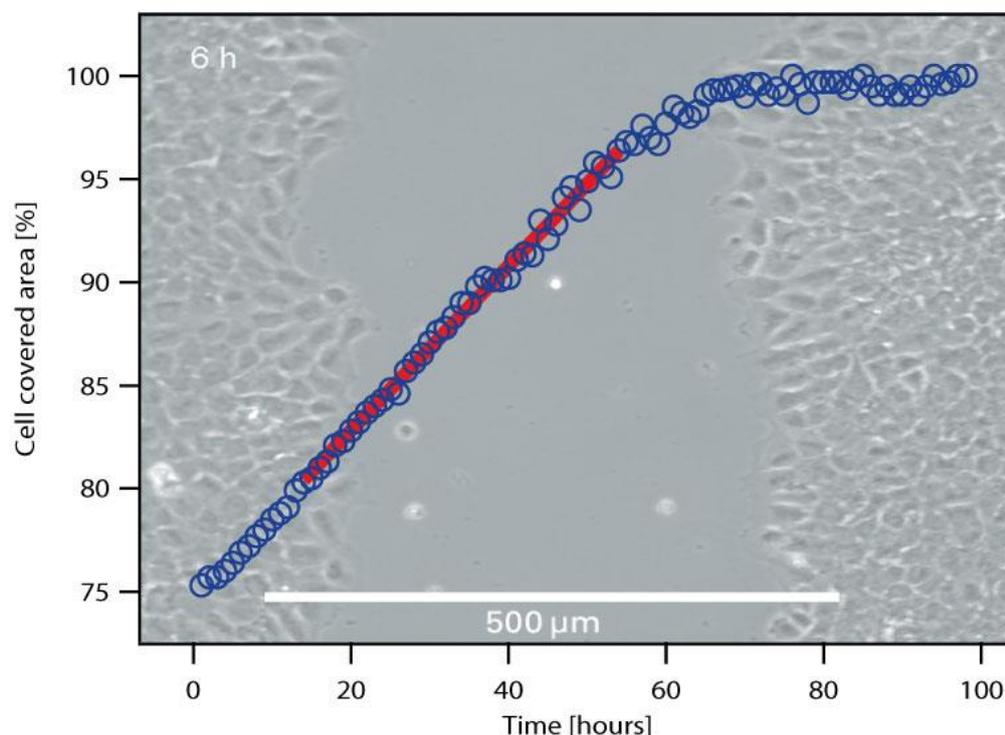


Experimental Setup Optimization and Data Analysis of Wound Healing Assays

1	Introduction.....	2
2	Choosing the Right Experimental System	2
3	Establishment of the Assay Parameters.....	3
3.1	Cell Seeding Density	3
3.2	Time Point of Gap Creation	3
3.3	Acquisition of Microscopic Images.....	3
3.4	Time Points for Measurement.....	4
3.5	Establishing Positive and Negative Controls	5
3.6	Experimental Plan and Number of Repeats	5
3.7	Documentation	5
4	Image Analysis	6
4.1	Image Analysis Output Parameters	6
4.2	Growth Rate Calculation.....	6
5	Data Normalization – Cell Front Velocity	7
5.1	Statistics	7
6	Appendix:	8
	Documentation Example.....	8



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. Imaging and analysis of the wound closure provides information about the migration characteristics. Further, either enhancing or inhibiting substances can be added to the growth medium to investigate their influence on cell movement and wound healing.

Typical readouts of a wound healing assay are the cell covered area, the starting and the end point characteristics, and the cell speed.

Performing wound healing assays requires the optimization of the practical protocol, as well as the establishment of a data acquisition that provides comparable data.

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

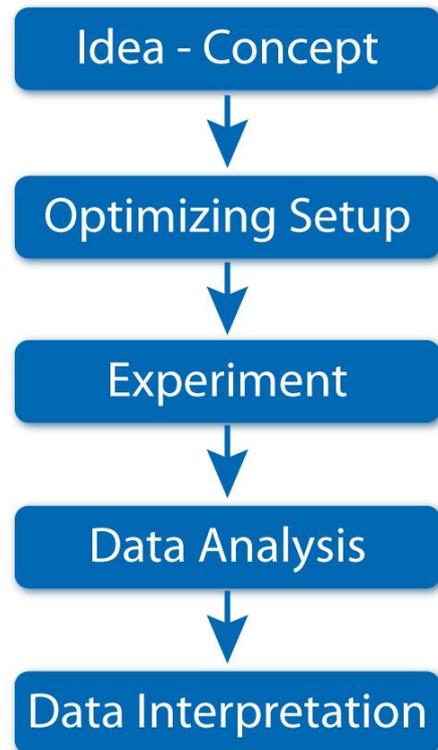
We refer to results and experience with the ibidi Culture-Insert, which is applicable on any flat, clean, and dry culture surface. Nevertheless, all wound healing assay data analysis can be handled in the same way.

For the specific handling of the ibidi Culture-Insert, please refer to the online [instructions](#) and the handling protocol in [Application Note 21 “Wound Healing Assay”](#).

2 Choosing the Right Experimental System

Before starting with wound healing experiments, the optimal experimental system must be determined. The system 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 creation
- Medium composition (growth factors, supplements, ...)
- Addition of mitotic inhibitors



Application Note 30

There are different principles for creating a cell-free gap:

- Insertion of a barrier for the cell growth, e.g. by using the ibidi Culture-Insert and removal after reaching confluency
- Mechanical scratching, e.g. by using a pipet tip
- Wound burning using electrodes

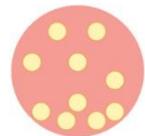
When using the Culture-Insert method (a barrier for cell growth), only a few cells are damaged at the borders of the gap. In contrary to that, mechanical or electrical wound generation will always result in some damage on the cell layer in the affected region.

3 Establishment of the Assay Parameters

Before starting with your screening experiments, the experimental parameters need to be defined. Some parameters, such as cell seeding density, time points, or serum concentration, strongly affect the measured values and have to be kept constant to ensure a reliable data acquisition system.

3.1 Cell Seeding Density

We recommend using a cell density that builds a confluent cell layer 24 hours after cell seeding. Depending on your cell type, apply a seeding concentration of $3-7 \times 10^5$ cells/ml (for the ibidi Culture-Insert you need 70 μ l cell solution per well).



3.2 Time Point of Gap Creation

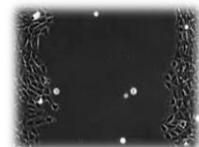
The cell layer should be optically confluent when generating the wound, optimally 24 hours after cell seeding. If the cell layer is not yet confluent, we recommend incubating the cells until confluency is reached.

Note: If the cells are more than 100% optically confluent, i.e. when a multilayer is building up, this could lead to detachment of single cells (or even the whole layer) when removing the Culture-Insert.



3.3 Acquisition of Microscopic Images

For a standard migration experiment, **phase contrast** images are sufficient. If you want to investigate further cell characteristics, such as protein expression or structural organization of molecules, then choose an adequate live stain, or an immunofluorescence staining after the migration monitoring is finished.



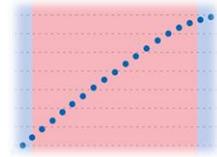
For most wound healing experiments, a low **magnification** will be adequate. It is important to observe the biggest field of view possible, in order to obtain the maximum amount of information. When using the ibidi Culture-Insert, we recommend using a 4x or 5x objective. The image will show you a section of around $2 \times 1.7 \text{ mm}^2$.

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

Application Note 30

3.4 Time Points for Measurement

Before determining the time points for measurement, it is crucial to know the characteristics of the growth curve.



A typical growth curve of a wound healing experiment is shown in Figure 1:

After creating the gap (time point zero), a **lag phase** might occur before the cells start migrating. This phase can be very short. The cells might also start directly into the linear growth phase.

In the **linear growth phase** the wound closure velocity is constant. The wound closure speed is the most important characteristic of your wound healing assay and should be used for data acquisition.

When reaching confluency, the velocity first slows down and then goes to zero when the coverage reaches 100%.

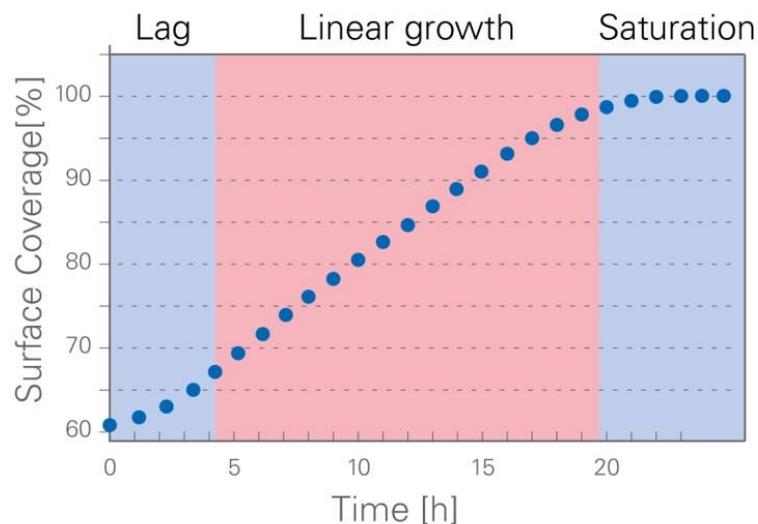


Figure 1: Typical growth curve of a wound healing assay. The wound closure is shown in percentage of surface coverage over time

Two-Point Measurement

The easiest and fastest way to monitor migration behavior is to use a two-point measurement. The cell layer is first imaged just after creating the gap (start point) and again after a defined time interval (end point). Note: Make sure that the end point lies in the linear growth phase!

To determine the end point, perform an experiment with optimal growth conditions and define the length of the linear growth phase. To have optimal results choose the end point just before the curve enters the saturation phase.

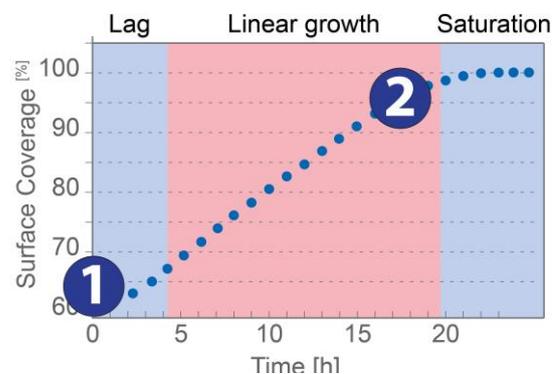


Figure 2: Setup of a two point measurement

Application Note 30

Ten-Point (or More) Measurement

Monitoring ten or more images will show a more precise image of the curve. More details of the different treatments can be discovered, such as the slope of the linear growth phase, a prolonged lag phase or the exact time point of wound closure.

With the exact determination of the linear growth phase, the calculation of the cell front velocity is possible (slope of the linear growth phase).

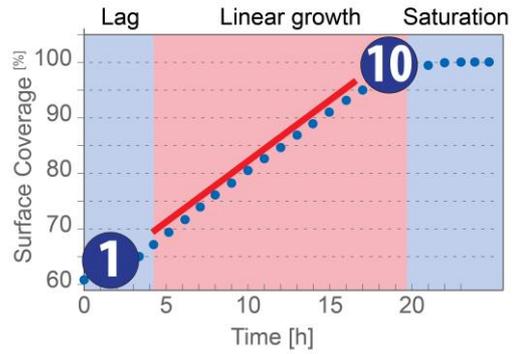
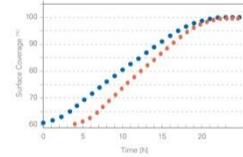


Figure 3: Setup of a ten point measurement

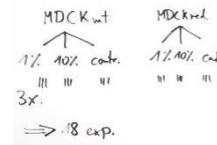
3.5 Establishing Positive and Negative Controls

For the comparison of data, it is crucial to establish suitable controls. The positive control represents optimal growth conditions, i.e. the fastest gap closure. For the negative control, a migration inhibitor is added to the cell medium, to be sure that the cells can be inhibited 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 performing at least three repeats per data point.



3.7 Documentation

It is important to record all of the experimental parameters, in order to generate reproducible and traceable results.

An example of the documentation is shown in the appendix.

Date	05.12.2014
Conducted by	AI
Experimental Parameters	
Cell type	MDCK
Medium	DMEM
Substrate	DMEM without FCS
Concentration Cell Suspension	2x10 ⁶ cells
Concentration Media Seeding	10%
Surface	96well
Time to Suspension (min)	15 min
Time between seeding and gap creation	20 h
Disposal number 01	Positive control
Disposal number 02	Positive control
Disposal number 03	Positive control
Disposal number 04	Positive control
Disposal number 05	inhibitor 1
Disposal number 06	inhibitor 1

4 Image Analysis

To quantify the characteristics of the cell migration, the images can be analyzed manually using an image processing software (e.g. ImageJ), or in an automated way (e.g. ACAS or Wimasis). This application note gives an overview of the wound healing image analysis output in general.

4.1 Image Analysis Output Parameters

The image analysis detects the **cell-covered and uncovered area** of the image. In the examples below (Figure 4) the cell covered area is colored in blue and green respectively, or the uncovered area is outlined with a cyan blue line.

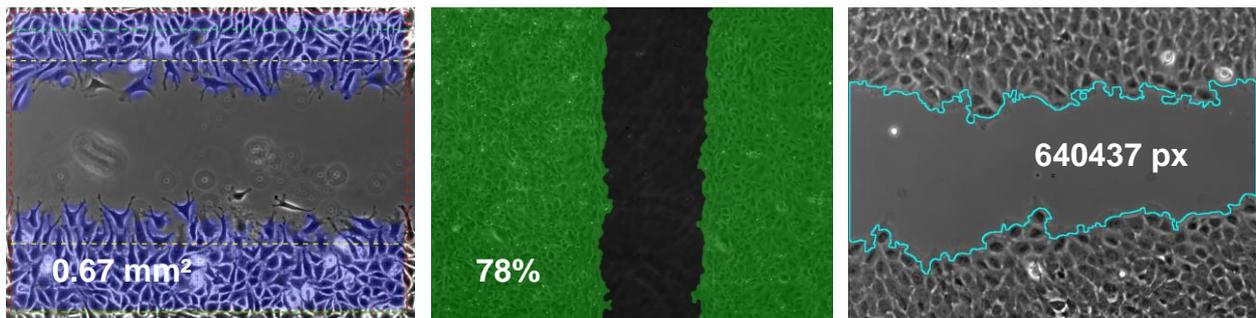


Figure 4: Analyzed example images of a migration assay. Note the output can be either in pixels, percent or mm².

The cell covered area is given in an absolute value (pixels, μm^2 or mm^2) or in a relative value (percentage of the whole picture).

The data can be saved as a .csv, .txt or Excel-file for subsequent analysis.

4.2 Growth Rate Calculation

To determine the wound closure speed (surface area per time), perform the following steps:

1. Load your data into a spreadsheet.
2. Create a graph showing the cell-covered area per time (Figure 5)
3. Define the linear part of the curve and let the program calculate the trend line for this section of the graph.
4. The slope of the trend line corresponds to the growth rate (surface area per time). In the example in Figure 5: $674 \mu\text{m}^2/\text{min}$

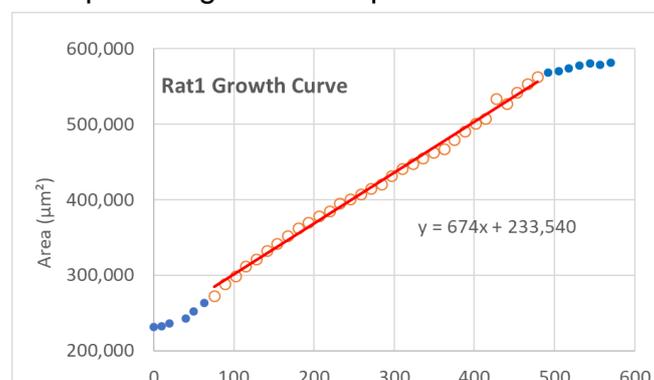


Figure 5: Growth curve example with experimental data from Rat1 cells

5 Data Normalization – Cell Front Velocity

There are two reasons, why the growth rate is not a parameter comparable to any setup and a data normalization must be done:

- The gap width in the beginning of the experiment can vary, changing the starting point of cell-covered area.
- Other setups are using a different camera, microscope, objective, etc. resulting in different observation areas.

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

Note: The cell front velocity is independent from the initial gap width, gap creation method, gap orientation, the microscopy settings and the objective lens` magnification.

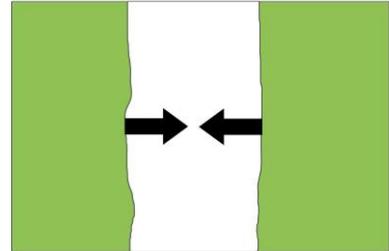


Figure 6: Visualization of the cell front velocity

Follow these steps to normalize the data:

1. Determine the length of your cell front, i.e. the height or length of the image, depending on the gap orientation.
2. Use the wound closure speed calculated in Section 4.2.
3. Divide the wound closure speed by the length of the cell front
4. Divide this number by 2 (in case of two cell fronts migrating towards each other).
The result is the normalized cell front velocity in $\mu\text{m}/\text{min}$ or $\mu\text{m}/\text{hour}$.

Example (Figure 6)

686.67 μm
674 $\mu\text{m}^2/\text{min}$
$674 \mu\text{m}^2/\text{min} / 686.67 \mu\text{m}$ $= 0.98 \mu\text{m}/\text{min}$
$0.98 \mu\text{m}/\text{min} / 2$ $= 0.49 \mu\text{m}/\text{min}$ $= 29.4 \mu\text{m}/\text{hour}$

5.1 Statistics

The normalized cell front velocities of different experiments, can now be compared. We recommend performing at least three experiments per treatment. The single experiments can be summed up to mean values and an error estimation should be performed (e.g. standard deviation, standard error, etc.). Display the values e.g. in a bar chart with the standard deviation shown as error bars.

Perform an adequate statistical analysis to show differences between the treatments.

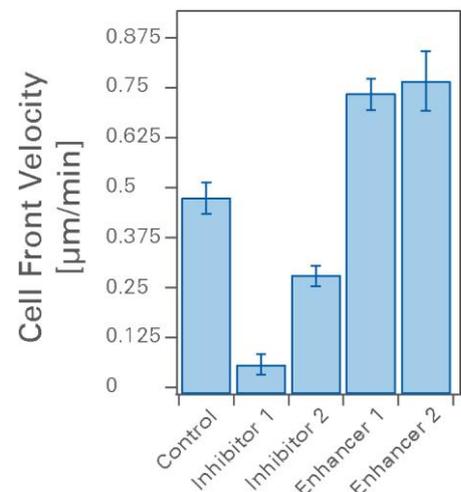


Figure 7: Example results of a wound healing assay

Application Note 30

6 Appendix:

Documentation Example

Date	12.04.2019
Conducted by:	AT
Experimental Parameters:	
Cell type:	Rat1
Passage:	P13
Medium:	DMEM without FCS
Concentration Cell Suspension	2×10^5 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:	Princeton
Image dimension in pixel:	1300x1030
Image dimension in μm^2 :	601,077
Objective:	10x
Large Image:	1x1
Dimension of pixel ($\mu\text{m}/\text{px}$):	0,6667 $\mu\text{m}/\text{px}$
Number of images	45
Time interval	10 min
Total time	9.5 h
Analysis	
Analysis date:	21.04.2019
Analyzed by:	ImageJ
Module:	MRI_Wound_Healing_Tool
Date	23.04.2019
Conducted by:	AT