SYNENTEC

Experiment Guide – Spheroid Quantification

Schaefer W[1], Willms A[1], Christmann T[1] and Geisen R[1]
[1] SYNENTEC GmbH

ABSTRACT

The purpose of this document is to guide the user through the experiment setup for the Spheroid Quantification application. It does not contain any procedure of setting up a spheroid assay itself and seeding the cells into a plate in an appropriate dilution, since this may vary due to customer requirements and internal procedures.

CONTENT

1 MATERIALS & PROCEDURES

This chapter lists the requirements for setting up NYONE® or CELLAVISTA® for Spheroid Quantification Application and for Spheroid Quantification (1F) and (2F) Application. As already mentioned, the kind of equipment may vary on individual user requirements. The following items are a suggestion, as they have shown to achieve reliable results.

1.1 Microplates

It is required to use microplates with clear bottom. The format depends on the method of spheroid generation and the desired size of the spheroids. The U bottom 96-well plates with a cell-repelling surface listed in Tab. 1 were tested and generated good results in our hands:

Tab. 1: 96 well U bottom plates

Name	Manufacturer	Cat. No.	comments
Costar®, Ultra-Low Attachment Microplate	Corning	7007	
BIOFLOAT™	faCellitate	F202001	
CELLSTAR®, CELL-REPELLENT SURFACE	Greiner	650970	
Nunclon™ Sphera™, Sphera-Treated	Thermo Scientific™ Nunclon™	174925	Centrifugation for spheroid formation necessary

For smaller spheroids or the generation of many spheroids at once, 24-well plates containing 750 microwells with a high-end ultra-low attachment nanocoating can be used, such as the Kugelmeiers sphericalplate 5D (sp5d). The ultra-low attachment coating allows ideal cell aggregation and avoids settling of cells in areas other than the microwells.

Handling of the microplates: Do not touch the transparent bottom of the microplates with your fingers (even with gloves) at any time before measurement. Place your thumb and fingers on the rim on the longer side of the plate. The transparent bottom is part of the optical path and can lead to erratic measurements if it is soiled with fingerprints or dirt.

1.2 Procedure

An example could be:

1. Aspirate culture medium
2. Wash cells with $PBS^{-/-}$
3. Aspirate $PBS^{-/-}$
4. Detach cells with trypsin
5. Suspend cells in medium
6. Count the cells with the Trypan Blue Application of YT®-software
7. Prepare a cell suspension with the optimal cell density
8. Add 200 µL of the cell suspension per well (recommended 1,000 – 20,000 cells/well). The optimal cell number must be determined in preliminary experiments. An increase in diameter decreases the concentration of oxygen and nutrients in the spheroid core, resulting in zones of proliferation, hypoxia, and necrosis as this is also the case in solid tumors [1], [2]. We have obtained spheroids with a necrotic core from a diameter of 400 - 600 µm

The optimum cell density depends on the cell line and the ability of the cell to form spheroids as well as the scientific question/hypothesis. Therefore, it has to be determined by the user. The spheroid diameter depends on the used cell number.

9. Incubate the cells for 4-24 h at 37 °C in the incubator (usually, loose aggregates have formed after 4 h, while tight spheroids/aggregates have formed after 24 h)
10. Image the cells/spheroids daily for 4 till 7 days (the best imaging interval depends on your cells and experimental settings)
11. Exchange half of the medium after 2 days (be careful, don't aspirate the spheroids)
12. Treat the cells by removing half of the medium and exchanging it with treatment solution (2x concentrated) after 4 days (recommended for the most cell lines) or apply assay (e.g. Calcein AM green and propidium iodide).

A medium change in the wells with spheroids can result in the removal of the spheroids. Be careful while removing the medium, we recommend exchanging only half of the medium.

2 EXPERIMENT SETUP

Click on the Generic Measurements icon to start a new experiment and select *Spheroid Quantification* as Image Processor.

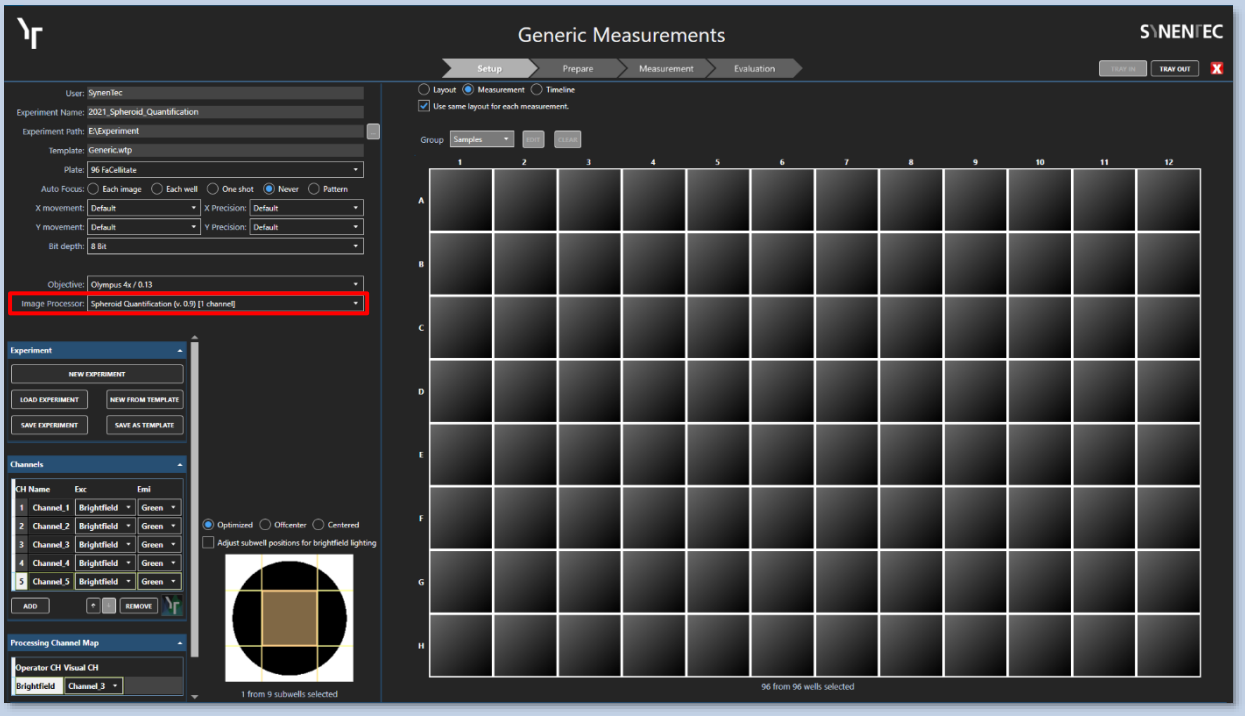


Fig. 1: Spheroid Quantification setup page

It is important to reduce the measurement motion to prevent agitation of the spheroids. When using the NYONE® Scientific or CELLAVISTA® Scientific, the mode of the X and Y movement should be set at *Very_Gentle* (Fig. 3).

Fig. 3: Movement of plate
Reduced velocity of the plate prevents spheroid disintegration.

When using another device without harmonic drive, the *Anti-Blur* setting can slow down the movement of the plate (Fig. 4) as well as table movement customizations conducted by SYNENTEC.

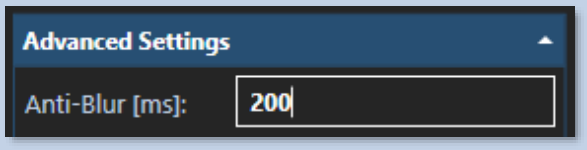


Fig. 4: Anti-Blur
The Advanced Setting *Anti-Blur* can be used to decelerate the velocity of the plate movement to reduce agitation of the spheroids.

2.3 Subwell selection

The recommended objective for spheroid imaging in 96 well plates with a U bottom is the 4x objective. Due to the shape of the well bottom, the spheroids are located in the center of the well in these plates. Therefore, the 4x objective enables imaging of the whole spheroid by selecting the centered subwell (Fig. 5). Imaging only one subwell per well is reducing the measurement time.

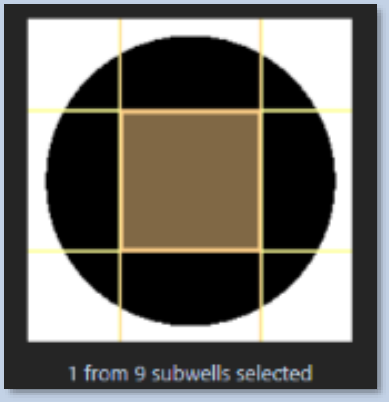


Fig. 5: Subwell selection

2.4 Optical Setup

For the U bottom 96 well plates, the recommended autofocus setting is different from the normal plates with flat bottom. The autofocus of NYONE® and CELLAVISTA® is based on reflection of the autofocus laser at the plate bottom. The U bottom can deflect the laser or the reflection is not at the lowest point of the U bottom, which can lead to different focus detection. When the plate settings cannot accurately be determined, you should use the autofocus setting *Never* (Fig. 6). In this case, the focus height is determined by yourself. This setting compensates for the fact, that plates are never 100% uniform. For plates with a very high accuracy, the *Each well* setting can be used (not recommended).

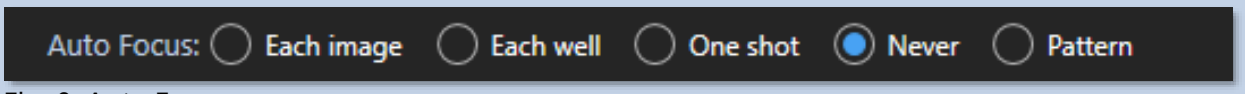


Fig. 6: Auto Focus

Due to the fact that spheroids are 3D cell formations, multiple brightfield channels provide the opportunity to observe different regions of the spheroids (Fig. 7) using multiple focus offsets (z-stacking). Using *Spheroid Quantification (1F)* and *(2F)*, you can do the same for the fluorescence channels.

When using the *Never* autofocus setting and multiple brightfield channels with different offsets,

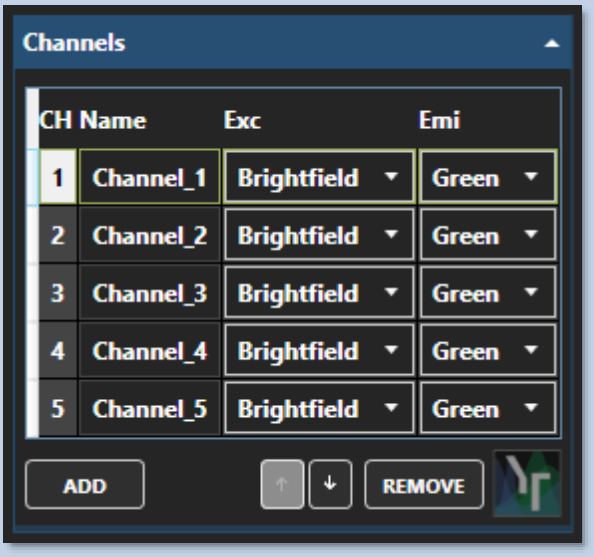


Fig. 7: Channels
For spheroids (3D), more than one channel can be used to obtain focused images of different regions of the spheroid.

the used focus heights should be saved in the comments on the setup page to check that the same focus height is always used.

3 PREPARATION

Click on the Prepare arrow to get to the tab for configuring the optical settings for the measurement (Fig. 8). The microplate will automatically be moved to the first well defined in the setup, and the experimental setup will automatically be saved.

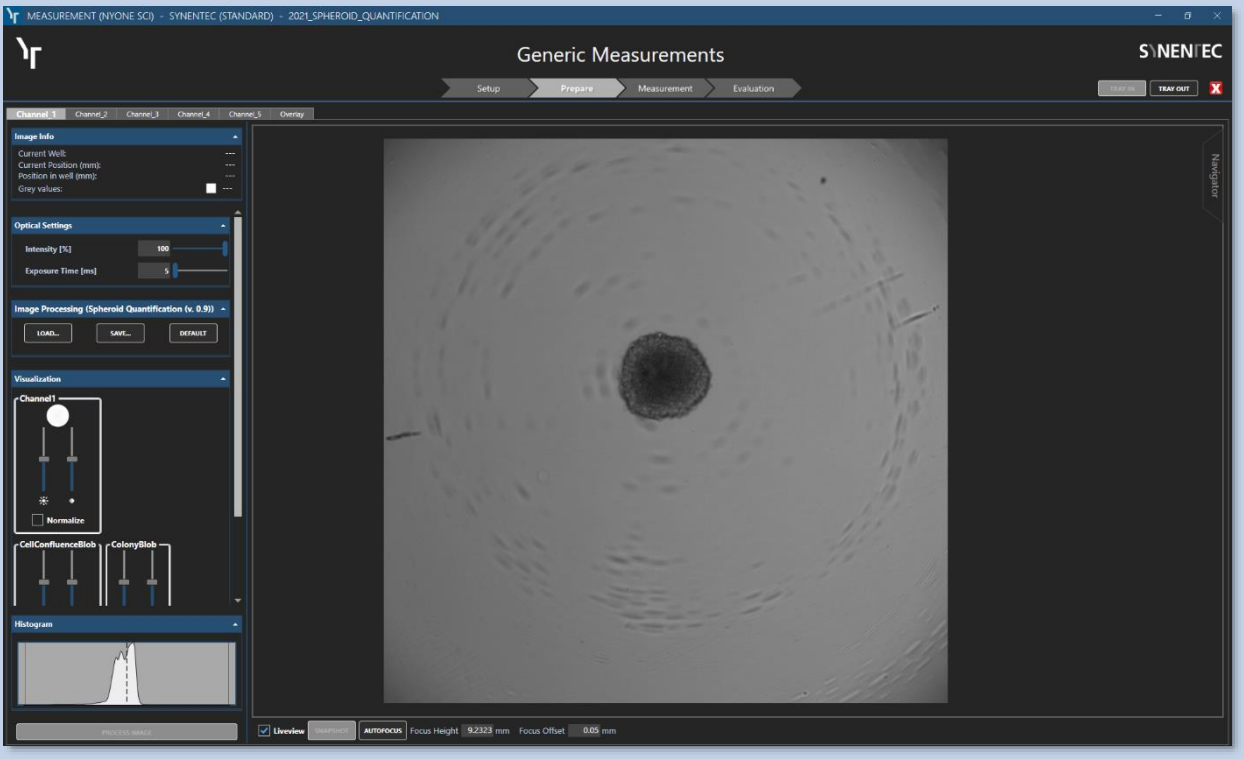


Fig. 8: Preparation

3.1 Focus

The preparation starts with the autofocus. Although you don't use autofocus for the measurement, the autofocus function is useful to find the plate bottom and set the desired focus height, as well as directly set the desired focus height. Turn on the *Liveview*, so you can see changes immediately, and set the *Focus Offset* to the desired sharpness of the spheroid (Fig. 9).

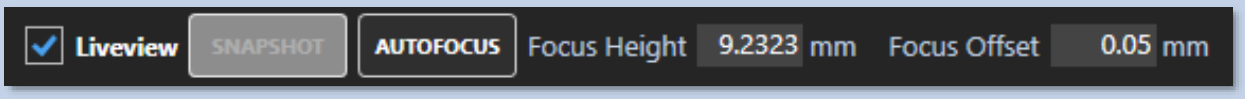


Fig. 9: Liveview, Autofocus, Focus Height and Focus Offset

When you are using more than one channel, start the preparation with *Channel_1* and use the *Focus Offset* to define the distance between the stacks (Fig. 10).

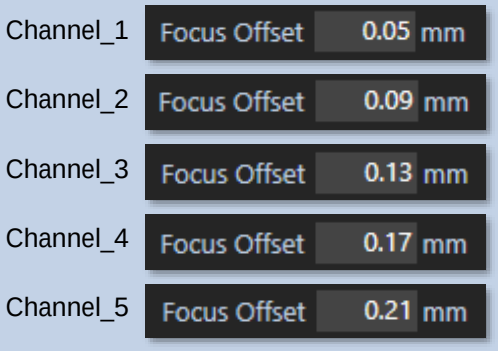


Fig. 10: Focus Offset
Example for measurement with *Focus Offsets* of five different channels with 0.04 mm between each.

The distance between the channels can be set according to the total size of the spheroids, determinable with the *Measurement Mode*, but also according to your requirements (Fig. 11). For a spheroid with a diameter of 500 µm I have chosen a distance of 40 µm between the *Focus Offsets* of the 5 channels. You can also choose 6 channels with 50 µm distance between the *Focus Offsets* to cover half the spheroid.

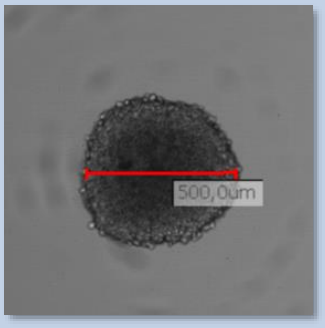


Fig. 11: Diameter of Spheroid
Measure the diameter of the spheroid, as a possible approach for choosing the distance between the focus offsets. Activate this with a click of the right mouse button and select Measurement Mode.

When using the same number of fluorescence channels, we recommend to use the same focus offsets as for brightfield, so that both brightfield and fluorescence can be viewed in each plane.

3.2 Exposure

In addition to the focus, the exposure has to be adjusted. Increase or decrease the brightness with the sliders for *Intensity* [%], *Exposure Time* [ms] and *Gain* [%] (if available) to a grey value of ~130 counts for the background around the spheroid (Fig. 12). Keep the exposure time as low as possible and the intensity as high as possible to achieve a shorter measurement time. The gray values are displayed in the *Image Info* when you move the mouse over the area of interest in the image.

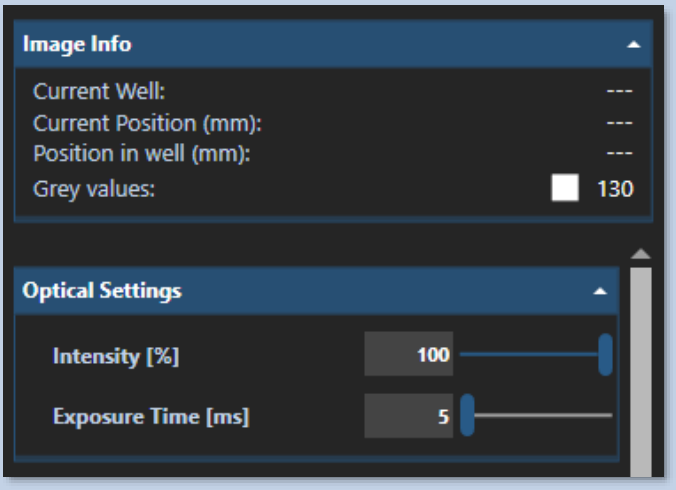


Fig. 12: Optical Settings for Exposure
The *Intensity* is at 100 % so that an *Exposure Time* of 5 ms is sufficient to achieve a *Grey value* of 130 counts around the spheroid and a fast measurement.

The same is recommended for fluorescence channels. When using fluorescent dyes as in live/dead staining, the optical settings should be made in the well where the highest signal is expected.

3.3 Image Processing

Before processing, tick the checkbox for *Internal 2x2 Binning* and *Multiple Cell Layers Expected* (Fig. 13). Both parameters are recommended for *Spheroid Quantification, (1F)* and *(2F)*. *Internal 2x2 Binning* uses 2 times 2 pixel and evaluates them as one pixel. This reduces the processing time and increases the sensitivity, but the accuracy is lower. *Multiple Cell Layers Expected* is for cell formations with a dark core surrounded with cell clusters.

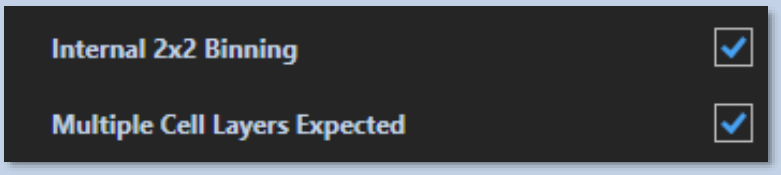


Fig. 13: Checkbox
Tick the check boxes for Internal 2x2 Binning and Multiple Cell Layers Expected.

After ticking the checkboxes and preparing focus and exposure, the proper detection of the spheroids can be checked by the *Spheroid Quantification* application of the YT®-software via *Process Image* button (Fig. 14).

Fig. 14: Process Image

After successful processing, the multiple cell layer is marked with a yellow line and the detected spheroid is circled with a green line (Fig. 15). If more than one channel is used, ensure that the channel to be processed is selected at the beginning of the setup under *Processing Channel Map*.

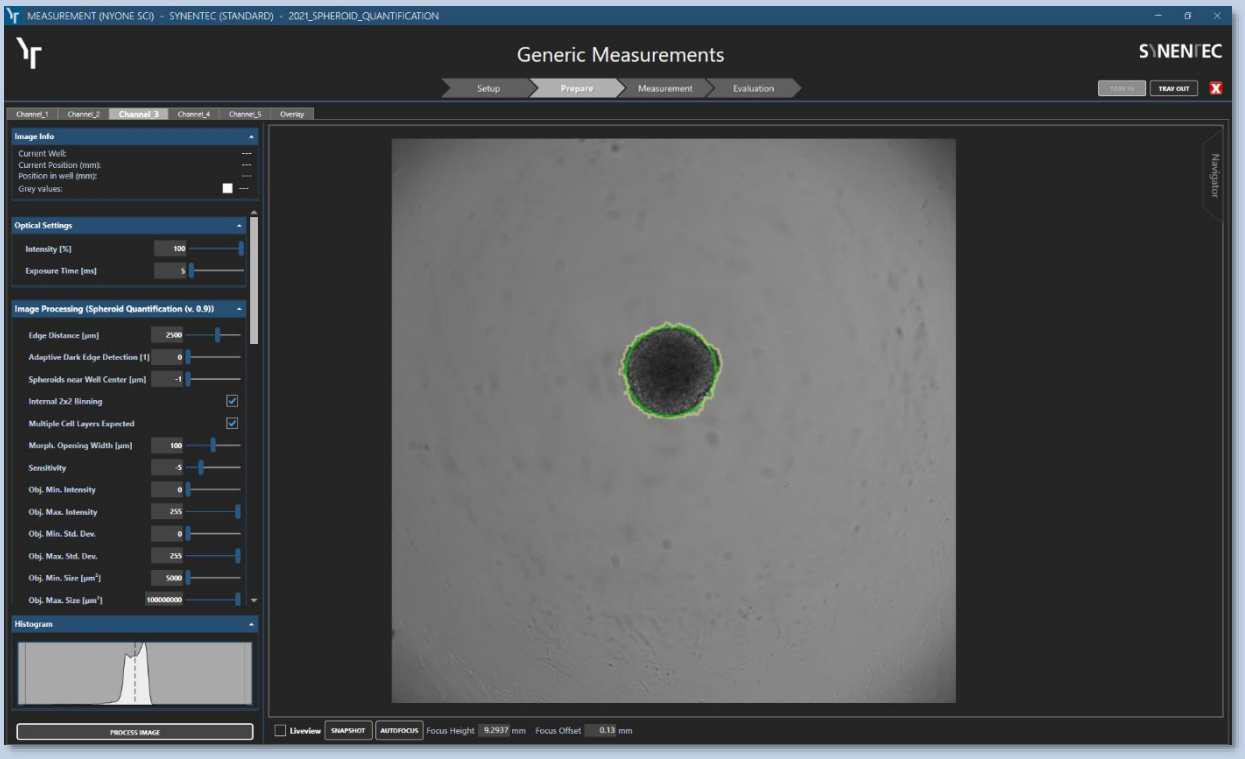


Fig. 15: Processed Image
The detected cell layer is marked with yellow lines and the determined spheroid is encircled green.

For *Spheroid Quantification (1F)* and *(2F)*, the selected brightfield channel can be processed individually, or select *Overlay*, then processing can be performed for all channels. In this case, the green circle marking the spheroid has a different color depending on the detected fluorescence. Thus, the circle is orange for fluorescence 1 positive spheroids, light blue for fluorescence 2, and red if both are detectable.

4 MEASUREMENT

After selecting the appropriate optical setting, focus, exposure and testing of image processing, the measurement (next arrow) can be started. You can choose between *None*, *During Measurement* and *Batch Processing*, if you have a batch processor (Fig. 16). Then you can start the measurement.

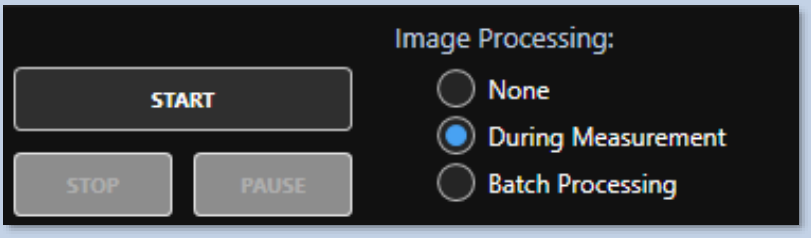


Fig. 16: Measurement

5 EVALUATION

After measurement, you can optimize the processing parameters for the detection of cell confluence and spheroids. The *Cell Confluence* should be recognized properly and marked by a yellow line (Fig. 17). The detected spheroid (*Colony*) is circled by a green circle.

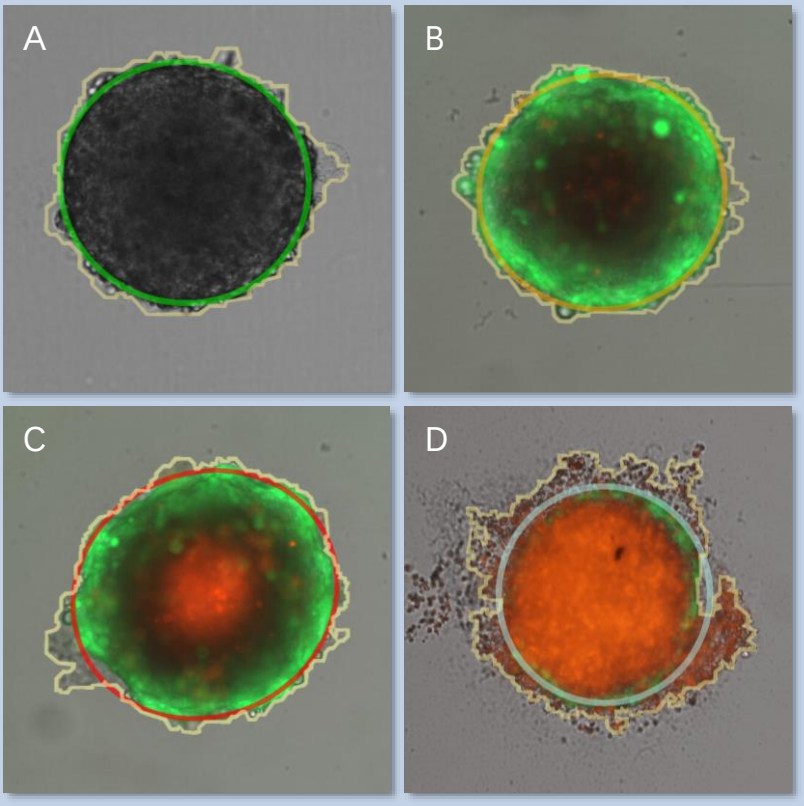


Fig. 17: Spheroid Quantification
Yellow: Cell Confluence
A. Green: Spheroid (*Colony*)
B. Orange: Fluorescence 1 positive spheroid
C. Light blue: Fluorescence 2 positive spheroid
D. Red: Fluorescence 1 and Fluorescence 2 positive spheroids

5.1 Image Processing Settings

The default settings of the *Spheroid Quantification Application* with ticked *Internal 2x2 Binning* and *Multiple Cell Layers Expected* provide a basis for image analysis (Fig. 18). Nevertheless, we recommend to check the results and alter the *Image Processing* parameter to more accurate results.

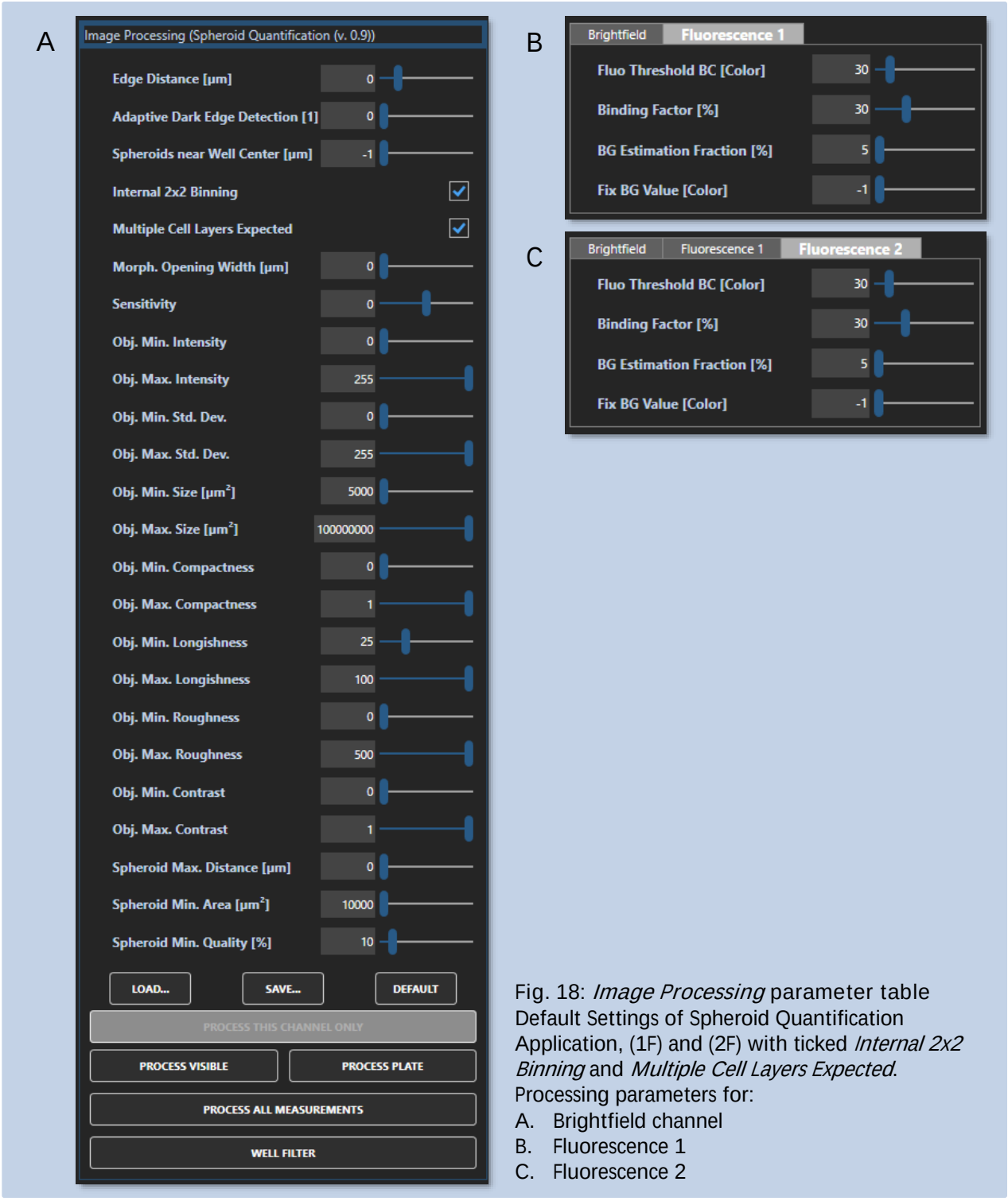


Fig. 18: *Image Processing* parameter table Default Settings of Spheroid Quantification Application, (1F) and (2F) with ticked *Internal 2x2 Binning* and *Multiple Cell Layers Expected*.
Processing parameters for:
A. Brightfield channel
B. Fluorescence 1
C. Fluorescence 2

You can check the new settings by cklicking *Process Visible*. In this case, only the visible images are processed. This has no influence on the results, but it is fast. Only after *Process Plate* (processing of visible measurement) or *Process All Measurements*, the results will be updated. Especially, when you perform the experiments for the first time, check if the image processing settings produce reasonable results. Have a look at different wells and check that the cells are properly recognized (indicated by yellow lines) and the spheroids are correctly detected (green circle), while background signals or debris are excluded. If this is not the case, modify the image processing setting until the results are satisfactory.

Most of the parameters are the same as for other applications of the YT®-Software and are explained when you move the mouse cursor over the parameter name. The special parameters for the *Spheroid Quantification* Application are explained below (5.1.1-5.1.5). The special parameters for *Spheroid Quantification (1F)* and *(2F)* are described afterwards (5.1.6).

5.1.1 Spheroids near Well Center

The *Spheroid near Well Center* parameter specifies the distance from the well center where the center of the spheroid must be located to be counted as a spheroid (Fig. 19) to avoid false positive detection events.

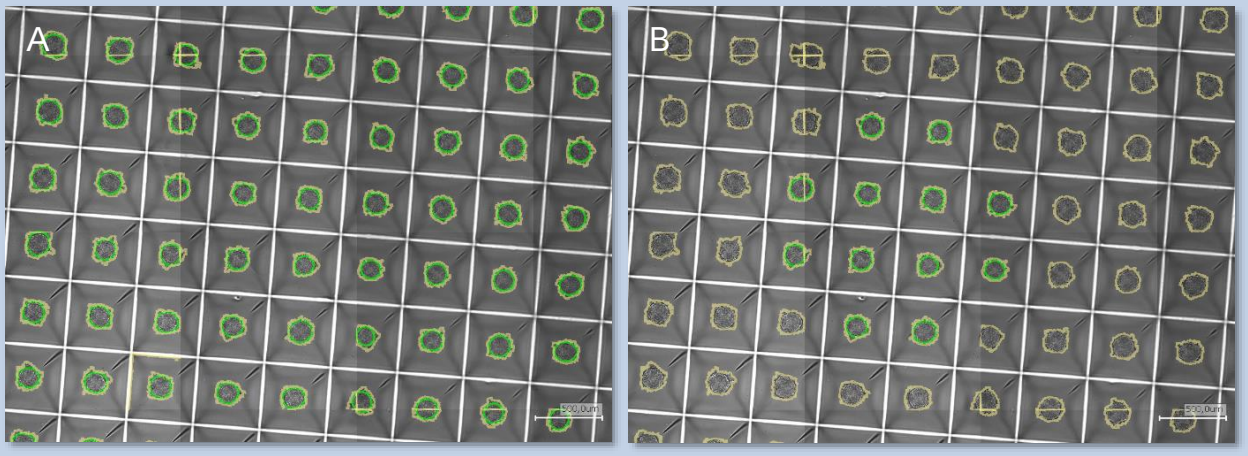


Fig. 19: *Spheroid near Well Center*
The *Spheroid near Well Center* has a range from -1 to 10,000 µm. Here, you can see how it affects the detection (scale bar: 500 µm). In this example, the counted spheroids are encircled green and have a distance to the well center of:
A. -1 µm (default, off)
B. 1000 µm

5.1.2 Morph. Opening Width

The *Morph. Opening Width* removes artefacts or cell debris by smoothing the outer boundary of detected object areas (Fig. 20).

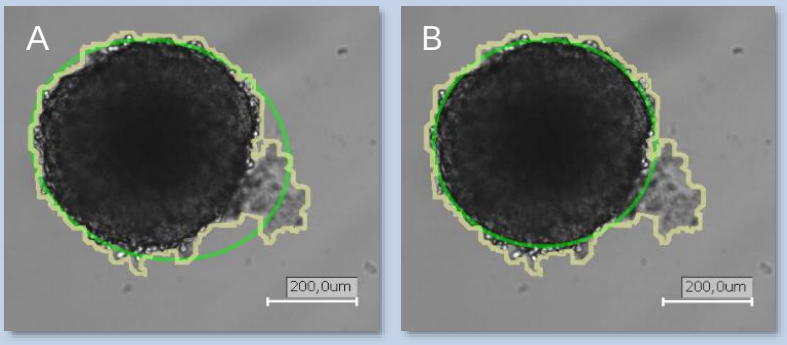


Fig. 20: *Morph. Opening Width*
The *Morph. Opening Width* has a range from 0 to 200 µm. If the *Morph. Opening Width* is increased, less cell debris around the spheroid is detected (green circle, scale bar: 200 µm):
A. 0 (default)
B. 120 µm

5.1.3 Spheroid Max. Distance

Spheroid Max. Distance defines the distance between two spheroids to determine if two or more spheroids are counted as one (Fig. 21)

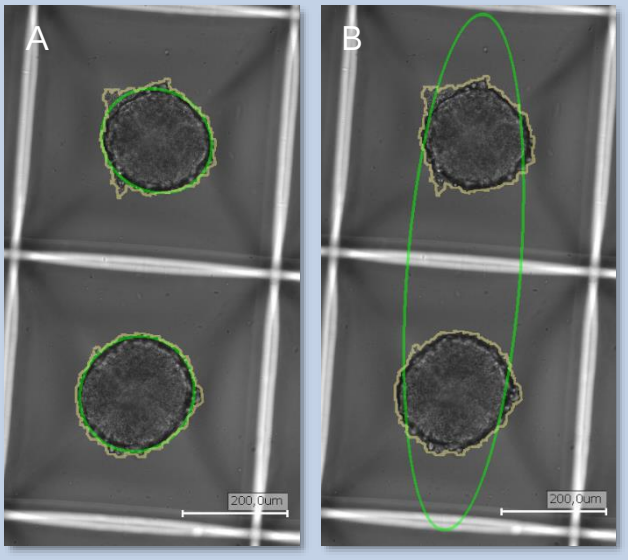
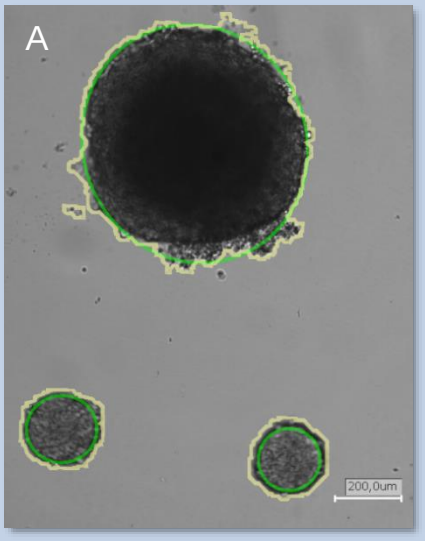


Fig. 21: *Spheroid Max. Distance*
The *Spheroid Max. Distance* has a range from 0 to 1000,000 µm. If the *Morph. Opening Width* is increased, the spheroids with less distance are counted as one spheroid (green circle, scale bar: 200 µm):
A. 0 (default)
B. 270 µm

5.1.4 Spheroid Min. Area

Spheroid Min. Area is the area a spheroid must have to be counted as a spheroid (Fig. 22).



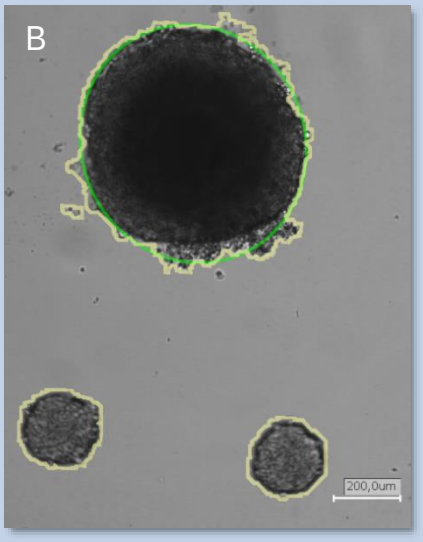
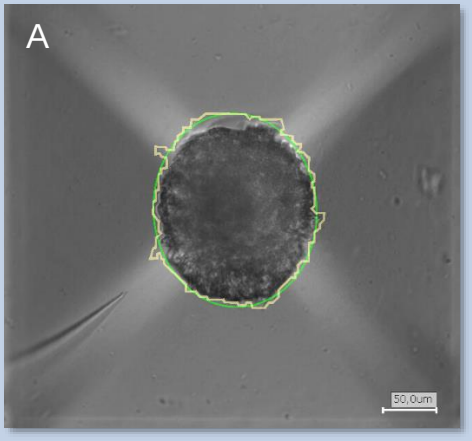


Fig. 22: *Spheroid Min. Area*
The *Spheroid Min. Area* has a range from 0 to 1000,000,000 µm². If the spheroid is smaller than the selected min. area the spheroid is not counted (green circle, scale bar: 200 µm):
A. 10,000 µm² (default)
B. 50,000 µm²

5.1.5 Spheroid Min. Quality

Spheroid Min. Quality is defined by the ratio of detected cells to the ellipse area (Fig. 23). The more of the ellipse (green circle) is filled by cell area (yellow lined) the higher the spheroid quality.



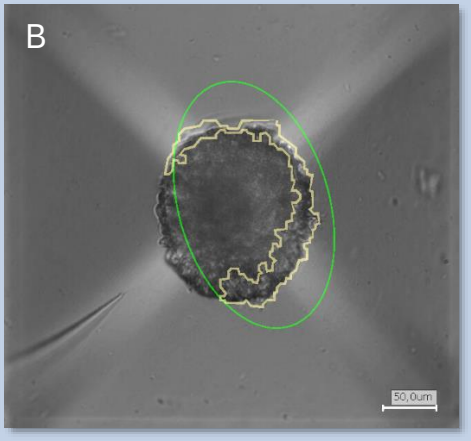


Fig. 23: *Spheroid Min. Quality*
The *Spheroid Min Quality* has a range from 0 to 100 %. The ratio of cell area (yellow) to ellipse defines the spheroid quality (green circle, scale bar: 50 µm):
A. 97 %
B. 27 %

5.1.6 Fluorescence

The *Binding Factor* is defined by the quantity of pixels in the circle (spheroid), that lie above the *Fluo Treshold BC* (background corrected, Fig. 24). The more pixels above the *Fluo Treshold BC* the higher the *Binding Factor* is. The spheroid in figureFig. 24 A (orange circle, Fluo CH1 positive but Fluo CH2 negative) has a *Fluo CH2 Intensity BC* of 26 and a *Fluo CH2 Binding* of 25 %, this means that under 30 % of the spheroid area has red fluorescence (Fluo CH1: green fluorescence, Fluo CH2: red fluorescence). The lower the *Fluo Treshold BC*, the more pixel are counted as Fluo CH2 positive and the spheroid will be marked with a red circle as Fluo CH2 positive (31 %, Fig. 24 B). The value of the binding factor, from which number of positive pixels a spheroid is evaluated as positive, can also be adjusted (Fig. 24 C). the same parameters are also adjustable for Fluo CH1.

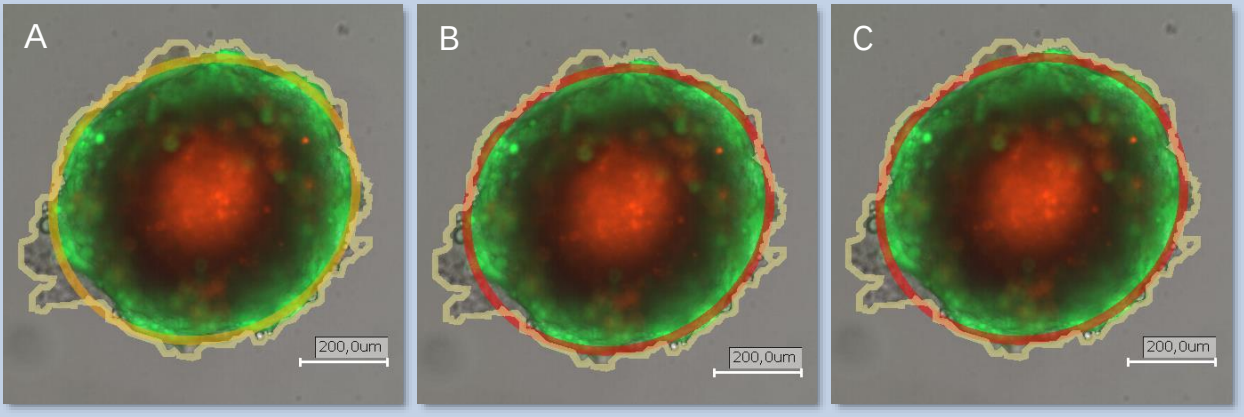


Fig. 24: *Fluo Threshold BC* and *Binding Factor*
The *Fluo Threshold BC* has a range from 1 to 255 and the *Binding Factor* has a range from 0 to 100 %. The quantity of pixels above the *Fluo Treshold BC* defines the *Binding Factor* of the spheroid. A spheroid with a *Binding Factor* of 25 % (orange circle – Fluo CH2 negative) can be detected as Fluo CH2 positive (red circle) by decreasing Fluo Threshold BC or Binding Factor (scale bar: 200 µm):
A. *Fluo Threshold BC* 30 and *Binding Factor* 30 % (default)
B. *Fluo Threshold BC* 25 and *Binding Factor* 30 %
C. *Fluo Threshold BC* 30 and *Binding Factor* 20 %

Furthermore, for the evaluation of the fluorescence, a fixed background value (*Fix BG Value*) or the portion of the background to be considered in the calculation of the background value (*BG Estimation Fraction*) can be set.

5.2 Results

The results are available after completion of image processing with the default image processing parameters and processing during measurement or after altering the image processing parameters and click *Process Plate* or *Process all Measurements* (Tab. 2). The most important result values are *Avg Spheroid Area* and *Avg Spheroid Diameter* as well as the *Spheroid Count*. The results can be checked by scrolling through the result table or by visual display as heat map and in the time chart.

Tab. 2: Result Parameters of *Spheroid Quantification* application

Parameter	Unit	Description
Evaluated Area	mm^2	Area that is evaluated by the image processing settings
Area of Spheroid	mm^2	Area of all spheroids combined (green circle)
Spheroid Count	#	Counted spheroids
Avg Spheroid Area	μm^2	Average area of all spheroids (green circle)
Avg Spheroid Diameter	μm^2	Average diameter of all spheroids (green circle)
Cell Area	µm	Area that is covered by cells (yellow lined)
Cell Confluence	%	Cell confluence in percent of the area that is covered by cells (yellow lined)
Process Duration	ms	Time needed for evaluating the area of this well
Processed Area	%	Percent of the well area that is evaluated

The Spheroid Quantification (1F) and (2F) applications have additional results regarding to the fluorescence (Tab. 3). These show whether or not fluorescence is present in the spheroids and which.

Tab. 3: Additional Result Parameters of *Spheroid Quantification* (1F) and (2F) application

Parameter	Unit	Description
Avg Fluo CH1/CH2 Intensity BC	-	Average fluorescence intensity in channel 1 or 2 over background
Avg Binding Fluo CH1/CH2	%	Average binding in Fluo CH1 or CH2
F1/F2 Marker positive	# or %	Number or percent of Fluo CH1 or CH2 positive spheroids
Spheroid-nn	# or %	Spheroid marker negative on Fluo CH1 and negative on Fluo CH2
Spheroid-pn	# or %	Spheroid marker positive on Fluo CH1 and negative on Fluo CH2
Spheroid-np	# or %	Spheroid marker negative on Fluo CH1 and positive on Fluo CH2
Spheroid-pp	# or %	Spheroid marker positive on Fluo CH1 and positive on Fluo CH2

5.2.1 Heat map

The *Heat map* visualizes the results (Fig. 25). This allows you to get a quick overview of the results. In the drop-down menu of the property box, you can select which parameter is displayed in the *Heat map*. The different colors of the *Heat map* represent the values of the chosen parameter. Blue represents a low value and red a high value (legend visible by clicking on the arrow below the *Heat map* name).

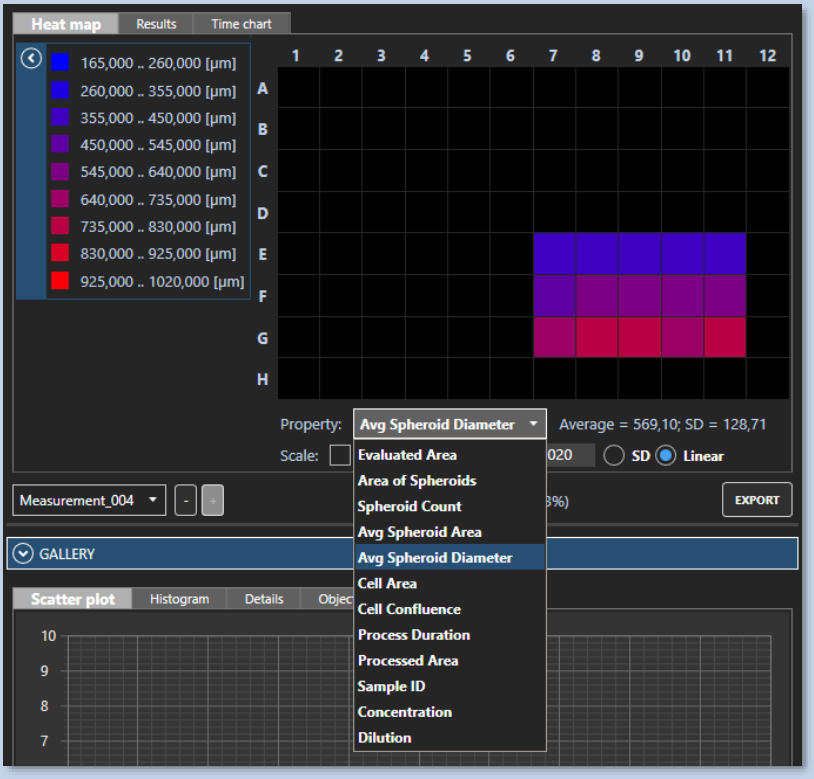


Fig. 25: Analyst with the heat map as one possibility of result presentations
Overview of the different presentation properties for the heat map and the heat map legend.

5.2.2 Time chart

The time charts, the scatter plot or histograms are available on the analyst tab. You can also choose which parameter is displayed in the graphs in the drop-down menu of the property box. The *Time chart* displays the values of the chosen parameter over all measurements. If you click on *Details* in the lower tab, and then select one or more wells in the upper chart (left click on it), only the selected curves will be displayed in the lower graph (Fig. 26). In the example, different cell numbers formed compact spheroid overnight and were growing over the next few days depending on cell number/spheroid diameter.

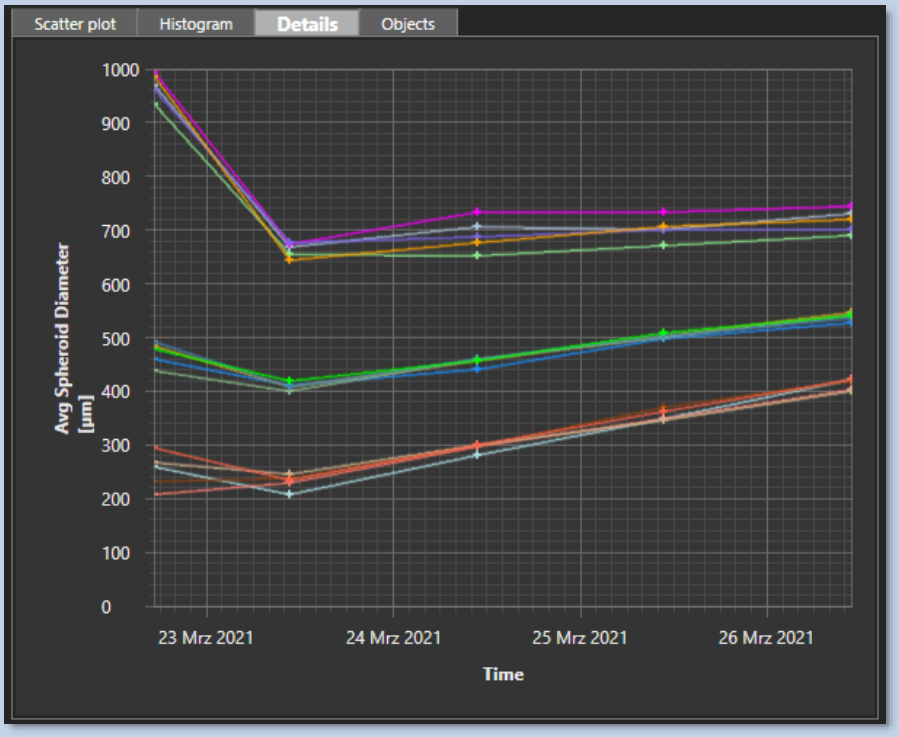


Fig. 26: Time chart
Single or several curves and fields may be selected by pressing CTRL and clicking on the required curve/field or dragging the mouse cursor to mark several fields at once and visualized under *Details*.

5.3 Gallery

Additional to the result values, you can generate a *Gallery* for each well that displays all images of this well (Fig. 29). To do this, activate the gallery in the middle of the *Analyst* tab below the Measurement box. You have to define the *Image size* in pixel. By activating *Preview*, you see the image section that will be exported to the gallery as a bold red box (Fig. 27). The value depends on the size of your spheroids. If the default value 768 pixel is too small and does not cover the entire cell area, you can increase the value. If the value is below 50, the software marks it with a red exclamation mark. However, the galleries will still be generated and can be exported. Disable the normalize check box of the brightfield channel in the visualization tab so that the dark core is clearly visible, and then click on *Create* to create the gallery. Once this is done, a *Gallery* tab appears on the left side and the pictures can directly be observed in the software. However, it only makes sense to open this tab if the image size is below 1024 pixel. Otherwise, the images are too big and cannot be properly displayed. Also, you can export the *Gallery*, this is described in the next section.

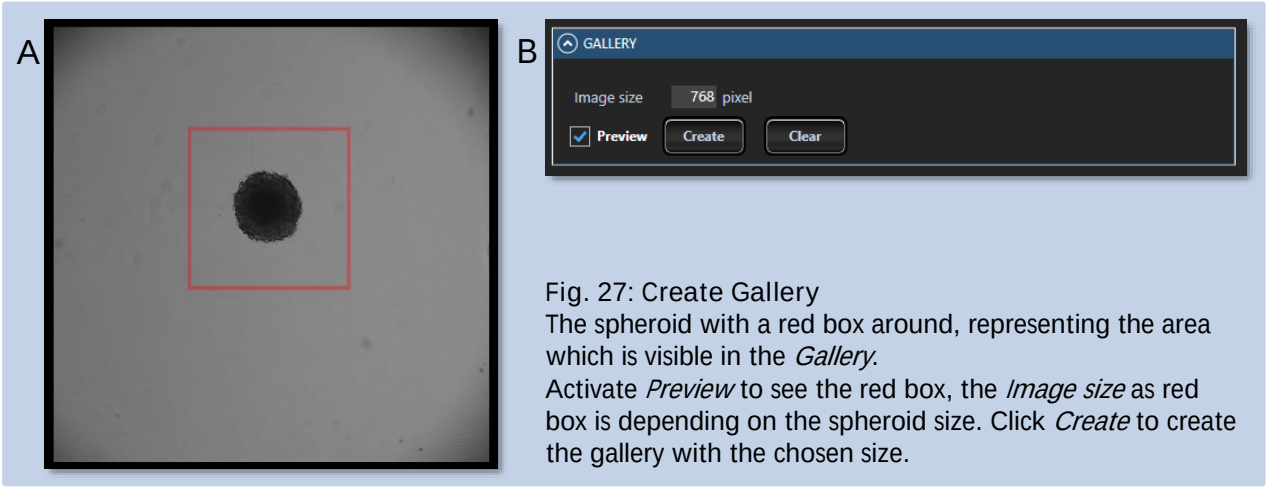


Fig. 27: Create Gallery
The spheroid with a red box around, representing the area which is visible in the *Gallery*.
Activate *Preview* to see the red box, the *Image size* as red box is depending on the spheroid size. Click *Create* to create the gallery with the chosen size.

5.4 Export

In case you are satisfied with your results, you can export your data and galleries using the *Export* button in the *Analyst* tab. You can either just export the results from the currently *Selected measurement* or from *All measurements* (Fig. 28). If you want to export all measurements, you can also choose between *All results in one file* or *One file per measurement*.

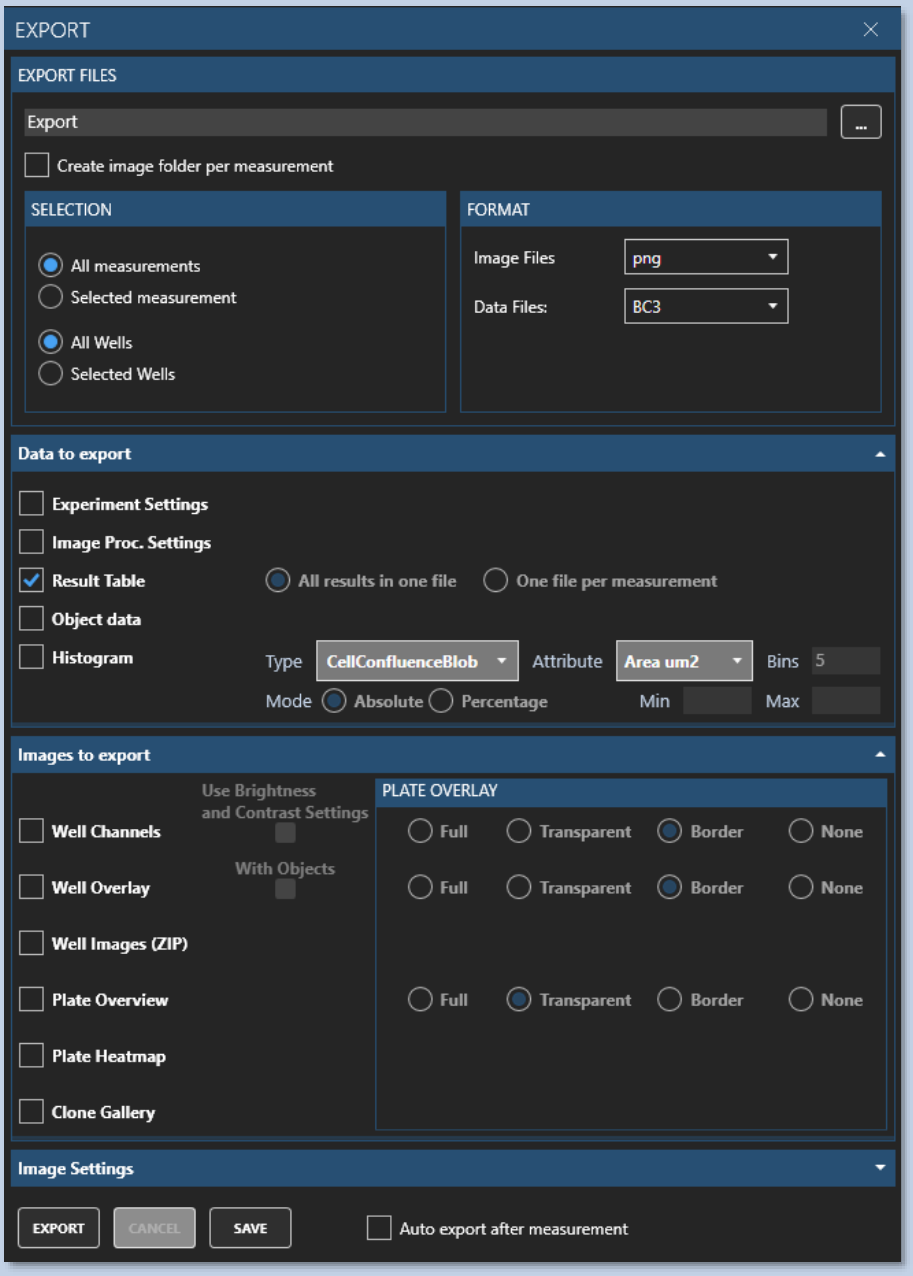


Fig. 28: Export
Click on *Export* in the *Analyst* tab. In the *Export* window you can choose which measurements you want to export and if you want one file for all measurements or single files for each measurement. You can also choose the format of the files and which data you want to export. If you want to export the *Gallery*, check *Clone Gallery*. When you have made your decision, click on *Export*.

You can export the galleries by clicking on *Clone Gallery* in the export window. You find the galleries also in the *Export Folder*. The filmstrip contains the images, the date of the experiment and the times of the different measurements (Fig. 29).

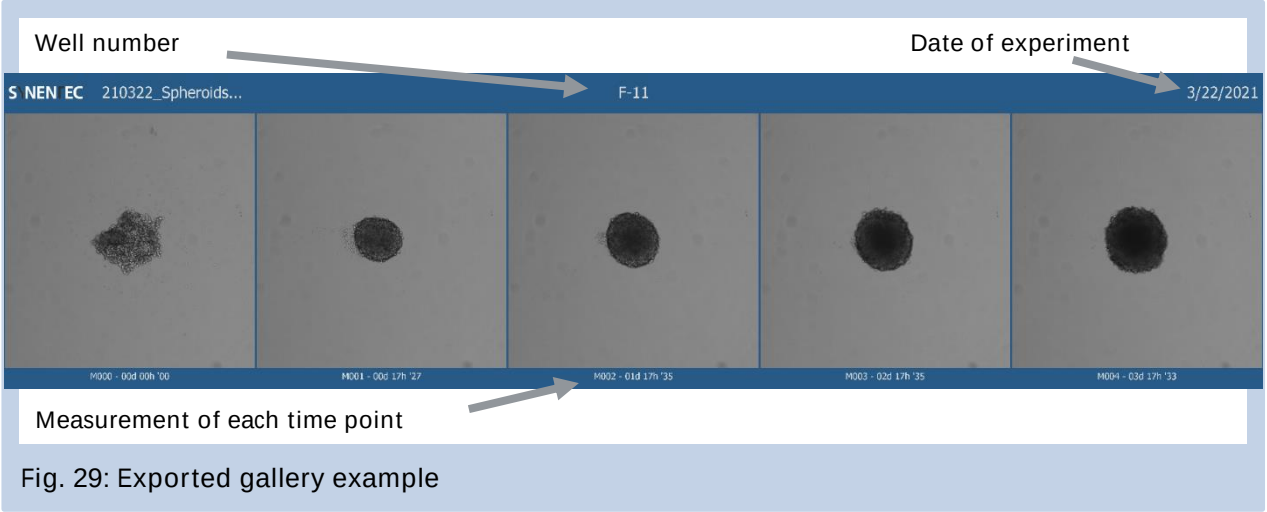


Fig. 29: Exported gallery example

References

[1] A. P. Kipp, S. Deubel, E. S. Arnér, and K. Johansson, "Time-and cell-resolved dynamics of redox-sensitive Nrf2, HIF and NF-κB activities in 3D spheroids enriched for cancer stem cells," *Redox Biol.*, vol. 12, pp. 403–409, 2017.

[2] N. Hedemann *et al.*, "ADAM17 inhibition increases the impact of cisplatin treatment in ovarian cancer spheroids," *Cancers*, vol. 13, no. 9, p. 2039, 2021.

SYNENTEC GmbH
Otto-Hahn-Str. 9A
25337 Elmshorn/Germany
Phone. +49 (0) 4121 463 11-0
Email. service@synentec.com