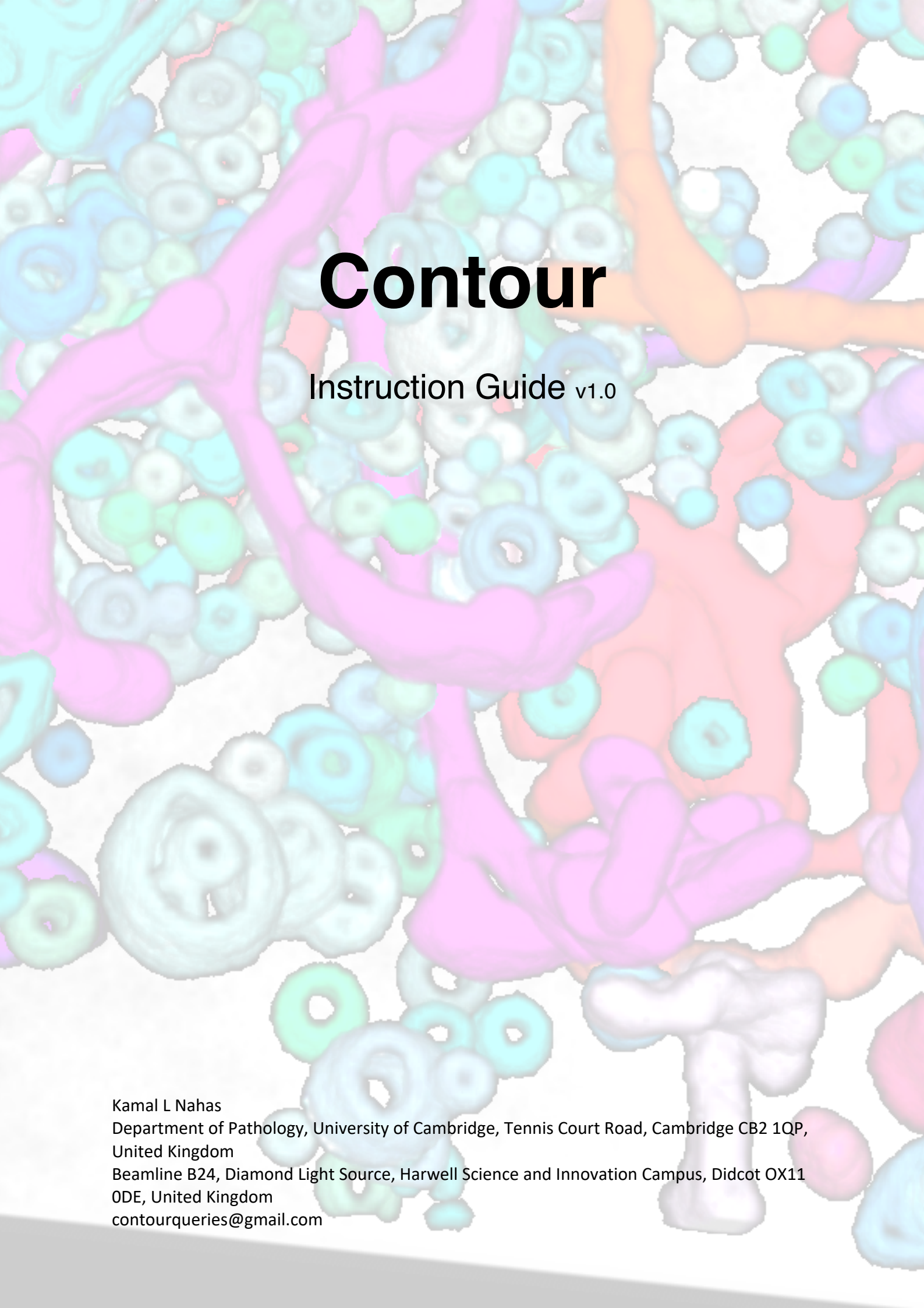

Contour

Instruction Guide v1.0

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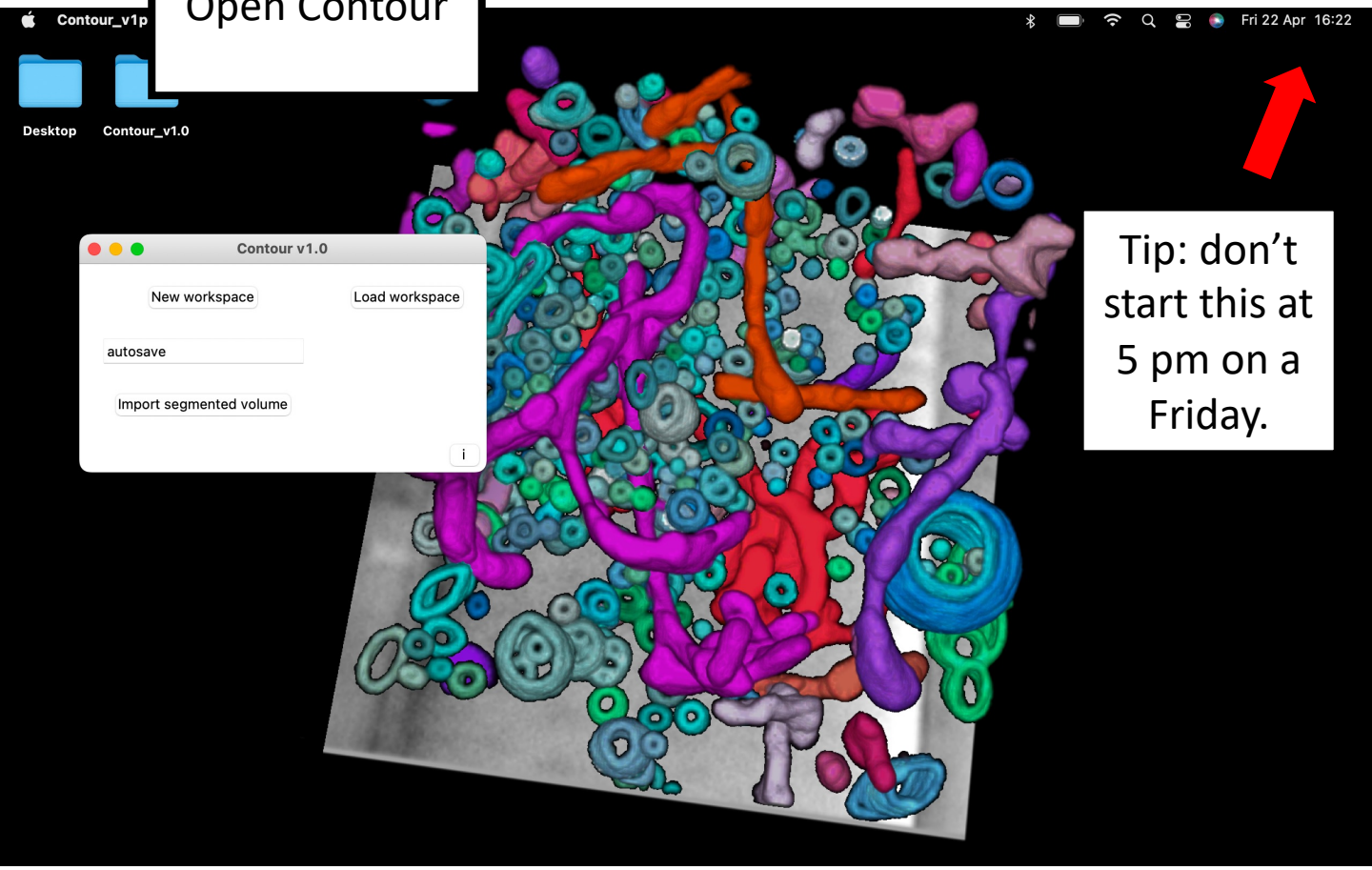
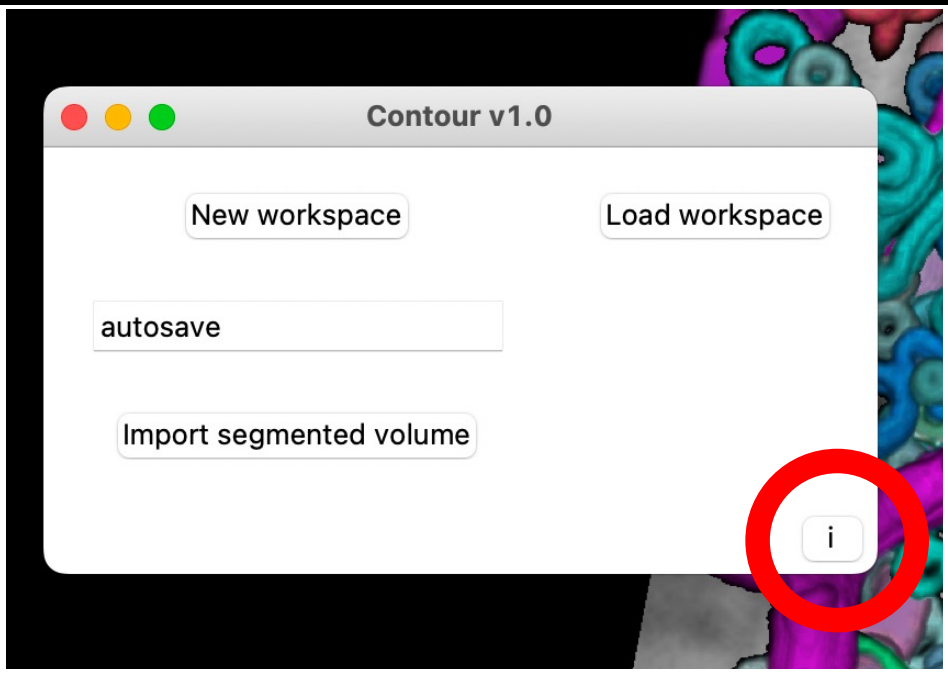
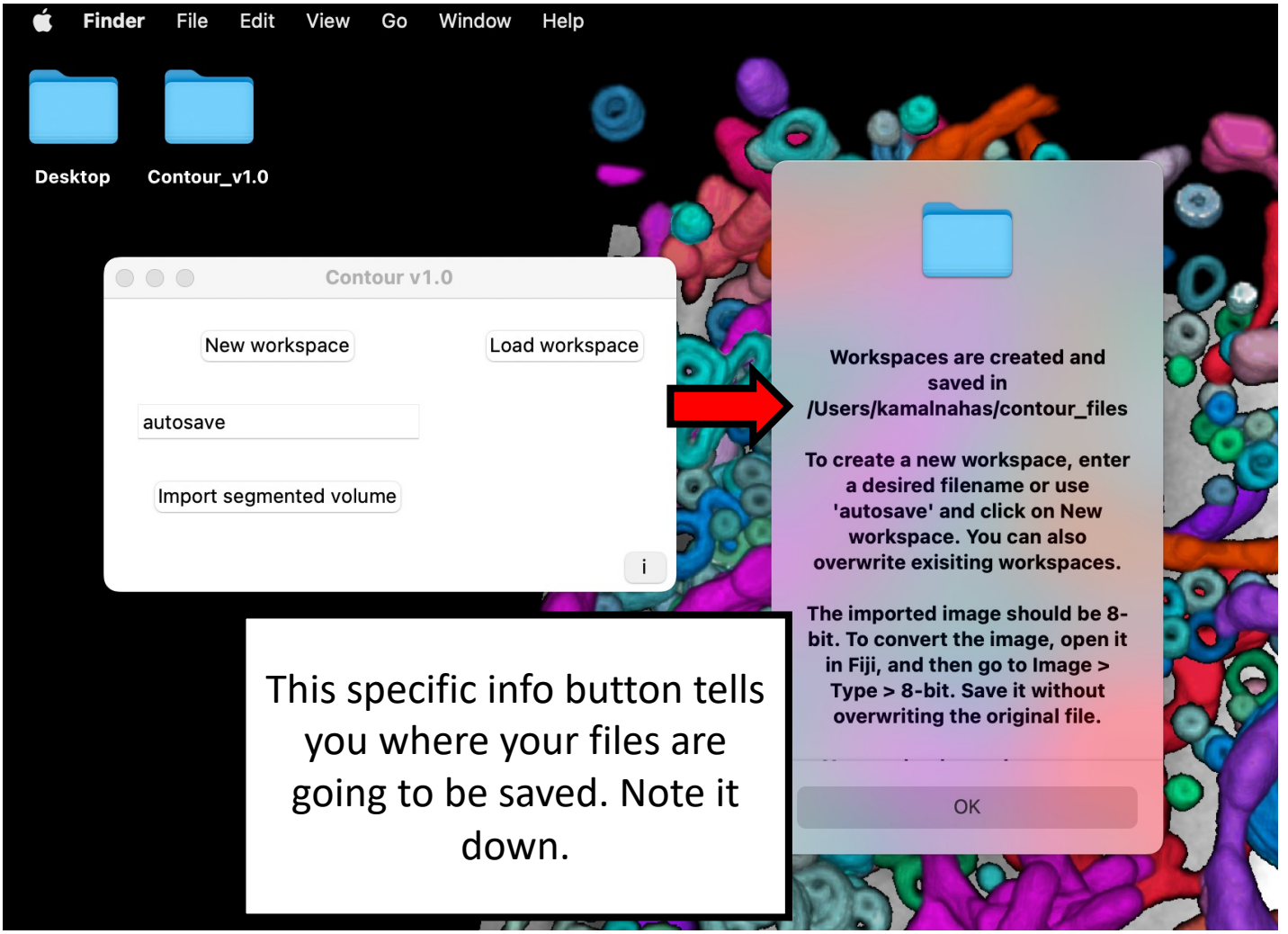
Open Contour



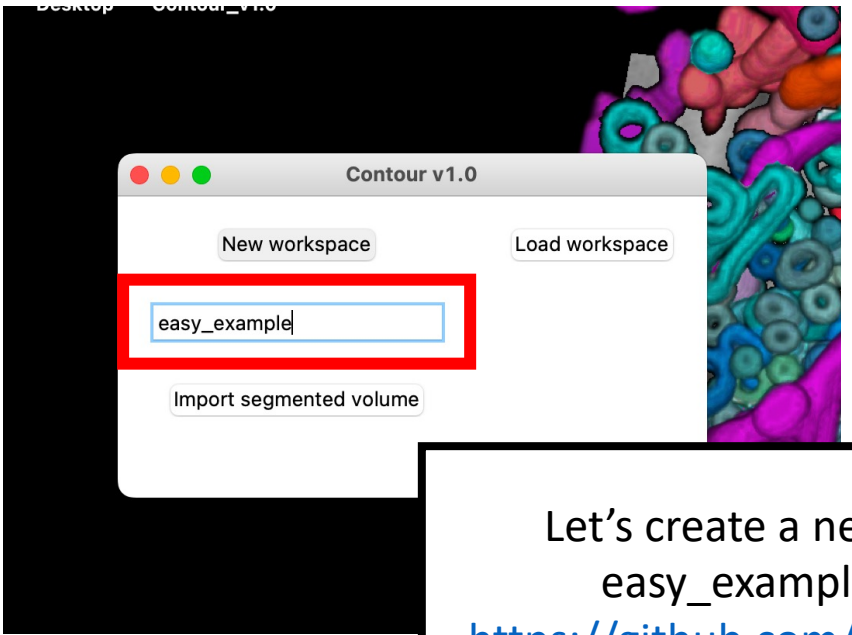
Tip: don't start this at 5 pm on a Friday.



Info buttons can be found throughout the program and can be helpful guides. Click on this one.



This specific info button tells you where your files are going to be saved. Note it down.

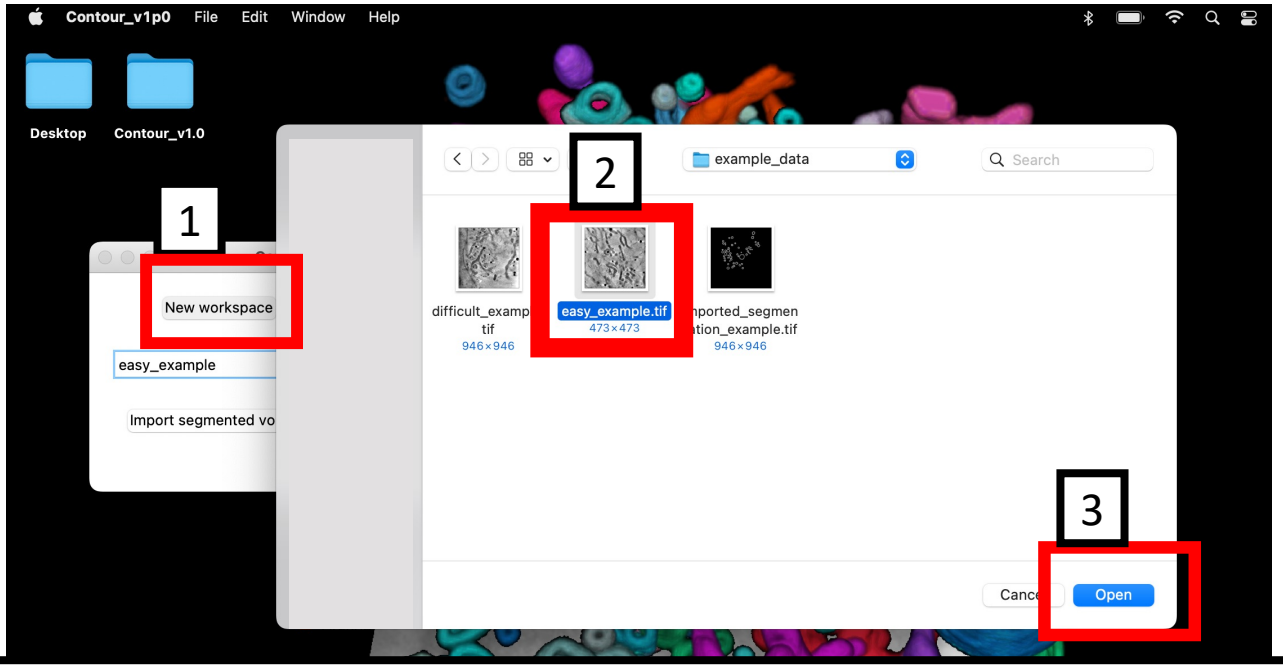


Let's create a new workspace with the easy_example.tif file available at https://github.com/kamallouisnahas/Contour.

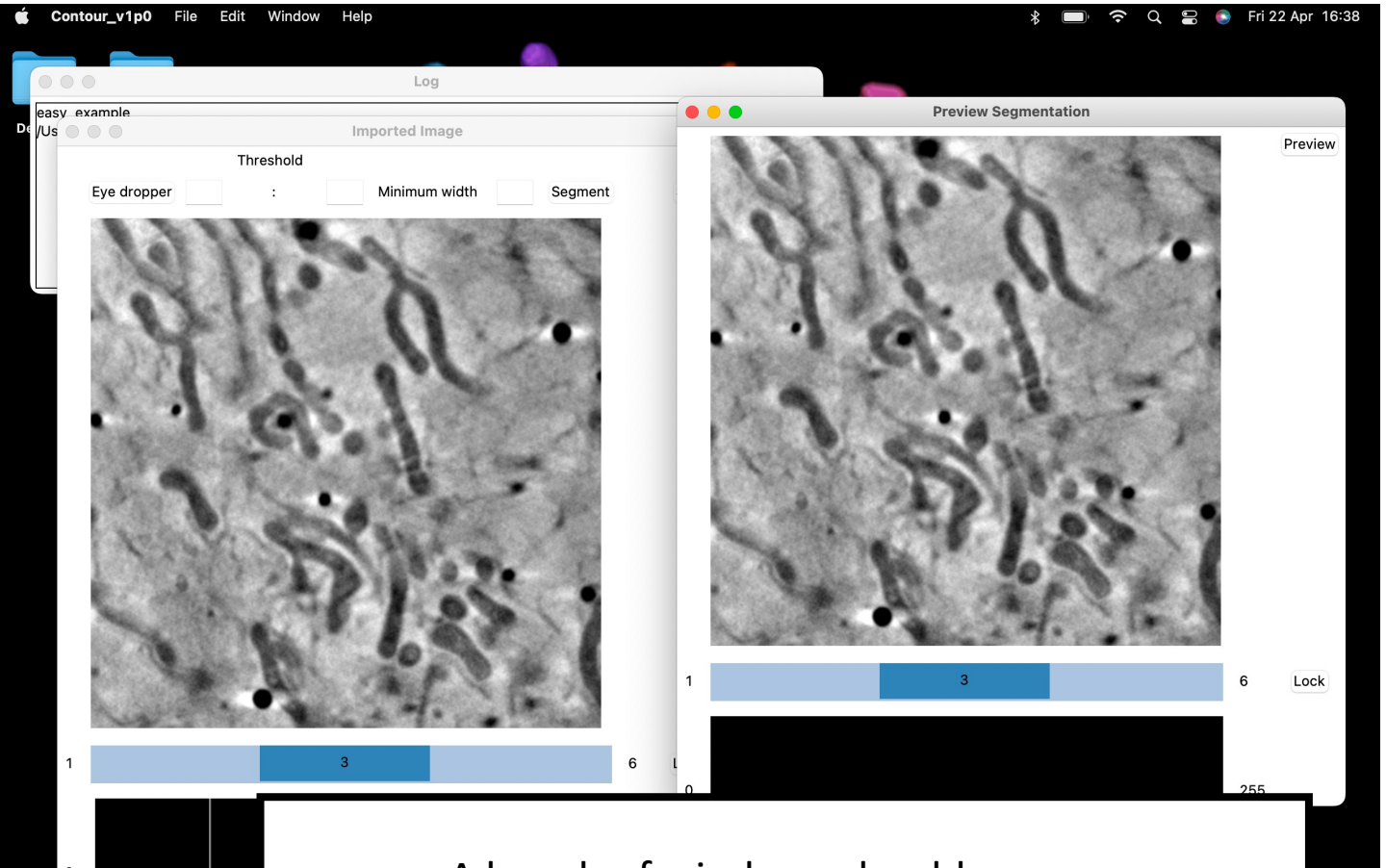
Download the file if you haven't already. Then change the workspace name.

Global segmentation

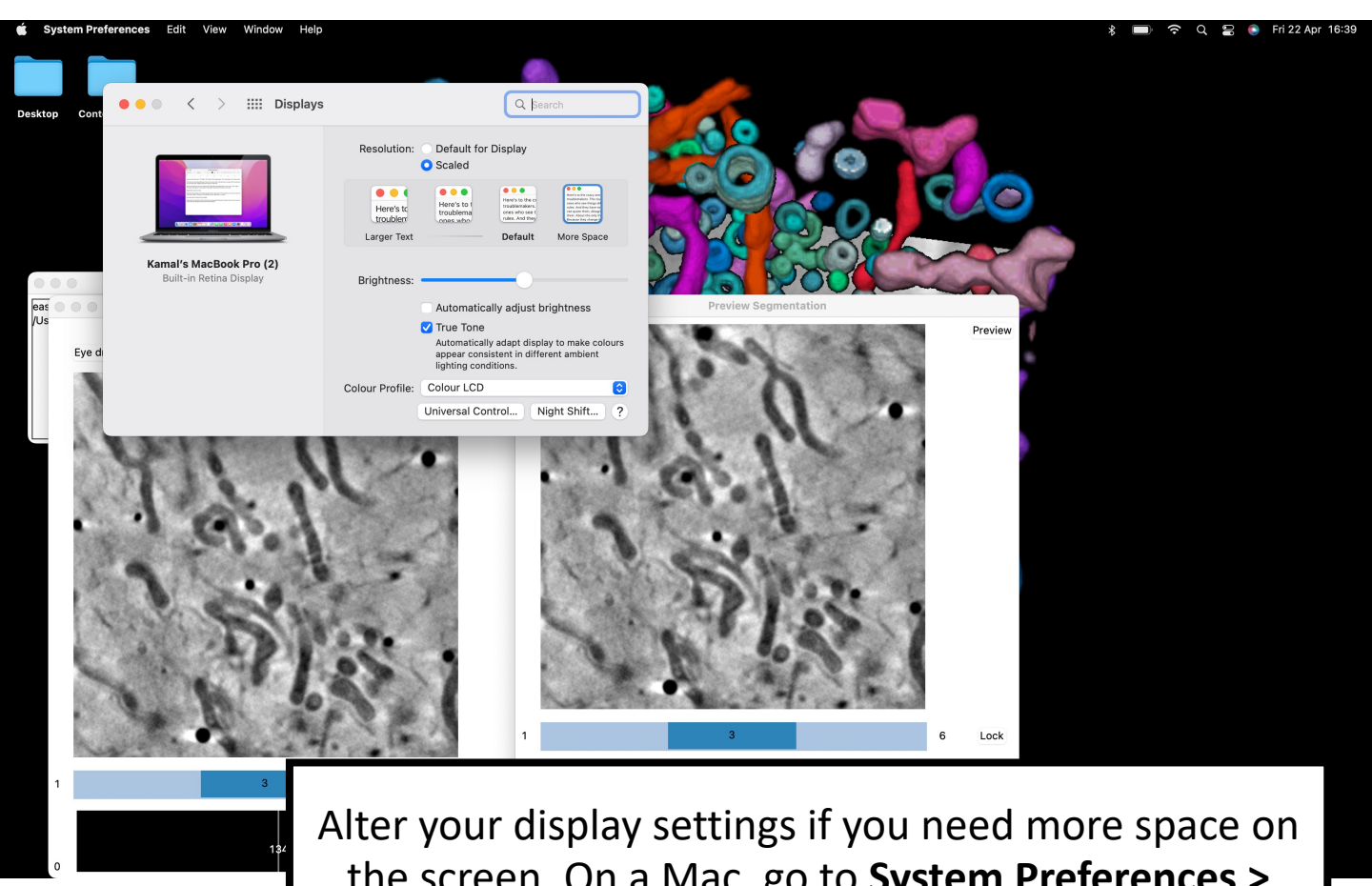
Now we're going to segment the whole tomogram in one go.



Click on New workspace, select the file, and open it.
The image must be an **8-bit tiff stack and not an mrc file (e.g. rec).**

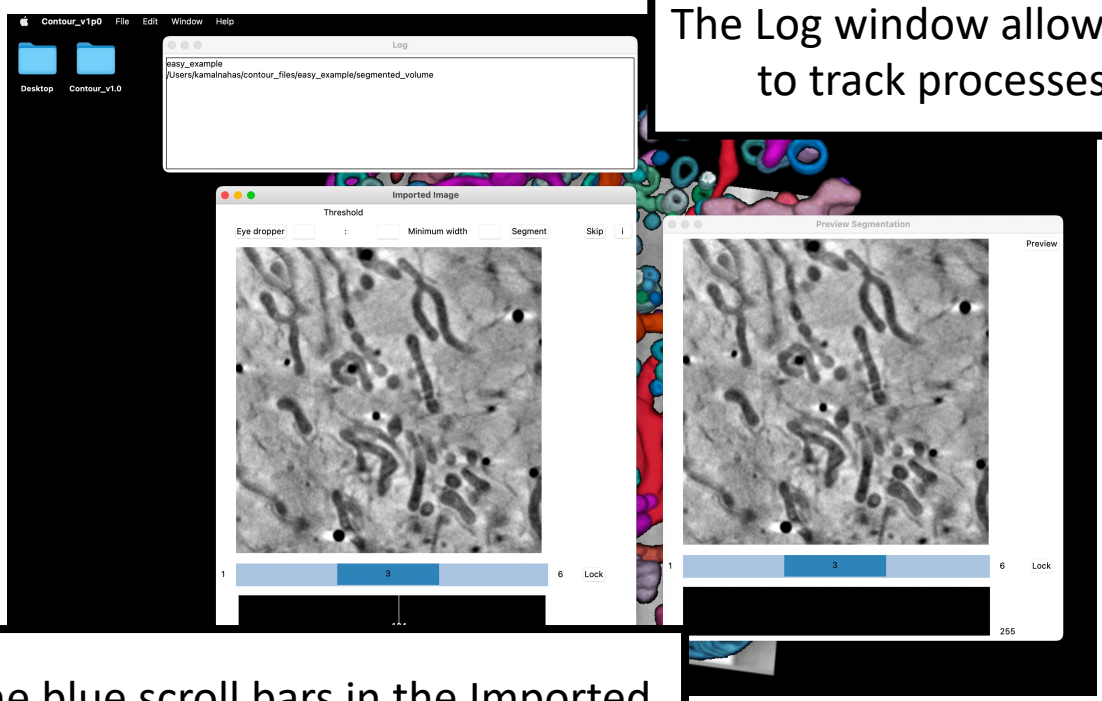


A bunch of windows should pop up.

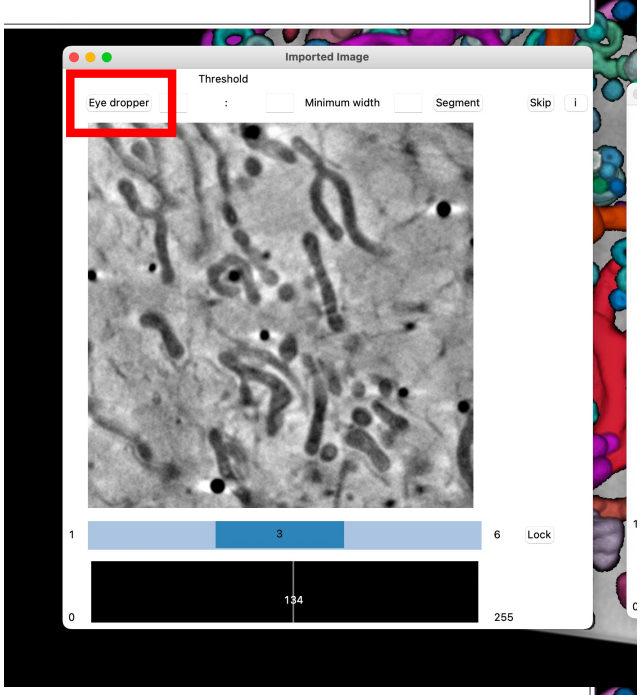


Alter your display settings if you need more space on the screen. On a Mac, go to **System Preferences > Displays > More Space**.

The Log window allows you to track processes.

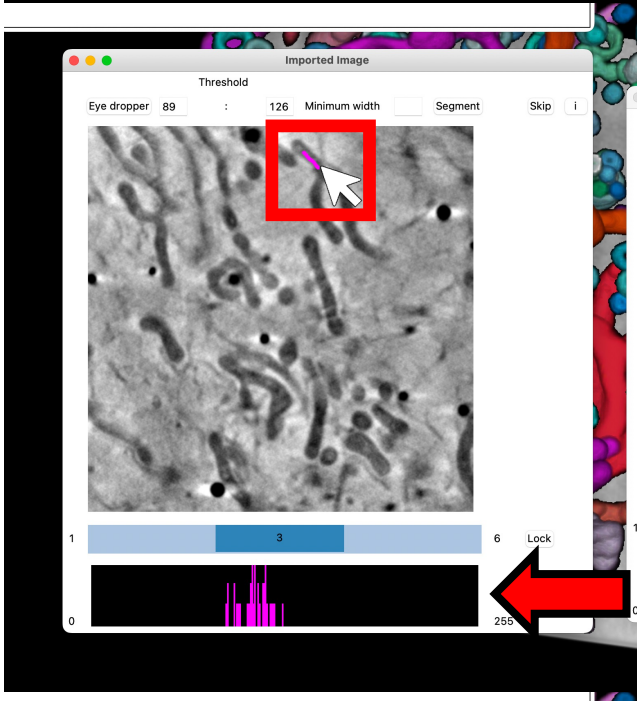


The blue scroll bars in the Imported Image window and Preview Segmentation window allow you to scroll through the Z stack.



We're going to segment the mitochondria based on their projection intensity.

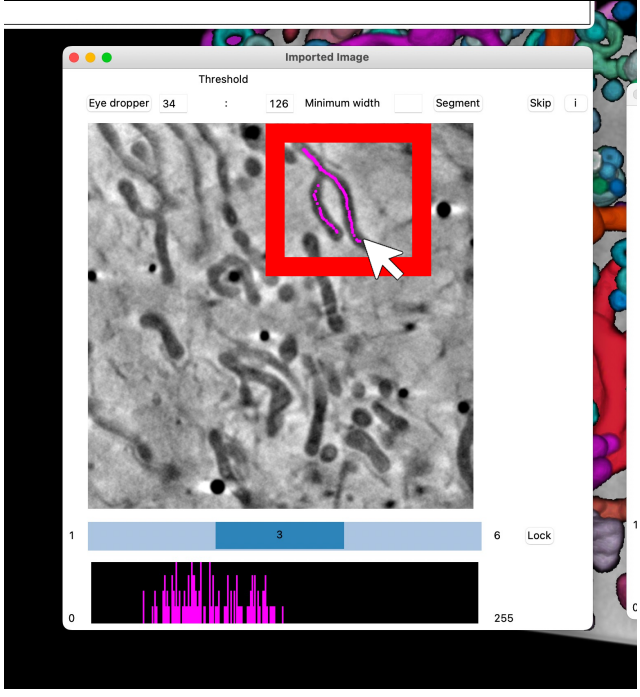
Click on **Eye dropper**.



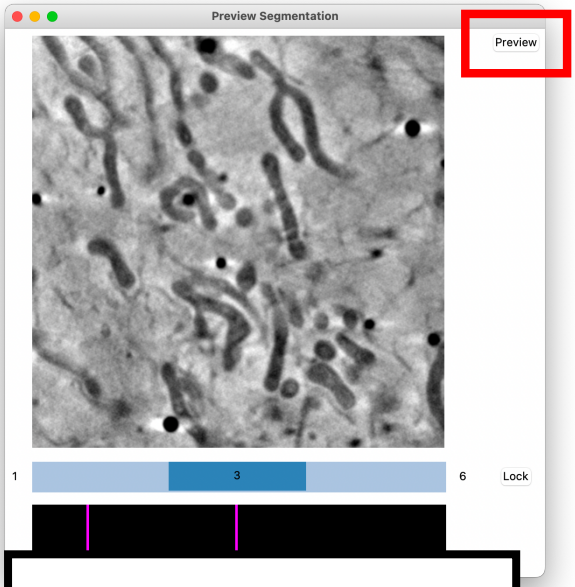
Now shade the inside of a mitochondrion – do not shade the edges.

Left click with the mouse and **hold down** to shade.

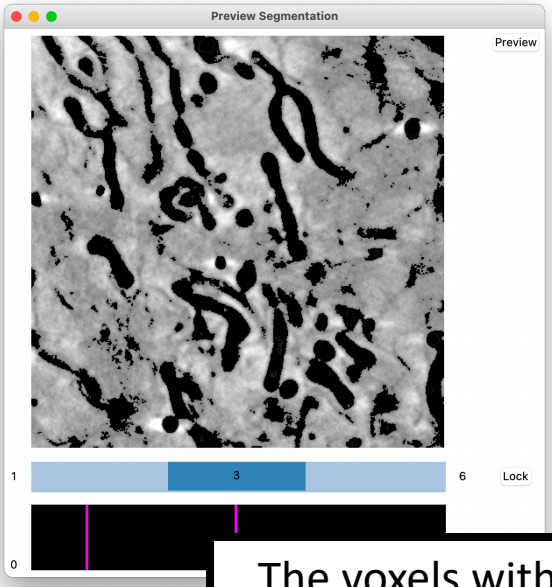
The intensity of the voxels being picked up by the eye dropper is displayed on a histogram at the bottom.



Shade in until you feel you've collected the full intensity range.

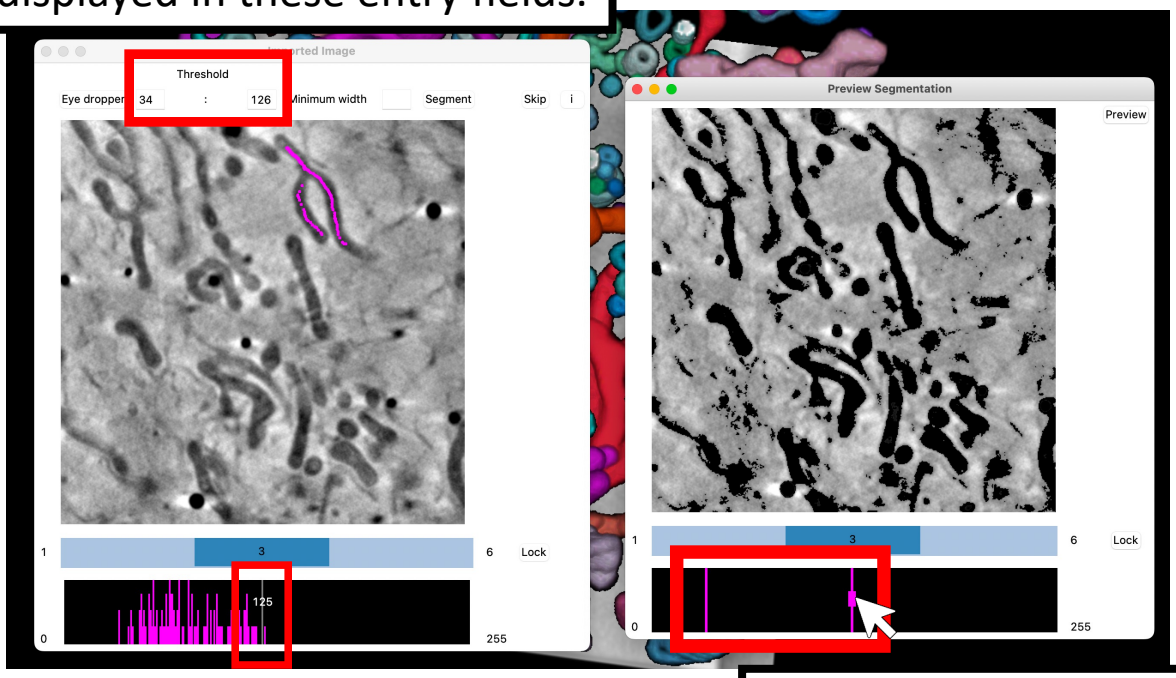


Click on **Preview** in the Preview Segmentation window.



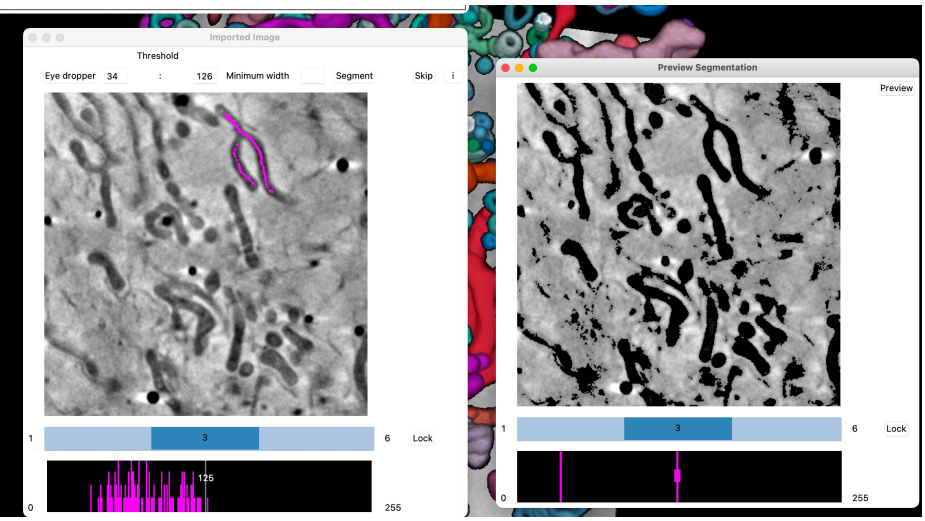
The voxels within the lower and upper limits of the threshold range will appear black.

The threshold range is displayed in these entry fields.



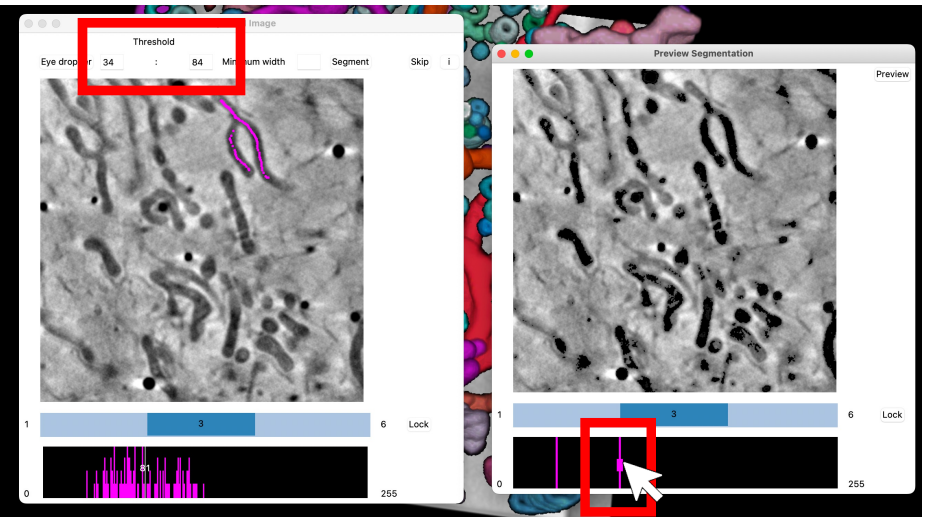
Hover over the pink lines in the Preview Segmentation window and you can see the intensity value in the Imported Image window

These pink lines show the threshold limits set by the eye dropper

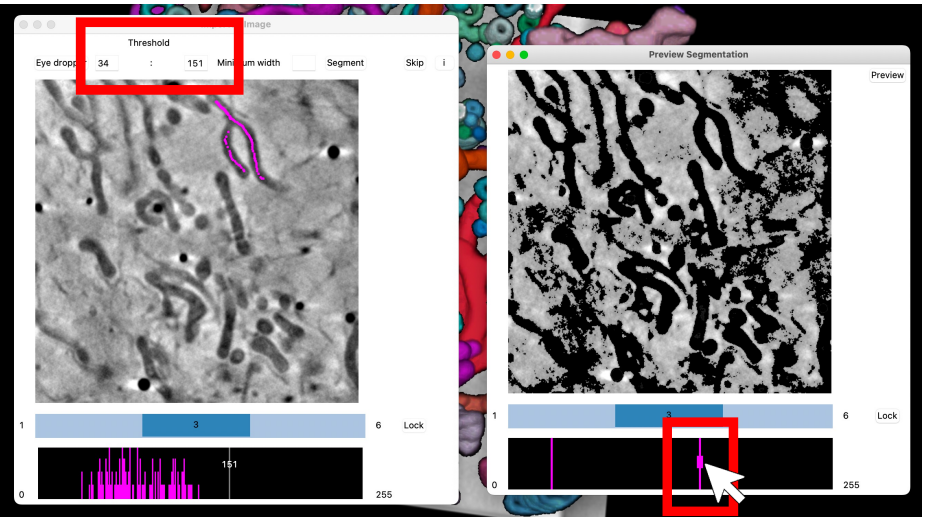


You can use the mouse to move the pink lines and alter the threshold range.

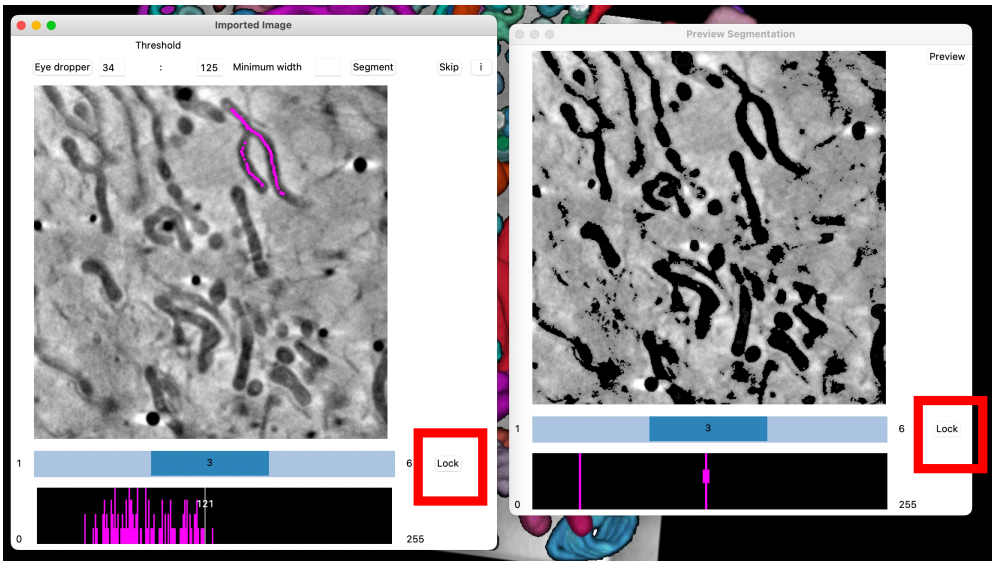
The threshold values readjust when you move the lines.



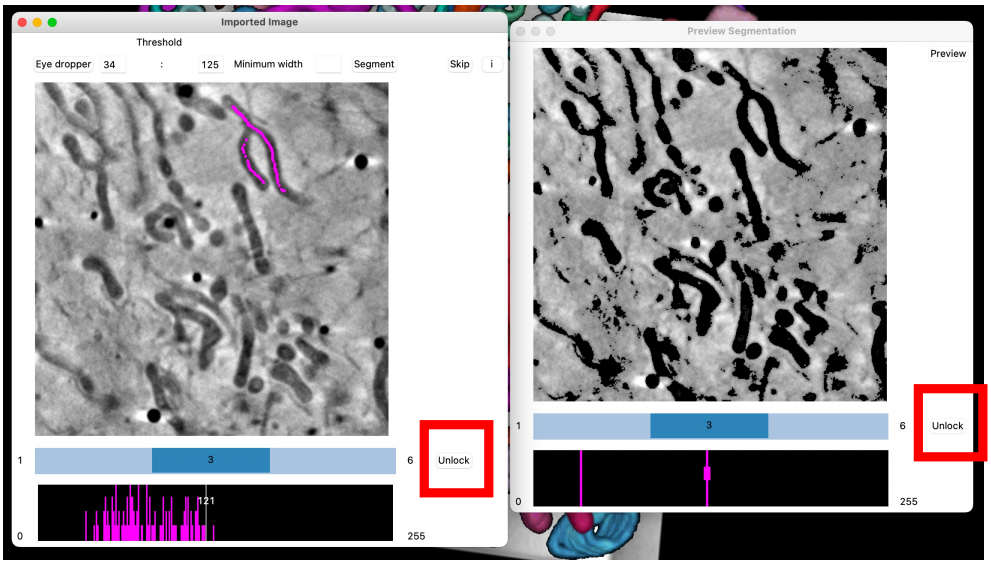
Here the upper line has been moved to the left, picking up a smaller range.



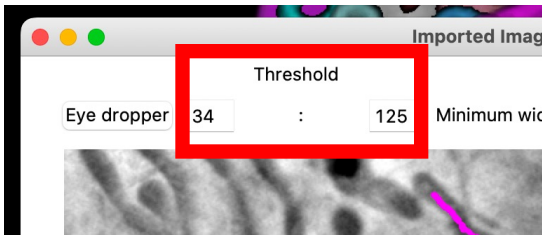
Here the upper line has been moved to the right, picking up a larger range.



If you click on the Lock button on either window, the z stacks will be synced. You can scroll through both at the same time with either blue scroller.

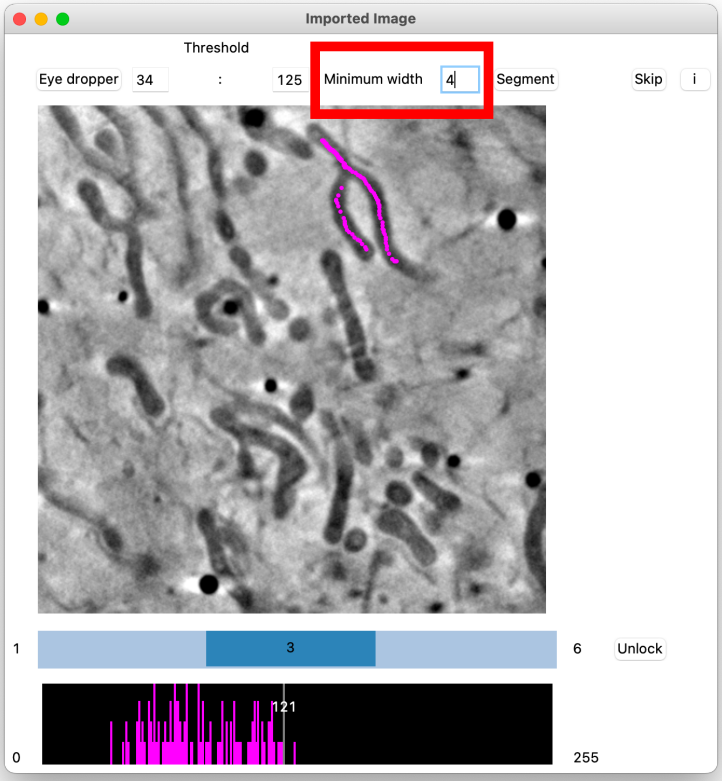


This is useful if you want to preview the segmentation in other slices and readjust the threshold range with the **Eye dropper**.



The values in the Threshold entry fields will readjust.

Whatever values are in these fields will be used in the segmentation. You can manually alter them if you wish.



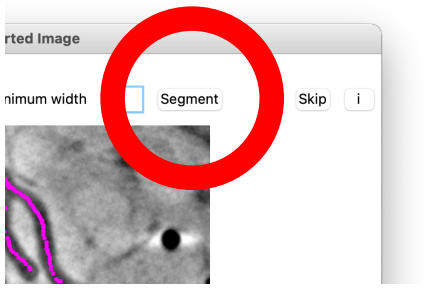
Once you're happy with the threshold range you've selected, move on to the next parameter: Minimum width.

Type in **4** for these mitochondria.

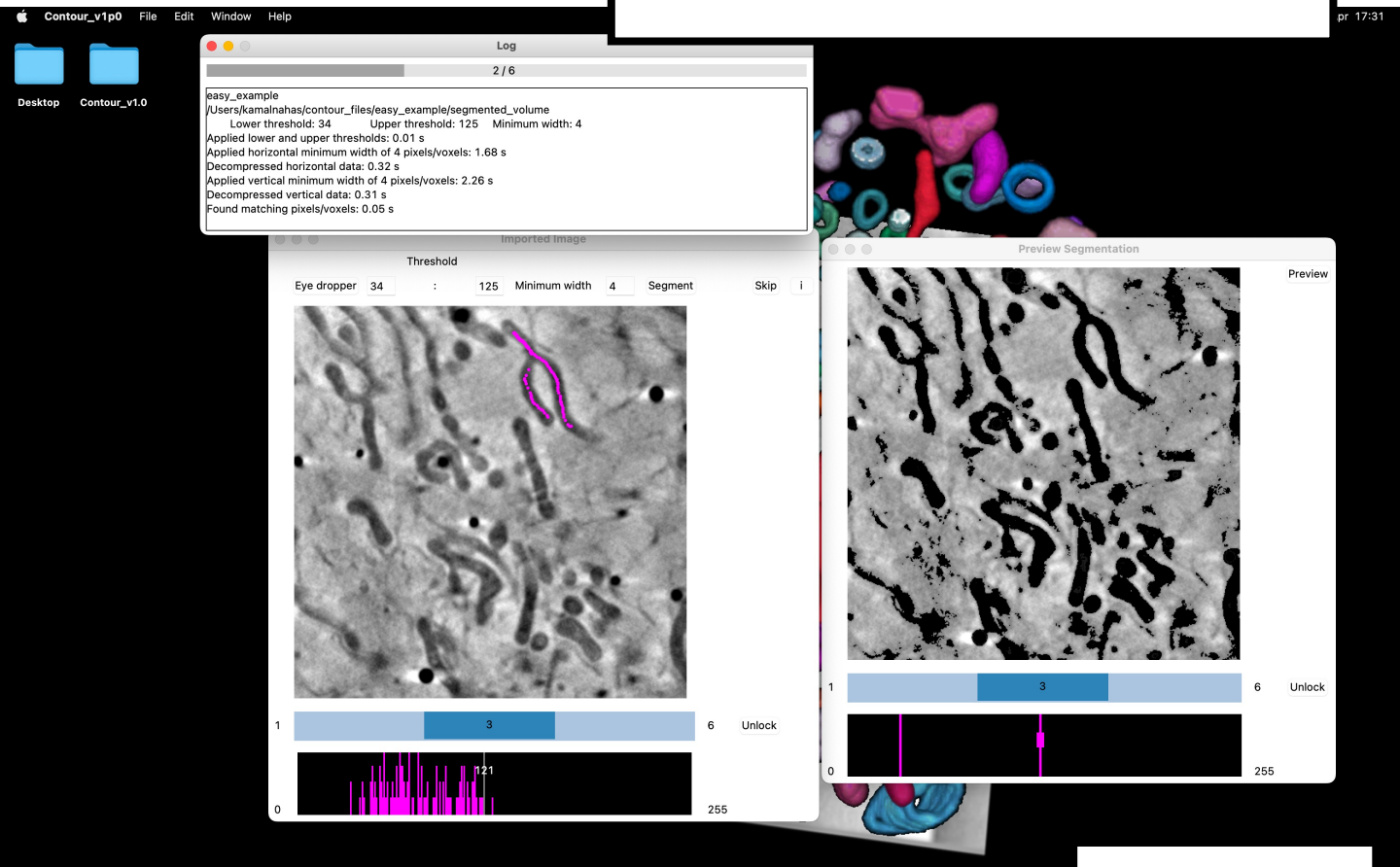
After the threshold range is used to segment the voxels, the minimum width will filter the data so that only 4X4 matrices will be included. This helps to reduce noise.

You don't have to use a minimum width. Try segmenting the lipid droplets later without a minimum width.

Click **Segment**

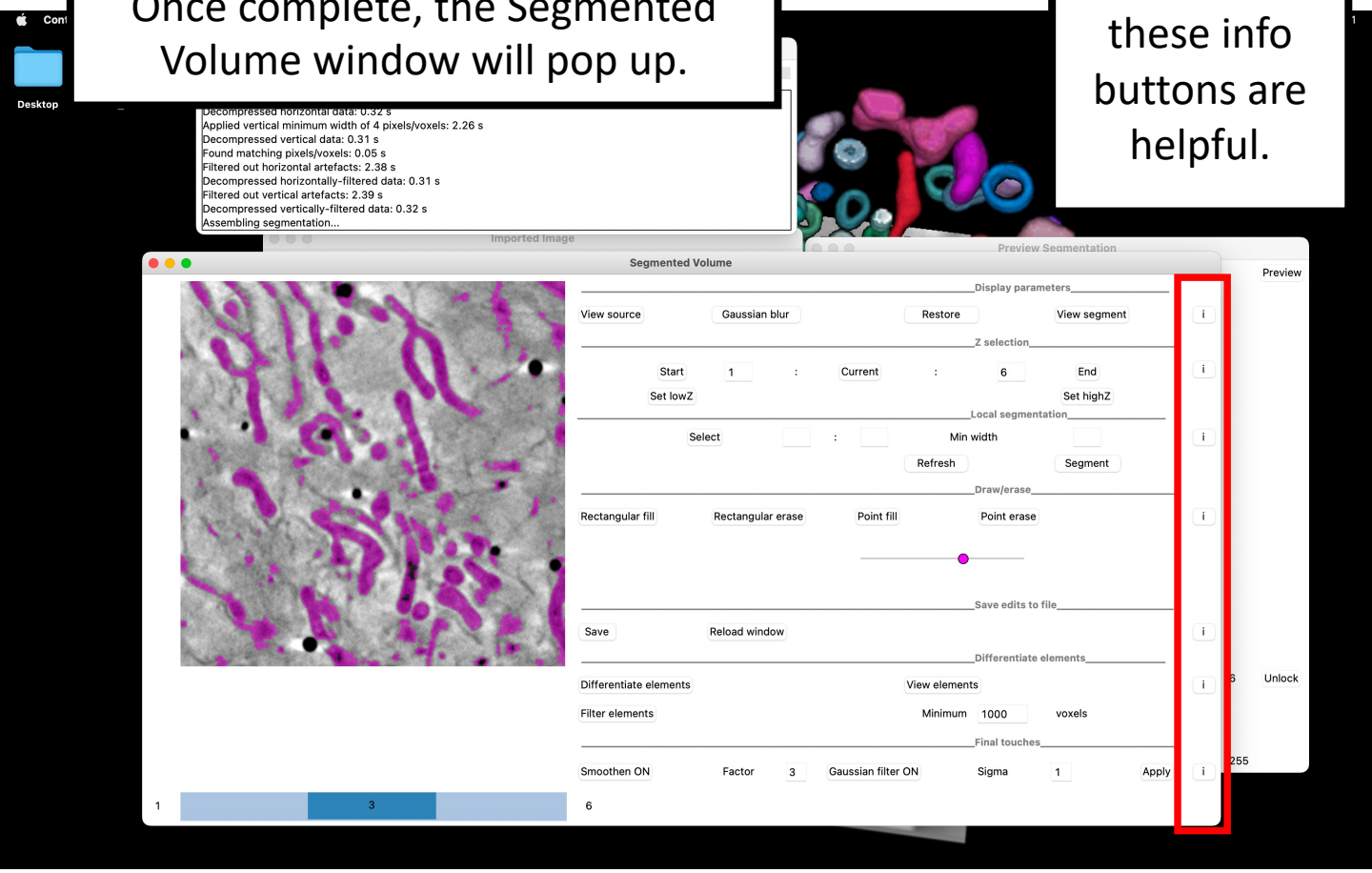


Check the status in the Log window.



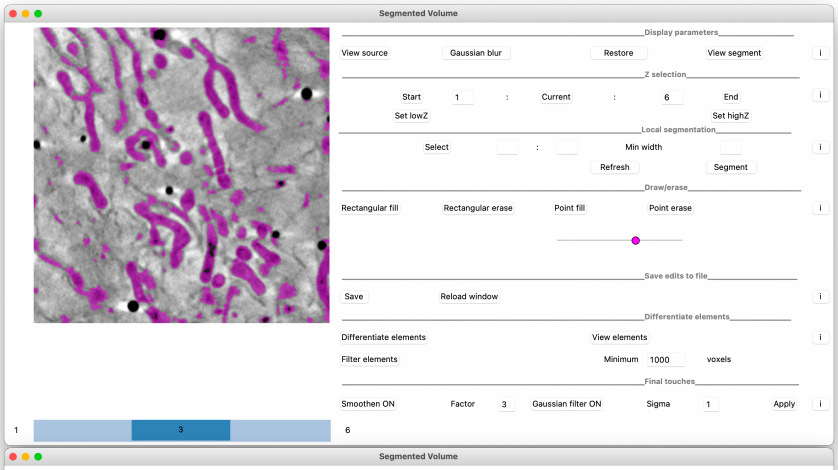
Once complete, the Segmented Volume window will pop up.

Reminder: these info buttons are helpful.

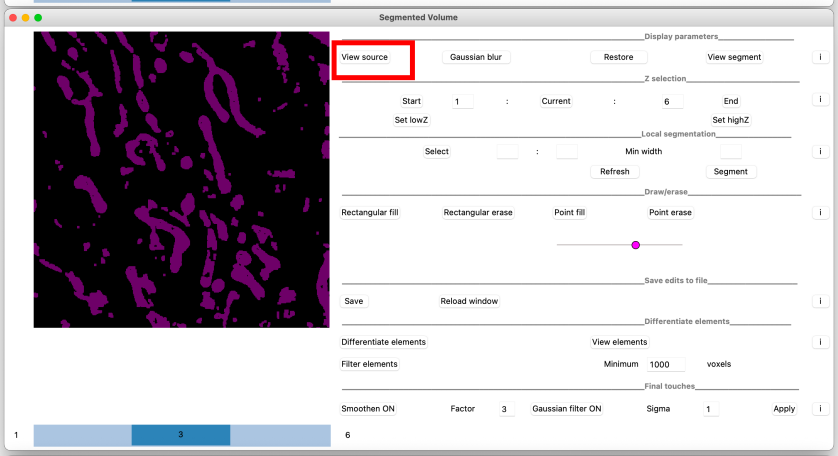


Local segmentations

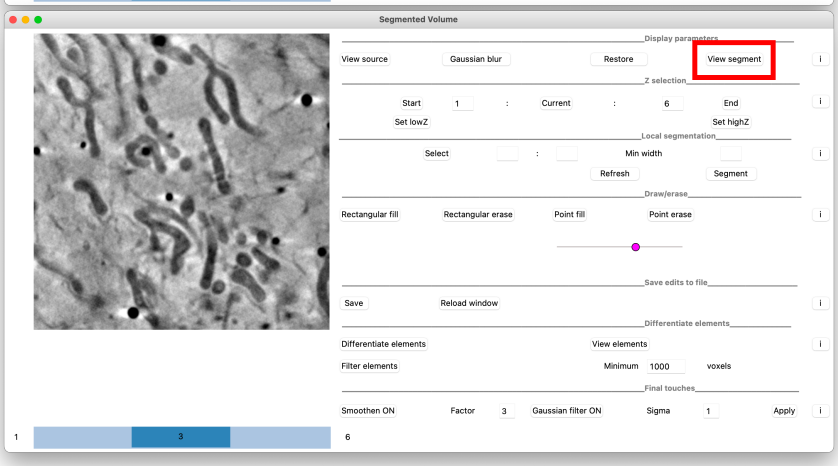
Now we're going to inspect the global segmentation and make corrections.

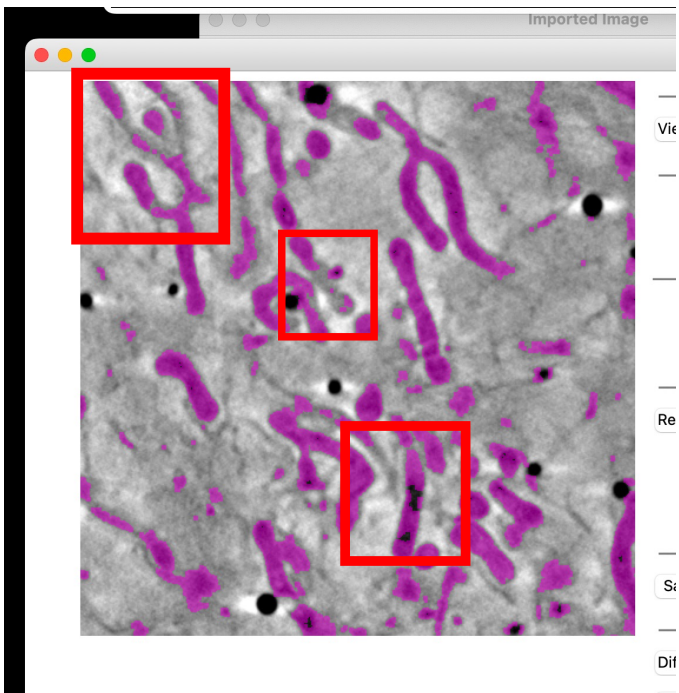


Click **View source** to toggle ON/OFF the display of the imported z stack.

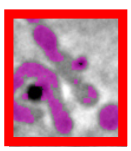


Click **View segment** to toggle ON/OFF the display of the segmented volume.

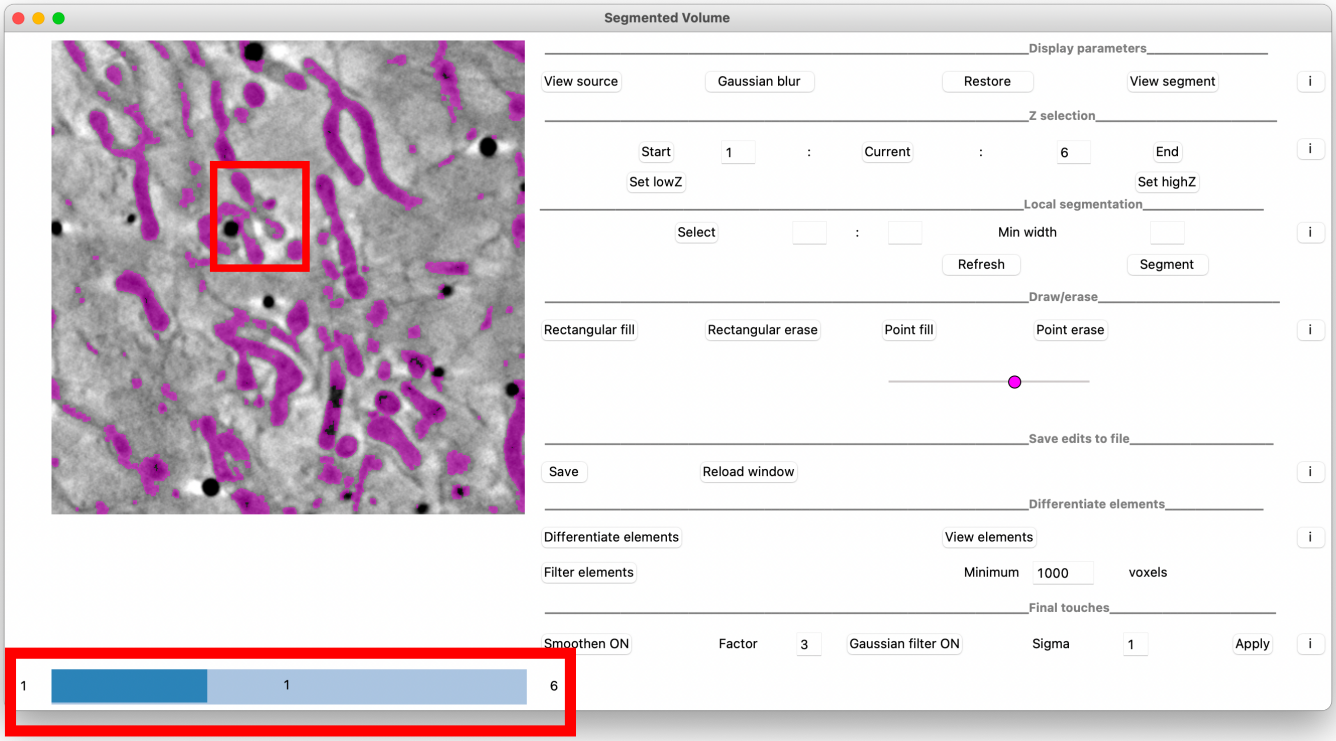




You can see that most of the mitochondria have been segmented but there are a few areas that have been missed.



Let’s fill in this area first.



First, use the scroll bar to find the Z limits of the feature you want to segment. In this case the limits are 1:6 (i.e. the whole Z stack) so we don't need to change any parameters.

If you did want to change the Z limits, you could do so with these tools.

Start resets the lower limit to slice 1.

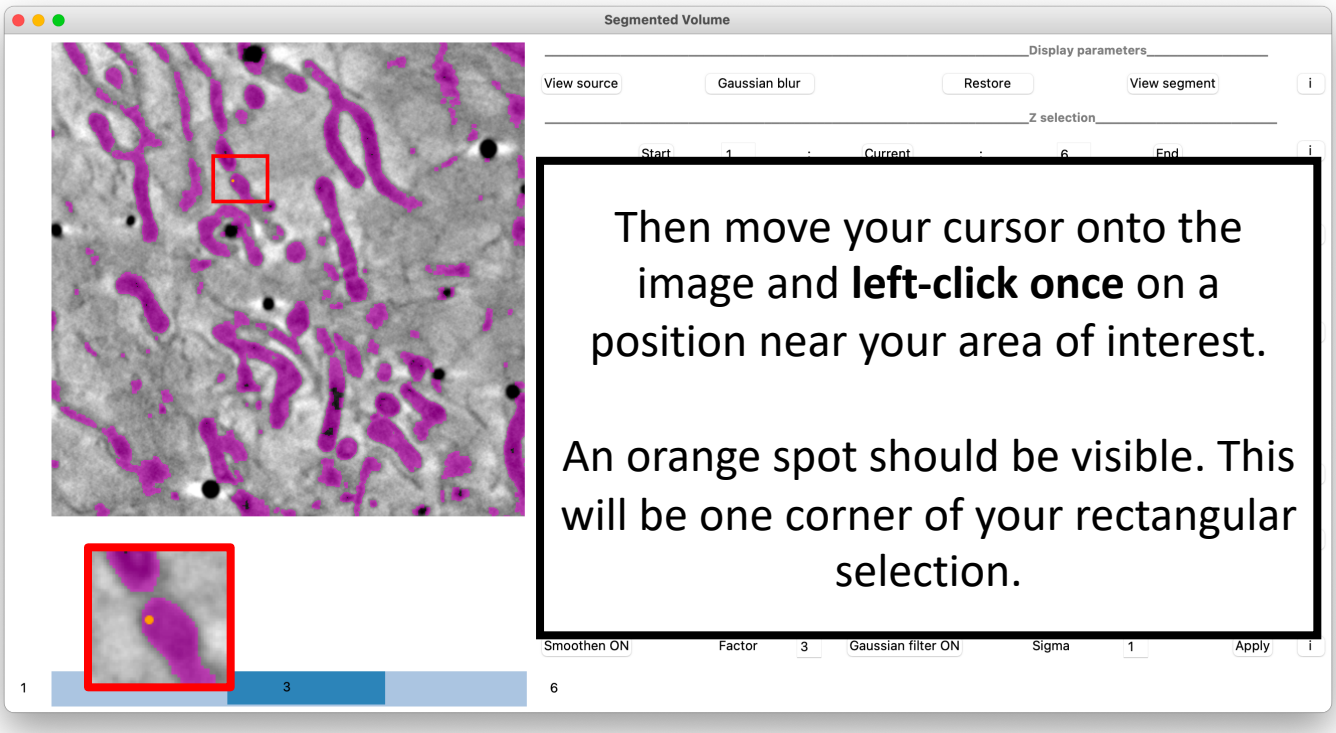
Current sets both limits to the currently-displayed slice.

End resets the upper limit to the last slice.

Set lowZ sets the lower limit to the currently-displayed slice.

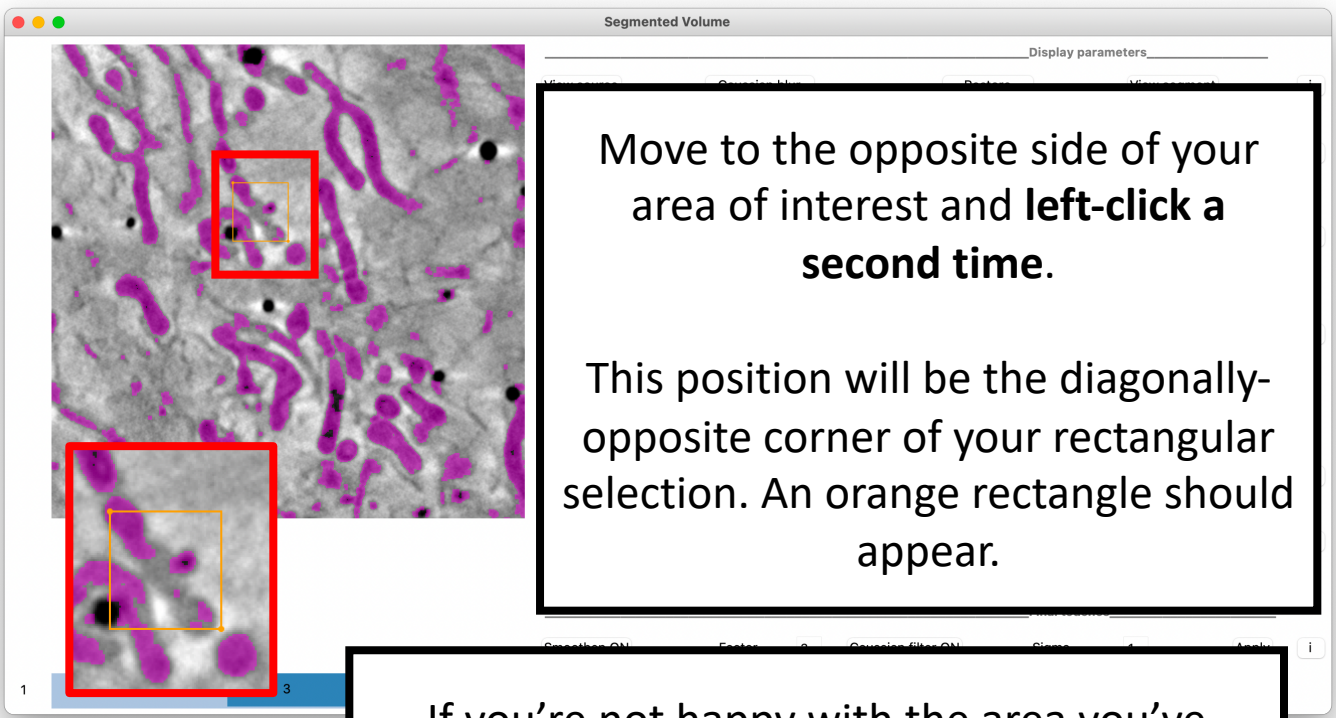
Set highZ sets the upper limit to the currently-displayed slice.

Now that you've selected your Z limits, you need to select the XY area in which you want to segment. Click **Select**.



Then move your cursor onto the image and **left-click once** on a position near your area of interest.

An orange spot should be visible. This will be one corner of your rectangular selection.

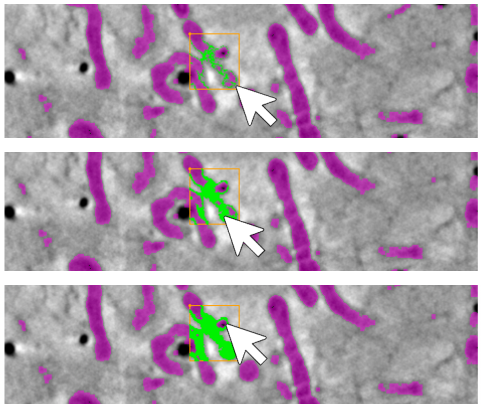


Move to the opposite side of your area of interest and **left-click a second time**.

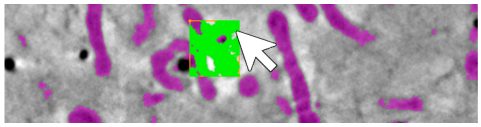
This position will be the diagonally-opposite corner of your rectangular selection. An orange rectangle should appear.

If you're not happy with the area you've selected. Click **Select** and try again.

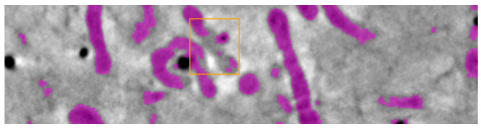
Your cursor is now an eye dropper. **Press down** on the image to pick up the threshold range. The entry fields will readjust simultaneously.

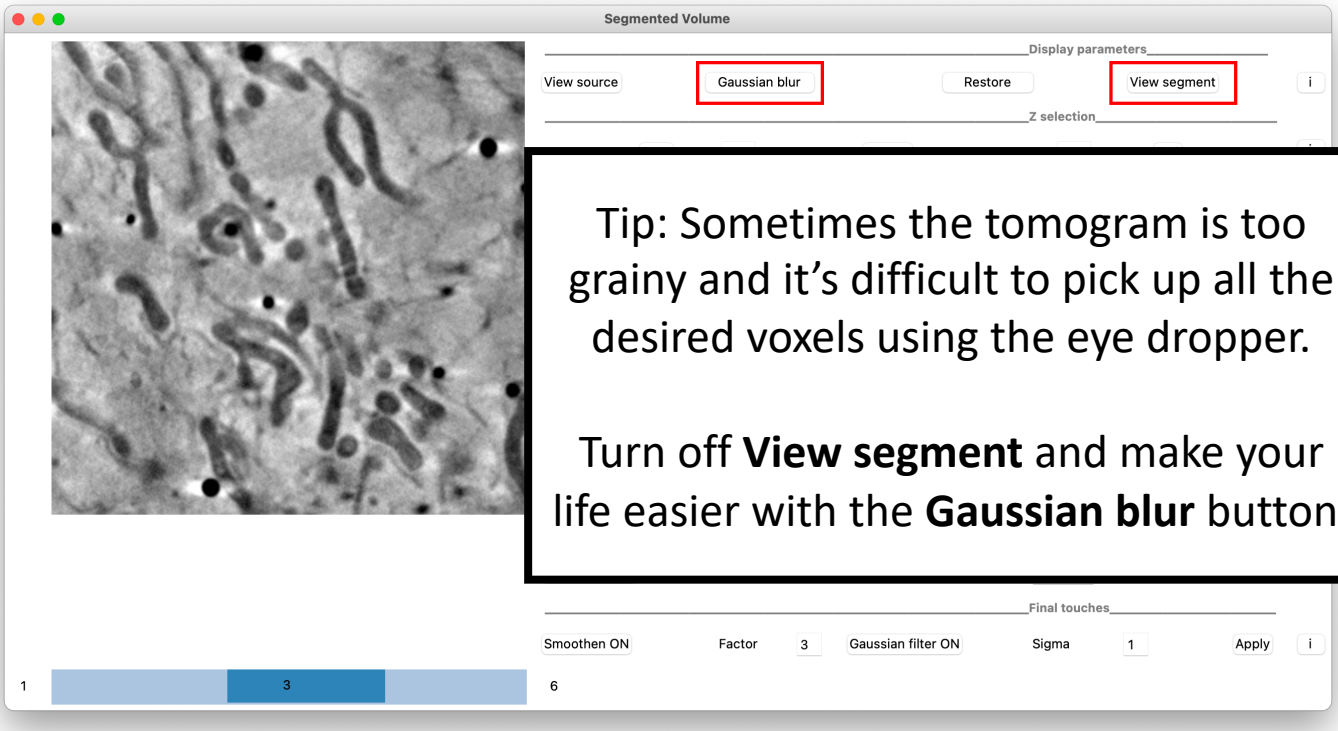


Try to pick up all the voxels of the mitochondria.



Oops! If you overshoot and pick up areas outside the mitochondrion, just click **Refresh** and reattempt the eye dropping.

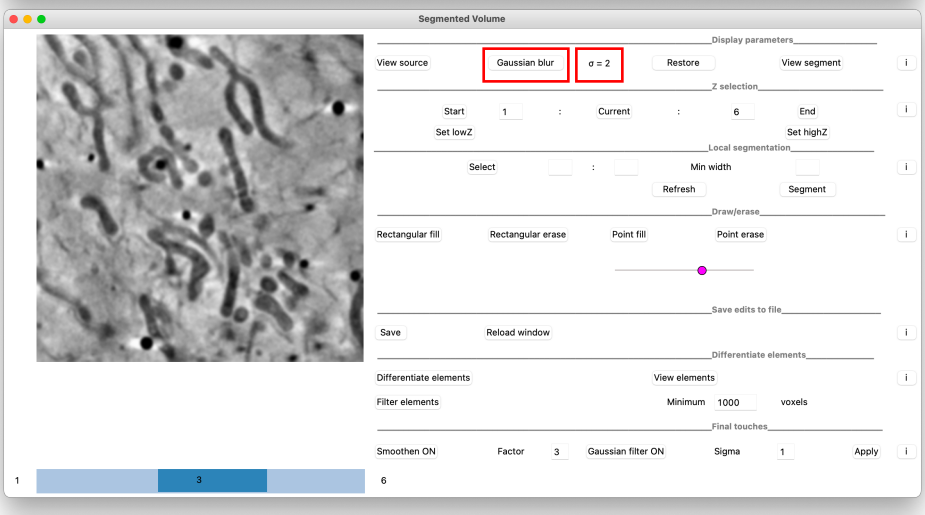




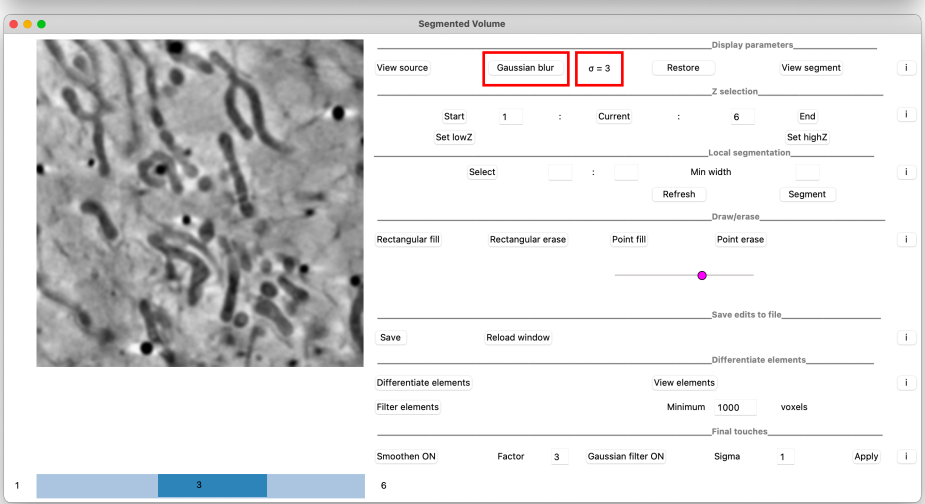
Tip: Sometimes the tomogram is too grainy and it's difficult to pick up all the desired voxels using the eye dropper.

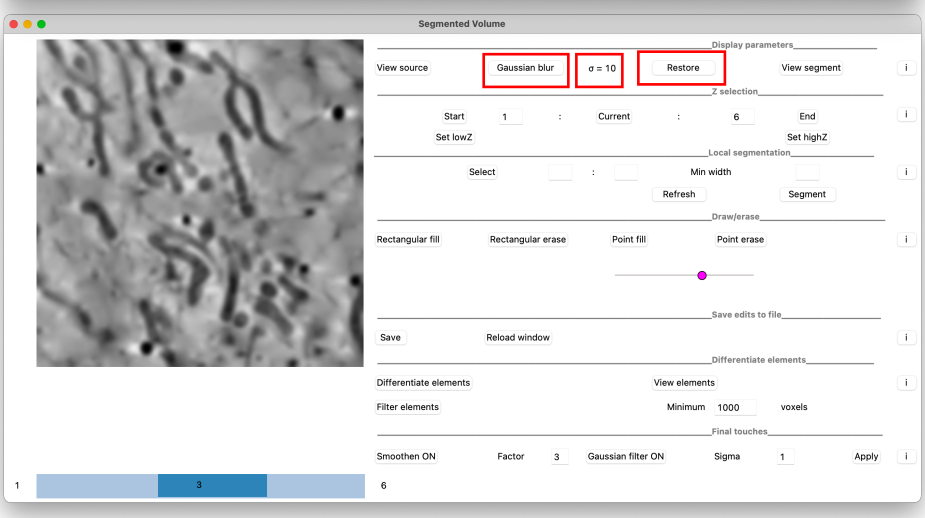
Turn off **View segment** and make your life easier with the **Gaussian blur** button.

Each time you click **Gaussian blur**, the source image will be blurred by one standard deviation.

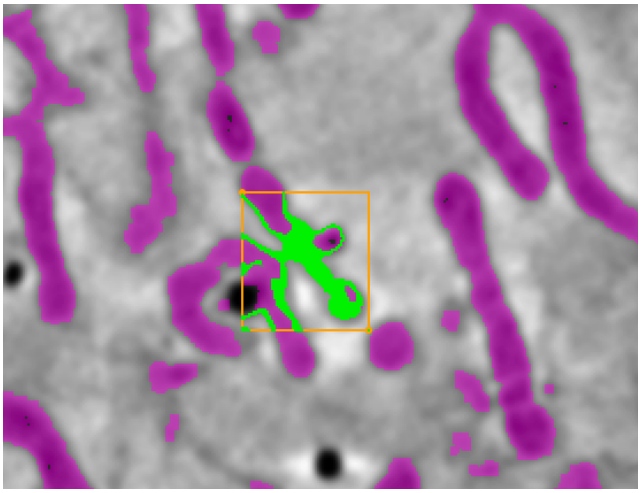


A σ of 1 or 2 is recommended.

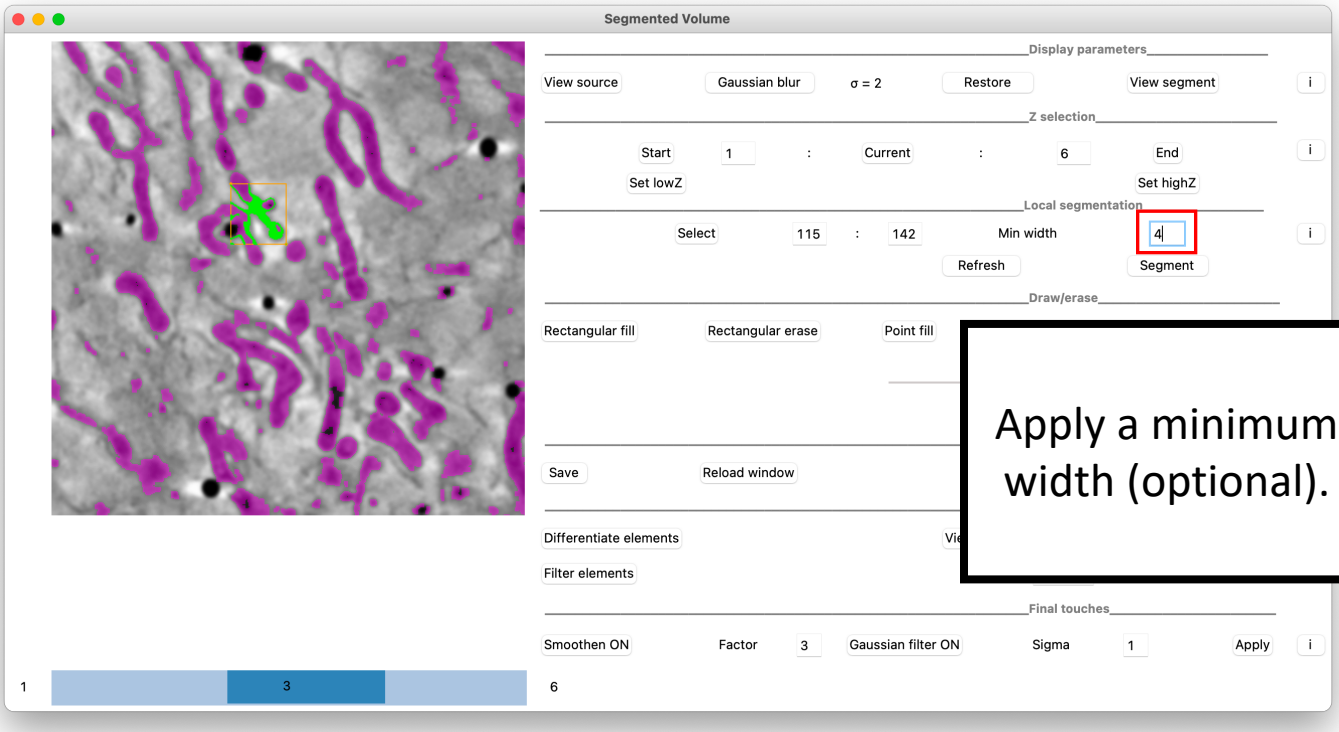




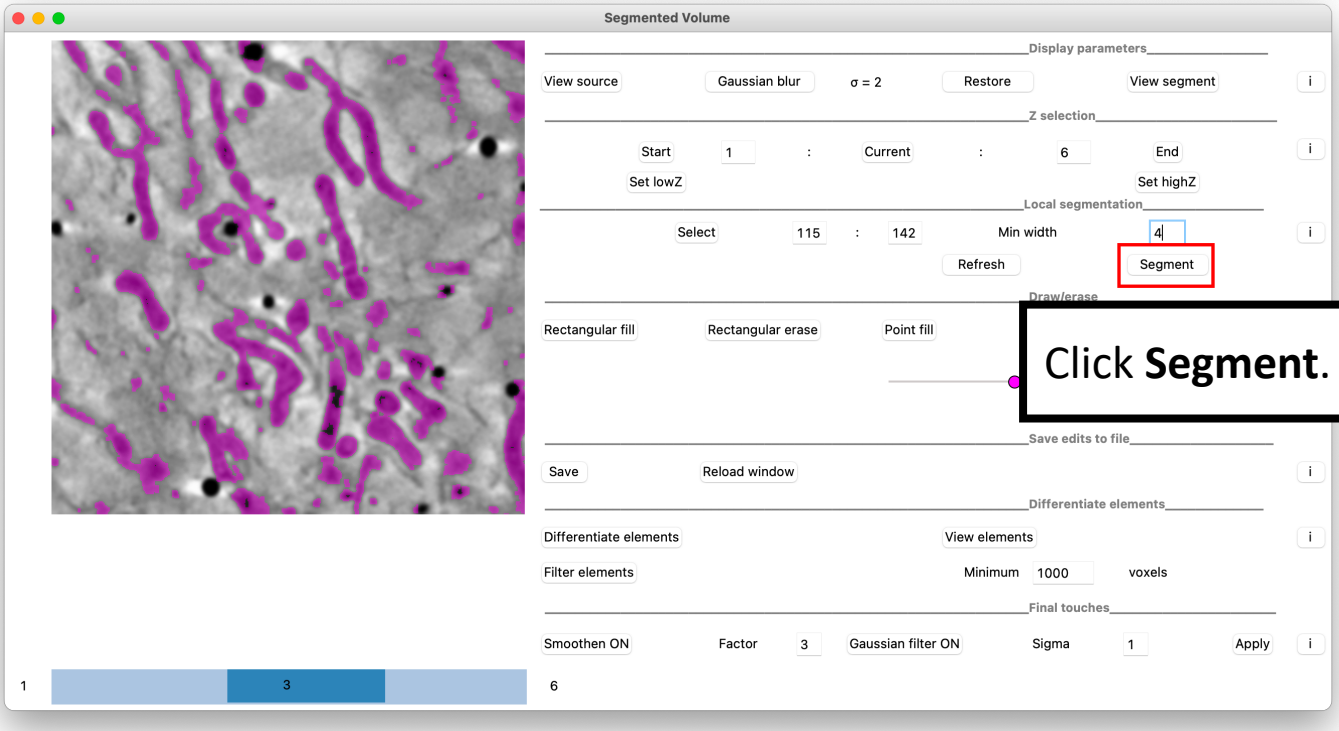
You can restore the original image with the **Restore** button.



Try using the eye dropper cursor after applying a Gaussian blur. It will be easier to pick up the threshold range.



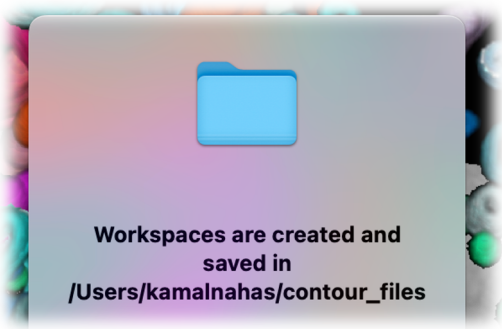
Apply a minimum width (optional).



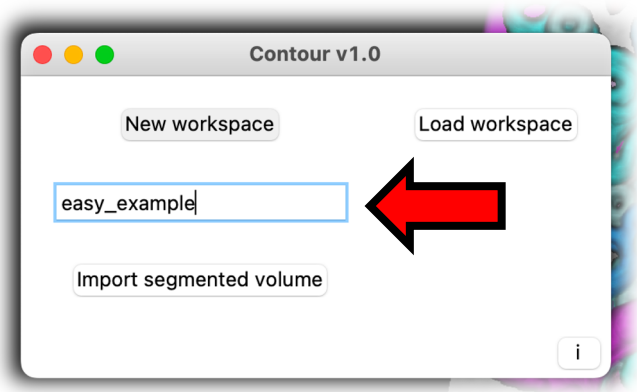
Click **Segment**.

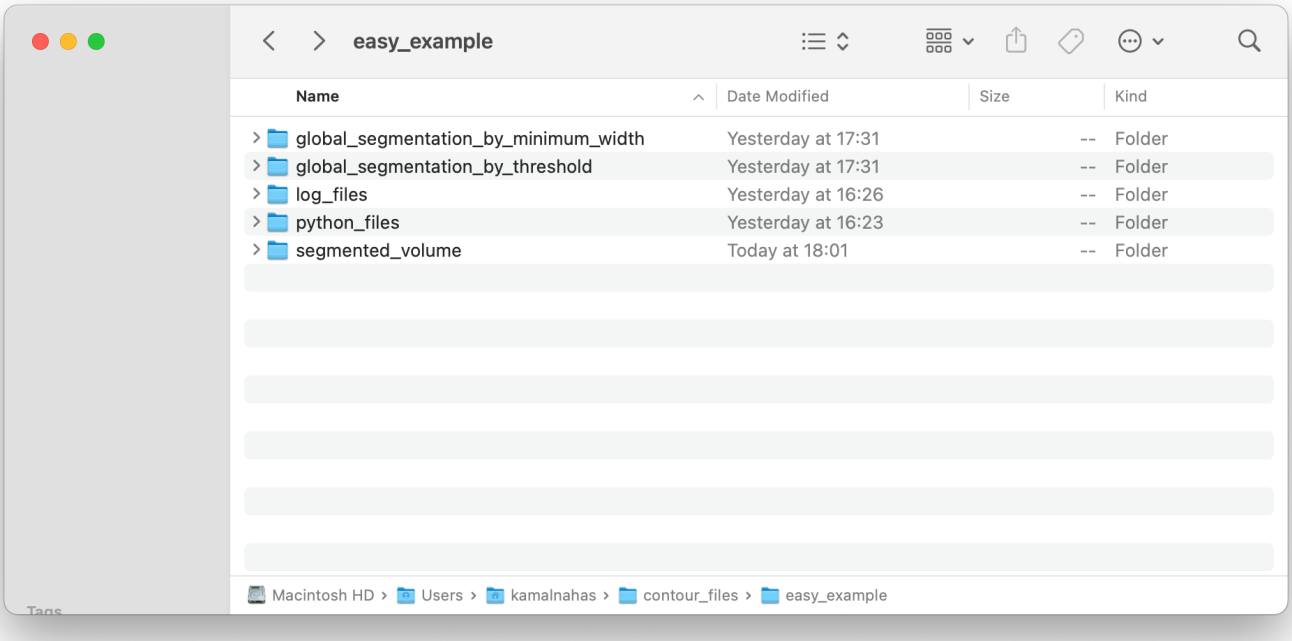
Fill in the remaining gaps and press **Save** frequently.

Navigate to the folder containing your output files. You should have noted it down at the start. If not, search for the folder **contour_files** on your computer.



It should contain a subfolder with the workspace name you created.

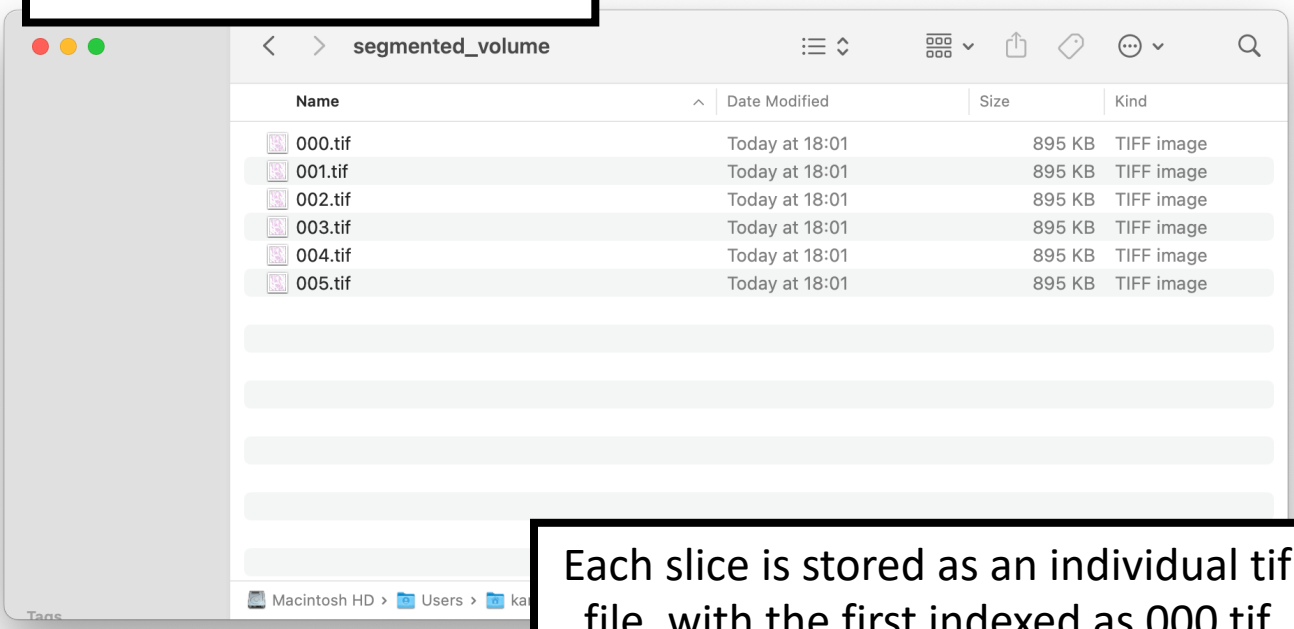




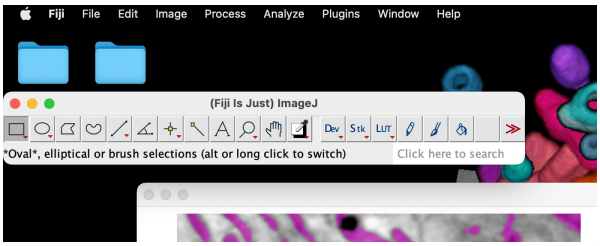
Open the folder to find these files.

global_segmentation_by_minimum_width	contains the global segmentation you ran initially (i.e. with the thresholding and minimum width parameters but without the local segmentations).
global_segmentation_by_threshold	contains the global segmentation you ran initially but with only the thresholding applied.
log_files	contains the log files of all processes. A new log file is created each time a workspace is reopened. This contains parameters such as the threshold range and minimum width.
python_files	contains files used by the program. Don't touch them.
segmented_volume	Contains the up-to-date segmentation.

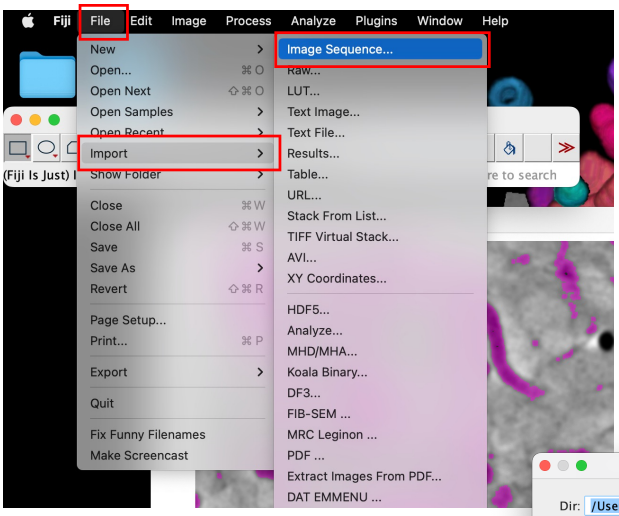
Open **segmented_volume.**



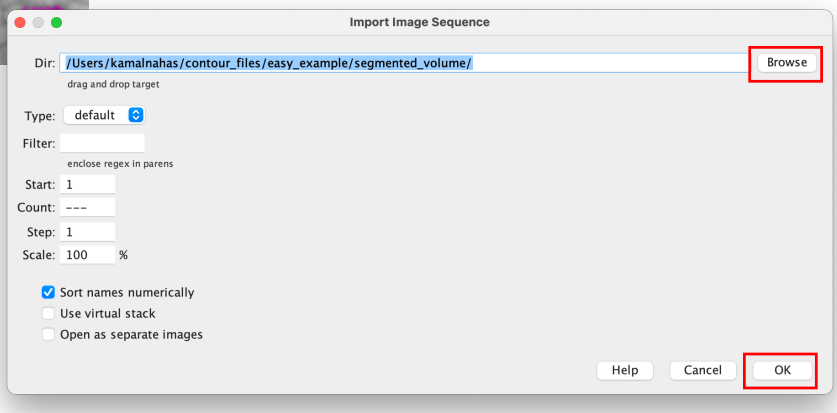
Each slice is stored as an individual tif file, with the first indexed as 000.tif



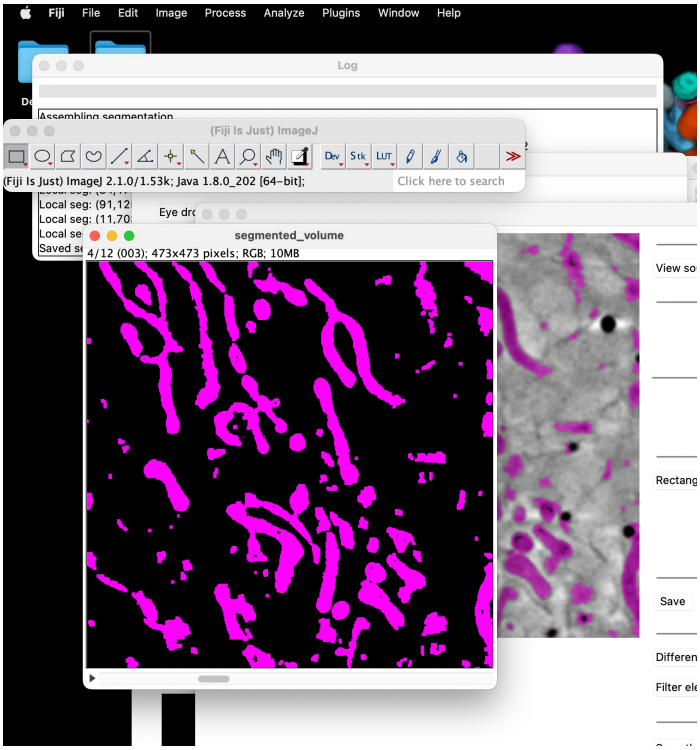
Open Fiji to view the slices together in a single stack.



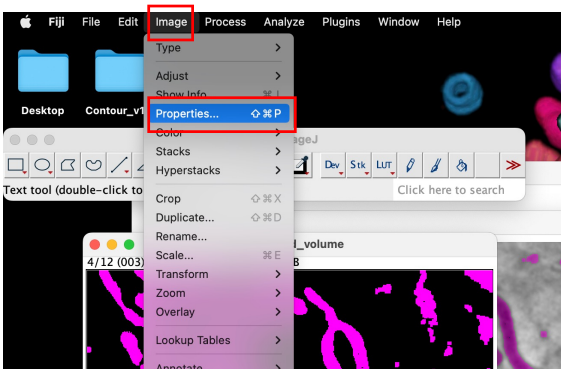
Go to **File > Import > Image Sequence...**



Then browse for the **segmented_volume** folder and click **OK**.

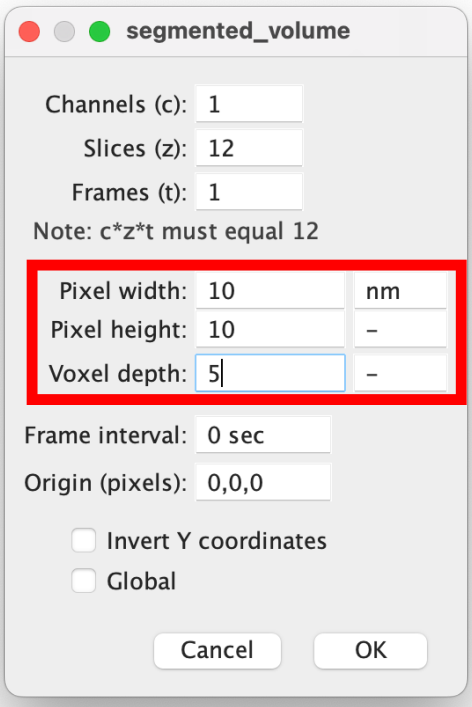


This will compile all the individual tif slices into one stack.



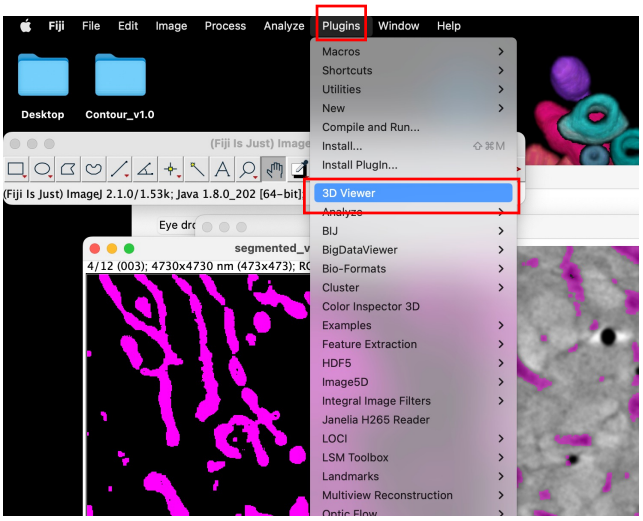
You can view the stack as a 3D volumetric rendering. First, we need to adjust the scale of the image.

Go to **Image > Properties…**

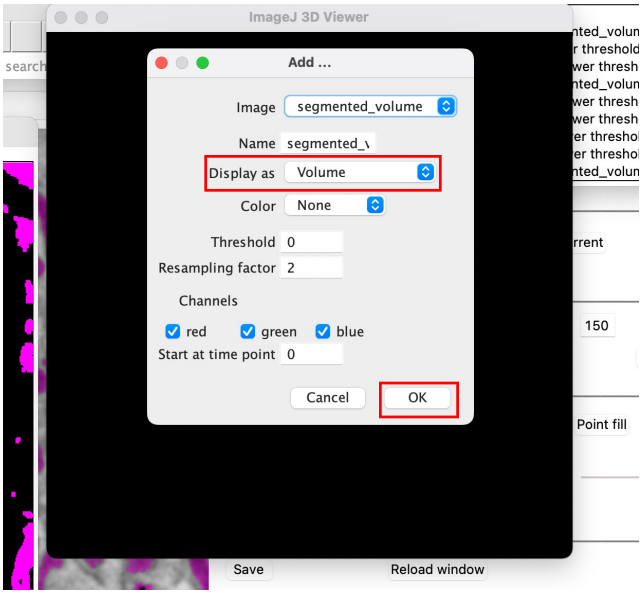


This tomogram was collected with a 9.46 × 9.46 µm field of view, with each voxel being 10 × 10 × 10 nm. Therefore the slices have dimensions of 946 × 946 voxels. However, this stack has been downsized by a factor of 2 and has the dimensions 473 × 473 voxels. To maintain the proportions between XY and Z, you need to downsize the Z scale by 2 as well –from 10 nm to 5nm).

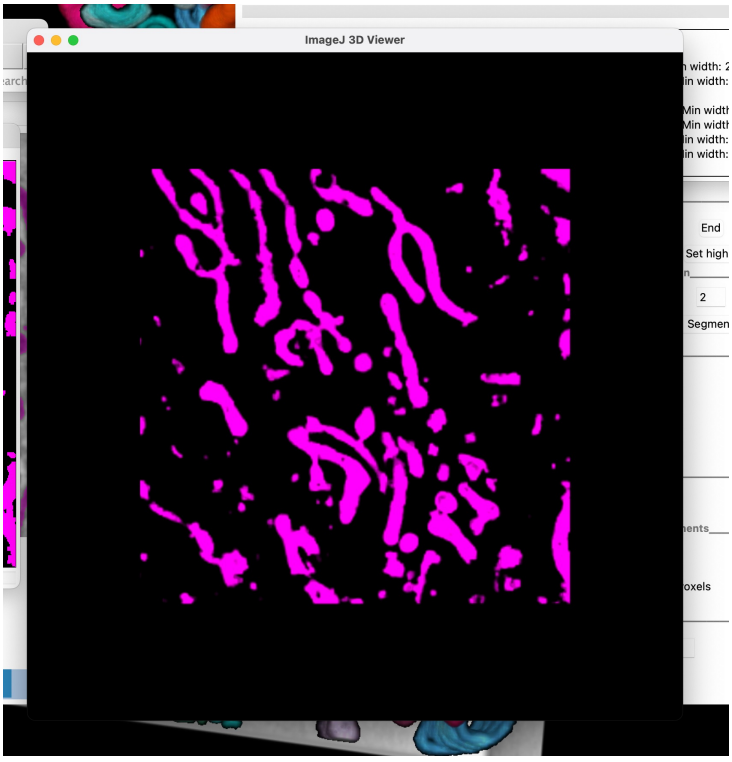
Fill in the dimensions as shown.



After you've adjusted the scale, go to **Plugins > 3D Viewer**.

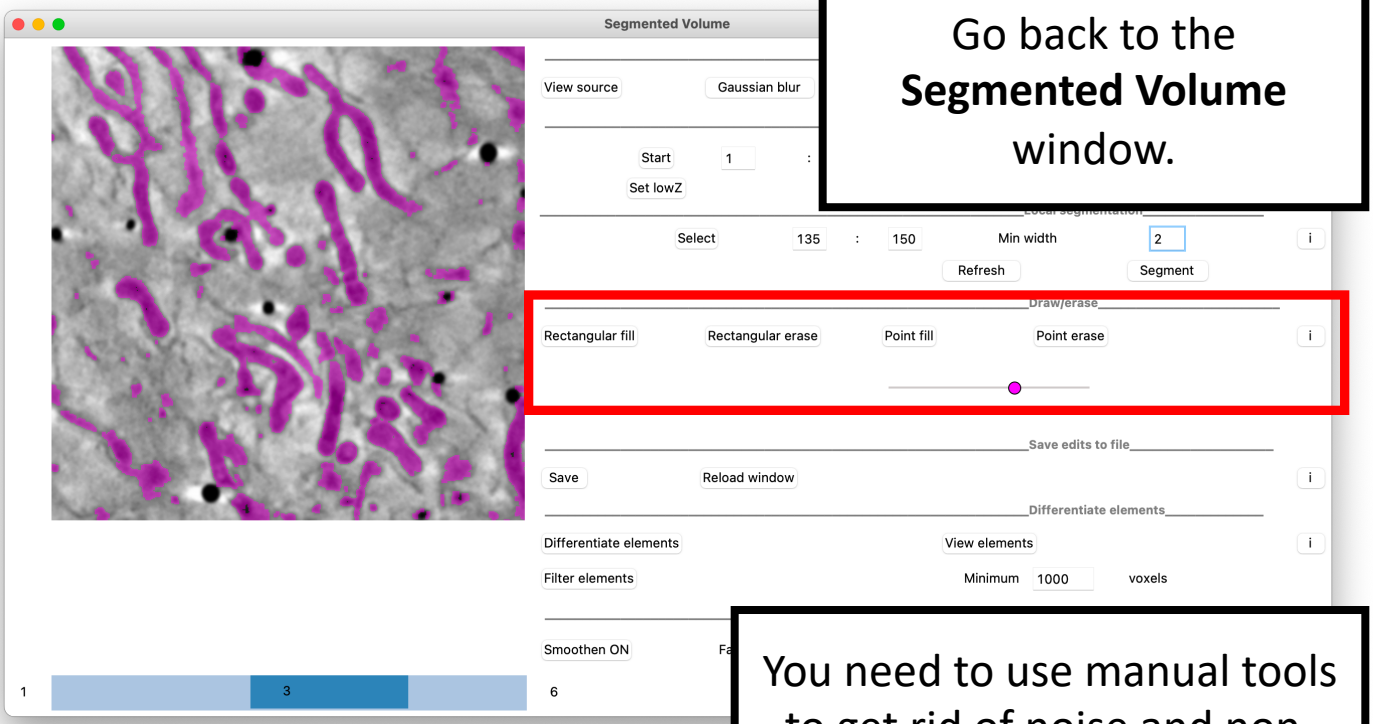


Make sure **Volume** is the display option and click **OK**.



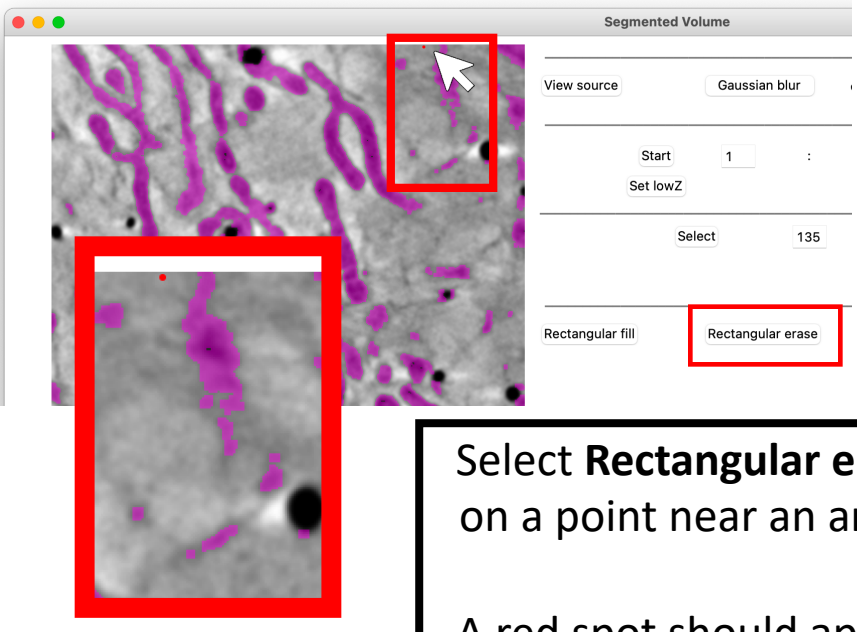
You can now view a 3D rendering of the segmentation and you can drag with your cursor to rotate the volume.

Although the mitochondria are complete, there is still a bit of noise in the segmented volume. Let's get rid of the noise/non-specific elements.



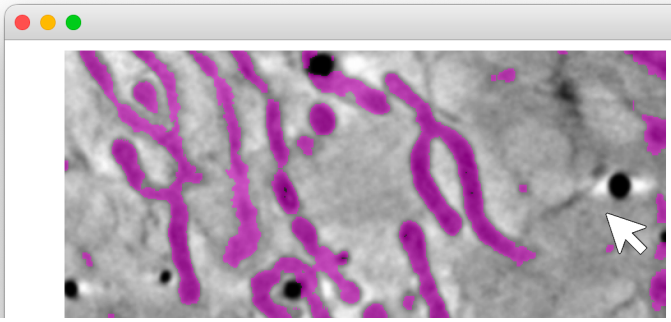
Go back to the **Segmented Volume** window.

You need to use manual tools to get rid of noise and non-specific material.



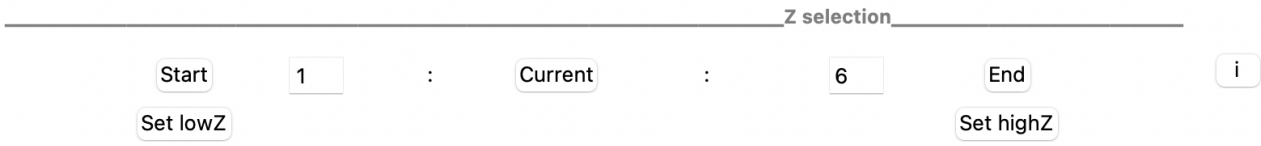
Select **Rectangular erase** and **left-click once** on a point near an area you want to delete.

A red spot should appear and will mark one corner of your rectangular selection.

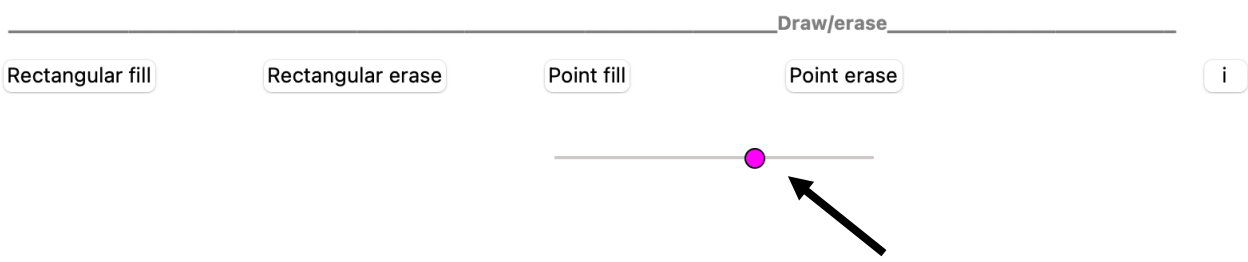


Left click a second time to mark the diagonally opposite point of your rectangular selection. This will delete the segmented elements within the rectangle.

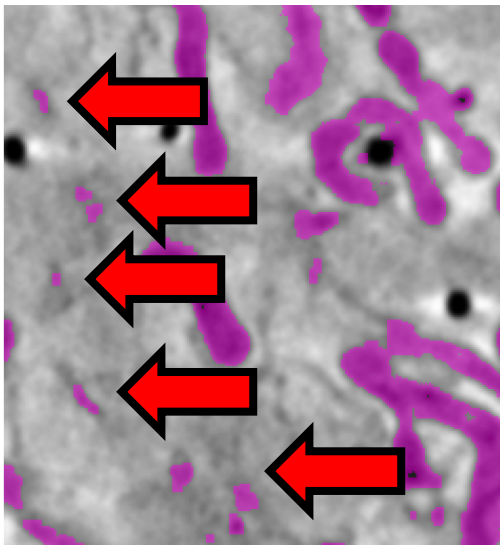
Reminder: you can use the Z selection to determine in which slices to manually erase segmented material.



You can also do other types of manual edits, including fill options.



If you use point options, you can adjust the size of the points with this bar.

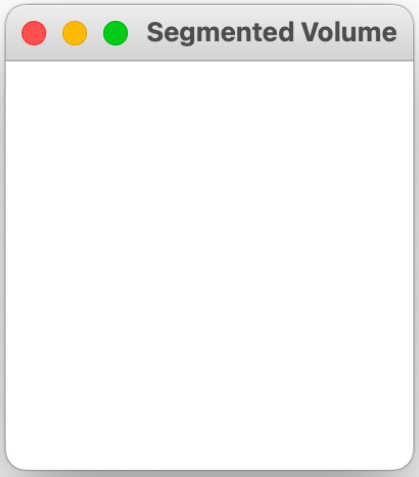


Erase as much of the noise as you can and remember to save frequently.

Tip: Don’t stress out over small bits of noise like these – they can all be deleted in one go later.

If the program starts to become sluggish as you make edits, you can reload the window to refresh the RAM. Changes will be automatically saved first.

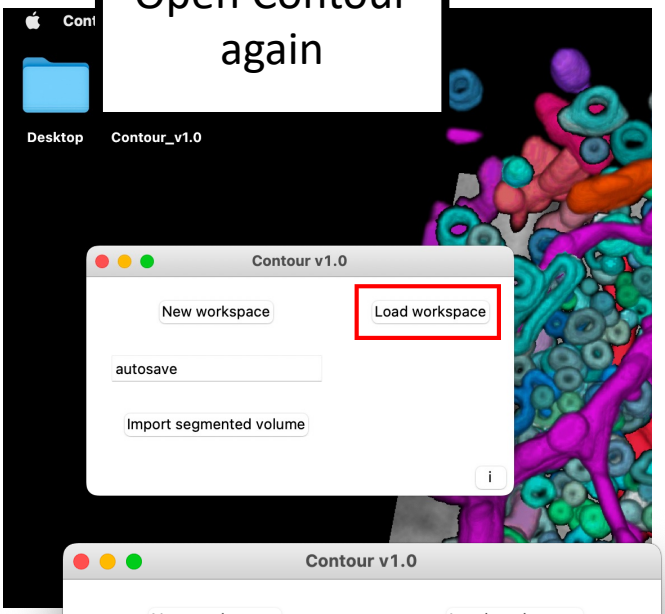
Click **Reload window**



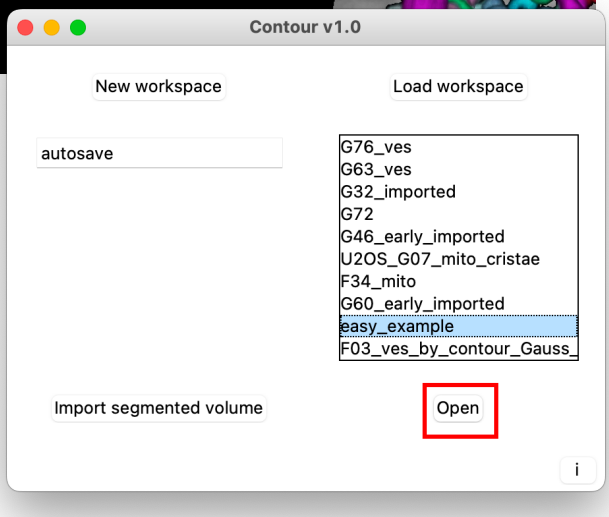
Well, this is embarrassing…

There's a tiny bug in the program that I haven't got around to fixing: sometimes when you click **Reload window** you get a blank window like this. This normally happens the first time you do it in a new workspace. No biggie. This won't happen again if we reload the workspace. Close all the windows.

Open Contour again

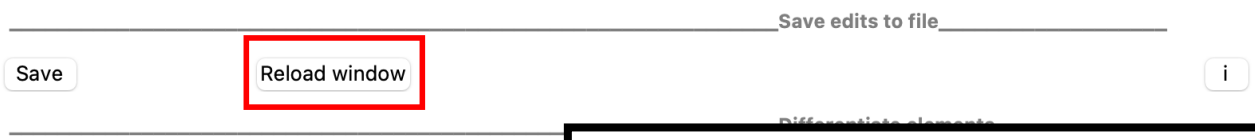


Click **Load workspace**



A scrollable dropdown list will appear with all your saved workspaces, although you probably only have one right now.

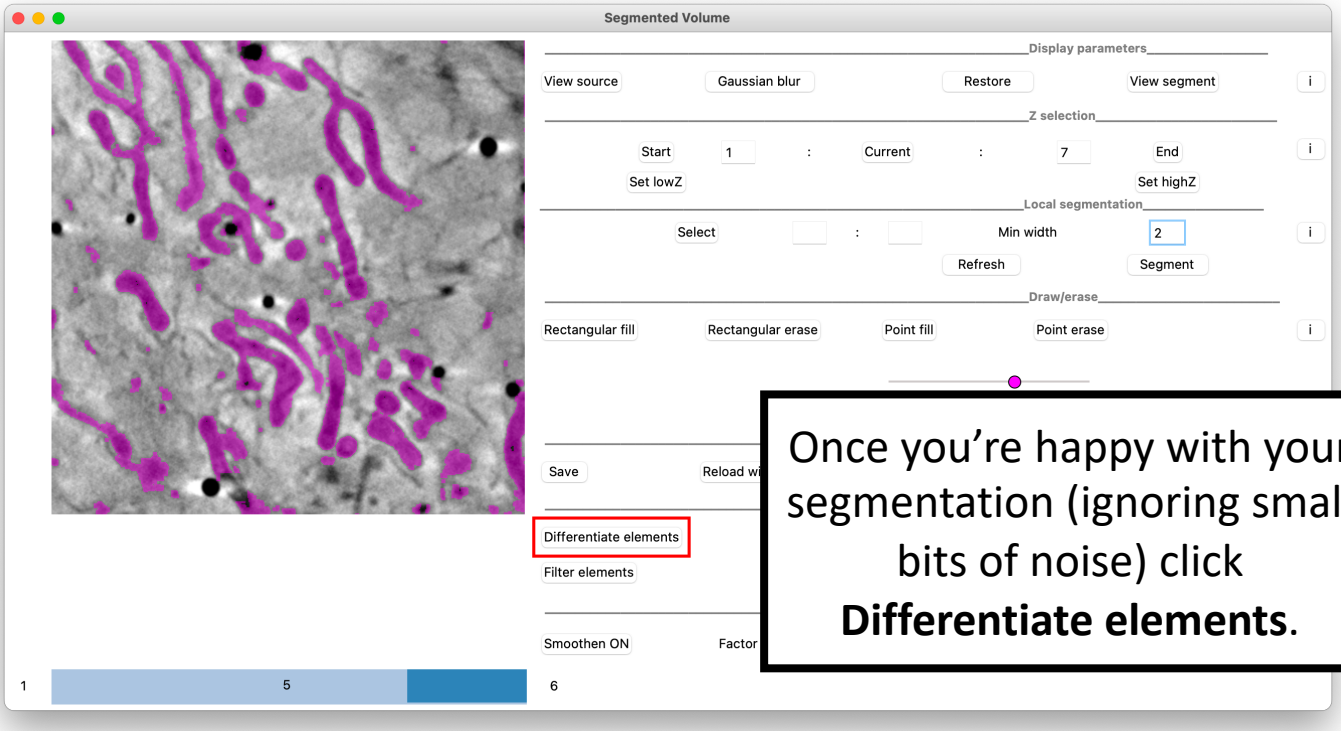
Select **easy_example** and **Open**.



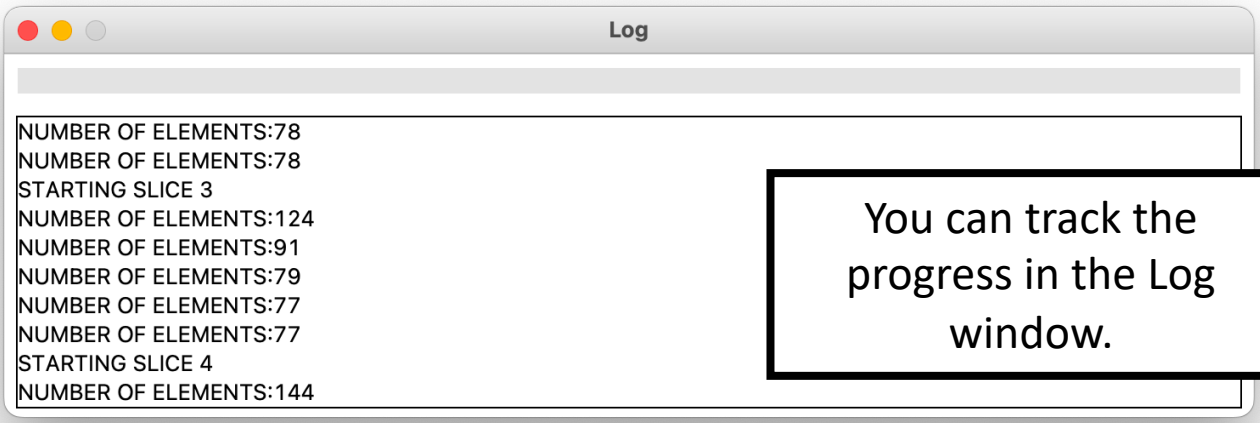
Great, try **Reload window** again.

Differentiate elements

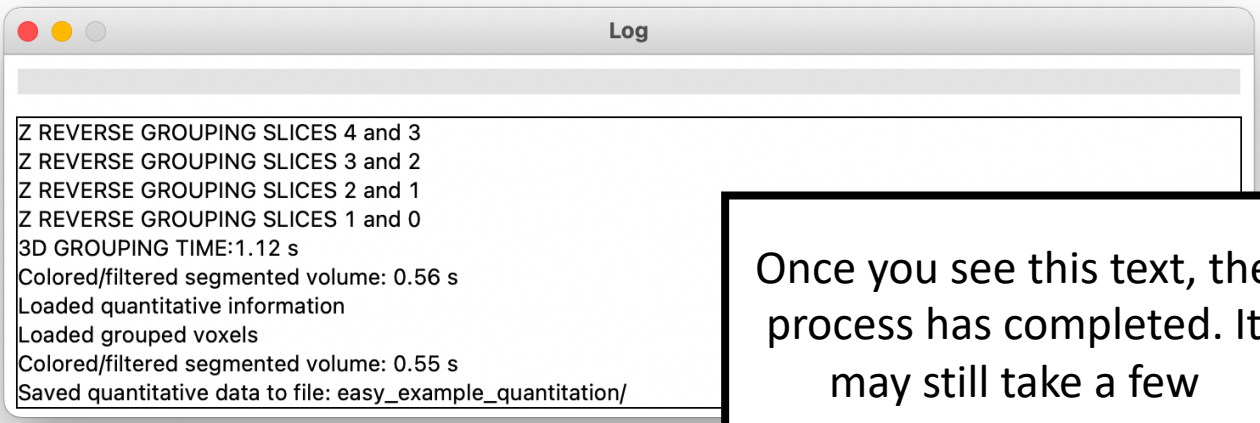
This tool allows you to differentiate separate mitochondria and quantitate them.



Once you're happy with your segmentation (ignoring small bits of noise) click **Differentiate elements**.



You can track the progress in the Log window.

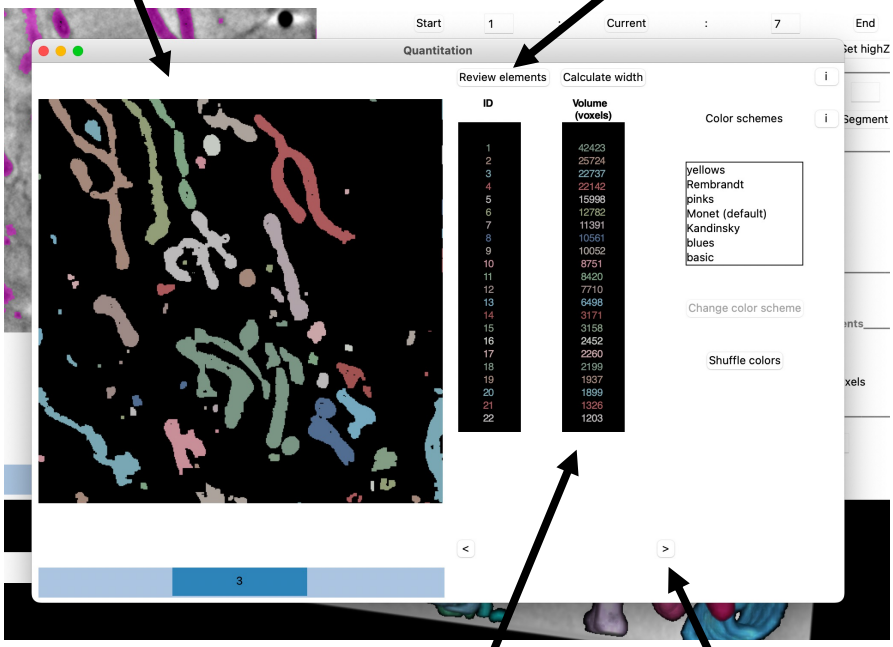


Once you see this text, the process has completed. It may still take a few moments for the next window to load.

The Quantitation window will pop up.

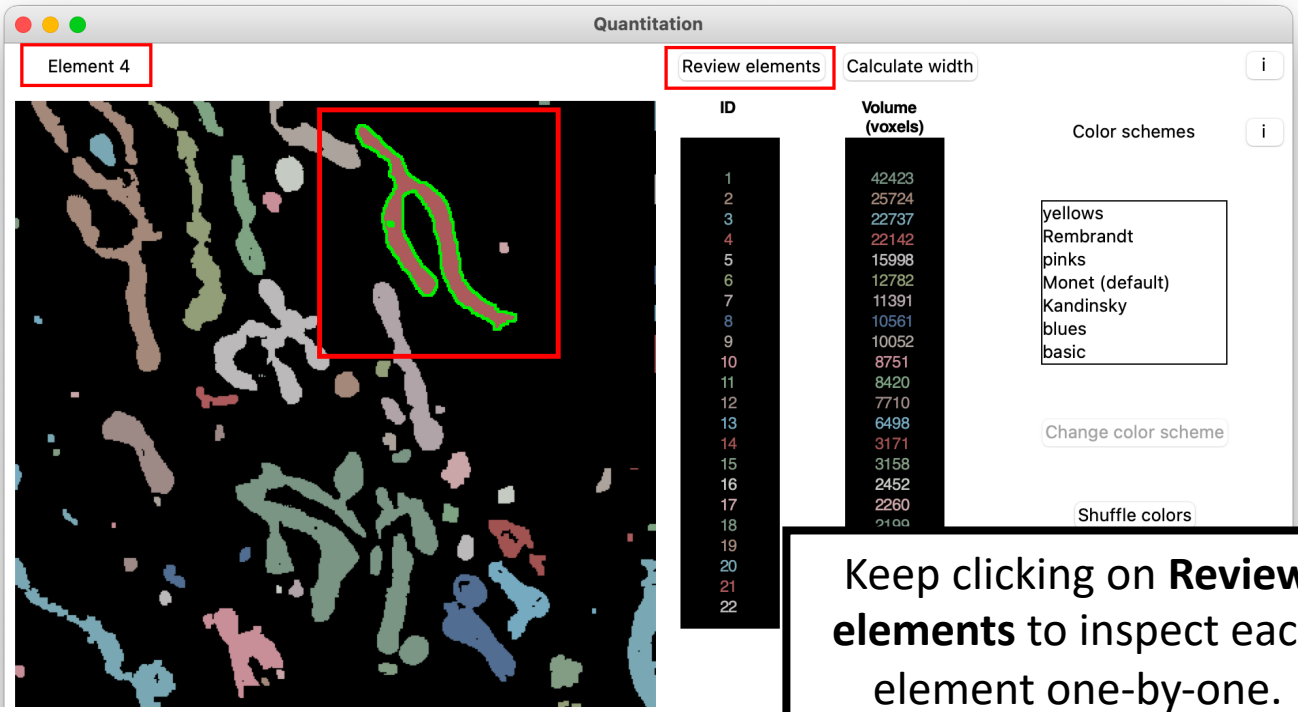
Separate mitochondria will be color coded.

You can use **Review elements** to inspect each element one at time in descending order of volume.

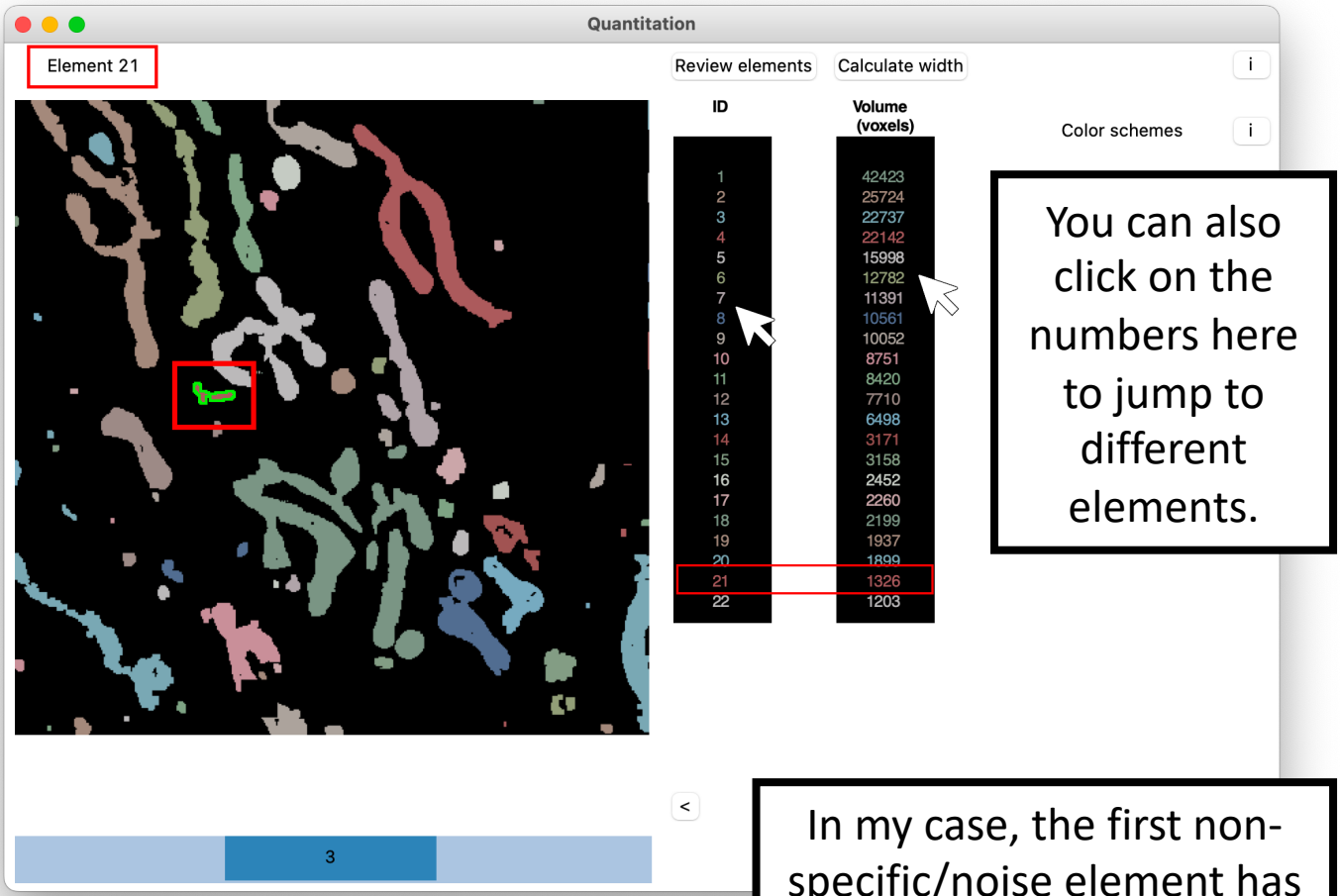


Volumes will be calculated (in voxel units).

Continue moving through the list of elements.

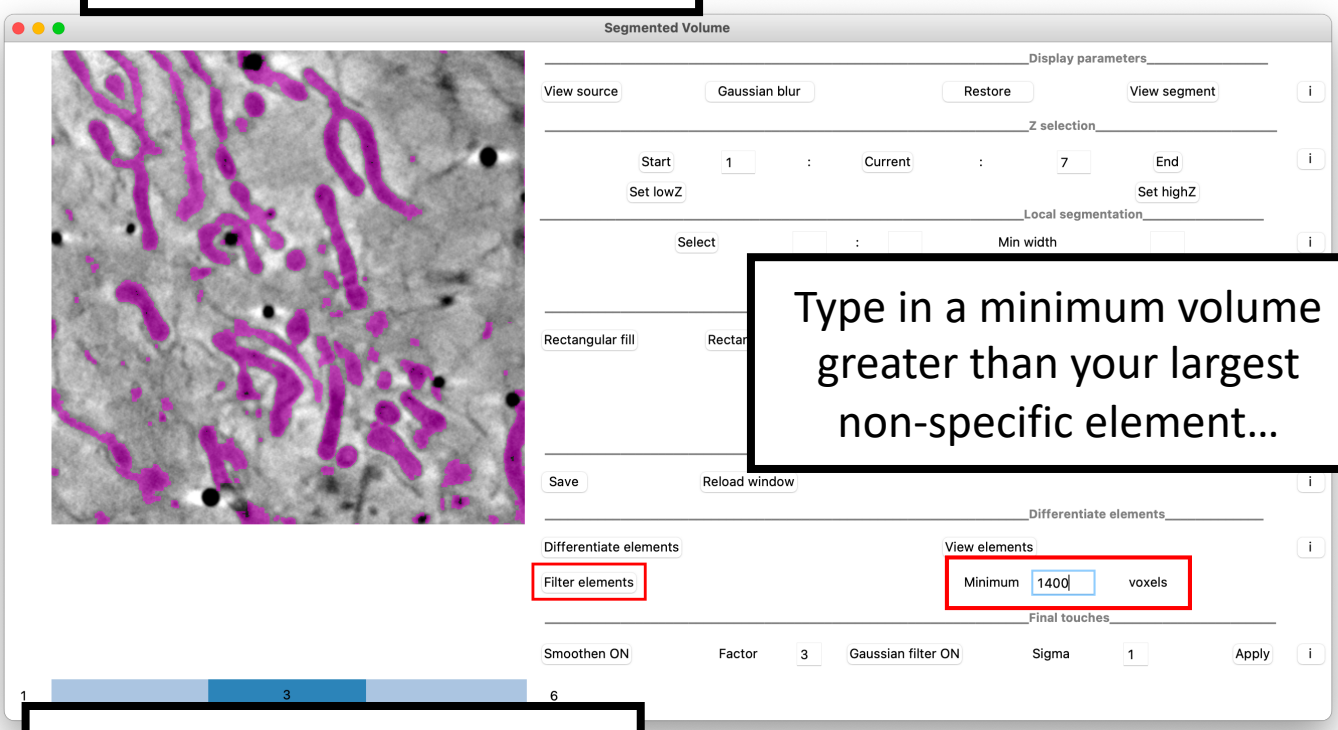


Keep clicking on **Review elements** to inspect each element one-by-one.



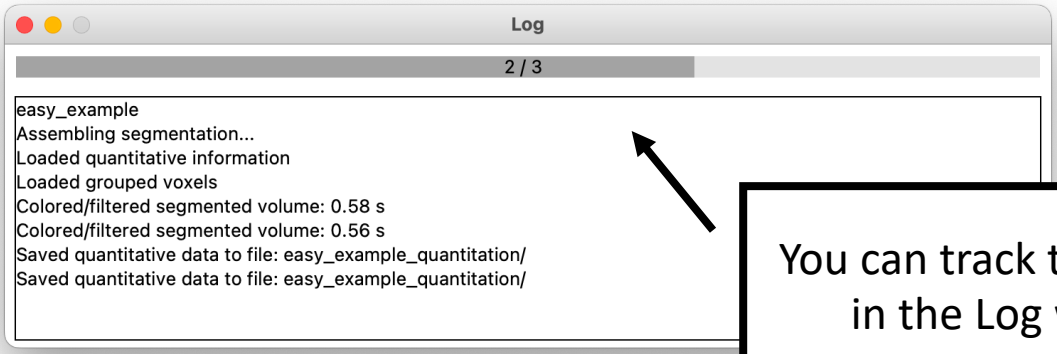
In my case, the first non-specific/noise element has a volume of 1326 voxels.

Go back to the Segmented Volume window.

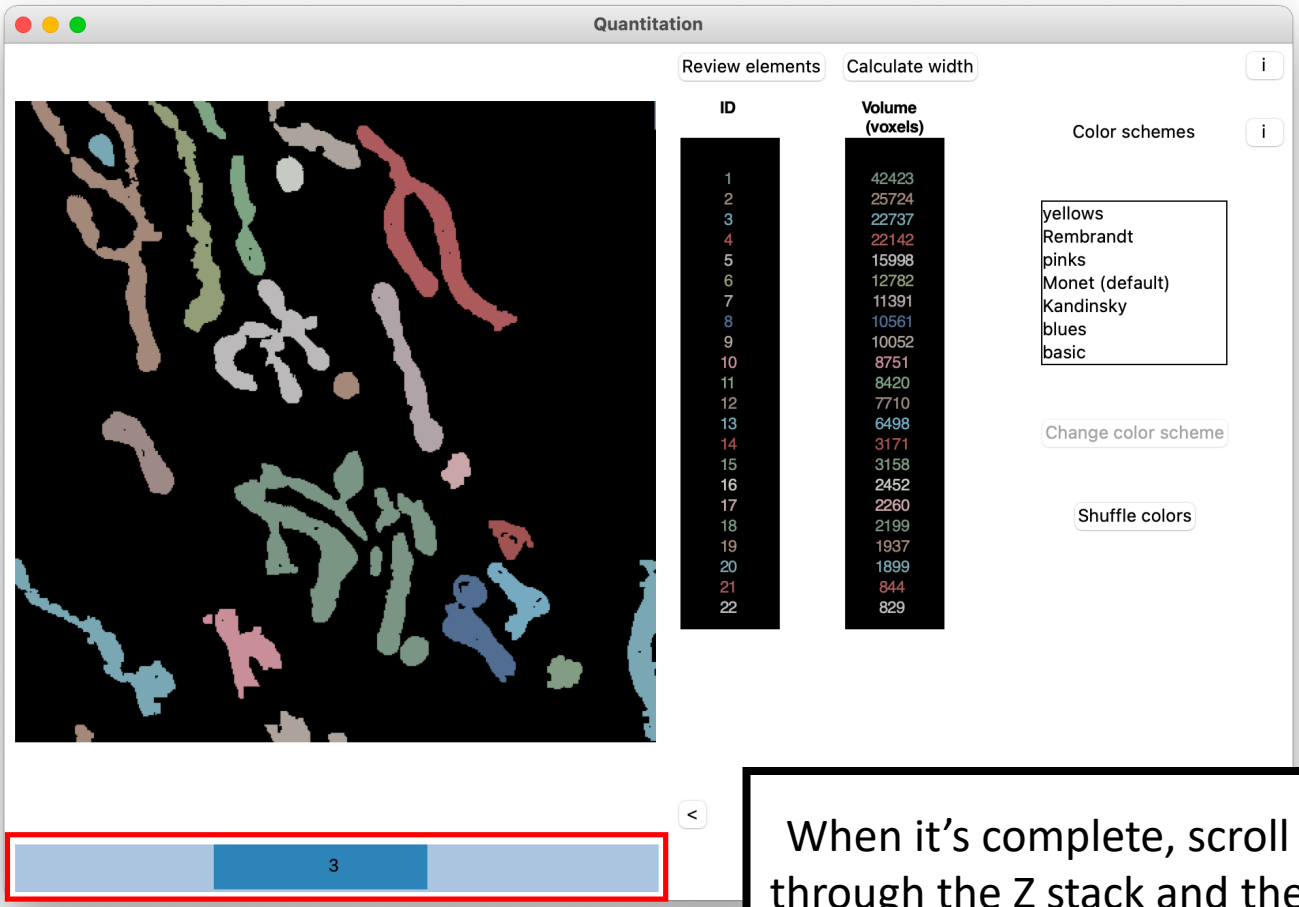


Type in a minimum volume greater than your largest non-specific element…

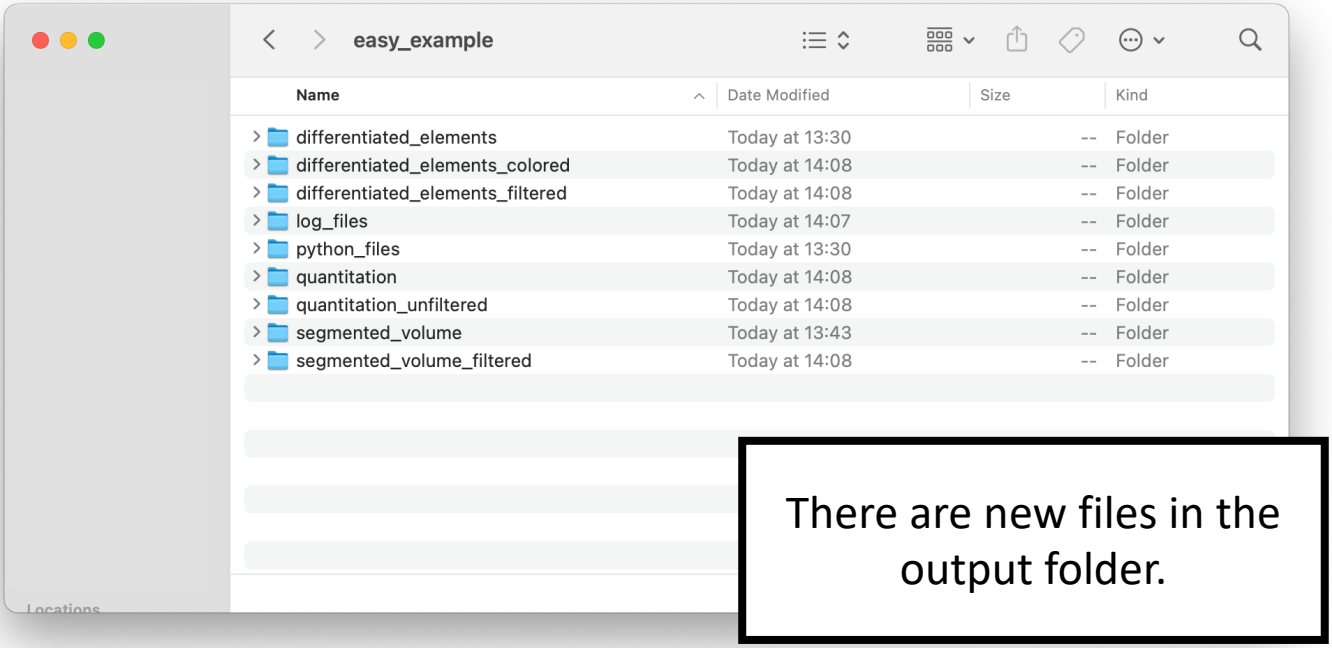
…then click **Filter elements**



You can track the progress in the Log window.



When it's complete, scroll through the Z stack and the small noisy elements should have disappeared.



differentiated_elements — contains grayscale 8-bit images of the differentiated elements.

differentiated_elements_colored — contains RGB images of the color-coded differentiated elements, including the size filter (if applied).

differentiated_elements_filtered — contains grayscale 8-bit images of the differentiated elements with the size filter applied.

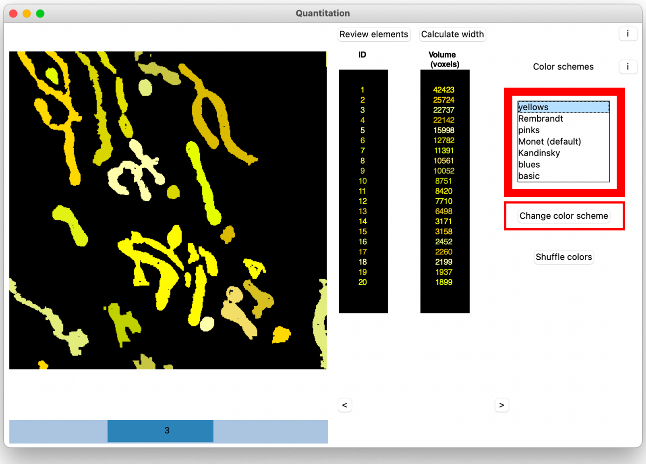
quantitation — contains a csv file of the quantitative information (volume by default and width if selected (more on width later)). If a size filter was applied, this folder would only contain data for the filtered elements.

quantitation_unfiltered — contains a csv file of the quantitative information (volume by default and width if selected (more on width later). This folder is generated if a size filter is applied to back up all the quantitative data with the filtered-out elements included.

segmented_volume_filtered — contains the undifferentiated segmented elements after applying the size filter.

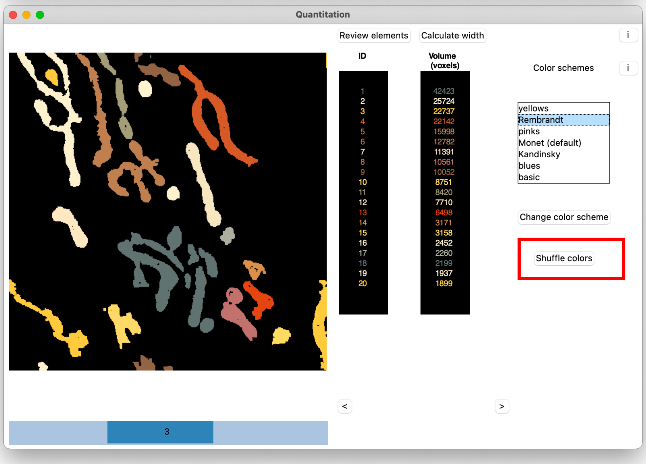
Final touches

Polish the segmented volume.

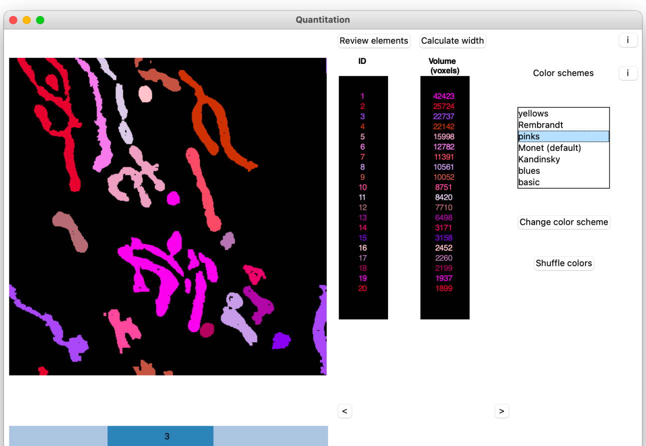


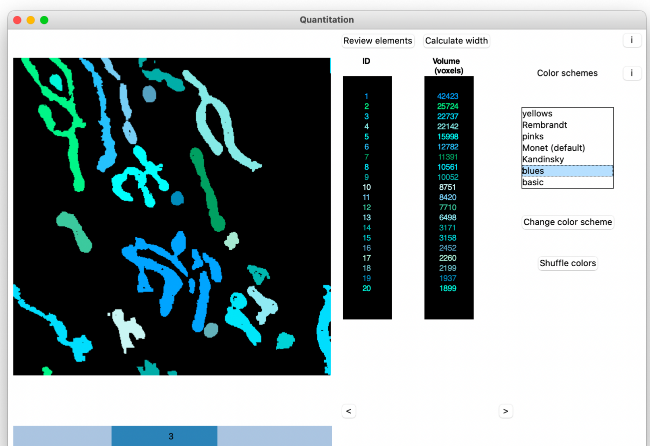
Select a color scheme from the scroll list and click **Change color scheme**.

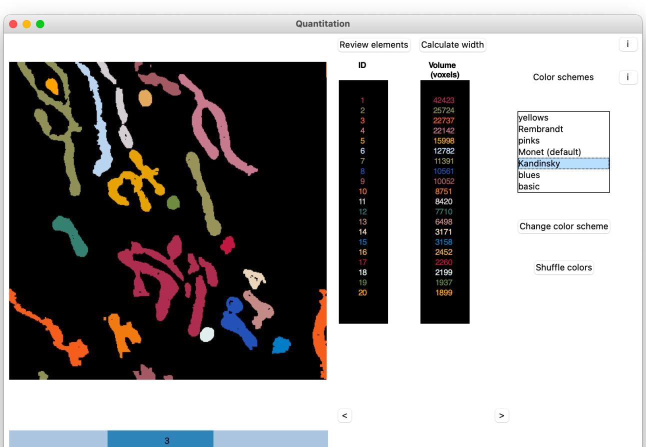
Monet is the default scheme.

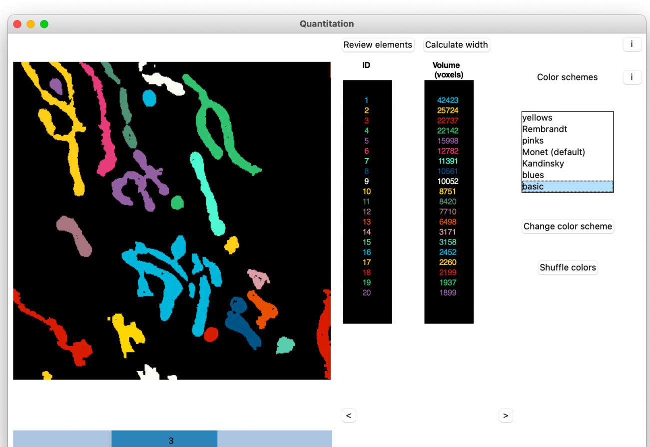


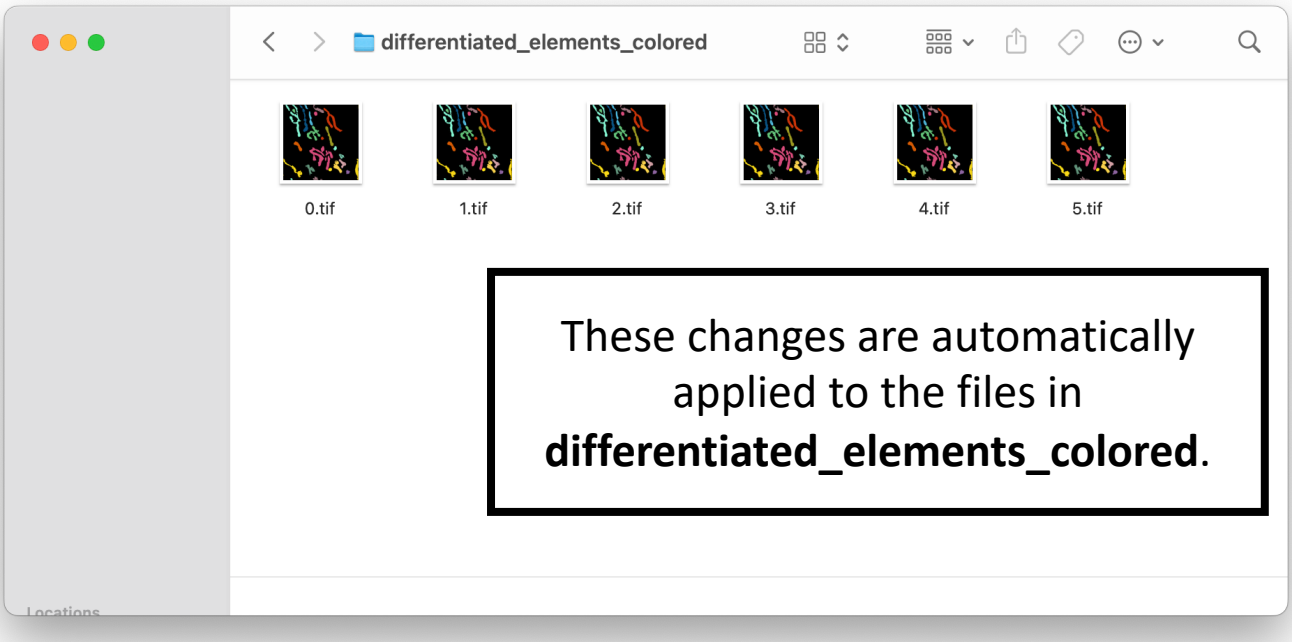
If two neighboring but separate mitochondria have a very similar shade of color, click **Shuffle colors** to mix things up and make the distinction clearer.











Once you're happy with the color scheme, go to the Segmented Volume window.

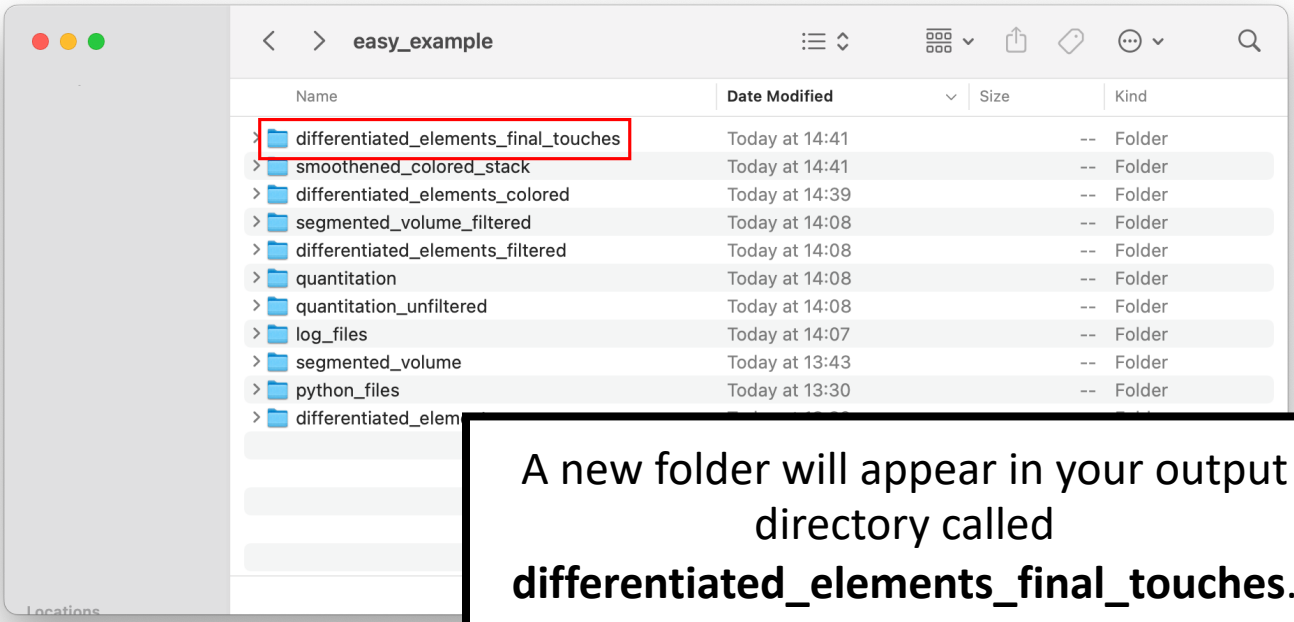
Toggle ON/OFF to smoothen the edges of the segmented volume.

Toggle ON/OFF to apply a Gaussian blur to the segmented volume.

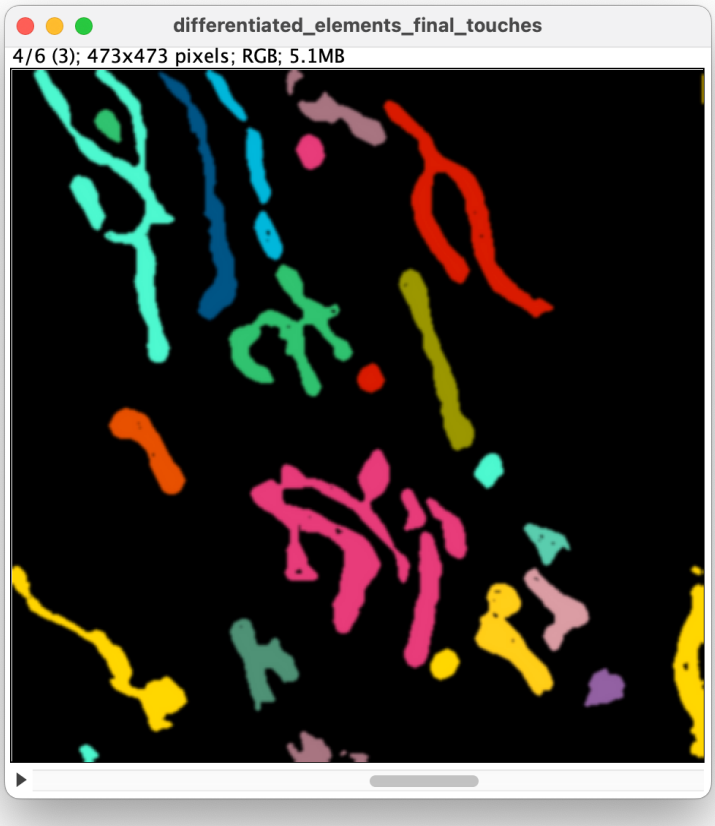
Higher smoothing factors lead to smoother edges but also erode the edges. A value of 1 to 3 is recommended. Go for Factor 3.

Higher standards of deviation lead to more blur, which can smoothen 3D renderings of the segmented elements. Go for Sigma 1.

Hit Apply



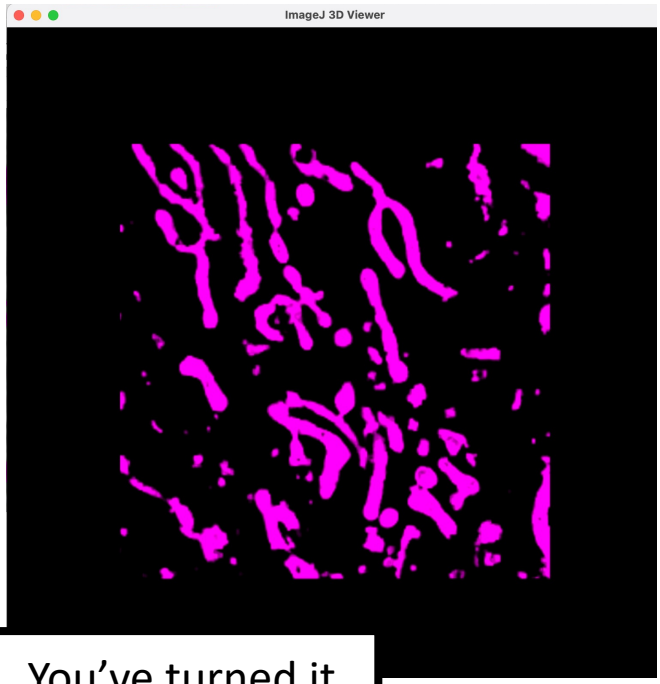
A new folder will appear in your output directory called **differentiated_elements_final_touches**. This will contain the applied smoothing and Gaussian blur.



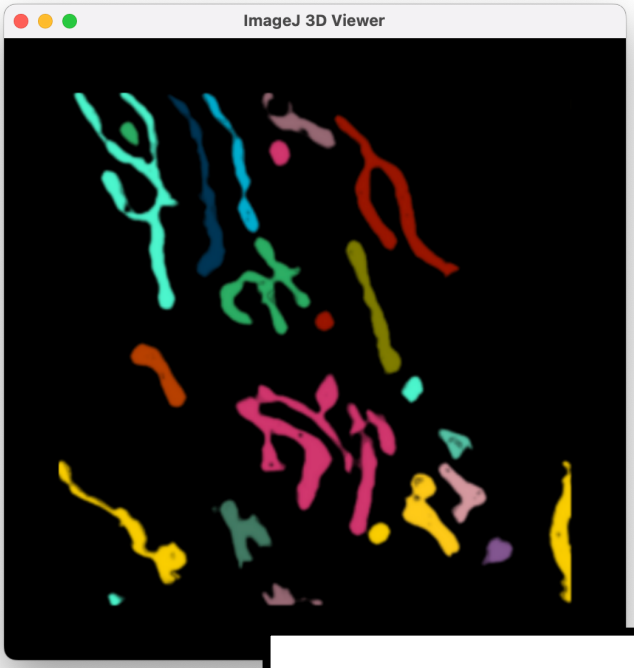
Import this image sequence into Fiji as you did previously.

Adjust the scale to 10 nm × 10 nm × 5 nm.

Then load it into 3D Viewer.



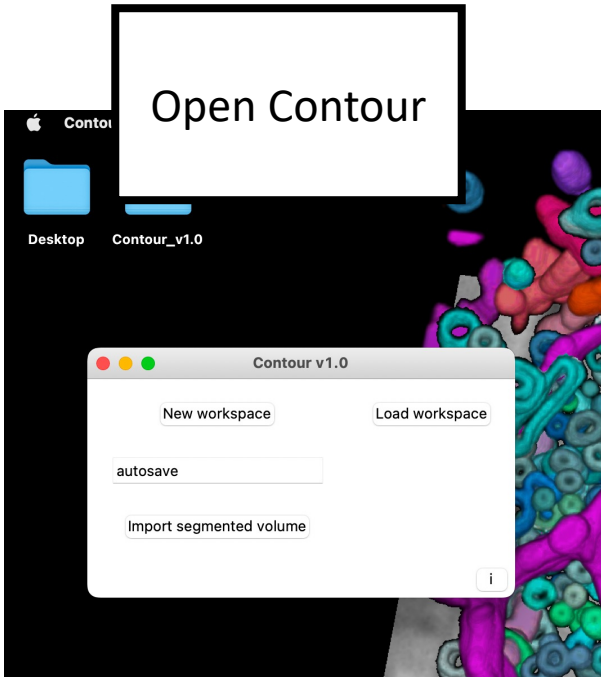
You've turned it from this…

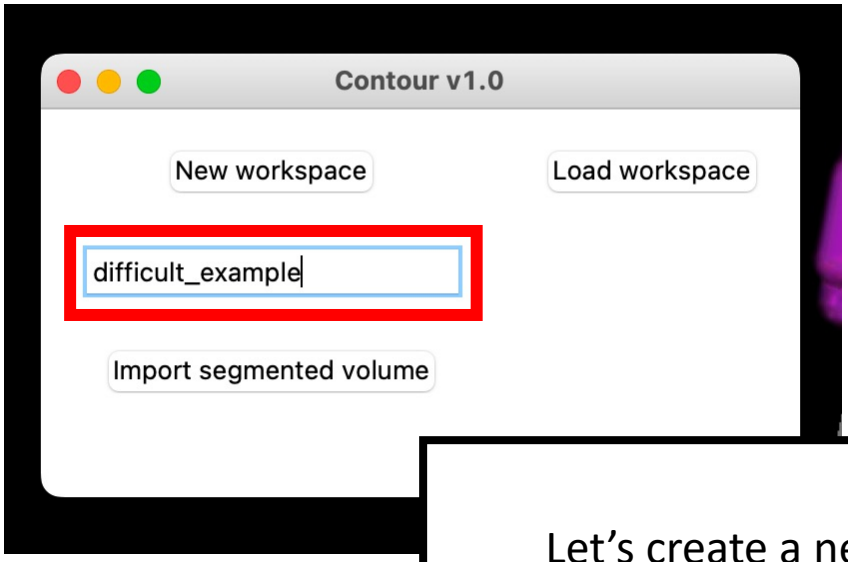


…into this!

Difficult example

Let’s try segmenting mitochondria from a more difficult example.

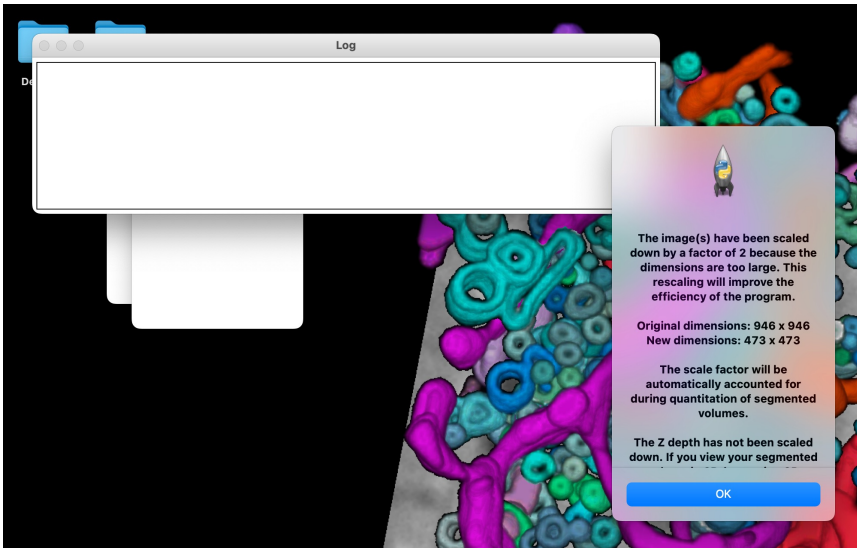




Let's create a new workspace with the difficult_example.tif file available at https://github.com/kamallouisnahas/Contour.

Download the file if you haven't already. Then change the workspace name.

Click **New workspace** and select the file.



This time we get a message we haven't seen before.

In order to improve the efficiency of the segmentation, any source stack larger than 512 × 512 pixels will be halved in size.

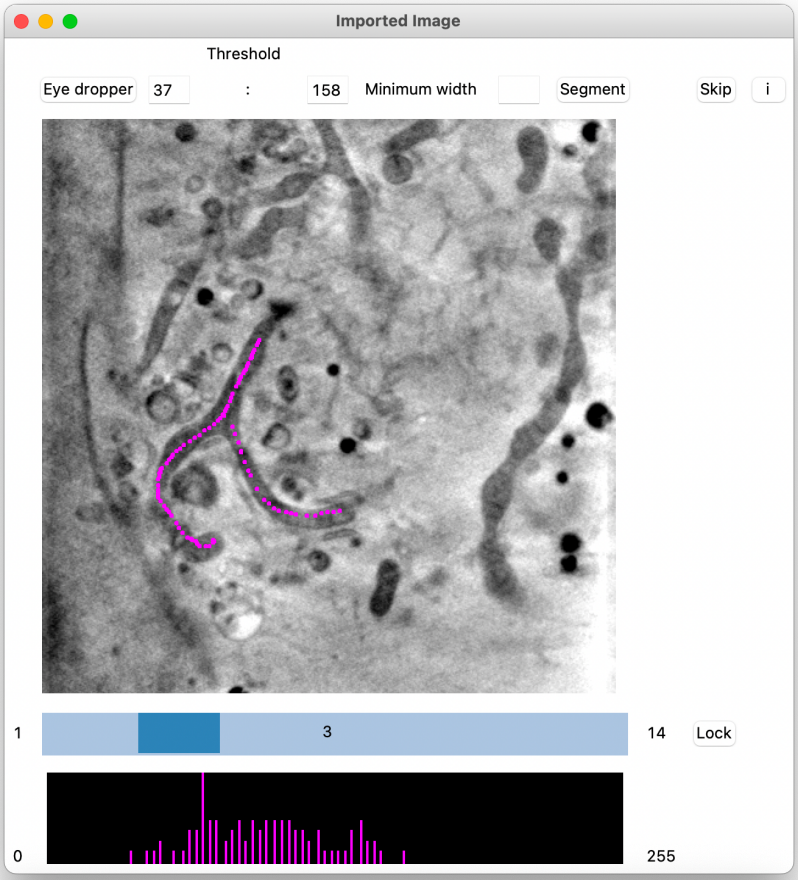
The example tomograms were collected with a 946 × 946 pixels field of view and need to be halved to 473 × 473.

We didn't see this message with **easy_example.tif** because it was downsized by a factor of 2 *before* we opened it in Contour. This was done to keep things simpler with the first example.

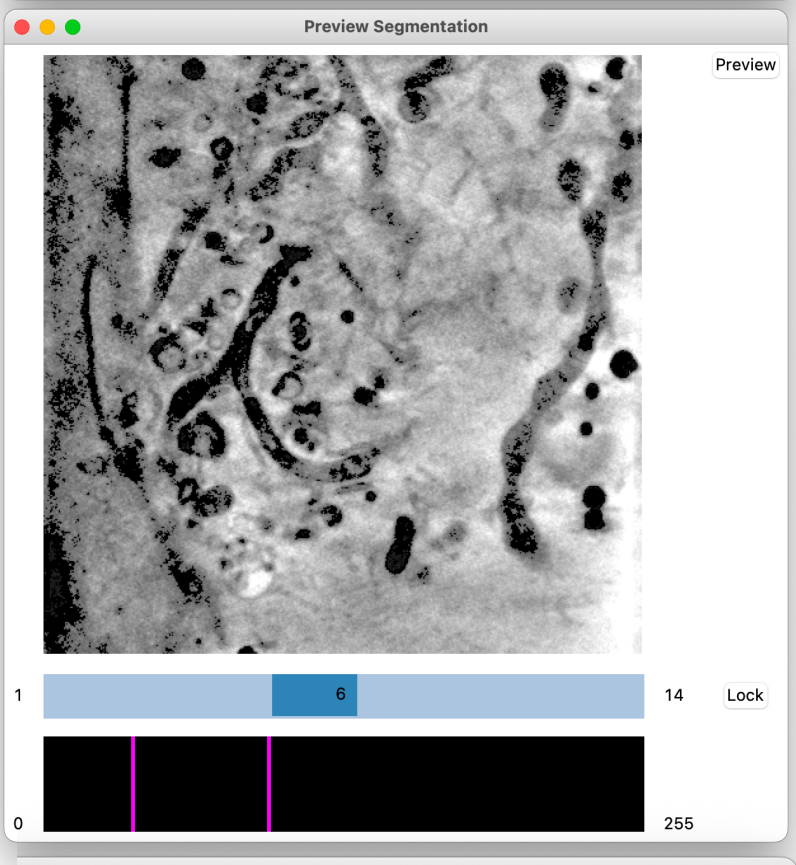
It's recommended that you don't downsize the stacks *before* opening them in Contour. If you allow Contour to downsize them, it will keep a record of the changes and will account for them when it quantitates the segmented elements.

There is only one thing you need to note: you will have to downsize the Z scale (from 10 nm to 5 nm) before you produce a 3D rendering in 3D Viewer with Fiji.

Click **OK**.



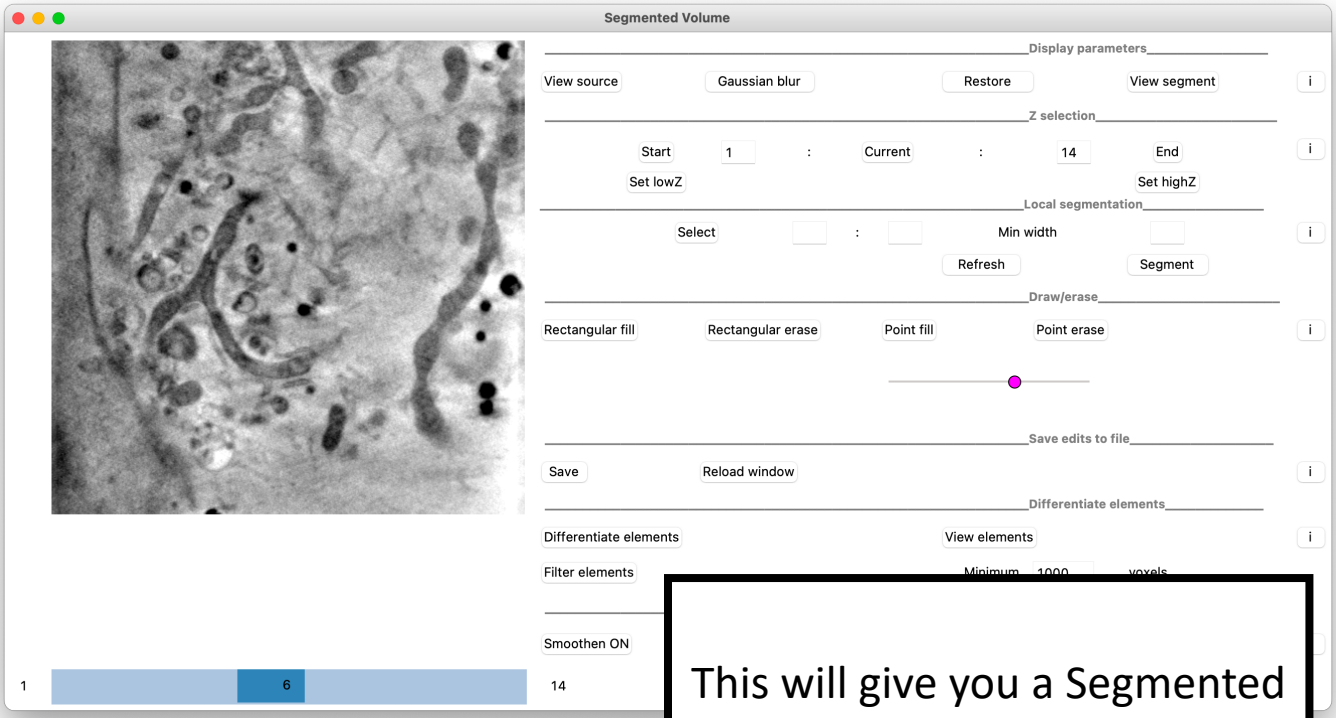
Try to select the intensity range of the mitochondria using the **Eye dropper.**



With this tomogram you should find it harder to run a mitochondria-specific *global segmentation* on the whole tomogram because

- the cytoplasm is crowded,
- numerous features (e.g. vesicles) have a similar intensity,
- and the mitochondria have a more variable intensity.

Don't attempt the **Segment** function. Click **Skip**.



This will give you a Segmented Volume window with a blank segmented volume.

In tomograms such as this, it would be easier and less time-consuming to use *local segmentations* to segment all the mitochondria.

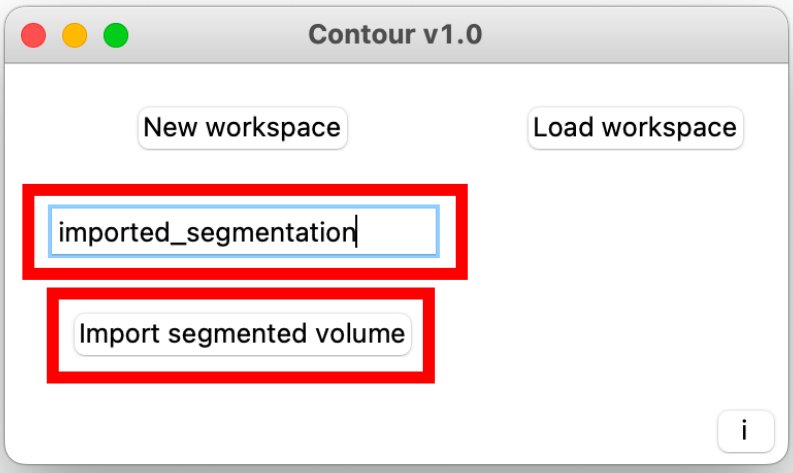
If you used global segmentation, you would generate a lot of noise and non-specific material that would be time-consuming to erase.

Importing volumes

Contour is useful for segmenting highly contrasting thick features, such as mitochondria or lipid droplets, but you might struggle to segment thinner features, such as vesicles.

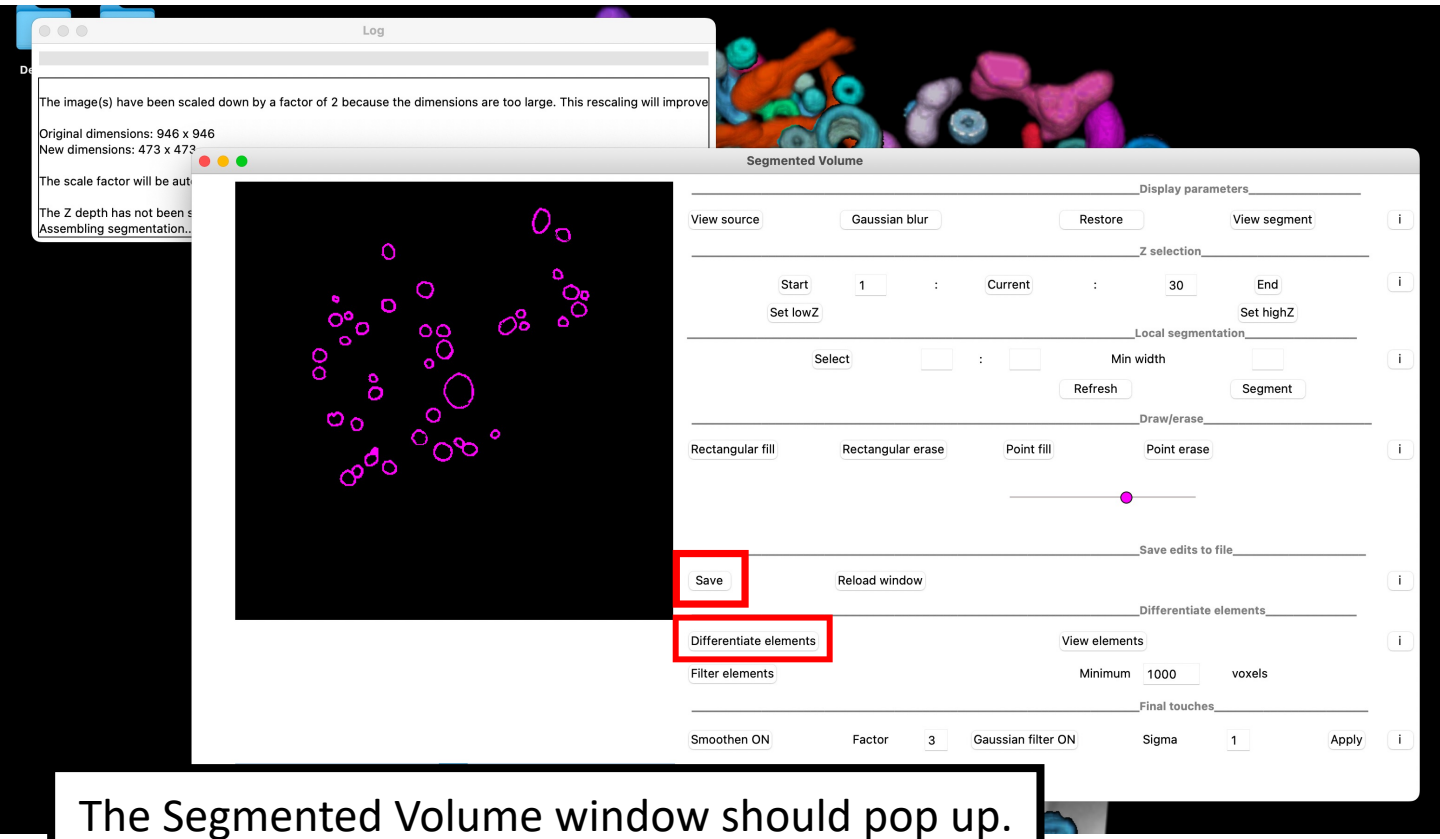
If you segment features in another program, you can import the segmented volume into Contour to quantitate the elements.

Just make sure you save it as an **8-bit tif file with background as value 0 and the segmented elements as any positive values**.

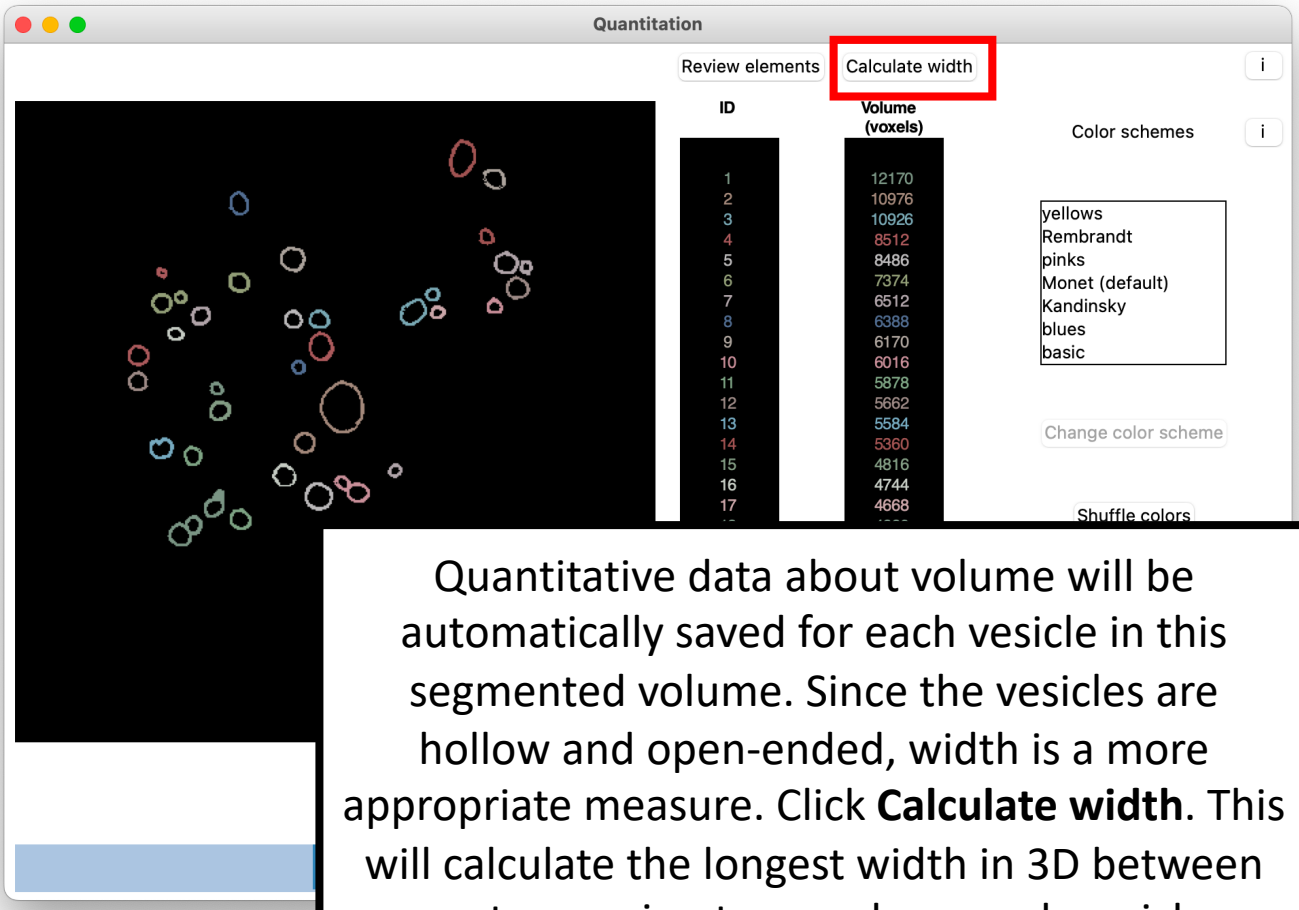


Download the imported_segmentation_example.tif file available at https://github.com/kamallouisnahas/Contour.

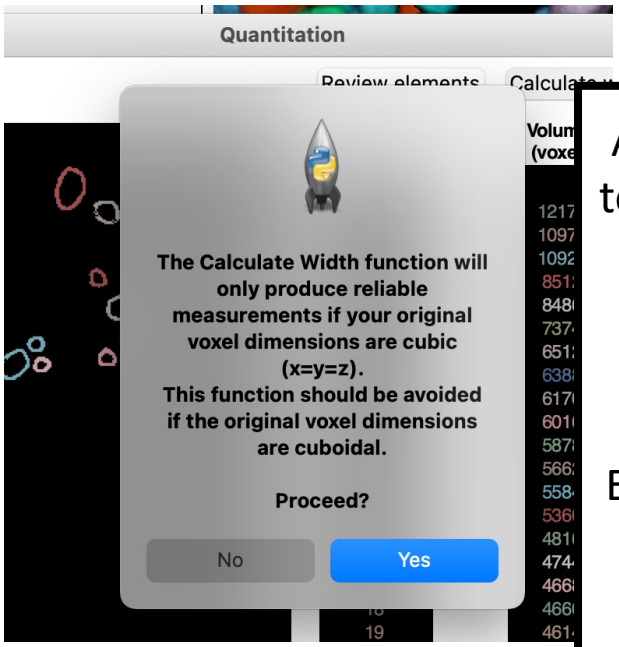
Change the workspace name and click **Import segmented volume**.



The Segmented Volume window should pop up. Click **Save** and run **Differentiate elements.**



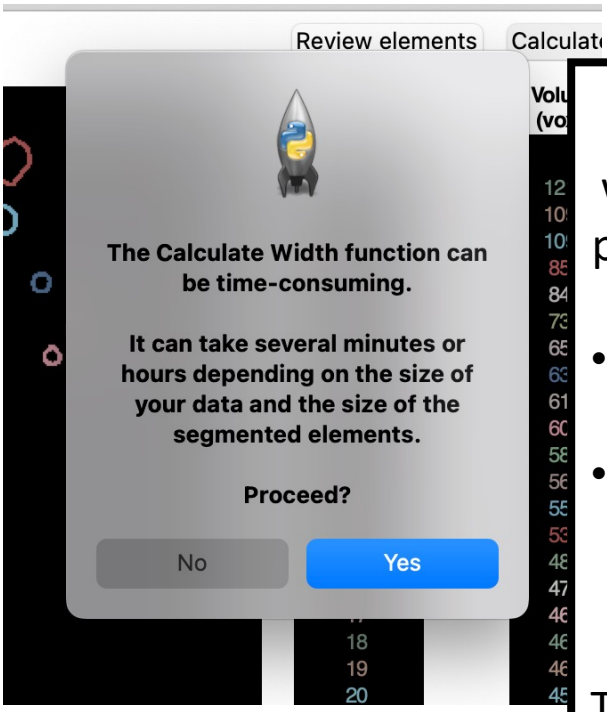
Quantitative data about volume will be automatically saved for each vesicle in this segmented volume. Since the vesicles are hollow and open-ended, width is a more appropriate measure. Click **Calculate width**. This will calculate the longest width in 3D between any two perimeter voxels on each vesicle.



An information window will pop to let you know that this function will only work reliably on data where the original voxel dimensions are cubic.

E.g. 10 nm × 10 nm × 10 nm for the tomograms.

Click **Yes**.

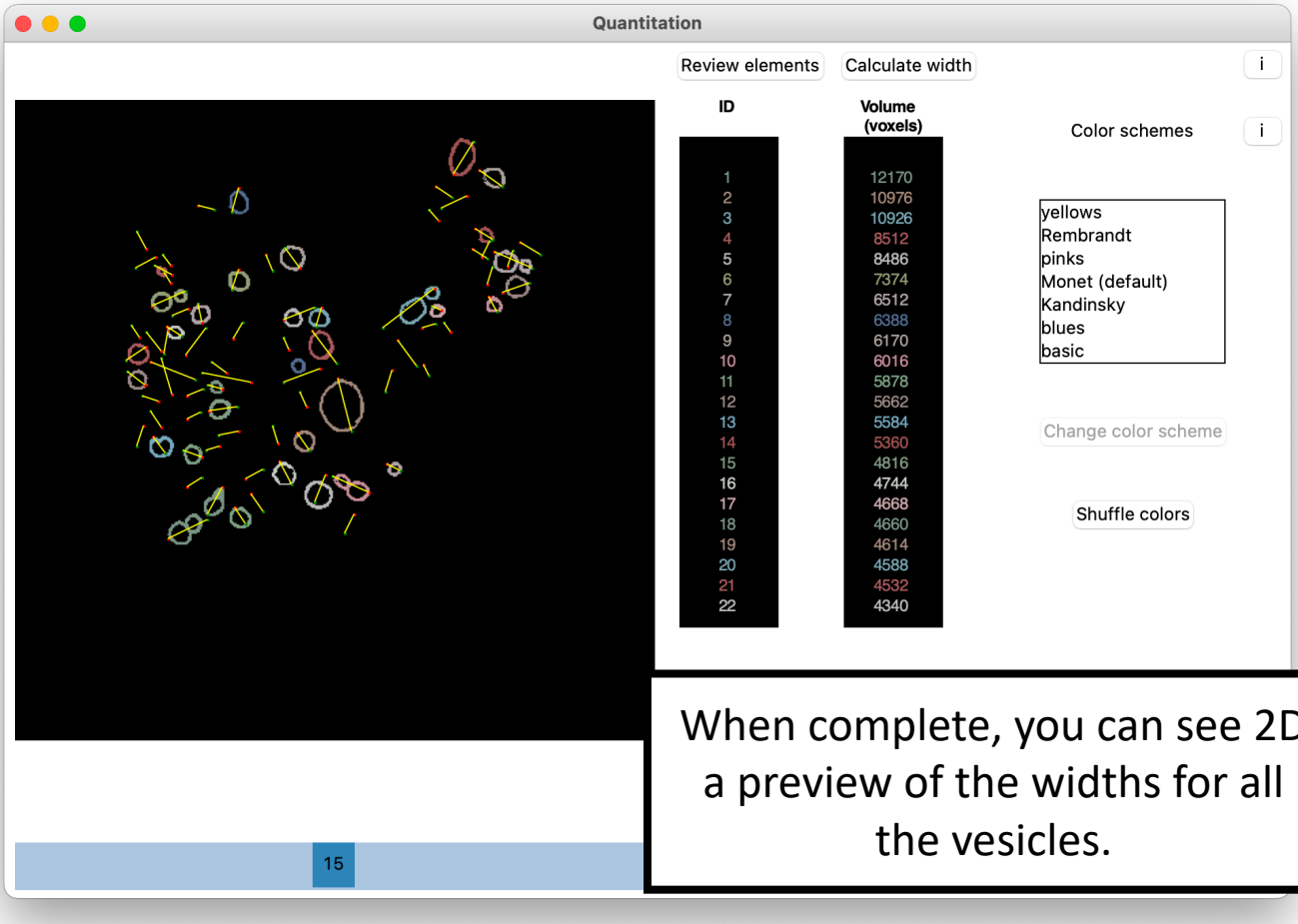


A second information window will pop up to warn you that this process can be time-consuming if

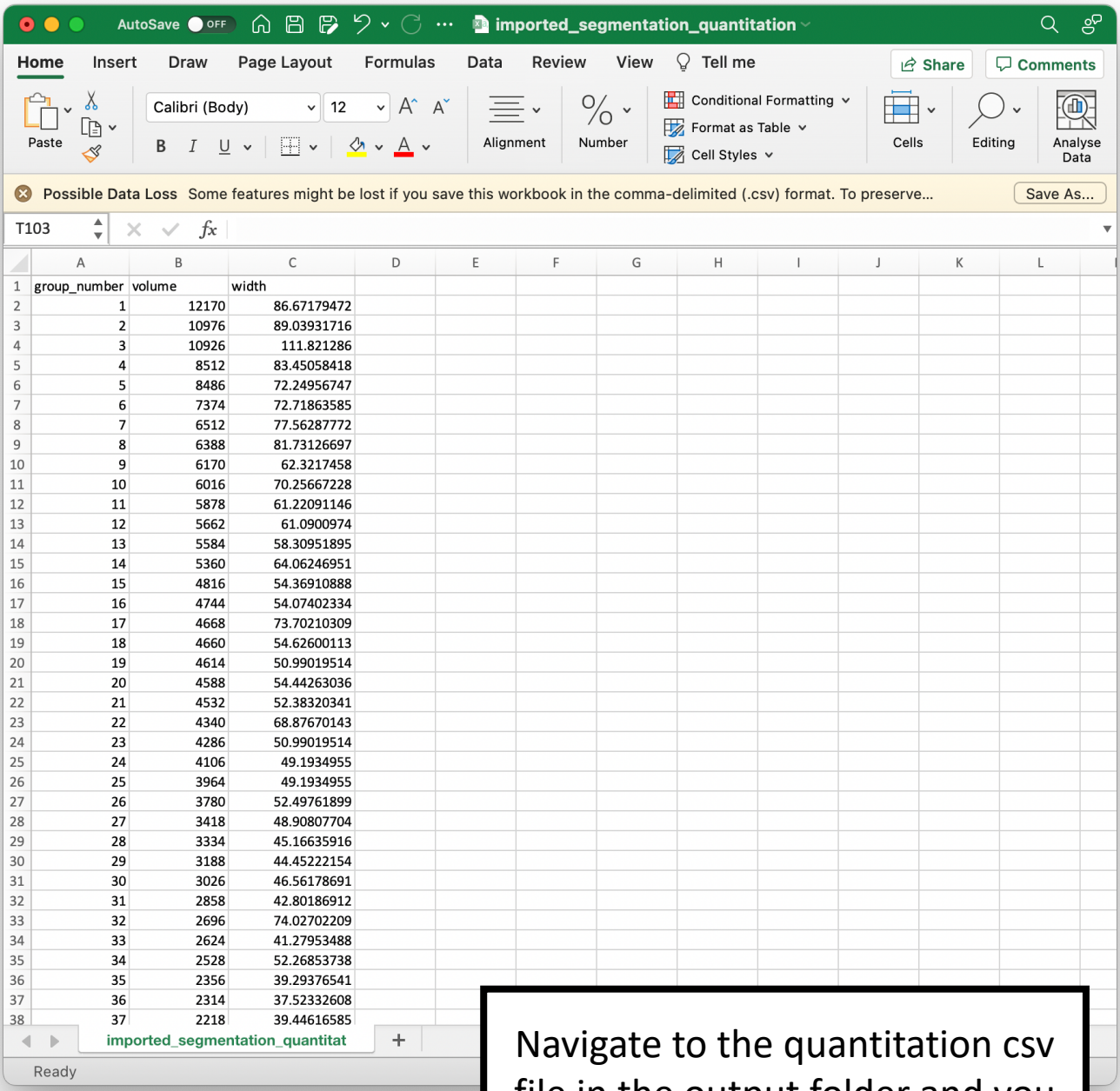
- there are a lot of segmented elements
- or there are large segmented elements with a lot of voxels at their perimeters.

This example data should take approximately 6 minutes to run.

Click **Yes**.



When complete, you can see 2D a preview of the widths for all the vesicles.

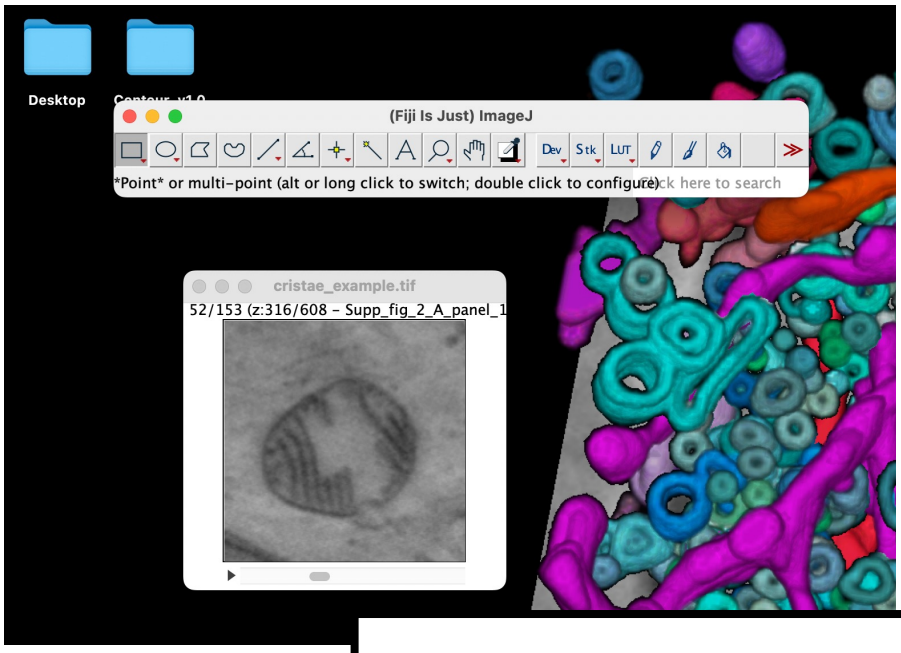


group_number	volume	width
1	12170	86.67179472
2	10976	89.03931716
3	10926	111.821286
4	8512	83.45058418
5	8486	72.24956747
6	7374	72.71863585
7	6512	77.56287772
8	6388	81.73126697
9	6170	62.3217458
10	6016	70.25667228
11	5878	61.22091146
12	5662	61.0900974
13	5584	58.30951895
14	5360	64.06246951
15	4816	54.36910888
16	4744	54.07402334
17	4668	73.70210309
18	4660	54.62600113
19	4614	50.99019514
20	4588	54.44263036
21	4532	52.38320341
22	4340	68.87670143
23	4286	50.99019514
24	4106	49.1934955
25	3964	49.1934955
26	3780	52.49761899
27	3418	48.90807704
28	3334	45.16635916
29	3188	44.45222154
30	3026	46.56178691
31	2858	42.80186912
32	2696	74.02702209
33	2624	41.27953488
34	2528	52.26853738
35	2356	39.29376541
36	2314	37.52332608
37	2218	39.44616585

Navigate to the quantitation csv file in the output folder and you can find the widths reported in voxel units.

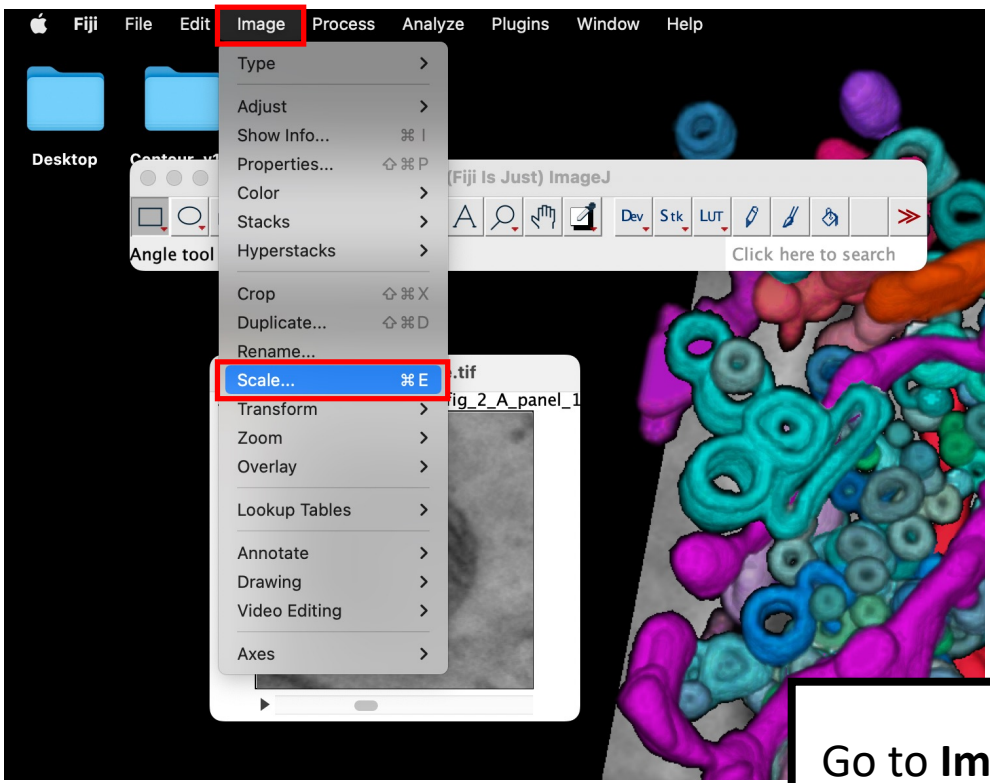
Segmenting cristae

It is possible to segment cristae using Contour but there are a few more steps you need to do first in Fiji.

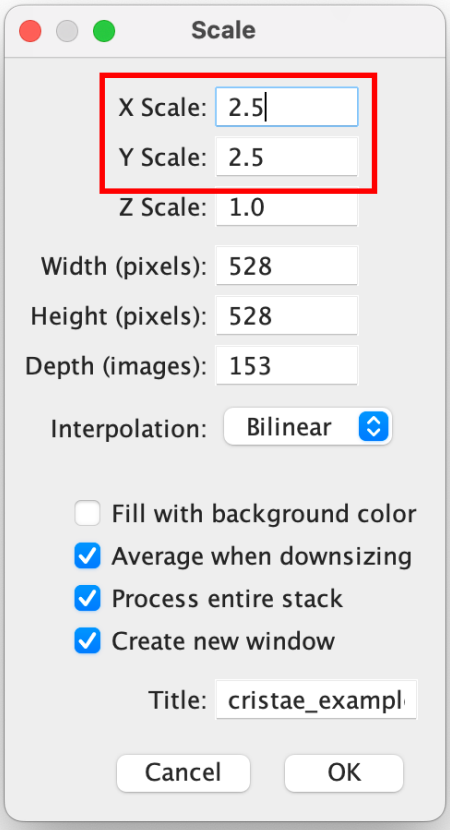


Download the file cristae_example.tif from https://github.com/kamallouisnahas/Contour.

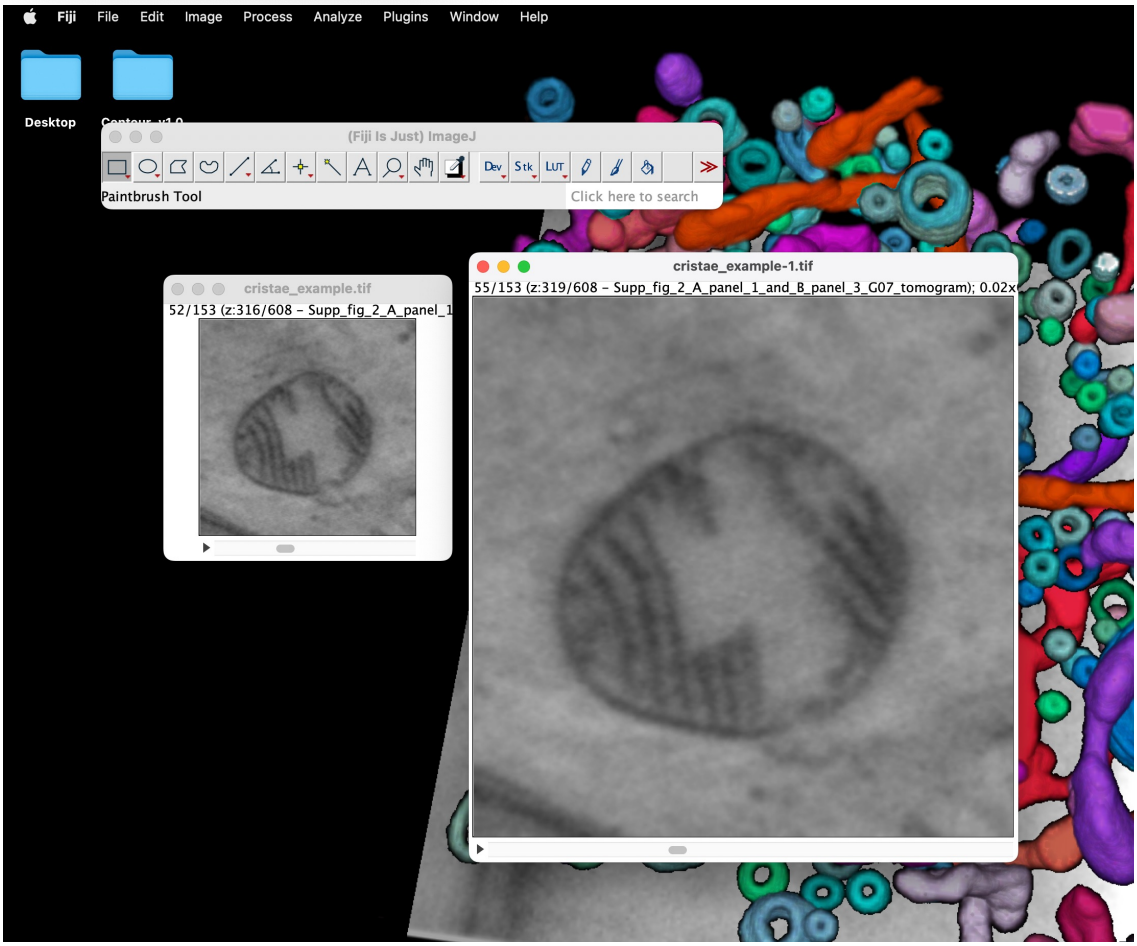
Open it in Fiji.



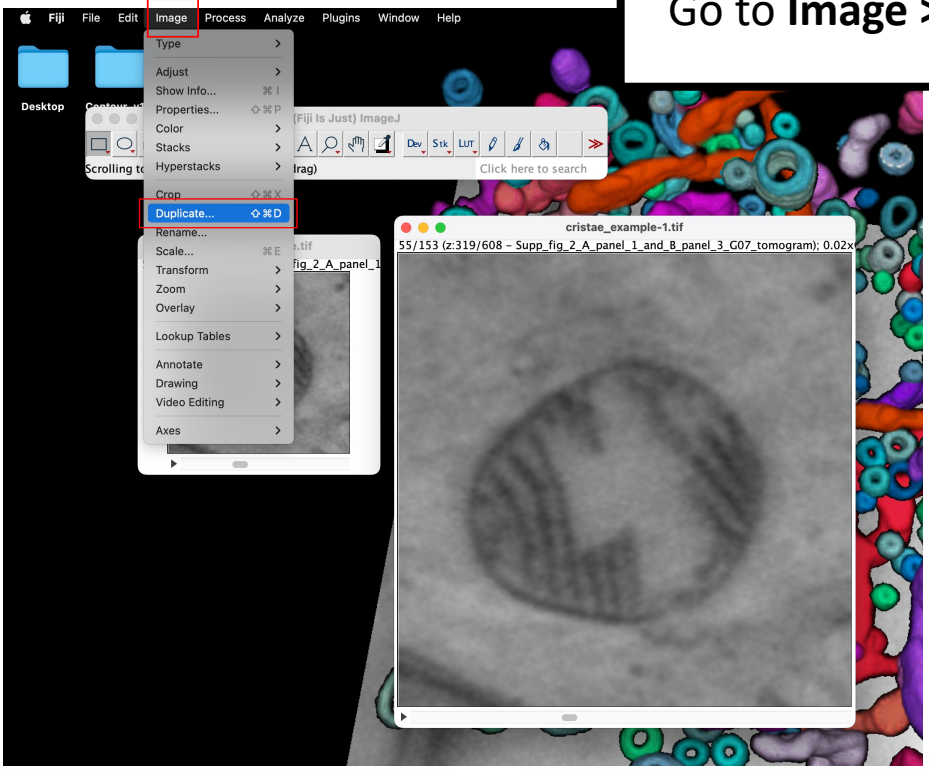
Go to **Image > Scale...**

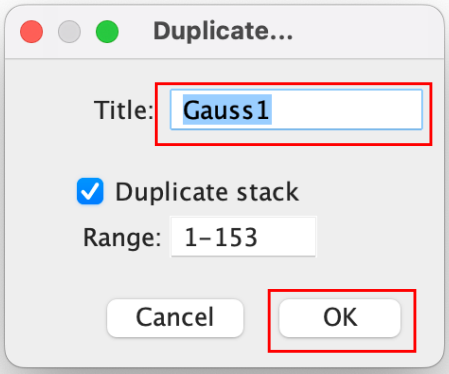


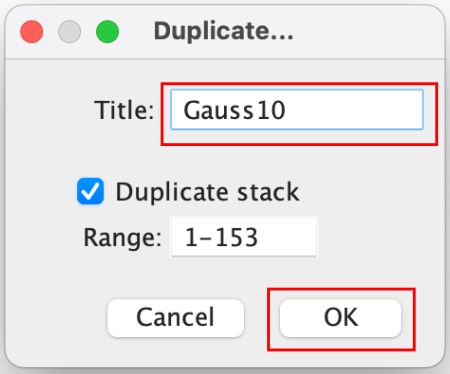
Increase the XY scale by 2.5 times. This will increase the voxel width of the cristae to make it easier to apply a minimum width.



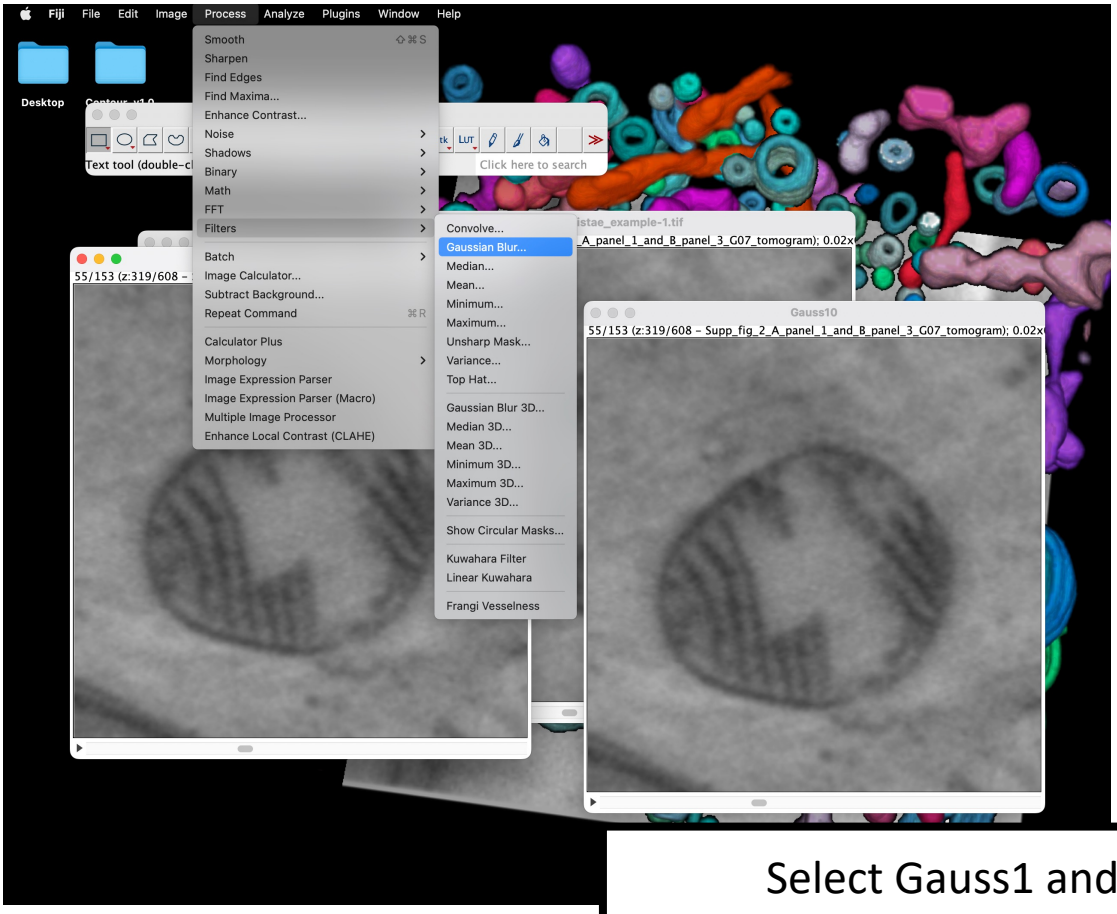
Go to **Image > Duplicate…**



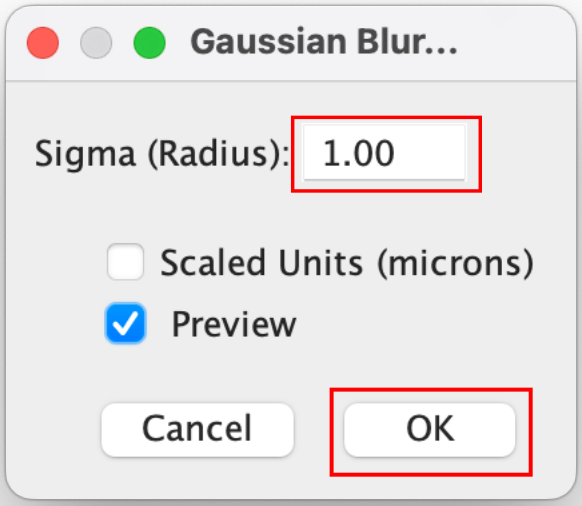




Duplicate the stack twice and rename them Gauss1 and Gauss10.

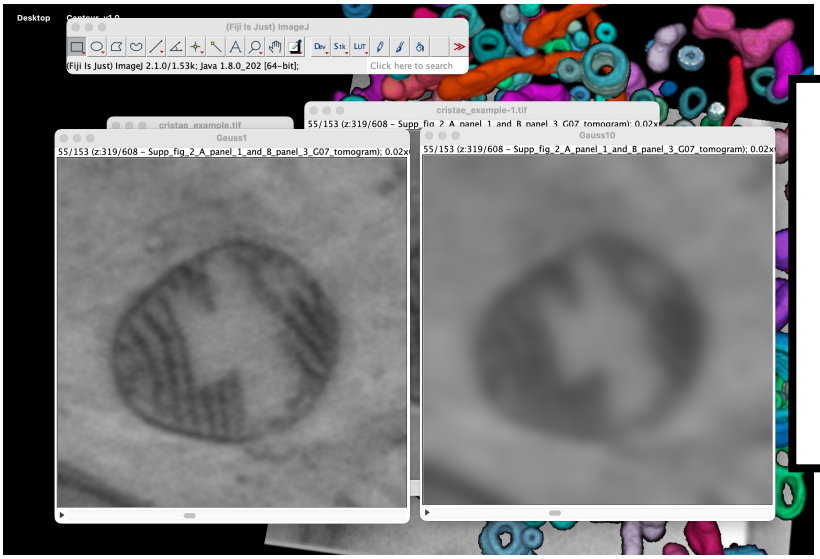


Select Gauss1 and go to
Process > Filters > Gaussian Blur…

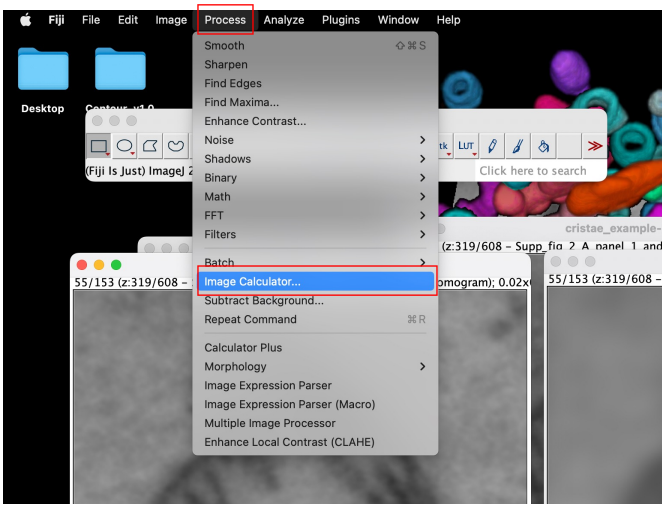


Apply a Sigma of 1.00
and click **OK**.

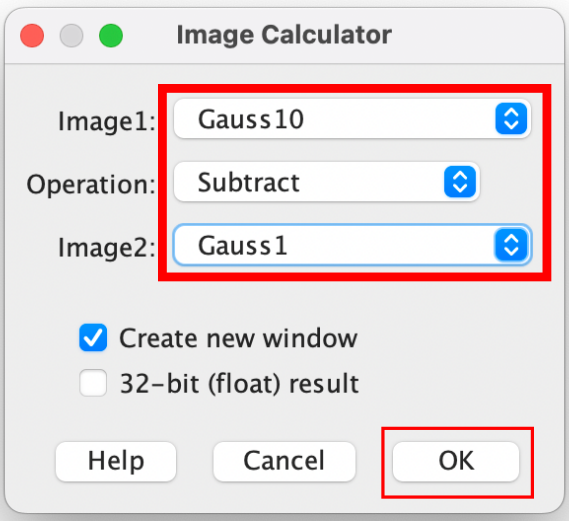
Repeat this for Gauss10 but apply
a Sigma of 10.



Now we're going to subtract Gauss1 from Gauss10 and this will increase the signal-to-background ratio.

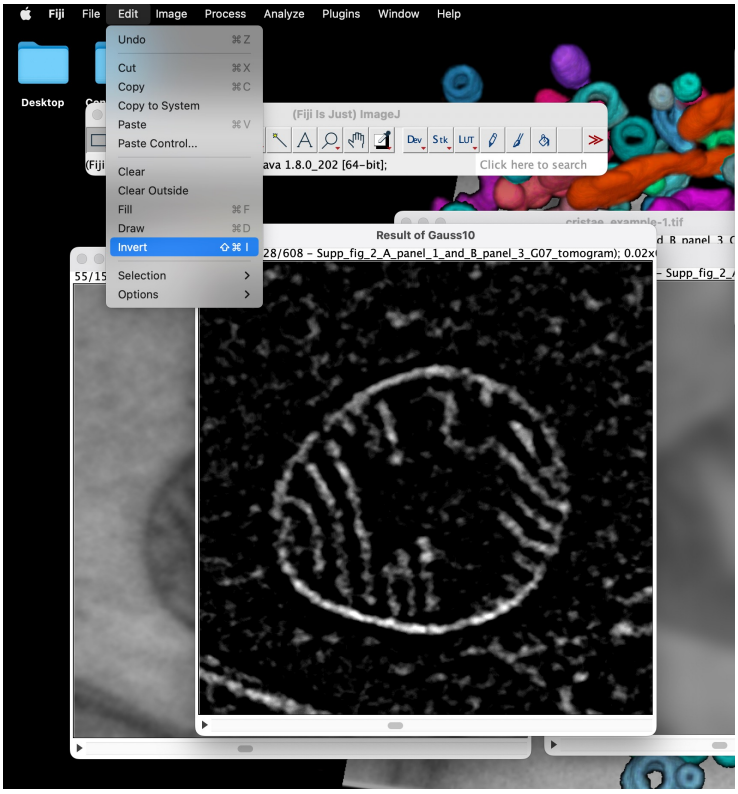


Go to **Process > Image Calculator...**

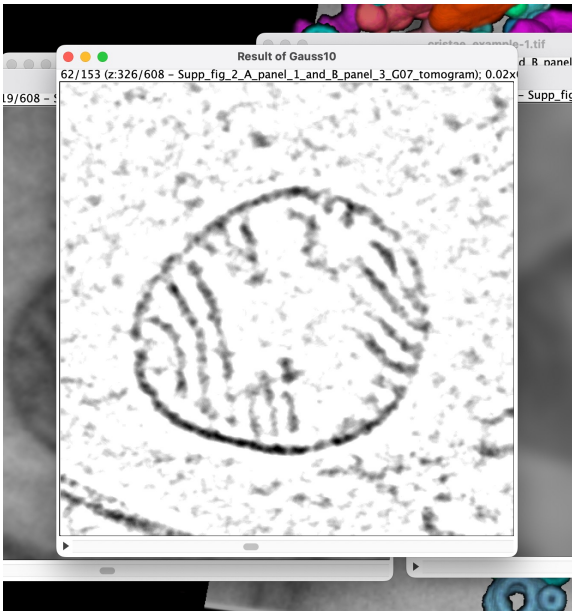


Fill in these fields with a Subtract operation.

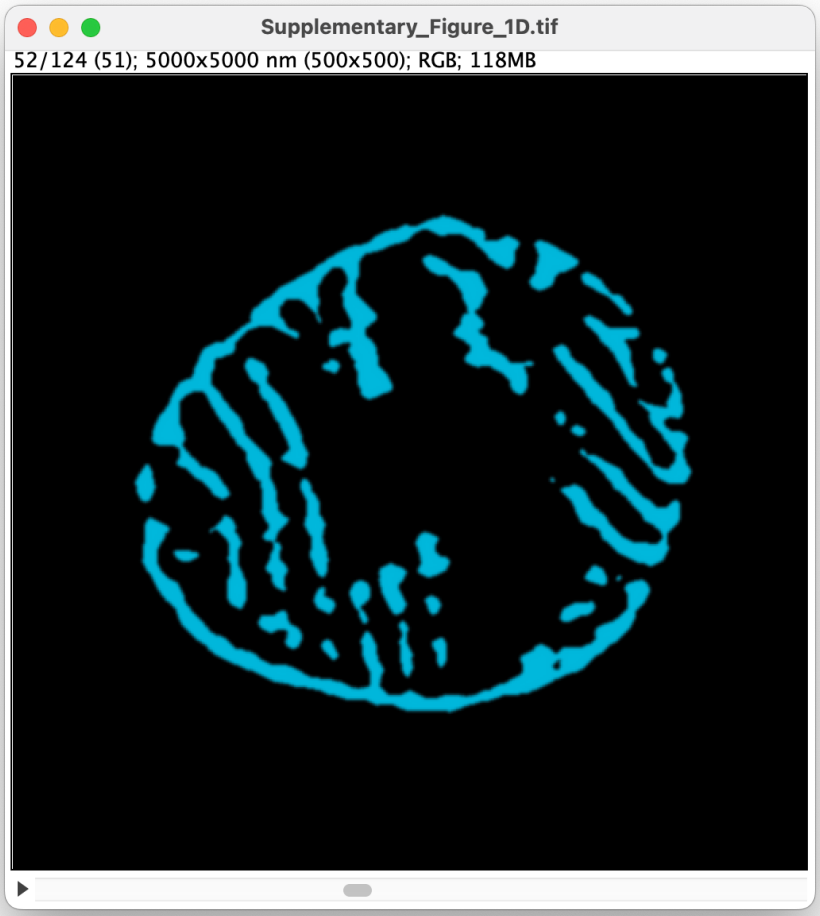
Adjust the brightness/contrast on the Result of Gauss10 window.



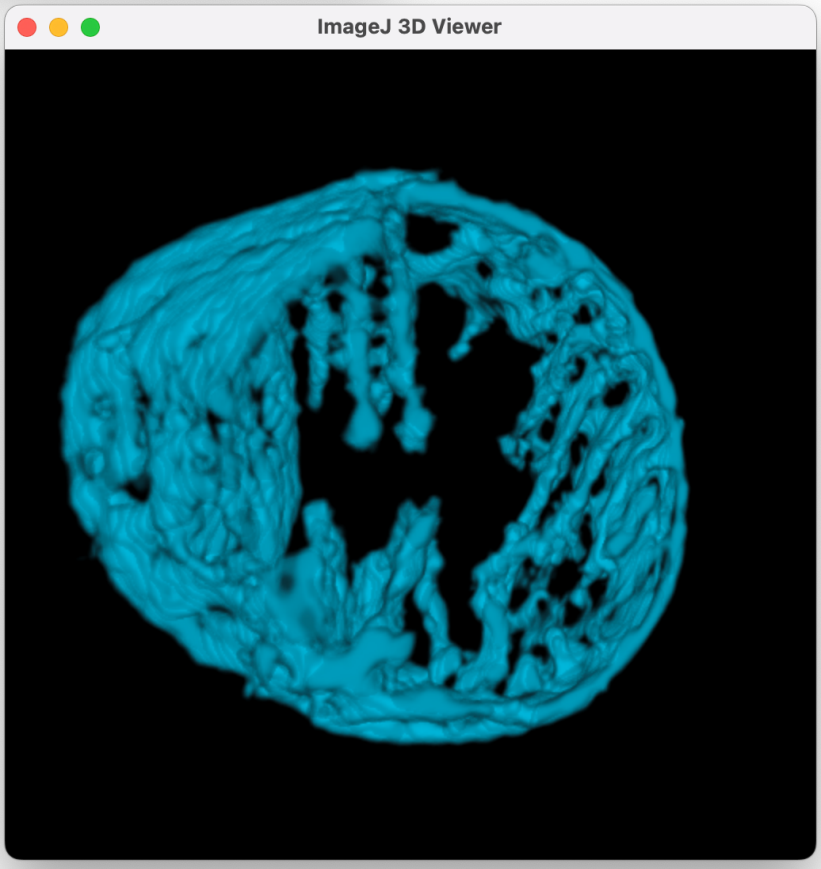
Go to **Edit > Invert**.



Try performing local segmentations on this stack.



You can segment the cristae by performing local segmentations and applying a minimum width.



Thank you for using Contour

If you have any queries or want to report bugs, please email contourqueries@gmail.com