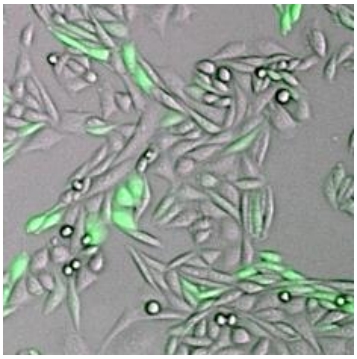
SYNENTEC

Experiment Guide – Confluence (1F)
Image Analysis



Technical Note
TN-B120-XVII-07

The purpose of this document is to guide the user through the *Confluence (1F)* image analysis to determine the fluorescent areas within an analyzed brightfield region and the ratio between them. Therefore the image analysis uses one brightfield and one fluorescence image in a sequence way which means that at first the area covered by cells in brightfield will be determined followed by an analysis procedure that examines these regions for fluorescence. This guide does not contain a complete procedure of setting up a Confluence (1F) assay itself since this may vary due to customer requirements. It includes just a few tips to help optimizing your results.

Content

1. Materials & Procedures

This chapter lists the equipment used to set up the NYONE® or CELLAVISTA® for the Confluence (1F) application. As already mentioned the kind of equipment may vary on individual user requirements. The following items are a suggestion, which have shown to achieve reliable results.

1.1 Material

1.1.1 Cells and Reagents

- Adherent cells (e.g. CHO-K1)
- Complete Culture Medium (e.g. DMEM/Ham's F12 1:1 with 5 % FBS and 1 % P/S)
- Phosphate Buffered Saline (w/o Ca^{2+} & w/o Mg^{2+} (PBS^{--}))
- Trypsin 0.25 %

1.1.2 Sample Carrier

The sample carrier used in this guide is a 96-well full area flat bottom plate, with black walls and clear foil bottom (µclear®; cat. #655090), supplied by Greiner Bio-One. It is also possible to use common transparent plates (e.g. Nunc #167008, nunclon™ Delta Surface).

1.1.3 Equipment

- Cell incubator (37°C, 5% CO_2)
- Pipettes and tips
- NYONE® or CELLAVISTA® microplate imaging microscope

Handling of the sample carrier:

The clear bottom of the sample carrier must never be touched with fingers (not even with gloves) at any time before measurement. Place your thumb and fingers at the rim on the longer side of the plate. The transparent bottom is part of the optical path and may lead to erratic measurements if stained with finger prints or dirt.

1.2 Procedure

1. Aspirate medium from the cells
2. Add 5 mL PBS, swing gently and aspirate
3. Add 500 µL Trypsin on to the cells (25 cm² T-flask)
4. Incubate cells at 37°C for 5 min.
5. Tap cells carefully off the bottle base.
6. Assimilate cells in 4.5 mL growth medium
7. Dilute cell suspension in 50 mL centrifuge tubes (Dilution factor depends on the number of cells in suspension and the required cell density).
8. Invert centrifuges several times to ensure a preferably even suspension.
9. Fill your microplate with the cell dilution (e.g. 200µL/well).
10. Place the plate under a lab bench for 30 min at room temperature to allow cells to settle at the bottom – preferably without agitation which ensures a more evenly spread of the cells.
11. Place the plate into the incubator at 37°C and 5% CO_2 until the measurement should start to allow cells to grow.

Plate handling:

Any rough handling of the filled plate before or after centrifugation may result in inhomogeneous distribution of the cells and should be avoided!

2. Getting Started and Experiment Setup

Double click on the NYONE® or CELLAVISTA® logo (Fig. 1) which opens the next window (Fig. 2).

Fig. 1: NyONE® logo on desktop
Double click to open the software.

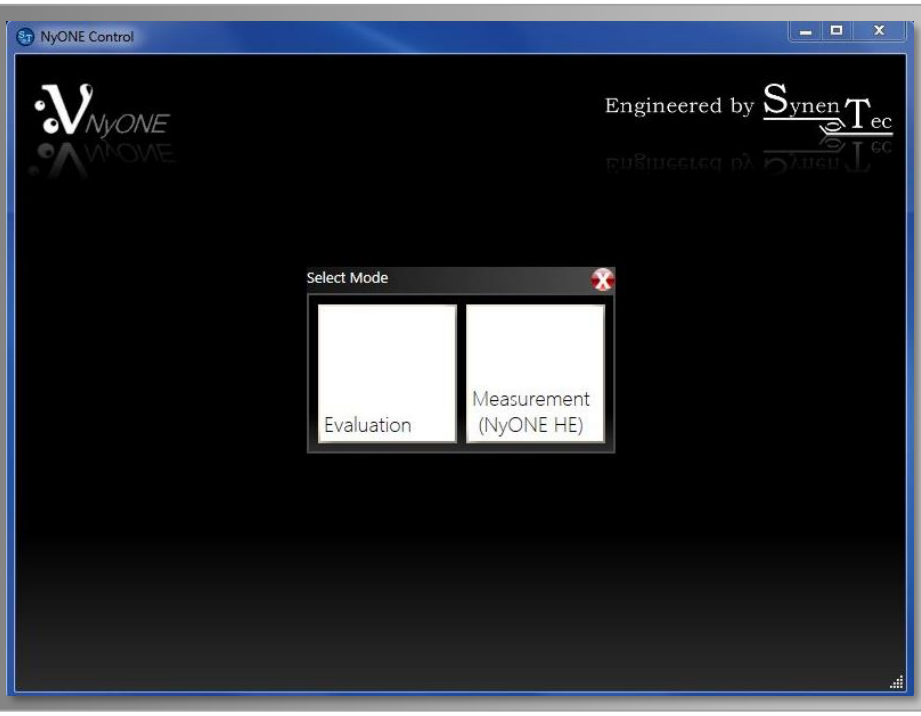


Fig. 2: 2nd window
With the 'Evaluation' button you can reprocess an existing experiment; with 'Measurement' you can generate a new one.

Click on 'Measurement' which opens the main window (Fig. 3a).

The system will now initialize which might take a few seconds. During this time, please take hands off the plate carrier area of the instrument. Click on the 'Tray in/Tray out' button in the upper right corner (red arrow in Fig. 3a, Fig. 3b) which will move the table into the right position for inserting the plate (please also refer to the Operating Guide for a correct placement of your plate). Now click on the 'Confluence (1F)' logo (red circle in Fig. 3a) to proceed with the application.

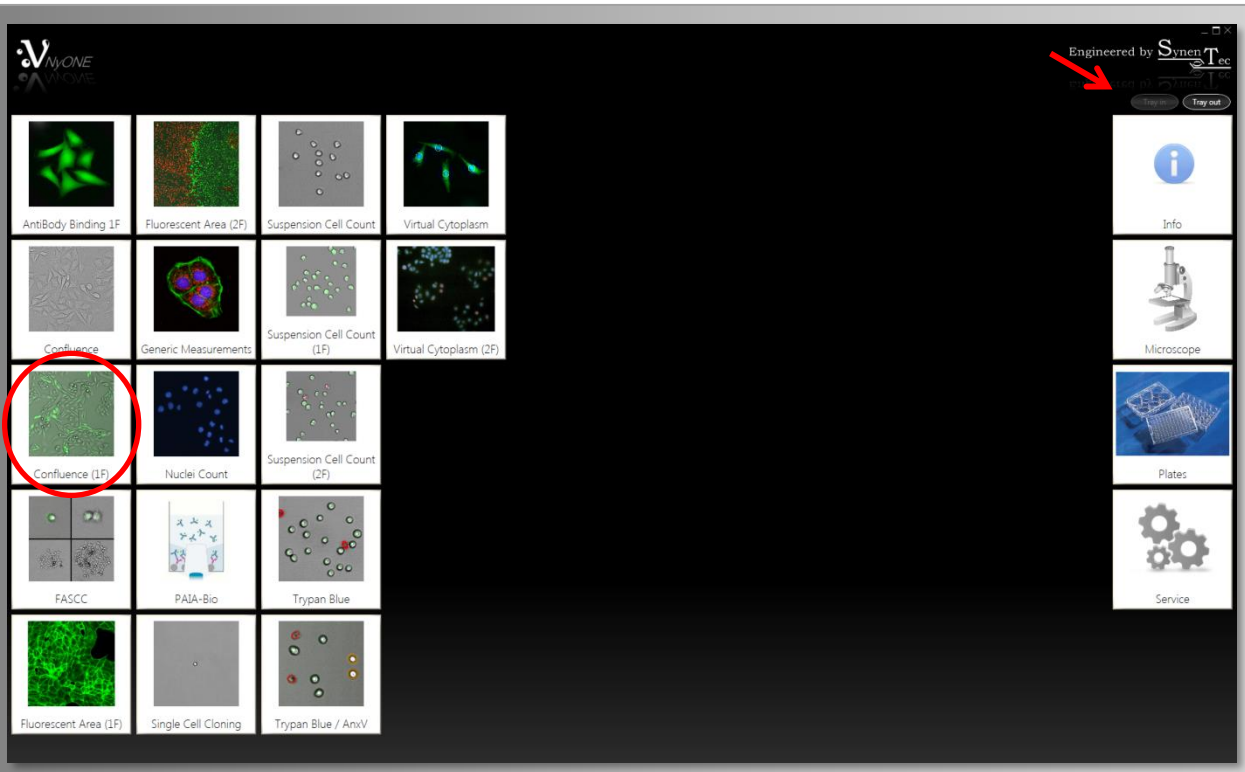


Fig. 3a: Main window with application logos (left) and special functions (right)

Fig. 3b: 'Tray in' and 'Tray out' button

Carry on completing the necessary fields (User, Experiment Name etc.), choose the required objective (10x was used for this experiment) and select the wells and subwells to be measured (Fig. 4). Please also refer to the 'Operating Guide' for further information about setting up an experiment.

There are many ways to measure an experiment and the associated plate. For this experiment the entire well was measured to achieve exact results as the cell distribution might be irregular (please also refer to 'Operating Guide' for well and subwell selection).

Contrary to the Confluence application a second channel for fluorescence is already added (Fig. 4, red arrow + Fig. 5). In case that an additional channel is required, simply click on the 'Add' button. Complete the 'Source', 'Dichro' (Cellavista® only) and 'Emi' fields according to your requirements. For this experiment LED_Blue and Emi_Green were used as parts of these cells fluoresce green (Fig. 5). It is recommended to use the same 'Emi' for the brightfield and fluorescence channel as this saves time and improves the overlay image quality unlike using two different dichros and/or filters. For further information regarding 'Processing Channel Map' and 'Advanced Settings' please refer to the 'Operating Guide'.

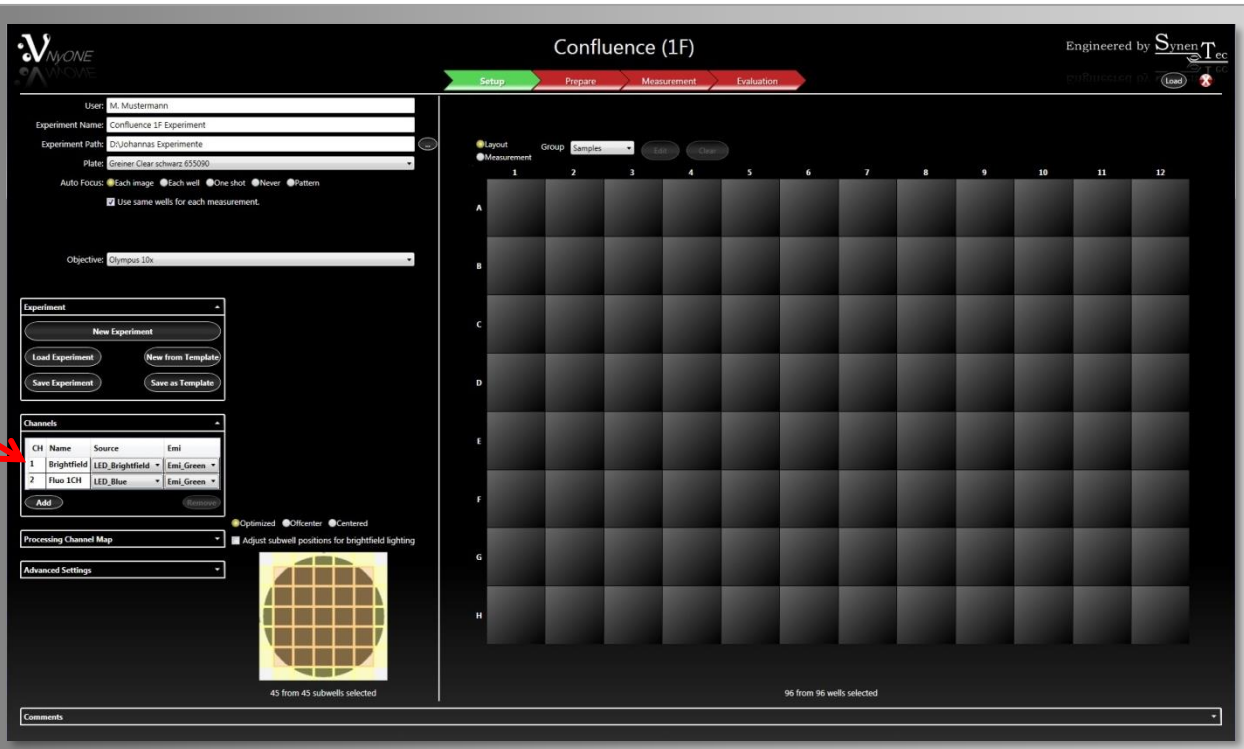


Fig. 4: Set up the Confluence (1F) experiment

Fig. 5: Channels
Choose your required light source, dichro (CELLAVISTA® only) and emission filter.

Also check if the 'Auto Focus' is set according to your requirements before measuring (Fig.6). For a measurement with a 10x objective 'Each well' is recommended as this option ensures a shortened measuring time, providing that the plate quality is sufficiently good.

Fig. 6: Auto Focus settings

To transfer the plate layout to your experiment, check the 'Layout' box, choose the mentioned wells and press 'Edit' (Fig. 7).

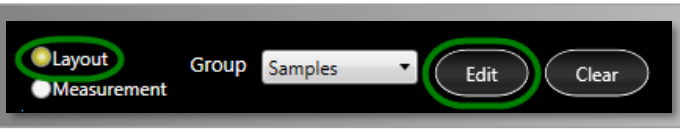


Fig. 7: Apply the plate layout

The 'Plate Layout Configuration' (PLC) dialog appears where the 'Final Dilution' and the 'Volume per Well' have to be completed (Fig. 8). Once all parameters are set, click on 'Apply' to activate same for the pre-selected wells. For further details regarding the PLC and setting up an experiment, please refer to the Operating Guide.

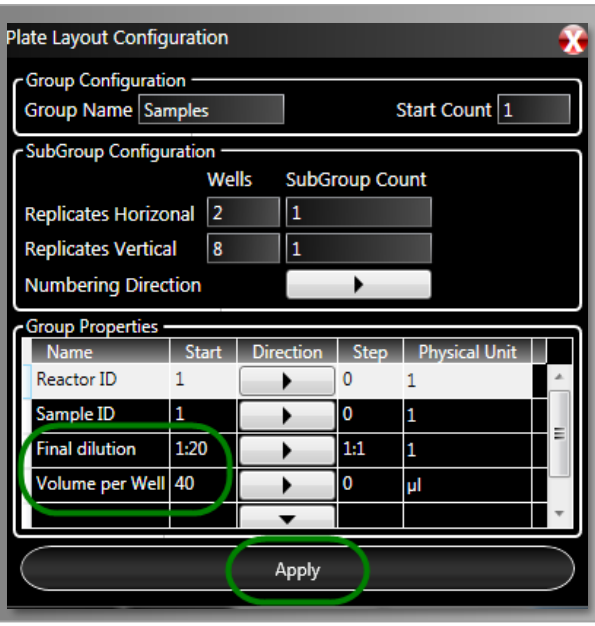


Fig. 8: Configure the plate layout
Enter your final cell dilution, the volume per well and e.g. the concentration of an added dye to save it for your experiment.

Once all experiment definition parameters are set, click on the 'Prepare' arrow (Fig. 9) to set up the optical and analysis parameters.

Fig. 9: Workflow

The following check box appears (Fig. 10). Click 'Yes' to move on to the 'Prepare' mode.

Fig. 10: Save Experiment

3. Optical Setup

3.1.1 Optical Settings

This application starts with adjusting the optical settings for the brightfield channel. Before an image can be obtained an autofocus must be carried out (click) followed by a snapshot to get an overview of the current image and to adjust the right optical settings (Fig. 11).

Fig. 11: Start with an autofocus

In case that the image is either too dark or bright, check the 'Histogram' on the lower left side and use (tick the box) 'Liveview' while adjusting 'Exposure Time' and 'Gain' accordingly for the brightfield (BF) channel (Fig. 12). Now deactivate 'Liveview' to cross check the 'Current grey value' in the upper left 'Image Info' box (abt. 130 counts) by simply moving the mouse curser over the image. Press 'Process Image' (lower left side) to get an actual overview of your settings.

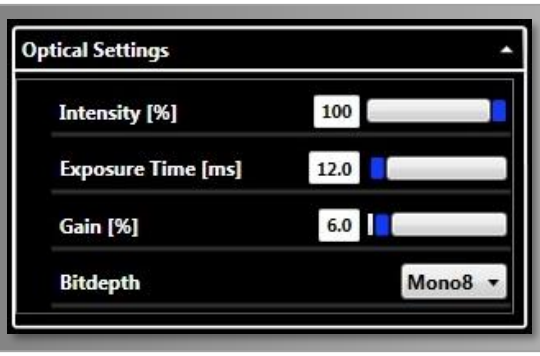


Fig. 12: Best Optical Settings in BF for this example

Continue by adjusting the 'Focus Offset'.

3.1.2 Focus Offset

To adjust the focus offset activate 'Liveview' first and either position the mouse cursor into the focus offset box and move the mouse wheel up/down or position the mouse cursor right into the image and press CTRL while turning the mouse wheel (Fig. 13). A good focus offset has been achieved when suspension cells appear bright inside and dark at the rim (Fig. 14), which is called the 'Lens Effect', and adherent cells are rich in contrast. The chosen value will be kept for the brightfield channel.

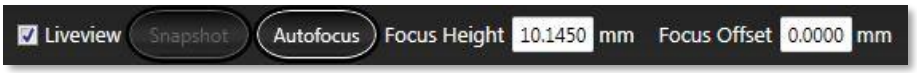


Fig. 13

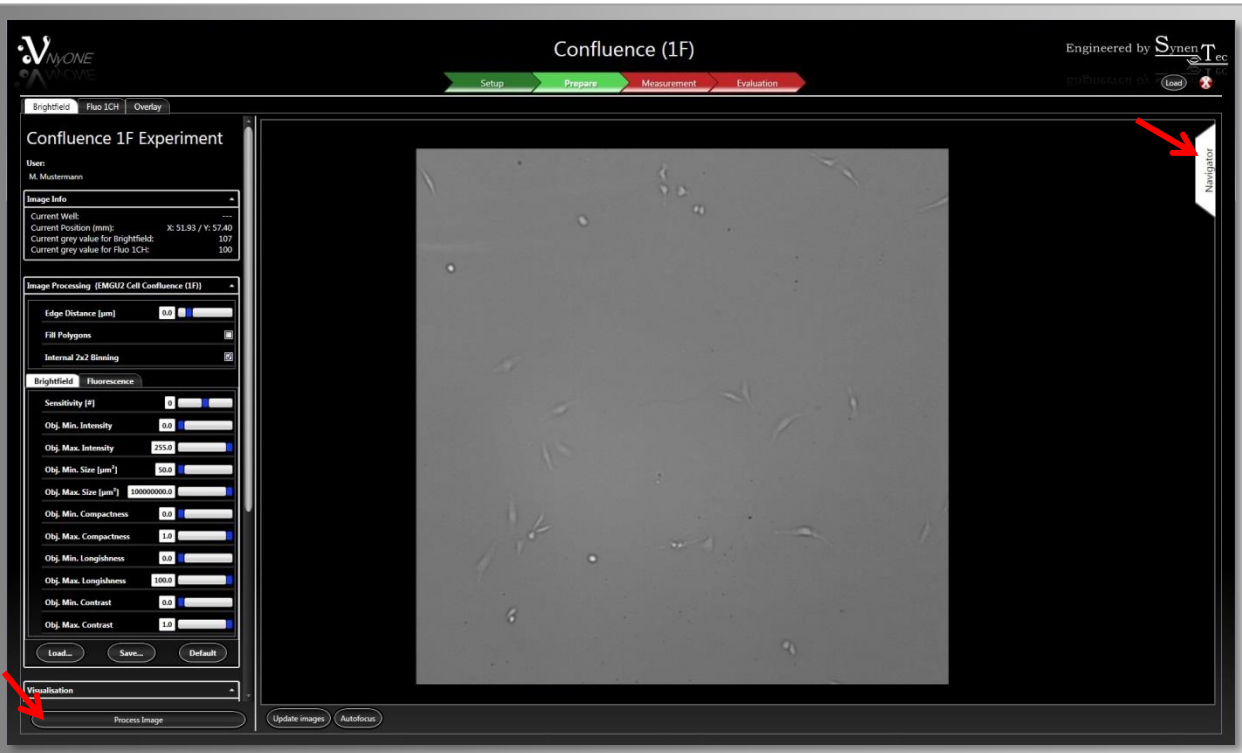


Fig. 14: Best 'Focus Offset' settings

Once all optical settings have been completed and the required image quality was found, the image processing should now be tested. It is recommended to start using the Default parameters which in general ensure to meet the desired results.

Click on 'Process Image' to test your settings (Fig. 14 - red arrow, lower left + Fig. 14b):

Fig. 14b

By zooming into the image more details as decisive criterion can be seen. Therefore position the mouse cursor right into the image and move the mouse wheel up/down. The entire image should be tested by zooming out of it completely (use 'F' button).

The Navigator (red arrow, right side Fig. 14) can also be used to click through the different wells for checking the optical settings and the focal plane.

3.2 Set up for Fluorescence

Setting up the parameters for the fluorescence channel differs compared to the brightfield channel. Please note that the histogram serves only adjusting the parameters for brightfield.

3.2.1 Optical Settings

Carry out an 'Autofocus' followed by a 'Snapshot' (Fig. 15) to obtain a first overview and image of your cells. In case that the image is either too dark or bright tick the 'Liveview' box and adjust 'Exposure Time' and 'Gain'. Here it should be noted that the gain is not set too high to keep a good image quality. We would recommend setting the 'Gain' not higher than 40 %. It is important that medium bright cells obtain a current grey value of abt. 130 counts and to also ensure that the number of cells in saturation (255 counts) is kept to a minimum.

3.2.2 Focus Offset

To adjust the focus offset activate 'Liveview' first and either position the mouse cursor into the focus offset box and move the mouse wheel up/down or position the mouse cursor right into the image and press CTRL while turning the mouse wheel (Fig. 15). The chosen value will be kept for the fluorescence channel.

Fig. 15

In case that the detection of your cells is not satisfactory 'Exposure Time' and 'Gain' should be amended accordingly.

The following images will show you how to achieve good results for the fluorescence illumination:

Fig. 16: Unaltered **'Focus Offset'**

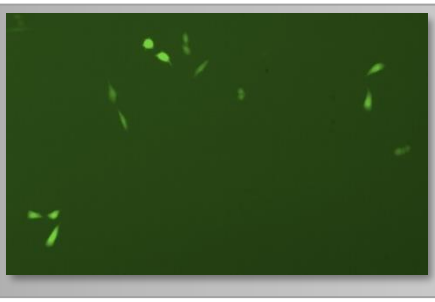


Fig. 17: Best 'Focus Offset' adjustment for this example

Once the result is satisfactory deactivate 'Liveview' as its warmth will affect the condition of the cells and the dye within the cells will be bleached, the fluorescence decreases.

If all optical settings have been completed and the required image quality was found, the image result for the fluorescence channel can be tested in the 'Overlay' mode by clicking on 'Process Image'.

3.3 Overlay

To test your parameter settings for brightfield and fluorescence, click on 'Overlay' (Fig. 18 – red arrow upper left).

Fig. 18: Overlay image - balanced illumination

In this mode you can also individually adjust the 'Image Processing Parameters' for the two channels by clicking on either 'Brightfield' or 'Fluorescence' (red ellipse). Further click on 'Process Image' to view and check the results. It is recommended to start using the default parameters which in general ensure to meet the desired results.

In case the result as shown in the 'Overlay' mode is not satisfactory the Image Processing Parameters for the brightfield and fluorescence channel can either be re-adjusted or previously saved parameters may be used or the parameters can be reset to default (Fig. 19). For further detailed information please refer to chapter 5. Evaluation, 5.2 Image Processing Parameters.

The Navigator (red arrow, upper right - Fig. 18) can also be used to click through the different wells for checking the optical settings and the focal plane.

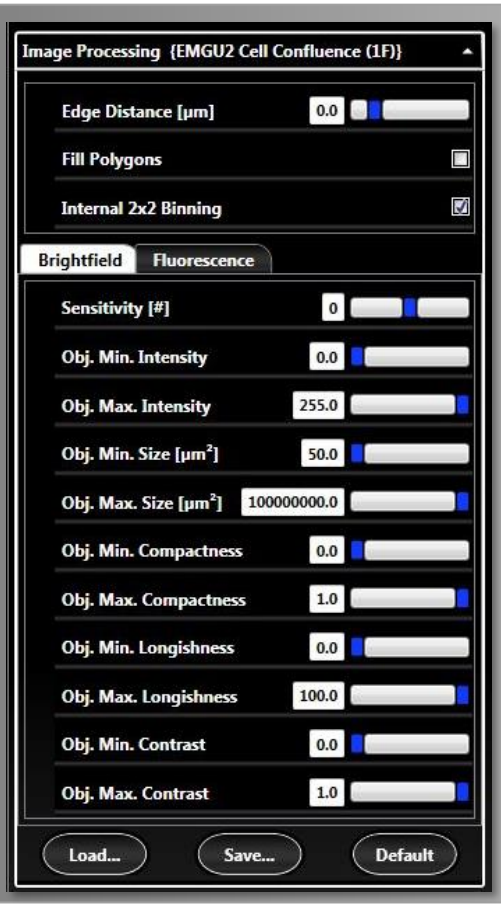


Fig. 19: Image Processing Parameters

An additional possibility to obtain a differing view of your cells and to concurrently accentuate the illumination of the channels is to click on 'Visualisation' (Fig.18 - red arrow, lower left). The following window appears (Fig. 20) which allows the user to adjust the brightness and contrast of the image separately. This option does not change the original image but changes the visualization of it.

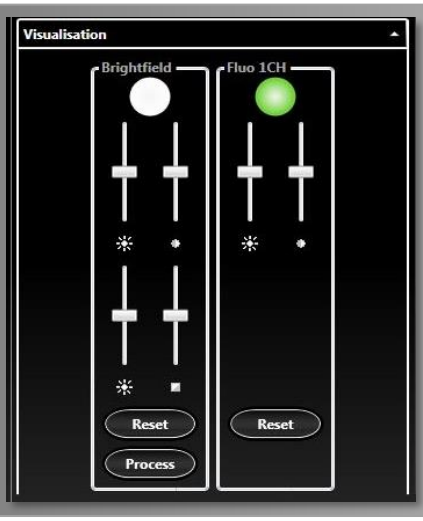


Fig. 20: Visualisation slider

Click on 'Process' to view the appropriate images and on 'Reset' to return to the default settings.

By zooming into the image more details as decisive criterion can be seen. The entire image should be tested by zooming out of it completely (use 'F' button).

Double-click in the middle of a particular object will open another window (Fig. 21) presenting more detailed information about objects of interest. This feature can be helpful to find the right parameter settings to include or exclude an object.

Click on 'Ok' to close the window again.

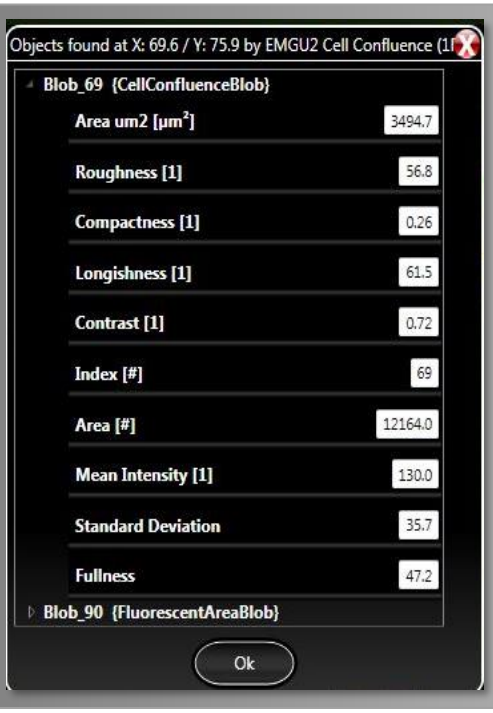


Fig. 21: particular object window

If the achieved results are satisfying, these parameters should be saved (Fig. 22).

Fig. 22

Now proceed with the Measurement (click – Fig. 23).

Fig. 23: workflow arrows

4. Measurement

There are two further options which may be chosen before you simply click on the 'Start' button on the lower left side to start the measurement.

It is recommended to tick both: 'Process Image During Measurement' (results will then also be available immediately in the 'Evaluation' mode) and 'Show Images' while the measurement will be carried out. These options allow to zoom into the appropriate wells and to watch the imaging process (Fig. 24).

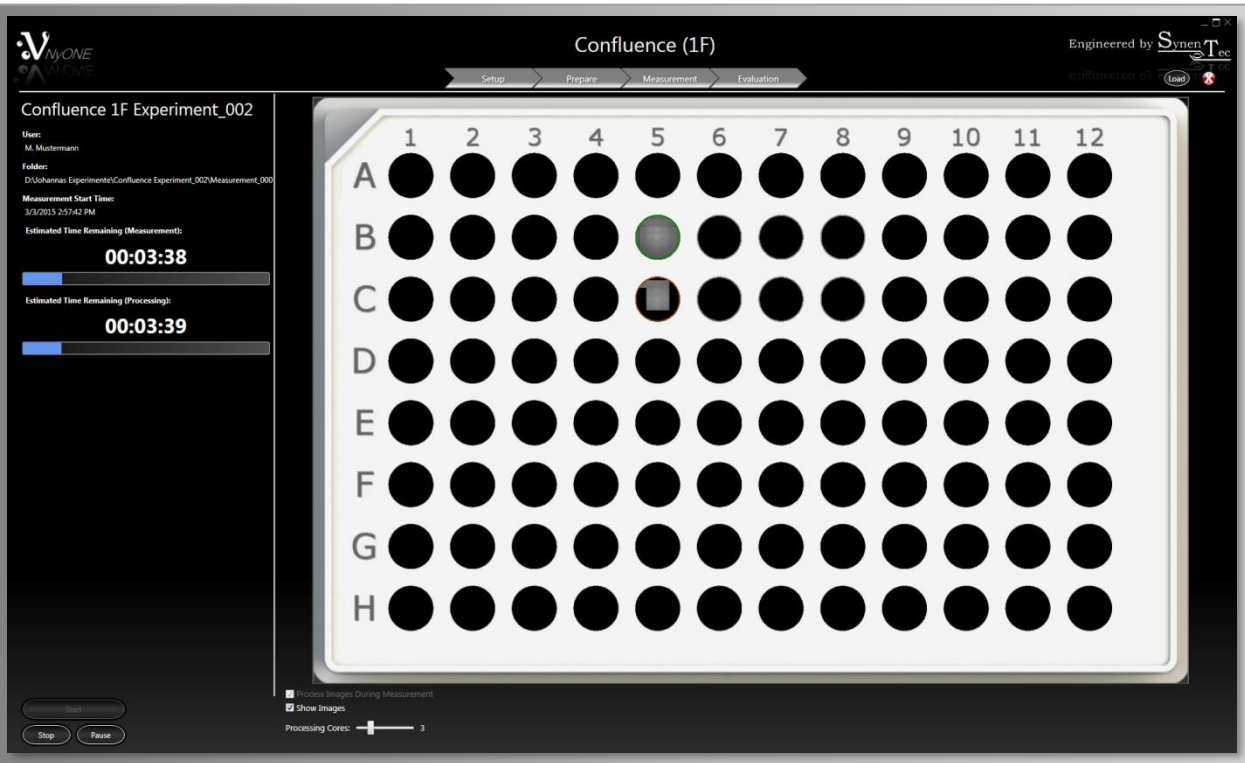


Fig. 24: Measuring Process

The following box appears once the measurement is done (Fig. 25) and 'Ok' needs to be clicked.

Fig. 25: Measurement Done

Proceed with 'Evaluation' to view the results of your experiment by clicking on the corresponding arrow (Fig. 26).

Fig. 26: workflow arrows

5. Evaluation

5.1 Result Presentations

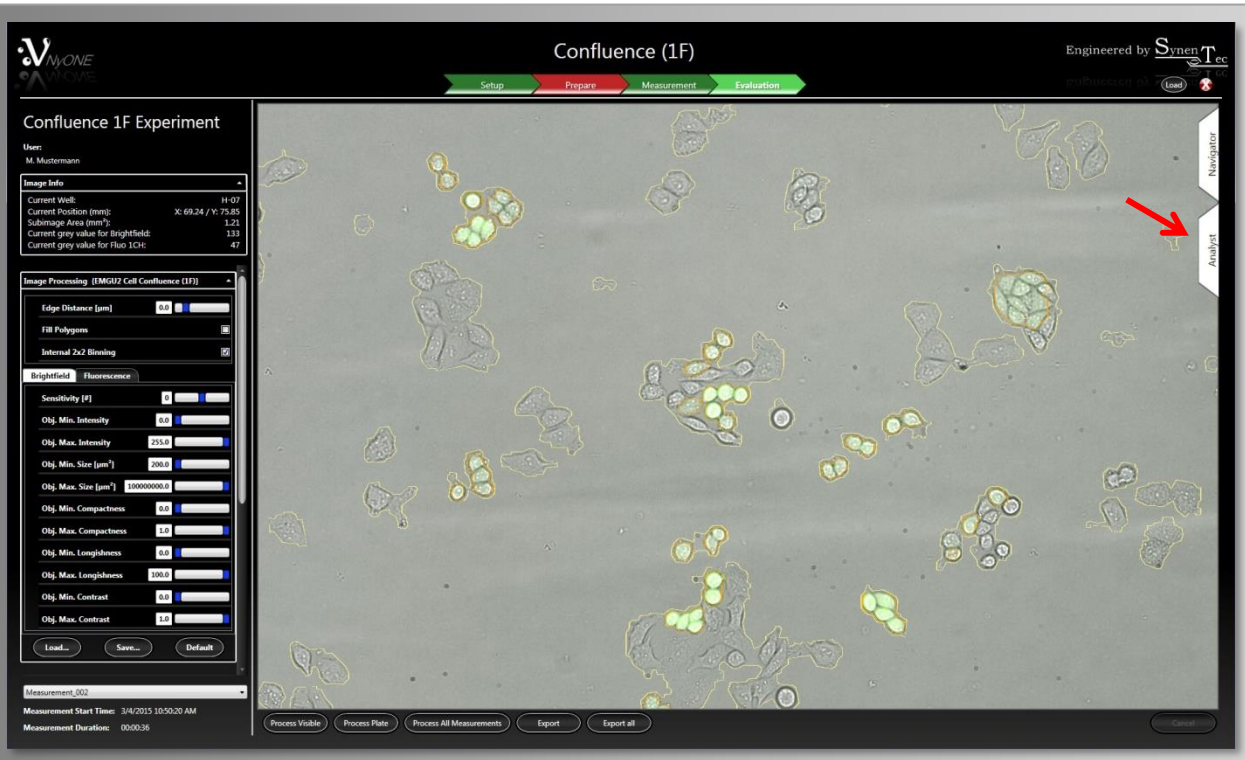


Fig. 27: Measuring Process

You can now check your results by scrolling through the result table, the Heat map, a timed chart graph, the Scatter plot and Histogram to obtain an overview (Fig.28). Therefore click on the 'Analyst' flag on the right (Fig. 27 - red arrow). For each type of result presentation many different properties like 'Cell Area' or 'Cell Confluence' can be chosen.

The below seen different colors of the 'Heat map' (Fig. 28) represent e.g. the cell confluence according to the covered area within each well. Blue represents a low and red a high cell confluence.

The result table as well as required images can be exported as a CSV file using the 'Export' buttons in the 'Results' tab.

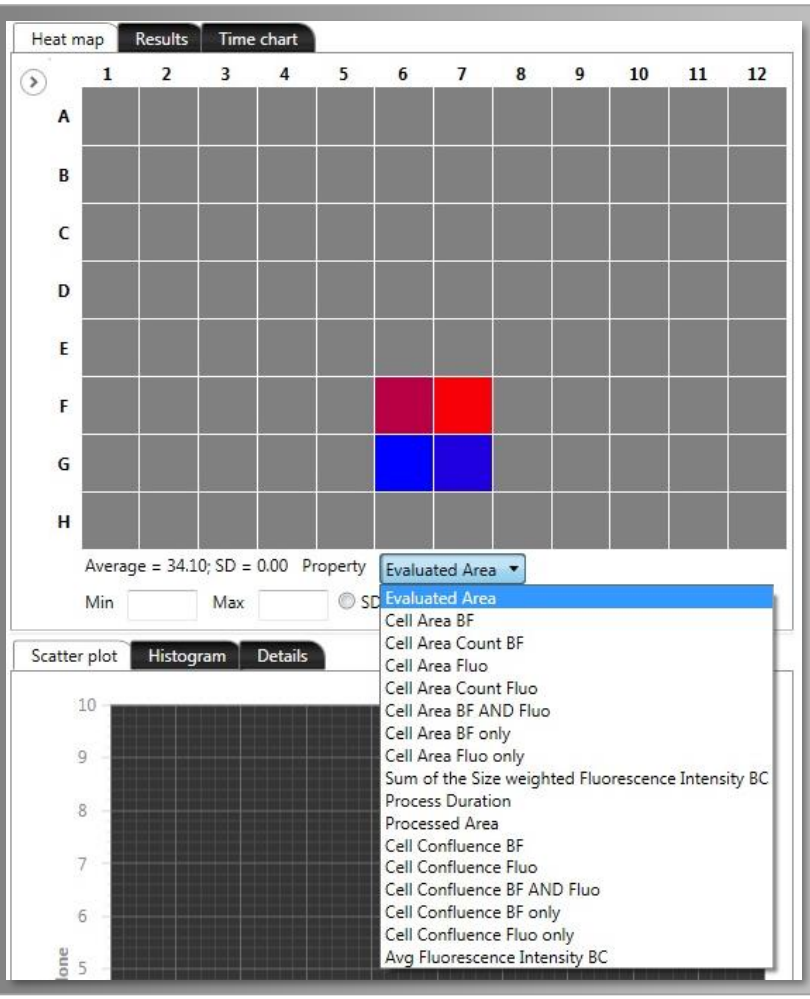


Fig. 28: Analyst with different forms of result

The following data are supplied by the result table:

Cell Area BF	Area covered with cells in brightfield
Cell Area Count BF	Number of separate cell clusters in brightfield
Cell Area Count Fluo	Number of separate cell clusters in fluorescence
Cell Area BF AND Fluo	Area covered with cells in brightfield AND fluorescence (coincidence)
Cell Area BF only	Area covered with brightfield only
Cell Area Fluo only	Area covered with fluorescence only
Cell Confluence BF	Percentage of Cell Area in BF and Evaluated Area
Cell Confluence Fluo	Percentage of Cell Area in fluorescence and Evaluated Area
Cell Confluence BF AND Fluo	Percentage of Cell Area BF AND Fluo and Evaluated Area
Cell Confluence BF only	Percentage of Cell Area in BF (without fluorescence) and Evaluated Area

The ‘Time chart’ allows and represents results of the measurement of cells over e.g. several days and also presents details for each well by simply clicking on the appropriate curve (Fig. 29).

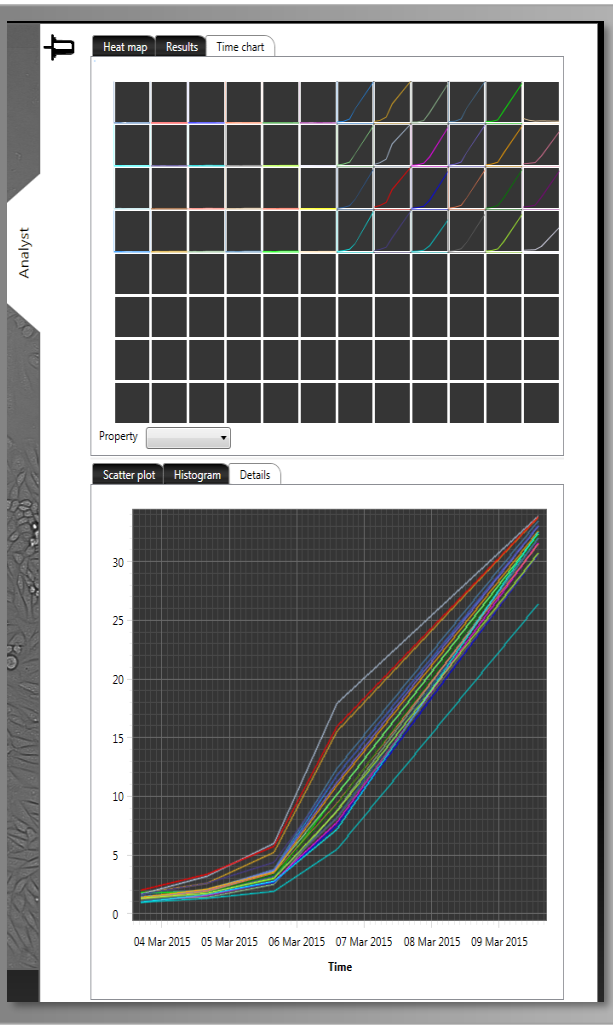


Fig. 29: Time chart
Single or several curves and fields may be selected by pressing 'Ctrl' and a) clicking on the required curve/field or b) dragging the mouse cursor to mark several fields at once.

If you are satisfied with the results you may 'Export' either just the results from the actual experiment or click on 'Export all' to export the data from previously carried out experiments (Fig. 30).

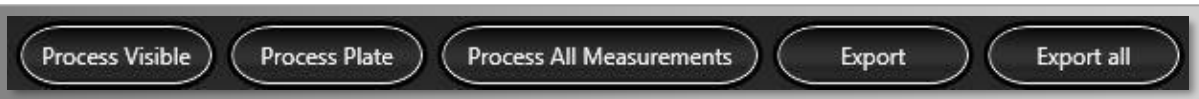


Fig. 30: Possible options after measurement is done

In case you are not satisfied with your results you can alter the image processing parameters accordingly, check same by clicking 'Process Visible' and further reprocess the whole plate by clicking on 'Process Plate' (Fig. 30).

In the following the different 'Image Processing Parameters' will be described which offer a variety of possible adjustments (P. 12, Fig. 19). In general only a few of them require adaption for the Confluence (1F) application. Experiences have shown that the parameters 'Compactness', 'Longishness' and 'Contrast' lead to proper results with the default settings.

5.2 Image Processing Parameters

5.2.1 Edge Distance

To ensure that only cells are counted and well edge artifacts and irregularities will not falsify the results, this parameter is to be used. The higher the value of edge distance the less area will be evaluated. If this parameter is set to 100 µm operator results within a distance of 100 µm of the well edge will be excluded (Fig. 31a – 31c).

Fig. 31a: Edge Distance **'0'**

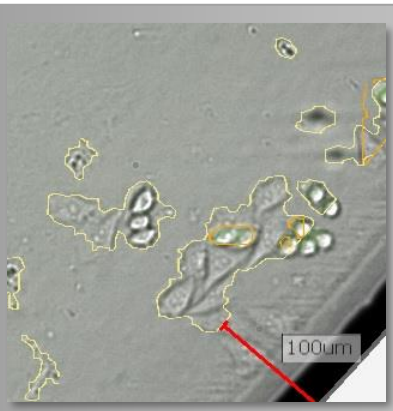


Fig. 31b: Edge Distance **'100'**

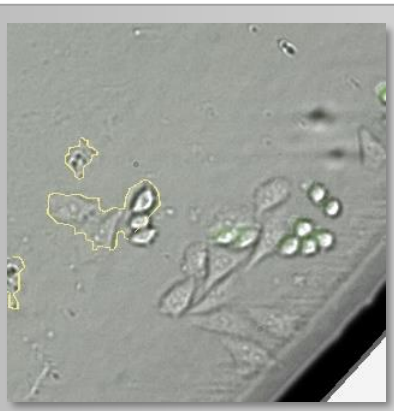


Fig. 31c: Edge Distance

5.2.2 Fill Polygons

Fills detected cells entirely with color and facilitates their visualization. Yellow marks cells detected in brightfield and orange shows cells in fluorescence (Fig. 32a + b).

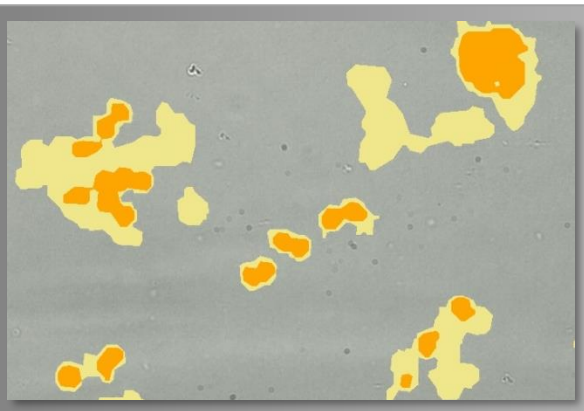


Fig. 32a: Fill Polygons **'on'**

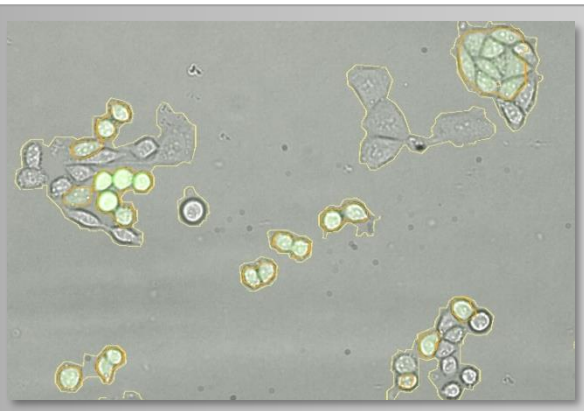


Fig. 32b: Fill Polygons **'off'**

5.2.3 Internal Binning (2x2)

Defines the amount of pixels within a cell to be detected (e.g. 2 x 2 pixels shown as one) and offers an advantage regarding weakly fluorescent cells. The processing will then be faster but a little more imprecise.

The following images show the Image Processing Parameters for the brightfield channel, if present, (grey background) followed by images for fluorescence (dark green background).

5.2.4 Sensitivity

This Parameter determines the sensitivity of the cell detection. According to the cell line or the cultural state, either unknown objects or debris may occur or cells may also be weakly contrasting in fluorescence depending on their condition.

A reduction of the parameter increases the insensitivity of the picture analysis, so that contrast weak objects (e.g., fragments of dead cells and other background objects) can be excluded from the analysis (Fig. 33a + b).

According to the analysis situation an increase of the parameter sensitivity tunes the image analysis in such a way that contrast weak objects can be counted up to a certain degree (Fig. 34c).

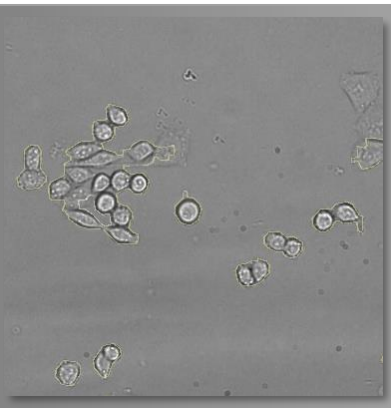


Fig. 33a: Sensitivity '-8'

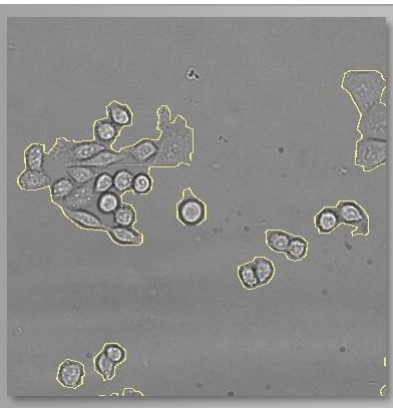


Fig. 33b: Sensitivity '0'

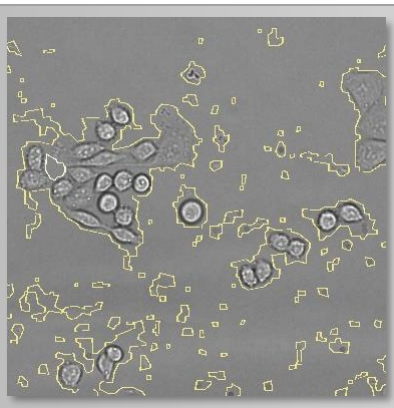


Fig. 33c: Sensitivity '6'

Fig. 34a: Sensitivity set on -6

Fig. 34b: Sensitivity set on 0

Fig. 34c: Sensitivity set on 6

5.2.5 Obj. Min./Max. Intensity

States the average grey value of the object in the image and offers a range from '0' to maximum '255' (background corrected minimum/maximum intensity of a single object that shall be detected). If min. intensity is set on '0' all objects above this value will be detected (Fig. 35a) whereas a value of '20' is needed as a minimum to exclude objects with low fluorescence (Fig. 35b). An increased value of '70' allows detection of very bright fluorescence cells only (Fig. 35c).

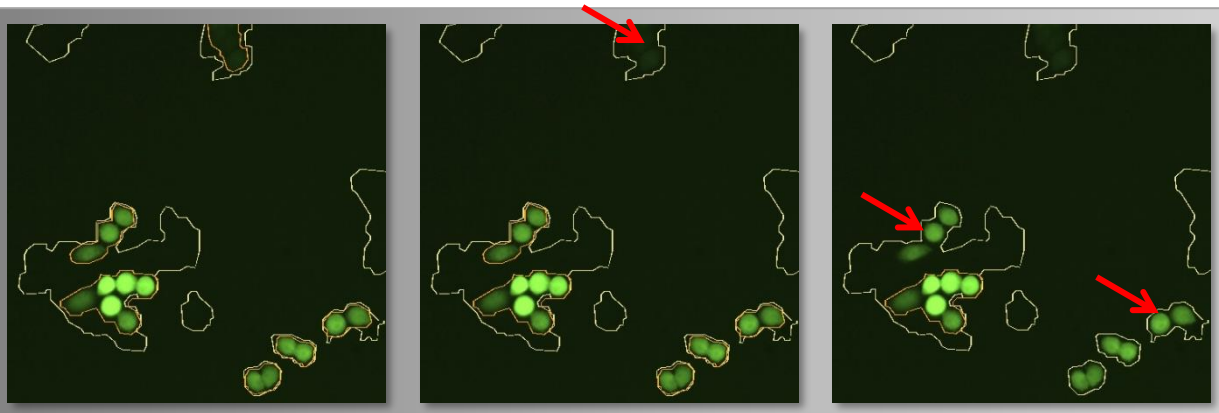


Fig. 35a: Min. **Intensity BC '0'** Fig. 35b: **Min. Intensity BC '20'** Fig. 35c: Min. Intensity BC **'70'**

A maximum intensity BC of '255' allows the detection including the brightest cells (Fig. 36c) whereas a value of '100' excludes them (Fig. 36b). A value of '50' detects even less bright cells (Fig. 36a).

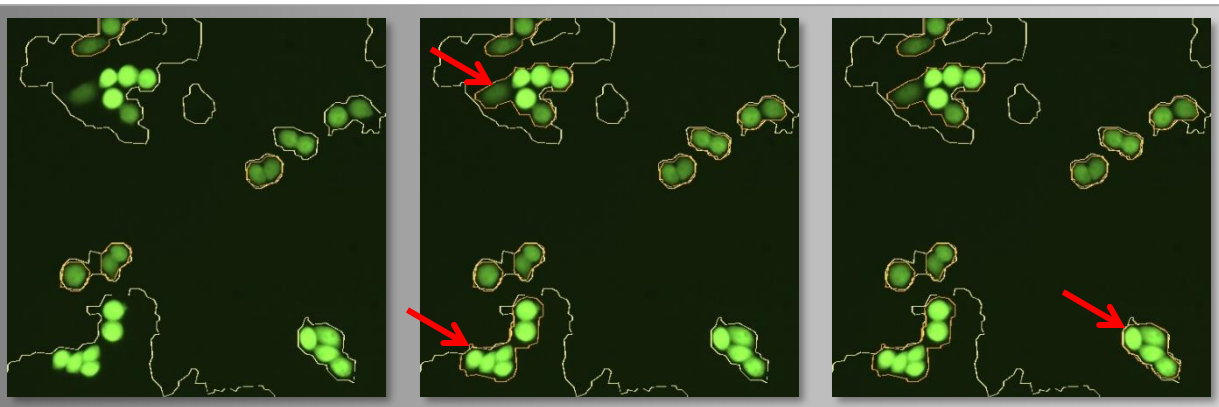


Fig. 36a: Max. Intensity BC '50' **Fig. 36b: Max. Intensity BC '100'** **Fig. 36c: Max. Intensity BC '255'**

5.2.6 Obj. Min./Max. Size

Confines the size of objects to be detected. Depending on the cell line and cultural state, 'Obj. Min. Size' is especially useful to exclude debris or smaller cells (Fig. 37a – c). Please note that a default value of '0' is more time consuming whereas a higher value of e.g. '200' or '1000' ensures detection of proper and/or required cells in brightfield and fluorescence (Fig. 37a vs. 37b).

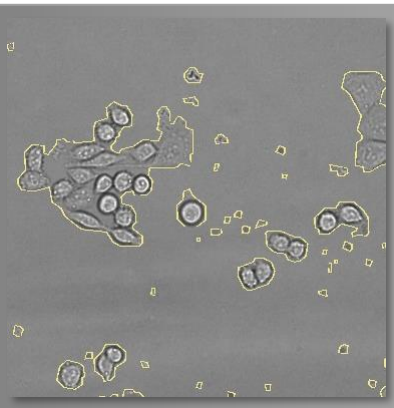


Fig. 37a: **Obj. Min. Size '0'**

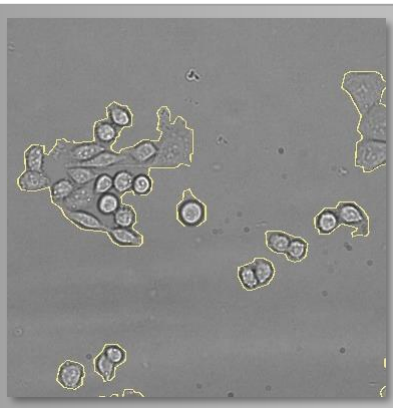


Fig. 37b: **Obj. Min. Size '200'**

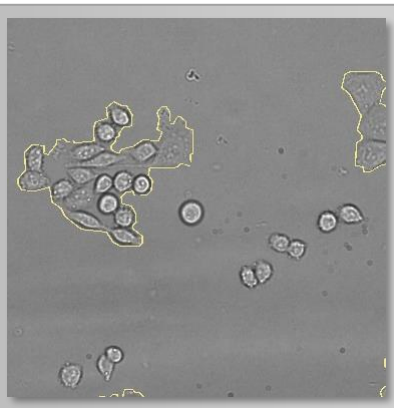


Fig. 37c: **Obj. Min. Size '1000'**

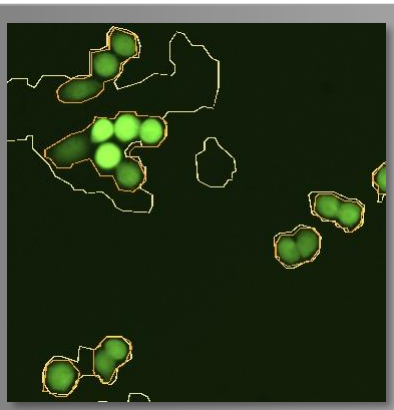


Fig. 38a: **Min. Size '0'**

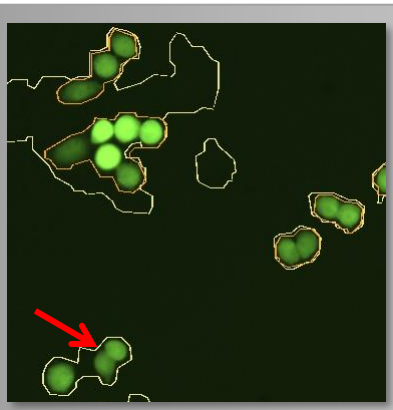


Fig. 38b: **Min. Size '500'**

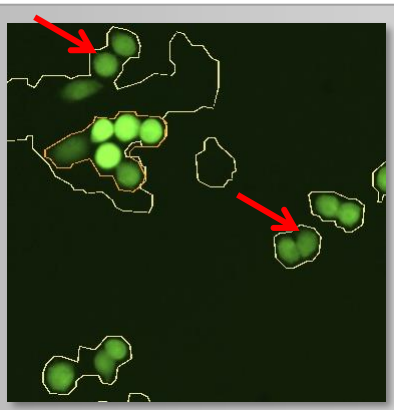


Fig. 38c: **Min. Size '2000'**

If 'Obj. Max. Size' is set on '100000000.0' even largest cells including merged cells will be detected (Fig. 39 + 40c) whereas a value of e.g. '600' and '1000' detects only smaller cells (Fig. 39 + 40a).

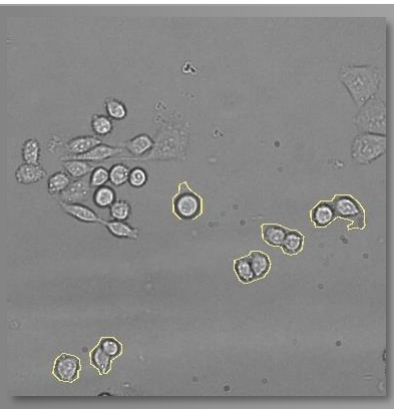


Fig. 39a: **Obj. Max. Size '1000'**

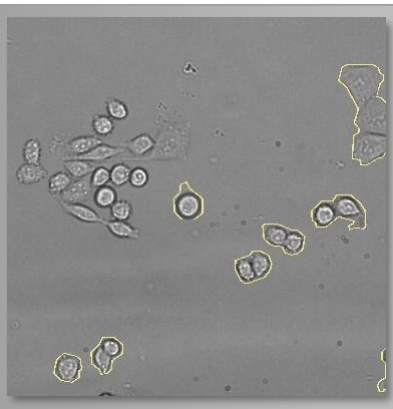


Fig. 39b: Obj. **Max. Size '5000'**

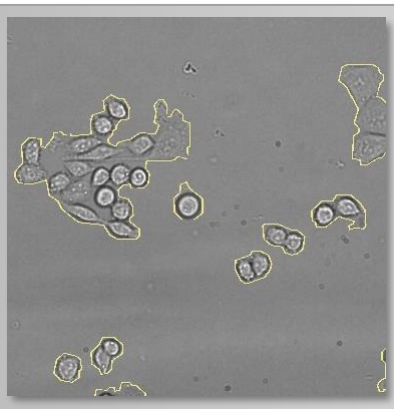


Fig. 39c: **Obj. Max. Size '100000000'**

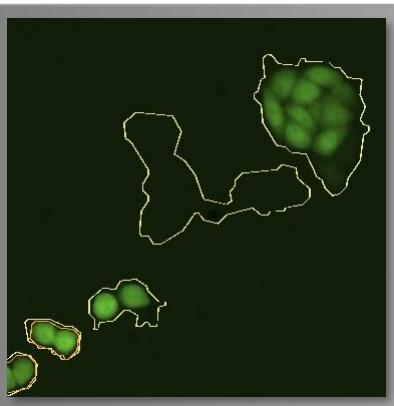


Fig. 40a: **Obj. Max. Size '600'**

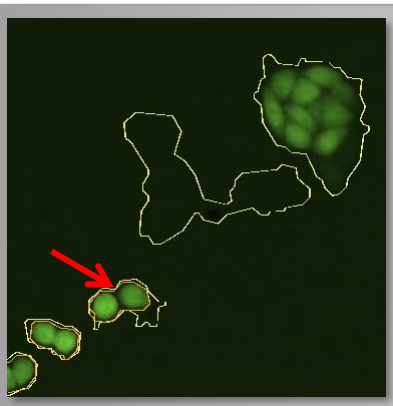


Fig. 40b: Obj. Max. Size '3000'

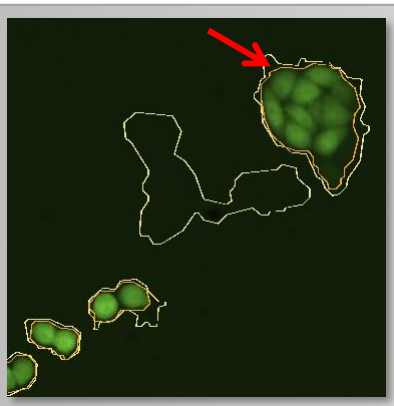


Fig. 40c: Obj. Max. Size '100000000'

A right mouse click into the image / onto a particular object enables to check the right size to either include or exclude this object.

5.2.7 Obj. Min./Max. Compactness

Serves the description of the 2D form of the object to be detected in the image in comparison to a perfect circular object. The less round an object, the closer the value is to '0'. The ideal form of a circle results in the maximum value of '1'.

5.2.8 Obj. Min./Max. Longishness

Describes the form of an object as well as the parameter 'Compactness'. Therefore an ellipse is determined which resembles a two-dimensional image of an object. The relation of the minor to the major axis of the ellipse as a percentage value results in the attribute Longishness.

5.2.9 Obj. Min./Max. Contrast

Describes the distribution of grey values of the object. It is calculated by the difference of the minimum and maximum grey value of the object in relation to the sum of both values. The value from '0' to '1' allows excluding cells which are rich or low in contrast. The images below show different settings for the brightfield channel (Fig. 41) and the fluorescence channel (Fig. 42 Obj. Min. Contrast).

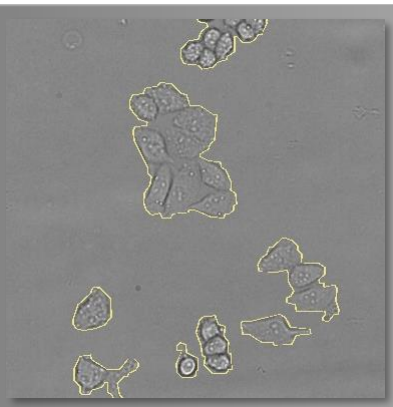


Fig. 41a: **Obj. Min. Contrast '0'**

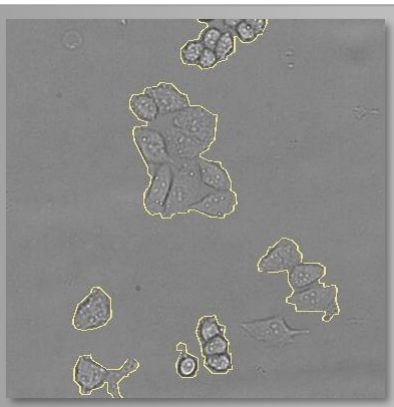


Fig. 41b: **Obj. Min. Contrast '0.4'**

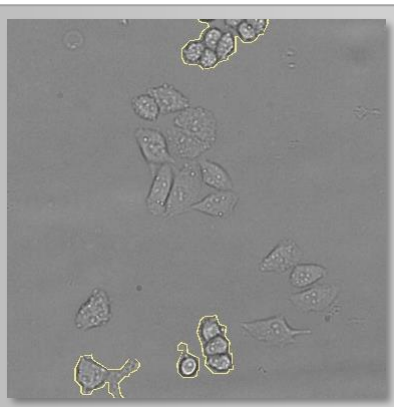


Fig. 41c: **Obj. Min. Contrast '0.6'**

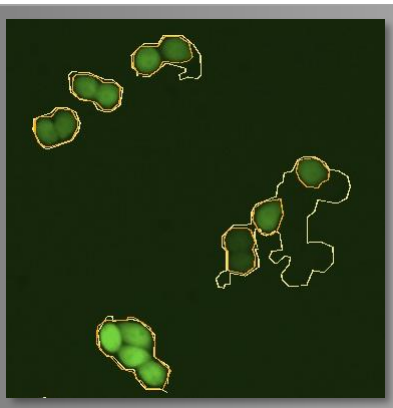


Fig. 42a: Obj. Min. Contrast **'0'**

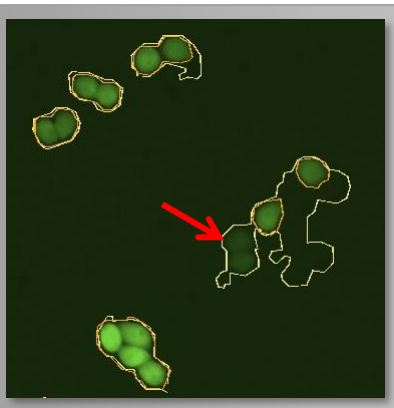


Fig. 42b: Obj. Min. Contrast '0.4'

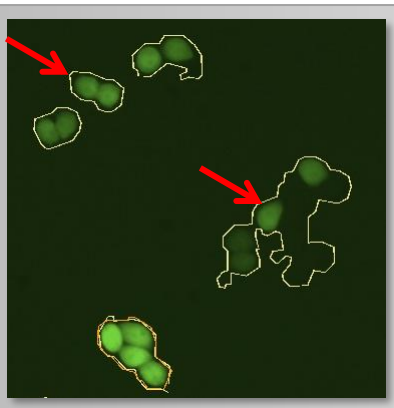


Fig. 42c: Obj. Min. Contrast '0.6'

The following images show examples for the 'Obj. Max. Contrast' for brightfield (Fig. 43) and fluorescence (Fig. 44).

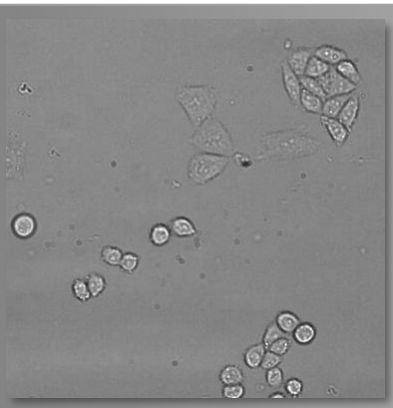


Fig. 43a: **Obj. Max. Contrast '0.5'**

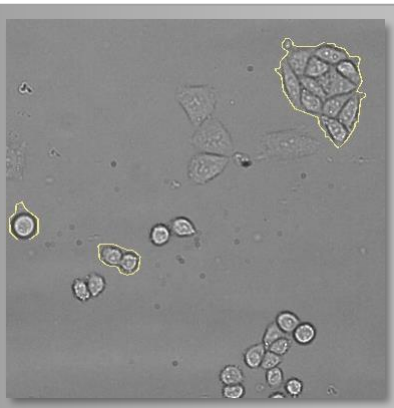


Fig. 43b: **Obj. Max. Contrast '0.7'**

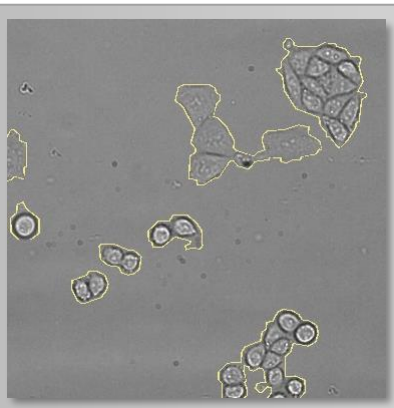


Fig. 43c: **Obj. Max. Contrast '1'**

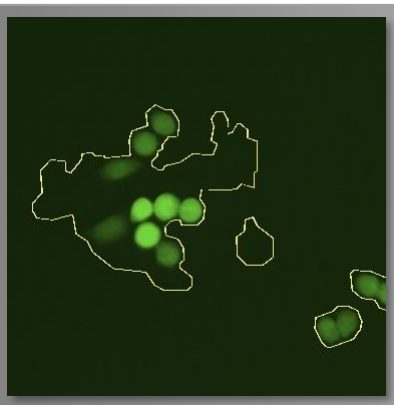


Fig. 44a: Obj. Max. Contrast **'0.5'**

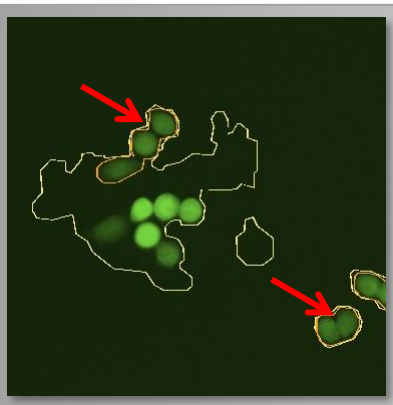


Fig. 44b: Obj. Max. Contrast '0.7'

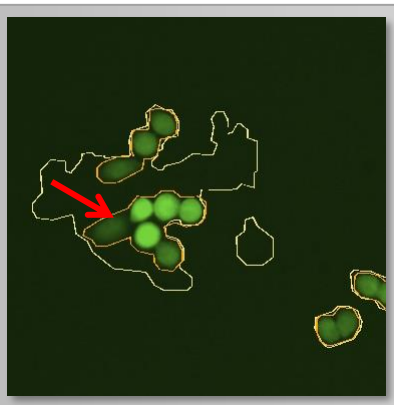


Fig. 44c: Obj. Max. Contrast '1'

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