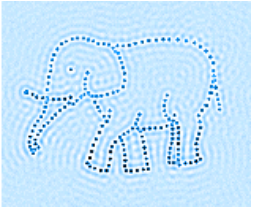
EMBO Practical Course on Image Processing for Cryo EM
1-11 September 2015



Practical 4: Optional part for experienced IMOD users - Reconstructing a cryo tomogram and sub-tomogram averaging of GroEL

IMOD was developed and is maintained by the EM group at Colorado University Boulder. They have extensive web help that contains installation instructions, tutorials and a tomography guide (http://bio3d.colorado.edu/imod/). This section was written by Dan Clare and Jonathan O'Driscoll and is for users already familiar with IMOD, or those who have already completed the main IMOD practical. You will learn how to align a cryo tomogram and extract particles for sub-tomogram averaging, which is single particle analysis in 3D but with the added complication that each particle has a missing wedge of data whose orientation depends on the orientation of the particle in the tomogram volume relative to the optical axis.

Open a new terminal window and in your home directory.

> cd PRAC-4

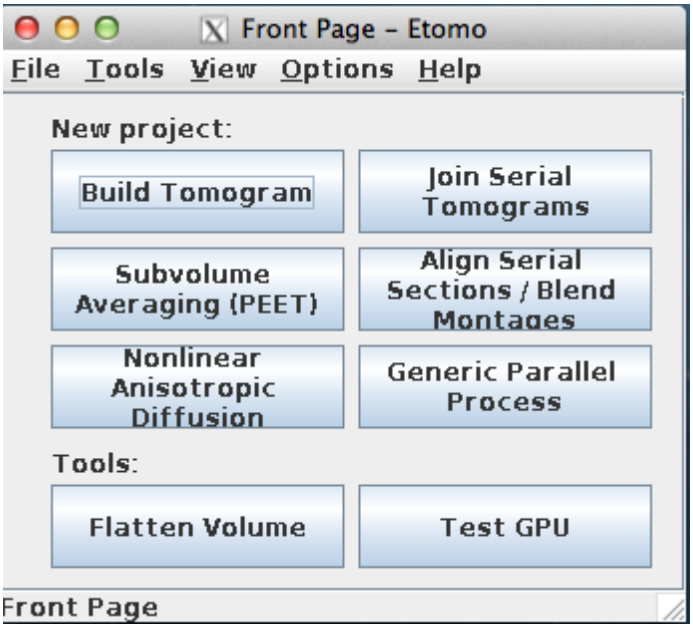
If you've already completed Part 1 then, go to the GROEL directory;

> cd GROEL

1) To start eTomo:

> etomo

When eTomo is first started, a **Front Page** panel will come up (shown below), allowing you to select which operation you want to perform with eTomo.



Press **Build Tomogram** and the **Setup Tomogram** panel will come up.

2) Setup Page

Enter the **Dataset name** containing the raw tilt series, in this case ELgpld4K_23K_3_2013bin.st. This data set is a single axis cryo tomogram of GroEL, so make sure **Single axis** is selected from the Data Type options menu. Selecting **Scan Header** will retrieve the **Pixel size** and **Image rotation** values from the MRC file header. Enter the fiducial size as 10 nm. Ensure that the Parallel Processing checkbox is enabled.

You must also specify that the tilt angles will be taken from an existing ELgpld4K_23K_3_2013bin.rawtlt file in the Axis A options menu. If you look at the raw stack you will notice how few grey levels are used to display the image. Select **Create Com Scripts** to begin processing the tilt series. Ignore the error message about the pixel spacing in the FEI extended header.

3) Pre-Processing

You will need to remove some of the extreme values from the tilt series so you should create a fixed stack for further processing. The default parameters are sufficient for this sample dataset. There are still a few outliers but they do not cause streaks in the back projection, so the effects of X-rays have probably been removed. Click **Create Fixed Stack** and then **Use Fixed Stack** once you are satisfied with the result.
For X-rays which are too large to be removed with the default parameters, you can increase the Maximum radius to encompass these large X-rays and try again. If the

larger radius causes inappropriate points to be corrected, then do the removal in two stages. First create a fixed stack with the smaller radius, and press Use Fixed Stack. Then increase both the radius and the criterion so that only the large X-rays are removed, and rerun the removal.

Coarse Alignment

The default parameters produce a reasonably well-aligned tilt series so there is no need to use midas.

Fiducial Model Generation Based on the Position of Gold Particles

Once again the contrast of the tilt series is good enough to automatically detect the gold particles. Select **Generate seed model automatically.** In the **Selection and Sorting Parameters** window, for **Seed Points to Select** next to **Total number**, enter 40. Click **Generate Seed Model** to automatically detect 40 representative gold particles on the section. Make sure Select beads on two surfaces is unchecked. Then switch to the **Track Beads** tab and fix gaps as usual. When fixing gaps, if the gold particle is out of frame, just skip it and move on to the next one. When finished, press **Done** to go on to the fine alignment step again.

***Note ***If you want to try manual picking of gold particles instead, select **Make seed model manually.** Press the **View Seed Model**. This will open ELgpld4K_23K_3_2013bin.preali in 3dmod and create an empty model file named ELgpld4K_23K_3_2013bin.seed. It will also bring up the Bead Fixer dialog box in **Make seed** mode with **Autocenter** checked. Check **Automatic new contour** as well. 3dmod will open the file to the middle section. In the Zap (image) window, place a model point in the center of 20-30 gold particles by centering the cursor in the middle of the gold particle and pressing the middle mouse button.
Because **Automatic new contour** is checked, a new contour for each new gold bead will be created. Because **Autocenter** is checked, 3dmod should make sure that the model point is positioned in the center of the gold bead, so you do not need to be as careful as when filling gaps. Save this seed model and closed 3dmod. Switch to the **Track Beads** tab. Press **Track Seed Model** to track your seed points. As before, press **Track with Fiducial as Seed** and then **Fix Fiducial Model** and fill in any gaps. When fixing gaps, if the gold particle is out of frame, just skip it and move on to the next one. Press **Done** to go on to the fine alignment step again.

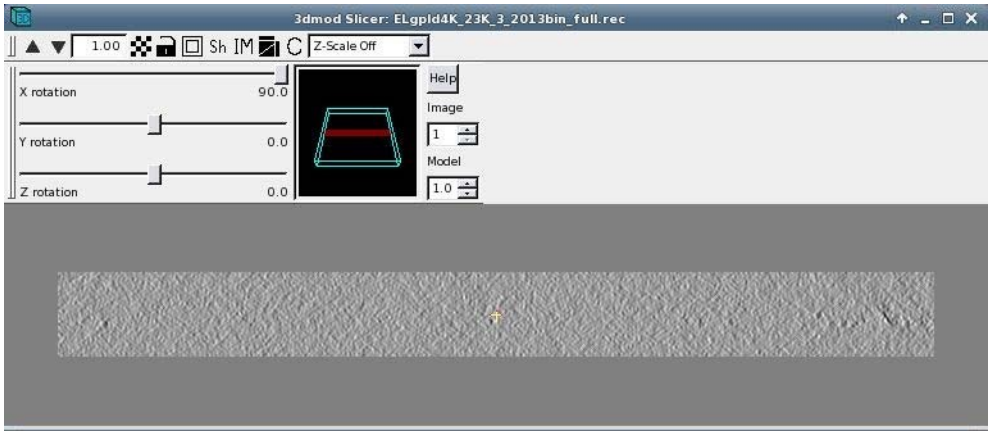
4) Fine alignment

The gold beads in this tomogram are distributed in the ice so you **must select** the '**Do not sort fiducials into 2 surfaces**' for analysis. Unfortunately, this means that you will not be able to correct for both X stretch and skew therefore **Distortion Solution Type** must be disabled in the Global Variables tab. Compute an alignment and refine it as normal.

5) Tomogram positioning

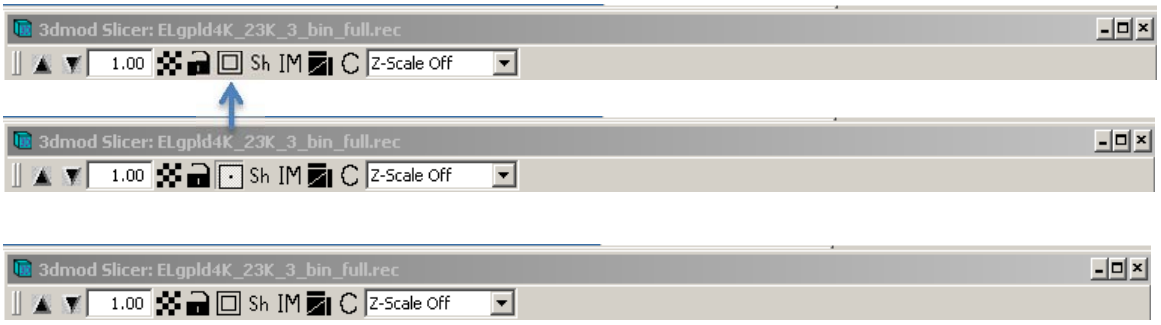
This part of the procedure is a little tricky so you are advised to **Create Whole Tomogram** binned by 3 (see below). When you press the **Create Boundary Model** button, 3dmod will open up the tomogram in the XY view in the ZaP Window (below).

You could try estimating where top and bottom surfaces are in this view but this can be difficult. Alternatively you can open a slicer window (Image – slicer) and rotate the reconstruction by 90° in X, so that you get the XZ view.

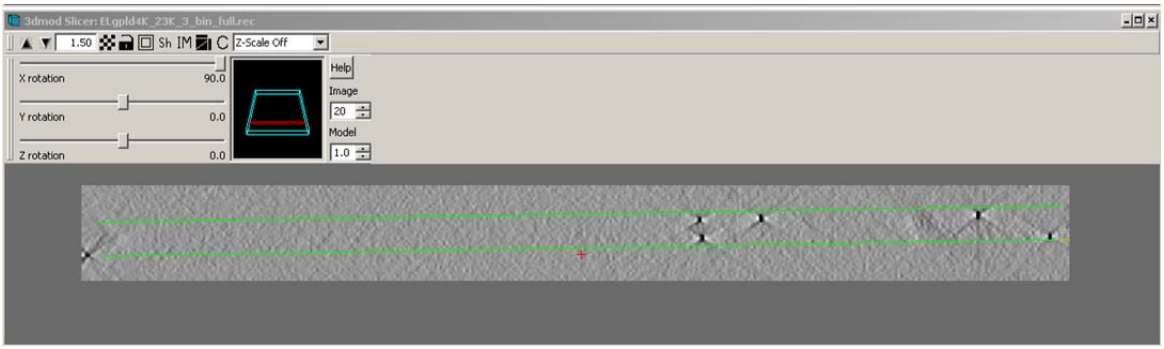


In XY view on ZaP window, click near the top of the tomogram:

Then, click and unclick the centering icon on the slicer window (see below).



This will center the XZ view of the Slicer window on the point you selected in the XY view from the ZaP window. You may want to average several slices to help you see the Z boundaries of the tomogram in the Slicer XZ view (see below). Then you can mark the top and bottom boundaries on the Slicer Window with two contours and repeat the process for an area near the bottom of the tomogram.



If you make a mistake while doing this you can delete a point by selecting it with the left mouse button then pressing delete or you can delete a whole contour by selecting it pressing shift-D. Remember to save the model then calculate the Z shift and pitch angles and create the final alignment.

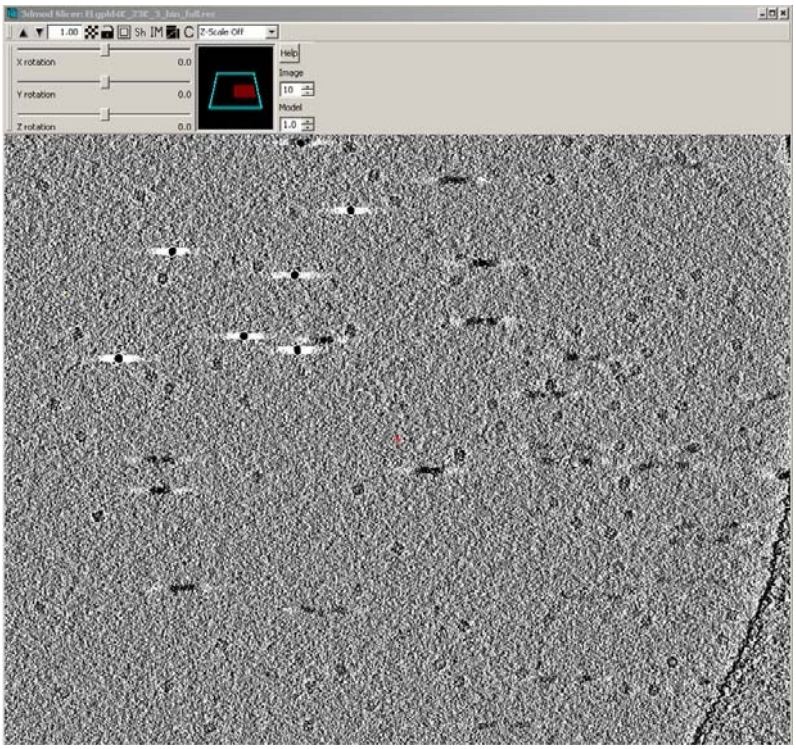
6) Create the Final Aligned Stack

Use linear interpolation to reduce noise.

7) Generate the Tomogram

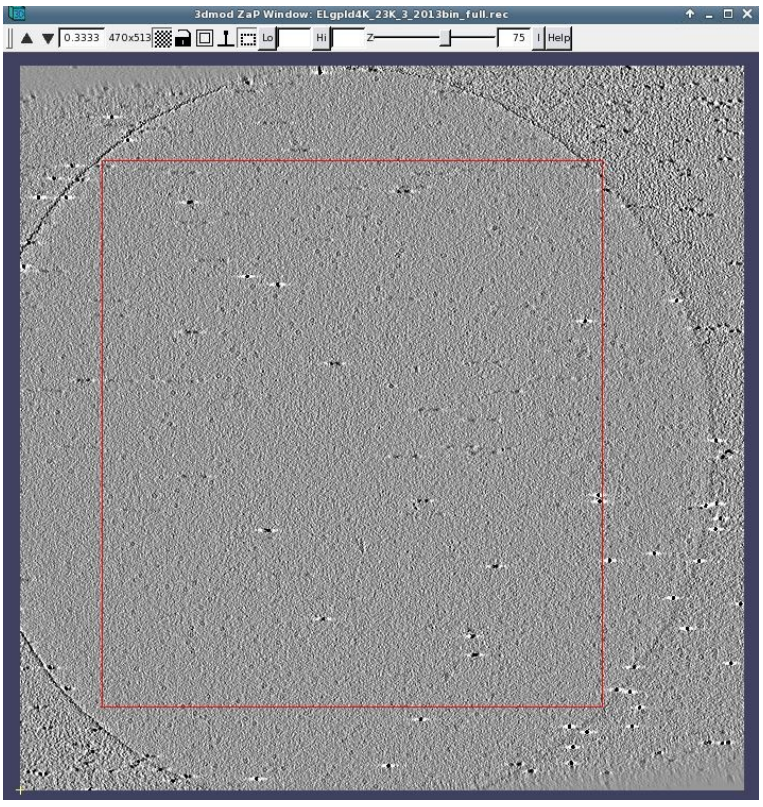
The default parameters for reconstruction assume that the numbers in your images are linearly related to the number of transmitted electrons caught by the detector. The logarithm of such values should then be proportional to projected mass density for imaging dominated by amplitude (aperture) contrast. Because of this, the backprojection program, Tilt, has an option to take the logarithm of the numbers after adding a base value. Our data was derived from unsigned 16-bit integers and has had 32768 subtracted for storage as signed integers in the MRC file. The program detects this and automatically sets the base value to add to 32768.
It is recommended that for predominantly phase contrast (cryoEM) data, the logarithm is turned off, allowing a separate set of factors to scale the backprojection data for output.
Unclick the logarithm checkbox, Click the 'A' to display advanced settings and enter 32768 in the Offset box for linear density scaling and generate the tomogram.

You may want to use slicer and average some Z sections so you can see GroEL complexes (slightly larger than a gold bead) in the reconstructed volume.

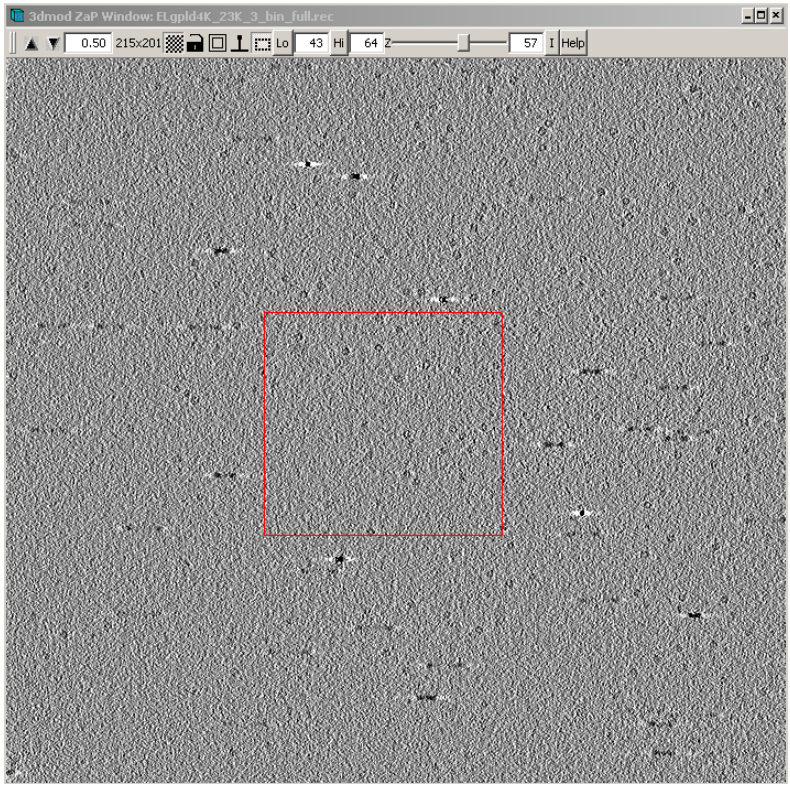


8) Trim Volume and scale grey levels

When trimming you should cut out a region containing just the ice/GroEL filled hole using the rubberband tool. Select as large an area as possible (see below).

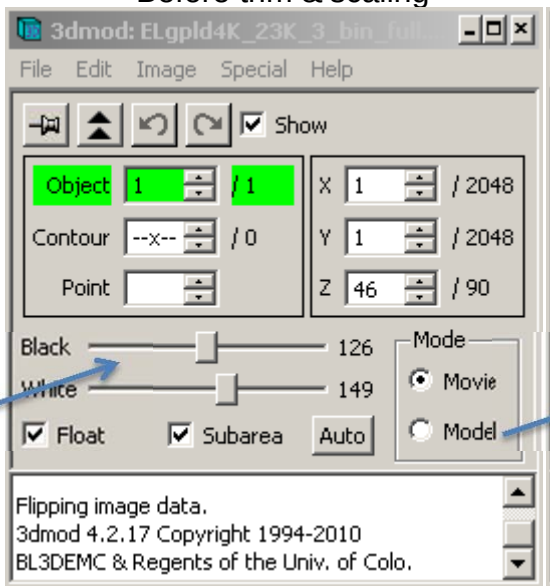


Be careful when scaling the grey levels; carefully select an area with GroEL complexes only and no gold beads (see below).

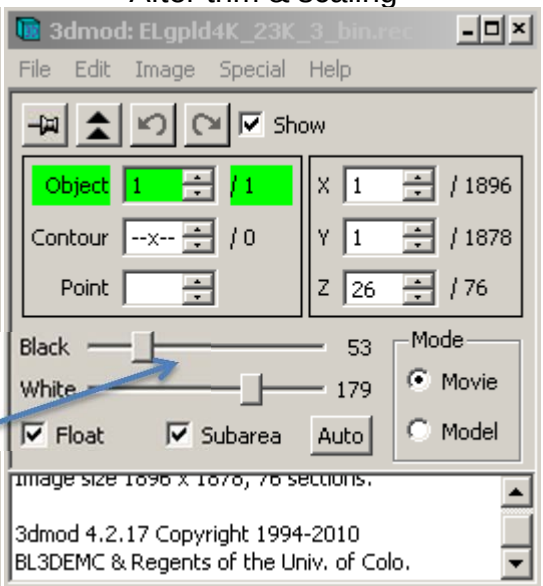


Once you have trimmed and scaled the tomogram you will notice that many more grey levels are used (see below; 23 before and 126 after scaling).

Before trim & scaling



After trim & scaling



9) Tidy Up

You can archive the original file and then delete all the intermediate files. Then close the eTomo window.

10) Tomogram Filtering and GroEL Subvolume Selection

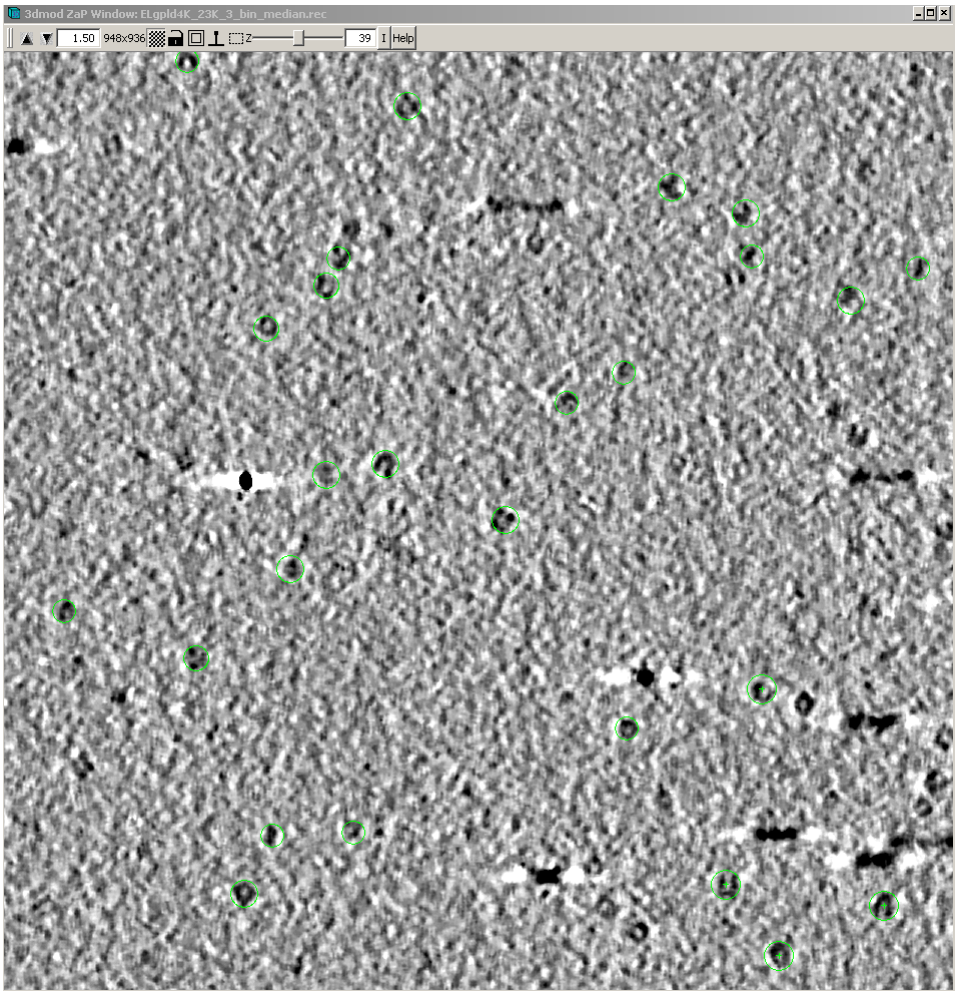
We will use a median filter on the tomogram so that the GroEL volumes are easily seen. On the command line enter:

> clip median -3d –n 5 ELgpld4K_23K_3_2013bin.rec Median.rec

This will take 5 or 6 minutes to run. Then, open the Median.rec volume in 3dmod:

> 3dmod Median.rec

Switch from Movie mode to Model in the 3dmod window. In the Edit – Object menu, select Type, and set the object type to **Scattered** and use a **Sphere radius for points** size of 12. You now need to create a model, selecting ~100 GroEL volumes by placing an object point on each volume (see below). Make a note of a nice volume that you may want to use as a reference later. It would be nice to use many more GroEL volumes but this would take too long on the computers being used for the practical. **Save the model** as, for example, EL_Model.mod, and close 3dmod.

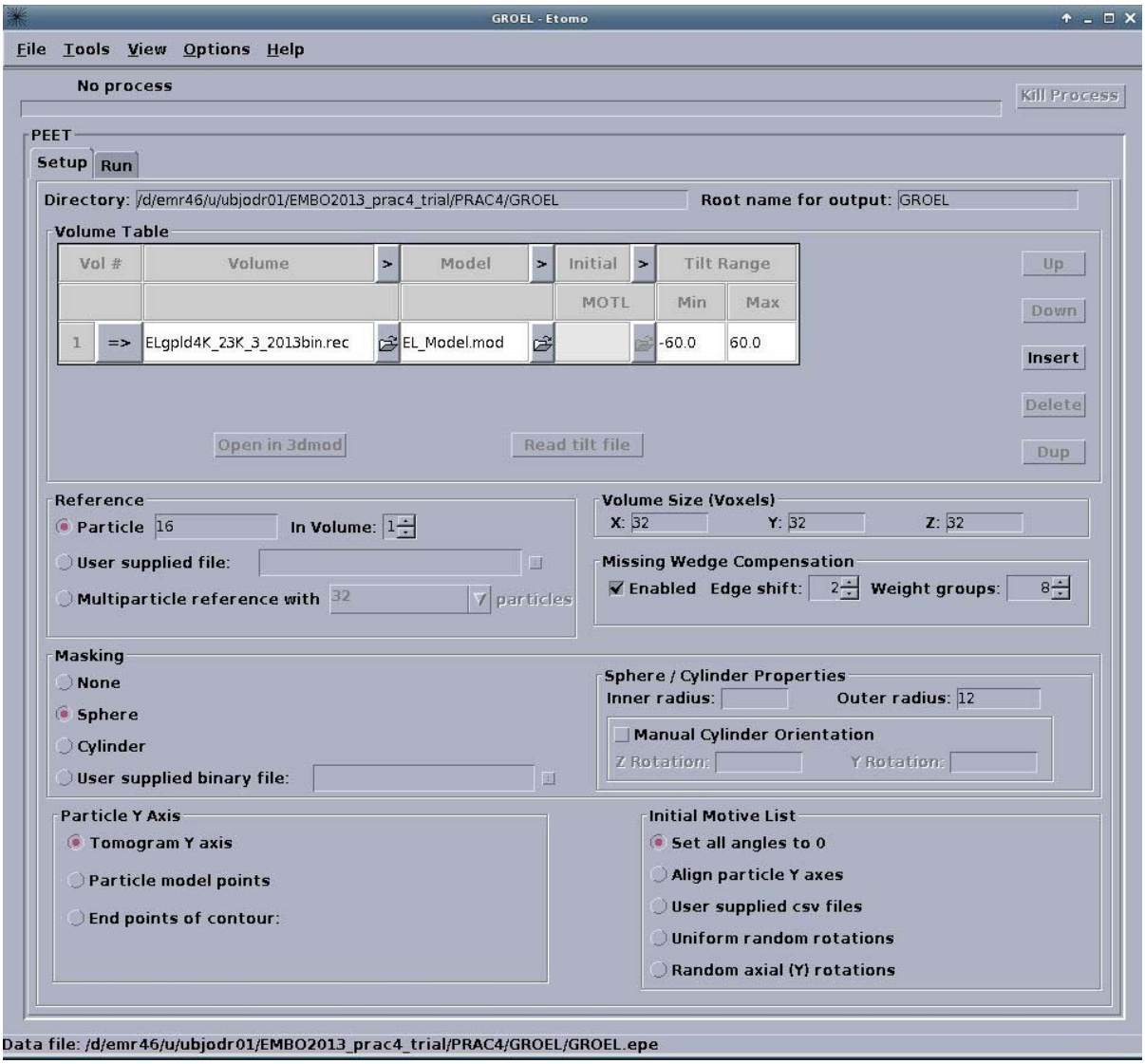


11) PEET

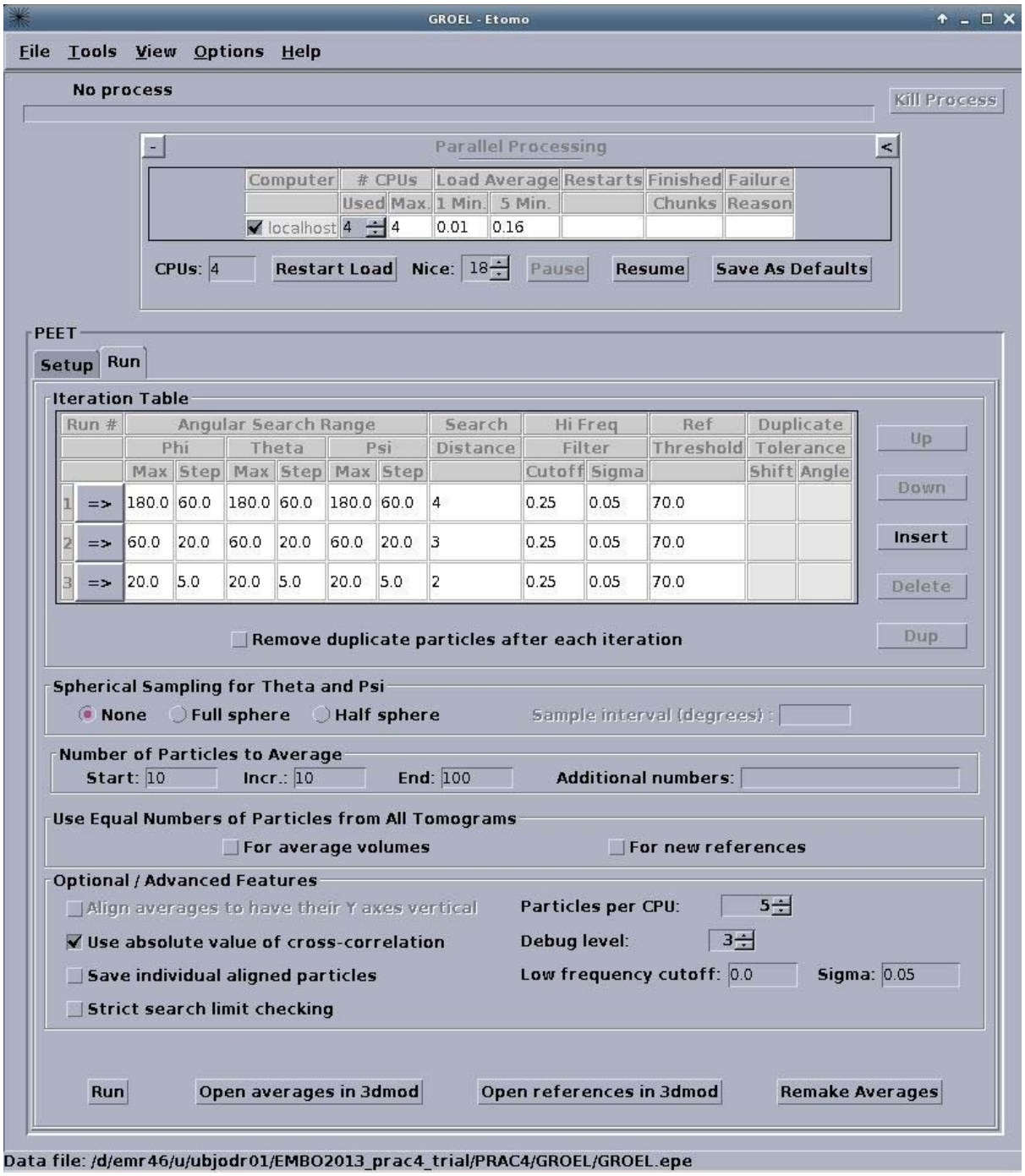
You can access detailed information about each of the processing steps during the practical by clicking on the **Help** tab on the **eTomo Main Window** and selecting **Peet Users Guide.**

Start etomo as normal from the command line. Initially, you should enter the **Options menu** and select **Settings.** Configure etomo ensuring parallel processing is enabled (for 4 CPUs) and the "Native look & feel" check box is selected. The latter option ensures a better display of the title bars when running this version of PEET on our Linux system. Apply your settings, exit etomo, and re-run it again for your changes to take effect.
Select **Subtomogram averaging (PEET)**. This will open a set-up page for PEET (see below).

The GROEL directory should be automatically selected. Enter a base name for all the PEET output, such as, GROEL. To save time we have given you an EMBO_EL.prm file which contains most of the parameters you will need to run PEET. To use it, enable the **Copy project from** checkbox, browse to the PRM directory by clicking the small search box on the right hand side (arrow above) and select the prm file. Click **OK**.
This will fill in a lot of the entries in both the setup page and the run page (see below). However, there may still be a few things that have to be entered/checked. Ensure the unfiltered volume (ELgpld4K_23K_3_2013bin.rec) and the correct model file (EL_Model.mod) containing the coordinates for the 100 GroEL volumes you picked are selected in the Volume Table. Press the **Fix Incorrect Paths** button if necessary. The tilt range min and max should be set to -60 and 60, respectively, and make sure that the Missing Wedge Compensation is enabled. Next, specify the number of the reference volume you would like to use for the first round of alignment (you should have written this down when picking them; if not just choose a number from 1-100).



Then, select the **Run** tab and the run page will be displayed. The parameter file you imported earlier has filled in all the details on this page (see below). The only thing you need to do is to check on the local host and select all 4 CPU's. After this you are ready to press the **Run** button at the bottom of the page. Ignore the error message about the IMOD version difference. The job will still proceed. The alignment should take 15 -20 minutes so you are strongly advised to go to the PEET manual webpage (http://bio3d.colorado.edu/PEET/PEETmanual.html) and look at the documentation for all of the parameters that PEET uses. In addition, a growing set of introductory tutorials can be viewed on the Boulder 3D EM YouTube channel http://www.youtube.com/BL3DEMC.



Once the PEET run has finished, you can view the results by selecting **Open averages in 3dmod**. When the 3dmod window opens you will notice that there is a 4D button, which will allow you to look through the 10 different averages (average 1 contains 10 volumes whereas average 10 contains 100 volumes). The Isosurface viewer will also open automatically. You may want to play around with the thresholding in the isosurface control box to get a nice looking GroEL complex.

A directory with the averages from one of Dan's PEET runs on the same sample (but with 500 volumes) has been provided. These files are located in the DAN directory. Navigate there and at the command line type:

> 3dmod DAN_TEST_AvgVol_*

This will open 5 averages containing between 100 and 500 volumes. To get the isosurface viewer go to the image menu and select isosurface.