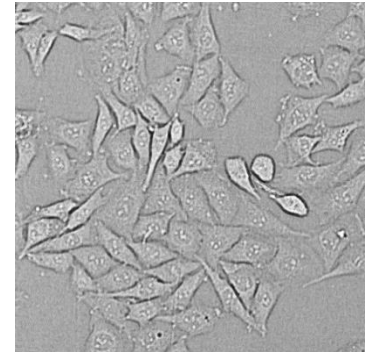


Experiment Guide – Confluence Image Analysis

The purpose of this document is to guide the user through the *Confluence* image analysis which monitors the area covered by cells using one Brightfield Channel only. It does not contain a complete procedure of setting up a Confluence assay itself since this may vary due to customer requirements. It includes just a few tips to help optimizing your results.



Technical Note
TN-B000-XVII-08

Content

1	Materials & Procedures	3
1.1	Material	3
1.1.1	Cells and Reagents	3
1.1.2	Sample Carrier.....	3
1.1.3	Equipment	3
1.2	Procedure.....	4
2	Getting started and Experiment Setup.....	5
3	Optical Setup.....	9
3.1	Optical Settings	9
3.2	Focus Offset	10
3.3	Testing ‚Image Processing Parameter‘	11
4	Measurement	13
5	Evaluation	15
5.1	Result Presentations.....	15
5.2	Image Processing Parameters	18
5.2.1	Edge Distance	18
5.2.2	Fill Polygons	18
5.2.3	Multiple Cell Layers Expected.....	18
5.2.4	Expected Hole Max. Intensity	19
5.2.5	Sensitivity	19
5.2.6	Obj. Min/Max. Size	19
5.2.7	Obj. Min./Max. Compactness.....	20
5.2.8	Obj. Min./Max. Longishness	20

5.2.9	Obj. Min./Max. Intensity.....	21
5.2.10	Obj. Min./Max. Contrast	21
5.2.11	Internal Binning (2^2).....	21

1. Materials & Procedures

This chapter lists the equipment used to set up the NYONE® for the Confluence 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 Ca²⁺ & w/o Mg²⁺ (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 % CO₂)
- Pipettes and tips
- NyONE® 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 μ L 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 number of cells in suspension and required cell density).
8. Invert centrifuges several times to ensure a preferably even suspension.
9. Fill your microplate with the cell dilution (e.g. 200 μ L/well).
10. Place the three plates 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% CO₂ 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.

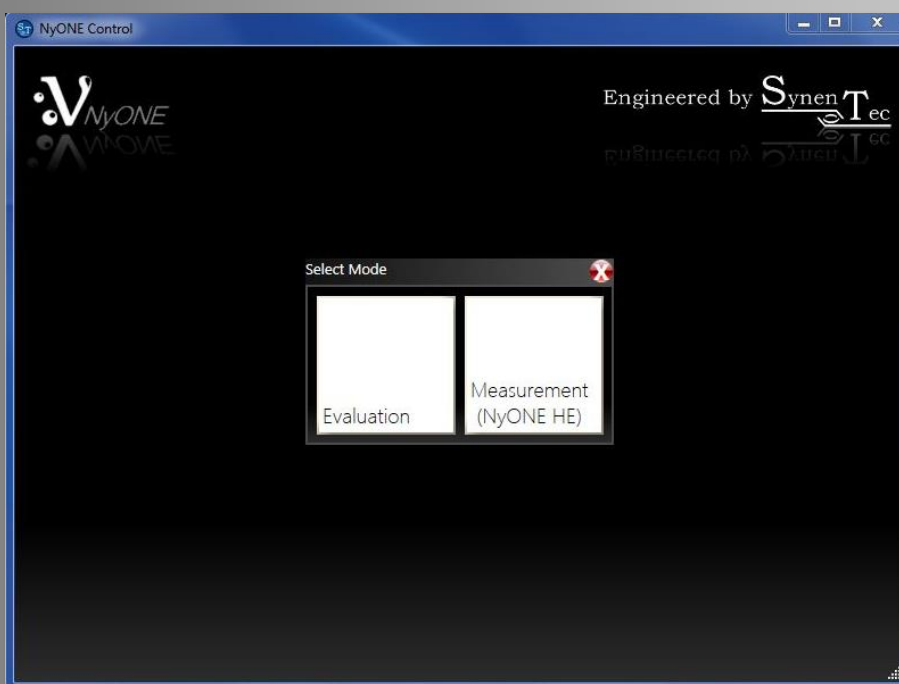


Fig. 2: 2nd window
With the 'Evaluation' button you can reprocess an existing experiment; with 'Measurement' you can generate a new one.

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

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' logo (red circle in Fig. 3a) to proceed with the application (Fig. 3).

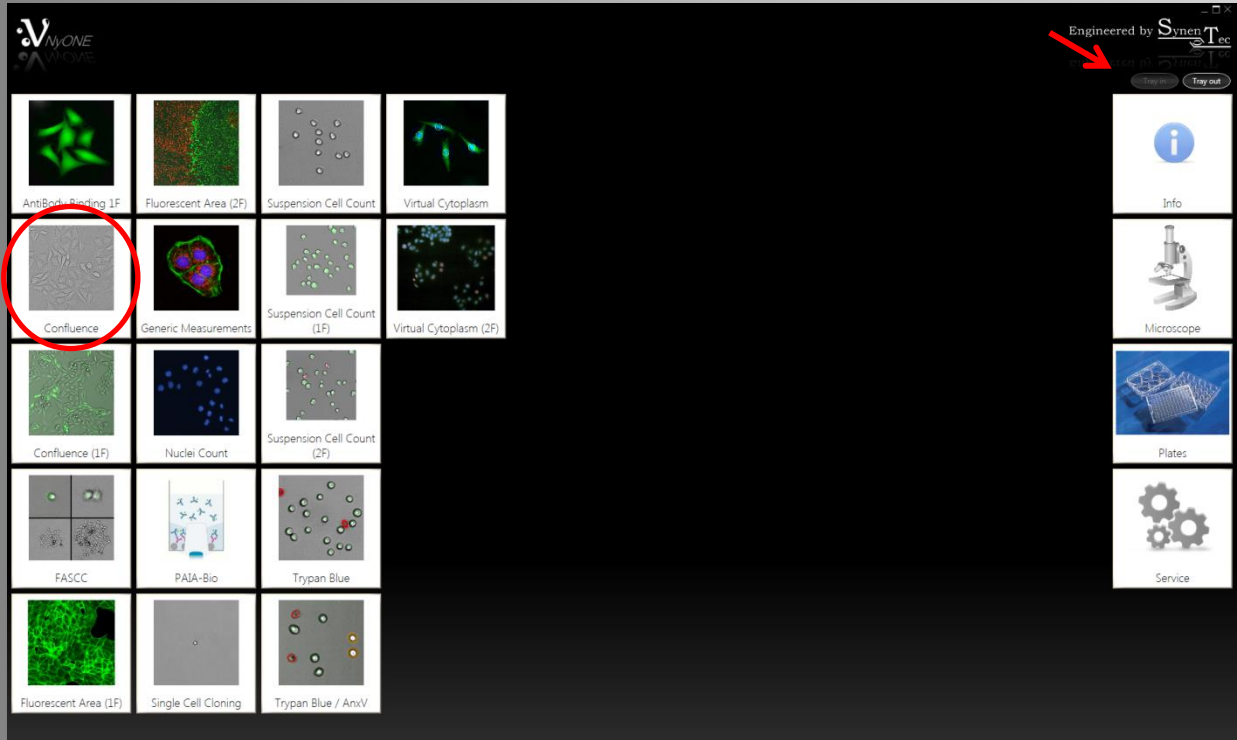


Fig. 3a: Main window with application logos (left) and special functions (right)



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 image your cells in the required well. 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).

In the channel area a brightfield channel is already added. In case that an additional channel is required, simply click on the 'Add' button. Complete the 'Source', 'Dichro' (only Cellavista®) and 'Emi' fields according to your requirements. For this experiment only one brightfield channel was used (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.

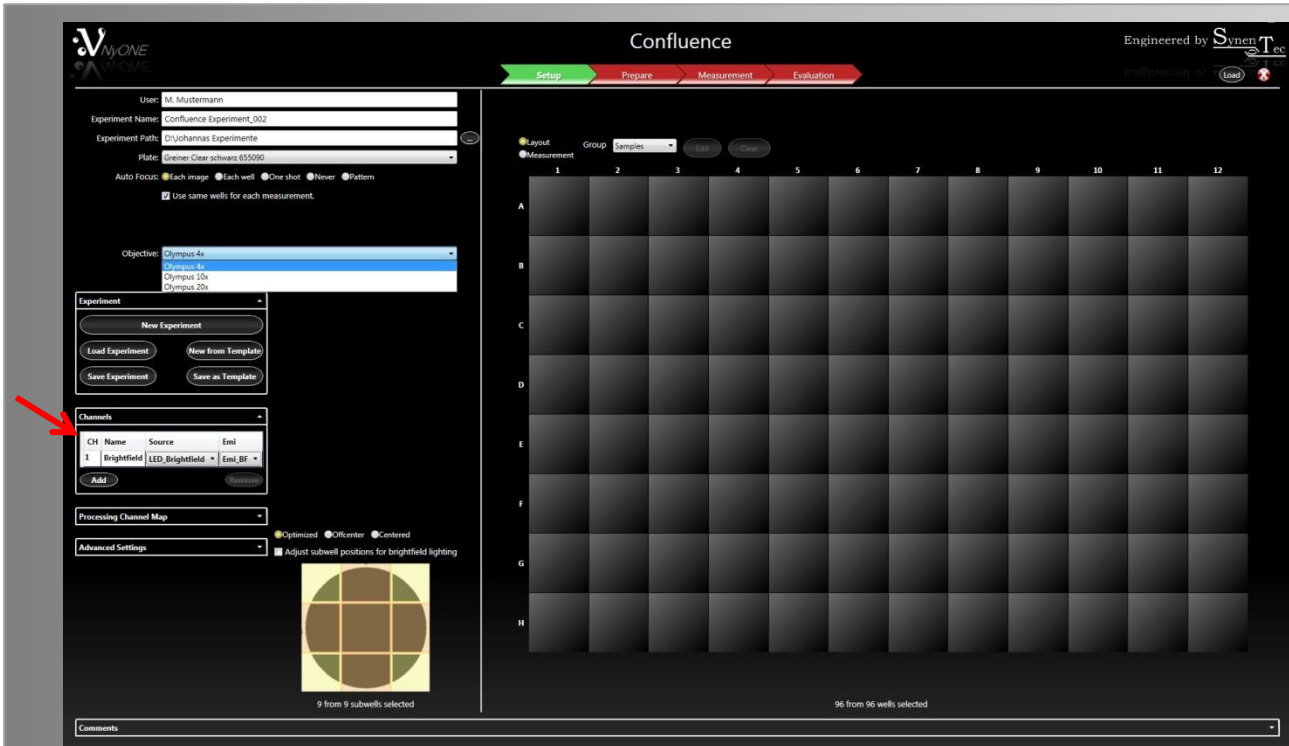


Fig. 4: Set up the Confluence experiment



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.

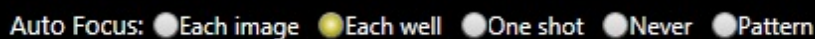


Fig. 6: Auto Focus settings

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

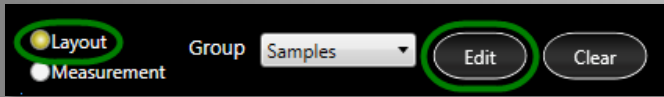


Fig. 7: Apply the plate layout

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.

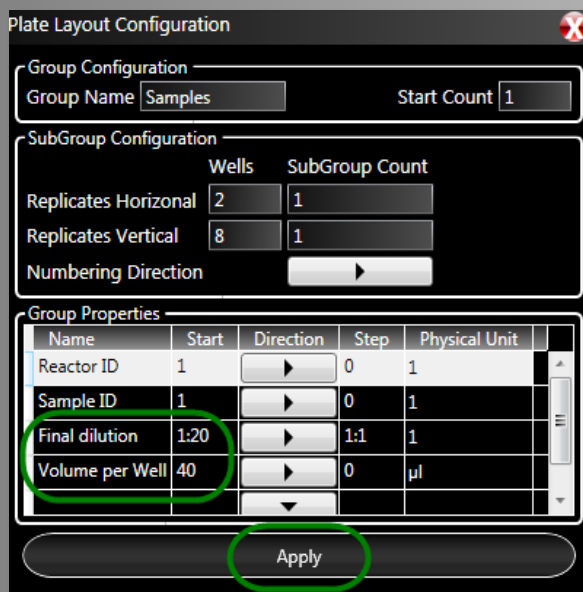


Fig. 8: Configure the plate layout
Enter your final cell dilution, the volume per well and e.g. the concentration of an added dye to save it for your experiment.

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 arrows

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

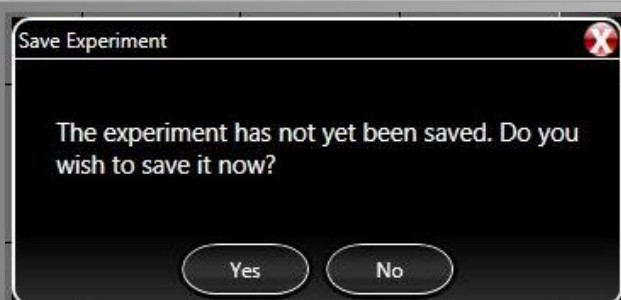


Fig. 10: Save Experiment

3. Optical Setup

3.1 Optical Settings

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 for the right optical settings (Fig.11).



Fig. 11: Start with an autofocus

The following illustrations will show you how to achieve good results.



Fig. 12: Image too dark

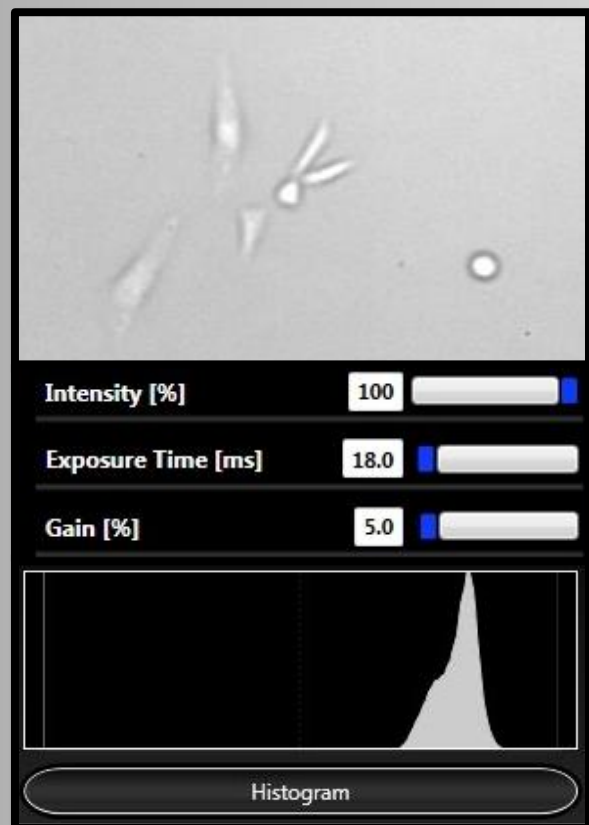


Fig. 13: Image too bright

In case that the image is either too dark or bright (Fig. 12 + 13), activate the 'Histogram' on the lower left side and use 'Liveview' while adjusting 'Exposure Time' and 'Gain' accordingly.

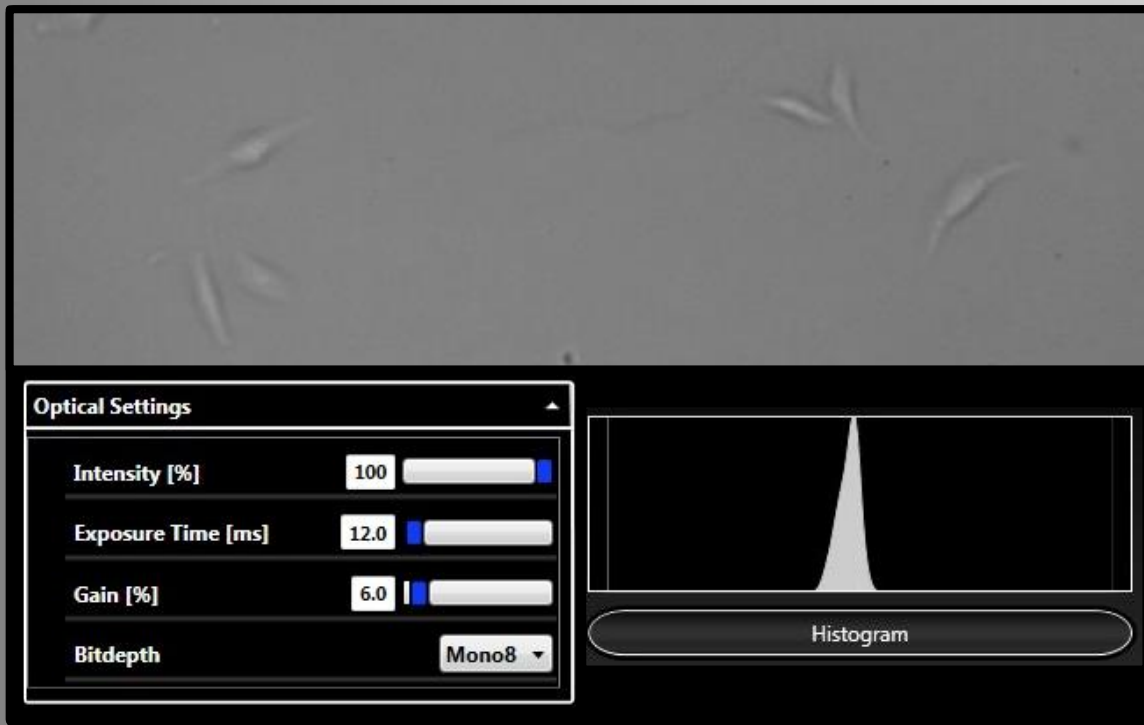


Fig. 14: Best Optical Settings for this example

Once the result is satisfactory deactivate 'Liveview' as its warmth will affect the condition of the cells and hide the histogram by clicking on the appropriate button. To cross check your settings, move the mouse cursor directly over the image while watching the 'Current grey value' in the upper left corner (Image Info – Fig. 15). A value at abt. 130 counts reflects good settings. Press 'Process Image' (lower left side of screen) to get an actual overview of your settings.

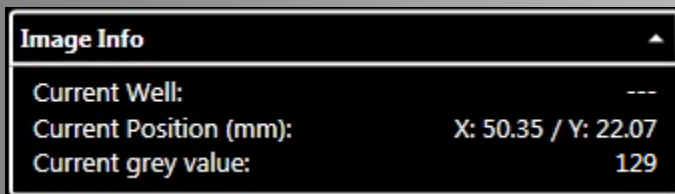


Fig. 15: Image Info – current grey value

Continue by adjusting the 'Focus Offset'.

3.2 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. 16). A good focus offset has been

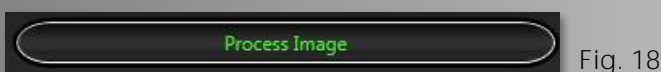
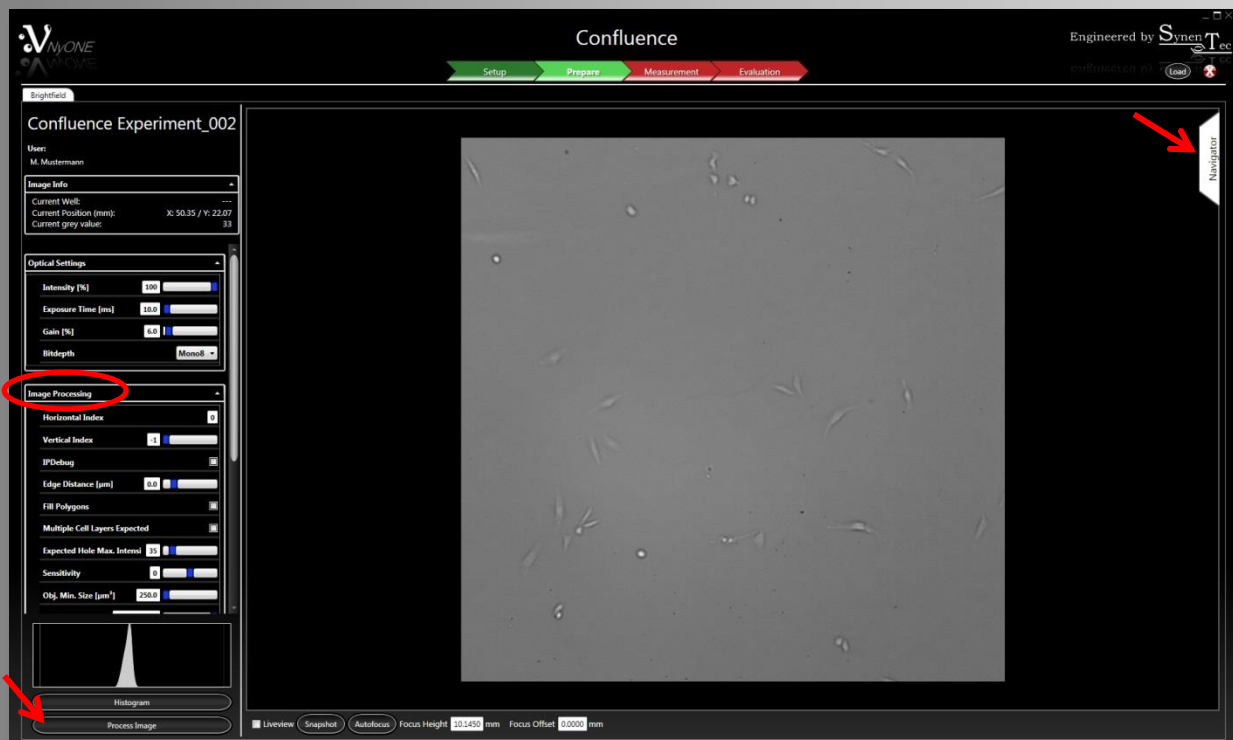
achieved when suspension cells appear bright inside and dark at the rim (Fig. 16), which is called the 'Lens Effect', and adherent cells are rich in contrast.

The chosen value will be kept for the measurement.



3.3 Testing 'Image Processing Parameter'

Once all optical settings have been completed and the required image quality was found, the image processing should now be tested (Fig. 17 - red ellipse). Click on 'Process Image' to test your settings and view the results (Fig. 17 - red arrow, lower left + Fig. 18). It is recommended to start using the Default parameters which in general ensure to meet the desired results.



In case the result is not satisfactory the Image Processing Parameters 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.

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. 17) can also be used to click through the different wells for checking the optical settings and the focal plane.

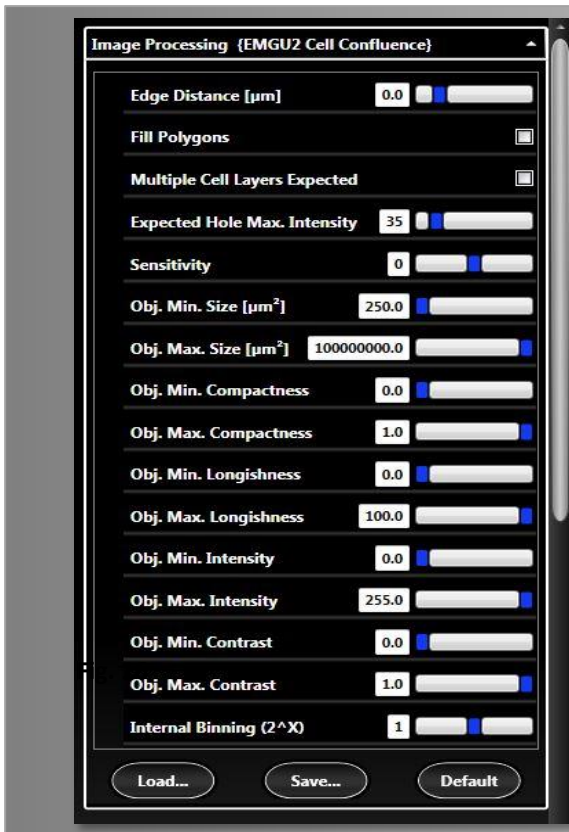


Fig. 19: Image Processing Parameters

Double-click in the middle of a particular object will open another window (Fig. 20) 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.

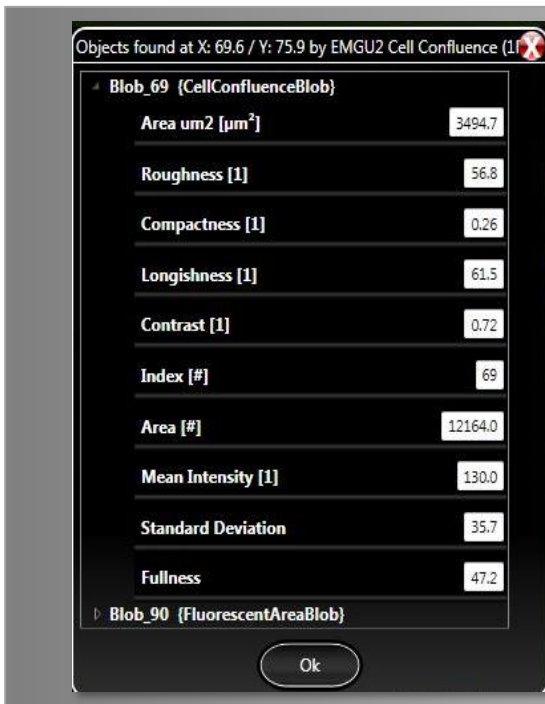


Fig. 20: Particular object

If the achieved results are satisfying, these parameters should be saved (Fig. 21).



Fig. 21

Now proceed with the Measurement (click – Fig. 22).

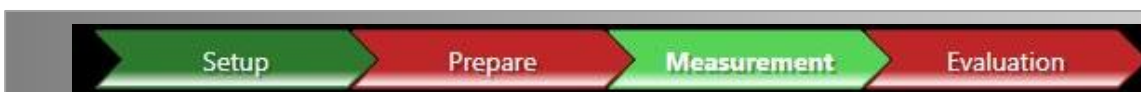


Fig. 22: Workflow arrows

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. 23).

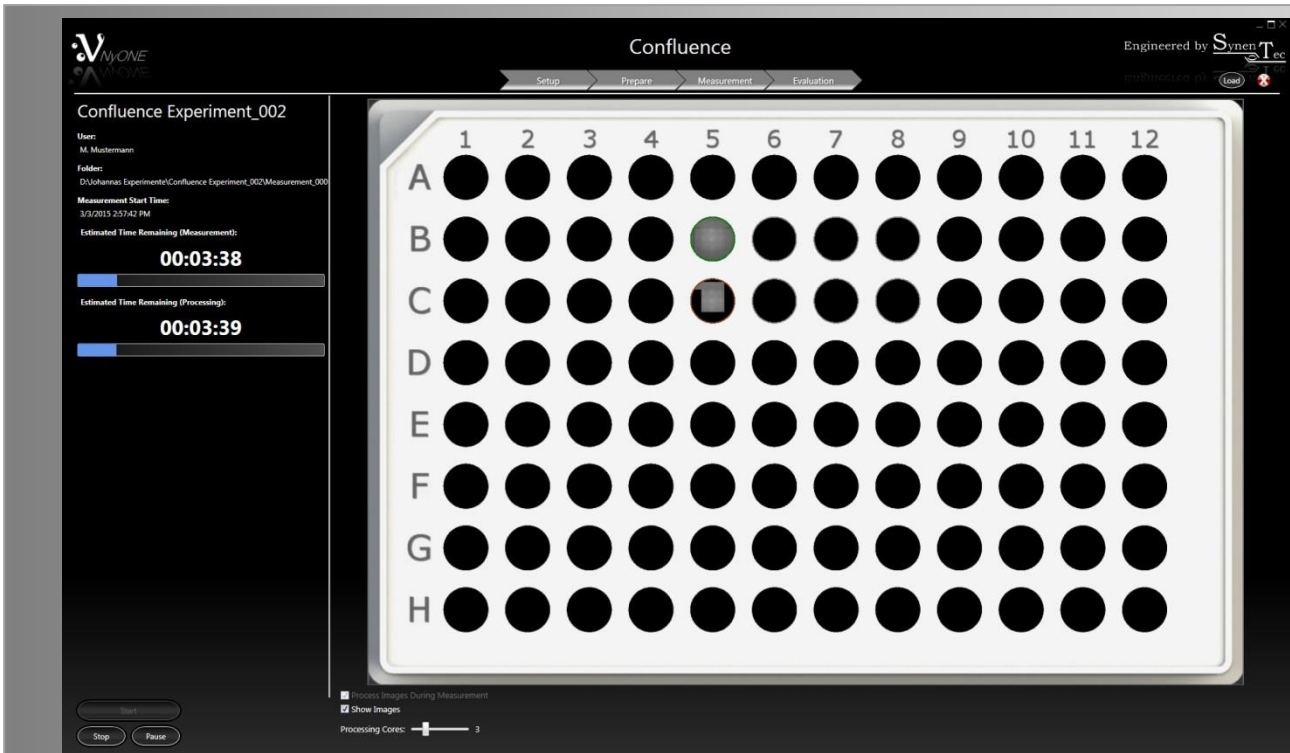


Fig. 23: Measuring process

The following box appears once the measurement is done (Fig. 24) and 'Ok' needs to be clicked.



Fig. 24: Measurement Done

Proceed with 'Evaluation' to view the results of your experiment by clicking on the corresponding arrow (Fig. 25).



Fig. 25: Workflow arrows

5. Evaluation

5.1 Result Presentations



Fig. 26: 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. Therefore, click on the 'Analyst' flag on the right (Fig. 26). For each type of result presentation many different properties like 'Cell Area' or 'Cell Confluence' can be chosen.

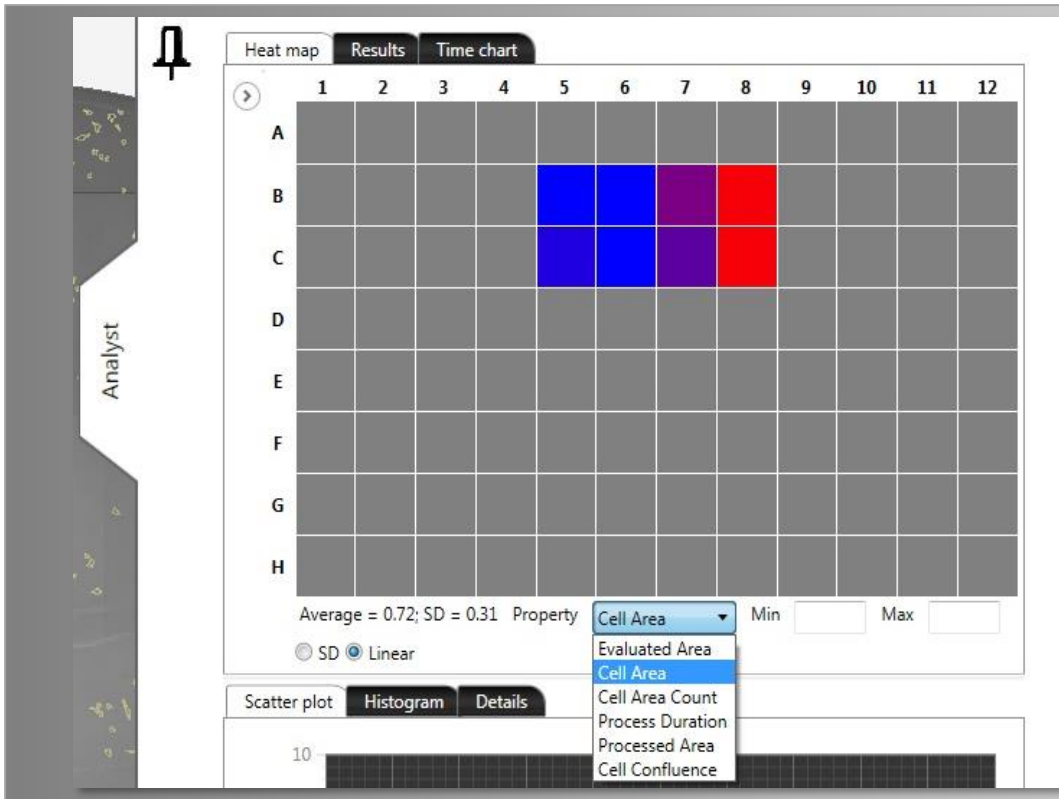


Fig. 27: Analyst with the variety of result presentations

The above seen different colors of the heat map represent 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.

The following data are supplied by the result table:

<i>Cell Area</i>	Area covered by cells
<i>Cell Area Count</i>	Number of separate cell clusters
<i>Cell Confluence</i>	Percentage of cell area and evaluated area

The time chart allows the measurement of cells over several days and also presents details for each well by simply clicking on the appropriate curve (Fig. 28).

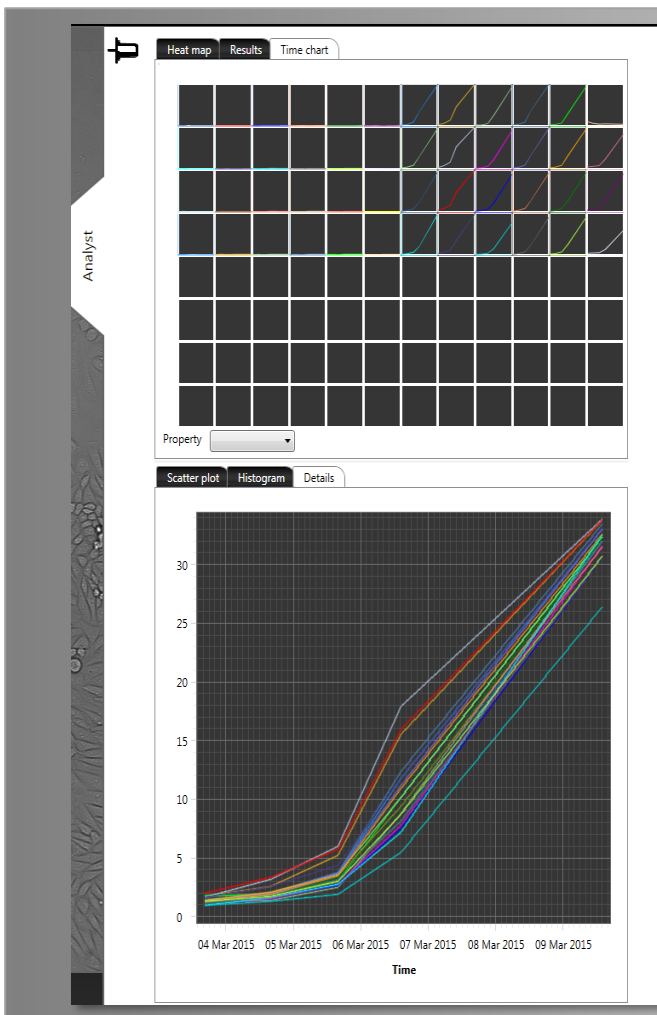


Fig. 28: Time chart
Single or several curves and fields may be selected by pressing 'Ctrl' and
a) clicking on the required curve/field
or b) dragging the mouse cursor to mark several fields at once.

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. 29).

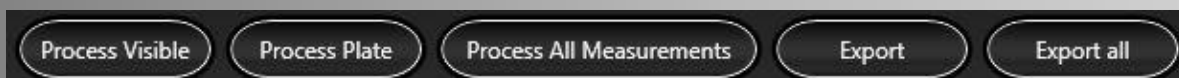


Fig. 29: Possible options after measurement is done

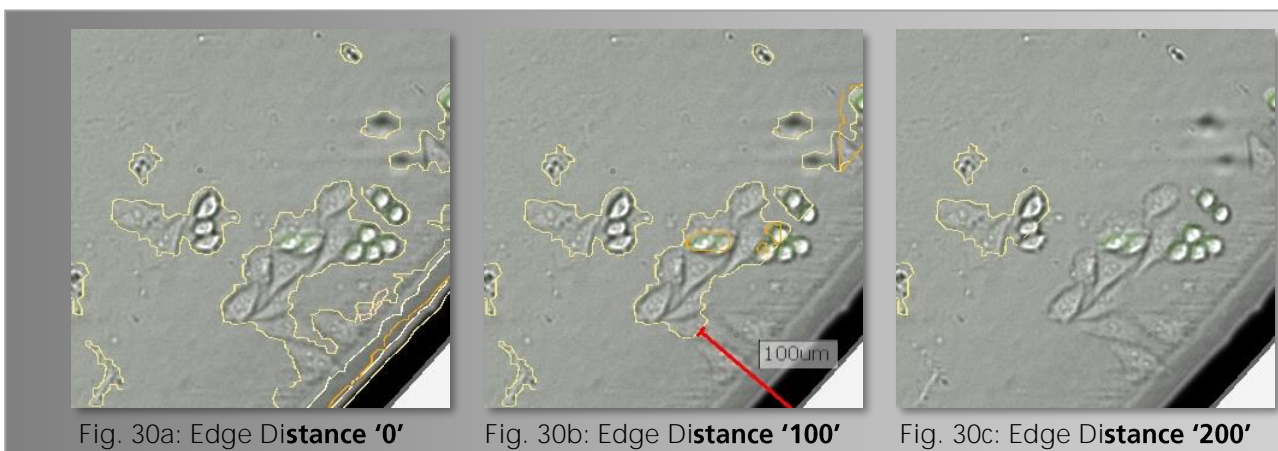
In case you are not satisfied with your results you can alter the image processing parameters accordingly, check same by clicking 'Process Visible' and further reprocess the whole plate by clicking on 'Process Plate' (Fig. 29).

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 application. Experiences have shown that the parameters 'Compactness', 'Longishness' and 'Contrast' lead to proper results with the default settings.

5.2 Image Processing Parameters

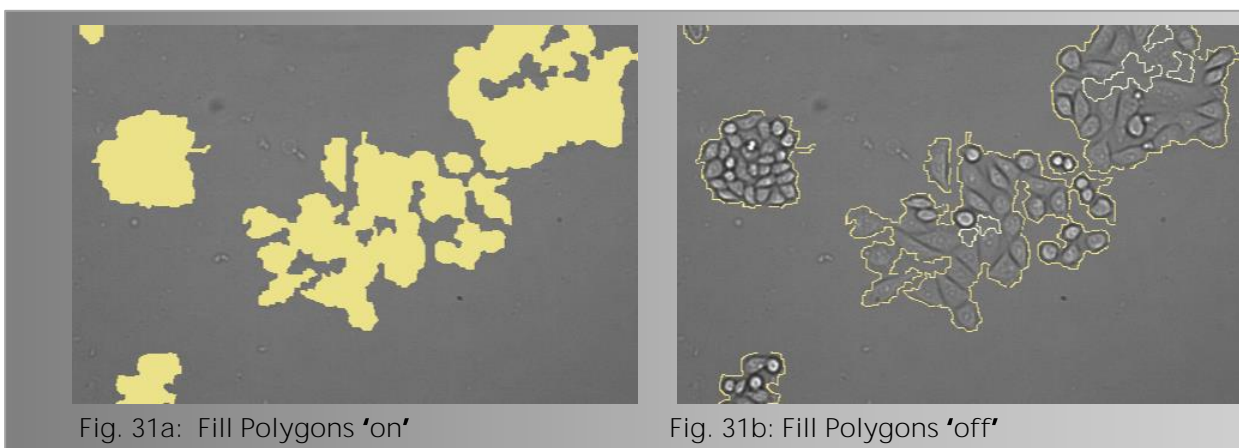
5.2.1 Edge Distance

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. 30a - c).



5.2.2 Fill Polygons

Fills detected cells entirely with color and facilitates their visualization (Fig. 31a + b).



5.2.3 Multiple Cell Layers Expected

Ensures that also cells lying on top of each other and therefore appear much darker will be detected.

5.2.4 Expected Hole Max. Intensity

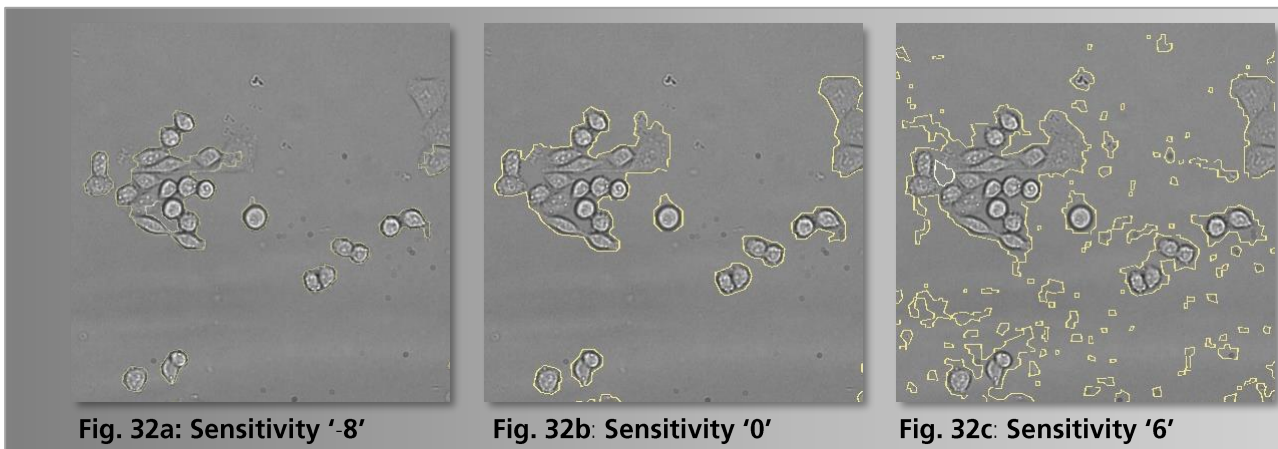
Defines the degree of darkness for detecting cells provided that 'Multiple Cell Layers Expected' has been marked.

5.2.5 Sensitivity

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 brightfield 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) can be excluded from the analysis up to a certain degree (Fig. 32a + 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. 32c).



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 and smaller cells (Fig. 33a – 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.

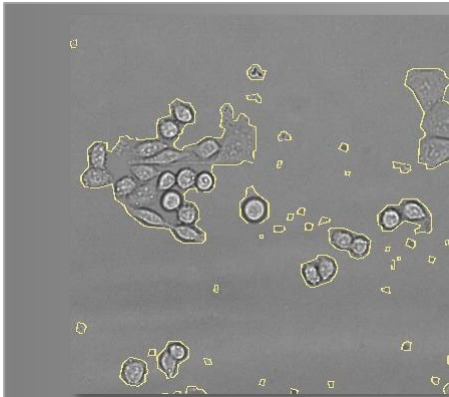


Fig. 33a: **Obj. Min. Size '0'**

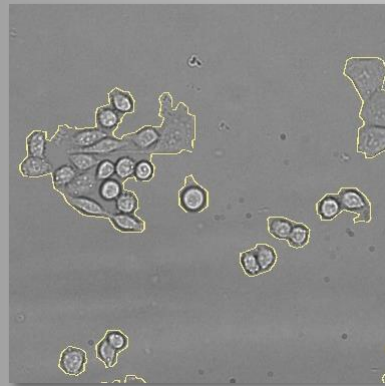


Fig. 33b: **Obj. Min. Size '200'**

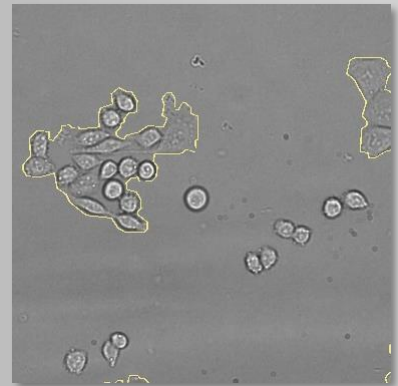


Fig. 33c: **Obj. Min. Size '1000'**

If 'Obj. Max. Size' is set on '100000000.0' even largest cells including merged cells will be detected (Fig. 34c) whereas a value of e.g. '100' and '5000' detects only smaller cells (Fig. 34a + b).

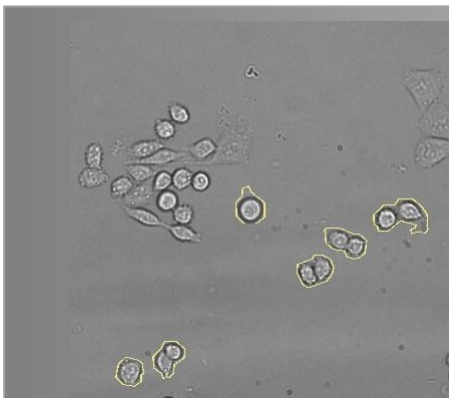


Fig. 34a: **Obj. Max. Size '1000'**

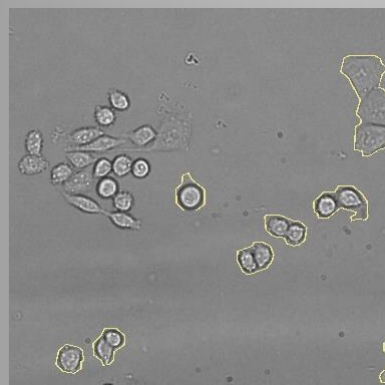


Fig. 34b: **Obj. Max. Size '5000'**

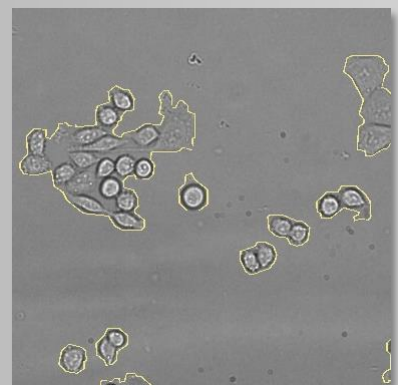


Fig. 34c: **Obj. Max. Size '100000000'**

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

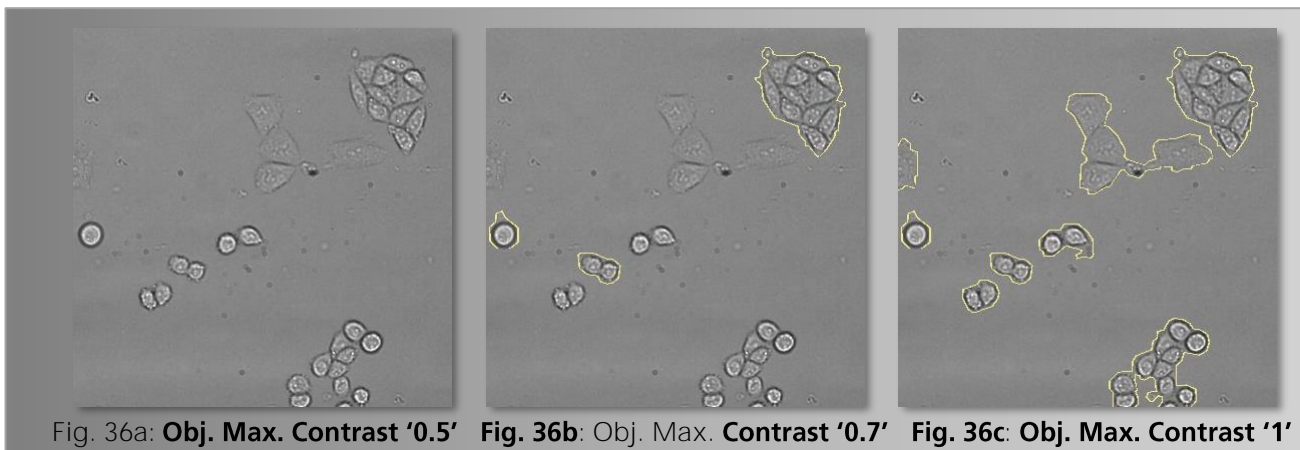
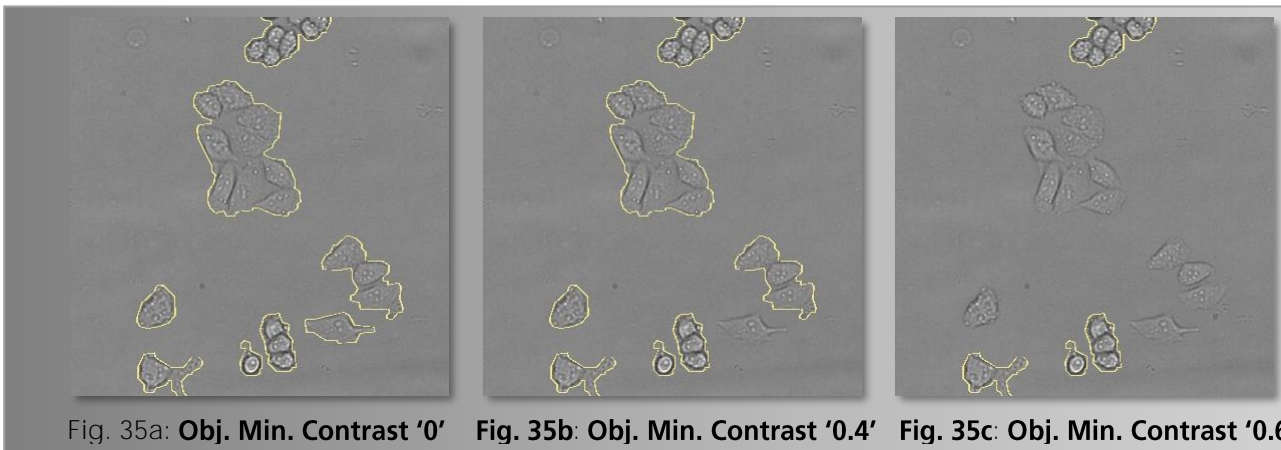
Describes the form of an object as well as the parameter 'Compactness'. Therefore an ellipse is determined which resembles a two-dimensional image of an object. The relation of the minor to the major axis of the ellipse as a percentage value results in the attribute 'Longishness'.

5.2.9 Obj. Min./Max. 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).

5.2.10 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 (Fig. 35a – c + 36a – c).



5.2.11 Internal Binning (2^2)

Defines the amount of pixels within a cell to be detected (e.g. 2 x 2 pixels shown as one). The processing will then be faster but also a little more imprecise.

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