Strategies for Cryo Tilt-Series Acquisition

The strategy for tilt-series acquisition for cryo-ET has several major differences compared to plastic section ET.

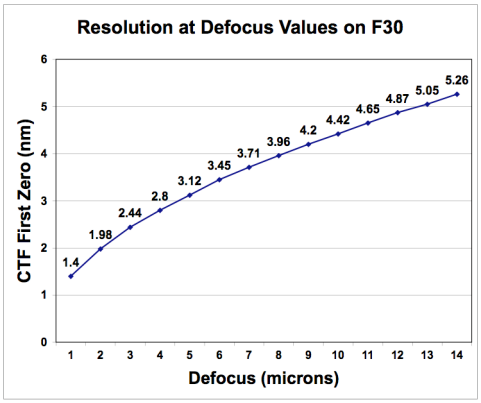
1) Plastic-embedded specimens can tolerate much higher dose without significant damage. A plastic tilt-series under "normal" conditions can get up to a total dose of 10,000 e^-/A^2; where in cryo we strive for a total dose of 100 e^-/A^2 or less (total dose means all ancillary imaging like eucentricity plus the tilt-series dose). In cryo we therefore use the "low-dose mode", where Focus and Trial/Tracking images are displaced along the tilt axis and don't overlap with the Record area.
2) There is essentially no dual-axis capability due to the low dose requirements. So, we are left with single-axis reconstructions. If the sample allows you to, taking advantage of sample orientation to the tilt-axis can become very important. The best resolution is parallel to the tilt-axis.
3) Cryo samples have no stain, so the contrast is very low. We use defocus to increase contrast, but at a price. The more defocus you have, the more resolution you lose (unless you can correct the data for phase inversions past the first zero of the CTF). It becomes a trade-off between how much you want to see vs. how much you want to resolve at the end. For example, if you knew your tilt-series was only for checking if you had full decoration of microtubules with motors, you might want to use a high defocus to visualize this. If however, the same sample was going to be averaged, you would want to use a low defocus since you will be trying for your best resolution in the 3D averaging (but you might hardly see the decoration in a close to focus image before averaging).

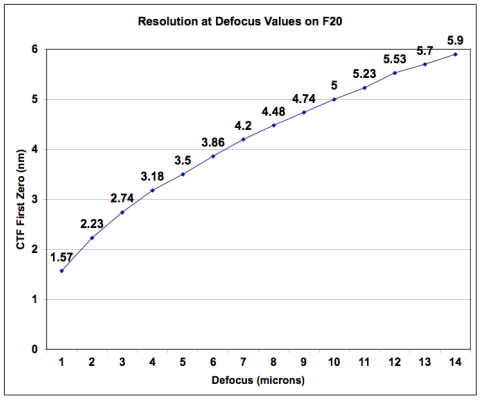
Other key points to consider in cryo-ET:

- Some samples bubble before others. It's a good idea to do a dose series (a whole bunch of 1 e^-/A^2 images until you see bubbling) and analyze the FFTs to see how your sample fairs in the beam.
- There is no guarantee that your sample is vitreous. Use diffraction to check if the ice is vitreous before wasting your time imaging. The ice is clearly not vitreous if you see it "flicker" during tilting. Flickering is caused when crystals, including ice, look lighter or darker depending on how the beam is oriented to the crystal lattice; so when the specimen is tilted, the crystalline area can appear light in one image and dark in the next i.e. the area "flickers".
- Cryo takes a long time (maps, searching for a good area, tilt series setup, 1-port read-out camera …), so plan accordingly.

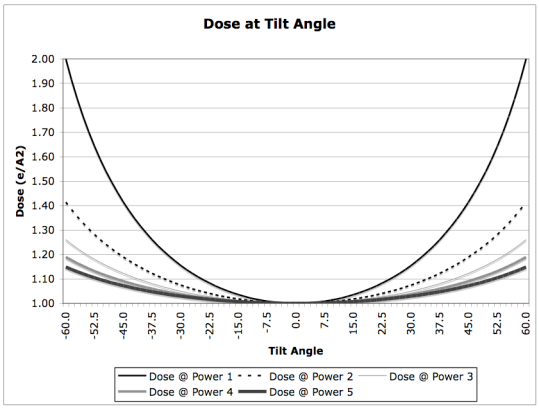
Setting up the tilt-series is a compromise between dose and resolution. The goal is to pick the tilt range, tilt increment, and defocus to optimize your final result. For instance, if you pick too many tilts (large tilt range and/or small tilt increment), you will have to choose a lower dose per image at each tilt to stay below your 100 e^-/A^2 limit, theoretically with better resolution. But, each individual image may be so noisy, that it becomes tricky and even impossible to align the tilt-series properly. In addition, picking the proper defocus is critical for the final resolution. If your final goal is averaging, it may be good to try a 2-6 micron defocus (on the F30), but if you are not going to average, you will never get the

theoretical resolution from that defocus, so use a larger defocus like 7-12 microns. The following graph shows the theoretical resolution (values on the line) for each defocus based on the first zero of the CTF if your tilt-series was taken on the F30 (left) or F20 (right).





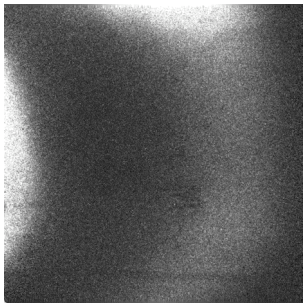
In addition, we use a different scheme to adjust the dose for the tilt-series acquisition. In plastic, you target the same number of counts per image, so the higher tilts get much more dose because a smaller fraction of the electrons hitting the specimen make it into the image. In cryo, we still give the higher tilts more dose and the tilts closer to zero less dose, but we need to make the variations more predictable in order to control the total dose. In the tilt-series setup window this is done with the option "Vary intensity as 1/cosine of tilt angle to the 1/X power" where X is a value from 1-5. This scheme allows you to have better pictures at the higher tilts where the sample is thicker due to the tilt, but it lets you avoid using up too much dose on the higher tilt pictures. You can see how the Total Dose changes when you adjust the power setting. You must know how much dose you have set up on the record area to know the total dose. Be careful here when starting your tilt-series. If you start at 0, the dose will be the product of the current Record dose and the number shown on the Total Dose line. However, if you walk-up to an extreme angle first, you MUST click the "use current intensity at zero tilt" option or it will only be a fraction of what you are targeting (the second value on the Total Dose line). As seen in the chart below, a 1/cosine scheme allows the dose to be higher at the high tilts and approach 1 e^-/A^2 at the lower tilts on a smooth curve. This tilt-series is set-up to have a tilt range of +/-60º with a 1.5º tilt-increment and a record dose at 0 at 1 e^-/A^2. With a power of 1, you get a nice, gentle curve with more of the zero tilts getting 1 e^-/A^2 per image. The total dose for that tilt-series would be very close to 100 e^-/A^2. However, if you selected power 2 without changing the tilt-increment or tilt range, you would get slightly less than 100 e^-/A^2 of total dose. The curves for higher powers with these tilting conditions are even less steep and the total dose for those tilt-series would be even less (84.5 e^-/A^2 with power=5). In those cases, you would have to change either your tilt range or tilt-increment to keep the dose at or below 100 e^-/A^2.



As a general rule, the thickness of your specimen will dictate what you can do.

	Thin Specimen	Thick Specimen
Tilt-Increment	High	Low
Defocus	Low	High
Total Dose	<100 e^-/A^2	~100 e^-/A^2
Resolution	Higher than Thick Specimen	Medium

If you are using the GIF, you need to make sure you "Refine ZLP" before you start your tilt-series and you may opt to have the program refine the ZLP during your tilt-series at an interval of your choosing. If you choose this option in the tilt-series setup controller, there is no need to refine the ZLP before you start, as the program will do that first thing when the tilt-series starts. When the slit comes in to the image it looks like this:



Tracking during a cryo-tilt-series can be very challenging. It is critical to choose your tracking area wisely. A good tracking area has enough features on it to cross-correlate. For instance, the edge of a quantifoil hole, gold on carbon, a piece of section, and MTs on carbon can make good tracking areas. Make sure a large crystal of ice, thick ice, or the grid bar does not get in the way at high tilt. Also, don't choose a tracking area over a potential future recording area. There are several options to deal with difficult tracking in the tilt-series setup tracking control parameters area.

- Stop when Autoalign gives a large shift. With this option selected, the program will examine all Autoalignments at the regular magnification and will stop the tilt-series if the alignment shift exceeds the specified percentage of the size of the

image being aligned. This may prevent the program from going astray on a wildly incorrect alignment—an easy thing to do in cryo. When the tilt series stops, you can revert to the previous specimen position by clearing the alignment using the button on the Image Alignment and Focus control panel. If the large image shift is correct, you can resume the tilt series instead.

- Repeat the record if a certain percentage of the field is lost. This option should have a high value (10-20). If the option is unchecked, the tilt-series will continue on even if you lose your area of interest entirely (or up to the limit set when "Stop when Autoalign gives a large shift" is set). A high value will allow some "slop" during image acquisition but still let you decide what to do when the program thinks you are losing a large portion of the area of interest. Note that in low-dose, the program will always ask you for confirmation before redoing the record image, so the tilt series will be paused, giving you an option to choose if you really want to re-take the; avoid taking a new record if possible as it adds more dose to your sample, especially at the higher tilts.
- Get tracking image when prediction error is high. This entry determines when the program will acquire a tracking image rather than relying on the prediction of the position on the next tilt. The criterion that is entered is expressed as a percentage of the size of the camera area that is being recorded. You may want to enter a larger value when working at high magnifications (or in cryo) where errors are inherently larger.
- Track before, after, or before and after autofocusing. The default is tracking before autofocusing, which will guarantee that focusing is done at the center of the field. However, when drift is bad, the Record image might then be displaced because of the time spent focusing. In this case, selecting tracking after autofocusing will keep the Record image from being off by much. When tracking occurs after autofocusing, SerialEM will adjust the focus as necessary to compensate for the lateral shift in position. If drift is really bad, one could select tracking both before and after focusing—an option often needed at high tilts in cryo.
- Align only with tracking images. This option is only available in low dose mode; SerialEM will ordinarily align with tracking images in the Trial area when necessary, and refine the alignment on every tilt with the Record image. In cryo the area you are interested in sometimes doesn't cross-correlate well (e.g. a thick bacterial cell), then the alignment with the Record images can throw everything off. This "align only with tracking option" allows you turn off the alignment on Record; it will cause a Trial image to be taken on every tilt and used for tracking.
- Get a new track reference if record alignment differs from track. The criterion entered in this text box comes into play in low-dose mode because it is possible for the alignment specified by the tracking images to diverge from the alignment of the Record images. When the program takes a tracking image and uses it for alignment, then finds that the position of the next Record image is in error by more than this criterion, it will take a tracking image after the Record alignment and use this image as a new tracking reference. This criterion should be set rather high, because minor misalignments are handled by the program shifting the existing reference.

- Align with preview before getting a new tracking reference. In low dose mode, it is sometimes necessary to take a new tracking reference after a Record image. If there is drift and time for acquiring the Record image is long, this reference will be in the wrong place. This option could help you when using the Ultracam because of the time it takes to average the dark reference on the first image. This will help you get started in the right place. Afterwards, you can turn the option off. With this option selected, the program will always take a Preview image and align it to the existing Record reference just before acquiring a new tracking reference. Since the Preview should take less time to acquire, the track reference location will be more accurate. This presumes that Preview is set up to take a fast, binned, low dose image that will not contribute much to the dose on the Record area.

The autofocus option called "autofocus offset" in the tilt-series setup is mainly used when you are recording your tilt series close to focus; in such a case the contrast might be so low that the auto-focusing fails. This option will "offset" the defocus by a certain value and the focus measurement is done at a higher defocus with better contrast; e.g. you want a defocus of –2μm, but the contrast is too low, use an offset of –5μm and the focus images will be taken around -7μm, after correlation the right focus is set—of course compensating for the additional 5μm. Don't choose the offset too high, as there is some inaccuracy in the focus adjustments over a long distance.

Even with choosing the proper parameters for your tilt-series from the beginning, problems still crop up. The most common is tracking errors. You can play around with the tracking settings to try and fix the problems. In addition, you can end/stop the tilt-series, reposition and resume. Be sure to check the "use image in buffer A for alignment" option if you stopped due to tracking issues. You can have either a Track or a Record area image in buffer A, and it will do the right thing. If you need to reset using the Record area, use a Preview, not a Record for your alignment.

At the end of your tilt-series, terminate and tilt to 0. Also, the record is now set to the dose of your last image in the tilt-series (which could be as high as 2-2.5 e^-/A^2) so change your record back to 1 e^-/A^2.