INSTRUCTION of *countPHICS*
(count & Plot HIstograms of Colony Size)

1. INTRODUCTION

countPHICS is a program that analyses images of cell colonies located on a cell culture dish. It counts the number of colonies and examines statistical distribution of their size.

2. SYSTEM REQUIREMENTS

countPHICS is dedicated for Windows 7, 8, 10 platforms (64-bit).

3. IMAGE REQUIREMENTS

3.1 Format

countPHICS supports the same file formats as the ImageJ program. A list of formats can be found here:

http://imagejdocu.tudor.lu/doku.php?id=faq:general:which_file_formats_are_supported_by_imagej

However to obtain size of colonies in SI units, information about pixel size must be embedded in the image information. Therefore, TIFF is the recommended format.

3.2 Resolution

Plates have to be scanned in colour mode, grey scale pictures will not work since this software uses the RGB colours during analysis. The higher resolution, the better accuracy is provided with countPHICS. It is not recommended to use images with a resolution below 200 DPI. While larger images (e.g. 1200 DPI) give better results, they cause the program to execute slower (this might be a problem only on slow computers).

3.3 Image quality

At the end of the document some sample images are shown with further description of their potential performance.
The general rule is to keep the image uniform, i.e. with the same intensity of background in the whole image. The higher contrast between the colonies and the background, the easier it is for countPHICS to locate them. In case images are acquired with a camera and not a scanner, photos should be taken perpendicularly to the dish surface to minimise the area where colonies overlap the dish edges.

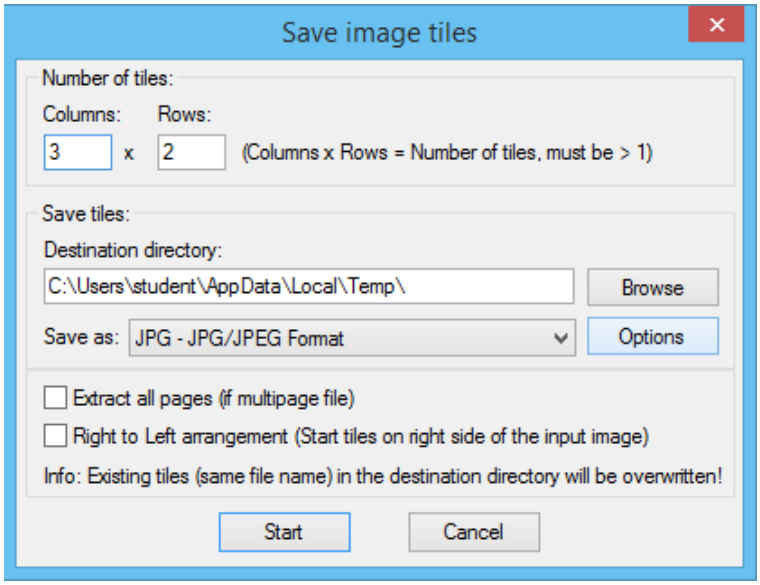
3.4 Processing multiple images

Sometimes the scanned image consists of many single dishes which can be split into single pictures. It can be done with IrfanView software (available from http://www.irfanview.com) according to the instruction given for the 6-well plate below:

- Open the scanned file: *File* → *Open*;

- Rotate the file: *Image* → *Rotate Right/Left* (depending on your scanner);

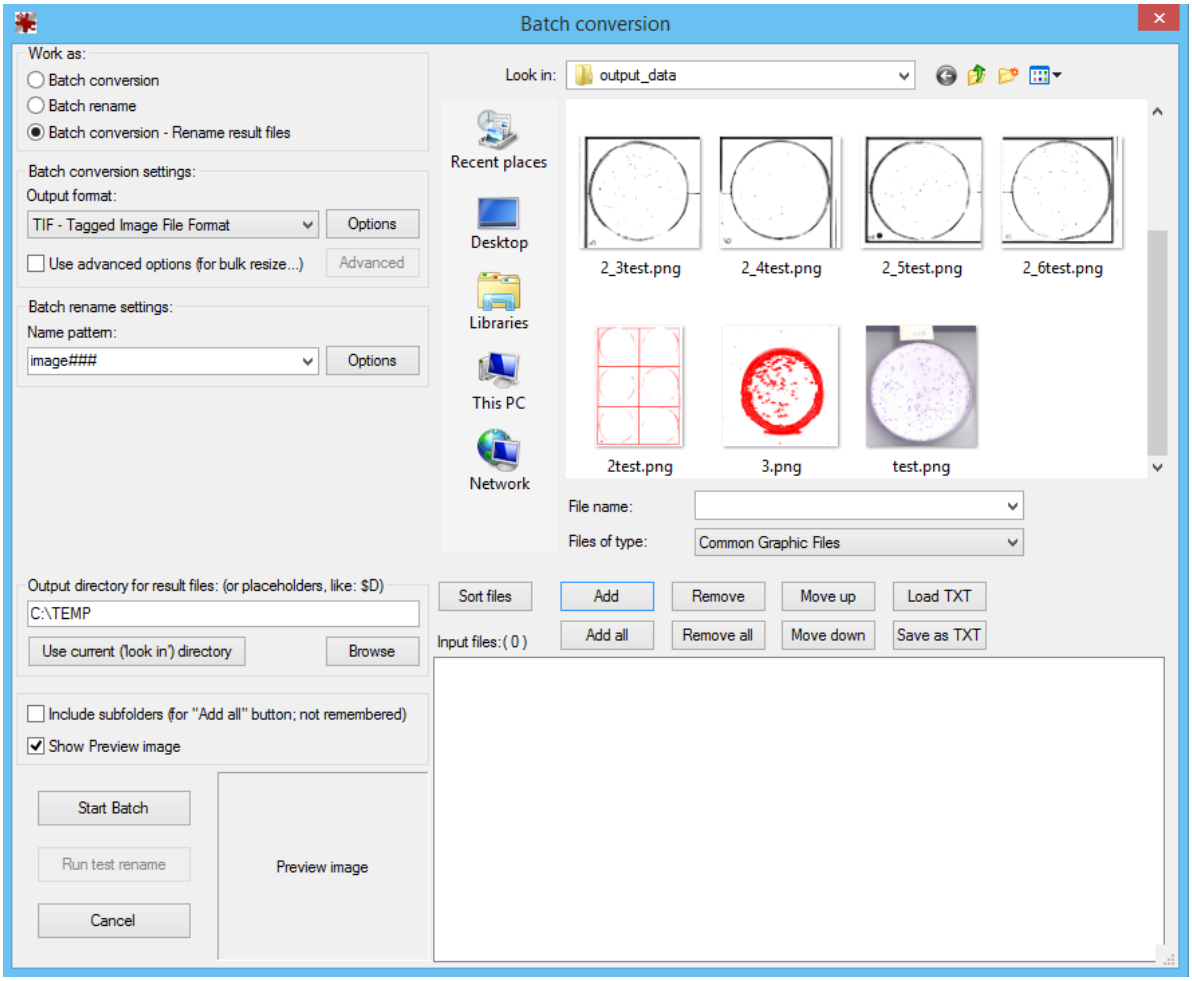
- Split into two rows and three columns: *Options* → *Export image tiles* (split image) as it's shown in figure below;



- Choose the place where you want to save the results: *Browse* and click *Start*.

As a result you will have six files with the names including information of their position (e.g. [Untitled]001 2x3.jpg means that it is the plate in the second row and the third column). The next step is to convert the files into TIF files and rename them as: 1.tif, 2.tif, 3 tif,..., respectively. To do that the IrfanView can be used again:

1. Choose from the menu: *File* → *Batch Conversion/Rename...* (batch conversion window appears as in figure below);
2. Choose *Batch conversion - Rename result files* (the third option in the left top corner), then TIF format and the name pattern (# - only one hashtag sign);
3. Going down still on the left side choose the place where you want to save the results by clicking *Browse*;
4. On the right side you can choose the directory where your files are located and mark those interesting for you;
5. Use the button *Add* or *Add all* to add them to the window in the bottom right corner;
6. Eventually you can click *Start Batch* (left bottom) and close the window clicking *Exit* batch;



3.5 File name

There are specific terms of file naming. Two conventions are available:

a) ImageNumber_*filename*.format e.g. 1_experiment.TIF, 13_OldExp.TIF etc.

or simply:

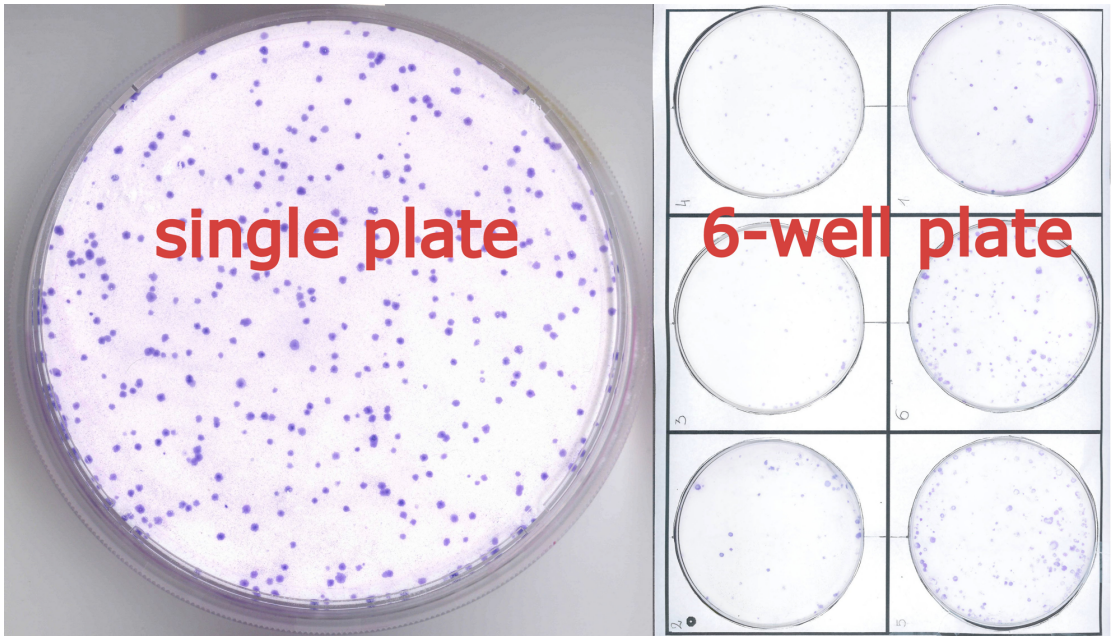
b) ImageNumber.format e.g. 1.TIF, 2.TIF … 99.TIF etc.

Other file naming conversions will NOT work.

It is extremely important to write all file names with the descriptive information into an excel file which can be used as a legend. All plates should be described there and the information about their localisation (the name of the folder and file names) of all photos should be included. The excel file can be used later for copying the results from ImageJ and final analysis of the data.

3.6 Single image or 6-well plate image

countPHICS supports one single dish or 6-well plate per image. 6-well plate should be 3x2 well axis as illustrated below. When using images of 6-well plates, it is important that they are cropped along the outer well edges to ensure symmetrical localisation of each well in the image.



4. CREATING A DESKTOP SHORTCUT *(optional)*

In order to create desktop shortcut to countPHICS follow these steps:

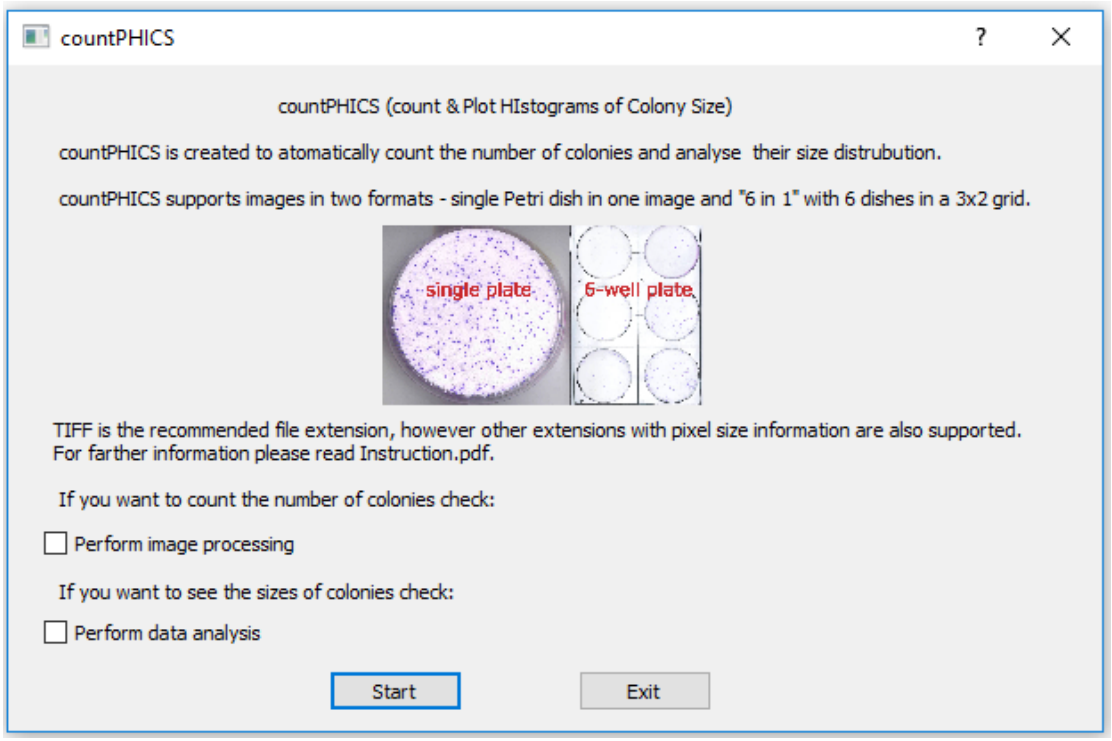
1. Right click on desktop and select *New* → *Shortcut*
2. A new window will open. Select *Browse,* find the countPHICS.bat in your countPHICS directory and then click *OK.*
3. In the new window, click *Finish.*

The shortcut is ready, but you can also add a proper icon:

4. Right click on the shortcut and choose *Properties*
5. Choose *Shortcut* tab and click *Change icon…*
6. Click *OK* in the new window.
7. Click Browse and find icon.ico. Select the file and click *Open.*
8. Click *OK* in both windows. The shortcut with the new icon is now ready.

5. USING countPHICS

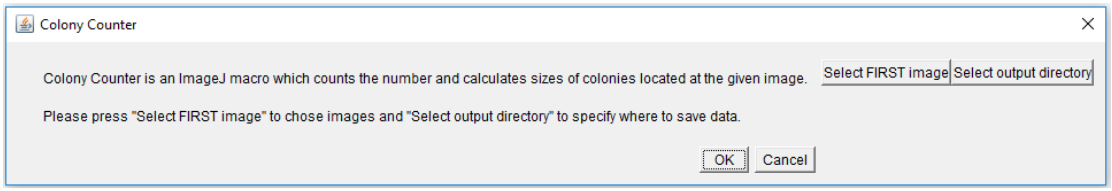
In order to run countPHICS double-click the countPHICS.bat file located in the main directory, or use the desktop shortcut if it has been created. The following window will appear:



In order to run only the colony counting part, check the first checkbox. In order to run size distribution analysis check the second checkbox. You can check both of them at the same time; in this scenario, once data is collected from the colony counter, the window for the size distribution analysis will automatically open. The text files containing colony size data will be saved, but you can still decide to perform the size analysis at a later time point.
Once the options are chosen click *Start.*

5.1 Instruction of Colony counter

Once *Start* is clicked the program loads the ImageJ macro *counter.txt*. Depending on computer performance it may take a while, from a few seconds to even 1-2 minutes. Once the ImageJ is opened it may ask you for an update. This will generally happen only at the first program usage on each computer. In this case simply accept the update. It is recommended to shut down the ImageJ after that and use the *Start* button once again. A new window will pop up:

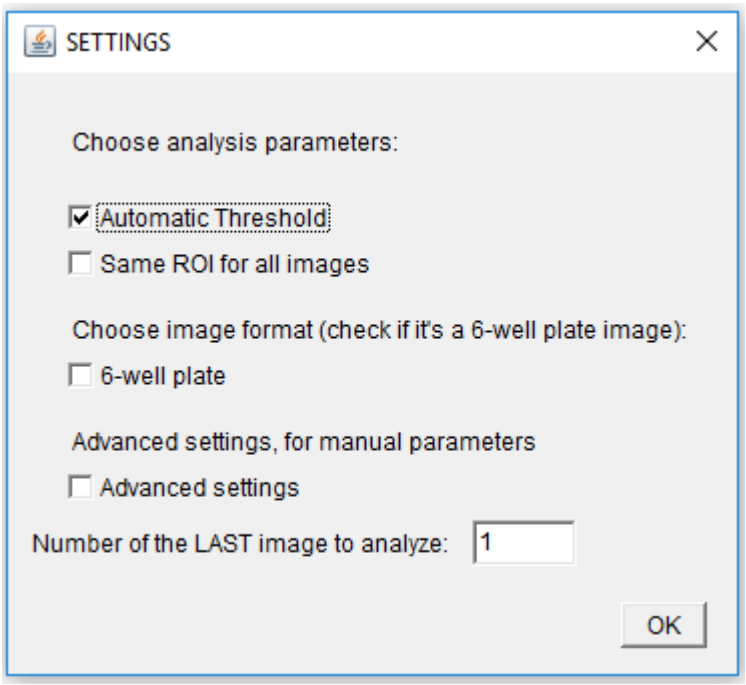


To select images from your computer, use the *Select FIRST image* button, then navigate to an image file which should be analysed. Colony counter is able to analyse a series of images located in a folder. If you want to use this option always select the image with the lowest number first.

Select output directory is an additional button that lets the user choose the output data

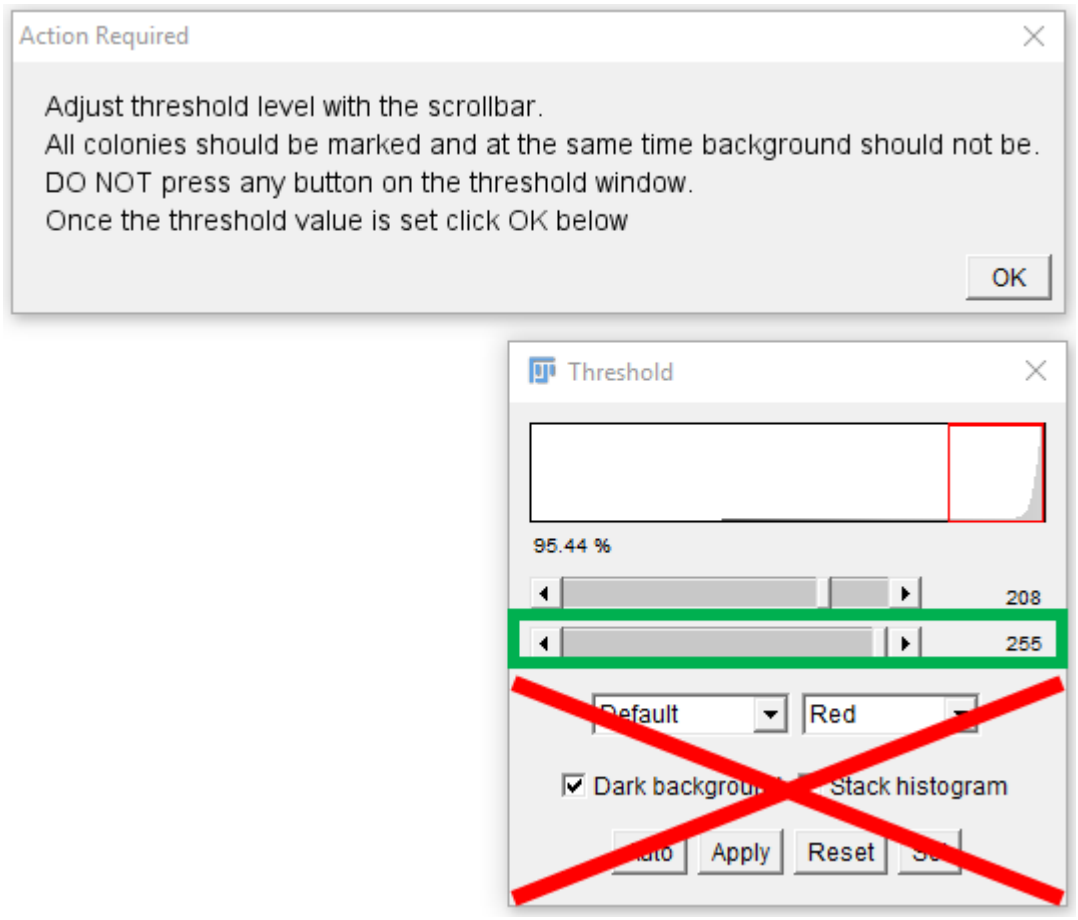
directory. If not specified, data will be saved in the *output_data* folder located in your *countPHICS* directory.

Once image and data directory are chosen click *OK*. The next window will pop up:



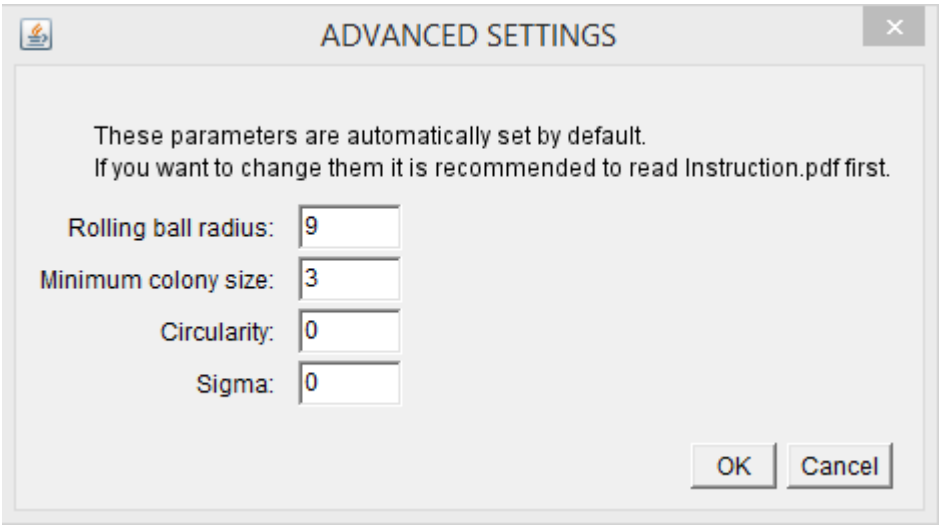
The user is asked to choose settings. Each of them are described below:

- **Automatic Threshold** – if checked, the image will convert to a binary (black-and-white) picture automatically. This option results in a slightly lower accuracy in most cases but it is recommended for inexperienced users. If Automatic Threshold is not selected, a manual threshold adjuster will be used. During the program execution the following windows will pop up:



Adjust the scrollbar (checked in the green box) to obtain the desired effect. All colonies should be marked with red colour and at the same time background should not be marked at all. Some examples of correct threshold-adjusted images are showed at the end of the instruction (section 7).

- **Same** region of interest **(ROI) for all images –** this option is useful if a series of images are processed. It is recommended to fit the ROI to each image one by one, but if the dish is located at the same spot on each image and you want to speed up the analysing process this option can be used.
- **6-well plate –** it should be checked if 6 in 1 images are analysed.
- **Advanced settings –** An advanced settings window will pop up. Usually this is not recommended because default parameters are optimised to give the best results. However sometimes it might be needed to choose parameters manually because every image is different.



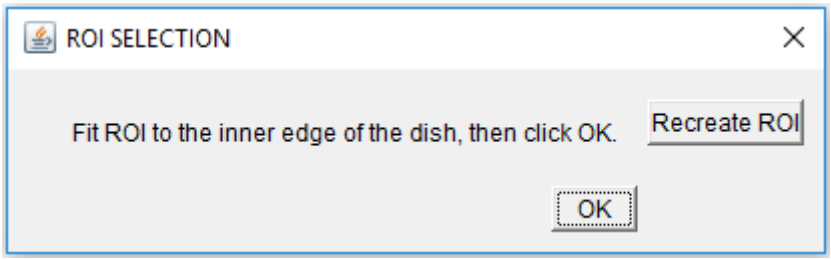
Rolling ball radius is the radius of curvature of the paraboloid. As a rule of thumb, for 8-bit or RGB images, it should be at least as large as the radius of the largest object (given in pixels) in the image that is not part of the background. Larger values will also work unless the background of the image is too uneven.
Visit http://imagejdocu.tudor.lu/doku.php?id=gui:process:subtract_background for more information.

Minimum colony size is the area (given in pixels) of the smallest object to analyse. Objects with size below this value will not be counted.

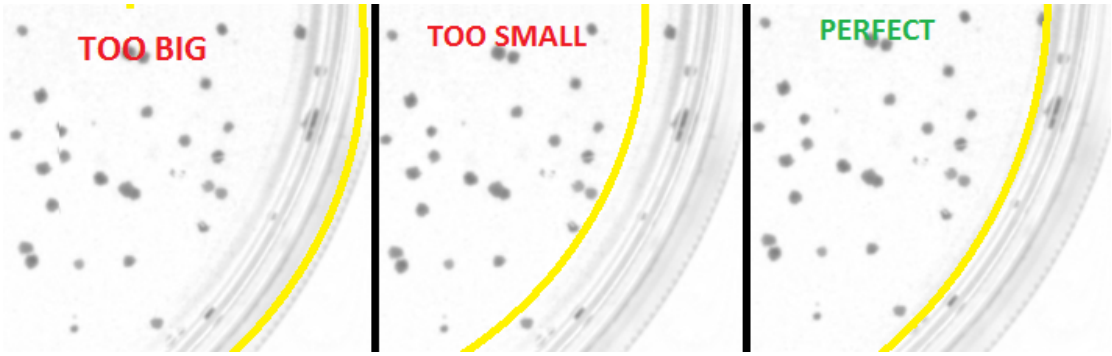
Circularity is a value between 0 and 1. By default it is set to 0.5. The higher the value, the more strict is the 'round shape' for colonies to be analysed.
For more information visit:
http://imagejdocu.tudor.lu/doku.php?id=gui:analyze:analyze_particles

Sigma is the standard deviation of the Gaussian blurring filter. It is chosen as default depending on the image resolution. More details can be found here: http://imagejdocu.tudor.lu/doku.php?id=gui:process:filters&s[]=sigma

Once all the settings are chosen the user is asked to fit the ROI to the image.

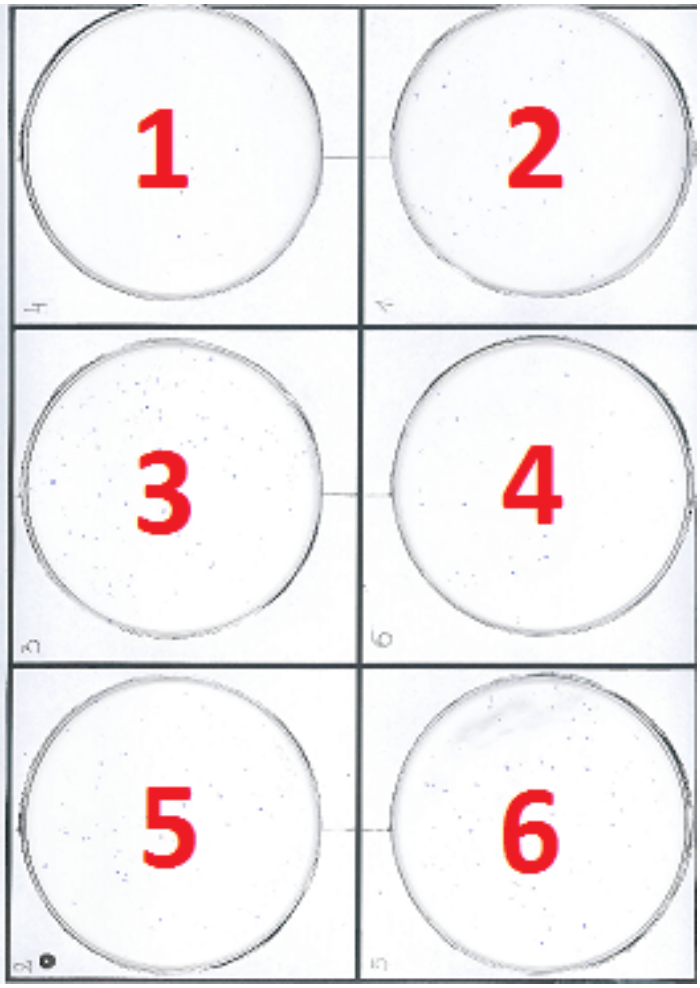


If ROI disappears *Recreate ROI* button can be used to show it again. ROI should not include dish edges and at the same time it should enclose as many colonies as possible. Some examples are given below.



Once the ROI is fit, click *OK*.

The result file which is created now in your output_data folder is called: ImageNumber_*filename*.txt. In case of 6-well plate images the numbering system is shown below:



The output files consist of the analysed image name, colony numbers and colony sizes given in mm^2.

5.2 Instruction of PHICS (Plot HIstograms of Colony Size)

The program menu consists of **File**, **Fit** and **Help** modules.

File:

With the File module you can open a file with colony size data (Open), then press Plot to show the histogram. Within this module you can also save the plot as PNG (Save PNG) and PDF format (Save PDF).

After you plot the histogram you can change the range of *X* axis setting x_{min} and x_{max} values. To create histograms the entire range of data should be divided into non-overlapping intervals called bins. It is possible to plot histograms with different number of bins by changing the number of bins in the designated place. The width of the bin is calculated as (x_{max}-x_{min})/No. of bins. To visualize your changes the Plot button (right bottom corner) should be clicked.

Fit:

To fit a function to your data choose Fit module from the program menu. There are two functions available at the moment: Gaussian and Weibull (recommended) distributions. The chosen function (listed in Fit module) will be fitted using parameters from the equations below. The least square method is used to fit and the distributions are described by the equations:

Gaussian distribution:

$$f(x) = \frac{1}{\sqrt{2\pi}\cdot\sigma}\cdot \exp\left(-\frac{(x-\mu)^2}{2\cdot\sigma^2}\right)$$

where μ is a mean value and σ - standard deviation.

Weibull distribution is described as follows:

$$f(x) = c\cdot\frac{a}{b^a}\cdot x^{a-1}\cdot \exp\left(-\left(\frac{x}{b}\right)^a\right)$$

where *x* is colony size, *c* is an amplitude, *a* and *b* – Weibull's parameters which are needed to calculate the mean size value and standard deviation (displayed in the figure as μ and σ, respectively).

Help:

To open this instruction click Open instruction, to close it - click the Quit button (right top corner).

Some definitions:

Bin – the interval of values with a given width including a certain amount of data, the width is calculated as (x_{max} -x_{min})/(no. of bins)

x_{min} – minimal value of the X-axis (the minimal colony size) plotted in the histogram

x_{max} – maximal value of the X-axis (the maximal colony size) plotted in the histogram

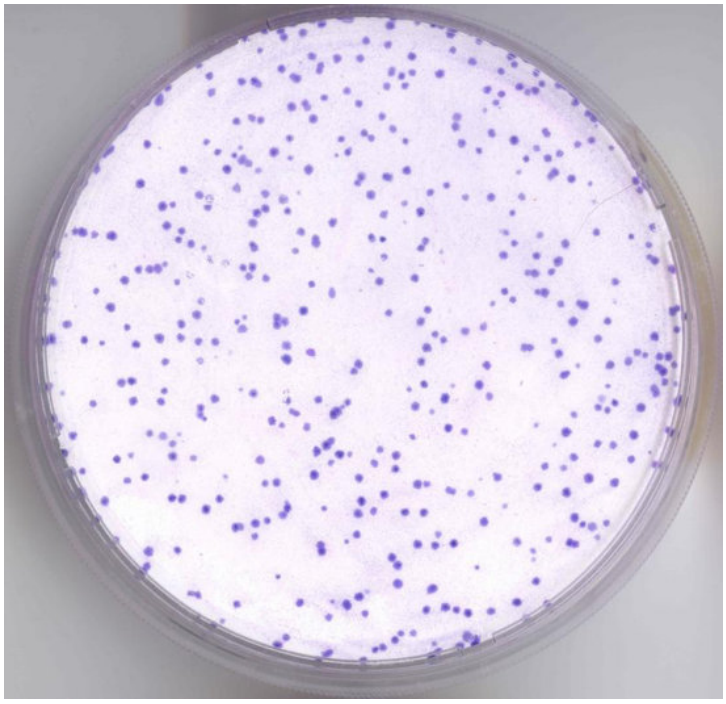
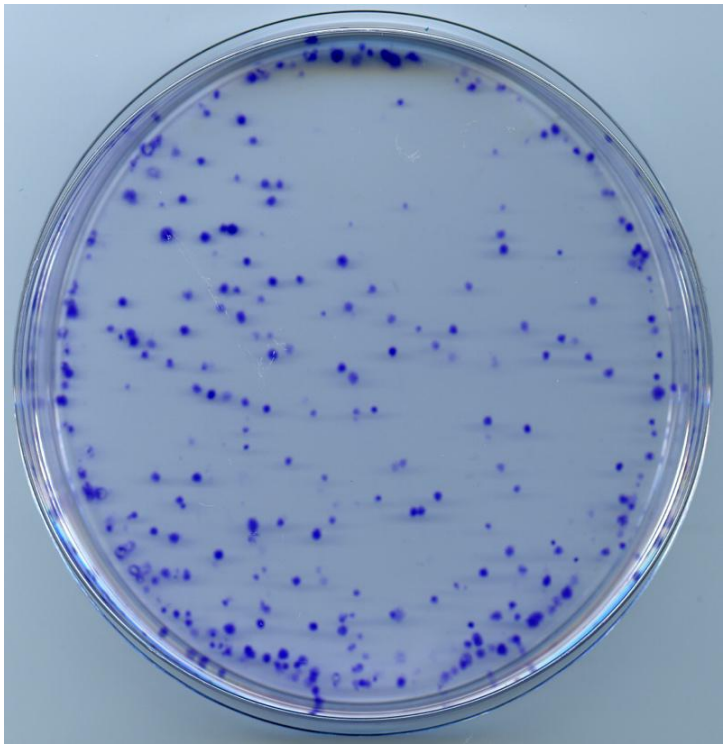
μ – mean value fitted from the chosen distribution

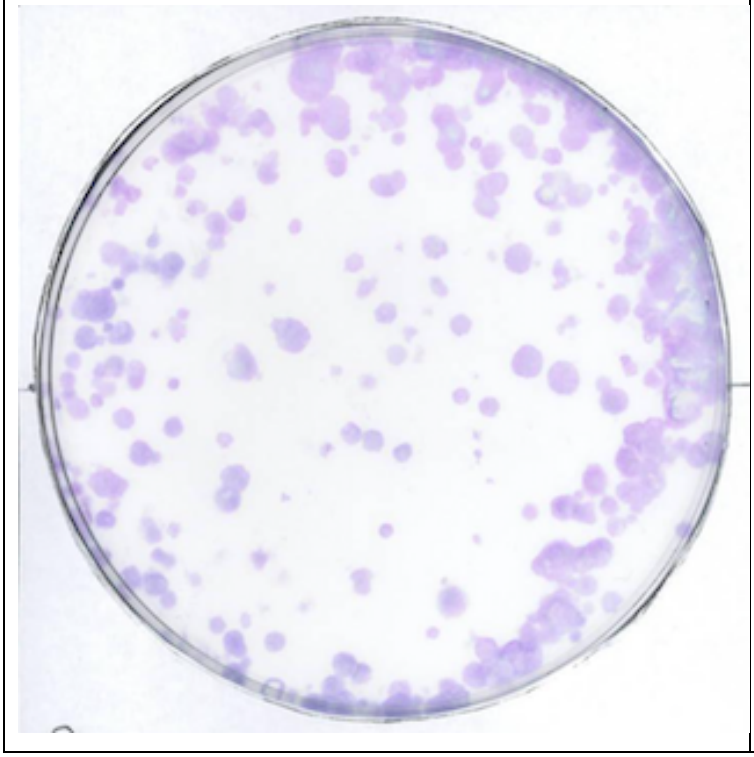
σ – the standard deviation

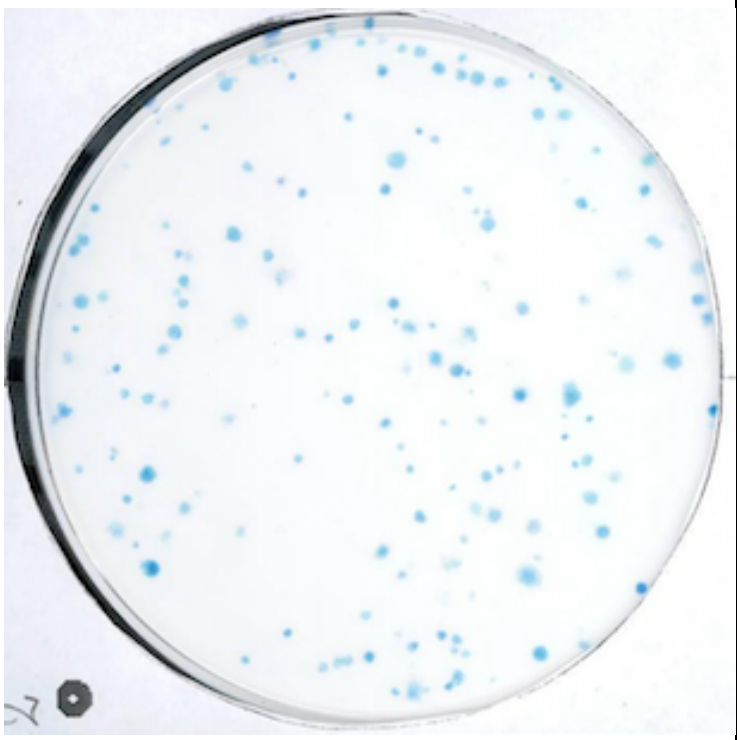
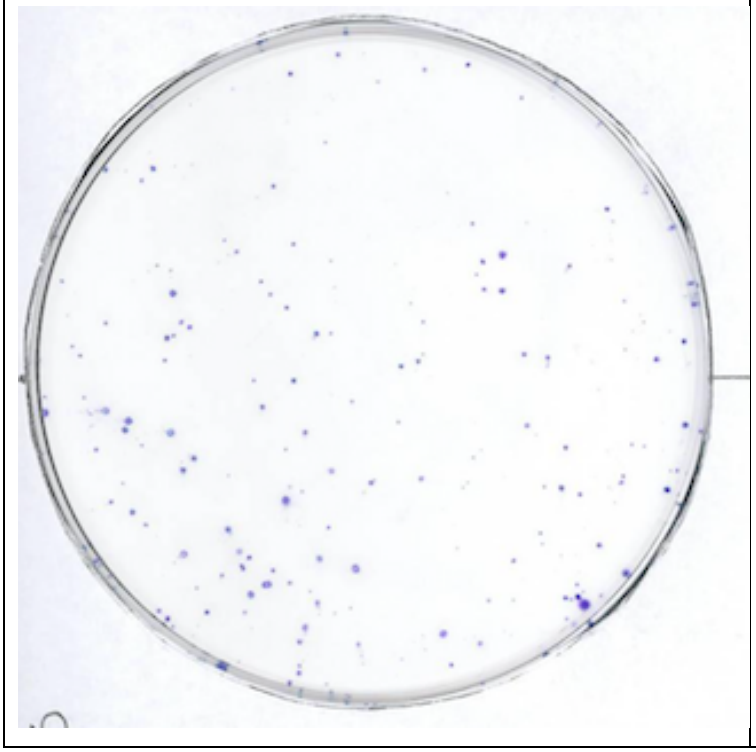
We recommend to analyse control plates first, and then use the same parameters (x_{min}, x_{max} and no. of bins) for all samples that should be processed.

6. Image examples

On the next pages some example images are shown.

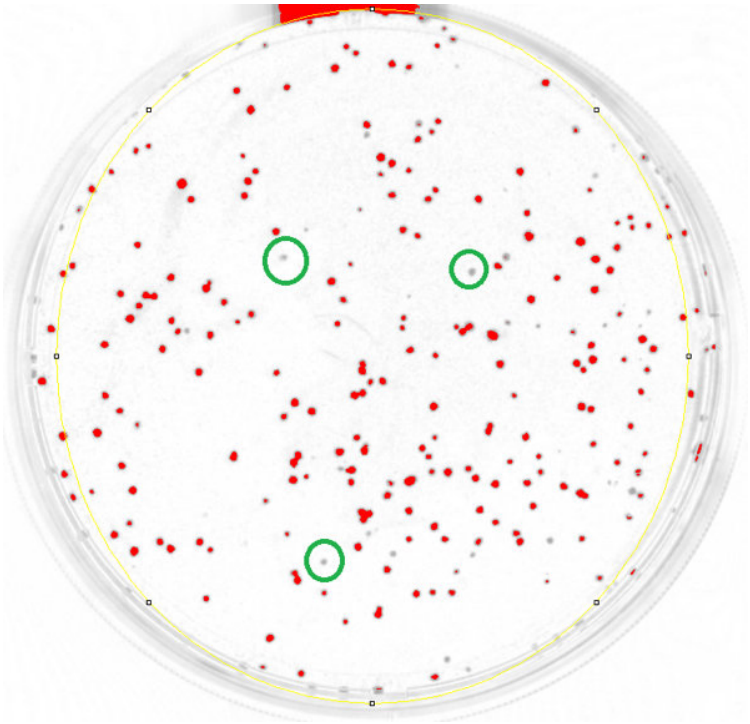
Image	Description
	Almost perfect image. Some colonies are hidden behind the “dish edge”.
	Overall good image, but the downside are too many colonies behind the dish edge, and shades created by colonies (the program might count them as additional colonies).

	Good image, but be careful! Once the resolution is too low colonies might disappear during the analysing process (binary erosion).
	Colonies are in a large conglomeration. It is difficult to count them in both ways: manually and automatically. Difficult image.

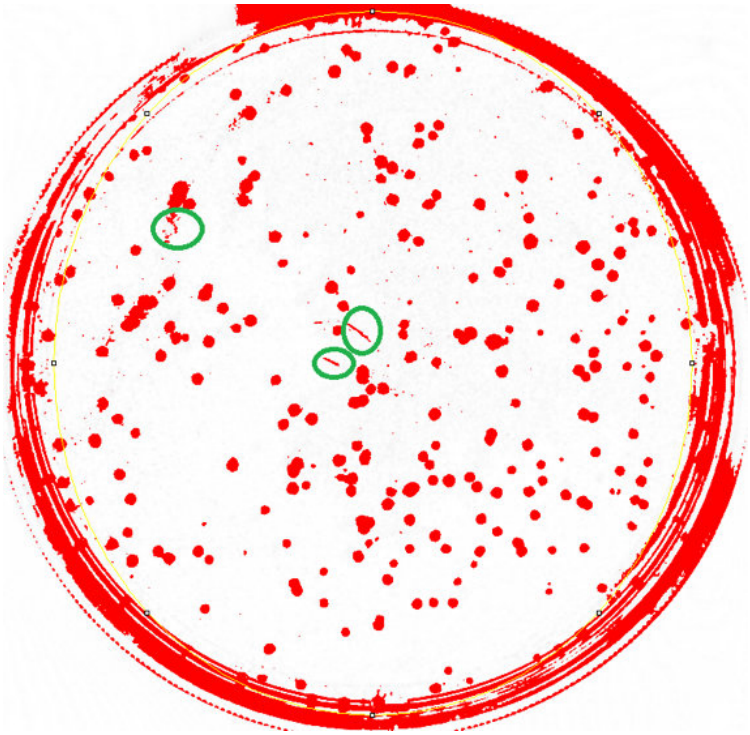
	The stain is not strong enough, the contrast is too low. The program will still count the colonies but less efficiently.
	Colonies are too small and they might disappear during the binary erosion operation.

7. Threshold tips

Threshold value is too low. Some colonies are not marked (green circles).



Threshold value is too high. Artefacts appear (green circles).



Threshold value is just fine!