# Optimizing Tomography for Plastic Sections

The setup:

- Align microscope
- Take gain reference (select calibrate dose with gain reference)
- Insert specimen and find area of interest

1. Make sure the area is adequately "baked". Go to lower mag (~2000X); spread the beam out to fill the screen. Open the dose meter and wait until the dose reaches 2000-3000 $e^-/Å^2$.

2. Go back to your working mag and do the eucentricity on or very near your area of interest.

3. Set your focus target to about –0.2 $\mu$m and then autofocus.

4. Reset image shift and lower the microscope's screen. Center the objective aperture and make the beam the smallest you are comfortable with and center it—don't forget to work on the right side of crossover. The beam should be about 1.5-2 cm larger in radius than the outer circle inscribed on the screen. This will aid in keeping the beam from entering the images at high-tilts (see step 6) and keep out any image aberrations due to the beam getting too small.

5. Using trial images, center your area of interest in SerialEM. It is a good idea to take a record image and see how many counts there are—especially if this is a new sample type. If the number of counts at zero degrees is lower than the optimal number of counts for an image, it will be even worse at high tilt. This is because at 60°, your sample is twice as thick as it is at 0°. You should aim for 1.3-1.4 times your optimal counts at zero tilt with the beam at the smallest you allow it to be (was set up in step 4). IF you don't have adequate counts at zero tilt, consider doing the following: go down to spot 1—recheck that the beam is centered and stigmated. Change the C2 aperture to a larger size—be sure to center it properly and check your condenser stigmation. Or as a last resort, change the objective aperture to a larger size—this is last resort because it will drop the resolution of your final tomogram.

6. Once the beam is setup and your area of interest is centered, you can open up the tilt-series setup/start menu. These are standard starting points:

- Input your tilt-range (typical is –60 to +60). Heavier rods should work better starting on the –60 side, but for most rods, it doesn't matter which side you start on.
- Input your increment (typical is 1 degree). The default of a 2 second tilt delay is good unless you are using larger increments, a heavy holder, or you have a very fast camera, in which case, you might want to increase that to 4 or 6 seconds.
- Make sure the controller has the appropriate mag and binning.
- Use low mag tracking when you are at 50K or higher in magnification.
- Image shift should be limited to 1-1.5 $\mu$m (for TF30) or 3 $\mu$m (for TF20).
- Set your target intensity to the optimal counts for your camera.
- Check "Keep intensity below current value (use if intensity already set up)". This was set up in step 4.
- Defocus target should be around –0.2 $\mu$m, beam tilt should be 1.6 milliradians, and autofocus offset should be 0.

- Autofocus every 4-6 degrees and focus every time above 50-55 degrees. The check autofocus option is used if it is an unfamiliar sample. This will tell you if you are having a difficult time getting accurate focus at high-tilt. Autofocus at high-tilt failures usually happen when something comes into the focus area that is too thick (i.e. plant cell wall) at high tilt. Otherwise, if the sample is familiar and there haven't been problems in the past, skip it since it does take time.
- No need to refine eucentricity since it was done in step 2.
- You can either choose to walk-up using the tilt-series controller or start at high-tilt. Either way, you have to decide to use a mid-tilt anchor or not. A mid-tilt anchor is used if the area of interest encompasses almost the entire image area.
- Use "Repeat record if percentage of field lost is more than X" when your area of interest fills the entire image area. A good place to start is 5%, but you may have to drop to 3%. If the number is very low, your tilt-series will lengthen in time significantly since it constantly has to redo records. If this happens to you, go to the camera pull-down menu and uncheck post-actions. This eliminates tilting until after a successful record has happened. The percentage you put here is magnification dependent—the higher the mag, the more often the program will have to back up to do a record. If it backs up a lot, you might want to increase the tilt delay to give the sample more time to settle. You may also consider going down in magnification or using montaging.
- Track before autofocus. Again, if your area of interest is large or the sample is very drifty, you might want to track before and after autofocus, but it's usually unnecessary.

Just to note, there are many other options in there, but these usually deal with difficult data—like cryo or unusual plastic embedded samples.

7. Go to the tilt series menu and check autosave log. The log file is very small—only a few KB—so it doesn't take up a lot of disk space and can only help you in the future. The only crucial thing to remember is to close the log file and open a new one between each tilt-series. After the first saved record of your tilt-series, the program will prompt you to name the log file and then will automatically save the file after each record.

8. It's really a good idea to stick around for the first 5 tilts or so. It's tempting to get up and leave right away, but many people have had to retake entire tilt-series because of a mistake in the first high tilt or two.

9. When your tilt-series is finished, terminate and say yes to the first question. Then you have the option of saving pre-rotated zero tilt images of the a-axis for reference in aligning the b-axis in buffers G and H. This is a good idea if you are going to rotate right away. However, if you are doing serial sections, it's generally easier to just do all of the a-axes and then rotate and do all of the b-axes.

10. After rotating the grid, find your area of interest again and repeat the steps 1-5 except for centering. Go to File-Read in SerialEM and load the middle section of your a-axis. This will load into a buffer (usually E). Then go to Process and rotate either left or right. This will put the rotated image in buffer A. Now you have to look at the "align to X" button in the alignment panel. Copy the image in buffer A to the "align to X" buffer (usually D), then click the "align to X" button and your image in A should now be aligned to the zero tilt of your a-axis. This should keep the a- and b-axes very close to the same area. NOTE: if you have the already rotated images in buffers G and H, you can just copy those directly into your align buffer without having to read in the a-axis zero tilt image.