Alignment and Other Challenges in Reconstructing Cryotomograms with IMOD

Challenges in Cryotomography

- Alignment, alignment, alignment
  - It can be hard to get fiducials onto/in the sample
  - The low SNR makes the fiducial positions more uncertain
  - Fiducials are often essentially in a plane, restricting the kind of alignment solution that can be sought
  - Energy filtering, good for cryoimaging, introduces geometric distortions that impair alignment (correctable in IMOD, minimal in new filters)
  - The ice does change (deform) over course of tilt series
  - Cryosections are even harder to get fiducials on, tend to change more under the beam, and fiducialless alignment works worse because of crevasses

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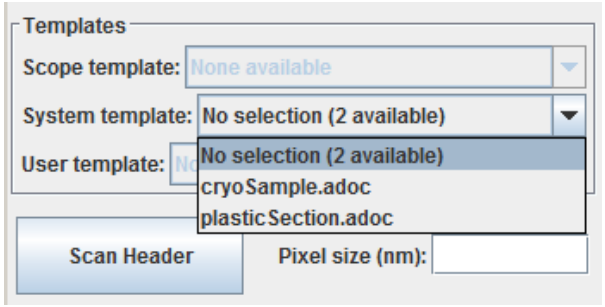
- Contrast is low and artifacts from gold become more prominent
  - Erasing gold may be particularly helpful
- SNR is low and filtering/denoising may be needed to see features of interest

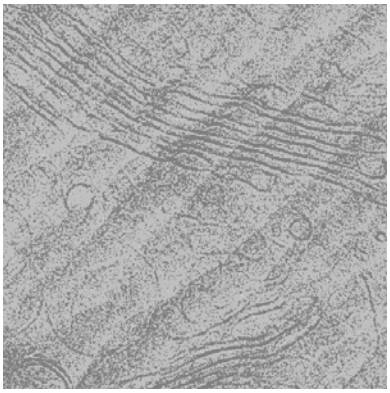
Use a Template in Etomo

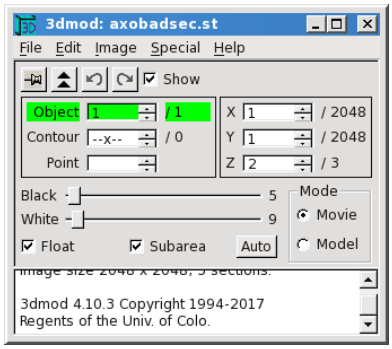
- The cryoSample template distributed with IMOD has ~10 useful default values for cryo reconstructions, e.g.,
  - Filter setting for centering gold beads better when tracking
  - Patch size for tracking image patches for alignment
- You can substitute a system template or make a user template with even better values, but in any case, you should use something



Looking at the Raw Stack

- Before preprocessing to remove X-rays (extreme values), the dynamic range of cryo data often appears poor when the data are loaded into 3dmod as bytes
  - 3dmod stores the data as bytes by default, without truncating extreme values





Default loading as bytes

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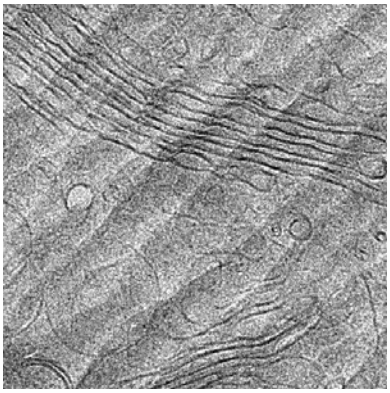
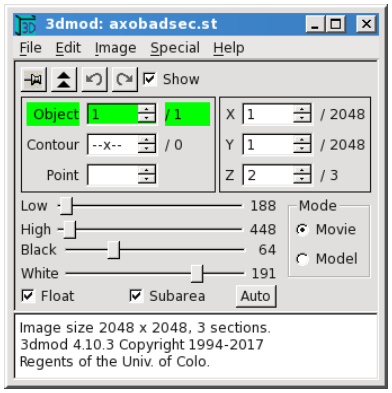
- Loading data into 3dmod as integers preserves the number of gray levels when contrast is stretched





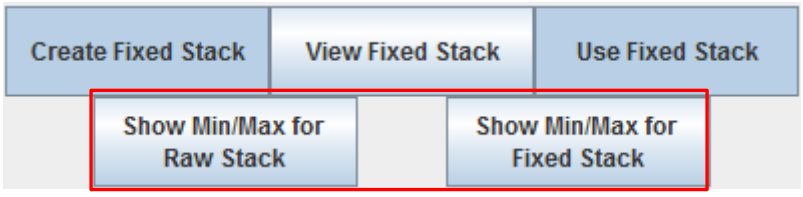
Loading as integers

Looking at the Raw Stack

- Before preprocessing to remove X-rays (extreme values), the dynamic range of cryo data often appears poor when the data are loaded into 3dmod as bytes
  - 3dmod stores the data as bytes by default, without truncating extreme values
- Loading data into 3dmod as integers preserves the number of gray levels when contrast is stretched
  - Etomo will load a raw stack this way to avoid initial problems
  - You can set default to load integers
- Better long-term solution: remove extreme values
  - This also avoids artefactual rays through tomogram

Extreme Value Removal

- X-ray events and extreme values in images are found in two ways:
  - Looking for pixels higher than background by extreme amounts (controlled by “peak criterion”)
  - Looking for pixels that differ from adjacent ones by extreme amounts (controlled by “difference criterion”)
- The default criteria in the cryo template are a good starting point.
  - If more points need to be removed, lower both criteria by 1
- The best way to judge if removal is sufficient is from output of Min/Max values



Statistics Before and After X-ray Removal

c127_bpv_2_4_09_1a.st_stats.log

view	min	(x, y)	max	(x, y)	mean	std dev.
1	-595.0000	(220,1130)	3821.0000*	(356, 1243)	56.6928	26.2009
2	-581.0000	(220,1130)	3199.0000	(10, 1038)	58.9421	26.3049
3	-583.0000	(220,1130)	263.0000	(1015, 378)	62.3637	26.6336
4	-604.0000	(220,1130)	1420.0000	(637, 922)	65.3897	26.4552
5	-559.0000	(220,1130)	2501.0000	(1991, 569)	68.5710	26.5550
6	-543.0000	(220,1130)	783.0000	(1919, 738)	70.3252	26.1427
7	-594.0000	(220,1130)	4199.0000*	(1866, 1526)	72.7389	26.1145
8	-569.0000	(220,1130)	618.0000	(1234, 971)	74.4710	25.5117
9	-591.0000	(220,1130)	2077.0000	(424, 296)	74.2311	25.6620
10	-567.0000	(220,1130)	633.0000	(1164, 760)	77.1407	25.0176
11	-578.0000	(220,1130)	645.0000	(252, 568)	78.3838	24.6603

Before

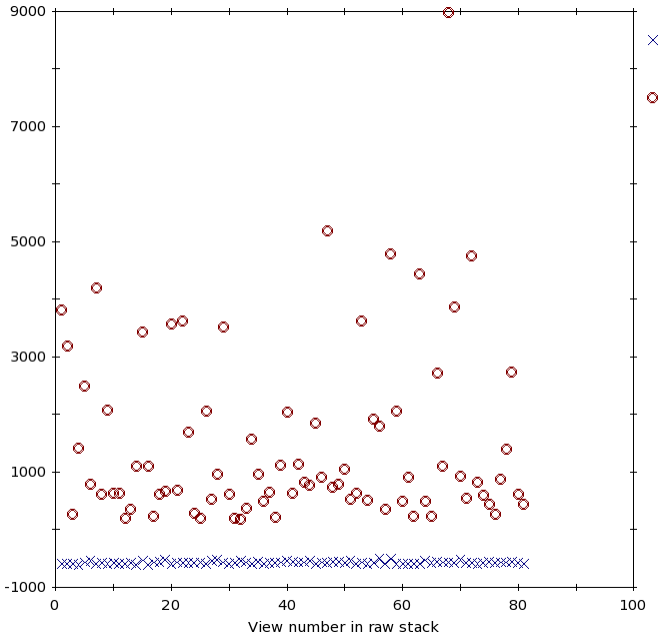
Min and max values that stand out are marked with *

c127_bpv_2_4_09_1a_fixed.st_stats.log

view	min	(x, y)	max	(x, y)	mean	std dev.
1	-6.0000	(90, 181)	182.0000	(1177, 996)	56.6901	26.0801
2	-7.0000	(1987, 367)	188.0000	(1164, 775)	58.9417	26.2543
3	-6.0000	(322, 115)	189.0000	(1135, 907)	62.3641	26.6285
4	-7.0000	(917,1920)	202.0000	(1229, 883)	65.3889	26.4300
5	-8.0000	(739,1931)	205.0000	(1271, 968)	68.5707	26.5228
6	-29.0000*	(369, 987)	197.0000	(1271, 899)	70.3254	26.1359
7	-6.0000	(709,1717)	220.0000	(1309, 890)	72.7376	26.0193
8	-5.0000	(355, 74)	208.0000	(1216, 875)	74.4712	25.5031
9	-5.0000	(364, 101)	195.0000	(1309, 1019)	74.2307	25.6368
10	-5.0000	(472, 126)	206.0000	(1348, 955)	77.1411	25.0100
11	-6.0000	(675,1898)	207.0000	(1054, 1029)	78.3840	24.6524

After

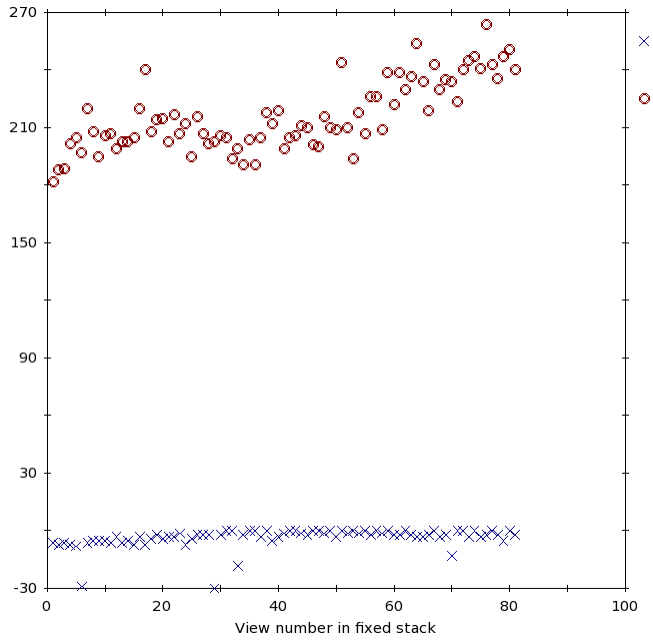
Min/Max in Raw Stack



Every view has large negative values, many views have extreme maximum values

Note the range of the graph: -1000 to 9000

Min/Max in Fixed Stack



The range of the graph is only -30 to 270

There are still some anomalous values, but they are only ~10% of range: not a concern

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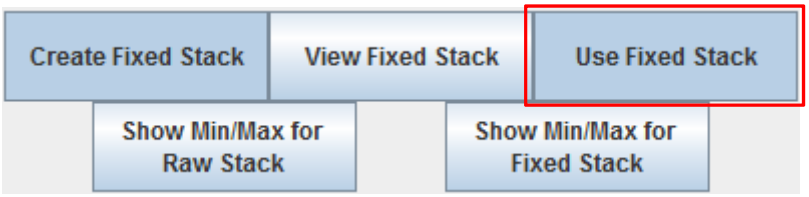
- The default criteria in the cryo template are a good starting point.
  - If more points need to be removed, lower both criteria by 1
- The best way to judge if removal is sufficient is from output of Min/Max values
- You need to push “Use Fixed Stack” to replace original stack with the fixed stack
- You could then iterate, but the program now does 3 iterations, so manual iteration should not be needed much



Points on Alignment Specific to Cryo

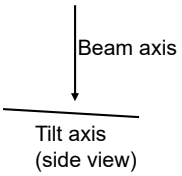
- Solving for distortion (stretching)
- Solving for beam tilt
- Local alignments
- Dealing with few beads

Distortion (Stretching) Solution is Rarely Helpful

- Solving for stretch requires a good distribution of fiducials in Z, which is usually not the case for cryoET
- There are fewer of the kind of changes that it can correct for than with plastic sections
  - Section thinning is corrected by this solution

Beam Tilt

- Beam tilt is shorthand for nonperpendicularity between beam axis and tilt axis
- Its effects are very similar to those of stretch along an oblique axis in specimen
  - In “aligned” images, features move up and down in Y through the tilt series
  - Solving for stretch will correct for beam tilt effects
  - Solving for variable rotation angles partially corrects for them



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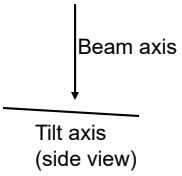
  - Solving for stretch will correct for beam tilt effects
  - Solving for variable rotation angles partially corrects for them
- Including beam tilt can significantly improve the alignment solution when variable rotation angles are not being solved for
- Including beam tilt only adds one unknown, not one per group of views
- Data sets from a given microscope tend to have similar values



Local Alignments

- The alignment equations can account for linear changes in the specimen (the same everywhere)
- Nonlinear changes in specimen have worse effects on alignment the larger the area
- With local alignments, different alignments are obtained from subsets of fiducials, thus allowing correction for nonlinear changes

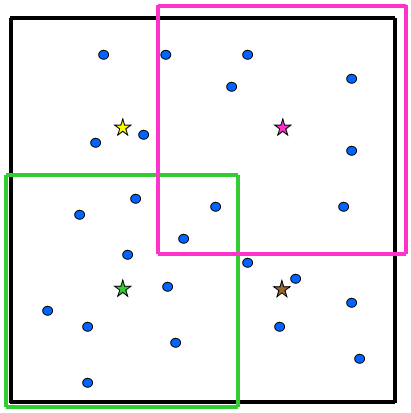
Local Alignments in Overlapping Local Areas

The centers of the local areas are defined by the number of overlapping areas.

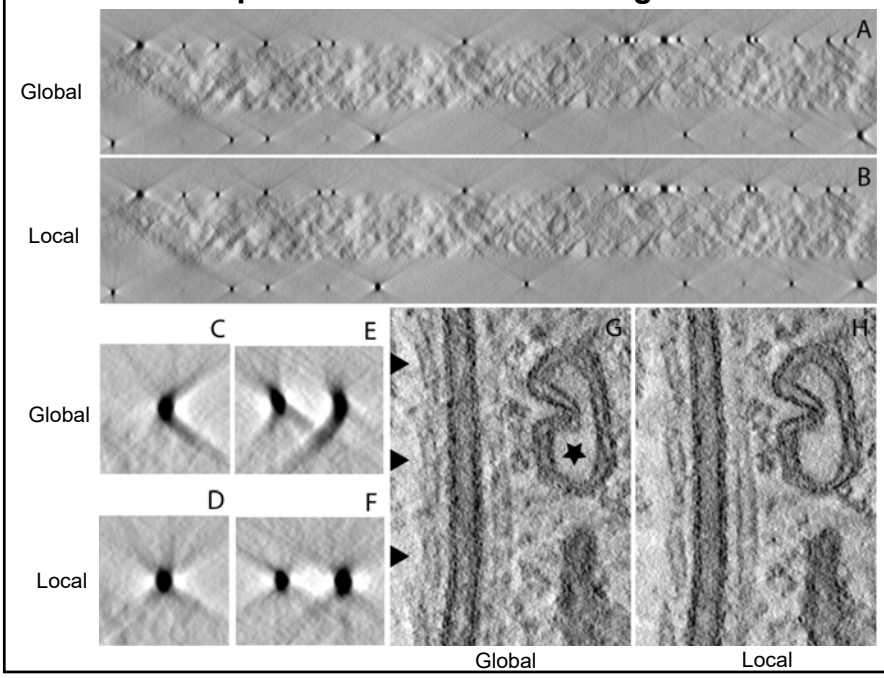
Here each local area is required to have 8 fiducials.

Area 1,1 does not need to be expanded from the minimum size to include 8 fiducials

Area 2,2 grows from its center to include 8 fiducials

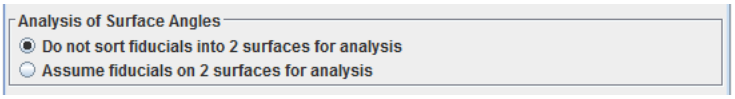


Improvements with Local Alignments

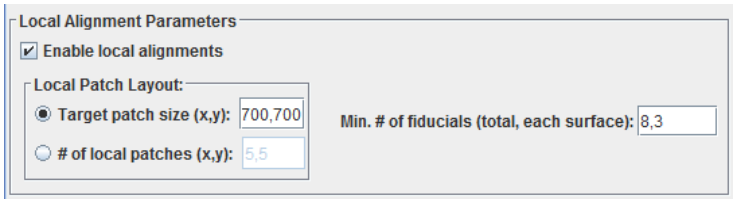


Local Alignments in CryoET

- Local alignments can be helpful with cryoET if there are enough fiducials, particularly for larger areas acquired with direct detectors
  - There should be at least 20 fiducials, preferably ~40-50
  - The average mean residual of local areas can be 20-40% less than that of global solution
- Just be sure not to solve for stretching and not to analyze for beads on two surfaces (use a template!)



- For 4K areas, increase the default target patch size or switch to specifying # of local patches

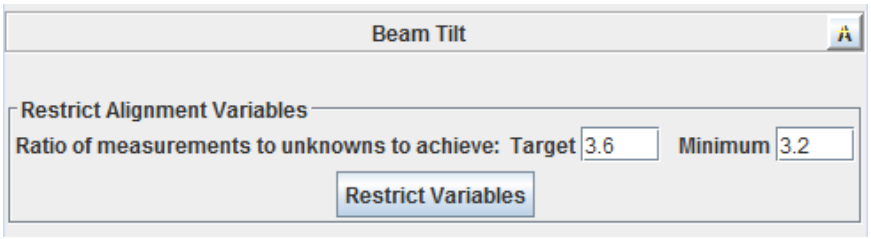


What to Do with Few Fiducials

- In general, try to keep the ratio of measured/unknowns >= 4; if points are well-distributed and well-centered, the ratio can go down to 3 or even lower
  - When the ratio is too low, there is insufficient averaging over the errors in fiducial positions and images become misaligned by these errors
  - How low a ratio you can get away with also depends on how good the distribution of fiducials is: do they cover the field, are some of them close together?

Restricting Alignment Variables

- The script Restrictalign will restrict variables automatically to reach a target value for the ratio of measurements to unknowns
  - It first groups variables that are not grouped, then fixes variables
  - It will start to solve for beam tilt if it drops to solving for one rotation
  - It handles all the way down to one point and solving for shifts only
  - It is used in the batch processing



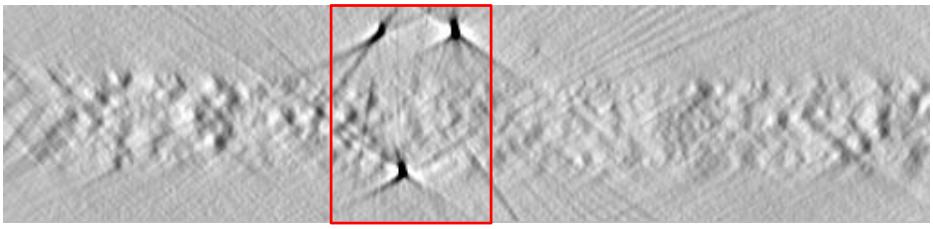
Guidelines for Adjusting Variables by Hand

- 5 Fiducials:
  - Group all variables (magnification and rotation).
  - Or solve for one rotation and beam tilt to get M/U near 4.
- 4 Fiducials:
  - You should be able to solve for one grouped variable, e.g., group magnification and solve for one rotation and beam tilt.
  - You may be able to solve for two grouped variables instead.
- 3 Fiducials:
  - You can certainly solve for one rotation angle and beam tilt (M/U near 3)
  - With well-distributed points you may be able to solve for one grouped variable (magnification or rotation would be more reliable).
- 2 Fiducials:
  - If points are well-separated you should be able to solve for one rotation angle and beam tilt (M/U near 2)
- 1 Fiducial:
  - You can solve for translation only – fix all variables (M/U = 1)

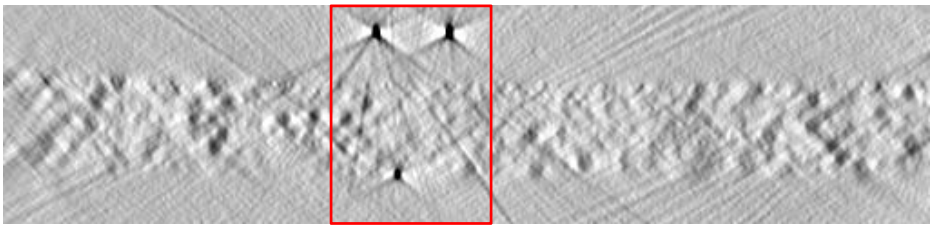
Avoid Overfitting with Few (or Many) Fiducials

- When comparing results with different variable selections:
  - A selection that give a substantial reduction in mean residual with only a small drop in M/U ratio is good
  - If a selection drops the M/U ratio substantially but gives only a small reduction in mean residual, the reduction is a direct consequence of fitting to more variables, which can do more harm than good

Initial Cross-Correlation Alignment is Inadequate



Cross-section of plastic section tomogram aligned by correlation alone

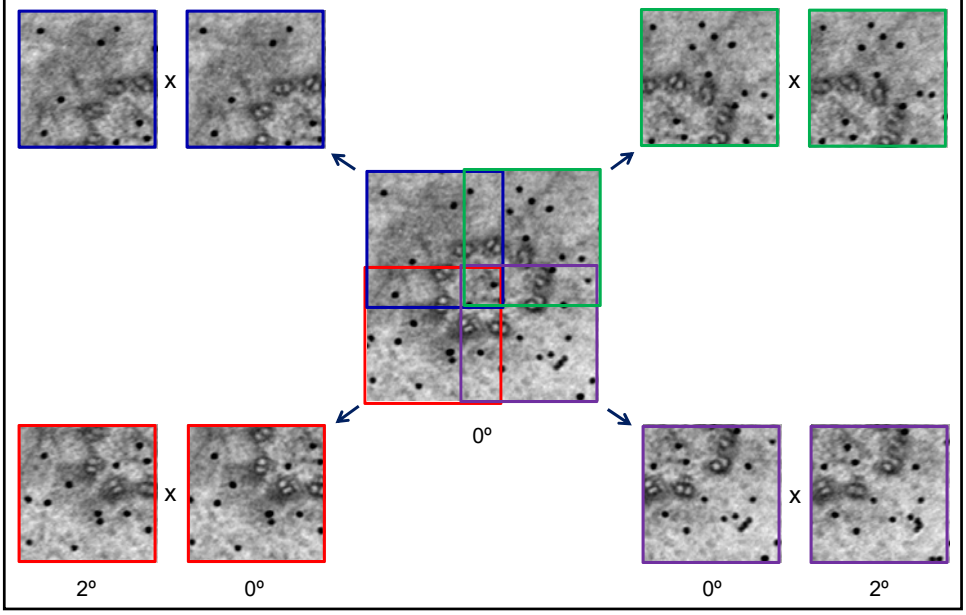


Cross-section of tomogram aligned with fiducial markers

Initial Cross-Correlation Alignment is Inadequate

- It fails to correct for effects like shrinkage of plastic-embedded specimens
- Errors can build up when aligning one image to the next, so that one end of tilt series is out of register with the other
- The angle of the tilt axis has to be determined accurately by other means
- There is no guarantee that it is aligning the same features through the whole series, especially for thick specimens

Local Patch Tracking from One View to Next



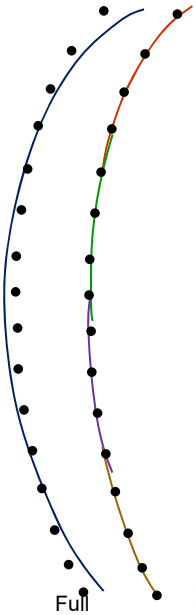
Local Patch Tracking from One View to Next

- Multiple subregions are independently correlated from one view to the next
- The positions of the patch centers are saved as a model that can be used for alignment
- The correlations are of a whole image area, do not localize or center any features in the area, and are vulnerable to the same problems of wandering off as whole image correlations
- Because there are multiple patches, we can derive alignment parameters such as rotation, and assess quality of fit

Local Patch Tracking Fiducial(less) Alignment

- The size of patches needed depends on the richness of image detail and its SNR in the images
  - For cryospecimens, 500-1000 pixels is typically needed (template default value is 680)
- Areas can be excluded from the coarsely aligned stack
- Run initially without breaking contours of tracked points into pieces
  - Mean residual from alignment with full-length contours can be validly compared to residual from true fiducial alignment

Breaking Tracking Contours into Pieces



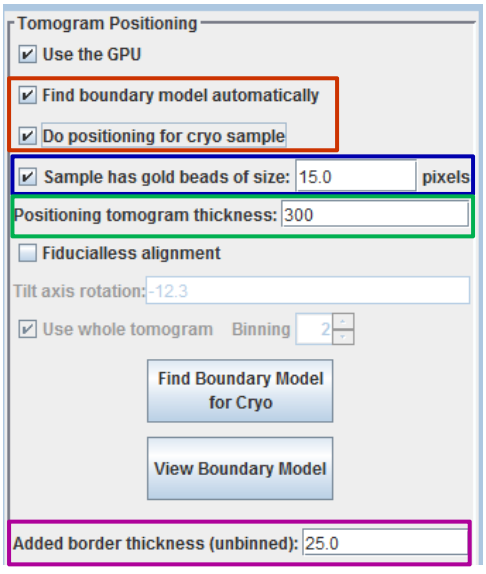
- Breaking patch tracking contours up may help in two ways
  - To extent that feature being tracked changes through series, each segment of contour tracks a feature more consistently
  - Subsets of the tracking that are least consistent can be identified and eliminated in Bead Fixer or with robust alignment
- But whenever there is a systematic misfit between fiducials and alignment model, fitting over shorter extents will reduce the error
  - So the **much** lower residual that occurs from breaking the contours is largely due to fitting over shorter segments

Tomogram Positioning

- Tomogram positioning (setting angles and thickness) is challenging for cryo tilt series because of low contrast and sparseness
- There is now automated positioning in Etomo
  - Works great for plastic sections
  - Completely different procedure implemented for cryo – worth trying
- With cryosamples, artifacts from the high-density structures (gold) interfere with the detection of ice boundaries
- The Cryoposition script builds a tomogram from tilt images with high-density features erased and analyzes that for structure to find surfaces of the ice

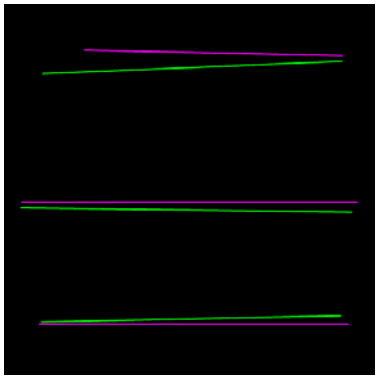
Automated Cryopositioning

- It is selected by turning on both the automatic and cryo options
- The procedure needs to know if there are gold beads even if they were not used for alignment
- It needs a generous thickness set, as usual for positioning
- Etomo automatically switches to a larger border to add when computing the thickness from the boundary model



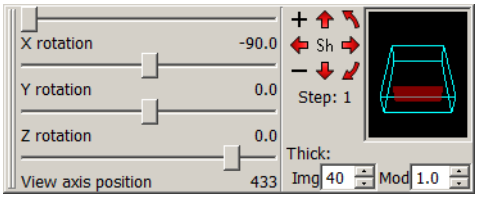
Whole Tomogram Positioning

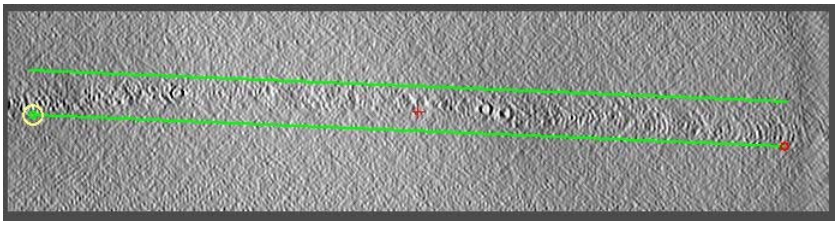
- The whole tomogram allows you to draw 2 or more pairs of lines at arbitrary positions in Y, instead of 3 pairs of lines at fixed positions
  - The lines need not be perfectly horizontal (viewed from top) and need not match up exactly between top and bottom surfaces
  - But it should be obvious how they are paired – open model view window to assess this
- The tomogram should be built with binning
  - This will be quick
  - Binning increases SNR a lot and makes it easier to see the surface features in a cryotomogram



The Easiest Way to Do Whole Tomogram Positioning

- Use Slicer to view X/Z slices as in conventional sampling
  - Set X angle to +90° or -90°
  - Scroll with the “View axis position” slider to a likely level in Y
  - Average slices until you can see the top and bottom surface
  - Repeat at 1-2 more places in Y



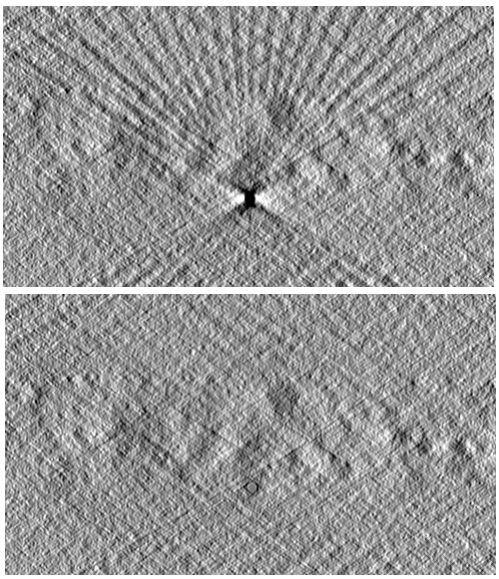


Harder Ways to Do Whole Tomogram Positioning

- View X/Y slices in Zap window
  - Looking at one Y level, scroll in Z until the surface becomes visible on left or right side of image, and add a point there
  - Step in Z until surface is reached on the other side and add point there
  - Scroll in Z to other surface of the material and add two points there
  - Repeat at 1-2 more Y levels
- View slices parallel to surface in Slicer
  - Scroll in Z until a surface is visible in middle
  - Rock angles until the surface appears parallel to the slice
  - Draw lines at surface, step in Z to bring each point to consistent location relative to surface if necessary

Erasing Gold Particles

- Erasing gold beads will eliminate undesirable rays in tomograms, whose effect is worse in cryotomograms due to lower contrast

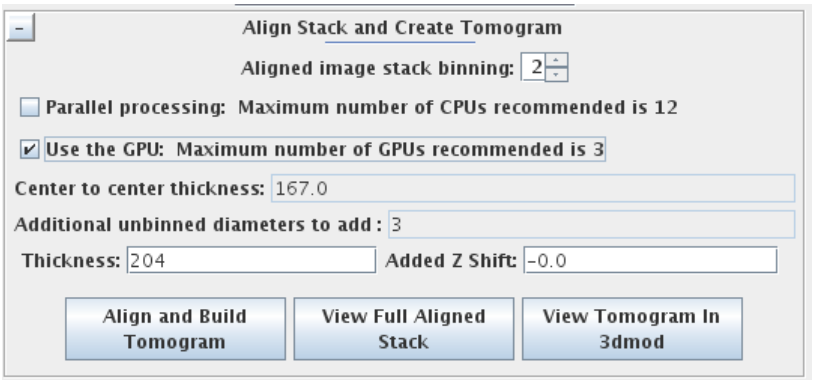


Erasing Gold Particles

- Erasing gold beads will eliminate undesirable rays in tomograms, whose effect is worse in cryotomograms due to lower contrast
- There are two different methods of getting positions for erasing gold beads
  - Transform the fiducial model itself
    - This is simple to do but only includes fiducials
    - A “completed” model with missing points filled in is used
  - Find gold in tomogram, project positions onto aligned stack
    - This can be the easiest way to remove all gold, but checking the 3D model is more difficult
    - If a position is correct in the 3D model, its projections will be present and (nearly) correct in all views of aligned stack

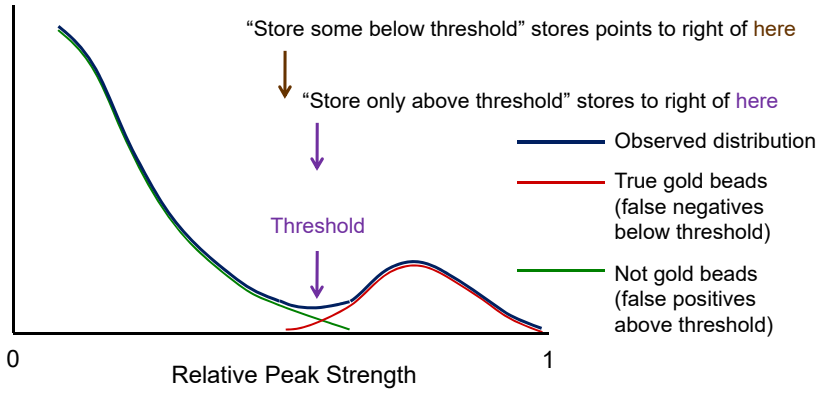
Erasing Gold Particles after Detecting in Tomogram (1)

- Build a tomogram specifically for detecting gold beads
  - Can and should be binned for speed, as long as beads are still >= 4 pixels
    - Etomo will pick the right binning
  - May need to be thicker, or shifted in Z, to hold all the gold
    - Etomo will initialize based on analysis of range of fiducial positions reported in align.log



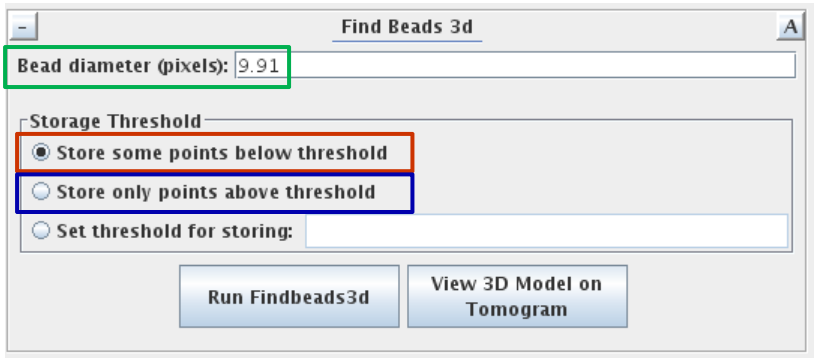
Findbeads3d Operations

- For every possible bead, an integrated density relative to background is computed
- Integrated densities are scaled from 0 to 1 to obtain “relative peak strengths”
- Histogram of peak strengths is analyzed to find dip between two peaks
- This dip is the threshold value that best separates densities that are probably gold from densities that are probably not



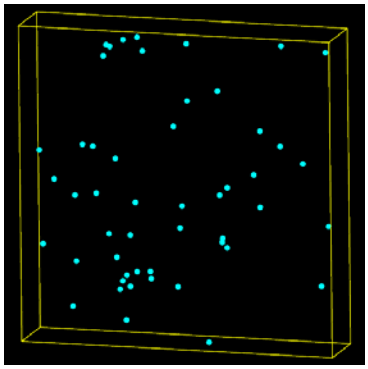
Erasing Gold Particles after Detecting in Tomogram (2)

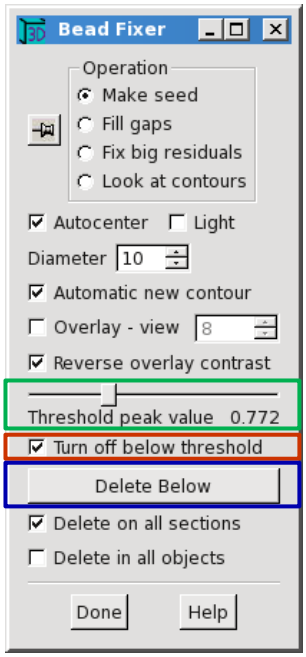
- Detect beads in tomogram with Findbeads3d using the true diameter
  - “Store some points below threshold” will save points in model that are probably not beads and will need to be deleted
  - Use “Store only points above threshold” if you do not plan to work with model or have trouble remembering to delete points below threshold



Erasing Gold Particles after Detecting in Tomogram (3)

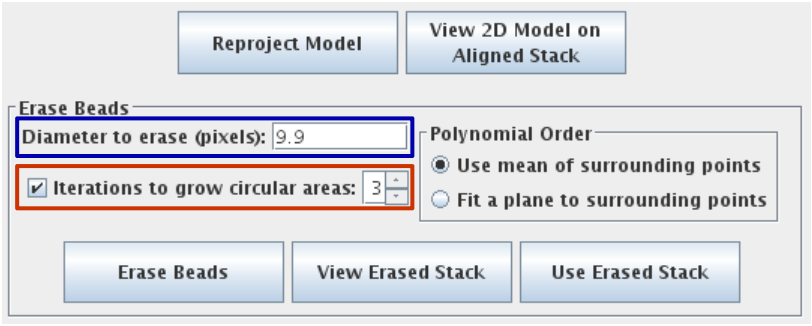
- "View 3D Model on Tomogram" opens tomogram, model view, and Bead Fixer with extra controls for this situation
  - The threshold slider lets you adjust the selection of points
  - Toggle "Turn off below threshold" to see the ones you could add
  - Right click on point to bring it up in Zap window
  - Press "Delete Below" to remove points below current threshold once you have set it optimally





Erasing Gold Particles after Detecting in Tomogram (4)

- Project model, erase stack, and view erased stack
  - It may be easier to see problems here; if so, adjust the 3D model, project, erase, and view again
  - Points are often not centered at high tilt; "Growing circular areas" will erase dark pixels on one side
  - Increase the diameter both to handle remaining deficiencies in centering and to remove underfocus fringes



Using Subtomosetup to Avoid Huge Unbinned Volumes

- Goal: “Extract” full-resolution particles from tomogram for alignment without making full unbinned tomogram
- Method:
  - Make binned aligned stack and tomogram
  - Select particles on binned tomogram or volume derived from it
  - Make unbinned aligned stack
  - Run Subtomosetup to make command files for generating subtomograms at each particle position
  - Run Processchunks (Parallel Processing in Etomo) on command files
- Flexibility with restrictions
  - “`The program allows considerable flexibility as long as several restrictions are followed`.”
  - I.e., you have to follow 7 rules!
    - This is easy if you work within IMOD – it can take account of coordinate changes from trimming and rotation/flipping
    - If you work with external software you can either preserve the coordinate information or provide a simple point list

Using Subtomosetup to Avoid Huge Unbinned Volumes

- Subtomosetup –root series4 –volume series4.rec –center particles.mod \
  -size 100,100,100 –dir subtomos –proc 16
  - -root: data set root name
  - -volume: name of volume that was modeled (at least its header must be present)
  - -center: model of particle centers (or point list)
  - -size: size of subtomograms to make, after final rotation if any
  - -dir: directory to put subtomograms in
  - -proc: expected number of processors, determines how it divides particles into command files