SerialEM data collection with Apollo camera

Updated on 1/18/2023
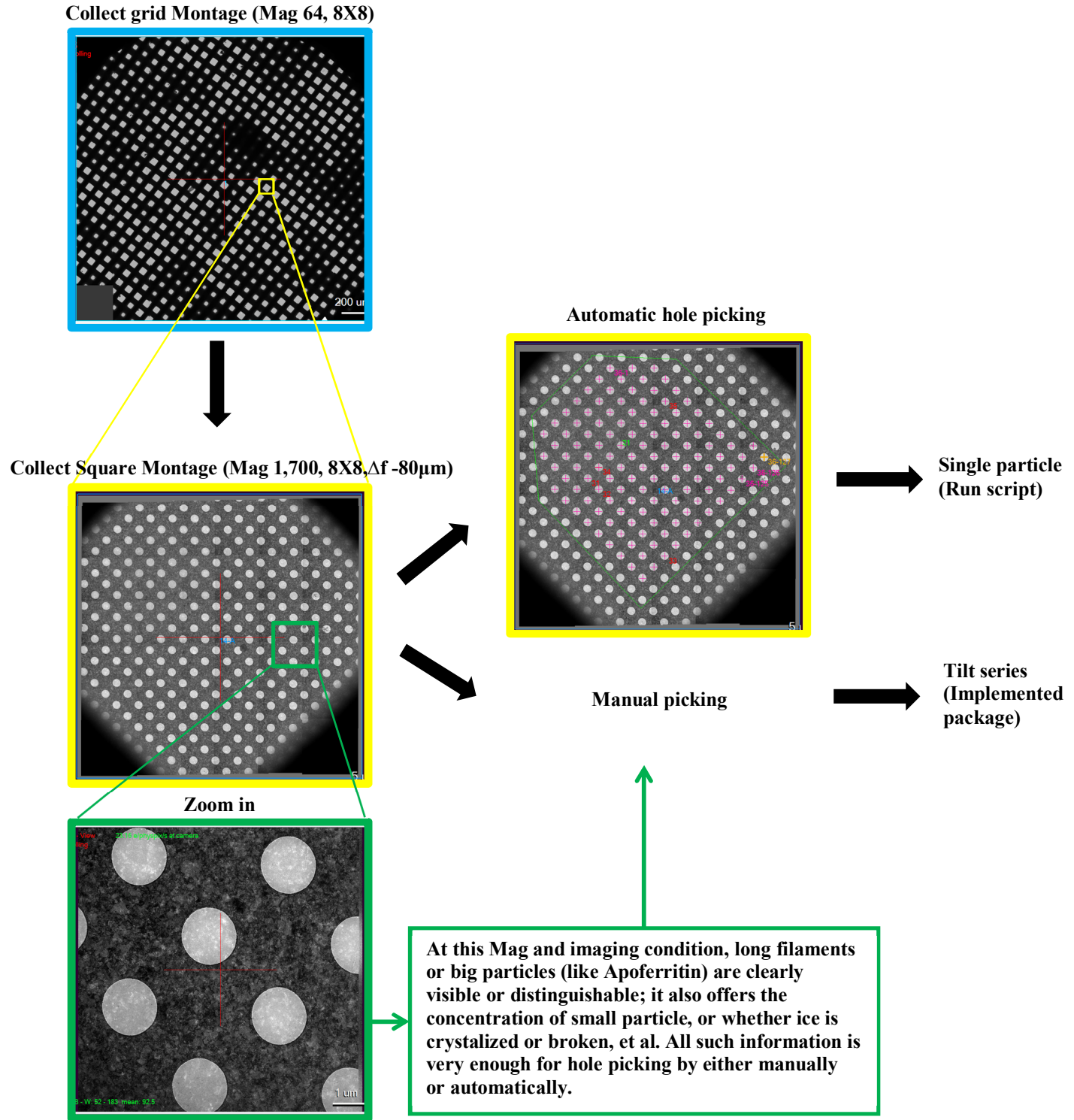**SerialEM version: 4.0.9**

This document illustrates cryo-EM data collection by using software SerialEM and DE Apollo camera at BSIR in Florida State University. It starts with general introduction of SerialEM GUI. Meanwhile some very basic cryoEM concepts of beam and camera are also included to help fresh user to catch up. Those experienced user can skip these and jump to data collection setup. Several unique hole targeting methods are addressed. The data collection splits into Tomography and Single Particle after targeting.

Index

1 Diagram of data collection

Collect grid Montage (Mag 64, 8X8)

Collect Square Montage (Mag 1,700, 8X8,Δf -80μm)

Zoom in

Automatic hole picking

Single particle (Run script)

Manual picking

Tilt series (Implemented package)

At this Mag and imaging condition, long filaments or big particles (like Apoferritin) are clearly visible or distinguishable; it also offers the concentration of small particle, or whether ice is crystalized or broken, et al. All such information is very enough for hole picking by either manually or automatically.

2   SerialEM GUI

1 Top bar

2 Menu bar

3 Control panel

4 Display Window

5 Navigation window

3 Control panel

Camera

Low dose

6 Log window

7 Ongoing actions

2.1 Top bar

The Only information displayed on "Top bar" is the opened files. As in the GUI example shows, `SerialEM - Titan Krios - LMM.st` "LMM.st" is the current opened file. You could have multiple files open at the same time, then they will be shown from left to right, and the rightest one is the current active one. If you save any image through Control Panel: Buffer Controls (2.3.3), it will be saved into the active one. Make sure the image you will save has the same size or binning as that in the active file.

2.2 Menu Bar

2.2.1 Files:
2.2.2 Settings:
2.2.3 Camera:
2.2.4 Calibrations:
2.2.5 Focus/Tune:
2.2.6 Script:
2.2.7 Tasks:
2.2.8 Tilt Series:
2.2.9 Process:
2.2.10 Navigator:
2.2.11 Window:
2.2.12 Help:

2.3 Control Panel

SerialEM has thirteen Control Panels on the left side of GUI; each one can be minimized `-` or floated `F` to relocate anywhere by user. SerialEM will automatically save the new arrangement in its Setting file in about every five minutes and load the arrangement back for the next time. Two Control Panels of Camera (2.3.11) and Low Dose (2.3.10) have been relocated on the right of GUI since they are widely used. The question mark `?` on the right top corner of each panel describes the meaning of each item inside the panel.

2.3.1 Buffer Status:
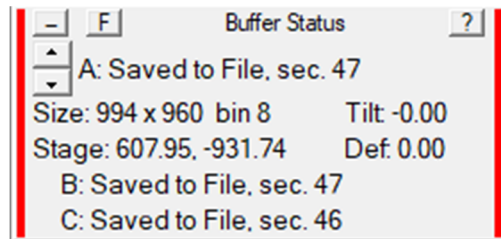
SerialEM saves images temporarily in computer memory and keeps them in a set of buffers for display. The buffer is a rolling system which always stores the newest image in buffer A and rolls previous buffer A to buffer B, and so on. You can use the spin button `▲▼` to navigate and display different buffer images. The information for active buffer image includes image size, binning, tilt angle, defocus value, and stage X&Y. Up to three additional buffer contents are described, for example buffer A, B and C are shown here.

2.3.2 Buffer Controls:
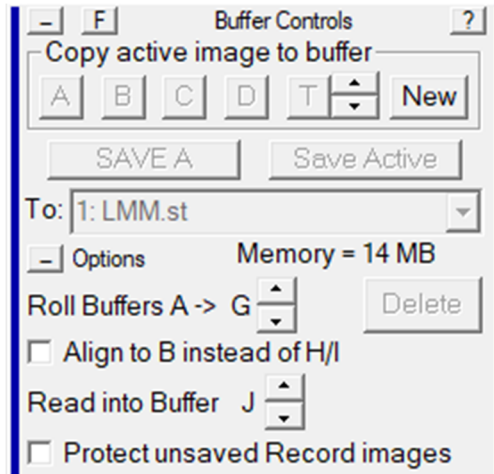
- "Copy active image to buffer" is to copy the currently active buffer to any other buffer.
- You can save buffer A or Active buffer (the one in display window, but maybe not buffer A) to a file. The file should have been opened already, and thus should be displayed on the Top bar (2.1). Opening a file is through Menu (2.2) → File →Open New or Old.
- SerialEM has total 20 buffers from A to T. In order to keep some buffers unchanged during the session, for example, a hole template image, SerialEM saves at least three buffers at the end of buffer system, and allow maximum 14 buffers (from A to N) for rolling. User has the freedom to adjust how many buffers for rolling, for example seven buffers here (A → G).
- "Read into Buffer J" is to allocate which buffer to store the image read from file when you click Menu (2.2) → File →Read. Remember in SerialEM opening a file is to just open a file which will wait for further display or saving more images, while reading a file is to open and display.

2.3.3 Image Display Controls:
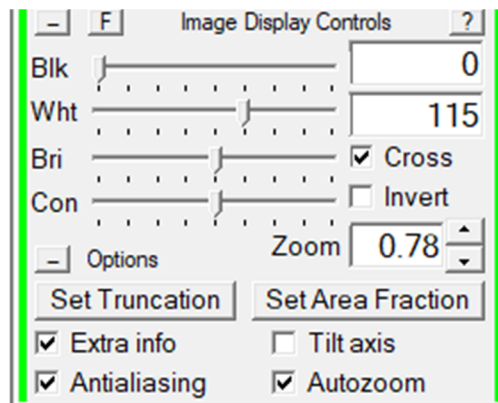
- "Zoom" shows the current zoom factor for image in Display Window (2,4) and it is also adjustable by rolling middle mouse or changing the value here.

- "Set Truncation": values for the percent of pixels that will have their intensities truncated at the low and the high end of the intensity range.
- "Set Area Fraction" : the faction of image will be analyzed.
- "Extra info"displays scale bars, min/max/mean, camera name, and dose rate.
- "Tilt axis" displays the tilt axis which is very useful for tilt series targeting since both Focus and Track area will locate along the tilt axis through Record area.
- "Antialiasing" applies filter to reduce aliasing effect when zooming out images.
- "Autozoom" adjusts zoom factor for different binning automatically.

2.3.4 Microscope: Display a few microscope status
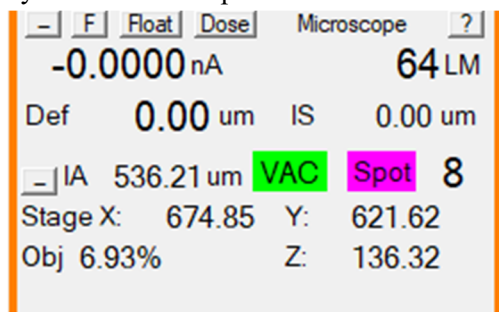
- Dose (nA), magnification, defocus (Def), image shift (IS), Illuminate Area (IA)
- Vacuum status (VAC, green means perfect, yellow means adequate for working, red means do not open the column valve); Spot size (Spot)
- Stage position (X, Y and Z)
- Object lens strength (Obj)

2.3.5 Microscope Control: A few microscope control function
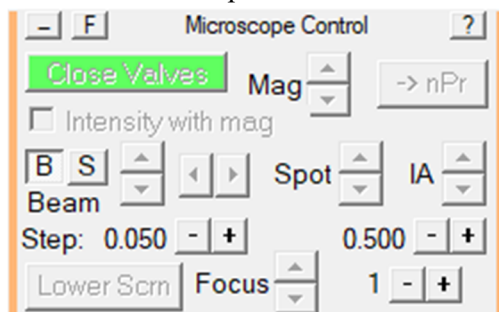
- "Open/Close Valves": green means microscope column valve is open, red is close.
- "Mag": increase or decrease Magnification.
- "Intensity with mag": Turn it on to maintain the same brightness when Mag is changed. It only works when intensities have been calibrated in SerialEM.
- "→nPr": switch between nPr (nano probe) and µPr (micro probe)
- "B" or "S": move beam (B) or stage (S) up or down, left or right, with the step below (0.050 in the example)
- "C2/C3/IA": change C2, C3 lens value or Illuminate Area with the step below(0.500 in the example)
- "Spot": change spot size

- "Raise/Lower Screen": retract or insert Flu screen or camera
- "Focus": change focus value with the step on its right (1µm in the example)
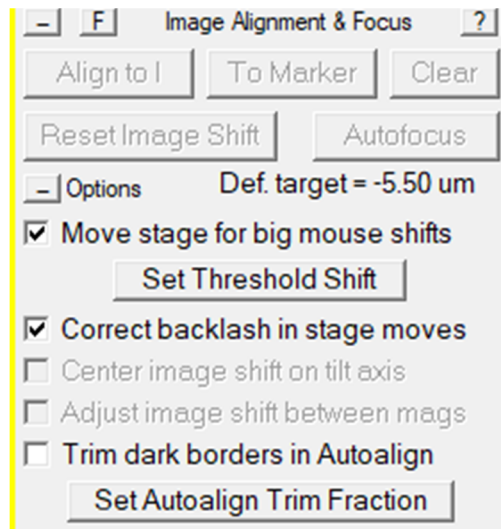
2.3.6 Tilt Control:
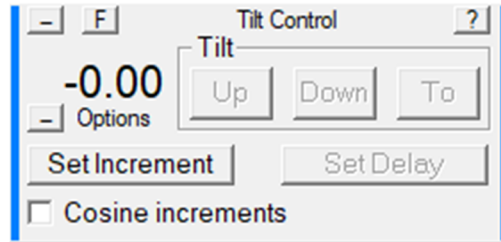
- Tilt microscope stage with value in "Set Increment" Up or Down, or directly to a certain angle with "To"
- "Set Delay": Stage will delay certain time before tilting for the next increment.
- "Cosine increments": select cosine tilt increments in which the increment is proportional to the cosine of the tilt angle.

2.3.7 Image Alignment & Focus:

- "Align to" will align the image in buffer A to the image in the indicated buffer.
- "To Marker": "Marker" here is usually a small green cross you add when you left click on image. It is often a feature spot or some places you would like to move to. "To Marker" will move image or stage so to center on the "Marker".
- Drag image with right mouse can shift image to anywhere you would like to. SerialEM has a combined mechanism for moving position: small amount of movement with image shift, big amount of movement with stage shift, by turning on "Move stage for big mouse shifts". "Set Threshold Shift" sets the threshold of a fraction of image size and an absolute shift value in microns to determine that image shift will be used when lower than the threshold, stage shift will be used when

higher than the threshold. Holding Shift key while shifting with the right mouse button will always trigger to use stage shift.

- Image autofocus in SerialEM has two steps: the first is to set up focus target in Menu → Focus → set target, the second is to Menu → Focus → Autofocus, or use the button Autofocus here.

2.3.8 Montage Control :
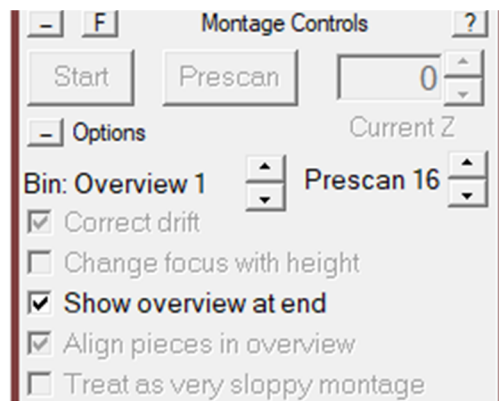
- Simply speaking, Montage is a super image composed of multiple pieces of images. Generally, SerialEM collects two Montages, one is low magnification montage, called LMM, which is to scan the whole grid with Search beam, usually in LM; the other is medium magnification montage, called MMM, which is to scan a grid square with higher magnification (View beam) where not only the ice thickness but also the sample details can be distinguished and targeted for data collection.
- "Start" button is to start a Montage collection at the current position. The Montage parameter should have been set in Menu → Files → Montage Setup, and Montage file should have been created and opened as seen at Top Bar.
- "Bin Overview" is to bin the Montage when it is too big. Change the binning factor here and double-click the corresponding map from Navigator window (2.5) to reload it.
- "Prescan" is to collect a quick scan of the region being montaged with the "Prescan Bin"
- SerialEM allows saving multiple images or multiple montages in a same file. For example when you run batch middle mag montage collection you will only need to open one file to save them all. The order of each montage is called "Z" section here, which starts from 0. Here "Current Z" number means the section number for the next montage to save. You can overwrite a montage by pointing to the Z number where the old montage file exists.
- "Correct drift" is only helpful when there is big drift during montage is collected, especially for plastic section sample (I guess), not for cryo sample. Basically it will use the misregistration between a pair of successive pieces to predict and correct for the error in position of the next acquired piece.

- "Change Focus with Height" will change focus for every piece that is acquired from a tilted specimen.
- "Show Overview at End" is to show the stitched overview in buffer B when the montage is completed.
- "Align pieces in overview" is always on. The individual piece of montage usually doesn't stitch perfectly without being aligned to each other. So each piece will be aligned to its neighboring pieces based on the overlap area which was set in Montage Setup (3.3.5). Sometime it is not only shift the image but also stretch the piece image to reach a seamless overview, so the overlap percentage in Montage Setup is very important.
- "Treat as very sloppy montage" sometimes helps for very badly aligned overview. Most of time it doesn't work.

2.3.9 Direct Electron Camera:
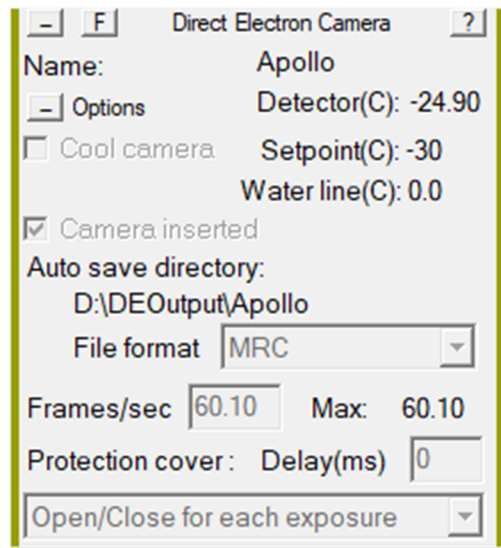
- All information is pulled out from DE server, so if you would like change anything go to DE computer for its server page.

2.3.10 Low dose control: low dose beam settings including intensity (Mag, spot size, illuminate area), focus position, beam alignment, and so on.
- "Low Dose Mode" has to be turned on to enable all beam settings below. The next line shows the setting of current active beam which corresponds to the beam in "Go To". The example here shows the View (Vie.) beam with its Mag, spot size, and illuminates area.
- "Continuous update" should be on whenever you are about to change your beam intensity, especially at the beginning when you start a new session. It will update the current beam immediately from the microscope. It is important to remember to turn it off when you finish adjusting beam intensity. It will save any changes on magnification, spot size, brightness (illuminate area), diffraction focus in diffraction mode, energy filter setting, probe mode (micro or nano probe).

- "Define position of area" is to define the position of Focus or Trial relative to Record. Focus is the beam for Autofocus. Trial is the beam for tomogram tracking, which is usually set as the same as Focus by selecting "Keep Focus and Trial identical" at the bottom Options. Obviously you have the freedom to set Focus and Trail with different beam and position under certain circumstance. Particularly for tomogram Focus has to set along microscope's tilt axis through Record area since only the tilt axis keeps unchanged on Z height when grid is tilted. So the next line shows the distance of Focus apart from Record by the value in "Position on tilt axis". You can either manually give the value here or click on buffer A image to define one. For single particle you are allowed to choose Focus not along the tilt axis by unchecking the option "Rotate inter-area axis" at the bottom of this panel. The next line is the value of "Maximum area separation" which tells the distance between Focus and Record before they will overlap.

- "Go To" is to send a beam setting to the microscope. A beam setting includes beam intensity, beam shift (relative to Record), beam defocus (for View). "Go To" is often used when you set up the beam at the beginning together with "Continuous update" is selected. The current beam setting will be saved before you click to switch another beam. Here is a list of beam setting for cryo-EM data collection (example for data collection at 7,5000x):
  - Sea. (Search) :   64x, sp 9, IA : 536 μm : LMM grid overview
  - Vie. (View)   : 1,700x, sp 9, IA : 20.3μm : MMM square overview, targeting
  - Foc. (Focus)   : 75,000x, sp 6, IA : 735μm : Autofocus
  - Tri. (Trail)   : 75,000x, sp 6, IA : 735μm : Tomogram tracking
  - Rec. (Record) : 75,000x, sp 6, IA : 735μm : Exposure of data collection

- "Additional beam shift" is to apply beam shift to current active beam (in green) to Record beam. It works for all beams except for Search. Usually this function is used to align View to Record since we often have same beam setting for Focus, Trial and Record. The current beam shift value usually has been shown on the next line right after Reset button. Reset button is to clear the existing beam shift. You would like to have "Set" checked and then center the beam on the microscope using MF-X&Y,
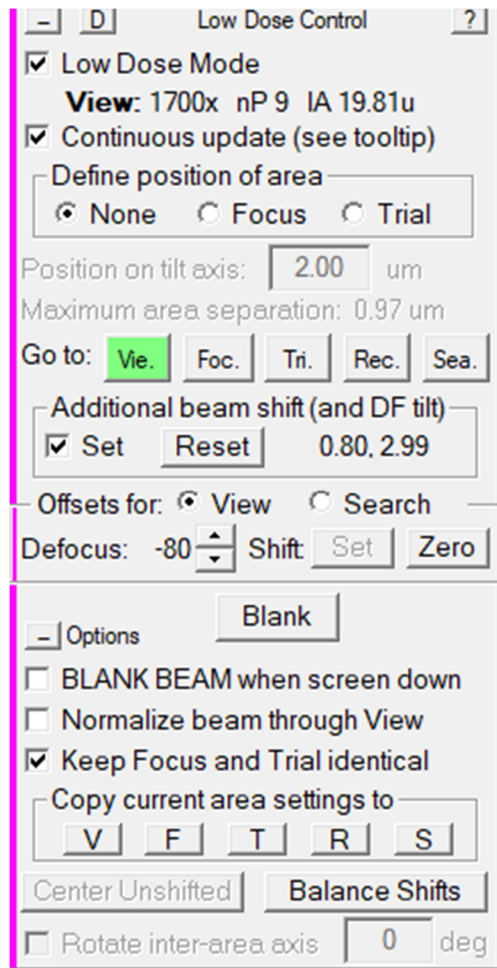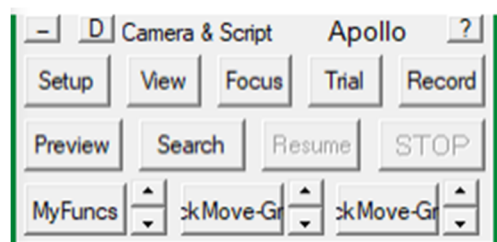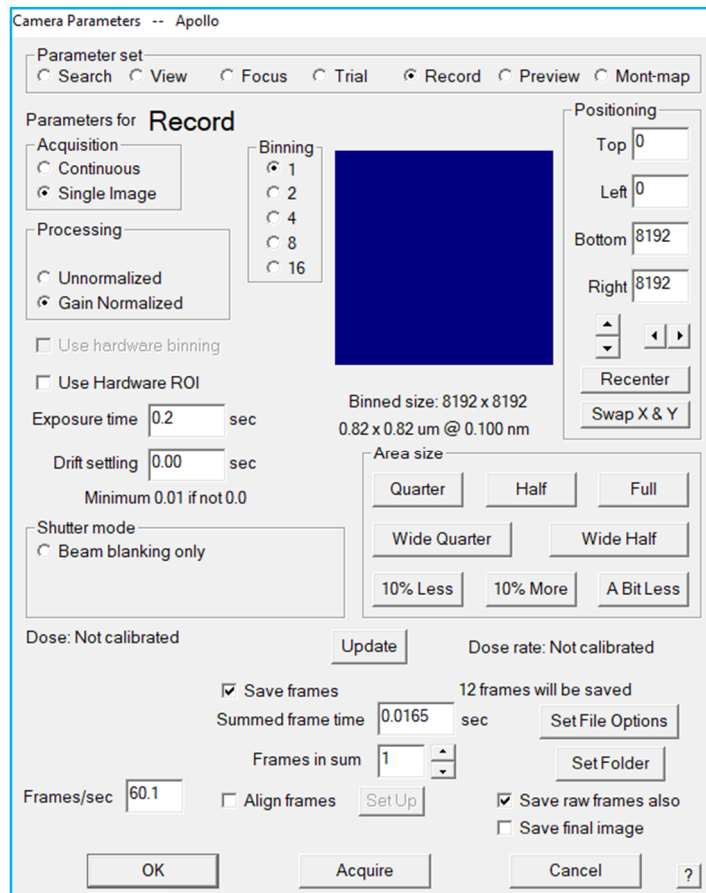
then the beam shift will be updated to the current active beam. We usually don't set beam shift for Record, if it is not centered; go microscope → Tune panel → Direct alignment → beam shift → center beam with MF-X&Y. Centering beam inside and outside Direct alignment is different.

- "Offset for" and "Defocus" below is to set up an extra defocus for the beam you choose here, View or Search. We usually set -80μm defocus to View. A lesson for fresh use is that SerialEM always assume current focus on the microscope is zero when you enter Low Dose Mode. Very often if you go to Low Dose Mode when the beam on the microscope is previous View with -80μm defocus, then SerialEM will assign -80μm to Record, Focus and Trial, and -160μm (additional -80 μm on -80μm or Record) to the View beam. It will cause a lot of odd phenomena. One way to correct defocus error for Record is to Go To the Record and click Eucentric Focus on the microscope right control panel.
- "Blanked or Unblank" shows the current status of beam blanker. You can manually switch it for certain purpose.
- "Blank beam when screen is down" will prevent specimen from exposure while Flu screen is inserted.
- "Normalize beam through View" means to compensate for the lens hysteresis that makes the beam position depend on the recent history of intensity changes. Usually turning it on can make beam more stabilized, especially switching between Mag and spot size.
- "Keep Focus and Trial identical" will have Focus and Trial the same.
- "Copy current area setting to" is copy the current beam to overwrite any other beam. It is useful when you have finished Record setting and can simply copy it to Focus (F) and Trial (T) here.
- "Balance Shifts" is to let Record and Focus&Trial share the image shift between them, which will cause one is on the left and the other on the right. Unclear the usage yet.
- "Rotate inter-area axis" shows the Focus angle if the Focus is set to not along the tilt axis.

2.3.11 Camera & Script: camera setting including binning, exposure time, camera area to use, and so on, and shortcuts of three serialEM scripts

Camera Parameters -- Apollo

Parameter set
○ Search ○ View ○ Focus ○ Trial ● Record ○ Preview ○ Mont-map

Parameters for **Record**

Acquisition
○ Continuous
● Single Image

Binning
● 1
○ 2
○ 4
○ 8
○ 16

Positioning
Top 0
Left 0
Bottom 8192
Right 8192
Recenter
Swap X & Y

Processing
○ Unnormalized
● Gain Normalized

☐ Use hardware binning
☐ Use Hardware ROI

Binned size: 8192 x 8192
0.82 x 0.82 um @ 0.100 nm

Exposure time 0.2 sec
Drift settling 0.00 sec
Minimum 0.01 if not 0.0

Area size
Quarter   Half   Full
Wide Quarter   Wide Half
10% Less   10% More   A Bit Less

Shutter mode
○ Beam blanking only

Dose: Not calibrated   Update   Dose rate: Not calibrated

☑ Save frames   12 frames will be saved
Summed frame time 0.0165 sec   Set File Options
Frames in sum 1   Set Folder
Frames/sec 60.1   ☐ Align frames Set Up   ☑ Save raw frames also
☐ Save final image

OK   Acquire   Cancel   ?

- "Setup" will open the "Camera Setup dialog box" (above) and allow you to set parameters like exposure time, field of view and binning for six different exposure modes: View, Focus, Trial, Record, Preview and Search.
- Clicking each button of "View", "Focus", "Trial", "Record", or "Search" will do two things: the first is to send the beam which is linked to its corresponding beam in "Low dose mode" (2.3.10) to microscope, for example "View" button will send "View" beam, "Focus" will send "Focus" beam, Record and Preview will send "Record" beam; the second is to take image with the its camera setting here (exposure time, binning, and so on). So be aware of the difference between here and in "Low dose mode" where it only send the beam to microscope but doesn't take exposure.
- Three buttons on the bottom of this panel are shortcuts of certain existing scripts you have set up. Again SerialEM is specifically written for tomography data collection, so only the tilt series has a complete setup page, while for single particle or any other particular data collection you will need to run it through Script. SerialEM has its own language system and its special terms to control microscope and cameral, which covers everything you need to complete a task. You can go to SerialEM Menu → Script → Edit script to write your own or copy from others to past here to run. Here is all commands you can have for SerialEM script (https:bio3d.colorado.edu/SerialEM/hlp/html/script_commands.htm)

- "Resume/Pause/End Nav": If a script has been running and was stopped, this button will resume the script at the next step. If the Tilt Series Controller is active, the button will open the Tilt Series Resume dialog box. When acquisition of tilt series is being run from the Navigator, the button is labeled End Nav while the tilt series is running and will stop the Navigator acquisition at the end of the current tilt series. If a tilt series run from the Navigator or a script prior to a tilt series is stopped, the button is labeled ResumeT if it will resume the tilt series, or ResumeS if it will resume the script. When other acquisitions are running from the Navigator, the button (labeled PauseN) will pause the acquisition after the current item, and it can then be resumed with the same button, labeled ResumeN.
- "Stop/End Nav/End All": This is a general Stop button for image acquisition, autofocus, montaging, scripts, tasks, tilt series, or any other extended sequence of actions. It will generally stop the activity as soon as possible after the current action. When you are running a tilt series or a script from the Navigator, the Stop button acts on the lowest level of operation only, so it can leave a tilt series or script in a suspended state. However, once such a tilt series is stopped, the button is labeled EndNav and will end the Navigator acquisition after the current item; and if a script is stopped, the button is labeled End All and will stop acquisition immediately. Try to not Stop SerialEM during Autofocus step which often crashes SerialEM!!!
- "Camera Setup dialog box"
  - Parameter set: Search, View, Focus, Trial, Record, Preview, Mont-map. Preview uses the same beam as Record but with very short exposure time, so it can take a very low dose exposure at the imaging area, which is very helpful for tomogram data collection that user can analyze if the area is what they want before proceeding. Mont-map takes a montage with an existing montage setting.
  - Acquisition: Usually all beams are set to take "Single image", but in certain case for example you would like to do live imaging for live FFT to check astigmatism or coma-free, it is helpful to set "Continuous" with very short exposure time. Remember to not use Focus beam for this purpose unless you have set Focus position as 0 in Low Dose Control Panel (2.3.10).
  - "Binning": value "1" points to super resolution mode. For example Apollo camera is physically 4K*4K, and its super-resolution mode is 8K*8K, so all binning factor is based on the super-resolution mode. Remember all pixel sizes or dose calculations are based on the real physical pixel size of 4K*4K. In general a pixel size we mention for certain Mag is the real physical pixel size, as is the one written on the white board in Titan room.
  - "Processing" allows taking exposure before or after gain correction (or normalization): Unnormalized or Gain Normalized. Since here gain correction is done on DE server for Apollo camera so serialEM always receive gain corrected integrated image and non-gain corrected frames according to DE server setting. The gain-corrected images is 32-bit float MRC, and non-gain corrected frame is 8-bit integer MRC of super resolution mode since it only saves the electron counts.

➢ "Exposure time" is the exposure time; "Drift settling" is a special setting that allows the sample being exposed to beam for certain time before exposure, it usually helps the plastic sectioning sample to stabilize, and can be as long as hours.

➢ "Shutter mode" and "Beam blank only". The microscope has two shutters, one is above the specimen called beam blanker, and the other is below the specimen and above the camera called post blanker. In general Low dose cryoEM only use beam blank meaning beam will only not be blanked during exposure to illuminate on specimen and camera.

➢ "Dose" is calibrated through DE server so no need to calibrate in SerialEM and the dose information will be borrowed from DE server and display on "Display Windows" (2.4).

➢ "Positioning" is to allow user to choose which camera area to image. You are able to manually adjust the range by "Top", "Left", "Bottom", and "Right". "Recenter" will keep the size you have chosen and center the area on the camera. Or, you can use any of those pre-defined area: "Quarter", "Half", "Full", "Wide Quarter", and "Wide Half". Furthermore you can make adjustment based on whatever you have selected by "%10Less", "10% More" or "A Bit Less". The blue windows at its left show the location and area you have selected on the full camera. The bottom of blue window tells the image size after binning, its image area on sample with its pixel size at that binning.

➢ At the bottom of this dialog box is the internal camera setting and frame setting. For Apollo camera "Frames/sec" is a fixed number of 60.1. SerialEM will give error if it is changed. Since the "Frames/sec" is fixed, the "Summed frame time" is 1/(Frames/sec) = 0.0165 is also fixed. User can choose whether to save frames and set up the folder by "Set Folder", and set up the format of file name by "Set File Options".

2.4 Display Window

2.4.1 Display Window shows the image from active buffer with extra information including:
- Does rate and scale bar if you choose "Extra info" in Image display control (2.3.3)
- Center cross in red if you choose "Cross" in Image display control (2.3.3)
- Tilt axis if you choose "tilt axis" in Image display control (2.3.3). Tilt axis is quite important when you set up focus along tilt axis for tilt series data collection.

2.4.2 Sometimes you need to see the FFT or Live FFT of image in buffer A, Go to Menu → Process → FFT or Live FFT (for continuous imaging in Camera Setup dialog box). You can choose to let the FFT displayed side by side to the image.

2.5 Navigator Window
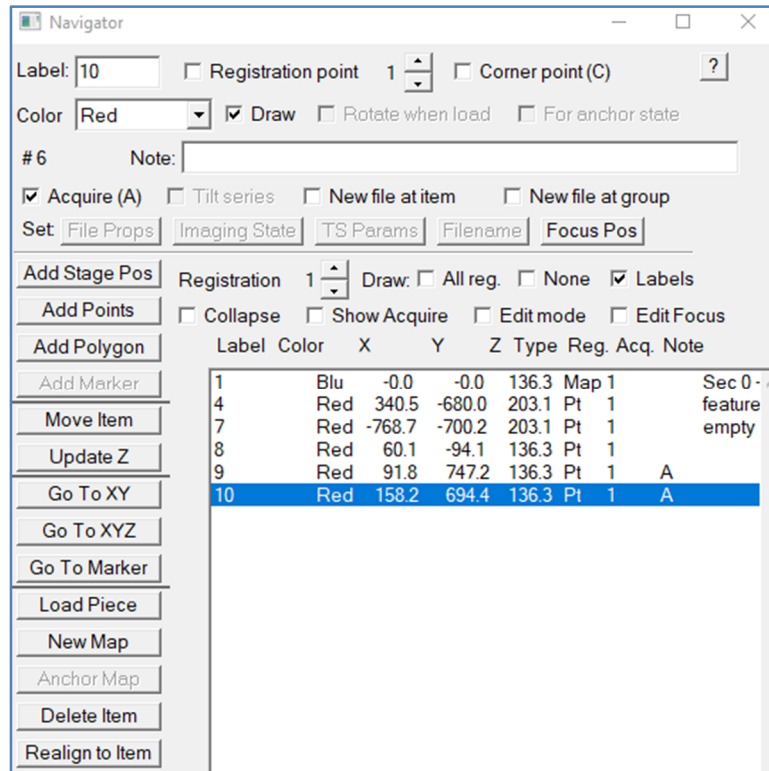
2.5.1 Navigator Window can be understood as simple as a list of stage coordinates, but the content linked to a coordinate can be a montage map, a polygon, or a point. Not only that, Navigator also keeps track of the relationship between items, for example, it can tell which MMM map the point is added from, the location of the MMM map on the LMM map, and so on. Navigator Window is the place where user do hole targeting as the starting point of data collection. Every object on the Navigator list is called "item" here.

2.5.2 Go SerialEM Menu → Navigator → Open to open a new empty Navigator window. You can't save the navigator as file unless you have at least one item in there. It is a good habit to Menu → Navigator → Save As a file after LMM is finished and a corresponding map is made (New Map) in Navigator Window, then SerialEM will constantly update the navigator file during your operation in Navigator Window, so that you won't loss all navigator content in case SerialEM crashes.

2.5.3 "Label" is assigned to item created by order as sequential numbers. User is free to edit it to anything else more meaningful. It will also be shown on the map in Display Window (2.4).

2.5.4 "Registration Point" is the registration number for grid. It is very helpful in case you unload and load back the same grid; you can use the "Registration Point" to shift the coordinates from the previous loading to current.

2.5.5 "Corner Point" is to make a point as a corner of an area for which you want to construct a montage.

2.5.6 "Color" is the label color which can be changed here. By default, single points are red, drawn polygons are green, maps are blue, and super-montage polygons are yellow. There is a special option "NoRealign" here either to prevent a map from being used for the first stage of the "Realign to Item" routine, or to prevent a higher magnification map from being aligned to in a second stage when realigning to that map. One time I had a good experience with this function, for certain reason I made two MMM map for the same square, then when I did "Realign to Item" for one point from one map it went to nowhere and probably even crashed SerialEM. The solution is just to select "NoRealign" for the previous MMM map so to avoid confusion.

2.5.7 "Draw" is whether or not to display the label.

2.5.8 "Rotate when load" will reload map automatically rotated into the same orientation as images at the current magnification.

2.5.9 "For anchor state" is to mark a map item as a template for acquiring further medium-magnification maps that will be suitable for realigning to.

2.5.10 "Item #" is different from "Label", the Item number is the real order number, is automatically assigned to, cannot be changed.

2.5.11 "Note" is a good place where you can put any notes during data collection setup.

2.5.12 Acquisition Controls: After points (or targets) are added into Navigator list user will set them either with "Acquire (A)" or "Tilt Series" for the next step of data collection. The next step is to SerialEM Menu → Navigator → Acquire at point →more options.

- "Acquire (A)": set points as further acquisition for Single Particle, montage, or any other batch acquisition through certain scripts.
- "Tilt Series": set points for tilt series collection. The tilt series setup page will show up.
- "New file at item"
- "New file at group"
- "(Set) File Props":
- "(Set) Imaging State":
- "(Set) TS Params":
- "(Set) Focus Pos":

2.5.13 Controls of General Behaviors:

- "Current Registration": starts at 1 and should be increased every time the grid is repositioned in the holder or the holder is replaced in the microscope.
- "(Draw:) All Reg.":
- "(Draw:) None"
- "(Draw:) Labels"
- "Collapse Groups": items that are added together by various means are marked as belonging to a group with a unique ID number. Use this check box to have each group summarized by a single line in the table.
- "Show Acquire Area": turn on drawing a yellow box representing the current acquisition area.
- "Edit Mode": go to edit mode where the mouse can be used for editing points in many useful ways. Go to help for more information.

- "Edit Focus": Turn on this check box to be able to visualize and adjust the position of the Low Dose Focus area.

2.5.14 Action Buttons:

- "Add Stage Pos": Add current stage point as a point item
- "Add Points": add point with left mouse click; press backspace to delete the points backwards one by one; adjust the last added point with right mouse, not forget to click this button again to Stop Adding.
- "Add Polygon": draw a polygon by clicking points in an image with the left or middle mouse. The polygon can be used to define an area to be montaged. Push the button again when you are done adding points. Press backspace to delete the points backwards one by one. Adjust the last added point with right mouse.
- "Add Marker": The marker is the green cross that is deposited on an image by clicking with the left mouse.
- "Move Item": Use this button to reposition the current item by clicking a new center position for it in an image with the left or right mouse button.
- "Update Z": update the current item Z with current Z on stage, you can update a whole group of items by one click.
- "Go To XY": move stage to the item X and Y
- "Go to XYZ": move stage to the item X, Y and Z.
- "Go To Marker": move stage to the maker (green cross)
- "Load Map": load in the image for a map
- "New Map": define the current image as a map
- "Anchor Map":
- "Delete Item": delete an item from the list
- "Realign to Item": start a routine to return accurately to the position of the current item by cross-correlation with an appropriate map.

2.5.15 Item List Box

2.6 Log windows

2.6.1 Log window record all log information when SerialEM starts to run. It saves very detailed information for any action happening in SerialEM. It automatically starts and saves a new log file for each tilt series collection. You can open a new one if you accidently close it at Menu → File → Open Log.

2.7 Ongoing action

2.7.1 It shows the coordinate where your current cursor is and any ongoing task. For example it will show which piece it is collecting for LMM, what is doing for Eucentricity. Most important it is a good place to tell whether SerialEM has crashed or is still running; the other is Stop button in red in Camera&Script (2.3.11) when SerialEM is running.

3   Set up beam and camera

3.1 Pre-actions

3.1.1 Close Leginon clients both on Titan computer and DE computer

3.1.2 Restart DE server (skip this step if you are continuing running SerialEM)

3.1.3 Titan computer: run SerialEM client (C:\Program Files\SerialEM\FEI-SEMserver.exe) to enable SerialEM to control Titan microscope.

3.1.4 DE computer: Desktop → SerialEM.exe → right click on the icon and "run as administrator").

3.1.5 Titan microscope: The right Control Panel → push button "Eucentric Focus" before entering Low dose in SerialEM. This step is not necessary but you must ensure the current focus on microscope is not too off from zero focus.

3.2 Set up beam condition and camera settings.

3.2.1 Go to Low Dose Control Panel (2.3.10); have "Low Dose Control" checked.
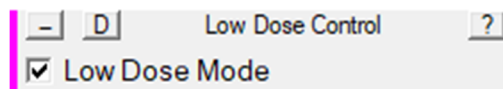
3.2.2 Have "Continuous update" checked in Low Dose Control Panel (2.3.10) to enable SerialEM update beam to any changes from microscope. Remember to uncheck it once you are satisfied with the beam setting afterward.
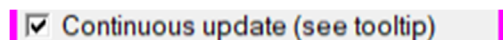
3.2.3 Click the "Rec." button in Low Dose Control Panel (2.3.10) to send Record beam to microscope, or load current beam from microscope to Record if it is the first time.
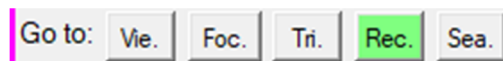
3.2.4 Adjust Record beam on microscope, specifically, the Intensity button (Left Control Panel on microscope), spot size (R3 button on Right Control Panel) to reach to the desired beam intensity and size (illuminate area, IA) after magnification is determined (Magnification butter on Right Control Panel).

- Beam intensity is usually determined as dose rate which can be measured on DE server on DE computer. 30e/p/s is recommended for Apollo camera.

- Beam Size (or illuminate Area) is set as small as possible to maximize number of exposure in one hole, and at least bigger than camera. One criterion to ensure that

beam size is big enough is to take an image on DE server and make sure no fringe rings shown up on the image.

- Center Record beam to camera by microscope GUI → Tune panel → Direct alignment → Beam shift with multi-function XY button on both Control Panels on the microscope. Apollo camera center is the green cross on Flu screen (or camera). Centering beam through Direct Alignment on microscope will make it more stabilized during data collection, and this centering method can only be used once in one set of magnifications. For example usually Record (Focus, Trial) and View both belong to SA set, thus once you have centered beam for Record from Direct alignment, you can no long use the same way to center View beam. You will need to center directly with multi-function button when they are assigned to beam shift.

- Adjust Condense astigmatism on microscope GUI → Stigma → Condense → Multi-function button if necessary. Copy Record beam to Focus and Trial by clicking F and T buttons in Low Dose Control Panel (2.3.10). Usually choose "Keep Focus and Trial identical" for tomogram data collection.
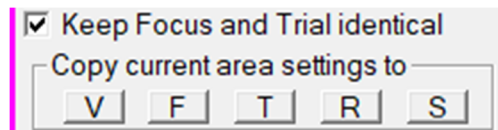
3.2.5 Click the "Vie." button in Low Dose Control Panel (2.3.10) to send View beam to microscope.

3.2.6 Adjust View beam (Mag: 1,700, IA: 20.3µm, Spot : 9; Δf = -80µm)

- View beam is pretty strict here and no much space to change since we have to satisfy two goals: one is beam is bigger than camera when Obj. aperture is inserted, the other is that -80µm defocus is applied. So the beam will very centered when Obj. aperture is inserted, but shift to very right when Obj. aperture is out. So keep Obj. aperture inserted when adjusting View beam.

- Adjust microscope to: Mag: 1,700, IA: 20.3µm, Spot : 9

- Center View beam directly with Multi-function button for beam shift. Remember to have "Additional beam shift: Set" checked before shift the beam so that SerialEM can remember the image shift value (0.8, 2.99 in the example below) in micron relative to Record beam. You don't have to uncheck Set before switch to other beam since SerialEM will automatically save it before beam switch.
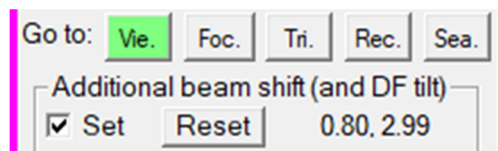
- Apply extra defocus (-80µm) for View beam to increase contrast for View image. In Low Dose Control Panel (2.3.10) have "Offset for View" checked, Use the up and

down scroll to -80, and click "Set" button. View beam will change a lot on position and size, so better do this step even before adjusting View beam intensity and centering.
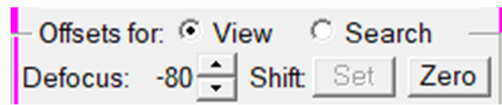

3.2.7 Adjust Search beam (for collecting low magnification montage, LMM, for whole grid overview)

- Sent Sea. beam to microscope after taking out Obj. aperture

- Set Mag 64, sp 9, IA 536 μm

- Center beam directly with Multi-function button for beam shift

3.2.8 Have "Continuous update" unchecked in Low Dose Control Panel (2.3.10) to avoid of beam adjustment by accident.
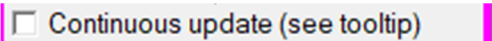
3.2.9 Set up binning, exposure, field on camera to use, and so on in Camera & Script control panel (2.3.11). Click "Setup" button to set up for all beams.
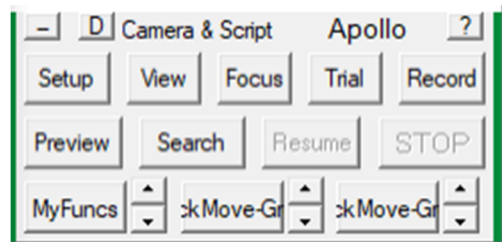
3.3 Collect LMM (overview) of whole grid

3.3.1 Load grid

3.3.2 Find an area close to grid center for rough Z Eucentricity.

3.3.3 Take a View image Camera & Script (2.3.11) → View button; make sure image shows enough details, not too strong or two weak.

3.3.4 Menu → Task → Rough Eucentricity: You will find microscope stage tilts and Z height changes in Log Window (2.6) and Ongoing Action (2.7). Occasionally if you haven't loaded grid on the right orientation in cassette or grid is very bent, auto Eucentricity might fail. In this case you can manually change Z to 100μm (microscope GUI → Stage panel → type 100 in Z and click Goto) and run this task again.

3.3.5 Menu → Navigator → Montaging & Grid → Setup Full Montage → Montage Setup page (as the example here); → File properties page: keep all as default, name output file as LMM.st in your project folder → then go to Montage Control Panel (2.3.8) → Start. One thing you should check is that in Montage Setup page you should be able to select "User Search parameters in low dose mode", otherwise it means that the previous montage collection was "User View parameter in low dose mode" which has been remember by SerialEM. The way to correct this is before collecting LMM, go to Menu → File → Montage setting → choose "Use Search parameter in low dose mode", ignore all other setting for this moment, click OK → then click Cancel on File Properties page → then SerialEM will remember "User Search parameter in low dose mode". Start over again you should see all parameters are correctly shown as the example; the only parameter you should change is "Minimum overlap", set it to least value 2% to save time. SerialEM will collect 8*8=64 pieces to complete the LMM in about 12mins. At the end you will be asked whether to make a new map, answer yes. Remember in SerialEM all hole targeting or point adding are based on map, which could be created from the LMM, MMM or even a single image. Once the map is created it will be shown on the Navigator list, and then you no longer need the LMM.st or MMM.st file, which can be closed once the map is created. Close file: Menu → File → Close and they will disappear from Top Bar (2.1). Save Navigator information as file: Menu → Navigator → save as. SerialEM will automatically update the navigator file once a while. It is a good habit to save this file so you can reload it (Menu → Navigator → open) since SerialEM may lose many setting including Navigator list when it rashes.
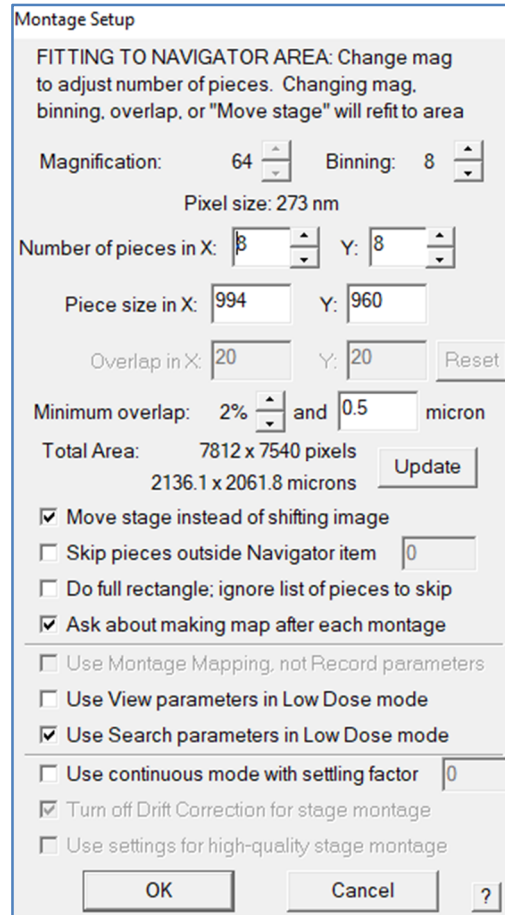
3.4 Align Search and View area: You can follow the same way as 3.5 Align View and Record area. The method 3.4.1 to 3.4.6 is for older version of SerialEM.

3.4.1 In Navigator window, find an area with a big junk which should be visible at both Search and View mode. Add Point at the big junk from LMM map. Remember to click "Stop Adding" otherwise you can't continue to operate in Navigator window.

3.4.2 Navigator Window → Go To XYZ → Menu → Task → Rough Eucentricity (can skip) →Take a View image by Camera & Script → View in Low Dose control Panel (2.3.10)

3.4.3 Uncheck "Move Stage for big mouse shifts" so any shift latter will be done only with image shift, not with stage shift, because we will need to calculate the image shift between Search and View area (the example below is reversed)
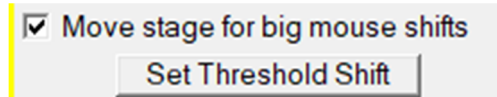
3.4.4 Hold right mouse to drag the View image towards the junk. Repeat dragging and View since sometime the junk is out of view then you will have to drag several times to have the junk appear on the View image. Click on the junk with left mouse will come up a green cross, called "marker" in SerialEM.

3.4.5 Make sure the point you added earlier in Navigator for the junk is highlighted (displayed on blue background compared to other items on the list), then Menu → Navigator → Shift to Marker. Then Search and View area are aligned.

3.4.6 Remember to check back "Move Stage for big mouse shifts"

3.5 Align View to Record area

3.5.1 At the same square, find a smaller feature which should be visible on both View and Record mode. You can go to other area but then do not forget to run Eucentricity again.

3.5.2 Find a way to center on the feature on Record mode. I prefer to find and center the feature on Flu screen by moving stage. If you try to center the feature in serialEM then make sure there is no image shift (IS 0.00 um on Microscope Control Panel 2.3.4). The way to clear image shift is to convert it to stage shift by "Reset Image Shift" in Image Shit Control Panel 2.3.7. You will have to repeat "Reset Image shift" → take Record image → drag image to center at Record image → "Reset Image shift" a few cycles until it stabilizes. Sometimes you might also need to run Autofocus before taking Record image (Menu→ Focus → Set target (-2~-3μm); → Autofocus).

3.5.3 Take a View image.

3.5.4 Hold right mouse to drag and center on the same feature, you will see a non-zero Image Shift created at (IS on Microscope Control Panel 2.4.5).

3.5.5 Go to Low dose control Panel (2.3.10) → Click "Set", the Image shift will be cleared to zero and a new centered image will be taken.
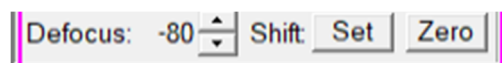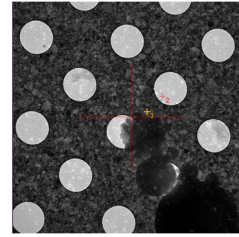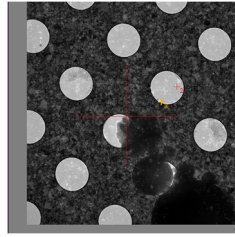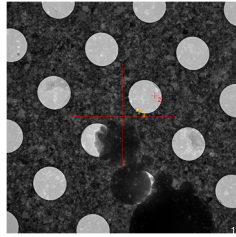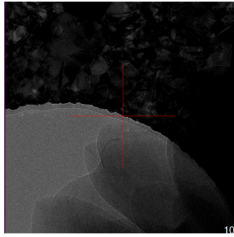
Take Record (IS=0) →   Take View   →   Drag to center; Set   →   Retake View itself

3.5.6 It is good idea to save the feature coordinate in case you would like to redo this alignment: Navigator Window → "Add Stage Point"

3.5.7 You can also center Obj. Aperture here.

3.5.8 You can also do beam alignment now, or wait after you future optimize your Record beam.

3.6 Do gain correction and further optimize Record beam

3.6.1 Gain correction has to be done through DE server on DE computer, as can be done earlier in Low dose Record beam setting (3.4.5), or here.

3.6.2 Obj. aperture can be inserted after LMM is complete, but it doesn't affect much for gain correction.

3.6.3 Add Point on an empty area from LMM map, better at the center of empty square; → "Go To XY" in Navigator Window.

3.6.4 Quite SerialEM, the beam will automatically go to Record beam in low dose mode.

3.6.5 Go to DE server → Imaging panel → Reference → Take Reference → Trial

3.6.6 Read dose rate from Image Stats → e⁻/pixel/s: adjust beam intensity to your target, which is usually 30 for Single Particle. The example is 20.048 e⁻/pixel/s.

3.6.7 Record the spot size and IA, and update them in low dose Record beam after you restart SerialEM later.

3.6.8 Calculate exposure time: for example for dose rate of 30e/p/s, pixel size of 0.92Å/p at Mag 4,7000, total target dose of 60e/$Å^2$ Exposure time = total dose*(pixel size)^2/dose rate = 60*0.92^2/30 = 1.69

second. Update it in SerialEM for Record beam later.

3.6.9 You can change the gain location at your own project by set it at DE server → Control Panel → Autosave Directory prior to gain correction, otherwise gain will be written at the default directory.
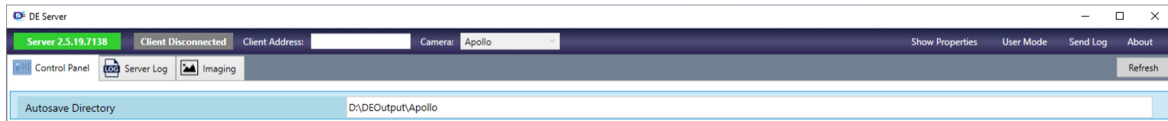
3.6.10 Restart SerialEM and update spot size or IA for Record and its exposure time.

3.6.11 Insert Obj aperture if you haven't and center Record beam again through Direct alignment.

3.7 Beam alignment: coma-free and Obj. astigmatism

3.7.1 Fine a decent area with thin ice, no crack and flat

3.7.2 Menu → Task → Rough Eucentricity, and Menu → Focus → Autofocus (target: ~-1μm)
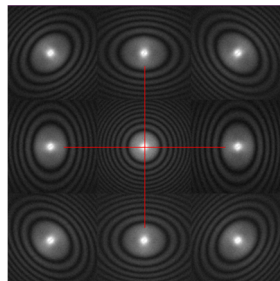
3.7.3 Menu → Focus/Tune → Correct Astigmatism by CTF
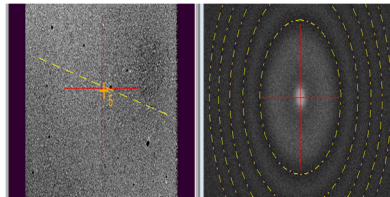
3.7.4 Menu → Focus/Tune → Coma-free Alignment by CTF; and repeat Correct Astigmatism by CTF. Both correction results will be displayed in Log Windows.
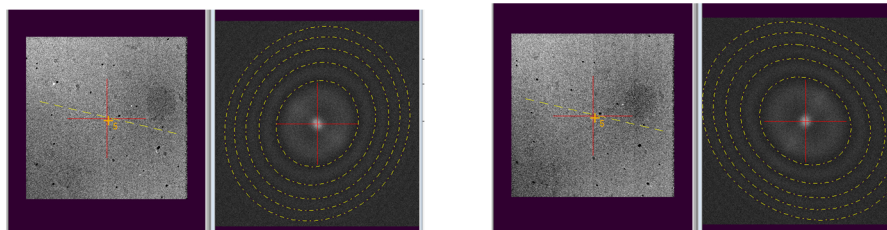
3.7.5 Display Coma-free result (optional): Menu → Focus/Tune → Beam-tilt FFT Array

25

4   Hole targeting

Except for manual targeting usually for tilt series or low concentration of filament, SerialEM offer four kinds of automatic (or semi-auto) methods for hole targeting, including fully automatic non-hole selection (not for hole, for negative staining sample), fully auto hole selection (single hole), semi-auto vector-define hole selection (single hole or 2x2, 3x3 hole pattern), auto multi-shot hole selection.

4.1 Medium Magnification Montage (MMM) collection

4.1.1 Add Points on center of squares which you choose for data collection from LMM map in Navigator Window.

4.1.2 Mark these points as "Acquire (A)" in Navigator Window (hot key: Ctrl + A).

4.1.3 MMM setup: Menu → File → Montage setup (you would need to close LMM.st file otherwise you won't be able to change montage settings)

- Choose "Use View parameters in Low Dose mode"
- Choose "Move Stage instead of shifting image"
- You will need to give Number of piece for X and Y, the "Total Area" calculates the area size when the piece number is given
- Give "Minimum overlap 20%", too small overlap might end up with bad overview map
- All other parameters will keep default or change by themselves correspondingly.

    → Click OK to the next page → File Properties: keep all as default; Click Ok → name output as MMM.st in your project folder.

4.1.4 Run the batch montage collection through Menu → Navigator → Acquire at items. Actually this is the only channel for all automatic collection in SerialEM. The next page will split into Single Particle or Montage collection if points have been marked as "Acquire (A)", and tilt series collection if points have been marked as "Tilt series (TS)".

4.1.5 "Acquire at Item" Page. You can skip reading this part and jump to the next 4.1.6. Here is the place to execute auto jobs. A job can be separated into three parts: before task, primary action, and after task. This page has five categories:

- Primary action: is the main action of batch job

  ➢ Acquire and save image or montage: it is for collect a single exposure image or montage. If you have montage file opened already, this option will be chosen automatically and collect montages.

- ➢ Acquire multiple Records: is to collect multiple exposures in one hole or multiple holes. You can use Setup button here or set up or at Menu → Navigator → Set Multi-shot Parameters. We usually use this one for Single Particle data collection.

- ➢ Acquire tilt series: for tilt series data collection. If points have been marked as "TS", this option will be automatically chosen. The tilt series setup page will come up once you click OK on this page.

- ➢ Run Script: Script is very powerful tool in SerialEM which users have all freedom to write their own scripts to perform special demands.

- Tasks before or after Primary:

  - ➢ Usually there are some tasks which can be done prior to Primary action, for example, check LN_2 level, run Eucentricity, Align item to template(single hole, 2*2 or 3*3 hole pattern), and so on. You can even run some special tasks through Script. Each task has its own Setup button here. Remember the order of running multiple tasks here is following the list order. You can define some tasks post to Primary actions as well. Particularly the first task here "Manager Dewars/Vacuum" is only suitable for Single Particle, since for tilt series the targeting point itself is considered as an item, so it will only check LN_2 level once at the beginning of tilt series. Checking LN_2 level for each tilt exposure during tilt series has been considered in SerialEM property, so user don't have to worry about it.

- Primary action related

  - ➢ Cycle defocus target in autofocus. Here is where you can define defocus range and steps. Usually it is good place to set up various defocus for tilt series data collection. For Single Particle we do it through script.

- Task-related options

  - ➢ Feel free to try them. I have never used any of them.

- General options

  - ➢ Close column valve at end. This is usually checked for auto data collection.

- General controls for task: Manage Dewars/Vacuum

  - ➢ Haven't tried yet

Acquire at Items ✕

Parameters for: **Mapping** | **Final Data**

Primary Action
- ⦿ Acquire and save image or montage
  - ☑ Make Navigator map
    - ☐ Skip saving to file
- ○ Acquire multiple Records [Setup]
- ○ Acquire tilt series
- ○ Run script # 26 ▾

Primary Action-related
- ☐ Cycle defocus target in autofocus, drift wait & TS
  - From -6 to -4 um in 3 ⏶⏷ steps
- ☐ Adjust beam tilt & astig for image shift
- Acquire non-montage images in Low Dose with
  - ⦿ Record ○ View ○ Search

No output file is open for first acquire item!
Open a file or Postpone to set one up to open

Task-related options
- ☐ Do hybrid Realign to Item/Align to Template
- ☐ Relax stage after final movement
- ☐ Skip Z moves in initial move and Realign
- ☐ Skip stage move to item if possible

General options
- ☐ Do subset from index 1 to 8
- ☐ No message box when errors occur
- ☐ Close column valves at end
- ☐ Retract cameras at end
- ☐ Send email at end
- ☐ Hide unselected options

[Save Parameters to File] [Read in Parameters]

Tasks before or after Primary
- ☐ Coma-free Alignment — Every item — [Setup]
- ☐ Correct Astigmatism — Every item — [Setup]
- ☐ Condition Phase Plate — Every item — [Setup]
- ☐ Update Dark Reference — Every item — [Setup]
- ☑ Rough Eucentricity — Every item
- ☐ Eucentricity by Focus — Every item — [Setup]
- ☐ Autocenter Beam — Every item — [Setup]
- ☐ Realign to Item — Every item — [Setup]
- ☐ Cook Specimen — Every item — [Setup]
- ☐ Fine Eucentricity — Every item
- ☐ Align to Template — Every item — [Setup]
- ☐ Autofocus — Every item — [Setup]
- ☐ Wait for Drift — Every item — [Setup]
- ☐ Run Script before Action — Every item
  - Script to run: LD_test ▾

Primary Action inserts
- ☐ Run Script after Action — Every item, after
  - Script to run: MyFuncs ▾

General controls for task: Coma-free Alignment
- How often to run task
  - ⦿ Every 1 ⏶⏷ items
  - ○ Only at start of group
  - ○ Only at end of group
  - ○ Every 15 minutes
  - ○ When moved > 40 um
- Position in sequence
  - [Move Up]
  - [Move Down]
- ☐ Run after Primary Action
- ☐ Run at nearest item with ⦿ label ○ note starting with [   ]

☐ Hide unused tasks   [Restore Default Order]

[GO] [Postpone] [Cancel] [?]

4.1.6 Acquire at Item: choose "Rough Eucentricity" for Task before and "Acquire and save image or montage" for Primary action.

4.1.7 Once it starts, it will move to the stage XYZ of the first Acquire item on the Navigator list, run Rough Eucentricity, and then collect montage. Then move to the next Acquire item, repeat until all items are finished. The MMM will be saved to the same MMM.st file as the order of Z=0, 1, 2…... each MMM map will be generated automatically and saved in Navigator list. After all montages are completed double-click the map in Navigator list will show the montage map in Display Window.

4.2 Fully auto non-hole selection
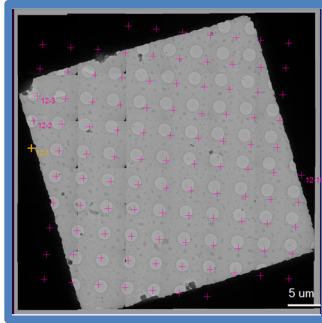
4.2.1 This method is to add points based on a given spacing. It works for collecting data on negative staining grid without asking for particular geometry. A regular hole grid is used here only as example. The idea is to work on non-hole negative staining grid.

4.2.2 Menu → Navigator → Montaging & Grids → Add Grid of Points → "Spacing between points in microns": give a number at least bigger than your beam size.

4.3 Fully auto hole selection
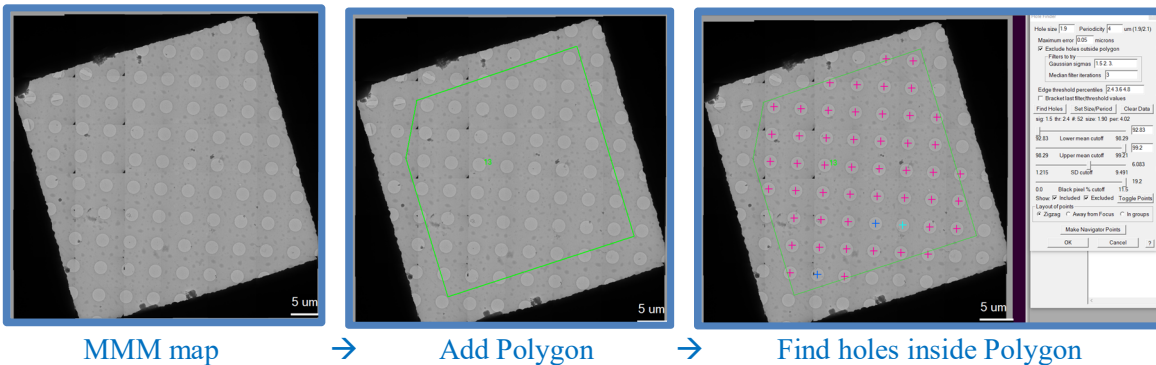
MMM map → Add Polygon → Find holes inside Polygon

4.3.1 This method finds all holes based on the given diameter and spacing, user can further clean up the selection by adjust the criterion of peak selection, stand derivation, and so on. It is a good method for Single Particle hole selection, but it can only target single hole.

4.3.2 Navigator Window → Add Polygon: draw a polygon to exclude the area close to square edge where is usually thick and crystalized ice.

4.3.3 Menu → Navigator → Montaging & Grids → Find Holes in Regular Grid

- Hole size (hole diameter in pixel) and Periodicity (distance from hole to hole)

- Measure distance in SerialEM: hold Shift Key; move left mouse from one to the other spot, the distance and angle information will be shown in Log Window. The measured hole size is usually accurate, but the measured distance from hole to hole is usually smaller than the program needs.

- Have "Exclude holes outside polygon" checked

- Click "Find Holes" button

- Adjust "Lower mean cutoff" and "Upper mean cutoff" to select right thickness of ice hole

- Adjust "SD cutoff" to exclude holes with cracks or big ice contamination

- Click "Make Navigator Points"

- You will need to repeat 4.2.2 and 4.2.3 for each MMM map.

4.4 Semi-auto vector-define hole selection

Add vector points →add points inside polygon   Or add 2*2 pattern   Or add 3*3 pattern
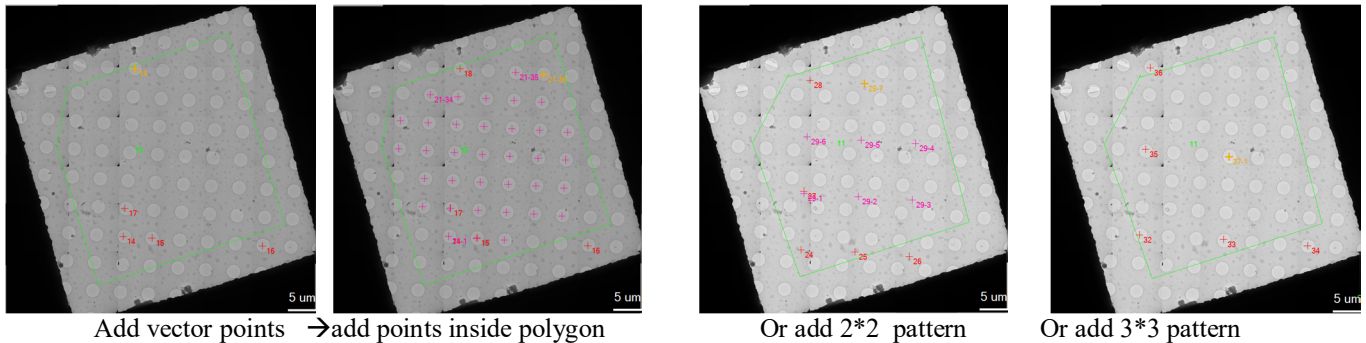
4.4.1 This method is semi-auto which needs user to manually define a few vector points to determine the hole geomoery.

4.4.2 Add five vector points: three adjcent points define two basic vector as #14, 15 and 17 in the examle, then add another two points along each vector'e elongation as #16 and #18 in the example. These five points needs to be added continuously during Add Points, meaning no Stop Adding between, to make them recognized as a group. Highlight on anyone before the next step.

4.4.3 Menu → Navigator → Montaging & Grids → Add Grid of Points → You will be asked for the polygon label → Answer "Zigzag" for point adding sequence → Answer Yes to turn on Acquire (A).

4.4.4 Once it is done, all these points can be considered as one group. Navigator Window → Have "collapse" checked to combine them into one group to make Navigator list more organized.

4.4.5 Again you will have to repeat these operation for all MMM maps

4.4.6 You can use the same strategy to set up 2*2 or 3*3 hole patterns.

4.5 Auto multi-shot hole selection (suitable for single particle data collection)

4.5.1 This method consists three steps: (1) set up template; (2) set up Multi-shot (a function implemented in SerialEM which user can define a hole pattern like 2*2 or 3*3 by given image shift, and how many exposure can be put in one hole); (3) find all suitable holes

on MMM maps (a function implemented in SerialEM to allow users to distinguish "good" and "bad" holes); (4) merge all suitable holes to match the multi-shot pattern.

4.5.2 Set up template for Multi-shot

- Find a clean area with View beam and center on the 3*3 pattern.

- Clear IS (image shift) with "Reset Image Shift" in Image Alignment & Focus Control Panel (2.3.7) and retake View image, repeat it multiple times until IS is zero and the 3*3 pattern is well centered.

- Open a new file called "template.mrc" and save the concerted image by "Save A" or "Save Active" in Buffer Control Panel (2.3.2); close the file. The reason to save the image to file is that you can reload it back in case SerialEM crashes.

- Copy the centered image to Buffer P which will be used as template for each Navigator item during data collection: Buffer Control Panel (2.3.2) "Copy active image to buffer" "P"

- Set View beam focus offset to 0 in Low Dose Control Panel (2.3.10) and retake a new View image for Multi-shot setting. The reason is that the relationship between View and Record has been acurrately calibrated around 0 focus, while the usual -50μm defocus of View which is only to increase image contrast now will break the relationship and thus introduce inaccuracy for targetting. Don't forget to change the focus offset back to whatever it was after Multi-shot setting. Below is a comparison of View image between with -50μm and with 0 focus offset. It is not a problem that the aperture shows up a little bit on the 0 foucs offset View image.
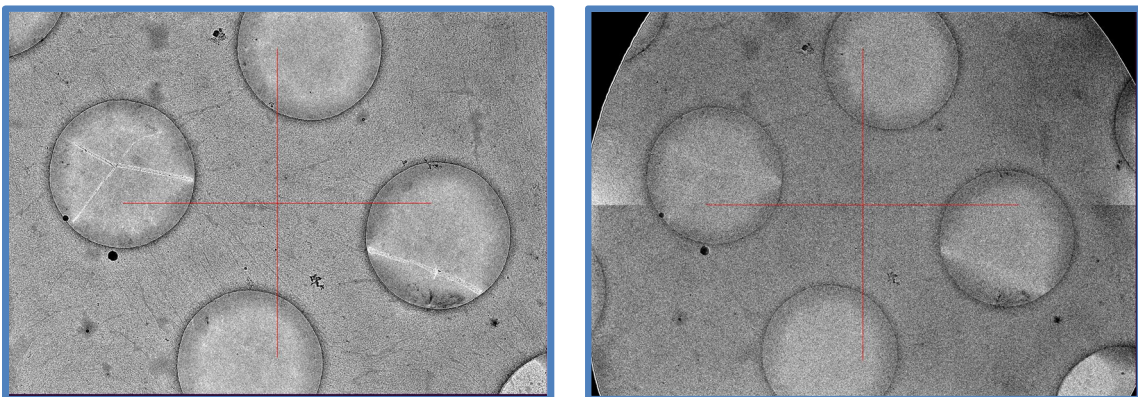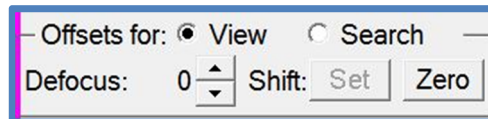
4.5.3 Set up Multi-shot of 2*2 hole pattern
- SerialEM Menu (2.2.10)→ Navigator→Montaging & Grids→Set Multi-shot Parameters

- Have "Do multiple Records within each hole" unchecked, and have "Do Records in multiple holes" checked, since we are focusing on setting up multiple holes and leave multiple records in each hole later.
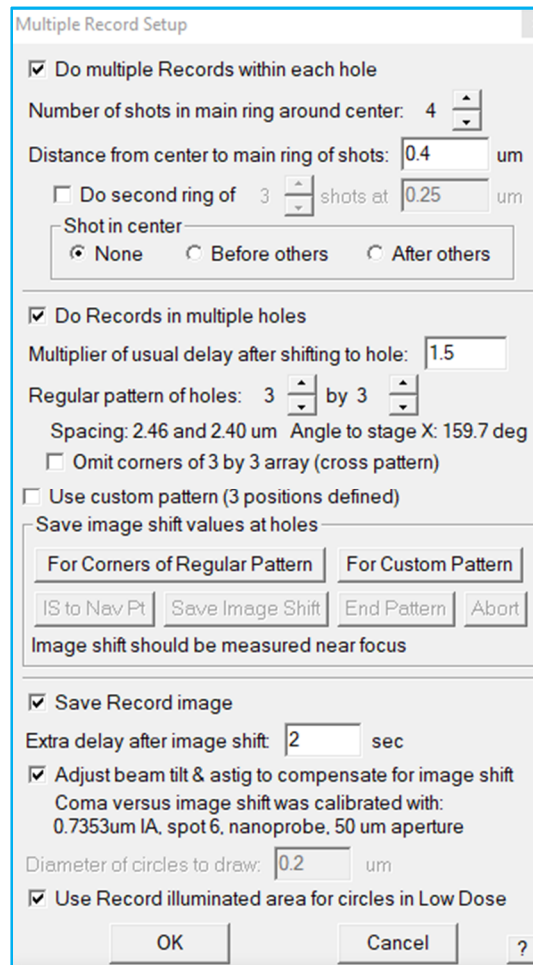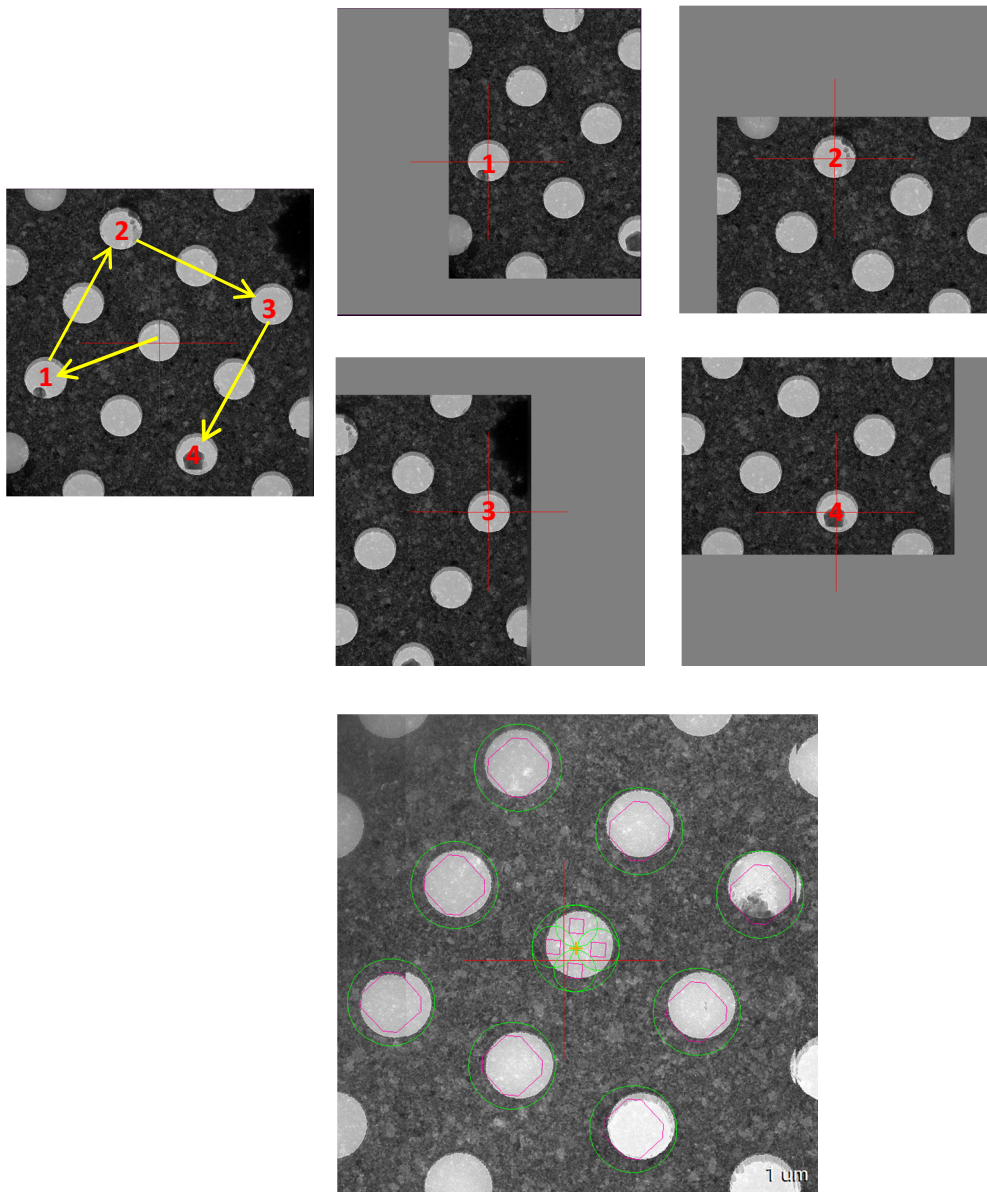

- Set "Regular pattern of holes 2 2"

- Two ways to define a hole pattern, we will use "Corners" as example, so uncheck "Use custom pattern"

- Click "For Corners of Regular Pattern" button

- Move View image (taken at 0 focus offset) to the center of hole #1 (hold right mouse to drag), click "Save Image Shift", and continue to repeat for other corner hole #2-4. You can compare the IS (image shift) value (2.3.4 Microscope Panel) created when you move to each hole and they should be very close to each other.

- Click "Clear" button on 2.3.7 Image Alignment & Focus Panel to bring the View image to its center. Left clicking mouse on the center will add a green "marker", so to display the Multi-hole setting result.

- Have "Do multiple Records with each hole" checked to set up multiple shots in one hole. You can adjust how many exposure in one hole ("Number of shots in main ring around the center") and the distance of the exposure to its hole center ("Distance from center to main ring shots"). You can also add "Shot in center" if possible. The criterion is that no camera area (the pink rectangular) should be double-exposed by beam (the green circle). The example here is 3 shots per hole with distance from center of 0.3μm, and no shot in center.

- Have "Adjust beam tilt & astig to compensate for image shift Coma ……" checked to enable image shift vs. coma-free calibration.

- Not forget to set View focus offset back to what it was (-50μm or -80μm)

View image with 0 defocus

2*2 pattern

3 shots per hole

View image with -50μm defocus
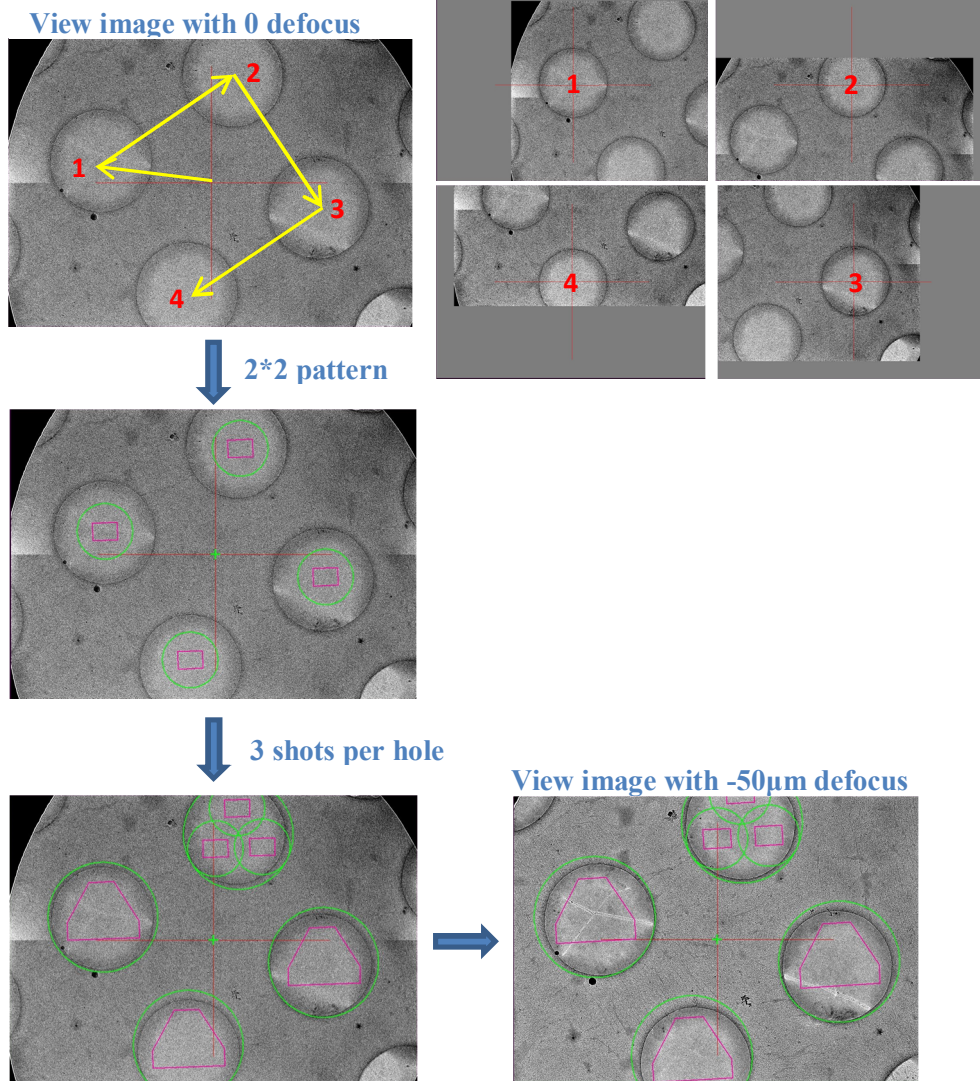
- Below is another example of 3*3 pattern

4.5.4 Find suitable holes from MMM
- SerialEM Menu (2.2.10)→Navigator Montaging & Grids→Find Holes in Regular grid. The "Hole Finder" page will show up.
- Measure hole diameter and hole-hole distance: On MMM drag mouse from one end to the other with Ctrl key pushed, a green line will show up and the line length will show up on Log Windows (2.6). Measure the hole diameter and the distance from a hole to its closest neighbor, and fill these two values to "Hole" and "Periodicity" in "How Finder" page

- An option is that you can define a polygon prior to "Hole Finder" to only include the center area of MMM to exclude those crystal ice hole close to the square edge. This is not necessary since in most cases those ice holes could be excluded by the parameter below, but it is useful in some cases, then you have to define polygon for each MMM, and have "Exclude holes outside polygon" checked.

- Click "Find Holes" button. No need to change any parameters above since the default works pretty well without any exception so far.

- All holes on MMM should be picked at this point. Then use the four parameters below to exclude those bad ice holes: "Low and upper mean cutoff" separates holes by ice thickness; "SD cutoff" separates holes by standard derivation since broken ice hole or holes with ice contamination gives higher SD; "Black pixel cutoff" can remove ice hole with big black ice crystals.

- Click "Make Navigator Points" button to create all suitable hole items in Navigator Windows.
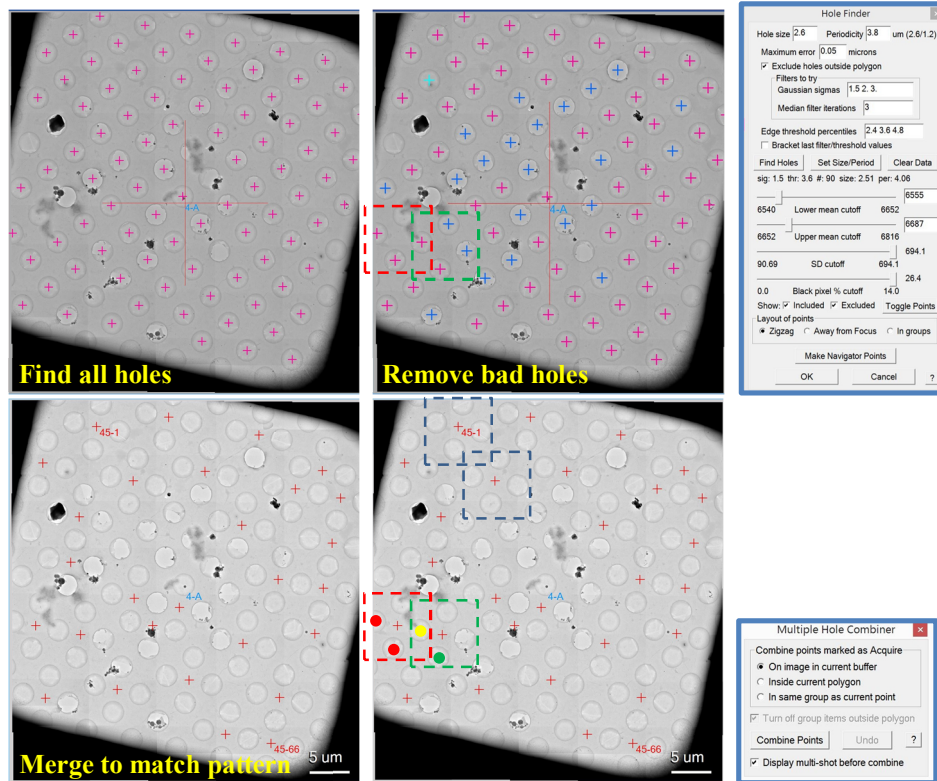
4.5.5 Merge suitable holes to match multi-shot pattern
- SerialEM Menu (2.2.10)→Navigator Montaging & Grids→Combine points for Multi-shots

- Choose "On image in current buffer" to combine points which matches the defined Multi-shot pattern (2*2 or 3*3) into new Navigator points, and replace those old points in Navigator Windows.

- After merging into pattern SerialEM has saved the information which is good hole for imaging, for example those two red points in red pattern item and the green point in green pattern item. The yellow one which locates in the overlapping area of red and green pattern will be only imaged once.

4.6 End

5   Tilt series setup

5.1 Pick points suitable for tilt series collection

5.1.1 Points suitable for tilt series collection are pickier than those for Single Particle.

- Be more careful for loading grid to cassette. If you load grid on the wrong side you might end up with very big Z eucentricity. If the Z is close to 300μm after Rough Eucentricity then it will be challenging to tilt grid because the grid itself might touch Obj. Lens pole piece. So always load your grid with its C-ring towards left or slot #1 direction on cassette.

- Always pick flat grid for tilt series collection, bent grids causes many unnecessary problems.

- Always choose intact grid squares without cracks or big junk ice contamination. The former will cause strong drift when grid is tilted to high angle, the latter may block beam at high tilt angle.

- Try to always pick square as much as close to grid center, or at least 3 or 4 squares away from grid edge, for the same reason that grid edge might block beam at high tilt angle.

- Roughly check whether the square can tilt to highest tilt angle as you set for data collection without being blocked.

- Always pick hole of 2~3 holes away from square edge. The grid bar may block beam as well.

- Remember which side the Focus area is relative to Record area. Go to Low Dose Control Panel (2.3.10) → Select "Focus" → the focus area will show up as 🔳. If Focus is on the left side of Record, then start to pick points from left to right on MMM map, otherwise you will have the worry that the next point could be just the Focus area of previous points.
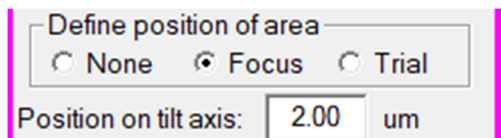
5.1.2 Mark points as "Tilt Series" instead of "Acquire (A)". You will be asked to give name for the first tilt series, for example "TS001.st" and set up tilt series parameters as 5.3. The second and all the rest will share the same tilt series parameters and their names will be automatically created as TS002.st, TS003.st, and so on.

5.2 Set Focus area

5.2.1 Have tilt axis shown on image: Go to Image Display Control Panel (2.3.3) to have "tilt axis" checked. The tilt axis will be displayed as yellow dash line cross the center.

5.2.2 Uncheck "Rotate inter-area axis" at the bottom of Low Dose Control Panel (2.3.10) , so to ensure Focus will be always along tilt axis

5.2.3 Go to Low Dose Control Panel (2.3.10) → select "Focus", you can either pick Focus area by left mouse click on image, or directly give a value here. Positive value is on the left relative to Record area. The criterion of Focus distance is not too far, and not too close to overlap with Record area. Remember to select "None" when it is done.
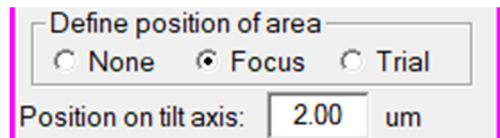
5.3 Set up tilt series parameters
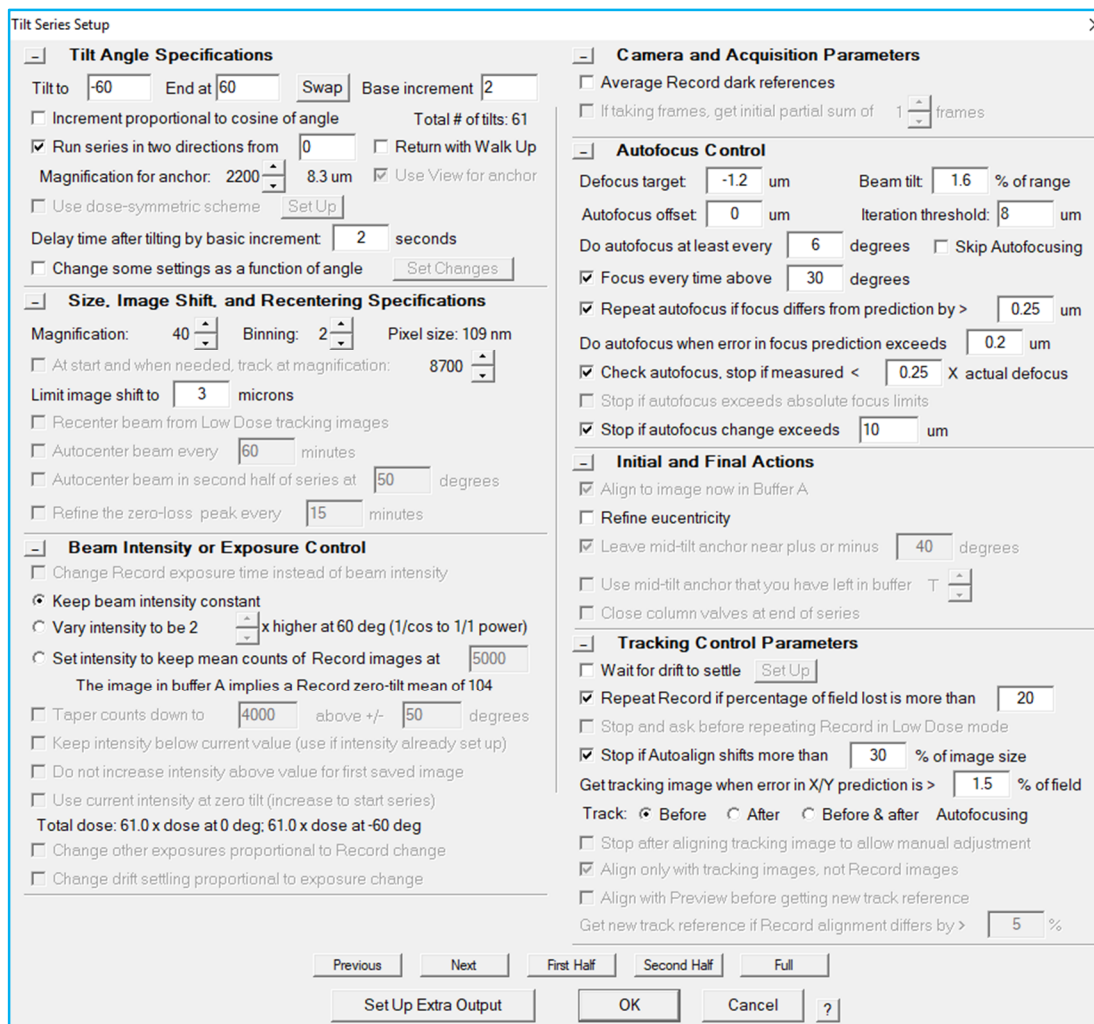
5.3.1 Three kinds of tilt schemes in "Tilt Angle Specifications"

- 1. Bidirectional series: tilt series collection starts from given initial angle, (usually 0 degree as in the example), tilts towards one direction (usually minus direction), then returns to 0 degree and continues to the other direction (usually plus direction). This is the one we usually use as in the example. One benefit of this scheme is your targeting point won't be lost during collection. One disadvantage is that tilt series may lose tracking when it returns to 0 degree and fail collection.

  Check "Tilt Angle Specifications" → "Run series in two directions from 0" to select this scheme.

- 2. Unidirectional series: tilt series collection starts from one side of highest tilt (usually minus highest tilt), tilts towards 0 degree, and continues to the other side. The targeting is the same as other two schemes that Add Point at 0 degree, it tilts up to one highest tilt angle before tilt series collection starts. The advantages of this scheme are every adjacent tilt image has similar dose damage and least shift between each other; both will be helpful for tilt series alignment. Two disadvantages are: the least dose damage image will be collected on high tilt angle with thickest ice, the Added point may lose or shift a lot when it initially tilts up to highest angle before data collection.

  Uncheck "→ "Run series in two directions from 0" and "Use dose-symmetric scheme" to select this scheme.

- 3. Dose-symmetric: collect tilt series at a symmetric manner. For example if the interval is 2 degree, it will collect 0; -2, -4; +2, +4; -6, -8; +6, +8; and so on. The idea of this scheme is to try the best to collect those least dose damage images at low tilt angles so the high resolution will be preserved at thin ice for low tilting. The biggest disadvantage is it is very slow (usually takes one hour and a half to complete one tilt series) due to tilt back and forth. It deserves a try if you aim for high resolution sub-tomogram average.

  Check "→ "Use dose-symmetric scheme"

5.3.2 Three ways to set Record exposure in "Beam Intensity or Exposure Control"

- "Keep beam intensity constant"

  Simply use the Record exposure in Camera Control Panel (2.3.11) for every tilt image, as in the example page

- "Vary intensity to be x higher at 60 deg (1/cos to 1/y power)"

  This option will give more exposure to higher tilt angle (thicker ice) so to have images collected at different tilt angles almost have a same contrast.

- "Set intensity to keep mean counts of Record images at"

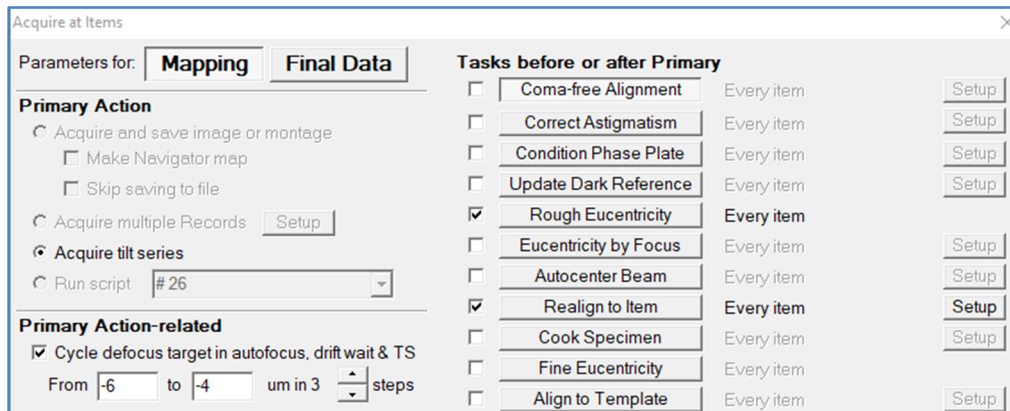Every tilt exposure will be adjusted to make image counts the same.

5.3.3 Autofocus Control

- Defocus target varies in batch tilt series, and the value is given not here, so ignore the value here.

5.3.4 More to come

5.4 Run tilt series collection

5.4.1 Menu → Navigator → Acquire at Items

- Check "Rough Eucentricity" and "Realign to Item".

  The point added may moves after "Rough Eucentricity" so run "Realign to Item" to re-center the point by align new View to point position from MMM map.

- Check "Cycle defocus target in autofocus"

  The example is from -6 to -4µm in 3 steps meaning it will cycle defocus value in -6, -5 and -4µm.

5.5 Batch tilt series policy

5.5.1 These policies are important for batch tilt series collection. Most of time the default setting is quite good, but you should be aware that sometimes it needs little modification. All content are self-explanatory, so no more comments here.

5.5.2 Menu → Tilt Series → Bach Polices

Batch Tilt Series & Other Policies ✕

General response to error during series
- ○ Stop with the usual message box and allow intervention
- ◉ Terminate series or take specific action selected below

After 3 images too dim or one dim Record in Low Dose mode
- ◉ Stop with message box   ○ Terminate

After failure to tilt to expected angle (possible pole touch)
- ◉ Terminate   ○ Continue

When Record image is too misaligned in Low Dose mode
- ○ Take again   ◉ Continue   ○ Terminate

When Record image is too dim in Low Dose mode
- ○ Take again   ◉ Continue   ○ Terminate

☑ Autosave log and start new log for each tilt series

───────── Other Policies ─────────

☑ Consider batch series successful or end first part of a
bidirectional series if dim image or bad autofocus occurs
above `55` or within `5` degrees of ending tilt

☐ Do similar at high tilt if intensity/exposure set for mean
exceeds `3` times its minimum value

☑ Close column valves after message box is open `30` min

[OK]   [Cancel]   [?]

5.6 Display collected tilt series

5.6.1 Menu → File → Open Old: open the complete tilt series file

5.6.2 Menu → File → Read, you will be able to check slide by slide. The tilt series has been re-organized from minus highest tilt to plus highest tilt, and starts from Z=0 for the minus highest tilt.

5.7 Problems you may come across

5.7.1 More to come

5.8 End

6   Single Particle setup

6.1 Set up Multi-shot by following 4.5.1 to 4.5.3 in 4.5 Auto Multi-shot hole selection

6.2 Find all suitable holes from MMM by following 4.5.4 to 4.5.4, and make Navigator points.

6.3 Open a file to save integrated Record images

Menu → Open new → File properties: enter a total image number bigger than that you may collect. SerialEM will stop data collection when it reaches to this number.

6.4 Acquire at Items

- Final Data→Primary Action→Run Script: LD_test

- The script LD_test is as below. When data collection starts, it will run the script LD_test through all Navigator items (points) from the top to the end.

```
MacroName LD_test

Echo ===> Running LD ...

RefrigerantLevel 1
RefrigerantLevel 2
Loop 10
AreDewarsFilling
If $repVal1 == 1
    echo LN2 is Refilling
Else
    Break
Endif
Delay 5 min
EndLoop


## position (X,Y)
RealignToNavItem 1

## CycleTargetFocus, range is set in macro "Parameters".
CallFunction MyFuncs::CycleTargetDefocus -0.8 -3.0 0.2

## Autofocus
G

MultipleRecords

ReportClock
echo -------------------------------------------------------------
```

Check LN2 level and wait if LN2

Align again after moving to the

Vary defocus in the given range

Focus and apply the defocus

Collect data

7 Script examples

Here is a script exmaple (downloaded) which automatically loads all twelve girds and collects LMM. Go to https://bio3d.colorado.edu/SerialEM/hlp/html/script_commands.htm to find out the meaning of each commands

```
MacroName mapgrids

# Dock a cassette, setup full montage, change cooldown delay time and start this script
# When reloading a previously mapped grid, see SerialEM help in Navigator menu, Align with Rotation.

Delay 15 min # change cooldown wait time to match your local autoloader cooldown time
LongOperation In

Loop 12 index
  echo $index
  ReportSlotStatus $index
  if $RepVal1 == 1
    ScreenUp
    LoadCartridge $index
    MoveStageTo 0 0 0
    SetColumnOrGunValve 1
    M
    NewMap
  endif
endloop

CLoseFile
SetColumnOrGunValve 0
```

8   Attentions

8.1   Don't stop serialEM during focusing, which very likely crashes SerialEM

8.2   Build up a habit to constantly save Navigator file in case SerialEM crashes you still have chance to load it back

8.3   Build up a habit to save SerialEM setting although it automatically saves it in every 5 minutes.

8.4   When SerialEM crashes, you might need to:
- Re-set focus position
- Re-set template in P buffer
- Double check your beams, especially Record beam.
- Check the Multi-Record setup

8.5   In case SerialEM wasn't closed properly sometimes it doesn't release Shutter control to microscope, so you will need to restart SerialEM and close it properly.