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. You should have already performed the alignment using the RT Single Tilt holder.

Low Dose Setup (for first time users)

1. Check Low Dose Mode in SerialEM and make sure the objective aperture is inserted.
2. For single particle data collection, the settings shown below are a good starting point, especially for beam sensitive materials and can be imported directly. Open the Settings menu, then click open, navigate to C:\ProgramData\SerialEM\ATiano\, and choose the file labeled SerialEMsettings_SPAcryo.txt.
   a. **Search**: mag = SA 5000, binning 4, Exp: 0.5 s, Spot 10, C2: 47.27%, full area
   b. **View**: mag = SA 20500x, binning 4, Exp: 0.5 s, Spot 11, C2: 42.02%, full area
   c. **Record**: mag = SA 29000x, binning 1, Exp: 4 s, Spot 10, C2: 41.70%, full area
   d. **Trial/Focus**: same mag as record but binning 2, Exp: 0.5 s, half area
      i. Checking the box for 'Keep Focus and Trial identical' in the Low Dose Control panel is helpful to keep these identical (Figure 1).
   e. If you have imported these settings directly, please immediately save them in your own folder before making any changes to prevent overwriting the original file (see Step 5). If you do not save them under your own file, they will automatically be overwritten when you close serialEM.
3. Cycle through each of the modes in low dose: check beam centering, collect an image and to ensure you are at your target electron dose (for more on this, see Step 4). Click and unclick 'Continuous update' to update settings for each imaging mode.
   a. If the beam is not well centered after capturing an image for a given mode, you can also use the Center Beam function and add an additional beam shift:
      i. In the Low Dose Control panel, check the 'Continuous update' box.
      ii. For Record mode open the Tasks menu, then click 'Center Beam'.
      iii. For other modes check the 'Set' box in the Additional beam shift section (Figure 1) then open tasks and click 'Center Beam'
      iv. Take a new image.
      v. If centering is good, uncheck both the 'Set' and 'Continuous update' check boxes.
      vi. Note: You may encounter some drift in the beam centering through your session, so it's good idea to recheck this a few times during your session.

4. Exposure dose must be calibrated daily in order to obtain the total electron dose, which will display in the top of the Low Dose Control panel (Figure 1):
   a. Take a Record Image of a blank beam (no sample inserted). The 'Spot' indicator in the Microscope control panel must be blue (Figure 1).
      i. Note: Electron doses can only be calculated where the beam intensity has been calibrated (indicated by the blue color) at a given spot size.
   b. Open the Calibration menu, then click 'Exposure Dose'. It will ask you to confirm the image in Buffer A is a Record image. Click OK.
   c. The dose should now be visible.
5. Once your imaging conditions have been set up, 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.
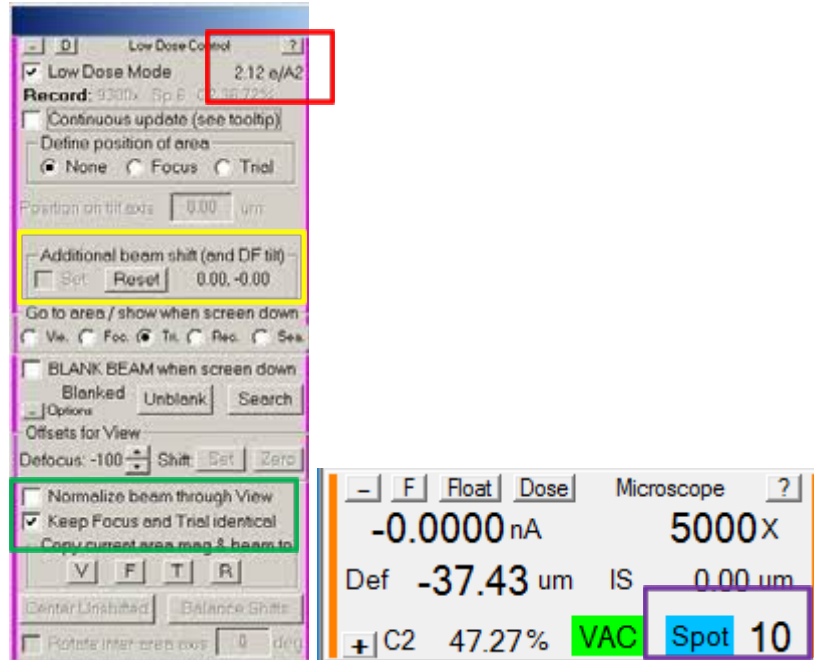   a. Settings should be saved under: C:\ProgramData\SerialEM**YOURUSERNAME**

Figure 1: Images showing the location of the electron dose (left, red box), Keep Focus and Trial identical box (left, green box), set additional beam shift (left, yellow), and calibrated Beam Intensity indicator (right, purple box).

Full Grid or Partial Low-Mag Montage

6. Open the navigator window by clicking on the Navigator drop down menu and clicking 'Open'.
7. The next step is to collect a Low Mag Montage (LMM) of the entire grid.
   a. Turn off Low Dose mode and remove the objective aperture.
   b. Change the magnification to LM 120x or LM 180x and move to roughly the middle of the grid.

      i.   For a partial LMM, move roughly to the center of the desired area you want to image.

c.   Check and adjust the beam intensity on the screen. Take a Record image to see that the captured image looks good. Adjust the beam intensity and/or exposure time as needed. If you want to avoid having to change the exposure time for Record, it is also possible to set separate exposure time under Montage Mapping in camera setup and then use those for the low mag montage (Figure 2)

      i.   Once you have ideal Low Mag settings, it's recommended to save these settings. Open the Navigator menu, then click Open Imaging States (Figure 2).

      ii.   Click on 'Add Current State', then use the text box to name the state (LMM is recommended for the name).

      iii.   Go back to Low Dose and go to your Search settings.

      iv.   In the Imaging States Dialog, click on 'Add Current State' and again use the text box to name it (MMM is recommended).

      v.   Now you can easily navigate between Low Mag (outside Low Dose) to Low Dose Search using the Imaging States Dialog by selecting the desired state and clicking 'Go to Selected State'.

      vi.   You can add as many additional states as desired, ensuring each is label to easily transition between them.
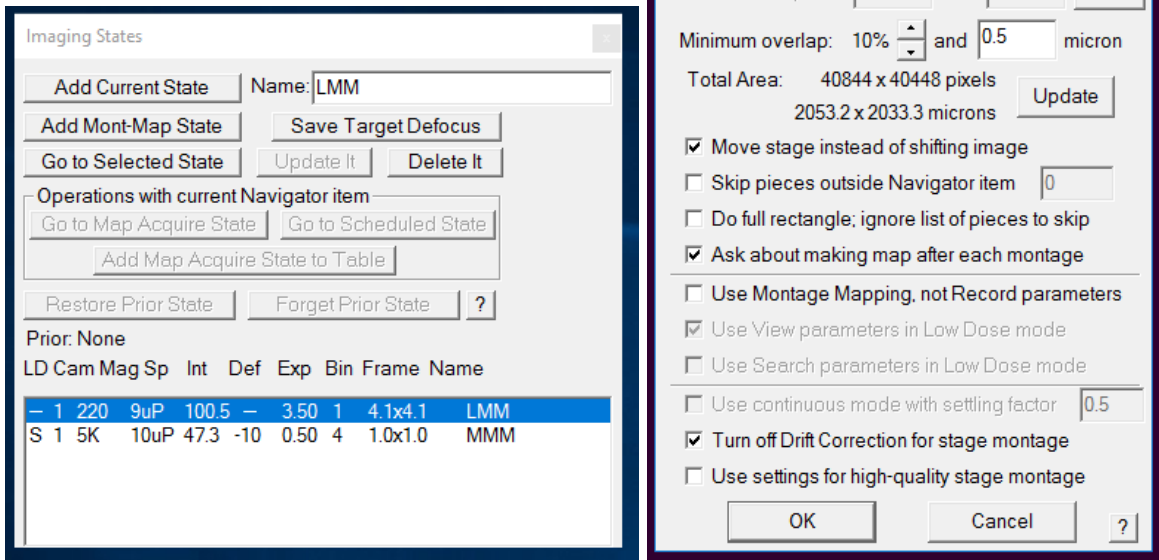
Imaging States

Add Current State   Name: LMM
Add Mont-Map State   Save Target Defocus
Go to Selected State   Update It   Delete It

Operations with current Navigator item
Go to Map Acquire State   Go to Scheduled State
Add Map Acquire State to Table
Restore Prior State   Forget Prior State   ?
Prior: None
LD Cam Mag Sp Int Def Exp Bin Frame Name

— 1 220 9uP 100.5 — 3.50 1 4.1x4.1 LMM
S 1 5K   10uP 47.3 -10 0.50 4 1.0x1.0 MMM

Montage Setup

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

Magnification: 220   Binning: 1

Pixel size: 50.3 nm

Number of pieces in X: 11   Y: 11

Piece size in X: 4084   Y: 4048

Overlap in X: 408   Y: 408   Reset

Minimum overlap: 10% and 0.5 micron

Total Area: 40844 x 40448 pixels   Update
               2053.2 x 2033.3 microns

☑ Move stage instead of shifting image
☐ Skip pieces outside Navigator item 0
☐ Do full rectangle; ignore list of pieces to skip
☑ Ask about making map after each montage
☐ Use Montage Mapping, not Record parameters
☑ Use View parameters in Low Dose mode
☐ Use Search parameters in Low Dose mode
☐ Use continuous mode with settling factor 0.5
☑ Turn off Drift Correction for stage montage
☐ Use settings for high-quality stage montage

OK   Cancel   ?

Figure 2: Image of the Imaging States Panel (left) and the Full Grid Montage Panel (right).

d. For a Full Grid Montage, open the Navigator menu, then click on Montaging & Grids, then select 'Setup Full Montage'. The Montage Setup dialog will open (Figure 2).

   i. The number of pieces will automatically be calculated based on the magnification chosen. Increasing the binning to 2 or 4 should help speed up the collection. When ready, click OK.

e. For a partial LMM, click 'File' then select 'New Montage'. Try a 6x6 mesh size for the montage and check the 'Ask about making a map' box. Under Metadata - Save in extended header check all the parameters. Type in the desired name for the montage. It's a good idea to have consistent naming conventions, such as: DATE_SAMPLE ID_MONTAGE DEPTH.mrc
Montage Depth: Use LMM or MMM for Low Mag Montage or Medium Mag Montage, respectively.
If you are unable to check the 'Ask about making a map' box, then you forgot to open the Navigator!

f. In the Montage Control panel, check 'show overview at end' and 'align pieces in overview', uncheck 'treat as very sloppy montage'. Then, click 'Start'.

8. 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.
   Note: Maps are now also indicated in blue framed area in images.
9. In the event of program crashes, it's a good idea to save the navigator (Navigator -> Save).
10. Close the full grid montage (File -> Close).

Medium Mag Montage

11. Turn on Low Dose imaging and insert objective aperture.
12. 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. Rough and Fine Eucentricity (under the 'Tasks' menu).
    c. Autofocus (make sure the drift is low).
    d. (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.
13. Collect a medium mag montage:
    a. Under 'File' menu, choose '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. Check "Move stage instead of shifting image"
    b. A 3x3 montage works well for M 2050x or a 5x5 works well for SA 5000x on a 400-mesh grid.
    c. 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
       Montage Depth: Use LMM or MMM for Low Mag Montage or Medium Mag Montage, respectively.
       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).

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.

14. 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 5x5 montage works well for SA 5000x (Search) on a 400-mesh grid.
15. 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).
16. Enable 'Acquire' for each of these points.
    To enable 'Acquire' for many points simultaneously, select 'Collapse' in the Navigator menu.
17. (*Optional*) Save the Navigator.
    This is important if SerialEM or the camera computer are prone to crashing.
18. Under the Navigator menu 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'.
19. Make sure you are selecting the parameters for Mapping on the top left (Figure 3). 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 acquired 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.
       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.
       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).
    g. You can click on the 'Setup' tab for additional settings
    h. See the screenshot below for reference.
20. 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.
21. Alternatively, you can also collect Medium Mag Montages individually in any given grid square, especially if you want to check the ice and particle conditions before collecting data:
    a. Add a point for each grid square of interest in the Navigator.
    b. Move to the grid square of interest by highlighting the point in the Navigator and clicking 'Move to XY'.
    c. Take a Search image (be sure 'Low Dose Mode' is checked in the control panel).
    d. Open the Tasks menu, then click Eucentricity, then select 'Rough Eucentricity'.
       i. You can also select 'Both Rough and Fine', but Rough Eucentricity is sufficient.

e. When completed, lower the screen to make sure you're roughly centered in the grid square.
f. Open the File menu, then click 'New Montage'. Repeat the same steps as you did for the LMM. Then click start in the Montage Control Panel.
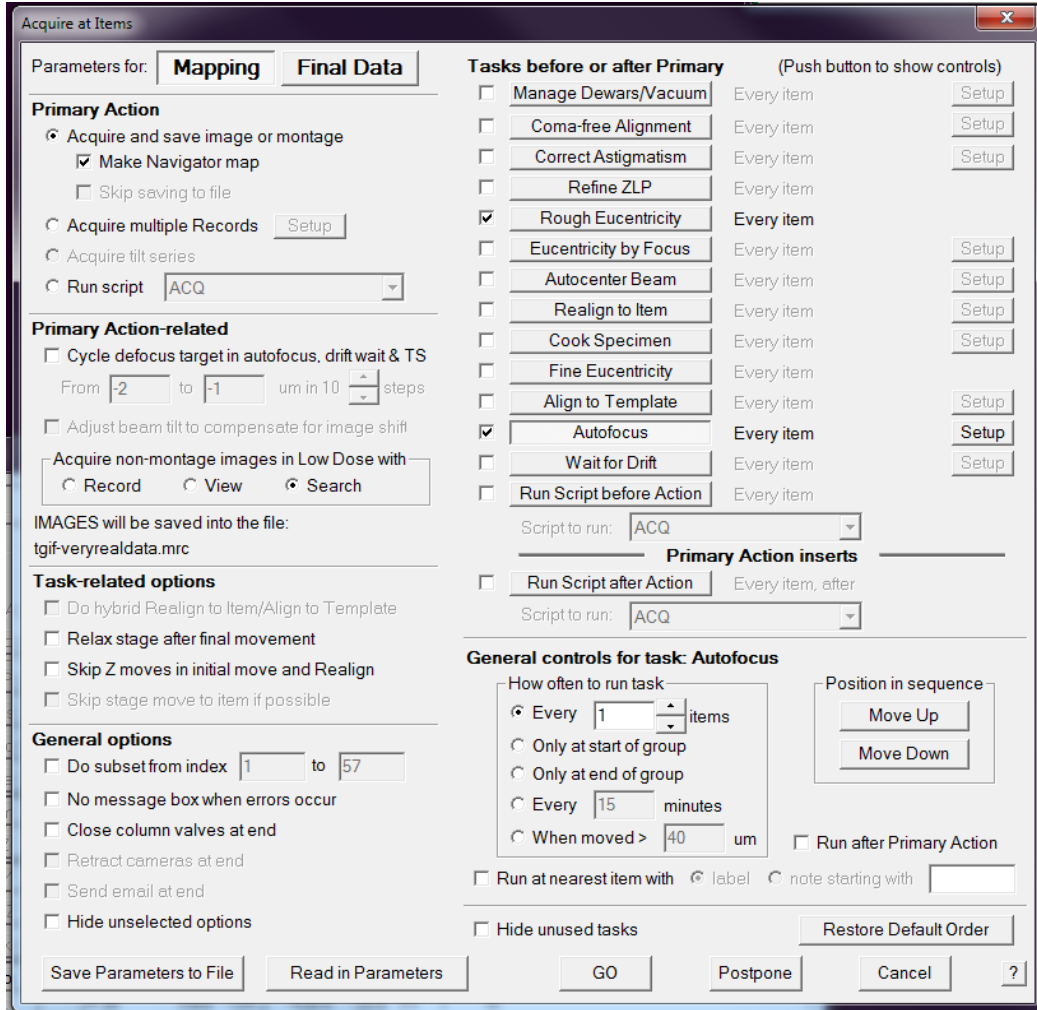   i. A 5x5 mesh size works well at SA 5000x (Search) on a 400-mesh grid.

Figure 3: The Acquire at Items dialog for Mapping.

Running the hole finder

22. Select one of the mapped grid squares. Identify a sacrificial area on a grid square (preferably one that isn't ideal for imaging). 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 with the right mouse button.
23. 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 the tilt axis to -1.71 um.
        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 grid holes.

b. Adjust the 'Rotate inter-area axis' as needed so that the yellow circle sits in the middle of four holes. This value will need to be updated for each new grid since the grid orientation will change each time.
Note: This should be set first, before running the hole finder, since we want to make sure holes are ordered away from the focus area.

24. For each grid square that was mapped:
a. Open the corresponding map from the Navigator by double clicking the entry
b. Optional: draw a polygon on the map (Navigator -> Add polygon) to exclude grid square edges, contamination, etc.
c. Run the hole finder (Navigator menu -> Montaging and Grids -> Find Holes in Regular Grid)
d. 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).
e. Enable 'Bracket last filter/threshold values' to speed up hole finding in subsequent runs.
f. Click 'Find Holes'.
g. Adjust the intensity 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'.
'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.

25. Now use the same sacrificial area used in Step 22 to check your image shift and defocus offsets for low dose mode and adjust any values as needed (see step 29 for instructions). It's recommended to always check these prior to data collection.

Final Data Collection via Acquire at Items

26. 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.
If the contrast is low, adjust the defocus offset (see next section).
i. Note: It is recommended to use a defocus offset of -40 um for View, otherwise the Align to Template routine will likely fail.
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. Once you have that, open the 'Acquire at Items' dialog. Make sure to switch to the 'Final Data' tab, check the 'Align to Template' box, then click 'Setup'.

d. Enter the numerical label for the template from the Navigator into the box (Figure 4). 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'.
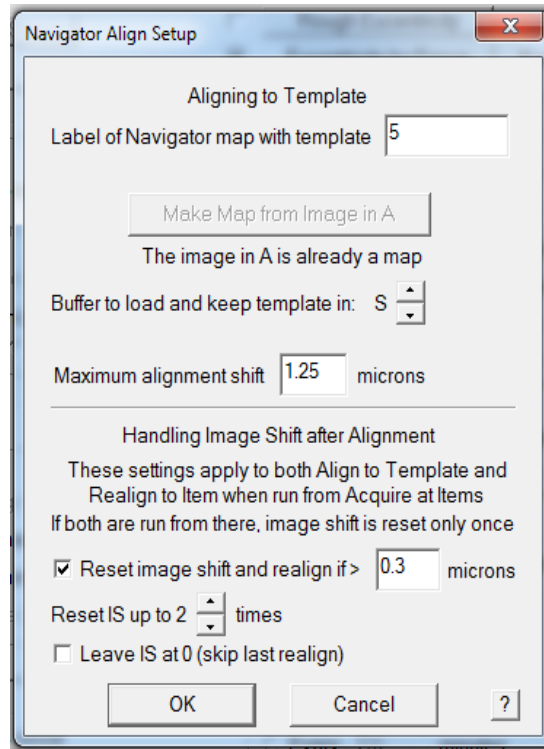

Figure 4: The Align to Template setup dialog.

27. Setup the remaining parameters for Final Data (Figure 5):

a. Manage dewars/vacuum should be 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, or if the Z is hight (close to +/-100um). This avoids the need to tilt the stage to obtain the eucentric height, which is ideal for data collection. Each user will need to calibrate:
    i. Make sure you are at eucentric height before running calibration
    ii. In the SerialEM main toolbar, under the 'Focus/Tune' menu, select 'Set Offset' and set to -5 um.
    iii. Under the 'Tasks' menu, select Eucentricity -> 'Calibrate Focus Targets' and run the calibration.
    iv. **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. This is also preferable 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 'Postpone' 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.
    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' to save changes to dialogue.

Figure 5: Acquire at Items Dialog for Data Collection

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

Setting Shift and Defocus Offsets for Low Dose Mode

29. In the Low Dose Control panel, it's recommended to set an offset defocus value for 'View' and 'Search'. This will help increase contrast for montages and templates. A -40 µm defocus offset is recommended for View to achieve good alignment with the template. A -10 µm defocus offset is a good starting point for Search.
    a. Note that if montaging does not work at high defocus values (unreliable alignment of montage pieces and navigation to stage positions), 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.
    Please see Amanda or Berith if you need to perform this calibration. Calibrations should only be done when you are certain of your imaging conditions and, unless you change conditions, only need to be performed once.
30. You absolutely must do the shift offsets (image shifts) for View and Search (Figure 6). 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. It doesn't matter in 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 (Figure 6)!
        v. First, Search and View:
            1. Choose a hole (preferably a landmark one) so it's easy to find
            2. Click on the 'Search' radio button in the 'Offsets for' 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.
            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. Click on the 'View' radio button in the 'Offsets for' dialog

2. This is the same process as above, but cycle between View, Record, and back to view, centering the hole each time. After centering the last View, click the 'Set' button in the offset dialog.
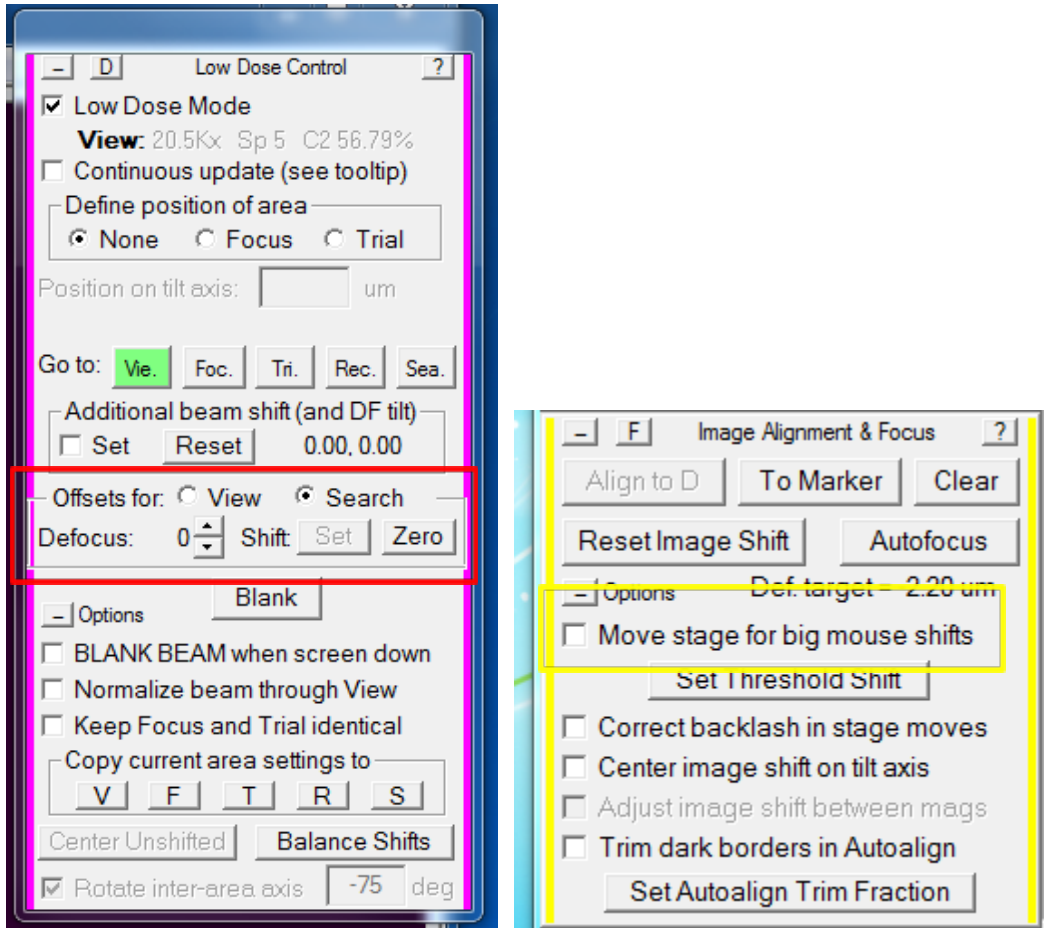3. Take images again to make sure they are centered.

Figure 6: Images highlighting the View and Search Defocus offsets (left, red box) and the Move stage for big mouse shifts checkbox (right, yellow box).

Section 2: Negative Stain Imaging

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 SPA cryo imaging protocol for the step by step process.
   b. Remember to re-insert objective aperture after collecting a low mag montage.
2. Open the Navigator ('Navigator' menu -> Open).
3. Set up Low Dose Mode in SerialEM if you have not done so already. Follow Low Dose setup instructions above.

4. In the 'Low Dose Control' menu, in the 'Define Area of' section, click 'Focus'. You should see a green box and a yellow circle.
   a. 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.
   b. This value will need to be updated for each new grid since the grid orientation will change each time.
5. 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. Rough and Fine Eucentricity (under the 'Tasks' menu).
   c. Autofocus (make sure the drift is low).
   d. (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.*
6. Collect a medium mag montage:
   a. Under 'File' menu, choose '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. Check "Move stage instead of shifting image".
   b. A 3x3 montage works well for M 2050x or a 5x5 works well for SA 5000x on a 400-mesh grid.
   c. 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
   Montage Depth: Use LMM or MMM for Low Mag Montage or Medium Mag Montage, respectively.
   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).
7. 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. (*Optional*) Save the Navigator.
8. Open a new file (File -> Open New) in which to save your images.

9. Open the Navigator menu and click 'Acquire at Items'. Make sure you selected the 'Final Data' tab 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 should have 'Record' selected.
    c. Verify 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. Under Tasks
        i. Check 'Manage Dewars/Vacuum'.click Setup and set to 120 mins.
        ii. Check Autofocus. Set to every 10 items. If you are cycling the defocus, you may want to change this value.
        iii. *'Eucentricity by focus' and 'Align to template' only necessary for cryo imaging. Wait for Drift is also useful if the sample drift is high, especially for longer exposure times. Other Tasks can be checked and set up at your discretion, if desired, but note that some options may require calibrations.*
10. When ready, click the 'GO' button.
11. After collection, close the file.
12. Move to the next area and repeat Steps 5 – 11 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 shown below and these settings can be directly imported. Open the Settings menu, then click open, navigate to C:\ProgramData\SerialEM\ATiano\, and choose the file labeled SerialEMsettings_RT-tomo.txt.:
    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.
    b. If you have imported these settings directly, please immediately save them in in your own folder before making any changes to prevent overwriting the original file. Settings should be saved under: C:\ProgramData\SerialEM**YOURUSERNAME**
3. When moving to a new square, do Tasks -> Eucentricity -> Rough and Fine.
    a. After refining the eucentricity, check the log to see what the lateral displacement is. If it's far from 0 microns (more than ±1 micron), it is recommended to open the Task menu, then click the 'Set Tilt Axis Offset' option.

b. SerialEM should autofill this based on the eucentric data information, so click OK.
c. Then, click the 'Reset Image Shift' button in the Image Alignment & Focus Panel.
d. Perform another Fine Eucentricity. The lateral displacement should be closer to zero now (between ± 1 micron). This will help SerialEM to keep better track of the sample during the tilt series.
4. Take a View image, then set the Area of Focus. Then click 'Autofocus'.
5. Click the Tilt Series menu, then click Setup/Start, which will open the Tilt Series Setup dialog (Figure 7). Below is an example of parameters that have worked for the collection of datasets of DNA origami, and is a good starting point:
    a. Be sure to carefully determine a suitable mean counts for your Record images in the Beam Intensity or Exposure Controls section (below an example of 5000 counts is shown at spot size 4), which will be dependent on your spot size and desired exposure dose.
    b. All be sure to carefully select your Tracking Control Parameters (red box). If you plan to walk away while the system collects the tilt series, you should uncheck the 'Stop and ask before repeating Record in Low Dose mode' box, so the series can continue without a clickable prompt. (This assumes you want the Record to be repeated if more than a certain percentage of the field is lost.)
        i. If the box is greyed out, it's because Low Dose is off!
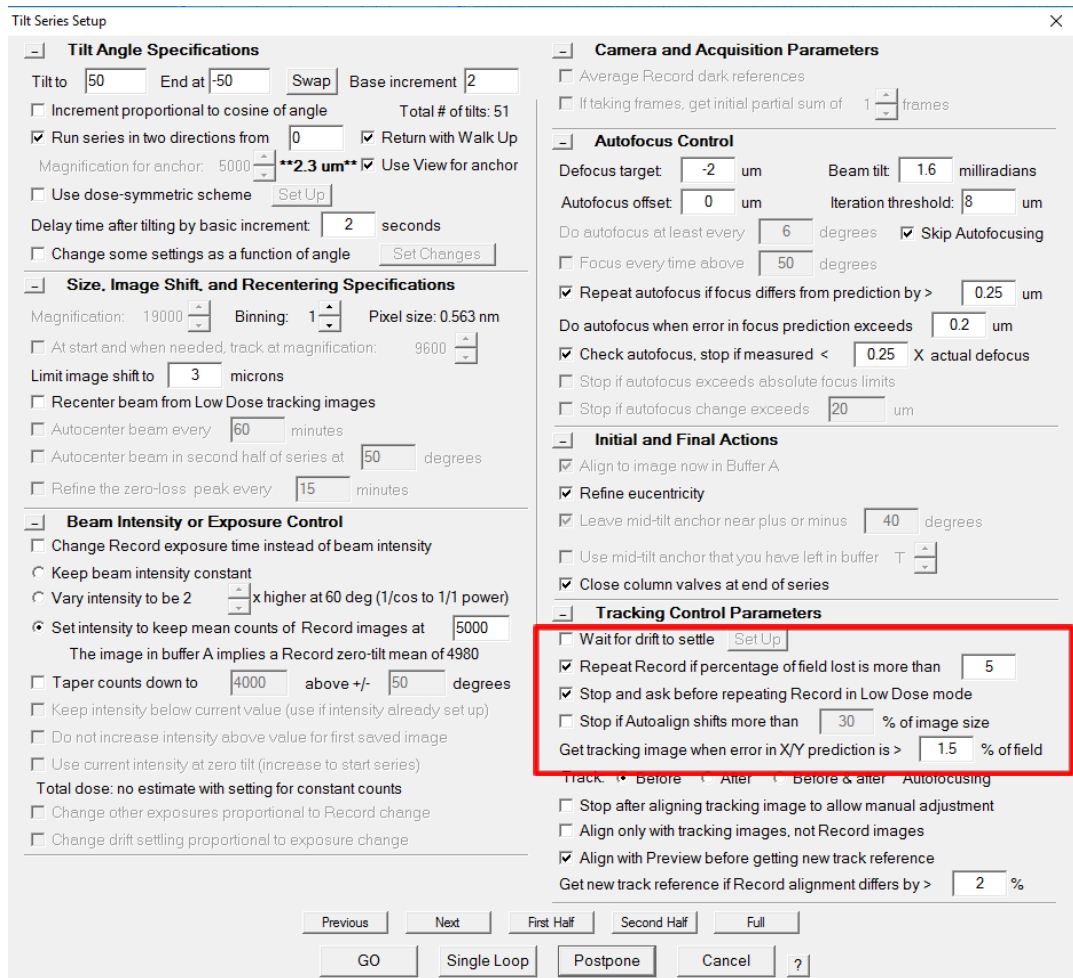
Figure 7: Tilt Series Setup Dialog

    ii. Note: If you get an error that says "The Record image has more than the specified fraction of data loss", this has to do with your settings in the Tracking Control Parameters. See step 5b, especially if you plan to walk away during the acquisition, otherwise the OneView may time out and retract.

6. It's also a good idea to check your global tilt series settings, by opening 'Batch Policies' in the Tilt Series menu (Figure 8) and adjust any of the settings as needed.
    a. Be sure that 'After failure to tilt to expect angle (possible pole touch)' is set to terminate.
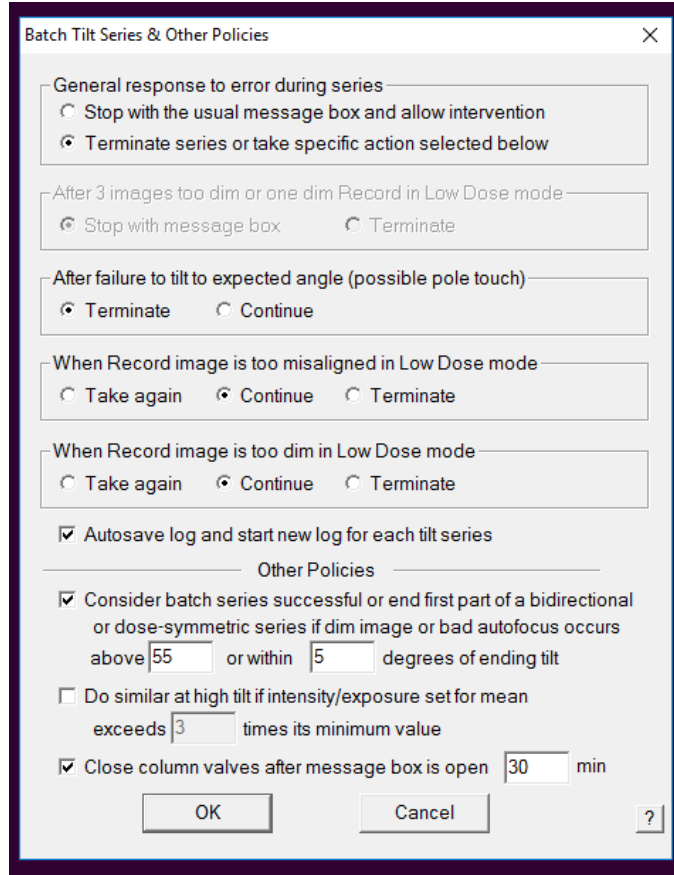
Figure 8: Batch Policies (global settings) for Tilt Series.

7. When ready click the 'GO' button.
8. 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, set alpha to 0.
9. 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 and grid square due to the tilt angle; samples too close to the edge will be cut off at higher tilt values.

Section 4: Miscellaneous

1. 'Image Display Controls': check the box that says 'Cross' so you always can see the red crosshair
   For tomography users, there is now an option to show you the tilt axis in the image. Click on + button for more options.
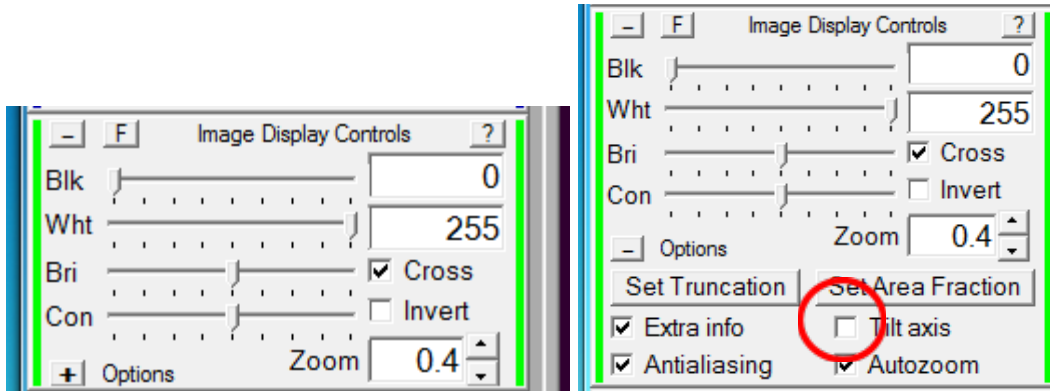
2. A new option in the 'Acquire at Items' dialog enables users to cycle the defocus while obtaining images.
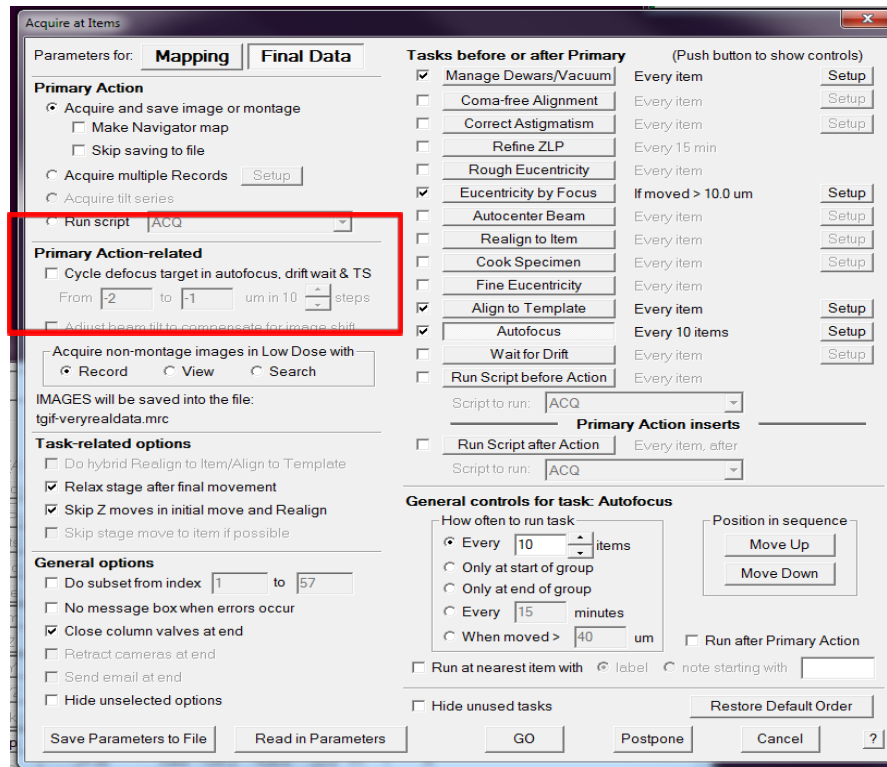
3. Another useful feature is the 'Note' section of the Navigator. This is helpful for keep track of grid squares of interest that you have already imaged, indicate poor ice, contamination, etc. Click on the desired point, then add any desired information in the 'Note' field.