

**Making Low Mag Maps:**

The most important thing to remember when working with cryo-microscopy is the concept of low-dose. You must always take care to put as little dose as possible on the sample. Frozen-hydrated samples are extremely beam sensitive. Because of this, SerialEM has a low-dose mode that will help to avoid sample damage and a low-dose method for mapping the entire imageable area of the grid or selected areas of the grid. It is also very useful to have the C2 lens value in your TUI workstation dock.

- To get started, make sure the screen is up and then open the column valves.
- Adjust the magnification to about 50X (LM mode) and set spot size to 7.
- Lower the screen and quickly look for an area with no sample—this is usually a grid square with no section, or slightly ripped carbon termed the “bad area” (preferably near center of grid).
- Mark the bad area using the stage “add” in TUI for easy access throughout your microscopy session.
- Go to a working mag in your bad area and do eucentricity and focus either manually or with SerialEM, then check your beam tilt alignments and rotation center. NOTE: The bad area is the ONLY safe place to have a full strength beam on your grid. It’s an area of sacrifice to the beam.
- Setup for a full low mag map montage: objective aperture=out, mag~170X, spot=8, record=binx4 (on 4K CCD), ~0.35s exposure, 0s drift settling, beam edges are just off the CCD screen—usually C2=100%.
- Take one record image to make sure the beam and the cryo-box is centered and the image looks good.
- Under the Navigator menu in SerialEM, open up a new navigator.
- Then, under the navigator menu, select “Setup Full Montage”. You should always be less than 10X10—assuming you are taking 1K images. The program should automatically pick for you the size it needs. name the file (i.e. LMM.st); the program goes to the center of the compustage and waits. Now press start in the Montage dialogue box. When the map is finished, under the navigator menu, select “new map from image”, close the montage (under the file menu), and save the navigator file (nav.nav).

**Making a Medium Mag Map:**

Making medium mag maps can be a bit difficult because the LMM is not necessarily accurate about the actual position of the sample in comparison to the map image (this is due to the usual inaccuracy of stage movements). In any event, it can be worked around.

- First, go to your “bad area” to set up the beam.
- Setup for medium mag map montage: objective aperture=40um, mag~3100X, spot=4, record=binx4, ~0.35 exposure, 0s drift settling, beam spread so that it just covers the CCD.
- In your bad area, you can center the aperture and beam without messing up your sample. Take a record and verify the image looks good.
- Using the navigator, draw a polygon on the LMM around your desired MMM area (no larger than a 200mesh grid square).
- Then, in the navigator menu, select “Setup Polygon Montage”. You should again be less than 10X10, name the file, (MMM\_X.st), and press start in the Montage

- dialogue box. When the map is finished, under the navigator menu, select “new map from image”, and save the navigator file. Often, the MMM does not line up very well with the LMM. The easiest thing to do, is to move the polygon on the MMM to the correct position and make a new MMM of that area—may not be a good idea if your sample is very dose sensitive. Otherwise, read David’s documentation on using registration points to transform the map.
- If you need to do multiple MMMs, you can either draw polygons around your other areas of interest, or add points to the center of your area of interest (keep in mind the shift you discovered on your first map). This only works if you have a montage already open. Then, make these acquire points and choose “Acquire Areas” in the navigator menu. This will save a single file with MMMs into one stack. The size of each MMM-map around the acquire point has the size of the last montage, so if you chose to just add points, it will use the size of the last polygon (caution, if no polygon was used, it will take just one image and that will be your map). When the acquire areas option is used, they all automatically become maps and so when it’s finished, close the montage and save the navigator file.

### **Setting Up Low-dose:**

The idea with low-dose is to not damage the sample with the beam before and while recording tilt-series. First, click on the low-dose dialogue box and if you are using the GIF, click on the EFTEM dialogue box in SerialEM (and turn EFTEM on so you are using the GIF camera). You should set up low-dose at a bad area of the grid where you can burn it if you need to. Make sure the objective aperture is in and centered. Make sure you are eucentric and in focus because if you are way off, you will have to do this all over again. Also, ALL imaging parameters should have 0 drift settling in the camera setup. Whenever you are changing magnification, spot size, or intensity (as measured by C2 lens), you must have “continuous update of mag & beam” checked. But keep it unchecked as default.

- Set up the record parameter first. You should be at bin=2 and exposure time ~0.3s. Go to your desired magnification (e.g. one that has a 1-0.8 nm pixel size). The goal is to make the beam just small enough that when you are at high-tilt, you don’t see the edge of the beam, but you aren’t so spread out that you are burning too much of a surrounding area. Try starting at spot size 8 and use the intensity to set a dose of  $2.5e^-/A^2$ . Center the beam and if you can see the edges, move to spot 7. Keep doing this until you no longer see the edge of the beam at that higher dose. Then, drop the dose down to  $1e^-/A^2$ . The beam is now centered and set for records (and previews which should be bin=8 and very low exposures of the record area). If you are using the GIF, make sure to have the slit in, with a width of 20eV.
- Next, set up the view parameter. You should be at bin=8 and exposure time=0.05s. Go to a lower magnification at around 3000X. Try starting at spot size 8 and make the beam small so that you can see the edges along the sides of the picture. Look at the dose and try to get it  $<0.020e^-/A^2$ . You may have to change the spot size to accomplish this. Now, using the “additional beam shift” feature, center the beam for view images by checking the set button first,

centering the beam, then unchecking the set button. If you are using the GIF, make sure the slit is out.

- Next, set up the trial/focus parameters. In almost all cases, the trial and focus will be exactly the same (i.e. same mag, same position on tilt-axis). The magnification should be the same as what you are taking for your records to get the best possible focus. If that mag is very high, you should make the trials at a lower mag to ease tracking. This will explain what to do if you are keeping trial/focus exactly the same. Take a trial image and set the mag in your trial area; then take a view image. To set the position of the focus and trial area in relation to the record area use the “Define position of area” option (set to focus or trial), and click on the view image: the green cross marks the chosen position—it will only move along the calibrated tilt axis; set the distance between record area (center of the view image) about 1 $\mu$ m away from the record area (number below position on tilt-axis value). You can either go above or below the record area. Then, take a trial image. The goal is to not allow the trial beam to overlap with the record area. So, if you are above the record area, you should see the edge of the beam in the bottom of your trial image. If you are below the record area, you should see the edge of the beam at the top of your trial image. The beam should barely cover the trial area and should give a good image for tracking and focus. Usually, bin=4 for focus and bin=8 for trial (sometimes you need to make your trial a smaller bin) with moderate dose (1-2 e<sup>-</sup>/A<sup>2</sup>). If you have the GIF, don’t have the slit in for trial/focus because you have to increase the dose that much more to get good pictures. Use the “additional beam shift” feature to center the beam for focus and trial images.
- Double-check everything by taking at least one picture of each type.

**Imaging in Low-Dose:** Imaging in low-dose is different than what you are used to in plastic. Here are some tricks of the trade that should help you:

- Never drop the viewing screen unless you are certain you are on your “bad area”. As an extra precaution, you should check the “BLANK BEAM with screen down” option. Then, when you lower the screen, you have to press the “Unblank” button to see the beam. When lowering the screen you can see the beam/aperture for any of the imaging parameters by choosing either view, focus, trial, or record.
- When searching for a sample, it’s easiest to use the navigator and your maps to go to an area that has potential for tomography.
- In the Buffer Controls options menu, it is helpful to roll your buffers to the max (A-->N) and turn off the “copy on save to X” and “align on save”. These will help keep you from having to take too many images on the same area and potentially ruin an area for tomography.
- Because in low-dose, small shifts are done with right clicking the mouse and dragging the image in A, it is critical to check “Move stage for big mouse shifts” in the Image Alignment & Focus options menu. You can also play around with setting the threshold shift to find what you like best. In this options menu there is also the ability to center the image shift on the tilt-axis instead of the optical axis. This should help keep focus levels during the tilt-series better.