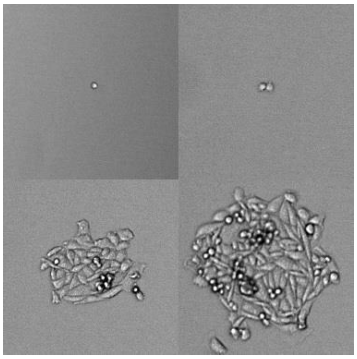
Experiment Guide – Single Cell Cloning with the CELLAVISTA®



Technical Note
TN-B002-XVII-08

The purpose of this document is to guide the user through the single cell cloning Image analysis. It does not contain any procedure of setting up a single cell cloning experiment itself since this may vary due to customer requirements.

Content

1. Measurement Setup

Please set up the measurement according to the operating guide for conducting brightfield measurements.

Here is just a short overview for the 'Prepare' dialog:

- Turn on the 'Liveview' and adjust the exposure with the sliders for Intensity [%], Exposure Time [ms] and Gain [%] while using the histogram (Fig. 1).
- The gain should be kept at lowest levels, unless necessary, to avoid digital noise.
- We recommend a grey value of 130 counts for the background in brightfield measurements.

Fig. 1 a: Underexposed image and corresponding histogram.

Fig. 1 b: Correctly exposed image and corresponding histogram.

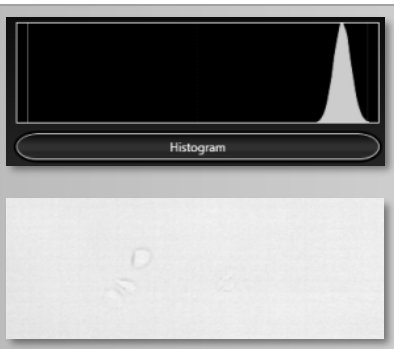


Fig. 1 c: Overexposed image and corresponding histogram.

- Define the focus-offset by moving the curser into the image and using the mouse wheel while holding CTRL.
- Cells are recognized in an optimal way when they appear with a dark rim at their edge and a bright center (Fig. 2).

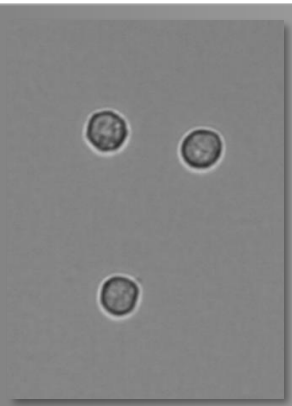


Fig. 2 a: No lens effect, cells appear dark with a bright rim.

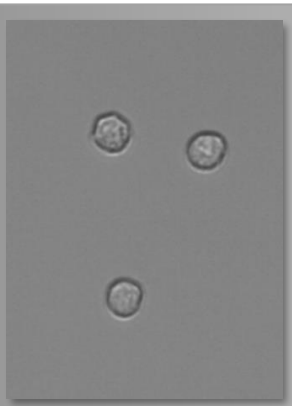


Fig. 2 b: No lens effect, cells are low in contrast and difficult to detect.

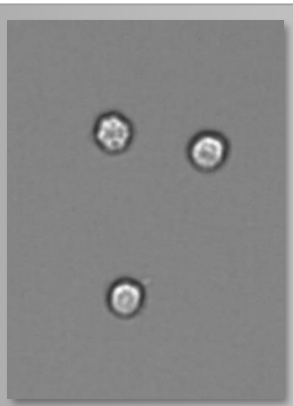


Fig. 2 c: Good lens effect, cells are bright with a dark rim, rich in contrast to the background.

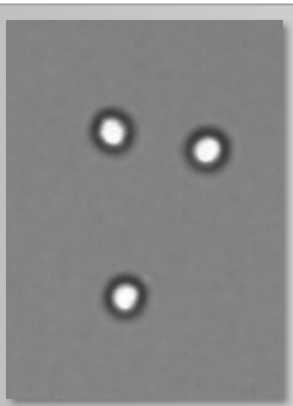


Fig. 2 d: Cells are bright with a dark rim, but out of focus.

- Use the navigator to check the exposure and focusing settings at the well edges and also for different wells across the plate, especially at later stages of incubation (evaporation).

1.1 Image Processing During Measurement

During routine usage, CELLAVISTA® performs the image processing in parallel to the image acquisition. In case that the first and second measurement is done for the first time, the best parameters have to be established post measurement and then to be stored as a template for future measurements.

2. Evaluation - Tips for Image Processing

Go to the evaluation tab by clicking on the appropriate arrow in the upper area of the screen (Fig. 3).

Fig. 3: Proceed with the evaluation.

In many cases the default parameters will lead to good results. But depending on the circumstances of culture, culture carriers and cell line, some tweaking might be necessary (Fig. 4).

2.1 Parameter Settings

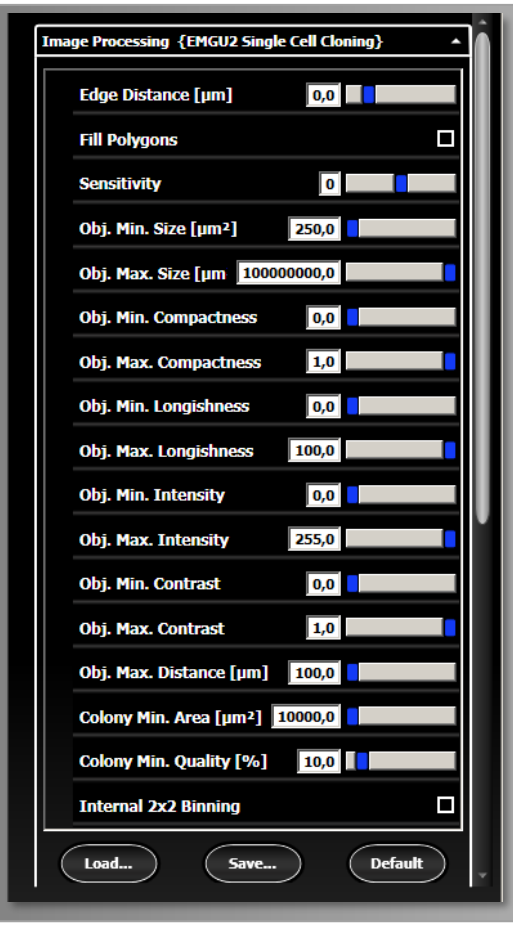


Fig. 4: Table of default parameters for single cell cloning.

In the following the most frequently used parameters will be explained.

2.1.1 Edge Distance

Sometimes artifacts (e.g. glue) at the edges of the wells cause wrong results for certain attributes. To overcome this Edge Distance can be used to exclude the objects within a given distance from the edge of the well (Fig. 5). The higher the value of Edge Distance the less area per well will be evaluated.

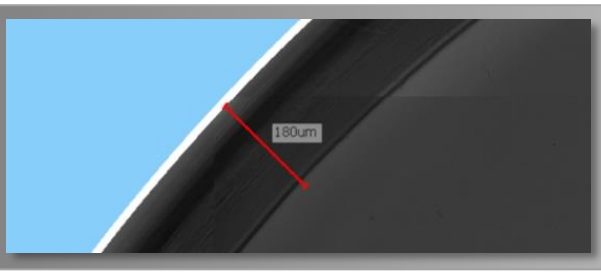


Fig. 5: Edge distance.

2.1.2 Fill Polygons

If the Fill Polygons option is selected, detected cells are filled entirely with color to facilitate their visualization (Fig. 6).

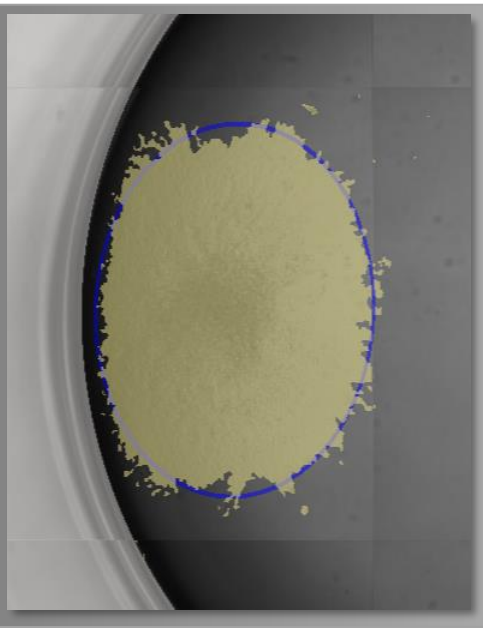


Fig. 6: Fill Polygons of a colony.

2.1.3 Sensitivity

This Parameter determines the sensitivity of the cell detection. In case the images have a too low intensity of the signal e.g. 70 it is possible to overcome this by changing Sensitivity. The average brightness of each image should be at 130 to get best results in image processing.

A reduction of the parameter increases the insensitivity of the image analysis, so that contrast weak objects (e.g., fragments of dead cells and other background objects) can be excluded from the analysis (Fig. 7a + 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.

The Sensitivity can be set from -10 to 10.

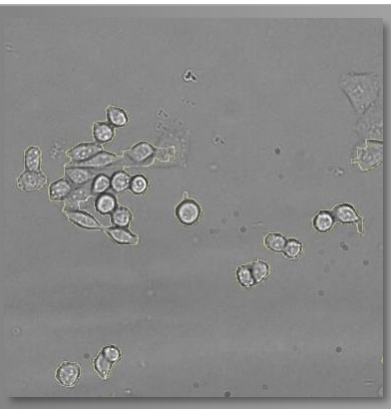


Fig. 7a: Sensitivity '-8'.

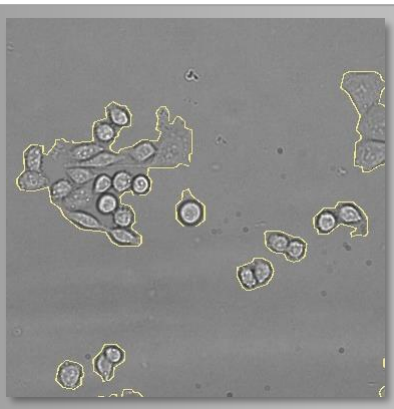


Fig. 7b: **Sensitivity '0'**.

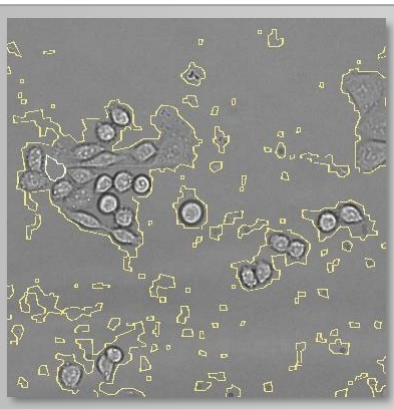


Fig. 7c: **Sensitivity '6'**.

2.1.4 Object Min. Longishness [%]

The Longishness parameter describes the form of an object and is used to exclude undesired objects e.g. lints in the plate bottom. Therefor 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. This parameter can be set from 0 to 100.

Fig. 8a: Obj. Min. Longishness. set to 25: Object excluded

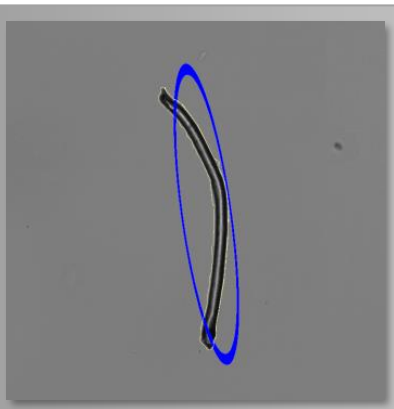


Fig. 8b: Obj. Min. Longishness. set to 10: Object detected

<u>Example</u>:
An object, e.g. lint, is 5 times as long as wide

→ The ratio is 1/5 ≙ 20 %
→ The Longishness of the object is 20 %
→ If the Obj. Min. Longishness parameter is set to 25 %, the object will be excluded.

2.1.5 Object Max. Distance [µm]

Object Max. Distance is used to merge separated colonies that have grown from a single cell. This is useful after tracking a colony back over time (an experiment with multi measurement is required) and sets up the image processing accordingly to detect a single colony or two colonies (Fig. 9a + b). The range is 0 to 1000000 µm.

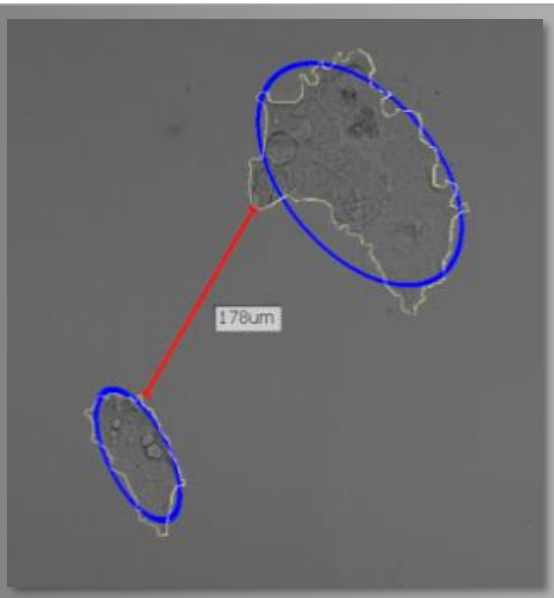


Fig. 9a: Unmerged colonies.

Fig. 9b: Merged colonies.

2.1.6 Colony Min. Quality [%]

This parameter is the ratio of the sum of all cell areas detected in a colony to an ellipse created around the colony. The Colony Min. Quality parameter indicates how much of the ellipse is covered by cells. The higher the parameter is set the more cell area has to cover the colony area (ellipse) to be counted as a colony (blue circle). If the parameter is set to 50, 50 % of the ellipse has to be covered with cell area. The range is 0 – 100 %.

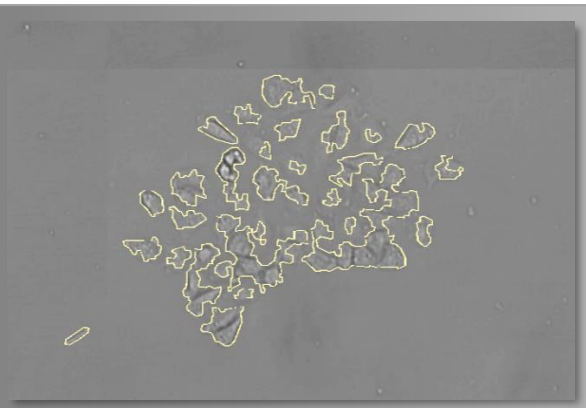


Fig. 10a: Colony Min. Quality set to 40.

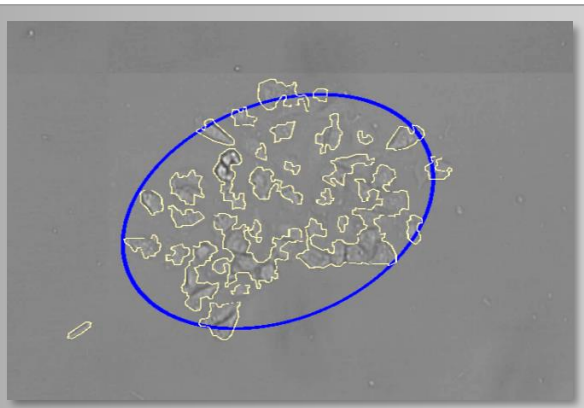


Fig. 10b: Colony Min. Quality set to 10.

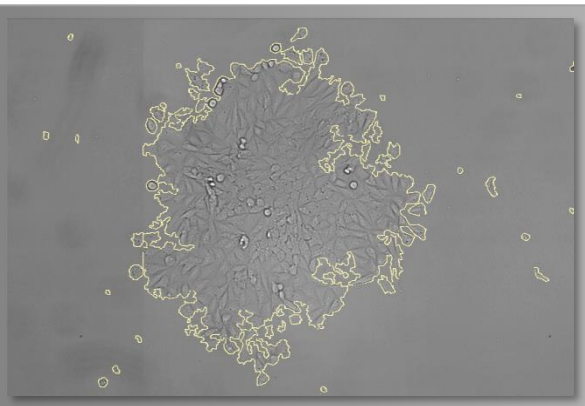


Fig. 11a: Colony Min. Quality set to 90.

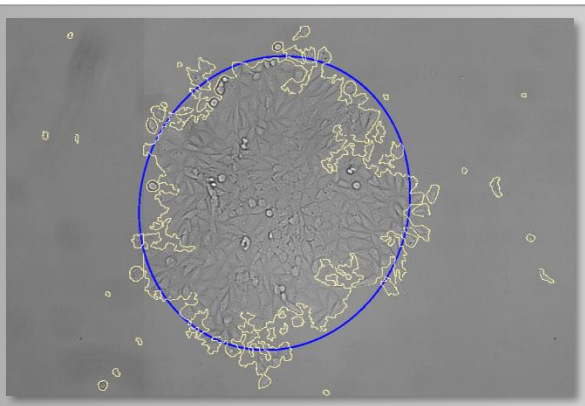


Fig. 11b: Colony Min. Quality set to 40.

2.1.7 Colony Min. Area [µm²]

This parameter defines the minimum threshold value (cell area in µm²) for the colony detection (blue circle). Different growth rates require different settings of Colony Min. Area. The value depends on the generation time (doubling time) and the period of time between imaging. This parameter can be set from 0 to 1000000000 µm². The images show a colony with a size of 256475 µm².

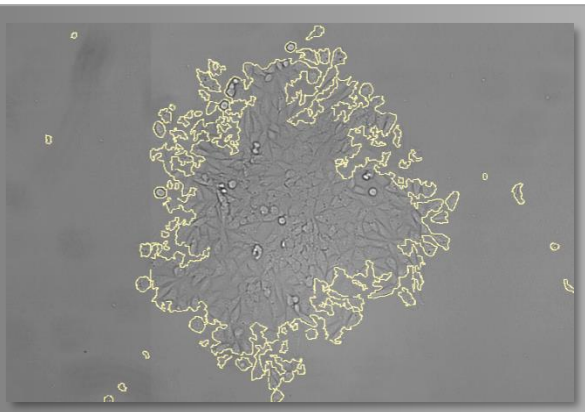


Fig. 12a: Colony Min. Area set to 300000.
The cell area is not detected as a colony.

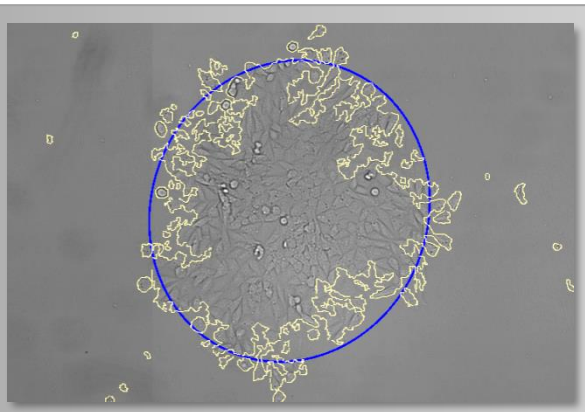


Fig. 12b: Colony Min. Area set to 20000.
The cell area is marked as a colony (blue).

3. Filtering and Exporting

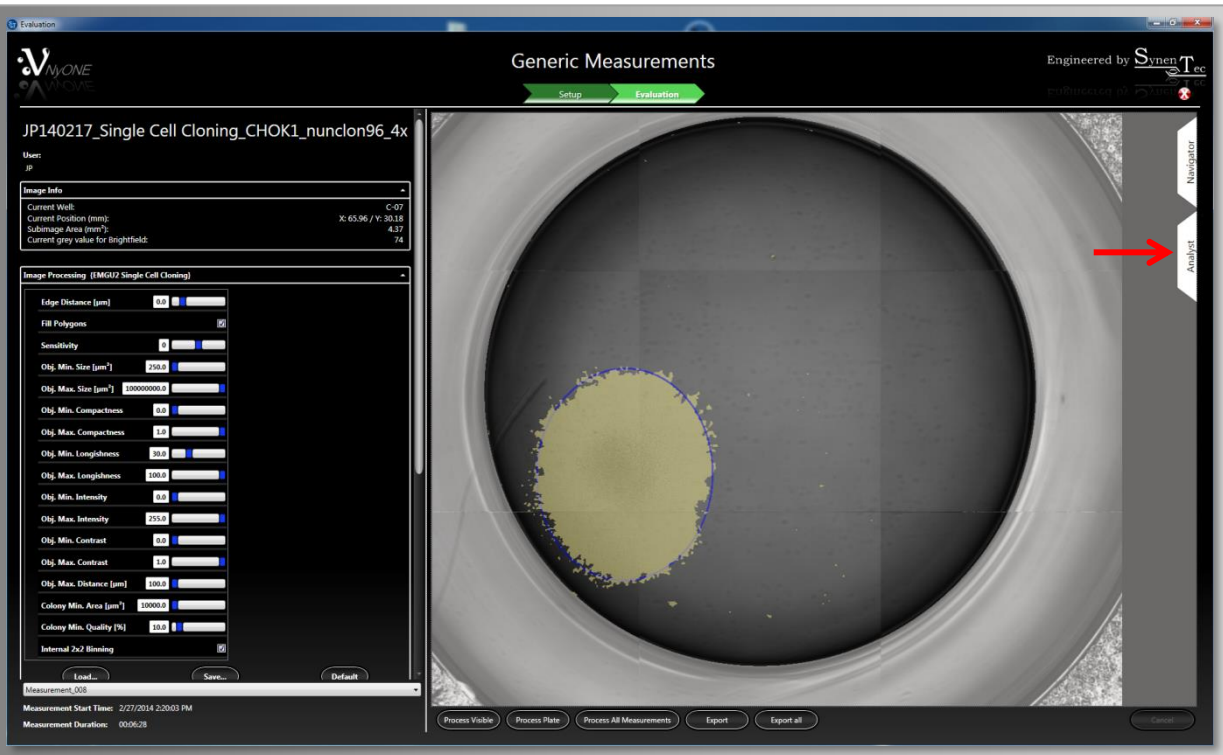


Fig. 13: An example for a single colony in a well.

3.1 Getting an Overview

By clicking on the 'Analyst' tab on the right upper side (Fig. 13 - red arrow) the heat map, the result table and the time charts are displayed. For each type of result presentation different properties like 'Cell Confluence' or 'Colony Count' can be chosen.

3.1.1 Heatmap

By choosing 'Heat map' tab and selecting 'Colony Count' in the dropdown menu underneath the heat map, a colored overview is displayed. Blue represents a low and red a high number of the chosen property. Now it is possible to click on the wells and quickly review the images (Fig. 14).

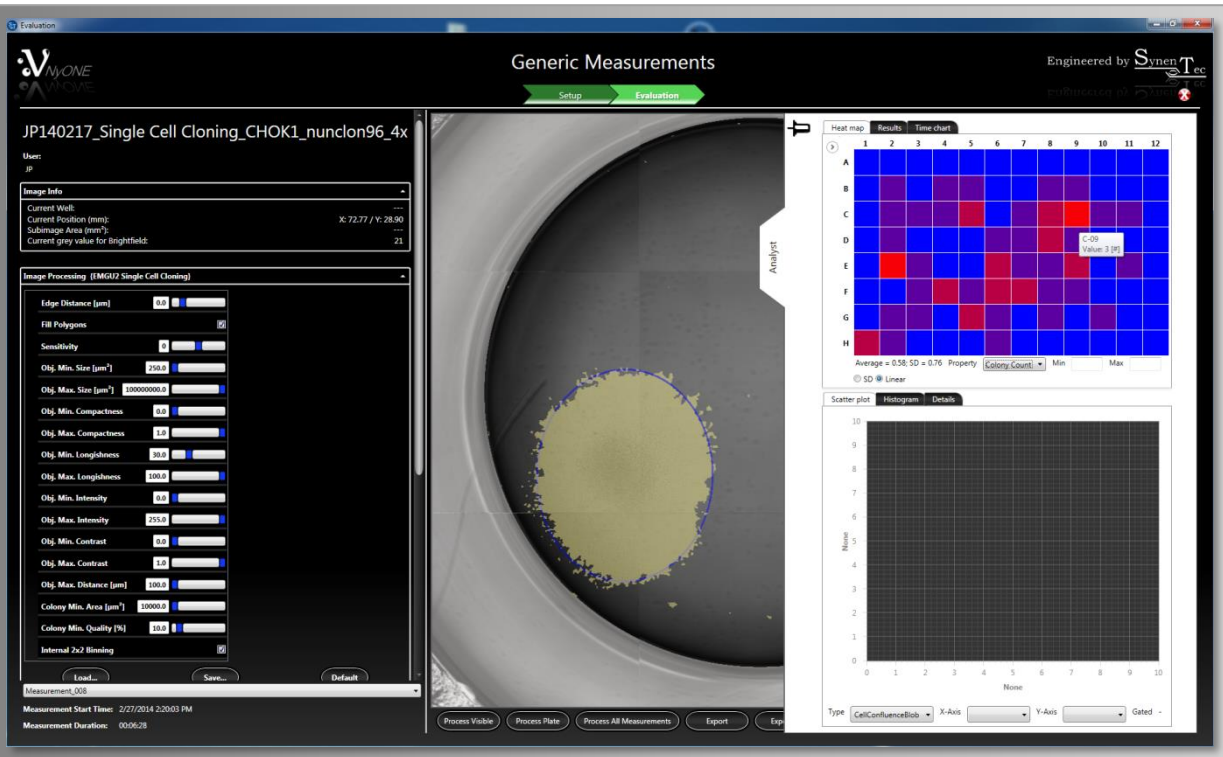


Fig. 14: The colored heat map overview and one chosen well.

3.1.2 Result Table

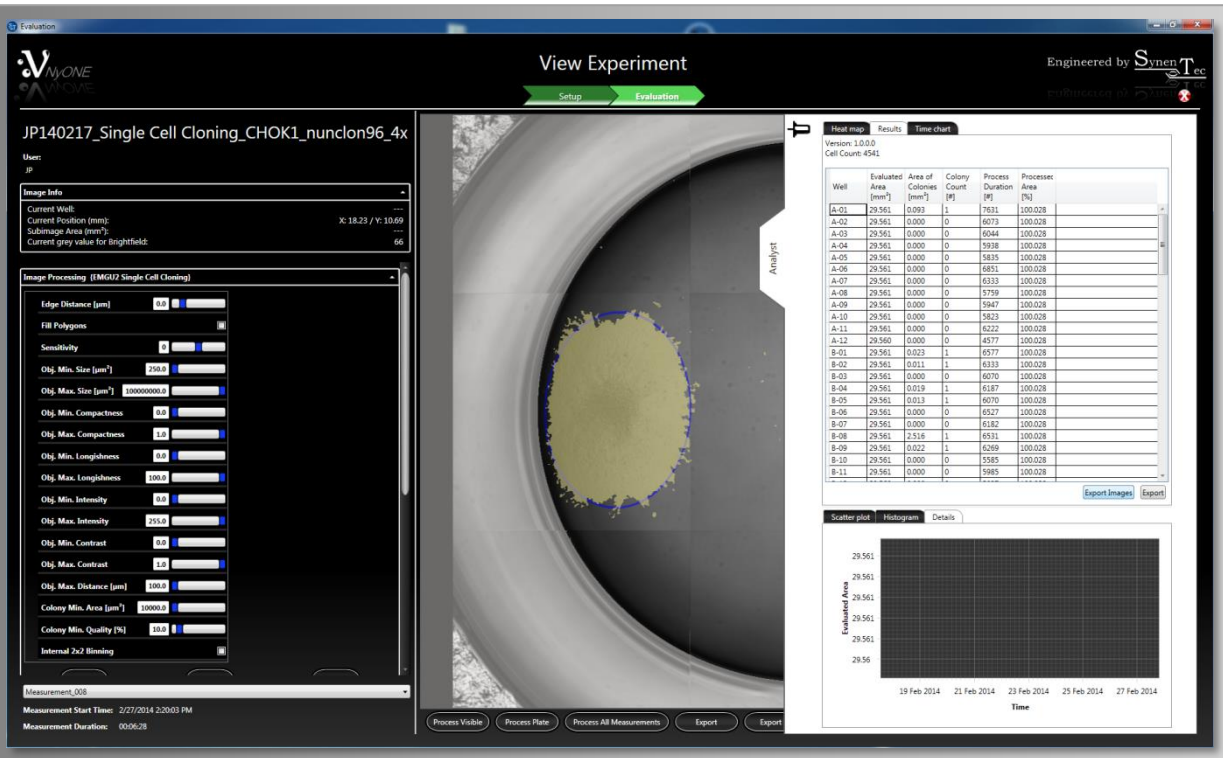


Fig. 15: Result Table.

The adjacent tab of the heat map shows a table with all attributes and results (Fig. 15). By right clicking on an attribute in the result table, a filter can be set to exclude undesirable wells (Fig. 16 – 18).

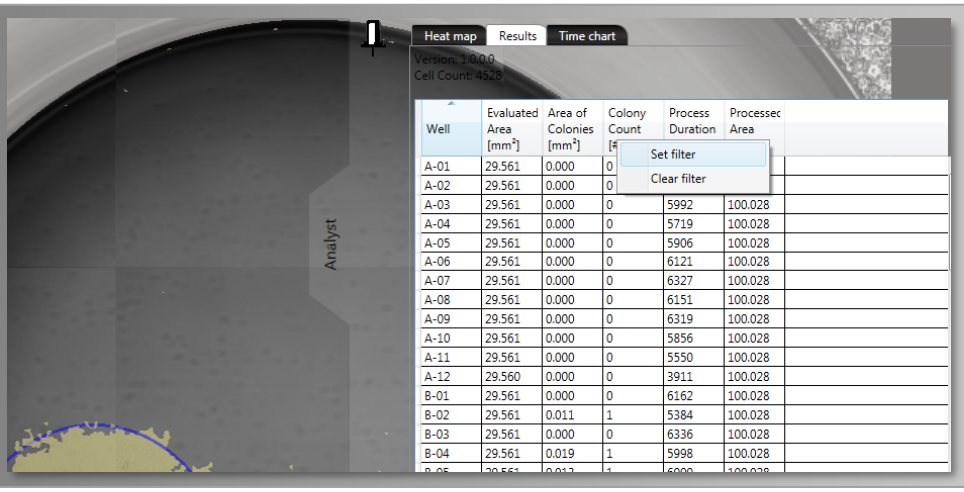


Fig. 12: Right mouse click for filtering.

Fig. 13: Setting filter criteria.

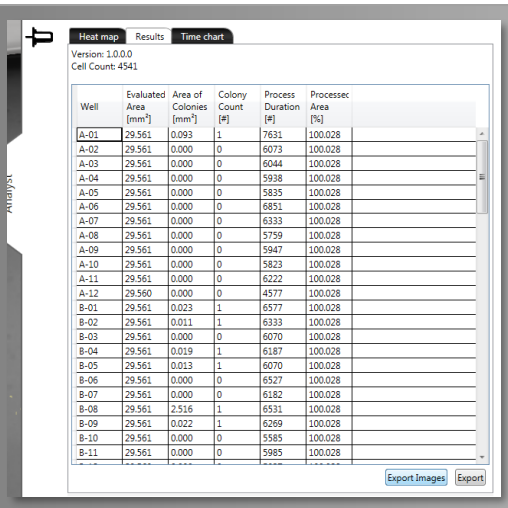


Fig. 14: Filtered result list.

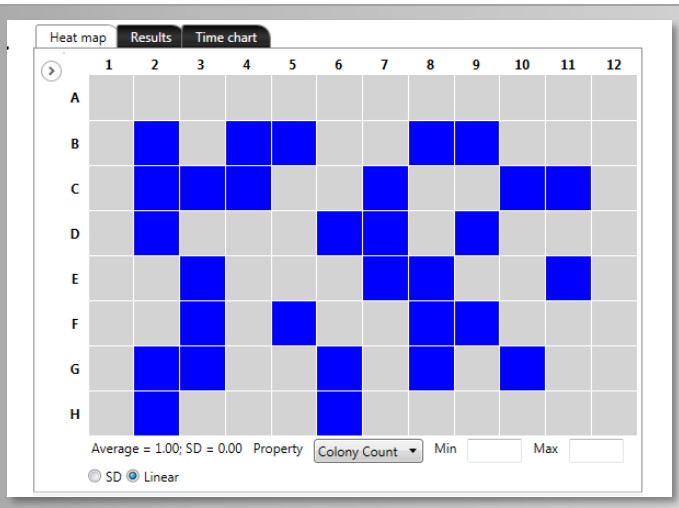


Fig. 15: The resulting **"Heat Map"**.

3.1.3 Time Charts

The YT-software® enables the compilation of measurements in an existing experiment. The third tab 'Time chart' allows and represents the results of all measurements over e.g. several days and also presents details in the lower right corner for each well by simply clicking on the appropriate curve (Fig. 20).

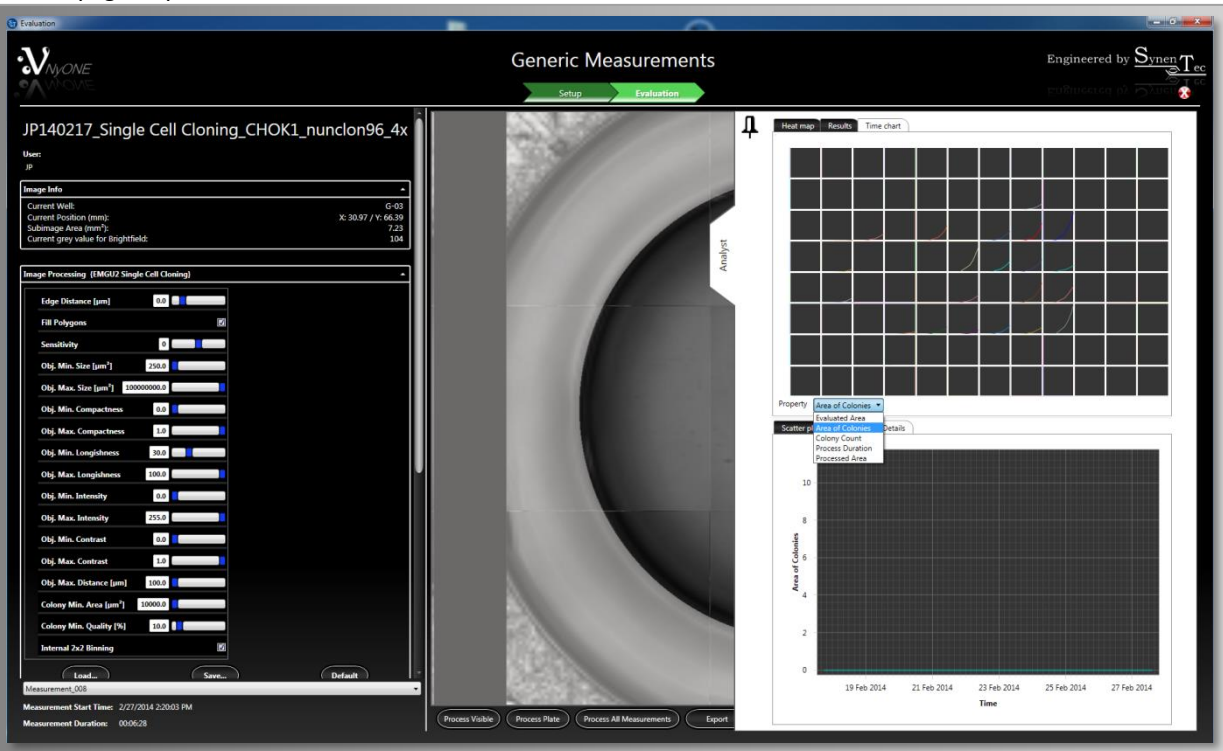


Fig. 20: Growth Curves and Measurements.

Select the attribute of interest to get a time chart of each well (Fig. 21).

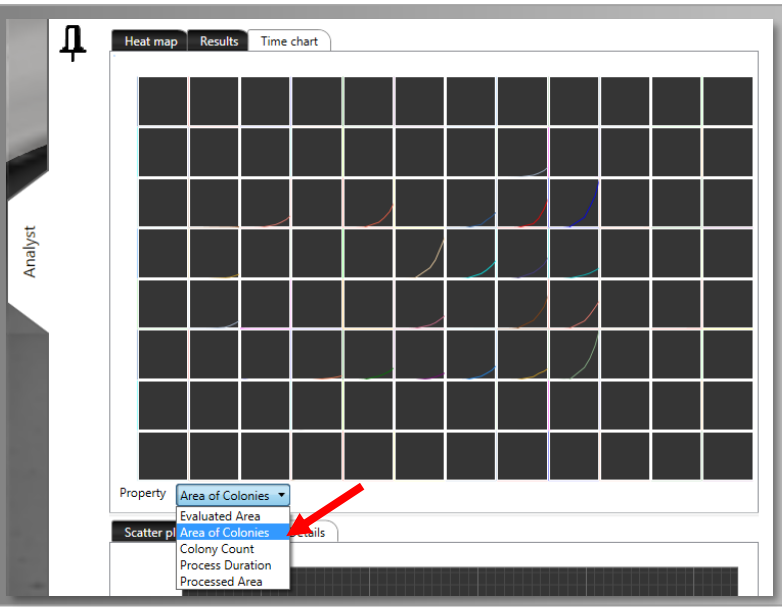


Fig. 21: Getting a time chart.

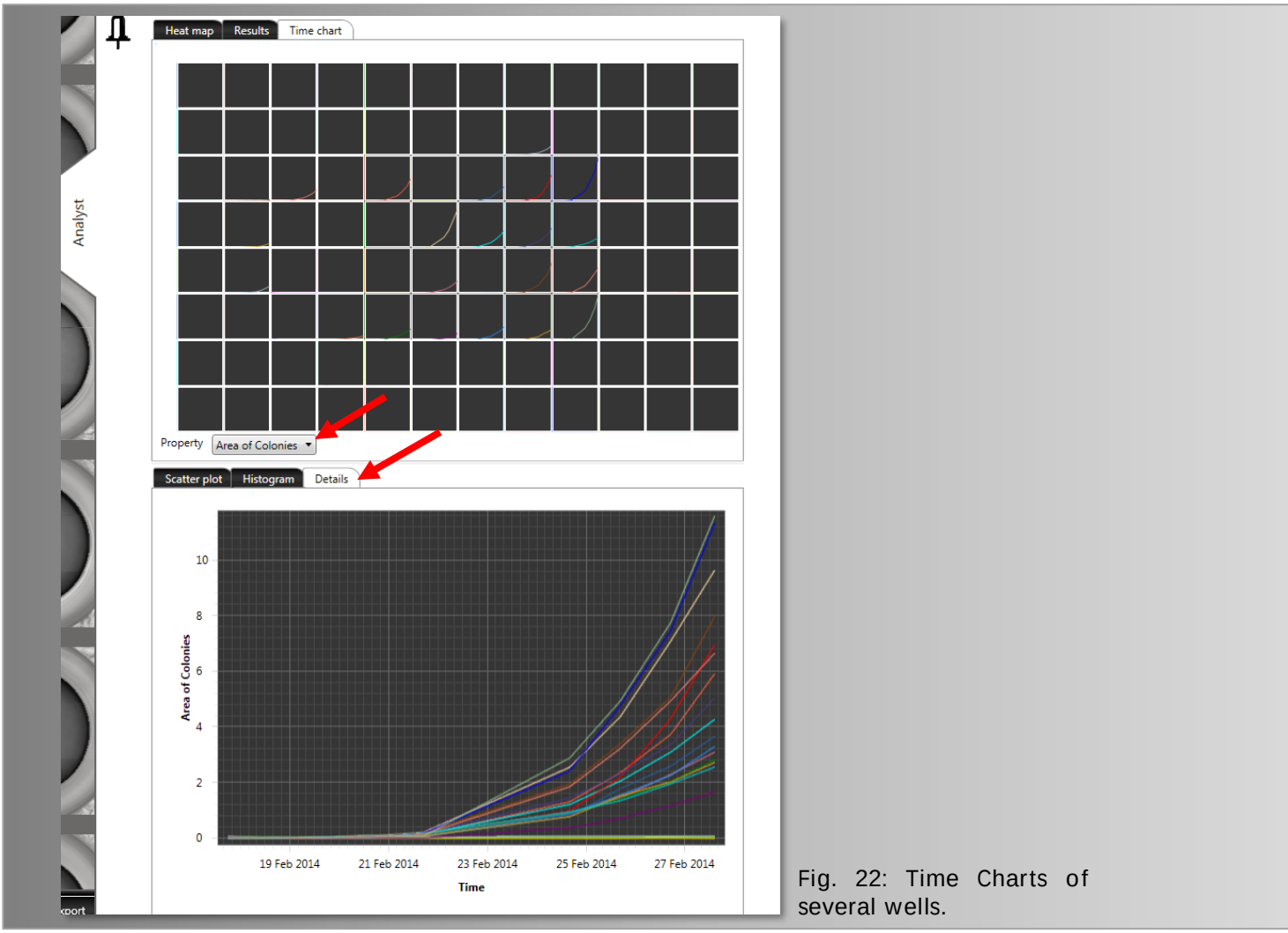


Fig. 22: Time Charts of several wells.

When clicking on one or more wells (to add more wells, drag the mouse or use "Ctrl" key) the charts will be shown in detail below the plate over view (Fig. 22). A typical attribute is "Area of Colonies", which represents the size of the colonies in square millimeters.

3.2 Excluding Wells for Subsequent Measurements

The amount of wells to be scanned for future measurements can be reduced by using the filter options shown in chapter 3.1.2 to save time and data storage space.

After getting the appropriate filtered well list, perform a right mouse click in the heat map and click on 'Use filtered wells for measurement' (Fig. 23) and save the experiment in 'Setup'. Now, in all subsequent measurements only the filtered wells will be imaged.

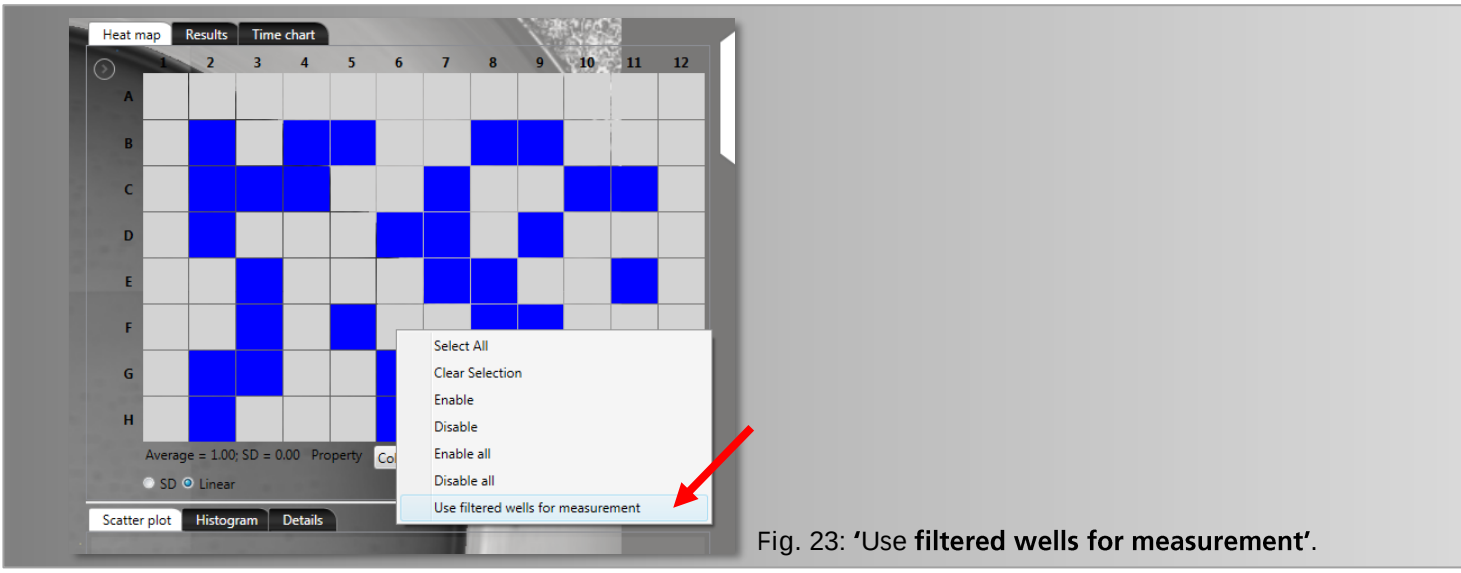


Fig. 23: **'Use filtered wells for measurement'**.

3.3 Creating a Clone Gallery

If the evaluation of all measurements is completed successfully, it is possible to create a so-called Clone Gallery. This 'Clone Gallery' shows one image of each measurement for each found colony in a survey. In that way it is possible to easily and quickly get an overview of all wells over time. The Clone Gallery feature determines the center of each colony found and displays images from this location from seeding day onwards. In this manner the backtracking of the colonies' origins is facilitated without switching between measurements. The Clone Gallery also provides the possibility to discard wells with non-clonal colonies (e.g. because of doublets).

The gallery is created in the 'Gallery' tab on the right hand side (Fig. 24 – red arrow).

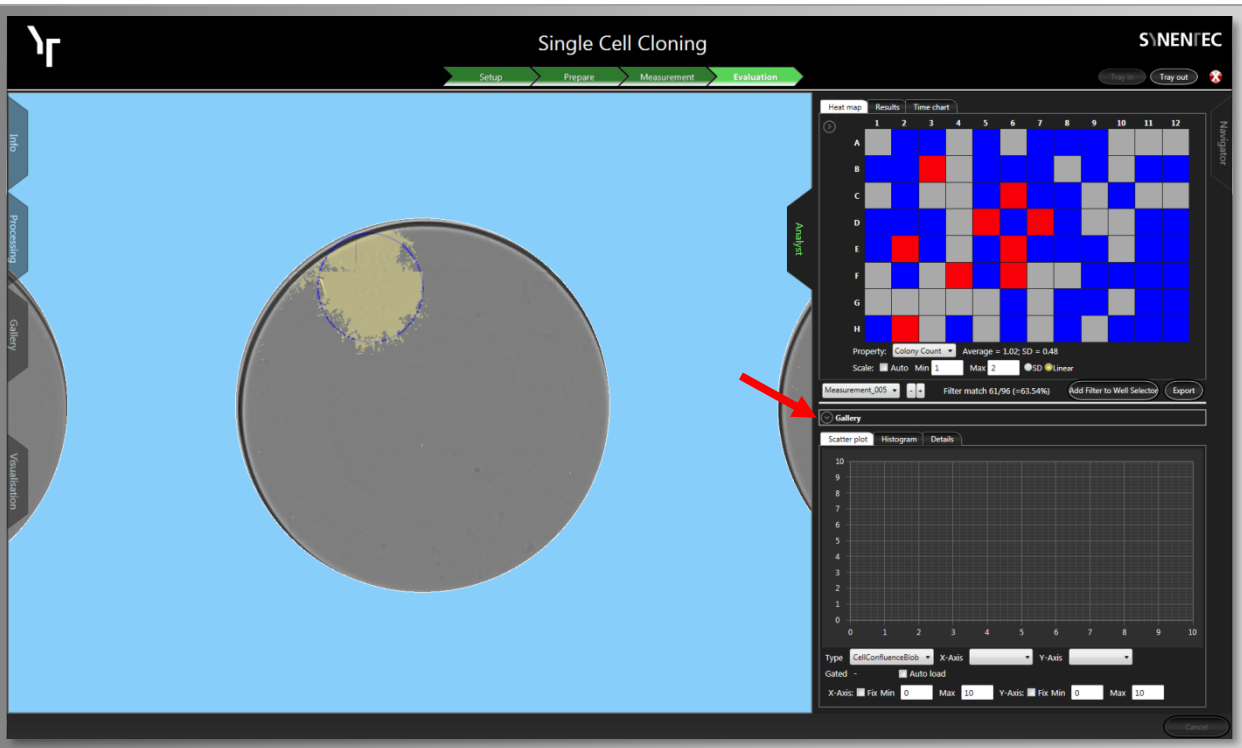


Fig. 24: Open the Clone Gallery creation tab.

It is possible to choose the size per image in pixel (Fig. 25). Images with a size of 1024 pixels are recommended to ensure a good and meaningful overview, e.g. displaying the single progenitor cell of the colonies. When 'Preview' is checked, a preview of the size is displayed on the colonies of the current image. Clicking on 'Create' starts the Clone Gallery creation.

Fig. 25: Opened gallery creation tab.

The duration/progress of the generation is displayed at the bottom of the bar (Fig. 26 – first red arrow). When it is finished the gallery can be opened with the tab on the left side of the software (second red arrow).

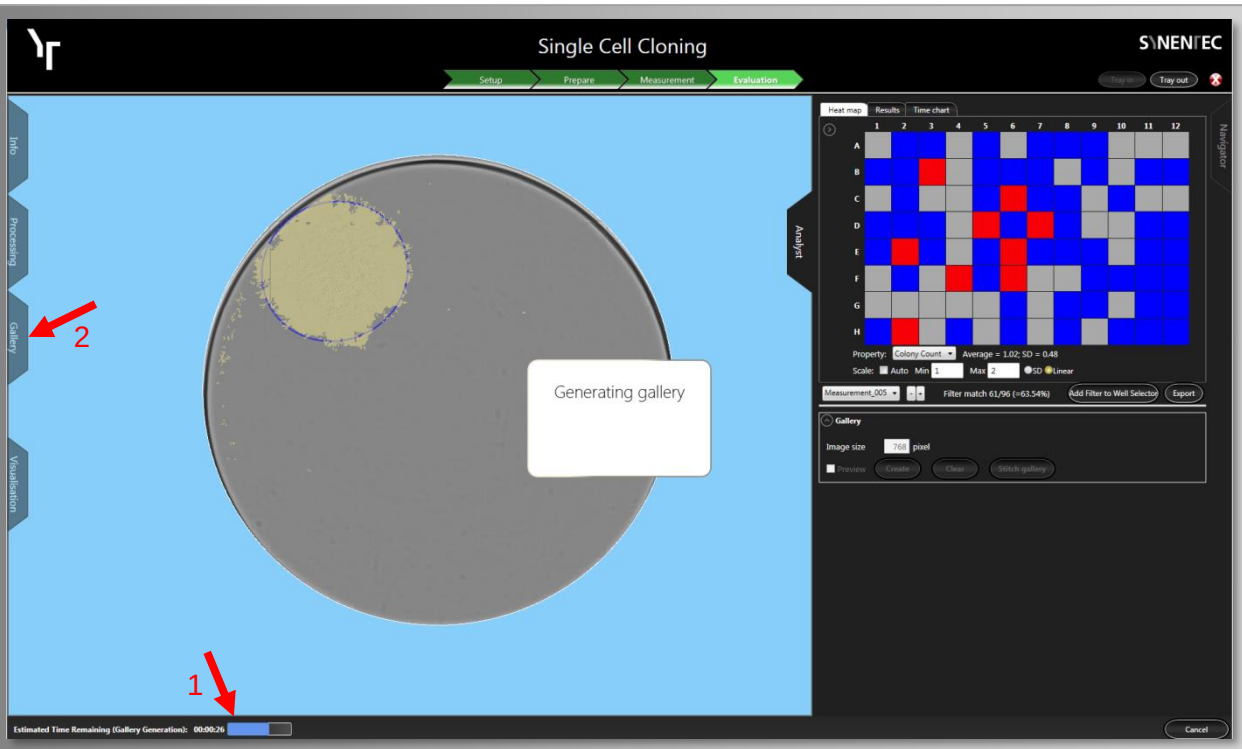


Fig. 26: Generating the gallery.

Each colony is displayed individually when the corresponding well is selected. On top of the gallery tab, the well is reported in which the colony is located (here, well A2) (Fig. 27 - red arrow and Fig. 28) showing the number of colonies within this well (last number, here 2) and which of the colonies is concerned (here 1).

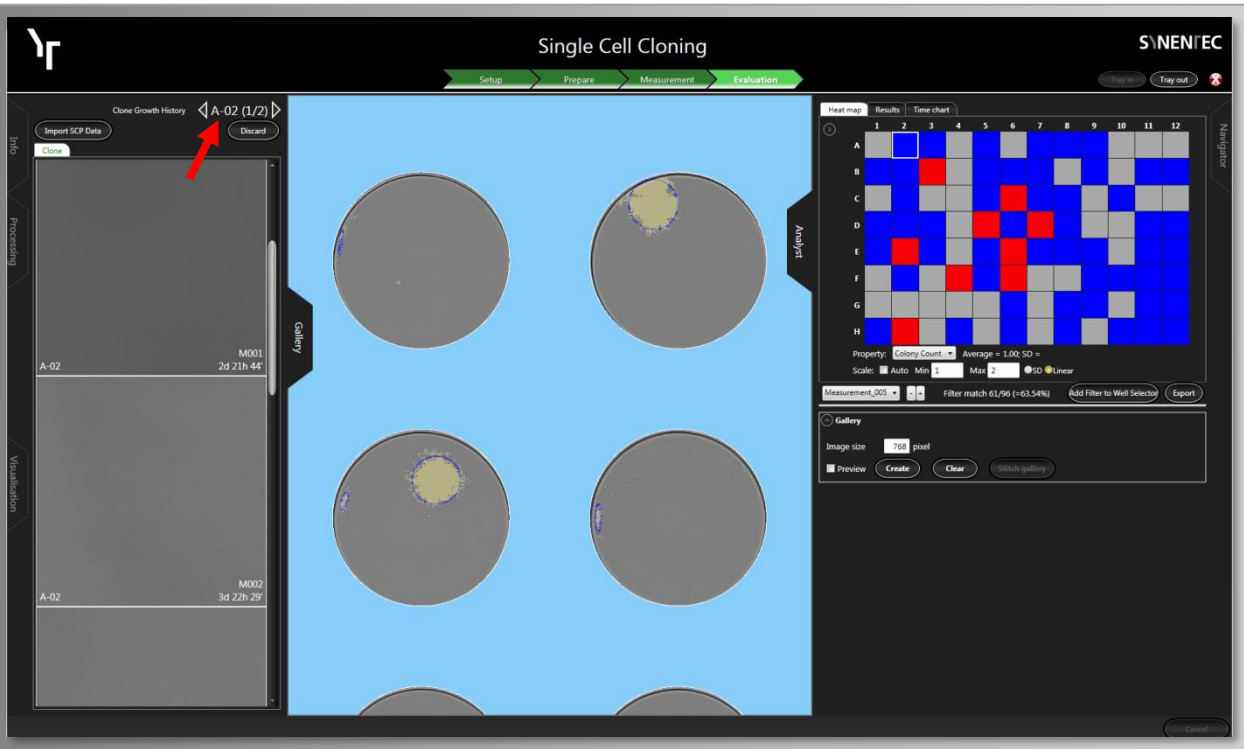


Fig. 27: Clone gallery.

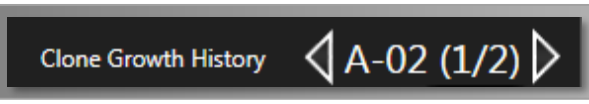


Fig. 28: Navigation through the gallery.

With the arrows on the left and the right hand side of the colony identification, it is possible to switch between colonies/wells to check whether there is a single cell in the seeding day images. The current colony is also shown in the plate overview in the middle of the software.

Upon a click on one of the images in the survey, the well is shown in the image area of the software. The software will navigate to the respective measurement, well and location within the well.

If a well in which a doublet can be seen at seeding day or if the images are doubtful, the well can be excluded by clicking on 'Discard' (Fig. 29). The images are then red-crossed (Fig. 30) and excluded from the results and the exporting.

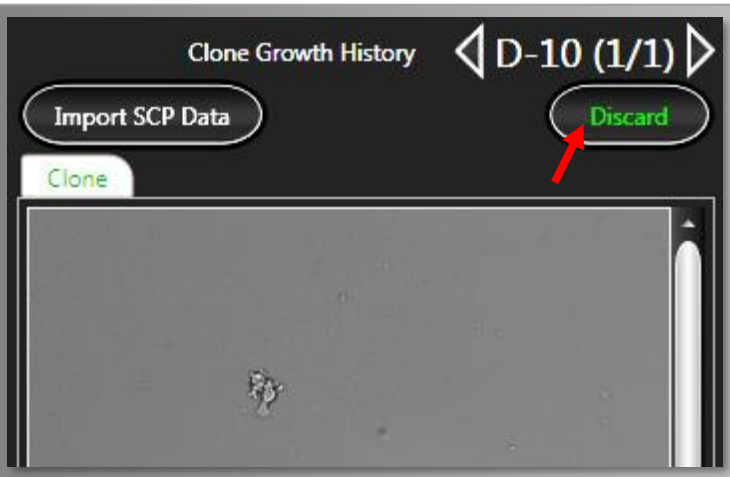


Fig. 29: Discard wells containing more than one cell.

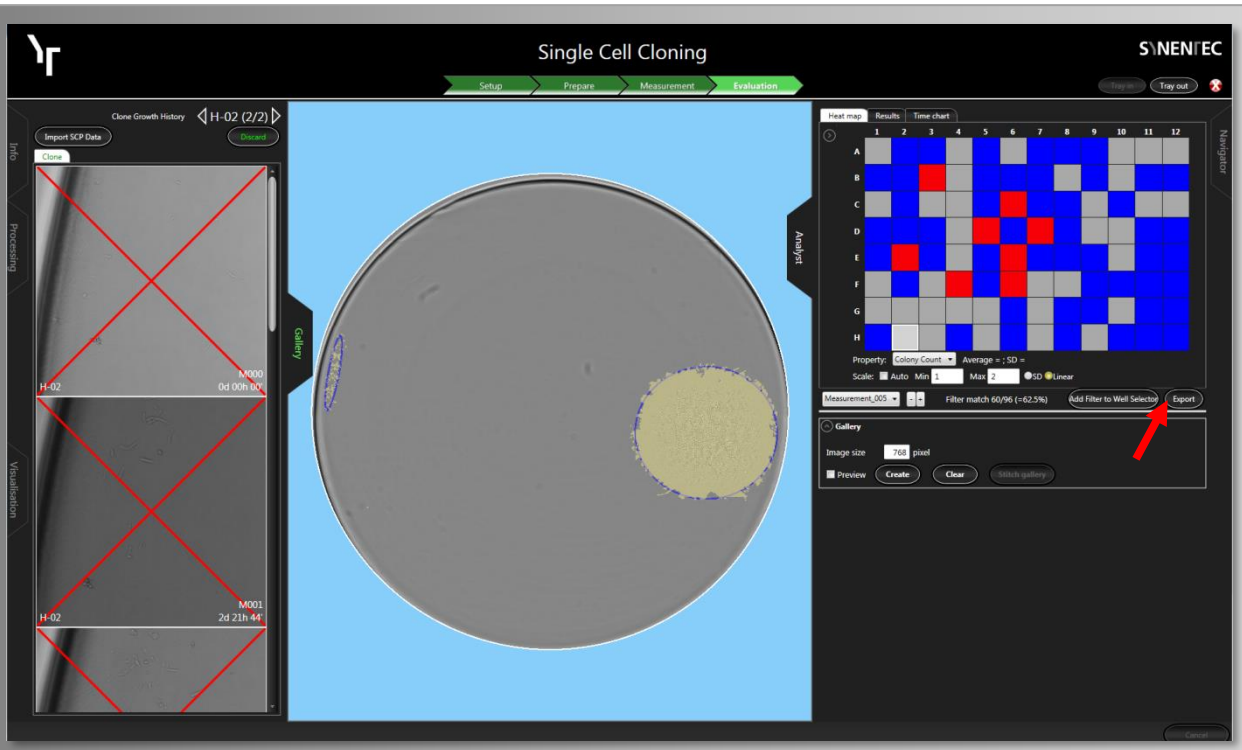


Fig. 30: A discarded well.

After review of the images and results it is possible to export e.g. the result table, the images or the gallery.

3.4 Export

On the right side, below the heatmap is an 'Export' button to export all results, data, images and overviews (Fig. 30 – red arrow). Clicking the button opens the following export dialog (Fig. 31):

Fig. 31: The export window

For more detailed information about selective exporting options, please refer to the operating guide.

3.4.1 Clone Gallery

If the 'Clone Gallery' feature was used, it is also possible to export comprehensive overviews for each well/colony (Fig. 32). These provide detailed information about the experiment, well, the date of the first measurement and the duration from seeding day per gallery image.

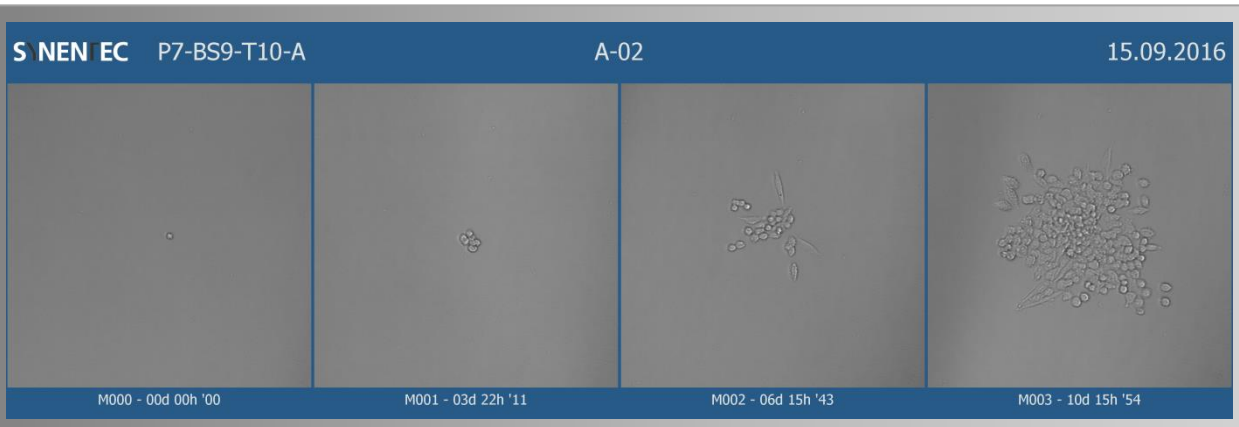


Fig. 32: Exported colony overview

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