Cryo EM - Data Collection Protocol

Scope

This procedure is based on a Titan-Krios system that has a K3-BioQuantum energy filter attached. The automated data collection program is Latitude S provided by Gatan.

Procedures

A. Clipping Grids

1. Prior to pouring liquid nitrogen, set up all the tools and apparatuses for clipping. This includes:
   a. Assembling the clipping stage,
   b. Putting clip rings into the clipping tools (and flattening the clip ring against the rim of the clipping tool),
   c. Placing cartridge rings in the 4 slots on the clipping stage,
   d. Putting empty cartridge boxes in the designated slots,
   e. Turning on the warming stage,
   f. Assembling all tools required for clipping on the warming stage, and
   g. Making sure all tools are currently dry and free of any liquid, water, ice, dust, and detritus.

B. Preparation

1. Find and identify the tube with the grids.
2. Fill both small (4 L) dewars with liquid nitrogen.
3. Make sure to wear a mask when handling cryo samples as it will prevent condensation and frost from forming from your own breath.
4. Cool down the sample-holding cup and clipping-stage assembly with liquid nitrogen. Make sure the clipping stage is entirely immersed. Exposed cold metal increases frost precipitation in the basin.
5. Once the sample cup is cool (you will know it is cool when there is minimal bubbling), remove the sample from the storage dewar.
6. Once the clipping assembly is cold, cool your forceps and transfer the grid boxes to the clipping stage. Ensure this is done as quickly as possible to minimize the grid box's contact with warm air.
7. Cool the screwdriver (or hex wrench) and unscrew the grid box until the top retention plate can be rotated fully. Do not remove the screw from the grid box.

C. Clipping

1. Cool 4 clipping tools (with clip rings inserted) by placing them against the clipping basin.
2. Cool the cartridge-handling forceps and insert it into the clipping stage rotator notch.
3. Next, cool the tips of the insulated forceps.
4. Once everything is cool, rotate the clipping stage to the first position.
5. Remove the first grid (the first slot clockwise from the notch) by grasping the grid edge (or as close to the edge you can manage) and place it into the cartridge. Do this delicately, without bending or deforming the grid. If necessary, gently tap the grid so that it settles into the cartridge.
6. Rotate the clipping stage by 1 notch to the clipping position.
7. Insert the clipping tool down to the bottom.
8. Gently depress the clipping tool to insert the ring. You should feel a soft click as the clip ring goes into place.
9. Remove the clipping tool.
10. Make sure the cartridge is settled in the slot; adjust if necessary.
11. Move to the second slot and repeat with the rest.
12. Once 4 grids are clipped, use the cartridge-handling forceps to place the grids into the cartridge box. Make sure the C-clip side is toward the notch of the grid box. Remember to track the grid condition when clipping.
13. If doing more than 4 grids, with a fresh pair of forceps, quickly place 4 new cartridges into the 4 slots. You must be quick to prevent frost buildup.

14. Get another pair of insulated forceps and repeat the clipping again. Do not let the frosted metal directly touch the grids at any time.

D. Loading of Grids

1. Put the newly clipped cartridges into the transfer chamber.
2. Cool down 2 (or however many are necessary) labeled cartridge boxes into the transfer chamber.
3. Using the cooled nanocab, undock the cassette from the Krios.
4. After the nanocab is released, make sure that the autoloader valves to the turbopump have opened.
5. Attach the nanocab to the transfer chamber and remove the cassette with the cassette-gripping tool.
6. Using the cartridge forceps, place the grids to be stored in the marked cartridge boxes. Make sure to note the order and placement of the cartridges within those boxes for future reference and imaging sessions.
7. Place the newly clipped cartridges into the cassette (note the order). Make sure the C-clip side of the cartridges is toward the lower numbers on the cassette; this happens automatically if you place them toward the notch in the box. Make sure they are firmly within their slots. There should be no movement of the cartridge when gently prodded by forceps.
8. Place the cassette back into the nanocab. Check that the cassette is all the way in the nanocab (push on it with the blunt end of a tweezers); the pin will only come out if the cassette is all the way at the bottom and the microscope will refuse to load the cassette if the pin does not stick out. Prior to docking, check that a high vacuum has been achieved and that no parts of the autoloader are warm (> –165°C) and the autoloader vacuum is low (< 29).
9. Dock the nanocab.
10. Make sure the turbo valves reopen after the docking cycle is complete. Additionally, keep the Krios door open until the autoloader reaches a high-vacuum state (after the vault opens when the vacuum reaches < 29). You may hear a hiss if the autoloader spontaneously shuts down, which sometimes occurs. If this happens, restart the vacuum and re-initialize the autoloader. If you do this quickly enough, the warm-up should not be bad enough to affect the sample grids.
11. Screw caps onto the cartridge boxes and place them in a pre-cooled storage tube, label the tube with the project number, and place the tube in the storage dewar.
12. Once all autoloader parts have cooled below –170°C, initiate the inventory routine.

E. Screening Grids

1. Open a new Latitude S project based on settings from a previous one, ideally a routine single-particle project (e.g., no tilt acquisitions). You will use these atlas (135x), hole (8,700x), and data (generally 64,000–105,000x in nanoprobe-EFTEM, which corresponds to 1.42–0.873Å pixel size) magnifications for screening, regardless of what is actually needed for the project. Make sure to set up the data mode with a reasonable exposure of 40–60 e-/Å2 at a 4-second exposure for 40 frames on a K3 camera.
2. If the cross grating was left on the column during loading procedures, unload the cross grating. Do so separately from loading; unload any grids from the previous session before loading new ones.
3. Load the first screening grid.
4. Scan the grid at atlas magnification. Note the quality of freezing at this magnification, though there is no need to be precise (specifically look for thick vs. thin ice, empty vs. filled holes, and cracking or tearing of grid squares). Based on the grid-square size, determine if you have enough spots for a run.
5. Select 1 or 2 grid squares and take a 0.5-second image (4 frames) for reference. It may be a good idea to do a “good” grid square and an area that is representative of the grid as a whole. If the whole grid is “good,” then these can be one and the same.
6. Switch to hole magnification.
7. Initiate the “Optimum Focus” procedure to get the eucentric height. If the height goes extremely in the negative, repeat the process and start at a higher height. If necessary, perform a rough manual eucentric-height adjustment with the stage wobbler and repeat the “Optimum Focus” procedure from there.

8. Switch back to hole magnification again, as defocus should have gone to 0. Switching will put it back at –40 µm.
9. Take a 1-second exposure (4 frames). Observe where the protein is distributed within the hole. On grid squares with freezing gradients, test a couple of representative holes.
10. Target an area of protein or likely imaging area. Ideally, this should be toward the center; however, in many projects, protein appears to gravitate toward the hole edge. When shifting to a spot, do a couple of small backlash movements to minimize stage drift.
11. Switch to data magnification. Allow the stage to settle for several seconds, adjust the defocus to about –4 µm, and take a 4-second exposure. If possible, take a data image on an empty area prior to this and note the percentage of electron loss with the GIF in. This is a good indication of ice thickness. <20% reduction in electrons by the GIF is considered "thin" ice, capable of information limits in the 3–4 Å range. 20% reduction corresponds to 55 nm ice thickness. The equation is: ice thickness = 250 nm * ln (dose of empty image/dose on image).
12. Often, images will be drifty if working too quickly. If that happens, go back to hole magnification and select another area. Extend the settling time. Make sure the stage drift is nonexistent in hole magnification mode. Sometimes, you can place the target area so the beam overlaps with the hole. All these things can reduce drift.
13. Once a grid is screened, close the column valves and then load another grid. While the grid is loading, name your screening files.
14. Write down your thoughts about the grid. You may want to describe the best areas but be honest about the general condition of the grid. If you do not think a 2-day or even a single-day session can be extracted, state that the grid is not recommended or unimageable. As you go, also rank your grids in order of imageability/quality.
15. Grid screening should only take 15–20 minutes per grid.
16. Once screening is finished, insert the cross grating.

F. Daily Alignments

These alignment instructions are based on a Krios microscope using a K3-BioQuantum camera/energy filter combination.

1. Proceed with daily alignments if everything looks fine. If you have a well-aligned microscope, you should be only doing minor touch-ups.
2. Over an empty area, at 81,000x magnification and 100 µm C2 aperture (corresponding to ~1 Å pixel size), on the flu screen, put the beam at crossover and eucentric focus.
3. From the "Daily Alignments" tab in the user interface, select the X pivot point and use the multifunction knobs to align the spots. (If the spots are different sizes, the condenser and other alignments may be off). Repeat with the Y pivot point.
4. Click "Beam Shift" and "Center Beam" (usually found over the GIF aperture, or depending on the camera you are using.)
5. Expand the beam to a reasonable size, move the stage to the cross grating, and bring the grating to eucentric height using the wobbler to go through +/– 15°. Adjust the z-height until there is minimal movement.
6. Select the rotation center and use the multifunction knobs to minimize the circle's movement. (It will never be completely still, but try to get it to a slight circular motion). You can use the focus-step knob to adjust the magnitude of the shifts. Adjust this alignment only if it is clearly off. The rotation center will be fine-tuned anyway during coma-free alignment.
7. Switching to live view on the K3 camera, select "Live Fast Fourier Transform" from the "Process" menu. Adjust the objective stigmation if the thon rings are very oblong. Otherwise, do a coma-free axis alignment.
8. Go to a small defocus, about –0.5 to –1.0 µm. Select "Coma-Free X Alignment." The beam should switch between 2 deflection states. The thon rings will be oblong, so hit the "–" button once to adjust the Y multifunction sensitivity and adjust the knobs until the rings are the same size and shape. Initially, the rings might rotate while being the same size and shape. If this occurs, switch to "Coma-Free Y Alignment" and adjust the Y multifunction sensitivity and repeat. Switch between these 2

states until the rings are the same size, shape, and direction while the microscope is deflecting between 2 states.

9. After the coma-free alignment, hit “Done” on the “Daily Alignment” page.
10. Switch to objective stigmation and go as close to focus as possible while visualizing the thon rings. Adjust the multifunction knobs as necessary to get a perfect circle. You can even use drawing tools in the Digital Micrograph (DM) program to follow along if you get confused or lost. You should be able to perform this function below –0.5 µm defocus (typically between –0.1 and –0.05 µm defocus).

G. Gain Reference

1. Once finished with objective stigmation, hit the “Done” button on the “Stigmation” page, go to “Focus,” go to the empty grid square, lower the flu screen, enter “100 µm C2 aperture,” go to spot size 3, and center the beam.
2. Raise the flu screen and, from the “Camera” menu, select “Collect Gain Reference.”
3. Follow the directions on the screen, adjust the intensity to 1280 counts for linear gain reference, and click “Ok.”
4. After adjusting the linear gain reference, collect the counting gain reference. Always follow this order because interrupting this process has negative consequences. When prompted, collect the hardware dark reference.
5. After the hardware dark reference is collected, lower the flu screen, raise the spot size to 7, and center the beam.
6. Click through the prompts and adjust the counts to 15. Once that is completed, acquire the counting reference. This should take ~15 minutes.
7. Once the gain reference is completed, adjust the beam settings for a 4-second exposure with a 40–50 e/Å2 dose and take an image. Select “Autocorrelation” from the “Process” menu. There should be a single black dot in a sea of even grey. If there are very white bands or significant lines, you will need to repeat the gain reference procedure.

H. Grid Setup for Latitude S Run

1. Be sure to obtain the following information on the run settings:
   - Magnification
   - Pixel size (super vs. counting)
   - Dose
   - Defocus range
   - Exposure settings (time, frames)
   - Template (if multi-, stage, or image shift)
   - GIF settings
2. With the targeted magnification, check the objective stigmation.
3. Set the dose over the empty hole. Adjust the intensity, exposure time, and C2 aperture as needed. As a last resort to setting the wanted dose, change the spot size. Additionally, do not make the beam too narrow; Fresnel fringes will appear at the edge of the image if the beam is too narrow.
4. Make sure that the focus and hole state are the same spot size. This will minimize beam settling times to speed up the run.
5. After setting the dose in exposure mode, change the settings to –2 µm defocus and then hit “Scope” on the data and focus states. This will save the beam settings for those states, which should be identical. Make sure to do this prior to any atlas being taken. If you are using a new magnification that has previously not been calibrated, upon hitting “Scope,” you will receive an error stating that “only a partial state has been read” and prompting you to do calibrations. Go to the “Calibrate” button on the state tab, which will enter you into the calibration wizard. Perform the requested calibrations. You will need an area with some features and an empty area on the grid in order to accomplish this. The calibration wizard will guide you with step-by-step instructions to perform the requested calibrations.
6. After determining the microscope state, determine the image state. Set the exposure time, binning, and number of frames and check “Save Stacks” from the DM settings. Using the gear icon in the DM menu, bring up advanced settings and select “Uncorrected” instead of “Gain Normalized” from the drop-down menu. Additionally, make sure that “Defect Corrected” remains checked.

7. Take an image with these settings, click on the image, and then click the "Image" button from the data state menu. This will save the image settings to the data state. After this is done, reset the imaging settings to the previous screening state (use "Gain Normalized," uncheck "Save Stacks," and set 1x binning).
8. Insert the imaging grid. Adjust the grid state to a magnification that properly encompasses the grid square. (If you select something too small, then you are not maximizing the grid square area. If it is too large, you will waste time deleting points with the autopicker. Additionally, the higher magnification you use here, the more easily you can spot and avoid defective areas). Hit "Scope" in the grid state when you have found the magnification you desire (somewhere between 360x and 1200x is recommended).
9. Over a grid square with some contamination, perform the course alignment. Make sure "On State Change Update from Microscope" is checked so that all adjustments are saved for each respective state automatically. When adjusting focus/data state, use the Beam Shift from the direct alignments.
10. Find a large piece of ice or other recognizable feature on a grid. Go to "Optimum Focus" in hole state. Center the feature in hole state, then data state. Go to the "Fine Alignment" tab and capture the fine alignment. Without adjusting focus and data positions (they should be identical; if not, then data state is not equal to focus state and you should correct that). Adjust the cursors to be on the feature you have selected and calculate the fine alignment.
11. In the "System Stability" tab, set hour time-points for certain automated functions, such as how often focus is performed (e.g., select every 12 µm of stage movement), how often a dark reference is collected (4 hours), how often eucentric height is assessed (80–100 µm, depending on grid square size and spacing), and how often the zero-loss peak of the energy slit is centered (generally every 4 hours). If the grid contains very thick ice, an opaquer support (such as gold foil grids), or any situation where it is reasonable that the dose on the camera will fall below 9–10 e-/pix/s, the zero-loss-peak (ZLP) centering will not perform correctly, and automated ZLP centering should be turned off. On a Titan Krios instrument, the ZLP remains centered on the slit for 16–24 hours, but it will have to be checked often and manually centered on an empty or thin area periodically (we recommend every 8 hours).
12. Go to the "Focus Alignment" tab and set the defocus range and the step size (typically –2.5 to –1.0 µm with a step size of 0.25 µm) for the defocus to cycle through. Larger particles and/or thinner ice conditions may warrant going to a defocus range closer to 0 µm.
13. On the transmission electron microscopy computer, move the stage to the center of the grid. Hit "Capture" from the "Latitude Experiment" tab and collect a 5 x 7 atlas (assuming an atlas magnification of 135x).
14. Select the grid squares and press "Schedule".
15. Add the hole position to 1 of the grid squares and hit "Acquire".
16. If the center of the hole is off, collect a centered hole image using the "Capture" button on the "Hole State" menu. After it is acquired, hit "Update" in the "Hole State" menu to save it as a reference. It is better to save a thinner hole than a thicker hole. Saving a thicker hole as a reference and then selecting thinner holes negatively affects hole targeting. Make sure the "Apply" box is checked (skip this for lacey carbon), which will force Latitude S to use this reference when targeting holes.
17. Select another position with the hole reference saved and hit "Acquire". Make sure that this hole is properly centered and then use that image to construct a template. Add acquisition positions and focus positions as necessary. Remember to arrange the focus position so that it will not be near any holes that are to be imaged in the future, only near past holes. That way, if the beam drifts, it will not negatively affect imaging. (Determining past vs. future holes is done in the "Auto Find" menu). The program raster goes in from "Center" to "A" and then up toward "B." Save your setup as a template.
18. Use "Auto Find" to set up hole grids. Be careful to center the markers and remember the direction of the raster. Take care to avoid the edges of the grid squares as these often contain hexagonal ice. Additionally, avoid areas of heavy ice contamination as it can affect hole targeting and lower image quality. A good

rule of thumb is to select ~150–180 images an hour for a standard image-shift multi-template run based on a 4-position image shift (more if using more targets during image shift or other means to boost output). However, Latitude S does not have a method for compensating beam-tilt aberration, so shifts should be kept to a minimum. ~6,000 spots need to be set up for a 2-day (40-hour) run, and 10,000 spots for a 3-day run. There should be a minimum of 3,000 spots to last overnight (~16 hours).

19. Prior to starting the run, go to the data state, collect the dark reference, and center the zero-loss peak (if using GIF). Additionally, in the "Latitude Tasks" tab, click the gear icon to bring up a selection menu. For sessions using image shift, make sure the "Image Shift/Beam Shift" box is checked under the "Center Data Position With" selection. You can also select "Reschedule Failed Tasks" and "Close Column Valves When Idle" here if you wish, although it may be beneficial to manually reschedule failed tasks depending on the nature of the failure.

20. To schedule all positions, select the "Schedule" button from the "Latitude Tasks" tab. All tasks you have selected will be acquired.

I. Modifications for Acquiring Data at A Fixed-Tilt Angle

Due to the preferred orientation of the sample, occasionally it is necessary to acquire images at a fixed-tilt angle. Latitude S does not offer this as an integrated feature; however, there is a work-around that functions well. There are a few modifications to the above procedure that will be outlined here.

1. Screen grids as normal. Perform all beam alignments and gain references. Speak with the user about imaging conditions and which grids to image and set those up. When you have selected a grid of interest, acquire an atlas. Find the areas of interest and perform the eucentric height and note the height of various areas of interest.

2. Make sure DM/Latitude S is closed before starting.

3. If the desired angle is not already calibrated, you will have to calibrate a new one. With the cross grating in and at grid-square magnification (generally the lowest grid square magnification, 360x) in the DM top bar, go to "Calibrations" and then "Edit Calibrations." From the calibration selection, select "Stage Orientation" and delete this calibration as you will be generating a new one at stage tilt.

4. In the microscope computer, go to your desired tilt angle using the stage control.

5. Reload the grid square magnification state. It should prompt you to complete calibrations. Perform the stage orientation calibration using the instructions from the calibration wizard prompts.

6. Select "File," then "Global Info." From the pop-up menu, select "Global Tags," then "Latitude," then "Latitude S," and then "Control." Right-click on "Control" and select "Add Child." Copy the following statement (without punctuation) taking care that this is case sensitive: "Simulate eucentric height tuning." This will be added to the control menu. Double-click on this value and enter the value "True." This will turn off automated eucentric-height tasks. You must take care to be at the eucentric height of your areas of interest as determined from the 0° atlas.

7. Go to "File," then "Save Preferences." Additionally, make a copy of the "Prefs" folder with the date and tilt angle.

8. Load your grid of interest, go to an area of interest, and set the eucentric height. Go to the desired tilt. Perform an autofocus and note the change in defocus. Adjust the height to compensate. Go to the center of the grid and acquire the atlas. The atlas will be the same in the x-direction as that is parallel to the stage axis (on various Krios machines, although this may be different for certain machines) but should be shrunk in the y-direction. Generally, a 5 x 6 or 5 x 5 (extreme tilt) is sufficient. Even with the stage-orientation calibration complete, grid squares will often appear more misaligned than 0°-tilt atlases. This is fine, provided you don't see the entire duplicated squares at the tile borders. Another sign of success is if the grid square selections are reasonably centered in the image during acquisition.

9. When setting up templates, make sure the focus position is on the tilt axis. This is not as much of a problem for 4 hole-four image templates as the focus will be centered.

10. Make sure this is a stage-shift session. From the "Latitude Tasks" tab, select the gear icon, and make sure the "Stage Shift" box is

checked. Multi-image templates will speed up the run; however, the data image will be off target by as much as 25% of the image frame because the template is not realigned after every individual data image, which is usually an acceptable compromise. If it is unacceptable, then create a single-hole image template.

11. If there are areas of interest that vary dramatically in their eucentric height (> 10 µm difference), schedule them in batches and adjust the eucentric height as necessary for each batch.

12. Tilted collections, while slower, can still achieve data rates of 100 images/hour.