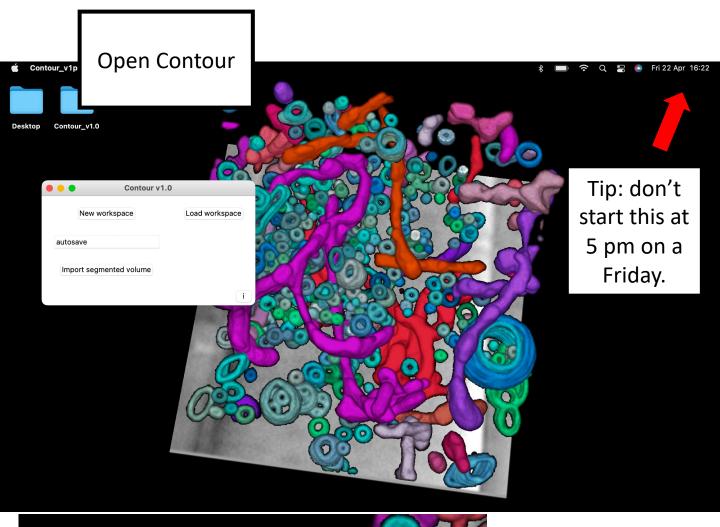
Contour

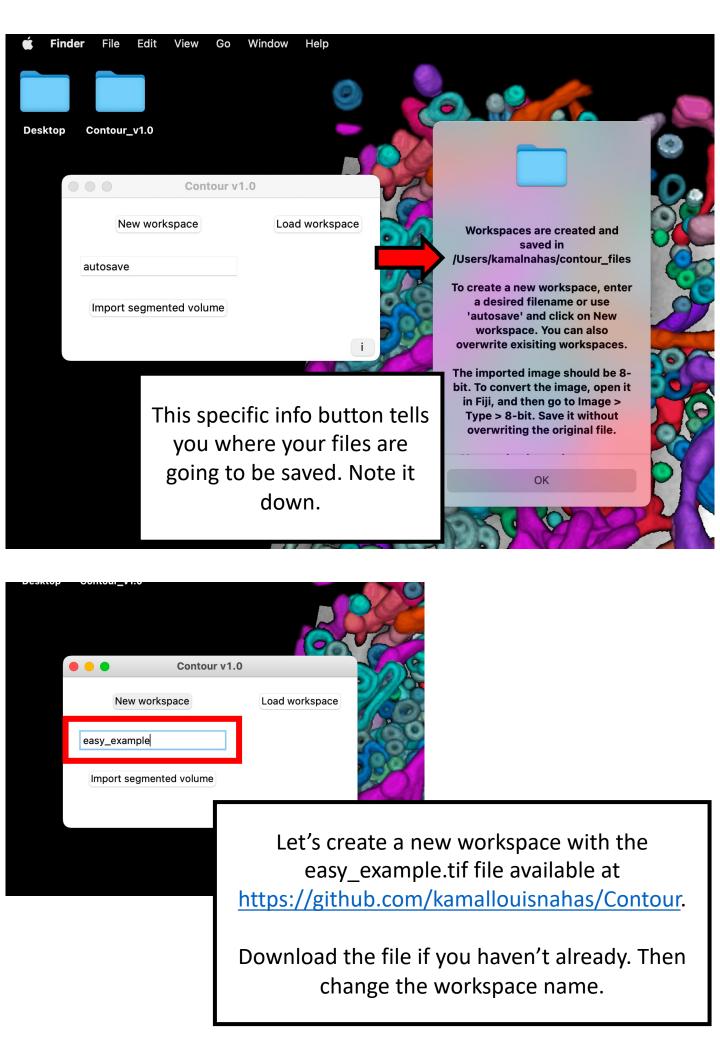
Instruction Guide v1.0

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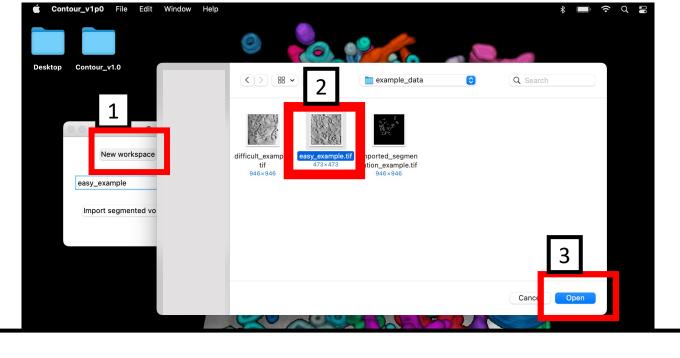
•••	Contour v	1.0
New w	orkspace	Load workspace
autosave		
Import segn	nented volume	23

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

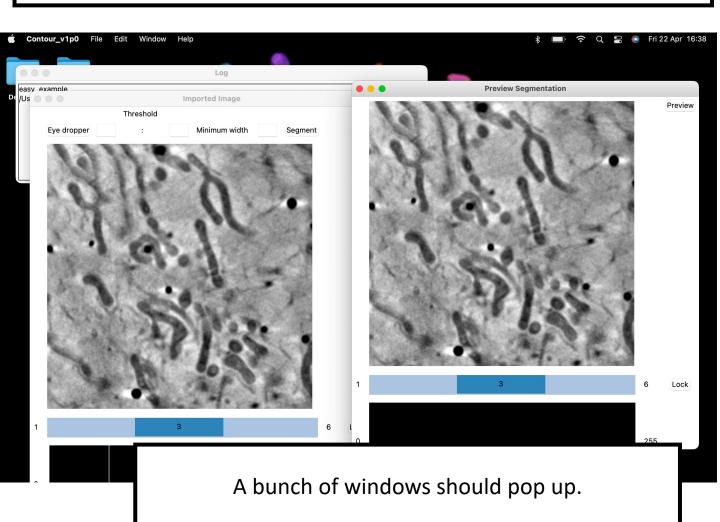


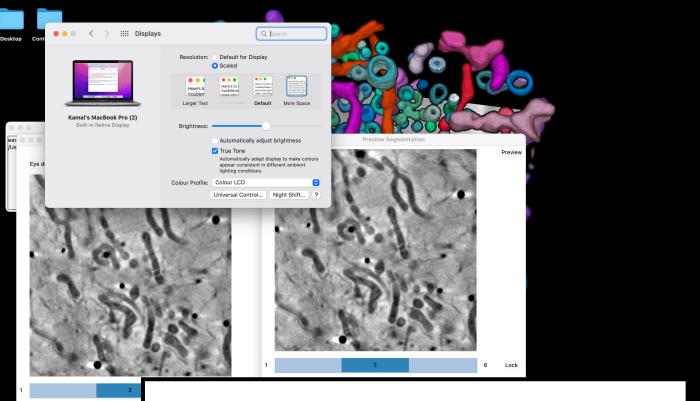
Global segmentation

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



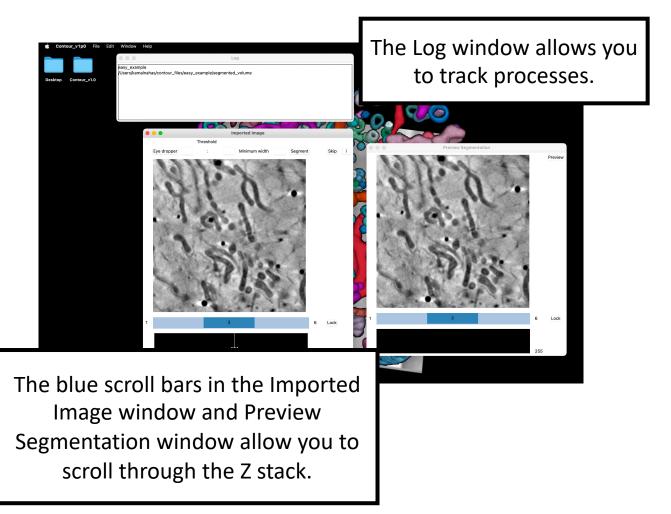
Click on New workspace, select the file, and open it. The image must be an **8-bit tiff stack and not an mrc file (e.g. rec)**.

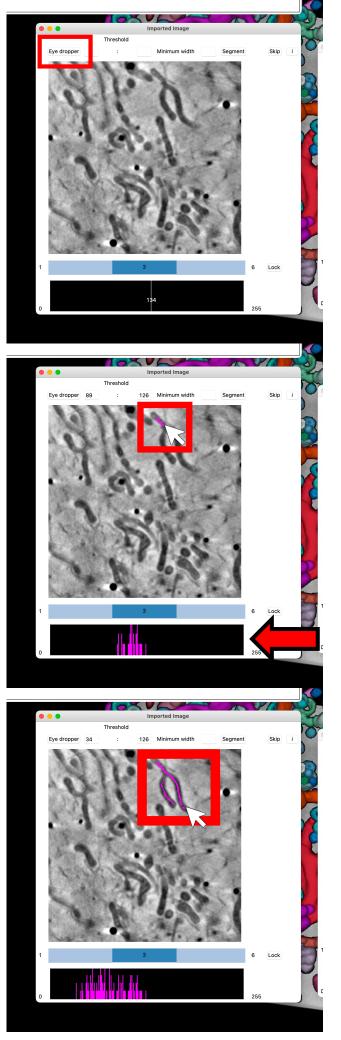




Alter your display settings if you need more space on the screen. On a Mac, go to **System Preferences > Displays > More Space**.

ri 22 Apr 16:39





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

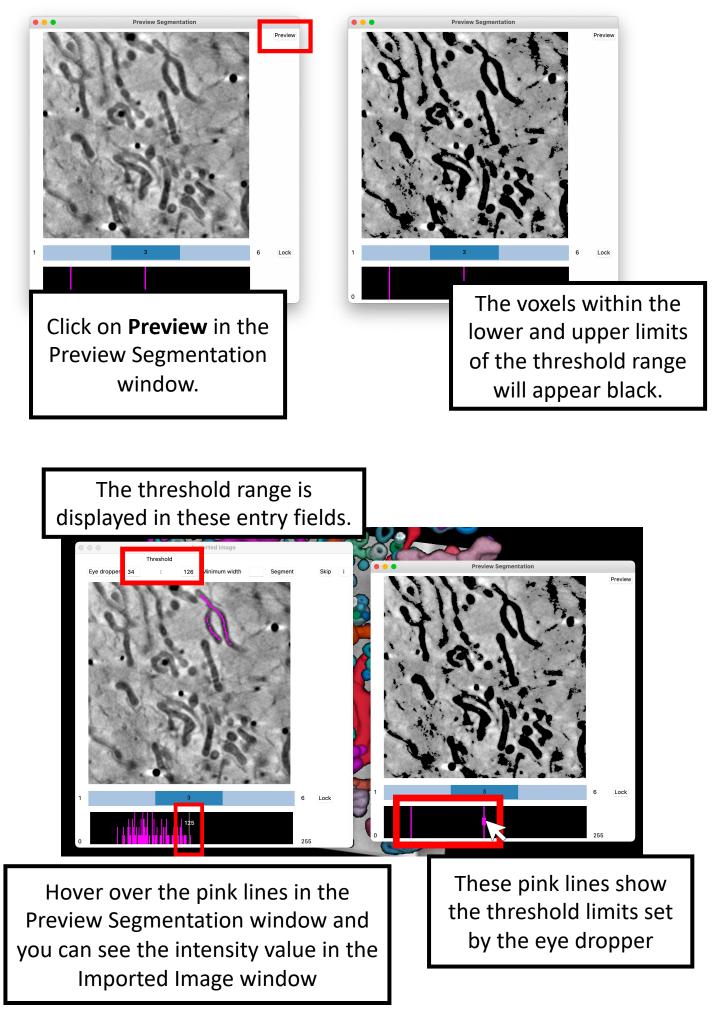
Click on Eye dropper.

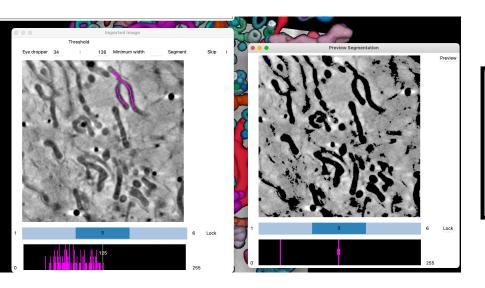
Now shade the <u>inside</u> of a mitochondrion – do not shade the edges.

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.

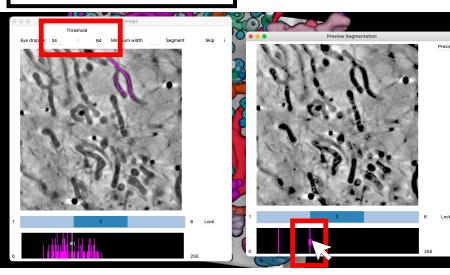
Shade in until you feel you've collected the full intensity range.



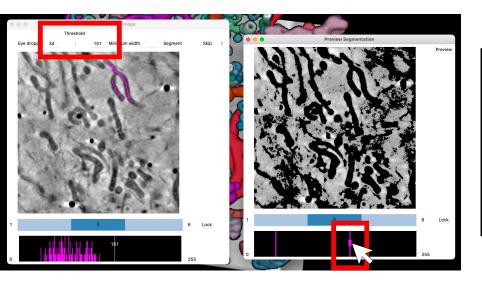


You can use the mouse to move the pink lines and alter the threshold range.

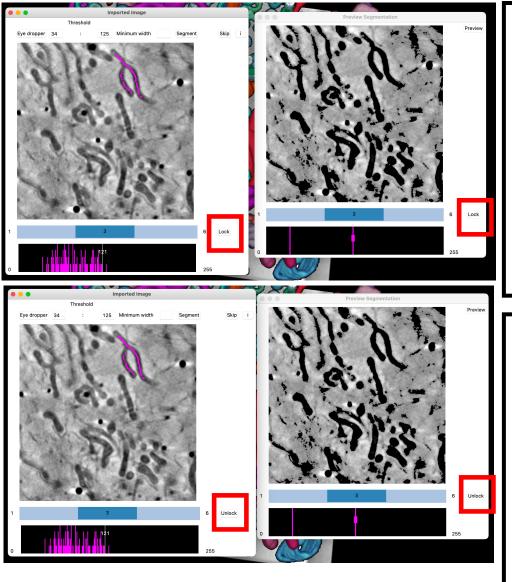
The threshold values readjust when you move the lines.



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.



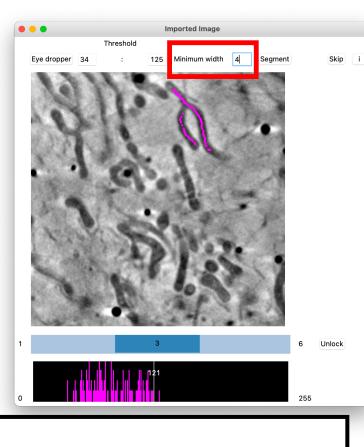
If you click on the Lock button on either window, the z stacks will be synced. You can scroll through both at the same time with either blue scroller.

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.

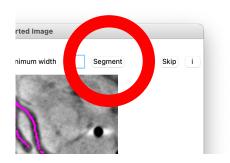


You don't have to use a minimum width. Try segmenting the lipid droplets later without a minimum width. Once you're happy with the threshold range you've selected, move on to the next parameter: Minimum width.

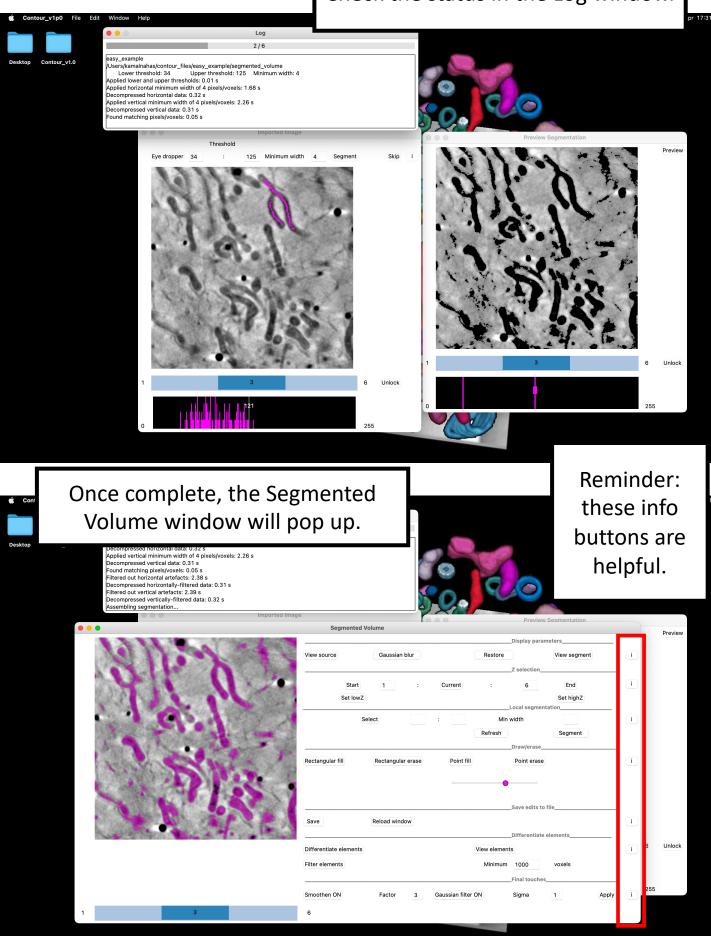
Type in **4** for these mitochondria.

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

Click Segment

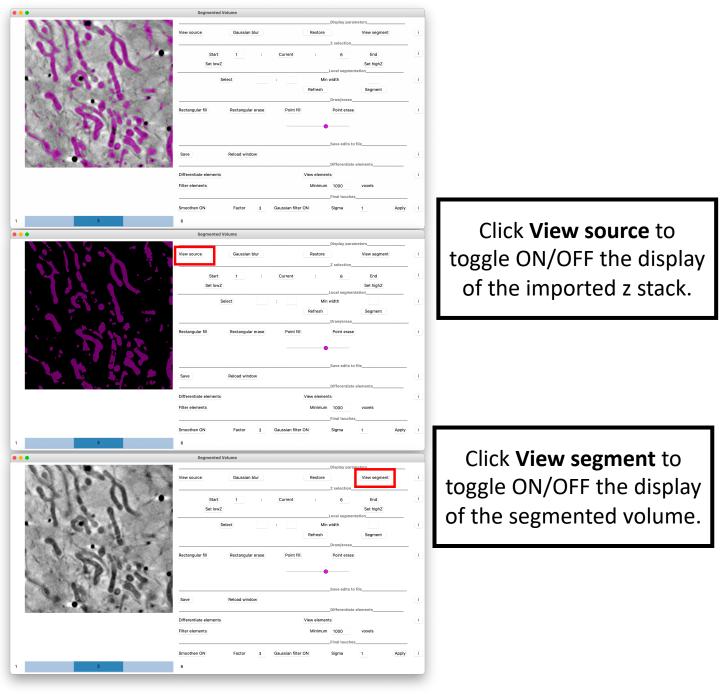


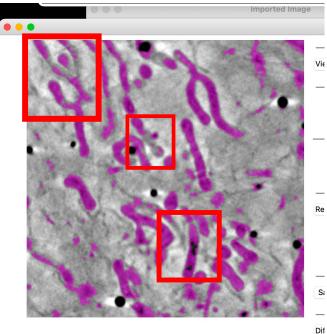
Check the status in the Log window.



Local segmentations

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





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

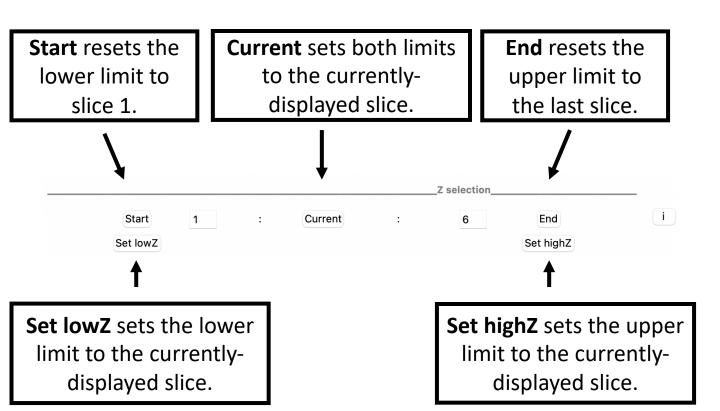


Let's fill in this area first.

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			Final touches
			Smoothen ON Factor 3 Gaussian filter ON Sigma 1 Apply i
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		1	6

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.



Select

__Local segmentation

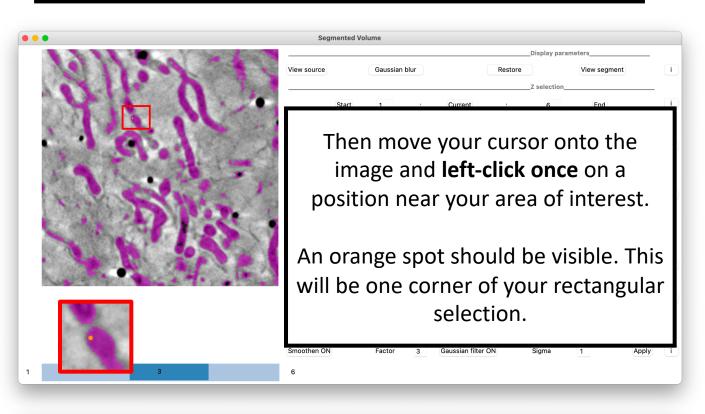
Min width

Refresh

Segment

i)

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



Segmented Volume

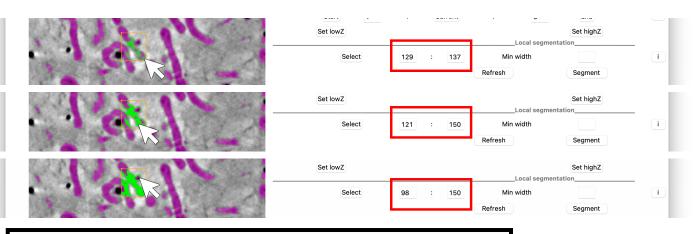
Move to the opposite side of your area of interest and **left-click a second time**.

Display parameters

This position will be the diagonallyopposite 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.



Set lowZ

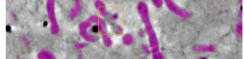
Try to pick up all the voxels of the mitochondria.



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

Set highZ

Local segmentation

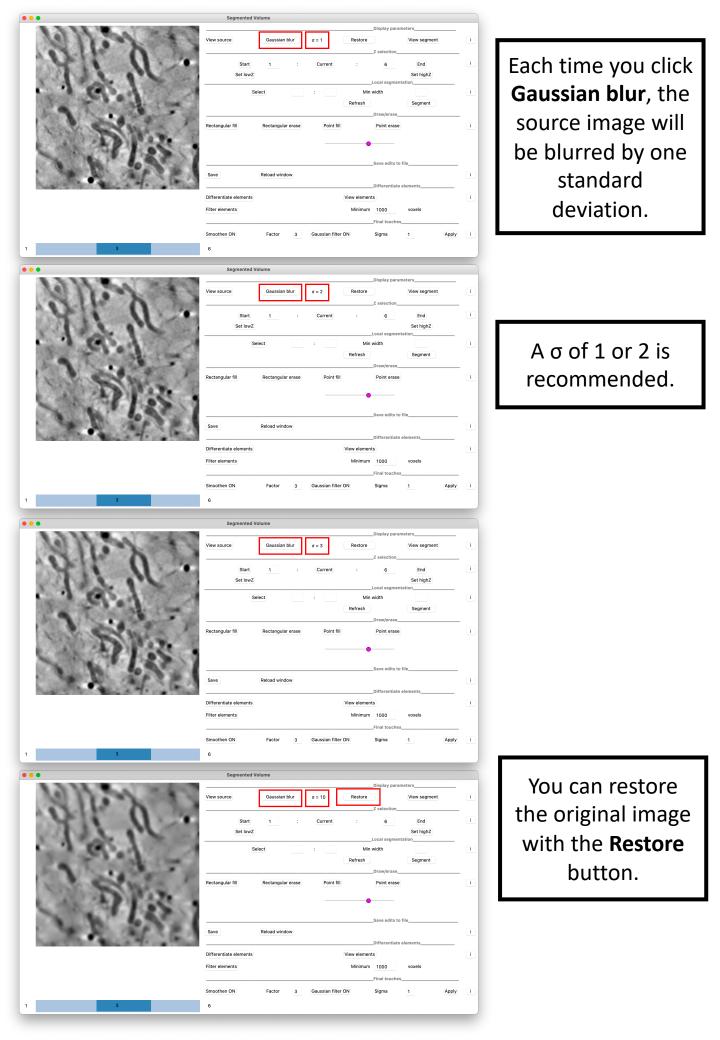


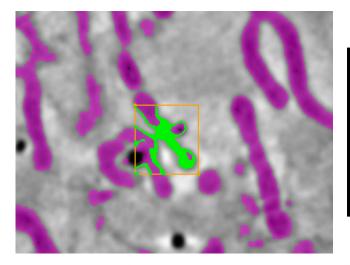


Tip: Sometimes the tomogram is too grainy and it's difficult to pick up all the desired voxels using the eye dropper.

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

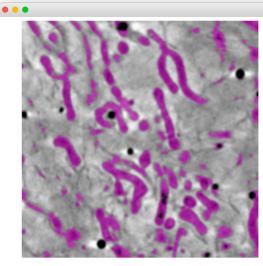
Final touches





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

	Segmented Volume	
10122	View source Gaussian blur σ = 2	Display parametersi Restore View segment i Z selection
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1 B 2/	Save Reload window	width (optional).
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		Final touches
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Segmented V	olume						
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				Refresh		Segment	
				D	raw/erase		
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Save Differentiate elements			Point fill	View elements Minimum	ave edits to f ifferentiate e 1000	file	-

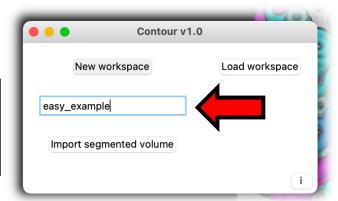
Fill in the remaining gaps and press **Save** frequently.

		● ● ○ Log		inequentity.	
Desktop	Contour_v1.0	Local seg: (3,86:61,103) in z (0:5) Lower threshold: 109 Upper thres Local seg: (35,72:77,97) in z (0:5) Lower threshold: 137 Upper thres Local seg: (18,52:203,261) in z (0:5) Lower threshold: 35 Upper thre Local seg: (20,40:247,272) in z (0:5) Lower threshold: 96 Upper th Local seg: (268,300:148,234) in z (0:5) Lower threshold: 96 Upper th Local seg: (383,367:236,277) in z (0:5) Lower threshold: 96 Upper th Local seg: (383,367:247,286) in z (0:5) Lower threshold: 114 Upper Local seg: (383,367:247,286) in z (0:5) Lower threshold: 22 Upper th Local seg: (387,412:265,289) in z (0:5) Lower threshold: 22 Upper th Local seg: (384,099:267,304) in z (0:5) Lower threshold: 20 Upper th Local seg: (394,099:267,304) in z (0:5) Lower threshold: 0.20 Upper th Local seg: (394,099:267,304) in z (0:5) Lower threshold: 0.20 Upper th Local seg: (394,099:267,304) in z (0:5) Lower threshold: 0.20 Upper th	hold: 147 Min width: 2 eshold: 150 Min width: 2 eshold: 151 Min width: 2 reshold: 132 Min width: 2 threshold: 154 Min width: 2 reshold: 62 Min width: 2	Display parameters_	
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		1 E.F -	Select 0	: 84 Min width 2 Refresh Segment Draw/erase_	
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			255		

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.



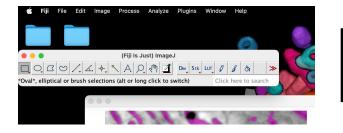
	< > easy_example	\equiv	· · · ·	···· ~	Q
	Name	Date Modified	Size	Kind	
	> 📄 global_segmentation_by_minimum_width	Yesterday at 17:31		Folder	
	>] global_segmentation_by_threshold	Yesterday at 17:31		Folder	
	> 🚞 log_files	Yesterday at 16:26		Folder	
	> 📄 python_files	Yesterday at 16:23		Folder	
	> 🚞 segmented_volume	Today at 18:01		Folder	
	🧾 Macintosh HD > 🧰 Users > 🛅 kamalnahas > 🚞 contour_	files > 🚞 easy_example			
Snet					

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.

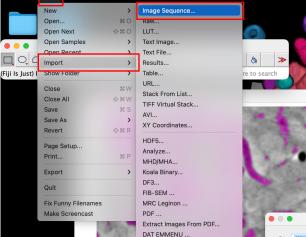
Open segmented_volume.

•••	< > segmented_volume	≔≎	······································	v ⊂	2
	Name	A Date Modified	Size	Kind	
	000.tif	Today at 18:01	895 KB	TIFF image	
	001.tif	Today at 18:01	895 KB	TIFF image	
	002.tif	Today at 18:01	895 KB	TIFF image	
	003.tif	Today at 18:01	895 KB	TIFF image	
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	005.tif	Today at 18:01	895 KB	TIFF image	
	Г				
Tans	💭 Macintosh HD > 🧰 Users > 🛅 kai	Each slice is stored file, with the first			



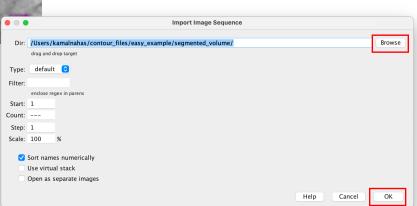
Analyze

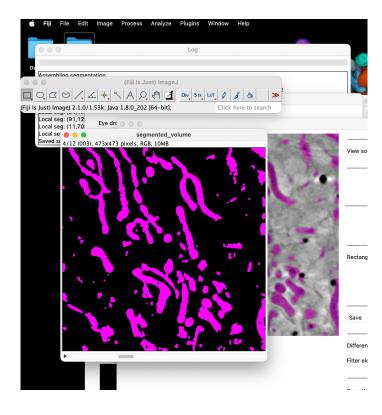
Open Fiji to view the slices together in a single stack.

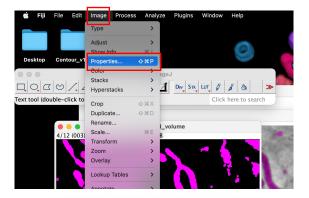


Go to File > Import > Image Sequence...

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







🛑 🔵 🌒 segme	ented_volume	•
Channels (c): Slices (z): Frames (t): Note: c*z*t mu	12 1	
Pixel width: Pixel height: Voxel depth:	10	nm _ _
Frame interval: Origin (pixels): Invert Y Global		
C	ancel	ОК

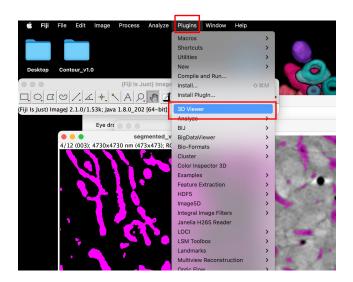
This will compile all the individual tif slices into one stack.

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 \times 9.46 µm field of view, with each voxel being 10 \times 10 \times 10 nm. Therefore the slices have dimensions of 946 \times 946 voxels. However, this stack has been downsized by a factor of 2 and has the dimensions 473 \times 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.



ImageJ 3D Viewer ted volun r threshold Add ... er thresh ted_volur er thresh Image segmented_volume 📀 er thresh r thresho Name segmented v r thresho Display as Volume ted_volur Color None Threshold 0 Resampling factor 2 Channels 150 🗸 red 🗹 green 🛛 V blue Start at time point 0 ОК Cancel Point fill Reload window

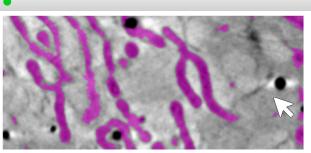
After you've adjusted the scale, go to **Plugins > 3D Viewer**.

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/nonspecific elements.

	Segmented Volume	Go back to the
31.9	View source Gaussian blur Start 1 : Set lowZ	Segmented Volume window.
1.12	Select 135	: 150 Min width 2 i Refresh Segment
Striz'	Rectangular fill Rectangular erase	Draw/erasei Point fill Point erase i
1 1 2 M	Save Reload window	Save edits to file
	Differentiate elements Filter elements	View elements i Minimum 1000 voxels
1 3	6	a need to use manual tools get rid of noise and non- specific material.
	Segmented Volume View source Gaussian blur Start 1 : Set lowZ Select 135	
in the second	Rectangular fill Rectangular erase]
CONTRACT CONTRACTOR AND	-	erase and left-click once area you want to delete.
A re	•	appear and will mark one rectangular selection.
•••		

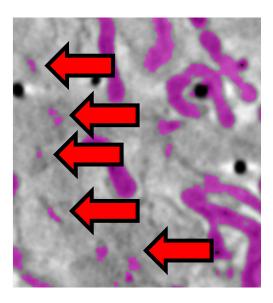


Left click a second time to mark the diagonally opposite point of your rectangular selection. This will delete the segmented elements within the rectangle.

Reminder: which slig	-						
					Z selection		
Start	1	:	Current	:	6	End	i
Set lowZ						Set highZ	

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

			Draw/erase	
Rectangular fill	Rectangular erase	Point fill	Point erase	i
	_			
		If you use the size	point options, you of the points with	ı can adjust 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.

Reload window

Save edits to file

Differentiate elements_

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

i

Segmented Volume

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

Contour v1.0

Load workspace

Load workspace

Open

autosave

Import segmented volume

New workspace

New workspace

autosave

Contour_v1.0

Desktop

G32_imported G72 G46_early_imported U2OS_G07_mito_cristae F34_mito G60_early_imported easy_example F03_ves_by_contour_Gauss_

G76_ves

G63_ves

Contour v1.0

Import segmented volume

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.

(i)

Save edits to file_

Save

Reload window

Great, try Reload window again.

Differentiate elements

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

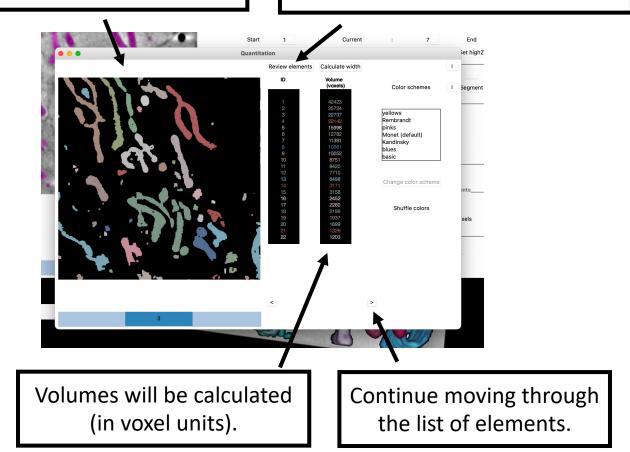
• •	•	Segmen	nted Volume				
	MWW -	View source	Gaussia	n blur	Display par Restore Z selection	ameters View segment	- I
	· M. M.		art 1 IowZ	: Current	: 7	End Set highZ	
	1.9.1		Select	:	Local segme Min width Refresh	2 Segment	1
C State		Rectangular fill	Rectangu	ar erase Point fill	Draw/erase		1
	S S S S S	Save Differentiate elements Smoothen ON	Reload wi ents Factor	segmen bi	ou're hap tation (ig ts of nois rentiate	gnoring se) click	small
1	5	6	•				

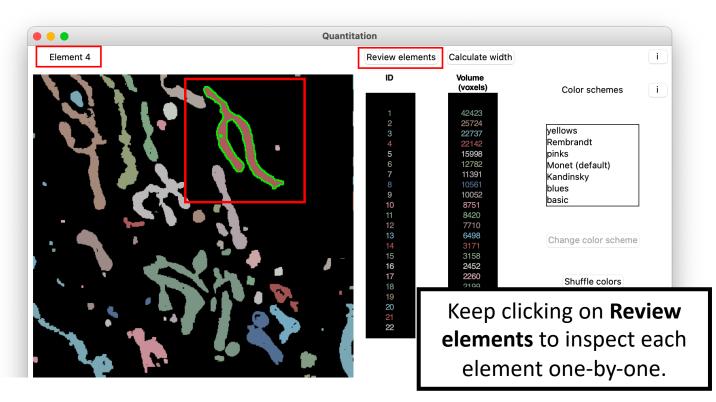
	Log
NUMBER OF ELEMENTS:78 NUMBER OF ELEMENTS:78 STARTING SLICE 3 NUMBER OF ELEMENTS:124 NUMBER OF ELEMENTS:91 NUMBER OF ELEMENTS:79 NUMBER OF ELEMENTS:77 NUMBER OF ELEMENTS:77 STARTING SLICE 4	You can track the progress in the Log window.
NUMBER OF ELEMENTS:144	

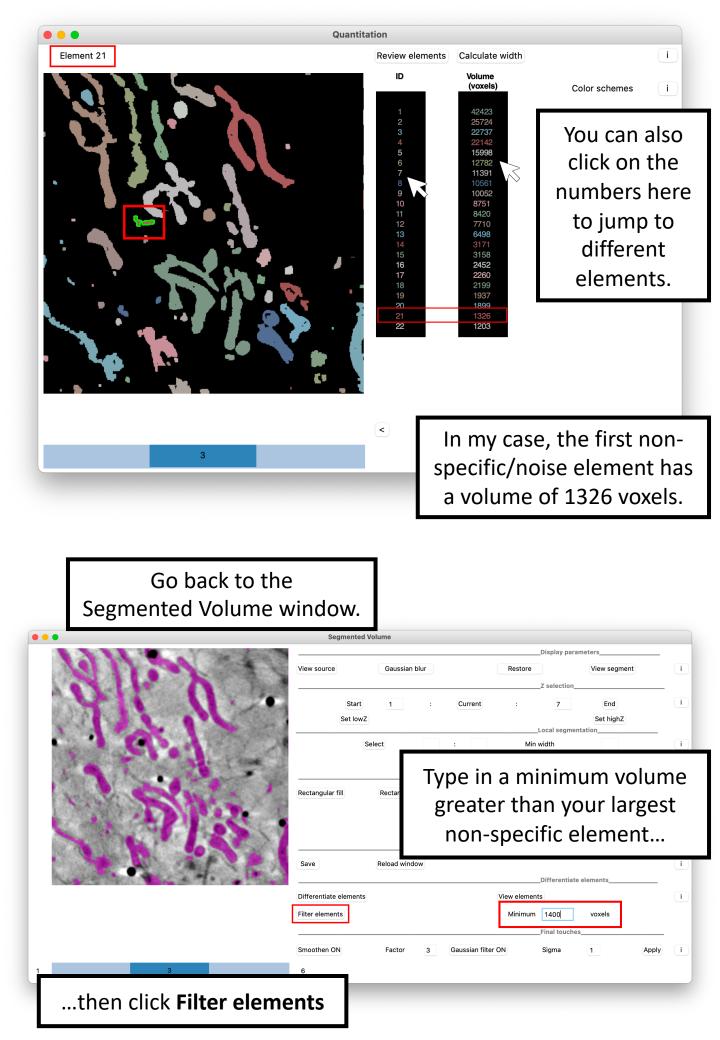
Log Z REVERSE GROUPING SLICES 4 and 3 Z REVERSE GROUPING SLICES 3 and 2 Z REVERSE GROUPING SLICES 2 and 1 Z REVERSE GROUPING SLICES 1 and 0 3D GROUPING TIME:1.12 s Once you see this text, the Colored/filtered segmented volume: 0.56 s Loaded quantitative information process has completed. It Loaded grouped voxels Colored/filtered segmented volume: 0.55 s may still take a few Saved quantitative data to file: easy_example_quantitation/ moments for the next window to load.

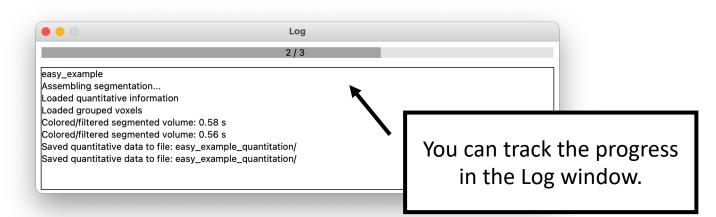
Separate mitochondria will be color coded.

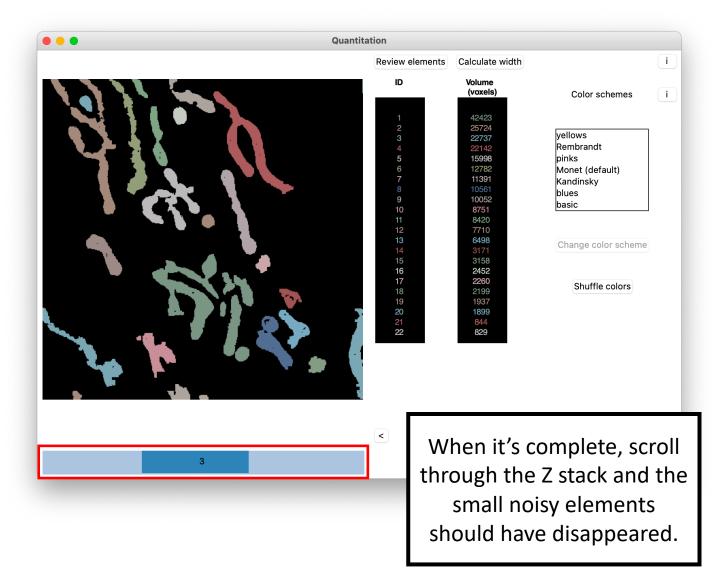
You can use **Review elements** to inspect each element one at time in descending order of volume.









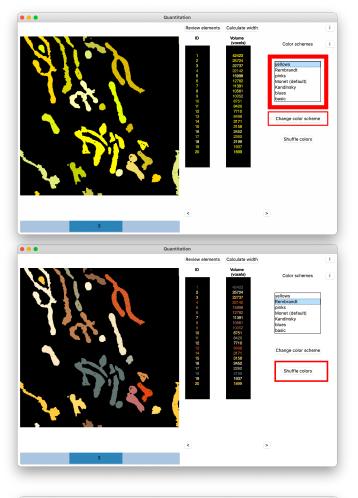


easy_example		• û ⊘ ⊙• Q
Name	A Date Modified	Size Kind
> 🚞 differentiated_elements	Today at 13:30	Folder
> indifferentiated_elements_colored	Today at 14:08	Folder
> indifferentiated_elements_filtered	Today at 14:08	Folder
> 🚞 log_files	Today at 14:07	Folder
> python_files	Today at 13:30	Folder
> 🚞 quantitation	Today at 14:08	Folder
> 🚞 quantitation_unfiltered	Today at 14:08	Folder
> 🚞 segmented_volume	Today at 13:43	Folder
> begmented_volume_filtered	Today at 14:08	Folder
ions		e new files in the tput folder.

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 <u>un</u> differentiated segmented elements after applying the size filter.

Final touches

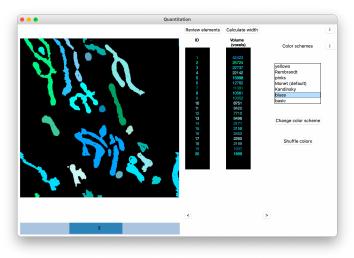
Polish the segmented volume.

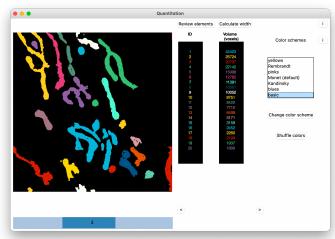


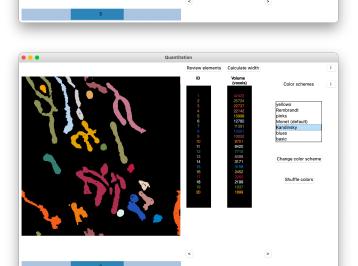
Select a color scheme from the scroll list and click **Change color scheme**.

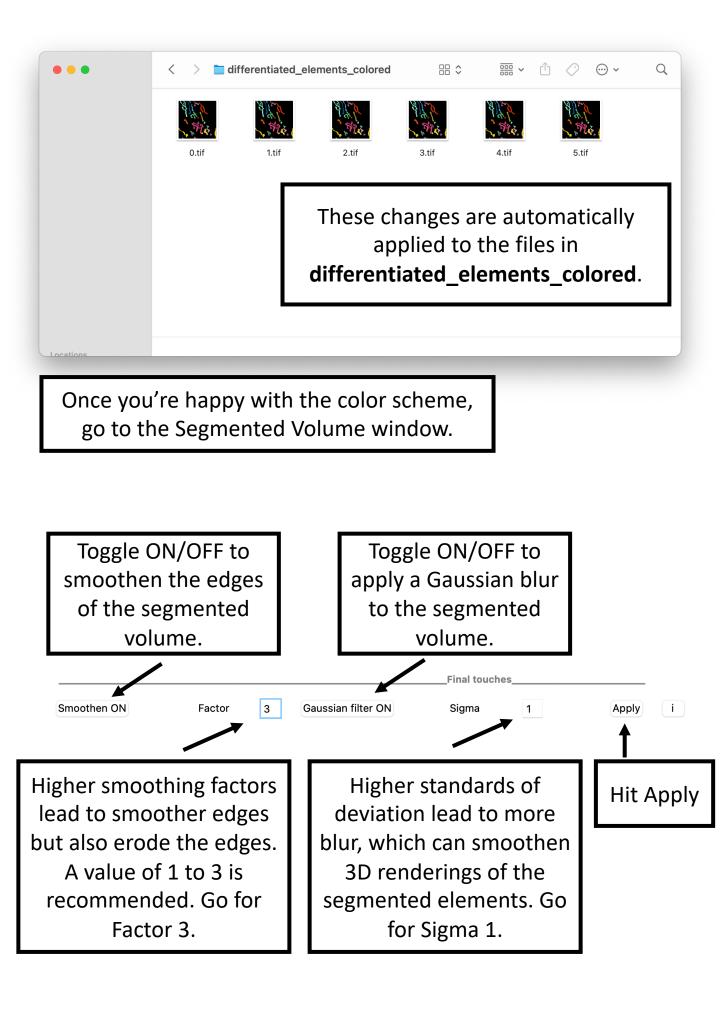
Monet is the default scheme.

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.









Name	Date Modified ~	Size Kind
differentiated_elements_final_touches	Today at 14:41	Folder
> smoothened_colored_stack	Today at 14:41	Folder
> differentiated_elements_colored	Today at 14:39	Folder
> segmented_volume_filtered	Today at 14:08	Folder
> differentiated_elements_filtered	Today at 14:08	Folder
> quantitation	Today at 14:08	Folder
> quantitation_unfiltered	Today at 14:08	Folder
> log_files	Today at 14:07	Folder
> segmented_volume	Today at 13:43	Folder
> in python_files	Today at 13:30	Folder
> differentiated_elem		
> differentiated_elem	v folder will appe directory c	ar in your outp alled
A new differentiated_elem	v folder will appe	ar in your out alled ts_final_toucl



Import this image sequence into Fiji as you did previously.

Adjust the scale to 10 nm \times 10 nm \times 5 nm.

Then load it into 3D Viewer.

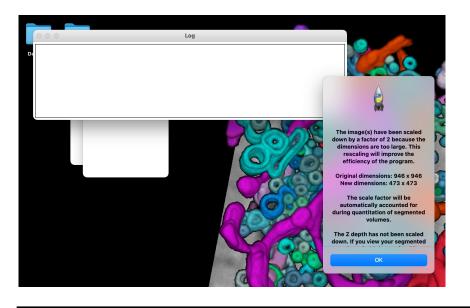


Difficult example

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

É Contoi Desktop	Open Cor	ntour
	 Contour v' 	1.0
	New workspace	Load workspace
	autosave	ī

Contou	r v1.0	
New workspace	Load workspace	
difficult_example		
Import segmented volume		
	Let's create a	new workspace with the
	difficult_exa	mple.tif file available at m/kamallouisnahas/Conto
		e if you haven't already. The he workspace name.
	Click New wor	kspace 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.

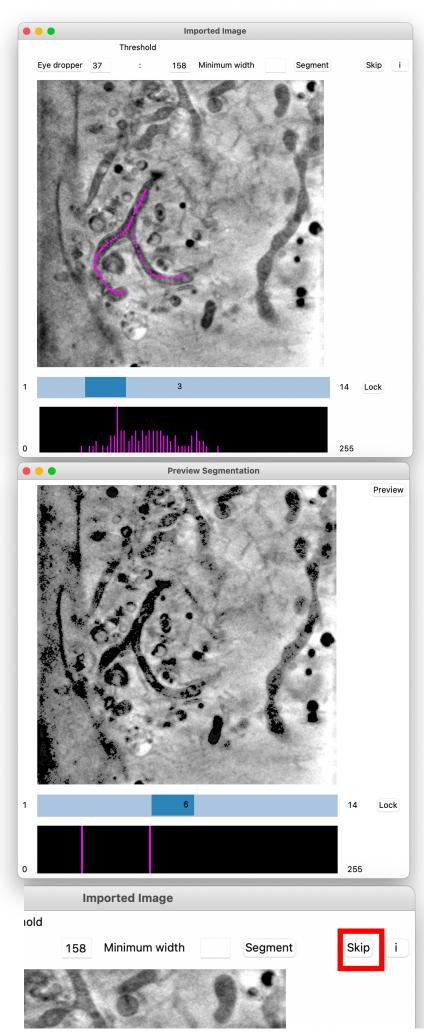
The example tomograms were collected with a 946 \times 946 pixels field of view and need to be halved to 473 \times 473.

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 mitochondriaspecific 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.

	Segmented	d Volume	
1 3 5 8 9	View source	Display param Gaussian blur Restore	View segment
· · · · · · · · · · · · · · · · · · ·	Start Set low		End i Set highZ
1. 3/3		Select : Min width	sationi
36 3	Rectangular fill	Draw/eraseDraw/eras	i
1 5 1 1 1		Save edits to f	ile
	Save	Reload windowDifferentiate e	lements
	Differentiate elements	s View elements	1
1 6	Filter elements Smoothen ON 14	This will give you a Se	gmented
		Volume window with segmented volume	a blank

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

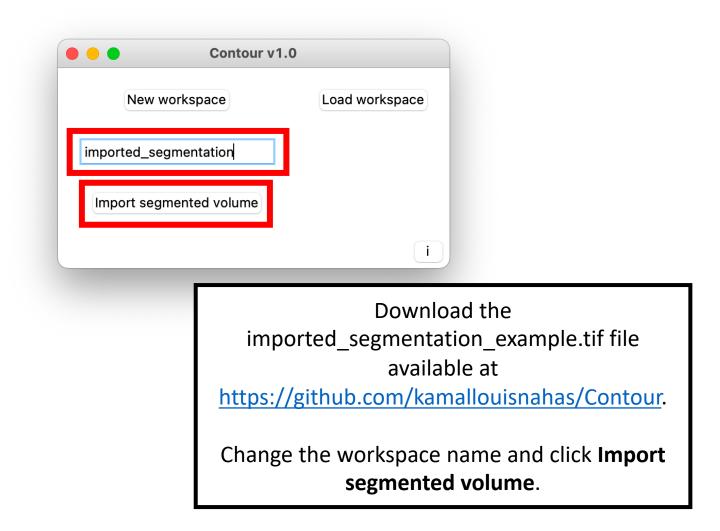
If you used global segmentation, you would generate a lot of noise and non-specific material that would be time-consuming to erase.

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.

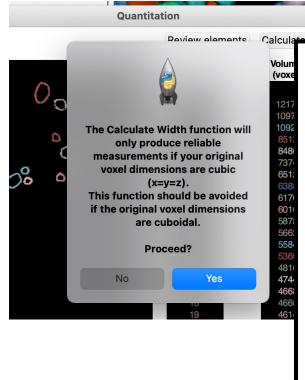
If you segment features in another program, you can import the segmented volume into Contour to quantitate the elements.

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.



Log The image(s) have been scaled down by a factor of 2 because the dimensions are too large. This rescaling will in Original dimensions: 946 x 946 New dimensions: 473 x 473	mprove Segmented Volume	
The scale factor will be aut	Display parameters	
The Z depth has not been s Assembling segmentation.	View source Gaussian blur Restore View segment	1
	Start 1 : Current : 30 End Set lowZ Set highZ	1
°°°°	Refresh Segment	1
	Draw/erase	1
	Save edits to file	i
	Filter elements Minimum 1000 voxels	Ŀ
	Smoothen ON Factor 3 Gaussian filter ON Sigma 1 Apply	1
The Segmented Volume windo Click Save and run Different		

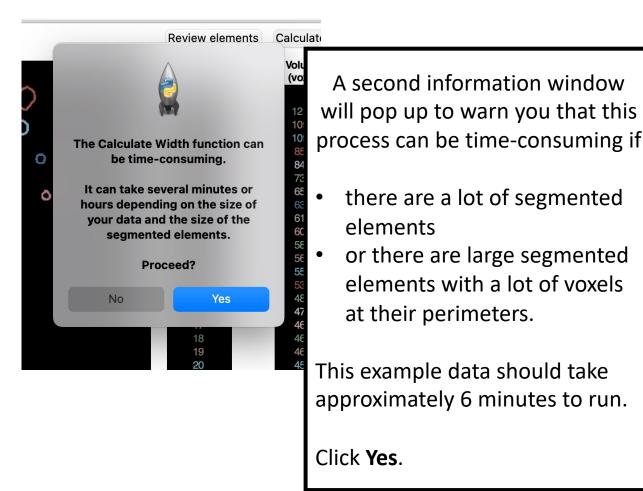
Quantitation • • • **Review elements** Calculate width i ID Volume (voxels) (i Color schemes 10976 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 0 yellows 10926 Rembrandt pinks 8486 7374 Monet (default) 0° 6512 Kandinsky blues 6170 basic 00 6016 5878 5662 5584 Change color scheme 0 CO 4816 \circ 4744 000 4668 Shuffle colors 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.

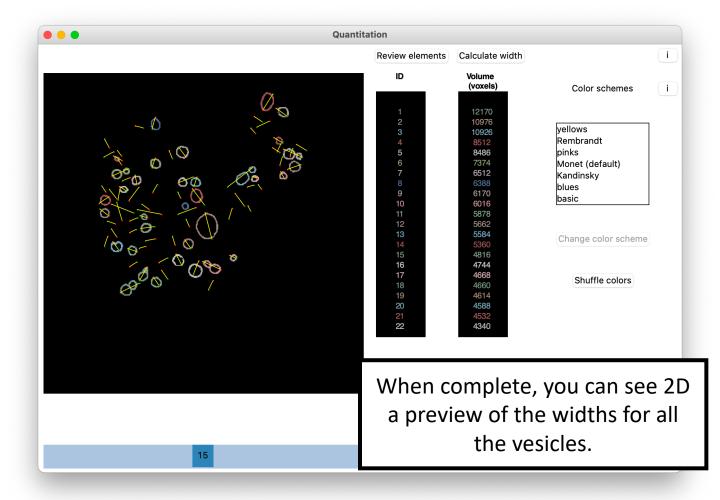


An information window will pop to let you know that this function will only work reliably on data where the original voxel dimensions are cubic.

E.g. 10 nm \times 10 nm \times 10 nm for the tomograms.

Click Yes.

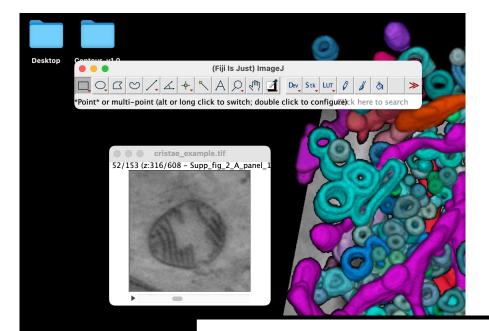




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	5	8486	72.24956747									
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-	10	6016	70.25667228									
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	15	4816	54.36910888									
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	17	4668	73.70210309									
	18	4660	54.62600113									
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-	20	4588	54.44263036									
-	21 22	4532 4340	52.38320341									
-	22	4340	68.87670143 50.99019514									
	23	4106	49.1934955									
	25	3964	49.1934955									
	26	3780	52.49761899									
	27	3418	48.90807704									
	28	3334	45.16635916									
-	29	3188	44.45222154									
F	30	3026	46.56178691									
	31 32	2858 2696	42.80186912									
	32	2696	74.02702209 41.27953488									
-	34	2528	52.26853738									
	35	2356	39.29376541									
	36	2314	37.52332608									
	37	2218	39.44616585									
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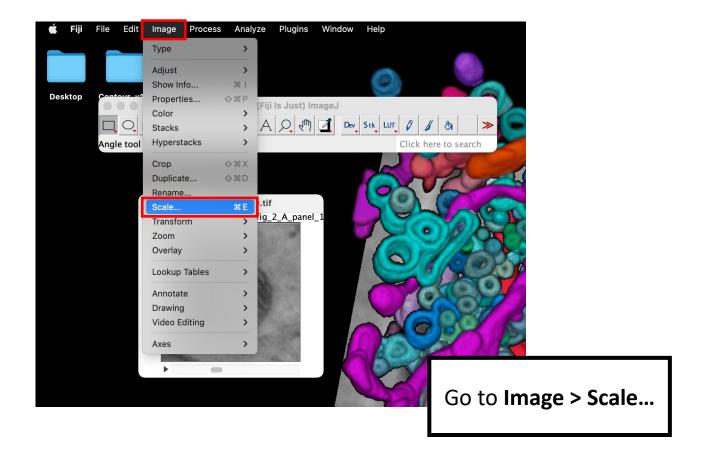
Segmenting cristae

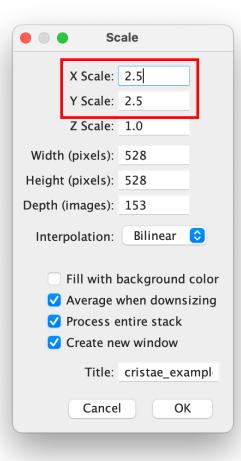
It is possible to segment cristae using Contour but there are a few more steps you need to do first in Fiji.



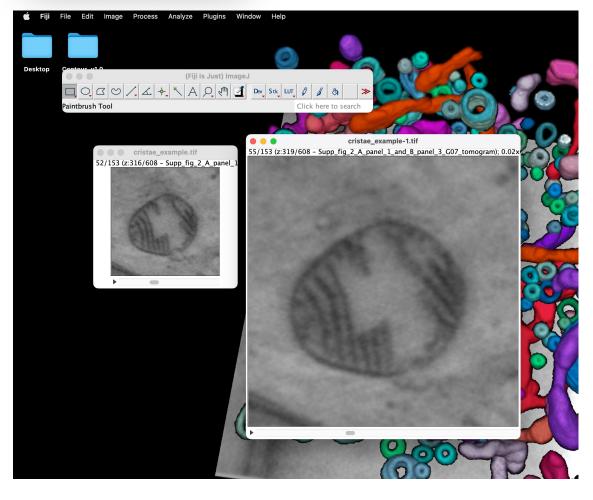
Download the file cristae_example.tif from https://github.com/kamallouisnahas/Contour.

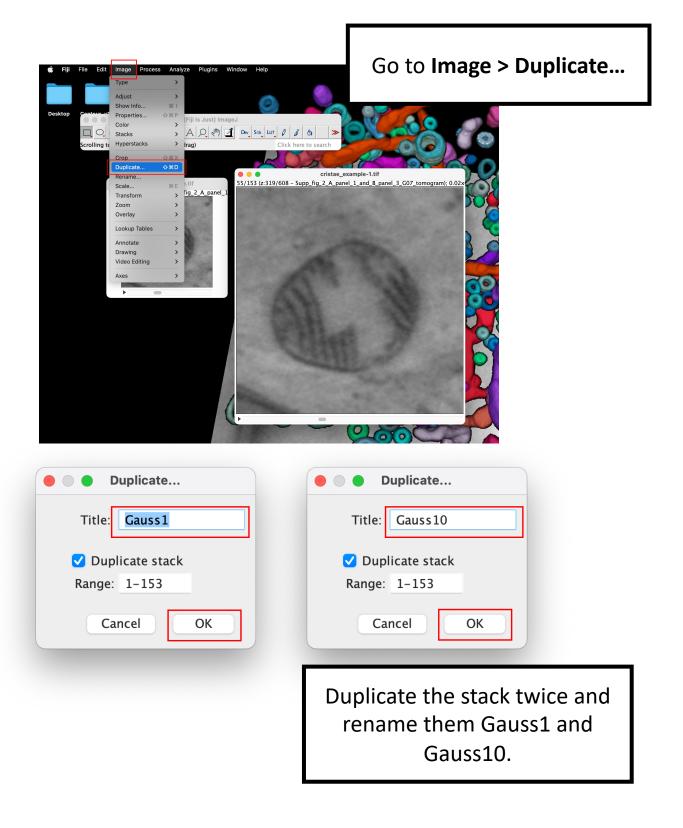
Open it in Fiji.

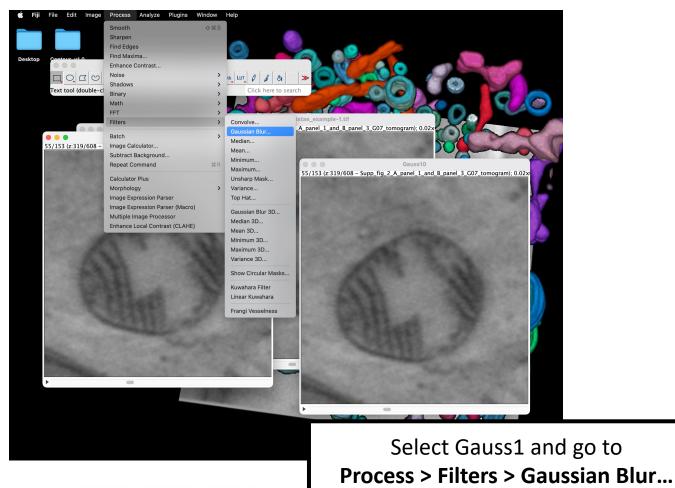


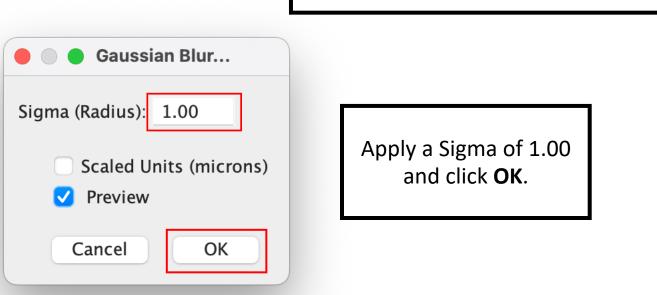


Increase the XY scale by 2.5 times. This will increase the voxel width of the cristae to make it easier to apply a minimum width.





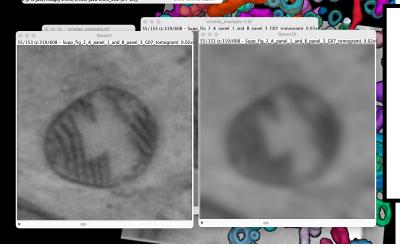




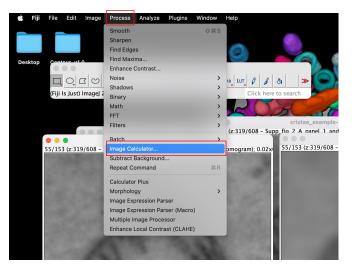
Repeat this for Gauss10 but apply a Sigma of 10.

 (Fiji Is Just) ImageJ

 ImageJ
 <t



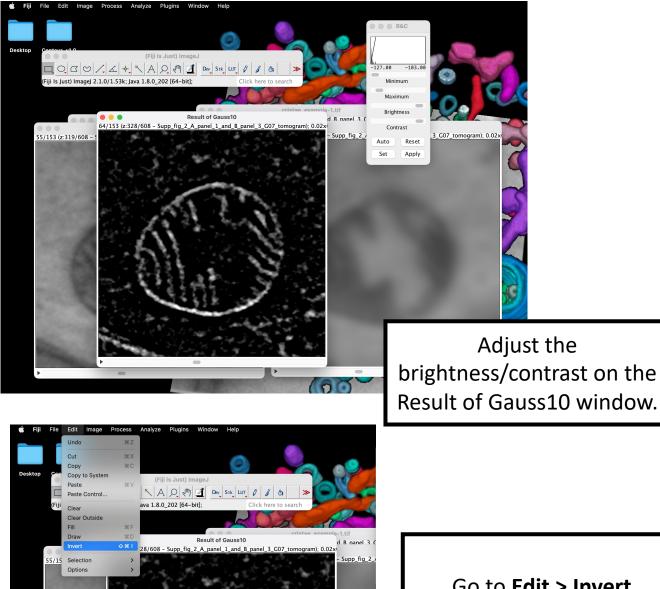
Now we're going to subtract Gauss1 from Gauss10 and this will increase the signal-tobackground ratio.



Go to Process > Image Calculator...

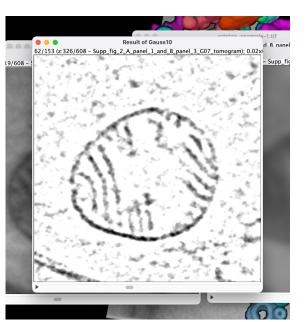
Image Calculator \bigcirc Gauss10 Image1: **Operation**: Subtract \bigcirc \Diamond Gauss1 Image2: 🗸 Create new window 32-bit (float) result Help Cancel OK

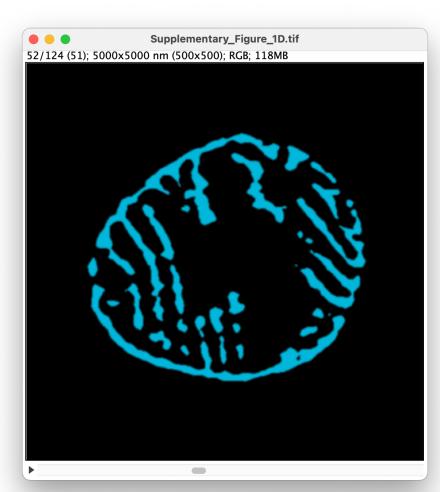
Fill in these fields with a Subtract operation.



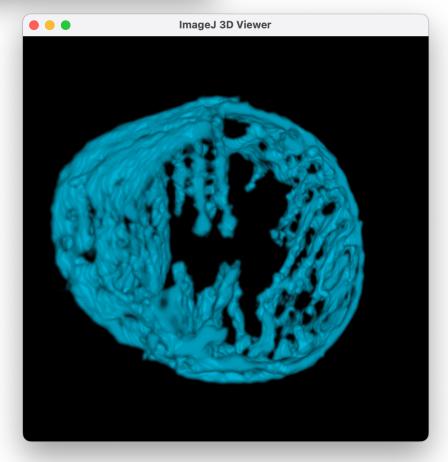
Go to Edit > Invert.

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 <u>contourqueries@gmail.com</u>