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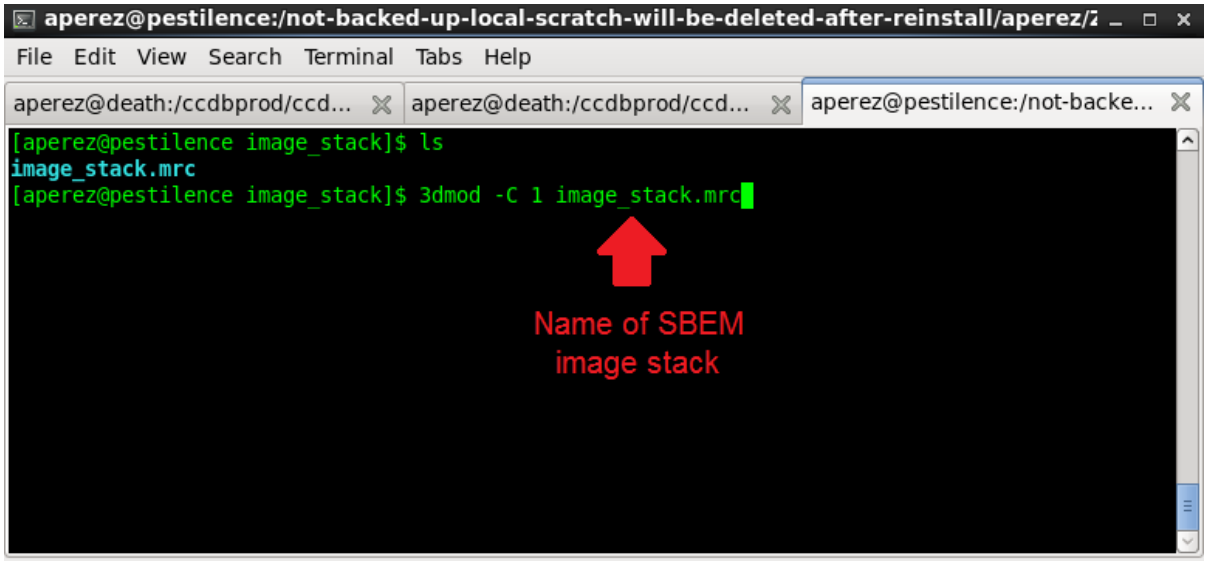
This protocol describes the steps required to create a training set for CHM that consists of 50 images of size 500 x 500 pixels each.

A. OPENING 3DMOD AND CREATING YOUR MODEL FILE

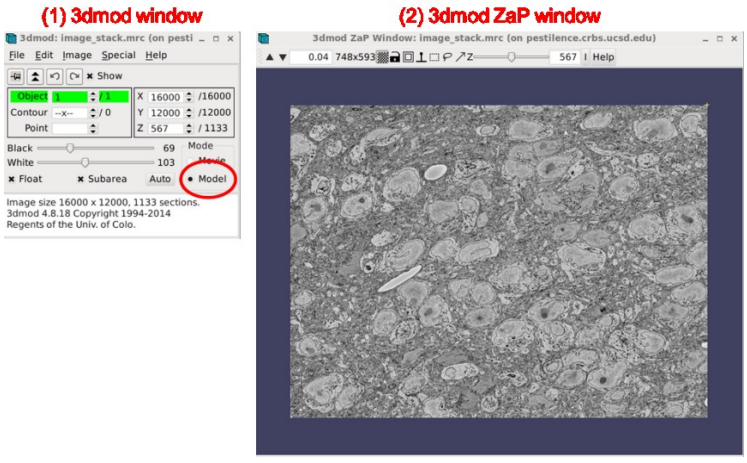
1. If necessary, source a recent version of IMOD. This will be necessary if, for example, you get an error when trying to open 3dmod. First, open a terminal. Then, enter the following at the command line. These steps assume a default installation path for IMOD on a Linux system. If you installed IMOD elsewhere, or are using a different OS, change the path accordingly.

   ```
   $ export IMOD=”/usr/local/IMOD”
   $ export IMOD_DIR=”/usr/local/IMOD”
   $ source /usr/local/IMOD/IMOD-linux.sh
   ```

2. Using the terminal, open the image stack in 3dmod. If the stack is large, load only one slice into memory at a time by supplying a value of one to the ‘-C’ argument:

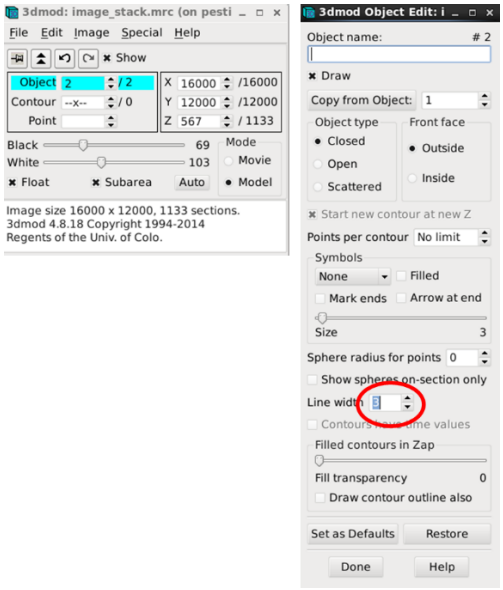


3. Two new windows will appear: (1) a 3dmod window and (2) a 3dmod ZaP window. In the 3dmod window, select the “Model” mode:

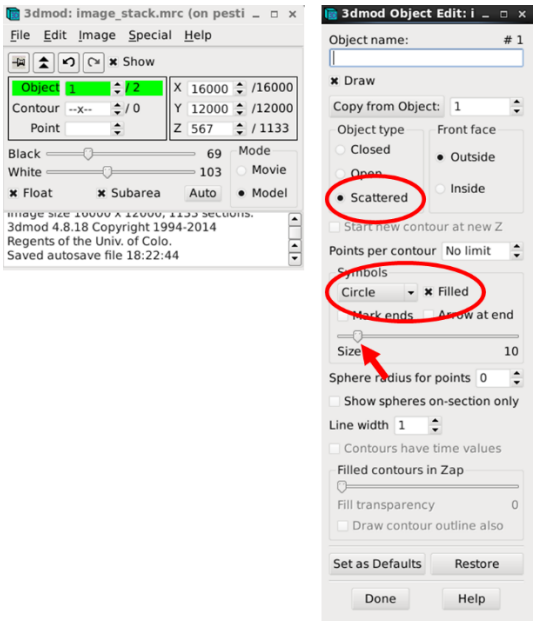


4. Make sure your mouse preferences are set properly. Select Edit → Options → Mouse, and choose the "Right, Left, Middle **" option. Click Done. (NOTE: This only needs to be done once, typically when running IMOD for the first time on a new machine).

5. From the 3dmod window, create a second object by selecting Edit → Object → New. A new object will be created with a color of cyan, and a "3dmod Object Edit" window will pop-up. For ease while segmenting, select a line width of 2-3. Leave all other parameters alone:



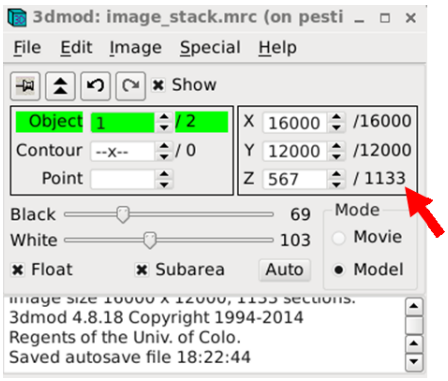
6. In the 3dmod window, use the Object arrows to select Object 1, which should be green. Edit Object 1's properties by selecting Edit → Object → Type. Change the Object type to 'Scattered' and the Symbol to 'Circle'. Check the 'Filled' box, and select a Symbol Size of ~10:



7. Save your model by selecting File → Save Model As, and select an appropriate name.

B. PLACING SCATTERED POINTS

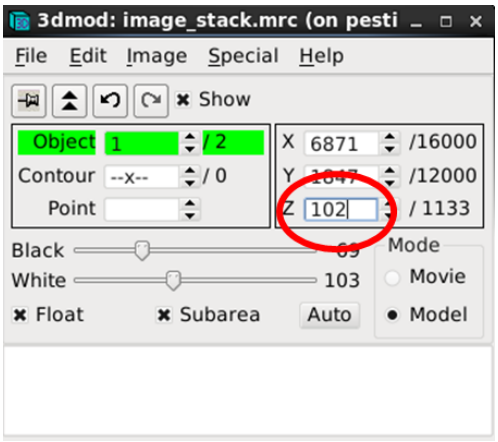
1. Determine which slices to generate training images from. The goal is to make 50 training images from well-distributed areas throughout the entire image stack. A good way to do this is by placing 5 scattered points on 10 different slices throughout the stack. The slices should be distributed as evenly throughout the entire depth of the image stack as possible. It's also a good idea to not use the first few and last few slices for training. The image stack used here contains 1,133 slices (denoted by the red arrow):



   Therefore, it would be reasonable to place 5 points on every slice numbered 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000.

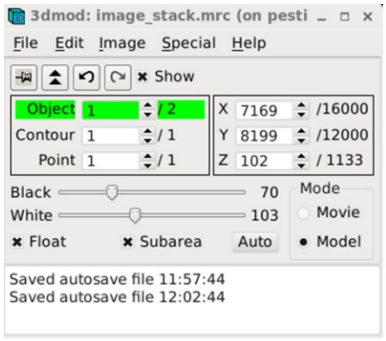
   It is important to note that these slice numbers are only suggestions. If, for example, a suggested slice has poor image quality (due to bad focus, section overlap, etc.), it should be skipped for a nearby slice of better quality.

2. Navigate to the first suggested slice by entering the slice number in the 3dmod window, as shown below:



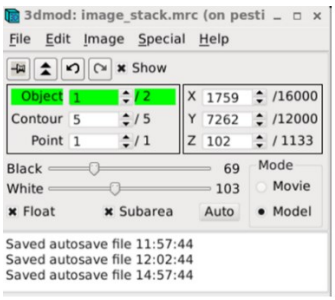
3. Bring up the 3dmod ZaP window. Zoom in many times by pressing the '+' button on your keyboard, until the organelle of interest (i.e., mitochondrion) is clearly visible. Pan around the image by clicking and dragging with the right mouse button. Find an area that contains many mitochondria, make sure Object 1 (green) is selected, and place a single point by left-clicking.

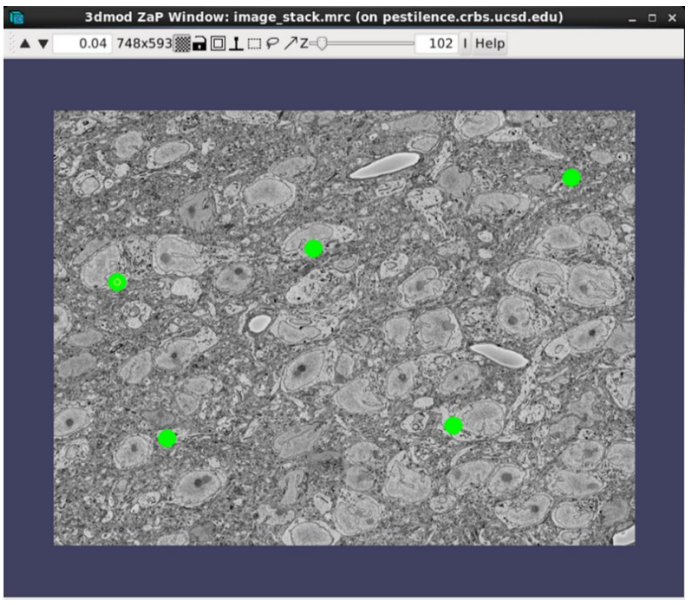
This will create a new contour with a single point that appears as a filled-in circle in the 3dmod ZaP window:



4. Press the ‘n’ button on your keyboard to create a new contour. Pan around the image and find another unique area containing many mitochondria. Place a new scattered point here by left-clicking once. The new area should be a significant distance away from the first one. Object 1 should now have two contours, with each contour containing a single point.

5. Repeat Step 4 until five points have been placed around the current image slice. The points should be sufficiently distributed around the slice, such that the ZaP window likes something like this when zoomed out:

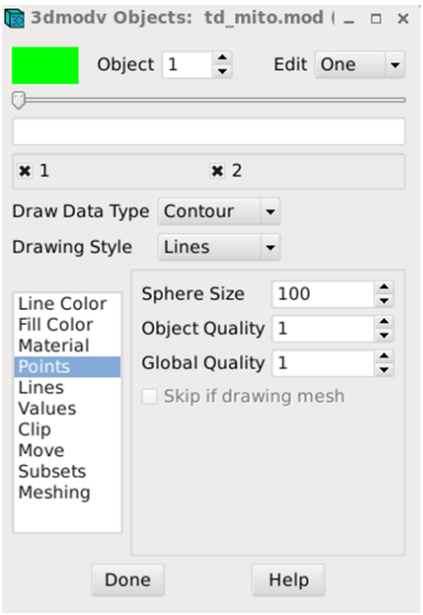


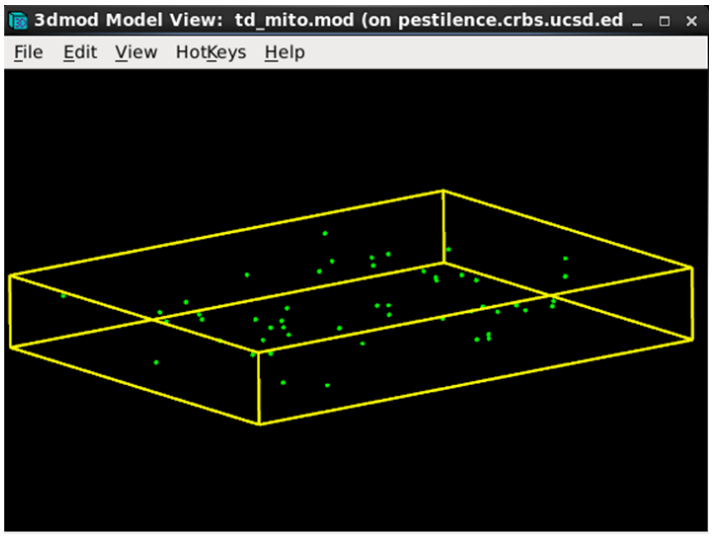


6. Navigate to all other suggested slices and repeat Steps 3-5 until you have placed five new points on each slice. At the end of this process, Object 1 should have 50 contours, and each contour should have exactly one point each.

7. To confirm the distribution of points, enter model view mode by selecting Image → Model View in the 3dmod window. In the 3dmod Model View window, do the following:

    - Select View → Bounding Box
    - Select Edit → Objects to open a 3dmodv Objects window. In this window, select Object 1, then select "Points" and set the "Sphere Size" to a value of ~100. You should see the set of points you just placed appear in 3D throughout the bounding box.
    - By clicking and dragging with the middle mouse button, look at different views of the bounding box and ensure the points are well-distributed throughout it. You should see something look this:





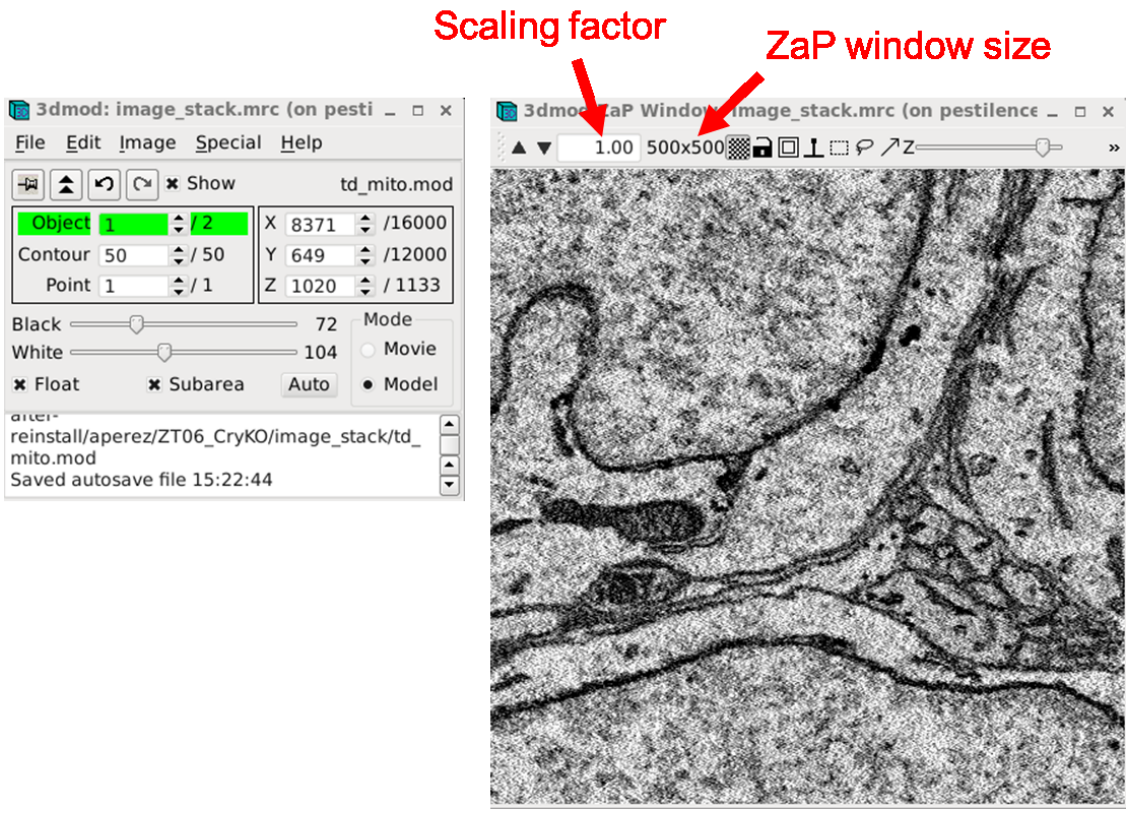
C. SEGMENTING FEATURES OF INTEREST

1. In the 3dmod Model View window, reset the Sphere Size of Object 1 to zero and click Done.

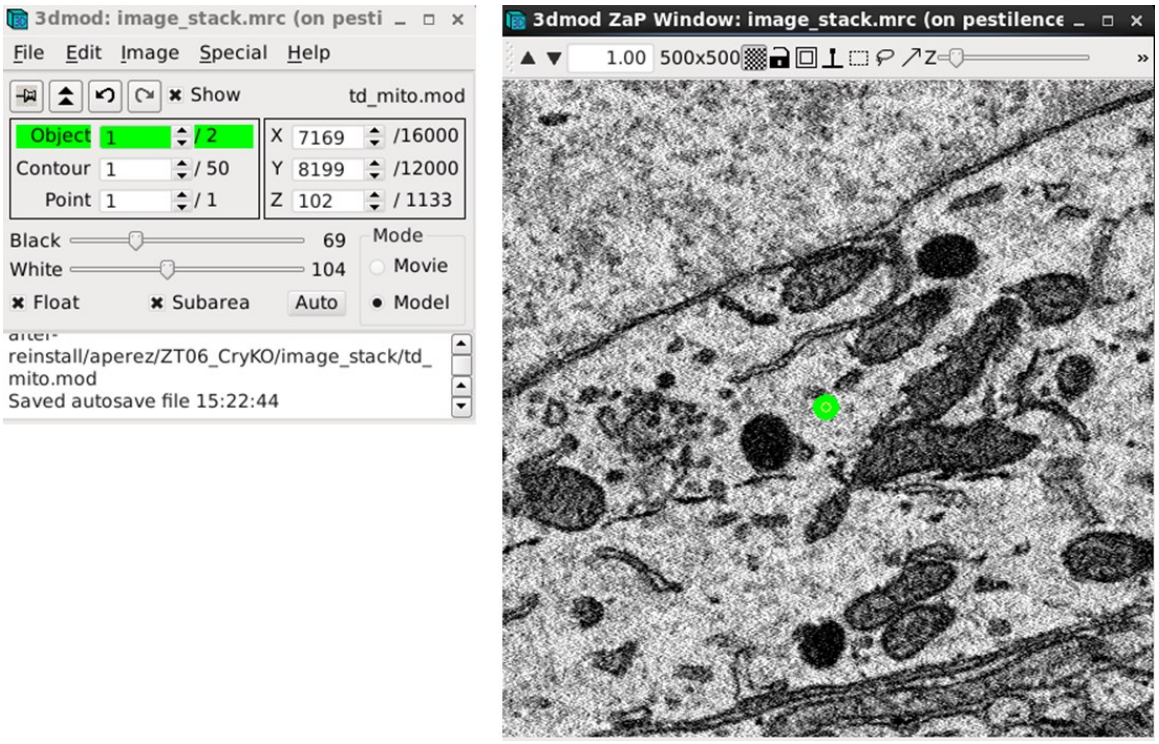
2. Close all windows except the original 3dmod window and 3dmod ZaP window.

3. In the 3dmod window, select Special → Drawing Tools open the Drawing Tools window.

4. Press the '+' button on the keyboard to zoom in on the image until the scaling factor is exactly 1.0. Then, drag the edges of the 3dmod ZaP window until the window is of size 500 x 500 pixels:



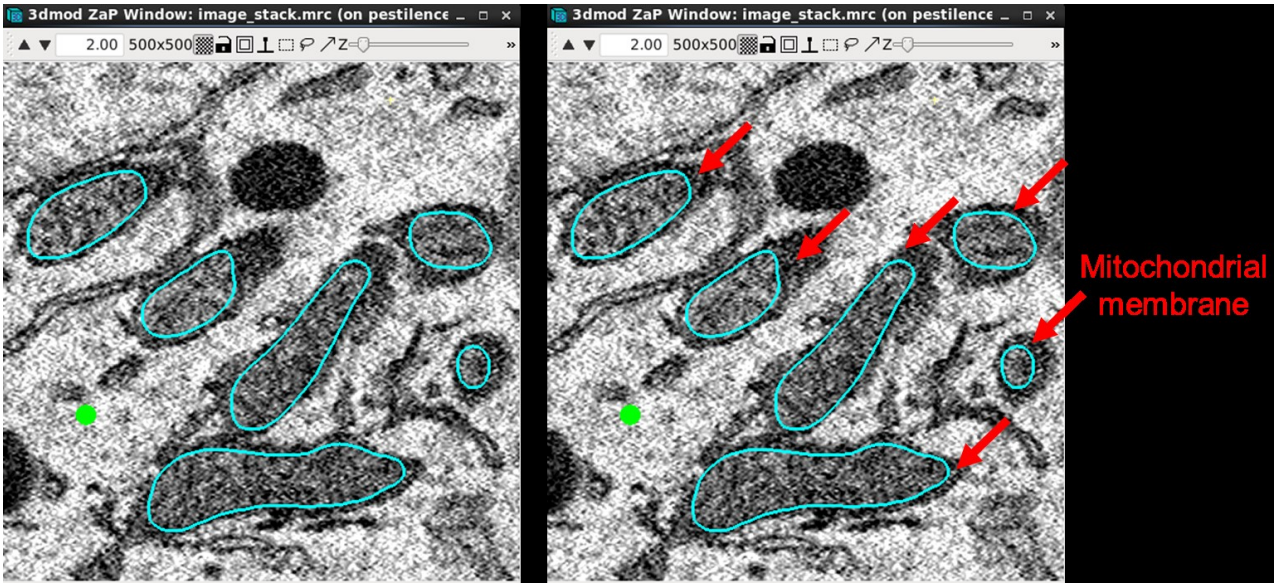
5. In the 3dmod window, select Object 1, Contour 1. You will see that the ZaP window has centered on the scattered point placed for Contour 1:



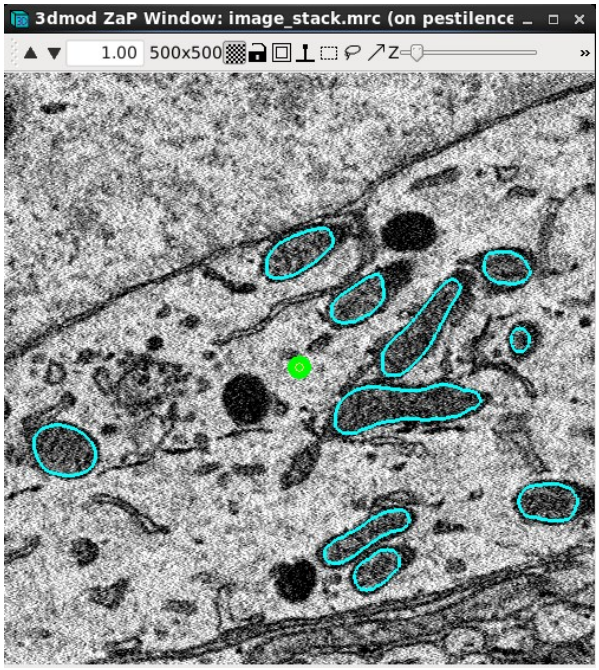
6. Right-click off of the point specified by Contour 1. Select Object 2.

7. Manually segment all instances of the feature of interest in the current box that is centered on Contour 1. This is best done by performing the following steps:

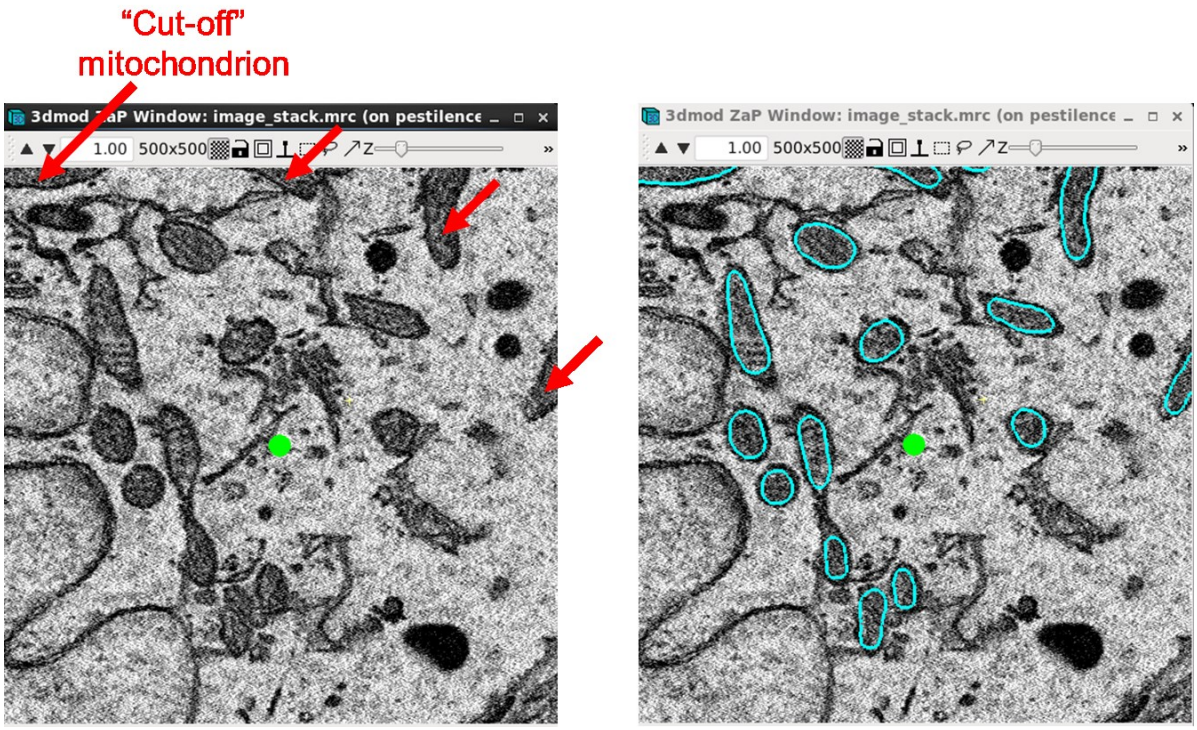
    - Zoom in to a Scale Factor of ~2-3 with the '+' button.
    - Pan to a mitochondrion by right-clicking and dragging.
    - Select the 'Curve' tool in the Drawing Tools window.
    - Sequentially left-click around the boundary of the mitochondrion until you have closed the loop.
    - After closing the contour, right-click on it and press the 'e' button a few times to smooth it.
    - Now, select the 'Warp' tool from the Drawing Tools window.
    - Make the diameter of the tool fairly large (slightly bigger than the diameter of the mitochondrion) by rolling the mouse wheel.
    - Left-click and drag the warp tool to nudge the contour such that it falls on the inside of the mitochondrial membrane.
    - Repeat these steps for each mitochondrion in the current boxed area.



8. Once you think you've segmented all the mitochondria in the box, zoom back out to a Scale Factor of 1.0 by pressing the '-' button on the keyboard. Right-click on the green point corresponding to Object 1, Contour 1. Press 'SHIFT + C' to go up one contour, then press 'C' to go back down to Contour 1. This will re-center the ZaP window on Contour 1. In the example shown below, you'll see that all mitochondria in the box have been properly segmented:



9. Zoom in and out a few times to make sure all mitochondria have been segmented. If you are unsure whether something is a mitochondrion or not, it can help to press “Page Up” and “Page Down” a few times to see how the object evolves in 3D. NOTE: Sometimes, a mitochondrion will be cut off at the boundary of the box. When this occurs, the mitochondrion should still be traced, as below:



10. Once you have confirmed that all mitochondria have been segmented, proceed to Object 1, Contour 2 and repeat Steps 6-9. Do this for all 50 contours in Object 1.