Theory and Principles of Electron Tomography

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Tomography Topics

1. Diverse biological examples of tomography
2. 3D reconstruction from tilt series images
3. Some factors limiting tomogram resolution
Reconstruction by Electron Tomography

3D object $\Rightarrow$ set of 2D projections $\Rightarrow$ 3D reconstruction
Biological Applications of Electron Tomography

Applications are diverse but most fall into three categories:

- Large scale cellular organization, such as of membrane compartments and cytoskeletal systems

Membrane compartments in mouse synaptic terminal
Four independent tubular systems with clathrin-coated buds are in different colors

Ferguson et al., 2007. Science 216:570
Biological Applications of Electron Tomography

• Applications are diverse but most fall into three categories:
  – Large scale cellular organization, such as of membrane compartments and cytoskeletal systems
  – Medium scale organelles, such as basal bodies


Assembly of centrioles in *C. Elegans*
Biological Applications of Electron Tomography

• Applications are diverse but most fall into three categories:
  – Large scale cellular organization, such as of membrane compartments and cytoskeletal systems
  – Medium scale organelles, such as basal bodies
  – Macromolecular structure, usually involving averaging of multiple copies

Microtubule doublet in flagellar axoneme of *Chlamydomonas*

Nicastro et al., 2005, PNAS 102:15889 and later work on mutant
Subvolume Averages Spanning a Range of Sizes

- a) Ribosomes on ER membrane
- b) COP1-coated vesicles
- c) Glycoprotein spike of HIV
- d) Human nuclear pore
- e) Microtubule doublet from a Chlamydomonas flagellum

Aligned Cryo-Tilt Series of Flagella of a Giardia Cell

±60° at 2° increments

Horizontal line of projection data
One line of projection data from one slice of the specimen, at 3 tilt angles
Backprojection

P - Projection of 3 points onto a line at various tilt angles

B – Backprojection of projection data into reconstruction volume

(From Radermacher, 1992)
Backprojection without and with R-weighting

Nearby lines of tilt series image at 0°

X/Z slice of backprojection without R-weighting

X/Z slice of backprojection with R-weighting
The Central Section Theorem

The 2-D Fourier transform of a projection image is a central plane through the 3-D Fourier transform of the object

(From Carazo, 1992)
Problems Revealed by Projection Data in Fourier Space

- Sample points from projection lines do not fall on rectangular lattice -> interpolation needed for inverse Fourier method
- Fourier space is oversampled near the origin -> R-weighting needed in backprojection
- Samples are too sparse at high frequencies -> resolution is limited by number of projections
Radial Filtering of Projection Line before Backprojection

- Raw input line
- Radially filtered input line

$$X = \text{R-weighted filter}$$

FFT → R-weighted filter → Inverse FFT
Backprojection Without and With R-weighting

R-weighting keeps low frequencies from dominating
Iterative Reconstruction

- In an iterative method, each iteration involves:
  - Reprojecting from the current estimate of the tomogram
Iterative Reconstruction

- In an iterative method, each iteration involves:
  - Reprojecting from the current estimate of the tomogram
  - Taking difference between reprojection and original data
Iterative Reconstruction

• In an iterative method, each iteration involves:
  – Reprojecting from the current estimate of the tomogram
  – Taking difference between reprojection and original data
  – Adjusting tomogram by distributing difference for one projection pixel into the pixels along the ray through reconstruction

Reconstruction
X/Z slice

Difference (error)
Iterative Reconstruction

- In an iterative method, each iteration involves:
  - Reprojecting from the current estimate of the tomogram
  - Taking difference between reprojection and original data
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- In ART (Algebraic Reconstruction Technique) this operation is done one angle at a time

- In SIRT (Simultaneous Iterative Reconstruction Technique) the reprojections are computed for all angles first, then the tomogram is adjusted by all the differences
  - Requires more iterations than ART but is more resistant to noise
Iterative Reconstruction

• In an iterative method, each iteration involves:
  – Reprojecting from the current estimate of the tomogram
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  – Adjusting tomogram by distributing difference for one projection pixel into the pixels along the ray through reconstruction
• In ART (Algebraic Reconstruction Technique) this operation is done one ray at a time
• In SIRT (Simultaneous Iterative Reconstruction Technique) the reprojections are computed for all angles first, then the tomogram is adjusted by all the differences
  – Requires more iterations than ART but is more resistant to noise
• The starting reconstruction is generally an unweighted backprojection, so it starts dominated by low frequencies, and higher frequencies are added in through the iterations
Cryotomogram Computed with Backprojection
Cryotomogram Computed with SIRT (16 iterations)
Cryotomogram Computed with SIRT (8 iterations)
Cryotomogram Computed with Backprojection
Cryotomogram Computed with SIRT (16 iterations)
Cryotomogram Computed with SIRT (8 iterations)
Factors Limiting Tomogram Resolution

Resolution here refers to the ability to resolve features within the tomogram reliably; this is independent of the resolution that can be achieved by averaging information above the tomogram resolution. Factors are:

- Number of projections relative to thickness of material
- Density of material within the volume
- Signal-to-noise ratio of input images, determined by electron dose and efficiency of camera
- Resolution of imaging system (microscope and camera)
- Quality of alignment of data entering into backprojection
The Point Spread Function and its Effects on Resolution

(From Radermacher, 1992)
The Crowther Resolution Formula
(Crowther, DeRosier, and Klug 1970)

1. For $D =$ diameter of reconstructed volume
   $\Delta \theta =$ tilt increment (radians)
   resolution $d = D \Delta \theta$

2. For $\Delta \theta =$ tilt increment (degrees)
   $f =$ resolution in frequency (reciprocal space) units
   $f = 57.3 / (D \Delta \theta)$

3. For $n =$ number of views
   $\theta_{max} =$ maximum tilt angle
   $f = 28.5 \ n / (D \ \theta_{max})$

4. For $\theta_{max} = \pm 60^o$
   $f = 0.48 \ n / D$
Tomograms Often Beat the Formulas

• Stained and especially frozen-hydrated material may be sparse – the “object” of diameter D is not the whole specimen but structural components in it

• The artifactual rays are not of paramount importance at relatively fine tilt intervals – they are just another source of noise
Factors Limiting Tomogram Resolution

- Number of projections relative to thickness of material
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Factors in Electron Tomography
Data Collection and Processing

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http://bio3d.colorado.edu/imod
http://bio3d.colorado.edu/SerialEM
http://bio3d.colorado.edu/PEET
Tomography Topics

1. Tilt series acquisition with SerialEM
2. X-ray and artifact removal
3. Cross-correlation alignment
4. Alignment with fiducial markers
5. Fiducialless alignment by patch tracking
6. CTF correction
7. Gold erasing
8. Post-processing and filtering
9. Subvolume averaging with PEET
The Problem of Tilt Series Acquisition

- The goal in acquiring a tilt series is to have every picture centered on the same specimen feature, and at the same focus.
- When the stage is tilted, the specimen position changes laterally and its height also changes, requiring a focus change.
- Specimen position can also change because of persistent drift.
- The basic sequence of operations required is thus:
  - Tilt to a new tilt angle
  - Correct change in specimen position
  - Correct change in specimen focus
  - Acquire final ("Record") image
Approaches to Automated Tomography

• Traditional approach: track and focus at every tilt
  – Used in early software
  – May have to track at low mag: time-consuming
• Precalibration: take a coarse tilt series to determine X, Y, and focus, then take full tilt series without tracking or focusing
  – Used in FEI software
  – Can be fast but is susceptible to drift and calibrations being off; hard to accommodate user intervention
• Robust prediction: predict X/Y/Z position on next tilt from changes in position on previous tilts
  – Adapts to non-ideal conditions and user interventions
  – Achieves the reliability of traditional approach and speed of precalibration
The Robust Prediction Method in SerialEM

- **Tilt 2** Y
  - Actual position
  - Predicted position

- **Tilt 3** Y
  - Make and use prediction but track

- **Tilt 4** Y
  - Make and use prediction but track

- **Tilt 5** Y
  - Rely on prediction – do not track
Low Dose Mode in SerialEM

- Low dose areas are tied to the different camera acquisition modes and are activated just by taking a picture.
- Each area can have separate magnification, beam, and energy filter settings.
- View area is used for operations (e.g., finding eucentricity) that require large (~5-10 µm) field of view.
Mapping Grid Locations

- SerialEM has a Navigator module that can be used to keep track of and return to stage positions
- A map is an image in a file that can easily be reloaded; the Navigator stores the stage position and acquisition parameters
  - Maps are often montages of overlapping frames
- The approach with SerialEM is to take maps at more than one resolution
Mapping Grid Locations

• SerialEM has a Navigator module that can be used to keep track of and return to stage positions
• A map is an image in a file that can easily be reloaded; the Navigator stores the stage position and acquisition parameters
  – Maps are often montages of overlapping frames
• The approach with SerialEM is to take maps at more than one resolution
  – Low-magnification for whole grid map,
  – Medium-magnification for seeing whether target is present or for aligning to
• The “Realign to Item” routine allows one to return reliably to a location marked on a map
Acquiring from Multiple Areas Automatically with Navigator

- Multiple tilt series can be acquired automatically
  - The user interface supports all parameter-setting and even allows parameters to vary between series
- Images and direct detector movies can also be acquired
  - Currently, efficient collection of high-quality data requires extensive use of the scripting capability ("macros")
  - A user interface comparable to that for tilt series is planned
Generating a Tomogram with IMOD

Preprocess
  • Convert to MRC stack
  • Remove X-rays/artifacts

Coarse Alignment
  • Cross-correlate successive images
  • Make pre-aligned stack

Make Fiducial Model
  • Make seed model
  • Track automatically
  • Complete model
  OR
  • Track overlapping image patches by correlation

Fine Alignment
  • Check/fix points with large errors and/or use robust fitting
  • Adjust parameters (stretching, local alignments)

Position Tomogram
  • Make 3 samples or binned down whole tomogram
  • Model borders of section
  • Get and use angle, Z-position, and thickness settings

Generate Tomogram
  • Make aligned stack, optionally filter, CTF correct, or erase gold
  • Compute tomogram by backprojection

Combine Two Axes
  • Trim, scale to bytes, reorient

Postprocess
  • Clean up intermediate files

(Many steps, can run automatically)
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Postprocess
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](image)
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
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 X-rays
  – This also avoids artifactual rays through tomogram
X-Ray Removal

• X-ray events 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 for cryo data sets 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

<table>
<thead>
<tr>
<th>View</th>
<th>Min (x, y)</th>
<th>Max (x, y)</th>
<th>Mean</th>
<th>Std Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>56.6928</td>
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<tr>
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<td>645.0000 (252,568)</td>
<td>78.3838</td>
<td>24.6603</td>
</tr>
</tbody>
</table>

Before

Min and max values that stand out are marked with *

<table>
<thead>
<tr>
<th>View</th>
<th>Min (x, y)</th>
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<th>Mean</th>
<th>Std Dev</th>
</tr>
</thead>
<tbody>
<tr>
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<td>207.0000 (1054,1029)</td>
<td>78.3840</td>
<td>24.6524</td>
</tr>
</tbody>
</table>

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.
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.
X-Ray Removal

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- The default criteria for cryo data sets 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 can iterate after replacing the original stacks.
Good Alignment is Key to Getting a Good Tomographic Reconstruction

- Images must be aligned so that each feature backprojects to the same point in the tomogram from all of the projections
  - If this is not the case, the backprojection of a feature is smeared out instead of summing at a single point
- The first step in alignment to use cross-correlation between pairs of images to find how to shift images into registration
Generating a Tomogram with IMOD

Preprocess
- Convert to MRC stack
- Remove X-rays/artifacts

Coarse Alignment
- Cross-correlate successive images
- Make pre-aligned stack

Make Fiducial Model
- Make seed model
- Track automatically
- OR Track overlapping image patches by correlation

Fine Alignment
- Check/fix points with large errors and/or use robust fitting
- Adjust parameters (stretching, local alignments)

Position Tomogram
- Make 3 samples or binned down whole tomogram
- Model borders of section
- Get and use angle, Z-position, and thickness settings

Generate Tomogram
- Make aligned stack, optionally filter, CTF correct, or erase gold
- Compute tomogram by backprojection

Combine Two Axes
- Trim, scale to bytes, reorient
- Clean up intermediate files

Postprocess
- (Many steps, can run automatically)
Pre-alignment by Cross-correlation

- Each image is cross-correlated with the one at the next lower tilt angle to find the shift that aligns the two images.
- One image is cosine stretched (by the ratio of the cosines of the two tilt angles) before correlating.
- Images are appropriately masked and padded before correlating.
Unaligned image – 58°
Unaligned image – 61°
Tapered, padded image – 58°
Stretched image – 61°
Pre-alignment by Cross-correlation

- Each image is cross-correlated with the one at the next lower tilt angle to find the shift that aligns the two images
- One image is cosine stretched (by the ratio of the cosines of the two tilt angles) before correlating
- Images are appropriately masked and padded before correlating
- The cross-correlation is low and high pass filtered and the position of the peak is computed with sub-pixel accuracy
Filtered Cross-correlation
Generating a Tomogram with IMOD

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**OR**
- Track overlapping image patches by correlation
Automatic Fiducial Tracking

- Beadtrack will track fiducials through the tilt series, starting from a “seed” model.
- It proceeds from one view to the next, trying to find all of the beads on the next view based on their positions on previous views.
- It solves for the location of fiducials in 3-D and uses this solution to predict the position on the next view and to keep points from getting off.
The goal is to find a well-distributed set of seed points suitable for tracking.
Automatic Seed Model Generation

1. Find all gold beads on three starting images near $0^\circ$

2. Track the beads independently from each starting image through 11 images

3. Use tracking information to sort onto two surfaces, if appropriate
Automatic Seed Model Generation

The goal is to find a well-distributed set of seed points suitable for tracking

1. Find all gold beads on 3 starting images near 0°
2. Track the beads independently from each starting image through 11 images
3. Use tracking information to sort onto two surfaces, if appropriate
4. Score beads based on how well and consistently they tracked
5. Select a well-distributed subset of highest-scoring beads to achieve the desired density
Seed Model for Cryo Tilt Series

- Requested 25 beads
- It avoided clusters, distinct beads too close to others, and elongated (overlapping) beads

Tilt series of frozen-hydrated mammalian cell infected with bovine papilloma virus, from Mary Morphew
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(Many steps, can run automatically)
Fiducial positions in every projection view are derived from a single set of positions in 3D by the following changes or operations:

1. The specimen shrinks uniformly in 3D
2. The specimen shrinks along one axis in the plane of the specimen
3. The specimen is tilted around the Y axis, which need not be perpendicular to the beam axis
4. An image is projected with a magnification that may vary among the views.
5. The image is rotated during projection (the tilt axis is not vertical on the camera) and may also stretch along an axis during projection
6. The image is shifted due to variations in specimen position
The Tiltalign Variables

- The specimen changes and imaging operations are expressed in terms of 5 variables that Tiltalign can solve for at each tilt:
  1. **Mag**: a uniform change in specimen size or microscope magnification
  2. **Tilt**: the tilt angle
  3. **Rotation**: the rotation of the tilt axis from the vertical
  4. **X-stretch (Dmag)**: a shrinkage/stretch along the X-axis in the plane of the specimen
  5. **Skew**: a change in the angle between X and Y axes

- X-stretch and Skew together represent a linear shrinkage along an arbitrary axis (distortion)

- Tiltalign can also solve for two variables that are the same for all views:
  1. **Beam tilt**: the angle between the tilt axis and the perpendicular to the beam axis
  2. **Projection skew**: a change in the angle between X and Y axes resulting from stretch during projection
Solving for Distortion (Stretching)

• Solving for distortion will correct for linear distortions in the plane of the specimen, indirectly for thinning of plastic sections, and for some imaging distortions.

• The fiducials MUST be distributed in Z (e.g., on two surfaces or distributed in throughout the ice) to solve for both tilt angle and distortion together – otherwise there is no unique solution.

• If the fiducials are all or predominantly in one plane, do not solve for distortion.
**Grouping of Views to Reduce Variables**

When grouping is used, a variable is constrained to change linearly over a set of contiguous views, or to have the same value for all of the views in the set.

No grouping: Tiltalign solves for an independent tilt angle at each view.

Grouping by 4: Tiltalign solves for tilt angle only at a subset of views (circles). At other views, the tilt angle is constrained to be a linear combination of two variables. Fiducial positions from all views are used to find these tilt angles.
Benefits of Grouping for Reducing Random Errors and Giving an Accurate Tilt Angle Solution
Grouping

- Grouping can dramatically reduce the number of variables being solved for.
- Grouping provides more averaging over errors in fiducial positions and keeps the solution from accommodating to random errors. (Mean residual may be higher but the solution should be more accurate.)
- Grouping is appropriate for slowly changing variables, especially for ones that are hard to solve for.
Number of cycles: 93
61 views, 149 geometric variables, 24 3-D points, 1464 projection points
Ratio of total measured values to all unknowns = 2928/338 = 8.66
Residual error mean and sd: 0.326 0.194

Measured position
Position predicted by fit
Residual error
| view | rotation | tilt  | delt| tilt | mag    | d| dmag    | skew | mean resid |
|------|----------|-------|-----|------|--------||--------|------|------------|
| 1    | -11.8    | 60.4  | 0.41| 1.000| 0.000  | | 0.000  | 0.00     | 0.98   |
| 2    | -11.8    | 58.9  | 0.42| 1.001| 0.000  | | 0.000  | 0.00     | 1.04   |
| 3    | -11.8    | 57.4  | 0.40| 1.000| 0.000  | | 0.000  | 0.00     | 0.92   |
| 4    | -11.8    | 55.9  | 0.39| 0.999| 0.000  | | 0.000  | 0.00     | 0.86   |
| 5    | -11.8    | 54.3  | 0.33| 0.998| 0.000  | | 0.000  | 0.00     | 0.74   |
| 6    | -11.9    | 52.9  | 0.38| 0.997| 0.000  | | 0.000  | 0.00     | 0.73   |
| 7    | -11.9    | 51.4  | 0.34| 0.996| 0.000  | | 0.000  | 0.00     | 0.70   |
| 8    | -11.9    | 49.9  | 0.34| 0.996| 0.000  | | 0.000  | 0.00     | 0.76   |
| 9    | -11.9    | 48.4  | 0.33| 0.996| 0.000  | | 0.000  | 0.00     | 0.74   |
| 10   | -11.9    | 46.9  | 0.38| 0.995| 0.000  | | 0.000  | 0.00     | 0.58   |
| 11   | -11.9    | 45.4  | 0.34| 0.994| 0.000  | | 0.000  | 0.00     | 0.58   |
| 12   | -11.9    | 43.9  | 0.37| 0.994| 0.000  | | 0.000  | 0.00     | 0.56   |
| 13   | -12.0    | 42.4  | 0.34| 0.993| 0.000  | | 0.000  | 0.00     | 0.56   |
| 14   | -12.0    | 40.8  | 0.30| 0.993| 0.000  | | 0.000  | 0.00     | 0.56   |
| 15   | -12.0    | 39.3  | 0.25| 0.992| 0.000  | | 0.000  | 0.00     | 0.51   |
| 16   | -12.0    | 37.8  | 0.28| 0.992| 0.000  | | 0.000  | 0.00     | 0.53   |
| 17   | -12.0    | 36.3  | 0.27| 0.992| 0.000  | | 0.000  | 0.00     | 0.49   |
| 18   | -12.0    | 34.9  | 0.28| 0.992| 0.000  | | 0.000  | 0.00     | 0.53   |
| 19   | -12.0    | 33.4  | 0.30| 0.992| 0.000  | | 0.000  | 0.00     | 0.52   |
| 20   | -12.0    | 31.9  | 0.30| 0.991| 0.000  | | 0.000  | 0.00     | 0.55   |
| 21   | -12.0    | 30.4  | 0.29| 0.991| 0.000  | | 0.000  | 0.00     | 0.47   |
| 22   | -12.1    | 28.9  | 0.28| 0.991| 0.000  | | 0.000  | 0.00     | 0.60   |
| 23   | -12.1    | 27.4  | 0.30| 0.991| 0.000  | | 0.000  | 0.00     | 0.54   |
| 24   | -12.1    | 25.8  | 0.24| 0.990| 0.000  | | 0.000  | 0.00     | 0.54   |
| 25   | -12.1    | 24.3  | 0.20| 0.990| 0.000  | | 0.000  | 0.00     | 0.50   |
| 26   | -12.1    | 22.7  | 0.13| 0.990| 0.000  | | 0.000  | 0.00     | 0.51   |
| 27   | -12.1    | 21.2  | 0.11| 0.990| 0.000  | | 0.000  | 0.00     | 0.49   |
| 28   | -12.1    | 19.8  | 0.17| 0.989| 0.000  | | 0.000  | 0.00     | 0.49   |
| 29   | -12.1    | 18.3  | 0.14| 0.989| 0.000  | | 0.000  | 0.00     | 0.54   |
| 30   | -12.2    | 16.7  | 0.06| 0.989| 0.000  | | 0.000  | 0.00     | 0.53   |
| 31   | -12.2    | 15.2  | 0.07| 0.989| 0.000  | | 0.000  | 0.00     | 0.48   |
| 32   | -12.2    | 13.7  | 0.06| 0.989| 0.000  | | 0.000  | 0.00     | 0.54   |
| 33   | -12.2    | 12.2  | 0.08| 0.989| 0.000  | | 0.000  | 0.00     | 0.49   |
| 34   | -12.2    | 10.8  | 0.18| 0.989| 0.000  | | 0.000  | 0.00     | 0.49   |
Views of 3-D Model of Fiducials

Top view shows distribution of points in plane (in two colors when sorted onto two surfaces)

Side view indicates distribution in Z

Do not solve for distortion

OK to solve for distortion
Tiltalign Does Not Need a Point on Every View

- When in doubt, leave it out - omit points that are obscured (e.g., by gold on opposite surface) or cannot be discerned (e.g., out of focus at high tilt)
Robust Fitting – A Substitute for Fixing Bad Points

- Robust fitting follows these steps:
  - Fit as usual and get residual for each point
  - Give each point a weight based on how extreme the residual is
  - Fit again, minimizing *weighted* sum of errors
  - Get new residuals, new weights, repeat until convergence

- About 5% of points end up with weights < 0.5, ~2% have zero weight
- If there is a sufficient excess of points, this should give as good an alignment as correcting positions manually
Tips on Alignment in Cryotomography (1)

• It is best to use fiducials over the hole and not to use any fiducials over the carbon
• The distortion (stretching) variables are rarely helpful (fiducials are often in or near a plane)
• Local alignments may be helpful if there are enough fiducials
  – Non-uniform behavior of the specimen or imaging system makes a single alignment over the whole area get worse, the larger the area
  – Local alignments involve solving for alignment in overlapping subareas and reconstructing with those alignments, thus avoiding this problem
  – They can be helpful for reconstructions from 4K direct detector images
Tips on Alignment in Cryotomography (2)

• If there are few fiducials, select variables and grouping to keep the ratio of measurements to unknowns at least 3-4
  – When the ratio is too low, there is insufficient averaging over the errors in fiducial positions and images become misaligned by these errors
  – In general terms, first group variables that are not grouped, then fix variables
  – The script RestrictAlign will restrict variables automatically following those rules
• Solving for beam tilt (beam axis not perpendicular to tilt axis) can be helpful if you have very few fiducials and solve for only one rotation
Generating a Tomogram with IMOD

- **Preprocess**
  - Convert to MRC stack
  - Remove X-rays/artifacts

- **Coarse Alignment**
  - Cross-correlate successive images
  - Make pre-aligned stack

- **Make Fiducial Model**
  - Make seed model
  - Track automatically
  - Complete model
  - OR
    - Track overlapping image patches by correlation

- **Fine Alignment**
  - Check/fix points with large errors and/or use robust fitting
  - Adjust parameters (stretching, local alignments)

- **Position Tomogram**
  - Make 3 samples or binned down whole tomogram
  - Model borders of section
  - Get and use angle, Z-position, and thickness settings

- **Generate Tomogram**
  - Make aligned stack, optionally filter, CTF correct, or erase gold
  - Compute tomogram by backprojection

- **Combine Two Axes**

- **Postprocess**
  - Trim, scale to bytes, reorient
  - Clean up intermediate files

(Many steps, can run automatically)
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
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.
- Specimens have thickness, and the features that dominate the correlation may shift to different heights during the series.

Images near $0^\circ$ align to put middle in tomogram center.
Images near $30^\circ$ align to put top in tomogram center.
Images near $-30^\circ$ align to put bottom in tomogram center.
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 is chosen based on richness of image detail and its SNR in the images
  - Stained sections: 150-250 pixels
  - Cryospecimens: 500-1000 pixels
- 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 lower residual that occurs from breaking the contours is largely due to fitting over shorter segments

Four separate contours overlapping in Z, made from same set of points
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Position Tomogram

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Generate Tomogram

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Combine Two Axes

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Postprocess

(Many steps, can run automatically)
Tomogram Positioning

• The goal of positioning is to find:
  1) The Z shift, tilt angle offset and X-axis tilt that will make specimen be level and centered in Z
  2) The thickness in Z required after those adjustments

• These values are computed from pairs of lines along the top and bottom of the specimen at three locations in Y

• In cryo-ET, a whole, binned-down tomogram is generally used instead of small, unbinned samples at three fixed locations
  – You can select three locations where borders can be perceived
  – In the 3dmod Slicer, you can average as many slices as needed to see a boundary
Generating a Tomogram with IMOD

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(Many steps, can run automatically)
Optionally Modifying the Aligned Stack

• Correction for phase inversions in the CTF
  – The effects of the microscope CTF must be corrected to have valid information past the first zero of the CTF
absorption + phase contrast = total contrast

- 2.0 µm defocus
What Makes CTF Correction of Tilt Series Hard?

- There is a different defocus gradient across each tilt series image
  - Not straightforward to invert phases in an FFT
  - Adding together data from different defocuses will blur the power spectrum, make it hard to detect zeros
Defocus and Thon Ring Variation in Tilted Sample

5 nm gold on carbon film, tilted to 65°

4.9 µm underfocus  6 µm underfocus  7.1 µm underfocus
What Makes CTF Correction of Tilt Series Hard?

- There is a different defocus gradient across each tilt series image
  - Not straightforward to invert phases in an FFT
  - Adding together data from different defocuses will blur the power spectrum, make it hard to detect zeros
- The dose is ~10-20 fold lower per micrograph than for single-particle averaging
  - Data must be averaged from multiple images to see CTF effect in power spectrum, unless camera is very efficient
- There is may be little information past the second zero (or even the first zero with a CCD camera)
  - This depends on relationship between pixel size and defocus
  - For cryoET with relatively low defocus, there may be few zeros in spectrum, unless very high resolution is being targeted
Rotational Averaging and Background Subtraction Are Essential for Seeing CTF Zeros in Tilt Series
Rotational Averaging and Background Subtraction Are Essential for Seeing CTF Zeros in Tilt Series

- The Ctfplotter program uses noise images to estimate the background
  - These are specific to camera, microscope, KV, binning.
  - Do this once, use images on many data sets
  - Take series of blank images increasing in counts by factor of 1.5-2
  - See Ctfplotter man page for instructions
- Ctfplotter interpolates between the nearest pair of noise images to find the noise background for a given image
To Compute Power Spectra, Each Image Is Divided into Overlapping Tiles

- This is periodogram averaging, standard method used to get power spectra for CTF correction
  - Tiles are typically 256 pixels square and overlap by 50%
  - Fourier transforms are taken separately and averaged
- For tilt series images, tiles are classified by distance from tilt axis in center:
  - Close to axis (Z height within 200 nm of center): central tiles
  - Farther from axis (Z height difference > 200 nm): off-center tiles
Adding Together Spectra from Different Defoci

- Power spectra from off-center tiles are scaled and shifted so that the first and second zeros are reinforced when they are added into spectra from central tiles.
Optionally Modifying the Aligned Stack

• Correction for phase inversions in the CTF
  – The effects of the microscope CTF must be corrected to have valid information past the first zero of the CTF

• Gold erasing
  – Gold beads cast undesirable rays in tomograms, and their effect is worse in cryotomograms due to lower contrast of material
  – To avoid an artifact, each gold being erased should be removed from every image
  – Erasing the gold used for fiducials is easy but will often be incomplete
  – Another approach supported in eTomo is to build a binned-down tomogram, detect gold there, reproject positions
Generating a Tomogram with IMOD

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Combine Two Axes

(Many steps, can run automatically)

Postprocess

- Trim, scale to bytes, reorient
- Clean up intermediate files
Cryotomogram Trimming

• Aside from removing unneeded slices or regions, there are two goals in the trimming step:
  1. Rotating the volume into a more usable orientation, with image slices being X/Y slices in plane of specimen
  2. Scaling the data to bytes for efficient storage

• Byte data should be sufficiently accurate for further analysis as long as the biological material occupies a good portion of the dynamic range (~100 gray levels)
  – It is necessary to exclude gold and other high densities from the region being analyzed for intensity scaling
  – Otherwise structures of interest will have very poor dynamic range (few gray levels)
Post-processing Filters in IMOD

• The 3dmod image processing dialog lets you experiment with several simple filters then run them on the full volume
  1. 2D smoothing by convolution with a Gaussian kernel in real space
  2. Median filtering in 2D or 3D
      • Each pixel is replaced by the median of surrounding ones
      • De-noises by removing extreme values, with tendency to preserve edges
  3. Low and high-pass frequency filtering in 2D in Fourier space

Use 3dmod menu entry File – Process File to apply current filters to file
Post-processing Filters in IMOD

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  1. 2D smoothing by convolution with a Gaussian kernel in real space
  2. Median filtering in 2D or 3D
     - Each pixel is replaced by the median of surrounding ones
     - De-noises by removing extreme values, with tendency to preserve edges
  3. Low and high-pass frequency filtering in 2D in Fourier space
- Filtering in 3D in Fourier space can also be applied at command line
- Filtering in 3D with Nonlinear Anisotropic Diffusion
Nonlinear Anisotropic Diffusion (NAD)

• What does that mean?
  – Diffusion: iterative kernel smoothing that diffuses densities between neighboring pixels
  – Anisotropic: not the same in all directions, but less in directions with bigger gradients in density
  – Nonlinear: amount of diffusion in a direction is controlled in nonlinear way by gradient relative to a threshold

• Is supposed to preserve/enhance edges by smoothing along edges and not across them
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• Is supposed to preserve/enhance edges by smoothing along edges and not across them

• Requires two parameters to be selected
  – Number of iterations is intuitive: the more, the smoother
  – K value sets the threshold for blocking diffusion: nonintuitive, has to be found by trying different values and picking the one that gives desired result
The Role of the K Parameter in NAD

- **Small K**: Smoothing rate slow in all directions
- **Medium K**: Smoothing rate faster where gradient is low
- **Large K**: Smoothing rate fast in all directions

Gradients at one point in image
Generating a Subvolume Average with PEET

1. **Starting Reference**
   - Can be a single particle

2. **Reference**

3. **Angular Search around 3 Axes**
   - For each orientation, apply rotation and cross-correlate to find CCC and shift
   - Adjust CCC for missing wedge and find orientation with best CCC

4. **Pick Particles for Average**
   - Use ones with highest CCC’s, or use various schemes to reduce missing wedge and other bias

5. **Rotate Particle and Wedge Mask**
   - Wedge mask is 1 where there are data in Fourier space

6. **Sum Particle FFTs and Wedge Masks**
   - Divide FFT sum by wedge mask sum and back-transform

7. **Compute Average**
   - Iterate with reduced angular range and step size
Setting up Intelligent Searches

- Exhaustive searches are expensive
  - Total steps = product of steps around the three axes
  - Thus do multiple iterations with decreasing step size instead of many smaller steps in one iteration
  - After the first iteration, it should be possible to do $7 \times 7 \times 7 = 343$ steps
- Take advantage of symmetry or constraints on orientation to reduce steps on first iteration
- Particles along filaments or arranged on stalks have one axis that is fairly well known
  - Set up this axis as the “Particle Y axis” around which first angle is searched
  - That axis needs the widest search range; the other two axes need much less initially
Minimizing Bias

- Missing wedge bias usually needs conscious effort to minimize, unless you have randomly oriented particles with little symmetry
  - Symmetrize or impose random initial rotations around symmetry axis
  - Combine data from multiple filaments/tomograms

No random rotation or symmetrization

15-fold symmetrization by aligning in all 15 orientations

Average of Eg5-decorated microtubule
Minimizing Bias

• Missing wedge bias usually needs conscious effort to minimize, unless you have randomly oriented particles with little symmetry
  – Symmetrize or impose random initial rotations around symmetry axis
  – Combine data from multiple filaments/tomograms

• Reference bias and overfitting to noise are less of a concern with larger structures; there are features to minimize these also
  – Multi-particle reference
  – Starting from highly filtered reference
  – Starting from different references
Recommendations on Resolution and Filtering

• Use Crowther formula as a guideline for dense material, but be aware that better resolution may be achieved in practice.

• Do not filter to theoretical resolution, filter to preferred appearance (tradeoff between noise and blurring).

• Some filtering in 1-D is applied by default during tomogram generation; this can be increased and images can be filtered in 2-D before this step.

• For cryoET, the preferred approach is to filter after tomogram generation, especially if separate volumes are needed for visualization and subtomogram averaging.