Application Note 30

Data Analysis of Wound Healing Assays
With Instructions for WimScratch

1 Introduction

The wound healing assay is a powerful tool for the investigation of the migration characteristics of cultured cells.

Cells are cultured in a confluent layer, then a wound or scratch gap is created either by removing a growth barrier or by damaging the cell layer. The subsequent image acquisition of the wound closure provides information about the migration characteristics. These may alter, depending on the addition of either enhancing or inhibiting substances to the growth medium.

The parameters that are measured to evaluate the effects are the cell covered area, the starting and the end point characteristics, and the speed calculations.

Performing wound healing assays requires the optimization of the practical protocol, as well as the establishment of a data acquisition that provides comparable data. The three critical key parameters are the time point of wound creation, the time points of data acquisition and the cell seeding density.

This application note will help determine the best wound healing assay parameters for your own cell line, thus ensuring the acquisition of reliable and reproducible results.

We rely on the results and experience with our ibidi Culture-Insert, which is applicable on any culture surface. Nevertheless, all wound healing assay data analysis can be handled in the same way.

For the specific handling of the ibidi Culture-Insert, please refer to the online instructions.

2 Choosing the Right System

The first step, when starting with wound healing experiments, is to find the right experimental system. It must be relevant, in context to your approach, and the data must be experimentally accessible. The most important considerations are:

- Cell type
- Cell culture surface
- Method of wound insertion
- Medium composition (growth factors requested?)
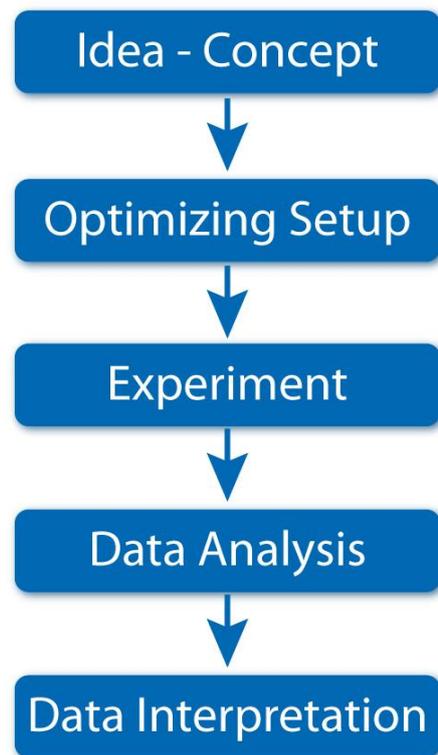
There are different principles for creating a cell-free gap:
- Barrier for the cell growth and removal after reaching confluency
- Mechanical scratching methods
- Wound burning with electrodes

When using the Culture-Insert method (a barrier for cell growth), only a few cells are damaged at the borders of the gap. In contrary to that, mechanical or electrical wound generation will always result in some damage on the cell layer in the affected region.

3   Establishment of the Experimental Parameters

In order to establish a reliable data acquisition system, you first have to define the experimental parameters before starting with your screening experiments. Some parameters, such as cell seeding density, time points, or serum concentration, strongly affect the data values.

3.1   Cell Seeding Density

We recommend using a cell density that leads to a confluent cell layer after 24 hours. Depending on your cell type, apply a seeding concentration of 3-7 x 10^5 cells/ml (applied in 70 µl per well of an ibidi Culture-Insert).
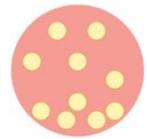
3.2   Time Point of Gap Creation

The cell layer should be optically confluent when generating the wound. Optimally, this should happen after 24 hours. If the cell layer is not yet confluent, we recommend waiting until confluency is reached.

Note: If the cells are more than 100% optically confluent, such that a multilayer is building up, this could lead to problems with detached cells (or the detachment of the whole layer) when removing the Culture-Insert.

3.3   Acquisition of Microscopic Images

For a standard migration experiment, **phase contrast** images are completely sufficient. If you want to investigate further characteristics of the cells, such as protein expression or structural organization of molecules, then choose an adequate live stain to measure at different time points, or make your immunofluorescence staining after migration monitoring is finished.
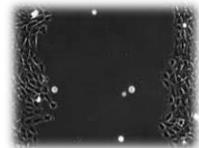
For most wound healing experiments, a low **magnification** will be adequate. It is important to observe the biggest field of view possible, in order to obtain the maximum amount of information. When using the ibidi Culture-Insert, we recommend using a 4x or 5x objective. The image will show you a section of around 2 × 1.7 mm.

The **orientation** of the wound area is not critical, but should be horizontal or vertical.

3.4   Time Points for Measurement

The graph below shows the characteristic growth curve of a wound healing experiment.
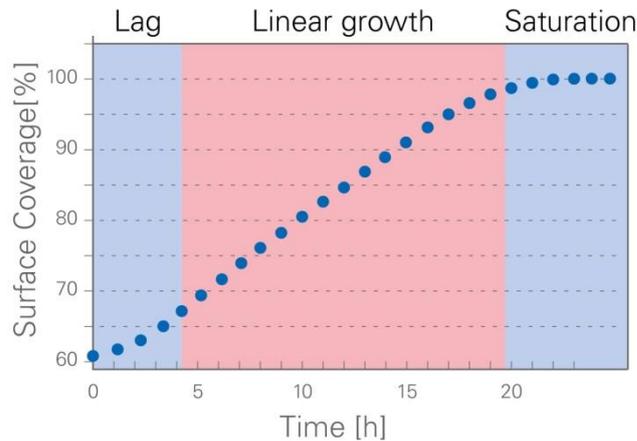
Figure 1: Wound closure measured in percentage of surface coverage over time

After creating the gap (time point zero), a lag phase might occur until the cells start migrating. This phase can be very short. The cells might also be entered directly into the linear growth phase, where the wound closure velocity is constant. When reaching confluency, the velocity first slows down and then goes to zero when the coverage reaches 100%.

Data Provided for the Different Types of Measurements

|	Unit	Available for
Cell covered area	% coverage	≥ 1 image
Scratch area (inverse cell covered area)	% coverage	≥ 1 image
Speed of wound closure	% coverage/time unit	≥ 5 images
Acceleration	[%/(time unit)^2]	≥ 5 images
Center piece approximation (slope of linear growth phase)	% coverage/time unit	≥ 10 images

Two-Point Measurement
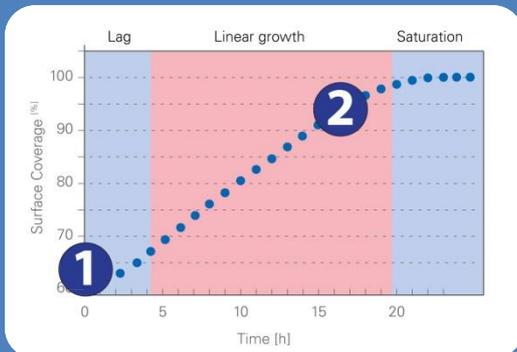
The easiest and fastest way to monitor migration behavior is to use a two-point measurement of a start and an end point. The cell layer is first imaged just after creating the gap and again after a defined time interval. Note: Make sure that the second time point (end point) does not sit in the saturation phase of the curve.

To determine the end point, perform an experiment with optimal growth conditions and define the time interval that is needed for complete wound closure. Choose the end point of your measurement just before the cell layer reaches 100% optical confluency.
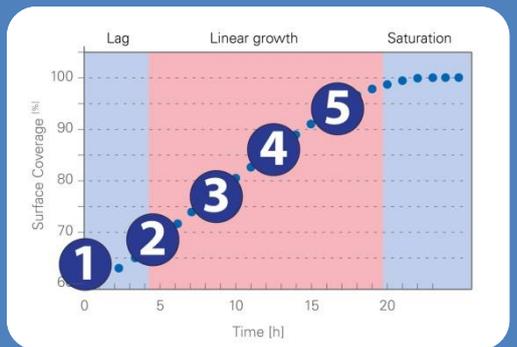
Five-Point Measurement

Making a five-point measurement will provide additional information about migration velocity and acceleration. Take five pictures, at equal time intervals, between the starting and end point. Optimally, the wound will not yet be closed in the final image.
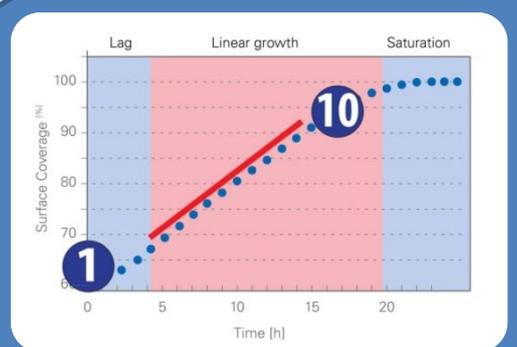

Ten-Point (or More) Measurement

Monitoring ten or more images will enable the software to make a "center piece approximation". For this value, the slope of the linear growth phase (shown as a red bar) is calculated.

3.5   Establishing Positive and Negative Controls

For the comparison of data, it is crucial to establish good controls. The positive control will represent the optimal growth conditions, which is when the cells are closing the gap the fastest. For a negative control, use a migration reaction inhibitor to be sure that the cells can be hindered in their migrational behavior.
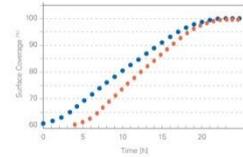
3.6   Experimental Plan and Number of Repeats

Before starting your experiments, make a project plan that specifies every single experiment and considers all of the required resources. We recommend making at least three repeats per data point.
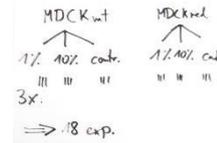
3.7   Documentation

It is important to register all of the experimental parameters, in order to generate reproducible and traceable results.
An example of the documentation is shown in the appendix.

4 Data Analysis

4.1 Online Analysis of the Images on the Platform—WimScratch

In order to quantify the characteristics of the cell migration, the microscopic pictures must be analyzed. This can be done by hand with image processing software, or in an automated way by sending the pictures to the Wimasis image analysis platform.

A free trial of 25 images is available, so that you can evaluate the image analysis performance of WimScratch. Please follow these steps to test your own images:

1. Create an account at: https://mywim.wimasis.com
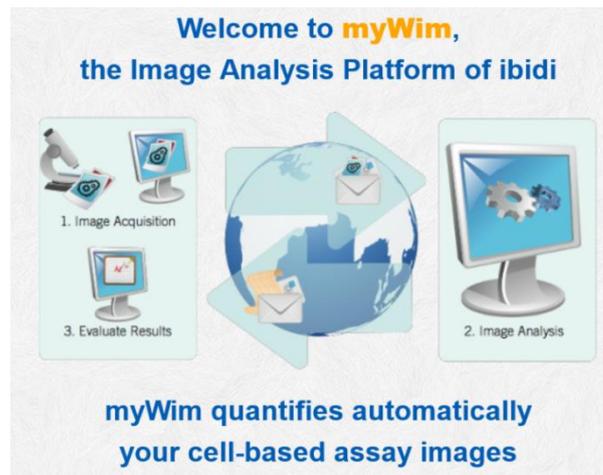
2. Activate your account by clicking on the confirmation link in the registration email.
3. Login at: https://mywim.wimasis.com
4. Upload your images after choosing the correct magnification range (see Section 3.3). Note: The magnification level will affect the expected values for the cell dimensions, in terms of the given thresholds.
5. The following graphical formats are accepted:
    *.jpg, *.jp2, *.png, *.gif, *.tiff, *.tif, *.bmp
6. After a few minutes, the results will be ready for download. You will receive the following files:
    *   Single images with the detected cell area in green (see Figure 2).
    *   Summary .csv-files containing the raw data of all the measured cell-covered areas, as well as the calculated values of velocities, acceleration, average values for the whole time period, and the characteristics of the central piece approximation
    *   A graph showing the migration progression

7. The output parameters are explained in detail in Section 4.2.

4.2 WimScratch Output Parameters

The automated image analysis detects the **cell-covered surface** of the image. The modified images (cell surface in green) are included in the results. Figure 2 shows an example.
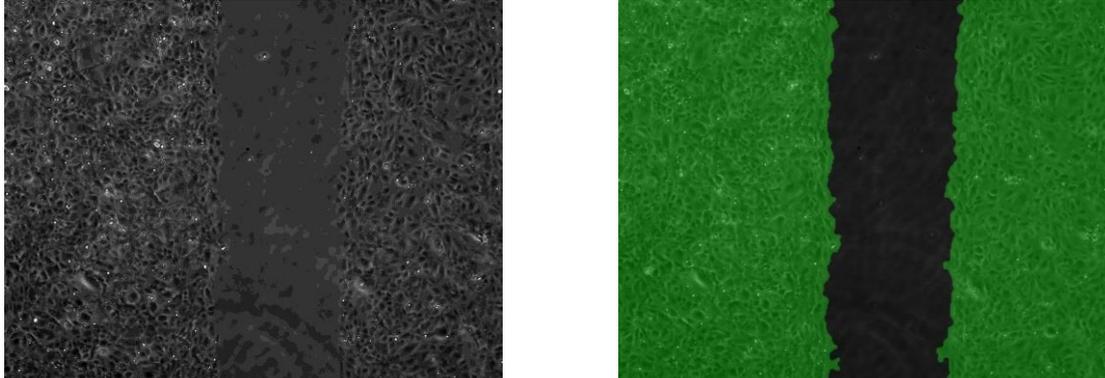
Figure 2: Sample images of a migration assay analyzed by WimScratch

The percentage of cell-covered area is visualized in a **graph**. The characteristics of the curve (for 24 hours) are shown in Figure 3. The time span of linear growth is the most important part of the curve. The velocity of wound closure for this phase (slope) will show a characteristic value, depending on the treatment of the cells.
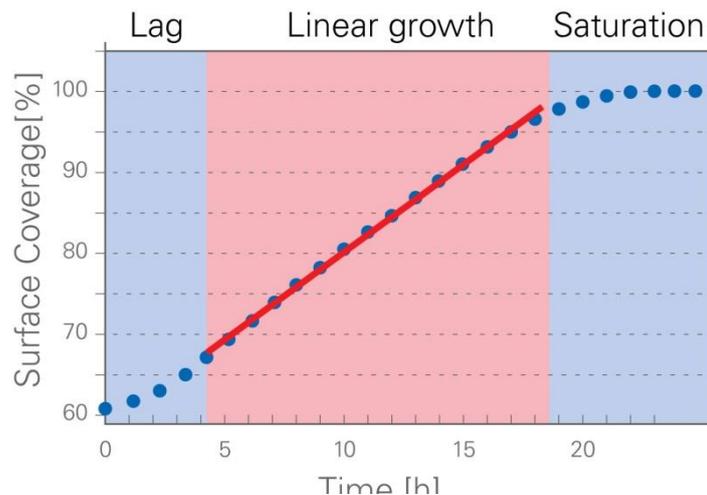
Figure 3: Cell-covered area over time. The red line shows the slope of the linear growth phase.

At the beginning, just after creating the gap, a **lag phase** might occur. This phase can be very short, in which case the cells would enter directly into the **linear growth** phase. The linear growth phase shows the characteristic migration speed of the wound closure. The cell front proceeds at a constant speed.

When reaching 100% optical confluency, the curve comes to saturation. This phase is not relevant for the experiment, because there is no change in the cell-covered surface.

The analyzed values can be downloaded as a .csv-file. An example of a ten-point measurement is shown in the graph below.

	Speed [%/ hour]	Start Point: Time [hours]	Start Point: Cell-covered Area [%]	End Point: Time [hours]	End Point: Cell-covered Area [%]
Overall Average	3.07	0	30.4	9	92
Center Piece Approximation	8.73	3	41.8	7	90.1
Image Name	Time [hours]	Scratch Area [%]	Cell-covered Area [%]	Speed [%/hour]	Acceleration [%/(hour)^2]
Control_01	0	69.6	30.4	0.6	2.5
Control_02	1	69	31	3.1	4.6
Control_03	2	65.9	34.1	7.7	17.9
Control_04	3	58.2	41.8	25.6	-14
Control_05	4	32.6	67.4	11.6	-4.5
Control_06	5	21	79	7.1	-3.1
Control_07	6	13.9	86.1	4	-3.1
Control_08	7	9.9	90.1	0.9	0.1
Control_09	8	9	91	1	
Control_10	9	8	92		

5 Data Normalization

The characteristic measurement for the whole migration experiment is the wound closure speed in the linear growth phase. The centerpiece approximation will give you the characteristic increase of the cell-covered area (in %) per time unit.

The cell-covered area, which is given for each analyzed picture, is an absolute value that is dependent on the observation area, magnification, and width of the gap. Thus, it must be normalized for comparison with other experiments.

To make all data comparable, the centerpiece approximation must be converted into a normalized value, which is the **cell front velocity**.

1. Determine the total area of your microscopic picture in µm².
   e.g. 2.400.000 µm²
2. Multiply the total area by the centerpiece approximation.
   e.g. 2.400.000 µm² x 0.0873/hour (8.73% per hour) = 209.520µm²/hour
3. Divide this number by the height/length* of the picture in µm.
   e.g. (209.520µm²/hour)/1200µm = 174.6 µm/hour
4. Divide this number by 2 (two cell fronts).
   e.g. 174.6 µm/hour / 2 = 87.3 µm/hour

The result is the normalized cell front velocity in µm/hours or µm/min.

*Depending on the gap orientation
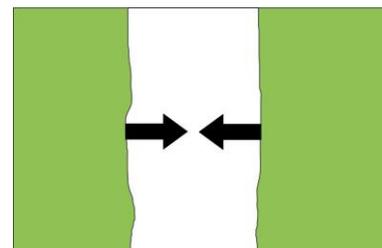
6 Data Interpretation

The normalized cell front velocities, after the different treatments of the cells, can now be compared. We recommend making at least three experiments per treatment. First, calculate the mean values from the single values, and then determine the standard deviation, which should not be more than 10% of the average. Display the values in a bar chart with the standard deviation shown as error bars.
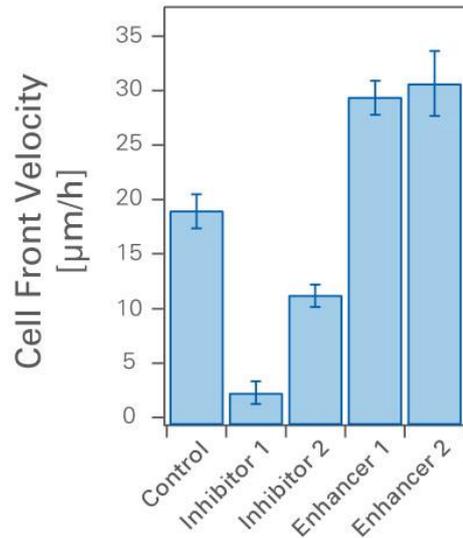
Use a Student's T-test, which will test the different populations of data points against each other, for statistical analysis. The Student's T-test gives evidence of whether or not the two separate samples, with t-distributed values, differ significantly from each other. This will depend on the number of repeats. It is recommended that the T-test be performed using a suitable statistical software or with Microsoft Excel.

7   Appendix:

Documentation Example

Date	06.02.2014
Conducted by:	AT
Experimental Parameters:	
Cell type:	MDCK
Passage:	P13
Medium:	DMEM without FCS
Concentration Cell Suspension	$2x10^5$ c/ml
Confluence before Seeding:	70%
Surface:	ibiTreat
Time in Suspension (max. 15 min):	5 min
Time between seeding and gap creation	20 h
Dish/well number 01	Positive control
Dish/well number 02	Positive control
Dish/well number 03	Positive control
Dish/well number 04	Positive control
Dish/well number 05	Inhibitor 1
Dish/well number 06	Inhibitor 1
Dish/well number 07	Inhibitor 1
Dish/well number 08	Inhibitor 1
Dish/well number 09	Inhibitor 2
Dish/well number 10	Inhibitor 2
Dish/well number 11	Inhibitor 2
Dish/well number 12	Inhibitor 2
Dish/well number 13	Inhibitor 3
Dish/well number 14	Inhibitor 3
Dish/well number 15	Inhibitor 3
Dish/well number 16	Inhibitor 3
Microscope/Imaging	
Microscope:	Nikon Ti
Camera:	DS-Qi1Mc
Image dimension in pixel:	1280x2048
Image dimension in mm²:	6.97
Objective:	4x
Large Image:	1x2
Dimension of pixel (µm/px):	1,61 µm/px
Number of images	10
Time interval	1 h
Total time	9 h
Analysis	
Analysis date:	09.02.2014
Analyzed by:	Wimasis
Module:	WimScratch low magnification
Date	09.02.2014
Conducted by:	AT