Lesson 9: Symmetrization

First, we'll practice using modifyMotive list to symmetrize the very simple "pi" data from the first exercise.

1) `cd $WORKSHOP_HOME/PEET_Labs/Intro-1/PEET/run1`
This directory should already be populated with files from running the introductory lab. If not, you can copy the files from or pre-computed version: "`cp ../run1Done/* .`".

2) `3dmod pi_AvgVol_2P3.mrc`
Recall that this is an output average (with 3 particles from Iteration 2) from our previous alignment. Note that the "pi" is centered and oriented vertically. The vertical alignment is because we selected Align averages to have their Y axes vertical under Optional / Advanced Features during the initial alignment. The vertical alignment angles are not included in the final motive list, so we'll need to find them in **finish.log* and create a motive list containing them. As noted in the lecture, when PEET 1.12.0 is released this step will no longer be necessary, since a **_Vertical.csv* motive list will be created automatically whenever vertical alignment is enabled. Exit 3dmod.

3) `grep Slicer *finish.log`
You should see something like: **Averages rotated by Slicer (Z-Y-X) angles rotX: -0.03 rotY: -0.00 rotZ: 3.49 degrees**. (Your numbers may vary if you've run an alignment with your own model). You can also locate this information near the end of the **finish.log* without running grep, if you prefer. Let's apply these angles to the original, output motive list. Notice that by default modifyMotiveList expects Z-Y-X Slicer angles, but they must be entered in the order X, Y, Z.

4) `modifyMotiveList pi_MOTL_Tom1_Iter3.csv \`
`pi_vertical.csv "-0.03,0,3.49"`
where "\" should be followed immediately by **Enter**. We now have a new motive list, *pi_vertical.csv*, reflecting an alignment in which the individual particles (and, as a result, the average) have been oriented vertically. You can also apply additional rotations and shifts sequentially using modifyMotiveList. To illustrate, let's choose to further rotate our average by 45° counterclockwise about the tomogram Z axis, and to shift it to the right by 5 voxels.

5) `modifyMotiveList pi_vertical.csv pi_shifted.csv \`
`"0,0,-45" "5,0,0"`

Next we'll use this new motive list, *pi_shifted.csv*, to create a corresponding new average. It's prudent to backup parameter files, motive lists, averages, etc. before modifying them. Create a backup directory by executing `mkdir backup` followed by `cp * backup`. Depending on the size of your directory, you may want to only copy specific files to the *backup* directory. In this tutorial there is already a backup copy of everything under *../run1Done*.

6) `cp pi_shifted.csv pi_MOTL_Tom1_Iter3.csv`
We are copying our modified motive list over the original generated during alignment, so it will be the one seen by Etomo and PEET.

7) `etomo *.epe`

8) Uncheck **Align averages to have their Y axes vertical** (on the **Run** tab under **Optional / Advanced Features**). This will prevent the new average from being automatically rotated back to vertical. Press **Remake averages**. Wait a few moments for the averages to complete. (Check the progress bar at the top of the Run tab).

9) Press **Open averages in 3dmod** and verify that all 3 averages have shifted and rotated as we've requested.

10) Exit Etomo and the 3dmod windows when finished. There are command-line versions of all these operations. "Remake averages" corresponds to "`averageAll pi.prm 2 averages`", for example. The keyword "averages" can be replaced with "reference" or "both", depending on the desired output. "Open averages in 3dmod" corresponds to the command "`3dmod -Z -V -E U pi_AvgVol*.mrc`".

SYMMETRIZING THE 15 PROTOFILAMENT MICROTUBULE

For the 15 PF microtubule, vertical alignment was used, so we'll again need to extract those angles from the **finish.log*. Additionally, we'll make sure the tube is well centered, and then we'll repeatedly apply 24° rotations around the Y axis combined with shifts of 16 nm / (15 * 0.906 nm / voxel) = 1.17734 voxels.

11) `cd ../../../MT/PEET/firstSearch/`

12) `grep Slicer *finish.log`

Note the resulting angles. We'll apply them with modifyMotiveList in step 22 using a shell script. Next, we'll check the XZ centering of the current average. (The tube is extended and periodic in Y, so we can ignore that dimension in this case).

13) `3dmod *AvgVol*.mrc`

14) Scroll to the final average with 243 particles, open a Slicer window (\) and rotate **-90** about **X**. As before, the Y axis now points into the screen and you're looking down on an XZ view.

15) Select **Model** mode and place a point (middle-click) near the center of the tube.

16) Press **Edit / Object / Type** and set **Sphere radius for points** to **12**.

17) In the Slicer window, set **Img** to **20** and **Mod** to **5**.

18) Right-click (repeatedly if necessary) to move the model point until you're happy with the centering of the green and yellow circles in the microtubule.

19) Note that the yellow circle (the current point) is now almost directly above of (*i.e.* +Z) the red cross (the center of the image where X,Y,Z in the 3dmod info window is 28, 28, 28).

20) To measure the actual displacement, zoom in the Slicer window to a **Zoom** of **16** so the yellow circle and the red cross are clearly separated, center the cursor over the red cross with the mouse, and then press **q**. Pixel coordinates (X, Y, and Z) of both the current point and the cursor as well as the total distance between them will be reported in the 3dmod info window. Take the difference between the 2 X coordinates to calculate the X shift required, and between the 2 Z coordinates to calculate the Z shift needed to properly center the particle. (Signs are best handled by remembering that a +X shift will move the average to the right, while a +Z shift will move it up toward the top of the page). In this case you'll see that we need to shift down by approximately 1 voxel in Z… *i.e.* a Z shift of -1.

21) Exit 3dmod without saving the changed model, `cd../full15Fold`, and run `gedit reorient.sh`. This is the script that we'll use to generate virtual particles for symmetrization. Examine its contents and notice the use of the shift from step 20 and rotations determined in step 12 above. Exit gedit when finished.

22) (Optional) Execute this script by running `./reorient.sh`. The script will take a few minutes to run. Open a new shell window from your terminal and proceed with the following steps in the new window without waiting for it to complete.

23) `cd $WORKSHOP_HOME/PEET_Labs/MT/PEET/full15Fold`

24) `etomo *.epe`

Let's examine the settings used for the symmetrization alignment. On the Setup tab, notice that we've selected File names are templates and specified the initial motive list as *initMOTL01-15.csv*. This is convenient shorthand equivalent to entering 15 separate rows in the Volume Table, with motive lists *initMOTL01.csv*, *initMOTL02.csv*, *etc*. Templates may also be used for the other filenames in the Volume Table. In this example that the entries in each "row" all refer to our single, original volume.

We've selected particle 120 of volume 1 (the same as in the firstSearch alignment) as our initial Reference. Alternatively, we could have chosen the output reference file from the previous run as our starting reference; in this case, we would also have copied / renamed the corresponding binary wedge mask so PEET would be able to use it for missing wedge compensation at iteration 1. Missing wedge compensation doesn't really matter in this case, because the first iteration will be no-search, and we've told PEET to use all the available particles (15 * 243 = 3645) for the new reference created after this iteration. *I.e.* the first iteration simply creates a new reference based on our initial, approximate symmetrization of the previous alignment.

On the Run tab, we've selected Use Equal Numbers of Particles From All Tomograms both for average volumes and new references, since each of the tomograms are equivalent (and in fact identical!). After the no-search first iteration, we do 2 quick refinement iterations to improve the alignment. After each of these, we use approximately 2/3 of the available particles to generate references. This is typical, and allows for the possibility that some particles may be damaged or have been poorly aligned. Symmetrization takes considerable time, so we won't run it during

the workshop; you're of course welcome to do so on your own. Instead, we've provided all the important output files for you.

25) Press **Open averages in 3dmod** and **Open references in 3dmod** and examine the results. Note that
   a. The initial reference (*full15Fold_Ref2.mrc*) looks good, indicating that we've applied the symmetry operations correctly.
   b. The mask is appropriately sized and positioned.
   c. The references improve monotonically at successive iterations. Remember that you can use the ZaP window's **4th D** left and right arrows to scroll through iterations. Reference 1 is simply a single particle, and is not actually used here, since iteration 1 is no-search.
   d. Averages with increasing numbers of particles look similar, with improving SNRs, indicating that damaged or misaligned particles are not a serious problem. Particles with higher cross-correlation coefficient are typically incorporated first, depending whether you've selected "Use equal numbers…" or "Randomized particle selection". When substantial numbers of damaged or misaligned particles are present, the best average may not be the one with the most particles.

26) Examine the other output files generated during the run. PEETCleanup has already been run, so many intermediate files have been deleted, and only output files deemed important remain. Exit all Etomo and 3dmod windows when finished.

SYMMETRIZING BINNED BPV

Symmetrization of BPV is similar in many respects. Vertical alignment was not used during the initial BPV alignment, so it's simpler in that regard. Instead of applying helical / screw symmetry, we will need to bring each of the twelve 5-fold vertices to top center in a standard orientation and then apply 72° rotations around the Z axis.

27) `cd ../../../BPV_-3/PEET/firstSearch`

28) `3dmod -S bpv_AvgVol_4P132.mrc myPentamers.mod`

Next, we will check if the average is adequately centered in all 3 dimensions, or if it needs to be translated before symmetrization. As before, select **Model** mode, click on **Edit / Object / Type**, set **Object Type** to **Scattered** and increase **Sphere radius for points** to **14** and **line width** to **3**. In the Slicer window, adjust **Img** to **10** and **Mod** to **2**. As in the previous exercise, place a temporary model point to check centering; now however, we will check for possible shifts in all 3 dimensions rather than just X and Y. X and Y shifts can be checked in the unrotated (XY) slicer view, while X and Z shifts can be checked after rotating -90° around X. You should find that the average needs to be shifted approximately -1 voxel in Y. (Don't worry about the exact results; anything within about 1 voxel is probably okay).

29) Restore the Slicer view to its original orientation (set all angles to **0**) and position (X=Y=Z=**27**) in the 3dmod info window.

30) Delete any temporary model points you may have added while checking centering.

31) Open an Isosurface view (**Shift+U** or **Image / Isosurface**). In the Model View window, select **Edit / Controls** and press **Link to top Slicer Angles**. The Isosurface and Slicer images will now rotate together. Zoom in the Model View with the mouse wheel until the virus nearly fills the image.

In the following, we will be rotating the average in the Slicer and Isosurface views. There are several ways to do this:

a) If your keyboard has a numeric keypad, you can rotate the particle around X using the 2 and 8 keys, around Y using 4 and 6 keys, and around Z using the 3 and 9 keys. For X and Y, it is

helpful to think of the keys as pointing toward the compass direction in which you would like to rotate.

b) Under the Model View window, you can choose Edit / Controls and then either click (and, optionally hold) on the X, Y, Z rotation arrows or type the desired angles directly in the corresponding text fields.
c) In the Slicer window, you can drag the X, Y, and Z rotation sliders, or click on the red rotation arrows near the upper right of the window, or you can Shift+middle-click and drag.
d) In Model View, you can middle-click and drag.

In each case, you can monitor the orientation in the Model and Slicer Views until the desired orientation is obtained. In the following, you are free to use whichever of these methods you prefer, subject, of course, to the constraint that most laptops lack a numeric keypad, so method "a" may not be available. Note that when you wish to change focus to a particular window (*e.g.* the Slicer or Model View) without changing anything else, you can do so by left-clicking in that window's title bar, rather than in the window's display area.

32) Notice that a pentamer / 5-fold axis happens to be located just to the right and above the center of the starting view. Rotate the average using one of the above methods until this pentamer is centered in the Model View and facing you.

33) Turn the Isosurface **Threshold** down to **80**. At this setting, the capsomeres should look like isolated particles, and you'll more easily be able to see their relation to one another. Refine the previous rotation so that the centered, front pentamer is directly over the one behind it, and so that one of the vertices / capsomeres is pointing directly to 12 o'clock. We'll define this as our "standard" orientation, and will seek rotations which position each pentamer in such an orientation.

34) You're now looking directly down a 5-fold axis, in standard orientation, and have located 2 pentamers. Finding pentamers is typically easy, but enumerating all of them can get confusing unless you carefully mark the position and orientation of previously located ones as you go. We'll illustrate one method for keeping track of this information.

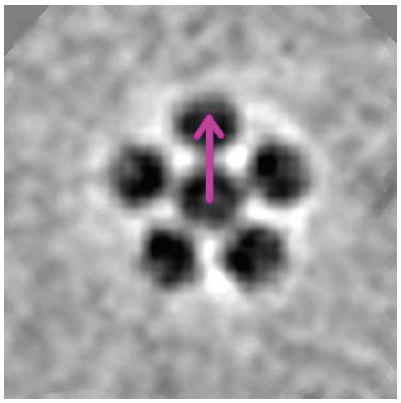
35) In the main 3dmod info window, press **Edit / Angles**. Arrange your screen so the Slicer, Model View, Slicer Angles, and 3dmodv Control windows are all visible.

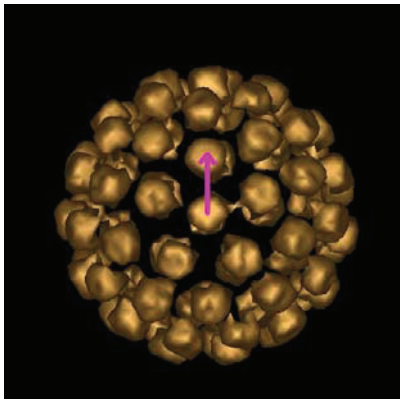
36) A pentamer should already be at the front of both the Slicer and Isosurface views, but the Slicer window is probably not at the correct slice height, so the pentamer may not be visible in it yet. Select the Slicer window by left clicking on its title bar (***not*** in the image itself!), and the press **PgUp / PgDn** or adjust the **View axis** position slider until the Slicer images is centered on the central capsomere of the front pentamer, and the 5 surrounding capsomeres are visible, but less dense. Be sure that you always use the front / upper-most pentamer! Adjusting slice height manually in this fashion will always suffice to locate the front / uppermost pentamer in the Slicer view. However, another 3dmod feature can be used to speed up this process, as we'll illustrate next.

37) With the Slicer window selected, press **PgDn** 10-20 times so the pentamer is no longer visible, simulating the state we were in at the start of the previous step. The pentamer will no longer be visible in Slicer. In the Slicer window, enable **centering** by clicking on the third control icon. Verify that this icon changes from a box within a box to a dot in a box, indicating that centering mode is enabled. ***The following will only work correctly in centering mode!!!*** In the Model View, right click on the capsomere at the center of the front pentamer. This will instantly change the all 3 Slicer coordinates, including slice height, to match the point you clicked on in the

Isosurface; the capsomere at the center of the pentamer should immediately become visible in the center of the Slicer window. Select the Slicer window by left clicking in its title bar, and refine the slice height as before until you are in the middle of the central capsomere with the 5 neighboring capsomeres are visible but less dense.

38) Regardless of which of the 2 previous methods you use, you should now adjust the orientation of the front pentamer, referring to both the Slicer and Model View windows. Rotate the pentamer until a line from the central capsomere to one of the surrounding vertices points toward 12 o'clock. Ensure that a vertex is oriented toward 12 o'clock and that the 5-fold axis is centered (as illustrated below) before proceeding. Next, we will add a model point to mark this pentamer and will save the angles used to bring it to this standard orientation.





In the Slicer window, add a model point by middle-clicking on the center of the central capsomere. Using 3dmod's **Edit / Object / Type** dialog, set **Sphere radius for points** to **3**, and verify in the Model View that the model point is located where you'd like. If not, you can move it in X, Y, or Z by right-clicking in the Slicer window.

39) Also in the Slicer window, press **New** to add a new row to the **3dmod Slicer Angles** table with the rotation required to bring this pentamer to the standard orientation.

40) Recall that there's another pentamer directly behind / underneath this one. Select the Model View, and rotate **180**° around X until this new pentamer is in front, directly over the green sphere marking the previous one.

41) Then rotate around Z until a vertex is pointed at 12 o'clock.

42) As before, locate the center of the new pentamer in the Slicer view either by right clicking in the Model View or by changing slice height. Refine the slice height and orientation as previously, place a new model point by middle-clicking in the Slicer window, and press **New** to store the corresponding angles. ***It's important that model points and stored angles remain in 1-to-1 correspondence!***

    By rotating the isosurface, search for another pentamer and its partner on the opposite side of the virus. Add the corresponding 2 model points and sets of angles, as above. To complete the model, you would simply repeat this procedure until all 12 pentamers had been located. There's little additional to be learned by doing so, however, so we've created such a model for you.

43) Save your partial model (**File / Save)**, and open our predefined *pentamers.mod* using **File / Open Model.** The resulting table of angles contains the remaining information needed to symmetrize our starting alignment. You can either make note of these manually, or you can find them by looking in the listing produced by running `imodinfo -a pentamers.mod`.

44) (Optional) If you wish, explore the geometry of the 5 fold axes in an icosahedral virus by setting the Isosurface **Threshold** so only the green spheres are visible. Can you detect a regular pattern?

45) Close any 3dmod windows when finished.

46) Quite a few steps are involved in actually applying all the symmetry operations, so we've placed the necessary commands in a script, *../iscosahedral/reorient.sh*, for you. Run `cd ../icosahedral` followed by `gedit reorient.sh` and see if you understand the various steps. If not ask your instructor for help. Exit gedit without changing anything when finished.

    The *reorient.sh* script itself is rather lengthy. The bash shell has built-in looping and integer arithmetic capabilities that allow for more concise scripting, if you're willing to take the time to learn the somewhat arcane syntax. I've provided an equivalent example in *reorientWithLoop.sh*. Examine this script with `geditreorientWithLoop.sh` now, exiting when finished. Good tutorials on bash shell scripting are available on the web. If you do choose to learn Linux / Unix shell scripting, bash is the Linux shell I'd recommend.

47) As before, we've already done the actual alignment run and removed most intermediate files. `etomo *.epe` to examine the parameter file settings, output averages, and references. You may also wish to examine the **.prm* file or other text files of interest with gedit, or to open other volumes (**.mrc*) with 3dmod.