SYNENTEC

Experiment Guide – Wound Healing

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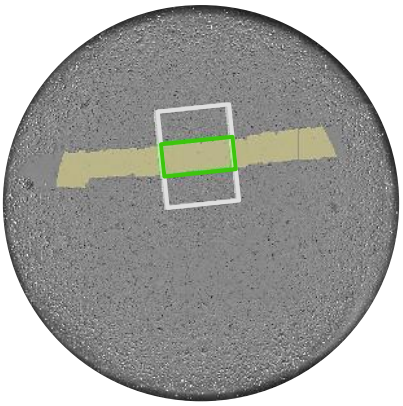
ABSTRACT

The purpose of this document is to guide the user through the experiment setup for the *Wound Healing* application. It does not contain any procedure of setting up a wound healing 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 Wound Healing Applications. 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 wound generation. The following formats generated good results in our hands:

Assay Type	Wound Generation	Insertion	Plate Format	Comments
Scratch	Pipette tip	-	96 well	
Oris™ Cell Migration Assay	Inserts	Pre-inserted	96 well	Different surface treatments available
Ibidi® culture-inserts	Inserts with 2, 3, or 4 wells (cavities)	Pre-inserted	µ-dishes	Carrier for µ-Dishes is available from SYNENTEC*
	2 well inserts	Pre-inserted	24-well	
	2 well inserts	Self-insertion	24-well	
	4 well inserts	Self-insertion	12-well	

* Six µ-Dishes can be fixed in this carrier and measured in NYONE® or CELLAVISTA®. Please contact us, if you are interested in this product.

Handling of the microplates: The clear bottom of microplates must never be touched with fingers (not even with gloves) at any time before measurement. Place your thumb and fingers at the rim on the longer side of the plate. The transparent bottom is part of the optical path and may lead to erratic measurements if stained with finger prints or dirt.

1.2 Devices to Generate Wounds

As mentioned above, different devices can be used for wound generation. Prepare your microplates for the wound healing assay as described in the following sections.

1.2.1 Scratch Assays

The easiest and cheapest way are scratch assays, in which the wound is generated by manual scratching. This can be done by using a 100 µL pipet tip. However, professional wound makers are also commercially available (e.g. IncuCyte™, WoundMaker™, BioTek®, AutoScratch™ or robotic pin tools).

1.2.2 Ready-to-use Sample Carriers with Pre-inserted Silicone Inserts

Examples are:

- Oris™ Cell Migration Assay (Platypus, Cat. No. CMA1.101, CMA5.101)
- Culture-Insert 2 Well µ-Dishes 35 mm (Ibidi®, Cat. No. 81176)
- Culture-Insert 4 Well µ-Dishes 35 mm (Ibidi®, Cat. No. 80466)
- Culture-Insert 2 Well 24-well plate (Ibidi®, Cat. No. 80241)

1.2.3 Silicone Inserts for Self-insertions

Examples are:

- 25 Culture-Inserts 2 Well for self-insertion (Ibidi®, Cat. No. 80209)
- 25 Culture-Inserts 4 Well for self-insertion (Ibidi®, Cat. No. 80469)

The surface of the microplate can be coated or tissue culture-treated and needs to be dry, flat, and clean. The inserts have a sticky underside. Transfer the inserts to empty wells by holding them at the upper side with sterile forceps. Push the insert with sterile forceps and ensure the inserts are sealed by turning around the plate and checking the bottom. After insertion, pre-warm the plate at 37 °C. The inserts have to be properly sealed to prevent the cells from growing under the inserts.

1.3 Procedure

After the previously described microplate preparation, perform the wound healing assay according to your protocols. 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 5 mL medium
6. Count the cells with the Trypan Blue Application of YT®-software
7. Prepare a cell suspension with the optimal cell density (example for H6c7-Kras: $1 \cdot 10^6$ cells/mL for 100 µL/cavity of a 4 well insert or a well of a 96-well plate and 66.7 µL/cavity of a 2 well insert)

The optimal cell density depends on the cell line and the assay format and has to be determined by the user. The cells should be 100 % confluent at the day of the wound healing assay.

8. Incubate the cells for 24 h at 37 °C in the incubator
9. Create the wound by scratching with a 100 µL pipet tip or by removing the inserts
10. Aspirate supernatant
11. Wash the cells twice with pre-heated serum-free medium
12. Add serum-free medium
13. Optional: remove air bubbles by centrifugation (200 x g, 3 min)
14. Image the cells shortly after creating the wounds/removing the inserts (time point 0 h)
15. Treat the cells e.g. with cytochalasin D
16. Image the cells in an interval of 2 h till the wound is closed (untreated cells)

The creation of the wounds leads to an increase of cell debris. Ensure to remove it by washing the cells. Otherwise, remaining debris or non-adherent cells in the wound gap will interfere with the image analysis and might lead to false results.

Further comments/tips to the different steps:

7. Make sure to use the same cell density for all your experiments, as it largely affects the results.

8. The time depends on the used cell lines. In our hands, 48 h with a lower cell density often generated a more uniform monolayer than 24 h with a higher cell density.

9. For scratch assays, create the wound by scratching with a yellow pipet tip and using the lid as a ruler. When you remove inserts, grab and remove them in a quick move using sterile forceps. Attention: be careful not to detach the cells by moving the insert over the bottom.

10. Make sure not to aspirate the cells.

11. This step is essential (see below) to remove remaining cell debris and non-adherent cells.

12. Serum-free medium reduces the effect of proliferation to ensure that mainly migration is analyzed. However, serum-containing medium can also be used if you are interested in the combination of both processes. Moreover, different supplements like cytokines etc. can be used depending on your research question.

16. The time until complete wound closure and the best imaging interval depends on your cells and experimental settings and has to be determined in preliminary experiments.

2 EXPERIMENT SETUP

Click on the *Wound Healing* icon to start a new *Wound Healing* experiment.

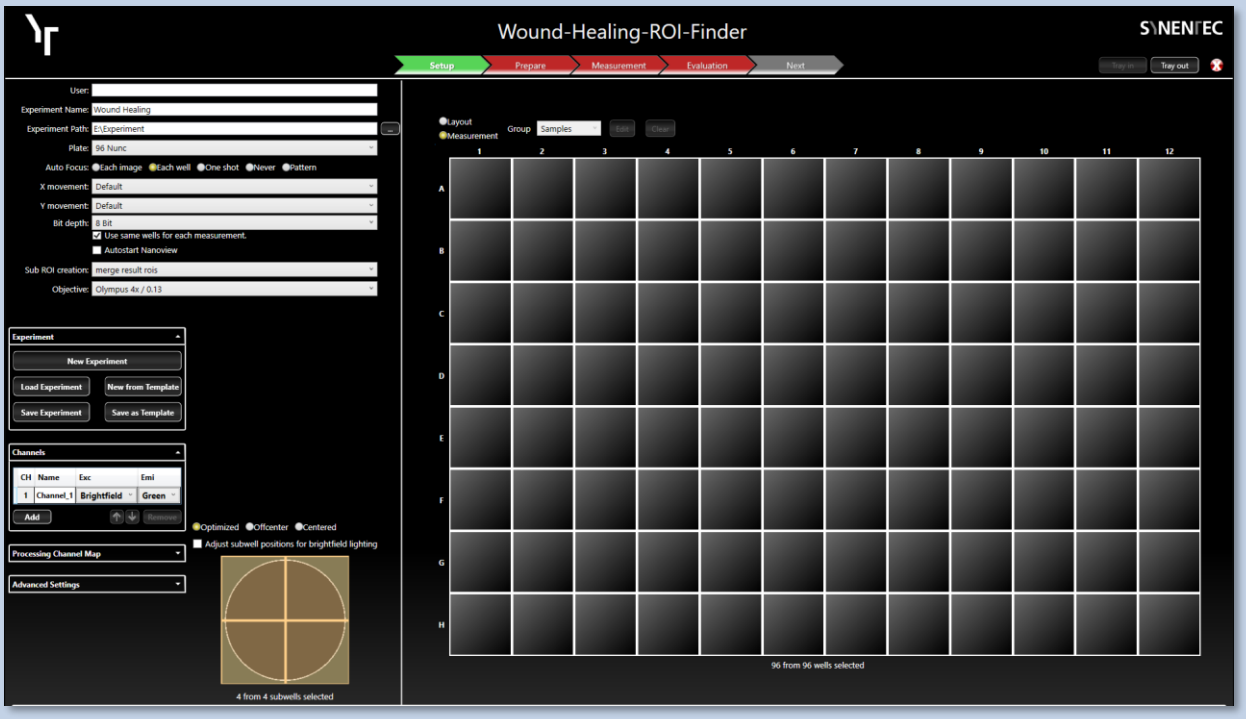


Fig. 1: Wound-Healing-ROI-Finder (Phase 1) setup page

2.1 Experiment Design

The Wound Healing experiment consists of two measurement steps in a single experiment (Fig. 2).

1. ROI-Finder (Phase 1)
   In the first phase, the cells are imaged with the 4x lens. The cell-free area is automatically determined by the software to find the wounds. If a cell-free area (meeting the specified requirements) is found, a region of interest (ROI) is placed inside this area.

2. Wound Healing (Phase 2)
   The Phase 2 scan will image the identified ROIs plus a periphery ROI in a higher resolution (10x). The images are automatically analyzed by the software resulting in different parameters like confluence on gap, confluence on periphery and average gap width.

Phase 1 is only needed for the detection of the original wounds at time point 0 h (t_0, directly after wound creation). For all subsequent time points, Phase 2 is sufficient (Fig. 2). This saves a lot of time, as only a very small part of the well needs to be imaged.

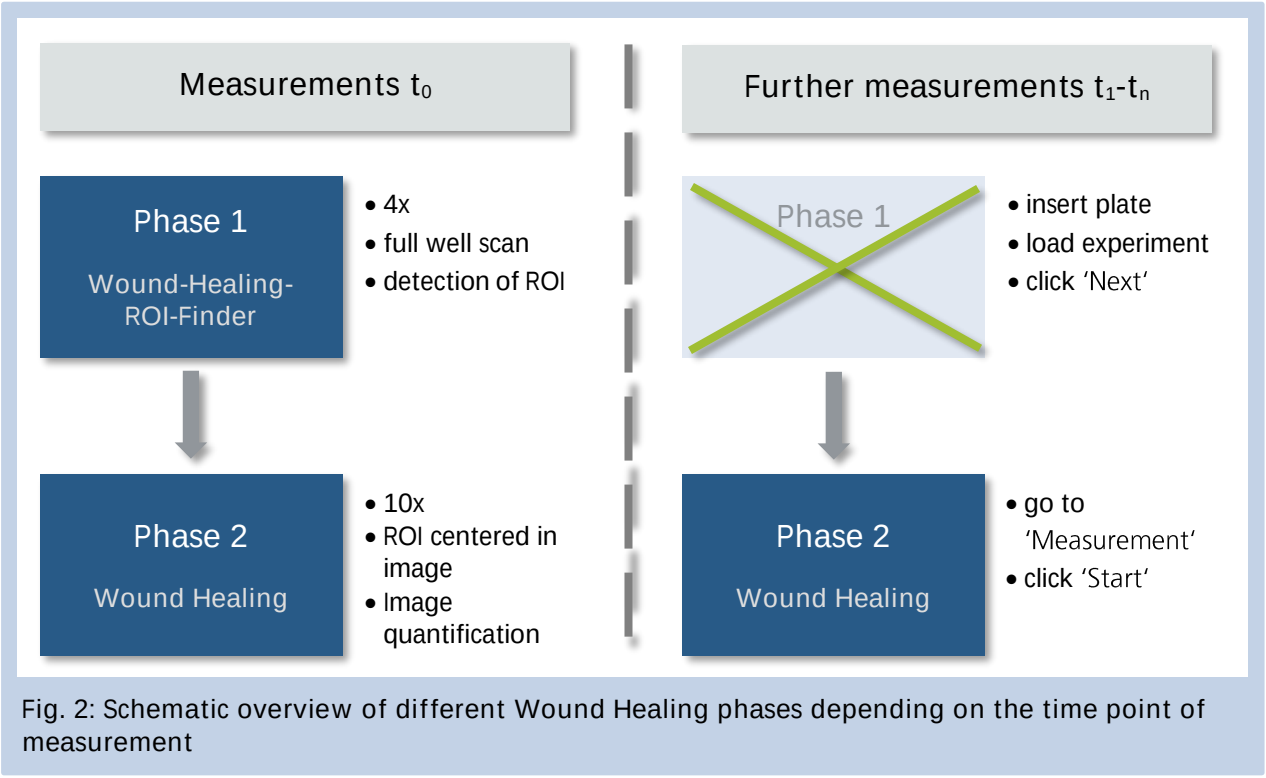


Fig. 2: Schematic overview of different Wound Healing phases depending on the time point of measurement

The page setup differs slightly to the normal experiment designs of the YT®-software. This difference is described in more detail in the following chapter.

2.2 Phases in Wound Healing Experiments

Fig. 3: Experiment phase overview

The Wound Healing Application comprises two phases: Phase 1 and Phase 2 (Fig. 3). The guiding arrows at the top of YT®-software have either a *Next* or a *Prev* step to move to the next or previous phase of the experiment. The first phase is only relevant at time point 0 h (t_0), the second phase has to be measured at t0 and is used for all subsequent time points.

Phase 1 must not be measured after t_0. To carry out a measurement on e.g. t_1, load the experiment in the setup dialogue (Fig. 1) and go directly to Phase 2 by clicking *Next* (Fig. 3). Then go to the measurement section and click *Start*.

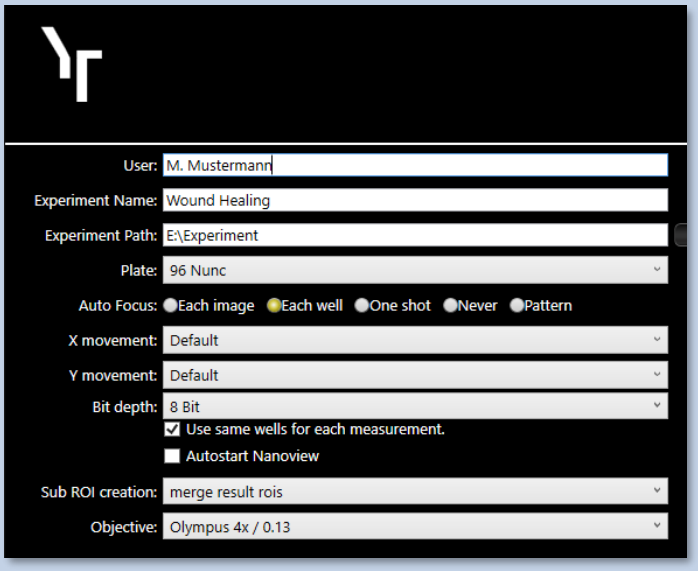


Fig. 4: Start to set up
Start with choosing an experiment name, experiment path, your used plate type, the autofocus setting and the required objective.

For every experiment, an individual experiment name has to be given and the experiment path as well as the plate type has to be chosen (Fig. 4). The autofocus setting is determining the quality of the images and the time for the measurement (see User Guide for details). For high-throughput, *Each well* is recommended.

Autostart Nanoview and *Sub ROI creation* are features needed for the correct imaging and analysis of the second phase.

2.2.1 Autostart Nanoview

When *Autostart Nanoview* is activated, the application will automatically enter the second phase and start the next measurement after completion of Phase 1 measurement and processing. This box should only be checked after you have established all optical and operator analyzing parameters for your cell line.

2.2.2 Sub ROI Creation

This parameter determines which regions of the well are imaged in Phase 2 and how these images are analyzed. Phase 1 determines the exact location of the ROIs inside the wells. This information is used to only image the ROI in Phase 2. You can choose between *copy*, *full well*, *merge result rois*, *use result rois*, and *single image* (Fig. 5). It is important to choose *merge result rois* to obtain proper imaging and analysis in Phase 2.

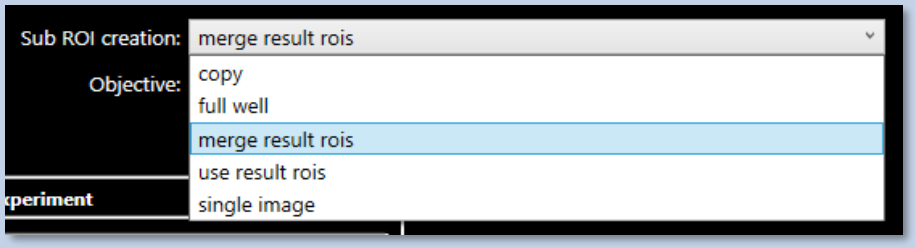


Fig. 5: Sub ROI creation
Choose the Sub ROI creation.

1. Copy:
   The ROI of Phase 1 is transferred 1:1 to Phase 2. This can be chosen when you want to use the 'Nanoview' but have the possibility to reprocess the Phase 1.

2. Full well:
   This option should be chosen in case you want to change the ROI parameters of Phase 1 at a later timepoint. It is usually only used in a validation phase because it will take a little more time due to the full well scan.

3. Merge result ROIs:
   If you choose this option, the objective lens will be directly positioned at the position of the ROI and will image it using the minimal number of subwells. Overlapping result ROIs of the single subimages are merged.

4. Use result ROIs:
   If you choose this option the objective lens will also be directly positioned at the position of the ROI and will image it using the minimal number of subwells. However, Result-ROIs of the single sub-images remain separate and thereby an overlapping of the result-ROIs is possible.

5. Single image:
   The objective lens will also be directly positioned at the position of the ROI with imaging of the minimal number of sub-images. The Result-ROIs of the single sub-images were also analyzed/measured as single ROI.

3 PHASE 1 MEASUREMENT

3.1 Optical Setup and Preparation Phase 1

An optical setup and preparation are required if the properties of the cells are unknown or no template is available.

At first, check that the correct channel settings are selected. For Phase 1, usually only the brightfield channel is needed. We recommend to use the green filter as an emission filter as it usually has a better contrast of the cells as the brightfield filter (Fig. 6).

Fig. 6: Channels
For Phase 1, only *Brightfield* is required.

Select the wells in the plate layout and the number of subwells (Fig. 7). In most wound healing assays, the wound is in the middle of the well, and the subwells at the edge do not need to be imaged. The reduction of subwells (e.g. 9 images per well instead of 25) influences the imaging time and thus the throughput.

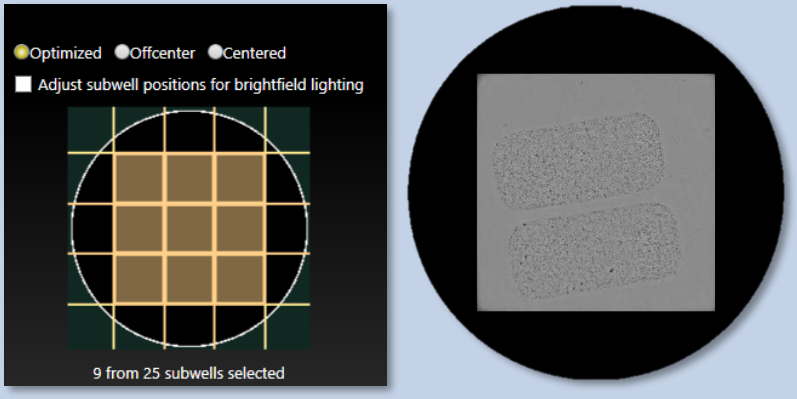


Fig. 7: Subwells
Minimize the number of subwells to decrease the duration of the measurement:
Example: culture-inserts 2 well in 24-well plate. The selected 9 subwells still image the whole area covered by the insert.

After selecting the appropriate optical setting, proceed with the preparation (next arrow) to set up the optical and autofocus settings. When you click on the *Prepare* tab, the microplate will automatically be moved to the first well defined in the setup, and the experiment setup will automatically be saved.

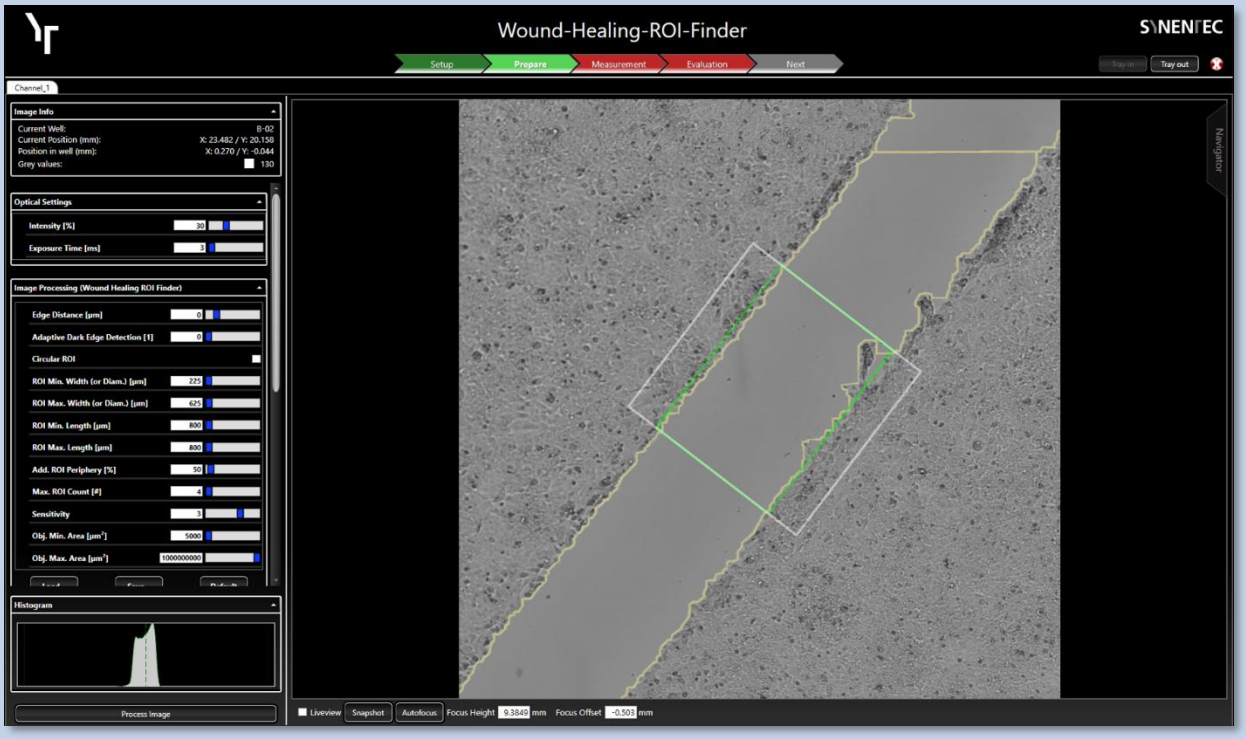


Fig. 8: Preparation of Phase 1: Start with *Autofocus* and end with *Process Image*
The cell-free area is marked with yellow lines, the ROI with the gap with a green box and the additional ROI periphery with a white box.

Start the preparation with a click on *Autofocus* (Fig. 8). For the further preparation, find a gap/wound in your image (Fig. 8) by opening the Navigator tab (right side) and changing the position in the well in the left bottom corner (Fig. 9). You can use this gap to find the optimal optical settings.

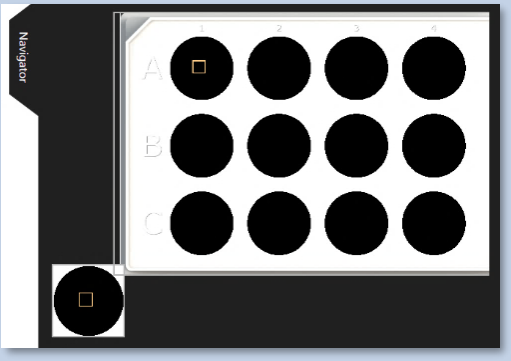


Fig. 9: Navigator tab
Plate layout to choose the well of the plate
Single well in the left bottom corner to choose the position within the well.

To achieve a good illumination of the image, activate the histogram by clicking on the histogram button and check if the peak is in the middle. The brightness can be adjusted by the *Intensity* and the *Exposure Time*. To find the best settings, activate the *Liveview* first (see user guide for details). Set the brightness of the image background (cell-free area) to an average grey value of approximately 130 counts. This value reflects good settings. The current grey value is shown in the Info tab by moving the mouse cursor directly over the image (Fig. 10).

Change the focus of the cells by the *Focus Offset*. To adjust the focus offset, position the mouse cursor into the focus offset box and move the mouse wheel up/down or position the mouse cursor right into the image and press CTRL while turning the mouse wheel. A good focus offset has been achieved when the cells are rich in contrast. The changes can be observed by activating *Liveview* or *Snapshot*. Once the result is satisfactory, deactivate *Liveview* as its warmth will affect the condition of the cells.

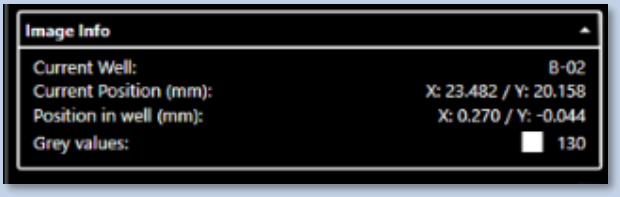


Fig. 10: Image Info
Current grey value of the Brightfield by moving the mouse cursor over the image.

Check the image processing settings by clicking *Process Image*. The cell-free area should now be marked with yellow lines and a green box (ROI) placed within this cell-free area (Fig. 11). Another white box surrounds the green one – it determines the cell periphery (Fig. 8).

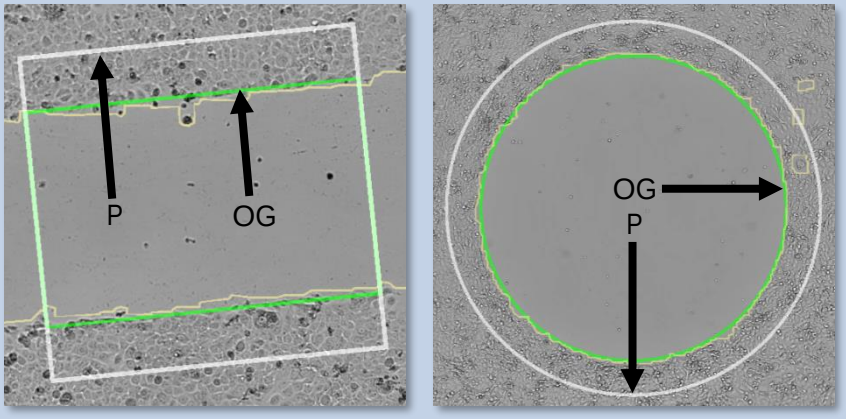


Fig. 11: ROI Finder description
Green – OG – Original Gap t_0
White – P – Periphery
Yellow – Cell-free Area

For most experiments, the default image processing settings of the operator will work properly. Depending on your assay type, you have to set the *Max. ROI Count* to the expected number of wounds. For example, set the value to 4 when using culture inserts 4 well from Ibidi. It is not possible to analyze more than one ROI per wound/cell-free area. The software only places one ROI into each cell-free area and the size of the shown ROI is defined by your settings. Therefore, make sure not to define more ROIs than the expected number of wounds.

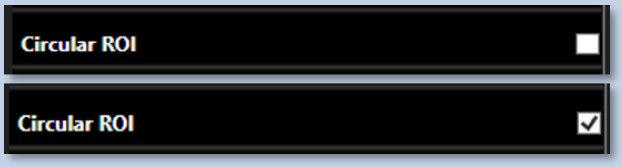


Fig. 12: Circular ROI
The default setting is for rectangular ROI. Ticking on the checkbox 'Circular ROI' activates the identification of round ROI.

As the default settings are for rectangular ROIs, activate the checkbox when using circular wounds (Fig. 12). Set the 'ROI Min./Max. Width (or Diam.)' as a range around the expected width (or diameter) to minimize further processing (Fig. 14). For scratch assays, the wound width may greatly vary. To get an idea about the width of your wounds, use the *Measurement Mode*. Right-click in the image and activate the *Measurement Mode* (Fig. 13).

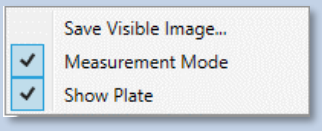


Fig. 13: Measurement Mode

Then you can measure by pressing CTRL and dragging the mouse from one side of the gap to the other. Adjust the maximum gap width accordingly (Fig. 20 C). Check the correct identification of the ROIs by clicking on *Process Image*. The image processing settings can further be re-evaluated at the evaluation step and are therefore more detailly described there (section 3.3).

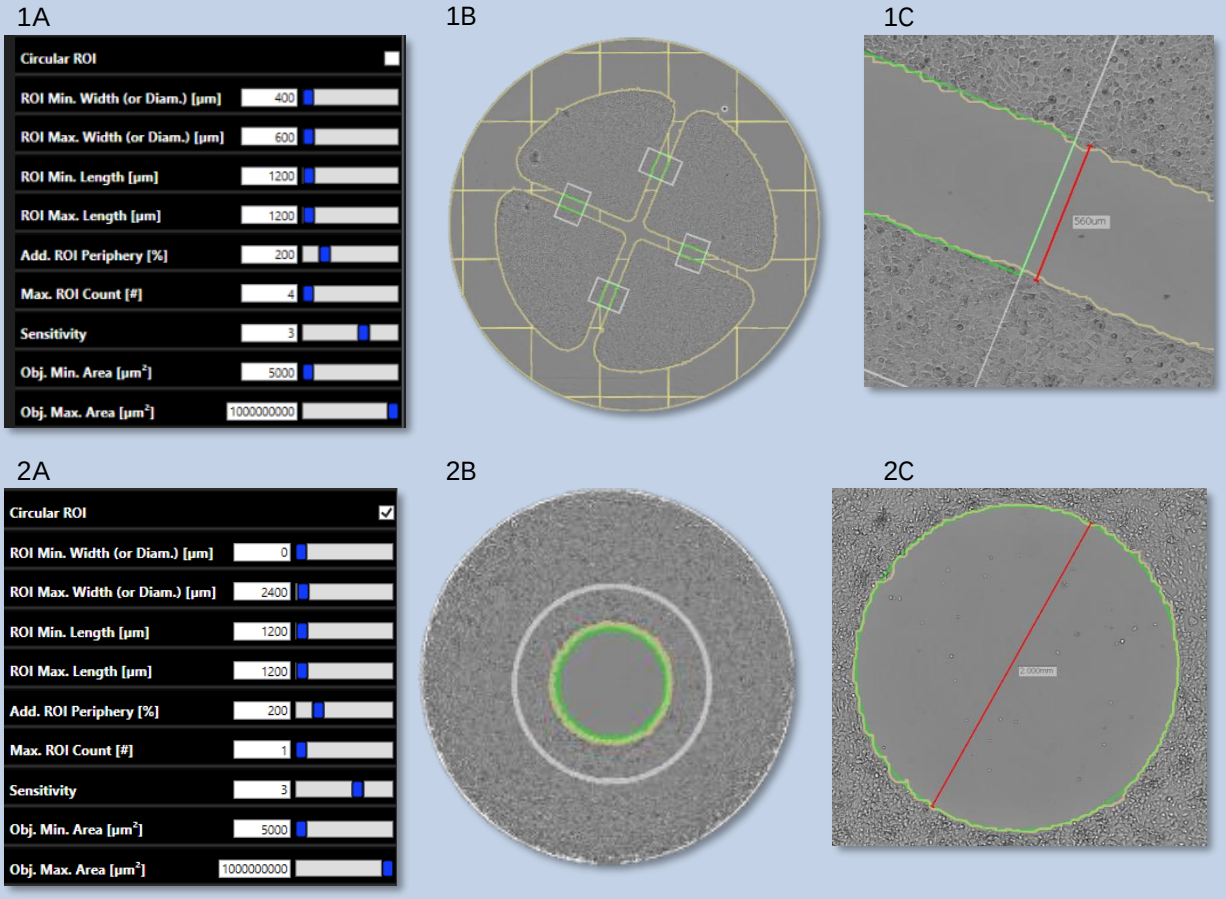


Fig. 14: ROI Min./Max. Width (or Diam.) example

1. Rectangular ROIs:
   A. Processing parameter
   B. imaged gaps of culture-inserts 4 well
   C. measured width of cell free gap (expected: 500 µm +/- 100 µm)
2. Circular ROI:
   A. Processing parameter
   B. imaged gaps of Oris Stoppers
   C. measured diameter of cell free gap (expected: 2000 µm)

3.2 Measurement Phase 1

Click on the arrow for measurement. Before you start the measurement by clicking the *Start* button on the lower left side, you can choose between the options for image processing. It is recommended to tick *Process Image During Measurement* (Fig. 15).

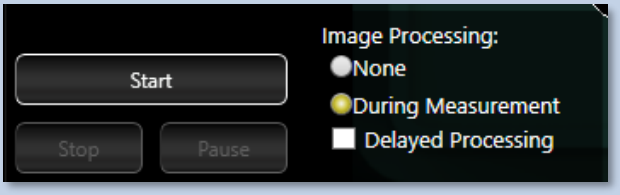


Fig. 15: Options for measurement
Image processing setting.

Proceed with *Evaluation* to view the results of Phase 1.

3.3 Evaluation of Phase 1

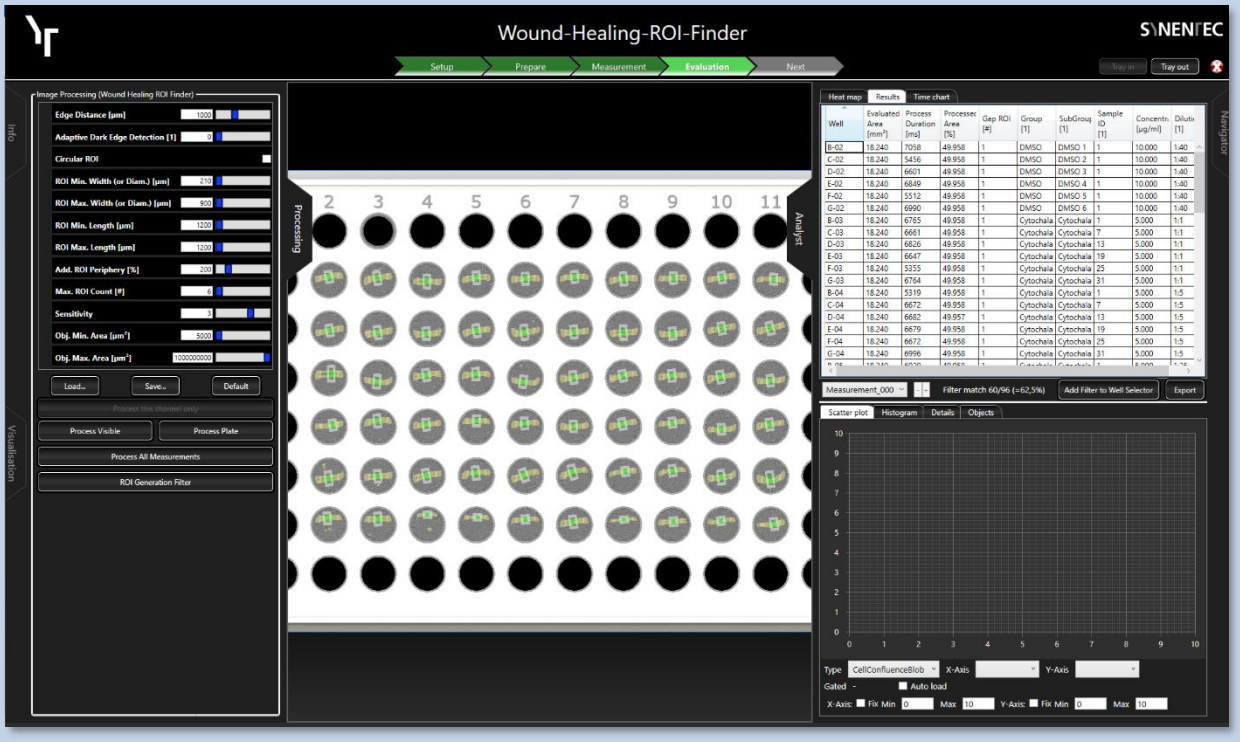
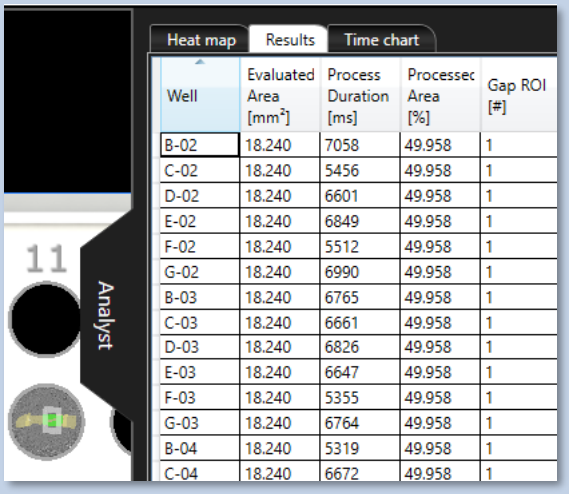


Fig. 16: Evaluation of Phase 1

In Phase 1 of the experiment, YT®-software automatically analyzes the images, identifies the cell-free areas and places ROIs in them (Fig. 16). Therefore, this phase is called *Wound-Healing-ROI-Finder*.

3.3.1 Result Table in Analyst

The result table gives an overview of the results. The number of identified *Gap ROI* will be displayed in the result table (Fig. 17). Now it's possible to check the results, alter *Image processing* parameters, and reprocess by *Process Plate*.



Well	Evaluated Area [mm²]	Process Duration [ms]	Processed Area [%]	Gap ROI [#]
B-02	18.240	7058	49.958	1
C-02	18.240	5456	49.958	1
D-02	18.240	6601	49.958	1
E-02	18.240	6849	49.958	1
F-02	18.240	5512	49.958	1
G-02	18.240	6990	49.958	1
B-03	18.240	6765	49.958	1
C-03	18.240	6661	49.958	1
D-03	18.240	6826	49.958	1
E-03	18.240	6647	49.958	1
F-03	18.240	5355	49.958	1
G-03	18.240	6764	49.958	1
B-04	18.240	5319	49.958	1
C-04	18.240	6672	49.958	1

Fig. 17: Analyst flag: Result table

3.3.2 Image Processing Settings Phase 1

As mentioned before, the *Wound Healing ROI finder* algorithm at first detects the cell-free area (yellow lines or area if *fill* is activated) in this phase. Then, it places a wound healing ROI within this area (green box) and adds a periphery (white box). The detection can be altered by changing the Image Processing settings, which are described in Tab. 1. By sliding the mouse cursor over each setting, you will also find a short description of each parameter.

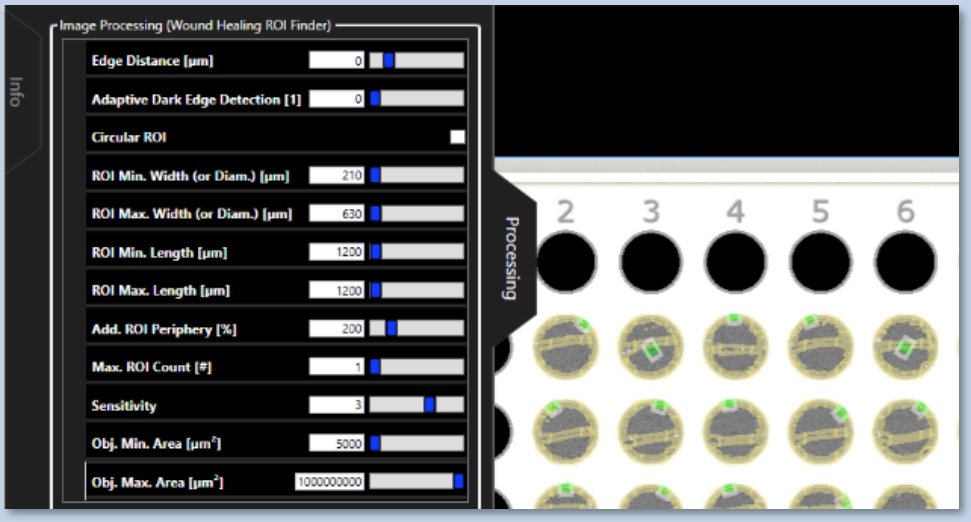


Fig. 18: Default ROI settings
In this scratch wound healing experiment, the default setting didn't give the correct result (*Max. ROI Count*: 1).

Tab. 1: Image Processing Parameter

IP parameter	Default	Refers to	Description
Edge Distance	0 µm	Cell-free area (yellow lines or area called CellConfluenceBlob in the Visualisation tab)	distance from the well edge until which the area is analyzed (see IP guide for details)
Adaptive Dark Edge Detection	0 (OFF)	Cell-free area	Threshold for detecting dark well edge regions
Circular ROI	unchecked	Wound healing ROI (green box)	Check this box, if you created circular wounds
ROI Min./Max. Width (or diameter)	210 µm – 630 µm	Wound healing ROI	Minimal/maximal width (for circular ROIs: diameter) of the wound healing ROI
ROI Min./Max. Length (or diameter)	1200 µm – 1200 µm	Wound healing ROI	Minimal/maximal length (for circular ROIs: diameter) of the wound healing ROI
Add. ROI periphery	200 %	Periphery ROI (white box called ROI Blob in Visualisation Tab)	Adds a periphery box depending on the width of the detected wound healing box (200 % means that the width of the wound is added to each side)
Max. ROI Count	1	Wound healing ROI	Max. Number of allowed ROIs per well
Sensitivity	3	Cell-free area	The higher the sensitivity, the more cell-free area should be detected
Obj. Min./Max. Area	5000 µm² – 1000000000 µm²	Cell-free area	Excludes cell-free areas that are smaller or bigger than the specified value

Make sure that all wounds are detected by choosing the correct settings. You can change the visualisation of them by clicking on the *Visualisation* tab (Fig. 19). The change of the visualisation has no influence on the processing. If the wounds are not correctly identified in Phase 1, they will not be imaged in Phase 2 as only the detected ROIs are imaged and evaluated in Phase 2.

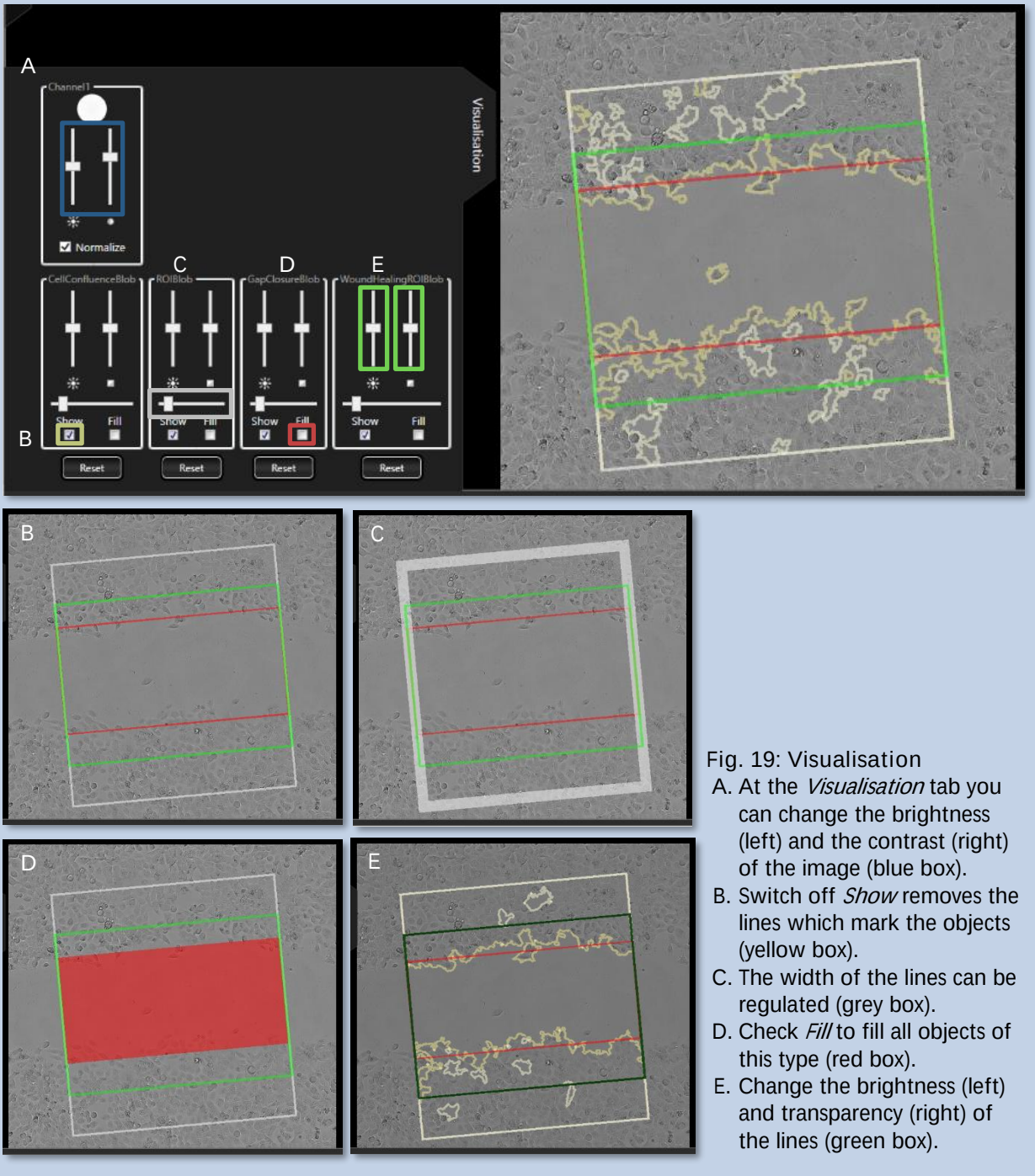


Fig. 19: Visualisation
A. At the *Visualisation* tab you can change the brightness (left) and the contrast (right) of the image (blue box).
B. Switch off *Show* removes the lines which mark the objects (yellow box).
C. The width of the lines can be regulated (grey box).
D. Check *Fill* to fill all objects of this type (red box).
E. Change the brightness (left) and transparency (right) of the lines (green box).

If the wounds are not properly detected (Fig. 18), first change the width/diameter of the gap as described in section 3.1. If ROIs are falsely detected at the well edge, because there are also cell-free areas, exclude the edge by increasing the edge distance (Fig. 20 B).

The ROI length defines how long the ROI will be. The default settings will work properly for most kinds of wounds, but you can optimize it. The *ROI Min. Length* should not be smaller than the real width of the gap and the *ROI Max. Length* can be set higher or equal to the minimum length. The length might influence the imaging time of Phase 2. For example, ROIs with a length of 800 µm might be scanned in one image while the default length of 1200 µm usually requires two images. Consequently, if your aim is to reduce scanning times for high-throughput imaging, reduction of ROI length could be an option.

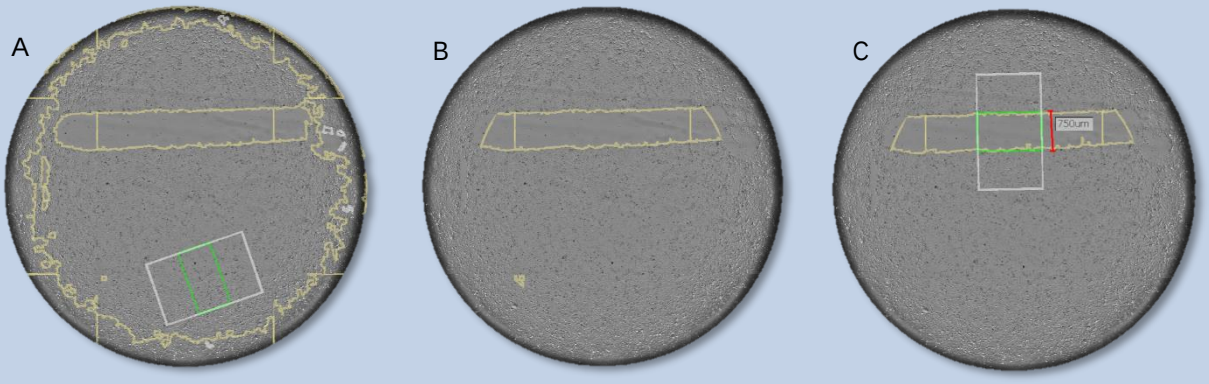


Fig. 20: Adjustment of the processing parameters by *Edge Distance* and *ROI Min./Max. Width (or Diam.)*
By using the measurement mode, you can set the edge distance and the max. width to include all scratches:
A. default settings.
B. edge distance: 1000 µm.
C. edge distance: 1000 µm and max. width: 900 µm (>750 µm).

Add. ROI Periphery (additional periphery to the ROI) depends on the width of the ROI (gap). The default value of 200 % adds the width of the gap to each side of it. The periphery is important if you are also interested in the confluence of the cells surrounding the gap, for example if you want to calculate the *relative wound density*. Phase 2 images this white periphery box (Fig. 21). Therefore, if you are interested in high-throughput screening, you might reduce imaging times by decreasing the periphery value.

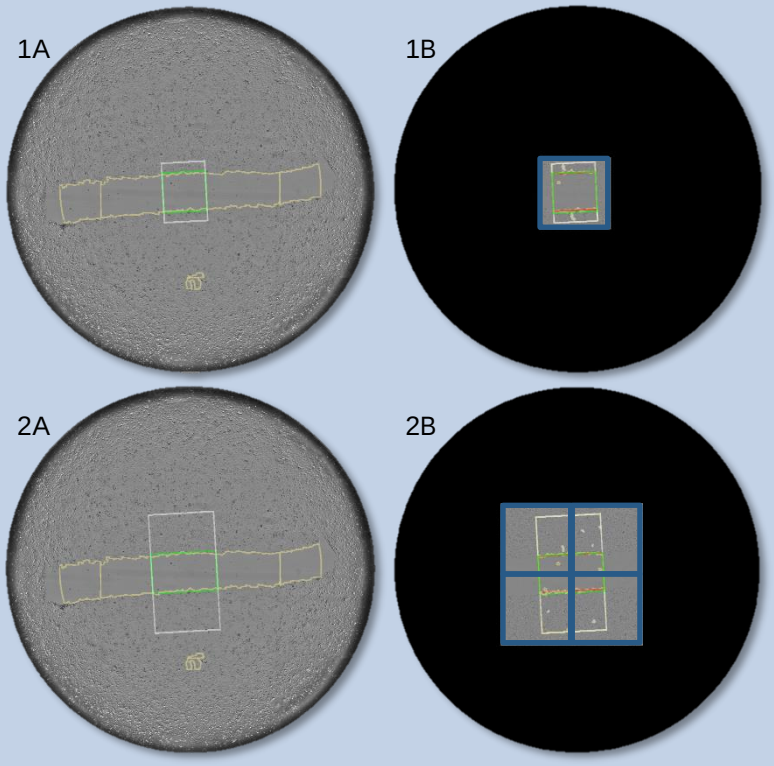


Fig. 21: ROI settings

The size of the ROI and periphery determines the number of pictures needed to image the ROI in phase 2 and by that the duration of the measurement.

1. Small ROI (800 µm) with small additional periphery (50 %):
   A. images of the whole well in Phase 1.
   B. one image in Phase 2.

2. Larger ROI (1200 µm) with larger additional periphery (200 %):
   A. images of the whole well in Phase 1.
   B. four images in phase 2.

The sensitivity has an influence on the detection of the cell free area. The higher the sensitivity, the more cell-free area will be detected (Fig. 22). In most cases, the default value offers good results.

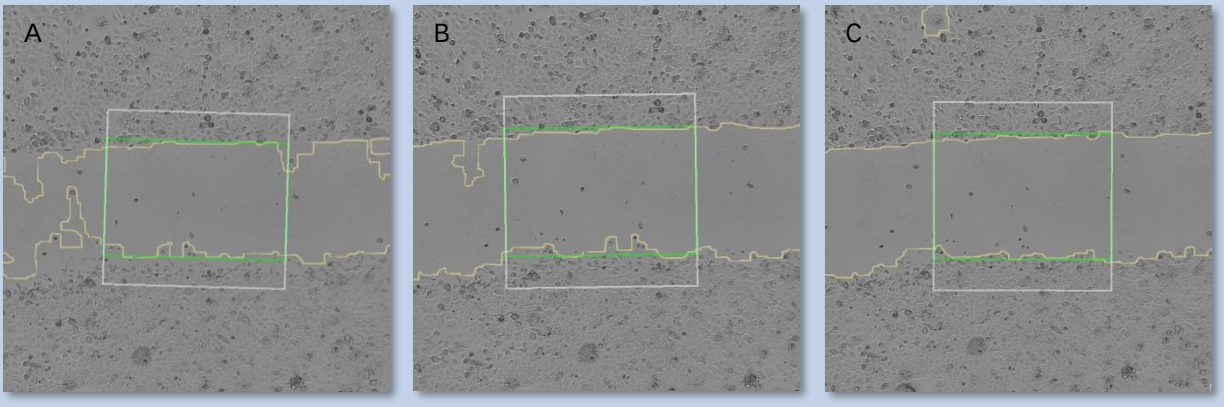


Fig. 22: Sensitivity
The sensitivity has a range from -10 to 10. If the sensitivity is increased, more cell-free area is detected.
Sensitivity of:
A. -5
B. 3 (default)
C. 7.

In Phase 1, the detection of non-adherent cells or cell debris has only a minor influence on the finding of the ROI and usually, the default setting give good results. However, you can exclude debris and single cells by increasing the *Obj. Min. Size* (Fig. 23).

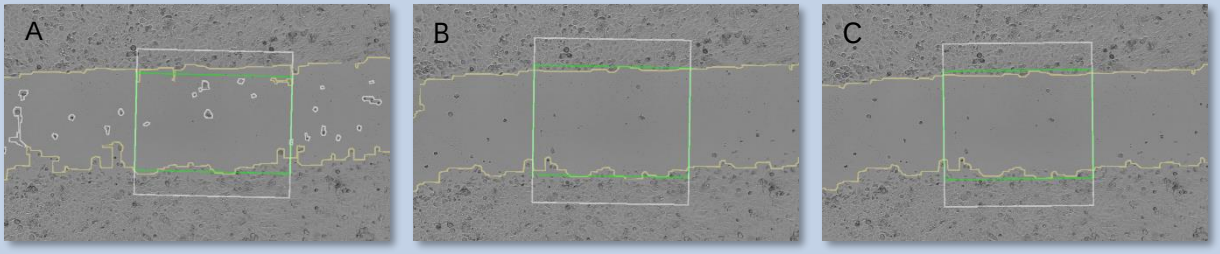


Fig. 23: *Obj. Min. Size*
The *Obj. Min./Max. Size* has a range from 0 to 1,000,000,000 µm². Here, you can see how it affects the detection. In this example, Object minimum size is:
A. 250 µm²
B. 5000 µm² (default)
C. 100,000 µm².

4 PHASE 2 MEASUREMENT

In Phase 2 of the *Wound Healing* application, only the ROIs determined in Phase 1 are measured at different time points. The *Wound healing* analysis monitors the cell confluence of the original gap ROI and of the additional periphery.

The first measurement has to be carried out directly after the adjustment of the ROI processing parameters of Phase 1 by clicking on *Next*. For further measurements, you don't need Phase 1 anymore. Open the *Wound Healing* application and load your experiment in the setup dialog. Directly click on the *Next* arrow to open the Phase 2 setup. Now you can proceed with *Prepare* (Fig. 24).

4.1 Optical Setup and Preparation Phase 2

The layout of the setup page looks very similar to the Phase 1 setup page. The major difference is that you can't choose any wells to be measured. The well selection has been determined automatically by the *ROI Filter* setting in the previous phase.

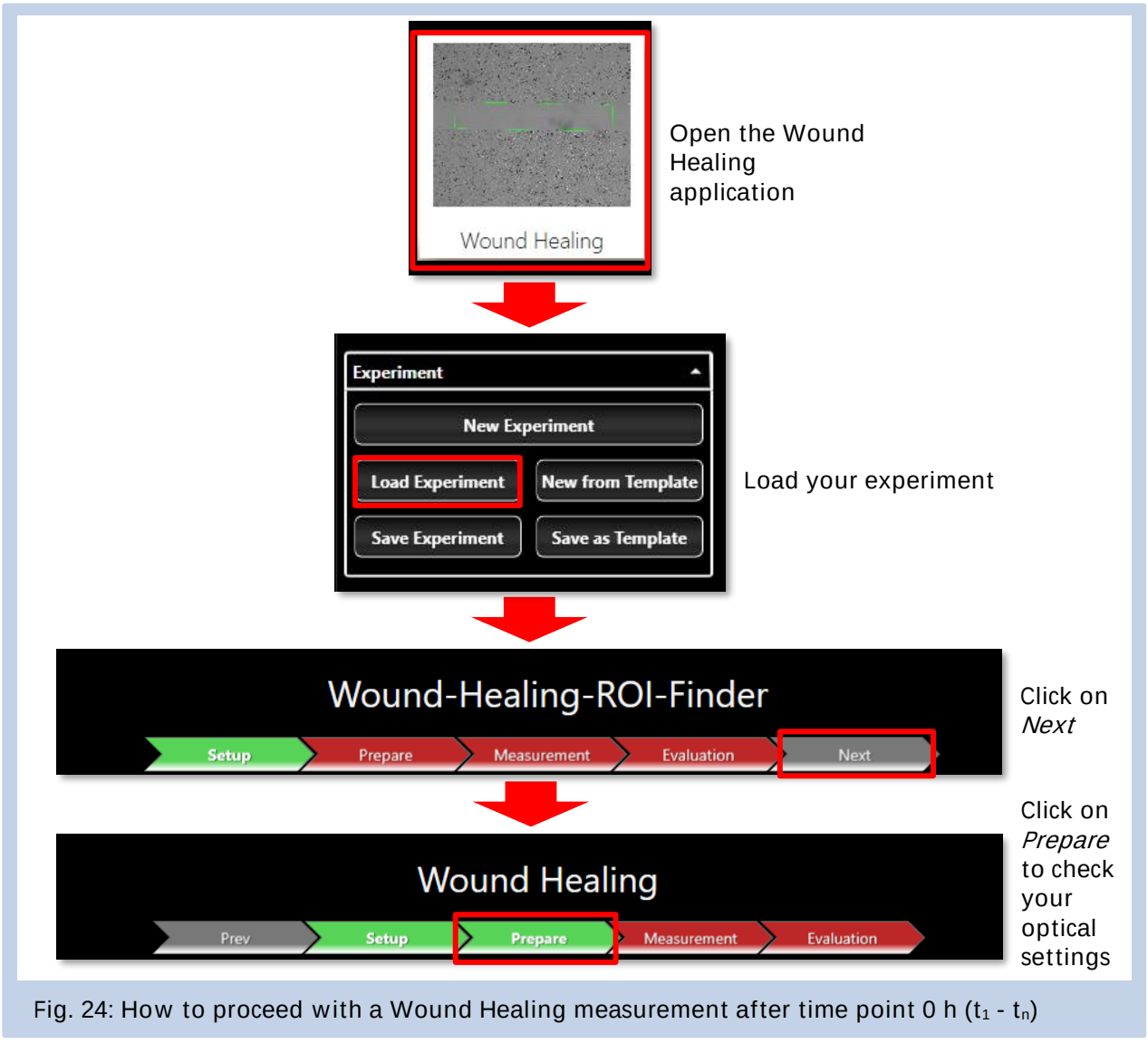


Fig. 24: How to proceed with a Wound Healing measurement after time point 0 h (t_1 - t_n)

The default objective is the 10x but other resolutions can be chosen, e.g. the 20x.

In case that the objective is changed, it is necessary to go back to the Evaluation page of the Phase 1 and set the ROI Filter again.

The next step is to go to the prepare page and to set up the optical and autofocus settings. If you click on the *Prepare* tab, the microplate will automatically be moved to the first well in which an ROI was detected in Phase 1.

Before an image can be obtained, again an autofocus followed by a snapshot should be carried out to get an overview of the current image for the right optical settings (Fig. 25).

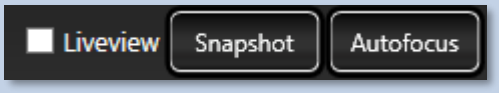


Fig. 25: Start with the autofocus

In case that the image is either too dark or bright, proceed as in section 3.1 by activating the *Histogram* on the lower left side and use *Liveview* while adjusting *Exposure Time* and *Intensity* accordingly. The grey value should be at 130 counts again.

The focus offset can also be set as described in section 3.1. At the optimal offset, the cells are rich in contrast.

Once all optical settings have been completed, and the required image quality was obtained, the image processing should be tested. Press *Process Image* (lower left side of screen) to test your settings and view the results (Fig. 26). The software will show the original gap and cell periphery as detected in Phase 1 as green and white box, respectively. Additionally, it will recognize the cell confluence in both boxes and mark it with a yellow line. Moreover, it recognizes the cell fronts and from that it calculates the average gap width, which is shown as a red box (Fig. 23). It is recommended to start using the default parameters which in general obtain the desired results. In case the result is not satisfactory the Image Processing Parameters can either be re-adjusted or previously saved parameters may be used.

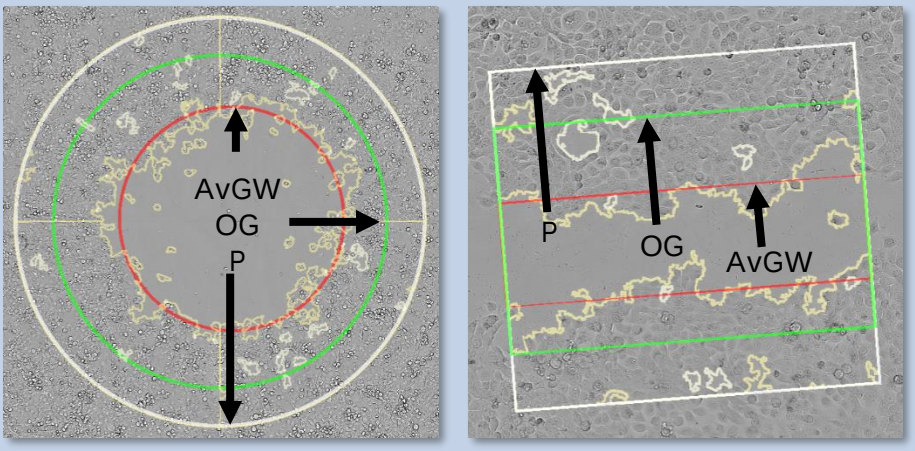


Fig. 26: ROI description
Red – AvGW – Average Gap Width
Green – OG – Original Gap t_0
White – P – Periphery
Yellow – Cell Confluence

Now proceed with the measurement (next arrow).

4.2 Measurement Phase 2

There are the same measurement options as for Phase 1. It is also recommended to tick *Process Image During Measurement* (results will then also be available immediately in the *Evaluation* mode). Proceed with *Evaluation* (next arrow) to view the results of your experiment (Fig. 27).

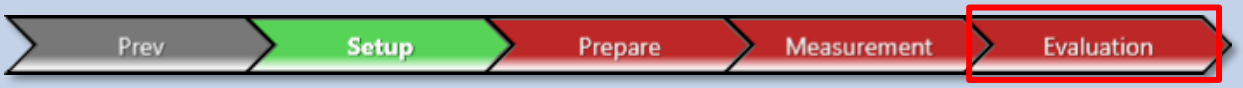


Fig. 27: Workflow arrow

4.3 Evaluation Phase 2

If you perform the experiments for the first time, check that the image processing settings give reasonable results. Have a look at different wells and check that the cells are properly recognized (marked by yellow lines) while background or debris is excluded. If this is not the case, modify the image processing setting until the results are satisfactory.

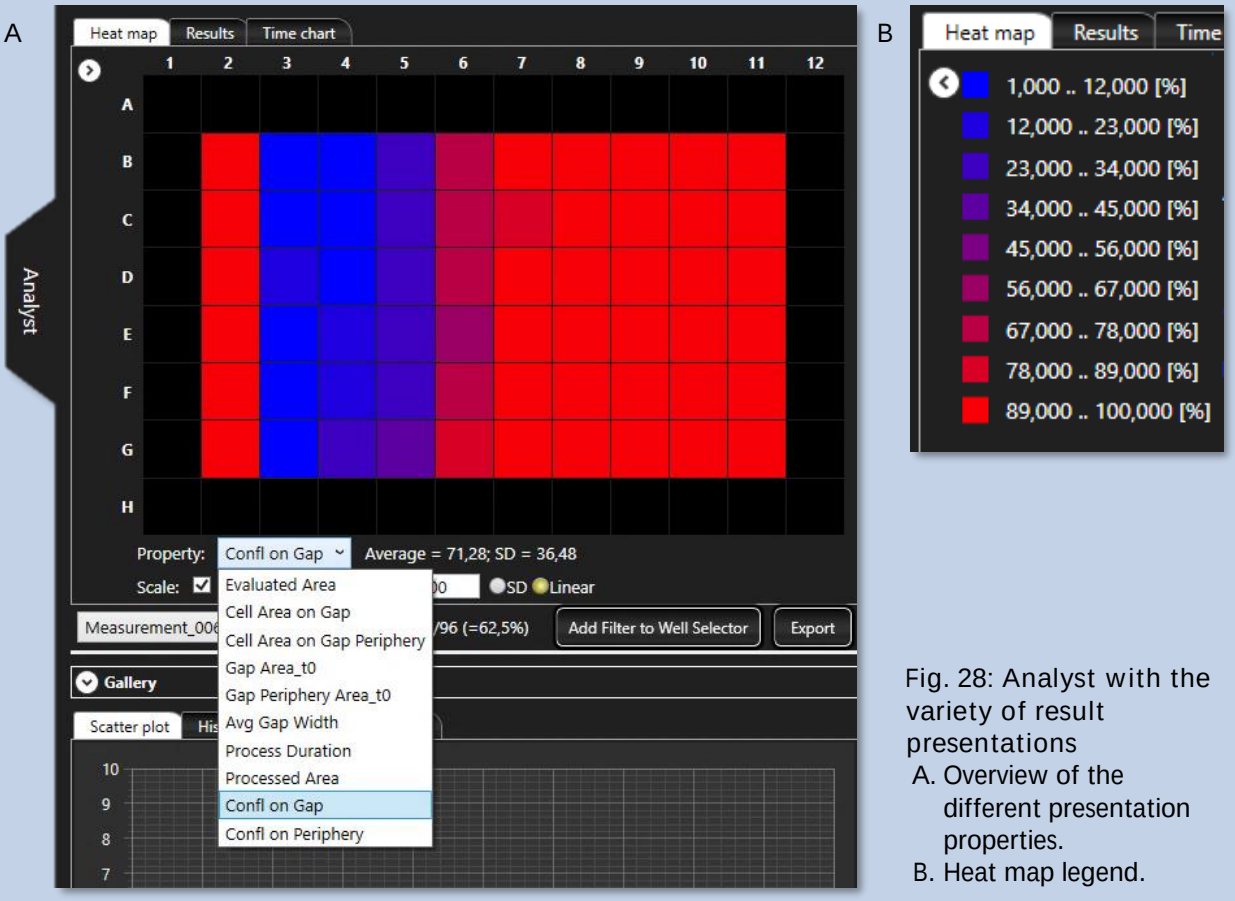


Fig. 28: Analyst with the variety of result presentations
A. Overview of the different presentation properties.
B. Heat map legend.

The result parameters are described in table (Tab. 2). The most important parameters are *Avg Gap Width* as well as *Confl on Gap*. During wound closure, *Confl on Gap* will increase as more cells migrate into the gap. Accordingly, *Avg Gap Width* will decrease, as the gap becomes smaller and is eventually closed.

Confl on Periphery can be used to calculate the relative wound density (RWD) using the following formula:

$$RWD\ [\%] = [(Confl\ on\ Gap\ t_x - Confl\ on\ Gap\ t_0)/(Confl\ on\ Periphery\ t_x - Confl\ on\ Gap\ t_0)] \cdot 100$$

with t_x = time x and t_0 = time 0 h. [1]

Tab. 2: Result Parameters

Parameter	Unit	Description
Evaluated Area	mm^2	Area that is evaluated by the image processing settings (corresponds to the area of the white periphery box)
Cell Area on Gap	mm^2	Area covered by cells within the gap recognized in Phase 1 (green box)
Cell Area on Gap Periphery	mm^2	Area covered by cells within the cell periphery as defined in Phase 1 (white box) minus the Cell Area on Gap
Gap Area_t0	mm^2	Area of the original gap as defined in Phase 1 (green box)
Gap Periphery Area_t0	mm^2	Evaluated Area (white box) minus Gap Area_t0 (green box) as defined in Phase 1
Avg Gap Width	µm	Average distance between the migrating/invading edges of the wound (width of red box)
Process Duration	ms	Time needed for evaluating the area of this well
Processed Area	%	Percent of the well area that is imaged?
Confl on Gap	%	Cell confluence in the original gap as defined in Phase 1 i.e. percent of the gap area that is covered by cells (Cell Area on Gap/Gap Area_t0 · 100)
Confl on Periphery	%	Cell confluence in the cell periphery i.e. percent of the periphery area that is covered by cells (Cell Area on Gap Periphery/Gap Periphery Area_t0 · 100)

To obtain an overview, you can now check your results by scrolling through the result table, the heat map, time charts, the scatter plot or histograms in the analyst tab. In the drop-down menu of the property box, you can choose which parameter is displayed in the graphs (Fig. 28 A).

The different colors of the Heat map represent the values of the chosen parameter (Fig. 28 B). Blue represents a low and red a high value (legend visible by clicking on the arrow under the Heat map tab).

The *Time chart* displays the values of the chosen parameter over all measurements. If you click on *Details* in the lower chart, 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. 29).

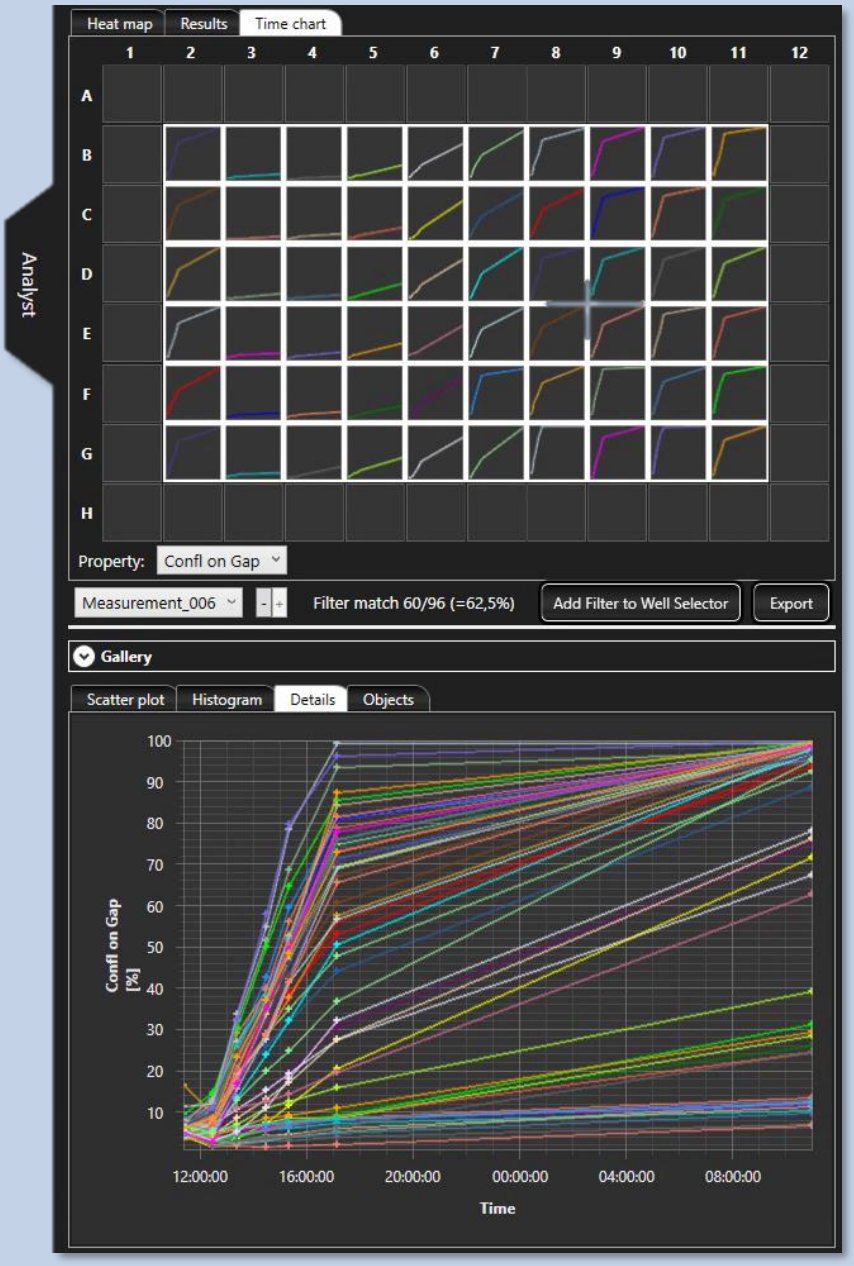
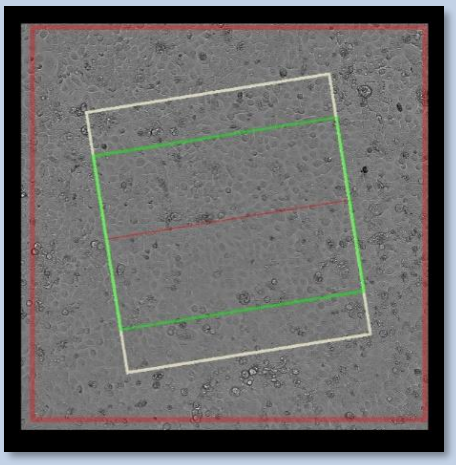


Fig. 29: Time chart
Single or several curves and fields may be selected by pressing CTRL and a) clicking on the required curve/field or b) dragging the mouse cursor to mark several fields at once.

Additional to the time chart (Fig. 29), you can generate a *Gallery* for each well, displaying all images of this well (Fig. 31). To do so, 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 fat red box (Fig. 30). The value depends on the size of your wounds. The default value is often too small and does not cover the whole gap. Usually, a value of 2000 pixel works well for scratch assays and Ibidi inserts and of 4000 pixel for

Oris inserts. If the value is below 50 or above 1024, the software marks it with a red exclamation mark. However, the galleries will still be generated and can be exported. 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 (hence the exclamation mark).

A



B

Fig. 30: Create Gallery
A. The ROI with fat red box around, the box represents the area which is visible in the *Gallery*.
B. Activate *Preview* to see the red box, the *Image size* as red box is depending on the pixel. Click *Create* to create the gallery with the chosen size.

When you are satisfied with the results, you may export your data using the *Export* button. You can either just export the results from the actual measurement or click on *Export all* to export the data from all measurements (Fig. 32). You can also export the galleries by clicking on *Clone Gallery*. The film strip contains the date of the experiment and the time points of the different measurements (Fig. 31).

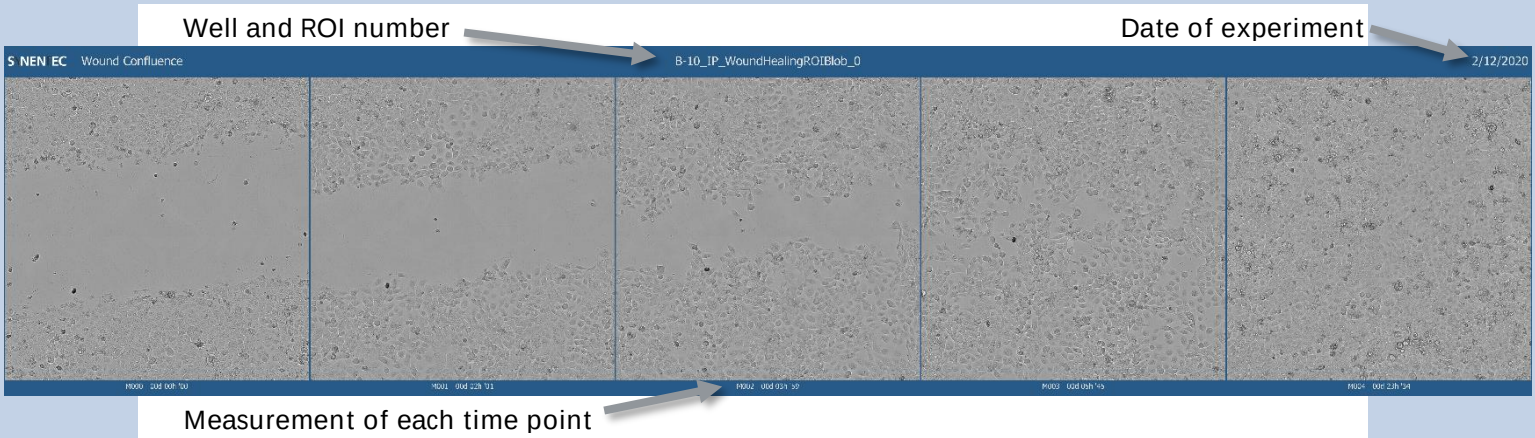


Fig. 31: Gallery example

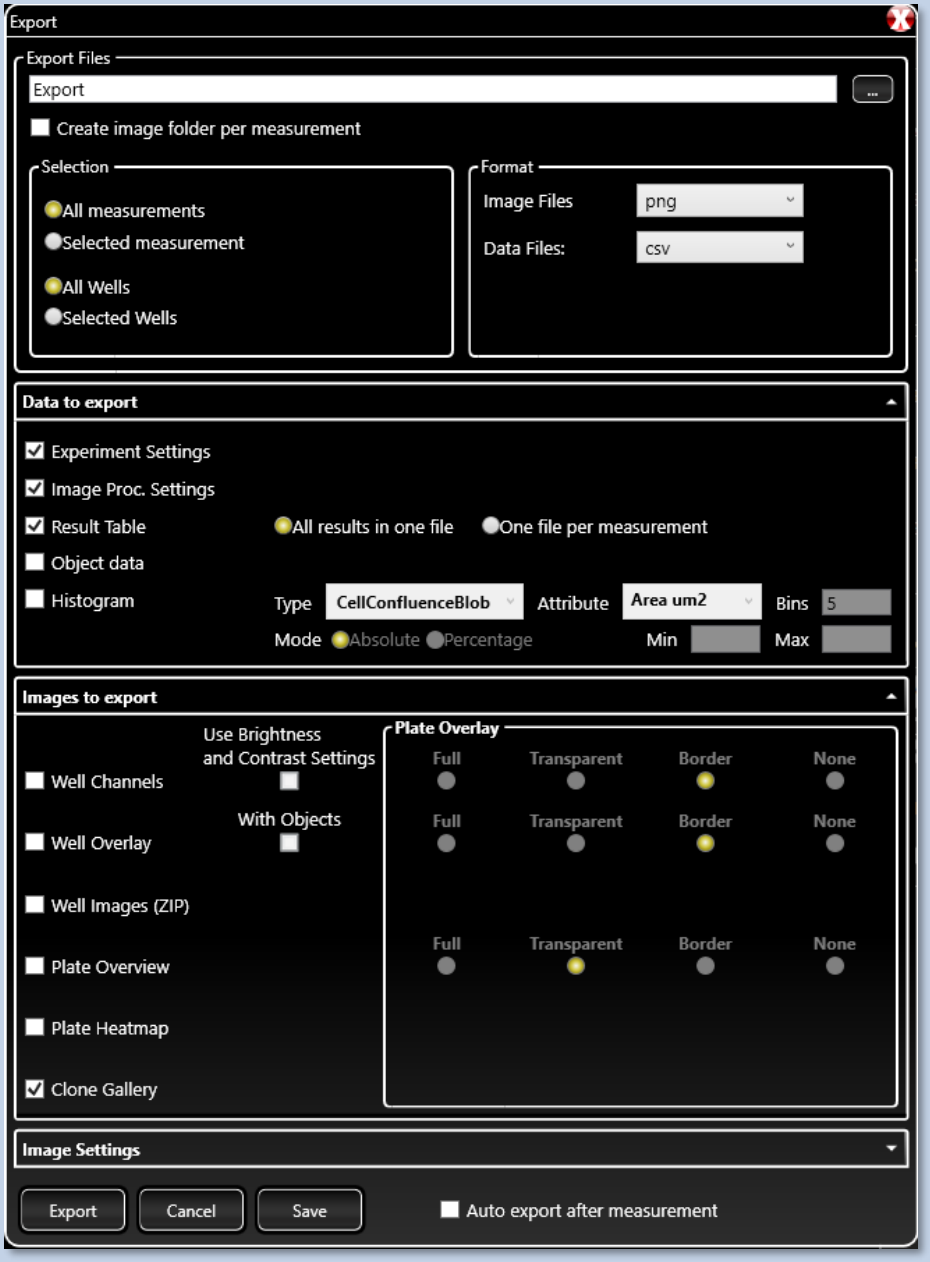


Fig. 32: Export
Click on *Export* in the *Analyst* tab. In the *Export* window you can choose which measurements you want to export and if you want one file for all measurements or single files for each measurement. You can also choose the format of the files and which data you want to export. If you want to export the *Gallery*, check *Clone Gallery*. When you have made your decision, click on *Export*.

In case you are not satisfied with your results, you can alter the image processing parameters accordingly (see section 4.4). After making your changes, check them by clicking *Process Visible* and further reprocess the whole plate by clicking on *Process Plate* as well as *Process All Measurements* (Fig. 33).

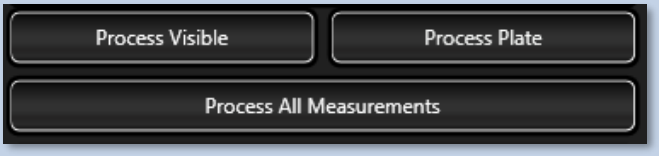


Fig. 33: Processing options

4.4 Image Processing Settings of Phase 2 (Wound Healing)

Fig. 34 shows the Image Processing settings of Phase 2. The principle of the algorithm is a cell confluence detection within the ROIs as defined in Phase 1. A detailed description of most parameters can be found in the *Image Processing Parameters Guide*, which you can find in your device folder. Here, we describe only the ones unique or relevant for the wound healing application:

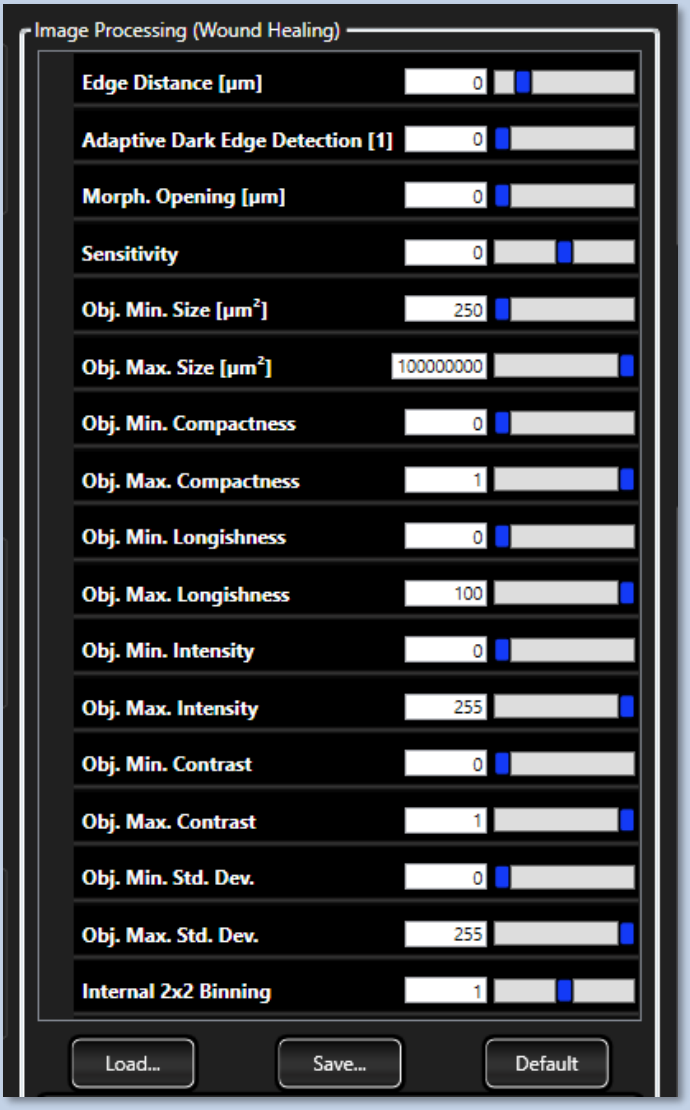


Fig. 34: *Image Processing* parameter table Processing parameters of phase 2.

You can see the parameter values by double clicking on a blob (marked cell area) in your image. The first parameters, which should be optimized, are the sensitivity and the internal binning. The other parameters are following to exclude unwanted objects.

4.4.1 Sensitivity

The *Sensitivity* is an important parameter for the processing of cells. The higher the sensitivity is, the more objects and cells are detected. For the detection of the confluence, choose the minimal sensitivity which is sufficient to detect all cells. A higher sensitivity means an increase in detection of cells, but also of unspecific objects (Fig. 35).

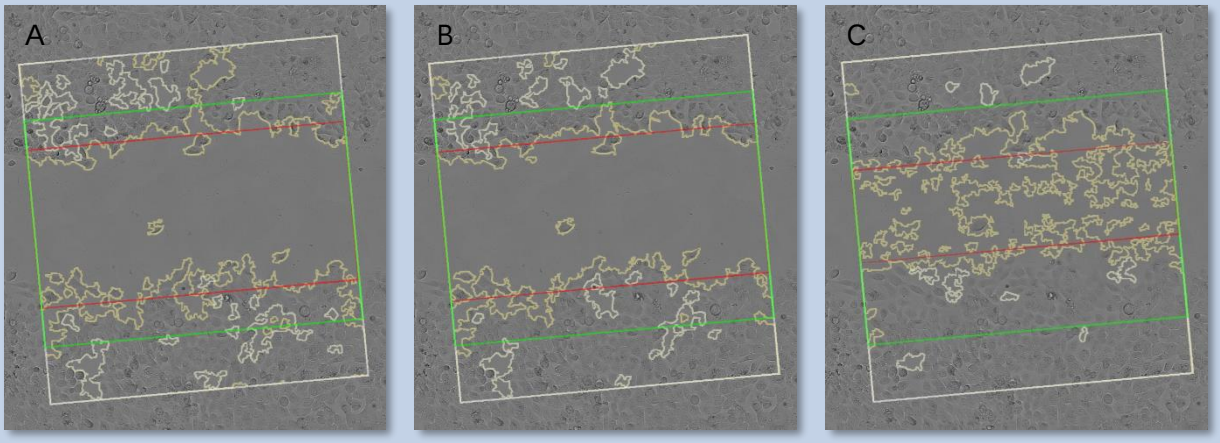


Fig. 35: Sensitivity
The higher the sensitivity is, the more cells are detected, but also the detection of unspecific objects is increased.
A. Default setting 'Sensitivity' of 0: The cell in the middle is not properly detected.
B. 'Sensitivity' of 5: the cell in the middle is detected.
C. 'Sensitivity' of 10: the cell in the middle is detected, but also some other unspecific objects.

4.4.2 Internal 2x2 Binning

The *Internal 2x2 Binning* defines the number of pixels of an image which are analyzed as one (2^x x 2^x with 0: none, 1: 2x2, 2: 4x4). By the internal pixel binning, images will be processed faster but with less accuracy. A value of 1 leads to an approximate halving of the process duration. The loss of accuracy is accompanied with an increase of sensitivity (Fig. 36). When you increase the internal binning, you maybe have to adapt other parameters as well.

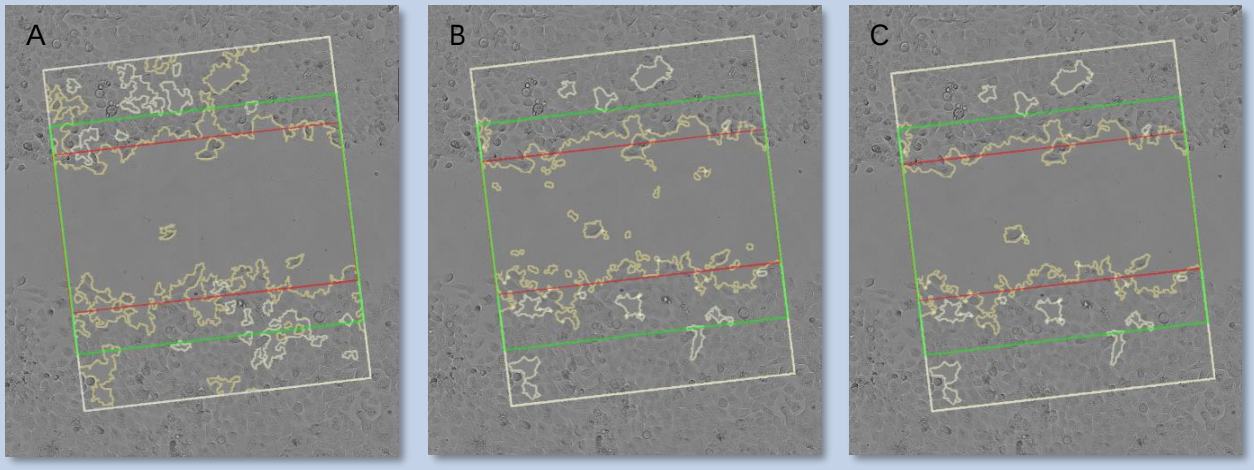


Fig. 36: Internal 2x2 Binning
The *Internal 2x2 Binning* means less accuracy and faster processing and by this an increase in detection of cells.

A. *Internal 2x2 Binning* of 1 (default setting): the cell in the middle is not properly detected.
B. *Internal 2x2 Binning* of 2: the cell in the middle is properly detected, but now also some debris is included.
C. *Internal 2x2 Binning* of 2 and *Obj. Min. Size* increased from 250 µm to 1000 µm: only the cell in the middle is detected.

4.4.3 Eliminating Non-Adherent Cells or Debris

The detection of unspecific objects like non-adherent cells or cell debris would lead to a cell confluence on the gap that is too high. There are different parameters available to eliminate the unspecific objects without eliminating the cells. In the example, *Obj. Min. Size* and *Obj. Min. Contrast* were used (Fig. 37).

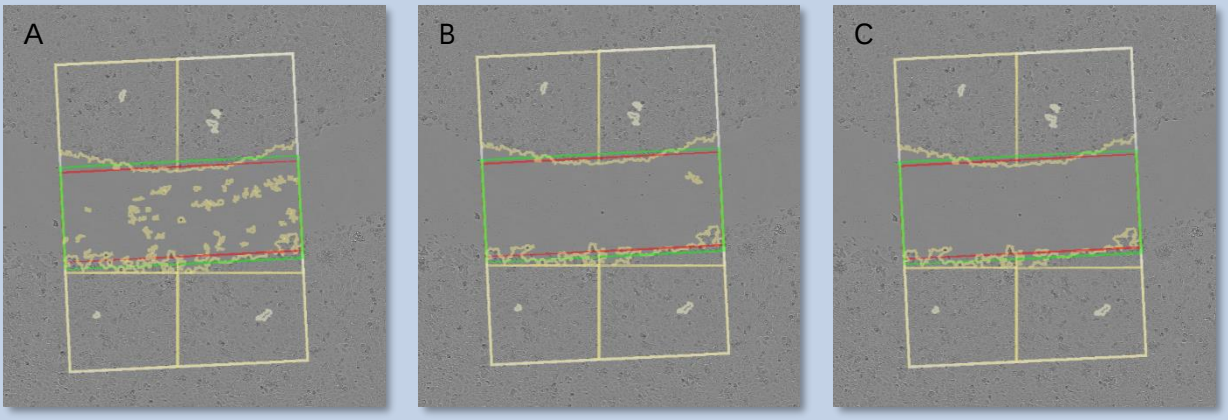


Fig. 37: Eliminating unspecific objects like non-adherent cells or cell debris
The detection of unspecific objects leads to a higher confluence on the gap.
A. *Obj. Min. Size* of 250 µm and *Obj. Min. Contrast* of 0 (default settings).
B. *Obj. Min. Size* of 1000 µm and *Obj. Min. Contrast* of 0.
C. *Obj. Min. Size* of 1000 µm and *Obj. Min. Contrast* of 0.31.

Additionally to the size and the contrast, an unspecific object can be eliminated by the compactness, the longishness, the intensity and the standard deviation. The values of the different parameters can be used to include (cells) or exclude (debris) the different objects.

5 CREATING A TEMPLATE FOR WOUND HEALING

Creating a template in Wound Healing differs from the conventional template creation. It makes no sense to use the *Save as Template* button in the experiment *Setup* of Phase 1, as only the first phase would be saved as a template. Therefore, instead of clicking on *Save as Template*, just click on *Save Experiment*.

To do so, you need an existing experiment, in which you have already carried out all steps through both phases (Wound-Healing-ROI-Finder and Wound Healing). Be sure that the *Image Processing Parameter* of the *Evaluation* of Phase 1 works fine, then you can use the *Autostart Nanoview* mode for further measurements. When the measurement in Phase 2 has been completed, go back to Phase 1 and save the experiment.

Now, generate a new experiment by pressing the button *New Experiment from Template* and use the previously saved experiment as template. This is now your new template without any imaging data. When you set the *Autostart Nanoview*, Phase 2 will be measured directly after Phase 1.

References:

[1] Grada A, et al. Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay, Journal of Investigative Dermatology (2017) 137, e11-e16; doi:10.1016/j.jid.2016.11.020