



Talos the living robot and guardian of the island Crete, forged from bronze and fueled by ichor...defeated by the sorceress Medea...

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I. Basic information before operating the microscope

This document is for trained users, contact the CryoEM Core Director Joshua Strauss to schedule a training session before using the microscope.

- Do not contact GTS or fix the problem without supervision.
- Do not make your settings files or change the settings files without supervision.
- Do not download or install software on any of the cryoEM core computers.
- Do not transfer data from the microscope computer to a flash drive.
- If you noticed an error or if the alignments are not ideal or the microscope is not working contact Joshua Strauss ASPA. If you think that there is a problem with the microscope chances are you correct.
- If you are sick do not go to the core.
- If you need help then ask.
- If you are uncertain about something then ask.
- If you have any questions please contact Joshua Strauss or Emily Robinson

It is best to think of this document as a guide for using the Talos Arctica. The purpose of this document is to help guide you. If you treat this document as a set of immutable instructions your ability to use the Talos Arctica will be limited by this document. Before you do anything with the microscope think about what you are doing. I am serious, really think about what you are doing.

During the session take good notes, for example....

Autoloader

12. XG
11. CA 20200922 A G1 – screened – Trash
10. CA 20200922 A G2 – too thick – Trash
9. CA 20200922 A G3 – good – collect 100 movies – saved Box CA 20200922 A slot 3 cain 3 puck 007 slot 6
8. CA 20200922 A G4 – did not screen – save 7.
7. CA 20200922 B G1 – autoloader error –

Notes

Gain reference for K3 at 9 AM

Direct alignments

Which serialEM settings file was used

Inventory started at 10AM

Map to grids started at 10:45 AM

Setup data collection 10 grid squares overnight, collected 1,000 movies

Saved data to X:/K3data/20200926_RM_CA/Data/

When collecting data indicated the imaging conditions:

Gatan K3

45,000 X magnification

Pixel size 0.874 Å

Spot size 3

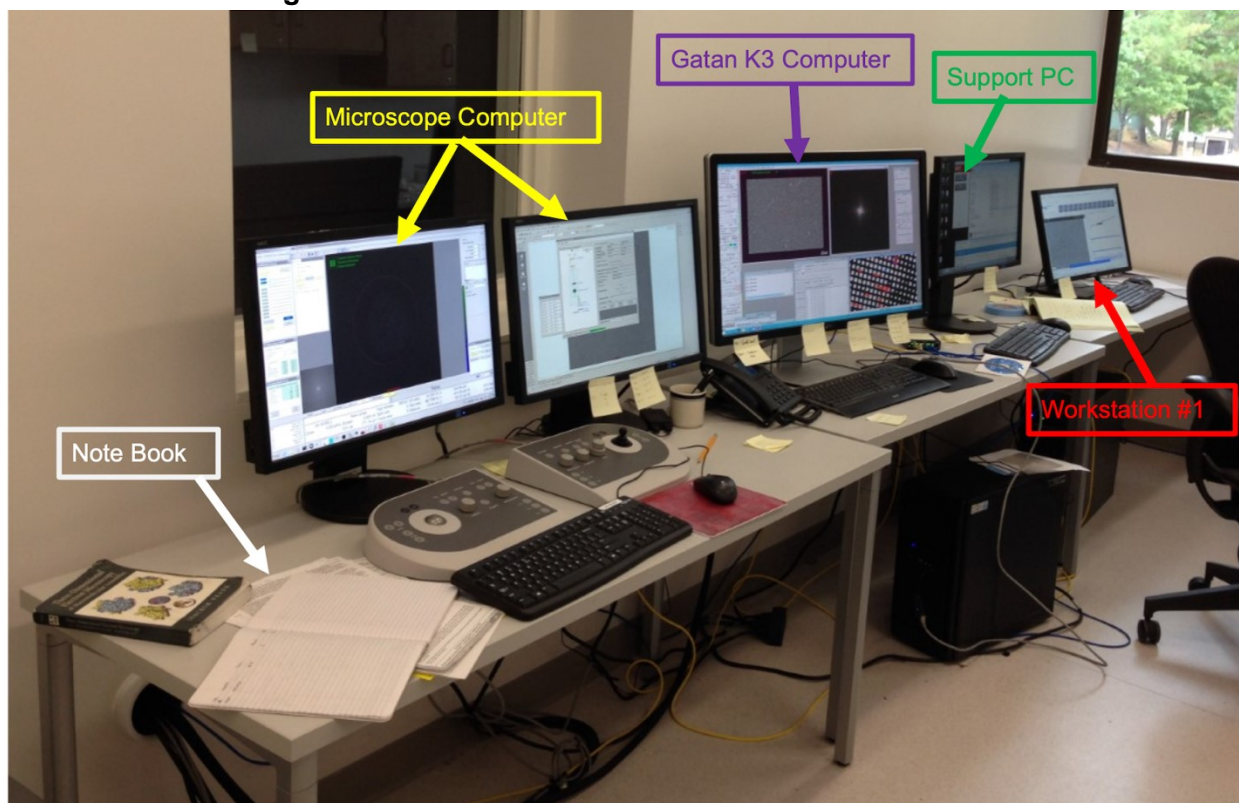
Flux 15/e-/pix/second

Total dose 54 e-/Å²

9 images per stage shift

Comma vs Image shift

II. Before starting:

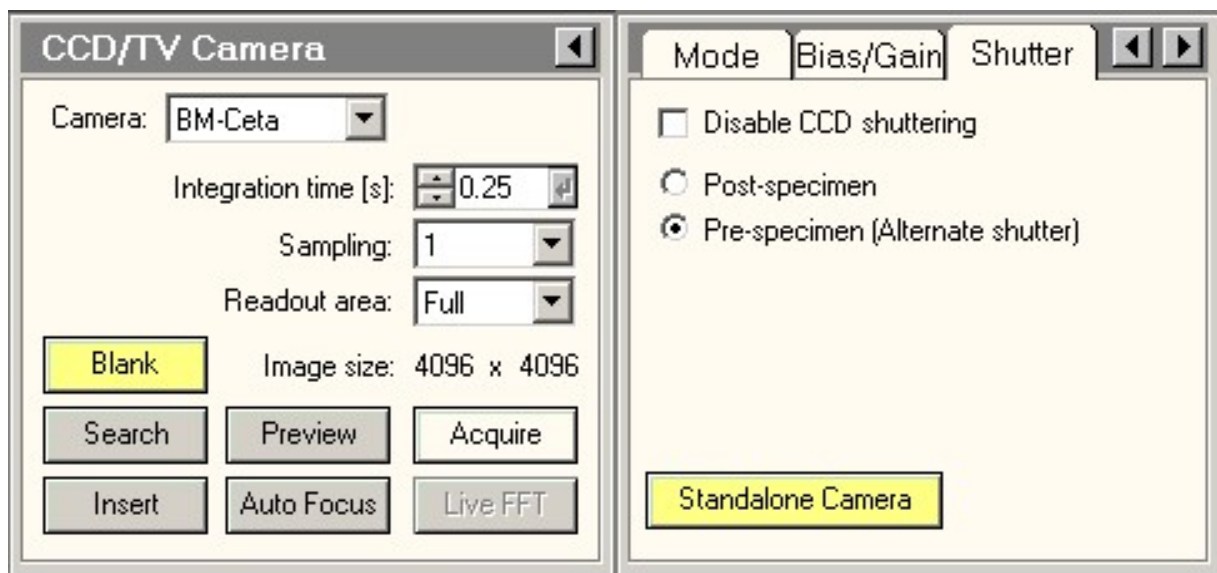


Enter your Information into the Talos Arctica Notebook:

Check the **microscope notebook** and enter the following information: data, your name/initials, PI name, time started/ended, the number of grids loaded, current, and notes on the imaging session.

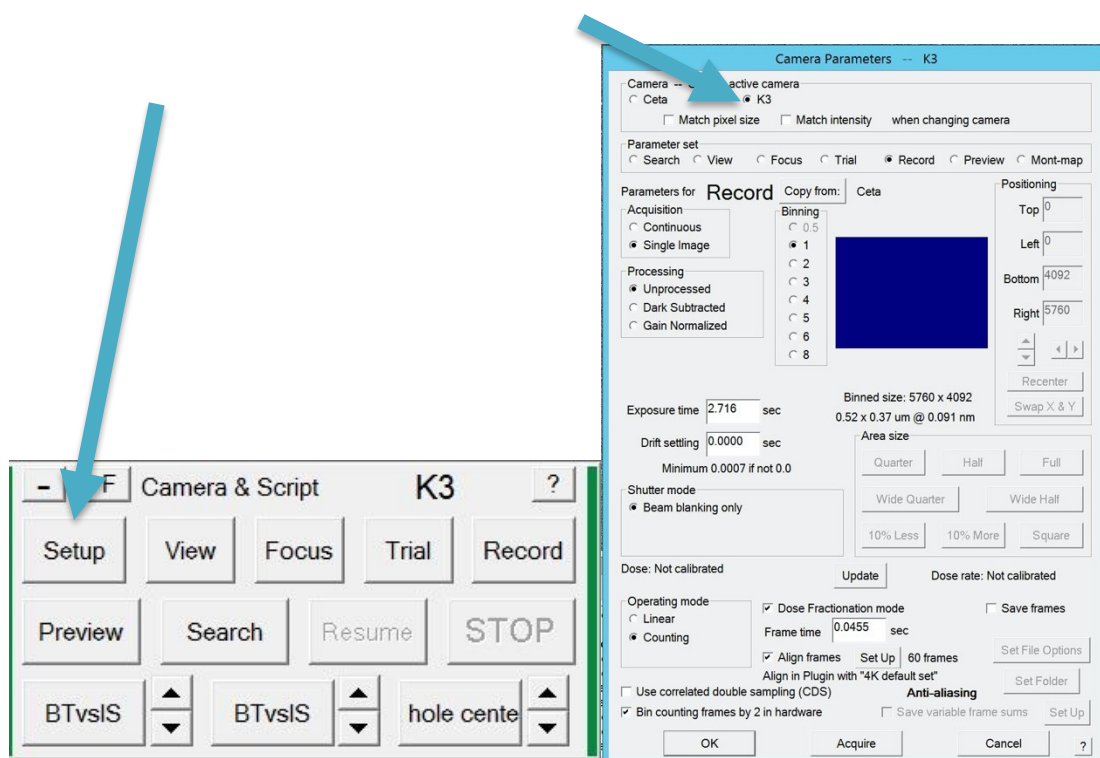
Check microscope computer:

- The Column Valves should be closed
- The FEG Gun should be on
- The Column and Autoloader vacuum and temperature should be green
- be closed, the FEG gun should be on, and the Autoloader Vacuums
- SerialEM SEM-Server should be running
- Gatan Remote TEM should be running
- No error messages
- Select "**Standalone**" in the CCD/TV Camera Panel, also make sure that the "**pre specimen** (Alternative Shutter) is checked in the Shutter tab.



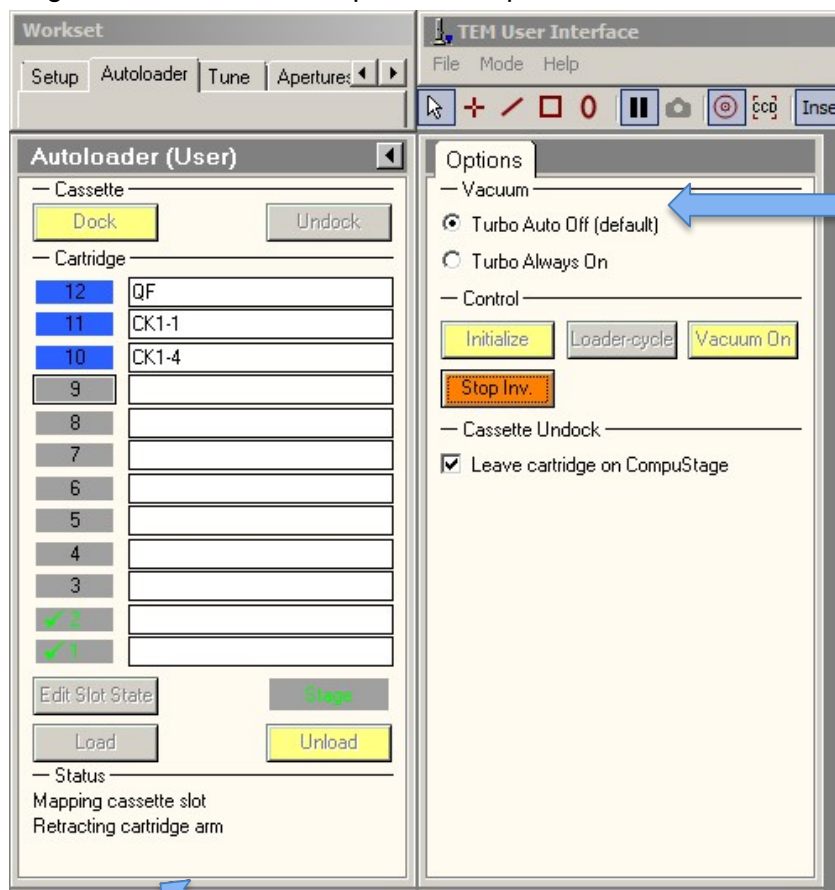
Check the SerialEM / Gatan K3 Computer:

- Digital Micrograph should be open and running
- SerialEM should be open and running
- Load current SerialEM Settings: File→ open “20200628_LD_K3_45K.txt”
- Select the Gatan K3 Camera in SerialEM Camera Panel →Setup



III. Autoloader Inventory

At this point, your cryo-grids have been clipped and loaded into the cassette and autoloader. Before starting the Autoloader Inventory, the vacuum and temperature of the autoloader should be green. On the microscope, the computer find the “Autoloader” tab.



Should always have the Turbo pump off.

Normally the stage is **Initialized**. If not contact the Core Director.

“Loader-cycle”.....don’t click on this button!!!!

“Vacuum on”, this should be on.

Status-----

This panel will display what the autoloader is doing. Keep an eye on it during the session. If the autoloader is doing something ie. loading a cartridge don’t interrupt this process. Once it is finished the Status panel should be empty.

Operation of the UNC Talos Arctica

- The **“Turbo Auto Off (default)”** should be checked.
- Click on **“Edit Slot State”**, then click on the Cartridges changing the color to BLUE if the Cassette slot is occupied by a Cartridge. If it is empty click on it twice to make it dark grey. Light grey (not shown) if you don’t know.
- Enter the **label** for each of the Cartridges in the Autoloader.
- Click on **“Inventory”** in the Options tab.
- The microscope will check to see if there is a Cartridges in the Cassette.
- The image above was taken during the Inventory, once the inventory is completed there should be a green check mark next to each Cartridge. A Red X on the Cartridge indicates an Autoloader error.

If you get an Autoloader error during the Inventory do not attempt to fix this, immediately tell the Core Director, and enter this information in the notebook.

IV. Collect Gain Reference Gatan K3 Camera

This protocol is only for CryoEM Core Staff

Gatan K3 Gain Reference

1. Find the area of the TEM grid with no sample and move the stage to that area or unload the cartridge.
2. Select Standalone Camera in the CCD/TV Camera Shutter tab.
3. Open the column valve and in SerialEM open data collection settings and go to Record Low-Dose imaging conditions and uncheck Low-Dose. Alternative adjust magnification on microscope computer. the
4. On FluScreen center the beam at K3 magnification was used for data collection.
5. Use 70 micron or a larger condenser aperture to collect the gain reference.
6. On Gatan Computer select Camera → prepare gain reference. Must be in either power user or service mode.
7. Change SPT 1 to collect gain reference in Linear mode. Use the Microscope Control Pads Multifunction L3 or R3 to do this.
8. Condense beam (on FluScreen, not K3 detector), the beam should be slightly larger than the largest circle, so the counts on the K3 detector are ~ 1500.
9. Follow instructions on GMS – will take a few mins to collect and gain reference in Linear mode.
10. Once a gain reference in linear mode is collected repeat steps 6-8 above procedure for collecting gain reference in counting mode but with SPT 3 or 5 to get ~15 e counts on the sensor.
11. Take a Record image (do not align frames) with SerialEM to get the Flux and verify that the gain reference is working. If not repeat, power-cycle the camera, and/or contact Gatan if an error.

Notes

- Do not condense the e-beam on the K3 sensor. Always use the FluScreen to the center or condense the beam.
- Collect gain reference for Linear and Counting mode.
- Use ALT key to select either Linear or Counting mode gain when taking a gain reference.
- Make sure SerialEM is not in Low-Dose Mode.
- Collect gain reference after annealing the camera or before collecting the data set (before aligning the TEM) if a gain reference has not been collected in 2-3 days.

V. Load a cross-line grating replica TEM grid and perform direct alignments

This will be performed each morning (or on a need-to-do basis) by a Core employee. If you believe that the alignment is off and are not comfortable adjusting it yourself, ask an employee to assist you. We sometimes will use a Quantifoil, C-Flat, or UltraAufoil TEM grid in place of a Cross line grating replica TEM grid, this can be done to save time during a session.

** This protocol is for use only by users who have received training in the microscope alignments process. Ask a Core staff member before doing alignments yourself. * We assume you understand what you are doing at each step –and know about optics.*

I. Load the cross grating replica TEM grid

- On the microscope computer, go to the autoloader at top left.
- Close column valves (bottom left).
- Click on slot and number that has cross grating grid, and then load.

II. Set eucentric height

- Open column valves and insert FluScreen (“insert screen” at top).
- Click “natural” (bottom left).
- Find a square in the middle of the grid with low contamination (use a joystick to move around).
- Click “wobbler” (bottom right) to rock the stage.
- Adjust the Z height of the stage using the control pads, the feature of interest should be centered as the stage wobbles.
- When movement is minimized, click “wobbler” again to turn it off.

III. Before Direct Alignments

- **Load serialEM settings file.** First go to serialEM computer and click “settings” (top left) and then the settings that you will use for data collection.
- On the serialEM computer, check that “rec.” is green in the low dose panel (bottom left), and uncheck “low dose” to **turn low-dose off**.
- Press the L1 button on the control pads to **normalize lenses**.

- Got o **true focus** by hit wobble button on control panel (objective lens wobbler) and adjust the focus to minimize movement. Hit L2 button to reset focus to zero
- Can check focus on the screen to the FFT inset and the image.

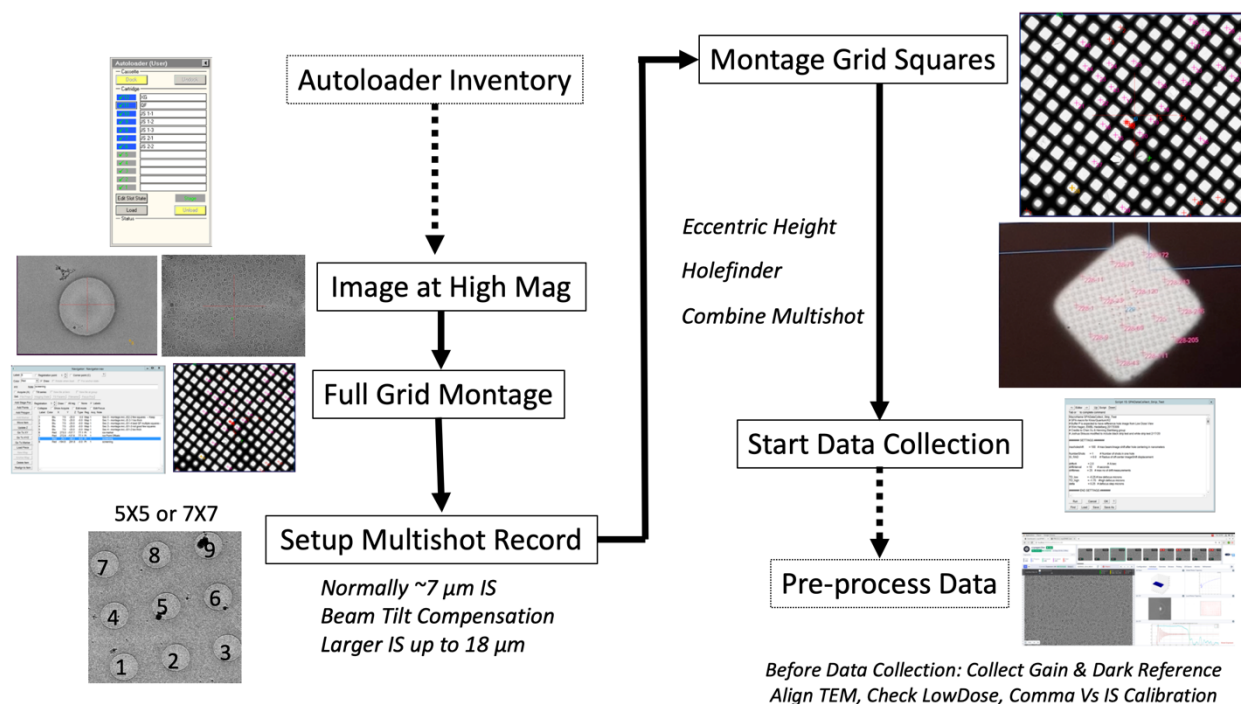
Direct Alignments

- **Center the beam.** Click “tune” tab (top left)→ “beam shift”. Use intensity knob to condense the beam to ~pencil size. Use the multifunction axis knobs to center beam. Click “done”
- **Adjust pivot points.** Click “np beam tilt pp X”. Use multifunction x knob to merge the two light spots. Click “done”
- **Adjust pivot points.** Click “np beam tilt pp Y” Use multifunction x knob to merge the two light spots. Click “done”
- **Condense and Center the beam** using beam shift again.
- **Selected C2 aperture and center it.** Normally use the 50 or 70 micron depending on the size of the hole. Click “aperture” tab → **adjust**. Enlarge the beam with intensity knob (on the right side of cross over), and make sure it’s centered. Spread the beam and adjust the mechanical position using multifunction knobs to center the aperture as you condense and spread the beam.
- **Condenser lens stigmatism.** Click “tune” tab → stigmator tab→ condenser. Adjust intensity knob to condense and spread the beam, make sure it appears round – if really astigmatic, adjust condenser lens. Use FFT inset and circle on FluScreen to tell if the beam is round or astigmatic.
- **Find parallel illumination.** In diffraction mode (click Diff button on control pads) Adjust with intensity knob until rings are sharp – C2 lens (bottom right) should read about 43.3%. Take note of C2 value.
- **Insert and center objective aperture.** This you can skip but it is fine to do this. Click diffraction button. Select the 100 micron objective aperture and center the beam, than the mechanical position of the aperture. Hit the diffraction button again to bring back to image mode.
- **Adjust Objective lens rotation center.** Go to “tune” tab → rotation center. Minimize movement multifunction XY knobs. To check adjust defocus to -10 microns the image should be centered.

Alignments on serialEM – Objective stigmatism, comma free alignment, and comma vs IS calibrations, in that order.

- Go to serial EM computer
- “Focus/tune” panel (top) → “correct astig by CTF”
- Close the navigation window and open the log
- Adjustment should be close to zero (on log), if not run correct astig by CTF again
- “Focus/tune” panel → “coma free alignment by CTF”
- Run twice if needed
- Turn on low dose (bottom left)
- Click “rec.” (bottom left) → “continuous update”
- Use intensity knob to change C2 lens
- Uncheck “continuous update” then click autofocus
- Under camera tab (left), click “setup” then select “align frames” and “fractionation” (bottom)
- “Calibration tab” (top) → “focus” → “coma vs. image shift”
- 7.5 is the default
- “Settings” tab (top left) → save
- “Focus/tune” tab → correct astig by CTF

Typical session screening and data collection



Note: If the grid has very thick ice don't image it just skip it and load another grid. If the grid has thin ice and the particles/samples look fine proceed with collecting a single full-grid montage. We sometime skip full grid montage to save time, or deviate from this workflow depending on the purpose of the experiment.

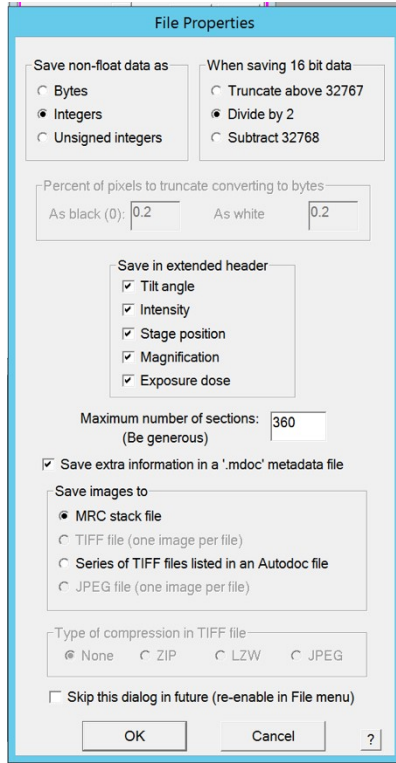
Before screening session check the following

- Apertures – For low mag imaging use the 150, for data collection, use the 70 or 50 micron condenser aperture
- Microscope should be aligned
- Check that standalone camera is selected when using the K3
- Turbo pump in autoloader should be always off by default
- Temperature of the autoloader, column, and vacuum – all should be green
- No errors on microscope computer
- TIA should be running
- Microscope computer, Gatan computer, and support PC should all be on and open
- Microscope computer – TIA should be running, no microscope errors
- Digital micrograph remote TM should be running on microscope computer
- SEM FEI server for serial EM should be open on microscope computer
- On Gatan computer, digital micrograph and serial EM should be running (in administrator mode)
- Check serialEM settings (will vary between single particle and tomography)
- Check camera and script setup
- Make sure no files are open in serialEM

V. Map to Grids or Single Full-grid Montage

On the SerialEM Computer

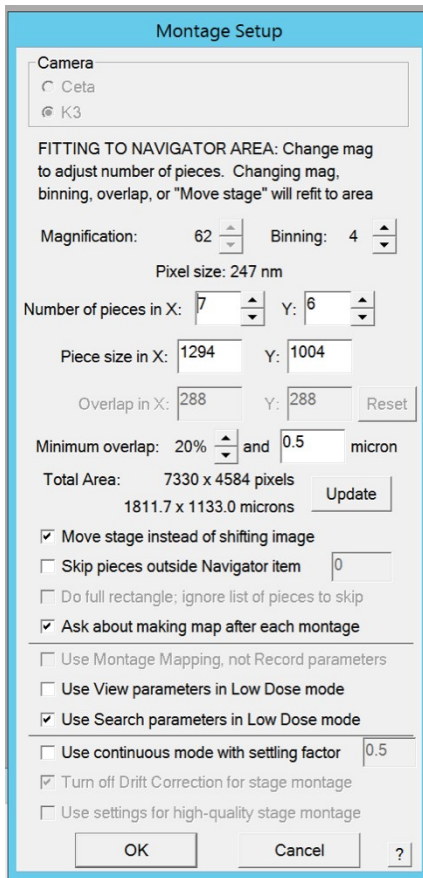
1. Open a new **Navigator** file
2. Setup **Full Grid Montage**, Navigator → Montage and Grids → Setup Full Montage



File Properties: normally don't need to change anything..

Click **"OK"**

Save the files as MRC stack in the directory where you are saving the data something like this
/k3data/20200606_JDS/Maptogrids/Fullgridmontage.mrc



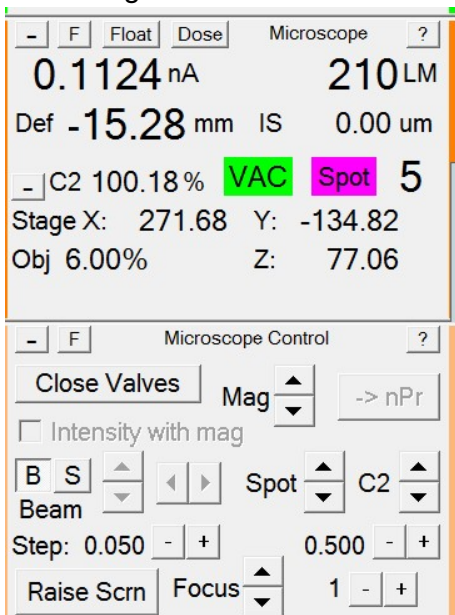
Montage Setup: Normally don't need to change anything..

Number of pieces in X and Y, usually set to 5 - 8 The more pieces the longer the montage takes.

Set Minimum overlap to 15-25% for **UltrAufoil TEM grids set this to 23-25%.**

Click "OK"

3. Change the Low-Dose Search Mode Magnification to 62X



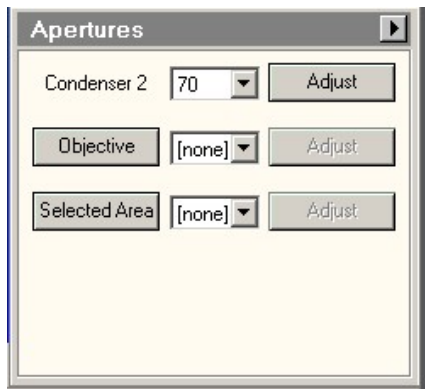
Microscope Control:

To change the magnification click on the Mag arrows.

The magnification is indicated in the Microscope panel, here in the image as LM 210.

4. On the microscope computer **Retract the Objective Aperture**

5. Insert and Center the **150 μm Condenser 2 Aperture**

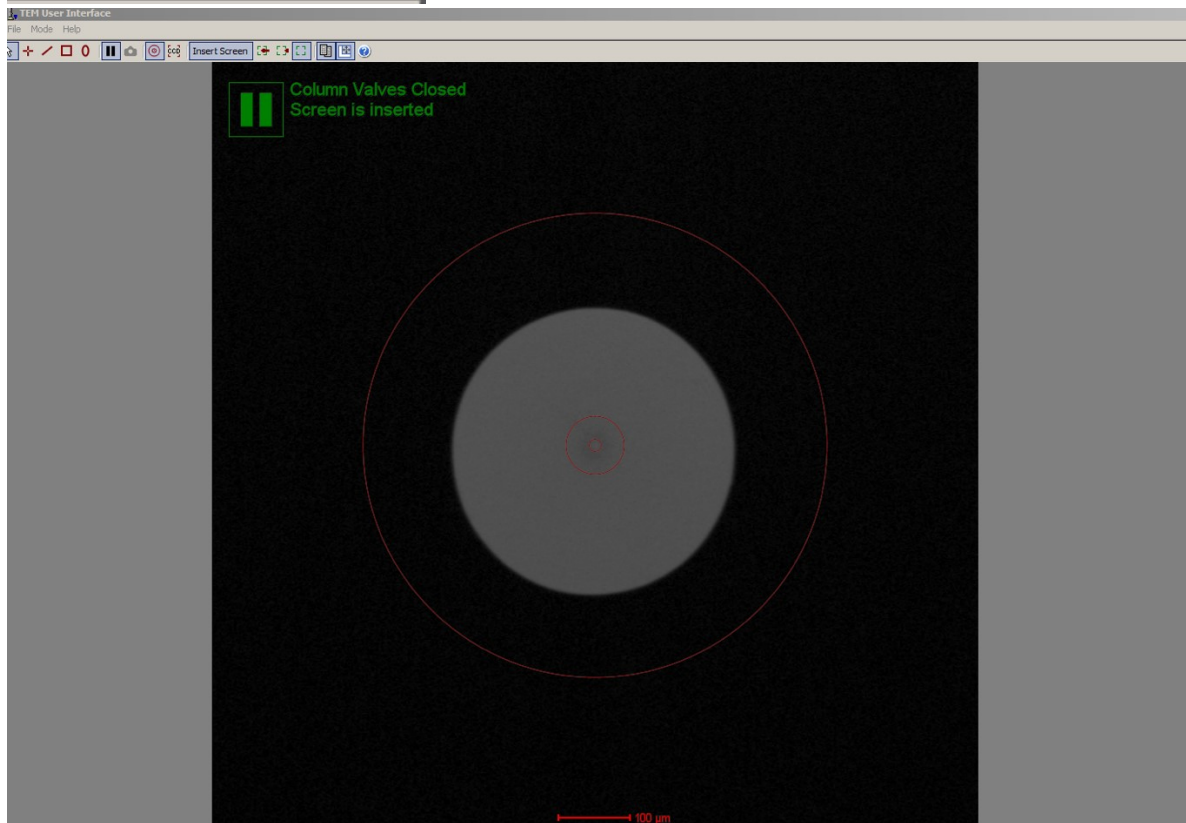


Apertures

To change apertures click on the pull-down tab.

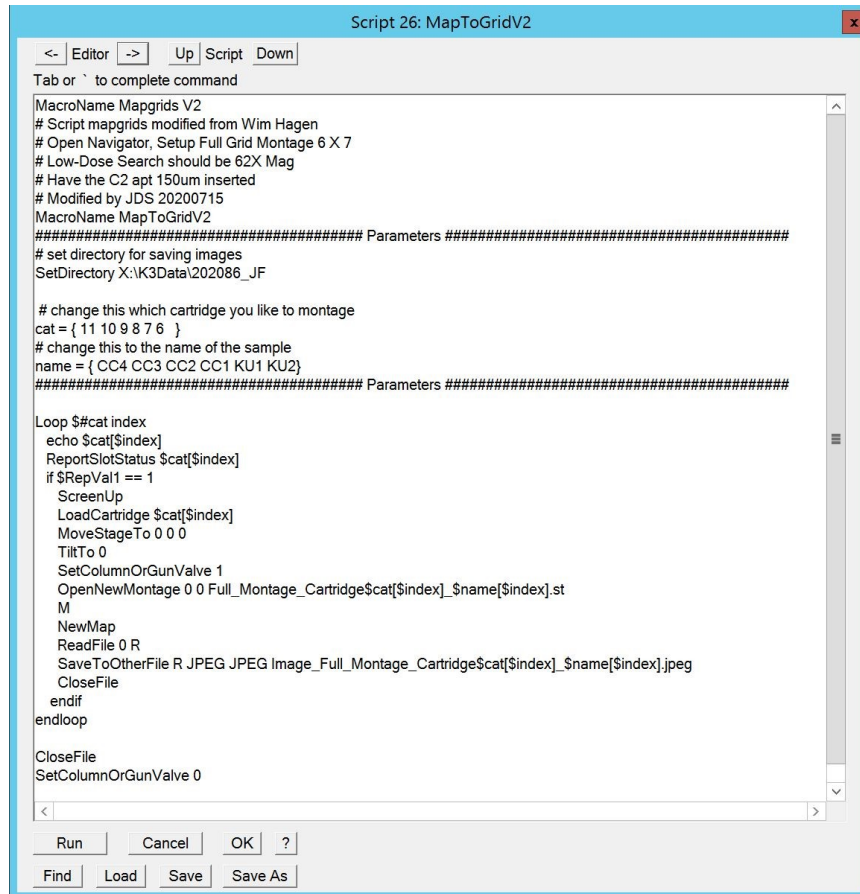
To center the aperture click on "**Adjust**" and use the **Multifunction X Y knobs** on the Microscope Control Panels to move it.

Lower the **FluCam** and to see if the beam and apertures are centered. See image below, note this is the 70 μm not the 100 or 150 but it is centered.



6. Open and **edit script 26 "MapToGridV2"**, or "**Single Montage**", Select the Script tab→ Edit

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```
Script 26: MapToGridV2
<- Editor -> Up Script Down
Tab or ` to complete command
MacroName Mapgrids V2
# Script mapgrids modified from Wim Hagen
# Open Navigator, Setup Full Grid Montage 6 X 7
# Low-Dose Search should be 62X Mag
# Have the C2 apt 150um inserted
# Modified by JDS 20200715
MacroName MapToGridV2
##### Parameters #####
# set directory for saving images
SetDirectory X:\K3Data\202086_JF

# change this which cartridge you like to montage
cat = { 11 10 9 8 7 6 }
# change this to the name of the sample
name = { CC4 CC3 CC2 CC1 KU1 KU2 }
##### Parameters #####

Loop $#cat index
  echo $cat[$index]
  ReportSlotStatus $cat[$index]
  if $RepVal1 == 1
    ScreenUp
    LoadCartridge $cat[$index]
    MoveStageTo 0 0 0
    TiltTo 0
    SetColumnOrGunValve 1
    OpenNewMontage 0 0 Full_Montage_Cartridge$cat[$index]_ $name[$index].st
    M
    NewMap
    ReadFile 0 R
    SaveToOtherFile R JPEG JPEG Image_Full_Montage_Cartridge$cat[$index]_ $name[$index].jpeg
    CloseFile
  endif
endloop

CloseFile
SetColumnOrGunValve 0

Run Cancel OK ?
Find Load Save Save As
```

You will need to change the **Directory** to the one you created to save your images.

Change the **Cartridge list** to reflect the autoloader grid slots you want to montage.

Change the **Name** of each grid, keeping the same order as the cartridge inventory order. This is how each montage file will be named so each grid is easier to keep track of.

Nothing below **Loop \$#cat index** will typically need to be changed.

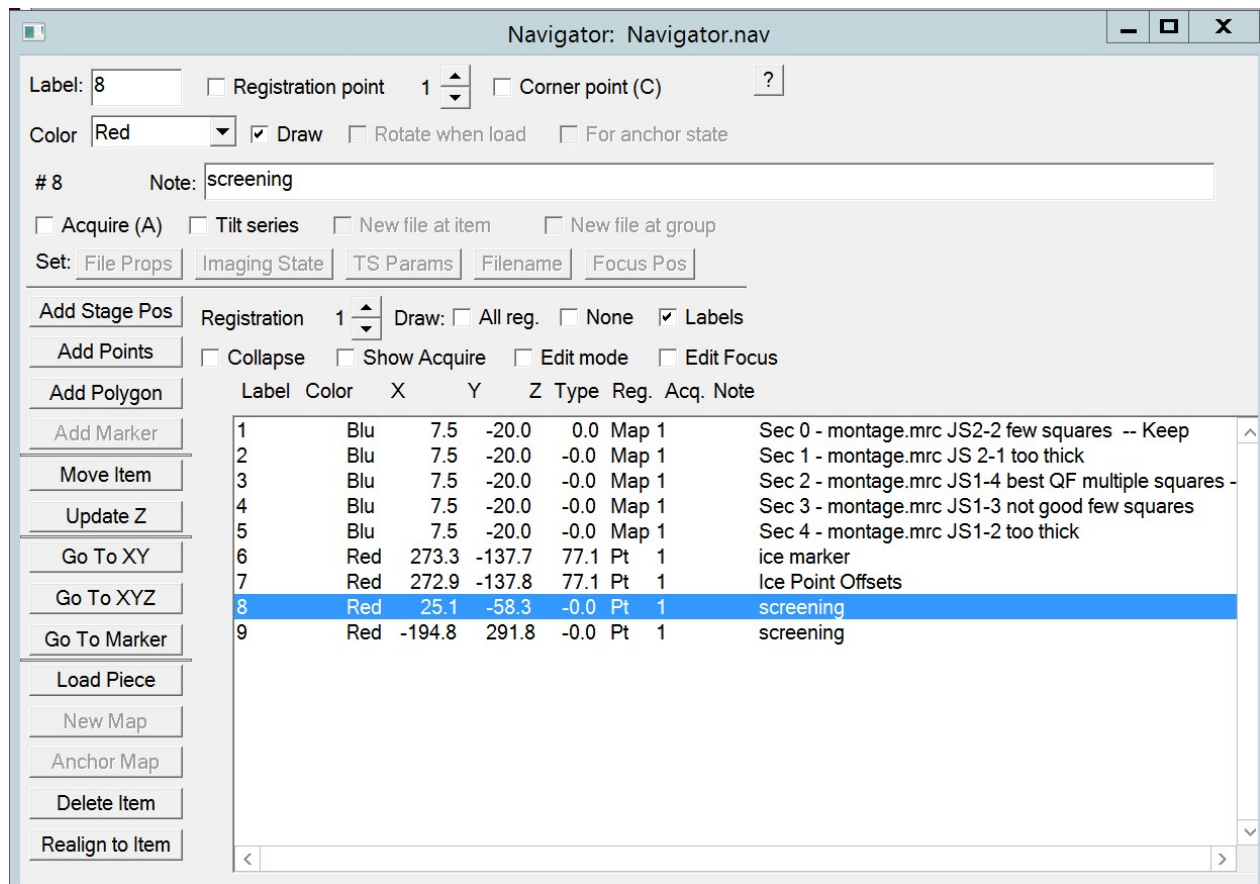
After changing the script click **“OK”**

To Run the Scrip click **“Run”**

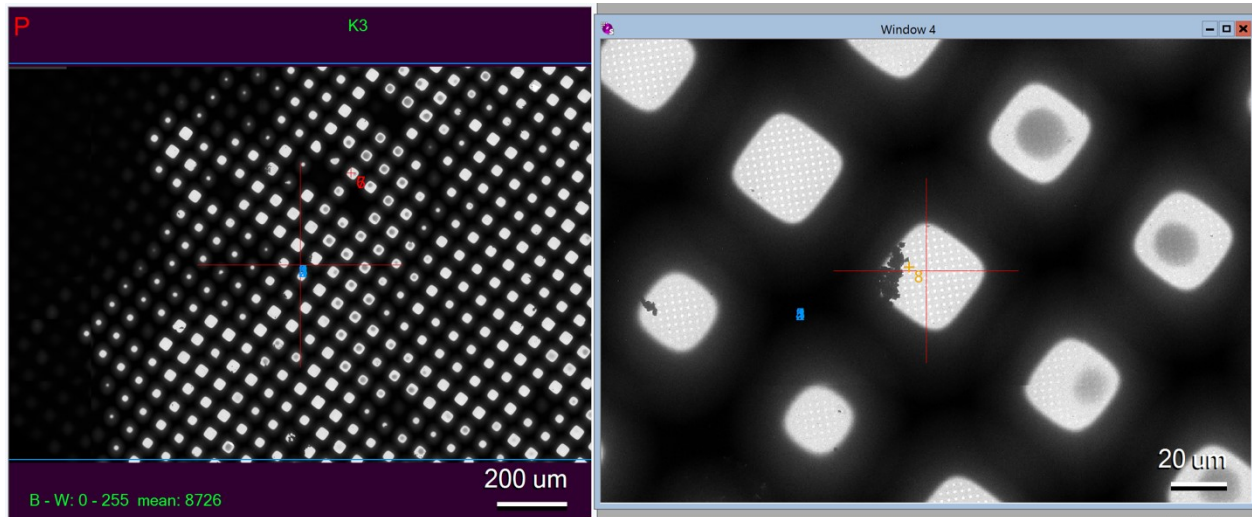
- Alternative you can set up a montage of the entire grid using Full-grid montage in SerialEM in the Navigator Tab.

VI. Screening Cryogrids

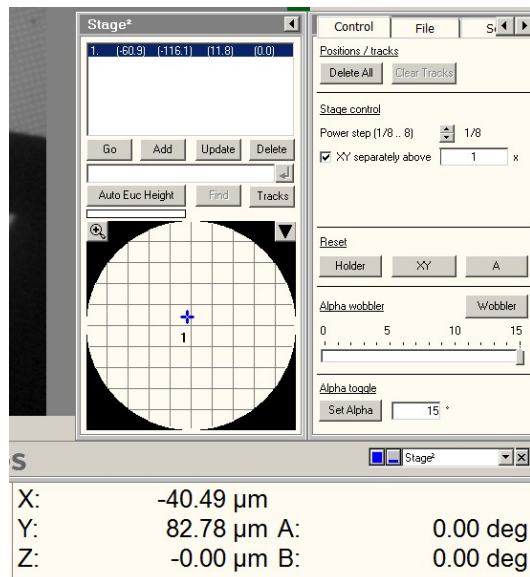
1. **Look at the montages (Maps) in the Navigator.** To open a Map **double click on it** in the Navigator, this will display it to the main window. You can open it in a separate window if you like in the Window tab. **If the grids do not contain thin vitreous ice or are too thick (completely black) don't spend time imaging these grids.** Pick the best one and take images at different magnifications to determine if the grid can be used for high-resolution data collection. You can annotate each of the Navigator items like Maps or points in the Note tab.



This is an **example of a good cryo-grid** with multiple grid squares with thin vitreous ice.



2. Change the Low-Dose Search mode to 210X (as you did before).
3. Insert and center the 70 μm Condenser aperture.
4. On the Microscope Computer load one of the grids with the Autoloader Panel. **Close the Column Valves**, on the Stage² panel Click on “XY” or “Holder” to **Center the XY (or Holder)** then wait for the stage to stop moving.



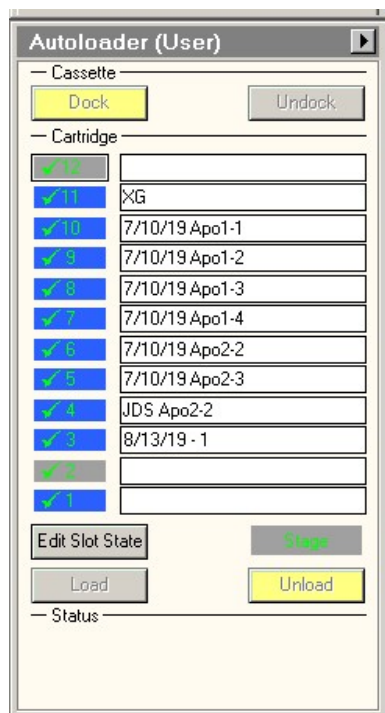
Holder – will reset X Y Z and Alpha to 0

XY – will reset X Y to 0

A – will reset Alpha to 0

When the Stage is moving or active the Reset icons will be grey and the text grey.

5. To load a cryogrid on The **Autoloader Panel** click on the Cartridge you want to load it should have a black outline over it then click **Load**.



Autoloader: Loading and Unloading Cartridges

To **select a Cartridge** to load click on it with the mouse, it will have a black outline on it, as show for Cartridge 12. Then click on “**Load**”

When the Cartridge in on the Stage it will be yellow.

You can put that Cartridge back into a Cassette by click on “**Unload**”

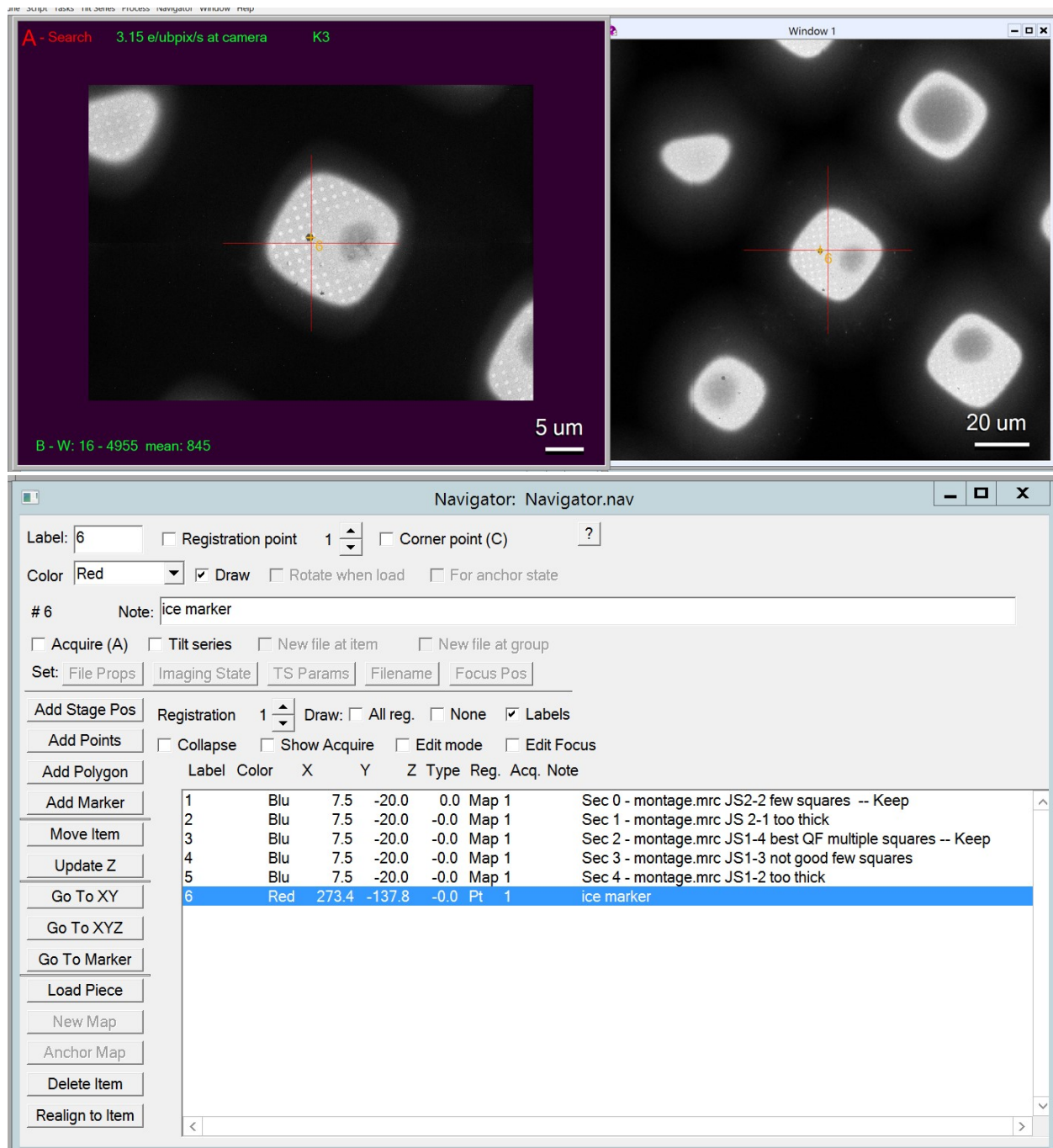
6. Once a cryogrid is on the stage you can image it. Open the Full Grid Montage (or Map) of that cryo-grid. **Add a point to the center of a grid square you want to image.** In the Navigator you can annotate if the area is good or not, this is helpful for keeping track of where the good area is on the grid. Typically, I'll pick 3 points on a grid to image to determine if the sample is good for data collection.

Typically, we collect low-dose images at Search, View and Record with the K3 and save them as MRC or JPG but you can save the images as TIFF if you like. MRC and TIFF are better for image processing or making measurements.

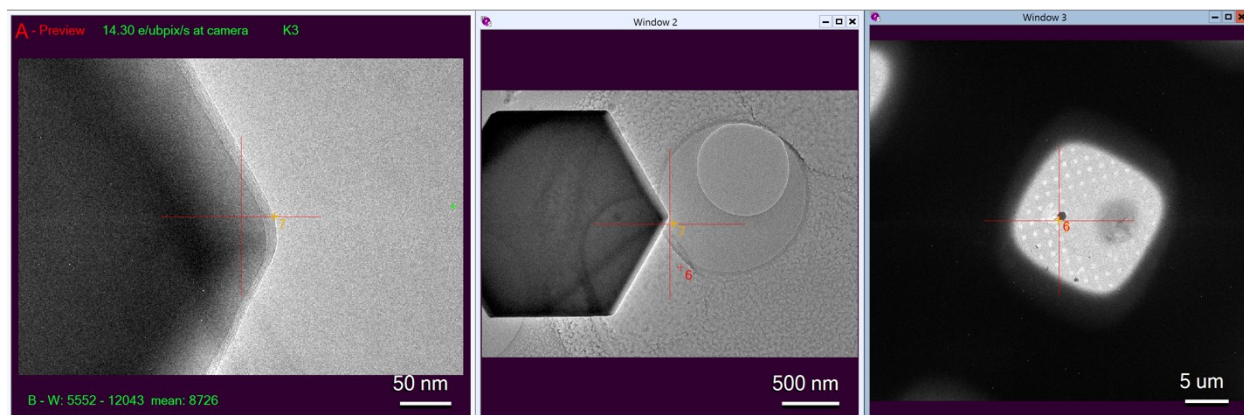
7. **When you load, unload then load a cartridge it will not be at the same position on the stage.** So the Map will be displaced slightly. To correct for run the **Shift to Marker** procedure. Find a feature on the Full Grid Montage Map like a chunk of ice or dirt, to the Full Grid Montage Map put a point on it. Move the stage to that point. Take a **Search Image**, then mouse click to put a marker (green +) on the Search Image displayed in the window. Then on the Navigator tab → shift to marker. It should look like this....

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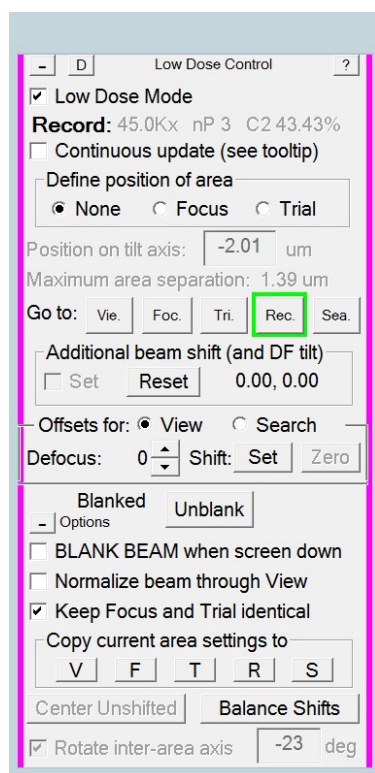
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8. **Adjust Offsets for View and Search** (optional, don't need to do this every time you use the microscope). Sometimes the Record, View and Search are off centered, to check for this find a chunk of ice and move to it in Record. Take Record, View and Search Image, the ice should be centered in each of the image.



9. If the images are not centered then you can apply an offset to the View and Search. This is done in the Low-Dose panel.



At this point the ice is centered in Record but not centered in View or Search or both.

Check "Offsets for ..View or Search depending on which one you want to change.

Take a View or Search image

With the mouse drag that image so the ice is in the center. You might need to uncheck "Move stage for large image shifts" option in the Image Alignment and Focus Panel.

Click Shift "Set" to set the offset.

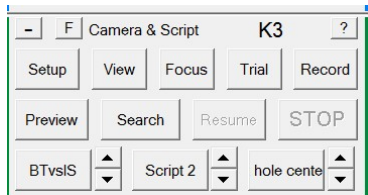
Do this for the View or Search.

10. To **Move to the point** click on it in the navigator file point, should be highlighted in blue, then click "**Go to XY**" or "Go to XYZ"

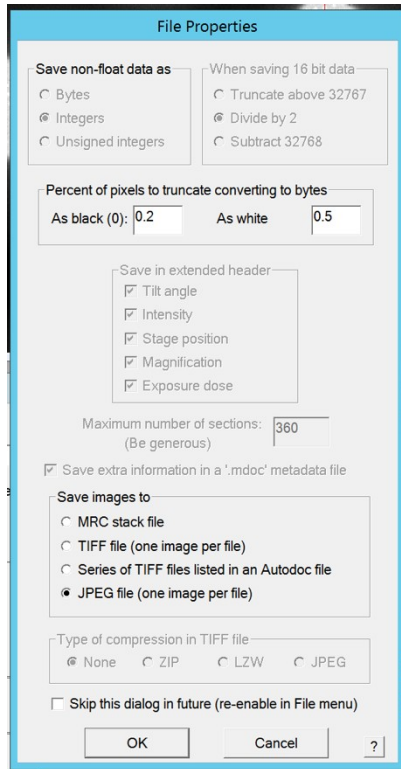
11. After moving to a point adjust the **eucentric height** using the Microscope Stage Wobbler or in SerialEM use Rough Eucentric, Task → Rough Eucentric. *Note the Rough Eccentric will not work if the Z height of the stage is very far (75-50 μm) from focus. If you change the Z height of the Stage you can save this to the Navigator point by clicking "**Update Z**".*

12. To take a **Search image**, click on the "Search" icon in the "Camera and Script" panel.

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13. To save this (and other images) select File → save to other. Then click ok. Usually just save as a JPEG if you just want a picture for documentation (not image processing). ***If you plan on measuring the particles, or preforming any image processing save as MRC or TIFF.***

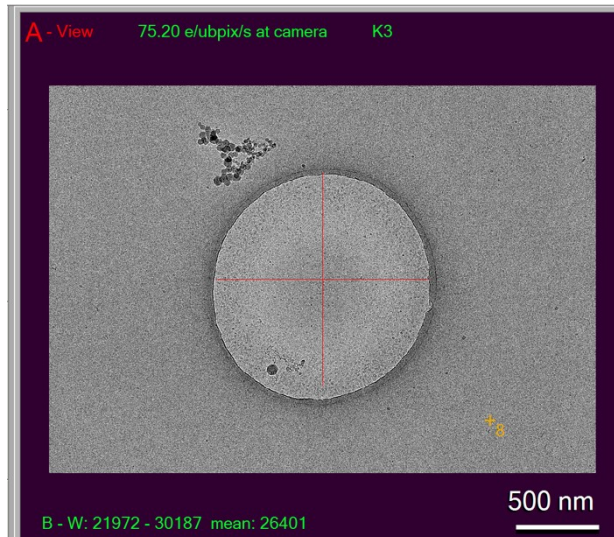


14. To take a **View image**, click on the “View” icon in the “Camera and Script” panel. To increase the contrast for the View image you can change the “**defocus offset**” on the “Low Dose” Panel, typically 10-20 μm is fine.

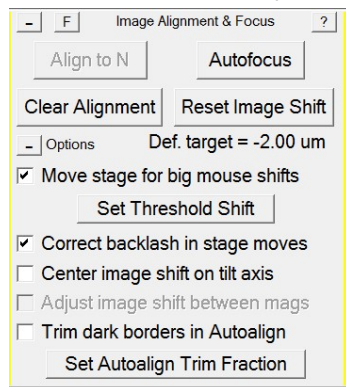
15. To take a **Record image**, set **Target Autofocus** to - 1 to -3 μm .

Note the Target Autofocus will not be precise if the C2 % of Focus is different from Record, or if the grid is not flat. Typically, we condense the Focus beam to minimize exposing the sample, which makes a ~ 0.5 to 1 micron offset in defocus. You can measure the defocus of the Record image using the CTFfind plugin in serialEM just click on the FFT window where the first CTF zero is) to determine the best Target Defocus to use.

16. **Move the stage or image shift over a hole**, by clicking on the View Image in the window with the mouse and dragging it to the center (will either stage shift or image shift depending on distance). Should look like this.



17. **Autofocus** by clicking on the “Autofocus” icon in the “Image Alignment and Focus” panel



Look at the SerialEM log, Window, and FFT of the image to judge if the autofocusing is working.

18. Take a record by clicking on the “Record” icon on the “Camera and Script” panel. *Note before collecting images check the **Camera Parameters**, can be selected by clicking on “Setup” icon in the “Camera and Scrip” panel.*

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Camera Parameters -- K3

Camera -- Selects active camera
☐ Ceta ☒ K3
☐ Match pixel size ☐ Match intensity when changing camera

Parameter set
☐ Search ☐ View ☐ Focus ☐ Trial ☒ Record ☐ Preview ☐ Mont-map

Parameters for **Record** Copy from: Ceta

Acquisition
☐ Continuous
☒ Single Image

Processing
☒ Unprocessed
☐ Dark Subtracted
☐ Gain Normalized

Sum will be gain-normalized

Binning
☐ 0.5
☒ 1
☐ 2
☐ 3
☐ 4
☐ 5
☐ 6
☐ 8

Positioning
Top 0
Left 0
Bottom 4092
Right 5760
Recenter
Swap X & Y

Binned size: 5760 x 4092
0.52 x 0.37 um @ 0.091 nm

Area size
Quarter Half Full
Wide Quarter Wide Half
10% Less 10% More Square

Exposure time 2.716 sec
Drift setting 0.0000 sec
Minimum 0.0007 if not 0.0

Shutter mode
☒ Beam blanking only

Dose: Not calibrated Update Dose rate: Not calibrated

Operating mode
☐ Linear
☒ Counting

☒ Dose Fractionation mode
Frame time 0.0455 sec
☒ Save frames
60 raw to TIF-LZW, binned

☒ Align frames Set Up
Align in Plugin with "4K default set"

☐ Use correlated double sampling (CDS) Anti-aliasing
☒ Bin counting frames by 2 in hardware ☐ Save variable frame sums Set Up

Set File Options
Set Folder

OK Acquire Cancel ?

Camera Parameters for Screening

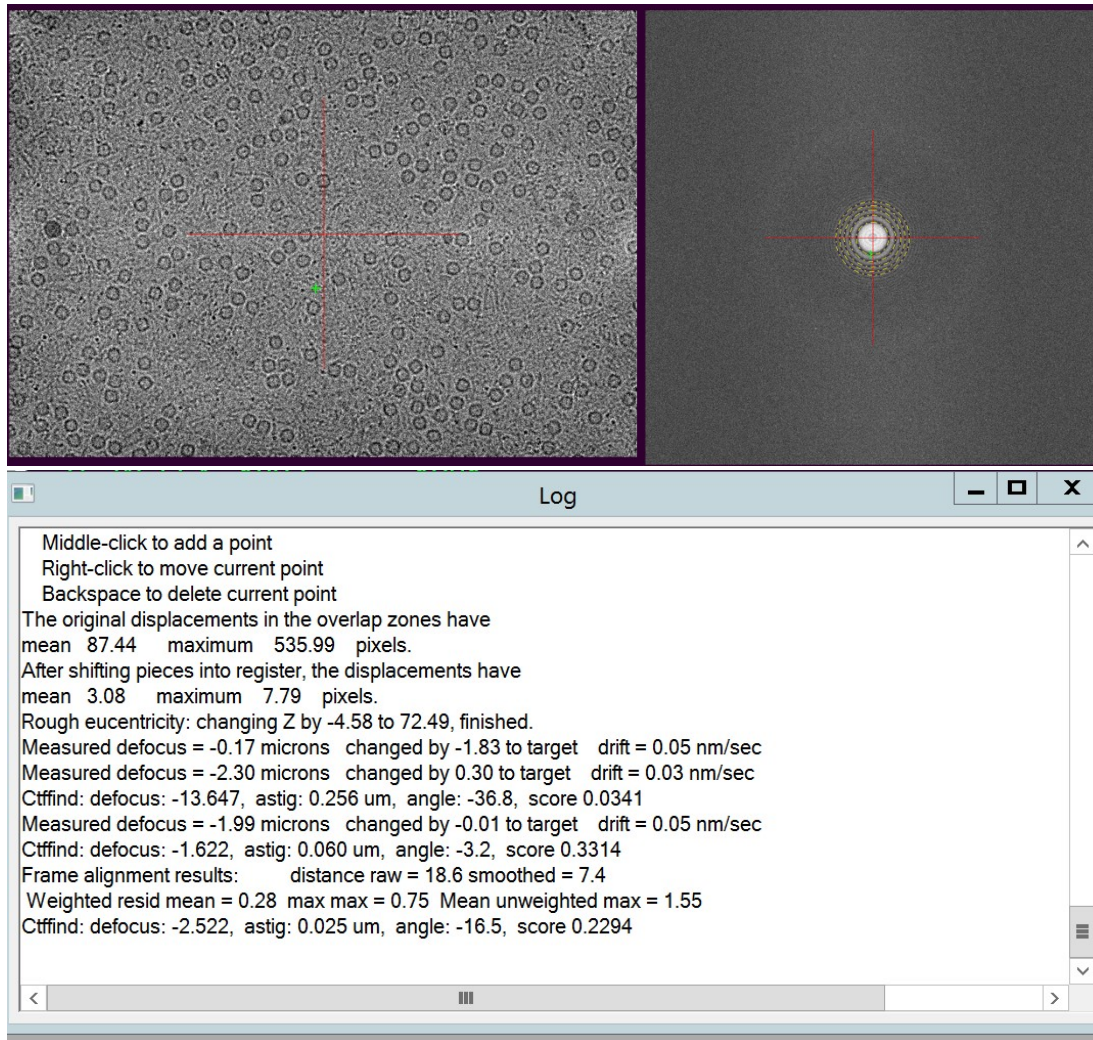
Don't need to change the parameters but ...

For screening / collecting a few images select the following:

Processing **"Gain Normalized"**

Uncheck **"Save frames"**

19. To take a record image click on the **"Record"** icon in the "Camera and Script" panel.

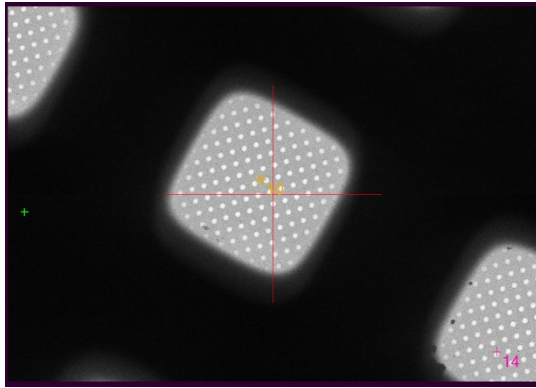


*You can click on the FFT to activate **Ctffind** plugin....will print out info in the serialEM log this is useful to checking image quality and measuring the defocus and astigmatism of the image.*

VII. Montage Grid Squares

At this point you have identified a cryo-grid for data collection, and it is on the stage.

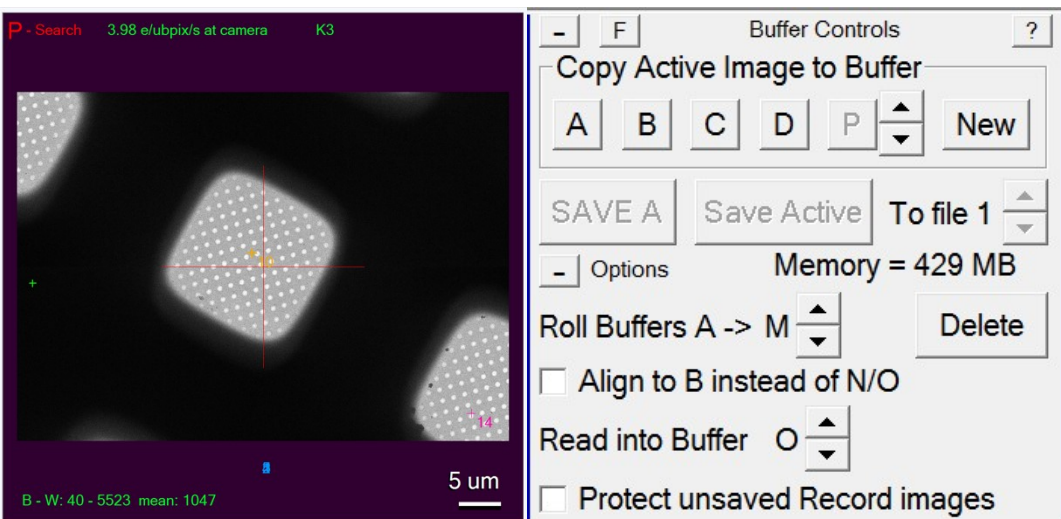
1. On the Full Grid Montage Map add points to the center of grids squares that you want to image. Try to estimate how many grids squares you will image, to do this count how many holes are on each grid square.



2. On the Navigator mark these points as **Acquire**) (check box), the points will have a "A"

10	Red	47.9	-39.1	72.5 Pt	1	A	collect
11	Red	-203.5	46.9	72.5 Pt	1	A	collect
12	Red	-182.0	-42.7	72.5 Pt	1	A	collect
13	Red	-67.3	-108.2	72.5 Pt	1	A	collect
14	Red	12.4	-90.6	72.5 Pt	1	A	collect

Take an **Search image** of one grid square make sure it is centered, save it as a MRC, or copy it to **Buffer P** in the "Buffer Control" panel by clicking on the Icon "P". The Buffer is displayed in the Window in red letter.



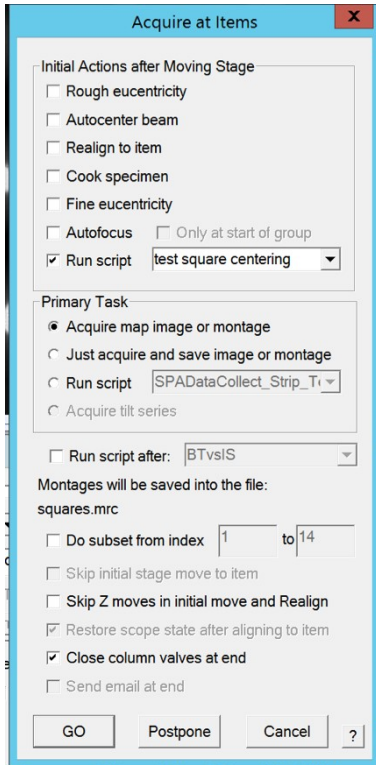
3. Make sure Full Grid Montage is not open in SerialEM. If it is open: File→Close 4. Setup Montage to take one image at 210 X Search

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The screenshot shows the 'Montage Setup' dialog box. At the top, the 'Camera' section has 'Ceta' and 'K3' selected. Below this, 'Magnification' is set to 210 and 'Binning' to 4. The 'Pixel size' is 40.4 nm. The 'Number of pieces in X' is 1 and 'Y' is 1. 'Piece size in X' is 1440 and 'Y' is 1022. 'Overlap in X' is 144 and 'Y' is 144, with a 'Reset' button. 'Minimum overlap' is 20% and 0.5 micron. 'Total Area' is 1440 x 1022 pixels (58.1 x 41.2 microns) with an 'Update' button. Checkboxes include: 'Move stage instead of shifting image' (checked), 'Skip pieces outside Navigator item' (unchecked, with a value of 0), 'Do full rectangle; ignore list of pieces to skip' (unchecked), 'Ask about making map after each montage' (unchecked), 'Use Montage Mapping, not Record parameters' (unchecked), 'Use View parameters in Low Dose mode' (unchecked), 'Use Search parameters in Low Dose mode' (checked), 'Use continuous mode with settling factor' (unchecked, with a value of 0.5), 'Turn off Drift Correction for stage montage' (checked), and 'Use settings for high-quality stage montage' (unchecked). At the bottom are 'OK', 'Cancel', and '?' buttons.

4. Select the Navigator tab --> **Acquire at items**

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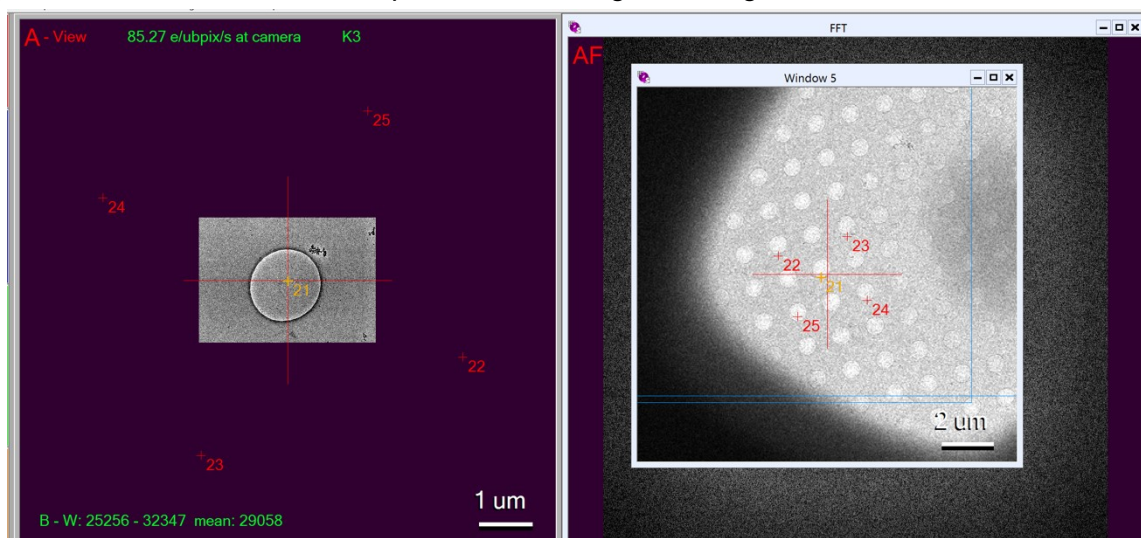
Make sure to select **Run Script** “square centering” or “square centering tilt 45.txt”. Depending if you want to collected tilted data ie. tilt the grid 45 degrees. Make sure the tilt angle is set to 0, or the desired tilt angle in the script (using the Script Editor).

It takes about 1 min to collect a montage of each grid square.

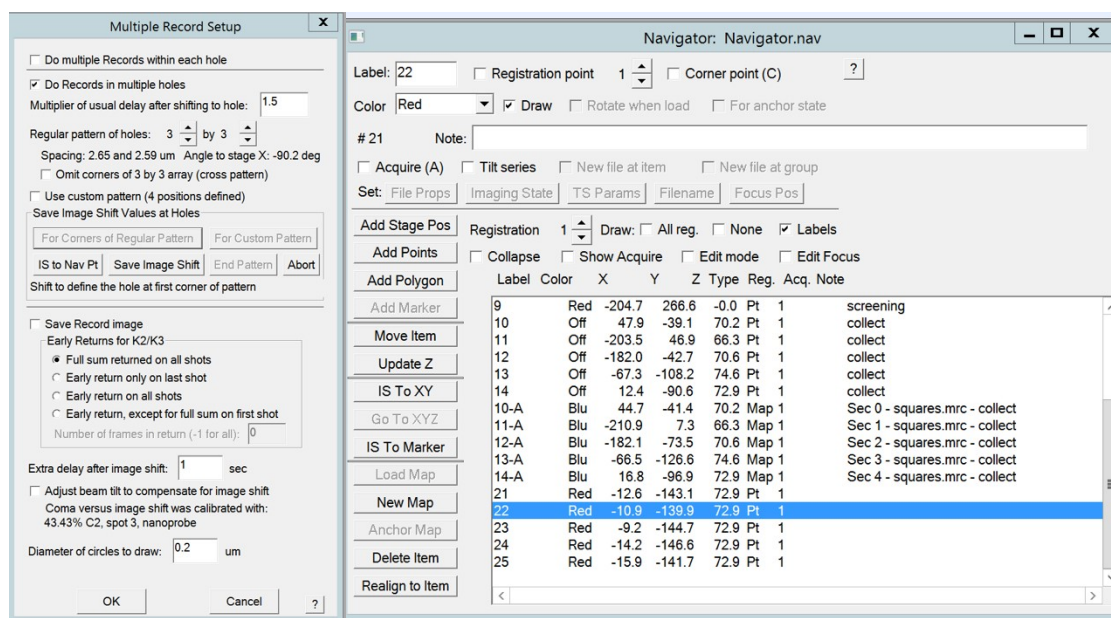
VIII. Setup Multishot Record

For large multishot (5X5 or larger) we typically just add the point manually, for a large grid squares or 3X3 multishot follow the procedure outlined below.

1. On one of the grid square montages **add 5 points**, a center point and 3 diagonal points. Typically, we collect 5X5 array, but a > 7X7 (15X15 is limit) are possible depending on the hole size and mesh. We routinely obtain ~1.8 Å Maps of Apoferritin using large multishot up to ~8 microns.
2. To ensure that each point is in the center of a hole, take an image in View, and move the points to the center of their respective hole using the Navigator "Edit" mode.



3. Once all the points are set, **Setup Multiple Record** in the Navigator → Montaging and Grids → Set Multishot Parameters



4. Return to the center point using **Go to XY**

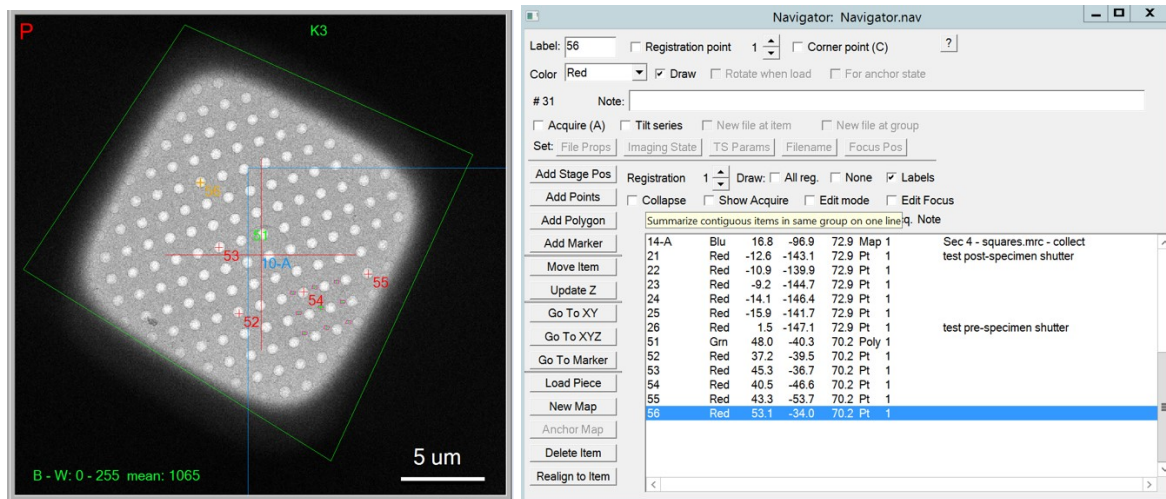
Select **For Corners of Regular pattern** in the Multiple Record Setup window. In the Navigator select the **corner points** (highlighted in blue), then click **"IS XY"**, to image shift to that point. Each image shift should be $\sim 3.5 \mu\text{m}$, this will be reported in the "Microscope Control" panel. Then in the Multiple Record Setup click on **"Save Image Shift"**. Repeat this for the other 3 corner points. The corner points should be ordered CW or CC.

5. **Double check** by mouse clicking on a hole in a search image, it should show a diagram of where each of the record image will be acquired.

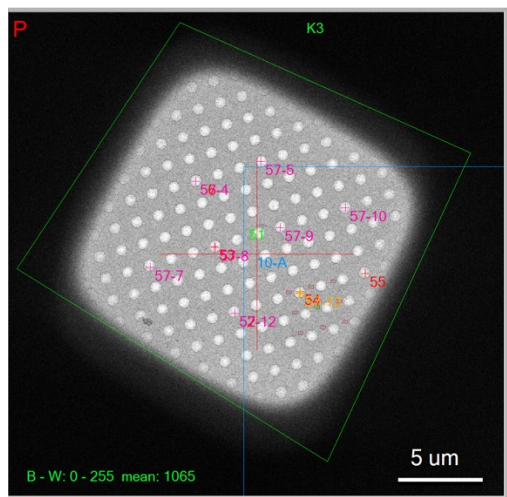
IX. Add Grid of Points

Note: for 5X5 or larger multishots we find it faster just to add the points manually on each grid square. For single shots per stage shift or 3X3 multishots on large <300 mesh TEM grids the Add Grid of Points works best.

1. **Open a grid square montage** in the main window, on the Navigator double click on the map.
2. Add a **polygon** around the grid square, shown in green. To do this on the Navigator click **"Add Polygon"**. On the grid square montage add 5 points, spaced 4 holes apart, as shown in the image, to do this in the Navigator click **"Add Points"**. Add grid of points in the Navigator → Montage → add grid of points.



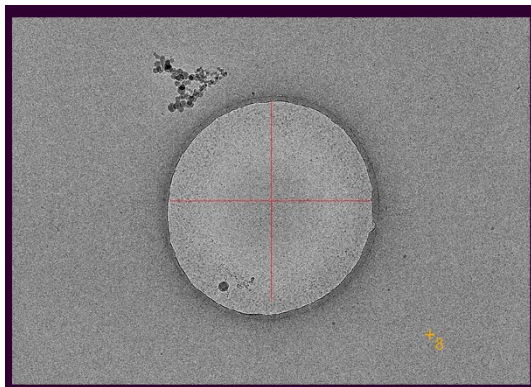
It should look like this



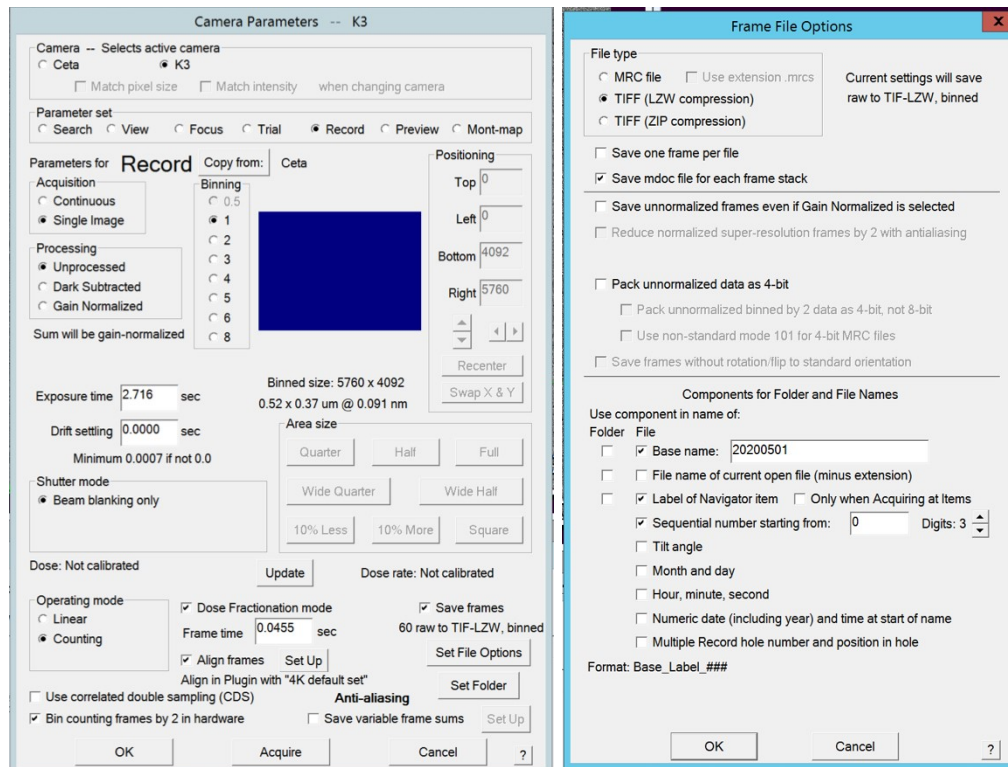
3. To add more point on the other grid square montages, open the grid square montage. Add a polygon around it. Add one point to the center. On the Navigator → montage → add grids like last one. This is add a grid of points within this polygon. Mark these points for data collection by click on the "Aquire (A)" in the Navigator. Make sure to save the Navigator file periodically.

XI. Data Collection

1. Acquire a **View Image of a Hole**, it should be centered.
2. Copy this image to **buffer P**, click on “P” in the Buffer Control panel. The script will use this image to move the stage/image shift to the acquired points over a hole.



3. Check the **Camera Record Parameters**, click on “Setup” in the Camera and Script panel.

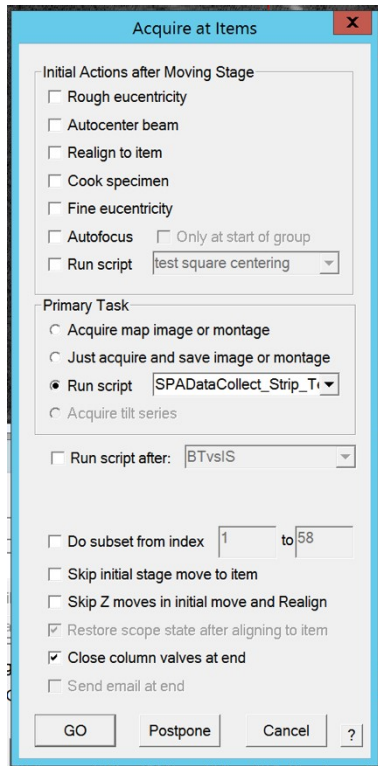


- Check the following options: “Unprocessed”, “Binning 1”, “Beam blanking only”, “Counting”, “Dose Fractional mode”, “Save frames”.
- Click on “Set File Options”, to open the “Frame File Options” panel. Change the “Base name” to the date.
- Uncheck “Align frames”, will speed up data collection, ~25% faster.
- Click “Set Folder” to save the moves to the correct directory. *Note, must save data to the (X):/k3data/ do not save the data to another hard drive.*

Note: to increase data collection speed when we set the IS Delay Factor to 0 either in the data collection script, serialEM menu, SerialEM properties file. Also, you can reduce the

number of frames or exposure time. The fastest we collected was 720 movies/hour and obtained EM Map of Apoferritin of 2.2 Å on a Lacey TEM Grid. Typically, ~500-400 movies an hour is normal for this microscope setup.

4. Check position of focus.
5. On the Navigator → **Acquire points**



6. Check “Run script” select script UpdatedDataCollection_V2.txt. *Note don’t usually need to modify this script, but feel free to look it over.* Then click “Go” to start data collection. If you are unsure which script to use check with Jared Peck or Joshua Strauss, we are constantly writing new scripts.

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XII. Saving data from a screening session

To save images and data (including serialEM log and navigator) follow the following naming conventions. Be sure to save the data on the K3 Server X: drive and not the C: drive!!!

Make directory in /K3data/
20191111_JS_TK/"date" "PI initials" "Users Initials"

For each grid make a new directory to save images and navigator 20191111_JS_TK_TK3_G1/

File naming...save as tif or mrc or jpg
20191111_JS_TK_TK3_G1_Montage.mrc Full grid montage
20191111_JS_TK_TK3_G1_Pt2_Search.mrc Pt2 "navigator point 2"
20191111_JS_TK_TK3_G1_Pt2_View.mrc
20191111_JS_TK_TK3_G1_Pt2_Record.mrc

Make sure to save the montages and Navigator files, and serialEM log file.

If you collect data on a grid, take a screenshot of the Full Montage in SerialEM Window indicating which areas were collected on and save it as a png.

XIII. List of SerialEM Scripts and Settings Files

Most Current Setting File

Settings file follow the naming convention of "Date"_LD_"Mag". For example
20210730_LD_45.txt was made July 30th in low-dose mode at 45,000 X magnification.

Ask the Core if the setting files is current, and make sure to check the low-dose and camera settings after loading a new setting file.

All scripts that are currently used will be in the directory /K3Data/SerialEM_Scripts/Current/

Name of Script

Chainsaw49.txt	SPA data collection one multishot per grid square
ChainsawTilted30.txt	SPA data collection one multishot per tilted grid square
HoleCenterTomo.txt	Hole centering for SPA data collection
MapAlign.txt	Stage align for tilt series of polymorphic object for tilt series collection
Mapgrids.txt	Map cartridges in cassette
Nothing2Fancy.txt	Screening: Collect Search, View and Record at multiple points on fullgrid montage
NothingFancy.txt	Screening: View and Record at multiple points on grid square
RotateMultiShot.txt	Quick way to adjust multishot parameters
SingleMontage.txt	Collect fullgrid montage of one grid

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square centering tilt 45.txt	Center grid square for SPA data collection "UpdatedDataCollection" script
UpdatedDataCollection_V2.txt	New SPA data collection
View_Hole_Center.txt	Hole centering for tilt series collection
SeekandDestroy	Hold finder script (<i>references to Metallica post 1986 are prohibited</i>)
MultishotFilter	Skips over acquire point with less than this threshold

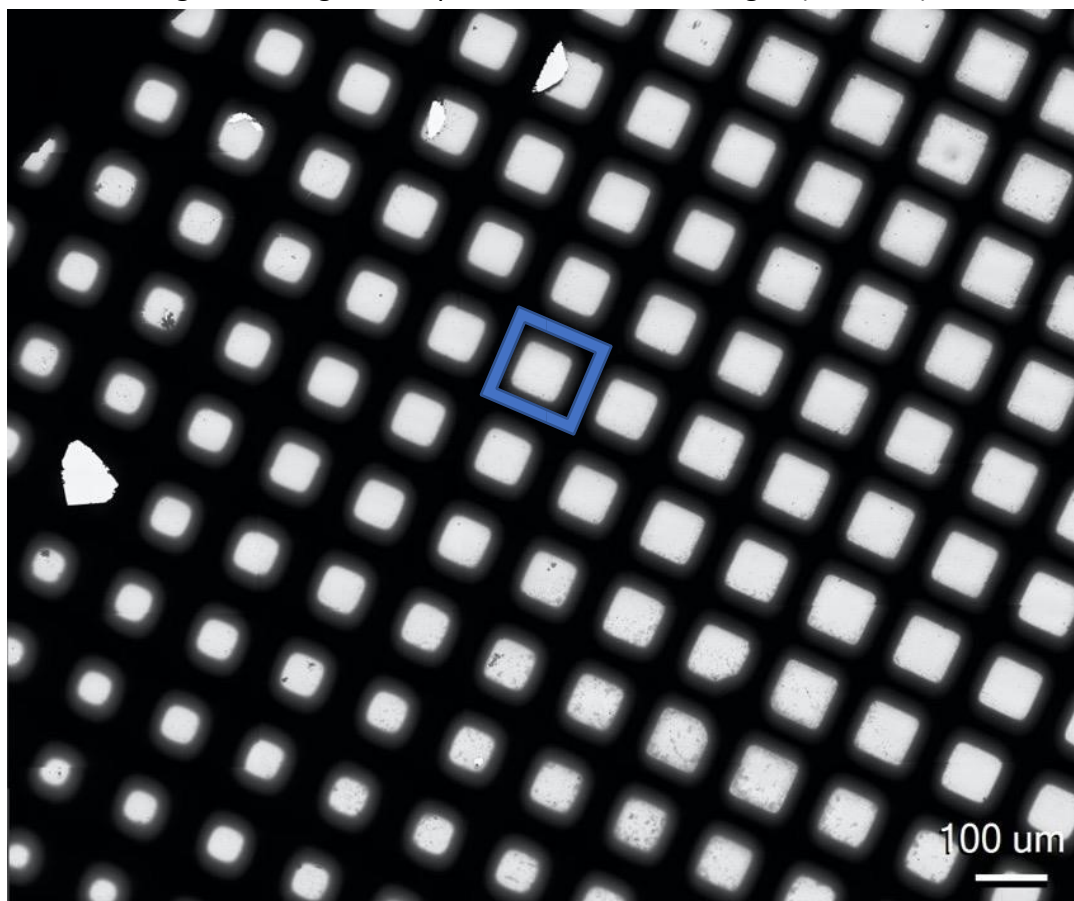
XIV. Tilt Series Setup

Load the SerialEM Settings File marked for tomography ""_tomo_".

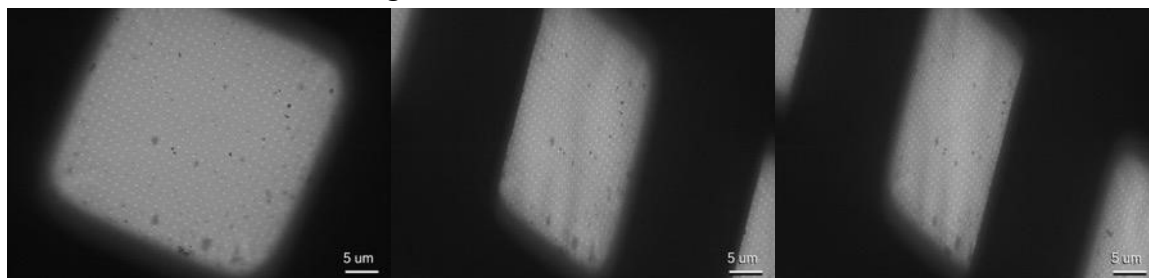
At this point the microscope should be aligned, the Gatan K3 should have a new gain and dark reference, and a area(s) of the grids should be identified that is suitable for collecting tilt series.

Go to a grid square (stay away from the edge of the stage/grid) and check eccentric height (manually or with SerialEM).

Collect a full-grid montage to keep track of the areas imaged (blue box).



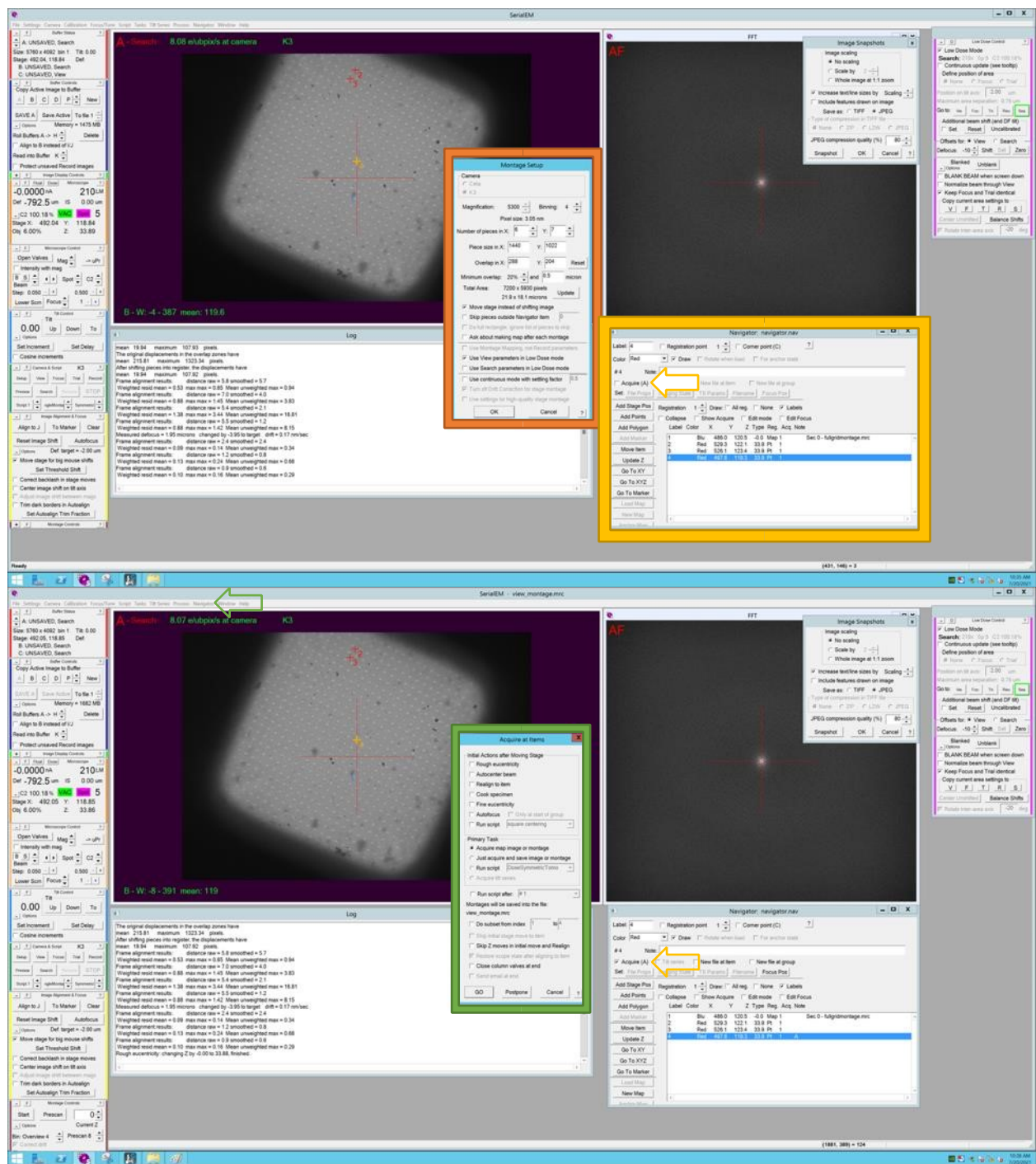
Verify that you can tilt the grid ± 60 degrees (or to the desired tilt range). Set Stage Tilt angle to 60, then take a Search Image, do this for -60 as well. Should look like this.



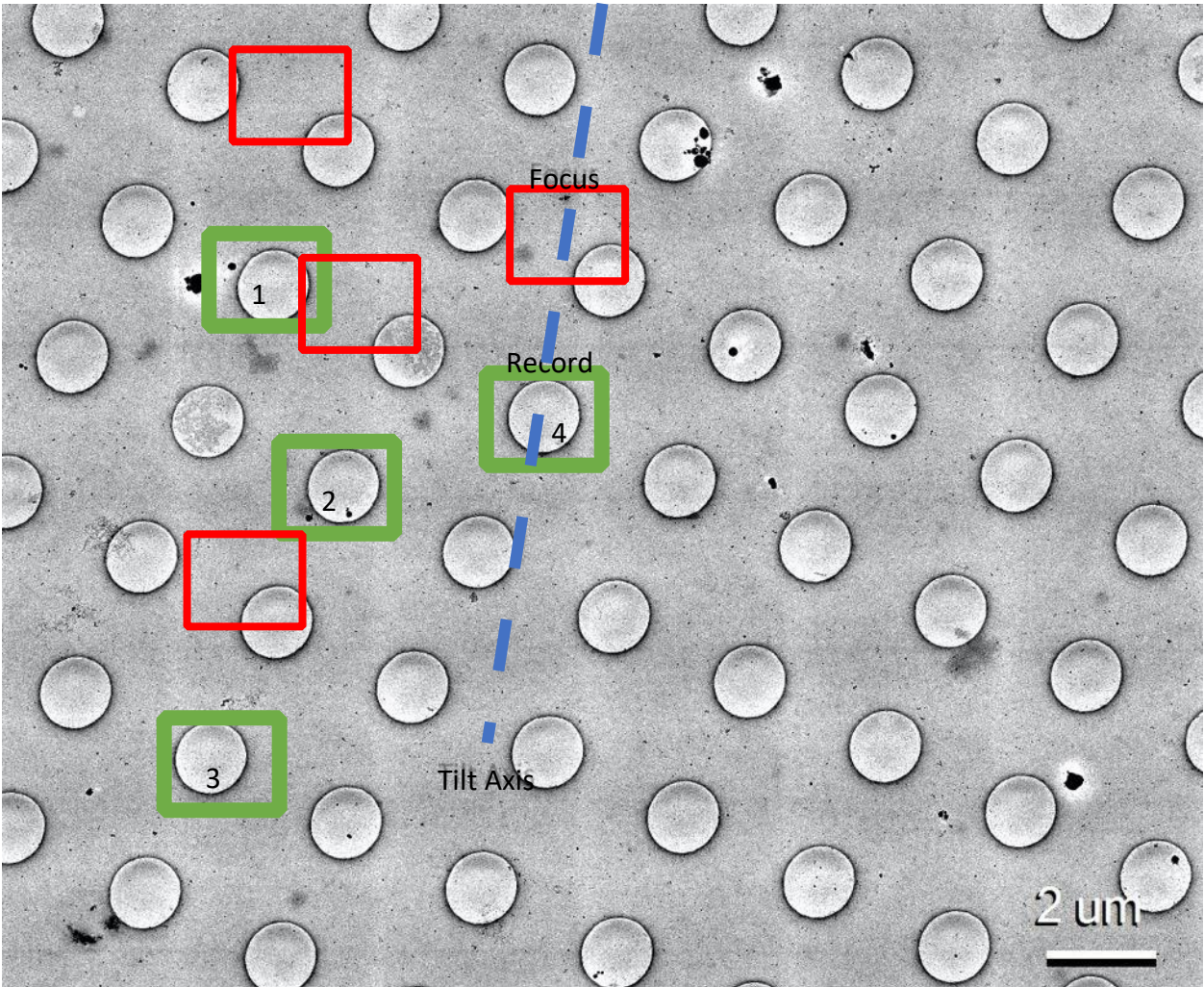
Notice that for the tilted Search images the carbon film is not perfectly flat. Try to avoid collecting over area of the grid that is very bent.

Collect View Montage (by shifting the stage) in the center of this grid square.

1. In the Navigator Window add a point to the center of this grid square (Point #4).
2. Mark it as Acquire (See Yellow Arrow).
3. Setup the montage parameters in File → Montage Setup make sure to check (See Orange Box).
 - a. “Move Stage Instead of shifting image”
 - b. “View Parameters in Low-Dose”
 - c. Minimum Overlap set to 15-25%
 - d. Number of pieces in X and Y depending on the size of grid square or area you wish to image.
4. In the Navigator Tab (Green Arrow) select Acquire at Items.
5. In the Acquire at Items Window (Green Box) check:
 - a. “Acquire Map or Image”
6. Click “Ok” to start the View Montage.



The View Montage should look like this...

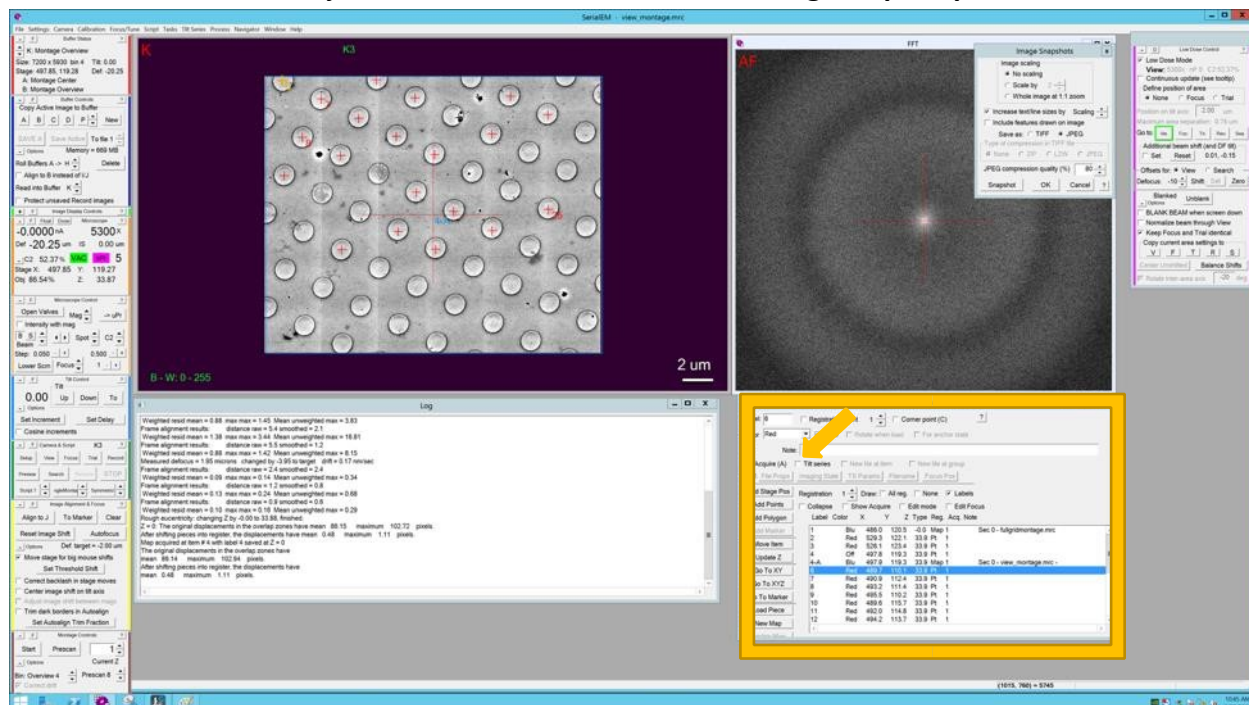


Now add points to the center of the Quantifoil Holes where you like to collect the Tilt Series.

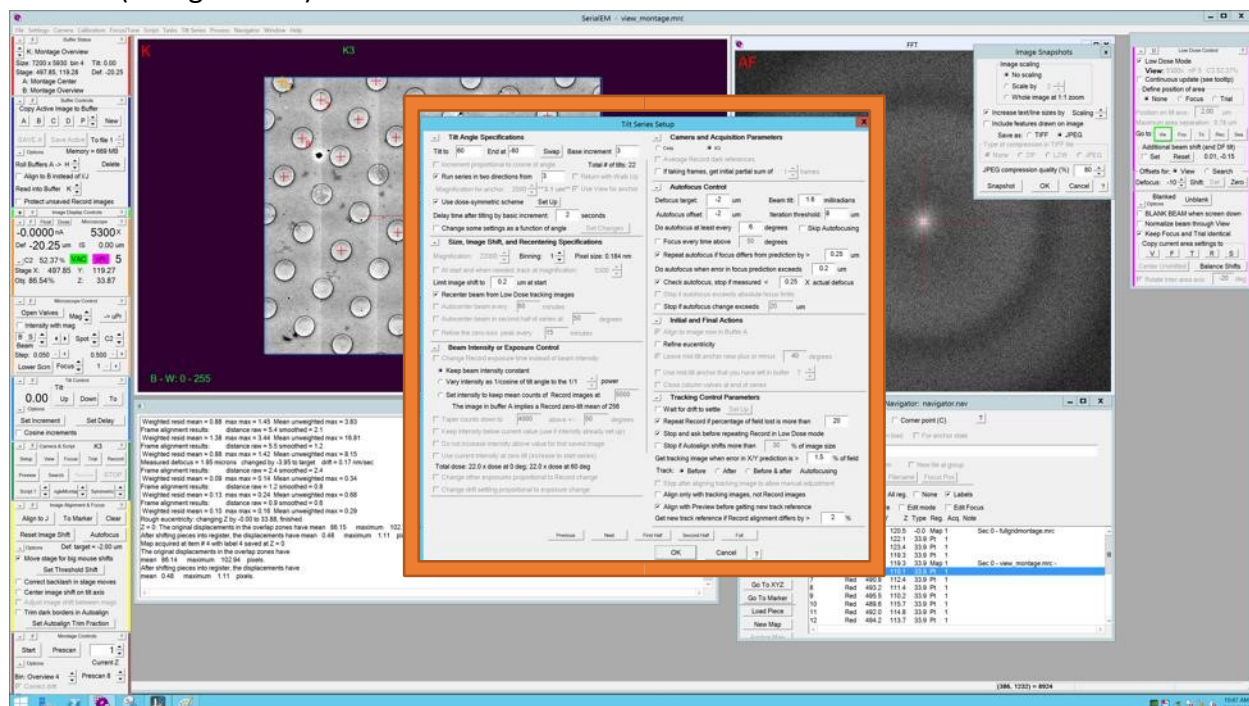
Keep track of the position area of Focus, and the order you add the points, to avoid over exposing the Record area to the Focus beam. Make sure the position area of Focus (red box) is on the tilt axis (set in the Low-Dose Panel as 0 or 180 degrees). Also, the Focus should be on an area of the grid which has enough features (ie. 10 nm gold, or ice, or edge Quantifoil hole) to track and predict the position of Record (by collecting Trial images) during tilt series. Avoid areas that have ice contamination, or cracks in carbon film. As shown above the tilt series will be collected right→to left, starting with 1...2...3. If you are imaging large (viruses, cells, nano-particles) the long axis of the object should be aligned with the tilt axis.

In this protocol, I have added the points to the center of the holes, since it is purified protein, however you don't need to collect over the center of a this if you are imaging cells or a polymorphic object. If you are collecting each tilt series in the center of the Quantifoil hole use

the **tomo-hole-centering** script. If you are collecting each tilt series away from the center of the hole, ie. on a cell, or just off-center of the hole use the **AlignMap** script.



After adding point to the View-Montage, mark the points as “Tilt series” in the Navigator window (orange arrow).



Now edit the Tilt Series Setup window, as shown above. Modify the following:

- **Tilt Angle Specifications**...for this I am using +/- 60 with 3 degree increment with the dose-weighted grouped scheme.
- **Size, Image Shift and Re centering Specifications**...fine as shown above.
- **Beam Intensity or Exposure Control**
 - DO NOT CHECK Change Record exposure time instead of beam intensity!!!!Critical!!!!!!
 - The Dose reported is not accurate in this window...you can estimate total dose of tilt series using this equation below.
- Autofocus Control: set defocus target.....other parameters are fine shown above.
- Initial and final actions...don't need to do anything
- Tracking control options....leave as above.

$$\text{Flux } [(e-/pix/second) / pixels\ size^2(\text{\AA})] * \text{Exposure (seconds)} = \text{Total Dose (e-/}\text{\AA}^2\text{) one image}$$

$$\text{Total Dose (e-/}\text{\AA}^2\text{) one image} * \text{Number of Tilts} = \text{Total Dose Tilt Series (e-/}\text{\AA}^2\text{)}$$

Assuming you are not changing exposure based on tilt angle.....

To measure the Flux take Record over empty hole...the number will be reported in SerialEM window #1 in buffer A.

To find or adjust Exposure in the Camera & Script panel (green) click Setup than click Record.

Typical total dose for tomography 60-200 e-/A².

Quick explanation about dose....this will depend on the object you are imaging as well as the microscope voltage. As different biological samples will have different sensitivities to e-. And the ratio of elastic/inelastic scattered electron will depend on voltage. This is well described in literature for proteins, nucleic acid and bacteria cellular appendages. If you are not sure what dose to use you can run a dose series to find the dose at which the sample bubbles or loses high-resolution features. Also I recommend collect a few tilt series with different total doses.

What I recommend...based on my experience....

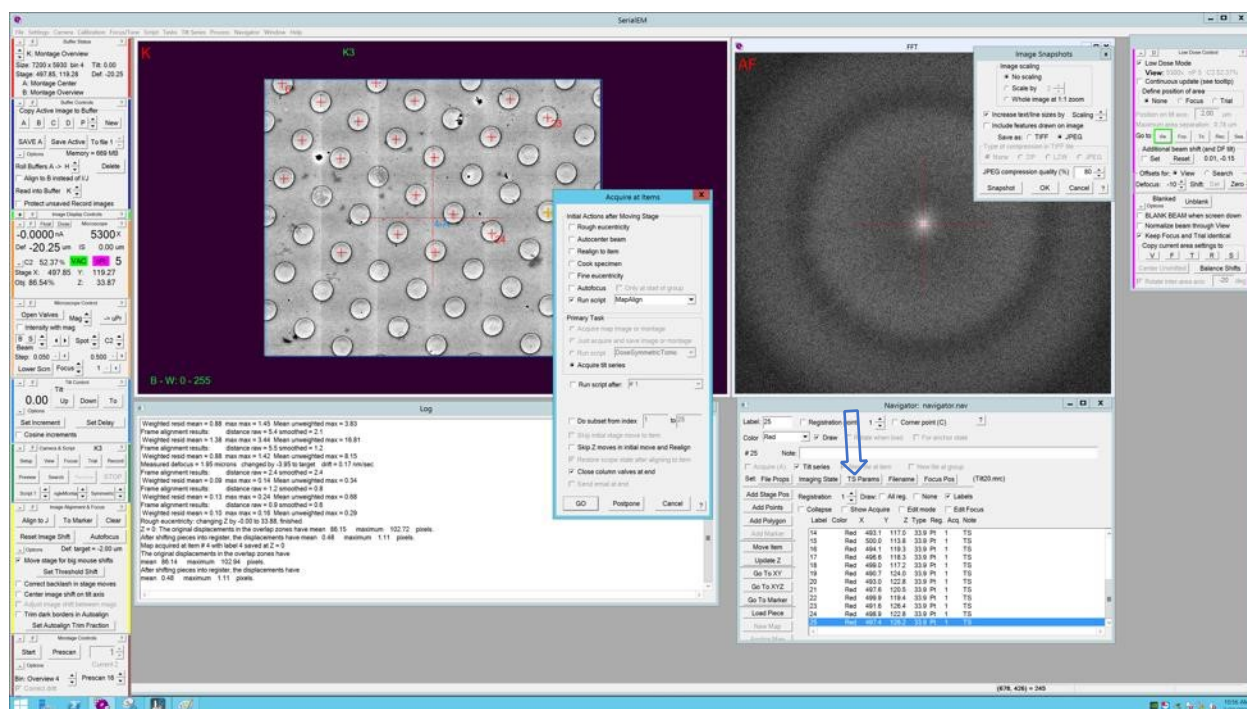
Protein complexes 60-80 e-/Å²

Bacteria 120 – 200 e-/Å² (but exceptions exist ie. *Vibrio V. flagellum* ---more like 60 e-/Å²)

Viruses like HIV 100-130 e-/Å²

Mammalian cells 120-150 e-/Å²

Now in the Navigator mark points as Tilt series, and change parameters like defocus if you wish to use different defocus by clicking on TS Params icon (blue arrow, below) in the Navigator window.

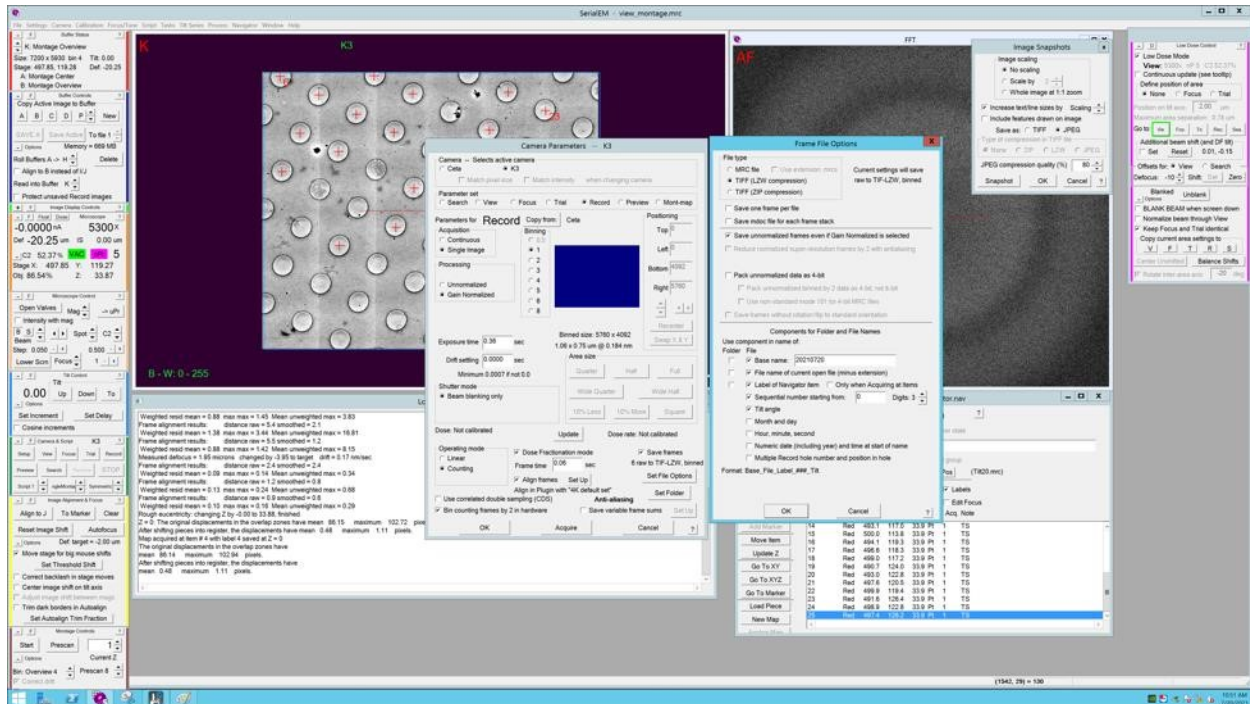


When you are ready to collect the tilt series click on **Navigator tab** → **acquire at items**. For Initial Actions after Moving Stage check Run script **MapAlign** (will work for holes as well as polymorphic objects).

Before setting up tilt series check Gatan K3 Record parameters (Camera & Scripts→ Setup). Check and set the following:

- **Single Image**
- **Gain Normalized** – if you are using mrc stack generated by SerialEM this is critical
- **Exposure time** – adjust this If you like, will depend on samples sensitive to e-, desired contrast in the final tomogram, reason you are collecting tomogram (ie. subvolume averaging, or not). The total dose for a single tomogram will be between 60-200 e-/Å². Can also truncate higher tilt angles in IMOD (ie. collect +/- 60 but only process +/- 45).
- **Counting** – Yes for the K3 always us counting...you can count on that.
- **Dose Fractionation mode**---yes check this
- **Align Frames** --- if you are using the mrc stack generated by serialEM yes check this ...if you are going to motion correct the movies using another program like Warp click no. Better to click yes.
- **Save frames** --- yes if you plan on processing movies...refer to Frame File Options window for recommended parameters.

- **Bin counting frames by 2 in hardware** – yes check this if you are not using SuperResolution. Keep the pixel size in mind as well as desired resolution. I have collected and processed tilt series with Binning 0.5X and 1X both work. On a separate note, I had better results using IMOD CTF plotter using Binning 1X as compared to 0.5X but might be related to background noise subtraction. I recommend collected Binning 1X if you not planning on doing high-resolution subvolume averaging and will correct CTF in IMOD.
- **CDS Mode** – have not tested for tomography—will need to do gain reference in DM



Run Warp on SerialEM Computer...will include more documentation on this...for now refer to screenshot and ask for assistance.

- Check GPU on left
- Refer to parameters in screenshot

DURCHLICHTELEKTRONENMIKROSKOPIEBILD-DATENENTZERRUNGSWERKZEUG 1.0.9

SAVE SETTINGS LOAD SETTINGS

Overview Fourier Space Real Space

EXPORT MICROGRAPH LIST ADJUST PARTICLE DEFOCUS EXPORT PARTICLES IMPORT PARTICLE COORDINATES MATCH TEMPLATE EXPORT BOXNET EXAMPLES

Input

Input: [XYZData\20190720_IDS178_Series1.tif](#)

Pixel X/Y: 0.9200/0.9200 Å 0.9 * 1

Bin: 0.00x (0.9200 Å/px)

Exposure: 2.40 e-/Å²/frame (group)

Preprocessing

☒ Correct gain using: [XYZData\20190720_IDS178_Series1Supernat.tif](#)

☐ Correct defects using: [Select defect map...](#)

☐ Flip X axis ☐ Flip Y axis ☐ Transpose

☒ CTF

Window: 512 px Range: 18.4–3.1 Å ☒ Use Movie Sum

Voltage: 200 kV Cc: 2.70 mm ☐ Phase Shift

Amplitude: 0.07 Defocus: 1.0–4.0 µm

☐ Motion

Consider 36.8–7.4 Å weight with B = -500 Å²

Models

Defocus: 2 x 2 x 1 Motion: 1 x 1 x 0

☐ Pick Particles

Use [BoxNet2Mask_20190918](#)

Expect 150 Å [crys](#) particles; use scores above 0.10

Maintain a minimum distance of 50 Å from

☒ Extract 256 px boxes, 0.9200 Å/px ☒ invert ☒ normalize

☐ Maintain a separate list of the latest 10000 particles

Output

Skip first 0, last 0 frames

☒ Average

☐ Aligned stack, collapse every 0 frames

Processing Status

Astigmatism (use up to 3.0 σ)

Defocus (use 0.50–3.00 µm) — average [CTF]:

Estimated resolution (use better than 15.0 Å)

(use up to 50 %)

STOP PROCESSING

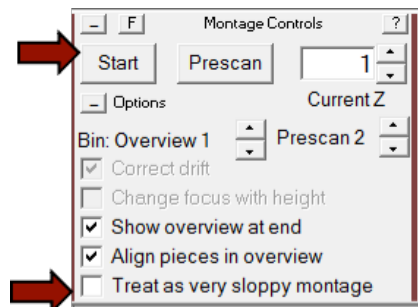
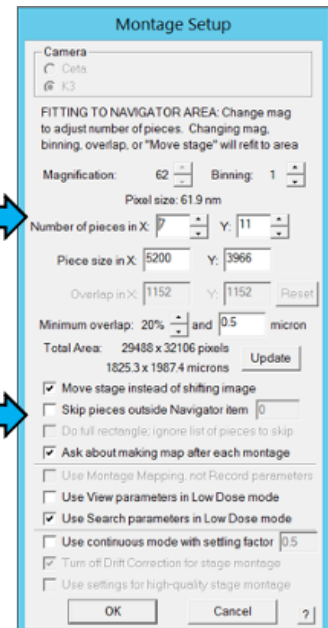
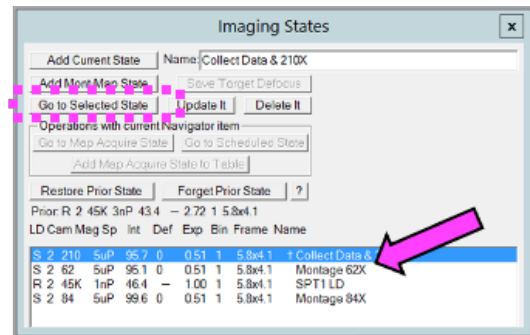
XV. Data Collection using SerialEM Version 4.00 or higher

Check before starting a data collection:

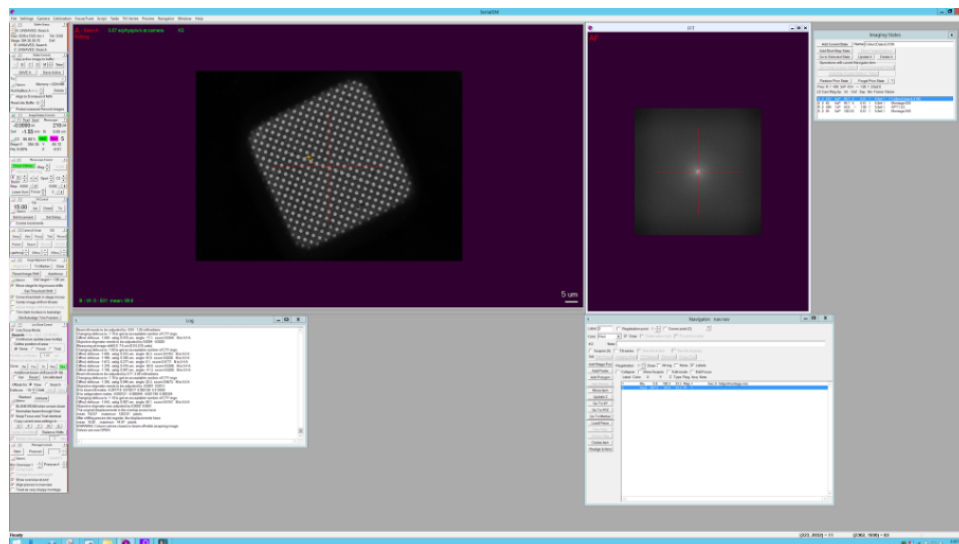
- Check liquid nitrogen tank – make sure there's enough for a session (if it's below 30, change the tank).
- Check hard drive – Raid X in Gatan computer – make sure there's enough space.
- Make sure microscope has been aligned and gain and dark references have been collected.
- Check condenser apertures (should use 70 or 50).
- Double check turbo pump is off.
- Make sure save frames is checked in camera parameters and the files are being saved to the correct directory.
- Before setting up big collection, run cryosparc live and make sure data quality is good.

1. Setup Full grid montage with SerialEM

- On the microscope computer use the Control Pads and Flue Screen to manually set **Eucentric height**.
- Make sure the **C2 150 μ m aperture** is inserted.
- Set **Imaging State to 62X**. SerialEM Tab Window \rightarrow Imaging states \rightarrow select "Montage 62X" \rightarrow click Go To Selected State. As shown with Yellow and Pink Arrow.
- Open a new **Navigator** (SerialEM Tab Navigator (Green Arrow) \rightarrow New). The Navigator is shown with the blue arrow.
- In Navigator Tab (Green Arrow) \rightarrow Setup Full Montage.
- In the **Montage Setup** window set (Blue arrows)
 - Number of pieces in X Y to 7 x 11
 - 15-20% overlap.
 - Move Stage instead of shifting image
 - Use Search parameters in Low Dose Mode
 - Save file as MRC and mdoc (not shown)
- Click **Start** in the Montage Controls panel (Dark Red Arrows).

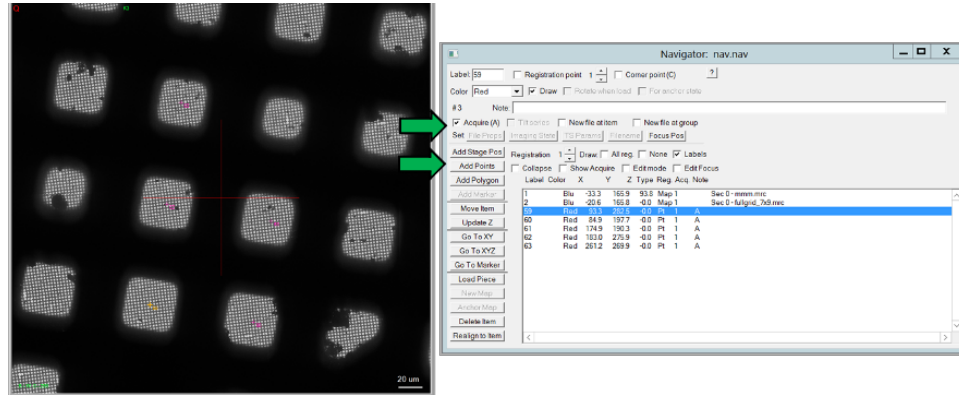


- After you have collected the montage, **close the montage file**, SerialEM Tab File→Close. The name of the montage is no-longer listed in top of the serialEM window.
- At this point the Full Montage should be saved to a Map in the Navigator Window. If the stitching fails try **“Treat as very sloppy montage”**, then double click on the Map in the Navigator to display it.
- **For tilted collection:** Go to tilt control panel (blue) and adjust the tilt to the desired degrees. Then take a search and save to buffer M. Typically we collect -40 degrees.

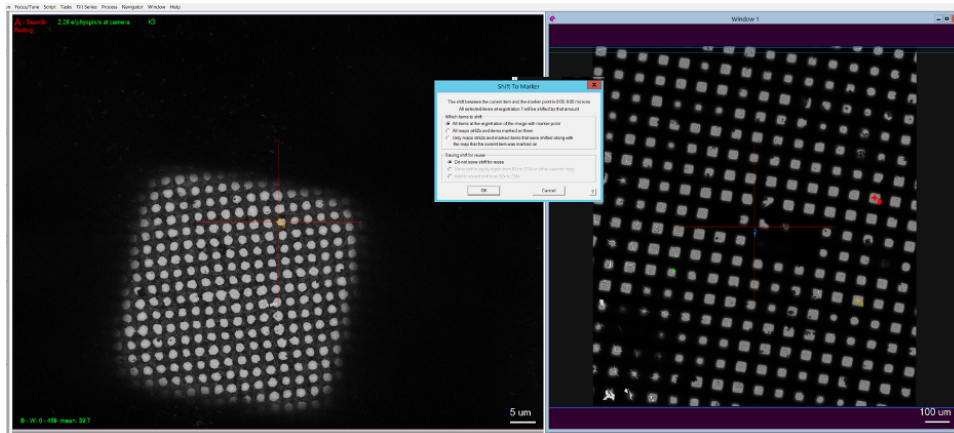


2. Setup Medium Magnification Montages

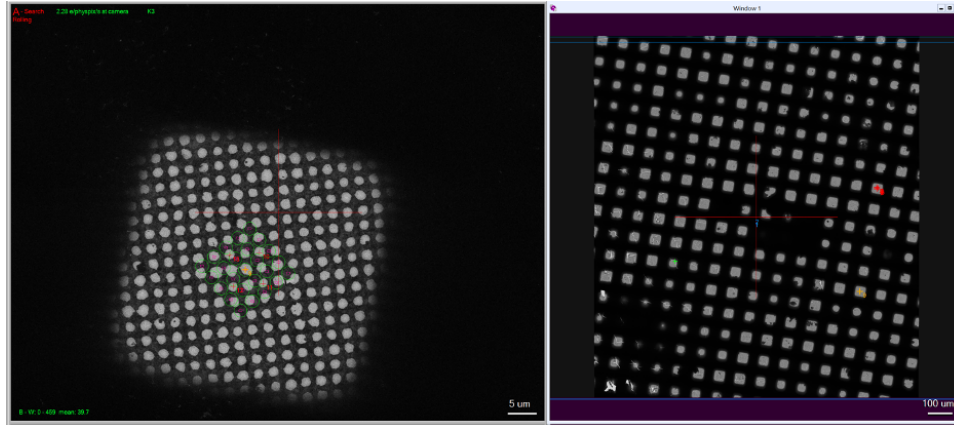
- Go to **“Collect Data 210 X”** imaging state.
- On the microscope computer under the Aperture tab **change the C2 aperture** to 70 or 50 μm (if you are using R0.6/1 grid use the 50 μm condenser aperture. The beam diameter should be ~ 2.3 or $1.6 \mu\text{m}$ in diameter for spot size 3 under parallel illumination ($C2 = \sim 43.3\%$). Check centering of the apertures before collecting data.
- Insert and center the **100 μm Obj Aperture**. This step can be omitted but make sure that the microscope alignments were done with the Obj Aperture retracted. If not, the images will be astigmatic.
- Click in the **Navigator Add Points**.
- Now on the Full Montage and **add points in the center of the squares** using the mouse clicker. Start with the best ones first. Avoid grid squares with cracks and with ice contamination, thick ice, broken carbon, or squares near the edge of the grid... When done Click Adding Points (green arrow). As an example in the image below we added points to 5 squares and marked as Acquired (green arrow).



- Typical collection speed for R1.2/1.3 hole spacing with 7 X 7 multishot BIS is 450 movies per hour at spot size 3, 45,000 X Magnification and 2.716 second exposure. **Estimates how much data you want to collect, and how many grid squares you will collect on** = (number hours to collect * movie/hour) / (number of holes in each grid square), = (12 hours * 450 movies/hour) / (300 holes per grid square) = 18 grid squares.
- On Navigator Go to the XY (green arrow) of the square that you want to start collecting on (as shown as point 9 in the Full Montage Window) this point is not in the center of the of the Grid square at 210X . The 62 X Full montage might be off-center (relative to 210 X image) ~50 microns offset or more is not uncommon.



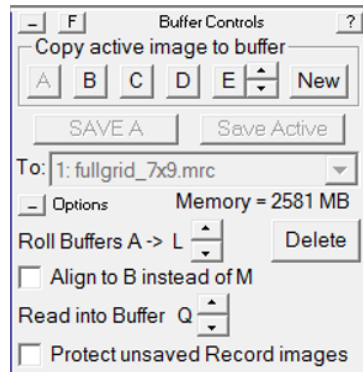
- To correct this offset add a marker (green + using mouse) on the 210X image to the center of the grid square. SerialEM Tab Navigator **Shift to Marker**. Now click Ok as shown in the image. Now the point is in the center of the grid square. The point should be shifted to the center of the square, as shown below.



- If you are using a Quantifoil grid, you can run through the alignments on one of the grid squares, if you are using UF then do the alignments beforehand with the Gradient Replica TEM grid.

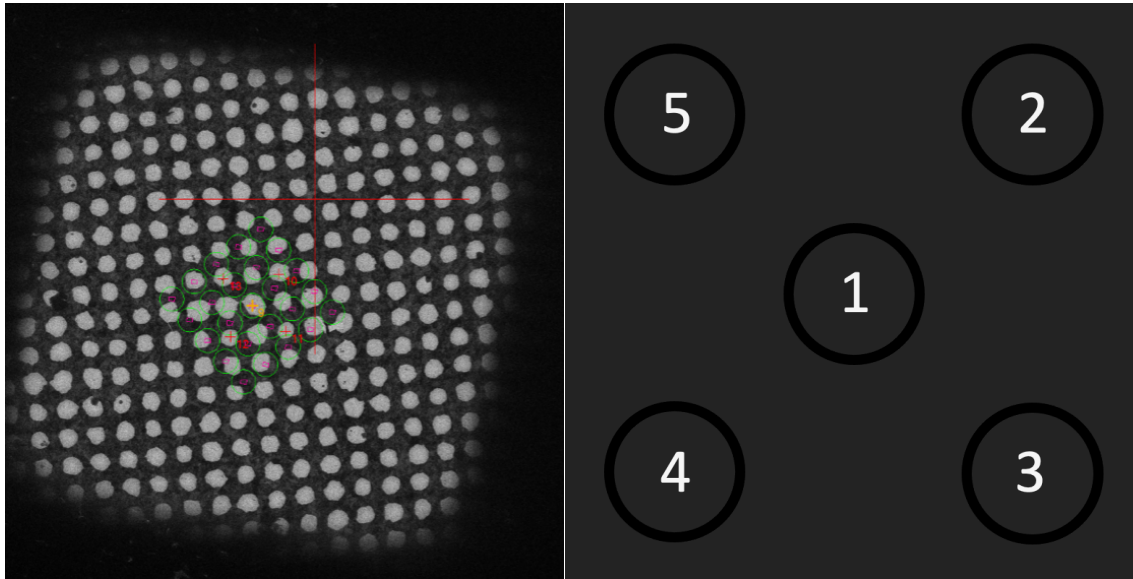
3. Save Images of Hole and Grid Square to Buffer P and M

- **Take View of the Hole and save it to Buffer P.** Click on Copy active image to buffer P, use the up/down arrows in the Controls pane down to select BufferP. This image will be used in the Data Collection scrip to move the stage or IS over each hole. Make sure the contrast is good and the image does not have a lot of ice contamination. *You can use a old image of a different grid if you like, assuming the hole size and magnification are the same.*
- **Take an Search and center it with the mouse, then copy it to Buffer M.** *The image of the grid square in Buffer M will be used in a script to center the stage over each grid square before collecting a map.*



4. Setup Multishot Record

- On a Search Image add points in the center of five holes, starting with a center point (1) and then four diagonal corners (2 to 5, added Clock-wise), like the five on a 6 sided dice.



- In the **Navigator Window**, select the center point and then select **Go to XY**, take a **View**, make sure it is centered. If not use Shift to Marker to move all the points. Also, check Offsets for Search and View in the Low-Dose Panel. Sometimes if this is off the multishot collection will collect image of the carbon/gold film for a R06/1 grid.
- Go to Navigator tab ☐ Montage and grids → Set Multishot Record
- On the **Multiple Record Setup** Window
 - Check Record in multiple holes
 - Make it a Regular pattern of holes 3 by 3, it is easier this way
 - Check Adjust beam tilt & astig to compensate for image shift
 - Diameter of circles to draw adjust set to the current beam diameter or just leave it as it is
- On the Navigator Window, Go to XY of first point, take a View, make sure it is centered, if not center using the mouse to move the stage over that hole.
- On the **Multiple Record Setup** Window
 - Then click for corners of regular pattern, this will start the procedure of setting up the Multishot, if you make an error click Abort to end and start over.

Multiple Record Setup

☐ Do multiple Records within each hole

☒ Do Records in multiple holes

Multiplier of usual delay after shifting to hole: 0.1

Regular pattern of holes: 3 by 3

Spacing: 1.31 and 1.29 um Angle to stage X: -261.1

☐ Omit corners of 3 by 3 array (cross pattern)

☐ Use custom pattern (NONE DEFINED)

Save image shift values at holes

For Corners of Regular Pattern For Custom Pattern

IS to Nav Pt Save Image Shift End Pattern Abort

Image shift should be measured near focus

☐ Save Record image

Early Returns for K2/K3

☒ Full sum returned on all shots

☐ Early return only on last shot

☐ Early return on all shots

☐ Early return, except for full sum on first shot

Number of frames in return (-1 for all): 0

Extra delay after image shift: 0 sec

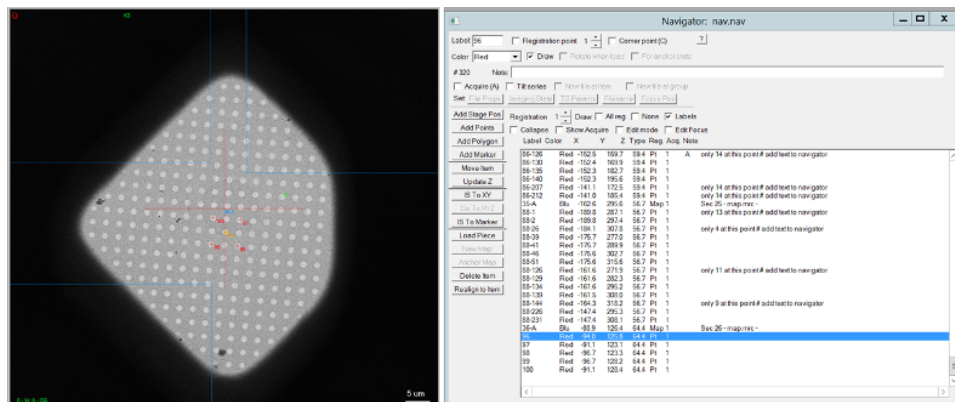
☒ Adjust beam tilt & astig to compensate for image shift

Coma versus image shift was calibrated with:
43.39% C2, spot 3, nanoprobe

Diameter of circles to draw: 2.2 um

OK Cancel ?

- On the **Navigator Window** (see the example image below)

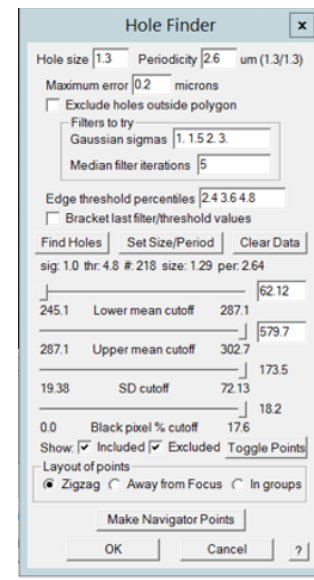
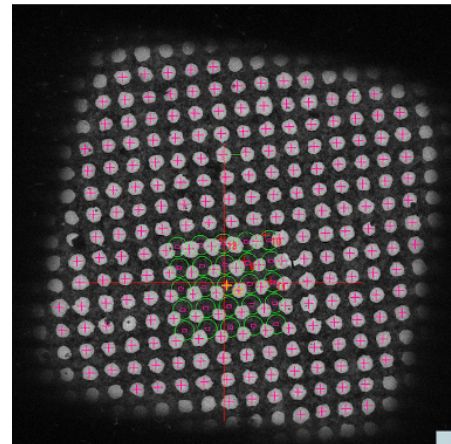


- On Select the next point, **click IS to XY**
- Take a **View Image** center it using the mouse.
- Keep track of the IS distance it should be the same for each of the holes, *unless you are tilting the grid.*
- On the Multiple Record Setup Window
 - Click **Save Image Shift**
- Repeat this for the other 3 holes in the multishot array**
- Once this is done on the Multishot Setup
 - Change Regular patter of holes to 5 x 5 or **7 x 7**
 - Make sure the Comma Vs IS calibration is within this range

- **Verify that the Multishot Parameters are accurate** by taking a View, and with the mouse click on the image to see the pattern of the multishot displayed on the screen. Or take a View montage.
- In the Low-Dose Panel check **Define position area for Focus**. Make sure that the autofocus circle is between your green selected circles. Go to Focus conditions. Verify on flu screen – make sure beam is centered and not hitting any quantifoil holes. If off center, on the Low-Dose Panel, next to additional beam shifts check set, and then use multifunction x y knobs to center it. When it's centered uncheck set. *This step is critical, you don't want the Focus / Trial / Autofocus to expose Record areas.*

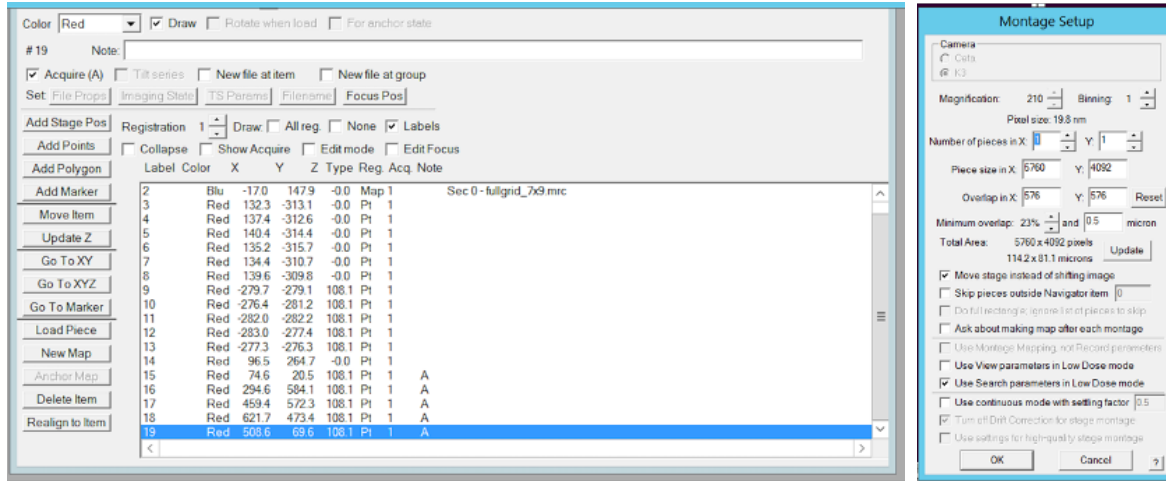
5. Setup and Test Hole Finder

- Take a **Search image** of a grid square
- Then go to Navigator Tab→Hole Finder
- **Test the Hole Finder**, click Find Holes, it should find all the holes in the grid square as Pink +s. If this works proceed, see example image all holes have a pink + and none are added over the grid bar. *Default is usually good but sometimes is helpful to adjust.*
- If this the Hole Finder fails click clear data in Hole Finder and check parameters.
 - Bracket last filter/threshold values.
 - *Make sure that hole size, period, max error, filters, medium iterations are correct.*
 - *Check hole size and period, if not working then adjust slide bars, if that doesn't work change max error and filter parameters...*
 - *Also, you can measure the hole size and period by holding shift and using the mouse to draw a line on the serialEM window, the distance is printed in the log window.*
 - *Try adjusting the sliders*
 - *Try changing Filters*
 - *If the Hole finder fails you can omit it from the Medium Mag Montage Collection by not running the "seek and destroy" script. If you do this you will need to add points manually.*

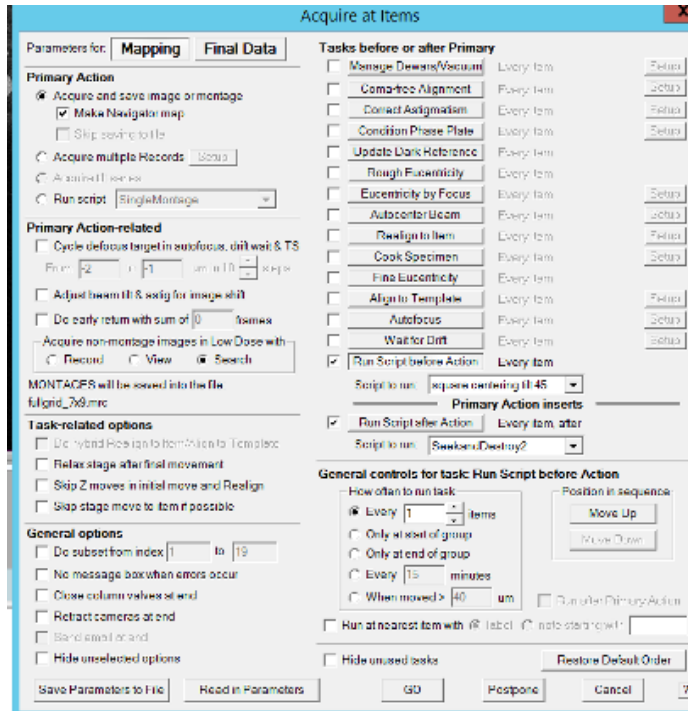


6. Setup Medium Mag Montages

- After marking all the points you want to collect on a grid mark each as Acquire (A) in the Navigator window.



- Setup the **Medium Mag Montage**, on SerialEM Tab File → Montage Setup → click okay
 - Should be Magnification 210X
 - Use Search Parameters in Low Dose
 - Name the file it MMM or something similar
- Navigator→Acquire at items→**Mapping Tab**
 - Check Primary Action→ **Make Navigator Map**.
 - Check Run Script Before Action – “**square centering tilt 45**”. You can modify this script to tilt the grid. This will center the stage and run Rough Eucentric.



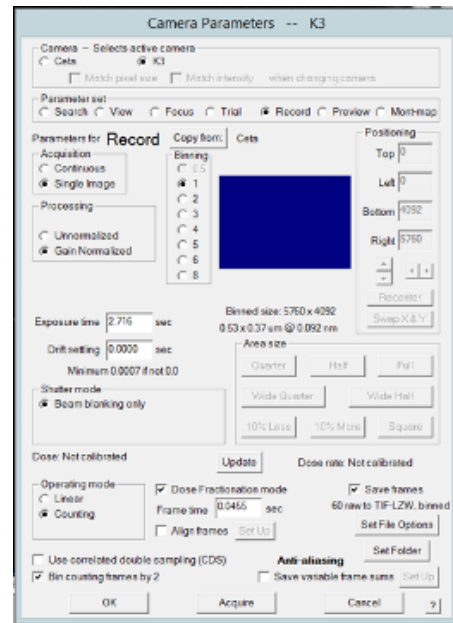
- Check run Script After Action → “**SeekandDestroy2**” this will run Hole Finder and combine points.
- Click “Go” to collect Montages.
- **Check for errors.** In the Navigator that Montages Click on each of the maps or scroll over acquire points to do this, delete false positives.
 - **For tilted collection:** use script 9 (titled script) and make sure that you have first edited the script to reflect your desired tilt angle.

7. Collect Data using BIS Method

- Check Camera Setup (Green Panel)→ Parameter set Record.

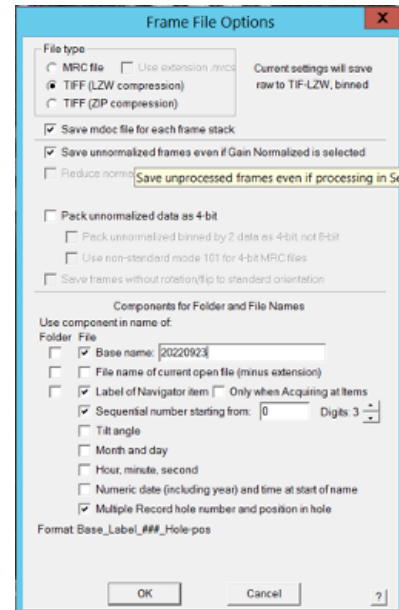
Parameter for Record:

- Single Image (always)
- Gain Normalized (does not matter if this is checked as long as **Set File Options** is done)
- Exposure time (normally 2.7316 seconds for 45K)
- Counting (always)
- Dose Fractional mode (always)
- Uncheck Align frames (to make it faster)
- Save frames (always)
- Check Bin counting frames by 2 (normally yes)
- We don’t normally use CDS mode, or Binning 0.5 X, however both will work.

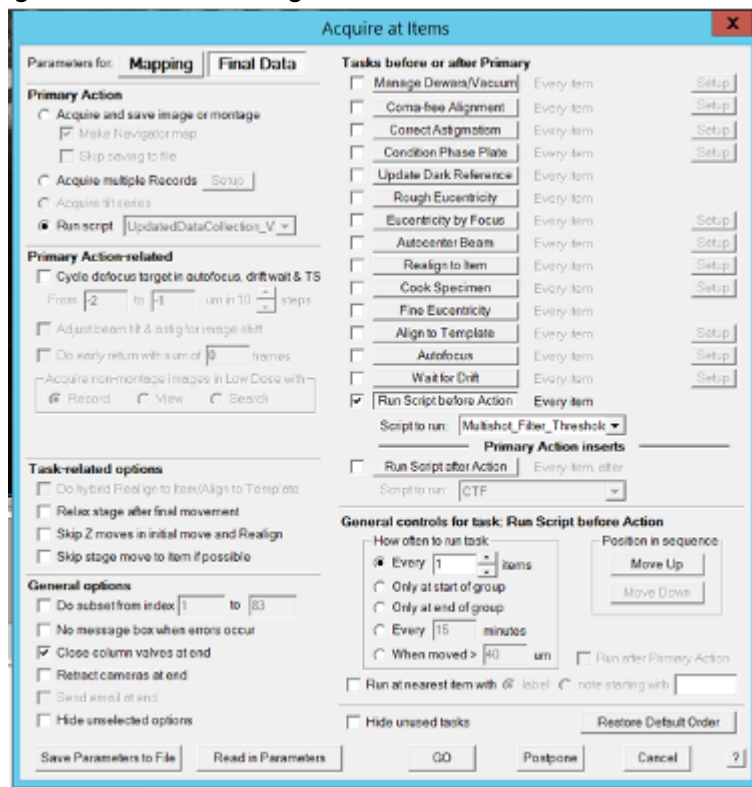


Frame File Options:

- **TIFF LZW compression** – other formats will work but this is preferred format.
- Save **mdoc file** for each frame stack – yes but not necessary.
- Save **unnormalized frames** even if Gain Normalized is selected – yes always check this.
- **Base name** you should name date.
- **Label of Navigator** item – yes.
- **Sequential number** starting from 0 – yes.
- Multiple Record hole number and position in hole – yes. helpful for making subsets and correcting for beam-tilt.
- To start data collectionin the SerialEM tab Tasks → Acquire at Items→**Final Data tab**
 - **Primary action Run Script for data collection**, we are using V3 at this time.
 - **Close column valves** at end



- **Run Script before Action** “Multishot_Filter_Threshold” you will need to modify this script to adjust the filter, will skip points with less than # number of multishots. Useful for avoiding thick ice and making data collection faster.



- Click “Go” to start data collection
- Before running data collection double check the data collection scripts modify if necessary

Data Collection Script check the parameters highlighted in yellow

MacroName UpdatedDataCollection_V3

SPA macro for Krios/Quantum/K2

Buffer P is expected to have reference hole image from Low Dose View

Wim Hagen, EMBL Heidelberg 20170309

Credits to Chen Xu & Henning Stahlberg group

Joshua Strauss modified to include black strip test

Joshua Strauss modified 20200824 hole center and defocus check

Joshua Strauss modified 201024 will save LastView for troubleshooting

Jared Peck modified to add SaveLog

JF mod touch on/off toggle in settings and colval on

JVP 36K has defocus offset of +0.5

JDS Black Strip View False + adjust threshold 980

JVP modified time reporting and trimmed out some delays, needs testing

JDS removed WH IS for Multishot

SETTINGS

#####

maxholeshift = 100 # max beam/image shift after hole centering in nanometers

driftcrit = 2.0 # A/sec

driftinterval = 10 # seconds of delay between drift measurements

drifttimes = 7 # max no of drift measurements

```
TD_low      = 0.25 # low defocus microns
TD_high     = -0.75 #high defocus microns
delta       = 0.25 # defocus step microns 0.1
```

```
touch       = 1 #set to 0 for off
bmsft       = 0.1
```

Multishot Filter Script check the parameters highlighted in yellow

MacroName Multishot_Filter_Threshold

Writen JDS on 4/10/22

Run this script before action when using Data_Collection_V3

change "numMultishot" before running, will skip points with have less Multishot holes than this

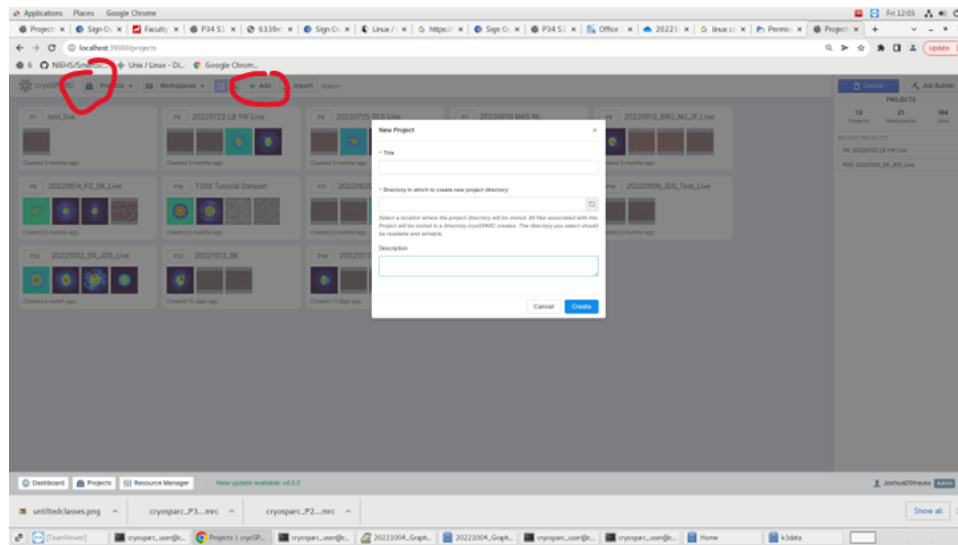
Settings

numMultishot = 15 # Threshold for aqiring multishot, less than this the item will not be aquired

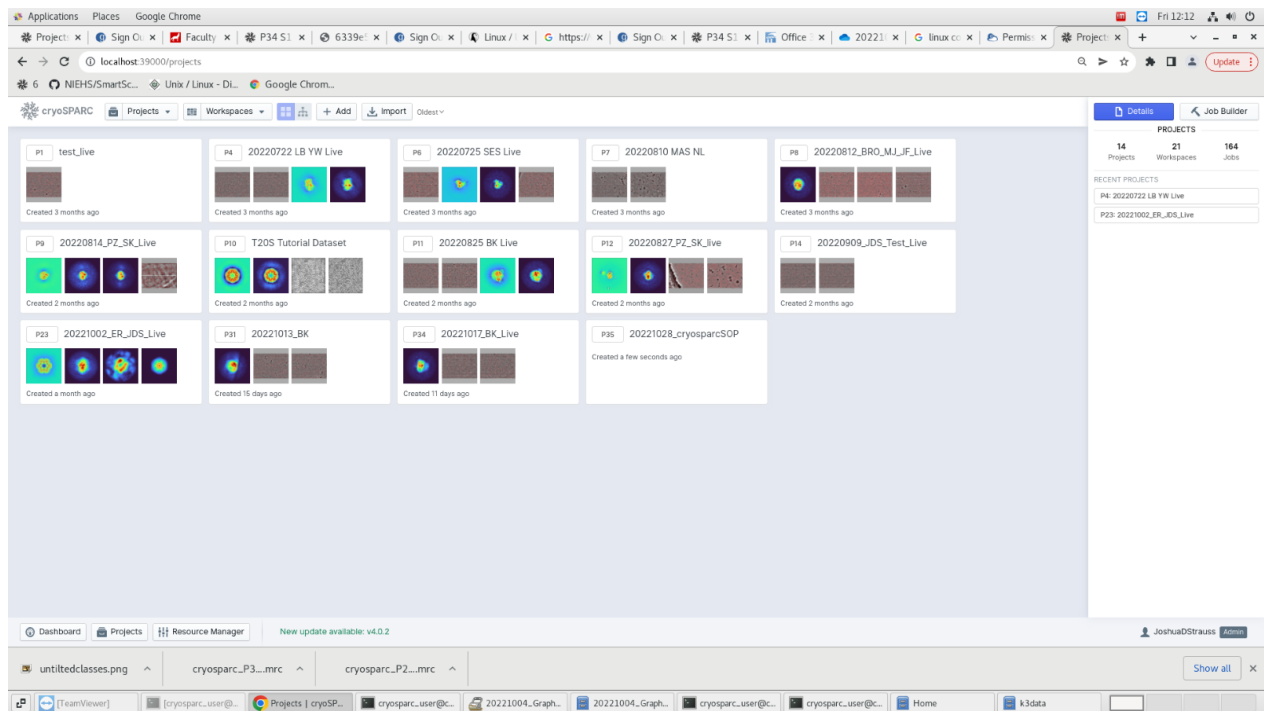
#####

XVI. Running CryoSPARC Live

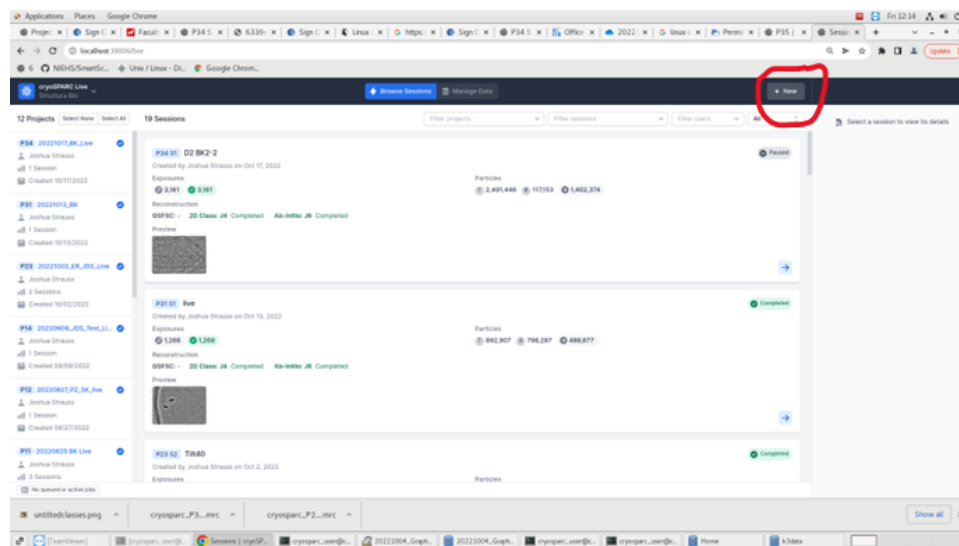
- Open Chrome web browser and type in localhost:39000 to open cryosparc
- Click the file drawer icon next to “projects” at the top left
- Click “+ Add” to start a new project



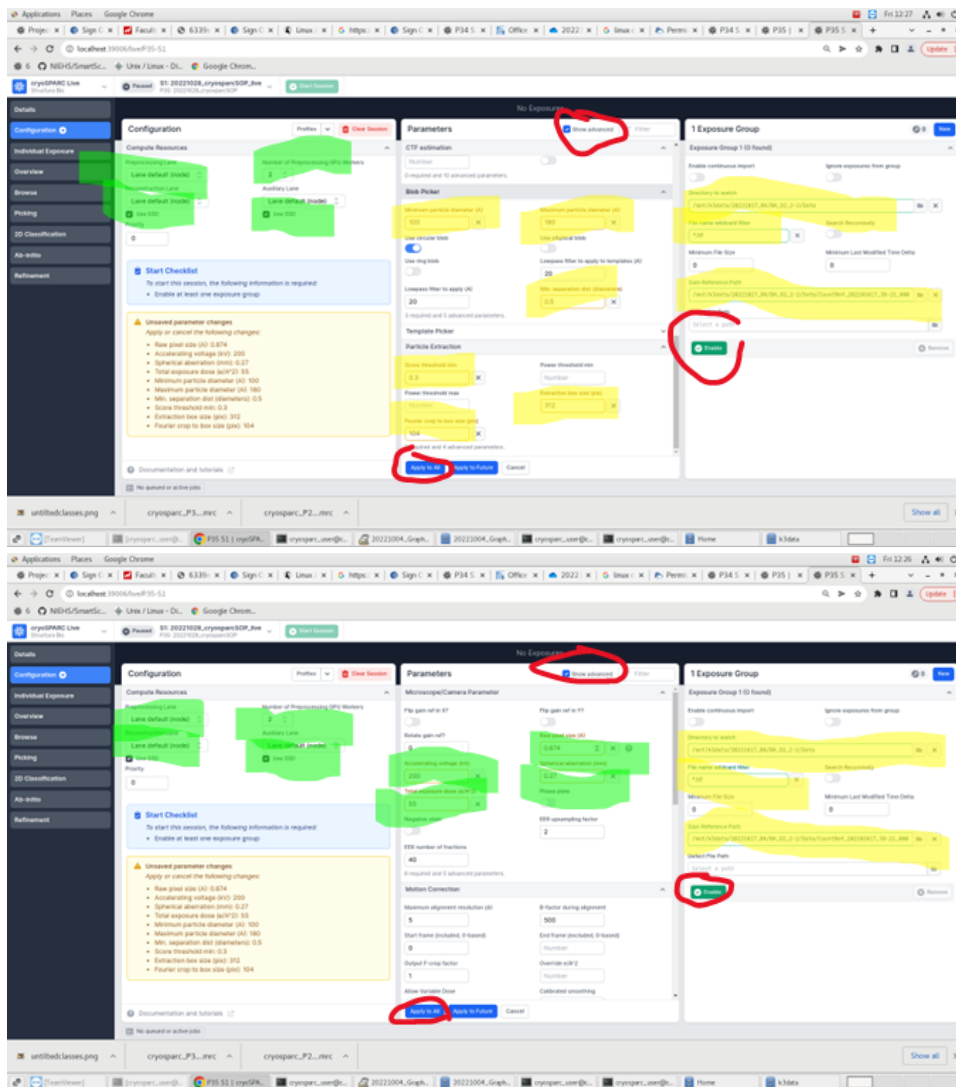
- You will be prompted to add a title and directory
- In the command terminal, go to the data directory and make a directory titled DATE_INITIALS_cryosparc
- Then enter DATE_INITIALS as title, and the directory you just created as the directory in which to create new project directory
- Your new project will show up with the others below - click on the number in the corner if you want to open it



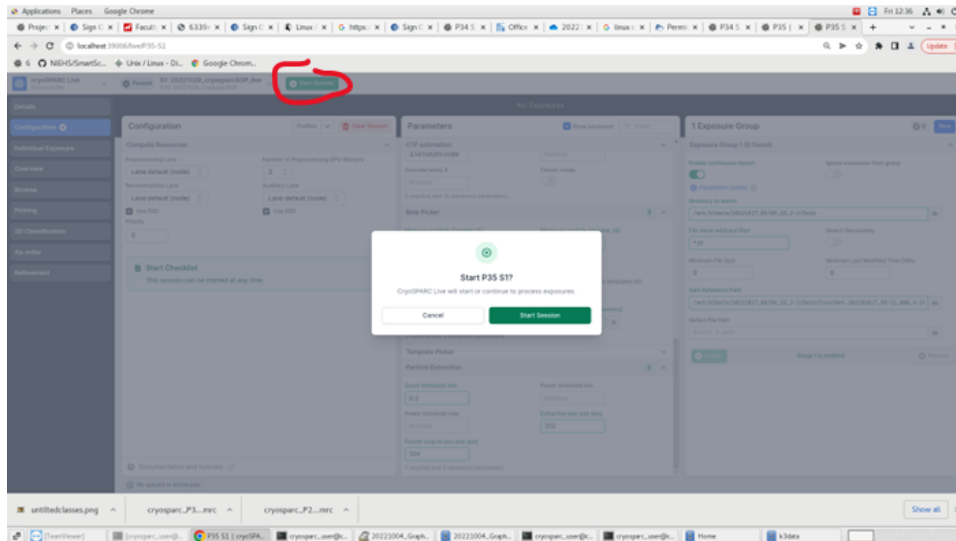
- In a new tab, type in localhost:39006 to go to cryosparc live
- In the top left, click “+ New”



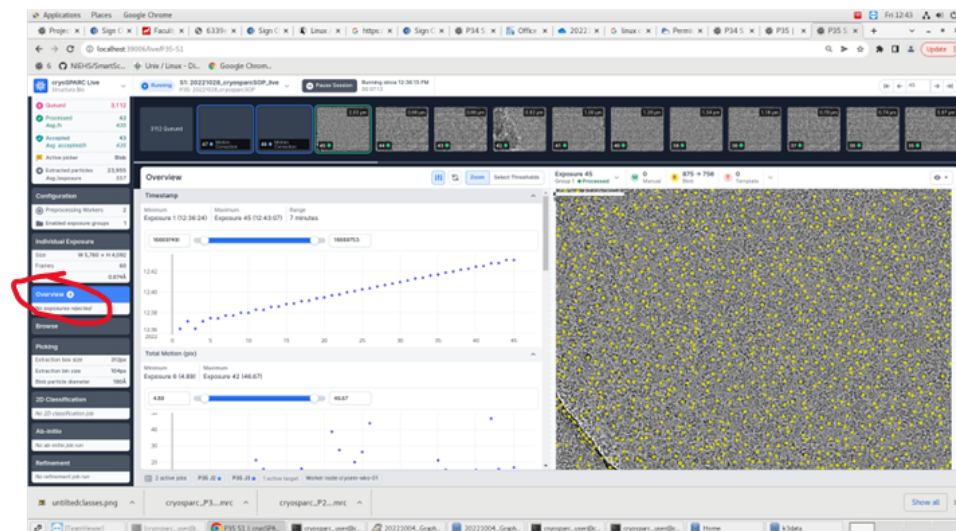
- You will be prompted to enter a project and title – enter the project you just created in crysparc, and title it DATE_INITIALS_live
- The configuration tab will open – click “show advanced” next to “parameters” at the top of the middle box, then input information in the text boxes highlighted below



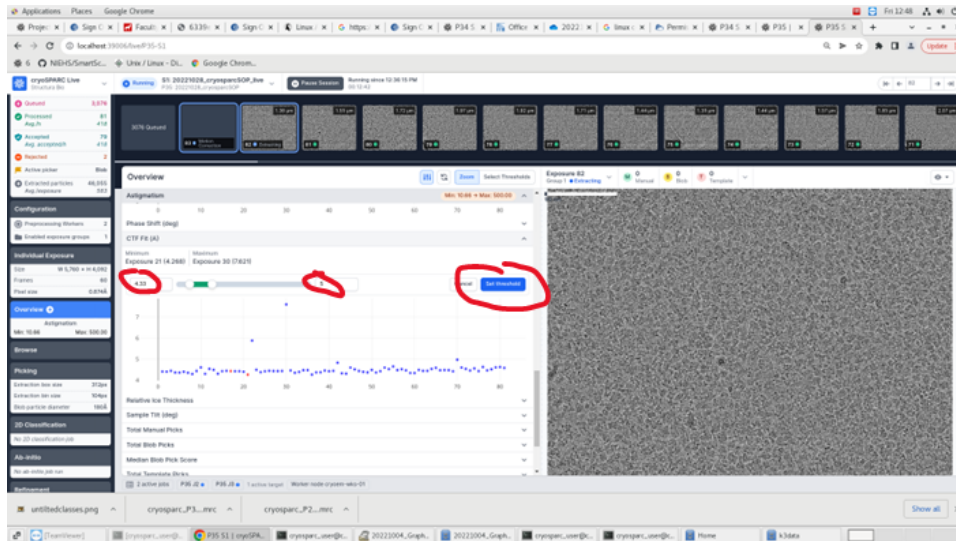
- The text boxes in green will be the same as the number on the screen except in unusual circumstances, the text boxes in yellow need to be tailored to correspond to your sample
- For the “1 exposure group” rectangle (right) - the directory to watch should be /mnt/k3data/DATE_INITIALS/data (or where ever you created the data folder when you set up your data collection) and the gain reference path should be the file in that folder that says “CountRef”
- The extraction box size should be ~2x the size of the particle, and the Fourier crop to box size should be about ~1/3 of that size
- Click “Apply to all” at the bottom of the “Parameters” rectangle, and “Enable” at the bottom of the “1 exposure group” rectangle when all the information is entered
- Click “start session” at the top middle, then confirm “start session” in the popup window



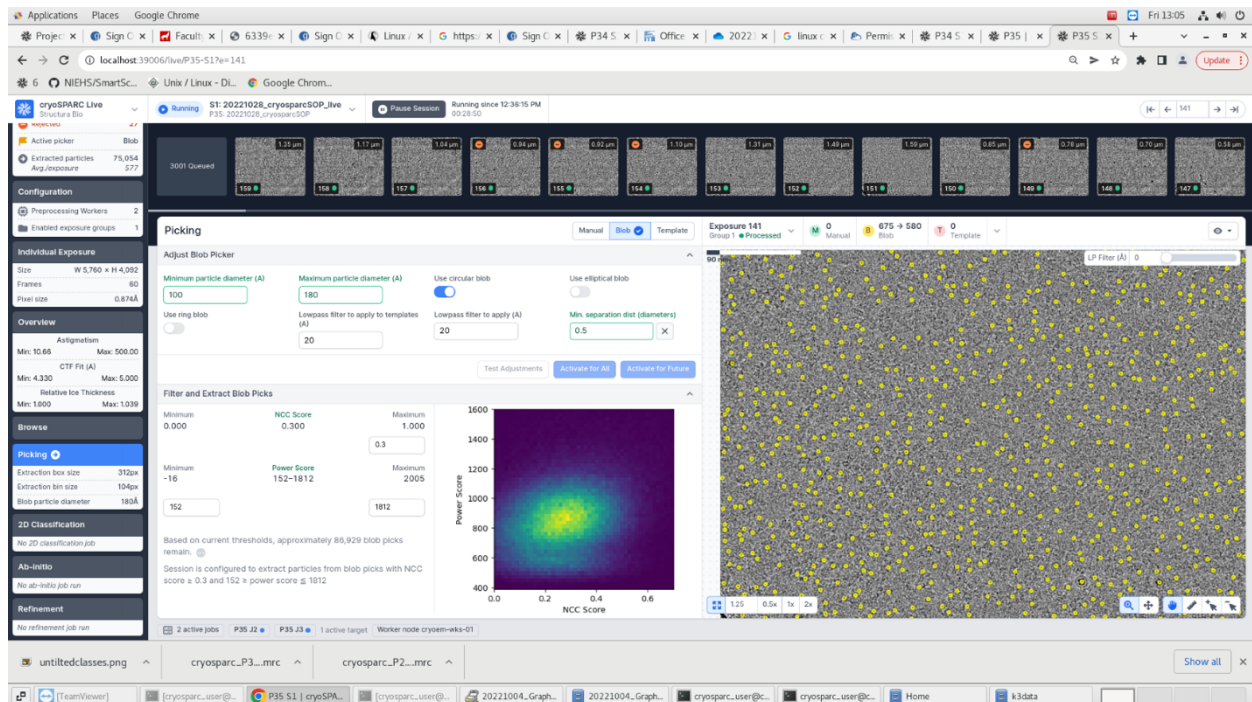
- Click the “overview” tab on the left side



- Scroll down to see all the parameters that you can set min/maxes for
- Set min/max for Astigmatism (~10 – 500), CTF Fit (~0.43 - 5) and Relative Ice Thickness (~0.989 - 1.039) by entering the numbers in on either side of the slider bar, and then clicking “set threshold.” Tailor as needed for your sample



- Click the “Picking” tab on the left side
- Adjust the NCC score and Power score until it seems like there are yellow dots on all of the particles in the image to the right
- When you are satisfied with the parameters you’ve set, click “confirm thresholds” and “extract for all”



- Click on the “2D Classification” tab on the left and change the number of classes to 40
- Click “start”
- Review 2D classes – look for clear feature.

XVII. Running a Cryo Cycle

Check before/after running a cycle:

- Specimen is not in autoloader or stage
- Make sure there's nitrogen to bring to liquid nitrogen temp after the cycle
- Make sure vacuum is in normal range (in the green)
- After cryocycle go to all vacuum
- After cryocycle make sure to switch autoloader to turbo pump always off

To start cycle

1. Open Nitrogen NST Temperature Controller, click "all room temperature" (top)
2. On microscope computer, go to "autoloader" tab --> vacuum options to the right --> click "turbo always on"
3. Open "Talos Vacuum Overview" --> click "condition column"

Then anneal the Gatan K3 camera

4. In digital micrograph, right click on k3 and select retract camera
5. Under camera tab, go to temp, then hit yes to changing temp, icon will come up and change temp to 50 degrees, duration to 12 hours, and nothing for delay.

To finish cycle

- Go to All Vacuum
- Look at autoloader tab on microscope computer --> check "column" under "vacuum supervisor" --> should be in single digits
- Open Nitrogen NST Temperature Controller --> turn temperature to "all nitrogen"
- On microscope computer, under "autoloader" --> click "turbo auto off"