General cell counter plugin for Fiji

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Contents

1 Purpose

The purpose of this plugin is to count objects on images. It offers a wide variety of methods to segment properly objects from background and allows a live preview of the result to help choose the parameters. In addition to the segmentation, it is possible to choose the range of size and circularity of the objects and to set a minimal distance below which two objects are counted as one. Finally, it pools the results from the images in a folder into a single file.

2 Setup

Move General_Cell_Counter-1.0.5.jar file into the folder Fiji.app/plugins/ (on MacOS X, right click Fiji.app > Show content folder). Keep only the latest version in that folder to prevent bugs.

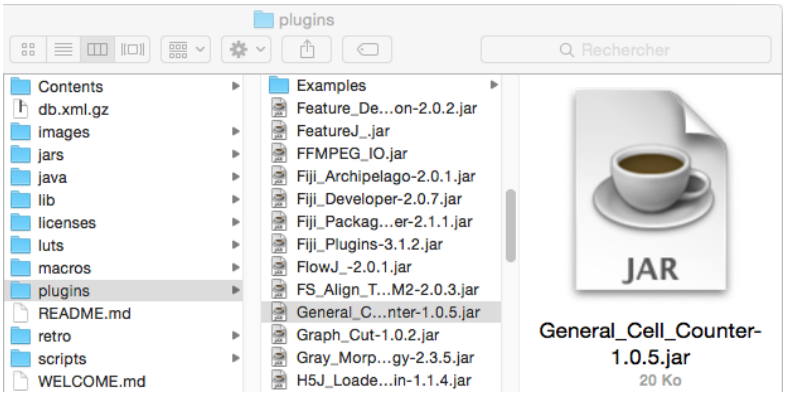


Figure 1 – Location to put .jar file.

Select *General Cell Counter* in the Plugins menu.

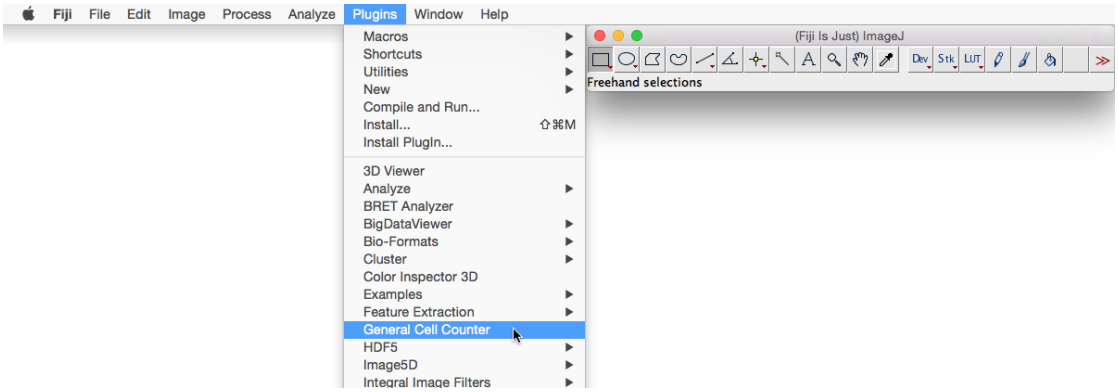


Figure 2 – Starting the plugin

3 Use

This plugin uses four windows: the main one (*General Cell Counter v1.0.5*) which manage images and parameters will pop up when the plugin starts; the second one is the Region Of Interest (ROI) Manager. In addition to this, there are two more windows dedicated to images: the image opened by the user and eventually (the preview of) the result.

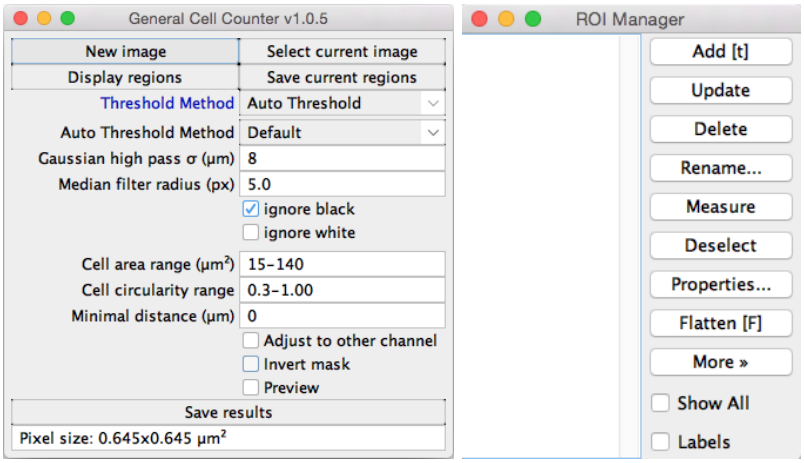


Figure 3 – Left: main window of the plugin. Right: Region Of Interest (ROI) Manager

3.1 Image management

GCC works on one image at a time and it has to be a saved one (in order to find its path to save subsequent files). To work with an image generated in Fiji, save it first. Two buttons can be used to change current image, they will both start by closing the previously chosen image and its result preview if they were opened. *New image* will prompt a dialog window asking to select an image in the file browser. The image will be opened and selected. *Select current image* will set the frontmost image window opened in Fiji as selected. *Preview* is automatically unchecked in both cases.

3.2 Regions Of Interest (ROI) Management

Analysis is done on the whole image. The result is then obtained for the whole image but can also in addition be obtained for one or more subregions of the image. To do so, the ROI Manager is used. Draw a region then hit T key (or click on Add in the ROI Manager window). Make sure there were no remaining ROIs when you start adding new ROIs, as they would be saved as well. Once all regions are in the ROI Manager, click on *Save current regions*. It will save a zip file containing the ROIs that are in the ROI Manager. Clicking on *Display regions* will clear the ROI Manager, load the zip file and display them on the original image.

3.3 Thresholding methods

This drop down menu offers different options to pre treat and threshold the images.

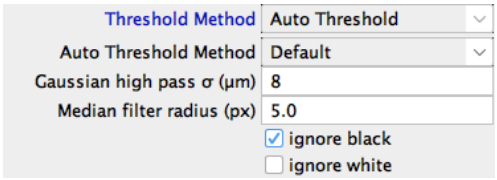
Auto Threshold

Automatic threshold methods generate a single threshold value that is used on the whole image to create the binarized image. The methods available are those present in Fiji version 1.52h. A description of these methods is available on ImageJ website.

Before applying this thresholding method, two optional pre treatment are available:

- a gaussian high pass filter to remove slow fluctuations of the background and have better contrast between cells and background. *Gaussian high pass σ (unit)* sets the range of the filter.

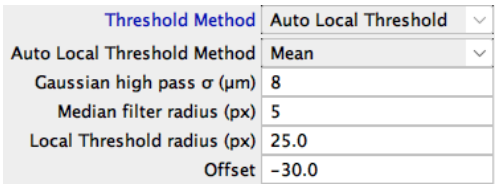
The unit is determined when the image is loaded. If it is calibrated, the unit of the image will be used (e.g. μm), else it will be in pixels. A value of 0 disables the gaussian high pass filter.



- a median filter to get a more homogeneous image and have potentially less pixelated cells. *Median filter radius (px)* sets the area of the filter. A value of 0 disables the median filter.

- *ignore black* and *ignore white* are used to ignore the extremities of the histogram (respectively 0 / 255 for 8-bit images, lowest / highest values for 16-bit images) during the Auto Threshold process.

Auto Local Threshold



Automatic local threshold methods generate a threshold value for each pixel to create the binarized image. The methods available are those present in Fiji version 1.52h. A description of these methods is available on ImageJ website.

Before applying this thresholding method, two optional pre treatment are available:

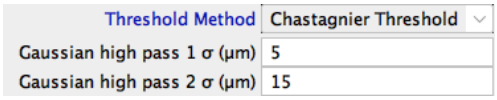
- a gaussian high pass filter to remove slow fluctuations of the background and have better contrast between cells and background. *Gaussian high pass σ (unit)* sets the range of the filter. The unit is determined when the image is loaded. If it is calibrated, the unit of the image will be used (e.g. μm), else it will be in pixels. A value of 0 disables the gaussian high pass filter.

- a median filter to get a more homogeneous image and have potentially less pixelated cells. *Median filter radius (px)* sets the area of the filter. A value of 0 disables the median filter.

All automatic local threshold methods share one parameter, the *Local Threshold Radius (px)*. It defines the area in which the threshold value is computed for each pixel.

Additionally, some methods have one or two parameters specific to them. Their descriptions can be found on ImageJ website. A parameter set to 0 will use the method's default value.

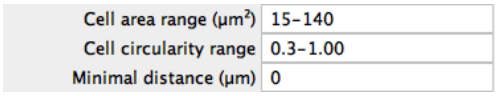
Chastagnier Threshold



Chastagnier Threshold uses a combination of global and local thresholding methods. Description of the method can be found in the paper.

The method has two parameters: two gaussian high pass filters (*Gaussian high pass 1 / 2 σ (unit)*). The unit is determined when the image is loaded. If it is calibrated, the unit of the image will be used (e.g. μm), else it will be in pixels.

3.4 Cells characteristics



Cell area range (unit²) defines the limits of area to count cells. If the size of a cell is below the first value or higher than the second one, it is ignored. The format of the field is either a range of two values separated by a "-" (e.g. 0-100, 20-40...), or a single value which will be the lower bound. Incorrect format might have unexpected behavior.

Cell circularity range sets constraints on the object's shape. The formula for circularity is $4\pi(area/perimeter^2)$. A value of 1.0 indicates a perfect circle, a value of 0.0 indicates a line. The format of the field is a range of two values separated by a "-".

Minimal distance (unit) can be used to count multiple objects detected as a single if they are too close. The bigger will be kept, the smaller ignored. On the resulting image, cells detected by the particle analyser will be represented in red. The ones ignored by the minimal distance criteria will be colored yellow instead.

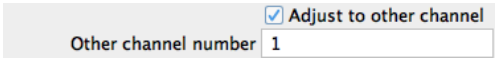
3.5 Channel adjustment

The idea behind the channel adjustment is to compare the regions found on a channel (e.g. a labelling of cells) to the regions found on another channel (e.g. nuclear labelling of the cells), to remove outliers which are not present on the other channel (e.g. cell without a nucleus). An other goal is to count a large region as more than one if it is an aggregate of multiple cells (e.g. cell with multiple nuclei).

In practice, it counts the cells as usual but removing the upper limit of area. It then counts, for each cell detected, how many objects detected on the other channel are located inside the cell (at least 50% of the pixels of the cell in the other channel located inside the cell in current channel). Table 1 tells whether the ROI should be removed, kept or count as more than one cell, based on the number of objects in the other channel (n). For n = 0, the ROI is discarded. For n = 1 it is kept as is. For n > 1, it depends on the fraction of the area (a) of the cell divided by n, compared to the area limits (a_{min} and a_{max}). If the fraction is between the limits, the cell count as n cells. If the fraction is below a_{min}, we count the maximum number of cells m for which $\frac{a}{m} > a_{min}$. It gives $m = \lfloor a/a_{min} \rfloor$. The idea behind the calculation of m is that the cell is big enough to be accounted for as a cell, but there are several objects in it which makes the average size too low. Taking the number of objects with the minimal size which fill the area is a trade-off between rejecting the ROI because it is composed of too small elements and counting all the objects because they appear small only because their signal overlap. If the fraction is above a_{max}, the cell count as 0, as the average area for each object would still be above the limit. Overall, if the result is above 1, the ROI is duplicated to have it the correct number of times.

	$\frac{a}{n} < a_{min}$	$a_{min} < \frac{a}{n} < a_{max}$	$\frac{a}{n} > a_{max}$
$n = 0$	0	0	0
$n = 1$	0	1	0
$n > 1$	$\lfloor a/a_{min} \rfloor$	n	0

Table 1 – Number of times the region is counted based on the number of regions found on the other channel (n) and the relation between the area (a) and its limits (a_{min} and a_{max}).



Adjust to other channel enables or disables the channel adjustment.

Other channel number is the id of the channel to use for channel adjustment.

Note that enabling channel adjustment will disable *Minimal distance*.

3.6 White background

By default, *General Cell Counter* expects white objects on black background. Check *White background* for GCC to inverse images with black objects on white background before doing the process of filtering and thresholding the image.

3.7 Preview

When enabling the preview, the original image is first thresholded with the method selected. Then, the *Analyse Particules...* is done with current parameters for cell size and circularity. Finally, the distance between objects found is checked to ignore objects too close to another object (count them as one). Once the process is done, a new image is opened with: in red the objects counted; in yellow the objects ignored because they are too close to another object; in white the objects not counted because of the size or circularity parameters; in black the background.

When the preview is enabled, parameters can be modified to see the effect with live effect. When updating a field, hit enter to take the modification into account.

In case the image has multiple frames, slices and/or channels, it is the currently selected one that is processed.

3.8 Save results

When hitting the *Save results* button, first the process described for the preview is executed. Then, for each region saved with the *Save current regions* button for the current image (or for the whole image if none has been saved), a count of the number of cells present in the region is done (a cell is counted if its center of mass is inside the region), and the area of the region is measured.

The results are saved in GeneralCellCount.csv. The preview image and the cells regions are also saved. See section 4.

In case the image has multiple frames, slices and/or channels, it is the currently selected one that is processed.

3.9 Log field

The field just below *Save results* button displays warning, errors and notifications to the user. Here is a list of possible messages and their meaning, ordered alphabetically.

- Display regions requires an image: when clicking *Display regions* while there is no image opened or selected.

- Failed to open image: when clicking on *New image* but Fiji doesn't manage to open it (if the image is corrupted or if it is not an image).

- Image already selected: when clicking on *Select current image* while it is already the one used by the plugin.

- Image must correspond to an opened file: when clicking on *Select current image* on an image that was not opened but created in Fiji. To use such an image, save it somewhere, then click again on *Select current image*, or use *Open image*.

- Image selected: when successfully selecting current image.

- Incorrect channel number or zip file doesn't exist: when process is done with *Adjust to other channel* is enabled but the zip file corresponding to the *Other channel number* doesn't exist, either because the channel number is incorrectly written or the analysis wasn't previously done for that channel.

- Method doesn't exist. Process canceled: when enabling the preview (or clicking *Save results*) while the method selected is incorrect. It shouldn't happen unless the code is changed to add new methods.

- No image to process: when enabling the preview (or clicking *Save results*) while there is no image opened or selected.

- No image to save regions for: when clicking *Save current regions* while there is no image opened or selected.

- No image to select: when clicking on *Select current image* while there are none opened in Fiji.

- No ROI to save: when clicking *Save current regions* while there is no region in the ROI Manager.

- No ROIs file to open for current image: when clicking *Display regions* while there is no existing .zip ROI file for current image.

- Open image canceled: when clicking on *New image* then canceling.

- Parameter changed: when modifying the value of a field.

- Parameter is not a number: when entering a badly formatted value in a field requiring a number.

- Pixel size: 1x1 $unit^2$: when successfully opening or selecting an image. With 1 and unit replaced accordingly.

- Preview displayed. n object(s) detected: when the preview is correctly generated. n represents the number of regions in the whole image (red and yellow included).

- Results saved: when *Save results* successfully process current image and saves the results.

- ROIs opened: when *Display regions* successfully opens ROIs.

- ROIs saved: when *Save current regions* successfully saves ROIs.

4 Files generated

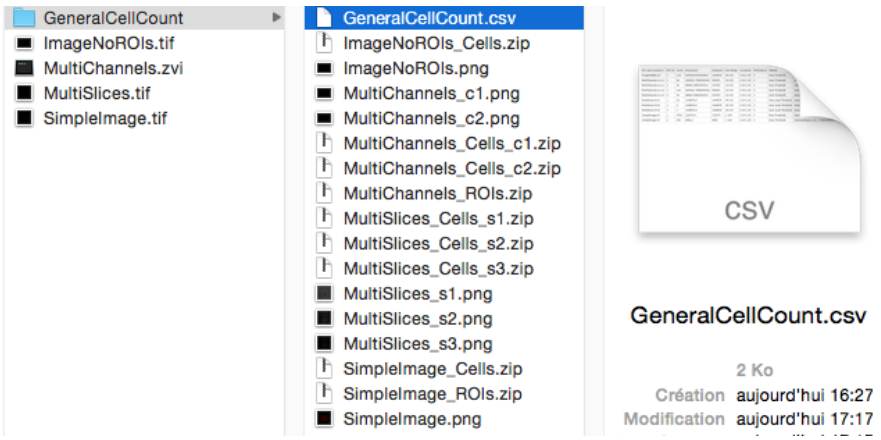


Figure 4 – Structure of the files. Left: folder containing raw images and the subfolder. Middle: content of the subfolder. Right: preview of the results file. In this example, there are four different image files. ImageNoROIs.tif: a single image on which no regions were selected by the user. MultiChannels.zvi: 2 channels and 2 regions selected. MultiSlices.tif: 3 slices and 0 region selected. SimpleImage.tif: simgle image with 2 regions selected.

All files generated are placed in a subfolder named GeneralCellCount and located in the same folder as the original image (let's call it ImageName.tif).

Save current regions saves the .zip file named ImageName_ROIs.zip containing the regions selected by the user for the image.

Save results saves multiple files:

File name [position]	ROI ID	Count	Area(unit2)	Area(px2)	Size Range	Circularity	MinDistance	Method					
ImageNoROIs.tif	1	119	600540.8704163812	1443519	20-100	0.00-1.00	0	Auto Threshold	GaussianSigma:10.0	MedianRadius:3.0	AutoThMethod:Default		
MultiChannels.zvi c1	1	62	229022.17852500002	550501	10-100	0.00-1.00	0	Auto Threshold	GaussianSigma:10.0	MedianRadius:5.0	AutoThMethod:Default		
MultiChannels.zvi c1	2	20	98806.76955000001	237502	10-100	0.00-1.00	0	Auto Threshold	GaussianSigma:10.0	MedianRadius:5.0	AutoThMethod:Default		
MultiChannels.zvi c2	1	122	229022.17852500002	550501	10-100	0.00-1.00	0	Auto Threshold	GaussianSigma:10.0	MedianRadius:5.0	AutoThMethod:Default		
MultiChannels.zvi c2	2	40	98806.76955000001	237502	10-100	0.00-1.00	0	Auto Threshold	GaussianSigma:10.0	MedianRadius:5.0	AutoThMethod:Default		
MultiSlices.tif s1	1	22	1048576.0	1048576	40-100	0.00-1.00	0	Auto Local Threshold	GaussianSigma:10.0	LocalThRadius:10.0	LocalThMethod:Phansalkar	k value:0.0	r value:0.0
MultiSlices.tif s2	1	2	1048576.0	1048576	40-100	0.00-1.00	0	Auto Local Threshold	GaussianSigma:10.0	LocalThRadius:10.0	LocalThMethod:Phansalkar	k value:0.0	r value:0.0
MultiSlices.tif s3	1	1	1048576.0	1048576	40-100	0.00-1.00	0	Auto Local Threshold	GaussianSigma:10.0	LocalThRadius:10.0	LocalThMethod:Phansalkar	k value:0.0	r value:0.0
SimpleImage.tif	1	4718	132370.0	132370	1-100	0.00-1.00	0	Auto Threshold	GaussianSigma:10.0	MedianRadius:1.0	AutoThMethod:Default		
SimpleImage.tif	2	245	6992.0	6992	1-100	0.00-1.00	0	Auto Threshold	GaussianSigma:10.0	MedianRadius:1.0	AutoThMethod:Default		

Figure 5 – Resulting file. In this example, there are four different image files. ImageNoROIs.tif: a single image on which no regions were selected by the user. MultiChannels.zvi: 2 channels and 2 regions selected. MultiSlices.tif: 3 slices and 0 region selected. SimpleImage.tif: simgle image with 2 regions selected.

- GeneralCellCount.csv: the results of all the images of the folder that have been analyzed, as a spreadsheet with one line for each region selected by the user containing: the file name (with eventual frame, slice or channel position), the ROI ID, the count of cells detected, the area in unit^2, the area in pixel^2, the size range, the circularity, the min distance, the method, and the other parameters for the method selected.

- ImageName_Cells.zip: the ROIs detected by *Analyse Particles....* In red the cells counted and in yellow the cells ignored by the min distance criteria, as seen on preview image. In case the image has multiple frames, slices and/or channels, "_fi" (i-th frame), "_sj" (j-th slice) and/or "_ck" (k-th channel) is added to the name before .zip. E.g. ImageName_Cells_f3_c2.zip for 3rd frame 2nd channel of ImageName.

- ImageName.png: the preview image with regions selected outlined in green. In case the image has multiple frames, slices and/or channels, a suffix is added, e.g. ImageName_f3_c2.png for 3rd frame 2nd channel of ImageName.

5 Closing General Cell Counter window

Closing the main window of the plugin (titled General Cell Counter v1.0.5) will do the following:

- Save current parameters in ij.Prefs so that they are automatically loaded the next time the plugin is started.
- Close original and preview images.
- Close the ROI Manager.
- Close the Results window.
- Save the current position of the General Cell Counter window and finally close it.