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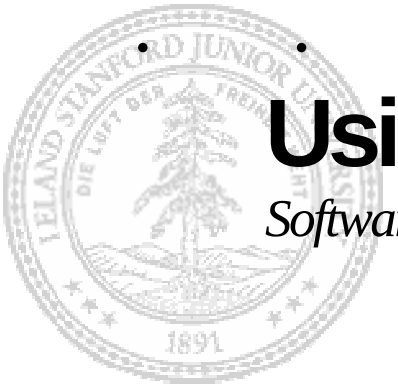

# Using EM3D©

*Software for Electron Microscope Tomography*

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# 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.

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. The software was initially developed by Dr. David Ress in this laboratory and is now being maintained, improved, and extended by David Yip, Cornelia Koch-Stoschek, and Mira Raman.

This tutorial is designed to instruct a new user in the analysis of EM tomography data. The tutorial takes the user from the beginning stages of importing a small synthetic dataset through the entire process of data analysis. Details on acquiring the EM3D application, Synthetic datasets, and IDL Virtual Machine can be found at http://em3d.stanford.edu.

This release of EM3D has the typical benefits and liabilities of early software releases. Nevertheless, major efforts are being made on many levels to make EM3D intuitive, stable, and easy to use, as well as to develop new tools and features. 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.

EM3D is implemented using IDL (Interactive Data Language), which makes use of an extensive graphics library as well as numerous tools for rapid applications development. Research Systems, Inc., provides a free version of IDL, called IDL Virtual Machine (**http://www.rsinc.com/idlvm/index.asp**), which can run EM3D. IDL VM is available from **http://www.rsinc.com/download**.

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

# 1. Getting Started

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

## 1.1. An introduction to this text

### 1.1.1. Main sections of text

This text is divided into 3 main sections and an Appendix.

1. **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.

2. **Tutorial** - This is a step-by-step introduction to the EM3D software that takes the user through all the steps involved in the successful analysis of TEM data.

3. **Reference Manual** - This section provides more in-depth information about each feature in EM3D.

4. **Appendix** – Contains a glossary of terms, acronym list, and a list of figures.

> ***Note**: Section numbers in the **Tutorial** and **Manual** correspond so that more information about a section can easily be found. For instance, Section 3.1 is the Tutorial section on loading data, and section 4.1 is the Manual section on loading data.*

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.

### 1.1.2. Syntax of text

Items in **bold** refer to elements of the EM3D User Interface, such as menu items, sliders, buttons, etc.

**Projection Number** slider, **File** menu

The first time a glossary name is used, it is written in ***bold italics***.

***Voxel***

EM3D and tutorial related definitions are written in Italics.

*State* file, *Binary* file.

## 1.2. Some precautions and recommendations

Although the synthetic dataset that accompanies this tutorial is designed to simulate real EM data, it does have some different characteristics. For instance, because of the simplicity of the artificial data some objects in the reconstructed volume exhibit unusually prominent reconstruction artifacts. Nevertheless, working through the tutorial with this synthetic data will enable the new user to quickly learn the features of EM3D.

The size of a dataset that can be loaded is dependent on both the capabilities of the computer and the memory restrictions of IDL. For instance we have found that on a Windows machine IDL is only capable of allocating up to 1.3GB of memory, regardless of greater hardware specifications. Additionally, IDL is a 32bit program so on any platform the process space available to EM3D is less than 3GB, even if the computer has far more memory. Here are some tips for dealing with large datasets:

- Decimate the projections as they are initially loaded
- Reconstruct a decimated volume
- Produce a core (subvolume) from the reconstructed volume
- After reconstruction, do not load the projections
- Decimate the rendering volume

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.

Slight variations in results have been observed based on platform and version of IDL used.

## 1.3. 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.

### 1.3.1. Import raw EM projections

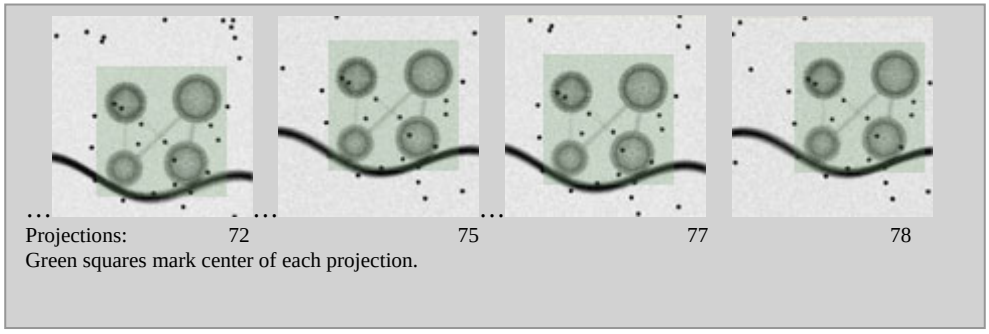



*Figure 1: Raw, unaligned EM projection images*

### 1.3.2. 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 rotate around a common axis.

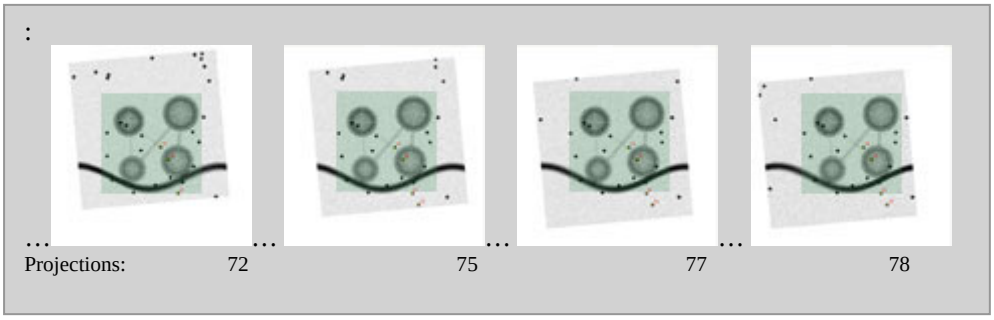



*Figure 2: Aligned projections*

### 1.3.3. 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 images corresponding to orthogonal cut planes.

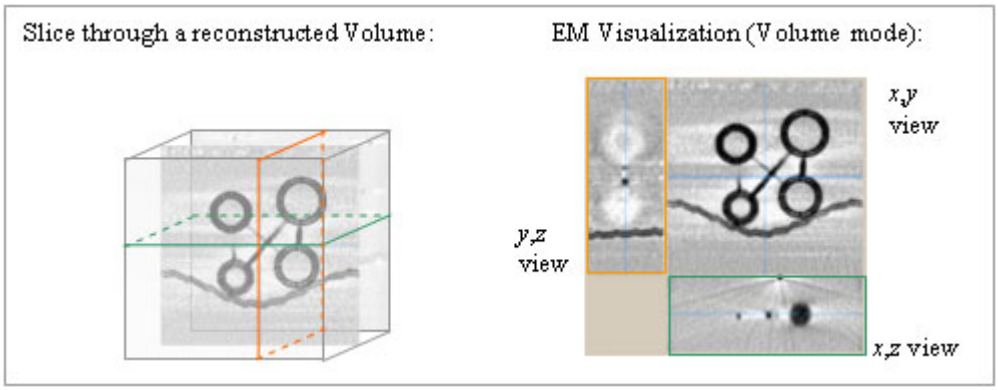
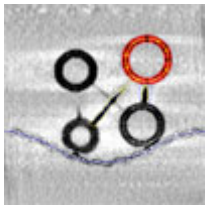


*Figure 3: Reconstruction*

### 1.3.4. 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*

### 1.3.5. 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.

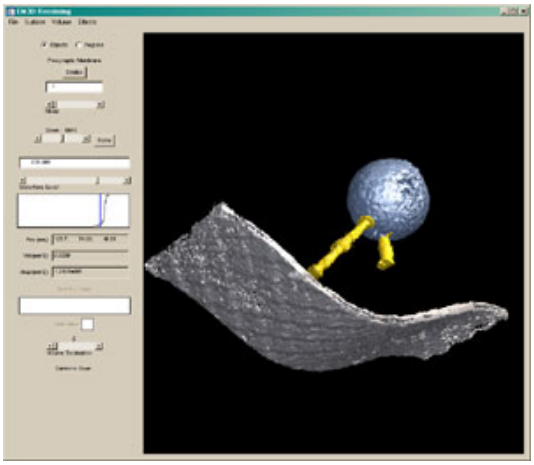


*Figure 5: Surface model of a synthetic synaptic vesicle (blue), presynaptic membrane (gray) and two filaments (yellow)*

### 1.3.6. Calculation

The surface models can be analyzed to measure their spatial uncertainty, proximity to neighboring objects, and other quantitative features. The results of these calculations can be visualized as a colormap overlaid on the rendered surface models.

## 1.4. EM3D file format and maintenance

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

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

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

The *State* file is a relatively small file that contains all the changes made with respect to the larger *Binary* file such as projection alignment and segmentation data. The *Binary* file 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., *dataset1.idl*, *dataset2.idl*, *dataset3.idl*, etc. By saving changes in the smaller *State* files, and retaining the larger *Binary* file, disk space is conserved with successive steps in the process. The *Binary* file needs only to be saved after a tilt series is reconstructed into a volume, or if the volume is filtered, decimated, or cropped. In the latter case, it is best to create a new directory that, once again contains only a single Binary file and its associated state files.

> ***Caution:*** *All State files contain a reference to the Binary file from which they were generated; so changing file names outside of EM3D will result in errors.* ***Do not*** *change file names by changing the file's icon name. Name or rename files only inside EM3D through the* ***File > Save > State*** *or* ***Binary and State*** *procedure.*

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 TIFF files or one, or more, MRC stack files.

## 1.5. Downloading EM3D

The newest release of EM3D is available for download at http://em3d.stanford.edu.

## 1.6. Launch EM3D

### 1.6.1. Windows platforms

Double-click the **em3d.sav** icon or double-click em3d.sav from a file browser.

Dismiss the IDL VM splash screen by pressing the button marked "*click to continue*".

### 1.6.2. Macintosh, Cygwin, Linux, and Unix platforms

From a terminal window launch the IDL virtual machine by entering ***idl –vm***

Dismiss the IDL VM splash screen, and a file-browser window will open.

Use the file browser to locate and select the *em3d.sav* file (Figure 6).

> *Note: If the IDL VM does not start on Mac, Cygwin, and Linux ensure that X11 is installed.*

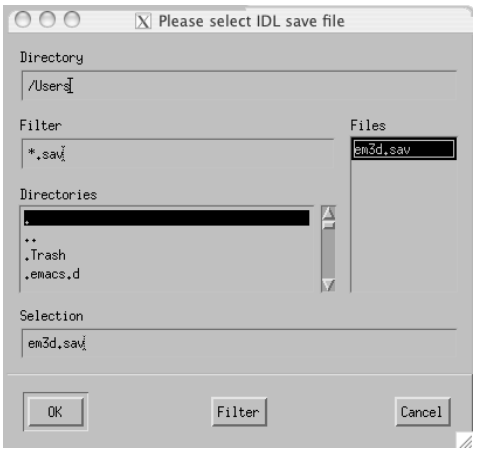



*Figure 6: Startup file-browser window*

# 2. Tutorial

## 2.1. Importing and saving datasets

Data recognition by the software is first accomplished by importing your images into EM3D, and saving the file in the proper format to an appropriate directory.

When you start EM3D, a file browser window appears. This will be used during later stages of processing to load files already in the EM3D format. To perform raw data import, dismiss the file browser window by pressing the **Cancel** button on its lower right corner.

**Summary**

1. Import TIFF files.
2. Describe data in the study.
3. Save in EM3D format.

### 2.1.1. Load TIFF files

Select **File > Import > TIFF**.

Make sure the **Filters** or **Files of type** field includes ***.TIFF**

Navigate to the *TIFFs* directory in the EM3D distribution software.

Select all of the syntheticA_###.TIFF files in the appropriate sequence. On Macintosh, Unix, or Linux platforms select *syntheticA_000.TIFF* with the left mouse button. Scroll to the bottom of the list, hold the <*shift*> key, and select the last file, *syntheticA_150.TIFF*. All files should be highlighted. On Windows platforms it is easiest to use the <ctrl-A> (select all) key.

Select **OK**, and the dataset import window will appear (Figure 7).

### 2.1.2. Describe data

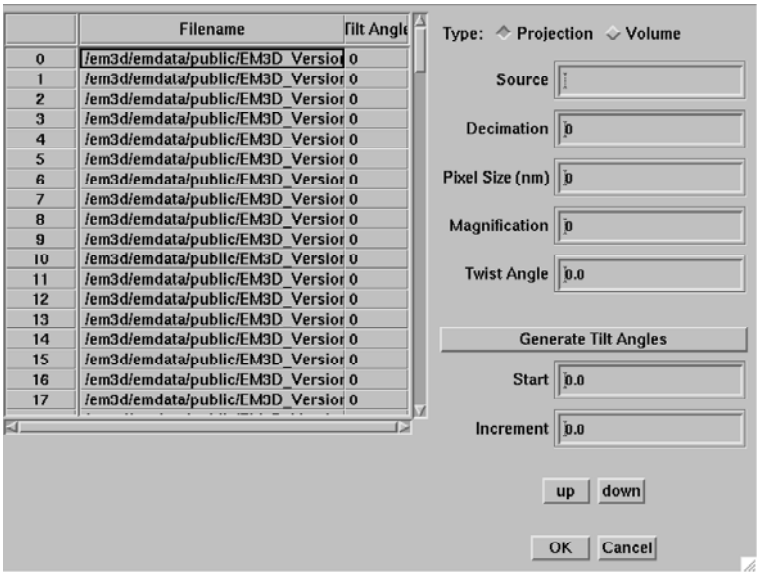



*Figure 7: Dataset import window*

Fill in the following fields:

- **Source:** Synthetic <Enter>
- **Pixel Size:** 1.0 <Enter>
- **Start:** -75.0 <Enter> (This corresponds to a ±75º rotation with respect to the center [zero-tilt] projection.)
- **Increment:** 1.0 <Enter> (Projections acquired at 1º intervals)

Press **Generate Tilt Angles**.

Press **OK**.

The center projection number 75 is displayed in the EM3D Visualization window. Explore the features of this window in the EM3D Visualization window – projection mode, but first save the imported projections.

### 2.1.3. Save data in EM3D format

From the EM3D menu select **File>Save>Binary and State**. A file browser appears.

Navigate to the EM3D distribution directory.

Save the file as *synthetic_start.idl*

> *Note: Both the State and Binary files are saved.*

## 2.2. EM3D visualization window – projection mode

Before we proceed with alignment, learn about the features of the EM3D visualization window in projection mode.

The upper-left corner of the *Visualization window* displays information about the loaded dataset: Name, Source, Magnification, and Pixel size, based on the data's header information or user-entered information.

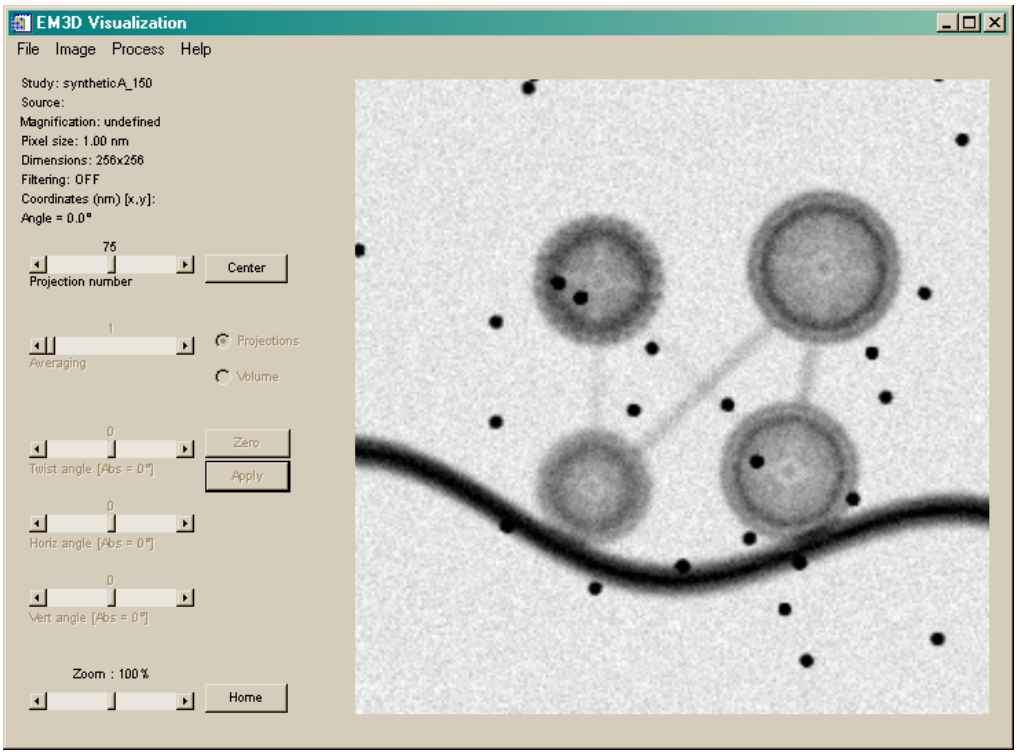



*Figure 8: Projections visualization window*

### 2.2.1. Projection number slider

The projection number slider is originally set to the zero-tilt projection "75".

Scroll through the projections by using the **Projection number** slider. Now return to projection 75 by clicking the **Center** button.

### 2.2.2. Zoom

The size of the displayed image can be controlled using the **Zoom** slider at the lower left corner of the window. Enlarge the display to 150%. Note that the image window gets horizontal and vertical scroll bars that allow navigation through the entire image. Use the **Home** button to return to the original resolution.

### 2.2.3. Exporting images

Export the non-aligned projections from 50 to 100 as 16 bit TIFF images:

1. Open the Image Write window by selecting the **File > Write**….

2. Specify the **Start** (50) and the **Stop** (100) projections using the sliders near the top of the window.

3. Leave the **Decimate** slider at 0.

4. Select the **Raw** button.

5. Select the **TIFF** button.

6. Select the **16-bit** button.

7. Press the **Write** button.

8. Select the directory in which to write the files.

9. Type a prefix for the 51 files to be written, for example “RawProj”. The selected projections are saved to 51 TIFF files. A numerical counter is automatically appended to every filename. Ex. RawProjA_050.TIFF.

### 2.2.4. Tilt series animation

Review the projections as a tilt-sequence animation. Open **Image > Review x,y**, the image window will be sequentially updated with the entire set of projections. This first review sequence serves to create an animation that is then displayed in a new window. This window provides extensive control over the animation. On platforms with a full IDL license, an MPEG movie can be generated. Click **End Animation** to close the window.

> *If an error occurs while generating the animation, exit EM3D, restart the program, and shrink the size of the graphics window, before retrying.*

## 2.3. Alignment

The raw EM projections are not aligned. Before reconstructing a volume the image-to-image offsets between individual projections have to be determined, using *fiducial markers* that are either deposited on the sample or contained within the sample itself using gold-labeled antibodies.

**Summary**

1. Select **Process** > **Align** in the EM3D Visualization window to open the **Projection Alignment** window.

2. Adjust the fiducial contrast-detection parameters and press the **Acquire** button (see Acquisition).

3. Review the acquired fiducial positions, make a few adjustments, and press the **Index** button (see Indexing the Fiducial Markers).

4. Review indexing results, **Delete** less-frequently occurring fiducials (see Review indexing results and Delete fiducials).

5. **Calculate** fiducial alignment offsets and residual errors (see Calculating alignment offsets).

6. Refine the alignment to reach a small residual alignment error (see Refining the alignment).

### 2.3.1. Open the projection alignment window

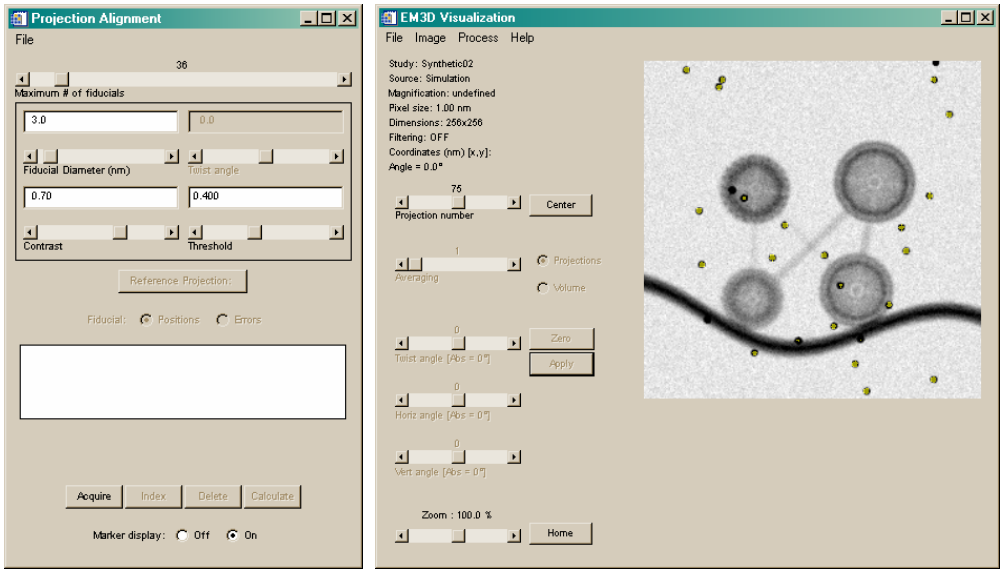



*Figure 9: Projection Alignment window and initial EM3D Visualization window appearance*

From the **EM3D Visualization** window, select **Process > Align** to open the **Projection Alignment** window (Figure 9). The main EM3D Visualization window will go into alignment mode, displaying the projections with a yellow overlay that indicates the likely positions of fiducial markers.

### 2.3.2. Acquisition

In this step we are going to set the parameters to locate all potential fiducials. Once we have decided on parameters, the program identifies all possible fiducials throughout the projections.

Adjust the acquisition parameters so that the majority of the fiducial markers visible on the zero-tilt image are marked with the yellow overlay. Adjustments can be made by either clicking in the text field or by moving the sliders. Please read the reference manual section 3.3 for information about choosing parameters. Recommended settings for the Synthetic dataset are

| | |
|---|---|
| Fiducial Diameter: | 3.0 nm |
| Contrast: | 0.7 |
| Threshold: | 0.4 |

In the Synthetic dataset, with the settings indicated above, the **Maximum # of fiducials** slider will adjust itself to 36.

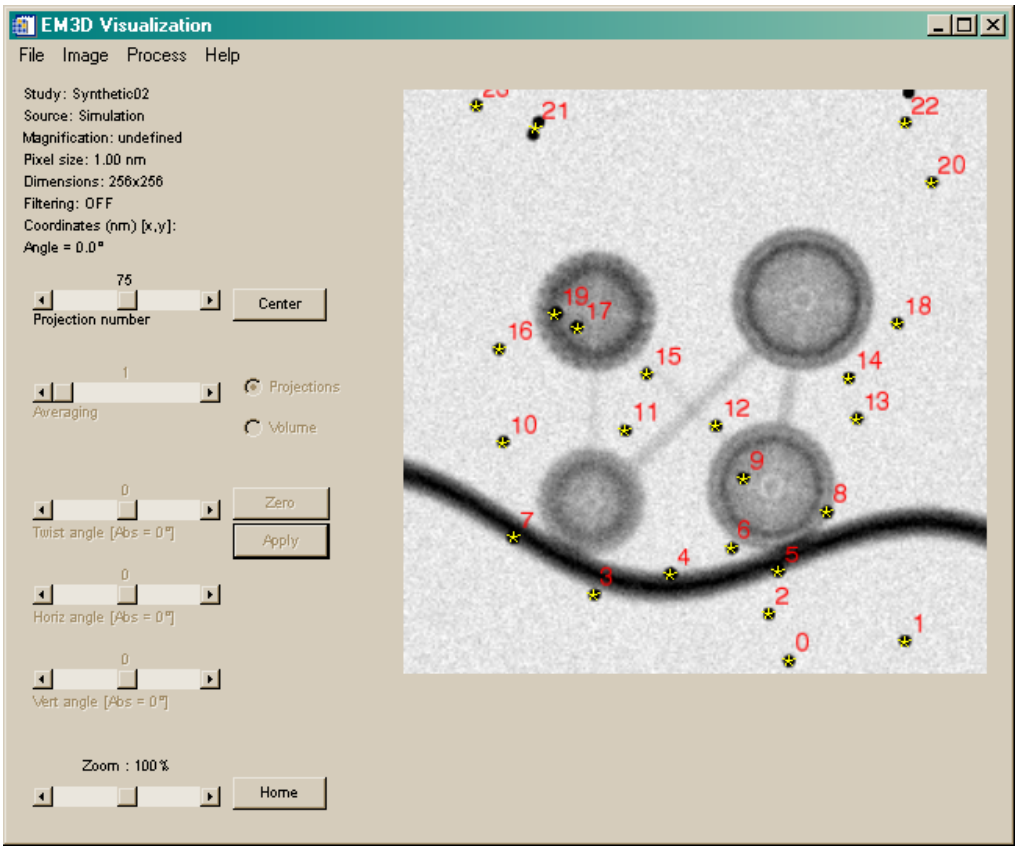



*Figure 10: Acquisition of 36 fiducials*

When the parameters have been adjusted, press the **Acquire** button. A Status window will open showing the progress of the acquisition, and the Visualization window will show the sequence of projection images from the tilt series, each marked with putative fiducial locations. On most desktop computers, acquisition will take <1 minute for the Synthetic dataset. When acquisition is complete, the Visualization display will appear as shown in Figure 10. Note that the yellow fiducial marker overlay is now replaced by yellow '*' symbols, each marked with a red numeral. These numerals are more-or-less arbitrary from projection-to-projection.

The **Twist Angle** is the rotation of the projection tilt axis about the depth axis. When the projections are aligned, the tilt axis will be oriented vertically, parallel to the *y*-axis of the Visualization display. Normally, this parameter is automatically adjusted during subsequent alignment steps to angles as high as 45°. The twist angle for the Synthetic dataset is small, ~5°, so it does not need to be adjusted manually.

### 2.3.3. Indexing the fiducial markers

During figucial indexing, 2 things happen: 1) A cross-correlation is done to roughly align the images, and 2) each fiducial is tracked through the projections, so that each number will correspond to the same fiducial marker in all projections.

To prepare for indexing, examine the acquired fiducial markers in the zero-tilt projection (center) of the Visualization of window. In general, one should now delete poorly positioned or false-positive markers.

In the Synthetic dataset, the marker labeled 21 in Figure 10 is poorly positioned because the associated gold beads are too close together. Delete this marker by positioning the cursor over the yellow '*' symbol and pressing the right mouse button. In a real data set it is often not necessary to do this because clustered fiducials will often have high error values and will subsequently be automatically eliminated.

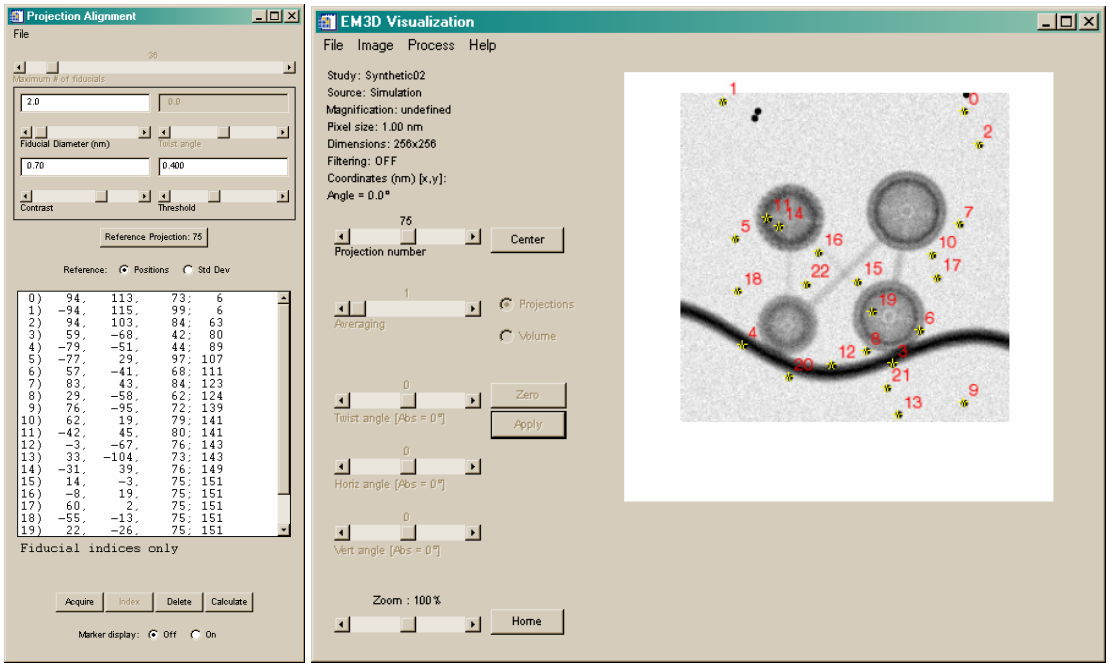



*Figure 11: Align and Visualization windows after indexing*

When the fiducial display has been edited as described, press the **Index** button on the **Projection Alignment** window. A Status window will indicate the progress of this calculation, which relates the identities of the fiducials locations together across the entire sequence of projections. Indexing should take only a few seconds for the Synthetic dataset. The indexed fiducial positions are then displayed as a list in the **Projection Alignment** window. The Visualization window display will also change to reflect the approximate offsets and indexed fiducial labels calculated during the indexing process.

### 2.3.4. Review indexing results and delete fiducials

Now that we have tracked individual fiducials through the tilt series, we need to assess which ones tracked well, and which failed.

Prepare for the alignment-offset calculation by examining the list of indexed fiducial positions in the Projection Alignment window. Each line shows one fiducial position. The number on the left side of each line now corresponds to the red index number displayed near each marker in the Visualization window. The mean fiducial positions are sorted by the frequency of their appearance throughout the projection tilt series, with the fiducials occurring least frequently appearing at the top of the list; the precise frequency is the number at the right side of each line in the list. For example, fiducial 0 has mean (*x*, *y*, *z*) pixel position (94, 113, 73) and was indexed on only 6 projections, while fiducial 10 has mean position (61, 19, 78) and was indexed on 142 of the projections.

While it is possible to perform calculations with the entire set of fiducials, it is neither necessary nor desirable to include the less-frequently occurring fiducials in the calculation, since they are not representative of the whole dataset. Ideally, fiducial markers should span the entire dataset, but in reality, this rarely occurs. Select the less-frequently occurring fiducials in the list, and press the **Delete** button to remove them. A warning dialog will ask for confirmation; select the **OK** button to initiate the deletion.

In the Synthetic dataset, select and delete markers 0—9.

After deletion, the markers will disappear from both the Alignment and Visualization windows.

### 2.3.5. Calculating alignment offsets

Now we check the quality of our alignment by calculating the mean error of each fiducial and eliminating ones with high error.

Start by pressing the **Calculate** button. A Status dialog will open to indicate the progress of this calculation. Several iterations or 'passes' are frequently necessary when this calculation is first performed, particularly if the twist angle is significant. When the calculation is complete, the Status window will close and the root-mean-square (RMS) residual alignment error (in pixels) for all the fiducials will be displayed near the bottom of the fiducial list (Figure 12). The list shows 3D fiducial positions by default; residual errors for each fiducial can also be displayed by selecting the **Fiducial: Errors** radio-button located above the list.

For the Synthetic dataset, the calculation requires 3 passes and should take less than 2 minutes. The total alignment error will be 2.27 pixels. In general, the goal is to obtain an alignment where the total RMS error is <1.0 pixel.

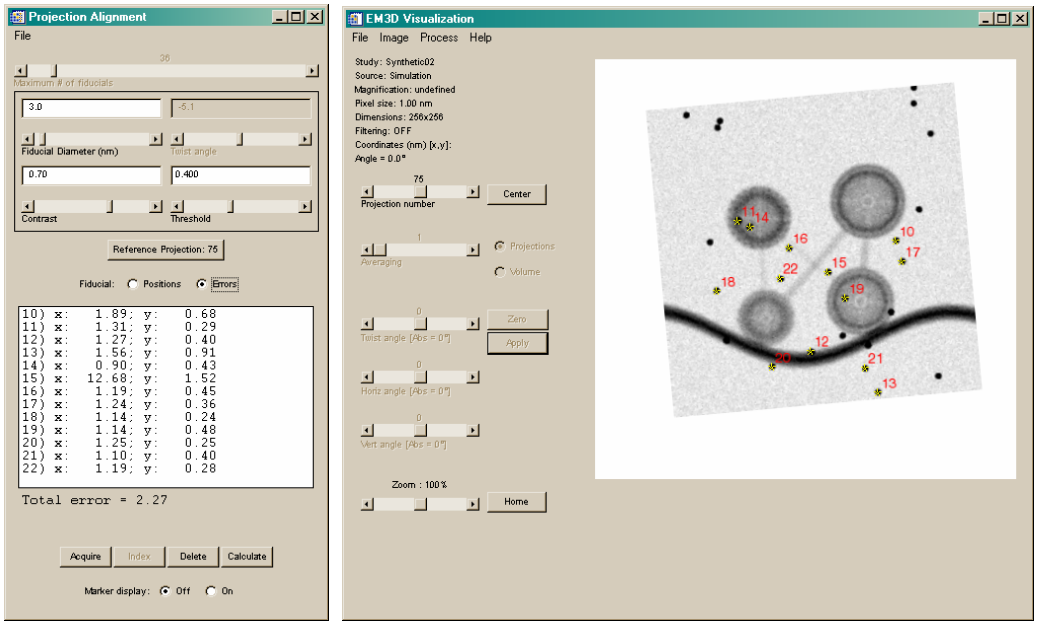



*Figure 12: Alignment and Visualization windows after calculating*

### 2.3.6. Refining the alignment

In many EM projection datasets, the initial alignment will not be satisfactory because the error is too high. There are two strategies for refining the alignment to reduce the error: 1) *deleting* fiducials and 2) *modifying* fiducials.

***Deleting fiducials*.** The first and simplest approach is to delete fiducials with high residual errors from the list. Begin by deleting the fiducials with large error. After deleting the fiducials with large error, press the **Calculate** button to determine the new alignment and its error. Review the resulting individual and total errors and if necessary delete more fiducials and recalculate until a satisfactory alignment is obtained.

During refinement, try to maintain fiducials from different regions of a projection. In most cases a better global alignement can be obtained by keeping widely separated fiducials with good error values, rather than by keeping a tight cluster of fiducials with better error numbers. Since the projections are not completely rigid bodies, we want to calculate an averaged alignment over the entire projection by basing our calculations on samples from as many regions of the projection as possible, not just one area. Otherwise, one area of the projections may be well-aligned and other areas may not be.

For the Synthetic dataset, there is one fiducial with a very large error; fiducial index 15 has an *x*-axis error >12 pixels. **Delete** this one fiducial and press the **Calculate** button. The **Total error** now drops to 0.62 pixels, which would be an excellent result for typical EM data. With the Synthetic data, however, it is possible to achieve a better result. **Delete** all fiducials with errors greater than 1 pixel, in this case fiducials 10 and 13; this will leave only the ten best markers. Press the **Calculate** button again; after two passes, the total alignment error will be 0.24 pixels.

If too many fiducials are deleted, a dialog window will warn that at least two marked fiducials per projection are needed for alignment. When this occurs, a **Bad Projections** field will become visible below the fiducial list in the Alignment window, including a drop-down list that indicates the projections with less than 2 fiducials. Either revert to the previous marker configuration using **File > Revert > Single Step**, or add the missing fiducials to the offending projections using the procedure described below.

***Modifying fiducials***. At times, it will be desirable to refine individual fiducial locations on projections to improve the alignment. The indexing procedure uses a quasi-2D approach, which sometimes causes errors on projections with larger tilt angles (>60). Fiducials positions on these projections may therefore need to be added or adjusted interactively.

To add or adjust a fiducial, select the desired fiducial by left-clicking it in the Alignment window list. If the fiducial is visible in the current projection, the corresponding marker will change from a yellow '*' to a green '+'. Using either the Projection Number slider or the Bad Projections drop-down list, select each projection that requires modification. Next, determine the location of the desired fiducial marker. Usually, the best strategy is to look at adjacent projections on which the desired fiducial has been properly marked. Once the fiducial is located, left-click on or near it to mark its position. It is often useful to turn on the fiducial marker overlay by selecting the **Marker Display: On** radio-button. It may also be necessary to adjust the **Threshold** slider so that the marker position is properly indicated by a discrete patch of yellow overlay. Repeat this process on additional projections and for additional fiducials as needed. To observe the effects of this interactive fiducial marking process, press the **Calculate** button again.

For example, fiducial 11 on projection 148 is missing. Select that fiducial in the list and view projection 149. Locate the fiducial marked with the green '+', and note its relationship to nearby image features. Now, move to projection 148, locate the same fiducial, and add a marker using the left mouse button.

If at any point the alignment goes awry (such as by deleting too many fiducials), previous marker configurations can be accessed by using commands located in the **File** > **Revert** drop-down menu:

- **Single Step** undoes the most recent deletion and calculation.
- **Calculation** removes all previous fiducial deletions and calculations, returning the process to its status immediately after the **Index** step.
- **Indexing** returns the process to its status immediately after the **Acquire** step.

The entire alignment can also be reset to start over using **File** > **Reset.**

At any point in the alignment, a review of the tilt series can be made as a visual check on the accuracy of fiducial marking and the quality of the alignment. In the **EM3D Visualization** window, select **Image > Review x,y**.

When satisfied with the alignment save it as a new EM3D pair (save *Binary* and *State* files); use the name "*synthetic_aligned.idl*".

## 2.4. Reconstruction

After successfully aligning the data we are ready to reconstruct it into a 3-dimensional volume.

**Summary**

1. Open the Reconstruction window.
2. Estimate z thickness (volume thickness in pixel) and initial z (slice, where reconstruction will start) on the basis of the specimen (see Estimate of z thickness and the initial z).
3. Perform a fast (2:1) decimation reconstruction. (see Reconstructing quickly a decimated volume).
4. Adjust **initial z** and **z thickness** as needed and recheck reconstruction parameters again (see Adjusting "initial z" and "z thickness") .
5. Reset **Decimation** and perform a full-resolution reconstruction (see Full Resolution Reconstruction).
6. Save EM3D data (see Full Resolution Reconstruction).

### 2.4.1. Open the Reconstruction window

Select **Process > Reconstruct** from the EM3D Visualization window.

### 2.4.2. Estimate of z thickness and the initial z

There are several things to consider before reconstructing the volume. The size of the volume is directly related to the size of the projections. A tilt-series with projection dimensions of 1024x1024 pixels can yield a reconstructed volume with dimensions 1024x1024x1024 pixels. We know the thickness of our sample is much smaller than the width of the projections, so to reduce time and memory usage, we will only reconstruct a slab of the volume that contains data. The *z*-axis corresponds to the thickness dimension. In an ideal example, we could assume that the specimen is directly centered in the volume, and ½ the data would be above the midline and ½ below. Since real data sets are less than ideal, let us start by reconstructing an additional ~10% on either side of our predicted volume. In order to do this we need to convert the slice thickness from nanometers to pixels, using the following equation.

$$z_{thickness} \approx \left[ 1.2 * \left( \frac{sample\_thickness}{resolution} \right) \right]$$

$$z_{initial} = \left[ \frac{n_{pixels} - z_{thickness}}{2} \right]$$

In the calculation for $z_{thickness}$, the value 1.2 accounts for the 10% oversample on either side of the specimen.

Calculation for the synthetic dataset:

The sample_thickness = 128 nm, resolution = 1 nm, and dimensions = 256×256 pixels, so $n_{pixels}$ = 256. Therefore $z_{thickness}$ = 1.2 * 128nm / 1nm = 153.6, which can be rounded down to 150 and $z_{initial}$ = (256 – 150) / 2 = 53, which we can round down to 50.

Set the **Initial z index** slider to: 50 pixels

Set the **z thickness** slider to : 150 pixels

### 2.4.3. Quickly reconstructing a decimated volume

The **Decimate** slider controls the size of the reconstructed volume. Projection images are often quite large, e.g., 2048 × 2048 pixels, an 8 MB short-integer image. Consequently, the corresponding size of an undecimated reconstructed volume can be very large, e.g., 1 GB for a 128 slice reconstruction. Such reconstructions are time consuming to compute, and can tax or overwhelm the memory (RAM) capabilities of many desktop computers. The table below illustrates the dramatic reduction in image volume (computation time) achieved through decimation.

| Decimation Level | In-plane dimension reduction | Volume reduction (Volume) |
|---|---|---|
| 0 | 0 | 0 |
| 1 | ½ | 1/8 |
| 2 | ¼ | 1/64 |

| | | |
|---|---|---|
| 3 | 1/8 | 1/512 |

The reconstructed volume size is indicated by the text field, **Volume array size = ## MB**, located near the middle of the Reconstruction window.

First, perform fast, decimated reconstructions to determine the $Z_{intital}$ and $Z_{thickness}$.

For the Synthetic dataset, perform a fast 2:1 decimation reconstruction by setting the **Decimate** slider to 1.

> *Note that the **Volume array size** goes from 15 to 2 MB.*

Select the **Reconstruct** button at the bottom left and a Status window will open to display the progress of the calculation. The Visualization window will display the center slice of the reconstruction as it builds up each projection.

> *A partial reconstruction can be created by pressing the **Cancel** button on the Status window after only a fraction (e.g., half) of the projections have been processed. Partial reconstructions exhibit substantial artifacts, but they are often satisfactory for determining the desired depth range of the reconstruction, and they save time.*

When the reconstruction is complete, the Visualization window will convert to **Volume** mode to display the new reconstruction (see Figure 13). For a description of the controls in Volume mode see 2D Volume Visualization,

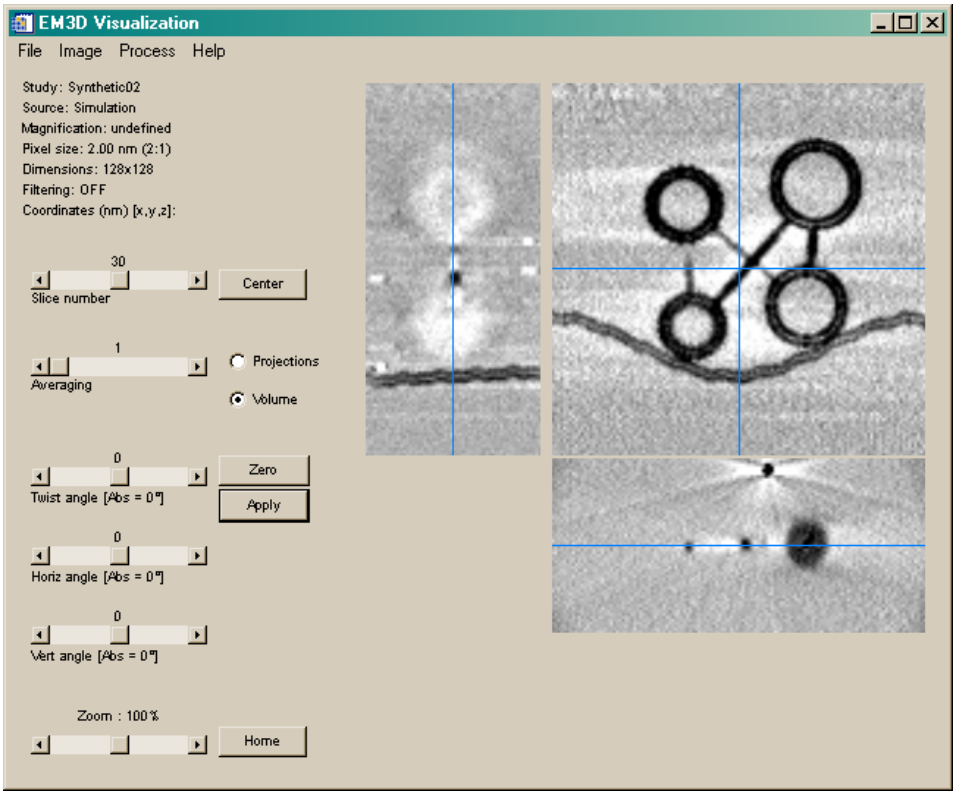


*Figure 13: Reconstructed volume, 2:1 decimation (Decimate = 1)*

The reconstructed Synthetic volume exhibits artifacts common in tomography data. The white streaks eminating from the fiducial markers and vesicles are a result of 'shadowing'; one structure blocking another behind it. It is also evident on other structures here because of the artificially dense nature of the synthetic data.

In addition to the original (*x*, *y*) window, the EM3D Visualization window displays two additional windows showing slices that display the *z* dimension of the reconstructed volume, the (*x*, *z*) at the bottom and (*z*, *y*) on the left. Note that the blue cross hairs are active in all windows. Use the left mouse button to 'slice' through one of the windows and notice how the other windows respond. This is a valuable tool used extensively in segmenting objects in the volume. Turn the crosshairs off or on with **Image > Show > Crosshairs**.

### 2.4.4. Adjusting "initial z" and "z thickness"

The Synthetic data was constructed so that its reconstruction would be perfectly centered in the volume cube. Consequently, this reconstruction is nearly ideal – it contains the full volume of the sample. However, it is too thick, extending beyond the fiducial markers on the surface. To reduce the memory requirements of the final, undecimated volume reconstruction, the depth should be reduced.

To do this, first determine a new setting for the **Initial z index**. In the (*x*, *z*) volume window of the EM3D Visualization window, use the cross hair to locate the slice where the gold fiducials begin and end. Here, they begin to disappear from the lower part of the volume around **Slice** number 16 [bottom edge of the (x,z) window]. Since this volume is decimated 2:1, each pixel is 2 nm, so begin the reconstruction at 32 slices (16 * 2) deeper into the volume than the original reconstruction start slice; that is, set the **Initial z index** to 50 + 32 = 82.

To determine a new **z thickness**, use the (*x*, *z*) window and determine where the fiducials on the top of the volume disappear. This is around slice number 65. The number of slices between the lower slice number limit, 16, and the upper slice number limit, 65, is 65 – 16 + 1 = 50. Again, because of the decimation, multiply by 2, so the new z thickness is 100.

Enter these new parameters into the **Reconstruction** window:

- Initial z index: 82
- z thickness: 100

### 2.4.5. Full resolution reconstruction

After setting the initial z and z thickness, adjust the **Decimation** from 1 to 0, and begin a new full-resolution reconstruction by selecting the **Reconstruct** button.

If satisfied with this reconstructed volume, save both *State* and *Binary*. Go to the EM3D Visualization window and click on **File > Save > *Binary* and *State***, Save files as *synthetic_reco.idl*. The reconstructed volume will be automatically saved as *synthetic_reco.data*. These new files will be used in the remainder of the tutorial.

## 2.5. EM3D visualization window – volume mode

The EM3D *Visualization window* operates in two modes, **Projections** and **Volume. Until** reconstruction is complete, only *Projections* mode has been available. However, once reconstruction is complete, *Volume* mode becomes available. The mode display is controlled by a pair of ***radio buttons*** to the right of the **averaging** slider. *Projections* mode is used to view individual EM projection images; when a dataset is initially imported, only this mode is available. Since the dataset is now reconstructed, both volume and projections mode are available.

### 2.5.1. Volume mode

In *Volume* mode the EM3D Visualization display adds 2 additional graphics panels., This allows you to see cuts through the volume at perpendicular (orthoganol) slices (Figure 14). The middle slice (slice 50) is displayed in the main (*x*, *y*) display panel, while the panel to the left shows the (z, y) plane and the panel below shows the (x, z) plane.

1. The blue crosshairs indicate the relative positions of the different slices within the volume. The crosshairs can be turned on/off by selecting **Show > Crosshairs** from the **Image** menu. The crosshairs provide a perspective of objects' relative positions within the volume, and they are useful for finding edges of objects during segmentation.

2. Navigate through 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.

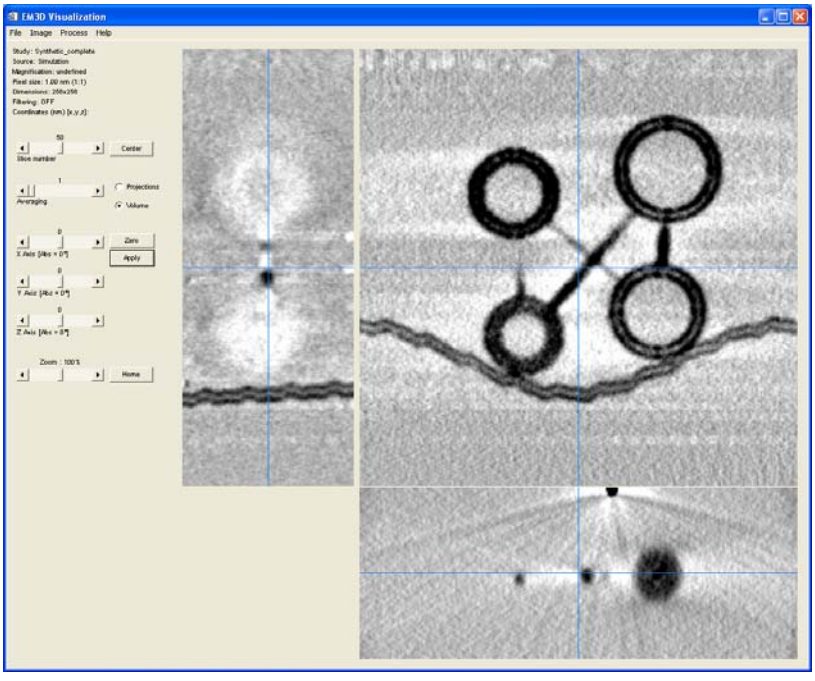



*Figure 14: Volume visualization mode*

3. The (x,y) slice can also be changed using the **Slice number** slider. Display several other slices of the volume using the **Slice number slider**. Click on the **Center** button to go back to the slice 50.

4. **Averaging** allows the selection of a different number of adjacent volume slices to be averaged and displayed as the current slice image. Set the **Averaging** slider to 5. The average gray scale of slices 48 to 52 is displayed in the (*x, y*) display. Averaging is also performed on the two transverse displays.

5. Virtual slices can be formed at any angle through the volume. The rotation sliders allow the user to adjust the view in order to see stained objects with better contrast, which is particularly important during segmentation. Rotate the volume using the 3 sliders and observe the images.

   - The **X Axis** is perpendicular to the (z, y) display, positive rotation is clockwise..
   - The **Y Axis** is perpendicular to the (x, z) display, positive rotation is clockwise.
   - The **Z Axis** is perpendicular to the main (x, y) display, positive rotation is counter-clockwise.

   The slice images are interpolated directly from the volume. To permanently rotate the volume, click on the **Apply** button. Use **File > Restore** to revert the volume to the original, non-rotated state.

6. A simple measurement tool is available to measure distances in any of the three coordinate windows. Right-click at the beginning point, then move the cursor to the end point. A yellow dotted line will show this distance and the **Distance (nm)** will read out just below the **Coordinates** readout on the left. The distance measurement disappears when the right mouse button is released.

   > *Distance measurement does not work when the Segmentation window is open.*

7. **Scaling** is used to adjust the image's contrast.

   Open the **Image Scaling Window** by selecting **Image > Scaling** from the menu. The gray scale range is shown as a ***cumulative distribution function***. Voxels (3D pixel) that have a gray value less than the **Min Clip** will be displayed in black and voxels with gray values above the **Max Clip** will be displayed in white. All values between the Min Clip and Max Clip will be linearly scaled to the full range of gray values.

   For example, if both fields are set at 1%, then the darkest 1% and the brightest 1% of the pixels in the image will be clipped at black and white, respectively, while the remaining 98% of the pixels will be linearly scaled between black and white. Increasing both clip values together increases the image contrast.

   On the lower left corner are two radio buttons, **Absolute** and **Relative**.

**Absolute** scales each image based on the range of data values in the entire tilt series or volume, while **Relative** scales each image based only on that projection or slice. Relative is the default mode for projections, while absolute is the default mode for volume slices. The **Invert** button reverse the grayscale, generating a negative image that is often useful for segmentation

8. In volume mode one can review any of the three orthogonal slice orientations by creating animation sequences. Select **Image > Review x, y** and review the volume along the z axis. Select **Image > Review x, z** and **z, y** to see the animation of the transverse views.

> *If a memory error occurs while generating the animation, exit EM3D, restart the program, and shrink the size of the graphics window, before retrying.*

9. During segmentation, it is often useful to invert the image. Select **Image > Invert**, the grayscale image is reversed and a negative image is displayed. Click again on **Image** > **Invert** to revert to the original.

## 2.6. Segmentation

Essentially, segmentation defines a volume of interest (VOI) containing a logically distinct structural component, allowing the user to 'carve out' or isolate individual structures for the purpose of surface modelling and 3D visualization.

> *If the synthetic dataset has been cored or decimated, the original reconstructed volume (with full resolution) should be reloaded for the remainder of this tutorial. Reloading is accomplished by exiting EM3D, followed by restarting and loading the Synthetic_reco dataset.*

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

**Summary**

1. Identify a structural component by viewing and navigating through the Visualization window in order to choose the optimal volume orientation for that object; it may be necessary to rotate the volume. The Synthetic dataset, however, is oriented to permit immediate segmentation without adjustment.

2. Find the central slice of the structural component.

3. Name the object to segment.

4. Select the type of segmentation – Manual or Automatic. If Automatic, choose Open, Closed, or Isolated.

5. Segment the slice, either automatically or manually.

6. Propagate the segmentation throughout the volume, either automatically or manually.

7. If desired, use a selection of the segmented objects to build a region map (see Regional Segmentation).

### 2.6.1. Segmentation modes

Since the nature 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, than the program finds logical extensions of the object on subsequent slices.

- **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.

Below are 3 distinct sub-classes of Automatic segmentation

- **Open – Used for 'open-ended' objects that stretch from one edge of the display to another edge, i.e. an ~planar cell membrane.**

- **Closed** – Used for objects that are not 'open-ended', i.e. the cross section of ~spherical vesicles.

- **Isolated**— Also used for 'open-ended' objects without the constraint of being 'anchored' to two edges of the image.

### 2.6.2. Automatic open segmentation

To illustrate open segmentation, consider the extensive trilaminar structure in the reconstructed volume of the Synthetic dataset, which is designed to have the appearance of a presynaptic plasma membrane. Use the slice-navigation tools in the Visualization window to examine this membrane. Note that it has an "open" ***topology***, extends throughout the volume, and touches 2 edges of the image in each slice. These characteristics make it an ideal candidate for **Automatic, Open** Segmentation.

Object slider →


*Figure 15: Segmentation Window*

1. Using the **object** slider (Figure 15) in the **Segmentation** window, select the first non-segmented object, this will have the default label "Object 1" and Status: clear. Name the new object by entering text to replace the "Object 1" label with "*Presynaptic Membrane*" <Enter>.

2. If not already selected, choose the **Automatic** segmentation mode by clicking on the tab.

3. Select the **Open** radio button if it isn't already selected.

4. Choose a reference slice to define the initial anchor points. Use the Visualization window navigation controls to find this slice. For the Synthetic dataset, any slice near the center of the volume can be used, e.g., 50.

5. Define the anchor path. Starting at one end or the other of the membrane, use the middle mouse button to define points along its center. At first, define only a few points that roughly define the entire length of the membrane on this slice; see Figure 16. The size, shape and colour of the anchor points can be defined in the segmentation window: **Drawing > Change Anchor Mark**.

   - Define point: middle mouse button.
   - Remove point: right mouse button.

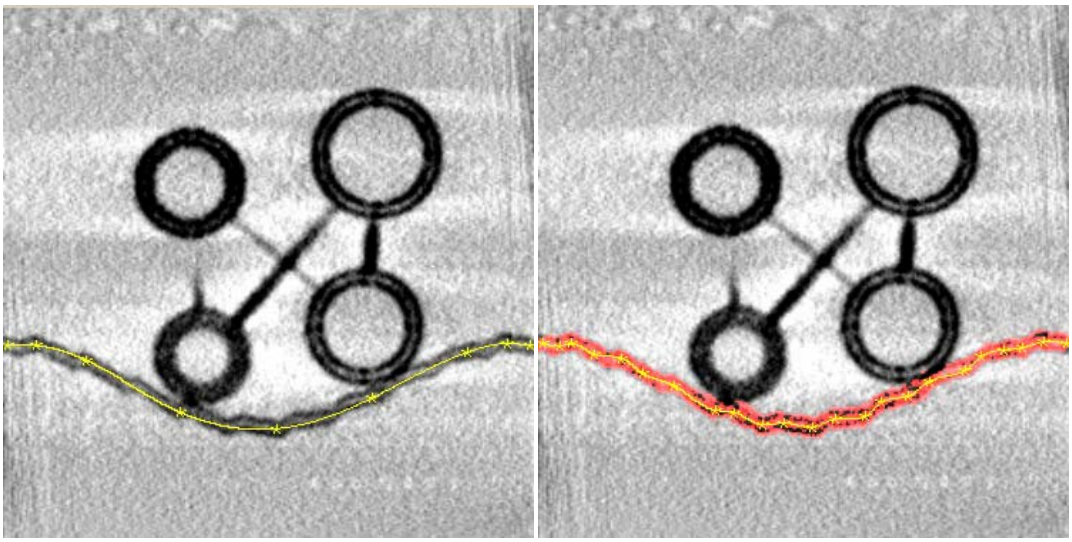


*Figure 16: Anchor path for the Synthetic presynaptic membrane, initially and after refinement and **search**.*

6. Refine the anchor path. Add points using the middle mouse button along the length of the membrane to more accurately capture the undulations of the membrane on this slice. It may be necessary to delete one, or more, of the anchor points using the right mouse button.

7. Press the **Search** button. Note that the **Minimum** and **Maximum** gray value sliders are automatically adjusted; values within this gray value range are indicated by a red overlay (Figure 16). Sometimes, it is useful to adjust these values to improve the segmentation and its propagation, but here it is satisfactory to accept the automatically calculated values.

8. Adjust the **Surface width** slider to 9 to encourage the segmentation to follow the center of the membrane, and not become attracted to opposing membranes with similar stain values. Note that the colored overlay narrows slightly.

9. Press the **Segment** button. The yellow line and points are replaced by a continuous green line. This is the *segmentation path* for this slice. The **Reference slice:** button becomes active and indicates the current slice index.

10. Adjust the segmentation smoothing. To avoid numerical instability during propagation of the segmentation path, some additional smoothing is necessary. Check the segmentation path. Does the line run accurately along the middle of the membrane? To adjust the smoothing,

    - Re-anchor the object (**File > Re-anchor**).
    - Adjust the segmentation anchor points (if necessary) using the mouse.
    - Adjust the smoothing level, set the **Smoothing** slider to 10, and the **Iterations** slider to 4.

- Re-determine the grayscale range, press **Search**
- Re-segment, press **Segment**.
- Repeat until the segmentation path accurately follows the middle of the membrane.

11. Adjust the propagation range. The synthetic presynaptic membrane extends throughout the volume, but normally it would terminate at the actual boundaries of a prepared tissue slice. Moreover, the reconstructed volume near the surface may show beam degradation. For illustration, restrict the propagation so that it excludes the fiducial markers. Do this by navigating upward through the Visualization window from the zero slice until the fiducials disappear, then press **Set Start** (near slice 10). Repeat this process on the other side of the volume, navigating downward from the maximum slice number until the fiducials again disappear, then press **Set Stop** (near slice 90).

12. When the controls have values similar to those shown in Figure 17, press the **Propagate** button. The Visualization window will display the results of the segmentation on each slice as the propagation proceeds.

> *Refreshing the display can take significantamounts of time on slower computers, it may be a good idea to reduce the size of the Visualization window before initiating propagation.*

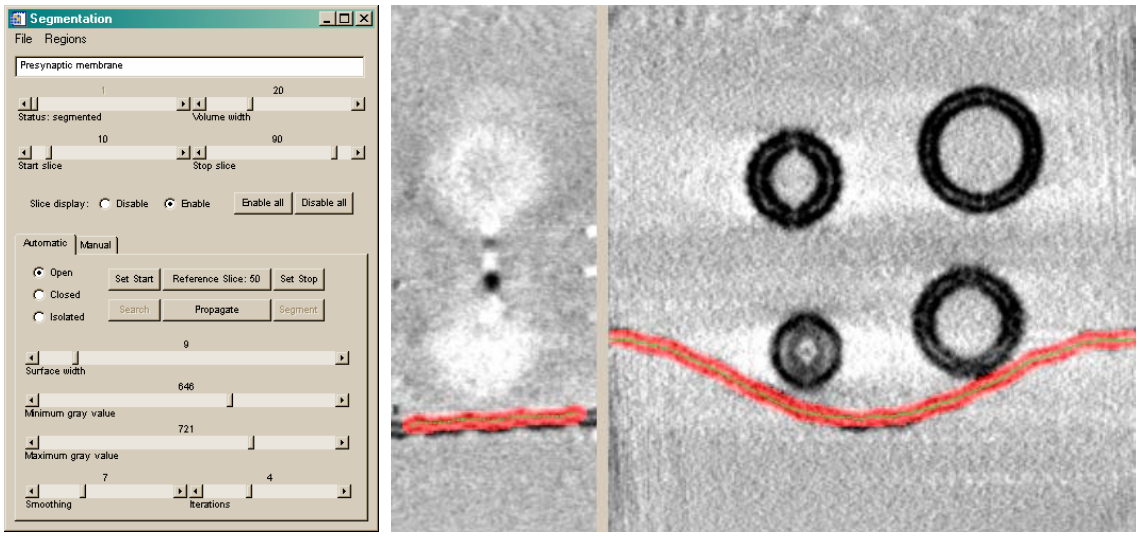



*Figure 17: Segmentation control window settings and corresponding propagation results.*

13. Use the navigation controls in the Visualization window to examine the segmentation results. The green segmentation path should closely follow the center of the membrane.

14. Adjust the **Surface width** slider, aiming to find the minimum value for which the red overlay (VOI) appears to fully enclose the membrane. For the synthetic presynaptic membrane, the final **Surface width** should be about 11. 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, as discussed in the Section 2.7.

> *Note: the red overlay is not an accurate depiction of the volume-of-interest that corresponds to the segmentation on each slice; to save time, the overlay is created in 2D only. The actual 3D VOI can be significantly more extensive. Use slice planes that are relatively orthogonal to the object to assess the coverage of the VOI.*

### 2.6.3. 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.

1. Move the object slider to Object 2, and name it "*Vesicle 1*" <Enter>. Select the **Closed** topology button. Segmentation should begin near the middle of the volume containing the object (slice 50).

2. Define a closed anchor path. Starting at any place along the membrane, use the middle mouse button to define points along the center of the membrane. After the first three points are defined, the path will close. Follow the same procedure as before, initially selecting just a few points to roughly define the membrane, then adding additional points to refine it.

3. Press the **Search** button to display the selected pixel overlay.

4. Adjust the **Surface width** slider to 9, so that the entire vesicle membrane is marked with the color overlay.

5. Press the **Segment** button to generate the segmentation path. Figure 18 shows the results of these operations.

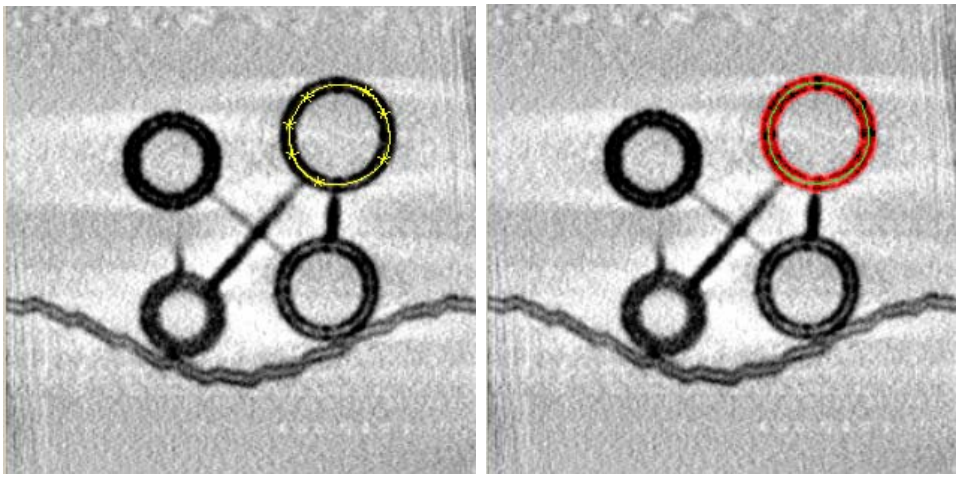


*Figure 18: Closed anchor path (left) and resulting segmentation path (right).*

6. Adjust the segmentation smoothing. Check the segmentation path. Does the line run along the middle of the object, or is the line perturbed? Adjust the **smoothing** slider, Re-anchor the object (**File > Re-anchor**), press **Search** and press **Segment**. Repeat until the segmentation path is in the middle of the membrane and even. For the Synthetic dataset the **Smoothing** to 10, and the **Iterations** slider to 4.

7. The automatic gray range detection is designed to be fairly generous, often it is desirable to adjust the automatic gray range. For example, when the ***SNR*** (Signal to Noise Ratio) is fairly high, as it is in the Synthetic dataset, it is useful to reduce the **Maximum gray value** (e.g., by 25)**.**

8. For automatic segmentation, it is not absolutely necessary to establish a start and stop slice in advance; propagation will automatically end when the specified gray levels are no longer present.

9. Press the **Propagate** button and observe the results. When propagation is complete, left-click on the center of the vesicle and examine the transverse displays. In the direction of smaller slice indices, the propagation automatically stopped near the end of the vesicle; in the direction of larger slice indices, the segmentation erroneously followed an artifact beyond the end of the vesicle (Figure 19).

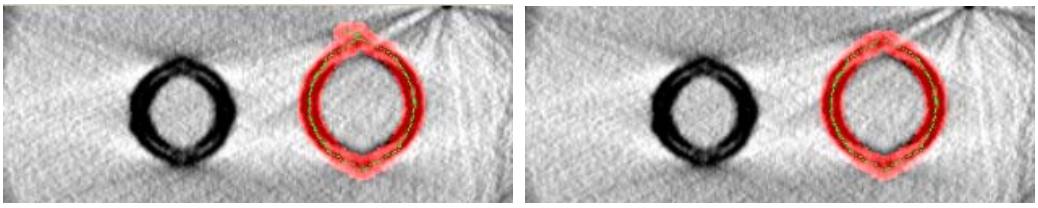


*Figure 19: Propagated segmentation with artifact-induced error (left) and after adjustment (right).*

10. 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 83**. Now, press the **Set Stop** button on the Segmentation control window. A query dialog will ask for confirmation of this action. Respond affirmatively and the erroneous portion of the segmentation will be deleted.

11. Another approach to this problem is to further reduce the maximum gray value and redo the propagation. To do this, select the *Re-anchor* from the *File* menu. This will return the process to the step immediately before the **Search** button was pressed. Press **Search** again, and reduce the **Maximum gray value** even more than before, so that the fainter gray values produced by artifact are ignored. Press **Segment** and set the **Start** / **Stop slice** sliders to their minimum and maximum values, respectively. Press the **Propagate** button and again examine the results. Adjust the Maximum gray value until the propagation terminates properly at slice 84.

12. 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.

> *Adjusting an Automatic segmentation:*
>
> *Sometimes, a single collection of settings is not satisfactory to propagate the segmentation throughout the volume of a complex or inhomogeneous structural component. In such cases, there are several tools available to adjust the segmentation. In addition to the slice-deletion discussed in the example above, one can also extend the slice range of a segmentation, which requires defining a new anchor path at the slice midpoint of extended range. One can also directly edit a segmented slice after propagation by clicking on it with the middle mouse button. This click will bring up a query dialog, and, if a positive response is selected a new anchor path can be initiated. Adjust parameters, and perform an additional segmentation and propagation over a new slice range for this same object.*

### 2.6.4. Automatic isolated segmentation

This is used for objects such as short membranes in the middle of a volume that don't 'close' to the edges, filaments, and so forth. Its use is similar to Open automatic segmentation.

For example, segment the synthetic, rod-like filament connecting the lower left to the upper right vesicle. The range of the filament through the volume is from about slice 43 to 56.

1. In the **Segmentation** window, select a new object (Object 3), name it *Filament 1<Enter>*, and select the **Isolated** topology button.

2. On slice 48, place a few points along the length of the filament and press the **Search** button. The gray range will automatically adjust to suitable values.

3. Leave the smoothing and propagation-range sliders at their default values:
   Surface width: 11
   Smoothing: 10
   Iterations: 4

4. Press the **Segment** button and then the **Propagate** button and examine the results. The propagation will automatically terminate over the slice range of 37—58.

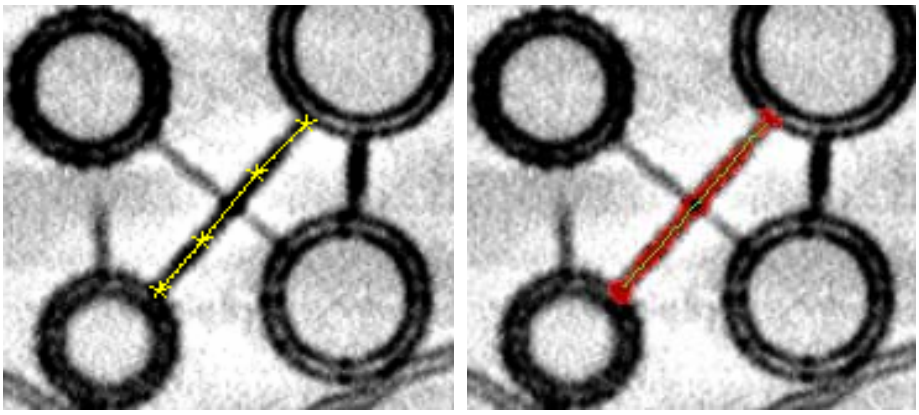


*Figure 20: Isolated segmentation, anchor path (left) and initial segmentation (right).*

### 2.6.5. 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. Adjust the object slider to find an unused object, e.g., Object 4. Name this object *Filament 2* <Enter>. Click on the **Manual** tab to display the manual segmentation controls.

2. Use the Visualization window to navigate to the center of the filament connecting the upper-right and lower-right vesicles (about slice 49).

3. 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; piecewise interpolation connects them with straight lines. Figure 21 shows the results using spline interpolation.

4. Press the **Segment** button. The interior of the volume is now marked with a red overlay and the anchor path turns green (see Figure 21). All points in the interior on this slice have now been added to the VOI for this object.

5. 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 as usual.

6. 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.*

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

8. Return to the 1st slice segmented using the *Visualization Window NavigationTtools*; **NOT** the **Down** button.

9. Navigate down one slice, using the *Visualization Window Navigation Tools* ; **NOT** the **Down** button. From here repeat steps 5 and 6 using the **Down** button to segment the lower half of the object.

10. 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

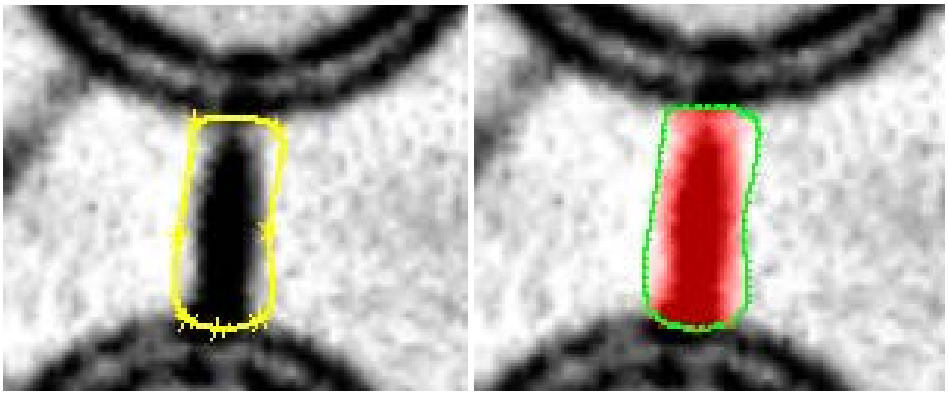


*Figure 21: Points Surrounding Volume (left), Segmented Object (right)*

### 2.6.6. Regional segmentation

After several objects have been segmented, it can be useful to divide the volume into regions, a set of smaller volumes delineated by boundaries of the existing segmentations. For example, in the reconstructed Synthetic volume, based on our current segmentations, one could divide the volume into three regions: the extracellular space, the cytoplasm, and the intralumenal portion of the vesicle. We will now perform this segmentation:

1. In the **Segmentation** control window, press the **Disable all** button. All of the segmentation color overlays will disappear.

2. Using the object slider, go to the "Presynaptic Membrane" object and click on the **Slice display: Enable** radio button. The segmentation color overlay will reappear. Also, adjust the **Volume width** slider so that its value matches the **Surface width**. Repeat this step for the "Vesicle 1" object.

3. In the **Segmentation** control window, select **Regions > Map.** After some brief calculations, the Visualization window display will change to what is indicated in Figure 22.

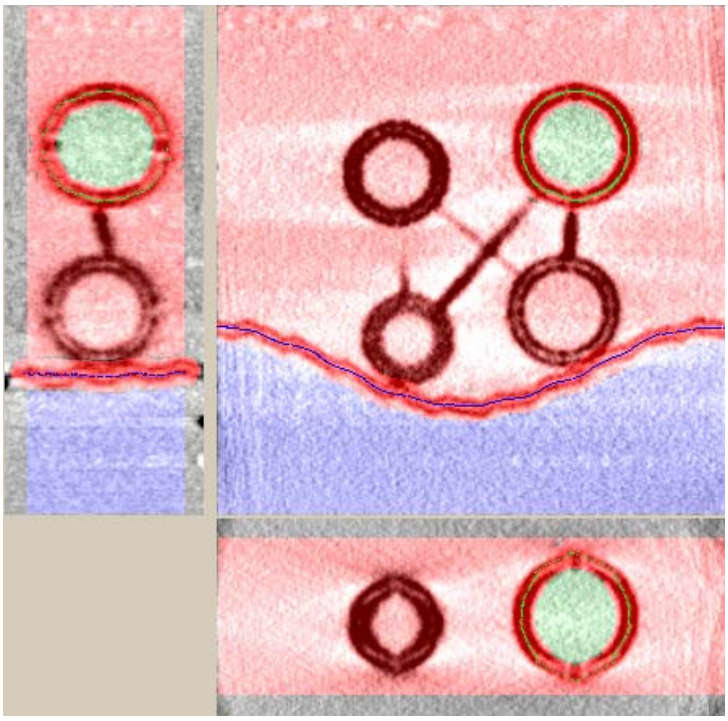


*Figure 22: Segmented volume after region mapping.*

4. Examine the volume using the Visualization window. A new, graduated color overlay divides the volume into three regions or compartments based on the two segmentation objects that were **Enabled** in the slice display: the extracellular space is indicated in light blue, the cytoplasm in light red, and the intralumenal space of Vesicle 1 in light green.

5. The region map can be removed by selecting **Region** > **Reset** on the **Segmentation** control window. However, the region map will be used later to illustrate the generation and visualization of models from regions. For the moment, hide the region-map overlay by toggling off **Image** > **Show** > **Regions** in the **Visualization** window.

6. Clean up. Press the **Disable all** button to hide all of the segmentation overlays. Select **File** > **Close** to hide the Segmentation control window.

7. Save the *State* file. From the EM3D Visualization window select **File > Save > State**. Save the file as *synthetic_segmented.idl*.

## 2.7. Model generation and 3D visualization

The segmentation process generates a set of volumes-of-interest (VOIs). From each VOI, one can create a model of the structure it encloses. Isodensity surface models are usually the most informative structural models, and EM3D provides a collection of tools to create and visualize these models. The VOIs are themselves volumetric structural models, and EM3D provides tools for volume rendering, which can be useful as an adjunct to the isosurface models.

**Summary**

1. Open the EM3D **Rendering** window.
2. Create surface models of selected objects and manipulate these models using the mouse as a virtual 3D trackball in the rendering window (see Basic Procedures).
3. Open the **Rendering Control** window.
4. Adjust colors and opacities of models (see Rendering control window operations)
5. Adjust isosurface levels to improve the reliability of the models (see Adjusting isosurface levels).
6. Create surface models of regions (see Working with regions).
7. Group sets of logically related objects and/or regions to groups for convenience (see Working with groups).

Use lighting, clip planes, cut planes, and spin-animation controls to better visualize and distinguish the models.

Open the EM3D Rendering window (RW) by selecting **Process > Render** from the EM3D Visualization window. The large 3D graphics window will be black and blank – no models have yet been generated.

### 2.7.1. Basic procedures

1. Make sure the **Objects** radio button is selected and the **Objects/Regions** slider is set to 1, the segmentation object previously labeled "Presynaptic Membrane."
2. Press the **Enable** button. A Notice window will open briefly, and then a surface model of the membrane will be created in the 3D graphics window. Note that the button label changes to **Disable**; pressing it a second time would remove the model from the display.
3. Adjust the Model slider to 2, to the object labeled "Vesicle 1." Press the **Enable** button again. A surface model of the vesicle will appear on the display.
4. Mouse Controls

   Examine these two models using the interactive mouse controls.

   **Rotate**: Position the cursor over the 3D graphics window, then depress and hold the left mouse button. Move the mouse, while continuing to hold the button down. The display will change to a lower-quality rendering. The models will rotate interactively, following the mouse movements. Release the mouse button; the rendering returns to full quality.

**Zoom**: Hold the middle mouse button down to control the zoom (magnification) of the rendered models; outward movements make the models larger, while inward movements make the models smaller.

**Translate**: Hold the right mouse button down to control translation of the models.

5. After the examination is complete, press the **Home** button that is next to the **Zoom** slider. The display will return to a standard viewing orientation.

6. This one-by-one generation and examination of models is useful to quickly visualize models of new segmentation objects or regions.

### 2.7.2. Rendering control window operations

1. The **Rendering Control (RC)** window is useful for applying model-generation and visualization commands to multiple segmentation VOIs. Open the Rendering Control window by selecting **RW > File > Control…**. As an example, select the other two segmentation objects in the list, "Filament 1" and "Filament 2." Now create models for both of these objects by selecting **RC > Surface > Enable**.

2. Adjust the color of one model. Select "Vesicle 1" from the Objects list. If it isn't already selected, select the **Color** tab to make this control panel visible at the bottom of the Rendering Control window. The RGB color sliders are all at midrange (128), and the opacity slider is at maximum (255), yielding the default mean gray, and fully opaque surface model appearance. To make the vesicle blue, adjust the Blue slider to a value of 255. Note that the **Current color** patch shows the original mean-gray color, while the **New color** patch now indicates a blue color. Press the **Apply** button to change the color of the model.

3. Give the two filaments a gold color. Select both filaments in the Objects list. Adjust the **Red** slider to about 230, the **Green** slider to about 180, and the **Blue** Slider to 0. Note the color in **New color** patch. Press the **Apply** button.

4. Hide the "Presynaptic membrane." Select it from the Objects list, and then select **RC > Surface > Hide**. The surface model is still present internally, but it is removed from the rendered image.

5. Make the filaments partially transparent. Select both of them in the Objects list, and adjust the Opacity slider to a value of 100. Press the **Apply** button. Use the mouse controls in the 3D rendering window to rotate the models so that the filaments are in front of the vesicle. The vesicle surface should be visible through the filaments. If not, press, the **Front** button (next to the **Apply** button at the bottom of the Rendering Control window).

### 2.7.3. Adjusting isosurface levels

Surface models have a default value for their isosurface level. This value should be adjusted, manually or automatically, to optimize its accuracy and reliability.

Hide all of the models except "Vesicle 1." Using the **Rendering Control** window, select the "Presynaptic membrane," "Filament 1," and "Filament 2" from the Objects list, and then

select **RC > Surface > Hide**. Press the **Home** button in the Rendering window so that this one model fills the 3D display.

**Manual adjustment** of isosurface level is performed using controls on the **EM3D Rendering** window. Use the following steps:

1. Select the "Vesicle 1" object using the **Model** slider in **EM3D Rendering** window.

2. Examine the **Isosurface level** slider, the text field above it, and the small plot window below. The isosurface gray level value can be adjusted using any of these three methods: adjust the slider, left-click in the plot window, or type a numeric value into the text field.

3. The current value for the isosurface level is the calculated default for Vesicle 1's segmentation VOI; it should be about 670. The units for isosurface level are normalized to the full spectrum of values in the volume, so that 0 is the darkest value and 1000 is the lightest. The plot shows the cumulative distribution function (CDF) for the entire volume on this 0—1000 scale. The blue line marks the current value of the isosurface level. Normally, the isosurface level should be somewhere on the steep slope of the CDF.

4. Examine the main **EM3D Visualization** window. Select **Image > Show > Segmentation** and confirm that **Objects** is checked; select it if not. Click in the center of "Vesicle 1" so that its cross-section is visible in all three views. The outlines of the surface model should be visible as blue lines. This display provides useful feedback between the 3D models and the 2D volume slices.

5. Adjust the **Isosurface level** slider downwards in several small steps of about 10 units each by using the mouse or by entering numbers into the text field pressing <Enter> key on the keyboard. After each change, a new surface is created, and the blue line moves leftwards on the plot. Note the changes in the models in both the 3D graphics and Visualization windows. The optimum value is probably somewhat smaller than the default setting, but after 5 or 6 downward adjustments, the surface model will become quite sparse and broken.

6. Return the isosurface level to its default level by entering 670 in the **Isosurface level** text field.

7. Repeat the sequence of incremental changes, this time moving upwards in isosurface level. Again note the changes in the surface model in both the 3D graphics and Visualization windows. After 5 or 6 small upward adjustments, the model will become increasingly truncated against the boundaries of the VOI.

**Automatic optimization** adjusts the isosurface level so that it occurs at the sharpest contrast boundary for the material within the VOI. Use the following steps:

1. Use the manual controls to adjust the "Vesicle 1" isosurface level to a relatively small value near the lower "tail" of the CDF, e.g., 620. Automatic optimization is an iterative search that begins from this value, and

convergence is more robust when starting from a smaller (darker) initial isosurface level.

2. In the Rendering Control window, select “Vesicle 1” again from the Objects list, and then select **RC > Surface > Optimize**.

3. A Notice window will open and display information about the progress of the optimization. About a dozen iterations will be necessary to find the optimal value for the vesicle model.

4. After the calculation is complete, the model will be displayed at its optimal isosurface level. Examine the results in the 3D and Visualization windows – the optimized model should appear to follow the sharpest contrast level.

5. As another example, optimize both of the synthetic filaments. Select both of them in the Objects list, and then select **RC > Surface > Optimize**. The two manual segmentation VOIs will be optimized one-after-the-other. After the calculation, the 3D display will update with the new filament models. Note that the new models are much better representations of the filaments than the default isosurface level model, which exhibited a great deal of noise.

### 2.7.4. Working with regions

Regions are segmentation VOIs derived by compartmentalizing the volume with one, or more, object VOIs (see Regional segmentation). Most of the same tools described for the object VOIs operate analogously upon region VOIs. Here are some common operations with region VOIs.

1. Examine the Regions list in the **Rendering Control** window. Three regions should be visible with default labels “Region 1,” “Region 2,” and “Region 3.” The regions are ordered by size, starting with the smallest. From inspection of the Regions color overlays in the Visualization window, it is possible to conclude that “Region 1,” the smallest, is the intralumenal space of “Vesicle 1,” while “Region 2” is the extracellular space, and “Region 3,” the largest, is the cytoplasm.

> *Note also the first label in the Regions list called “Null”; this region is always created, and corresponds to those portions of the volume that were outside of the slice range of all of the selected objects. For the Synthetic dataset at this point, the “Null” region corresponds to slices 0—9 and 91—99.*

2. Hide all of the object models by selecting **RC > File > Select all > Objects**, and then **RC > Surface > Hide**.

3. Regions can be dealt with one-by-one using the controls in the **EM3D Rendering** window in much the same fashion as segmentation objects were handled. Begin by selecting the **Regions** radio button, and then adjust the slider so that “Region 1” is selected. Press the **Enable** button. A small spheroidal surface model of the intralumenal space will appear.

4. Regions can also be dealt with using the **Rendering Control** window. Rename the three regions by either double-clicking each one and naming it in turn, or by selecting all three regions and then selecting **File** > **Rename**.

Change the name of “Region 1” to Intralumenal space,” “Region 2” to “Extracellular space,” and “Region 3” to “Cytoplasm.”

5. Regions can also be optimized. Select “Intralumenal space” from the Regions list in the **Rendering Control** window, then select **RC > Surface > Optimize**. The optimized version of this surface model won’t look much different than the default, because this model is formed from the artificial noise within the synthetic lumen.

### 2.7.5. Working with groups

Sets of logically related objects and/or regions can be collected into named groups for convenience.

1. In the **Rendering Control** window, select “Filament 1” and “Filament 2” from the Objects list, then select *File* > *Group*. A new entry will appear in the Groups list called “Group 1.”

2. Rename this group by double-clicking it; name it “Filaments.”

3. Regions can also be grouped. Select “Extracellular space” and “Cytoplasm” from the Regions list, then again select *File* > *Group*. A second entry will appear, “Group 2.”

4. Double-click this group and name it “Large volumes.”

5. Whenever a group is selected, all of its corresponding members will be selected in the *Objects* and/or *Regions* lists. This permits convenient adjustment of color, visibility, or for the application of calculations as described in the next chapter.

6. To remove a group, rename it with an empty string. Double click each of the two groups. In each case highlight the entire text label for the group and press the <Backspace> key on the keyboard to clear the label, then press the <Enter> key. The groups will disappear from the list as they are removed.

## 2.8. Calculation

**Summary**

**Spatial uncertainty**

1. Open the **Segmentation** window.

2. Create a manual SNR reference object and close the **Segmentation** window.

3. Open EM3D Rendering and Rendering Control windows.

4. Select the **Uncertainty** tab.

5. Select the previously defined SNR reference in the **Objects** list and press the **SNR Reference** button.

6. Select object(s) and/or regions in their respective lists.
7. Press the **Calculate** button.
8. Examine the results histogram, or create a colormap overlay.

**Thickness**

1. Open EM3D Rendering and Rendering Control windows.
2. Select the **Thickness** tab.
3. Select objects and/or regions in lists.
4. Adjust parameters if necessary.
5. Press the **Calculate** button.
6. Examine the results histogram, or create a colormap overlay.

**Proximity**

1. Open EM3D Rendering and Rendering Control windows.
2. Select the **Proximity** tab.
3. Select a single reference object or region and press the **Reference** button.
4. Select object(s) and/or regions and press the **Destination** button.
5. Press the **Calculate** button.
6. Examine the results histogram, or create a colormap overlay.

**Automatic Regional Segmentation (ARS)**

1. Open the EM3D Segmentaion Window.
2. Press **Disable All**.
3. **Enable** the Presynaptic Membrane.
4. Select **Regions** > **Map**.
5. Close Segmentation window and open the Render Window and the Render Controls window.
6. Select the **A.R.S.** tab.
7. Select the Green Region in the Regions: list above.
8. Press **Set Region** in the ARS tab.
9. Set **Threshold** = 650, **Decimation**=10, and **Minimum Size**=100

10. Press **Calculate**.

11. Enable the RegionGreen Auto Group from the Groups: menu.

12. Examine the results in the 3D graphics window.

**Overlays**

1. Open EM3D Rendering and Rendering Control windows.

2. Select the **Overlay** tab.

3. Select the calculated result from the **Overlay** pull-down menu.

4. Select object(s) and/or regions in lists.

5. Adjust the **Minimum** / **Maximum** text fields if necessary.

6. Press the **Apply** button.

7. Examine the results in the 3D graphics window.

### 2.8.1. Spatial uncertainty

EM3D estimates spatial uncertainty by calculating the gradient scale length, *l*, in the vicinity of each vertex of the model. The scale length is measured, specifically, along the outward normal of the model vertex. This is a measure of how rapidly the gray level is changing as one moves away from the model surface at that point – a measure of the sharpness of the contrast boundary. This measure is combined with an estimate of grayscale uncertainty, $\sigma_g$, to convert grayscale uncertainty to spatial uncertainty: $\sigma_n = l / \sigma_g$.

The steps involved in performing the spatial uncertainty calculation are as follows:

1. Identify a background region.

   - Examine the volume using the navigation controls in the Visualization window. Choose a region corresponding to ‘background,’ a bright and uniform area that appears to contain no significant biological material. Perform a manual segmentation of that region.

   - Open the Segmentation window Process > Segment….

   - Choose a distinct, unused Object # such as 999, and label it “SNR Reference.”

   - Select the **Manual** tab to initialize the segmentation. Select **Piecewise** mode and outline a small portion of the ‘background’ region. Select either the **Up** or **Down** buttons to create a volume of ‘empty’ space in the background area (see Figure 23). It is necessary to select only a few volume slices; the goal is select about 5,000—10,000 voxels so that the noise estimate is not significantly affected by counting statistics.

- On the last slice, press the **Segment** button to complete the manual segmentation.

- Close the **Segmentation** window.

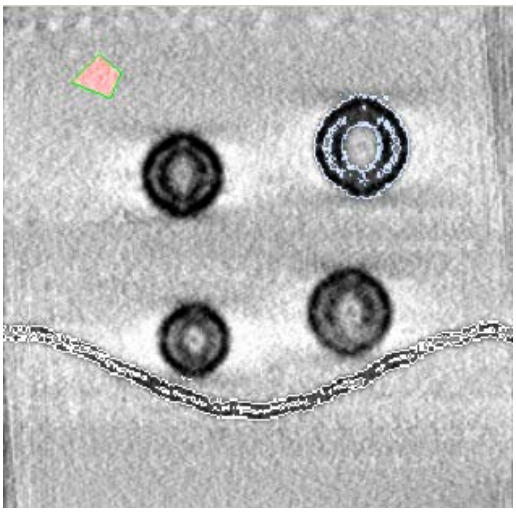


*Figure 23: Manual segmentation to establish a signal-to-noise ratio reference.*

2. Open the **EM3D Rendering** and **Rendering Control** windows. Note that the new "SNR Reference" object is now present in the Objects list.

   - Click on this object to select it.

   - Select the **Uncertainty** tab.

   - Press the **SNR Reference** button to establish the reference.

   - The *Reference object* and *Reference signal +/– noise* fields will update appropriately. The reference signal should be approximately 10x larger than the reference noise, i.e. the signal-to-noise ratio should be close to 10.

2. Once the SNR reference has been established, it becomes possible to perform spatial uncertainty calculations on any collection of Objects or Regions. To illustrate these calculations, select "Vesicle 1" and "Filament 1" from the Objects list, and press the **Calculate** button.

3. A sequence of **Notice** dialogs will pop-up as the calculations proceed in three steps: 1) a volume mask is created for each VOI; 2) spatial gradients are calculated within the VOI; 3) the spatial uncertainty is calculated. After each uncertainty calculation, a histogram of uncertainty values is displayed in the plot area at the bottom of the **Uncertainty** tab.

4. The results of the calculation can be examined by selecting each object or region in turn. Select "Filament 1." The histogram is displayed in the plot window, and the minimum, maximum, median, and percentage of "bad" values are displayed as text below the plot window. Because of noise, the spatial uncertainty calculation inevitably produces a long sparse "tail" of

large values, and even some negative values where the gradients point inward rather than outward. The latter are flagged as “bad.” At locations where large or bad values occur, the surface model is not a reliable description of the structure, either because the noise is too large or the structural component does not have a sharp boundary at that point.

5. The results of the spatial uncertainty calculation can be visualized as a colormap overlay on each surface model. See Overlays, below.

> ***Note: There was an error in a constant used in the proximity calculations prior to Version 1.3. All earlier values should be adjusted by a factor of 2/3 (0.6667). All versions from 1.3 onward have this correction applied.***

### 2.8.2. Proximity

EM3D calculates precise Euclidian distance maps between model vertices. The calculation is exact, so it can be quite time consuming; faster, more approximate results can be obtained by selecting the **Quick** radio button instead of the **Exhaustive** radio button. The quick method creates a distance, volume map which contains the distance of every voxel in the volume to the closest point on the surface of the Reference object. Thus the same map can be applied to multiple objects at virtually no additional expense in time or computing. The quick method suffers from less precision because calculations are done by whole voxels instead of floating point Euclidean measures.

1. Open the **Rendering Control** window and select the **Proximity** tab.

2. Specify the reference model; distance will be calculated with respect to the nearest-neighbor vertices on this model. For example, select “Vesicle 1” in the Objects list and press the **Reference** button; the label of this object will appear in the **Reference model** field.

3. Specify the destination model(s). Nearest-neighbor distances will be calculated for each vertex on these models. For example, select the “Filament 1” and “Filament 2” models in the Objects list and press the **Destination** button; the labels of these objects will appear in the **Destination models** list. Note that exhaustive proximity calculations can take a long time; whereas, quick proximity calculations take approximately the same time to calculate for small objects as well as large objects. In addition the quick calculation takes virtually no additional time to calculate the proximity of additional objects.

4. Press the **Calculate** button. A **Status** window will open to indicate the progress of the calculation. As the proximity calculation for each object is completed, its histogram of distance values appears in the plot window in the lower part of the **Proximity** tab.

5. Examine the results by selecting each model in the Objects or Regions lists. For example, select first “Filament 1,” then “Filament 2.” The histogram and statistical information (minimum, maximum, median) will appear for each model as it is selected.

6. The results of the proximity calculation can be visualized as a colormap overlay on each surface model. See Overlays, below.

### 2.8.3. Thickness

EM3D calculates thickness at each vertex by searching along the direction of the inward surface normal for opposing vertices that fall within a specified angular aperture. The "thickness" is defined as the mean distance between these vertices and the original vertex. This calculation can be time consuming for extensive surfaces; faster, more approximate results can be obtained by increasing the decimation level of the surface models.

1. Open the **Rendering Control** window and select the **Thickness** tab.

2. Choose model(s) for the calculation by selecting from the Objects or Regions list. For example, select "Vesicle 1". Warning: it takes a very long time to perform calculations for the "Presynaptic membrane" and "Vesicle 1".

3. The **Minimum** and **Maximum Thickness Slider**s have 2 purposes, 1) to limit the range of values considered in calculating the Thickness, and 2) to isolate values once the Thickness has been calculated. For instance, with a Vesicle that has a membrane thickness of 7nm and a diameter of 60nm, you can calculate either by setting the Minimum and Maximum Slider values correctly. To get the diameter of the Vesicle leave the Slider values at 0 and 99. To get the membrane thickness set the slider values at 3 and 15. By limiting the slider values to distances roughly half to twice the expected thickness you will only be considering points in the right range.

4. Press the **Calculate** button. A **Status** window will open to indicate the progress of the calculation. As the thickness calculation is completed, its histogram appears in the plot window in the lower part of the **Thickness** tab.

5. Adjust the **Minimum** and **Maximum Thickness Sliders** to identify only reasonable points. For instance choose values around the histogram peak you are interested in.

6. Note that the statistical information (minimum, maximum, median) will appear at the bottom, these values will only be calculated for values within the Min-max range shown on the sliders.

7. The result of the thickness calculation can be visualized as a colormap overlay on a surface model. See Overlays below.

### 2.8.4. Overlays

The results of any of the three calculations described above, uncertainty, proximity, and thickness, can be visualized on its corresponding surface model as a colormap overlay. The overlay represents the numerical value of the calculation at each vertex as a specific color.

1. Open the **Rendering Control** window and select the **Overlay** tab.

2. Near the top of this tab is the **Overlay** pull-down menu. (If this menu displays "NONE", perform the tutorial calculations described in the previous three subsections (Uncertainty, Proximity, and Thickness); the following assumes this has been done.) Select "Uncertainty" from this menu.

3. In the **Rendering Control** window, select **File > Select all > Objects**; all of the object models will be highlighted.

4. The **Available models** list will display that subset of the selected models for which Uncertainty data is available. In this case, “Obj: Vesicle1” and “Obj: Filament 1” appear, corresponding to the two objects for which uncertainty was previously calculated.

5. Below the list are text fields labeled **Minimum** and **Maximum**. By default, these fields are set to the actual minimum and maximum values for the selected collection of overlay data; these values are also shown for reference as labels just to the right of the text fields. Presently, the minimum value should be small, <1, while the maximum value should be a large number, ~$10^4$.

6. To display a map of uncertainty that spans the range 0—5 nm, select the text in the **Minimum** field and enter 0 (zero), then press <Enter> on the keyboard. In a similar fashion, enter the number 5 into the **Maximum** field. Press the **Apply** button near the bottom of the tab.

7. Examine the 3D graphics window (**Error! Reference source not found.**). A colormap overlay is now visible on the simulated vesicle and one of the filaments. The color bar near the bottom of the Overlay tab indicates the mapping between the colors and the uncertainty values; the bar is set so that it spans the range 0—5 nm. The majority of the vesicle and filament surfaces show a dark blue color indicating small uncertainty values; here the model surface is interpolated through regions where the gradient scale length was very small, so the model’s vertex locations are insensitive to grayscale noise in the volume and therefore very reliable. There are a few punctuate regions of larger spatial uncertainty near the equator of the simulated vesicle because the larger amount of surface texture here increased the gradient scale length, making the model vertex locations in these more sensitive to grayscale noise in the volume and therefore less reliable.

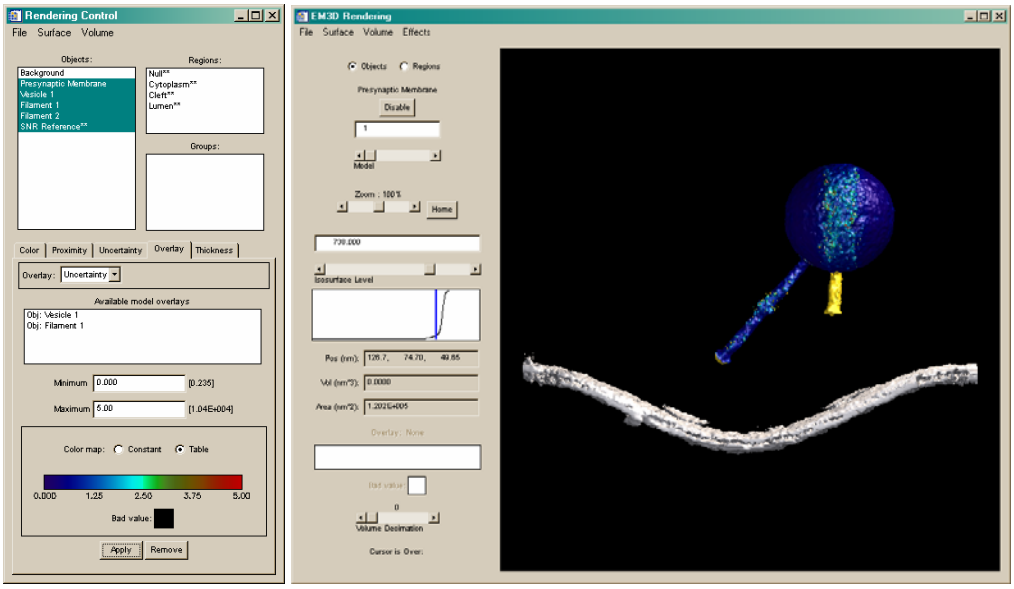



*Figure 24: Spatial uncertainty overlays*

8. Examine the **EM3D Rendering** window. Use the **Model** slider to select "Vesicle 1." Information about the spatial uncertainty overlay will now appear near the lower left corner of the window. This feature provides convenient information about whatever overlay has been applied to a particular model.

9. Many colormaps are available, and each can be customized in various ways. Click the left mouse button directly on the color bar to bring up a dialog window with an extensive list of colormaps and tools for their individual customization (see Figure 25). For example, select the "Rainbow" colormap from the list, then press the **Done** button. The new colormap will now appear on the colorbar in the **Overlay** tab of the **Rendering Control** window. Press the **Apply** button to display the new colormap on the surface models.

10. The **Bad value** patch below the color bar can be used to mark such values with a distinctive color. By default, the patch shows black (RGB value = {0, 0, 0}). With this setting, bad values are not colormapped; they remain at whatever color was set for the model before the overlay was applied. Change this setting to mark the bad values. For example, in the Rendering Control window, select only "Filament 1," then click the left mouse button on the **Bad value** patch. A small dialog will pop up with a text field showing the current settings. Change these values to "255, 255, 255" and press the <Enter> key on the keyboard. The **Bad value** patch will now turn white. Press the **Apply** button to apply this white marking to the vertices with bad spatial uncertainty values on model.

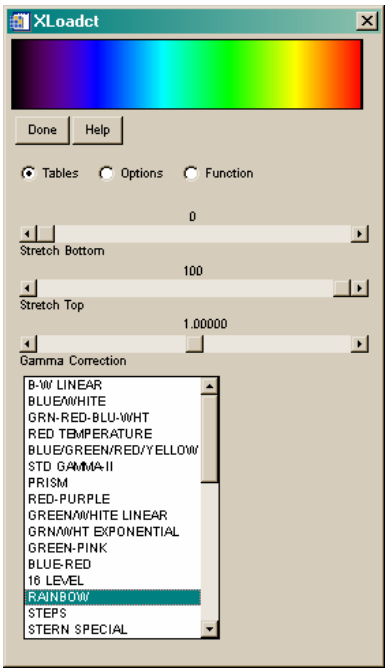



*Figure 25: Color-map dialog window*

11. Using the mouse controls in the 3D graphics window, rotate the models so that the tip of "Filament 1" distal to the vesicle is visible. This tip will have several white vertices (see Figure 26). This region of the model cuts into another synthetic vesicle, so the gradients in this region are inverted, creating "bad values" by definition.

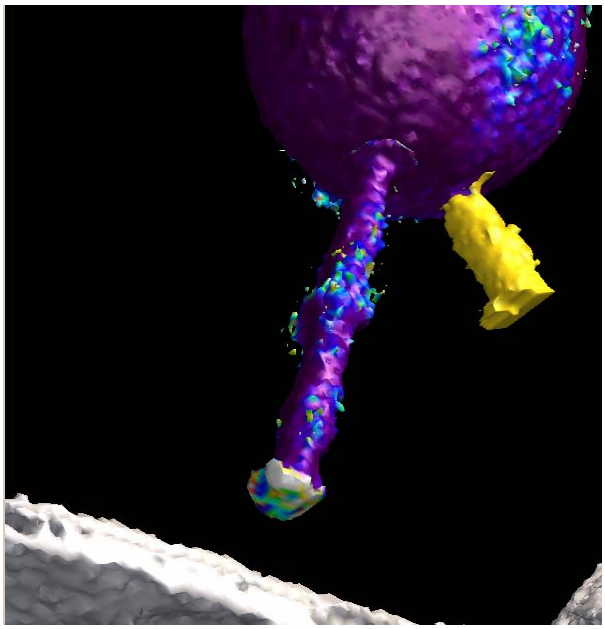


*Figure 26: Marking bad values*

12. It is also possible to remove colormap overlays from models. On the **Overlay** tab of the **Rendering Control** window, press the **Remove** button to remove the overlay from "Filament 1." On the **EM3D Rendering** window, press the **Home** button to return the display to its default viewpoint.

13. Any of the other calculated quantities can also be rendered as an overlay. In the **Overlays** tab of the **Rendering Control** window, select "Proximity" from the pull down menu. Press the **Apply** button. A color-map overlay will appear upon "Filament 1" that indicates the proximity of its vertices to the vesicle.

14. Select "Thickness" from the **Overlays** pull-down menu. In the Objects list, select "Filament 2." Click on the color bar, and use the color bar dialog to change the color bar to "Blue-Red." Press the **Apply** button to visualize the thickness values on "Filament 2." Use the mouse controls in the 3D graphics window to examine the results (see Figure 27).

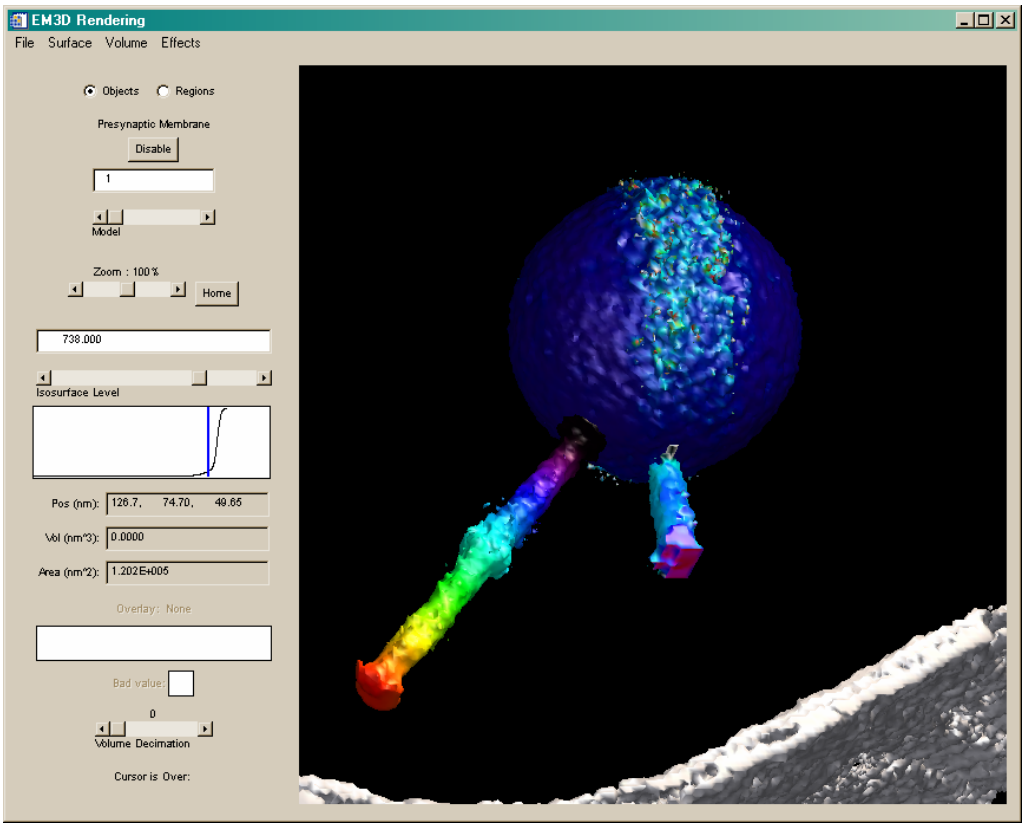



*Figure 27: Color overlays showing spatial uncertainty on the vesicle, proximity to the vesicle on Filament 1, and thickness on Filament 2.*

# 3. Reference Manual

## 3.1. Loading data

To use EM3D, raw data from the EM must first be imported into EM3D. The imported data then will be saved in an EM3D specific format (.idl and .data files). Current raw data formats include sets of individual TIFF files or multiple MRC stack files.

### 3.1.1. Opening EM3D datasets

Select **File** > **Open** from the menu. A file browser opens. Select the *State* file that corresponds to the dataset.

### 3.1.2. Importing raw EM projection images and reconstructed volumes into EM3D

To access the file importation features, press **Cancel** on the initial File open Dialog. This will reveal the main **EM3D Visualization** window. Use the **File** menu to access the import features.

### 3.1.3. Importing TIFF projection images

Select **File** > **Import** > **TIFF** from the EM3D Visualization window.

Locate the directory containing the TIFF files. On all platforms except Windows, select all the images by clicking on the first file, then the last file while pressing the shift key. On Windows platforms, reverse the procedure by first clicking on the last file in the sequence, then the first, or press ctrl+A to select all files. Press **OK** to start the file import process.

Because TIFF images have no header information regarding tilt, magnification, pixel size, etc., EM3D will pop up the **Specify Parameters** window to allow this information to be specified (Figure 28).

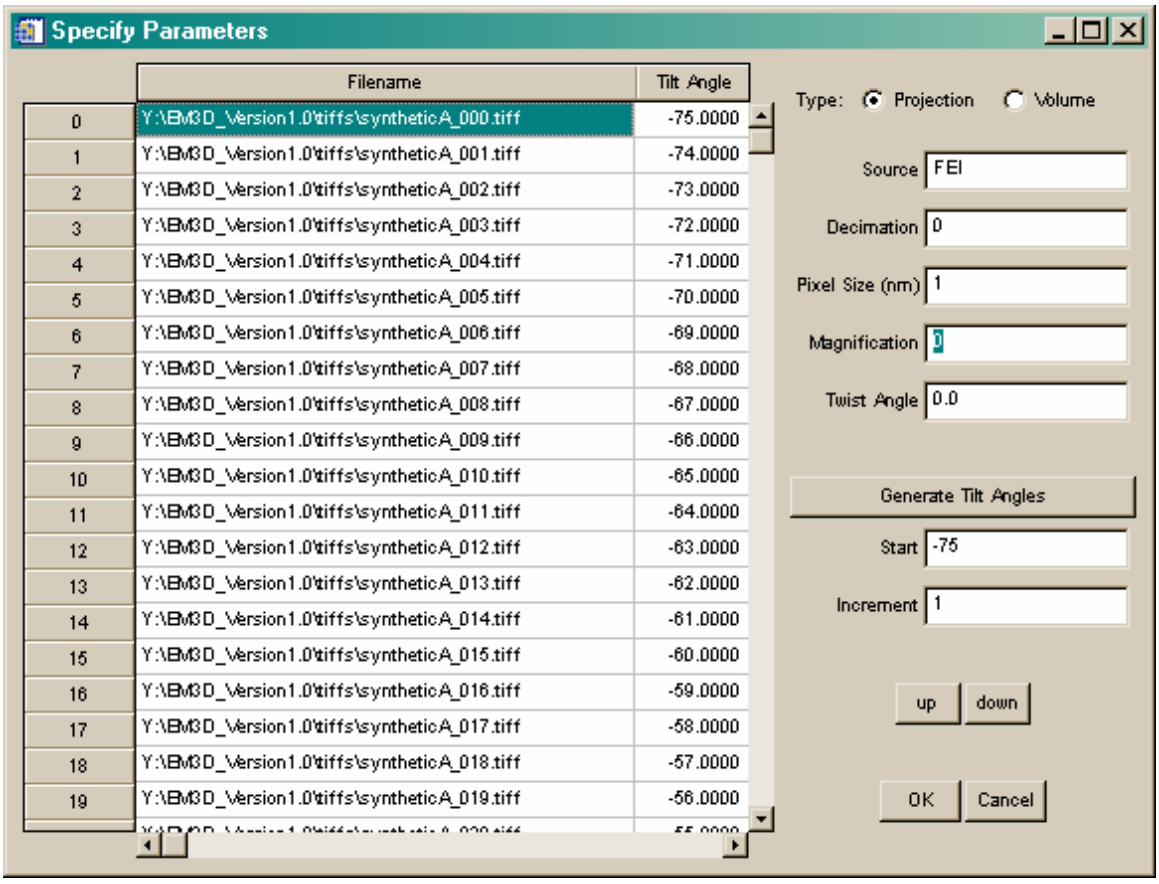



*Figure 28: Specify parameters for loading raw EM projection data*

### 3.1.4. Importing MRC stack files

Select **File > Import** > **MRC Stack** from the **EM3D Visualization** window. A file browser window will open; use it to select one MRC Stack files. After EM3D loads the MRC files, it will open the **Specify Parameters** window. Because the MRC files include header information, generally only Source information needs to be entered, although the other fields can be edited if necessary (see Figure 28).

> *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 TIFF files and import these in EM3D.*

### 3.1.5. Saving the imported data

When projections are loaded, the zero degree tilt will appear in the **EM3D Visualization** window. Save the dataset with a new name (**File > Save > *Binary* and *State***) to generate new .idl and .data files.

### 3.1.6. Importing Reconstructed Volumes from TIFF images

It is possible to import already reconstructed volumes into EM3D. The volume must be saved as a series of TIFF images with 16 bit precision.

Select **File > Import > TIFF** from the EM3D Visualization window.

Locate the directory containing the TIFF files. On all platforms except Windows, select all the images by clicking on the first file, then the last file while pressing the shift key. On Windows platforms, reverse the procedure by first clicking on the last file in the sequence, then the first, or press ctrl+A to select all files. Press **OK** to start the file import process.

The **Specify Parameters** window will appear. The default **Type** for TIFF images is Projection, so select the Volume radio button to the right of the file list. The parameters to specify will change from Figure 28 to Figure 30. Enter the appropriate data and press **OK.**

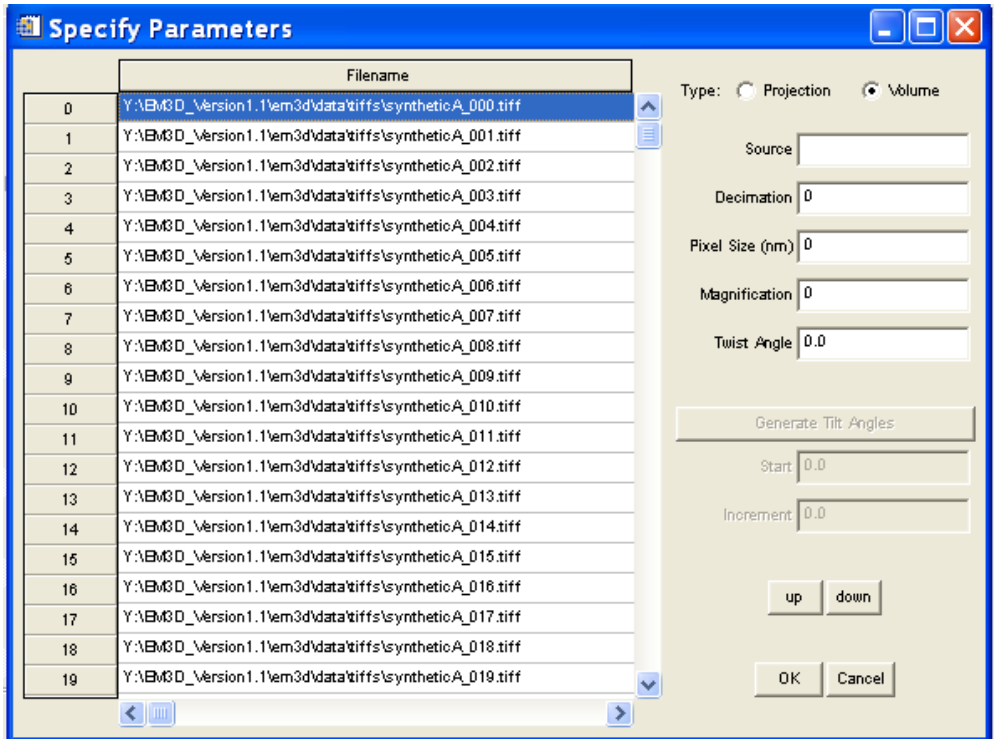



*Figure 29: Importing Reconstructed Volume from TIFF*

## 3.2. EM3D visualization window

### 3.2.1. Overview

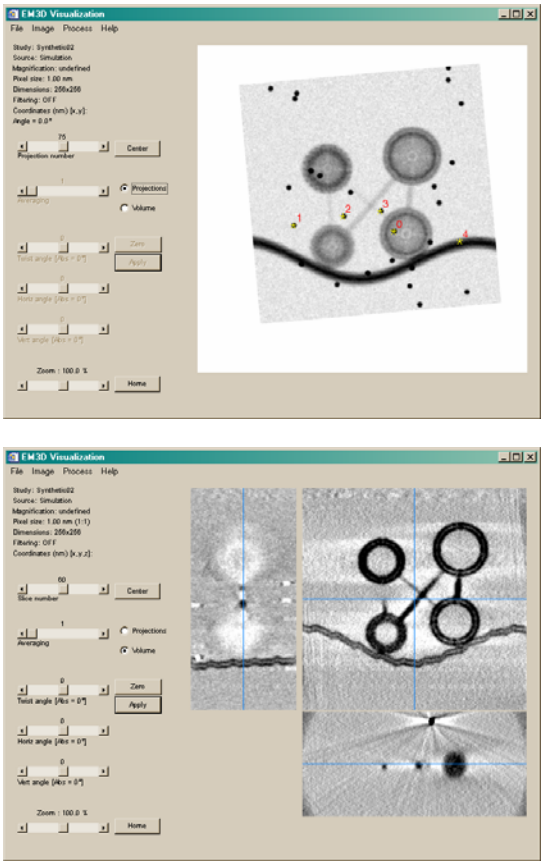


*Figure 30: EM3D visualization window, projection and volume modes*

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-entered information. The EM3D Visualization window operates in two modes, **Projections** and **Volume**, controlled by the pair of radio buttons at the left-center of the window. The size of the displayed image can be controlled using the **Zoom** slider at the lower left corner of the window. Zooming can be particularly useful during segmentation (described below); note that large zoom factors will significantly slow the image display update.

**Projections** mode is used to view individual EM projection images. When a dataset is initially imported, only this mode is available. The **Projection number** slider, just left of the mode-control buttons, controls which tilt-angle projection is displayed. The tilt angle and cursor pixel coordinates are displayed just above the slider.

> *Note: For Macintosh and UNIX/LINUX platforms, 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.*

**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 becomes available only after a volume has been reconstructed. 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.

Several additional controls, which are grayed out and inactive in Projection mode, become available in Volume mode. **Averaging** allows the selection of adjacent volume slices to average with the current slice to form a smoothed slice (for display purposes only).

Virtual slices can be formed at any angle through the volume, as 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 directly from the volume. To permanently rotate the volume, click on the **Apply** button. These rotation tools allow the user to adjust the view in order to see structural components with better contrast, which is particularly important during segmentation.

> *Note: The segmentation and alignment windows must both be closed to rotate the volume.*

A simple measurement tool is available to measure distances in any of the three coordinate windows. Right-click at the beginning point, then move the cursor to the end point. A yellow dotted line will show this distance and the **Distance (nm)** field will read out just below the **Coordinates** readout on the left. The distance measurement disappears when the right mouse button is released.

The three main pull down menus of the EM3D Visualization window are: **File, Image,** and **Process**; these are each described in detail in the next chapters.

### 3.2.2. The file menu

**Open** is used to open .idl/.data files. It is grayed out after a dataset is loaded, to load a new dataset, one must exit and restart EM3D.

**Import** is used to import raw data files, e.g. TIFF, MRC stack, or volume projections from other sources. It is also grayed out after a dataset is loaded.

**Restore** is used to revert to the original reconstructed volume data after changes such as rotation or smoothing are made, that is, **Restore** returns the data to the original, non-modified state. It operates only in volume mode.

Restore doesn't work if you want to undo a decimation or core. These are irreversible steps. If not satisfied with decimation or core operations, quit EM3D without saving.

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

**Save > Binary and State** writes new State and Binary files. This is necessary after one first generates a volume or after making changes to an existing volume. The .idl and .data files will be saved. To preserve the previous files, choose a new name for the .idl file different than the previous name. The .data file automatically gets the same filename as the .idl file. (It's not necessary to add .idl after the new file name.)

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

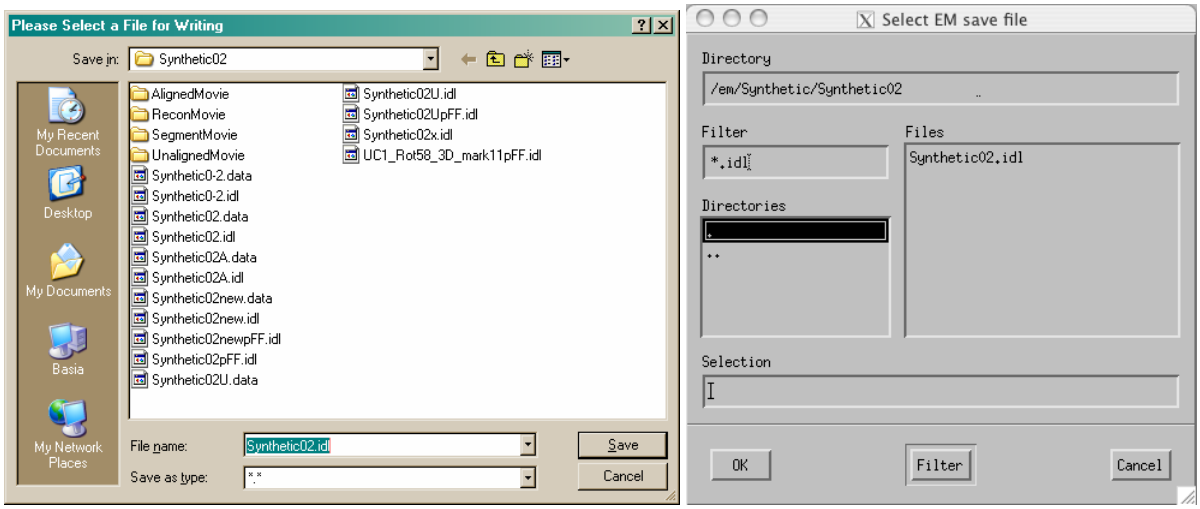



*Figure 31: File save dialog boxes for Windows(left) and Unix platforms (including Macintosh)*

In the **Save in** / **Directory** field, enter the pathway to the directory where the files will be saved and select <Enter> (on the keyboard) or **Filter** to set it. In the **File name** / **Selection** field, enter the name of the new file(s) and select **Save** / **OK**. (One can also navigate these dialogs by left-clicking and double-clicking with mouse in accordance with the standard practices of that particular platform.)

**Write…** opens a window that permits creation of a set of files, each containing an individual projection or volume image in either PICT or TIFF format. Use this tool to save sequences of projections or slices for illustration or animation purposes.

> *We recommend writing 16 bit TIFF images unless you want to print the TIFF files.*

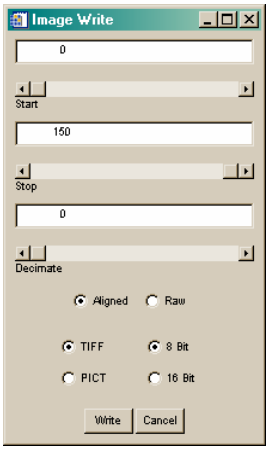



*Figure 32: Write window*

Adjust the **Start** and **Stop** sliders for the range of projections or slices, and select the Write button. A file dialog will be displayed; the output sequence of files will have names that start with the filename, each with an appended sequence number corresponding to the projection or slice number of the image it contains.

**Delete Proj/Slice** removes an undesirable projection or volume slice from the series, which can be necessary because of bad focus, noise, or other image problems.

**Quit**, exits EM3D. A warning dialog may ask if you want to save EM3D file(s).

### 3.2.3. The image menu

**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. It changes the image scaling 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 red lines on the graph. For example, if both fields are set at 1%, then the darkest 1% and the brightest 1% of the pixels in the image will be clipped at, respectively, black and white, while the remaining 98% of the pixels will be linearly scaled between black and white. Increasing both clip values together increases the image contrast.

On the lower left corner are two radio buttons, **Absolute** and **Relative**. **Absolute** scales each image based on the range of data values in the entire tilt series or volume, while **Relative** scales each image based only on that projection or slice. Relative is the default mode for projections, while absolute is the default mode for volume slices. The **Invert** button reverse the grayscale, generating a negative image that is often useful for segmentation.

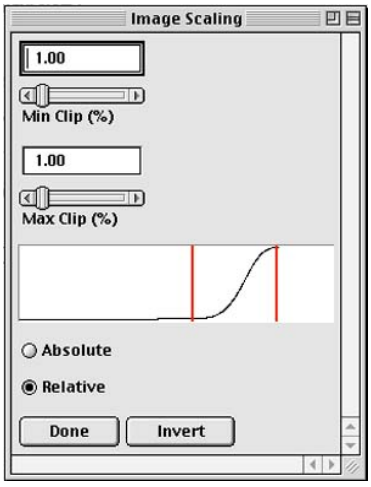



*Figure 33: Image Scaling*

**Review x,y/x,z/z,y** create animation sequences of the projection or volume-slice data. In projection mode, only the x,y review is available; in volume mode one can review any of the three orthogonal slice orientations. When selected, the appropriate image window will be sequentially updated with the entire set of projection or slice images. This first review sequence serves to create an animation that is then displayed in a new window (see Figure 34).

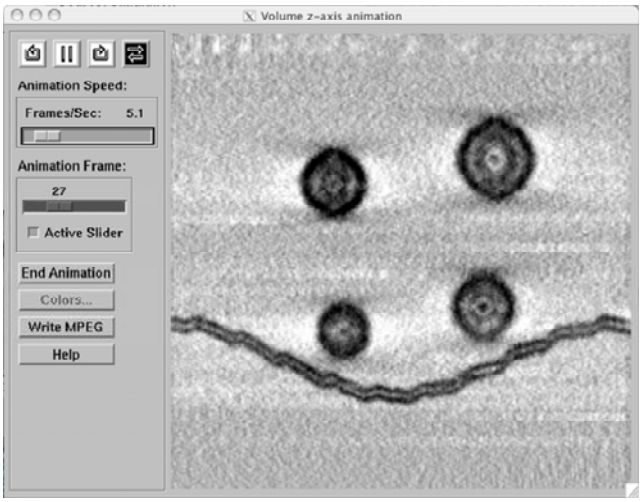



*Figure 34: Review animation window*

This window provides extensive control over the animated review. On platforms that have fully licensed versions of IDL, MPEG movies may be written.

**Average x,y** is a toggle that provides a 3D extension of the slice averaging controlled by the **Averaging** slider (see above); it only functions in Volume mode. When selected, the averaging slider now controls a volumetric boxcar smoothing of the data, with a setting of 1 corresponding to a 3×3×3 smoothing kernel, 2 corresponding to a 5×5×5 kernel, and so forth.

**Invert** provides the same function as **Invert** in the **Scaling** window.

**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.
- **Segmentation** > **Objects** / **Regions**: toggles on or off color overlays that indicate segmentation boundaries or locations (see Segmentation).

### 3.2.4. The process menu

#### *Decimate*

The **Decimate** slider controls the size of the reconstructed volume. Projection images are often quite large, e.g., each 2048 × 2048 pixel image requires 8 MB of memory. Consequently, the corresponding size of an undecimated reconstructed volume can be huge, e.g., 1 GB for a 128 slice reconstruction. Such huge reconstructions are time consuming, and can tax or overwhelm the memory (RAM) capabilities of many desktop computers. The table below illustrates the dramatic reduction in image volume (computation time) achieved through decimation.

| Decimation Level | In-plane dimension reduction | Volume reduction (Projections) | Volume reduction (Volume) |
|---|---|---|---|
| 0 | 0 | 0 | 0 |
| 1 | 1/2 | 1/4 | 1/8 |
| 2 | 1/4 | 1/16 | 1/64 |
| 3 | 1/8 | 1/64 | 1/512 |

The reconstructed volume size is indicated by the text field, **Volume array size = ## MB**, located near the middle of the Reconstruction window. Note that setting the reconstructed volume **Data type** to **Real** will double the size of the volume array.

Use Decimate judiciously — it is an irreversible step. In order to preserve the original data, save the decimated file with a new name. If it is decided that a decimated file is not appropriate **Cancel** and do not save.

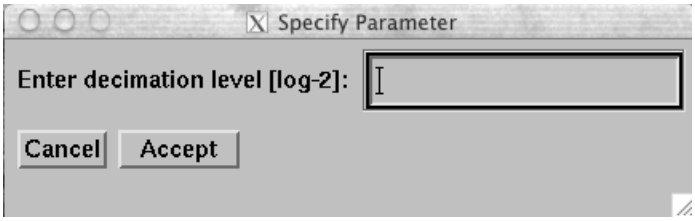



*Figure 35: Decimate data*

## *Core*

Use **Core** to permanently cut a subvolume from the existing volume. Select **Process** > **Core…** to open the **Core Volume** window (Figure 36).

Define the core area by holding down the right mouse in the (*x*, *y*) view of the EM3D Visualization window. Drag the mouse to create a square box that will define the boundaries of the "cored" subvolume.

Next, define a start slice and stop slice to specify the depth limits of the new volume. This can be done by manually adjust the Start slice and Stop slice sliders in the Core Volume window. Alternatively, use the navigation controls in the Visualization window to display the slice where the new volume should start, press **Set Start** and then navigate to the last slice for the new volume and press **Set Stop**.

Create the new volume by pressing the **Core** button. Press **Cancel** to abort the entire process.

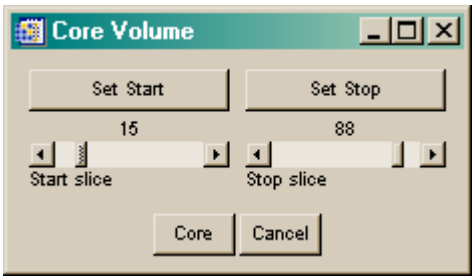



*Figure 36: Core Volume window*

Use Core judiciously — it is an irreversible step. In order to preserve the original data, save the "cored" subvolume using a file with a new name.

### *Filter*

The Filter menu controls a simple boxcar filter to reduce noise

- **Smooth…** opens a simple dialog containing two numbers. The first is the size of the boxcar filtering kernel ; the second is the number of smoothing iterations to apply. Smoothing can be applied to the projections (2D smoothing) or the volume (3D smoothing). By default these numbers are 3, 1 – that is, in volume mode a 3×3×3 boxcar applied once. Like decimation, smoothing should be used judiciously.

- **Commit** makes the filtering permanent, so that it cannot be Reset or Restored.

- **Reset** removes the filtering by rereading the projections or volume from the Restore file. One can also remove smoothing by selecting **File** > **Restore**.

The other **Process** menu selections, **Align…, Reconstruct…**, **Segment…**, and **Render…** are described in the next chapters.

## 3.3. Alignment controls

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

The Projection Alignment window contains one drop-down menu, the **File** menu:

- **Revert** allows convenient error handling during alignment-offset calculations.

    - **Single-step** "undoes" the previous alignment set of fiducials deletions and alignment-offset calculations.

    - **Calculations** removes all calculations and deletions, bringing the alignment procedure back to the results of the Index step.

    - **Indexing** brings the process back to the results of the Acquire step.

- **Reset** removes all alignment data.

- **Close** hides the Alignment window.

### *Acquisition controls*

Floating-point slider values can be adjusted directly, or by entering a specific number in the text field and hitting the <Enter> button on the keyboard.

- **Acquire** button initiates acquisition.

- **Fiducial Diameter** slider controls the smoothing. Larger numbers increase the marked areas and reduce noise, but excessive numbers will merge together closely spaced fiducials. Typical range: one-half to one-quarter of the actual diameter of the fiducials.

- **Contrast** slider controls the fiducial marker contrast enhancement. Larger numbers increase fiducial visibility, but also increases noise. Typical range: 0.5—0.9.

- **Threshold** slider controls detection threshold. Larger numbers increase the number of detected fiducial positions. Typical range: 0.25—0.5.

- **Maximum # of fiducials** slider will adjust automatically as changes are made to the three parameters above. It can also be manually adjusted to deal with unusual circumstances.

### *Index controls*

- **Index** button initiates fiducial indexing, that is, correlating the identities of fiducials between projection images.

- **Twist angle** slider controls the rotation angle of the tilt axis around the depth axis of the zero-tilt projection. As long as the twist angle is < 45º, it will normally be automatically calculated during subsequent alignment steps.

- **Reference projection** button indicates which projection was the indexing reference. Pressing this button makes the Visualization window display that projection.

### *Calculation Controls*

- **Calculate** button initiates the iterative calculation of alignment offsets, 3D fiducial positions, and the twist angle.

- **Delete** button removes fiducials selected in the Fiducial list, removing previous alignment offset data.

- **Fiducial positions** / **errors** radio button controls the information displayed by the fiducial list.

- **Fiducial list** displays detailed information about fiducial positions or errors, and can be used to select fiducials for deletion or adjustment.

## 3.4. Reconstruction controls

From the main Visualization window, open the **Reconstruction** window by selecting **Process > Reconstruct**; see Figure 37. Near the top of the window are five sliders that control the position of the reconstructed volume within the 3D coordinate system established by the **Alignment** process, while controls in the lower half of window adjust various numerical aspects of the reconstruction. The specific controls are:

- **Initial z index, z thickness (pixels)** – Initial position and thickness of the reconstruction, in pixels, along the depth (*z*) axis. Default values approximately center a slab that is half as thick as it is wide and high.

- **Tilt offset** – Features within the specimen volume may sometimes be tilted around the *y*-axis with respect to the zero-tilt slice. This control adds an offset to the specified tilt angles, and can be used to make a particularly specimen feature more closely normal to the depth axis.

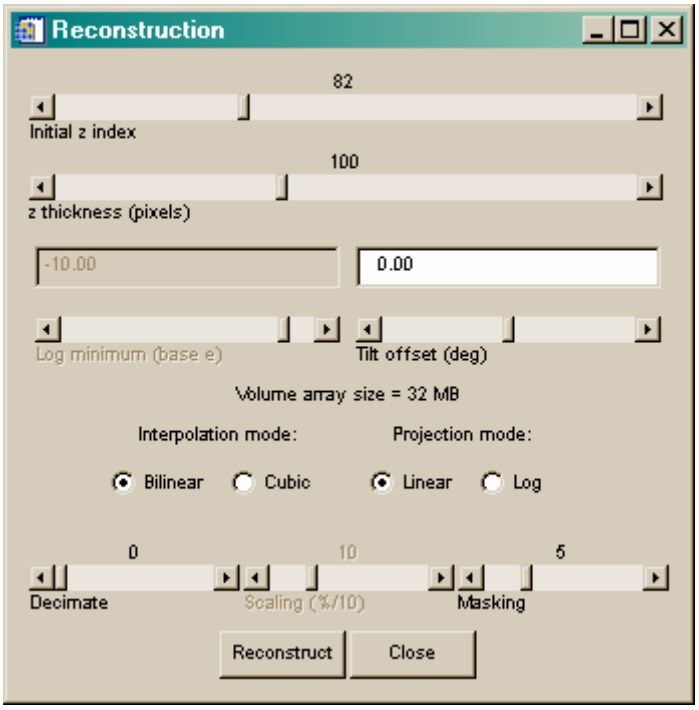



*Figure 37: Reconstruction window*

- **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 by $2^N$, so that decimation of zero (default) is 1:1, decimation of 1 is 2:1, decimation of 2 is 4:1, etc.. 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 1%.

- **Masking** – Controls the masking of linear artifacts produced near the edges of the projections associated with finite twist angle and alignment offsets. Default value is 5 pixels.

## 3.5. EM3D visualization controls – volume mode

This chapter was included to preserve the correspondence of sections numbers in the Tutorial and Reference Manual. The Volume mode of the EM3D Visualization window is covered in chapter EM3D Visualization Controls.

## 3.6. Segmentation controls

High-contrast structures with a simple topology, such as most trilaminar membranes, can be segmented automatically. Lightly stained structures, such as the rod-like filaments in the Synthetic dataset, will often require manual segmentation. All structures with complex topologies will require manual segmentation, although regional segmentation can be used to develop approximate models.

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

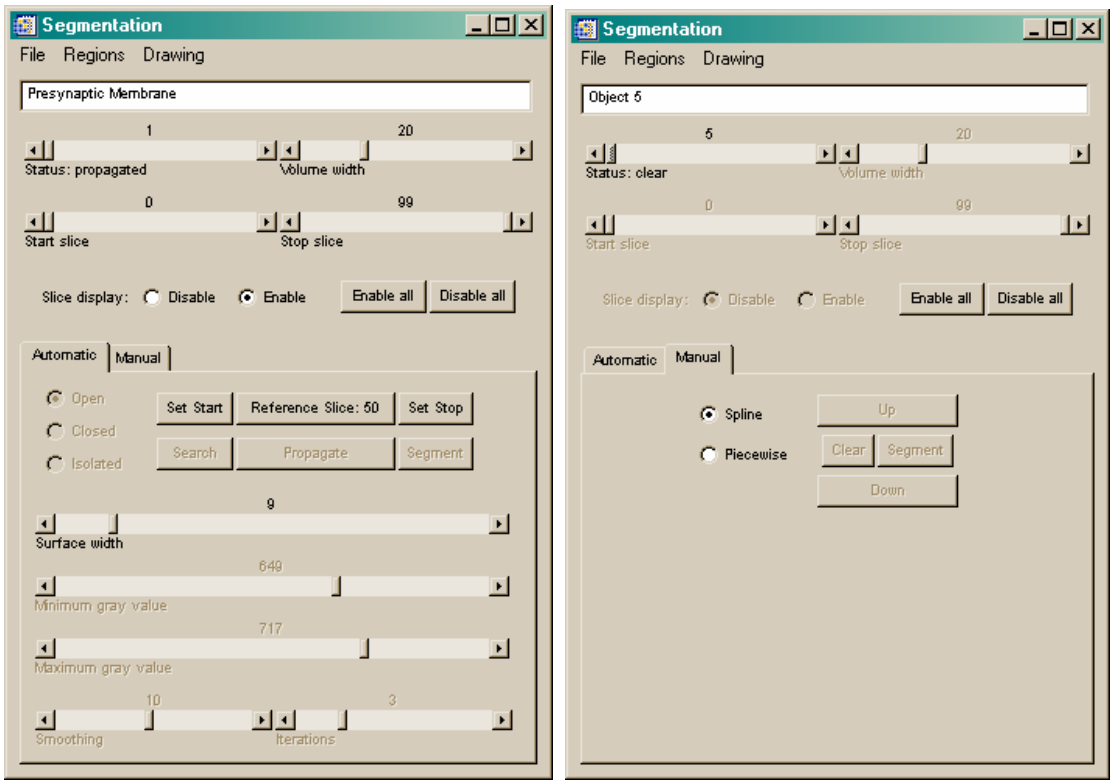



*Figure 38: Segmentation window, automatic and manual modes*

### *File menu*

- **Re-anchor** removes automatic segmentation and reverts to the original user anchor points.
- **Clear Object** removes all segmentation of the selected object.
- **Close** hides the segmentation window.

### *Regions menu*

- **Map** initiates the regional segmentation mapping process.
- **Reset** removes any regional segmentation map.

### *Drawing menu*

- **Change Anchor Mark** – The color, shape and size of the segmentation marks can be changed.

### *Selecting and identifying objects*

All segmentation objects have a name, which is displayed and edited in the text field at the top of the window. Objects are specifically identified by a unique index number, which is

selected using the slider control that is below the left edge of the text field. The text label below this slider indicates the segmentation **Status** of each object. Immediately right of this slider is the Volume width slider, which controls the size of any volume-rendering VOI that is associated with an object.

### *Visualization display*

A colored overlay in the Visualization window provides feedback about the segmentation process. Display controls are:

- Enable / Disable radio buttons turn the display on or off for the selected object.
- Enable / Disable all buttons turn the display on or off for all objects.

### *Segmentation modes*

Objects can be defined using two modes, Manual and Automatic, accessed by clicking on the "tabs" below the Status field.

**Manual mode**

In this mode, a closed path is manually defined on every slice containing a structural component. Define a path by clicking on several anchor points using the middle mouse button. The point locations are indicated by '*' symbols. After three points have been selected, a closed path is interpolated between them. Continue placing points so that the path encloses that portion of the structure visible on this slice. Use the right mouse button to delete points as needed. Use the control buttons described below to continue the segmentation onto other slices.

- **Spline/Piecewise** radio buttons control how a path is interpolated between user-selected anchor points. Spline mode uses cubic-spline interpolation; piecewise uses linear interpolation.
- **Segment** adds all the points interior to the path to the segmentation object VOI.
- **Up** copies the current set of anchor points into the next higher slice after first segmenting the current slice.
- **Down** copies the current set of anchor points into the next lower slice after first segmenting the current slice.
- **Clear** removes the current anchor points and deletes any contents of this slice from the segmentation object VOI.

**Automatic mode**

In this mode, an initial anchor path must be defined on a single reference slice. Define the path with a sequence of middle-mouse button clicks to create anchor points. Automatic software will use this path and other parameters to segment the reference slice and propagate this segmentation throughout the volume.

- Topology radio buttons

- **Open** objects extend out of the image field.
- **Closed** objects close within the image field.
- **Isolated** objects are segments or blobs within the image field.

- Search controls
  - **Search** button initiates a pixel search around the path. The minimum and maximum gray values for the search will be set based on gray values along the anchor path.
  - **Surface width** slider sets the diameter of the search region around the anchor path (in a fashion similar to setting line-width in a drawing program).
  - **Minimum** / **Maximum gray value** sliders allow manual control of gray values selected by the pixel search. It is scaled from 0 to 1000 in which 0 represents black and 1000 represents white.

- Segmentation controls
  - **Segment** button finds the median axis of the pixels selected by the search around the anchor path. This axis is converted to a new segmentation path.
  - **Smoothing** / **Iterations** sliders set the parametric smoothing applied during the segmentation process. The smoothing value should be set so that the path generated by pressing the Segment buttons follows the center of the structural component. Default value for iterations is 4.
  - **Reference slice** button indicates which slice was used to define the initial anchor path. Pressing the button returns the Visualization display to that slice.

- Propagation controls
  - **Propagate** button initiates automatic propagation of the segmentation through the volume.
  - **Start** / **Stop slice** sliders set the range for propagation.
  - **Set Start** / **Stop** buttons offer an alternative means to set the propagation range when used together with slice navigation controls in the Visualization window.

## 3.7. Model generation and 3D visualization controls

All features for model generation and visualization are accessed from the **EM3D Rendering** window, which is opened by selecting **Process > Render** in the **EM3D Visualization** window. In general, it is also necessary to open the **Rendering Control** window by selecting *File > Control* in the **Rendering** window.

### 3.7.1. EM3D rendering window

The **EM3D Rendering** window is a visual interface to the rendered models, and contains controls to modify the isodensity level of individual surface models. It also provides geometric or analytic information about each model.

#### *Model control and information*

- **Objects** / **Regions** radio buttons determine which type of isodensity surface model to operate upon.
- **Model** slider determines which object or region is currently selected. The label field over the slider gives its name.
- **Enable** / **Disable** toggle button above the **Model** slider can be used to quickly create or remove surface models.
- **Isosurface level** slider provides precise manual control over the gray value that defines each surface model. The slider can be adjusted directly for immediate control. Alternatively, a specific number can be typed into the text field above the slider; hit <Enter> to create the surface model at the new level.
- The small **isodensity plot window** below the slider provides a graphical interface for isosurface gray level control. The plotted curve shows the cumulative distribution function for the entire reconstructed volume. Models will generally be constructed at some point along the slope of this curve, with smaller (darker) values producing sparser models, and larger (lighter) values producing more extensive models. The vertical blue line indicates the current isodensity level for the selected model. Isosurface gray level can be adjusted by clicking with the left mouse button within this plot window.
- The information display fields, **Pos** (center position of the object), **Volume**, and **Area**, provide information about the currently selected model.
- The **Overlay** fields indicate the name, color bar, and scaling of any 3D colormap overlay that is presently applied to the selected model.

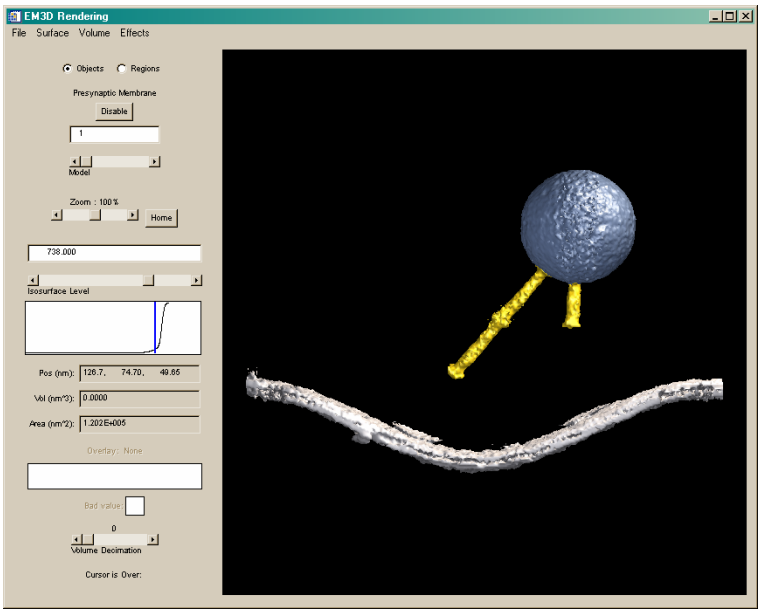



*Figure 39: EM3D rendering window*

## *3D Rendering Display*

The large **3D graphics window** (see Figure 39) permits interactive visual display of the models. When the mouse is positioned over this window, the **Cursor is Over:** label field indicates the label of the model closest to the mouse cursor.

- ***Mouse buttons*** access different visualization control features. Specifically, click and hold the mouse buttons to access the following features:
    - Left mouse button-down initiates a virtual 3D trackball. Moving the mouse in this mode will rotate all the models about the center of the current rendering volume.
    - Middle mouse button initiates scaling of the models. An outward movement makes the rendered models appear larger; inward movements make them smaller.
    - Right mouse button initiates translation of the models.
    - Double-click the left mouse button to select that object for control or information display.
- **Zoom** slider provides more precise control over the scaling of the rendered models. The number shown represents the relative change from the last zoom.
- **Home** button restores the rendering to a standard viewpoint and scale.
- **Volume Decimation** slider controls the quality of renderings with respect to the reconstructed volume. Specifically, a setting of zero (0) corresponds to full resolution, a 1:1 mapping between the models and the volume.

Larger numbers correspond to $\log_2$ reductions, i.e., a setting of 1 corresponds to a 2:1 reduction, a setting of 2 corresponds to a 4:1 reduction, and so forth.

### *File menu*

- Control… opens the **Rendering control** window.
- **Save VRML** brings up file dialog so that the current 3D rendering can be output in a file format readable by various VRML viewers, or imported into other 3D rendering applications such as AVS.
- **Close** hides the EM3D Rendering window.

### *Surface menu*

- **Shading** offers two options that have an effect in **Solid** rendering style.
  - **Flat** uniform color shade across each surface polygon.
  - **Gouraud** interpolated shading across each polygon (default).
- **Style**
  - **Points** renders every vertex of the model as a colored point in space.
  - **Wire** renders the model as a set of colored lines connecting the vertices.
  - **Solid** renders each polygon as a colored, shaded surface (default).
  - **Hidden points** renders only those vertices that are visible from the current viewpoint.
  - **Hidden wire** renders only those lines that are visible from the current viewpoint.

### *Volume menu*

- **Show / Hide** toggles the display of volume-rendering model.
- **Scaling…** opens the **Volume scaling** window.
- **Interpolation** toggles the interpolated display of the model. Interpolation improves the quality of the display, but takes longer to refresh.
- **Coarse / Fine** toggles the spatial resolution of the volume rendering.
- **Z-buffering** toggles use of the z-buffer. The use of the buffer controls the ordering of surface and volume rendering, allowing different visibility effects.

- **Compositing** controls how the volume render is created; the various modes create different appearances. Default mode, **alpha**, is the most useful and popular. **MIP** (maximum-intensity projection) can also be useful. The other two modes, **alpha-sum** and **average**, produce other appearance effects that can be useful under unusual circumstances.

### *Effects menu*

- **Drag quality** controls the display detail while interacting with the 3D Rendering display. Lower quality settings, **Medium** and **Low**, offer less detail but a faster refresh.

- **Perspective** toggles the rendering of perspective in the current view.

- **Spin** controls animation of the 3D display.

    - **x- / y- / z-axis** initiates a rotational animation around the corresponding axis.

    - **Axis…** opens a dialog to allow specification of an arbitrary axis, and then initiates rotational animation around that axis.

    - **Isodensity** initiates an animation of isosurface level for the currently selected object or region.

    - **Increment…** allows specification of the rotation increment in degrees or the isosurface level increment (in grayscale units) used for the rotational/isosurface level animations.

    - **Rock** toggles the animation mode between looping (default: 1 → N, 1 → N, …) and rocking (1 → N → 1 …).

    - When an animation is terminated, a query window opens asking if the user wants to write the frames of the animation sequence. A 'yes' response initiates the writing of the frames as a set of TIFF image files.

- **Lighting…** opens the **Lighting** control window, which controls the color, illumination pattern, and placement of up to seven light sources for the 3D display.

- **Clip Planes…** opens the **Clipping planes** control window, which controls the placement of clipping planes in the 3D display.

- **Stereo…** opens the **Stereo** control window, which controls the display of stereo information using red-green anaglyphics. The user must wear stereo red-green glasses.

- **Cut…** opens the Cut control window, which displays one or more principal-axis cut planes overlaid on the 3D-model rendering.

### 3.7.2. Rendering control window

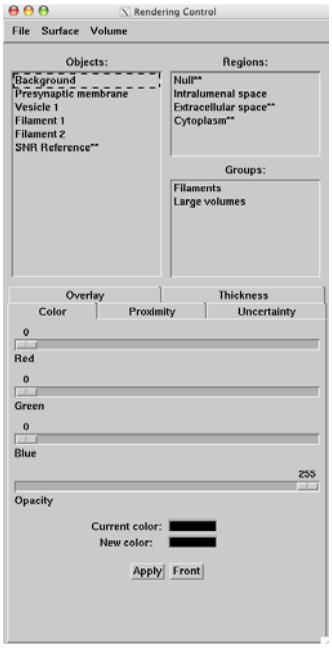



*Figure 40: Rendering control window*

This window provides lists of segmented VOIs, both objects and regions. Various functions can be applied to one, or more, of the VOIs selected in these lists. Subsets of these features are as follows:

#### *Segmentation VOI lists*

- **Objects / Regions** lists show all available segmentation VOIs to create and be operated upon as models. Any combination can be selected using the mouse, to allow application of various visualization commands, model-generation commands, and calculations. Double-clicking any list element opens a dialog window to name or rename it.

- **Groups** list provides access to user-generated collections of objects or regions. Clicking on a group selects all its members in the **Object / Regions** lists. Double-clicking a group element opens a dialog window to name or rename that group.

#### *Tabbed control panels*

- **Color** controls the color and opacity of all selected surface models. The current RGB color and opacity values are displayed on the sliders and the **Current color** patch near the bottom of the panel shows the mean color for all objects selected. As the sliders are adjusted, the **New color** patch shows the changes. Use the **Apply** button to modify the color and opacity of the selected models. The **Front** button controls rendering order; when making surfaces transparent (opacity < 255), use the **Front** button so that the models behind them become visible.

- **Uncertainty, Proximity, and Thickness** panels control various calculations that can be applied to the selected surface models (see chapter 3.8).

- **Overlay** controls the display of calculated results as vertex colormap overlays (see chapter 3.8.5, below).

### *File menu*

- **Rename** provides another way to change the name of segmentation VOIs.

- **Select all** permits convenient selection of all objects or regions.

- **Group** provides a mechanism to label and collect logically related models. New groups appear in the Groups list with a default name (e.g., Group 1), where they can be renamed by double-clicking. To delete a group, double-click it and enter an empty name.

- **Combine** creates a new segmentation VOI by performing logical operations upon the selected VOIs. **Union** corresponds to the logical OR of the VOIs by creating a new volume that contains all of the points in the selected VOIs. **Intersect** corresponds to the logical AND creates a new volume that contains points where all of the selected VOIs coexist). The **Volume width** slider controls the dilation of these VOIs during these operations.

- **Delete** removes the selected segmentation VOIs, clearing them from the Segmentation window. Any associated models are also deleted.

- **Close** hides the window.

### *Surface menu*

- **Show / Hide** controls the visibility of surface models in the Rendering Window.

- **Enable** creates surface models by the default polygonal surface generation algorithm.

- **High quality** creates surface models by true tessellation rather than with variable-sided polygons. Tessellated models are more accurate, but take much longer to generate.

- **Disable** destroys surface models.

- **Optimize** initiate an iterative search for the isosurface gray value that produces the most reliable surface model. Before optimization, it is generally best to manually adjust the isosurface value to a relatively small value so that the initial surface is relatively sparse.

### *Volume menu*

- **Enable** creates a volume model containing the currently selected object and region VOIs.

- **Disable** destroys the volume model.

### 3.7.3. Lighting control window

From the EM3D Rendering window open the Lighting Control Window by selecting **Effects > Lighting…**

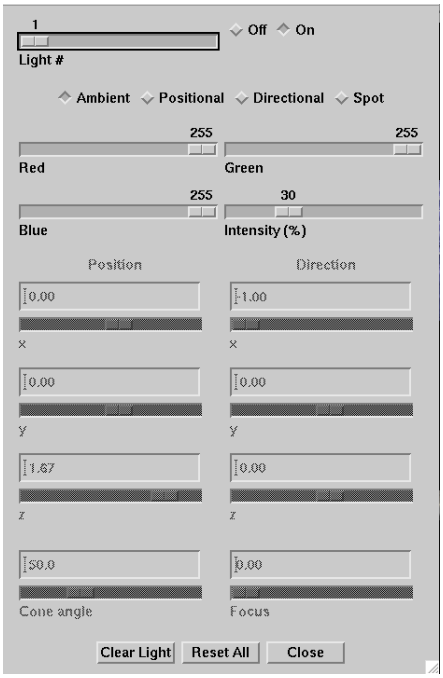



*Figure 41: Lighting control panel*

- **Light #** selects one of the seven light sources.
- Use the **On/Off** radio buttons to switch the selected light source on/off. By default the first 3 light sources are on.
- Four types of light sources are available. By default the first light source is ambient, the second and third are positional light sources.
    - **Ambient**: The light source emits its light everywhere and in all directions.
    - **Positional**: The light source is a point light source at a specific (x,*y*,*z)* location and emits light in all directions from that point. Use the **x, y** and **z** sliders under **Position** to specify the location of the light.
    - **Directional**: The light is located at infinity and shines in a particular direction with parallel rays like the sun, for example. Specify the **direction** with the corresponding **x, y and z sliders**.
    - **Spot**: The spot light, like the positional light, has a specified location but emits a cone of light in one direction only, like a flashlight. Specify the direction and position of the light using the

corresponding sliders. Use the **Cone angle** slider to change the cone angle of the light source, same procedure with **Focus**.

- The **Red, Green** and **Blue** sliders change the color of the light source.
- **Intensity** controls the luminosity of the light.
- The **Clear Light Button** resets the selected light source ambient with 30% intensity.
- The **Reset All Button** resets all light sources to its default values.

### 3.7.4. Clipping planes window

The Clipping Planes window controls a maximum of 4 clipping planes. Clipping planes cut away the portion of the model on one side of the plane.

From the EM3D Rendering Window open the Clipping Planes window by selecting **Effects > Clip Planes…**

- Use the Plane drop down list to selects a plane. The Enable checkbox initiates clipping for the currently selected plane in the Rendering window.
- X, Y, Z sliders define the position of the clipping plane in the volume. The Theta slider defines the rotation angle about the y axis, and the Phi slider defines the rotation angle about the z axis.

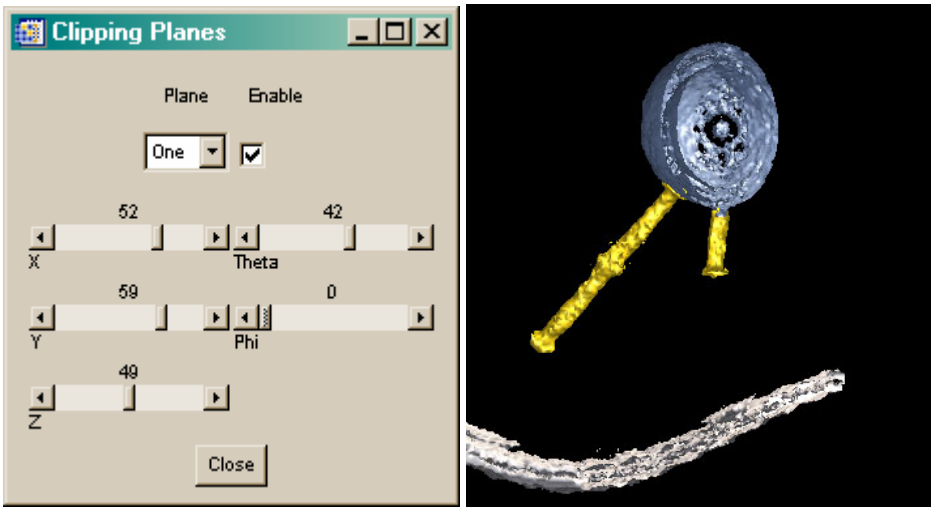



*Figure 42: Clipping planes window and clip through vesicle and presynaptic membrane*

### 3.7.5. Cut control window

The Cut Control Window displays one or more principal-axis cut planes overlaid on the 3D-model rendering (see Figure 43).

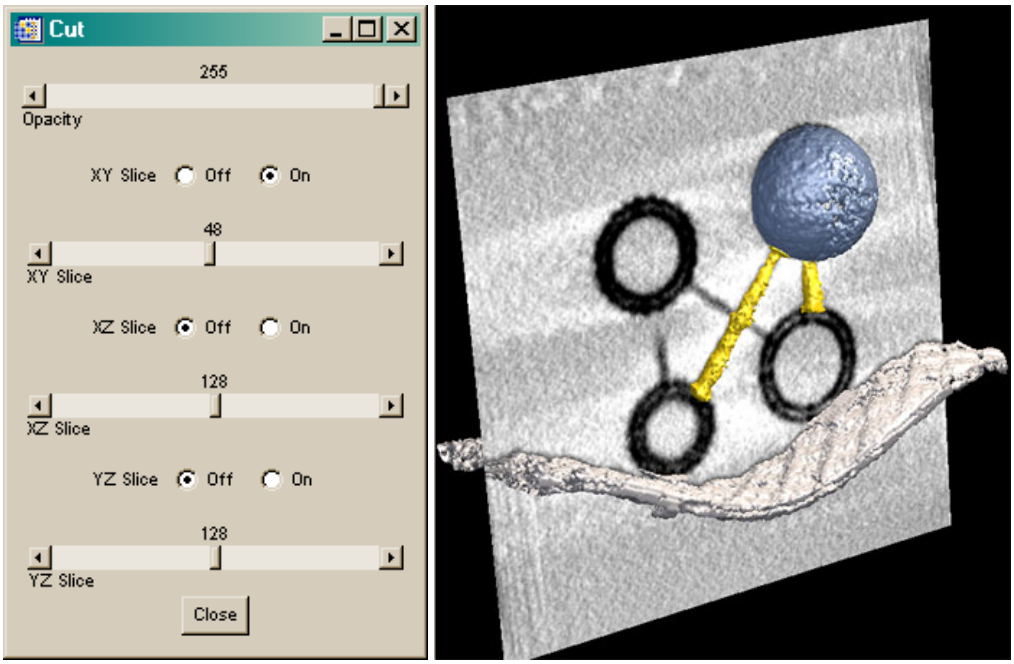



*Figure 43: Cut control window and visualization in rendering window*

- **Opacity** controls the opacity of the objects within the 3D rendering.
- The **XY**, **XZ**, and **ZY Slice** radio buttons turn on or off the corresponding principal-axis cut-plane display.
- The **XZ** and **ZY Slice** sliders specify the volume index of the slice to be displayed.

## 3.8. Calculations

### 3.8.1. Calculation controls

Controls are accessed from the tabbed controls panels located at the bottom of the **Rendering Control** window. Three of these tabs, **Uncertainty**, **Thickness**, and **Proximity**, control those three calculations. Surface models must be created before initiating any of these calculations. The **Overlay** tab controls the display of the various calculated results as a colormap overlay on the rendered surface models.

> *It should be noted that although decimation dramatically increases the speed of calculations, it also reduces the reliability of the results.*

### 3.8.2. Uncertainty

This tab controls the spatial uncertainty calculation, a measure of the reliability of an isosurface model.

- **Reference object** field displays the current signal-to-noise ratio manual reference object, and the current SNR estimate.

- **Reference signal +/– noise** field reports the current maximum signal and noise values, in arbitrary grayscale units, based on the currently selected Reference object and current minimum clip values for the Visualization window.

- **SNR Reference** button sets the currently selected manual object as the SNR reference.

- **Calculate** button initiates the spatial uncertainty calculation.

- The plot window below the buttons displays a histogram that combines all of the spatial uncertainty results from the currently selected models.

- The Min, Max, Med, and Bad fields display, respectively, the minimum value, maximum value, median value, and percentage of bad values for the combined calculated data ensemble.

### 3.8.3. Thickness

This tab controls the membrane thickness calculation.

- **Minimum** and **Maximum thickness** slider set the range of the thickness.

- **Calculate** button initiates the thickness calculation.

- The plot window below the buttons displays a histogram that combines all of the thickness results from the currently selected models.

- The Min, Max, Med, and Bad fields display, respectively, the minimum value, maximum value, median value, and percentage of bad values for the combined calculated data ensemble.

### 3.8.4. Proximity

This tab controls proximity calculations among the currently enabled surface models. These calculations require that one surface 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 neighbor vertex on the reference surface.

- **Reference** field displays the current reference surface model. Nearest-neighbor distances are calculated with respect to the vertices of this surface.

- **Destination** list displays all object or region models selected for the calculation. Distance values will be calculated for every vertex on these surfaces.

- **Reference** button sets the reference surface model. Only a single object can be set as reference.

- **Destination** button sets the currently selected object or region surface models as destinations.

- **Calculate** button initiates the proximity calculation.

- The plot window below the buttons displays a histogram that combines all of the proximity results from the models currently selected in the **Rendering Control** window.

- The Min, Max, Med, and Bad fields display, respectively, the minimum value, maximum value, median value, and percentage of bad values for the combined calculated data ensemble.

### 3.8.5. Overlay

This tab controls the display of calculated data as a colormap overlaid upon their associated surface models.

- **Overlay** pull-down menu selects which calculated quantity, uncertainty, proximity, or thickness will be overlaid.

- **Selected objects/regions** list indicates which of the currently selected models have the currently selected calculated quantity available for overlay.

- **Minimum** / **Maximum** fields set the numerical range for the colormap. The actual minimum and maximum values for selected models are displayed to the right of the editable text fields.

- **Colormap** radio buttons controls the mapping between the numerical values and vertex colors. **Constant** imparts a single, user-selectable color the specified minimum—maximum range. **Table** associates a colormap with the current minimum—maximum range.

- The **Colormap** graphics patch displays the current map. Click on the map to change the color or colormap.

- The **Undefined value** graphics patch displays the color used to mark vertices with “bad” values; such values are produced when the calculation fails to produce a meaningful result. Click on this patch to enter RGB color values. The default setting is black, RGB values all zero. With this setting, bad values are simply ignored and “bad-flagged” model vertices appear at the previously set model color.

- **Apply** button adds the overlay to the selected models.

- **Remove** button removes the overlay from the currently selected models.

# 4. Appendix

## 4.1. 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 gray scale of an image to create a negative image.

**Isosurface/isodensity:** A region or surface in which the image gray scale 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.

## 4.2. 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 $1x10^{-9}$m
- RMS: root mean square
- SNR: signal to noise ratio
- TEM: Transmission Electron Microscope
- TIFF: Tagged Image File Format
- VOI: Volume of Interest

## 4.3. Table of Figures