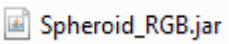
version 1.0

Spheroid RGB

by Maximilian Maske

This document provides a brief guide to the first version of the Spheroid RGB ImageJ/Fiji plugin, released in January 2017 for analysing microscopy images of cancer spheroids which are labelled with up to three different fluorescent markers. The tool provides a user interface to manipulate certain parameters, to use the software in a variety of different ways.

The software has been developed as part of a project with *Auckland Cancer Society Research Centre* and *Bio Imaging Research Unit* at the University of Auckland, New Zealand.

Install the software by simply drag and drop 

into the plugins folder of ImageJ/Fiji. Restart ImageJ and go to *Plugins --> Spheroid RGB* to run the plugin. If the is no image already open, an example image will be shown for demonstration. You are now also able to open more images via the plugin.

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Basic Functions

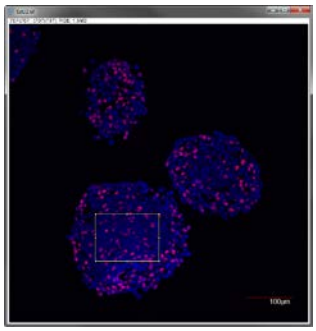
Select colour channels

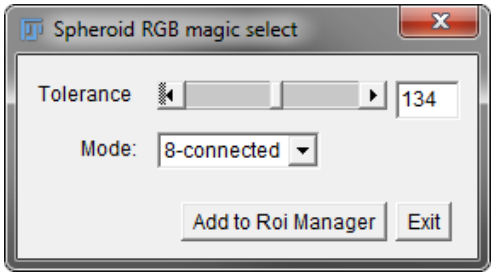
Use a merged RGB image as input and select the channel(s) you want to analyse. If you select more than two channels and you want to display the value ratios and/or ratio mean, you need to select the channel which represents the total number of cells. For example you analyse the area of red and blue marked cells, where the blue marker ideally represents all cells. The ratio would be the red area divided by the blue area, not the other way around.

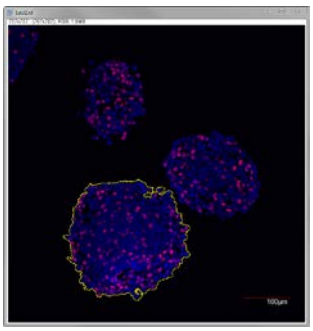
You can also use a grey-scaled image. In this case select none of the three channels. “Count and mean” requires an 8-bit image. “Multi plot” will take any format.

Using magic select

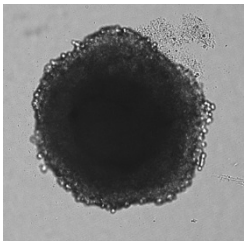
To create a Region of Interest that fits to the Spheroid, use magic select. It is looking for similar pixels related to a seed value. If the current image has no selection the seed value will be a pixel with the maximum intensity in the image. If you use an area selection, the seed value will be the brightest point in that selection. You can also use a point selection to determine the seed manually. From here you need to adjust the tolerance and the kernel mode until you get a suited polygon selection.

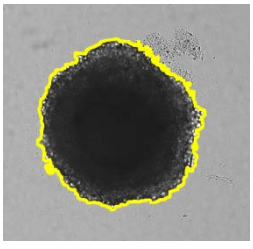






In case the segmented phase is inverted (bright field images) select the checkbox light background (dark peaks). The magic selection will now start from the pixel with the lowest intensity value.





Count and Mean

Using automatic threshold

Depending on what colour channel is selected the threshold slider will change as well. The default threshold slider (no channel selected) is for grey-scaled images. If you click auto the slider will be set to the result of the automatic threshold calculation depending on the selected threshold mode and channel.

If your image has a light background, the threshold value will be subtracted from the maximum of 255. So 15 means that all pixels in the value range from 240-255 will be above threshold instead of 0-15.

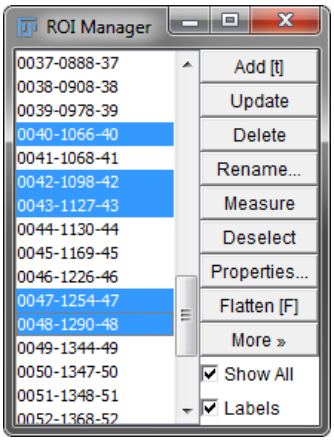
Which method segments your data best? To answer this question split the image with the show button and process this images with the ImageJ threshold plugin to have a better understanding what the threshold is segmenting.

More detailed information: http://imagej.net/Auto_Threshold

With max you can set the slider to the maximum/minimum noise value from an area selection, depending of light background is selected. It makes sense to select a region from the background.

Dependency to the ROI Manager

The plugin will iterate through all ROIs in the ROI Manager and list the results for each ROI in a row in the Results table. If the ROI Manager is empty yet, it will save the active ROI to the list for future analysis.

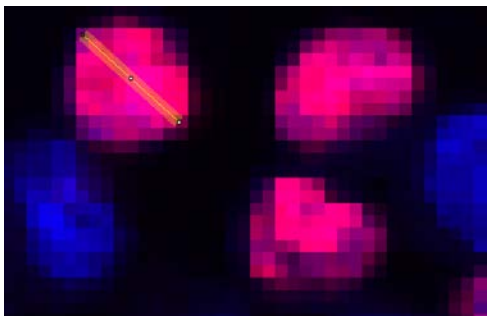


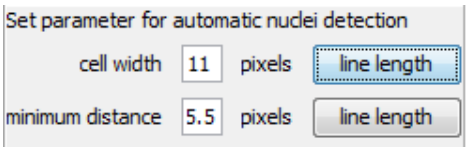
In case you have more ROIs than you want to run through the analysis, just select the ROIs by holding the Ctrl key.

By the way: The default name of an ROI of interest is made up as follows: 0003-200-500-02 is a ROI from a Stack on the 3. Slice and has its centroid coordinates at y = 200 and x = 500 and is the second of its kind. You can also rename the ROI as you wish.

Measuring the cell width and minimum distance

To give the automatic count algorithm an estimation of the diameter of the cells, select the straight line tool from ImageJ and draw a line over a representative cell. The minimum distance will be automatically set to the half of the cell width. If the cells are further apart from each other you can increase this value manually or by measuring a line length similar as above.





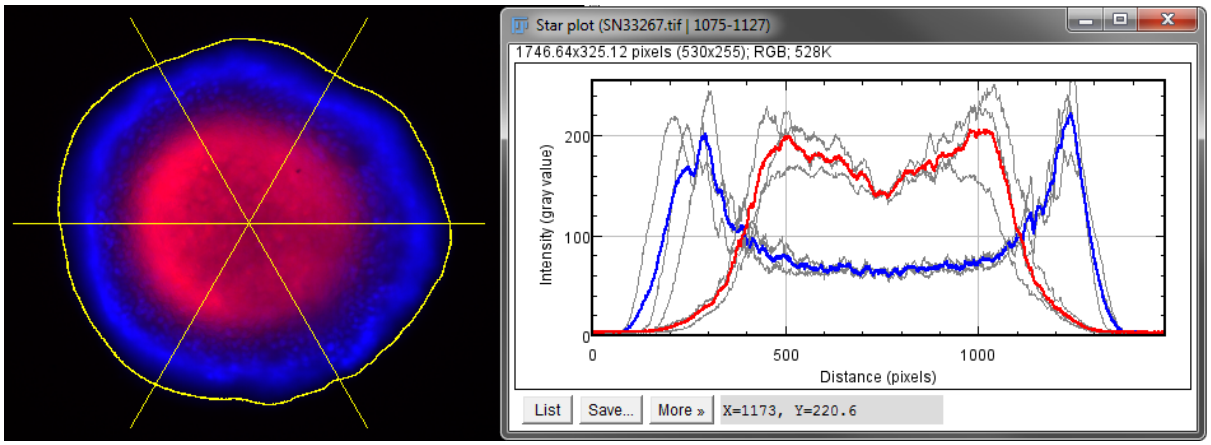
Multi Plot

Plot the intensity of an Area ROI in various ways. If there is no active ROI pointing to the image the plugin will take the first element from ROI Manager.

Star Plot

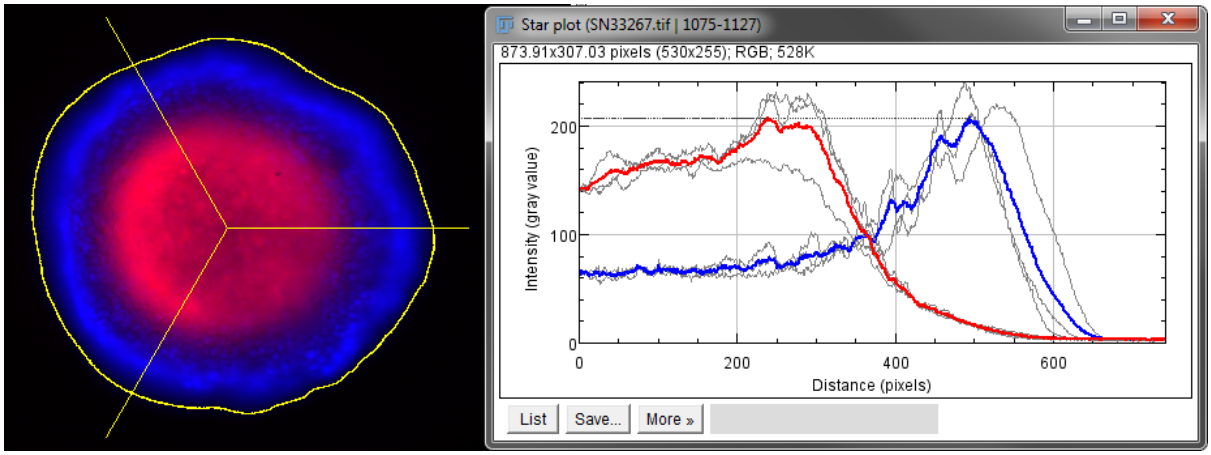
This method will plot the profile through the centroid of an active area and it will turn the angle of the lines up to 10 times and then averaging the results. The angles between all lines are equal. You can vary the number of lines from 1 to 10 and the length of the line from -50% to +100% of the radius. The radius is either the width or height of the bounding box of the selection, depending which is greater.

Diameter
A plotted line goes from one edge (±variance) of the selection through the centroid to the other edge (±variance).



Example of diameter star plot with 3 lines and +10% line length.

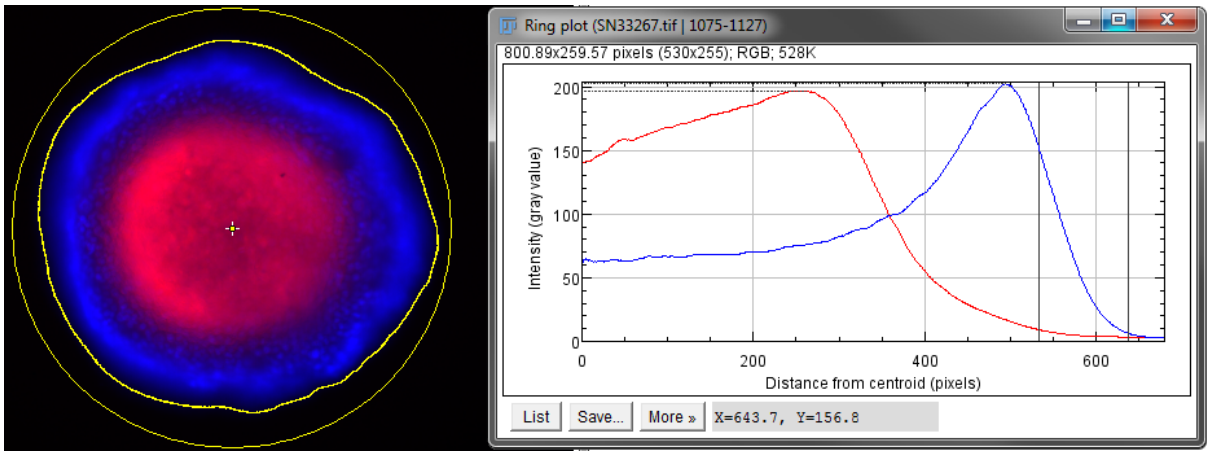
Radius
A plotted line starts always from the centroid of the selection and ends at the edge (±variance). Additional you get a results table of maxima and optional bounds and area under the plot.



Example of radius star plot with 3 lines and +10% line length.

Ring Plot

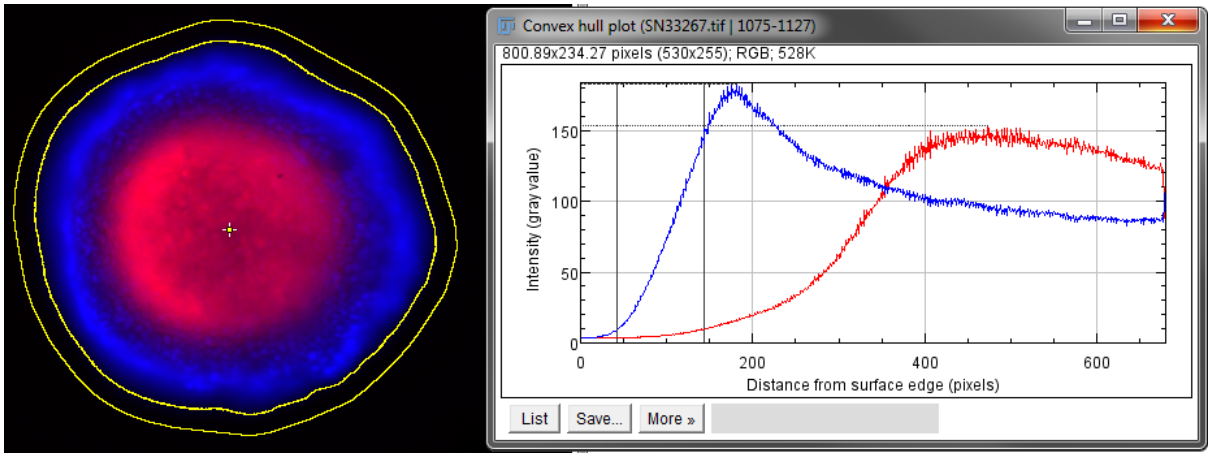
This method calculates the average of the intensity from each pixel in the selection with the same distance to the centroid of the selection. You can vary the maximum distance by the variance slider, to make sure you see the background noise in the plot.



Example ring plot with +5% variance. The yellow ring represents the maximum distance.

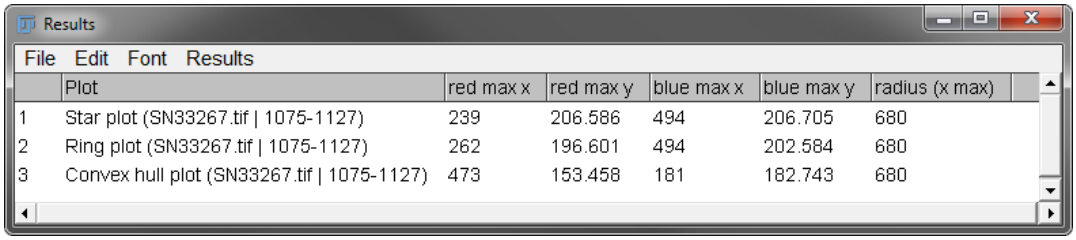
Convex Hull Plot

This method calculates the average intensity from each pixel in the selection with the same distance to the adge of the convex hull of the selection. You can enlarge or shrink the convex hull by the variance slider.



Example convex hull plot with +5% variance.

The results table could look like this:

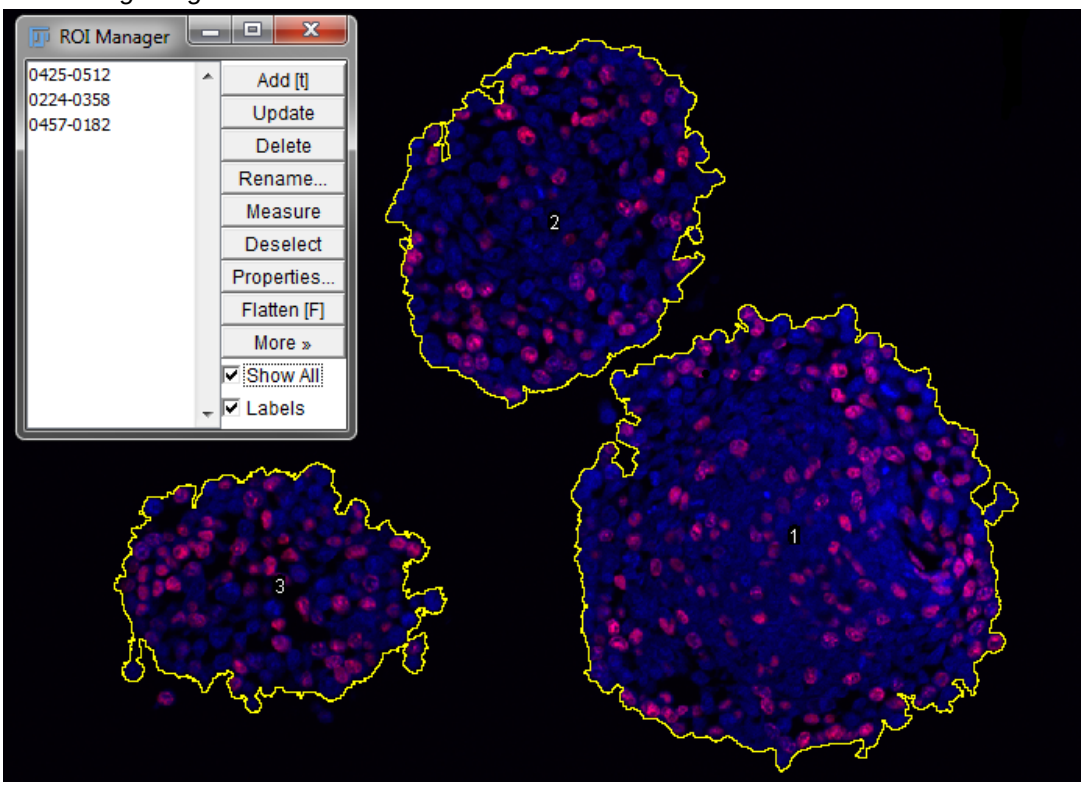


	Plot	red max x	red max y	blue max x	blue max y	radius (x max)
1	Star plot (SN33267.tif | 1075-1127)	239	206.586	494	206.705	680
2	Ring plot (SN33267.tif | 1075-1127)	262	196.601	494	202.584	680
3	Convex hull plot (SN33267.tif | 1075-1127)	473	153.458	181	182.743	680

Example Use Cases

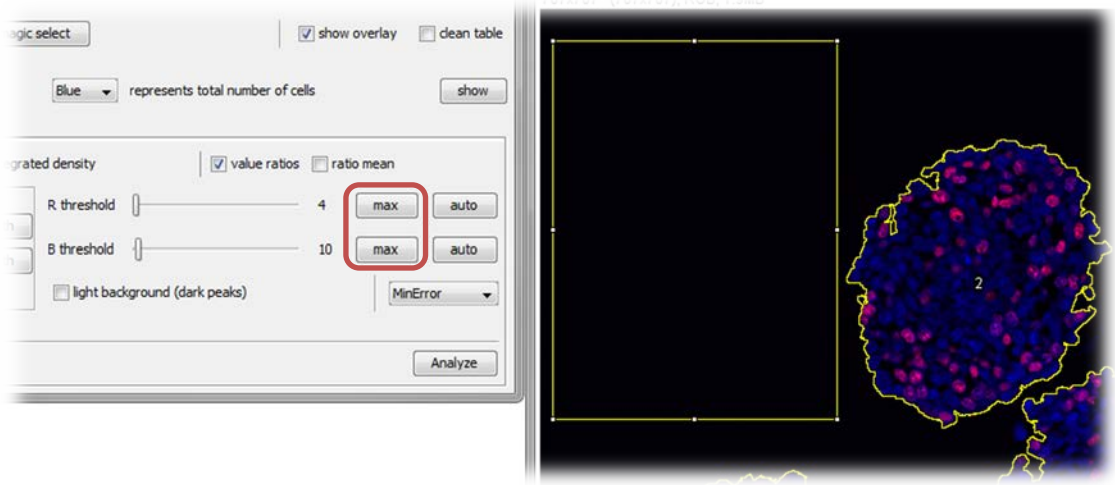
Count the cells of multiple Spheroids on one image at once

1. Define your selection by magic select and add them to the ROI Manager. See *Using magic select* for details.

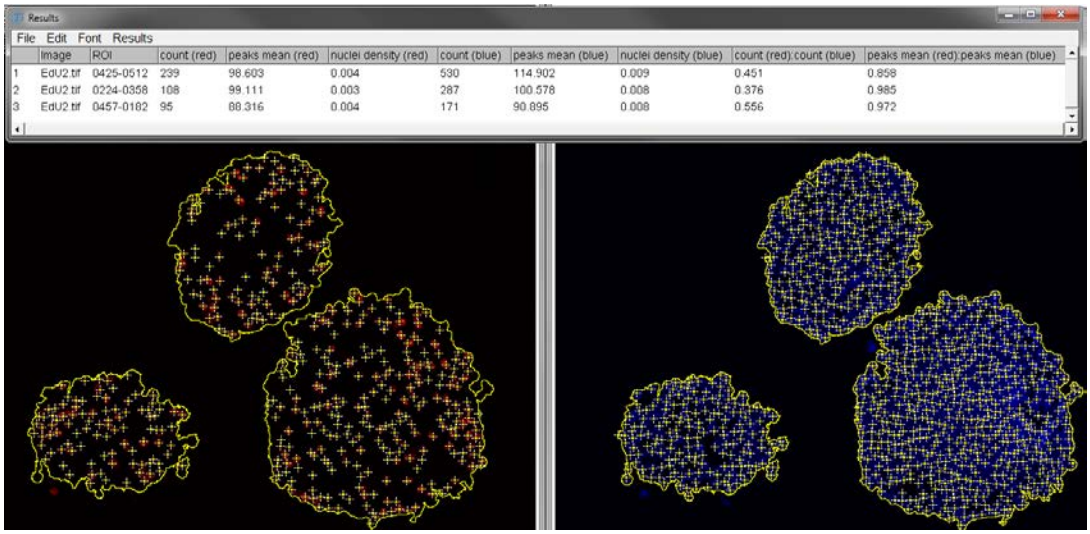


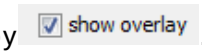
2. Select the colour channel you want to analyse and which channel represents the total number of cells.

3. Select count cells and measure the diameter of a representative cell. It is recommended to set the minimum distance to the half of the cell width.
4. Chose a relatively low threshold to just avoid background noise. You may measure the maximum background noise by selecting an area and click *max*.



5. If you are interested in the actual ratios of the counted cells, make sure you select the option *value ratios*
6. Hit Analyse and wait a moment. Depending of the accuracy of the estimation of the cell with and the threshold the algorithm will count the cells. The output will be the number of cells, the mean intensity of the peaks and the nuclei density for each selected ROI and channel.
    - **Count** is the number of all peaks (local maxima depended by cell width, minimum distance and threshold).
      Peaks Mean is calculated by the sum of all intensity by all local maxima divided by the number of cells (peaks)
    - **Nuclei Density** is calculated by number of cells divided by the calibrated area of the ROI (total area/number of pixels).



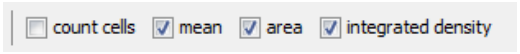
All ROI and position of the counted cells will be saved as an overlay, which you can switch on and off by 

. If you save the result images the overlay will by attached to the file. You can see the overlay by reopen the file in ImageJ.

Measure mean intensity, area, integrated density of each ROI

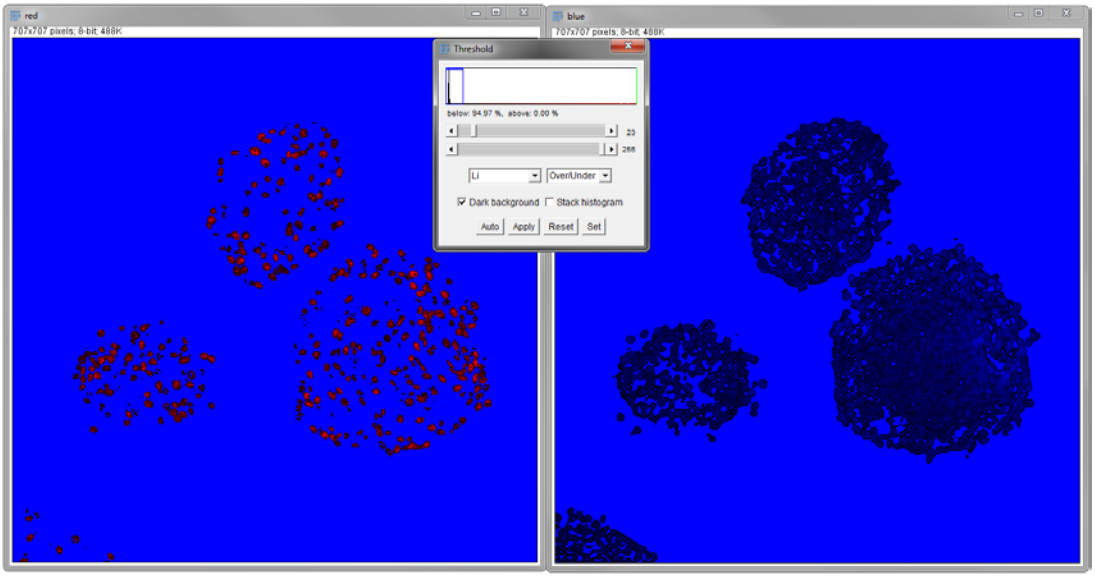
1. Define your selection by magic select and add them to the ROI Manager. See *Using magic select* or example above for details.
2. Select the colour channel you want to analyse and which channel represents the total number of cells.

3. Select your output.

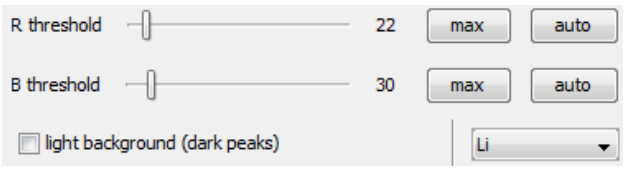


All components related to the count algorithm will be faded out.

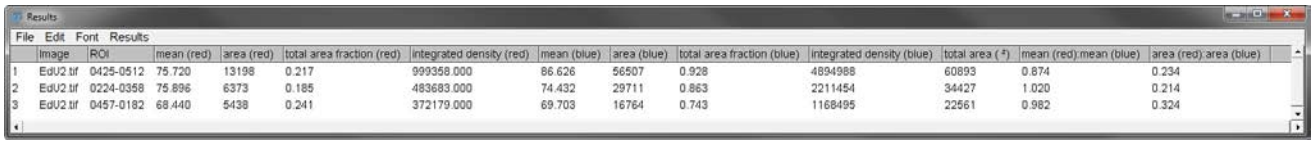
4. Find the best automatic threshold mode. To do so click *show* and try various options by *Image – Adjust – Threshold…* (*Ctrl+Shift+T*). Li looks fine for both channels here. The most importand thing is to get the actual area of the cells. Every pixel above the threshold will define the area.



5. Now set the threshold on the user interface of the plugin by choosing the automatic threshold and apply it by clicking the auto button next to the threshold slider.

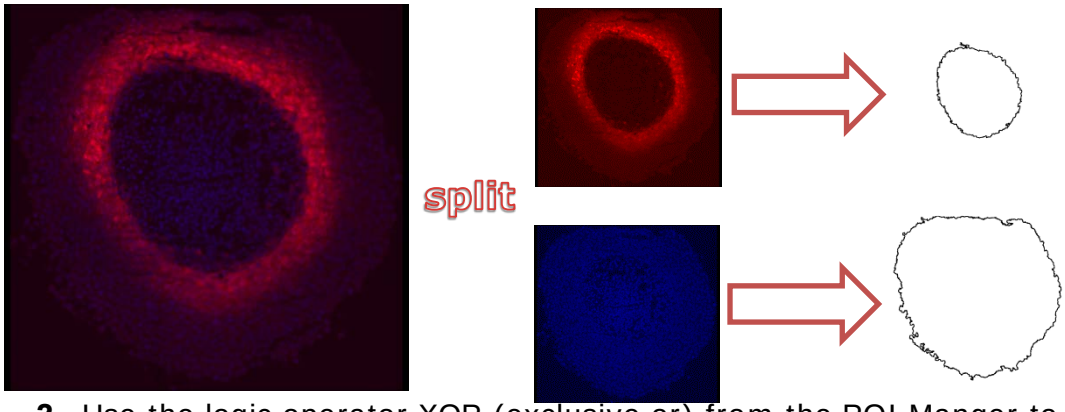


6. If you also select values rations you get the following output after clicking analyse:
    - **Mean** is the sum of the intensity of every pixel above the threshold divided by the number of pixels above the threshold
    - **Area** is the number pixels above the threshold multiplied by the calibration factor in x and y direction. The result is the actual calibrated area without background (set by threshold)
    - **Total Area Fraction** is the Area divided by the total area of the ROI without threshold
    - **Integrated Density** is the mean value multiplied by the area

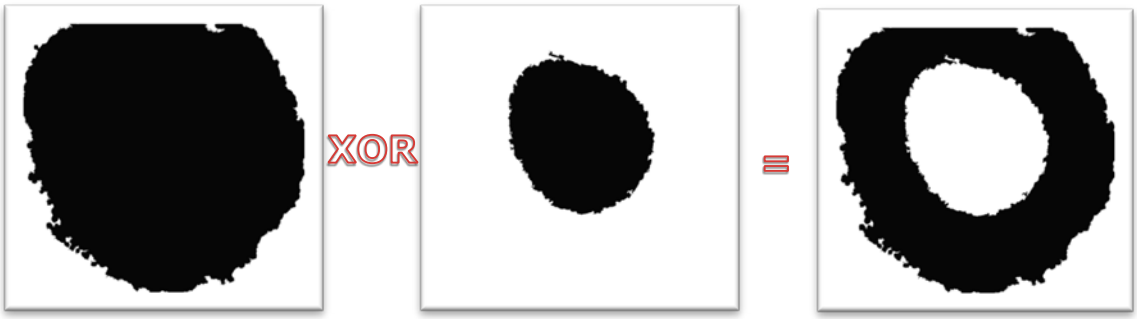


Compare the core with the rim of a spheroid

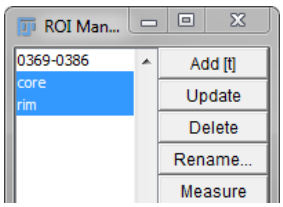
1. Define a selection that includes the entire spheroid and another of the core. Add those selections to the ROI Manager.
   Tipp: split the image in case some channels might be better for selection. In the example below the red channel defines the core, not the blue. For that reason it is better to perform magic select on the separated channel.

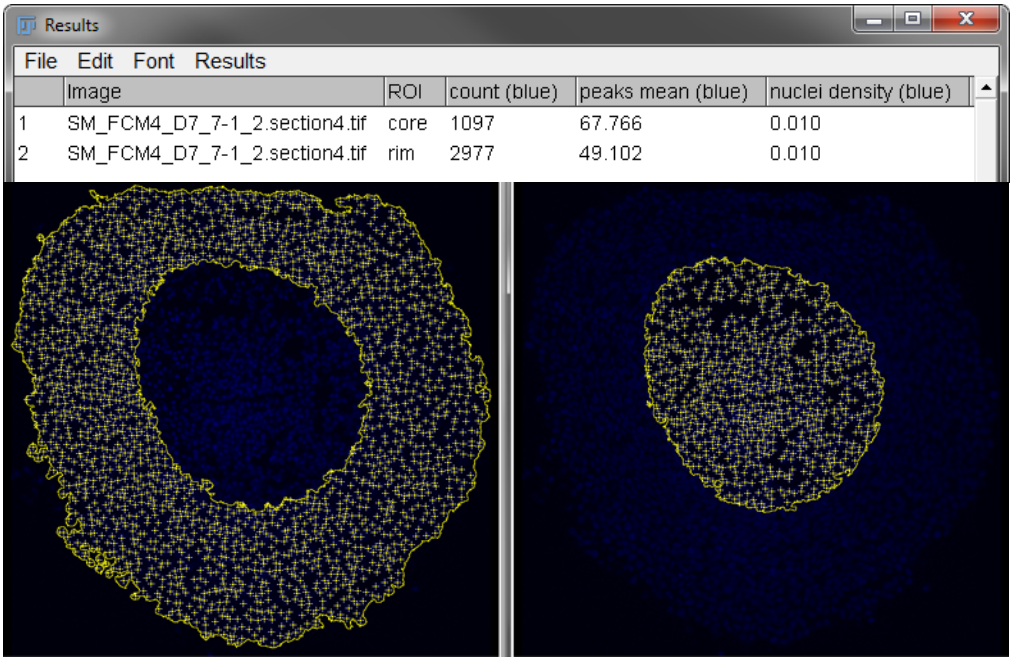


2. Use the logic operator XOR (exclusive or) from the ROI Manger to define the rim. Add this new created ROI to the RO Manager.



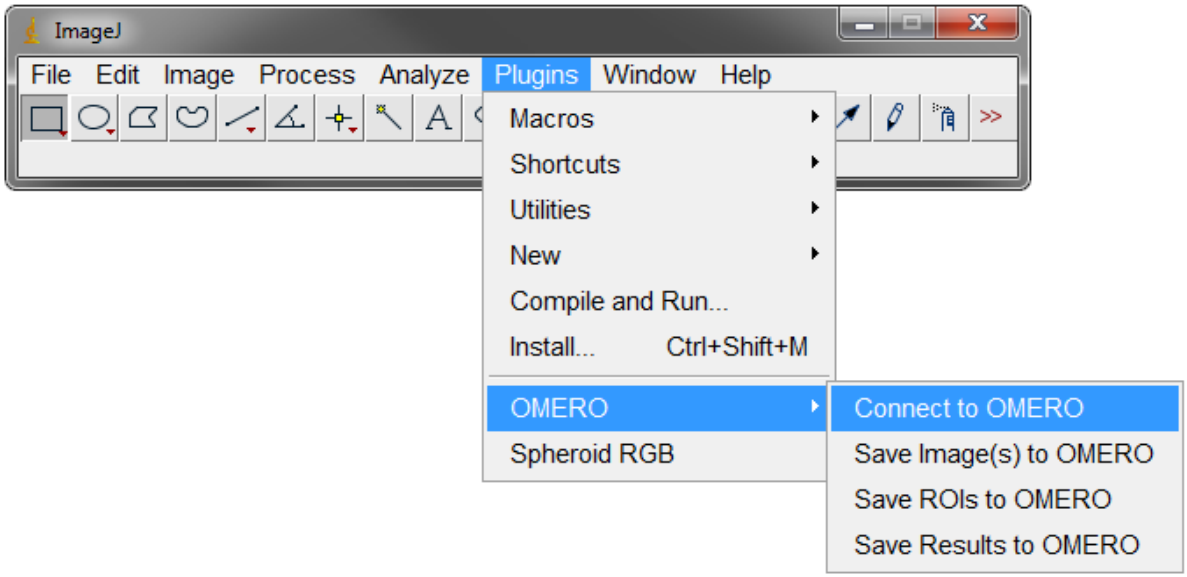
3. You may want to rename the ROI, since they have the same centroid. There are 3 ROI in the ROI Manager now.
4. Select the core and the rim and analyse this ROIs in the same way you would do it with regular shaped selections
5. Example: Count the nuclei from the blue channel:





ImageJ – OMERO – Spheroid RGB Workflow

In order to connect to an OMERO server and use the data stored there directly in ImageJ and Fiji, you need to install the OMERO plugin for ImageJ. Connect to the OMERO server via the plugin menu and open an image via OMERO insight by just double clicking it. In case you want to analyse an image which is not yet in the database, just save the image to OMERO first and then attach ROIs and results to the image.



Get the latest Omero ImageJ plugin here:
http://downloads.openmicroscopy.org/omero/

Documentation for Developers

This is a Maven project implementing an ImageJ 1.x plugin. Clone the project https://github.com/IamMM/Spheroid_RGB to develop this ImageJ plugin in the IDE of your choice.

- In Eclipse, it is as simple as *File>Import...>Existing Maven Project*.
- In IntelliJ and NetBeans, it is even simpler: *File>Open Project*.
- If jEdit is your preferred IDE, you will need the Maven Plugin.

However you build the project, in the end you will have the `.jar` file (called *artifact* in Maven speak) in the `target/`subdirectory.

Developing plugins in an IDE is convenient, especially for debugging. To that end, the plugin contains a `main` method which sets the `plugins.dir` system property (so that the plugin is added to the Plugins menu), starts ImageJ, loads an image and runs the plugin. See also this page for information how ImageJ makes it easier to debug in IDEs.

I recommend InetlliJ IDEA because of its easy handling with XML based GUI forms with the UI designer. You can just drag and drop components without breaking already working code. This is using the Java *Swing* Framework.