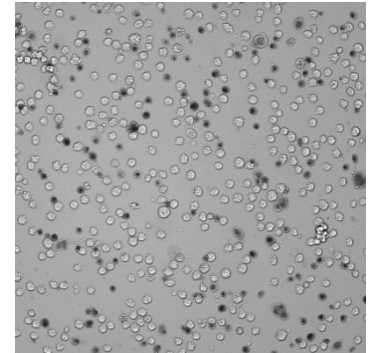


Experiment Guide – Trypan Blue Image Analysis

The purpose of this document is to guide the user through the Trypan Blue Image analysis. It does not contain any procedures of setting up a Trypan Blue assay itself since this may vary due to customer requirements.



Technical Note
TN-B004-XVII-19

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1. Materials & Procedures

This chapter lists the equipment used to set up NYONE® for high throughput viability monitoring using Trypan Blue. 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 Liquids

- Cell suspension
- PBS (w/o Ca^{2+} w/o Mg^{2+}) (e.g. Biochrom)
- Trypan Blue 0.02 % w/v in PBS (e.g. made of Gibco 0.4 % stock solution)

1.1.2 Sample Carrier

The sample carrier that is used in this Guide is supplied by Corning Costar. It is a 96-well half area flat bottom plate, Catalog number 3695 or other suitable half area plates (e.g. corning black walled).

Refer to:

http://www.corning.com/lifesciences/us_canada/en/technical_resources/product_guid/eq_cp/drawings.aspx

Handling of the sample carrier: The clear bottom of the sample carrier must never be touched with fingers (even not 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

To achieve high throughput and to ensure equal distribution of the cells in the well it is required to spin down the samples. Aspirate your suspension cells/Trypan Blue solvent in a sample carrier and apply this 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 spin time. Furthermore it is required to use acceleration and deceleration at a maximum (centrifuge brake) to optimize a uniform distribution.

1.2 Procedure

1. Make sure that all liquids have room temperature at minimum. Pre-incubation at 37 °C is not mandatory but can also be used.
2. The assay works with final cell concentrations up to $2.5 \cdot 10^5$ cells/mL. Apply a 1:20 dilution with PBS⁻ if you prefer a much higher cell concentration in your initial cell suspension. When diluting, mix well but gently. Other dilution factors are possible, depending on your expected initial cell concentration of your culture.
3. Pipet a minimum of 40 µL of the diluted cell suspension into a vessel for mixing e.g. an e-cap or an ordinary microtiter plate with sufficient volume capacity.
4. Add the same volume as used in point 3 (min. 40 µL) of 0.02 % Trypan Blue solution into each vessel containing cells and gently mix by pipetting for several times. This step is a further 1:2 dilution of your cell sample. Ensure that pipetting is done gently.
5. Pipet 40 µL of that mixture into a half area microtiter plate in one step and ensure that no bubbles or droplets form inside the wells.
6. Centrifuge the plate for one minute at 30 x g in the swing-out rotor with maximum acceleration and deceleration (brake).
7. Remove the plate carefully out of the centrifuge and incubate the mixture for 5 minutes at room temperature to ensure homogeneous Trypan Blue staining.
8. Place it into the sample holder of the NYONE® imager.
9. The measurement should be done within one hour after Trypan Blue addition. The cells remain stable during this period due to the low Trypan Blue concentration used in this assay.

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

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 four, which achieves high accuracy for determining the viability (refer to NYONE® Operating Guide for subwell selection).

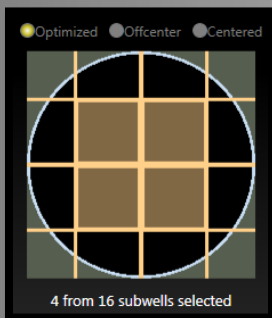


Fig. 1: Subwell selection
The number of subwells depends on the well size and the magnification.

To transfer the plate layout to your experiment, check the “Layout” box and press “Edit”

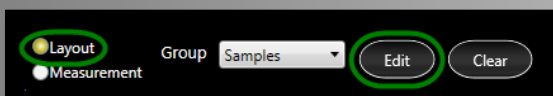


Fig. 2: Apply the plate layout
This enables to define the cell dilution and the volume per well.

A Dialog appears where you have to fill in the “Final Dilution” and the “Volume per Well”

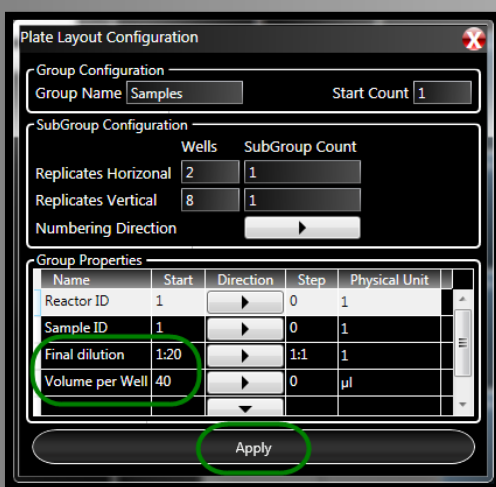


Fig. 3: Set the experiment attributes
Based on this values and the performed measurement the YT-software® can determine the exact cell number/well.

Please refer to the NYONE® Operating Guide to get more information for setting up an experiment.

3. Optical Setup

One of the decisive settings for a Trypan Blue application is the position of the appropriate focal plane as well as the illumination of the sample.

The following illustrations will show you how to achieve this.

3.1 Focal Plane

Setting up the focal plane is a crucial task in this application. A significant lens effect has to be achieved (for viable cells only) to distinguish between viable and dead cells. The following images will illustrate this mechanism.

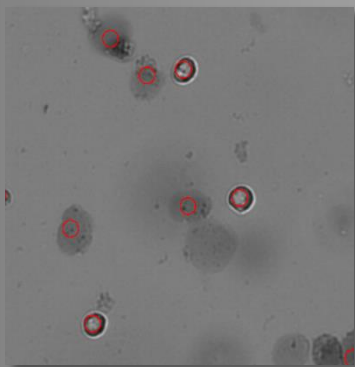


Fig. 4: Focal plane too high no lens effect visible on viable cells.

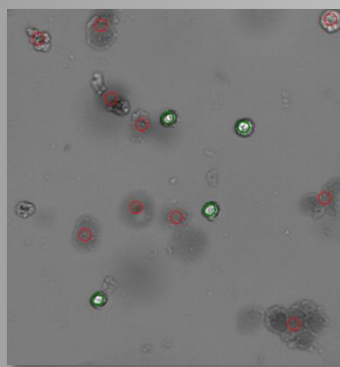


Fig. 5: Focal plane o.k. viable cells show a slight lens effect.

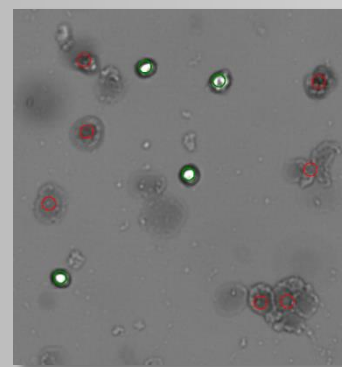


Fig. 6: Best setting of the focal plane with a strong lens effect for the viable cells.

3.2 Brightness

Just like the focal plane has to be adjusted, the illumination of the image has to be balanced to supply the best image for the analysis.



Fig. 7: too dark

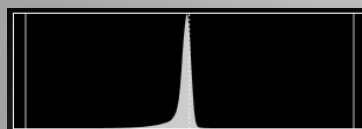


Fig. 8: Illumination balanced



Fig. 9: too bright

4. Image Analysis

This chapter guides you through the image processing/analysis with its settings and how to achieve the best results for your cell line.

4.1 Edge Distance

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 will exclude operator result within a distance of 100 μm off the well edge.



Fig. 10: Edge Distance set to 100 μm

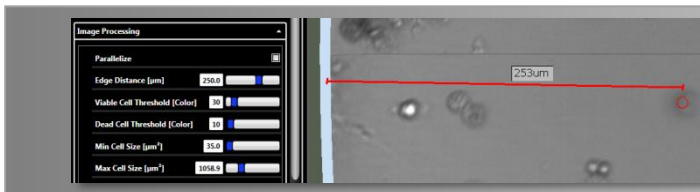


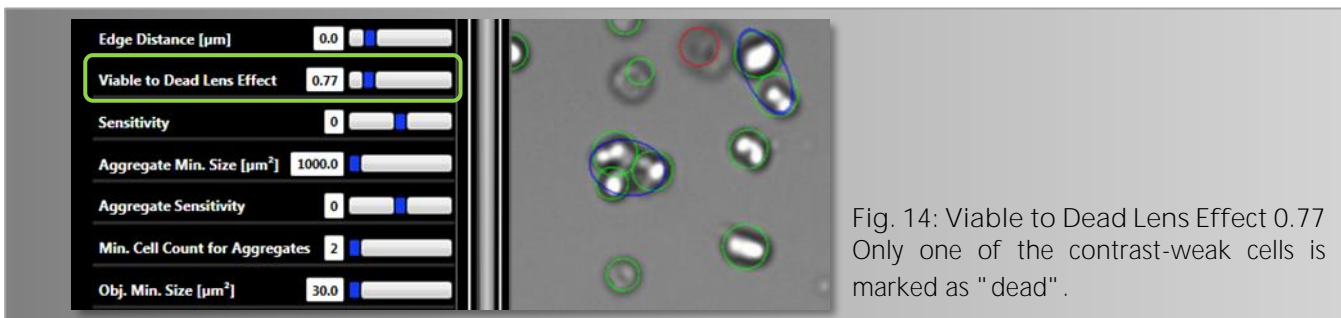
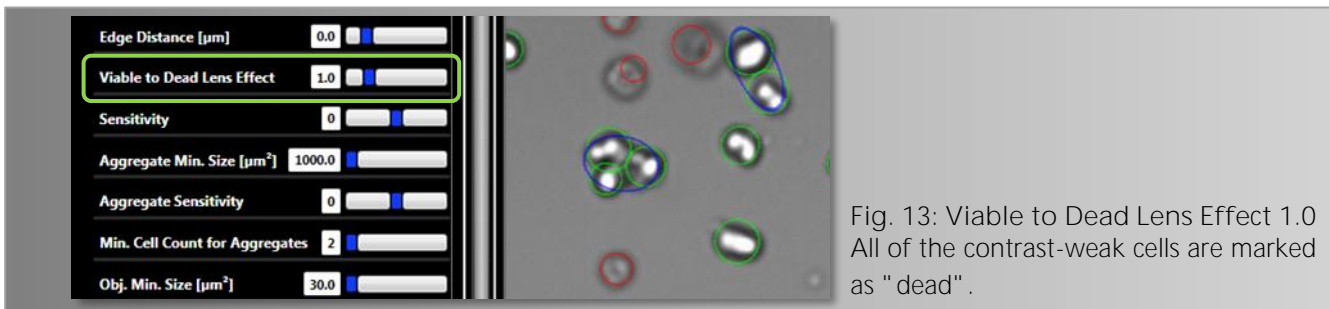
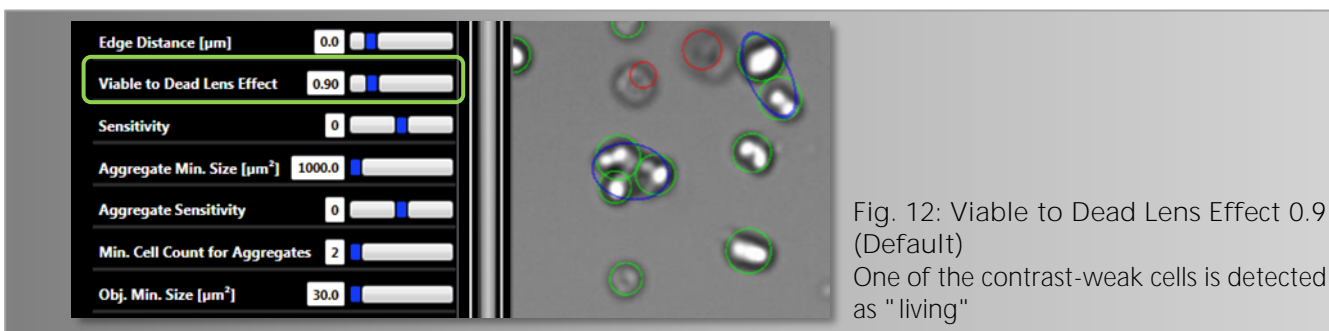
Fig. 11: Edge Distance set to 250 μm

4.2 Viable to Dead Lens Effect

With the parameter "Viable to Dead Lens Effect" the classification border is fixed between living and dead cells. Living cells show a distinctive lens effect while dead cells by the addition of Trypan Blue appear dark.

Nevertheless, according to the used cell line and used Trypan Blue, the classification border from living to dead cells may vary, so that this parameter must be modified. A higher value leads to the effect that less living but more dead cells tend to be counted. A lower value causes the contrary effect.

Examples:



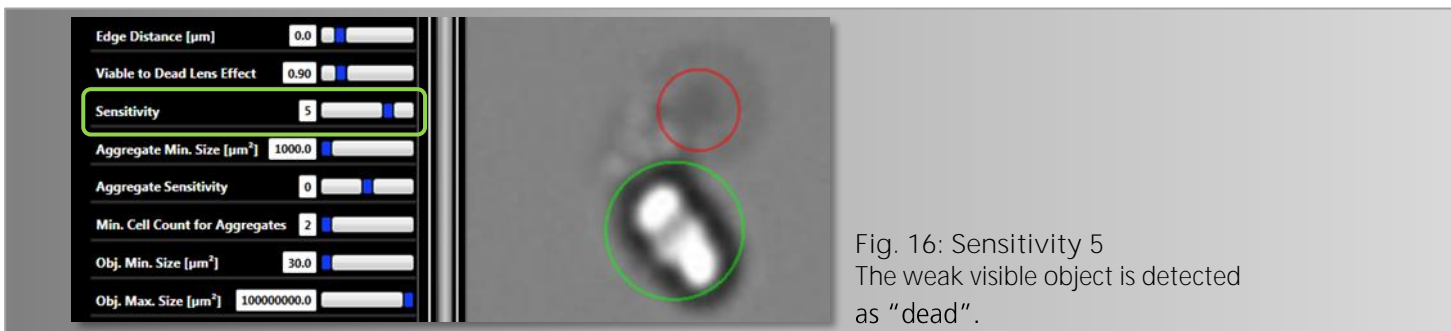
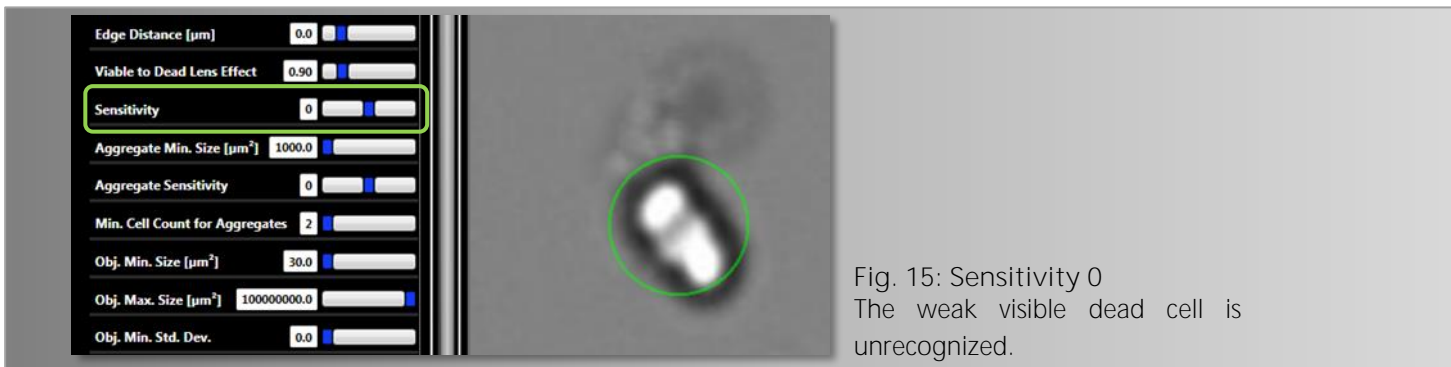
4.3 Sensitivity

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:



4.4 Aggregate Min. Size and Aggregate Sensitivity

Depending on the state of the cell culture, cells can also exist in cell aggregates. Cell aggregates are more difficult to analyze than single cells. If the analysis of cell aggregates is not satisfactory when using the default settings of the operator, "Aggregate min. Size" and "Aggregate Sensitivity" can be altered to influence the cell counting in aggregates according to your requirements.

With the parameter "Aggregate min. Size" the minimum size of the (potential) aggregates is defined which should be analyzed by the setting of the parameter "Aggregate Sensitivity". The higher the value of the parameter "Aggregate Sensitivity", the more cells will be counted, according to the morphological appearance of the cells. The lower the value, the fewer cells are counted. If "Aggregate Sensitivity" is set on "0", the default image analysis is applied.

The cell counting in potential aggregates is affected by the parameter "Aggregate Sensitivity" only if this is possible morphologically.

Examples:

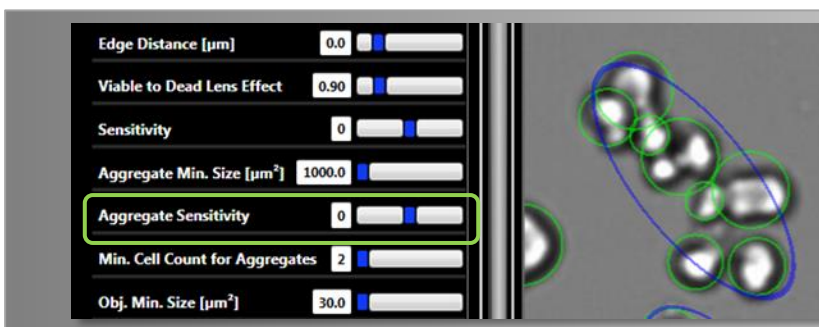


Fig. 17: Aggregate Sensitivity 0
8 cells are counted inside an aggregate.

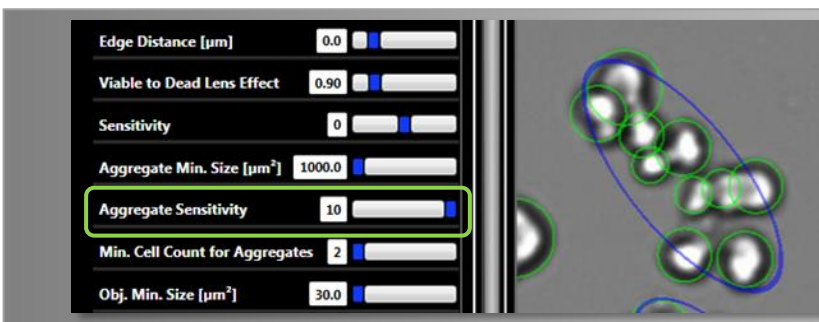


Fig. 18: Aggregate Sensitivity 10
10 cells are counted inside an aggregate.



Fig. 19: Aggregate Sensitivity -10
6 cells are counted inside an aggregate.

4.5 Min. Cell Count for Aggregates

The image analysis enables to count aggregates from a certain cell number. By a default setting of "2", two relating cells are already evaluated and counted as an aggregate. In case smaller aggregates shall not be counted this value can be raised accordingly. A counted aggregate is marked in the picture with a blue ellipse. The sum of the counted aggregates appears as "Aggregate Count" in the result table.

Examples:

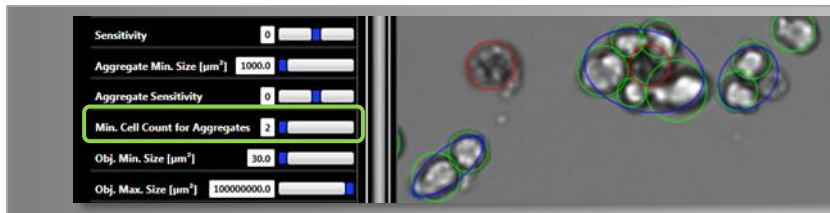


Fig. 20: Min Cell Count for Aggregates 2
Aggregates from up to 2 cells are counted.

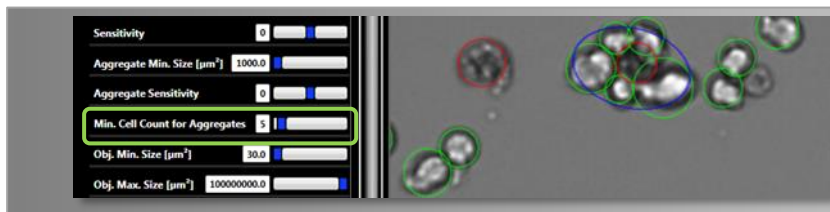


Fig. 21: Min Cell Count for Aggregates 5
Aggregates from up to 5 cells are counted.

4.6 Objekt 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 detected 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].

4.6.1 Obj. Min. Size and Obj. Max. Size

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.

Examples:

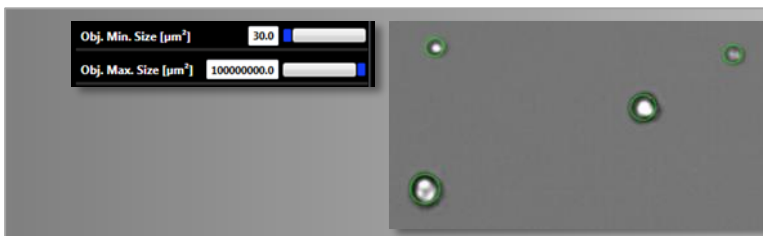


Fig. 22: Obj. Min Size 30 μm^2
2 vesicles are detected as living cells.

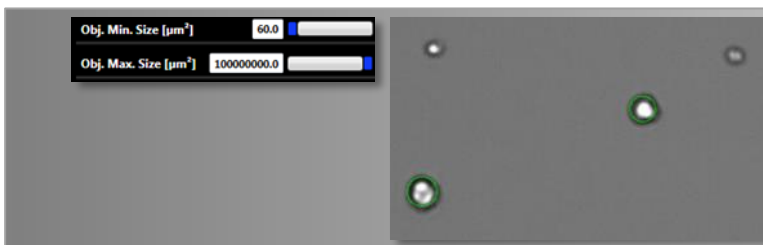


Fig. 23: Obj. Min Size 60 μm^2
The 2 vesicles are undetected because of their small size.

4.6.2 Obj. Min. Std. Dev. and Obj. Max. Std. Dev.

The attribute "Standard Deviation" (Std. Dev.) indicates the standard divergence of grey values of the object in the picture. Shadowy or blurred illustrated objects have a low standard deviation; sharply illustrated suspension cells have a high standard deviation.



Fig. 24: The adjustable range of the Obj. Std. Dev.

4.6.3 Obj. Min. Compactness and Obj. Max. Std. Compactness

The attribute "Compactness" serves the description of the 2D form of the object to be recognized in the picture in comparison to a perfect circular object. The less round the object, the closer this value is to "0". The ideal form of a circle results in the maximum value 1.

This value results from the perimeter² divided by the object area. For the ideal circle a value of 4/4 arises, so that the attribute borders lie in a limited range between 0 and 1. This value is formed standardized on 4/4 and the reciprocal value.



Fig. 25: The adjustable range of the Obj. Compactness

4.6.4 Obj. Min. Longishness and Obj. Max. Longishness

The attributes "Longishness" and "Compactness" describe the form of the object. Therefore an ellipse is determined which resembles the two-dimensional picture of the object. The relation of the minor axis to the major axis of the ellipse as a percentage value results in the attribute "Longishness".

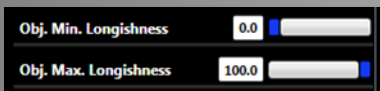


Fig. 26: The adjustable range of the Obj. Longishness

4.6.5 Obj. Min. Intensity and Obj. Max. Intensity

The attribute "Intensity" states the average grey value of the object in the image. By the use of pictures with 8-bit color a maximum interval of 0 to 255 arises.

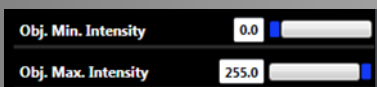


Fig. 27: The adjustable range of the Obj. Intensity

4.6.6 Obj. Min. Contrast and Obj. Max. Contrast

The attribute "Contrast" describes the distribution of grey values of the object. It is calculated by the difference of maximum and minimum grey value of the object in relation to the sum of these both values.

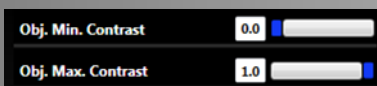


Fig. 28: The adjustable range of the Obj. Contrast

5. Software Readout

• Viability	Percentage of viable cells in your sample
• VCD	Viable Cell Density [#/ml]
• CD	Cell Density [#/ml]
• Aggregates per ml	Number of aggregates per ml
• Ratio of Aggregates	Percentage ratio of aggregates in the sample
• Reactor ID	Name of the Reactor
• Sample ID	Name of the Sample
• Avg Cell Size	Average of the Cell Size [μm^2]
• # of Aggregates	Number of aggregates
• Final Dilution	Dilution factor
• Volume per Well	Sample Volume per well
• Cell Count	Number of cells listed per well



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