

The screenshot displays the BioFilmAnalyzer software interface, which is used for the semi-automatic processing of fluorescent microscopic images. The interface is divided into several main sections:

- Top Panel:** Contains two histograms. The top histogram shows 'Npixels' (log scale) vs. 'Multi-threshold histograms: Npix'. The bottom histogram shows 'Nobjects' (log scale) vs. 'Multi-threshold histograms: Nobj'. Both histograms include a legend for various cell size ranges (e.g., 1-260, 261-520, etc.).
- Channel Selection:** A section with three colored grids (Red, Green, Blue) and a 'Now count...' section with radio buttons for 'RED', 'GREEN', and 'BLUE'. It also includes 'Detect color if...' options (R-G-G-R > T, R.G.B > T, R+G+B > T) and 'Sub-population fraction is given by...' options (Red + Green + Blue).
- Statistics Panel (Left):** Provides detailed statistics for each channel:
  - Red channel statistics:** Total pixels above the threshold in the "RED" channel: 142329; 56 cells within the given range of average size; The image contains effectively 369.2575399 cells.
  - Green channel statistics:** Total pixels above the threshold in the "GREEN" channel: 338995; 77 cells within the given range of average size; The image contains effectively 918.5239988 cells.
  - Blue channel statistics:** Total pixels above the threshold in the "BLUE" channel: [blank]; cells within the given range of average size: [blank]; The image contains effectively [blank] cells.
- Processing Controls (Right):** Includes 'Iterations of cell selection' (set to 5x), 'Re-analyze current image', 'Effective range of single cell sizes' (Min: 261, Max: 520), 'Re-apply cell size range', 'Fix current effective cell sizes', and 'Multi-threshold analysis' (Run analysis, Normalized). It also features 'Sstep = 260' and 'Smax = 2607' fields, and an 'Export results to Excel >>>' button.
- Main Image Area (Right):** A large central area for image processing, labeled 'Drag and drop image(s) here'. It shows a processed image with colored spots (red, green, blue, black) representing different cell populations. A '99%' label is visible at the top of this area.
- Adjustability:** A 'Adjust sensitivity threshold' slider is located at the bottom right, currently set to 92.

Numbered callouts (1-18) highlight specific features and controls throughout the interface.

1 Start here. Fill the column headers with some parameters characterizing your experimental setting, e.g. drug concentrations. Choose which color staining you would like to analyze first and the initial cell where to paste the cell count data, e.g. the upper left corner of the red table like in this example. Please note that choosing an active cell in another table (e.g. green) will automatically adjust the choice of the color in 2.

3 Choose the selection rule. By default, the color channel rule is applied, i.e. the point is considered as red-stained when the intensity of the red channel exceeds T. Alternatively, you can select rules based on either differential or overall (normalized) intensities exceeding T which can be tuned between 0 and 255 using 4.

5 Now try to process your sample image by drag&drop in this area. The processing will start instantly, indicated by the progress bar just above the image. As it is completed, you will see only parts of your image that satisfy the rule 3 for chosen channel 2 and sensitivity threshold 4. If you are not satisfied with the choice of the parameters, you can always adjust them by 2 3 4 and repeat the image processing using 6. At any given time you can switch between the processed and the original image by single-click anywhere in 5.

7 Here you will see the results of your image segmentation. The segmentation procedure selects all isolated objects, e.g. cells from the processed image above. You should aim at selecting those fragments that represent typical single cells. For that you can specify the range of the typical cell sizes by 8 and repeat the

segmentation procedure using 9. Image segmentation is generally an iterative procedure, with the number of iterations always being a certain compromise between accuracy and performance. While the default settings work fine in most typical scenarios, for some complex-shaped objects such as stem cells you may eventually see that the segmentation is inaccurate and thus need to increase the number of iterations by 10. Alternatively, for simple-shaped cells you can decrease it this way gaining better performance.

Please note that for larger T values the same cells are usually represented by objects of smaller sizes. One may find the proper balance between the sensitivity threshold T and the single cell size range empirically in a trial-and-error fashion. However, a more scientific approach would be to learn a bit more about the statistical properties of your images before making a final choice. For that, set up size resolution and maximum size of objects of interest in and run multi-threshold analysis 11. Shortly the histograms of object sizes and counts for all possible T values will be depicted in 12. Expand histograms by 13 to see also object count. Choose the size ranges of interest by 14, in most cases excluding very small object sizes, and find the histogram maximum 15. In most cases, it should correspond to the maximum 16 in the object count plot. By clicking on the histogram point, the chosen threshold T and single size range will be automatically copied to 4 and 8. Try whether the chosen settings work fine in your case by 6. Eventually, choosing a wrong peak may lead to the selection of other objects than single cells, e.g. cell clusters or noise

speckles, then try with another one. If you feel that the despite of all efforts the range-based selection is not accurate enough, you can remove redundant entries by selecting them directly in 7 (here selected items are shown in red) followed by a double-click anywhere in 7. Please note that you should not aim at selecting all or nearly all cells at this step. They could be just a few but should be of the representative size of single cells.

17 Results of single image processing are summarized in these panels, separately for each color channel. Briefly, the effective number of cells is obtained as the total area above the threshold in 5 divided by the effective cell size that in turn is determined from 7. Of note, non-viable cells often exhibit smaller sizes compared to viable cells that may require using different effective cell size ranges 8 for different color channels.

18 In order to calculate the relative fractions of sub-populations, you can define the rule according to your experimental setting. The results will appear in the sub-populations table and will be automatically adjusted every time you change the sub-population rule.

Once you are satisfied with the results for a single or few representative images, you can turn to **automatic processing of image series**, given that they are rather homogeneous. Hide the histograms by 13 and keep all other settings that worked well for your sample image(s). Choose the starting cell for the series in the respective table for given color 1 and drag&drop multiply selected images into 5. The entire series of up to 100 images will be processed consecutively, with the results filling the

chosen column in 1 downwards from chosen starting point. Repeat the same procedure for other color channel(s) if necessary by choosing corresponding target cells in the color tables.

For several series of experiments with changing parameter, e.g. drug concentration, choose each column for a series of measurements obtained for each concentration. When several columns are filled, the results will also appear in the graphical form 19 including data points and trend lines. Eventually missing data in 1 may indicate that some of the images in your series lack statistics of cells of respective color to estimate the effective single cell size. In this case, given that the image series is rather homogeneous, you may like to fix the effective cell size obtained from representative image(s) by 20 and repeat calculations for missing data.

You can also specify explicitly which data you would like to display within 19 where the sub-population fraction is determined by the rule 18. For further statistical analysis and dissemination of the results, you can export the data accumulated so far in all four tables, including color channels data and sub-population data, as an Excel workbook at any time using 0. Note that each export procedure creates a new instance of Excel that has to be closed manually after saving the necessary data, especially on intermediate steps in order to save memory.

The screenshot displays the BioFilmAnalyzer software interface. At the top, it shows the title bar and menu options. The main window is divided into several sections:

- Top Panel:** A multi-threshold histogram showing cell size distributions for Red, Green, and Blue channels. A callout '19' points to the histogram area.
- Table Panel:** A grid of data tables for each channel (Red, Green, Blue) and a summary table. The Red channel table is highlighted in red, Green in green, and Blue in blue.
- Statistics Panel:** Detailed statistics for each channel, including total pixels, cells within a size range, and effective cell count. For example, the Red channel has 35263 total pixels and 925.4285714 cells within the given range.
- Image Processing Panel:** Controls for cell selection, including iterations (5x), re-analysis, and cell size range (Min: 602, Max: 1202). A callout '20' points to the 'Effective range of single cell sizes' section.
- Image View:** A large image showing the processed cell population. A callout '0' points to the 'Export results to Excel' button at the bottom.

## BioFilmAnalyzer v.1.2 – Semi-automatic processing of fluorescent microscopic images with multi-layer visualization option (amendment)

An additional tab in version 1.2 provides a multi-layer visualization option to analyze the biofilm structure based on the fluorescence confocal laser scanning microscopy (CLSM) imaging providing with multi-layer representation of samples, including both individual color channels (upper panel) and the calculated sub-population fractions determined by the user (lower panel). The analysis procedure is similar to the processing of image series is v.1.0, while images in the series should correspond to consecutive Z-stacks. Switching between different samples is performed by the selection of the corresponding column by clicking on the head row **1**. That applies both to the multi-layer biofilm structure visualization and to the cells sub-population statistics tabs. Other image analysis and visualization options remain unaffected by this amendment.

