Lesson 14:
Fourier Shell Correlation

In this exercise, we'll explore the various options in PEET for creating and examining FSC curves.

CALCFSC / PLOTFSC

1) `cd $WORKSHOP_HOME/PEET_Labs/MT/PEET/firstSearch`

2) `calcFSC *.prm`
The program should complete within a minute or 2. Wait for it to finish before proceeding. calcFSC generates an "odd / even" FSC from a previously completed PEET alignment. Outputs from calcFSC (and the other FSC programs to be discussed below) will be written to a number of **.txt* files. These can be useful if you wish to use a different plotting program or to analyze the data further.

3) plotFSC is one of the rare PEET commands that does not require any mandatory arguments. (PEETHelp is another). As a result, if you want to see what optional arguments are available, you'll need to refer to the man page. Do this now with `man plotFSC`

4) Run `plotFSC`. By default, both a linear Spatial Frequency axis at the bottom and a non-linear Resolution (1 / Spatial Frequency) axis at the top will be shown. Both will be adjusted automatically as you pan, zoom, resize, or maximize the plot. Try using the **Magnifying Glass** and **Hand** icons from the toolbar. Since the default graph is linear in spatial frequency, interpolation to find frequency / resolution is best done using the frequency axis in this mode.

Practice this by finding the frequency at which the FSC is 0.5. Zoom and pan until the points on either side of **0.5** in the green curve are clearly visible. Select the **data cursor** (the 3d icon from the right in the toolbar, which looks like a curve with a visible "+" and a note above it). Click on the points on either side of 0.5 with this tool, and you'll see that the corresponding FSC values are 0.5204 at a frequency of 0.2441 lp/nm and 0.3437 at 0.2653 lp/nm. Interpolating linearly, we see that an FSC of 0.5 would occur at 0.2465 lp/nm, corresponding to a resolution of 4.1 nm. Close the plot window when finished.

5) `calcFSC *.prm 3`

The optional "3" means that the calculation will be repeated 3 times with different pseudo-random assignment of particles to the 2 subsets at each repetition. This gives a measure of repeatability and will allow plotting error bars on the FSC curves. Naturally, the calculation will take 3 times as long. Note that 3 is the minimum number of repeats which permits computation of error bars. Typically, you should use 5 or more repeats for reliable estimation. Wait for the computation to complete.

6) `plotFSC`

Zoom in and you should see the error bars corresponding to 95% confidence intervals for the various points. Keep in mind that these error bars indicate only the variability due to sampling errors nad not absolute accuracy. Close the plot window when finished.

7) `plotFSC 1 0`

The first argument says to label the plot as Figure 1 (must be an integer), and the 2nd argument says to plot versus resolution rather than frequency. Error bars are suppressed by default when plotting versus resolution, since they tend to be scaled inappropriately. There is an optional argument, which will allow you to manually turn them on if you insist, but you probably won't like the results! Notice that you can read off resolution at a given FSC value directly

in this mode; don't expect the values to exactly match those found with a linear frequency axis. Close the plot window. For information on additional options, consult the plotFSC man page. calcSSNR is somewhat similar to calcFSC but computes Spectral signal-to-noise ratio rather than Fourier Shell Correlation.

CALCUNBIASEDFSC

Next, we'll explore how to compute a "gold standard" FSC, which eliminates one common source of bias, namely that all the data used by calcFSC had previously been aligned jointly against a common reference.

8) `cd ../../../BPV_-3/PEET/odd`

To compute a gold standard FSC, you first split your particles into an even number (typically 2) of equal-sized subsets, which must be aligned separately using independent references. Normally, you would split the initial model into 2 (or more) new models to do this. In this example, I've taken a shortcut. We've already aligned the complete data set using random rotations for the initial motive list. I've taken the initial motive list (containing just the random initial rotations) from that run (*../firstSearch/bpv_MOTL_Tom1_Iter1.csv*), copied it to this directory as *initMOTL.csv*, and modified it by inserting alternating class numbers 1 and 2 in column 20. Examine *initMOTL.csv* (using cat, gedit, head, or less) to see these class assignments.

9) `etomo *.epe`

Examine the settings for this project, and you'll see they're identical to those we previously used for the initial BPV firstSearch run except that under Advanced / Optional Features I've set Average only members of class to 1, and I've approximately halved the number of particles to be used for references and the final average. Recall that class 1 contains the odd numbered particles; since these are the only particles allowed to enter averages or new references,

we'll effectively be using only the odd-numbered half of the data. All of the particles will still be aligned, and represented in motive lists, so this isn't the most efficient way to do things; this data set is small and runs quickly, however, so this isn't an issue. More typically, you would use createAlignedModel twice with Average only members of class set to split the data set into halves. I've specified an initial reference from the odd half of the data. The run has already been done and intermediate files deleted. Press **Open averages in 3dmod** to see what the final average from this half set looks like.

10) `cd ../even`, run `etomo *.epe`, and again press **Open averages in 3dmod** to view the corresponding settings and average from the even half set. Notice that the 2 averages are in different orientations since they were aligned to different starting references. We'll need to take care of that next. Close all the 3dmod and Etomo windows.

11) `cd ../alignOddAndEven`

12) `etomo *.epe`

13) Press **Run**

The run will only take a few minutes to complete. While it's running, examine the various settings. We're going to align the average from the even half-set against that from the odd half-set. I've specified no reference refinement, so only tomogram 2 (the even average) should show non-zero translations and rotations in the output motive list. After the alignment completes, check that this is true by running `cat bpv_MOTL_Tom*Iter4.csv`.

For this alignment, we need a model with a single point in the center of the volume to use with each of the 2 averages. Since the averages are 52^3, I created a file, center.txt, containing "26 26 25.5" and then ran `point2model center.txt center.mod` to generate such a model. In the Etomo parameter settings, notice that I specified the number of tilt axes as "2 or more" so I could supply

the binary wedge masks automatically generated by PEET during the odd and even alignment runs. In this case, there is little remaining missing data in Fourier space, so it would also have been reasonable to simply disable missing wedge compensation. I selected Save individual aligned particles in the Etomo settings, so we will generate mrc volumes corresponding to the odd and even aligned half-set averages for verification.

You may wonder why we're using the odd average as the reference / template rather than the larger reference generated at the end of the original odd alignment. That indeed would seem more appropriate; in practice, however, doing so typically results in small rotations translations to volume 1 in addition to the larger ones for volume 2. When the run completes, exit Etomo.

14) `3dmod aligned*.mrc`

Verify that the alignment appears to have worked correctly, bringing the two averages to a common orientation. Exit 3dmod when finished.

15) `cat bpv_MOTL_Tom2_Iter4.csv`

Make note of the corresponding Euler angles in columns **17**, **19**, and **18**, and translations in columns **11-13**. You'll see that the Euler angles are 113.413°, 34.918°, and -106.582°, and the translations are -1.35, 1.8, and 0.75. Next, we'll incorporate these rotations into the output motive list for the odd half-set, after backing up the original motive list first.

16) `cd ../even`

17) `cp bpv_MOTL_Tom1_Iter5.csv bpv_MOTL_Tom1_Iter5.csv.bkp`

As usual, we backup the original motive list before modifying it.

18)
```
modifyMotiveList bpv_MOTL_Tom1_Iter5.csv \
bpv_MOTL_Tom1_Iter5.csv.modified \
"113.413,34.918,-106.582" "-1.35,1.8,0.75" 0 1
```

Here, and subsequently, the "\" escape characters should be followed immediately by **Enter.** The optional "0 1" arguments say that we're specifying the transform we wish to apply (rather than its inverse), and that we're specifying Euler rather than Slicer angles. Note that in this case, we're directly applying the angles found for volume 2 (the even average) to volume 1. Alternatively, we could equally well have applied the inverse of this transform to volume 2. It's easy to get confused about whether an inverse transform is needed or not. When in doubt, do a no-search alignment with Save individual aligned particles to verify that things have gone as expected.

19) `cp bpv_MOTL_Tom1_Iter5.csv.modified \`
`bpv_MOTL_Tom1_Iter5.csv`
(Once again, "\" should be followed immediately by **Enter**).

20) `cd ..`

21) Run `calcUnbiasedFSC odd/*.prm even/*.prm`, wait for the calculation to complete, and then run `plotFSC` to display the gold standard FSC curves. Leave this figure window open for now.

SIMPLEFSC

PEET also has a simpleFSC program, which will compute and plot an FSC curve from two corresponding volumes. For example, we could generate the gold standard FSC corresponding to using all available particles in step 20 using this program.

22) `cd alignOddAndEven`

23) `simpleFSC aligned_tom1_P0001.mrc aligned_tom2_P0001.mrc`
Notice that the results are identical to the green curve generated in step 20. simpleFSC is applicable whenever you wish to compare 2 volumes with common position and alignment, and is not

restricted to computation of gold standard FSCs. For example, it can be used to compute the FSC between a subvolume average and a PDB model. Unlike calcFSC and calcUnbiasedFSC, it requires only the 2 volumes and no other information from a PEET alignment. Close both plot windows.