

Lesson 1:

Manual Reconstruction of a Cryo Tomogram

RECONSTRUCTING A CRYO TOMOGRAM USING FIDUCIALS

In this exercise, you will build a tomogram from scratch using Etomo.

- 1) `cd $WORKSHOP_HOME/IMOD_Labs/cryo-manual`
- 2) `etomo`
- 3) In this initial step, we define some features of the data set and create the files needed for processing. Press **Build Tomogram** in the Etomo **Front Page**.

Press the yellow file chooser icon on the **Datset name** line and select *cryo.st*. For **SystemTemplate**, select *cryoSample.adoc*. This will set several parameters appropriately for cryo reconstructions; you should generally select a template.

Press **Scan Header** to retrieve the pixel size and rotation angle of the tilt axis from the image file. Enter **15** for **Fiducial diameter (nm)**. Select **Parallel Processing** to use all of the CPU cores when possible. These laptops do not have GPUs, however, it is best to check this box if you do have an IMOD compatible GPU. For **Image distortion field file**, select *27.5kGIF2007-03-24.idf*. These data were taken with an energy filter which introduces significant geometric

distortions. The distortions were measured and can be corrected with this file.

Press **View Raw Image Stack** and scroll through the images to see that they are not well-aligned. Notice the 4 contrast control sliders on the IMOD info window, which are present because the data have been loaded as integers. The top two sliders are very close together, which means the image data occupy a very small fraction of the full data range; the rest of the data range is taken up by image artifacts due to X-rays. In general, if you see bad views that you know that you want to exclude from alignment and reconstruction, you can list these in the Exclude views box. Close 3dmod. Press **Create Com Scripts**.

- 4) Click on **Pre-processing Not Started**. Pre-processing is needed to remove artifacts in the images, generally produced by X-ray events in the camera. These artifacts will produce streaks in a reconstruction and can also make it harder to see the image features, which have a much smaller dynamic range than the artifacts.

Press **Show Min/Max for Raw Stack** to see the range of the data; both a plot of minimum and maximum values and a table with more detailed information will open. Every view has a large negative minimum because there was an X-ray artifact in the dark reference.

Press Create Fixed Stack to run the program that finds and erases artifacts. Press **View Fixed Stack**. Now there are only 2 contrast sliders because data can be loaded as bytes after removing most of the artifacts.

Press **Show Min/Max for Fixed Stack** to see the new range of data. Deviations of 50-100 from the rest of the data will not matter in the reconstruction. Close all 3dmod, graph, and table windows.

Press **Use Fixed Stack**. Press **Done** to advance to the next step.

- 5) In the Course Alignment step, we use image cross-correlation to align successive images, which makes it easier to track fiducial markers.

Press **Calculate Cross-Correlation**

When done, press **Generate Coarse Aligned Stack**. When done, press **View Aligned Stack in 3dmod**. Scroll through the images to see that they now look aligned. Press **Done** to go on.

- 6) In the Fiducial Model Gen. step, the positions of selected gold markers are found on all of the images, which allows a more accurate alignment to be obtained.

Check that **Make seed and track** is selected. On the **Seed Model** tab, select **Make seed model manually**. The option to make the seed model automatically works quite well; we are not using it so that you learn how to do this step manually if necessary. Press **Seed Fiducial Model**, which opens the Bead Fixer window. Turn on **Automatic new contour** in the Bead Fixer window if it is not on already. A contour is a set of connected points. We need to put these points in separate contours because a) we don't want to see them connected and b) each contour will be added to with the corresponding points on other views.

Make sure you are on the central view (view **31**) and place the cursor very near each bead and add a point by middle-clicking. It will be automatically centered (the Autocenter option is turned on by Etomo in seeding mode). Press the **s** key to save the model.

Switch to the **Track Beads** tab and press **Track Seed Model**. The **Project Log** window shows the number of missing points when done. It may be 0. Press **Fix Fiducial Model**, which will load the tracked model into 3dmod and switch the Bead Fixer to Fill gaps mode. You can scroll

through the views and also press the **v** key to see the tracks of the beads in 3D.

If there are missing points, you can now use the Bead Fixer to step from one gap to the next (using the space bar as a hot key) and add a point, if appropriate. A bead does not need to be marked on every view, and you should not add a point if the bead's position is not clear. If you add points, be sure to save the model with the **s** key. Another way to complete the model is to press Track with Fiducial Model as Seed. It is a good idea to look at the model in 3D first and make sure there are not deviant points at the ends of contours that should be fixed first. Press **Done** to go on.

- 7) During Fine Alignment, the bead positions are fit to a mathematical model of specimen movements. The model predicts a position for each bead on each view, and the mean distance between the predicted and actual positions is referred to as the "mean residual error". These errors will let you find and correct badly modeled points. The need to do so has been much reduced by the recent addition of a method called "robust fitting", which automatically gives less weight or even eliminates the points most likely to be at incorrect positions. However, it is good to learn how positions can be fixed manually.

Select **Do not sort fiducials into 2 surfaces for analysis** and press **Compute Alignment**. When it is done, the Mean residual error will appear in the **Project Log**.

Press **View 3D Model** to open the model of solved positions in 3D and assess whether to solve for distortion (stretching and skew). Press the **r** key in the Model View window for a side-view of the model. If you middle-click and move the mouse to the right, the model will rotate until the three lower beads are very close together. To solve for distortion, fiducials should be well-distributed in Z: not all on one plane, more than a few at a different

Z height, and the ones at a different height distributed over the area. This is not often the case for cryo data sets, and this one fails the latter two tests.

Press **View/Edit Fiducial Model**. The Bead Fixer will open in (or be switched to) Fix big residuals mode and read in the log from alignment. Zoom up in the ZaP window to 2 or so. It is also helpful to switch the ZaP window to keeping the current model point centered, by pressing the concentric squares in the toolbar.

On the **Bead Fixer** window, press **Go to Next Big Residual** or the single quote (') key, it will move to each point with a big residual in order by decreasing value. If a point is not centered on the gold bead, you can move it to the correct position by right-clicking to the correct position. The red arrow points to the position predicted by the alignment solution; it is not based on any knowledge of what is in the image. This predicted position may not be correct, but if it is, you can move the point to that predicted position with **Move Point by Residual** or the semicolon (;) key. All of the biggest residuals occur at very high tilt, and each point has been correctly modeled, so there is nothing to fix. The reason for this is evident if you examine the output from the alignment program.

Right-click in Etomo over the **Fine Alignment** panel and select **Align log file**. The error summary and the ratio of measured values to variables solved for are in the **Errors** tab. Switch to the **Solution** tab to see the alignment parameters. Various columns from the solution table can be plotted by right-clicking in Etomo and selecting a particular parameter. Right-click and select **Plot global mean residual**. Notice that the mean residual gets much higher at high tilt, which is why we saw only those points having the biggest residuals. To find possible erroneous points at lower tilts in this case, in Etomo, under **Residual Reporting**, select **Relative to Neighboring views**. Press **Compute Alignment** again.

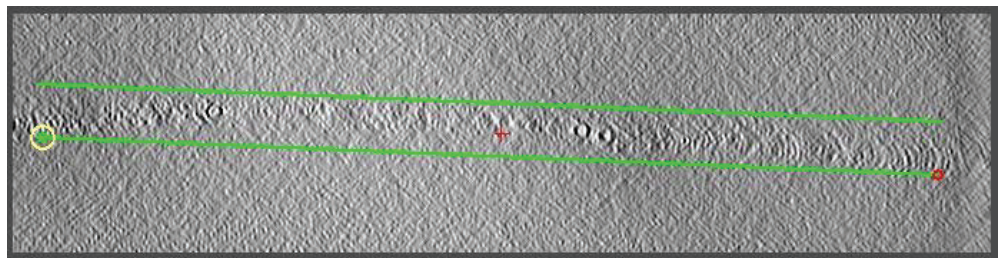
There are no new points to fix as reported in the 3dmod info window. Press **Done** to go on. When Etomo asks “coarsely aligned stack is open in 3dmod. Should file be closed?” Press **Yes**. Etomo knows when a file is no longer needed and will prompt you to close them as necessary.

- 8) The goal of Tomogram Positioning is to set angles and an offset in Z so that the specimen is flat and centered in Z in the computed volume, thus minimizing the computational effort.

Set **Positioning tomogram thickness** to **500**. Press **Create Whole Tomogram** to build a whole, binned-down tomogram.

Press **Create Boundary Model** to open the tomogram. Open a Slicer window with **Image / Slicer** from the 3dmod info window. Set **X-rotation** to **90** and **Thick: Img** to **20**, which makes it average 20 slices. The goal is to draw a pair of lines enclosing the sample at ~3 different Y locations in the tomogram. For this specimen we can see the boundaries of the sample well in cross-section, so this is the easiest way to draw them.

Use the **View axis position** slider to scroll through Y and get a sense of how the boundaries change. Go to the middle (~340) and draw two lines along the top and bottom surfaces of the sample. 3dmod will automatically start a new contour after two points. Move the current point by right-clicking to the new position; left-click to select a new current point. See the image below.



Do the same near the lower end of the tomogram (axis position ~70) and the upper end (~610). Your final model should contain 6 contours with 2 points each. Save the model with the **s** key.

Press **Compute Z Shift & Pitch Angles**. The Angle offset, Z shift, X-axis tilt, and tomogram thickness are shown; these will give the thinnest, most level tomogram containing the area you outlined. The required Final Tomogram Thickness is also shown.

Close 3dmod windows, and press **Create Final Alignment**, which reruns the fine alignment. Press **Done** to go on.

- 9) Next we will create a Final Aligned Stack, which applies a rotation to make the tilt-axis vertical. Press **Create Full Aligned Stack**. When done, press **View Full Aligned Stack**. Notice how the image has rotated and the corners are now cut off. Close 3dmod windows and press **Done** to move on.
- 10) On the Tomogram Generation page, you will at last compute the tomogram. In the **Parallel Processing** box, change the **# of CPUs Used** to 4 and press **Save As Defaults**.

Press **Generate Tomogram**. Press **View Tomogram in 3dmod** when it is done. After reviewing the tomogram, press **Done** to go on.

- 11) In the Post-processing step, you can trim away unneeded regions, convert the tomogram to bytes to save time and space, and reorient the tomogram so that the slices stored in the file are in X/Y planes instead of X/Z planes. Even if you do not want to trim or convert to bytes, you should always go through this step to get a reoriented tomogram, which will work better with other programs.

Turn on the rubber band (rectangle to the left of the lasso) in the ZaP window and select an area of about 1500x1500 pixels, i.e., at a window zoom of **0.25**, the rubber band size on the screen of about **375x375** pixels, as shown in the ZaP toolbar. You can move an edge or corner of the rubber band by left-clicking, or shift the whole band by middle-clicking with the cursor over an edge. Scroll to the first slice that you want to keep and press **Lo**, then find the last slice you want to keep and press **Hi**. In Etomo, press **Get XYZ Volume Range from 3dmod** to import these limits.

In the ZaP window, move to where you can clearly see the gold particles. Reposition the rubber band square so that no gold particles or associated rays are within the box, then select a narrower range of slices with **Lo** and **Hi** that will be used to determine scaling; exclude the dense contamination on the surface. In Etomo, press **Get XYZ Sub-Area from 3dmod** in the **Scaling** section to import these limits. Close all 3dmod windows.

Press **Trim Volume**. When done, press **3dmod Trimmed Volume** to see the result. Press **Done** to go on.

- 12) Next we will Clean Up the directory. In most cases, there is no need for the intermediate files from processing. This step allows you to remove these files and leave all of the information from which they could easily be recreated if necessary.

The original raw tilt series stack can also "archived" by compressing its difference from the current stack; this operation is reversible. Press **Archive Original Stack** and press **Delete** to confirm the deletion of the original stack when it is done.

All intermediate files are now shown in the box. Click in the box and type **Ctrl+A** to select all files. You could then click on individual files while holding down the Ctrl key to unselect them.

You might wish to leave *cryo.preali* (coarse aligned stack), *cryo.ali* (final aligned stack), or *cryo_full.rec* (raw reconstruction, which could be trimmed differently).

Press **Delete Selected** to remove the intermediate files that are still selected. Press **Done** to finish and close Etomo.

RECONSTRUCTING A CRYO TOMOGRAM USING PATCH TRACKING

13) `cd $WORKSHOP_HOME/IMOD_Labs/cryosection`

14) `etomo`

15) Press **Build Tomogram**. Press the file chooser icon on the **Datset name** line and select *cryosection.st*. For **System template**, select *cryoSample.adoc*. Press **Scan Header** and enter **0** for **Fiducial diameter**. Some parameters for X-ray removal are set based on the fiducial size to avoid erasing fiducials, so it is important to enter 0 rather than an arbitrary size when there are no fiducials. Select **Parallel Processing** to use multiple CPUs to compute the reconstruction. In the **Axis A** group box, select **Series was bidirectional from** and set the angle to **0**. Press **Create Com Scripts**.

16) Select **Pre-processing** and press **Create Fixed Stack**. Press **Show Min/Max for Fixed Stack**. Press **Use Fixed Stack**, then **Done**.

17) Press **Calculate Cross-Correlation**. When done, press **Generate Coarse Aligned Stack** and then **View Aligned Stack in 3dmod**. Notice the slight 'shift' at zero degrees. Press **Done**.

18) In the Fiducial Model Generation step, patches of image will be tracked from one view to the next, and the center positions of the patches will be placed into a model file. This model can be used to solve for the tilt series alignment, much as a model of true fiducial

markers can. Near the top of the window, select **Use patch tracking to make fiducial model**. Change the **Size of patches(X, Y)** to **680,680** (if it is not already set to that); this will give 4x4 patches. Press the **Advanced** button at the bottom and change the **High frequency cutoff radius** to **0.125** (if it is not already set to that). The parameters for patch tracking are mostly based on the ones for coarse alignment since the same program is being run. However, the correlations for coarse alignment are done on almost the full image and are generally binned down, so the high frequency filter cutoff of 0.25 is adequate for filtering out noise. The patches being correlated are smaller and will not be binned, so stronger filtering is needed. Press **Basic** to make it easier to see the basic controls. Press **Track Patches**.

When done, press **Open Tracked Patches** to see the tracked locations on the image and in Model View (use hotkey **v** to open). No specific feature has been tracked, just the whole patch area. Notice that there are just 16 contours going all the way through the tilt series. Press **Done**.

- 19) For this data set, the **Fine Alignment** process will involve cycling back to the Patch Tracking section on the Fiducial Model Gen. page.

Press **Compute Alignment**. Look at your **Project Log** window, the mean residual is fairly high, ~1.5 pixels. Such a high residual occurs because some of the positions tracked through the patches do not correspond to projections from single points in 3D. A better fit to the points can be gotten by breaking the contours into pieces.

Return to the **Fiducial Model Generation** page and select **Break contours into pieces with overlap 4**. Press **Recut or Restore Contours**. Return to the **Fine Alignment** page and press **Compute Alignment**. The mean residual is much lower because much smaller errors build up over the shorter tilt ranges in each contour.

Press **View/Edit Fiducial Model**. In the Model View window, rotate the model so that you can see all the tracks well by pressing the middle mouse button and moving the mouse at a steep diagonal. Each track consists of 7 overlapping segments, which you can see by right-clicking near a track to select a contour; only a small segment is highlighted.

Right-click in Etomo to bring up the **Align log file** and switch to the **Coordinates** tab. The "mean resid" column shows the mean residual in each contour or segment of the track. The range is large, from 0.2 to 1.2. When patch tracking was first developed, the approach was simply to delete the contours with the highest residuals, and the Bead Fixer has a Look at contours mode to assist this process. Not only is this somewhat arbitrary, but it would lead to trouble in this case, since 10 of the highest 11 residuals occur for contours passing through zero degrees. The robust fitting that is now available is a better approach. Close the log file. In Etomo, turn on **Do robust fitting with tuning factor 1.0** and then turn on **Find weights for contours, not points**. Press **Compute Alignment**.

Open the **Align log** and go to **Coordinates** again. Each contour has been given a weight between 0 and 1, where a weight of 0 means that the points in the contour are ignored in the fitting. On the **Robust** tab, a summary line indicates 6-8 contours have weights under 0.5. On the **Coordinates** tab, there is a new column with the weights. The contours that have small weights are ones that have large residuals relative to other comparable contours (i.e., ones at similar tilt angles).

For your own data sets with patch tracking, you will probably want to select the robust fitting whenever you break contours into pieces. If contours are not broken into pieces, there are usually too few contours to allow robust fitting. Close all 3dmod windows and log window. Press **Done** in Etomo.

20) This sample can be positioned by viewing X/Z slices in the binned-down tomogram. Set **Positioning tomogram thickness** to **800**. Press **Create Whole Tomogram** to build a whole, binned-down tomogram. Press **Create Boundary Model** to open the tomogram

Open a Slicer window with **Image / Slicer**. Set **X-rotation** to **90** and **Thick: Img** to **20**. Draw 3 pairs of lines: in the middle of the tomogram (axis position ~**380**) and at axis positions near **115** and **555**. Save the model with the **s** key. Close 3dmod.

Press **Compute Z Shift & Pitch Angles**. Note the large angle offset (~11), which means that the tilt angles assumed during patch tracking were significantly different from the actual angles, enough to affect the correlations. Do the following steps to incorporate this knowledge.

Return to the **Fiducial Model Generation** page and press **Advanced**. Enter the **Tilt angle offset** from the positioning page in the **Tilt angle offset** field. Press **Track Patches**. Go to the **Fine Alignment** page and do **Compute Alignment**. Note the substantial improvement in the weighted mean residual, from ~0.41 to ~0.37. You now have a final alignment that incorporates the positioning information, so you can go back to **Tomogram Positioning** and press **Create Final Alignment**. Press **Done**.

21) Press **Create Full Aligned Stack**. When done, press **View Full Aligned Stack**. Close 3dmod and press **Done**.

22) Press **Generate Tomogram**. Press **View Tomogram in 3dmod** when the program is done. Press **Done**.

23) Turn on the rubber band in the ZaP window and select a subarea area if desired. Scroll to the first slice that you want to keep and press **Lo**, then find the last slice you want to keep and press **Hi**.

You should be able to trim off most of the crevasses on the bottom. In Etomo, press **Get XYZ Volume Range from 3dmod** to import these limits.

In the ZaP window, select a narrower range of slices with **Lo** and **Hi** that will be used to determine scaling. In Etomo, press **Get XYZ Sub-Area from 3dmod in the Scaling** section to import these limits.

Press **Trim Volume**. When done, press **3dmod Trimmed Volume** to see the result.

24) Press **Archive Original Stack** and **Delete** when asked. Select all **Intermediate files, SIRT Iteration** files and press **Delete Selected**. Press **Done** and close Etomo.