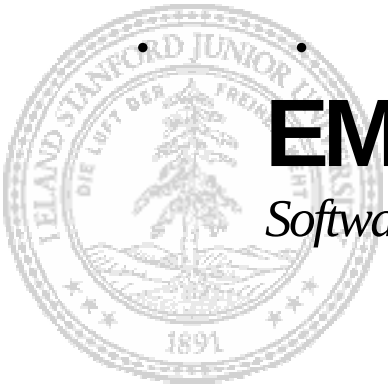
Department of Neurobiology
Department of Structural Biology

Stanford University
Laboratory of Uel Jackson McMahan



EM3D Manual

Software for Electron Microscope Tomography

EM3D Version 2.0 (preview release l), January 2007 Edition

Mira Raman, Cornelia Koch-Stoschek, David Yip, David Ress, Mark Harlow, Robert Marshall, Jing Xu, Joseph Szule, and U. J. McMahan

This Human Brain Project/Neuroinformatics research is funded by the National Institute of Mental Health

Table of Contents

1 Preface

EM3D is a multi-platform application designed to align, reconstruct, segment, model, and analyze electron microscope tomography data. EM3D is compatible with UNIX, Macintosh, Linux, and Windows. Each dataset consists of a series of two-dimensional transmission electron microscope (TEM) images recorded on a CCD camera at regular tilt intervals, for instance, ±70º at 1º increments; fiducial markers are visible on each image to facilitate subsequent alignment. Dual axis datasets contain two series of projections taken at perpendicular angles to one another.

EM3D is being developed in the Laboratory of Dr. U. J. McMahan (Professor of Neurobiology and of Structural Biology at Stanford University School of Medicine), and is funded by National Institute of Mental Health (MH068065). EM3D software is the joint effort between cellular and molecular biologists, who use it daily, and the computational biologists and engineers who develop it.

EM3D 1.X was developed by Dr. David Ress, David Yip, Mira Raman and Cornelia Koch-Stoschek in the IDL environment. EM3D 2.X is being developed by David Yip in Java/C++.

In addition to this manual a tutorial is available for using EM3D 2.X. The tutorial is designed to instruct a new user in the analysis of EM tomography data using a small synthetic dataset. Details on acquiring the EM3D application and the Synthetic dataset can be found at http://em3d.stanford.edu.

EM3D has the typical benefits and liabilities of early software releases. Nevertheless, major efforts are being made on many levels to make this new version of EM3D intuitive, stable, and easy to use. The IDL version of EM3D has been used since 1997 with excellent results in discovering new structures at the 2—3 nm scale, and efforts to improve features and performance continue.

This software is released expressly for **Research Purposes Only.**

2 Getting Started

All users should read this section before running EM3D for the first time.

2.1 An introduction to this text

2.1.1 Main sections of text

This text contains the following sections:

- **Getting Started** - This section contains all the information necessary to download, install, and run EM3D. It also contains a brief description of the steps used to analyze data from importation to 3D surface rendering.
- **Reference Manual** - This section provides more in-depth information about each feature in EM3D.
- **Appendix** – Contains a glossary of terms, acronym list, and a list of figures.

Most of the images in this document are taken from the Windows version of EM3D. The detailed appearance of the graphical user interface varies somewhat from platform-to-platform.

2.2 Some precautions and recommendations

We highly recommend going through the tutorial with synthetic data before using real data.

The size of a dataset that can be loaded is dependent on the capabilities of the computer and effects the speed of operation. Here are some tips for dealing with large datasets:

- Decimate the projections as they are initially loaded
- Reconstruct a decimated volume
- After reconstruction, do not load the projections
- Decimate the objects when rendering

A three-button mouse is very useful when using EM3D. On most platforms, the mouse will work properly in its default mode. Alternatively, a one-button mouse can be used, substituting ctrl-click (option-click on Macintosh) for the middle mouse button and alt-click (command-click on Macintosh) for the right mouse button.

Set display color quality to “Highest” for PC platforms; on Macintosh, set monitor colors to millions. A single large monitor or dual monitors are recommended to accommodate the many windows used with EM3D.

2.3 Operational features overview

EM3D provides all the process steps necessary for working with EM tomography data. The starting point for these operations is the raw, unaligned EM projection images (Figure 1). Note that the images show random spatial offsets from one another.

2.3.1 Import raw EM projections

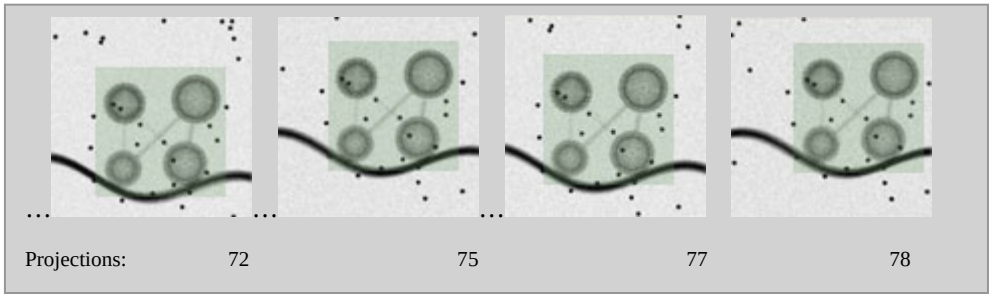


Figure 1: Raw, unaligned EM projection images

2.3.2 Alignment

Calculate and remove the image-to-image offsets between individual projections using *fiducial markers* deposited on the sample or contained within the sample. After alignment, the collection of images now appears to tilt along a common axis.

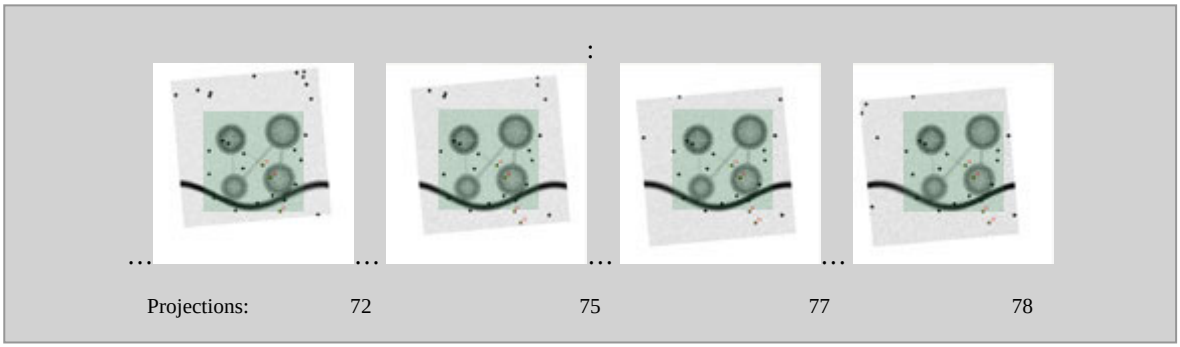


Figure 2: Aligned projections

2.3.3 Reconstruction

Filtered back-projection converts the aligned projections into a volumetric reconstruction of the data. The initial EM3D interface to the volume consists of three 2D views corresponding to orthogonal cut planes.

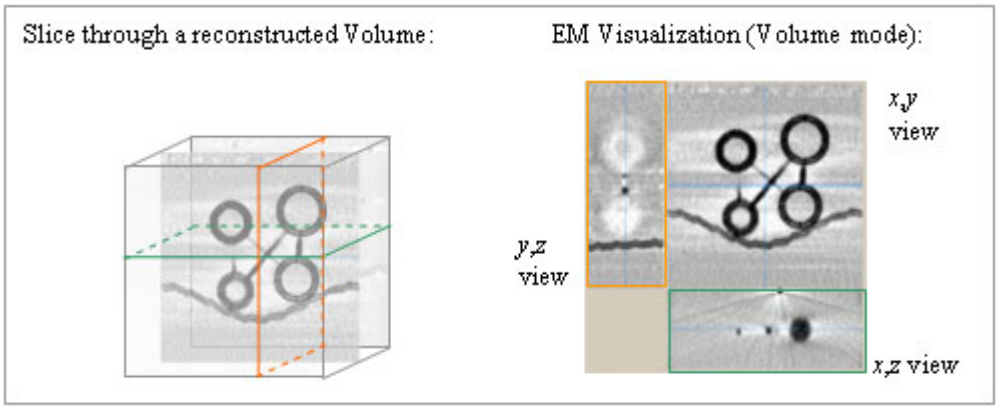


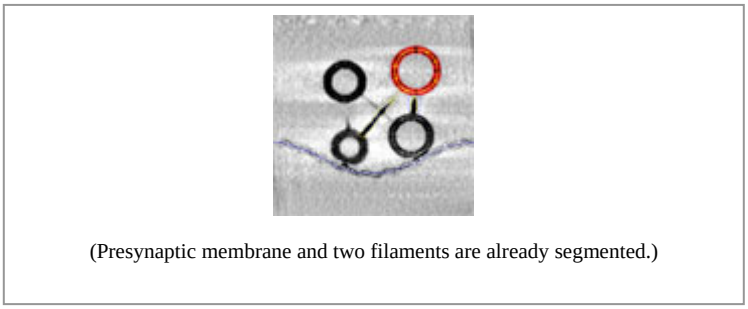
Figure 3: Reconstruction

2.3.4 Combination of dual axis datasets (optional)

If two orthogonal sets of projections were collected and reconstructed they are combined in this step into one volume.

2.3.5 Segmentation

Tools are provided to semi-automatically or manually isolate individual structures based on their image grayscale density. This process creates volumes-of-interests (VOIs), each containing a logically distinct structural component (Figure 4).



(Presynaptic membrane and two filaments are already segmented.)

Figure 4: Segmentation of a synthetic synaptic vesicle

2.3.6 Model generation and visualization

A structural model, usually an ***isodensity*** surface, can be created from each ***volume-of-interest*** (VOI). The models can be visualized in 3D renderings, turned on or off, given different colors and opacities, etc.

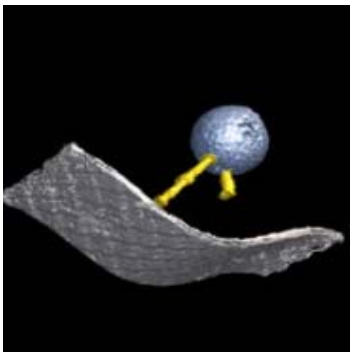


Figure 5: Surface model of a synthetic synaptic vesicle (blue), presynaptic membrane (gray) and two filaments (yellow)

2.4 EM3D file format and maintenance

The EM3D file format consists of two types of data files:

1. The *State* file, distinguished by its '.xml' extension: <filename>.xml

2. The *Binary* file, distinguished by its '.data' extension: <filename>.data.

The *State* file initially is a small file that contains all the information about the larger *Binary* file such as projection alignment and segmentation data. As objects are identified (**Segmented**) the *State* file grows, and may eventually become larger than the *Binary* file that contains the projection and volume data.

These two files are best kept together in named directories. For example, create a directory called **EM** to contain all EM data, e.g., /EM, and a subdirectory that corresponds to a single dataset, e.g., /EM/dataset. This directory, in turn, should contain a single *Binary* file, e.g., *dataset1.data*, and one, or more, *State* files, e.g., *dataset1xml*, *dataset2.xml*, *dataset3.xml*, etc.. The *Binary* file only needs to be saved after a tilt series is reconstructed into a volume, or if the volume is filtered, or decimated

> ***Caution:*** *All State files contain a reference to the Binary file from which they were generated; so changing file names outside of EM3D may result in errors.*
>
> ***Do not edit the State file by hand.***

To use raw data from the EM, images must first be imported into EM3D and converted to a pair of *State* and *Binary* files. Current raw data formats supported by EM3D include sets of individual image files or an MRC stack file.

2.5 Downloading EM3D

The newest release of EM3D is available for download at http://em3d.stanford.edu. Please check regularly the website for new releases of EM3D.

2.6 Installing EM3D

2.6.1 Java

Java 1.5.x or higher is needed to run EM3D. If an appropriate version of Java is not installed on

your computer, it can be downloaded at http://java.sun.com/j2se.

> *Note: EM3D will work with either the SDK or JRE installed. The JRE is much smaller.*

If Java is not installed on your computer EM3D will NOT run.

2.6.2 EM3D for Windows 2000 or greater

Double click on the **Setup.exe** icon

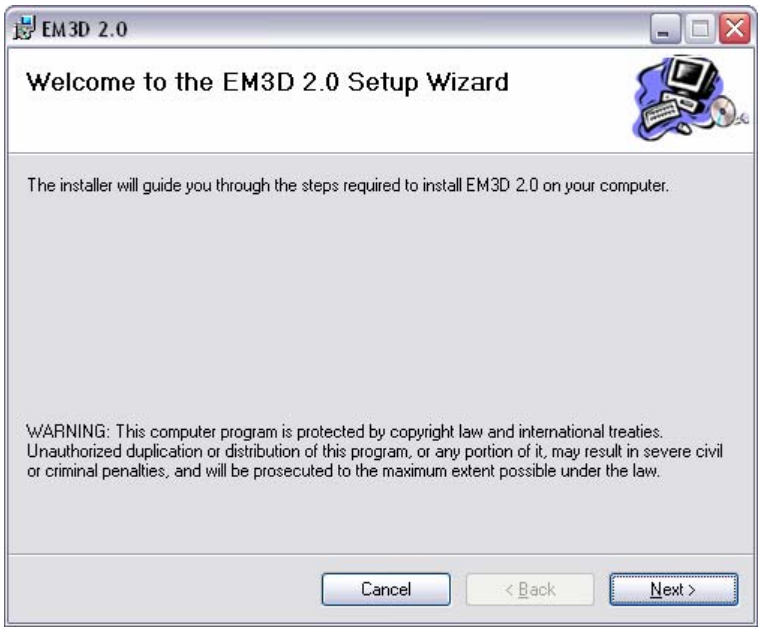


*Figure **6**: Windows Install Wizard*

Press **Next>**

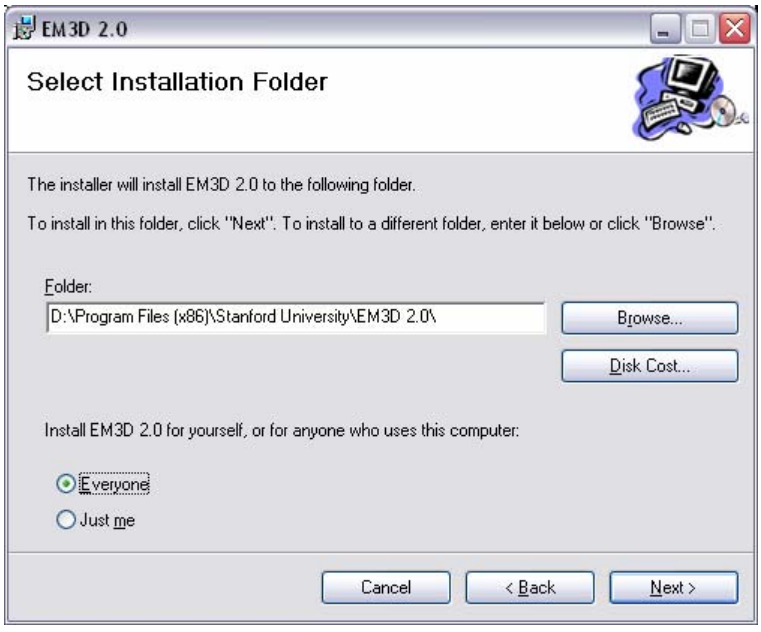


Figure 7: Select program location

Choose the location where you want EM3D to Install.

If more than one user will use EM3D on the computer select **Everyone**.

Press **Next >** on this screen and the next screen.

EM3D will be installed and can be run from the **Window's Start Menu**.

2.6.3 EM3D for Linux

Uncompress the file em3d.tar.gz.

> gunzip em3d_v2.0_linux.tar.gz

Extract the tar file into a directory.

> tar –xvof em3d_v2.0_linux.tar

Change to the directory you just untarred.

> cd em3d_v2.0i_linux

To run EM3D type

> ./em3d

2.6.4 EM3D for Mac OS X

Uncompress the file em3d.tar.gz.

> gunzip em3d.tar.gz

Extract the tar file into a directory.

> tar –xvof em3d.tar

Double click **em3d.command** to run or type

>./em3d/em3d.command

3 EM3D Visualization window

When starting EM3D the Visualization window opens.

3.1 File menu

Figure 8: File menu of the Visualization window

3.1.1 Open

Open is used to load datasets that are in the EM3D format (.xml/.data files). The **Open** button becomes grayed out after a dataset is loaded. Restart EM3D to load or reload data.

> *Note: The .xml file and the .data file need to be in the same directory.*

3.1.2 Import

Import is used to import data that are not in the EM3D format or to combine segmented objects from the same data saved in different files

Import MRC – The data can be imported from either a single MRC stack or from multiple files containing one projection/slice each. The data can be either a series of projections or a reconstructed volume.

> *Note: EM3D explicitly supports only the UCSF version of the MRC file format. If you have problems loading your MRC stack, convert the data stack to a series of image files and import these in EM3D.*

Import Image – The preferable image file format is 16bit TIFF; however, most standard image formats are supported. The data can be either a series of projections or a reconstructed volume.

> *In order to load a Dual Axis dataset, you must check the **Dual Axis** menu item BEFORE selecting **Import**. After the first dataset has been loaded you will be prompted to select the second dataset.*

Import Segmentation – This is used to combine segmented objects from different saved datasets. First load the first dataset. Then Import the segmentation data from the other dataset into the currently loaded dataset. The second dataset can be a cored version of the first dataset or a differently saved version of the first dataset with other segmented objects.

3.1.3 Export

Export is used to export an MRC stack that can be imported into earlier versions of EM3D for analysis.

Export MRC - If the projections are visible in the **EM3D Visualization** window then the unaligned projections will be written in the MRC file. If the 3D volume is visible in the **EM3D Visualization** window then the volume will be exported.

Export aligned MRC – This menu item is available when projections are visible in the EM3D Visualization window. It is used to write aligned projection images in the MRC file.

> ***No** information about segmentation will be exported into the MRC file.*

3.1.4 Save

Save > State… writes a new *State* file to make a record of changes to the dataset such as alignment or segmentations. Only the .xml file will be saved.

Save > Binary and State writes new *State* and *Binary* files. It is only necessary to write a binary file under one of the following conditions:

1. After first **Import**ing data into EM3D.
2. After **Deleting Projections**.
3. After **Reconstruct**ing a volume.
4. After a successful **Dual Combine**.

> ***NOTE:** When saving files, on UNIX platforms (including Macintosh), the directory to which they are to be saved must already exist.*

3.1.5 Delete Projection

Delete Projection removes an undesirable projection from the series, which can be necessary due to bad focus, noise, or other image problems.

> *It is necessary to recalculate the CDF after you have finished removing projections, in order to readjust the grayscale to appropriately represent the data, otherwise Alignment and Reconstruction will be compromised.*

3.1.6 Compute CDF

Compute CDF is used to recalculate the cumulative distribution function for the projections or images after a slice or projection has been removed. If the grayscale range of the image looks unusual at any point try selecting Compute CDF.

3.1.7 Load Projections

Load Projections is used in combination with **Open**. If **Load Projections** is checked, it means that both the volume and projections will be available once the dataset is loaded. Often once the volume has been reconstructed there is no need to go back to the projections (they use a great deal of memory). This option was added strictly to reduce the amount base memory used by the dataset.

> *Note as long as the original dataset contained projections, they will always be available with any state file, simply by checking Load Projections. In other words they are never removed from the binary file.*

3.1.8 Load Single Volumes

Load Single Volumes is used in combination with **Open.** When loading an already combined dual tilt dataset, check **Load Single Volumes** to load the 2 single volumes A and B in addition to the combined volume. This is necessary when recombining the two dual tilt reconstructed volumes. For segmenting and rendering it is recommended not to load the single volumes for saving memory.

3.1.9 Dual Axis

Dual Axis must be checked to load both sets of projections for a dual axis dataset.

> *Dual axis must be selected before importing images or MRCs .*

3.1.10 Unload from RAM

Unload from RAM is used to remove projection or volume data from memory. This is useful when working with big datasets to avoid memory issues. When projections are selected in the Visualization window projections are unloaded from RAM and only the currently selected projection slice is loaded into memory. When volume is selected the volume is unloaded from memory and the currently selected volume slice is loaded into RAM.

3.1.11 Load into RAM

Load into RAM is the reverse step of “Unload from RAM”. Once projection or volume data were unloaded from RAM this menu item becomes available to load data back to memory.

3.1.12 Quit

Quit, exits EM3D. Before quiting don't forget to save your data.

3.2 Visualization display

The main graphics window is called the **EM3D Visualization display**. The visualization display has 2 modes, **Projections** and **Volume**. In **Projections** mode the tilt series of images can be explored (Figure 9), while in **Volume** mode the reconstructed volume can be visualized in as a series of XY slices through the volume (Figure 10, left) or in 3 orthogonal views (Figure 10, right.)

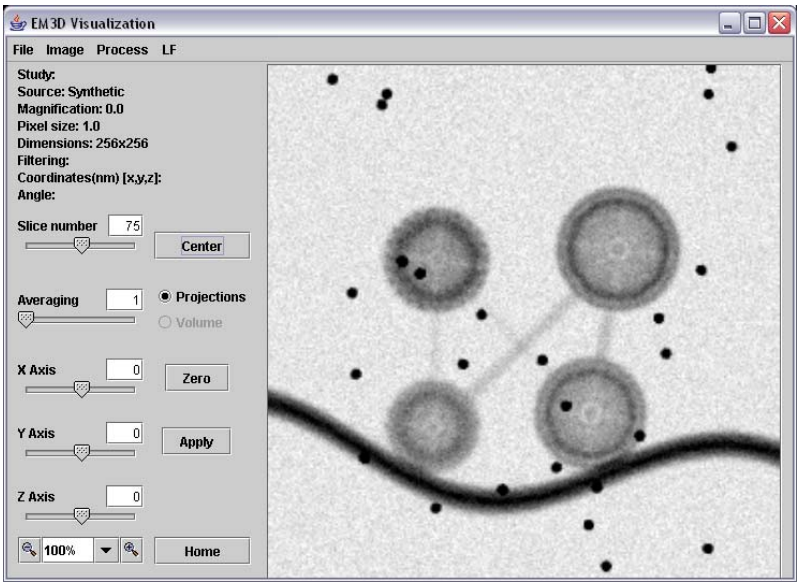


Figure 9: EM3D Visualization Window – Projections mode

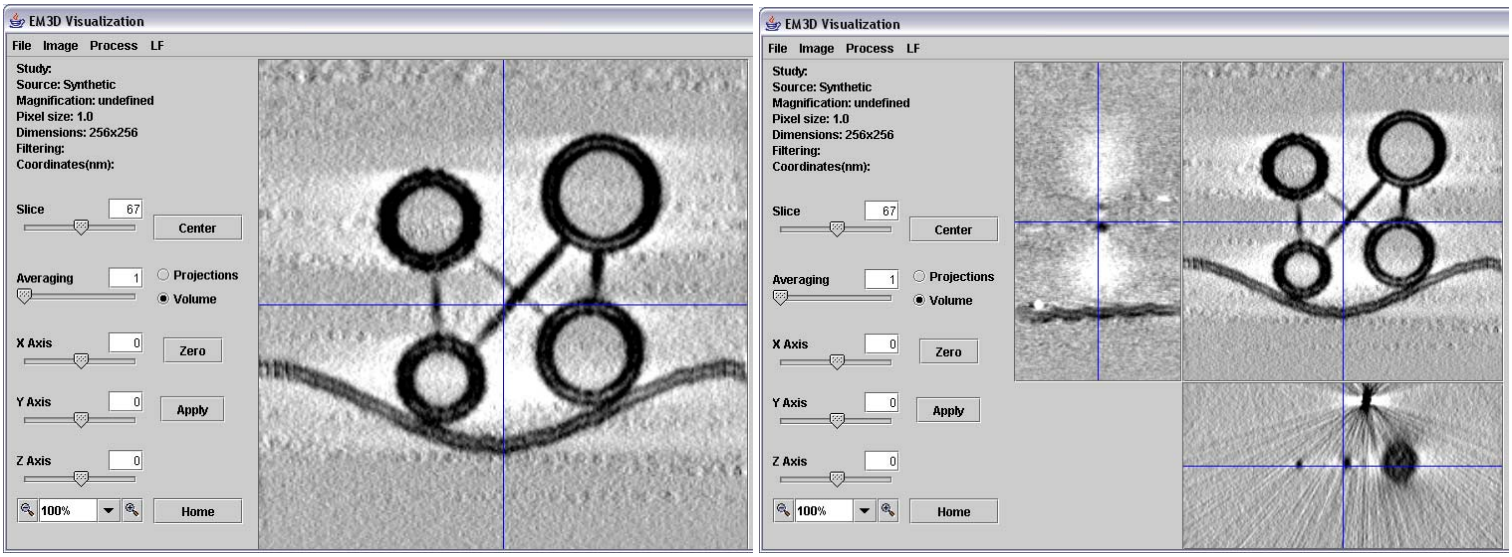


Figure 10: EM3D Visualization Window – Volume mode XY view (Left) and with transverse views (Right)

3.3 The controls

3.3.1 Dataset information

The upper-left corner of the **EM3D Visualization** window displays information unique to the dataset such as its name, source (produced by which computer or microscope), magnification, and pixel size, based on the data's header information or user-specified information.

3.3.2 Display mode

The EM3D Visualization display operates in two modes, **Projections** and **Volume**, controlled by the pair of radio buttons to the right of the **Averaging** slider.

- **Projections** mode is used to view the individual EM projections. When projections are initially imported, only this mode is available. The **Slice** slider controls which tilt-angle projection is displayed. The tilt angle and cursor pixel coordinates are displayed just above the slider.

- **Volume** mode is used to view virtual slices through a reconstructed volume.

  > *The following coordinate system will be used to describe the volume. The (x, y) plane corresponds to original zero-tilt plane of the EM images, and after alignment, the y-axis is the tilt axis. The z-axis is the reconstructed depth axis.*

  Volume mode is available only after a volume has been reconstructed or if the initial dataset is a volume. The display windows take on a different character in volume mode, showing three orthogonal slices through the volume, with blue crosshairs indicating the relative positions of the different slices within the volume. The crosshairs provide a reference for objects' relative positions within the volume, and they are useful for finding edges of objects during segmentation. One can navigate the volume display using the left mouse button: clicking on the (*x*, *y*) display moves the transverse (*x*, *z*) and (*z*, *y*) displays. Similarly, clicking on either of the transverse displays changes the slice number of the (*x*, *y*) display as well as the position of the other transverse display.

3.3.3 Image magnification

The size of the displayed image can be controlled using the **Magnification** or **Zoom** drop list located at the lower left corner of the window. Zooming can be particularly useful during segmentation (described below).

> *Note: Large zoom factors can significantly decrease the rate at which the display updates.*

3.3.4 Averaging and Rotating

Several additional controls, which are grayed out and inactive in Projection mode, become available in Volume mode.

> *Note: All sliders in EM3D can be controlled with the keyboard arrows. Select the slider and use the left arrow (←) to decrease, and right arrow (→) to increase values.*

Averaging allows the selection of adjacent volume slices to average with the current slice to form a smoothed slice in the (x, y) plane (for display purposes only).

Rotating - Virtual slices can be formed at any angle through the volume. Rotation of the volume is controlled by three sliders:

- X Axis rotates about the *x*-axis [in the (*z*, *y*) plane]
- Y Axis rotates about the *y*-axis [in the (*x*, *z*) plane]
- Z Axis rotates about the *z*-axis [in the (*x*, *y*) plane

These slice images are interpolated from the volume. To permanently rotate the volume, click on the **Apply** button.

3.4 Image menu

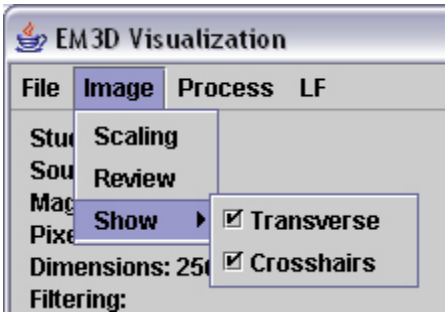


Figure 11: Image menu

3.4.1 Scaling

Scaling is used to set which part of a linear contrast range visually best suits the data, e.g., for better fiducial acquisition with noisy data or to better view images during segmentation. Scaling changes the visible gray scale range for either projections or volume slices (depending upon which mode is selected) based on the cumulative distribution function, which is shown as a plot versus gray value.

- The **Min Clip** and **Max Clip** fields set the percentage of pixel values that will be clipped; the corresponding gray values are displayed as the vertical blue lines on the graph. For example, if **Max Clip** is set to .99 then all of the pixels that have gray values in the highest 1% will be shown in white. If **Max Clip** is set to .85 then the highest 15% of the pixels will be displayed in white. Conversely the pixels below **Min Clip** will be displayed black. Increasing both clip values together increases the image contrast.
- **Absolute** scales each image based on the range of data values in the entire tilt series or volume.
- **Relative** scales each image based only on the currently displayed projection or slice. Relative is the default mode for projections, while absolute is the default mode for volume slices.
- The **Invert** button reverses the grayscale, generating a negative image that is often useful for segmentation.

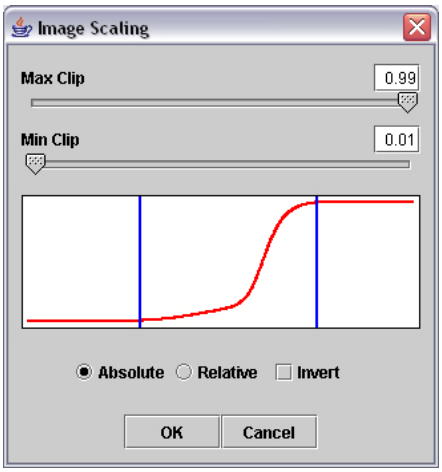


Figure 12: Image Scaling

3.4.2 Review

Review creates a movie by scrolling through projections or slices in the **EM3D Visualization** X, Y display. The movie's parameters are defined in the **Review** control (Figure 13).

Figure 13: Review Control

- The **Start Slice** and **Stop Slice** determine, which X, Y slices are used to generate the movie.
- **Review Start Slice** and **Review Stop Slice** determine the range of slices that are shown on the screen, when **Review** is pressed.
- After the movie is generated it plays in a continual loop, until **Stop** is pressed. By pressing **Bounce**, you can switch from displaying the movie in a loop to playing it forward then backward, then forward again

Review requires a large amount of memory, the following are suggestions for getting the best movie of the area you want to see.

- Turn off **Transverse** slices.
- Only add slices you want in the movie by selecting **Start Slice** and **Stop Slice** carefully.
- Finally reduce the size of the **EM3D Visualization** window.

3.4.3 Show

Show controls the display of various image properties in Volume mode:

- Crosshairs: toggles on and off the blue crosshairs in the three volume windows.
- Transverse: toggles off or on the (*x*, *z*) and (*z*, *y*) views.

3.5 LF menu

The LF menu controls the look and feel of the User Interface. Figure 14 gives an example of the different UI's on a Windows XP machine with Java 1.4.2.

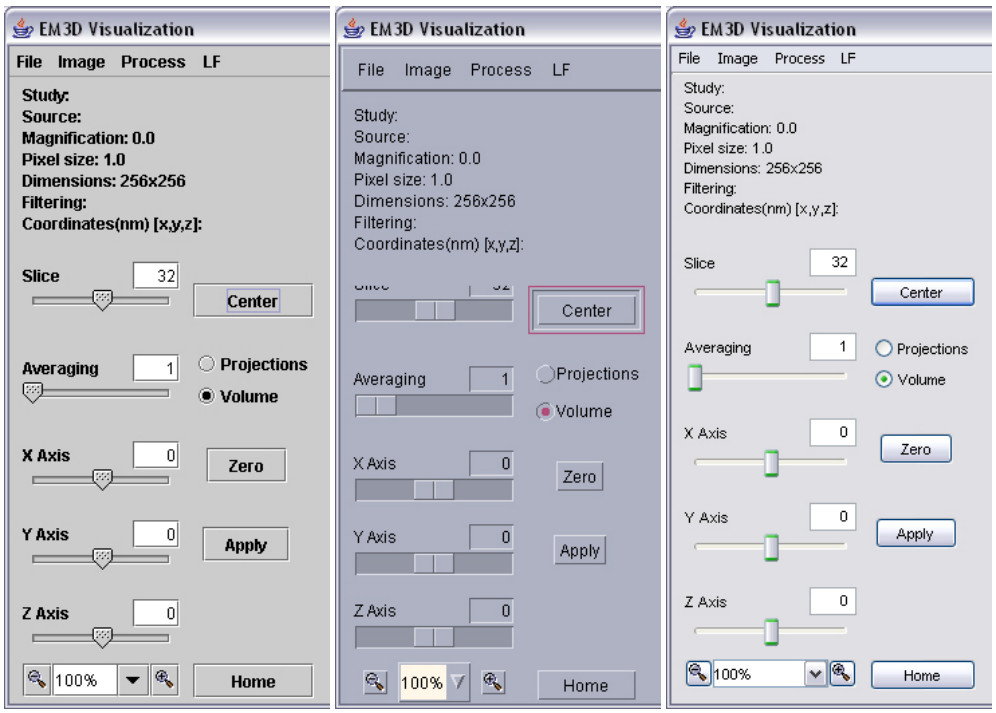


Figure 14: Look and Feel (LF) Java, Motif, and Native on Windows XP

4 Alignment

4.1 Why Align

This is a step that is unique to Electron Microscope tomography. In x-ray CT or nuclear medicine studies the resolution is such that the hardware can accurately and repeatablely find the same center from projection to projection; whereas with Electron Microscopy the projections are shifted slightly from projection to projection. The alignment process is a way to shift the projections into the correct position for reconstruction.

> *In a correctly aligned set of projections, each fiducial will move smoothly in a horizontal line during a **Review**.*

4.2 Alignment controls

From the EM3D Visualization window, select **Process > Align** to open the Projection Alignment window.

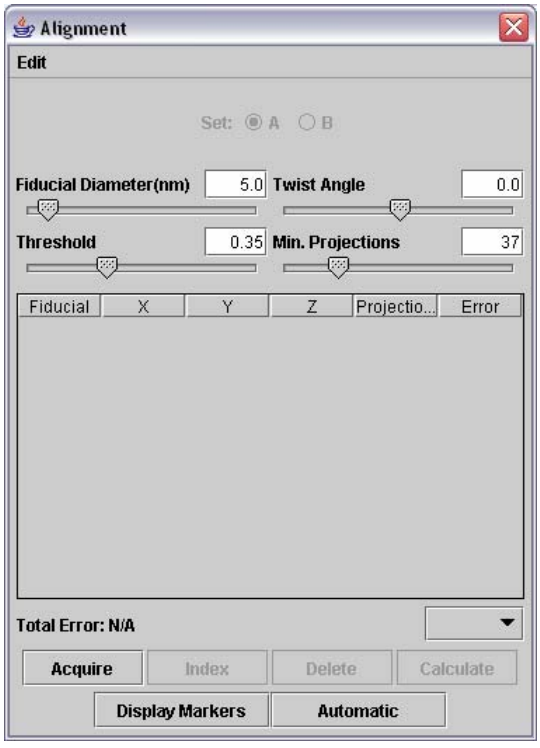


Figure 15: Alignment control

4.2.1 Select Projections set

The **Set:** radio buttons are used with a dual axis dataset to choose which set of projections to align.

Both sets must be aligned. It is grayed out for single axis reconstructions.

4.2.2 Sliders

Slider values can be adjusted by sliding the tab or by entering a specific number in the text field and pressing the <Enter> key on the keyboard.

- The **Fiducial Diameter** slider controls the size object for which the algorithm searches. Larger numbers can remove small noisy areas from consideration, but excessive numbers will merge together closely spaced fiducials. If the size of the fiducial is known use that as the initial value.

- The **Twist Angle** is the angle the projections are rotated around the Z axis. If the twist angle is known, it should be entered and then locked. Locking is achieved by pressing the left mouse button over the Twist Angle Label (Figure 16). By locking the angle you reduce the number of iterations in the Calculate step.

Figure 16: Twist angle locking

- The **Threshold** slider controls the strictness with which fiducials are identified, with smaller numbers corresponding to stricter definition of possible fiducial markers. Large numbers increase the number of possible detected fiducial positions on each projection. Typical range is 0.25—0.5.

- **Min Projections** is used in Indexing to determine which fiducials tracked through enough projections to remain.

> *Be careful when reducing the number of projections. It is ideal to have several fiducials track across the entire volume; however, this is not always possible. When you reduce the minimum number of projections a discontinuity may form, where below a given projection one group of fiducials is used and above that projection, a different set of fiducials is used. The lack of overlap could yield a poor alignment that appears to have a low **Total Error**. That is why it is always advisable to **Review** the projections once the alignment is complete.*

4.2.3 Buttons

- **Acquire** - The acquire button finds all areas in each projection that meet the criteria of a fiducial. The criteria for a fiducial are established using the sliders described above. There is no correspondence between the fiducial numbers on different projections at this stage.

- **Index** - Indexing establishes a relationship between the fiducials throughout the projection series. An example: Fiducial 8 refers to the same fiducial in projection 30 and projection 90 after indexing. Indexing also generates a list of fiducials as seen in Figure 17.

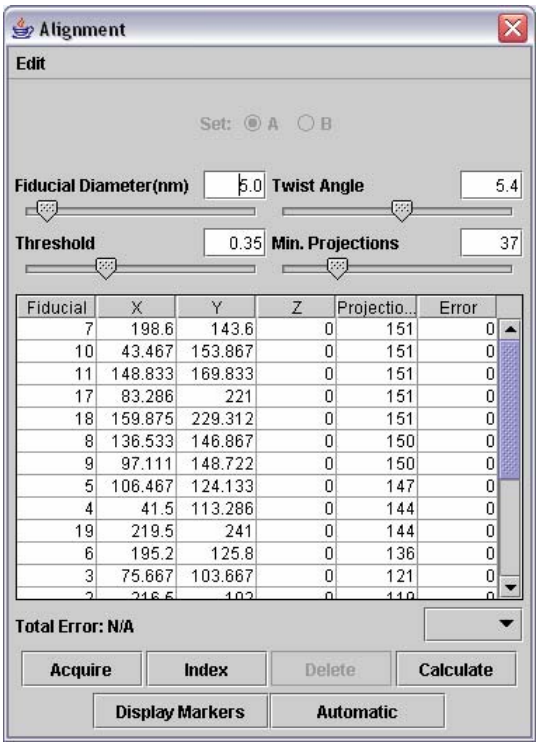


Figure 17: List of indexed fiducials sorted by Projections

- **Delete** removes selected fiducials from the fiducial list.
- **Calculate** does a cross correlation and returns the composite Error for each fiducial and the Total Error for the entire alignment.
- **Display Markers** overlays yellow on possible fiducials in the EM3D Visualization window.
- **Automatic** performs all steps to achieve the best alignment possible from the current step. If no actions have been taken Automatic does an Acquire, Index, and then iteratively does Calculate and Delete to minimize the error while still meeting all criteria. If an Acquire has been done than Automatic starts with Index. If Index has been done than a series of Calculate and Delete operations are done. If you are unsatisfied with the results, select Override from the Edit menu, then press Acquire, followed by Automatic. If you are still unsatisfied with alignment change the Acquisition/Indexing Parameter sliders, and press Acquire followed by Automatic.

4.3 Fiducial list

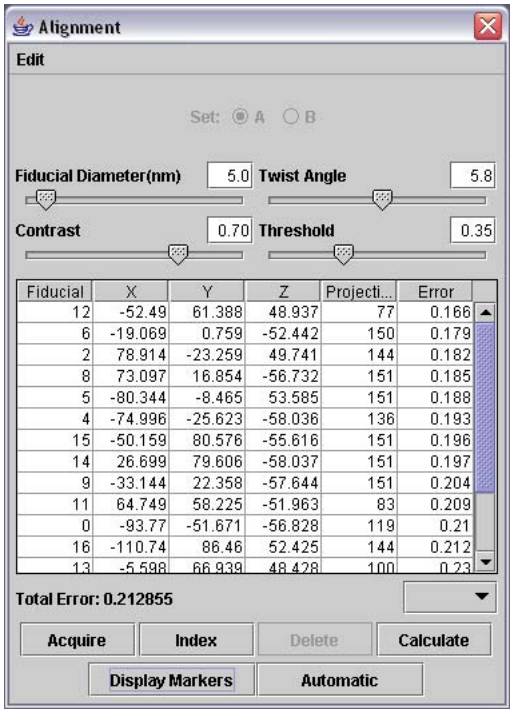


Figure 18: Alignment after calculation

- The **Fiducial** column contains a list of the unique ids associated with each fiducial. When a single row is highlighted that fiducial can be changed in the EM3D Visualization window. When the middle mouse button is pressed in the EM3D Visualization window, the fiducial number that is highlighted in the fiducial list will be placed at that location on the current projection, provided a possible fiducial exists in the vicinity.

- The **X**, **Y**, and **Z** columns are an estimate of the fiducial's location in the reconstructed volume. Z will not be filled in until the first calculation has been done.

- The **Projections** column states on how many projections each fiducial was identified.

- The **Error** column gives the composite error for each fiducial based on the calculations.

> *A good alignment will often yield a **Total Error** of less than 1.0 pixels per 512 pixels of resolution. i.e. 0.5 for a 256, 1.0 for a 512, 2.0 for a 1024….*

- All columns in the fiducial list can be sorted in ascending or descending order by pressing the column heading. In Figure 17 the fiducials have been sorted in descending order by Projections and in Figure 18 the fiducials have been sorted in ascending order based on Error.

4.4 Adjusting Fiducials on the EM3D Visualization Window

After Indexing it is possible to **Move**, **Add**, and **Delete** fiducials from *individual* projections using the mouse.

4.4.1 Adding or moving a fiducial on a single projection

1. Select the fiducial to add/move in the Fiducial List.
2. Navigate to the desired projection in the EM3D Visualization window.
3. Click the middle mouse button on the projection where it should go.
4. If the fiducial does not go in the right place turn on the Display Markers, and adjust the fiducial parameters until a small yellow region appears where you want to place the fiducial and press the middle mouse button.

4.4.2 Removing a fiducial from a single projection

Click the right mouse button next to the fiducial you want to remove.

> *The right mouse button removes whichever fiducial it is closest to, regardless of which one is selected in the **Fiducial List**.*

4.5 Alignment methods

4.5.1 Automatic steps

These are the steps that work in an ideal situation:

1. Turn on Display Markers.
2. Navigate to the center slice in the EM3D Visualization window. Adjust the Alignment sliders until the fiducials in the image are fully highlighted. Scroll back and forth through the projections in the Visualization window to ensure that the parameters are good for all projections.
3. Turn off Display Markers.
4. Lock the Twist Angle (Optional).

   > *Although not locking the twist angle is more time consuming it may be more accurate.*

5. Press Automatic.
6. Un-lock the Twist Angle.
7. Press Calculate.

If at any point there are any projections with too few fiducials (bad projections) change the slider parameters (particularly Threshold). Press Acquire and then press Automatic to restart the alignment process. You may also add fiducials on the bad projections by hand as described in Section 4.4.

4.5.2 Semi-automatic steps

These are the steps for aligning datasets with less defined fiducial markers:

1. **Acquire** – Identify potential fiducials
2. **Index** – Find correspondence between the identified fiducials
3. **Calculate** – The error associated with each fiducial
4. **Adjust** – Add, move or delete fiducials on individual projections using the mouse.
5. **Delete** – Remove fiducials from the alignment that have high error or are not present on a large number of projections
6. **Refine** – Repeat the Calculate and Delete steps until an acceptable alignment is obtained.

5 Reconstruction

From the main **EM3D Visualization** window, open the **Reconstruction** window by selecting **Process > Reconstruct…**.

5.1 Understand the reason for slab reconstruction

There are several things to consider before reconstructing the volume. A tilt-series with projection dimensions of 1024x1024 pixels can yield a full size reconstructed volume with dimensions of 1024x1024x1024 pixels. We know the thickness of our sample is always substantially smaller than the width of the projections. In order to reduce the memory and time needed to perform the reconstruction, we will only reconstruct a slab of the volume that contains useful data.

Below is an example using the Synthetic dataset.

In Figure 19 we see a series of slices through the volume. The fiducial markers that rest on the top and bottom of the sample are visible on slices 75 and 180 respectively; indicating the range of useful data. This is additionally evident in slices 40 and 216 which only contain noise. Thus reconstructing only the slices between the fiducial markers (slices 75 and 180) will greatly reduce the amount of time and memory used.

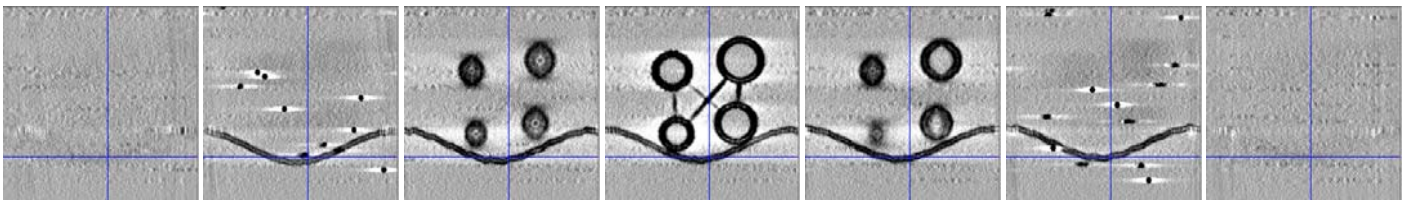


Figure 19: Slices 40, 75, 110, 128, 149, 180, 216

5.2 Reconstruction controls

5.2.1 Defining the area of the volume to reconstruct.

Near the top of the window are two sliders that control which slab of the entire volume will be reconstructed.

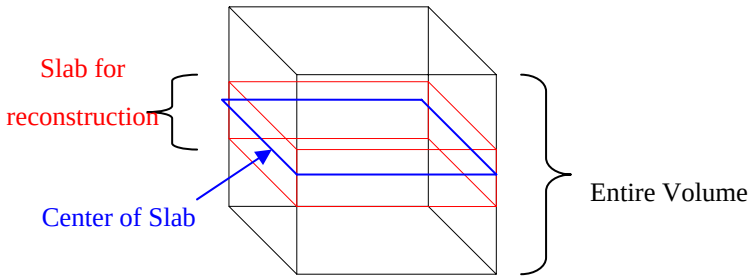


Figure 20:Selecting slab for reconstruction

- **Center** (pixels) – This determines where the center of the reconstructed volume will be with respect to the entire volume. The location of the center of the reconstructed volume. The default value for Center is ½ the dimension of the projections in pixels.

- **Thickness** (pixels) – The final thickness of the reconstructed volume. Defaults to ¼ the dimension of the projections in pixels.

- **Tilt offset** – Features within the specimen volume may sometimes be tilted around the *y*-axis with respect to the zero-tilt slice.

5.2.2 Controls of numerical aspects of the reconstruction

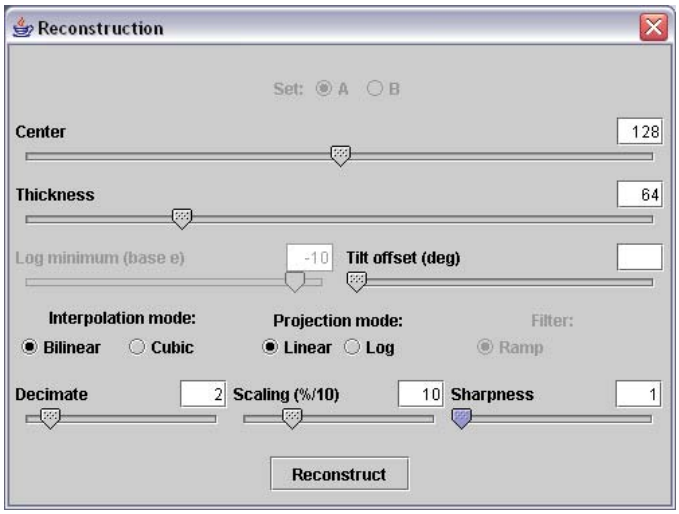


Figure 21: Reconstruction control window

- **Interpolation** mode – Controls the form of interpolation used for rotating the projections. Bilinear (default) is faster but less accurate than Cubic.

- **Projection** mode – Controls how the image data is interpreted before reconstructed. Linear (default) takes the EM data at "face value," the optically thin approximation. Log mode treats the EM data as optically gray, taking the logarithm of the image data before forming the back-projections. Use linear mode for thin samples, log mode for thick samples. In Log mode, the Log minimum slider becomes active; adjust this value to control the contrast of the reconstruction.

- **Decimate** – Combines pixels to reduce size of data. A Decimate value of 1 yields a full size reconstruction. Use large decimation values to perform fast reconstructions that permit convenient assessment of parameter settings.

- **Scaling** – Controls the treatment of artifacts created by the high-pass weighting process in Filtered Backprojection. The default value is 10%.

- **Sharpness** – Controls the blurring of the final volume. Low values are blurrier, but less noisy, and high values have better resolution and more noise. In addition the Sharpness level corresponds to the speed at which the reconstruction progresses, with low numbers being faster that high numbers. The default value of 4 is a good compromise between speed, resolution, and noise for most real datasets. When doing a quick, decimated reconstruction, it is advisable to set Sharpness to 1, and then return it to 4 for the final reconstruction.

5.3 Reconstruction process

These are the steps for reconstructing a 3D volume:

1. Define the area of interest using the center and the thickness sliders.

   Decimate the volume to preview the 3D volume.

   To calculate the center and thickness use these formulas:

   $center_{new} = center_{current} + (decimation * (max_{slice} + min_{slice}) - thickness_{current}) / 2$

   $thickness_{new} = (max_{slice} - min_{slice} + 1) * decimation$

2. Set Interpolation and Projection mode, Scaling, Sharpness, and Tilt Offset according your dataset.

3. Hit the Reconstruct button and evaluate your reconstructed volume.

6 Dual axis combine

6.1 Requirements for dual axis volumes

It is required that both volumes must have exactly the same size and contain the fiducials (preferably on both sides of the sample).

6.2 Dual Combile Controls

Select **File > Process > Dual Combine**… to open the **Dual Combine** window.

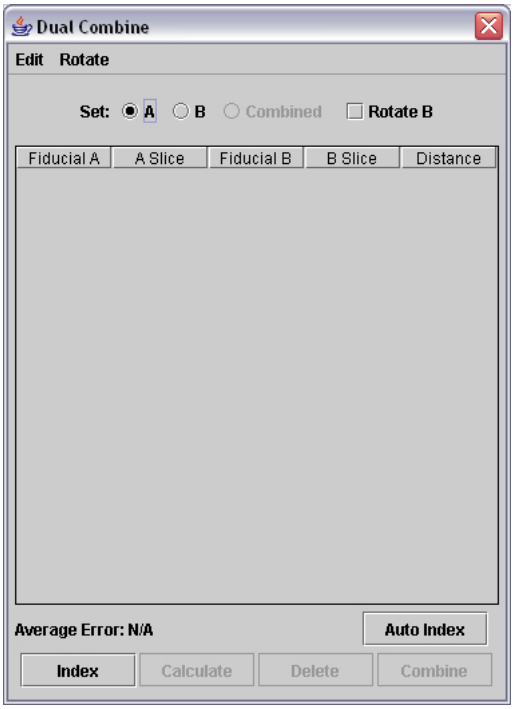


Figure 22: Dual Combine

- **Set** - The Set buttons indicate which volume is being displayed in the EM3D Visualization window. By toggling between the buttons you can navigate from volume A to volume B.
- **Rotate B** - Rotate volume B by 90 degrees either clockwise or counter. Choose which direction it rotates through the Rotate menu.
- **Index** – Find for every single fiducial its location in set A and B and list the pair with its identification and location in each volume and the distance between them after a least squares fit.
- **Auto Index** – Index all fiducials and remove all fiducucial pairs with a high distance.

- **Calculate** – Calculate the gross registration of the 2 volumes.
- **Delete** – Remove the incorrect fiducial pairs from the list. These fiducials are not taken for calculating the gross registration.
- **Combine** – The 2 volumes are merged. The Set: Combined radio button becomes available to switch to the combined volume. The Average Error: is set to the average RMS error for all fiducials in the list after this fine alignment.
- **File > Reset** – Start over dual combine.

6.3 Procedure of combining

These are the steps for combining two dual axis volumes:

1. Rotate Set B into same orientation as set A.
2. Identify possible fiducial pairs.
3. Select fiducial pairs to use in calculating combination.
4. Calculate the alignment of the volumes.
5. Revise the fiducial pairs to get a better gross registration of the volumes.
6. Recalculate the registration.
7. Combine the volumes (the fine registration is done as part of the combination step).

6.3.1 Rotate set B

The first step in combining the volumes is to get them in roughly the same orientation. We do this by rotating volume B by 90 degrees. Adjacent to the **Set** buttons is the **Rotate B** check box. Press this and volume B will rotate by either 90 degrees clockwise or counter – clockwise. You can choose which direction it rotates through the **Rotate** menu.

6.3.2 Automatically identify possible fiducial pairs

Once the volumes are in the same orientation, press the **Auto Index** button in the lower right of the **Dual Combine** window. Five columns will appear in the panel. Each row represents a *single* fiducial with its identification and location in each volume and the distance between them after a least squares fit.

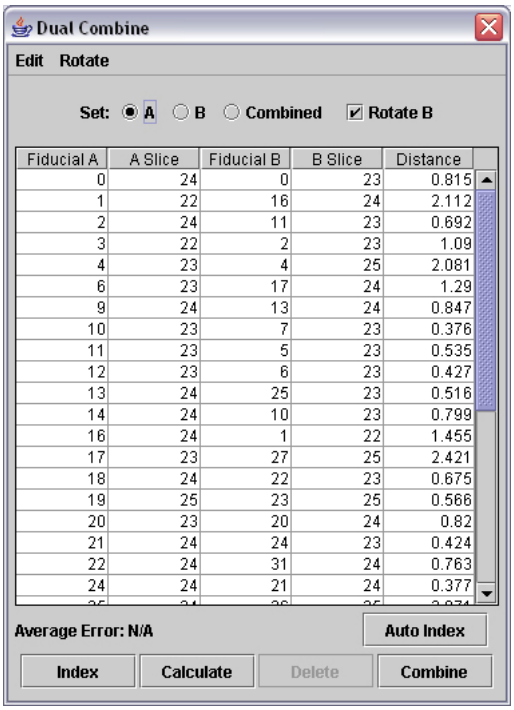


Figure 23: Auto Indexed

6.3.3 Indexing

If **Auto Index** fails or yields too few pairs, use **Index**. Auto Index has an arbitrary distance threshold, and immediately removes all pairs with a higher distance. By using **Index**, those pairs will not be thrown out, but you must be even more careful in ensuring that pairs are valid, see the next section.

6.3.4 Select fiducial pairs to use in the combination

We want to use as many fiducials as possible to give the most information about the correspondence between the volumes; however, we do not want to use areas of the volume with heavy stain that appear like fiducials to the algorithm.

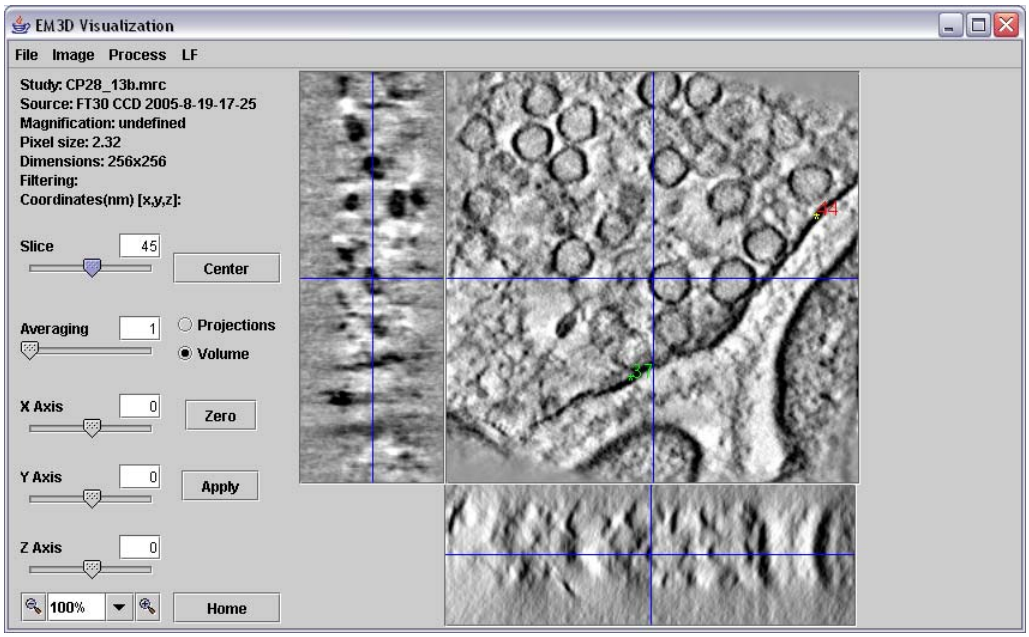


Figure 24: False fiducials

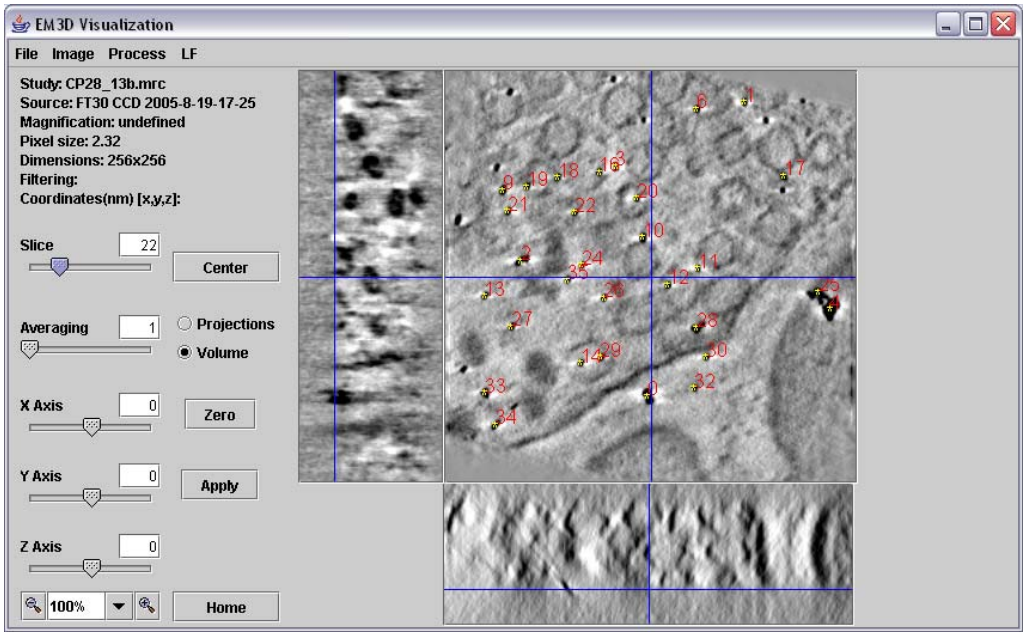


Figure 25: Correctly identified fiducials

By sorting the fiducials based on their **A Slice** number, it is easy to identify which fiducials are on either the top or bottom of the volume and which are really stain within the volume. **Delete** the incorrect fiducials.

> *Anytime fiducials are deleted the **Calculate** button should be pressed to recalculate the gross registration of the 2 volumes.*

6.3.5 Combine

When the **Combine** button is pressed the 2 volumes are merged. The algorithm uses the **Distance** information generated during the **Calculate** process to create a regional map for local warping, in order to generate the fine alignment for **combined volume**. After the volumes have been combined, the **Set: Combined** radio button becomes available so you can toggle to the combined volume. After **Combine** is pressed the **Average Error**: is set to the average RMS error for all fiducial pairs in the list after this fine alignment.

7 Segmentation

Segmentation allows the user to define or 'carve out' volumes of interest (VOIs). Each VOI should contain a distinct structural component. Each segmented object is the basis for a surface model, which can be displayed and manipulated in 3D.

> *In order to visualize the segmentation process, this manual will reference the synthetic dataset distributed with EM3D.*

7.1 Segmentation modes

Since the natures of structures observed by EM tomography vary widely, EM3D offers an assortment of ways to segment each object. All object descriptions here refer to the 2D shape in the (x, y) display.

There are two classes of segmentation available in EM3D, automatic and manual.

- **Automatic segmentation** requires the user to define an object on a single slice of the volume, then the program finds logical extensions of the object on subsequent slices.

  Below are 3 distinct sub-classes of Automatic segmentation

  - **Closed** – Used for objects that are topologically closed, such as the cross section of a sphere.
  - **Isolated**— Used for most 'open-ended' objects. The drawback to Isolated segmentation is that the algorithm used for propagating an Isolated object often contracts the anchor line as it propagates.
  - **Pinned –** Used for 'open-ended' objects that have endpoints that occur in the same X, Y position in each slice, such as a cell membrane that stretches from one edge of the volume to another edge.

- **Manual segmentation** is required for any object that is too complex for the automatic segmentation to follow, so the user must define the object on each slice.

7.2 Segmentation procedures

In this section we will outline the basic procedures for segmenting objects, using the synthetic dataset as an example. (Most of this information was taken from the EM3D Tutorial)

7.2.1 Automatic closed segmentation

The four spherical membranes are designed to simulate synaptic vesicles. Closed segmentation is the appropriate method to use for these objects because they can be fit by a closed polygon and do not vary substantially from slice to slice.

Open the Segmentation window by selecting **Process** > **Segment…** from the **EM3D Visualization** window.

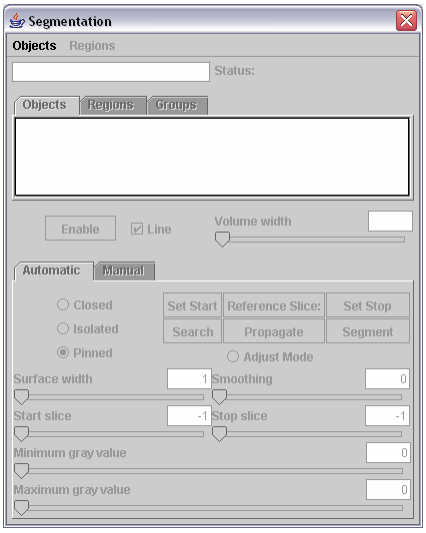


*Figure **26**: Segmentation Window*

7.2.2 Segmenting the upper right vesicle

Select **New Object** from the **File** menu.

*Figure **27**: Segmentation File Menu*

1. A default object name appears, such as Object 1, in the name field. The name should be replaced with a more descriptive name, in this case, Vesicle 1.

> *Note: All new segmentations default to the Automatic, Closed type.*

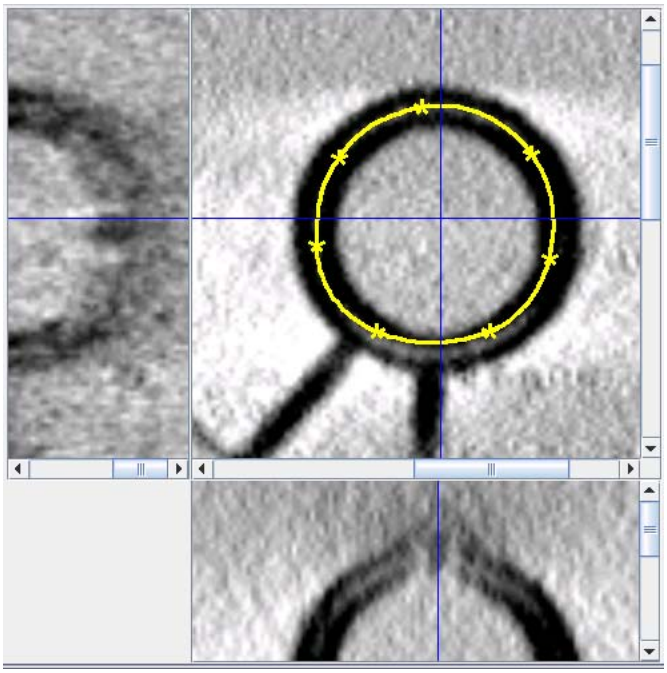


*Figure **28**: Initial segmentation anchor*

1. Use **Zoom** on the **EM3D Visualization** window to magnify the image by 250%. Then center the upper right vesicle in the X, Y display using the sliders.

2. Using the middle mouse button, select several points in the membrane of the vesicle to create a rough anchor path for the vesicle (Figure 29a). Poorly placed points can be removed by pressing the right mouse button.

> *All segmentations of the same object will vary slightly, due to the selection of the anchor points.*

3. Press **Search** (Figure 29b). Notice that the area around the yellow anchor line fills with red, and the **Minimum gray value** and **Maximum gray value** sliders in the Segmentation window adjust.

4. Reduce the **Maximum gray value** slider to a value that defines the stain only (Figure 29c) and press **Segment** (Figure 29d).

> *Note: All numbers are rough guides, since segmentations will vary based on anchor points.*

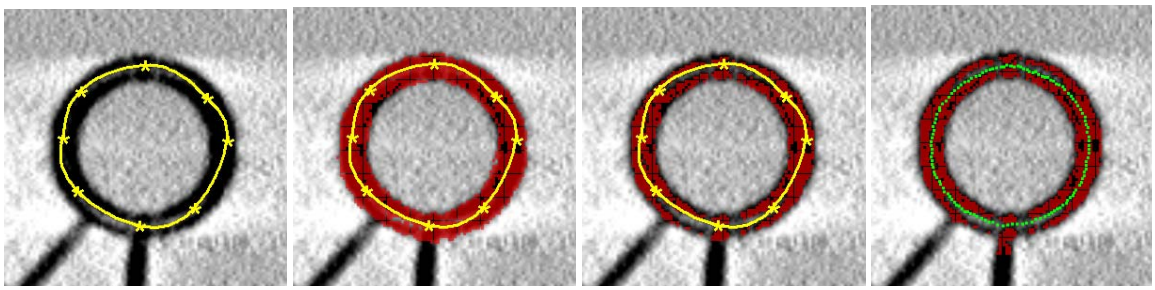


Figure 29: a) Outlined, b) searched area, c) reduced Maximum Gray value, and d) Segmented center slice of Vesicle 1.

4. Press **Propagate** to segment the remaining slices in the vesicle.

5. Press the **Home** button to return the Zoom to 100%, in order to see the segmentation in all 3 windows.

6. Left click inside the segmented vesicle in order to center that vesicle in the transverse displays.

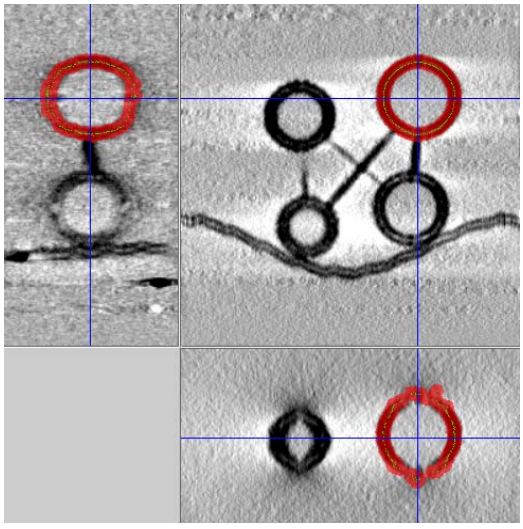


*Figure **30**: Initial propagated segmentation of vesicle 1*

7. From the 2 transverse views we see that the propagation worked well for most of the vesicle, but needs some adjustment at the top and bottom slices. (Figure 31). Adjust the propagated segmentation to correct this problem. Use the navigation controls in the **Visualization** window to find the slice where the vesicle ends, which should be around slice 87. Now, press the **Set Stop** button on the **Segmentation control** window.

> *Note: Your propagation may not look the same depending on the initial points chosen.*

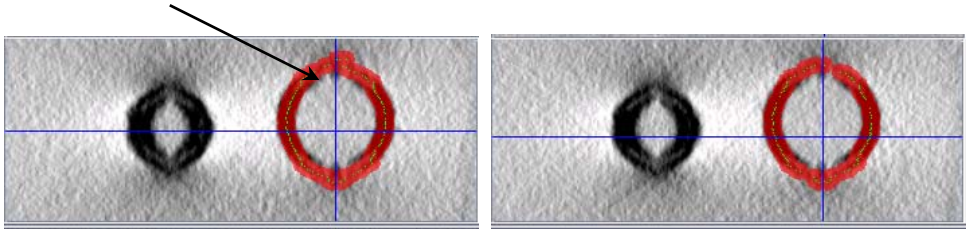


Figure 31: Propagated segmentation with artifact-induced error (left) and after adjustment (right).

8. Finally, adjust the Surface width slider until the entire structural component is enclosed (approximately 15). If possible, the surface width should always be set generously so that the entire object is enclosed by the VOI. This permits accurate generation of surface models, discussed in the next Section.

7.3 Automatic isolated segmentation

This is used for objects such as membranes and filaments.

For example, segment the synthetic, rod-like filament connecting the lower left to the upper right vesicle.

1. In the Segmentation window, select **File > New object**.
2. Name the object.
3. Select the **Isolated** radio button.
4. Navigate to center of the object in the **EM3D Visualization Window**.
5. **Zoom** into the image by 250%.
6. Place several anchor points along the rod, using the middle mouse button (Figure 32a).

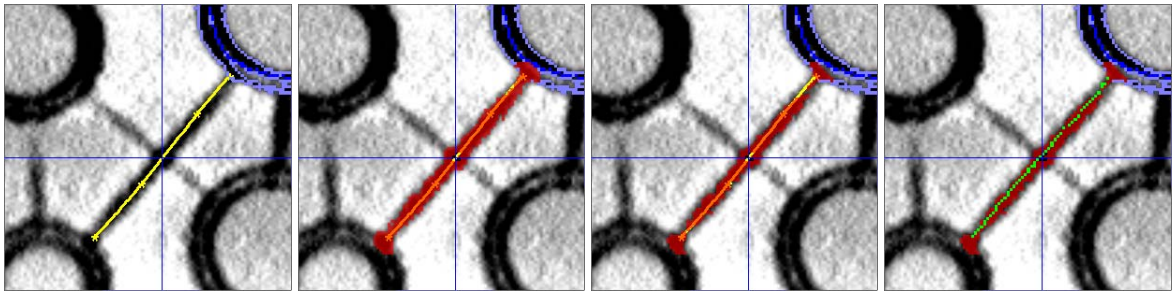


Figure 32: Isolated anchor: a) anchor line. b) searched. c)maximum grey reduced. d) Segmented.

7. Leave the **Surface width** and **smoothing** sliders at their default values:
8. Press the **Search** button (Figure 32b).

9. Reduce the **Maximum gray value** (Figure 32c).

10. Press the **Segment** button (Figure 32d).

11. Press the **Propagate** button.

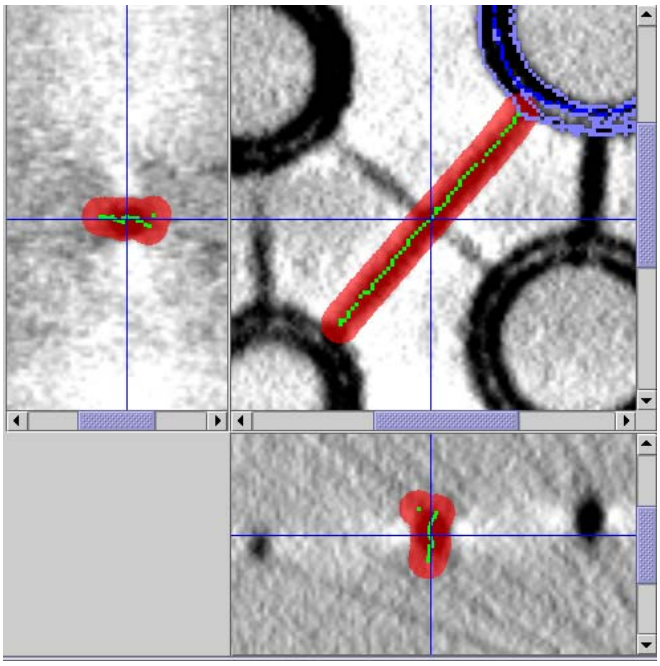


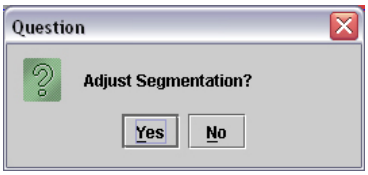
Figure 33: Propagated isolated filament.

12. Examine the segmentation in the EM3D Visualization Window.

13. If you are unsatisfied with the segmentation try the following techniques

    - Adjust the start / stop slice for the propagation and re-**propagate**.
    - Adjust individual segmentation lines by pressing the middle mouse button over the segmented filament.

      The following message will appear



      Select **Yes**.

      The yellow anchor line will reappear for the object. Adjust the anchor line and press **Segment**.

      Repeat for all slices that need adjusting, either individually or through re-propagation.

7.4 Automatic pinned segmentation

The endpoints of a pinned object occur in same x, y position throughout all the slices. This type of segmentation works well for objects like the membrane in our synthetic dataset.

1. In the **Segmentation Window** select **File > New Object**.
2. Name the object.
3. Select **Pinned** segmentation.
4. Use the middle mouse button to select points for the anchor, and the right mouse button to remove points from the anchor line.
5. Press **Search**.
6. Reduce the **Maximum gray value**.
7. Press **Segment**.
8. Press **Propagate**.

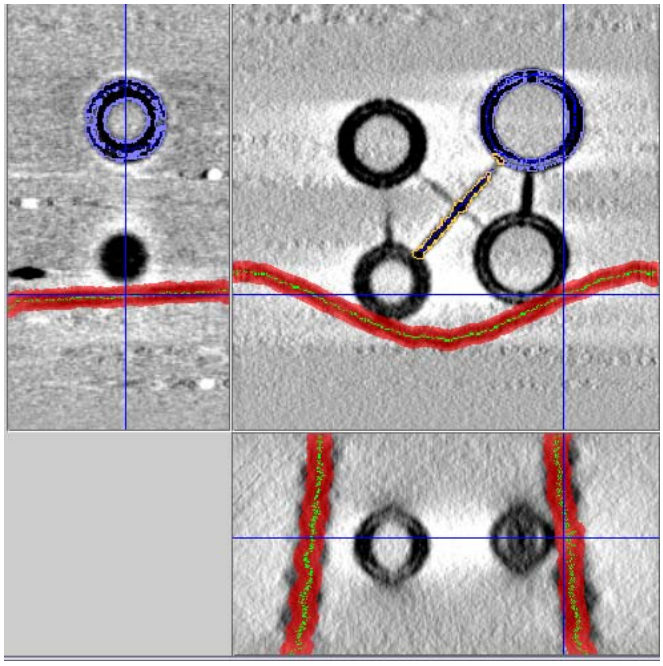


Figure 34: Propagated pinned segmentation

7.5 Strategies for adjusting automatic segmentations

7.5.1 Setting the start/stop slice

If the segmentation propagates beyond the edge of the object, then use the **Start Slice** and **Stop Slice** sliders to identify the range of the object.

7.5.2 Adjust minimum/maximum gray value

Usually it is a good idea to reduce the **Maximum gray value** to only include values that correspond to the stained object. This helps the algorithm follow the line of interest and not get attracted to noise.

7.5.3 Smoothing

The smoothing slider determines how fast the curve can deviate. By increasing the smoothing value, you can reduce the likelihood of wild fluctuations in the anchor line. Conversely if you have a shape that is changing rapidly, you will need to reduce the smoothing to allow the anchor line more flexibility.

7.5.4 Adjusting the anchor line and re-propagating

Often a segmentation will propagate smoothly for many slices, but then begin to deviate, especially with convoluted membranes. A good way to approach such a situation is to allow the object to propagate.

1. Scroll through the slices to where the segmentation begins to deviate.
2. Right click the mouse button to move into adjust mode.
3. Adjust the anchor line.
4. Press **Segment**.
5. Depending on direction you want to propagate set the **Start** or **Stop slice** to the current value.
6. Press Propagate.

Example: Initial Reference slice is 50. The segmentation starts to go awry at 60. Adjust the segmentation on slice 60 and set the Start Slice to 60. Press Propagate.

> *Note: Uncheck the **Adjust Mode** button before rendering the object.*

7.5.5 Using a thinner search width

When objects with similar grayscale are adjacent it is often a good idea to use a smaller search width when propagating the segmentation. This will help the algorithm stay in the desired object.

> *After the propagation is complete be sure to increase the width to include the entire object.*

7.6 Manual segmentation

When structural components have a complex topology or are too noisy for automatic segmentation, it is necessary to perform a manual segmentation. In manual segmentation, the goal is to literally define the boundaries of a VOI that encloses the structural component. In the following section a manual segmentation will be performed for the small filament that connects the two vesicles on the right-hand

side of the reconstructed Synthetic volume.

1. **File > New Object**.
2. Select the **Manual** tab.
3. Name the object.
4. In the **EM3D Visualization window** navigate to slice 70.
5. **Zoom** and center the object in the window.
6. This time instead of drawing an anchor line in the center of the object we are going to draw a closed path around the object.

> *Be sure to include the entire object inside the path, because only points inside the outline will be considered.*

7. Use the middle mouse button to define anchor points, creating a closed path around the object. Only a few points are necessary. The path can be defined using either the **Spline** or **Piecewise** interpolation-mode radio buttons. Spline interpolation connects the anchor points with smooth curves (Figure 35a); piecewise interpolation connects them with straight lines.

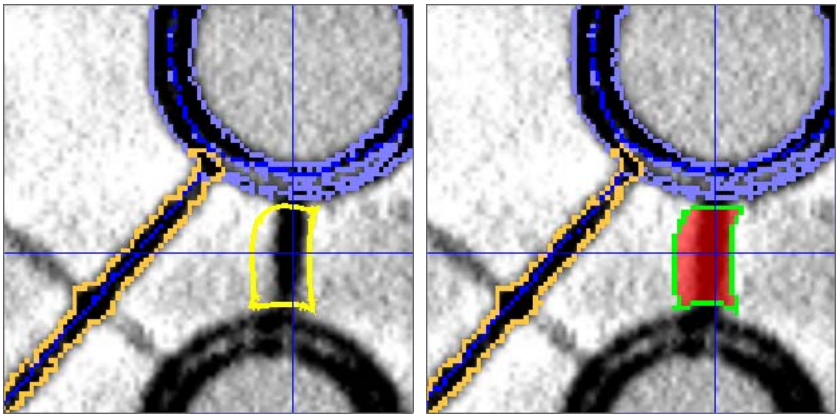


Figure 35: a) Outline for manual segmentation. b) Segmented region.

8. Press the **Segment** button. The interior of the volume is now marked with a red overlay and the anchor path turns green (Figure 35). All points in the interior on this slice have now been added to the VOI for this object.
9. Press the **Up** button to move to the next slice. The same anchor points that were defined on the previous slice appear and can be edited using the mouse with middle button to add, and right button to delete points.
10. When satisfied with the anchor path, press the **Up** button again.

> *Pressing the Up or Down button is equivalent to pressing the Segment button and moving the slider one slice.*

11. At the topmost slice containing the object, select **Segment.**

12. Return to the 1st slice segmented using the *Visualization Window Navigation Tools*; **NOT** the **Down** button.

13. Navigate down one slice, using the *Visualization Window Navigation Tools*; **NOT** the **Down** button. From here repeat steps 7 and 8 using the **Down** button to segment the lower half of the object.

14. Continue editing points as necessary (little editing will be necessary for this simple object) and pressing the Down button until the lowest slice containing the object is reached; select **Segment** again. This completes the manual segmentation of this object.

7.7 The Segmentation and Object Control Panel

The first thing to realize about this panel is that it controls BOTH the generation of **Objects**, and how those Objects are displayed in the **EM3D Visualization Window**.

7.7.1 Objects Menu

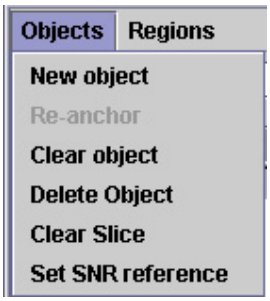


Figure 36: Segmentation Object Menu

- **New Object** – Generates an object.
- **Re-anchor** – After an object has been segmented and propagated you may want to adjust the anchor line, this can be done by either selecting Re-anchor or pressing the middle mouse button in the EM3D Visualization Window.
- **Clear Object** – Removes all segmentation that has been done in the object, as well as its name. The object still exists, it is just empty. This roughly equivalent to the combination of Delete Object, followed by New Object; however, it holds its place in the Object List.
- **Delete Object** – Completely removes the object from the Object List.
- **Clear Slice** – Removes any anchor or segmentation on the current slice.
- **Set SNR Reference** – not currently used.

7.7.2 Objects List

The Objects List is used to control which objects are overlaid on the volume in the **EM3D Visualization** Window. In order to segment or adjust segmentation, an object must first be selected in the Objects List.

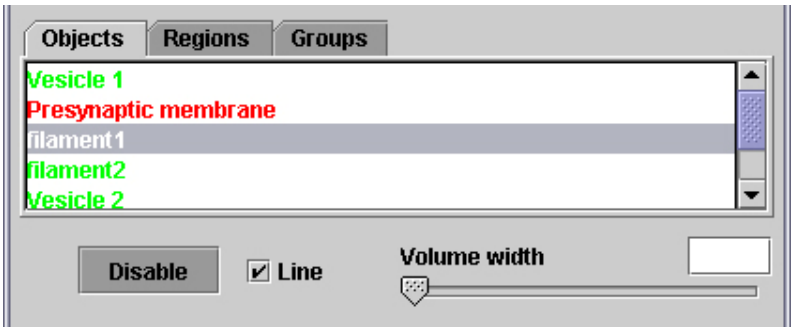


Figure 37: Object List

An object name is written in **green** if it is displayed in the **EM3D Visualization Window**.

An object name is **red** if it is hidden.

> *If an isosurface has been created for an object, the isosurface will be shown in the **EM3D Visualization Window** when it is **Enabled**. It can still be adjusted by clicking the middle mouse button inside the **EM3D Visualization Window**. This will delete the isosurface and allow segmentation adjustment as normal.*

The highlighted object is available for segmentation or segmentation adjustment.

Multiple objects can be selected for display purposes, but only one can be segmented at a time.

- The button in Figure 37 that says **Disable** is a toggle button and will change its text to **Enable** depending on whether the first highlighted object is Enabled or Disabled.
- The **Line** check box is used to display the anchor lines of *all* the objects.
- **Volume Width** is not currently used.

7.7.3 Automatic Controls

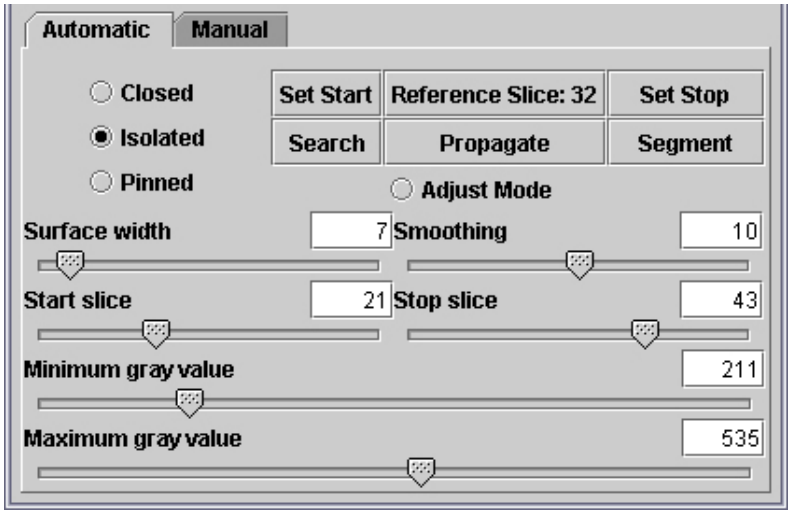


Figure 38: Automatic segmentation controls

- **Closed, Isolated, and Pinned radio buttons** are used to select which type of Automatic segmentation is to be preformed.

The **Set Start, Reference Slice, and Stop Slice** buttons are used to interact with the EM3D Visualization Window.

- **Set Start** sets the Start slice slider to the current slice in the **EM3D Visualization Window**.

  If the object has already been segmented and the new start slice is higher than the current start slice a question similar to Figure 39 will appear.

  If the **Adjust** button is selected this new start value will be used as the start for the new **Propagation** region and anything below this value will remain unchanged.

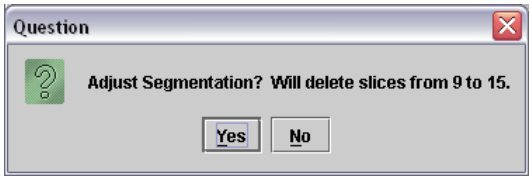


Figure 39: Adjust Start

- **Reference Slice** moves the EM3D Visualization Window slice to the Reference slice.

- **Set Stop** sets the Stop slice slider to the current slice in the **EM3D Visualization Window**.

  If the object has already been segmented and the new stop slice is lower than the current stop slice, a message similar to Figure 39 will appear.

  If the **Adjust** button is selected this new stop value will be where future **Propagations** will end; leaving the slices above it un-altered.

The Search, Propagate, and Segment buttons:

- The **Search** button finds the grayscale range in the area defined by the anchor line and the **Surface width**. It then adjusts the **Minimum** and **Maximum gray value** sliders accordingly.

- The **Segment** Button creates the segmentation on the *current* slice based on the **Minimum gray value**, **Maximum gray value**, **Surface width**, and the **Smoothing** slider values. The anchor line will change from the original hand selected points to a smooth green line that was fit to the grayscale image.

- The **Propagate** button uses the parameters defined by the **Surface width**, **Smoothing**, **Maximum gray value**, and **Minimum gray value** to define the object on slices between the **Start slice** and **Stop slice**. It stops "propagating" when no values meet the criteria. After the object has been propagated the **start** and **stop slice** slider values are set to the limits found during propagation.

- Multiple propagations can be done on the same object by defining a new anchor line, or adjusting an old one. Simply click the middle mouse button on the slice you want to be the new reference, define the anchor, and adjust the **Start** and **Stop** slice sliders. Press **Search**; adjust the **Minimum/Maximum Gray**. Press **Segment**. Press **Propagate**.

The Sliders:

- The **Surface Width** slider determines the outer boundaries of the object. Although it is only shown in 2D on the image, it is applied in 3D to the object when an isosurface is generated. It is often useful to make the **Surface width** low during the **Segmentation** and **Propagation** phases, then increase it to cover the entire volume for the isosurface generation phase.

- The **Smoothing** slider determines how fast the anchor line can vary. It is often necessary to have a high smoothing value with noisy data, to prevent the anchor line from wildly drifting.

- The **Start slice** slider sets the lower slice in the propagation range.

- The **Stop slice** slider sets the upper slice in the propagation range.

- The **Minimum gray value** and **Maximum gray value** slider determine which voxels in the volume are considered part of the object for segmentation purposes. Because the **Search** button sets these values to the extremes within the search region, it is often necessary to adjust these values so that only the stain is highlighted in red in the **EM3D Visualization Window**.

- The **Adjust Mode** button is turned on anytime a Segmentation is altered. When selected it affects how the **Start** and **Stop slice** sliders work. See **Set Start, Reference Slice, and Stop Slice** above**.**

7.7.4 Manual Controls

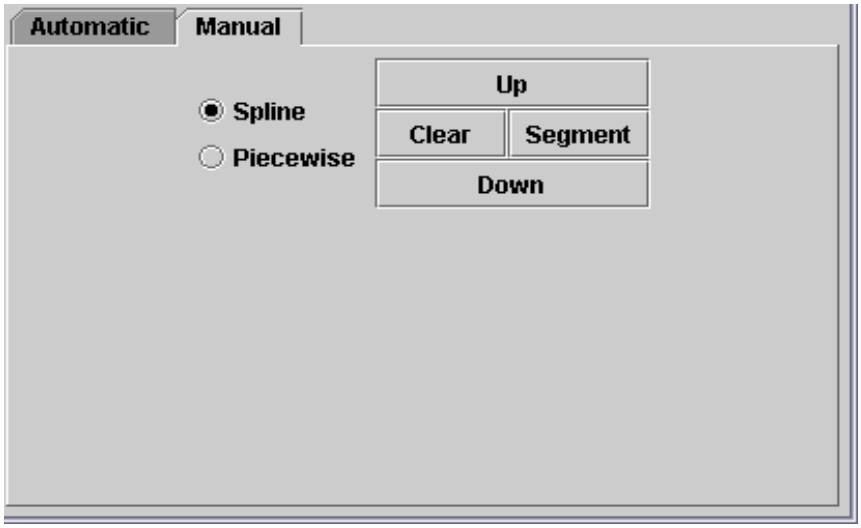


Figure 40: Manual Segmentation Control

- The **Spline** method connects the anchor points with curved lines which are influenced by multiple neighbors; whereas, the **Piecewise** method draws straight lines between points. Figure 41 shows the exact same points with Spline and Piecewise connectivity.

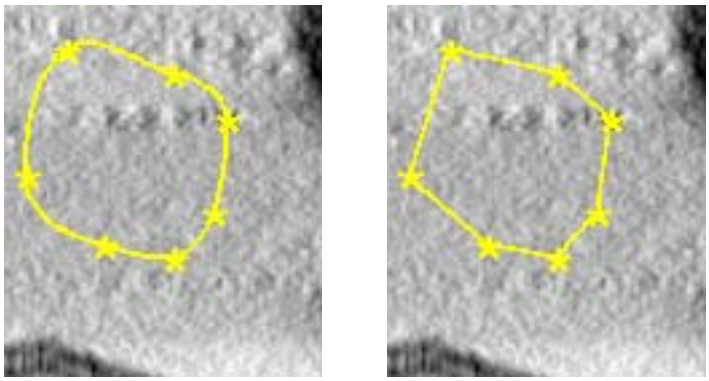


Figure 41: Spline connectivity (right) and Piecewise (left)

- The **Segment** button fills the entire area within the anchor with red overlay and adds the pixels to the segmentation region.

- The **Up** button is equivalent to pressing the **Segment** button on the current slice, then moving the **EM3D Visualization Window** to the next slice *up*, and copying the anchor line from the previous slice.

- The **Down** button is equivalent to pressing the **Segment** button on the current slice, then moving the **EM3D Visualization Window** to the next slice *down*, and copying the anchor line from the previous slice.

- The **Clear** button removes the anchor line (and the segmentation) from the current slice. It does not clear the entire object. In order to clear the entire object, select **Clear Object** from the **Objects** menu.

8 Model generation and 3D visualization controls

All features for 3D model generation and visualization are accessed from the **EM3D Render window**, which is opened by selecting **Process > Render** in the **EM3D Visualization window**. The **Render window** contains three parts, the **Render menu**, the **Render controls**, and the **Render display**. The **Render controls** allows you to select which objects are **Enabled** and the characteristics used to display them in the **Render display.** The Render Menu controls both the global appearance of the **Render display**, and provides some additional interaction with the objects and the original image volume. The **Render display** is the window where the 3D objects are displayed.

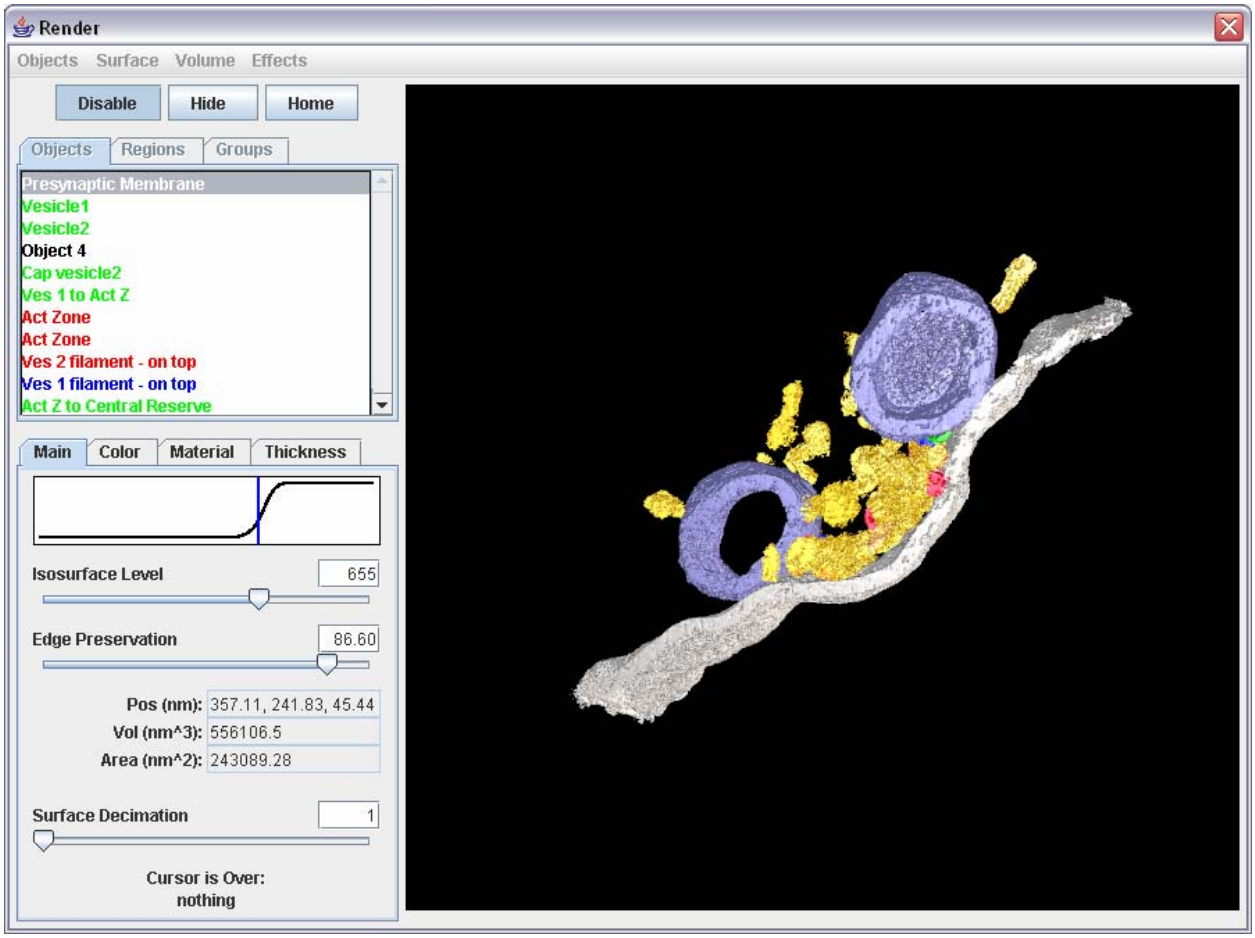


Figure 42: Render Window

8.1 Object rendering states

An object can be either **Enabled** or **Disabled**. When an object is **Enabled**, the isosurface is calculated using the region of the volume that was selected during **Segmentation** and the **Isosurface Level** selected in the **Main** tab of the **Render controls**. **Enabled** objects can be **Shown** or **Hidden**. The difference between Hide and Disable is subtle, but using the right one can save you a lot of time. If you are happy with your isosurface level, but do not want to see a particular object you can **Hide** it by highlighting it and selecting **Surface > Hide** from the **Render menu**. By doing this, the isosurface is preserved; the object is just not drawn in the **Render display**. If you press **Disable**, the isosurface will be deleted and a new isosurface will be calculated.

8.2 The Render controls

8.2.1 Object tab

This tab contains a full list of all of the objects that have been segmented. Objects can be shown in

Green – Enabled and currently visible in the **Render display**

Red – Disabled but available for Enabling.

Blue – Hidden, so the isosurface exists, but it is not visible in the **Render display**

Black – Something is wrong with the Segmentation, and it is not available for Enabling, check the Segmentation.

Multiple Objects can be selected and whatever action is performed will apply to all objects that are selected. Use Select All from the Objects menu to select all objects in the list.

Double click on an object to rename the object.

8.2.2 Group tab

This tab contains a full list of all of the groups that have been created. A group is a selection of objects arranged together. See Objects menu on how to create a group. Objects can be shown in

Multiple Groups can be selected and whatever action is performed will apply to all objects that are selected. Use Select All from the Objects menu to select all groups in the list.

Double click on a group in the list to rename the group.

8.2.3 Main control tab

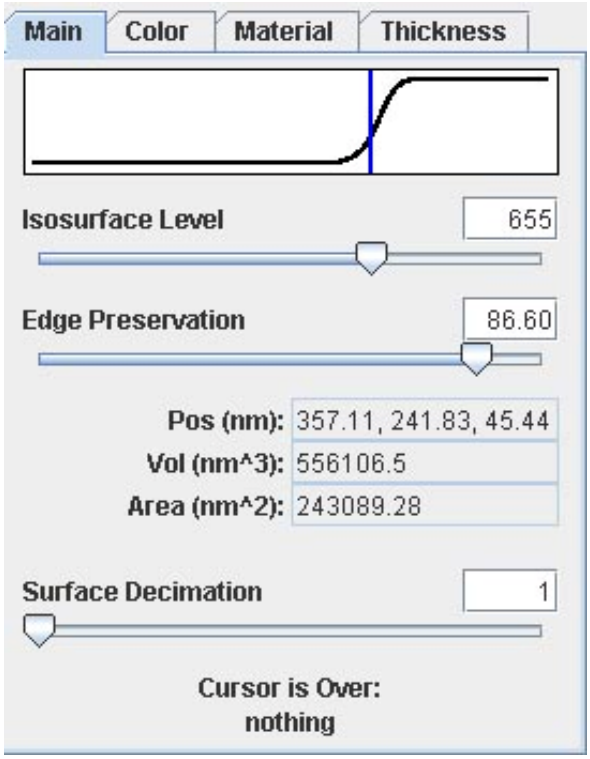


Figure 43: Render controls - Main tab

- **Isosurface level** - An isosurface is created by connecting points in the volume that have the same grayscale value. The surface that is generated does not have to be connected.

  Each level corresponds to 0.1% of the grayscale range of the segmented object.

$$grayScaleRange = mxGrayScale - mnGrayScale$$

$$level = 0.001 * (grayValue - mnGrayValue) / grayScaleRangle$$

- Edge Preservation
- Position, Volume and Area

  The **Position** value gives the centroid of the surface points for the selected object(s).

  Surface area is the total area of *all* triangles used to create the 3D surface model, and is highly dependent on the isosurface level chosen.

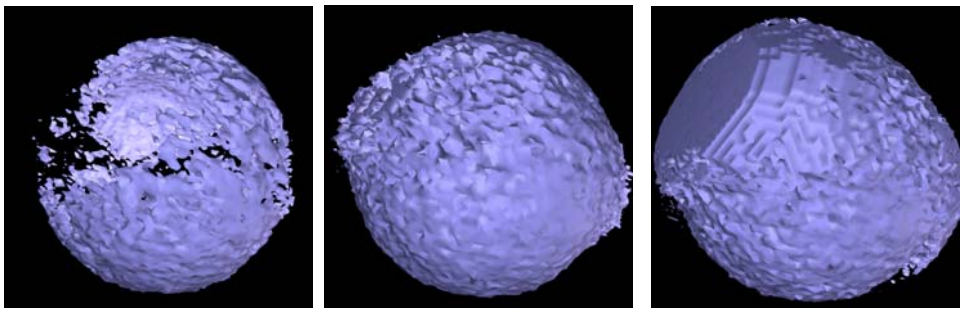


Figure 44: Surface area and Volume range based on isosurface level

Isosurface level	650	675	700
Surface Area ($nm^{\wedge 2}$)	24366	35514	27394
Volume ($nm^{\wedge 3}$)	21624	59583	96236

*Please remember that the **surface area (SA)** and **Volume (V)** are based on the triangles of the object model. This usually results in SA's more than twice the exterior surface area of the object, and volumes far less than the volume of the membrane.*

Example: Spherical Vesicle in Figure 44 with outer radius of 32nm and inner radius of 21nm.

Outer SA: $4 * \pi * r^2 = 4 * \pi * 32^2 = 12868nm^2$

Inner SA: $4 * \pi * 21^2 = 5542nm^2$

Total SA: $12868+5542 = 18410nm^2$

Outer Volume: $4 * \pi / 3 * r^3 = 4* \pi /3 * 32\wedge 3 = 137258nm^3$

Inner Volume: $4* \pi /3 * 21^3 = 38792nm^3$

Total Volume: $38792-137248 = 98466nm^3$

Isosurface level	650	675	700
Surface Area % calculated	1.32	1.93	1.49
Volume %	.21	.61	.98

calculated

- **Surface Decimation** combines vertices for rendering the selected surface model to reduce data. Use a high decimation for speeding up the enabling of a surface model.

8.2.4 Color control tab

EM3D uses an RGB color model. Figure 45 represents how red, green, and blue blend together to form other colors. Each color has an intensity range from 0-255. Figure 45 represents the 6 sides of a cube created with blue, red, and green as the axes. In each of the 3 surfaces shown on the left, one color is set to 255. For instance on the top surface green is set to 255 and contributes its maximum intensity to all colors blended on that surface, while in the image on the right there is no green contribution to the bottom surface.

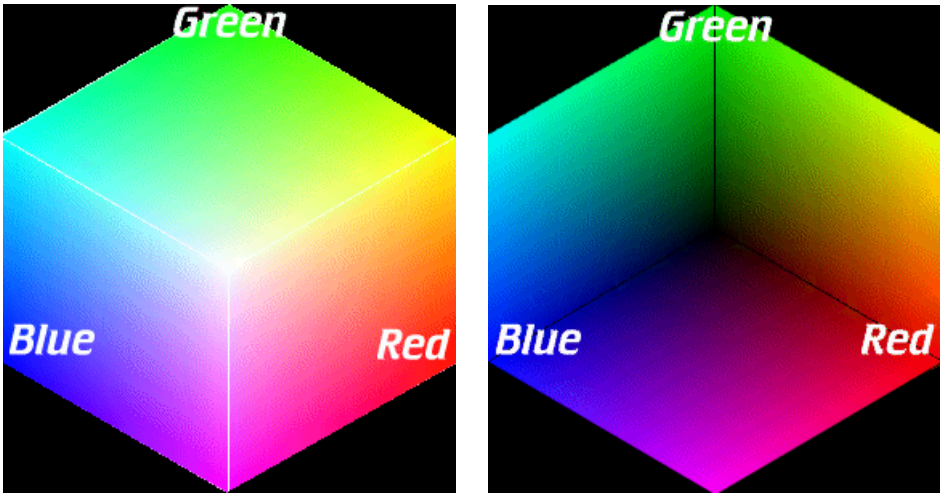


Figure 45: RGB color volume

As you move the sliders in the **Color** tab, the **New:** color swatch will immediately change. If **Auto Apply** is selected then the object in the **Rendering** window will also change color.

> *Note: The color change may lag in the Rendering window if Auto Apply is selected with large datasets.*

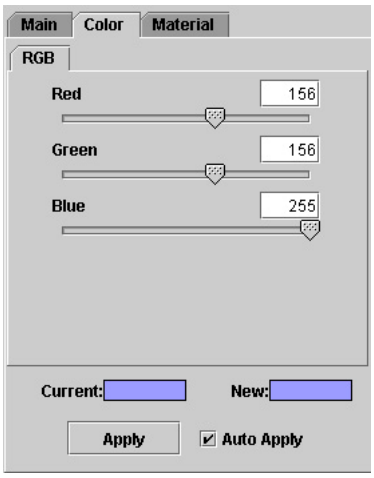


Figure 46:Color Tab

8.2.5 Material tab

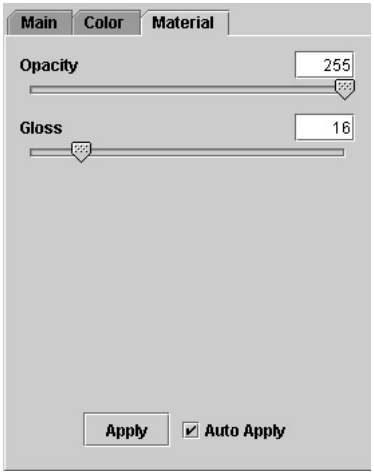


*Figure **47**: Material tab*

- The **Opacity** slider controls the transparency of the object(s). If it is set to 255 it is completely opaque and if it is set to 0 it is invisible.

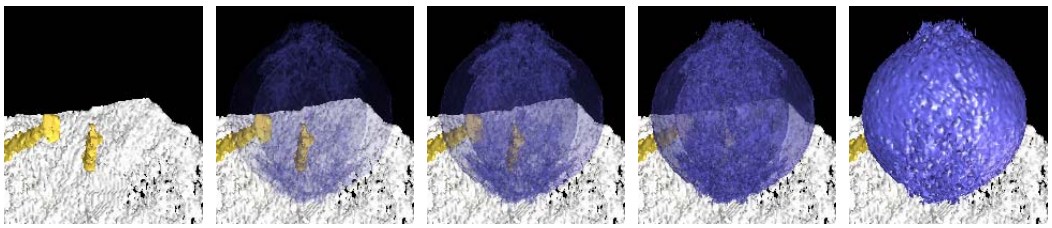


Figure 48: Vesicle opacity of 0, 25, 50, 100, and 255

- The **Gloss** slider controls how reflective the surface is.

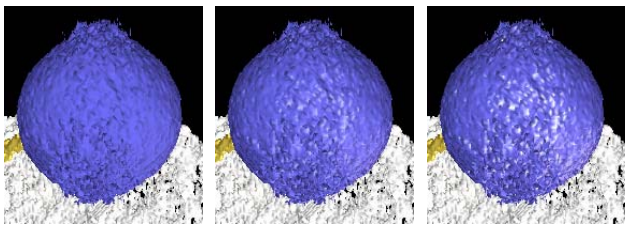


Figure 49: Gloss of 0, 50, and 100

8.2.6 Thickness, Proximity and Uncertainty tab

For a detailed description of the Thickness, Proximity and Uncertainty control tab see Calculations.

8.3 Objects menu

- The **Select All** menu selects all objects in the objects list. When the group tab is open all groups are selected.
- **Group** arranges selected objects together to a group. First select objects for grouping. Second go to Group in the Objects menu and type in a name for the group.
- **Auto 2D Enable** – Check that box to mark the object in the 2D Visualization window when objects get enabled in the Rendering window.
- **Delete** erases the selected group from the group list.
- Combine – not implemented yet.
- Export Object – not implemented yet.

8.4 Effects menu

8.4.1 Anti-Alias

Anti Alias - Display the models at 4 levels of alias reduction: 2x, 4x, 8x, 16x or with out reduction (off).

8.4.2 Lighting

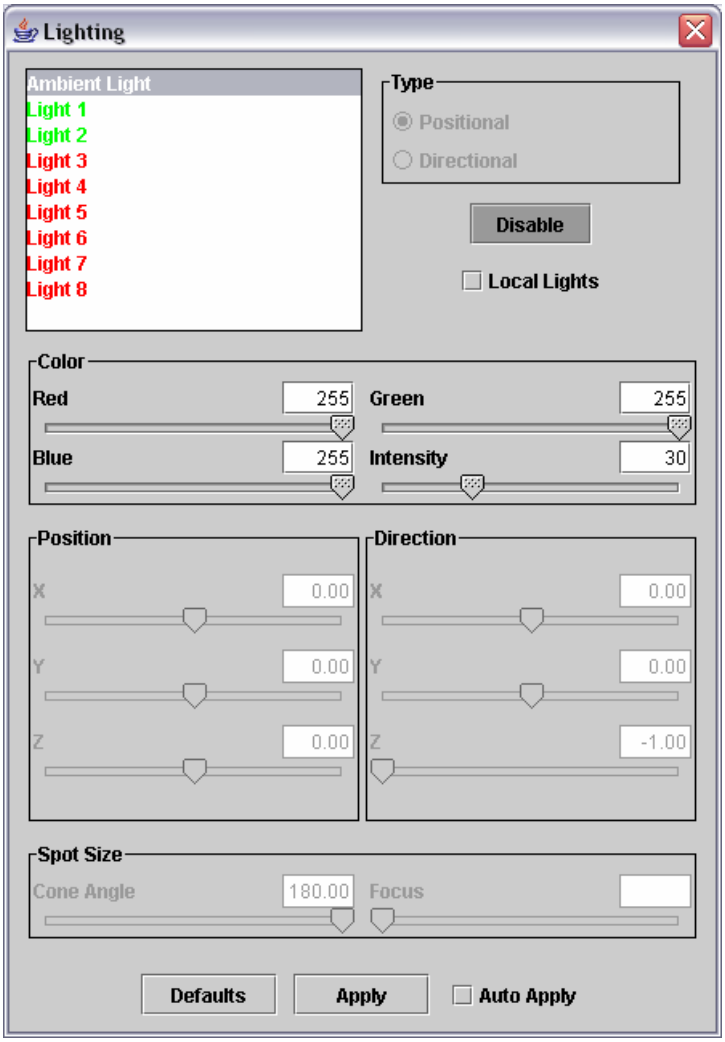


Figure 50: Lighting control

- **Ambient** lighting is the most general kind of lighting. It is the background light, Think of ambient light as having no direction and no source (technically not true, but a good way to visualize it). By adjusting the **color** and **intensity** of the ambient lighting you can change the general appearance of the rendering. Because ambient lighting refers to all background light there is no need for multiple ambient lights.

- **Directional** lighting is the second most generic form of lighting we will use. Think of a plane or sheet source of light with all the rays pointing parallel to the vector *from* the **X, Y, Z** position towards the center of the volume. You can control the color, intensity, and direction of a Directional light.

- **Positional** lighting is a cone beam of light beginning at a specified point. This is our spot light. For a positional light you can pick its, **color**, **intensity**, **origin** (position), **cone angle** spread, and the **direction** it points. Additionally, if **Local Lights** is checked then Positional lights move with the Rendering, continuing to spot light the areas at which they were

originally targeted. If Local lights is unchecked, the region of space that is illuminated does not change, similar to a street light on a freeway, it lights up whatever car goes underneath it.

8.4.3 Clip Panes

The Clip Planes window controls a maximum of 6 clipping planes. A Clip Planes cuts away the portion of the models on one side of the plane.

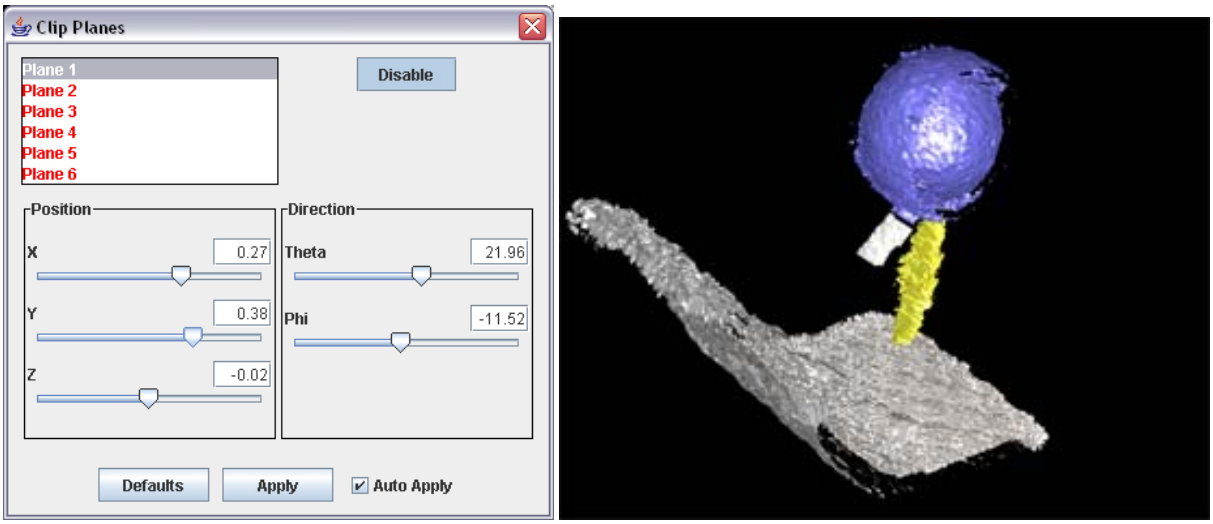


Figure 51: ClipPlanes control and example

- **Clip Planes list** – 6 different cut planes can be selected.
- **Position** - X, Y, Z sliders define the position of the selected clipping plane in the volume.
- **Direction** - The Theta slider defines the rotation angle about the z axis, and the Phi slider defines the rotation angle about the x axis.
- **Enable** - The Enable button initiates clipping for the currently selected plane in the Rendering window. The label of the button changes to Disable. Press the button to unclip the models along the plane in the Rendering window.
- **Apply** - It applies changes of the clip planes settings (Position and Direction) in the Rendering window. Check the Auto Apply check box to automatically apply any change in the settings of enabled clip planes.
- **Defaults** - Disables all clip planes and sets Position and Direction to default settings.

8.4.4 Cut Planes

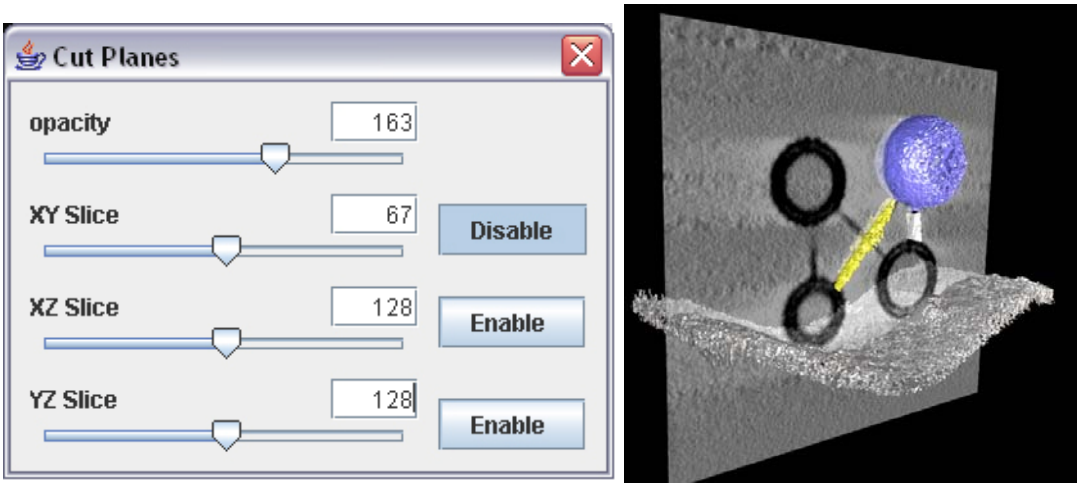


Figure 52: Cut Planes Control and example of XY Slice Cut Plane enabled

The Cut Planes window controls the display of up to 3 principal-axis cut planes overlaid on the 3Dmodel rendering.

- **Opacity** controls the opacity/light resistance of the planes within the 3D rendering.
- The **XY**, **XZ**, and **ZY** Slice sliders specify the cut plane slice displayed in the corresponding axis.
- **Enable**/**Disable** enables or disables the corresponding cut plane.

9 Calculations

Thickness, Uncertainty and Proximity are available calculations. The controls for these calculations are accessed from the tabbed controls panels located at the middle of the Rendering Window. Surface models must be created (see Object rendering states) before initiating any of these calculations.

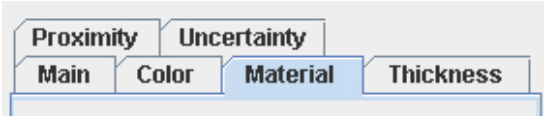


Figure 53: Thickness, Proximity and Uncertainty control tabs

9.1 Thickness

The Thickness tab controls thickness calculation of the selected surface model objects.

- The **Calculate** button initiates the thickness calculation. Select one-or-more objects in the object list for which the thickness should be calculated.
- The **plot window** displays a histogram of the frequency of vertices per measured thickness. Move the cursor to the graph or color bar to show the exact thickness at the current point.
- The **Min** and **Max** sliders eliminate vertices smaller and greater the minimum/maximum thickness value (outliers) and recalculate statistics.
- The **min**, **max**, **med**, **mean** and **bad fields** located under the histogram, display, respectively, the minimum value, maximum value, median value, mean value and percentage of bad values for the thickness data.
- The **Overlay** checkbox controls the display of the thickness data as color map overlaid upon the associated surface model.

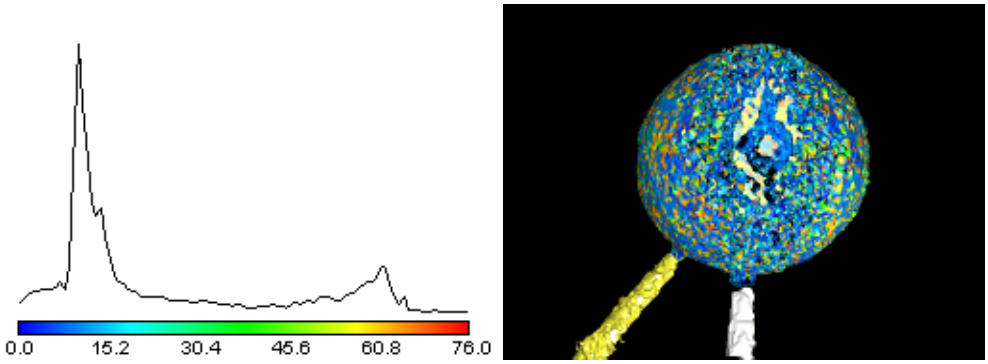


Figure 54: Histogram of vesicle thickness and colored overlay in vesicle

9.2 Proximity

The Proximity tab in the Rendering Window controls proximity calculations among the currently enabled objects. These calculations require that one object be designated as a reference, while one-or-more models be designated as destinations. Proximity values are distances between each destination vertex and its nearest vertex on the reference object surface.

- **Set Reference** button sets the selected object from the object list as reference.
- **Calculate** initiates the calculation of the proximity. One-or-more objects have to be selected for which the proximity to the reference surface model will be calculated.
- The plot window displays a histogram of the frequency of voxels per measured distance to the reference object. Move the cursor to the graph or color bar to show the exact distance at the current point.
- The **Min** and **Max** sliders eliminate voxels smaller and greater the thickness value in the fields (outliers) and recalculate statistics and overlay.
- The **min**, **max**, **med**, **mean** and **bad fields** located under the histogram, display, respectively, the minimum value, maximum value, median value, mean value and percentage of bad values for the proximity data.
- The **Overlay** checkbox controls the display of the proximity data as color map overlaid upon the associated surface model.

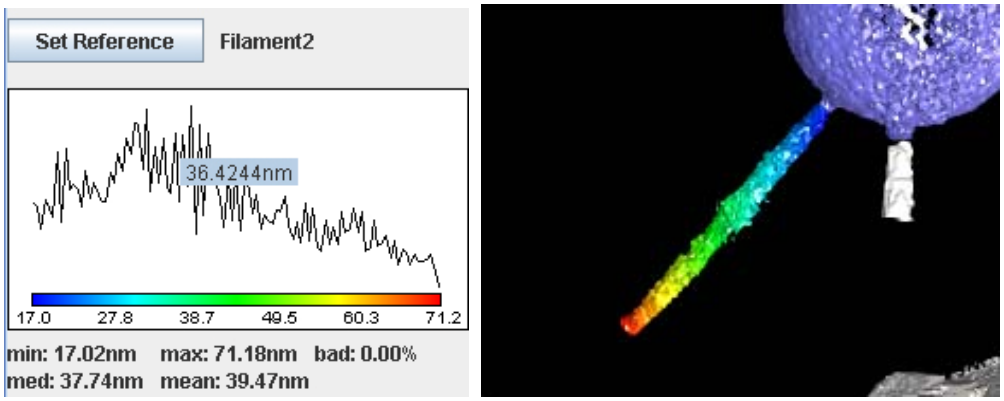


Figure 55: Proximity histogram of filament1 and filament2 and visualization in Rendering window

9.3 Uncertainty

The Uncertainty tab in the Rendering Window controls the spatial uncertainty calculation, a measure of the reliability of an isosurface model. Before calculating the uncertainty it is required to manually segment an area of noise as a reference object.

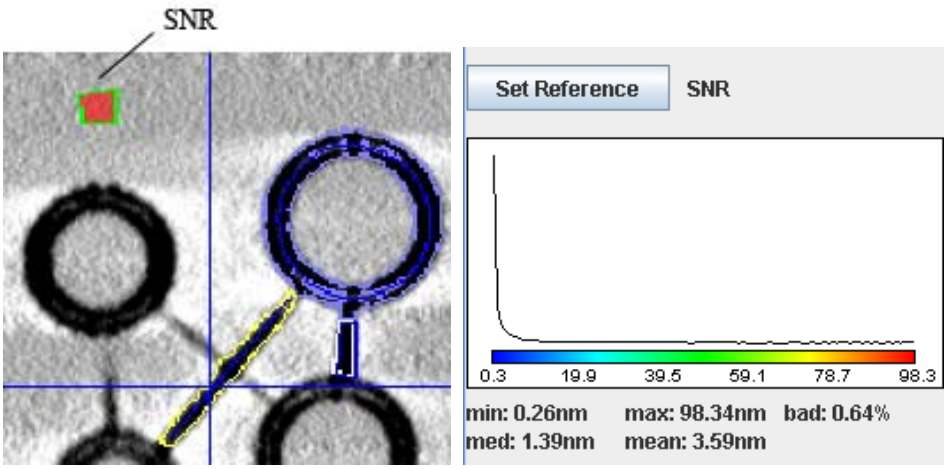


Figure 56: Manually segmented signal-to-noise ration and special uncertainty histogram

- The **Set Reference** button sets the selected object as the SNR reference.
- **Calculate** initiates the calculation of the uncertainty. One-or-more objects have to be selected for which the uncertainty will be calculated.
- The plot window displays a histogram that combines all of the special uncertainty result from the currently selected models.
- The **Min** and **Max** sliders eliminate voxels smaller and greater the value in the Min/Max field (outliers) and recalculate statistics and overlay.
- The **min**, **max**, **med**, **mean** and **bad** fields located under the histogram, display, respectively, the minimum value, maximum value, median value, mean value and percentage of bad values for the combined calculated data ensemble.
- The **Overlay** checkbox controls the display of the thickness data as color map overlaid upon the associated surface model.

10 Appendix

10.1 Acknowledgments

We would like to acknowledge the following groups whose code is either used in this program or has provided inspiration:

- Paul Bourke

  Algorithm from "Polygonising a scalar field" by Paul Bourke
  http://astronomy.swin.edu.au/~pbourke/modelling/polygonise

- Takuya Ooura

  "General Purpose FFT (Fast Fourier/Cosine/Sine Transform) Package"

  http://momonga.t.u-tokyo.ac.jp/~ooura/fft.html

- Lee Thomason and Yves Berquin

  "TinyXml"

  http://sourceforge.net/projects/tinyxml

- LAPACK

  Linear Algebra PACKage

  http://www.netlib.org/lapack

- Fautré , Tanguy

  Tri Stripper

  http://users.pandora.be/tfautre/softdev/tristripper/

- Ken Martin, Will Schroeder, and Bill Lorensen

  vtkThinPlateSplineTransfrom algorithm from "VTK"

  http://public.kitware.com/VTK

  Copyright (c) 1993-2002 Ken Martin, Will Schroeder, Bill Lorensen

  All rights reserved.

  THIS SOFTWARE IS PROVIDED BY THE COPYRIGHT HOLDERS AND

CONTRIBUTORS ``AS IS'' AND ANY EXPRESS OR IMPLIED WARRANTIES,INCLUDING, BUT NOT LIMITED TO, THE IMPLIED WARRANTIES OFMERCHANTABILITY AND FITNESS FOR A PARTICULAR PURPOSE AREDISCLAIMED. IN NO EVENT SHALL THE AUTHORS OR CONTRIBUTORS BE LIABLE FOR ANY DIRECT, INDIRECT, INCIDENTAL, SPECIAL,EXEMPLARY, OR CONSEQUENTIAL DAMAGES (INCLUDING, BUT NOT LIMITED TO, PROCUREMENT OF SUBSTITUTE GOODS OR SERVICES;LOSS OF USE, DATA, OR PROFITS; OR BUSINESS INTERRUPTION)

HOWEVER CAUSED AND ON ANY THEORY OF LIABILITY, WHETHER IN CONTRACT, STRICT LIABILITY, OR TORT (INCLUDING NEGLIGENCE OR OTHERWISE) ARISING IN ANY WAY OUT OF THE USE OF THIS SOFTWARE, EVEN IF ADVISED OF THE POSSIBILITY OF SUCH DAMAGE.

10.2 Glossary

CCD: A device made up of semiconductors arranged in such a way that the electric charge output of one semiconductor charges an adjacent one. Used to acquire images like a camera.

Cumulative Distribution Function: The probability that an image value is less than or equal to the current value, given the sum (numerical integral) of the image histogram *f*(*i*):

$$F(x) = \sum_{i=0}^{x} f(i)$$

Decimation: The image or volume size reduction expressed as a $\log_2$ exponent, so a decimation of 0 means no decimation, full resolution. A decimation of 1 is 1/2, decimation of 2 is $1 / 2^2$ or 1/4, decimation of 3 is $1 / 2^3$ or 1/8, etc.

Fiducial Markers: also referred to as fiducials, colloidal gold beads placed on the surface of the TEM sample or incorporated into the sample used to align the projections.

Intersection: Equivalent to Logical AND, meaning that a point is not added to the new region unless ***all*** selected VOI's exist at that point

Invert: Invert the grayscale of an image to create a negative image.

Isosurface/isodensity: A region or surface in which the image grayscale is the same, similar to topographic lines on a map.

Projections: Electron micrographs of the individual tilts in the dataset used to reconstruct a 3D volume.

Radio Buttons: A group of buttons in which exactly one button is selected at a time.

Signal-to-Noise Ratio: The ratio between 'signal', the desired quantity measured by an image, to the 'noise,' the random variations in that quantity. In EM tomography, the signal is usually the contrast between a structural component and its adjacent background, while the noise is a complex function of the EM projection image formation and subsequent tomographic processing steps, so SNR is usually estimated empirically from the standard deviation of a relatively uniform background portion of the reconstructed volume.

Slices: These are the 2D images that are virtually sliced (interpolated) from the reconstructed volume.

Tessellation: Creation of small triangles that represent a surface and can be used in surface rendering.

Transverse: The 2 orthogonal views to the *x*,*y* plane – the (*x*,*z*) and (*z*, *y*) planes.

Topology: Properties of geometric figures or solids that are not changed by stretching or bending. Donuts and picture frames have equivalent topologies.

Twist Angle: The rotation of the projection tilt axis about the depth axis

Union: The logical OR of two or more objects, i.e. considered true if ***any*** object lies in the space

Vertex: A point in 3D space that defines a corner of one or more polygons.

Volume of interest: A subset of the volume containing defined by the user or an object.

Voxel: Short for volume element, the smallest distinguishable cube in a three-dimensional image.

10.3 Acronyms

CCD: Charged-Coupled Device

EM: Electron Microscope

MIP: Maximum Intensity Projection

MPEG: Moving Pictures Experts Group, digital video format

MRC stack: output dataset from the microscope

nm: nanometer 1x10-9m

RMS: root mean square

SNR: signal to noise ratio

TEM: Transmission Electron Microscope

TIFF: Tagged Image File Format

VOI: Volume of Interest

10.4 Table of Figures