgxH2k>*

Binning/Sampling: Binning is the process of combining the signal from multiple adjacent pixels on the detector into a single pixel. This process increases the signal-to-noise ratio but reduces the overall resolution.

Ceta (Detector/Camera): TFS Ceta™ is a 4096 x 4096-pixel CMOS high-sensitivity, high-speed camera for image acquisition that detects electrons and represents them as an image.

*For more information refer to this Grant Jensen's lecture on Detectors here:
<https://www.youtube.com/watch?v=-0bjbWgxH2k>*

Chromatic Aberration: Chromatic aberration is an aberration of lens due to which the rays of different energies converge at different focal planes. In an electromagnetic lens, more energetic electrons ray has a longer focal point than does the less energetic electrons ray.

CMOS/Direct Detector: CMOS stands for **C**omplementary **M**etal **O**xide **S**emiconductor. It is a detector that uses CMOS technology to detect individual electrons directly. The incident electrons impinge directly on the CMOS detector and are directly converted into charge. As they are directly detected, the count they produce within the CMOS chip is more localized. The chip is also back-thinned, so there is a reduced chance that an electron scattered through the materials below it will return through the chip at an unrelated position. Additionally, the CMOS detectors can be read out hundreds of times per second, providing an advantage for building an image in which each electron carries the same weight, reducing shot noise. The hundreds of read-out frames during a single exposure allow one to track the specimen movement during the exposure and deblur the image due to this movement.

*For more information refer to this Grant Jensen's lecture on Detectors here:
<https://www.youtube.com/watch?v=-0bjbWgxH2k>*

Column Valves: Column valves are a set of valves in the column separating the column from the projection chamber and the column from the gun. The Column valves are mainly under user control, and it is suggested to close them when the microscope is not actively in use or while exchanging the sample holder to prevent air from entering the gun area.

Coma: Coma is an aberration that causes off-axis image points to appear distorted and elongated, resembling a comet's tail. This is due to variation in the lens's magnification with the position of the electron beam relative to the optical axis.

CompuStage: CompuStage is a motor-driven goniometer that provides computer-controlled movement of the specimen on five axes (X, Y, Z, α , β).

Condenser Aperture: Condenser aperture is an opening in the electron beam pathway positioned below the condenser lens to control the illumination area on the specimen.

Condenser Lens System: Condenser lens system is a set of electromagnetic lenses located below the Gun. It collects electrons from the first crossover image of the gun and focuses them onto the specimen. There are two/three condenser lenses in the condenser lens system, which control the amount and illumination area of electrons striking the specimen. The first condenser lens is referred to as the spot size, the second as the intensity, and the third is used to maintain the electron radiation parallel over a range of intensities.

*For more information refer to this Grant Jensen's lecture on The Column here:
<https://www.youtube.com/watch?v=hc2s4uSbpyI>*

Crossgrating: Crossgrating is a standard grid used to double-check magnification accuracy and align the microscope.

Crossover: Crossover refers to the point where the converging electrons focus and cross over each other's path.

Cryo Cycle: Cryo cycle is a special vacuum procedure for removing the water vapor from the microscope column that is released when the cold trap or cryo blades are being warmed up when the liquid-nitrogen cooling is removed at the end of a cryo-microscopy session.

CTF: CTF stands for **C**ontrast **T**ransfer **F**unction. A CTF is an oscillatory curve of phase contrast transferred to an image as a function of spatial frequency. It is a mathematical function that describes how microscope aberrations affect the contrast of features in an image.

For more information refer to this Grant Jensen's lecture on The Contrast Transfer Function here:
<https://www.youtube.com/watch?v=mPynoF2j6zc>

Deflector: A deflector is an electromagnetic coil used to tilt and shift the electron beam. The deflector coils are set for each lens system of an electron microscope and positioned perpendicular to the electron beam.

Defocus: Defocus is the phenomenon of bringing the image out of focus to impart phase contrast to the specimen image. It is done by varying the current in the objective lens system using the defocus knob on the hand panel.

For more information refer to this Grant Jensen's lecture on Defocus and Its Effects here:
<https://www.youtube.com/watch?v=izLp5vpoFyQ>

Diffraction: Diffraction is a phenomenon of the reflection of the electron beam at certain angles by a crystalline lattice.

DQE: DQE stands for **D**etective **Q**uantum **E**fficiency, which is a measure of how well an electron detector converts the signal from incoming electrons into a recorded image, essentially indicating how much noise the detector adds to the image, with a higher DQE signifying better image quality at a given electron dose; a perfect detector would have a DQE of 1.

Electromagnetic Lens: An Electromagnetic lens is a device that refracts or bends electromagnetic radiation to converge at a certain distance from the lens. It produces a magnetic field by the flow of electric current through electromagnetic coils termed solenoids. Because electrons carry charge, they can be affected by magnetic fields.

For more information refer to this Grant Jensen's lecture on EM Lenses here:
https://www.youtube.com/watch?v=SKjxp-al_Ul

Eucentric Focus: The Eucentric focus button on the hand panel sets the objective lens currents to the pre-aligned value for focus at the Eucentric height and normalizes the objective lens.

Eucentric Height: Eucentric height is a location along the optical axis of the microscope where the placed specimen, when tilted, remains centered in the field of view.

Exposure Time: Exposure time is the length of time for which the camera is exposed to the electrons transmitted from the specimen during the image recording.

Filament Heat: The filament heat setting controls the current passing through the filament and, hence, how hot the filament gets.

Flucam Viewer: Flucam viewer is a software that displays the live image from the Fluscreen. It is helpful during microscope alignments and checking imaging states.

Fluscreen: A Fluscreen is a phosphor-coated fluorescent screen detector positioned below the column that emits green photons of light upon electron collision. It aids in performing specific microscope alignments and in checking imaging states.

Focus: A specimen is said to be in focus when the crossover point of the electron beam is precisely on the image plane. When the beam crossover point is slightly above the image plane, then the condition is overfocussed, and when it is slightly below the image plane, then the condition is underfocussed.

(Fast) Fourier Transform: The Fourier transform is a mathematical technique that numerically transforms spatial-domain information into the frequency domain by applying the equations developed by Joseph Fourier. This converts the image into a frequency display. The term “fast” in Fast Fourier Transform (FFT) refers to the accelerated algorithm used. FFT is helpful during microscope alignment at the steps of finding focus, centering the objective aperture, correcting the objective astigmatism, and detecting drift.

For more information refer to this Grant Jensen's lecture on Fourier Transforms for Beginners here:
<https://www.youtube.com/watch?v=-EAQm8wgLbc>

Gain Reference: Gain reference is a reference image acquired usually at the data collection setup over vacuum and is used to normalize the recorded images during data collection.

Grid: Grids are screens or fine, mesh supports upon which sections and liquid suspensions may be deposited for transport and viewing in the electron microscope. The standard size grid is 3.05 mm in diameter. Grids are available in a variety of mesh designs depending on their intended use.

Gun Tilt: Gun tilt is an alignment step in an electron microscope to adjust the angle of the electron beam emitted from the electron gun by using deflection coils located below the gun, ensuring it enters the microscope column precisely along the optical axis.

Gun Shift: Gun shift is an alignment step in an electron microscope that adjusts the X and Y shifts of the electron beam emitted from the electron gun using deflection coils located below the gun, ensuring it enters the microscope column precisely along the optical axis.

High Tension: High tension is the total voltage drop across the accelerator stack, which accelerates electrons by that potential difference. This results in a high-energy electron beam, allowing for high-resolution imaging. In Talos L120C, the high tension voltage is set to 120 kV.

For more information refer to this Grant Jensen's lecture on Electron Guns here:
<https://www.youtube.com/watch?v=C6DSFK7zJo0>

Imaging States: An imaging state (IS) consists of the state of the microscope together with the parameters used to acquire an image. It includes the magnification, spot size, intensity, probe mode, and camera settings.

For more information refer to this link on Imaging States here:
https://bio3d.colorado.edu/SerialEM/hlp/html/hidd_statedlg.htm

Intermediate Lens System: The Intermediate lens system is a set of electromagnetic lenses located below the objective lens system. It magnifies the image from the objective lens system and projects it onto the projection lens system.

For more information refer to this Grant Jensen's lecture on The Column here:
<https://www.youtube.com/watch?v=hc2s4uSbpYI>

Intensity: Intensity is the brightness of the electron beam over the specimen. It is controlled by the intensity knob on the hand panel which controls the current in the second condenser lens.

Ion Getter Pump: An Ion getter pump is a type of vacuum pump in which high-voltage cathodes generate a flow of electrons toward high-voltage anodes. The electrons collide with gas molecules, ionizing them, causing the positive ions to flow to the cathode while the electrons continue along their collision path towards the anode. The gas molecules collide with the cathode surface and are chemically adsorbed onto it by a reactive coating of titanium or zirconium. Ion pumps are commonly used in ultra-high vacuum (UHV) systems, as they can achieve ultimate pressures below 10^{-11} mbar. In contrast to other standard UHV pumps, such as turbomolecular pumps and oil-diffusion pumps, ion pumps have no moving parts and use no oil. They are therefore clean, need little maintenance, and produce no vibrations.

LaB₆ filament (Gun/Accelerator): LaB₆ (Lanthanum hexaboride) filament is one of the three most common types of filaments used in the electron microscopes, others being Tungsten filament and Field Emission Gun. It is a small crystal of LaB₆ in which electrons are emitted from the tip of the crystal when it is heated to high temperatures, a process called thermionic emission. Comparison of the three major filaments:

	Tungsten Filament	Lanthanum Hexaboride (L120C)	Schottky FEG (Arctica/Krios G3i)	Cold Field Emission (Glacios2/Krios G4/Krios5)
Brightness (A/cm ² • sr)	10 ⁶	10 ⁷	10 ¹¹	10 ⁹
Source Diameter (nm)	>10 ⁴	10 ⁴	10-20	<10
Energy Spread (eV)	1.0-2.0	1.0-2.0	0.5-2 (usually 0.5)	0.2-0.3
Service Life (hours)	40-100	1000-2000	10 ⁴	>2000
Vacuum Required (Pa)	10 ⁻³	10 ⁻⁵	10 ⁻⁷	10 ⁻⁸

For more information refer to this Grant Jensen's lecture on Electron Guns here:
<https://www.youtube.com/watch?v=C6DSFK7zJo0>

Low-dose mode: Low-dose mode is a high-resolution data collection workflow that cycles between three modes: search, focus, and record. The search step centers the microscope stage over an imaging area at low magnification. After the area is centered, a focusing step is performed to set the image's target defocus. The focusing is performed at a position away from the target to protect the particles from radiation damage. After the defocus is set, a multi-second exposure is recorded over the target.

For more information refer to these link on Low dose mode here: <https://cryoem101.org/chapter-4/> & https://bio3d.colorado.edu/SerialEM/hlp/html/about_low_dose.htm

Magnification: Magnification is the degree of enlargement of the diameter of the final image compared to its original size in the specimen. In practice, it is the distance measured between two points on an image divided by the distance measured between these same two points on the original object.

Mechanical (rotary) pump: A Mechanical (rotary) pump is a type of vacuum pump. It has an off-center rotating cylinder with sliding spring-mounted vanes that, while rotating, create an area of lowered pressure to draw air from the inlet and an area of elevated pressure at the exhaust, thereby moving air from inside the closed microscope system to the outside. It is the first pump in a vacuum system as it is often used to establish a starting vacuum (10^0 to 10^{-1} Pa) before the oil-diffusion pump can be used. Mechanical pumps have moving parts; therefore, their operation causes vibration.

Objective Aperture: Objective aperture is an opening in the electron beam pathway positioned in the back focal plane of the objective lens, which helps in enhancing image contrast by trapping more of the peripherally deflected electrons.

Objective Lens System: The Objective lens system is a set of electromagnetic lenses located around the specimen. It forms, focuses, and magnifies the specimen's first image. It is controlled by the defocus known in the hand panel.

For more information refer to this Grant Jensen's lecture on The Column here: <https://www.youtube.com/watch?v=hc2s4uSbpyI>

Oil-diffusion pump: Oil-diffusion pump is a type of vacuum pump. It consists of a series of stacked towers with umbrella-like caps, situated inside a water-cooled cylindrical enclosure that is connected to the microscope. A heater heats oil at the bottom of this pump, and the pressurized vapor is forced up the towers, where it is deflected downward by the umbrella-like caps, carrying any diffused air molecules in the pump to the bottom, from where they are removed by a rotary pump. It contains no moving parts and can create a vacuum of 10^{-3} to 10^{-4} Pa.

Parallel Illumination: Parallel illumination is the condition in which the specimen is illuminated with a parallel electron beam. This condition is satisfied when the condenser lens forms the crossover at the front focal plane of the upper objective lens so that the angle with which the electron beam interacts with the specimen is zero. Parallel illumination of the specimen minimizes deterioration in image quality.

Pivot Point: Pivot point is a point in the electron path through the specimen where the beam appears to rotate or tilt around when deflection coils are used to manipulate its direction.

Projection Lens System: The Projection lens system is a set of electromagnetic lenses located below the intermediate lens system. It magnifies the image from the intermediate lens system and projects it on a viewing screen or a camera.

For more information refer to this Grant Jensen's lecture on The Column here:
<https://www.youtube.com/watch?v=hc2s4uSbpyI>

Radiation Damage: Radiation damage is the phenomenon of transfer of energy from the irradiating electrons to the specimen under observation, causing changes to its chemical bonds and atomic arrangement, leading to its structural damage.

For more information refer to this Grant Jensen's lecture on Dose Limitations here:
<https://www.youtube.com/watch?v=4BEXfZo-zDs>

Resolution: Resolution is the ability to discriminate between two closely placed structures that might otherwise appear as one and to see more details within the objects.

Rotation Center: The Rotation Center is a point in the electron path through the specimen at which the image rotates without lateral movement when the beam is tilted, or the lens strength is changed.

SA mode: SA mode is the high-magnification imaging mode for high-resolution data collection in an electron microscope.

SerialEM: SerialEM is a program that can acquire a variety of data from electron microscopes: tilt series for electron tomography, large image areas for 3-D reconstruction from serial sections, and images for reconstruction of macromolecules by single-particle methods. It is described in the articles by David N. Mastronarde at the University of Colorado, Boulder.

For more information refer to this link on SerialEM here:
<https://bio3d.colorado.edu/SerialEM/>

Single-tilt holder: A single-tilt holder is a specimen holder that allows the sample to be tilted (alpha tilt) only along one axis.

Spherical Aberration: Spherical aberration is an aberration of a lens due to which the rays passing through the periphery of the lens are refracted more than rays passing along the axis, leading the various rays to not come to a sharp common focal point.

Spot Size: Spot size controls the current in the first condenser lens and, therefore, the brightness of the first focused spot of the electron beam in the microscope.

Stigmator: A stigmator is an electromagnetic coil used to correct for the asymmetries in the lens's magnetic field. Stigmators add field strengths in one direction relative to another to compensate for asymmetries in the main magnetic field produced by the lens. The stigmator coils are set with each lens system of an electron microscope and are positioned perpendicular to the direction of the electron beam.

TEM: TEM stands for **T**ransmission **E**lectron **M**icroscope. A TEM projects electrons through a thin specimen to produce a two-dimensional projection on a detector. Since the illuminating electrons pass through the specimen, the image is said to be transmitted.

For more information refer to this Grant Jensen's lecture on Basic Anatomy of the EM here:
<https://www.youtube.com/watch?v=GBU1eA1PqeQ>

For simulation of TEM refer to this simulator: https://myscope.training/TEM_simulator.html

TEM UI: TEM UI stands for **T**ransmission **E**lectron **M**icroscope **U**ser **I**nterface. It is a graphical user interface software for operating a Transmission Electron Microscope manufactured by Thermo Fisher Scientific.

TFS: TFS stands for **T**hermo **F**isher **S**cientific Inc. It is the manufacturer and supplier of the Talos L120C microscope, the TEM UI software, and the TIA.

Thon rings: Thon rings are concentric rings in the Fourier transform of an image that describe the information content of the image as a function of spatial frequency, or resolution. The white rings represent spatial frequencies that contain information, while the gaps between rings and otherwise dark areas represent spatial frequencies with low or zero information.

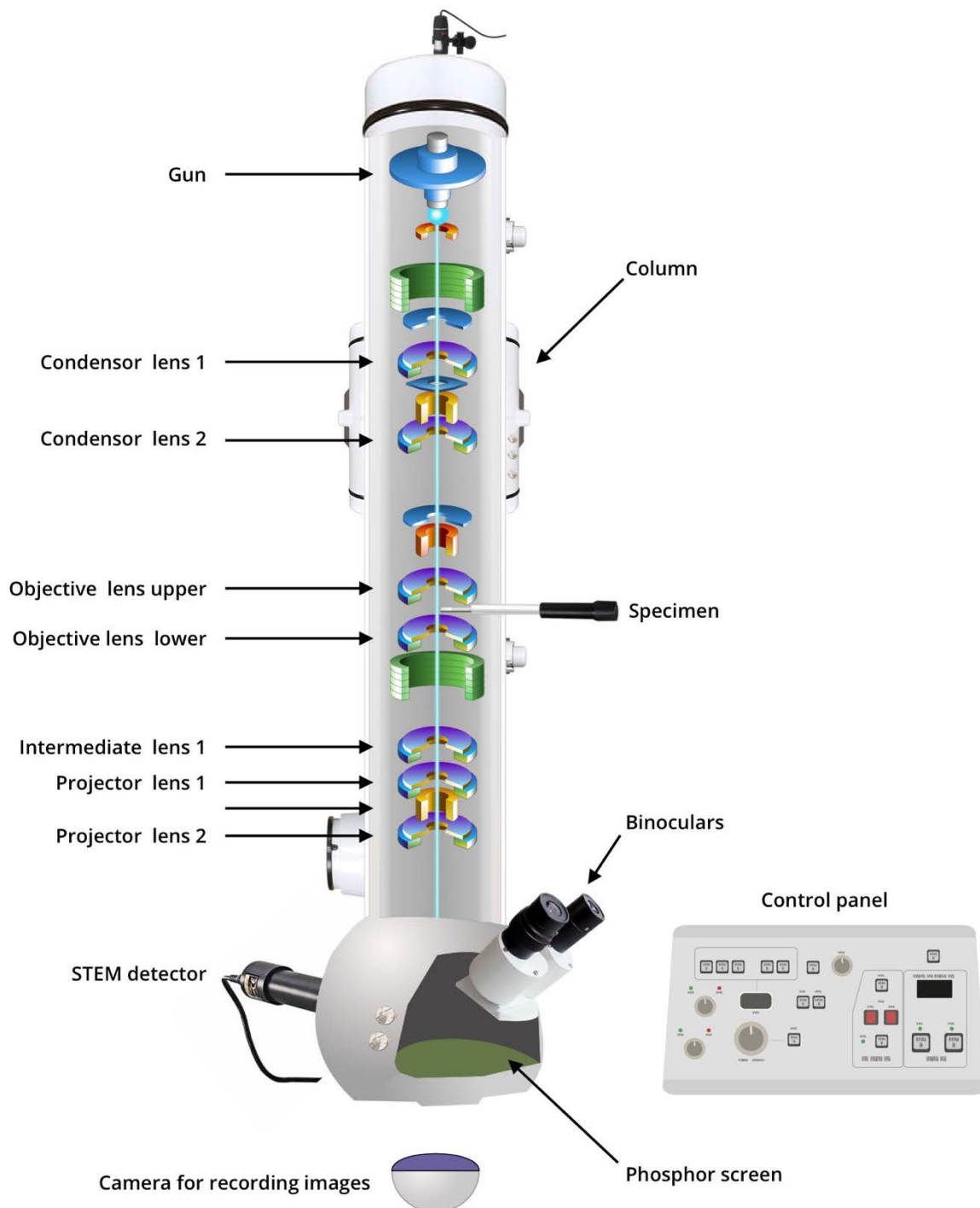
TIA: TIA stands for **T**EM **I**maging and **A**nalysis. It is a software for the acquisition and analysis of images generated by the TEM.

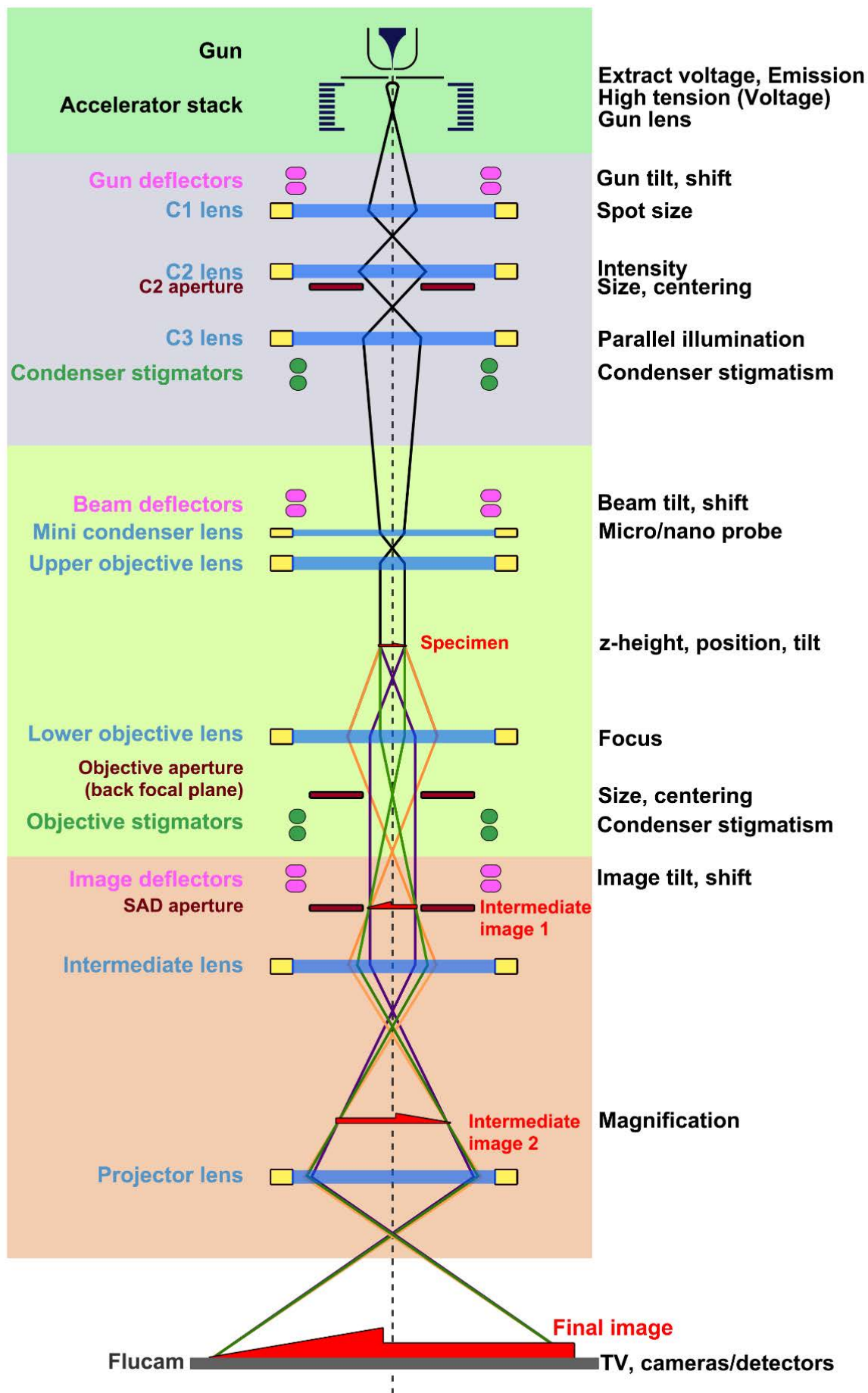
Turbo Pump (Turbo Molecular Pump): A Turbo molecular pump is a type of vacuum pump that has a series of closely mounted propellers, which, when they rotate, hit gas molecules from the inlet of the pump towards the exhaust to create or maintain a vacuum. The turbomolecular pump is versatile. It can generate vacuum levels ranging from intermediate vacuum ($\approx 10^{-2}$ Pa) to ultra-high vacuum ($\approx 10^{-8}$ Pa).

Vacuum System: A Vacuum system refers to any pumps used in the electron microscope to achieve the ultra-high vacuum levels required for operation. There are four types of pumps used: mechanical (rotary) pump, Oil Diffusion pump, Turbo molecular pump, and Ion getter pump.

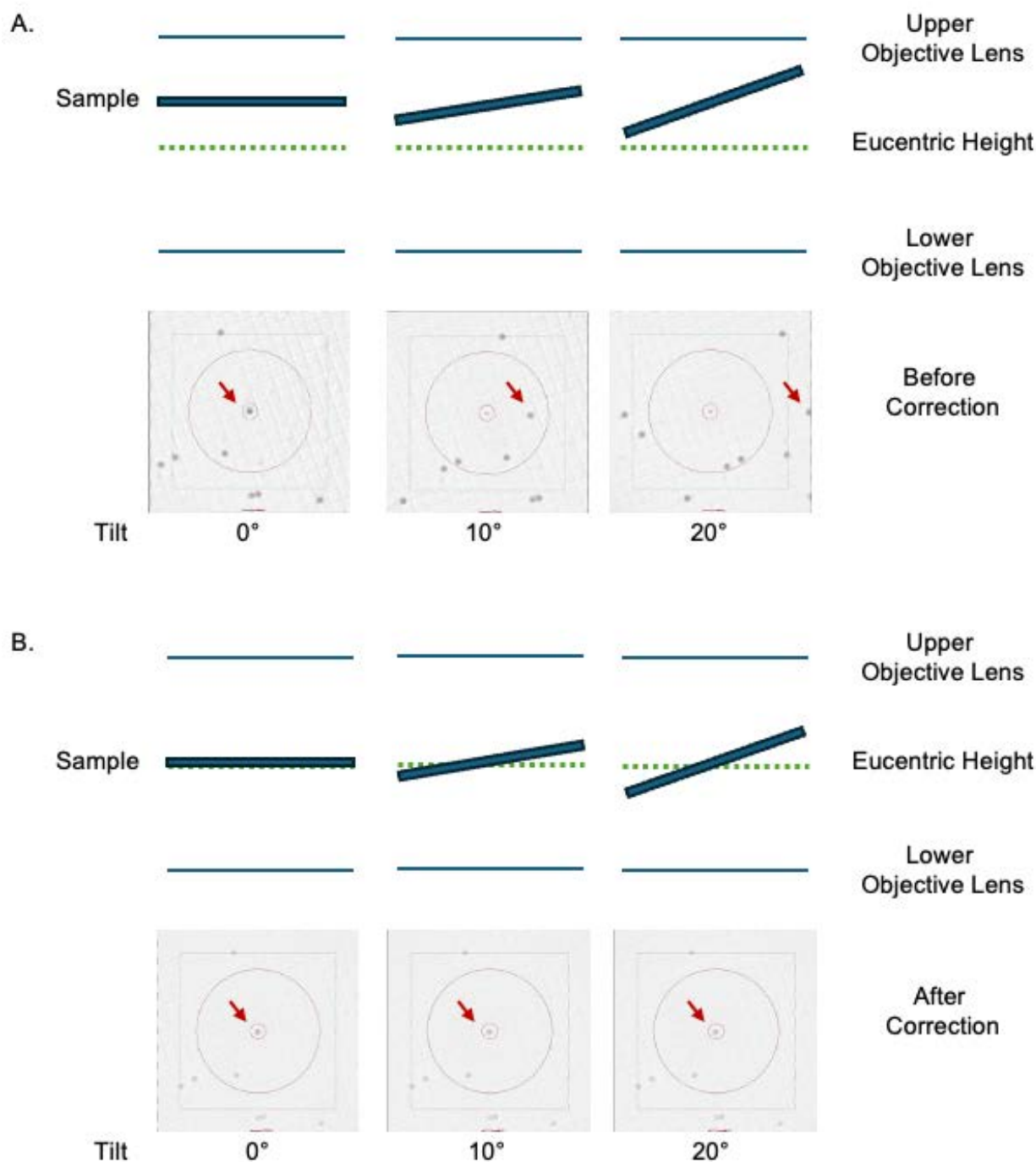
For more information refer to this Grant Jensen's lecture on Vacuum Systems here:
<https://www.youtube.com/watch?v=LUIM0kl6Lp0>

Appendix 4: Microscope Schematics



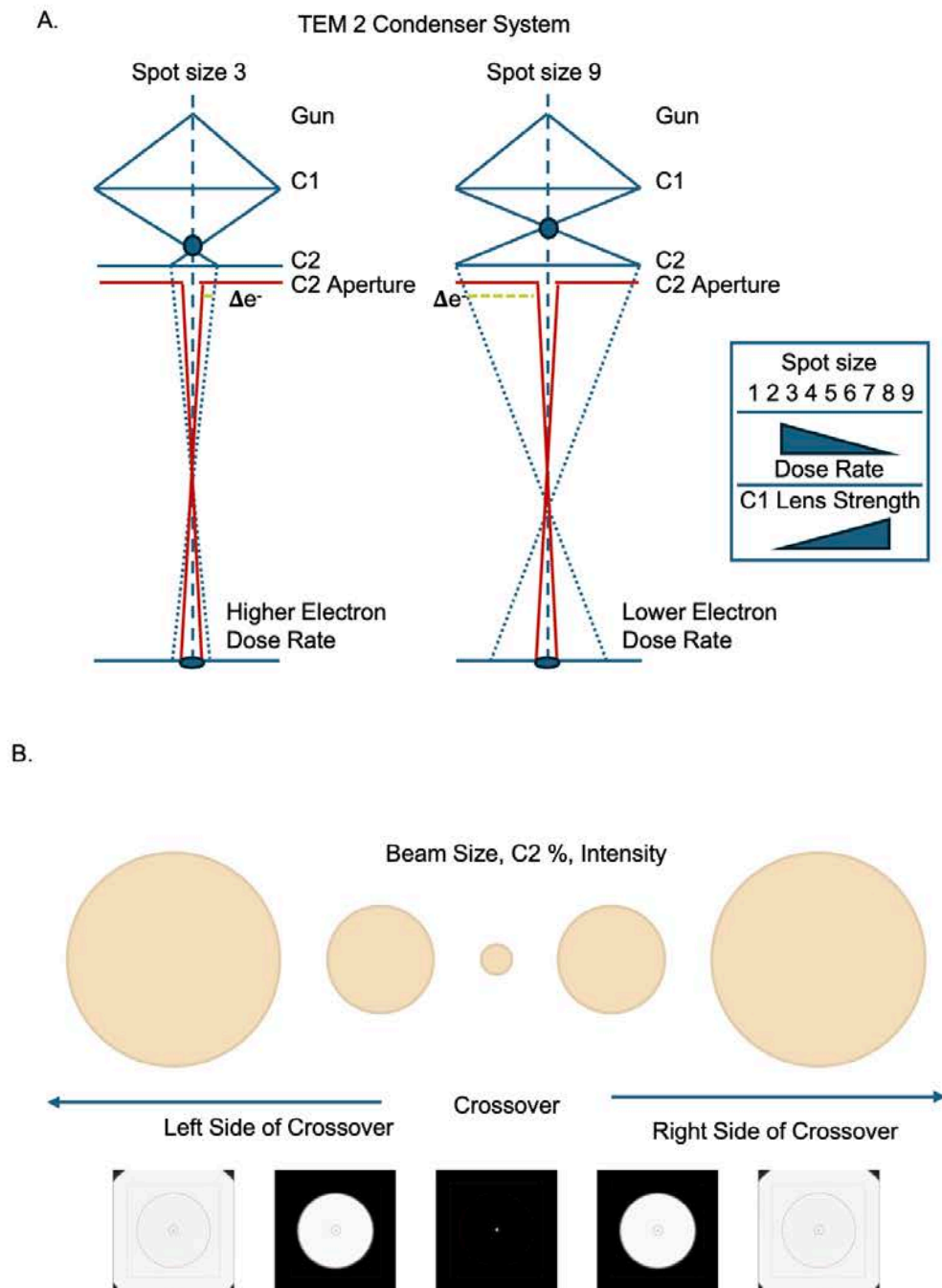


Appendix 5: Eucentric Height Schematics



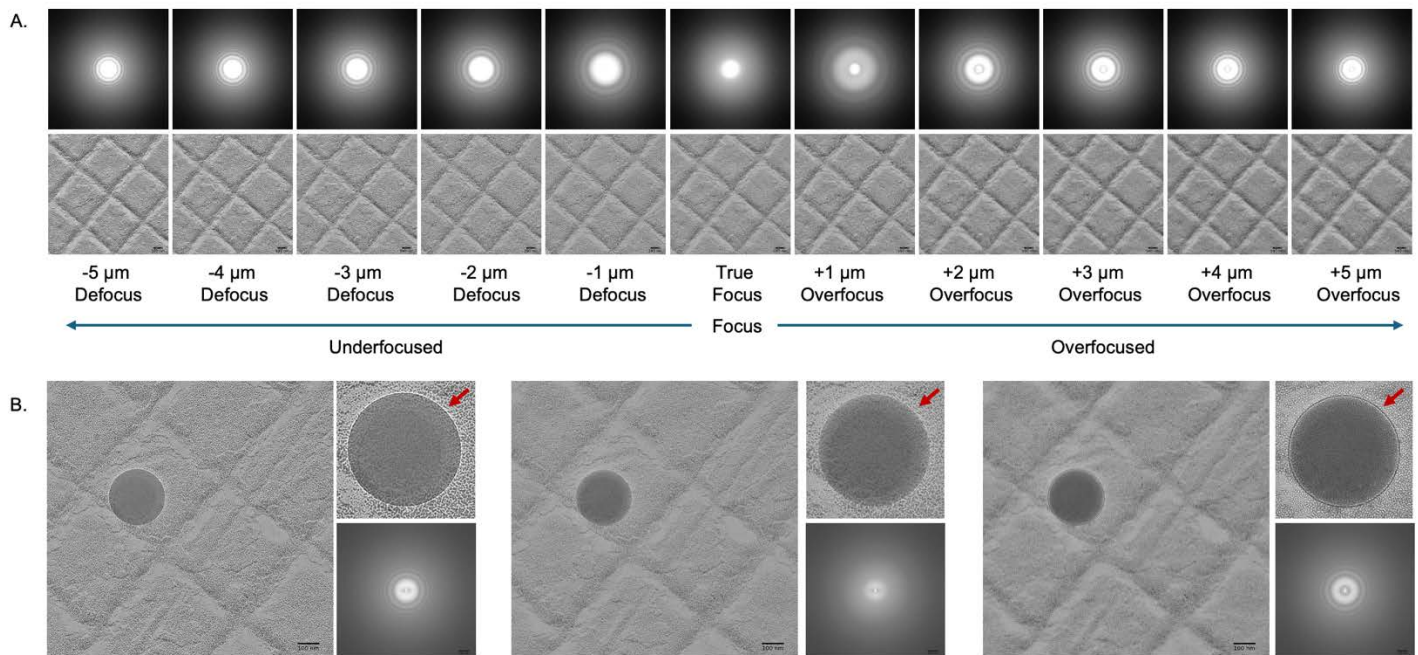
Eucentric Height: Eucentric height is achieved when the specimen lies on the microscope tilt axis and remains centered during stage tilting. This position occurs within the magnetic field of the objective lens and is not always located at the physical midpoint between the pole pieces. Determining the eucentric height requires iteratively adjusting the stage Z-position while tilting the stage through different angles. Because Z movement and stage tilt are independent motions, the only reliable confirmation of eucentric height is that the object exhibits minimal lateral movement during tilt.

Appendix 6: Condensing System Schematics



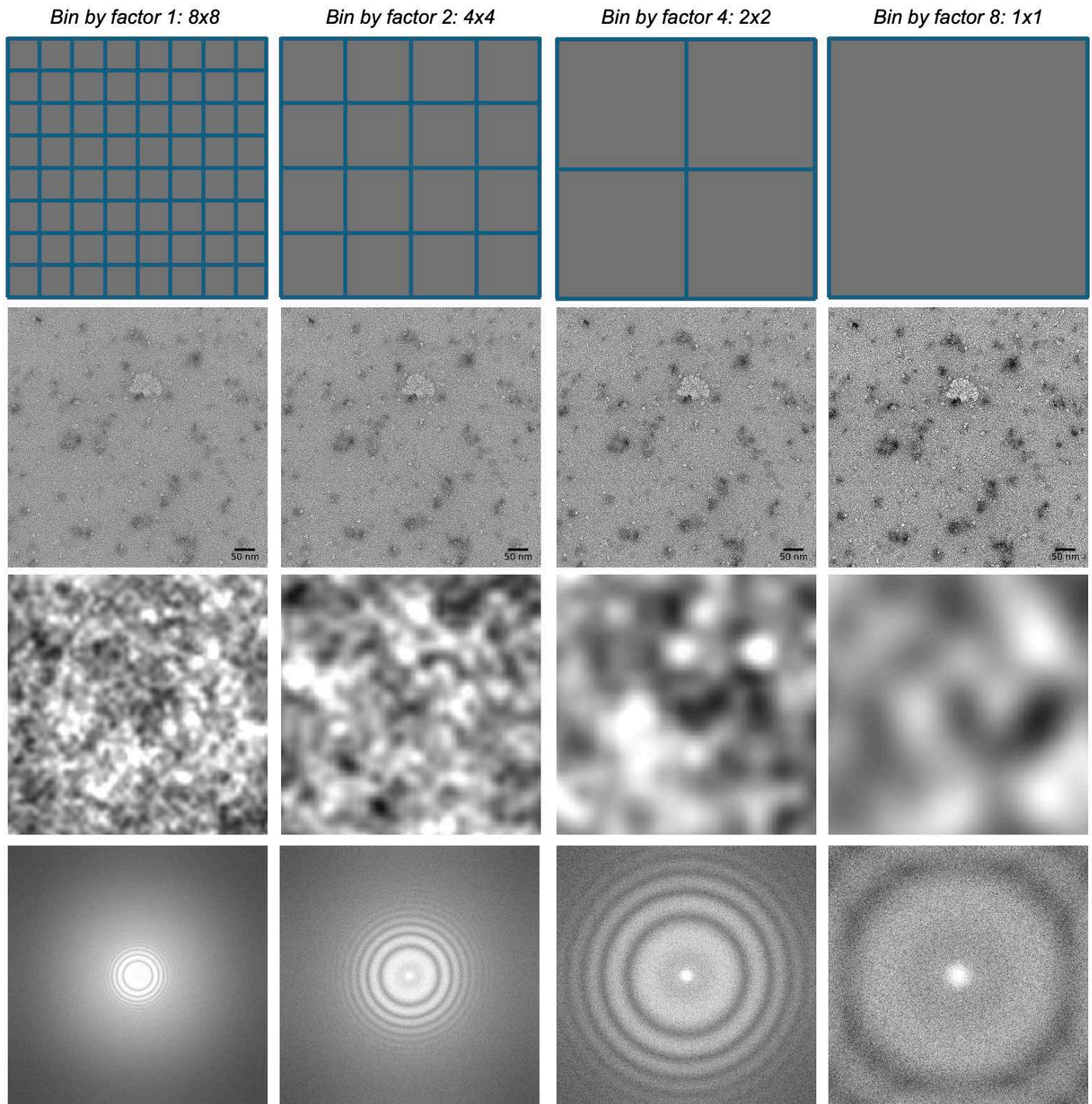
The Condensing System: The interplay between the C1 and C2 condenser lenses in a two-condenser system establishes the properties of the electron beam. **A.** A schematic of the two-lens condenser system is shown. Increasing the excitation of the C1 lens reduces the emission angle of electrons from the source and shifts the position of the first crossover along the optical axis. Different C1 excitation conditions correspond to different spot size settings, which determine the demagnified source size. The C2 lens further converges the beam emerging from C1 and controls the convergence angle and illuminated area at the specimen plane. **B.** A schematic illustrates variations in beam diameter, including the minimum diameter at the crossover point and the progressive increase in beam diameter with distance from the crossover along the optical axis. Representative images of different beam diameters acquired on the Talos L120C are shown.

Appendix 7: Effects of Defocus on Images



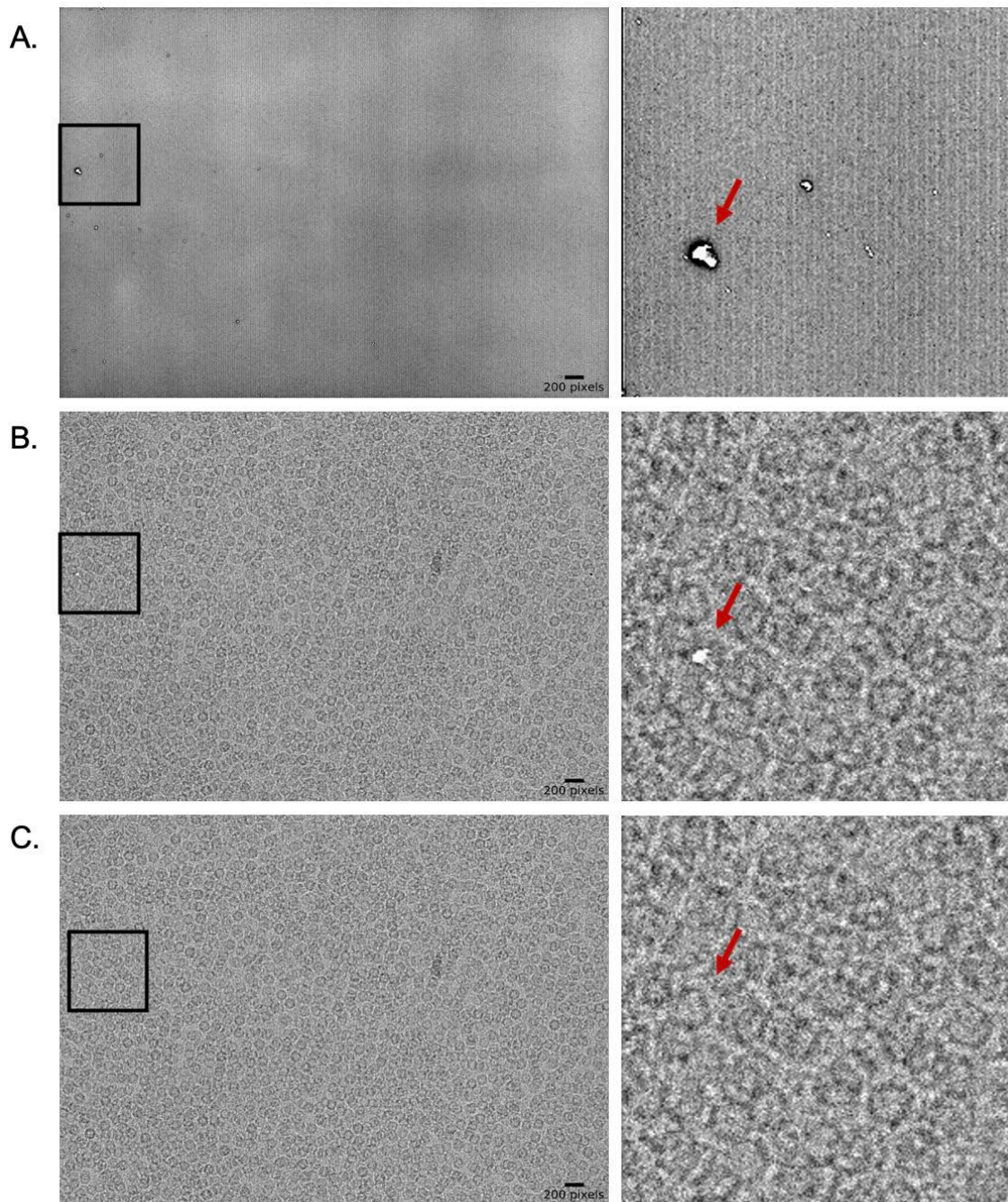
Defocus: Effects of defocus on the contrast transfer function (CTF) and corresponding real-space images. **A.** FFT and real-space images for a series of defocus and overfocus values illustrate changes in the CTF. Scale bar reads 100 nm. **B.** Real-space images of a silicone bead on a crossed-grid sample demonstrate contrast variations with focus. Underfocus produces a bright outline around the bead, true focus results in minimal outline, and overfocus produces a dark outline, reflecting the expected phase contrast behavior. Fresnel fringes arise when the objective lens is slightly defocused, causing interference between scattered and unscattered electrons at the edge of a specimen. This produces a thin bright or dark outline surrounding high-contrast features such as particles or contamination. As the focus is adjusted through underfocus and overfocus, the contrast of this outline reverses, resulting in a characteristic light or dark halo that is commonly used as a visual cue during focusing.

Appendix 8: Effects of Binning on Images



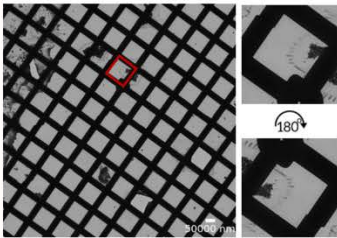
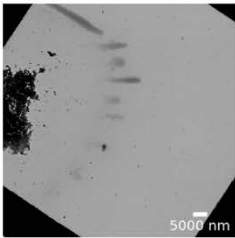
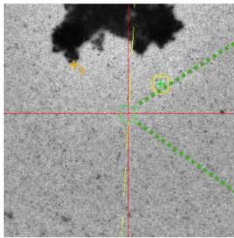
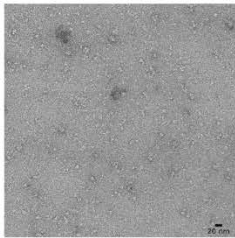
Binning: Effect of binning on data acquisition images collected at 92,000x magnification (1.57 Å/pixel). Representative micrographs acquired from the same specimen area are shown at progressive binning levels (1, 2, 4, and 8) to illustrate the impact of binning on image sampling and apparent resolution. Corresponding power spectra (FFT) are shown for each micrograph, highlighting the progressive degradation of visible Thon rings as binning increases.

Appendix 9: Effects of Gain References on Images



Gain Reference: Impact of gain reference application on Arctica collected apoferritin data. A. Gain reference image of the K3 detector used for this dataset, highlighting low-response or inactive pixels across the sensor. **B.** Micrograph of apoferritin after application of an incorrectly oriented gain reference, showing amplified pixel artifacts and uneven background intensity. **C.** Micrograph of apoferritin after application of the correctly oriented gain reference, producing a uniform background and restored particle contrast. This comparison demonstrates the critical role of proper gain reference application for accurate detector normalization and high-quality micrographs.

Appendix 10: SerialEM Workflow for L120C

SerialEM Low Dose Mode	Low Magnification Montage (LMM)	Medium Magnification Montage (MMM)	View	Record
				 Data Acquisition Focus
Magnification	155x (4x4)	2600x (4X4)	2600x	Varies: 45kx, 57kx, 73kx, <u>92kx</u> , 120kx
Bin	2	2	2	1

L120 Workflow: Workflow of low-dose imaging stages in SerialEM used on L120C. Representative images acquired at low-dose magnifications are shown for the Low Magnification Montage (LMM), Medium Magnification Montage (MMM), View, and Record mode. Additional panels illustrate focusing performed at the View magnification level prior to high-magnification data acquisition. Notice the 180° rotation between the LMM image and the MMM image.

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