Lesson 4:
Finding Defocus with Ctfplotter and Gold Erasing

ESTIMATING CTF ON A K2 CAMERA

This tilt series is of a flagellum of a Giardia cell, taken with a K2 camera on a Krios microscope at Janelia Farm. The total dose was 26 electrons/square Angstrom. Images were taken in superresolution mode with an exposure time of 0.5 sec to avoid having to save and align subframes, and reduced by a factor of 4 with antialiasing. With this protocol, they may have somewhat better high-frequency information than a typical tilt series taken in counting mode without binning, so the power spectra may be particularly good here.

1) `cd $WORKSHOP_HOME/IMOD_Labs/K2-ctf`

2) `etomo *.edf`

3) Go to the **Final Aligned Stack** page and press **Create Full Aligned Stack**. When done, switch to the **Correct CTF** tab. Notice Spherical Aberration can be 0; this is appropriate here because the data were collected on an aberration-corrected micrscope. The Expected defocus (microns) has already been set to 6.0 in this data set. The Config file has already been selected to access a configuration file listing noise files in the *Janelia* subdirectory of the data set directory (*K2-ctf*).

4) Press **Run Ctf Plotter**. The magenta curve shows the rotationally averaged power spectrum plotted versus spatial frequency. The green curve is a fitted curve, which in general may not fit well until

fitting parameters have been adjusted. The units of spatial frequency along the X axis are reciprocal pixels and range from 0 to 0.5/pixel.

5) Power is always very high at low frequencies, so the first operation is to zoom up the part of the curve that shows the CTF effect. Click the mouse just under the hump in the curve after 0.1/pixel and drag the zoom area out to the right edge, just under the curve, so that the Y axis range is about -0.1 to 0.5.

6) Since the fitting looks good already, on the **Angle Range & Tile Selection** window, switch to use the **Current defocus estimate**. In the **Initial tiles to include** section, select **All tiles** in and Press **Apply**.

7) In the upper left corner of the **ctfplotter** window, press **Fitting** to open the dialog for setting fitting parameters. The frequency of the first zero determined from the fit is shown at the top of the plotter window after Z:, and the corresponding defocus is shown. It is ~4.6 microns instead of the nominal 6 microns. The fitting range starts at a low frequency appropriate for the higher nominal defocus but too early for this lower actual defocus. It is best if the fitting is done to a linearly falling part of the curve and excludes the portion before that curving away from a line. Change **X1 Starts** to **0.16.** The fitting can go to the third zero or even the fourth, so change **X2 Ends** to **0.38** or **0.43**. Press the **Enter** key or **Apply** to fit with a changed value. Turn on **Vary exponent of CTF function**; the fit looks better, so that is an appropriate parameter to include when fitting these data.

8) To assess whether fitting to a single image is possible, go back to the **Angle Range and Tile Selection** window, change the **Starting tilt angle** to **0** and the **Ending tilt angle** to **1.8**, and press **Apply**. The curve looks good. To see whether this is still the case at high

tilt, change the **Starting tilt angle** to **60** and the **Ending tilt angle** to **62** and press **Apply**. The curve still looks good. Turn on **Fit each view separately** and press **Autofit All Single Views**. Resize the **Angle Range & Tile Selection** window so that you can see more of the table and scroll through the values to see how much defocus varies. Note the changes of over a micron up and down above 40 degrees. Double-click a series of lines in the table to check the curve-fitting in this region. It is clearly correct; the defocus changes from image to image have been measured accurately. Press **Save to File** and exit ctfplotter.

9) To apply the CTF correction, in Etomo, press **Correct CTF.** When done, **right-click** on the **View CTF Correction** button and select **Open with startup window**. **In Image file(s)**, press **Select** and choose both *WTI042413_1series4_ctfcorr.ali* and *WTI042413_1series4.ali* and press **Open**, then press **OK**. Compare the 2 files in the ZaP window by toggling the **4th D**. They look very similar, although there are some subtle changes. Close 3dmod and press **Use CTF Correction** in Etomo. Close Etomo.

10) `header WTI042413_1series4.ali`
At the bottom of the printout, you will see that ctfPhaseFlip has been used on these data.

GOLD ERASING USING FINDBEADS3D

11) Go to the **Erase Gold** tab. Under **Model Creation Method**, make sure **Use findbeads3d** is selected. Change **Aligned image stack binning** to **3** and press **Align and Build Tomogram**. When done, press **View Tomogram in 3dmod**. Verify that it appears that both surfaces containing gold particles are completely within the tomogram. In this case, a thickness of **350** worked, but in other cases, you may have to increase the Thickness value and try again.

12) Press **Run Findbeads3d**. When done, press **View 3D Model on Tomogram**. Bead Fixer will automatically pop up. Move through the tomogram and look for gold beads that do not have model points and model points that are not actually on a gold bead. If the model looks reasonable, press **Delete Below** on the **Bead Fixer** window and then **Yes**. This action deletes points whose correlation score was below the value set with the **Threshold** slider and were likely to be incorrect. Be sure to save your model (**s**).

13) Press **Reproject Model**. When done, press **View 2D Model on Aligned Stack**. Movie through the aligned stack and watch for any gold beads you may have missed that do not have model points. Also, there may be contours that do not follow gold beads. In this 2D model, you can easily delete contours that are not on gold beads and save the model. However, if there are any gold beads that are missing contours, you need to add them to the 3D model on the tomogram, save the model, and reproject that model onto the aligned stack. This can become an iterative process. It is often difficult to tell if gold beads are modeled properly when there are clumps of gold as in these data.

14) In the **Erase Beads** section of Etomo, **change Iterations to grow circular areas** to **3**. Press **Erase Beads**. When done, press **View Erased Stack**. Movie through the stack and you will probably notice some gold beads within the clumps that did not get erased. At this point, you would need to return to View 3D Model on Tomogram and add model points to those gold beads (centered in X, Y, and Z) and continue through until when you view the erased stack, you no longer see any gold beads. You may also determine that the gold beads are not over an area of interest and therefore it is OK if they remain. When you are satisfied, press **Use Erased Stack**. We are finished with this exercise, so close Etomo.

ESTIMATING CTF ON A DE-12 CAMERA

This tilt series is of a preparation of microtubules decorated with the motor protein Eg5, taken with a DE-12 camera during a demo on the F20 microscope in Boulder. The tilt series had a 2 degree increment and the total dose was 79 electrons/square Angstrom. Parts of the series have good signal for determining CTF, but not all of it.

15) `cd $WORKSHOP_HOME/IMOD_Labs/DE-ctf/`

16) `etomo MTEg5series6D.edf`

17) Open the **Final Aligned Stack** page press **Create Full Aligned Stack**. When done, switch to the **Correct CTF** tab. The Expected defocus has already been set to 6.0 in this data set. The Config file has already been selected to access a configuration file listing noise files in the *DE12-Div2* subdirectory of the data set directory (*De-ctf*).

18) Press **Run Ctf Plotter**.

Zoom the power spectrum by clicking on the magenta curve to the left of 0.1/pixel and dragging the selection region to a point before a frequency of 0.4/pixel and just below the baseline. The power spectrum shows a clear signal out to the third zero and the green fitted curve matches the location of the zeros fairly well, so the defocus estimate is good. On the **Angle Range & Tile Selection** window, select both **All Tiles** and **Current defocus estimate** and press **Apply**.

19) Press **Fitting** to open the Fitting Range dialog. Set **X1 Starts** to **0.1**, since the fitted curve deviates before this point. Set **X2 Ends** to **0.25** to fit out to the third zero, and press the Enter key or **Apply**. Turn on **Vary exponent of CTF function**; the fit does not look any better so there is no reason to leave this option on. Turn it off for now; below you will see how it is inappropriate in some cases.

20) To see if single images can be fit, set the **Ending tilt angle** to **-18** and press Enter or **Apply**. This curve is noisy but the fitting still seems reasonable. To see if fitting is still good at high tilt, change the **Starting tilt angle** to **-60** and the **Ending tilt angle** to **-58**. The curve is even noisier but fitting is still plausible. Turn on **Fit each view separately** and press **Autofit All Single Views**.

21) Resize the **Angle Range** dialog so that you can see more of the table and scroll through the values. Notice that there are some big jumps at positive tilt angles, particularly from **25** to **27** and from **41** to **43**. Double-click these lines in the table to see these fits. The power spectra at **27** and **43** are particularly low in signal, so fitting to the noisy data from single views is just not reliable. To get a better sense for how often the fitting looks good, double-click other lines through the series. At most (but not all) negative tilt angles, the fit looks fairly reliable; but at positive tilt angles it often is not. Since inaccurate defocus values can do more harm than good, we need to fit to multiple views instead, with the reduced goal of estimating the trend in defocus through the series.

22) Now switch to fitting sets of 4 views (8 degree ranges) by entering a **Starting tilt angle** of **0**, an **Ending tilt angle** of **8**, and **4** for **Step angle range by**. Turn off **Fit each view separately** and press **Autofit All Steps**. The program will ask you to confirm that you want to replace all of the existing values in the table; press **Yes**.

23) Double-click the lines at positive tilt angles to see how reliable these fits look. The signal gets rather low at the highest tilts, but the noise is low enough to allow you to see that the defocus is being found adequately. To see the potential problems with adding the exponent of the CTF function as a variable in the fit, double-click the line for **21** to **29** degrees. Press **Apply** 5-10 times and watch the defocus value (**D:**) in the plotter window. It varies from 5.26 to 5.29, which means that fitting is self-consistent and not too sensitive to the current assumed defocus value. On the **Fitting Range and**

Method window, turn on **Vary exponent of CTF function** and press **Apply** many times in the **Angle Range** dialog. The estimated defocus jumps around between 5.15 and 5.3, a 5-fold bigger range. When the signal is too weak, adding this fifth parameter to the fit can significantly reduce the reliability and stability of the fits. Turn off **Vary exponent of CTF function** one more time to go back to the stable fit. Press **Save to File** and close ctfplotter.

24) Press **Correct CTF**. When done, press **Use CTF Correction**.

GOLD ERASING USING EXISTING FIDUCIAL MODEL

25) Go to the **Erase Gold** tab. Under **Model Creation Method**, make sure **Use the existing fiducial model** is selected, and press **Transform Fiducial Model.** When done, press **View Transformed Model**. Notice the fiducial model used the 2-3 gold beads found very near the microtubules among others within the tilt-series.

26) In the **Erase Beads** section of Etomo, **change Iterations to grow circular areas** to **3**. Press **Erase Beads**. When done, press **View Erased Stack**. Press **Use Erased Stack** and then **Done**. We are finished with this exercise, close Etomo.