OpenSegSPIM

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OpenSegSPIM

A Quick User Guide

Version 1.1

Contents

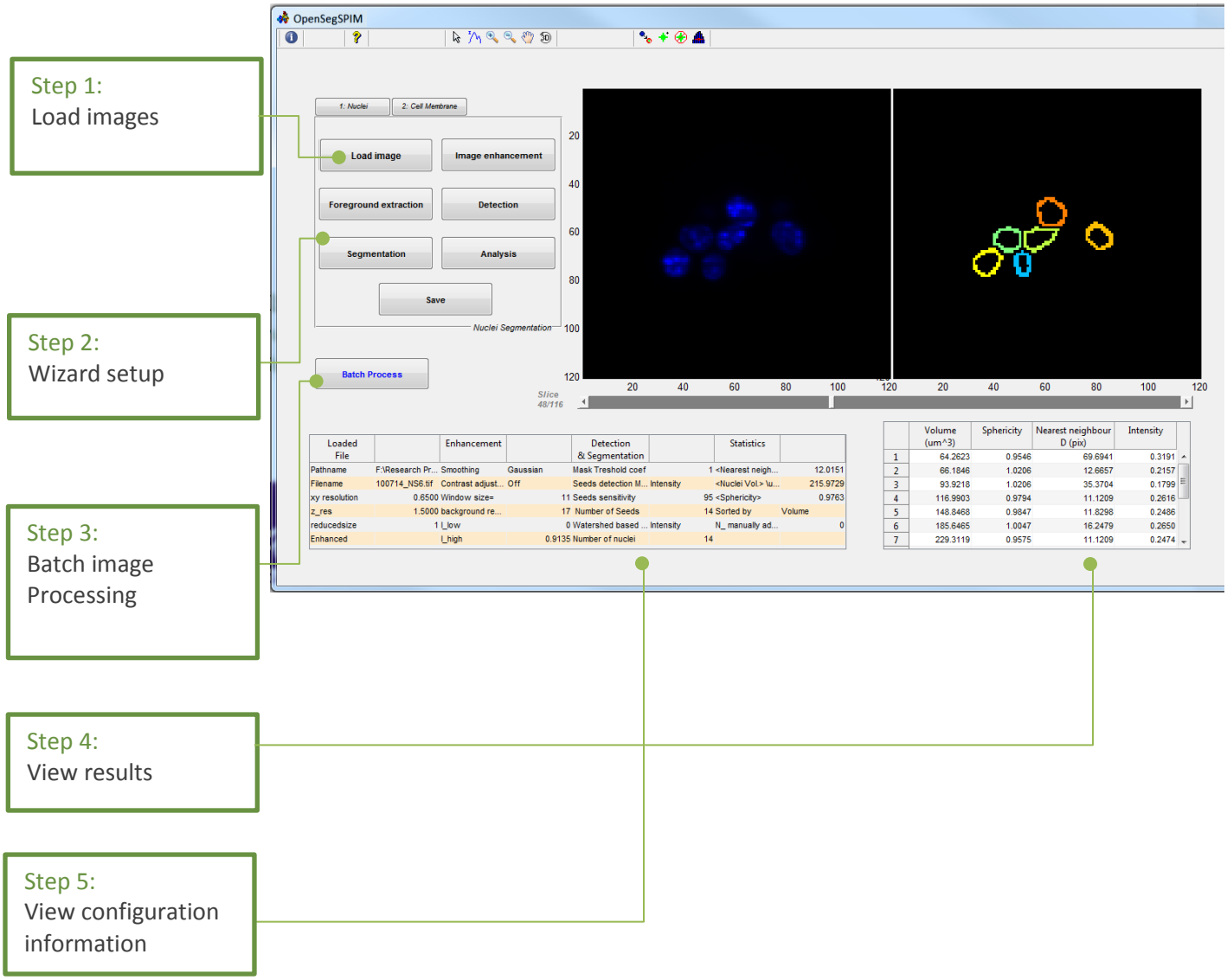
Tips: *A useful hint to the user of OpenSegSPIM when they operate the application.*

Image Processing Note: *A piece of technical information for readers, who are interested in more details of undertaking image processing operations.*

Important Note: *An important message required reader's attention during the operation of OpenSegSPIM.*

Chapter 1: OpenSegSPIM interface

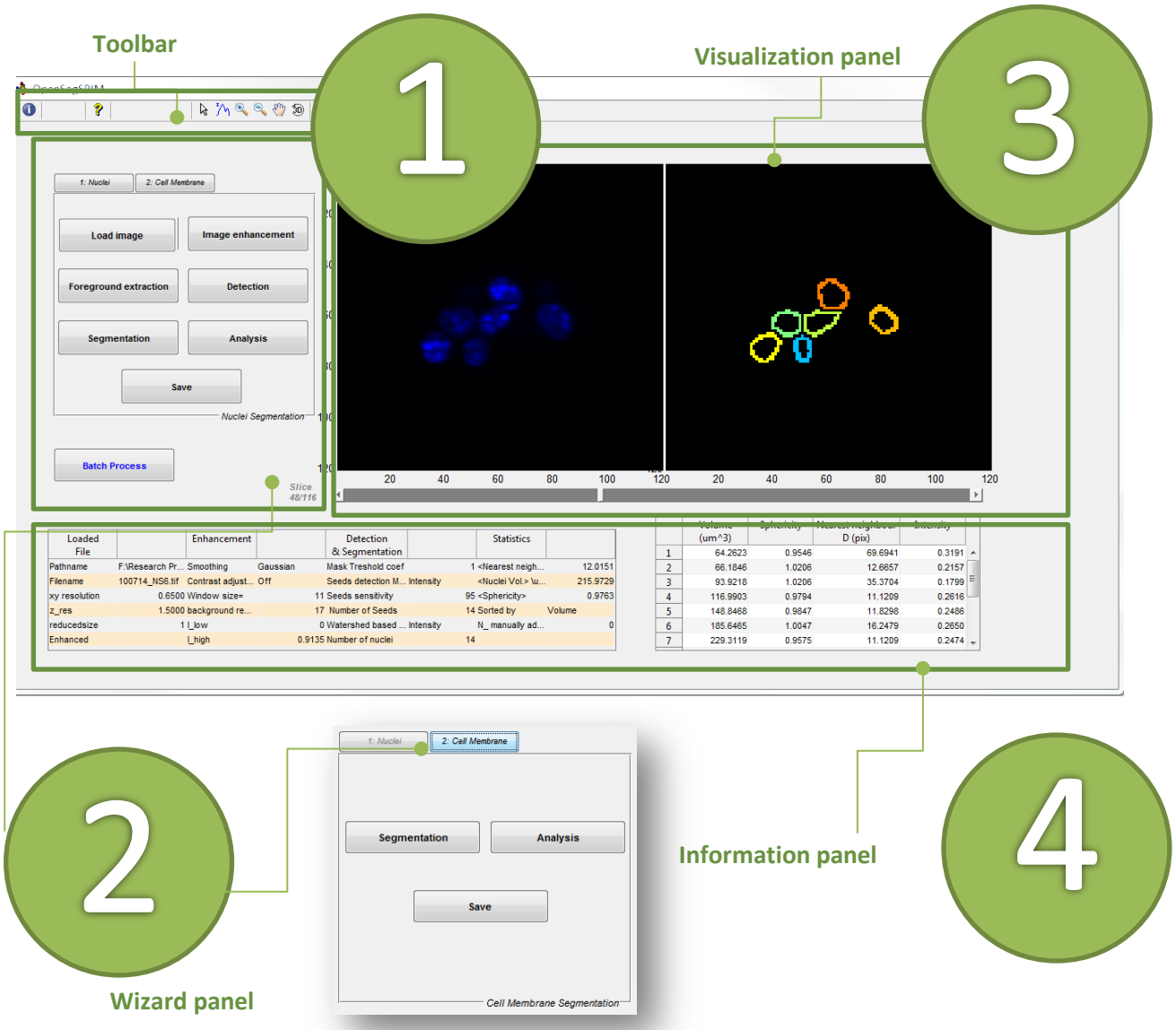
OpenSegSPIM at a quick start : Five main components



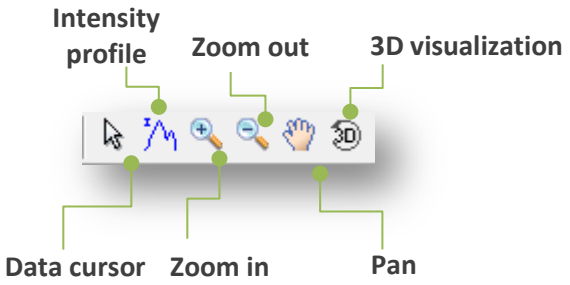
OpenSegSPIM overview

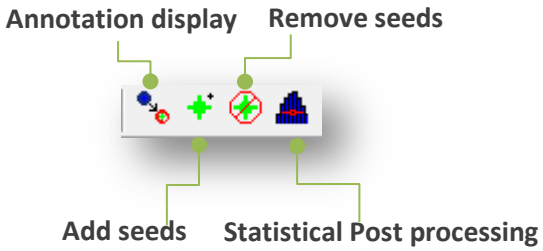
In the past decades, we have seen a rapid development of microscopy imaging techniques. Recent fast growth of SPIM allowed us to produce large amount of 3D data. However, this is probably the first step. In general, user-friendly, flexible and reliable tools are still a bottleneck of data quantitative analysis in this field.

OpenSegSPIM is a 3-D fully automatic quantitative analysis tool for the nuclei in cell aggregate acquired using SPIM or traditional confocal. The software mainly designs to extract quantitative relevant information from SPIM image stacks, such as the number of nuclei/cells and measure the volume and sphericity of stained nuclei and etc on Light Sheet Microscopy (LSM) images. Typically it is useful quantitative analysis tool to different biological problems such as Neurospheres, Zebrafish embryos, Drosophila embryos, Skin sample, Mouse embryos and Organotypic cell culture.



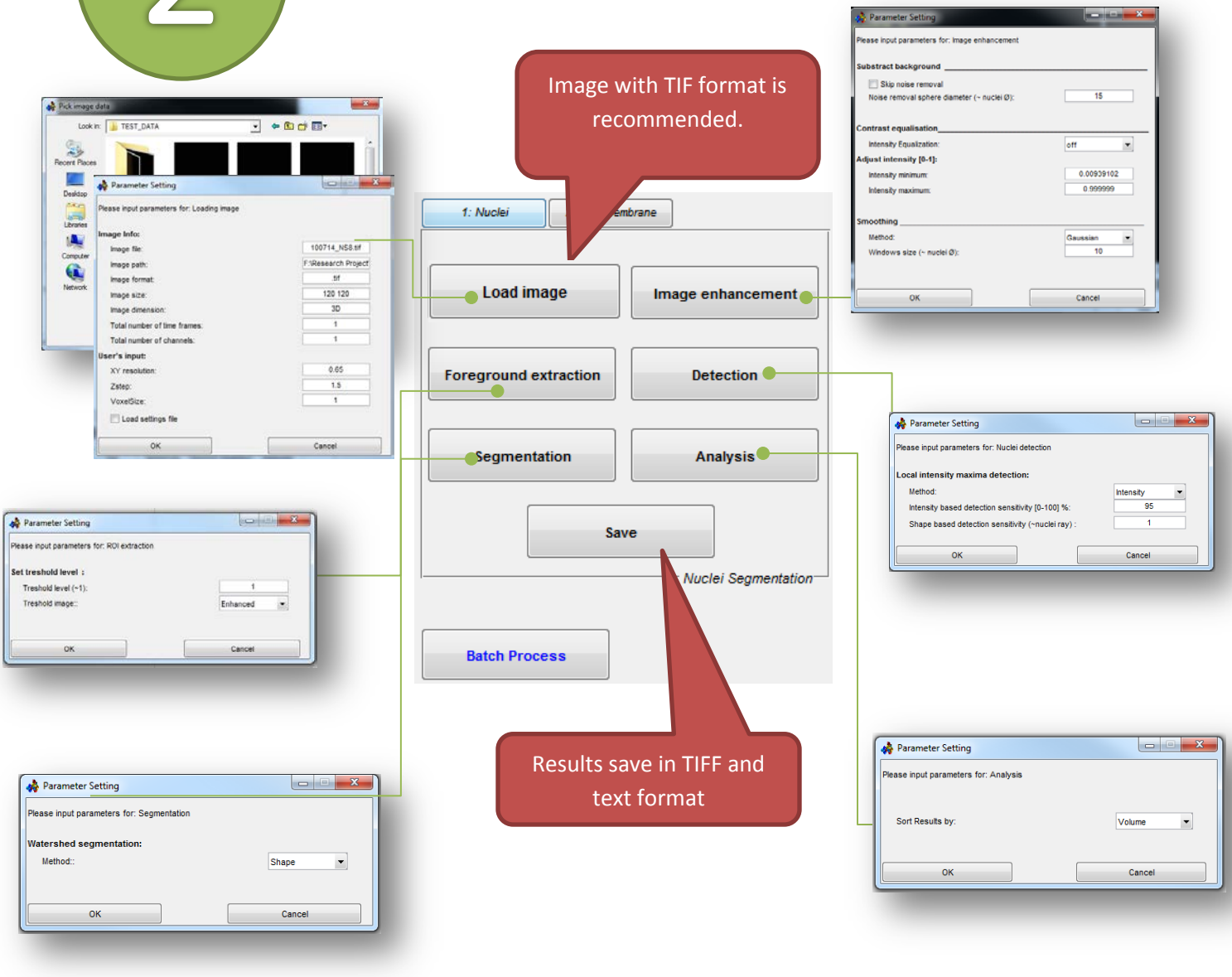
1 Toolbar of OpenSegSPIM





Menu item	Description
Data cursor	Activate ruler to measure nucleus in the viewer and intensity brightness/contrast adjustment.
Zoom in	Enlarge the image by clicking the area of the axes where you want to zoom in, or drag the cursor to draw a box around the area you want to zoom in. ***Tips:*** *When in zoom in mode, you can double click to restore the view and use Shift+click to zoom out (i.e., press and hold down the Shift key while clicking the mouse). You can also right-click and zoom out or restore the plot to its original view using the context menu.*
Zoom out	Reduce an image by clicking the area of the axes where you want to zoom out, or drag the cursor to the area you want to zoom out.
Pan	When the image is enlarged, click and drag the image and move the current region to another region.
3D visualization	Click to open the 3D viewer to visualize original nucleus and segmented nucleus in 3D space.
Intensity profile	Analytical tool used to show the intensity value in along the line draw by user
Annotation display	Select different mode of viewer to display outline of segmented nucleus.
Add seeds	Insert missing seeds in the visualization viewer at the right hand side.
Remove seeds	Remove an unreliable seed from the visualization viewer at the right hand side.
Statistical Post processing	Open histogram viewer to perform data filtering.

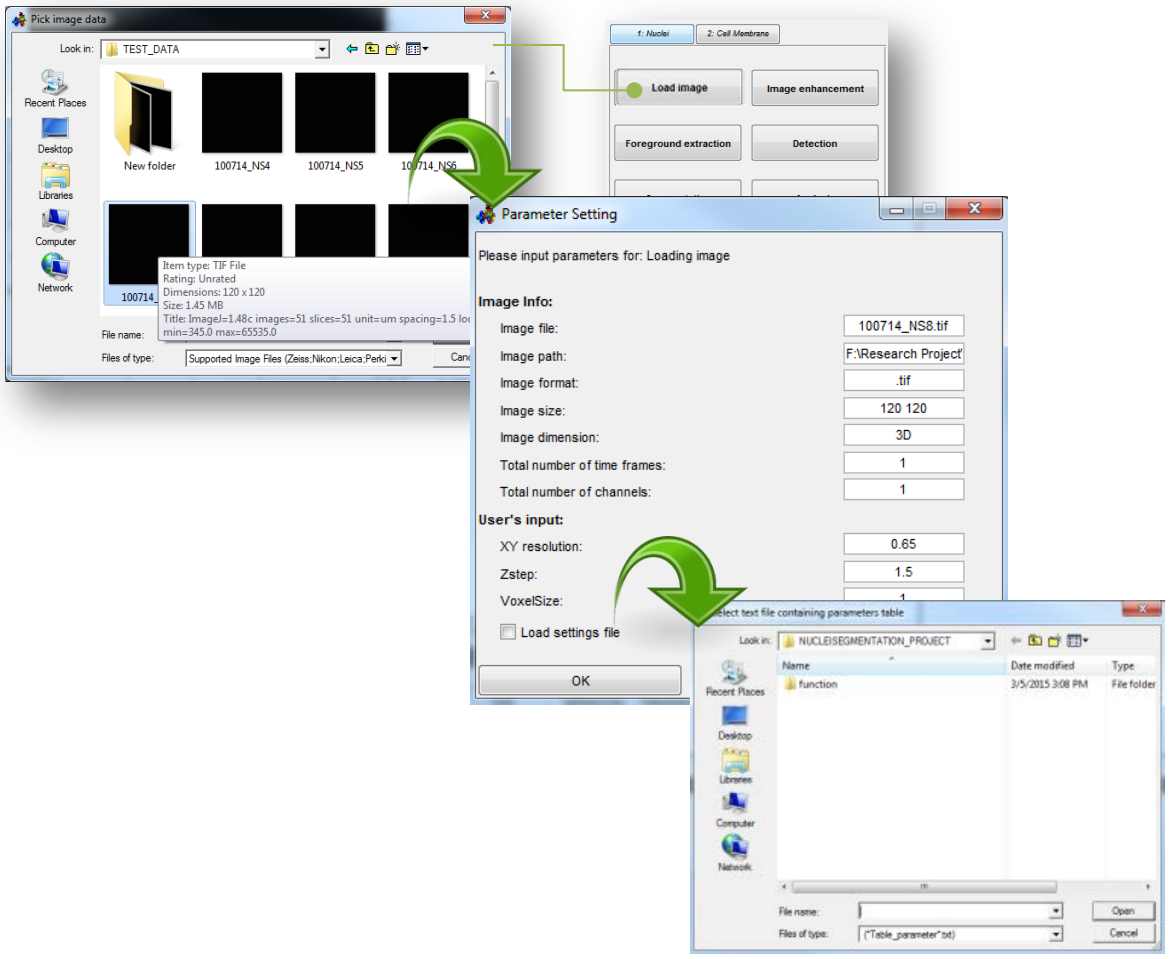
2 Wizard panel – Nuclei segmentation



Nuclei segmentation wizard panel

Components	Description
LoadImage	Load image stacks to start the quantitative analysis
Image enhancement	Enhance the loaded image stacks by using the following approaches: • Artefact and noise removal • Brightness and contrast adjustment
Foreground extraction	Extract the outline of targeted nucleus.
Detection	Identify each nucleus with seed detection.
Segmentation	Segment the nucleus aggregate (separate each of them)
Analysis	Count nucleus and compute the quantitative information such as volume and sphericity of each nucleus.
Save	Click to save the configured parameter value and segmented image stacks.

Load image



Load image parameter setting	Description
Pick image data	Load image stacks to start the quantitative analysis
Image info	The information is automatic collected via image metadata and display in the parameter setting dialog.
User' input	XY resolution and Z-step are extracted from image metadata. However, users are allowed to input according to their need. ***Tips:*** *User can load back the parameters that have been saved previously. Hence, tick on "Load setting file" and click ok button will prompt a configuration pickup dialog to select required setting file.*

Important note: The actual metadata might be lost or inaccurate when the original microscopy image stacks are transfer to TIF. Users need to pay extra attention.

Image enhancement

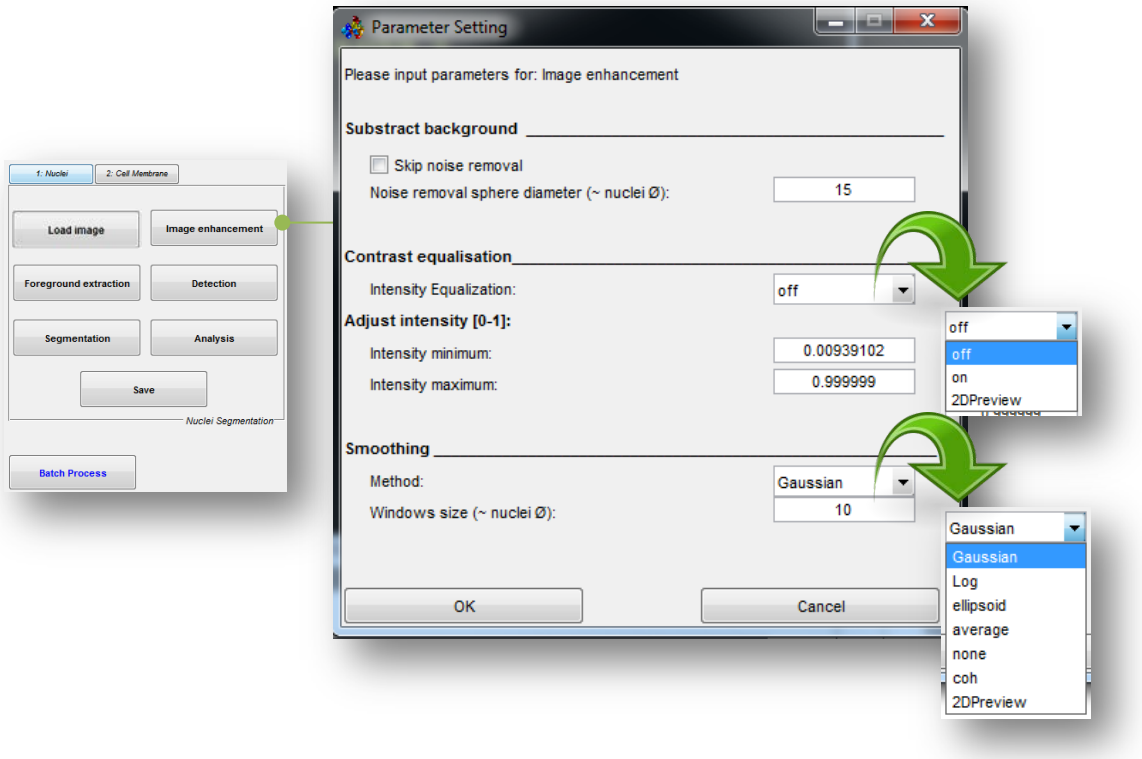


Image enhancement parameter setting	Description
Subtract background	Remove big artifacts, such as non-uniform background by knowing the approximate diameter of nucleus.
Contrast equalization	Increases the global contrast of images, especially when the usable data of the image is represented by close contrast values. Default value is off
Adjust intensity	Increase the visibility of nuclei\cells by adjusting the brightness (min and max intensity value). ***Tips**: min and max intensity value can be obtained automatically during adjusting the brightness contract at cell viewer (Please refer to visualization panel)*
Smoothing	Remove noise and periodic components from data sets while preserving underlying patterns.
2DPreview	Select 2DPreview option from combo box to visualize the original image and processed images based on the configuration value setting accordingly. (Please refer to image processing note)

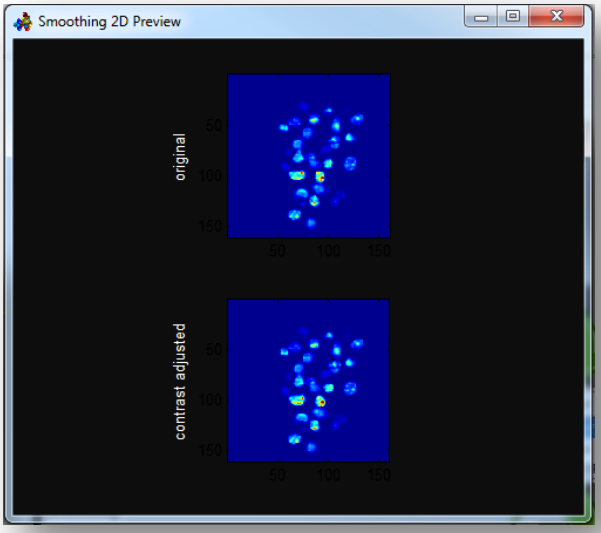
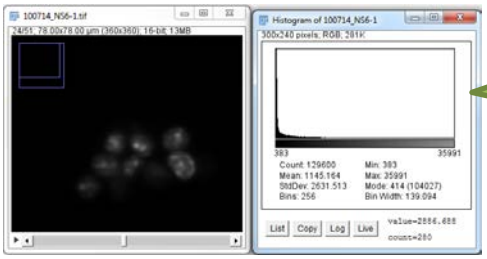
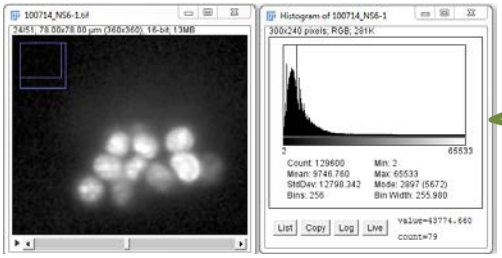


Image processing note: ***Contrast equalization*** *is based on* ***histogram equalization algorithm*** *for adjusting image intensities to enhance the contrast by stretch out the intensity range.*



Original image with intensity histogram

For instance, from the image above, you can see that the pixels seem clustered around the middle and right of the available range of intensities. Histogram Equalization stretch out intensity range. Take a look at the image below: After applying the equalization, we get an histogram like the figure in the below. The resulting image is shown in the picture at left.



Processed image with stretched intensity histogram

Implementation of this algorithm, readers is advised to follow the tutorial at following:

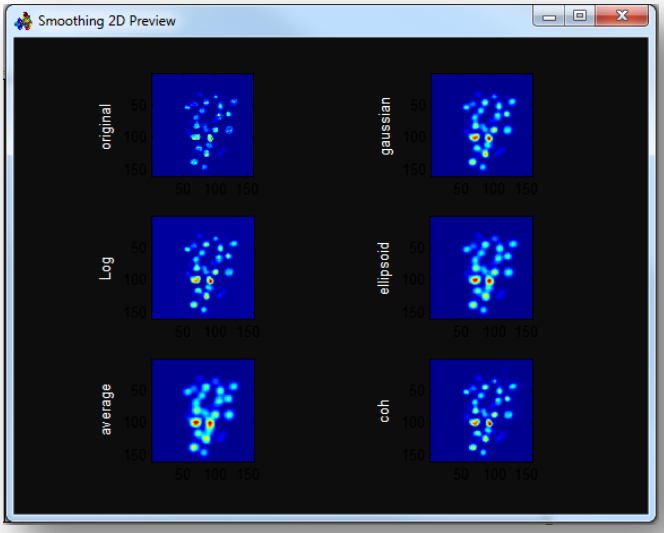


Image processing note:

Smoothing also known as blurring, it is a simple and often used for image processing operation.

Gaussian Filter: *The filter compute with a m x n mask that computed based on Gaussian function.*

$$G(x,y,z) = Ae^{\frac{-(x-\mu_x)^2}{2\sigma^2{}_x}+\frac{-(y-\mu_y)^2}{2\sigma^2{}_y}+\frac{-(z-\mu_z)^2}{2\sigma^2{}_z}}$$

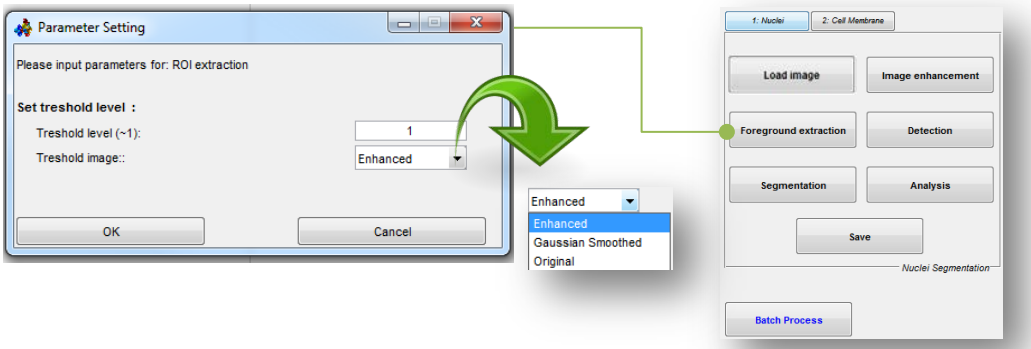
Where ***μ*** *is the mean and* ***σ*** *represents the variance.*

LoG filter*: is Laplacian of Gaussian (LoG) where Gaussian filter required to be applying before Laplacian. It is derivative filters used to find areas of rapid change (edges) in images.*

$$LoG(x,y,z) = -\frac{1}{\pi\sigma^4}\left[1-\frac{x^2+y^2+z^2}{2\sigma^2}\right]e^{\frac{-x^2+y^2+z^2}{2\sigma^2}}$$

Ellipsoid filter: *The filter computes with a ellipsoid as a mask that generates the data using the following equation:*

$$E(x,y,z) = \frac{(x-xc)^2}{xr^2}+\frac{(y-yc)^2}{yr^2}+\frac{(z-zc)^2}{zr^2}$$



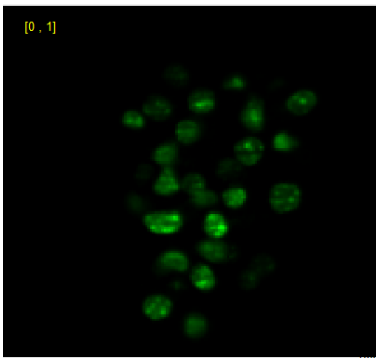
Foreground Extraction parameter setting	Description
Threshold level	Set the threshold floating value from 0 to 1 to extract the foreground. ***Tips**: We recommended user to set the threshold value approximate to 1 in order to obtain a good ROI.*
Threshold image	Optional to let user processed the ROI extraction based on original image, Gaussian smooth image or image enhanced since beginning.

Image processing note:

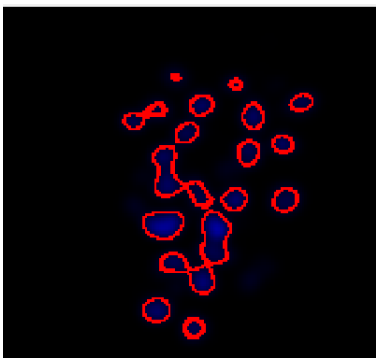
***Thresholding approach**: ROI extraction using Thresholding approach is in-expensive computation and strait forward approach. Thresholding operation define as*

$$f = \begin{cases} 1, & t > f(x,y) \\ 0, & otherwise \end{cases}$$

Where t denotes the threshold value, f is the grayscale image, and f(x,y) is intensity value of coordinate x and y. For example:

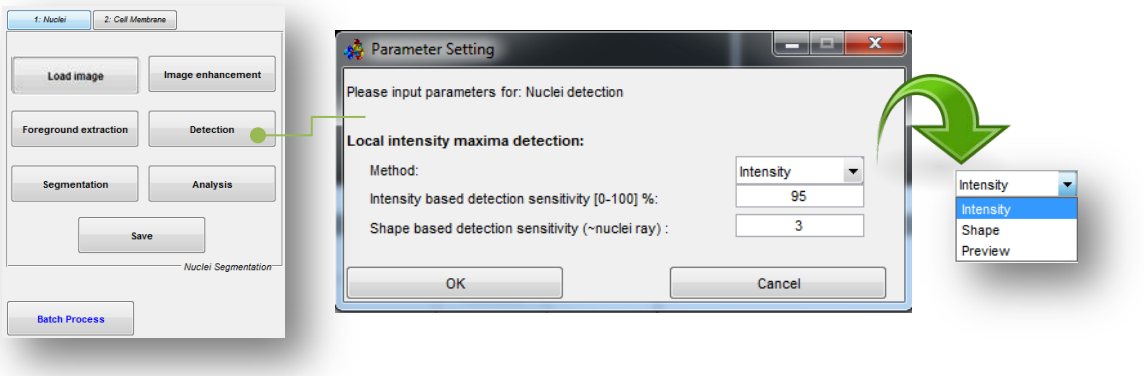


Grayscale Image (cell)



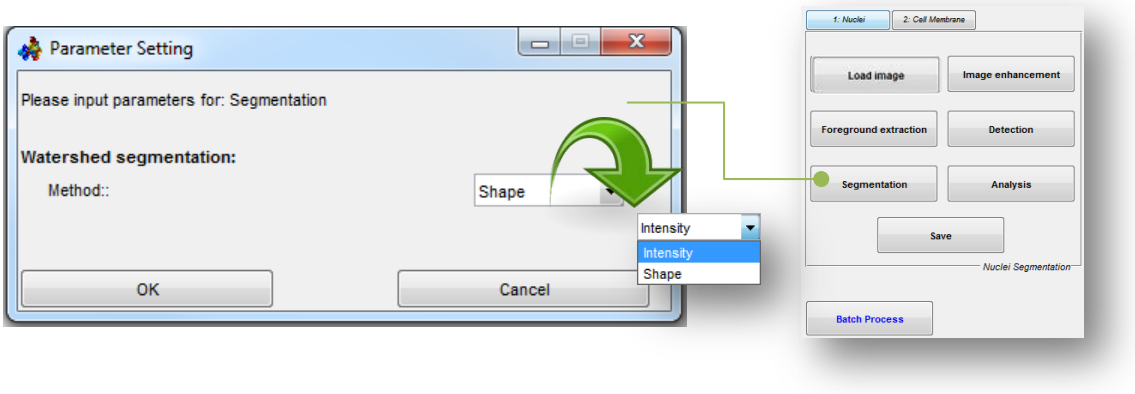
After thresholding process, cells are automatically identify

Detection

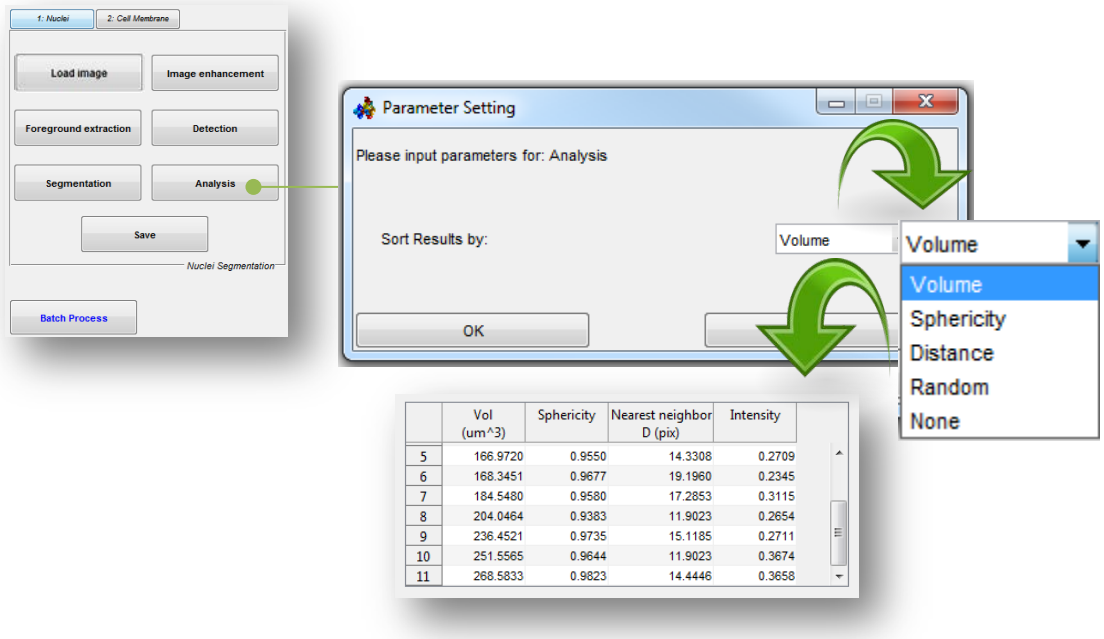


Detection parameter setting	Description
Local intensity maxima detection	Set the local intensity maxima method to detect seed of cells. The features used to seed detection are included: • Intensity • Shape
Intensity based detection sensitivity	Percentage of threshold level used to finding peak and valleys of intensity value.
Shape based detection sensitivity	Percentage of threshold level used to finding peak and valleys of distance value of shape.
	Important note: *If the seeds are not detected correctly, users are advised to add annotation using the seeds editing tool as described at toolbar section and page. 32.*

Segmentation

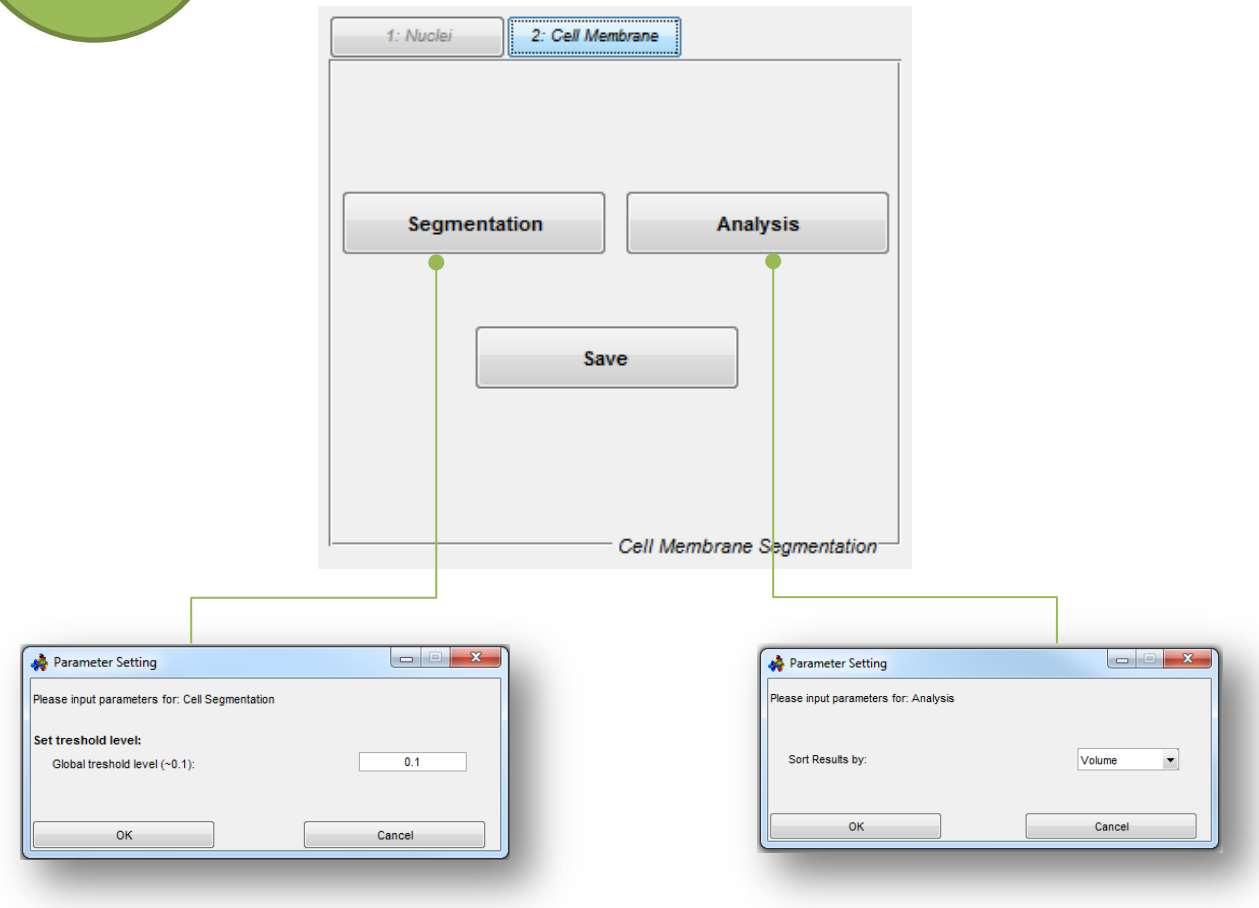


Segmentation parameter setting	Description
Segmentation method	Set the feature of watershed segmentation to segment cells. The features used to segmentation are included: • Intensity • Shape
Intensity	Apply watershed algorithm based on intensity value.
Shape	Apply watershed algorithm based on distance (shape) value.

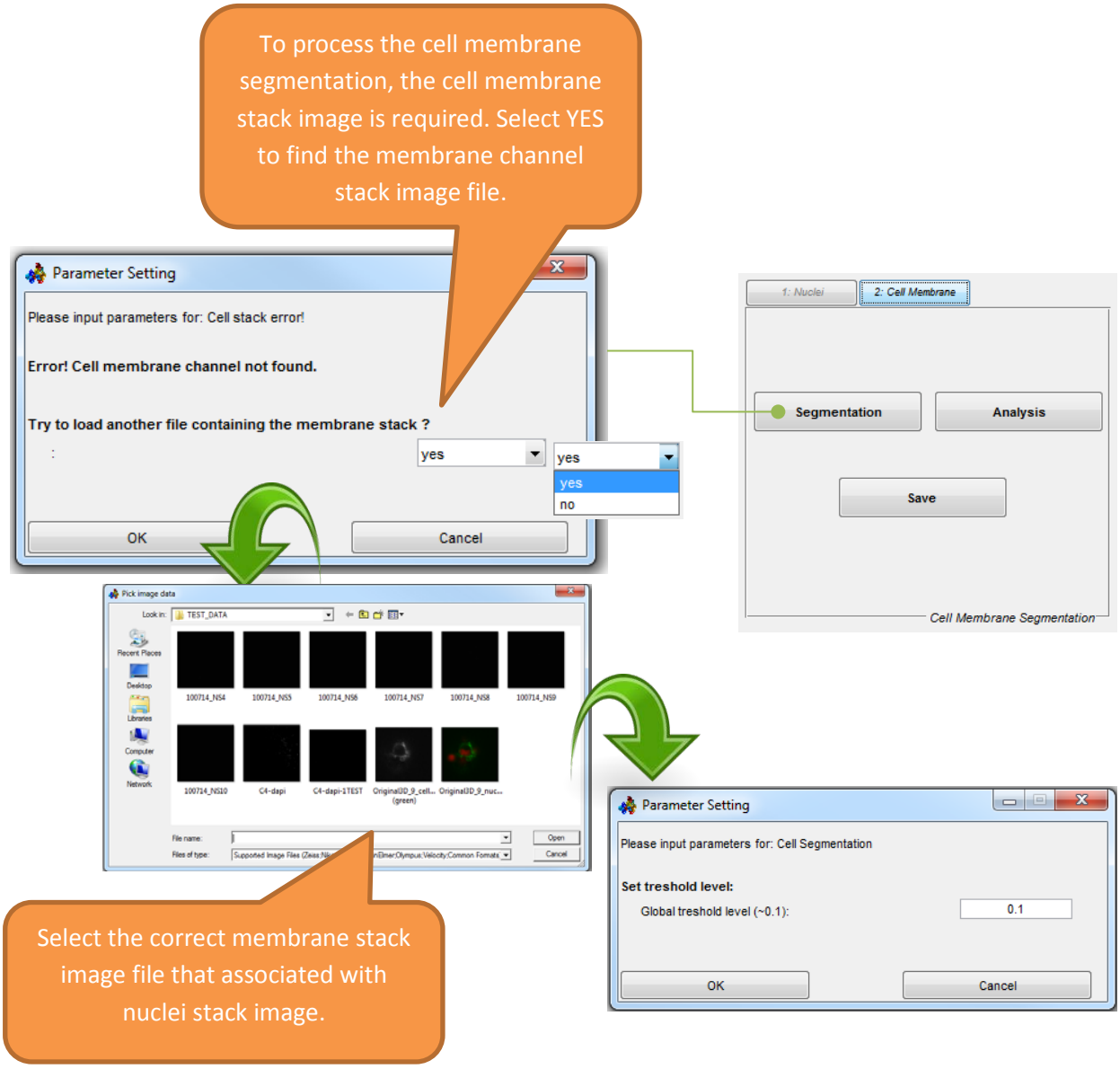


Analysis parameter setting	Description
Sort results by	Select the preferred quantitative measurements of nuclei for quick analysis.

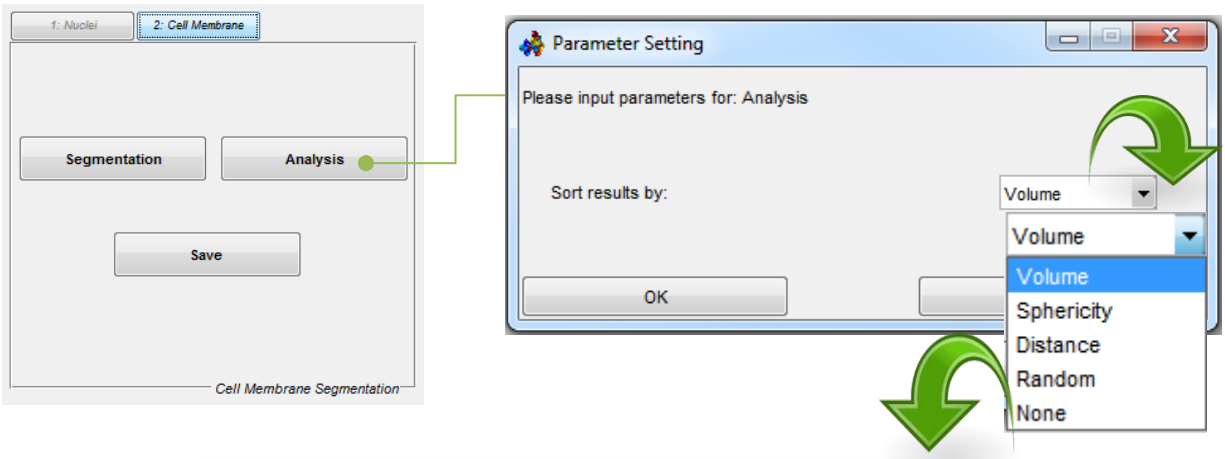
Wizard panel – Cell membrane segmentation



Components	Description
Segmentation	Segment the cell membrane.
Analysis	Count cell and compute the quantitative information such as volume and sphericity of each cell.
Save	Click to save the configured parameter value and segmented image stacks.



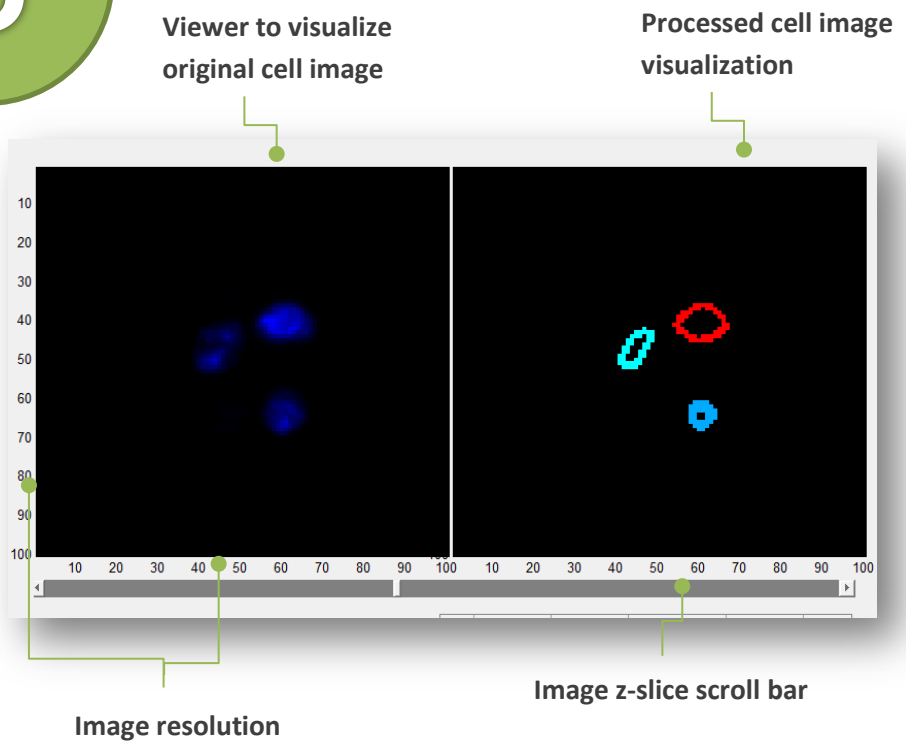
Components	Description
Global threshold level	User set the threshold level in between 0 and 1. Threshold level with 0.1 is recommended to be set.



	Cell Volume (um^3)	Cell Sphericity	Nearest neighbor D (pix)	Total Intensity
20	0.0208	0.7698	15.6849	727.6549
21	0.0260	0.9242	19.6936	2.1574e+03
22	0.0288	0.7861	21.2882	716.1765
23	0.0304	0.6711	13.2008	1.1963e+03
24	0.0304	0.8588	18.3376	1.1249e+03
25	0.0318	0.8031	15.8673	1.0843e+03
26	0.0414	0.7313	13.2008	1.7994e+03

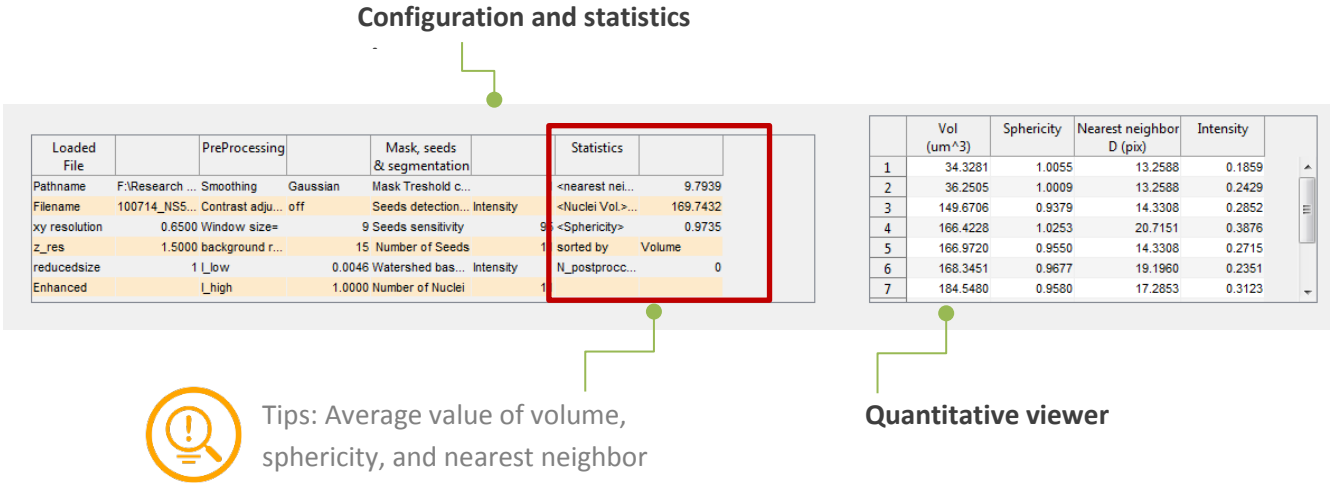
Analysis parameter setting	Description
Sort results by	Select the preferred quantitative measurements of cell for quick analysis.

Visualization panel



Components	Description
Cell viewer	Visualize original cell image.  ***Tips**: Single left clicks and drags on the cell image viewer to measure the targeted cell. Left click and drags on the cell to adjust the brightness contrast.*
Preprocessed viewer	Preview all processed cell image after parameters change.  ***Tips**: User can insert and remove the seed to the processed image viewer if the seed detection is not accurate.*
Image z-slice scroll bar	Scroll along the scroll bar to select and view the original cell image and processed image (both viewer is synchronized).
Image resolution	Numbering indicate to the 2D image resolution (width and height).

4 Information panel

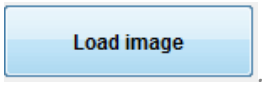


Components	Description
Configuration and statistics viewer	List of the parameters setting value for reference ***Tips**: All setting value will be saved in the results directory and named as Table_parameter.txt*
Quantitative viewer	List of quantitative information for each segmented cells. The measurements included volume, sphericity, nearest neighbor and intensity. ***Tips**: All quantitative results of each segmented cell will be save and named as Table_Results.txt*

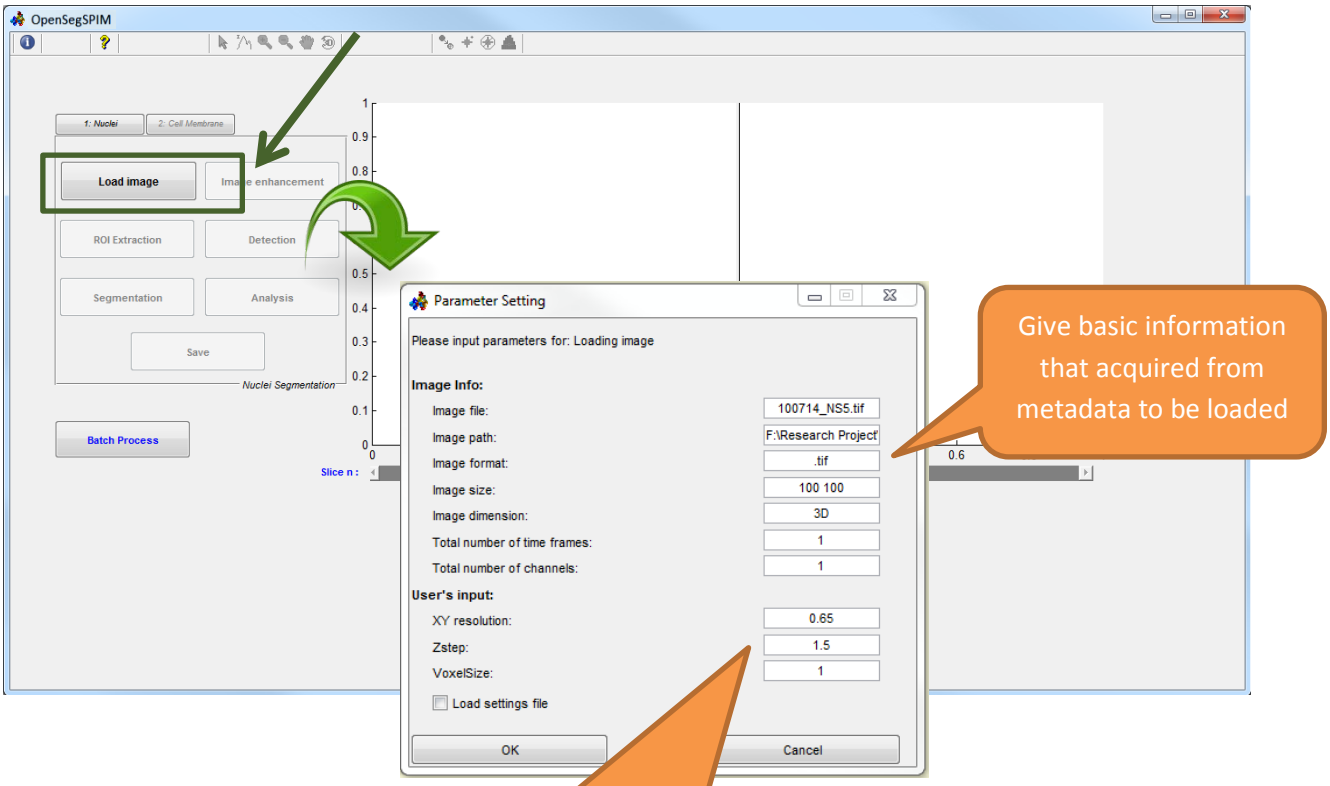
Chapter 2: Nucleus segmentation in action

Step 1: Select entrances of cell and nucleus image

The image processing pipeline in OpenSegSPIM is a linear design. Therefore, **each following steps is not accessible** (gray shaded) before the previous step is complete.

First step is to load the Nuclei 3D stack image by clicking Load Image 

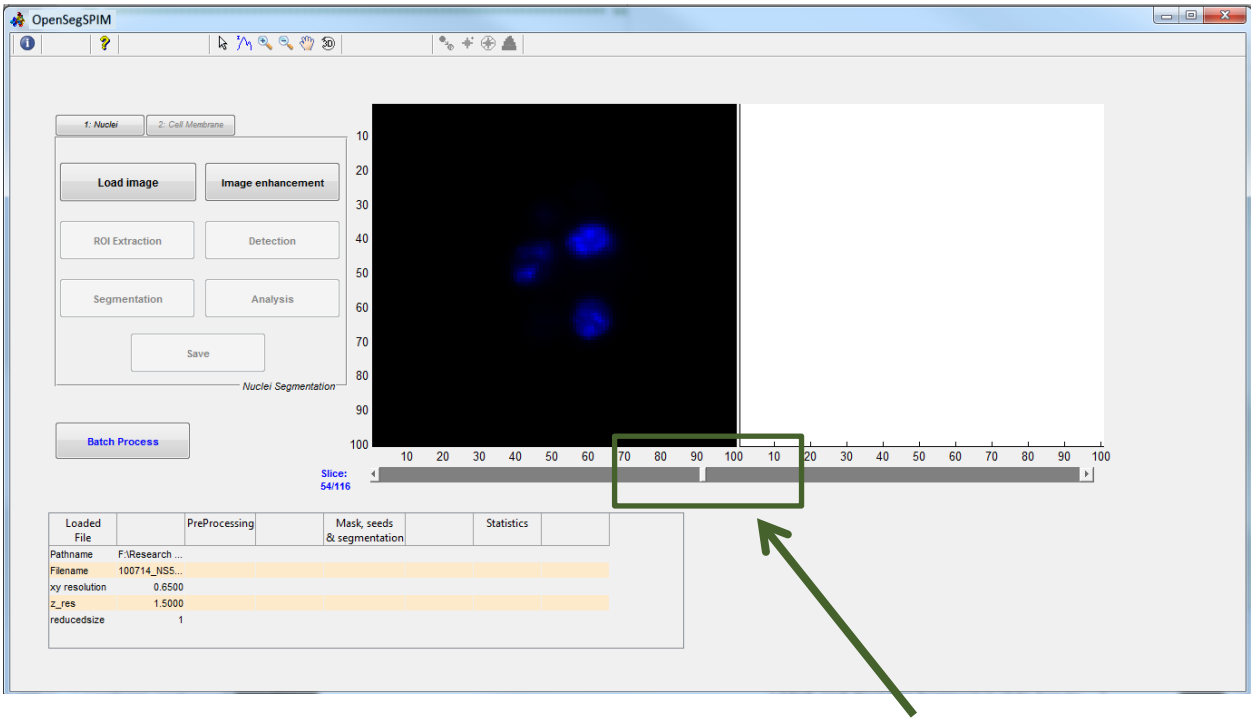
. Each push button contains a tooltip attached when the cursor stays over it giving the user quick tips to progress through the software.



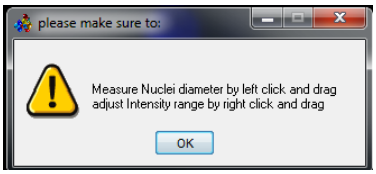
- Enter the x-y resolution (For example: 0.65 µm / pixel)
- Enter the Z-step of the 3D stack (1.5 µm step here)
- Change Voxel size to any value higher than 1 voxel will interpolate data with the specified step and reduce the size of the 3D image (uniformly along the 3 dimensions).

Step 2: Cell measuring and image enhancement

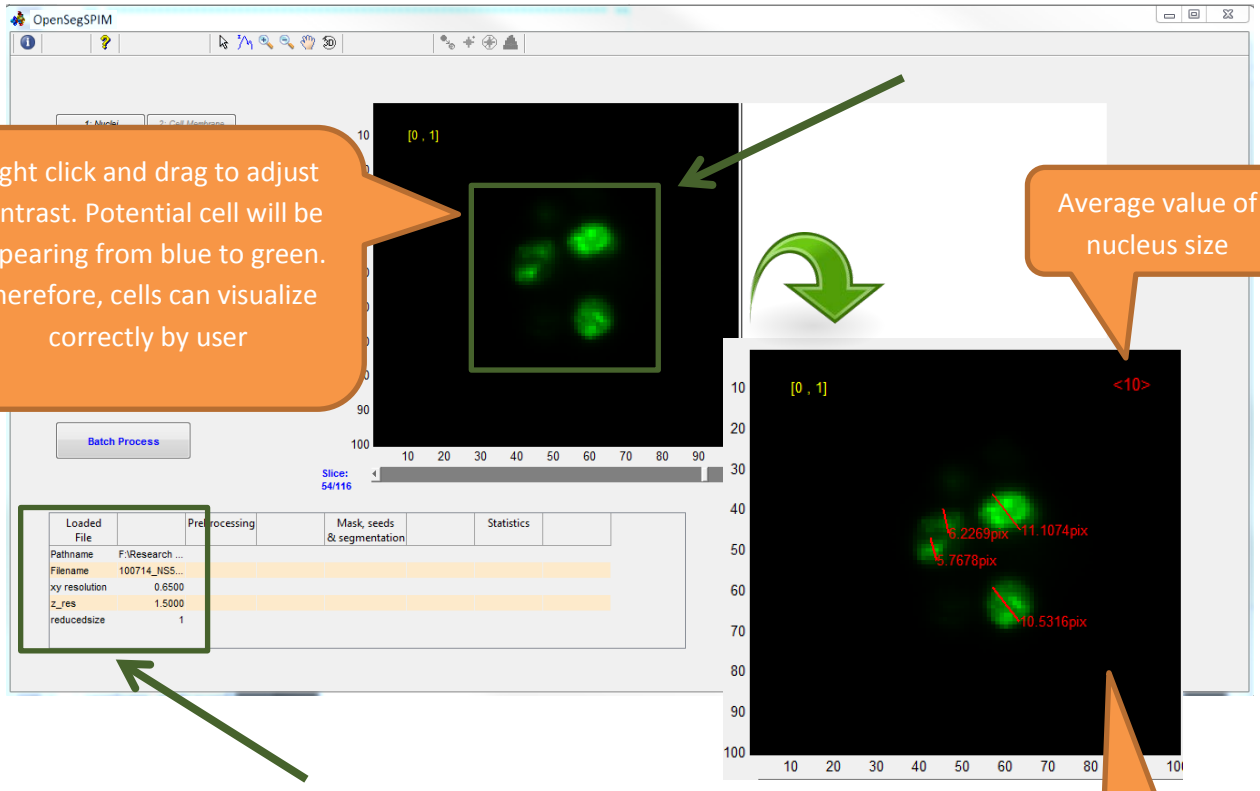
Interactive GUi design enable user fill up parameters in the quick way. Besides, user can select and view the image by scrolling the scroll bar as shown in following figure:



Please make sure the size of nucleus is determined before image enhance button is push.



"User needs to adjust brightness by right click and drag. Once the nucleus appear clearly then user required to measure the nucleus size by right click and drag (preferred at [0,1]) in the original image viewer as shown in following. Both information will be automatically fill up the parameter dialog when the image enhancement button is clicked"



Right click and drag to adjust contrast. Potential cell will be appearing from blue to green. Therefore, cells can visualize correctly by user

Average value of nucleus size

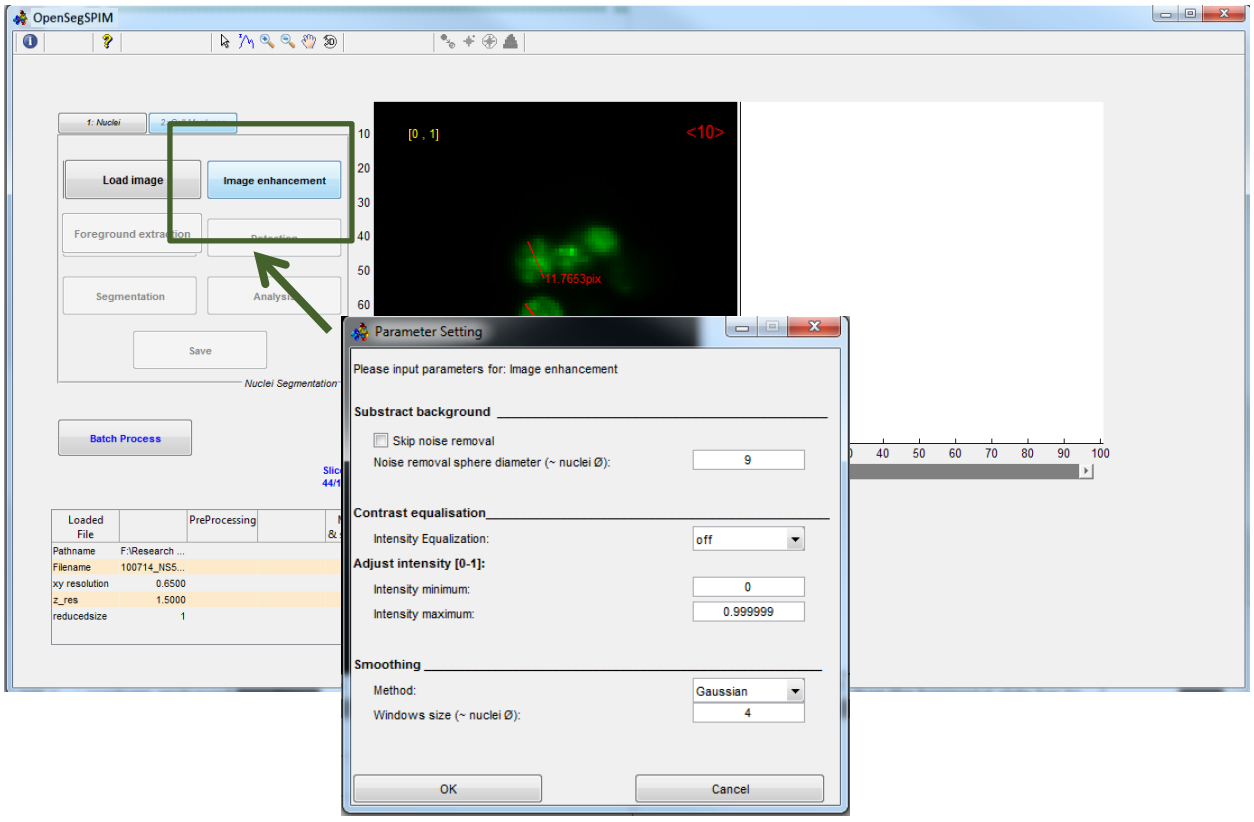
Tips: Parameters used during each processing steps will be store in this table and save as a text file.

Left click and drag to draw a red line as rules. Size of all cells (median value) will be automatic filled in the dialog in the next process.

Once the several sample cells have been measured. Click on 

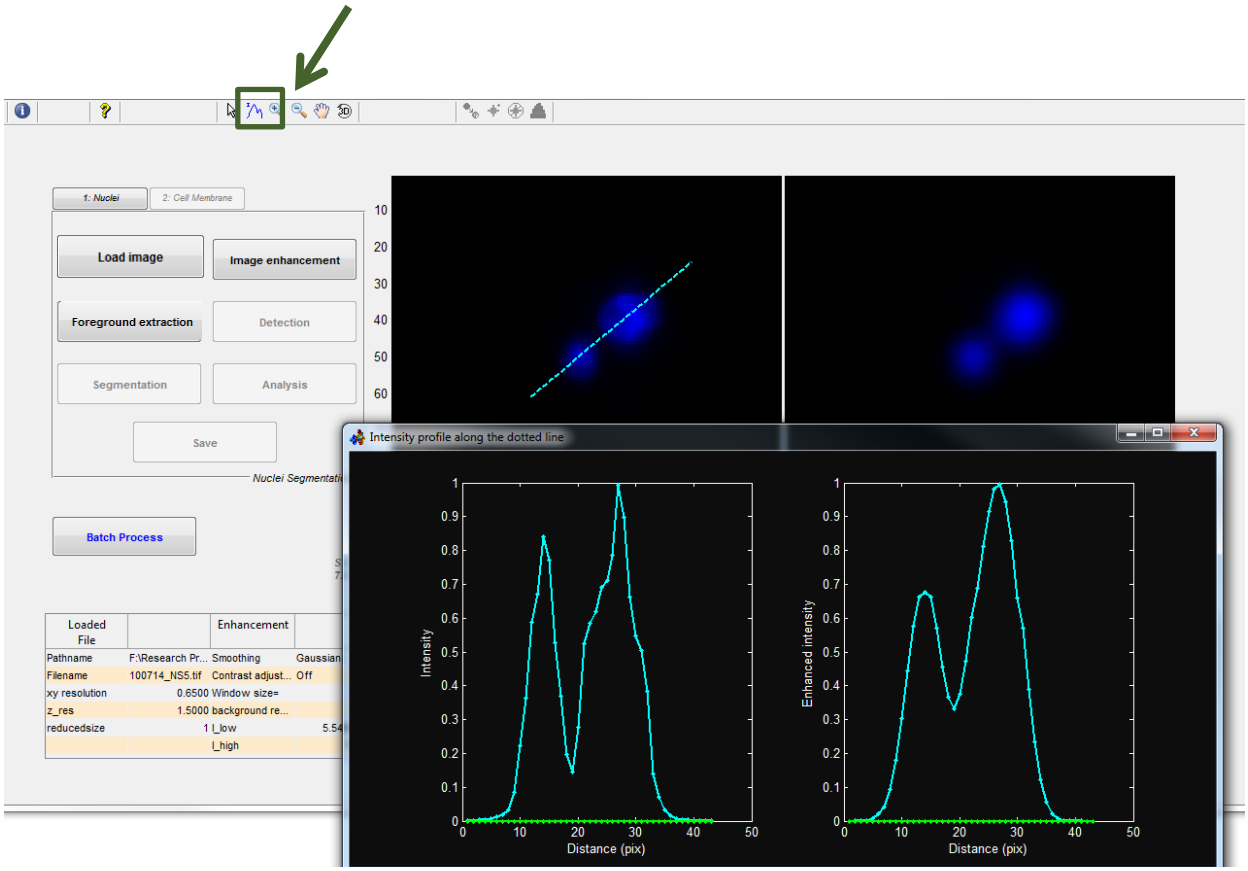
button to start following process.

Tips: Parameter setting dialog of image enhancement are filled automatically, For example: intensity minimum and maximum acquired from brightness contrast (right click and drag) interactive approach. Noise removal parameter is automatically filled based on the diameter all cells (median value).



*Typically the **non-uniform background removal diameter** should be slightly larger than the nuclei size whereas the **smoothing window size** should be slightly smaller than the nucleus diameter (odd integers only). A wide range of different type of 3D smoothing can be applied. Most useful are **Gaussian** and **ellipsoid** ones.*

Image processing note: Click on intensity profile button  *allow the user to visualize the 2D intensity profile along a line to have an idea of the intensity range (between 0 and 1). Line is drawn by a left click anywhere on the image , right click to end the line and display the intensity profile.*

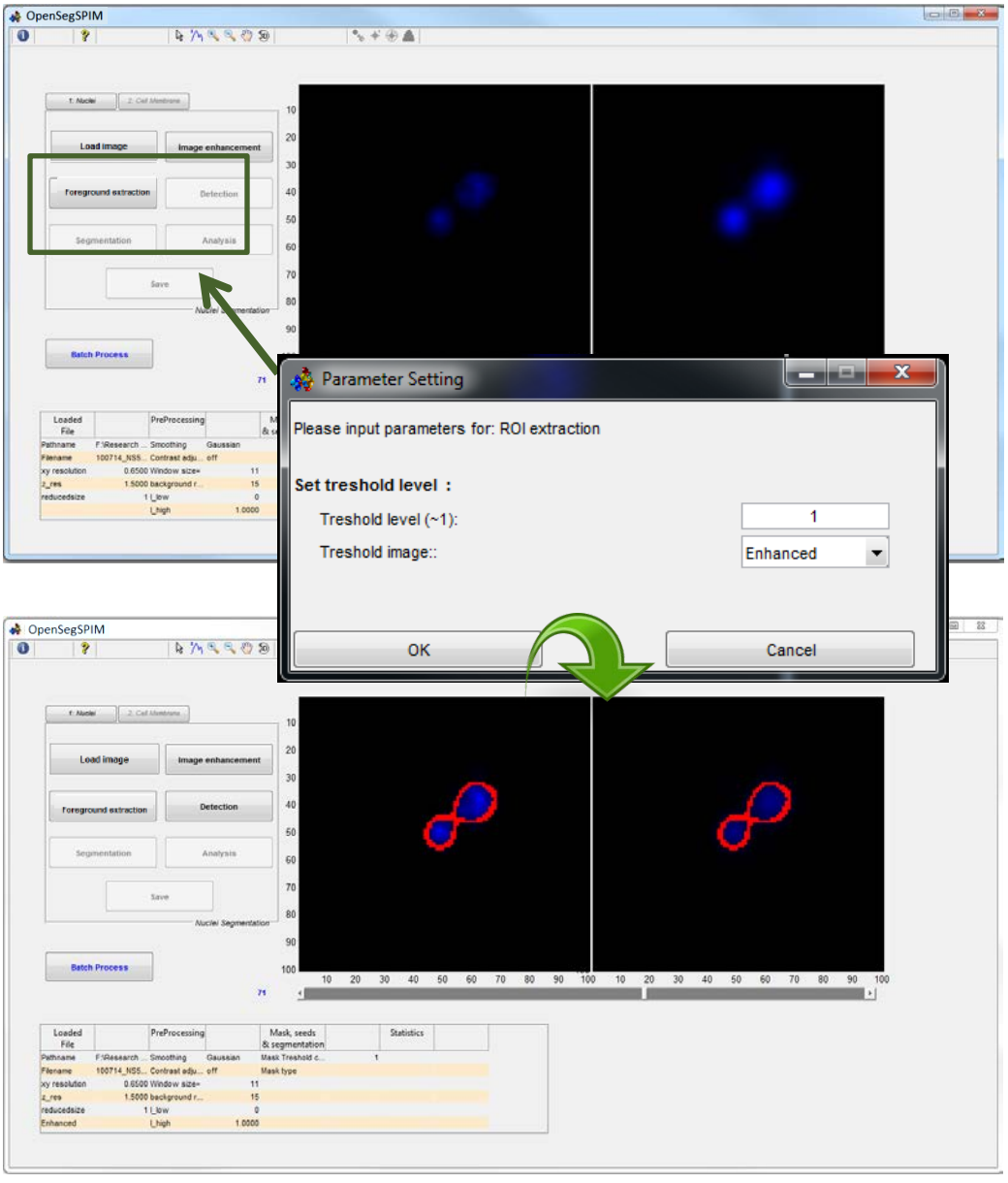


Tips: Intensity profile tool is provided to help user understand intensity value crossing along the dotted line and plot intensity profile. It is useful and a guideline to set the low and min intensity for preprocessing adjustment.

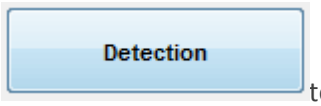
Step 3: Foreground extraction

Enhanced image in previous step will be displayed in the right viewer. We further process the foreground extraction using the enhanced image by click on **Foreground extraction** button. Click ok once the parameters value has been decided.

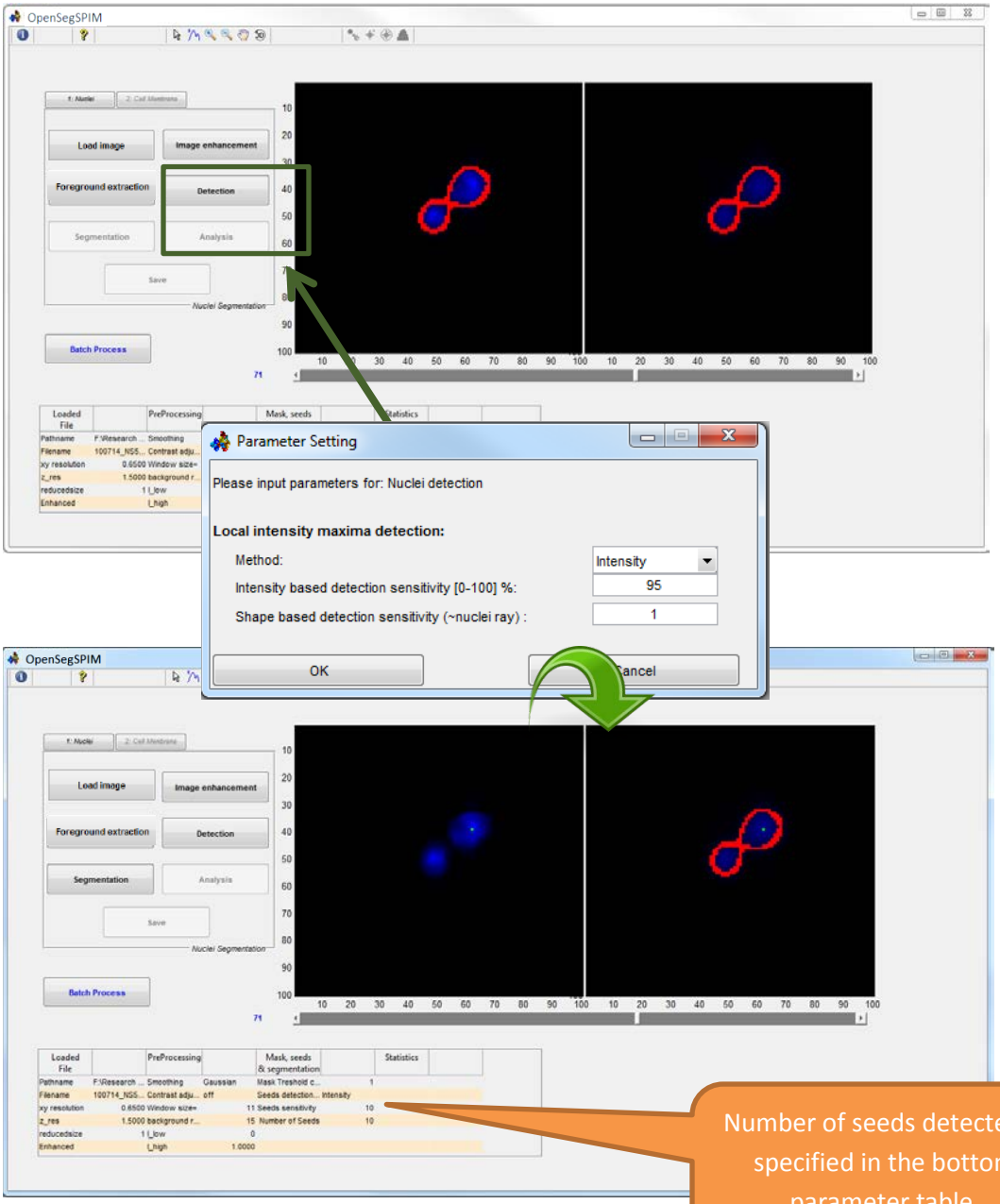
Foreground Extraction use a Otsu Method Tresholding . User can adjust The Treshold level value. It is a coefficient multiplied to the Treshold value found automatically (can be any positive value). 1 is typically a good value.



Step 4: Detection

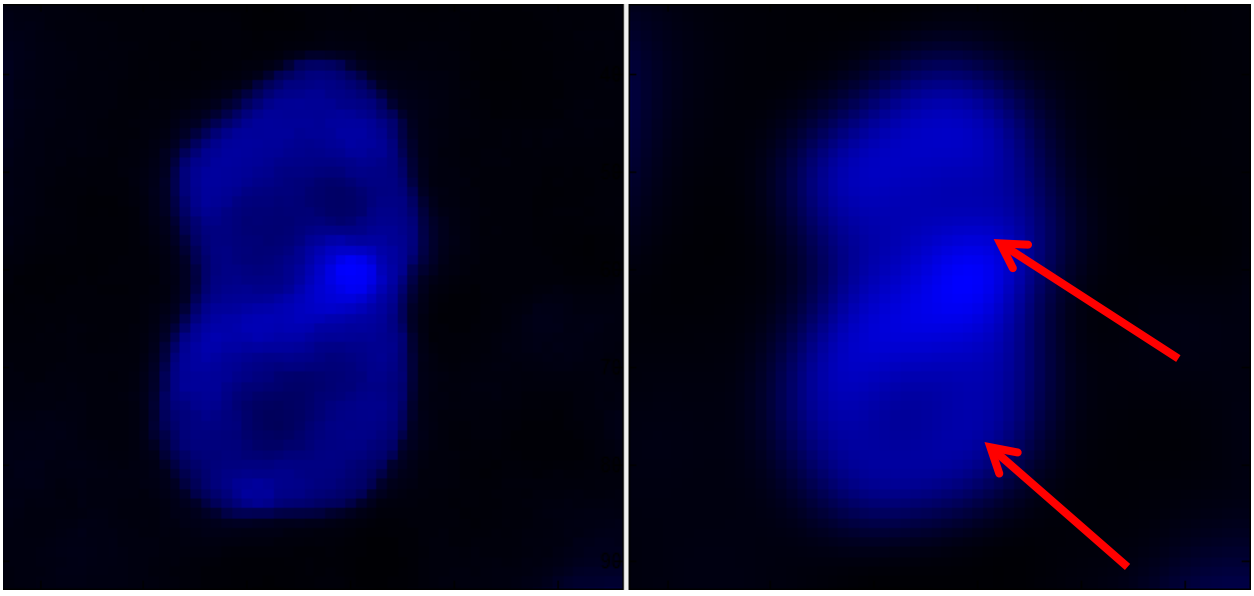
A boundary annotation is overlaid in red on the processed image. Click on 

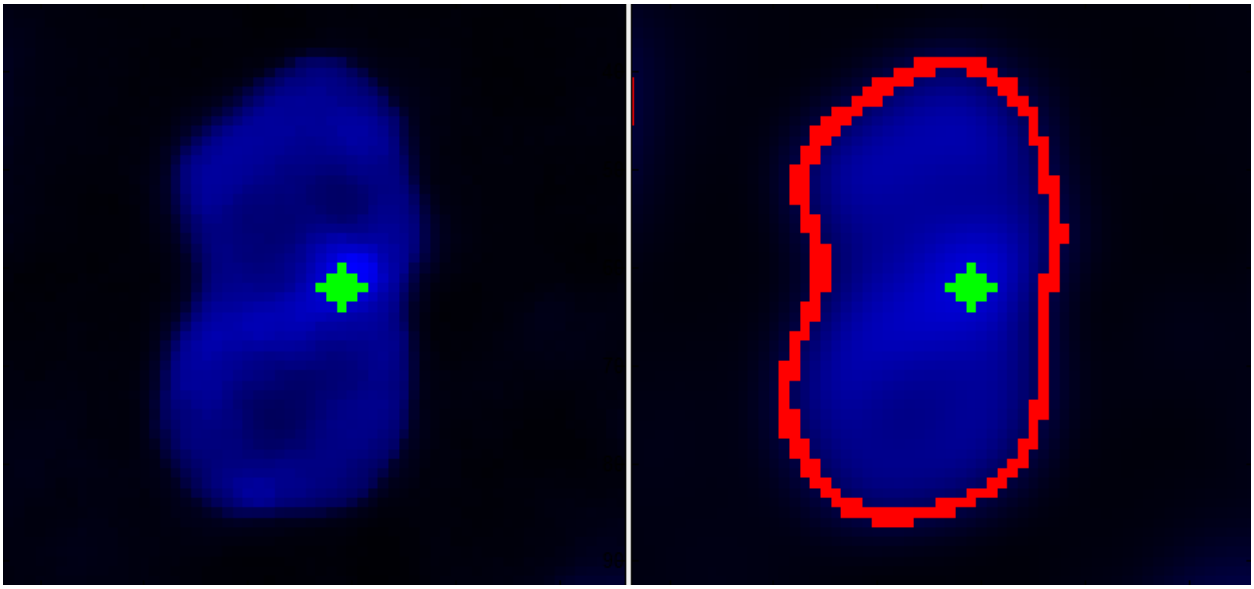
to open seeds detection parameter setting dialog. Click ok to process the seeds detection once the parameters value defined. This seeds detection process will produce reliable seeds shown as green dots as following figure.



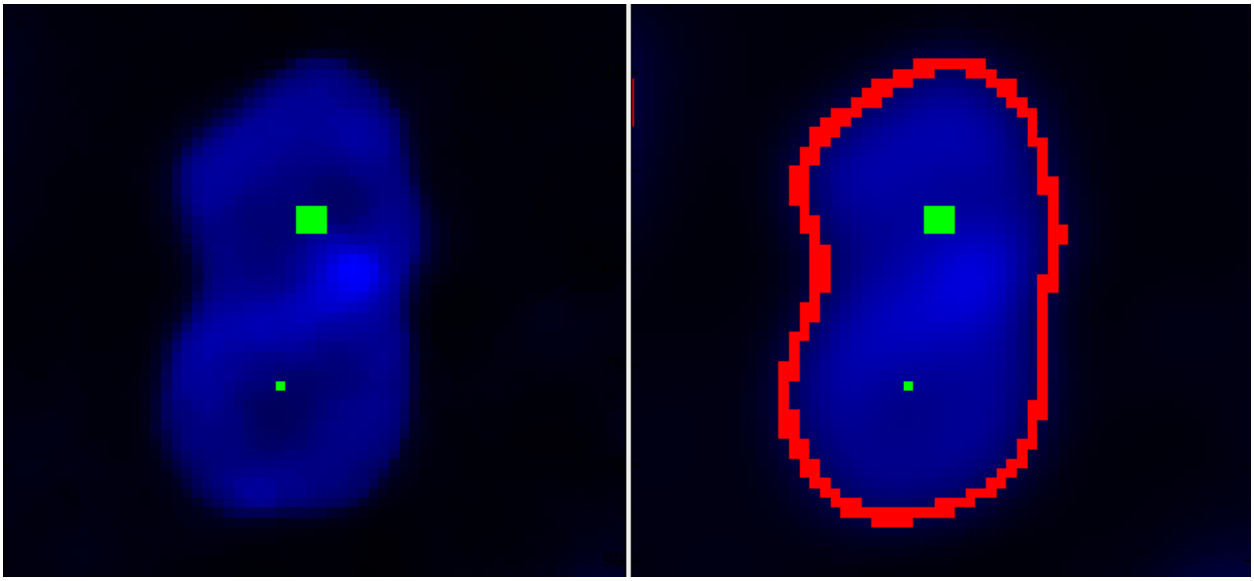
Tips: using intensity or shape is very depend to the images, if you find the nuclei with visible "hole" in the image as shown in following. This is because of nuclei stain is not distribute evenly. You should consider using shape instead of intensity. Visible "hole" in the object is because of low value of pixel intensity compare to high value of pixel intensity surrounding. Therefore, distance of shape is computed to finding the good seed during detection process.

For example: Given image in following is containing two nucleuses, Two "holes" is visible after enhancement process as pointed by red arrow. In the detection process, choosing intensity might not appropriate seem it only can detect one seed but in fact, it is two nucleus in this case. User is advised to perform detection method based on shape if image in above as input. Following are the example of detection results based on shape detection



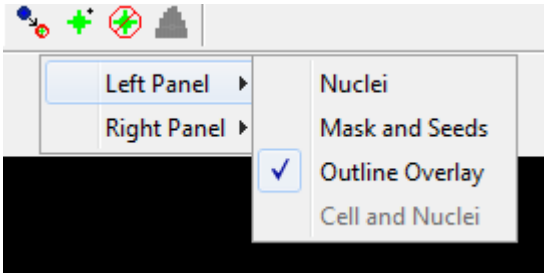


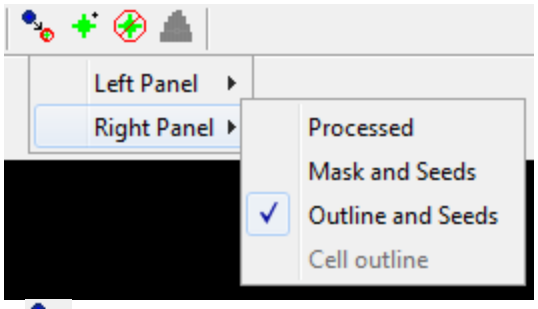
*Detection result based on **intensity** method that is consider wrong detection since there are two nucleuses.*



*Detection result based on **shape** method.*

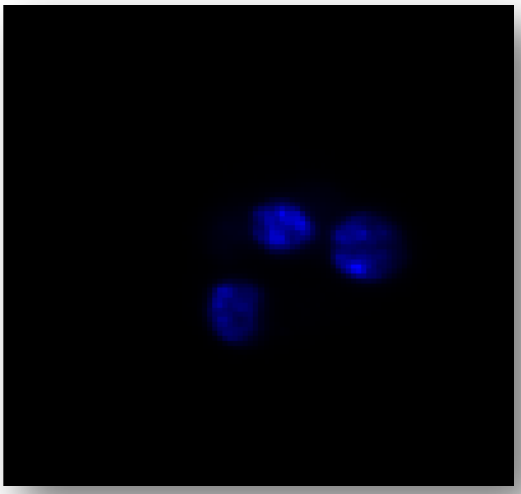
Tips: user can define the view panel based on their preferred display modes. Five different display modes are provided to uses. They are included Nuclei, Processed foreground and seeds, Outline Overlay, Cell and Nuclei. Example of these modes provided in following:



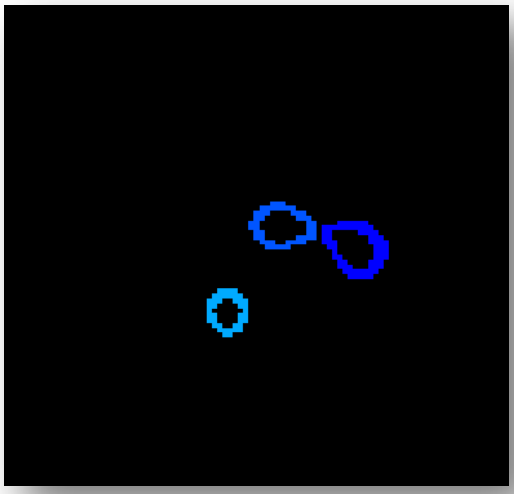


*User can choose the display mode from the **change display** from menu bar.*

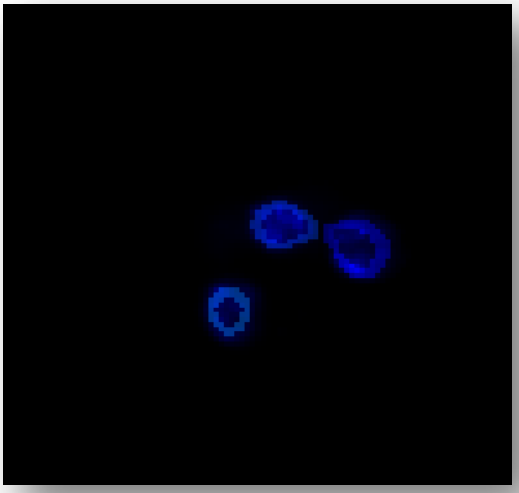
Left panel ->Nuclei



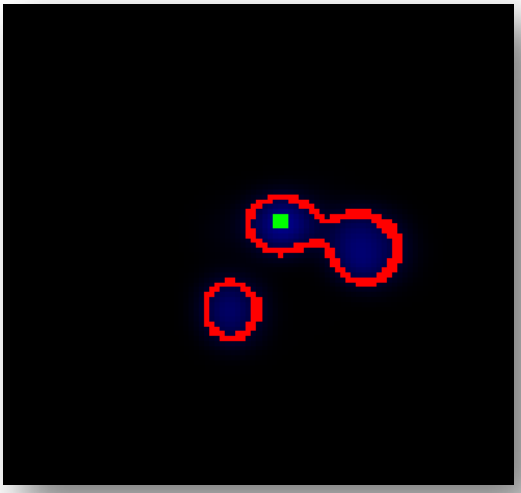
Right panel -> Processed



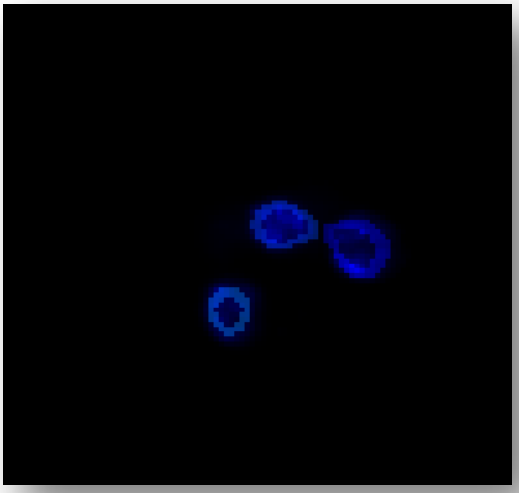
Left panel ->Mask and seeds



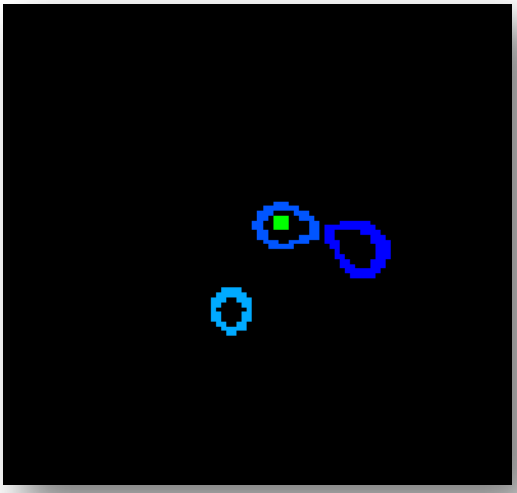
Right panel ->Mask and seeds



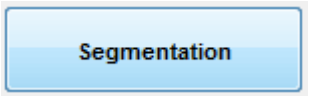
Left panel ->Outline overlay



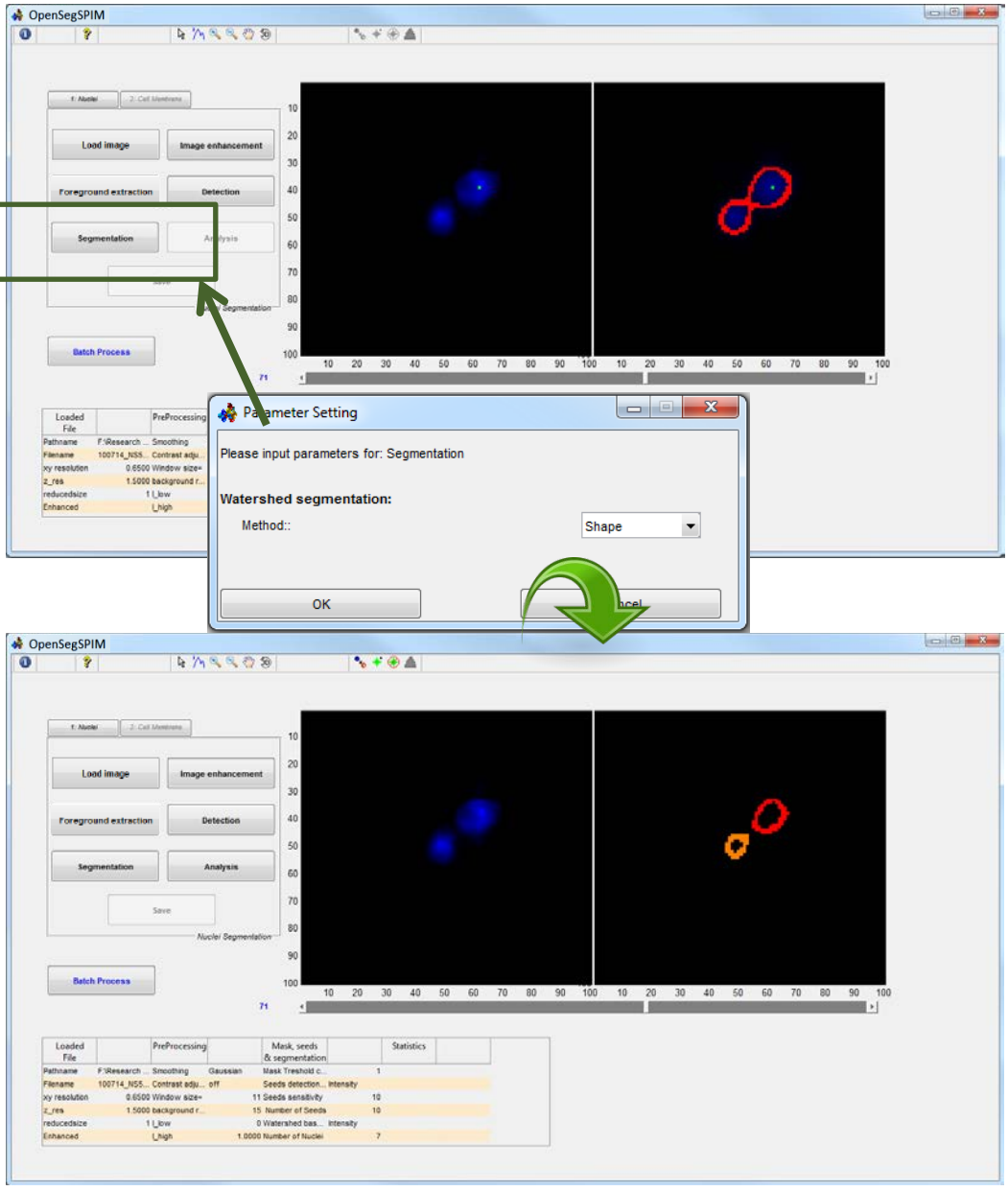
Right panel->outline and seeds



Step 5: Segmentation

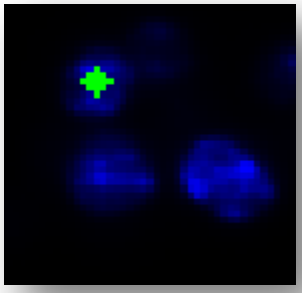
Click on the 

button to prompt a dialog to select proffered approach (Watershed with intensity feature or shape distance feature) to segment the nucleus.

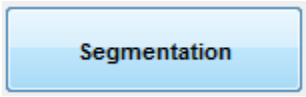


Tips: User can annotating the seed by using seed editing tool (It is only available after analysis process is complete), in order to improve the quality of segmentation. It is strongly recommender user should base on statistical information (Step 6: Analysis) to add/remove the missing/unreliable seeds respectively. This is because missing/incorrect seeds easy to identify from the result list instead of detecting every slice images.

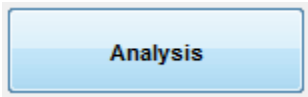
To add a new seed



Click and select then click and locate at the nucleus that is suspected missing the seed (Please ensure this by sliding a few slice, z-position to ensure no seed is allocated during detection process). Click

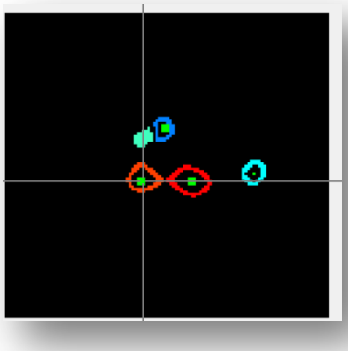


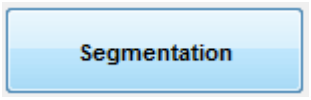
to re-run the results to segment the missing nucleus. Click on

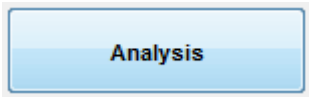


to check the update results.

To remove a seed

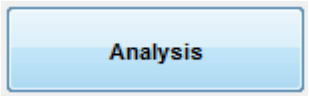


Click and select  *then click and locate at the nucleus that is false positive. The green dot will be disappearing. User required performing segmentation process to remove selected entire nucleus by click on* 

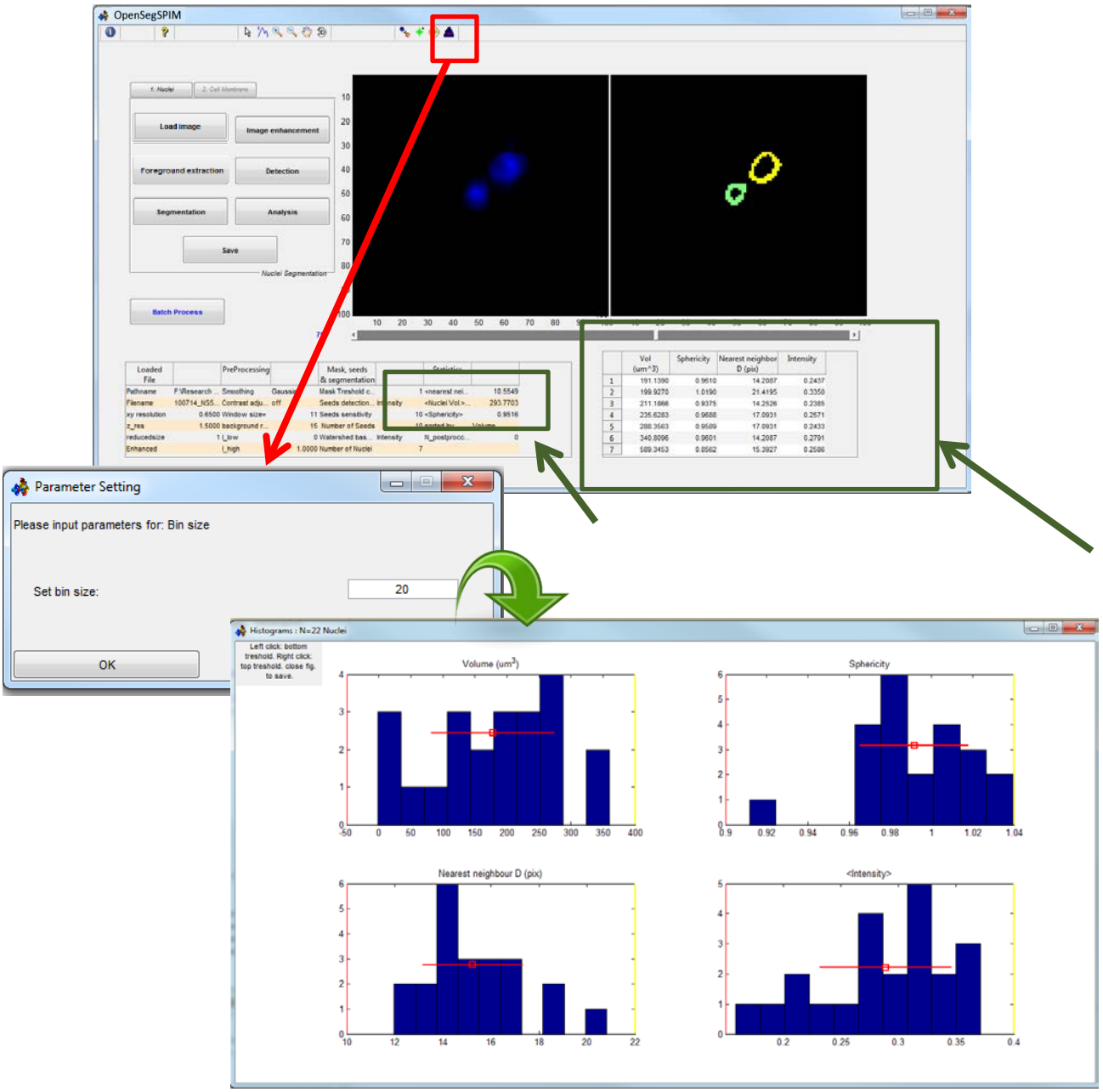
. Click on 

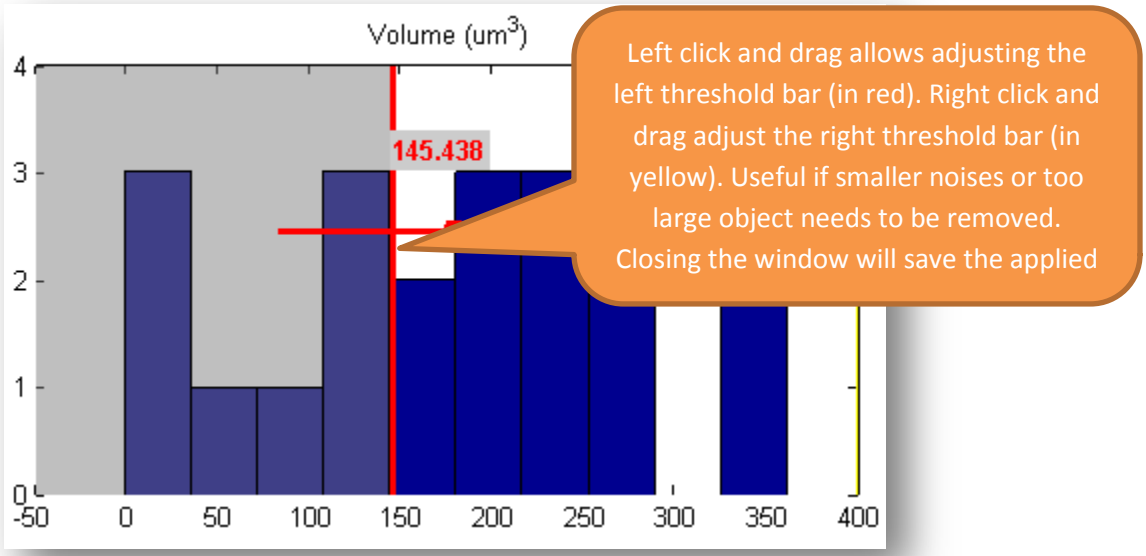
to check the update results.

Step 6: Analysis

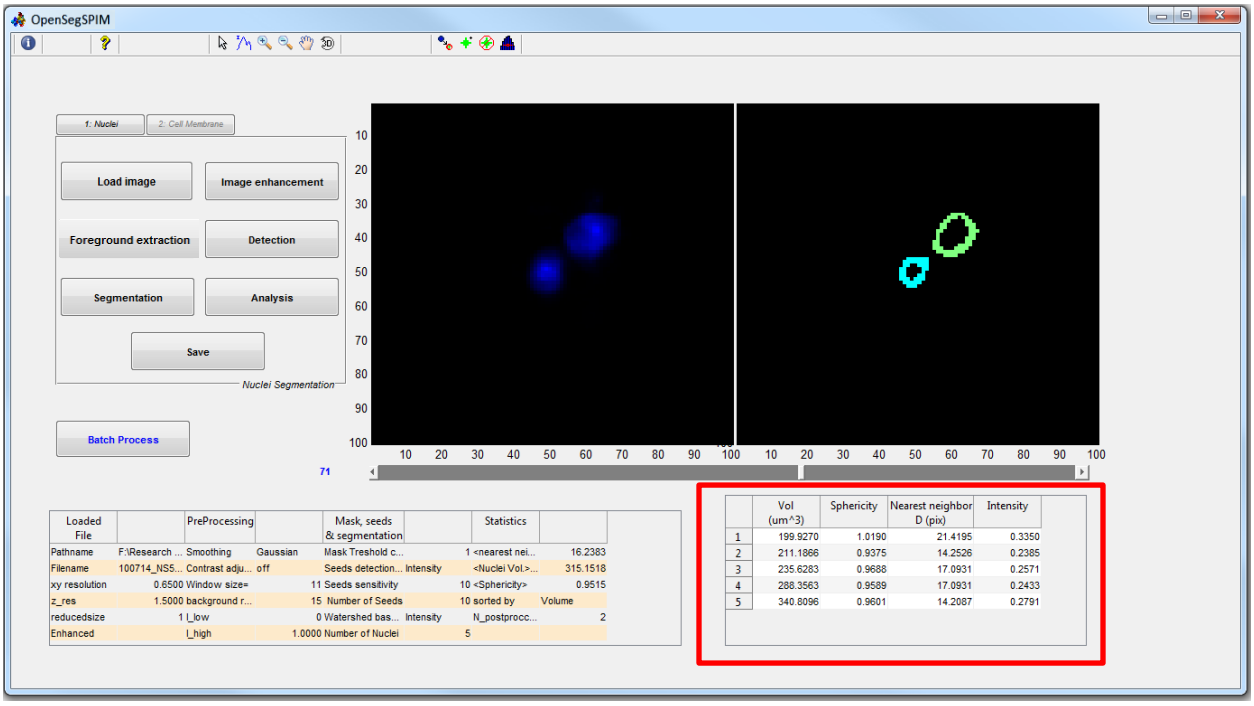
Click on the 

button to prompt a dialog to set the number of bins for histogram. Results can be sorted according to different preferred quantitative information. Histogram analysis is then enabling to let user to access for further analysis.



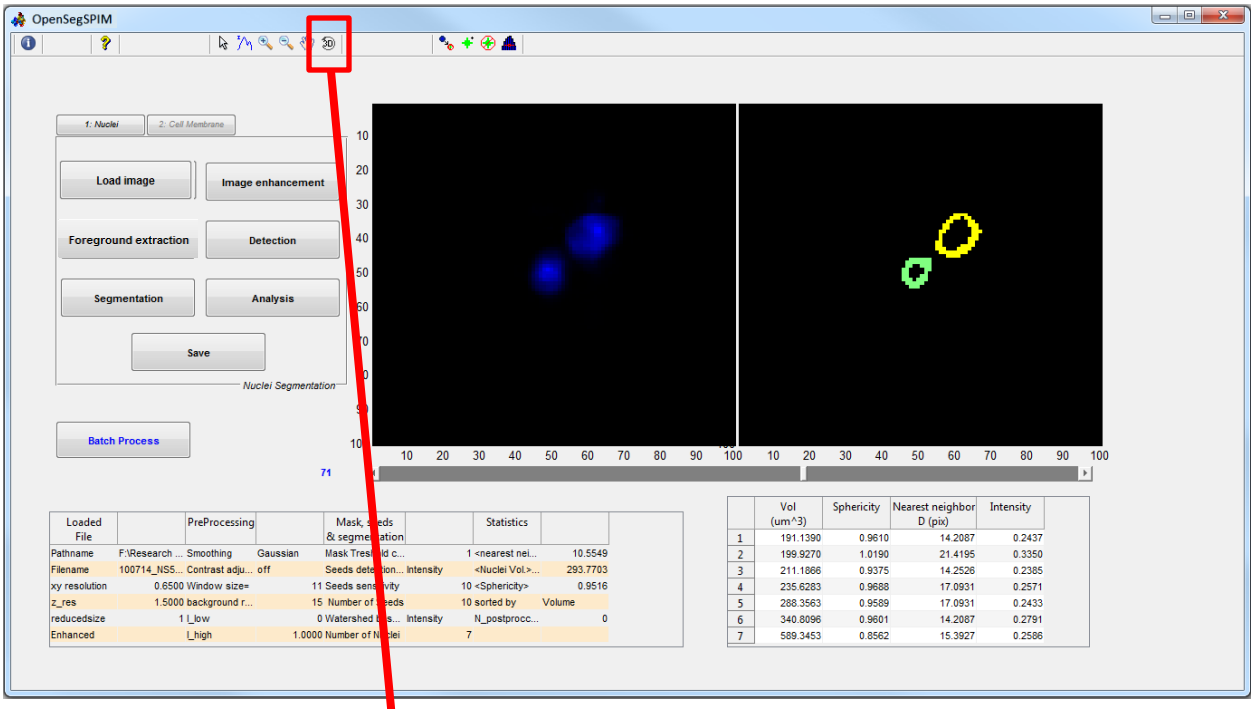


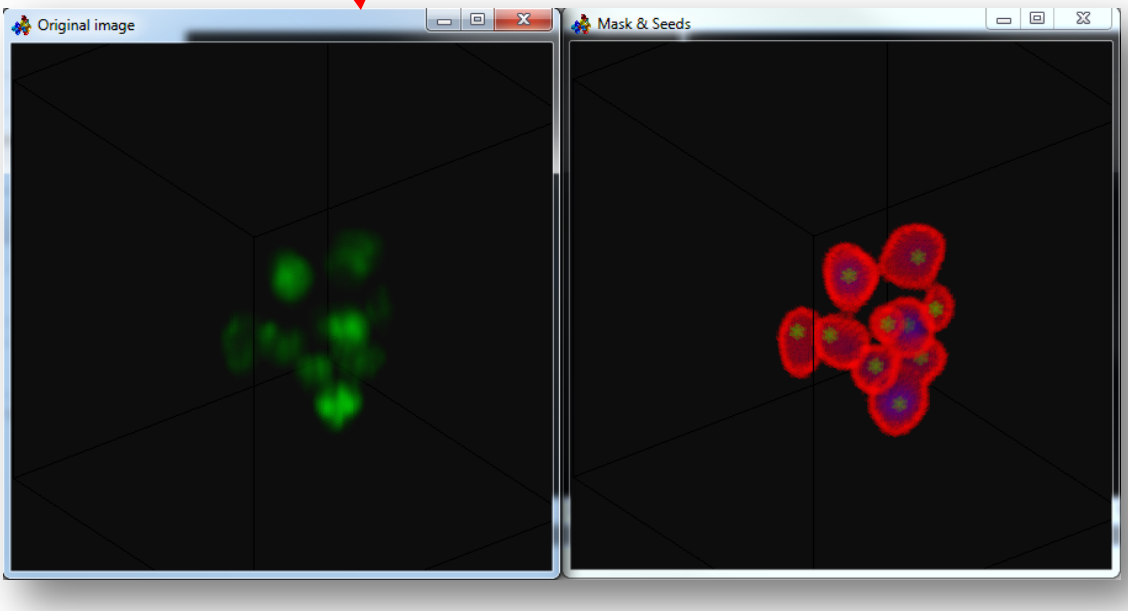
Close the histogram dialog, you will notice that quantitative information (**red rectangle**) by volume is filter according to the threshold value **145.4** as shown in figure above.



Step 7: 3D visualization

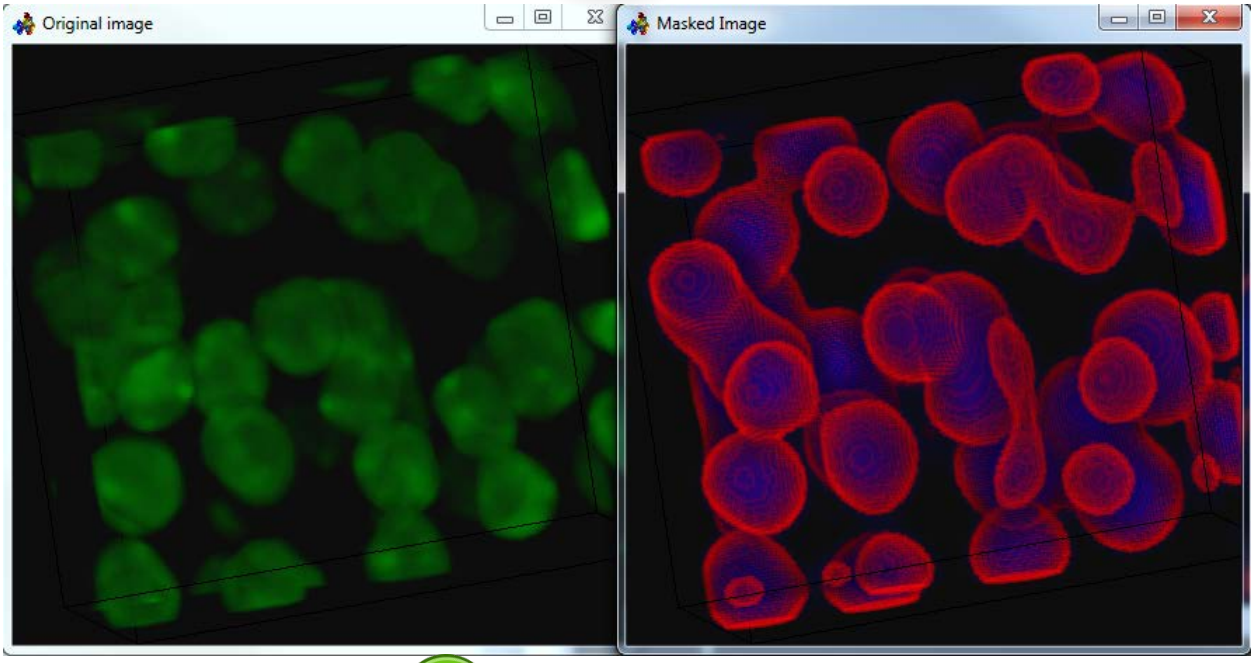
Click on  to perform 3D reconstruction and visualize the segmented cells in 3D space as shown in following:



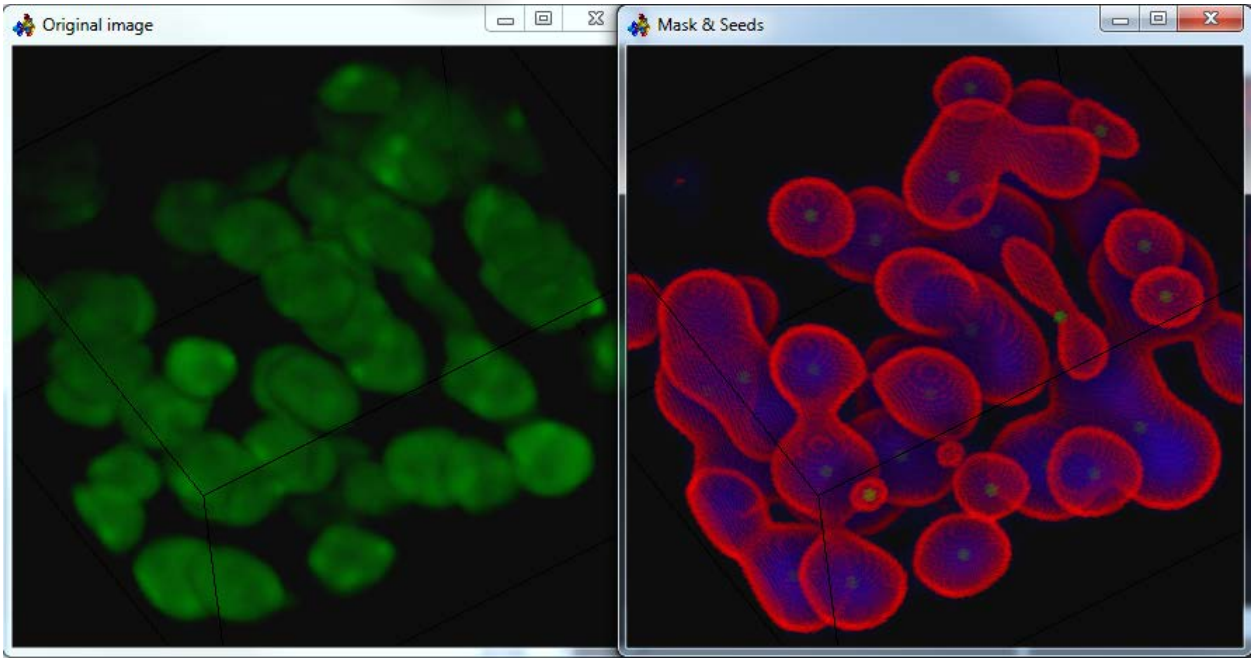


Tips: Visualize the 3D object in different processing state

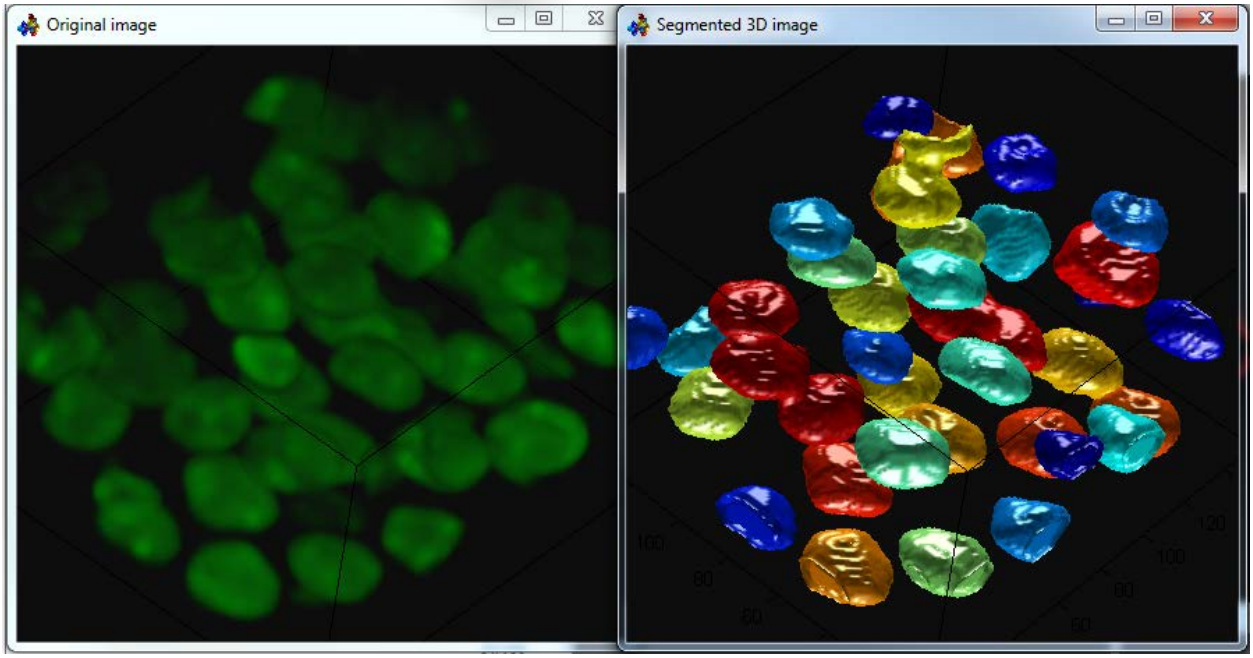
After foreground extraction processing state



After detection processing state



After segmentation processing state

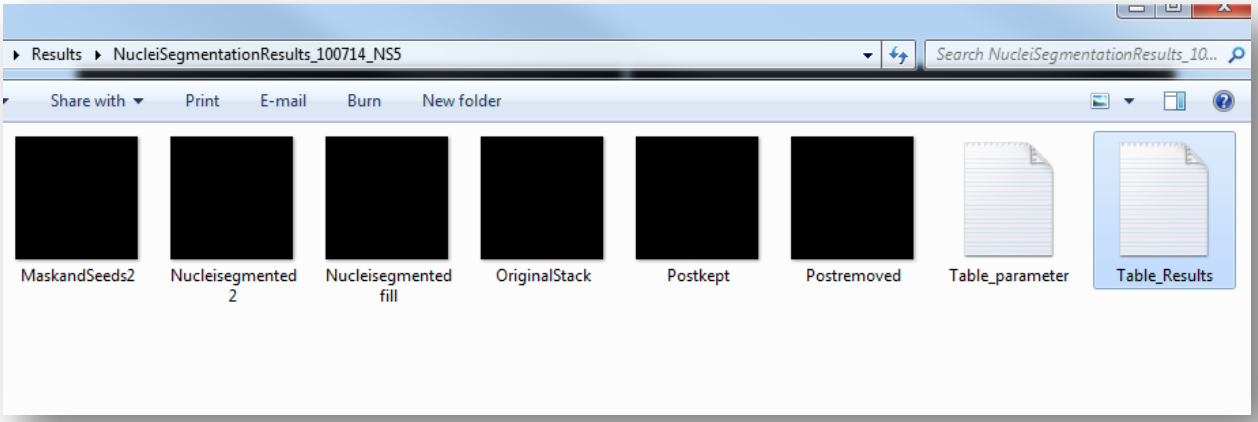


Step 8: Save (Export results)

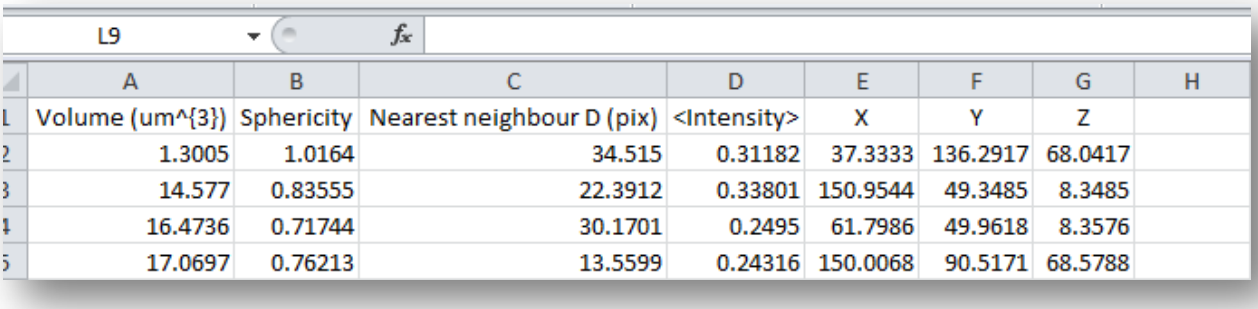
Click on the 

, image of tiff format are exported and saved at the directory selected by users. The several saved files as listed in following:

- 2 text files containing the applied parameters and the measurements of each nucleus are saved.
- 1 original interpolated image.
- 1 smoothed image with overlaid mask and seeds
- 2 segmented labelled image (fill and outlines)
- 2 binary images that contained with filtered nucleus and non-filtered nucleus.



*The computational quantitative result and configuration file will be **saved as a txt file under the same folder**. The name of the result file will be "Table_Results.txt" and their configuration file containing parameters setting named as" Table_parameter.txt"*



L9

	A	B	C	D	E	F	G	H
1	Volume (um^{3})	Sphericity	Nearest neighbour D (pix)	<Intensity>	X	Y	Z	
2	1.3005	1.0164	34.515	0.31182	37.3333	136.2917	68.0417	
3	14.577	0.83555	22.3912	0.33801	150.9544	49.3485	8.3485	
4	16.4736	0.71744	30.1701	0.2495	61.7986	49.9618	8.3576	
5	17.0697	0.76213	13.5599	0.24316	150.0068	90.5171	68.5788	

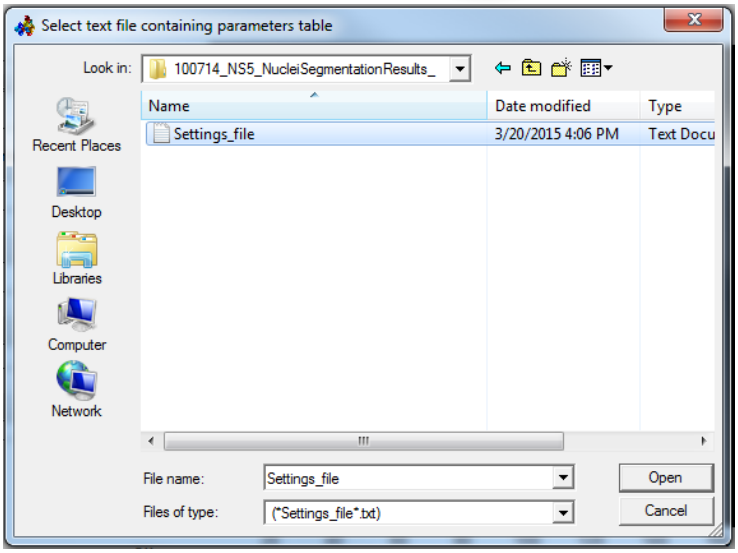
Tips: x, y, z coordinate value is indicate the centroid of each nucleus that only saved in text files.

Step 9: Batch processing

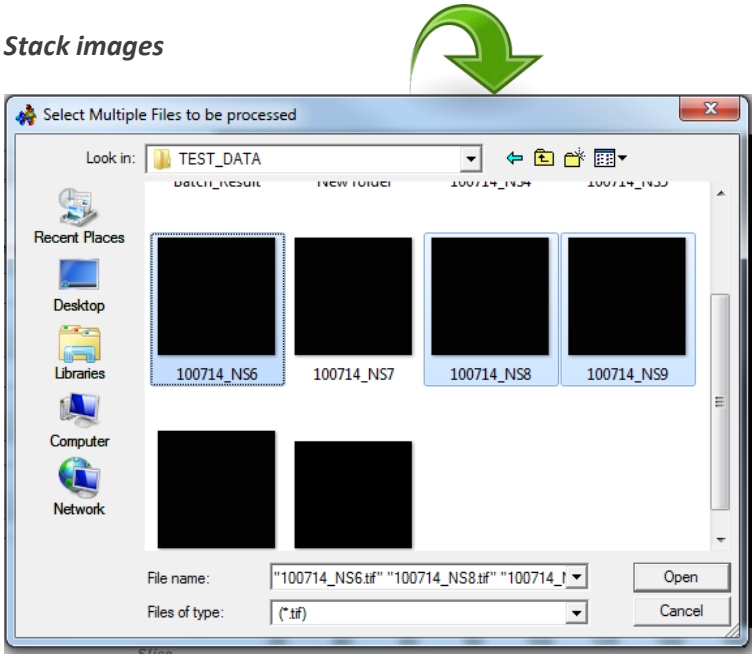
Click on 

button to select the configuration file and stack images need to perform the quantitative analysis.

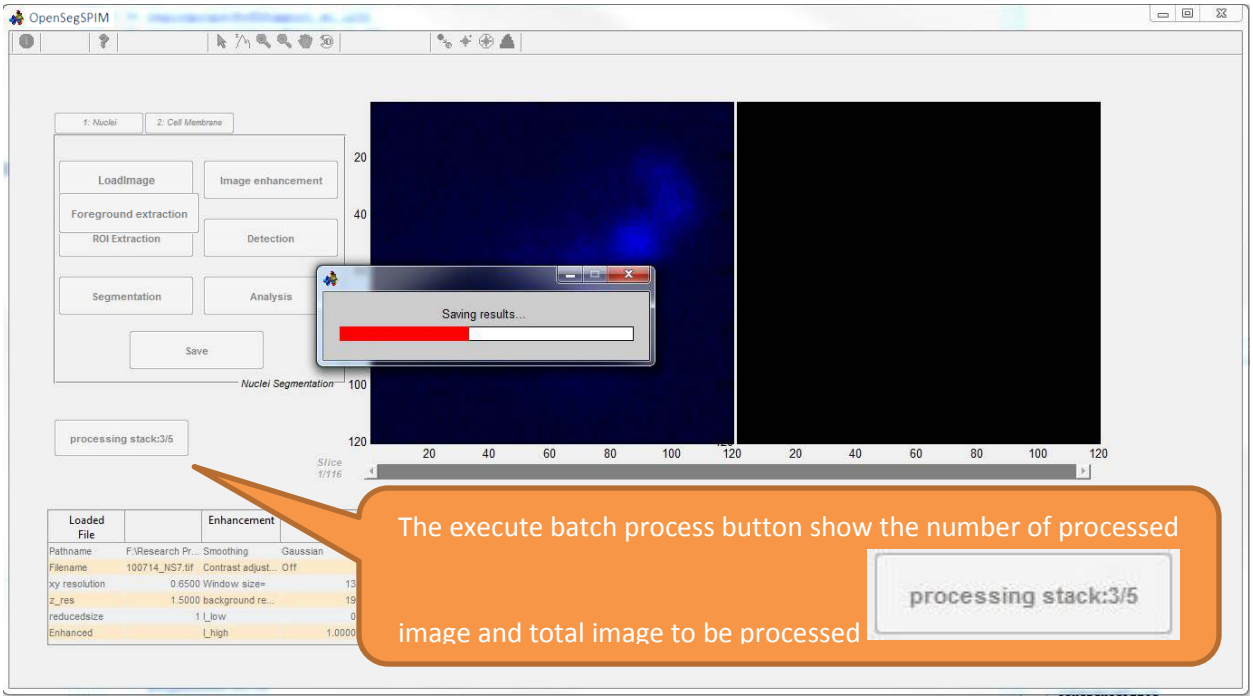
Configuration file



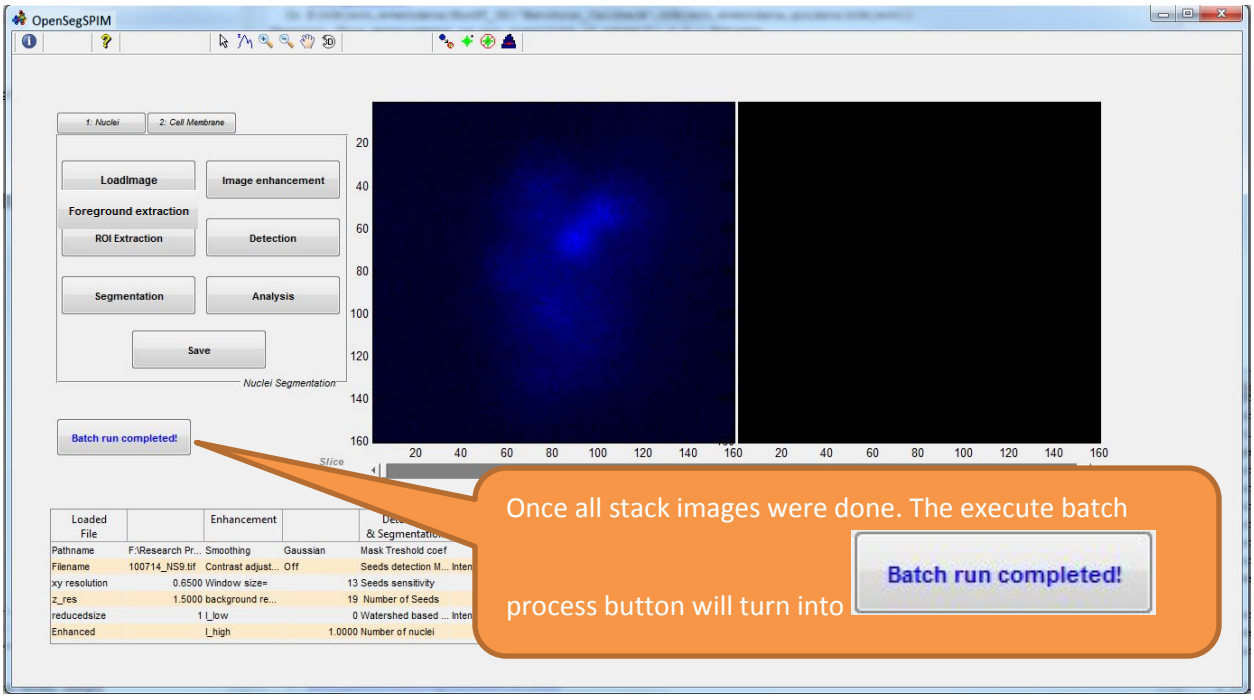
Stack images



Process is execute



Process is complete

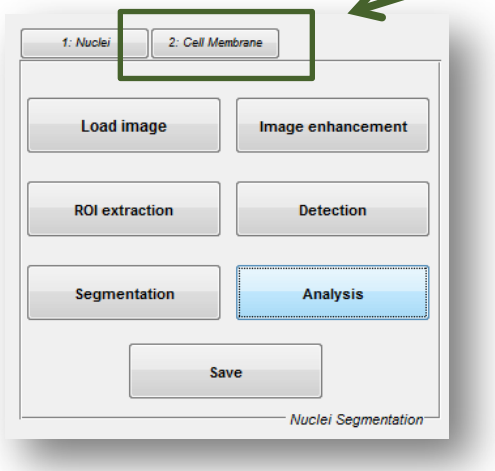


Chapter 3: Cell segmentation in action

Cell membranes segmentation

OpenSegSPIM provide additional features to do membranes segmentation if the membrane cell data is available. This function only available after the nucleus segmentation is complete. The 

button will be available to be accessed as shown in following figure:

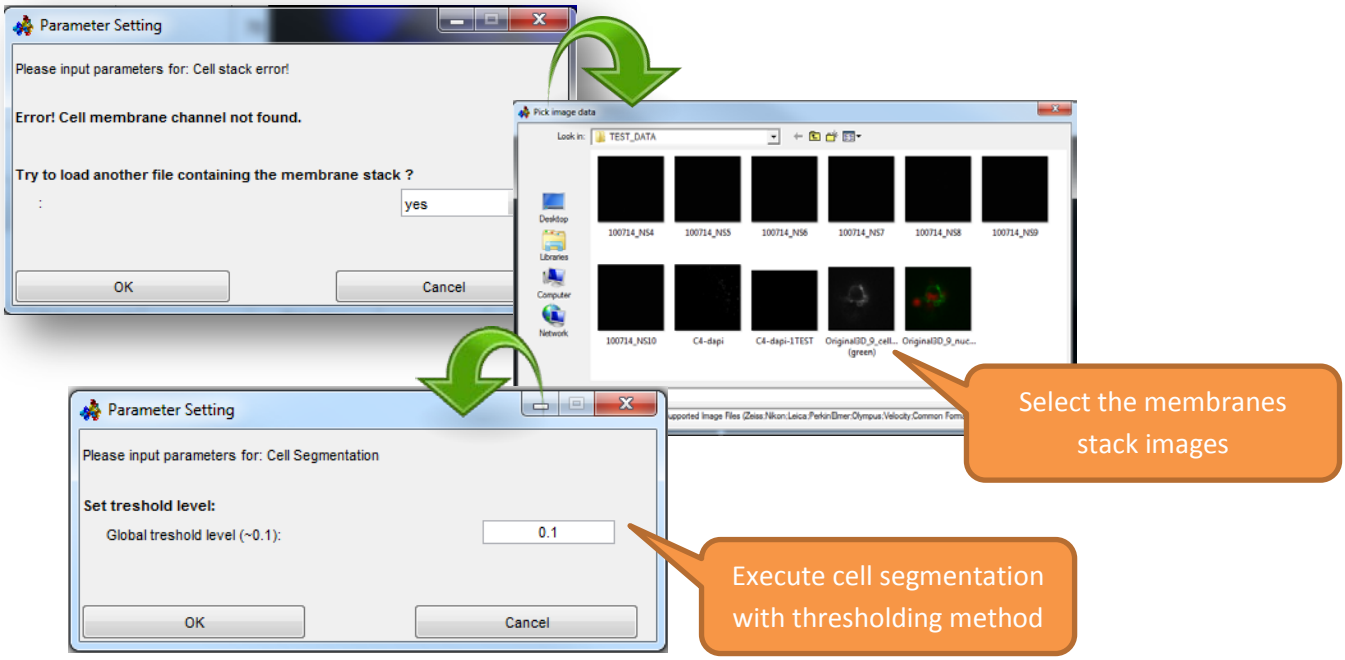


Click on 

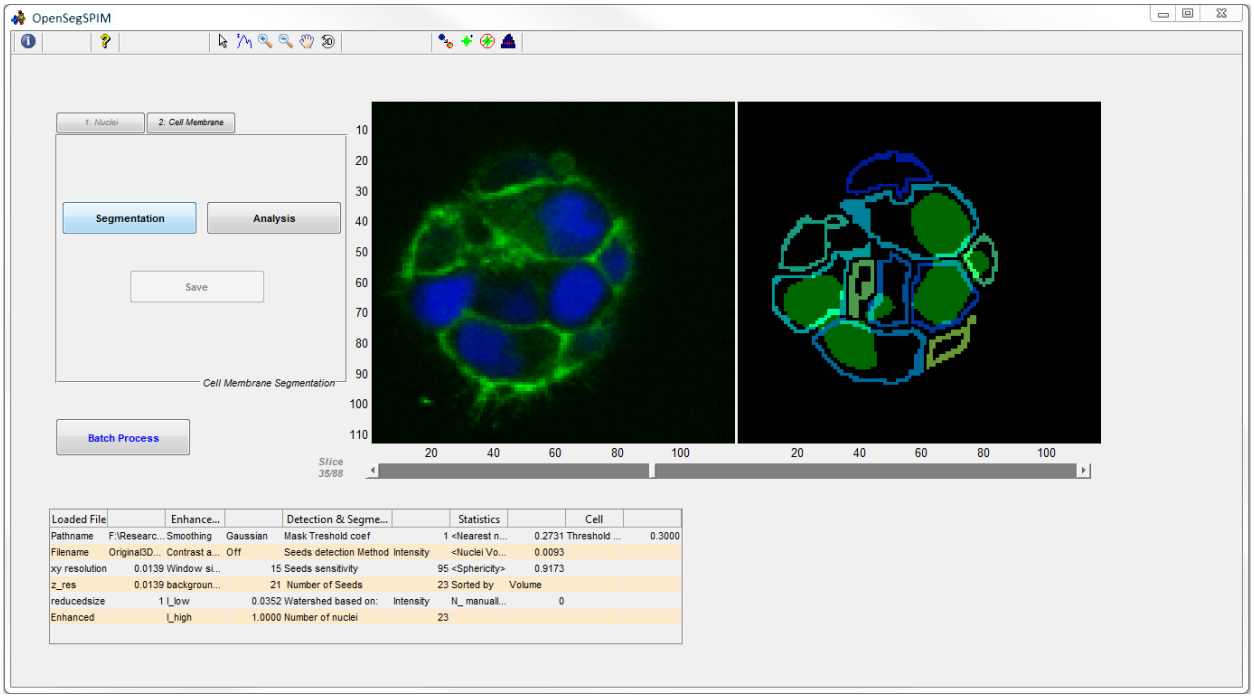
tab, following interface will be changed accordingly:

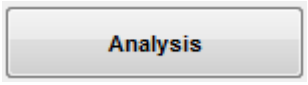
Click on the 

to load and select the membrane cell stack images as shown in following figure:

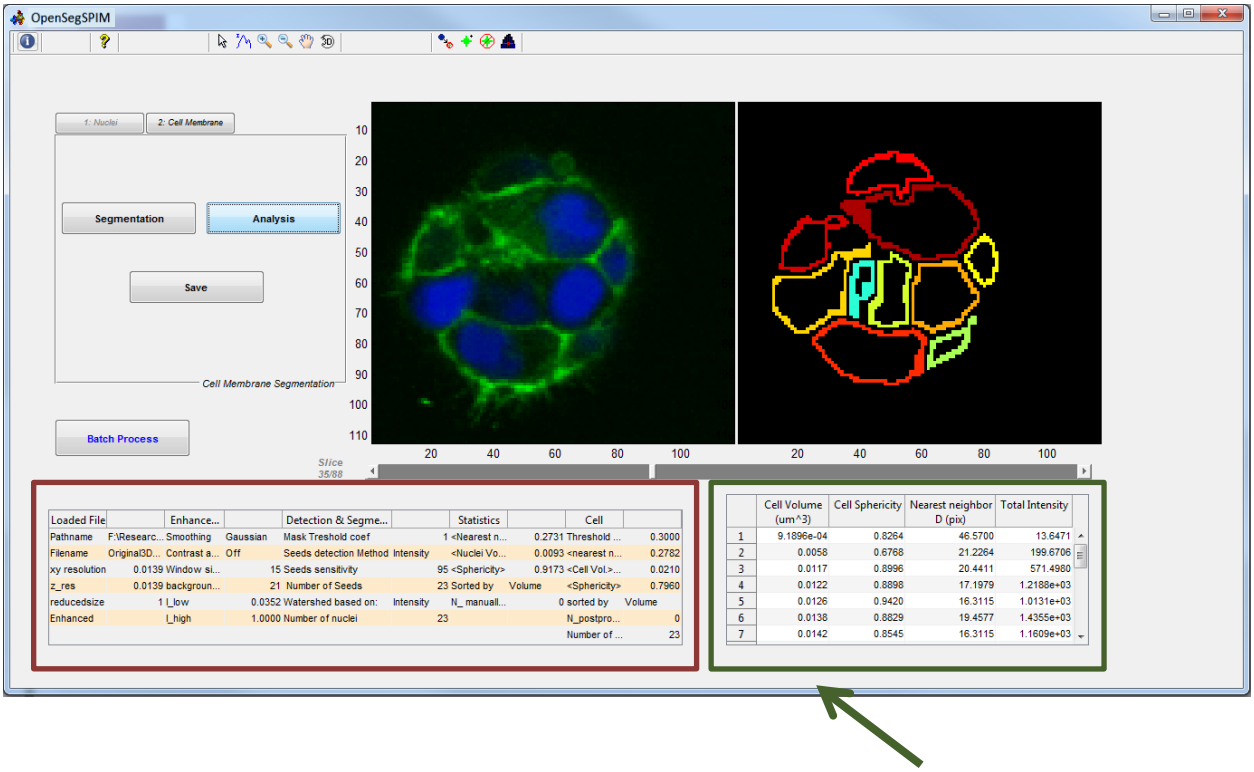


Once the cell segmentation is done, results of segmented cell will be displayed on the right visualization viewer.



Click on 

to extract quantitative information from cell as shown in green rectangle in following figure:



The 

button is similar to nucleus segmentation, it allow user to export and save the binary image and results in text file. The files including:

- Cell_measurement_results.txt – Quantitative results from cell.
- Cellsegmentedfill.tiff – labeled grayscale images that containing cell objects.
- CellSegments.tiff – labeled grayscale images that containing boundary of cell object.
- MaskandSeeds2.tiff – labeled color images that containing cell and seed object.
- OriginalStack.tiff – Original stack images.
- Settings_file.txt – a text file that store the information as shown in red rectangle.

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