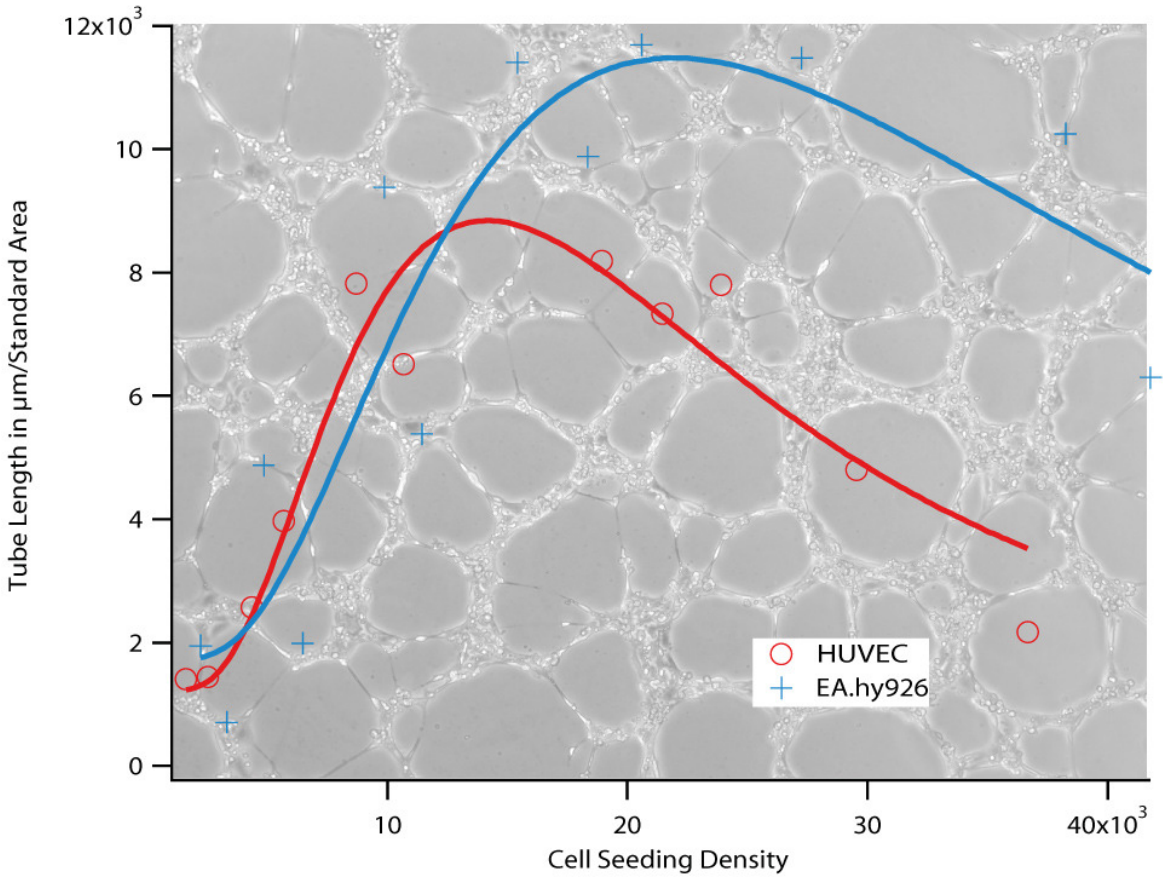
Data Analysis of Tube Formation Assays



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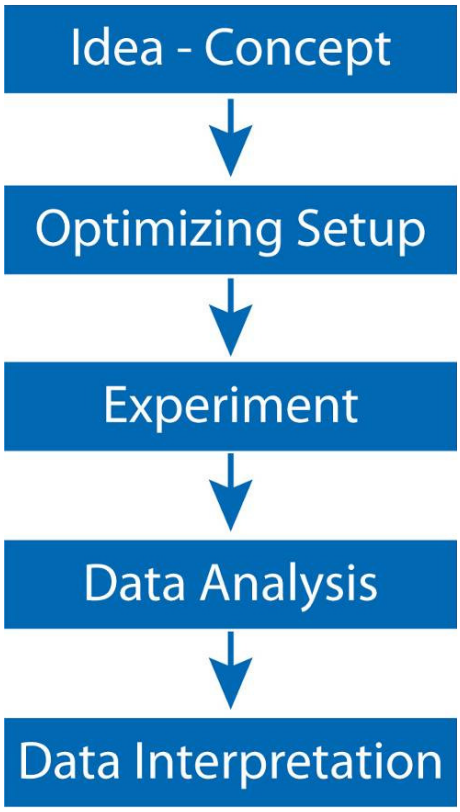
1. Introduction

The tube formation assay is a powerful tool for the screening of substances in order to discover their anti- or pro-angiogenic effect on cultured cells. In comparison to control cell cultures, the effect of a substance can be measured in parameters, like such as the length of the tubes build developing on the gel surface or number of loops.



Performing tube formation assays requires 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 data acquisition, the cell seeding density, and the serum concentration in the cell culture medium.

This application note will help determine the best parameters for tube formation assays using your own cell line, thus ensuring the acquisition of reliable and reproducible results.

In some parts of this document, we discuss µ-Slide Angiogenesis, but all types of tube formation assays will show the same characteristics and can be handled in the same way. For the specific handling of µ-Slide Angiogenesis, please refer to Application Note 19 "Tube Formation Assays in µ-Slide Angiogenesis".

NOTE: The expression „tubes" describes the cords of cells that are visible in a formed network. It does not mean, specifically, that the cords have a lumen.

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2. Choosing the Right System

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

- Cell Type—tube formation is physiologically observed in endothelial cells
- Gel Matrix—it needs to be compatible with the cells and it must provide binding motifs for cell attachment
- Medium Composition (growth factors requested?)

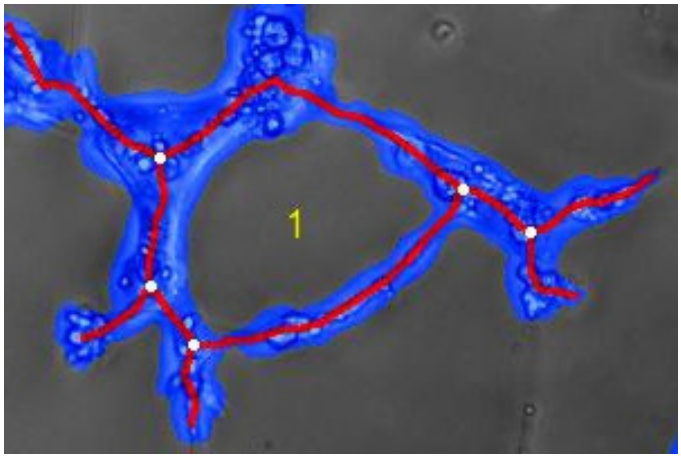
A setup that should work with most endothelial cells consists of Matrigel™, Growth Factor-Reduced, and any endothelial cell medium with 2% serum or less.

3. Data Analysis Overview

For a better understanding of the next steps of the protocol optimization, an overview is needed of the parameters included in the data analysis.

A microscopic picture, such as the one below, shows a tube formation network with four detectable key parameters:

- Cell covered area (blue)
- Tubes (red)
- Loops (yellow)
- Branching points (white)



By using those values, a range of further values can be calculated, such as the mean tube length, the total tube length, the mean area of loops, and others.

There are several possible ways to analyze your pictures. One way is to do this manually with an image manipulation software, such as ImageJ.

Another option is to use the WimTube image analysis tool, which calculates the key parameters of the network by an automated algorithm. We recommend using this web-based tool for maximum reproducibility and objectivity. In addition, it gives you results within minutes. For a free trial of WimTube image analysis, please visit the WimTube platform.

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4. Pre-Experimental Work

Before starting with your screening experiments, it is absolutely necessary to optimize the experimental procedure and setup.

4.1. Optimization of Parameters

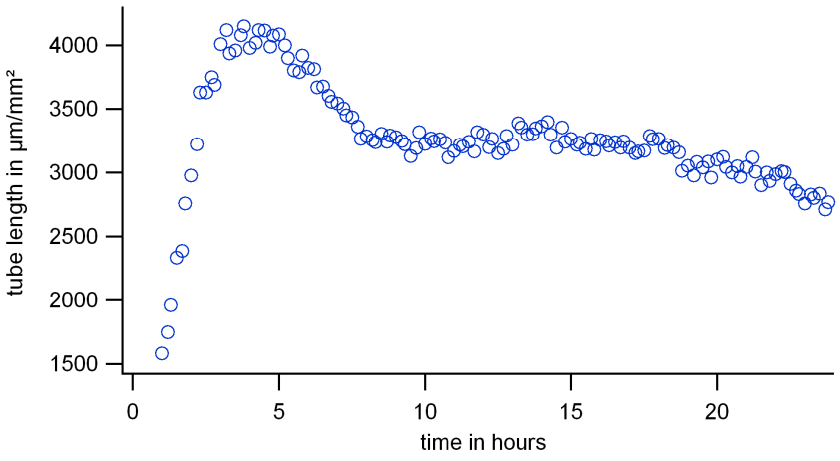
Some parameters, such as cell seeding density, time points, or serum concentration, strongly affect the data values. Therefore, the creation of a strict protocol is crucial for comparability of data.

4.1.1. Time Intervals for Measurement

First, choose a medium cell concentration and a tube formation-promoting setup to record a time curve. For HUVEC ~10,000 cells per well, using Matrigel with reduced growth factors and a medium without serum or growth factors is adequate.

Next, prepare the gel and cell suspension as described in Application Note 19, "Tube Formation Assays in µ-Slide Angiogenesis". Immediately after seeding the cells on the gel surface, place the slide into an incubation chamber on the microscope and start a time-lapse recording. Take a picture every 10 minutes by adjusting the focus at each time point with a software-based tool. Alternatively, if live cell imaging isn't possible, then take a picture every hour, for at least the first 8 - 10 hours, and then a final picture after an overnight incubation period.

Finally, analyze the pictures with WimTube and visualize the curve with suitable software. The time curve of a parameter (e.g., total tube length) typically looks like the chart shown below. In the first hours the curve rises to a maximum, then declines to a plateau phase (beginning at ~7 hours), and finally then slowly flattens out (>20 hours). The characteristics of all four key parameters look similar. For this reason, the evaluation of only one parameter is sufficient. We chose the tube length as a parameter, because it is controlled easily by comparing the original pictures to the evaluated ones.



The best results are found at the maximum phase and also in a stable phase that does not decline too fast. In the example above, the 5-hour mark and 10-hour mark would be good reference points for your measurements.

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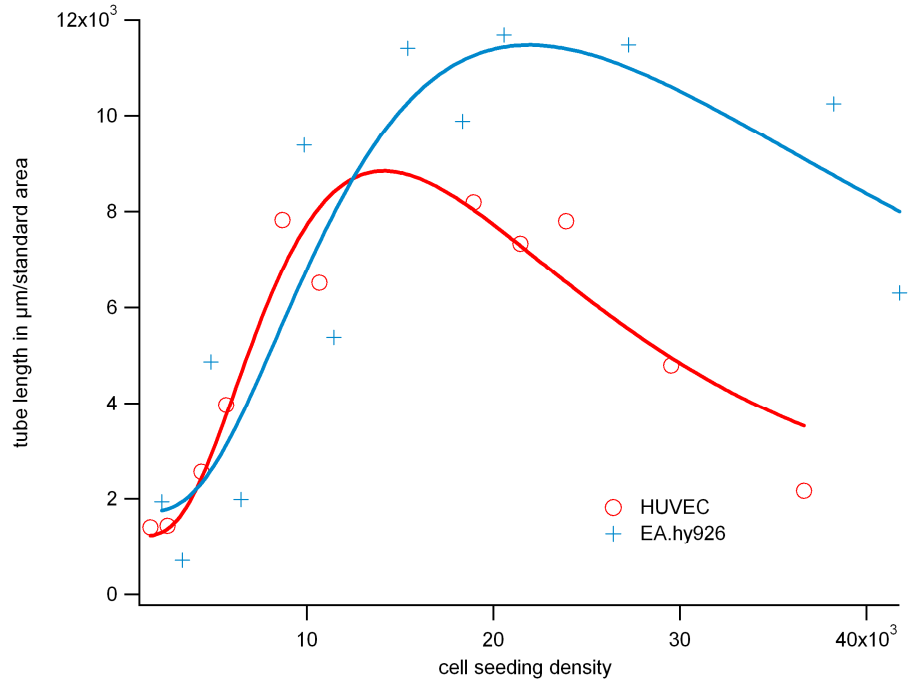
4.1.2. Cell Seeding Density

For determining the optimal cell seeding density, record the characteristics of your cell line.

First, make a dilution series in the range of 5-40 x 10^3 cells/ml and then seed the cells onto the gel surface. Incubate the slide for the determined length of time, after following the instructions in section 4.1.1., and then take phase contrast pictures. Make at least five repeats per cell concentration.

Then, evaluate the images, as mentioned in Sections 3 and 5, respectively. Visualize the data in a graph, as shown below. The data will once again show a characteristic curve with a maximum value. This maximum marks the best seeding density for your cell type.



4.1.3. Serum Concentration

The addition of serum to the culture medium might influence the tube formation behavior. In most cases, the serum inhibits tube formation. To avoid this problem, try different serum concentrations, from 0% up to 20%, with the parameters you defined in Sections 4.1.1 and 4.1.2. This will help you decide which concentration is best for tube formation and cell survival.

4.1.4. Magnification

The magnification determines the dimension of the representative screen section that will be shown on the chip of the camera. Because tube formation is a process that uses the whole surface of the well, it is important to image the widest possible section. If you are not interested in detecting cellular details, it is recommended to use a small magnification of 4x or 5x. When this is not possible, you can also stitch images that have a higher magnification.

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4.2. Establishing Positive and Negative Controls

To create a positive control, choose a setup where tube formation is assured (e.g., Matrigel with reduced growth factors and serum-free medium for HUVEC). The positive control will ensure that the cells are healthy and also that any anti-angiogenic effects that are observed are being caused by the investigated substances.

To create a good negative control, choose a substance that is proven to have an inhibiting effect on tube formation development (e.g., suramine for HUVEC). The negative control will ensures that the cells are not inert to inhibition and will give a comparable value to the values of your experiment.

4.3. Experimental Plan and Number of Repeats

Before starting the whole experimental series, calculate the amount of the required material, such as cells, media, gel matrix, and substances, as well as the laboratory requirements and the time schedule.

For a stable statistical analysis, a minimum of four independent experiments is recommended, each with a minimum of 8 single wells. The required number of repeats is dependent on the homogeneity of the data. Following a strict protocol is crucial for data acquisition. The p-value of the Students-T-test will provide feedback, if your data is homogeneous enough.

4.4. Documentation

A reasonable documentation includes all of the parameters that where determined in this section. Generate a tabular chart in which every experiment is recorded in one column. An example is given in the appendix.

5. Data Analysis

5.1. Evaluation of Pictures

The microscopic pictures need to be analyzed, in order to quantify the characteristics of the network. This can be done by hand with an image processing software, or by sending the pictures to the automated image analysis at Wimasis GmbH.

A free trial of 10 images is available, so that you can evaluate the image analysis performance. Please follow these steps to test your own images:

1. Create an account at:
   https://mywim.wimasis.com/index.php?page=Launch&select=Tube_Formation&gr=ibidi
2. Activate your account by confirming of the registration email.
3. Login at: https://mywim.wimasis.com/index.php?page=Home&gr=ibidi
4. Upload your images after choosing the right magnification range (see Section 4.1.4). The magnification level affects 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

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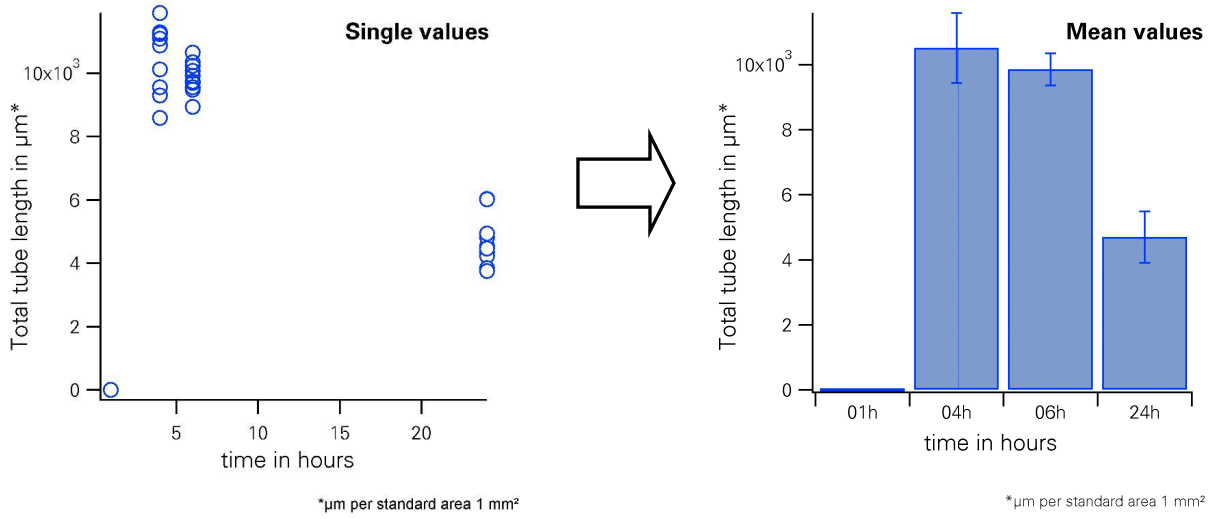
6. After a few minutes, the results will be ready to be downloaded. You will receive the following:
   - single images with colored cell area, tubes, nodes, and loops
   - a summary.csv-file with all of the measured and calculated values

The key metrics are
- Cell covered area [%]
- Total tube length [px]
- Total number of branching points
- Total number of loops
- Total number of nets (connected network of tubes)

All of the key metrics will show the same characteristic over the experimental span of time, therefore it is sufficient to evaluate only one parameter. The most stable and traceable value is the total tube length. Therefore, we recommend evaluating only the tube length as a representative value for the formation of tubes (see also 4.1.1).

5.2. Data Processing

Each analyzed well generates one tube length value. The values of one experiment (minimum 8 wells per condition) are then summed up and a standard deviation is calculated. The standard deviation should not be more than 10%. This is just one experimental data point.



The left graph shows the distribution of single well results for one experimental condition, at four different time points.

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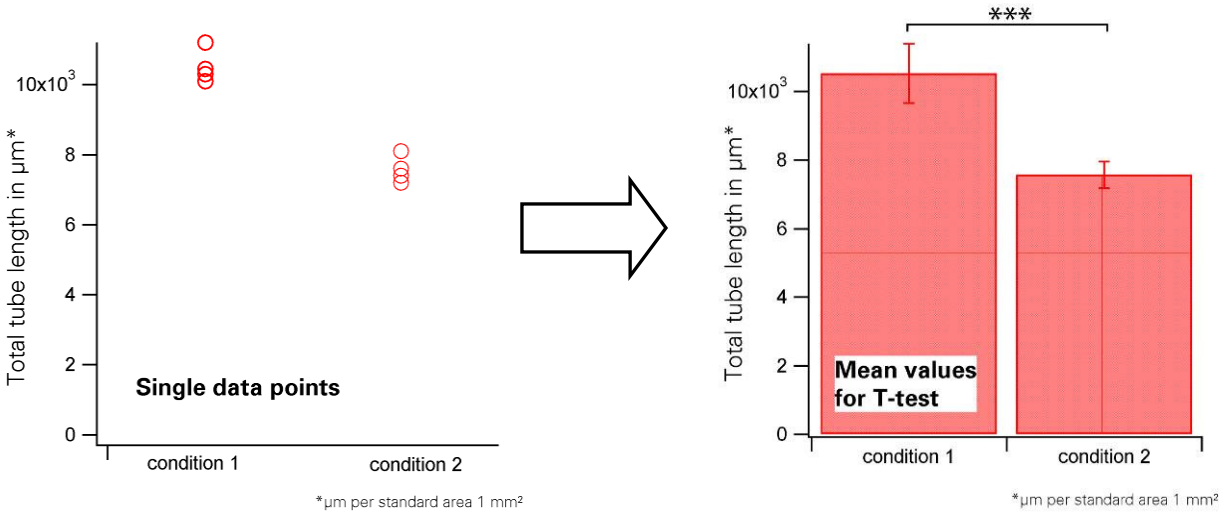
For a stable statistical analysis, at least four data points for each condition are needed. Please keep in mind that each data point consists of 8 individual wells.



The single data points are grouped into a mean value and can be displayed in a bar plot. Statistical analysis is done by a Student's T-test, in which the different populations of data points are tested against each other.

6. Data Interpretation

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 to perform the T-test with suitable statistical software or in Microsoft Excel.

It is important to consider what can realistically be predicted with the tube formation data. Tube formation is a very complex process that combines a huge variety of biochemical reactions and pathways. In our opinion, this experimental setup is suitable for a screening of substances, and for a preliminary prediction of whether or not the substance influences tube formation processes. It does not explain how this influence works. For further investigation, more profound biochemical analysis will need to be done.

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Appendix:
Documentation Example

Date	08.12.2011
Conducted by:	HW
Experimental Parameters:	
Cell type:	HUVEC
Passage:	P2
Medium:	EC Medium without supplements
Concentration Cell Suspension	2x105 c/ml
Confluence before Seeding:	70%
Gel matrix:	Matrigel with GFs
Time in Suspension (max. 15 min):	5 min
A1	negative control
A2	negative control
A3	negative control
A4	negative control
A5	negative control
B1	positive control
B2	positive control
B3	positive control
B4	positive control
B5	positive control
C1	
C2	
C3	
C4	
C5	
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:	3
Time points:	1h, 6h, 24h
Analysis	
Analysis date:	09.06.2011
Analyzed by:	Wimasis
Modul:	wimtube low magnification
Date	08.12.2011
Conducted by:	HW

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