Protocol: Automatic Quantification of Fluorescence-Imaged Live Dead Assays Using Fiji (ImageJ)

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Requirements:

- **Multi-channel** images (at least 2 channels (live & dead), additional channels like nucleus staining or brightfield can be contained in the source images but will be ignored in the analysis and not shown in the validation overlay.
- Developed and tested for **8-bit format** images but also applicable with 16-bit and 32-bit, but then higher prominence values will be needed (compared to 8-bit) if noise intensity scales with the bit format (see Figure 1, below).

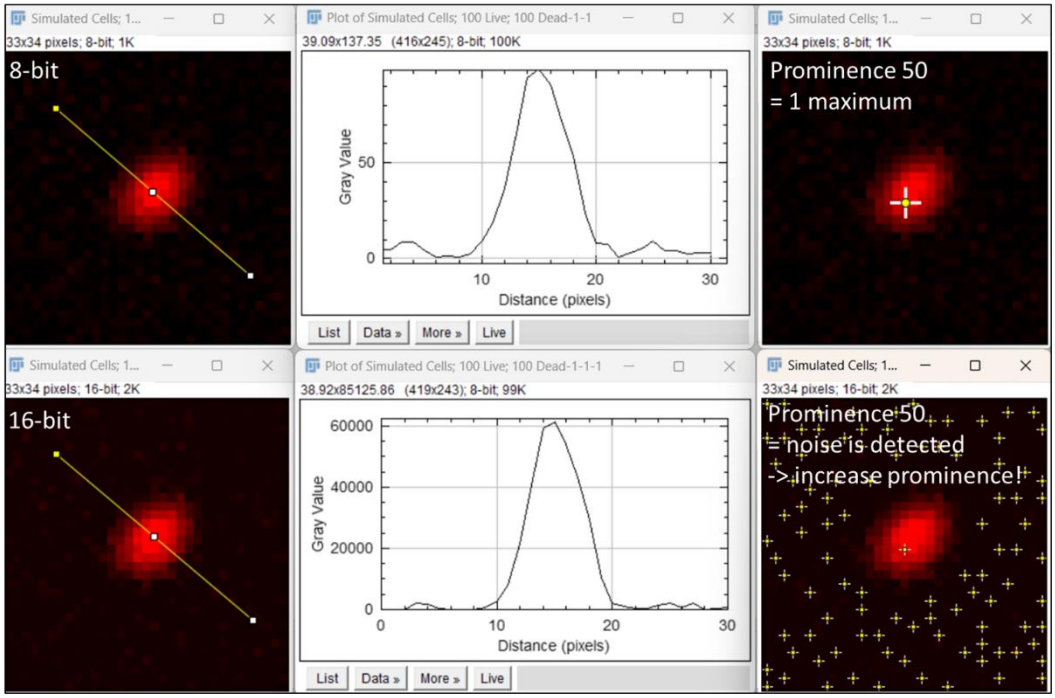

Figure 1: Determination of a suitable prominence with a line plot on an 8-bit image (top) and the same image as 16-bit (bottom). The higher intensity range of 16-bit leads to higher intensity of noise pixels and thus the prominence for analyzing the 16-bit image must be increased from ~ 50 (for 8-bit) to ~ 20.000 to avoid the detection of background noise as cells.

- All images to be analyzed must be contained in one **input folder**. The macro will analyze all images in this folder and sub-folders if present. The naming of that folder is unrestricted.
- The output data (validation overlay (tif) & summary data table (txt/csv)) will be stored in a single **output folder** that must be created and selected before the analysis. The naming of that folder is unrestricted.

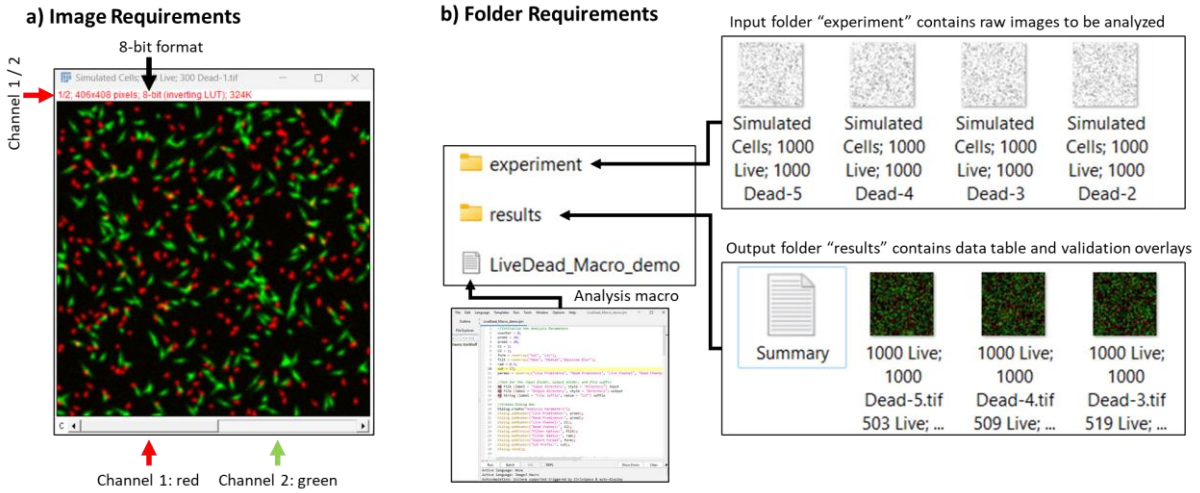

Figure 2: Graphical representation of the image and folder requirements to run the macro smoothly. Multi-Channel images must be contained in one input folder and results will automatically be saved in an output folder.

Tipps:

- Stacks can be converted to Multi-Channel images by (*Image > Stacks > Stack to Images; Image > Color > Merge Channels…*)
- RGB images can be converted to Multichannel image (*Split Channels & Merge channels*)
- 16-bit and 32-bit images can be converted to 8-bit images (*Image > Type > 8-bit*)

How to Use the Automatic Quantification Macro:

1. If not already done, install the latest version of Fiji: https://imagej.net/software/fiji/downloads
2. Download the macro (LDA_macro.ijm) from Zenodo
3. Open Fiji, drag and drop the macro-file in the action bar or Use *File > Open…*
    a. Do not use *Plugins > Macros > Run…* as it will not recognize the file directory input command and show an error
4. Make sure you have prepared the input and output folders
5. Press *Run*

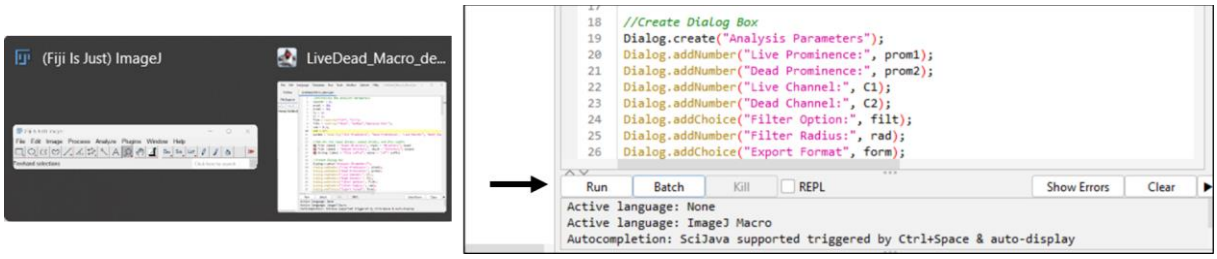

Figure 3: Fiji must be opened, and the macro loaded into the action bar. Then the macro can be started by pressing Run.

6. Now choose the paths of the input and output folder and the suffix of the images you want to analyze (e.g., tif, czi, lif, …)

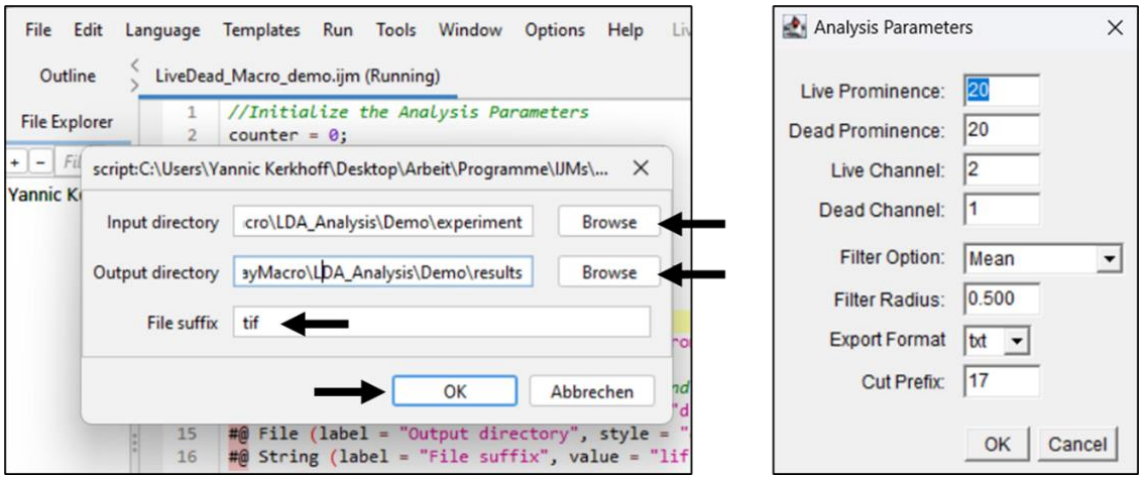

Figure 4: Choose the input and output directory and the file suffix as well as suitable analysis parameters.

7. Now you have to choose the few analysis parameters which is the prominence of the *Find Maxima* Function to detect local intensity maxima in the two channels of the Live Dead Assay (These can be assessed by making a line plot on the images to see the intensity difference (prominence) of the cells compared to the background).

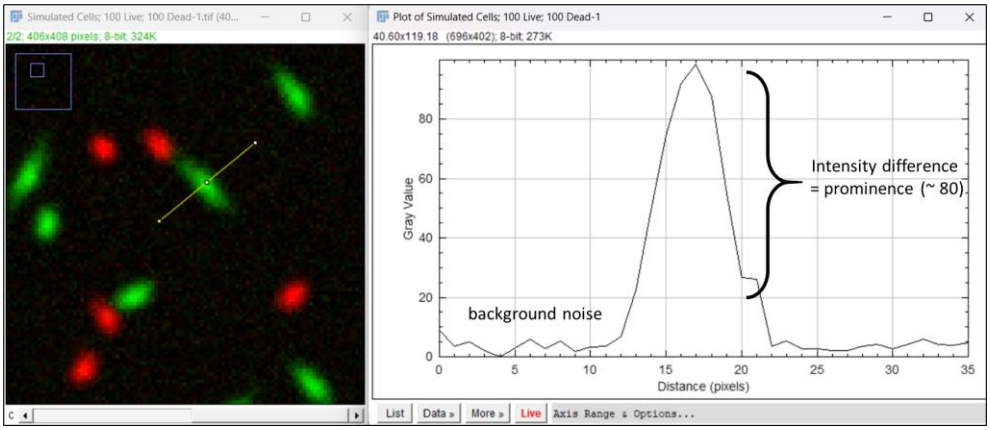

Figure 5: A line plot on the raw images can help to evaluate the background noise and the intensity difference of the cells to the background. In this example the prominence is ~ 80 a.u. but also a prominence of 20 would be sufficient to detect cells and no background.

8. You also have to select which channel contains the Live and which the Dead staining. You can also select a Filter to reduce noise. If you do not want to apply a filter just set the filter radius to 0. You can also choose the format of the data table (txt or csc) and cut a prefix of the images. This is helpful if each image name begins for example with a date and experiment description (this is often the case for images from lif files). In our case all input images started with “Simulated cells; “, so we choose a cut of 17 so that the first 17 characters of the images names are not contained in the output image names. If you do not want to cut a prefix set it to 0.
9. Press OK to start the batch analysis

Macro Output:

- The macro works on default in the batch mode, so no image processing steps are shown. However, you will see that the summary table opens and is subsequently filled with the analysis results while the Results table shows the number of Live and Dead Cells for each image. The Cell Viability in % is automatically calculated from the number of Live and Dead Cells.

- After the analysis is complete the data table will be saved in the output folder, together with validation overlays.

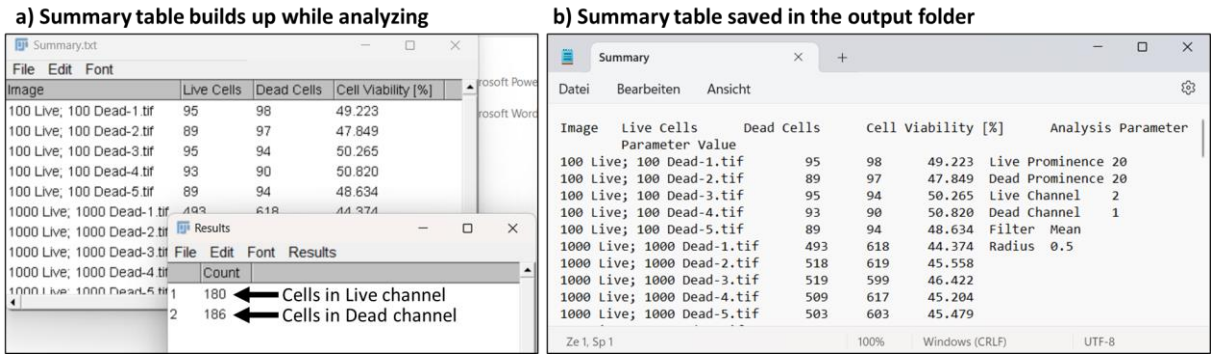

Figure 6: While analyzing progresses the Summary data table is updated with the Results data table from the images. After the analysis is completed the data table is saved as txt or csv in the output folder.

- The validation overlay will give you a hint if the analysis worked as expected. It shows the image after the filter was applied and with magenta crosses on all detected dead cells and with cyan crosses on all detected living cells. If background noise was detected as cells, then the prominence was too low. If cells are undetected then the prominence was set too high.

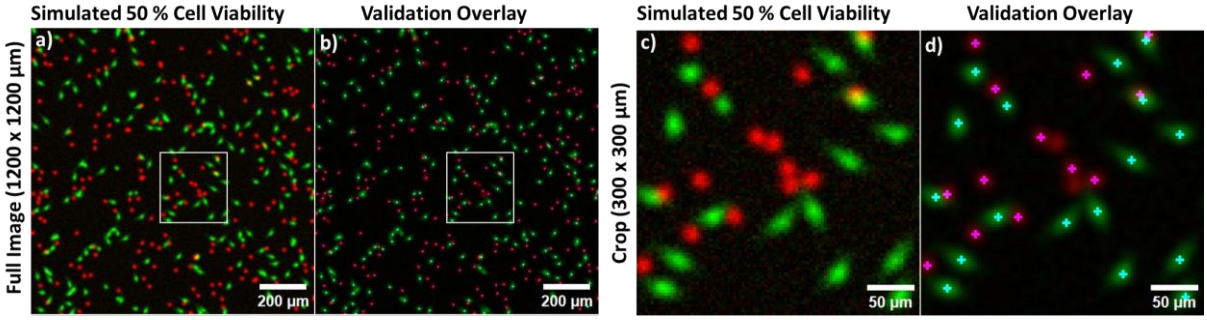

Figure 7: Full size and zoomed simulated raw images and the corresponding validation overlay saved as tif file in the output folder. The crosses show where live (cyan) and dead (magenta) cells were detected based on the prominence given in the analysis parameters. Touching cells will sometimes be counted as one cell so the overall number of detected cells can deviate from the real cell number (see validation part below). As long as this is the case for both channels the determined cell viability will still be correct.

Validation:

The macro was developed based on real confocal image data of Live Dead Assays performed with A549, HeLa, and McF7 cells with a pixel size of 3 µm and was validated with synthetically generated image data. While the simulated images are not perfect, they regard the intensity profile and shape of cells as well as the presence of background noise (Gaussian noise with a 5 % standard deviation) to be as realistic as possible.

On the simulated images the cells are randomly distributed like if freshly seeded and they can overlap by chance (just like in real images). However, it must be noted that incubated real cells may start to migrate and proliferate and build clusters so the grade of cellular overlap may be underrepresented in the simulated images. Manual comparison of real and synthetic images (Figure 8) however shows that they are quite similar and showed no obvious severe differences. The images for validation were analyzed with a prominence of 20 for each channel and a mean filter with a radius of 0.5. The images are provided in a zip file in this zenodo entry.

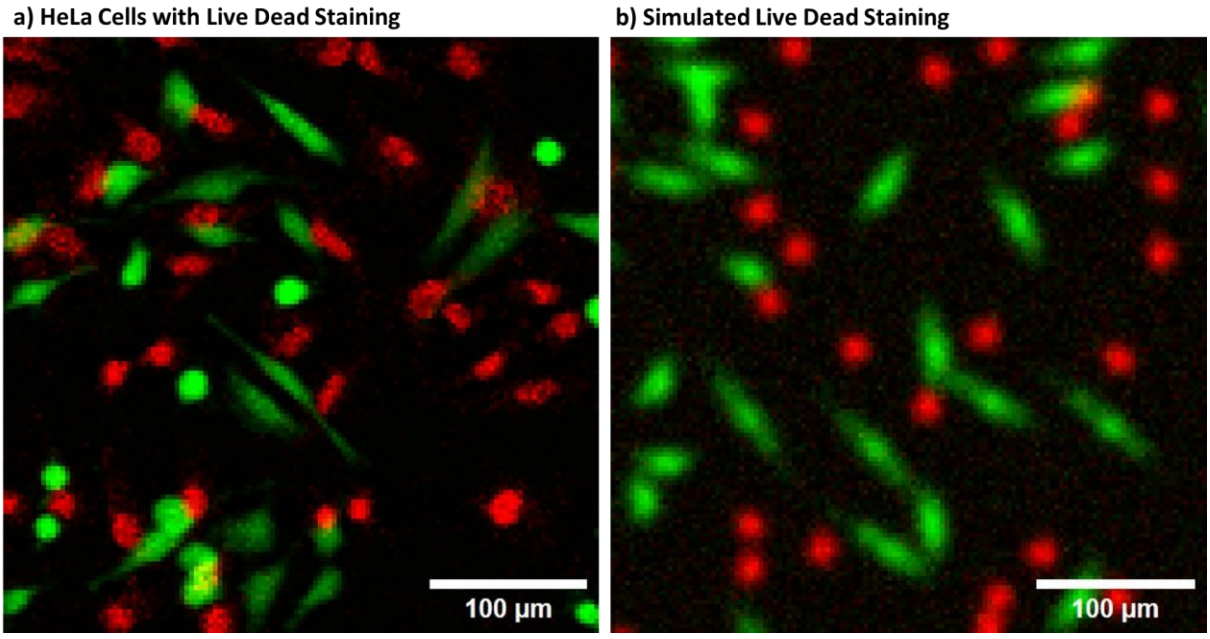

Figure 8: Direct comparison of real (a) and simulated (b) confocal fluorescence images of living (green) and dead (red) HeLa cells.

In general, a high cell density leads to an underrepresentation of detected cells because of clustering and overlapping of cells (Figure 9a). This is the case for dense living and dead cells (Figure 9b) but as living cells are often bigger and elongated and thus more overlapping it will lead to a minor bias of approximately - 4.5 % in cell viability if 1000 dead and 1000 live cells are randomly distributed on 1200 x 1200 µm sized images (Figure 9c).

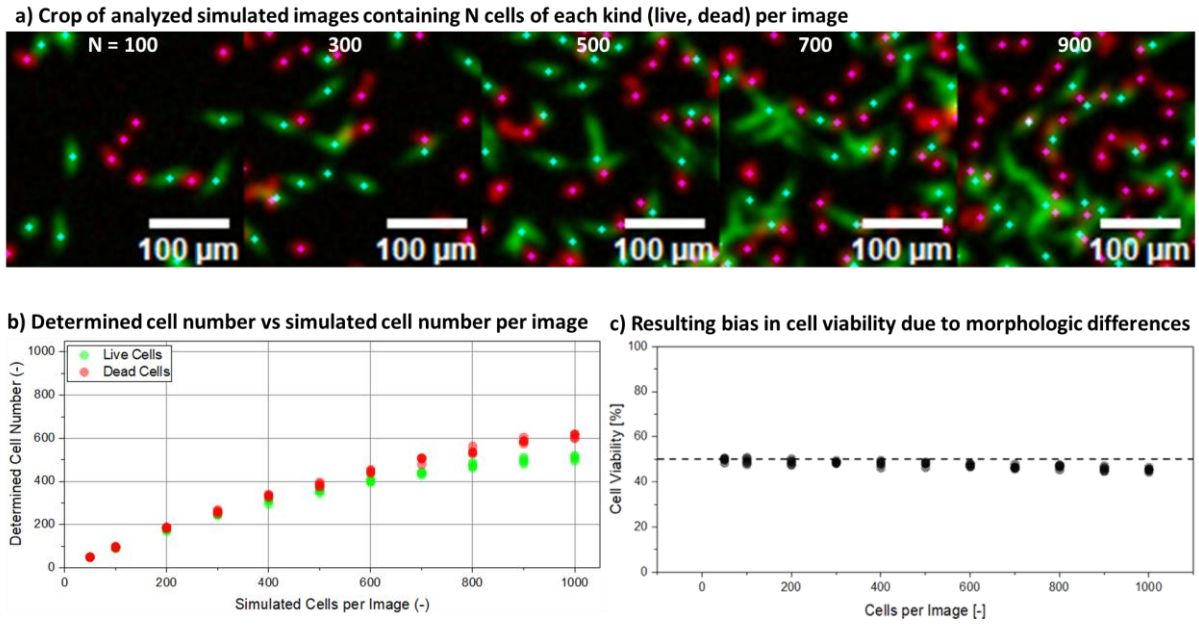

Figure 9: a) shows some zoomed region of the validation overlay of analyzed simulated images with cells in different densities (100 to 900 living and dead cells each) to show the effect of underrepresentation due to clustering and overlapping cells. b) shows how both living and dead cells are underestimated at high densities. At 1000 cells per image, approximately only half of all cells were detected. c) shows that the overall bias in cell viability (which was set to be 50 % in each simulated density) is only minor (~ 4.5 %). For b) and c) 5 synthetic images were generated and analyzed per condition (cell density).

If living and dead cells are not equally dense then the bias is increased but still relatively small. In simulations with 10 % and 90 % cell viability (Figure 10a) with 1000 total cells in one image the bias is 4 % and -6.4 % respectively (Figure 10b).

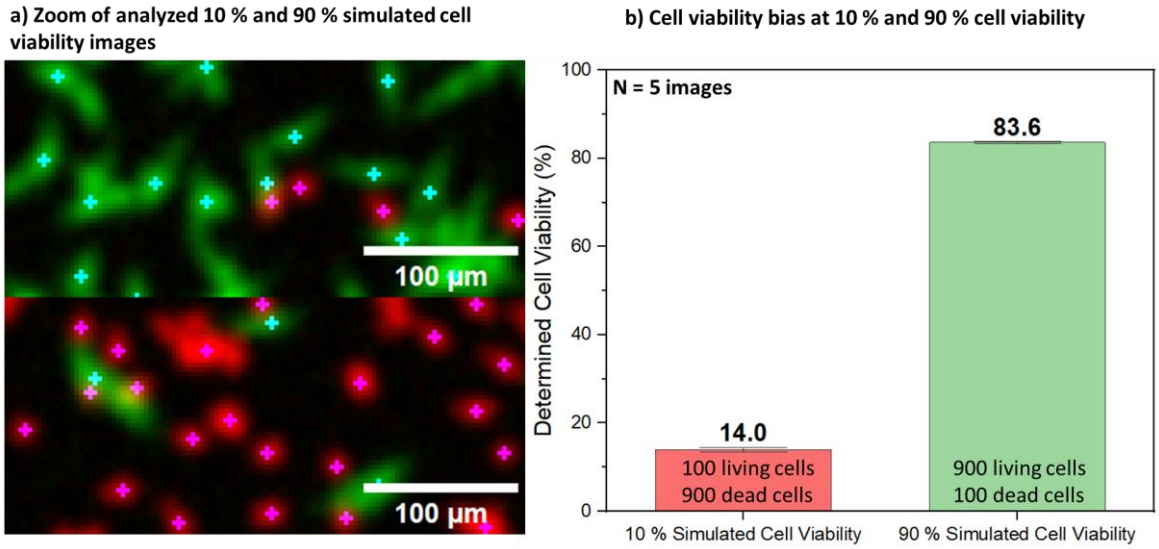

Figure 10: Determination of the cell viability bias due to high cell densities with 1000 total cells on 1200 x 1200 µm images (n = 5 each). The bias is 4 % for 10 % cell viability and -6.4 % for 90 % cell viability.

Based on our validation we therefore conclude that if correctly used, any difference in cell viability of more than about ± 5 % can be considered a real cytotoxic effect when using this macro for the automated analysis of Live Dead Assays.