MANUAL OF SEGMENTATION TOOLS FOR SYNAPTOSOMES

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1   INTRODUCTION

This is a manual for the whole set of tools designed for automatizing the segmentation of the structures of interest of synaptosomes in cryo-ET tomograms. The set of tools have been programmed in MATLAB® and can be run from Matlab's command window. The package contains two graphic tools **membseg2** for supervising the segmentation of the cleft and pre-synaptic membrane and **vesseg** for supervising the segmentation of the vesicles in the pre-synaptic region, they start from the results generated by package TomoSegMemTV.

The tools has been designed for segmenting well-posed synaptosomes, these tomograms have defined the pre- and post-synaptic membranes with a reasonable good contrast and a big size (surface). But even in the cases where the SNR is low or the post-synaptic density is hidden or broken, the tools may generate a satisfactory result.

This package is still under revision so any feedback is welcomed (an.martinez.s.sw@gmail.com).
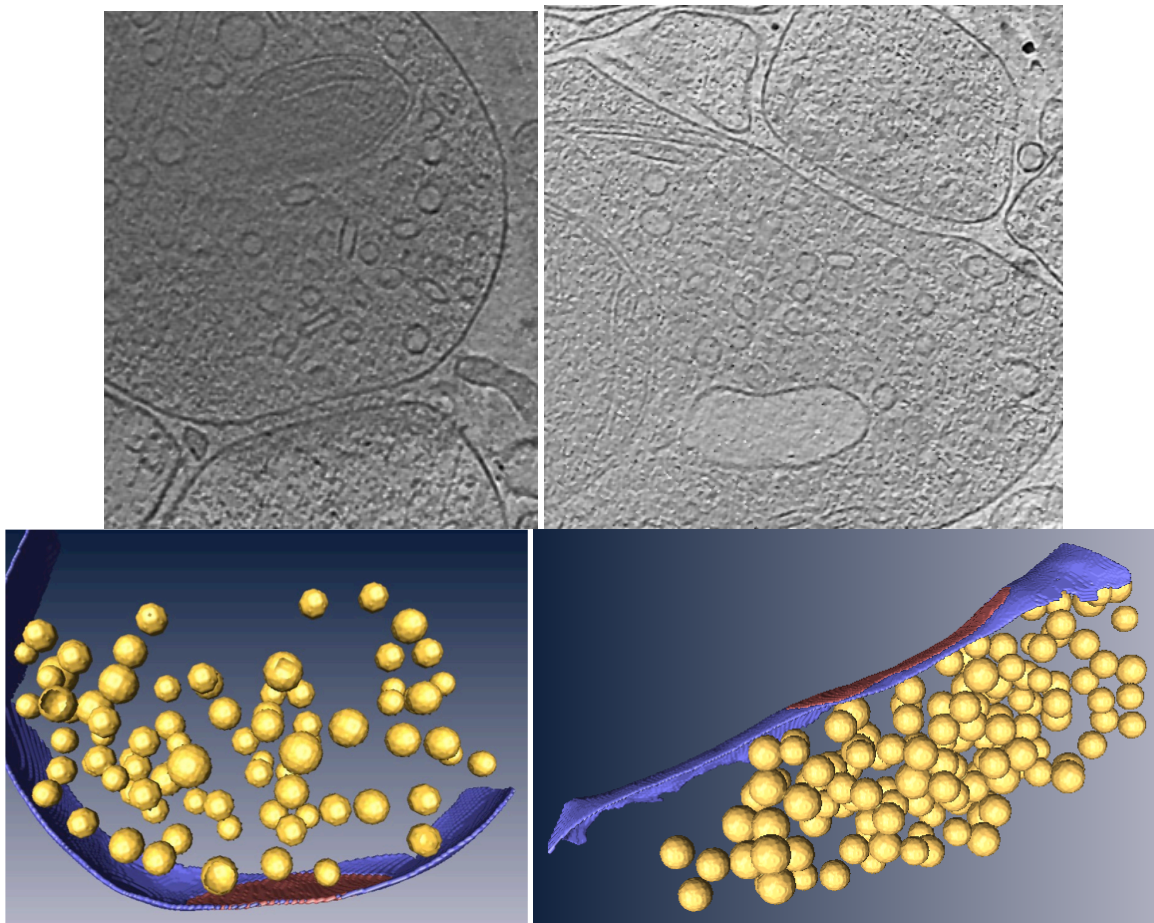

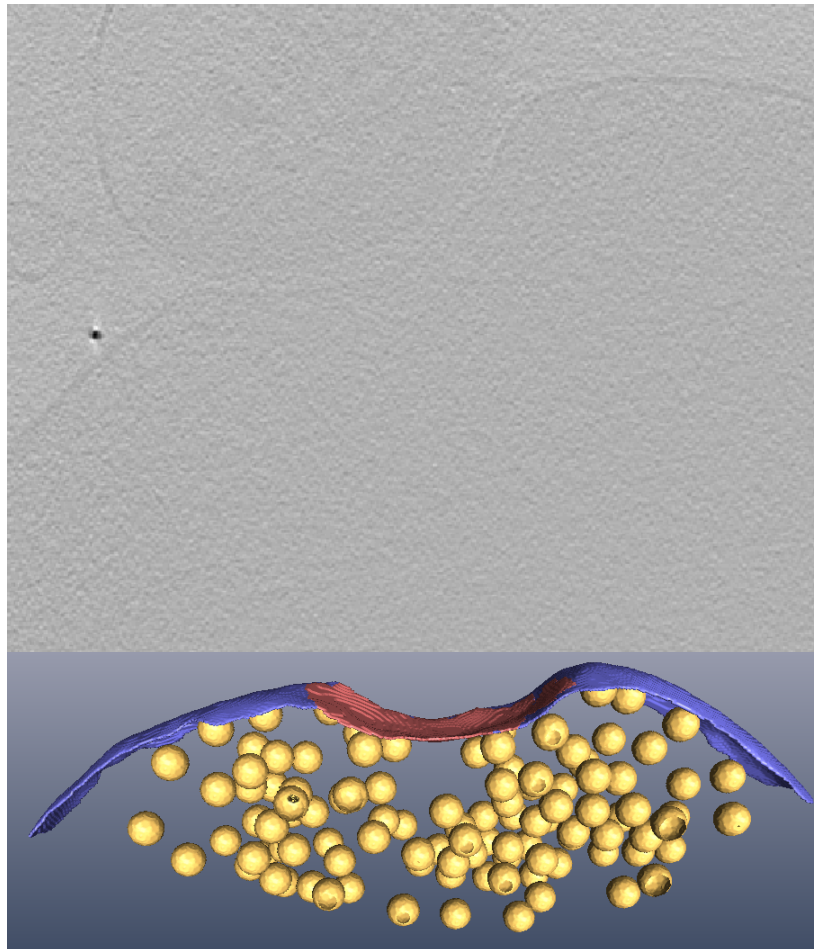Figure 1. Segmentation off well-posed synaptosomes

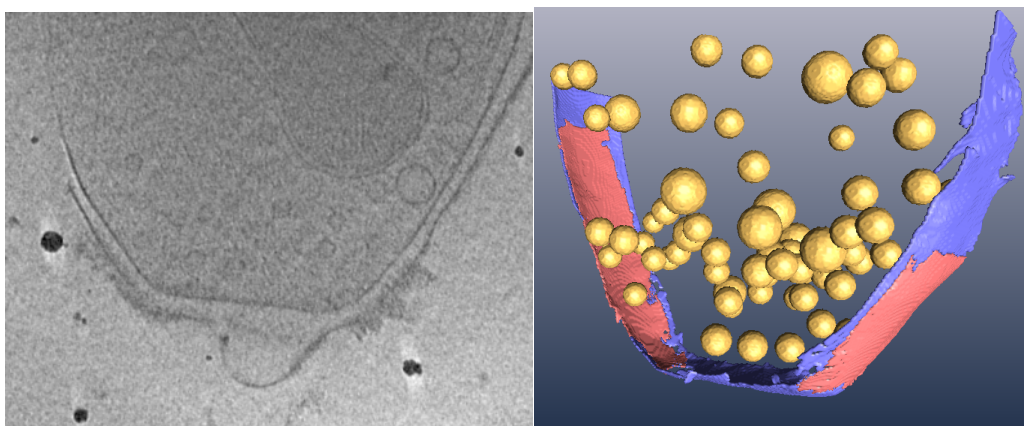**Figure 2. Segmentation of a tomogram with low SNR.**

Figure 3. Segmentation of a synaptosome with the post-synaptic membrane broken.

2   INSTALLATION

This package requires the previous installation of TOM toolbox, which allows loading and saving tomograms in standard formats such as MRC or EM.

Make the directories *source/memseg2* and *source/vesseg,* which contain the tools of the package, accessible from Matlab. Now the functions of the package are ready to be run in the Matlab environment from the Command Window.

This package has been tested for MacOS 64 bits and Linux 64 bits platforms, but it should work in any platform with Matlab and TOM Toolbox installed.

3   MEMBSEG2

This is a Matlab's graphical tool for labelling the membranes previously filtered with **tomosegmemtv** package. Call the command **membseg2** in Matlab command window from 'membseg2' directory for running this tool, the rest of functions are dependences of this tool.

The intention of this tool is to segment the pre-synaptic membrane and the part of this membrane in the active zone (or synaptic cleft).

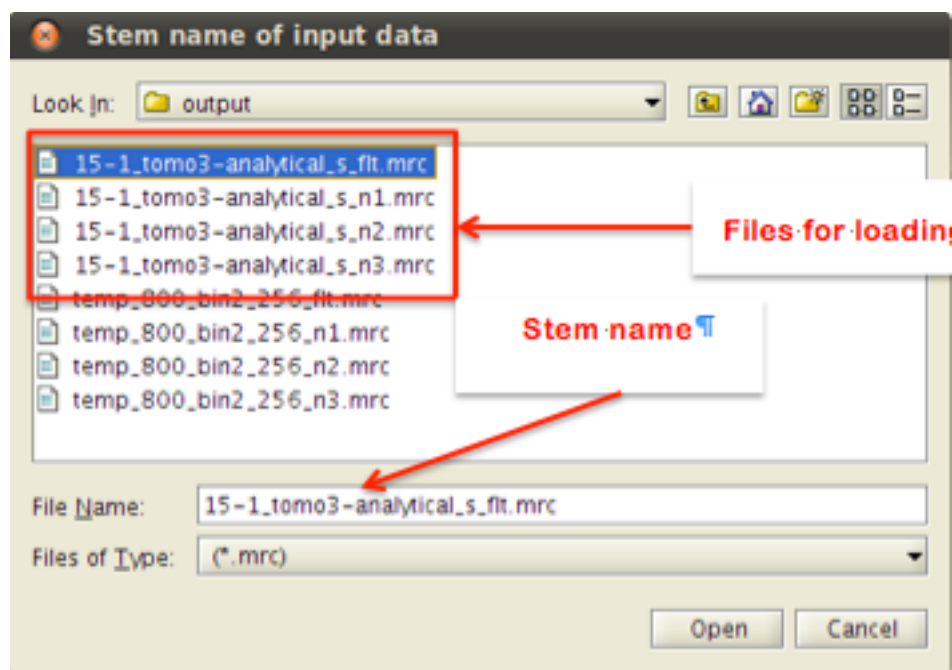When **membseg2** starting asks for the input data:

Figure 4. Window for loading data.

The user must choose the name of the original dataset, without being processed by **tomosegmemtv**, then the software automatically loads the output files generated by **tomosegmemtv**, they must have the string '_ftl', '_n1', '_n2' and '_n3' at the end of their names, see the figure above, and must be in the same directory as the original.

Once the input data are loaded the main window is showed. At the beginning, this view shows an slice of the original data set and has a slider bar for navigation along the tomogram slices in Z axis, it has also a radio button panel where the user can change view between the original data an the intermediate results generated by the tool.
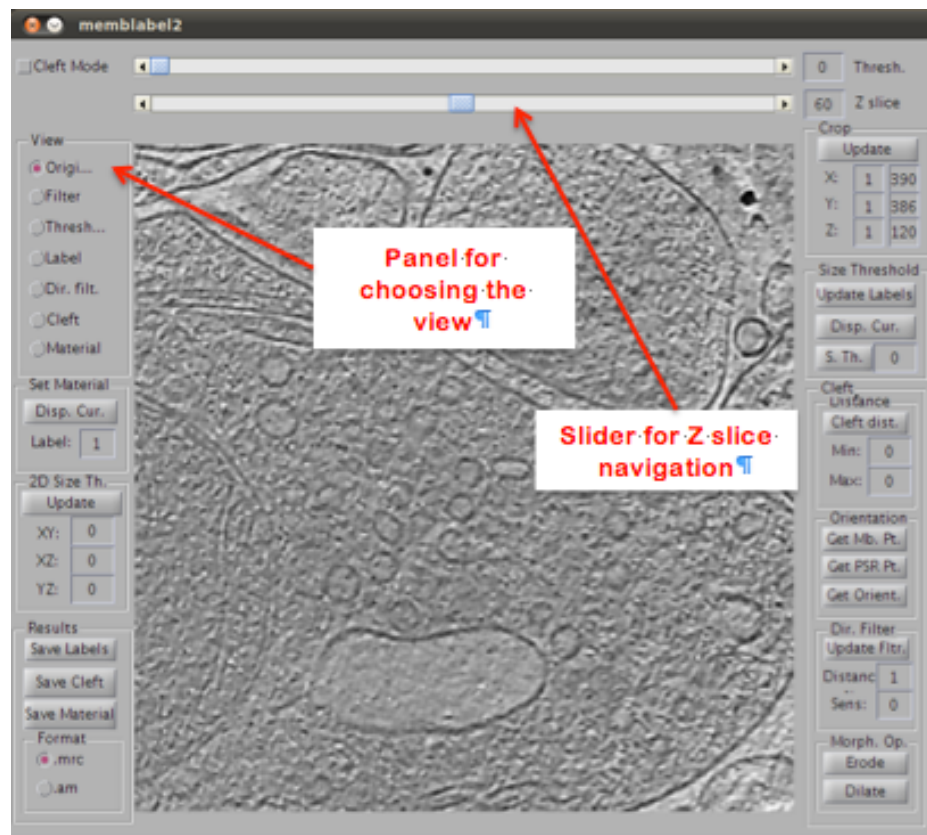

Figure 5. Main window of memblabel2 tool.

3.1   Density thresholding

Thresholding is the starting point of every labelling procedure, the "Thresh." slider carries it out. When the user changes the "Thresh." slider by default it takes the output with extension '_flt' generated by **tomosegmemtv** and applies the threshold value showed by the text box besides the slider.
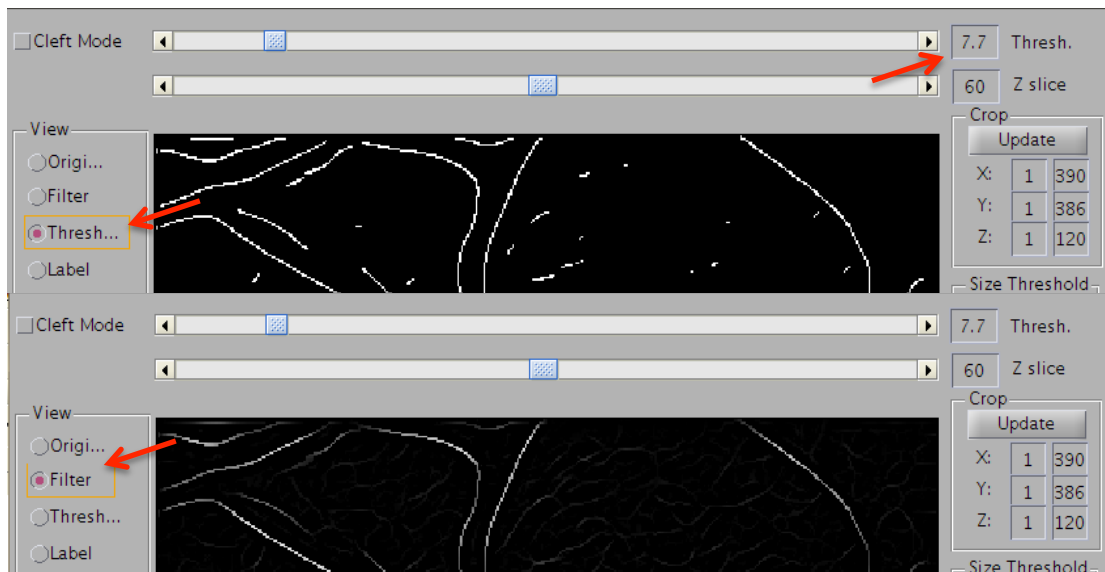
Figure 6. "Thresh" slider for binarizing the output generated by tomosegmemtv (below).

The input of this process is the "Filter" view and the output is the "Threshold" view.

3.1.1 Cleft Mode

When the check box "Cleft Mode" is selected, the input for thresholding is the "Dir. filt." (see section 3.3.2) instead of "Filter" view. But the output is stored in "Threshold" view.
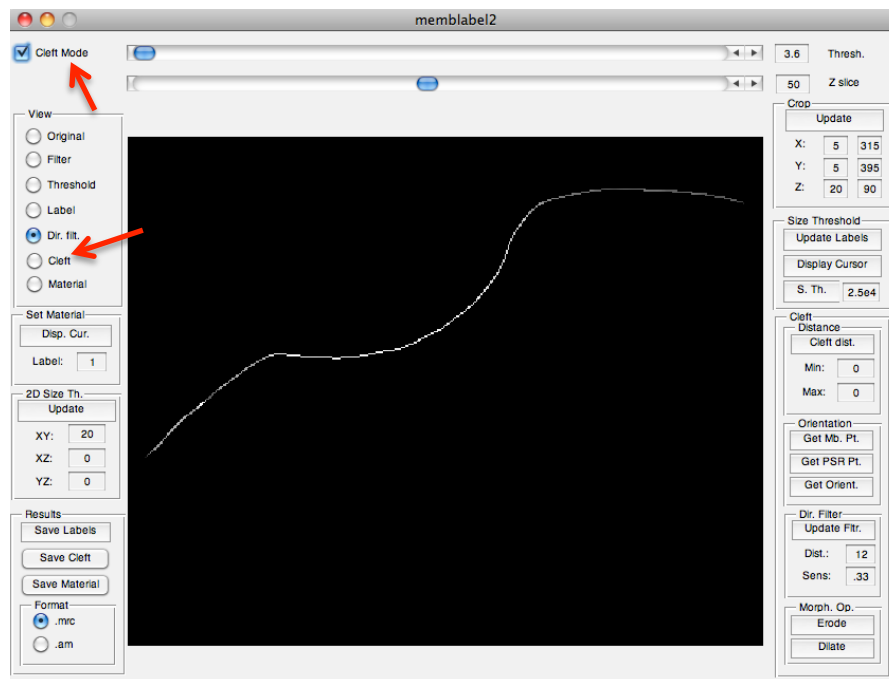

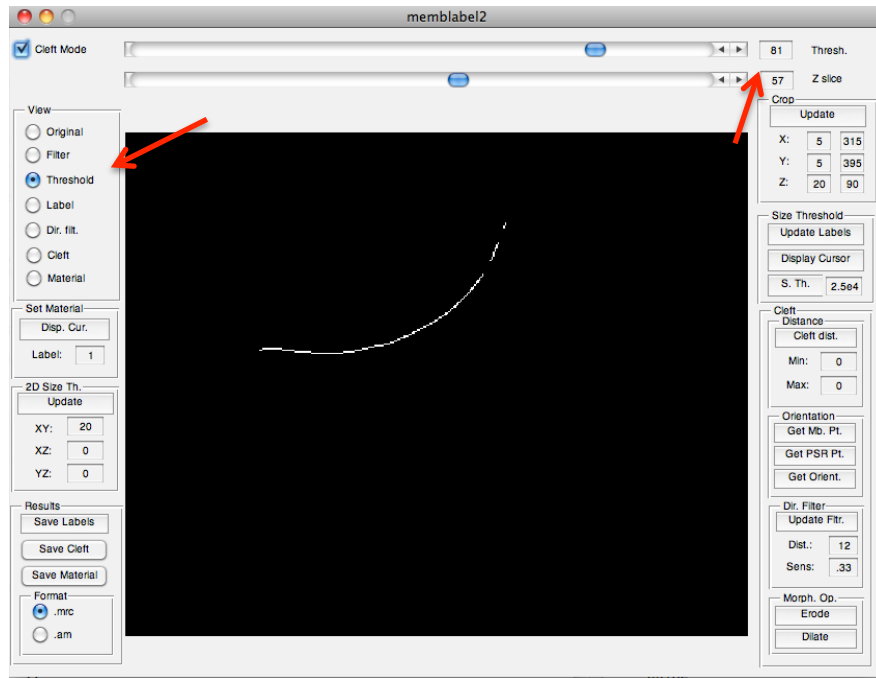**Figure 7. Example of "Dir. filt" view ready for being thresholded.**

Figure 8. Thresholding output from "Dir. filt" view.

3.2 Labelling

The output given by the threshold procedure needs be labelled in order to separate the interesting membranes.

3.2.1 Size threshold

The panel "Size Threshold" allows to perform an automatic labelling procedure, it is based on the size of every connected component in "Threshold" view.

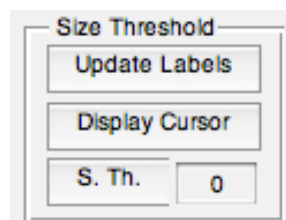**Figure 9. "Size Threshold" panel.**

The button "Update Labels" takes as input the "Threshold" view and measures the number of voxel of every connected component, finally it assigns them their size as label. The result is showed in "Label" view.
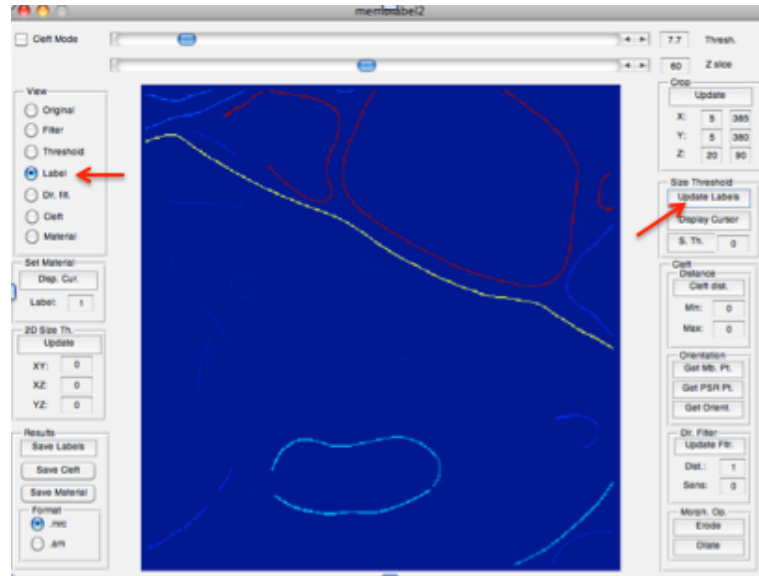
Figure 10. Labelling of previously thresholded structures by their size.

The segmentation of synaptosomes is only interested on pre-synaptic and post-synaptic membranes, even sometimes the post-synaptic is not necessary (see section 3.3.2). In the most of the cases these structures are the biggest ones so thresholding by size the rest of structures can be rejected.

The first step is to know what is the size of the structures of interest, the user can use the "Display Curson" button for doing that, when the user presses it a cursor is displayed over the view and it shows ("Index" number) the size of the structure pointed. When the cursor is displayed the button change its text to "Stop Cursor". Once the user knows the sizes he wants, the cursor should be released by pressing the "Stop Cursor" button. Now the user should know the size threshold that has to write in the box of the "Size Threshold" panel, this threshold is applied when the button "S. Th." is pressed.

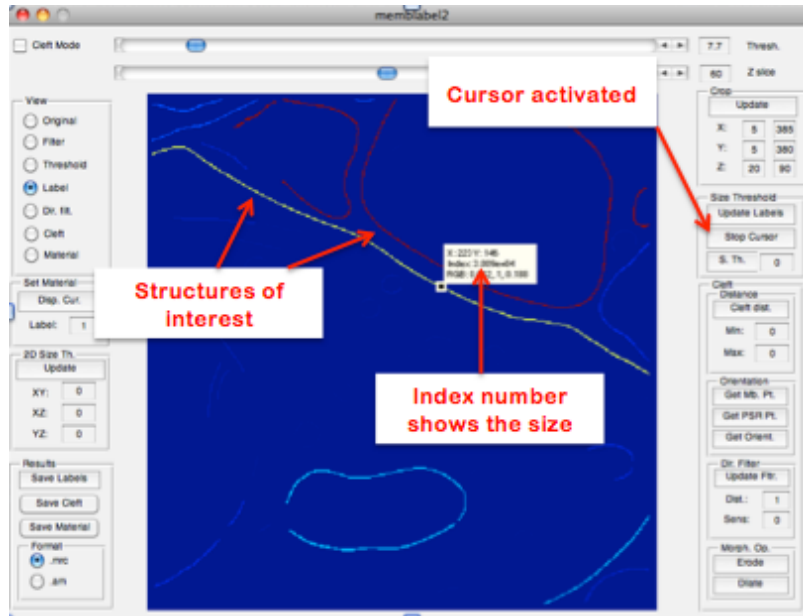**Figure 11. Color map used for code the size labels.**

Figure 12. Knowing the structure of interest size with "Disp. Cur." tool.
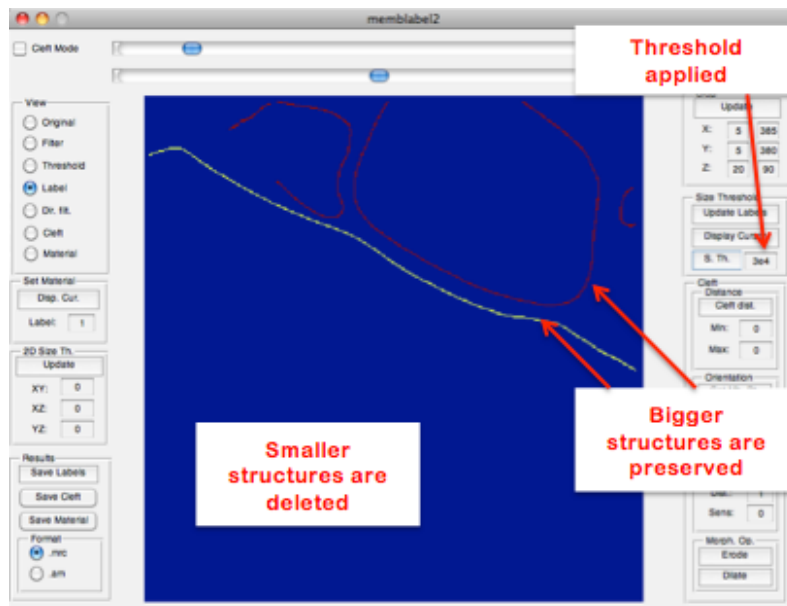

Figure 13. After size thresholding.

3.2.2 Manual Labelling

This tool has been designed to generate the input data for the **vesseg** tool, nevertheless the user may want to label in a different way the structures detected by **tomosegmemtv**. With the panel "Set Material" the user can display a cursor ("Disp. Cur." button), the cursor is placed over the structure of interest and then press again the button (now its text is "Change Lbl."), then this structure is stored in "Material" view with the label in "Label:" box. This tool only works over "Label" and "Cleft" (see section 3.1.1) views.
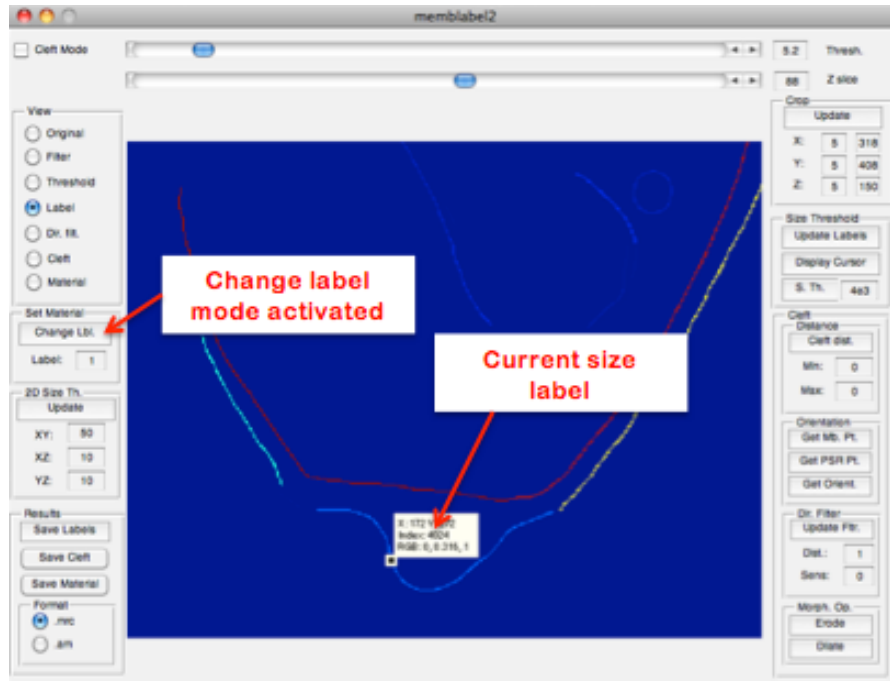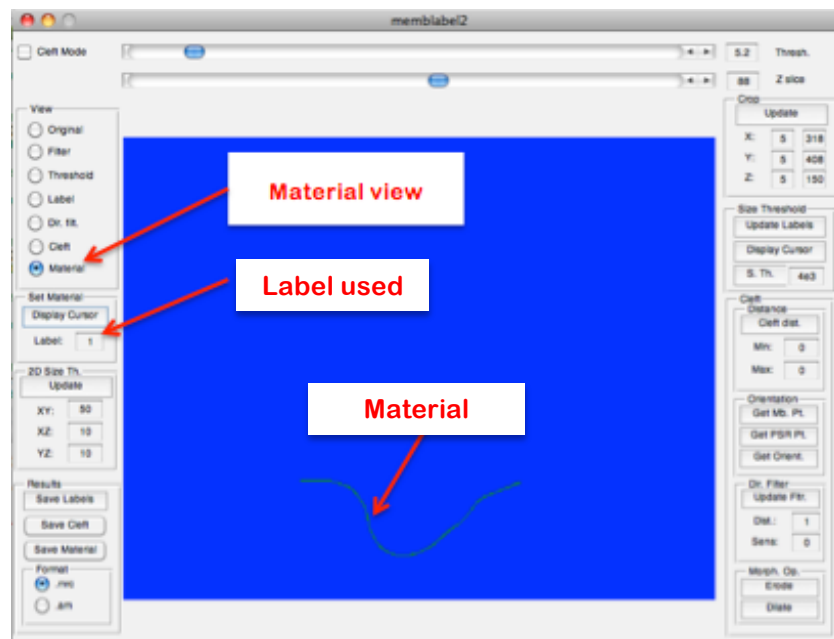
Figure 14. Selection a material.

Figure 15. View of the material labelled.

3.3   Cleft

The way of segmenting may differ depending on the result obtained in "Label". It is recommended to use Distance Based Segmentation (see section 3.3.1) as first option and only try Directional Filters (see section 3.3.2) when the first one is not possible.

3.3.1 Distance based segmentation

If both pre-synaptic and post-synaptic membranes have been correctly segmented in previous step ("Label" view). The "Distance" subpanel (enclosed in "Cleft" panel) allows separating structures that potentially are a synaptic cleft, in a synaptic cleft the normal distance between two membranes is approximately fixed and known. Therefore, setting the proper value (in voxels) for the range of distances in "Distance" subpanel and pressing "Cleft dist.", the tool turns to "Label" view and keeps all membranes whose closest normal distance to another (different label) is in range. By the way, the tool labels by their size all the membranes preserved in "Cleft" view.
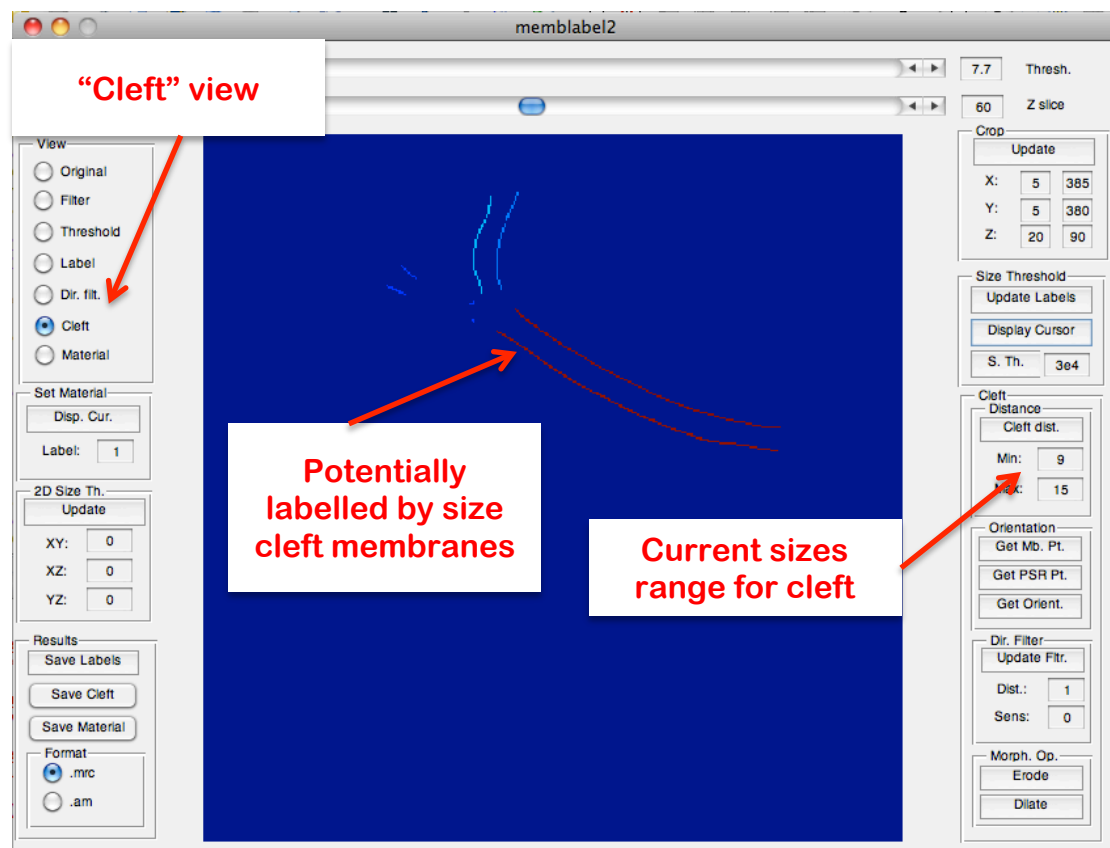

Figure 16. Output of "Cleft dist." processing from "Label" view in Figure 13.

At this point the tools of panel "Size Threshold" (see section 3.2.1) can be used for isolating only the desired cleft.
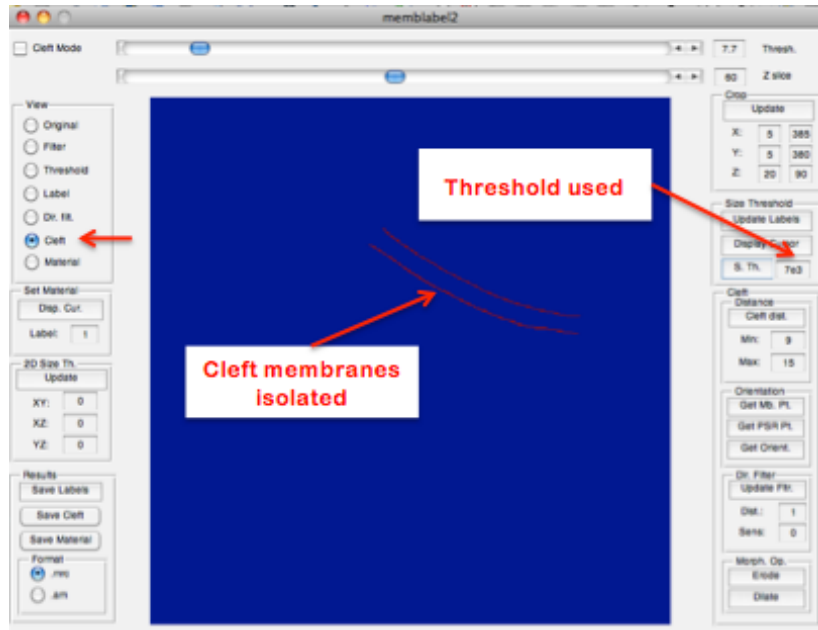
Figure 17. Synaptic cleft isolated by size thresholding from "Cleft" view.

As Figure 17 shows the output ("Cleft" view) given by this procedure may contain both pre and post-synaptic membranes of the synaptic cleft. It is not a problem save cleft at current state (see section 3.5), **vesseg** tool is going to ignore the post-synaptic membrane.

3.3.2   Directional Filters

Sometimes it is not possible to preserve post-synaptic membrane in the cleft as whole because it is hidden by a very high post-synaptic density, a low SNR, is broken or a combination of these factors. In these cases Directional Filter should be used.
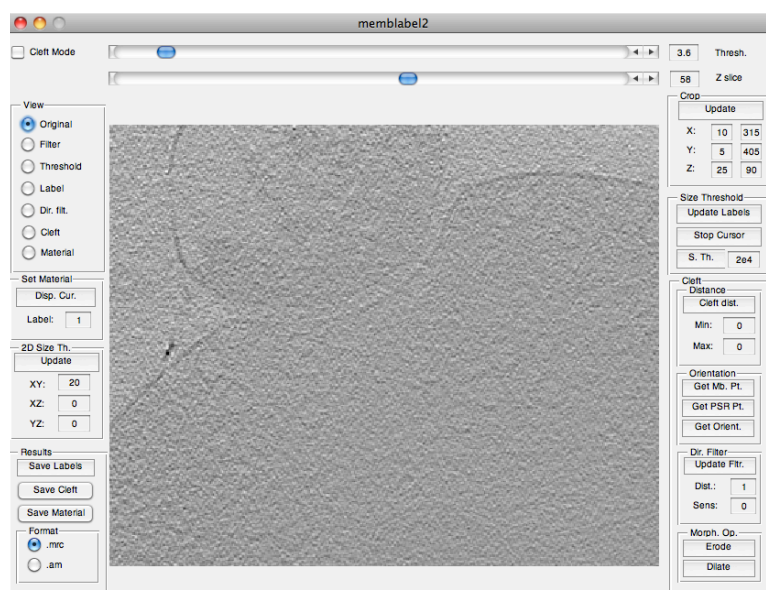

Figure 18. Tomogram with low SNR where the post-synaptic membrane in the cleft is partially hidden.
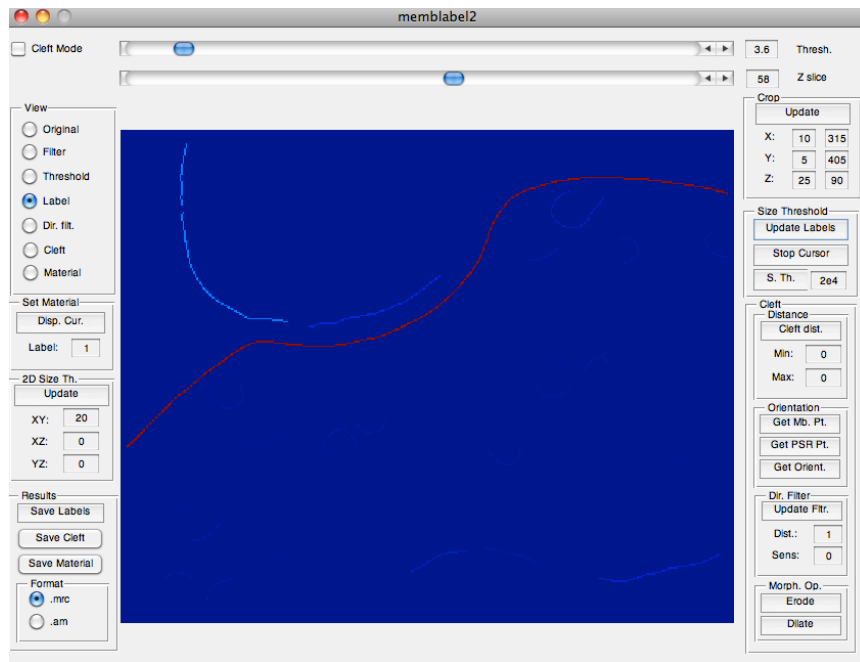
Figure 19. "Label" view obtained from tomogram in Figure 18, the post-synaptic membrane in the cleft has not completely been segmented.

Directional filters need orientation information so the first step is to provide this orientation by using "Orientation" subpanel tools. Firstly, the user presses "Get. Mb. Pt." (the text of this button is replaced by "Capture") and then a cursor is displayed for selecting the pre-synaptic membrane, once the cursor points this membrane, press the "Capture" button in order to retain this information.
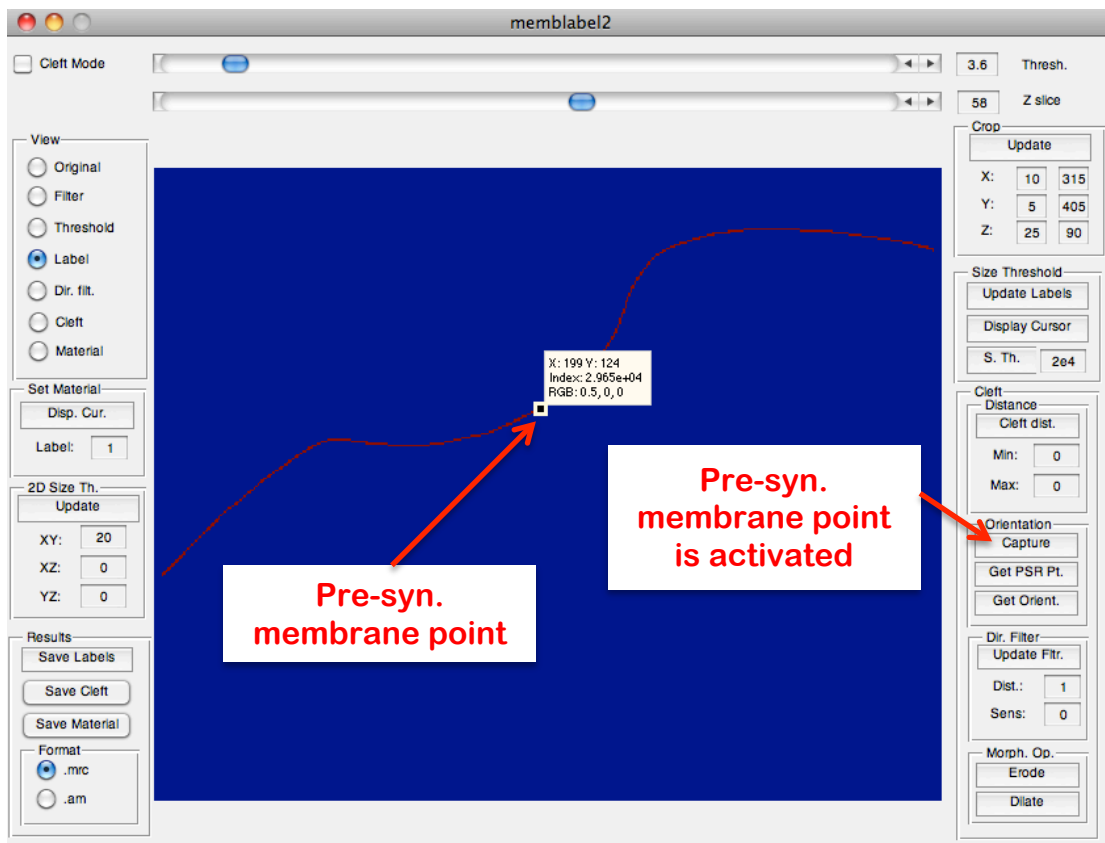

Figure 20. Getting pre-synaptic membrane point for directional filter.

For completing the orientation information a similar scheme must be applied but now it starts by pressing the button "Get PSR Pt.", and choosing a point in the pre-synaptic cytoplasmic region. The line formed by point in pre-synaptic membrane and the point in pre-synaptic region should be approximately normal to the pre-synaptic membrane.
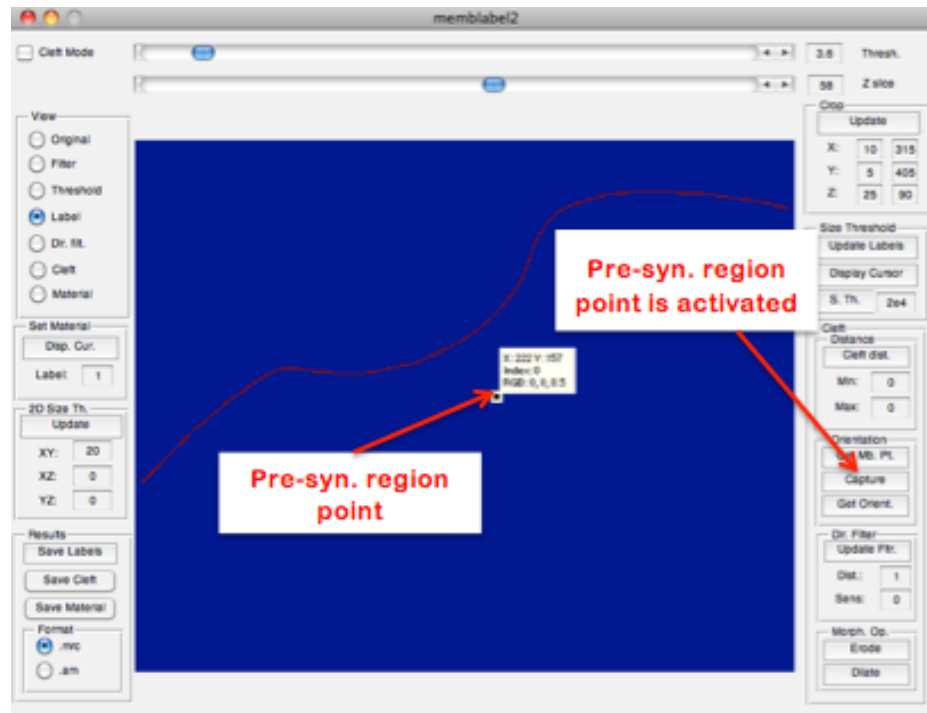

Figure 21. Selection of the point in pre-synaptic region.

Finally, once the user has successfully completed the previous steps, the orientation is propagated along the whole pre-synaptic membrane by pressing button "Get Orient.".

Now the tool is ready for applying the Directional Filter by choosing the proper input parameter and pressing "Update Filtr." button. The parameter "Dist." set the size of the filter and must be greater than zero, it should be set similar to synaptic cleft distance (or slightly greater). The parameter "Sens:" is related with the sensitivity of the filter to changes in the synaptic cleft distance, it must be in range (0,1] (0.33 could be a good starting point). This procedure can take a long time.
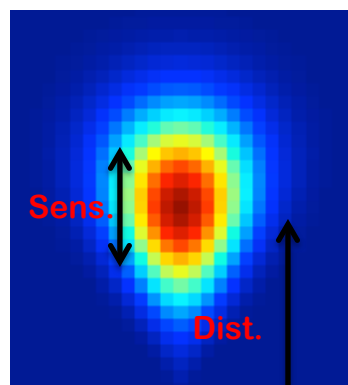

Figure 22. 2D slice of the 3D directional filter used with its parameters.

The output of the Directional Filter is stored in "Dir. Filt" view and it is made up by the voxel of pre-synaptic membrane coded with the probability of being a part of synaptic cleft, the higher value the higher probability.
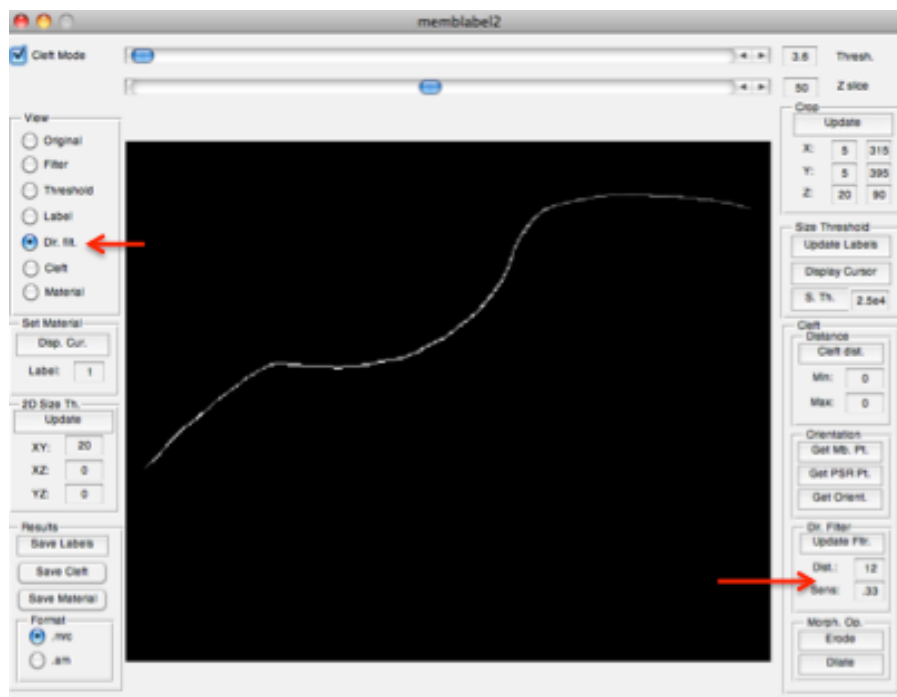
Figure 23. Directional filter output.

In order to separated the cleft from the rest of the membrane a thresholding procedure can be done, the user must activate "Cleft Mode" check box so as to the slider takes as input "Dir. filt." and view.
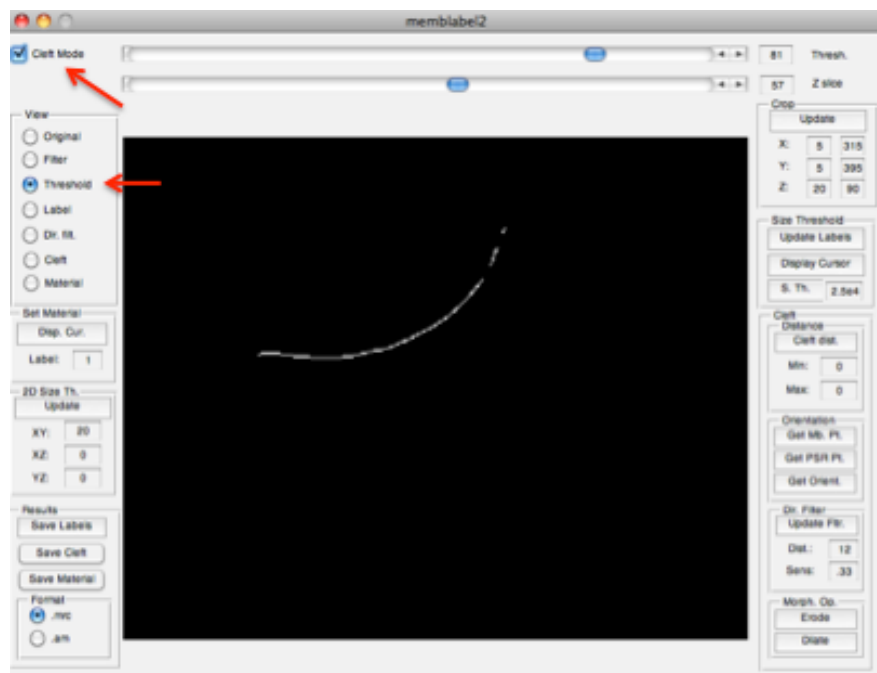

Figure 24. Thresholding output given by directional fiters.

The thresholded cleft can be refined by using the post processing tools (see section 3.4) and labelled by "Size Threshold" and/or "Set Material" panel.

3.4   Post-processing

Membseg2 provides some tools for improving the result in "Threshold" view. Sometimes the binary output generated by thresholding needs a post-processing before size labelling. That is because there are spurious structures connected to the binarized membranes, they can generate errors in the final output or even stop the segmentation procedure.

3.4.1   Crop panel

The crop panel has three pairs, X, Y and Z of editable boxes. For every pair the left box contains the low border and right the high one for this coordinate. When the user press "Update" button then all voxels with coordinates lower or higher than those specified in the panel are set to zero. By default these boxes contain the limits of the original data set.
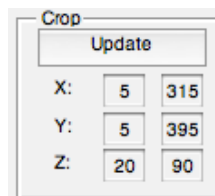

Figure 25. The crop panel.

Cropping is useful for deleting artefacts generated by **tomosegmemtv** tool in the border of datasets.
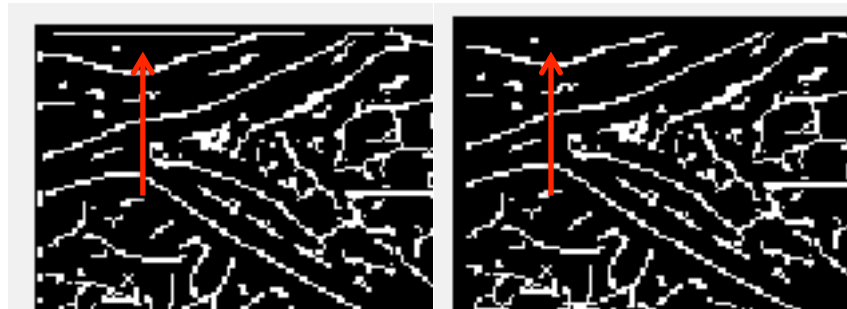

Figure 26. Spurious structure (left) deleted by cropping tool (right).

3.4.2   Erosion and Dilation

These tools have specially been designed for refining the output obtained when "Dir. filt." view is thresholded by setting "Cleft Mode" (see section 3.1.1). By combining these morphological operations holes in the cleft can be filled. These operations take "Label" view as background and "Threshold" (after thresholding the output of directional filters) view as foreground, so only holes present in "Threshold" view but not in "Label" view can be filled.
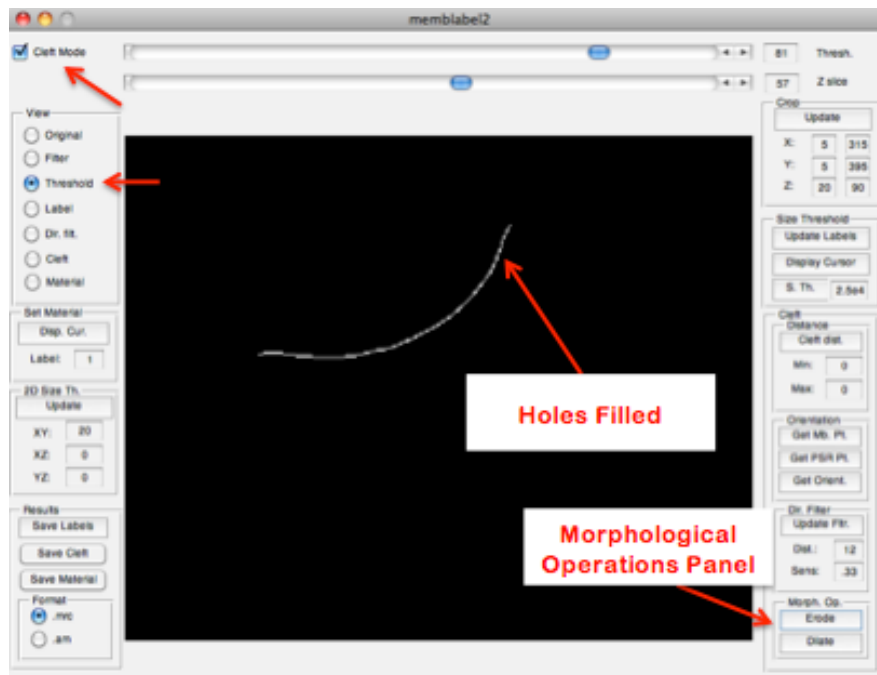
Figure 27. Post-processed "Threshold" view for filling cleft holes, Figure 24 is the input.

3.4.3   2D Size threshold

The panel "2D Size Th." allows rejecting some spurious non-membranous thresholded structures obtained when no optimal threshold can be selected. It is supposed that pre and post-synaptic membranes present a high area in all the 2D slices that cut them parallel to planes XY, XZ and YZ. This panel let define thresholds for rejecting all the structures that do not fulfil these requirement (see [2]). This a good tool for rejecting some non-membranous structures connected to a true membrane that survived to **tomosememtv**.
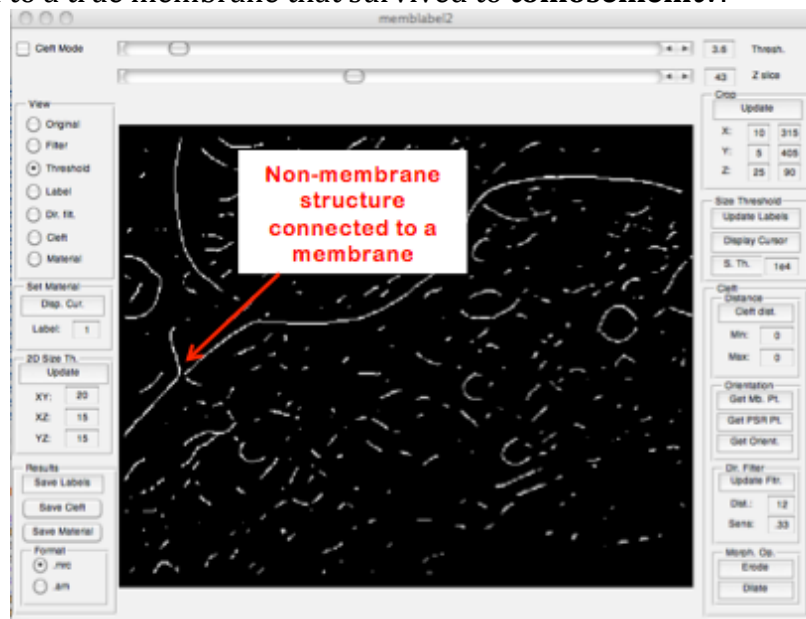

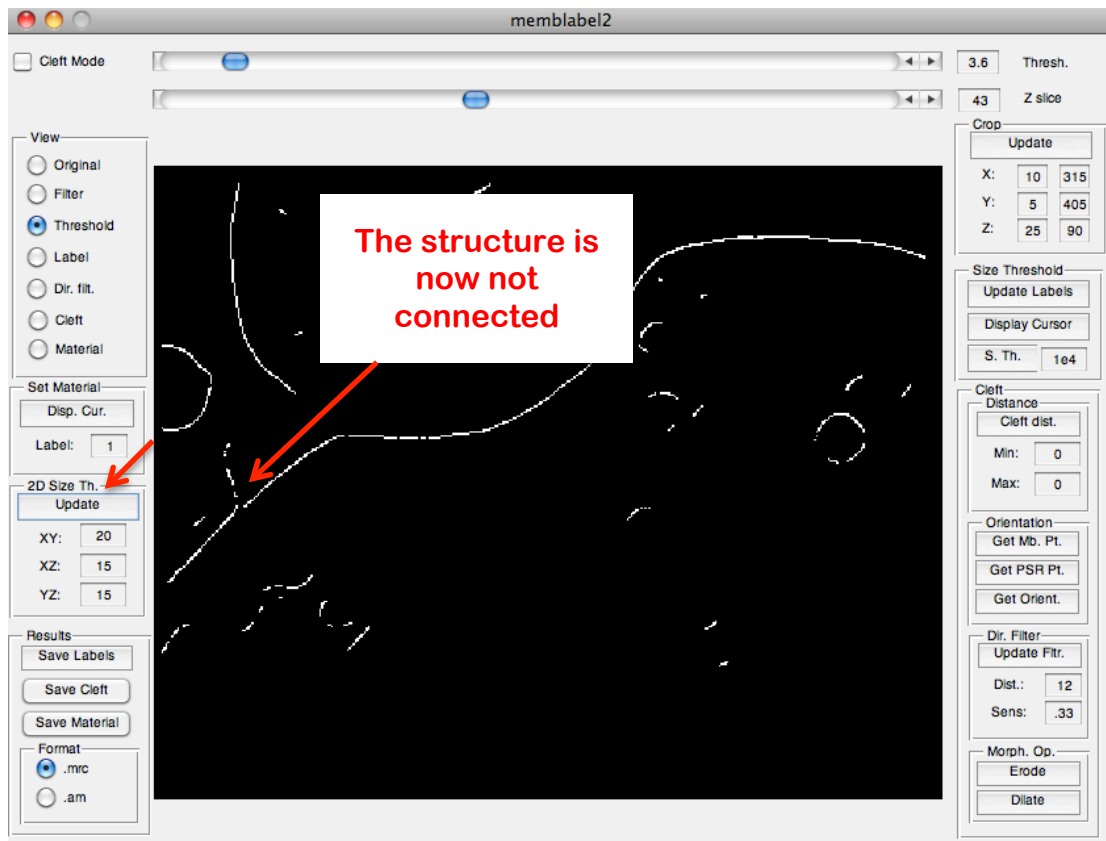**Figure 28. Example of underthresholded tomogram.**

Figure 29. Refined "Threshold" view obtained after 2D size thresholding from Figure 28.

3.5 Saving the Results

At every moment the views "Label", "Cleft" and "Material" can be stored as a file. The user has to press the correspondent button in "Results" panel and a "Save File" window will be opened, then he must choose the name and the path of the output file.
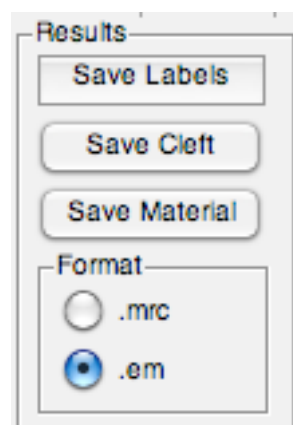

Figure 30. Save result panel.

At this point it is important consider that **vesseg** needs a file with, at least, the pre-synaptic membrane labelled and other file with, at least, pre-synaptic part of

the cleft. As example of correct labelled views can be found in Figure 13 and Figure 17 respectively. The first file should have the stem name of the original tomogram and the extension '_lbl', and the second the extension '_clft'. For example if the name of the original file is '18-2_tomo2_crop2.mrc' then the output generated by **memseg2** must be called '18-2_tomo2_crop2_lbl.mrc' and '18-2_tomo2_crop2_clft.mrc'.

If after following correctly the workflow of **membseg2** no satisfactory pre-synaptic membrane or cleft is segmented, consider to processes again the tomogram with **tomosegmemtv** but changing its parameters.

4 VESSEG

This tool has the intention of segmenting the vesicles present in the pre-synaptic region of a synaptosome. It takes as input the original tomogram, the cleft and pre-synaptic membrane segmented with **membseg2**.

When **vesseg** command is called from Matlab command window, firstly the tool asks for the input data. The user must choose the name of the original dataset, then the software automatically loads output generated by **tomosegmemtv** and **membseg2** (extensions to stem name '_n1', '_n2', '_n3', '_lbl' and '_clft') so they must be in the same directory as the original.

After loading data the main **vesseg** window starts and shows a slice in Z-axis with the original data and the segmentation done by **membseg2** superimposed. In green the regions segmented in the '_lbl' file and in red the regions in '_clft', these regions should overlap in the cleft, in this region the colour red is kept.
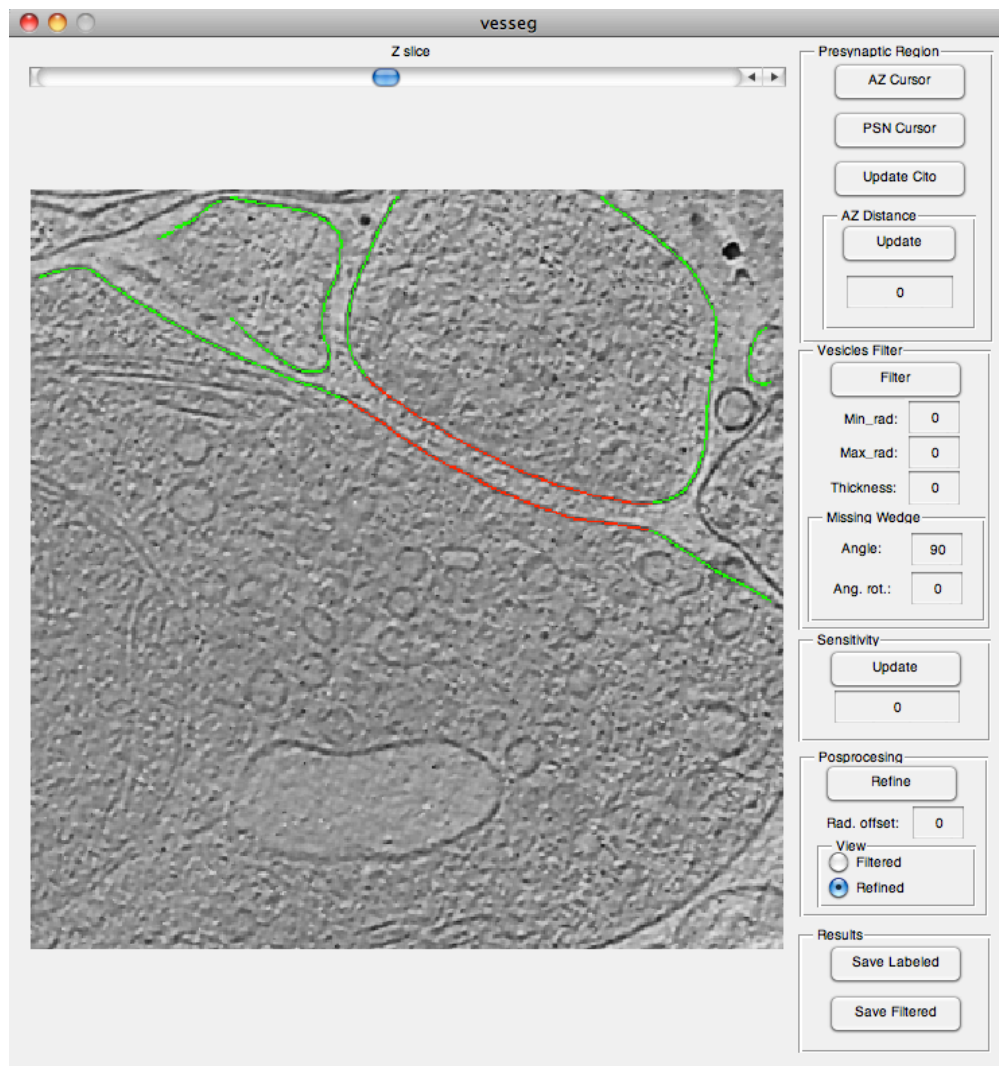

Figure 31. Initial aspect of vesseg main window.

4.1 Pre-synaptic Region

The first step in vesicles segmentation is to determine the region where they are going to be searched, the pre-synaptic region, the panel "Presynaptic Region" has been designed for this task.

When the button "AZ Cursor" is pressed its text changes to "Capture" and a cursor appears, then the user must point it over the pre-synaptic membrane in the cleft (Active Zone, AZ), the RGB indicator of the cursor must show 1,0,0, the value for red. Then the user presses "Capture" button for storing the information, now the button recovers its original text.
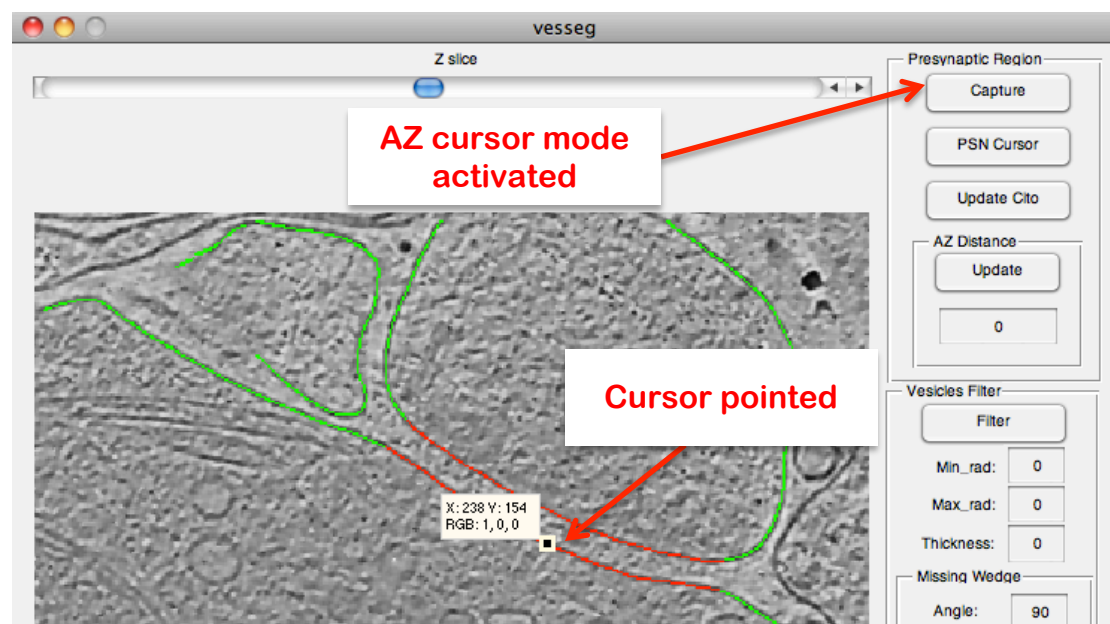

Figure 32. Cursor correctly pointing the pre-synaptic cleft membrane.

A similar is scheme is followed by "PSN Cursor" button but now the pointer must be pointed to one point in the pre-synaptic region. The line formed by point in AZ and point in pre-synaptic region should be approximately normal.
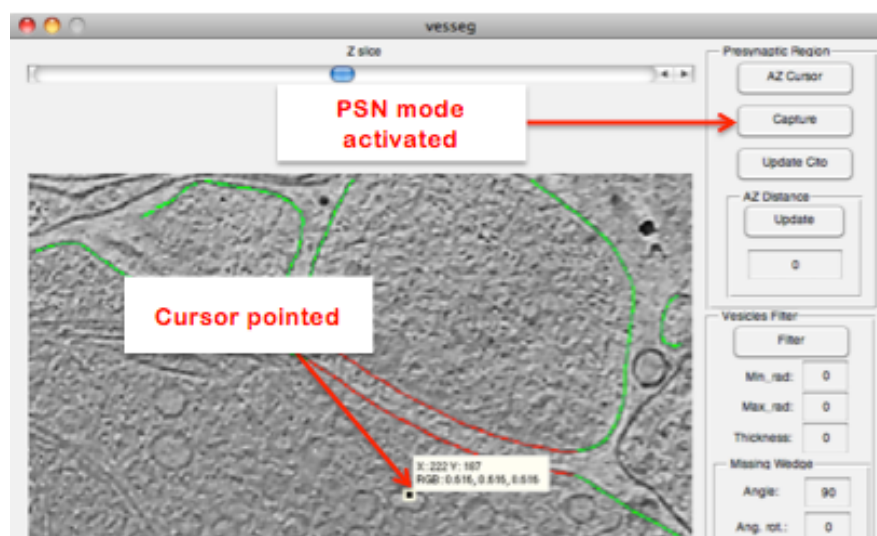

Figure 33. Cursor correctly pointing the pre-synaptic region.

Once the tool knows where is the pre-synaptic AZ and the direction of the pre-synaptic region, the user must press "Update Cito" to fill up the space contained by pre-synaptic region, now voxels contained by this space will be painted with dark blue. It is noticeable that post-synaptic membranes (if they where segmented by **membseg2**) have disappeared.
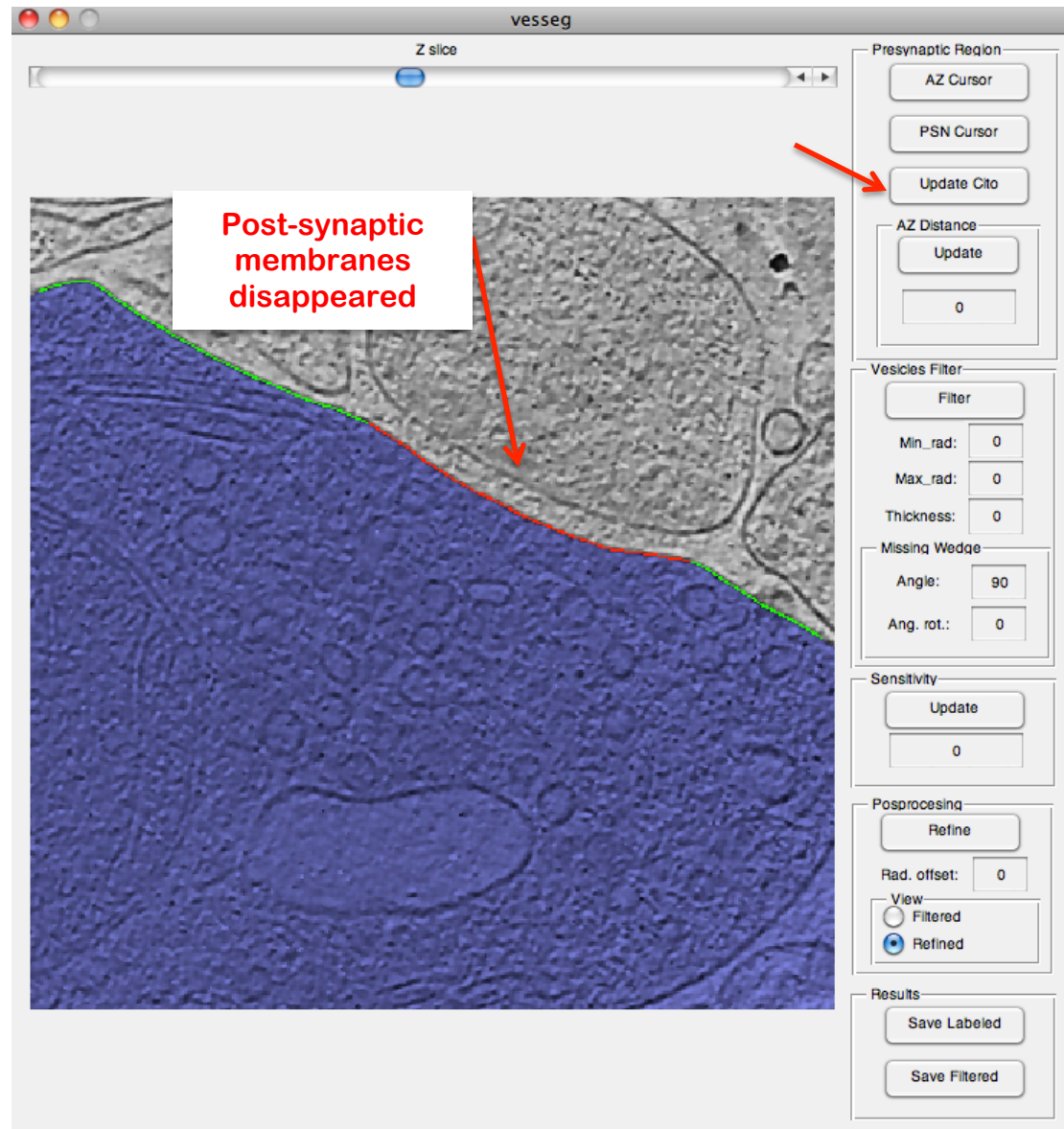

Figure 34. The pre-synpatic region.

Now the user must define the maximum distance allowed between a vesicle and the AZ by writing it in "AZ Distance" editable box (in voxels), and then press "Update" button. After this action the region of interest, where the vesicles filter is going to work, is painted with light blue.
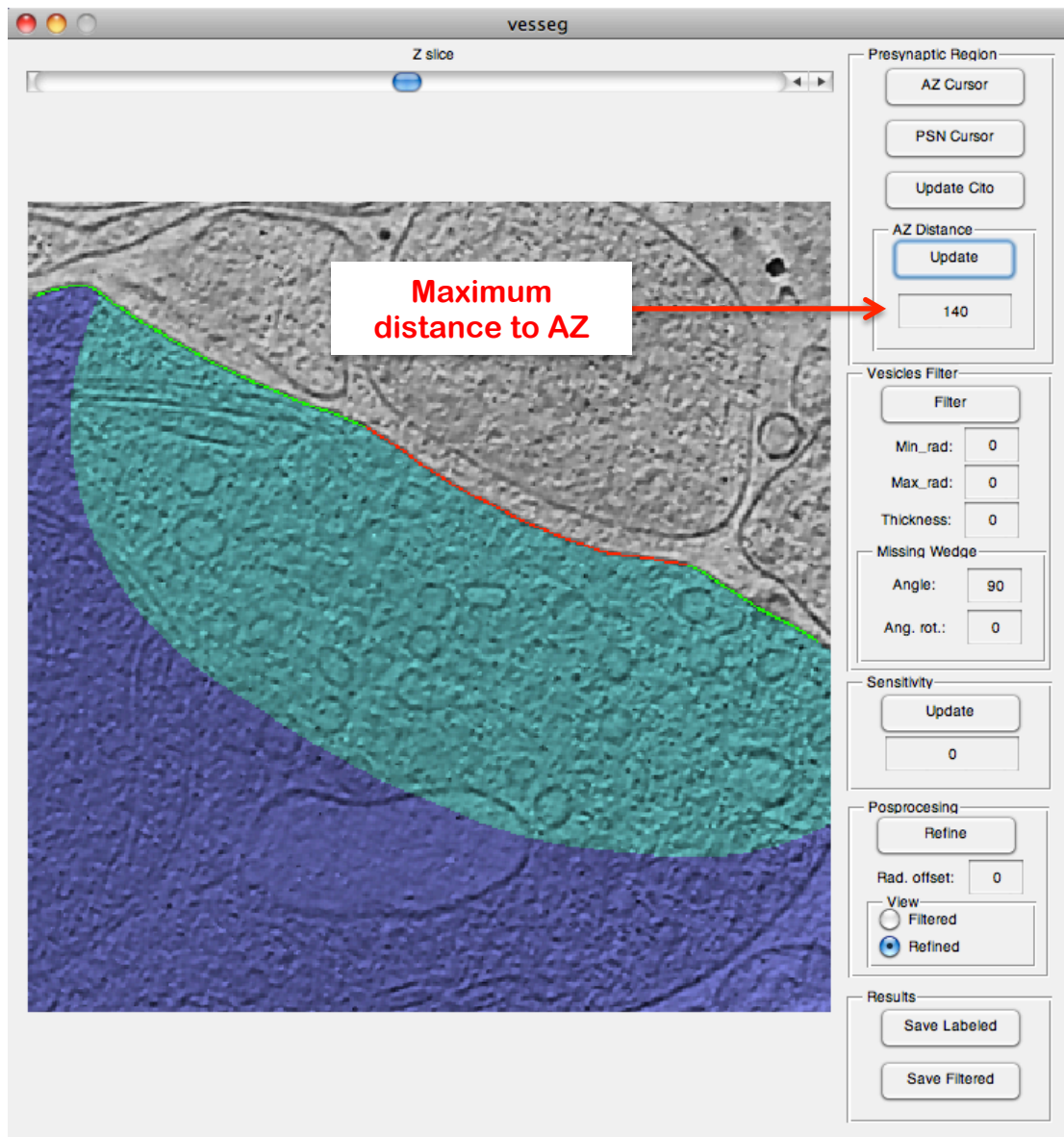
Figure 35. Vesicles region of interest in light blue.

4.2   Vesicles Filter.

This is the key point of **vesseg** and "Vesicles Filter" panel controls it. This filter is only going to consider the pre-segmented (see section 4.1) light blue region. The editable boxes allow defining the minimum and maximum radius for searching vesicles and the thickness of their membranes, all these metrics are in voxels. The user can also specify the missing wedge parameters in "Missing Wedge" subpanel, the semi-angle of wedge in degrees and the angle in which this wedge is rotated. Once the parameters have been set, the user must press "Filter" button to start the filtering process.
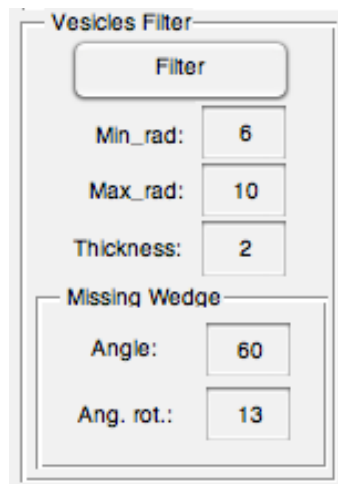
Figure 36. "Vesicles Filter" panel with possible valid configuration for parameters.

4.3 Sensitivity thresholding

After vesicles filter has been applied the user must choose the proper threshold for the output of this filter that is controlled by "Sensitivity" panel. The user must write a value in the editable box, the sensitivity range must be in range [1,255], and then press the "Update" button. The detected vesicles (centre and radius) will be painted on the display as yellow. The user will play with several values of sensitivity until find the optimum.
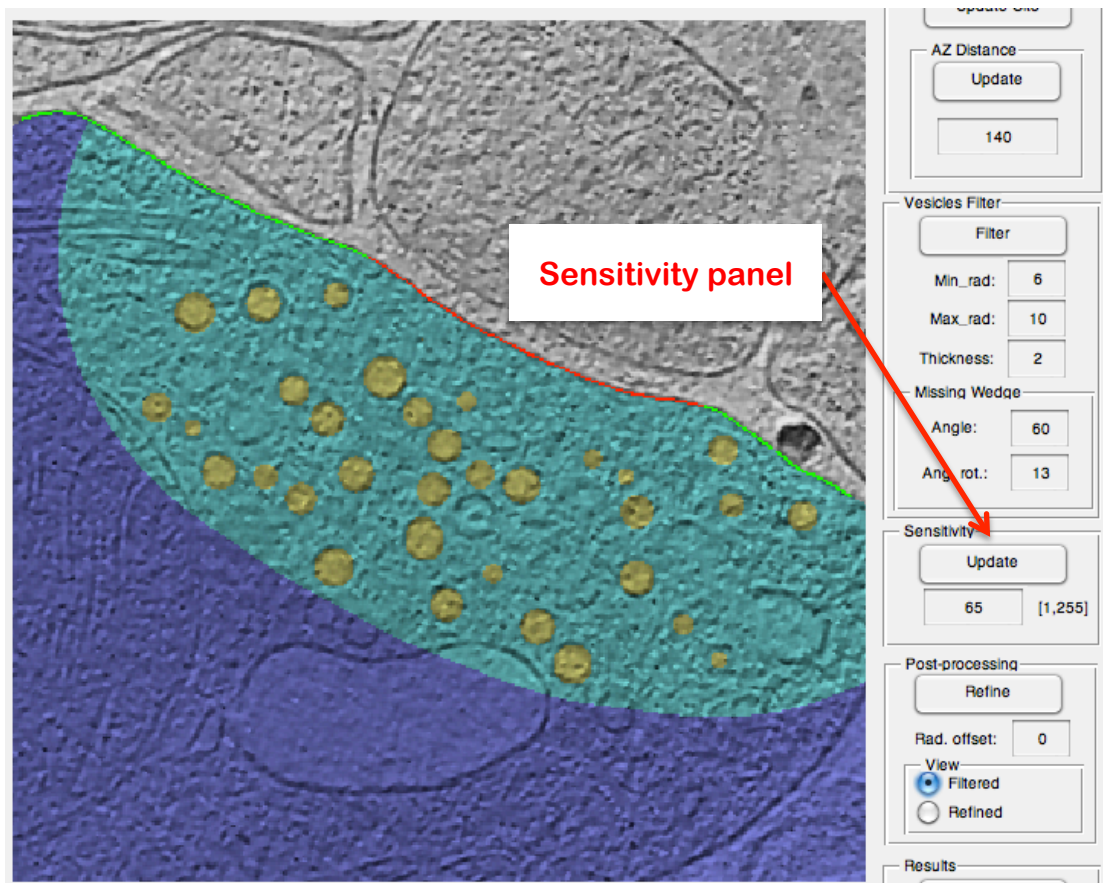

Figure 37. Vesicles filter output after a good selection of sensitivity threshold.

24

4.4   Post-processing

Using "Post-processing" panel the user can refine the output given by vesicles filter. Pressing "Refine" button the vesicles that touch a membrane are deleted, if two vesicles are touching each other the one that needs higher sensitivity will be deleted. By the way the user can define an offset in voxels applied to measured radius, "Rad. offset" editable box. This feature is useful because sometime the output should ensure that segmentation completely encloses the vesicles when they are aspherical. Using radio buttons in "View" subpanel the user can move between unrefined ("Filtered") and refined ("Refined") outputs.
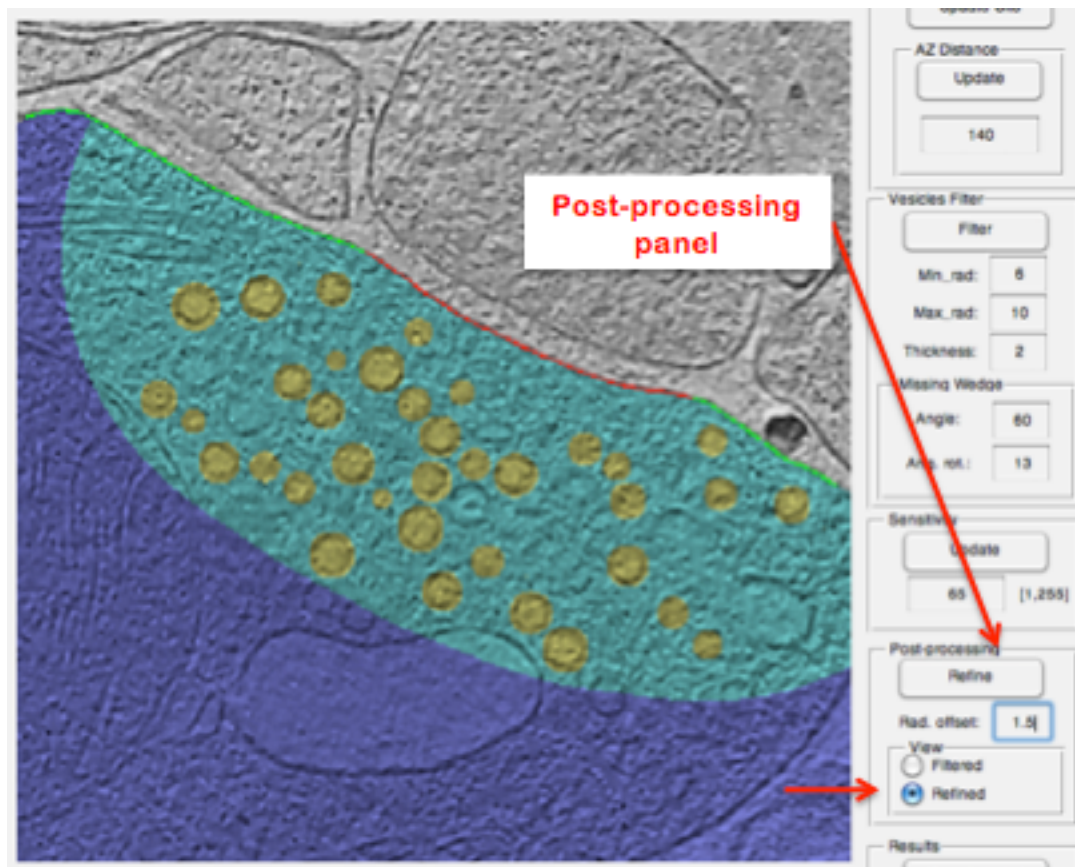

Figure 38. Refined output generated after post-processing.

4.5   Save Results

The vesicles segmentation can be saved in a file by pressing "Save Labelled" in "Results" panel. If the view "Refined" is selected when "Save Labelled" is pressed then the refined output is saved (recommended), in other case unrefined output is saved. The output is labelled as follows: 1 AZ pre-synaptic membrane, 2 the rest of the pre-synaptic membrane, 3 pre-synatic region far from AZ membrane, 4 pre-synaptic region close to AZ membrane where vesicles are segmented and finally each vesicle has a different label but greater than 4.

The "Save Filtered" allows saving the output generated by vesicles filter it has only debugging meanings.

5   REFERENCES

The publication of a paper that describes the algorithms used by these tools is in progress [1]. Here are also two references related with the segmentation problem in electron tomography:

[1]   *Martinez-Sanchez A., et al. Robust membrane detection based on tensor voting for electron tomography. sent to J. Struct. Biol. (2013)*

[2]   *Martinez-Sanchez, A., et al. A differential structure approach to membrane segmentation in electron tomography. J. Struct. Biol. (2011), doi:10.1016/j.jsb.2011.05.010*

[3]   *Martinez-Sanchez, A., et al. A ridge-based framework for segmentation of 3D electron microscopy datasets. J. Struct. Biol. (2012), http://dx.doi.org/10.1016/j.jsb.2012.10.002*