

SerialEM Instructions by Mode

This document provides protocols and guidance for using SerialEM for the following:

- SPA cryo imaging
- Negative staining imaging
- Tomography
- Miscellaneous

Section 1: SPA Cryo Imaging

The best practice is to collect an overview of the entire grid and identify the grid squares that look the best. Once those are identified, you can collect data from the squares of interest.

Full Grid Montage

1. Open a navigator window by clicking on the Navigator drop down menu and clicking 'Open'.
2. The next step is to collect a Low Mag Montage of the entire grid.
 - a. If you haven't done so already, you'll need to import the updated EFTEM calibration using the 'Align' tab in the Technai software. Choose the file labeled 'ATiano_300-4-4500' and make sure to select 'EFTEM LM' and load that. Once loaded, select your calibration file and save it. You will not need to repeat this step unless a new EFTEM calibration is performed.
 - b. Turn off Low Dose mode and switch over to the GIF. The GIF usually cannot be selected from the Technai software, so use the Filter Control Panel in SerialEM to switch between the GIF and the Falcon (EFTEM mode for GIF; uncheck for Falcon.)
 - c. Change the magnification to LM 120x or LM 180x and move to roughly the middle of the grid.
 - d. Check and adjust the beam intensity on the screen. Take a View image to see that the captured images look good.
 - i. **If you notice that there are hexagonal cells in your image, those are from the CCD. This means your intensity is too high.**
 - e. In the 'Navigator' menu, click open to open the Navigator.
 - f. Click 'File' then select 'New Montage'. Try an 8x8 mesh size for the montage and check the 'Ask about making a map' box. Then type out the desired name for the montage. It's a good idea to have consistent naming conventions, such as:
DATE_SAMPLE ID_MONTAGE DEPTH.mrc
 - i. Montage Depth: Use LMM or MMM for Low Mag Montage or Medium Mag Montage, respectively.
 - ii. If you are unable to check the 'Ask about making a map' box, then you forgot to open the Navigator!
 - g. In the Montage Controls panel, enable 'show overview at end' and 'align pieces in overview', and disable 'treat as very sloppy montage'. Then, click 'Start'.

- h. After the montage is done, click 'Yes' when asked about making a map. This will allow you to click on the map image, save positions, and reload it when necessary using the Navigator.
 - i. *Note: Maps are now also indicated in blue framed area in images.*
- i. In the event of program crashes, it's a good idea to save the navigator (Navigator -> Save).
- j. Close the full grid montage (File -> Close).

Low Dose Setup

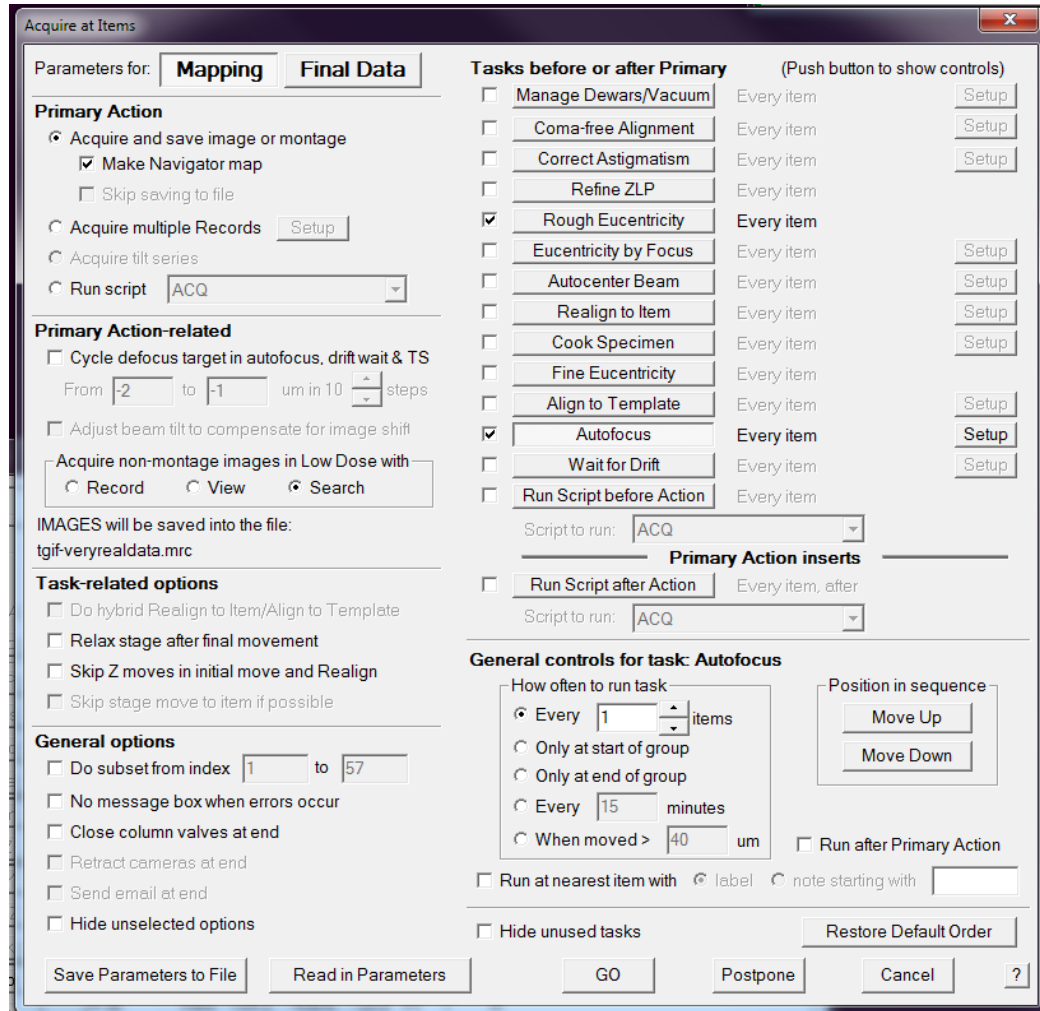
3. Check Low Dose Mode in SerialEM (be sure that EFTEM mode is unchecked in the Filter Control panel) and reinsert the objective aperture.
4. If you haven't done so already, set up Low Dose Mode. For single particle data collection, the following settings are a good starting point:
 - a. **Search:** mag = M 2050x, binning 4, Exp: 0.5 s, Spot 5, C2: 83.74%, full area
 - b. **View:** mag = SA 20500x, binning 4, Exp: 0.5 s, Spot 5, C2: 56.97%, full area
 - c. **Record:** mag = SA 35000x, binning 1, Exp: 3-4 s, Spot 5, C2: 54.5, full area
 - d. **Trial/Focus:** same mag as record but binning 2, Exp: 0.5 s, half area
 - e. Cycle through each of the modes (even if you have Low Dose Mode set up already).
 - f. Once your imaging conditions have been setup, it's recommended to save your settings. Open the 'Settings' menu and click 'Save As' and save the settings with a distinctive name. For example: tomo_RT, tomo_cryo, RT SPA, cryo SPA, etc. This enables you to easily switch between different imaging modes without needing to adjust Low Dose Mode each time.
5. Identify a sacrificial location on the full grid montage. In the Navigator, click 'Add Points' and then use the left mouse button to add a marker. Click 'Stop Adding' in the Navigator. Then click Go to Marker and take a Search image (keyboard control: Shift P). Center a hole in the middle of the image.
6. In the Low Dose control panel, click the 'Define Area of Focus' button. You should see a green box, indicating the record area and a yellow circle indicating the area of focus.
 - a. Adjust the value for the position on tilt axis to -1.71 um.
 - i. This value assumes using R 1.2/1.3 grids. If you are using different grids, adjust this value with rotation value (see point b below) so that the yellow circle sits in the middle of four holes.
 - b. Adjust the 'Rotate inter-area axis' as needed so that the yellow circle sits in the middle of four holes.
 - i. This value will need to be updated for each new grid since the grid orientation will change each time.

Medium Mag Montages via Acquire at Items (Mapping Grid Squares)

Using the 'Mapping' part of the 'Acquire at Items' dialog allows you to collect medium mag montages of the best squares selected from the full grid montage all at once, rather than collect on each grid square individually, and you can obtain the rough eucentricity at each grid square and that information will be saved for data collection later on.

7. Open a New Montage from the File menu and set up an appropriate montage size to cover a whole grid square. Make sure the 'Use Search Parameters for Low Dose Mode' box is checked.
 - a. A 3x3 montage works well for M 2050x (Search) on a 400-mesh grid.
8. Load the full grid montage from the Navigator and place a Navigator point on to the center of each grid square of interest (Navigator window -> Add points followed by Navigator window -> Stop adding).
9. Enable 'Acquire' for each of these points.
 - a. To enable 'Acquire' for many points simultaneously, select 'Collapse' in the Navigator menu.
10. Add another point in the vicinity the other points, containing a distinctive feature easy to identify over a large range of magnification (e.g. ice contamination; ideally a small feature near a bigger, easily identifiable structure), and mark it as a reference point.
 - a. Click on the reference point in the Navigator and check the 'Registration point' box.
11. Move to the reference point (Navigator window -> Move to XY), take a Search image. Optimize eucentric height at that location, then take a Search image. If the reference feature is not in the field of view, move the stage to center it (right mouse click and drag), then take another Search image.
12. On this image, add a green cross marker (left mouse click) to the exact structure that served as the reference point. While the reference point is selected in the Navigator, click the Navigator menu and select 'Shift to Marker' and then click 'Yes' to apply the shift.
13. (*Optional*) Save the Navigator.
 - a. This is important if SerialEM or the camera computer are prone to crashing.
14. Click the Navigator menu and select 'Acquire at Items'. If it's not available to select then you either forgot to add points or there are no points that are toggled to 'Acquire'.
15. Make sure you are selecting the parameters for Mapping on the top left. You should check the following:
 - a. The primary action should be to 'Acquire and save image or montage'.
 - b. 'Make Navigator map' should be checked.
 - c. Make sure images will be acquire in Low Dose mode with Search and that the images are set to save into the correct file.
 - d. Disable 'Relax stage after final movement' in the Task-related options section.
 - e. In general options, check the 'Close column valves at the end' if the system will be left unattended.
 - i. Optional to check 'no message box when errors occur'.
 - f. Under Tasks before or after the primary, both rough eucentricity and autofocus should be checked and set to run at every item.
 - i. Clicking on the name of the task in the dialog box will allow you to control how often that task will occur (general controls show on bottom right).
 - ii. You can click on the 'Setup' tab for additional settings
 - g. See the screenshot below for reference.

16. When ready, click 'Go' to start the acquisition; the progress and estimated remaining time can be monitored in the Navigation window.
- If you're not ready to start, click 'Postpone' to close the window and save your settings.



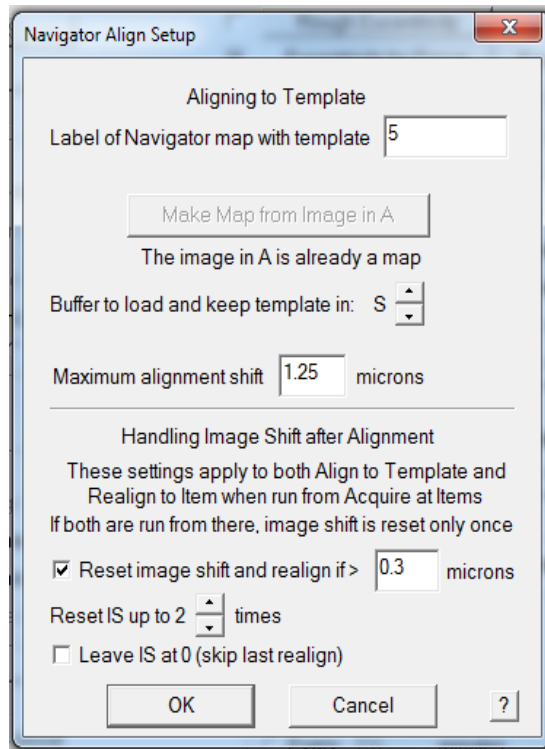
Running the hole finder

17. For each grid square that was mapped:
- Open the corresponding map from the Navigator by double clicking the entry.
 - Run the hole finder (Navigator menu -> Montaging and Grids -> Find Holes in Regular Grid)
 - Enter details about the film geometry. Note that the second value is the periodicity, not the spacing.
 - Periodicity is the hole size + spacing (i.e. for a 1.2/1.3 grid, the periodicity would be 2.5).
 - Optional: draw a polygon on the map (Navigator -> Add polygon) to exclude grid edges, contamination, etc.

- e. Enable 'Bracket last filter/threshold values' to speed up hold finding in subsequent runs.
- f. Press 'Find Holes'.
- g. Adjust the cutoffs until only the holes you want to target are displayed with magenta crosses and labels (magenta = included; blue = excluded).
- h. In the layout of points, select 'away from focus'.
 - i. 'Zigzag' can also be selected but only if autofocusing is run very infrequently.
- i. Click 'Make Navigator Points'. Delete any unwanted points manually or disable 'Acquire' for these points.
- j. (*Optional*) Save the Navigator.

Final Data Collection via Acquire at Items

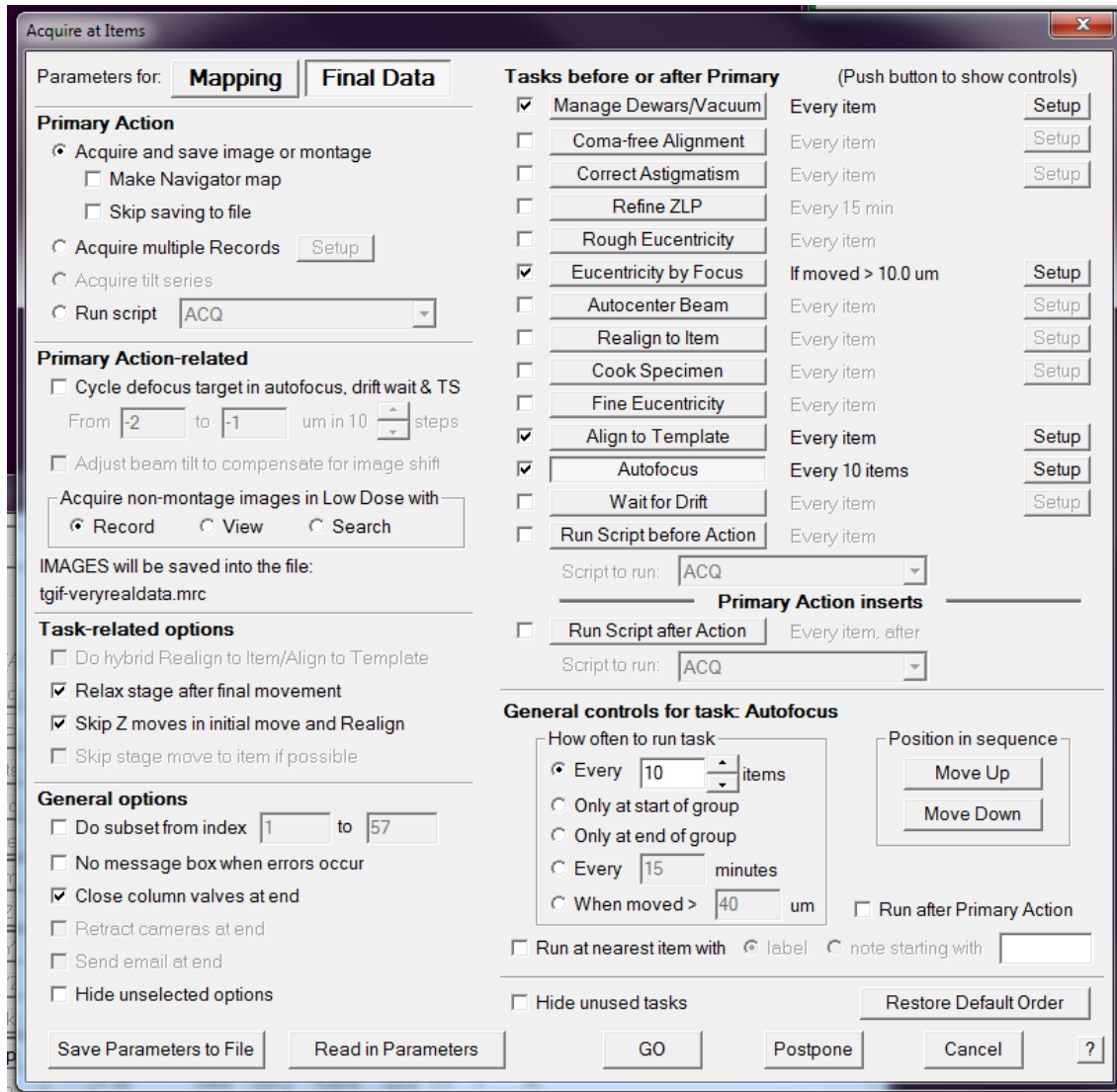
18. Create a template image of a hole in View mode. The hole should not have any contamination or gradient.
 - a. Close any open files. Then open a new file that you can save the template map into. It will not work if you use the LMM or MMM files.
 - b. Take a View image. If necessary, center it and retake the image.
 - i. If the contrast is low, adjust the defocus offset (see next section).
 - c. When you're happy with the image, under the Navigator menu, click 'New Map from Image'. You should see the new map in the Navigator with a new label.
 - d. Once you have that, open the 'Acquire at Items' dialog. Make sure the parameters for Final Data is showing, then check the 'Align to Template' box, then click 'Setup'.
 - e. Enter the numerical label for the template from the Navigator into the box. Set the maximum alignment shift to half the periodicity of your grid and set the reset image shift to 0.3 microns to be conservative. Then click 'OK'.



19. Setup the remaining parameters for Final Data (see screenshot below):

- a. Manage dewars/vacuum should used to set the time for the PVP buffer cycle, to prevent it from running while you are collecting data. Set to 120 minutes.
- b. Eucentricity by focus: recommended if moving more than 10 μm ; each user will need to calibrate this
 - i. Make sure you are at eucentric height before running calibration
 - ii. This avoids the need to tilt the stage to obtain the eucentric height, which is ideal for data collection
 - iii. Under the 'Focus/Tune' menu, select 'Set Offset' and set to -5 μm .
 - iv. Under the 'Tasks' menu, select 'Calibrate Focus Targets' and run the calibration.
 - v. **Important: Only do calibrations when you are certain of your imaging conditions!** The calibration only needs to be done once, unless you change your 'Record' conditions.
- c. Autofocus: not necessary at every hole, can set to every 10 items
 - i. This is good because you want different defocus values for data analysis
- d. Autocenter Beam and Drift: this will depend on the scope; if the scope loses the beam a lot then we may want to use the autocenter function, and same for drift
- e. If you make any changes to the dialog, but aren't ready to run yet, click the 'Postpose' button at the bottom so it saves your changes. If you click 'Cancel', your new changes won't be saved.
- f. Make sure you're saving into an appropriate file and not the previous map file before running.

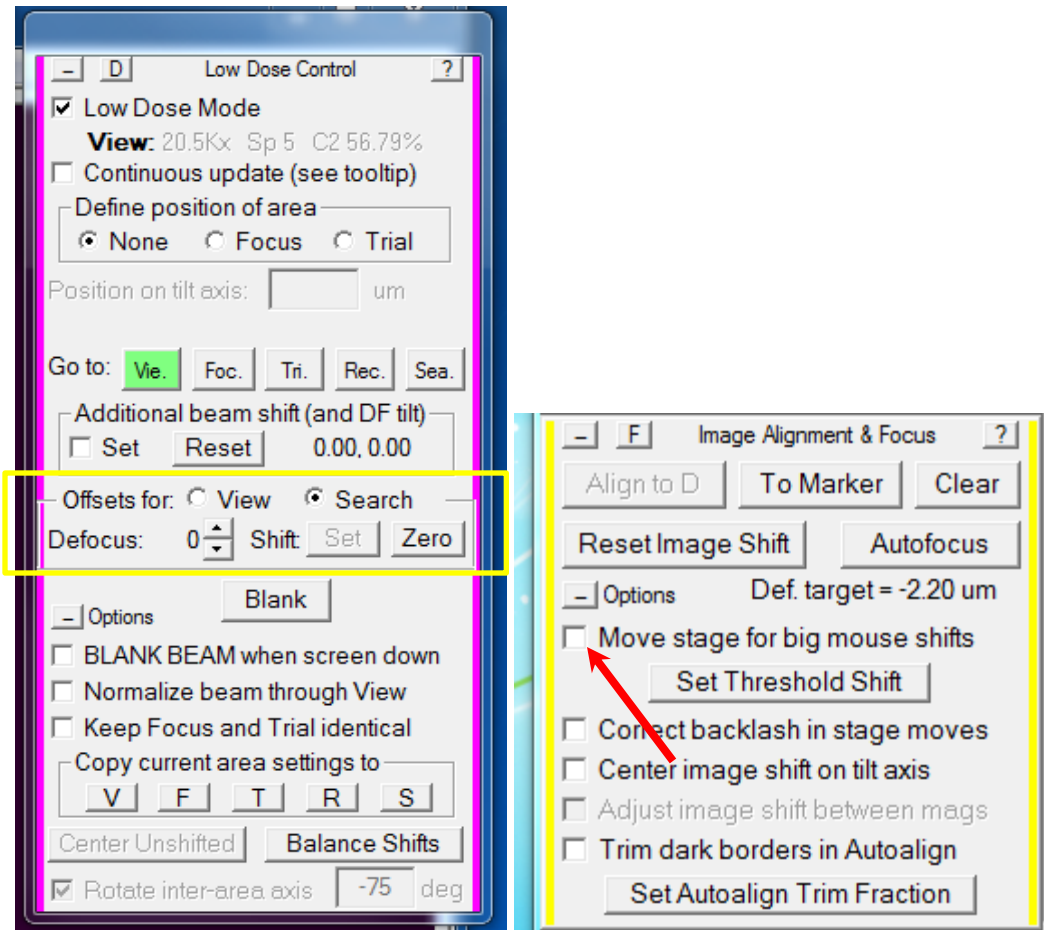
- i. Note: Acquire Multiple Records would enable you to obtain multiple images in a hole at a higher magnification than where we are currently collecting data and would likely require the coma-free alignments. Visit https://bio3d.colorado.edu/SerialEM/hlp/html/hidd_multi_shot_setup.htm to learn more.
- g. If you have already set up your shift offsets for View and Search, you are ready to collect your data. Click 'Go'. Otherwise, click 'Postpone' skip to the next section of the instructions.



Setting Shift and Defocus Offsets for Low Dose Mode

20. You may want to set an offset defocus value for 'View' and 'Search':
 - a. This will help increase contrast for montages and templates.
 - b. A -10 μm defocus offset is recommended for View to achieve good alignment with the template.

- c. Note that if the montage does not work at high defocus values, you may need to do the 'High-Defocus Mag' Calibration under the 'Calibration' menu. First lower the defocus value and try it again before doing that.
 - i. **Important: Only do calibrations when you are certain of your imaging conditions!!!** Calibrations only need to be performed once, unless you change your imaging conditions.
21. You absolutely must do the shift offsets (image shifts) for View and Search. This is sort of like the 'Shift to Marker' adjustment that you do between LM and SA, but it is between specific sets of images. It should not need to be adjusted after you initially set it, but you should check that it's still centered each time. If not centered, then zero the value and go through the process below.
- i. View: between View and Record
 - ii. Search: between Search and View
 - iii. Doesn't matter which order you do them in but be sure to do both!
 - iv. First make sure that 'Move stage for big mouse shifts' in the 'Image Alignment & Focus Control' box is unchecked!!!
 - v. First, Search and View:
 1. Choose a hole (preferably a landmark one) so it's easy to find.
 2. Click 'Search' in the Offset dialog of the Low Dose Control Panel.
 3. Take a search image. Center it. Take another search to make sure it is centered.
 4. Take a view image. Center it. Take another view to make sure it is centered.
 5. Take a search image. Center it. Take another search to make sure it is centered.
 6. Click the 'set' button in the Offset dialog of the panel.
 7. Take another image in search and view and make sure they are both centered.
 - vi. Then, View and Record:
 1. In the dialog box, make sure you're in the offsets for View.
 2. This is the same process as above, but cycle between View, Record, and back to view, centering the hole each time. After the last View, click the 'Set' button in the offset dialog.
 3. Take images again to make sure they are centered.



22. When you're ready, you can open the 'Acquire at Items' dialog and click 'Go' to collect your data.

Section 2: Negative Staining Imaging (work in progress)

1. After aligning the scope and obtaining a gain reference for the camera, identify squares of interest on the grid. Mark the squares in the 'Search' tab of the scope software.
 - a. Alternatively, you can collect a low mag montage of your grid, if desired. See F30 cryo imaging protocol for the step by step process.
2. Open the Navigator ('Navigator' menu -> Open).
3. Set up Low Dose Mode in SerialEM if you have not done so already.
 - a. Check 'Low Dose Mode' in the control panel and reinsert the objective aperture.
 - b. If you are using the F20, make sure the 'View' is off in Digital Micrograph or SerialEM won't work.
 - c. A good starting point for larger samples like DNA origami tubules is:
 - i. **View:** mag = SA 17000x, Binning 1, Exp: 0.5 s, Spot 4, C2: 54.06%, full area
 - ii. **Record:** Same as view
 - iii. **Focus:** Same as View but Binning: 2, half area

- iv. **Trial:** Same as View but Binning: 8, Exp: 0.05 s, C2: lower intensity
 - v. **Search:** mag = M 2050x, Binning: 4, Exp: 0.5 s, Spot 5, C2: 83.74%, full area (in case you want to do a medium mag montage)
 - vi. Cycle through each of the modes (even if you have Low Dose Mode set up already).
 - vii. Once your imaging conditions have been setup, it's recommended to save your settings. Open the 'Settings' menu and click 'Save As' and save the settings with a distinctive name. For example: tomo_RT, tomo_cryo, RT SPA, cryo SPA, etc. This enables you to easily switch between different imaging modes without needing to adjust Low Dose Mode each time.
4. Move to a square of interest using the stage controls (or the low mag map, if used). Every time you move to a new square you should perform the following tasks:
- a. Take a 'Search' image.
 - b. In the 'Low Dose Control' menu, in the 'Define Area of' section, click 'Focus'. You should see a green box and a yellow circle.
 - i. Adjust the value for the position on tilt axis and the rotation value so that the yellow circle (focus area) is away from the record area (green box). Once set, click 'None' in the control menu.
 - ii. This value will need to be updated for each new grid since the grid orientation will change each time.
 - c. Rough and Fine Eucentricity (under the 'Tasks' menu).
 - d. Autofocus (make sure the drift is low).
 - e. (Optional) You may want to obtain a Record image in a corner of the grid square to get a sense of the particle density and how many images you need to obtain. *Be sure to mark the location by adding it to the Navigator, so that you don't collect data over an area already imaged in Record mode.*
5. Collect a medium mag montage:
- a. Open the 'File' menu, then click 'New Montage'. Set up an appropriate montage size to cover a whole grid square. Make sure the 'Use Search Parameters for Low Dose Mode' box and the 'Ask about making a map' boxes are checked.
 - b. A 3x3 montage works well for M 2050x (Search) on a 400-mesh grid.
 - c. Then type out the desired name for the montage. It's a good idea to have consistent naming conventions, such as: DATE_SAMPLE ID_MONTAGE DEPTH.mrc
 - i. Montage Depth: Use LMM or MMM for Low Mag Montage or Medium Mag Montage, respectively.
 - ii. If you are unable to check the 'Ask about making a map' box, then you forgot to open the Navigator!
 - d. In the Montage Control Panel, click start.
 - e. Once the montage is finished, close the file. (File -> Close).
6. Next, you'll want to pick points at which to collect the data:
- a. Be sure the map is loaded using the Navigator.

- b. Draw a polygon around the area of interest (in the Navigator, click 'Add Polygon'). Even if you are interested in the whole area, you need to exclude any visible grid bars for the next step.
- c. Next, open the 'Navigator' menu, then click 'Montaging & Grids', then click 'Add Grid of Points'.
 - i. Set the desired spacing. This will depend on the settings you are using for Search and Record.
 - ii. Make sure that the points are selected Away from Focus.
 - iii. Add points to the Navigator with the 'A' flag for Acquire.
 - iv. (*Optional*) Save the Navigator.
7. Open a new file (File -> Open New) in which to save your images.
8. Open the Navigator menu and click 'Acquire at Items'. Ensure that you are selecting parameters for 'Final Data' at the top left. Then, set the following:
 - a. The primary action should be to 'Acquire and save image or montage'.
 - b. Acquire non-montage images in Low Dose with should have 'Record' selected.
 - c. Check that images are being saved into the correct mrc file.
 - d. Check the 'Relax stage after final movement' box.
 - e. If you will be leaving while data is being collected, check the 'Close column valves at end' box.
 - f. The following tasks should be checked:
 - i. Manage Dewars/Vacuum: click Setup and set to 120 mins.
 - ii. Autofocus: set to every 10 items. If you are cycling the defocus, you may want to change this value.
 - iii. *Other Tasks can be checked and setup at your discretion, if desired, but note that some options may require calibrations.*
9. When ready, click the 'GO' button.
10. After collection, close the file.
11. Move to the next area and repeat Steps 4 – 10 until the desired number of images have been collected.

Section 3: Tomography

1. Make sure the 'View' is off in the GMS3 software or SerialEM won't work.
2. Use Low Dose Mode. A good place to start is:
 - a. **View:** 19kx, Spot 5, C2: 52.63%, Binning: 1, Exp: 0.5 s, Full size
 - b. **Focus:** same as View but Binning 2, Half size
 - c. **Record:** Same as View, but larger C2 value
 - d. **Trial:** same as View, but Binning: 4, Exp: 0.05 s, larger C2 value
 - e. **Search:** Same as View but Binning: 8, Exp: 0.05 s
 - f. You may need to adjust the magnification and C2 value based on your sample, but your setup should otherwise follow the above settings.
3. When moving to a new square, do Tasks -> Eucentricity -> Rough and Fine.
4. Take a View image, then set the Area of Focus. Then click 'Autofocus'.

5. Click the Tilt Series menu, then click Setup/Start. Parameters should be set as shown below using a Spot Size of 4, except the Intensity should be set to a mean counts of 3500. If using a different spot size, the counts target may need to be adjusted accordingly.
6. When ready click the 'GO' button.
7. At the end of the series, click the Tilt series menu, then click 'Terminate' and close the file. Then in the scope software, click on the Search tab, then set alpha to 0.
8. Locate the next area of interest.
 - a. It may be more helpful to lower the screen and adjust the defocus by -10 μm to easily locate the sample.
 - b. Will need to work within the center area of the grid due to the tilt angle; samples too close to the edge will be cutoff at higher tilt values.
9. If you get an error that says "The Record image has more than the specified fraction of data loss", press 'Retake' to acquire the image again.
 - a. This may happen a few times while acquiring the series, so it's ideal to stick around during the acquisition, otherwise the OneView may time out and retract.

Tilt Series Setup

Tilt Angle Specifications

Tilt to End at Swap Base increment

Increment proportional to cosine of angle Total # of tilts: 51

Run series in two directions from Return with Walk Up

Magnification for anchor: ****2.3 μm **** Use View for anchor

Use dose-symmetric scheme

Delay time after tilting by basic increment: seconds

Change some settings as a function of angle

Size, Image Shift, and Recentering Specifications

Magnification: Binning: Pixel size: 0.563 nm

At start and when needed, track at magnification:

Limit image shift to microns

Recenter beam from Low Dose tracking images

Autocenter beam every minutes

Autocenter beam in second half of series at degrees

Refine the zero-loss peak every minutes

Beam Intensity or Exposure Control

Change Record exposure time instead of beam intensity

Keep beam intensity constant

Vary intensity to be 2 higher at 60 deg (1/cos to 1/1 power)

Set intensity to keep mean counts of Record images at

The image in buffer A implies a Record zero-tilt mean of 4980

Taper counts down to above +/- degrees

Keep intensity below current value (use if intensity already set up)

Do not increase intensity above value for first saved image

Use current intensity at zero tilt (increase to start series)

Total dose: no estimate with setting for constant counts

Change other exposures proportional to Record change

Change drift settling proportional to exposure change

Camera and Acquisition Parameters

Average Record dark references

If taking frames, get initial partial sum of frames

Autofocus Control

Defocus target: μm Beam tilt: milliradians

Autofocus offset: μm Iteration threshold: μm

Do autofocus at least every degrees Skip Autofocusing

Focus every time above degrees

Repeat autofocus if focus differs from prediction by > μm

Do autofocus when error in focus prediction exceeds μm

Check autofocus, stop if measured < X actual defocus

Stop if autofocus exceeds absolute focus limits

Stop if autofocus change exceeds μm

Initial and Final Actions

Align to image now in Buffer A

Refine eucentricity

Leave mid-tilt anchor near plus or minus degrees

Use mid-tilt anchor that you have left in buffer

Close column valves at end of series

Tracking Control Parameters

Wait for drift to settle

Repeat Record if percentage of field lost is more than

Stop and ask before repeating Record in Low Dose mode

Stop if Autoalign shifts more than % of image size

Get tracking image when error in X/Y prediction is > % of field

Track: Before After Before & after Autofocusing

Stop after aligning tracking image to allow manual adjustment

Align only with tracking images, not Record images

Align with Preview before getting new track reference

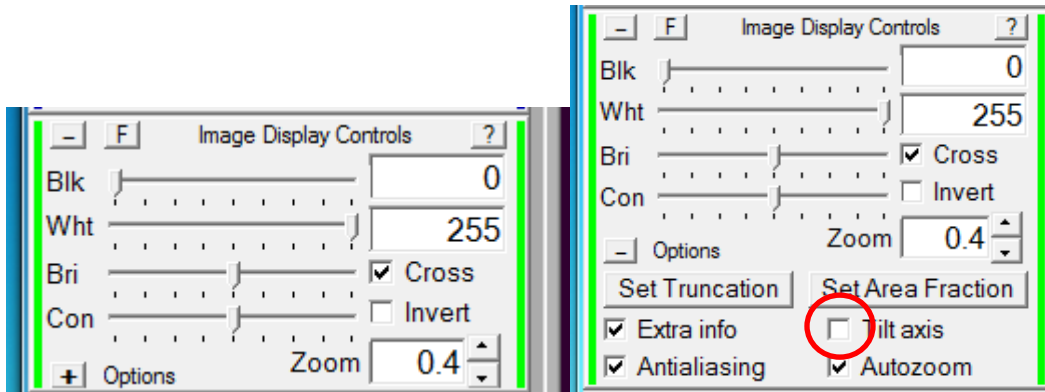
Get new track reference if Record alignment differs by > %

Previous Next First Half Second Half Full

GO Single Loop Postpone Cancel ?

Section 4: Miscellaneous

1. 'Image Display Controls': check the box that says 'Cross' so you always can see the red crosshair
 - a. For tomography users, there is now an option to show you the tilt axis in the image. Click on + button for more options.



2. A new option in the 'Acquire at Items' dialog is the option to cycle the defocus while obtaining images.

