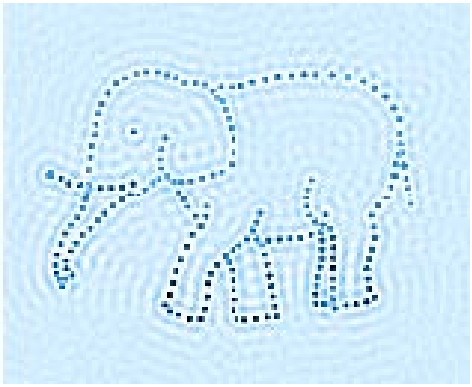


EMBO Practical Course on Image Processing for CryoEM

5th September 2019

Practical 3 part 1: Tomography with IMOD (version 4.9.12) Reconstructing a tomogram of a human neuronal cell

AIMS

Today you will be using the IMOD suite of programs to reconstruct a cryo-tomogram of the periphery of a U87MG cell. The tilt series has been collected on FEI Polara 300 kV cryo-electron microscope.

By the end of this practical, you should be able to:

- reconstruct a cryo-tomogram step by step, using ETomo, the IMOD's Tomography Graphical User Interface (GUI)
- inspect image data and place fiducial landmarks using the 3dmod GUI.

You can visit http://bio3d.colorado.edu/imod/#Guides to learn more about IMOD and discover where most of the documentation from this tutorial comes from (http://bio3d.colorado.edu/imod/doc/tomoguide.html).

During this tutorial you can also access detailed information about every step in the processing by clicking on the **Help** tab in the ETomo Main Window and selecting **Tomography guide**. You may also consider joining the IMOD mailing list https://bio3d.colorado.edu/imod/joinlist.html

Getting started

Log into the server using the instructions provided. Open a terminal window and in the path `/d/embo2019/u/embo##` type:

```
> cd Prac-3
```

and list the contents:

```
> ls
```

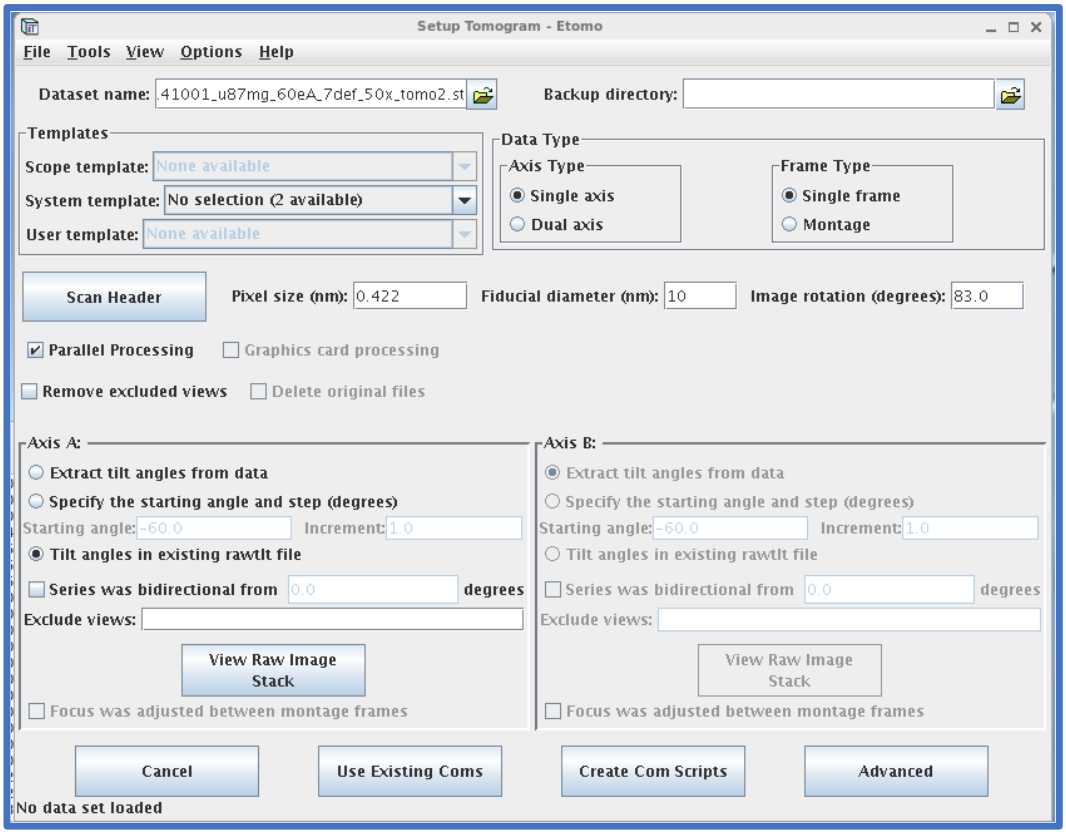
You will find two files:

1. ***u87mg_60eA_7def_50x_tomo2.st*** → tilt series as a stack of 2D images, acquired at different tilt angles,
2. ***u87mg_60eA_7def_50x_tomo2.rawtlt*** → a plain text file describing at what angle each 2D image was acquired.

Open up ETomo by typing:

```
> etomo
```

The front page panel of ETomo will appear. Press **Build Tomogram,** the **Setup Tomogram** panel will appear (shown below).



In this panel you will provide ETomo with information of our data.

Fill in the fields as follows:

- **Dataset name:** click on the folder icon near the text box and browse for the named ***u87mg_60eA_7def_50x_tomo2.st*** file.
- **Axis Type:** make sure the axis type is single axis. This should grey out the 'Axis B' section in the bottom right corner.
- **Pixel size/Image rotation:** click on the **Scan Header** button. This will look for the information in the header of the tilt stack and automatically fill in the pixel size in nm, 0.422, and image rotation degrees, 83.0, the expected tilt axis of the tomogram.
  IMPORTANT: please note that the image rotation degree of the raw tilt stack depends on the setup and magnification of the microscope, therefore in some cases can be closer to 0° (almost vertical), and in other cases, like here, close to 90° (almost horizontal). In any case, ETomo will automatically rotate final aligned stack for the output to be vertical.
- **Fiducial diameter:** this refers to the diameter of the gold particles used as fiducials to align the stack. In this tomogram they are 10 nm in diameter, so fill it in as required.
- **Axis A:** select **Tilt angles in existing rawtlt file** radio button to tell ETomo that the tilt angles for this stack are stored in the .rawtlt file. (note: ETomo will search for a file with the same base name as the tilt stack, but with a .rawtlt extension.)

Leave everything else as default. Backup directory can be left blank, Frame Type should be set to Single frame and Remove excluded views can be left unticked. No templates have been previously created, so the Template section can be ignored.

If you haven't already done so, you can look at the tilt series using the **View Raw Image Stack** button. This will open up 3dmod and two windows will pop up:

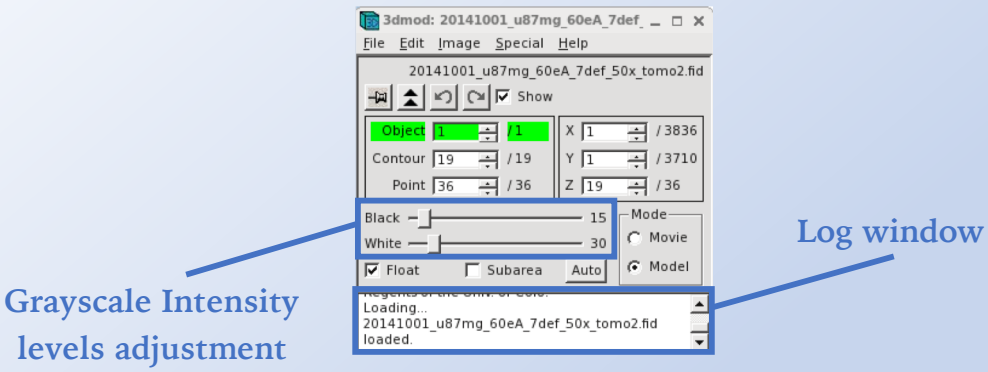
1. The "3dmod control window", a small window containing menus for performing file operations, selecting model editing functions, and opening various kinds of image windows.
2. the "ZaP window", which is a large window displaying the stack of images.

To scroll through the 2D slices of the raw stack use the cursor in the bar at the top of the ZaP window, or the **[pgUp] [pgDown]** keys, or right click anywhere in the ZaP

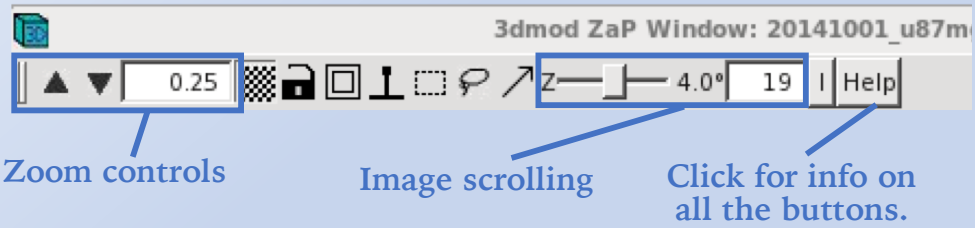
window to start the automatic scroll. Note that just closing the large ZaP window does not also close 3dmod (nor ETomo). In fact, you can re-open the ZaP window from the 3dmod control window by pressing “Z” or selecting “image>ZaP”.

3dmod

The control window is a small window containing menus for performing file operations, selecting model editing functions, and opening various kinds of image windows:



The “ZaP window”, a large window displaying the stack of images. Here is a brief description of key controls at the top of the window:



Click **Create Com Scripts** to start the reconstruction workflow, which will appear in a new window. **Create Com Scripts** will also trigger the creation of `.com` scripts in the working directory, that are necessary for advanced batch processing. Luckily, ETomo is a friendly user interface, that updates the `.com` scripts at each step of our reconstruction, allowing conventional users to ignore them.

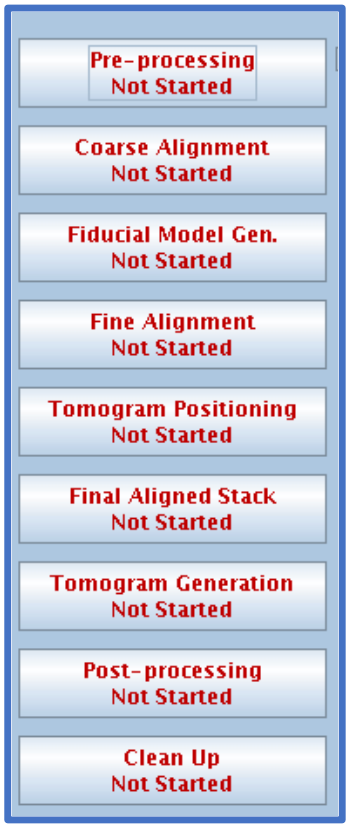
Common problems - setup

When working with your own data, you may run into one of these problems:

- The **Scan Header** does not fill out some fields or fills them out incorrectly, this usually happens when the header in your stack file is inaccurate. Always make sure these values are what you expect them to be. Getting them wrong can mess up a lot of the process, and IMOD might not be able to tell you why it is wrong.
- The **Parallel Processing** checkbox is greyed out. To fix this you will need to go to the options menu and select settings. Turn parallel processing on and set the # CPUs to a number appropriate for your system. Hit **Apply**. You will then have to exit eTomo and restart it.
- The `.rawtlt` file may not be provided. Therefore, other options for defining the tilt angles include attempting to read them from the tilt stack header (**Extract tilt angles from data**) or defining them manually (**Specify the starting angle and step and Series was bidirectional from…**). **Exclude views** allows you to ignore certain views, in case you might need to do that.
- You may have many more tomograms to reconstruct, all with similar parameters. One option is to set up a template, so that variables common to all the tomograms are filled in automatically. Details on how to setup a template can be found at https://bio3d.colorado.edu/imod/doc/UsingEtomo.html#Templates.
  Another option is to try automated batch tomography using *batchruntomo*. Details can be found **in** https://bio3d.colorado.edu/imod/doc/batchGuide.html.

The Reconstruction Workflow

On the left panel of the ETomo window, you should now see the pipeline of steps required to perform reconstruction. A brief description of each step is given below.



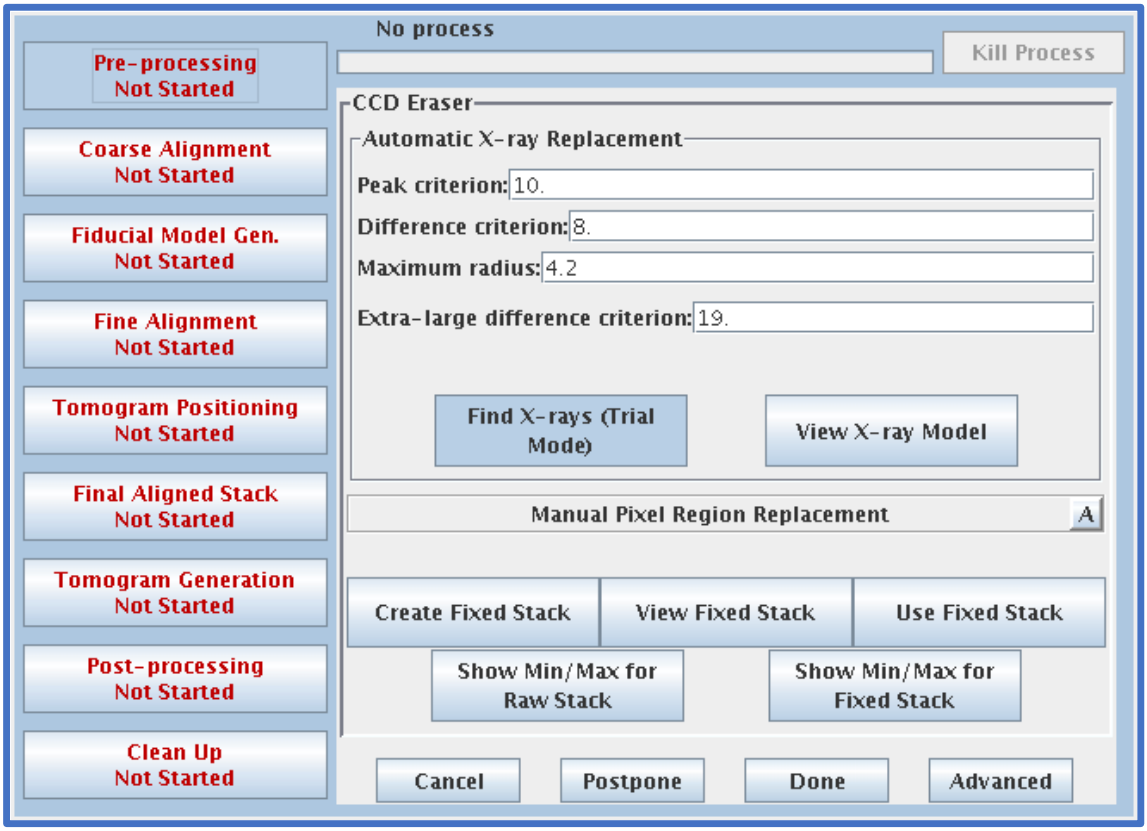
- Pre-Processing using *ccderaser*.
- Perform a rough alignment using cross-correlation.
- Generate an initial fiducial model.
- Refine the initial Fiducial model by minimizing residual error distances.
- Re-position the tomogram, in order to identify the appropriate tilting axis direction.
- Producing a final aligned stack.
- Reconstruct the actual tomogram.
- Trim and scale the tomogram.
- Remove intermediate files.

We will be working through each of these steps in order. Click on **Pre-processing** to continue.

1. Pre-processing

This step uses the IMOD program *ccderaser* to correct for pixels with unwanted extreme high or low intensity values, erroneously recorded by the detector during image acquisition. This may be due to a range of unpredictable events, including random X-rays in CCD images, glitches in CMOS camera, or other sources of noise. Unfortunately, these extreme values can cause artifacts not only in viewing and aligning but can also locally affect the quality of the reconstructed tomogram. Thus, if you are unsure whether not you need to perform pre-processing, it is always a good idea to perform it.

In this practical, however, the tilt stack does not seem have any major pixel correction to perform, so here pre-processing is not strictly needed. The procedure is anyways described below, but you can press **Done** and continue to the coarse alignment (next page).



The basic steps involved in *ccderaser* pre-processing are:

- Press **Find X-rays (Trial Mode)** to find potentially anomalous pixels.
- Press **View X-ray Model** to check whether or not are you satisfied with the selection of allegedly erroneous pixels.
- Press **Show Min/Max for Raw Stack** to run clip stats, a program which displays the minimum and maximum densities for each section, on the raw stack.
- Press **Create Fixed Stack** to create a second stack where X-rays are removed.
- Press **View Fixed Stack** to view that stack and again **Show Min/Max for Fixed Stack** to run *clip stats* on the fixed stack.

If the outlier pixels from the raw stack *clip stats* output are gone or improved from the fixed stack output, then press **Use Fixed Stack** and **Done**.

2. Coarse alignment

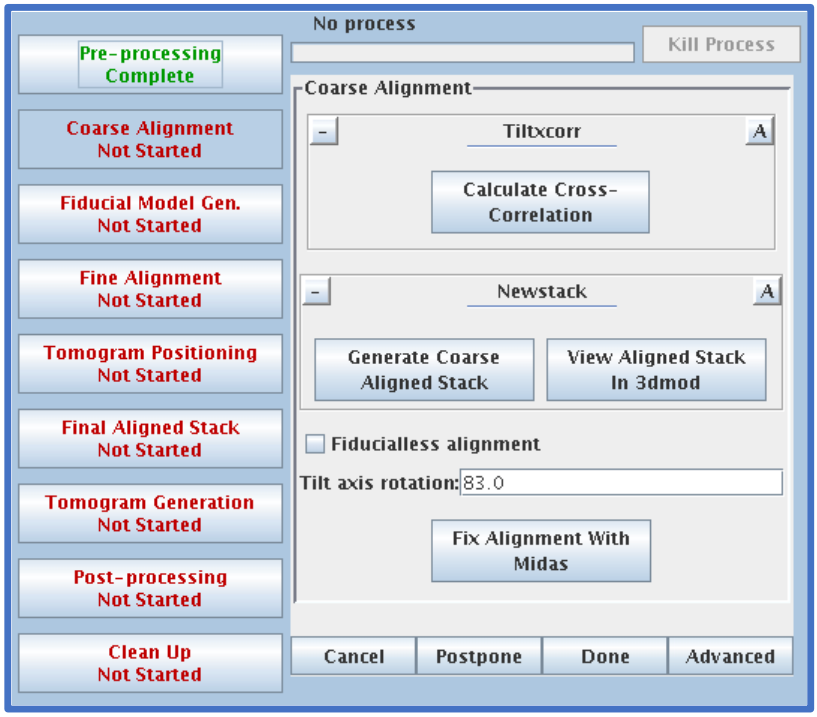
Summary

In this step, we are doing a quick alignment of our tilt stack using cross-correlation.

- Click **Calculate Cross-Correlation**
- Click **Generate Coarse Aligned Stack**
- Click **Done**

You can also click **View Aligned Stack** in 3dmod to check the alignment.

Press **Coarse Alignment** to create the coarse aligned stack. The default parameters are sufficient for aligning the tilt series in this practical. **Calculate Cross-Correlation** runs the program *tiltxcorr*. The program uses cross-correlation to find an initial translational alignment between successive images of a tilt series (i.e. just shifts in x and y). The output file will have the extension `.prexf`, and will be a list of transformations (recommended shifts) that will be applied to the image data in the next step.



Pressing **Generate Coarse Aligned Stack** will run two programs:

1. *xftoxg*, that takes the transforms created by *tiltxcorr* to obtain a single consistent or 'global' set of alignments.
2. These new transforms are then applied to the image data using the program *newstack*. The output file created has the extension `.preali`. To view the pre-aligned stack, press **View Aligned Stack in 3dmod**.

You should be able to see a smooth alignment among 2D slices (i.e. no large shifts between adjacent slices). Incorrect shifts can be fixed manually with the interactive program **Midas**, activated by pressing **Fix Alignment With Midas**. There is no need to do it in this data set.

If you are satisfied with the pre-aligned stack, press **Done** and proceed to the fiducial model generation to fine tune the alignment of the stack using gold fiducials.

Troubleshooting

- If something does go wrong with your coarse alignment, click the little **A** button in the top-right corner of the **Coarse Alignment** window. This will bring up the advanced options with an extensive set of parameters to play with. It is always possible to go back to the basic set of parameters by clicking the **B** button.
- The first step to fix bad coarse alignments is using the **Coarse aligned image stack binning** in the *newstack* advanced options.
- Sometimes you may need extra information regarding advanced options. Keep in mind that all of the programs mentioned here are IMOD commands that can be run in a terminal. For example, typing:

➢ `tiltxcorr`

will start the *tiltxcorr* program.

IMOD commands have also exhaustive manual pages. For instance, typing in a terminal:

➢ `man tiltxcorr`

will tell you the list of mandatory and optional parameters that can be used. More information about the algorithm used is also provided. Press **q** to leave the manual page.

3. Fiducial Model Generation

Summary

In this step, we are locating the positions of our gold fiducials, so we can use them to do a more accurate alignment in the next step.

1. Make sure the **Make seed and track** and **Make seed model manually** options are selected. Click **Seed Fiducial Model**.
2. In the Beadfixer dialog box that opens, make sure to check the **Automatic new contour** option.
3. Use the middle mouse button to pick as many gold beads as you can from a low angle tilt image (the gold beads are the black dots disperse in the image). Save the model file (**File ➜ Save Model**, or type **s**) and close 3dmod.
4. Switch to the Track Beads tab and click **Track Seed Model**.
5. Click **Fix Fiducial Model**. In the Bead Fixer window, click **Go To Next Gap**. If you see a down arrow over the bead in the ZaP window, press **[Page Down]** and use the middle mouse button to add the missing point. If you see an up arrow, click **[Page Up]** instead.
6. Repeat the previous step until the no more gaps are found message appears. Save the model file (**File ➜ Save Model**, or type **s**) and close 3dmod.

Gold beads are excellent landmarks to be used as fiducials for aligning a 2D tilt projections stack. This is because gold beads are approximately spherical, homogeneous and isotropic. They appear as high contrast dark disks even in projections acquired at high tilt angles. Therefore, the center of each 2D disk can be easily identified and tracked throughout the tilt projections stack. The high contrast of gold beads is due to their greater electron density compared to the rest of the sample, which is vitrified biological matter.

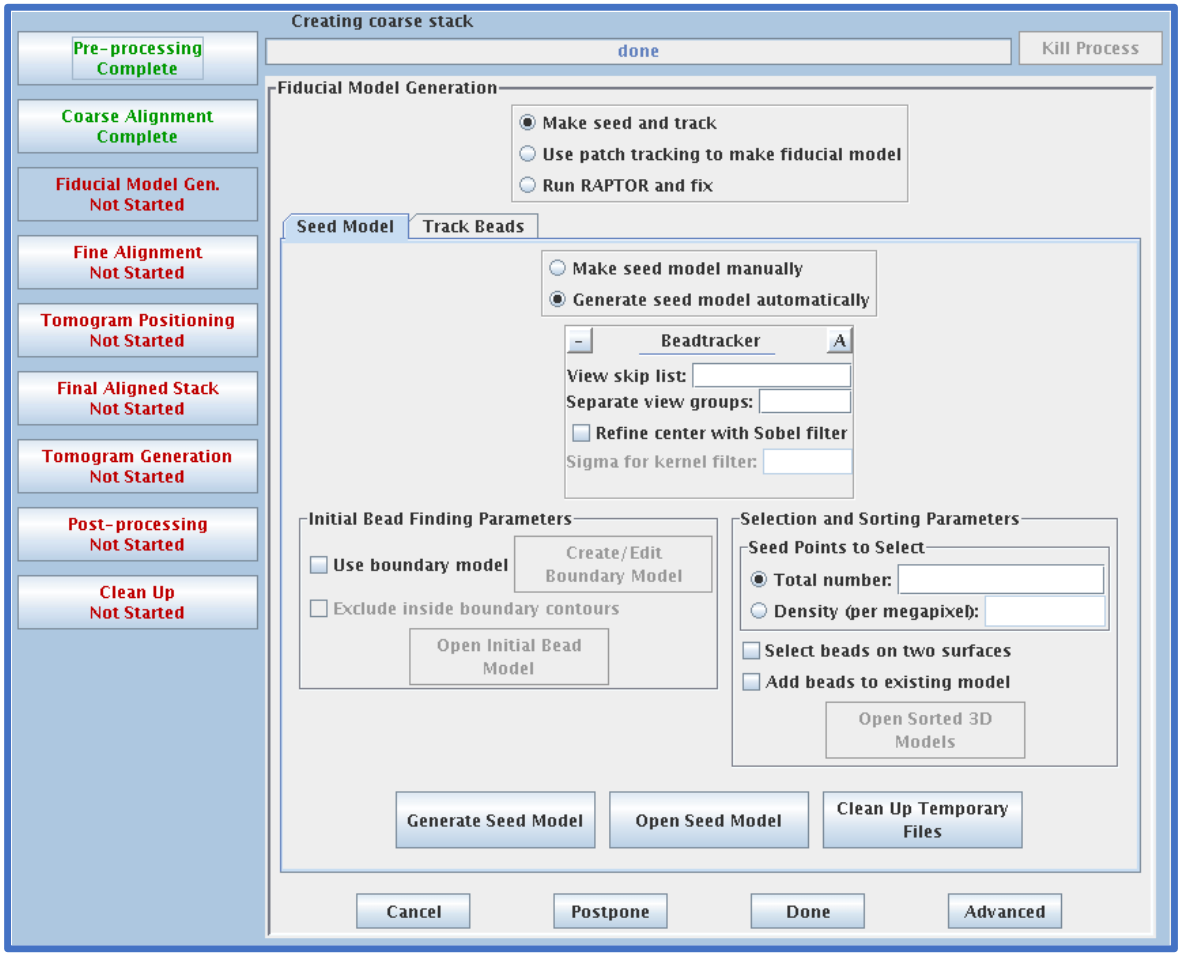
The aims of this step are:

1. locate gold bead fiducials in the lowest tilt image of the stack (near zero degrees), to create a ‘seed’, or starting positions, useful for the creation of an initial fiducial model (**Seed Model** tab).

2. Create an initial fiducial model. Track the same beads in all the other tilt images, based on the angle information and on their distance from the tilt axis (**Track Beads** tab). The resulting initial fiducial model will be refined in the next step.

There are the three alternatives to create an initial fiducial model:

- **Make seed and track:** this is the standard method for creating fiducial models using gold beads, or any other highly electron dense, spherical object.
- **Use patch tracking to make fiducial model:** this is the method used when there are no beads available. Patch tracking cross-correlates overlapping patches from your tilt images to build a model. This method is generally more difficult and prone to problems. It is almost always better to use beads.
- **Run RAPTOR and fix:** this is an old program to automatically find and track gold fiducial markers through the tilt series. We will ignore this option, because the IMOD fiducial model generation works fine enough for our purposes.



Make sure the **Make seed and track** radio button is selected.

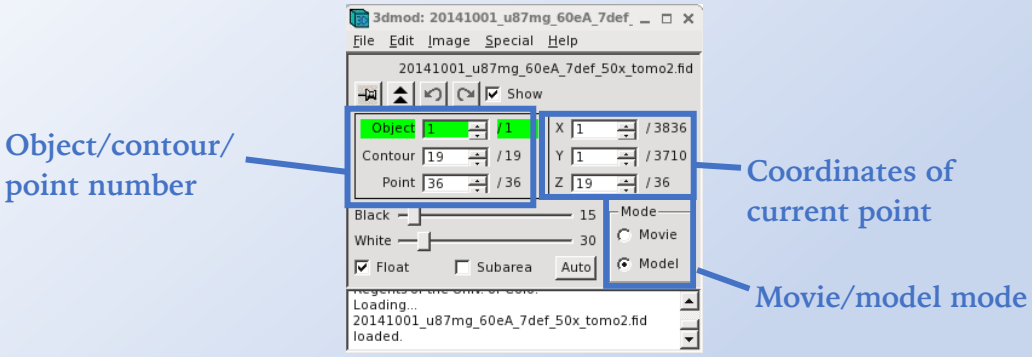
In the **Seed Model** tab there are two options for making our seed model:

1. **Make seed model manually** and
2. **Generate seed model automatically**. We will be doing this manually, but the procedure for generating the seed model automatically will also be described.

Select **Make seed model manually** and click **View Seed Model**. 3dmod will open and three windows will appear: the big ZaP window, that displays the image stack, the 3dmod control window, and the **Bead Fixer** dialogue box. In this new window, check the **Automatic new contour** option. This will generate a new contour for every point in a new position. The rest of the parameters can be left as default.

3dmod model mode tips

When making a model in 3dmod, everything you picked is grouped into a hierarchical structure. Your **Points** are grouped into **Contours**, and your **Contours** are grouped into **Objects**.



- **[Page Up]** and **[Page Down]** move you through the image stack.
- **Left-click** the mouse to select the nearest point.
- **Left-click and drag** to pan the image around.
- **Right-click** to reposition the current point.
- **[Ctrl + right-click]** to delete the current point.
- The **[Up]** and **[Down]** arrow keys step the zoom factor up and down.
- Press **'V'** to see a 3D view of the models.

In the ZaP window, scroll to the slice closest to the 0° angle and select as many gold beads as you can by using the middle mouse button. They appear as dark disks in the image. Zoom in and out to facilitate selection.

Try to pick at least **10** gold beads evenly distributed over the whole field of view. The more you have, the better your alignment will be, even if more beads also mean more work to align all of them.

Once you are done picking gold fiducials for the seed model, save it (**File ➜ Save** or type **S**) and close 3dmod.

A brief overview of automatic picking

Selecting **Generate seed model automatically** will allow you to use the *autofidseed* program to automatically find your gold beads.

- In the Selection and Sorting Parameters window, enter '20' in the Seed Points to Select, as the Total number of gold fiducials to find. Make sure **Select beads on two surfaces** is checked.
- Click **Generate Seed Model** to automatically detect most of the gold particles on top and at the bottom of the section.
- When done, click Open Seed Model to display the gold particles selected in the tilt series.

Autofidseed does a good job on most occasions, but it will struggle with beads near the edge of the image and overlapping beads.

Now click on the **Track Beads** tab and **Track Seed Model** button. This will run the *beadtrack* program to find the previously picked gold beads in all other tilt images. The resulting fiducial model needs to be manually refined, to correct for incomplete tracking by either deleting a fiducial from the model or completing the tracking.

If bead tracker is unable to find any of the beads through the tilt series, they will be listed in the ETomo Project log file. If you have any gaps, before moving to **Fix Fiducial Model**, you can try the **Track with Fiducial Model as Seed**, as this may automatically fill in some of the gaps.

Press **Fix Fiducial Model** (even if you have no gaps you should still use this option). This procedure will display magenta and green points, corresponding to the position of

the picked gold beads either on top or at the bottom of the tomogram. You can switch to **movie mode** in 3dmod and click the middle mouse button to scroll through the tilt series and check that *beadtrack* program has worked. Then, get back to **model mode** in 3dmod before continuing.

The **Bead Fixer** dialog box will appear in **Fill gaps** mode. **Go to Next Gap** (or **[Spacebar]**) will bring you to the first gap. In the ZaP window you will see a point highlighted with a yellow circle and a yellow arrow indicating the adjacent section that has a missing model point. Using the keys **[Page Up]** for the arrow pointing up, or **[Page Down]** for the arrow pointing down and find the slice where the point is missing. Middle mouse click in the center of the gold particle will add the missing point. It is useful to increase the magnification of the image with the **[+]** key and adjust the contrast on the sections, especially at high tilts or at the edge of the slices. In case the missing point ends up being outside of the image, the easiest fix is to delete that contour by going back to the section where the point is selected in yellow and click Edit Contour Delete in the 3dmod control window, or type **[Shift+d]**.

It is helpful to open the **Bead Helper** from 3dmod main window by clicking **Special ➜ Bead Helper**. This will trace a trajectory line for each contour of picked gold fiducials, to better visualize the fiducial model and spot misplaced or wrongly placed contours right away.

Repeat this procedure for all missing points in the fiducial model by clicking on **Go to Next Gap** until the message, 'No more gaps are found' comes up in the main 3dmod window. Repeat this procedure for any other missing points in the fiducial model by clicking on **Go to Previous Gap** until the message, 'No more gaps are found' comes up in the main 3dmod window.

Press **[V]** to open a 3D view of the models – they should be visible now as smooth curves. If any point seems out of position, select it by left-clicking on it in the ZaP window, then use right-click to reposition it. Once done, save the model file (**File ➜ Save** or type **S**), close 3dmod, and click the **Done** button to advance to the fine alignment step.

4. Fine Alignment

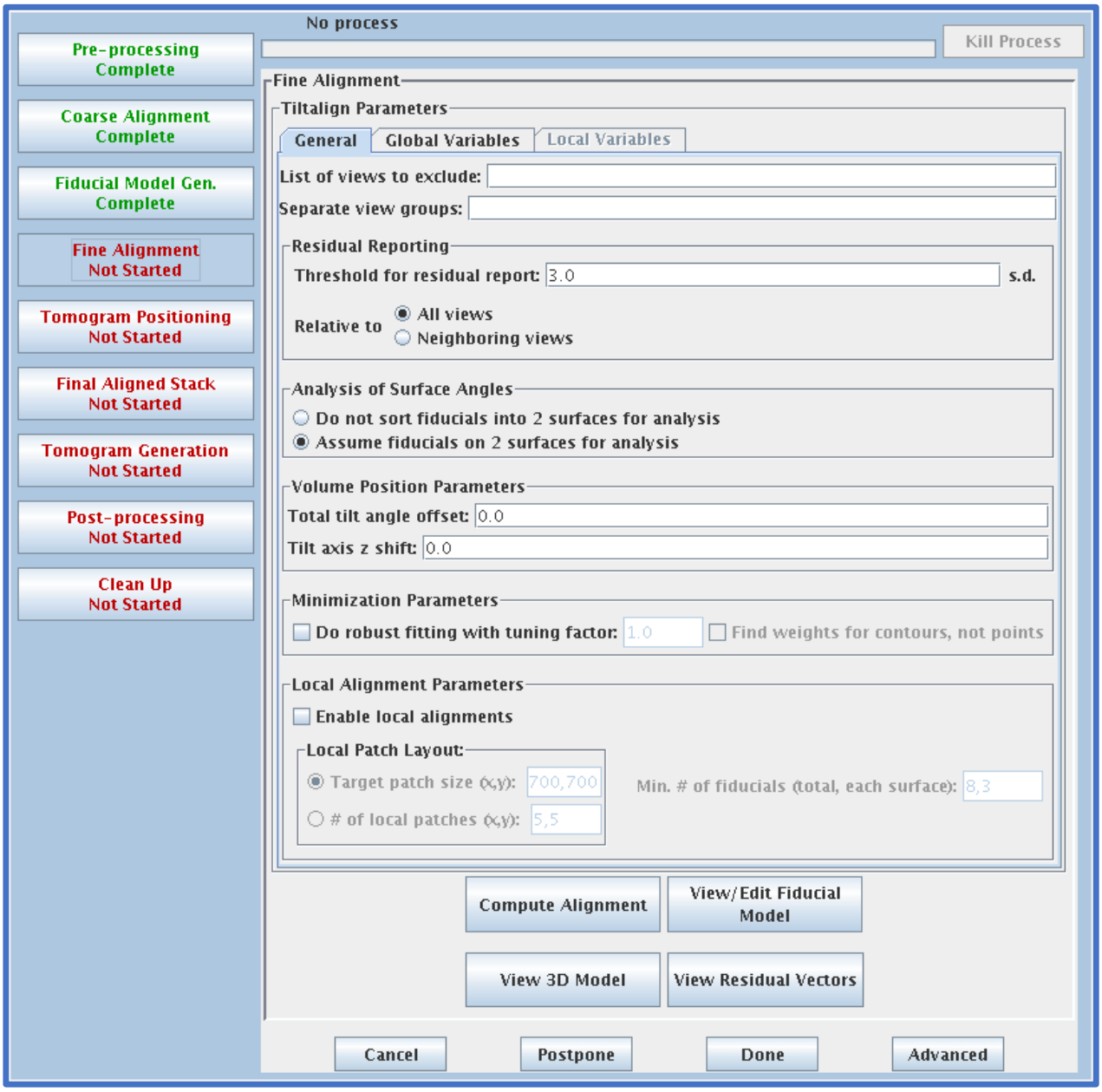
Summary

In this step, you will use the fiducial model created to align as precisely as possible the tilt stack.

1. Click **Compute Alignment**.
2. When the alignment is computed, click **View/Edit Fiducial Model**.
3. In the Bead Fixer dialogue box, click **Go To Next Big Residual**.
4. Click **Move Point by Residual**. If this produces an inaccurate shift from the center of the gold fiducial, then manually adjust it using the right mouse button (zoom if necessary).
5. Repeat the steps 3 and 4 until the no more residuals are found.
6. Press the **Save & Run Tiltalign** button in the Bead Fixer dialog box. In the Project Log window, check the mean residual error has decreased. The aim is to have a mean residual error between 0.6 and 0.2.
7. Repeat steps 3-6 until the mean residual error stops decreasing, or there are no more residuals.
8. Save the model file (**File ➜ Save** or type **S**) and close 3dmod .

In the previous step you used gold beads as landmarks for creating an initial fiducial model. In this step you will use the "residual" measure of error to refine the alignment. In fact, IMOD assigns to each fiducial in each projection a value called "residual", which represents the distance in pixels between the expected and the actual position of the fiducial. In general, the smaller is the residual error, the better is the alignment.

The **Fine Alignment** panel is organized within three tabs, each containing the parameters for different type of alignments. Ensure that the **Assume fiducials on 2 surfaces for analysis** option is selected in the Analysis of Surface Angles menu box. A general alignment is done when you press **Compute alignment** at the bottom of the Fine Alignment box.

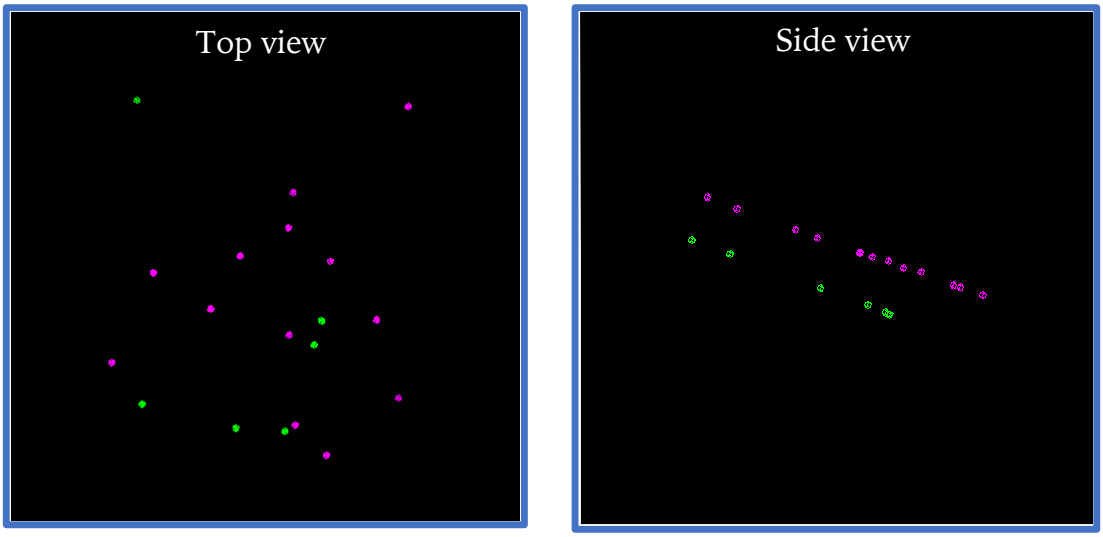


This runs the program *tiltalign* to solve for the displacements, rotations, tilts and magnification differences in the tilted views. The program uses the position of the gold beads in the fiducial model and a variable metric minimization approach to find the best fit. The residual error and the standard deviation for the alignment solution are also displayed in the ETomo Project Log file (this log window opens when ETomo starts).

tiltalign also creates two model files that provide useful information about the fiducial model. The first file (***20141001_u87mg_60eA_7def_50x_tomo2.3dmod***) displays a 3D model of the fiducials based on their solved positions. Fiducials are represented as

magenta and green spheres according to which surface they are located. Examine this model by hitting the View 3D Model button on the bottom of the Fine Alignment box:

You should see a nice distribution of magenta and green spheres across the field of view. Rotate the model from the top view to the side view by toggling between the **[F]** and **[T]** keys. You will see the separation of the two surfaces with this view. Close the 3dmod window.



If the distribution of the gold beads is fairly even, you can move to the **Global Variables** tab and select the **Full solution** for Distortion Solution Type. This option will allow the program to correct for both X-axis stretch and Skew distortions.

Note that solving for distortion effects can lead to improper, overfitted solutions, and should only be used if you think it is really necessary. You can find more information in the IMOD website: https://bio3d.colorado.edu/imod/doc/tomoguide.html#FINAL%20ALIGNMENT. There is no need to use it in the specific case of this tomogram.

Once done, go back to the previous tab (**General**) and click **Compute alignment** again.

Press **View/Edit Fiducial Model** to reload the fiducial model for editing. This will bring up the Bead Fixer dialog box in Fix big residuals mode (see below) and load the relevant details from the align.log file (reported in the 3dmod dialogue box below).

The goal of the fine alignment step is to reduce the mean residual error to a subpixel value (<1 pixel). The following iterative steps involve fixing fiducial points with large residuals:

- Click **Go to Next Big Residual** in the Bead Fixer dialog box. This will select the model point that had the biggest residual and you may see that it is not centered properly on the gold bead. You can also see a red arrow pointing in the direction of the recommended move (if not, use the zoom controls in the top left corner of the ZaP window or press **[+]** key until it is big enough for you to see).

- If you click **Move Point by Residual** in the Bead Fixer dialog box, it will move the model point by the recommended amount. This works most of the time, but if the suggested position looks wrong, you can move it by manually by centering the cursor in the center of the gold bead and then clicking the right mouse button to shift the point in the right position.

Repeat these two steps until the **Go to Next Big Residual** shows that no more residuals are found in the 3dmod control window. At this point you can save the model by clicking on **Save and Run Tiltalign** in the Bead Fixer dialogue box.

If you check the ETomo log file you should see that the residual and standard deviation have decreased. Repeat this process until there are no more residuals or until the mean residual error does not decrease any further. Save the model (**File ➜ Save** or type **S**) and close 3dmod. Then press **Done**. If ETomo asks you if you want to save the model select 'yes'.

Hints for a better fine alignment

In general, the smaller the residual error, the better the alignment. Please note that the mean residual error is an integrative measure of self-consistency of the fiducials model. This means you can have a subpixel mean residual error (<1.0) and yet an unaligned model. For example, this may be the case if you click **Move All by Residual**.
Furthermore, you may have a good subpixel alignment and yet a high mean residual error. This may happen if some of your gold beads sit in unstable locations, like at the edge of your grid, and move during acquisition.
Moreover, the mean residual error tends to increase proportionally to the number of gold beads used.

The take-home messages are:

1. always check what gold bead are you picking

2. never use **Move All by Residual**, unless you have an extremely good reason to do so, and

3. think of the mean residual error as a relative indicator of how your alignment is progressing, rather than an absolute indicator of how your alignment is.

5. Tomogram Positioning

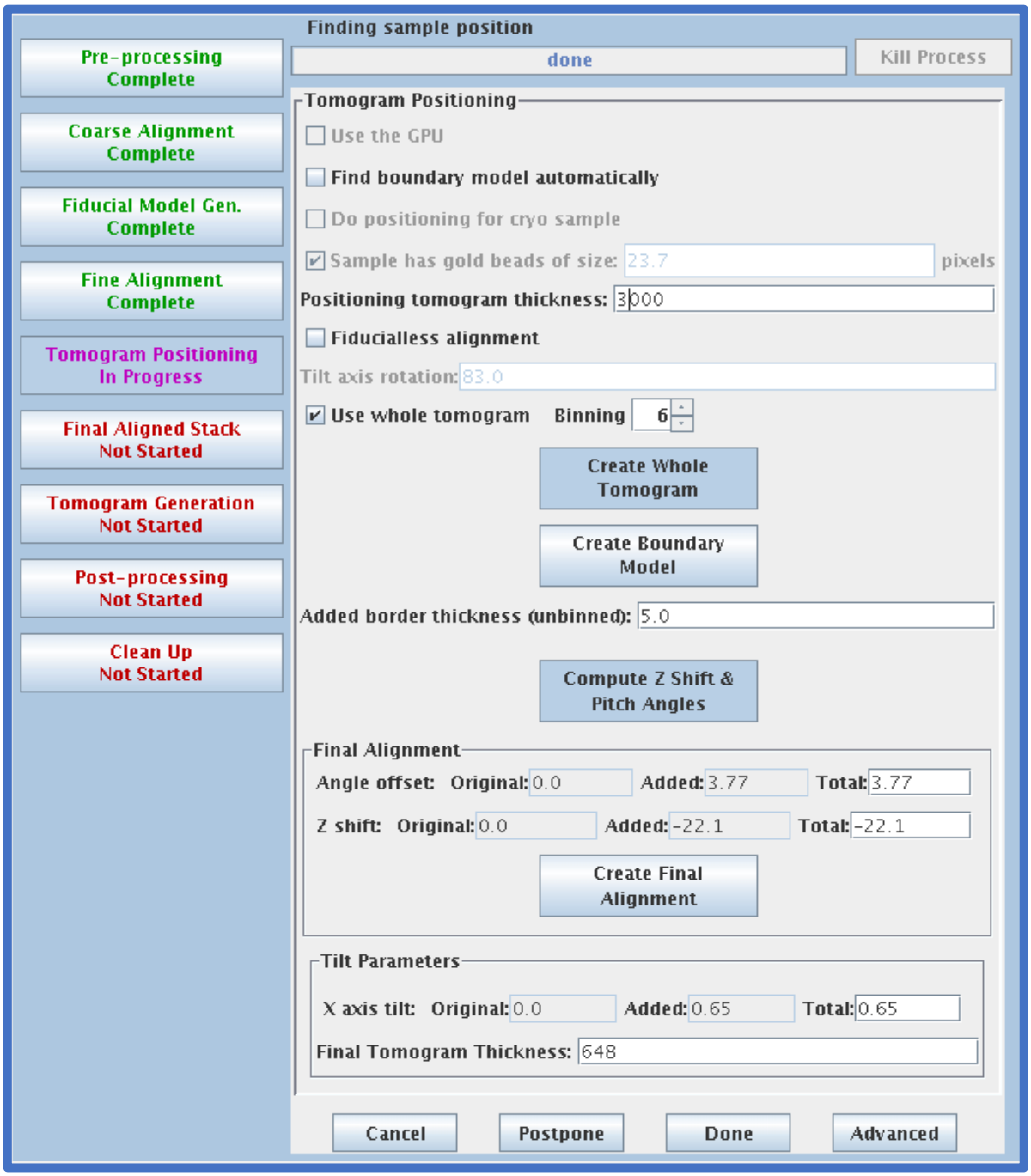
Summary

1. Enter '3000' in the **Positioning tomogram thickness** box, to have a very large view of the tomogram position.
2. Make sure **Use whole tomogram** is selected, and **Binning** is set to '6'.
3. Click **Create Whole Tomogram**. Wait until this is complete, then click **Create Boundary Model**.
4. In the opened 3dmod window, go to "image>XYZ" or press **[Ctrl-X]** to open the XYZ view. Use this to create 12 model points (4 per each position at the top, middle and bottom) using the middle mouse button. The points should form 2 parallel lines in each position, 3 above and 3 below the tomogram. Save the model (**File ➜ Save** or type **S**) and close 3dmod.
5. Click **Compute Z-shift and Pitch Angles**.
6. Click **Create Final Alignment**.
7. Click **Done**.

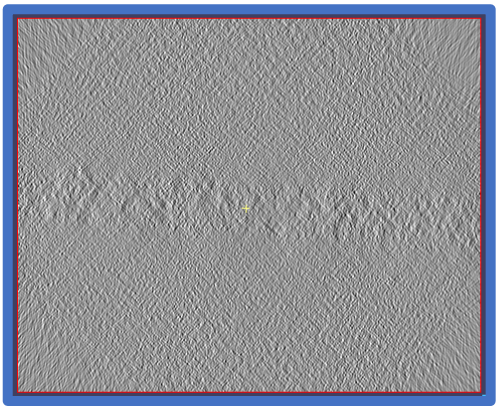
In the previous step, you probably noticed that the position of the gold beads in the side view is slightly tilted in respect of the coordinate axes. In this step, we are calculating the shifts and angles needed to re-position the tomogram correctly in the coordinate space, thus identify the exact tilting axis direction.

Make sure the **Positioning tomogram thickness** is set to a very large number, like '3000'. This number describes the side of a cubic volume (voxels) that will surround the tomogram, you want it much larger than needed to better locate the tomogram data. Check the **Use whole tomogram** option and set the **Binning** to '6'. Click on **Create Whole Tomogram** and wait for it to complete. It may take a while and the loading bar will get completed twice: first to make an aligned stack and second to create the actual tomogram.

Next click on **Create Boundary Model**. This will open 3dmod and give you a first glimpse of the actual tomogram. Note that it is binned, and therefore much smaller (and with higher contrast) than your final tomogram will be.



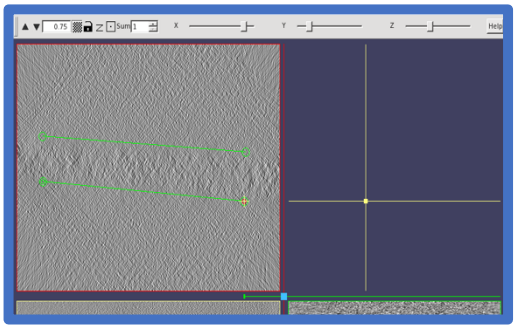
The figure on the right shows the tomogram viewed from the side (i.e: the view of the tomogram when the Y axis is perpendicular to the page). The mottled band across the center is the actual tomogram, and you can easily see that it is not perfectly parallel to its bounding box.



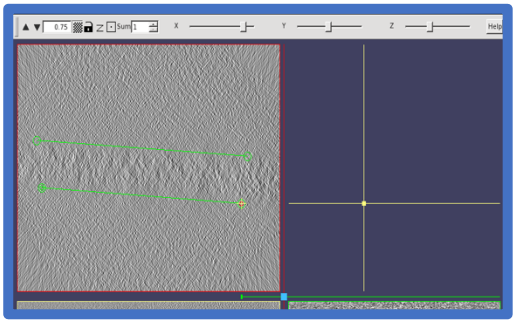
To allow ETomo to correct for that, press **[Ctrl-X]** in the ZaP window to open an XYZ view. Play with the sliders at the top to view different slices of the tomogram from the three different axes, X, Y and Z.

If the tomogram data is not easy to locate, close 3dmod and increase the **Positioning tomogram thickness** value. As before, click **Create whole tomogram** and, once done, **Create Boundary model** to have a larger bounding box. You can also increase the value of "Sum" in the XYZ view, this will average several adjacent views, increasing their signal-to-noise-ratio (SNR).

In this step of the reconstruction, 3dmod expects you slice the tomogram until one of its ends and create 2 horizontal lines, to delimit the actual tomographic data (as shown on the right).

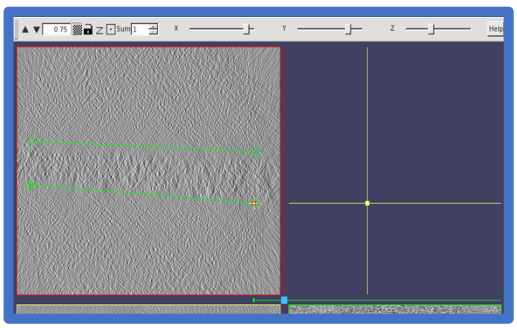


In the top left window of the XYZ viewer (XZ plane), use the Y-slider to go to one end of the tomogram and middle click to place two connected points above and two below the data (they will be automatically connected in pairs as different contours).



Once done, slice the tomogram somewhere in the middle and, placing points as before, create another two lines to delimit the tomogram.

Finally, slice the tomogram to the other end and repeat this step for the last time.



You should now have 6 lines that delimit the top and bottom of the tomographic data in 3 different positions of the tomogram (end, middle and opposite end), using a total of 12 model points and 6 contours (2 points in each contour).

Once done, save the model file (**File ➜ Save** or type **S**) and close 3dmod.

Click **Compute Z Shift and Pitch Angles** to find the pitch of the section on the three regions using the IMOD program *tomopitch*. *Tomopitch* analyes simple models of the boundaries of the section in slices from a tomogram and recommends how much to change tilt angles to make the section flat, how much to shift the tilt axis in Z to produce centered slices, and how thick to make the slices. It can also recommend how much X-axis tilt is needed to make the section flat in the orthogonal direction as well. It can also be used with a model drawn on a whole tomogram, possibly binned down.

Press **Create final alignment** to apply these values to the alignment solution.

Finally, press **Done**.

. Final Alignment

Summary

In the final alignment step, the final aligned tilt stack will be created, last alignment step before the final tomographic reconstruction. Although there are multiple useful options here, we will be skipping some of them for the interests of time.

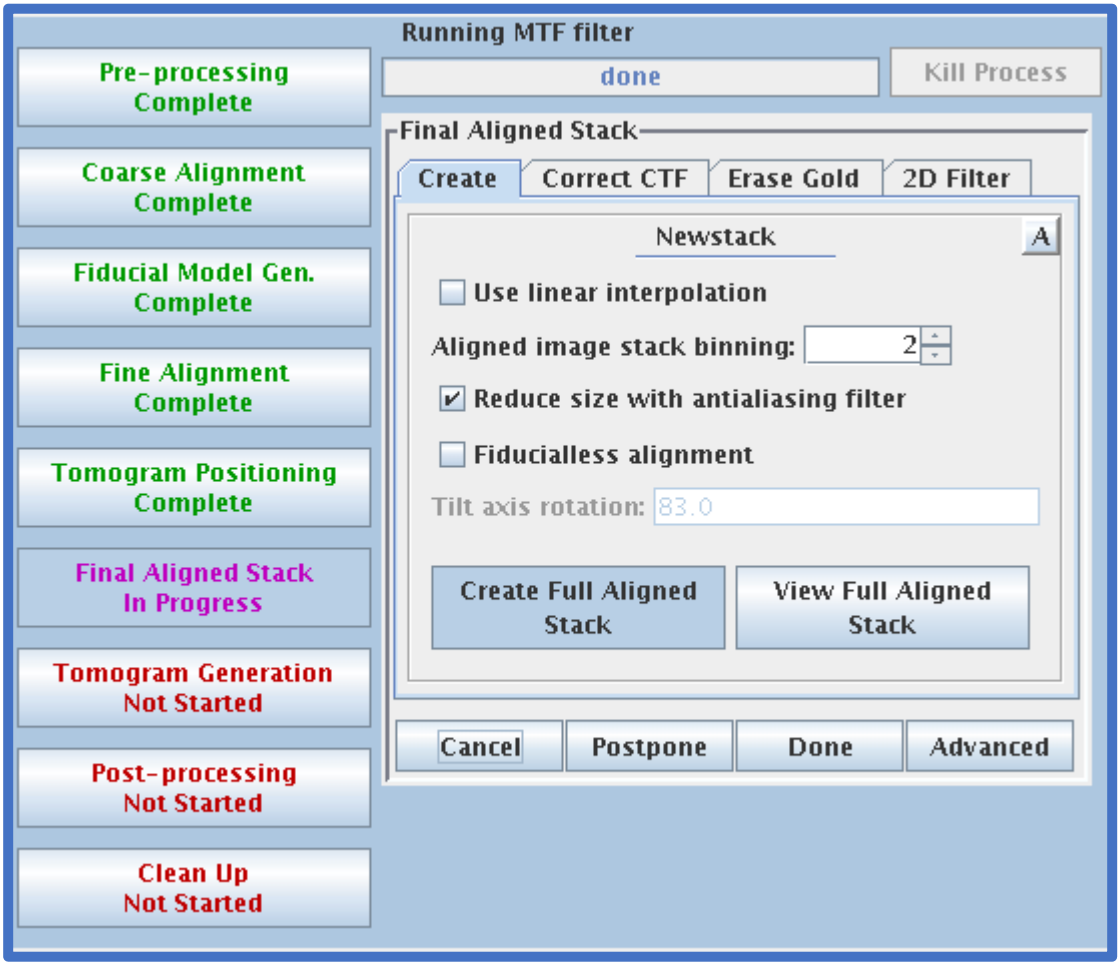
1. In the **Create tab**, change the **Aligned image stack** binning to '2'.
2. Turn on the **Reduce size with anti-aliasing filter**.
3. Click **Create Aligned Stack**.
4. Click **Done**.

If you want to know more about the other options, check the IMOD tomography guide (https://bio3d.colorado.edu/imod/doc/tomoguide.html#FinalAligned). For more info on how to use *ctfplotter* to correct for CTF effects, check out the CTF guide as well (https://bio3d.colorado.edu/imod/doc/ctfHelp/ctfguide.html).

The final alignment step will finalize and create the best aligned tilt stack to be used in the tomogram generation step.

You may consider binning the data, to reduce the size of the reconstructed tomogram and to speed up the actual reconstruction. Binning the data may also be recommended if your aim is to quickly inspect your reconstruction. In fact, binning increases the SNR, and the contrast of the reconstructed volume. However, binning reduces the maximum resolution you can achieve with your data. Thus, the decision of whether or not you may want to bin the data depends on the aim of your investigation.

For this practical, we bin the tomogram by a factor of **2**, in the interest of time. To do so, change **Aligned image stack binning** to '2' and make sure that **Reduce size with antialiasing** filter is selected.



Finally, click **Create Full Aligned Stack** and wait for program to finish running. Once done, click **View Full Aligned Stack** to open 3dmod and have a look.

Click **Done** to finish this step and move to the tomogram generation.

However, further processing of final aligned stack may be important for obtaining higher quality tomograms. For example, it has been shown in literature that local regularization of tilt projections can reduce artifacts in electron tomography. Contrast transfer function (**CTF**) correction may also be useful if you are aiming to achieve high resolutions. As you might have noticed, there are three more tabs after the **Create** one. Although we will be skipping them in this practical, they contain useful functions for cryo-tomography reconstruction and it is worth to know what each of them does.

Below is a brief description of each one.

Correct CTF: this tab leads to the *ctfplotter* program, where the CTF correction is calculated needed to compensate for defocus-based aberrations. Note that this is slightly different to CTF correction for single particle images, as it also deals with the variation in defocus across the images in the stack caused by the tilt. Note that the version of IMOD we are using here, and older versions, require a config file containing the locations of noise images. Newer versions of IMOD (4.10 onwards) have eliminated this requirement. For more information on CTF correction and using the *ctfplotter* user interface, visit: https://bio3d.colorado.edu/imod/doc/ctfHelp/ctfguide.html.

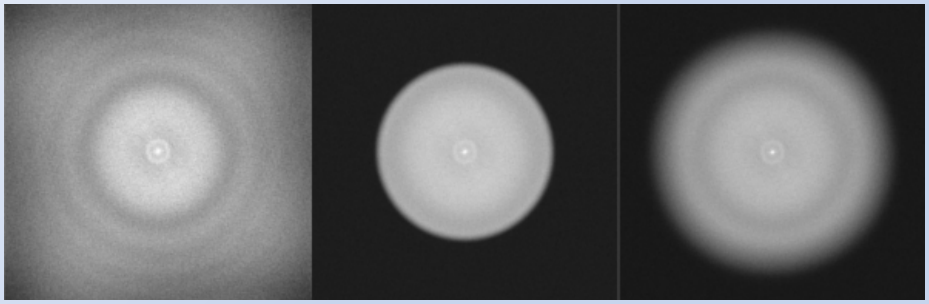
Erase Gold: gold nanoparticles are extremely useful in aligning the tilt stack. However, they are highly electron dense, thus they can hide proximal biological structures and degrade the overall quality of the reconstructed tomograms. This tab will locate gold beads and replace their intensity level with a constant value (either the average value of surrounding pixels or the mean intensity value of the whole image). As a result, this process drastically decreases the contribution of artifacts in the reconstructed volume. A more general result can be achieved using the IMOD command *preNID*, which aims to reduce gold-related artifacts without erasing the gold particles.

2D Filter: allows you to apply pre-reconstruction low-pass filters to each image in the tilt stack. See the '2D Lowpass Filtering in IMOD' box below to see what the filtering parameters mean. However, IMOD provides more effective pre-reconstruction filters, such as *preNAD* (see `man preNAD` for a more accurate description).

2D Lowpass Filtering in IMOD

Low pass filters in IMOD require you to input two values. The first number is the cutoff – frequencies higher than this value will be attenuated. The second number is the sigma value for the Gaussian dropoff – this describes how 'quickly' the attenuation occurs. This is needed because a sharp cutoff in Fourier space leads to odd artefacts in real space. The cutoff value can be translated into Ångstroms by dividing the pixel size of your image by the cutoff. For example, if your pixel size is 4.22, a cutoff value of 0.35 means you are attenuating frequencies higher than ~12Å (4.22/0.35). A cutoff value of 0.5 is therefore the same as the Nyquist limit of your image.

Below are three Fourier transforms of an image showing the effects of different filtering values.



No filtering Cutoff 0.25 Cutoff 0.25

7. Tomogram Generation

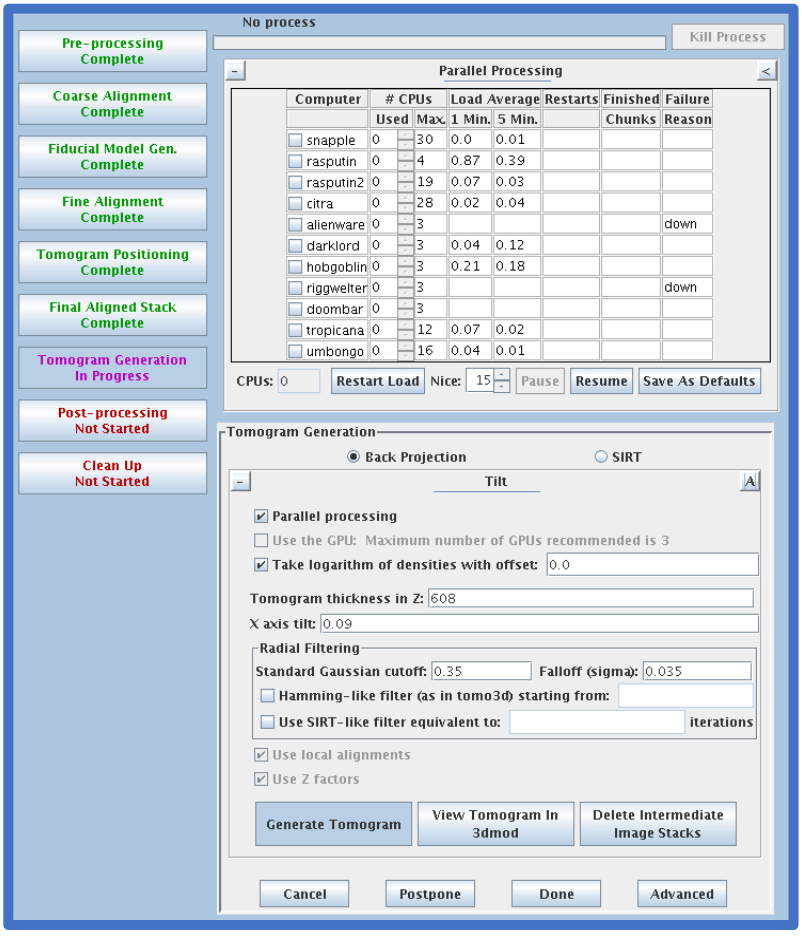
Summary

A few final adjustments to scaling and position.

1. Deselect **Convert to bytes**.
2. Click **Trim Volume** and wait for it to finish.
3. **Click Done.**

In the tomogram generation step, most of the parameters will be left as default.

The **Parallel Processing** section at the top displays a list of computers available to run the computationally demanding reconstruction algorithm, along with some information about the computational load on each computer. This can be useful for SIRT reconstruction, as we are planning to perform Back Projection reconstruction, this option can be ignored.



In the **Tomogram Generation** box there are two options for the reconstruction algorithms available: Back Projection and SIRT. As mentioned above, today, we will be using back projection, so make sure that it is selected.

The **Take logarithms of densities with offset** option is used to reduce the range of density values in your 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. Deselecting it increases the dynamic range of the density but can sometimes cause it to take values that are too large – if this happens, a warning will pop up telling you so. For this tomogram, either option should work fine.

The **Tomogram Thickness in Z**, **Z shift** and **X axis tilt** options should already be filled in, since these values were calculated in the **Tomogram Positioning** step, therefore there is no need to change them.

The **Radial Filtering** options are related to the final filtering of the tomogram. It is fine to leave them as they are, or you can play with parameters to see different results.

For instance, leave everything as default and click **Generate Tomogram**. Wait for it to finish, then click **View Tomogram in 3dmod** to see the final tomographic volume

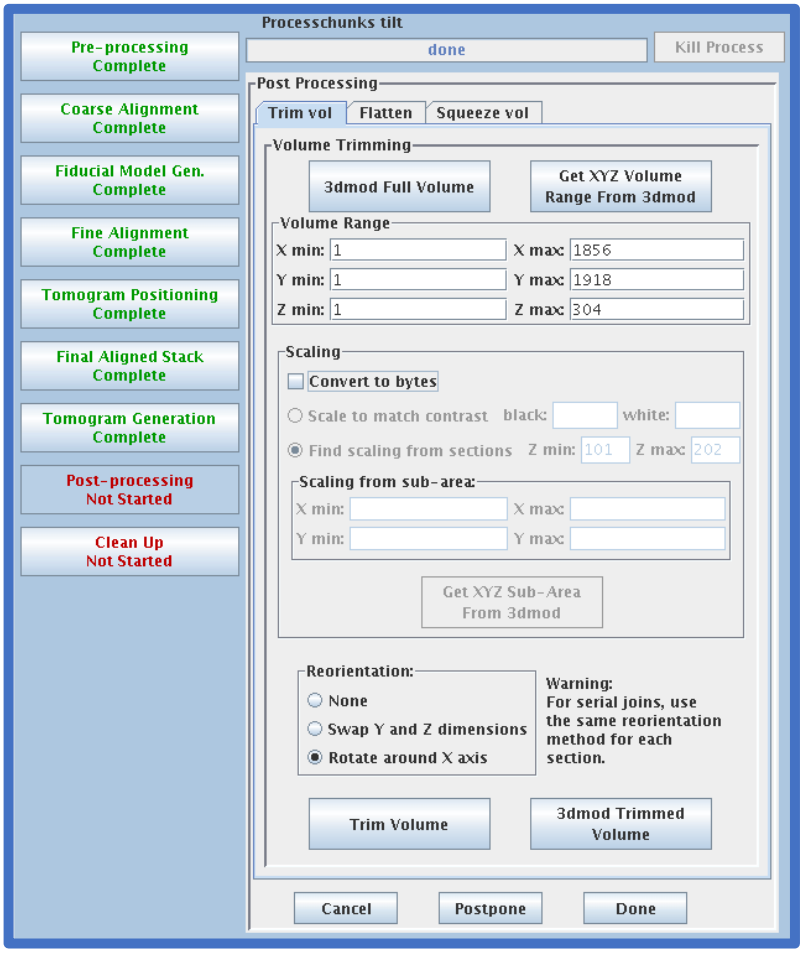
reconstructed. However, you may not be satisfied with the contrast of the reconstructed tomogram. Set the Use SIRT-like filter equivalent to option to 5 iterations and click again on "generate tomogram". This mimics a low iteration SIRT reconstruction, and acts similarly to a low-pass filter, thereby increasing the contrast at the expense of some high frequency information.

Once you are satisfied with the reconstruction, click **Done** to move to the post-processing.

8. Post-processing

This step allows to apply some final modifications to the final tomogram. We will ignore the **Flatten** and **Squeeze vol** tabs, as those options are particularly helpful when reconstructing serial sections, and not necessary in cryoEM.

The **Trim vol** tab gives you the option to trim the tomogram to focus on the information you want. Since we are interested in all the data within our region of interest, this is not necessary for us. Step through the reconstruction and determine the X,Y and Z ranges for the final volume.



A convenient way to set the X and Y range is to turn on the **rubberband** tool (the dashed rectangle in the toolbar of the Zap window), press the left mouse button over the upper left corner of the desired area, and drag the mouse to the lower right corner. Z can also be set, if desired; press **Lo** in the Zap window to set the minimum Z and **Hi** to set the maximum Z. When you press

Get XYZ Volume Range from 3dmod, Etomo will retrieve the X and Y values of the **rubberband** (and Z values if they are set) from 3dmod. Trimming the volume is performed by the IMOD command *trimvol*.

The **Scaling** section allows you to convert your tomogram from integer or float values to byte values, thereby reducing the dynamic range of your tomogram, and impair further processing of the data. The only reason to do this is if you want to save disk space (tomographic data can end up being very heavy and this can sometimes be an issue). We are not using this option, so deselect the **Convert to bytes** option.

The final box is for **Reorientation**. The default reorientation option will rotate the final volume around the X axis so that it can be read in easily by 3dmod and other programs without special options. Leave this option. Click **Trim Volume** and wait for it to finish.

You can now click **3dmod Trimmed Volume** to see your final, completed tomogram. Click **Done** to move to the very final step, which is a clean up of the intermediate files created in every step of the reconstruction.

9. Clean up

Summary

Final step to delete all intermediate files created during every step of the reconstruction.

1. Select all the files.
2. Click **Delete Selected**.
3. Click **Done**.

Reconstructing a tomogram in ETomo creates several intermediate files, some of them are superfluous after the final reconstruction. ETomo keeps of all the important files necessary to replicate any of the reconstruction steps, as well as the raw stack input files.

Note: your final non-binned aligned stack may be useful in the future, for subtomogram averaging, or for applying further pre-reconstruction filters (for example preNAD or

preNID), or for reconstructing the tomogram with other reconstruction software. If so, don't delete the file ending in `.ali`.

Use **[Shift+left mouse click]** to select all the files you see, and press **Delete Selected**. Click **Done** to complete the final clean up.

You can now close ETomo and type in your terminal:

```
> ls -lrt
```

This will list all the files in your reconstruction directory. Your final reconstruction will have the .rec extension.

Type:

```
> 3dmod u87mg_60eA_7def_50x_tomo2.rec
```

to open your tomogram in 3dmod and move on to Part 2 of the Tomography Practical to learn how to segment your tomogram.