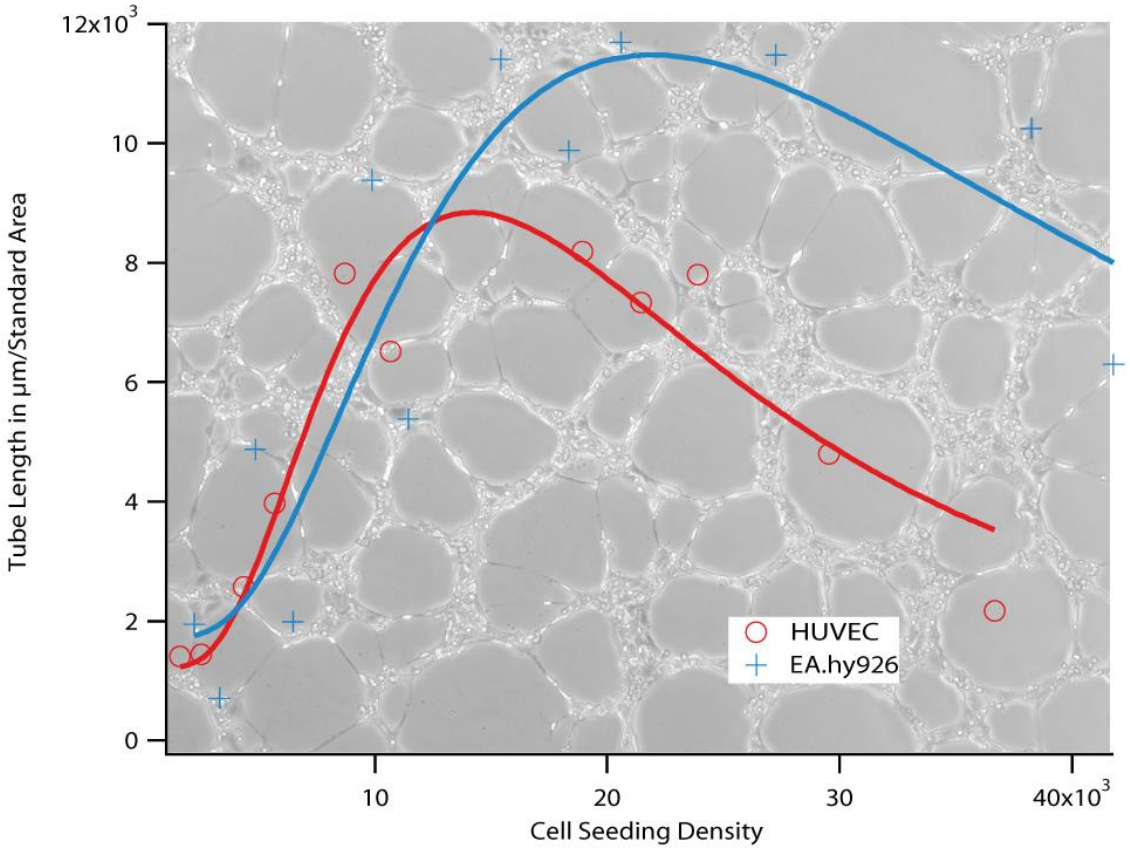
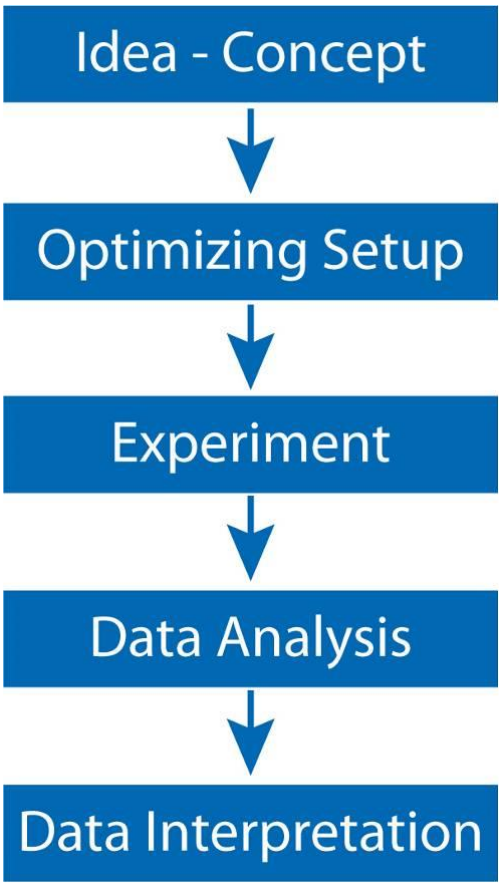
Experimental Setup Optimization and Data Analysis of Tube Formation Assays



Cell seeding density characteristics for different cell types (see Section 4.1.2.).

1. Introduction

The tube formation assay is a powerful tool for screening substances to discover their anti- or pro-angiogenic effect on cultured cells. By comparing cells treated with substances to control cell cultures, the substances' effect can be measured by parameters like tube length and the number of loops formed on the gel (i.e., Matrigel™) surface.



Performing tube formation assays requires optimizating the experimental protocol and establishing a data acquisition method that provides comparable data. The three critical components of the experimental setup are the data acquisition time point, cell seeding density, and serum concentration of the cell culture medium.

This application note will help optimize the experimental setup for tube formation assays using your own cell line, ensuring reliable data acquisition and reproducible results.

In some parts of this Application Note, we discuss the µ-Slide Angiogenesis, but tube formation assays performed in any vessel will show the same characteristics and the data can be handled in the same way. For the specific handling of the 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.

2. Choosing the Right System

The first step in performing a tube formation assay is to define the experimental system. 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 endothelial cell medium with 2% serum or less.

3. Data Analysis Overview

To better understand the next steps of the protocol optimization, an overview of the data analysis parameters is needed.

A microscopic image, 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)

Further values, such as mean tube length, total tube length, and mean area of loops, can be calculated from these parameters.

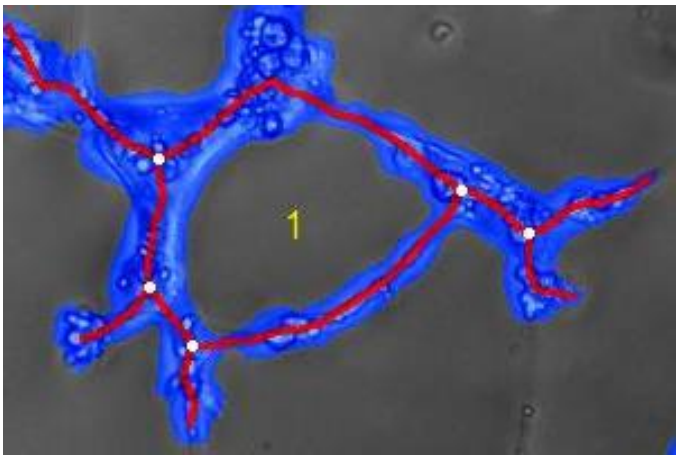


Figure 1 Key metrics of tube formation analysis

There are several ways to perform image analysis. One way is to do this manually with an image manipulation software, such as ImageJ “Angiogenesis Analyzer”.

Another option is to use an automated image analysis platform such as ACAS.

4. Pre-Experimental Work

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

4.1. Optimization of Experimental Setup

Cell seeding density, data acquisition time points, and serum concentration strongly affect the data values. Therefore, the creation of a strict protocol is crucial for generating reproducible data and comparability between data sets.

4.1.1. Time Intervals for Measurement

First, choose a cell concentration and a tube formation-promoting setup to record a time curve. For Human Umbilical Vein Endothelial Cells (HUVEC) ~10,000 cells per well, using Matrigel™ with reduced growth factors and a cell culture 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. Record an image every 10 minutes, adjusting the focus at each time point with a software-based tool. Alternatively, if an incubation chamber isn't available on your microscope, record an image every hour, for at least the first 8 - 10 hours. Place the sample in the cell culture incubator immediately after recording each image. Record a final image after an overnight incubation period.

Finally, analyze the images 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. The curve rises to a maximum (at ~5 hours), then declines to a plateau phase (beginning at ~7 hours), and then slowly flattens out (>20 hours). Since the characteristics of all four key parameters look similar, evaluating one parameter is sufficient. We chose total tube length as a parameter, because the original images provide a control for an easy comparison to the evaluated images.

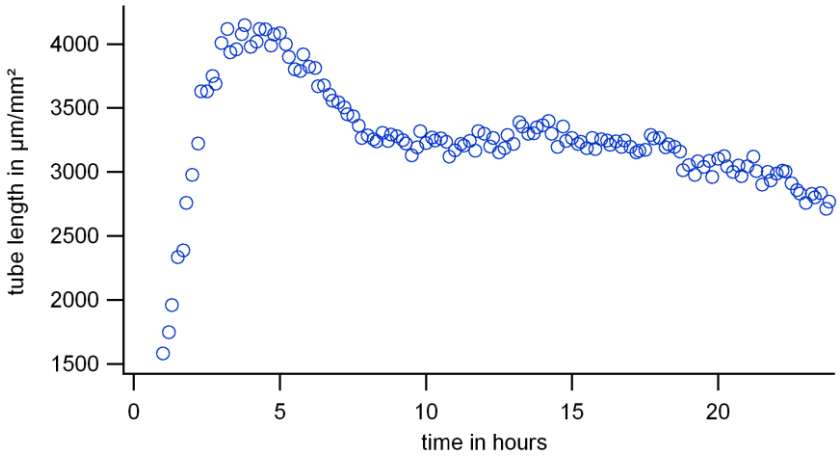


Figure 2 Time response of tube length during 24 hours

The optimal results are found at the maximum phase and also in a stable phase that does not decline rapidly. In the example above, the 4-hour time point and 10-hour time point are good reference points for measurement.

4.1.2. Cell Seeding Density

To determine 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 sample for the determined length of time, following the instructions in section 4.1.1., and record phase contrast images. Perform five replicates per cell concentration.

Evaluate the images, as mentioned in Sections 3 and 5, respectively. Visualize the data in a graph, as shown below. The data will show a characteristic curve with a maximum value. This maximum value is the optimal seeding density for your cell type.

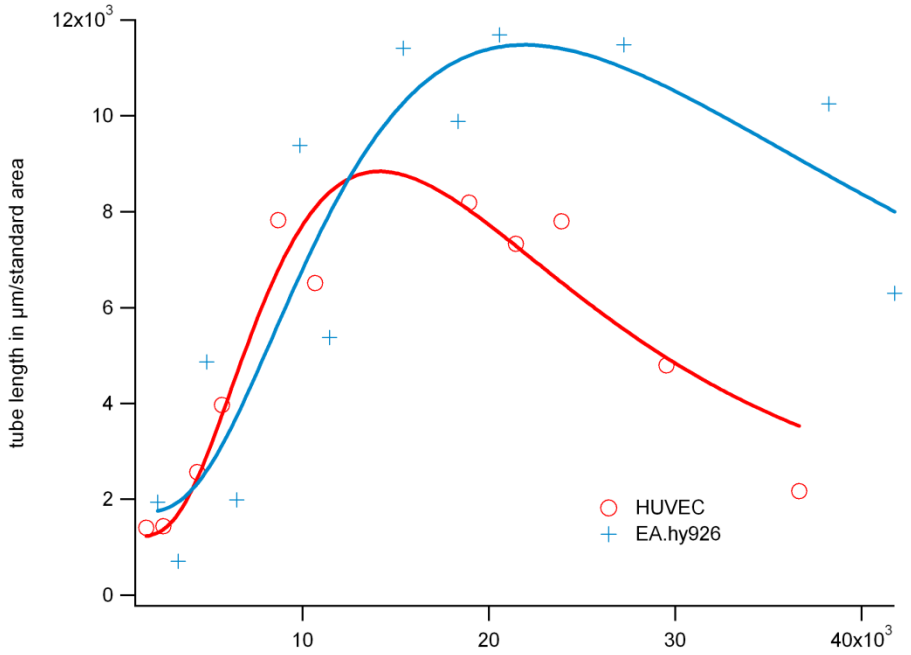


Figure 3 Seeding density response of HUVEC and EA.hy926 after 4 hours

4.1.3. Serum Concentration

The addition of serum to the culture medium may influence tube formation behavior. In most cases, serum inhibits tube formation. To avoid this problem, test different serum concentrations, from 0 - 20%, with the conditions you defined in Sections 4.1.1 and 4.1.2. This will determine which serum concentration is optimal for tube formation and cell survival.

4.1.4. Magnification

The magnification determines the area of the well that is able to be imaged by the camera. Since tube formation occurs over the entire surface area of the well, it is important to image the widest section possible. If detecting intracellular details is not necessary, use a low magnification (4x or 5x). If detecting intracellular details is necessary, it's recommended to take several higher magnification images of each well and stitch them together.

4.2. Establishing Positive and Negative Controls

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

To create a 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 ensures that tube formation can be inhibited in your cells and provides a point of comparison for your experimental results.

4.3. Experimental Plan and Number of Replicates

Prior to conducting the experiment, calculate the amount of the required material, such as cells, media, gel matrix, and substances. Also calculate the laboratory equipment and space requirements and the time schedule.
To properly power the statistical analysis, a minimum of four independent experiments is recommended, with a minimum of 8 single wells each. The required number of experiments is dependent on the homogeneity of the data. Following a strict protocol is crucial for data acquisition.
Perform the Student's-T-test on the data set. A p-value ≤ 0.05* (≤ 0.01**) indicates that the model is sufficiently powered to continue without additional experiments .

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 Images

The microscopic images need to be analyzed, to quantify the characteristics of the tube formation. This can be done manually with an image processing software (e.g. ImageJ "Angiogenesis Analyzer"), or by sending the pictures to an automated image analysis (ACAS).

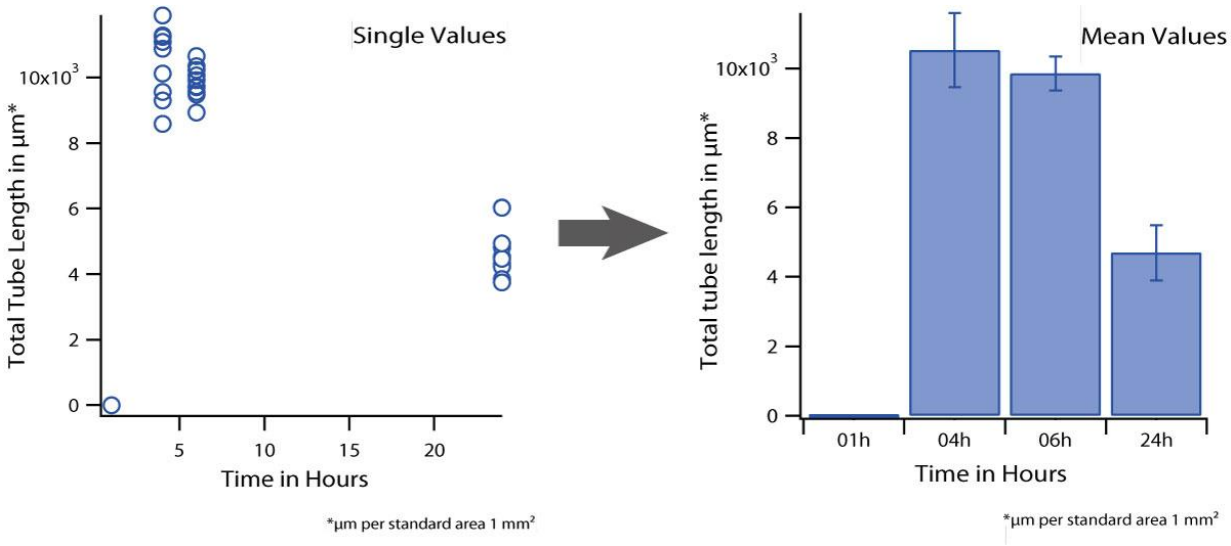
The key metrics of tube formation analysis are (see Figure 1):

- Cell covered area [%]
- Total tube length [px]
- Total number of branching points
- Total number of loops

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).

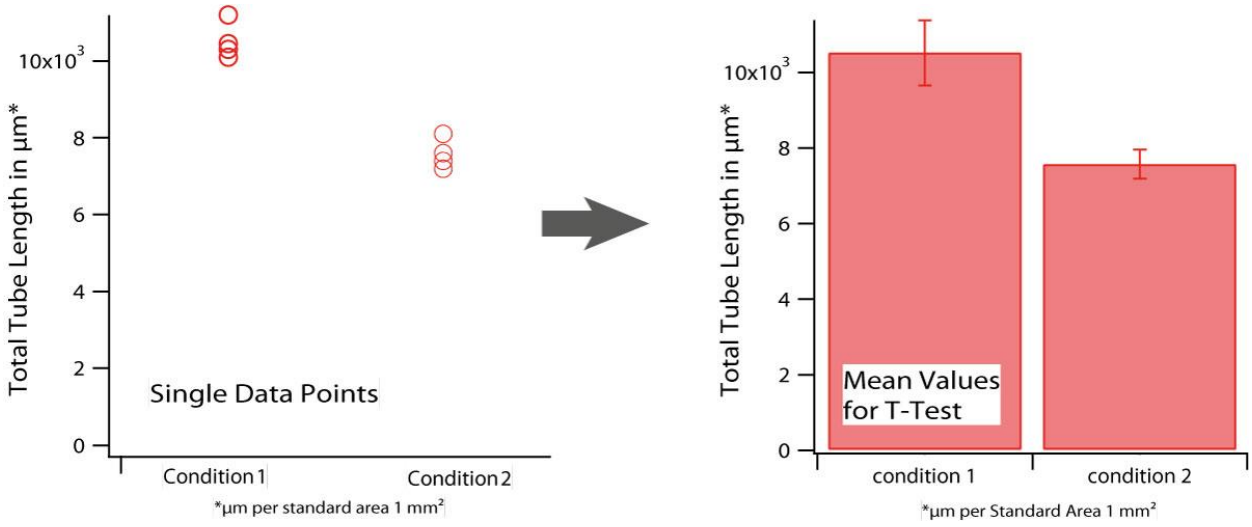
5.2. Data Processing

Each analyzed well generates one tube length value. The values of one experiment (minimum 8 wells per condition) are then summed and a standard deviation is calculated. The standard deviation should be less 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. The right graph shows the mean results of one experimental condition at four different time points.

To properly power the 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 pooled 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 data sets, with t-distributed values, show a statistically significant difference from each other. The number of replicates within an experiment and the number of experiments will affect the analysis. The Student's-T-test can be performed using statistical analysis software (i.e., SAS) or 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 screening substances and providing preliminary prediction about the substances' anti- of pro-angiogenic effect. It does not explain how any observed effect works. Additional biochemical analysis is necessary to determine this.

Appendix:
Documentation Example

Date	08.12.2011
Conducted by:	AA
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
Module:	wimtube low magnification
Date	08.12.2011
Conducted by:	HW