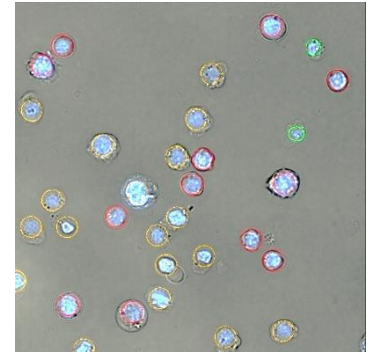# Experiment Guide – JC-1
Mito Potential Image Analysis

The purpose of this document is to guide the user through the *JC-1 Mito Potential* image analysis. It does not contain a complete procedure of setting up a JC-1 assay itself since this may vary due to customer requirements. It includes just a few tips to help optimizing your results. For a detailed protocol look at the application note AN-F342-XVII-09.

Content

1. Materials & Procedures

This chapter lists the equipment used to set up CELLAVISTA® or NYONE® for high quality apoptosis monitoring in combination with JC-1, a mitochondrial potential probe. As already mentioned the kind of equipment may vary on individual user requirements. The following items are suggestions, which have shown to achieve reliable results in an individual experimental set up.

1.1 Material

1.1.1 Liquids

- Cell suspension
- Complete Culture Medium
- Phosphate Buffered Saline (w/o Ca^{2+} & w/o Mg^{2+} (PBS^{-}))
- H_2O_2, 30 % stock-solution as positive control (e.g. Perhydrol®; MerckMillipore)
- JC-1 (e.g. 200 µM stock-solution in DMSO; Santa Cruz Biotechnology)
- Hoechst 33342 (e.g. 8.9 mM stock-solution in H_2O_{dest}; Santa Cruz Biotechnology)

1.1.2 Sample Carrier

The sample carrier that is used in this guide is supplied by Greiner Bio-One. It is a 96-well full area flat bottom plate, with black walls and clear foil bottom (µclear®; cat. # 655090). Black plates are usually recommended for fluorescence microscopy.

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.

1.1.3 Centrifuge

It is required to spin down the samples to ensure that all cells are in the focal plane. Fill your sample carrier (e.g. 100 μL/well of a 96-well plate) and apply it to a centrifuge. A swing out rotor is required to guarantee a fast sedimentation process with a uniform distribution of the cells. Setup the centrifuge to ca. 30 x g and 1 minute spinning time. Furthermore it is required to use maximum acceleration and maximum deceleration level (centrifuge brake) to optimize uniform distribution.

1.2 Tips for Assay Procedure

1. Usually the JC-1 concentration is between 0.25 and 2 μM
2. The assay works with final cell concentrations up to 2.5 10^5 cells/mL (viability of min. 90 % is recommended). If you prefer a much higher cell concentration in your initial cell suspension, apply a 1:20 dilution with PBS⁻⁻. When diluting, mix well but gently. Other dilution factors are possible, depending on your expected initial cell concentration of your culture.
3. Warning: a too low cell concentration increases the systematic error and gives no indication of your total cell population!
4. All incubation steps can be done in full culture medium
5. Make sure that all liquids have room temperature as minimum. Pre-incubation at 37 °C is not mandatory but can also be used.
6. After adding the dyes protect the sample/plate from light.
7. Pipet a minimum of 80 μL of that mixture into a well of a 96-well full area microplate.
8. Prepare 1 well, for example with a cell-sample, with half the concentration of the toxin to prepare your optical settings. Thus the bleaching effect of the optical preparation does not affect your real samples and results. This well can be outside the measured range.
9. Centrifuge the plate for one minute at 30 x g in the swing-out rotor with maximum acceleration and deceleration (brake).

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!

SYNENTEC

2. Experiment Setup

There are many ways to measure an experiment and the associated plate. In this guide we recommend a subwell configuration of the inner 9 (fig. 1), which achieves high accuracy for determining the viability (refer to (CELLAVISTA®/NYONE® Operating Guide for subwell selection).
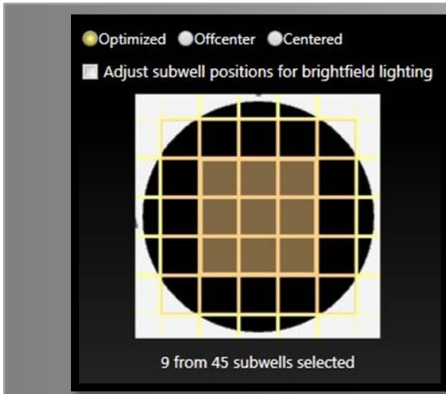

Fig. 1: Subwell selection.

To transfer the plate layout to your experiment, check the "Layout" box and press "Edit" (fig. 2).
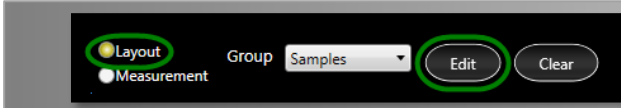

Fig. 2: Apply the plate layout.

A Dialog appears where you have to fill in the "Final Dilution" and the "Volume per Well" (fig. 3).
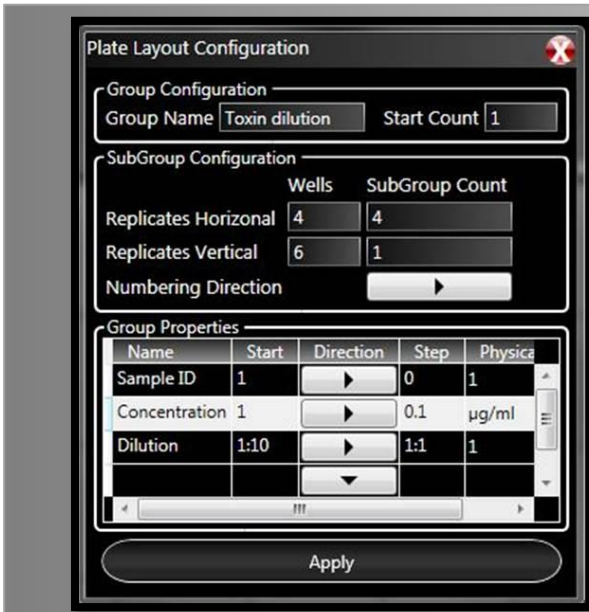

Fig. 3: Configure the plate layout.

Please refer to the Instruments Operating Guides to get more information about setting up an experiment.

One of the decisive settings for a JC-1 application is the excitation of the different stainings as well as the position of the appropriate focal plane. The following illustrations shows how to achieve good results.

3.1 Focal Plane & Brightness

Setting up the focal plane in the first detection channel – the Hoechst channel - is a crucial step in this application. A sharp image is needed to distinguish between two different nuclei which lie close together. Otherwise the blurring let two or more nuclei appear maybe as one. The following images will illustrate this mechanism (fig. 4).

As well as the focal plane has to be adjusted, the illumination of the image has to be balanced to supply the best image for the image analysis algorithm. Attention: the histogram on the lower left panel is just for brightfield image adjustments (fig. 5-7)!

Fig. 4: Wrong focal plane. Signal from each nucleus is blurred and weak in contrast.

Fig. 5: Too dark. Increasing exposure time or gain may help. Or modify staining protocol. Only the brightest objects are visible.

Fig. 6: Too bright. Decreasing exposure time or gain may help. Or modify staining protocol.

Fig. 7: Ideal illumination, staining and focal plane. No high background, most of the objects have an intensity around 130 grey-scale, objects are sharp and rich in contrast.

3.2 Recommended channel settings

The following information for optical settings is based on the protocol specified in the application note AN-F342-XVII-11 and is merely a recommendation! The respective focus offset can take a positive or negative value for the different channels and must be adjusted according to stipulations shown in chapter 3.1.

Hoechst-channel:		JC-green channel:		JC-red channel:	
Intensity	100%	Intensity	100%	Intensity	100%
Exp. Time	20 ms	Exp. Time	150 ms	Exp. Time	50 ms
Gain	50 %	Gain	30 %	Gain	50 %

This chapter guides through the image processing/analysis with its settings and how to achieve the best results for your cell line. Here are only parameters listed, which need to be modified frequently. Experiences have shown that the parameters "Compactness", "Longishness" and "Contrast" lead to proper results with the default settings.

4.1   Edge Distance [µm]

In case of unusual occasions it is useful to measure the whole well. To ensure that only cells are counted and that well edge effects will not falsify the results, the image analysis operator will provide a parameter called "Edge Distance". The higher the value of edge distance the less area is evaluated. Setting "Edge Distance" to 100 µm excludes operator results within a distance of 100 µm of the well edge.
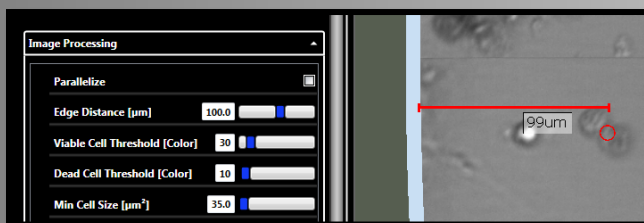
Fig. 8: Edge Distance set to 100 µm.
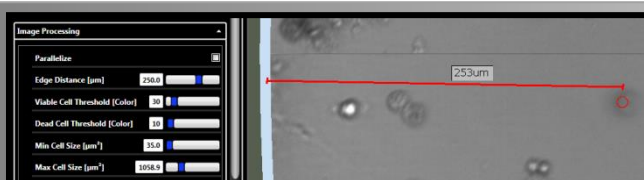
Fig. 9: Edge Distance set to 250 µm.

4.2   Sensitivity

The parameter 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.

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.

Whereas 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.
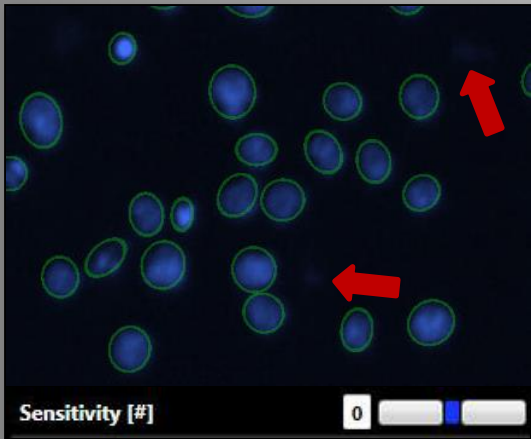
Examples:

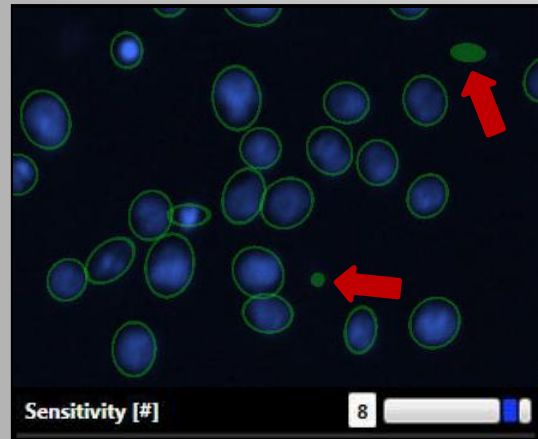Fig. 10: Sensitivity 0
The weak visible object is unrecognized.

Fig. 11: Sensitivity 8
The weak visible object is detected.

4.3 Nuclei Region Merging [μm^2]

This parameter defines how close objects need to be together for counting them as one nucleus. The higher this parameter is the more segmented neighboring regions shall be detected as one nucleus.
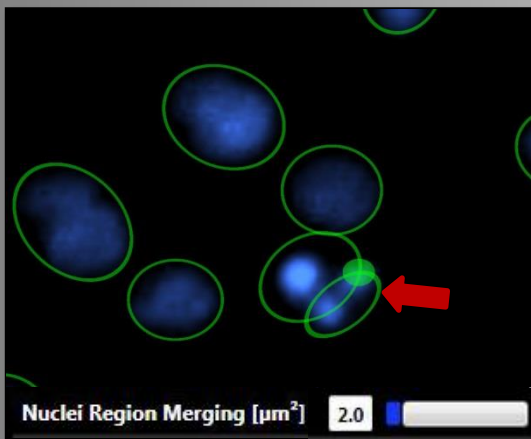

Fig. 12: Nuclei Region Merging 2.0 μm^2.
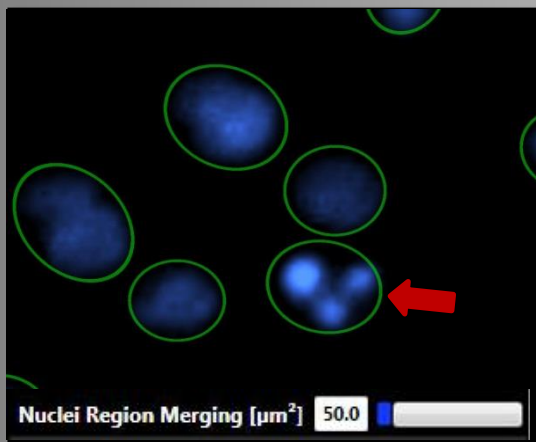In this case a fragmented Nucleus is counted as 3 nuclei.

Fig. 13: Nuclei Region Merging 50.0 µm². Objects in a radius of 50 µm² are counted as one nucleus. This value is close to the default value of 20, which is suitable for most samples.
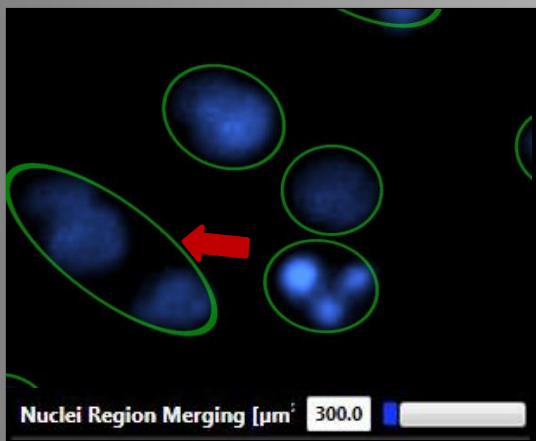

Fig. 14: Nuclei Region Merging 300.0 µm². Objects in a radius of 300 µm² are counted as one nucleus.

4.4 Object Attribute Parameter

In addition to the objects to be detected (cells), several attributes are calculated, which characterize the objects more precisely. These attributes can be used to exclude undesirable objects (e.g., Debris) from detection in difficult situations.

An object is only considered as "recognized" or as a cell to be counted if all attribute parameters are met in the range set by the user. The borders of an attribute are defined by the parameters "Obj. Min [attribute]" or ". Obj. Max [attribute]" besides which the most important attribute is the object size [Size].

4.4.1 Obj. Min. Intensity BC and Obj. Max. Intensity BC

The attribute "Intensity" is the average grey-value of the object in the image. When using pictures with 8-bit a maximum range of 0 to 255 arises (background corrected minimum intensity of a single object that shall be detected) (16-bit corresponds to 65536 grayscales).
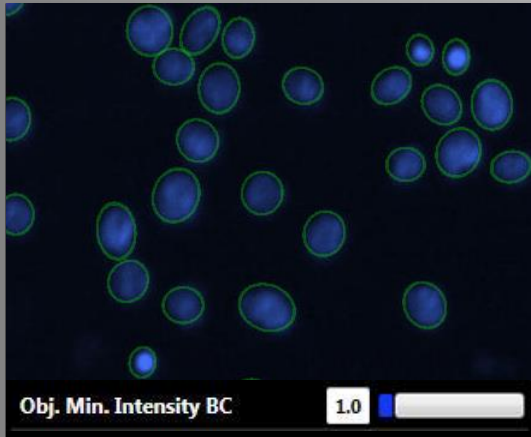
Examples for Obj. Min. Intensity BC (default 1.0):

Fig. 15: Obj. Min. Intensity BC=1
The default value; all objects with a background corrected grey-value at a minimum of 1 are detected.
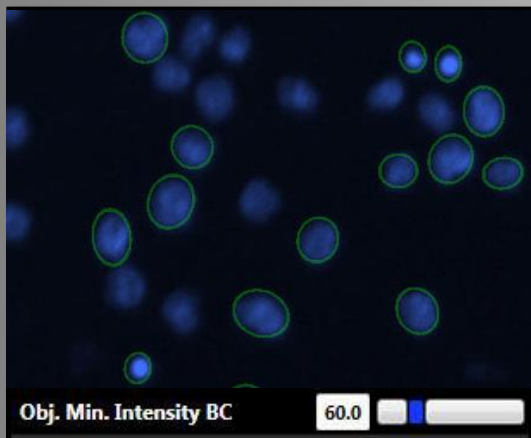

Fig. 16: Obj. Min. Intensity BC = 60.
All objects with a background corrected grey-value at a minimum of 60 or brighter will be detected.
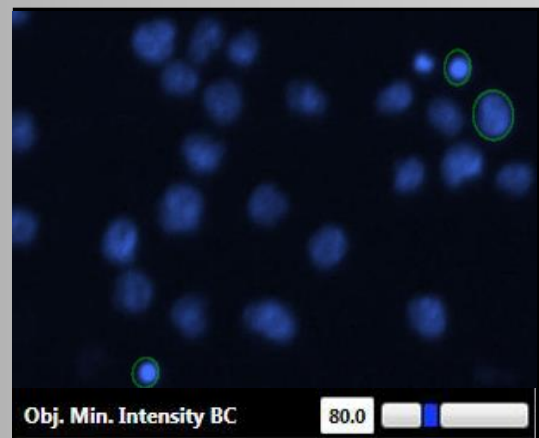

Fig. 17: Obj. Min. Intensity BC = 80.
All objects with a background corrected grey-value at a minimum of 80 or brighter will be detected.
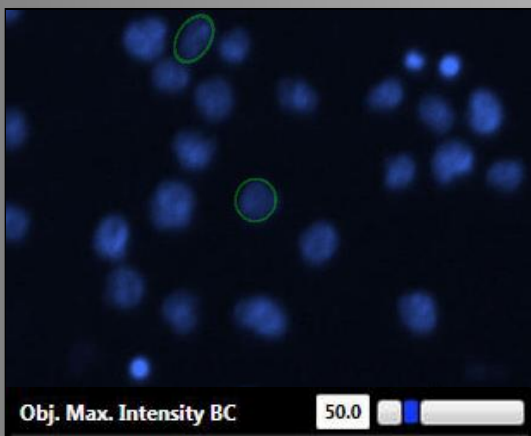
Examples for Obj. Max. Intensity BC (default 255.0):

Fig. 18: Obj. Max. Intensity BC = 50.
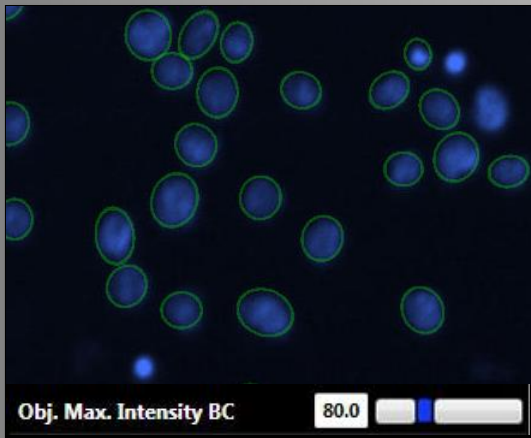All objects with a maximum background corrected grey-value of 50 are detected.

Fig. 19: Obj. Max. Intensity BC = 80.
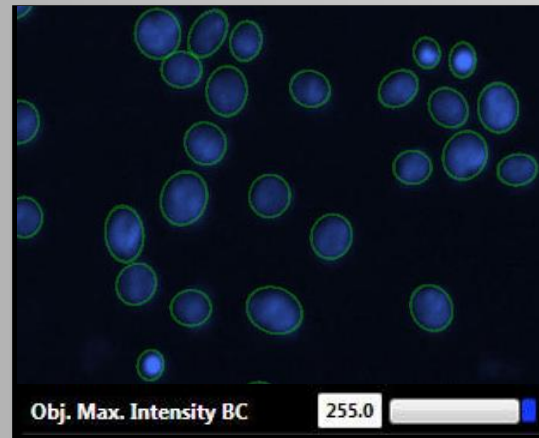All objects with a background corrected grey-value of 80 or darker are detected.

Fig. 20: Obj. Max. Intensity BC = 255.
The default value; all objects with a maximum background corrected grey-value of 255 or darker are recognized.

4.4.2 Obj. Min. Size and Obj. Max. Size [µm²]

With the parameters "Obj. Min Size" and "Obj. Max Size" the range is set for the allowed size of the objects to be detected. Depending on the cell line and cultural state, the parameter "Obj Min Size" is especially useful to exclude debris (=minimum area size of a single object, which shall be detected).

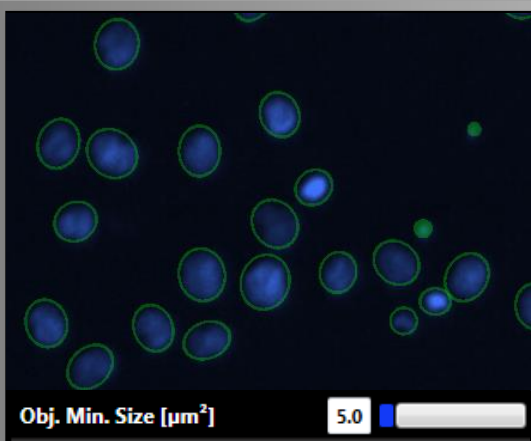Examples for Obj. Min. Size (default 5.0):

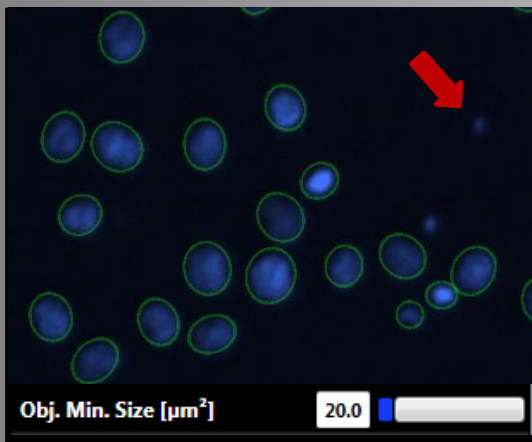Fig. 21: Obj. Min. Size = 5.
All objects are detected.

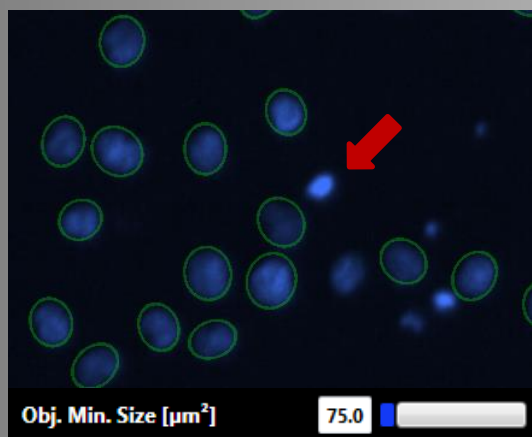Fig. 22: Obj. Min. Size = 20.
Some objects are missed.

Fig. 23: Obj. Min. Size = 75.
Many objects (smaller than 75 µm^2)
are not detected.
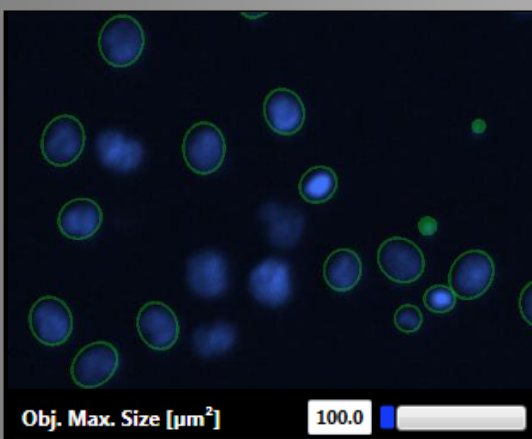
Examples for Obj. Max. Size (default 350.0):

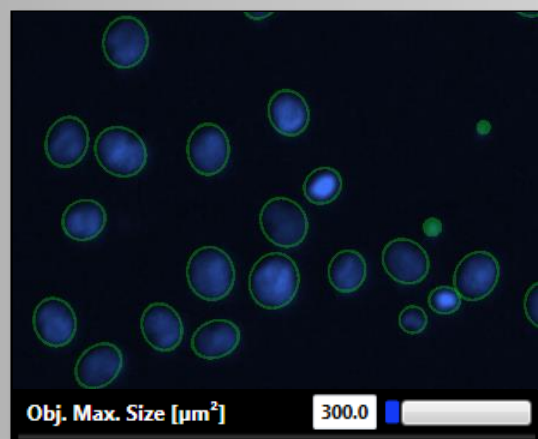Fig. 24: Obj. Max. Size = 100.
Some objects are missed.

Fig. 25: Obj. Max. Size = 300.
All nuclei in this image are detected.

4.4.3 Parameters for the JC-1 fluorescence detection

There are three parameters for the detection of the fluorescence channels – "Circle Diameter", "Fluo "Threshold BC" and "Binding Factor". These three parameters interact and their correct settings are important for the perfect detection of JC-dyes and the discrimination between living (JC-red) and apoptotic/dead cells (JC-green).

Circle Diameter [μm] *(exemplarily for JC-red channel, the green channel works in the same way):* This parameter determines the size of the evaluated area starting from the center of the object found in the brightfield channel. The "Circle Diameter" indicates in which area the parameters "Fluo Threshold BC" and "binding factor" should be applied. It needs to be defined seperately for the fluorescence channels 1 and 2. The range is 0.0 – 50.0, the default setting is 10.5.
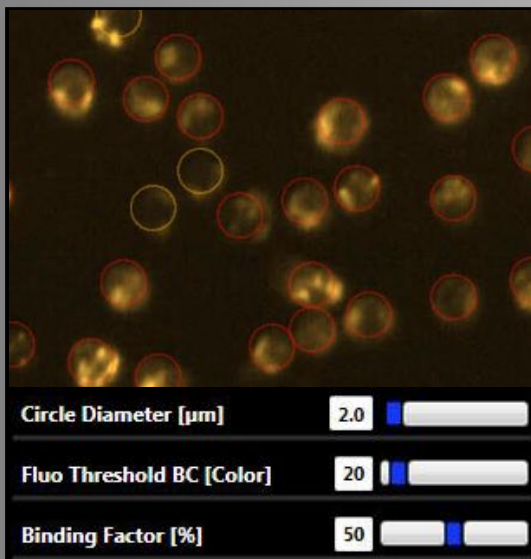
Examples:

Fig. 26: Circle Diameter = 2.0. This means that in an area of 2.0 microns, 50 % of all pixels must have a minimum grey-value of 30 (background corrected), as only then the objects are detected as JC-red (viable; red circles).
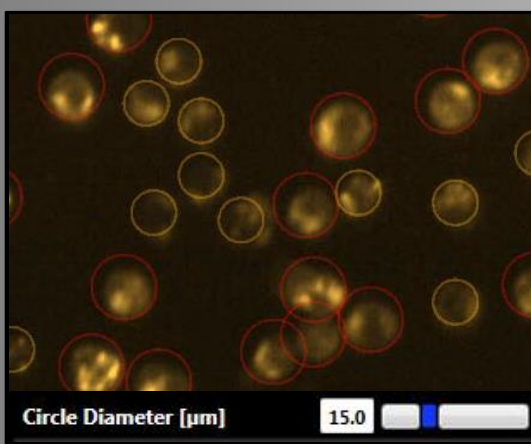

Fig. 27: Circle Diameter = 15.0. In a diameter of 15.0 μm the object needs to fulfill the definitions of "Fluo Threshold" and "Binding Factor" is to be marked as JC-red (viable; red circles). Otherwise it is counted as JC-green (orange circles).
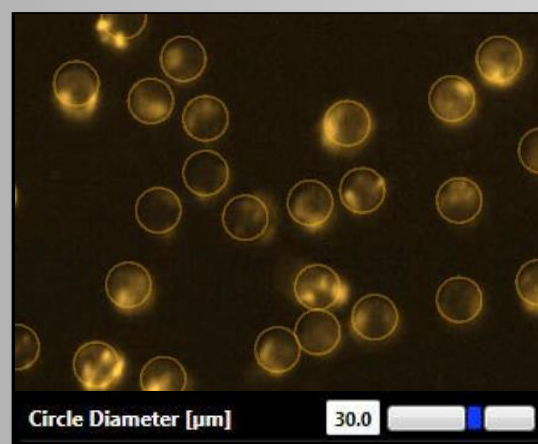

Fig. 28: Circle Diameter = 30.0. In this image are no objects, which fulfill the definitions of "Fluo Threshold" and "Binding Factor" in a "Circle Diameter"of 30, marked as JC-red. All objects are counted as JC-green (orange circles).

Fluo Threshold [Colour] (here: the JC-red channel)
The Fluo Threshold specifies the minimum grey-scale value (above background) which the pixel must have to be detected within the "Circle Diameter" as JC-red. The range is between 1 – 255 and the default setting is 6.

Examples:

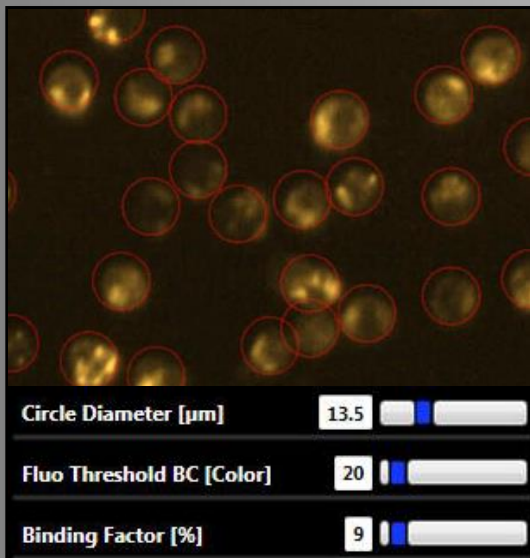| Circle Diameter [µm] | 13.5 |
| Fluo Threshold BC [Color] | 20 |
| Binding Factor [%] | 9 |

Fig. 29: Fluo Threshold BC = 20. This setting means that in a diameter of 13.5 microns 9 % of all pixels must have a minimum grey-value of 20 (background corrected). Only then the objects are detected as JC-red (viable; red circles).
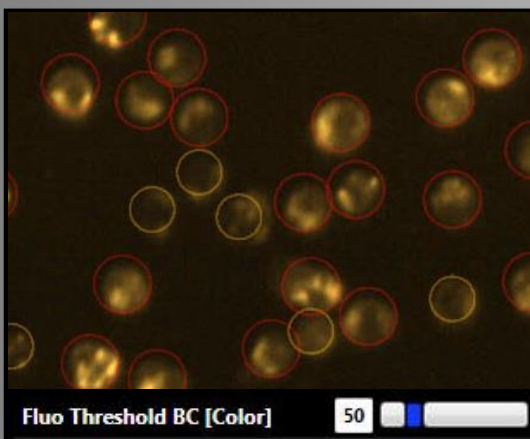

| Fluo Threshold BC [Color] | 50 |

Fig. 30: Fluo Threshold BC = 50.0. In the required diameter the desired amount of pixels need to have a background corrected minimum grey-value of 50, to be marked as JC-red (viable; red circles). Otherwise they are marked as JC-green (orange circles).
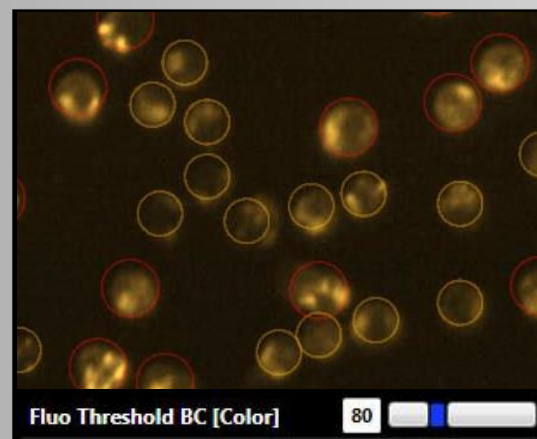

| Fluo Threshold BC [Color] | 80 |

Fig. 31: Fluo Threshold BC = 80.0. In the required diameter the desired amount of pixels need to have a background corrected minimum grey-value of 80, to be marked as JC-red (viable; red circles). Otherwise they are marked as JC-green (orange circles).

Binding Factor [%] (here: the JC-red channel)
As described before, this value determines the amount of pixels to be detected according to the required and set "Circle Diameter" and "Fluo Threhshold". The range is 1 – 100 % and the default value is 7 %.
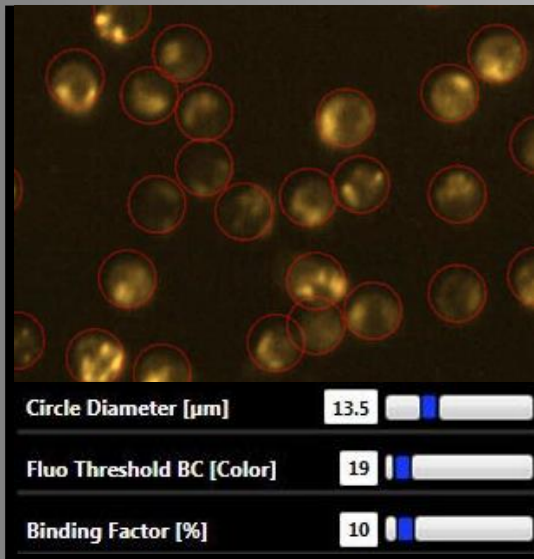
Examples:

Fig. 32: Binding Factor = 10.
With these settings all cells with 10 % of pixels and a minimum grey-value of 19 (background corrected) in a diameter of 13.5 are detected as JC-red (viable; red circles).
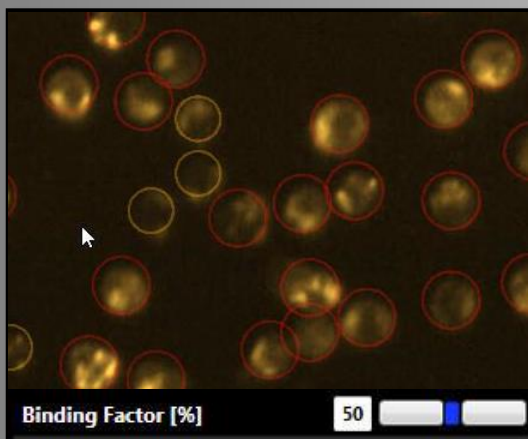

Fig. 33: Binding Factor = 50.0.
In the desired diameter 50 % of the pixels need to have the desired Threshold to be marked as JC-red (viable; red circles). Otherwise they are marked as JC-green (orange circles).
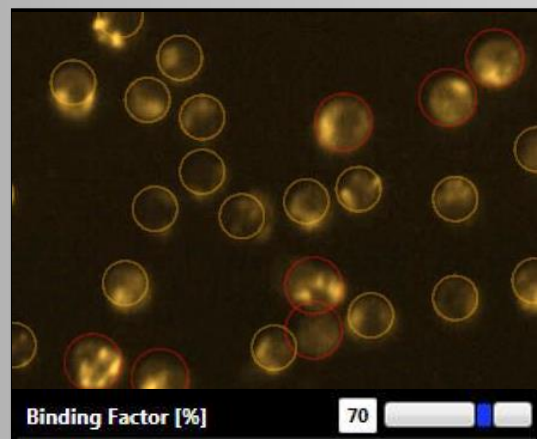

Fig. 34: Binding Factor = 70.0.
In the desired diameter 70 % of the pixels need to have the required Threshold to be marked as JC-red (viable; red circles). Otherwise they are marked as JC-green (orange circles). There are only a few JC-red stained objects left that fulfil these settings.

4.5 Result table

All relevant results are listed below and are to be found in the result-table, visible in the right tab of the "Evalution" window of the YT-software®. These results can be exported as a CSV file using the Export button.

Nuclei Count:	Sum of all detected Cells
JC-green positiv:	% of Cells containing JC-green fluorescence
JC-red positiv:	% of Cells containing JC-red fluorescence
TC-nn:	Total cells unstained
TC-pn:	Total cells stained with JC-green or stained with JC-green AND JC-red
TC-np:	Total cells with no JC-green staining; only stained with JC-red
TC-pp:	Total cells stained with JC-green AND stained with JC-green
Ratio red:green	Ratio between JC-red and JC-green stained cells

4.6 Circle colors

The detected objects within an image are marked with circles. Each color of the circles is a coding for a different population within the total cell population. When using the *JC-1 Mito Potential* operator the following color codes apply:

Marked green	= (TC-nn) Hoechst staining only
Marked orange	= (TC-pn / TC-pp) Hoechst staining AND JC-green staining, an additional JC-red staining is possible (apoptotic/dead cells; loss of mitochondrial membrane potential)
Marked red	= (TC-np) nuclei staining AND JC-red staining (viable cells)