Workflow of Low dose SerialEM setup for Talos Arctica

- **Direct alignment using cross grating grid:**
  - Eucentric height/focus, C2, zero defocus, ppX/Y, rotation center
  - Parallel beam (under collection Mag and spot size)
  - CTF-Stig, CTF-comaFree (under SerialEM)

- **Load sample grid to be collected:**
  - Eucentric height
  - LMM at 46x with C2=150
  - Set Mag for MMM
  - Align features between MMM Mag and LMM map using "shift to marker"
  - Choose squares to be mapped for MMM
  - Run MMM with "rough_eucentricity"

- **Turn on Low Dose Mode under SerialEM**
  - Set up Record and View Mags and illumination
  - Set "offset for view"
  - Align features between View Mag and MMM maps (shift to marker)
  - Define focus/trial area
  - Define hole template
  - Setup Multi-shots, and calibration "Coma vs. ImageShift"
  - Run a quick test with multi-shots matrix
  - Select a few points for acquisition (polygon)
  - Setup data collection parameters (exposure time, dose, etc.)
  - Start collecting
  - Verify data transfer is working
  - Use the "dummy SerialEM" to add more targets

SBL Low dose SerialEM setup for Talos Arctica
(Based on SBL and PNCC procedure, edited by Weiwei Wang, Victor Cruz Ruiz and James Chen)

A. Preparation

A1. Start the SerialEM server (SEMserver.exe) on Arctica PC located at C:/ProgramFiles/SerialEM/.

A2. Start SerialEM on the Gatan PC.

A3. C2 aperture: 150 μm.   Objective aperture: out.   SS: 6.   C2: 100%. Mag: 46 X. Defocus: about 100 um.

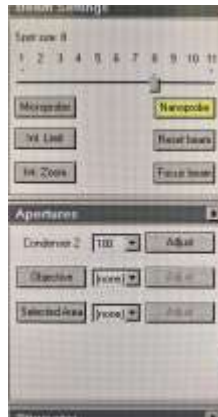   On Arctica PC: under Camera->"un-insert" ceta->flapout->Shutter->highlight "standalone camera"

   On Gatan PC: make sure K3 camera is inserted (could take up to 50 seconds).

A4. Panel *Camera & Script Control* → *Setup* → *Record*: exposure 0.5 s, binning 4

   Make sure K3 is selected. Uncheck "Save Frames".

   Use "Counting mode", not "Dose Fractionation".

A5. Take a "Record" image (just testing out the camera).

Follow Talos Arctica Alignment Protocol to align the microscope with the cross grating grid.

-   Eucentric height, C2 aperture at recording mag, ppX/Y (with "Euc.Focus"), rotation center.
-   Check MicroProbe or NanoProbe.
-   decide which Mag and spot size is going to be used for data collection, based on the resolution expected.
-   Set up parallel beam illumination under the desired record Mag and spot size. !Write down C2!
-   Run CTF-stigmatism and CTF-coma-free under SerialEM with the cross grating grid. If UltraAu grids are used, may have to run beam shift vs. coma-free as well with cross grating grid.
    o   under "Focus/Tune"
    o   will be using "record" settings. set bin=4, exposure=2.5 seconds. defocus close to zero.
-   Load sample grid to be tested.

B. Set up LMM (Low Mag Montage) at C2=150, 46x.

B1. check C2=150

B2. Menu *Navigator* → *Open*

B3. Menu *Navigator* → *Montaging & Grids* → *Setup Full Montage*
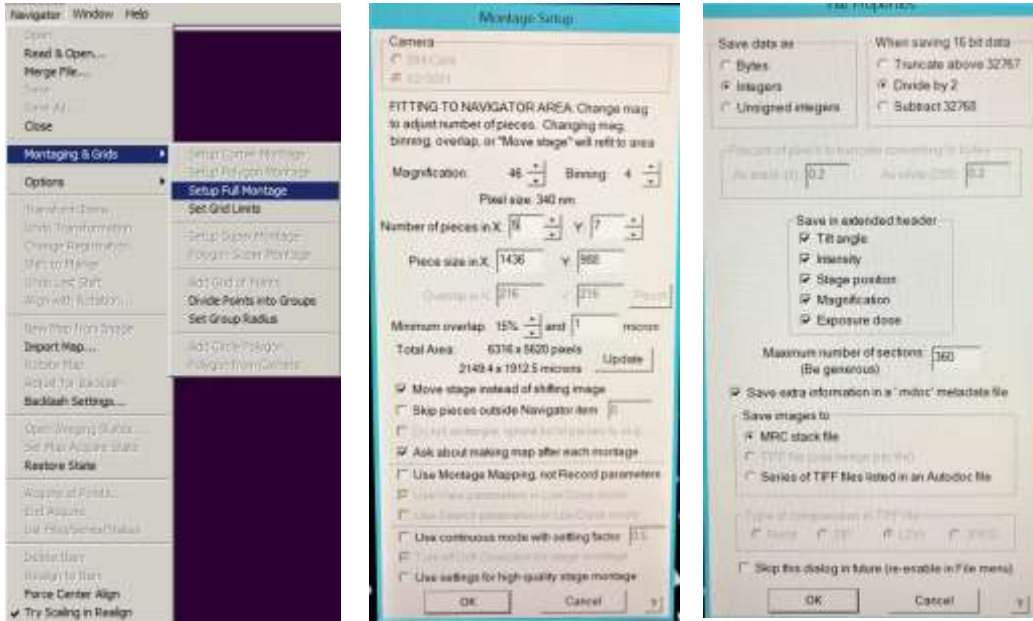
the default settings should work just fine. no need to make changes here.
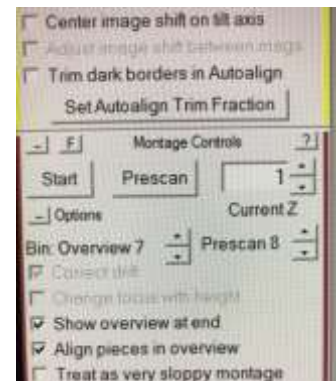
B4. Save as LMM.st in KEEP folder,

RAID_X:\DoseFractions\"BioHPC_User_Named_folder"\KEEP

B5. Panel *Montage Controls* →

Verify "Treat as very sloppy montage" is unchecked (default)

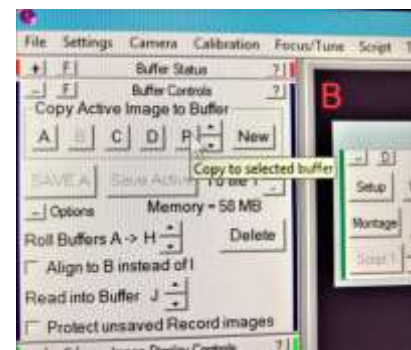*Make sure "Align pieces in overview" and "show overview at end" are checked*

Click "start" -> "yes"

B6. Save navigator.

Optional: Put the map in Buffer P for later use.

- *Poor stitching? check "Treat as very sloppy montage", then double click the map item in the navigator window.*
- Optional: Save the LMM as JPEG file.

Set C2 back to 50.

Set Mag for MMM:

- change, on the Arctica PC, the Mag to a value (200-700x) that allows the K3 detector to barely cover a representative mesh that is going to be mapped (as MMM).
    - o 400 mesh grid, 510x;
    - o 300 mesh grid, 380x;
    - o 200 mesh grid, 210x.
- decrease C2 value so that the beam covers the mesh (not too large).
- Take a "Record" exposure (0.5 seconds, bin 4) to verify with different sizes of squares. look out for corner clipping (may be hard to spot).

Align between LMM image and the MMM Mag (for example 380x):

- open the LMM map by double clicking on the navigator item.
- find a mesh/square that has a viewable feature.
    - o add a point there and "Go To XYZ";
    - o take a "Record" exposure with the updated MMM Mag;
    - o locate the same feature on the MMM image (may have to put down FluCAM and move stage to find the feature, and re-take the record image);
    - o add a marker to the feature, then "Navigator" -> "Shift to Marker".
    - o Repeat 3x with different mesh/feature.

E. Select squares and set up MMM (Medium Mag Montage) with single images

do NOT pick squares that are too close to the edge of the grid/stage

*Need to select a bad square (with features, not cracked) and good ones (~200 holes per hour).*

On Arctica PC: under View mode, center a hole (with expected ice thickness), verify "focus/Trial" area under View, then click "Screen" button under "Camera & Script Control". This would run script #20, for a quick check of particle density/quality, without hole alignment or saving of images. Repeat for another square for consistency.

E1. Double click on the LMM map. Add points to the centers of squares.

Check "Acquire" while adding points.

E2. Sub-window Navigator → tick "New file at item" for the first point
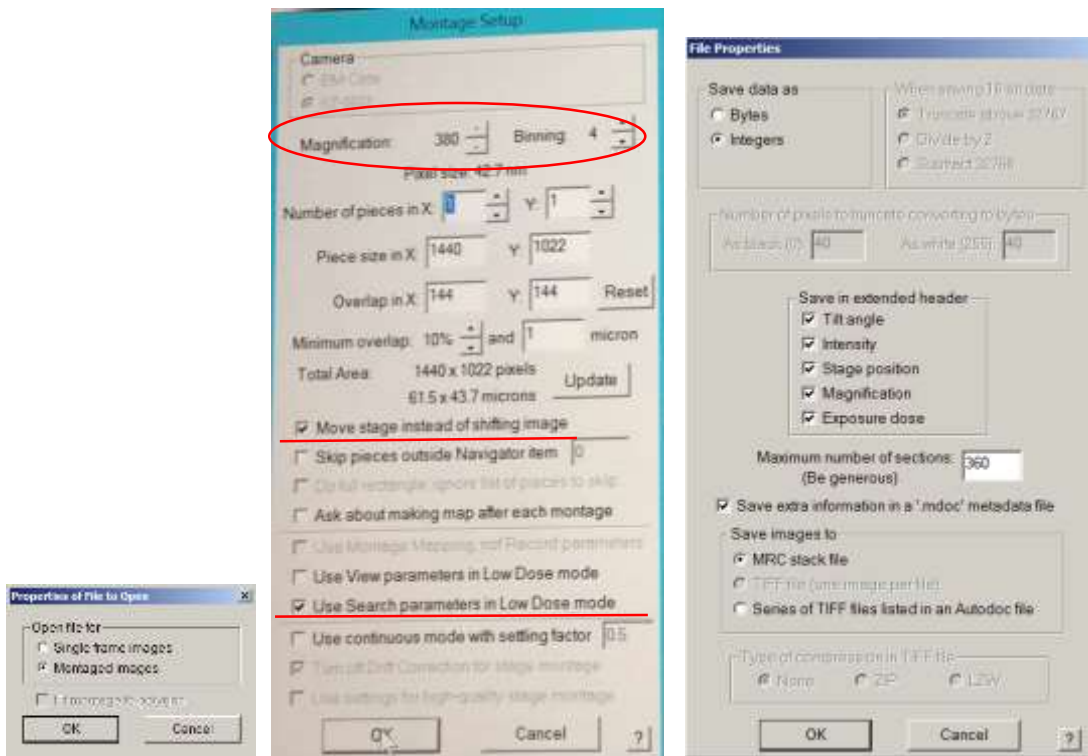
- **Mag should be matching the updated Search Mag (like 380x);**
- **Number of pieces in X: 1, Y: 1; Minimum overlap: irrelevant;**
- **Uncheck "Ask about making map after each montage".**
- **check "Move Stage instead shifting image",**
- **check "Use Search parameters in Low Dose mode" (not the View parameters).**

E3. Save as MMM.st in KEEP folder.

E4. Make sure "File" -> "close" option is grey (nothing to be closed).

Under "Setup" -> "Record" window, change Binning to match the Montage Binning.

Exposure time 0.2-0.5 s.

Counting mode (not Dose Fractionation).

E5. Menu *Navigator → Acquire at Points*

If images are grainy: try condense the beam more, test settings with record; can try lowering spot size.

"Rough eucentricity" or not: Verified with Weiwei, absolutely YES!!!

- Make sure Low Dose Mode is NOT checked (or turned on) under SerialEM.
- Rough encentricity will be using the MMM mag set here;
- Make sure "Low Dose Mode" is NOT checked, otherwise, Rough Euc. would switch to the View mag at 6700 (not recommended);
- however, one could also manually adjust eucentric height for each square/mesh and "update Z" for each before acquiring MMM maps.
    - Make sure "view" is checked under "Area to show when screen is down"
    - Select a line corresponding to a square in the Navigator window
    - Click "Go to XYZ"
    - Press R1 to insert screen
    - Set Z height (use any feature on square, set alpha 30 and adjust Z using buttons on right control board)
    - Click "Update Z" in Navigator on the line corresponding to that square
    - Repeat step for all the squares
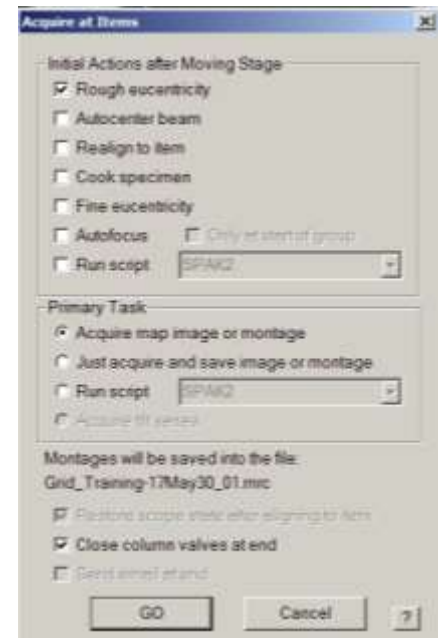
E6. Save navigator.

Quick setup under "Low Dose Mode" of SerialEM

Turn on "Low Dose Mode".

1. *SerialEM will load the settings from the last or default user settings, which may reset some (Mags, spot size, beam center, etc.) settings of the direct alignment you just finished.*

2. **Set up various Mag under View / Record**

   Go to each mode, set up the Mag:

   - check "Continuous update" box.
   - **View**:
       o Recommended: lowest Mag that gives a single hole (6700x for R1.2/1.3, 3600x for R2/2);
       o or the Mag that gives 4 holes (for setting up 4-hole pattern);
       o Offset for View: Defocus set to -100 (some may prefer -20 um);
   - **Record**: based on desired resolution (Nyquist frequency = 2x pixel size). low defocus value (zero).
   - Copy Record settings to Focus, make sure "Always keep F and T the same" is checked.
   - uncheck "Continuous update" box.
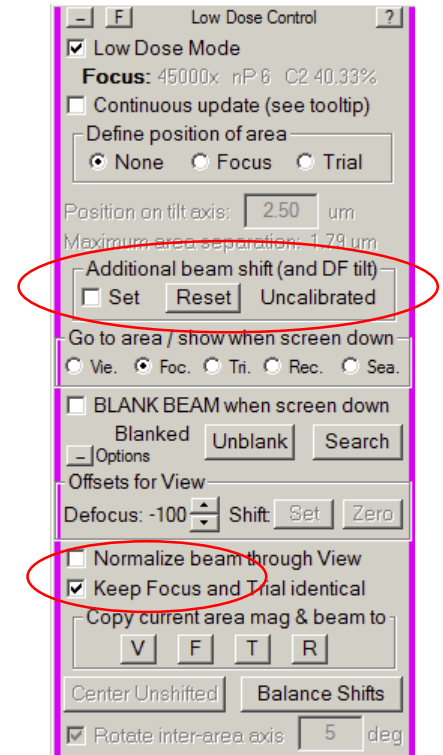
3. **Adjust illumination areas under each mode (Record -> Trial -> View).**
   a. Check "Continuous update" box.
   b. Go to Record mode: Task->Eucentric Rough, C2 aperture as desired, move to carbon area ppX/Y (with "Euc.Focus"). double check C2 condensor value, make sure it matches the parallel beam illumination. Re-adjust if needed. Center the beam at this C2 condensor value, using beam shift under Direct Alignment.
   c. Copy current area & beam to "F" (Focus).
   d. check "Keep Focus and Trail Identical".
   e. *optional: reduce the "Trial" beam size.*
   f. Go to View mode: adjust C2 if needed, to cover the detector. Move/center beam using the trackball. check then uncheck the "SET" button to apply "Additional beam shift under" under "View" mode.
   g. uncheck "Continuous update" box.
   h. Save settings: "Settings" -> "Save as". Give it a unique name.

# From this point on, uncheck "Move stage for big mouse shift"

o **for "stage shift": use "SHIFT" + "Right-mouse click and drag";**

o **for "Image Shift": "Right-mouse click and drag";**

o **There is an "IS" field to the left of the SerialEM interface**

      o **Make sure that it stays at ZERO (0 um);**

      o **monitor the value during desired image shift;**

   o **"Reset Image Shift" button will reset it back to zero.**
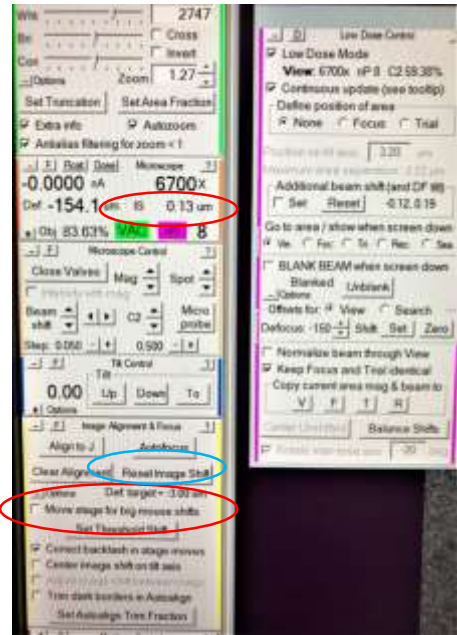
Set Offsets for View (*between Record mode at zero defocus and View mode at -100um defocus*)

- Find in MMM a square with a noticeable feature. Add a MARKER here and GO TO XYZ. Alternatively, Stage shift (SHIFT + right click and drag) to center the square.
- Check "Continuous update".
- Take a *View* image: first do Task->Eucentric Rough. Make sure it's not carbon. "UPDATE Z".
- Make sure defocus at View Mag is identical to the one used for hole centering, ie. at -100 um.
- Center a feature in *View* image with stage shift (SHIFT + right click and drag).
- Take a *Record* image (at close to zero defocus). Locate the feature. If can't find it, put down the FluCAM and stage shift (with the control pad big knob) to the feature.
- Take another *view* image:
  - Panel *Alignment & Focus* → uncheck "Move stage for big mouse shifts". Check "Continuous update". Center the feature with image shift.
  - Click *Set* under Label *"Offsets for View"*.
  - check and make sure "IS" value is zero.
  - Repeat setting if necessary.
 - uncheck "Continuous update".
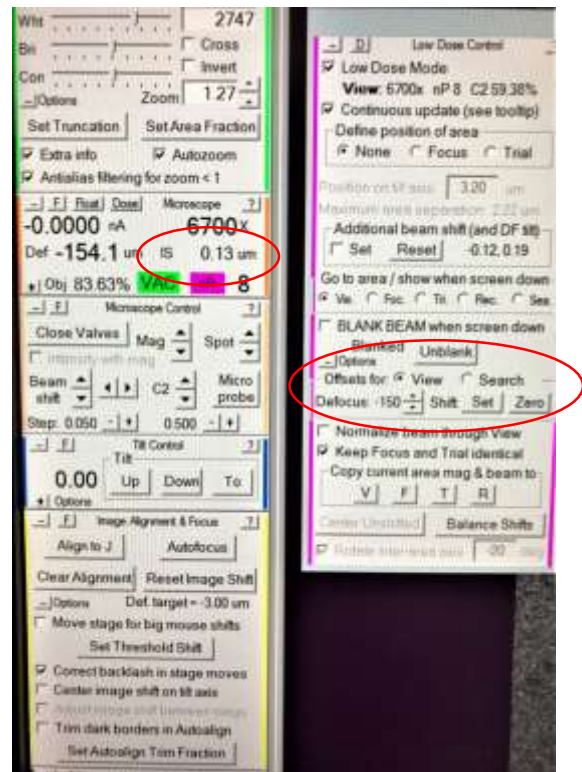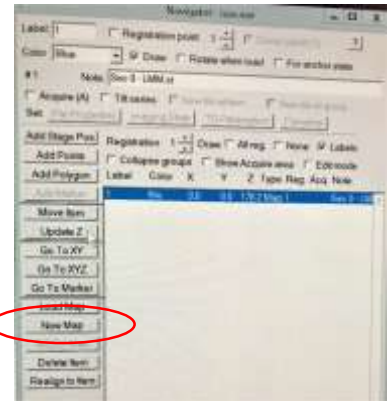
Align _View and MMM map_ (taken at lower LM Mag)

- Check "Continuous update".

- Find in MMM maps a square with a noticeable feature.

    - add a point to the feature;

    - Go to XYZ.

- Take a _View_ image (at -100 um defocus). Locate the feature and add a Marker to it.

    - If can't find it, put down the FluCAM and stage shift (with the control pad big knob) to the feature.

    - Navigator -> Shift to Marker.

    - Navigator -> GoTo XYZ, take a view image to verify centering.

    - Repeat 3x if necessary.

    - uncheck "Continuous update".
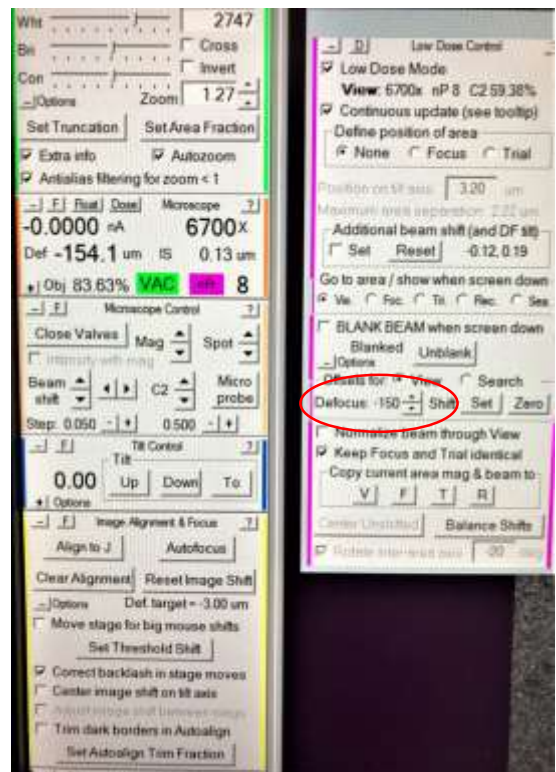
Define Focus and Trail area

- o Check "Continuous update".

- o Take a view and set the "Define position of area" for *Focus* and *Trial*.

- o Center (stage shift) to a hole.

- o Make sure that the "focus/trial" point is located at the center of 4 neighboring holes.

- o one may lower the Mag under View to estimate the position.

- o To adjust the position, you can change the two number in low dose control panel: "Rotate inter-area axis" and "Position on tilt axis" (position can be set by simply left-click).

- o uncheck "Continuous update".
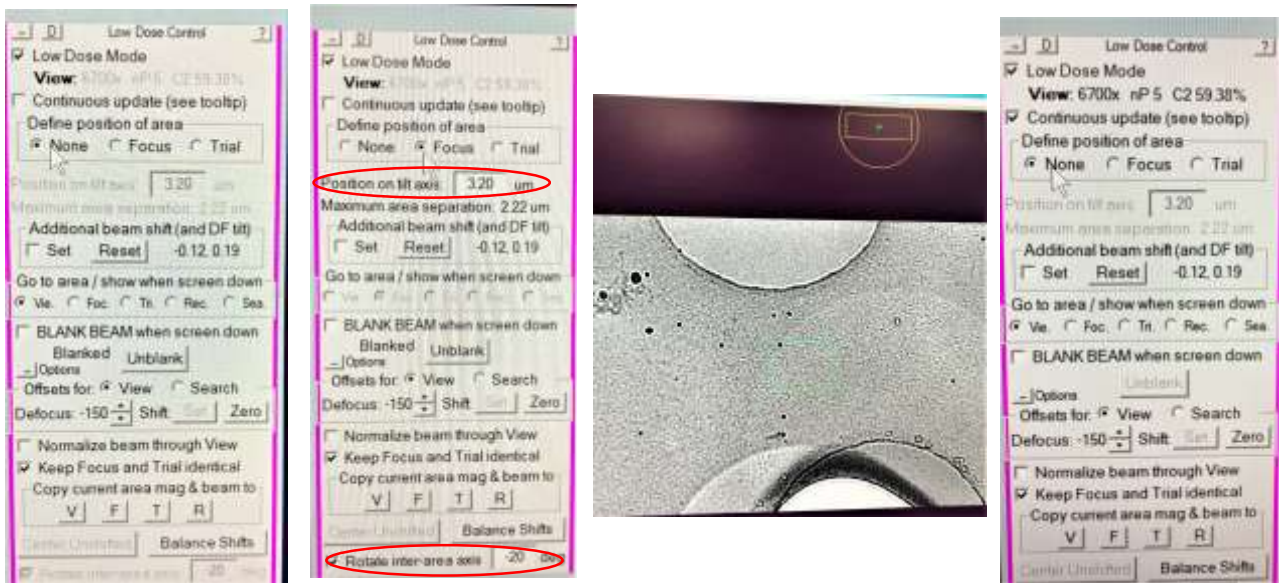
J. Save a centered hole for V align to P

J1. Use the first MMM map.

J2. Take a *View* image and center the hole.

J3. Save the centered hole in buffer P (under Buffer Control).

J4. Make image off-center. Run script of "Edit 2" to test the centering action. Go to a hole at a distance from current spot, test run script 2 again.

This is optional, could be done later

Set up low dose mode (Change C2=50, spot size = desired for data collection, between 5-8)

check "low dose mode" is turned on. Check "continuous update" for saving.

Change Mag only on Arctica computer

Check box to "keep F & T identical"

If "Binning=0.5" not available, make sure under "File" the "Close" option is greyed out.

Under "Camera Parameters"

Mode	Mag	Exp time	Binning	Size	C2	Dose
View	_6,700_	0.2	4	Full	_User defined_	No
Focus	45,000	0.2	2 ~ 4	Wide Half	40 %	No
Trial	45,000	0.2	4	Full	~38 %	No
Preview	45,000	0.2	4	Full	40 %	No
Record	45,000	1.5 – 2.0	0.5	Full	40 %	Yes

Make sure the "Trial" beam is NOT covering the hole!!!

uncheck "Continuous update".

Multi-Records (Multi-shots/Multi-hole) Setup under SerialEM

- when setting up the "pattern" under the view mag, one has to adjust the defocus close to what's used under data collection under "record" mode, which is closer to zero. Normally we set this at -100 um or similar under view by default (for hole centering), and if not changed, this would seriously mess up the setup that may lead to mismatch between view and record mags.

- Center the "center point/hole" (Point C) under -100 um defocus;
- then switch to **ZERO** defocus under View mode. (your view image for Point C, if taken again, may drift/off-center, do NOT re-center under zero defocus).
- Setting up the four corner hole/points (Point 1 through 4) at **ZERO** defocus.
- When finished, reset defocus for View mode back to -100um.

- what's registered (in XY) under the MMM map is often misaligned under the View mag, no matter how carefully one tried to align the two mags beforehand. This is more so when one uses a low mag (in LM lens group) such as 210x or 510x to capture MMM maps (avoiding the time consuming MMM maps using M/SA lens group). What Weiwei did was to further refine positions of the four "corner" points between MMM and View mags as follows:

      - before setting up the multi-shots: "Go to XYZ" on one of the 4 points,
      - take a view at -100um defocus;
      - it's likely not matching the hole well;
      - add a marker to the center of the hole under view;
      - Nav->Shift to marker.
      - He did this for all 4 corner points, before setting up the multi-shots.

- when not sure, it's always a good idea, after image shift to the center of a hole under view during the setup (before click on the "save image shift" button), to switch to "preview" just to make sure that it's hitting the hole. one could even fine tuning between preview and view modes here to register additional image shift if needed. we didn't have to do this, yet.

- I start keeping the "move stage for big mouse shifts" unchecked, and using strictly SHIFT+right mouse click for "stage move" and right click alone for image shift. because only image shift should be used during the multi-shots setup (most of the time), it's important that one doesn't "accidentally" trigger the stage shift.

- there's an "IS" field on one of the panels to the left. one should keep a close eye on that to make sure it's at zero unless an image shift is warranted. Reset IS back to zero otherwise.

- always run, under "Calibration" -> "Focus and tuning" -> "Image Shift vs. coma", before start of multi-shots data collection. One may have to increase binning, change the defocus, or increase exposure time to get the Thon rings to show up properly. **we used 2.5 seconds exposure, binning of 4, under 45k mag ss=6. For R1.2/1.3 grids, the 3x3 setup has a max image shift about 3.6um. for R2/2 grids, this becomes 5.7um. After finishing this calibration, make sure to change the "Record" settings back to the original exposure time and binning.**

Highly recommended:

**After one finishes setting up the mutl-shots pattern:**

- Add a point to the MMM map (testing MMM map).
- Make it "Acquire".
- check "Record" camera settings, make it simple (1s exposure, no dose fraction)
- Navigator -> Acquire at points
  - uncheck "Save Frames"
- Make sure hole centering and multi-shots works before adding grid points to acquire.

Weiwei's protocol for setting up 9-hole (3x3) pattern:

a. Pick an MMM map of one square in the navigator (either go to the square line or the MMM map of it) and click "go to XYZ" (Note: better if the ice is not too thin to see the holes well on view image)

b. Set the defocus to -100um, identical to the value used during hole centering step during data collection.
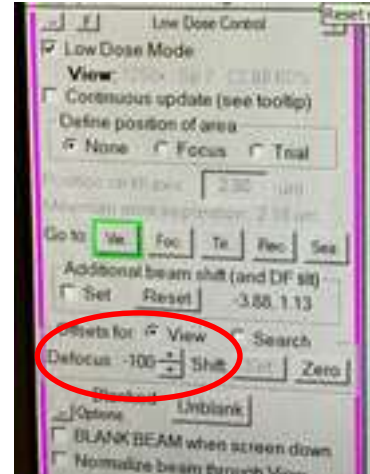
c. Add a point to the center of the 3x3 pattern (hereafter referred to as Point C). Take a view shot, if not seeing the hole well, adjust the view setup to 2s (usually at 1s).

d. Click "Add points" in the navigator window and pick the four corner holes of a 3X3 array in a clockwise order.

e. "Stop adding" and better note the 4 points (e.g. template pt1/2/3/4), so that they will not be mistakenly used.

- what's registered (in XY) under the MMM map is often misaligned under the View mag, no matter how carefully one tried to align the two mags beforehand. This is more so when one uses a low mag (in LM lens group) such as 380x or 700x to capture MMM maps (avoiding the time consuming MMM maps using M/SA lens group). What Weiwei did was to further refine positions of the four "corner" points between MMM and View mags as follows:

   - before setting up the multi-shots: "Go to XYZ" on one of the 4 points,
   - take a view at -100um defocus;
   - it's likely not matching the hole well;
   - add a marker to the center of the hole under view;
   - Nav->Shift to marker.
   *- Do this for all 4 corner points, before setting up the multi-shots.*
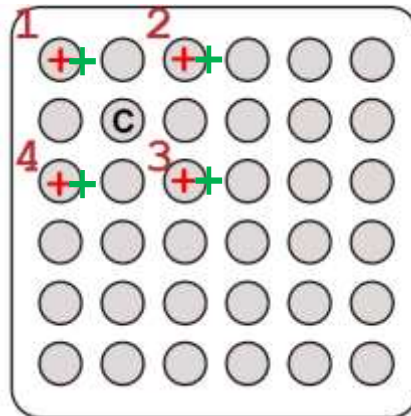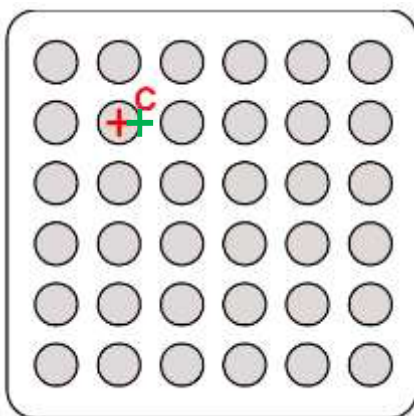
for more precisely alignment between "view" and "record/preview", add the four corner points to the edge of the holes (green markers)

***. burn with beam the 4 corner positions. *** (noticed that the image flips between the LM and SA lens groups, so your MMM images looks upside-down from the "view" mag images at 6700x)

84	Red	228.0	-69.3	226.5 Pt	1	
85	Red	216.0	-74.2	226.5 Pt	1	ct
86	Red	219.5	-74.9	226.5 Pt	1	1
87	Red	215.3	-77.7	226.5 Pt	1	2
88	Red	212.5	-73.5	226.5 Pt	1	3
89	Red	216.8	-70.7	226.5 Pt	1	4
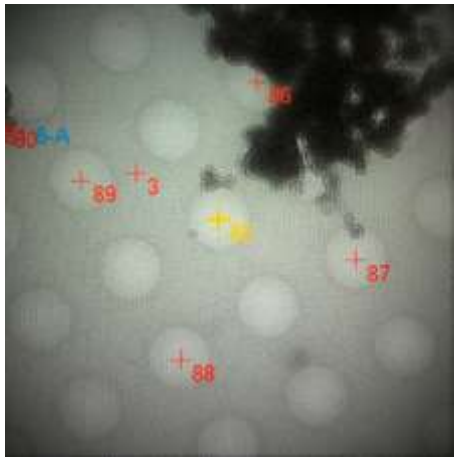
MMM map showing the 5 points selected (taken with LM lens group)
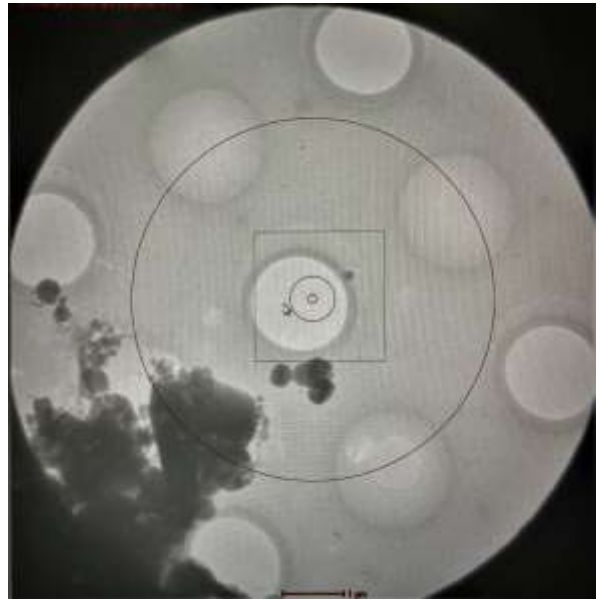
View mag (at 6700x) showing the 5 points intentionally "burned" empty

***. _Adjust the defocus from -100 um to 0_ using downward arrowhead. ***
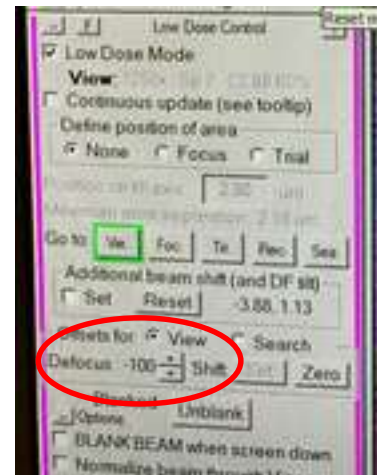
g. Highlight Point C line in the navigator

h. Navigator -> Montage & grids -> set multi-shot parameters

i. A "Multiple Record Setup" window will pop out:

    a). Click "For corners of regular pattern" -> Then the two panels below it will be clickable.

    b). First click "IS to Nav Pt" (note: IS = image shift")

    c). Ignore the warning and hit OK.

    it would read underneath "Go to first corner"

    d). The First of the 4 picked points will turn from red to yellow (see the figure below)

    e). Take a view shot and center to the hole using **image shift (right-click, manually drag to the target Point 1), using the burned beam mark as reference.** confirm centering with another view shot.
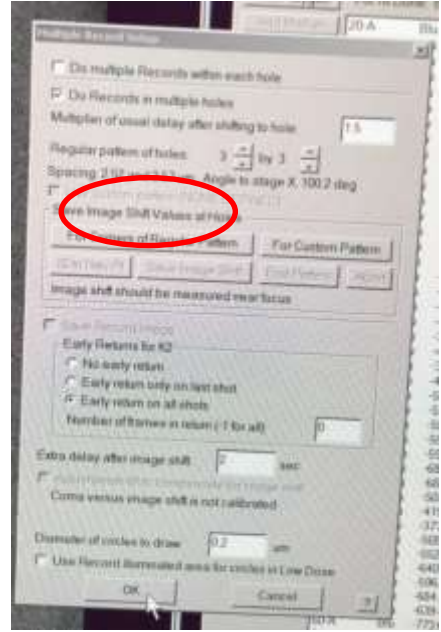
- when not sure, it's always a good idea, after image shift to center of hole under view during the setup (before click on the "save image shift" button), to switch to "preview" just to make sure that it's hitting the hole. one could even fine tuning between preview and view modes here to register additional image shift if needed.
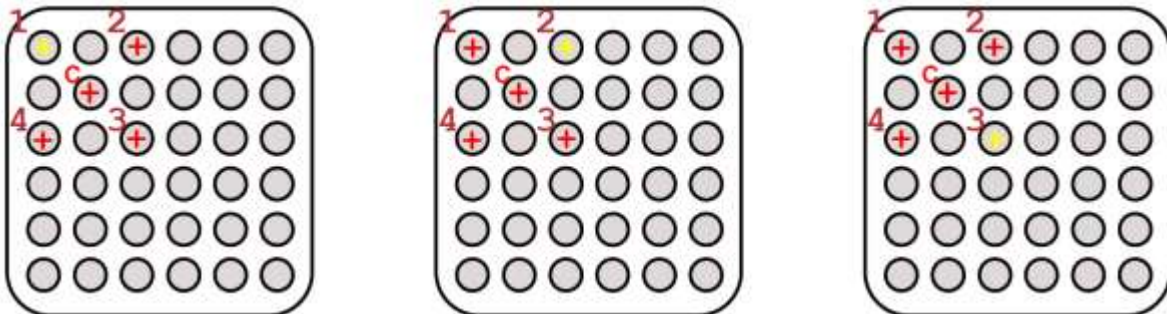
    f). Then click "Save image shift".

    Click "IS to Nav Pt", now **Point 1** would turn back to red, **Point 2** would become yellow.

    now it would read underneath "Shift by 2 holes in first direction"

    follow step e) to image shift to Point 2

    g). repeat steps f), for Point 3 and Point 4.

    h). When done with all four holes, will see an update of the numbers in one of the lines above the "For corners of regular pattern" button: Spacing: 3.8 and 3.6 um Angle to stage X: 100 deg

   make sure to check the box to "Adjust beam tilt to compensate…. "

    i). Click "ok" at the bottom of the Multiple Record Setup window.

    j). Very important: adjust the defocus back to **-100 um** using upward arrow head! And change the view setup back to **1s.**

    k). Save navigator.

-    **Reset Image Shift!!! Make sure "IS" is zero!!!**

- Click on "Show Acquire area" will bring up the pattern.

- **Calibration -> Focus & Tuning -> Coma vs. Image Shift, Set the MAX amount of image shift between the furthest two hole centers (could be measured by SHIFT+left click and drag)**

- One may have to increase binning, change the defocus, or increase exposure time to get the Thon rings to show up properly. **we used 2.5 seconds exposure, binning of 4, under 45k mag ss=6. For R1.2/1.3 grids, the 3x3 setup has a max image shift about 3.7um (set Max at 4 um). for R2/2 grids, this becomes 5.7um (set Max at 6 um).** Wactch out for beam "clipping" during the process.

- **After finishing this calibration, make sure to change the "Record" settings back to the original exposure time and binning.**

- check then uncheck "Continuous update" when done.
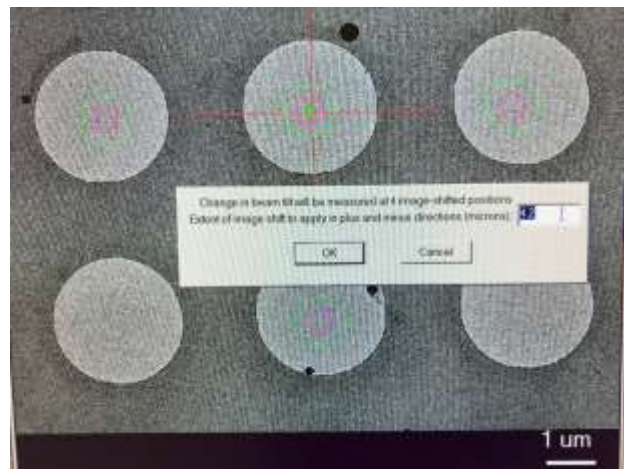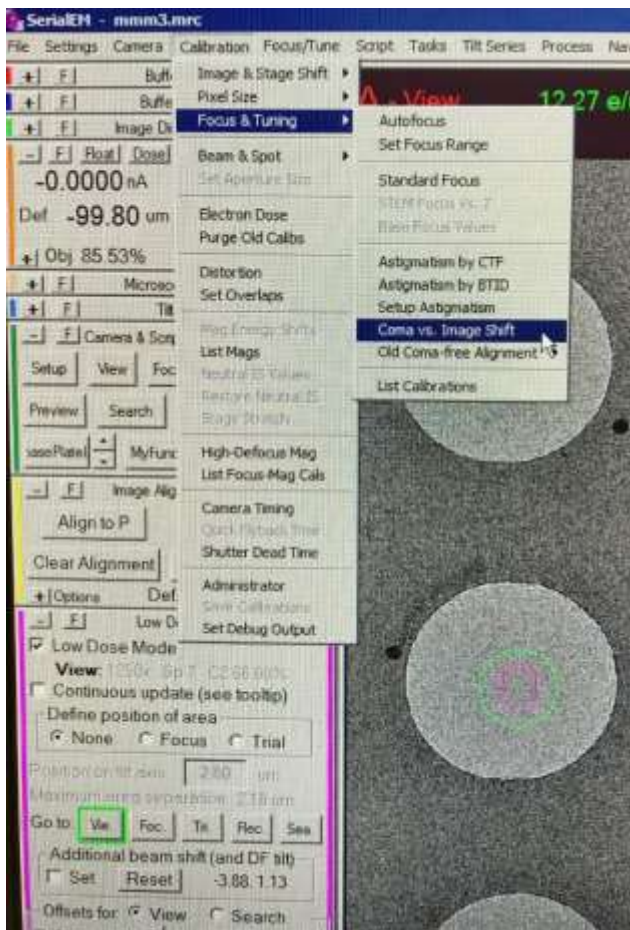
PNCC's protocol for setting up 5-hole cross pattern:

Take a View image, and precisely center a hole using stage shift (SHIFT+right click and drag)

- Add central point C, and five additional points (1 through 5) as shown below
- Point C and Point 1 should overlap.
- Center the center hole (Point C) at -100um defocus with stage shift.
- then switch to ZERO (0) defocus. Do NOT re-center the center hole (Point C) at zero defocus.
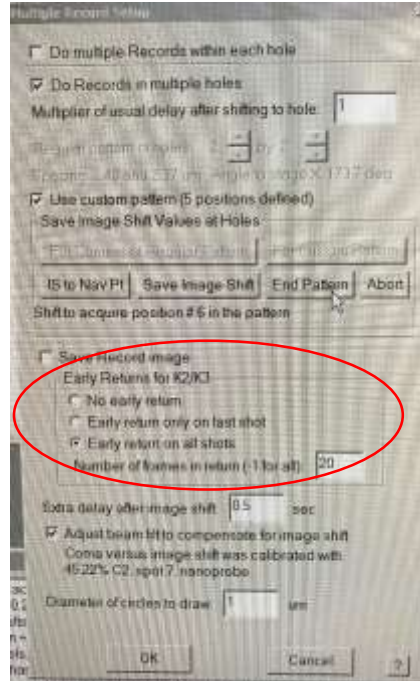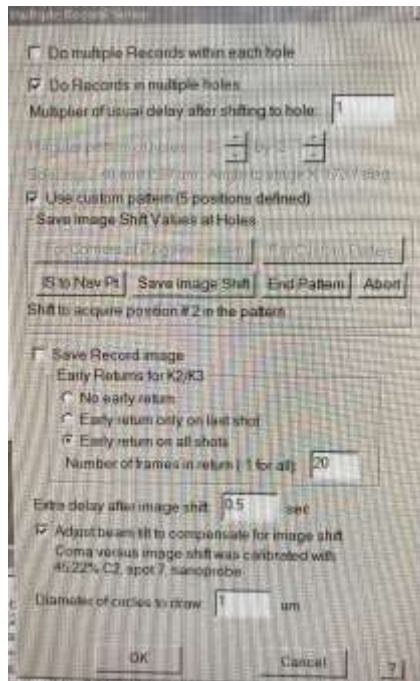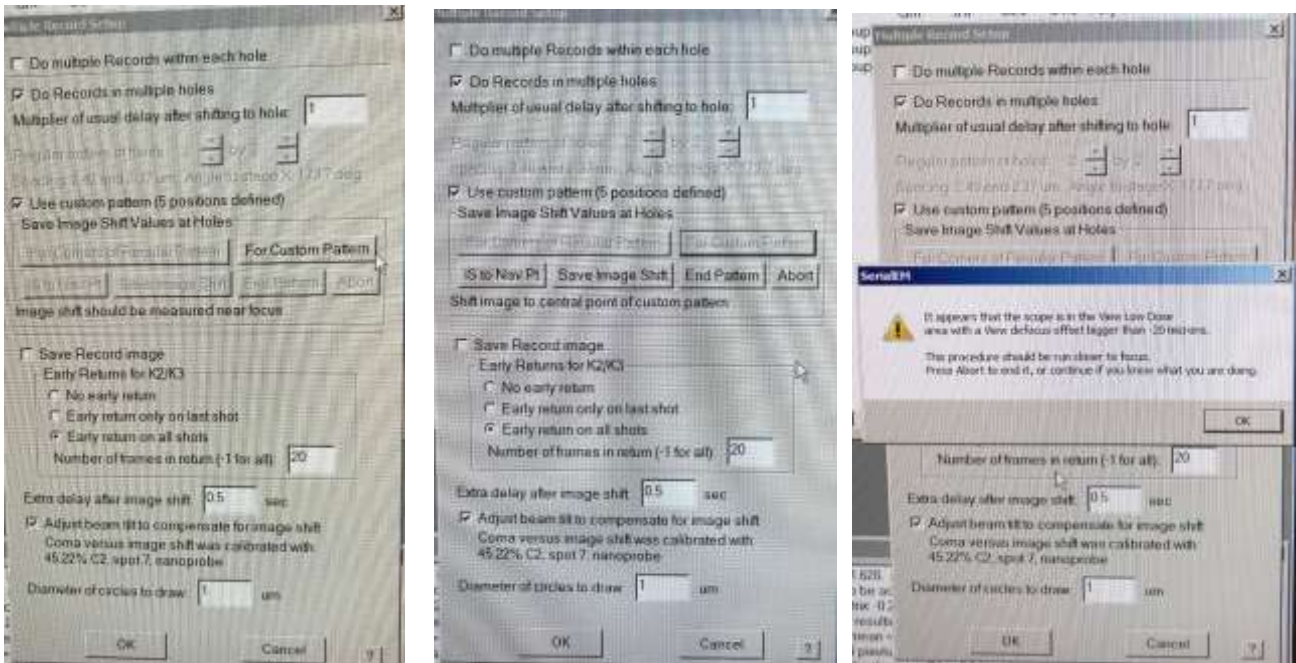- the setup of the 5-point cross pattern should be done under zero defocus!
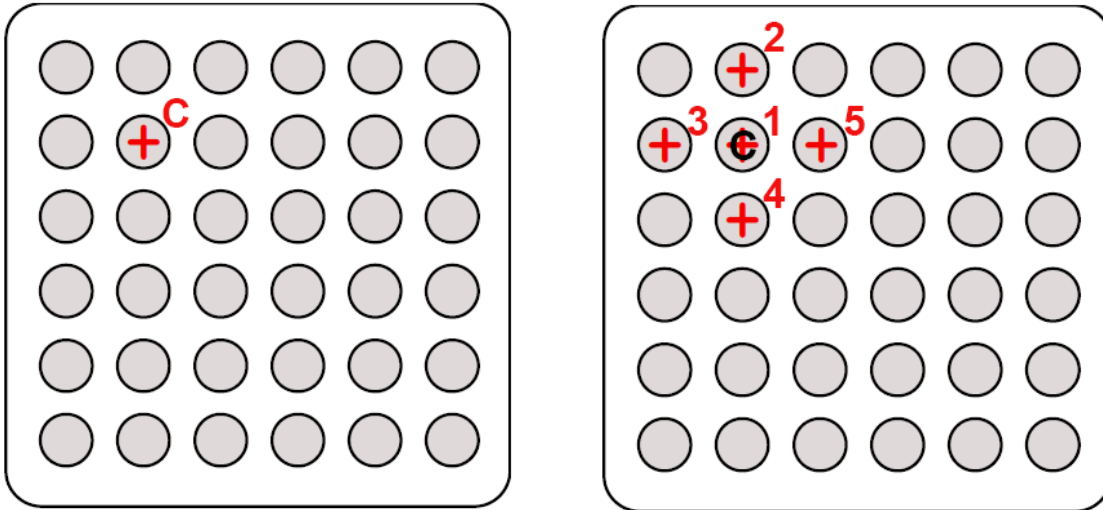
1. **Navigator -> Montaging & Grids -> Set Multi-shot Parameters**
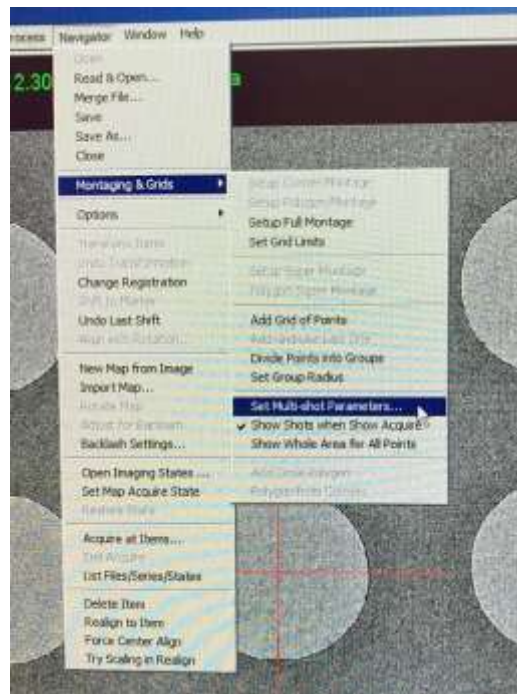
2. For Custom Pattern -> follow on-screen instructions (ignore the warning)

    a.   <u>follow the protocol described earlier on "IS to Nav Pt" and "Save Image Shift"</u>


Only select "Early return on all shots" at the very beginning of the data collection to make sure the script runs as predicted. this would slow down the data collection, so you would like to change it to "No early return" once everything is set.

**3. Reset Image Shift!!! Make sure "IS" is zero!!!**

Reset defocus under View mode back to -100 um!!!

Click on "Show Acquire area" will bring up the pattern.
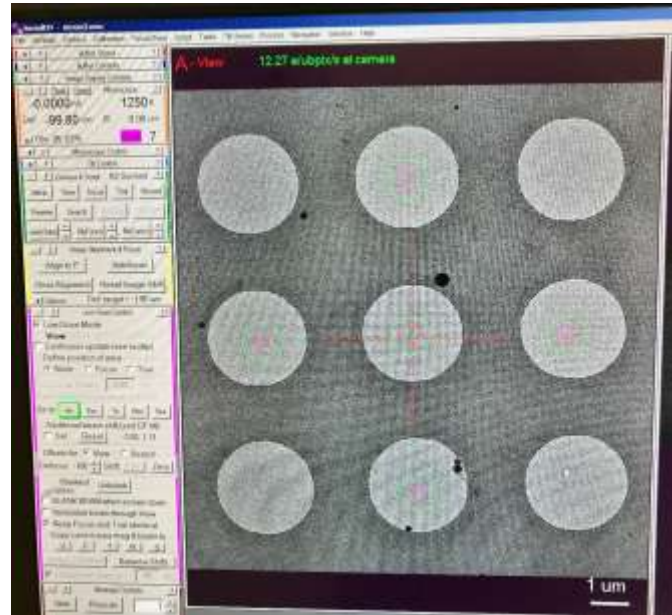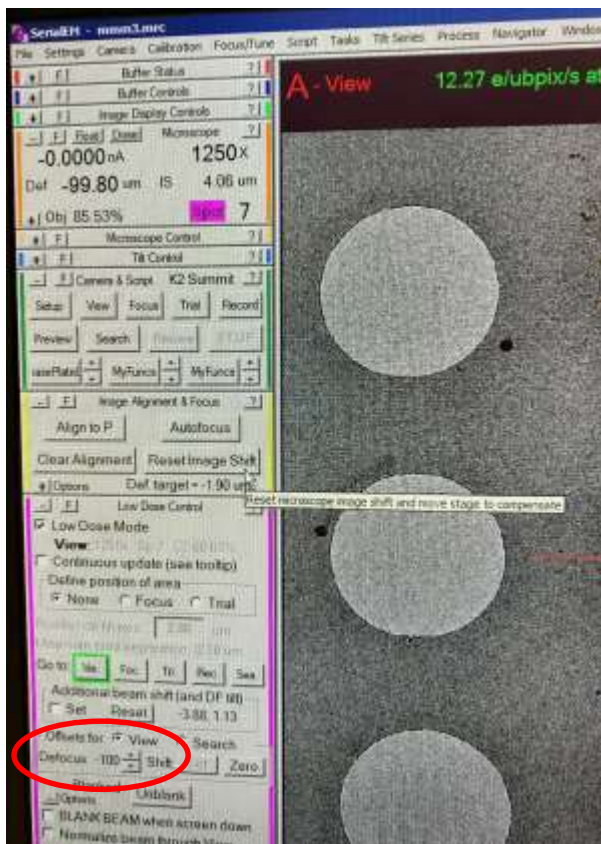
4. Calibration -> Focus & Tuning -> Coma vs. Image Shift

Set the MAX amount of image shift between the furthest two hole centers (measured by SHIFT+left click and drag). under "Calibration" -> "Focus and tuning" -> "Image Shift vs. coma", before start of multi-shots data collection. One may have to increase binning, change the defocus, or increase exposure time to get the Thon rings to show up properly. **we used 2.5 seconds exposure, binning of 4, under 45k mag ss=6. For R1.2/1.3 grids, this setup has a max image shift about 2.5um. for R2/2 grids, this becomes 4um. After finishing this calibration, make sure to change the "Record" settings back to the original exposure time and binning.**
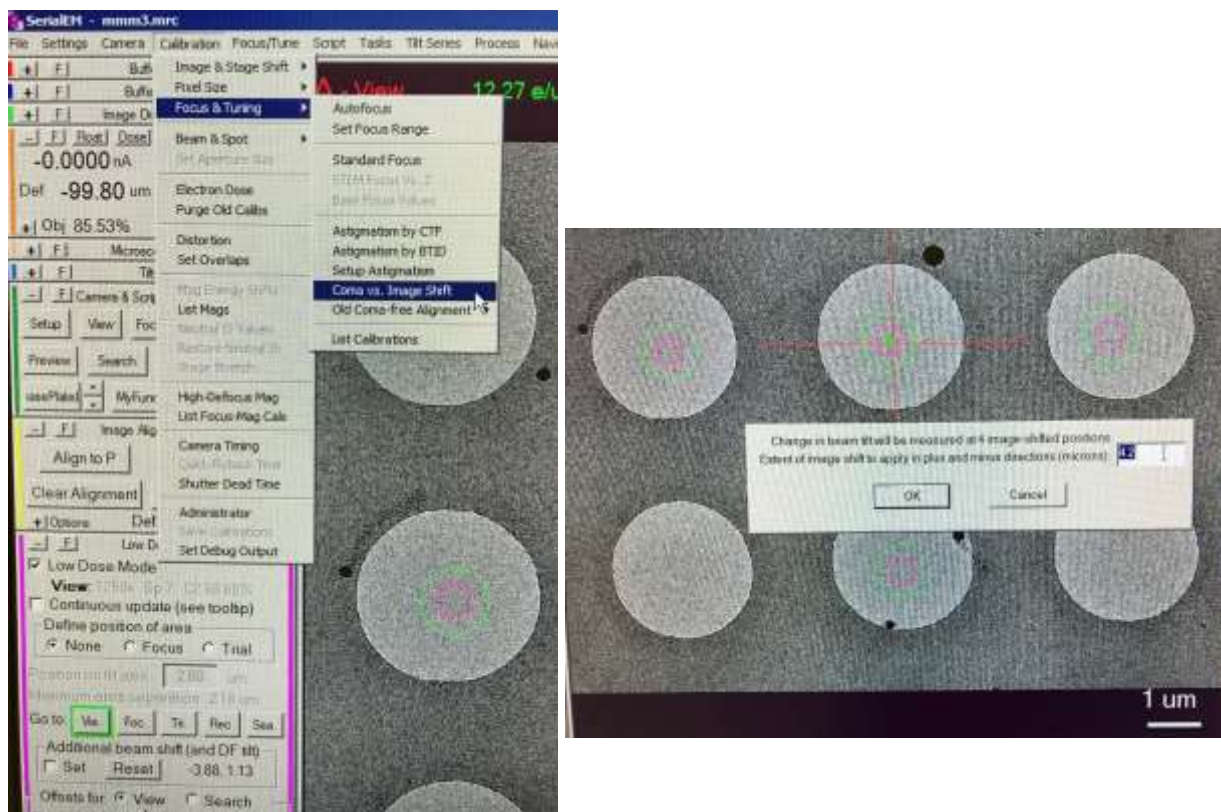

One may have to make changes to the CTF settings under "Focus/Tune", and/or beam intensity, defocus values, etc., to get this to work. also make sure the stage or beam isn't drifting during measurement (cropped/un-even CTF thon rings).

Highly recommended:

- *Add a point to the MMM map (testing MMM map).*
- *Make it "Acquire".*
- *check "Record" camera settings, make it simple (1s exposure, no dose fraction)*
- *Navigator -> Acquire at points*
    - *uncheck "Save Frames"*
- *Make sure hole centering and multi-shots works before adding grid points to acquire.*

Multi-shots per hole settings are similar.
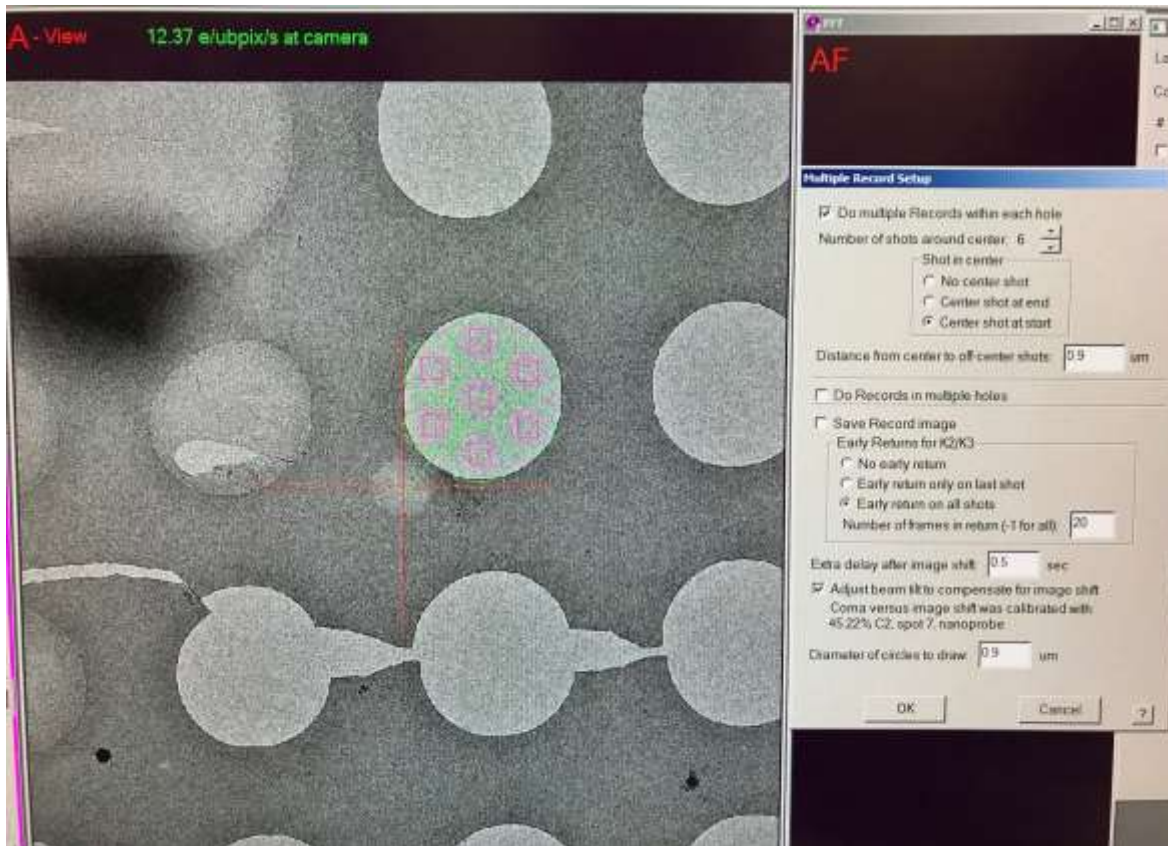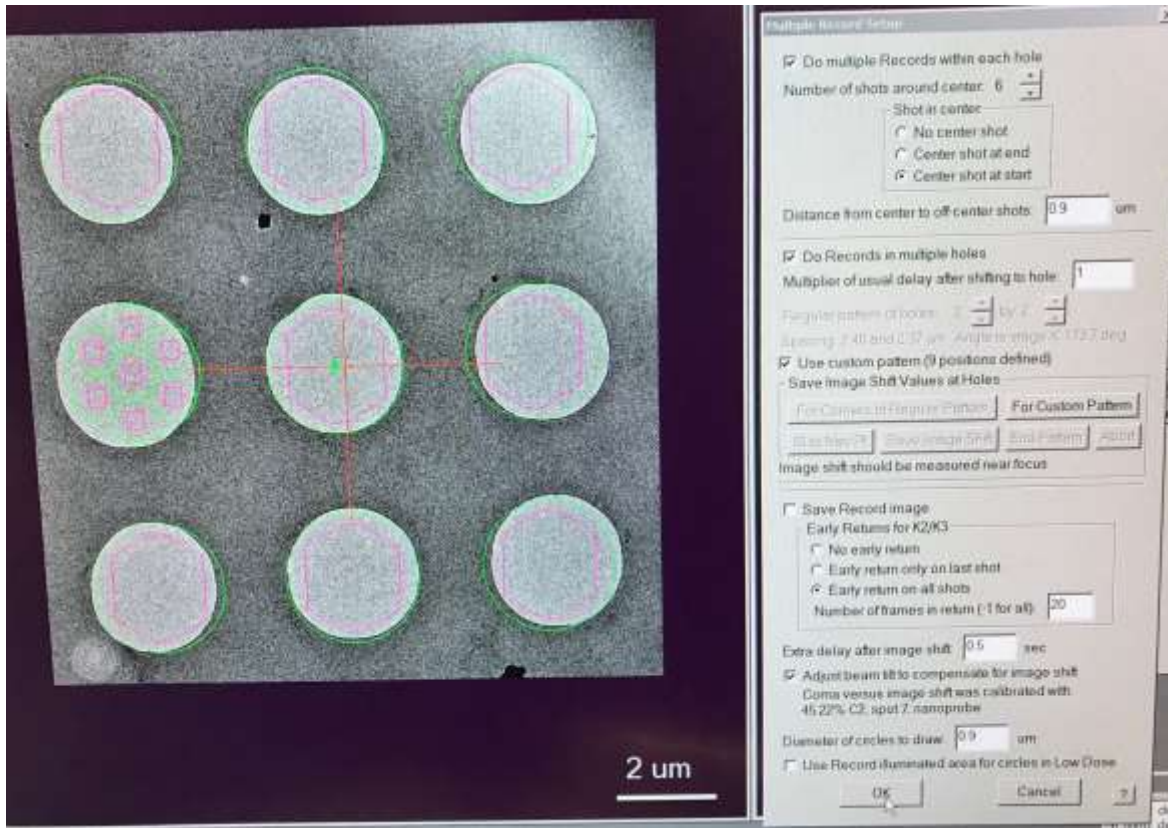
Select holes for data collection

> *Old way of doing things: manually pick 5 points at the corners, then "Add", and repeat for every single square.*

I1. Load a MMM map selected for micrograph acquisition.

I2. Sub-window *Navigator* → "add points", to add 5 points at the corners of the desired area

!!!Please follow the selection rules for the 5 points!!! (don't add more than 5).

Menu *Navigator* → *Montaging & Grids* → *Add Grid of Points (may have to Collapse group first)*

I3. Repeat adding holes for other squares.

I4. Save the navigator.

Select holes for data collection using Polygon

I1. Load a MMM map selected for micrograph acquisition.
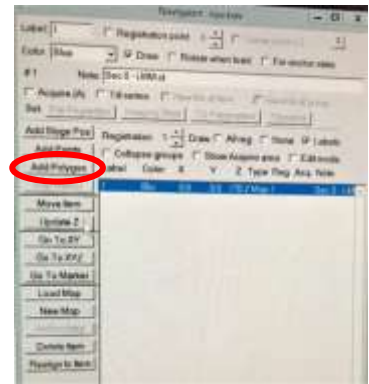
I2. Sub-window *Navigator* → Add Polygon

   Draw a polygon (in green color) on the MMM map.

   click "Stop Adding" when done.

   remember/write down the "label" number for the newly defined polygon.

   Save the navigator.

I3. Sub-window *Navigator* → "add points", to add 5 points within the polygon (no more than 5)

   No need to go to the extreme corners, but maybe a few holes apart.

   !!!Make sure the points are well positioned at the center of the holes!!!

   Click on "Stop adding" when finished. Points should be collapsed by default.

I4. Menu *Navigator* → *Montaging & Grids* → *Add Grid of Points*

   SerialEM will prompt if one would like to use a "polygon"

   - **Enter the "label number" corresponding to the defined polygon;**
   - **Click "OK"**
   - Save the navigator.

I5. Load the next MMM map selected for micrograph acquisition.

   - Select the polygon that was drawn earlier;
   - To use the same pre-defined polygon (from Step I2):
     - ○ Click on "Move Item", and drag/move the polygon to cover the new map;
     - ○ Click on "Stop Moving" when finished;
   - If the pattern of holes to be collected changes too much, then one may define a new polygon
     - ○ Follow steps listed under I2;
     - ○ Remember/write down the "label" number of the new polygon;
     - ○ Save the navigator.
   - "Add Points": Only need to add ONE (1) point at the center of a hole within the polygon;
   - Click on "Stop Adding" when finished;

   Menu *Navigator* → *Montaging & Grids* → *Add Grid of Points*

   SerialEM will prompt if one would like to use a "polygon"

   - Enter the "label number" corresponding to the defined polygon;
   - Click "OK"
   - Save the navigator.

To remove a hole from the selection:

- check "Edit mode"

- Left-click to select a point

- right-click to move a point

- Double click on the holes to be de-selected

- Uncheck "Edit mode" when finished.
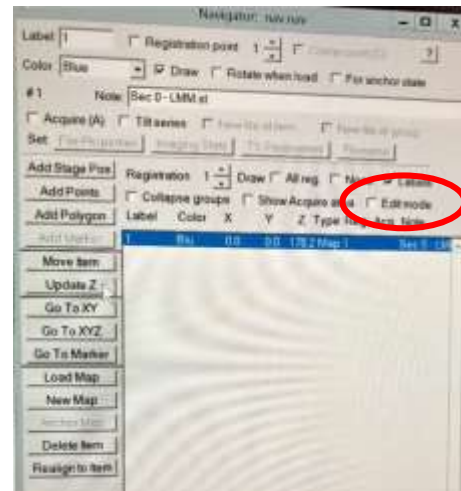
K. Calibrate the dose rate

Go to a square that's NOT going to be collected, with similar ice thickness of the target squares.

- o Task -> Eucentric Rough;
- o take a *Preview*;
- o calculate the dose rate as follows:
  - o ie., displayed dose = 20 e / ubpix/sec;
  - o divide that by the pixel size at the mag, ie., dose / 0.8 A(2)/pix = 25 e / A(2) / sec;
  - o target dose is around 50 e / A(2), this is detector independent;
  - o then exposure time should be 50 / 25 = 2 seconds.
- o Please note: **do NOT change C2 condensor settings, this should remain identical to the parallel beam illumination condition!!!**
- o if needed, increase or lower the spot size (each step is about 2x more or less intensity). **But will have to redo alignment with the new spot size applied.**
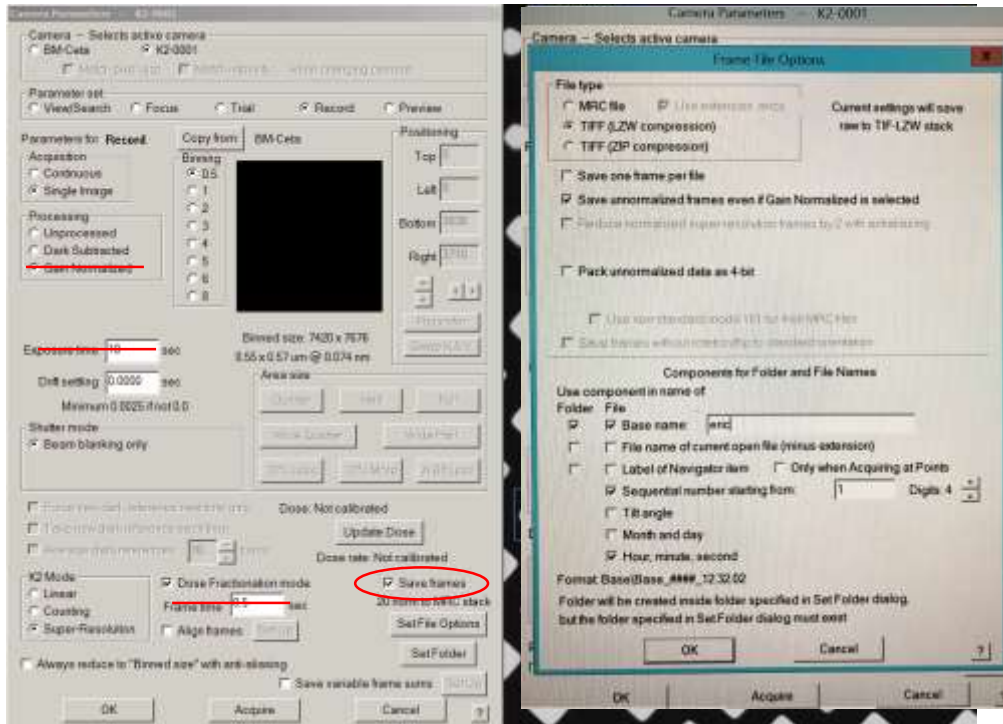

Old way as stated in protocol: In DM take an image in the counting mode (binning 1, 1 s). Find the dose rate.

L. Set up data acquisition

L1. Exposure dose: ~50 e⁻/A². Exposure time: ~ 2 s. Dose Fractionation mode: frame time 0.05 s.

L2. Under "Camera Parameters":

Set File Options and *Set Folder*. Directory X:\DoseFractions\"user"\Images-Disc*\

**Make sure "Save frames" is checked!!!**

Exposure time: 2 s; Frame time: 0.05 s; Check "TIFF (LZW compressed)" instead of "MRC file"; Check "Save unnormalized frames even if Gain Normalized is selected".

Check "Save mdoc file" (alternatively, IMOD "clip stat" gives dose/frame as well).

L3. Edit script 10 (for single hole) or script 13 (for multi-shots) to change storage directory

Default defocus range of -3.0 to -1.5 um, step = 0.2;

- Use -2.5 to -0.9 um for lower contrast, better quality data.
- PNCC uses -2.2 to -0.8 um.
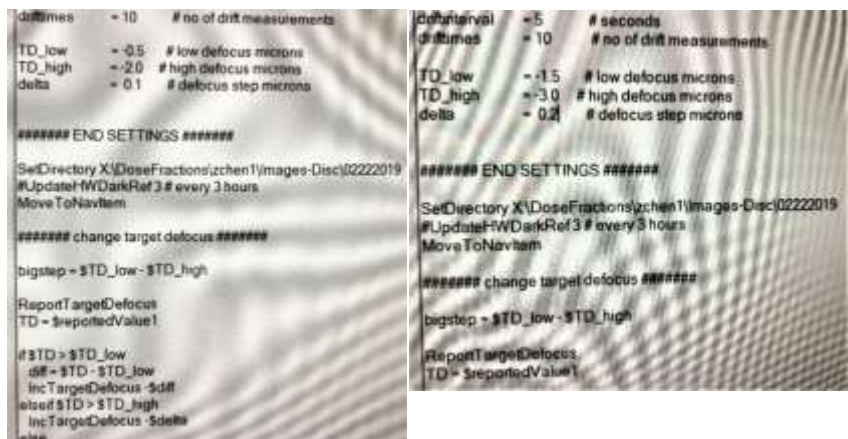
Check, and if needed, re-do direct alignment:

   - *Stigmatism, coma-free on carbon area;*

   - *Preferably with SerialEM;*

   - *Can be done on Arctica PC as well.*

Recheck alignment of features between Record and View mode, and between View mode and the MMM maps (do NOT go to Search/LM mode).

L4. Make sure "File" -> "close" is greyed out.

Make sure "Low dose mode" is checked.

Save navigator.

L5. Menu *Navigator → Acquire at Items*

   - uncheck "Rough eucentricity";

   - Run script "####".

Some pointers:

opening "Imaging States" window under "Nav"->"Open Image states", and add frequently used Mags to the list for easy switching between settings.

If you have to close SerialEM, remember to REDO the hole image -> Buffer P step for the view script (Step J).

SerialEM will blank the beam under Low Dose mode, whenever other programs such as DM tries to take a picture. make sure low dose mode is turned off under SerialEM.

Switching between LM and M/SA lens setup should be avoided during data collection.

Z-height (eucentric height) can be adjusted with FluCAM down. make sure to hit "UPDATE Z" after done on the navigator window.

Objective aperture is only needed when setting up parallel illumination beam (PNCC and Genentech).

- o NanoProbe mode;
- o insert 100um Objective aperture;
- o center the beam;
- o hit "Diffraction Mode";
- o offset the objective aperture (off center to view the Thon rings);
- o use the focus dial to sharpen the edge of the objective aperture;
- o then use the intensity dial to sharpen the rings.
- o repeat the above two steps if necessary.

Objective aperture will filter out scatterings at higher frequencies, which may reduce the highest resolution. it may be useful for increasing particle contrast with thick ice during screening (or particle picking at early stages), for really small particles (under 40kDa), or when using the UltraAU grids (to reduce excessive scattering from gold).