RGB-Counter

v. 1.0

Welcome to "RGB-Counter"

What is this for an ImageJ-macro and what can it do?

This is an automatized counter that just counts single spots/particles in the different channels of an **RGB**-image. You are asked to select a folder containing your images and the macro will process all files of a specified file-type (see later how to change this) in that folder.

After counting, the results are stored as text-file in an automatically created subfolder, also containing time and date of the evaluation along with the settings applied; furthermore, all recognized particles are stored separately as image files (JPG) for every single channel in order to gain confirmability of the whole evaluation process.

Just copy the results from the data table and paste them into something like "Excel" where they will be immediately distributed into columns like this (tabulators are not visible in text-files opened in Windows editor):

Filename	Part. blue Chan.	Part. green Chan.	Part. red Chan.
SC_KO_NT-6.bmp	275	283	287
SC_WT_NT-3.bmp	503	533	533
Zellbild.bmp	80	30	10

"*Part. blue Chan.*" stands for "*Particles counted in the blue Channel*" and returns an integer (also for both the green and red channel). Thus, it becomes quite easy to do statistics with the resulting data.

In order to make the evaluation more transparent, the particles recognized in every single channel are stored as JPEG-images (labelled like "filename blue-particles", stored in the same new subfolder like the results) during evaluation. If the particles are not properly recognized, you may change the setting of the macro (see later).

And what is it fit for?

For example, if you have fluorescence or confocal microscopic images with two different channels like DAPI (stains nuclear DNA and thus, the number of visible cells can be easily counted) and the immunochemical staining of Ki-67 (a proliferation factor that indicates dividing cells), you can quickly determine the ratio of "*all cells*" to "*just dividing cells*" by comparing the amount of particles counted in the blue (DAPI) and green (here: Ki-67) channel. Just to be sure that you don't miss anything, all particles from all three channels (**R**, **G**, and **B**) are counted and listed. Consequently, 3 different channels/stainings are quantified at the same time.

What do you need to run this macro?

It works for sure in “ImageJ” (v.1.50b); it also works in FIJI – at least for the moment, until they change labelling of the different channel-windows again, that are created after splitting the original image into **R**, **G**, and **B**. Though, this issue should be easy to fix by adapting the code.

How to use this counter?

Just copy the text of the macro (included as text-file in this zipped package) and paste it into ImageJ like this:

Start the software > “Plugins” > “Macros” > “Record” > Paste the Script into the “Record”-window > click the “Create”-button > a “Macro.ijm”-window opens > “Macros” > “Run Macro” (or press String + R).

Then it should ask you for the folder containing the images to process. Select the folder (don’t open it!) and click “okay” in order to start the evaluation.

The resulting data are finally stored as text file in a subfolder, just copy and paste them into your statistic software.

The settings do not match my images – how can I adapt them?

The main lines to adjust/adapt the macro are found right at the beginning:

Codeline	What it does
filetype=".bmp";	This specifies the file type of your images. You have only TIF(F) images? Just replace ".bmp" with ".tif"; images with lossy compression, like JP(E)G are strictly not recommended, since this type of compression causes loss of information, as well as both structural and color artefacts, that may end up falsifying the evaluation of those data.
Thresholdlowblue = 35;	This defines the lower threshold for the blue channel only; setting the threshold values allows you to separate fluorescent structures from the "uninteresting" background you want to be ignored by ImageJ. The lower threshold defines the minimal pixel intensity (brightness) that is recognized as part of the structure you are interested in. If you have an 8-bit greyscale image (covering an intensity range from 0 to 255) and the lower threshold is set to 35, the upper one to 255, every pixel in the brightness-range from 35-255 will be recognized as structure, while the range from 0-34 is considered as "background" and ignored. Setting the lower threshold to 0 and the upper one to 255 will result in masking of the whole image by covering the complete intensity range of an 8-bit greyscale image.
Thresholdlowgreen = 43;	This defines the lower threshold for the green channel only.
Thresholdlowred = 17;	This defines the lower threshold for the red channel only.
Nucsize=50;	This defines the minimal(!) size of structures to be recognized as particles in pixel; 50 is in fact a quite low area, thus, very small particles will be recognized. If you use images with higher resolution, this number may has to be increased.
Thresholdhighblue = 255;	Upper threshold for the blue channel only; in most cases, there should be no need to change this value.
Thresholdhighgreen = 255;	Upper threshold for the green channel only; in most cases, there should be no need to change this value.
Thresholdhighred = 255;	Upper threshold for the red channel only; in most cases, there should be no need to change this value.

Okay, but how exactly can I get an idea of the correct settings at all?

The main "problem" using this macro is setting the individual lower threshold for the according single channels.

For example, to adjust the blue channel you may proceed like this:

- Open an image that is representative(!) for your whole set of data.
- Select "Image" > "Color" > "Split Channels" from the menu, in order so split the image in its three separate channels (**R**, **G**, and **B**).
- Select the blue channel (the according window should be labelled "filename (blue)").
- Select "Image" > "Adjust" > "Threshold" from the menu. Check the "Dark background" box (important!).

Now your screen should look something like this (**Figure 1-5**):

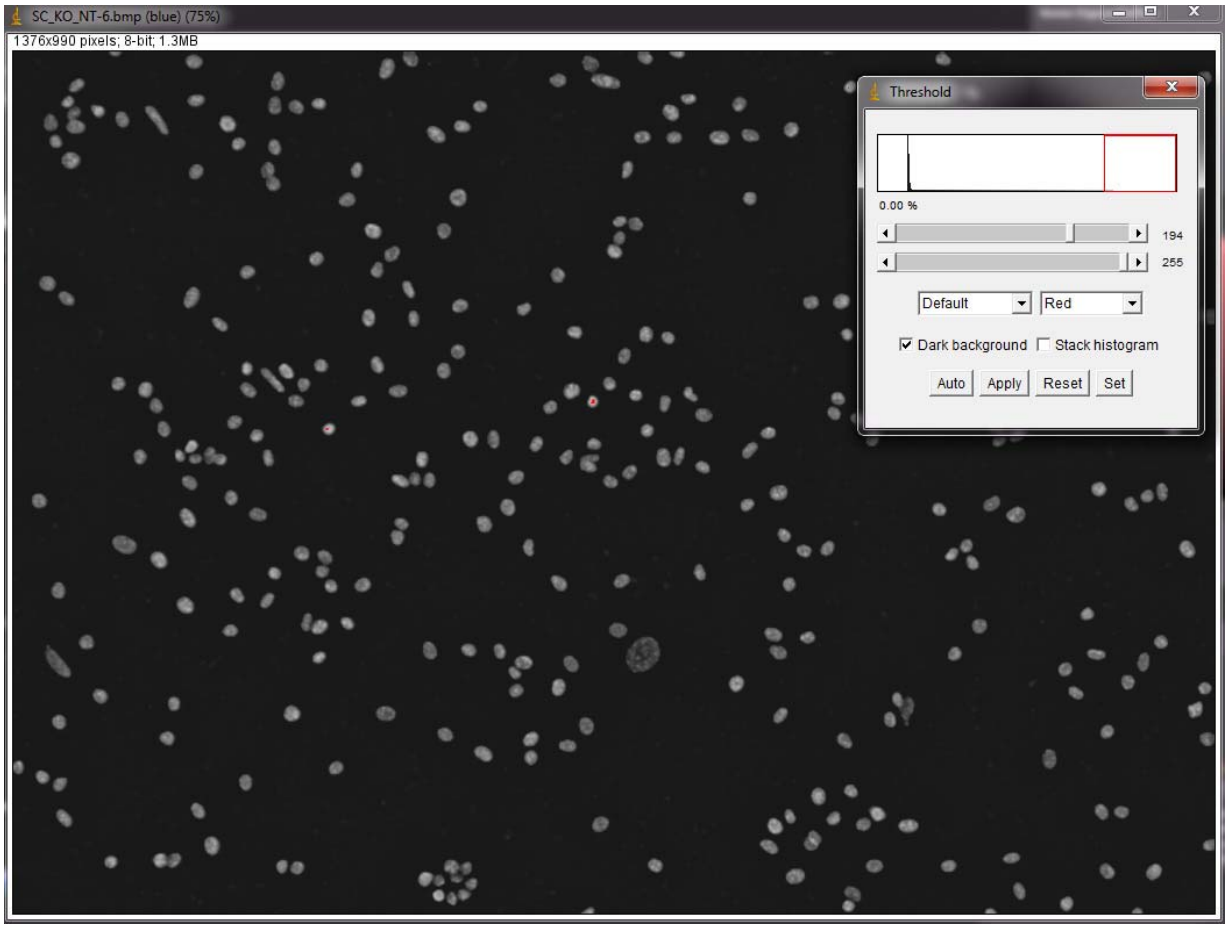

Fig. 1: Adjusting the lower threshold for an image for particle-counting.
Adjust the lower threshold by moving the upper slider, while leaving the upper threshold fixed at 255 (lower slider). In this case, the lower threshold of 194 seems to be much too high, because most of the particles are not masked (in red) and are consequently not recognized and also not counted.

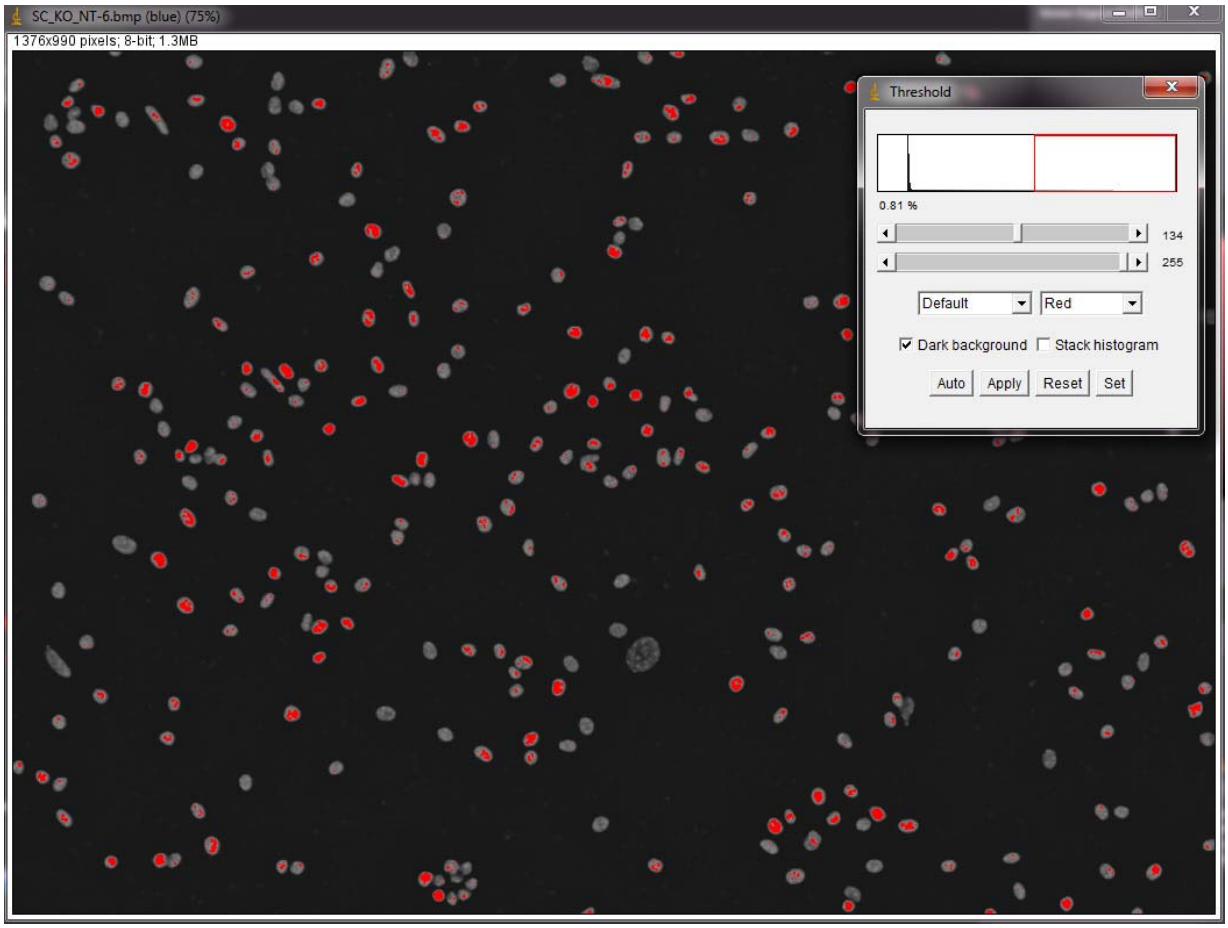

Fig. 2: Adjusting the lower threshold for an image for particle-counting.
Adjust the lower threshold to 134 results in (sometimes only partially) recognition of the brightest particles of the image. Thus, the lower threshold of 134 is still much too high and has to be further decreased.

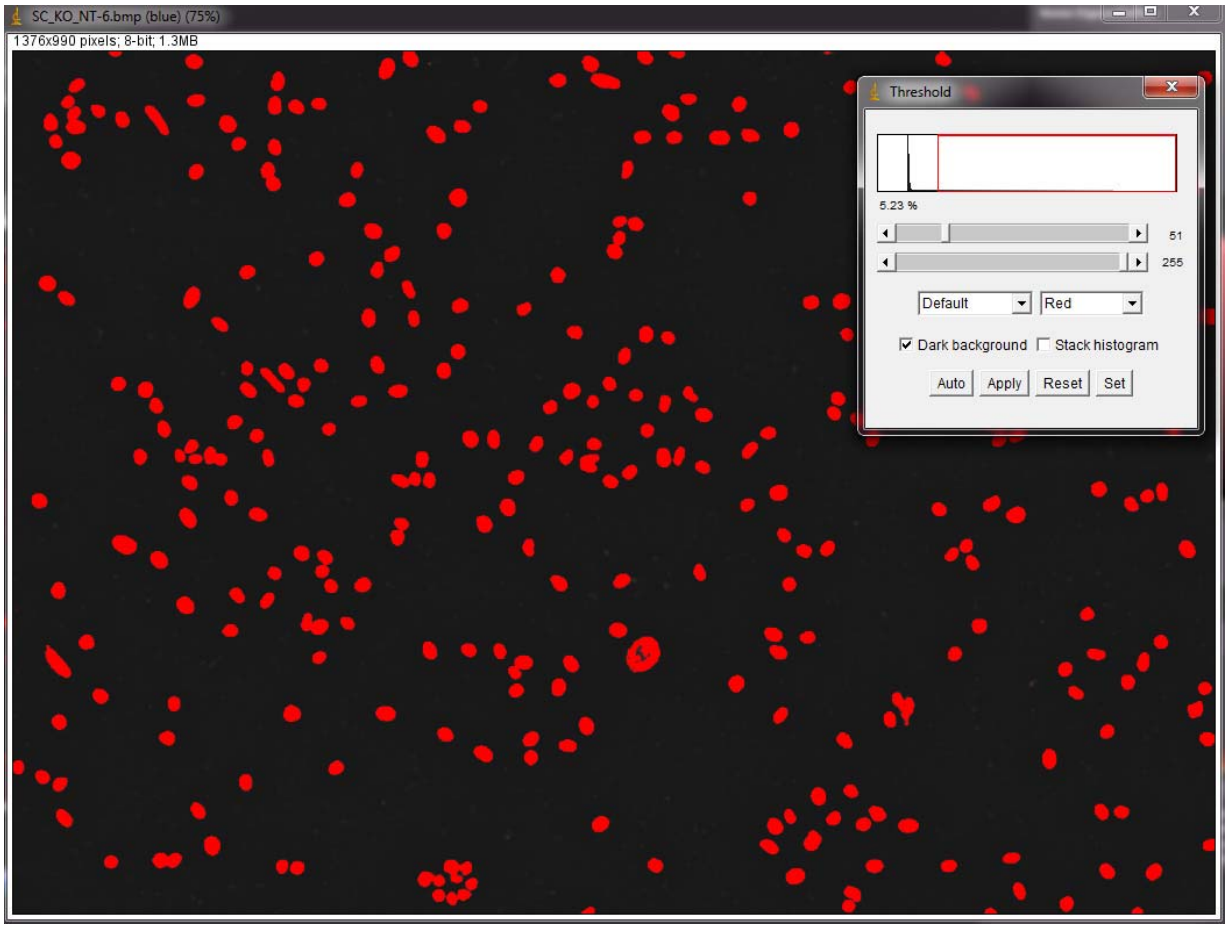

Fig. 3: Adjusting the lower threshold for an image for particle-counting.
Adjusting the lower threshold to 51 works pretty fine, all of the particles are completely masked (in red), while the darker background is still "ignored". In this special example here, a lower threshold of 51 would be the choice for evaluation of the blue channel.

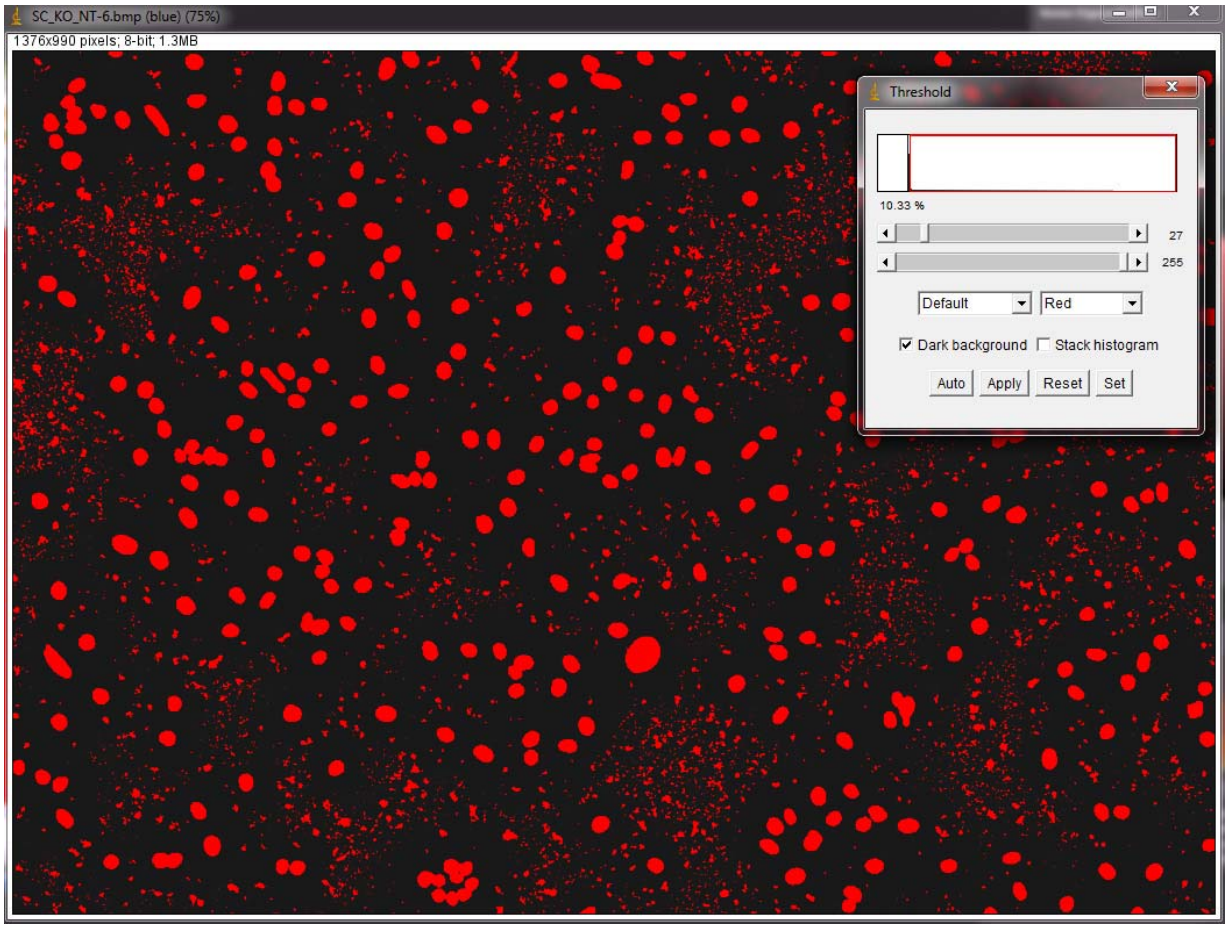

Fig. 4: Adjusting the lower threshold for an image for particle-counting.
Here, the lower threshold is already too small, particles in the background that are much darker than the DAPI-stained nuclei, are also recognized. Most of them would be "eliminated" by the minimal particle size, since they are also much smaller than the nuclei, but a few may be counted anyway and can end up falsifying the evaluation.

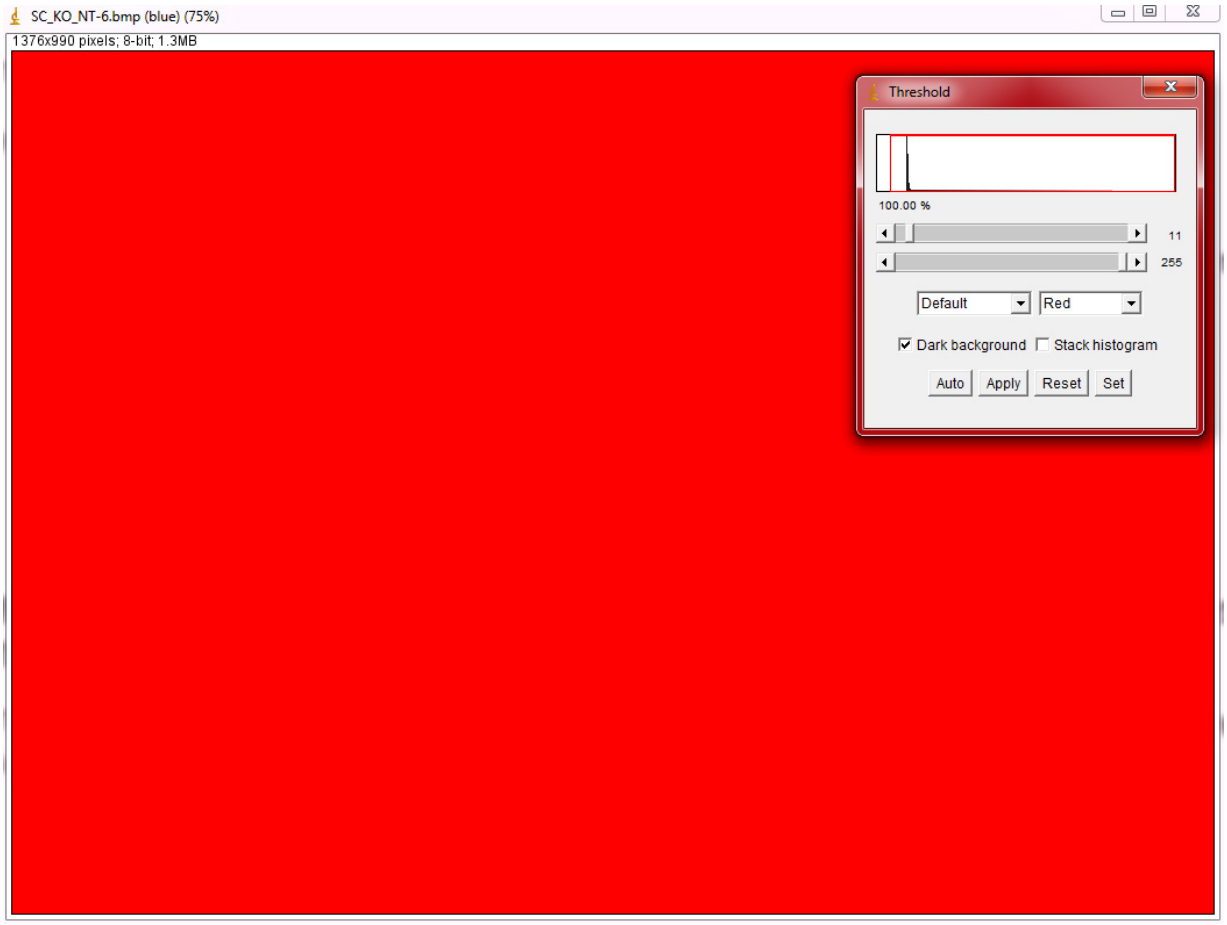

Fig. 5: Adjusting the lower threshold for an image for particle-counting.
With a very small lower threshold, the whole image is completely masked. In this special case, only a single particle would be recognized and counted in the blue channel.

The adequate lower thresholds of the other two channels (green and red) are estimated via the same strategy.

How the correct "**particle size**" can be quickly estimated is shown in the **figures 6-10**. First, open an image that is representative for your data.

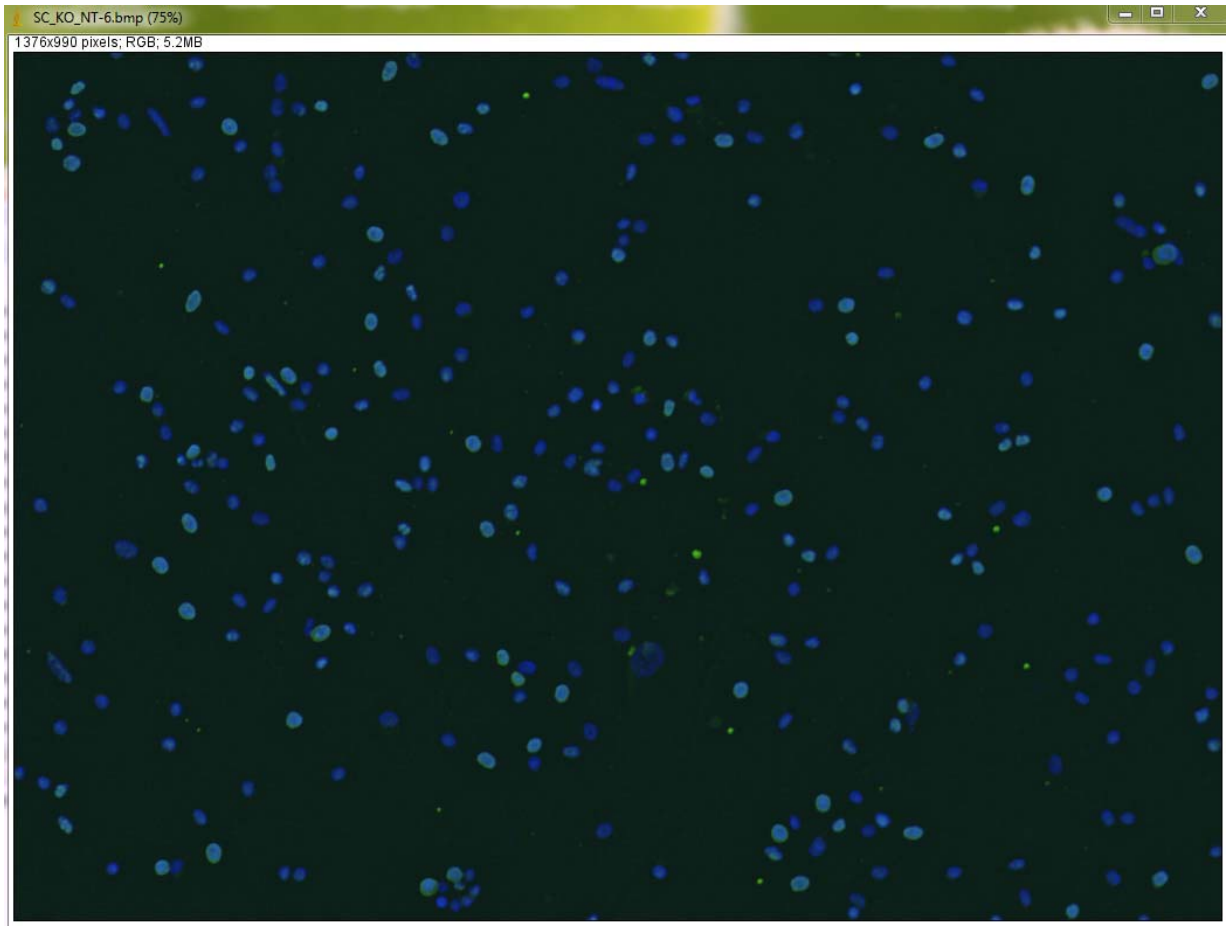

Fig. 6: Estimating an adequate particle-size for evaluation of your data.
This is a representative image, using the blue (DAPI staining of the nuclear DNA) and the green (Ki-67, immunostained) channel.

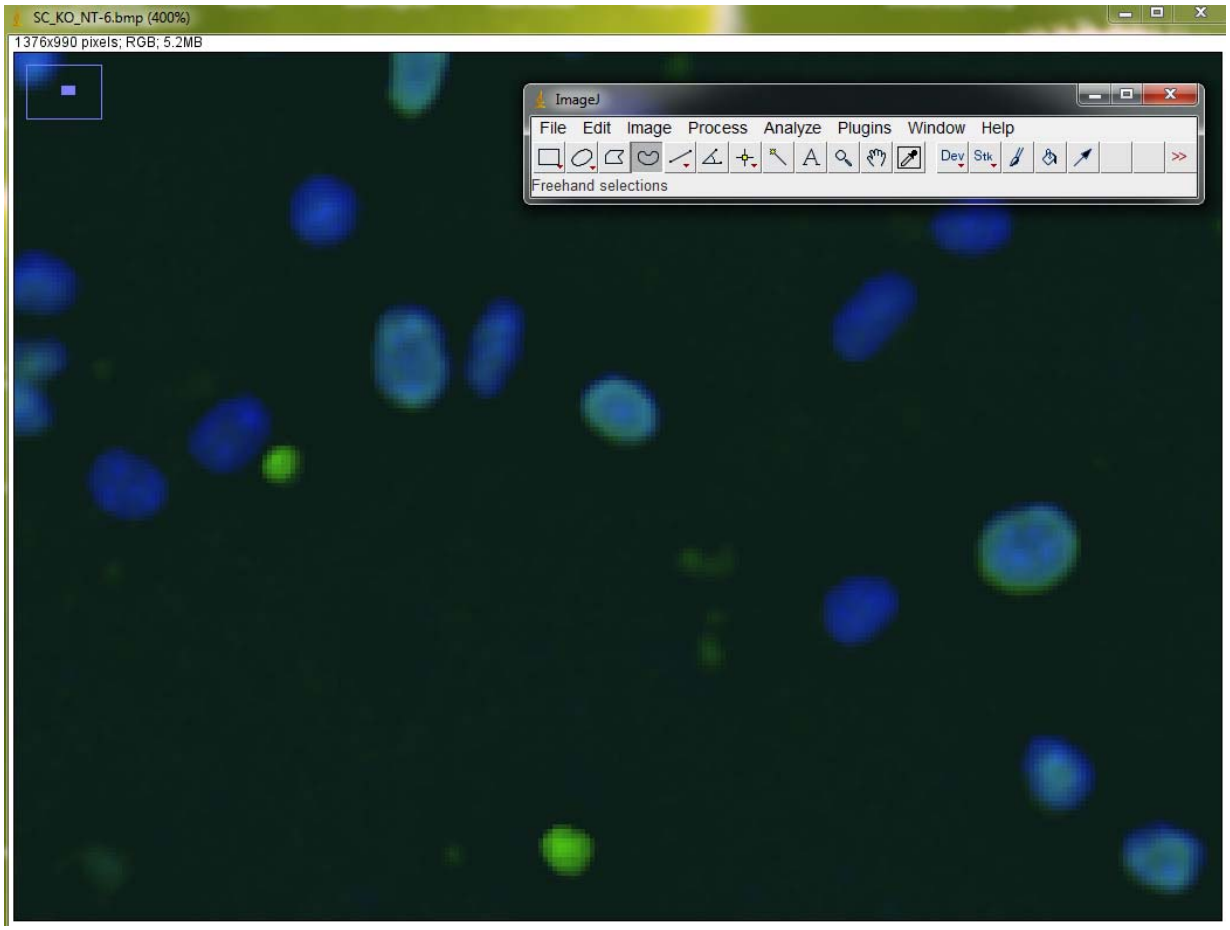

Fig. 7: Estimating an adequate particle-size for evaluation of your data.
You may also zoom in by holding down “STRG” and moving the mouse-wheel at the same time until several single nuclei are clearly visible. After zooming in, your image may look something like this.

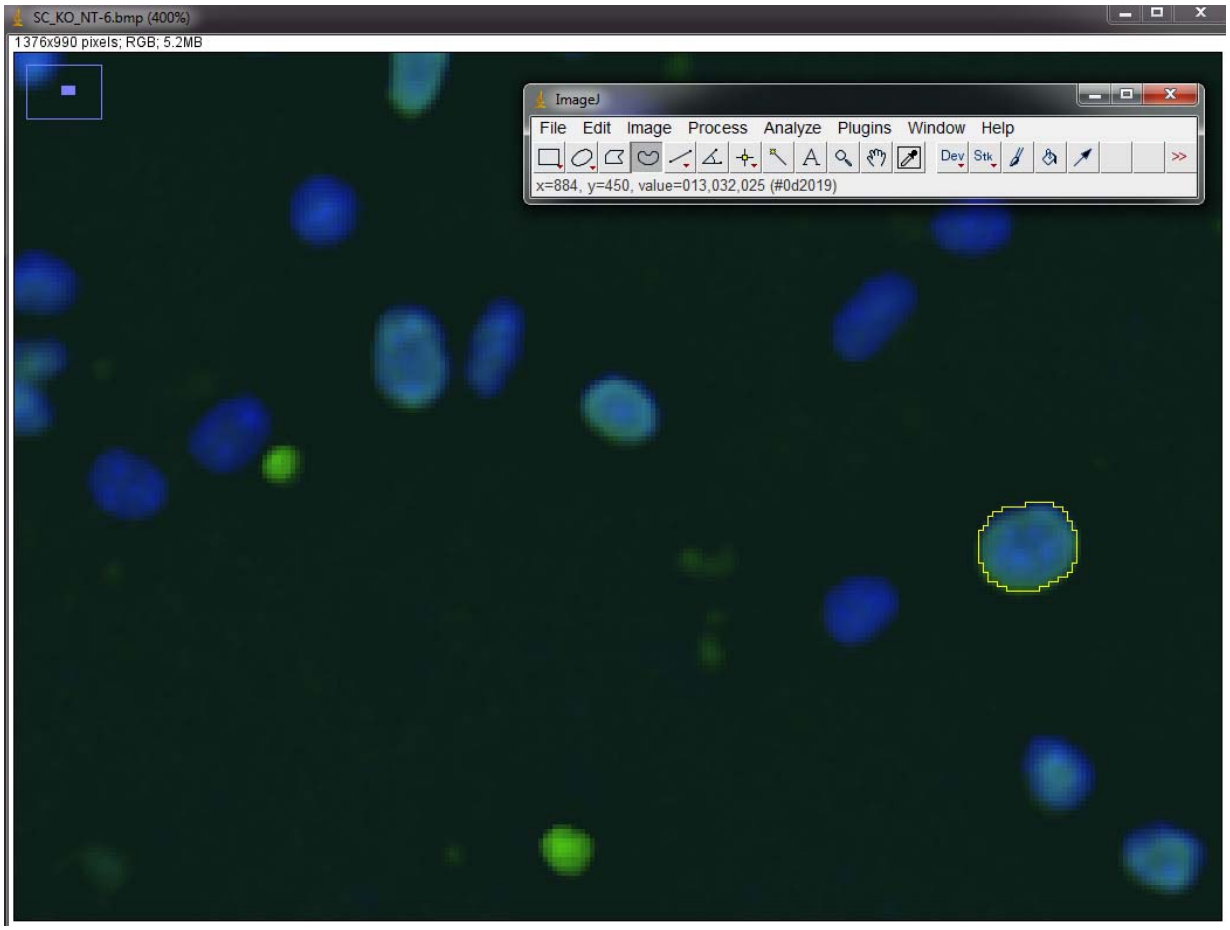

Fig. 8: Estimating an adequate particle-size for evaluation of your data.
Use the "Freehand Selection"-tool (4th button from left) to outline (yellow circle) a single representative nucleus.

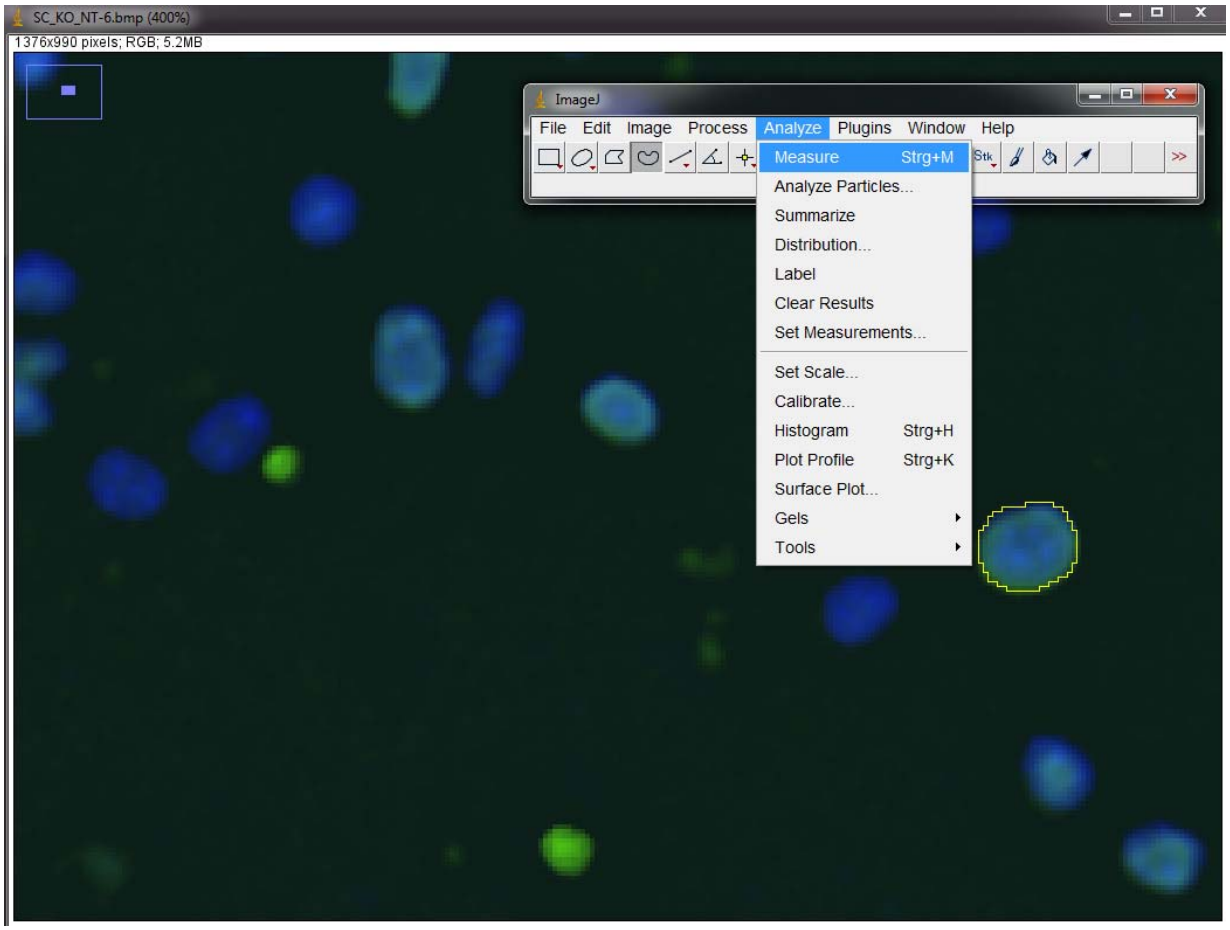

Fig. 9: Estimating an adequate particle-size for evaluation of your data.
Now, you select “Analyze” from the menu and click “Measure”.

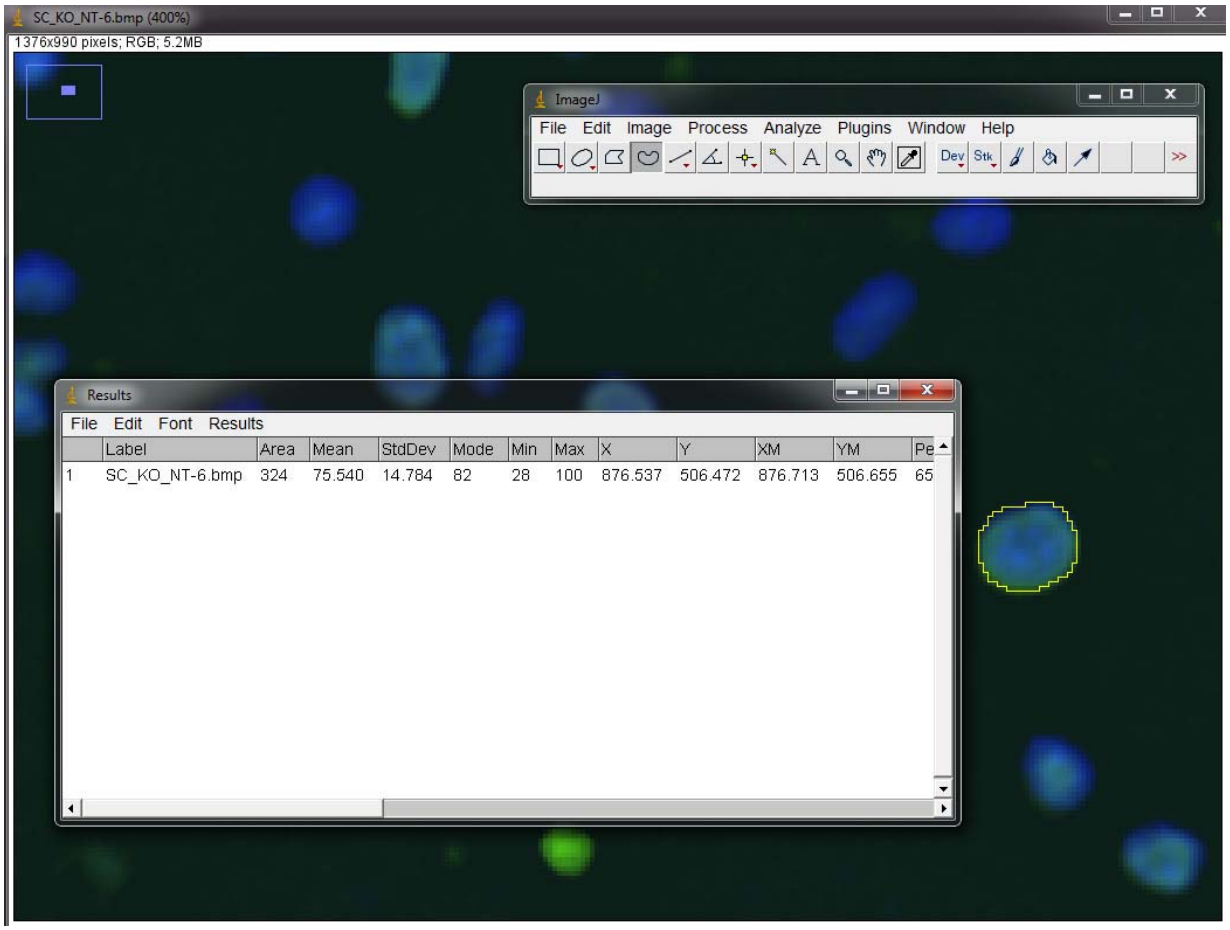

Fig. 10: Estimating an adequate particle-size for evaluation of your data.
Now, you should see the "Results"-table. "Area" returns you the number of pixels you just outlined. "Mean" represents the mean pixel intensity (brightness) and "StdDev" its standard deviation. If an 8-bit greyscale image is processed, "Mean" will be a value between 0 and 255. If an RGB-image is processed, the results are calculated using brightness values. RGB pixels are converted to brightness values using the formula brightness=(R+G+B)/3, or brightness=0.299R+0.587G+0.114B if the option "Weighted RGB Conversions" is checked (this can be done under Edit > Option > Conversions). In this case not necessary, because the RGB-images are split into separate channels that are represented as greyscale images.

In this special case, the selected nucleus covers an area of 324 pixels. Thus, a value of about 250 may be appropriate for the minimal particle size, depending on your images (and gut feeling). Maybe you have to play with the setting a little bit. The main function of the lower particle size is to get rid of (auto)fluorescent artefacts that are in most cases (significantly) smaller than the nuclei.

But my images are highly various! Setting a single threshold works for just a few, but not for all of them.

This issue may be addressed (in a restricted manner) by using the second macro (also included) "**RGB-Counter (AutoThrs).txt**".

This version applies an "automatic threshold" for every single image. Though, you still have to define the minimal particle size. Thus, the macro does not fit stacks of images that are produced using different magnifications (that may result from changing the objective during microscopy).

Remarks:

After evaluation of your data, please check carefully, if the nuclei/particles are reliably recognized by taking a look at the according images in the subfolder created.

Feel free to use and/or modify this macro. Though, we are not responsible for any software- or hardware-damage that may result (especially after changing the code).

If you use this macro, please refer to my homepage:

www.scyrus.de

This is version 1.0 of the "**RGB**-Counter"-manual (16.03.2018).